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The Interaction of the Carboxyl Terminus-binding Protein with the Smad Corepressor TGIF Is Disrupted by a Holoprosencephaly Mutation in TGIF*

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The homeodomain protein TGIF represses transcription in part by recruiting histone deacetylases. TGIF binds directly to DNA to repress transcription or interacts with TGF-β-activated Smads, thereby repressing genes normally activated by TGF-β. Loss of function mutations in *TGIF* result in holoprosencephaly (HPE) in humans. One HPE mutation in *TGIF* results in a single amino acid substitution in a conserved PLDLS motif within the amino-terminal repression domain. We demonstrate that TGIF interacts with the corepressor carboxyl terminus-binding protein (CtBP) via this motif. CtBP, which was first identified by its ability to bind the adenovirus E1A protein, interacts both with gene-specific transcriptional repressors and with a subset of polycomb proteins. Efficient repression of TGF-β-activated gene responses by TGIF is dependent on interaction with CtBP, and we show that TGIF is able to recruit CtBP to a TGF-β-activated Smad complex. Disruption of the PLDLS motif in TGIF abolishes the interaction of CtBP with TGIF and compromises the ability of TGIF to repress transcription. Thus, at least one HPE mutation in *TGIF* appears to prevent CtBP-dependent transcriptional repression by TGIF, suggesting an important developmental role for the recruitment of CtBP by TGIF.

Homeobox genes were first identified in *Drosophila* where they regulate segment identity. The homeodomain is an approximately 60-amino acid DNA-binding domain consisting of three α-helices, which is conserved among numerous proteins from diverse species (1–3). Members of the atypical TALE[1] homeodomain family have a three amino acid loop extension between helices one and two of the homeodomain (4, 5). Despite the similarity of their homeodomains, outside this region TALE proteins are considerably divergent. Members of this family bind to DNA in association with other homeodomain proteins (6–9) and can activate and repress transcription (10–12).

The TALE homeodomain protein TGIF (TG-interacting factor) was identified by its ability to bind a specific retinoid responsive element and has been suggested to compete with retinoid receptors for binding to this element (4, 13). However, it is not clear what role TGIF plays in the regulation of retinoid-responsive transcription. TGIF can also bind to an element within the upstream of the *D1A* dopamine receptor gene and is able to compete for binding with Meis2, another TALE family homeodomain protein (14). TGIF is a transcriptional repressor that contains multiple repression domains and interacts with histone deacetylases (HDAC) (15, 16). Transcriptional repression by TGIF is dependent only in part on its ability to recruit HDACs, and TGIF contains at least one HDAC-independent transcriptional repression domain (16).

The transcriptional response to transforming growth factor TGF-β is dependent on the intracellular mediators of TGF-β signaling, the Smad proteins. Following TGF-β receptor-mediated phosphorylation and activation, the activated Smad complex moves to the nucleus (17–20). A Smad complex is recruited to specific DNA elements via interactions with other DNA-binding proteins (21, 22) and by direct Smad-DNA contacts (23–25). Gene activation by Smads is, in part, dependent on interactions with coactivators, such as p300/CBP (26–29). A TGF-β-activated Smad complex can also interact with TGIF, resulting in transcriptional repression. This repression by TGIF is dependent on competition with coactivators for interaction with the Smads and on the intrinsic repression functions of TGIF (15). It appears that both HDAC-dependent and -independent repression domains of TGIF play a role in repression of a TGF-β response.

In humans, deletion or mutation of a single copy of the *TGIF* gene, which maps to the *HPE4* locus, causes holoprosencephaly (HPE), a prevalent defect of craniofacial development (30–32). The primary brain malformations in HPE consist of incomplete cleavage of the forebrain and are frequently accompanied by facial anomalies including cyclopia. It is not clear whether mutations in *TGIF* result in HPE because of effects on the TGF-β signaling pathway or on repression of other TGF-β-independent gene responses.

CtBP (carboxyl terminus binding protein) was first identified as a protein which binds the carboxyl terminus of the Adenovirus E1A protein (33, 34), and this interaction is required for transcriptional repression by E1A. Within E1A, the five-amino acid motif, PLDLS, was shown to be required for interaction with CtBP (33). This motif (or sequences related to it) is present in other transcriptional repressors that bind CtBP (35–38). Recent evidence has demonstrated that PLDLS-like motifs present within a subset of vertebrate polycomb-group proteins bind CtBP (39). Thus CtBP may interact not only with gene-specific repressors but also with components of general transcriptional repression complexes.

We demonstrate that CtBP and TGIF interact via a PLDLS motif in the amino-terminal repression domain of TGIF. In

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[1] The abbreviations used are: TALE, three amino acid loop extension; HPE, holoprosencephaly; GBD, Gal4 DNA binding domain; HDAC, histone deacetylase; CtBP, carboxyl terminus-binding protein; HA, hemagglutinin; PCR, polymerase chain reaction; PcG, polycomb group.

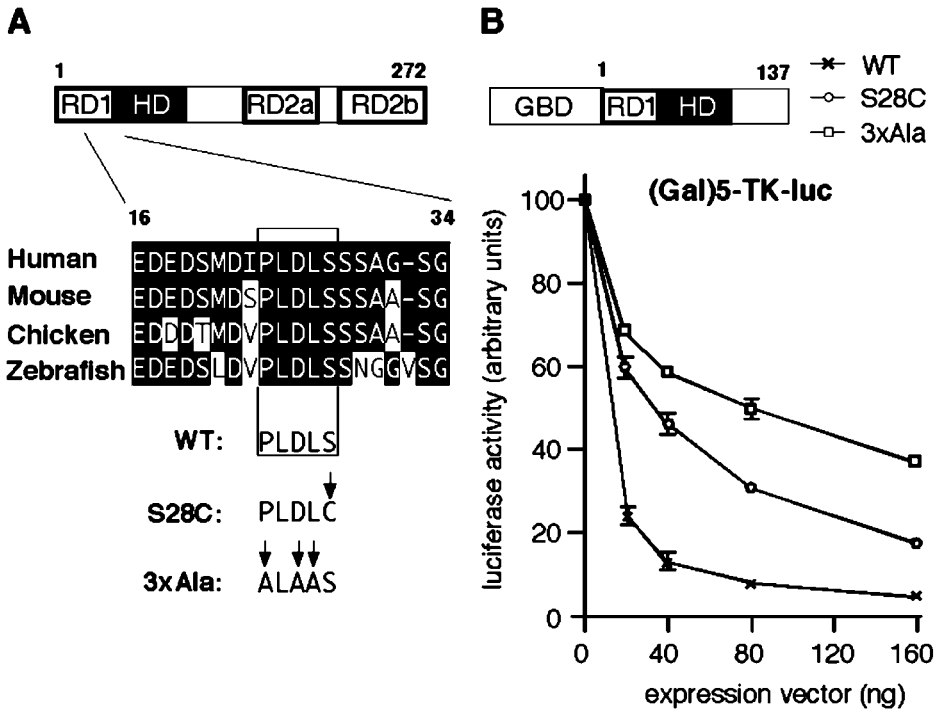

FIG. 1. **The PLDLS motif in TGIF is required for full transcriptional repression.** *A*, TGIF is shown schematically (*HD*, homeodomain; *RD*, repression domain) with an alignment of the amino acid sequences from human, mouse, chicken, and zebrafish TGIF of a region immediately amino-terminal to the homeodomain. Identities to human sequence are shaded, and the sequence of two mutant versions of human TGIF is shown below. *B*, the amino-terminal half of TGIF (amino acids 1–136) was fused to the Gal4 DNA binding domain (*GBD*). TGIF was either wild type (*WT*) or contained one of the mutations shown in *A*. Increasing amounts of plasmids encoding GBD·TGIF fusions were transfected into L17 cells with a reporter in which luciferase is driven by the TK promoter and five Gal4p binding sites. Luciferase activity was assayed 36 h later and is presented in arbitrary units as the mean ± S.D. of triplicate transfections.

response to TGF-β, TGIF can recruit CtBP to an activated Smad complex. The interaction of TGIF with CtBP is required for full repression by TGIF and is disrupted by a single amino acid missense mutation, which results in HPE.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Mink lung epithelial L17 cells were maintained in minimum essential medium with 10% fetal bovine serum and were transfected in six-well plates as described previously (15). COS-1 cells were grown in Dulbecco's modified essential medium with high glucose and 10% fetal bovine serum and were transfected using LipofectAMINE (Life Technologies).

Plasmids—$(Gal)_5$-TK-luc has five Gal4p binding sites upstream of the TK promoter from pBLCAT2 (40). 3TP-lux contains a TGF-β-inducible promoter region from the *PAI-1* gene and three TPA-response elements (41). TGIF was expressed from a modified pCMV5 plasmid, which has two HA-epitope tags inserted into the polylinker. TGIF mutants were created by PCR and verified by sequence analysis. Gal4p·TGIF fusions were created by PCR within the GBD fusion vector pM (CLONTECH). FLAG·HDAC1 is as described previously (42). T7-tagged CtBP (33) is present within a modified pRcCMV (Invitrogen) and was a gift of G. Chinnadurai (St. Louis University). FLAG·CtBP was created in pCMV5 by PCR. LexA·TGIF fusions were created in pBTM116 by PCR, and the Gal4p activation domain fusion to CtBP was expressed from pGAD424 (CLONTECH). The zebrafish TGIF sequence was obtained from an expressed sequence tag (EST) clone (GenBank 228 /EBI AI416090.1) and was verified by automated sequencing.

Luciferase Assays—36–40 h after transfection, cells were lysed in Promega passive lysis buffer and assayed for luciferase activity with a Berthold Flash n Glow automated luminometer. A Renilla luciferase reporter was included in all transfections to monitor transfection efficiency. Renilla luciferase activity was assayed with 0.09 μM coelenterazine (Biosynth) in 25 mM Tris, pH 7.5, 100 mM NaCl.

Yeast Two-hybrid Assays—LexA and Gal4p fusions were transformed into L40 cells (43) and interactions were assessed by streaking onto plates lacking histidine with 2 mM 3-aminotriazole, to score for activation of the *HIS3* gene, which is regulated by multiple LexA binding sites.

Immunoprecipitation and Western Blotting—36 h after transfection, COS-1 cells were lysed by sonication in 75 mM NaCl, 50 mM HEPES, pH 7.8, 20% glycerol, 0.1% Tween 20, 0.5% NP40 with protease and phosphatase inhibitors. Immunocomplexes were precipitated with FLAG M2-agarose (Sigma), or with HA (12CA5, Roche Molecular Biochemicals) monoclonal antibodies or a TGIF-specific rabbit antiserum and a mixture of protein A and protein G-Sepharose (Pierce). Following SDS-polyacrylamide gel electrophoresis, proteins were electroblotted to Immobilon-P (Millipore) and incubated with FLAG-, HA-, T7- (Novagen), or TGIF-specific antisera. Proteins were visualized with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit Ig (Pierce) and ECL (Amersham Pharmacia Biotech).

RESULTS

The PLDLS Motif of TGIF Is Required for Transcriptional Repression—TGIF contains multiple transcriptional repression domains, at least one of which appears not to require HDAC activity (16). This domain resides at the extreme amino terminus of TGIF and, when fused to a heterologous DNA binding domain, is able to repress transcription in isolation. Recent evidence has demonstrated that mutation of *TGIF* in humans can cause HPE (30). Such mutations are generally deletions of a single copy of the *TGIF* gene; however a number of missense mutations have also been identified. One of these causes a single amino acid substitution (serine 28 to cysteine, S28C) within the amino-terminal repression domain resulting in decreased transcriptional repression (30). The S28C substitution affects the integrity of a five-amino acid motif (PLDLS, Fig. 1*A*), which in other transcriptional repressors has been shown to bind CtBP. This motif is conserved in human and mouse TGIF (4, 13), in a highly related factor (AKR2) from chicken (44) and in zebrafish ESTs encoding a TGIF homolog. To determine whether other amino acid substitutions that affected the integrity of this motif decreased repression by TGIF, we created a mutant version of TGIF in which the PLDLS was altered to ALAAS (Fig. 1*A*, *3×Ala*). In addition to placing this mutation in the context of full-length HA-epitope-tagged TGIF, we also created a fusion of amino acids 1–137 of TGIF harboring this mutation to the Gal4p DNA binding domain (GBD). L17 cells were transfected with a luciferase reporter in which transcription is regulated by the HSV TK promoter and five Gal4p binding sites. Cells were cotransfected with increasing amounts of GBD·TGIF-(1–137) or mutant versions of this fusion containing either the S28C mutation or the 3×Ala. Both mutant forms of the TGIF fusion protein repressed transcription significantly less well than the wild type (Fig. 1*B*). It, therefore, appears that the PLDLS motif is required for efficient repression by this domain of TGIF.

CtBP Interacts With TGIF—To determine whether TGIF and

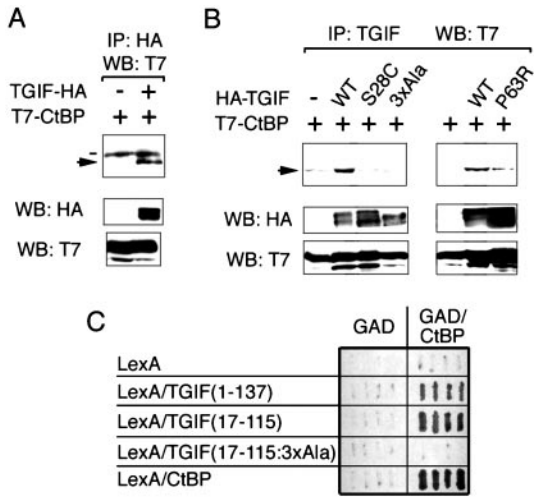

FIG. 2. **CtBP interacts with TGIF.** *A*, COS-1 cells were transfected with a T7 epitope-tagged CtBP expression construct, together with either a control vector or one expressing TGIF with a carboxyl-terminal HA tag. Complexes were immunoprecipitated via the HA epitope and analyzed for the presence of T7-CtBP by Western blotting. A portion of each lysate was analyzed separately (*below*) to monitor expression levels. The position of the CtBP band is indicated with an *arrow* and the immunoglobulin heavy chain with a *line*. *B*, COS-1 cells were transfected with T7-CtBP and HA·TGIF, either wild type or with one of the mutations indicated. Complexes were isolated using a TGIF antiserum and analyzed for T7-CtBP by Western blot. Expression levels of each of the constructs are shown below. *C*, L40 yeast cells were cotransformed with DNA binding domain (*LexA*) and Gal4p activation domain (*GAD*) fusions to the indicated regions of TGIF, or to full-length CtBP. To test for interaction, colonies were transferred to plates lacking histidine with 2 mM 3-amino triazole to assess activation of the *LexA* operator-HIS3 reporter.

CtBP interact, COS-1 cells were cotransfected with epitope-tagged versions of both proteins. T7-tagged CtBP was clearly detectable in immunocomplexes precipitated via the HA epitope present at the carboxyl terminus of TGIF (Fig. 2*A*). In contrast, no coprecipitating CtBP was detectable in the absence of TGIF·HA. Similar experiments were performed using either wild-type TGIF or each of the two mutant forms in which the PLDLS motif is disrupted. Complexes were precipitated using a TGIF-specific antiserum and analyzed for the presence of T7-CtBP. No CtBP was detectable in immunocomplexes from cells transfected with either of the TGIF mutants (Fig. 2*B*). Additionally, in a yeast two hybrid assay, a fusion of amino acids 17–115 of TGIF to the LexA DNA binding domain was able to interact with CtBP fused to the Gal4p activation domain (Fig. 2*C*). This interaction was clearly disrupted by altering the PLDLS to ALAAS, further demonstrating the importance of this motif for interaction with CtBP.

A second HPE mutation in *TGIF* results in the alteration of proline 63 to arginine (P63R). This mutation lies within the homeodomain and has been shown to prevent DNA binding by TGIF and to result in impaired transcriptional repression by the amino-terminal half of TGIF (30). As shown in Fig. 2*B*, the P63R mutation decreased interaction of TGIF with CtBP, but did not abolish the interaction all together, suggesting that this mutation may have multiple effects on TGIF function.

Together, these results demonstrate that TGIF interacts with the corepressor CtBP and that this interaction is dependent on the presence of the PLDLS motif within TGIF.

TGIF Interacts With Both CtBP and HDAC1—We have previously demonstrated that TGIF interacts with HDACs (16). To determine whether TGIF could be present in the same complex together with both HDAC and CtBP, COS-1 cells were cotransfected with expression vectors encoding all three proteins and complexes precipitated via the FLAG epitope present on

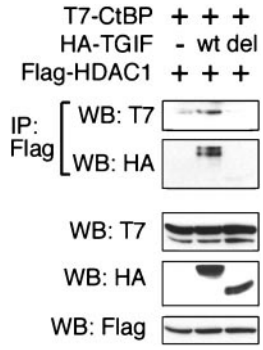

FIG. 3. **CtBP and HDAC interact with TGIF together.** COS-1 cells were cotransfected with expression vectors encoding FLAG-tagged HDAC1, T7-CtBP, and either a control vector or one encoding wild-type HA·TGIF or HA·TGIF with a deletion of the region that interacts with HDACs (*del*). Complexes were isolated on FLAG-agarose and analyzed for the presence of TGIF and CtBP by Western blotting. A portion of each lysate was analyzed by direct Western to assess expression levels (*below*).

HDAC1. As shown in Fig. 3, a very low level of T7-CtBP was detectable in FLAG-HDAC1 precipitates. However, on cotransfection of HA·TGIF, the amount of HDAC·CtBP complex was clearly increased. In contrast, coexpression of a deletion mutant of TGIF, which is unable to interact with HDAC failed to enhance the interaction of HDAC1 with CtBP. Thus, it appears that TGIF is able to interact with multiple transcriptional repressors, perhaps acting to coordinate a larger complex of corepressors with various mechanisms of action.

CtBP Interaction Is Required for Repression of TGF-β Responses by TGIF—The S28C mutant TGIF is impaired in its ability to repress the TGF-β-inducible transcriptional reporter, 3TP-lux (30). L17 cells were transfected with 3TP-lux and increasing amounts of expression vectors encoding wild-type or mutant TGIF. As shown in Fig. 4*A*, as compared with wild type, both the S28C and 3xAla mutations resulted in decreased repression of TGF-β-induced activation of the 3TP-lux reporter. Little repression by TGIF of basal activity was observed, and mutation of the CtBP binding site in TGIF had no effect in the absence of TGF-β (data not shown). In addition, we also tested the effect of the S28C mutation on repression of two other TGF-β transcriptional reporters. A3-lux contains three copies of the activin-response element (ARE) from the *Xenopus mix.2* gene (21). The ARE is bound by FAST winged helix transcription factors, and when FAST2 is expressed in mammalian cells, A3-lux is highly TGF-β inducible (45, 46). The *Smad7* promoter is induced by TGF-β signaling, and luciferase reporters in which transcription is driven by a region of the *Smad7* promoter show TGF-β-dependent activation (47). When cotransfected into L17 cells, wild-type TGIF repressed both the A3-lux reporter and a luciferase reporter containing the Smad7 promoter (Fig. 4, *B* and *C*). Importantly, in both cases the amount of repression by TGIF was significantly reduced by the presence of the S28C mutation. Thus, this HPE mutation appears to result in decreased transcriptional repression via several TGF-β-inducible response elements.

TGIF Recruits CtBP To Activated Smad Complexes—To investigate the possibility that TGIF can recruit CtBP to a TGF-β-activated Smad complex, COS-1 cells were transfected with FLAG-tagged CtBP, HA-tagged TGIF, and Smad2. Immunocomplexes were precipitated via the FLAG epitope present on CtBP and analyzed for the presence of TGIF and Smad2. As shown in Fig. 5*A*, Smad2 co-precipitated together with CtBP, but only in the presence of co-transfected TGIF. Importantly, this interaction was also dependent on the addition of TGF-β, suggesting that both TGIF and CtBP are recruited to an acti-

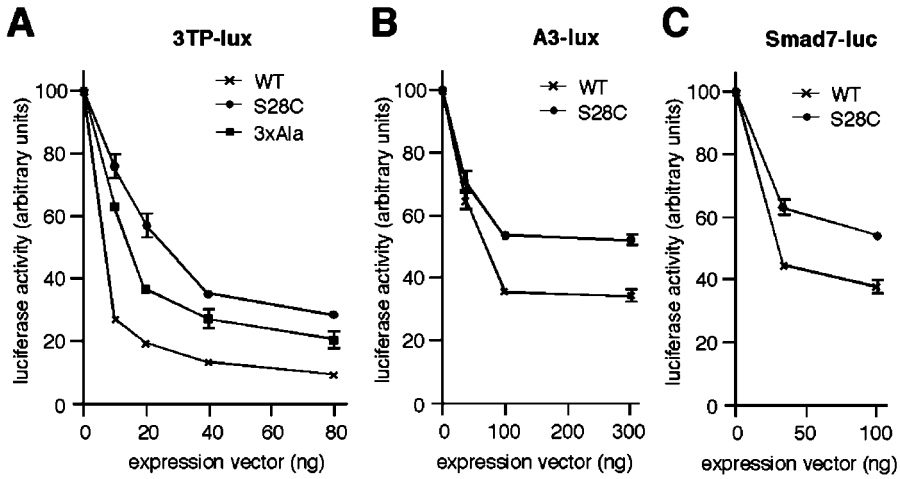

FIG. 4. **Repression of TGF-β transcriptional responses by TGIF requires interaction with CtBP.** *A*, L17 cells were transfected with the 3TP-lux reporter together with increasing amounts of expression vectors encoding wild-type (*WT*) TGIF or TGIF with mutations in the CtBP interaction motif (S28C or 3×Ala). 18 h after transfection TGF-β (100 pM) was added and luciferase activity was assayed after a further 18 h. Luciferase activity (arbitrary units) is presented as the mean ± S.D. of triplicate transfections. *B* and *C*, L17 cells were transfected and assayed as in *A* except that in *B* the A3-lux reporter and a FAST2 expression vector were used in place of 3TP-lux, and in *C* a reporter in which the luciferase gene is driven by the Smad7 promoter was used. For clarity, in *panels B* and *C*, the activities of wild-type TGIF and only the S28C mutant are shown.

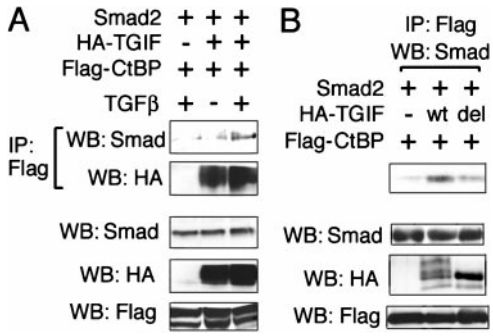

FIG. 5. **TGIF recruits CtBP to TGF-β-activated Smad complexes.** *A*, COS-1 cells were transfected with expression constructs encoding Smad2, FLAG-tagged CtBP and either HA-TGIF or a control vector. TGF-β (100 pM) was added for 1 h prior to cell lysis, and proteins were captured on FLAG-agarose. Complexes were analyzed for the presence of HA-TGIF and Smad2 using an HA-specific antibody or a Smad2/3 specific antiserum. Expression levels were monitored by direct Western (*below*). *B*, COS-1 cells were transfected, and protein complexes were analyzed as in *A*. The HA-tagged TGIF expressed was either wild-type (*wt*) or contained a deletion of amino acids 148–176 (*del*), as indicated.

vated Smad complex in response to TGF-β activation. Deletion of amino acids 148–176 from TGIF abolishes interaction with HDAC (16) and decreases interaction with TGF-β-activated Smads (15). In contrast to expression of wild-type TGIF, this deletion mutant was clearly compromised in its ability to bridge an interaction between CtBP and Smad2 (Fig. 5*B*). Together, these results suggest that following TGF-β stimulation, TGIF can recruit CtBP to an activated Smad complex and that the formation of this complex contributes to TGIF-mediated repression of TGF-β transcriptional responses.

DISCUSSION

TGIF is a transcriptional repressor with multiple repression domains, which appears to act in a context-independent manner (15, 16). TGIF represses transcription when bound to DNA via its cognate site or when brought to DNA by interactions with TGF-β-activated Smads. Repression by the carboxyl-terminal half of TGIF is dependent on the ability of the central repression domain of TGIF to recruit HDACs. We show here that the amino-terminal repression domain of TGIF, which represses transcription independent of HDAC activity, interacts with the corepressor, CtBP. This interaction is dependent on the presence of a conserved motif within TGIF (PLDLS, amino acids 24–28). Similar motifs have been identified in many transcriptional repressors and have been demonstrated to be required for recruitment of CtBP (35). In addition, this motif is conserved in all direct homologs of TGIF from other species, including the chicken AKR2 protein and zebrafish TGIF. Direct homologs of TGIF do not appear to be present in either *Drosophila* or *Caenorhabditis elegans*, although a *Drosophila* protein with a highly related homeodomain exists. It is likely that this *Drosophila* protein will bind to the same DNA sequence but may function differently from vertebrate TGIFs, because it lacks homology to TGIF repression domains.

As well as interacting with sequence-specific transcriptional repressors, CtBP is able to interact with a subset of human polycomb group (PcG) proteins via a PLDLS-like motif present within these proteins (39). PcG proteins in Drosophila are chromatin-associated proteins, which play a role in the stable repression of gene expression (48, 49). An analogous complex of PcG-related proteins is present in mammalian cells, although its precise mechanism of transcriptional repression is not clear (50–52). Because CtBP is able to homodimerize as well as interact with the related CtBP2 (Fig. 2 and Ref. 39), this raises the possibility that a dimer of CtBP may bridge an interaction between a gene-specific repressor and a complex of general transcriptional repressors including PcG proteins.

Recent evidence has suggested that CtBP represses via an HDAC-independent mechanism (53). Here we show that TGIF is able to interact with both CtBP and HDAC1 together, suggesting that TGIF may be able to recruit both of these transcriptional repressors to a specific gene. These results further strengthen the idea that TGIF may be part of a larger complex of transcriptional repressors, which acts to target such complexes to specific DNA elements, either via direct DNA binding or by interaction with TGF-β-activated Smads. However, we cannot rule out the possibility that different TGIF-containing corepressor complexes exist.

Interaction of TGIF with CtBP results in the incorporation of CtBP into a TGF-β-activated Smad complex, suggesting that TGIF can recruit both HDAC and CtBP to a Smad complex in response to TGF-β or activin signaling. It is also possible, therefore, that this would result in the recruitment of PcG

proteins to an activated Smad complex. It will now be of interest to further clarify the composition of the corepressor complex which TGIF is able to bring to TGF-β-activated Smads. Despite the fact that HDAC and CtBP can interact with TGIF together, it is possible that TGIF recruits only one of these corepressor activities to a specific target gene. Each of these modes of transcriptional repression may play a role either at different promoters or at the same promoter under different conditions, perhaps acting sequentially. However, it appears that at least for repression of some TGF-β-inducible gene responses by TGIF, both the CtBP and HDAC interaction domains are required.

In addition to the ability of the amino terminus of TGIF to recruit CtBP, it is possible that this region of the protein represses transcription by a second CtBP-independent mechanism. Several homeodomain proteins have been shown to repress transcription via direct interactions with general transcription factors. Transcriptional repression by Eve and Msx-1 is mediated by interactions of the homeodomain with the TATA-binding protein (54, 55), and repression by Msx-2 may require interactions with TFIIF (56). Mutations of the PLDLS motif in TGIF block interaction with CtBP but have only a modest effect on transcriptional repression by the amino-terminal half of the protein. In contrast, the P63R homeodomain mutant appears not to block interaction with CtBP but impairs transcriptional repression. Thus, it is possible that the TGIF homeodomain represses transcription by a distinct mechanism, possibly via interactions with general transcription factors.

Deletion or mutation of a single copy of *TGIF* in humans results in HPE (30). One previously identified HPE-associated mutation (S28C) in *TGIF* results in a single amino acid change within the PLDLS motif, which we show prevents interaction with CtBP. It, therefore, appears that disruption of the interaction between TGIF and CtBP can have a dramatic effect on craniofacial development in humans. As yet, it remains to be determined whether such mutations in *TGIF* cause HPE by affecting TGF-β gene responses or by impairing repression of distinct set of genes.

In summary, we demonstrate that TGIF interacts with CtBP via a conserved motif within the amino-terminal repression domain of TGIF and that the integrity of this motif is required both for interaction with CtBP and for full transcriptional repression by TGIF. The importance of this interaction is underscored by the fact that an HPE mutation in *TGIF* disrupts the PLDLS motif in TGIF and the interaction with CtBP.

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