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# TLE4 regulation of wnt-mediated inflammation underlies its role as a tumor suppressor in myeloid leukemia

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## Abstract

The presence of AML1-ETO (RUNX1-CBF2T1), a fusion oncoprotein resulting from a t(8;21) chromosomal translocation, has been implicated as a necessary but insufficient event in the development of a subset of acute myeloid leukemias (AML). While AML1-ETO prolongs survival and inhibits differentiation of hematopoietic stem cells (HSC), other contributory events are needed for cell proliferation and leuke-mogenesis. We have postulated that specific tumor suppressor genes keep the leukemic potential of AML1-ETO in check. In studying del(9q), one of the most common concomitant chromosomal abnormalities with t(8;21), we identified the loss of an apparent tumor suppressor, TLE4, that appears to cooperate with AML1-ETO to confer a leukemic phenotype. This study sought to identify the molecular basis of this cooperation. We show that the loss of TLE4 confers proliferative advantage to leukemic cells, simultaneous with an upregulation of a pro-inflammatory signature mediated through aberrant increases in Wnt signaling activity. We further demonstrate that inhibition of cyclooxygenase (*COX*) activity partly reverses the pro-leukemic phenotype due to *TLE4* knockdown, pointing towards a novel therapeutic approach for myeloid leukemia.

### Keywords

AML1-ETO; Acute myeloid leukemia; Tumor suppressor; TLE4; Inflammation; Wnt signaling

## 1. Introduction

Despite many advances leading to increased rates of remission, leukemia continues to be the 6th leading cause of cancer-related deaths in the United States. Acute myeloid leukemia

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**Conflict of interest**

The authors declare no conflicts of interest.

(AML) is accountable for approximately 32% of all new cases of leukemia and responsible for about 50% of all leukemia-caused deaths [1]. One of the most common subtypes of AML is associated with a t(8;21) chromosomal translocation, which creates the *AML1-ETO*/*RUNX1-CBF2T1* fusion gene. This gene has been implicated as a key, but insufficient, oncogenic driver of myeloid leukemia [2–4]. The presence of the *AML1-ETO* fusion gene has been demonstrated in the blood spot of a newborn who did not develop leukemia for over 10 years [5]. We have postulated the leukemic potential of this clone is held in check by potential tumor suppressor genes, the discovery of which might have significant therapeutic implications. Earlier studies demonstrated *AML1-ETO* can confer a survival advantage to HSCs, but induces cell cycle arrest and apoptosis in myeloid progenitors [6–8]. Previous studies suggest that *AML1-ETO* requires additional mutations that overcome this detrimental effect and contribute towards leukemogenesis. In search of such mutations, we noted that, other than losses or gains of chromosomes X,Y or 8, an interstitial deletion of chromosome 9q is the most common secondary cytogenetic change associated with t(8;21) AML [9–11]. This finding prompted our initial efforts to identify a potential AML tumor suppressor gene on 9q. After mapping a commonly deleted region in del(9q) AML, we used shRNA to target all candidate genes located in the commonly deleted region. This revealed knockdown of two neighboring genes, *TLE1* and *TLE4* conferred significantly reduced cell apoptosis and proliferation by *AML1-ETO* [10,11].

The Groucho/TLE family of proteins has been characterized as master regulators in Drosophila development because of their regulation of multiple signaling pathways and critical roles in many cell fate decisions, including receptor tyrosine kinase/Ras/MAPK, Notch, and Wnt signaling [12,13]. These pathways are dysregulated in many malignancies. An increasing role of *TLE*s in the pathogenesis and prognosis of various cancers, including synovial cell sarcoma, glioblastoma, and leukemia have been described [10,14–16]. Moreover, our previous work has shown the importance of *Tle4* in regulating normal bone metabolism and hematopoiesis, as indicated by significant bone mineralization defects, hematopoietic stem cell maintenance, and terminal differentiation of various hematopoietic compartments in *Tle4* knockout mice [17]. In leukemia, we previously demonstrated that proliferation and apoptosis of Kasumi-1 cells, a human myeloid leukemia cell line harboring t(8;21), is sensitive to TLE levels, especially TLE4. We further showed that loss of the TLE homologue, Gro3, in zebrafish cooperated to create a myeloid leukemia phenotype [10]. This indicated that TLE4 might be a critical gatekeeper blocking the oncogenic potential of AML1-ETO and underscored the importance of elucidating the mechanism behind the synergistic effects of AML1-ETO and loss of TLE4. Here, we identify upregulation of inflammatory genes related to prostaglandin metabolism and downstream effectors as a consequence of *TLE4* knockdown in leukemia cells. We further determined that increased Wnt signaling due to decreased inhibition is, at least in part, responsible for the inflammatory gene signature and concomitant proliferative, drug resistance, and differentiation block phenotype associated with *TLE4* knockdown.

## 2. Materials and methods

### 2.1. Cell culture, shRNA construction, and lentiviral infection

Kasumi-1 cells (ATCC, Manassas, VA) were cultured in RPMI-1640 (Lonza, Walkersville, MD) supplemented with 10% FBS (Sigma, St Louis, MO) and 1% Penicillin/Streptomycin (Invitrogen, Carlsbad, CA). HL60 cells (ATCC) were cultured in MEMα (Invitrogen) supplemented with 20% FBS and 1% Penicillin/Streptomycin. All cells were maintained at 37 °C and 5% $CO_2$ at concentrations of $10^6$ cells/mL. When indicated, cells were also cultured with the following: 10 uM all-trans retinoic acid (Sigma), 50 uM indomethacin (Sigma), 100 uM cytarabine (Abcam, Cambridge, MA), 0.1 uM vitamin D3 (Sigma), 10 nM recombinant human Wnt3a (R&D Systems, Minneapolis, MN), or 10 nM ICG-001 (Selleck Chemical, Houston, TX). Non-targeting scramble control and *TLE4*-specific shRNA constructs were developed and delivered to cells via lentiviral delivery as previously described [10]. The shRNA used and their target sequences were: shTLE4 1 (AGTGATGACAACTTGGTGG) and shTLE4 2 (GGCATTATGTCATGTATTA). Data in figures were obtained using shTLE4 2 unless otherwise indicated. Infected cells were identified by GFP fluorescence detected using FACS LSRII or selected for via cell sorting with FACS Aria (BD, San Jose, CA). Full-length *TLE4* (a.k.a.KIAA1261 kind gift of Dr. Ohara [18]) cDNAs were cloned into the MSCV-IRES-GFP retroviral vector.

### 2.2. Cell cycle, annexin V, and flow cytometry analysis

Cell cycling and death in Kasumi-1 cell populations were determined using DAPI cell cycle and Annexin V assays as previously described [10]. When indicated, the following fluorescent anti-human antibodies were used: CD11b-APC (ICRF44; eBiosciences, San Diego, CA), CD14-APC-Cy7 (M5E2; Biolegend, San Diego, CA), APC-Annexin V (BD, San Jose, CA), DAPI (Invitrogen, Carlsbad, CA). All flow cytometry data was analyzed using FlowJo X (Treestar, Ashland, OR) and ModFit LT 3.0 (Verity Software House, Topsham, ME). Cells were analyzed using FACS Aria or LSRII (BD).

### 2.3. Detection of Wnt signaling

Wnt activity was also measured in 293T cells (ATCC) using a TOPFLASH/FOPFLASH assay. Briefly, 293T cells were transfected with the following reporter constructs using TransIT reagent (Mirus): TOPFLASH construct containing 8x promoter binding sites followed by firefly luciferase, FOPFLASH firefly luciferase control, and pRL renilla luciferase transfection control. 293T cells were additionally transfected with abovementioned shRNA constructs and/or expression vectors containing *AML1-ETO*, *CBFb*, and *TLE4*. Treated cells were subsequently analyzed using MicroLumat PLUS LB luminometer (Berthold Technologies, Bad Wildbad, Germany) 24 h post-transfection.

### 2.4. Western blotting

Kasumi-1 and 293T cells treated with *TLE4*-specific or scramble control shRNA were lysed for protein. Western blots were run and probed as previously described [17] using the following antibodies: anti-human TLE4 (sc13377x; Santa Cruz Biotechnology, Dallas, TX), anti-Actin (sc10731; Santa Cruz Biotechnology), donkey anti-goat IgG-HRP (SC2020;

Santa Cruz Biotechnology), and goat anti-rabbit IgG-HRP (ab97051; Abcam). TLE4 band intensities quantified using ImageJ and standardized to Actin band intensity (NIH, Bethesda, MD).

### 2.5. Expression analysis via RNAseq and qRT-PCR

RNA was harvested from Kasumi-1 cells using TRIzol (Invitrogen) 7 days after lentiviral spinoculation with scramble control or *TLE4*-specific shRNA. Library construction was performed after polyA-tail selection and used for 50 bp paired-end reads that were aligned to human genome GRCh37.75 using STAR and quantified using HTSeq. DESeq was used for normalization and identification of differentially expressed genes. Gene Set Enrichment Analysis (GSEA; Broad Institute, Cambridge, MA) was performed via Java application by ranking genes using t-scores. Expression levels of select differentially expressed genes and others of interest were performed using qRT-PCR as previously described [17]. Primer sequences for SYBR Green and Taqman assays are listed in supplemental table.

### 2.6. Primary human AML patient samples

Diagnostic bone marrow specimens from AML patients with del(9q) or t(8;21) del(9q) and normal CD34+ samples were obtained from patients at Massachusetts General Hospital or from the Children's Oncology Group as described previously with informed consent and assent from parents and patients in accordance with protocols approved by Massachusetts General Hospital Institutional Review Board and Children's Oncology Group [10].

### 2.7. Statistics

Unless otherwise specified, analyses used student's unpaired *t*-test or two-way ANOVA with Graphpad Prism (Graphpad Software, La Jolla, CA). For RNAseq analyses, significantly differentially expressed genes were identified by false discovery rate < 0.2.

## 3. Results

### 3.1. *TLE4* knockdown increases proliferation and inhibits induced differentiation of leukemia cells

To determine the effects of *TLE4* knockdown (T4KD), Kasumi-1 cells were treated with scramble control or two different *TLE4*-specific shRNA via lentivirus. qRT-PCR and Western blotting confirmed 60–72% *TLE4* message reduction and 75–82% TLE4 protein expression reduction, respectively, in T4KD cells (Fig. 1A). shRNA treated cells were tracked over a period of 18 days to monitor cell growth. Consistent with previous studies [10], T4KD cells had significantly faster growth than their control counterparts (Fig. 1B). DAPI cell cycle and Annexin V analysis with T4KD Kasumi-1 cells seven days post-infection showed increased S and G2/M-phase cells concomitant with decreased dead cell populations (Fig. 1C and D). qRT-PCR further shows significant reduction of AML1-ETO repressive targets *CEBPa* and *GATA1*, suggesting T4KD enhances AML1-ETO activity [19,20]. (Fig. 1E).

Flow cytometric analysis revealed T4KD was able to repress ATRA induction of CD14+ populations compared to control (Fig. 2A). The differences in CD14+ populations suggests

*TLE4* may regulate the differentiation potential of t(8;21) leukemia. To better understand the effect of *TLE4* on differentiation of myeloid leukemia cells, T4KD using lentiviral-based shRNA was also achieved in HL60 cells, a human promyelocytic leukemia cell line harboring the *PML-RARα* oncoprotein. Treatment with vitamin D3 induces HL60 differentiation with increased surface marker CD14-positive cells [21,22]. T4KD was able to blunt vitamin D3 differentiation of HL60 cells, as indicated by reduced induction of CD14+ populations (Fig. 2B). T4KD was also able to reduce expression levels of *PU.1* and *MYB* compared to controls in HL60 cells, quantified by qRT-PCR (Fig. 2C). Our results illustrate the ability of *TLE4* to regulate the differentiation potential of leukemia cells independent of AML1-ETO function.

### 3.2. Low expression of *TLE4* and *TLE1* is not unique to t(8;21) AMLs

Del(9q) has a relatively unique association with t(8;21) and is not seen to any appreciable extent with any other AML translocation. As expression may be affected by other means such as methylation we sought to determine if low *TLE1* or *TLE4* expression was characteristic of other AML subtypes. We examined the curated data sets of over 2000 AML samples using the Blood-Spot web interface, (http://servers.binf.ku.dk/bloodspot), which includes over 2000 AML samples including those from the TCGA Research Network (http://cancergenome.nih.gov) as well as from the International Microarray Innovations in Leukemia Study Group [23]. As compared to AML samples with normal karyotype, which have a level of expression of *TLE1* and *TLE4* that is about average among AMLs, expression levels of *TLE4* are significantly lower in t(8;21), t(11q23)/MLL, and del(9q), while expression of TLE1 was significantly lower in t(8:21), Trisomy 8, Trisomy 13, and HSC (see Supplemental Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.leukres.2016.07.002). The overall levels of *TLE1* expression were lower in AML samples compared to *TLE4*.

### 3.3. *TLE4* knockdown leads to upregulation of inflammatory and immune response pathways, a hallmark of t(8;21) leukemia

To better understand the mechanism behind the oncogenic effects of T4KD, we performed RNAseq analysis of Kasumi-1 cells seven days after lentiviral delivery of control or *TLE4*-specific shRNA. Results are summarized in a heatmap (Fig. 3A) and were verified by qRT-PCR query for select genes with $\log_2$ fold change greater than 0.5 and false discovery rate (FDR) of less than 0.2 in an independent experiment (Fig. 3B). After filtering differentially expressed genes for FDR < 0.2, GSEA analysis using upregulated geneset showed enrichment of immune and inflammation-related pathways (Fig. 3C). Additionally, the same analysis identified enrichment of genes found to be upregulated in *AML1-ETO* expressing monocytes, further suggesting the contribution of *TLE4* knockdown enhancing AML1-ETO function [24]. Interestingly, qRT-PCR using RNA harvested from primary human AML diagnostic bone marrow samples show increased levels of *FOS*, *CEBPb*, and *PTGER4* expression in del(9q) samples, higher in those with t(8;21) del(9q), and even higher levels in non-del(9q) t(8;21) as compared to CD34+ controls (Fig. 4A). These findings emphasize the importance of inflammatory pathways in t(8;21) myeloid leukemia and the ability of del(9q), and apparently other cooperating mutations, to augment these pathways.

### 3.4. Increased inflammation signature is mediated through interplay between *TLE4* and a *COX*-Wnt signaling axis

Previous work by Zhang et al. describes AML1-ETO-driven upregulation of *COX* that contributes to increased b-Catenin stability and activity, possibly through inhibition of *GSK3β* [25]. Given the known repressive role of TLE4 on Wnt signaling [12], T4KD is expected to upregulate and intensify Wnt signaling due to AML1-ETO. qRT-PCR using samples from shTLE4- treated Kasumi-1 cells seven days after knockdown revealed significantly increased *COX1* and *COX2* expression as well as those genes associated with prostaglandin metabolism and inflammation identified from above RNAseq, including *FOS*, *PTGER4*, and *CEBPb* (Fig. 4B). T4KD-mediated changes in myeloid-associated gene expression in HL60 cells were concomitant with similar increases in inflammatory genes *FOS*, *CEBPb*, *PTGER4*, and *IL1b* (Fig. 4C).

To confirm the induced inflammatory signature is a Wnt-responsive effect, naïve Kasumi-1 cells were serum starved for 24 h and cultured in media with rhWnt3a for 48 h qRT- PCR analysis of these cells identified various Wnt-responsive genes, including those previously identified as Wnt targets [26–28], such as *LEF*1 and *CCND3*, as well as those previously described in the T4KD-associated inflammatory gene signature, such as *COX1*, *COX2*, *FOS*, *CEBPb*, and *PTGER4* (Fig. 5A). To further determine the role of Wnt signaling with T4KD, 293T cells were transduced with *AML1-ETO* to better characterize the relationship between *TLE4* and the *AML1-ETO*-*COX*-Wnt signaling axis. Ectopic expression of *AML1-ETO* in 293T cells was able to increase *COX1* and *COX2* expression by at least seven-fold as determined by qRT-PCR (Fig. 5B). TOPFlash/FOPFlash Wnt reporter assay shows *AML1-ETO* was able to increase Wnt signaling activity in 293T cells by two-fold. This was abrogated with ectopic overexpression of full length *TLE4*, verifying 293T cells as an alternative system for modeling the *AML1-ETO*-*COX*-Wnt signaling axis (Fig. 5C). Subsequently, 293T cells were transduced with *AML1-ETO* and either control or *TLE4*-specific shRNA. T4KD was able to increase Wnt signaling in 293T cells with *AML1-ETO* expression (Fig. 6A). qPCR using RNA harvested from these cells confirm upregulation of *COX1* and *COX2* expression in *AML1-ETO*-expressing 293T cells treated with *TLE4*-specific shRNA compared to control (Fig. 6B).

Additionally, T4KD and control Kasumi-1 cells were cultured in media supplemented with 10 uM ICG-001, a small molecule inhibitor that binds with CBP (CREB binding protein) to block b-Catenin/TCF-mediated Wnt signaling [12,29–32]. Activating interactions between CBP, b-Catenin, and TCF require clearance of TLE/TCF binding [12], thus ICG-001 is predicted to inhibit T4KD-mediated Wnt signaling activation. Fold change of GFP+ lentiviral treated T4KD and control Kasumi-1 cells significantly diminished over 15 days in presence of ICG-001 (Fig. 7A). qRT-PCR analysis further demonstrated that ICG-001-mediated Wnt inhibition was able to abrogate expression of not only *COX1* and *COX2*, but also the aforementioned inflammation and Wnt target genes (Fig. 7B). These results suggest that, while *COX* inhibition does indeed block Wnt signaling and consequent upregulation of inflammatory genes, the effects mediated by T4KD are largely dependent on release of Wnt regulation. Therefore, aberrant increases in Wnt signaling due to T4KD may explain its contributory leukemic effects to *AML1-ETO* function through a Wnt-induced inflammation.

### 3.5. *COX* inhibition is able to inhibit the proliferative and chemotherapy resistance effects conferred by *TLE4* knockdown

Despite many current efforts to develop Wnt inhibitors, direct blockade of Wnt signaling remains challenging to clinical therapeutics due to its ubiquitous and integral function in normal development and stem cell maintenance [33]. However, studies have shown a number of clinically approved drugs antagonize Wnt signaling indirectly, including cyclooxygenase inhibitors [34]. We found that the addition of 50 uM indomethacin (INDM), a non-selective *COX* inhibitor, was able to significantly reduce Wnt signaling in AML1-ETO-expressing 293T cells treated with shTLE4 compared to DMSO control, suggesting COX inhibition is able to abrogate T4KD-induced increases in Wnt signaling (Fig. 6A). qPCR analysis showed that decreased Wnt signaling in these cells was concomitant with reductions in *COX1* and *COX2* expression as well (Fig. 6B). Kasumi-1 cells treated with control or *TLE4*-specific shRNA were cultured in 50 uM INDM for 16 days (Fig. 8A). *COX* inhibition significantly reduced growth of T4KD cells compared to DMSO control by four days. By day 13, growth of INDM-treated T4KD Kasumi-1 cells plateaued and fell below that of control cells.

Similarly treated Kasumi-1 cells were cultured in 100 uM cytarabine (AraC), a conventional chemotherapy agent used at a level comparable to serum concentrations in patients undergoing high-dose therapy [35]. Prior studies have shown that b-Catenin and Wnt signaling activity are associated with chemotherapy resistance in various cancer models, including colon, breast, and pancreatic cancer [36–38]. We found that T4KD was able to confer relative resistance to AraC treatment, indicated by significantly reduced dead cell populations (Fig. 8B). Interestingly, the addition of INDM was able to overcome T4KD-induced AraC resistance and resulted in even higher levels of cell death compared to control, supporting the potential use of INDM as adjuvant therapy for conventional chemotherapeutics.

Furthermore, qRT-PCR assays queried changes in expression levels of the inflammatory signature and various myeloid differentiation markers in Kasumi-1 cells due to T4KD and INDM treatment. qRT-PCR assays demonstrate upregulation of multiple Wnt targets in Kasumi-1 and 293T cells treated with *TLE4*-specific shRNA compared control, which can be significantly inhibited, and in some instances blocked, with the addition of INDM (Fig. 9). The expression levels of *ELANE*, *MPO*, and *PU.1* were repressed in T4KD Kasumi-1 cells (Fig. 10) consistent with inhibition of differentiation. INDM was able to increase expression of these genes, suggesting that *COX* inhibition is able to relieve repression due to T4KD. The effects of combination ATRA and INDM therapy on differentiation could not be assessed due to severe lethality (data not shown).

## 4. Discussion

About half of all pediatric AMLs have one of four specific balanced translocations that are primary, but insufficient drivers of leukemogenesis [6–8,39]. One such translocation t(8;21) creates an *AML1-ETO* fusion gene [2–4]. We previously identified the TLE/Groucho co-repressors as apparent gatekeepers preventing leukemia progression of *AML1-ETO* transformed HSCs. We found increased *TLE4* expression slows the proliferation of *AML1-*

*ETO* expressing leukemia cells, while T4KD significantly increases growth rate and cell cycling while decreasing cell death. In a zebrafish model we found expression of AML1-ETO in the presence of knockdown of the TLE/Gro homologue leads to accumulation of blast-like hematopoietic cells [10]. These observations support TLE4 functioning as a tumor suppressor, whose absence confers pro-survival and blocked differentiation in t(8;21)-harboring leukemia. This effect may also extend to t(11q23)/MLL AMLs that also have low TLE4 expression (see Supplemental Fig. S1 at the online version at DOI: http://dx.doi.org/10.1016/j.leukres.2016.07.002).

Our findings extend a previous observation that expression of AML1-ETO in U937 cells leads to induction of AES, a truncated member of the TLE/Groucho family thought to repress TLE function in a dominant fashion. The loss of AES was found to antagonize stem cell self-renewal and immortalization effects by AML1-ETO [40]. This upregulation of AES or TLE methylation could provide alternative ways of providing cooperativity between AML1-ETO and loss of TLE function in cases without del(9q) [14].

In this current study, we sought to understand the mechanism behind the cooperation between loss of *TLE4* and AML1-ETO. Our expression analyses indicate T4KD-induced growth is concomitant with upregulation of pathways related to inflammation and immune responses. This is consistent with previous studies that propose *AML1-ETO*-dependent survival of leukemic cells is dependent on a *COX*/b-Catenin axis [25]. T4KD leads to significant upregulation of *COX1*, *COX2*, and select inflammatory genes related to prostaglandin metabolism; implicating TLE4 as an important regulator of the relationship between AML1-ETO, COX, and b-Catenin (Fig. 11).

We also provide evidence that an important effect of TLE4 loss is the maintenance of leukemia cells in an undifferentiated state resistant to triggers of differentiation. In Kasumi-1 cells T4KD was associated with inhibition of ATRA induction of CD14. T4KD in these cells resulted in reduced expression of *CEBPa* and *GATA1,* regulators of myeloid and erythroid differentiation. T4KD also reduced expression of the differentiation genes *ELANE*, *MPO*, and *PU.1*, an effect overcome by INDM (Fig. 10), suggesting that *COX* inhibition is able to counteract some of the effects of T4KD in Kasumi-1 cells. We could also demonstrate effects in HL60 cells, which possess a different fusion gene, PML-RARA. In these cells, T4KD blocked vitamin D3 induction of CD14. In HL60 cells this was coincident with decreased expression of myeloid transcription factors and increased expression of previously identified inflammatory genes in the Kasumi-1 cells with T4KD. This suggests TLE4 can have effects independent of AML1-ETO. Although not explored in detail in the current study, PML-RARA, similar to AML1-ETO, has also been shown to activate Wnt signaling [41], which might point to a common mechanism. We found that combining ATRA or Vitamin D3 with INDM was extremely lethal and left no viable cell populations for meaningful analysis; warranting additional studies to investigate potential of ATRA/Vitamin D3 and INDM combination therapy. Interestingly, Wnt signaling regulates hematopoiesis and its aberrant constitutive activation has been associated with defective hematopoietic stem cell maintenance and differentiation [42,43]. Previous studies have shown the importance of *Myb* in myeloid leukemia, where *Myb* null mice demonstrate increased disease-free survival in models of AML-ETO9a and MLL-AF9 leukemia [44]. While our

results show T4KD is associated with decreased *Myb* expression in leukemic cells, our experiments did not fully abolish *Myb* expression. Low levels of TLE4 may activate other pathways that compensate for low Myb. Further investigations are warranted to clarify how modulations of *TLE4* levels may affect the oncogenic effects of *Myb*.

In an attempt to induce leukemia we transplanted *Tle4* knockout mouse bone marrow transduced with *AML1-ETO* but were unable induce frank leukemia in wild-type mouse recipients (data not shown). This may represent the need for additional cooperating leukemic cell mutations or failed engraftment into a suboptimal niche. Recent developments have described the critical role of inflammation and cytokine signaling by leukemic myeloid cells on bone marrow niche remodeling and subsequent leukemia development [45]. Having identified the association between T4KD, proliferation, and inflammation in Kasumi-1 cells, future investigations into how TLE4 regulation of inflammation exerts intrinsic and extrinsic influences on leukemic stem cells in the bone marrow are underway.

Wnt signaling has been extensively studied in the context of various malignances, including colon cancer, as a key regulator of cancer cell proliferation and survival [26]. Consistent with the known repressor function of TLE4 on TCF/LEF activity [46], we demonstrate that T4KD increases Wnt signaling and Wnt-responsive gene expression in Kasumi-1 and *AML1-ETO*-expressing 293T cells, with simultaneous upregulation of a pro-inflammatory signature. T4KD-induced upregulation of these inflammatory Wnt targets are partially reversible by INDM, suggesting that the other inflammatory genes are downstream of both *COX* and Wnt signaling. Interestingly, repression of one Wnt signaling target, *CCND3,* is thought to contribute to growth suppression in *AML1-ETO* expressing cells and release of such repression has been associated with proliferative leukemia, especially in the *AML-ETO9a* model [42,47,48]. The importance of Wnt signaling on Kasumi-1 growth and AML1-ETO function is suggested by the extreme lethality of ICG-001, which repressed growth of Kasumi-1 cells regardless of *TLE4* status. ICG-001 was able to block the induction of *COX1* and *COX2* as well as other inflammation-related gene expression due to T4KD. We have not, however, excluded the possibility that ICG-001 may have inhibitory effects on other signaling pathways, such as NF-kB, through its inhibition of CBP [49].

Decreased Wnt signaling and target gene expression due to INDM was correlated with reduced cell proliferation and increased sensitivity to AraC treatment; indicating anti-inflammatory interventions can reverse the pro- leukemic effects of T4KD in t(8;21) AML cells. However, INDM treatment was unable to completely eliminate T4KD-induced increases in inflammatory gene expression. Our results do not preclude the possibility that loss of TLE4 may affect other pro-inflammatory pathways. While our results suggest *COX* as a Wnt-responsive target under TLE4-mediated regulation, they do not exclude the possibility of other regulators of *COX* and inflammation which may independently contribute towards Wnt signaling in t(8;21) leukemia. Recent work from our laboratory has shown loss of TLE1, a closely related isoform of TLE4, confers a pro- inflammatory predisposition associated with increased NF-kB activation [50]. Thus, it is possible that TLE4 is involved in the regulation of other parallel pro-inflammatory processes that contribute towards AML1-ETO leukemia. There may be additional effects of TLE4 on AML1-ETO function, not explored in this study, given our previous demonstration of

binding interactions between the two proteins [10]. We found that upregulation of pro-inflammatory genes such as *CEBPb*, *FOS*, and *PTER4* appears to be a characteristic of t(8;21) AMLs, even those without del(9q). This could indicate other cooperating mutations besides loss of 9q with TLE1 and TLE4 may be able to serve this role. Alternatively, since low levels of *TLE1* and *TLE4* were seen in a large number of t(8;21) samples the majority unlikely to have del(9q) (see Supplemental Fig. S1 in the online version at DOI: http://dx.doi.org/10.1016/j.leukres.2016.07.002), *TLE1* and *TLE4* expression may be repressed in these samples, e.g, through methylation. Such methylation of *TLE1* in myeloid leukemias has been previously shown [14]. The low levels of TLE1 expression observed in HSCs (see Supplemental Fig. S1 at the online version at DOI: http://dx.doi.org/10.1016/j.leukres.2016.07.002) may needed to maintain high levels of Wnt signaling and repressed differentiation in this population. This study demonstrates that the relationship between TLE4, Wnt signaling, and downstream inflammation targets is integral to the role of TLE4 as a tumor suppressor in Kasumi-1 cells. The demonstration that COX inhibition is able to reduce the pro-leukemic phenotype due to T4KD points towards a potential therapeutic approach for myeloid leukemia.

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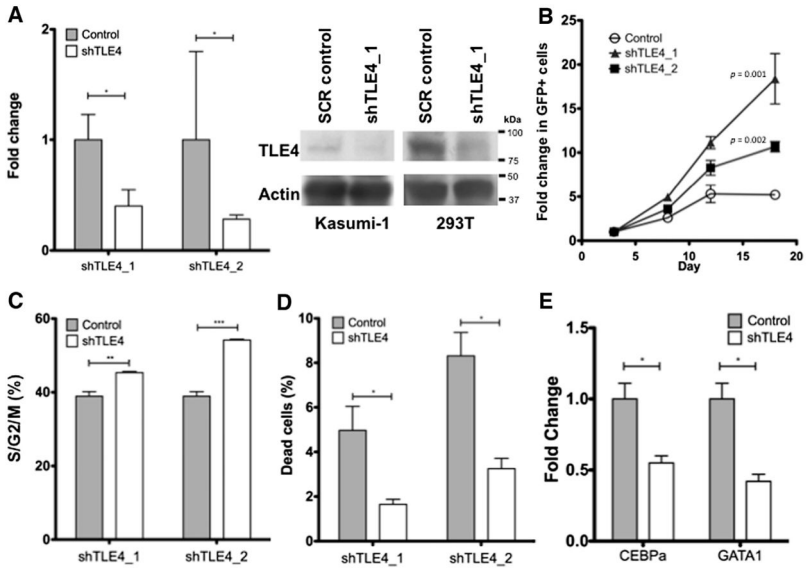


**Fig. 1.**

Kasumi-1 proliferation, apoptosis, and differentiation are sensitive to *TLE4* expression levels. (A) qRT-PCR using RNA from Kasumi-1 cells treated with two TLE4-specific shRNA confirms knockdown of *TLE4* message by at least 60% (n = 3 biologic triplicates with technical triplicates. *: $p < 0.05$). Western blot confirms over 75% decrease in TLE4 protein in Kasumi-1 and 293T cells treated with shTLE4 1. (B) Cell count of GFP+ Kasumi-1 cells treated with scramble control or two unique *TLE4*-specific shRNA coexpressed with IRES-GFP in lentivirus were tracked over 18 days. (C) Similarly treated cells were fixed and stained with for DAPI cell cycle or (D) stained for Annexin V analysis 7 days post-lentiviral infection. Dead population was defined as Annexin V+ and DAPI+ cells. (E) qRT-PCR query of two repressive targets of *AML1- ETO*, *CEBPb* and *GATA1*, reveal decreased levels of expression in *TLE4* knockdown Kasumi-1 cells. (All experiments carried out in biologic triplicates and repeated at least twice with technical triplicates. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

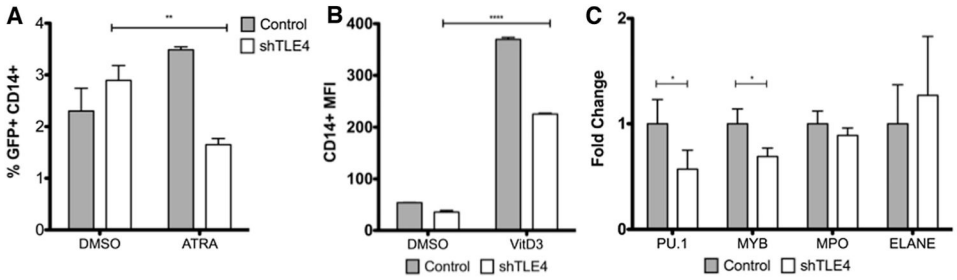


**Fig. 2.**

*TLE4*-mediated increases in inflammatory gene expression are concomitant with inhibition of pharmacologically induced myeloid differentiation in Kasumi-1 and HL60 cells. (A) Flow cytometry shows percentage of control and T4KD GFP+ Kasumi-1 cells that are CD14+ after culture in media supplemented with DMSO or 10 uM ATRA. (B) Flow cytometry analysis shows blunted induction of CD14+ populations in shTLE4-treated HL60 cells when cultured in media supplemented with 0.1 uM vitamin D3. (C) RNA harvested from shTLE4 or control shRNA treated HL60 cells were used in qRT-PCR seven days post- spinoculation to query for myeloid transcription factors. (All experiments carried out in biologic and technical triplicates. *: $p < 0.05$, ***: $p < 0.001$, ****: $p < 0.0001$).

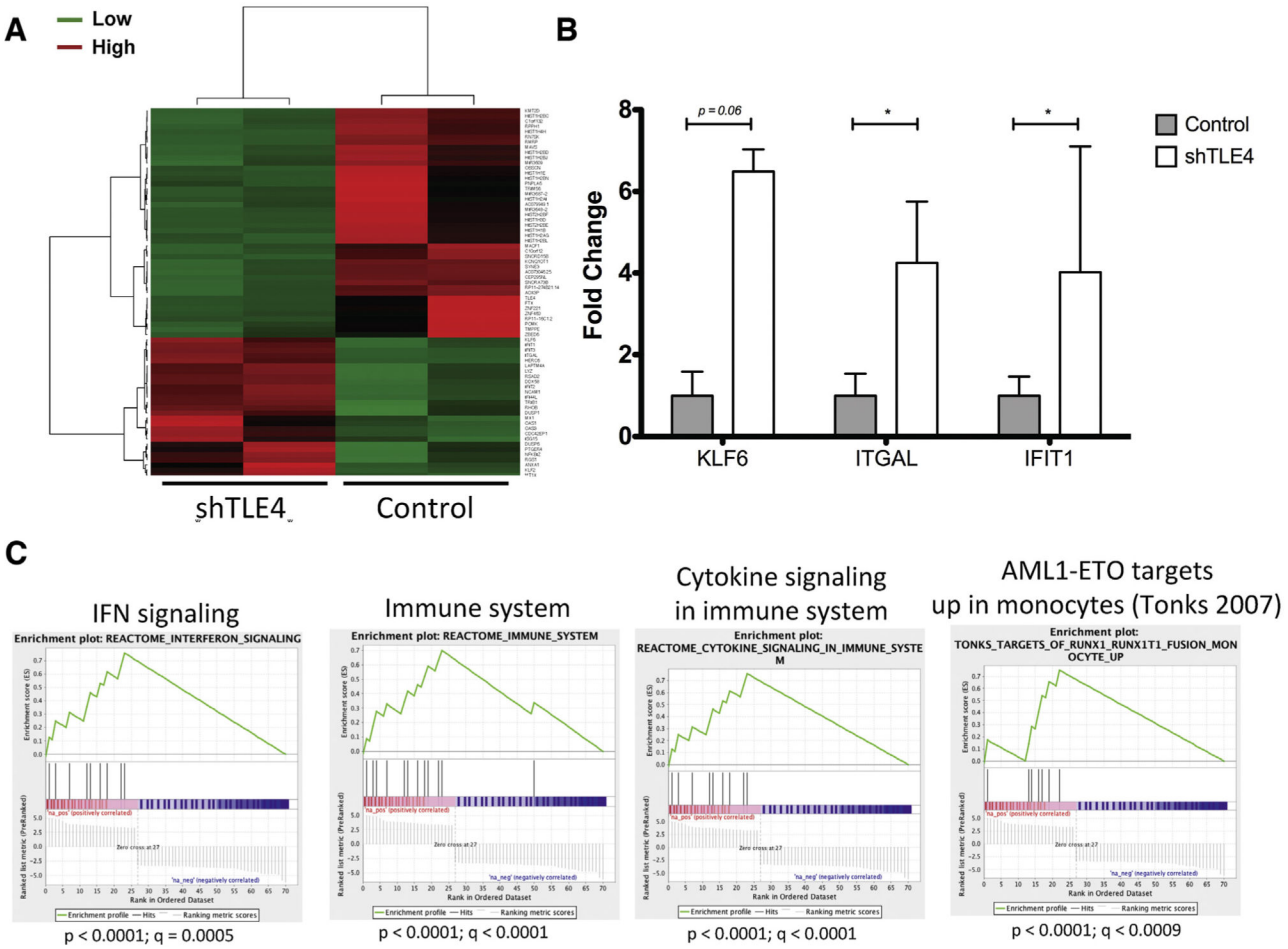


**Fig. 3.**

RNAseq identifies enrichment of immune system and inflammatory response pathways in genes upregulated in T4KD Kasumi-1 cells seven days post-spinoculation. (A) Heatmap summarizing differentially expressed genes between T4KD and control Kasumi-1 cells (n = 2 biologic replicates, filtered for FDR <0.2). (B) qRT-PCR verification of select upregulated genes with FDR <0.2 and log2 fold change >0.6 identified from RNAseq (n = 3 biologic replicates with technical triplicates, *: $p < 0.05$). (C) GSEA plots from analysis identifying pathways enriched in upregulated geneset (p and q values obtained from GSEA analysis).

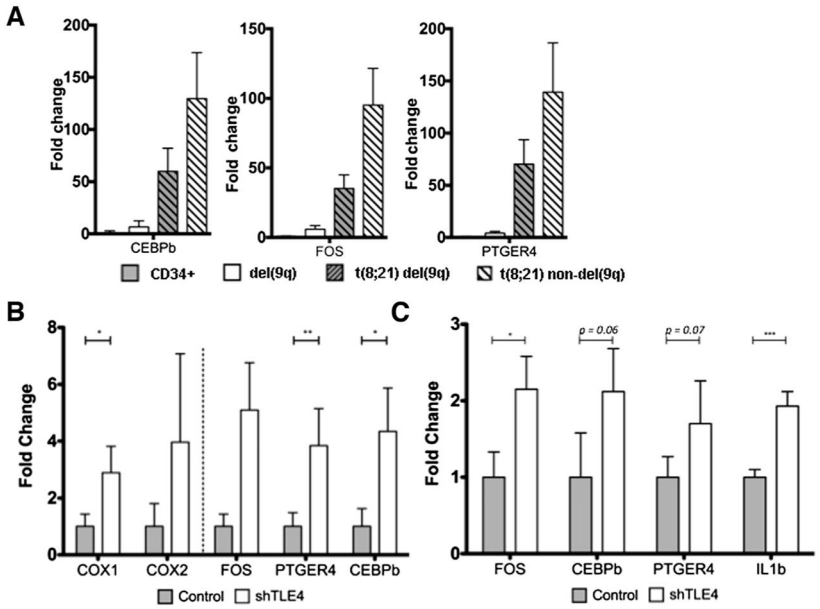


**Fig. 4.**

Elevated expression of inflammatory genes is characteristic of t(8;21) AML and is seen concomitant with increased Wnt signaling in AML cell lines with T4KD. (A) qRT-PCR query of *CEBPb*, *PTGER4*, and *FOS* using RNA harvested from primary human bone marrow samples reveal similar increases in inflammatory gene expression in del(9q) and t(8;21) del(9q) AML versus healthy CD34+ cells. qRT-PCR using RNA harvested from (B) Kasumi-1 cells and (C) HL60 cells seven days after lentiviral T4KD reveals increases in *COX1*, *COX2*, and inflammatory genes related to prostaglandin metabolism and downstream mediators. (A: n = 10–12 biologic replicates with technical triplicates per arm. B–C: n = 3 biologic replicates with technical triplicates. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

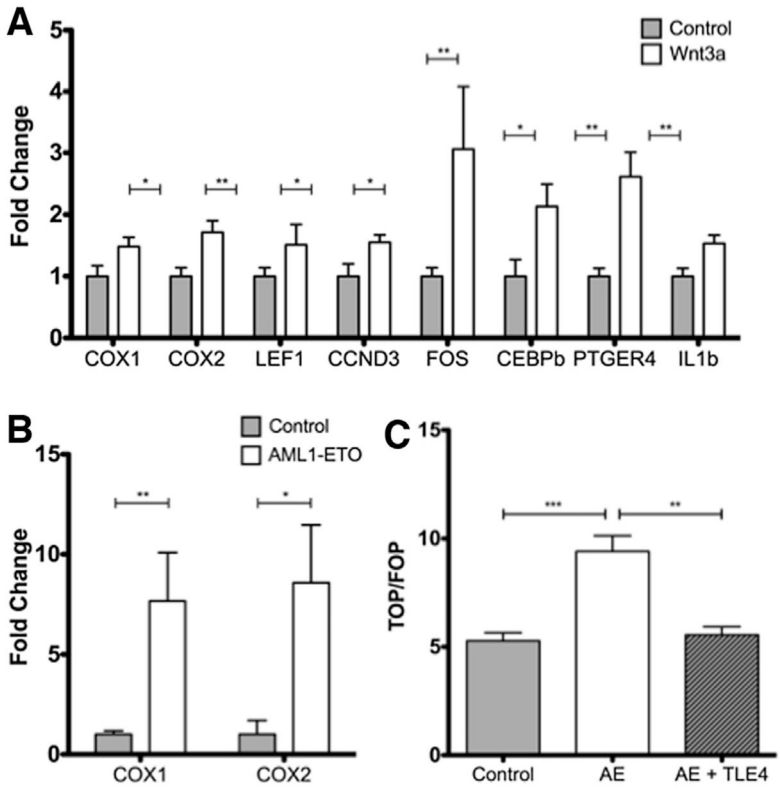


**Fig. 5.**

Wnt signaling in the context of AML1-ETO is sensitive to *TLE4* levels. (A) qRT-PCR with probes for inflammatory genes identified by RNAseq and other Wnt targets was performed using RNA harvested from Kasumi-1 cells cultured in media supplemented with either 10 nM recombinant human Wnt3a or DMSO. (B) qRT-PCR using RNA from AML1-ETO expressing 293T cells shows significant increases in COX1 and COX2 expression compared to control naive 293T cells (C) TOP/FOP ratios calculated from firefly and renilla luciferase activity of AML1 -ETO expressing 293T cells co-transfected with TOPFlash/FOPFlash. Presence of AML1-ETO increases Wnt signaling activity. Addition of TLE4 expression vector is able to abrogate AE-induced Wnt signaling, consistent with other studies confirming role of TLE4 as a regulator of Wnt signaling.

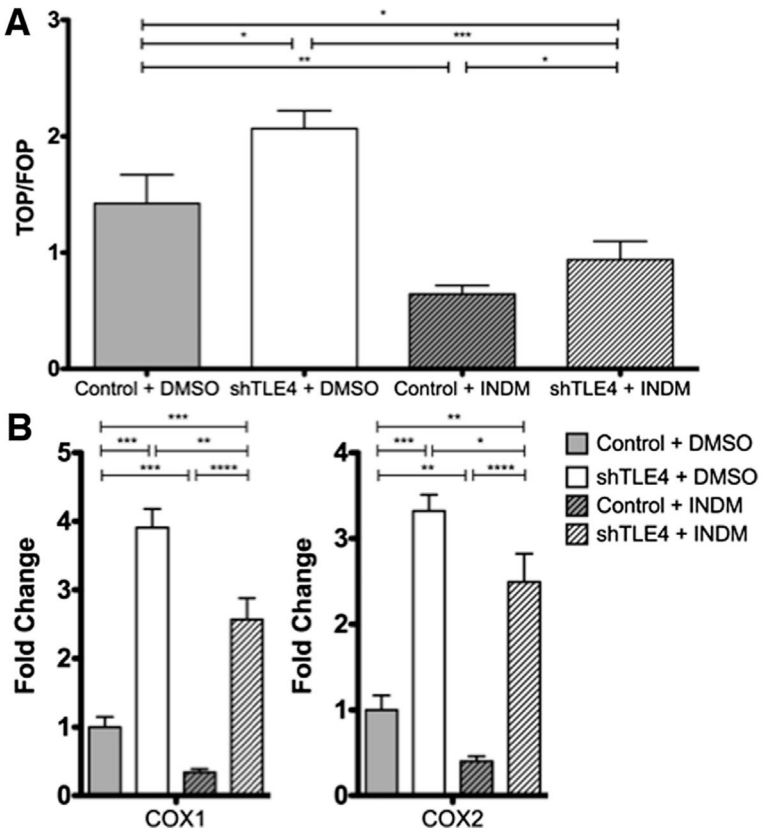


**Fig. 6.**

*COX* inhibitor INDM is able to modulate T4KD- dependent Wnt signaling in *AML1-ETO* expressing cells. (A) TOP/FOP ratios were calculated using fire-fly and renilla luciferase activity of 293T cells 48 h after nucleoporation with TOPFlash/FOPFlash reporter constructs, *AML1-ETO* expression vector, and either control or *TLE4*-specific shRNA. Assay reveals increased Wnt signaling activity in T4KD 293T cells compared to control, which is inhibited by addition of 50 uM indomethacin in culture media. (B) qRT-PCR analysis of cells used in (A) reveals significant increases in COX1 and COX2 expression due to T4KD and subsequent blunting with INDM treatment (All experiments carried out in biologic triplicates with technical triplicates for all experiments. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

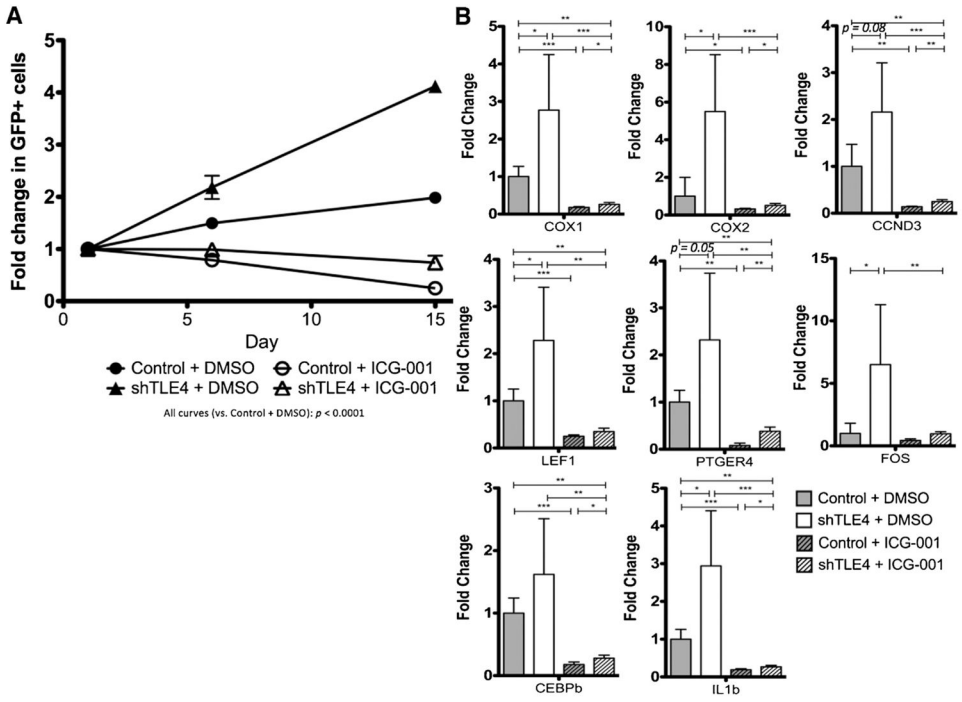


**Fig. 7.**

Wnt inhibitor ICG-001 is able to suppress T4KD-induced cell growth and expression of inflammatory genes and Wnt targets. (A) Kasumi-1 cells treated with lentiviral *TLE4*-specific or control shRNA delivery were tracked for fold change in GFP+ cells in the presence of either 10 nM ICG-001 or DMSO. While T4KD confers increased cell proliferation, ICG-001 is able to stunt growth of Kasumi-1 cells regardless of *TLE4* status (n = 3 biologic replicates; statistics shown for comparison of growth rates to Control + DMSO arm). (B) RNA from aforementioned cells was harvested at day 15 of culture for qRT-PCR. Assays reveal ICG-001 is able to block T4KD-induced increases in inflammatory and Wnt target gene expression. (n = 3 biologic triplicates with technical triplicates. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$).

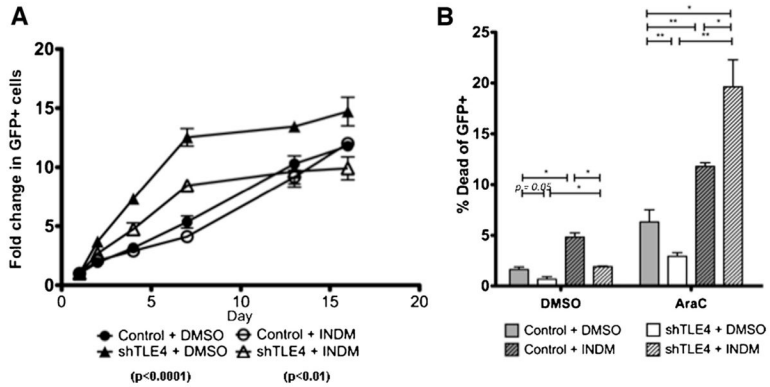


**Fig. 8.**

Indomethacin is able to reverse cell proliferation and drug resistance due to T4KD in Kasumi-1 cells. (A) T4KD and control Kasumi-1 cells were tracked for fold change in GFP+ cells in the presence of either 50 uM INDM or DMSO. Presence of INDM is able to stunt T4KD-induced cell growth (n = 3 biologic replicates; statistics shown for comparison of growth rates to Control + DMSO arm). (B) T4KD and control Kasumi-1 cells were cultured in DMSO, 50 uM INDM, 100 uM araC, or combination of 50 uM INDM and 100 uM araC. After seven days of treatment, cells were stained for Annexin V analysis and checked for dead GFP+ cell populations, which revealed INDM is able to reverse T4KD-induced resistance to araC treatment. Dead population was defined as Annexin V+ and DAPI+ cells. (n = 3 biologic replicates with technical triplicates. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

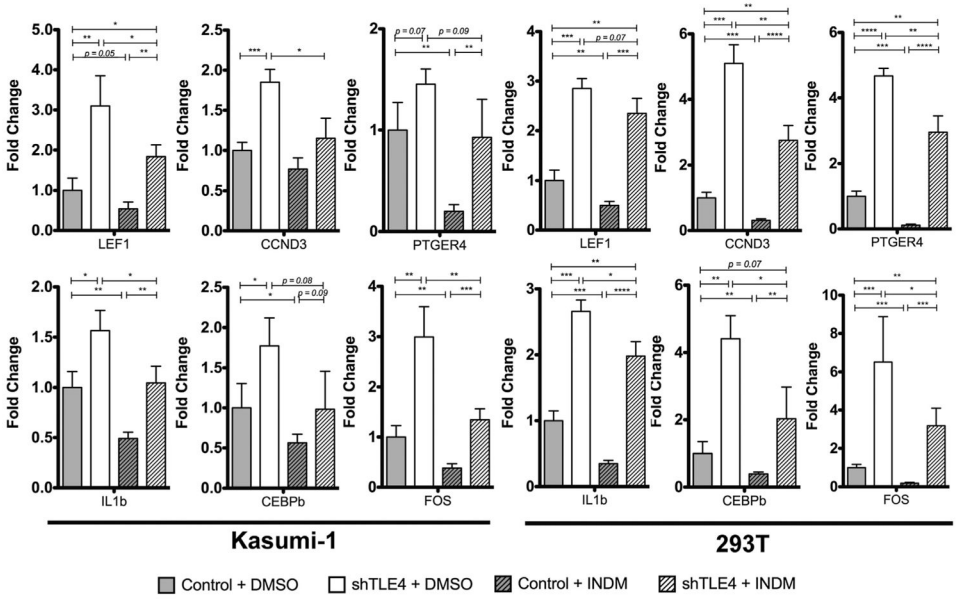


**Fig. 9.**

*COX* inhibition is able to partially reverse T4KD-mediated induction of inflammatory genes in Kasumi-1 and *AML1-ETO*-expressing 293T cells. qRT-PCR was performed using RNA harvested from either Kasumi-1 or *AML1-ETO*- expressing 293T cells cultured in media supplemented with either 50 uM indomethacin or DMSO. Query reaffirms induction of inflammatory genes with T4KD, which is abrogated in cells cultured in indomethacin. (n = 3 biologic replicates with technical triplicates. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

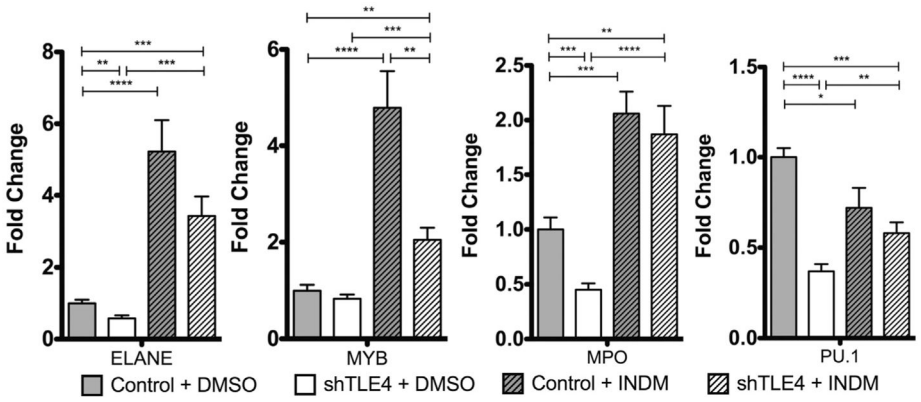


**Fig. 10.**

T4KD-induced suppression of myeloid transcription factors is reversible by COX inhibition. RNA for qRT-PCR was harvested from Kasumi-1 cells treated with 50 uM INDM or DMSO after lentiviral delivery of TLE4 or control shRNA. Assays reveal T4KD-induced suppression of myeloid transcription factors is relieved when cells were cultured in 50 uM INDM. (n = 3 biologic replicates with technical triplicates. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$).

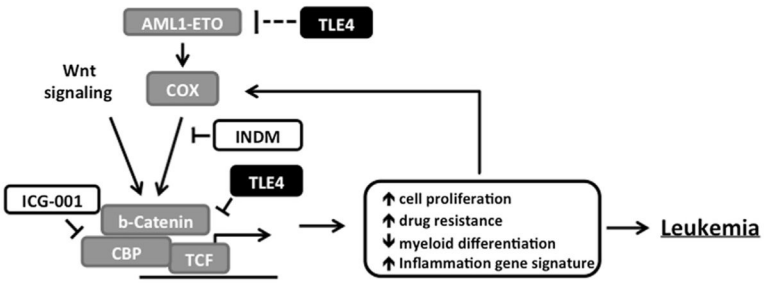


**Fig. 11.**

Schematic diagram summarizing proposed *TLE4* regulation of *AML1-ETO*/*COX*/Wnt axis. *TLE4* regulates AML1-ETO-mediated inflammatory signature by functioning as an "engine break"-like repressor of Wnt signaling. Blockade of the COX-Wnt signaling axis by Wnt inhibitor ICG-001 or COX inhibitor indomethacin is able to abrogate T4KD-induced pro-leukemic effects in t(8;21) leukemia cells. Direct interactions between TLE4 and AML1-ETO may provide an additional level of regulation.