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TBL1X: AT THE CROSSROADS OF TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL REGULATION

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Abstract

Over the past two decades, the adaptor protein transducin beta-like 1 (TBL1X) and its homologous TBL1XR1 have been shown to be upregulated in solid tumors and hematologic malignancies and their overexpression associated with poor clinical outcome. Moreover, dysregulation of the TBL1 family of proteins has been implicated as a key component of oncogenic pro-survival signaling, cancer progression, and metastasis. Herein, we discuss how TBL1X (and TBL1XR1) are required for the regulation of major transcriptional programs through the SMRT/NCOR/BCL6 complex, Wnt/β catenin and NF-kB signaling. We outline the utilization of tegavivint (Iterion Therapeutics), a first-in-class small molecule targeting the N terminus domain of TBL1, as a novel therapeutic strategy in preclinical models of cancer and clinically. While most of the published work focuses on TBL1X transcriptional role, we recently showed that in diffuse large B-cell lymphoma (DLBCL), the most common lymphoma subtype, genetic knockdown of TBL1X and treatment with tegavivint resulted in the decreased expression of critical (onco)-proteins in a post transcriptional/β catenin independent manner by promoting their proteasomal degradation through a Skp1/Cul1/F-Box (SCF)/TBL1X super-complex and potentially through the regulation of protein synthesis. However, given that TBL1X controls multiple oncogenic signaling pathways in cancer, treatment with tegavivint may ultimately results in drug resistance providing rationale for combination strategies. While many questions related to TBL1X function remain to be answered in lymphoma and other diseases, these data provide a growing body of evidence that TBL1X is a promising therapeutic target in oncology.

Keywords

TBL1X; adaptor protein; transcriptional regulation; protein stability

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The TBL1 Family of Proteins

Located on the X chromosome, the *TBL1X* gene was first identified in 1999 when its deletion was discovered to be associated with an X-linked human hearing defect, ocular albinism with sensorineural deafness (OASD) [1]. Additional TBL1 family members have since been identified and include TBL1X-related 1 (*TBL1XR1*) located on chromosome 3 and *TBL1Y*, the Y-linked homologue of *TBL1X* [2]. These genes encode for a family of highly evolutionarily conserved proteins sharing high structural and functional similarities from yeast to human. Sequence alignment studies demonstrate high (>80%) sequence similarity between the different TBL1 homologues [3]. Structurally, TBL1 proteins consist of an N-terminal Lis homology (LisH) domain (for hetero- and homodimerization and chromatin localization), an F-Box domain (for E3 ligase recruitment), and a C-terminal WD40 repeat domain (for protein-protein and protein-DNA interactions) [4].

TBL1 family members share a diverse set of binding partners, however the degree of affinity is often specific for each homologue [4-8]. In general, these protein-protein interactions occur in the context of large, multi-protein complexes, however, the mechanisms of complex formation and regulation are not fully delineated. The functions of TBL1X and TBL1XR1 are regulated by phosphorylation at Ser173, Thr334 and Ser420 for TBL1X and Ser123 and Ser199 for TBL1XR1 [4]. Additionally, SUMOylation by SUMO1 is also known to regulate the localization of TBL1X and TBL1XR1 [9]. In addition, SUMOylation regulates the complexing of TBL1X/TBL1XR1 with certain targets, including β-catenin (discussed below). Conversely, SUMO-specific protease I (SENP1) deSUMOylates TBL1X/TBL1XR1 resulting in complex destabilization [9].

TBL1X expression and correlation with oncogenesis and clinical outcomes

Immunohistochemical and transcriptome analyses reveal that TBL1X is expressed ubiquitously with low tissue specificity (https://www.proteinatlas.org/ENSG00000101849-TBL1X) [10]. TBL1X the relatively high RNA levels in the brain, endocrine tissues, and female and male reproductive tissues and lower levels in other tissues. Aberrant overexpression of the *TBL1X* gene and homologues has been correlated with more aggressive clinical behavior and poor prognosis in several cancers. In our recently published work, we showed that *TBL1X* overexpression correlates with poor survival in *de novo* diffuse large B cell lymphoma (DLBCL) patients treated with standard frontline immuno-chemotherapy assessed by standard immunohistochemistry (IHC) on an established tissue microarray (TMA) of 83 patient samples [11]. Specifically, we showed that the percentage of TBL1 positive lymphoma cells inversely correlate with progression free survival and overall survival regardless of DLBCL molecular subtype. Similar correlations between high *TBL1X* expression and poor prognosis have been shown in breast cancer, nasopharyngeal carcinoma (NPC), and pancreatic ductal adenocarcinoma [12-14]. These studies have also demonstrated that aberrant *TBL1X* expression is associated with tumor proliferation, migration, and invasion. For example, in breast cancer, TBL1X promotes epithelial-to-mesenchymal transition (EMT) by functioning as a cofactor to ZEB1 in the regulation of *CDH1* expression, thereby ultimately contributing to the metastasis of breast cancer [12]. In NPC, TBL1X acts cooperatively with Flot2 to promote cancer cell migration and invasion

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and thus metastasis [14]. Moreover, *TBL1XR1* expression has also been correlated to poor clinical outcomes in NPC, gastric cancer, serous epithelial ovarian cancer, lung squamous cell carcinoma, and colorectal cancer [15-21]

TBL1X-mediated regulation of selected transcriptional programs

Shortly following its discovery, TBL1X was shown to be a core component of the nuclear receptor co-repressor (NCoR) and silencing mediator for tetanoid and thyroid hormone receptors (SMRT) transcriptional repression complexes [6,8]. These large protein super complexes contain structural, chromatin-binding, and DNA/histone-modifying enzymes. Specifically, in addition to TBL1X, the NCoR/SMRT complex also includes TBL1XR1, HDAC3, GPS2, and actin-binding protein IR10 [6,8,22,23].

The BCL6 transcriptional repressor is also part of the SMRT/NCOR complex [6,8,22,23]. BCL6 is required for the formation of germinal centers (GC) during immune responses [24]. Specifically, the N-terminal BTB-POZ domain of BCL6 mediates homodimerization and recruitment of co-repressor molecules – SMRT, NCoR and BCoR – and collectively, this protein assemblage complexes with TBL1X, TBL1XR1, GPS2 and HDAC3 to promote HDAC3-mediated H3K9 acetylation [25-28]. This results in the silencing of genes required for exiting the GC reaction and for differentiation of GC B-cells to plasma cells (e.g. IRF4, PRDM1) [29]. BCL6 has also been shown to antagonize enhancer activation by p300 histone acetyltransferase through SMRT/HDAC3-dependent H3K27 de-acetylation, thus functioning as an on/off “switch” for enhancers [28]. This dynamic and reversible “toggling” of enhancers is speculated to permit physiologic recycling of B-cells for additional rounds of affinity maturation. Eventual downregulation of BCL6 in normal GC B-cells facilitates differentiation of B-cells to memory B-cells (MB) or plasma cells [25,29-32]. Deregulated expression of BCL6, on the other hand, drives malignant transformation of GC B-cells, promoting lymphomagenesis [33-35]. Inactivation of the BCL6-mediated transcriptional repression by targeting its BTB domain to disrupt activity with its corepressors has been demonstrated genetically (point mutation) and with specific peptides or small molecules [25,26,36-38], and BTB-corepressor interactions have been proven essential for survival of both malignant and normal B-cells [28].

Interestingly, mutations in TBL1XR1 have recently been shown to significantly impair differentiation of GC B-cells, thus driving lymphomagenesis [39]. Specifically, TB1XR1 mutations shift GC B-cells differentiation toward an abnormal immature MB cell phenotype by inducing preferential interaction of the SMRT/HDAC3 co-repressor complex with memory B-cell transcription factor BACH2; this shift occurs at the expense of the BCL6 resulting in impairment plasma cell differentiation and lymphomagenesis [39].

TBL1X/TBL1XR1 have also been demonstrated to possess co-activating functions in gene expression. Upon ligand-binding of RAR (retinoid acid receptor), estrogen receptor (ER), androgen receptor (AR), thyroid hormone receptor (THR), and peroxisome proliferator-activated receptor (PPAR), TBL1X/TBL1XR1 mediate the exchange of NCoR/SMRT for co-activators by recruiting the E3 ubiquitin-conjugating /19S proteasome machinery

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to degrade the co-repressors, thus controlling the transcriptional switch between gene repression and gene activation [4,31].

The canonical Wnt/β-catenin signaling pathways promotes cell proliferation, survival, and oncogenesis. TBL1X plays a key role in enhancing this signaling cascade by interacting with the transcription factor β-catenin at its N-terminus (residues 1-142) [7]. In the absence of Wnt signaling, cytoplasmic β-catenin undergoes Siah-1-mediated ubiquitination and subsequent proteasomal degradation [40,41]. Upon Wnt stimulation, on the other hand, SUMO1-mediated SUMOylation of TBL1X facilitates its translocation to the nucleus and direct binding to β-catenin, where this complex localizes to the promoters of target genes to promote the Wnt transcriptional program and oncogenesis [9]. Similarly, TBL1X is also required for nuclear factor κB (NF-κB) subunit p65 transcriptional activity. Upon tumor necrosis factor alpha (TNF-α) stimulation, TBL1X binds p65 facilitating its recruitment to the promoter regions of NF-κB targets [31,42] and thus regulating a wide range of cellular functions, including inflammation, tumor progression, and invasion [42].

Hepatic deficiency in TBL1X has been implicated in the development of hypertriglyceridemia, hepatocellular steatosis, and obesity-induced non-alcoholic fatty liver disease via upregulation of miR-367 [43-45]. Given the association between obesity, related metabolic dysfunction, and the pathogenesis of pancreatic ductal adenocarcinoma (PDAC) [46], further studies revealed that TBL1X is overexpressed in human PDAC cells that genetic inactivation of TBL1 both prevented and reversed pancreatic tumor growth via reduction of phosphatidylinositide 3 kinase (PI3K) signaling, glucose uptake, and glycolytic flux [13]. Specifically, this study showed that depletion of TBL1X in PDAC cells lead to transcriptional downregulation of the p110α catalytic subunit of PI3K, while transcription of canonical Wnt targets remained unchanged. These data suggest that TBL1X-driven tumorigenesis in PDAC stems from its role in transcriptional regulation of p100α, leading to enhanced oncogenic PI3K signaling.

Targeting TBL1X with tegavivint

Tegavivint (BC2059, Iterion Therapeutics) is a first-in-class small molecule targeting TBL1X's N-terminal domain, a region which contains a hydrophobic pocket that binds β-catenin, members of the NCoR complex, and the NF-κB subunit p65 [42,47,48]. When this pocket is occupied by tegavivint, TBL1X is unable to interact with some of its binding partners. For example, via disruption of the TBL1X-β-catenin interaction, treatment with tegavivint leads to degradation of β-catenin, consequently resulting in abrogation of the oncogenic Wnt-transcriptional program, cell cycle arrest, and tumor growth inhibition [49]. Activity of tegavivint has been demonstrated in preclinical models of multiple cancer types with active β-catenin signaling, including desmoid tumor, osteosarcoma, acute myeloid leukemia (AML), and multiple myeloma (MM) [9,50,51]. in these cancers, aberrant Wnt/β-catenin signaling develops as a result of germline mutations in the β-catenin gene (*CTNNB1*) or adenomatous polyposis coli (*APC*) gene [52-54]. APC is an essential component of a cytoplasmic protein complex that targets β-catenin for destruction; consequently, inactivating mutations in APC are associated with high levels of nuclear β-catenin and activation of the Wnt transcriptional program [55]. In preclinical models of chemoresistant

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osteosarcoma, tegavivint exhibited significant antiproliferative activity through attenuation of Wnt/β-catenin signaling and decreased activity of aldehyde dehydrogenase 1, an enzyme overexpressed in osteosarcoma cells that regulates tissue self-renewal, a feature of tumor stem cells [56]. Recently, tegavivint has also been shown to affect the tumor immune microenvironment in osteosarcoma by increasing numbers of CD8+ T cells and NK cells and enhancing checkpoint blockade in preclinical models of this disease [57].

By serving as transcriptional cofactors for β-catenin, the T-cell factor/lymphoid enhancer factor (TCF/LEF) family of nuclear DNA-binding proteins are the primary downstream effectors of Wnt signaling [58,59]. In both AML and MM, dysregulated Wnt signaling and impaired degradation of β-catenin lead to nuclear translocation of β-catenin and increased co-factor transcriptional activity with TCF4/LEF1. In preclinical models of these diseases, treatment with tegavivint disrupts the interaction between TBL1X and β-catenin [51,60]. This enhances the proteasomal degradation of β-catenin, attenuates TCF4/LEF1 transcription activity, and reduces expression of key target oncogenes, including *CCND1, c-MYC*, and *BIRC5*. Recently, it has been shown that tegavivint has significant activity in preclinical models of AML overexpressing EVI1, a transcription factor associated with poor prognosis in this disease through attenuation of the TBL1X-nuclear β-catenin-TCF4 axis resulting in reduced levels of EV1 level and other TCF4 targets [61]. In further studies in MM, tegavivint in combination with the pan-histone deacetylase inhibitor, panobinostat, has recently been shown to have significant activity in preclinical models of this disease by targeting aerobic glycolysis and mitochondrial respiration [62].

Clinically, in a recently completed Phase 1 clinical trial (NCT03459469), a total of 24 patients with progressive, unresectable desmoid tumors, where nuclear β-catenin is a critical oncogenic driver, were enrolled in six dose levels from 0.5 mg/kg to 5 mg/kg and treated on day 1, 8, and 15 of a 28 day schedule [63]. Tegavivint was overall well-tolerated. Treatment-related adverse events occurring in 20% of patients were mostly grade 1 and 2 (fatigue, nausea, headache, constipation, decreased appetite and dysgeusia), and there were no treatment-related Grade 4 or 5 adverse events or dose-limiting toxicities. Grade 3 TRAEs of hypophosphatemia, stomatitis, ALT increased, and headache occurred in 5 patients. One serious adverse event of an infusion site extravasation occurred. The maximum tolerated dose was not determined, and the recommended phase 2 dose (RP2D) was declared at 5 mg/kg based on pharmacologically relevant plasma concentrations and preliminary efficacy. Four objective responses were observed according to WHO/RECIST criteria (17% across all dose levels and 25% at the RP2D) with a median duration of response of 8.1 months with all responses ongoing at data cut-off. Nine months progression-free survival rate was 76%.

Two additional clinical trials with single agent tegavivint are ongoing: the first one is a Phase 1 study in patients with relapsed/refractory acute myeloid leukemia (NCT04874480). The second is a Phase 1/2 trial for recurrent or relapsed solid tumors including lymphomas and desmoid tumors (NCT04851119). A Phase 1b study in combination with osimertinib, an irreversible epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, for metastatic EGFR-mutant non-small cell lung cancer (NCT04780568) has been approved but it is now recruiting according to clinicaltrial.gov.

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TBL1X modulates protein stability in diffuse large B-cell lymphoma (DLBCL)

Diffuse large B cell lymphoma (DLBCL) is the most common non-Hodgkin's lymphoma (NHL) subtype and is characterized by remarkable heterogeneity with diverse variants that can be identified histologically and molecularly [64-67]. Large-scale gene expression profiling studies have identified the germinal center B-cell (GCB) and activated B cell (ABC) subtypes [65]. Chemoimmunotherapy with R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) remains the standard front-line therapy, curing approximately 40% of patients with ABC-DLBCL, which is associated with worse outcome compared to GCB-DLBCL [66]. GCB- and ABC-DLBCL subtypes are characterized by different genetic lesions, which result in distinct constitutively activated oncogenic pathways.

GCB-DLBCL oncogenesis is highly variable, with approximately 45% of cases having a t(14;18) translocation, resulting in juxtaposition of the *BCL2* gene and *IgH* locus [68]. *MYC* encodes for a global transcription factor with profound influence on many different cellular functions such as proliferation, metabolism, DNA replication, and protein synthesis[69,70]. Similar to *BCL2, MYC* can also be translocated under the control of the immunoglobulin promoter, which occurs in 5-15% of DLBCL cases[71-73]. In a smaller number of cases, *MYC* rearrangements may involve a non-IG partner such as *BCL6, BCL11A*, or *PAX5*. The presence of *MYC, BCL2*, (+/− *BCL6*) define double hit or triple hit lymphomas (high grade B-cell lymphoma). Patients with DHL and THL have a significant worse prognosis compared to the c-Myc negative counterpart following standard front-line chemotherapy[71-75]. In the absence of translocations, protein expression assessed by immunohistochemistry (IHC) of both c-Myc (>40%) and Bcl2 (>50%) define double-expressor DLBCL (DEL), which are significantly more common than DHL/THL, accounting for ~25% of DLBCL patients[75]. While other cases of GCB-DLBCL are characterized by amplification or gain in copy number of *MDM2*, encoding a negative regulator of tumor suppressor p53, and others by deletions in other tumor suppressor proteins, namely p73 or ING1 [68]. Translocations involving BCL2, MYC and mutations of EZH2 are almost exclusively seen in GCB-DLBCL [65,76]. In addition, up to 55% of GCB-DLBCL are characterized by loss of the tumor suppressor phosphatase and tensin homolog (PTEN), the major negative regulator of PI3K/AKT signaling [77].

ABC-DLBCL is characterized by constitutive activation of multiple oncogenic signaling pathways resulting from structural alterations disrupting terminal B-cell differentiation (*PRDM1*) and mutations of *TNFAIP3, CARD11, CD79A/B* and *MYD88*[78-81]. These changes ultimately result in the constitutive activation of NF-κB, B-cell receptor, and/or PI3K/AKT signaling pathways, which are responsible for the inferior clinical outcomes, as compared to GCB-DLBCL [78,82].

Previous work has shown that Wnt signaling is variably activated in DLBCL, and that β-catenin expression and nuclear localization are inconsistently increased in this disease [83,84]. Targeting Wnt-signaling has been shown to suppress growth of some DLBCL subtypes *In vitro* [85-87]. Generally, the importance of and potential for targeting this pathway in

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DLBCL is unclear. In our investigations, we found that unlike normal human immune cells, DLBCL cells express abundant levels of TBL1X, and its overexpression has prognostic significance, regardless of DLBCL molecular subtype [11]. We showed that genetic deletion of TBL1X or pharmacological targeting with tegavivint induces significant DLBCL cell death *In vitro* and *In vivo*. We further showed that in DLBCL, TBL1X interacts with a Skp1/Cul1/F-Box (SCF) super-complex, which controls the proteasome-mediated degradation of critical onco-proteins such as c-Myc, PLK1, and the autophagy regulatory protein, Beclin-1. While tegavivint was initially developed as an inhibitor of the TBL1X/β-catenin interaction, our data showed that genetic deletion of TBL1X and treatment with tegavivint reduces c-Myc expression in a post-transcriptional/β-catenin independent manner. We demonstrated that tegavivint induces conformational changes in the SCF complex, resulting in dissociation of CAND1 (an SCF inhibitor) [88] from CUL1 and subsequent enhanced degradation of SCF targets. Collectively, these data highlighted the role of TBL1 in controlling critical (onco)-proteins stability rather than their transcription and established the rationale for targeting TBL1X as a novel therapeutic strategy in DLBCL, particularly in patients with c-Myc-driven disease.

New Perspectives

In both the GCB and ABC subtypes of DLBCL, PI3K/mTOR signaling supports tumor cell proliferation and growth [68,89,90]. The $SCF^{\beta TrCP}$ complex, which interacts with TBL1X in lymphoma [11], also modulates the stability of proteins regulating mTOR signaling, including the Ras homolog enriched in brain (Rheb), an upstream direct activator of mTORC1 [91]. Interestingly, in GCB and ABC DLBCL cell lines, shRNA-mediated TBL1X knock-down (Fig.1A) and treatment with tegavivint (Fig.1B) resulted in decreased Rheb protein expression without significant decrease in Rheb transcript levels (Fig.1C), confirming that TBL1X is involved in controlling Rheb protein turn-over rather than its transcription. Co-treatment with the translational inhibitor cycloheximide (CHX) and tegavivint significantly shortened the half-life of Rheb compared to CHX alone (Fig1.D), while adding the proteasomal inhibitor MG132 to tegavivint efficiently rescued Rheb level (Fig.1D). Furthermore, treatment with tegavivint did not significantly affect the levels of mTORC1 components, mTOR and Raptor (Fig.1E). Immunoblot analysis of all tested cell lines after a 12 hour incubation with tegavivint showed evidence of decreased mTORC1 activity, as measured by decreased levels of phosphorylated p70S6 kinase (p70S6K Thr389) and eukaryotic initiation factor 4E binding protein 1 (p4E-BP1 Ser65) (Fig.1F)[92]. We hypothesize that tegavivint functions as a selective mTORC1 inhibitor, as treatment of DLBCL cells induces compensatory hyperphosphorylation of AKT at its serine 473 residue, followed by downstream inactivating phosphorylations of GSK-3β (Ser9) and FOXO1 (Ser256) (Fig.2A and Suppl. Fig.1)[93].

High levels of phosphorylated AKT are associated with poor prognosis in DLBCL, and several studies support the PI3K/AKT pathway as a therapeutic target in DLBCL [94-96]. The oral, ATP-competitive pan-AKT inhibitor AZD-5363 (capivasertib) has been shown to have significant activity in preclinical models of DLBCL and is currently in several clinical trials, including a trial for advanced solid tumors and lymphoma (NCT02465060) [97-101]. The combination of tegavivint and AZD-5363 resulted in synergistic killing of PTEN-deficient

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DLBCL cells, while additive cell death was seen in PTEN-wild type DLBCL cells (Fig.2B). Moreover, similar results were obtained with other AKT inhibitors, MK2206 and ipatasertib, in combination with tegavivint (not shown). These data suggest that co-targeting TBL1 and AKT may represent a promising novel combination strategy in AKT-addicted DLBCL.

Conclusion

As outlined here, there is a growing body of data demonstrating that TBL1X is overexpressed in several types of cancer and that its upregulation is associated with disease progression and poor prognosis. Moreover, this adapter protein is being established as a critical regulator of multiple oncogenic signaling networks, playing key roles in both transcriptional and post-transcriptional regulation of crucial mediators of pro-survival signaling. Together these data support further investigations aimed at targeting TBL1X as a novel therapeutic strategy in oncology.

While our published data demonstrated that genetic knockdown of TBL1X and treatment with tegavivint leads to significantly enhanced survival in preclinical models of DLBCL, cure is not observed. Although we have demonstrated the central role of TBL1X in modulating the stability of key oncoprotein such as c-Myc, PLK1 and Beclin-1 through the SCF complex, there are other potential SCF targets that may be affected by tegavivint. For example, as shown in Fig.1, the TBL1X-binding SCF complex ($SCF^{\beta TrCP}$) regulates the turnover of Rheb, a potent activator of the mTOR signaling pathway [103,104]; resulting in inhibition of this pathway.

Two important considerations arise from this: 1) while our data support the hypothesis that TBL1X is involved in controlling protein turn-over through the ubiquitin-proteasome system, our data on mTORC1 signaling additionally suggest that tegavivint may also affect targeted protein levels through inhibition of translation. 2) These data highlight the complexity of oncogenic signaling pathways regulated by TBL1X and suggest that treatment with tegavivint may induce compensatory mechanisms ultimately leading to drug resistance. For example, while additional work is needed, our data suggest that tegavivint functions as a selective mTORC1 inhibitor, evinced by decreased phosphorylation of downstream S6K and 4E-BP1. In turn, this may result in inhibition of 5’ cap-dependent mRNA translation and compensatory hyperphosphorylation of AKT at the serine 473 residue via mTORC2 [92], thus providing us rationale for co-targeting of TBL1X and PI3K/AKT/mTOR signaling in this disease. When combined with an AKT inhibitor, tegavivint treatment resulted in enhanced cell killing in PTEN-deficient DLBCL cell lines, demonstrating an approach to maximizing its therapeutic potential in selected patients.

While we have characterized the role of TBL1X in controlling oncoprotein stability through the SCF complex in DLBCL [11], its transcriptional role is yet to be characterized in this disease. This is particularly important in DLBCL, where TBL1X is part of a SMRT/NCoR complex which contains BCL6, a repressive transcription factor that serves as a scaffold for recruiting chromatin-modifying complexes. It is well established that deregulation of BCL6 plays a pathogenetic role in a subset of DLBCL [105]. In addition, the histone deacetylase, HDAC3, which deacetylates H3K27 and regulates enhancer-dependent promoter activation,

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is also part of this complex, emphasizing the genome-wide transcriptional impact that these complexes possess. It is also likely that TBL1X may play a BCL6-independent transcriptional role in DLBCL. For example, survivin is a member of the inhibitor of apoptosis family of proteins [106], and its high expression inversely correlates with 5-year overall survival in DLBCL patients treated with frontline anthracycline-based chemoimmunotherapy [107]. In addition, YM155, a small molecule inhibitor of survivin, has shown significant activity in preclinical models of DLBCL. Interestingly, our published and unpublished data show that, in addition to the decrease in *BIRC5* transcript and survivin protein levels, proteasomal inhibition partially restored survivin levels in the context of both tegavivint treatment as well as TBL1X knock-down (Suppl. Fig.2A and B). These results suggest a model in which TBL1X regulates survivin expression transcriptionally as well as in a post-transcriptional manner.

In sum, while targeting TBL1X with tegavivint alone and in combination represents an effective therapeutic strategy in DLBCL, many exciting questions remain related to TBL1X function in lymphoma and other diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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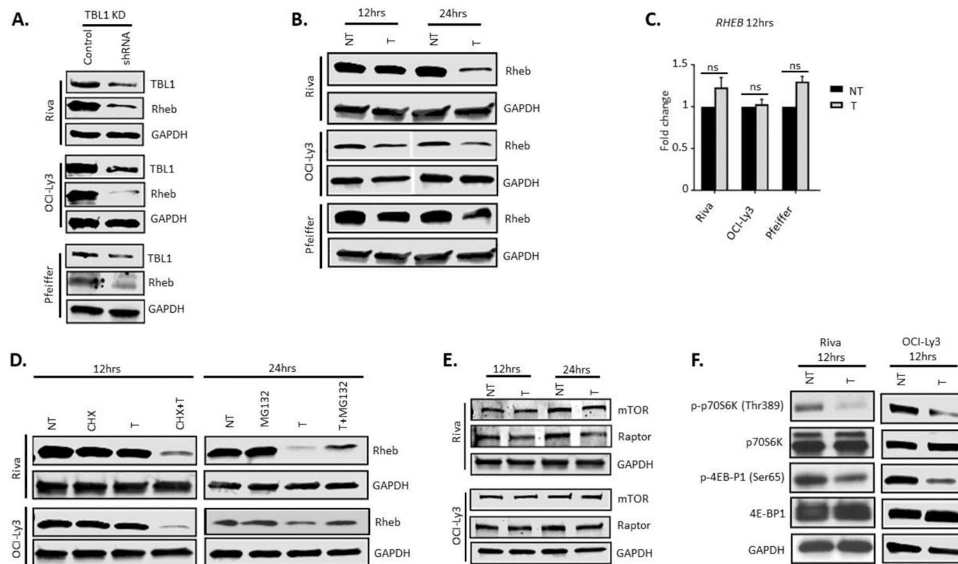

Figure 1. TBL1X regulates Rheb turnover in DLBCL.

Immunoblots showing Rheb protein levels in DLBCL cell lines (ABC-DLBCL cell lines: Riva and OCI-Ly3; GCB-DLBCL: Pfeiffer) following **A.** shRNA knockdown of TBL1 and **B.** incubation with tegavivint (T) or vehicle control (DMSO, NT) for 12-24hrs. For this experiment and the following, tegavivint was dosed at each cell lines' 24hour IC50 (Riva and Pfeiffer: 70nM, OCI-Ly3: 50nM) according to our previous publication[11]. **C.** *RHEB* mRNA transcript levels following 12hrs incubation with tegavivint. **D**. Immunoblots showing Rheb levels following treatment with either the translation inhibitor cycloheximide (CHX), tegavivint (T) or the combination (CHX+T) for 12hrs, and after treatment with either DMSO control (NT), tegavivint (T), the proteasome inhibitor MG132 or the combination (T+MG132) for 24 hours. CHX (70 mg/mL) was added for 1 hour and then washed before adding tegavivint. MG132 (Riva and OCI-Ly3: 0.5uM and Pfeiffer: 0.3uM) was added 6 hours after tegavivint treatment was started. Immunoblots showing protein levels of **E.** mTORC1 components, mTOR and Raptor, and **F.** mTOR targets, p70 S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1) in DLBCL cell lines following treatment with tegavivint (T).

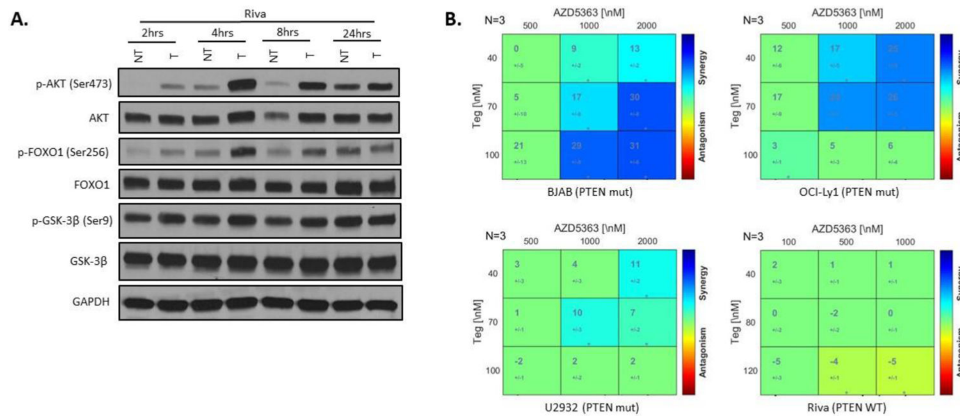

Figure 2. Tegavivint activates AKT signaling and the combination of tegavivint with AZD-5363 results in synergistic cell killing in PTEN-deficient DLBCL cell lines.

A. Immunoblots showing AKT protein levels, its phosphorylation status at the serine 473 residue, and its downstream targets, FOXO1 and GSK-3β, in an ABC DLBCL cell line following treatment with tegavivint (T) or vehicle control (DMSO, NT) for the indicated timepoints. **B.** Isobologram analysis and synergism determination by Loewe additivity synergy modelled using Combenefit[102]. Matrices display calculated Loewe additivity scores ± SD from dose concentration combinations of tegavivint (Teg) and AZD-5363 in PTEN mutant (mut) (BJAB, OCI-Ly1, and U2932) and PTEN wildtype (WT) DLBCL cell lines (Riva). Matrices are color-coded; blue indicates synergistic cell killing, red antagonism, and green represents dose combinations for which additive effect was found. Following 48 hours incubation with indicated concentrations AZD-5363, tegavivint was added for an additional 24 hours incubation. Viability was determined by annexin V/propidium staining and flow cytometry. Experiments were performed in triplicate.

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