Tetrameric Oligomerization Mediates Transcriptional Repression by the BRCA1-dependent Kruppel-associated Box-Zinc Finger Protein ZBRK1

Wei Tan, Seokjoong Kim, and Thomas G. Boyer‡

Received for publication, September 22, 2004, and in revised form, October 5, 2004
Published, JBC Papers in Press, October 20, 2004, DOI 10.1074/jbc.M410926200

The Kruppel-associated box (KRAB)-zinc finger protein ZBRK1 has been implicated in the transcriptional regulation of DNA damage-response genes that function in cell growth control and survival. Recently, we described a novel BRCA1-dependent C-terminal transcriptional repression domain (CTRD) within ZBRK1, the mode of repression of which is functionally distinguishable from that of the N-terminal KRAB repression domain within ZBRK1. The identification of BRCA1 binding-competent but repression-defective CTRD mutants further revealed that BRCA1 binding is necessary, but not sufficient, for ZBRK1 CTRD function. During an unbiased search for possible co-regulators of the CTRD, we identified ZBRK1 itself, suggesting that ZBRK1 can oligomerize through its CTRD. Herein we explore the physical and functional requirements for ZBRK1 oligomerization in ZBRK1-directed transcriptional repression. Protein interaction analyses confirmed that ZBRK1 can homo-oligomerize both in vitro and in vivo and further mapped the ZBRK1 oligomerization domain to the CTRD C terminus. Biochemical analyses, including protein cross-linking and gel filtration chromatography, revealed that ZBRK1 homo-oligomers exist as tetramers in solution. Functionally, ZBRK1 oligomerization facilitates ZBRK1-directed transcriptional repression through ZBRK1 response elements; requirements for oligomerization-dependent repression include the ZBRK1 CTRD and KRAB repression domains but not the DNA binding activity of ZBRK1. These observations suggest that higher order oligomers of ZBRK1 may assemble on target ZBRK1 response elements through both protein-DNA and CTRD-dependent protein-protein interactions. These findings thus reveal an unanticipated dual function for ZBRK1 in both DNA binding-dependent and -independent modes of transcriptional repression and further establish the CTRD as a novel protein interaction surface responsible for directing homotypic and heterotypic interactions necessary for ZBRK1-directed transcriptional repression.

ZBRK11 (zinc finger and BRCA1-interacting protein with a KRAB domain-1) is a member of the Kruppel-associated box-zinc finger protein (KRAB-ZFP) family of transcriptional repressors (1, 2). The ~300 members of this family comprise a significant proportion of the transcription factor complement of the human proteome and are believed to occupy important regulatory roles in development, differentiation, and transformation (3–9). In addition to its identification as the product of a gene up-regulated in senescent fibroblasts, ZBRK1 was independently identified as a BRCA1-dependent transcriptional repressor of the gene encoding GADD45a, a functionally important DNA damage-response effector that functions in G2/M cell cycle checkpoint control and the maintenance of genomic stability (2, 10, 11). Previous functional analyses revealed that ZBRK1 represses GADD45a gene transcription through an intron 3 DNA-binding site in a manner dependent upon direct interaction with BRCA1 and further suggested a model in which ZBRK1 and BRCA1 function coordinately to repress GADD45a gene transcription in the absence of genotoxic stress (2). Evidence to support this model derives from the recent observation that DNA damage-induced ubiquitin-mediated proteolysis of ZBRK1 leads to de-repression of GADD45a (12). Although the DNA damage-induced degradation of ZBRK1 is a BRCA1-independent phenomenon, familial breast cancer-derived mutants of BRCA1 that disrupt its interaction with ZBRK1 nonetheless abrogate its co-repressor activity, suggesting that its ZBRK1 co-repressor function may be important for the tumor suppressor properties of BRCA1 (2, 12).

In addition to GADD45a, potential ZBRK1 binding sites have been identified in other DNA damage-response genes that are also regulated by BRCA1, including p21, Bax, and GADD153 (2). This observation suggests a potentially broader role for BRCA1 and ZBRK1 in the coordinate transcriptional control of functionally diverse DNA damage response genes. As part of our initial effort to explore the mechanism by which BRCA1 mediates sequence-specific transcriptional repression through ZBRK1, we recently identified and functionally characterized a novel C-terminal transcriptional repression domain (CTRD) within ZBRK1 (13). Structurally, the CTRD comprises the last four zinc fingers and an atypical C-terminal extension within the eight-fingered ZBRK1 protein (13). Functionally, the CTRD represses transcription in a BRCA1-dependent, histone deacetylase-dependent, and promoter-specific manner and is thus distinguishable from the N-terminal KRAB repress-

‡ To whom correspondence should be addressed: Dept. of Molecular Medicine and Inst. of Biotechnology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78245-3207. Tel.: 210-567-7258; Fax: 210-567-7377; E-mail: boyer@uthscsa.edu.

1 The abbreviations used are: ZBRK1, zinc finger and BRCA1-interacting protein with a KRAB domain-1; BF, broken finger; CTRD, C-terminal transcription repression domain; DSS, disuccinimidyl suberate; KRAB, Kruppel-associated box; MBP, maltose-binding protein; TK, thymidine kinase; ZFP, zinc finger protein; ZRE, ZBRK1 response element.
sion domain in ZBRK1, which exhibits no BRCA1 dependence and broad promoter specificity (13).

During the course of our functional dissection of ZBRK1, we identified CTRD mutants that are competent for BRCA1 binding but nonetheless defective for transcriptional repression (13). This observation suggests that BRCA1 binding is necessary but not sufficient for the ZBRK1 CTRD repression function and implies a role for additional co-regulators of the CTRD. During an unbiased search for CTRD-interacting proteins and, therefore, potential CTRD co-regulators, we unexpectedly identified ZBRK1 itself, suggesting that ZBRK1 can homo-oligomerize through its CTRD. Herein, we explore the physical and functional requirements for ZBRK1 oligomerization in ZBRK1-directed repression. Our results reveal the ZBRK1 CTRD to be a novel interaction surface responsible for directing both homotypic and heterotypic interactions essential for ZBRK1 repression and further suggest an unanticipated dual role for ZBRK1 in both DNA binding-dependent and -independent modes of transcriptional repression. We discuss the implications of these findings for BRCA1-dependent ZBRK1 repression.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Maturation—Plasmid pMAL-C2-TEV-ZBRK1 ΔK for expressing maltose-binding protein (MBP)-ZBRK1 ΔK in *Escherichia coli* has been described previously (13). pCS2+–FLAG-ZBRK1 for *in vitro* transcription/translation and mammalian expression of FLAG epitope-tagged ZBRK1 was constructed by subcloning a BamHI-HindIII fragment carrying sequences encoding an amino-terminal FLAG-tagged ZBRK1 from pFastbac-FLAG-ZBRK1 into pCS2+ (14). pCS2+–FLAG-ZBRK1 broken finger (BF) and KRAB domain (D12A,V13A) mutants were generated by site-directed mutagenesis using the QuikChange II site-directed mutagenesis kit following the manufacturer's instructions (Stratagene, La Jolla, CA). Each broken finger mutant bears a histidine (CAT codon) to asparagine (AAT codon) substitution mutation at the first of the two conserved histidine residues within the targeted C_{12}H_{15}Zn finger. The D12A,V13A KRAB domain double mutation bears an aspartic acid (GAT codon) to glutamine (GAG codon) substitution at amino acid position numbers 12 and 13, respectively, of the 532-amino acid ZBRK1 protein. pCS2+–FLAG-ZBRK1 carboxyl-terminal truncation mutants (ΔC1, ΔC2, ΔC3) were generated by PCR-based subcloning. In brief, a PstI-XbaI fragment encompassing the carboxyl terminus of ZBRK1 in pCS2+–FLAG-ZBRK1 was replaced with PCR-amplified fragments carrying corresponding carboxyl-terminal truncations. Individual pCS2+–FLAG-ZBRK1 carboxyl-terminal deletion mutants were generated by replacing the four-amino-acid-encoding P4-0 (by T7 transcript) by four amino acids to create amino acids 1–523; ΔC2, amino acids 1–503; and ΔC3, amino acids 1–483. Plasmid pCS2+–T7-ZBRK1 for *in vitro* transcription/translation and mammalian expression of T7 epitope-tagged ZBRK1 was generated by replacing a BamHI-Eco47III fragment from pCS2+–FLAG-ZBRK1 with a PCR-amplified BamHI-Eco47III fragment carrying sequences encoding a T7 epitope tag in-frame with the ZBRK1 amino terminus.

Reporter plasmid pZRE4-TK-Luc was constructed by replacing a HindIII-BglII fragment including 5XGAL4 DNA-binding sites and the TK promoter with a PCR-amplified BamHI-Eco407III fragment carrying sequences encoding an amino-terminus GST epitope for mammalian expression of FLAG-ZBRK1 and reporter plasmid pG5TK-Luc has been described previously (13). This observation suggests that BRCA1 binding is necessary but not sufficient for the ZBRK1 CTRD repression function and further suggest an unanticipated dual role for ZBRK1 in both DNA binding-dependent and -independent modes of transcriptional repression. We discuss the implications of these findings for BRCA1-dependent ZBRK1 repression.

**Protein Expression and Purification—**MBP-ZBRK1 ΔK, MBP-ZBRK1 CTRD, and MBP proteins were each expressed in and purified from *E. coli* strain BL21 Star (DE3) pLysS (Invitrogen). The procedures for protein induction, extraction, and purification of MBP-ZBRK1 ΔK have been described previously (13). For MBP-ZBRK1 CTRD and MBP, cells were grown at 37 °C to an *A_{600}* of 0.6. Isopropyl-1-thio-β-d-galactoside was added to a final concentration of 0.3 mM, and the cells were transferred to 25 °C for another 3.5 h. Cells were pelleted, washed once with phosphate-buffered saline, and then resuspended in MBP binding buffer (20 mM HEPEs, pH 7.5, 1 mM EDTA, 50 mM NaCl, and 10 mM β-mercaptoethanol) supplemented with protease inhibitors (0.4 μg/ml aprotinin, 0.5 μg/ml chymostatin, 0.5 μg/ml leupeptin, 0.5 μg/ml phenylmethylsulfonyl fluoride, 0.5 μg/ml benzamidine-HCl). Resuspended cells were frozen and thawed once followed by sonication (three times for 1 min each on ice) and clarification by centrifugation at 30,000 × *g* for 30 min. MBP-ZBRK1 CTRD and MBP proteins were purified from clarified lysates by affinity chromatography on amylose resin (New England Biolabs, Beverly, MA). Briefly, clarified lysates were incubated with amylose resin in batch at 1 h at 4 °C, washed with MBP binding buffer, and eluted with MBP binding buffer containing 0.5% maltose in column format.

**MBP Binding Assays—**MBP-ZBRK1 ΔK was purified from clarified bacterial cell extract (150 μl) by incubation with amylose resin (15 μl) in batch for 30 min at 25 °C, followed by washing twice for 5 min each at 25 °C in MBP binding buffer supplemented with protease inhibitors and three times for 5 min each at 25 °C in lysis 300 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine-HCl) and visualized by PhosphorImager analysis (Amersham Biosciences).

**Co-immunoprecipitation Analyses—**U2OS cells at 70% confluency in 10-cm dishes were transfected with 12 μg of DNA using the following expression plasmids (with amounts in parentheses): pCS2+–T7-ZBRK1 (6 μg) and pCS2+–FLAG-ZBRK1 (6 μg); pCS2+–T7-ZBRK1 (6 μg) and pCS2+–FLAG-ZBRK1 BF mutants (6 μg); pCS2+–T7-ZBRK1 (6 μg) and pCS2+–FLAG-ZBRK1 ΔC1 (5 μg); pCS2+–FLAG-ZBRK1 ΔC2 (2 μg), or pCS2+–FLAG-ZBRK1 ΔC3 (2.25 μg) (the total amount of DNA was fixed by supplementing these transfections with pCS2+). Thirty-six hours post-transfection, 2.5 × 10^5 transfected U2OS cells were harvested, washed in phosphate-buffered saline, and lysed in 300 μl of lysis 150T buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.1% Nonidet P-40) by sonication four times for 30 s at 150 °C in a Branson 450 sonifier (Branson Ultrasonics) at the lowest setting. The lysate was clarified by centrifugation at 20,000 × *g* for 20 min at 4 °C. Clarified lysates were subjected to co-immunoprecipitation with either 1 μg of anti-T7 antibody (Novagen, Madison, WI) or 5 μg of anti-FLAG M2 monoclonal antibody (Sigma-Aldrich) for 4 h at 4 °C. Immunocomplexes were precipitated with 20 μl of protein A-Sepharose (Amersham Biosciences) (for anti-T7 antibody immunoprecipitation) or protein G-Sepharose (Roche Diagnostics) (for anti-FLAG antibody immunoprecipitation) for 2 h at 4 °C. Immunoprecipitates were pelleted and washed with lysis 150T buffer at least four times for 10 min each at 4 °C. Immunoprecipitated proteins were eluted by boiling in 2× Laemmli sample buffer for 3 min, and eluates were subsequently resolved by SDS-PAGE (10% gel) and processed for immunoblot analysis.

**Chemical Cross-linking Assays—**U2OS cells at 70% confluency on 10-cm plates were transfected with either 12 μg of pCS2+–FLAG-ZBRK1 or 6 μg of pCS2+–FLAG-ZBRK1 ΔC1 in combination with 6 μg of pCS2+. Thirty-six hours post-transfection, cells were harvested and lysed in 300 μl of lysis 150T buffer (20 mM HEPEs, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 0.1% Nonidet P-40) by sonication at four times for 30 s at 150 °C in a Branson 450 sonifier (Branson Ultrasonics) at the lowest setting. The lysate was clarified by centrifugation at 20,000 × *g* for 20 min at 4 °C. Clarified lysates (36 μl) were subjected to chemical cross-linking by the addition of disuccinimidyl suberate (DSS) (25 mM) to a final concentration of 2.25 mM. Cross-linking reactions were performed at 25 °C and terminated by the addition of 2× Laemmli sample buffer followed by boiling for 3 min (16). Cross-linked products were resolved by SDS-PAGE (10% gel) and processed for immunoblot analysis.
**RESULTS**

**ZBRK1 Homo-oligomerizes in Vitro**—To identify novel interaction partners and potential co-regulators of the ZBRK1 CTRD, we employed two independent experimental approaches. First, we used the CTRD (amino acids 319–532 of ZBRK1) as a bait protein to screen a human fetal brain cDNA library by a yeast-two hybrid assay. As reported previously, deletion of the KRAB domain permits ZBRK1 homo-oligomerization through its CTRD C terminus. (Fig. 1). A set of FLAG-tagged ZBRK1 and MBP-ZBRK1 CTRD mutant derivatives for their respective abilities to bind to MBP-ZBRK1 ΔK in an MBP pull-down assay (Fig. 1). As reported previously, deletion of the KRAB domain permits the expression and purification of an otherwise insoluble full-length recombinant ZBRK1 protein (13). MBP-ZBRK1 ΔK bound full-length ZBRK1 efficiently, thus confirming that ZBRK1 can directly self-associate in vitro (Fig. 1, A and B). Next, we examined the structural determinants within the CTRD required for ZBRK1 self-association. Analysis of C-terminal ZBRK1 truncation derivatives revealed that the deletion of only nine amino acids from the CTRD C terminus was sufficient to eliminate ZBRK1 oligomerization (Fig. 1A). By contrast, individual disruption of CTRD zinc fingers 5–8 by substitution mutagenesis revealed that each “broken finger” mutant (13) bound MBP-ZBRK1 ΔK comparably to wild-type ZBRK1, thus revealing that the CTRD zinc fingers are individually dispensable for ZBRK1 oligomerization (Fig. 1B). Taken together, these results demonstrate that ZBRK1 can homo-oligomerize in vitro through a direct interaction involving its CTRD C terminus.

**ZBRK1 Homo-oligomerizes in Vivo**—To determine whether ZBRK1 can homo-oligomerize in vivo, we examined the ability of ZBRK1 derivatives independently tagged with either T7 or FLAG epitopes to reciprocally co-immunoprecipitate one another following their transient over-expression in U2OS human osteosarcoma cells. T7-tagged ZBRK1 could be efficiently precipitated by T7-specific antibodies only in the presence of FLAG-tagged ZBRK1 (Fig. 2A). Reciprocally, FLAG-tagged ZBRK1 could be efficiently precipitated by T7-specific antibodies only when T7-tagged ZBRK1 was co-expressed (Fig. 2B). These results confirm that ZBRK1 can homo-oligomerize in vivo. Next, to map the relevant interaction surface(s) required for ZBRK1 oligomerization in vivo, we examined a set of FLG-tagged ZBRK1 CTRD mutant derivatives for their respective
Oligomerization Mediates ZBRK1 Transcriptional Repression

Fig. 2. ZBRK1 homo-oligomerizes in vivo through its CTRD C terminus. A and B, T7 and FLAG-tagged wild-type ZBRK1 proteins were ectopically expressed either alone or together in U2OS cells, and whole cell extracts from transfected cells were subjected to immunoprecipitation (IP) using antibodies specific for either the FLAG (A) or T7 (B) epitopes. Immunoprecipitates were resolved by SDS-PAGE (10% gel) and processed by immunoblot analysis using both T7 and FLAG epitope-specific antibodies as indicated. Input represents 10% of the total whole cell extract subjected to immunoprecipitation. C and D, T7-tagged wild-type (WT) ZBRK1 was ectopically expressed in U2OS cells along with FLAG-tagged WT ZBRK1 or its indicated ΔC (C) or BF (D) derivatives. Whole cell extracts from transfected cells were subjected to immunoprecipitation (IP) using antibodies specific for the T7 epitope. Immunoprecipitates were resolved by SDS-PAGE (10% gel) and processed by immunoblot analysis using both T7- and FLAG epitope-specific antibodies as indicated. Input represents 10% of the total whole cell extract subjected to immunoprecipitation.

abilities to co-immunoprecipitated along with T7-tagged wild-type ZBRK1 following their transient co-expression in U2OS cells. Consistent with the results of in vitro binding analyses, these in vivo binding studies revealed the CTRD C terminus to be critical and the CTRD zinc fingers to be dispensable for ZBRK1 self-association. Thus, deletion of only nine amino acids from the ZBRK1 C terminus largely abolished ZBRK1 self-association (Fig. 2C), whereas individual ZBRK1 derivatives bearing targeted disruptions of CTRD zinc fingers 5–8 bound to T7-tagged ZBRK1 as efficiently as wild-type ZBRK1 (Fig. 2D). Taken together, these results demonstrate that ZBRK1 can homo-oligomerize both in vitro and in vivo and furthermore delimit the oligomerization interface to the CTRD C terminus.

The ZBRK1 CTRD Mediates Tetrameric Oligomerization—To characterize the oligomeric state of ZBRK1 in vivo, we first employed the chemical cross-linker DSS to covalently capture ZBRK1 protein complexes present in extracts of U2OS cells transfected with FLAG-tagged ZBRK1 (16). Cross-linked samples were analyzed by SDS-PAGE and immunoblot analysis using FLAG epitope tag-specific antibodies. In the presence of DSS, a significant proportion of FLAG-tagged ZBRK1 migrated with an apparent molecular mass of ~240 kDa, consistent with the size of a ZBRK1 tetramer (Fig. 3A). Importantly, no cross-linked products were observed when an oligomerization-defective ZBRK1 derivative (ΔC1) was over-expressed in U2OS cells, demonstrating that DSS-induced formation of high molecular mass ZBRK1 complexes is strictly dependent upon the intact CTRD C terminus (Fig. 3A).

Although the electrophoretic properties of FLAG-tagged ZBRK1 in DSS-cross-linked U2OS cell extracts suggests the formation of tetramers, we could not exclude the possibility that additional cellular proteins were also incorporated into the ZBRK1 complex, particularly because we know that ZBRK1 interacts with other proteins in the cell, including BRCA1 (2, 13). The concentration of ectopically expressed ZBRK1 in these transfection experiments considerably exceeds that of its endogenous counterpart, and these conditions are likely to drive ZBRK1 self-association and mask the influence of endogenous interacting proteins on the size of a cross-linked ZBRK1 complex. Nonetheless, we sought independent confirmation of the stoichiometry of ZBRK1 oligomerization through its CTRD. To this end, we therefore subjected highly purified recombinant MBP-ZBRK1 CTRD to DSS cross-linking. Cross-linked samples were analyzed by SDS-PAGE followed by silver staining to visualize MBP-ZBRK1 CTRD oligomers. In the presence of DSS, a significant proportion of MBP-ZBRK1 CTRD migrated with an apparent molecular mass of ~270 kDa, consistent with the size of an MBP-ZBRK1 CTRD tetramer (Fig. 3B). Importantly, no cross-linked products were observed when MBP alone was treated with DSS, indicating that DSS-induced formation of a high molecular mass MBP-ZBRK1 CTRD complex is strictly dependent upon the ZBRK1 CTRD (Fig. 3B). Furthermore, immunoblot analysis using ZBRK1-specific antibodies confirmed that the high molecular mass complex observed upon DSS cross-linking of highly purified MBP-ZBRK1 CTRD is derived from MBP-CTR D oligomerization (data not shown).

As an independent approach to determine the stoichiometry of ZBRK1 oligomerization through its CTRD, we examined the respective gel filtration profiles of ectopically expressed wild-type ZBRK1 and its oligomerization-defective mutant (ΔC1) present in extracts of transfected U2OS cells. Immunoblot analysis of column fractions using a FLAG epitope-specific antibody revealed a peak of wild-type ZBRK1 protein in fractions corresponding to an apparent molecular mass of ~240 kDa, most consistent with the size of a ZBRK1 tetramer (Fig. 3C). We also noted a minor peak of wild-type ZBRK1 protein in a fraction corresponding to an apparent ZBRK1 monomer of 58 kDa. By contrast, an oligomerization-defective ZBRK1 derivative (ΔC1) peaked exclusively in column fractions corresponding to an apparent monomer of ~57 kDa (Fig. 3C). Taken together, these results indicate that ZBRK1 tetramerizes both in vitro and in vivo in a manner that is strictly dependent upon the integrity of its CTRD C terminus.

ZBRK1 Homo-oligomerization Potentiates ZBRK1-directed Transcriptional Repression—To explore the functional consequence of ZBRK1 homo-oligomerization, we initially exploited our previous observation that the ZBRK1 CTRD can function as an autonomous transcriptional repression domain when tethered to a heterologous DNA-binding domain from the yeast transcription factor GAL4 (13). First, we examined the ability of full-length ZBRK1 to potentiate transcriptional repression...
directed by GAL4-ZBRK1 CTRD in a transient repression assay using a reporter template bearing multimerized GAL4 DNA-binding sites located upstream of the herpes simplex virus TK promoter in U2OS cells (Fig. 4 A) (13). Consistent with our previous studies (13), we observed that GAL4-ZBRK1 CTRD repressed transcription from this reporter template at least 10-fold in a dose-dependent manner (data not shown). To examine the influence of wild-type ZBRK1 on repression directed by GAL4-ZBRK1 CTRD and MBP, purified recombinant MBP-ZBRK1 CTRD and MBP were subjected to cross-linking with 0.75 mM DSS for 0, 1.5, or 3 min as indicated, and cross-linking reactions were terminated with Laemmli sample buffer. Cross-linked proteins were resolved by SDS-PAGE (10% gel) and visualized by silver stain analysis. Arrowheads indicate un-cross-linked monomers of MBP-ZBRK1 CTRD complexes. Molecular weight markers (M_r) are indicated. Analysis of ZBRK1 derivatives bearing targeted disruptions of CTRD zinc fingers 5–8 (BF 5–8) revealed these mutants to be partially defective for the ZBRK1 co-repressor function (Fig. 5 B). Because these CTRD zinc finger mutations do not disrupt ZBRK1 oligomerization (see Figs. 1 and 2) but do disrupt CTRD interactions with additional co-repressors including BRCA1 (13), this result suggests that CTRD-mediated recruitment of additional co-repressors also likely contributes to the ability of ZBRK1 to function as a DNA binding-independent co-repressor. Finally, we examined the contribution of the

![Diagram A](image1.png)

**Fig. 3. ZBRK1 tetramerization requires its CTRD C terminus.** A, schematic representation of FLAG-tagged wild-type ZBRK1 and its oligomerization-defective ΔC1 deletion derivative. U2OS cells were transfected with expression vectors for either FLAG-tagged WT ZBRK1 or its ΔC1 deletion derivative as indicated. Whole cell extracts from transfected cells were subjected to cross-linking with 2.25 mM DSS for 0, 1.5, or 3 min as indicated, and cross-linking reactions were terminated with Laemmli sample buffer. Cross-linked proteins were resolved by SDS-PAGE (10% gel) and visualized by immunoblot analysis using FLAG epitope-specific antibodies. Arrowheads indicate un-cross-linked monomers of FLAG-tagged ZBRK1 and its ΔC1 deletion derivative as well as cross-linked ZBRK1 complexes. Molecular weight markers (M_r) are indicated. Asterisk denotes a cross-reacting cellular protein recognized by the FLAG antibody. B, schematic representation of recombinant MBP-ZBRK1 CTRD and MBP. Purified recombinant MBP-ZBRK1 CTRD and MBP were subjected to cross-linking with 0.75 mM DSS for 0, 1.5, or 3 min as indicated, and cross-linking reactions were terminated with Laemmli sample buffer. Cross-linked proteins were resolved by SDS-PAGE (10% gel) and visualized by silver stain analysis. Arrowheads indicate un-cross-linked monomers of MBP-ZBRK1 CTRD and MBP as well as cross-linked MBP-ZBRK1 CTRD complexes. Molecular weight markers (M_r) are indicated. C, whole cell extracts from U2OS cells transfected with FLAG-tagged wild-type (WT) ZBRK1 or an oligomerization-defective mutant derivative (ΔC1) were independently subjected to Superdex 200 gel filtration chromatography. Individual column fractions were resolved by SDS-PAGE (10% gel) and analyzed for the presence of FLAG-tagged ZBRK1 by immunoblot analysis with FLAG epitope-specific antibodies. Arrowheads indicate the relative positions of marker protein peaks. Input represents 5% of the total whole cell extract subject to gel filtration chromatography.

Oligomerization Mediates ZBRK1 Transcriptional Repression

55157
N-terminal KRAB repression domain within ZBRK1 to its co-repressor activity. Relative to wild-type ZBRK1, a ZBRK1 mutant derivative bearing two substitution mutations (DV → AA) within the KRAB domain previously shown to disrupt KRAB repression function (17) was significantly defective for co-repressor activity (Fig. 5C). As this mutation does not affect ZBRK1 oligomerization, this result suggests that KRAB domain-associated co-repressors also contribute to the ability of ZBRK1 to function as a DNA-binding-independent co-repressor of the CTRD. Taken together these results reveal that ZBRK1, in a DNA-binding-independent manner, can potentiate the repression function of DNA-bound CTRD through oligomerization and the consequent recruitment of co-repressors through both its KRAB and CTRD repression domains.

We next asked whether ZBRK1 CTRD-mediated oligomerization contributes to ZBRK1-directed transcriptional repression from ZBRK1 response elements (ZREs). To this end, we employed a reciprocal transient repression assay in which we examined the ability of the CTRD to potentiate ZBRK1-directed repression from a reporter template bearing multimerized ZREs (Fig. 6A). The fact that the CTRD by itself has no ZRE binding activity permitted us to assess the influence of the CTRD on ZBRK1-directed repression solely by virtue of its ability to oligomerize with DNA-bound ZBRK1. Consistent with our previous studies (2), we observed that wild-type ZBRK1 repressed transcription from a reporter template bearing multimerized ZREs located upstream of the HSV TK promoter in U2OS cells at least 10-fold in a dose-dependent manner (data not shown). To examine the influence of the ZBRK1 CTRD on repression directed by wild-type ZBRK1, we transfected a sub-optimal nanogram quantity of the ZBRK1 expression vector that supports only ~2.5-fold repression of transcription from this reporter template (Fig. 6B). Under these conditions, ectopic expression of GAL4-CTRD enhanced this level of repression up to 4-fold in a dose-dependent manner (Fig. 6B). Importantly, GAL4-CTRD had minimal influence on reporter activity in the absence of ZBRK1, confirming that the CTRD potentiated ZBRK1 repression in this assay by virtue of its direct recruitment to ZBRK1 and not to DNA (Fig. 6B). Furthermore, an oligomerization-defective CTRD mutant (GAL4-CTRD ΔC1) bearing a nine amino acid deletion from the CTRD C terminus was completely defective in its ability to potentiate ZBRK1 repression in this assay, confirming that CTRD-mediated oligomerization is a requirement for its ZBRK1 co-repressor activity (Fig. 6C). Collectively, these experiments reveal an unanticipated dual function for ZBRK1 in both DNA-binding-dependent and -independent modes of repression, the latter of which derives from the ability of ZBRK1 to homo-oligomerize through its CTRD.

**DISCUSSION**

The identification of ZBRK1 itself during an unbiased search for ZBRK1 CTRD co-regulators prompted us to characterize the structural requirements for and the functional consequences of ZBRK1 homo-oligomerization. Herein we demonstrate that ZBRK1 undergoes tetrameric oligomerization both *in vitro* and *in vivo*, and we further identify the ZBRK1 CTRD C terminus to be both necessary and sufficient for tetrameric oligomerization. Functionally, we demonstrate that ZBRK1 oligomerization facilitates ZBRK1-directed transcriptional repression and concomitantly identify several unique and biologically significant features of ZBRK1 relevant to its role as a KRAB-ZFP transcriptional repressor. First, by virtue of its ability to homo-oligomerize, we identify an unanticipated role for ZBRK1 as a DNA-binding-independent co-repressor in addition to its well characterized role as a sequence-specific DNA-binding transcriptional repressor (2). In this regard, we identify both the KRAB and CTRD repression domains within ZBRK1 to be necessary for oligomerization-dependent repression. Whether or not these domains are sufficient for oligomerization-dependent repression remains to be established. Second, we reveal the ZBRK1 CTRD to be a novel protein interaction surface responsible for directing both homotypic and the heterotypic interactions necessary for BRCA1-dependent ZBRK1 transcriptional repression.

In general, oligomerization enhances regulatory diversity among sequence-specific DNA-binding transcription factors by expanding the number of potential response elements to which oligomers can effectively bind and/or by increasing the number and type of transcriptional regulatory domains that can effectively function at a core promoter. Presently, we do not know whether and how ZBRK1 oligomerization might influence the choice of DNA sequences recognized by ZBRK1. The 15-base pair consensus ZRE was originally identified by sampling a random pool of double-stranded oligonucleotides with a recombinant ZBRK1 derivative comprised solely of the eight ZBRK1 zinc fingers but lacking the C-terminal extension that we show here to be critical for oligomerization (2). Future studies will be required to determine whether and how oligomerization-competent forms of ZBRK1 preferentially recognize an expanded ZRE. Interestingly, our most recent studies revealed that the inherent DNA binding activity of ZBRK1 zinc fingers 1–7 on a consensus ZRE is constrained by the ZBRK1 C terminus, suggesting a potential role for the ZBRK1 oligomerization domain in regulating the
sequence-specific association of ZBRK1 with a consensus ZRE (13). Although our previous studies clearly revealed that ZBRK1 can bind to and repress transcription from a single ZRE (2, 13), it nonetheless remains to be established whether and how ZBRK1 oligomerization might facilitate synergistic binding and/or repression from multimerized ZREs.

Our work reveals a unique and unanticipated dual function for ZBRK1 in both DNA binding-dependent and -independent modes of transcriptional repression. With respect to the latter, we demonstrate that ZBRK1 can function as a co-repressor in a manner dependent upon the integrity of its CTRD and KRAB repression domains but not upon its inherent DNA binding activity. This observation raises the possibility that the regulatory potential of ZBRK1 on certain target genes may derive from its recruitment via protein-protein rather than protein-DNA interactions. To our knowledge, this represents the first
example of a KRAB-ZFP that can function dually as both a DNA-binding repressor as well as a DNA binding-independent co-repressor. Thus, we propose that higher order oligomers of ZBRK1 may assemble on a target ZRE through protein-DNA and protein-protein interactions, the latter involving oligomerization through the CTRD C terminus.

Our work further provides unique insight into the structural and functional organization of ZBRK1 as a member of the KRAB-ZFP family. Like other KRAB-ZFPs, ZBRK1 harbors an N-terminal KRAB repression domain followed by a C$_2$H$_2$ zinc finger DNA-binding domain. However, ZBRK1 also harbors an atypical C-terminal extension that is generally absent from the larger family of KRAB-ZFPs. Our previous work revealed this C-terminal extension to comprise part of a novel BRCA1-dependent CTRD that also includes zinc fingers 5–8 within ZBRK1 (13). Herein, we describe an additional and unique role for the CTRD as an oligomerization interface sufficient for directing homotypic interactions critical for ZBRK1 repressor function. Within the CTRD we demonstrate that zinc fingers 5–8 are dispensable, whereas the C-terminal extension is essential for ZBRK1 oligomerization. This C-terminal extension shows no obvious sequence similarity to the SCAN domain that mediates selective oligomerization between certain KRAB-ZFPs (1, 18). However, a BLAST search of available protein databases reveals several additional KRAB-ZFPs (ZNF577, ZNF613, and FLJ12644), each of which carries an extended C terminus of variable length with sequence homology to the ZBRK1 C-terminal extension. Interestingly, these proteins exhibit the same rank order with respect to their degree of homology and physical proximity to ZBRK1 on chromosome 19q, suggesting that they may have arisen from gene duplication (19–21). It will be of future interest to determine whether and how these KRAB-ZFPs functionally oligomerize with ZBRK1 and/or BRCA1. Although zinc fingers 5–8 within the CTRD are dispensable for oligomerization, our previous studies have nonetheless shown them to be essential for mediating interactions with additional co-repressors, including BRCA1 (2, 13). Thus, our studies collectively reveal the CTRD to be a novel protein-protein interaction surface responsible for directing both homotypic and heterotypic interactions necessary for BRCA1-dependent ZBRK1 transcriptional repression.

Finally, our identification of a novel oligomerization domain within ZBRK1 suggests that the regulatory potential of ZBRK1 could extend to genes that do not contain ZREs. For example, ZBRK1 could repress transcription through its hetero-oligomerization with other sequence-specific DNA binding transcription factors through direct protein-protein interactions involving its C terminus. This, in turn, could expand the repertoire of target genes subject to coordinate transcriptional control by both ZBRK1 and BRCA1, because the repressive potential of ZBRK1 is dependent upon BRCA1 (2, 13). Consistent with this possibility, our yeast two-hybrid screen for CTRD-interacting proteins identified, in addition to ZBRK1 itself, the oligomeric transcription factors SRF and ATF-1 (22–26). Interestingly, these transcription factors control the expression of genes with diverse functions in cell growth control and survival, and future studies will seek to establish whether and how ZBRK1 physically and functionally interacts with these transcriptional regulators. Nonetheless, our work here sheds new light on the structural and functional organization of ZBRK1 as a KRAB-ZFP and further defines the multifunctional nature of its unique CTRD.

Acknowledgements—We thank our laboratory colleagues for advice, discussion, and comments.

REFERENCES
1. Collins, T., Stone, J. R., and Williams, A. J. (2001) Mol. Cell. Biol. 21, 5599–5615
2. Zheng, L., Pan, H., Li, S., Flesken-Nikitin, A., Chen, P. L., Boyer, T. G., and Lee, W. H. (2000) Mol. Cell 6, 757–768
3. Bellefroid, E. J., Marine, J. C., Bied, T., Lecocq, P. J., Riviere, M., Aminjama, C., Poncelet, D. A., Coulie, P. G., de Jong, P., Siprizer, C., Ward, D. C., and Sibbald, J. A. (1993) EMBO J. 12, 1363–1374
4. Bellefroid, E. J., Poncelet, D. A., Lecocq, P. J., Revelant, O., and Martial, J. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 88, 3608–3612
5. Jheon, A. H., Ganss, B., Cheifetz, S., and Sodek, J. (2001) J. Biol. Chem. 276, 18282–18289
6. Krebs, C. J., Larkins, L. K., Price, R., Tullis, K. M., Miller, R. D., and Robins, D. M. (2003) Genes Dev. 17, 2664–2674
7. Gebelein, B., Fernandez-Zapico, M., Imoto, M., and Urrutia, R. (1998) J. Clin. Investig. 102, 1911–1919
8. Gebelein, B., and Urrutia, R. (2001) Mol. Cell. Biol. 21, 928–939
9. Wagner, S., Hess, M. A., Ormonde-Hanson, P., Maldonado, J., Hu, H., Chen, M., Keffer, R., Frodsham, M., Schumacher, C., Beluch, M., Honer, C., Skolnick, M., Ballinger, D., and Bowen, B. R. (2000) J. Biol. Chem. 275, 15685–15690
10. Sheikh, M. S., Hollander, M. C., and Fornance, A. J., Jr. (2000) Mol. Cell 5, 551–558
11. Ran, Q., Wadhwa, R., Bischof, O., Venable, S., Smith, J. R., and Pereira-Smith, O. M. (2001) Exp. Cell Res. 263, 156–162
12. Yun, J., and Lee, W. H. (2003) Mol. Cell. Biol. 23, 7305–7314
13. Tan, W., Zheng, L., Lee, W. H., and Boyer, T. G. (2004) J. Biol. Chem. 279, 6576–6587
14. Turner, D. L., and Weintraub, H. (1994) Genes Dev. 8, 1434–1447
15. Zheng, L., Annab, L. A., Afsari, C. A., Lee, W. H., and Boyer, T. G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9587–9592
16. McCarty, A. S., Kleiger, G., Eisenberg, D., and Smale, S. T. (2003) Mol Cell 11, 459–470
17. Margolin, J. F., Friedman, J. R., Meyer, W. K., Vissing, H., Thiesen, H. J., and Rauscher, F. J., III (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4509–4513
18. Schumacher, C., Wang, H., Honer, C., Ding, W., Koehn, J., Lawrence, Q., Couls, C. M., Wang, L. L., Ballinger, D., Bowen, B. R., and Wagner, S. (2000) J. Biol. Chem. 275, 17173–17179
19. Shannon, M., Ashworth, L. K., Muenesci, M. L., Lamerdin, J. E., Brancomb, E., and Stubb, L. (1996) Genes Dev. 10, 112–120
20. Shannon, M., Kim, J., Ashworth, L., Brancomb, E., and Stubb, L. (1998) DNA Seq. 8, 303–315
21. Shannon, M., Hamilton, A. T., Gordon, L., Brancomb, E., and Stubb, L. (2000) Nucleic Acids Res. Mol. Biol. 26, 434–436
22. Treisman, R., and Ammerer, G. (1992) Curr. Opin. Genet. Dev. 2, 221–226
23. Buchwaltor, G., Gross, C., and Wasyleyk, B. (2004) Genes 324, 1–14
24. Shore, P., and Sharrock, A. D. (1995) Eur. J. Biochem. 229, 1–13