Metformin inhibits the senescence-associated secretory phenotype by interfering with IKK/NF-κB activation

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Summary

We show that the antidiabetic drug metformin inhibits the expression of genes coding for multiple inflammatory cytokines seen during cellular senescence. Conditioned medium (CM) from senescent cells stimulates the growth of prostate cancer cells but treatment of senescent cells with metformin inhibited this effect. Bioinformatic analysis of genes downregulated by metformin suggests that the drug blocks the activity of the transcription factor NF-κB. In agreement, metformin prevented the translocation of NF-κB to the nucleus and inhibited the phosphorylation of IκB and IKKα/β, events required for activation of the NF-κB pathway. These effects were not dependent on AMPK activation or on the context of cellular senescence, as metformin inhibited the NF-κB pathway stimulated by lipopolysaccharide (LPS) in *ampk* null fibroblasts and in macrophages. Taken together, our results provide a novel mechanism for the antiaging and antineoplastic effects of metformin reported in animal models and in diabetic patients taking this drug.

Key words: cellular senescence; cytokines; metformin; NF-κB; senescence.

Introduction

Aging is characterized by a general decline in the functions of all tissues and an increased incidence of chronic disease. A large body of evidence has correlated age-associated diseases with chronic inflammation leading to an aging theory known as inflammaging (Bonafe *et al.*, 2012). Alzheimer disease, cancer, sarcopenia, atherosclerosis, and diabetes are common diagnoses in the aging population associated to chronic inflammation (Cesari *et al.*, 2003; Bonafe *et al.*, 2012).

It has been proposed that the process of cellular senescence represents a general response to various forms of damage that may accumulate with age. Senescence is a permanent cell cycle arrest triggered by a variety of stressors, including short telomeres, oncogenes, DNA damage, and reactive oxygen species (Coppe *et al.*, 2010). The evidence correlating cellular senescence with aging and age-related diseases is abundant (Coppe *et al.*, 2010). Remarkably, elimination of senescent cells attenuates signs of accelerated aging in mice (Baker *et al.*, 2011). The mechanisms by which senescent cells accelerate organismal aging are not known but senescent cells secrete a large variety of inflammatory cytokines that can impair tissue homeostasis and promote chronic inflammation (Coppe *et al.*, 2010).

Cellular senescence is also a tumor suppressor mechanism and inhibiting key mediators of senescence such as p53, and the retinoblastoma protein leads to accelerated tumor formation (Serrano *et al.*, 1997). It has been proposed that cellular senescence was selected during evolution for tumor suppression and that its pro-aging effect was not eliminated by natural selection because it mainly acts after reproduction (Ferbeyre & Lowe, 2002). High cytokine gene expression by senescent cells is associated with persistent DNA damage signals (Rodier *et al.*, 2009), and in some situations, it can promote tumorigenesis in neighboring cells (Krtolica *et al.*, 2001; Gilbert & Hemann, 2010). Retrospective studies have suggested that the antidiabetic drug metformin has unanticipated cancer prevention activity in patients (Pollak, 2010; Formentini *et al.*, 2012) and inhibits the generation of reactive oxygen species and DNA damage in normal cells expressing oncogenic *ras* or treated with paraquat (Algire *et al.*, 2012). However, metformin did not prevent RAS-induced growth arrest (Algire *et al.*, 2012). We thus decided to investigate whether metformin could suppress the production of inflammatory cytokines by senescent cells.

Results

Effects of metformin on the gene expression profile of oncogene-induced senescence

To study the effects of metformin on the gene expression profile of oncogene-induced senescence, we infected normal human diploid fibroblasts IMR90 with a retroviral vector pBABE or its derivative expressing oncogenic *ras* (Ha-RAS-V12). In this protocol, cells expressing RASV12 entered a hyperproliferative phase quickly after selection but arrested their growth and senesced 4 days after, as

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shown by the increase in the percent of flat blue cells positive for senescence-associated β-galactosidase (SA-β-Gal) (Fig. 1A). We treated a fraction of RAS-expressing cells with 5 mM metformin 1 day postinfection during the hyperproliferative phase. Metformin arrested the growth of the cells (not shown) and both metformin-treated and untreated cells entered senescence at day four postinfection. We collected RNA from both cell populations at day six postinfection, when more than 90% of the cells were positive for SA-β-Gal (Fig. 1A). The RNA was then used to prepare probes for Afymetrix microarrays. Data from three independent experiments were analyzed with Flexarray 1.6.1.1 using robust multiarray average (RMA) as normalization method. The data discussed were deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE33612 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE33612). All gene expression changes showing 2 or more fold differences were considered for a pathway analysis using FatiGO single enrichment from the bioinformatics platform Babelomics 4.3 (Medina *et al.*, 2010). We found that metformin mostly inhibited gene expression with its largest effect being on genes controlling immunity and metabolism (Fig. 1B). The drug affected the expression of genes controlling hypoxia and oxidative stress, extracellular matrix, regulation of cell death, ion homeostasis, cell cycle, cell migration, cell signaling, metabolism, and immunity (Table 1). These global categories were derived from the most significant Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) term names (Fig. S1).

We previously showed that metformin may prevent RAS-expressing cells from escaping senescence by reinforcing growth arrest (Algire *et al.*, 2012), and this can be explained by the repression of cell cycle, cell signaling, cell migration, and metabolic genes (Fig. S1). For example, metformin represses the expression of growth factor receptors such as PDGFRA, ACVRL1, and BDKRB1, which can transduce signals from extracellular factors that stimulate cell proliferation (Pietras *et al.*, 2003), the cell cycle enzyme CDK6 (Malumbres *et al.*, 2000), and the key signaling enzyme PDK1, which acts in the AKT signaling pathway (Raimondi & Falasca, 2011). Metformin also suppresses the expression of kynureninase (KYNU) and quinolinate phosphorybosyl transferase (QPRT), enzymes important for *de novo* NAD biosynthesis (Rongvaux *et al.*, 2003). Limiting NAD levels may make cells more susceptible to metabolic stress and limit the use of NAD as a source of ADP-ribosylation reactions that control many signaling pathways (Rongvaux *et al.*, 2003). The drug also inhibited the expression of PTGS2 (also known as COX-2), an enzyme that links inflammation to cancer considered to be a potential target for cancer chemoprevention (Fischer *et al.*, 2011). Another intriguing downregulated gene is GPAM (also known as GPAT), coding for the mitochondrial enzyme glycerol-3-phosphate acyltransferase, critical for the synthesis of triglycerides and phospholipids (Ericsson *et al.*, 1997). However, the most dramatic effect of metformin on gene expression involved genes controlling inflammatory cytokines (Fig. 1C). We confirmed by qPCR that metformin inhibited the expression of multiple cytokines, chemokines, and serpin genes in the context of RAS-induced senescence (Fig. 1D–L). These changes in gene expression were translated into a reduction in the ability of the supernatant of metformin-treated senescent cells to stimulate the growth of the PC3 prostate cancer cells (Fig. 1M) as recently reported (Sun *et al.*, 2012). A dose–response experiment indicated that metformin inhibited cytokine expression at doses of 1 mM or higher (Fig. 2). Intriguingly, at doses of 1 mM, metformin reduced cytokine gene expression in cells expressing oncogenic *ras* but did not affect cell proliferation in the control cells (Fig. 2). At 0.5 mM, the effects of metformin on RASV12 expressing cells were moderately stimulatory on IL6 and IL8 and inhibitory on CXCL5. Taken together, the results suggest that high doses of metformin can block potentially maladaptive effects of the senescence program without compromising its anticancer effects.

Analysis of downregulated genes with the algorithm SPEED (Signaling Pathway Enrichment using Experimental Datasets) identified genes in Toll-like receptor (TLR), IL-1 and TNFα signaling pathways as the most downregulated genes in cells treated with metformin (Fig. S2A,B). TLR signaling regulates the expression of inflammatory cytokines via the transcription factors AP1/Jun and NF-κB but also the antiviral and antiproliferative interferon pathway through the transcription factors IRF3 and IRF7 (Hacker *et al.*, 2006). Using gene set enrichment analysis (GSEA), we found that genes regulated by AP1/JUN and NF-κB were significantly enriched among the downregulated genes in metformin-treated cells but not in the control (Fig. S2C,D). In contrast, genes regulated by the interferon pathway (IRF3/7) were not significantly altered by metformin (Fig. S2E). Next, we analyzed the gene expression data with the bioinformatics programs TFacTS and DIRE to look for transcription factors that can regulate the affected genes. The transcription factors TCF4, NFKB1, and RELA were the most important associated with gene expression changes caused by metformin (Fig. S3A,B). When we considered only downregulated cytokine genes, gene set enrichment analysis (GSEA) indicated a significant enrichment of cytokines genes targeted by NF-κB (Fig. 3A) and as shown in heat maps (Fig. 3B), this downregulation was consistent in three replicas of the same experiment. When we used this list of cytokines genes downregulated by metformin with the programs TFacTS and DIRE, NFKB, and CEBPδ were the most common transcription factors regulating those genes (Figs 3C and S3C). NF-κB is recognized as a master regulator of cytokine gene expression in senescent cells (Chien *et al.*, 2011; Jing *et al.*, 2011; Ohanna *et al.*, 2011) and aging (Adler *et al.*, 2007; Donato *et al.*, 2008). Therefore, we hypothesized that metformin inhibited cytokine gene expression in senescent cells by inhibiting NF-κB activation.

Effects of metformin on the NF-κB pathway

NF-κB is a transcription factor formed by homo or heterodimers of five distinct subunits RELA(p65), RELB, c-REL, NFKB1, and NFKB2 and is sequestered in the cytoplasm by the ankyrin repeat containing IκB proteins (Hoffmann *et al.*, 2002). During senescence both canonical and noncanonical NF-κB pathways are activated because DNA binding by RELA, RELB, NFKB1, and NFKB2 was found increased (Jing *et al.*, 2011). A key event in the activation of the NF-κB pathway is the phosphorylation of the cytoplasmic inhibitor IκB (Mercurio *et al.*, 1997) and metformin dramatically inhibited this

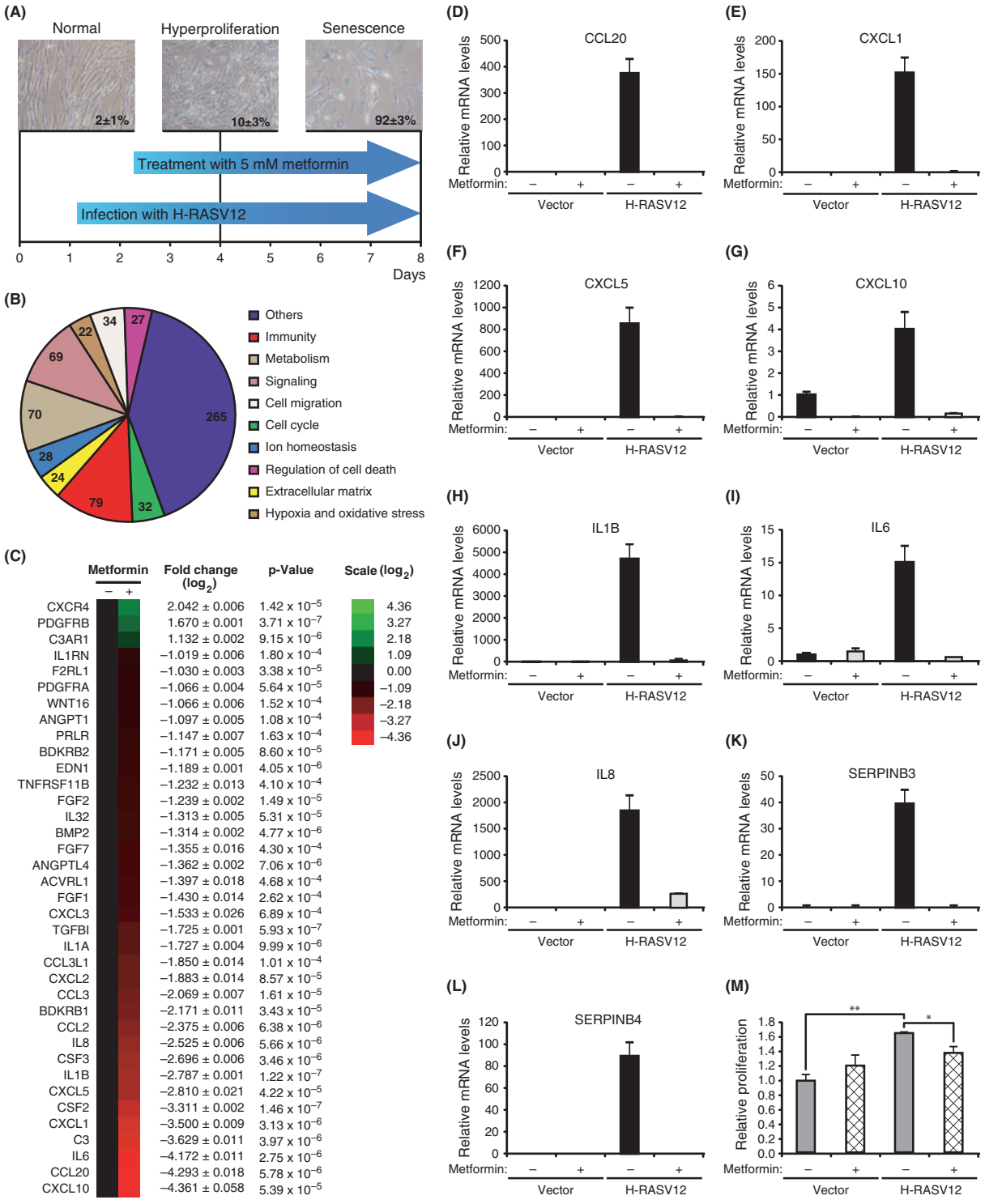

Fig. 1 Effect of metformin on gene expression in RAS- expressing cells. (A) Time course of RAS-induced senescence and treatment with metformin. IMR90 cells were infected with retroviruses allowing expression of H-RASV12. Twenty-four hours after infection, cells were treated with 5.0 mM metformin or vehicle for 6 days. RNA was collected at day 7 for microarray gene expression analysis (GEO accession number: GSE33612). (B) Pie chart of most important biological functions affected by metformin in senescent H-RASV12 expressing cells. A FatiGO single enrichment analysis was performed using transcripts with a fold change higher or equal to 2 and a *P*-value < 0.05 according to the two-sample Student's *t*-test. (C) Heat Map of fold change (log_2) of genes coding for cytokines and their receptors identified by a combination of Gene Ontology (GO) analysis and literature-based curation. The fold change and standard deviation (SD) are calculated from the mean expression values of three replicates of each condition. The *P*-value is determined according to the two-sample Student's *t*-test. (D–L) QPCR validation of several cytokines genes that are highly expressed in senescent cells expressing oncogenic *ras* and are repressed by metformin. RNA extracts from IMR90 fibroblasts expressing H-RASV12 or an empty vector and treated with 5 mM metformin or vehicle for 6 days were obtained 7 days after infection. Error bars represent ± SD of replicates. (M) Conditioned medium from senescent fibroblasts promote the growth of PC3 prostate cancer cells but pretreatment of senescent cells with metformin decreased the effect: $*P < 0.05$ and $**P < 0.005$ according to a two-sample Student's *t*-test, $n = 3$.

Table 1 Biological functions and genes affected in oncogenic ras expressing cells

Categories	Genes
Immunity	ACVRL1, ADM, ANGPT1, ANGPTL4, BDKRB1, BDKRB2, BIRC3, BMP2, C3, C3AR1, CCL2, CCL20, CCL3, CCL3L1, CD36, CDK6, CFH, COL1A1, CSF2, CSF3, CXCL1, CXCL10, CXCL2, CXCL3, CXCL5, CXCR4, DPP4, EDN1, EREG, F2RL1, F3, FABP4, FGF1, FGF2, FGF7, GCH1, GGCX, HIST2H2BE, ICAM1, ID3, IKBKE, IL1A, IL1B, IL1RN, IL32, IL6, IL8, IRAK2, KYNU, MAP3K8, NFKBIZ, NRG1, OLR1, PAPSS2, PDGFRA, PDGFRB, PIK3R3, PLD1, PLXDC2, PRLR, PSG4, PSG7, PTGS2, SERPINA1, SERPIND1, SERPINF1, SNCA, SOD2, STAT4, TGFBI, THBS1, THY1, TNFAIP3, TNFRSF11B, TNIP1, UACA, VCAN, VNN1
Metabolism	ADM, AMPD3, ANGPT1, ANGPTL4, APLP1, BDKRB1, BDKRB2, BMP2, CCL2, CD36, COL1A1, CSF2, CYB5R2, DHRS1, EDN1, EGR2, ENO2, ENTPD3, EREG, F3, FABP4, FGD4, FGF2, FGF7, GALNT5, GCH1, GDPD3, GPAM, HMGB2, HMOX1, IL1B, IL1RN, IL6, IRS2, KYNU, LPAR1, LPCAT3, LRRN3, MMP10, MMP8, NRG1, NRK, PDE5A, PDGFRA, PDPK1, PIK3R3, PLCXD1, PLD1, PPARG, PRKD3, PRLR, PTGS2, PTPRN, QPRT, RDH8, RETSAT, SERPINA1, SERPINB4, SLC25A20, SNCA, SOD2, SPP1, SULT1B1, TACR1, THBS1, THY1, TNFRSF11B, UACA, VGF, VLDLR
Signaling	ACVRL1, ADM, ANGPT1, ANGPTL4, BDKRB2, BDNF, BIRC3, BMP2, CCL2, CCL20, CCL3, CD36, CDK6, CHRNA1, COL1A2, CSF2, CXCL1, CXCL10, CXCL5, DCN, DDIT4, EDN1, EDNRA, EREG, F3, FABP4, FGD4, FGF1, FGF2, FGF7, FST, GABRA2, HIST1H4C, HMGB2, HOMER1, ID1, ID3, IKBKE, IL1B, IL1RN, IL6, IL8, IRAK2, JUP, L1CAM, LPAR1, LRRN3, NRG1, NRK, OLR1, PDGFRA, PDPK1, PIK3CG, PIK3R3, PLD1, PRLR, PTGS2, RAB3A, ROS1, RPS6KA2, SNCA, STAT4, TACR1, THBS1, THY1, TNFAIP3, TRAF3IP2, ULK2, WNT16
Cell Cycle	ACVRL1, ADM, BDKRB2, BDNF, BMP2, CCL2, CCL3L1, CDK6, COL8A1, CSF2, CSF3, CXADR, CXCL1, CXCL10, CXCL5, EDN1, EREG, F3, FABP4, FGF1, FGF2, FGF7, ID3, IL1A, IL1B, IL6, IL8, MMP12, MYOCD, PDGFRA, PTGS2, SOD2, THBS1
Cell migration	ACVRL1, BDKRB1, C3, CCL2, CCL20, CCL3, CCL3L1, CXCL1, CXCL10, CXCL2, CXCL3, CXCL5, EDN1, F2RL1, F3, FABP4, FGF2, ICAM1, ID1, IL1B, IL6, IL8, NRG1, PDGFRA, PLD1, PSG2, PTGS2, SEMA3C, SERPIND1, SERPINF1, SNCA, THBS1, THY1, UACA
Ion homeostasis	ADCY3, ADM, BDKRB1, BDKRB2, CCL2, CCL3, CHRNA1, EDN1, EDNRA, F2RL1, IL1B, IL6, ITPKA, ITPR3, LPAR1, NRG1, PDGFRB, PTGS2, SERPINA1, SLC11A2, SLC5A3, SLC8A3, SNCA, SOD2, TACR1, THBS1, THY1, TNFRSF11B
Regulation of cell death	ADM, ANGPT1, ANGPTL4, BDKRB2, BDNF, BIRC3, CCL2, CSF2, F3, FGF2, GCH1, ID3, IL1A, IL1B, IL1RN, IL6, IRAK2, PIK3R3, PRLR, PTGS2, SNCA, SOD2, THBS1, TMEM132A, TNFAIP3, UACA, VNN1
Extracellular matrix	ADAMTS9, ANGPTL4, APLP1, CD248, COL1A1, COL1A2, COL6A1, COL8A1, DCN, F3, FGF1, LUM, MMP10, MMP12, MMP8, NID2, PDGFRA, PI3, SERPINA1, TGFBI, THBS1, TNFRSF11B, VCAN, WNT16
Hypoxia and oxydative stress	ADM, ANGPT1, ANGPTL4, CCL2, COL1A1, DPP4, EDN1, GCH1, ICAM1, IL1B, IL6, OLR1, PDE5A, PDGFRA, PDLIM1, PTGS2, SERPINA1, SNCA, SOD2, THBS1, TRPA1, VNN1

event in RAS-expressing cells (Fig. 3D). IκB is phosphorylated by the IκB kinase, a complex of two catalytic subunits (IKKα and IKKβ) and one regulatory subunit (IKKγ also known as NEMO). This complex is activated by a mechanism involving several protein kinases including the ubiquitin-dependent kinase TAK1 (Wang *et al.*, 2001) and the serine/threonine kinase RIP1 (Meylan *et al.*, 2004) that phosphorylate IKKα/β in their activation loop at S176/177 and S180/181, respectively. Metformin also inhibited the phosphorylation of IKKα/β in RAS-senescent cells (Fig. 3D), suggesting that oncogenic *ras* activates the IKK kinases in primary cells but these kinases are inactive in metformin-treated cells. Of note, metformin did not prevent the activation of p38 MAP kinase in RAS-expressing cells (Fig. 3D), suggesting that its activity is confined to the NF-κB pathway. Finally, we found that oncogenic *ras* induced the nuclear translocation of RELA and RELB and metformin dramatically inhibited this effect (Fig. 3E,F). Of note, RELB localized to distinct nuclear foci in senescent fibroblasts and metformin reduced the intensity of staining of these foci.

Next, we investigated whether the main effect of metformin inhibiting the senescence-associated secretory phenotype (SASP) depended on NF-κB inhibition. We introduced previously validated shRNAs against RELA (Chien *et al.*, 2011) into primary human fibroblasts IMR90 together with oncogenic *ras* to induce cell senescence. Of note, neither metformin nor shRNAs against RELA reduced RAS mRNA expression (Fig. 4A). However, the two shRNAs used did efficiently reduced RELA mRNA: shRELA(a) more than shRELA(b) (Fig. 4B). Then, we measured the expression of several cytokines genes in these cells after treatment with metformin or vehicle. We found that knocking down RELA inhibited the expression of all tested cytokines and that metformin did not significantly further this effect in cells with shRELA(a) (Fig. 4C–F). Metformin did improve the ability of shRELA(b) to inhibit cytokine gene expression (Fig. 4C–F) and this can be explained because this shRNA was not as efficient as shRELA(a) on knocking down RELA levels. Notably, shRNAs against RELA did not affect growth as seen with the levels of the KI67 cell cycle gene, and RAS did induce growth arrest independently of their presence (Fig. 4G). Taken together, these results strongly suggest that metformin and the shRNAs against RELA are targeting the same pathway.

The exact activation mechanism of the IKK/NF-κB pathway during RAS-induced senescence is not yet fully characterized. For this reason, we investigated whether metformin could inhibit NF-κB activation in cells treated with lipopolysaccharide (LPS), which activate NF-κB by a pathway starting from Toll-like receptors leading to activation of the ubiquitin-dependent kinase TAK1 (Dong *et al.*, 2006). We found that metformin also inhibited RelA nuclear translocation in response to LPS in the murine macrophage cell line RAW264.7 (Fig. 5A,B). We also measured the phosphorylation of IκB in response to LPS in MEFs from *ampk* wild-type and *ampk* null animals and observed a similar inhibition of NF-κB pathway by metformin (Fig. 5C,D). We thus conclude that the inhibition of the TLR/NF-κB signaling pathway by metformin is independent of AMPK.

We previously used metformin at a dose of 5 mM to inhibit production of ROS in human and rodent primary cells (Algire *et al.*, 2012). We treated RAW264.7 cells with 1, 2, and 5 mM metformin and observed a dose-dependent inhibition of LPS-stimulated phosphorylation of IκB (Fig. 5E). A similar result was also obtained in

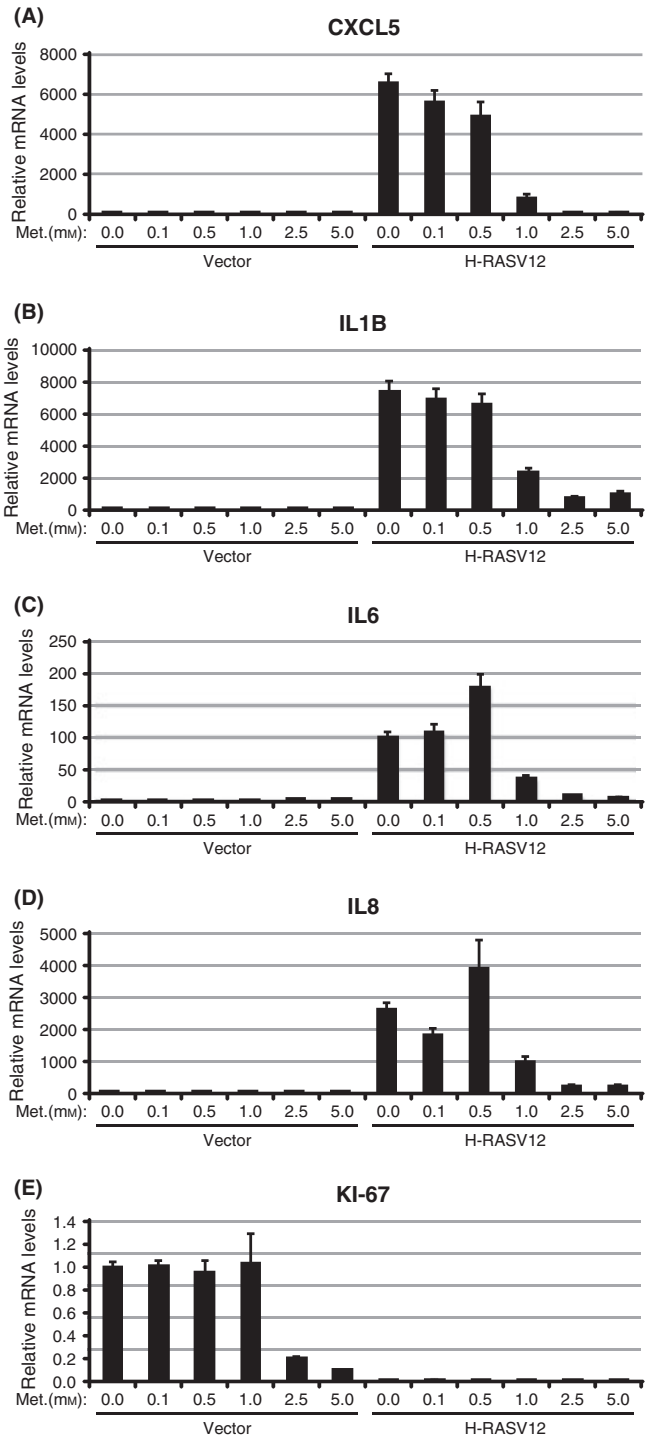

Fig. 2 Metformin inhibits the senescence-associated secretory phenotype at concentrations that do not affect the growth of normal cells. QPCR validation of several cytokines genes (A–D) that are highly expressed in senescent cells expressing oncogenic *ras* and are repressed by 1.0, 2.5, and 5.0 mM metformin. RNA extracts from IMR90 fibroblasts expressing H-RASV12 or an empty vector and treated with metformin at the indicated concentrations or vehicle for 6 days. (E) QPCR for the proliferation marker KI-67. Error bars represent ± SD of replicates.

senescent IMR90 cells expressing oncogenic *ras* (Fig. 5F) where we previously showed that a dose of 1 mM was sufficient to reduce cytokine gene expression (Fig. 2). These concentrations are considerably higher than serum concentration achieved in the treatment of diabetes (~16 μM) (Graham *et al.*, 2011). However, dose–response relationships relating serum level to activity are complex as different cell types concentrate metformin to different extents based on expression of cell surface transporters such as OCT-1 (Graham *et al.*, 2011). Thus, it will be necessary to investigate *in vivo* inflammatory endpoints in relationship to administered metformin dose to determine the clinical relevance of our findings.

Discussion

Epidemiological and laboratory studies have suggested that metformin has antineoplastic activity (Memmott *et al.*, 2010; Pollak, 2010; Bhalla *et al.*, 2012; Pollak, 2012). These findings are intriguing but require further study, as the population studies are retrospective, and thus hypothesis-generating rather than definitive, and the laboratory evidence does not fully take into account pharmacokinetic factors in humans.

One major risk factor in cancer is age and at the cellular level aging has been linked to the process of cellular senescence (Baker *et al.*, 2011). Paradoxically, cellular senescence is a tumor suppressor mechanism (Serrano *et al.*, 1997) and inhibiting cellular senescence may increase overall cancer incidence as seen in animals with disabled mutations in senescent regulators such as p16INK4a and p53 (Serrano *et al.*, 1997). One important question in the aging field is whether it is possible to reduce the pro-aging effects of cellular senescence without compromising tumor suppression. It was recently reported that glucocorticoids reduced the secretion of some but not all cytokines produced by senescence cells without interfering with the growth arrest program (Laberge *et al.*, 2012), suggesting that the pro-aging and anticancer effects of senescence can be experimentally dissociated.

Here, we report that metformin decrease the production of inflammatory cytokines in senescent cells and in response to LPS by interfering with the master regulator of inflammatory gene expression, the transcription factor NF-κB. In fact, analysis of the gene expression data in metformin-treated senescent cells indicates that the drug reduced the expression of proinflammatory cytokines mostly regulated by NF-κB but did not affect the expression of anticancer cytokines such as interferons and interferon target genes (Fig. 6). Consistent with this explanation, it was found in an experimental model of uveitis in rats that metformin prevented the production of inflammatory cytokines and the phosphorylation of RelA (Kalariya *et al.*, 2012). There is evidence that metformin can inhibit the production of ROS in senescent cells (Algire *et al.*, 2012) and mitochondrial ROS have been implicated in NF-κB activation (Formentini *et al.*, 2012). The exact mechanism by which ROS may activate the NF-κB pathway is unknown. Metformin inhibits IKKα/β kinases but not the p38MAPK, and as these two pathways are downstream of TAK1, it seems that the drug interferes only with the IKK activation pathway (Fig. 6). Also, metformin does not affect the expression of interferon target genes in RAS-expressing cells, suggesting that it does not interfere with NEMO functions, which are required for activation of both NF-κB and IRF3/7 in response to viral RNA (Zhao *et al.*, 2007).

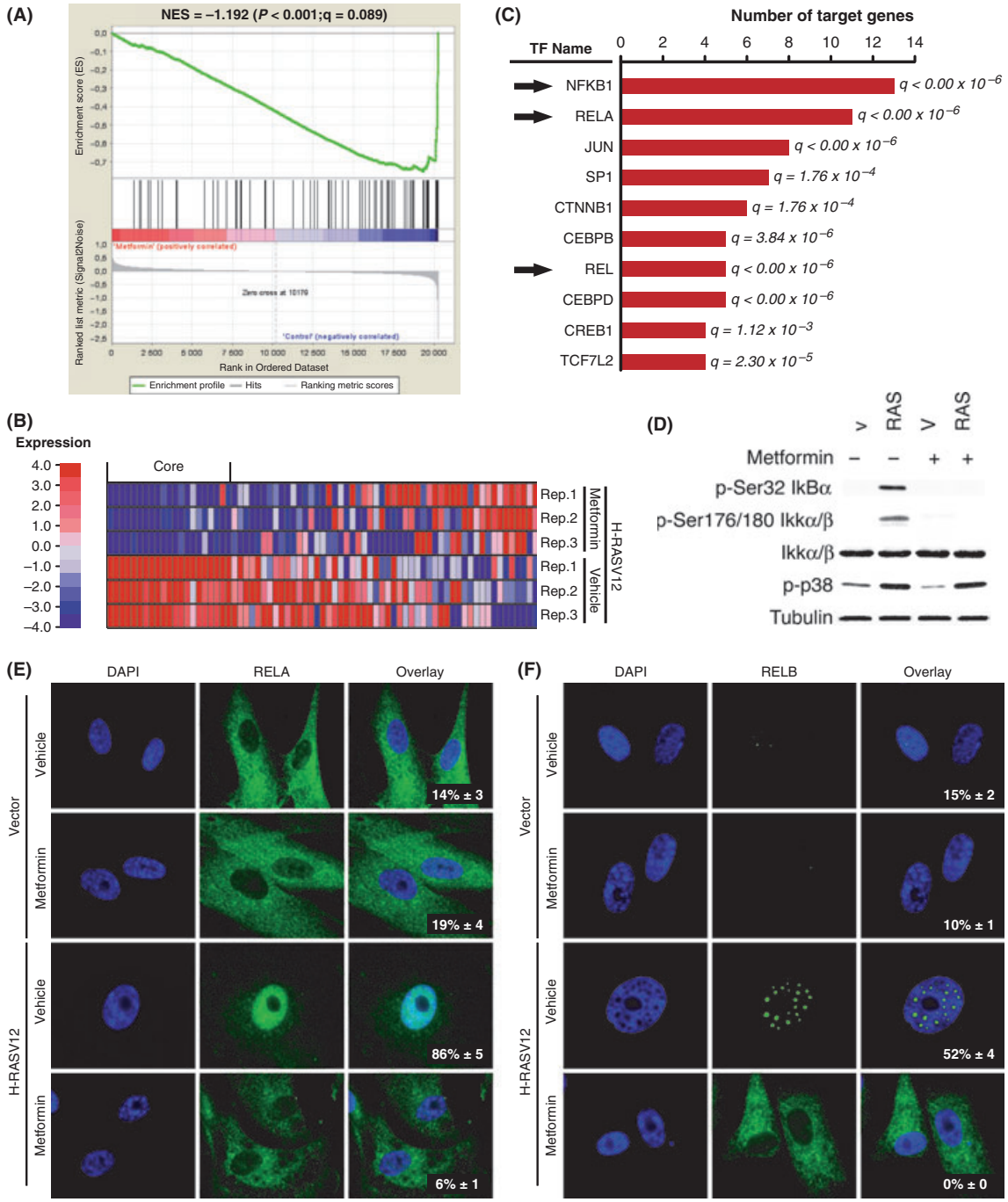

Fig. 3 Metformin inhibits the IKK/NF-κB signal transduction pathway. (A) Gene Set Enrichment analysis (GSEA) revealed that a gene set related to cytokines regulated by NF-κB was significantly decreased following treatment of H-RASV12 expressing cells with 5.0 mM metformin. The Normalized Enrichment Score (NES), the nominal *P*-value determined by an empirical phenotype-based permutation test procedure and the false discovery rate (FDR; q-value) are indicated. (B) Heat map of relative expression levels of the genes used in the GSEA analysis (Red = upregulated, blue = downregulated). The microarrays data obtained from three replicates are shown. (C) TFactS prediction of downregulated transcription factors in H-RASV12 expressing cells treated with 5.0 mM metformin for genes coding for cytokines and their receptors as identified in Fig. 1C. For each transcription factor, the number of target genes found in the list of 26 submitted genes present in the TFactS database is shown. The minimum False Discovery Rate (FDR) is indicated (q-value). (D) Immunoblots for the indicated proteins from extracts of IMR90 cells expressing an empty vector (V) or oncogenic *ras* (RAS) and treated with 5 mM metformin or vehicle for 6 days. (E–F) Indirect immunofluorescence against RELA (E) or RELB (F) of cells as in (D). The percent of cells with RELA or RELB in the nucleus is indicated at the bottom right of the overlay panel, standard deviations of three counts of a 100 cells each is indicated, $n = 3$.

NF-κB, ROS, and inflammatory cytokines have all been implicated in the physiopathology of aging. In fact, bioinformatics analysis of microarray data from several aged tissues indicated that NF-κB was the transcription factor most associated to changes in gene expression during aging (Adler *et al.*, 2007) and reducing NF-κB activity attenuated the accelerated aging phenotype of a mouse model of progeria (Tilstra *et al.*, 2012). Metformin can extend life span in rodents (Anisimov *et al.*, 2011) and worms (Onken &

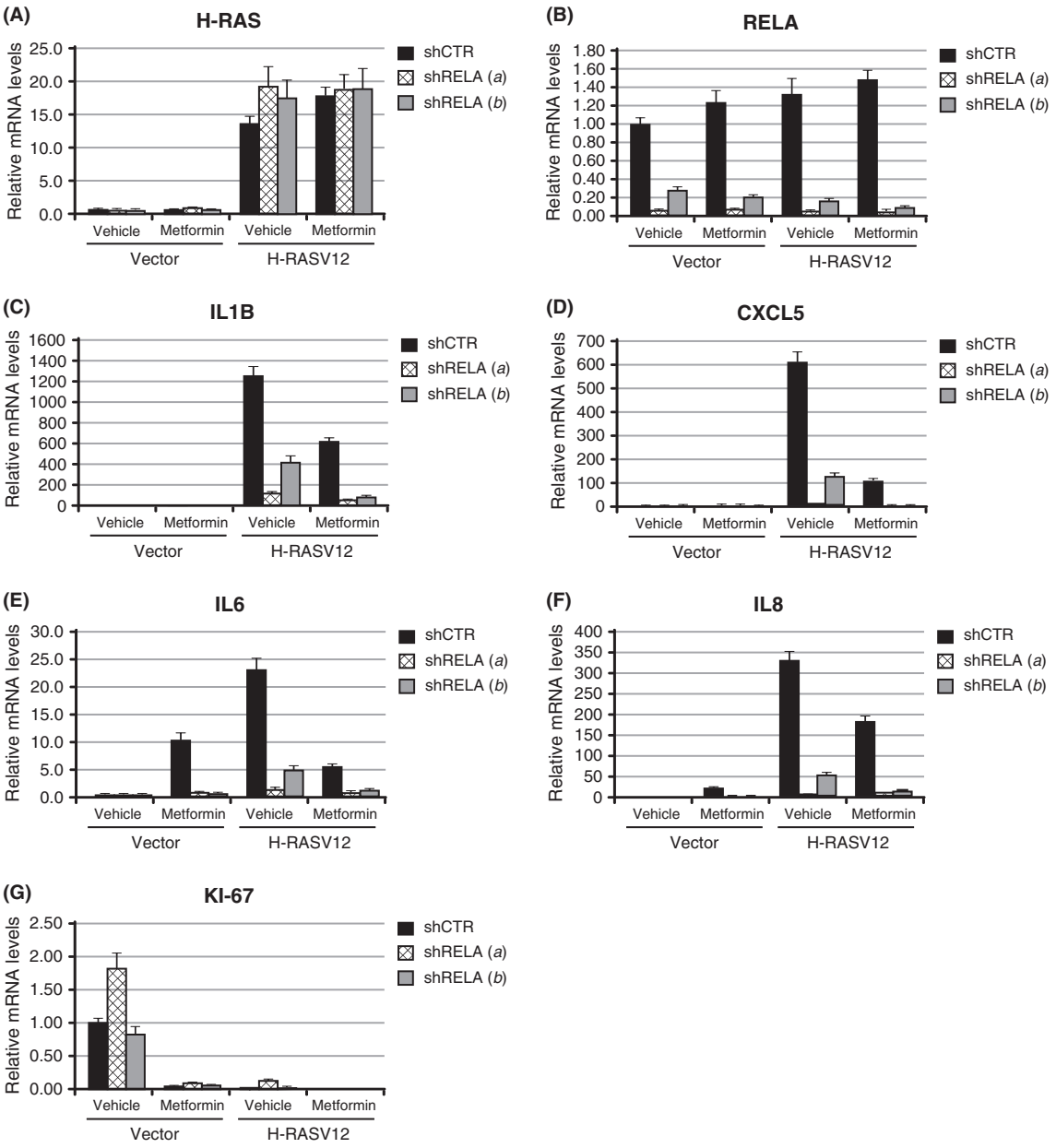

Fig. 4 Metformin and RELA act on the same pathway to control the SASP. QPCR of HRAS, RELA and several cytokines genes that are highly expressed in senescent cells expressing oncogenic *ras* and are repressed by shRELA(a), shRELA(b) or shRELA (a or b) plus 5.0 mM metformin for 6 days. Error bars represent ± SD of replicates.

Driscoll, 2010). In patients with impaired fasting glucose, high doses of metformin (3 g/day for 90 days) reduced the levels of several inflammatory cytokines accompanied by an improvement in insulin sensitivity and a reduction of blood free fatty acids (Krysiak & Okopien, 2012). Our data suggest that these beneficial clinical effects can be explained by metformin-mediated inhibition of NF-κB. In addition, the secretion of cytokines by senescent cells may have pro-cancer effects (Krtolica *et al.*, 2001) and inhibit the action of anticancer chemotherapy (Sun *et al.*, 2012). Hence, anti-inflammatory actions of metformin may provide novel opportunities for cancer prevention or treatment. However, the dose of metformin used for the treatment of diabetes was chosen to improve hyperglycemia, and this exposure level may or may not be optimal for antineoplastic or anti-inflammatory effects. Thus, our work motivates study of metformin pharmacokinetics in the context of endpoints beyond glycemic control.

Materials and methods

Cell Growth and retroviral infection

Normal lung human diploid fibroblasts IMR90 (CCL-186, ATCC, Manassas, VA, USA) and the murine macrophage cell line RAW264.7 (ATCC) were cultured in DMEM (Invitrogen, Logan UT,

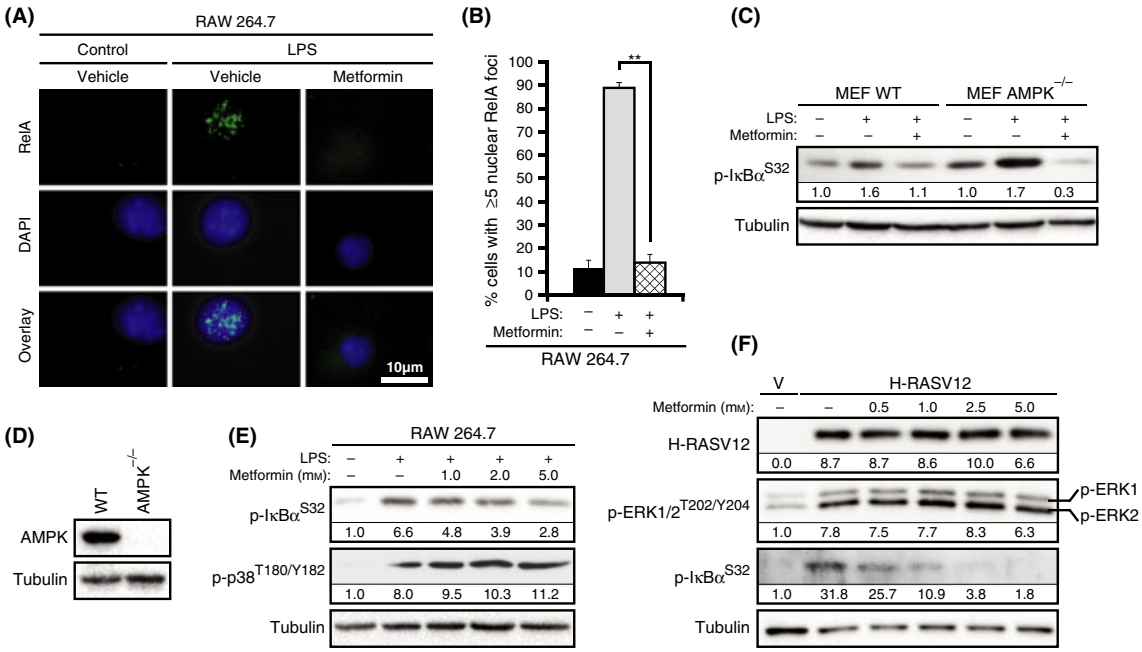

Fig. 5 Metformin inhibits NF-κB activation by lipopolysaccharide (LPS) in fibroblasts and macrophages. (A) Indirect immunofluorescence against RelA in murine macrophages (RAW 264.7) treated with LPS (500 ng/mL) and 5.0 mM metformin or vehicle for 24 h. (B) Quantitation of immunofluorescence data in (A), the percentage of cells and the standard deviation were calculated with three independent counts of 100 cells; $^{**}P < 0.005$ according to a two-sample Student's *t*-test. (C) Immunoblots for phospho-IκBα from extracts with wild-type and *ampk* null MEFs treated with LPS as in (A) and 5.0 mM metformin or vehicle for 48 h. (D) Immunoblots for Ampk from extracts with wild-type and *ampk* null MEFs. (E) Immunoblots for phospho-IκBα and phospho-p38 MAPK in RAW 264.7 macrophages treated with LPS or vehicle and metformin for 24 h at the indicated concentrations. (F) Immunoblots for phospho-IκBα and RAS/ERK signaling proteins in fibroblasts expressing RASV12 or an empty vector (V) control treated with the indicated concentrations of metformin for 6 days.

USA) supplemented with 10% FBS (Wisent, Montreal, QC, Canada) and 1% penicillin G/streptomycin sulfate (Wisent). AMP-activated protein kinase α (AMPKα)+/+ and AMPKα−/− mouse embryonic fibroblasts (MEFs) were provided by Dr. Russell Jones (McGill University) and were cultured in 10% FBS DMEM (Wisent) with glutamine. LPS (Sigma, Oakville, ON, USA) was used at 500 ng/mL. Retroviral vector pBabe, pWZL, pBabe H-RASV12, pWZLH-RASV12, and shRNAsRELA(a) and (b) were from S.W. Lowe (Chien *et al.*, 2011). IMR90 cells were infected with retroviruses expressing RASV12 and selected during 2 days with 2.5 μg/mL puromycin or 75 μg/mL hygromycin depending on the vectors. Twenty-four hours after infection, cells were treated with five different concentrations of metformin (Sigma) or water (vehicle) for 6 days. Infection and senescence protocols were described in (Moiseeva *et al.*, 2009).

Effects of conditioned medium from senescent cells on prostate cancer cells

Senescent IMR90 (H-RASV12) or control IMR90 (vector) were treated with 5 mM metformin for 48 h. Fresh medium without metformin was used the last 24 h to generate conditioned medium (CM). CM was then filtered (with 45-μm, to prevent cell transfer and added on PC3 cells (8×10^4 cells per 6 cm plates). PC3 cells were then grown for 3 days and fixed in 1% glutaraldehyde. Relative cell number was estimated using a crystal violet incorporation assay as previously described (Ferbeyre *et al.*, 2000). Each condition was carried out in triplicate.

RNA analysis

Total mRNA extracts were prepared in TRIzol (Invitrogen), and their purification was performed according to the manufacturer's instructions. For cDNA preparation, 2 μg of total RNA was reverse transcribed using the RevertAid H minus first strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada). Reverse transcription products were amplified by real-time quantitative PCR using TaqMan PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with TBP gene as endogenous control in Fig. 1 or with SyberGreen for all other figures with both TBP and HMBS as endogenous controls as previously described (Vernier *et al.*, 2011). The lightCycler 480 Real-Time PCR System (Roche Applied Science, Laval, QC, Canada) was used to detect the amplification level and was programmed to an initial step of 10 min at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 min at 60 °C. The TBP (TATA-binding protein) was used as endogenous control (Applied Biosystems, #4326322E-0705006). The relative quantification of target genes was determined using the ΔΔCT method. Primers used for qPCR and detailed procedure for microarrays and bioinformatics are presented in the supplementary data.

Western blot

For Western blot analysis, total protein extracts were separated on SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). Membranes were incubated with the primary

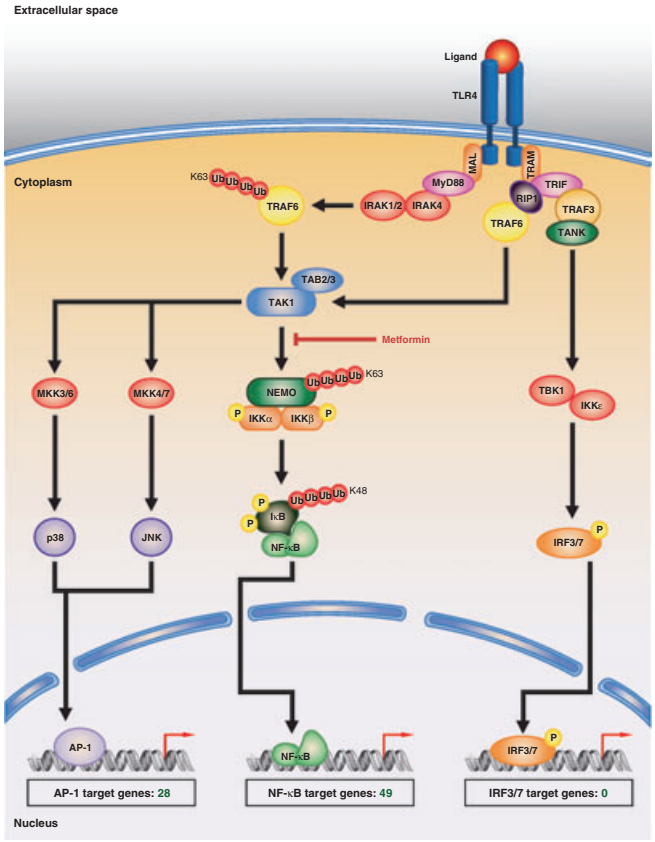

Fig. 6 Model of metformin action of the TLR signaling pathway. The drug blocks the proinflammatory NF-κB but does not affect the antiproliferative interferon pathway. In addition, as p38MAPK is not affected by metformin, its site of action must be between TAK1 and IKK kinases. Number of genes affected by AP1, NF-κB, and IRF3/IRF7 are shown at the bottom. Interferon genes were not affected by metformin, while multiple NF-κB and AP1 genes were affected. However, 16 of 29 AP1-affected genes were also NF-κB genes.

antibodies: anti-AMPKα (#2532, 1:1000, Cell Signaling Technology, Pickering, ON, Canada), antiphosphoThr202/Tyr204 ERK1/2 (1:2000; clone D13.14.4E, #4370, Cell Signaling), anti-H-RAS (1:250; clone F235, Sc-29, Santa Cruz Biotechnology, Santa Cruz, CA, USA), antiphosphoSer32 IkBα (1:1000, #2859 Cell Signaling), antiphospho-Ser176/180 Ikkα/β (1:1000 #2694 Cell Signaling), antiphospho Thr180/Tyr182 p38MAPK (1:1000, #9211 Cell Signaling), and antitubulin (1:5000, T5168, Sigma). Signals were revealed after incubation with anti-mouse or anti-rabbit secondary antibodies, coupled to peroxidase (BioRad, Hercules, CA, USA) using enhanced chemiluminescence (ECL, Amersham, Baie d'Urfe, QC, Canada).

Immunofluorescence

Cells were fixed as described (Vernier *et al.*, 2011). After washing with PBS and 3% bovine serum albumin (BSA) coverslips were incubated with anti-p65 RELA antibody (1:200, #8242 Cell Signaling) for 2 h at room temperature. Then, coverslips were washed with PBS and incubated with goat anti-mouse AlexaFluor 488 (1:1000; A-11001, Molecular Probes, Invitrogen, Logan, UT, USA) for 1 h at room temperature. Finally, coverslips were rinsed with PBS and incubated with 300 nM DAPI for 10 min. Images were acquired with an inverted microscope Nikon TE2000U and processed with the software Metamorph.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 Biological functions analysis of microarrays data obtained from oncogenic *ras* expressing cells treated with metformin.

Fig. S2 Metformin inhibits Toll-like receptors (TLRs) signaling to NF-κB.

Fig. S3 Metformin inhibits genes regulated by NF-κB and C/EBPδ.

Data S1. Materials and methods.