C3G down-regulates p38 MAPK activity in response to stress by Rap-1 independent mechanisms: Involvement in cell death

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Abstract

We present here evidences supporting a negative regulation of p38α MAPK activity by C3G in MEFs triggered by stress, which can mediate cell death or survival depending on the stimuli. Upon serum deprivation, C3G induces survival through inhibition of p38α activation, which mediates apoptosis. In contrast, in response to H_2O_2, C3G behaves as a pro-apoptotic molecule, as its knock-down or knock-out enhances survival through up-regulation of p38α activation, which plays an anti-apoptotic role under these conditions. Moreover, the C3G target, Rap-1, plays an opposite role, also through regulation of p38α MAPK activity.

Our data also suggest that changes in the protein levels of some members of the Bcl-2 family could account for the regulation of cell death by C3G and/or Rap-1 through p38α MAPK. Bim/Bcl-x_L ratio appears to be important in the regulation of cell survival, both upon serum deprivation and in response to H_2O_2. In addition, the increase in BNIP-3 levels induced by C3G knock-down in wt cells treated with H_2O_2 might play a role preventing cell death.

Therefore, we can conclude that C3G is a negative regulator of p38α MAPK in MEFs, while Rap-1 is a positive regulator, but both, through the regulation of p38α activity, can promote cell survival or cell death depending on the stimuli.

Introduction

p38 mitogen-activated protein kinases (MAPKs) are mainly activated by stress and inflammatory cytokines, although several non-stressful stimuli can also activate them, leading to the regulation of different cellular functions such as proliferation, differentiation, apoptosis or development [1-2]. p38α is ubiquitously expressed and is the most abundant isoform, being essential for embryonic development [3-4].

p38 MAPKs has been proposed to mediate either cell death or survival, depending on the cell type, the stimuli and the p38 isoform [1]. In particular, p38α plays an important role as a mediator of apoptosis in response to different pro-apoptotic stimuli [5]. Cardiomyocytes and MEF-derived cell lines deficient in p38α are more resistant to apoptosis due to downregulation of the expression of proapoptotic proteins [5] and upregulation of survival pathways [5-6]. p38α MAPK plays also an important role in the apoptosis induced by some chemotherapeutical drugs. For example, it is essential for cisplatin-induced apoptosis in the colon carcinoma derived cell line, HCT116, where the p53/ROS/p38αMAPK cascade mediates this process [7]. Moreover, p38α can be considered as a tumor suppressor in the transformation induced by oncogenic H-Ras [8], owned to its ability to induce apoptosis. In this line, mice deficient in p38α has been shown to be more susceptible to cancer development in some tissues such as lung [9] or liver [10].

C3G is a guanine nucleotide exchange factor (GEF) for Rap1 and R-Ras proteins [11-13] and is essential for mouse embryonic development [13]. C3G can also suppress malignant transformation induced by several oncogenes, including Hras$^{\text{Lys12}}$, by a mechanism not dependent on its GEF function [14-15], which involves PP2A-mediated down-regulation of ERKs activity [16]. In addition, C3G has been shown to play a role either as a mediator of apoptosis in hematopoietic cells [17] or of cell survival in a neuroblastoma cell line [18]. Moreover, we have

recently shown that although C3G overexpression increases apoptosis in the chronic myeloid leukemia (CML) cell line K562, C3G silencing also enhances STI-571 induced apoptosis through Rap-1-mediated p38α activation [19]. This represents a new functional relationship between C3G and p38α able to regulate apoptosis in chronic myeloid leukemia. However, in CML cells, a truncated C3G isoform is overexpressed and phosphorylated by the oncogenic protein Bcr-Abl [20], which could lead to a specific and cell-type dependent regulation of p38α by C3G. Therefore, it is important to know whether this cross-talk between C3G and p38α can operate in adherent (non-hematopoietic) non-tumoral cells, where the truncated C3G isoform is not present, in response to stress stimuli known to activate p38α. In this way, we will be able to establish the physiological role of C3G in a normal cell as a potential regulator of p38α MAPK and cell death upon stress stimulation. If C3G has any effect on p38α under these conditions, it would be important to know whether Rap-1 mediates the C3G effect, as some data from the literature show a crosstalk between Rap-1 and p38 [21-24].

Taking into account all this, in order to study whether C3G/p38α also collaborate regulating stress responses in non-tumoral cells, we have analyzed this potential cross-talk in mouse embryonic fibroblasts (MEFs) upon stress stimulation, as well as its role in cell death. We have also determined whether Rap-1 is the mediator of C3G actions. Our results support a negative regulation of p38α MAPK activity by C3G, which is essential to regulate cell death, mediating either cell survival (upon serum-deprivation) or cell death (upon stimulation with H_2O_2) depending on the stimulus. The effect of C3G is not mediated by Rap-1, which indeed plays an opposite role, also through regulation of p38α MAPK activity.

Materials and Methods

Cell lines, culture conditions and inhibitors

Wt, p38α-deficient and C3G knock-out mouse embryonic fibroblasts (MEFs) as well as those with permanent C3G silencing or expressing Rap-1 dominant negative were grown in DMEM medium containing 10% FBS (Gibco), at 37ºC in a humidified atmosphere of 5% CO_2. When indicated, the chemical p38 inhibitor, SB203580 (Calbiochem), was used at 5 μM.

C3G silencing

A construct containing a C3G shRNA in the pSuper.retro.puro vector (OligoEngine) was generated. This contained a 19 nucleotide specific insert corresponding to the C3G sequence (Acc. Num: AF348669) plus the nucleotides located just before the complementary sequence: 5'GATCCCC**GCCCTCTCCTCCTGTTATA**TTCAAGAGA**TATAACAGGAGGAGAGGC** TTTTTGGAAA3'. Restriction sites for Bgl II and Hind III were included in this sequence. Annealing of 3'-5´ complementary oligonucleotides was performed according to manufacturer specifications (OligoEngine). The presence of the insert was verified by a double digestion (EcoRI and HindIII) and by sequencing. Transfections of wt and p38α-deficient MEFs were performed with Metafectene-Pro (a polycationic liposomal transfection reagent, obtained from Biontex Laboratories GmbH (Munich, Germany) following the protocol supplied by the manufacturer (7μl metafectene/1μg of DNA). 72h later, cells were selected with 1μg/ml puromycine (Sigma). Clones with stable expression of C3G shRNA were picked, expanded and analyzed for C3G expression. Then, in those clones with a clear C3G knock-down expression, further analysis were carried out to characterize the effect of C3G silence on signalling and cell death, although in the results section we just show data from a representative clone.

***In vitro* generation of C3G knock-out MEFs**

C3G deficient MEFs were generated from C3G knock-out MEFs previously rescued with human C3G cDNA (C3G-/-hC3G) [13]. These parental cells were infected with an adenovirus expressing the Cre recombinase under the CMV promoter (Ad-CMV-Cre) (Vector Biolabs, Philadelphia, USA) to delete the human C3G exons 15 and 16, flanked by loxP sequences. Cells were plated 24h prior to infection at a density that guaranteed 80% confluence at the day of infection. Virus was diluted in growth medium supplemented with 2.5 µg/ml polybrene at a multiplicity of infection (MOI)=20. Original cell culture medium was replaced with the virus-containing medium (0.5 ml/well in a 12 well plate), cells were incubated for 1h with occasional shacking, and then fresh medium was added to complete volume. After 48 h of infection, cells were trypsinized and replated following the limiting dilution protocol. A total of 50 cell clones were selected, expanded and genotyped to identify C3G deletion by PCR using specific oligonucleotides [13]: for mouse C3G exon 15, forward: 5′-AAGGAATGGCTGCAGATGGTCAAGC-3′ and reverse: 5′-GGGACTCACTCTACAAGGAACTCCA-3′ and for human C3G (n1896-n1919), forward: 5′-CCGGATCCTCATGGGGTATACGGCTTCA-3′ (n1757-n1777) and reverse: 5′-CGGAATTCCCAGGGACGCCGCTGACCGCT-3′. Those clones (8 clones) with a clear C3G deletion were used for further characterization. In parallel, we got some clones after infection with no deletion, which were used as additional wt controls to discard any unspecific effect of the infection procedure. Those clones behaved as the original parental cells (data not shown).

Generation of MEFs expressing a dominant negative Rap-1 mutant

A Rap-1 dominant negative (with Ser 17 mutated to Asn) cloned into pCEP4 (Invitrogen, Carlsbad, CA) as a 1,2 kb BamHI fragment was transfected into wt and p38α-deficient MEFs using Metafectene-Pro (7µl/1µg of DNA per dish (20,000 cells)). Then, cells were selected with 2µg/ml hygromycin and different clones were picked, expanded and analyzed.

Re-expression of p38α MAPK in MEFs lacking p38α

To re-express p38α MAPK in p38α-/- MEFs, transient transfections with a p38α construct containing human p38α cDNA cloned into the EcoRI site of the pEFmlink expression vector [5] were performed using Metafectene-Pro. The protocol supplied by the manufacturer was followed using 7μl/1μg of DNA per dish (20,000 cells). Cell assays were performed 48 h after transfection.

Western-blot analysis

Western-blot analysis was carried out as previously described [6]. Proteins were separated by electrophoresis using Anderson gels and transferred to nitrocellulose membranes that were probed with the following antibodies: active cleaved caspase 3 (Cell Signaling, 9661), p38α (Santa Cruz, sc-535), phospho-p38 (Cell Signaling, 9211), phospho-MKK3/6 (Cell Signaling, 9231), C3G (H-300) (Santa Cruz, sc-15359), Rap-1 (Santa Cruz, sc-65), Bim (BD Bioscience, 559685), Bcl-x_L (BD Bioscience 610211), BNIP-3 (Cell Signaling, 3769), β-actin (Sigma A5441) and α-tubulin (Sigma T-5168).

Rap-1 activity

Rap-1 activity assay was performed with total cell lysates from preconfluent cells using pGST-RalGDS-RBD construct to pull down Rap-1-GTP, which was analyzed by Western-blot as described previously [19].

Analysis of cell viability and apoptosis

Cell viability was assayed using the crystal violet method. Cells were washed with PBS, incubated with a crystal violet solution (0.2%, w/v in ethanol) for 20 min, washed and dried. Stained (viable) cells were lysed in 1% SDS and absorbance at 560nm was measured.

Apoptotic cells were quantified by flow cytometric analysis of the cell cycle. Cells were trypsinized, washed with PBS and fixed with cold ethanol (70% v/v). The cells were then washed and resuspended in PBS and incubated with RNAase (25 μg/10^6 cells) for 30 min at

37ºC. After addition of 0.05% propidium iodide (PI), cells were analyzed in the cytometer. The percentage of cells in the different phases of cell cycle was determined. Cells in subG1 were considered as apoptotic.

Apoptosis was also measured by Western-blot analysis of the levels of active cleaved caspase 3.

To analyze condensed and fragmented nuclei, characteristic of apoptosis, nuclei were stained with propidium iodide (5μg/ml in PBS; 0,1% Triton X-100; EDTA 0,1M supplemented with 5μg/ml RNAse) [25] and visualized by fluorescent miocroscopy. Apoptotic indices were calculated after counting 500-1,000 cells per treatment in an inverted fluorescence microscope (Eclipse TE300, Nikon).

Statistical analysis

Statistical analysis was carried out by Student's t test.

Results

C3G down-regulates p38 MAPK activity in response to stress

We have recently found a new functional relationship between C3G and p38α able to regulate STI-571-induced apoptosis in chronic myeloid leukemia [19]. In order to study whether C3G and p38 also collaborate regulating stress responses in non-tumoral cells in a stress context, we have analyzed the potential cross-talk between C3G and p38MAPK in MEFs upon stress stimulation with NaCl or H_2O_2. First, we studied the effect of C3G knock-down through stable gene silence in wt and p38α deficient MEFs. As shown in figure 1A, the transfection of a shC3G construct decreased C3G protein levels and mRNA in both, wt and p38α deficient MEFs as shown in a representative clone. Next, we measured activation of p38MAPK pathway upon treatment with different stress stimuli. As shown in figure 1B for a representative clone,

stimulation of wt MEFs with H_2O_2 and NaCl induced a strong phosphorylation of two p38 MAPKs isoforms, p38α and another one with a higher mobility. This phosphorylation was enhanced by C3G knock-down (figure 1B), specially, upon NaCl treatment. In p38α-deficient MEFs, the effect of C3G down-regulation was similar, although, due to the lack of p38α, only the activation of the other p38 isoform was enhanced. According to this, the levels of phospho-MKK3/6 were also increased upon C3G silencing. Similar results were obtained in other C3Gi clones (data not shown). It should be noted that in p38α-deficient cells the activation of MKK3/6 is always higher due to the known upregulation of MKK6 protein levels [26].

To confirm this regulation of p38 by C3G, we used an additional experimental approach. We generated C3G knock-out MEFs by infection with an adenovirus expressing the Cre recombinase (see details under methods section) to delete human C3G exons 15 and 16 in MEFs C3G-/- rescued with human C3G cDNA [13]. As shown in figure 1C, the analysis of C3G expression by PCR in a representative C3G knock-out clone, demonstrated that human C3G cDNA was not expressed. Similarly, C3G protein expression (see upper band) was also absent. As expected, mouse C3G DNA was not expressed either (Fig. 1C). Using this representative clone (Fig. 1D) we observed that the level of p38 phosphorylation was upregulated upon treatment with oxidative stress (H_2O_2) and NaCl in C3G knock-out cells, as compared to wt cells. Similar results were obtained with other clones (data not shown). According to this, the levels of phospho-MKK3/6 were also increased in cells lacking C3G upon treatment with H_2O_2 or NaCl. Therefore, we confirmed the negative regulation of p38MAPK activity (mainly, p38α) by C3G in response to stress stimuli.

Rap-1 is not the mediator of C3G effects on the regulation of p38 MAPK pathway

Rap-1 is the main C3G target [review in 21], although C3G can also act through Rap-1 independent mechanisms [14-15, 17, 19], including through R-Ras [27]. Some data from the literature point out to a regulation of p38 MAPK by Rap-1, which could be either positive or

negative [20-24]. We show here that C3G regulates p38 MAPK activity upon triggering with different stress stimuli, so we wanted to know if C3G was acting through Rap-1. First, we analyzed Rap-1-GTP levels in C3G silenced cells, as a measure of Rap-1 activity (see supplementary figure). We found that basal Rap-1 activity was not decreased or even increased by C3G knock-down, both in wt and p38α-/- cells as it was previously described for C3G deficient MEFs [13]. Treatment with H_2O_2 slightly increased Rap-1-GTP levels in cells expressing C3G, while it had the opposite effect upon C3G silencing. In contrast, NaCl slightly reduced Rap-1-GTP levels in cells expressing C3G, while increased them upon C3G knock-down. Therefore, it was not a clear correlation between C3G knock-down and Rap-1 activity. So, we decided to study the effect of the inhibition of Rap-1 activity through transfection with a dominant negative Rap-1 construct.

As shown in figure 2A, Rap-1-GTP levels were highly decreased, both in wt and p38α-deficient cells permanently expressing a dominant negative Rap-1 construct, as well as in transiently transfected cells (data not shown).

Using representative clones expressing this dominant negative mutant of Rap-1, we analyzed p38 MAPK activity. As shown in figure 2B, in wt cells, p38α MAPK activation induced by H_2O_2 and NaCl was decreased by the expression of the dominant negative Rap-1. The activation of the other p38 isoform was similar or slightly reduced by dominant negative Rap-1. According to this, the levels of phospho MKK3/6 were also decreased by the expression of the Rap-1 dominant negative mutant.

All these results indicate that inhibition of C3G function, either by shRNA or by knocking out the gene, does not exert the same effect on p38 as Rap-1 inhibition. Moreover, they appear to play opposing roles upon stress stimulation, so that C3G would be a negative regulator of p38α MAPK, while Rap-1 would be a positive regulator.

Regulation of cell death by C3G/p38 MAPK and Rap-1

We show here that the absence of C3G or its silencing enhances p38 activation (mainly p38α) in response to some stress stimuli. In addition, it is well known that p38α MAPK plays an important role mediating apoptosis, including that induced by serum-deprivation [5-6]. Therefore, it is very likely that inhibition of C3G function can induce apoptosis through p38α MAPK.

Based on all this, we analyzed the role played by C3G in the regulation of cell death induced by serum-deprivation. As show in figure 3A, C3G knock-down increased the number of apoptotic cells in a significant way only in wt MEFs, but not in cells deficient in p38α. Moreover, activation of caspase 3 by serum deprivation was higher in C3G knock-out cells and it was decreased upon inhibition of p38α/β with SB203580 (Fig. 3B). It should be noted that in C3G deficient MEFs, caspase 3 was also highly activated even in the presence of serum, suggesting a basal anti-apoptotic role of C3G in these cells. Therefore, all these data indicate that C3G would mediate survival upon serum-deprivation through a mechanism involving down-regulation of p38α activity.

Although we have shown that Rap-1 and C3G had opposite effects on p38 MAPK activity (Fig. 1B, 1D and 2B), we determined whether Rap-1 could be a mediator of C3G survival effects upon serum-deprivation. As shown in figure 3C, activation of caspase 3 by serum-deprivation was highly reduced by the expression of Rap-1 dominant negative in wt cells. In p38α deficient cells, the levels of active caspase 3 were very low and remained unchanged upon expression of Rap-1 dominant negative. Therefore, all these data are in favour of a model where C3G would mediate survival through the negative regulation of p38α activation upon serum deprivation, while Rap-1 would play an opposite role.

To establish whether C3G/p38α MAPK cascade could play a similar role in the process of cell death induced by oxidative stress, we studied the effect of H_2O_2 on cell viability and on apoptosis in the cell lines where C3G had been silenced or knocked-out. We found that H_2O_2 decreased cell viability in wt MEFs, as expected, but surprisingly, in p38α-deficient cells, cell viability was significantly lower (Fig. 4A). C3G knock-down partially prevented cell death induced by H_2O_2 in wt and p38α-deficient cells, though changes in cell viability were always much higher and significant in wt cells. The reconstitution of p38α in p38α−/− MEFs increased cell viability, but more efficiently in C3G knock-down cells, demonstrating the survival role of p38α in C3G silenced cells. We confirmed the effect of C3G in C3G knock-out cells. As shown in figure 4B, cell viability upon H_2O_2 treatment was higher in cells lacking C3G than in wt cells. It should be noted that C3G-/- and their wt control MEFs were obtained from a different embryonic stage than p38α-deficient MEFs and its respective wt MEFs, which might explain the differences in cell viability between the two different wt clones. Altogether, these data indicate that C3G is involved in mediating H_2O_2-induced cell death through a mechanism mainly dependent on the attenuation of p38α MAPK activity.

To determine the contribution of apoptosis in this process of cell death, we analyzed the number of condensed and/or fragmented (apoptotic) nuclei. As expected, they were higher in p38α-deficient than in wt MEFs and C3G knock-down reduced them, particularly in wt cells (Fig. 4C). Thus, in our system, p38α would prevent from the apoptotic cell death induced by H_2O_2 and C3G through downregulation of p38α MAPK activity would mediate cell death.

To establish the role of Rap-1 as a potential mediator of C3G actions in this process of cell death, we examined the effect of the dominant negative Rap-1 construct in cells treated with H_2O_2. As shown in figure 5A, cell viability was highly reduced in wt MEFs expressing the dominant negative Rap-1 construct, reaching a lower level than that observed in p38α-deficient

cells, where dominant negative Rap-1 had no significant effect. According to this, the number of apoptotic nuclei was highly increased upon expression of dominant negative Rap-1 in wt cells (Fig. 5B), while in p38α-deficient cells the expression of the dominant negative Rap-1 had no significant effect.

Regulation of pro- and anti-apoptotic proteins from Bcl-2 family by C3G and Rap-1 through p38 MAPK dependent and independent mechanisms

It is known that p38 MAPKs can regulate the expression and/or function of different pro- and anti-apoptotic proteins from Bcl-2 family. Thus, we analyzed some of these proteins, which are relevant in cell death induced by serum-deprivation and/or in oxidative stress such as Bim, Bcl-x_L and BNIP-3. In addition, our data from DNA microarray analysis had revealed an upregulation of BNIP3 mRNA levels by C3G knock-down (not shown).

As shown in figure 6A and 6B, the levels of the pro-apoptotic protein Bim were upregulated upon serum-deprivation in both wt and p38α-deficient MEFs, which is accompanied by a reduction in the levels of the anti-apoptotic protein Bcl-x_L only in wt cells. C3G knock-down enhanced the effect of serum-starvation inducing Bim in wt cells, while in p38α-deficient cells the effect was opposite (Fig 6A). In addition, cells lacking p38α maintained high Bcl-x_L levels regardless of the presence of serum and C3G silencing (Fig 6A). Therefore, these data suggest that the parallel increase in Bim levels along with the decrease in Bcl-x_L levels might account for a higher level of apoptosis in wt MEFs (Fig 6C). Moreover, C3G knock-down would enhance serum-withdrawal induced cell death in wt MEFs up-regulating Bim through a p38α-dependent mechanism. According to this, the expression of a dominant negative Rap-1 construct led to a reduction in Bim protein levels upon serum-deprivation in wt cells, while Bcl-x_L levels remained high, which favours cell survival (Fig. 6B and 6C). In p38α-deficient cells, either

expressing dominant negative Rap-1 or not, high Bclx$_L$ levels were maintained in serum-deprived cells in parallel with moderate levels of Bim (Fig. 6B and 6C), which would also explain the low levels of apoptosis.

On the other hand, BNIP-3 protein levels decreased upon serum-deprivation in all the conditions tested (Fig. 6A and B), except in p38α-deficient MEFs expressing dominant negative Rap-1 or upon C3G silencing, where BNIP-3 levels were already very low. As a consequence, it is unclear the relevance of BNIP-3 as a regulator of cell death under these conditions.

We also analyzed whether changes in these proteins could play a role in the process of cell death induced by H$_2$O$_2$ and how it could be modulated by C3G and/or Rap-1 through p38α dependent and independent mechanisms. As shown in figure 6D and 6E, Bim was slightly up-regulated by H$_2$O$_2$ in wt MEFs and significantly increased in p38α-deficient cells after 4h of treatment. C3G knock-down induced a high increase in BNIP-3 levels upon treatment of wt cells with H$_2$O$_2$, while Bim and Bclx$_L$ protein levels were already up-regulated by C3G silencing under basal conditions and maintained high upon H$_2$O$_2$ treatment (Fig. 6D). In contrast, in p38α-deficient MEFs C3G knock-down did not significantly change BNIP-3 levels and slightly increased basal Bim and Bcl-x$_L$ protein levels, which remain constant upon H$_2$O$_2$ treatment (Fig 6E). Thus, all these data point out to a potential relevant role of BNIP-3 and Bcl-x$_L$ as mediators of cell survival in response to oxidative stress upon C3G knock-down of wt cells. According to this potential function of BNIP-3, the expression of the dominant negative Rap-1 did not induce any significant increase in BNIP-3 levels, either in wt or p38α-deficient cells (Fig. 6E). In addition, a decrease in Bcl-x$_L$ levels is produced in MEFs expressing dominant negative Rap-1 upon treatment with H$_2$O$_2$, which is more evident in cells lacking p38α. Moreover, the expression of Bim is highly increased in these cells with the dominant negative Rap-1 construct, which would also favour H$_2$O$_2$ induced cell death (Fig. 6E). Therefore, based on all these data,

we can propose that the p38α dependent sustained Bcl-x$_L$ expression and the up-regulation of BNIP-3 might be responsible for the enhanced survival observed in wt cells treated with H$_2$O$_2$ upon G3G knock-down. In contrast, Rap-1 appears to exert the opposite effect also through a p38α-dependent mechanism.

Model of regulation of apoptosis by C3G and Rap-1 through p38α MAPK

All these data indicate that C3G is a mediator of the apoptotic cell death induced by H$_2$O$_2$ through the negative regulation of p38α activation, while Rap-1 would play an opposite role depending on the stimuli (serum deprivation or H$_2$O$_2$). So, we propose a model to explain the function of C3G and Rap-1 in the regulation of p38 and cell death under stress stimulation (Fig. 7). Upon serum-deprivation, C3G inhibits p38α activation leading to cell survival (Fig. 7A). In contrast, activation of Rap-1 by alternative GEFs, different from C3G, mediates p38α activation, which results in apoptotic cell death. On the other side, in response to oxidative stress (H$_2$O$_2$), C3G through down-regulation of p38α activity induces apoptosis (Fig. 7B), while Rap-1, which is again not activated by C3G but by other GEFs, mediates cell survival via p38α.

Discussion

p38α plays an important role as a mediator of apoptosis in response to different pro-apoptotic stimuli and in different cell types [5-7]. Moreover, p38α functions as a tumor suppressor in the transformation induced by oncogenic H-Ras [8], owned to its ability to induce apoptosis upon stimulation with the reactive oxygen species (ROS) that are generated. In contrast, we present here evidences supporting a survival effect of p38α upon activation with H$_2$O$_2$, an oxidative stress stimulus. Moreover, C3G knock-down through the enhancement of p38α activation, increases MEFs survival under these conditions. In contrast, in serum starved

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MEFs, C3G silencing mediates apoptosis through increasing p38α activation, which agrees with previous data on the pro-apoptotic effect of p38α. But overall, these data indicate that C3G can mediate cell death or survival in MEFs depending on the stimuli and it does so through the negative regulation of p38α MAPK activity. This C3G effect is independent of its GEF activity on Rap-1 which, in fact, plays an opposite role. Therefore, our data point out to an important role for C3G as a modulator of cell death through p38α regulation. Moreover, although the effect of Rap-1 is the opposite, its function on cell death also requires p38 as intermediate, but in contrast to C3G, Rap-1 positively regulates p38.

We have described here a dual role for C3G as a regulator of cell death in MEFs, acting both as a pro- and anti-apoptotic molecule, depending on the stimulus. This is in agreement with previous data from the literature showing that C3G played a role either as a mediator of apoptosis in hematopoietic cells when over-expressed [17] or of cell survival in a neuroblastoma cell line upon serum deprivation [18]. Moreover, these data partially agree to what we found in the chronic myeloid leukemia (CML) cell line K562 [19]. In those cells, we have recently shown that C3G overexpression increased apoptosis and C3G silencing also increased STI-571 induced apoptosis through enhancement of p38α activation [19]. However, in contrast to our finding in MEFs, Rap-1 mediated the effect of C3G on apoptosis in K562 cells. Therefore, in the two different cell models, there is a cross-talk between C3G and p38α, which is essential to regulate cell death. However, depending on the cell type, Rap-1 can be a mediator of C3G or play an opposite role. In addition, depending on the stimuli, the effects of C3G can also be different.

The distinct effect of C3G/p38α cascade in the CML cell line K562 and in MEFs can depend on the differences between these two cell lines and/or the stimuli. On the other hand, the opposite effect of Rap-1 in these two cell lines is more likely to be a consequence of the differences between these two cell models. In contrast to MEFs, K562 is a tumor derived cell

line with a number of alterations such as the overexpression of a truncated C3G isoform, which is phosphorylated by the oncogenic protein Bcr-Abl [20]. In addition, the constitutively activation of Bcr-Abl contributes to activation of proliferation and survival. Therefore, it is possible that endogenous C3G (the full length and/or the truncated isoform) can be acting as an oncogene in these cells under certain circumstances. This, together with other differences between these two cell lines, including the ability of K562 cells to grow in suspension (as all the hematopoietic cells), can explain the different actions of C3G and/or Rap-1 on cell death.

Another interesting point is why Rap-1 does not always mediate the effects of C3G on p38 MAPK. There are different potential reasons for that. On one side, it is known that C3G can act through Rap-1 independent mechanisms as demonstrated with a C3G form lacking the guanine nucleotide exchange factor (GEF) domain [14-17]. In addition, there are other Rap-1 GEFs, different from C3G, able to activate Rap-1. These GEFs can be differentially activated by distinct signals and may have a different pattern of expression depending on the cell type [21, 28-29]. This might also explain the discrepancies in the functional interactions between Rap-1 and p38 MAPK. Thus, Rap-1 can inhibit Ras-induced p38 MAPK activation in a thymoma cell line [21-22], while FGF2 induced p38 MAPK activation in endothelial cells is mediated by Rap-1 [23], as we have observed in MEFs stimulated with H_2O_2.

Looking for potential molecular mechanisms involved in the regulation of cell death by C3G and/or Rap-1 through p38α regulation, our data suggest that Bim/Bcl-x_L ratio might play a role upon serum deprivation and in response to H_2O_2. In serum-starved cells, this ratio is higher in the presence of p38α and C3G knock-down, which enhances apoptosis; while Rap-1 inhibition reduces Bim/Bcl-x_L ratio in wt MEFs favouring cell survival (see fig. 6C). In response to oxidative stress, the regulation of cell death based on Bim/Bcl-x_L ratio appears also to operate, but the up-regulation of BNIP-3 upon C3G knock-down might be relevant to explain the enhanced survival observed in wt cells. However, there is not an easy explanation to all the

changes in the levels of Bim detected under the different conditions, since they might be a consequence of both, Bim expression and degradation. In fact, p38 MAPK can increase Bim transcription upon arsenite treatment [30], which could explain the increase in Bim levels observed in C3Gi wt cells, but not under other conditions. So, changes in Bim stability and degradation might play a role and might be a consequence of Bim phosphorylation. It is known that Bim can be phosphorylated in different residues, leading to its stabilization or degradation depending on the phosphorylated residue(s) [31]. Therefore, in our experimental conditions, the balance between the activation of p38 MAPK, ERKs and other kinases, that can phosphorylate Bim [31], could be important to regulate Bim stability, as changes in Bim mobility can be observed in the different blots (see fig. 6). Because different kinases are activated under the different experimental conditions, it is not a simple issue to explain all the changes observed in Bim levels.

Another interesting point refers to Bcl-x_L changes, which might result from the balance between Bcl-x_L expression and/or degradation that is regulated by different signalling pathways. For example, it is noticeable that Bcl-x_L highly decreases in H_2O_2 treated cells expressing DNRap-1, while in C3Gi wt cells, it is up-regulated and there is just a slight reduction after 4h of treatment.

In addition to the changes in Bim/Bcl-x_L ratio, other pro-apoptotic proteins, such as Bax, are down-regulated in MEFs lacking p38α [5], which will contribute to the differences in cell death regulated by p38α, as previously established [5]. In fact, Bax plays an important role in the process of apoptosis induced by serum-starvation [5].

It is also noticeable the up-regulation of BNIP-3 in response to H_2O_2 in G3Gi wt cells as a potential relevant mediator of the enhanced survival, according to some data from the literature [32-33]. Thus, although the classical function of BNIP-3 was a pro-apoptotic one, it is now known that it can play a dual role in the regulation of cell death, either mediating apoptosis or

cell survival [32]. So, in some settings such as ischemia/reperfusion [32] or hypoxia [32-33] BNIP-3 is up-regulated, which leads to survival [33].

Concerning the function of p38α in response to oxidative stress, there are data in favor of both, a pro-survival and a pro-apoptotic function that might depend on the stimuli, dose, cell type and other factors. Sustained activation of p38α by ROS has been implicated in apoptosis induction [34], although low levels of oxidative stress can also induce a p38 MAPK dependent cell cycle arrest [35]. In addition, it has been recently demonstrated that oncogene-induced ROS activate p38α, leading to apoptosis, which contributes to inhibition of tumor initiation [8]. In contrast, we have shown here that activation of p38α in MEFs by H_2O_2 results in cell survival, so cells lacking p38α are not able to survive. In agreement with this, H_2O_2-induced p38 MAPK activation was shown to promote cell survival [36-37] through different mechanisms, including the increase in the expression of some enzymes involved in the anti-oxidant defense of the cell such as catalase [36] or heme-oxygenase-1 [37]. However, the dose of H_2O_2 used for these studies was much lower and the period of treatment was also different. In any case, the oxidative stress is able to activate p38α in several model systems and then, this activation can regulate in a different way some cellular functions such as apoptosis/survival and proliferation, probably as a consequence of the cell context. Regarding this, it is important that the MEFs cell lines, which are non-tumorigenic cell lines, can respond in a different way to ROS than Ras-transformed MEFs [8].

In addition to the regulation of p38 by C3G and Rap-1, we have also found (unpublished data) that C3G knock-down upregulates the activity of Akt and ERKs survival pathways in response to oxidative stress, through a mechanism partially dependent on p38α. In contrast, Rap-1 is a mediator of Akt activation in cells expressing p38α. Thus, this can raise the possibility that Akt and/or ERKs could play a role in the regulation of the balance between cell survival and

apoptosis under our experimental conditions. However, preliminary data from our lab (data not shown) indicate that p38α would be more relevant mediating cell survival.

Conclusions

In conclusion, we have described here that C3G through down-regulation of p38α MAPK activity can play a dual role regulating cell death in MEFs depending on the stimuli. Thus, C3G mediates cell death in response to oxidative stress, while it induces cell survival upon serum-deprivation. However, we have found that in MEFs, in contrast to CML cells, C3G does not do so through Rap-1. Moreover, Rap-1 plays an opposite role in the regulation of cell death, although its effect is also dependent on p38α MAPK activity, which is positively regulated by Rap-1. Therefore, taking into account all this, our data point out to a central role of p38α MAPK as a mediator of either cell death or survival in a stress context, where C3G and Rap-1 converge in order to regulate the balance between cell death and survival. It would be important to know in the future whether the effect of Rap-1 on p38α described here in MEFs is dependent on cell-type, stimuli or even on the tumorigenic potential of the cells. This last possibility could open new therapeutical perspectives for cancer treatment.

Acknowledgements

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Figures legends

Figure 1-C3G knock-down and knock-out upregulates p38 MAPK pathway activity in response to stress. MEFs (wt, p38α-deficient with (C3Gi) or without C3G shRNA and C3G knock-out) maintained in the presence of serum were triggered with H_2O_2 (1mM) or NaCl (0, 5M) for 20 min as indicated. (A) Decrease in C3G expression upon C3G silencing: left panel, western-blot analysis of C3G and p38α protein levels normalized with β-actin; right panel, RT-PCR analysis of C3G mRNA normalized with GADPH. (B) and (D), Representative Western-blot analysis of phospho-p38 MAPK, p38α MAPK and phospho-MKK3/6 levels normalized with β-actin. (C) Absence of C3G expression in C3G knock-out MEFs. Left panel, PCR analysis of either human or mouse C3G normalized with GADPH, showing expression of human C3G in wt (C3G-/-hC3G) but not in C3G-/-, and absence of mouse C3G in both. Mouse C3G is expressed in wt p38α MEFs (wt mC3G) used as a positive control. Right panel, western-blot analysis of C3G protein levels normalized with β-actin. Quantifications indicate the mean values of the densitometric analysis of the blots from three different experiments normalized with β-actin.

Figure 2- Role played by Rap-1 in the regulation of p38 MAPK, Akt and ERKs pathways by C3G. MEFs (wt, p38α-deficient transfected with a dominant negative Rap-1 mutant

(DNRap-1) or untransfected) maintained in the presence of serum were triggered with H_2O_2 (1mM) or NaCl (0, 5M) for 20 min as indicated. (A) Rap-1 activity under growing conditions (in the presence of serum). Representative Western-blot analysis of Rap-1 GTP levels normalized with total Rap-1 protein levels. p38α expression was determined as a control. (B) Western-blot analysis of phospho-p38 MAPK and phospho-MKK3/6 levels normalized with β-actin. Total p38α levels were determined as a control. Quantifications indicate the mean values of the densitometric analysis from three different experiments normalized with β-actin.

Figure 3- C3G mediates cell survival through a p38α MAPK dependent mechanism in serum-deprived cells. Rap-1 plays an opposite effect. (A) Effect of C3G silencing (C3Gi) on apoptosis of wt and p38α-deficient MEFs. Cells were serum deprived for 24h and then, the number of apoptotic cells (sub-G0/1 peak) was determined by flow cytometric analysis of the cell cycle. Results are expressed as the fold increase of the control value. (**p<0,01) wt-C3Gi versus wt MEFs. (B) Effect of C3G knock-out on apoptosis. MEFs were maintained in the presence of serum (Left panel, FBS) or were serum-deprived for 24h (left panel, -FBS and right panel). When indicated, cells were treated with the p38α/β inhibitor, SB203580 (5μM). Representative Western-blot analysis of the levels of active (cleaved) caspase 3 normalized with β-actin. (C) Effect of dominant negative Rap-1 on apoptosis. Cells were serum-deprived for 24h. Representative Western-blot analysis of the levels of active (cleaved) caspase 3 normalized with β-actin.

Figure 4- C3G knock-down and knock-out induces cell survival in cells treated with H_2O_2 through a p38α MAPK dependent mechanism. Cells maintained in the presence of serum were treated with H_2O_2 (1mM) for 6h. (A) Effect of C3G silencing on cell viability in wt, p38α-deficient and p38α-deficient MEFs upon reconstitution of p38α expression (Rec p38α). (**p<0,01) wt-C3Gi versus wt MEFs and (*p<0,05) p38α-/- as compared to wt MEFs. Results

are expressed as the percentage of corresponding control cells, which was considered as 100% in all cases. There were no significant differences between wt and p38α reconstituted cells. Upper panel, analysis of p38α expression by Western-blot is shown as a control of p38α rescue. (B) Effect of C3G knock-out on cell viability. (***p<0,001) C3G -/- versus wt MEFs. (C) Effect of C3G silencing on apoptosis. Histograms show the number of apoptotic nuclei expressed as the fold increase of that from wt cells treated with H_2O_2. (*p<0,05) wt-C3Gi and p38α-/- as compared to wt MEFs.

Figure 5- Rap-1 protects from cell death induced by H_2O_2. MEFs (wt and p38α-deficient transfected with dominant negative Rap-1 mutant (DNRap-1) or untransfected) maintained in the presence of serum were treated with H_2O_2 (1mM) for 6h. (A) Cell viability analysis. (***p<0,001) wt expressing DNRap-1 versus wt MEFs and (*p<0,05) p38α-/- as compared to wt MEFs. (B) Quantification of apoptosis. Histograms show the number of apoptotic nuclei expressed as the fold increase of that from wt cells treated with H_2O_2. (**p<0,01) wt expressing DNRap-1 versus wt MEFs and (*p<0,05) p38α-/- as compared to wt MEFs.

Figure 6-Regulation of proteins from Bcl-2 family by C3G and Rap-1 through p38α-dependent and independent mechanisms. Effect of shC3G (C3Gi) and dominant negative Rap-1 mutant (DNRap-1) on the expression of Bim, Bcl-x_L and BNIP-3 proteins in wt and p38α-deficient MEFs. Cells were serum deprived for 24h when indicated (–FBS, in A, B and C) or maintained in the presence of serum and treated with H_2O_2 (1mM) for 2-4h (in D and E). Bim, Bcl-x_L and BNIP-3 proteins were analyzed by Western-blot using tubulin to normalize. (C) Histogram showing the ratio between Bim and Bcl-x_L protein levels from a representative experiment, determined by densitometric analysis of the blots normalized with tubulin. Results are expressed as relative values considering as 1 wt cells Bim/Bcl-x_L ratio.

Figure 7-Regulation of apoptosis by C3G and Rap-1 through p38α MAPK. Model showing the regulation of p38α by C3G and Rap-1 in MEFs and their effects on the balance between

apoptosis and cell survival. (A) Upon serum deprivation C3G through the negative regulation of p38α mediates cell survival, while activation of Rap-1 by other GEFs, leads to p38α activation inducing apoptosis. (B) Upon treatment with H_2O_2 C3G mediates apoptosis through inhibition of p38α activity and Rap-1 (activated by other GEFs) induces cell survival through p38α.
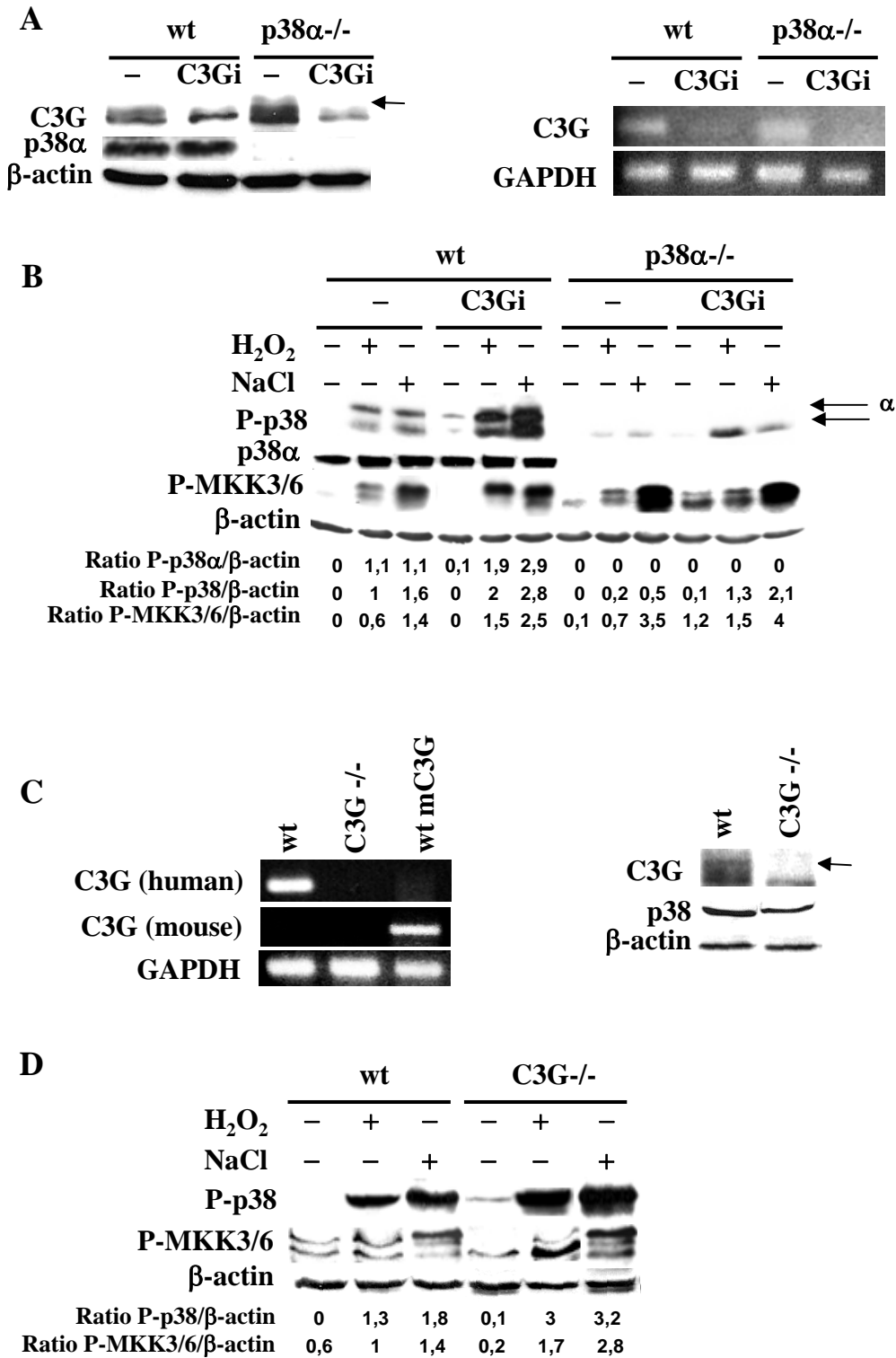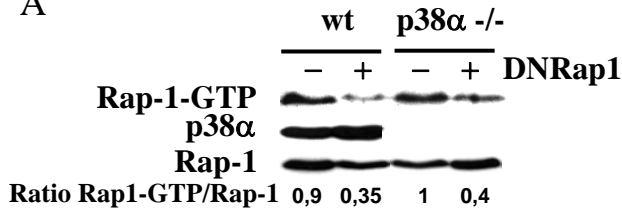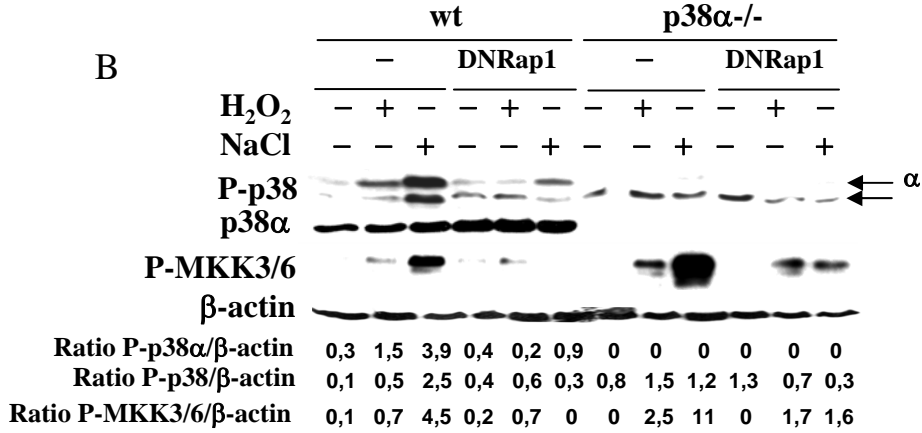
Figure 1

A

	wt		p38α-/-	
	–	C3Gi	–	C3Gi

C3G
p38α
β-actin

	wt		p38α-/-	
	–	C3Gi	–	C3Gi

C3G
GAPDH

B

	wt						p38α-/-					
	–			C3Gi			–			C3Gi		
H₂O₂	–	+	–	–	+	–	–	+	–	–	+	–
NaCl	–	–	+	–	–	+	–	–	+	–	–	+

P-p38
p38α
P-MKK3/6
β-actin

Ratio P-p38α/β-actin	0	1,1	1,1	0,1	1,9	2,9	0	0	0	0	0	0
Ratio P-p38/β-actin	0	1	1,6	0	2	2,8	0	0,2	0,5	0,1	1,3	2,1
Ratio P-MKK3/6/β-actin	0	0,6	1,4	0	1,5	2,5	0,1	0,7	3,5	1,2	1,5	4

C

	wt	C3G -/-	wt mC3G
C3G (human)			
C3G (mouse)			
GAPDH			

	wt	C3G -/-
C3G		
p38		
β-actin		

D

	wt			C3G-/-		
H₂O₂	–	+	–	–	+	–
NaCl	–	–	+	–	–	+

P-p38
P-MKK3/6
β-actin

Ratio P-p38/β-actin	0	1,3	1,8	0,1	3	3,2
Ratio P-MKK3/6/β-actin	0,6	1	1,4	0,2	1,7	2,8

Figure 2

A

	wt		p38α -/-		
	−	+	−	+	DNRap1
Rap-1-GTP					
p38α					
Rap-1					
Ratio Rap1-GTP/Rap-1	0,9	0,35	1	0,4	

B

	wt						p38α-/-					
	−		DNRap1				−		DNRap1			
H₂O₂	−	+	−	−	+	−	−	+	−	−	+	−
NaCl	−	−	+	−	−	+	−	−	+	−	−	+
P-p38												
p38α												
P-MKK3/6												
β-actin												
Ratio P-p38α/β-actin	0,3	1,5	3,9	0,4	0,2	0,9	0	0	0	0	0	0
Ratio P-p38/β-actin	0,1	0,5	2,5	0,4	0,6	0,3	0,8	1,5	1,2	1,3	0,7	0,3
Ratio P-MKK3/6/β-actin	0,1	0,7	4,5	0,2	0,7	0	0	2,5	11	0	1,7	1,6

α

Figure 3

A

B

C

Figure 4

A

B

C

Figure 5

A

B

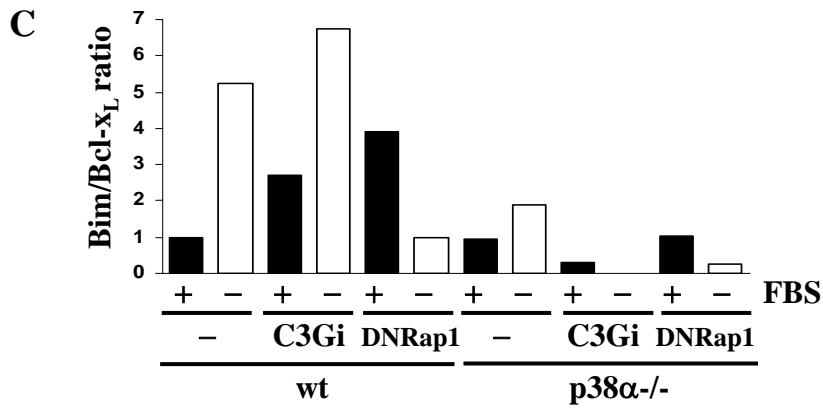**Figure 6**

Figure 7

A

Serum
starvation

GEFs

Rap-1   C3G

p38α
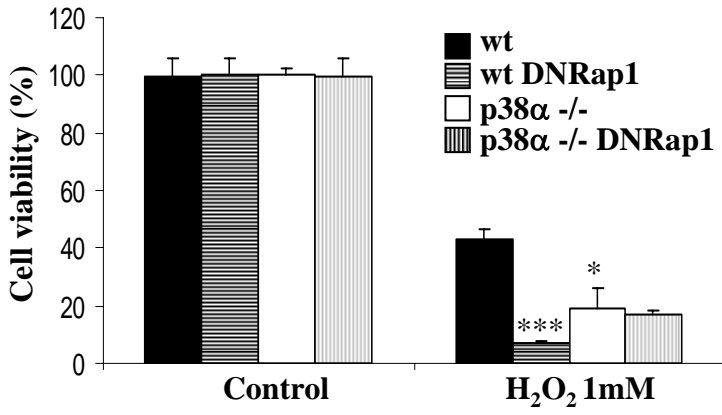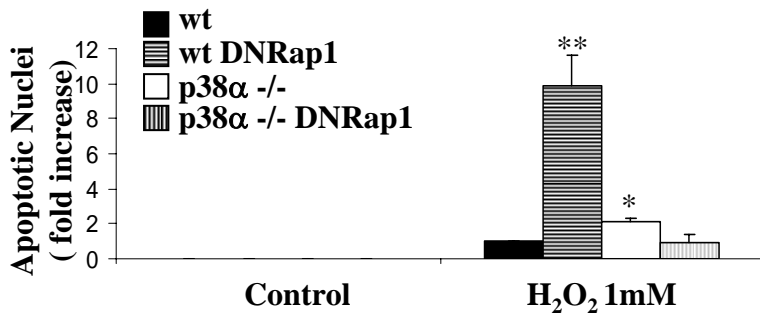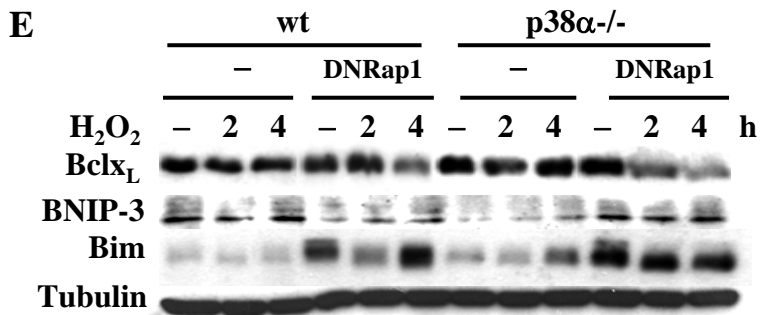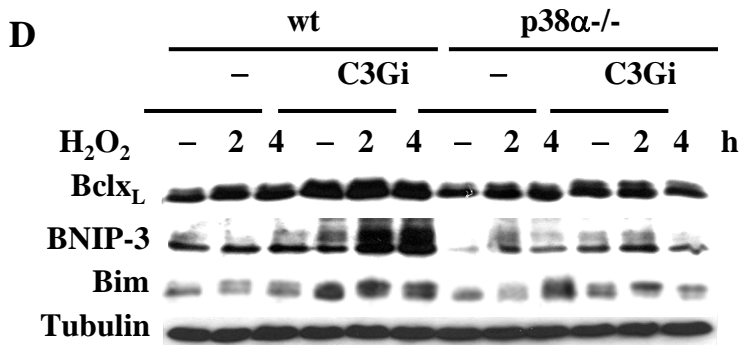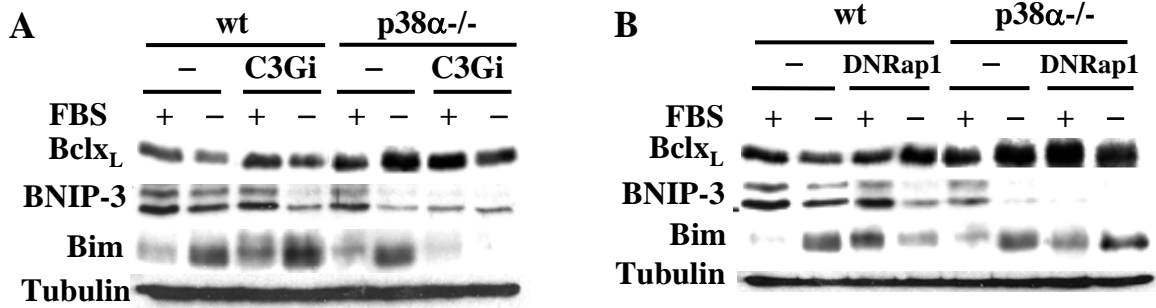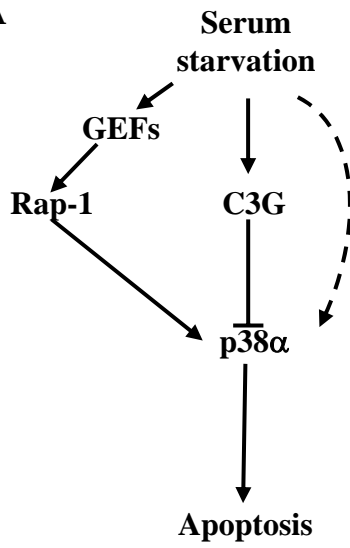
Apoptosis

C3G induces survival
Rap-1 induces apoptosis

B

H_2O_2

GEFs

Rap-1   C3G

p38α
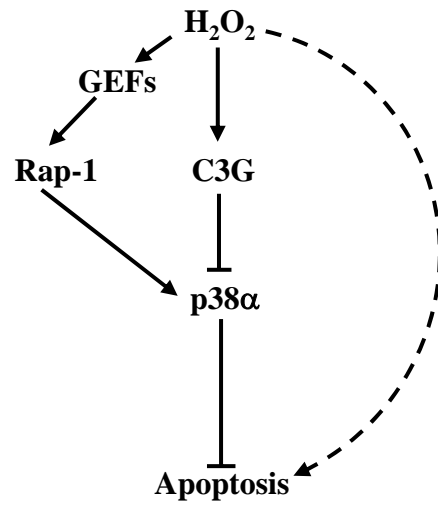
Apoptosis

C3G induces apoptosis
Rap-1 induces survival

Supplementary figure-Regulation of Rap-GTP levels by C3G knock-down in response to stress. MEFs (wt, p38α-deficient with (C3Gi) or without C3G shRNA) maintained in the presence of serum were triggered with H_2O_2 (1mM) or NaCl (0, 5M) for 20 min as indicated.

	wt						p38α-/-					
	−			C3Gi			−			C3Gi		
H_2O_2	−	+	−	−	+	−	−	+	−	−	+	−
NaCl	−	−	+	−	−	+	−	−	+	−	−	+