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Tumor and Stem Cell Biology

Cancer Research

Modulation of Gene Expression and Tumor Cell Growth by Redox Modification of STAT3

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Abstract

Reactive oxygen species (ROS) promote tumor cell proliferation and survival by directly modulating growth-regulatory molecules and key transcription factors. The signal transducer and activator of transcription 3 (STAT3) is constitutively active in a variety of tumor cell types, where the effect of ROS on the Janus kinase/STAT pathway has been examined. We report here that STAT3 is directly sensitive to intracellular oxidants. Oxidation of conserved cysteines by peroxide decreased STAT3 binding to consensus serum-inducible elements (SIE) *in vitro* and *in vivo* and diminished interleukin (IL)-6–mediated reporter expression. Inhibitory effects produced by cysteine oxidation in STAT3 were negated in redox-insensitive STAT3 mutants. In contrast, ROS had no effect on IL-6–induced STAT3 recruitment to the c-*myc* P2 promoter. Expression of a redox-insensitive STAT3 in breast carcinoma cells accelerated their proliferation while reducing resistance to oxidative stress. Our results implicate STAT3 in coupling intracellular redox homeostasis to cell proliferation and survival. *Cancer Res; 70(20); 8222–32. ©2010 AACR.*

Introduction

Reactive oxygen species (ROS) contribute to the development and progression of numerous cancers. Originally based on observations of elevated ROS levels in tumor cells (1), this consensus is now supported by compelling genetic and biological data. For example, mice lacking *prdx1*, the gene for peroxiredoxin-1, a ROS scavenger, display elevated nuclear ROS levels and succumb prematurely to cancer (2), whereas one role of BRCA1, expressed from the primary susceptibility marker for breast carcinoma (BC), is the reduction of cellular ROS (3).

Despite the range of correlative data, the mechanisms by which ROS drive tumorigenesis remain largely uncharted. One scenario invokes DNA damage, but alternative models implicate signaling events (4). ROS act as intracellular mediators of growth factor receptor signaling with direct effects on several regulatory molecules, including lipid and tyrosine phosphatases (5) and SUMO conjugating and deconjugating enzymes (6, 7). Redox-dependent regulation of DNA binding by transcription factors NFκB and AP-1 has also been reported (8), as has upregulation of c-Myc, a transcription factor involved in cell cycle progression (9).

Inappropriate activation of signal transducer and activator of transcription 3 (STAT3) has been observed in tumors from several tissues, notably BC (10, 11). A subset of such tumors seems to result from elevated levels of active Src serving to activate STAT3 (12), whereas other evidence points to autocrine control of STAT3 involving interleukin (IL)-6–type cytokines (13–16). Consistent with these observations, the potential to transform cells was ascribed to a "constitutively active" version of STAT3 (17) subsequently shown to resist dephosphorylation rather than eschew activation by upstream signals (18). Thus, the predominant role for STAT3 in tumor progression seems to be as a mediator of cell proliferation or enhanced survival in response to aberrant IL-6–type signals.

Several reports have highlighted an effect of ROS on STAT3 activity. Although ROS scavengers and inhibitors of NOX enzymes generally inhibited STAT3 activity (19, 20), the otherwise divergent reports indicated that the effects of ROS may be tissue specific and manifested at several levels (e.g., by their actions on tyrosine phosphatases and Janus kinases). Conversely, STAT3 seems to participate in intracellular ROS homeostasis: Its target genes include several involved in the hypoxic response, it is essential for ischemic preconditioning in the mouse heart, and it was recently shown to be present in mitochondria and to influence electron transport chain function (21–23). STAT3 itself is susceptible to oxidation in cells under oxidative stress (24) and was shown to be modified and repressed by cysteine glutathionylation (25).

Here, we report that oxidation of conserved cysteines in the DNA-binding domain (DBD) and COOH-terminal transactivation domain (C-TAD) of STAT3 by peroxide blocked binding to consensus serum-inducible elements (SIE) *in vitro* and

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impaired SIE reporter gene expression. Furthermore, peroxide decreased IL-6–induced STAT3 recruitment to the c-*fos* promoter, whereas a redox-insensitive STAT3 mutant was unaffected. In contrast, oxidative stress had no effect on IL-6–induced STAT3 binding to the c-*myc* P2 promoter *in vivo* and enhanced expression from a c-*myc* reporter gene, underlining the anomalous behavior of this STAT3-responsive gene. The direct regulation of STAT3 by ROS is also implicated in coupling intracellular redox homeostasis to cell proliferation and survival because expression of a redox-insensitive STAT3 increased the growth rate of BC cells but compromised their resistance to oxidative stress.

Materials and Methods

Cell culture, DNA transfections, and immunoblotting

HEK293 and HepG2 cells were cultured in DMEM with 10% FCS, 100 units penicillin, and 0.1 mg/mL streptomycin. BR293 and MCF-7 cells were maintained in Eagle's MEM with 10% FCS, 100 units/mL penicillin, 0.1 mg/mL streptomycin, 1% nonessential amino acids, and 2 mmol/L L-glutamine. HEK293 cells were transfected by DNA–calcium phosphate coprecipitation (14); HepG2, BR293, and MCF-7 cells were transfected with polyethyleneimine (26). For immunoblotting from reducing and nonreducing gels, standard protocols were used throughout.

Plasmids and oligonucleotides

The $(SIE)_2$ and $(P2E)_3$ reporters have been described (27). The $(SIE)_4$ reporter and vectors for FLAG-tagged STAT1, STAT3, and STAT3-Y/F were from Curt Horvath (Northwestern University, Evanston, IL) and James Darnell (Rockefeller University, New York, NY). The v-Eyk vector was from Daniel Besser (MDC, Berlin, Germany). Expression vectors for STAT1/STAT3 chimeras have been described (24). Cysteine substitutions were generated by site-directed mutagenesis and verified by DNA sequencing. Sequences of oligonucleotides for mutagenesis, electrophoretic mobility shift assay (EMSA) probes, and chromatin immunoprecipitation (ChIP) assays are available on request.

DNA-binding and gene reporter assays

DNA-binding assays (14) and luciferase reporter assays (18) have been described.

Chromatin immunoprecipitation

ChIP assays were performed as described (28) with modifications (29).

Cell proliferation assays

Cells transfected with vectors for STAT3 or STAT3-C3S were seeded in 96-well plates at 1×10^3 per well in 100 μL full medium and cultured at 37°C overnight. Proliferation was measured daily by MTT assay (30). Alternatively, cells were seeded in 24-well plates at 1×10^4 (BR293) and 3×10^4 (MCF-7) per well and cultured with or without administration of peroxide daily for 2 days at the concentrations indicated. After 72 hours (BR293) or 108 hours (MCF-7), cells were counted in a Neubauer chamber.

Wound-healing assay

BR293 cells were seeded in six-well plates, transfected, and grown to confluence. Monolayers were scarred with a micropipette and photographed at the beginning of the assay ($t = 0$) and at the times indicated. Images are from a single representative experiment ($n = 3$).

Flow cytometry

Cells transfected with vectors for STAT3 or STAT3-C3S were cultured for 16 hours with or without peroxide treatment, trypsinized, dispensed at 1×10^5 to 1×10^6 per tube, and fixed in ice-cold 70% ethanol. Cells washed and resuspended in PBS, 0.1% bovine serum albumin, and 0.1% azide were incubated with RNase (0.1 mg/mL) and propidium iodide (50 μg/mL) before flow cytometry (Coulter FC500). Cell cycle analysis was performed with Cylchred. Apoptosis was analyzed by Annexin V–FITC assay (AbD Serotec) and flow cytometry (Coulter Altra).

Results

DNA binding by STAT3 is sensitive to peroxide

To determine if ROS influence STAT3 directly, we examined the effect of peroxide on DNA binding *in vitro*. Nuclear extracts from HEK293 cells transfected with vectors for STAT1 and the oncogenic tyrosine kinase v-Eyk, or STAT3 followed by IL-6 stimulation, were treated with peroxide for 5 minutes, followed by addition of radiolabeled m67/SIE probe and EMSA, as outlined in Fig. 1A. STAT1 was unaffected by peroxide (Fig. 1B, lanes 1–4), whereas STAT3 binding was extinguished at 3 mmol/L peroxide (Fig. 1B, lane 7). Peroxide had no effect on tyrosine phosphorylation of STAT3 (Supplementary Fig. S1A).

Optimum detection of STAT3 DNA binding by EMSA requires reducing conditions (≥1 mmol/L DTT), as shown in Supplementary Fig. S1B and reported previously (18). To determine if the high peroxide concentration required to inhibit STAT3 DNA binding was due to added DTT (1.4 mmol/L final concentration), nuclear extracts supplemented with 0.2 or 1 mmol/L DTT were challenged with increasing concentrations of peroxide. Indeed, when five times less DTT was used, the inhibitory concentration of peroxide was also reduced 5-fold (Supplementary Fig. S1C, compare lanes 5 and 12), confirming the dependency of peroxide dose on the redox buffering capacity of DTT in the reactions. For consistency and optimum STAT3 binding, all subsequent EMSAs are benchmarked at 1.4 mmol/L DTT, necessitating inhibitory peroxide concentrations in the millimolar range.

To define regions of STAT3 conferring peroxide sensitivity, we used STAT1/STAT3 chimeras as designated in Fig. 1C. STAT1/3S (STAT1 NH_2 terminus fused to DBD and remainder of STAT3) was as sensitive to peroxide as STAT3 (Fig. 1D, third panel), and the reciprocal chimera (STAT3/1S) was as insensitive as STAT1 (Fig. 1D, fourth panel). A third chimera, STAT1/3H (NH_2 terminus and DBD of STAT1 fused to SH2

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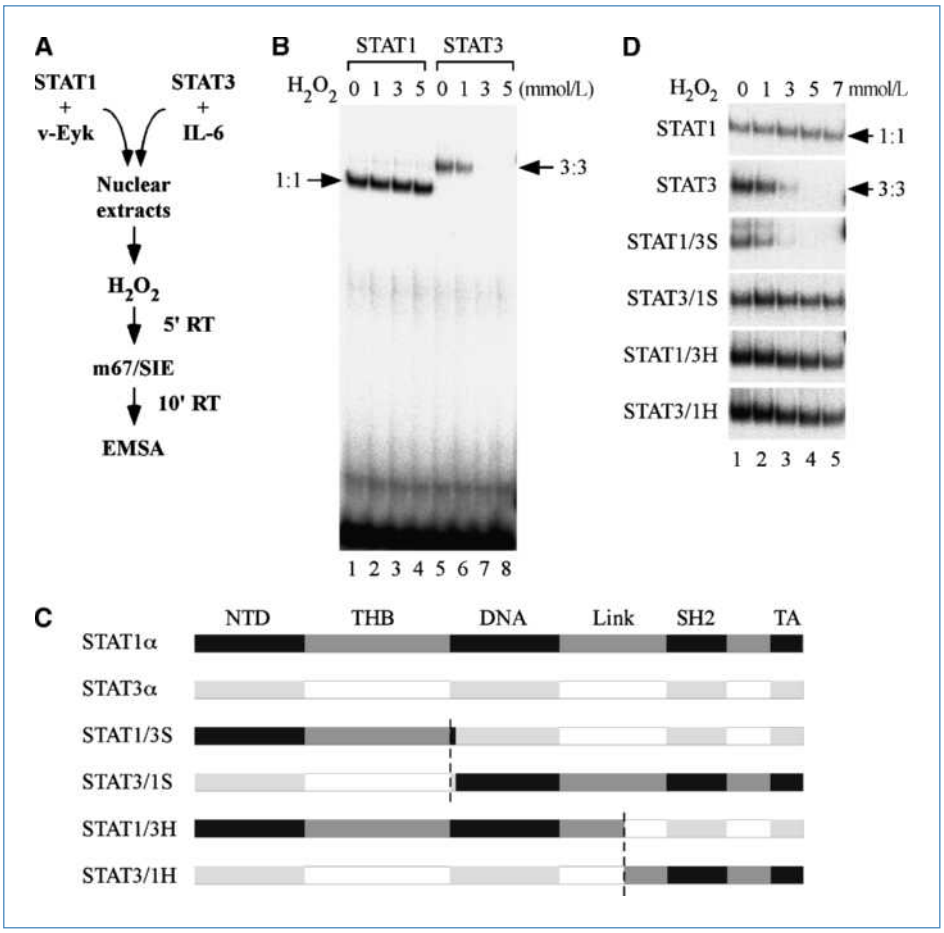

Figure 1. Differential sensitivity of STAT1 and STAT3 DNA binding to ROS. A, flow diagram for *in vitro* analysis of DNA binding by STAT1 and STAT3. B, nuclear extracts containing active STAT1 or STAT3 were treated with peroxide at concentrations indicated for DNA-binding analysis by EMSA. C, depiction of STAT1/3 chimeras used. Vertical dotted lines highlight transition points between STAT1 (dark) and STAT3 (light). NTD, NH_2-terminal domain; THB, tetrahelical bundle; Link, linker domain; SH2, SH2 domain; TA, transactivation domain. D, chimeras were activated and analyzed as described in A, with peroxide at concentrations indicated. Panels show bands corresponding to STAT-protein complexes.

and C-TAD of STAT3), showed little sensitivity to peroxide, and DNA binding was still detectable after treatment with 7 mmol/L peroxide (Fig. 1D, fifth panel). This implied that the critical region of STAT3 was the DBD. However, the reciprocal chimera (STAT3/1H) displayed a similar profile. Thus, the peroxide sensitivity seemed to map either to the linker region disrupted in both STAT1/3H and STAT3/1H chimeras or to multiple elements within the COOH-terminal half of STAT3.

As cysteines commonly confer redox sensitivity on proteins, similar experiments were performed with the cysteine-specific reagent *N*-ethylmaleimide (NEM). STAT3 was also sensitive to NEM, whereas STAT1 was not (Supplementary Fig. S2A). Moreover, chimeras STAT1/3S, STAT3/1H, and STAT1/3H showed relative levels of NEM sensitivity comparable with those seen with peroxide, indicating that STAT3 sensitivity to peroxide was due to oxidation of cysteines, although conceivably the effect was indirect, involving oxidation of another protein(s) in the nuclear extracts.

Generation of a peroxide-insensitive STAT3 by cysteine substitutions

STAT3 contains 14 cysteines, of which 5 are conserved in STAT1. Clusters of nonconserved cysteines lie within the DBD and the SH2/C-TAD (Fig. 2A). Substitution of C367 with serine had no effect on DNA binding (data not shown). Substitutions at C418, C426, and C468 showed slight decreases in sensitivity; double mutants were less sensitive and substitution of all three (C418, C426, and C468) produced a STAT3 mutant substantially resistant to peroxide (C3S; Fig. 2B, bottom).

Within the COOH-terminal region of STAT3, only the single substitution C765S decreased peroxide sensitivity (Fig. 2C, fifth panel) and its combination with other substitutions in that region had no further effect (see, for example, bottom panel). We also compared combinations of substitutions in both regions of STAT3. For example, addition of the C765S substitution to C418/26S conferred further resistance to peroxide (Fig. 2D, third panel). The resistance of the C4S mutant to NEM indicated that oxidation or modification of these cysteines was the likely cause for the loss of STAT3 DNA binding (Supplementary Fig. S2B). Together, these data indicate that the DBD of STAT3 contains up to three reactive cysteines with one other (C765) present within the C-TAD. Their oxidation or subsequent modification as a consequence of peroxide treatment is sufficient to inhibit DNA binding by STAT3 *in vitro*.

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STAT3 redox complexes detected in cells under oxidative stress

C418 and C426 lie on a flexible loop between βe and βf of the eight-stranded β-barrel that contributes to the STAT3 DNA-binding interface (Supplementary Fig. S3A). This loop makes DNA backbone contacts, with hydrogen bonds between R417 and phosphates between ±3 and ±4 of the consensus m67/SIE (31). C468 is at the NH_2-terminal end of helix α5, which is part of the four-helix link between β-barrel and SH2 domain, and contributes to base pair recognition, specifically the methyl groups of thymines in the ±3 and ±4 positions (31). Thus, modification of C418, C468, and C426 by NEM or other redox event could block STAT3 DNA binding. These cysteines show extensive species conservation in STAT3 (Fig. 3A) but are absent from other STAT proteins (Supplementary Fig. S3B), implying that only DNA binding by STAT3 is redox sensitive.

We previously described STAT3 redox dimer formation (24). From HEK293 cells treated with peroxide and quenched with NEM, three high–molecular weight species are observable under nonreducing (Fig. 3B) but not reducing SDS-PAGE (Supplementary Figs. S4A and S5). Treatment for 1 minute with 10 μmol/L peroxide induced formation of these complexes (Fig. 3C; Supplementary Fig. S4B). Similar complexes formed by endogenous STAT3 were detected in several cell lines (Supplementary Fig. S4C). Single point mutations had little effect on redox complex formation (100 μmol/L peroxide, 2 minutes), but substitutions at C418 and either C426 or C468 prevented formation of the larger complexes (Fig. 3C, lanes 6 and 7). These complexes were also absent with STAT3-C3S (Fig. 3C, lane 9). Formation of the larger complexes was unaffected by substitutions C687S, C712S, or C718S but impaired by C765S (Fig. 3D). However, C712S and C718S decreased formation of the smaller complex (Fig. 3D, lanes 3 and 4). Peroxide did not induce tyrosine phosphorylation of STAT3 or the cysteine mutants (Supplementary Fig. S5). Thus, cysteines involved in redox inhibition of STAT3 DNA binding *in vitro* participate in formation of discrete redox complexes in cells under oxidative stress. Based on migration under nonreducing SDS-PAGE, the sizes of these complexes are approximately 180, 270, and 360 kDa (Supplementary Fig. S6). As mass spectrometry (MS) analyses detected no other proteins in these complexes (data not shown), they may reflect the formation of STAT3 redox multimers.

Peroxide modulates STAT3-mediated reporter gene expression

The effect of ROS on STAT3-dependent gene expression was examined with reporter genes driven by consensus SIEs. HepG2 cells transfected with an $(SIE)_4$ reporter plasmid alone yielded low levels of IL-6–inducible expression. Cotransfection of an expression vector for STAT3 significantly

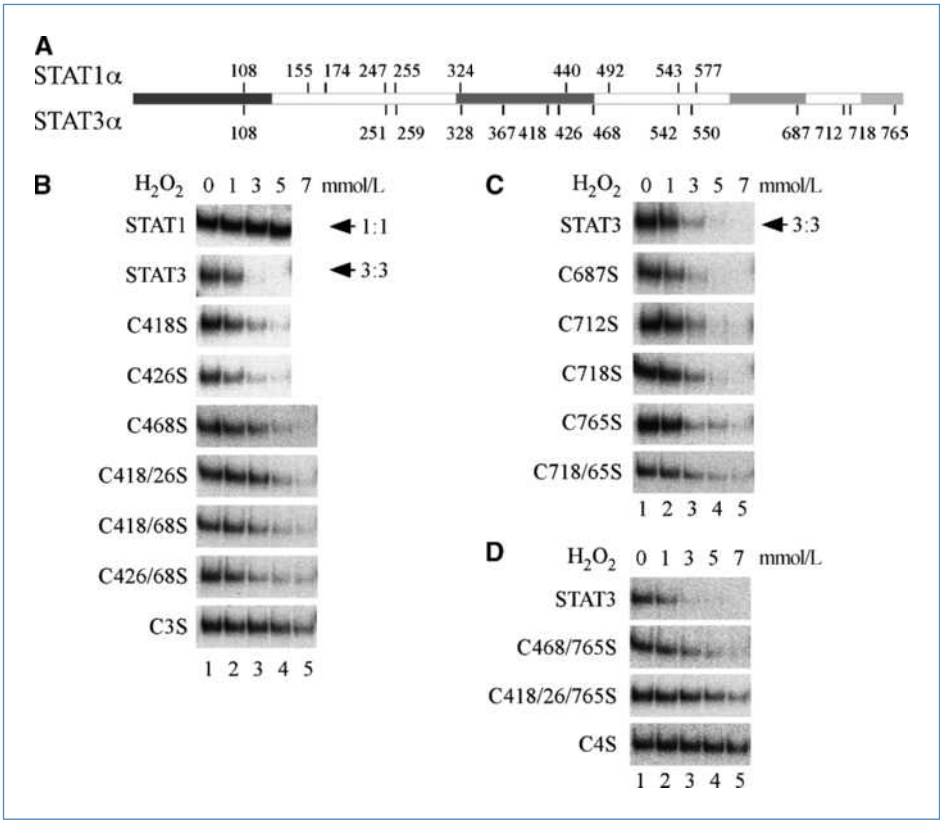

Figure 2. Redox-sensitive cysteines in STAT3 affecting DNA binding. A, diagram showing cysteines in STAT1 (above) and STAT3 (below) in relation to STAT domain structure. B to D, nuclear extracts containing active STAT1, STAT3, or the indicated STAT3 cysteine substitution mutants were treated with peroxide at concentrations from 1 to 7 mmol/L, and DNA binding was analyzed by EMSA. Panels show STAT-DNA complexes from gels comparable with the one in Fig. 1B.

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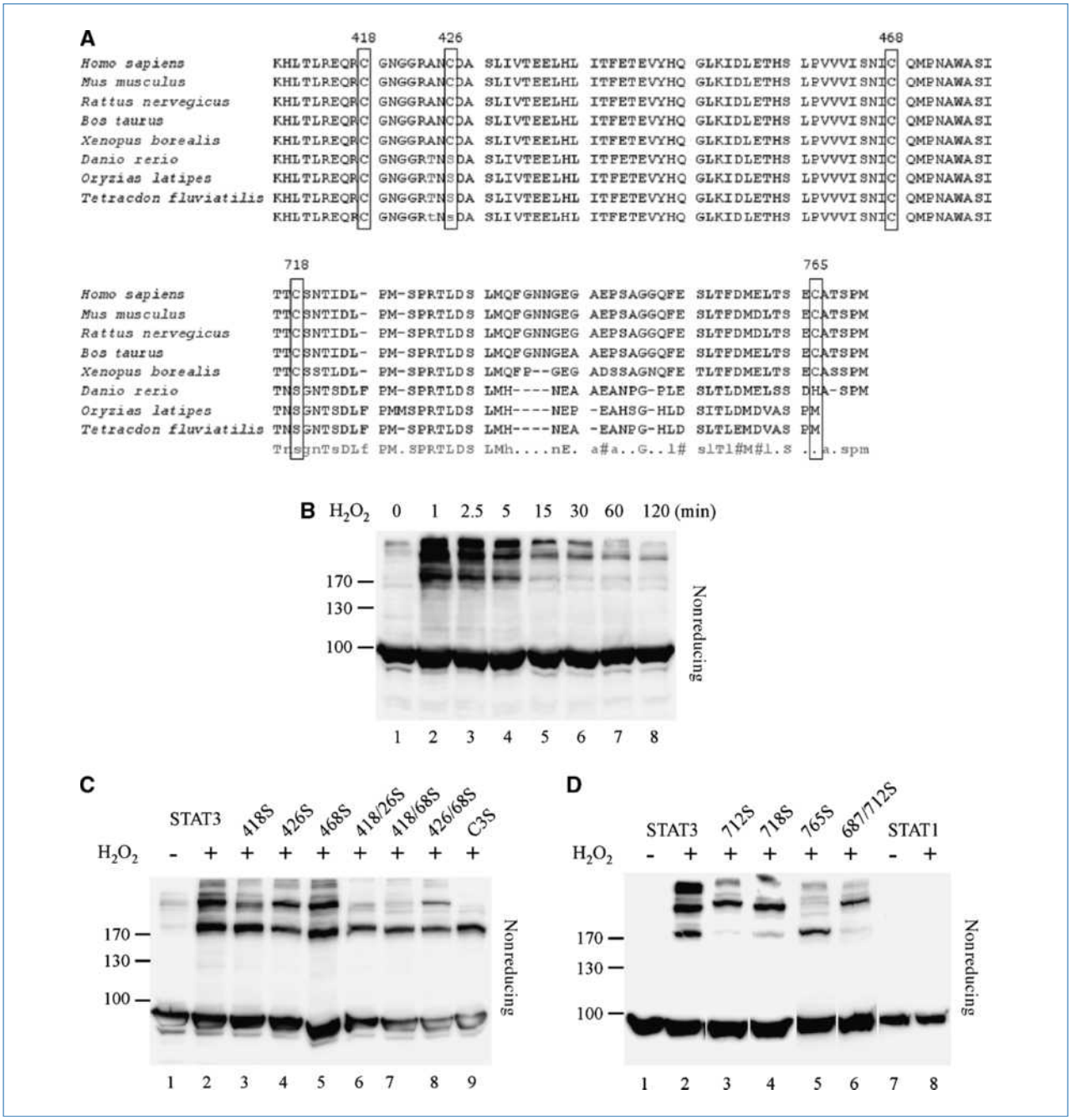

Figure 3. Modification of redox-sensitive cysteines in STAT3. A, STAT3 protein sequences from eight vertebrate species highlight conservation of cysteines at positions 418, 426, 468, and 765 (open boxes). The consensus (bottom line) indicates high conservation of DBD and relatively poor conservation of C-TAD. B, serum-starved HEK293 cells expressing FLAG-STAT3 were treated with 100 μmol/L peroxide and harvested at times indicated, and STAT3 redox complexes were detected by nonreducing SDS-PAGE and immunoblotting with an anti-FLAG antibody. C, serum-starved HEK293 cells expressing FLAG-STAT3 or indicated mutants were treated with 100 μmol/L peroxide and harvested after 2 min, and STAT3 redox complexes were detected as in B. D, serum-starved HEK293 cells expressing FLAG-STAT1, FLAG-STAT3, or the indicated STAT3 mutants were treated and processed as in C.

enhanced expression, whereas a STAT3-Y705F (Y/F) mutant reduced expression (Fig. 4A), confirming the response to exogenous STAT3 proteins. Treatment with increasing amounts of peroxide before IL-6 stimulation progressively reduced expression (Fig. 4B). This effect was reproducible at 400 μmol/L peroxide; pretreatment of cells with 800 μmol/L peroxide caused a 30% reduction in STAT3-dependent reporter activity stimulated by IL-6. In cells transfected with a vector for STAT3-C4S, inhibition by peroxide was lost, suggesting that the effect on STAT3-dependent reporter

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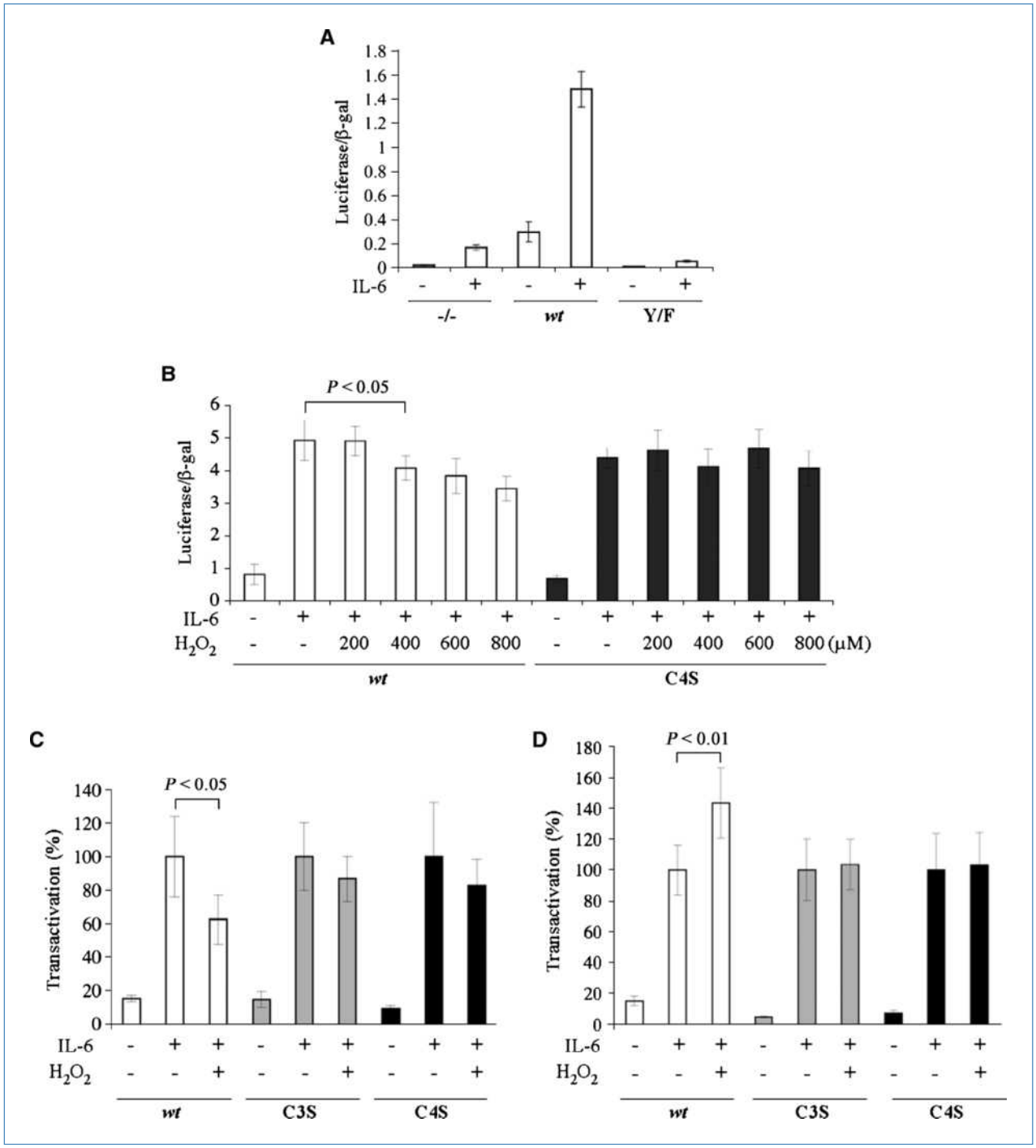

Figure 4. Effect of oxidative stress on STAT3-dependent reporter expression. A, HepG2 cells were cotransfected with a $(SIE)_4$ reporter plasmid and control plasmid (–/–) or expression vectors for STAT3 (wt) or inactive STAT3 (Y/F). Following serum deprivation for 24 h, cells were given fresh serum-free medium (–) or medium containing 10 ng/mL IL-6 (+) 6 h before harvesting and processing. Luciferase values were normalized against expression from a cotransfected β-gal vector. Columns, mean from unpaired Student's *t* test of three experiments performed in triplicate; bars, SD. B, HepG2 cells were cotransfected with a $(SIE)_4$ reporter and expression vectors for STAT3 or STAT3 C4S. Following serum deprivation for 24 h, cells were stimulated directly with IL-6 (10 ng/mL) or pretreated for 10 min with 200 to 800 μmol/L peroxide followed by IL-6. After 6 h, cells were processed as in A. Columns, mean evaluated by unpaired Student's *t* test from five experiments with duplicate points; bars, SD. C, HepG2 cells were cotransfected with a $(SIE)_2$ reporter and expression vectors for STAT3, STAT3 C3S, or STAT3 C4S. Following serum deprivation for 24 h, cells were stimulated with IL-6 (10 ng/mL) or pretreated for 10 min with 800 μmol/L peroxide medium followed by IL-6 stimulation. Cells were processed after 6 h. D, HepG2 cells were cotransfected with a $(P2E)_3$ reporter and expression vectors for STAT3, STAT3 C3S, or STAT3 C4S and processed as in C. C and D, columns, mean from unpaired Student's *t* test of three experiments with duplicate points; bars, SD.

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expression was due, at least partly, to oxidation of cysteines participating in redox complex formation and inhibiting DNA binding. In further experiments, with an $(SIE)_2$ reporter, peroxide reduced IL-6–induced expression by 40% in the case of STAT3, whereas with STAT3-C3S and STAT3-C4S no statistically significant reduction was observed (Fig. 4C). These data corroborate the negative effect of peroxide on consensus SIE-mediated reporter gene expression.

Several STAT3 target gene promoters contain nonconsensus SIEs, one example being the c-*myc* P2 promoter, with a low-affinity STAT3-binding element (P2E), where inducible STAT3 binding has been detected *in vivo* by ChIP assay but not reproducibly *in vitro* (27, 32, 33). It was also shown previously in HepG2 cells that a $(P2E)_3$ reporter responded to IL-6 stimulation in a STAT3-dependent manner (27, 32). In contrast to the downregulation of consensus SIE reporters by peroxide, inducible $(P2E)_3$ reporter activity was enhanced ~50% by peroxide (Fig. 4D). However, as before, the effect of peroxide was lost with STAT3-C3S or STAT3-C4S. IL-6–induced tyrosine phosphorylation of STAT3, C3S, or C4S was not affected by peroxide (Supplementary Fig. S7). These data indicate that STAT3-dependent gene expression can be modulated by direct action of peroxide on key cysteines in STAT3, but the STAT3 response element determines the outcome.

Peroxide reduces STAT3 binding to c-*fos* promoter

To assess STAT3 binding to endogenous promoters, ChIP assays were performed on HepG2 cells. STAT3 levels at the c-*fos* promoter were low in unstimulated cells but readily induced by IL-6 (Fig. 5A, compare lanes 9 and 11), as previously reported (27). Peroxide had no effect on STAT3 in unstimulated cells, but decreased STAT3 recruitment in response to IL-6 (Fig. 5A, compare lanes 11 and 12), in line with the effect of peroxide on IL-6 induction of SIE reporter expression, without decreasing the STAT3 tyrosine phosphorylation induced by IL-6 (Fig. 5B).

If the ROS-dependent reduction in STAT3 binding to the c-*fos* promoter were caused by oxidation of STAT3, a ROS-insensitive STAT3 should be refractory to peroxide and bind to the promoter. Thus, HepG2 cells were transfected with FLAG-tagged versions of STAT3 and STAT3-C3S and ChIP assays were performed with an anti-FLAG antibody. Recruitment of FLAG-STAT3 to the c-*fos* promoter could be detected on IL-6 stimulation (Fig. 5C, top, lane 7), and as with endogenous STAT3, this was decreased by pretreatment with peroxide (Fig. 5C, top, lane 8). In contrast, IL-6 stimulated the recruitment of STAT3-C3S to the c-*fos* promoter equally well regardless of peroxide pretreatment (Fig. 5C, third panel, compare lanes 7 and 8), confirming

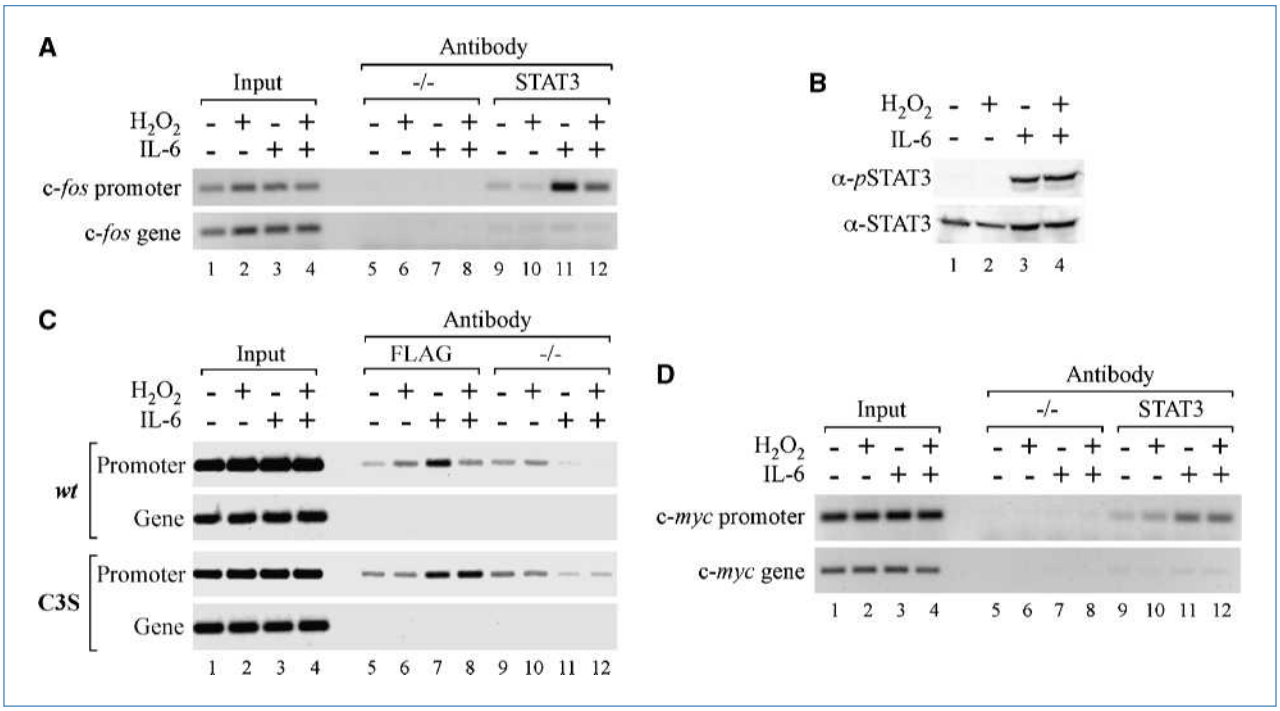

Figure 5. Peroxide modulates STAT3 binding to endogenous SIE promoter. A, serum-starved HepG2 cells were untreated, pretreated with peroxide (600 μmol/L), and/or stimulated with IL-6 (10 ng/mL) for 20 min (+). Binding of STAT3 to c-*fos* promoter was determined by ChIP assay. Primer pairs amplified the SIE promoter region (top) or part of the c-*fos* gene (bottom) after immunoprecipitation of DNA complexes with a STAT3 antibody or mock control, as indicated. B, serum-starved HepG2 cells were treated as in A, and STAT3 tyrosine phosphorylation was monitored by SDS-PAGE and immunoblotting. C, HepG2 cells transfected with vectors for FLAG-tagged wt STAT3 or STAT3 C3S were untreated, pretreated with peroxide, and/or stimulated with IL-6 (10 ng/mL) for 20 min (+). Binding of STAT3 and STAT3 C3S to the c-*fos* promoter was determined by ChIP assay as in A after immunoprecipitation of DNA complexes with a FLAG antibody or mock control, as indicated. D, as in A, except binding of STAT3 to the c-*myc* P2 promoter was determined by primer pairs for the c-*myc* P2E promoter (top) or a region of the c-*myc* gene (bottom).

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that direct modification of STAT3 decreases its recruitment to target gene promoters with consensus SIEs, as exemplified by c-*fos*.

IL-6–inducible recruitment of endogenous STAT3 was also detected at the c-*myc* P2 promoter, but here, there was no discernible decrease with peroxide (Fig. 5D, lanes 11 and 12). Thus, peroxide differentially modulates the binding and activity of STAT3 at consensus SIEs and nonconsensus sites such as the c-*myc* P2E.

STAT3 cysteine oxidation modulates proliferation of BC cells

To seek an effect of STAT3 oxidation on tumor cell growth, proliferation assays were performed with BC cell lines displaying constitutive STAT3 activity (14). Compared with STAT3, expression of STAT3-C3S increased proliferation of BR293 and MCF-7 cells under normoxic conditions (Fig. 6A). Moreover, BR293 cells expressing STAT3-C3S were more responsive in wound-healing assays than control cells (Fig. 6B). In cell cycle progression assays, we observed that after 16 hours more cells expressing STAT3-C3S had reached G_2-M than control cells expressing STAT3 (BR293 = +45%; MCF-7 = +20%; Supplementary Fig. S8), suggesting that the advantage conferred by STAT3-C3S was linked to contraction of the cell cycle.

As DNA binding by STAT3-C3S was ROS insensitive, we predicted that oxidative stress would exacerbate the growth advantage its expression conferred on BC cells. However, cells expressing STAT3-C3S were less resistant to peroxide than cells expressing STAT3; BR293:STAT3 cell numbers were reduced 3-fold over 72 hours by treatment with 30 μmol/L peroxide, whereas BR293:STAT3-C3S cells were reduced 10-fold (Fig. 6C). Similarly, MCF-7:STAT3 cell counts were reduced 6-fold, whereas MCF-7:STAT3-C3S cells were reduced ~17-fold. Cell cycle analysis revealed fewer STAT3-C3S cells than STAT3 cells in G_2-M after 16 hours (Supplementary Fig. S8). In Annexin V–binding assays, there were no significant differences in the levels of apoptosis between cells expressing STAT3 and STAT3-C3S (Supplementary Fig. S9). Together, the data indicate that proliferation of these BC cells is coupled to redox modulation of STAT3 activity (Fig. 6D).

Discussion

In this study, we have shown STAT3 sensitivity to peroxide and provided evidence that STAT3 is involved in a mechanism whereby ROS modulate its activity to influence gene expression and cell proliferation.

Redox control of DNA binding by STAT3

Initial experiments addressed the effect of peroxide on DNA binding by active STAT3. At the optimum DNA-binding conditions established for STAT3 *in vitro*, which include ≥1 mmol/L DTT (Supplementary Fig. S1B; ref. 18), the effective peroxide concentration lay in the millimolar range. However, at lower DTT concentrations, inhibition by peroxide occurred in the micromolar range (Supplementary Fig. S1C). On treatment of unstimulated cells with micromolar concentrations of peroxide, three STAT3 redox complexes were observed. Raising the concentration did not induce additional complexes, an indication that sensitivity was confined to specific residues and confirmed with STAT3 mutants, as the same cysteines in the DBD and C-TAD were highlighted in DNA-binding assays and by formation of the two larger redox complexes. Importantly, serine substitutions at these cysteines had no discernible effect on tyrosine phosphorylation or DNA binding per se, although substitution of C712 does reduce STAT3 DNA-binding affinity (18). Thus, loss of DNA binding can be attributed to formation of interchain cysteine disulfides to generate STAT3 redox multimers.

Redox control of STAT3 reporter gene expression and promoter recruitment

In HepG2 cells, which, compared with BR293 and MCF-7 cells, are relatively resistant to peroxide (34, 35), IL-6–induced SIE reporter expression was reduced by peroxide in a dose-dependent manner. The effective peroxide concentration was cell number dependent, and statistical reproducibility required accurate plating of cells and close adherence to a time schedule. However, under these conditions, 30% to 40% reductions in the activities of two different SIE reporters were consistently seen on peroxide pretreatment of cells expressing STAT3 but not STAT3-C3S or STAT3-C4S. These effects were not due to a reduction in the pool of phospho-STAT3, as assessed by Western blotting, and nuclear accumulation seemed normal, although minor alterations in the intracellular distribution of STAT3 could not be excluded.

ChIP assays confirmed that peroxide decreased the IL-6–induced recruitment of STAT3 to endogenous promoters, exemplified by c-*fos*, an immediate-early and acute-phase response gene (36). Rather than the integrated response revealed by reporter assays, the ChIP assays reflected short-term changes in promoter recruitment, indicating that an acute effect on STAT3 DNA binding underlies the cumulative effect of peroxide on reporter gene expression. Changes in STAT3 recruitment as a consequence of direct redox modification were confirmed by IL-6–induced promoter recruitment of STAT3-C3S being refractory to oxidative stress.

In striking contrast to the behavior of consensus SIE reporters, IL-6–induced c-*myc* P2E reporter expression was enhanced by peroxide treatment, and this response was lost with STAT3-C3S and STAT3-C4S. Consistent with these observations, cytokine-induced binding of STAT3 to the c-*myc* promoter, which has been well documented (27, 32, 37), was unaffected by peroxide treatment. These results confirm our contention that STAT3 recruitment to the nonconsensus c-*myc* P2E involves an unconventional binding modus (27). One interpretation is that STAT3 accesses the c-*myc* promoter not by direct DNA interactions but via another transcription factor. This is not without precedent as enolase (MBP1) interacts with YY1 on the c-*myc* promoter in the Notch signaling pathway (38). Together, these data imply that expression of consensus and nonconsensus STAT3 target genes may change inversely in response to oxidative stress.

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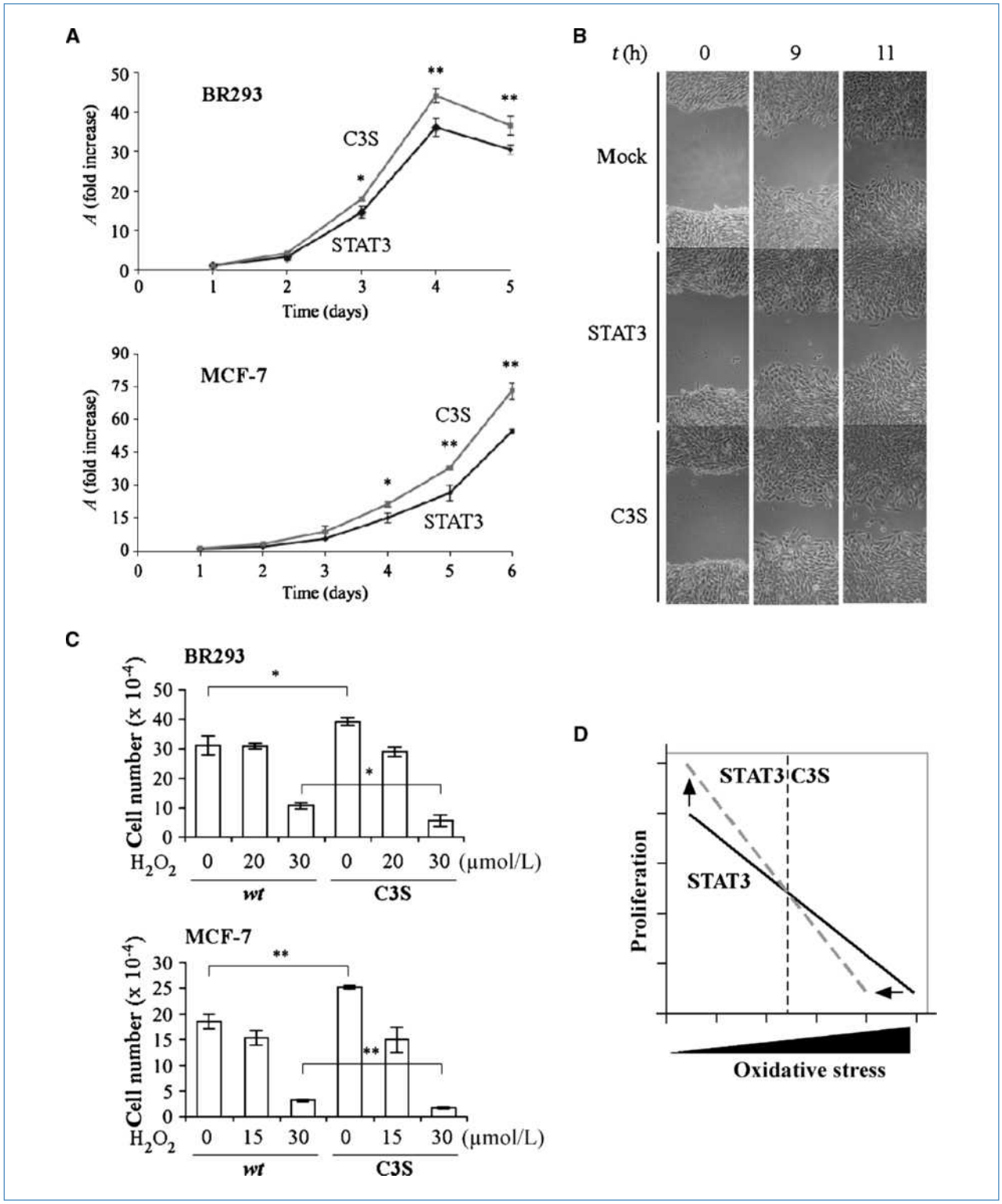

Figure 6. A redox-insensitive STAT3 alters BC cell proliferation and resistance to oxidative stress. A, BR293 and MCF-7 cells transfected with expression vectors for STAT3 or STAT3 C3S were seeded in 96-well plates and cultured for times indicated. Cell proliferation was measured by MTT assay. B, BR293 monolayers were scored, and regrowth was monitored over time by light microscopy. Images are representative of three independent experiments. C, BR293 and MCF-7 cells transfected with vectors for STAT3 or STAT3 C3S were cultured in the absence of peroxide or daily administration at concentrations indicated. Data represent average values ($n = 4$) from one of three comparable experiments. Bars, SD. **, $P < 0.01$; *, $P < 0.05$. D, diagram indicating effect of STAT3 C3S versus STAT3 expression on relationship between oxidative stress and BC cell proliferation.

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Redox-dependent formation of covalently linked STAT3 tetramers

At 180 kDa, the smallest STAT3 redox complex migrates as a dimer. Formation involves C712/C718 within and close to the phosphotyrosine loop, respectively, suggesting that the complex is formed by their oxidation when juxtaposed in the parallel conformation. The two larger complexes have apparent molecular weights of approximately 360 and 270 kDa (Supplementary Fig. S6), and from MS analyses, they seem to contain only STAT3 (data not shown). Formation of the 360/270-kDa complexes in cells requires C418 and C426 or C468 and C765, indicative of an interaction between DBD and C-TAD in a multimeric arrangement. The requirement for different subsets of cysteines indicates that 180- and 360/270-kDa redox complexes reflect oxidation of at least two distinct STAT3 conformations.

The simplest explanation for the two larger redox complexes is that they represent STAT3 redox trimers and tetramers. Structures of inactive STAT1 and STAT5α dimers revealed antiparallel alignment of the core domains forming a shallow W with the DBDs exposed at the lower apices, albeit with the βe-βf loops (carrying C418 and C426) unresolved (39, 40). Interestingly, the capacity of STAT1 N-domains to dimerize contributes to formation of inactive STAT1 tetramers involving the upper face of the dimer (39), but STAT3 N-domains have a significantly lower propensity for dimerization (41). Conceivably, interactions between DBD and C-TAD could direct the formation of analogous STAT3 tetramers. Interactions between DBDs and TADs are known to occur in other transcription factors, for example, in the inactive conformation of SMADs, which is released on phosphorylation of an SSxS motif in the C-TAD (42). Formation of three interchain disulfides, each between a C-TAD (C765) and a juxtaposed DBD (C418/C426/C468) would suffice to link four protomers, whereas two disulfides could link a trimer.

STAT3 and redox control of cell proliferation

Although links between tumor cell growth and elevated ROS production are well established, the positive effect of low peroxide levels on cell survival and proliferation is less widely appreciated. Given that high oxidative stress induces temporary senescence or even apoptosis, this implies that cells maintain intracellular ROS levels within a concentration window, and that in tumor cells this window may be shifted or expanded, which may accompany the switch from oxidative phosphorylation to glycolysis. Intriguingly, STAT3 inhibition can be achieved by lowering (via antioxidants) or raising sufficiently the intracellular oxidative potential (19). Moreover, inhibition of STAT3 by other means has been shown to induce apoptosis (43, 44). This correlation implies that the adverse effects of peroxide are manifested when STAT3 activity is compromised. Although ROS impinge on signaling cascades that could affect STAT3 indirectly, the present findings suggest a direct mechanism whereby elevated peroxide inhibits STAT3 DNA binding, leading to downregulation of cell cycle and prosurvival genes such as *cyclin D1, Bcl-2, Bcl-xL*, and *survivin* (11).

STAT3 is also associated with mitochondria, a potent source of peroxide (23, 45). Thus, mitochondria could play a role in modulating STAT3 target gene expression. However, the ability of tyrosine phosphorylation–defective STAT3 mutants to upregulate oxidative phosphorylation implies that cytokine-induced gene expression plays no part in this novel STAT3 function. Conceivably, STAT3 plays a dual role in intracellular ROS homeostasis, allowing peroxide-dependent modulation of target genes and participating in a feedback mechanism to control mitochondrial respiration.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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