Disruption of a Nuclear NFATc2 Protein Stabilization Loop Confers Breast and Pancreatic Cancer Growth Suppression by Zoledronic Acid*§

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The aminobisphosphonate zoledronic acid has elicited significant attention due to its remarkable anti-tumoral activity, although its detailed mechanism of action remains unclear. Here, we demonstrate the existence of a nuclear GSK-3β-NFATc2 stabilization pathway that promotes breast and pancreatic cancer growth in vitro and in vivo and serves as a bona fide target of zoledronic acid. Specifically, the serine/threonine kinase GSK-3β stabilizes nuclear NFATc2 through phosphorylation of the serine-rich SP2 domain, thus protecting the transcription factor from E3-ubiquitin ligase HDM2-mediated proteolysis. Zoledronic acid disrupts this NFATc2 stabilization pathway through two mechanisms, namely GSK-3β inhibition and induction of HDM2 activity. Upon nuclear accumulation, HDM2 targets unphosphorylated NFATc2 for ubiquitination at acceptor lysine residues Lys-684/Lys-897 and hence labels the factor for subsequent proteasomal degradation. Conversely, mutagenesis-induced constitutive serine phosphorylation (Ser-215, Ser-219, and Ser-223) of the SP2 domain prevents NFATc2 from HDM2-mediated ubiquitination and degradation and consequently rescues cancer cells from growth suppression by zoledronic acid. In conclusion, this study demonstrates a critical role of the GSK-3β-HDM2 signaling loop in the regulation of NFATc2 protein stability and growth promotion and suggests that double targeting of this pathway is responsible, at least to a significant part, for the potent and reliable anti-tumoral effects of zoledronic acid.

Initially discovered due to its potential to inactivate osteoclastic bone resorption, the third generation bisphosphonate, zoledronic acid (ZOL), has become an attractive agent in the treatment of benign and malignant skeletal diseases related to increased bone loss, e.g. osteoporosis, Paget disease of bone, and tumor-associated hypercalcemia (1, 2). In addition, a beneficial effect of ZOL has been extensively demonstrated in the treatment of advanced cancer with bone metastasis (3, 4). Over the past decade, ZOL has become the standard therapy for breast cancer patients with skeletal metastases (1, 2). Furthermore, besides these well characterized effects on skeletal metastasis, increasing evidence from preclinical and clinical trials demonstrate that ZOL exhibits strong anti-tumor functions outside of the bone. In certain epithelial cancers, ZOL has a high selectivity for targeting tumor cells, resulting in inhibition of tumor outgrowth, reduced incidence of visceral metastasis, and increased overall survival (5–8). In fact, a recent large study reported a substantial reduction of local breast cancer recurrence after surgery when endocrine therapy was combined with ZOL (9). Therefore, ZOL may not only become the drug of choice for many translational and clinical studies in cancer but may also serve as a platform to develop novel therapeutic strategies in cancer treatment. Consistent with clinical data, in vitro and in vivo studies have identified marked growth suppression activities of ZOL in tumors from different origins (10, 11). However, the molecular mechanisms underlying the anti-tumoral functions of this highly promising drug in cancer therapy remain poorly understood.

Here, we describe a new NFATc2-dependent mechanism modulating cell growth in breast and pancreatic cancer and identify this novel pathway as a bona fide target of ZOL. We demonstrate a role for GSK-3β-dependent phosphorylation in NFATc2 protein stabilization and growth promotion in cancer. ZOL interferes with this phenomenon by acting as a GSK-3β inhibitor. In addition, by inducing HDM2-mediated polyubiquitination and degradation of NFATc2, ZOL operates as a new...
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functional antagonist of NFATc2, which acts via a different mechanism than the well established calcineurin-NFAT inhibitors. This double interference with the same pathway is responsible, at least in part, for the potent and reliable growth suppression effects of ZOL in cancer. In conclusion, the current study of a novel biochemical mechanism that regulates the lifetime and growth-promoting functions of oncogenic NFATc2 as well as the identification of this signaling loop as an important target for ZOL-mediated breast and pancreatic cancer cell growth inhibition carry significant potential implications for both basic science and clinical medicine.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfection—Human breast cancer cell lines MDA-MB-435 and MDA-MB-231 and pancreatic cancer cell lines IMIM-PC1, Suit-028, and PaTu8988t were maintained in Dulbecco’s modified minimal essential medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% FCS. Transient transfection was performed by using transfection reagent (Promega, Madison WI). Small interfering RNAs to human NFATc2 (siRNA 1: 5’-gcuagagacaggacucuuaat-3’; siRNA 2: 5’-ccauuaacaggagcaag-3’) obtained from Ambion Applied Biosystems (Austin, TX), HD2M (siRNA 1: 5’-cccaacucugauagauuu-3’; siRNA 2 5’-guagaguccuuaauu-3’), or SMARTpool siRNA (SMARTpool siRNA) obtained from Dharmacon (Lafayette, CO) were transfected into the indicated cell lines by using siLentFectTM (Bio-Rad) or Effectene (PAA Laboratories GmbH, Austria) supplemented with 10% FCS. Transient transfection was performed by using transfection reagent (Promega, Madison WI). Small interfering RNAs to human NFATc2 (siRNA 1: 5’-gcuagagacaggacucuuaat-3’; siRNA 2: 5’-ccauuaacaggagcaag-3’) obtained from Ambion Applied Biosystems (Austin, TX), HD2M (siRNA 1: 5’-cccaacucugauagauuu-3’; siRNA 2 5’-guagaguccuuaauu-3’), or SMARTpool siRNA (SMARTpool siRNA) obtained from Dharmacon (Lafayette, CO) were transfected into the indicated cell lines by using siLentFectTM (Bio-Rad) or Effectene (PAA Laboratories GmbH, Austria) supplemented with 10% FCS. Transient transfection was performed by insertng a stop codon into the open reading frame by using the following primers: 5’-gagagctctggctgagag-3’ and 5’-gctgttggactttgatttatttggagcgcg-3’. The pSP2 mutation was generated by site-directed mutagenesis using the ΔSP2 mutant. Alanine 215 and 219 were exchanged to glutamic acid using the primers: 5’-gctcatgaccgaccgaccgacg-3’ and 5’-catttggatctttggctataacg-3’ and alanine 223 was exchanged using the primers: 5’-cgcctcataaatggagaccaaccggc-3’ and 5’-gctgttggactttgatttatttggagcgcg-3’. The K684R/K897R construct was generated by mutation of lysine Lys-684 (5’-cctgctcattagggagacgagcc-3’ and 5’-gctgtgctctgtctgtttgagggccgcg-3’) and lysine Lys-897 (forward, 5’-gagagctctggctgagag-3’ and reverse, 5’-cgtttcctctgtctctct-3’) to arginine.

The C-terminal deletion of NFATc2 (1–460) was generated by inserting a stop codon into the open reading frame by using the following primers: 5’-gagagctctggctgagag-3’ and 5’-gagagctctggctgagag-3’. All mutations and deletions in NFATc2 were verified by DNA sequencing.

Subcellular Fragmentation, Co-immunoprecipitation, and Immunoblotting—Protein analyses were performed as described previously (12). The following antibodies were used for immunostaining. Monoclonal antibodies against cyclin D1, Cdk4, Cdk6, HA, and β-actin were purchased from Cell Signaling Technology (Danvers, MA). Anti-NFATc2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antiserum against lamin a/c, GSK-3β, and phospho-GSK-3β were obtained from Cell Signaling Technology. A polyclonal antisera against ubiquitin was purchased from Biomi (Biomi GmbH, Hamburg, Germany), a polyclonal antisera against phospho-glycogen synthase was from Cell Signaling Technology (Danvers, MA), and a polyclonal antisera against ORC-2 was from Upstate Biotech Millipore (Lake Placid, NY).

Immunohistochemistry and Fluorescence Microscopy—Cancer cells grown on chambered coverslips were transfected with the indicated constructs and treated with 10 μM ZOL or cycloheximide or left untreated for the indicated time periods. Cells were washed, fixed with 4% paraformaldehyde, blocked, and probed with anti-HA antibody (1:250; Cell Signaling) as described previously (13). Immunoreactive proteins were visualized with a fluorochrome-conjugated secondary antibodies, and nuclei were counterstained with 4’-diamino-2-phenylnidole (DAPI). Coverslips were mounted on glass slides and subjected to fluorescence microscopy (Carl Zeiss Inc., Oberkochen, Germany). To enlarge the axial resolution, we used the structured illumination microscopy (Carl Zeiss Inc.). Immunohistochemical analysis of tumors explanted from nude mice or human cancer tissues (provided by the Institute of Pathology, University of Marburg, Germany) was performed as described previously (13). Briefly, paraffin sections were stained with anti-NFATc2 (1:250; Santa Cruz Biotechnology), GSK-3β, phospho-GSK-3β (both 1:200), Cell Signaling), or anti Ki-67 (1:700, Thermo Fisher Scientific). Antibody binding was visu-
alized using a biotinylated secondary antibody, with avidin-conjugated peroxidase (ABC method; Vector Laboratories, Burlingame, CA) and 3,3′-diaminobenzidine tetrachloride as a substrate and hematoxylin as counter staining, and evaluated by standard light microscopy.

**Proliferation Assays and Flow Cytometry Analysis** — The indicated cell lines were seeded in 24-well plates, transfected with the indicated siRNA oligonucleotides, and treated with 10 μM ZOL or left untreated for the indicated time periods. [3H]Thymidine (0.5 μCi/well) was added during the last 6 h of incubation. Cells were washed with 5% trichloroacetic acid (TCA) and incubated with 1 M NaOH for 30 min at 37 °C. Radioactivity was determined with a scintillation counter. All proliferation assays were performed in triplicates in at least three independent experiments. For flow cytometry analyses, cells were seeded in 6-well plates and treated with ZOL (10 μM) for 72 h or left untreated. Cells were then resuspended in 1 ml of PBS containing 20 μl of propidium iodide (2 mg/ml) and 50 μl of RNase (100 µg/ml) for 3 h. The DNA content of 10^6 cells was analyzed with a BD Biosciences FACSCalibur flow cytometer using Cell Quest software from BD Biosciences.

**RT-PCR** — Cells were treated with ZOL (10 μM) for the indicated time periods. After treatment, total RNA was extracted using the RNaseasy mini kit (Qiagen), and first-strand cDNA was synthesized from 2 μg of total RNA using random primers and the SuperScript first-strand synthesis kit (Invitrogen) according to the manufacturer’s instructions. The quantitative RT-PCR was performed using a 7500 fast real-time PCR system from Applied Biosystems (Foster City, MA). Specific primer pairs were designed with the PrimerExpress 3.0 (Applied Biosystems, Wellesley, MA) system. The following primers were used for our studies: human NFATc2, forward, 5′-gtgcttaccacctgcatcatcag-3′, and reverse, 5′-cccgagtgattacctcttgttg-3′; HDM2, forward, 5′-gggagccgcatgaacctc-3′, and reverse, 5′-atccaaacatcctgaatgt-3′; XS-13, forward, 5′-gtcggaggagctgccaggag-3′, and reverse 5′-gcctttcttgcttggtgcaaa-3′.

**Luciferase Assays** — For reporter gene studies, 10^6 cells were seeded into 12-well tissue culture dishes and transfected after 24 h with the indicated constructs. Cells were treated with ZOL (10 μM) for 72 h before cells were harvested, and luciferase assays were performed using a Lumat LB 9501 luminometer (Berthold Technologies) and the Dual-Luciferase® reporter assay system (Promega). Firefly luciferase values were normalized to Renilla luciferase activity and were expressed either as relative luciferase activity or as mean “fold induction” with respect to empty vector control. Mean values are displayed ± S.D.

**In Vitro Kinase Assay** — Murine wild-type HA-NFATc2 construct was transfected in PaTu8988t cells, and 48 h after transfection, cells were lysed and immunoprecipitated using anti-HA antibody. To perform in vitro kinase assays, protein G-agarose-bound HA-NFATc2 proteins were incubated with recombinant GSK-3β (500,000 units/ml; New England Biolabs) for 30 min at 30 °C in a buffer containing 20 mM Tris HCl, pH 7.5, 10 mM MgCl₂, 5 mM DTT, 200 μM ATP (cold), and 3 μCi of [γ-32P]ATP (PerkinElmer Life Sciences). The beads were spun down, resuspended in an equal volume of 2× SDS-PAGE loading buffer, and boiled for 5 min. The proteins were resolved on SDS-PAGE, and the protein phosphorylation state was detected by autoradiography.

**Mouse Experiments** — 6–8-week-old pathogen-free female athymic nu/nu mice were obtained from Harlan Winkelmann (Harlan Winkelmann GmbH, Borchen, Germany). We injected 1 × 10⁶ IMIM-PC1 or MDA-MB-435 cancer cells mixed with Matrigel (BD Biosciences) subcutaneously into both flanks of nude mice. After the establishment of visible tumors, mice were randomized into two groups and received either 1 mg/kg of ZOL (i.p. three times a week) or 10% DMSO as vehicle control. Mice and tumor size were monitored weekly. The tumor volume (V) was determined by using the formula: (4/3π × (L/2) × (W/2) × (H/2)); (L = length, W = width, H = height). After 4 weeks of treatment, mice were sacrificed, and tumors were explanted and analyzed by size and weight and subjected to immunohistochemistry. Animal experiments were carried out using protocols approved by the Institutional Animal Care and Use Committee at the University of Marburg.

**Statistical Analysis** — Mean values and S.D. were calculated for all countable results. For statistical analysis, Student’s t test was used, and p < 0.05 was considered significant.

**RESULTS** — Zoledronic Acid Suppresses Cancer Growth through Induction of a G₁ Cell Cycle Arrest — Previous studies performed in cultured cell lines in vitro have shown that both breast and pancreatic cancer cells serve as suitable models for observing the growth-suppressing effect of zoledronic acid (10–11, 14). For this reason, we utilized a series of established human epithelial cancer cell lines derived from ductal pancreatic and breast cancers as models to search for cellular and molecular mechanisms underlying the growth suppressor activities of ZOL. Initially, we performed [3H]thymidine incorporation assays and flow cytometry analysis to determine the effect of ZOL on tumor cell growth in vitro. Treatment of cancer cells with ZOL (10 μM) led to a time-dependent reduction of cancer cell proliferation, which was evident after 24–48 h and reached maximum 72 h upon treatment (Fig. 1A). Depending on the tested pancreatic and breast cancer cell line, ZOL caused a 60–80% inhibition of cell proliferation (Fig. 1B). Flow cytometry analysis further showed that inhibition of cancer cell proliferation originated from induction of a G₁ cell cycle arrest, as evidenced by the shift of cancer cells from the S to the G₁ phase (Fig. 1C). Consistently, we found reduced expression levels of cyclin D1 and its partner kinases CdK4 and CdK6 over time (Fig. 1D). To assess whether tumor growth suppression by ZOL can be recapitulated in vivo, we carried out growth studies in a xenograft tumor model following injection of either IMIM-PC1 pancreatic cancer cells or MDA-MB-435 breast cancer cells into the flanks of athymic nude mice. We allowed the development of sizable subcutaneous tumors before we started with ZOL or DMSO control treatment (i.p. three times a week). Tumor volumes were measured once a week for a treatment period of 4 weeks, animals then were sacrificed, and tumor masses were extracted for volume measurement and immunohistochemical analyses. In congruence with our in vitro data, ZOL treatment caused a significant reduction of tumor mass over time (Fig. 1E, top panel; supplemental Fig. 1A), and this
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FIGURE 1. Zoledronic acid blocks cancer cell growth through induction of a G1 cell cycle arrest. A, time curve analysis of cell proliferation in human breast MDA-MB-231 cancer cells following treatment with ZOL (10 μM) for 24, 48, and 72 h. B, cell proliferation was determined by [3H]thymidine incorporation in human breast (MDA-MB-435 and MDA-MB-231) and pancreatic cancer cells (IMIM-PC1 and PaTu8988t) after treatment with ZOL (10 μM) for 72 h. Data are representative of triplicate experiments and displayed ± S.D. C, flow cytometry analyses performed in MDA-MB-435 breast cancer cells. Cells were left untreated or treated with ZOL (10 μM) for 72 h and analyzed by propidium iodide staining and flow cytometry. The percentages of cells in the G1, S, and G2 phases, respectively, are indicated. ZOL treatment results in a profound cell cycle arrest, as indicated by a shift from S to G1 phase. D, Western blot analysis of cyclin D1 and its associated kinases Cdk4 and Cdk6 following treatment with ZOL (10 μM) over time. E, mice bearing IMIM-PC1 cells were treated i.p. with either 1 mg/kg of ZOL (n = 6) or 1 mg/kg of DMSO (n = 6) for 4 weeks before mice were sacrificed and tumors were extracted. Top panel left shows an example of ZOL-treated and untreated mice tumors. Calculated tumor volumes are presented as mean tumor volume, and error bars represent S.E. (top right). The lower panel illustrates immunohistochemistry staining for the expression of the proliferation marker Ki-67 in ZOL-treated and untreated cancers.

growth retardation resulted from reduced cell proliferation, indicated by decreased Ki-67 staining (Fig. 1E, bottom panel). Together, the combined data from our in vitro and in vivo analysis suggested that G1 cell cycle arrest is a key cellular response underlying ZOL-induced cancer growth suppression.

ZOL Targets the Growth-promoting Transcription Factor NFATc2 for Proteasomal Degradation in Cancer Cells—ZOL controls cellular responses through modulation of intracellular Ca2+ fluxes (15). NFATc2 is a prototypic Ca2+-signaling regulated transcription factor with strong growth-promoting activities in cancer (12, 16). For instance, NFATc2 controls promoter induction of the c-Myc proto-oncogene and stimulates pancreatic cancer cell growth in response to increases in Ca2+ concentrations. We found high expression levels and a predominant nuclear localization of NFATc2 in both pancreatic and breast cancer tissues (supplemental Fig. 1B) and in the analyzed cancer cell lines (12). Consistent with a key role of NFATc2 in G1/S cell cycle progression, genetic depletion of the nuclear factor caused a profound reduction of cancer cell growth and diminished expression of G1/S-phase-promoting genes (supplemental Fig. 1, C and D). Initial experimental evidence supporting NFATc2 as a molecular target of ZOL in cancer was provided by reporter gene assays using an NFAT-responsive promoter construct (cis-NFAT) harboring three consensus binding sites for the transcription factor fused to the luciferase gene. Cancer cells were transfected with cis-NFAT reporter plasmids along with NFATc2 and treated with the bisphosphonate for 48 h. Interestingly, ZOL treatment caused a complete blockade of NFATc2 promoter transactivation, and this effect was associated with a dramatic and time-dependent reduction of NFATc2 protein expression levels in all cancer cell lines derived from the breast and pancreas (Fig. 2, A–C). Importantly, loss of NFATc2 expression upon treatment resulted from accelerated protein turnover of the transcription factor rather than inhibition of its gene transcription. Therefore, no significant changes in NFATc2 promoter activity (data not shown) or mRNA expression were found up to 48 h after treatment (Fig. 2D). However, application of MG-132, a cell-permeable and reversible inhibitor of proteasomal degradation, prevented NFATc2 down-regulation by ZOL, and this was paralleled by a strong ubiquitination signal of the factor with a characteristic ladder indicative of polyubiquitination (Fig. 2, E and F). Together, these studies demonstrated that ZOL modulates NFATc2 expression and activity in breast and pancreatic cancer cells and suggested that proteasomal degradation of the pro-proliferative factor is an underlying mechanism mediating cancer growth suppression by this bisphosphonate.

ZOL Inhibits GSK-3β Kinase-mediated NFATc2 Stabilization in Cancer Cells—Inactive NFAT proteins reside in the cytoplasm in a highly phosphorylated version. Dephosphorylation of the factor through the action of the Ca2+-regulated phosphatase calcineurin allows its shuttling into the nucleus, where NFAT binds target gene promoters for transcriptional regulation (17). NFAT activation can be reversed by phosphorylation through the action of distinct export kinases, e.g. GSK-3β, which induce the cytosolic translocation of the transcription factor so that it is poised for the next activating stimulus. Interestingly, an additional role of the export kinase GSK-3β in NFATc2 regulation and function has most recently been postulated in breast cancer, suggesting that the kinase might cooperate with the transcription factor to stimulate cancer cell migration (18). It is noteworthy that, consistent with a cooperative oncogenic function of both molecules in cancer, we found concomitant expression of NFATc2 and GSK-3β in cancer tis-
suces and cell lines (Fig. 3, A and B). In addition, genetic depletion of GSK-3β exerted an effect on NFATc2 expression and function similar to ZOL treatment, namely transcriptional inactivation and accelerated protein turnover of the transcription factor (supplemental Fig. 2, A–D). These findings emphasized a pro-proliferative activity of the GSK-3β-NFATc2 pathway in both cancer cell models and suggested that ZOL treatment targets this oncogenic pathway for inactivation. In fact, ZOL treatment inhibits GSK-3β activity in a dose-dependent manner both in vitro and in vivo, as evidenced by increased Ser-9 phosphorylation and/or reduced levels of phospho-glycogen synthase, and as a consequence of this, treatment caused a profound loss of endogenous NFATc2 in cultured cancer cells and in their corresponding xenograft tumors (Fig. 3, C–E; supplemental Fig. 2F).

To provide direct evidence of the effect of ZOL on GSK-3β kinase activity to phosphorylate NFATc2, an in vitro kinase assay was performed with recombinant GSK-3β and immunoprecipitated wild-type HA-NFATc2 in the presence or absence of ZOL. As shown in Fig. 2F, GSK-3β efficiently catalyzed the incorporation of phosphate into the NFATc2 substrate, whereas ZOL did not alter this kinase reaction, suggesting that ZOL indirectly blocks GSK-3β activity. Finally, introduction of a constitutively active GSK-3β version protected the GSK-3β-NFATc2 pathway from ZOL-induced disruption and hence prevented NFATc2 from proteasomal degradation (Fig. 3F). Thus, these findings emphasized that ZOL inhibits the GSK-3β-mediated signaling pathway under normal physiological conditions. Taken together, these studies revealed the existence of a pro-proliferative GSK-3β-NFATc2 phosphorylation and stabilization pathway in cancer and identified this pathway as a prime target of ZOL anti-tumor function.

**ZOL-induced NFATc2 Degradation Requires Dephosphorylation of GSK-3β-Phospho-serines at the SP2 Motif**—Next, we set out a bioinformatics-based analysis to identify putative GSK-3β phosphorylation sites within the NFATc2 sequence. These studies revealed three consensus GSK-3β serine phosphorylation residues located in the SP2 motif of the NFAT homology region (Fig. 4A). These phospho-serine residues, previously implicated in NFAT nuclear export, are highly conserved among species and match with the “phospho-degron” sequence, a key identification code for GSK-3β to label other transcriptional regulators (e.g. β-catenin, SRC-3, and Notch-1) for phosphorylation-dependent ubiquitination (19, 20). These data led us to hypothesize that GSK-3β also targets NFATc2 through conserved phospho-degron sequences: in this case, however, to stabilize the transcription factor in cancer cells. To verify this hypothesis, we generated mutations of murine NFATc2 pathway from ZOL-induced disruption and hence prevented NFATc2 from proteasomal degradation (Fig. 3F).
NFATc2 in which the phospho-degron elements were modified through substitution of phospho-serines for either alanine to obtain a non-phosphorylatable NFATc2 mutant (referred to as ΔSP2) or glutamic acid to generate a mutant that mimics constitutive phosphorylation by GSK-3β (referred to as pSP2), respectively (Fig. 4B). We then determined the significance of the GSK-3β phospho-serines for NFATc2 stability and inactivation by ZOL in cancer cells. The results shown in Fig. 4 demonstrate that wild-type NFATc2 behaved similar to its endogenous counterpart, and therefore, it was sufficiently ubiquitinated and metabolized following ZOL treatment (Fig. 4, E and F). Mutational inactivation of the GSK-3β-targeted phospho-serines Ser-215, Ser-219, and Ser-223 (ΔSP2) further increased susceptibility of NFATc2 and led to accelerated protein turnover and degradation of the transcription factor in cancer (Fig. 4, C and D). In addition, expression of a constitutively active version of GSK-3β was not able to prevent degradation of NFATc2 when present in its non-phosphorylatable form (data not shown). Most importantly, however, constitutive phosphorylation of the GSK-3β serine residues enhanced NFATc2 stability (pSP2) (Fig. 4, C and D; supplemental Fig. 3A) and rendered the factor fully resistant to ZOL-mediated polyubiquitination and degradation (Fig. 4, E and F), as evidenced by Western blot analysis, ubiquitination assays, and immunofluorescence microscopy. Together, these data demonstrate that GSK-3β-dependent phosphorylation of serine residues Ser-215, Ser-219, and Ser-223 stabilizes NFATc2. Moreover, these results indicate that dephosphorylation of these GSK-3β phospho-serines is required for ZOL-induced ubiquitination and proteasomal degradation of NFATc2 in cancer cells.

ZOL Triggers Ubiquitination of Unphosphorylated NFATc2—To investigate how ZOL induces ubiquitination of unphosphorylated NFATc2 in cancer, we searched its amino acid sequence for putative ubiquitin acceptor lysine residues. Protein sequence analysis revealed multiple lysine residues within the N-terminal regulatory domain. Consequently, to determine whether the N terminus is indeed the target for ubiquitination, cells were transfected with wt-NFATc2 or a deletion construct that encompasses the lysine-rich N-terminal half (1–460) (Figs. 4A and 5A). Unexpectedly, treatment with ZOL failed to induce ubiquitination and degradation of NFATc2 N terminus (1–460) (Fig. 5, A and B), indicating that this was not the region responsible for this molecular event. Subsequently, we focused on the C terminus, which displays two additional lysine residues (Lys-684 and Lys-897) that have most recently been identified as target sites for the small ubiquitin-related modifier (SUMO) (21). Because recent findings indicate that the small ubiquitin-like modifier and ubiquitin can actually target the same lysine residues of common substrates (22), we tested the relevance of Lys-684 and Lys-897 in NFATc2 ubiquitination by zoledronic acid. Although single point mutation of the first lysine residue (Lys-684) only marginally reduced the degradation of NFATc2 (Fig. 5C), introduction of double mutations to inactivate both lysines (NFATc2K684R/K897R) completely prevented ubiquitin transfer and consequently abolished degradation by zoledronic acid (Fig. 5D). In addition and most importantly, even non-phosphorylatable and highly susceptible NFATc2 that lacks protection by GSK-3β became resistant to ZOL upon mutational disruption of the two ubiquitin acceptor sites (ΔSP2/K684R/K897R), clearly underscoring the relevance
of Lys-684 and Lys-897 in ZOL-induced degradation of the factor (Fig. 5, E and F).

ZOL Induces HDM2 Ubiquitin E3 Ligase to Promote Degradation of Unphosphorylated NFATc2 in Cancer Cells—Ubiquitination of target lysines is accomplished through the action of E3 ligases, which conjugate ubiquitin to target proteins and thus label them for subsequent degradation by the 26 S proteasome. Because HDM2, the human homolog of the RING finger ligase MDM2 (murine double minute 2), has been recently shown to target NFATc2 for degradation, this E3 ligase was an obvious candidate for acting on NFATc2 during zoledronic acid-mediated tumor growth suppression (23). Interestingly, we found that HDM2 is highly induced by ZOL in cancer cells, where it serves as an E3 ubiquitin ligase for NFATc2. In fact, ZOL treatment enhanced the mRNA and protein levels of HDM2 in a dose-dependent manner in breast and pancreatic...
cancer cells displaying a progressive loss of NFATc2 expression
(Fig. 6, A and B). In line with a role of HDM2 in NFATc2 degrada-
tion, transfection of increasing amounts of HDM2 caused a
dose-dependent reduction of endogenous NFATc2 levels (Fig.
6C), and conversely, HDM2 silencing prevented the transcrip-
tion factor from ZOL-induced degradation, and as a conse-
quency of this, rescued cancer cells from growth suppression by
the compound (Fig. 6D; supplemental Fig. 3, B and C). Consis-
tent with our findings described above, the capacity of HDM2
to induce NFATc2 degradation was strictly dependent on the
level of NFATc2 phosphorylation, and therefore, NFATc2 was
resistant to HDM2 when present in its phosphorylated form
(Fig. 6E). Subcellular fractionation experiments defined the
nucleus at a candidate cellular site of HDM2-mediated
NFATc2 turnover upon ZOL treatment (Fig. 6F). Accordingly,
co-immunoprecipitation studies revealed HDM2-NFATc2
complex formation in the nucleus of ZOL-responsive cancer
cells (Fig. 6G). Surprisingly, however, this protein-protein

FIGURE 5. Degradation of unphosphorylated NFATc2 requires ubiquitination of Lys-684 and Lys-897. A and B, MDA-MB-231 cells were transfected with
the indicated NFATc2 expression constructs and treated with ZOL (10 μM) for 72 h. Total cell lysates were immunoblotted with anti-HA-NFATc2 or anti-β-actin
antibodies (A) or treated with ZOL (10 μM), MG132 (10 μM), or a combination of both agents (B). NFATc2 was precipitated using an anti-HA antibody, and
ubiquitination was detected with anti-ubiquitin antibodies. Total lysates were immunoblotted with anti-HA antibody to reveal the expression of transfected
NFATc2. IP, immunoprecipitation. C and D, cells were transfected with the indicated NFATc2 expression constructs and treated with ZOL (10 μM) for 72 h. Total
lysates were immunoblotted with anti-HA-NFATc2 or anti-β-actin antibodies (C) or treated with ZOL (10 μM), MG132 (10 μM), or a combination of both agents
(D) before immunoprecipitation was performed using anti-HA antibodies. Ubiquitination was detected using an anti-ubiquitin antibody. E, cancer cells were
transfected with the indicated HA-tagged NFATc2 constructs and treated with ZOL (10 μM) for 72 h. HA-NFATc2 expression upon treatment was detected by
immunoblotting. Note that unstable ΔSP2 becomes resistant to ZOL-mediated degradation upon inactivation of the ubiquitin acceptor lysines K684R/K897R.
F, immunofluorescence staining was performed upon transfection of PaTu8988t cells with the indicated NFATc2 constructs and subsequent treatment with
ZOL (10 μM). NFATc2 was visualized (in red) using anti-HA and Cy3-labeled antibodies, and transfection was controlled by EGFP co-transfection (in green). Nuclei
are visualized by DAPI (in blue). Magnification, 630×; detection time for Cy3, 250 ms; detection time for GFP, 100 ms; detection time for DAPI, 15 ms.
interaction was independent of the SP2 phosphorylation status, as evidenced by co-immunoprecipitation showing a sufficient interaction between HDM2 and NFATc2 even when present in its phosphorylated form (pSP2) (Fig. 6H). Together, these findings demonstrated that GSK-3β-mediated phosphorylation of the SP2 domain does not protect from HDM2 binding but clearly controls the functional consequence of this interaction, namely the subsequent ubiquitination and degradation of NFATc2 by the ligase.

Degradation of Unphosphorylated NFATc2 Is Required for ZOL-mediated Cancer Growth Suppression—To determine the biological relevance of NFATc2 degradation in ZOL-mediated cancer growth suppression, we generated two different pancreatic cancer cell lines (PaTu8988t and Suit-028 cells) with stable expression of either wild-type NFATc2 or ZOL-resistant pSP2 NFATc2 mutations. In line with a strong growth-promoting function of NFATc2 in cancer, [3H]thymidine incorporation assays showed increased cell proliferation in NFATc2 overexpressing cancer cells when compared with mock transfected controls. The growth-promoting effect of NFATc2 overexpression is illustrated for PaTu8988t cells in Fig. 7A. Moreover, treatment with ZOL caused a significant reduction of cell proliferation in controls and in wt-NFATc2 overexpressing Suit-028 cells (Fig. 7B), and to a similar degree, in PaTu8988t cells (data not shown). In contrast and most importantly, however, constitutive phosphorylation of the SP2 motif to prevent NFATc2 degradation not only caused increased cell proliferation but rendered cancer cells significantly less responsive to ZOL-induced growth suppression (Fig. 7B). Together, these experiments confirmed the functional relevance of the GSK-3β-NFATc2 stabilization pathway in cancer growth, and in addition, demonstrated that proteasomal degradation of NFATc2 is of utmost importance for successful cancer growth suppression by ZOL.

DISCUSSION

The present study reports, for the first time, a novel mechanism mediating the anti-tumoral effect of zoledronic acid. In particular, our investigations reveal that zoledronic acid works, at least in part, by blocking the GSK-3β-mediated phosphorylation-dependent NFATc2 stabilization, thus promoting its proteasomal degradation following HDM2-mediated polyubiquitination. Notably, we demonstrate that successful disruption of the GSK-3β-NFATc2 pathway by zoledronic acid under physiological conditions involves two key mechanisms: 1) inhibition of GSK-3β activity, which impairs NFATc2 phos-
phorylation at the SP2 domain, thereby increasing the levels of the unphosphorylated form of this protein; and 2) nuclear accumulation of HDM2, which targets this unphosphorylated NFATc2 for Lys-684 and Lys-897 ubiquitination and subsequent degradation. Notably, disruption of the GSK-3β-NFAT pathway by zoledronic acid is ultimately linked to growth suppression, as evidenced by \textit{in vitro} and \textit{in vivo} studies of cell growth and tumorigenesis in breast and pancreatic cancer. Collectively, these findings expand our understanding of how the GSK-3β-NFATc2-HDM2 pathway modulates cancer cell growth by coupling NFATc2 phosphorylation to degradation, as well as identify this signaling loop as an important target for zoledronic acid to inhibit neoplastic cell growth.

The fact that zoledronic acid inhibits GSK-3β activity has significant implications. GSK-3β is abnormally up-regulated and active in some types of epithelial and hematopoietic malignancies, is required for the maintenance of a specific subtype of human leukemia with poor prognosis, is genetically defined by mutations of the MLL proto-oncogene, and confers resistance to conventional chemotherapy in certain malignancies such as pancreatic cancer (24–26). Together with our findings, these studies underline a key mechanism by which zoledronic acid indirectly blocks GSK-3β activity, leading to destabilization of NFATc2. Therefore, our results demonstrate that zoledronic acid can be used as a potent therapeutic drug in cancer treatment.

Interestingly, zoledronic acid ultimately leads to the modulation of NFATc2 activity. NFATc2 is frequently expressed in epithelial and non-epithelial tumors (16, 27–30). For instance, chromosomal rearrangement is the cause of EWSR1 and NFATc2 fusion in a portion of Ewing sarcomas, the second most common and highly aggressive bone sarcoma in childhood (31). Breakpoint cloning has revealed an in-frame fusion between the EWSR1 transactivation domain and the NFATc2 DNA-binding domain through which the oncogene contacts target gene promoters. Moreover, genomic DNA-chip hybridization has revealed chromosomal amplification of the NFATc2 gene in the majority of pancreatic cancer tissues, as well as in fast-growing pancreatic cancer cells, in which NFATc2 confers mitogen-induced c-Myc promoter induction and growth (12, 32). These findings have spurred great interest in developing novel therapeutic strategies to target NFAT pathways. Cyclosporin A and FK506 show severe toxic side effects, and their long term administration is associated with cancer development, most likely due to suppression of tumor immunosurveillance mechanisms (35, 36). These side effects clearly prevent their application in cancer medicine. Therefore, new treatment strategies that specifically switch off NFAT signaling in tumor cells, without affecting immunosurveillance, are urgently needed. Based on our work, zoledronic acid fulfills these criteria through disruption of the pro-proliferative GSK-3β-NFATc2 pathway and subsequent induction of NFATc2 degradation. Although we have not analyzed the effects of zoledronic acid on the local or systemic immune response, it appears unlikely that application of the drug compromises immunosurveillance. In fact, with more than a million women treated worldwide, an increased incidence of tumor diseases upon long term application of zoledronic acid has never been reported (37). Therefore, our results demonstrate that pharmacologically, zoledronic acid can also be conceived of as a new NFAT inhibitor.

Consequently, it becomes important to provide a perspective regarding the scientific relevance of these results. For instance,
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medically, this work constitutes a highly detailed molecular study on the mechanism by which zoledronic acid achieves its tumor suppression activity. Alone, the interpretation of these results within this theoretical framework of human treatment suggests that the knowledge provided by this study significantly expands the understanding of the biochemical basis underlying one of the most promising and exciting new treatments for malignant disease. Biochemically, by outlining a defined pathway mediating the effects of zoledronic acid, we have discovered novel mechanisms for growth suppression that designate several molecules as potential additional targets for therapies that may complement zoledronic acid treatment. For instance, treatment with this drug leads to an accumulation of nuclear HDM2 and inhibition of GSK-3β activity to disrupt phosphorylation-dependent NFATc2 stabilization. HDM2 targets unphosphorylated NFATc2 for ubiquitin transfer and induces its proteasomal degradation, resulting in loss of NFAT transcriptional responses and a subsequent block of cancer cell growth. Consequently, this study should fuel future investigations on how zoledronic acid and similar newly designed compounds can also work via this pathway.

Collectively, the results of this study contribute to both expanding the basic biochemical knowledge of novel mechanisms for the regulation of the GSK-3β-NFATc2-HDM2 pathway as well as describing medical information of paramount importance to patients by providing a well characterized example of a pathway that underlies the beneficial effect of zoledronic acid.

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