

# Scaffold Attachment Factor SAFB1 Suppresses Estrogen Receptor $\alpha$ -Mediated Transcription in Part via Interaction with Nuclear Receptor Corepressor

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Activity of the estrogen receptor (ER) is regulated through interaction with coactivators and corepressors. These proteins are present in large complexes, suggesting functional interactions among various cofactors. Scaffold attachment factors B1 and B2 (SAFB1/2) and nuclear receptor corepressor (N-CoR) function as ER $\alpha$  corepressors—they directly interact with ER $\alpha$ , and repress transcription via repression domains. We asked the question whether SAFB1/2 and N-CoR could directly interact with each other, and whether this interaction results in altered repressive activities. Employing coimmunoprecipitation, cofractionation, and colocalization experiments, we have shown that SAFB1/2 interact with the nuclear receptor corepressor N-CoR. This interaction was direct, and was mediated *in vitro* and *in vivo* through the C-terminal region of SAFB1 (amino acids 600–915 and the N-terminal region of N-CoR (amino acids 1–373). Decrease of SAFB1 or N-CoR expression by small interfering RNA resulted in an increase of the estrogen response in reporter assays, confirming prior data that both proteins are attenuating

estrogen-mediated induction of genes. Importantly, the effect of SAFB1 on this attenuation was significantly decreased in the presence of N-CoR small interfering RNA. Using chromatin immunoprecipitation assays, we observed that SAFB1/2 and N-CoR were recruited to the pS2 promoter in the absence of estrogen, and this recruitment was enhanced in the presence of Tamoxifen. Detailed kinetic studies showed that the addition of estrogen resulted in the concurrent release of SAFB1/2 and N-CoR from the promoter. Finally, we measured expression of SAFB1/2 and N-CoR in 289 clinical breast cancer specimens, and detected a strong and highly significant correlation between their expression levels. Taken together, our studies demonstrate that SAFB1/2 and N-CoR interact, and that this interaction is, at least in part, necessary for SAFB1's repressive activities. The coexpression of these proteins in breast cancer specimens, and the combined recruitment (and release) of SAFB1/2 and N-CoR furthermore suggests that this interaction has functional relevance. (*Molecular Endocrinology* 20: 311–320, 2006)

**E**STROGEN PLAYS a key role in growth and development of a number of organs. Estrogen mediates its effect through binding to estrogen receptor (ER), and subsequently altering transcription (1). ER $\alpha$ -mediated transcription has been an area of extensive study for many years, and it has become clear that it is very complex. For example, there are two ERs, termed

ER $\alpha$  and ER $\beta$ , and there is evidence that both receptors can function not only by directly affecting transcription but also through modulating signaling pathways in the cytoplasm. Furthermore, ER $\alpha$  activity can be regulated through interaction with a multitude of coregulatory factors, termed coactivators and corepressors (2).

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Abbreviations: aa, Amino acids; AD, activation domain; AIB1, amplified in breast cancer 1; ChIP, chromatin immunoprecipitation; CSS, charcoal-stripped serum; 3D, three-dimensional; E2, estradiol; ER, estrogen receptor; ERE, estrogen response element; GST, glutathione-S-transferase; HDAC, histone deacetylase; N-CoR, nuclear receptor corepressor; NF $\kappa$ B, nuclear factor  $\kappa$ B; SAFB1/2, Scaffold attachment factors B1 and B2; SDS, sodium dodecyl sulfate; siRNA, small interfering RNA; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; Src, steroid receptor coactivator; Tam, 4-hydroxytamoxifen; Tk, thymidine kinase.

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Coactivators interact with ER $\alpha$  to increase target gene transcription. A number of coactivator complexes contain histone acetyltransferase and/or methyltransferase activities, suggesting that the modification of histone and thus chromatin reorganization are crucial for activation. Other mechanisms of coactivators include the direct interaction with members of the basal transcription machinery, effects on protein degradation, and others (3).

In contrast to coactivators, little is known about the nature and role of ER $\alpha$  corepressor complexes. There is increasing evidence that corepressors are involved in antiestrogen-mediated inhibition of ER $\alpha$ , in the fine-tuning of the estrogen response, in repression of unliganded receptor, and in estrogen-mediated down-

regulation of target genes (4). Just like coactivation, corepression seems to involve changes in chromatin organization. For example, the corepressors receptor-interacting protein 140 (RIP140) (5), ligand-dependent corepressor (LCoR) (6), metastasis-associated protein 1 (MTA1) (7), and testicular receptor 2 (TR2) (8) mediate repression, at least in part, through recruitment of histone deacetylases (HDACs). Similarly, the best characterized corepressors, N-CoR and silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), interact with a number of HDACs to repress transcription (9). A series of elegant studies (10–12) have shown that N-CoR/SMRT primarily exist in large complexes containing HDAC3, TBL1/TBLR1, and other less well-characterized proteins.

Our laboratory has identified the scaffold attachment factors SAFB1 as a transcriptional repressor (13). SAFB1 directly interacts with ER $\alpha$  in the DBD-Hinge domain. It can bind to ER $\alpha$  in the absence or presence of estrogen, and the interaction is enhanced in the presence of antiestrogen. Overexpression of SAFB1 strongly inhibited ER $\alpha$ -mediated transcription in cultured cell lines, whereas deletion of SAFB1 in mouse embryo fibroblasts resulted in increased ER $\alpha$  activity (14). Recently, we have identified a domain in the SAFB1 C terminus that, when transferred to a heterologous DNA-binding protein, can confer strong repressive activity (15). Interestingly, SAFB1-mediated repression was partially relieved by treatment with HDAC inhibitors (16); however, a yeast two-hybrid assay using the C-terminal repression domain did not reveal any direct interaction with HDACs. Instead, we discovered an interaction with TAFII68, suggesting a direct interaction with the basal transcription machinery (15). More recently, we have identified a second family member, termed SAFB2 (17). The two proteins share a high degree of homology (74%), and, although limited, our studies have indicated that the repression functions might be redundant in SAFB1 and SAFB2.

Considering that N-CoR has been shown to function in large protein complexes, that SAFB1/2 might interact with HDAC complexes, and that a number of cofactors have been found to interact, we asked the question whether SAFB1/2 would directly interact with N-CoR. Using a variety of different approaches, we here define a novel and direct interaction between SAFB1/2 and N-CoR, and show that this interaction is, at least in part, necessary for SAFB1-mediated transcriptional repression.

## RESULTS

### SAFB1/2 Proteins Interact and Show Partial Colocalization with N-CoR

To test whether SAFB1/2 proteins interact with N-CoR, we performed coimmunoprecipitation assays using MCF-7 breast cancer cell extracts. For the SAFB1/2 immunoprecipitation we used a polyclonal

pan-SAFB1/2-antibody that detected both SAFB1 and the closely related SAFB2 protein. Because the full-length SAFB1 and SAFB2 proteins are indistinguishable in size on an SDS-PAGE, the detected band reflects the combined expression levels of SAFB1 and SAFB2. We were able to detect a weak but reproducible interaction between SAFB1/2 and N-CoR (data not shown), which was significantly enhanced when cells were exposed to a cross-linking reagent before coimmunoprecipitation (Fig. 1A).

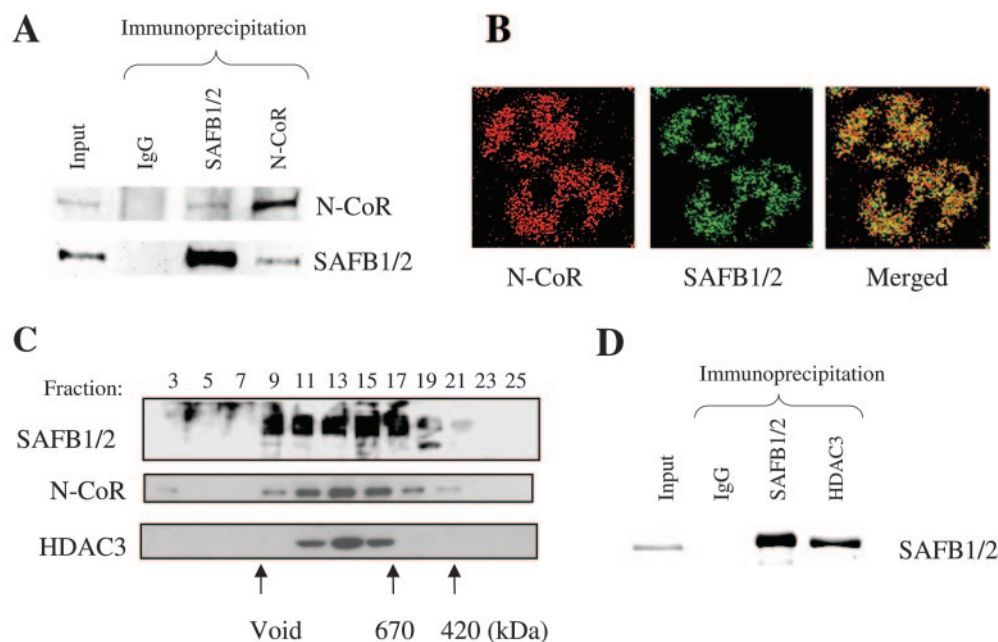
To confirm this interaction, we analyzed a potential colocalization of SAFB1/2 and N-CoR endogenous proteins in MCF-7 cells. Cells were fixed, stained with antibodies for N-CoR and SAFB1/2, and analyzed by confocal microscopy followed by three-dimensional (3D) deconvolution (Fig. 1B). In agreement with previous studies (18, 19), punctate nuclear staining patterns (except in the nucleoli) were observed. The merged image indicated a significant (but not complete) colocalization as reflected by the yellow color.

We have previously used gel filtration analysis to examine the presence of other proteins (HDAC3, TBL, and TBLR1) in the N-CoR complex (10). To further test the interaction between SAFB1/2 and N-CoR, we examined whether they cofractionated in gel filtration experiments. Therefore, HeLa cell nuclear extract was fractionated with a Superose 6 gel filtration column, fractions were collected, and analyzed by immunoblotting. As shown in Fig. 1C, SAFB1/2 was detected in fractions 9–17. N-CoR was also eluted in fractions 9–17, and HDAC3 in fractions 11–15. Thus, cofractionation of SAFB1/2 with the N-CoR/HDAC3 complex further suggests a possible functional connection between these proteins.

We also tested whether SAFB1 would interact with HDAC3, as one would expect based on SAFB1—N-CoR and previously described N-CoR—HDAC3 (10, 12) interactions, as well as on the cofractionation results. Therefore, we performed coimmunoprecipitation in MCF-7 cells and detected interaction between SAFB1 and HDAC3 (Fig. 1D). Collectively, these data suggested that SAFB1/2 and N-CoR are interacting partners.

### Direct Interaction between SAFB1 C-Terminal Repression Domain and N-CoR Repression Domain RD1 in *in Vitro* and *in Vivo* Systems

To analyze the interaction between SAFB1/2 and N-CoR further, we next examined their interaction *in vitro*. Given the high degree of homology between SAFB1 and SAFB2, we decided to focus our studies on one family member, SAFB1. To test a possible direct interaction between SAFB1 and N-CoR, we performed glutathione-S-transferase (GST)-pull-down assays using *in vitro*-transcribed and -translated N-CoR and four GST-SAFB1 fusion proteins [full-length, amino acids (aa) 1–600, 260–915, and 600–915]. The results in Fig. 2A clearly show that N-CoR directly interacted with the SAFB1 C terminus, which was



**Fig. 1.** SAFB1/2 Associate with N-CoR

A, MCF-7 cell lysate was immunoprecipitated followed by immunoblotting using antibodies as indicated. B, MCF-7 cells were grown on coverslips, fixed, and stained with N-CoR and SAFB1/2 antibodies followed by incubation with fluorescein isothiocyanate-conjugated antimouse and Texas Red-conjugated antigoat IgG, respectively. Images were taken on a Nikon Confocal Eclipse E1000 scanning laser microscope using a  $\times 60$  objective at 0.25- $\mu\text{m}$  steps (Z sections), followed by 3D deconvolution using ImageQuant software, and analysis using Metamorph. The *yellow areas* in the merged picture show areas of SAFB1/2 and N-CoR colocalization. C, HeLa cell nuclear extract was fractionated with a Superose 6 gel filtration column. Fractions were collected, and analyzed by Western blotting using the antibodies as indicated. D, MCF-7 cell lysate was immunoprecipitated followed by immunoblotting using antibodies as indicated.

previously shown to harbor a strong transcriptional repression domain (15).

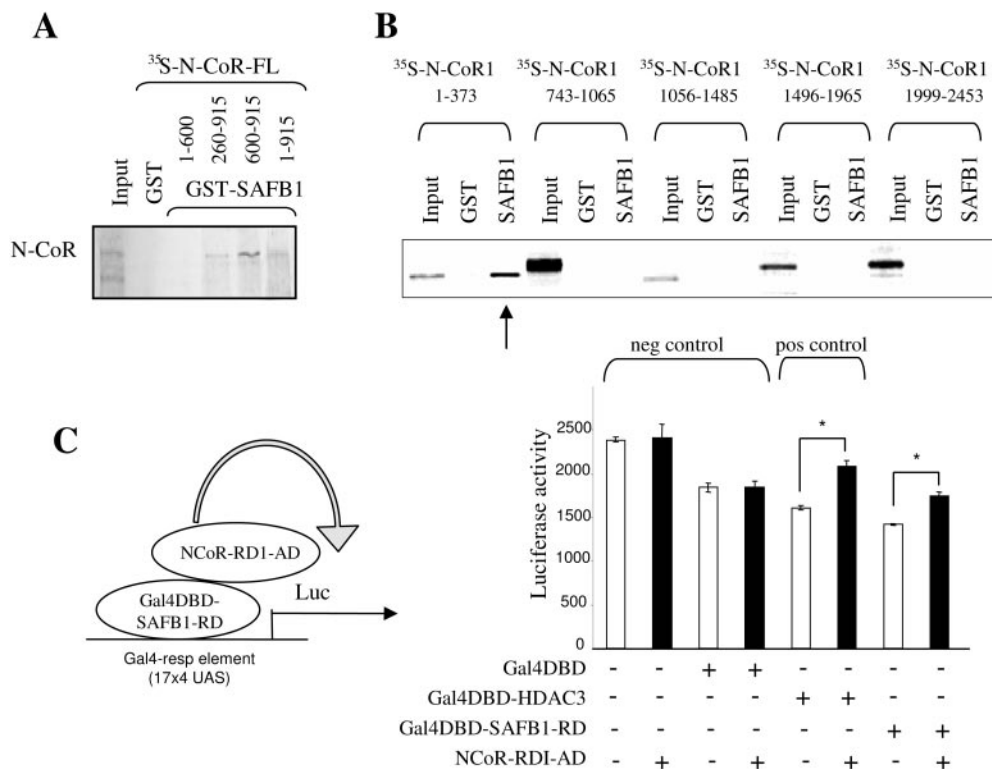
To determine the SAFB1 interaction region(s) in N-CoR, we used N-CoR expression constructs harboring the four independent repression domains (RD1: aa 1–373, RD2: 743–1065, RD3: 1056–1485, and RD4: 1496–1965) and the nuclear receptor-interacting domain (NRID: 1999–2453). As shown in Fig. 2B, SAFB1 strongly interacts with RD1, whereas it failed to interact with any other region in N-CoR. Taken together, SAFB1 directly interacts with N-CoR RD1 through its C-terminal repression domain.

To verify this interaction in an *in vivo* system, we used a mammalian two-hybrid system. In this assay, we used Gal4DBD-SAFB1-RD (600–915 aa), in which the SAFB1 C-terminal repression domain was fused to a Gal4DBD, and N-CoR RD1, fused to the transcriptional activation domain (AD) from nuclear factor  $\kappa\text{B}$  (NF $\kappa\text{B}$ ) (NCoR-RD1-AD). These plasmids were transfected into MCF-7 cells, along with Gal4-thymidine kinase (Tk)-Luc reporter construct that contains four 17-mer Gal4 DNA binding elements (illustrated in Fig. 2C). For a negative control, we transfected NCoR-RD1-AD with the reporter plasmid only, and with the reporter plasmid and Gal4DBD (lanes 1–4). At the concentrations used in our assays, we did not observe a significant effect of NCoR-RD1-AD. For a positive control, we used HDAC3 fused to Gal4DBD because it

has been shown to interact with N-CoR RD1 (10). As expected, cotransfection of NCoR-RD1-AD and Gal4DBD-HDAC3 (lane 6) resulted in a significant increase of activity as compared with Gal4DBD-HDAC3 only (lane 5). The addition of Gal4DBD-SAFB1-RD resulted in a decrease of transcriptional activity (lanes 7), as previously reported (15). Importantly, cotransfection of NCoR-RD1-AD resulted in significant increases of transcriptional activity (lanes 7 and 8), indicating that N-CoR RD1 and the SAFB1 C-terminal repression domain interact *in vivo*, similar to what has been shown for HDAC3 and N-CoR RD1.

#### SAFB1-Mediated Repression Is Diminished in the Presence of N-CoR Small Interfering RNA (siRNA)

Next, we set out to study the functional significance of the SAFB1-N-CoR interaction. First, we wanted to confirm that both proteins were indeed involved in the attenuation of the estrogen induction of genes. Therefore, we used N-CoR and SAFB1 siRNAs, which we showed were efficient in down-regulating N-CoR and SAFB1 expression in MCF-7 cells, respectively (Fig. 3A). Next, we measured estrogen response element (ERE)-Tk-Luc reporter activity in cells in which N-CoR (Fig. 3B, *left panel*) or SAFB1 levels were decreased (Fig. 3B, *right panel*). There was a significant increase in luciferase activity in the presence of estrogen after



**Fig. 2.** Direct Interaction between SAFB1-C-Terminus and N-CoR RD1 *in Vitro* and *in Vivo*

A, Full-length N-CoR was *in vitro* translated and labeled with [<sup>35</sup>S]-cysteine, and incubated in GST-pull-down assays with GST only, and GST-SAFB1 (aa 1–600, 260–915, 600–915, and 1–915). Input was 20% of the sample used for pull-down assays. B, N-CoR deletion mutants were used as indicated, and incubated in pull-down assays with GST only or GST-SAFB1 (aa 260–915). The *pointed arrow* indicates the specific binding of SAFB1 with N-CoR RD1. C, Mammalian two-hybrid assays of SAFB1-C terminus and N-CoR RD1. MCF-7 cells were transfected with Gal4DBD (200 ng), Gal4DBD-SAFB1-C terminus (10 ng), or Gal4DBD-HDAC3 (200 ng) (as positive control) in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of N-CoR-RD1-AD (200 ng) and cell lysates were measured for luciferase reporter activity. \*, Significant differences ( $P < 0.05$ ) (*t* test).

addition of siRNA, providing strong evidence that both genes function in attenuating the cellular estrogen response.

Next, we measured the effect of SAFB1 in the attenuation of the estrogen response in the presence and absence of N-CoR. Therefore, we transfected increasing amounts of SAFB1 along with either nonspecific control or N-CoR siRNA, and measured reporter activity (Fig. 3C). Upon cotransfection of N-CoR siRNA, the SAFB1-mediated repression was not completely abolished; however, it was significantly reduced when compared with control siRNA ( $P < 0.0001$ ; test for interaction). To better illustrate the effect of N-CoR siRNA on the SAFB1-mediated repression, we also presented the data as percentage of control (Fig. 3D). In addition, we tested the effect of N-CoR siRNA on SAFB1's attenuation of the estrogen response in MCF-7 cells containing a reporter construct that is stably integrated into the genome. Similar results were obtained (data not shown), *i.e.* SAFB1's repressive effect was significantly decreased in the presence of N-CoR siRNA.

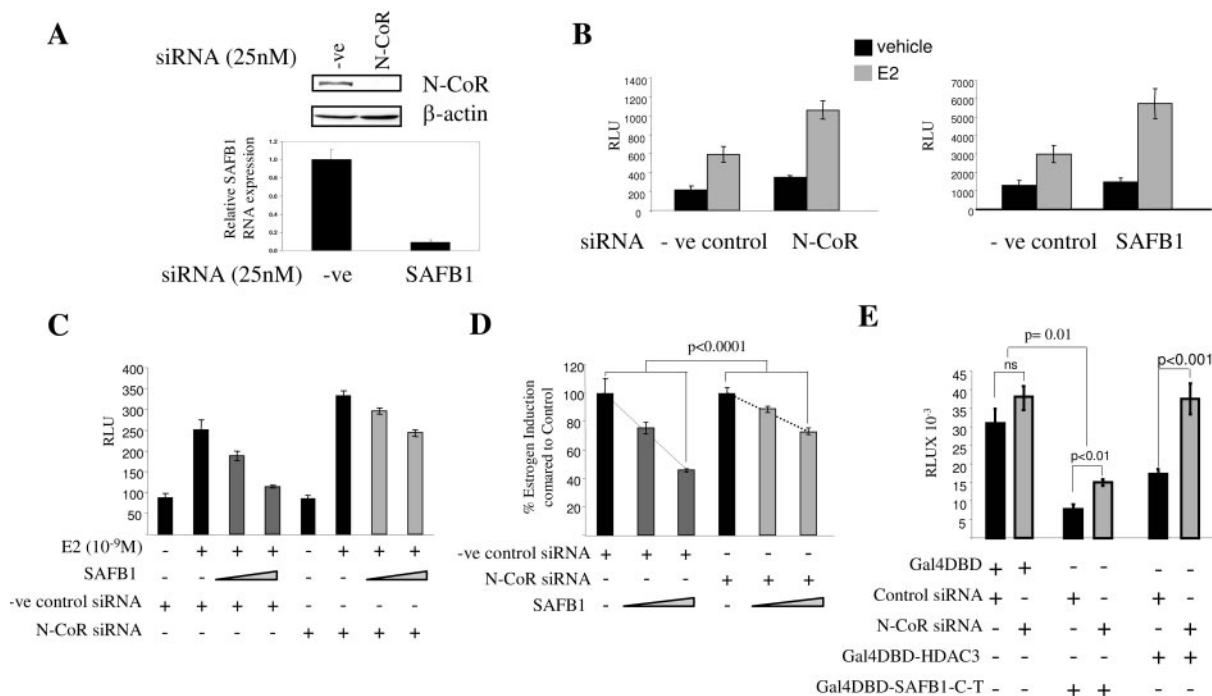
Finally, we performed similar assays in which we tested the effect of N-CoR siRNA on SAFB1 RD fused

to Gal4DBD (Fig. 3E), instead of full-length SAFB1. In contrast to the negative control where the addition of N-CoR siRNA did not result in a significant increase in luciferase activity, SAFB1 RD-mediated repression was significantly decreased. Again, test for interaction showed that there was a significant difference between the effect of N-CoR siRNA in the control group vs. the SAFB1-RD transfected cells. As a positive control, we included Gal4DBD-HDAC3, for which repression was also significantly relieved in the presence of N-CoR siRNA.

Taken together, these results suggest that SAFB1-mediated attenuation of ER $\alpha$ 's transcriptional activity is mediated, at least in part, through N-CoR.

#### Similar Kinetics of SAFB1/2 and N-CoR Release and Recruitment on an Endogenous Estrogen-Responsive Promoter

Next, we wished to determine whether the kinetics of SAFB1/2 on an estrogen target-promoter would mirror that of N-CoR, a finding that would provide further support for our hypothesis that SAFB1/2 and N-CoR can exist in a functional complex. To address this question, we per-



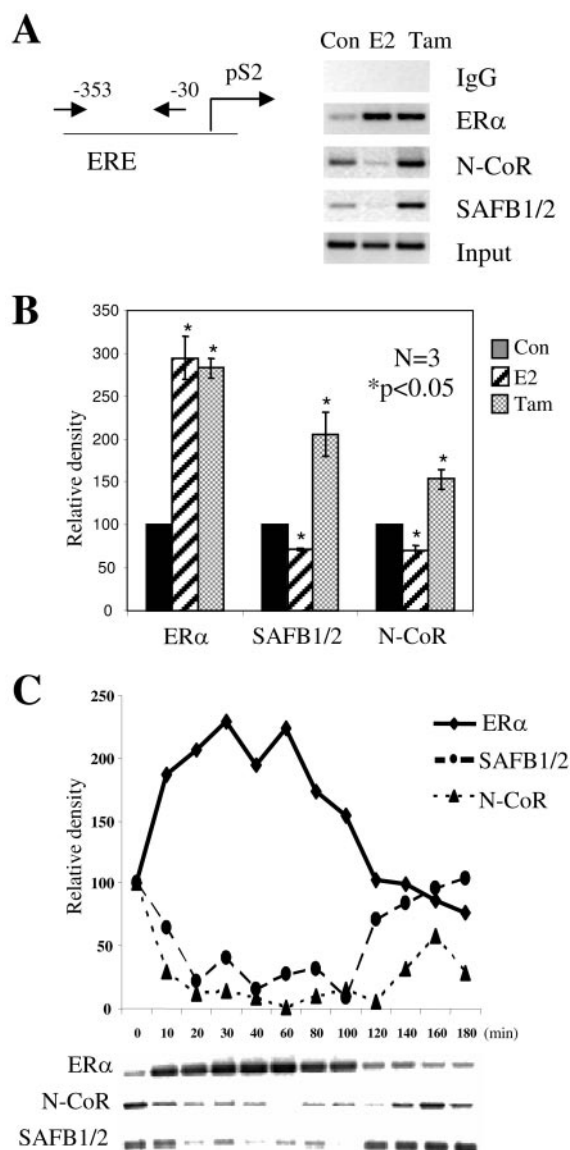
**Fig. 3.** SAFB1 Repression Activity Correlates with N-CoR levels

A, MCF-7 cells were transfected with control or N-CoR siRNA (25 nM) (*top panel*), and lysed 48 h later. SDS-extracts were immunoblotted using N-CoR and  $\beta$ -actin antibodies. Similarly, MCF-7 cells were transfected with control or SAFB1 siRNA, and RNA was isolated 48 h later. Q-PCR was performed using SAFB1-specific primers and probe, and data were analyzed using the  $\Delta\Delta Ct$  method. B, MCF-7 cells were first transfected with N-CoR (or SAFB1 -, *right panel*) or nonspecific siRNA (25 nM), and 2 d later were transfected with ERE-Tk-Luc reporter (100 ng), and  $\beta$ -gal (20 ng) plasmids. Estradiol ( $10^{-9}$  M) was added as indicated, and 24 h later, cells were lysed, and RLU was determined. The *bars* represent mean  $\pm$  SD. C, MCF-7 cells were first transfected with N-CoR siRNA (25 nM) or nonspecific siRNA (25 nM), and 2 d later were transfected with SAFB1 (20 and 50 ng), ERE-MAR-Luc reporter (100 ng), and  $\beta$ -gal (20 ng) plasmids. Ligand was added as indicated, and 24 h later, cells were lysed, and luciferase activity was determined. The *bars* represent mean  $\pm$  SD, and the results are representative of three independent experiments. D, Different graphical presentation of data from experiments shown in panel C. Here, the values are presented as percentage of estrogen induction as compared with the control values to visualize the difference in repression in the absence or presence of specific N-CoR siRNA. An analysis of variance model on log RLU values was performed to examine repression levels across three groups (control, low, and high SAFB1) with and without N-CoR siRNA. The test for interaction was highly significant (the pair-wise tests between the different SAFB1 concentrations were all significant). E, 293 Cells were transfected with N-CoR or nonspecific siRNA (25 nM), and subsequently with 17x4Tk-Luc and Gal4DBD-fusion vectors as indicated. The *bars* represent mean  $\pm$  SD, and the results are representative of three independent experiments. There are significant differences in repression by SAFB1 and HDAC3 with or without N-CoR siRNA (but not in the control group). The test for interaction also shows that the effect of N-CoR siRNA is significantly different between negative control and SAFB1-RD transfection ( $P = 0.01$ ).

formed chromatin immunoprecipitation assays (ChIP) in MCF-7 cells, using the well-defined estrogen-responsive pS2 promoter as a model (Fig. 4A, *left panel*). MCF-7 cells were exposed to vehicle (control), estradiol (E2), or 4-hydroxytamoxifen (Tam) for 45 min, and chromatin was isolated after chemical cross-linking. After fragmentation by sonication, and immunoprecipitation using control IgG, ER $\alpha$ , N-CoR, or SAFB1/2 antibodies, DNA was purified, and amplified using primers flanking the estrogen-responsive sites (Fig. 4A). Figure 4A (*right panel*) shows a representative ChIP assay, in which E2 treatment resulted in recruitment of ER $\alpha$ , and release of N-CoR. In agreement with previously reported data (21, 22), Tam treatment resulted in recruitment of ER $\alpha$  and N-CoR. SAFB1/2 showed similar behavior as N-CoR, *i.e.* E2 treatment resulted in release, and Tam treatment in recruitment of SAFB1/2.

Three independent assays yielded similar data, which are graphically presented in Fig. 4B.

We next investigated the time course of SAFB1/2 and N-CoR corepressor release from the promoter after E2 treatment. As shown in Fig. 4C, SAFB1/2 and N-CoR were released at the earliest time point studied (10 min), and were recruited back onto the promoter 120–140 min later. As expected, ER $\alpha$  recruitment was seen within 10 min of E2 addition, and a decline in ER $\alpha$  recruitment mirrored the increased recruitment of the corepressors starting at 120 min. We did observe a cycling at later time points where ER $\alpha$  reassociated with the promoter (between 4 h and 5 h) (data not shown). Interestingly, in the presence of Tam, we did not see any release and reassociation of ER $\alpha$  after the initial promoter recruitment (data not shown). Most



**Fig. 4.** Coordinated Recruitment and Release of SAFB1/2 and N-CoR at the pS2 Promoter

A, MCF-7 cells, which were kept in 5% CSS for at least 3 d, were treated with vehicle [control (Con)],  $10^{-8}$  M E2, or  $10^{-6}$  M 4-hydroxytamoxifen (Tam) for 45 min, and subjected to ChIP assays using antibodies as indicated. For the PCR analysis, we used primers in the pS2 promoters that amplify a region containing the ERE. The results are representative of three experiments. B, The values, expressed as percentage of density relative to vehicle-only treatment, represent the mean  $\pm$  SD of three separate experiments. C, Kinetic ChIP experiments were performed as described in panel A except that cells were only treated with E2 ( $10^{-8}$  M). The experiment is representative of three experiments.

importantly for our question, release and subsequent recruitment of SAFB1/2 to the promoter coincided that of N-CoR, again supporting our hypothesis that at least a fraction of these proteins can coexist in a complex.

### Strong Correlation between SAFB1/2 and N-CoR Protein Expression in Human Breast Tumors

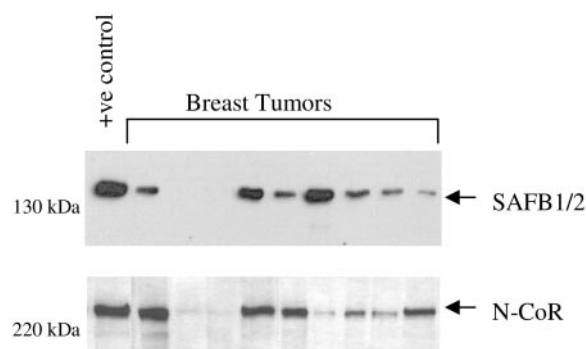
Finally, we wished to extend our findings from breast cancer cells in tissue culture to clinical specimens. We asked the question whether the corepressors are co-expressed, *i.e.* tumors with low SAFB1/2 levels express only low N-CoR levels, and *vice versa*. To address this question, we measured SAFB1/2 and N-CoR protein levels in 289 tumors from breast cancer patients. The patient and tumor characteristic for the entire study population is displayed in Table 1. For this set of clinical specimens, we also had data available for expression of ER $\alpha$ , progesterone receptor, steroid receptor coactivator (Src)-3/amplified in breast cancer 1 (AIB1), human epidermal growth factor receptor 2, B-cell leukemia/lymphoma 2, p53, which have been described elsewhere (23).

Expression of N-CoR and SAFB1/2 in different tumors varied widely; representative blots are shown in Fig. 5. After normalizing band intensity, using the MCF-7 reference standard, SAFB1/2 expression ranged from 0 (completely absent) to a maximum of 3.02, with a mean score of 0.48. For N-CoR, expres-

**Table 1.** Patient and Tumor Characteristics

Variable	All Patients	
	n	%
Age		
$\geq 50$ yr	249	85
<50 yr	40	15
Nodes		
1–3	161	56
>3	127	44
Missing	1	
Tumor size		
$\leq 2$ cm	68	23
2–5 cm	169	59
$\geq 5$ cm	51	18
Missing	1	
S-phase fraction		
Low (6%)	65	22
Intermediate (6–10%)	97	34
High (>10%)	122	44
Missing	5	
ER		
Positive	257	88
Negative	32	12
PgR		
Positive	170	60
Negative	107	40
Missing	12	
Ploidy		
Diploid	102	36
Aneuploid	183	64
Ploidy	4	
Therapy		
None	117	40
Endo	172	60

PgR, Progesterone receptor.



**Fig. 5.** Wide-Ranging Expression Levels of SAFB1/2 and N-CoR in Breast Tumors

Representative blots are shown that were immunoblotted with SAFB1/2 (*top panel*) or N-CoR antibodies (*bottom panel*). In the *left lane*, MCF-7 cell extracts was loaded as a reference standard.

sion ranged from 0–3.0, with a mean score of 1.87. Interestingly, we found that there was a strong and significant correlation between SAFB1/2 and N-CoR expression (Table 2). The Spearman correlation coefficient was 0.39, with a  $P$  value of  $P < 0.0001$ . This correlation was stronger than that with any other marker measured in this data set. It is of note that we also detected significant correlations between SAFB1/2 and SRC-3/AIB1 and ER $\alpha$ . Such associations among cofactors and with ER $\alpha$  expression levels have previously been described (24). In summary, in human breast cancer specimens, SAFB1/2 and N-CoR levels were highly correlated, providing *in vivo* evidence that these proteins could indeed function in a complex.

## DISCUSSION

We present several lines of evidence that SAFB1/2 and N-CoR corepressors physically and functionally

interact. We have used multiple approaches to confirm interaction between the proteins and have shown functional significance of the interaction. First, SAFB1/2 and N-CoR can be coimmunoprecipitated, cofractionated using gel filtration analysis, and the proteins partially colocalize in the nucleus. Secondly, we detected direct interactions between the SAFB1 C terminus and N-CoR N terminus, which was confirmed *in vivo*. Thirdly, decrease of N-CoR via siRNA resulted in partial loss of SAFB1-mediated repression. In addition, the kinetics of recruitment and release of SAFB1/2 and N-CoR on the pS2 promoter coincided. Finally, we have shown that SAFB1/2 and N-CoR expression are highly correlated in clinical breast cancer specimens, providing a correlative basis for possible interactions *in vivo*.

A number of studies have shown that nuclear receptor cofactors exist in large multiprotein complexes. For example, N-CoR and SMRT complexes were estimated to have a size of 1.5–2 MDa, containing HDAC3, TBL1, and the related TBLR1 (10–12). More recently, N-CoR was shown to interact with the coactivator SRC-3/AIB1 (25), a finding that also suggests a close link between transcriptional repression and activation. We show here that SAFB1/2 proteins can also interact with N-CoR. Although it might seem obvious, we would like to stress that this interaction is only partial, *i.e.* our data suggest that only a fraction of the total SAFB1/2 and N-CoR molecules in the cell interact. This is evident, for example, in the partial colocalization and the relatively weak coimmunoprecipitations. It is feasible that the complex containing SAFB1/2–N-CoR proteins is only formed on a subset of genes, which could be determined by a number of factors including the promoter sequence, and the availability of other cofactors in the cell. We predict that there will be promoters that are affected by N-CoR without any involvement of SAFB1/2, and *vice versa*. Our data also suggest that SAFB1 not only interacts with N-CoR but that there are clearly other interacting proteins that can mediate repression by SAFB1. This becomes most obvious in the experiments in which N-CoR siRNA results in partial but not complete loss of SAFB1-mediated repression.

One question that arises is why it would be necessary to have more than one corepressor in a repressive complex? The existence of redundant functions is a common phenomenon in biological processes. One reason for redundancy is to prevent errors and to ensure that the final goal is achieved, possibly using various closely related strategies. In the case of recruitment of SAFB1/2 and N-CoR, we believe that this would give the cell the advantage of controlling transcription at multiple levels. N-CoR recruits HDAC complexes, whereas SAFB1/2 directly interacts with members of the basal transcription machinery. As a result, a more robust repression can be achieved, securing the inhibition of transcription. Alternatively, the presence of a number of corepressors in a repressive complex would allow for responses to different

**Table 2.** Patient and Tumor Characteristics

Variable	SAFB Correlation Coefficient	$P$ Value
N-CoR	0.39	<0.0001
SRC-3	0.28	<0.0001
ER	0.19	0.0006
Her-2	0.16	0.004
Bcl-2	0.12	0.04
Tumor Size	–0.11	0.06
S-Phase	0.07	0.23
P53	–0.06	0.29
Age	0.04	0.45
PgR	0.04	0.55
Nodes	–0.004	0.95

PgR, Progesterone receptor.

intra- and extracellular signals. For example, post-translational modifications in SAFB1/2 and N-CoR might be activated by different pathways, providing the flexibility to respond to various signals.

The interaction between SAFB1 and N-CoR maps to the C terminus in SAFB1 (aa 599–915), and the N terminus in N-CoR (aa 1–373). In detailed structure-function studies, we have previously identified a transferable and receptor-independent repression domain in the SAFB1/2 C terminus (15). Using the C terminus in yeast-two hybrid assays, we have identified an interaction with TAFII68, suggesting a direct interaction with the basal transcription machinery. However, in these assays, we failed to detect a direct interaction with members from the chromatin remodeling complex. Because SAFB1-mediated repression was partially released using HDAC inhibitors (trichostatin A) (16), we were somewhat surprised by the lack of interactions with HDACs. Our findings described here provide explanations for the trichostatin A-mediated relief of repression. We provide evidence that there is an indirect association of SAFB1/2 with HDAC3 through the interaction with N-CoR. Interestingly, the interaction domain in N-CoR for HDAC3 differs from that for SAFB1/2; thus, these interactions are not mutually exclusive, allowing simultaneous binding of both HDAC3 and SAFB1/2 to N-CoR.

As part of our studies we employed ChIP technology to study the dynamics of SAFB1/2, N-CoR, and ER $\alpha$  on a well-defined estrogen-responsive promoter. As previously described, Tamoxifen treatment resulted in the recruitment of both N-CoR and ER $\alpha$  (21, 22). As expected, we observed similar behavior in SAFB1/2, *i.e.* Tam treatment resulted in its recruitment. We did detect some binding of the corepressors in the absence of ligand (control), which decreased after the addition of estradiol. The release of SAFB1/2 and N-CoR precisely coincided, further supporting the idea that they interact, and that they behave in a very similar manner. It should be added that the release of corepressors as a prerequisite for ER $\alpha$  activation has not been previously suggested [as was done for other receptors such as retinoic acid receptor  $\alpha$  (26)] but might be a common mechanism that deserves further investigation. Although not analyzed in more detail in our study, we did see that both SAFB1/2 and N-CoR reassociated with the promoter after the original release. There are a number of studies showing that not only ER $\alpha$  but also its cofactors undergo dynamic cycling at the promoter (21, 27). Although the exact reasons are still under intense investigations, one hypothesis is that the constant promoter clearance (and reassociations) allows for efficient sensing of altered ligand concentration.

Finally, we have shown here that SAFB1/2 and N-CoR expression levels are highly correlated in primary breast cancers, *i.e.* breast tumors that express high levels of SAFB1/2 are likely to express high levels of N-CoR (and *vice versa*). Interestingly, coexpression between a series of coactivators has

been previously reported (24), but this is the first report showing that there is also a positive correlation between different corepressor expression levels. Whereas this finding only shows an association between SAFB1/2 and N-CoR levels, their coexpression lends support to the idea that they may function cooperatively.

## MATERIALS AND METHODS

### Plasmid Constructions

The generation of SAFB1 and N-CoR full-length and truncated constructs has been previously described in detail (13, 15, 17, 28). To clone an estrogen-inducible *Xenopus* vitellogenin A2 (XVA2) promoter that contains not only a consensus ERE but also nearby sequences that might be involved in estrogen regulation, we amplified *Xenopus* testis DNA by PCR using the forward primer: 5'-AGTTTTCTGGATAACG-GATTTCGG-3' and the reverse primer with an inserted *Nco*I digest site: 5'-GGACGATCCCTCCATGGTGATGG-3'. These primers amplify a 1049-bp region covering the ERE (–319/–331 bp), scaffold/matrix attachment regions (S/MARs) (–81/–571 bp), and the TATA box (–26/–31 bp). The PCR product was cut with *Bam*HI and *Nco*I, and inserted into pGL3 basic to generate ERE-MAR-Luc-reporter vector.

To construct N-CoR-RD1-NF $\kappa$ B-AD, we amplified N-CoR RD I (aa 1–373) using the forward primer 5'-GGCGAATTCATGTCGAAGTTCAGGTTATCCTCCC-3', and the reverse primer 5'-CCCCTCGAGCTAAATGGTGGCTGAAAGACCAGC-3'. The PCR product was digested with *Eco*RI and *Xho*I, and inserted into pCMV-AD (Stratagene, La Jolla, CA), which contains the NF $\kappa$ B AD. All plasmids were verified by sequencing.

### Mammalian Cell Transfection

MCF-7 cells were maintained in DMEM, 5% fetal bovine serum, and antibiotics in 5% CO<sub>2</sub>. MCF7-XVA2-Luc stable cell lines were generated by cotransfection of ERE-MAR-Luc and pSV-Neomycin, and selection with antibiotics for three weeks. Positive clones were tested for estrogen induction, and clone no. 11 showed approximately 30- to 35-fold estrogen induction was used for the experiments described in this study. To measure SAFB1-mediated effects on ERE-MAR-luciferase activity in the stable clone, the cells were transfected with a EGFP-tagged SAFB1 expression construct (17), and fluorescent cells were isolated by fluorescence-activated cell sorting using a Beckman Coulter (Miami, FL) Altra before measuring luciferase activity.

For transient transfections, cells were plated in six-well plates at a density of  $2\sim 4 \times 10^5$ /well in phenol red-free DMEM with 5% charcoal-stripped serum (CSS), incubated for 2 d, and transfected using lipofectamine. Reporter constructs used are ERE-Tk-luciferase, ERE-MAR-luciferase, or 14x4UAS (Gal4-responsive element)-luciferase. The luciferase activities were measured using the luciferase assay kit from Promega (Madison, WI), and normalized to  $\beta$ -galactosidase activity.

### siRNA Experiments

For the siRNA experiments, we transfected cells with N-CoR (28) or SAFB1 (Dharmacon, Lafayette, CO; M-005150-00) siRNA. For a negative control, we used commercially available nonspecific siRNA control duplex X. All siRNAs were purchased from Dharmacon. For SAFB1, efficiency of siRNA was measured by Q-PCR using an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) and

specific primers and FAM-BHQ1-labeled taqman probes [SAFB1; forward (F): 5'-AGA AGC GGT CCG TCG TGT C-3', reverse (R): 5'-TTC ACG CAC CCT GCT ATG G-3' and probe (P): 5'-AAG GTC AAG GAG CCT CGG AAG TCA AGA GA-3';  $\beta$ -actin: F: 5'-CCC TGG CAC CCA GCAC-3', R: 5'-GCC GAT CCA CAC GGA GTA C-3' and P: 5'-ATC AAG ATC ATT GCT CCT CCT GAG CGC-3'].

### Analysis of Tumor Specimens

The tumor specimens have been described previously (23). Briefly, 289 breast cancer specimens from patients who had positive axillary lymph nodes at the time of initial surgery were analyzed. The studies were approved by the Baylor College of Medicine Institutional Review Board. For each sample, we run 25  $\mu$ g of sodium dodecyl sulfate (SDS) protein extract on a gel, along side with MCF-7 human breast cancer cell extracts as a standard to correct for gel-to-gel variations in band intensity. Protein bands were visualized on a Fluorchem digital imaging system (Alpha Innotech Corp., San Leandro, CA) using an enhanced chemiluminescence detection system. Band intensities were measured densitometrically using AlphaEase FC software (Alpha Innotech Corp.).

### GST-Pull-Down Assays

pCMX-N-CoR full-length or pCMX-N-CoR I to V domains (28) were labeled with [<sup>35</sup>S]-cysteine using the TNT T7 coupled transcription/translation kit from Promega. GST-SAFB1 fusion proteins (aa 1–600, 260–915, 600–915, and 1–195), which have been described previously (15), were expressed in *E. coli* BL21 strain, and affinity purified using Glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. The binding assays were performed following standard procedures, as described previously (17).

### Immunoblotting, Immunoprecipitation, and ChIP Assay

SDS lysates from MCF-7 cells were separated on a 6% SDS-PAGE gel, and immunoblotted as described (17). Antibodies against N-CoR (C-20, sc1609) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), SAFB1/2 (Upstate Biotechnology, Lake Placid, NY), HDAC3 (Upstate) and  $\beta$ -actin (Sigma) were used at dilutions of 1:1000, 1:1000, and 1:4000, respectively. For the tumor study, we used a rabbit anti-N-CoR antiserum (raised against human NCoR, aa 2239–2653), kindly provided by R. M. Lavinsky and M. G. Rosenfeld (University of California, San Diego, CA). To detect interactions of endogenous proteins, MCF-7 cells were lysed in NETN [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 0.1 mM PMSF], and immunoprecipitations were performed as described (13, 17). In some experiments, cells were exposed for 10 min to a cross-linking reagent (1% formaldehyde) before coimmunoprecipitations.

The ChIP assays were essentially performed as previously described (22, 29). Briefly, MCF-7 cells ( $3 \times 10^6$ ) were plated in 15-cm dishes in phenol red-free DMEM supplemented with 10% CSS and incubated for at least 3 d. The cells were treated with vehicle only,  $10^{-8}$  M  $E_2$  or  $10^{-6}$  M 4-hydroxytamoxifen for 45 min. After washing the cells with PBS (three times), they were cross-linked with 1% formaldehyde for 10 min at room temperature. Cells were rinsed three times with ice-cold PBS, collected in 100 mM Tris-HCl (pH 9.4)–10 mM dithiothreitol, incubated for 15 min at 30 C, and centrifuged for 5 min at 2000  $\times$  g. Subsequently, the cells were washed sequentially with 1 ml of ice-cold PBS, buffer I [0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES (pH 6.5)] and buffer II [200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 10 mM HEPES (pH 6.5)]. Cells were then resuspended in 0.3 ml of lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1), protease inhibitors], sonicated three times for 10 sec each time, followed by centrifugation for 10 min. Supernatants

were diluted in 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl (pH 8.1), and 250  $\mu$ g were precleared with 2  $\mu$ g of sheared salmon sperm DNA and protein G-Sepharose (40  $\mu$ l of 50% slurry) for 2 h at 4 C. Immunoprecipitations were performed overnight at 4 C with ER (H-184, Santa Cruz), N-CoR (C-20, sc1609, Santa Cruz), SAFB1/2 (Upstate Biotechnology), or rabbit IgG control (Santa Cruz) antibodies (2  $\mu$ g protein/each). Subsequently, 50  $\mu$ l of protein G-Sepharose and 2  $\mu$ g of salmon sperm DNA were added, and the incubation was continued for 1 h. Precipitates were washed sequentially, each in TSE I [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 150 mM NaCl], TSE II [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.1), and 500 mM NaCl], and buffer III [0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris-HCl (pH 8.1)]. Precipitates were then washed three times with TE buffer [10 mM Tris (pH 8) 1 mM EDTA], extracted with 1% SDS–0.1 M NaHCO<sub>3</sub>, and heated at 65 C for at least 6 h to reverse the formaldehyde cross-linking. After DNA purification (QIAquick Spin kit; QIAGEN, Germantown, MD), the proximal pS2 promoter was amplified (28 cycles) using the forward primer, 5'-GGCCATCTCTCACTATGAATCACTTCTGCAG-3' (–353/–333), and the reverse primer 5'-GGCAGGCTCTGTTTGCTTAAAGAGCGTTAGATA-3' (–63/–30).

### Purification of Protein Complexes from HeLa Nuclear Extract

HeLa nuclear extracts were prepared as described previously (30). Gel filtration analysis of HeLa nuclear extracts was performed essentially as described (20). Proteins in each fraction were bound to glass beads (Stratagene) and then separated by a 7.5% SDS-PAGE and analyzed by Western blotting using different antibodies as indicated.

### Immunofluorescent Staining and Confocal Microscopy

MCF-7 cells grown on coverslips were washed with cold PBS, fixed with 4% formaldehyde for 10 min at room temperature, washed twice with PBS, permeabilized with 1% Triton in PBS for 10 min, and again washed twice with PBS. After blocking with 3% BSA for 30 min, control IgG (Santa Cruz SC-2025), SAFB1/2 (Upstate), and N-CoR (C-20) (Santa Cruz) antibodies were added at a 1:250 dilution for 1 h. After washing, fluorescein isothiocyanate-conjugated donkey antimouse IgG and Texas Red-conjugated donkey antigoat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were added at a 1:250 dilution for 1 h. Subsequently, the cells were mounted on glass microscope slides using Dako (Carpinteria, CA) Fluorescent Mounting Medium. Confocal images were obtained using a Nikon Confocal Eclipse E1000 scanning laser microscope (Nikon, Melville, NY) with a  $\times$ 60 objective lens, and applying 3D deconvolution using ImageQuant software (Molecular Dynamics, Piscataway, NJ).

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