

c-Myc Phosphorylation Is Required for Cellular Response to Oxidative Stress

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Summary

Aside from the well-established roles of c-Myc in the regulation of cell cycle, differentiation, and apoptosis, a recent picture is beginning to emerge linking c-Myc to the regulation of metabolic pathways. Here, we define a further function for c-Myc in determining cellular redox balance, identifying glutathione (GSH) as the leading molecule mediating this process. The link between c-Myc and GSH is γ -glutamyl-cysteine synthetase (γ -GCS), the rate-limiting enzyme catalyzing GSH biosynthesis. Indeed, c-Myc transcriptionally regulates γ -GCS by binding and activating the promoters of both γ -GCS heavy and light subunits. Exposure to H₂O₂ enhances c-Myc recruitment to γ -GCS regulatory regions through ERK-dependent phosphorylation. Phosphorylation at Ser-62 is required for c-Myc recruitment to γ -GCS promoters and determines the cellular response to oxidative stress induced by different stimuli. Thus, the c-Myc phosphorylation-dependent activation of the GSH-directed survival pathway can contribute to oxidative stress resistance in tumor cells, which generally exhibit deregulated c-Myc expression.

Introduction

c-myc is the most investigated among the nonredundant *myc* gene family members and encodes a transcrip-

tional factor implicated in many cellular processes such as proliferation, differentiation, transformation, and apoptosis (Nilsson and Cleveland, 2003). The c-Myc protein belongs to the basic helix-loop-helix leucine-zipper protein family (b/HLH/Z) and, by dimerizing with the ubiquitously expressed protein Max, binds to the specific CAC(G/A)TG motif (E box) and regulates transcription both positively and negatively. c-Myc can also bind to DNA sites that differ from the palindromic hexanucleotide canonical sequence (Blackwell et al., 1993). A number of cellular target genes have been identified that are directly or indirectly regulated by c-Myc, and this list is rapidly increasing (Zeller et al., 2003; Adhikary and Eilers, 2005). A c-Myc Target Gene Database assembling the c-Myc-responsive genes has been launched (<http://www.mycncancer.org/site/mycTargetDB.asp>).

Thus far, most of the literature has focused on the relationship between modulation of c-Myc protein expression levels and induction or repression of target gene transcription. Recently, a growing number of groups have started to explore c-Myc posttranslational modifications, which affect c-Myc transcription factor capabilities by activating and stabilizing the protein (Sears et al., 2000; Kamemura et al., 2002). In this context, many serine and threonine sites have been identified along the c-Myc protein as phosphorylation target residues, although the functional consequences of these modifications have not been fully elucidated. The known effects include changes in the stability of the protein and its ability to elicit either transformation or apoptosis (Pulverer et al., 1994; Noguchi et al., 1999; Gregory and Hann, 2000; Sears et al., 2000; Chang et al., 2000; Yada et al., 2004).

Aside from the well-established involvement of c-Myc in the regulation of cell cycle, differentiation, and apoptosis, a recent picture is beginning to emerge that identifies further functions of c-Myc in metabolic pathways such as amino acid and nucleotide synthesis, regulation of lipid metabolism, glycolysis, and mitochondrial homeostasis (Dang, 1999). Whether c-Myc regulates cellular homeostasis in response to oxidative stress remains to be determined. Glutathione (GSH) is the most important low molecular thiol involved in cellular detoxification, redox balance, and stress response (Deneke and Fanburg, 1989). γ -glutamyl-cysteine synthetase catalyzes the first rate-limiting step in GSH biosynthesis. γ -GCS is a heterodimer composed of a heavy catalytic (γ -GCS_H) and a light regulatory (γ -GCS_L) subunit, which are encoded by two different genes in both rat and human (Gipp et al., 1995). The γ -GCS enzyme exerts a key role in the maintenance of intracellular redox balance and in determining cellular response to several different stimuli, including oxidative stress, xenobiotic and drug exposure, hormones, and growth factors (Wild and Mulcahy, 2000). The 5' flanking regions of both γ -GCS subunits have been cloned and sequenced, identifying putative NF- κ B, Sp-1, AP-1, and AP-2 binding sites together with metal response (MRE), antioxidant response (ARE), and electrophile-responsive (EpRE) elements (Moinova and Mulcahy, 1998; Mulcahy et al., 1997; Wild et al., 1998; Moinova and Mulcahy, 1999).

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Previous work from our group has implicated c-Myc in modulating the expression of γ -GCS genes and, consequently, the intracellular GSH content (Biroccio et al., 2002), but the mechanism(s) involved in this facet have not been uncovered. Here, we demonstrate that c-Myc oncoprotein transcriptionally regulates γ -GCS genes by binding and activating the promoters of both γ -GCS heavy and light subunits. Exposure to H_2O_2 enhances c-Myc recruitment to γ -GCS regulatory regions through ERK-dependent phosphorylation, thus activating a c-Myc-mediated response to oxidative stress.

Results

c-Myc Transcriptionally Regulates γ -GCS Expression and Determines Cellular Response to Oxidative Stress

To investigate a possible involvement of c-Myc in the regulation of redox balance and response to oxidative stress, c-Myc levels were modulated in two different human melanoma lines (M14 and SbCl), which displayed different endogenous c-Myc expression, and in immortalized human fetal fibroblasts (HFF), which showed almost no detectable c-Myc protein levels. Specific inhibition of c-Myc expression in the M14 line by RNA interference strategy (siMyc) produced a decrease of intracellular GSH content, whereas an increase of GSH was observed in SbCl and HFF cells when transfected with the pcDNA-c-myc expression vector (Figure 1A). Moreover, when cells were exposed to increasing doses of H_2O_2 , inhibition of c-Myc significantly enhanced H_2O_2 -induced reactive oxygen species (ROS) production and cell death, whereas its overexpression protected cells from H_2O_2 -induced damage, indicating that c-Myc is determinant in cellular response to oxidative stress (Figure 1B). The c-Myc-dependent stress response was reverted by preincubating siMyc M14 cells with GSH ethyl-ester (GSHEst), an exogenous source of GSH, and c-Myc overexpressing HFF/SbCl cells with L-buthionine-sulfoximine (BSO), a specific inhibitor of GSH synthesis (Figure 1B). To investigate the mechanism(s) underlying c-Myc-dependent regulation of cellular redox balance, we analyzed whether c-Myc controlled the expression of γ -GCS. mRNA expression and promoter activity of both heavy and light subunit genes were evaluated in melanoma and HFF cells after treatment with H_2O_2 . Inhibition of c-Myc in M14 cells decreased the endogenous mRNA expression of both γ -GCS_H and γ -GCS_L and abrogated the effect on γ -GCS gene expression induced by H_2O_2 treatment (Figure 1C). Conversely, overexpression of c-Myc in SbCl and HFF cells increased transcript levels of both subunits and potentiated the H_2O_2 -induced γ -GCS mRNA expression (Figure 1C). Consistent with these results, changes in c-Myc protein levels, by either knockdown or overexpression, led to a modulation of both γ -GCS_H and γ -GCS_L promoter activity (Figure 1D). More interestingly, after oxidative stress, γ -GCS transcriptional activity was markedly impaired in siMyc M14 cells and significantly enhanced in c-Myc overexpressing SbCl and HFF lines (Figure 1D).

Oxidative Stress Recruits c-Myc to γ -GCS Promoters

Upon analysis of the nucleotide sequence of both γ -GCS promoters, we identified five CACATG nonca-

nonical c-Myc binding sites. Two E boxes, mapping at -559/-554 (E box 2) and -500/-495 (E box 1), are located in the region proximal to the transcription starting point of the γ -GCS_H promoter (Figure 2A), and three E boxes, mapping at -2340/-2335 (E box 3), -2039/-2034 (E box 2), and -1609/-1604 (E box 1), are placed on the γ -GCS_L gene promoter (Figure 2B). When either the single or both E boxes on the γ -GCS_H promoter were mutated (mut M1, mut M2, and mut M1-2), the basal luciferase activity was significantly impaired (Figure 2A). More interestingly, the response to oxidative stress was strongly affected and even more compromised upon double mutation of both E boxes (Figure 2A). Consistent with previously reported data, deletion of the 5' distal portion of the γ -GCS_H promoter (Δ 597 mutant), including several transcription factor binding sites and four AREs, led to a dramatically decreased basal and stress-induced transcription activity and, together with the double E box mutation (Δ 597-mut M), completely abolished the ability to respond to oxidative damage (Figure 2A). Because the distal ARE4 site is reported to be crucial in regulating both basal and stress-induced promoter activity (Mulcahy et al., 1997), we evaluated its contribution in relation to the c-Myc binding sites. Upon single ARE4 mutation (mut A4), the luciferase activity driven by the γ -GCS_H promoter significantly decreased after oxidative damage when compared to wild-type (wt), with the degree of reduction being comparable to that observed in the double E box mutant. Simultaneous disruption of both ARE4 and c-Myc binding sites (mut M-A) led to a more severe impairment of the luciferase activity when compared to each single mutant (mut A4 and mut M1-2), even if the promoter still retained the ability to elicit a response to stress. In the γ -GCS_L promoter, the deletion of the region containing both distal E boxes (Δ 1633 mutant) did not affect γ -GCS_L promoter activity (Figure 2B). When mutating the c-Myc E box 1 (Δ 1633 mut M), the luciferase activity decreased and the relevance of the putative c-Myc binding site was more evident after oxidative stress (Figure 2B). A similar effect was observed by introducing a single base mutation in the ARE element located in the proximal region (Δ 1633 mut A) and, together with E box 1, it further decreased both basal and oxidative stress-triggered response (Figure 2B).

To evaluate whether c-Myc actually binds to the regions covering the two closed E boxes on the γ -GCS_H gene promoter and the unique consensus site on γ -GCS_L, which are crucial in regulating basal and stress-modulated transcriptional activity, ChIP experiments were carried out. Specific c-Myc antibody exclusively immunoprecipitated the region covering both E boxes on the γ -GCS_H promoter and the region that includes the c-Myc consensus on γ -GCS_L regulatory sequence, whereas no PCR amplification was observed by targeting the 5' and 3' γ -GCS E boxes' flanking regions (Figure 2C). Interestingly, H_2O_2 exposure significantly increased the recruitment of c-Myc to γ -GCS_H and γ -GCS_L promoters and, consistent with these results, H_2O_2 -induced binding of c-Myc to both γ -GCS regulatory sequences was completely abolished after siMyc (Figure 2D). Treatment with H_2O_2 also led to a modulated recruitment to some specific c-Myc target genes. A higher binding of c-Myc protein to the hTERT promoter

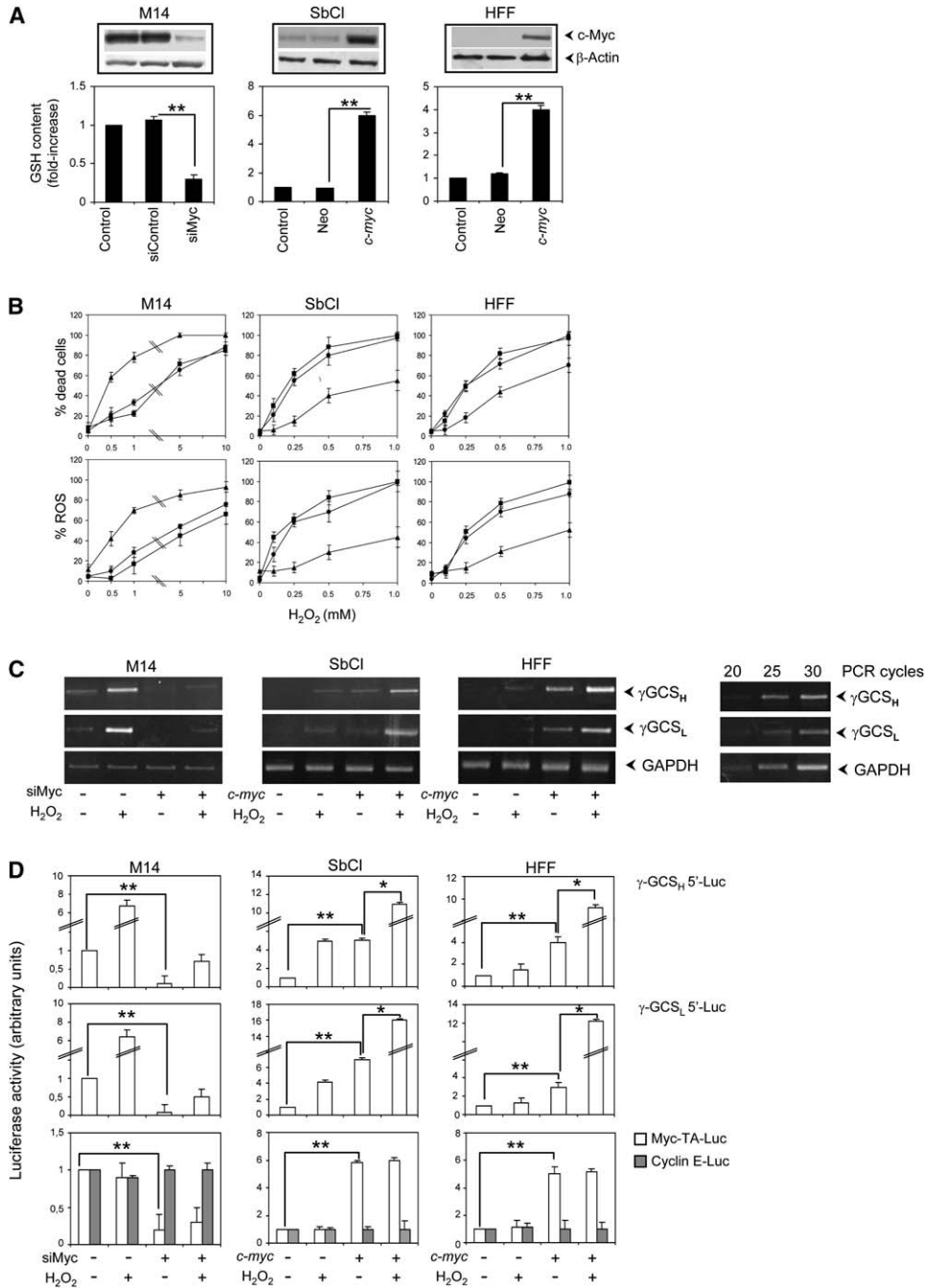


Figure 1. c-Myc Transcriptionally Regulates γ -GCS Expression and Determines Cellular Response to Oxidative Stress

M14 cells were treated with either control (siControl, ●) or c-Myc RNA interference pools (siMyc, ▲), whereas SbCl and HFF cells were transiently transfected with either pcDNA3-Neo (Neo, ●) or pcDNA3-c-myc (c-myc, ▲). Cells were exposed to H₂O₂ (1 mM for M14, 0.25 mM for SbCl and HFF) for 30 min.

(A) Western blot analysis of c-Myc protein expression (upper panels) and intracellular GSH content evaluation (lower panels). Error bars indicate \pm standard deviation (SD).

(B) Flow cytometry evaluation of the percentage of both cell death and ROS production in cells treated with the indicated doses of H₂O₂. M14 cell line was also preincubated with GSHest (■), whereas c-Myc overexpressing SbCl and HFF cells were preexposed to BSO (■). Error bars represent \pm SD.

(C) Evaluation of γ -GCS_H and γ -GCS_L mRNA expression by RT-PCR assay. GAPDH transcript amplification has been used for loading normalization.

(D) Evaluation of γ -GCS_H and γ -GCS_L transcriptional promoter activity performed by luciferase assay. Transfection with either Myc-TA-Luc or cyclin E-Luc expression vectors was included in the assay as c-Myc-specific positive and negative controls, respectively. The data represent the mean of three independent experiments with standard deviation (SD). p values are *p < 0.05 and **p < 0.01.

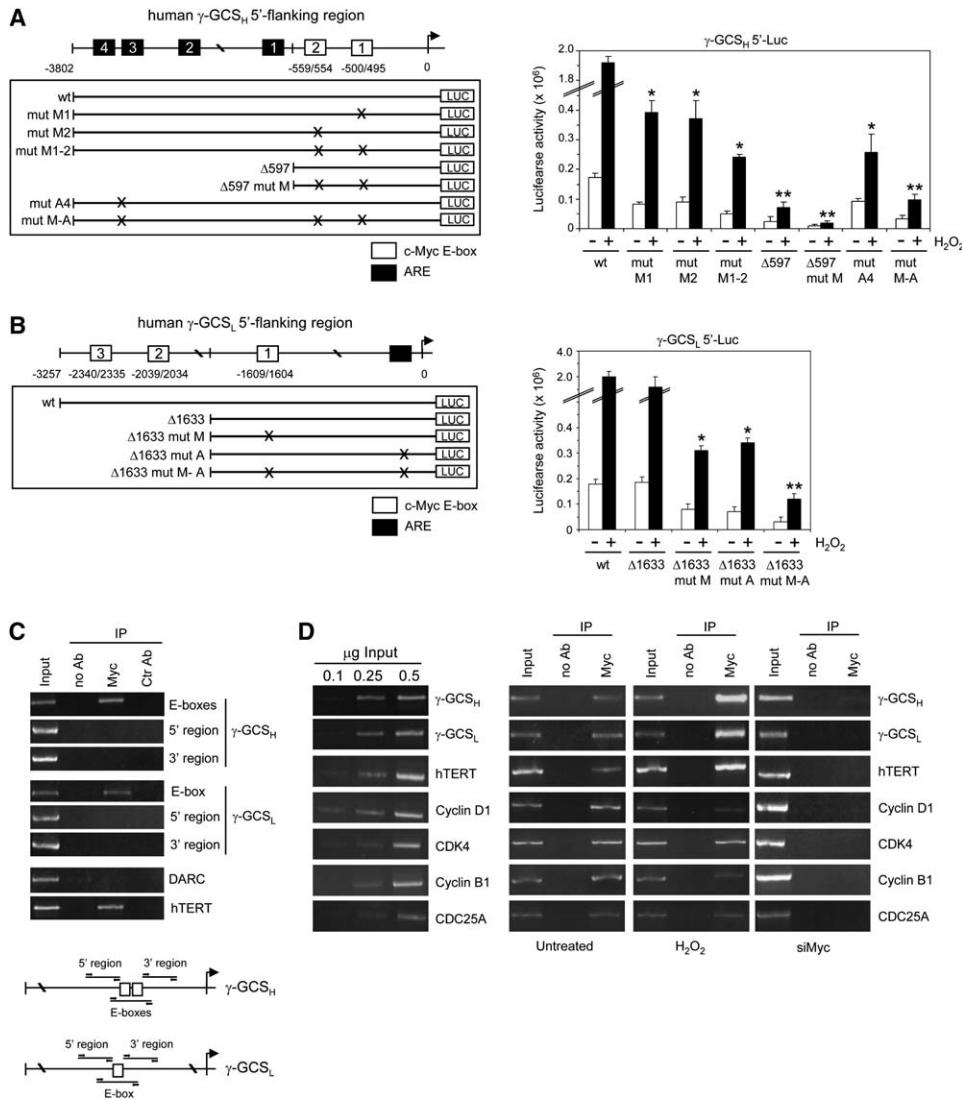


Figure 2. Oxidative Stress Increases c-Myc Recruitment to γ -GCS Promoters

(A) Schematic representation of wild-type (wt), deleted (Δ), and point-mutated (mut) γ -GCS_H promoter (left panel) and evaluation of the promoter activity by luciferase assay (right panel) carried out by transfecting M14 cells with either wt or the indicated mutant γ -GCS constructs. The data represent the mean of three independent experiments with \pm SD.

(B) Schematic representation of wild-type (wt), deleted (Δ), and point-mutated (mut) γ -GCS_L promoter (left panel) and evaluation of promoter activity by luciferase assay (right panel) carried out by transfecting M14 cells with either wt or the indicated mutant γ -GCS promoters. The data represent the mean of three independent experiments with \pm SD. p values are *p < 0.05 and **p < 0.01.

(C) ChIP assay was carried out in untreated M14 cells as reported in the [Experimental Procedures](#). Amplification with oligos specific for hTERT (region -254/+31) and DARC (region -477/-189) promoters was included as positive and negative controls of c-Myc DNA binding, respectively. The DNA fragments used in the ChIP experiments on both GCS regions have been indicated on each promoter diagram reported below the ChIP data.

(D) ChIP assay carried out as described above in the untreated, H₂O₂-treated, and c-Myc-interfered (siMyc) M14 cells. Amplification was performed with oligos specific for γ -GCS_H, γ -GCS_L, hTERT, cyclin D1 (region -638/-396), CDK4 (region -161/+66), cyclin B1 (region -192/-22), and CDC25 (region +1020/1336) genes.

was observed after oxidative stress (Figure 2D). Conversely, its recruitment to both cyclin D1 and cyclin B1 regulatory regions was significantly decreased after treatments with the prooxidant agent, whereas no modulation of c-Myc binding to CDK4 and CDC25A promoters was reported (Figure 2D).

We then verified the hypothesis that the increased binding of c-Myc to γ -GCS promoters might strictly depend on the higher GSH synthesis requirement dis-

played by H₂O₂-treated cells. GShest incubation led to a 3-fold increase in the intracellular GSH content, without affecting c-Myc protein expression levels (Figure 3A). Exposure to the exogenous GSH completely abolished the increased binding of c-Myc to γ -GCS E boxes upon oxidative stress (Figure 3B). A similar modulation was reported for hTERT promoter, whereas no change in c-Myc binding to cyclin B1 and CDC25 target genes was observed in the H₂O₂-treated cells after

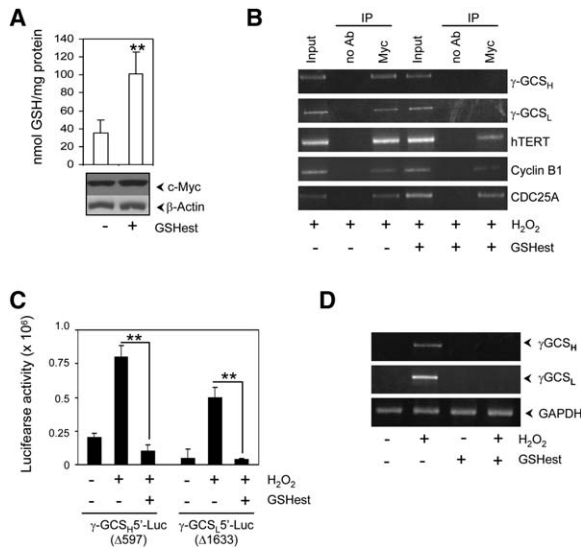


Figure 3. GSH Requirement upon Oxidative Stress Recruits c-Myc to γ -GCS Promoters
M14 cells were incubated with GSH ethyl ester (GSHest) prior to exposition to H₂O₂.
(A) Intracellular GSH content evaluation and Western blot analysis of c-Myc protein expression. The data represent the mean of three independent experiments with \pm SD. p values are **p < 0.01.
(B) ChIP assay.
(C) Evaluation of γ -GCS_H and γ -GCS_L promoter activity by luciferase assay. The data represent the mean of three independent experiments with \pm SD. p values are **p < 0.01.
(D) Analysis of γ -GCS_H and γ -GCS_L mRNA expression by RT-PCR assay.

GSHest preincubation (Figure 3B). Moreover, ester exposure blocked the ability of both γ -GCS promoters to respond to oxidative stress (Figure 3C) and inhibited both γ -GCS_H and γ -GCS_L mRNA expression (Figure 3D).

ERK-Mediated Phosphorylation Drives c-Myc to γ -GCS Promoters upon Oxidative Stress

Exposure to H₂O₂ did not change the expression level of c-Myc protein, but instead triggered its phosphorylation at Thr-58/Ser-62, the critical sites regulating c-Myc protein stability after mitogenic stress (Sears et al., 2000; Yeh et al., 2004) (Figure 4A). Among the different kinases reported to be responsible for c-Myc phosphorylation, activation of p38, ERK, JNK, AKT, and GSK-3 β was evaluated. Treatment with the prooxidant agent triggered cellular activation of both p38 and ERK, without affecting JNK, AKT, and GSK-3 β phosphorylation (Figure 4B). To identify the kinase(s) involved in c-Myc posttranslational modification, ERK and p38 phosphorylation was inhibited by either chemical inhibitors (UO126 and SB202190) or by specific RNA interference. Western blot analysis revealed that only UO126 and siERK were effective in completely inhibiting c-Myc phosphorylation (Figure 4C), indicating that ERK is necessary for the posttranslational modification of c-Myc in response to H₂O₂-induced stress.

Next, we evaluated whether phosphorylation was required for c-Myc recruitment to γ -GCS promoters. The data obtained by ChIP analysis, carried out with an anti-phospho-c-Myc antibody, demonstrated that the

increased recruitment to both γ -GCS promoters upon oxidative damage was attributable to c-Myc phosphorylation (Figure 4D). Oxidative stress elicited by H₂O₂ treatment triggered a posttranslational modification of the c-Myc protein that enabled it to respond to the damage by increasing its binding to GSH genes. Furthermore, preincubation with UO126 abolished the recruitment of phospho-Myc to γ -GCS regulatory regions, whereas p38 inhibitor SB202190 did not have an effect on phospho-c-Myc binding to γ -GCS_H and γ -GCS_L promoters (Figure 4D). In agreement, UO126 reduced both γ -GCS_H (Δ 597) and γ -GCS_L (Δ 1633) transactivation, whereas SB202190 did not (Figure 4E), and, consequently, γ -GCS mRNA expression underwent significant impairment exclusively upon ERK phosphorylation inhibition (Figure 4F). Finally, upon H₂O₂ exposure, the percentage of nonviable cells increased in UO126-treated versus untreated cells (Figure 4G). p38 kinase inhibition by SB202190 slightly modified cell viability after H₂O₂, highlighting that other pathways are involved in the regulation of the response to stress damage. The same experiments were performed by using siERK and sip38 instead of UO126 and SB202190, respectively, giving comparable results (data not shown).

Serine-62 Phosphorylation Is Necessary for c-Myc Recruitment to γ -GCS Promoters upon Oxidative Stress

With the aim of evaluating the precise residue of phosphorylation, between Ser-62 and/or Thr-58, responsible for the higher c-Myc recruitment to γ -GCS target promoters, we studied the c-Myc-dependent response to oxidative stress in c-myc knockout mouse embryonic fibroblasts (MEF^{myc^{-/-}}). As shown in Figure 5A, reintroduction of wt c-myc cDNA in knockout cells significantly reduced stress-induced ROS production and cell death. Upon H₂O₂ exposure, MEF^{myc^{-/-}} activated the ERK-mediated c-Myc phosphorylation pathway (Figure 5B), thus harboring protection from the oxidative damage-triggered cell death (Figure 5C). After site-directed mutagenesis of Ser-62 and/or Thr-58 residues in the pcDNA3-c-myc expression vector, either the wt or each different c-myc mutant was transfected into the MEF^{myc^{-/-}} cells. Upon H₂O₂ exposure, phosphorylation of c-Myc protein was only reported in the wt c-myc and T58A c-myc-transfected cells, whereas no posttranslational modification was observed after transfection with either the S62A or the T58A/S62A c-myc mutant cDNA (Figure 5D). The inability to be phosphorylated at Ser-62 made c-Myc protein unable to respond to oxidative stress in terms of increasing binding to both γ -GCS promoters, whereas c-Myc^{T58A}, as the c-Myc^{wt}, was highly recruited to both target genes upon H₂O₂ exposure (Figure 5E). When mutated at both sites, c-Myc behaved as the c-Myc^{S62A} form. Consistent with these data, c-Myc^{S62A} and c-Myc^{T58A/S62A} were unable to transactivate both γ -GCS promoters after oxidative damage (Figure 5F), thus indicating a master role played by Ser-62 in the c-Myc phosphorylation-driven response to H₂O₂ stress. The impaired ability of c-Myc^{S62A} and c-Myc^{T58A/S62A} to be recruited to γ -GCS promoter E boxes significantly affected the expression of both heavy and light gene transcripts (Figure 5G). Consistent with these results, the γ -GCS enzymatic activity and

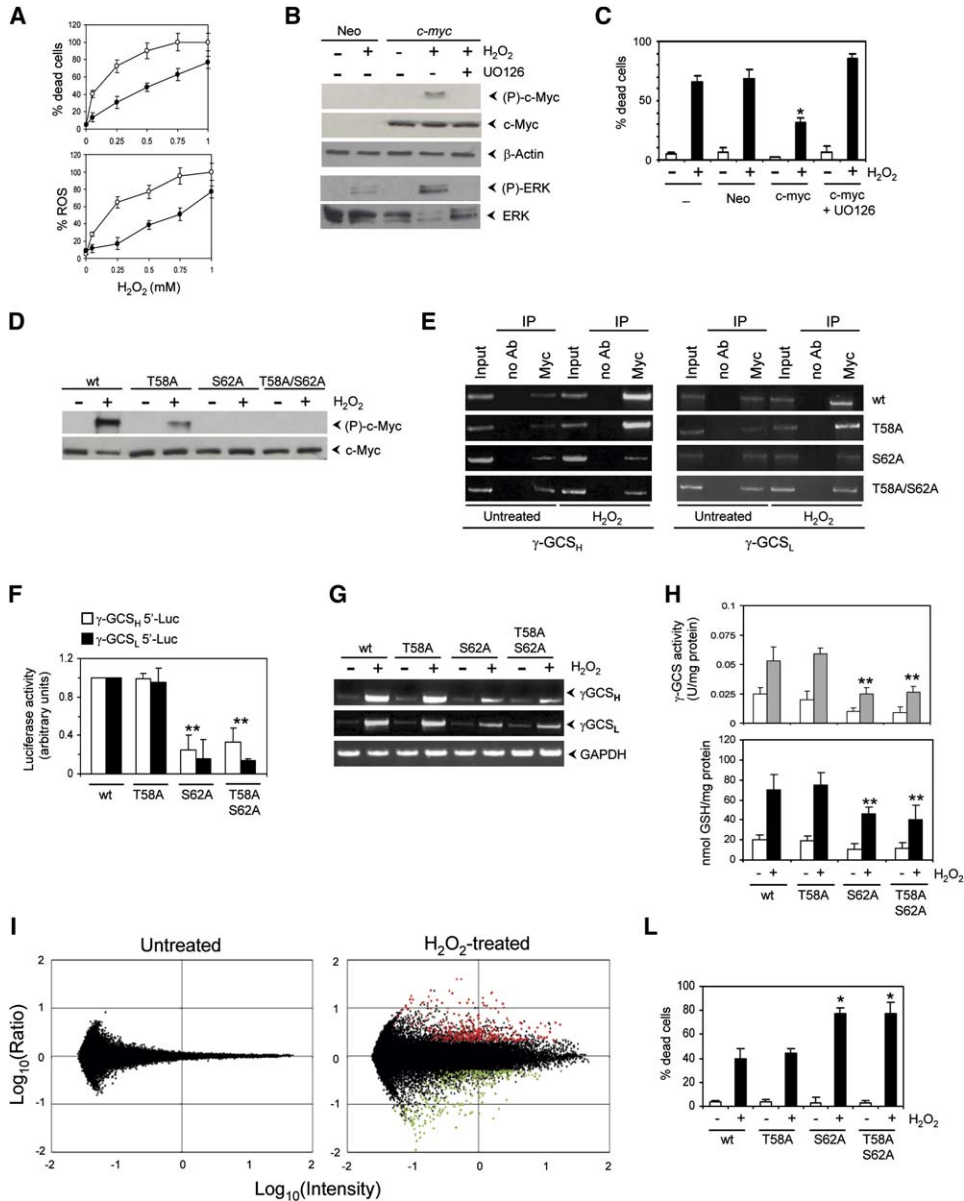


Figure 5. Serine-62 Phosphorylation Is Necessary for c-Myc Recruitment to γ -GCS Promoters upon Oxidative Stress

MEF^{myc-/-} cells were untransfected (-) or transiently transfected with either Neo (○) or wt (●) or mutant (T58A, S62A, T58A/S62A) *c-myc* cDNA cloned into the pcDNA3 expression vector. Where not specified, data represent H₂O₂-treated cells.

(A) Flow cytometry evaluation of the percentage of both cell death and ROS production. Error bars indicate \pm SD.

(B) Western blot analysis of phospho-Myc and phospho-ERK after exposure to the indicated treatments.

(C) Cytofluorimetric evaluation of cell death after transfection with the indicated vectors and treatment with UO126. The data represent the mean of three independent experiments with \pm SD. *p* values, calculated in *c-myc*-transfected versus Neo-transfected cells, are **p* < 0.05.

(D) Immunoblot analysis of phospho-Myc expression levels.

(E) ChIP assay.

(F) Analysis of γ -GCS_H and γ -GCS_L promoter activity carried out by luciferase assay. Error bars represent \pm SD.

(G) Evaluation of γ -GCS_H and γ -GCS_L mRNA expression by RT-PCR assay (GAPDH transcript amplification has been used for loading normalization).

(H) Analysis of γ -GCS enzyme activity (upper panel) and intracellular GSH content (lower panel). Error bars indicate \pm SD.

(I) Plots of log₁₀(Ratio) against log₁₀(Intensity) obtained from untreated and H₂O₂-treated cells. In the plots, Ratio = S62A/wt, and Intensity = $\sqrt{(S62A \times wt)}$. The red and green spots correspond to genes overexpressed and underexpressed between the two mRNA sources.

(L) Cytofluorimetric evaluation of cell death. The data represent the mean of three independent experiments with SD. *p* values, calculated in S62A and T58A/S62A versus wt-transfected cells, are ***p* < 0.01.

to several apoptotic triggers (Hoffman and Liebermann, 1998; Thompson, 1998). However, protection from apoptosis by c-Myc has been observed in other models

by different groups (Waikel et al., 1999; Liu et al., 2000; Biroccio et al., 2001, 2002; D'Agano et al., 2001; Ceballos et al., 2005). Several lines of evidence indicate that the

role of c-Myc on apoptosis may depend on the cellular genetic background (Nilsson and Cleveland, 2003; Ceballos et al., 2005). However, the protective effect of c-Myc reported here cannot be attributable to cell type, because it also occurs in MEF cells, where overexpression of c-Myc is reported to induce apoptosis (Hoffman and Liebermann, 1998; Thompson, 1998). The reliability of the experimental model used in this paper is based on data reported in Figure S3, demonstrating that c-Myc-overexpressing MEF cells undergo apoptosis under serum deprivation, whereas the same cells became resistant to oxidative stress in the presence of growth factors. The observed dual effect of c-Myc ensures that its role in cell death is influenced by the presence and/or absence of growth factors. The involvement of c-Myc in apoptosis may also depend on mechanism of action and dose or time-exposure of the cellular insult. For instance, deregulated c-Myc expression can sensitize cells to some stimuli, but not to others (Biroccio et al., 2004; Grassilli et al., 2004). Moreover, c-Myc seems essential for the induction of apoptosis by sublethal doses of drug, but not required in cases when the apoptotic stimuli are sufficient to trigger apoptosis (Grassilli et al., 2004; Soucie et al., 2001). This is consistent with data demonstrating that low drug doses can have a cytostatic rather than a cytotoxic effect, a condition in which endogenous c-Myc expression is usually downregulated and overexpression of c-Myc generally triggers apoptosis. In our experiments, the dose and/or time exposure of the oxidative damage is not able to reduce endogenous c-Myc expression or to decrease cell proliferation, and, in these circumstances, c-Myc induces resistance to oxidative stress by upregulating the γ -GCS gene expression. Similarly, Park and coworkers (Park et al., 2002) demonstrated that when the apoptotic stimulus is able to increase endogenous c-Myc levels, the overexpression of c-Myc could promote survival through activation of the *ornithine decarboxylase* gene. The protective effect of c-Myc from oxidative stress reported here seems also to contrast with some results showing that c-Myc increases ROS generation (Tanaka et al., 2002; Vafa et al., 2002). Data reported in Figure S3 demonstrated that MEF cells transfected with an inducible *c-myc* expression vector show an increase in ROS production at a very early time in c-Myc induction. Then, when the cells are cultured in growth medium, ROS progressively decrease and the cells do not die. On the contrary, activation of c-Myc in the same cells maintained under serum deprivation, a condition known to give rise to oxidative stress, markedly increases ROS production and death. These results are in agreement with those of the Kanacura and Cleveland groups, showing that c-Myc overexpression alone does not induce cell death (Tanaka et al., 2002; Maclean et al., 2003). The divergence with the paper from Vafa et al. (2002), which shows, rather, that c-Myc directly induces DNA damage, could reflect the difference in cell type and/or the fact that their studies were carried out in the absence of survival factors. Finally, the functional role of c-Myc in determining cellular response to oxidative stress appears to be p53-independent, as it occurs both in wild-type (SbCl, HFF, and MEF) and mutated (M14) cell lines. Moreover, data reported in Figure S4 indicate that even in conditions where p53 is activated, the c-Myc-mediated protection from oxidative damage oc-

curs to the same extent in the p53 wild-type HCT116 and its knockout derivative line.

γ -GCS Heavy and Light Subunits Are Stress-Induced c-Myc Target Genes

By studying the mechanism(s) by which c-Myc induces γ -GCS genes and mediates the response to oxidative damage, we found that c-Myc transcriptionally regulates the γ -GCS genes by binding and activating the promoters of γ -GCS heavy and light subunits. The transcriptional control of γ -GCS gene expression by c-Myc occurs through its binding to the noncanonical c-Myc consensus sites identified on γ -GCS_H and γ -GCS_L gene promoters. However, not all the different E boxes identified on both regulatory sequences were bound and activated by c-Myc, in agreement with data demonstrating that accessory factors may play a role in effective binding to E box elements (Fernandez et al., 2003; Patel et al., 2004). Our results give functional relevance to the two E boxes located in the region proximal to the transcription starting point of the γ -GCS_H promoter. These data differ from most available literature data that describe several distinct binding elements in the distal genomic fragment (Mulcahy et al., 1997; Wild et al., 1998). On the contrary, the distal region of the γ -GCS_L promoter, which includes two putative c-Myc consensus sites, is not involved in the regulation of the transcription rate, as previously described (Moinova and Mulcahy, 1998). The E box 1 on the γ -GCS_L promoter is determinant in c-Myc-dependent transcriptional regulation, even though deletion of this region still retains the ability to respond to oxidative stress, consistent with data demonstrating that some consensus sites for other transcriptional factors are located in this region (Moinova and Mulcahy, 1998). Furthermore, simultaneous mutation of the E boxes and ARE consensus strongly compromises the transcriptional activity of both γ -GCS promoters, indicating that the transcriptional factors binding these regions work independently in regulating γ -GCS expression. We also found that the increased transcriptional rate and mRNA expression of γ -GCS genes after oxidative stress is attributable to a higher recruitment of c-Myc to both γ -GCS promoters. Moreover, the stress-dependent regulation of c-Myc recruitment to target genes occurs quickly and strongly depends on the higher GSH synthesis requirement displayed by the stress-exposed cells.

Phosphorylation at Ser-62 Confers Promoter Specificity to c-Myc

The fast c-Myc-mediated response to stress raised the question as to whether it could be tightly regulated by posttranslational modification. Indeed, exposure to H₂O₂ did not change the expression levels of c-Myc protein but instead triggered ERK-dependent Thr-58/Ser-62 phosphorylation. Thr-58/Ser-62 phosphorylation has been previously observed during Ras-dependent mitogenic stimulation, and it was also observed that this posttranslational modification of c-Myc exerts opposing roles in controlling protein stability: phosphorylation at Ser-62 stabilizes c-Myc, whereas phosphorylation at Thr-58 promotes c-Myc degradation through the ubiquitin-mediated proteasome pathway (Sears et al., 2000; Yeh et al., 2004). The data obtained here by using

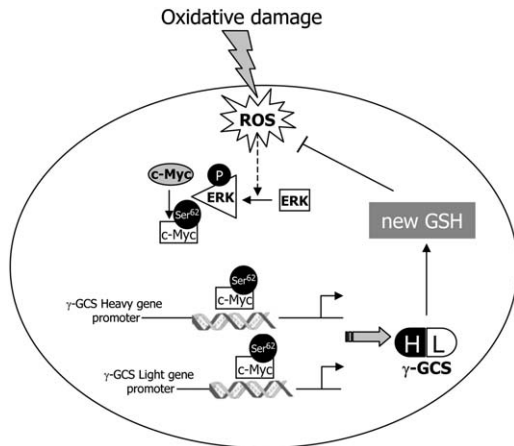


Figure 6. Proposed c-Myc-Dependent Survival Pathway Activated by Oxidative Damage

Oxidative stress triggered by ROS-generating agents induces ERK-dependent phosphorylation of c-Myc at Ser-62. Ser-62-phosphorylation drives c-Myc to both γ -GCS promoters, thus inducing GSH neo-synthesis. c-Myc-dependent GSH increase, acting as ROS scavenger, protects cells from oxidative damage.

c-myc knockout MEF cells transfected with the different *c-myc* mutants suggest that stress-induced phosphorylation of c-Myc at Thr-58 is dependent upon Ser-62, as occurs after mitogenic stimulation (Sears et al., 2000; Yeh et al., 2004). However, the findings reported in this paper add mechanistic insights into this c-Myc phosphorylation function. Indeed, Ser-62 phosphorylation not only regulates protein stability (Sears et al., 2000; Yeh et al., 2004), but also can dictate the choice of target genes. Specifically, c-Myc phosphorylation at Ser-62 is required for activation of γ -GCS genes. Like c-Myc, other important transcription factors, such as p53, have been found to be phosphorylated in sites far away from the DNA binding domain, and these posttranscriptional modifications regulate the binding ability to target genes (Saito et al., 2003). Moreover, microarray analysis revealed that besides both γ -GCS genes, other genes were differentially regulated in the *c-Myc*^{S62A} compared to *Myc*^{wt} cells, exclusively upon H₂O₂ treatment, supporting the key role of this c-Myc phosphorylation site during oxidative stress response. However, array data need to be confirmed to verify if c-Myc directly modulates their transcription or if their alteration is a consequence of the upstream c-Myc-mediated γ -GCS regulation. The relevance of the Ser-62 residue in the transcriptional regulation of each gene will be the object of further studies.

Finally, in this paper we attribute a biological function to c-Myc phosphorylation at Ser-62 in determining cellular response ROS-triggering agents, including H₂O₂ and anticancer drugs.

In summary, we identify an oxidative stress-induced survival signal pathway (Figure 6), depending on c-Myc phosphorylation, that can contribute to resistance to oxidative damage in tumor cells, which generally exhibit deregulated c-Myc expression. Our data support the hypothesis that regulation of c-Myc phosphorylation by both mitogenic stimuli and oxidative stress may be critical for functional Ras/Myc cooperation in cancer development.

Experimental Procedures

Antibodies and Reagents

The antibodies against c-Myc (clone 9E10 and clone N-262) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibodies against phospho-Myc (Thr58/Ser62), recognizing c-Myc phosphorylated at Thr58 and/or at Ser62, and the nonphosphorylated and phosphorylated forms of p38 (Thr180/Tyr182), ERK (Thr202/Tyr204), JNK (Thr183/Tyr185), AKT (Ser473), and GSK-3 β (Ser9) were obtained from Cell Signaling (Beverly, MA, USA). The control (siControl) and sequence-specific short RNA duplex pools for interference were purchased from Upstate (Lake Placid, NY, USA) for c-Myc and from Cell Signaling for p38 and ERK.

H₂O₂, BSO, GShest, propidium iodide (PI), and 4-hydroxy-tamoxifen (4-HT) were purchased from Sigma-Aldrich (Milan, Italy), and kinase inhibitors UO126 and SB202190 were purchased from Promega Corporation (Madison, WI, USA).

Cell Cultures and Treatment

Human melanoma cell lines (M14 and SbCl) were maintained in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum (FCS, Gibco-BRL). Human fetal immortalized fibroblasts (HFF) and mouse embryonic fibroblasts (MEF) were cultured in DMEM medium (Gibco-BRL) containing 10% and 15% FCS, respectively. In the experiments with H₂O₂, cells were incubated with increasing doses of the prooxidant agent for 30 min, and all of the analyses except for the evaluation of cell death were performed at the end of the treatment. Where not indicated, the dose of H₂O₂ used was as follows: 1 mM for M14 and 0.25 mM for SbCl, HFF, and MEF. BSO (10 mM) and GShest (5 mM) were added to cells 24 hr before H₂O₂ exposure. Kinase inhibitors UO126 (10 μ M) and SB202190 (25 μ M) were added to the cell medium 24 hr before H₂O₂ treatment. Growth curve analysis was performed by counting the total number of viable cells by trypan blue dye exclusion.

Plasmid Constructs

pcDNA3-Neo and pcDNA3-*c-myc* have been previously described (Biroccio et al., 2004). pMyc-TA-LUC vector, containing six tandem copies of c-Myc E box, was from Clontech (Palo Alto, CA, USA). Deleted pGL3b- γ -GCS_H (Δ 597) was created by digesting wt pGL3b- γ -GCS_H with BstPI. Deleted pGL3b- γ -GCS_L (Δ 1633) was obtained by cutting pGL3b- γ -GCS_L (Δ 1927) with NdeI restriction enzyme. All the other mutants were created by the PCR-based QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. All mut-M mutants carry a mutated E box site where the *cacatg* c-Myc binding region has been replaced by a scrambled *tcgac* sequence. All mut-A mutants carry a point-mutated ARE element in which the *gtgacNNNgc* core sequence has been replaced by a *gggacNNNgc* site.

Transfection and Infection

Transient transfection experiments were carried out by lipofectamine reagent (Gibco-BRL). Transfected cells were then maintained in FCS-containing medium and harvested 24 or 48 hr after the end of transfection. Transfection efficiency was assessed for each cell line and in each single experiment by using a plasmid carrying the gene either for the green (GFP) or the red fluorescent protein (RFP). Only experiments with transfection efficiency higher than 70% were considered valuable and included in the reported data.

To delete the *c-myc* gene and generate knockout mouse embryonic fibroblasts (MEF^{*c-myc*-/-}), *c-myc*^{fl/fl} fibroblasts were infected with a retroviral-based vector carrying the GFP gene fused to the *Cre* locus. After 48 hr of infection, GFP positive cells were sorted by cytofluorimetric analysis and cultured in FCS-containing DMEM for just one passage before further transfection with either wild-type or mutant *c-myc* cDNA. MEF^{*c-myc*-/-} cells have been freshly prepared by infection and sorting procedure before each experiment.

Western Blot Analysis

Western blot analysis was performed as previously reported (Biroccio et al., 2002). Briefly, cells were lysed on ice for 30 min in lysis buffer supplemented with phosphatase inhibitors. Forty μ g of total

proteins was then loaded onto denaturing sodium dodecyl-sulfate-polyacrylamide gels. ECL (Amersham Biosciences, Piscataway, NJ, USA) was employed for chemoluminescence detection.

Glutathione Determination and γ GCS Enzyme Activity

Intracellular GSH content was measured as previously described (Biroccio et al., 2004). γ GCS enzyme activity was evaluated by using a coupled assay with pyruvate kinase and lactate dehydrogenase (Seeling and Meister, 1984) and assaying the rate of decrease in absorbance at 340 nm at 37°C. Enzyme activity was expressed as μ mol of NADH oxidized per minute (U)/mg protein.

Flow Cytometry

Cell viability was assessed by staining cells with PI (1 μ g/ml). Sixty minutes after the end of H₂O₂ treatment, adherent cells were harvested, washed twice in PBS, stained with PI, and immediately analyzed by flow cytometry. PI positive cells were defined as nonviable cells. The evaluation of ROS was performed as previously described (Biroccio et al., 2004).

Reverse Transcriptase-Polymerase Chain Reaction

For semi-quantitative RT-PCR analysis, total RNA was isolated by TriZol (Gibco-BRL). First-strand cDNA synthesis and amplification of specific DNA sequences were performed by using ThermoScript RT-PCR System (Invitrogen, Carlsbad, CA, USA). cDNAs were amplified with each specific pair of forward/reverse primers (sequences available upon request). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript amplification has been used as an internal control for normalizing data to RNA loading. The ability of reverse transcriptase assay to detect semiquantitative changes in mRNA expression was verified by amplifying target mRNAs with 20, 25, and 30 PCR cycles.

Luciferase Assay

For promoter activity assay, cells were cotransfected with PEQ-176 (Promega Corporation) and pGL3basic- γ -GCS promoters. Luciferase activity was measured with a Luciferase Assay System kit (Promega Corporation), as specified by the manufacturer, and normalized to β -galactosidase expression.

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) assay was performed by using a Chromatin Immunoprecipitation Assay kit (Upstate). Briefly, formaldehyde crosslinked chromatin fragments were immunoprecipitated with no antibody, anti-c-Myc (clone N-262), anti-phospho-Myc^{Thr58/Ser62}, or β -actin antibodies and assayed by PCR (the sequences of each pair of forward/reverse oligos is available upon request). To verify that an equivalent amount of chromatin was used in the immunoprecipitates, a sample representing 0.01% of the total chromatin (input) was included in the PCR samples. Linearity of the signal was insured by amplifying increasing amounts of template DNA (0.1, 0.25, and 0.5 μ g).

Microarray and Data Analysis

Total RNA was extracted from wt and S62A c-myc-transfected MEF^{myc-/-} cells, both exposed and unexposed to H₂O₂, by TriZol reagent followed by additional purification with an RNeasy kit from Qiagen (Valencia, CA, USA). Preparation of cRNA, oligonucleotide array, hybridization to MOE430A2.0 GeneChip arrays (Affymetrix, Santa Clara, CA, USA), and scanning of the arrays were carried out at the Dana-Farber Microarray Core Facility.

Raw chip data were loaded into the Rosetta Luminator software, and intensity profiles (one for each chip analyzed) were obtained after application of the Affymetrix Error Model implemented by Rosetta Biosoftware. Intensity values, together with intensity errors and p values, were then associated to each probe set contained in the Mouse Affymetrix Chip for the different experimental conditions. Ratio experiments were generated by combining replicate chips and selecting couples of mRNA sources to be compared. Successively, log₁₀(Ratio) against log₁₀(Intensity) plots were created (Bolstad et al., 2003). Differentially expressed genes were extracted by selecting probesets with a Log₁₀(Ratio) p value of less than 0.01 and Absolute Fold Change greater than 2. Successively, information about modulated genes was extracted with the Batch Search option

available within the SOURCE website (<http://smd.stanford.edu/cgi-bin/source/sourceBatchSearch>).

Statistical Analysis

The experiments have been repeated from three to five times, and the results obtained are presented as means \pm SD. Significant changes were assessed by using the Student's t test for unpaired data, and p values <0.05 were considered significant.

Supplemental Data

Supplemental Data include four figures and one table and can be found with this article online at <http://www.molecule.org/cgi/content/full/21/4/509/DC1/>.

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