Revised Version JOCES/2013/128835

IκBζ is a regulator for the senescence-associated secretory phenotype in DNA damage- and oncogene-induced senescence

Eva Alexander[1], Dominic G. Hildebrand[1], Anna Kriebs[1], Kerstin Obermayer[1], Marianne Manz[1], Oliver Rothfuss[1], Frank Essmann[1*], Klaus Schulze-Osthoff[1,2*]

[1]Interfaculty Institute for Biochemistry, Eberhard Karls University Tübingen, 72076 Tübingen, Germany; [2]German Cancer Consortium (DKTK) and German Cancer Research Center, 69120 Heidelberg, Germany

[*]equal senior authorship

Running title: IκBζ-dependent SASP induction

Address correspondence to: Klaus Schulze-Osthoff, Interfaculty Institute for Biochemistry, University of Tübingen, D-72076 Tübingen, Germany; Phone: +49+7071-2973399; Fax +49+7071-294017; Email: kso@uni-tuebingen.de

JCS Advance Online Article. Posted on 18 June 2013

Summary

Cellular senescence, a state of sustained cell cycle arrest, has been identified as an important anti-tumor barrier. Senescent cells secrete various growth factors and cytokines, such as IL6 and IL8, which collectively constitute the senescence-associated secretory phenotype (SASP). The SASP can signal to the tumor environment and elicit the immune-mediated clearance of tumor cells or, depending on the context, could potentially promote tumor progression. Despite the importance of the SASP to tumor biology, its regulation remains rather unknown. Here, we show that IκBζ, an atypical member of the inhibitor of NFκB proteins and selective coactivator of particular NFκB target genes, is an important regulator of SASP expression. Several models of DNA damage- and oncogene-induced senescence revealed a robust induction of IκBζ expression. RNAi-mediated knockdown of IκBζ impaired IL6 and IL8 expression, whereas exogenous IκBζ expression resulted in enhanced SASP cytokine expression. Importantly, during senescence of IκBζ knockout cells induction of IL6 and IL8 but not of the cell cycle inhibitor $p21^{WAF/CIP1}$ was completely abolished. Thus, we propose a distinguished and hitherto unappreciated role of IκBζ for SASP formation in both DNA damage- and oncogene-induced senescence.

Keywords: Cytokines, DNA damage, IκBζ, NFκB, SASP, senescence

Abbreviations: CDK, cyclin-dependent kinase; C/EPB, CCAAT/enhancer-binding protein; HP, heterochromatin protein; IκB, inhibitor of NFκB; IKK, IκB kinase; IL, interleukin; MCP, monocyte chemoattractant protein; MEF, mouse embryonic fibroblast; NFκB, nuclear factor kappaB; SA-β-gal, senescence-associated β-galactosidase; SAHF, senescence-associated heterochromatin foci; SASP; senescence-associated secretory phenotype; WT, wildtype.

Introduction

In addition to apoptosis, cellular senescence has been recognized as a potent tumor-suppressive mechanism that is induced by various stress stimuli including the exposure to chemotherapeutic drugs or irradiation, telomer erosion or abnormal oncogene activation (Adams, 2009; Kuilman et al., 2010; Campisi, 2013; Acosta and Gil, 2012). A hallmark of senescent cells is a sustained cell cycle arrest that is established by the p53 and retinoblastoma tumor-suppressive pathways and maintained by an upregulation of the $p16^{Ink4a}$ and $p21^{WAF/CIP1}$ cyclin-dependent kinase (CDK) inhibitors. Cells undergoing senescence display a number of characteristic features, such as a flattened and enlarged morphology and increased senescence-associated β-galactosidase (SA-β-gal) activity, which is often used as a marker to detect senescent cells (Dimri et al., 1995). Senescent cells also show widespread chromatin modifications, known as senescence-associated heterochromatin foci (SAHF), which contain chromatin repression marks, such as phospho-HP1γ, HMGA1, histone H3 trimethylation at lysine-9, or the histone variant macroH2A (Narita et al., 2003; Adams, 2007; Sulli et al., 2012).

In addition, it has been identified that senescent cells secrete a number of growth and immune factors, a feature termed senescence-associated secretory phenotype (SASP) or senescence-messaging secretome (Coppe et al., 2008; Coppe et al., 2010; Kuilman and Peeper, 2009). Secreted factors include several interleukins, chemokines, growth factors and matrix metalloproteinases. It was proposed that the SASP may act as a doubled-edged sword and, depending on the physiological context, can exert tumor-promoting or suppressive activities. Several SASP factors have the ability to promote tumor cell proliferation, invasion or angiogenesis (Krtolica et al., 2001). On the other hand, more recent evidence shows that interleukin-6 (IL6) or IL8, which are conserved key factors of the SASP, could reinforce the senescent growth arrest by promoting reactive oxygen production or by enhancing the DNA

damage response in a positive feedback loop (Kuilman et al, 2008; Acosta et al., 2008). Finally, these cytokines or other secreted factors have been shown to attract immune cells in an inflammatory response, leading to the elimination of senescent tumor cells (Kang et al., 2011; Xue et al., 2007; Hoenicke and Zender, 2012).

Despite the potential importance of the SASP, so far little is known about its mechanism of regulation, its relation to DNA damage or its impact on different cancer types. It was observed that inhibition of the DNA damage-responsive kinases ATM and Chk2 prevents the release of some SASP components, whereas p53 obviously restrains SASP formation (Rodier et al., 2009). Important transcriptional activators of the SASP are NFκB and C/EBPβ as well as p38 kinase, which appears to be required for sustained NFκB activation (Kuilman et al., 2008; Rovillain et al., 2011; Chien et al., 2011; Jing et al., 2011; Freund et al., 2011). Furthermore, membrane-bound IL1α has been proposed as a general autocrine regulator of the SASP, because blockade of IL1 signaling reduced IL6 and IL8 secretion in senescent human fibroblasts (Orjalo et al., 2009). It was reported that transcription of these cytokines was dependent on IL1-mediated NFκB activation. Yet, it is largely unknown how NFκB, which is certainly a master regulator of the SASP, is activated during senescence.

NFκB is classically activated by the IκB kinase (IKK)-induced phosphorylation and subsequent proteasomal degradation of its cytoplasmic inhibitor IκBα, which enables NFκB's nuclear translocation and transcriptional activation of a diverse array of target genes involved in various biological processes (Oeckinghaus et al., 2011). Recent evidence, however, suggests that the activation of NFκB target genes is more complex and dependent on the particular target gene context or stimulus, which is thought to facilitate a selective gene regulation in distinct physiological settings (Smale, 2011). Whereas rapid activation of primary response genes is directly induced by the classical NFκB pathway, expression of so-called secondary response genes is delayed and requires prior protein synthesis of additional

co-regulators. In this context, we and others have recently identified the inhibitor of NFκB ζ (IκBζ), an atypical nuclear IκB protein, which is not regulated by phosphorylation-induced degradation and can act as a repressor but, more importantly, also as an activator of a selective subset of NFκB target genes (Totzke et al., 2006; Yamazaki et al., 2001, Kitamura et al., 2000). IκBζ itself is a primary response target gene and, by association with the NFκB subunit p50, is thought to exert its transcription-enhancing activity on secondary response genes mainly at the level of chromatin remodeling (Kayama et al., 2008; Yamazaki et al., 2008).

In the present study, we found that IκBζ is upregulated in several models of senescence. Moreover, we demonstrate that expression of IL6 and IL8, two highly conserved SASP cytokines, requires IκBζ in both DNA damage- and oncogene-induced senescence, thus establishing IκBζ as an essential novel regulator of SASP formation.

Results

IκBζ expression is induced in DNA damage and oncogene-induced senescence. Previously, we reported that ionizing radiation induces senescence rather than apoptosis in the p53 wild type-expressing human breast cancer cell line MCF7 (Essmann et al., 2005). In order to establish MCF7 cells as a model for irradiation-induced senescence, we investigated further senescence markers, in addition to the enlarged morphology and SA-β-galactosidase activity (Fig. 1A). Immunofluorescence microscopy of γ-irradiated MCF7 cells revealed typical punctuate structures of phosphorylated heterochromatin protein 1γ (pHP1γ) in the nuclei (Fig. 1A), and real-time PCR (RT-PCR) demonstrated an approximately 10-fold higher expression of the CDK inhibitor p21 (Fig. 1B) 5 days post irradiation. Furthermore, mRNA expression of the central SASP cytokines IL6 and IL8 (Fig. 1B) as well as of other SASP components (data

not shown) was significantly enhanced in γ-irradiated cells. We confirmed cytokine secretion by analyzing cell culture supernatants in flow cytometric bead assays that also showed enhanced expression of IL6 and IL8 (Fig. 1C). Interestingly, in addition to the established SASP factors, also expression of IκBζ, which was almost undetectable in control cells, was increased about 5-fold in senescent MCF7 cells (Fig. 1B).

In order to verify the results from irradiation-induced senescence, we additionally investigated therapy-induced senescence and therefore incubated MCF7 cells with bleomycin, a DNA-damaging chemotherapeutic drug. Similar to irradiation, bleomycin treatment induced the typical senescence alterations, including an enlarged morphology, increased SA-β-gal activity as well as a strong nuclear recruitment of pHP1γ (Fig. 1D). Moreover, compared to control cells, RT-PCR analyses revealed a more than 15-fold higher expression of p21 as well as a strong induction of IL6 and IL8 mRNA and protein expression (Fig. 1E, F). Importantly, the expression of IκBζ was increased 3.6-fold, indicating that upregulation of IκBζ is independent of the senescence-inducing stimulus.

To confirm that IκBζ is generally induced during senescence, we further investigated oncogene-induced senescence by Ras^{G12V} expression (Serrano et al., 1997). To this end, we took advantage of the pInducer lentiviral vector system (Meerbrey et al., 2011) and generated MCF7/Ras^{G12V} cells that expressed oncogenic Ras^{G12V} protein under the control of the tetracycline-responsive element (TetOn). Doxycycline treatment resulted not only in a strong induction of Ras^{G12V}, but concomitantly induced senescence alterations including increased p21 expression, SA-β-gal activity and the characteristic pHP1γ staining (Fig. 2A-C). Furthermore, in concert with p21, IL6 and IL8 mRNA expression were significantly induced (Fig. 2D). The expression of the senescence markers was again associated with a strongly increased IκBζ expression, indicating that, regardless of the stimulus, IκBζ is induced in both DNA-damage and oncogene-induced senescence.

IκBζ modulates expression of SASP components. IκBζ has previously been identified as an important regulator of IL6 expression in the Toll-like receptor/IL1 receptor pathway (Yamamoto et al., 2004; Seshadri et al., 2009). To investigate a functional role of IκBζ for SASP formation, we transfected MCF7/Ras^{G12V} cells with an siRNA targeting IκBζ. Due to the long time course of the experiments, siRNA transfection only resulted in an approximately 50% reduction of IκBζ expression (Fig. 2E). Nevertheless, despite the incomplete knockdown, downregulation of IκBζ upon Ras-induced senescence resulted in a significantly reduced IL6 and IL8 expression compared to a non-targeting control siRNA (Fig. 2E). In contrast, mRNA expression of Ras and p21 remained unaffected by the IκBζ-specific siRNA during doxycycline treatment (Fig. 2E).

In order to corroborate IκBζ-dependent expression of SASP components, we next generated a HeLa cell line for the doxycycline-inducible expression of IκBζ. Doxycycline treatment of the resulting cell line HeLa/TetOn-IκBζ triggered a robust protein expression of IκBζ (Fig. 3A) At the mRNA level, IκBζ expression was increased more than 20-fold, which was further induced during irradiation-induced senescence (Fig. 3B, C). In line with the previous experiments, exogenous expression of IκBζ resulted in strongly enhanced IL6 and IL8 expression (Fig. 3D, E). Unlike the SASP components, p21 was only slightly but not significantly affected by doxycycline-induced IκBζ expression (Fig. 3F). Thus, in different senescence models modulation of IκBζ levels affects SASP cytokine expression.

To further substantiate the role of IκBζ for SASP formation, we employed mouse embryonic fibroblasts (MEFs) from IκBζ$^{-/-}$ mice. MEFs from wildtype (WT) and IκBζ$^{-/-}$ mice were induced to undergo senescence by irradiation or incubation with bleomycin. Both treatments resulted in senescence as verified by a β-galactosidase assay of cytosolic extracts (Fig. 4A, D). In line with the results in MCF7 cells, neither irradiation nor bleomycin

treatment resulted in a significant difference in p21 induction in IκBζ-deficient and WT MEFs (Fig. 4B, E). However, IκBζ-deficient MEFs were almost completely unable to upregulate IL6 and the murine IL8 orthologue CXCL1, whereas both cytokines were strongly induced in WT MEFs upon irradiation. In addition, IκBζ deficiency completely abolished the induction of IL6 and CXCL1 upon bleomycin treatment. These results were additionally confirmed by the measurement of IL6 and CXCL1 cytokines in the culture supernatant of control and senescent WT and IκBζ$^{-/-}$ MEFs. The amount of IL6 and CXCL1 protein was exclusively increased in supernatants from senescent WT MEFs but not from IκBζ$^{-/-}$ MEFs (Fig. 4C, F). Thus, these data further underscore our results of the knock-down experiments in MCF7 cells and unambiguously establish IκBζ as an indispensable regulator for the central SASP components IL6 and IL8/CXCL1.

IκBζ mediates SASP formation independently of IL1α. In human fibroblasts expression of SASP cytokines was previously proposed to depend on autocrine IL1α signaling (Orjalo et al., 2008). To delineate the position of IκBζ in IL1α signaling and SASP formation, we first incubated MCF7 cells with IL1α, which clearly induced IκBζ as well as IL6 and IL8 (Fig. 5A). IL1α-mediated induction of IκBζ as well as of the cytokines was strongly impaired by the clinical IL1 receptor antagonist Anakinra (Dinarello et al., 2012). In contrast, blockade of the IL1 receptor pathway by Anakinra did neither affect induction of IκBζ nor IL6 and IL8 expression in irradiated MCF7 cells (Fig. 5B). In addition, upon incubation of MCF7 cells with bleomycin, the induction of IκBζ or the SASP components remained largely unaffected by Anakinra (Fig. 5C), indicating that IL1α is not required for senescence-induced IκBζ or cytokine expression.

As IL1α has been proposed to mediate SASP induction (Orjalo et al., 2009), we wished to further corroborate our data in another experimental system and therefore used MEFs

deficient in MyD88, an essential component of the IL1 signaling pathway (Gay et al., 2011). As expected, IL1α induced upregulation of IκBζ, IL6, and CXCL1 in WT MEFs, but not in MyD88$^{-/-}$ cells (Fig. 5C). In contrast, we detected no significant differences in the upregulation of IκBζ and the cytokines between WT and MyD88$^{-/-}$ MEFs upon senescence induction by either irradiation or bleomycin treatment (Fig. 5D, E). Hence, our data suggest that not only IκBζ can be regulated independently of IL1 signaling, but that also for expression of the SASP components IL1 signaling is dispensable.

IκBζ modulates a subset of SASP factors. In addition to NFκB, other transcription factors might be involved in the regulation of SASP formation. To identify which SASP components are modulated by IκBζ, we performed RT-PCR analyses for additional SASP genes in WT and IκBζ$^{-/-}$ MEFs. Both bleomycin treatment as well as irradiation led to strong induction of the mRNA levels of monocyte chemoattractant protein-1 (MCP1, CCL2) in WT but not IκBζ-deficient MEFs (Fig. 6A), indicating that MCP1 is regulated by IκBζ. In contrast, there were no significant differences in the senescence-associated induction of IL15 mRNA levels (Fig. 6B). Moreover, we found that, in addition to IL6, CXCL1/IL8 and MCP1, bleomycin-induced transcription of MCP2 and MIF was suppressed in IκBζ-deficient as compared to WT cells (Fig. 6C). In contrast, the mRNA levels of other SASP products, such as TIMP1, TIMP2 and fibronectin, were even more strongly induced in IκBζ-deficient cells, which might be due to the fact that IκBζ can additionally function as an inhibitor of particular target genes (Totzke et al., 2006; Yamazaki et al., 2001). In conclusion, IκBζ drives the expression of a subset of SASP factors, but these include in particular potent chemokines.

Discussion

The present study demonstrates that the atypical nuclear IκB protein, IκBζ, is an essential mediator required for the induction of conserved SASP cytokines. Although a few recent studies have already suggested an involvement of NFκB, in particular of its subunit p65, in SASP formation (Rovillain et al., 211, Chien et al., 2011; Jing et al., 2011; Freund et al., 2011), it has become clear that NFκB target genes can be classified in two groups, namely primary and secondary response genes, of which the latter require additional co-factors for transcriptional activation (Smale, 2011). Our results suggest that several SASP components, including IL6, IL8, MCP1 and others belong to those secondary response genes that require prior induction of IκBζ. IκBζ itself is a primary NFκB target, whose transcriptional induction depends on the proteasomal degradation of cytoplasmic IκBα and subsequent NFκB p65 activation, suggesting that expression of the SASP components requires a two-step mechanism (Fig. 7). It is currently thought that such gene-specific regulation and mechanistic diversity between a primary and secondary NFκB response ensure a selectivity and precise control of NFκB target gene activation in distinct physiological settings, including apoptosis, inflammation, senescence and other NFκB-controlled processes.

We studied different models of senescence in human and murine cells and show that IκBζ is induced in both oncogene- and DNA damage-induced senescence. Although we have tried several commercial antibodies, immunoblot analyses did only yield reliable data on IκBζ protein expression in the HeLa overexpression system. This might be caused by the fact that, in contrast to acute proinflammatory stimulation with e.g. IL1 or LPS, SASP formation requires a more sustained but weaker inflammatory and DNA damage response (Adams, 2009; Kuilman et al., 2010; Campisi, 2013; Acosta and Gil, 2012). In fact, the induction of secondary response genes is generally delayed compared to primary NFκB response genes that are immediately induced. Nevertheless, we show that even a partial RNAi-mediated

knockdown of IκBζ is sufficient to reduce mRNA and protein levels of IL6 and IL8 during SASP formation. *Vice versa*, the inducible expression of IκBζ led to a strong potentiation of IL6 and IL8 expression during senescence. Even more convincingly, we show that during senescence of IκBζ-deficient MEFs induction of IL6 and IL8 expression was completely abolished.

Although the secretory features of the SASP are probably cell type- and stimulus-specific, IL6 and IL8 have been identified as the most conserved and robustly expressed SASP cytokines (Campisi, 2013; Kuilman and Peeper, 2009). In tumors these factors can attract immune cells leading to immune surveillance and subsequent elimination of senescent cells. In addition to the proinflammatory effects, IL6 and IL8 have been shown to reinforce the growth arrest and thereby contribute to the senescence process. For instance, it was demonstrated that depletion of IL6 abolished oncogene-induced senescence and suppressed SAHF formation and $p15^{Ink4b}$ expression (Kuilman et al., 2008). In addition, IL8 can increase reactive oxygen production and exacerbate DNA damage (Acosta et al., 2008). Although we have not studied such amplification loops in detail, we found no significant difference in the expression of β-galactosidase or p21 in the presence or absence of IκBζ. Thus, autocrine or paracrine effects might also depend on the senescent tumor cell type.

In addition to IL6 and IL8/CXCL1, MCP1 was identified as an IκBζ-dependent SASP cytokine, a finding that is in line with our observation that MCP1 expression in macrophages strictly requires IκBζ (unpublished data). MCP1 is one of the most potent chemokines and involved in the recruitment of macrophages, favoring tumor cell clearance and cancer regression (Deshmane et al., 2009). On the other hand, MCP1 expression has been identified as a prometastatic factor during senescence of melanoma cells (Ohanna et al., 2011).

The SASP comprises more than 40 different factors that are certainly not all controlled by IκBζ. In fact, we found that senescence-associated expression of TIMPs and fibronectin

was apparently even suppressed by IκBζ. In addition to NFκB, C/EBPβ has been implicated in SASP expression (Kuilman et al., 2008). Interestingly, NFκB and C/EBPβ often show synergistic effects in diverse settings of gene regulation (Oeckinghaus et al., 2011). Moreover, there is a close interdependence of C/EBPβ and IκBζ in the regulation of secondary response genes. Recent analyses surprisingly revealed that IκBζ is required for the recruitment of C/EBPβ to the lipocalin-2 promoter, which is an established IκBζ target gene (Yamazaki et al., 2011). In addition, Kuilman *et al.* (2009) showed by chromatin immunoprecipitation that C/EBPβ was present at both the IL6 and IL8 promoters, suggesting a cooperative role of IκBζ and C/EBPβ for SASP induction.

The mechanism of how DNA damage triggers NFκB activation and subsequent induction of primary target genes such as IκBζ is unclear. Although p53 is not required for SASP formation, available evidence suggests that NFκB activation is mediated by the DNA damage-responsible ATM kinase, resulting in IKK activation and IκBα degradation (Miyamoto, 2011). In this context, however, different mechanisms of ATM activation, involving its interaction with IKK subunit NEMO as well as with SUMO ligase PIASγ, RIG1, PARP1 and presumably additional molecules have been proposed (Miyamoto, 2011; Liu et al., 2011; Hinz et al., 2011; Ohanna et al., 2011). Thus, although the detailed mechanisms of NFκB activation during senescence are unclear, genotoxic stress seems to be linked to NFκB activation via ATM. Interestingly, it was reported that ATM signaling does not regulate the entire SASP, although ATM was required for the secretion of IL6 and IL8 (Rodier et al., 2009).

Senescence is accompanied by massive alterations in chromatin structure and the epigenetic silencing of E2F-regulated genes driving cell proliferation. It is worth mentioning that IκBζ-mediated gene expression largely depends on histone modification and nucleosome remodeling of secondary target genes (Kayama et al., 2008; Yamazaki et al., 2008). It will

therefore be interesting to investigate whether IκBζ also influences repressive chromatin marks, such as H3K9 trimethylation in genes of SASP factors.

So far, NFκB has been implicated in tumor biology mainly due to its anti-apoptotic effect involving the transcriptional activation of several apoptosis inhibitors, such as FLIP, XIAP and several anti-apoptotic Bcl2 proteins (Perkins, 2012). In view of this anti-apoptotic but potentially pro-senescent role, activation of NFκB might be considered as a double-edged sword. Using gene expression arrays of WT and IκBζ-deficient cells we interestingly found that, in contrast to chemo- and cytokines, IκBζ is not involved in the transcriptional regulation of anti-apoptotic genes (unpublished data). Thus, it is tempting to speculate that also in this respect IκBζ might confer specificity to the NFκB response.

Materials and Methods

Cell lines, reagents and antibodies. MCF7 cells were maintained in RPMI-1640 medium (PAA Laboratories, Linz, Austria), supplemented with 10% fetal calf serum (FCS; PAA Laboratories) and antibiotics (MycoZapPlus-CL; Lonza, Cologne Germany). WT, IκBζ$^{-/-}$ (Shiina et al., 2004) and MyD88$^{-/-}$ (Adachi et al., 1998) MEFs were cultured in DMEM (PAA Laboratories) containing 10% FCS and antibiotics. HeLa/TetOn-IκBζ and MCF7/TetOn-Ras^{G12V} cells were maintained in RPMI-1640 medium supplemented with 10% tetracycline-free FCS (PAA Laboratories), 400 μg/mL neomycin (PAA Laboratories) and antibiotics. For IL1α stimulation, MEFs were cultured in the presence of murine IL1α (100 ng/mL; ImmunoTools, Friesoythe, Germany) for 1 h and collected by scraping, before mRNA expression was analyzed by quantitative (q) RT-PCR. All chemicals were purchased from Sigma (Munich, Germany). Monoclonal mouse anti-β-actin (AC-74, Sigma), monoclonal mouse anti-p21 (Becton Dickinson, Heidelberg, Germany) and polyclonal rabbit antisera

against Ras (C-20, Santa Cruz Biotechnology, Heidelberg, Germany) and IκBζ (Hildebrand et al., 2013) were used for immunoblot analysis. Immunofluorescence staining was performed using polyclonal rabbit anti-phospho-serine-83 HP1γ (Abcam, Cambridge, UK) and monoclonal mouse anti-α-tubulin (Sigma). Horseradish-coupled secondary antibodies to mouse, rabbit and goat IgG were purchased from Promega (Mannheim, Germany). Secondary 488-/594-Alexa Fluor-coupled antibodies to mouse and rabbit IgG were purchased from Molecular Probes (Life Technologies, Darmstadt, Germany).

Cloning of pInducer vectors. IκBζ and Ras^{G12V} cDNAs were PCR-amplified using the primers IκBζ (5'-CACCATGATTGTGGACAAGCTGCTGGAC-3'; 5'-CTAATACGGTGG AGCTCTCTGCTGAATGG-3') and Ras^{G12C} (5'-CACCATGACGGAATATAAGCTGGT-3'; 5'-TCAGGAGAGCACACACTTGCAGCTC-3'). PCR products were ligated into pENTR-D-TOPO (Life Technologies) according to the manufacturer's protocol yielding pENTR/IκBζ and pENTR/Ras^{G12V}. The pInducer20/TetOn-IκBζ and pInducer20/TetOn-Ras^{G12V} lentiviral vectors were generated using gateway cloning technology (Life Technologies) by recombining pInducer20 plasmid (Meerbrey et al., 2011) with pENTR/IκBζ and pENTR/Ras^{G12V}, respectively, using LR-Clonase II (Life Science Technologies) according to the manufacturer's protocol. Vector sequences were confirmed by sequencing.

Transfection and viral transduction. HEK293FT cells were cultured in DMEM supplemented with 10% FCS, 1% L-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, neomycin (400 μg/mL) and antibiotics. Cells were seeded 24 h before transfection in culture dishes at a density of 6 x 10^5 cells/cm^2. The pInducer20/TetOn-IκBζ and pInducer20/TetOn-Ras^{G12V} plasmids were cotransfected with lentiviral assembly ΔR8.9 and envelope (VSV)g plasmids cells using jetPEI reagent (PEQLAB, Erlangen, Germany). 48 h post transfection

virus-containing culture supernatant was collected and concentrated to 250 μL with filter vials (Sartorius AG, Göttingen, Germany). MCF7 or HeLa cells were transduced with virus-containing supernatant in the presence of polybrene (4 μg/mL; Sigma, Munich, Germany) for 48 h. Transduced cells were selected in the presence of neomycin (600 μg/mL) for 7 days and analyzed for target gene overexpression upon addition of doxycycline (2 μg/mL; Fagron, Barsbüttel, Germany) by immunoblotting.

For siRNA transfection of MCF7/Ras^{G12V} cells 1x10^5 cells/cm^2 were seeded in culture dishes in the presence of doxycycline (200 ng/ml) to induce Ras^{G12V} expression and either IκBζ ON-TARGET Plus Smartpool siRNA or non-targeted (NT) Smartpool ON-TARGET plus control siRNA (Thermo Fisher Scientific, Bonn, Germany) was delivered using Dharmafect I reagent (Thermo Fisher Scientific) after 24 h and 72 h. Cells were grown for additional 48 h, collected by scraping and analyzed for mRNA expression by qRT-PCR. For IL1α stimulation 1x10^5 MCF7/wt cells per cm^2 were seeded and transfected with siRNA after 24 h. 48 h post siRNA transfection recombinant IL1α (100 ng/mL, ImmunoTools) was added to the culture medium for 1 h. Then, cells were harvested by scraping and mRNA expression was analyzed by qRT-PCR. Anakinra (Swedish Orphan Biovitrum; Stockholm, Sweden) was added at a concentration of 1 µg/mL 24 h prior to irradiation or treatment with bleomycin or IL1α and replenished daily.

Senescence induction. Cells were γ-irradiated with 20 Gy (MCF7), 15 Gy (MEF), and 10 Gy (HeLa). Therapy-induced senescence was achieved by treatment with bleomycin (50 µg/mL). Cells were collected 5 days post senescence induction by scraping. For Ras-induced senescence MCF7/TetOn-Ras^{G12V} cells were incubated for 3 days in the presence of doxycycline (200 ng/mL) and for further 4 days in the absence of doxycycline. Samples from cells cultured tetracycline-free medium for identical periods of time served as control.

Immunoblot analysis. Cells were washed in ice-cold PBS and resuspended in lysis buffer [1% Nonidet P-40, 20 mM HEPES (pH 7.9), 2 mM PMSF, 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA and 0.5 mM DTT] supplemented with complete protease inhibitor cocktail (Roche, Mannheim, Germany). Protein concentrations were determined using the BCA assay (Thermo Fisher Scientific), and 15 μg of protein per lane were loaded onto standard SDS-PAGE gels. After electrophoresis proteins were transferred onto polyvinylidenedifluoride membranes (Amersham Biosciences, Freiburg, Germany) by tank blotting. Membranes were blocked in PBS containing 4% BSA and 0.05 % Tween-20) for 1 h, followed by an overnight incubation with the primary antibody in blocking buffer at 4°C. After washing the membrane thrice in blocking buffer, the secondary antibody (1:5000) was applied for 1 h. Proteins were visualized using ECL reagents (Amersham Biosciences).

Quantitative real-time PCR. Total RNA was isolated from cells using the RNeasy kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. Complementary DNA synthesis and qRT-PCR were performed as described (Graupner et al., 2011). Primers for SASP analyses were purchased from Qiagen (QuantiTect Primer Assays) or Sigma. For IκBζ the following primers were used: human IκBζ (5'-CCTTTCAAGGTGTTCGGGTA-3', 5'-CAAGCAGGTCCATCAGACAA-3'), mouse IκBζ (5'-TATCGGGTGACACAGTTGGA-3', 5'-TGAATGGACTTCCCCTTC AG-3'). Results were normalized to GAPDH and analyzed by the $\Delta\Delta C_t$ method to give fold mRNA expression compared with untreated control samples.

Analysis of senescence-associated β-galactosidase (SA-β-gal) expression. Cells were seeded 24 h before senescence induction by γ-irradiation, bleomycin stimulation or Ras^{G12V} overexpression in six-well plates at a density of 5 x 10^4 cells/cm^2. 5 days later SA-β-gal

activity was assessed using the Senescence Cells Histochemical Staining Kit (Sigma) according to the manufacturer's protocol. Alternatively, SA-β-gal activity was assayed in cell lysates. To this end, cells were harvested by scraping, washed in ice-cold PBS and lysed for 30 min on ice in lysis buffer (20 mM Tris-HCl pH 7.4, 0.2% Triton-X-100) supplemented with complete protease inhibitor cocktail. 5 μl of protein lysate were incubated with 45 μl reaction buffer (66 mM Na_2HPO_4, 66 mM NaCl, 33 mM citric acid, freshly prepared 2 mM 4-methylumbelliferone) at 37°C for 1 h. Reaction was stopped by adding 200 μl 0.2 M Na_2CO_3 followed by centrifugation at 14000 rpm for 5 min. Fluorescence of samples was detected at 355 nm excitation and 460 nm emission in an Infinite M200 plate reader (Tecan, Männerdorf, Switzerland). Based on a methylumbelliferone standard curve, enzyme activity was calculated and normalized to protein concentrations.

Immunofluorescence microscopy. Cells were seeded 24 h before senescence induction onto coverslips (5 mm^2) in 12-well plates. 5 days after senescence induction by γ-irradiation or bleomycin treatment and 7 days after oncogene-induced senescence induction the cells were washed with PBS and fixed with ice-cold fixation solution (50% acetone, 50% methanol) for 5 min. Cells were washed twice with PBS, followed by incubation for 1 h in blocking buffer (4% BSA and 0.05% saponin in PBS) at room temperature. The primary antibody diluted (1:500) in blocking buffer was incubated at 4°C overnight. After washing the cells thrice in blocking buffer, the appropriate Alexafluor-coupled secondary antibody (1:500 in PBS) was applied for 1 h. The cells were washed thrice in PBS and incubated afterwards in PBS containing 100 ng/mL 4',6-diamidino-2-phenylindol (DAPI; Life Technologies) for 5 min. Coverslips were mounted in fluorescence mounting medium (DAKO, Hamburg, Germany) and analyzed using a DMI6000 fluorescence microscope (Leica, Wetzlar, Germany).

Statistical analysis. Data are presented as the mean ± SD or SEM from at least three independent experiments. Statistical significance was calculated using Student's t-test. Values of $p < 0.05$ were considered significant. *: $p<0.05$; **:$p<0.005$, ***: $p<0.0005$.

Acknowledgements

We thank Stephen J. Elledge, Roland Piekorz, Masami Morimatsu and Gudrun Totzke for providing various materials. This work was supported by grants from the Deutsche Forschungsgemeinschaft (SFB773, SFB685), BMBF (AID-NET) and the Deutsche Krebshilfe.

Conflict of interest

The authors declare no conflict of interest.

References

Acosta, J. C. and Gil, J. (2012). Senescence: a new weapon for cancer therapy. *Trends Cell Biol.* **22,** 211-219.

Acosta, J. C., O'Loghlen, A., Banito, A., Guijarro, M. V., Augert, A., Raguz, S., Fumagalli, M., Da Costa, M., Brown, C., Popov, N., Takatsu, Y., Melamed, J., d'Adda di Fagagna, F., Bernard, D., Hernando, E. and Gil, J. (2008) Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell* **133,** 1006-1018.

Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K. and Akira, S. (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9,** 143-150.

Adams, P. D. (2007). Remodeling of chromatin structure in senescent cells and its potential impact on tumor suppression and aging. *Gene* **397,** 84-93.

Adams, P. D. (2009). Healing and hurting: molecular mechanisms, functions, and pathologies of cellular senescence. *Mol. Cell* **36,** 2-14.

Campisi, J. (2013). Aging, Cellular Senescence, and Cancer. *Annu. Rev. Physiol.* [Epub ahead of print].

Chien, Y., Scuoppo, C., Wang, X., Fang, X., Balgley, B., Bolden, J. E., Premsrirut, P., Luo, W., Chicas, A., Lee, C. S., Kogan, S. C. and Lowe, S. W. (2011). Control of the senescence-associated secretory phenotype by NF-kappaB promotes senescence and enhances chemosensitivity. *Genes Dev.* **25,** 2125-2136.

Coppe, J. P., Desprez, P. Y., Krtolica, A. and Campisi, J. (2010). The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu. Rev. Pathol.* **5,** 99-118.

Coppé, J. P., Patil, C. K., Rodier. F., Sun, Y., Muñoz, D. P., Goldstein, J., Nelson, P. S., Desprez, P. Y. and Campisi, J. (2008). Senescence-associated secretory phenotypes

reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS Biol*. **6,** 2853-2868.

Deshmane, S. L., Kremlev, S., Amini, S. and Sawaya, B. E. (2009). Monocyte chemoattractant protein-1 (MCP-1): an overview. *J. Interferon Cytokine Res.* **29,** 313-326.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O. and Campisi, J. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc. Natl. Acad Sci. USA* **92,** 9363-9367.

Dinarello, C.A., Simon, A. and van der Meer, J. W. (2012). Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nat. Rev. Drug Discov.* **11,** 633-652.

Essmann, F., Pohlmann, S., Gillissen, B., Daniel, P. T., Schulze-Osthoff, K. and Jänicke R. U. (2005). Irradiation-induced translocation of p53 to mitochondria in the absence of apoptosis. *J Biol Chem* **280,** 37169-37177.

Freund, A., Patil, C. K. and Campisi, J. (2011). p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype. *EMBO J.* **30,** 1536-1548.

Gay, N. J., Gangloff, M. and O'Neill, L. A. (2011). What the Myddosome structure tells us about the initiation of innate immunity. *Trends Immunol.* **32,** 104-109.

Graupner V., Alexander, E., Overkamp, T., Rothfuss, O., De Laurenzi, V., Gillissen, B.F., Daniel, P.T., Schulze-Osthoff, K. and Essmann, F. (2011). Differential regulation of the proapoptotic multidomain protein Bak by p53 and p73 at the promoter level. *Cell Death Differ.* **18,** 1130-1139.

Hildebrand, D. G., Alexander, E., Hörber, S., Lehle, S., Obermayer, K., Münck, N. A., Rothfuss, O., Frick, J.-S., Morimatsu, M., Schmitz, I., Roth, J., Ehrchen, J. M., Essmann, F., and Schulze-Osthoff K. (2013). IκBζ Is a Transcriptional Key Regulator of CCL2/MCP-1. *J. Immunol.* **190,** 4812-4820.

Hinz, M., Stilmann, M., Arslan, S. C., Khanna, K. K., Dittmar, G. and Scheidereit, C. (2010). A cytoplasmic ATM-TRAF6-cIAP1 module links nuclear DNA damage signaling to ubiquitin-mediated NF-kappaB activation. *Mol. Cell* **40,** 63-74.

Hoenicke, L., and Zender, L. (2012) Immune surveillance of senescent cells--biological significance in cancer- and non-cancer pathologies. *Carcinogenesis* **33,** 1123-1126.

Jing, H., Kase, J., Dörr, J. R., Milanovic, M., Lenze, D., Grau, M., Beuster, G., Ji, S., Reimann, M., Lenz, P., Hummel, M., Dörken, B., Lenz, G., Scheidereit, C., Schmitt, C. A. and Lee, S. (2011). Opposing roles of NF-kappaB in anti-cancer treatment outcome unveiled by cross-species investigations. *Genes Dev.* **25,** 2137-2146.

Kang, T. W., Yevsa, T., Woller, N., Hoenicke, L., Wuestefeld, T., Dauch, D., Hohmeyer, A., Gereke, M., Rudalska, R., Potapova, A., Iken, M., Vucur, M., Weiss, S., Heikenwalder, M., Khan, S., Gil, J., Bruder, D., Manns, M., Schirmacher, P., Tacke, F., Ott, M., Luedde, T., Longerich, T., Kubicka, S. and Zender, L. (2011). Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature* **479,** 547-551.

Kayama, H., Ramirez-Carrozzi, V. R., Yamamoto, M., Mizutani, T., Kuwata, H., Iba, H., Matsumoto, M., Honda, K., Smale, S. T. and Takeda, K. (2008). Class-specific regulation of pro-inflammatory genes by MyD88 pathways and IkappaBzeta. *J. Biol. Chem.* **283,** 12468-12477.

Kitamura, H., Kanehira, K., Okita, K., Morimatsu, M. and Saito, M. (2000). MAIL, a novel nuclear Ikappa B protein that potentiates LPS-induced IL-6 production. *FEBS Lett.* **485,** 53-56.

Krtolica, A., Parrinello, S., Lockett, S., Desprez, P. Y. and Campisi, J. (2001). Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc. Natl. Acad. Sci. USA* **98,** 12072-12077.

Kuilman, T. and Peeper, D. S. (2009). Senescence-messaging secretome: SMS-ing cellular stress. *Nat. Rev. Cancer* **9,** 81-94.

Kuilman, T., Michaloglou, C., Mooi, W. J. and Peeper, D. S. (2010). The essence of senescence. *Genes Dev.* **24,** 2463-2479.

Kuilman, T., Michaloglou, C., Vredeveld, L. C., Douma, S., van Doorn, R., Desmet, C. J., Aarden, L. A., Mooi, W. J. and Peeper, D. S. (2008). Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell* **133,** 1019-1031.

Liu, F., Wu, S., Ren, H. and Gu, J. (2011). Klotho suppresses RIG-I-mediated senescence-associated inflammation. *Nat. Cell Biol.* **13,** 254-262.

Meerbrey, K. L., Hu, G., Kessler, J.D., Roarty, K., Li, M. Z., Fang, J. E., Herschkowitz, J. I., Burrows, A.E., Ciccia, A., Sun, T., Schmitt, E. M., Bernardi, R. J., Fu, X., Bland, C. S., Cooper, T. A., Schiff, R., Rosen, J.M, Westbrook, T. F., and Elledge, S. J. (2011). The pINDUCER lentiviral toolkit for inducible RNA interference in vitro and in vivo. *Proc. Natl. Acad. Sci. USA* **108,** 3665-3670.

Miyamoto, S. (2011). Nuclear initiated NF-kappaB signaling: NEMO and ATM take center stage. *Cell Res.* **21,** 116-130.

Narita, M., Nunez, S., Heard, E., Narita, M., Lin, A. W., Hearn, S. A., Spector, D. L., Hannon, G. J. and Lowe, S. W. (2003) Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* **113,** 703-716.

Oeckinghaus, A., Hayden, M. S. and Ghosh, S. (2011). Crosstalk in NF-kappaB signaling pathways. *Nat. Immunol.* **12,** 695-708.

Ohanna, M., Giuliano, S., Bonet, C., Imbert, V., Hofman, V., Zangari, J., Bille, K., Robert, C., Bressac-de Paillerets, B., Hofman, P., Rocchi, S., Peyron, J. F., Lacour, J. P., Ballotti, R. and Bertolotto, C. (2011). Senescent cells develop a PARP-1 and nuclear factor-kappaB-associated secretome (PNAS). *Genes Dev.* **25,** 1245-1261.

Orjalo, A. V., Bhaumik, D., Gengler, B. K., Scott, G. K. and Campisi, J. (2009). Cell surface-bound IL-1alpha is an upstream regulator of the senescence-associated IL-6/IL-8 cytokine network. *Proc. Natl. Acad. Sci. USA* **106,** 17031-17036.

Perkins, N. D. (2012). The diverse and complex roles of NF-kappaB subunits in cancer. *Nat. Rev. Cancer* **12,** 121-132.

Rodier, F., Coppé, J. P., Patil, C. K., Hoeijmakers, W. A., Muñoz, D. P., Raza, S. R., Freund, A., Campeau, E., Davalos, A. R. and Campisi, J. (2009). Persistent DNA damage signalling triggers senescence-associated inflammatory cytokine secretion. *Nat. Cell Biol.* **11,** 973-979.

Rovillain, E., Mansfield, L., Caetano, C., Alvarez-Fernandez, M., Caballero, O. L., Medema, R. H., Hummerich, H. and Jat, P. S. (2011). Activation of nuclear factor-kappa B signalling promotes cellular senescence. *Oncogene* **30,** 2356-2366.

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. and Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* **88,** 593-602.

Seshadri, S., Kannan, Y., Mitra, S., Parker-Barnes, J. and Wewers, M. D. (2009). MAIL regulates human monocyte IL-6 production. *J. Immunol.* **183,** 5358-5368.

Shiina, T., Konno, A., Oonuma, T., Kitamura, H., Imaoka, K., Takeda, N., Todokoro, K. and Morimatsu, M. (2004). Targeted disruption of MAIL, a nuclear IkappaB protein, leads to severe atopic dermatitis-like disease. *J. Biol. Chem.* **279,** 55493-55498.

Smale, S. T. (2011). Hierarchies of NF-kappaB target-gene regulation. *Nat. Immunol.* **12,** 689-694.

Sulli, G., Di Micco, R. and d'Adda di Fagagna, F. (2012). Crosstalk between chromatin state and DNA damage response in cellular senescence and cancer. *Nat. Rev. Cancer* **12,** 709-720.

Totzke, G., Essmann, F., Pohlmann, S., Lindenblatt, C., Janicke, R. U. and Schulze-Osthoff, K. (2006). A novel member of the IkappaB family, human IkappaB-zeta, inhibits transactivation of p65 and its DNA binding. *J. Biol. Chem.* **281,** 12645-12654.

Xue, W., Zender, L., Miething, C., Dickins, R. A., Hernando, E., Krizhanovsky, V., Cordon-Cardo, C. and Lowe, S. W. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* **445,** 656-660.

Yamamoto, M., Yamazaki, S., Uematsu, S., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Kuwata, H., Takeuchi, O., Takeshige, K., Saitoh, T., Yamaoka, S., Yamamoto, N., Yamamoto, S., Muta, T., Takeda, K. and Akira, S. (2004). Regulation of Toll/IL-1-receptor-mediated gene expression by the inducible nuclear protein IkappaBzeta. *Nature* **430,** 218-222.

Yamazaki, S., Matsuo, S., Muta, T., Yamamoto, M., Akira, S. and Takeshige, K. (2008). Gene-specific requirement of a nuclear protein, IkappaB-zeta, for promoter association of inflammatory transcription regulators. *J. Biol. Chem.* **283,** 32404-32411.

Yamazaki, S., Muta, T. and Takeshige K. (2001). A novel IkappaB protein, IkappaB-zeta, induced by proinflammatory stimuli, negatively regulates nuclear factor-kappaB in the nuclei. *J. Biol. Chem.* **276,** 27657-27662.

Figure legends

Figure 1. IκBζ expression is induced in DNA damage-induced senescence. MCF7 cells were induced to undergo senescence by γ-irradiation (γIR; *A-C*) or bleomycin treatment *(D-F)*. 5 days post γ-irradiation *(A)* or bleomycin treatment *(D)* cells show enhanced β-galactosidase activity (upper panels), pHP1γ-positive nuclear foci (lower panels) and enlarged morphology. RT-PCR analysis of senescent MCF7 cells reveals enhanced expression of IκBζ, the CDK inhibitor p21, and the SASP cytokines IL6 and IL8 5 days post γ-irradiation *(B)* or bleomycin treatment *(E)*. Cytometric bead assays confirm enhanced levels of IL6 and IL8 in supernatants of senescent MCF7 cells 5 days after irradiation *(C)* or bleomycin treatment *(F*). Results of the RT-PCR analyses and cytokine measurements represent mean values ± SEM from three independent experiments. Scale bars: 100 µm (β-galactosidase staining); 35 µm (fluorescent staining).

Figure 2. Oncogenic Ras^{G12V}-induced senescence involves the expression of IκBζ and SASP components, which is reduced by the knockdown of IκBζ. *(A)* MCF7/TetOn-Ras^{G12V} cells were incubated in absence or presence of doxycycline to induce Ras^{G12V} expression and analyzed after the indicated time for the expression of Ras^{G12V} and the CDK inhibitor p21 by immunoblotting. ß-actin served as control for equal protein loading. After 5 days of doxycycline treatment MCF7/TetOn-Ras^{G12V} cells reveal enhanced β-galactosidase activity *(B)*, pHP1γ foci *(C)* and enlarged morphology. *(D)* qRT-PCR analysis of MCF7 cells induced to undergo senescence reveals enhanced expression of p21, the SASP cytokines IL6 and IL8 and IκBζ. Data represent the mean values relative to cells incubated in the absence of doxycycline from three experiments. *(E)* Knockdown of IκBζ results in reduced expression of IL6 and IL8 mRNA, whereas Ras and p21 mRNA levels remain unaffected. MCF7/TetOn-Ras^{G12V} cells were treated with an IκBζ-specific siRNA or a non-targeted control siRNA and

induced to undergo senescence by 5 days of incubation with doxycycline. Mean values of mRNA expression were calculated as the ratio of mRNA levels in cells transfected with the non-targeted (NT) siRNA. Scale bars: 100 µm (β-galactosidase staining); 35 µm (fluorescent staining).

Figure 3. Inducible expression of IκBζ potentiates irradiation-induced expression of IL6 and IL8 but not p21. HeLa/TetOn-IκBζ cells were incubated for 24 h in the presence or absence of doxycycline and subsequently γ-irradiated or left untreated. *(A)* After three days of further incubation, protein levels of IκBζ were determined by Western blotting. An unspecific protein band (asterisk) served as loading control. *(B)* Increased β-galactosidase activity in doxycycline-treated HeLa/TetON-IκBζ cells confirmed induction of senescence three days post-irradiation (scale bars: 100 µm). In addition, mRNA levels of IκBζ *(C)*, IL6 *(D)*, IL8 *(E)* and p21 *(F)* were determined three days post-irradiation by RT-PCR and calculated as the mean values from 3 independent experiments.

Figure 4. Expression of the SASP cytokines IL6 and IL8 is strongly reduced in the absence of IκBζ. *(A)* Irradiated and *(D)* bleomycin-treated WT and IκBζ$^{-/-}$ MEFs show enhanced SA-β-galactosidase activity after irradiation and bleomycin treatment. qRT-PCR analyses show reduced mRNA expression of IL6 and CXCL1 in IκBζ$^{-/-}$ as compared to WT MEFs 5 days post-irradiation *(B)* and bleomycin treatment *(E)*. *(C, F)*. IL6 and IL8 secretion is reduced in IκBζ$^{-/-}$ as compared WT MEFs 5 days after senescence induction by irradiation *(C)* or incubation with bleomycin *(F)*.

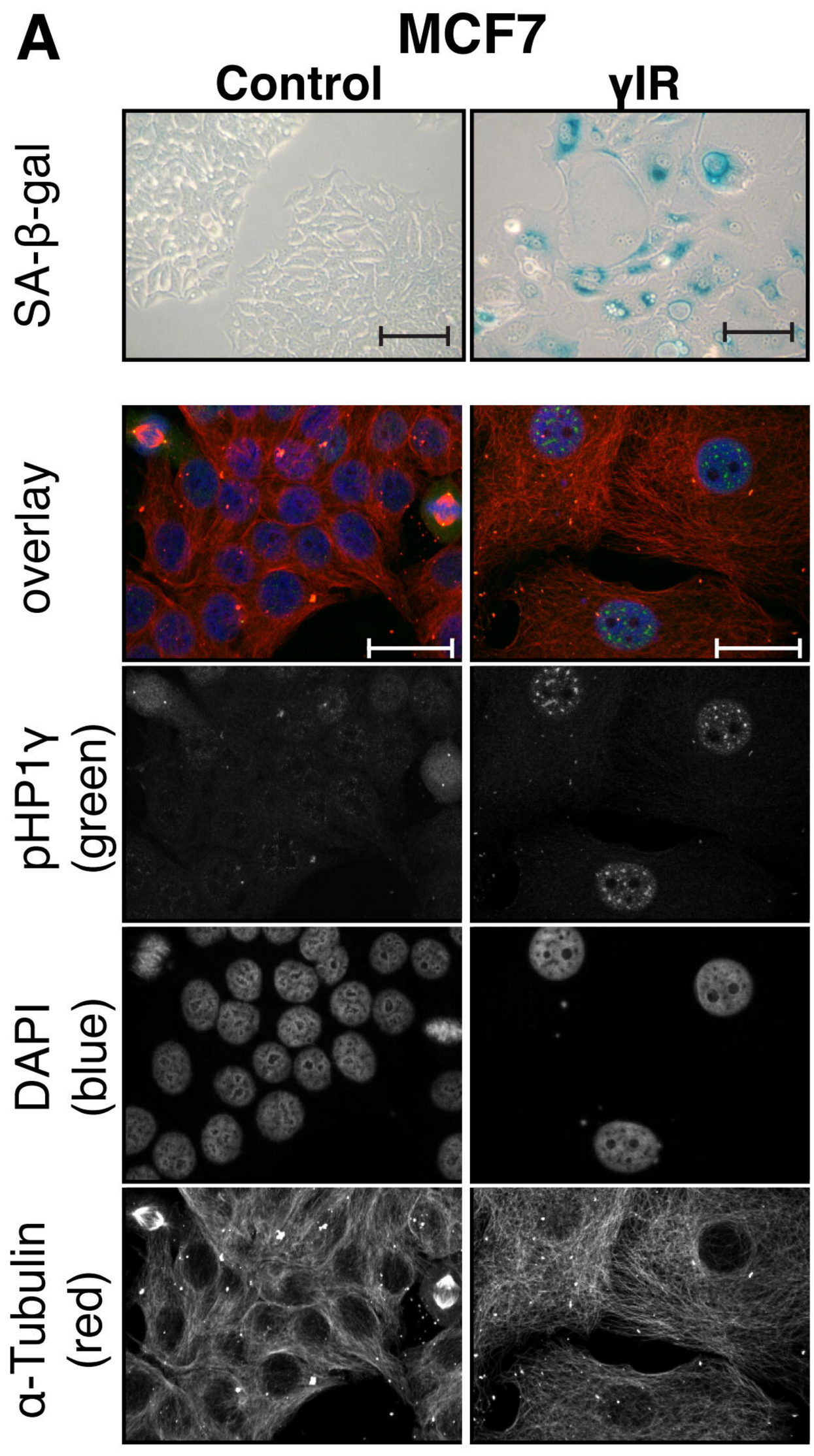
Figure 5. IL1 signaling is dispensable for the expression of SASP cytokines. *(A-B)* The IL1 receptor antagonist Anakinra prevents induction of IκBζ, IL6 and IL8 mRNA after

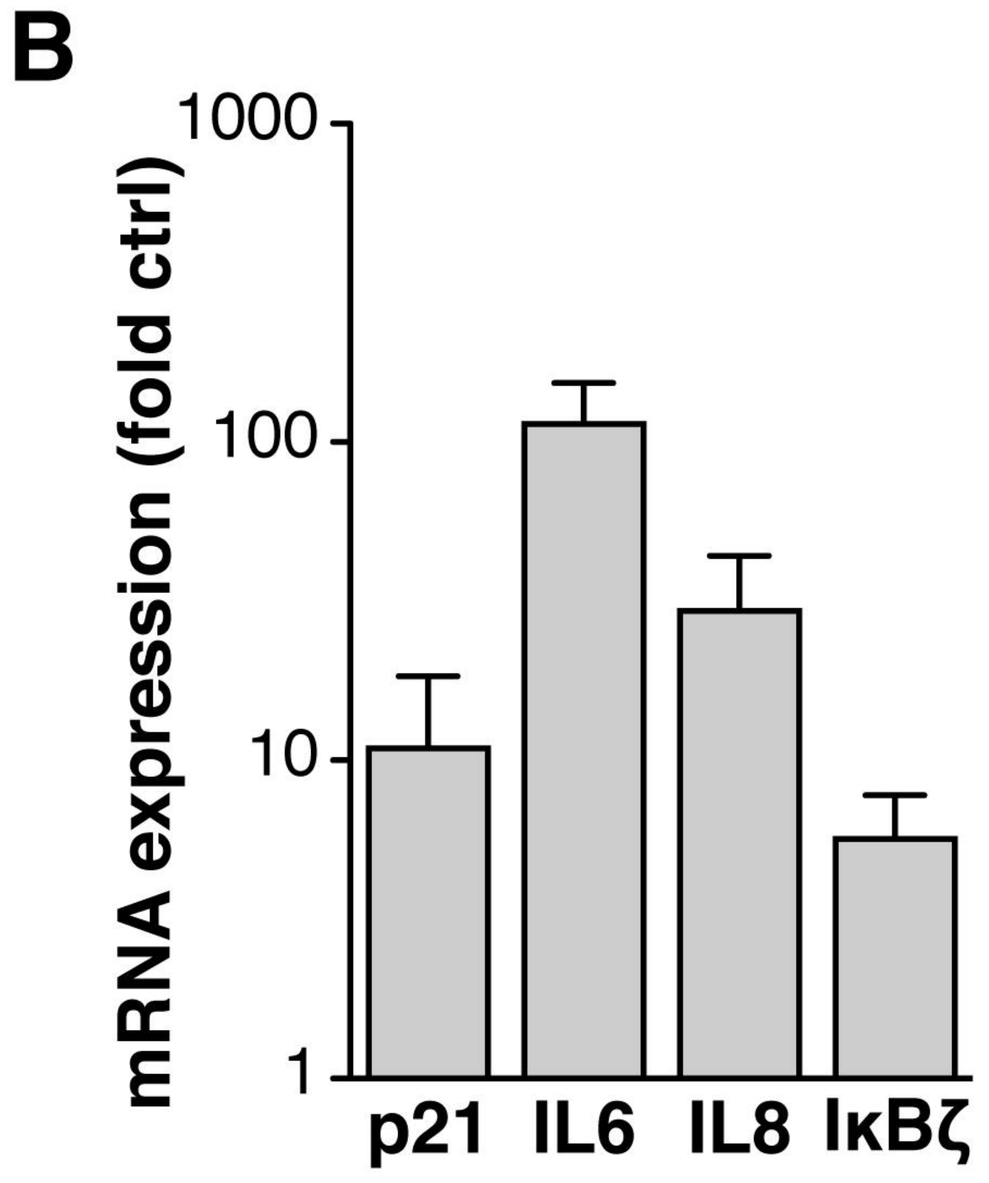
stimulation of MCF7 cells with IL1α but not after senescence induction by γ-irradiation or bleomycin treatment. Cells were either left untreated (Ctrl) or pretreated with Anakinra (Ana) and analyzed by qRT-PCR 5 days after irradiation or bleomycin treatment. *(C)* IL1α- but not bleomycin- or irradiation-induced expression of IκBζ, IL6 and CXCL1 is abolished in MyD88$^{-/-}$ MEFs. WT and MyD88$^{-/-}$ MEFs were stimulated for 1 h with IL1α or induced to undergo senescence after 5 days of bleomycin treatment or irradiation.

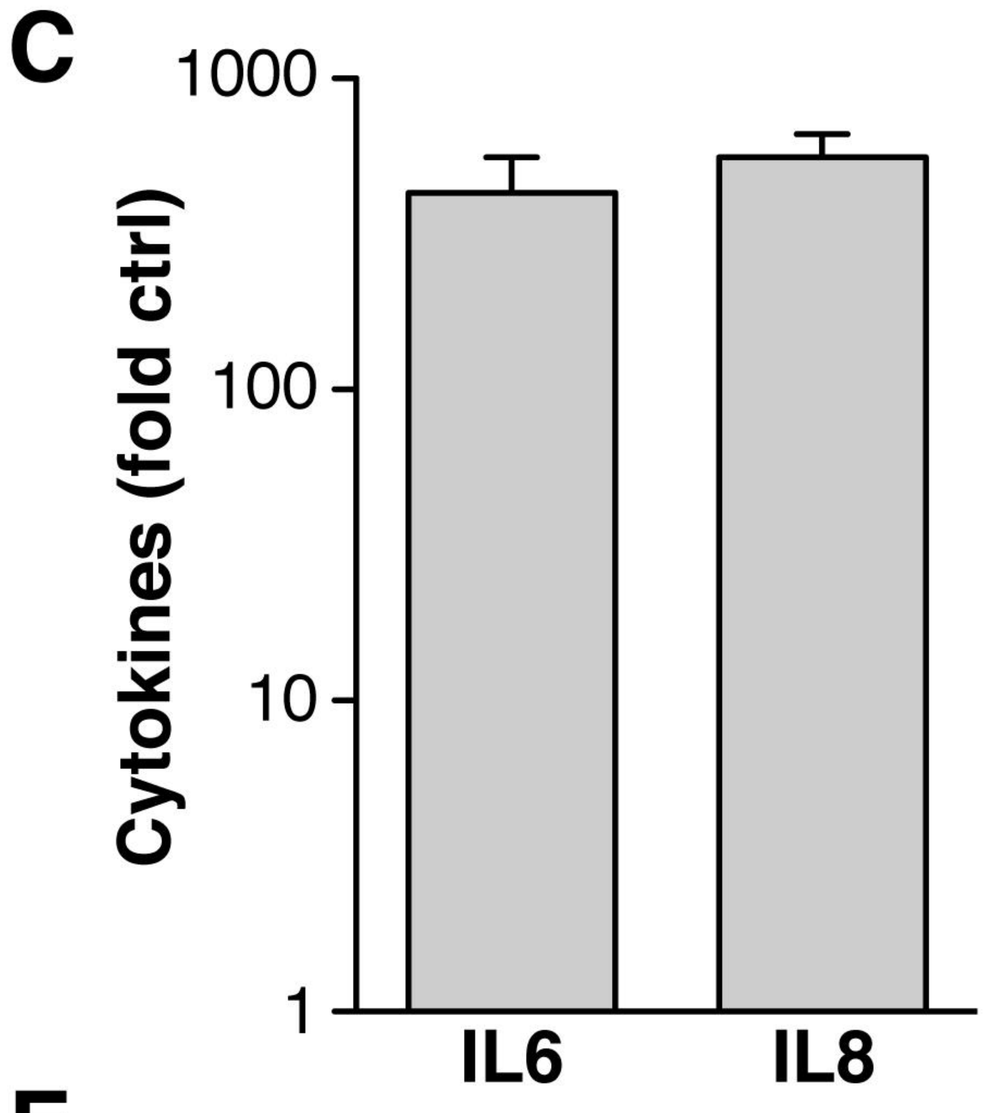
Figure 6. IκBζ modulates a subset of SASP factors. *(A-B)* WT and IκBζ$^{-/-}$ MEFs were incubated with bleomycin or γ-irradiated. After 5 days qRT-PCR analyses were performed showing a strong reduction of senescence-associated MCP1 *(A)* but not IL15 *(B)* expression in IκBζ$^{-/-}$ as compared to WT MEFs. Mean values of three independent experiments were calculated as fold induction compared to untreated cells. (C) Heatmap of several SASP factors. Relative mRNA levels were determined by qRT-PCR in IκBζ$^{-/-}$ and WT MEFs after 5 days of bleomycin treatment. mRNA levels above baseline values are shown in red, levels below baseline are shown in green.

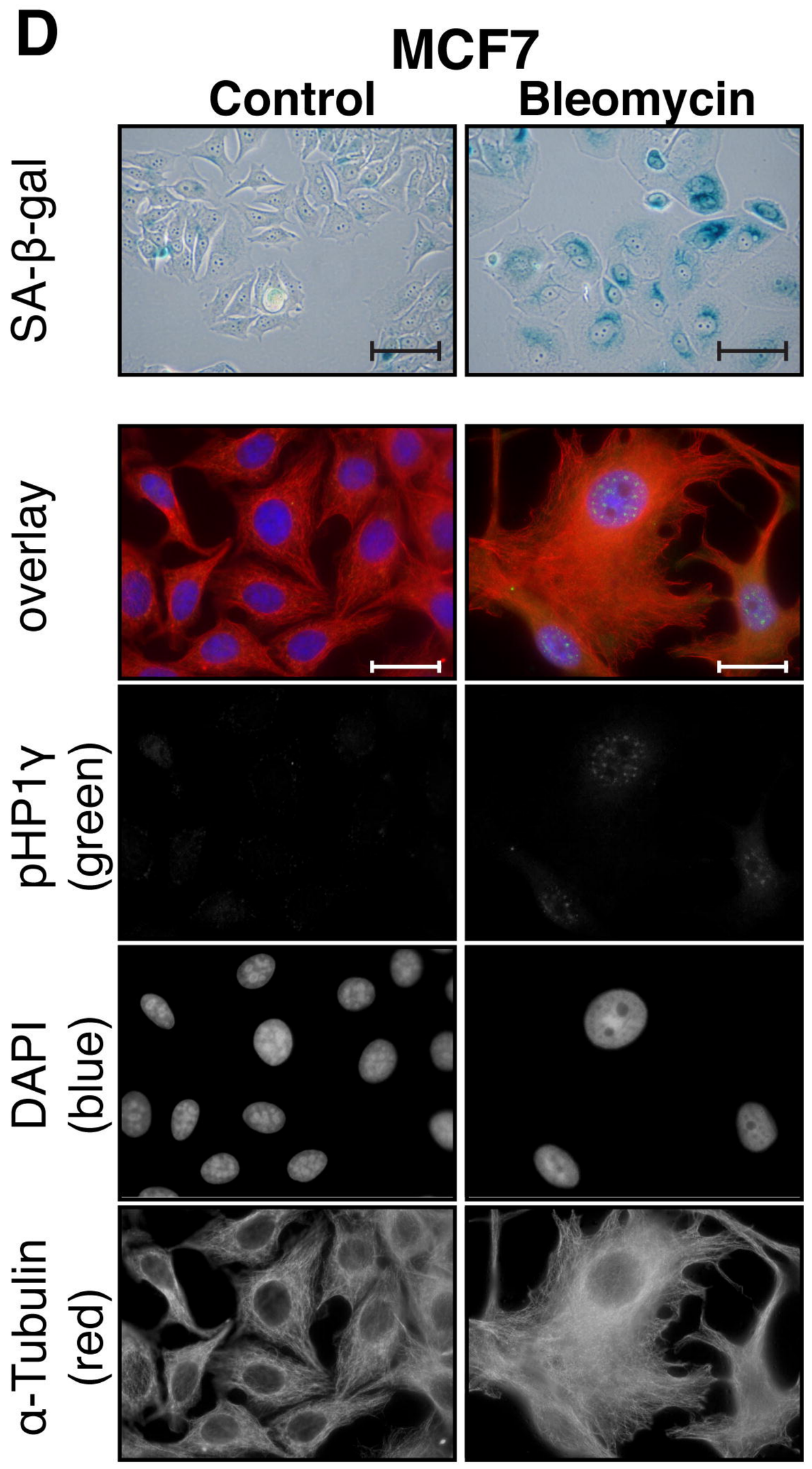
Figure 7. Role of IκBζ in SASP formation. Diverse senescent stimuli trigger the activation of the classical NF-κB p65/p50 dimer resulting in the activation of primary response genes and synthesis of IκBζ. Several SASP components, such as IL6, MCP1 and others, are encoded by secondary genes, which require IκBζ and its interaction with the p50 DNA-binding subunit for chromatin remodeling and transcriptional activation.

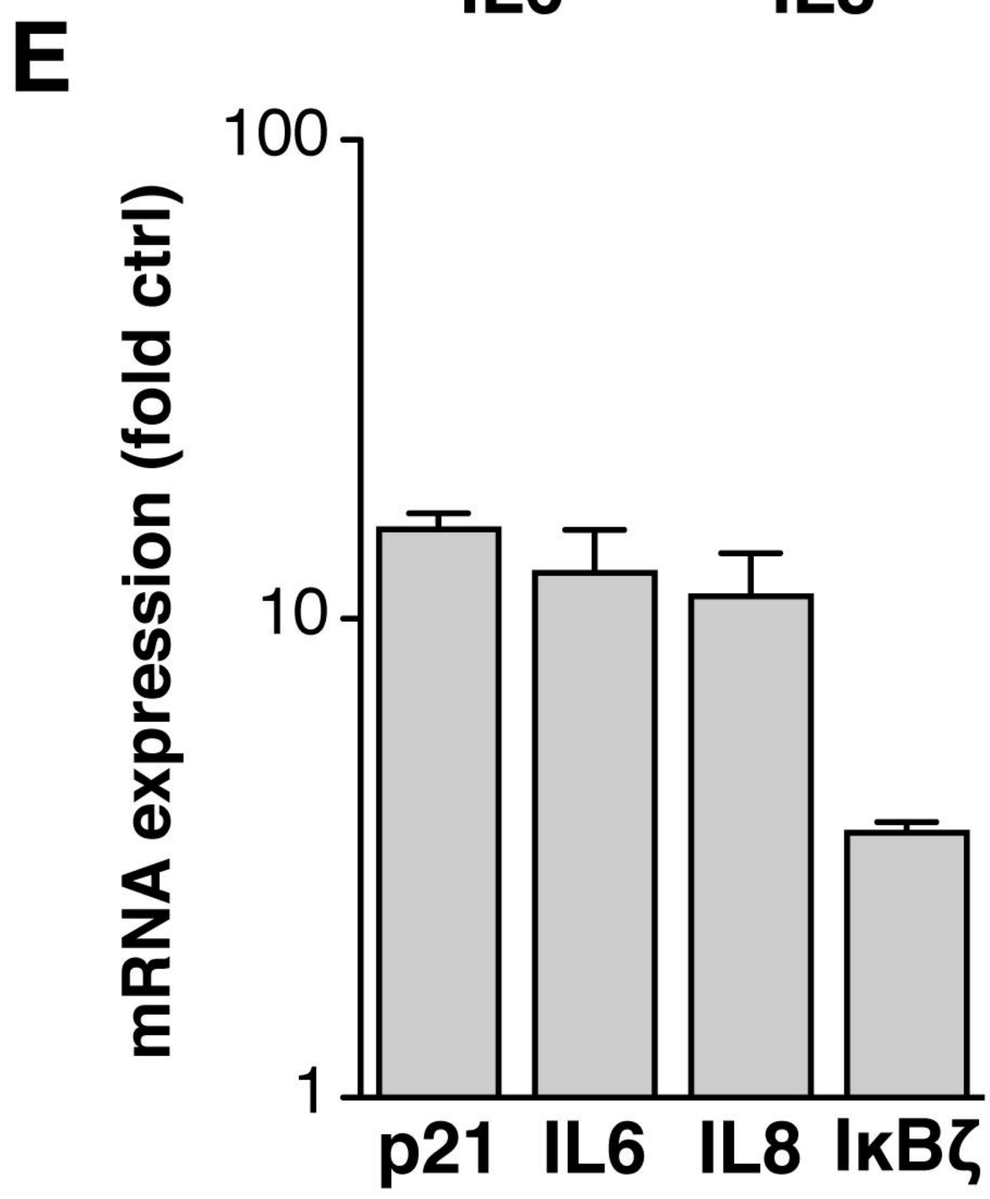
Figure 1











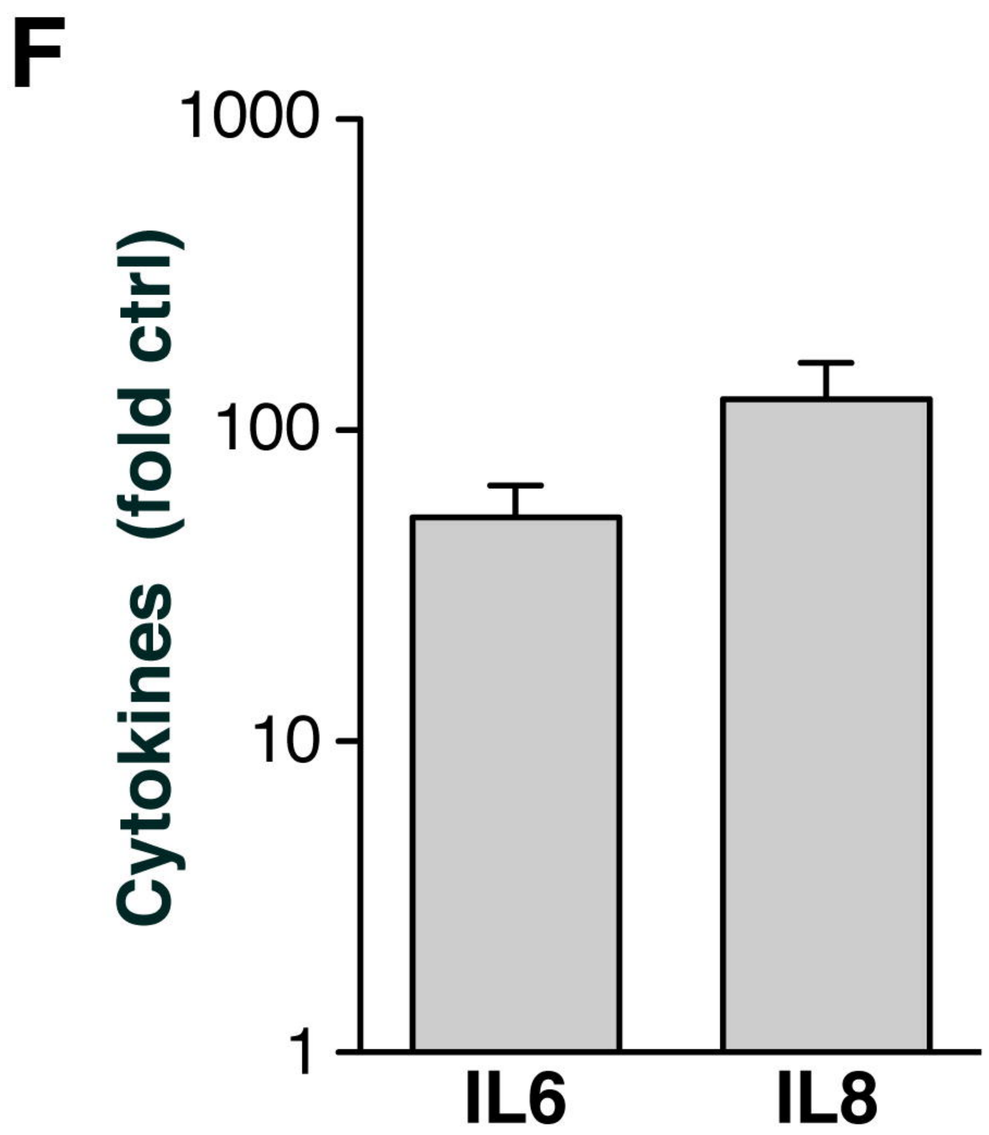
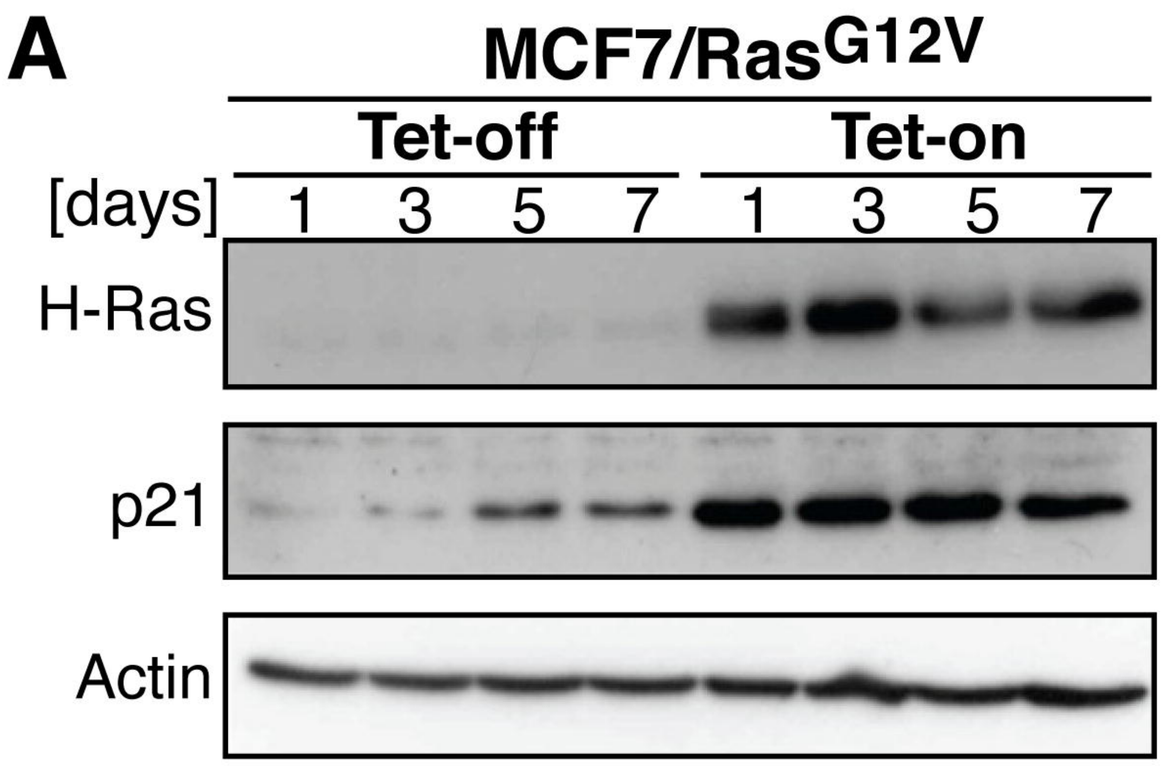
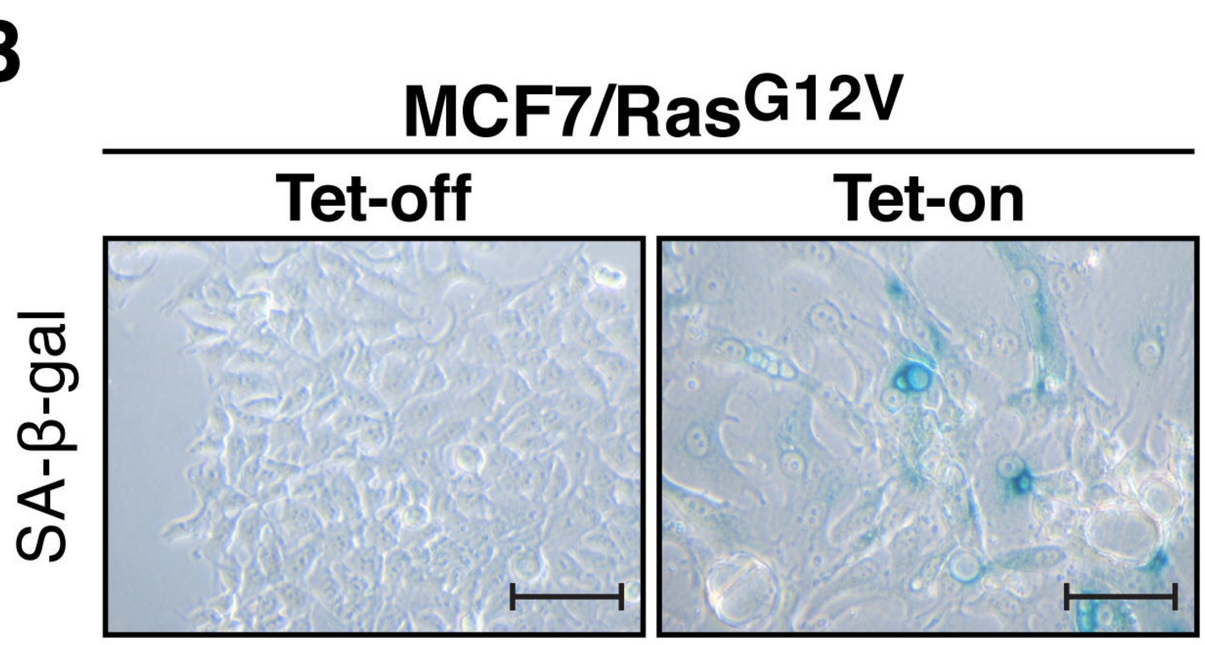
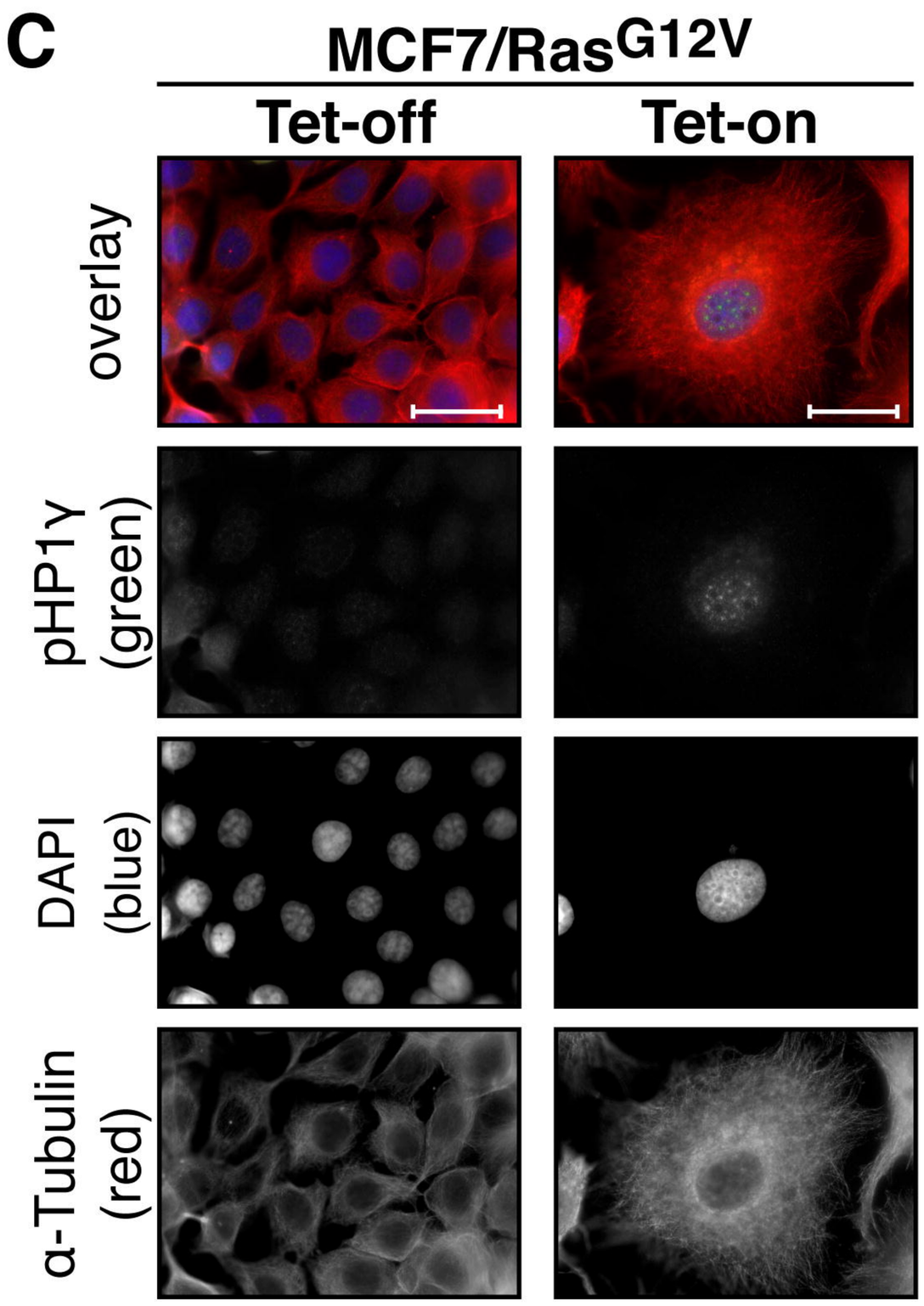
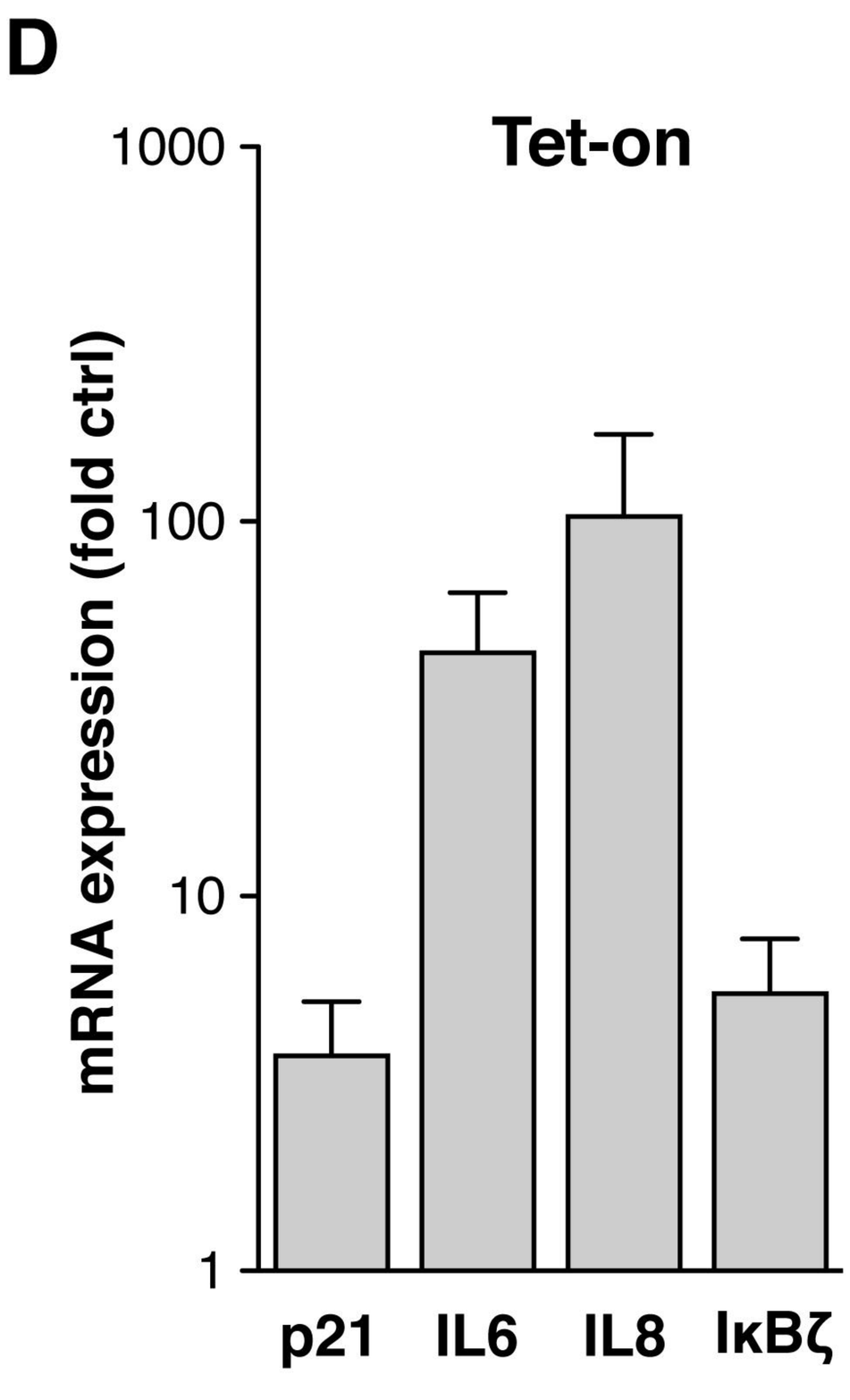


Figure 2









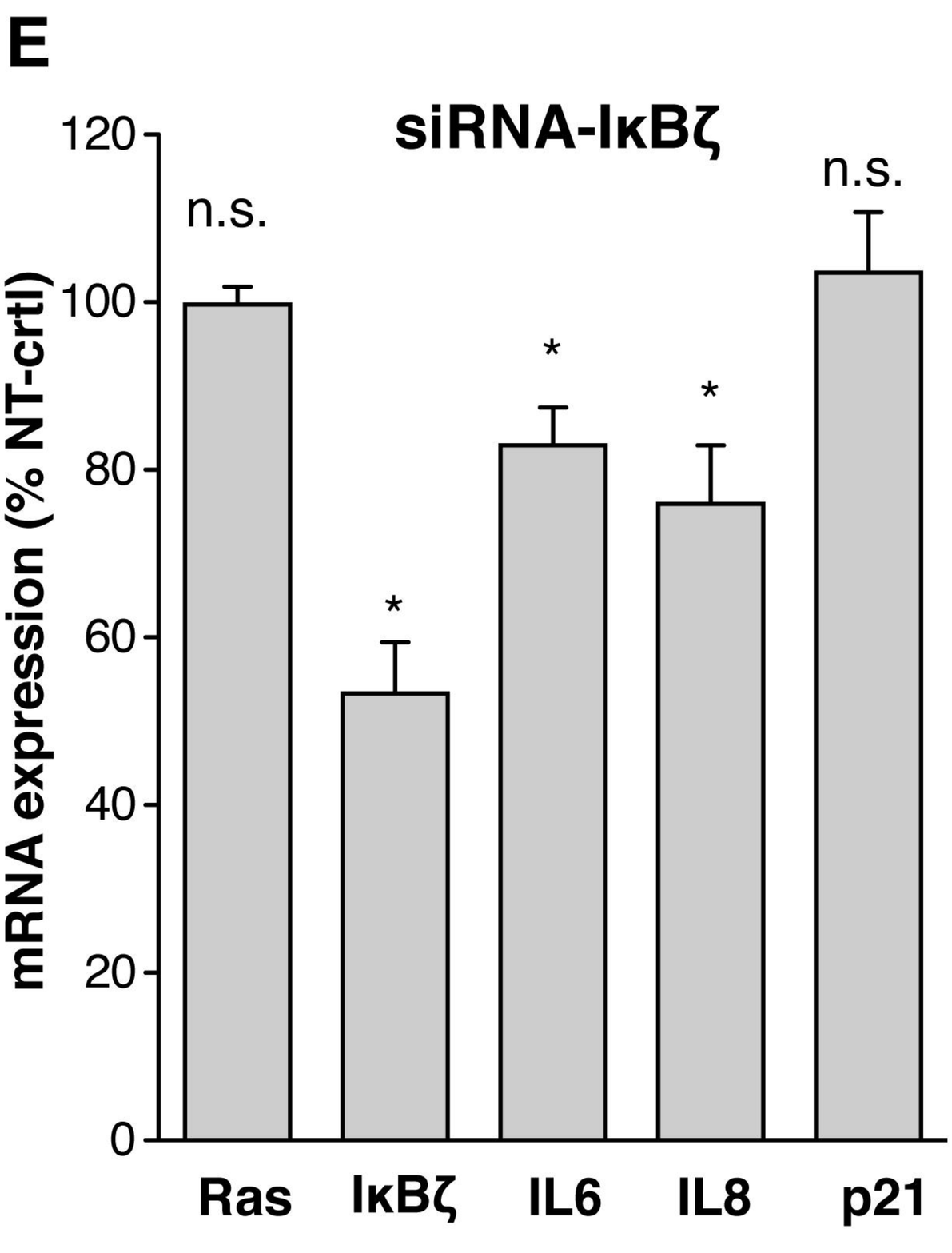


Figure 3

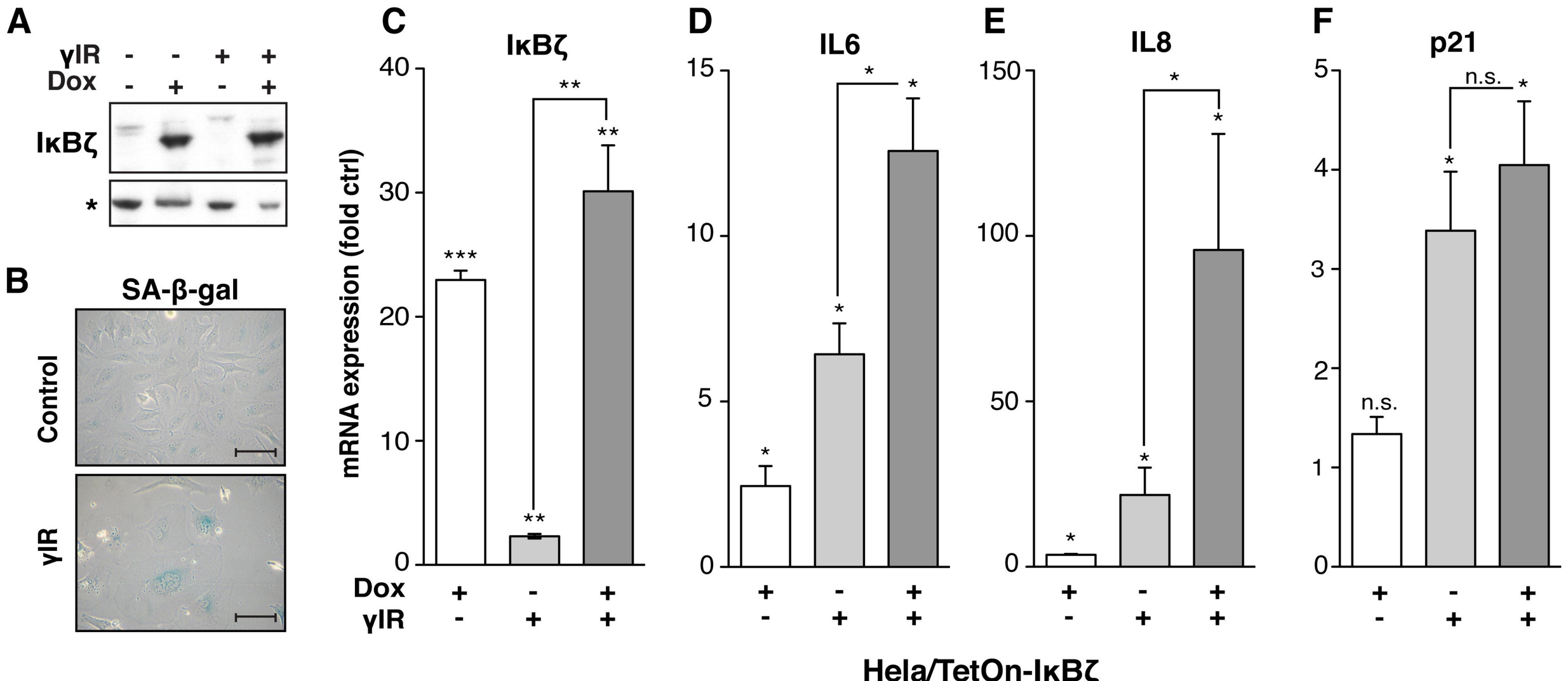


Figure 4

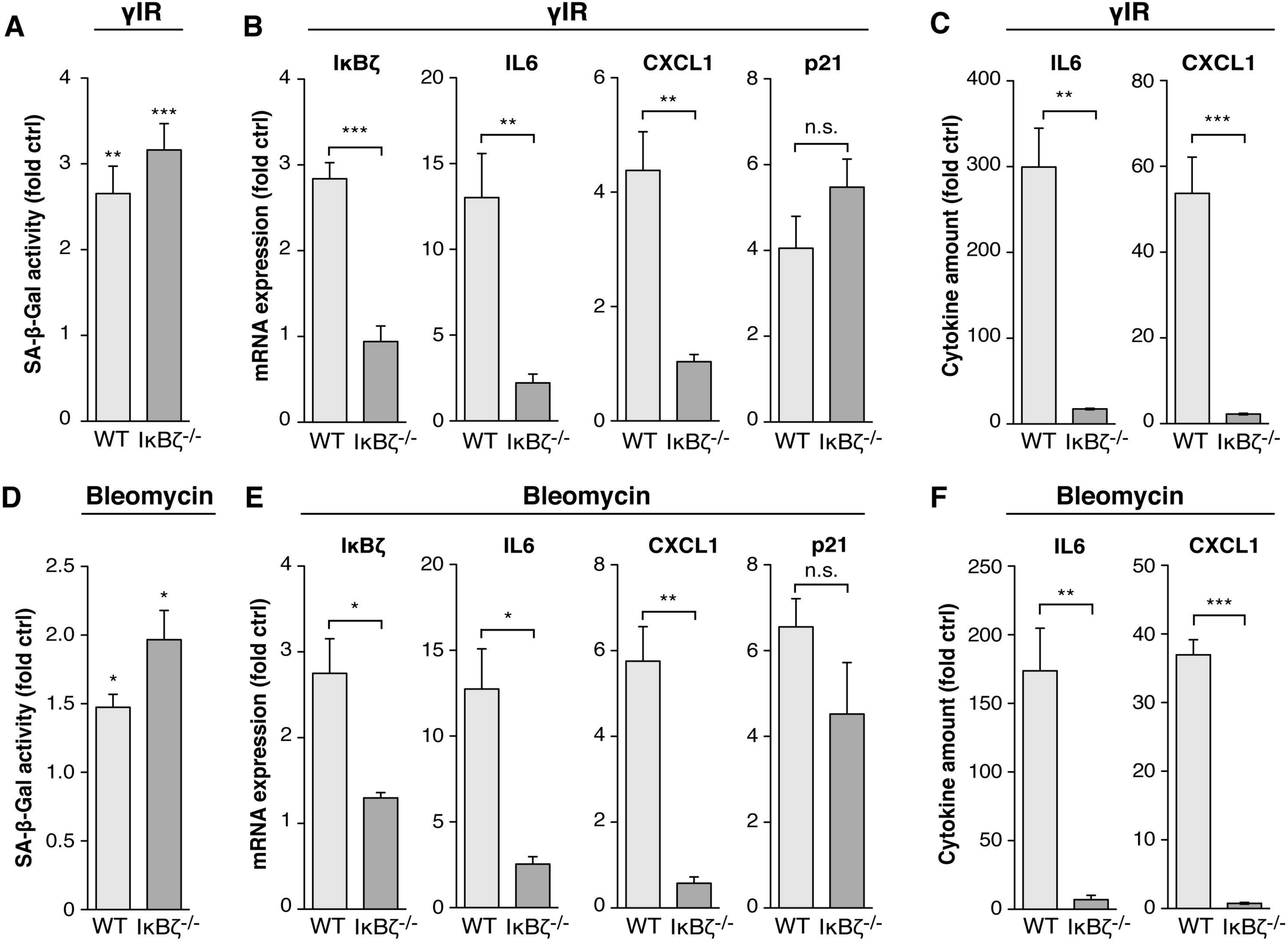


Figure 5

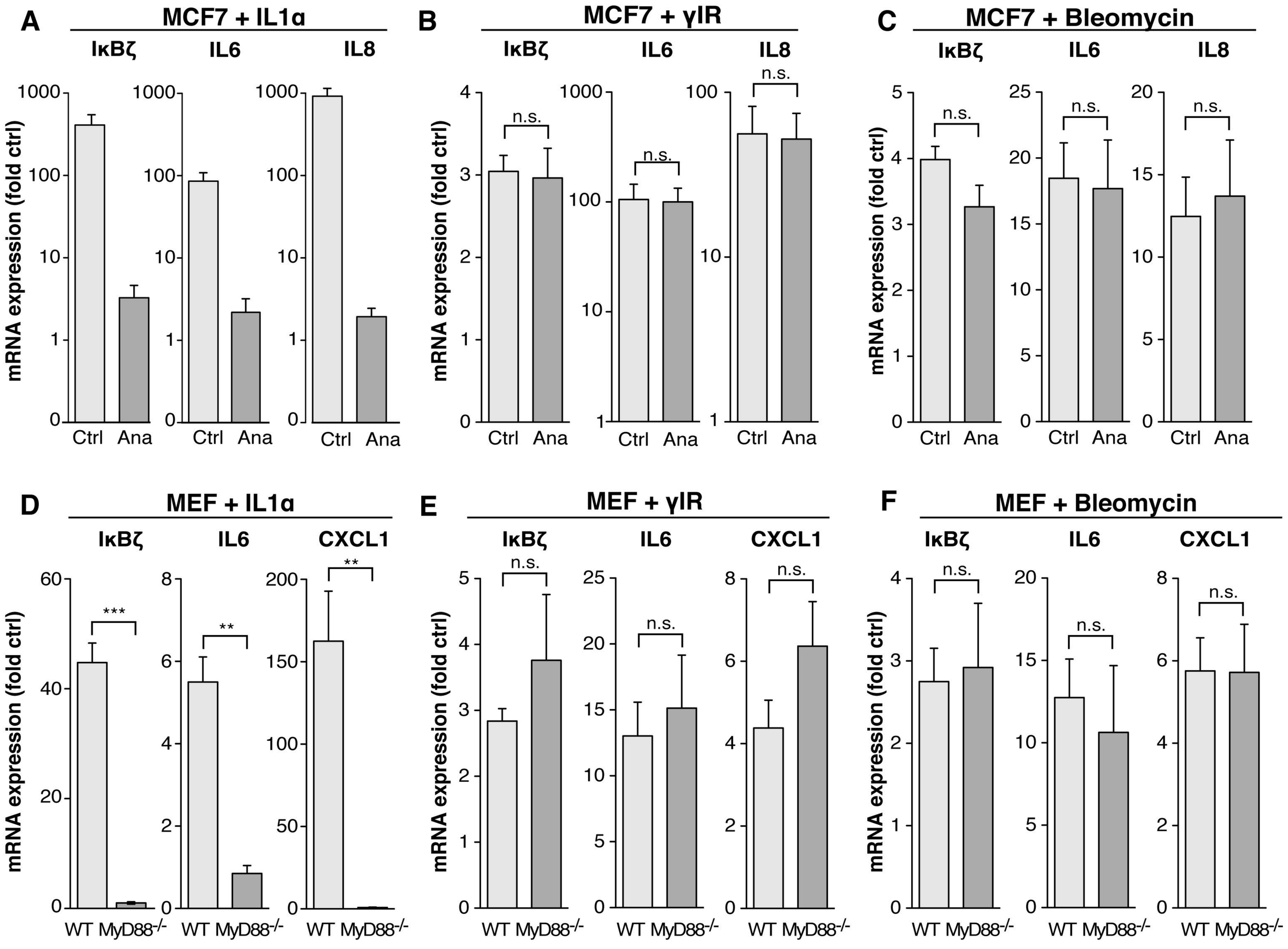
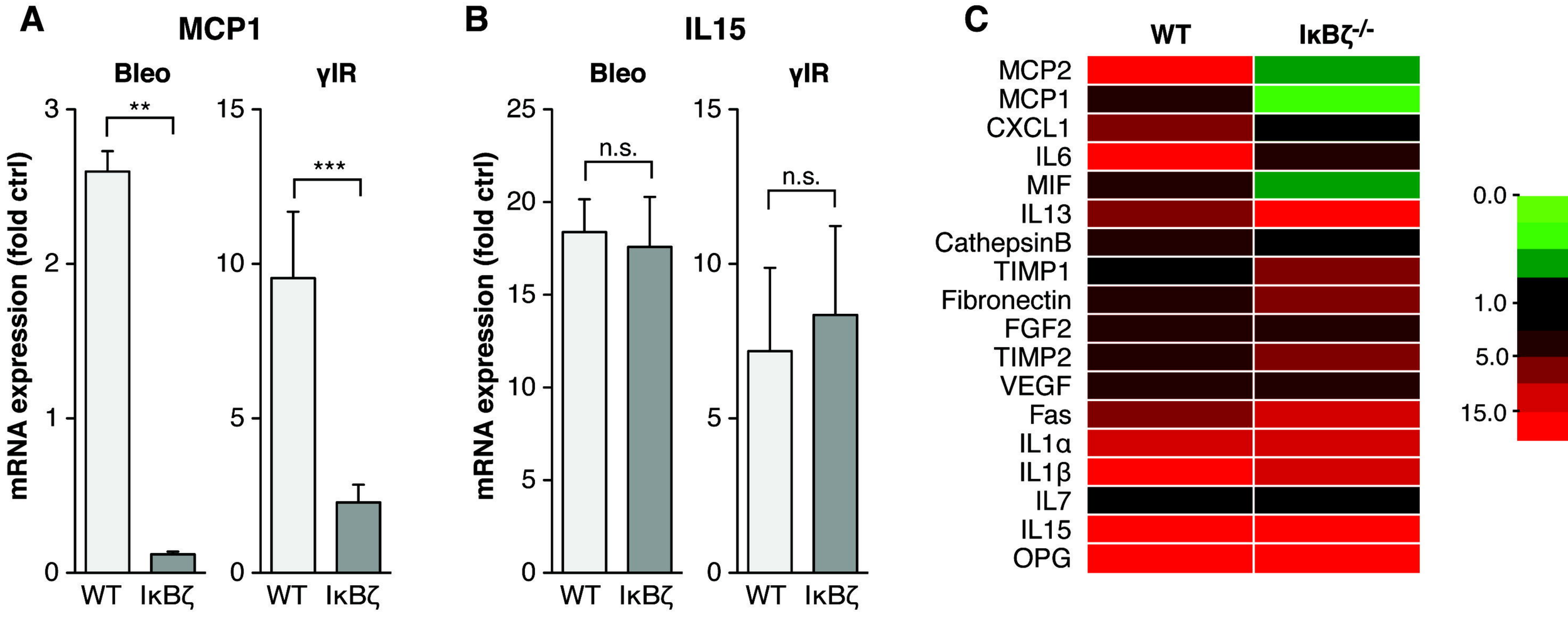


Figure 6



Senescence Stimuli

Primary response

NFκB p50/p65

IκBζ synthesis

SASP

Secondary response

IκBζ

IL6, IL8, MCP1 etc.

p50/IκBζ