Recruitment of a Repressosome Complex at the Growth Hormone Receptor Promoter and Its Potential Role in Diabetic Nephropathy

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Received 29 May 2002/Returned for modification 29 August 2002/Accepted 29 October 2002

The growth hormone (GH)-GH receptor (GHR) axis modulates growth and metabolism and contributes to complications of diabetes mellitus. We analyzed the promoter region of the dominant transcript (L2) of the murine GHR to determine that a cis element, L2C1, interacts with transcription factors NF-Y, BTEB1, and HMG-Y/L. These proteins individually repress GH expression and together form a repressosome complex in conjunction with mSin3b. The histone deacetylase inhibitor trichostatin A increases expression of the murine GHR gene, enhances association of acetyl-H3 at L2C1, inhibits formation of the repressosome complex, and decreases NF-Y’s association with L2C1. Our studies reveal that murine models of experimental diabetes mellitus are characterized by reduced hepatic GH expression, decreased acetyl-H3 associated with L2C1, and increased formation of the repressosome complex. In contrast, in the kidney diabetes mellitus is associated with enhanced GH expression and lack of alteration in the assembly of the repressosome complex, thus permitting exposure of kidneys to the effects of elevated levels of GH in diabetes mellitus. Our findings define a higher-order repressosome complex whose formation correlates with the acetylation status of chromatin histone proteins. The delineation of the role of this repressosome complex in regulating tissue-specific expression of GHR in diabetes mellitus provides a molecular model for the role of GH in the genesis of certain microvascular complications of diabetes mellitus.

The growth hormone (GH)—insulin-like growth factor 1 (IGF-1) axis plays a critical permissive role in the pathogenesis of chronic microvascular complications of diabetes mellitus (DM). The absence of functional GH receptor (GHR) confers a protective effect against diabetic nephropathy and retinopathy in murine models of insulin-dependent DM (IDDM) (2, 29). For humans, acquired hypopituitarism ameliorated retinopathy and iatrogenic hypopituitarism was the standard care for proliferative retinopathy until the advent of laser coagulation therapy (24). In contrast there is resistance to GH’s actions in promoting linear growth and IGF-1 generation in poorly controlled IDDM (30); animal and human studies indicate that decreased hepatic expression of GHR contributes to this resistance to GH’s actions (1, 19, 21). However, the molecular mechanisms underlying the paradox of the liver becoming resistant but the kidney and retina retaining sensitivity to GH in DM is not known.

The GHR gene is characterized by sequence heterogeneity in the 5′ untranslated region (UTR) (8). For the mouse, three 5′ UTRs (L1, L2, and L5) have been characterized in some detail (20, 34, 36). The L2 transcript constitutes 50 to 80% of the hepatic GHR transcripts in the nonpregnant adult animal (8, 34). The 5′ flanking region of the L2 transcript exhibits promoter activity. A cis element, L2A, interacts with the Sp family of proteins and plays a role in the development-specific expression of the GHR gene (34). Recent studies have focused attention on the role of chromatin in modulating the transcriptional activity of genes (4). The present model for involvement of chromatin in gene activation or gene repression envisions the effects of a diverse array of posttranslational modifications that impinge on the histone amino terminus, thereby modulating access of transcription factors and other regulatory proteins to the underlying DNA. Whereas the role of chromatin structure and function in DNA template-dependent cellular processes such as transcription, DNA replication, DNA recombination, cell cycle progression, and epigenetic silencing is recognized, there is paucity of information regarding the significance of chromatin structure and function in specific disease states such as DM. Here we present evidence that a second cis element in the promoter of the murine GHR gene, which we designate L2C1, interacts with transcription factors NF-Y, HMG-Y/I, and BTEB1 and that these factors in conjunction with the corepressor mSin3b form a higher-order repressosome complex that decreases transcriptional activation of the mouse GHR. Our studies demonstrate novel in vivo associations between members of this quartet. We further demonstrate that alterations in the interaction of this repressosome complex with L2C1 play a role in the tissue-specific dysregulation of expression of the GHR gene in DM. To the best of our knowledge this is the first report demonstrating a direct correlation between the acetylation status of histone proteins and the transcriptional activity of a specific gene in DM.

MATERIALS AND METHODS

Oligonucleotides. The following synthetic oligonucleotides (Invitrogen) were used in this study (residues altered in mutant oligonucleotides are indicated in
lowercase): L2C1, 5′-TCTAGGAGGAGGCCCAGCCTATGAGAGCGACAC-3′; NF-Y consensus, 5′-ATTTTTTCATTTGTTAAGGT-3′; NF-Y mutant, 5′-GGCGCGGCCCAGCAGGCGAGACAC-3′; BTEB1 mutant, 5′-TCTCCTAGGAGGAGGCAGTAgataaCCATTGAGAGCGACAGC-3′; L2C1 reverse, 5′-GGTGGTTGTCCTGCTCACTAT-3′; L2C1 probe, 5′-ATCGAGAGGGAGCCGGCCCCGCC-3′TAMRA.

Where necessary, double-stranded oligonucleotides were generated by annealing the synthetic oligonucleotides with respective complementary sequences.

Phage in vitro transcription and luciferase assays. BNL CL.2 cells (mouse liver; American Type Culture Collection, Manassas, Va.) were cultured and transiently transfected by using Lipofectamine as described previously (20). Three or four independent transfections were performed in triplicate, and transfection results are represented as means ± standard deviations (SD).

Cell culture, transient transfections, and luciferase assays. BNL CL.2 cells were suspended in ice-cold phosphate-buffered saline, minced, washed, and homogenized. The homogenate was centrifuged, and the pellet was resuspended in cell dissociation buffer (Invitrogen) and incubated for 15 min at room temperature. The cells were resuspended in culture medium, and proteins were prepared by using standard protocols.

Fluorescent 5′-nucleotide RT-PCR. Total RNA was extracted using TRI-reagent (Molecular Research Center). Real-time quantitative reverse-transcription (RT)-PCR was performed and analyzed following protocols described previously (20).

DNase I footprinting. The PCR-derived radiolabeled probe was incubated with nuclear extracts from adult mouse liver and processed for DNase I treat ment, electrophoresis, and analysis as previously described (22). The exact locations of the DNase I footprints were determined by comparison to a concurrently electrophoresed sequence ladder.

EMSAs. Electromobility shift assays (EMSAs) were performed with 2 μg of nuclear proteins by using standard protocols and samples resolved on a 4% nondenaturing polyacrylamide gel. For supershift experiments, anti-BTEB1 (kindly provided by H. Imataka, McGill University, Montreal, Canada), R. Mantovani (University of Milan, Milan, Italy), and A. Fusco (Università degli Studi di Catanzaro, Catanzaro, Italy), respectively.

RESULTS

Proteins regulating expression of the L2 transcript. Deletion analysis of the 5′-flanking region of the L2 transcript of the murine GHR identified a regulatory region located 344 to 210 bp 5′ of the transcription start site. Extension of this region enhanced the activity of the L2 promoter (Fig. 1a), indicating that the proteins interacting with a cis element(s) within this region repress transcription of GHR. Extending the analysis via the DNase footprint assay identified a 72-bp stretch within this region that is protected by adult mouse liver extract (Fig. 1b).

Computer-based analysis for consensus binding sites revealed the presence of CCAAT and GC box motifs within this putative cis element. A 37-bp oligonucleotide encompassing these CCAAT and GC boxes, which we designated L2C1, was tested for protein binding activity by EMSA. Addition of nuclear extracts from adult mouse liver to an aliquot of 32P-labeled L2C1 resulted in the formation of two distinct sequence-specific protein-DNA complexes, C1 and CII (Fig. 2a). Supershift assays demonstrated the presence of the canonical CCAAT box binding protein NF-Y in the CI DNA-protein complex (Fig. 2a). Upon excluding the canonical members of the GC box/Kruppel-like family of proteins, Sp1, Sp2, Sp3, and Sp4, as candidates for protein(s) binding to the GC box (data not shown), we established another member of this family, BTEB1, to be a component of the CII protein-DNA complex (Fig. 2a). The presence of NF-Y and BTEB1 in the CI and CII complexes, respectively, was also investigated by analyzing the effect of mutations within the L2C1 sequence on the ability to compete for protein binding. Hence, altering the NF-Y binding site in the L2C1 sequence abolished competition for binding to the CI complex (Fig. 2b) and a mutation in the putative BTEB1 binding site resulted in a failure of the mutant oligonucleotide to compete for binding at the CII complex (Fig. 2b). These results demonstrate that NF-Y is a component of the CI complex and BTEB1 participates in the formation of the CII protein-DNA complex. Overlapping the NF-Y binding site on L2C1 is a recognition site for HMG. The presence of HMG in the CI complex was revealed by a concentration-dependent inhibition of the formation of the CI complex following preincubation with graded concentrations of either poly(dI-dC)·poly(dI-dC) or HMG-Y/I antibody (Fig. 2c) and by direct binding of recombinant HMG-Y/I to L2C1 (Fig. 2d). Inhibition of NF-Y binding following depletion of HMG-Y/I from the CI complex (Fig. 2c) indicates that HMG-Y/I is necessary for the participation of NF-Y in the assembly of the CI complex at L2C1.

Mutation of the composite L2C1 element comprising NF-Y, HMG-Y/I, and BTEB1 binding sites resulted in a significant increase in transcriptional activity (Fig. 3a). We next tested for the individual effects of these factors on GHR transcription. Inhibition of endogenous NF-Y by overexpression of a D/N NF-YA resulted in increased activity of the L2 promoter (Fig. 3b). Overexpression of the HMG-Y/I sense construct decreased L2 activity, and conversely overexpressing antisense
HMG-Y/I increased L2 promoter activity (Fig. 3b). Since the effects of HMG-Y/I were absent when the D/N NF-YA was coexpressed, we conclude that NF-Y binding is obligatory for the effect of HMG-Y/I at the L2CI site (Fig. 3b). The specificity of the actions of the D/N NF-YA and HMG-Y/I was confirmed by the loss of these effects when the L2CI element was mutated (Fig. 3c). Overexpression of BTEB1 resulted in decreased L2 promoter activity (Fig. 3d). Altering the BTEB1 binding site within L2CI abolished the effect of overexpressing BTEB1 on L2 promoter activity, indicating that the action of BTEB1 was dependent on an intact L2CI-BTEB1 site (Fig. 3d). NF-Y and HMG-Y/I binding are a prerequisite for BTEB1 activity, since mutation of the NF-Y binding site that abolished both NF-Y and HMG-Y/I binding (data not shown) abolished the response to BTEB1 (Fig. 3d). These data indicate that collectively NF-Y, HMG-Y/I, and BTEB1 proteins repress L2 promoter activity via interaction with the L2CI element.

Role of chromatin in the transcriptional activation of the L2 promoter in BNL CL.2 cells. NF-Y and HMG-Y/I can alter chromatin architecture via changes in the acetylation status of histone (3, 18, 31). To investigate the possible effects of chromatin structure and in particular histone acetylation on the expression of L2, we engineered BNL CL.2 (murine liver) cell lines stably expressing the L2-promoter construct pGL3B-L2, containing approximately 2.0 kb of the 5′-flanking region of L2. Incubation of these cells with TSA, a histone deacetylase-specific inhibitor, resulted in increased activity of the L2 promoter (Fig. 4a). To ascertain that this effect of histone acetylation is transduced by interaction(s) at the L2CI site, BNL CL.2 cells stably transfected with a mutant L2-reporter construct (pGL3B-L2[mCCAAT]) that incorporated a mutation within the NF-Y binding site proven to obviate NF-Y binding were exposed to TSA. The effect of TSA on cells stably transfected with pGL3B-L2[mCCAAT] was significantly less than that on the wild-type construct (Fig. 4a). Thus, the effect of histone acetylation on the L2 transcript is dependent on the interaction(s) at the L2CI site. To validate the biological significance of these results, RNAs from TSA-treated BNL CL.2 cells were analyzed by real-time RT-PCR. Compared with control cells, cells exposed to TSA exhibited increased levels of L2CI mRNA (Fig. 4b). Furthermore, EMSA revealed that exposure to TSA decreased binding of CI and CII complexes at L2CI (Fig. 4c). To correlate transcriptional activation of GHR with changes in chromatin at the L2CI site, BNL CL.2 cells exposed to TSA were subjected to ChIP with anti-acetyl-H3, anti-NF-YA, and anti-HMG-Y/I antibodies. In parallel with EMSA results, TSA treatment resulted in decreased association of NF-Y to chromatin at the L2CI region (Fig. 4d). In contrast, significantly higher levels of acetylated histone H3 were associated with L2CI in TSA-treated cells than in control cells (Fig. 4d). We did not observe a significant change in the
FIG. 2. Identification of the proteins interacting at L2C1. (a) Nuclear proteins from mouse liver interact with the L2C1 site in the promoter of the L2 transcript of the murine GHR gene. 32P-labeled L2C1 was incubated with (lanes 1 to 3) or without (lane P) nuclear extracts prepared from liver tissues of adult mice in the absence (lane 1) or presence of antibodies against the NF-YA (lane 2) or BTEB1 (lane 3), electrophoresed, and subjected to autoradiography. The bands representing specific DNA-protein complexes (CI, CII), supershifted complexes (SS), and the free probe (FP) are indicated. (b) NF-YA and BTEB1 interact with L2C1. 32P-labeled L2C1 was incubated with nuclear extract from adult mouse liver without (lane NC) or with excess unlabeled oligonucleotide (lanes a [25×], b [50×], and c [100×]). The unlabeled oligonucleotide used was either the wild-type sequence (L2C1) or oligonucleotides with mutations in the NF-Y (NF-Ym), BTEB1 (BTEBm), or both (L2C1m) binding sites. The bands representing specific DNA-protein complexes (CI, CII) and the free probe (FP) are indicated. (c) HMG Y/I is a component of the CI complex. 32P-labeled L2C1 was incubated with (lanes 1 to 6) or without (lane P) nuclear extracts prepared from liver tissues from adult mice in the presence of either poly(dI-dC)·poly(dI-dC) (0 μg [lane 1], 1 μg [lane 2], and 2 μg [lane 3]) or anti-HMG-Y/I antibody (20 ng [lane 4], 40 ng [lane 5], 100 ng [lane 6]). The bands representing specific DNA-protein complexes (CI, CII) and the free probe (FP) are indicated. (d) L2C1 binds HMG Y/I. 32P-labeled L2C1 was incubated without (lane P) or with recombinant HMG-Y/I protein (lane 1), electrophoresed, and subjected to autoradiography. The bands representing L2C1-HMG complex (●) and the unbound 32P-labeled L2C1 (FP) are indicated.
levels of HMG-Y/I associated with chromatin at the L2C1 site (data not shown). We conclude that transcriptional activation of the L2 promoter due to perturbation of the chromatin architecture results from decreased binding of NF-Y to the L2C1 region.

Establishment of a repressive role for the L2C1 cis element indicated a possible association of canonical corepressor proteins with the DNA-protein complex at L2C1. In EMSA, preincubation of nuclear proteins from BNL CL.2 cells with anti-mSin3b antibody resulted in the inhibition of the CI and CII complexes and the appearance of a new DNA-protein complex (Fig. 5a). This new complex could either represent a supershift of the CII complex or the faster migration of the CI complex devoid of mSin3b protein. In cells exposed to TSA, mSin3b antibody failed to alter the formation of either complex (Fig. 5a), suggesting that TSA prevents the association of mSin3b with CI and CII. Prior studies have demonstrated physical interaction between NF-Y and HMG-Y/I (5, 15) and between BTEB1 and Sin3a (35). In coimmunoprecipitation experiments we established an in vivo association of NF-Y with BTEB1 and of NF-Y+HMG-Y/I or BTEB binding sites at L2C1. Results represent means ± SD of three or four independent transfections performed in triplicate. *, P < 0.05 in comparison to respective transfections carried out without BTEB1 (control).

FIG. 3. L2C1 represses the activity of the murine GHR gene promoter. (a) Transient transfection of luciferase reporter plasmids whose expression was controlled by wild-type GHR promoter (L2) or GHR promoter with a mutation designed to abolish the L2C1 binding sites for NF-Y, HMG-Y/I, and BTEB1 (L2C1 mutant) in BNL CL.2 cells. Results represent means ± SD of three or four independent transfections performed in triplicate. For luciferase activity, P < 0.05 compared to that of the empty vector (pGL3B) (*) or the wild-type GHR promoter (#), respectively. (b) NF-Y, BTEB1, and HMG-Y/I individually repress transcriptional activation. Transient cotransfection assays in BNL CL.2 cells with L2 luciferase reporter plasmid with constructs expressing the dominant negative form of NF-YA (DN/NF-Y, 250 ng), sense HMG-Y/I (HMG-S, 250 ng), or antisense HMG-Y/I (HMG-AS, 250 ng). Results represent means ± SD of three or four independent transfections performed in triplicate. *, P < 0.05 (luciferase activity compared to that of the wild-type GHR promoter); ns, statistically not significantly different from DN/NF-Y. (c) BNL CL.2 cells transiently cotransfected with the L2C1m-GHR reporter construct and expression plasmid for either D/N NF-Y, BTEB1, or sense HMG-Y/I. Results represent means ± SD of three or four independent transfections performed in triplicate. (d) Cotransfection of BTEB1 (500 ng) expression plasmid with luciferase constructs whose expression was controlled by wild-type promoter or GHR promoter with mutated NF-Y+HMG-Y/I or BTEB binding sites at L2C1. Results represent means ± SD of three or four independent transfections performed in triplicate. *, P < 0.05 (luciferase activity compared to that of the wild-type GHR promoter); ns, statistically not significantly different from D/N NF-Y.
FIG. 4. Role of chromatin in modulating the activity of L2C1. (a) Effect of TSA exposure on BNL CL.2 cells stably transfected with L2- and mCCAAT reporter constructs. Results represent mean ± SD of three independent experiments. *, P < 0.05 in comparison to the effect of TSA on cells stably transfected with an intact L2C1 site. (b) Fluorescent 5′-nuclease (TaqMan) real time RT-PCR analysis of expression of endogenous L2 transcript after exposure of BNL CL.2 cells to vehicle (alcohol) or TSA. The results are depicted as mean and range. *, P < 0.05, in comparison to the cells exposed to vehicle only. (c) 32P-labeled L2C1 was incubated with or without nuclear extracts prepared from BNL CL.2 cells exposed to vehicle or TSA in the absence or presence of antibodies against the NF-YA or BTEB1, electrophoresed, and subjected to autoradiography. The inset shows total NF-Y content in these cells as ascertained by EMSA with 32P-labeled NF-Y consensus oligonucleotide. The bands representing specific DNA-protein complexes (CI, CII), supershifted complexes (SS), and the free probe (FP) are indicated. (d) ChIP assay on BNL CL.2 cells treated without (vehicle) and with TSA. Immunoprecipitation with anti-NF-YA (top panel) and anti-acetyl-H3 (middle panel) antibodies. The input DNA and the sample without addition of the antibody (unbound) are also shown. The linearity of the PCR assay was established by assaying serial concentrations of the input DNA (bottom panel).
The two major findings of this study are that expression of the dominant transcript of the GHR in postnatal life is regulated by a higher-order repressosome complex consisting of NF-Y, HMG-Y/I, BTEB1, and mSin3b proteins and that the effect of insulinopenic DM on hepatic GHR gene transcription is mediated by the mechanisms involving chromatin remodeling at a defined site on the GHR promoter. The differential effects of DM on NF-Y's interactions at the GHR promoter in the liver and kidney results in tissue-specific regulation of ex-
pression of GHR in the liver and kidney in DM and thus plays a role in the genesis of diabetic nephropathy.

Our studies indicate that NF-Y is a critical member of the DNA-binding protein complex at the L2C1 site of the GHR promoter. NF-Y is a ubiquitous heterotrimeric complex (18), and its binding to DNA brings about structural changes resembling nucleosome organization (14). Whereas NF-Y usually plays a role as an activator of gene transcription (13, 16), the present report provides evidence that NF-Y participates in the assembly and action of a repressosome complex and thus adds to the select examples of NF-Y inhibiting gene transcription (9, 12, 17, 28). NF-Y forms partners with a disparate group of proteins (3, 5, 6, 15, 18). The present report establishes that NF-Y also associates in vivo with the transcription factor BTEB1 and corepressor Sin3b. Like NF-Y, BTEB is a bimodal transcription factor and can function either as an activator or as a repressor of transcription (27), and a previous report has demonstrated that BTEB can interact with mSin3a to repress transcription (35). Physical interactions between NF-Y and HMG-Y/I modulate NF-Y activity through stabilization of NF-Y binding (5). It is noteworthy that previous studies have demonstrated that acetylation of HMG results in the disruption of an enhanceosome complex (25). Thus, posttranslational modifications of HMG in response to alterations in the cellular milieu, such as in DM, could modulate NF-Y binding at the L2C1 site. In the context of the L2 transcript of the GHR gene, the ability of NF-Y to act as a repressor possibly reflects the association with other members of the repressosome complex and also the spatial relationship with cis elements in the minimal promoter.

Activation of GHR transcription by TSA is associated with an increase in acetylated histone H3 at the L2C1 site. Conversely, inhibition of GHR transcription in DM is associated with decreased levels of acetylated histone H3 at the L2C1 site. These results are in agreement with the contemporary model wherein acetylation of histone H3 and/or H4 correlates with the opening of the nucleosome and activation of transcription (13). Based on the ability of TSA to alter the activity of the GHR promoter, our results indicate that histone acetyltransferases and deacetylases influence GHR promoter activity and that this effect is mediated in part due to interaction(s) at the L2C1 site. Both NF-Y and HMG-Y/I are known targets for acetylation by proteins possessing histone acetyltransferase activity (18, 31). Although TSA treatment results in activation and/or derepression of the GHR promoter, the effect of acetylation on the function of NF-Y is unclear. Thus, acetylation of NF-Y could alter its DNA-binding affinity, influence its interactions with other transcription factors or with general factors of the holoenzyme machinery, or modulate its association with nucleosomes, resulting in modification of nearby chromatin structures. Alternatively, the activity of nonhistone proteins like HMG-Y/I or NF-Y could be influenced by patterned display of modifications on one or more histone tails (“histone code” [32]). The inverse relationship between levels of NF-Y and acetylated histone H3 at L2C1 supports such a model, wherein modifications of histone at the L2C1 site initiate alterations in the binding activity of cognate DNA-binding proteins resulting in the dissolution of the repressosome complex with consequent activation of GHR transcription.

One paradox of the role of GH/IGF-1 in the genesis of
microvascular complication of DM is that the liver is resistant to GH while GH action is required for the genesis of retinopathy and nephropathy (1, 19, 21, 23, 30). Tissue-specific differences in the expression of the GHR may play a role in this differential sensitivity of organs to the effects of DM (21). Our studies indicate that lack of recruitment of the repressosome complex to the L2C1 site of the GHR promoter plays a permissive role in the sensitivity of kidney to GH in DM. The increase in NF-Y binding to L2C1 in diabetic liver is specific to DM, since under other circumstances that lead to decreased expression of the L2 transcript of the GHR gene such as fasting, altered binding of NF-Y at the L2C1 site is not observed (data not shown). Our findings are also consistent across both the NOD and STZ models of insulinopenic DM. One of the questions raised by our study is the mechanism(s) involved in recruitment of the repressosome complex to the GHR promoter in the diabetic state. The regulation of gene transcription via effects of cellular redox on DNA-binding activity of NF-Y is known (26). Experimental and clinical DM are associated with altered cellular redox (7, 33), and the potential role of these metabolic changes in transducing the effects of DM on NF-Y activity at L2C1 remains to be defined. It will be of interest to investigate if drugs that either alter cellular redox (7) or modulate the activity of NF-Y (18) are potential targets for drug discovery in the prevention and/or therapy of diabetic nephropathy. Another potential target for DM-induced changes in chromatin is histone proteins. Thus, in addition to the various posttranslational modifications that histones are exposed to in the nondiabetic state (4), histones could also be the target of modification by advanced glycation end products in DM (11). The roles of these myriad mechanisms in modulating expression of GHR in DM remain to be clarified.

In summary, we have defined the role of a repressor complex in the control of transcription of the murine GHR gene. The
The differential effects of DM on the assembly of this higher-order repressor complex in liver and kidney provide a molecular basis for the tissue-specific regulation of expression of GHR in DM. The understanding of these mechanisms may permit the identification of pharmacophores designed to alter expression of the GHR in a tissue-specific manner and thus potentially abolish the development of certain complications of DM such as nephropathy.

ACKNOWLEDGMENTS

This study was supported by grants from the NIH (DK49845 [to R.K.M.] and T32DK07729), Children’s Hospital of Pittsburgh, and the Vira I. Heinz Foundation.

We thank M. Trucco and S. Bertera for the murine models of IDDM and M. K. Bennett (University of California, Irvine) for advice regarding ChIP assays. We also thank Clayton Mathews and Lee Denson for critically reviewing the manuscript.

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