Regulation of Glucocorticoid Receptor Activity by a Stress Responsive Transcriptional Cofactor

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The activity of the glucocorticoid receptor (GR) is modulated by posttranslational modifications, protein stability, and cofactor recruitment. In this report, we investigated the role of the stress-responsive activator of p300/tetratricopeptide repeat domain 5 (TTC5), in the regulation of the GR. TTC5 is a member of the TTC family of proteins and has previously been shown to participate in the cellular response to DNA damage and heat shock. Here, we demonstrate that TTC5 is an important cofactor for the nuclear hormone receptors GR and estrogen receptor. GR and TTC5 interact through multiple tetratricopeptide repeat and LXXLL motifs. TTC5 stabilizes GR and increases its half-life, through a proteasome-dependent process and by inhibiting the actions of the ubiquitin ligase murine double minute 2. Cellular stress, including DNA damage, proteasome inhibition, and heat shock, modulates the interaction pattern of GR/TTC5, thereby altering GR stability and transcriptional activity. Furthermore, GR transcriptional activity is regulated by TTC5 in both a positive and negative fashion under DNA damage conditions in a target gene-specific way. In this report we provide evidence supporting the notion that TTC5 is a novel cofactor regulating GR function in a stress-dependent manner. (***Molecular Endocrinology*** **25: 58–71, 2011)**

The glucocorticoid receptor (GR) is a member of the nuclear hormone receptor superfamily that is activated as a transcription factor upon binding glucocorticoids. Glucocorticoids are lipophilic hormones released from the adrenal cortex in response to stress that bind to the GR in the cytoplasm of cells and trigger its release from an inactive complex containing heat shock proteins and immunophilins. Once active, GR regulates the transcription of a large range of genes, both in a positive and negative fashion, regulating processes such as metabolism, inflammation, and the immune response (1). Due to the diverse range of GR actions, its activity is regulated at multiple levels, including protein stability, posttranslational modifications, and cofactor interactions.

The stability of the GR is an important factor in regulating its transcriptional activity. Upon long-term exposure to hormone, GR is down-regulated in a proteasome-dependent process (2). GR proteasomal degradation is signaled by ubiquitination of the receptor. Indeed, GR has been shown to interact with members of the ubiquitin pathway, and in particular, the PEST motif in the vicinity of the DNA-binding domain has been shown to be targeted for this modification (2). In addition, the phosphorylation status of the receptor is thought to have an effect on its protein stability (3). Murine double minute 2 (Mdm2), CHIP (C terminus of Hsp70-interacting protein), and E6-AP (E6-associated protein) are ubiquitin ligases that play a role in the proteasomal degradation of GR (3–9). The proteasome machinery, apart from regulating GR degradation, has also been shown to regulate its transcriptional activity. The proteasome is required for the rapid exchange of GR at the promoter, and its inhibition affects the transcriptional function of the receptor in a gene-specific manner (10–12).

Many GR cofactors have so far been identified that either remodel chromatin or recruit the basal transcrip-

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Abbreviations: AF-2, Activating function 2; AR, androgen receptor; ATM, ataxia telangiectasia mutated; CBP, cAMP response element-binding protein binding protein; cdc, cell division cycle; ChIP, chromatin immunoprecipitation; Dex, dexamethasone; DHT, dihydroxytestosterone; DRIP, vitamin D receptor interacting protein; ER, estrogen receptor; Est, estradiol; Eto, etoposide; β-gal, β-galactosidase; GILZ, glucocorticoid-induced leucine zipper; GR, glucocorticoid receptor; HA, hemagglutinin; HAT, histone acetyl transferase; Hdm2, human homologue of Mdm2; Hsp, heat shock protein; JMY, junction mediating and regulatory protein; MCJ, methylation controlled DNA J; Mdm2, murine double minute 2; MMTV, mouse mammary tumor virus; NR, nuclear receptor; qRT-PCR, quantitative real-time PCR; SCR-1, steroid receptor coactivator 1; siRNA, small interfering RNA; SLC19A2, solute carrier 19A2; TAT, tyrosine amino-transferase; TPR, tetratricopeptide repeat; TTC5, tetratricopeptide repeat domain 5; WT, wild type.

tional machinery to the GRE sites within the promoters of the receptor's targets. The major corepressors identified for GR are NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid hormone receptor), which bind the receptor in the absence of hormone and repress GR-dependent transcription by recruiting histone deacetylases to the transcriptional complex (13). NCoR and SMRT both contain nuclear receptor (NR) interaction domains, called CoRNR boxes, or LXXI/HIXXXI/L helix motifs (14). These motifs form extended helices, which bind NRs in the hydrophobic pocket within their C-terminal activating function 2 (AF-2) domains (15). The conformational change occurring in this domain upon ligand binding inhibits the attachment of a corepressor and triggers NRs release from the cytoplasmic complex (16). Coactivators interact with GR via either the AF-1 or AF-2 domains. Binding to the AF-2 domain is hormone dependent, and coactivators such as members of the p160 family, p300/cAMP response element-binding protein binding protein (CBP), *BRG1* (Brahma-related gene 1), PCAF (p300 CBP associated factor), and vitamin D receptor interacting protein 205 (DRIP205)/TRAP220 (thyroid receptor associated protein 220), interact with this domain (17–20). The GR conformational change taking place upon hormone binding generates a surface capable of binding to the LXXLL motif present in its coactivators. The LXXLL motif has been shown to be necessary for the interaction between NR and numerous coactivators (21).

AF-1 is a hormone-independent activation function located in the N-terminus of the receptor, but it is not conserved in sequence or structure among the NR family (22). The coactivators' motif necessary for binding to AF-1 has not yet been characterized. However, proteins such as members of the p160 family, DRIP150, and the tumor susceptibility gene 101 (TSG101) have been shown to interact with this domain (22–26). Because members of the p160 family, as well as the DRIP/TRAP complex of proteins, can interact with both AF-1 and AF-2 domains, it is possible that the two domains can be cooperatively linked (20). The p160 coactivator family is a well-characterized group, including steroid receptor coactivator 1 (SRC-1), SRC-2, and SRC-3, which bind AF-2 of GR in a ligand-dependent manner. These proteins coactivate GR using their intrinsic histone acetyl transferase (HAT) activity and through recruitment of other cofactors, including the HATs p300/CBP (18). p300/CBP is also able to bind GR directly, in a hormone-dependent fashion, and serves as a coactivator through acetylating histones and recruiting other HATs and RNA polymerase II to transcription start sites. p300 is an important coactivator for many transcription factors, including p53, where it works as part of a multiprotein complex containing other transcriptional cofactors, such as PCAF, tetratricopeptide repeat domain 5 (TTC5), and junction mediating and regulatory protein (JMY) (27). TTC5 in particular is stabilized in response to DNA damage after phosphorylation by ataxia telangiectasia mutated (ATM) and aids the formation of the p300/JMY/TTC5 complex (28–31).

TTC5 is composed of six protein-protein interaction tetratricopeptide repeat (TPR) motifs distributed throughout its sequence and also contains one LXXLL NR interaction motif between TPR4 and TPR5. TPR motifs were first identified in the yeast cell division cycle (cdc) proteins cdc16 and cdc23, which form the anaphase promoting complex and have been found in functionally unrelated proteins (32–34). The motif, which often occurs in tandem arrays, is made up of 34 amino acids, which are not generally conserved but show similarities in size, hydrophobicity, and spacing (34). However, there are eight consensus residues, conserved more often throughout TPR motifs, which are possibly required for the maintenance of the TPR structure or for specific protein-protein interactions. TPR motifs are involved in many cellular functions, including transcriptional regulation, protein degradation, protein folding, protein transport, and phosphate turnover. Interestingly, with regards to nuclear hormone receptors, heat shock protein (Hsp)90 and Hsp70 interact with cochaperones bearing TPR motifs, such as the FKBP52 (FK506-binding protein 52) and Hop (Hsp70/Hsp90 organizing protein) or the PP5 (protein phosphatase, 5) which contribute to the regulation of GR (35).

Here, we present evidence suggesting that the chaperone-like and stress-responsive protein TTC5 is a GR coregulator. In particular, TTC5 adjusts GR function in response to hormone and DNA damage by modulating the receptor's protein stability and transcriptional activity. Our data suggest that TTC5 coordinates the glucocorticoid and estrogen receptors (ERs)-mediated response to hormonal and cellular stress signals.

Results

TTC5 interacts with the GR

To explore the possibility that the stress-responsive TTC5 cofactor of p53 serves as a regulator of the GR function, we investigated the interaction between TTC5 and GR. For this purpose, various recombinant versions of TTC5 were expressed and purified from *Escherichia coli* [wild type (WT); derivatives carrying amino acids 1-220 or 220-440] (Fig. 1A). *In vitro* pull-down assays using these purified derivatives and extract from A549 cells expressing endogenous WT GR indicated that the full-length and the C-terminal domain (220-440) of TTC5 interacted specifically with GR,

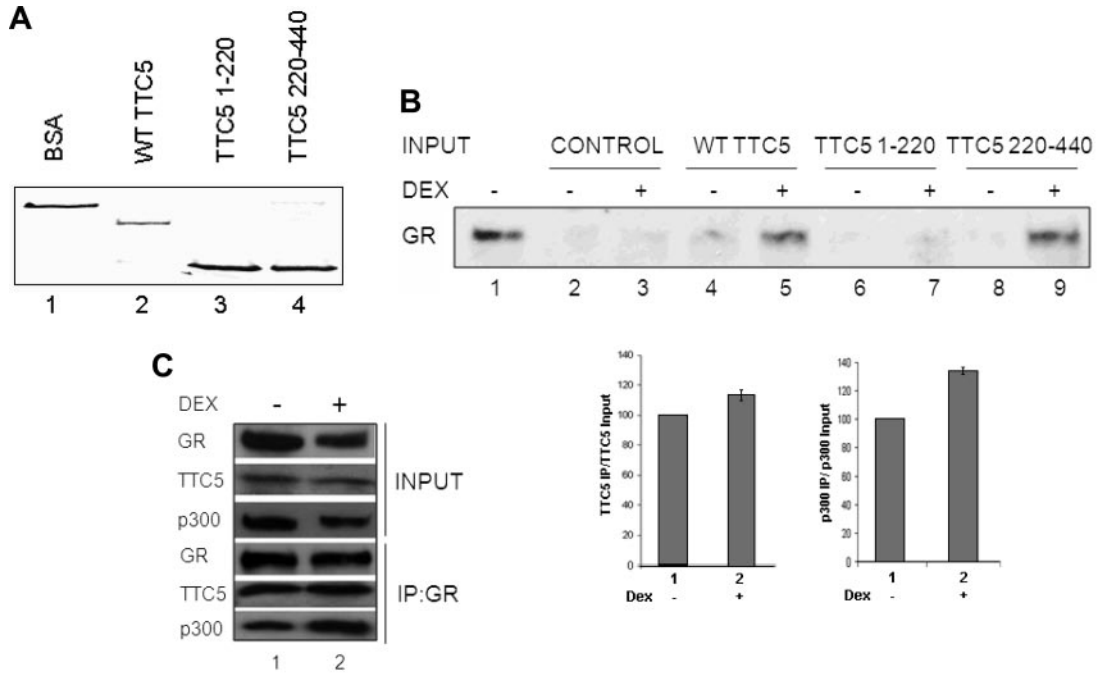

FIG. 1. TTC5 interacts with the GR. A, Nickel beads carrying WT TTC5 fusion protein (lane 2), the first three TPR motifs (1-220) (lane 3), or the last three TPR motifs (220-440) (lane 4) were expressed and purified from *E. coli* and resolved on SDS-PAGE and Coomassie blue stained. Indicated proteins were used in *in vitro* pull-down assays. B, A549 cell lysates were incubated with nickel beads (lanes 2 and 3), his-tagged WT TTC5 attached to the beads (lane 4 and 5), TTC5 derivative with the deletion of the three C-terminal TPR motifs (TTC5 1-220) (lanes 6 and 7), or TTC5 derivative with the deletion of the three N-terminal TPR motifs (TTC5 220-440) (lanes 8 and 9) in the presence or absence of 1 μM Dex, for 1 h. Levels of GR pulled down by TTC5 were detected by Western blot analysis using GR specific antibody; 5% of input sample was loaded in the lane 1. C, A549 cells treated with (lane 2) or without (lane 1) Dex (1 μM, 4 h) were used in an endogenous coimmunoprecipitation, where GR was immunoprecipitated with GR specific antibody, and protein levels were analyzed by Western blotting, using antibodies specific for the indicated proteins. *Upper panels* show input levels of proteins, and *lower panels* show the coimmunoprecipitation. *Graphs* show quantification of TTC5 and p300 protein levels coimmunoprecipitated with GR and normalized to input from C. *Error bars* show SD obtained from three independent quantifications.

and this interaction occurred in a glucocorticoid hormone-dependent manner (Fig. 1B, compare lanes 4 and 5 with 8 and 9, respectively). To investigate whether this interaction occurred in cells with physiologically relevant GR and TTC5 protein levels, we performed coimmunoprecipitation experiments in A549 cells (Fig. 1C). Interaction between endogenous GR and both TTC5 and p300 cofactors was observed in A549 cells and moderately but consistently increased with dexamethasone (Dex) treatment (Fig. 1C), supporting the view that GR and TTC5 interact and that this interaction can be increased by hormone treatment in A549 cells.

To test whether TTC5 interacts with multiple nuclear hormone receptors, we followed the interaction between ER and TTC5 in Cos-7 cells overexpressing either GR (Fig. 2A, lanes 1 and 2), ER (Fig. 2B, lanes 1 and 2), or androgen receptor (AR) (Fig. 2C, lanes 1 and 2), together with hemagglutinin (HA)-TTC5. Both GR and ER interacted with TTC5, and the presence of hormone increased the affinity of this interaction, whereas hormone didn't significantly change AR/TTC5 complex formation. Taken together, these results indicate that TTC5 interacts with multiple members of the nuclear hormone receptor superfamily, and their binding is regulated in a complex manner.

To investigate possible variations in the interaction between GR and TTC5 under conditions of cellular stress, such as DNA damage, we followed the pattern of this interaction in Cos-7 cells exposed to various types of stress (Fig. 3). The interaction of GR with TTC5 in-

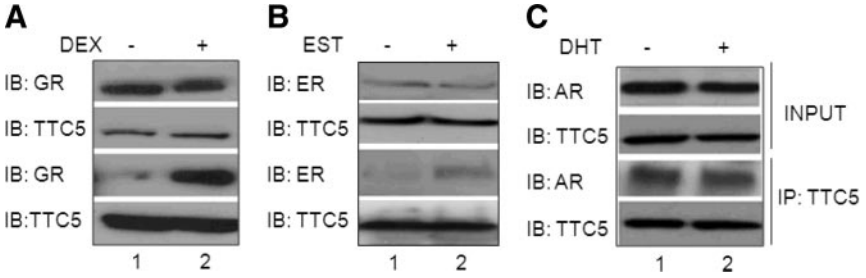

FIG. 2. Regulation of TTC5/NR interaction by hormone. Cos-7 cells were transfected with pSG5-WTGR and pCDNA3-HA-TTC5 (lanes 1 and 2) (A), pSG5-HEOα and pCDNA3-HA-TTC5 (lanes 1 and 2) (B), or pSV-AR and pCDNA3-HA-TTC5 (C). Cells were treated with 100 nM Dex (lane 2) (A), 100 nM β-Est (lane 2) (B), or 100 nM DHT (lane 2) (C) for 4 h. HA polyclonal antibody was used to immunoprecipitate HA-TTC5, and membranes were probed with anti-GR antibody (lanes 1 and 2), anti-ER antibody, anti-AR antibody, and HA monoclonal antibody to detect TTC5. *Top panels* show input levels of protein, and *lower panels* coimmunoprecipitation results. IB, Immunoblot; IP, immunoprecipitation.

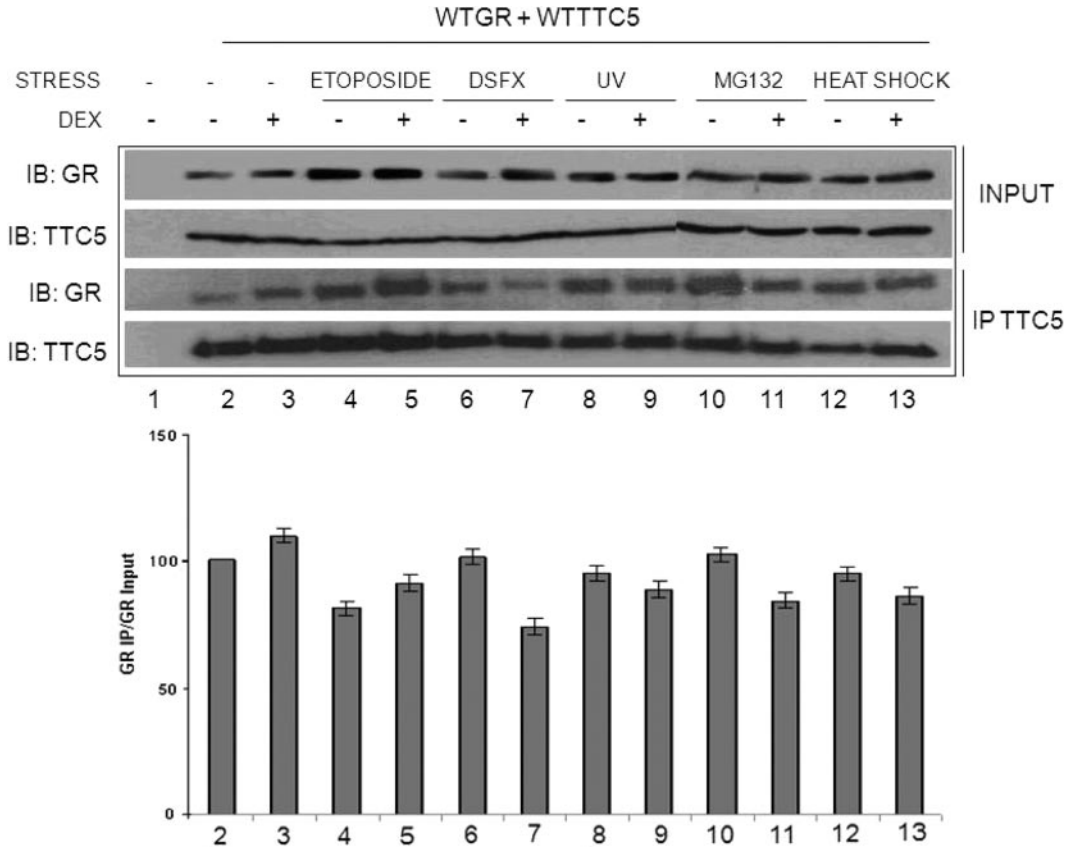

FIG. 3. Regulation of TTC5/GR interaction by stress signals. Cos-7 cells were transfected with pcDNA3 (lane 1) and pSG5-WTGR and Flag-TTC5 (lanes 2–13). Cells were treated with no hormone (lanes 1, 2, 4, 6, 8, 10, and 12), 100 nM Dex for 4 h (lanes 3, 5, 7, 9, 11, and 13), no stress (lanes 1–3), 25 μM Eto (lanes 4 and 5), 25 μM desferoxamine (lanes 6 and 7), 60 J/m^2 UV radiation (lanes 8 and 9), 1 μM MG132 (lanes 10 and 11), or heat shock (43 C, 15 min) (lanes 12 and 13). *Error bars* show SD obtained from three independent quantifications.TTC5 was immunoprecipitated using Flag beads and Westerns blottings probed with antibodies specific for GR and TTC5. *Upper panels* show input levels of proteins, and *lower panels* show the coimmunoprecipitation. *Graphs* show quantification of GR protein levels coimmunoprecipitated with TTC5 and normalized to input. IB, Immunoblot; IP, immunoprecipitation; DSFX, desferoxamine.

creased in Dex-treated and nonstressed cells (Fig. 3, lanes 2 and 3) and increased when cells were exposed to DNA damage conditions induced by etoposide (Eto) in a hormone-dependent manner (Fig. 3, lanes 4 and 5). Hypoxia-mimicking conditions seemed to reduce the affinity of the interaction between GR and TTC5 in the presence of hormone (Fig. 3, compare lane 7 with lane 6). When cells were treated with the proteasome inhibitor MG132, the interaction increased in the absence, but was weaker in the presence, of hormone (Fig. 3, lanes 10 and 11). Cells treated with UV irradiation and heat shock did not exhibit a hormone-dependent increase in GR-TTC5 interaction (Fig. 3, lanes 8 and 9 and lanes 12 and 13, respectively).

Overall, these results suggest that cellular stress is able to modulate the interaction between GR and TTC5, in a stress-specific manner. Also, the hormone dependency of the interaction appears to change under different types of cellular stress.

Mapping the interaction surfaces of TTC5 with GR

Next, to elucidate the molecular details of the GR-TTC5 interaction, we determined the domains involved in the association between the two proteins. For this purpose, we mutated crucial residues in each one of the TPR domains, as well as in the LXXLL motif of TTC5 (Fig. 4A). The last two leucine residues in the LTSLL motif were mutated to alanines (LTSAA), a mutation which should be sufficient to inhibit the GR-TTC5 interaction if this motif was important for the binding between the two proteins (21). Immunoprecipitation of TTC5 and subsequent blotting to identify interacting GR showed that the LTSAA mutant TTC5 derivative was capable of interacting with GR to a greater extent in the absence and to a lesser extent than the WT TTC5 in the presence of hormone (Fig. 4B, *third panel*). These findings were quantified and confirmed by densitometric scanning (Fig. 4B, *lower panel*). This suggests that although LTSLL motif might be involved in the interaction between TTC5 and GR, this motif is not the only TTC5 domain responsible for the complex formation between the two proteins.

To identify other potential interaction domains between TTC5 and GR, we tested whether the individual TPR motifs could play any role. Mutants of TTC5 were made, where a crucial residue in each motif (either residue 8 or 20) (34) was mutated to an alanine or a glycine residue, and these TTC5

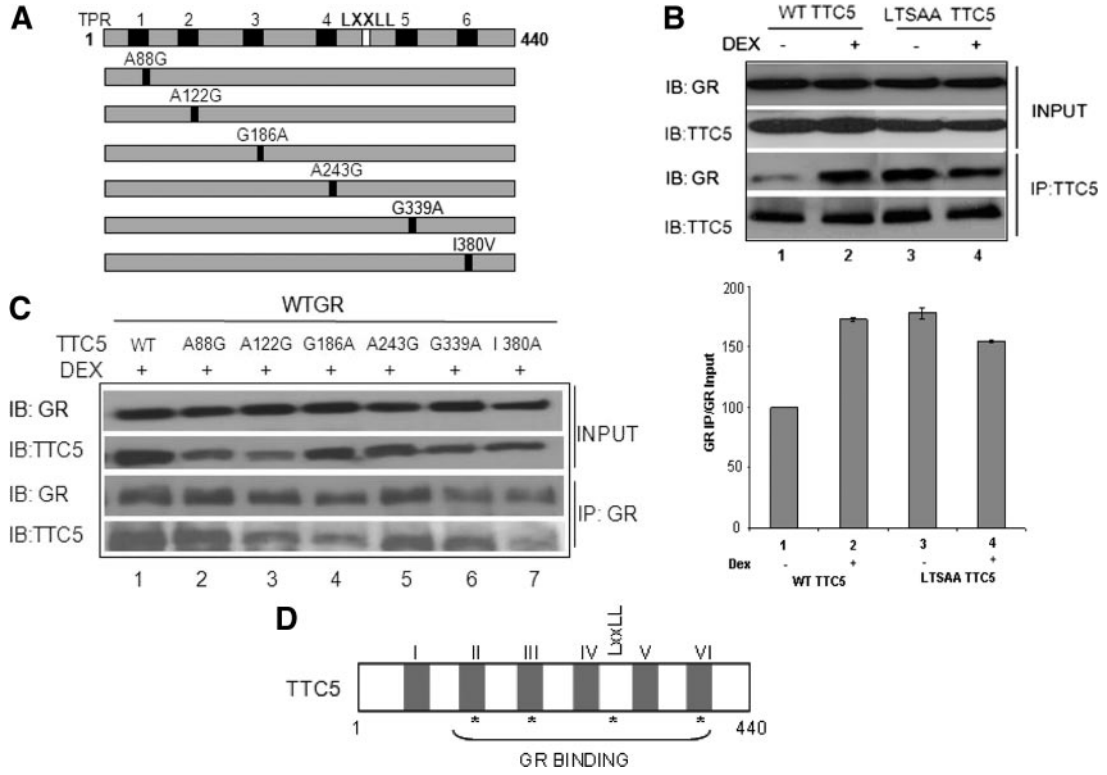

FIG. 4. Multiple domains of TTC5 are required for GR interaction. A, TTC5 is composed of six TPR motifs and a LXXLL motif; each TPR motif had a crucial residue mutated as indicated. B, Cos-7 cells were transfected with pCDNA3-WTGR and pCDNA3-HA-WT-TTC5/HA-LTSAA-TTC5 and treated with Dex (100 nM for 4 h; lanes 2 and 4). TTC5 was immunoprecipitated, and interacting proteins were detected by Western blot analysis using anti-GR and anti-TTC5 antibodies. Lanes 1 and 2 show levels of proteins coimmunoprecipitated with WT TTC5, and lanes 3 and 4 show levels of proteins coimmunoprecipitated with LTSAA TTC5. Densitometry of indicated blots is shown *below the blots*. *Error bars* represent SD obtained from three independent quantifications. C, Cos-7 cells were transfected with pCDNA3-WTGR and pCDNA3-HA-TTC5 variants containing mutations within TPR motif as indicated. GR was immunoprecipitated from cells treated with 100 nM Dex for 4 h, coprecipitated proteins resolved on SDS-PAGE and detected by Western blot analysis using GR and TTC5 specific antibodies. *Top panels* show input levels of proteins, and *lower panels* show immunoprecipitation. IB, Immunoblot; IP, immunoprecipitation. D, *Schematic diagram* showing GR interaction surfaces on TTC5.

mutants were used in binding reactions with GR (Fig. 4C). Mutations in TPR motifs 2: A122G (lane 3), 3: G186A (lane 4), and 6: I380A (lane 7) seemed to reduce the most the binding affinity between GR and TTC5. This implied that the LXXLL motif and the TPR motifs 2, 3, and 6 were mainly responsible for the interaction between GR and TTC5 (Fig. 4D).

TTC5 regulates the stability of the GR

Taking into account the fact that TTC5 is important in the regulation of p53 protein stability, we hypothesized that the interaction between TTC5 and GR could have as a functional consequence the stabilization of GR. We investigated whether TTC5 stabilizes GR through reducing proteasomal degradation in Cos-7 cells treated with or without the proteasome inhibitor MG132 (2). Treating cells with MG132 considerably reduced GR degradation in the presence of hormone (Fig. 5A, compare lane 3 with lane 4 in the *first and third panels*). Transfection of TTC5 and treatment of cells with MG132 did not have an additive effect on the levels of GR (+MG132) (Fig. 5A, compare lanes 5 and 6 with lanes 3 and 4 in the *third panel*), suggesting that both TTC5 and MG132 work through a common mechanism, *i.e.* inhibiting proteasome-mediated degradation of GR. However, the fact that there is less GR in MG132-treated cells as compared with untreated cells transfected with TTC5 (Fig. 5A, lane 6, compare *third* with the *first panel*) suggests that MG132, together with TTC5, operates through a complex mechanism to regulate the stability of GR.

To substantiate these observations, we sought to assess the half-life of endogenous GR in A549 cells transfected with either a control vector or TTC5 and treated with or without Dex in the presence of cycloheximide. A more rapid decline in GR levels was seen in the presence of Dex in accord with previously published observations (3). Transfection of TTC5 increased GR half-life in the absence of hormone from 16 to 24 h (Fig. 5, B *top panels* and C). Transfection of TTC5 in Dex-treated A549 cells resulted in increased GR half-life from 8 to 9 h (Fig. 5, B *lower panels* and C). Moreover, in the presence of hormone, GR was stabilized in cells transfected with the WT and the TPR 5 mutant G339A TTC5 that binds to GR, whereas the receptor was not stabilized in cells expressing the mutant TPR 6 I380A TTC5 that binds inefficiently to

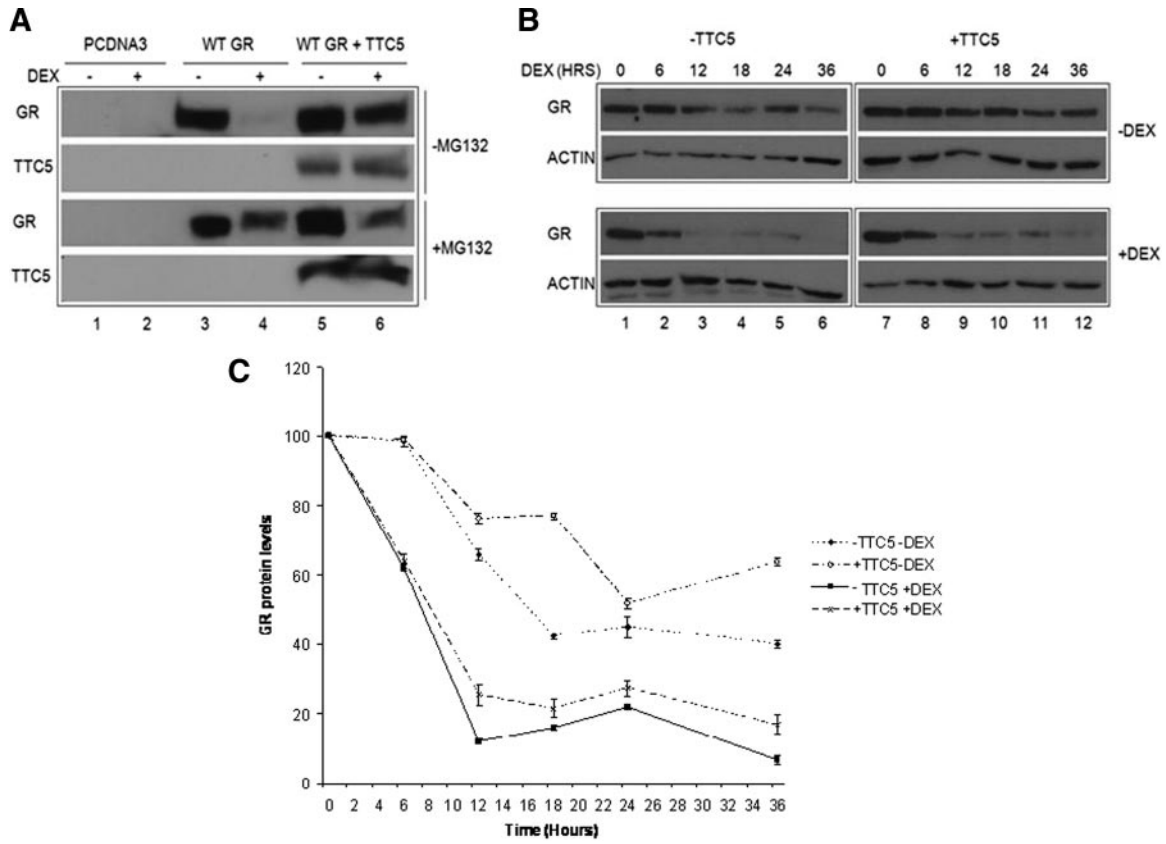

FIG. 5. TTC5 increases the half-life of the GR protein. A, Cos-7 cells were transfected with pcDNA3 (lanes 1 and 2), pSG5-WTGR (lanes 3 and 4), and pSG5-WTGR and pcDNA3-HATTC5 (lanes 5 and 6). Cells were treated with or without 100 nM Dex for 16 h. Experiments were carried out either in the absence (*top panels*) or presence (*lower panels*) of MG132 (1 μM, 17 h). Proteins were detected by Western blot analysis using GR-specific (*first and third panel*) and HA antibodies (*second and fourth panel*). B, A549 cells were transfected with either pcDNA3 (*left hand panels*) or TTC5 (*right hand panels*) and treated with cyclohexamide (30 μM), 1 h before the addition of Dex. Only cells in the *lower panels* received hormone treatment (100 nM for the indicated times) in addition to cyclohexamide. Endogenous GR protein levels were detected by Western blot analysis with GR specific antibody, and actin served as a loading control. C, Western blottings obtained in B were densitometrically scanned and GR normalized to actin protein levels were plotted. *Error bars* show SD obtained from three independent quantifications.

GR (Figs. 4C, lanes 6 and 7, and 6A, compare lanes 1 and 2 with lanes 3 and 4, respectively). Furthermore, TTC5 is essential for the stabilization of endogenous GR, as shown in cells transfected with small interfering RNA (siRNA) against TTC5. GR was down-regulated in hormone-treated cells when TTC5 expression was silenced (Fig. 6B, compare lanes 6 and 8 with lanes 2 and 4). These findings suggest that binding of TTC5 to GR through TPR 6 is necessary for complex formation and receptor protein stabilization.

TTC5 has been shown to stabilize p53 by inhibiting Mdm2 E3 ligase activity (28), and a role of Mdm2 in GR degradation has been proposed (4). To determine whether TTC5 can stabilize GR in the presence of Mdm2, GR protein levels were followed in Cos-7 cells transfected with WT GR, in the presence or absence of TTC5 and Mdm2. GR levels decreased both in the presence and absence of hormone, when Mdm2 was present (Fig. 6C, lanes 3 and 4 compared with lanes 1 and 2). Mdm2-mediated GR degradation was limited in the presence of TTC5 (Fig. 6C, lanes 5 and 6 compared with lanes 3 and 4), implying that Mdm2 down-regulates GR, and this effect can be limited by TTC5.

TTC5 is a transcriptional coregulator of the GR

The effect of TTC5 on GR transcriptional activity was studied by luciferase reporter assays employing three known GR-responsive promoters, namely tyrosine aminotransferase (TAT), mouse mammary tumor virus (MMTV), and glucocorticoid-induced leucine zipper (GILZ) reporters in A549 cells. GR transcriptional activity increased in the presence of TTC5 in hormone-treated cells in the case of TAT and GILZ reporters (Fig. 7, A and C, respectively, compare *bar* 2 with *bar* 4) but had a weak repressive effect on MMTV reporter gene activity (Fig. 7B, compare *bar* 2 with *bar* 4), suggesting that TTC5 regulates GR in a promoter-specific manner. Furthermore, GR transactivation on GILZ reporter by TTC5 (Fig. 7C) implied that this coactivator is involved in the induction of the antiinflammatory function of GR. Similar results were obtained when this assay was performed in Cos-7 cells (data not shown).

To investigate whether TTC5 had a coregulatory function for other members of the steroid receptor family, we cotransfected Cos-7 cells with the ER expression vector together with an ER responsive reporter (Fig. 7D). TTC5

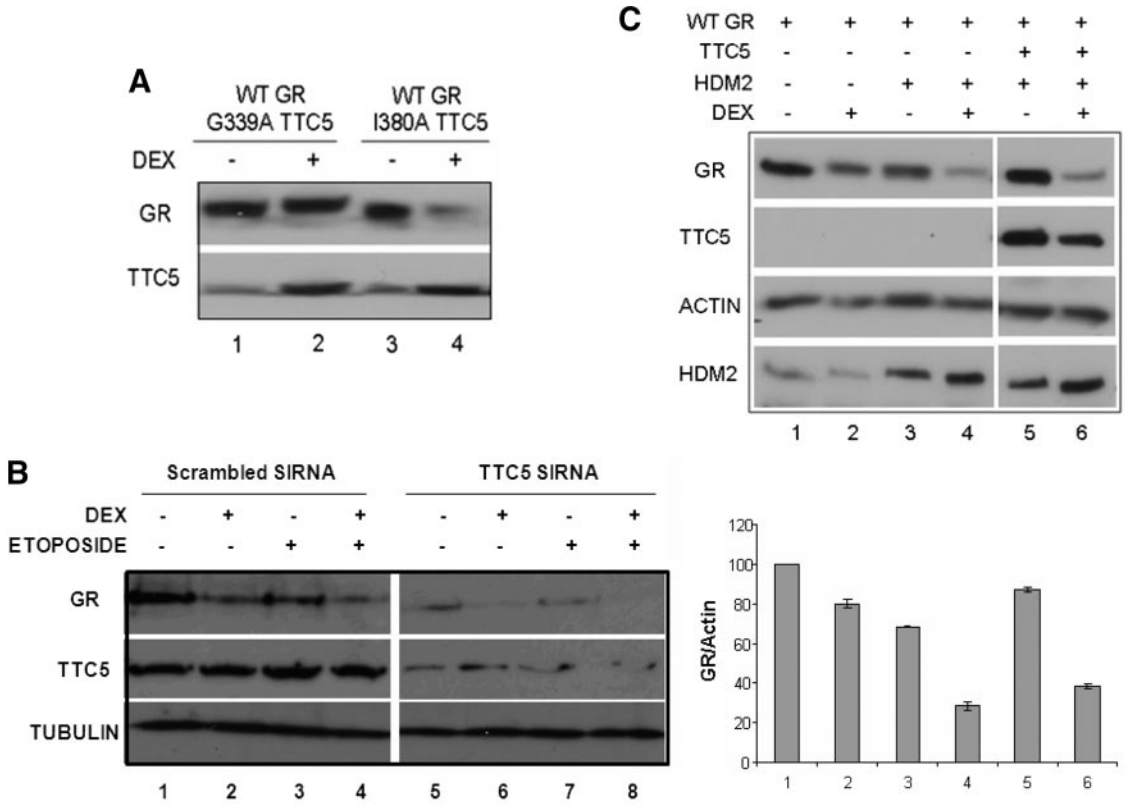

FIG. 6. TTC5 stabilizes GR by competing with Mdm2. A, Cos-7 cells were transfected with pcDNA3-HA G339A TTC5 (lanes 1 and 2) or I380A TTC5 (lanes 3 and 4) in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 100 nM Dex for 16 h. GR and TTC5 protein levels were analyzed by Western blotting with anti-GR and HA antibodies, respectively. B, A549 cells were transfected with either scrambled siRNA (lanes 1–4) or TTC5 siRNA (lanes 5–8). Cells were treated with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) 100 nM Dex for 16 h, and cells in lanes 3, 4, 7, and 8 were treated with 25 μM Eto for 16 h. Protein levels were analyzed by Western blotting using antibodies specific for GR, TTC5, and tubulin. C, pSG5-WTGR (all lanes), pcDNA3-HATTC5 (lanes 5 and 6), and Hdm2 (lanes 3–6) were transfected into Cos-7 cells. Cells were treated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) 100 nM Dex for 16 h. Proteins were detected by Western blot analysis using antibodies specific for the indicated proteins. *Graph* shows quantification of GR protein levels normalized to actin levels from the C. *Error bars* show SD obtained from three independent quantifications.

increased ER transcriptional activity by 2-fold in estradiol (Est)-treated cells on this reporter (Fig. 7D, compare *bar* 4 with *bar* 6). On the other hand, TTC5 had no effect on AR activity in dihydroxytestosterone (DHT)-treated Cos-7 cells, as monitored by the AR-responsive promoter MMTV (Fig. 7E, compare *bar* 4 with *bar* 6).

TTC5 differentially regulates GR transcriptional response under diverse conditions of cellular stress

Because TTC5 is a stress-responsive cofactor and its interaction with GR is regulated by the type of extracellular stress, we investigated the effect of cellular stress on GR-mediated transcriptional regulation. The effect of TTC5 under diverse types of stress on endogenous GR-directed transcription was assessed using A549 cells transfected with the TAT3, MMTV, and GILZ luciferase reporters and treated with the indicated type of stress (Fig. 8). Eto treatment reduced GR-dependent transcription at all promoters tested, but to varying extents (Fig. 8, A–C, *bars* 5 and 6). Inhibiting the proteasome through MG132 treatment induced TAT transactivation but repressed MMTV and GILZ (Fig. 8, *bars* 9 and 10), whereas heat shock had a weak repressive effect on GR transcriptional activity (Fig. 8, *bars* 13 and 14). The cofactor function of TTC5 was altered by stress in a promoter-specific manner. In Eto-treated cells, TTC5 further increased hormone-dependent transcription on TAT3 and GILZ reporter genes and had no effect on MMTV reporter expression (Fig. 8, compare *bar* 6 with *bar* 8). MG132 augmented the effect of TTC5 on all three reporters (Fig. 8, compare *bars* 2 and 4 with *bars* 10 and 12). In heat-shocked cells, the effect of TTC5 was blunted at TAT3 and MMTV and resulted in a further increase of GILZ promoter activity (Fig. 8, compare *bars* 2 and 4 with *bars* 14 and 16, respectively).

TTC5 silencing affects the transcriptional activity of GR in a target-specific manner

To investigate whether TTC5 exerted endogenously the same effect on GR target genes, we silenced TTC5 expression in A549 cells using siRNAs and analyzed the expression of various GR target genes using quantitative real-time PCR (qRT-PCR) (Fig. 9, A–D). In addition to

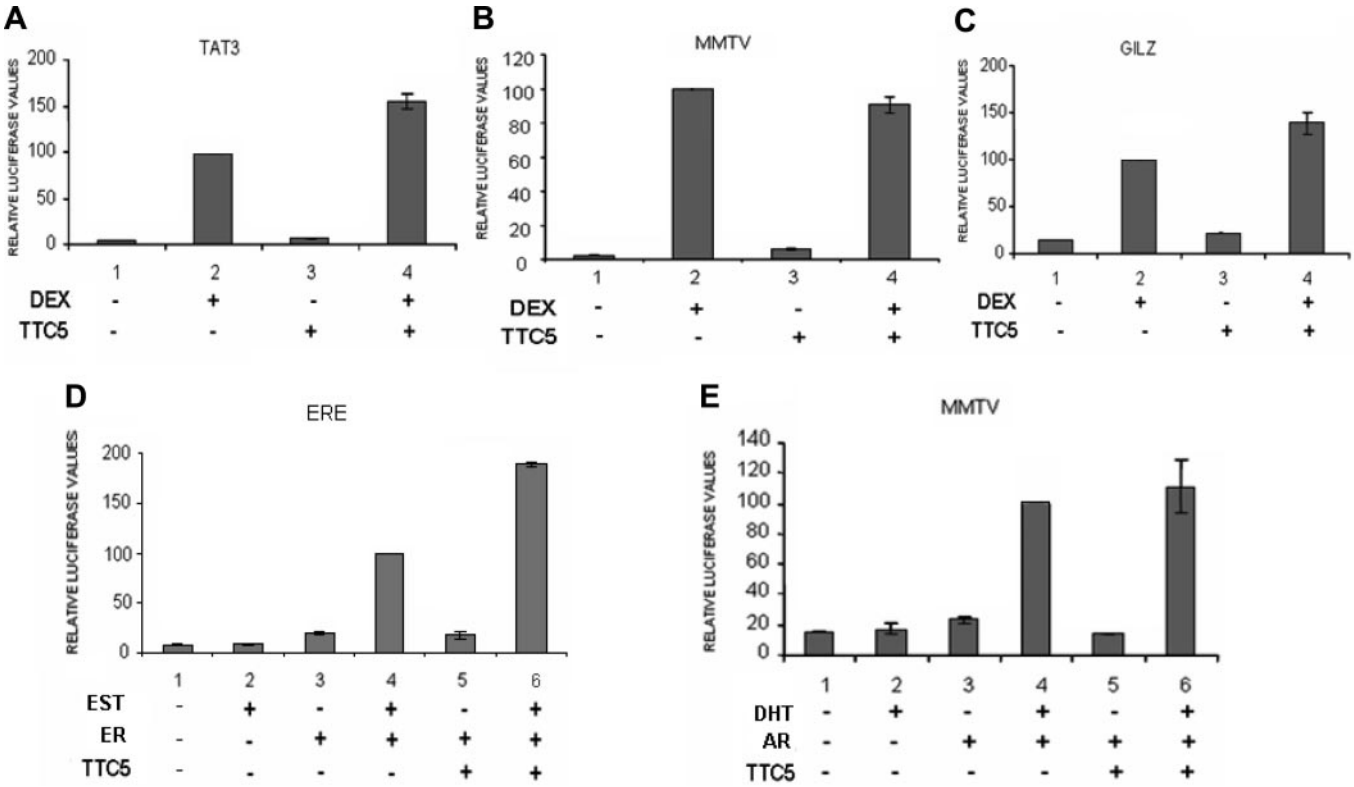

FIG. 7. TTC5 is a transcriptional coregulator of the GR. A549 cells were transfected with TAT-luciferase reporter construct (TAT3) (A), MMTV-luciferase (MMTV) (B), or GILZ-luciferase (GILZ) (C) constructs, as well as CMV-β-gal, without (lanes 1 and 2) or with (lanes 3 and 4) TTC5. Cells were treated with or without 100 nM Dex for 16 h. Luciferase readings were normalized to the β-gal readings. D, pSG5-HEO (lanes 3–6), ERE-Luc, CMV-β-gal, and TTC5 (lanes 5 and 6) were transfected into Cos-7 cells, which were treated with 100 nM β-Est for 16 h. E, Cos-7 cells were transfected with MMTV-luciferase, CMV-β-gal, pSG5-AR (lanes 3–6), and TTC5 (lanes 5 and 6). Cells were treated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) 100 nM DHT for 16 h. Data are representative of three or more independent experiments showing similar results. *Error bars* represent SD.

Dex, cells were treated with Eto to assess the effect of DNA damage on TTC5's role as a GR transcriptional cofactor. Silencing of TTC5 resulted in substantial reduction of hormone-dependent induction of GILZ and p57/KIP2 (kinase-inhibitory protein 2) target gene expression (Fig. 9, A and B, compare *gray* with *black bars* 1 and 2). In contrast, TTC5 corepressed GR in the case of solute carrier 19A2 (SLC19A2) gene (Fig. 9D, compare *gray* with *black bars* 1 and 2). Inhibition of TTC5 by siRNA did not have a significant effect on methylation controlled DNA J (MCJ) target gene in hormone-treated cells.

Eto treatment did not significantly affect the expression of any of the GR target genes that we studied in the absence of hormone (Fig. 9, A–D, *gray bars* 3). In cells treated with both hormone and Eto, enhancement of GILZ and SLC19A2 gene expression was observed (Fig. 9, A and D, respectively, compare *gray bar* 2 with *gray bar* 4), whereas mRNA levels of p57/KIP2 and MCJ decreased (Fig. 9, B and C, respectively, compare *gray bar* 2 with *gray bar* 4). Transfection of siRNA against TTC5 had an inhibitory effect on SLC19A2 gene expression in hormone and Eto-treated cells (Fig. 9D, compare *black bar* 2 with *black bar* 4) and weak or no effect on the rest of the genes studied.

Taken together, these results show that TTC5 is required for the regulation of several GR targets, and it can exert both positive and negative effect depending on the specific gene (Fig. 9). Because the main differences are seen in the presence of Dex, this suggests that the effect of TTC5 is dependent on active GR.

TTC5 is present on the GILZ promoter and occupancy depends on proteasome activity

To analyze the effect of glucocorticoids on both GR and TTC5 recruitment to a GR regulated promoter, chromatin immunoprecipitation (ChIP) assays were carried out in A549 cells treated with either Dex or a proteasome inhibitor individually or in combination (Fig. 10). Analysis of GILZ promoter occupancy showed increased GR and TTC5 recruitment to this promoter in Dex-treated cells (Fig. 10, *gray and black bars*, respectively, lanes 1 and 2). To assess the role of the proteasome in GR TTC5 interaction and promoter recruitment, cells were treated with MG132 and Dex (Fig. 10). MG132 treatment inhib-

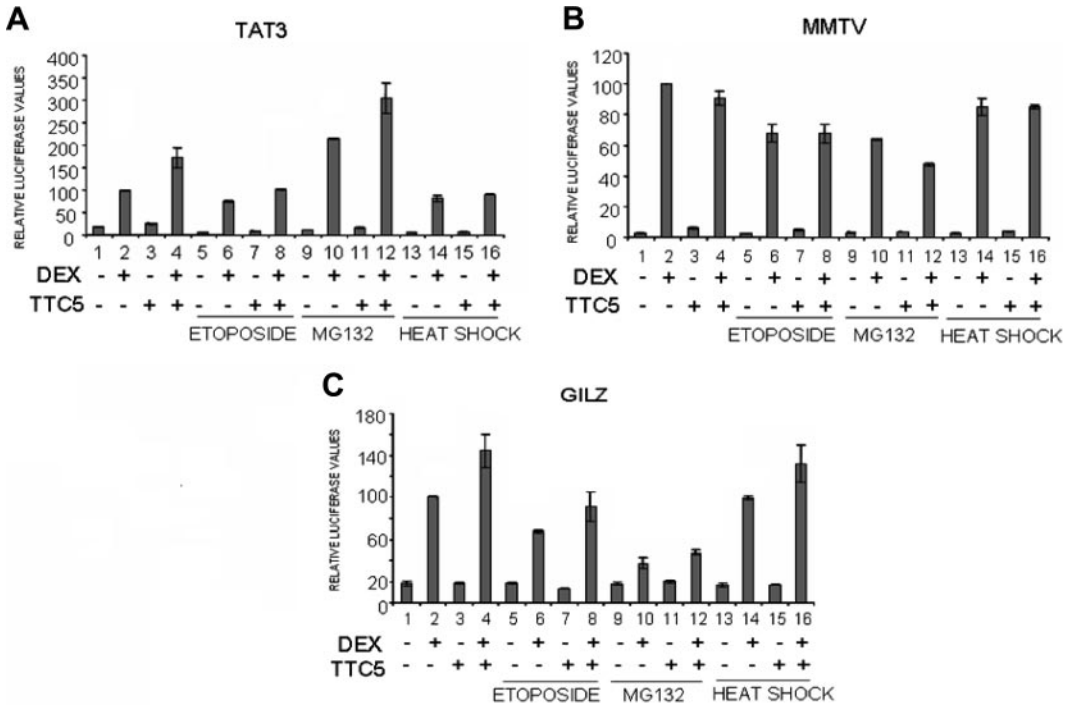

FIG. 8. Cellular stress affects the transcriptional response of the GR. A549 cells were transfected with TAT3-luciferase (A), with MMTV-luciferase (B), or with GILZ-luciferase (C) as indicated in Fig. 7. Cells were transfected with either pcDNA3 (lanes 1, 2, 5, 6, 9, 10, 13, and 14) or TTC5 (lanes 3, 4, 7, 8, 11, 12, 15, and 16). Cells in lanes 2, 4, 6, 8, 10, 12, 14, and 16 were treated with 100 nM Dex for 16 h, whereas the other lanes received no hormone treatment. Cells in lanes 5–8 were treated with 25 μM Eto, whereas lanes 9–12 were treated with 1 μM MG132 for 16 h. Cells in lanes 13–16 were heat shocked at 43 C, for 15 min, followed by recovery at 37 C overnight. Data are representative of three or more independent experiments showing similar results. *Error bars* represent SD.

ited both the recruitment of GR and TTC5 to the GILZ promoter, in the absence and presence of Dex (Fig. 10, lanes 3 and 4). The action of the proteasome is therefore important for GR promoter binding, in accord with previous reports (10, 12). The finding that TTC5 recruitment is also affected by proteasome inhibition suggests that the action of the proteasome is linked to TTC5's activity as a transcriptional cofactor.

Discussion

This work demonstrates that TTC5 is an important cofactor for GR, because it interacts with and stabilizes GR protein and regulates its transcriptional activity, particularly in response to stress. Many cofactors have been identified for the GR, but TTC5 is unusual in that it links the stability and transcriptional regulation of GR to the cellular stress response, and further to this, it regulates GR in a gene and stress-specific manner.

Our data suggest that TTC5 interacts with GR and ER in a hormone regulated manner, whereas its interaction with AR is not hormone inducible (Fig. 2). It is possible that TTC5 regulates a subset of the superfamily of nuclear hormone receptors and other transcription factors (28, 29, 36). However, the role of hormone in regulation of the interaction of TTC5 with members of this superfamily will have to be further investigated. Multiple motifs of TTC5 are involved in the interaction with GR (Fig. 4). The LXXLL motif of TTC5 is necessary for the binding with GR, because mutation of this sequence caused a modest but reproducible alteration in the interaction with the receptor. However, several TPR motifs of TTC5 are also required for the interaction with GR, including TPR motifs 2, 3, and most significantly 6, because mutation of crucial residues in these motifs reduces GR binding (Fig. 4). These findings indicate the involvement of the LXXLL motif and TPR motif 6 in binding to GR. Investigations to pinpoint the domains of GR involved in binding TTC5 suggested that multiple regions of GR or perhaps other factors could be involved (data not shown).

Binding between TTC5 and GR occurs in both the absence and presence of hormone, but higher affinity was observed upon hormone addition. The structure of TTC5 suggests that it may be important as a molecular scaffold protein and could recruit other cofactors to the GR transcriptional complex, with various enzymatic activities. TPR motifs are known for their role in protein-protein interactions, and TTC5 has six of these motifs found throughout its structure (28). TTC5 and GR have been reported to associate with other cofactors, such as p300,

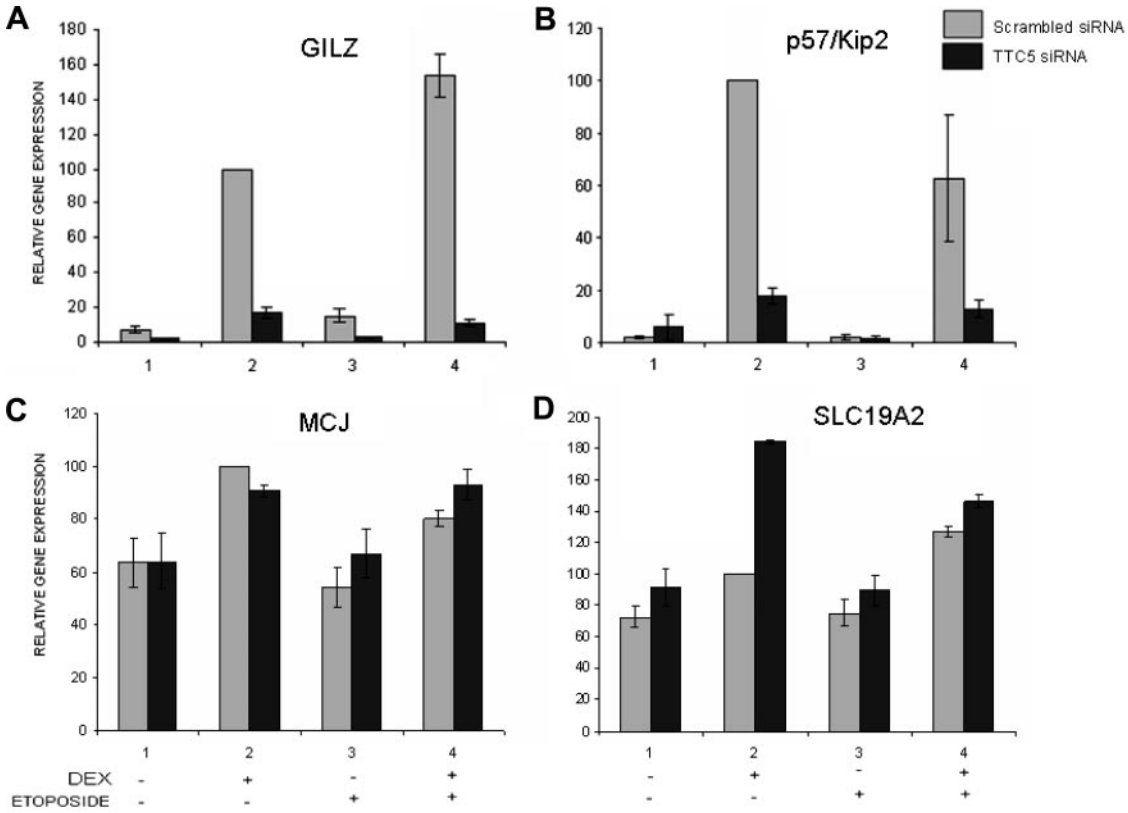

FIG. 9. TTC5 coregulates GR target gene expression in a gene-selective manner. A, A549 cells were transfected with either 100 nM scrambled siRNA (*gray bars*) or TTC5 siRNAs (*black bars*). Cells received either no treatment (lanes 1) or were treated with 100 nM Dex (lanes 2 and 4) and 25 μM Eto, both for 6 h (lanes 3 and 4). RNA was extracted from cells, reverse transcribed, and mRNA levels determined using specific primers in qRT-PCRs. All mRNA levels were normalized to the housekeeping gene Rpl19 to show relative gene expression of the GILZ. B, p57/Kip2. C, MCJ. D, SLC19A2. Data are representative of three or more independent experiments showing similar results. *Error bars* represent SD.

as well as with Hsps (28, 36). The fact that multiple interaction motifs are involved in the GR-TTC5 interaction suggests that complex formation could be flexible and that different components could be recruited in a context-dependent manner, possibly related to the different transcriptional surfaces of GR required at each promoter (26).

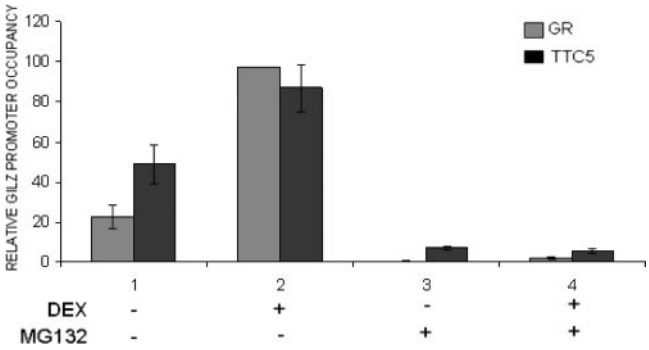

FIG. 10. GR and TTC5 are recruited to the GILZ promoter. GR (*gray bars*) and TTC5 (*black bars*) antibodies were used to precipitate chromatin from A549 cells that had received no treatment (lane 1) or were treated with 100 nM Dex (lanes 2 and 4) or 1 μM MG132 (lanes 3 and 4). Rabbit IgG antibody was used as a negative control (data not shown). Quantitative PCR was used to analyze DNA bound by the immunoprecipitated proteins. Data are representative of three or more independent experiments showing similar results. *Error bars* represent SD.

Our data shown in Figs. 5 and 6 demonstrate that TTC5 is an important regulator of GR stability. TTC5 is known to be involved in regulating the stability of p53, and these results suggest that it plays a similar role in the regulation of GR. We have shown that TTC5 increases the half-life of GR and plays a significant role in the stabilization of endogenous GR in a proteasome-related manner (Fig. 5). The interaction of TTC5 with GR is necessary for the stabilization effect, because the TTC5 mutant I380A that does not interact with GR is also incapable of stabilizing GR in the presence of hormone (Fig. 6A). Our results support the notion that TTC5 possibly shields the receptor from the actions of the ubiquitin ligases, such as Mdm2, resulting in reduction of the proteasome-mediated degradation of GR.

The stability of transcription factors and NRs is closely linked to their transcriptional activity (2, 37). Ubiquitination of Est-bound ER is delayed when transcription is inhibited, and proteasomal degradation of ubiquitinated ER is crucial for promoter cycling and efficient transcription (38). GR promoter cycling was also shown to be proteasome dependent, which suggests that ubiquitination is involved in transcriptional regulation (10). The ubiquitin ligases Mdm2, CHIP, and E6-AP, as well as

other components of the ubiquitin proteasome pathway, have been reported to affect GR function (4–9). p300 has been demonstrated to have ubiquitin ligase activity with p53 as a substrate, but other substrates are yet to be identified (39). Ubiquitination often licenses transcription factors and cofactors for transcriptional activation (40, 41), and through altering the GR stability, TTC5 may affect its transcriptional activity. Different promoters may vary in their sensitivity to actively cycling GR, and as such, TTC5 could affect transcription in a gene-specific manner (Fig. 8) (11).

TTC5 control of the GR transcriptional activity could be achieved by stabilizing the receptor, but it is unlikely that this is the only mechanism, because treatment of cells with MG132 augmented the effect of TTC5 on GR transcriptional activity but not on GR protein levels (Fig. 8, lanes 9–12, and Fig. 5A, lanes 5 and 6, respectively). The finding that inhibition of the proteasome interfered with the TTC5 recruitment to the GILZ promoter suggests a complex link between the proteasome, TTC5, and regulation of GR transcriptional activity (Fig. 10).

Treatment of cells with heat shock abolished the hormone-dependent interaction between GR and TTC5 (Fig. 3) and moderately inhibited the effect of TTC5 on GR transcriptional activity on TAT3 and MMTV luciferase reporters (Fig. 8 and data not shown). TTC5 levels have been shown to increase under heat shock conditions, but the mechanism of this is not clear (36). Through binding to chaperone proteins, TTC5 could also affect GR protein function, because Hsps, such as Hsp90, are known to be important in regulating GR stability (35, 42–44).

Eto treatment, used to induce single- and double-stranded DNA damage (45), caused an increase in the GR-TTC5 interaction (Fig. 3). However, the expression of GR target genes in Eto-treated cells seemed complex, perhaps because many different transcription factors, apart from GR, share the coregulatory function of TTC5 (Figs. 8 and 9 and data not shown).

ChIP assays indicated that TTC5 is present on the GR-regulated promoter GILZ, and an increase of this promoter occupancy by TTC5 correlated with increased GR binding, whereas decrease in TTC5 promoter recruitment coincided with a decrease in GR binding, upon proteasome inhibition (Fig. 10). Therefore, it is possible that proteasomal inhibition alters the efficiency of TTC5 recruitment to GR target promoters, which is consistent with the potential coordinating role of TTC5 between proteasome function and GR transcriptional activity (10).

A limited number of reports indicates that DNA damaging agents, such as UV or ionizing radiation, affect GR function. UV stress activates the c-Jun N-terminal kinase family of kinases, which are known to phosphorylate GR and repress its transcriptional activity (46, 47). Phosphorylation of GR in response to UV activation has also been reported to increase GR nuclear export (48). Ionizing radiation, which activates ATM kinase and is used in cancer treatment, has been seen to trigger a change in GR subcellular localization and cause an increase in IL-6 transcription, which GR represses (49). However, little is known about the effect of this stress on GR. It has been reported that the DNA-PK (DNA-dependent protein kinase) phosphorylates the rat GR at serine 527, located between the ligand and DNA-binding domains. DNA-PK is a member of the same family as ATM kinase, and it is important in double-strand DNA break repair (50). Finally, cross talk between GR and the stress-responsive tumor suppressor p53 could be mediated by TTC5 (51). The protein stability of GR and p53 have been linked together through their forming a complex with the ubiquitin ligase human homologue of Mdm2 (Hdm2) (4). The relationship between GR and p53 is clearly a complex one, but it could play a role in GR's response to stress, and TTC5 could be involved.

It seems likely that TTC5 employs multiple mechanisms to regulate the transcriptional activity of GR, as illustrated in Fig. 11. The role of TTC5 in the regulation of GR appears to be modulated by stress. This could reflect the action of differentially posttranslationally modified versions of TTC5, which could alter TTC5's localization, structure, available binding sites for GR or other cofactors, or TTC5's protein levels. Therefore, the cellular response to glucocorticoids under stress conditions could be primarily dictated by the type of stress rather than hormone.

The regulation of GR by TTC5 is likely to contribute to the physiological role of glucocorticoids and GR. TTC5 regulates the expression of GR target genes involved in a range of processes, including inflammation (Fig. 9) (1). Given the significance of glucocorticoids in treating inflammatory conditions and in wider clinical practice, TTC5 is a novel potential target for specific compounds that will allow better control of glucocorticoid-dependent treatments.

Materials and Methods

Cell culture and transfection

Cos-7 and A549 cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin (Cambrex, Charles City, IA) and were seeded into 60-mm plates for luciferase assays, RNA extraction, and Western blot analyses or 100-mm plates for immunoprecipitations 24 h before transfection. If cells were treated with hormone, the media was changed to 10% charcoal stripped FBS (Thermo Fisher Scientific, HyClone, Northumberland, UK) in DMEM, before treatment. Cells were treated with 100 nM Dex, Est, or dihy-

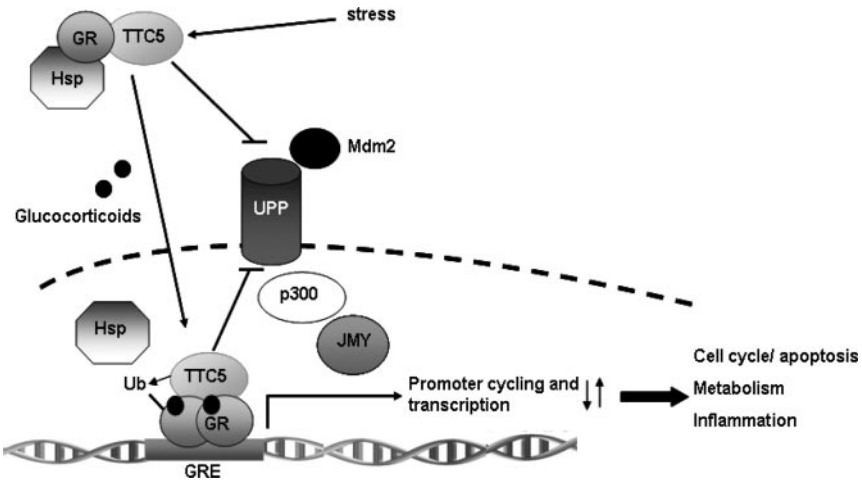

FIG. 11. Model summarizing the regulation of GR by TTC5. Cellular stress regulates the complex formation between GR and TTC5. Through binding to GR, TTC5 stabilizes it by preventing the action of the ubiquitin proteasome pathway (UPP), possibly by preventing the action of the ubiquitin ligase Mdm2. Upon hormone binding, GR translocates to the nucleus and binds to glucocorticoid response elements in the regulatory regions of its target genes. TTC5 is present at GR regulated promoters and can regulate the transcriptional activity of GR, acting as a coactivator or corepressor, in a gene-specific manner. Hsps, p300, and JMY have all been shown to interact with TTC5 and could regulate GR transcriptional activity. In being sensitive to cellular stress, TTC5 can link the response of cells to glucocorticoids with cellular stresses, such as heat shock and DNA damage.

drotestosterone (Sigma, St. Louis, MO) or 25 μM Eto (Sigma) for indicated times. To determine the role of proteasome, 1 μM MG132 was added, 1 h before hormone treatment. The protein half-life was determined by treating the cells with 30 μM cycloheximide, 1 h before Dex treatment, for the indicated times. Cells were harvested 48 h after transfection. Before cells were lysed, they were washed twice in cold PBS. Transfections were carried out using Polyfect (QIAGEN, Valencia, CA), following the manufacturer's guidelines, or the calcium phosphate method (28, 29).

Plasmids and constructs

pSG5-WTGR and derivatives, TAT3-luciferase, GILZ-luciferase, were gifts from K. Yamamoto. pSG5-HEO was obtained from D. Ray, as was MMTV-luciferase (52). pSVAR was from A. Brinkmann and pERE-Luc was from J. Davis. CMV-β-galactosidase (β-gal), HA-TTC5 and derivatives, Flag-TTC5, pET-TTC5, and derivatives have been previously characterized (28, 29). WT-Hdm2 (pcHDM1A) was obtained from K. Vousden.

In vitro pull-down assay

WT, TTC5 (1–220), or TTC5 (220–440) pET-TTC5 derivatives were purified using His-tag chromatography, then analyzed by SDS-PAGE and Coomassie staining as described previously (28). A549 cells were harvested in TNN buffer [50 mM Tris (pH 7.5), 240 mM NaCl, 5 mM EDTA, 0.5% Igepal, 1 μg/ml protease inhibitor cocktail, 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 2 mM sodium orthovanadate, 5 mM sodium pyrophosphate, and 20 mM β glycerophosphate]. Lysate protein concentrations were normalized using the Bradford assay; 5% of each lysate was saved for input analysis. The remainder of each lysate was used in the pull-down experiments, using versions of His-TTC5 attached to nickel beads. Pull downs were diluted in TNN buffer, and hormone was added to tubes at 1 μM concentration. Samples were rotated at 4 C for 1 h, and the beads were washed four times in TNN buffer and once in PBS. Beads were resuspended in 30 μl of sodium dodecyl sulfate loading buffer and analyzed by SDS-PAGE and Western transfer. M20 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to detect GR.

Immunoblotting and immunoprecipitation

Immunoprecipitations and immunoblotting procedures were as described previously with the following modifications (47). For immunoprecipitation, cell extracts were incubated with antibody and protein-A sepharose (Sigma), or Flag beads (Sigma), and rotated at 4 C overnight. Samples were analyzed by Western blotting using indicated antibodies. HA, M20, ER, AR, and Mdm2 polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc.; HA (monoclonal) antibody was obtained from Babco (Richmond, CA); TTC5 antibody, tubulin, and β-actin loading controls were purchased from Abcam (Cambridge, MA).

Small interfering RNA transfection

A549 cells were plated into six-well plates for Western blotting and qRT-PCR analysis and transfected using Dharmafect 1 reagent following the manufacturer's guidelines. A mixture of two siRNA duplexes against TTC5 was used (TTC5 1 sequence, ccagccuccuugagaguaa; and TTC5 2, ggaaaggacuauuccuuuu) at a total final concentration of 100 nM. These and a control-scrambled siRNA duplexes were purchased from Eurogentec (Fremont, CA). Cells were treated with the transfection mixes for 24 h in 10% serum DMEM, then media was replaced with 10% serum and 1% penicillin-streptomycin DMEM. Cells were treated as described in figure legends before being harvested.

Luciferase assay

Cells were split into 60-mm plates and transfected with indicated plasmids using Polyfect reagent (QIAGEN). Cells were treated with the indicated ligands, washed with PBS, and lysed using reporter lysis buffer (Promega, Madison, WI). Luciferase activity was measured using luciferase assay reagent (Promega), and the TD-20/20 Turner design luminometer. β-gal activity was measured as previously described (28). Luciferase readings were normalized to β-gal as an internal control.

Quantitative RT-PCR

Cells were harvested and RNA extracted using the RNeasy Plus mini kit and QIAshredder (QIAGEN), following manufacturer's guidelines. RNA samples were normalized using NanoDrop measurements, and equal amounts of RNA were reverse transcribed using the two-step protocol, with Anchored oligos and Reverse-iT RTase Blend reverse transcriptase (Thermo Scientific, Northumberland, UK). Quantitative RT-PCR was then performed on samples and analysis carried out using Opticon

monitor 3.1 software. Primers used were described in Wang *et al.* (1) or are available upon request.

ChIP assay

A549 cells were treated as required, and cells were harvested for ChIP following the method described by Nelson *et al.* (53). Briefly, samples were immunoprecipitated using N499 antibody for GR (described in Ref. 1) (gift from K. Yamamoto) or TTC5, and nonspecific IgG antibody as a negative control. Occupancy of the immunoprecipitated proteins at the GILZ promoter was determined using qRT-PCR and analysis carried out using the software Opticon monitor 3.1.

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