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Original Article

# The generation of a ZBTB16-inducible expression system in the ACHN adenocarcinoma cell line

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**الملخص**

**أهداف البحث:** لتكوين خط جديد من خلايا الكلية الغُديْة السرطانية القابلة للتحفيز، التي يمكن من خلالها التحكم بمستوى البروتين "ZBTB١٦".

**طرق البحث:** استخدم نظام الجيل الثالث للتحكم بالبروتينات القابلة للتحفيز "Tet-On" من شركة كلونتك لتكوين خط من الخلايا الغُديْة السرطانية الناتجة من ورم في الكلية لتحفيز إنتاج بروتين "ZBTB١٦" باستخدام المضاد الحيوي تيتراسايكلين.

**النتائج:** لقد قام نظام الجيل الثالث للتحكم بالبروتينات القابلة للتحفيز "Tet-On" بتنظيم إنتاج البروتين "ZBTB١٦" في الوضع الطبيعي للخلايا عند انعدام وجود المضاد الحيوي دوكسي سيكلين من الوسط الغذائي للخلايا السرطانية، وعند إضافة كميات صغيرة تصل إلى ٢٠ نانوغرام/ملل من الدوكسي سيكلين قامت بتحفيز نظام التحكم لانتاج البروتين بنجاح.

**الاستنتاجات:** إن التحكم الدقيق في كمية إنتاج البروتينات وخاصة معاملات الاستنساخ الخلوي للحمض الوراثي مهم جدا لدراسة تأثير هذه المعاملات على الأنظمة الحيوية داخل الخلية السرطانية. وبما أن معظم معاملات الاستنساخ مثال "ZBTB١٦" تقوم بأداء عملها داخل الخلية حتى ولو تم إنتاجها بكميات ضئيلة جدا. لذا فإن نظام الجيل الثالث للتحكم بالبروتينات القابلة للتحفيز "Tet-On" كان ضروريا للتحكم بآلية إنتاج البروتين بطريقة محكمة وخالية من التسرب للتحكم بكميته دخل الخلية لتكون متقاربة مع الوضع الفيسيولوجي داخل جسم الإنسان.

**الكلمات المفتاحية:** بروتين ""ZBTB١٦؛ خلايا الكلية الغُديْة السرطانية؛ تحفيز؛ المضاد الحيوي دوكسيسيكلين

**Abstract**

**Objectives:** To establish an inducible system for controlled ZBTB16 protein expression in a renal adenocarcinoma cell line.

**Methods:** The Tet-On® 3G Inducible Expression System from Clontech was used to generate a tetracycline-regulated ZBTB16 expression system in the ACHN renal adenocarcinoma cell line.

**Results:** The Tet-On® 3G system successfully controlled the basal level of ZBTB16 with no detectable protein expression when doxycycline was absent from the culture medium. The addition of doxycycline induced ZBTB16 protein expression with as low as 20 ng/ml of doxycycline.

**Conclusion:** The firm regulation of transcription factor expression in cancer cell models is very important in investigating their biological roles. Many transcription factors, such as ZBTB16, will carry on their biological activities even with minimal expression levels. The Tet-On® 3G system was essential in controlling the basal expression to prevent transcription leakage and providing a tightly regulated method for expressing ZBTB16 in a manner resembling physiological conditions.

**Keywords:** ACHN; Dox; Inducible-system; ZBTB16



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## Introduction

Globally, cancer is one of the leading causes of death, and finding a suitable treatment option for cancer cells is a major challenge for researchers in the medical field. Many cancer-based therapeutics are cytotoxic and induce death in actively proliferating cells. This, however, is beneficial only in high-grade tumours. In many cancers, such as early stage prostate cancer[1] or primary lesions of renal carcinoma,[2] malignant cells proliferate almost like normal cells, making them a difficult target for chemotherapy. Developing therapies by targeting certain molecules that are crucial for the proliferation, metastasis and survival of cancer cells is a major focus in cancer research. Several approaches have emerged in the field, including targeting cell surface antigens,[3] proteins that are over-expressed in specific malignant cells[4] and transcription factors that positively drive the cell cycle.[5]

The promyelocytic leukaemia zinc finger (ZBTB16, PLZF or ZNF145) protein is a transcriptional repressor belonging to the Krüppel-like zinc finger family of proteins.[6] It plays a key role in various developmental and biological processes and has been implicated in leukaemogenesis and oncogenesis.[7–11] ZBTB16 expression is required to maintain the proliferative as well as the self-renewal capacity of many stem and early progenitor cells. This requirement is maintained even after maturation to regulate the differentiation of these cells.[8] A genome-scale high-density oligonucleotide array showed that ZBTB16 was expressed in many tissue types. The study also showed that ZBTB16 expression was present under normal physiological conditions in various tissue types, but the level of expression was significantly decreased in many cancer cell types.[12] It was also shown that ZBTB16 expression is required to sustain the pluripotency of stem cells, but when those cells differentiate toward more functional and mature cells, the expression of ZBTB16 decreases, allowing certain signalling pathways to be activated.[13]

ACHN is human renal carcinoma cell line that was developed from a pleural effusion of a 22-year-old Caucasian male diagnosed with renal cell carcinoma. It grows as an adherent cellular group with a doubling time of approximately 16 h and shows no detectable ZBTB16 expression.[14] This cell line has been widely used as a cell model for invasive renal adenocarcinoma.[15–18]

The study of transcription factor activity in cancer cells is usually accompanied by the overexpression of that transcription factor using molecular cloning to study its effect on different biological systems. The overexpression process is mainly carried out by cloning the open reading frame (ORF) of the protein of interest to an expression plasmid that is transfected into cancer cells. This might overwhelm the cells with large amounts of that specific protein because the plasmid is constitutively expressed by the cell's transcriptional machinery. Therefore, a better system of controlled protein expression is required for use in different biological experiments or assays.

The Tet-On system is a molecular technique for inducible gene expression which utilizes specific operator motifs in the promoter sequence that bind certain proteins in order for transcription to be activated.[19] Another molecular technique for controlling protein expression is the addition of a destabilizing domain (DD) that can be fused to either end of the protein of interest. The DD would cause the direct targeting of DD-fused protein by proteasomal degradation complexes in the cell unless the DD is neutralized with a small molecule called Shield1 (Clontech, USA). Shield1 works by masking the DD binding domain and making it inaccessible to the degradation complex.[20] In this study, I have utilized both approaches to generate a stable renal adenocarcinoma cell line in which the expression of ZBTB16 can be tightly regulated.

## Materials and Methods

We began by constructing the ZBTB16 expression plasmid that would be under control of the tet-induced promoter from Clontech (Figure 1). First, the ZBTB16 ORF from pLv129 (GeneCopoeia, USA) was cut with EcoRI and NotI-HF (NEB, USA) and purified using gel electrophoresis. pcDNA3.1-B (Life Technologies, USA) was also linearized using the same restriction enzymes and purified using gel electrophoresis. ZBTB16 ORF was then ligated to the linearized pcDNA3.1-B to fuse ZBTB16 with the V5 epitope and was subsequently transformed into DH5α cells (Invitrogen, USA) that were cultured in Carbenicillin-containing plates. The plasmid DNA was extracted from the DH5α *E. coli* using column chromatography (Promega, USA) and colony PCR was performed to verify the ZBTB16 ORF orientation.

Next, ZBTB16-V5 was amplified using Phusion PCR (NEB, USA) with primers that would add both BglII and EcoRI restriction sites to the 5′ and 3′ ends, respectively. The PCR product was purified and directly ligated to pJet1.2 (Thermo Scientific, USA) and then transformed in DH5α that were grown in Carbenicillin-containing plates. The plasmid DNA was extracted from the DH5α *E. coli* and digested with BglII and EcoRI to release ZBTB16-V5 with sticky ends. pTagRFP-N (Evrogen, Russia) was linearized with the same enzymes and ZBTB16-V5 was fused to the N-terminal end of TagRFP. The ligation product was transformed in DH5α, which was grown in kanamycin-containing plates.

Once again, TagRFP-ZBTB16-V5 was amplified using Phusion PCR with primers that would add both BamHI and EcoRV restriction sites to the 5′ and 3′ ends, respectively. The PCR product was purified and directly ligated to pJet1.2 and then transformed in DH5α, which was grown in Carbenicillin-containing plates. The plasmid DNA was extracted from the DH5α *E. coli* and digested with BamHI and EcoRv to release TagREFP-ZBTB16-V5 with sticky ends. pTRE-Cycle3 (Clontech, USA) was linearized with the same enzymes and TagRFP-ZBTB16-V5 was ligated into the BamHI and EcoRV sites. The ligation product was transformed in DH5α, which was grown in Carbenicillin-containing plates.

Generating the ZBTB16 inducible-expression system required two steps of transfections and selections: the first to introduce the tetracycline 3G trans-activator needed for Dox binding and the second to introduce the ZBTB16 expression construct. To generate stable ACHN cells expressing the trans-activator protein, 1 μg of pCMV-3G plasmid was transfected in $5 \times 10^4$ ACHN cells using the X-fect reagent


**Figure 1:** ZBTB16 inducible fusion protein construct. ZBTB16 ORF fused to TagRFP and V5 and cloned into the tet-inducible pTight plasmid. This plasmid has a bi-directional promoter in which ZsGreen is also induced upon Dox treatment.

(Clontech, USA) at 80% confluency for 48 h. Twelve different clones were isolated after G418 selection (800 µg/ml) of the transfected cells over two weeks. The amount of expression of the trans-activator protein was assessed by transfecting the 12 clones with the tet-inducible luciferase construct pTRE-Luc and comparing the basal expression levels of luciferase before Dox treatment to that after Dox treatment for 24 h. Accordingly, the cells were either untreated or treated with 500 ng/ml Dox and then lysed and processed for luciferase activity. Clone #5 designated ACHN:3G, which was selected as the best candidate from the other 11 clones, was further transfected as previously discussed with 1 µg of pTRE-Cycle3-pTagRFP-ZBTB16-V5 and selected by puromycin (3 µg/ml) for 10 days before isolating monoclonal colonies.

## Results

To test the expression pattern of ZBTB16 after creating the TagRFP-ZBTB16-V5 expression plasmid, it was co-transfected with the trans-activator plasmid into HeLa cells for microscopic examination and for western blot analysis. The expression of ZBTB16 protein was comparable to that of the original plasmid pLv129 by using both Anti-ZBTB16 and anti-V5 antibodies (Figure 2). ZBTB16 expression also showed typical nuclear-focused expression in HeLa cells after 500 ng/ml Dox induction for 24 h (Figure 3). Moreover, lower doses of Dox were also able to induce a detectable protein expression of ZBTB16 protein, while no protein expression was seen in the absence of Dox (Figure 4).

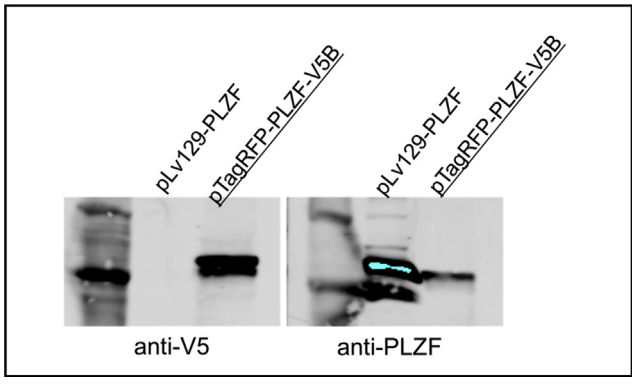


**Figure 2:** Protein expression of different ZBTB16 constructs. Western blot analysis showing ZBTB16 expression in HEK293 cells after transfection with the designated plasmid to test the tet-inducible promoter. pTagRFP-ZBTB16-V5 expression was detected using V5 antibodies as well as ZBTB16-specific antibodies.

Furthermore, ZBTB16 was strongly expressed in the absence of Shield1, which indicates that the destabilizing domain does not promote the proteasomal degradation of the ZBTB16 protein.

To test the clones expressing the 3G trans-activator that are to be chosen for stable cell selection, luciferase levels were compared between the induced state after Dox treatment and the baseline levels without Dox treatment (Figure 5) and the clone with the highest induction rate was chosen. ACHN clone #5 showed minimal basal luciferase activity and a high induction rate when compared to other ACHN clones, the cells also showed no significant morphological changes to the parental ACHN cells.

ACHN clone #5 was further transfected with the ZBTB16-expression plasmid pTRE-Cycle3-TagRFP-ZBTB16-V5-His. Twelve different clones were isolated after double selection with puromycin and G418 of the transfected cells over 10 days. Western blot analysis showed that clone #5-7 expressed the highest amount of ZBTB16 protein after 1 µg/ml Dox induction for 24 h (Figure 6). Furthermore, the

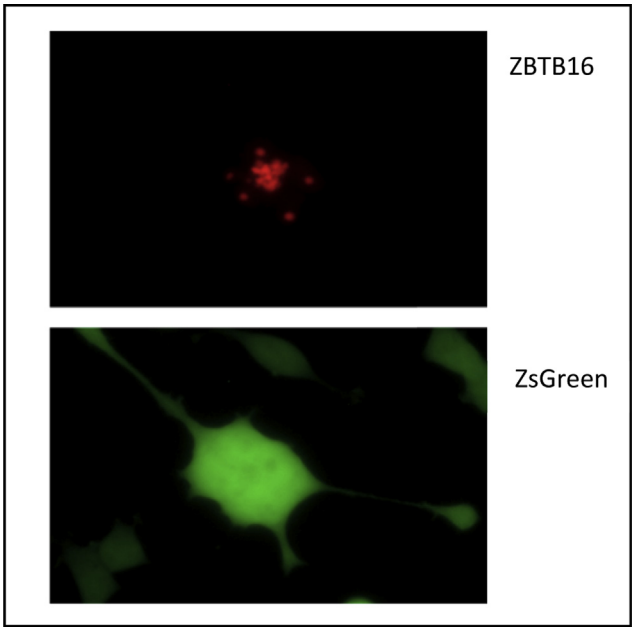


**Figure 3:** Fluorescence microscopy examination of the ZBTB16 inducible system in HeLa cells. pTagRFP-ZBTB16-V5 plasmid was transfected in HeLa cells and the cells were treated with 500 ng/ml of Dox for 24 h and imaged under confocal microscopy. The cells exhibited nuclear-focused ZBTB16 expression seen in red for the expression of TagRFP (top) as well as green fluorescence for the expression of ZsGreen (bottom).

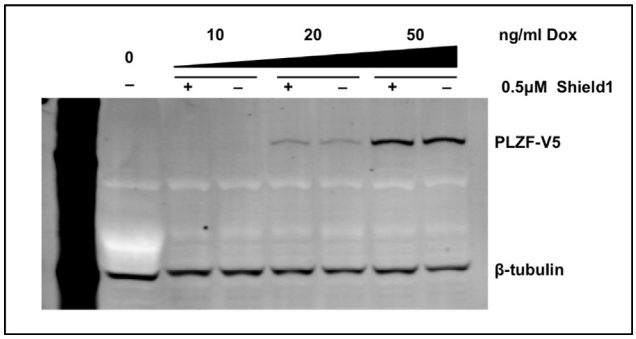


**Figure 4:** ZBTB16 inducible expression system in HeLa cells. pTagRFP-ZBTB16-V5 plasmid was transfected into HeLa cells and treated with increasing doses of Dox (10, 20 and 50 ng/ml) for 24 h. The cells were lysed and ZBTB16 expression was detected by western blot using V5 antibodies.

amount of expression of ZBTB16 and ZsGreen1 was assessed by treating the 12 clones with 500 ng/ml Dox and comparing the fluorescence intensity of the cells before Dox treatment to that after Dox treatment for 12 h. Ten out of twelve clones showed no detectable fluorescence without Dox treatment and two clones showed minimal amounts of fluorescence and were excluded from the experiment. ACHN clones #5-7 showed the highest level of inducibility in terms of green fluorescence (Figure 7) after Dox treatment with detectable fluorescence in Dox absence. This double selected ACHN clone was designated ACHN:ZBTB16. ACHN clone #5, which was only selected by G418 and expresses the trans-activator, was used as a control and was designated ACHN:control, as it did not harbour the ZBTB16-expression plasmid and would not manifest any Dox-mediated ZBTB16 effects. This would also be helpful in determining the biological effects of Dox on

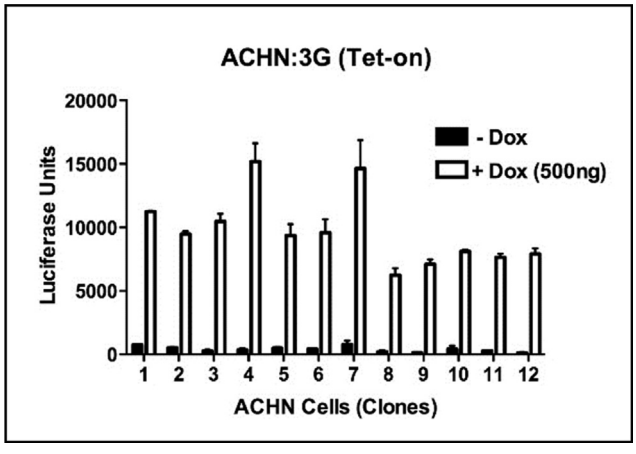


**Figure 5:** Inducibility of ZBTB16 in different clones of ACHN:3G. Twelve single-cell-derived clones from ACHN cells were isolated and tested for the expression of the 3G trans-activator by transfecting the pTRE-Luc construct and quantifying luciferase activity. Basal as well as Dox-induced luciferase was assessed and used to select the best clone for antibiotic selection to generate a stably selected cell line.

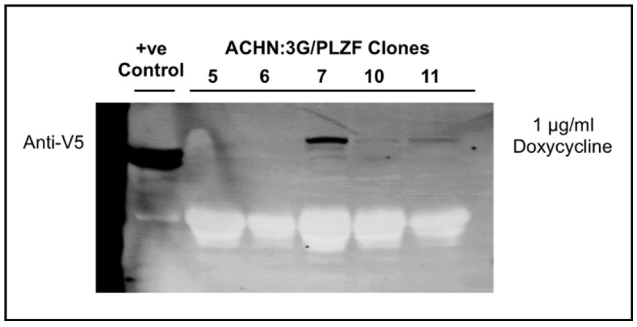


**Figure 6:** ACHN clones expressing different amounts of ZBTB16 after Dox treatment. ACHN clones (originating from Clone #5) were treated with 1 μg/ml Dox and lysed for western blot analysis. ACHN clone #5-7 showed maximal expression of PZLF after 24 h of treatment and was subsequently used as the cell model and designated ACHN:ZBTB16.

ACHN cells when comparing ACHN:control to parental ACHN cells.

The ability of Shield1 to control the expression levels of ZBTB16 in the double-selected ACHN:ZBTB16 cells was tested again by treating the cells with 500 ng/ml Dox in the absence or presence of 0.5 μM Shield1 protein for 24 h. Again, there was no noticeable effect of Shield1 on the amount of ZBTB16 protein in our cell model (Figure 8).

## Discussion

Transcription factors play a very important role in many intertwined biological functions. Understanding the extent of their involvement in certain molecular signalling pathways is key to discovering novel ways to tackle their targets. Additionally, the firm regulation of their expression in cancer cell models is very important in investigating their biological involvement in carcinogenesis. Many transcription factors including ZBTB16 will carry on their biological activities, such as driving the cell cycle or promoting cellular growth, even with minimal expression levels. Many transcription factors, including ZBTB16, are under heavy spatial and temporal regulatory mechanisms. The overexpression of transcription factors by regular transfection methods may result in high protein expression levels that do not represent the physiological condition of actively growing cells. This would prevent researchers from studying the effect of that transcription factor on certain signalling pathways without sacrificing reliability.

In this study, I used the Clontech Tet-inducible system to regulate the expression of ZBTB16 in order to study the biological roles of this transcription factor on different aspects of cancer cell biology. ZBTB16 is required for stem cell self-renewal and survival, and its expression regulates the cell cycle through many key mediators including Cyclin-D1 and Myc.[21] However, for some cancer cells to escape the effect of ZBTB16, its expression is down-regulated throughout carcinogenesis and those signalling pathways are relieved from its repression.[13] This required a biological system in which the expression of ZBTB16 protein can be manipulated and adjusted but with tight regulation that would produce a complete transcriptional silencing when required.

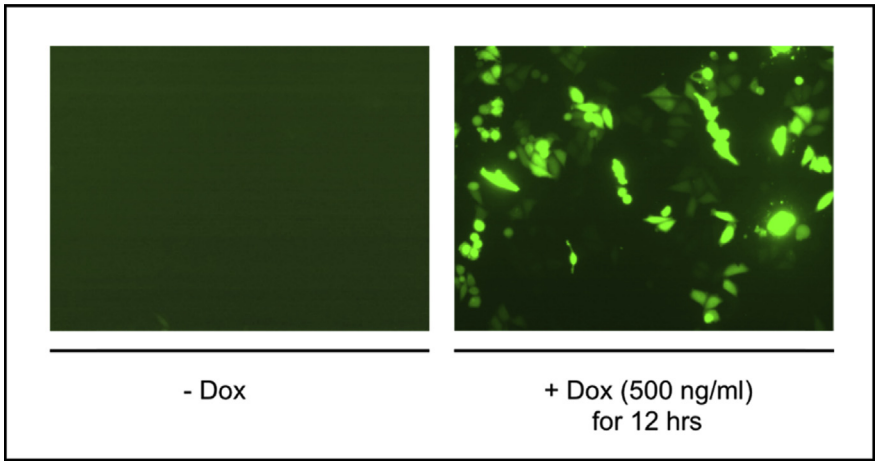


**Figure 7:** ACHN:ZBTB16 showing ZsGreen expression under fluorescence microscopy. Stably selected ACHN:ZBTB16 cells were either un-treated (left) or treated with 500 ng/ml Dox (right) for 12 h and then imaged under confocal microscopy. ACHN:ZBTB16 showed a similar morphological phenotype to the parental cells.

The Tet-On 3G system from Clontech was used to utilize the 3rd generation tet-promoter that was shown to provide a very low background activity in the absence of Dox.[22] The bi-directional pTRE plasmid was used to clone ZBTB16 ORF and produce the ZBTB16 expression plasmid (Figure 1). The pTRE plasmid also contained another layer of control offered by Clontech, namely the ProteoTuner™ Shield System.[23] The system works on the protein level by adding a DNA sequence, called the destabilization domain, to the sequence of the protein-of-interest that makes it targeted by proteasomal degradation machineries inside the cell.[24] The addition of a small molecule called Shield1 would protect the protein from proteasomal degradation and allow the levels of the protein to rapidly increase with the continued addition of Dox to the culture medium. Unfortunately, the second layer of control using the ProteoTuner™ Shield System was not effective in our study. The absence of Shield1 in the culture medium did not decrease the protein levels as it should have, and ZBTB16 was effectively expressed by ACHN:ZBTB16 cells with the addition of Dox alone (Figure 8). Similar results were obtained even prior to the stable selection because HeLa cells that were transiently transfected with the ZBTB16 expression plasmid did not respond to the absence of Shield1 and because ZBTB16 was also expressed by the addition of Dox alone (Figure 4). This shows that the Shield system was not effective after adding the ZBTB16 ORF to the original plasmid. Due to time constraints, the destabilization domain was not transferred to C-terminus of ZBTB16 ORF to check whether it would successfully control the protein expression. I believe that the overall 3D structure of the fused protein Destabilization Domain-TagRFP-ZBTB16-V5 (Figure 1) may have masked the destabilization domain and render it unreachable to the proteasomal degradation signals.

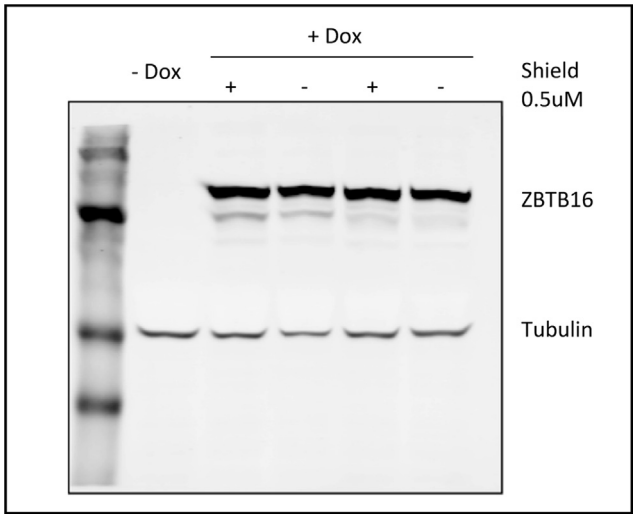


**Figure 8:** No effect of Shield1 on ACHN:ZBTB16 cells after Dox treatment. Stably selected ACHN:ZBTB16 cells were treated with 500 ng/ml of Dox either in the presence or absence of 0.5 μM Shield for 24 h and then analysed for ZBTB16 expression using V5 antibodies. Shield showed no effect on PZLF expression levels and was considered ineffective in this system.

ACHN cells that were used as a cell model in this study were first transfected by the pCMV-3G plasmid to express the 3G trans-activator that is required to bind to the tet promoter. The selection of the best clone was based on a luciferase assay to detect the sensitivity of each clone to Dox induction (Figure 5). The luciferase protein was under control of the same tetracycline responsive elements that are part of the pTRE plasmid, the same plasmid into which ZBTB16 was cloned. Based on responsiveness to Dox and morphological characteristics, clone #5 was chosen for the second round of transfection. Subsequently, this clone was transfected with the ZBTB16 expression plasmid pTRE-Cycle3-TagRFP-ZBTB16-V5 and selection was based on the ability to respond to Dox treatment by expressing the ZBTB16 fused protein. Based on protein expression, as demonstrated by western blot, and on fluorescence intensity, as demonstrated by fluorescence microscopy, clone #5-7 was chosen as the cell model for this study and was designated ACHN:ZBTB16.

Eventually and regardless of the ineffectiveness of the Shield1 system, the ZBTB16 expression plasmid was transfected into ACHN cells that passed a double selection process and was able to regulate the expression of ZBTB16 protein remarkably.

## Conclusion

The Tet-On® 3G system that was used in this study was essential in controlling the basal expression of ZBTB16 to prevent transcription leakage and to provide a tightly regulated method for expressing the ZBTB16 protein in a fashion resembling physiological conditions. The addition of doxycycline enabled us to carefully control ZBTB16 expression in a dose- and time-dependent manner. Shield1 was not able to produce its desired effect of giving a second line of post-transcriptional control; nevertheless, the Tet-On 3G system alone was sufficient to block ZBTB16 transcriptional in the absence of Dox. This inducible expression system of ZBTB16 in the ACHN renal adenocarcinoma cell line will be a very valuable tool to investigate the role of ZBTB16 in effecting the carcinogenic behaviour of these cells and possibly other cancer cell lines.

## Conflicts of interest

The authors have no conflict of interest to declare.

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