Amplified lipid rafts of malignant cells constitute a target for inhibition of aberrantly active NFAT and melanoma tumor growth by the aminobisphosphonate zoledronic acid

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Nuclear factors of activated T cells (NFAT) are critical modulators of cancer cell growth and survival. However, the mechanisms of their oncogenic dysregulation and strategies for targeting in tumors remain elusive. Here, we report coupling of anti-apoptotic NFAT (NFAT2) activation to cholesterol-enriched lipid raft microdomains of malignant melanoma cells and interruption of this pathway by the aminobisphosphonate zoledronic acid (Zol). The pathway was indicated by capability of Zol to promote apoptosis and to retard in vitro outgrowth of tumorigenic melanoma cell variants through inhibition of permanently active NFAT2. NFAT2 inhibition resulted from disintegration of cholesterol-enriched rafts due to reduction of cellular cholesterol by Zol. Mechanistically, raft disruption abolished raft-localized robust store-operated Ca2+ (SOC) entry, blocking constitutive activation of protein kinase B/Akt (PKB) and thereby reactivating the NFAT repressor glycosynthetic kinase 3β (GSK3β). Pro-apoptotic inactivation of NFAT2 also followed reactivation of GSK3β by direct inhibition of PKB or SOC, whereas GSK3β blockade prevented Zol-induced NFAT2 inhibition and cell death. The rescuing effect of GSK3β blockade was reproduced by recovery of entire SOC/PKB/GSK3β cascade after reconstitution of rafts by cholesterol replenishment of Zol-treated tumorigenic cells. Remarkably, these malignant cells displayed higher cholesterol and lipid raft content than non-tumorigenic cells, which expressed weak SOC, PKB and NFAT2 activities and resisted raft-ablative action of Zol. Together, the results underscore the functional relevance of amplified melanoma rafts for tumor-promoting NFAT2 signaling and reveal these distinctive microdomains as a target for in vitro and in vivo demise of tumorigenic cells through NFAT2 inhibition by the clinical agent Zol.

Introduction

Ca2+-dependent nuclear factors of activated T cells proteins (NFAT1-4) are emerging as attractive targets for cancer therapy. These transcription factors drive expression of distinct sets of genes, associated with aggressive cancer phenotype (1,2). Consequently, one of the NFAT isoforms, i.e. NFAT2, readily transforms non-neoplastic cells and renders these cells tumorigenic when expressed in permanently active form (1,2). Pertinent to human pathology, constitutive nuclear localization and activation of NFAT2 and/or NFAT1 occurs in hematological malignancies (1,2), pancreatic (1–3), breast (1,2,4) and hepatocellular carcinomas (1,2,5), glioblastoma (6) and in malignant melanoma cells (7,8). Rare incidence of mutations in these isoforms (1,2) suggests linking of their hyper-activity to up-stream signal events that either drive to or retain NFAT in the nucleus. For instance, robust function of store-operated Ca2+ (SOC) channels, stimulating nuclear translocation of NFAT through the action of calcineurin phosphatase (1,2), has been reported in cancer types with NFAT abnormalities (9–13). Cancer cells also frequently display elevated basal activity of protein kinase B/Akt (PKB) (14), which is a negative modulator of the NFAT repressor glycosynthetic kinase 3β (GSK3β) (15,16). However, the exact mechanisms of oncogenic NFAT dysregulation and strategies for their targeting in tumors are largely unknown. In fact, the available blockers of NFAT activity, the calcineurin inhibitors cyclosporine A and tacrolimus, impair growth and survival of malignant cells in vitro, but suppress anti-cancer immunity and promote tumor development in vivo (1,2,17,18).

The structural and biochemical properties of cholesterol-enriched plasmalemmal microdomains (lipid rafts) result in selective retention of certain classes of membrane-associated molecules, such that rafts promote interactions between discrete subsets of signal intermediates and thereby signal transduction (19). In cancer cells, lipid rafts are viewed as important linchpins, from which signals essential for invasive growth, resistance to death-inducing stimuli and other malignant characteristics are launched (20,21). Thus, we reported that in malignant melanoma cells, hyper-activity of PKB is sustained by robust SOC-mediated Ca2+ entry, occurring in raft microdomains (22,23). Ablation of rafts with the membrane cholesterol-extracting oligosaccharide methyl-β-cyclodextrin interrupted the SOC/PKB axis and impaired survival of melanoma cells and their tumorigenicity in animals (22,23). In the present research, we address coupling of this raft-driven pathway via GSK3β to NFAT and feasibility of its targeting with the clinical agent zoledronic acid (Zol). Our study focused on Zol for the following reasons. Besides the efficacy against bone disorders including cancer-induced osteolysis, Zol has been reported to directly inhibit skeletal and extra-skeletal outgrowth of certain experimental malignancies through induction of apoptosis and/or growth arrest in neoplastic cells (24–29). In preclinical models of pancreatic and breast carcinoma models, Zol readily inhibited NFAT in both cancer cell types (28), though the precise mode of interference with tumor-promoting signaling by this drug is poorly understood. Nevertheless, it is known that at cellular level, Zol blocks the mevalonate pathway enzyme farnesyl diphosphate synthase and depletes cholesterol among other mevalonate pathway metabolites (24,29). Cholesterol depletion is expected to alter raft integrity, but capability of Zol to ablate rafts and thereby raft-driven signal events has not previously been explored. Here, we report amplification of lipid rafts in tumorigenic melanoma cell variants that maintains constitutive NFAT (NFAT2) activity through enhancement of raft-localized SOC, SOC-dependent PKB activity and PKB-mediated GSK3β blockade. By disrupting amplified rafts of these malignant cells but not microdomains of non-tumorigenic cells, Zol inactivates NFAT2 in vitro and in vivo that facilitates apoptosis and significantly attenuates melanoma tumor growth.

Materials and methods

Cell culture and plasmids

Murine melanoma cells (B16BL6-8, Kb30, JB/RH1) (9,22,30) were provided by Prof. E.Gorelik (University of Pittsburgh, Pittsburgh, PA) and human GA melanoma clone (31) by Prof. J.Gopas (Ben-Gurion University, Beer-Shea, Israel). GA-PLXR cells were established by treating GA cells with PLX4720 (3 μM) for 90 days. Cells were grown in RPMI medium supplemented by 10% bovine serum and antibiotics. Tissue culture reagents were purchased from ThermoFisher Scientific. We used human GA-LVU, a stable cell line expressing HA-tagged wild-type c-Jun (31) by Prof. J.Gopas and a stable cell line expressing HA-tagged wild-type caspase-3 (31) by Prof. J.Gopas. The luciferase reporter plasmid pGL3 basic (Promega) was used for determination of NFAT activity and cell viability. All experiments were performed using 10% TrypLE solution (ThermoFisher Scientific) and 10% bovine serum and antibiotics.

Abbreviations: GSK3β, glycosynthetic kinase 3β; HA, haemagglutinin; NFAT, nuclear factors of activated T cell; PKB, protein kinase B; SOC, store-operated Ca2+.

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from Biological Industries (Beth Haemek, Israel) and Zol and PLXL4720 from MedKoo Biosciences (Chapel Hill, NC). Plasmid encoding for the NFAT-driven firefly luciferase reporter (NFArLuc) was provided by Prof. G.R.Crabtree (Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA; Addgene 17870); Stim1-YFP and the haemagglutinin (HA)-tagged constitutively active NFAT2 constructs were provided by Prof. A.Rae (La Jolla Institute for Allergy and Immunology, La Jolla, CA; Addgene 11102 and 19754); the HA-tagged dominant-negative PKB construct was provided by Prof. W.R.Sellers (Novartis Institute for Biomedical Research, Cambridge, MA; Addgene 9006) and the HA-tagged dominant-negative and constitutively active GSK3β constructs were provided by Prof. K.Woodgett (Samuel Lunenfeld Research Institute, Toronto, Canada; Addgene 14755, 14754). NFAT2 (murine: TRCN0000008123; human: TRCN0000017333) and Stim1 (murine: TIS36190) small hairpin RNA (shRNA)-encoding plasmids were purchased from Sigma and Origene (Rockville, MD), respectively. Renilla luciferase construct was purchased from Promega (Madison, WI). All transfections were carried out using the Arrest-In reagent according to the protocol provided by the manufacturer (Open Biosystems, Huntsville, AL). Cells were maintained in serum-supplemented media for 24 h post-transfection and then under serum-free conditions for additional time intervals (48–96 h) detailed in the text. The amount of each plasmid was ~0.5 µg/0.3–0.5 × 10^5 cells, except anti-murine and anti-human shRNA constructs applied at ~0.8 µg/0.3–0.5 × 10^5 and ~1 µg/0.3–0.5 × 10^5, respectively, and Renilla luciferase construct applied at ~0.1 µg/0.3–0.5 × 10^5. Co-transfection of more than three distinct plasmids reduced specificity in all melanoma cell types, except B16BL6-8 cells (S.Fedida-Metula, unpublished observation). Therefore, such experiments (Figures 4 and 5) employed B16BL6-8 cells only.

Analyses of cell cycle, SOC function, cholesterol content and levels of liquid-ordered rafts by flow cytometry

Cellular DNA measurements were performed by staining cells, fixed in 70% ethanol, with propidium iodide (10 µg/ml; Sigma), as described (9,22). To assess SOC function, Fluor-4-AM-loaded (2.5 µM, 30 min, room temperature; Invitrogen, Eugene, OR) cells were suspended in Ca^2+-free Ringer solution (20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4, 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl_2, and 15 mM glucose). Samples were run for ~60 s, thapsigargin (2 µM; Sigma) was added and CaCl_2 (2 mM) applied at the end of thapsigargin-induced fluorescence peak, as described (9,23). Instantaneous fluorescence changes were normalized to the resting values before thapsigargin addition and plotted. For cholesterol measurements, cells fixed in 4% of paraformaldehyde (Sigma) were probed to filipin (20 µg/ml; Sigma), as described (33) and its tumorigenic GA-PLXR variant (GA-PLXR, GA) cells in 0.03 M but not lower doses of Zol led to constructs were provided by Prof. K.Woodgett (Samuel Lunenfeld Research Institute, Toronto, Canada; Addgene 14755, 14754). NFAT activity was expressed as a ratio of NFArLuc firefly and Renilla luciferase luminescent signals.

Preparation of total cellular extracts and sub-cellular fractions for western blotting and dot blot analyses

Total cellular extracts were prepared, as described (22), and subjected to western blotting analysis using anti-NFAT1 (1:200; #7296, Santa-Cruz Biotechnology, Santa-Cruz, CA), anti-NFAT2 (1:200; #7094, Santa-Cruz), anti-NFAT3 (1:200; #8405, Santa-Cruz), anti-NFAT4 (1:200; #721597, Santa-Cruz), anti-p-Akt (1:800; #4068, Cell Signaling), anti-p-GSK3β (1:1000; #9832, Cell Signaling), anti-PK-B (Akt1) (1:200; #5298, Santa-Cruz), anti-GSK3β (1:1000; #9336, Cell Signaling), anti-α-Actinin (1:500; #7392, Santa-Cruz) and anti-actin (1:5000; #69010, MP Biomedicals, Aurora, OH) antibodies. Band intensities were assessed using the ImageJ software. For sub-cellular fractionation (23), cells suspended in ice-cold buffer (1% Triton X-100, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 6.9, 100 mM NaCl, 2 mM ethylenediaminetetraacetic acid, 0.2 mM sodium orthovandate and protease inhibitors) were homogenized using a Dounce glass homogenizer at ice. Large debris and nuclei were removed by centrifugation (400g, 5 min, 4°C). Clear supernatant (0.3 ml) were mixed with equal volume of lysis buffer containing 85% sucrose (vol/ vol), overfilled with 1 ml of 35% sucrose and 0.3 ml of 5% sucrose. Following ultracentrifugation (200 000 g, 16 h, 4°C), nine fractions (0.2 ml each) were collected from the top of the gradient and subjected to western blotting and dot blot analyses using anti-Stim1 antibodies (1:500; #52458, Abcam, Cambridge, UK) and horseradish peroxidase-conjugated cholaera toxin B subunit (CTXB) (5 µg/ml; Merck, Schwalbach, Germany), respectively.

Fluorescence confocal microscopy analysis of NFAT2 compartmentalization

For NFAT2 studies, cells seeded onto glass cover-slips and fixed in 4% paraformaldehyde (10 min, room temperature), permeabilized in phosphate-buffered saline containing 0.1% of Triton X-1 (1 min, room temperature) and probed to anti-NFAT2 antibodies (1:100, 16 h, 4°C; #2796, Abcam) and cy2-conjugated secondary antibodies (1:200, 3 h, 4°C; Jackson ImmunoResearch, PA). Samples were counter-stained with 7-aminoactinomycin-D (Invitrogen) and analyzed using the LSM510 system (480 excitation laser, 505–545 band pass (cy2) and 560 long pass (7-aminoactinomycin D) filters) (Carl Zeiss, Jena, Germany).

Statistical analysis

All analyses were performed using the SPSS software (SPSS for Windows, Version 15.0., SPSS, Chicago, IL). Student’s t-test was applied to calculate statistical significance of differences between parameters measured in experimental samples versus controls at time intervals indicated for each particular experiment.

Results

Zol promotes apoptosis in cultured and animal-engrafted tumorigenic melanoma cells and retards melanoma tumor growth

Since tumorigenic melanoma cells can grow in the absence of serum and resist apoptosis triggered by serum starvation (32), we assessed melanoma-suppressive activity of Zol by testing its effect on growth and survival of selected melanoma lines under serum-supplemented and serum-free conditions. For this purpose, we employed murine tumorigenic (B16BL6-8, JB/RH1) and non-tumorigenic (KB3) melanoma cells (22,30). Our study also utilized human non-tumorigenic GA melanoma line (31) and its tumorigenic GA-PLXR variant (Figure 1D and data not shown). The latter cells were generated by treating GA cells with the Braf inhibitor PLX4720 (3 mM, 90 days) and were insensitive to PLX4720-induced cytotoxicity (Supplementary Figure 1, available at Carcinogenesis Online). Concentrations of Zol were in range of 10–50 µM, sufficient for eliciting biological response in cell types other than melanoma (24–26,28,29,33). Figure 1A shows steady increase of cell count in serum-supplemented and serum-starved cultures of tumorigenic cells, whereas deprivation of serum arrested growth of KB3 cells at 72 h and markedly retarded growth of GA cells. Application of 50 µM but not lower doses of Zol led to ~2-fold reduction of cell counts in serum-supplemented B16BL6-8, JB/RH1 and GA-PLXR cultures, but not in KB3 and GA cultures, at 72 h of treatment (Figure 1A). The effect was more prominent under serum-starved conditions, as it became apparent at 48 h and reached the maximum of ~5-fold inhibition compared to control 72 h.
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upon Zol treatment. The observed reduction of cell counts was paralleled by accumulation of cells with sub-G1 DNA content (apoptotic cells), the percentages of which were approximately two times higher in serum-starved than in serum-supplemented samples (Figure 1B).

No consistent changes in G1, S and G2/M phase distribution accompanied Zol-induced apoptosis under serum-starved conditions, while...
a moderate delay in G\textsubscript{1} phase progression in serum-supplemented cultures was unavoidably followed by appearance of sub-G\textsubscript{1} cells (Figure 1B). The results indicated that impaired survival manifested by apoptosis and compromised resistance to deprivation of serum-derived growth factors was a key response of tumorigenic cells to Zol. Apoptotic nature of cell death was confirmed by detection of collapsed inner mitochondrial membrane potential (ΔΨ\textsubscript{m}) and DNA fragmentation (Supplementary Figure 2, available at Carcinogenesis Online). To determine the implications of pro-apoptotic action of Zol for \textit{in vivo} melanoma growth, we employed isograft and xenograft tumor models. Murine (B16BL6-8, Kb30) and human (GA-PLXR, GA) cells were subcutaneously inoculated into the footpads of C57BL/6 and Balbc nu/nu\textsuperscript{mice, respectively. Neither Kb30 nor GA cells generated tumors in animals under our experimental conditions (data not shown). B16BL6-8 and GA-PLXR cells formed visible melanin-producing lesions at day 7 after cancer cell implant when vehicle (saline) or Zol (0.1 and 1 mg/kg, twice a week, intraperitoneal) was administered. Both doses were in agreement with sub-acute toxicity data (34) and previous reports on anti-cancer activity of Zol in experimental malignancies (24–28). Tumor development was monitored by measuring foot diameter until day 28, when lesions were excised for histochemical evaluation of apoptosis. Remarkably, 1 mg/kg of Zol strongly attenuated melanoma development, as reflected by slower growth and significantly smaller sizes of tumors compared to the control group (Figure 1C). Furthermore, small tumors from Zol-treated animals displayed higher extent of apoptotic TUNEL staining relative to large tumors from control mice (Figure 1D). Together, the results underscore capability of Zol to promote apoptosis in cultured and animal-engrafted tumorigenic melanoma cells and suggest that apoptotic response mediates suppression of melanoma tumor growth by this drug.

Zol promotes apoptosis by abolishing constitutively nuclear localization and activation of the NFAT2 isoform of NFAT in tumorigenic melanoma cells

Recent study implicated Zol in modulation of NFAT (28). Since these transcription factors elicit pro-survival function in many cancer types (1,2), we asked whether Zol promotes apoptosis by inhibiting NFAT. By transfecting cells with the NFAT-driven luciferase reporter construct (NFATLuc) and assaying luciferase 48 h thereafter (Figure 2A), we recorded >4-fold higher basal NFAT activity in tumorigenic than in non-tumorigenic melanoma variants (Figure 2B). Application of Zol (50 μM, 24 h) reduced this enhanced NFAT activity to the levels detected in non-tumorigenic cells but did not alter weak NFAT function in the latter cells (Figure 2B; Supplementary Figure 3, available at Carcinogenesis Online). Western blotting analysis revealed that NFAT2 dominated over other NFAT isoforms and was expressed at comparable levels in all melanoma variants with no protein loss in Zol-treated tumorigenic cells (Figure 2C). By confocal microscopy, NFAT2 was detected mostly in the nuclei of these but not Kb30 and GA cells, and, consistently with inactivation of NFAT reporter, relocated to the cytosol upon Zol treatment (Figure 2D). Nuclear localization of NFAT2 was also evident in B16BL6-8 and GA-PLXR tumors from vehicle-treated animals, while Zol-treated tumors displayed predominantly cytosolic NFAT2 staining (Figure 2E). The results suggested that Zol reduced elevated basal NFAT activity in tumorigenic cells by abolishing constitutively nuclear localization of the NFAT2 isoform of NFAT. Indeed, silencing of NFAT2 expression in murine tumorigenic cells mimicked Zol-induced inhibition NFAT reporter and apoptosis 48 and 72 h after transfection of NFAT2 shRNA, respectively (Figure 3A and C, Supplementary Figure 4, available at Carcinogenesis Online). Inactivation of NFAT and apoptosis also followed NFAT2 silencing in GA-PLXR cells 72 and 96 h post-shRNA transfection, respectively (Figure 3B and D; Supplementary Figure 4, available at Carcinogenesis Online). On the contrary, over-expression of the constitutively active NFAT2 mutant (Figure 3E–G), indicating that hyper-active NFAT2 promotes survival of tumorigenic cells and its inhibition mediates pro-apoptotic action of Zol in our melanoma model. Consistently, NFAT2 silencing did not alter weak NFAT activity and survival in non-tumorigenic cells, both control and shRNA-transfected samples of which underwent apoptosis 72 h (Kb30 cells) and 96 h (GA cells) upon serum starvation (Figure 3A–D; Supplementary Figure 4, available at Carcinogenesis Online). We addressed the relevance of our findings for human pathology and assessed sub-cellular compartmentalization of NFAT2 in melanoma specimens from patients. Nuclear immune reactivity of this protein was apparent in five of six specimens (Supplementary Figure 5, available at Carcinogenesis Online), implying its constitutive activation and feasibility of targeting by Zol in proportion of human melanoma cases.

Constitutive activation of NFAT2 is sustained in the SOC-driven double-negative PKB/GSK3β pathway, up-regulated and sensitive to inhibition by Zol in tumorigenic cells

NFAT2 can be excluded from the nuclei and inactivated through the action GSK3β, which constitutes a substrate for inhibitory phosphorylation by PKB (15–17). Using western blotting analysis and antibodies, reactive to the S\textsuperscript{9}S phosphorylated GSK3β (inactive, pGSK3β) and S\textsuperscript{9}A phosphorylated PKB (active, pAkt), we detected up-regulation of both phosphoproteins in cultured tumorigenic cells and a ~6-fold reduction of their levels by NFAT2-inhibitory dose of Zol (50 μM, 24 h) (Figure 4A; Supplementary Figure 6, available at Carcinogenesis Online). Similarly, histochemical analysis revealed diminished pGSK3β and pAkt immune reactivity in melanoma lesions from Zol-treated but not control animals (Figure 4B). Downregulation of pGSK3β also followed over-expression of the K179M dominant-negative PKB mutant (DN-Akt) in tumorigenic cells for 48 h (Figure 4C), suggesting that Zol inhibits NFAT2 through elimination of GSK3β repression by permanently active PKB. Indeed, over-expression of the S9A constitutively active (CA-GSK3β) GSK3β mutant or DN-Akt in tumorigenic B16BL6-8 cells for 48 h reproducibly inhibited the inhibitory effect of Zol on NFAT reporter and nuclear retention of NFAT2 (Figure 4C–F). Inactivation of NFAT2 in Zol-treated (24 h) and DN-Akt-transfected (48 h) cells was prevented by overexpression of the K85A dominant-negative (DN-GSK3β) GSK3β mutant (Figure 4C–F), DN-GSK3β also rescued Zol-treated (48 h) and DN-Akt-transfected (72 h) B16BL6-8 cells from apoptosis, whereas CA-GSK3β by itself evoked cell death at 72 h (Figure 4C and G), thus, confirming the proposed action of Zol on the double-negative PKB/GSK3β/NFAT2 cascade. Since hyper-activity of PKB in melanoma is promoted by robust function of the SOC (9,22,23), we examined if Zol alters SOC-mediated Ca\textsuperscript{2+} influx. By monitoring cytosolic Ca\textsuperscript{2+} changes after SOC-stimulating depletion of intracellular Ca\textsuperscript{2+} stores with the sarco/endoplasmic reticulum ATPase blocker thapsigargin and addition of extracellular Ca\textsuperscript{2+}, we recorded SOC-evoked Ca\textsuperscript{2+} rises of significantly higher rates and amplitudes in tumorigenic than in non-tumorigenic cells, and their ~3.5- to 4.5-fold inhibition by Zol in tumorigenic cells only (Figure 5A). Direct blockade of enhanced SOC function in tumorigenic B16BL6-8 cells by silencing the SOC regulator Stim1 inactivated PKB, reactivated GSK3β and inhibited NFAT2 48 h after transfection of Stim1 shRNA (Figure 5B–E). Furthermore, 72 h post-shRNA transfection, ~50% of cells underwent apoptosis (Figure 5F), as it occurred upon application of CA-GSK3β, DN-Akt or Zol. In contrast, co-transfection of Stim1 shRNA with DN-GSK3β did not alter NFAT2 activity and cell viability (Figure 5C–F), indicating that anti-apoptotic constitutive NFAT2 activity is sustained in a SOC/PKB/GSK3β pathway, up-regulated in tumorigenic cells and sensitive to SOC inhibition by Zol.

Up-regulation of the SOC/PKB/GSK3β/NFAT pathway is mediated by amplified tumorigenic cell lipid rafts, which are vulnerable to disintegration by Zol

Activation of SOC in malignant melanoma cells is promoted by Stim1 recruitment to and aggregation in cholesterol-enriched lipid rafts upon depletion of intracellular Ca\textsuperscript{2+} stores (23). Therefore, we examined if Zol inhibits the SOC by depleting cellular cholesterol and thereby altering raft integrity. Cholesterol measurements were
Conducted using the fluorescent cholesterol probe filipin and flow cytometry (23). For quantitative raft analysis, we employed the fluorescent di-4-ANEPPDHQ (ANE) probe, which emits distinct signals from ordered (560 nm, raft) and disordered (620 nm, non-raft) membrane phases (35). The degree of ordered microdomains was calculated as a ratio between these signals, flow cytometrically recorded at two separate channels (Supplementary Figure 7, available at Carcinogenesis Online). Raft integrity was also studied by analyzing distribution of the glycolipid raft marker GM1 in the low-density (raft-enriched) versus high-density (non-raft) sub-cellular fractions isolated by ultracentrifugation in a gradient of sucrose. Using this paradigm, we detected ~1.5- to 2-fold higher cholesterol (Figure 6A) and raft levels (Figure 6B) in tumorigenic versus non-tumorigenic melanoma cells. Application of Zol (50 µM, 24 h) reduced cholesterol content of both cells types but, remarkably, decreased lipid raft levels (Figure 6A and B) and triggered relocation of GM1 from the low-density to high-density fractions (Figure 6C) in tumorigenic cells only. The results indicated amplification of tumorigenic cell rafts, which, most probably due to high cholesterol content, are particularly sensitive to cholesterol-depleting action of Zol. Indeed, raft integrity was reconstituted by replenishing Zol-treated tumorigenic cells with exogenous cholesterol (50 µg/ml, 1 h), as reflected by recovered ANE fluorescence and GM1 distribution in the sucrose gradient (Figure 6A–C). Consistently, cholesterol replenishment restored Stim1 recruitment to raft-enriched fractions (Figure 6D) and formation of GM1-co-localized Stim1 clusters (Supplementary Figure 8, available at Carcinogenesis Online), altered by Zol in thapsigargin-stimulated B16BL6-8 cells. These cholesterol-replenished cells also re-established robust SOC function, up-regulated pAkt and pGSK3β expression, retained NFAT2 in their nuclei and displayed diminished apoptosis (Figure 6E–H) compared to non-replenished Zol-treated samples. The rescuing effect of the cholesterol replenishment on NFAT2 and cell survival was blunted by CA-GSK3β (Figure 6G and H), consistently with proposed GSK3β-mediated link of hyper-active NFAT2 to raft-dependent enhanced SOC and PKB events in tumorigenic cells. In contrast in non-tumorigenic Kb30 cells, Stim1 expression and clustering were barely detectable in the rafts and not affected by Zol (Figure 6D, Supplementary Figure 8, available at Carcinogenesis Online). Together, our data underscore the functional relevance of amplified melanoma rafts for sustained activation of the SOC/PKB/GSK3β/NFAT2 cascade in tumorigenic cells and reveal these distinctive microdomains as a novel target for...
Fig. 3. Inactivation of NFAT2, either directly or by Zol, is sufficient for evoking apoptosis in tumorigenic melanoma cells. (A) Protein levels of NFAT2 (left panel) and NFAT-driven luciferase activity (right panel) were determined in murine melanoma cells at indicated time intervals after co-transfection of NFATluc with NFAT2 shRNA or shRNA control constructs. Western blotting analysis (100 µg of protein per lane) and luciferase assay were applied, respectively. (B) NFAT2 expression (left panel) and NFAT activity (right panel) in human melanoma cells at indicated time intervals after co-transfection of NFATluc with NFAT2 shRNA or shRNA control. In (A) and (B), luciferase assay was performed when decrease in NFAT2 levels became apparent, i.e. 48 and 72 h post-shRNA transfection into murine and human melanoma cells, respectively. Luciferase assay data were expressed as means + SD of triplicates in three (murine cells; n = 9) and two (human cells; n = 6) independent experiments. (C and D) Samples of murine (C) and human cells (D) described in (A) and (B) were utilized for cell cycle analysis at indicated time intervals post-transfection. Left panels: typical cell cycle distribution histograms obtained in one experiment of three performed in triplicates for murine and human cells. Right panels: percentages of cells with sub-G1 DNA content and cells in G1, S, and G2/M phases of cell cycles (mean + SD; n = 9). (E) Left panel: layout of experiments shown in (F and G). NFATluc was co-transfected with construct encoding for the HA-tagged constitutively active NFAT2 mutant (CA-NFAT2) and 24 h thereafter treated with 50 µM of Zol for indicated time intervals. Right panel: expression of the CA-NFAT2 transgene was determined 48 h post-transfection by western blotting analysis. (F and G) NFAT activity (F; 48 h) and cell cycle analysis (G; 48 and 72 h) were assessed in thereby treated cells. Both assays were performed in triplicates and data expressed as means + SD obtained in three independent experiments (n = 9). *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test.
Fig. 4. Constitutive activation of NFAT2 is sustained in a double-negative PKB/GSK3β pathway. (A) Upper panel: western blotting analysis of pGSK3β and pAkt expression in Zol-treated (50 µM, 24 h) and control melanoma cells. Each blot (50 µg of protein/lane) represents one of three independent experiments performed. Lower panel: quantification of pGSK3β and pAkt band intensities normalized to actin. Data are expressed as mean ± SD of three independent experiments (n = 3). (B) Paraffin-embedded sections of tumors collected from Zol-treated (1 mg/kg) and control animals (D) were subjected to immunohistochemistry with anti-pGSK3β and anti-pAkt antibodies. Typical images of pGSK3β and pAkt immune reactivity are shown. (C) Left panel: western blotting analysis (20 µg of protein per lane) of pGSK3β and pAkt expression in melanoma cells transfected with the HA-tagged dominant-negative PKB mutant 48 h post-transfection. Right panel: quantification of pGSK3β and pAkt band intensities normalized to actin. Data are expressed as mean ± SD of three independent experiments (n = 3). (D) Left panel: layout of experiments depicted in (E–G). B16BL6-8 cells were transfected with indicated plasmids and assayed at depicted time intervals. Right panel: western blotting analysis of expression of the HA-tagged GSK3β mutants and the DN-Akt mutant performed 48 h post-transfection of appropriate plasmids. (E–G) NFAT-driven luciferase activity (E), NFAT2 compartmentalization (F) and cell cycle distribution (G) were determined, using luciferase assay, confocal microscopy and flow cytometry, respectively. Data of luciferase assay (E) and cell cycle analysis (G) are expressed as mean ± SD of three independent experiments performed in triplicates (n = 9). (E) Typical fluorescent images acquired in one experiments of three performed are shown. Magnification ×100; scale bar 20 µm. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test.
inhibition of dysregulated NFAT2 by the clinically available generic drug Zol.

Discussion

Figure 7 summarizes two principal findings of our study. First, we revealed a novel pathway, in which amplified cholesterol-enriched rafts of malignant melanoma cells promote anti-apoptotic constitutive activation of NFAT2 through enhancement of SOC- and SOC-dependent PKB activities, maintaining repression of the negative NFAT modulator GSK3β. Second, high sensitivity of amplified melanoma rafts to cholesterol-depleting action of Zol enables inhibition of NFAT2 by interrupting the above pathway with this agent that facilitates apoptosis in malignant cells and retards their in vivo outgrowth. The pathway was indicated by the mechanism of suppressive action of Zol on murine and human tumorigenic melanoma cell variants. Striking correlations between over-activation of NFAT2 in these malignant cells and their vulnerability to Zol and between Zol-induced apoptosis and NFAT2 inhibition (Figures 1 and 2) suggested that dysregulated NFAT2 elicits pro-survival function and constitutes
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a target for Zol in melanoma. The rescuing effect of the constitutively active NFAT2 mutant on NFAT function in Zol-treated cells and the fact that NFAT2 inhibition did not result from its protein loss but, rather, from nuclear exclusion (Figures 2 and 3) pointed to interruption of signal events up-stream of NFAT2. Based on the following findings (Figures 4–6), we concluded that nuclear exclusion of NFAT2 by Zol ultimately resulted from elimination of PKB-mediated repression of GSK3β due to disintegration of lipid rafts and subsequent inhibition of SOC. Namely, (i) Zol destabilized raft integrity, abolishing raft recruitment of Stim1 and thereby Ca\(^{2+}\) influx via the SOC. This inactivated PKB and reactivated GSK3β. (ii) Reactivation of GSK3β, either directly or by blocking SOC or PKB, reproduced the inhibitory effect of Zol on NFAT2 and cell survival. Similar extent of NFAT2 inhibition and apoptosis, triggered by each of the above manipulation, further indicated that the lipid raft-, SOC- and PKB-driven repression of GSK3β maintained NFAT2 in constitutively active state without a need for concomitant stimulation through canonical calcineurin pathway. Finally, (iii) reconstitution of microdomains restored SOC, PKB, GSK3β and NFAT2 activities to their basal levels and diminished apoptosis, confirming that loss of raft integrity was a trigger for Zol’s mechanism of action.

Fig. 6. Up-regulation of the SOC/PKB/GSK3β/NFAT pathway is mediated by amplified tumorigenic cell lipid rafts, which are vulnerable to disintegration by Zol. (A and B) Melanoma cells were treated with Zol (50 mM, 24 h) and then replenished with cholesterol (50 µg/ml) for additional 1 h; cellular cholesterol content (A) and degree of ordered rafts (B) were flow cytometrically determined using filipin and ANE staining, respectively. Left panels: typical histograms obtained in one experiment of three performed in triplicates. Punctuated lines, corresponding to median of filipin and ANE ratio fluorescence peaks in non-tumorigenic cells, were added to illustrate that both parameters are increased in tumorigenic versus non-tumorigenic cells. Right panels: means of filipin fluorescence intensity or ANE ratio values ± SD (n = 9). (C–G) Cells were treated as described in (A). Total B16BL6-8 and Kb30 cell extracts were fractionated by ultracentrifugation in a gradient of sucrose and fractions subjected to dot blot (C) and western blotting analyses (D) of distribution of GM1 and Stim1 in sub-cellular fractions, respectively. Each blot represents one of three independent experiments performed. (E–G) The effects of cholesterol replenishment on Zol-treated B16BL6-8 cells. (E) The effect on SOC function. Left panel: changes of mean of [Ca\(^{2+}\)]\(_{\text{cyt}}\) ± SD in three independent experiments performed in triplicates. Right panel: bar graphs show means ± SD of SOC entry rates (n = 9) measured in three independent experiments. (F) The effect on pGSK3β and pAkt expression. Left panel: each blot represents one of three independent experiments performed. Right panel: quantification of pGSK3β and pAkt band intensities normalized to actin. Data are expressed as mean ± SD of three independent experiments. (G) The effect on NFAT2 compartmentalization. Magnification ×100; scale bar 20 µm. (H) The effect on cell survival. Cells were treated by Zol, as described in (A), except Zol-containing medium was returned to culture after cholesterol replenishment for additional 24 h. Some samples were transfected with CA-GSK3β 24 h before Zol treatment. Cell cycle was analyzed by flow cytometry and data expressed as mean ± SD of three independent experiments performed in triplicates. *P < 0.05, **P < 0.01, ***P < 0.001, Student’s t-test.
for down-regulation of the entire pathway in Zol-treated tumorigenic melanoma cells.

The proposed mechanism of NFAT2 dysregulation in melanoma is supported by several lines of evidence. For instance, a series of histopathology reports demonstrated repression of GSK3β in tumors, displaying elevated PKB and NFAT activities. Examples include melanoma (7,14,36), lymphoma (1,14,37) and carcinomas of breast and hepatocellular origins (1,14,38,39). Consistently, studies in benign osteoclasts (16) and renal carcinoma cells (40) highlighted the capability of ectopically expressed hyper-active PKB to maintain GSK3β-dependent nuclear retention and activation of NFAT2, similarly to the action of endogenous hyper-active PKB in our melanoma model (Figure 4). Furthermore, triggering of PKB-induced inhibitory phosphorylation of GSK3β by cytosolic Ca^{2+} rises revealed in neurons (41) is in agreement with persistent GSK3β repression by permanently active PKB, coupled in tumorigenic melanoma cells to robust function of the SOC [Figure 5 and (9,22,23)]. NFAT2 is a prototypic down-stream effector of the SOC (1), the predominant Ca^{2+} entry route in all non-excitable cells, stimulated by many membrane receptors and mediated by depletion of intracellular Ca^{2+} stores (42). A multitude of studies implicated strong Ca^{2+} fluxes through these channels in aggressive cancer cell growth, invasion and resistance to apoptotic stimuli (9–13,19,43–45). Up-regulation of the Stim1 and Orai1/3 protein constituents of the SOC have been evidenced in certain malignancies (10–13,43), but, importantly, increased levels of each particular constituent do not necessarily mirror tumor-promoting acceleration of Ca^{2+} influx, as we (9) and Motiani et al. (10) reported in melanoma and glioblastoma, respectively. On the contrary, over-expression of Orai1 without concomitant over-expression of Stim1 reduces channel sensitivity to store-depleting signal and thereby inhibits the SOC and cancer cell growth (46). Notably, due, in part, to abnormal accumulation of cholesterol, many types of transformed cells express increased amounts and changed composition of cholesterol-enriched lipid raft microdomains (20,47–49). Liquid-ordered rafts can modulate the SOC by limiting lateral diffusion and promoting interactions between different SOC-forming proteins, recruited to these microdomains (50). Specifically, Stim1 might be targeted to the rafts, where its binding to Orai and TRPC channel constituents triggers Ca^{2+} permeation (50). Dysregulation of SOC and NFAT by modified lipid rafts of malignant cells are, therefore, predictable events, though this issue has not been explored in any cancer type. Thus, not only do we show now that cholesterol-enriched rafts are highly expressed in tumorigenic versus non-tumorigenic melanoma cell variants. Most importantly, our data provide for the first time evidence for enhanced Stim1 recruitment to these amplified rafts that augments rates and amplitudes of SOC-evoked Ca^{2+} rises, linked via PKB and GSK3β to anti-apoptotic sustained activation of NFAT2 (Figures 5 and 6).

Clearly, a number of questions regarding this pathway remain unresolved. At present, we do not know which of the raft-associated molecules are enriched and increase docking sites for Stim1 in expanded microdomains of tumorigenic cells. It yet remains to be determined whether these molecules are Stim1-binding phosphoinositide lipids (51) and/or protein components of Ca^{2+} permeation pore of the SOC (50), or seiptin GTPases, which drive formation of Orai1 complexes with Stim1 at the plasma membrane (52). We also do not know how tumorigenic melanoma cells maintain their high cholesterol and lipid raft content. Remarkably, cytosolic Ca^{2+} rises and PKB by themselves promote cholesterol synthesis through up-regulation of lipogenic enzymes (53,54). If so, it would be of the particular interest to examine whether a positive feedback loop between SOC-dependently activated PKB and biogenesis of amplified rafts exists and boosts pro-survival SOC/PKB/GSK3β/NFAT2 signaling in tumorigenic cells. Pertinent to human pathology, such a loop could be the part of aggressive melanoma phenotype, associated with insensitivity to pharmacological inhibition of BrAraf. Approximately 50% of melanomas harbor oncogenically mutated BrAraf, which co-operations with PKB (55) and NFAT (15,16) in its tumor-promoting activities. Tumors, containing BrAraf mutations, respond to BrAraf inhibitors but shortly become drug resistant, whereas deregulation of PKB has been revealed as one of the core resistance pathways (56). It is also noteworthy that lipid raft- and Ca^{2+}-dependent activation of PKB has been reported in neoplastic cells other than melanoma, that is in lung (13,57) and colon carcinoma cells (20,58). Given our present data, it would as well be of the interest to determine whether this PKB activity contributes to NFAT- and nuclear factor-KB-driven transcription of pro-metastatic genes, coupled in both latter cell types to SOC influx (44,45).

It is possible that mechanisms alternative to reduction of cellular cholesterol and raft ablation might be proposed to explain interruption of the SOC/PKB/GSK3β/NFAT2 pathway by Zol, at least in part. For instance, blockade of the Zol target enzyme farnesyl diphosphate synthase impairs production of isoprenoid lipids and function of prenylation-dependent small GTPases (24), acting as modulators of PKB and NFAT activities (14,59). In addition, accumulation of the farnesyl diphosphate synthase substrates is followed by their conversion to mitochondria-toxic ATP analogues and dissipation of ΔΨ_m (24). Arrest of the ΔΨ_m-driven mitochondrial Ca^{2+} transport could...
diminish removal of incoming Ca\(^{2+}\) from the SOC vicinity, leading to Ca\(^{2+}\)-dependent inactivation of the SOC and thereby PKB and NFAT (9). Two sets of data argue against the involvement of the above potential actions of Zol in our proposed mechanism. First, the effects of Zol were reversed by loading cells with cholesterol but not with the isoprenoid precursor geranylgeraniol (Figure 6 and data not shown). Second, Zol-treated cells preserved intact \(\Delta \psi_m\) within the time interval, needed for inactivation of the SOC/PKB/GSK3β/NFAT2 cascade (Supplementary Figure 2, available at Carcinogenesis Online; Figures 2–5). Another concern is connected to systemic application of Zol, since sequestration of this mineral-affine bisphosphonate derivative in the bone could diminish its availability for non-osseous tumors (24). Opposite to this notion, preclinical studies demonstrated attenuated growth of epithelial malignancies and reduced incidence of visceral metastases in Zol-treated tumor-bearing animals (24–28).

Furthermore, Zol substantially decreased local cancer recurrence after surgery in breast carcinoma patients when added to conventional endocrine therapy (60). Although data on distribution of this drug to extra-skeletal tumors are limited (24), it might be envisaged that enhanced blood flow, permeability of tumor neovascularization, necrosis and deposition of minerals facilitate bisphosphonate uptake by neoplastic tissues (61). Indeed, accumulation of bisphosphonate-based probes for bone imaging in soft tissue malignancies is widely recognized (61–63), supporting previously (24–28) and presently (Figure 1) reported anti-cancer action of Zol outside of the bone. Close association of this action with inhibition of aberrantly active NFAT2 and apoptosis in our melanoma model warrants further clinical evaluation of Zol for capability to switch-off NFAT function specifically in tumorigenic cells through disruption of distinctive rafts, and thereby to improve melanoma outcome.

Supplementary material
Supplementary Materials and Methods and Figures 1–9 can be found at http://carcin.oxfordjournals.org/

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