TOX3 is a neuronal survival factor that induces transcription depending on the presence of CITED1 or phosphorylated CREB in the transcriptionally active complex

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Summary

TOX3 is a nuclear protein containing a high mobility group (HMG)-box domain, which regulates Ca^{2+}-dependent transcription in neurons through interaction with the cAMP-response-element-binding protein (CREB). TOX3 appears to be associated with breast cancer susceptibility and was previously shown to be expressed downstream of a cytoprotective cascade together with CITED1, a transcriptional regulator that does not bind directly to DNA. In the present study we show that TOX3 is predominantly expressed in the brain, forms homodimers and interacts with CITED1. TOX3 overexpression protects neuronal cells from cell death caused by endoplasmic reticulum stress or BAX overexpression through the induction of anti-apoptotic transcripts and repression of pro-apoptotic transcripts, which correlates with enhanced transcription involving isolated estrogen-responsive elements and estrogen-responsive promoters. However, both functions cannot be inhibited with the anti-estrogen fulvestrant and are only attenuated by mutation of estrogen-responsive elements. TOX3 also interacts with native CREB and induces the CREB-responsive *BCL-2* promoter, which can be inhibited by coexpression of CITED1. Coexpression of CREB, by contrast, abolishes TOX3-mediated transcription from the estrogen-responsive complement C3 promoter. Our results suggest that TOX3 can enhance transcriptional activation from different cytoprotective promoters and that this is dependent on the predominance of either phosphorylated CREB or CITED1 within the transcriptionally active complex.

Key words: TOX3, CITED1, CREB

Introduction

TOX3, also known as TNRC9 (trinucleotide-repeat-containing 9), was first identified in a screen for transcripts containing trinucleotide (CAG) repeat expansions (Margolis et al., 1997). A variety of neurodegenerative diseases are caused by the expansion of translated CAG repeats, and many of the proteins involved have a role in the regulation of transcription (Riley and Orr, 2006), which is also the case for TOX3 as it contains a nuclear localization signal (NLS) and a high mobility group (HMG)-box domain followed by a C-terminal polyglutamine stretch. HMG-box proteins can modify chromatin structure by bending and unwinding DNA, which is primarily mediated through contacts of the HMG box with the minor groove. This potentially allows simultaneous binding of other transcriptional regulators to the DNA. HMG-box proteins can be divided into subfamilies that recognize DNA either in a sequence-dependent or sequence-independent manner, and a previous bioinformatical analysis suggested that TOX, a close homologue of TOX3 involved in T cell differentiation, is a sequence-independent HMG-box DNA-binding protein (O'Flaherty and Kaye, 2003). Recently, TOX3 was identified as a novel Ca^{2+}-dependent neuronal transcription factor, which contributes to Ca^{2+}-induced activation of *c-fos* expression by direct interaction with a transcriptionally active complex consisting of cAMP-response-element-binding protein (CREB) and CBP (CREB-binding protein) (Yuan et al., 2009). There is little further data on TOX3 but it is known that a single-nucleotide polymorphism near its 5′ end appears to be strongly associated with breast cancer susceptibility (Easton et al., 2007; Huijts et al., 2007; Stacey et al., 2007).

We recently found TOX3 to be expressed in concert with CITED1 (for 'CBP/p300-interacting transactivator with glutamic-acid- and aspartic-acid-rich C-terminal domain 1') in human embryonic kidney HEK-293 cells overexpressing the constitutively active orphan G-protein-coupled-receptor GPR39 (Dittmer et al., 2008). GPR39 overexpression protects against a wide variety of cellular stressors by induction of serum-response element (SRE)-mediated transcription (Dittmer et al., 2008). CITED1 is a transcriptional regulator, which, on its own, lacks DNA-binding properties but enhances transcription mediated by diverse transcription factors, such as estrogen receptors (Yahata et al., 2001), SMAD (Shioda et al., 1998), or early growth response (EGR) 2 (also known as Krox20) (Dillon et al., 2007). The role of

CITED1 in the brain is unclear, but its expression parallels that of estrogen receptors ERα and ERβ (Gerstner and Landry, 2007).

In the present study, we investigated the expression of TOX3 in human tissues, the effect of TOX3 and CITED1 on cell death, and whether and how this includes transcriptional activation.

Results

TOX3 is an HMG-box transcription factor which is predominantly expressed in the brain

TOX3 contains three separable domains: an N-terminal domain with an NLS, the HMG box and a C-terminal polyglutamine stretch (Fig. 1A). TOX3 has not been extensively characterized; we therefore first investigated its expression in a variety of human primary cell lines and normal tissues using quantitative real-time PCR. In normal human tissues, TOX3 expression was most prominent in the central nervous system (CNS), in the ileum, and within the brain in the frontal and occipital lobe (Fig. 1B). In primary human cell lines, TOX3 was expressed mainly in epithelial cells but not in cells of the endothelial or mesenchymal lineage (supplementary material Fig. S1A). We conclude that, despite the recently published role of TOX3 in breast cancer susceptibility and its expression in three out of five established breast cancer subtypes (Nordgard et al., 2007), TOX3 is not expressed in normal mammary tissue (i.e. it is not expressed in primary cell lines or in tissue from adult human origin). Our results instead suggest a role for TOX3 in the brain, which is in accordance with the recent report that TOX3 is a Ca^{2+}-dependent neuronal transcription factor (Yuan et al., 2009).

TOX3 is co-regulated and interacts with the transcriptional activator CITED1

We became interested in TOX3, when we found it to be induced in concert with CITED1 downstream of a cytoprotective signal transduction cascade in HEK-293 cells stably overexpressing the constitutively active receptor GPR39 (Dittmer et al., 2008). We aimed to reproduce this induction at the protein level in neuronal cells and transiently overexpressed either empty vector or GPR39 in Neuro2a cells (which express TOX3, see supplementary material Fig. S1B) for 24 hours and immunoblotted the cell lysates using anti-TOX3 and anti-CITED1 antisera. Although the basal expression of both proteins was rather low, GPR39 overexpression clearly induced expression of both TOX3 and CITED1 protein (Fig. 2A).

On the basis of the function of TOX3 and CITED1 in the regulation of transcription and their concerted expression, we hypothesized that TOX3 and CITED1 might interact with each other to induce synergistically the expression of cytoprotective transcripts, thereby mediating the protective effect of GPR39. We transfected vector encoding Myc–TOX3 and hemagglutinin (HA)–CITED1 or a similarly HA-tagged control protein (a nonfunctional mutant of the pro-apoptotic protein Puma, also known as BCL-2-binding component 3) into Chinese-hamster ovary (CHO) cells and evaluated whether the two proteins could be co-immunoprecipitated. Immunoprecipitation with anti-Myc antibodies followed by an anti-HA antibody immunoblot revealed that the two proteins are indeed in the same complex. The reverse experiment, immunoprecipitation with anti-HA antibodies and immunoblotting with anti-Myc antibodies gave the equivalent results (Fig. 2B). Overexpressed TOX3 migrated slower, probably owing to the Myc tags; the predicted mass is approximately 65 kDa, as shown for native TOX3 (Fig. 2A). Pulling down native TOX3 was unsuccessful, probably owing to the low amounts of TOX3 in these cells and the rather low sensitivity of the anti-TOX3 antiserum. However, when we transfected neuronal Neuro2a cells with Myc–TOX3 we were able to co-immunoprecipitate endogenous CITED1 (Fig. 2C). To reproduce

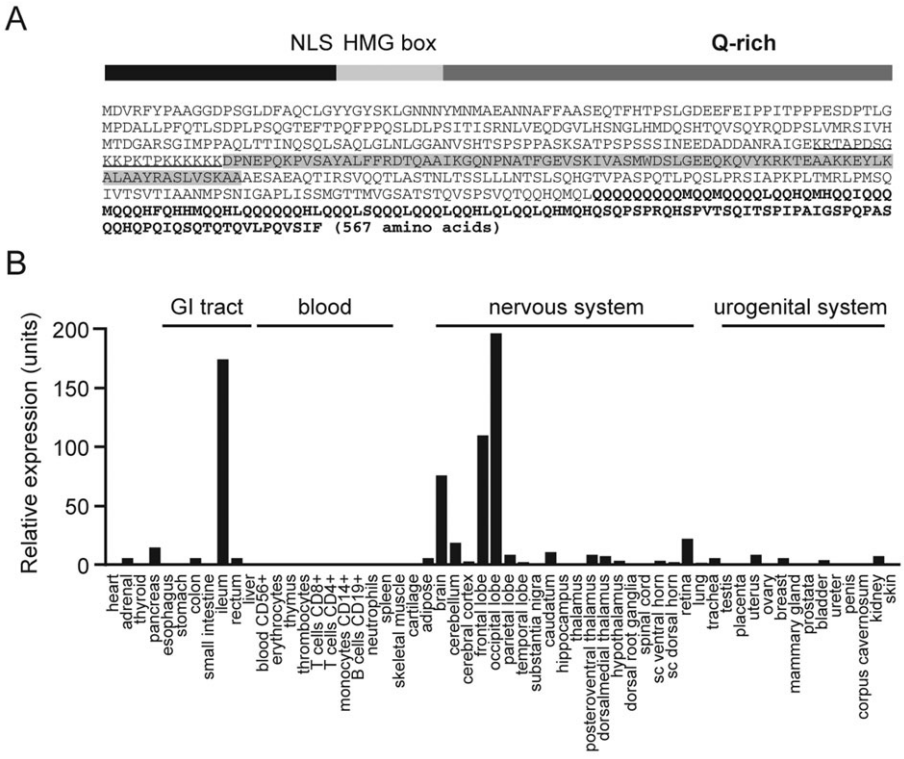

Fig. 1. TOX3 is an HMG-box transcription factor with a predominant expression in the brain. (**A**) The protein sequence of TOX3 and a schematic depicting its structure. The NLS is underlined, the HMG domain is shown on a gray background and the glutamine-rich C-terminal domain (Q-rich) is in bold text. (**B**) Quantification of *TOX3* levels in human tissues by quantitative real-time PCR. Expression was normalized to the expression of housekeeping genes encoding hypoxanthine phosphoribosyltransferase, GAPDH and β-actin and is shown in arbitrary units.

this interaction, in order to investigate homodimerization of TOX3, and to test the strength of interaction, we then tagged TOX3 at its N-terminus with *Renilla* luciferase and pulled down Protein-A-tagged CITED1 and TOX3 using IgG-coated magnetic beads. This assay, called LUMIER (for 'luminescence-based mammalian interactome mapping'), is amenable to automated and unbiased interaction studies (Barrios-Rodiles et al., 2005) and confirmed the binding of TOX3 to CITED1 and to itself with *z* scores of 1.39 for CITED1 and 1.75 standard deviations different from the mean of *n*=80 control proteins (Fig. 2D). Together, these experiments suggest that the two proteins are not only regulated in concert but also present within a complex. The interaction was specific for CITED1 as the homologous proteins CITED2, CITED3 and CITED4 could not be co-immunoprecipitated with TOX3 (supplementary material Fig. S2). This and the fact that it was possible to pull down endogenous CITED1 strongly suggests that a coincidental interaction caused by overexpression is unlikely.

We then used CITED1 mutants lacking either the SMAD4-interacting domain (ΔSID, amino acids 30–60) or the acidic C-terminal transactivation domain (ΔCR2) (Shioda et al., 1998) for immunoprecipitation with full-length TOX3 to pin down the interaction domain. TOX3 precipitated together with full-length CITED1 and the ΔSID mutant but only weakly with the ΔCR2 mutant (Fig. 2E). The reverse experiment using TOX3 mutants encompassing the N-terminus (amino acids 1–237), the N-terminus plus the HMG domain (amino acids 1–290), the HMG domain plus the C-terminus (amino acids 238–570) or the C-terminus (amino acids 291–570) alone showed that CITED1 bound only to those mutants containing the HMG domain and, among these, bound most prominently to the construct also containing the N-terminus (Fig. 2F). We therefore conclude that TOX3 and CITED1 are present in a protein complex, linked through the HMG domain of TOX3 and the CR2 domain of CITED1, and that this interaction is enhanced by the TOX3 N-terminus or by proteins binding to the N-terminus.

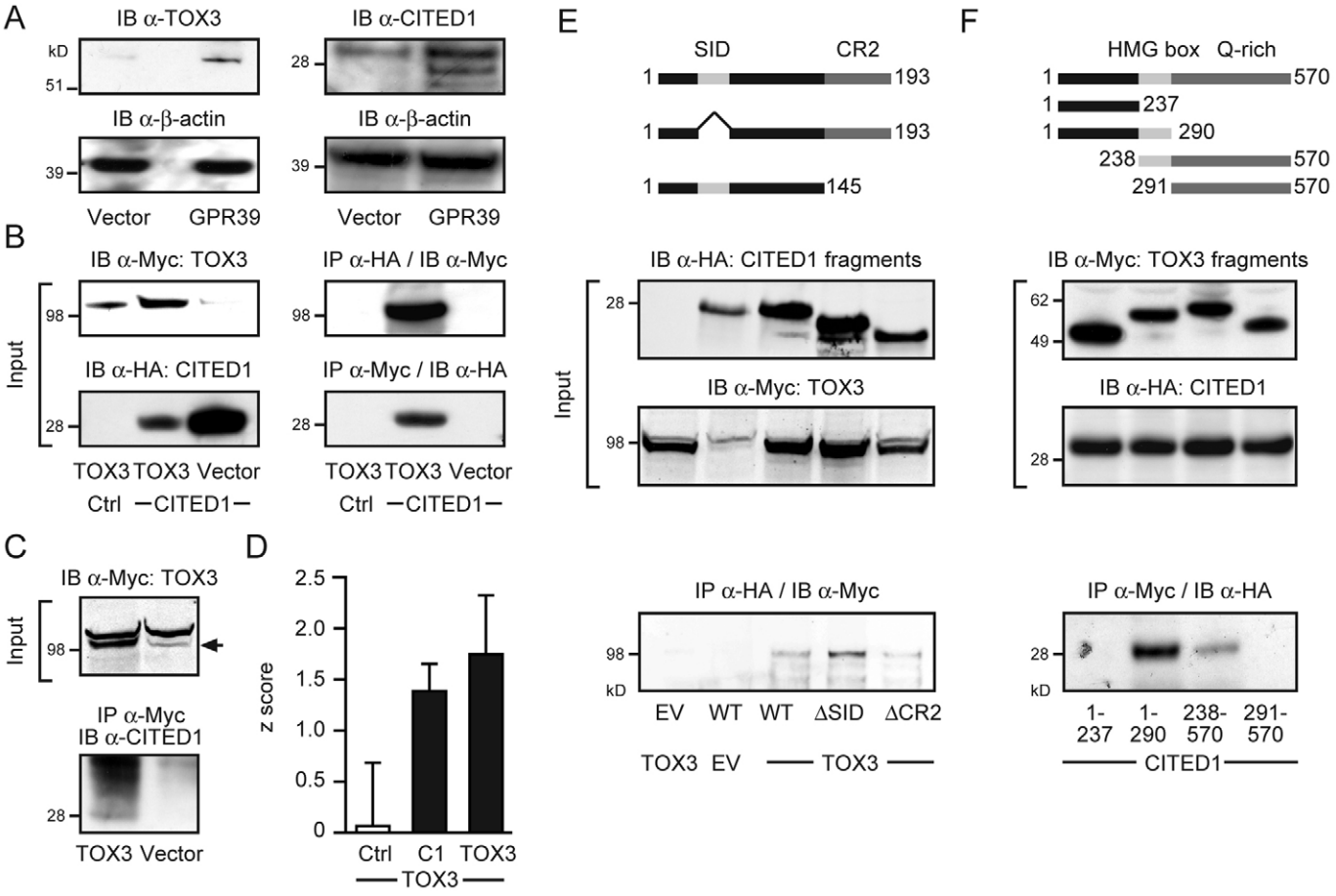

Fig. 2. TOX3 is co-regulated and interacts with the transcriptional activator CITED1. (**A**) Immunoblot (IB) showing induction of TOX3 and CITED1 in Neuro2a cells transiently overexpressing the cytoprotective receptor GPR39. The molecular mass in kDa is indicated. β-Actin served as the loading control. (**B**) Myc–TOX3, transfected into CHO cells, immunoprecipitates (IP) transfected HA–CITED1 and vice versa. The input is shown as the control (Ctrl) and the molecular mass in kDa is indicated. (**C**) Myc–TOX3, transfected into Neuro2a cells, immunoprecipitates endogenous CITED1. The anti-Myc antibody stained a double background band; the position of TOX3 is indicated with an arrow. CITED1 always runs as multiple bands owing to multiple phosphorylation. (**D**) LUMIER assays showing the interaction of TOX3 with CITED1 and with itself in HEK-293 cells. TOX3 was expressed as a fusion with *Renilla* luciferase and tested for co-purification with coexpressed Protein-A-tagged CITED1 (C1), TOX3 or control proteins. The bar graphs represent the means+s.d. from hexuplicate samples for CITED1 and TOX3, respectively or from 80 randomly chosen proteins. (**E**) Co-immunoprecipitation of Myc–TOX3 with the indicated HA-tagged CITED1 deletion mutants or (**F**) of HA–CITED1 with the indicated Myc-tagged TOX3 deletional mutants in CHO cells.

TOX3 protects against cell death by inducing anti-apoptotic and repressing pro-apoptotic transcripts

We next investigated the effect of TOX3, CITED1 or both of these proteins on cell death caused by two different stress stimuli. Tunicamycin inhibits the synthesis of all N-linked glycoproteins and causes cell death by endoplasmic reticulum stress. Overexpression of the pro-apoptotic protein BAX, by contrast, leads to release of cytochrome *c* from mitochondria and direct activation of the caspase cascade. GPR39, which is upstream of TOX3 and CITED1, protects against both stressors (Dittmer et al., 2008). Cell death was quantified by flow cytometry by gating for early plus late apoptotic cells [cells double-positive for annexin V and 7-aminoactinomycin D (7-AAD)]. Both proteins protected against cell death; TOX3 appeared to be slightly more effective against tunicamycin (TOX3 gave ~16.5% protection compared with ~14.1% for CITED1), whereas CITED1 proved to be more effective against BAX-induced cell death (TOX3 gave ~14.2% protection compared with ~19.9% CITED1). Coexpression of TOX3 and CITED1 together increased the protective effect of either one against BAX- and tunicamycin-mediated cell death (~28.6% against tunicamycin and ~27.7% against BAX, Fig. 3A). This effect was of a similar magnitude to the protection conferred by the anti-apoptotic protein BCL-2, which we used as the positive control.

To substantiate these findings at the transcriptional level, we then transiently overexpressed TOX3 in Neuro2a cells for 24 hours and examined the expression levels of several anti-apoptotic (Xiap, Mcl-1, BCL-2L12, Bcl-XL and BCL-2) and pro-apoptotic (Puma, Diablo, several caspases, Bok, Bnip31, Bnip3, Bim, Bid, BAX, Bak, Bad and Apaf1) transcripts using quantitative real-time PCR. In line with the pro-survival effect of TOX3, overexpression mainly induced the expression of anti-apoptotic transcripts (all except Xiap) and repressed the expression of pro-apoptotic transcripts (all except Diablo) (Fig. 3B).

TOX3 induces transcription from estrogen-responsive promoters

Being DNA-interacting proteins, we hypothesized that TOX3 and CITED1 might mediate the activation of SRE-mediated transcription conferred by GPR39 overexpression in Neuro2a cells (Dittmer et al., 2008). We also investigated activation of cAMP-response elements (CREs) as TOX3 was shown to interact with CREB and CBP (Yuan et al., 2009). As a third possibility, we investigated the activation of estrogen-response element (ERE)-dependent transcription because CITED1 binds to ERα (Yahata et al., 2001) and both proteins are expressed in similar regions of the brain (Gerstner and Landry, 2007). We overexpressed TOX3, CITED1 or both proteins with luciferase reporter constructs for SRE-, CRE- and ERE-dependent transcription in Neuro2a cells. In these cells, TOX3 alone only increased ERE-dependent transcription with a statistically significant effect of ~3.5-fold. We observed no effect on SRE- and CRE-dependent transcription, although all of these experiments were performed in parallel and the transfection efficiency was controlled. The combination of TOX3 and CITED1 resulted in a statistically significant increase in ERE-dependent transcription of ~15-fold but had no effect on SRE- or CRE-dependent transcription (Fig. 4A). To evaluate the contribution of endogenous TOX3 to ERE basal transcriptional activity, we then used three small hairpin RNA (shRNA) constructs targeting different regions of TOX3. All three constructs were effective in knocking down expression of endogenous TOX3

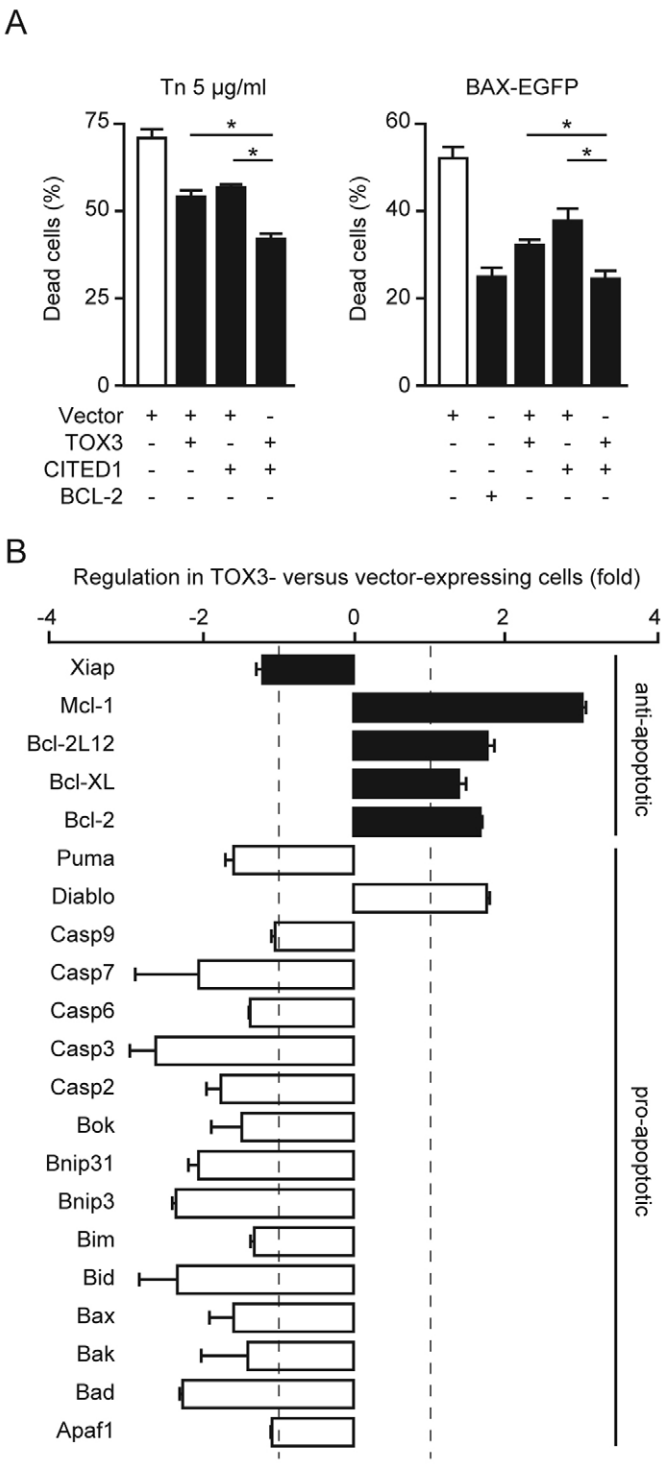

Fig. 3. TOX3 protects from cell death by inducing anti-apoptotic and repressing pro-apoptotic transcripts. (**A**) Neuro2a cells were transfected with the indicated constructs and treated with tunicamycin (Tn) or co-transfected with Bax–EGFP. Viability was quantified 24 hours later by gating for 7-AAD- and annexin-V-positive (EGFP)-fluorescent cells. (**B**) Expression of the indicated anti- or pro-apoptotic transcripts in Neuro2a cells transiently transfected with TOX3 for 24 hours. Expression was normalized to the expression of housekeeping genes hypoxanthine phosphoribosyltransferase and *GAPDH*. The bar graphs represent the means+s.e.m. for at least three (A) or two (B) independent transfections performed in triplicate. **P*<0.05 as determined by one-way ANOVA with Dunnett's multiple comparison test.

protein (Fig. 4B) and mRNA (supplementary material Fig. S3). When co-transfected along with the ERE reporter plasmids into Neuro2a cells, all TOX3 shRNA constructs, but not the control shRNA, significantly reduced the basal transcriptional activity from this promoter (Fig. 4C).

TOX3 contributes to endogenous ERE-dependent transcription

To prove the transcriptional activity of TOX3 on estrogen-responsive promoters, we then used three estrogen-responsive constructs previously used in the study of CITED1-mediated induction of estrogen-responsive transcription (Yahata et al., 2001). Namely, ERE-tk containing a single ERE in front of the herpes simplex virus thymidine kinase (tk) promoter, ERE-E1B, where three EREs are followed by the adenovirus E1B TATA box, and a reporter consisting of one ERE followed by the promoter of the gene encoding β-globin. We also used the endogenous promoter for complement 3 (C3), which has been used previously as a tool to study estrogen-dependent transcription (Fan et al., 1996; Yoon et al., 2000). We transiently transfected TOX3 or empty vector with these four promoters driving the expression of the gene encoding firefly luciferase and a *Renilla* luciferase control construct into Neuro2a cells and quantified transcriptional activation by dual luciferase assays. TOX3 significantly induced transcription from all four promoters; the β-globin, thymidine kinase and E1B promoters were induced twofold and the C3 promoter eightfold (Fig. 4D). Similar to the results obtained with the ERE reporter, transfection of the shRNA constructs targeting TOX3 effectively knocked down the basal transcriptional activity from the C3 promoter (Fig. 4E) proving the contribution of endogenous TOX3 to C3-promoter-dependent transcription. We then mutated the ERE elements in the C3 promoter to prove the specificity of TOX3 induction. Specifically, the ERE elements of the C3 promoter identified previously were mutated (Fan et al., 1996), from –235

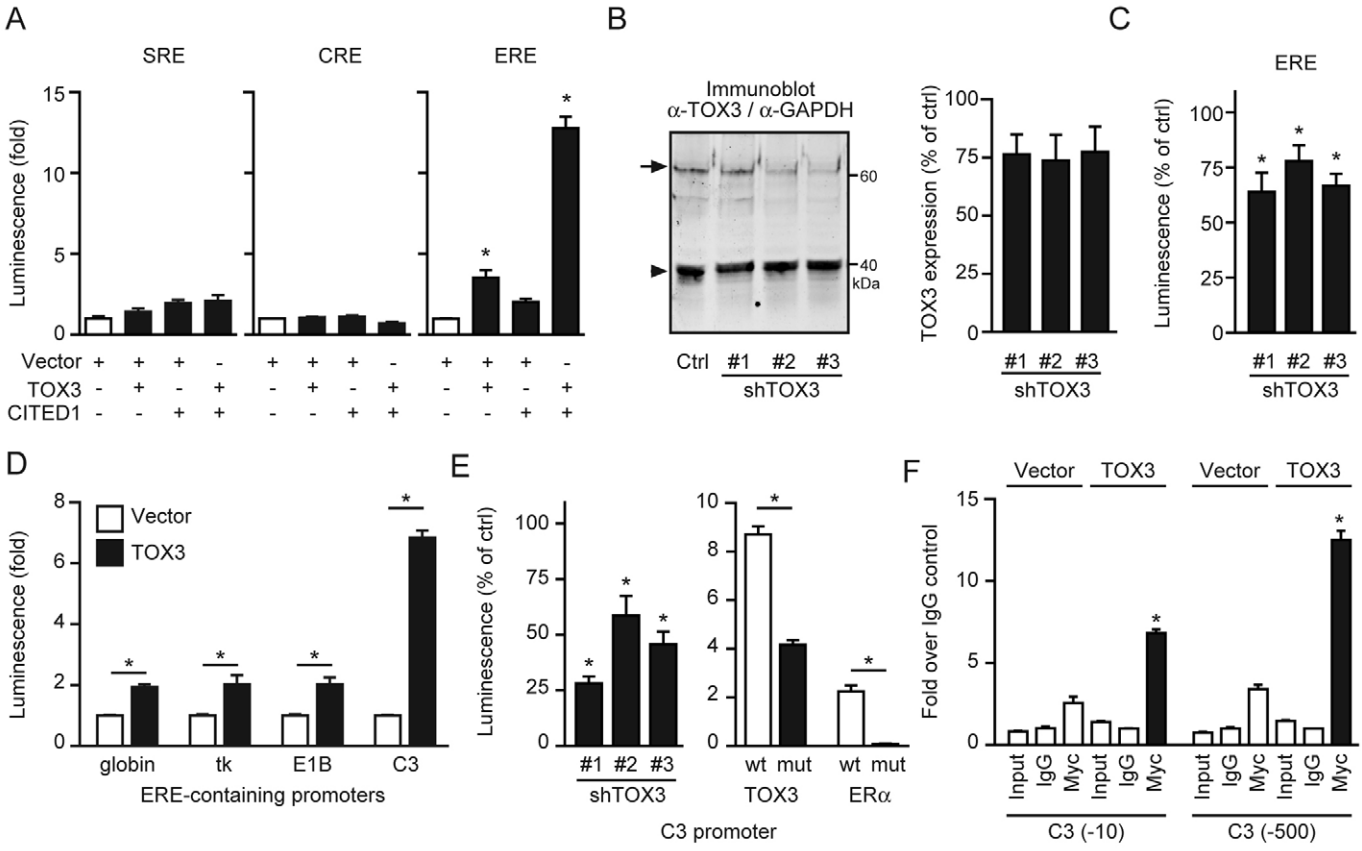

Fig. 4. TOX3 induces transcription from estrogen-responsive promoters. (**A**) TOX3 and CITED1 increase transcription from reporter constructs containing an ERE, but not a CRE or SRE. (**B**) Immunoblot of protein lysates from Neuro2a cells transfected with shRNA plasmids against TOX3 (shTOX3) or control shRNA (Ctrl) were probed with mixed antisera against TOX3 (arrow) and GAPDH (arrowhead). The right-hand panel shows a quantification of TOX3-knockdown by shRNA normalized to the endogenous control. (**C**) TOX3 shRNAs suppress endogenous ERE-dependent transcription. (**D**) TOX3 overexpression also induces transcriptional activity from the estrogen-responsive β-globin (globin), thymidin kinase (tk), E1B and complement C3 promoters. (**E**) TOX3 shRNAs also suppress transcriptional activity from the C3 promoter and mutation of two EREs in the C3 promoter attenuates TOX3 and abolishes ERα-mediated transcriptional activation. (**F**) TOX3 interacts with the genomic C3 promoter. A ChIP assay with HEK-293 cells transfected with Myc-tagged TOX3 or empty Myc vector. Chromatin was incubated with an anti-Myc or anti-IgG antibody, as a control, and precipitated. Two regions of the C3 promoter (the primer start position is indicated in brackets) and a reference sequence were amplified and quantified by real-time PCR and normalized to the IgG control. All luciferase assays were performed by transfecting Neuro2a cells with the indicated constructs. Emitted luminescence was quantified 24 hours later and normalized to the EGFP fluorescence of a co-transfected EGFP plasmid (for A and C) or dual luciferase assays (for D and E). Values are given as the fold luminescence over empty vector. The bar graphs represent the means+s.e.m. for at least three independent transfections performed in triplicate. **P*<0.05 as determined by one-way ANOVA with Dunnett's multiple comparison test (for A, C, E right-hand panel and F), or by Student's *t*-test (D, and E left-hand panel).

to –223, GGTGGcccTGACC to GGTGGcccTtACt, and from –149 to –137, GGACAtgtTGGCC to tGAtAtgtTGGCt (lowercase represents intervening, non-binding sequences). The induction by TOX3 was indeed significantly attenuated by these mutations, whereas induction induced by ERα, which we used as positive control, was almost abolished (Fig. 4E, right-hand panel). These observations indicate that endogenous TOX3 contributes substantially to the expression of complement C3 in neuronal cells. Taken together with the results from the ERE reporter, we conclude that this induction is partly mediated through ERE elements in the C3 promoter.

TOX3 interacts with the human C3 promoter

In order to show binding of TOX3 to the C3 promoter region, we performed a chromatin immunoprecipitation (ChIP) assay with HEK-293 cells transfected with Myc-tagged TOX3 or empty Myc vector. Chromatin was incubated with an anti-Myc antibody or, as a control, anti-IgG antibody. After immunoprecipitation, two regions of the C3 promoter and a reference sequence were amplified and quantified by real-time PCR, which showed a relative enrichment over the IgG control of 6.8-fold for primers amplifying a region starting at –10 (relative to the transcriptional start site) and a 12.5-fold enrichment for primers amplifying a region starting at –500 (Fig. 4F). This suggests that TOX3 binds to the genomic C3 promoter region.

The effects of TOX3 on cellular survival and transcription cannot be inhibited with the anti-estrogen fulvestrant

To investigate whether the effect of TOX3 and CITED1 could be inhibited with an anti-estrogen agent, we transiently transfected Neuro2a cells with a vector encoding the pro-apoptotic protein BAX and empty vector, or TOX3 plus CITED1, and treated half of the cells with the anti-estrogen fulvestrant (ICI-182780) and the other half with vehicle only. We then quantified the amount of dead cells by flow cytometry by gating for early and late apoptotic (annexin-V and 7-AAD double-positive) cells. Although the combination of TOX3 and CITED1 was again protective against this insult, we observed no statistically significant effect of fulvestrant (Fig. 5A), which was otherwise effective in our model system, as it suppressed transcriptional activation of the ERE reporter and the C3 promoter in ERα-overexpressing Neuro2a cells (Fig. 5B). Similar to the results on cell death above, we also observed no effect of fulvestrant on TOX3-mediated increases in ERE-dependent transcription or induction of the C3 promoter (Fig. 5C). The increase in ERE-dependent transcription conferred by co-transfection of CITED1 seemed to be slightly attenuated, but this was not statistically significant (Fig. 5C). We therefore conclude that the effect of TOX3 on ERE-mediated transcription is ligand and/or receptor independent.

TOX3 resides in a complex with phosphorylated CREB

The above results show that TOX3 interacts with CITED1 (Fig. 2), and, furthermore, CITED1 is known to interact with ERα (Yahata et al., 2001). We have also shown that TOX3 increased transcription from estrogen-responsive promoters and interacted with the estrogen-responsive human C3 promoter (Fig. 4). We therefore aimed to investigate whether TOX3 is present in a complex with ER receptors and whether such a complex mediates the induction of ERE-dependent transcription despite the results obtained with fulvestrant-treated cells. However, both known estrogen receptors ERα and ERβ could not be detected by immunoblotting in TOX3-responsive Neuro2a cells (supplementary material Fig. S4). We therefore turned to the published interaction with CREB and investigated the interaction of TOX3 with CREB or the transcriptionally less active S133A mutant (for a review, see Johannessen et al., 2004), which inhibits CBP binding to CREB after phosphorylation at S133 by Ca^{2+}/calmodulin-dependent protein kinases (Lee et al., 1995). We co-transfected vectors encoding Myc–TOX3 with CREB or S133A-CREB into CHO cells. Immunoprecipitation with anti-Myc antibodies followed by immunoblotting with an anti-CREB antibody revealed that TOX3 and CREB are indeed in the same complex, whereas the S133A mutant did not interact with TOX3 and even reduced binding to native CREB, suggesting a dominant-negative function of this mutant on TOX3 function (Fig. 6A, note the reduced amount of native CREB immunoprecipitated in the presence of the S133A mutant compared with the empty vector control). From these

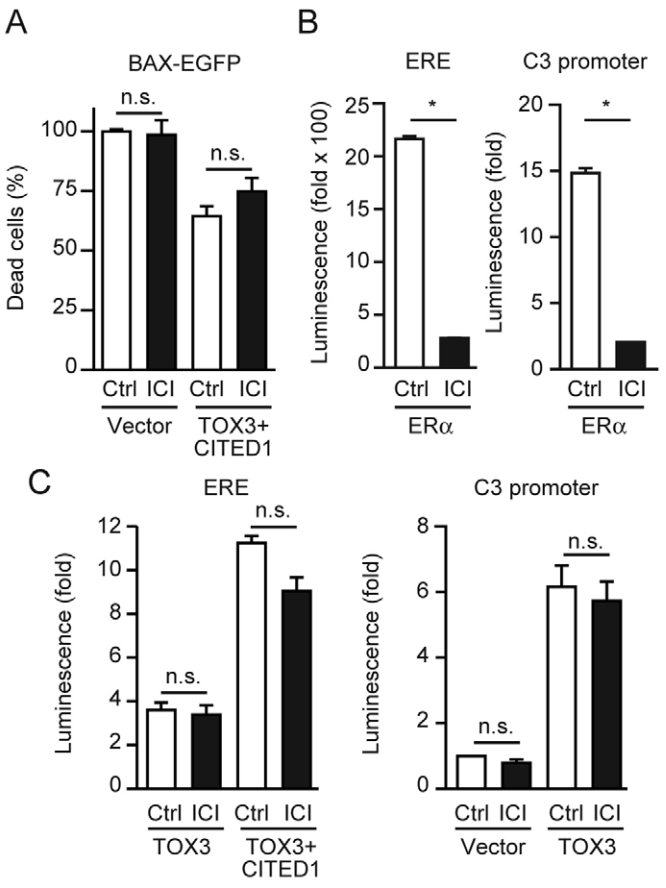

Fig. 5. TOX3 effects on cell survival and transcription cannot be inhibited with the anti-estrogen fulvestrant. (**A**) Neuro2a cells were transfected with vector encoding Bax–EGFP and empty vector, or TOX3 plus CITED1 in the presence of fulvestrant (ICI) or vehicle (Ctrl). Viability was quantified 24 hours later by gating for 7-AAD- and annexin-V-positive (EGFP)-fluorescent cells. (**B** and **C**) Neuro2a cells were transfected with the indicated reporter constructs and emitted luminescence quantified 24 hours later. Transcriptional activity of transiently transfected ERα (B), but not TOX3 or TOX3 plus CITED1 on ERE elements or the estrogen-responsive C3 promoter (C), was significantly attenuated by ICI. Values are given in fold luminescence over empty vector and normalized to the EGFP fluorescence of a co-transfected EGFP plasmid. The bar graphs represent the means+s.e.m. for at least three independent transfections performed in triplicate. **P*<0.05 as determined by Student's *t*-test. n.s., not significant.

experiments, we concluded that TOX3 interacts with phosphorylated CREB.

TOX3-mediated induction of the C3 promoter is not dependent on CREB

To investigate whether the interaction with phosphorylated CREB mediates the transcriptional induction of the endogenous estrogen-responsive C3 promoter by TOX3, we co-transfected TOX3 with CREB (or the noninteracting and nonfunctional S133A or ACREB mutants) into Neuro2a cells and quantified the transcriptional activation of the C3 promoter using luciferase assays. The wild-type (wt) control and both CREB mutants completely abolished the prominent effect of TOX3, suggesting that induction of the C3 promoter by TOX3 is not dependent on its interaction with phosphorylated CREB and that it was even inhibited by these proteins (Fig. 6B). We then repeated these experiments using the *Bcl-2* promoter, which (1) can be induced by TOX3 overexpression (Fig. 3B and Fig. 6C), (2) is CREB responsive (Wilson et al., 1996) and (3) might participate in the positive effect of TOX3 on cell survival. We co-transfected TOX3 alone or together with CREB, S133A-CREB or CITED1 into Neuro2a cells and quantified transcriptional activation from the *BCL-2* promoter using luciferase assays. TOX3 indeed induced transcription from this promoter ~12-fold. This was even increased twofold by co-transfection of CREB but there was no induction of transcription by TOX3 and S133A-CREB co-transfection, suggesting an effect mediated by phosphorylated CREB in line with the co-immunoprecipitation studies, where S133A-CREB attenuated binding of TOX3 with endogenous CREB (Fig. 6A). Even more pronounced was the effect of co-transfected CITED1, which completely abolished the transcriptional effect of TOX3 on CREB-mediated transcription from the *BCL-2* promoter (Fig. 6D). These results suggest that TOX3 can mediate cytoprotective transcription from the *BCL-2* promoter or the complement C3 promoter, depending on the predominance of either phosphorylated CREB or CITED1 within the transcriptionally active complex.

Discussion

Our results suggest a model whereby TOX3 assembles a transcriptionally active complex with either phosphorylated CREB or CITED1 to increase transcription from different promoters (see the model in Fig. 6E). CBP probably also participates in this complex as it can bind to CITED1 (Yahata et al., 2001) and/or to the C-terminus of TOX3 (Yuan et al., 2009). We observed that the effect of TOX3 on cell survival and activation of ERE-dependent transcription could not be inhibited by the anti-estrogen fulvestrant, which leads to degradation of the receptors by the proteasome and inhibits binding of ligand. This, together with the fact that Neuro2a cells contain only trace amounts of both classical ERs, suggests that the activity of TOX3 in these cells is receptor independent. It is noteworthy that we observed a faint interaction with overexpressed ERα in CHO cells, which could not be reproduced with native ERα in MCF7 cells. Moreover, mutation of the ER elements in the C3 promoter attenuated, but did not abolish, the transcriptional activation of TOX3. It is possible that TOX3 interacts with other ERα-like proteins, such as estrogen-related receptor (ERR) α or ERRγ, which have a high similarity to the estrogen receptors but are ligand independent (Xie et al., 1999) and more promiscuous with regard to DNA sequence recognition requirements (Razzaque et al., 2004). Alternatively, TOX3 could

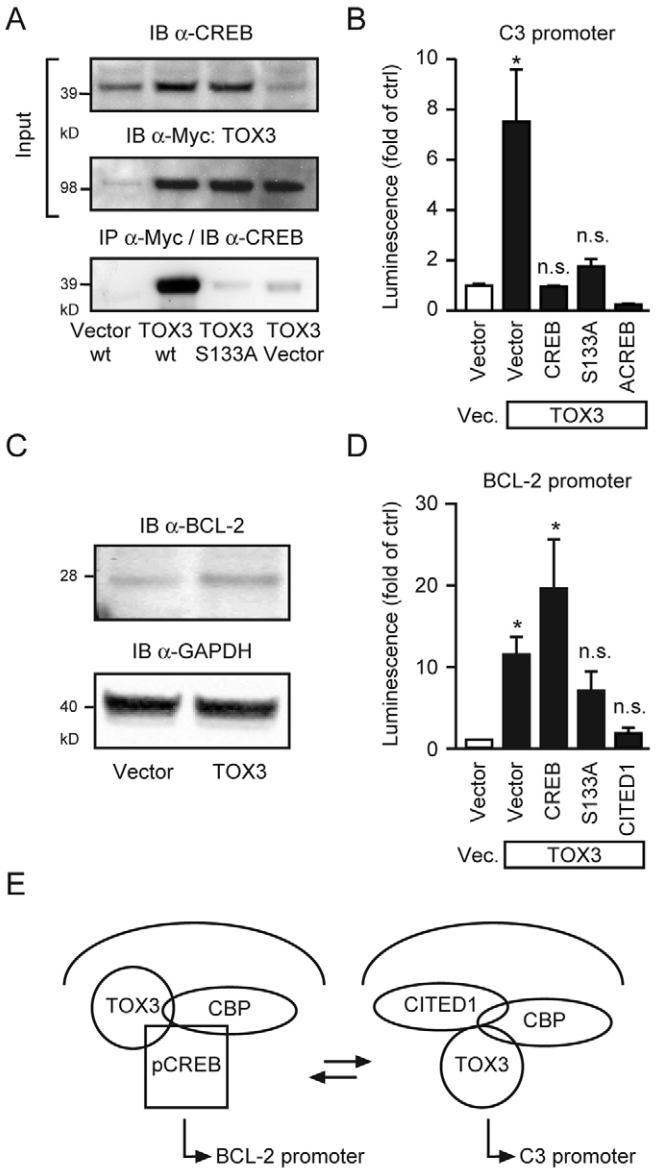

Fig. 6. TOX3-mediated induction of the C3 promoter is not dependent on CREB. (**A**) Myc–TOX3 transfected into CHO cells immunoprecipitates (IP) CREB but not S133A-CREB. The input is shown as a control and the molecular mass in kDa is indicated. Note the immunoprecipitation of endogenous CREB and its attenuation by S133A. IB, immunoblot. (**B**) TOX3-mediated induction of the estrogen-responsive C3 promoter is abolished by CREB and mutated S133A-CREB or ACREB. Neuro2a cells were transfected with the indicated constructs and emitted luminescence was quantified 24 hours later. Values are given as the fold luminescence over empty vector and normalized to the EGFP fluorescence. Data are the means+s.e.m. for three independent transfections performed in triplicate. *P<0.05 as determined by one-way ANOVA with Tukey's multiple comparison test. n.s., not significant. (**C**) Immunoblot showing induction of BCL-2 in Neuro2a cells transiently overexpressing TOX3 for 24 hours. The molecular mass in kDa is indicated; GAPDH served as a loading control. (**D**) TOX3-mediated induction of the CREB-responsive *BCL-2* promoter is abolished by CITED1 and increased by wild-type, but not mutated, CREB. Neuro2a cells were transfected with the indicated constructs and emitted luminescence quantified 24 hours later. Normalization and statistical analysis were performed as described in B. (**E**) Cartoon depicting the proposed mechanism whereby TOX3 can mediate CREB- or hormone-independent ERE-dependent transcription, depending on the presence of either phosphorylated CREB (pCREB) or CITED1 within the transcriptionally active complex.

interact directly with DNA in a sequence-dependent manner, as shown for other HMG-box domain transcription factors (O'Flaherty and Kaye, 2003).

We should emphasize that, although our experiments support the association of TOX3 with either CREB or CITED1, we do not yet know whether the interactions are direct. A definitive resolution of this will require purification of the endogenous complexes, as well as binding assays with purified proteins.

Estrogen-dependent transcription was shown previously to protect a large variety of neuronal cells against an equally large variety of stressors (for a review, see Behl, 2002). The fact that coexpression of CITED1 abolished the effect of TOX3 on CREB-dependent transcription from the *BCL-2* promoter but additively increased the protection conferred by TOX3 against endoplasmic reticulum stress or direct activation of the caspase cascade suggests that the protective activity of TOX3 is possibly mediated through the induction of hormone-independent ERE-dependent transcription and less through the induction of CREB-mediated transcription in these cells. However, as we only studied one CREB-dependent promoter, we cannot exclude the induction of other CREB-dependent neuroprotective proteins, as CREB, similar to estrogen, has a well-known function in cell survival and induces a large variety of protective proteins (for a review, see Walton and Dragunow, 2000).

The hormone-independent survival function of TOX3 is of utmost interest because of the recently documented role of TOX3 in breast cancer susceptibility (Easton et al., 2007), especially as CITED1 has also been implicated in breast cancer. In human breast cancer samples, CITED1 expression parallels that of ERα (McBryan et al., 2007). Therefore, both the proteins TOX3 and CITED1 seem to be implicated with breast cancer and estrogen-dependent signal transduction. It is easy to conceive that TOX3 confers a risk due to enhanced survival of cancer cells. However, this needs to be investigated in more detail in breast cancer cells and models, which is beyond the scope of the present study.

We conclude that TOX3 is a nuclear protein containing a C-terminal polyglutamine stretch, which is mainly expressed in the brain, and protects from cell death by inducing transcription from different cytoprotective promoters, depending on the co-factors present in the transcriptionally active complex. This is of utmost interest considering that a large variety of neurodegenerative diseases are caused by expansion of polyglutamine stretches, which leads to subsequent tinkering with transcriptional control.

Materials and Methods

Cells, proteins, shRNA and plasmids

Neuro2a and COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (PAA laboratories) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 µg/ml streptomycin, and CHO cells in DMEM with Ham's F12 (Gibco) supplemented with L-glutamine, 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin. Human CITED1–4 in pRC/CMV, pHA-CITED1-ΔCR2, pHA-CITED1-ΔSID, pGERE-Luc, pERE-tk-Luc, 3×ERE-E1B-TATA-Luc have been described previously (Shioda et al., 1998; Yahata et al., 2001). Myc-tagged full-length rat TOX3 and TOX3 deletion mutants were generated by PCR and cloned into a pBOS backbone. HA-tagged human Puma lacking its cell-death domain in pcDNA3 was a kind gift from Andreas Villunger (Innsbruck Medical University Biocenter, Austria). BAX–EGFP (enhanced green fluorescent protein) in pcDNA3 was a gift from Pawel Kermer (University of Göttingen, Germany), CREB and S133A-CREB in pcDNA3 were a kind gift from Ugo Moens (University of Tromsø, Norway), ACREB in pCAGGS was a gift from Hermann Rohrer (MPI Hirnforschung, Frankfurt, Germany). SRE–Luc and CRE–Luc reporter constructs were purchased from Clontech, and ERE–Luc (Addgene plasmid no. 11354), C3–LUC (Addgene plasmid 11358), *Bcl-2*-promoter–luciferase (Addgene plasmid no.15381), and VP16–ERα (Addgene Plasmid no 11351) were from Addgene. HuSH shRNA constructs against TOX3 in pGFP-V-RS were from Origene. The C3 mutant promoter was synthesized by Mr. Gene and cloned into the original C3 plasmid through internal restriction sites *Avr*II and *Bam*HI. Tunicamycin was purchased from Calbiochem and ICI-182780 from Tocris.

Transfections

High-purity plasmids were prepared using Nucleobond AX 500 columns (Machery & Nagel). For transient transfections, cells were grown to 80%–90% confluence and transfected with Lipofectamine 2000 (Invitrogen) or Attractene (Qiagen).

LUMIER assays

For LUMIER assays, proteins were transiently expressed in HEK-293 cells as hybrid proteins with the *Staphylococcus aureus* Protein A tag or *Renilla reniformis* luciferase fused to the indicated termini. A total of 20 ng of each expression construct was transfected into 10,000 HEK293 cells using 0.05 µl of Lipofectamine 2000 (Invitrogen) in 96-well plates. After 40 hours, the medium was removed and cells lysed on ice in 10 µl of ice-cold lysis buffer [20 mM Tris-HCl, pH 7.5, 250 mM NaCl, 1% Triton X-100, 10 mM EDTA, 10 mM dithiothreitol (DTT), protease inhibitor cocktail (Roche), phosphatase inhibitor cocktail (Roche), 100 units/µl benzonase (final concentration) (Novagen)] containing sheep anti-(rabbit IgG)-coated magnetic beads (Invitrogen; Dynabeads M280, 2 mg/ml final concentration). Lysates were then incubated on ice for 15 minutes. Next, 100 µl of washing buffer (PBS with 1 mM DTT) was added per well, and 10% of the diluted lysate was removed to determine the luciferase activity present in each sample, before washing. The rest of the sample was washed six times in washing buffer in a Tecan Hydroflex plate washer. Luciferase activity was measured in the lysate as well as in the washed beads. Negative controls were transfected with a plasmid expressing the respective luciferase fusion protein and a vector expressing a dimer of Protein A instead of the Protein A fusion protein. For each sample, four values were measured: the luciferase present in 10% of the sample before washing (input), the luciferase activity present on the beads after washing (bound), and the same values for the negative controls (input nc and bound nc). Normalized interaction signals were calculated as follows: log(bound)/log(input)–log(bound nc)/log(input nc). Normalized interaction signals were *z*-transformed by subtracting the mean and dividing by the standard deviation. The mean and standard deviation were calculated from large datasets of protein pairs that were not expected to interact, i.e. from negative reference sets.

Polymerase chain reaction

TOX3 mRNA levels in human tissues and regulation of pro- and anti-apoptotic transcripts were quantified using a TaqMan real-time PCR assay on a 7900 HT sequence detection system (Applied Biosystems). First-strand cDNA synthesis was primed with random hexamer primers. Human TOX3 primers were 5′-ATACAGGGCCAGCCTCGTT-3′ and 5′-TCTGCTGAACAGAACGGATG-3′ together with a 6-carboxyfluorescein (FAM)- or 6-carboxytetramethylrhodamine (TAMR)-labeled probe 5′-TGCTGAGTCAGCAGAAGCCCAGAC-3′. For mouse *TOX3* and pro- and anti-apoptotic transcripts, we used probes from Universal Probe Library (Roche) and primers according to supplementary material Table S1. cDNA was normalized to the expression levels of the housekeeping genes hypoxanthine phosphoribosyltransferase, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin. Relative expression was calculated using normalized expression values. ERα from Neuro2a cells was amplified with 5′-TCCTGTTTGCTCCTAACTTGC-3′ and 5′-GGTGCTGGACAGAAACGT-3′ generating an exon-spanning product of 191 bp (1398–1571) of the mouse ERα mRNA.

Immunoblotting and co-immunoprecipitation assays

Total cell lysates or eluates were separated on 8–16% polyacrylamide gels (Thermo Scientific), transferred onto nitrocellulose membranes (Invitrogen) and blocked in 3% non-fat dried milk powder for 1 hour at room temperature before overnight incubation with a primary hamster polyclonal anti-BCL-2 antibody (BD Bioscience, 1:1000), a mouse monoclonal anti-Myc antibody (clone 4A6, Upstate, 1:1000), a rabbit polyclonal anti-HA antiserum (Abcam, 1:4000), a rabbit polyclonal anti-VP16 antibody (Abcam, 1:2000), a rabbit polyclonal anti-CITED1 antibody (I51904K, 1:500) (Shi et al., 2006), a rabbit polyclonal anti-TOX3 antibody (1:1000) (Yuan et al., 2009), a rabbit monoclonal anti-β-actin antibody (Millipore, 1:5000), a mouse monoclonal anti-GAPDH (Cell Signaling, 1:5000), a rabbit polyclonal anti-ERα (Epitomics, 1:500), a rabbit polyclonal anti-ERβ (Santa Cruz Biotechnology, 1:500) or rabbit monoclonal anti-CREB antibody (Cell Signaling, 1:1000) followed by an anti-(mouse IgG) or anti-(rabbit IgG) secondary antibody (Fc), as appropriate, conjugated to a fluorophore with infrared fluorescence (Licor, 1:30,000). The membranes were scanned for infrared fluorescence at 680 or 800 nm using the Odyssey system (Licor). Co-immunoprecipitations were performed using the ProFound HA or Myc tag IP/Co-IP kit (Pierce) according to the manufacturer's instructions. Briefly, CHO, COS7 or Neuro2A cells were transfected with the indicated constructs and lysed 48 hours later in M-PER mammalian protein extraction reagent (1000 µl per10-cm-diameter plate) containing protease inhibitors. A total of 200 µl of the lysate (~500 µg of total protein) was incubated with 10 µl of anti-HA (or anti-Myc) agarose slurry at 4°C overnight, eluted with 40 µl of nonreducing sample buffer, and 20 µl of each sample was then separated by SDS-PAGE, transferred onto a nitrocellulose membrane and probed with antibodies as described above.

Chromatin immunoprecipitation assay

Chromatin precipitation was performed using the SimpleCHIP enzymatic chromatin IP Kit (Cell Signaling) according to the manufacturer's protocol. Briefly, 10^7 HEK-293 cells transfected with vector or Myc-tagged TOX3 were crosslinked by incubation with 1% paraformaldehyde (PFA) for 10 minutes and lysed. Chromatin in the nuclear fraction was digested by incubation with micrococcal nuclease, nuclear membranes were broken by sonication, and the mixture was cleared. To determine the chromatin concentration, 15 µg of chromatin was incubated with an anti-Myc antibody, IgG or an anti-(histone H3) antibody at 4°C overnight and subsequently with Protein-G–agarose beads for 2 hours. The chromatin was eluted, reverse crosslinked and digested with Proteinase K. After purification, samples were analyzed by quantitative real-time PCR using primers for ribosomal protein L30 as a control and two primer pairs recognizing the C3 promoter region. Details of primers and probes are given in supplementary material Table S1.

Cell death and viability assays

For cell-death analysis by flow cytometry, Neuro2a cells were plated in 24-well plates and transfected with 0.4 µg of BAX–EGFP and the indicated constructs. After 24 hours, cells were resuspended in 100 µl of annexin-V-binding buffer (BD-Pharmingen) and stained with 5 µl of annexin-V–PE (phycoerythrin) (BD-Pharmingen) and 5 µl of 7-AAD. Single EGFP-positive cells were gated at 488 nm and analyzed for annexin V and 7-AAD staining. Data were acquired with a FACSCalibur flow cytometer and quantified using Cell Star software (Becton Dickinson). For the analysis of tunicamycin-mediated cell death, 5 µg/ml tunicamycin was added after 24 hours and cell death assessed as above 24 hours later.

Reporter assays

Neuro2a cells were transiently transfected in a 48-well plate with the indicated luciferase reporter plasmid, a SRE–*Renilla* or EGFP control plasmid, and the indicated expression constructs. At 24 hours after transfection, cells were washed and lysed in 200 µl of passive lysis buffer (Promocell), the lysate was centrifuged at 12,000 ***g*** for 1 minute and 20 µl of supernatant was transferred to a white 96-well microtiter plate. Then, 100 µl of luciferase assay buffer (Promocell) was injected into each well directly before measurement. Luminescence was measured by a Genios Pro microplate reader (Tecan) and integrated for 10.000 ms. Normalization was performed by EGFP fluorescence or by dual luciferase assays; 20 µl of lysate was transferred to a second white microtiter plate, and 40 µl of *Renilla* assay enhancer solution was added to the wells. Coelenterazine (Promocell) in the appropriate assay buffer was then injected into the well and luminescence was measured as described above.

Statistical analysis

Data were summarized as means±s.e.m. and the statistical significance assessed using two-tailed Student's *t*-tests or ANOVA with Tukey's or Dunnett's multiple comparison test, as indicated.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/2/252/DC1

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