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Upregulation of endogenous farnesyl diphosphate synthase overcomes the inhibitory effect of bisphosphonate on protein prenylation in Hela cells

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ABSTRACT

Nitrogen-containing bisphosphonates (N-BPs) such as zoledronic acid (ZOL) are the gold standard treatment for diseases of excessive bone resorption. N-BPs inactivate osteoclasts via inhibition of farnesyl diphosphate synthase (FPPS), thereby preventing the prenylation of essential small GTPases. Not all patients respond to N-BP therapy to the same extent, and some patients, for example with tumour-associated bone disease or Paget's disease, appear to develop resistance to N-BPs. The extent to which upregulation of FPPS might contribute to these phenomena is not clear. Using quantitative PCR and western blot analysis we show that levels of FPPS mRNA and protein can be upregulated in HeLa cells by culturing in lipoprotein deficient serum (LDS) or by over-expression of SREBP-1a. Upregulated, endogenous FPPS was predominantly localised to the cytosol and did not co-localise with peroxisomal or mitochondrial markers. Upregulation of endogenous FPPS conferred resistance to the inhibitory effect of low concentrations of ZOL on the prenylation of the small GTPase Rap1a. These observations suggest that an increase in the expression of endogenous FPPS could confer at least partial resistance to the pharmacological effect of N-BP drugs such as ZOL in vivo.

1. Introduction

Over the last 4 decades, nitrogen-containing bisphosphonates (N-BPs) such as zoledronic acid (ZOL), have become the gold standard of treatment for post-menopausal osteoporosis as well as Paget's disease of bone (PDB) and tumour-associated bone diseases [1–4].

The molecular target of N-BPs is farnesyl diphosphate synthase (FPPS), a crucial enzyme at the branch point of cholesterol and isoprenoid synthesis in the mevalonate pathway [5]. Because they bind calcium ions, N-BPs accumulate quickly in the skeleton and are internalised by osteoclasts, leading to rapid inhibition of FPPS. The subsequent lack of intracellular isoprenoid lipids prevents the post-translational prenylation of small GTPase signalling proteins such as Rap1a, leading to disruption of osteoclast function [5,6]. The accumulation of unprenylated Rap1a can therefore be used as a biochemical indicator of the pharmacological activity of N-BPs [7–11].

Resistance of patients to treatment with N-BPs has been recognized as a clinical issue in cancer-associated bone disease and PDB [1,12] where assessment of biochemical remission has been used as a measure of unresponsiveness [13]. This phenomenon is observed even in patients who have received intravenous BP therapy, thus ruling out lack of adherence to oral N-BP treatment. The molecular mechanism underlying this apparent drug resistance is unclear; upregulation of multidrug resistance protein 1, the antiapoptotic bcl-2 gene [14] and heat shock protein 27 [15] have been observed in tumour cell lines that have developed N-BP resistance, but these observations have yet to be shown to be clinically relevant. Given that FPPS is the major molecular target for N-BP drugs (reviewed in [5]), we explored the possibility, using a cellular model, that resistance to N-BPs could be acquired as a consequence of upregulation of this enzyme.

Although prolonged N-BP treatment can result in increased FPPS expression in cultured cells [16–18], a model system in which endogenous FPPS expression can be rapidly modulated, with the intention of altering responsiveness to N-BPs, has not been examined. Expression of endogenous FPPS, as well as other enzymes of the mevalonate pathway, is

Abbreviations: BMD, bone mineral density; FPPS, farnesyl diphosphate synthase; HMG CoA reductase, 3-hydroxy 3-methyl-glutaryl CoA reductase; LDS, lipoprotein deficient serum; N-BP, nitrogen-containing bisphosphonate; SREBP, sterol regulatory element binding protein; ZOL, zoledronic acid

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upregulated by sterol regulatory element binding proteins (SREBPs) under conditions of sterol depletion [19,20], for example by culturing cells in the presence of lipoprotein deficient serum (LDS) [20,21]. We therefore examined whether upregulation of endogenous FPPS, by culturing cells in LDS-containing medium, conferred resistance to the N-BP ZOL, measuring the accumulation of unprenylated Rap1a as a surrogate marker for inhibition of protein prenylation.

The exact subcellular location of endogenous FPPS is not clear, with evidence for both cytosolic and peroxisomal locations [22–25]. In 2007, an isoform of FPPS was identified that contains a mitochondrial targeting sequence, raising the possibility of a mitochondrial location for the enzyme [26]. We therefore examined the subcellular localisation of LDS-induced endogenous FPPS in peroxisomes, mitochondria or the cytosol. Since HeLa cells are reported to have intact peroxisomal import pathways [27], these cells are an ideal model with which to study the subcellular localisation of FPPS.

2. Materials and methods

2.1. Reagents

All reagents were purchased from Sigma Aldrich (Poole, UK), unless otherwise stated. Zoledronic acid (ZOL, hydrated disodium salt), provided by Novartis Pharma AG (Basel, Switzerland), was dissolved in PBS, adjusted to pH 7.4 and sterilized with 0.2 μm filter. Other reagents were MitoTracker-CMX-ROS 579–599 (Cat. No. M7512, Invitrogen), anti-myc antibody (9B11, mouse monoclonal antibody, Cat. No. 2276, Cell signalling), anti-PMP 70 antibody (Cat. No. ABT12, Millipore) and pDsRed2-Peroxi vector (Cat. No. 632418, Clontech). The 1470 bp cDNA encoding human SREBP-1a was cloned into the pcDNA3.1 vector to create the SREBP-1a-FLAG plasmid, kindly provided by Dr T. Osborne, University of California.

2.2. Cell culture

HeLa cells were maintained in DMEM (Life Technologies, Paisley, UK), containing 10% [v/v] foetal calf serum (FCS) and 1 mM glutamine. For each set of experiments, FPPS expression was manipulated by culturing cells in medium containing either 10% [v/v] foetal calf serum (FCS) or batch-matched lipoprotein-deficient foetal calf serum (LDS), or by transfecting the cells with FLAG-tagged SREBP-1a.

2.3. Quantitative RT-PCR

Cells were seeded into 6-well plates at a density of 2×10^5 cells per well and incubated overnight. The cells were washed once with PBS, fresh medium with 10% [v/v] FCS or LDS was added and the plates were incubated for 24 h. The cells were then treated with 0–50 μM ZOL for a further 24 h. Total RNA was extracted using TRIzol®. cDNA was reverse transcribed using SuperScript ™ II Reverse Transcriptase (18064-022, Invitrogen). In a 20 μL reaction volume, 2 μg of RNA, 2 μM of random primer and 200 U of SuperScript ™ II reverse transcriptase were mixed and incubated at 70 °C for 10 min, 42 °C for 50 min and finally 95 °C for 5 min.

For quantitative real time PCR, 3 μL of cDNA was added to 10 μL of LightCycler® 480 Probe Master (Roche), 0.2 μL of TaqMan® probe (Probe no. 46, Universal Probe Library), 0.2 μL of forward and reverse primers (20 μM) each and 1 μL of human GAPDH probe dye VIC-MGB (Applied Biosystems, 4326317E). The human FPPS gene specific intron spanning primers between exons 2 and 3 were designed using Universal Probe library software and purchased from Sigma-GenoSys (forward primer: 5′-CAGCTTTCTACTCCTTCTACCTTCC-3′, reverse primer: 5′-GCTC CTTCTCGCCATCAAT-3′). The results were analysed using LightCycler® 480 software 1.5 and FPPS was normalised to GAPDH and presented as a ratio (Ratio $= E^{\text{targetΔCT(treated-control)}} / E^{\text{GAPDHΔCT(treated-control)}}$).

2.4. Western blot analysis

Cells were seeded into 6-well plates at a density of 2×10^5 cells per well and incubated overnight. The cells were washed once with PBS, fresh medium with 10% [v/v] FCS or LDS was added. The cells were incubated for 24 h then treated with 0–50 μM ZOL for a further 24 h and cell lysates were analysed by western blotting with antibodies specific for unprenylated Rap1a (goat polyclonal, Santa Cruz Biotechnology; SC-1482) and total Rap1 (rabbit monoclonal, Cell Signalling Technologies; 2399) as previously described [28], or for FPPS (1/1000 dilution of affinity-purified rabbit anti-serum), FLAG-SREBP1a using M2-antiFLAG antibody (1/1000 dilution; Sigma, Poole, UK) together with infrared-labelled secondary antibodies (LI-COR, Cambridge, UK). The blots were analysed on an infrared scanner (LI-COR, Cambridge, UK) with Odyssey software.

2.5. Immunofluorescence staining of FPPS

FPPS cDNA containing an apparent mitochondrial targeting sequence in exon 2 [26] (Ensembl, transcript: FDPS-001 (ENST00000356657)) was inserted into pcDNA™ 3.1/myc-His A vector (Invitrogen) using T4 DNA ligase. The ligated product was transformed into XL-1 Blue super-competent *Escherichia coli* and the plasmid was purified and sequence verified.

Hela cells were seeded on coverslips into 48 well plates. After overnight incubation they were either treated with medium containing 10% [v/v] LDS or 10% [v/v] FCS. Some cultures were transfected with the FPPS-encoding plasmid using jetPRIME™ transfection reagent. To label peroxisomes, cells were also transfected with pDsRed2-Peroxi vector. Following transfections, cells were incubated for 24 h before transferring to LDS or FCS containing medium for a further 24 h. Mitochondria were labelled by incubating cells with 100 nM MitoTracker® Red CMXRos for 30 min at 37 °C at the end of the culture period. The cells were fixed in 4% [v/v] paraformaldehyde, washed and then permeabilised with 0.1% [v/v] Triton-X-100 for 20 min. The cells were incubated with 10% [v/v] normal goat serum in PBS for 30 min and then with affinity-purified rabbit polyclonal FPPS anti-serum ([29]; 1/1000 dilution) followed by goat anti-rabbit 488 antibody (1/150 dilution; Invitrogen). To confirm that the pDsRed2-Peroxi vector labelled peroxisomes, some cells were co-stained with anti-PMP70 antibody (1/1000 dilution). Cells were analysed and images captured on a Carl Zeiss LSM510 META Confocal microscope with Zen 2009 software.

2.6. Transfection with SREBP-1a plasmid

HeLa cells were seeded in 6 well plates at a density of 2×10^5 cells per well and incubated overnight in DMEM containing 10% [v/v] FCS. Cells were then transfected with FLAG-tagged SREBP-1a (a generous gift from Dr Timothy Osborne, University of California) using jetPRIME™ transfection kit (114–15, Polyplus transfection™). The culture medium was replaced after 4 h and cells were incubated for 18 h. The medium was replaced with fresh medium containing 0–25 μM ZOL and cultures were incubated for a further 6 h with or without ZOL, after which the cells were lysed for western blot analysis.

2.7. Statistical analysis

Mean densitometric measurements from western blots of FCS- and LDS-treated cells were compared using an unpaired *t*-test (SPSS, version 20).

3. Results and discussion

The expression of FPPS is upregulated in the absence of sterols via binding of SREBP-1 protein to the FPPS promoter [30–32]. Consistent with this, in Hela cells, as in other cell lines such as THP-1, HepG2 and

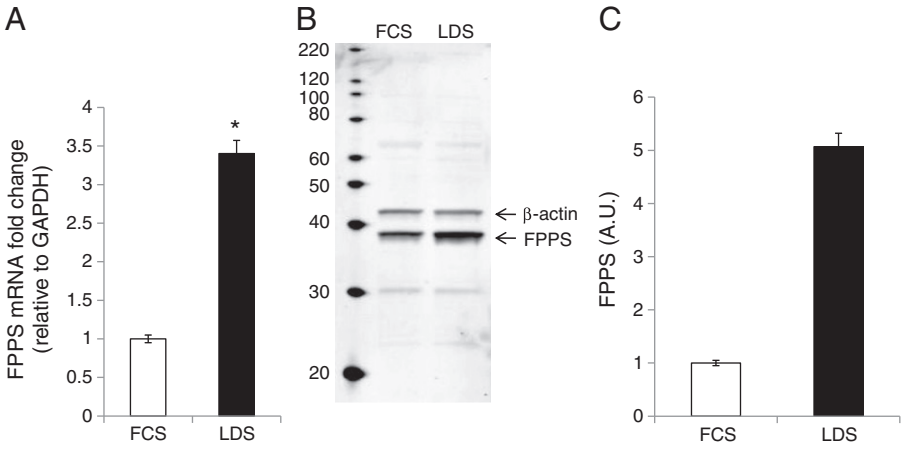

Fig. 1. Culturing Hela cells in 10% LDS upregulates FPPS. A) HeLa cells were cultured in 10% LDS or 10% FCS for 48 h then FPPS expression and GAPDH expression were measured using real time qPCR. B) HeLa cells were cultured in 10% FCS or 10% LDS for 48 h then FPPS expression was assessed by western blotting. Markers show molecular mass (kDa). C) Band intensities were quantified using Licor Odyssey software and normalised to β-actin. Data are representative of at least 2 independent experiments, * $p < 0.05$, values significantly different from cells cultured in 10% FCS (Student's unpaired *t*-test).

Huh7 [20,21], the absence of extracellular sterols (by culturing cells in LDS) led to an increase in the expression of endogenous FPPS (mRNA and protein). After 48 h of culture in 10% [v/v] LDS, FPPS mRNA increased approximately 3.4-fold in HeLa cells compared to cells cultured in 10% [v/v] FCS, when assessed by quantitative RT-PCR (Fig. 1A). Densitometric analysis of western blots (Fig. 1B) demonstrated a 5-fold increase in 39 kDa FPPS protein, when normalised to β-actin (Fig. 1C). The LDS-induced increase in FPPS was confirmed by immunofluorescence staining. FPPS was barely detectable in HeLa cells that had been cultured in 10% FCS (Fig. 2B), whereas in cells that had been cultured in 10% LDS, FPPS was clearly visible in punctate structures throughout the cytosol (Fig. 2C).

By measuring the accumulation of unprenylated Rap1a as a surrogate marker for the inhibition of FPPS [8–11], we found that upregulation of FPPS expression in Hela cells reduced the accumulation of unprenylated Rap1a in response to 24 h' treatment with 0–50 μM ZOL. As expected, cells cultured in 10% FCS showed robust accumulation of unprenylated Rap1a following treatment with 2 μM ZOL (Fig. 3A), whereas in cells cultured in LDS (in which endogenous FPPS levels were increased; Fig. 3B), unprenylated Rap1a was not detectable in

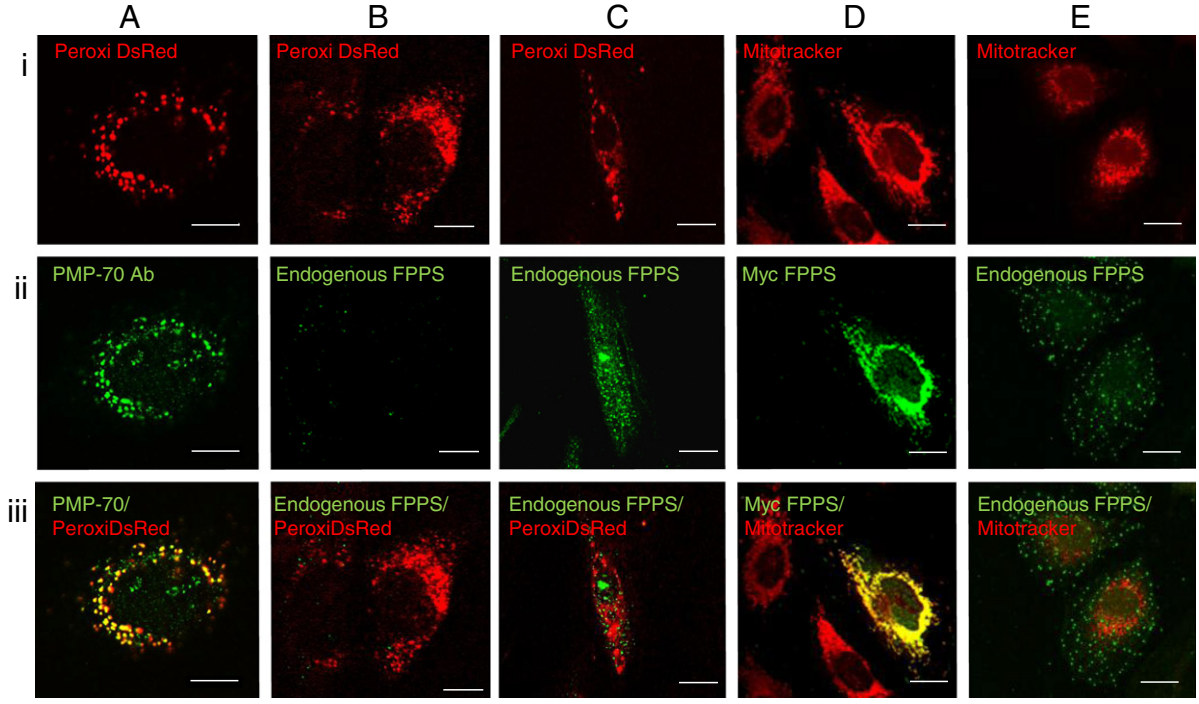

Fig. 2. Endogenous FPPS is not localised to peroxisomes or mitochondria in HeLa cells. A) Distribution of the peroxisomal markers, pDsRed2-peroxi (i; red) and anti-PMP-70 (ii; green); scale bars = 10 μm. B) and C) Hela cells were transfected with pDsRed2-peroxi (i; red), incubated in medium containing 10% FCS (B) or 10% LDS (C) for 48 h and immunostained for FPPS (ii; green); scale bars = 10 μm and 20 μm. D) Hela cells were transfected with the myc-tagged mitochondrial isoform expression construct and incubated for 48 h, including a 30 minute incubation in MitoTracker (i; red) and immunostained for myc expression (ii; green); scale bars = 10 μm. E) Hela cells were incubated in 10% LDS for 48 h, the cells were stained with (i) MitoTracker (red), and immunostained for FPPS (ii; green); scale bars = 20 μm. Images showing both green and red signals are shown in iii) throughout.

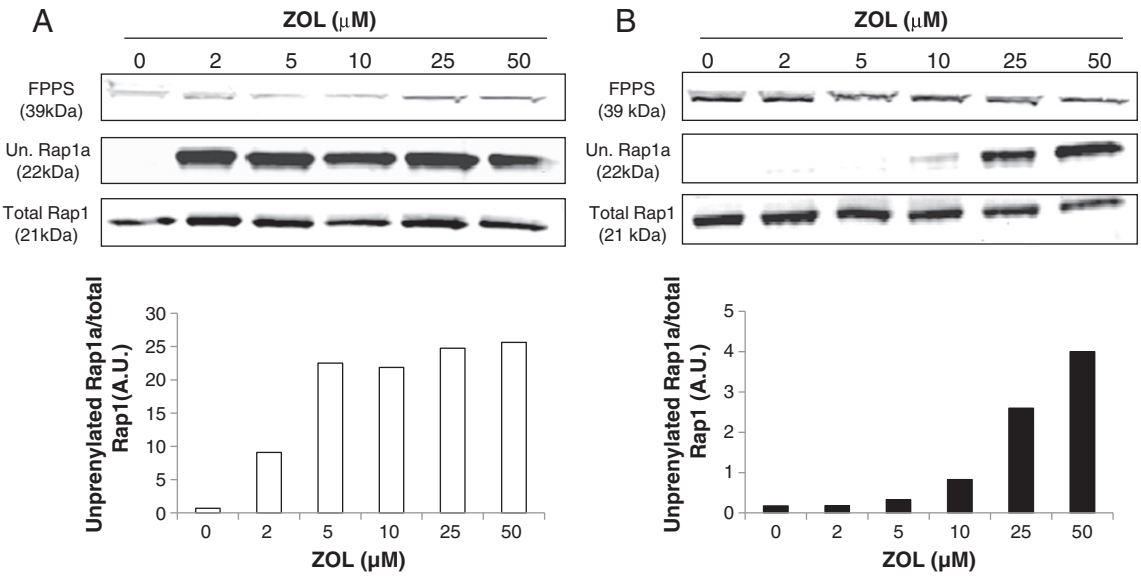

Fig. 3. Culture in LDS reduces the inhibitory effect of ZOL on Rap1a prenylation. HeLa cells were cultured in A) 10% FCS or B) 10% LDS for 24 h then treated with 0–50 μM ZOL. The cells were lysed 24 h later for western blot analysis using anti-FPPS, anti-unprenylated Rap1a and anti-Rap1 antibodies. Unprenylated Rap1a band densities were normalised to total Rap1 and compared using Licor Odyssey software. Data shown are representative relative band densities from three independent experiments.

cells treated with 5 μM ZOL and was barely detectable in cells treated with 10 μM ZOL (Fig. 3D). Hence, the upregulation of endogenous FPPS conferred partial resistance to the pharmacological effect of ZOL.

Intracellular cholesterol and fatty acid metabolism are regulated by SREBP-2 and SREBP-1a respectively, although in cultured cells the effect of SREBP-1 predominates [33]. Overexpression of SREBP-1a in HeLa cells

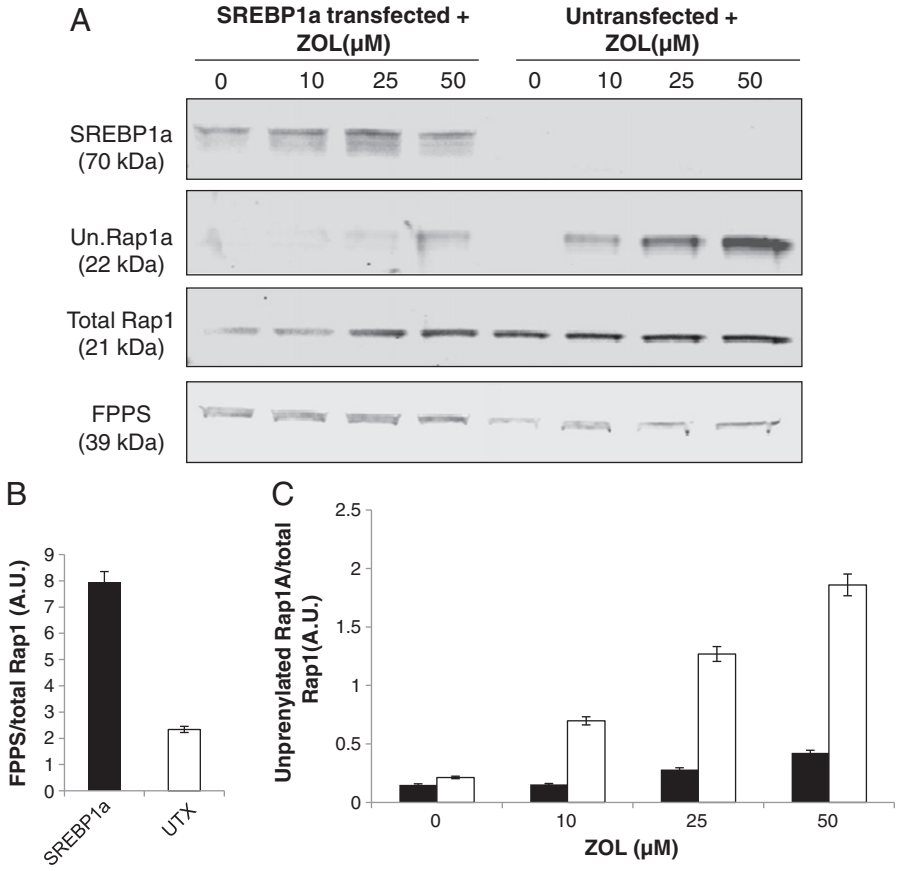

Fig. 4. Overexpression of SREBP-1a reduces the inhibitory effect of ZOL on Rap1a prenylation. A) HeLa cells were transfected with SREBP1a plasmid or were untransfected (UTX). After 18 h cells were treated with 0–50 μM ZOL for a further 6 h. 20 μg of cell lysate were analysed by western blotting using anti-FLAG (to detect SREBP1a), anti-FPPS, anti-unprenylated Rap1a and anti-Rap1 antibodies. B) and C) Unprenylated Rap1a and FPPS band densities were normalised to total Rap1 and compared using Licor Odyssey software (black bars, SREBP1a transfected; white bars, UTX). Values are the mean ± SD of three independent experiments.

caused upregulation of endogenous FPPS (Figs. 4A,B), similar to the effect of culturing cells in LDS. Furthermore, the inhibitory effect of ZOL on Rap1a prenylation was reduced in HeLa cells overexpressing SREBP-1a compared to untransfected cells (Figs. 4A,C). Together, these studies demonstrate that increasing the level of endogenous FPPS (either by removal of exogenous sterols, or by overexpression of SREBP-1a) can confer at least partial resistance to the pharmacologic effects of N-BP drug.

A single nucleotide polymorphism (rs2297480) within the promoter region of FPPS was recently reported to be associated with low BMD [34] and reduced response to BP treatment in post-menopausal women [34,35], with carriers of the rare allele demonstrating significantly less improvement in BMD with long term BP treatment compared to homozygous carriers of the common allele [35]. It was suggested that the variant allele creates a Runx1 binding site, likely leading to reduced FPPS transcription [34] although this was not studied. Our observations indicate that reduced expression of FPPS would actually lead to increased sensitivity to BP therapy rather than decreased responsiveness. Consequently the relevance of this polymorphism to BP treatment remains to be determined.

Despite the punctate appearance of FPPS following immunofluorescence staining, the LDS-induced, endogenous FPPS did not colocalise to peroxisomes (Fig. 2C), which were labelled in cells transfected with the pDsRed2-Peroxi vector (the product of which colocalised with the peroxisomal marker PMP-70; Fig. 2A). A myc-tagged isoform of FPPS that includes exon 2 (containing an apparent mitochondrial targeting sequence; [26]) colocalised with MitoTracker (Fig. 2D). However, by comparison with the distribution of MitoTracker, the LDS-induced endogenous FPPS did not localise to mitochondria (Fig. 2E). This is consistent with a previous study in *Trypanosoma* species [25], in which the presence of FPPS was not detected in mitochondria. Taken together, these results suggest that endogenous FPPS is predominantly cytosolic, consistent with a proposed model of the mevalonate pathway in which FPPS, together with other enzymes of the mevalonate pathway, is predominantly cytosolic rather than peroxisomal [23].

In conclusion, we demonstrate that increases in the level of endogenous cytosolic FPPS in cultured cells decrease the effectiveness of N-BPs for inhibiting protein prenylation, the major pharmacological action of these drugs. If such variations in FPPS levels occur in human osteoclasts in vivo, this may contribute to the variability in clinical response to these drugs, and to the phenomenon of resistance to BP therapy.

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