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PRINCIPAL INVESTIGATOR: Tso-Pang Yao, Ph.D.

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, NC 27710

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Tso-Pang Yao, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Duke University Medical Center
Durham, NC 27710

E-Mail: Yao00001@mc.duke.edu

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13. ABSTRACT (Maximum 200 Words)
Wnt signaling is mediated by a multi-components cascade that relays the signal from cell membrane to nuclear TCF- β -catenin transcriptional complex. Genetic study has implicated CREB-Binding Protein (CBP) as a negative regulator of Wnt signaling. We initially found that CBP can acetylate TCF4, the key transcriptional effector in the wnt signaling pathway. Furthermore, we also found that CBP and β -catenin, another key element of the wnt signaling, forms a complex. We proposed that that CBP-mediated acetylation negatively regulates TCF-dependent transcription in the absence of Wnt signaling. Wnt signaling then relieves this repression and converts CBP to a classical transcriptional-coactivator by inducing its complex formation with β -catenin. Supporting this possibility, by analyzing TCF reporters integrated chromosomally, we have obtained evidence that TCF binding sites indeed confer transcriptional repression activity. Furthermore, we have found that CBP potentiates TCF- β -catenin transcriptional activity in a reporter assay. However, whether or not acetylation of TCF plays a critical role in establishing the repression remains to be established, as we have evidence that there are more than one acetylation sites present in TCF4. The identification of the novel acetylation sites, which is still being pursued in the lab, will be critical for analyzing the function of TCF acetylation and how CBP regulates Wnt signaling.

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Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	13
Reportable Outcomes.....	13
Conclusions.....	14
References.....	15
Appendices.....	

Progress Report:

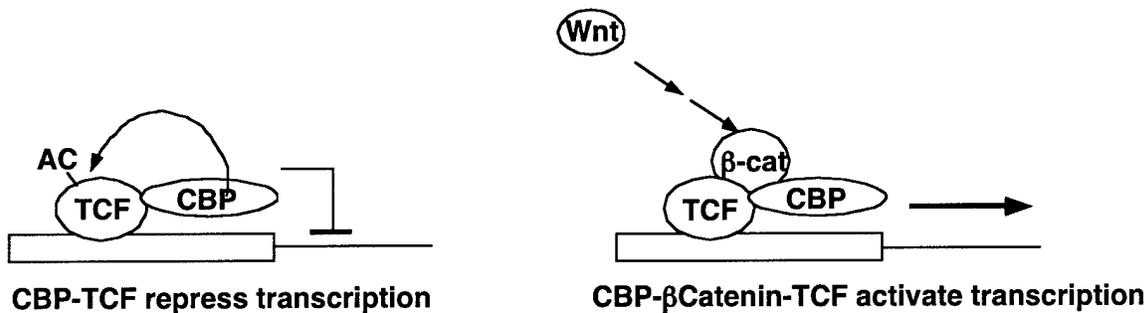
Title: Functional analysis of the transcriptional co-activator CBP in Wnt-signaling dependent mammary carcinogenesis

Introduction

The long-term objective of this proposal is to analyze the role of the transcription co-activator CBP in Wnt-signaling dependent carcinogenesis. In mouse model, wnt over-expression in mammary gland results in the development of breast carcinoma. Genetic study in *Drosophila* has implicated CBP as a negative regulator of TCF transcription factor, the major downstream effector of wnt signaling (Waltzer and Bienz, 1998). As heterozygous mutant CBP mice develop mammary gland hyperplasia, these observations suggest that CBP might play a negative role in regulating wnt-dependent mammary gland carcinogenesis. Molecularly, TCF can function both as a transcription repressor and activator (Cavallo et al., 1998). In the absence of wnt, TCF actively suppresses the wnt-responsive genes. However, wnt activation leads to the formation of β -catenin –TCF complex, which functions as a transcriptional activator on wnt-responsive genes. In our proposal, we hypothesized that CBP negatively regulates wnt-signaling by promoting the transcriptional repressor function of TCF, possibly by acetylating TCF, in the absence of Wnt. Upon Wnt activation; CBP is converted to a classic transcriptional co-activator by the complex formation with β -Catenin, the key partner for TCF in activating gene transcription (Figure 1).

Hypothesis/Model

Figure 1.



Body

The first phase of the work is trying to accomplish the Technical Objective number 1, which aims at “establishing whether CBP regulates the transcriptional activity of TCF- β -catenin complex”. As it will be discussed further, we have now firmly established the functional relationship between wnt- β -catenin-TCF dependent transcription and CBP/p300 family members.

Based on our hypothesis that CBP/p300 might negatively regulate TCF4 dependent transcription, we first test whether over-expression of CBP/p300 can suppress TCF- β -catenin-dependent transcription. As shown in Figure 2, expression of TCF4 and β -catenin together results in an increase of TCF-reporter gene. However, co-expression of CBP does not suppress this activity. In contrast, CBP increases the reporter activity in a dose-dependent manner. This result demonstrates that CBP does not repress TCF4- β -catenin transcription activity; rather it functions as a co-activator in this context. To examine whether CBP has similar effect on endogenous TCF- β -catenin activity, we analyze SW480, which contains constitute active-TCF- β -catenin complex due to a mutation on APC tumor suppressor (Korinek et al., 1997). As shown in Figure 2B, CBP can similarly enhance the transfected TCF reporter activity in Sw480, supporting that CBP can enhance the activity of TCF- β -catenin transcriptional complex.

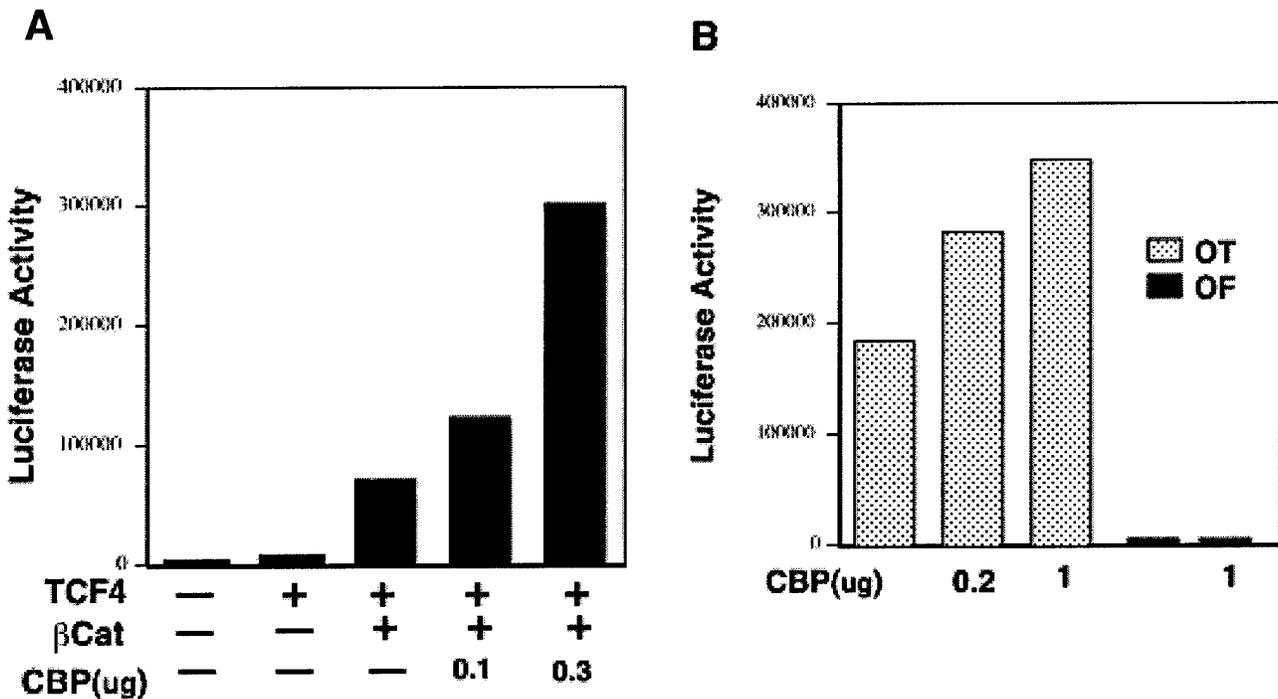


Figure 2. Co-expression of CBP potentiates TCF4- β -catenin transcriptional activity. (A) Expression plasmids for TCF4, stable form of β -catenin (Δ N90) and CBP was

transfected into 293T cells alone or in combination as indicated. Note that CBP increases the TCF4- β -catenin dependent transcription activity in a dose-dependent manner. (B) Expression plasmids for TCF-responsive reporter (OT) or a mutant reporter (OF) was co-transfected with indicated amount of CBP. Note that only OT but not OF display high activity in response to CBP.

To further substantiate the positive effect of CBP on TCF4- β -catenin-dependent transcription, we inhibit CBP by the co-expression of adenovirus E1A, which binds CBP and inhibit its co-activation activity (Arany et al., 1995; Kwok et al., 1994). As shown in Figure 3, consistent with the positive role of CBP in TCF4- β -catenin transcriptional activity, co-expression of E1A dominantly suppress the reporter activity. Supporting this conclusion, expression of E1A mutant that does not bind CBP (Δ N) fails to inhibit TCF- β -catenin mediated transcription. Based on these results, we conclude that CBP positively regulates TCF4- β -catenin transcriptional activity.

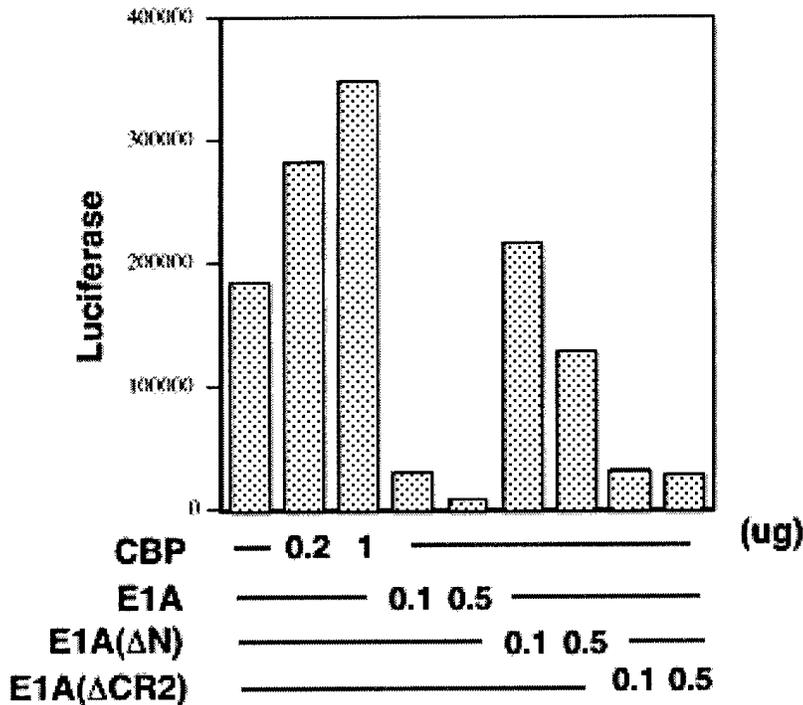


Figure 3. CBP binding protein E1A inhibits TCF- β -catenin mediated transcription. Expression plasmid for CBP, E1A, E1A mutant which can not bind CBP (Δ N) or pRB(CR2) were co-transfected into Sw480 cells as indicated. Note that wild type and CR2 mutant E1A but not CBP binding deficient (Δ N) mutant E1A can repress TCF- β -catenin dependent transcription.

To investigate how CBP might regulate TCF- β -catenin transcriptional activity, we investigate whether CBP forms a complex with TCF and/or β -catenin. As shown in figure 4, when the stable form of β -catenin (Δ N90) was co-expressed with CBP in 293T cells, a complex of β -catenin (Δ N90) and CBP can be detected. We note that a significant portion of Δ N90- β -catenin is localized in nuclei as judged by immunostaining on the transfected cells (data not shown), which allows its interaction with nuclear CBP.

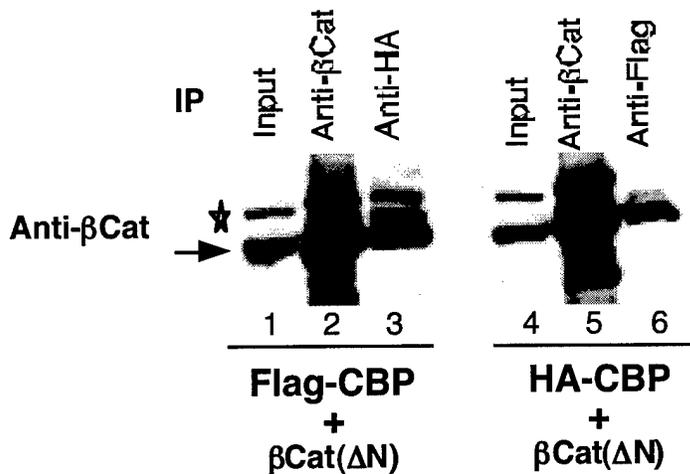


Figure 4. CBP interacts with β -catenin. Expression plasmids for the stabilized form of β -catenin (Δ N90) and for either flag-tagged or HA-tagged CBP were transfected into 293T cells. Co-immunoprecipitation was performed using antibodies against HA (12CA5), Flag (M2) or β -catenin and then blotted with antibody against β -catenin. 2.5% of the total protein used in immunoprecipitation were loaded in the input lanes. We note that a Δ N90- β -catenin (arrows) was co-immunoprecipitated by both HA and Flag antibodies. The slower migrated band (asterisk) is the full-length endogenous β -catenin, which does not appear to interact with CBP strongly.

The results obtained so far shows that it is unlikely that CBP negatively regulates wnt signaling by suppressing TCF- β -catenin transcriptional activity. We thus consider the alternative hypothesis wherein CBP might potentiate TCF transcriptional repression. To test this idea, one need to first establish a TCF-dependent repression assay. However, in a typical transient transfection system, TCF-4 does not show any appreciable transcriptional repression activity (data not shown). One possibility for this observation might be due the nature of transient transfection. In the transient transfection system, the reporter gene is generally not properly assembled into chromatin-like structure, which might be essential for active transcriptional repression to occur. Indeed, it has been observed that TCF can only repress reporter gene that is stably integrated into chromosomes (H. Clevers, personal communication).

Although our initial attempt to address the role of CBP in TCF-mediated transcription repression is unsuccessful due to the assay limitation, we are now in the process of developing TCF mediated repression assay using properly chromatinized reporter template. To achieve this goal, we are taking two approaches. First, we will use Xenopus oocyte to perform the transcription repression assay. Reporter plasmids injected into oocyte have been shown to be quickly assembled into chromatin. Under this configuration, we have shown that the histone acetyltransferase activity (HAT) of CBP is specifically required for the transcription activity of nuclear hormone receptor (C.H. Lai, Z. Q. Huang, J.M.Wong and T.P.Yao, Manuscript in preparation). However, this activity is not required under the transient transfection system (data not shown). We have now transferred all TCF, β -catenin and CBP related reagents to expression plasmids that are suitable for assays in Xenopus oocytes. The assays are currently being performed in Dr. Wong's lab. We hope to use this system to determine if CBP-mediated acetylation is necessary for the active repression mediated by TCF. Meanwhile, we have taken a second approach that utilizes cell line stably expressing TCF reporter genes. As these reporters are stably integrated into chromosomes, it is likely that they will be properly chromatinized. By stably transfection followed by antibiotic marker selection, we have successfully generated several clones with wild type and mutant TCF reporter stably integrated. We will describe the characterization of these cell lines in the next few sections.

-To investigate the potential role of CBP-TCF mediated transcriptional repression.

Genetic results from both Drosophila and Xenopus have suggested that TCF might actively repress wnt-target gene expression (Waltzer and Bienz, 1998). Furthermore, this repression appears to involve CBP (Waltzer and Bienz, 1998). We have proposed to reconstitute this transcriptional repression system in tissue culture system to further dissect out the molecular details of this repression. Despite the repeated effort, however, a TCF-reporter assay (TOP-flash, OT) in a transient transfection setting failed to reveal any TCF dependent transcriptional repression. As transcriptional repression often involves specific repressive chromatin configuration (reviewed in (Wade et al., 1997)), we reasoned that it might be necessary to utilize chromatinized reporter to establish the transcriptional repression assay. It is generally believed that reporter plasmid in a transient transfection setting will not be efficiently or properly assembled into chromatin. As discussed previously, we have taken three independent approaches to establish chromatinized reporter system to assess TCF, CBP and β -catenin function. The proposed study on Xenopus oocyte system is still being investigated, here we will describe the other two approaches.

1. To generate cell lines with TCF reporter stably integrated to genome. The stably integrated reporter genes are likely to be organized into proper chromatin structure and therefore confer TCF-dependent repression. To achieve this, we have transfected TCF response element driven luciferase (OT) or mutant reporter (OF) with a selectable marker: puromycin resistant gene, and selected for the puromycin-resistant stable clones. We analyzed more than 10 independent clones for cells stably transfected with either OT or OF reporter. Although, as expected, there are variations in the transcriptional levels among clones, in general, OT stable lines express much lower basal transcriptional activity when compared with OF. We have found that more than 50% of the OT

expressing stable lines display considerable lower basal transcriptional activity. The analyses of a few stable lines are shown in Figure 5, wherein OT lines show lower basal transcriptional activity. This observation is consistent with the idea that TCF binding sites can mediate transcriptional repression in the absence of β -catenin. It is important to note that OT, but not OF, can be activated by the introduction of TCF4 and β -catenin, demonstrating that these clones have functional TCF-reporter gene integrated.

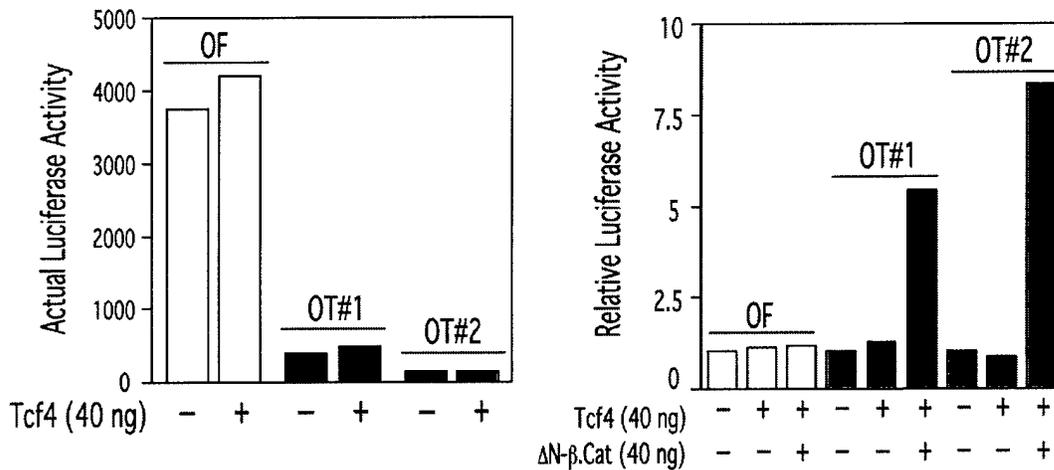


Figure 5. Transcription repression by Tcf and activation by ΔN - β .Catenin in H1299 stably expressing TCF reporter gene. H1299 cells with pGL3-OF/OT luciferase reporter stable expression were transiently transfected with Tcf4 alone or plus activated form of β .Catenin (ΔN). Forty-eight hours post-transfection, cell extracts were subjected to luciferase assay. Luciferase activity is shown in fold induction and pGL3-OF stable line without transfection of Tcf4 or/and ΔN - β -catenin is arbitrarily designated as 1.

2. To assess a TCF-reporter plasmid which can actively replicate. We also took second approach to establish a chromatinized TCF-luciferase reporter. We took advantage of the fact that plasmid DNA undergoes replication is known to be assembled into nucleosome, we cloned the TCF response element into an episomal vector that contains Epstein-Barr virus replication origin and encodes the nuclear antigen EBNA-1 (PREP-4 luciferase) (Liu et al., 2001). As shown in Figure 6, wild type TCF response element (OT) driven luciferase cloned into this vector (pREP4) does display much lower transcriptional activity when compared with that of the mutated reporter (OF). This result provides another line of evidence that TCF does repress transcription in the absence of active wnt signaling. Surprisingly, however, additional expression of TCF4 by transfection did not confer further transcriptional repression. There are at least two testable explanations for this observation. First, other TCF family member, such as TCF1 or LEF1 but not TCF4 is responsible for the repression. This will be addressed by assessing whether transfection of TCF1 or LEF1 expression plasmids can lead to a further reduction of the reporter activity. Second, the endogenous level of TCF4 is

sufficient to mediate repression. This possibility can be examined by knocking down endogenous TCF4 by SiRNA technique and determine if this mutant can lead to the de-repression of this reporter. These series of experiments are still being developed.

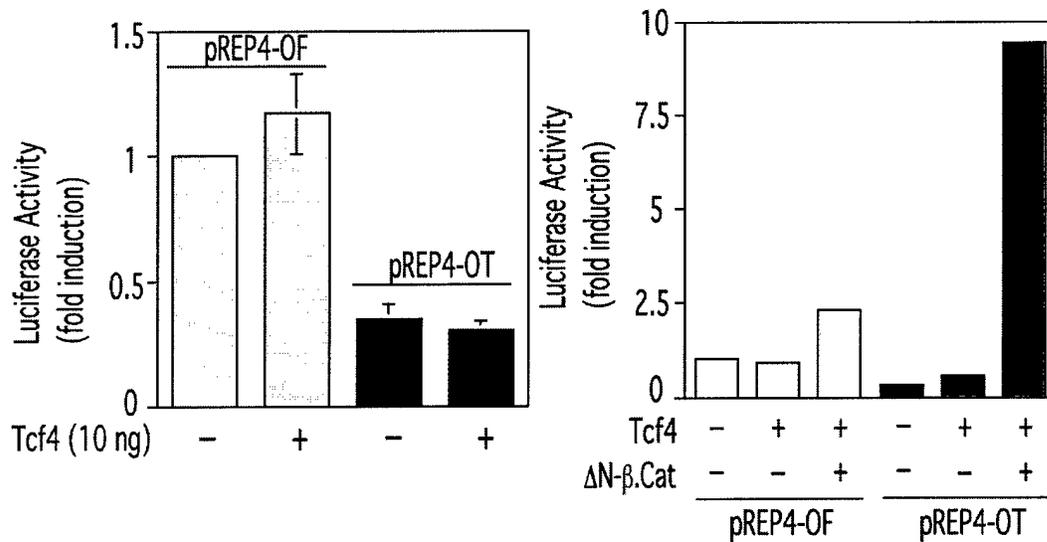


Figure 6. TCF binding sites confers transcriptional repression in the context of pREP4. Tcf4 responsive elements contained three copies of the optimal Tcf motif CCTTTGATC (designed as OT), or three copies of the mutant motif CCTTTGGCC (designed as OF) were cloned into pREP4-Luciferase episomal vector that contains Epstein-Barr virus replication origin and encodes nuclear antigen EBNA-1. These plasmids can be incorporated into nucleosomes upon its replication. H1299 cells were transiently transfected with pREP4-OF/OT luciferase reporter with or without Tcf4 or β -catenin as indicated. Forty-eight hours post-transfection, cell extracts were subjected to luciferase assay. Luciferase activity is shown in fold induction by arbitrarily designating transfection with pGL3-OF luciferase reporter alone as 1. Note that PREP4-OT has much lower basal transcriptional activity than PREP -OF, suggesting an active repression mediated by TCF binding element (Left Panel). Both PREP4-OT and PREP4-OF responds to TCF4 and Δ N- β -Catenin normally (Right Panel).

-To assess the function of TCF4 acetylation.

The evolutionarily conserved Lysine 22 in TCF4 is believed to be acetylated by CBP (Waltzer and Bienz, 1998). To begin to investigate the function of this specific acetylation, we have mutated this lysine to alanine (KA(22)). However, as shown in Figure 7, this mutation has no apparent effect on the transcriptional property of TCF4. We thus suspect that there might be more than one lysine residues subject to modification by CBP. Therefore, mutation on one lysine might not be sufficient to create a dominant phenotype. A similar conclusion was obtained by our previous study on p53 acetylation (Ito et al., 2001).

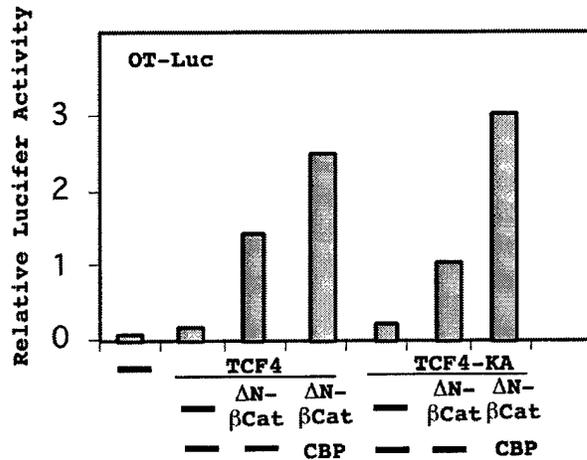
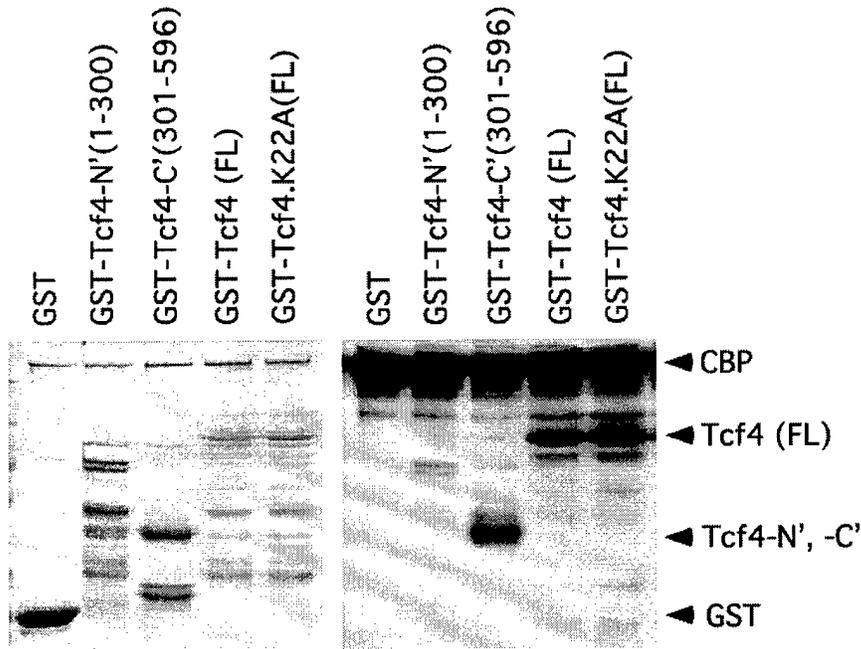


Figure 7. TCF4-KA (22) mutant retains wild type TCF4 transcriptional activity. Expression plasmid for wild type TCF4 or acetylation deficient KA(22) mutant are co-transfected with reporter plasmid in the presence or absence of β -catenin and CBP. Note that TCF4-KA has similar transcriptional activity under the condition tested.

To further explore the possibility that there might be additional lysine residues, other than lysine 22, can be acetylated by CBP, we generated recombinant TCF4-KA(22) protein and test whether it is still a substrate for CBP. As shown in Figure 8, TCF4-KA(22) is efficiently acetylated by CBP in vitro. This result indicates that there are additional lysine residues that can be acetylated by CBP. To begin to map the additional acetylated lysine residues, we evaluate recombinant fragments of TCF4 in CBP-mediated acetylation assay. To our surprise, C-terminal TCF4, which does not contain K22, is strongly acetylated. This result indicates that novel acetylation site(s) present at the C-terminus of TCF-4. In collaboration with Dr. Eppella's lab at NIH, with whom we have mapped the MDM2 acetylation sites, we will begin to identify the acetylated lysine residues in TCF4. Our observation is also consistent with a recent report that identified additional lysine residues that are acetylated by CBP in the *C. elegans* homologue of TCF, pop-1 (Gay et al., 2003). We are currently investigating whether the lysine residues found to be acetylated in pop-1 is also a target of CBP mediated acetylation. Mapping the acetylated lysine residues will be essential to dissect the function of TCF4 acetylation.



Coomassie Blue Stain Autoradiography

Figure 8. CBP-mediated TCF4 acetylation in vitro. The substrates, purified recombinant GST fusion Tcf4 N'-terminal fragment (1-300), C'-terminal fragment (301-596), full length wild-type Tcf4, or full length Tcf4.K22A mutant from *E. coli*, were incubated with baculoviral recombinant CBP in HAT reaction buffer with [¹⁴C]-acetyl-CoA. The reaction products were subjected into SDS-PAGE and then followed with Coomassie brilliant blue staining (left panel) and autoradiograph (right panel). Note that TCF4KA (22) is efficiently acetylated.

Key Research Accomplishment:

- We have uncovered an unexpectedly that CBP co-activates TCF- β -catenin dependent transcription, suggesting a dual role of CBP in modulating wnt signaling.

-CBP physically interacts with β -catenin. This observation provides a molecular mechanism explaining how the recruitment of β -catenin activates TCF-dependent transcription. This result suggests that tripartite complex of TCF- β -catenin –CBP is likely the functional transcriptional complex mediating wnt-dependent transcription.

-Establish the chromatinized wnt/TCF responsive reporter system for the study of the roles of various TCF family member and CBP-mediated acetylation in active repression of TCF/wnt target genes.

-Establish that TCF binding sites can confer repressive transcriptional activity in chromatinized but not the non-chromatinized configuration. This result support the hypothesis that TCF can repress transcription in the absence of wnt signaling.

-Establish that the acetylation of lysine 22, the putative major acetylation site in TCF4, is not necessary for its function as a transcription activator. Furthermore, we found that lysine 22 of TCF4 is not the only lysine residues that can be acetylated by CBP. This result suggests that, similar to most of known acetylated proteins, there will be additional lysine residues can be modified by acetylation.

Personnel

Tso-Pang Yao, Ph.D.

Akihiro Ito, Ph.D.

Chun-Hsiang Lai, MS

Reportable Outcome:

Manuscript:

Lai, C.-H, Ito, A. and Yao, T.-P. Dual functions of Transcriptional co-activator CBP in regulating TCF- β -catenine transcriptional activity. **(In Preparation).**

Presentation

Invited Presentation:

Society of Chinese Biologist Association Annual meeting (Hongkong): The regulation of TCF-B-catenin by CBP acetyltransferase.

Cell lines established:

U2OS cells with stably integrated wild type or mutant TCF luciferase reporter.

Training:

The training of a postdoctoral fellow Dr. Akihiro Ito was in part supported by this grant.

Conclusion:

Our study has led to an unexpected model in which CBP both positively and negatively regulates TCF-dependent transcription. We propose that the TCF-mediated repression is achieved by the CBP-mediated acetylation of TCF while activation is accomplished by the recruitment of β -catenin and the formation of the CBP- β -catenin complex in response to Wnt signaling. Although our study fell short of demonstrating this model, our observation provides two important insights into the wnt-signaling pathway. First, As CBP is a known transcriptional co-activator, our study provides a molecular basis on how the recruitment of β -catenin by TCF can activate transcription. Second, it suggests a role of reversible acetylation in regulating TCF activity. Future work will be focused on in verifying the hypothesis that acetylation of TCF does contribute to its repressive activity and that the complex formation of β -catenin and CBP leads to a change in TCF acetylation, and consequently, transcriptional activity.

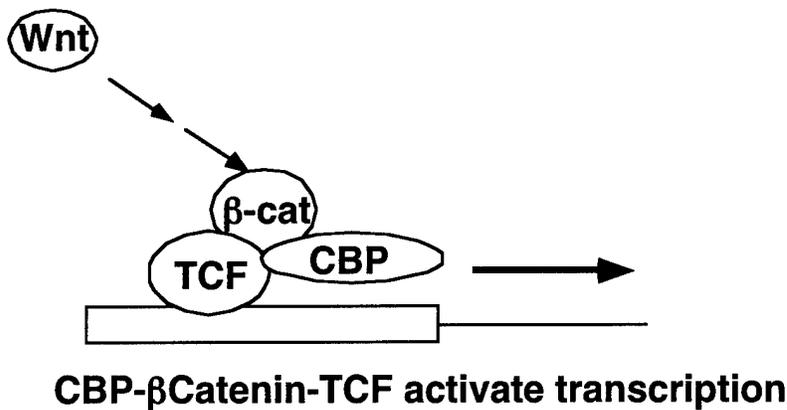
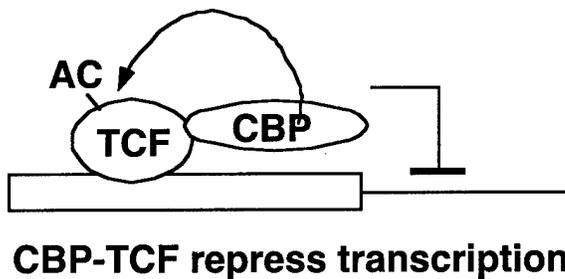


Figure 9. Model for the functional consequence of CBP- β -catenin-TCF interaction. In the absence of wnt signaling, CBP facilitates TCF to function as a transcriptional repressor, possibly by acetylating TCF. Upon wnt signaling, β -catenin is stabilized, translocates into nucleus and subsequently binds to CBP and TCF. The binding of β -catenin converts CBP to the transcriptional co-activator and consequently, TCF can now function as a transcriptional activator.

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