The mitochondrial ATPase Inhibitory Factor 1 (IF1) triggers a ROS-mediated retrograde pro-survival and proliferative response

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Abstract

Recent findings indicate that prevalent human carcinomas overexpress the mitochondrial ATPase Inhibitory Factor 1 (IF1). Overexpression of IF1 inhibits the synthase activity of the mitochondrial H^+-ATP synthase and plays a crucial role in metabolic adaptation of cancer cells to enhanced aerobic glycolysis. Herein, we demonstrate that IF1 overexpression in colon cancer cells triggers mitochondrial hyperpolarization and the subsequent production of superoxide radical, a reactive oxygen species (ROS). ROS are required to promote the transcriptional activation of the NFκB pathway via phosphorylation-dependent IκBα degradation. Activation of NFκB results in a cellular adaptive response that includes proliferation and Bcl-xL mediated resistance to drug-induced cell death. Quenching the mitochondrial production of ROS prevents the activation of NFκB and abolishes the IF1-mediated cellular adaptive response. Overall, our findings provide evidence linking the activity of a mitochondrial protein with retrograde signaling to the nucleus to promote cellular proliferation and survival.

Highlights:

1.- IF1-mediated inhibition of the H^+-ATP synthase activates NFκB via ROS signaling.

2.- IF1 signals metabolic reprogramming, proliferation, invasion and survival.

3.- IF1-mediated overexpression of Bcl-xL confers apoptotic resistance.

4.- IF1 switches mitochondrial activity from a tumor suppressor to a tumor promoter.

Introduction

The enhanced aerobic glycolysis of tumors (Cuezva et al., 2009; Vander Heiden et al., 2009) is a phenotypic trait acquired by cancer cells in their progression to malignancy (Hanahan and Weinberg, 2011; Hu et al., 2011). The boost in glucose consumption is required to fuel the pentose phosphate and glycolytic pathways which provide the NADPH and metabolic intermediates needed as building blocks for the biosynthesis of nucleic acids, proteins and the phospholipids required for proliferation (Formentini et al., 2010; Frezza and Gottlieb, 2009). Glutamine is also utilized by cancer cells to replenish with carbon skeletons the TCA cycle (Frezza and Gottlieb, 2009). In order to regenerate NAD^+, aerobic glycolysis produces large amounts of lactate which is secreted from the cell to favor metastasis and angiogenesis (Gatenby and Gillies, 2004). Several mechanisms directly promoting glycolysis, inhibition of mitochondrial function or both have been proposed, aiming to explain the metabolic hallmark of cancer cells and tumors (for review see (Cairns et al., 2011; Formentini et al., 2010; Frezza and Gottlieb, 2009)). However, the mitochondrial contribution to the enhanced aerobic glycolysis of cancer cells has been questioned (Dang, 2010; Funes et al., 2007; Li et al., 2005). In any case, some properties of cancer cells cannot be explained only by mutations. In fact, it is accepted that the tumor microenvironment (Cairns et al., 2011; Nyberg et al., 2008) and other epigenetic events (Iacobuzio-Donahue, 2009) contribute to cancer development and its behavior.

A master regulator of energetic metabolism is the mitochondrial H^+-ATP synthase, a reversible engine of oxidative phosphorylation that catalyzes the synthesis of ATP using as driving force the proton gradient generated by the respiratory chain (Boyer, 1997). Oxidative phosphorylation is also required for efficient execution of cell death (Dey and Moraes, 2000; Matsuyama et al., 1998; Santamaria et al., 2006) and cells that are unable to perform oxidative phosphorylation and rely heavily on aerobic glycolysis have an apoptotic-resistant phenotype (Dey and Moraes, 2000; Santamaria et al., 2006). Conversely, activation of oxidative phosphorylation prevents tumor development (D'Errico et al., 2011; Schulz et al., 2006). The catalytic β-F1-ATPase subunit of the H^+-ATP synthase carries out the synthesis or hydrolysis of ATP upon changes in the physiological conditions of the cell. Genomic (Sheffer et al., 2009), transcriptomic (Lu et al., 2007; Sanchez-Arago et al., 2010), proteomic (Cuezva et al., 2002; Cuezva et al., 2009) and functional studies (Holm et al., 1995; Lopez-Rios et al., 2007) in prevalent human carcinomas indicate that repressed bioenergetic activity of mitochondria is involved in tumor progression. Likewise, a lower bioenergetic activity of mitochondria is also linked to chemotherapy resistance (Li et al., 2010; Santamaria et al., 2006; Shin et al., 2005). Masking the translation of β-F1-ATPase mRNA through the binding of specific mRNABPs onto the mRNA (Ortega et al., 2010) or by silencing of the ATP5B gene promoter (Li et al., 2010) is known to affect the mitochondrial content of β-F1-ATPase in human cancer. Moreover, metabolic reprogramming of tumors also encompass the inhibition of the H^+-ATP synthase activity through the overexpression of the ATPase Inhibitory Factor 1 (IF1) (Sanchez-Cenizo et al., 2010), which is a physiological inhibitor of the mitochondrial synthase.

The biological relevance of IF1 in human pathology is largely unknown (Campanella et al., 2008; Jennings et al., 1991) and specifically in the cancer field (Sanchez-Cenizo et al., 2010). In this study, we have addressed the mechanisms and signaling pathway by which IF1 could participate in the biology of cancer cells. Remarkably, we illustrate that a ROS-mediated retrograde response generated in mitochondria as a result of IF1 interference with oxidative phosphorylation switches on proliferation, invasion and cell survival. The IF1-mediated adaptive response of cancer cells is mediated by the activation of the canonical NFκB pathway.

Results

IF1 is overexpressed in colon cancer and regulates energy metabolism of HCT116 cells. As revealed by immunohistochemistry, and in agreement with previous findings (Sanchez-Cenizo et al., 2010), IF1 is highly overexpressed in mitochondria (see punctuate cytoplasmic staining in right panel of Figure 1A) of human colon carcinomas (Figure 1A) when compared to the normal tissue (Figure 1B). The overexpression of IF1 in HCT116 cells (Figure 1C) resulted in decreased oxidative phosphorylation (OSR) (Figure 1D), mitochondrial hyperpolarization (Figure 1E) and a concurrent enhancement of the rate of aerobic glycolysis (Figure 1F) in agreement with previous findings in other cells (Sanchez-Cenizo et al., 2010). Likewise, siRNA-mediated silencing of IF1 in HCT116 cells (Figure 1C) promoted an increase in oxidative phosphorylation (Figure 1D) and a reduction of the rates of aerobic glycolysis (Figure 1F) with marginal effect on the mitochondrial membrane potential ($\Delta\Psi m$) (Figure 1E).

Basal cellular ATP concentrations were not affected by the regulated expression of IF1 (Figure 1G). However, the inhibitory effect of IF1 on the synthase activity of the H^+-ATP synthase was further demonstrated by the significant drop in ATP content when aerobic glycolysis of the cells was inhibited with 2-deoxyglucose (2DG) in IF1 overexpressing cells (Figure 1H). Likewise, the silencing of IF1 promoted a relevant increase in ATP content when glycolysis was inhibited (Figure 1H). Treatment of the cells with oligomycin indicated that oxidative phosphorylation provides most of the ATP determined in the presence of 2DG (Figure 1H), thus supporting that the IF1-

mediated changes in respiration are linked to the production of ATP by the H^+-ATP synthase. These findings indicate that IF1 is used by cancer cells to inhibit the activity of H^+-ATP synthase to favor the switch of energy metabolism toward aerobic glycolysis (Sanchez-Cenizo et al., 2010).

IF1 regulates the rates of cellular proliferation and invasion. We studied the role of the expression level of IF1 in the rate of proliferation of HCT116 cells by assessing the incorporation of 5-ethynyl-2'-deoxyuridine (EdU) into DNA (Figure 2A). IF1 overexpression significantly augmented the rate of DNA replication when compared to control cells (Figure 2A). On the contrary, siRNA-mediated silencing of IF1 resulted in a lower rate of cellular proliferation when compared to control (Figure 2A). Moreover, the over-expression of IF1 increased cell invasiveness when compared to controls (Figure 2B) whereas its silencing significantly reduced invasion (Figure 2B). These results suggest a role for IF1 in switching on a mitochondrial retrograde signal to the nucleus (Butow and Avadhani, 2004).

IF1 regulates the cell death response to toxic agents. Overexpression of IF1 significantly diminished the percentage of dead cells (sub-G0) after treatment with 10μM 5-fluorouracil (5FU) or 1μM staurosporine (STS) (Figure 2C). On the contrary, downregulation of IF1 significantly augmented the percentage of dead cells in response to 5FU- or STS-treatment when compared to their matched controls (Figure 2D). Similar results were obtained by determining the percentage of pyknotic nuclei in ToPro3-stained cells (see supplementary Figure S1), supporting a role for IF1 in controlling the fate of cancer cells.

IF1 controls the release of apoptogenic molecules from mitochondria. Consistent with the role of IF1 in cell death (Figure 2C and D), the exposure of phosphatidylserine at the plasma membrane was significantly reduced (Figure 3A, left panel) or increased (Figure 3A, right panel) in HCT116 cells treated with STS when IF1 was over-expressed or silenced, respectively. IF1 overexpression in HCT116 cells was not able to prevent the rapid fragmentation of the mitochondrial reticulum after STS treatment but ameliorated the swelling of mitochondria (Supplementary Figure S2). On the other hand, IF1 downregulation in HeLa and T47D cells (both expressing high levels of IF1 (Sanchez-Cenizo et al., 2010)) aggravated the fragmentation and swelling of mitochondria after STS treatment (Supplementary Figure S2). Moreover, we noted that the loss of AIF immunoreactivity in mitochondria after STS treatment was delayed in HCT116 cells overexpressing IF1 when compared to controls (Figure 3B). Conversely, siRNA-mediated silencing of IF1 in HeLa cells accelerated the release of AIF after STS treatment (Figure 3C), supporting a role for IF1 in regulating mitochondrial-geared apoptosis (Figure 3A). Overall, the effects of IF1 overexpression on HCT116 cells mimicked the effects of oligomycin in delaying apoptosis (Matsuyama et al., 1998; Santamaria et al., 2006), further supporting a role for the activity of the H^+-ATP synthase in signaling the efficient execution of cell death.

Overexpression of IF1 generates a mitochondrial ROS signal. Inhibition of the activity of the H^+-ATP synthase, mediated either by treatment of the cells with the ATPase inhibitor oligomycin (Sanchez-Cenizo et al., 2010) or by the overexpression of IF1 (Sanchez-Cenizo et al., 2010) (and see Figure 1E), promotes mitochondrial hyperpolarization due to the interruption of the backflow of H^+ into the matrix. In this situation, mitochondria produce the superoxide radical (Brand et al., 2004). Consistently, we observed that IF1 upregulation in HCT116 cells triggered a significant increase in the production of superoxide (Figure 4A). The mitochondrial scavenger MitoQ (MQ) (Kelso et al., 2001) was able to quench superoxide production in IF1 overexpressing cells (Figure 4A) as well as to ameliorate basal mitochondrial ROS levels in controls (Figure 4A).

One of the targets of oxygen radicals is the covalent modification of cellular proteins. Indeed, a significant increase in the carbonylation of specific cellular proteins was noted in HCT116 cells overexpressing IF1 when compared to controls (Figure 4B and 4C). Remarkably, incubation of the cells with MQ diminished the IF1-triggered carbonylation of these proteins (Figure 4B and 4C). Interestingly, the overexpression of IF1 did not affect cellular hydrogen peroxide levels or the GSH/GSSG ratio (see Supplemental Figure S3) supporting that the IF1-mediated superoxide signal is of mild intensity and localized in mitochondria.

Cellular adaptive response to the regulated expression of IF1. IF1 was either overexpressed or silenced in HCT116 cells (Figure 5A) to study the expression of known targets of oxidative stress involved in the regulation of energy metabolism, apoptosis and proliferation (Klaunig and Kamendulis, 2004). We did not find relevant changes in the content and/or in the phosphorylation status of the serine/threonine kinases Akt, c-Jun N-terminal kinase (JNK) and p38 kinase, which play important roles in adaptation to stress, inhibition of apoptosis and induction of proliferation (Cairns et al., 2011). Likewise, the transcription factor HIF1α was not affected by IF1 expression (Figure 5A). Interestingly, overexpression of IF1 triggered the upregulation of the anti-apoptotic Bcl-xL (Boise et al., 1993) (Figure 5A) whereas silencing of IF1 mediated its downregulation (Figure 5A). The changes in Bcl-xL expression occurred in the absence of relevant changes in the cellular content of the pro-apoptotic Bax (Figure 5A). The transcription factor NFκB regulates a large number of genes related to inflammation, cell proliferation and apoptosis (Guha et al., 2010; Karin, 2006) and specifically Bcl-xL

(Lee et al., 1999). Therefore, we next studied the putative ROS-mediated activation of the NFκB signaling pathway in response to IF1 overexpression.

Upregulation of IF1 triggers the activation of the NFκB pathway. A significant increase in the activity of the NFκB promoter was observed in HCT116 cells treated with H_2O_2 or overexpressing IF1 (Figure 5B). Interestingly, the enhanced activity of NFκB promoter was obliterated in the presence of the mitochondrial ROS scavenger MQ (Figure 5B), indicating the relevance of ROS signaling for IF1-mediated activation of NFκB.

An increased activity of NFκB was further confirmed by assessing the expression of some of its targeted genes (Figure 5C). Significantly, IF1 overexpression triggered the downregulation of *NFKBIA*, the IκBα inhibitor of NFκB, and of the signaling molecules and receptors involved in the innate immune response such as complement factor B (*CFB*), interleukin 8 (*IL8*), the toll-like receptor 7 (*TLR7*) and the toll-like receptor adaptor molecule 1 (*TICAM1*) (Figure 5C). Likewise, IF1 triggered the upregulation of NFκB targeted genes such as the mitogen-activated MEK kinase (*MAP3K1*), which integrates cellular responses to mitogenic and metabolic stimuli, as well as the transcription factors and co-activators *EGR1*, *c-FOS, STAT1, BCL3* and *NFKB2* (Figure 5C). Furthermore, IF1 led to the upregulation of the signaling molecules (*IL* and *LTA*) and receptors (*LPAR* and, *TLR2*) that promote proliferation and tumor invasion (Figure 5C). Determination of the expression of NFκB targeted genes in the presence of the ROS scavenger MQ confirmed for most of them their ROS-dependent (inhibition/activation) response (Figure 5C). It should be noted that MQ did not revert the IF1-mediated changes in gene expression in those cases where treatment with MQ alone induced a significant change compared to controls (Figure 5C).

Consistent with the ROS-mediated activation of the NFκB pathway, cells overexpressing IF1 showed an increased phosphorylation of the repressor IκBα (Figure 5D) and the concurrent reduction of its cellular content (Figure 5D). The incubation of the cells with the ROS scavenger MQ minimized IκBα phosphorylation and its downregulation (Figure 5D).

ROS mediate transcriptional activation of Bcl-xL. Next, we studied the mechanisms controlling the cellular accumulation of Bcl-xL in response to IF1-mediated ROS signaling (Figure 6A-C). The overexpression of IF1 triggered the transcriptional activation of the Bcl-xL promoter (Figure 6B) resulting in the cellular accumulation of Bcl-xL mRNA (Figure 6C) and protein content (Figure 6A). Quenching the IF1-mediated ROS signal abolished transcriptional activation of Bcl-xL (Figure 6B) and the accumulation of Bcl-xL at both the protein (Figure 6A) and mRNA levels (Figure 6C). Interestingly, and consistent with the lack of changes in Bax content (Figure 5A), we observed no changes in Bax promoter activity by IF1 overexpression (Figure 6D), suggesting that the IF1-mediated resistance to cell death is accomplished by increasing the cellular content of Bcl-2 anti-apoptotic members.

Mitochondrial ROS signaling links IF1 with proliferation and cell survival. To establish a link between IF1-mediated ROS production and cellular proliferation and the prevention of cell death, the effect of quenching the ROS signal was studied in proliferation (Figure 7A) and cell death after exposure to STS (Figure 7B). Treatment of IF1-overexpressing cells with MQ inhibited the positive effect of IF1 on cellular proliferation (Figure 7A), suggesting that it represents a ROS-mediated response. As shown previously (Figure 3A), IF1 overexpression significantly reduced cell death after STS treatment (Figure 7B) while quenching the IF1-induced ROS signal with MQ reverted this effect (Figure 7B). Moreover, the silencing of Bcl-xL in IF1 overexpressing cells resulted in a large increase in cell death (Figure 7C), further supporting that IF1-mediated up-regulation of Bcl-xL is critical for restraining apoptosis in this situation.

IF1 mediates similar signaling events in SW620 colon carcinoma cells. The overexpression of IF1 in SW620 cells corroborated the role of IF1 in mediating the inhibition of oxidative phosphorylation (OSR) and the subsequent production of mitochondrial ROS (Supplemental Figure S4). Likewise, IF1 overexpression also triggered the ROS-dependent activation of NFκB, of cellular proliferation and ameliorated STS-induced cell death in SW620 cells (Supplemental Figure S4). Consistently, quenching the IF1-induced ROS signal with MQ reverted these effects (Supplemental Figure S4), strongly supporting the essential role of IF1 in retrograde signaling the oncogenic phenotype in colon cancer.

Discussion

The switch from oxidative phosphorylation to glycolysis is a metabolic feature of proliferating cells. Consistently, human carcinomas show downregulation of the H^+-ATP synthase concurrently with the induction of enzymes of glycolysis (Cuezva et al., 2002; Willers and Cuezva, 2011). Moreover, carcinomas have an additional strategy to promote the metabolic switch required for proliferation that is mediated by upregulation of IF1 (Sanchez-Cenizo et al., 2010). Indeed, here we show that IF1 overexpression in colon cancer cells inhibited the activity of the H^+-ATP synthase and promoted the upregulation of aerobic glycolysis. Conversely, siRNA-mediated silencing of IF1 triggered an increase in the activity of the H^+-ATP synthase and the downregulation of aerobic glycolysis. The inhibition of oxidative phosphorylation and subsequent mitochondrial hyperpolarization have further consequences in cellular homeostasis by generating a ROS signal (Figure 7D). ROS activate the canonical NFκB pathway, which finally signals to the nucleus the activation of proliferation and the evasion of cell death (Figure 7D). Since all human breast, colon and lung carcinomas ((Sanchez-Cenizo et al., 2010), and unpublished data) show a very large increase in the mitochondrial content of IF1 we support that IF1 is mastering the epigenetic acquisition of several hallmarks of the oncogenic phenotype via the regulation of energy metabolism in mitochondria.

Inhibition of the black flow of H^+ generated by respiration into the mitochondrial matrix promotes an increase in ΔΨm (Brand et al., 2004). In fact, oligomycin that binds the proton channel of the H^+-ATP synthase promotes mitochondrial hyperpolarization (Sanchez-Cenizo et al., 2010). Similar findings are obtained when cancer (Figure 1C) and non-cancer (Sanchez-Cenizo et al., 2010) cells overexpress IF1. Cancer cells display unusually high ΔΨm (Modica-Napolitano and Singh, 2002) and a direct consequence of mitochondrial hyperpolarization is the production of superoxide radical (Brand et al., 2004). Consistently, cancer cells are known to have higher basal levels of ROS (Szatrowski and Nathan, 1991). In agreement with these findings, we have observed that the overexpression of IF1 triggers mitochondrial hyperpolarization, a higher basal production of superoxide radical as well as increased carbonylation of cellular proteins. Therefore, we suggest that the high ΔΨm and ROS levels observed in cancer cells most likely result from the IF1-mediated inhibition of the H^+-ATP synthase due to the high expression level of IF1 observed in most cancer cells (Sanchez-Cenizo et al., 2010). In this regard, it has been shown that IEX-1, a stress-inducible gene involved in IF1 degradation, also suppresses the production of reactive oxygen species (Shen et al., 2009).

Previous studies have shown in carcinomas with mutations in mtDNA or in Kras (Ishikawa et al., 2008; Weinberg et al., 2010) that an enhanced mitochondrial activity is required to generate the ROS involved in tumorigenicity. In contrast, the ROS-mediated oncogenic function of IF1 is exerted by inhibition of the H^+-ATP synthase and thus by restraining the bioenergetic activity of mitochondria. Since IF1 is highly up-regulated in breast, colon and lung carcinomas ((Sanchez-Cenizo et al., 2010) and unpublished data) it is likely that its mechanism of action could be generalized to all these tumors. We suggest, in light of recent findings (Mullen et al., 2011), that carcinomas with IF1-limited oxidative phosphorylation could also have glutamine-dependent reductive carboxylation as a major pathway for citrate formation.

Mitochondrial retrograde signaling is a pathway of communication from the mitochondria to the nucleus that influences many adaptive responses under both normal and pathophysiological conditions (Butow and Avadhani, 2004). It has been documented that the collapse of $\Delta\Psi m$ triggers a Ca^{2+}-mediated signaling cascade that leads to the activation of the Ca^{2+}-responsive phosphatase calcineurin, which regulates the activity of several transcription factors including the NFκB pathway (Guha et al., 2010). Although we cannot exclude Ca^{2+}-signaling in cells overexpressing IF1, its participation seems unlikely due to the transient nature of Ca^{2+} signals and to the opposite changes noted in $\Delta\Psi m$. Likewise, the absence of IF1-mediated changes in HIF1α expression supports the idea that mitochondrial ROS signaling might activate a cellular adaptation program unrelated to the hypoxia associated pathway (Weinberg et al., 2010). We believe that sustained retrograde ROS signaling by mitochondrial hyperpolarization in non-hypoxic conditions explains the adaptive phenotype of cells overexpressing IF1. Indeed, we show that quenching the mitochondrial production of superoxide radical prevented both the proliferative and pro-survival activities of IF1.

The effect of ROS on cell fate depends on the level at which ROS are present (Hamanaka and Chandel, 2010). Interestingly, the ROS-mediated activation of the NFκB pathway triggered by IF1 appears not to correlate with the induction of the antioxidant defense because we observed a higher basal level of oxidation of cellular proteins, suggesting that the ROS-signaling event is of moderate intensity and able to promote proliferation and survival. Consistent with a mild IF1-mediated ROS signal, we observe no relevant changes in cellular hydrogen peroxide content and in the GSH/GSSG ratio. These observations suggests that cancer cells have additional strategies to overcome the effects of excessive ROS signaling, which might be related to the expression of uncoupling proteins in their mitochondria (Brand et al., 2004).

The transcription factor NFκB has emerged as a crucial regulator of cellular adaptive responses involving proliferation and survival (Karin, 2006). The NFκB pathway is also involved in the regulation of the immune response (Vesely et al., 2011) and in some situations promotes the activation of mitochondrial respiration (Mauro et al., 2011). Alterations in these activities contribute to cancer development and progression. ROS can interact with NFκB at various places within its signaling pathway (Morgan and Liu, 2011). One main pathway that cross-talks with NFκB and ROS is JNK. However, we observed no discernible changes in JNK at both the protein and mRNA levels as well as in the extent of phosphorylation of the kinase. It has been argued that mitochondrial retrograde signaling regulates both the canonical and non-canonical NFκB pathways (Guha et al., 2010). Our data demonstrate that IF1 mediates the activation of the canonical pathway because we observe the phosphorylation and cellular reduction of the repressor IκBα. Moreover, we show that quenching mitochondrial ROS production abolished NFκB activation by preventing IκBα phosphorylation and degradation.

It is well established that oxidative phosphorylation is required for efficient execution of cell death (Dey and Moraes, 2000; Matsuyama et al., 1998; Santamaria et al., 2006). In fact, the inhibition of the activity of the H^+-ATP synthase by oligomycin delays apoptosis in response to different stimuli (Matsuyama et al., 1998; Santamaria et al., 2006). Fragmentation, swelling and the release of apoptogenic molecules from mitochondria are primary events in signaling the execution of cell death after STS treatment (Santamaria et al., 2006). Accordingly, we observe that IF1-mediated inhibition of the activity of H^+-ATP synthase also contributed to ameliorating mitochondrial swelling, delaying the release of AIF from mitochondria and the execution of apoptosis, very much mimicking the effects of oligomycin (Santamaria et al., 2006). In addition to the inhibition of the activity of the H^+-ATP synthase by IF1, human carcinomas decrease the activity of oxidative phosphorylation by promoting the downregulation of the expression of the H^+-ATP synthase (Cuezva et al., 2002; Li et al., 2010). These findings indicate that active oxidative phosphorylation functionally operates as a tumor suppressor, in agreement with previous suggestions (for review see (Cuezva et al., 2009)) and recent findings (D'Errico et al., 2011).

The IF1-mediated activation of NFκB promotes the expression of anti-apoptotic genes such as Bcl-xL, which is an additional strategy of the tumor cell to evade immunosurveillance (Vesely et al., 2011). Accordingly, IF1 signaling to the NFκB pathway promoted the repression of the expression of genes involved in the innate immune response (Figure 5). Likewise, IF1-mediated NFκB activation was observed to lead to the upregulation of mitogenic genes and genes involved in proliferation and tumor invasion (Figure 5). Thus, IF1-signaling to the nucleus promotes the activation of multiples pathways for the perpetuation of the cancer cell. Therefore, IF1 could be envisioned as the molecular switch in the cancer cell that turns mitochondrial activity from a tumor suppressor into a tumor promoter.

The number of cancers related with genetic alterations of mitochondria are very limited (Frezza and Gottlieb, 2009). However, the impact of cancer genes in energy metabolism has been amply demonstrated (Cuezva et al., 2009; Hu et al., 2011; Li et al., 2005; Vander Heiden et al., 2009). Although the role of mitochondrial activity in cancer cells has been questioned (Dang, 2010; Funes et al., 2007; Li et al., 2005) the study of large cohorts of most prevalent human carcinomas sustain the alteration of the bioenergetic function of mitochondria (Cuezva et al., 2002; Cuezva et al., 2009; Lopez-Rios et al., 2007). We should emphasize that mitochondrial dysfunction in tumors is not "irreversible" (Michelakis et al., 2008; Sanchez-Arago et al., 2010) because energy metabolism depends on many factors that could influence at short- or long-term the activity of the enzymes involved (Formentini et al., 2010). The over-expression of IF1 in human carcinomas is an additional epigenetic factor that contributes to the peculiar energy metabolism of mitochondria in cancer. In fact, IF1 directly promotes the acquisition of three hallmarks of the cancer phenotype: (i) the switch in energetic metabolism, (ii) increased proliferation and invasion and (iii) the evasion of cell death (Figure 7D). We suggest that studies aimed at characterizing the regulation of IF1 expression will provide profitable targets for therapeutic intervention.

Experimental Procedures

Cell cultures, transfections and siRNA silencing. Human colorectal carcinoma HCT116 and SW620 cells were grown in McCoy's 5A or RPMI media, respectively. HeLa and T47D cells were grown in DMEM and RPMI, respectively. Transfections were performed using Lipofectamine and Plus Reagent (Invitrogen™). The plasmids encoding human IF1 and a mitochondrial version of GFP were used (Sanchez-Cenizo et al., 2010). Suppression of IF1 (Qiagen S100908075) and Bcl-xL (Qiagen s1922) expression was exerted by small interfering RNA (siRNA). An inefficient siRNA sequence, Silencer® Select Negative Control #1 plasmid (Ambion/Applied Biosystems), was used as a control. Where indicated, 20 nM of the mitochondrial ROS scavenger MQ (Kelso et al., 2001), kindly provided by Dr. Murphy (University of Otago, Dunedin, New Zealand), was added to the incubation.

Determination of aerobic glycolysis and oxygen consumption rates. The initial rate of lactate production and the oxygen consumption rates (XF24 Extracellular Flux Analyzer, Seahorse Bioscience) were determined (Sanchez-Cenizo et al., 2010).

Determination of the mitochondrial membrane potential ($\Delta\Psi_m$). Cells were incubated with 0.5 μM TMRM$^+$ (Molecular Probes) and processed for flow cytometry (Sanchez-Cenizo et al., 2010).

Determination of ATP. Cells were incubated for 1 h with or without 100 mM 2-deoxy-D-glucose and cellular ATP concentrations determined using the ATP Bioluminescence Assay Kit CLS II (Roche).

Assessment of cell growth and invasion. The incorporation of 5-ethynyl-2′deoxy-uridine (EdU) into cellular DNA was determined using the Click-iT EdU Flow Cytometry Assay Kit (Molecular Probes). To quantify cell invasion a QCM invasion assay kit (Millipore) was used.

Cell cycle and cell death assays. Cells were treated with 1μM STS or 10μM 5FU during 24 h and processed for flow cytometry after propidium iodide staining.

Exposure of phosphatidylserine on the cell surface was analyzed using the annexin V-FITC assay (Sigma-Aldrich) (Sanchez-Arago et al., 2010).

Western blot and analysis of protein carbonylation. Details of the antibodies used in western blots are provided in Supplemental information. For the determination of protein carbonylation, the Oxyblot Oxidized Protein Detection kit (Chemicon International) was used (Santamaria et al., 2006).

Determination of promoter activities. Cells were co-transfected with IF1, CDL-GFP-β-3'UTR (Sanchez-Cenizo et al., 2010) and NFκB, Bcl-xL and Bax promoter reporter plasmids (Switchgear Genomics). Luciferase activity was determined with the Luciferase Assay System kit (Promega) using a FLUOstar OPTIMA (BMG Labtech) plate luminometer.

Gene expression analysis by qPCR. Reverse transcription reactions were performed using 1 μg of total RNA and the High Capacity Reverse Transcription Kit (Applied Biosystems). Gene expression analysis was performed using the cDNA on Human NFκB Signaling Pathway RT^2*Profiler* PCR Array plates (SABiosciences) and real-time polymerase chain reaction (PCR) with an ABI PRISM 7900HT SDS (Applied Biosystem) and Power Sybr Green PCR Master Mix (Applied Biosystems). Bcl-xL expression was assessed using the following primers: F, 5'-AGCCTTGGATCCAGGAGAA-3' and R, 5'-AGCGGTTGAAGCGTTCCT-3' as detailed (Ortega et al., 2010).

Statistical Analysis. Statistical analyses were performed using a two-tailed Student's t-test. ANOVA with post hoc Dunnett's test were used for multiple comparisons to the control, using the SPSS 17.0 software package. The results shown are means ± SEM. A $P < 0.05$ was considered statistically significant.

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Figure Legends

Figure 1. IF1 is upregulated in colon cancer and regulates energy metabolism. A and **B,** Representative immunohistochemistries of IF1 expression in human colon carcinomas (**A**) and in normal tissue (**B**). Bars, 35 µm. **C-H**, HCT116 cells transfected with CDL-GFP-β-3'UTR were co-transfected with control (CRL and siCRL, closed bars), IF1 plasmid (IF1, open bars) or siIF1 siRNA (siIF1, grey bars) to regulate the expression of IF1. **C,** Representative blots of IF1 and β-F1-ATPase (β-F1) expression. The mitochondrial content of IF1 is shown by the IF1/β-F1 ratio. Bars are the mean ± s.e.m. of four experiments. **D,** The oligomycin sensitive respiratory (OSR) rates were determined in a XF24 Seahorse equipment after the addition of 6µM OL. Bars are the mean ± s.e.m. of three experiments. **E,** The mitochondrial membrane potential (ΔΨm) was determined in cells expressing gfp. Bars are the mean ± s.e.m. of three experiments. **F,** Rates of aerobic glycolysis. Bars are the mean ± s.e.m. of three experiments. **G** and **H**, Cellular ATP concentrations were determined in the absence (**G**) or presence (**H**) of 100 mM 2-deoxy-D-glucose (2DG) for 1 h. The effect of 6 µM oligomycin (OL) is shown (**H**). Bars in **G** and **H** are the mean ± s.e.m. of three experiments. See also Supplemental Figure S4-A.

Figure 2. IF1 triggers cellular proliferation, invasion and survival. HCT116 cells transfected with CDL-GFP-β-3'UTR were co-transfected with control (CRL and siCRL, closed bars), IF1 plasmid (IF1, open bars) or siIF1 siRNA (siIF1, grey bars) to regulate the expression of IF1. **A,** Cellular proliferation was assessed by the incorporation of EdU into cellular DNA. Black (CRL and siCRL) and grey (IF1 and siIF1) traces are shown. **B**, Cellular invasion was assessed with the QCM cell invasion assay**.** In **A** and **B**, the panels show representative data from overexpression (IF1) and silencing (siIF1)

experiments. Bars are the mean ± s.e.m. of four experiments. **C** and **D**, Twenty four h after transfection with IF1 (**C**) or siIF1 (**D**), the cells were treated with 1μM STS or 10μM 5FU. After 24 h of treatment the cells were stained with propidium iodide and the green population of cells was analyzed by flow cytometry to evaluate the cell cycle (panels). Bars show the percentage of sub-G0 cells. Bars are the mean ± s.e.m. of three experiments. See also Supplemental Figure S1.

Figure 3. IF1 modulates the execution of apoptosis. HCT116 (**A,B**) or HeLa (**C**) cells transfected with CDL-GFP-β-3'UTR were co-transfected with control (CRL and siCRL, closed bars), IF1 plasmid (IF1, open bars) or siIF1 siRNA (siIF1, grey bars) to regulate the expression of IF1. Twenty four h after transfection with IF1 (**A, B**) or siIF1 (**A, C**), the cells were treated with 1μM STS. **A,** After 24 h of treatment the cells were stained with annexin V-FITC to evaluate the percentage of apoptotic cell death by flow cytometry. Bars show the percentage of apoptosis in transfected cells. The results shown are the mean±s.e.m. of five experiments. **B** and **C,** At the indicated time after STS treatment HCT116 (**B**) or HeLa (**C**) cells were processed for AIF immunostaining. The loss of the mitochondrial staining of AIF was evaluated in 30 different fields taken at 20x magnification per condition. Note the redistribution of AIF into the cytoplasm and nucleus in STS-treated cells. The plots show the delayed (**B**) or accelerated (**C**) release of AIF from mitochondria mediated by IF1 overexpression or its downregulation, respectively. The results shown are the mean±s.e.m. Bars, 30 μm. See also Supplemental Figure S2.

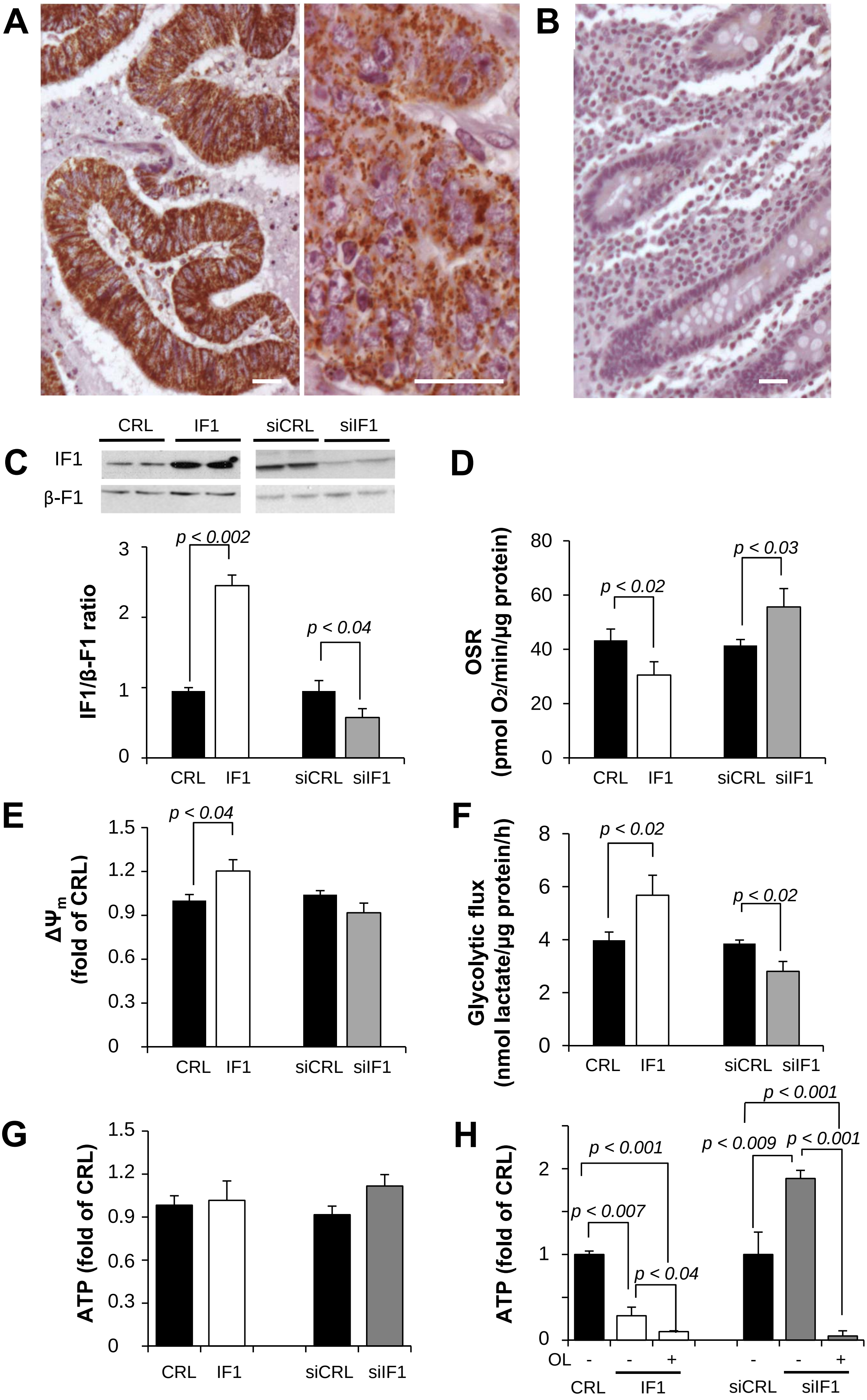
Figure 4. **IF1 triggers mitochondrial ROS production.** HCT116 cells transfected with CDL-GFP-β-3'UTR were co-transfected with control (CRL, closed bars) or IF1 plasmid (IF1, open bars) in the absence (-) or presence (+) of 20 nM of the mitochondrial ROS scavenger MitoQ (MQ). **A,** The superoxide radical was determined by FACS analysis using MitoSox. The data shown are mean±s.e.m. of four experiments. **B,** A representative experiment of the extent of carbonylation of cellular proteins treated in the absence (-) or presence (+) of DPNH for the identification of protein carbonyls. Protein loading of the samples was verified by western blotting with anti-tubulin antibody. The migration of molecular mass markers is indicated to the right. Arrows (to the left) identify the migration of the four proteins used in quantification of protein carbonylation in the histograms shown in **C**. The data shown are mean±s.e.m. of three experiments. See also Supplemental Figure S3 and Figure S4-B.

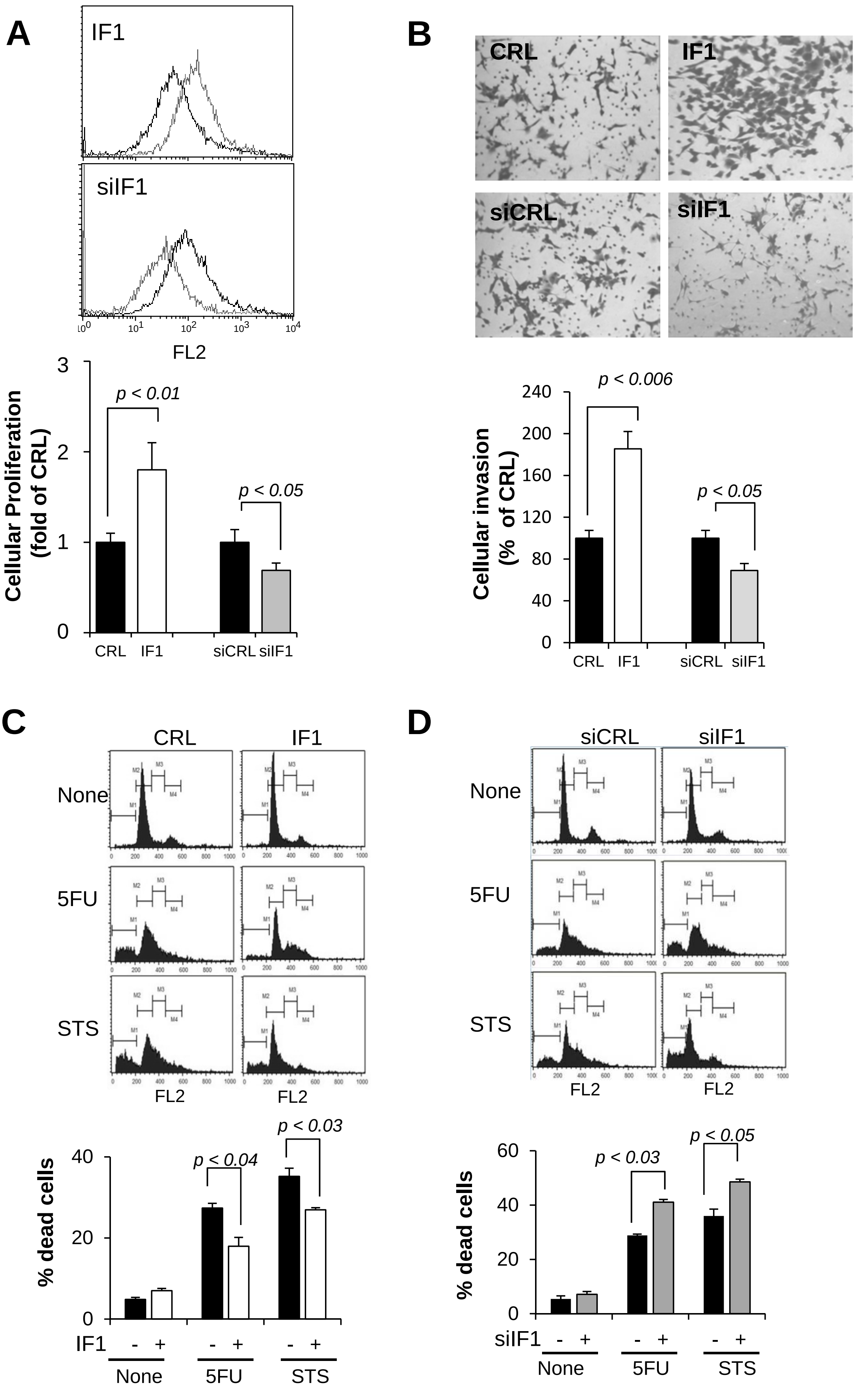
Figure 5. **IF1-dependent activation of the NFκB pathway.** HCT116 cells transfected with CDL-GFP-β-3'UTR were co-transfected with control (CRL and siCRL, closed bars), IF1 plasmid (IF1, open bars) (**A-D**) or siIF1 siRNA (siIF1, grey bars) (**A**) in the absence (-) or presence (+) of 20 nM of the mitochondrial ROS scavenger MQ (**B, C, D**). **A** and **D**, Cellular proteins were fractionated on SDS-PAGE and processed for Western blotting with the indicated primary antibodies. Representative blots of one (**A**) or two different samples (**D**) per condition tested are shown. The results shown are the mean±s.e.m. of three experiments. **B,** A luciferase reporter plasmid of the NFκB promoter was co-transfected and the luciferase activity was determined in cellular extracts after 24h transfection. Cells incubated with 10 μM H_2O_2 were used as positive control of the activation of NFκB promoter. The data shown are mean±s.e.m. of five experiments. **C**, The expression of genes of the NFκB pathway was determined by qPCR in RNA extracted from cells treated as indicated using the RT²*Profiler* PCR Array. The schematic summarizes some of the genes up-regulated (closed boxes), non-affected (grey boxes) and downregulated (open boxes) by the overexpression of IF1. The data shown are mean±s.e.m. of four experiments. See also Supplemental Figure S4-C.

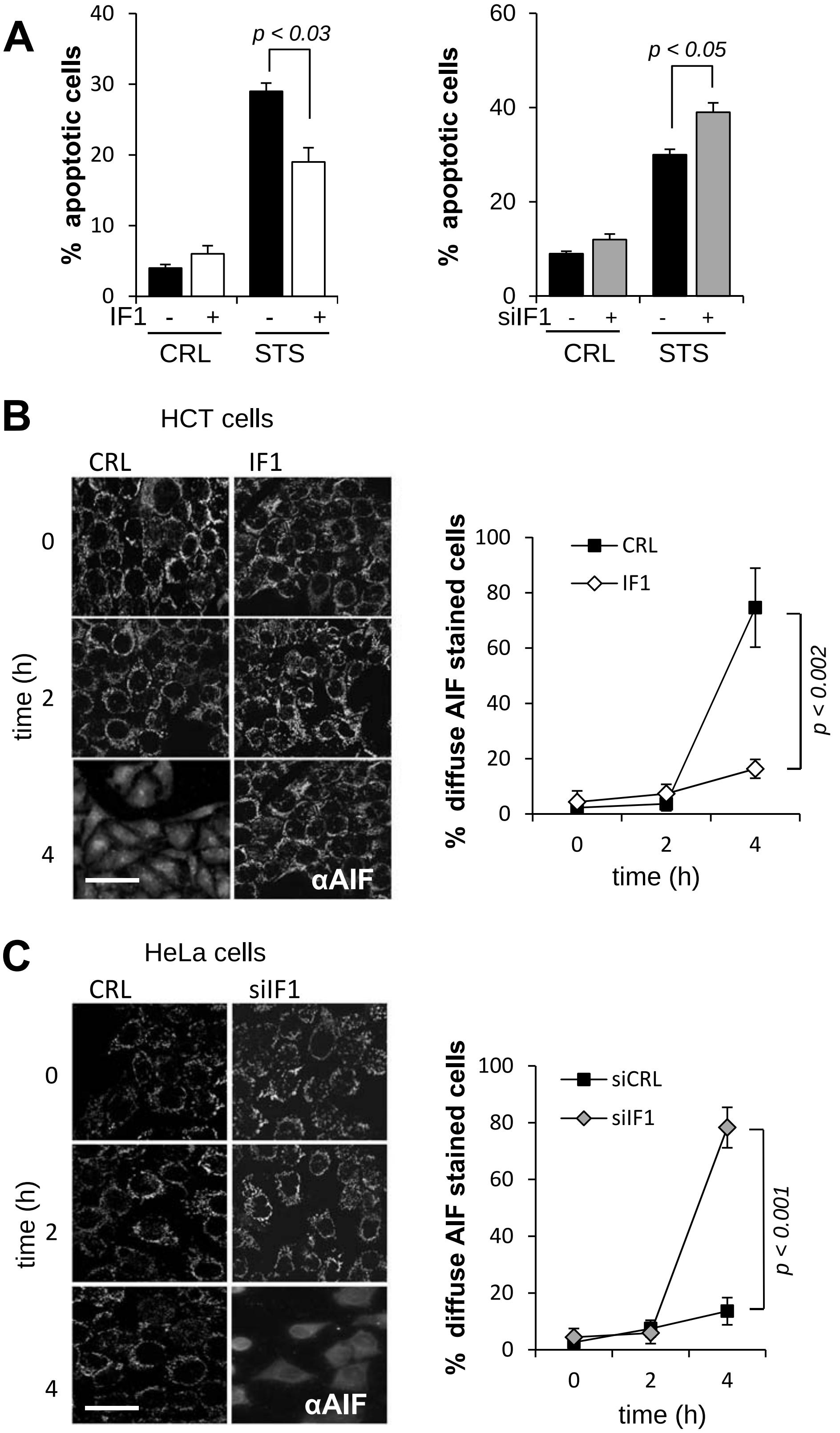
Figure 6. **ROS-mediated transcriptional activation of Bcl-xL.** HCT116 cells transfected with CDL-GFP-β-3'UTR were co-transfected with control (CRL, closed bars) or IF1 plasmid (IF1, open bars) in the absence (-) or presence (+) of 20 nM of the mitochondrial ROS scavenger MQ (MQ). **A**, A representative Western blot of control (CRL) and IF1 over-expressing (IF1) cells shows the ROS-dependent up-regulation of Bcl-xL in response to IF1 overexpression. Two different samples per condition tested are shown. The data shown are mean±s.e.m. of four experiments. **B** and **D**, Cells were co-transfected with luciferase reporter plasmids of the Bcl-xL (**B**) or Bax (**D**) promoters and the luciferase activity determined. The data shown are mean±s.e.m. of four experiments. **C**, Bcl-xL mRNA expression was determined by reverse transcription and quantitative polymerase chain reaction (qPCR). The data shown are mean±s.e.m. of three experiments.

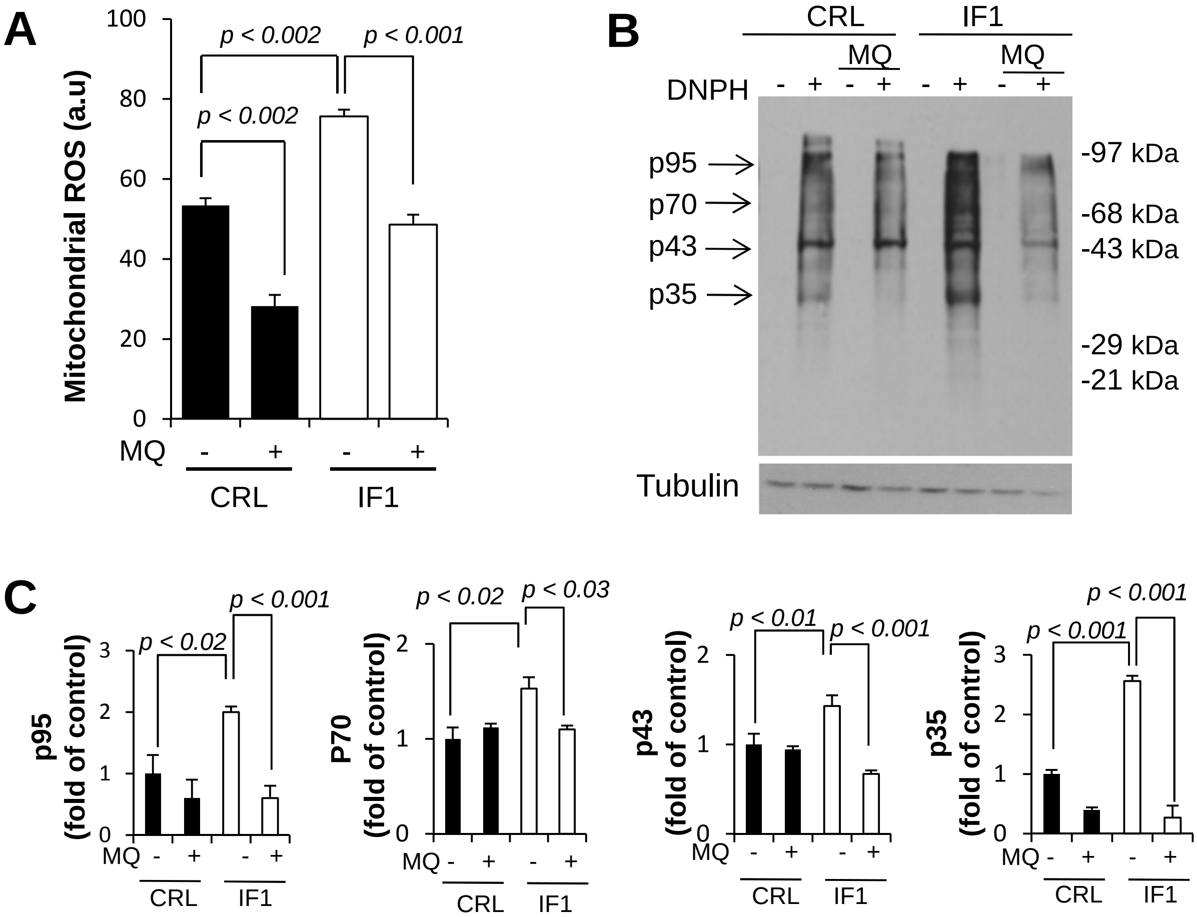
Figure 7. IF1 mediates metabolic adaptation, cellular proliferation and survival. HCT116 cells transfected with CDL-GFP-β-3'UTR were co-transfected with control (CRL, closed bars) or IF1 plasmid (IF1, open bars) in the absence (-) or presence (+) of 20 nM of the mitochondrial ROS scavenger MQ (MQ). **A,** Cellular proliferation was assessed by the incorporation of EdU into DNA by FACS analysis**.** Upper and lower panels show representative data from control (CRL) and IF1-overexpression (IF1)

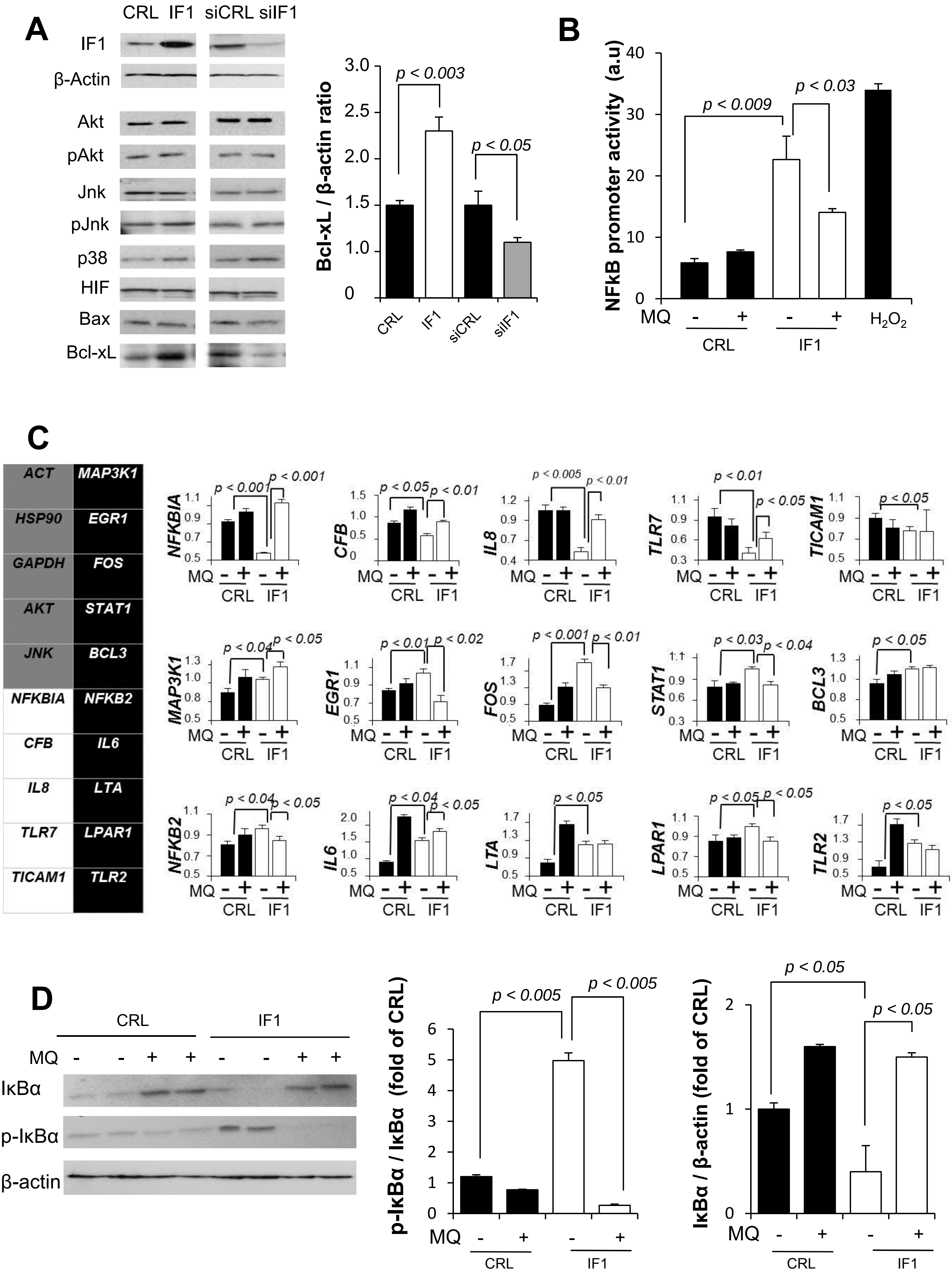
experiments, respectively. Traces: pink (CRL), blue (CRL+MQ), brown (IF1) and green (IF1+MQ). The results shown are the mean±s.e.m. of three experiments. **B**, Cells were treated with 1μM STS. Twenty four h post-treatment the percentage of sub-G0 cells was determined by flow cytometry. The data shown are mean±s.e.m. of three experiments. **C**, IF1-transfected cells were co-transfected with control (siCRL) or Bcl-xL (siBcl-xL) siRNA and processed as in **B**. The data shown are mean±s.e.m. of three experiments. **D,** The schematic summarizes the IF1-mediated inhibition of the synthase activity of the mitochondrial H^+-ATP synthase and the further activation of aerobic glycolysis in cancer cells. Inhibition of the H^+-ATP synthase also triggers mitochondrial hyperpolarization ($\uparrow\Delta\Psi$) and the subsequent increase in superoxide radical production (ROS). The increase in cellular ROS leads to the activation of the NFκB pathway which is involved in activation of cellular proliferation and survival by the inhibition of cell death. Metabolic reprogramming to aerobic glycolysis (Warburg Effect) is also known to confer a cellular phenotype that offers advantages for proliferation and minimizes the chances of cell death. See also Supplemental Figure S4-D and Figure S4-E.

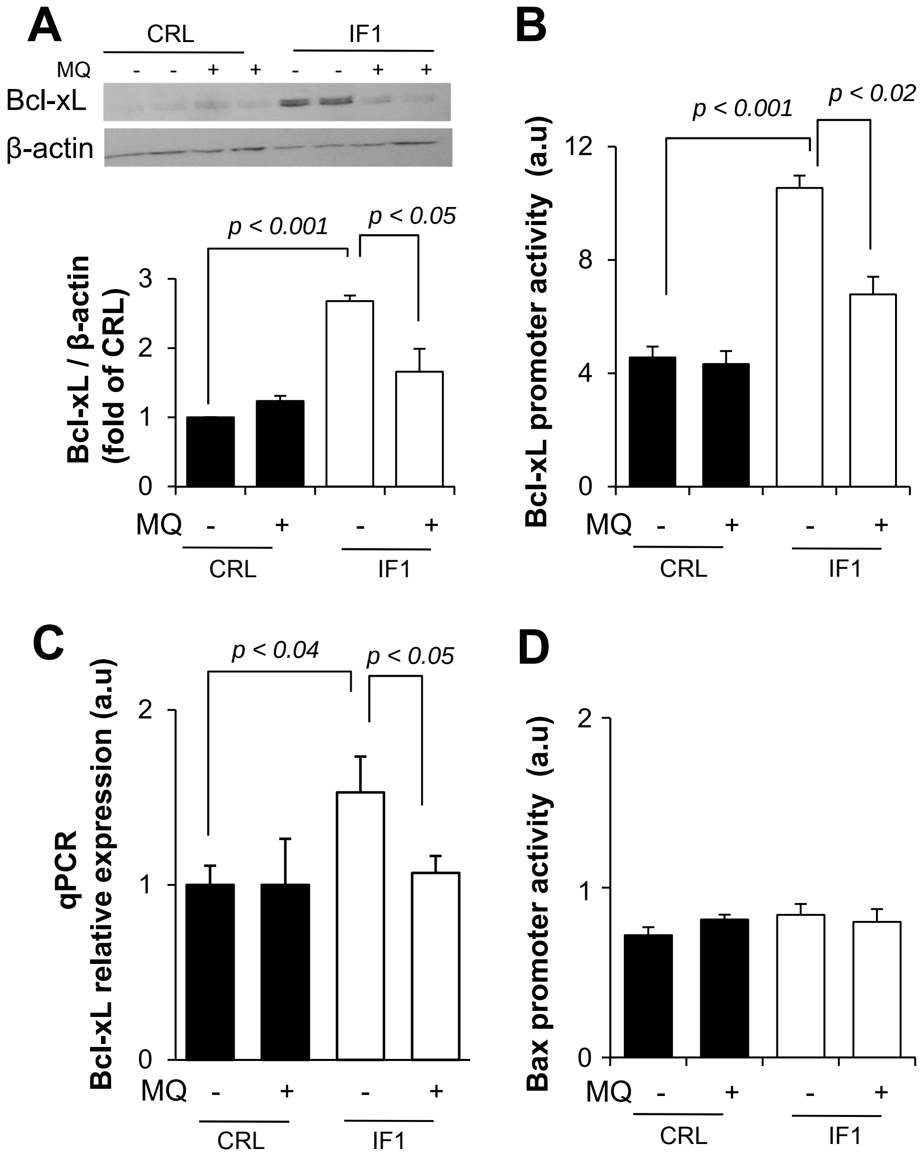

A
B
C
CRL
IF1
siCRL
siIF1
IF1
β-F1
IF1/β-F1 ratio
3
2
1
0
p < 0.002
p < 0.04
CRL
IF1
siCRL
siIF1
D
OSR
(pmol O₂/min/µg protein)
80
60
40
20
0
p < 0.02
p < 0.03
CRL
IF1
siCRL
siIF1
E
ΔΨm
(fold of CRL)
1.5
1.2
0.9
0.6
0.3
0
p < 0.04
CRL
IF1
siCRL
siIF1
F
Glycolytic flux
(nmol lactate/µg protein/h)
8
6
4
2
0
p < 0.02
p < 0.02
CRL
IF1
siCRL
siIF1
G
ATP (fold of CRL)
1.5
1.2
0.9
0.6
0.3
0
CRL
IF1
siCRL
siIF1
H
ATP (fold of CRL)
2
1
0
p < 0.001
p < 0.007
p < 0.04
p < 0.001
p < 0.009
p < 0.001
OL
-
-
+
-
-
+
CRL
IF1
siCRL
siIF1

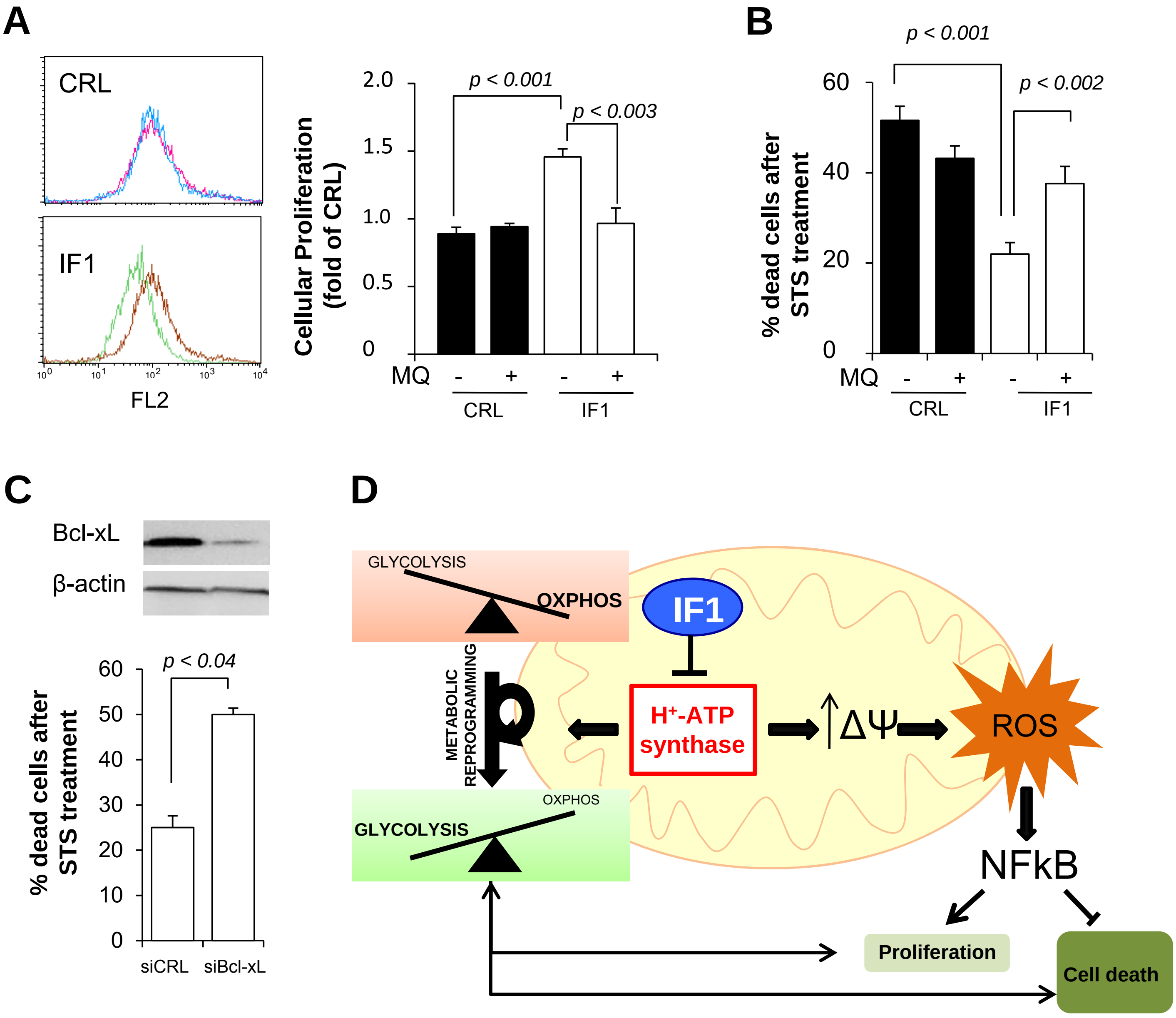

A
IF1
siIF1
10^0
10^1
10^2
10^3
10^4
FL2
Cellular Proliferation (fold of CRL)
3
2
1
0
p < 0.01
p < 0.05
CRL
IF1
siCRL
siIF1
B
CRL
IF1
siCRL
siIF1
Cellular invasion (% of CRL)
240
200
160
120
80
40
0
p < 0.006
p < 0.05
CRL
IF1
siCRL
siIF1
C
CRL
IF1
None
5FU
STS
M1
M2
M3
M4
0
200
400
600
800
1000
FL2
FL2
% dead cells
40
20
0
p < 0.04
p < 0.03
IF1
- +
- +
- +
None
5FU
STS
D
siCRL
siIF1
None
5FU
STS
M1
M2
M3
M4
0
200
400
600
800
1000
FL2
FL2
% dead cells
60
40
20
0
p < 0.03
p < 0.05
siIF1
- +
- +
- +
None
5FU
STS

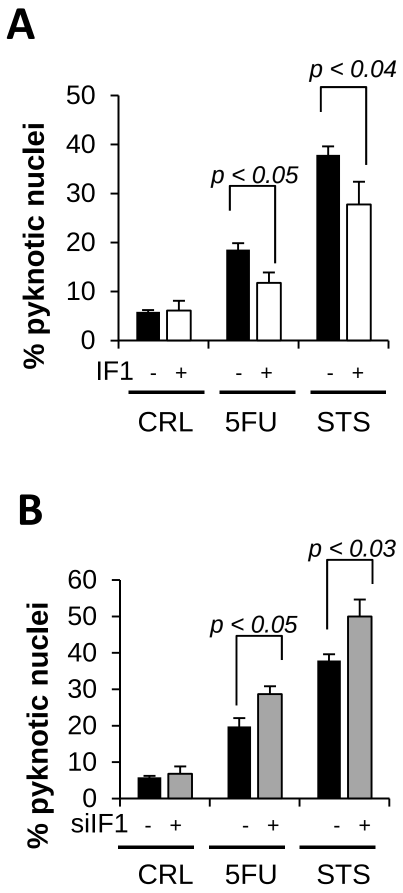

A
% apoptotic cells
40
30
20
10
0
p < 0.03
IF1 - + - +
CRL
STS
% apoptotic cells
60
40
20
0
p < 0.05
siIF1 - + - +
CRL
STS
B
HCT cells
CRL
IF1
0
2
4
time (h)
αAIF
% diffuse AIF stained cells
100
80
60
40
20
0
CRL
IF1
p < 0.002
0
2
4
time (h)
C
HeLa cells
CRL
siIF1
0
2
4
time (h)
αAIF
% diffuse AIF stained cells
100
80
60
40
20
0
siCRL
siIF1
p < 0.001
0
2
4
time (h)

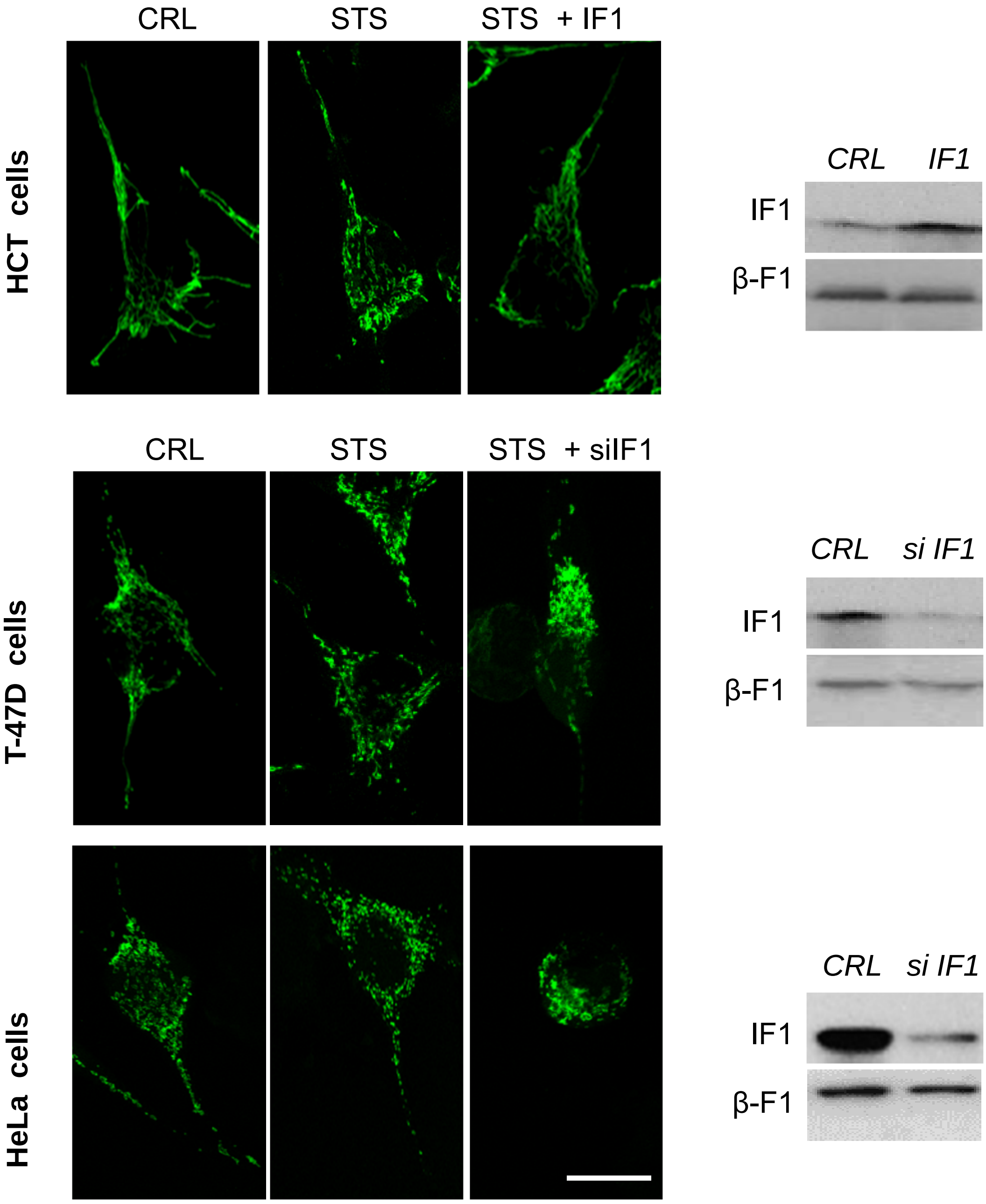

A
Mitochondrial ROS (a.u)
100
80
60
40
20
0
p < 0.002
p < 0.001
p < 0.002
MQ
-
+
-
+
CRL
IF1
B
CRL
IF1
MQ
MQ
DNPH
- + - + - + - +
p95
p70
p43
p35
-97 kDa
-68 kDa
-43 kDa
-29 kDa
-21 kDa
Tubulin
C
p95 (fold of control)
p < 0.02
p < 0.001
P70 (fold of control)
p < 0.02
p < 0.03
p43 (fold of control)
p < 0.01
p < 0.001
p35 (fold of control)
p < 0.001
p < 0.001
MQ - + - +
CRL
IF1

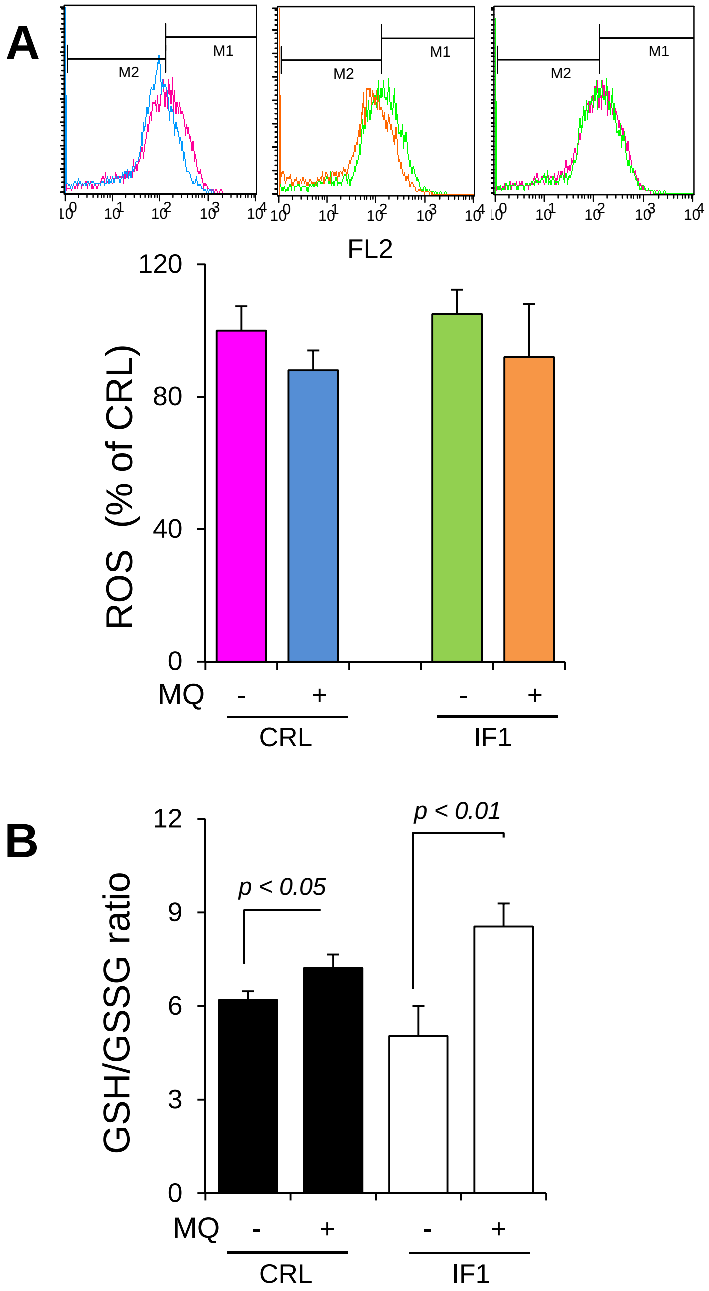
A
CRL IF1 siCRL siIF1
IF1
β-Actin
Akt
pAkt
Jnk
pJnk
p38
HIF
Bax
Bcl-xL
Bcl-xL / β-actin ratio
3.0
2.5
2.0
1.5
1.0
0
p < 0.003
p < 0.05
CRL
IF1
siCRL
siIF1
B
NFkB promoter activity (a.u)
40
30
20
10
0
p < 0.009
p < 0.03
MQ - + - +
H2O2
CRL
IF1
C
ACT
HSP90
GAPDH
AKT
JNK
NFKBIA
CFB
IL8
TLR7
TICAM1
MAP3K1
EGR1
FOS
STAT1
BCL3
NFKB2
IL6
LTA
LPAR1
TLR2
NFKBIA
p < 0.001
p < 0.001
CFB
p < 0.05
p < 0.01
IL8
p < 0.005
p < 0.01
TLR7
p < 0.01
p < 0.05
TICAM1
p < 0.05
MAP3K1
p < 0.04
p < 0.05
EGR1
p < 0.01
p < 0.02
FOS
p < 0.001
p < 0.01
STAT1
p < 0.03
p < 0.04
BCL3
p < 0.05
NFKB2
p < 0.04
p < 0.05
IL6
p < 0.04
p < 0.05
LTA
p < 0.05
LPAR1
p < 0.05
p < 0.05
TLR2
p < 0.05
MQ - + - +
CRL IF1
D
CRL
IF1
MQ - - + + - - + +
IκBα
p-IκBα
β-actin
p-IκBα / IκBα (fold of CRL)
6
5
4
3
2
1
0
p < 0.005
p < 0.005
MQ - + - +
CRL
IF1
IκBα / β-actin (fold of CRL)
2
1
0
p < 0.05
p < 0.05
MQ - + - +
CRL
IF1

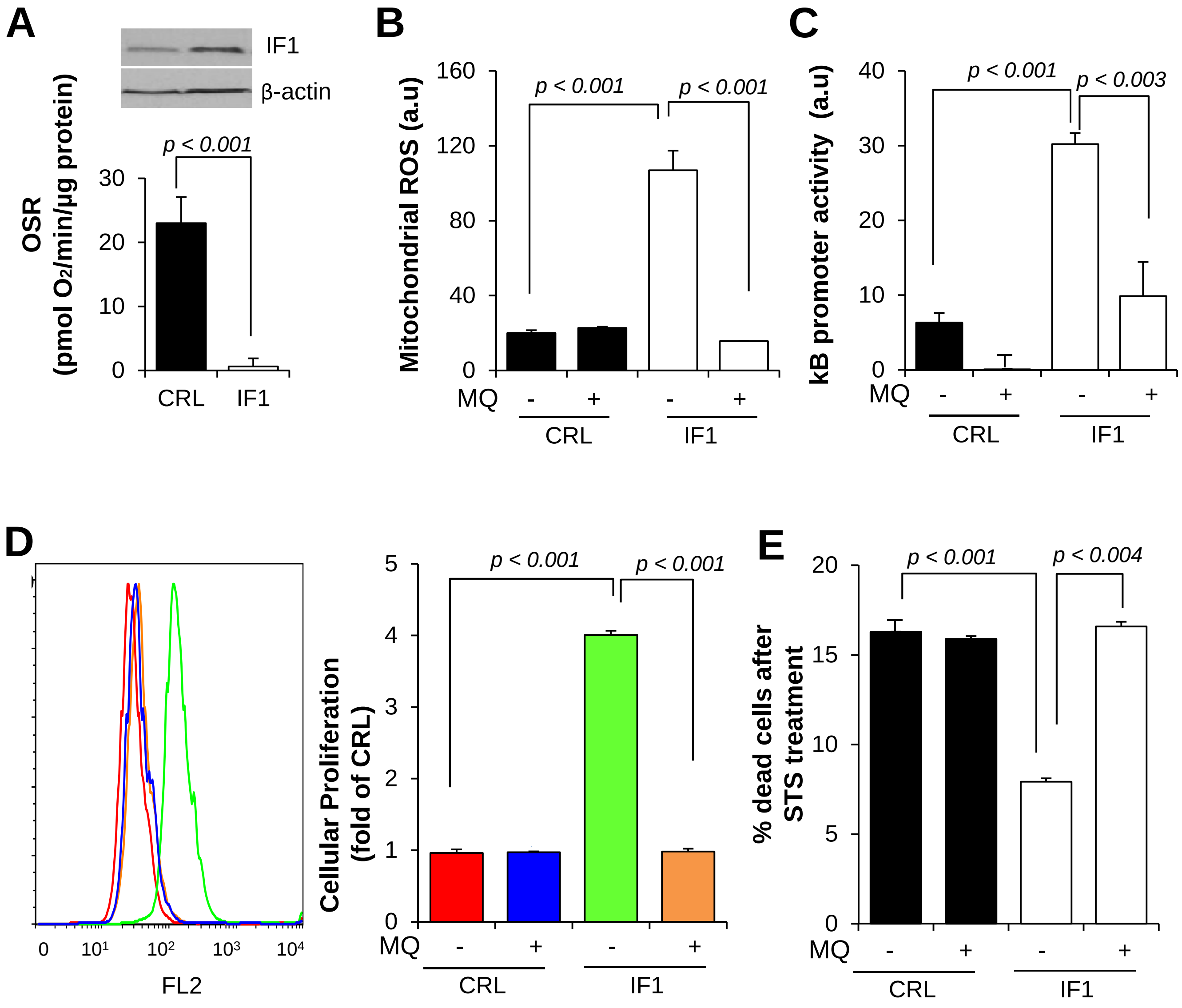
A
CRL
IF1
MQ - - + + - - + +
Bcl-xL
β-actin
p < 0.001
p < 0.05
Bcl-xL / β-actin (fold of CRL)
0
1
2
3
MQ - + - +
CRL
IF1
B
p < 0.001
p < 0.02
Bcl-xL promoter activity (a.u)
0
4
8
12
MQ - + - +
CRL
IF1
C
p < 0.04
p < 0.05
qPCR Bcl-xL relative expression (a.u)
0
1
2
MQ - + - +
CRL
IF1
D
Bax promoter activity (a.u)
0
1
2
MQ - + - +
CRL
IF1


A
CRL
IF1
10^0
10^1
10^2
10^3
10^4
FL2
Cellular Proliferation (fold of CRL)
2.0
1.5
1.0
0.5
0
p < 0.001
p < 0.003
MQ
-
+
-
+
CRL
IF1
B
p < 0.001
p < 0.002
% dead cells after STS treatment
60
40
20
0
MQ
-
+
-
+
CRL
IF1
C
Bcl-xL
β-actin
% dead cells after STS treatment
60
50
40
30
20
10
0
p < 0.04
siCRL
siBcl-xL
D
GLYCOLYSIS
OXPHOS
METABOLIC REPROGRAMMING
IF1
H^+-ATP synthase
↑ΔΨ
ROS
GLYCOLYSIS
OXPHOS
NFkB
Proliferation
Cell death


A
% pyknotic nuclei
50
40
30
20
10
0
p < 0.05
p < 0.04
IF1
-
+
-
+
-
+
CRL
5FU
STS
B
% pyknotic nuclei
60
50
40
30
20
10
0
p < 0.05
p < 0.03
siIF1
-
+
-
+
-
+
CRL
5FU
STS


CRL
STS
STS + IF1
HCT cells
CRL
IF1
IF1
β-F1
CRL
STS
STS + siIF1
T-47D cells
CRL
si IF1
IF1
β-F1
HeLa cells
CRL
si IF1
IF1
β-F1


A
M2
M1
10^0
10^1
10^2
10^3
10^4
FL2
120
80
40
0
ROS (% of CRL)
MQ
-
+
-
+
CRL
IF1
B
12
9
6
3
0
GSH/GSSG ratio
p < 0.05
p < 0.01
MQ
-
+
-
+
CRL
IF1


A
IF1
β-actin
OSR
(pmol O2/min/μg protein)
p < 0.001
0
10
20
30
CRL
IF1
B
Mitochondrial ROS (a.u)
p < 0.001
p < 0.001
0
40
80
120
160
MQ
-
+
-
+
CRL
IF1
C
kB promoter activity (a.u)
p < 0.001
p < 0.003
0
10
20
30
40
MQ
-
+
-
+
CRL
IF1
D
0
10¹
10²
10³
10⁴
FL2
Cellular Proliferation
(fold of CRL)
p < 0.001
p < 0.001
0
1
2
3
4
5
MQ
-
+
-
+
CRL
IF1
E
% dead cells after
STS treatment
p < 0.001
p < 0.004
0
5
10
15
20
MQ
-
+
-
+
CRL
IF1