MTG16 and Zbtb Family Members Interact to Repress the Wnt Target Matrilysin

Lauren C. Lu, Caitlyn W. Barrett, Michael E. Engel, and Christopher S. Williams

KEYWORDS. Colon cancer, Wnt pathway, MTG16, Zbtb family members

BRIEF. Determination of the interaction between MTG16 and Zbtb family members and their ability to repress the Wnt target matrilysin.

ABSTRACT. MTG16 and Zbtb33 (Kaiso) are transcriptional corepressors that form complexes implicated in colon cancer development. MTG16 can only interact with DNA by forming complexes with other proteins, such as Kaiso, that can directly bind DNA. While both proteins have been shown to target known cancer pathways, such as the WNT pathway, Kaiso may work by interacting with MTG16 and targeting the MTG16 repression complexes to specific promoter. Prior experiments demonstrated MTG16/Kaiso colocalization and identified similar transcriptional targets. The purpose of this study is to determine the whether MTG16 and the Zbtb family members interact or not and to determine the regions of interaction important for colon cancer formation. The essential MTG16 domains for binding to Zbtb family members were determined by co-transfecting MTG16 mutants and Zbtb family members into COS-7 cells and performing co-immunoprecipitations. The findings confirmed that full-length MTG16 co-immunoprecipitates with Kaiso and demonstrated that the first 363 amino acids of MTG16 are required for the Kaiso interaction. This study also determined that expression of the Kaiso target matrilysin (Mmp-7), which is important in colon cancer development, is repressed upon overexpression of MTG16. The MTG16/Kaiso interaction inhibits expression of proteins that promote colorectal tumorigenesis, so mutations to their binding sites or loss of expression of either may result in increased expression of pro-tumorigenic MTG16/Kaiso targets such as Mmp-7. Understanding the MTG16/Kaiso interaction could lead to novel therapeutics for colon cancer and other epithelial malignancies.

INTRODUCTION.

Colon cancer is the second leading cause of cancer-related mortality affecting both men and women [1]. This suggests that a more intimate knowledge of colon cancer is needed to develop novel therapeutics. Cancers, including colorectal cancer, develop when genes are turned on or off incorrectly. Knowledge of specific proteins or DNA domains could help determine predisposition to colon cancer and create enhanced treatment.

Inside each cell, genes encode the instructions for the production of specific proteins. Gene expression occurs when these genes are transcribed into RNA and then translated to form proteins. Controlling gene expression is vital because cells must not waste energy and essential materials used in protein synthesis. Gene regulation is also necessary and critical in cell differentiation, growth, and death, functions often undermined in cancer [2]. The regulation of genes can be achieved by transcriptional activators and repressors. Transcriptional activators promote the expression of specific genes, and repressors prevent genes from being expressed. However, mutations or alterations in these transcription factors can alter gene expression, leading to uncontrolled cellular proliferation and cancer formation.

Two transcriptional repressors believed to contribute to colorectal cancer when altered are Zbtb33 (Kaiso) and a myeloid translocation gene, MTG16. Kaiso is a protein that can directly bind DNA at specific promoters to turn off transcription [3]. As a negative regulator of gene expression, Kaiso recruits other repressors which form complexes and aid in dampening the transcription of the target gene. This complex blocks the binding of transcriptional activators [4]. Similarly, MTG16 forms repression complexes consisting of the same transcription factors. However, in contrast to Kaiso, MTG16 cannot directly bind DNA, but requires an interaction with a DNA-binding transcription factor, such as Kaiso, which targets MTG16 to the specific promoters [5]. The complexes formed with MTG16 and Kaiso are shown in Figure 1.

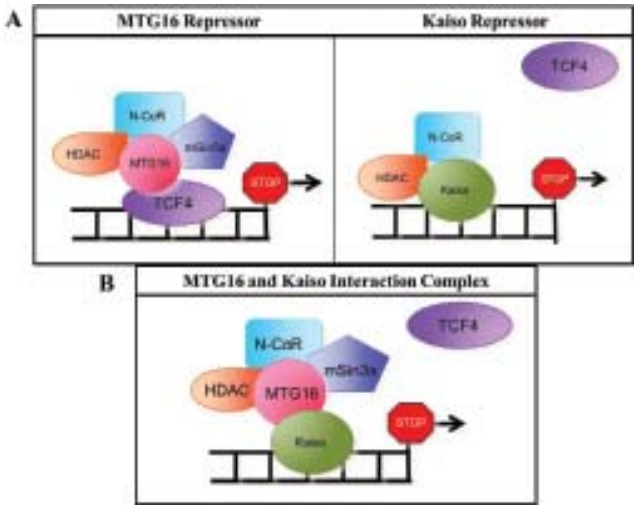

Figure 1. MTG16 and Kaiso repression pathways and complex. A) MTG16 and Kaiso repressors both recruit N-CoR and histone deacetylases in order to block the binding of transcription activators. However, unlike Kaiso, MTG16 is incapable of binding directly to DNA. B) Since MTG16 and Kaiso have many similar properties and target the Wnt target, it is hypothesized that MTG16 and Kaiso interact together in a complex in order to repress transcription.

Although Kaiso and MTG16 have functional similarities, there have been no studies describing their interaction to date. Preliminary unpublished experiments have demonstrated that the MTG16 and Kaiso colocalize. Colocalization suggests that since the two proteins are in the same proximity within the cell, they are more likely to interact with each other. Furthermore, Kaiso and MTG16 have been shown to target the same cancer pathways such as the Wnt signaling pathway and matrilysin (Mmp-7) [6]. Mmp-7 is an established Kaiso target, which is known to play a critical role in tumor development and progression [7]. Complete loss of Mmp-7 within a model of colorectal cancer leads to a significant reduction in tumors. Thus, if Kaiso and MTG16 are linked to the repression of Mmp-7, they may serve as targets for Mmp-7 control in colorectal cancer development.

Because of these properties, Kaiso and MTG16 are hypothesized to form a complex together to regulate gene expression (Figure 1). Preliminary studies support this hypothesis and has also identified two other Kaiso family members, Zbtb4 and Zbtb38, as MTG16-interacting proteins. The objective of this study was to determine the region of MTG16 that is necessary for the interaction with Kaiso and the other Zbtb family members. The knowledge of the protein-interaction domains can potentially allow scientists and physicians to screen for colon cancers that contain mutations in the determined regions. A second objective was to determine whether MTG16 possesses the ability to repress expression of the Kaiso target Mmp-7. If so, it would link MTG16 repression to a protein known to be deregulated in colon cancer. By genetically deleting sections of MTG16, the regions of MTG16 important for interacting with

Kaiso are determined. This information will help determine how mutations in these proteins could lead to the development of colorectal carcinoma. Thus, it could also provide the ability to modify the region of interaction between the two proteins and it would be essential in colon cancer repression, which could potentially produce novel therapeutics.

MATERIALS AND METHODS.

Cloning.

In order to successfully see the overexpression of the Zbtb family members in the COS-7 cells, entry clones, which contain the digested DNA fragment of the Zbtb family member and an entry vector, were cloned into a triple flag tagged destination vector. A triple flag tagged destination vector is a plasmid containing a polypeptide protein tag used to separate overexpression of certain proteins in a number of assays requiring recognition by an antibody such as co-immunoprecipitation. The protein tag is attached to the protein of interest upon translation. Refer to the supplemental methods for the cloning method utilized. The plasmid DNA for the clones was purified according to manufacturer's protocol using the Qiagen Maxiprep kit. MTG16 constructs that are myc tagged were provided by a collaborating research laboratory (Engel).

Cell Culture.

COS-7 cells, which derived from monkey kidney epithelium, were maintained in DMEM media supplemented with Fetal Bovine Serum (FBS) and Penicillin/Streptomycin (P/S). Cells were grown in 100mm dishes in a 37°C incubator and were split at 80% confluency.

Transfection.

Zbtb family members and full-length MTG16 or truncated mutants were over-expressed exogenously using Superfect transfection reagent according to manufacturer's protocol. Figure S1 shows the MTG16 constructs with the wild type or full-length constructs con containing all 620 amino acids, Δ5C containing amino acids 1-371, Δ7N containing amino acids 364-620, and ΔNHR2, which is missing 365-402 from the full length MTG16. Kaiso, already cloned into an expression vector, was co-transfected. Co-transfection methods are found in the supplementary methods section.

Retroviral Transfection.

Zbtb4 and Zbtb38 within the LZRS-3xFlag-GW-Stop-IRES-Neo retroviral vector were transfected into the packaging cell line GP2-293, which produces virus capable of infecting COS-7 cells. The method used to perform the retroviral transfection can be found in the supplementary methods section.

Co-immunoprecipitation.

Co-immunoprecipitation was performed in order to determine if the Zbtb family members and MTG16 are indeed bound to each other. Furthermore, it allowed for isolation of the complex containing the two interacting proteins. The COS-7 cells that were lysated, post-transfection, were co-immunoprecipated following the methods in the supplementary methods section.

Western Blotting.

Samples were run on a 10% SDS-PAGE gel, transferred onto a nitrocellulose membrane, and blocked at room temperature for an hour using Odyssey blocking buffer (Li-Cor). Proteins were then visualized using methods referring to the supplementary methods section. The blot was then analyzed using the Odyssey system, which uses near-infrared (NIR) fluorescence detection.

Real-Time Polymerase Chain Reaction.

Real-time polymerase chain reaction was performed to amplify and quantify the RNA of Mmp-7 with the overexpression of both MTG16 and Kaiso. RNA was isolated from cells using the RNEasy MiniKit (Qiagen). Taqman real-time polymerase chain reaction (qRT-PCR) using Mmp7 and GAPDH specific primers was performed in triplicate according to manufacturer's protocol. Fold-change analysis was performed using the ΔΔCt method.

RESULTS.

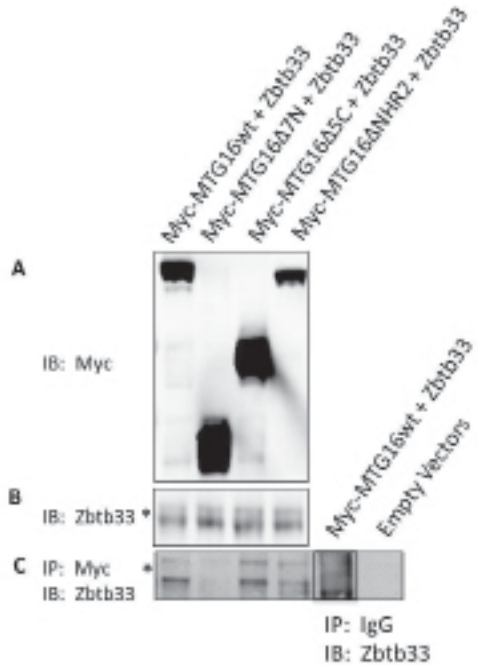

Figure 2. MTG16 requires the first 363 amino acids to interact with Zbtb33. (A) Overexpression of MTG16 and Zbtb33 from transfection is shown to have been successful on the immunoblot. (B) The co-immunoprecipitation shows success in transfection of Zbtb33 into the cells. (C) The immunoblot shows that the N terminal, which is the first 363 amino acids, is needed for the binding of MTG16 and Zbtb33.

MTG16 and Kaiso/Zbtb33 Interaction Domain.

Since both MTG16 and Kaiso (Zbtb33) exist in low quantities in cells, transfections of the two proteins were performed in order to better visualize the effect of mutated MTG16 constructs. An immunoblot established the overexpression of the protein, MTG16, in the transfected COS-7 cells (Figure 1A). Kaiso was also identified in all co-transfected cells as demonstrated by Western blot (Figure 1B). Finally, an immunoprecipitation was performed to determine the region of MTG16 that is necessary for the MTG16/Kaiso interaction. After probing and using antibodies to detect Zbtb33, binding was observed between all the MTG16 mutants and Zbtb33 except for MTG16 Δ7N, which lacks the first 363 amino acids (Figure 1C). The empty negative control lane shows that there was no contamination in the experiment.

MTG16 and Zbtb4 Interaction Domain.

In order to determine the region of MTG16 that is required to bind to Zbtb4, the MTG16 mutants and Zbtb4 were co-transfected into COS-7 cells. A Western blot using myc antibodies was performed for the corresponding MTG16 construct and analyzed to determine whether the proteins were successfully overexpressed. The immunoblot establishes the overexpression of the MTG16 mutants in the transfected COS-7 cells (Figure S2A). Zbtb4 was also identified in the cells as demonstrated by Western blot using the flag-tagged antibody. As shown, Zbtb4 was present in all of the combinations of constructs (Figure S2B). Finally, immunoprecipitation with myc antibody was analyzed and blotted for Zbtb4. Binding was shown to be maintained between all the MTG16 mutants and Zbtb4 (Figure S2C).

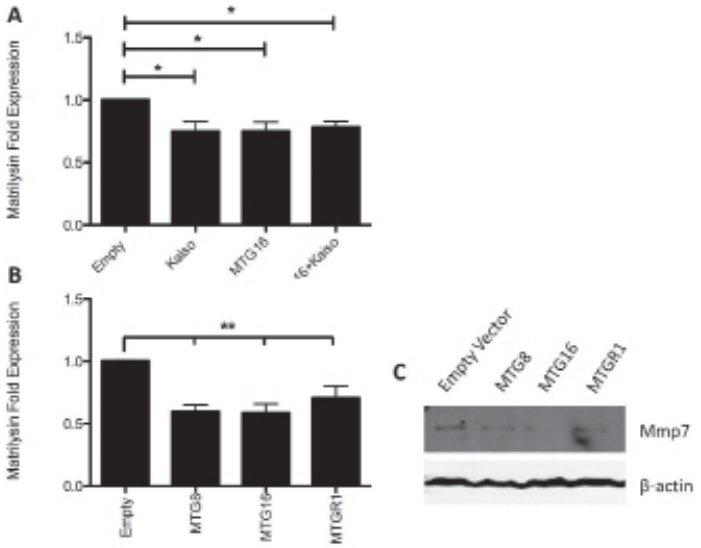

Figure 3. MTG16 and Kaiso repress matryliysin. (A) Matrilysin (Mmp-7) RNA expression was repressed by both MTG16 and Kaiso examined with qRT-PCR. Line above each bar represent significance compared to empty vector. (B) MTG16 and other MTG family members, MTG8 and MTGR1, repress Mmp-7 expression as measured by qRT-PCR. (C) Blot showing loading control of β-actin of similar volumes. Western blot of Mmp-7 demonstrates that protein levels also decrease.

Human Matrilysin Expression Repressed by MTG16 and Kaiso.

Real-time quantitative reverse transcription PCR (qRT-PCR) was performed to quantify Mmp-7 expression. The level of RNA expression of Mmp-7 demonstrates the result of the proteins of interest on the expression. Mmp-7 expression decreases after MTG16 and Kaiso are overexpressed (Figure 3A). The expression of Mmp-7 is shown to decrease as MTG16 and the other MTG family members, such as MTG8 and MTGR1, were added (Figure 3B). In order to confirm that Mmp-7 protein levels also decrease when MTG16 and Kaiso are overexpressed, a western blot was performed on lysates from transfected cells (Figure 3C). This result shows that MT6 family members' repression of Mmp-7 RNA results in decreased protein production.

DISCUSSION.

In this project, MTG16 and Zbtb family members are tested to confirm that they could exist in a complex. This hypothesis ish is driven by the observations that both protein families are important in colon cancer pathogenesis. Our findings demonstrate that full-length MTG16 co-immunoprecipitates with Kaiso, indicating that Kaiso and MTG16 do exist in a complex (Figure 2). Zbtb4 is shown to form a complex with MTG16 as well (Figure S2). However, because the blot did not show clear bands, the interaction of Zbtb38 with MTG16 cannot be confirmed (Figure S3). The study demonstrates that the first 363 amino acids of the MTG16 are required for the Kaiso interaction and binding. During the immunoprecipitation, MTG16 was pulled down by the myc antibody and the rest of the unbound antibodies and proteins in the lysate were removed. Later, the immunoblot that showed that Kaiso remained in the lysate in all of the combinations with MTG16 mutants and Kaiso except for MTG16 Δ7N, which lacks the first 363 amino acids of MTG16.

Zbtb4 also co-immunoprecipitated with all MTG16 mutant constructs, which indicate that there are two or more independent interaction domains between Zbtb4 and MTG16 (Figure S2). Mmp-7 expression was repressed with the overexpression of both Kaiso and MTG16. Furthermore, MTG16 and other MTG family members are established to repress the Mmp-7 expression (Figure 3). These findings indicate that MTG16 and the Zbtb family members are important in the regulation of the repression of the Mmp-7. The MTG16/Kaiso interaction inhibits expression of proteins that promote colorectal tumorigenesis so mutations to their binding sites or loss of expression of either may result in increased expression of pro-tumorigenic MTG16/Kaiso targets such as Mmp-7 thus promoting cancer development.

In future studies, Zbtb38 co-immunoprecipitation needs to be optimized. Also, finer mapping or smaller deletions of the MTG16 is required to further determine the exact domain that interacts with Zbtb4, Zbtb33, and Zbtb38. Understanding the MTG16/Zbtb family member interaction could lead to novel therapeutics for colon cancer such as targeting a specific peptide in the region of MTG16 that is needed to interact with the Zbtb family members. Those who are missing the necessary domain for the MTG16 and Kaiso interaction would be genetically predisposed to colon cancer. After performing potential targeted gene therapy, the necessary peptide using the MTG16 and Zbtb family member can then interact to repress the Mmp-7. Thus, colon cancer would be less likely to be induced.

ACKNOWLEDGMENTS. I would like to thank Dr. Christopher S. Williams for the use of his lab and Caitlyn W. Barrett for her mentorship throughout the research project. Also, I would like to thank Dr. Michael E. Engel for providing the MTG16 mutant constructs. Furthermore, I would like to thank Dr. Angela Eeds as well as the rest of the School for Science and Math at Vanderbilt instructors for their support. The project described was supported by Award Number R25RR024261 from the National Center For Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center For Research Resources or the National Institutes of Health.

SUPPORTING INFORMATION.

Figure S1. MTG16 mutant constructs
Figure S2. MTG16 mutants and Zbtb4
Figure S3. MTG16 mutants and Zbtb38
Supplementary Methods Section

REFERENCES.

1. L. Ricci-Vitiani. *et al*., Iden.. Nature, 445, 111-115. (2007)
2. J. Davis, L. McGhee & S. Meyers. ETO. Gene, 303, 1-10 (2003).
3. F.M. van Roy. P. D. McCrea. Rol. Nat Rev, 5, 956-964 (2005)
4. H.G. Yoon *et al.,* N-CoR, 12, 723-734 (2003).
5. J. A Martinez *et al*., Deletion. Gastroenterology, 131, 579-588. (2006)
6. J.I. Park *et al*., Kaiso. Dev Cell, 8: 843-54 (2005).
7. C.M. Spring, K.F. *et al*., The catenin. Exp Cell Res. 305: 253-65. (2005)

Lauren C. Lu is a student at Martin Luther King Jr. Magnet High School and enrolled in the School for Science and Math at Vanderbilt.