Active-site tryptophan, the target of anti-neoplastic CtBP inhibitors, mediates inhibitor disruption of CtBP oligomerization and transcription coregulatory activities


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# Equal Contribution
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Active-site tryptophan is target of CtBP-inhibitors

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List of non-standard abbreviations
1. CtBP: C-terminal Binding Protein
2. EMT: Epithelial-Mesenchymal transition
3. MTOB: 4-(methylthio)-2-oxobutanoic acid
4. HIPP: 2-(hydroxyimino)-3-phenylpropanoic acid
5. 4-Cl-HIPP: 4-Chloro-2-(hydroxyimino)-3-phenylpropanoic acid
6. 2-OMe-HIPP: 2-Methoxy-2-(hydroxyimino)-3-phenylpropanoic acid
7. GST: Glutathione-S-Transferase epitope
8. His: Hexa-histidine epitope
9. Apc\textsuperscript{min/+}: Adenomatous Polyposis Coli, multiple intestinal neoplasia
10. EV: empty-vector
11. wt: wild-type CtBP2
12. mt: Mutant
13. NBD: Nucleotide-binding domain
14. CD: Catalytic domain
15. TS: Tetramer Switch
16. W324: Tryptophan-324 in CtBP2
17. W318: Tryptophan-318 in CtBP1
18. TIAM1: T-lymphoma invasion and metastasis-inducing protein 1
ABSTRACT

C-terminal Binding Proteins (CtBP1/2) are oncogenic transcriptional coregulators and dehydrogenases often overexpressed in multiple solid tumors, including breast, colon and ovarian cancer, and associated with poor survival. CtBPs act by repressing expression of genes responsible for apoptosis (ex. PUMA, BIK) and metastasis-associated epithelial-mesenchymal transition (EMT; ex. CDH1); and activating expression of genes that promote migratory and invasive properties of cancer cells (ex. TIAM1) and genes responsible for enhanced drug resistance (ex. MDR1). CtBP’s transcriptional functions are also critically dependent on oligomerization and nucleation of transcriptional complexes. Recently, we have developed a family of CtBP dehydrogenase inhibitors, based on the parent 2-hydroxyimino-3-phenylpropanoic acid (HIPP), that specifically disrupt cancer cell viability, abrogate CtBP’s transcriptional function, and block polyp formation in a mouse model of intestinal polyposis that depends on CtBP’s oncogenic functions. Crystallographic analysis revealed that HIPP interacts with CtBP1/2 at a conserved active site tryptophan (W318/324; CtBP1/2) that is unique among eukaryotic D2-dehydrogenases. To better understand the mechanism of action of HIPP-class inhibitors, we investigated W324’s contribution to CtBP2’s biochemical and physiological activities utilizing mutational analysis. Indeed, W324 was necessary for CtBP2 self-association, as shown by analytical ultracentrifugation and in vivo cross-linking. Additionally, W324 supported CtBP’s association with the transcriptional corepressor CoREST, and was critical for CtBP2 induction of cell motility. Notably, the HIPP derivative 4-chloro-HIPP biochemically and biologically phenocopied mutational inactivation of CtBP2 W324. Our data support further optimization of W318/W324-interacting CtBP
dehydrogenase inhibitors that are emerging as a novel class of cancer cell-specific therapeutic.

**Keywords:** CtBP, transcriptional coregulators, dehydrogenase inhibitor, 4-Cl-HIPP, Active-site tryptophan.
INTRODUCTION

CtBP1/2 play central roles as transcriptional coregulators in metazoan development and human diseases alike (Chinnadurai, 2000). Although a precise mechanism of coregulation is yet to be described, a plausible mechanism involves CtBP oligomerization in the presence of NAD(H), while one or more CtBP monomers interact with DNA-interacting proteins (ex. ZNF516 (Li et al., 2017). Following oligomerization, chromatin-modifiers can be recruited to the CtBP oligomer to form a promoter-bound “supercomplex” that actively regulates transcription (Shi et al., 2003).

Mounting evidence also suggests that CtBP plays a central role in multiple diseases, including cancer (Dcona et al., 2017). Both CtBPs act as oncogenic transcriptional co-regulators and are overexpressed and genomically amplified in multiple cancer types including breast, colon and ovarian cancer. Elevated expression levels of CtBP is associated with worse prognosis and survival (Barroilhet et al., 2013; Straza et al., 2010). The causal effect of CtBP on cancer severity could be due to CtBP hyperactivity, which has been found to modulate genes that regulate apoptosis, epithelial-mesenchymal transition, and DNA repair (Stankiewicz et al., 2013). Additionally, CtBP2 has also been described as a transforming oncoprotein that is central to the evolution of neoplasia driven by Apc mutation (Sumner et al., 2017). In light of this evidence, CtBP could be considered a potential therapeutic target in cancer. Our group has developed CtBP inhibitors targeting the dehydrogenase domain that are able to modulate CtBP target genes, induce selective cytotoxicity in cancer cells, and attenuate polyposis in the Apc min/+ mouse model of human Familial Adenomatous Polyposis (Straza et al., 2010; Korwar et al., 2016; Sumner et al., 2017; Chawla et al., 2018).
Chemically, CtBP dehydrogenase-domain inhibitors were designed to assimilate the structural attributes of MTOB (2-keto-4-methylthiobutyrate), an α-ketoacid substrate of CtBP that acts as inhibitor of its dehydrogenase activity at higher concentration (Figure 1A) (Straza et al., 2010). Additionally, the co-crystal structure of CtBP1/2 and MTOB revealed that MTOB’s thioether functional group interacts with the indolyl ring of CtBP1/2 tryptophan residue 318/324 (denoted as W324 in this paper), while a hydrophilic water-filled cavity links MTOB with the phosphate group of NAD(H) (Hilbert et al., 2014). This led to rational design of a more potent competitive inhibitor, hydroxyl-imino phenylpyruvate (HIPP) that included replacement of the oxo-group with a non-reducible imino-hydroxyl functional group, and replacement of the thioether group with a phenyl ring that enhanced binding efficiency of the inhibitor with W324 as a result of strong π-π* stacking between the phenyl and indolyl rings (Hilbert et al., 2015). Notably, the W324 residue is unique to CtBP among all eukaryotic NAD(H) dependent D2-dehydrogenases and has been previously hypothesized to be critical for the formation of higher-order oligomers of CtBP (Madison et al., 2013).

In light of this evidence, it is imperative to understand the physiologic role for conserved W318/324 in CtBP1/2 activities, as this residue is the key affinity target of the parent substrate competitive inhibitor HIPP that has already exhibited promising pre-clinical anti-neoplastic activity (Sumner et al., 2017; Chawla, et al 2018). To better understand the biologic effect of W318/324 targeted inhibitors on CtBP function, we have used site-directed mutagenesis, wherein W324 was mutated to simulate the conditions of inhibitor interaction/inhibition of W324 biochemical activity. Using this tool, we investigated the changes in the intrinsic activities of CtBP2 such as oligomerization,
transcriptional co-regulation and induction of cell migration, correlating the effects of W318/324 mutation with the observed biophysical and biologic effects of HIPP-class inhibitors.
MATERIALS AND METHODS

Antibodies and immunoblot analyses

Antibodies used in immunoblotting (IB) and immunoprecipitation (IP) assays are CtBP2 Antibody* ((E-16): sc-5966), CtBP2 (BD-Biosciences), His-probe Antibody* ((H-15): sc-803), GST Antibody ((B-14): sc-138) from SCBT, Vinculin (Cell Signaling Technology, 4650T), CoREST Antibody (Bethyl Antibody, A300-130A). All antibodies were used at dilutions suggested by the manufacturers. For immunoblot analysis, indicated mass of total protein-extract was boiled at 95°C in sample buffer, followed by separation on SDS–PAGE (Novex gels) and then transferred onto nitrocellulose membrane (0.45 microns porosity) (GE Healthcare). The membrane was incubated for 1-2 h in blocking buffer (Tris-buffered saline, 0.1% Tween (TBS-T), 5% non-fat dry milk) followed by incubation overnight at 4°C with the primary antibody solubilized in blocking buffer with sodium azide (0.01%). After 3X washes of 5 minutes with TBS-T, the blot was incubated with Alexa Fluor 680 or 790 nm secondary antibodies (Invitrogen) for 1 h in TBS-T and visualized on a Bio-Rad imager. (*discontinued by SCBT)

Cell culture

Cells (wild-type or CRISPR) were maintained according to the ATCC’s recommendation. HCT116 and MDA-MB-231 cells were maintained in regular RPMI-1640 or DMEM media supplemented with 10% (v/v) FBS and penicillin-streptomycin unless specified. Cells were maintained in a humidified incubator equilibrated with 5% CO2 at 37°C. All of the cells were authenticated by examination of morphology and growth characteristics and were confirmed to be mycoplasma-free using the Mycoplasma Detection Kit and DAPI staining.
Site-directed Mutagenesis and PCR:

A pET28a vector containing the truncated, 31-364 a.a., his-tagged CtBP2 cDNA was used as a template for site directed mutagenesis using round the horn amplification technique. Briefly, non-overlapping primers amplifying in opposing directions were designed to create point mutations. Primers were phosphorylated and PCR was performed followed by DpnI digestion and overnight ligation with T4 DNA ligase. DH5α chemical competent cells (Invitrogen) were transformed using the ligation products and selected on LB plates supplemented with 30µg/ml Kanamycin. Successful mutants were confirmed by sequencing. Informative mutations, as determined by NAD(H) reaction curves were substituted for wild type codons into full length CtBP2, carrying C-terminal V5 tag, in a modified pcDNA3.0 mammalian expression vector that also expresses GFP and puromycin acetyltransferase (PAC) as a chimeric protein. Mutants included in the study are G189A, R272A, H321A, W324G/A/F in addition to the empty vector and plasmid encoding wt-CtBP2 (Morris, 2016).

Protein Expression and purification.

Standard culture procedures were followed as reported in our previous work (Hilbert et al., 2015). Briefly, BL21-DE3 (RII) cultures were induced at an OD600 of 0.5-0.6 by addition of 0.5 mM IPTG and the temperature was reduced to 30°C from 37°C. Cells were harvested 5 hours after induction, pelleted and resuspended in 30 ml of buffer containing Complete EDTA-Free protease inhibitors (Roche), 50 mM Tris (pH 7.3) and 100 mM NaCl. Cells were then lysed using high pressure homogenizer and treated with DNasel. After pelleting, the soluble fraction was collected and incubated with 5 ml of nickel beads (Goldbio/Thermo-Scientific) with gentle stirring at 4°C for 90 minutes. The lysate and
beads were applied to a disposable column (Bio-Rad), and the lysate allowed to flow through. Then, beads were washed with 25 ml of Wash Buffer (WB) (50 mM Tris pH 7.3, 300 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 40 mM Imidazole) followed by 50 ml Wash Buffer supplemented with 0.5% Triton-X 100, 25 ml of Wash Buffer, 50 ml WB supplemented with 2.0 M NaCl and then 25 ml of WB. To remove excess cofactor (by converting NAD(H) to the weaker binding NAD+), 10 ml WB supplemented with 250 mM sodium pyruvate was incubated for 5 minutes on the column at 4°C. Sodium pyruvate incubation was repeated 5 times. Beads were washed with a final 25 ml of WB before elution with 300 mM imidazole. Eluted protein was dialyzed into dialysis buffer (50 mM Tris pH 7.3, 300 mM NaCl, 2 mM DTT, 1 mM EDTA) supplemented with 10% glycerol, flash frozen and stored at -80°C for long term or stored at 4°C for usage within a day or two.

**Deletion of CtBP2 by CRISPR-Cas9 in MDA-MB-231 cells:**

The co-ordinates of the targeted sequence are 124998142 to 124998161 (TGCAGACGGGATGTTGCACA) in Homo sapiens chromosome 10, GRCh38.p7 Primary Assembly, Sequence ID: NC_000010.11. Annealed DNA oligonucleotides that coded for the target specific crRNA were ligated with linear GeneArt CRISPR Nuclease Vector with Orange fluorescence Protein (OFP) (Cat No: A21174; Thermo Fisher Scientific). Electrocompetent *E. coli* (DH10B) were transformed overnight on 100 µg/ml Ampicillin supplemented LB-Agar plates. The plasmid DNA was verified by sequencing. MDA-MB-231 cells at 70% confluency, grown in DMEM supplemented with 10% FBS, in a 100mm dish were transfected with 6µg plasmid DNA and incubated at 37°C (5%CO₂) for 72 hours. OFP positive cells were single cell sorted in a 96 well plate using an Aria-
BD FACSAria™ II High-Speed Cell Sorter at $\lambda_{\text{exc}}=488\text{nm}$ and allowed to grow to confluency before splitting them in triplicate plates to screen for mutant clones. Clones were lysed in 96 well plates using 50 µL RIPA buffer (150mM NaCl, 50mM Tris HCl, pH 8.0, 1.7% NP-40, 0.17% sodium dodecyl sulfate (SDS), 0.5% Na-deoxycholate, 5mM EDTA) supplemented with Protease inhibitor cocktail (Roche). Samples were clarified by centrifugation and analyzed using immunoblot technique. HCT116 CtBP2-KO using CRISPR protocol is published elsewhere (Chawla et al., 2018).

**Sedimentation Velocity Analysis**

Sedimentation velocity experiments were carried out using a Beckman Optima XL-I analytical ultracentrifuge (Beckman Coulter Inc.) equipped with an eight-position AN-60Ti rotor. The protein samples were loaded in the cells, using, in all cases, the reference buffer. Samples in double sector cells were centrifuged at 40000 rpm. For all experiments, temperature was kept at 20 °C. Sedimentation profiles were recorded using interference scanning optics. For analysis of the data, the program Sedfit was used to calculate sedimentation coefficient distribution profiles using the Lamm equation (Schuck, 2000).

**GST pull-down assay**

Recombinant GST-CtBP2 were produced in BL21 (DE3)-RIL competent cells following treatment of cells with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h, lysed and incubated with glutathione-agarose beads (Thermo Scientific) for 2 h in immunoprecipitation (GST-IP) buffer consisting of 20 mM HEPES, pH 7.3, 200 mM NaCl, 0.5 mM EDTA, 1% IGEPAL CA-630, 0.5 mM DTT and protease inhibitors (Roche) (added just prior to the experiment). After binding of protein to the beads and
thorough washing (4X), the modified beads were incubated with relevant recombinant proteins (glycerol-free) overnight at 4°C in GST-IP buffer. After binding of protein to the beads and thorough washing (4X) following that, and resin-bound proteins were eluted by heating, resolved by SDS-PAGE and were detected by immunoblotting with anti-GST and anti-His antibodies.

**In-vivo Crosslinking**

Disuccinimidyl glutarate (DSG) crosslinker was purchased from Thermo Scientific and appropriately stored with desiccants at -20°C. A stock solution of 10 or 50mM in DMSO was made fresh for each experiment. In vivo crosslinking was carried out in cells that were collected by scraping and washed with PBS, and later resuspended in PBS (pH ~8.2) with 1X Complete Protease Inhibitor Mixture, EDTA-free (Roche Applied Science). Samples were incubated with cross-linker (250 µM) for 30 min at 37 ºC with rotation. The reaction was quenched with the addition of 1 M Tris, pH 7.6, to 20 mM final concentration and incubated for 15 min at RT. After quenching, in vivo cross-linked samples were lysed using RIPA buffer, followed by ultracentrifugation for 30 min at 15000 rpm at 4 ºC. The quantified samples were then loaded and analyzed on SDS-PAGE gel for efficiency of inter-protein crosslinking.

**Immunoprecipitations**

For immunoprecipitation experiments, HCT116; CtBP2-/- cells were transfected with expression vector that encodes either vector, wt or relevant mutants (6 µg) using lipofectamine 2000 as recommended by the manufacturer (Invitrogen). After 48 h, cells were lysed in IP buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% NP-40, protease inhibitor-added fresh). Whole cell lysates (1.0–2.0 mg) were pre-cleared by incubation with
protein G Agarose Fast Flow (EMD Millipore) for 1 h on a rotator at 4 °C. 2 micrograms of relevant antibodies were added to the pre-cleared lysates and incubated on a rotator at 4 °C for 1h. Then, washed beads were added to these mixtures and incubated overnight. Following incubation, beads were washed three times in IP buffer and resuspended in 40 μl of 4X loading buffer (NuPage-Invitrogen) and heated to 95 °C for 10 min to isolate protein and beads, and then centrifuged for clear supernatant, which were then analyzed by SDS–PAGE using standard western blotting technique. For endogenous drug assays, the cells were treated with 500 or 1000 μM of inhibitor for 24 h before cellular lysis, post transfection of either EV or wt-CtBP2 and the IP protocol was followed.

**RT-PCR and real-time RT-PCR**

48 h post transfection of 6 μg of empty-vector, wt, G189A, R272A, W324F and W324A plasmids, total cellular RNAs were isolated from samples using QIAGEN RNeasy kit and instructions therein. Later, cDNA synthesis was carried out using SensiFAST cDNA Synthesis Kit from BIOLINE. Quantitation of all gene transcripts was done by qPCR using SYBR Green (Applied Biosystems, Foster City, CA) and an ABI 7300 (Applied Biosystems) machine.

18srRNA expression was used as an internal control. The primer pairs used were:

- 18srRNA: 5’-CGCCGCTAGAGGTGAAATTC -3’ (forward) and 5’- TGGCAAATGCTTTCGCTCTG-3’ (reverse);
- TIAM1: 5’- CGCTGGAGTCGTACCTCATC-3’ (forward) and 5’-GGTCAACACACAGCCCCAACC-3’ (reverse)
Relative amounts of the mRNA transcripts were calculated using the ΔΔCT method and reported as fold change with respect to empty-vector transfection (Schmittgen and Livak, 2008).

**In Vitro Wound Healing Assay (mutant and inhibitor)**

*Mutants:* MDA-MB-231 cells (CtBP2-KO) were seeded into six-well dishes at a density of $5 \times 10^5$ cells/well. The dishes were cultured as confluent monolayers and were then further transfected with 3μg of empty-vector, wt, G189A, R272A, W324F and W324A plasmids. *Inhibitor:* MDA-MB-231 cells (CtBP2-wt) were seeded into six-well dishes at a density of $5 \times 10^5$ cells/well. The dishes were cultured as confluent monolayers and were then further treated with 4-Cl HIPP at 156, 312, 625, 1250 and 2500 μM concentration, while a vehicle treated dish served as control.

Following the 24 h incubation time in either case, a scratch was made once per well with a 200-μL pipette tip to create an artificial wound. Wounded cell cultures were then incubated in the presence of DMEM after thorough, but gentle washes. The migration of cells was monitored over a duration of 24 h for transfection and, 24, 40 and 48 h for inhibitor treatment, as a function of how far from the scratch line the cells had progressed. The scratch closures were quantified using ImageJ (NIH). The area at a time-point is normalized relative to 0 h time and reported as absolute value.

**Data analysis and statistical methods**

All experiments were carried out three times (represented as N=3 in their legends). Student t-test was used for statistical analysis of Figure 3B and Supplemental figure 6. Statistical evaluation of the differences between the groups from the qPCR and mutant CtBP2 migration data (Figure 5A and 5B) were conducted initially using a one-way
analysis of variance (ANOVA), followed by multiple comparisons with false discovery rate (FDR) adjustments for handling multiplicity issues (Hochberg, 1995). The FDR adjustment is usually a less stringent control of Type-1 error, compared to family-wise error controls, via the Bonferroni adjustment, leading to higher power to detect truly significant results. For the migration data of inhibitor treated cells (Figure 5C) where measurements were taken at 4 time points, we initially conducted a one-way repeated measures ANOVA, followed by FDR adjusted multiple comparisons to test group differences. We used SAS statistical software, Version 9.4 of the SAS System for Windows, for all analyses. The error bars refer to standard deviation (SD) for all calculations.
RESULTS

Active-site tryptophan 324 contributes to CtBP2 self-association that can be abrogated by 4-Cl HIPP in vitro

To understand the role of W324 in CtBP function, we first investigated the effect of W324 mutation on CtBP2’s self-association capability, focusing on CtBP2, as the CtBP1 and CtBP2 dehydrogenase domains are structurally nearly identical (Hilbert et al., 2014). Oligomerization of CtBP contributes directly towards CtBP’s transcription coregulatory activity (Dcona et al., 2017). The efficiency of oligomerization is intensified in the presence of NAD(H), which further influences CtBP’s ability to associate with other coregulators (Bellesis et al., 2018; Bhambhani et al., 2011). Previous studies have shown that mutations in the CtBP1 nucleotide-binding domain (NBD) diminish CtBP1’s ability to oligomerize, and also influence its transcriptional activities (Mani-Telang et al., 2007). Having previously engineered a His-tagged truncated version of wild-type (wt) CtBP2 protein (Figure 1B) (Hilbert et al., 2014) active for dehydrogenase and oligomerization activities, we compared the biophysical and functional effects of a series of W324 mutations to control mutations known to directly affect CtBP nucleotide binding (G189A) or catalysis (R272A, and H321A) (Dcona et al., 2017). W324 was then mutated to G, A, and F (Figure 1C): W324F partially tests the requirement for the indolyl ring at position 324, as opposed to a phenyl ring, which is predicted to also engage in a parallel-displaced π-π* interaction with substrate bound within the catalytic domain (McGaughey et al., 1998); W324A and G are hydrophobic mutations that should plausibly abrogate CtBP2 dehydrogenase activity by weakening the affinity for substrate, as π-π* interaction of the MTOB thioether with the 324 residue would not be
possible, with W324G further testing the effect of conformational freedom not available in W324A (de Groot et al., 1997).

Prior work has demonstrated that truncated CtBP2(31-364) (st. CtBP2), dimerizes in accordance with crystallographic data; whereas in solution, full-length CtBP1 tetramerizes as a dimer of dimers (Kumar et al., 2002; Madison et al., 2013). For the purpose of our studies, we used st. CtBP2, due to the highly unstructured nature of the C-terminus of CtBP1/2 arising from a proline/glycine rich sequence (Nardini et al., 2006). Hence, for the purpose of simplicity and to maintain consistency with previous reports (Hilbert et al., 2014, 2015; Kumar et al., 2002), in vitro studies utilized the residue 31-364 truncated versions of wt and mt CtBP2 recombinant proteins.

After affinity-based purification, the oligomeric behavior of wt and mt His-tagged st. CtBP2 proteins were analyzed by analytical ultracentrifugation (AUC) using sedimentation velocity. The sedimentation coefficient distribution of the wt protein at 27.2 μM indicated the presence of a predominant dimeric population (s=6-6.7), which was further intensified in the presence of 10 μM NAD(H) (Figure 2A-C). The range of values representing the CtBP monomer/dimer species are a result of the presence of the His-tag and the multiple conformations that it can acquire. The values are consistent with the theoretical sedimentation velocity coefficients calculated using the CtBP2 crystal structure and two different conformations of His-tail using the program SOMO as part of the ultrascan III (Demeler, 2010; Schuck, 2000). Mutations in any relevant functional domain of CtBP2 (G189A, R272A, H321A; see Figure 1C) impaired self-association, resulting in a mixed population of monomer and dimer (Figure 2D, 2E and Supplemental Figure-1) (Kumar et al., 2002; Madison et al., 2013). Interestingly, W324F and W324G
mutants exhibited monomeric or mixed monomer/dimer populations without NAD(H), but yielded patterns similar to that of wild-type in the presence of NAD(H). In contrast, W324A was primarily (~60%) monomeric, despite addition of NAD(H) (Figure 2D, 2E and Supplemental Figure-1). Taken together, these results demonstrate that the indolyl moiety of the W324 residue is pivotal for CtBP2 oligomerization, and suggest that this residue functions to stabilize the oligomeric interface (Madison et al., 2013).

Given the strong interaction of the substrate-competitive CtBP inhibitor HIPP with W324, (Hilbert, et al., 2015) we examined the effect of the more potent 2nd generation CtBP inhibitor 4-Cl-HIPP (Korwar et al., 2016) on CtBP2 self-association, to determine if disruption of the oligomer could be part of this inhibitor’s mechanism of action. For this purpose, CtBP2 was incubated with increasing concentration of 4-Cl-HIPP (10 to 1000 μM) with 10 μM NAD(H), and the oligomerization state probed using sedimentation velocity. Surprisingly, only 10 μM of 4-Cl-HIPP (only 58.4-fold higher than the Kd for HIPP/CtBP interaction was sufficient to robustly disrupt CtBP2 oligomerization, indicating that HIPP-class CtBP dehydrogenase inhibitors disrupt not only catalysis, but also CtBP2 homo-dimerization (Figure 2F-H and Supplemental Figure-2) (Korwar et al., 2016).

**W324 mutation disrupts self-association with wild-type CtBP2**

To further illustrate that W324 is important for CtBP2’s self-association, we investigated the ability of mutant CtBP2 (mt-CtBP2) to hetero-dimerize with wt-CtBP2 in the presence of NAD(H), using a glutathione-S-transferase (GST)-based pulldown assay. GST-CtBP2 (31-364) protein was immobilized onto glutathione (GSH)-agarose beads, and incubated
with wt or mt CtBP2 proteins tagged with His6-epitope at different concentrations (Figure 3A-B). As predicted by the AUC homodimerization analyses, dimerization between two differently tagged wt-CtBP2 proteins proceeded as expected, and the interaction increased with an increase in the concentration of His6-wt-CtBP2 protein (Figure 3A). In contrast, His-tagged CtBP2 W324A and G189A mutant proteins were both similarly defective for hetero-dimerization with wt-GST-CtBP2 as evidenced from the blots and densitometry calculations (Figure 3A-B and Supplemental Figure-3).

**Determination of IC50 for 4-CI-HIPP disruption of oligomerization**

After understanding that W324A impairs hetero-dimerizing capability in the pulldown assay, and knowing that 4-CI-HIPP can disrupt CtBP oligomerization at or near its Kd, we utilized the pull-down assay to more quantitatively measure the IC50 for disruption of oligomerization of 4-CI-HIPP vs. an inactive (for catalytic inhibition) control HIPP derivative, 2-methoxy-HIPP (Figure 3C, Supplemental Figure-4) (Korwar et al., 2016). Using log-scale titration of 4-CI-HIPP from 0.01 to 500 µM, we found that CtBP2 dimerization diminished between 1 and 10 µM of 4-CI-HIPP, and disappeared after further titration of the drug above 10 µM (Figure 3C). The calculated IC50 value of inhibition of dimerization by 4-CI-HIPP was 10.52 µM (Figure 3D), consistent with AUC analyses above (Supplemental Figure-2), whereas 2-methoxy-HIPP displayed an IC50 for disruption of hetero-dimerization >100 µM (Supplemental Figure-4) (Korwar et al., 2016). Thus, in addition to inhibiting CtBP’s dehydrogenase activity, 4-CI HIPP acts as a high affinity inhibitor of CtBP oligomerization—which is required ultimately for its transcriptional function (Dcona et al., 2017).
Cross-linking of CtBP2 in vivo reveals that W324 is central to the formation of physiologic oligomeric species that can be disrupted using 4-Cl HIPP

To analyze the physiologic oligomeric state of full-length CtBP2 \textit{in situ} in living cells, we next performed chemical protein cross-linking of whole cells in culture (Figure 3E, Supplemental Figure-5). For this purpose, we transfected expression plasmids encoding \textit{wt}, G189A, W324F and W324G mutated CtBP2 cDNA into HCT116 cells homozygously deleted for CtBP2 using CRISPR techniques (see S.I). Intact transfected cells were then incubated with DSG (disuccinimidyl glutarate) at 37°C for 30 mins, and total cellular protein immunoblotted for CtBP2. CtBP2 was absent, as expected, from vector transfected cells, and \textit{wt}-CtBP2 exhibited distinct forms consistent with monomer and dimer based on predicted molecular weight (Figure 3E). Notably, W324F and G proteins exhibited a lower efficiency of dimer and higher oligomer formation as did G189A, consistent with our \textit{in vitro} AUC and pulldown data (Figures. 2-3). All of the above results indicate that W324 is an essential residue that contributes significantly toward CtBP2’s oligomeric capability, including in the setting of full length CtBP2 in the physiologic cellular environment.

After understanding that the oligomer formation of CtBP2 is strongly dependent on W324, we performed a chemical cross-linking experiment using DSG in HCT116; cells with wild-type CtBP2 expression, treated with either 500 or 1000 μM of 4-Cl HIPP. Here again (Figure 3F), we found that the ability of CtBP2 to dimerize was abrogated in the presence of the 4-Cl-HIPP, similar to the effect of the drug \textit{in vitro} in the AUC and
pull-down experiments, albeit at higher concentration, due presumably to either poor cellular penetration or rapid intracellular metabolism.

**Interaction of CtBP2 with other transcriptional coregulators is facilitated by W324**

CtBP associates with CoREST among other chromatin modifier proteins, to transcriptionally co-regulate (repress and activate) numerous cancer-related tumor suppressor and oncogenes, including CDH1 and EGFR (Li et al., 2017; Quinlan et al., 2006). Having established that W324 influences CtBP2’s oligomerization capabilities, we further inquired whether physiologic functions, such as CtBP co-association with CoREST (Choi et al., 2013) depend on intact W324. For this purpose, plasmids encoding FL. wt-CtBP2, CtBP2-G189A, and CtBP2-W324G were transfected into HCT116; CtBP2/- cells along with empty-vector control, and 24 hr post-transfection, cell lysates were immunoprecipitated (IP’d) with anti-CtBP2 antibody, followed by immunoblotting (IB) with antibodies against CtBP2, CoREST and vinculin (loading control). Whereas wt-CtBP2 exhibited robust coIP with CoREST, neither the W324G nor G189A mutants were able to coIP CoREST (Figure 4A). Despite the close sequence similarity of CtBP1 and CtBP2, these data do however conflict with prior work showing that a cluster of mutants in the CtBP1 NBD, namely G183A and G186 (G189A and G192A in CtBP2), did not have any influence on the interaction between CtBP1 and CoREST(Kuppuswamy et al., 2008). Nevertheless, mutations which have in common disruption of CtBP2 oligomerization all disrupt CoREST/CtBP2 interaction, and these data may point to heretofore unknown distinct functions for CtBP1 and CtBP2 in transcriptional
regulation, similar to distinct functions already described in Golgi regulation, mitosis, and ribbon synapse formation (Dcona et al., 2017).

Given the strong influence of W324 on CtBP2/CoREST interaction, we further investigated whether 4-Cl HIPP was able to disrupt CtBP2/CoREST complex formation. 24 h post transfection of FL-CtBP2 or empty-vector, HCT116; CtBP2/-/- cells were incubated with 500 and 1000 μM of 4-Cl-HIPP for an additional 24h and cell lysates were IP’d with anti-CtBP2 antibody, followed by immunoblotting (IB) with antibodies against CtBP2, CoREST and vinculin (loading control). 4-Cl HIPP at 1000 μM, but not 500 μM, effectively disrupted CtBP2/CoREST complexation in a manner similar to the disruption provided by mutation of W324 to glycine (Figure 4B).

Thus, genetic or pharmacologic targeting of CtBP2 W324 inactivated a key biochemical correlate of its transcriptional activity, namely interaction with a member of the CtBP transcriptional supercomplex. (Shi, et al., 2003)

W324 mutation or inhibitor abrogates CtBP2 transcriptional regulation and induction of cellular motility

A key pro-oncogenic function of CtBP is to induce cell migratory behavior that is related to CtBP’s role in induction of EMT and metastasis. (Dcona, et al., 2017) Previously, we have shown that CtBP2 activates the TIAM1 gene that mediates CtBP-dependent cell migration (Minard et al., 2004; Paliwal et al., 2012). Therefore, we inquired whether intact W324 is required for CtBP2 to transactivate TIAM1, and also affect cell migration. Plasmids encoding vector control, wt CtBP2 or CtBP2 mutants G189A, R272A and W324F/A were transfected into HCT116; CtBP2/-/- cells, followed by extraction of total...
mRNA, and QPCR determination of TIAM1 mRNA abundance. Expression of wt and mt CtBP2 proteins was equivalent as determined by CtBP2 immunoblot (Supplemental Figure-6). Whereas wt-CtBP2 activated TIAM1 4.5-fold, all CtBP2 mutants were defective for TIAM1 induction (Figure 5A). This effect could be explained by disruption of oligomerization, where monomeric CtBP2 mutant proteins are unable to form the active CtBP transcriptional supercomplex that bridges chromatin modifying enzymes with DNA interacting transcription factors (Dcona et al., 2017).

Next, we investigated whether cellular motility is affected by mutation of W324. MDA-MB-231;CtBP2-/- (CRISPR deletion) cells, which migrated at slower-rate than HCT116; CtBP2-/- cells, were transfected with vector, FL. wt-CtBP2, or CtBP2 G189A, R272A, or W324F/A plasmids, and after 24 h, a scratch was made and cells were allowed to migrate to fill the scratch. Expression of wt- and mt-CtBP2 proteins was equivalent as determined by CtBP2 immunoblot (Supplemental Figure-7). Whereas wt CtBP2 transfection significantly accelerated wound-closure within 24 h, all mutants were significantly defective for promoting wound closure, indicating that W324, CD, and NBD are all required for both TIAM1 transactivation and for the key physiologic cellular CtBP function of promoting migration (Figure 5B).

To chemically phenocopy genetic inactivation of W324, MDA-MB-231 cells were treated with 0-2500 µM 4-Cl HIPP, followed by a scratch assay time course over 48 hrs. With increasing dosage of the inhibitor, the ability of cells to migrate diminished, and then nearly ceased, at the highest dosage, despite minimal loss of cell number during this time frame (Figure 5C). Thus, pharmacologic targeting of W324 and catalytic function impairs
CtBP2 oligomerization, transactivation of a key migration gene, and ultimately CtBP2’s ability to effectively drive cell migration.
DISCUSSION

In an attempt to understand the reasons behind the anti-neoplastic efficacy of CtBP dehydrogenase inhibitors, we have analyzed the contribution of the key inhibitor interacting residue, CtBP2 W324 (Hilbert et al., 2015; Madison et al., 2013), to oligomerization and key biochemical/functional correlates of transcriptional complex formation, activation of a key migration gene and cell migration. Prior work reported that the paralogous CtBP1 W318 residue acts as a switch that links NAD(H) binding to dimerization through a strand exchange mechanism, which then facilitates tetramer (dimer of dimer) formation and though F.L. CtBP1 (W318F) exhibited diminished ability to form tetramers, it dimerized partially (Madison et al., 2013). Our results corroborate and extend this hypothesis, wherein we witnessed that mutation or chemical targeting of CtBP2 W324 impaired dimerization as observed from altered sedimentation coefficients (s) and pull-down assays.

It is worth noting that despite extensive purification, the wt-CtBP2 protein existed as a dimer in the absence of added NAD(H) as evidenced from AUC (Figure 2A), which could be due to the presence of NAD(H) nucleotide from E. coli, whereas most mt-CtBP2 existed as mixed population of monomer and dimer, and underwent conformational rearrangement in the presence of excess nucleotide (Figure 2E). Of note, the H321A, G189A, and W324A mutants remained largely unaltered in oligomeric state despite the addition of NAD(H), which could be due to an impaired strand-exchange oligomerization mechanism. Contrary to our expectations, wherein we assumed that all non-aromatic mutants of W324 would have impaired oligomerizing capabilities, W324G exhibited a substantial dimer population, even in the absence of the NAD(H). Therefore, it is
reasonable to suppose that there could be an equilibrium between monomeric and dimeric populations of CtBP2 W324G protein, as is the case presumably for W324F, which though impaired for dimerization in the absence of NADH, readily dimerized with NADH addition. Exactly why W324G dimerizes, while other non-aromatic mutants are impaired is unclear, and will require further in silico modeling and/or X-ray crystallographic analysis of the W324G dimer.

Consistent with our previous hypothesis that the HIPP-CtBP complex may be trapped in an inactive abortive ternary complex (Hilbert et al., 2015), we found that addition of increasing concentrations of 4-Cl-HIPP inhibited oligomerization of monomeric-CtBP2, despite the presence of NAD(H). This is explained by CtBP-NAD(H) complex assuming a closed conformation at 4-Cl-HIPP concentrations ≳ ~10 µM, the IC50 for CtBP2 oligomer disruption.

Mutation of CtBP2 W324, or substrate competitive inhibition with 4-Cl-HIPP, debilitated CtBP2 co-association with CoREST (Choi et al., 2013; Cowger et al., 2006; Kuppuswamy et al., 2008; Li et al., 2017). Interestingly, mutation of W324 and the CtBP2 NBD and CD domains disrupted TIAM1 transcription and migration. Specifically, transcriptional analysis using QPCR showed that the robust transcriptional induction of TIAM1 by CtBP2 was abolished by mutations W324A and W324F as well the NBD and CD mutations G189A and R272A (Figure 5A). Consistent with a key role of TIAM1 expression in the cellular migration phenotype, the same mutations also abolished the robust induction of cell migration observed with wt CtBP2 (Figure 5B). Though in both the TIAM1 and migration assays, certain mutants seemed to cause a possible dominant negative effect with measured values going below control baseline values (Figure 5A-B),
the experiments were not sufficiently statistically powered to test this possibility, which will be explored in the future. Intriguingly, possible dominant negative effects were particularly seen in the mutants that displayed diminished self-association characteristics not restorable with additional NAD(H) as evidenced from AUC and crosslinking experiments (Figures 2 and 3), consistent with these specific mutations more potently disengaging the activities of CtBP dependent on oligomerization.

The dosage of 4-Cl-HIPP to achieve efficacy in cellular assays remains well beyond its \textit{in vitro} effective concentration, and higher than desirable for a true therapeutic molecule. This need for high micromolar concentrations of inhibitor \textit{in vivo} derives from the need to achieve stoichiometric excess over NAD(H) cellular concentrations, combined with likely inefficient cellular entry of 4-Cl-HIPP (Korwar \textit{et al.}, 2016). Using the mechanistic knowledge gained in this work, we are focused on creating more potent inhibitors that target W324 with improved cellular entry, to thus achieve therapeutic effect at doses within the range of practicality for clinical development.
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Performed data analysis: Dcona, Escalante, Bandyopadhyay, Deng, Grossman

Wrote or contributed to the writing of the manuscript: Dcona, Bandyopadhyay, Escalante, Grossman
REFERENCES


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Morris, B.L. (2016). Understanding and targeting the C-terminal Binding Protein (CtBP) substrate-binding domain for cancer therapeutic development. Virginia Commonwealth
University.


FOOTNOTES

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FIGURE LEGENDS

**Figure 1.** (A) MTOB (1) HIPP-derivatives (2). (B) Graphical representation of full length (F.L) and short truncated (st.) versions of CtBP2. (C) Table listing mutants and their functions. TS/IB (Tetramer Switch/ Inhibitor binding residue).13

**Figure 2.** Normalized ratio of area under the curve for monomer and dimer calculated using SedFit20 and sedimentation coefficient distribution profile of native wt-CtBP2 (A, B) or mt-CtBP2 (D); and wt-CtBP2 (A, C) or mt-CtBP2 (E) in the presence of 10 µM NAD(H). (F) Graphical representation of area under the curve for AUC assay of wt-CtBP2 incubated with varying 4-Cl-HIPP concentrations and 10 µM NAD(H). (G-H) Sedimentation coefficient distribution of wt-CtBP2 (G) native or incubated with (H) 10 µM 4-Cl-HIPP and 10 µM NAD(H). The protein concentration for each assay is 27.2 µM. Red-monomer, Blue-dimer.

**Figure 3.** (A-D) GST-pulldown assay using GST-CtBP2(31-364) incubated with (A-B) wt- or mt-His-st. CtBP2 and 10 µM NAD(H) or (C-D) wt-CtBP2 incubated with increasing doses of 4-Cl-HIPP and 10 µM NAD(H). The letter “m” denotes protein standard marker on top of the representative gel for GST-wt-CtBP2 and * = p value <0.05. Triangles indicate increasing dosage of wt or mt proteins (N=3). (E) HCT116; CtBP2-/- cells were transfected with either wt- or mt-CtBP2 expression plasmids and cells treated with DSG for 30 mins, followed by lysis and CtBP2 immunoblot. (F) HCT116 cells with wt. CtBP2 expression were treated with 4-Cl HIPP at 500 and 1000 µM for 24 h, and cells then exposed to DSG for 30 mins, followed by lysis and CtBP2 immunoblot. (N=3). Yellow arrow represents monomer (Mol. Wt. ~ 49 KD) and red arrow indicates dimer (Mol. Wt. ~98 KD). * = p<0.05 (student t-test)
**Figure 4.** Co-IP between CoREST and (A) wt- or mt-CtBP2 or (B) wt-CtBP2 transfected into HCT116; CtBP2-/- cells treated with vehicle, 500 or 1000 µM of 4-CI-HIPP, followed by immunoblotting with indicated antibodies. (N=3).

**Figure 5** (A) Fold-change of TIAM1 mRNA expression after transfection of either wt- or mt-CtBP2, relative to empty-vector transfection. (N=3). (B-C) Graph representing quantified area of scratch after 24 h measured relative to area at 0 h time point in cells transfected (B) with either wt- or mt-CtBP2, (N=3). or (C) with wt-CtBP2 and treated with 4-CI HIPP at concentrations between 0-2500 µM for 24 h. (N=3). False discovery rate procedure was applied and pairwise comparisons were conducted to determine the significance of these experiments. * = p<0.05; ** = p<0.01; *** = p<0.001.
Figure 1

A

\[
\begin{align*}
1 & \quad \text{S-S} \quad \text{O} \quad \text{OH} \\
2 & \quad \text{R} \quad \text{N} \quad \text{OH}
\end{align*}
\]

B

Full length CtBP2 (F.L.)

<table>
<thead>
<tr>
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<th>Dehydrogenase domain</th>
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<tbody>
<tr>
<td>1</td>
<td>Short truncated CtBP2 (st.CtBP2)</td>
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C

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<tr>
<td>CtBP2-G189A</td>
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<tr>
<td>CtBP2-R272A</td>
<td>Catalysis</td>
</tr>
<tr>
<td>CtBP2-H321A</td>
<td>Catalysis</td>
</tr>
<tr>
<td>CtBP2-W324G/A/F</td>
<td>TS/IB</td>
</tr>
</tbody>
</table>

R = 4-Cl, 2-OMe
Figure: 2

A

Ratio of area under curve
Native:Cp2g
NASH+NADH

B

s = 6

C

s = 6.38

D

Ratio of area under curve
Monomer:Dimer

E

s = 5.82

F

s = 4.32

G

H

Vehicle
+10 μM NAD(H)

10 μM 4-Cl HIPP
+10 μM NAD(H)

Monomer

Dimer
Figure: 3

A) GST-CtBP2 (31-364) and Ab-GST

B) Normalized densitometry values relative to GST-CtBP2:His CtBP

C) 10% Input and 4-Chloro HIPP

D) IC50 = 10.52 μM

E) α-CtBP2

F) 4-Chloro HIPP and α-CtBP2
Figure: 4

A

<table>
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<tbody>
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<td>-</td>
</tr>
<tr>
<td>pCtBP2-Wt</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CtBP2-G189A</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>CtBP2-W324G</td>
<td>-</td>
<td>+</td>
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</table>

\[\alpha-CtBP2\]

\[\alpha-CoREST\]

\[\alpha-Vinculin\]

B

<table>
<thead>
<tr>
<th></th>
<th>Input (pEmpty, pCtBP2-wt)</th>
<th>IP-CtBP2 (pEmpty, pCtBP2-wt)</th>
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<tbody>
<tr>
<td>4-Cl HIPP *</td>
<td>[\text{Image}]</td>
<td>[\text{Image}]</td>
</tr>
</tbody>
</table>

\[\alpha-CtBP2\]

\[\alpha-CoREST\]

\[\alpha-Vinculin\]
Figure: 5

A

Fold Change of T1AM1 mRNA expression (normalized to 18sRNA)

B

% Wound Closure (Absolute Values)

C

% Wound closure (Absolute value)

4-ClHIPP (μM)

Vehicle 156 312 625 1250 2500

0h 24h 40h 48h