# A PRECLINICAL ASSAY PIPELINE TO IDENTIFY AND OPTIMIZE TBXT TARGETED THERAPIES IN CHORDOMA AND OTHER TUMORS

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## ABSTRACT


- Chordoma is a rare bone cancer of the skull base and axial spine that arises from remnants of the embryonic notochord.
- Disease incidence is 1 per million, with median survival from diagnosis of 8 years.
- There are no approved systemic therapies for the treatment of chordoma. Standard care is maximal surgical resection +/- radiation, which cures ~30% of patients.
- A nearly universal hallmark of chordoma is the expression of *TBXT*, which encodes the T-box transcription factor Brachyury (TBXT).
- TBXT is a key driver and genetic dependency of chordoma.
- TBXT has been associated with tumor progression, chemotherapy resistance, and poor patient outcomes in other cancers including lung, colon, breast, and prostate.
- The objectives of this study are to establish a preclinical assay pipeline that enables the identification and optimization of TBXT therapies and assess the role of TBXT in the progression and response to standard of care therapy of other solid tumors.


## BACKGROUND

- TBXT expression is the diagnostic hallmark of chordoma.
- Functional genomics identified *TBXT* as the most selectively essential gene in chordoma[1].
- The Structural Genomics Consortium (SGC) recently solved the crystal structure of TBXT DNA-binding domain in complex with DNA, revealing new binding pockets.
- Multiple structure-guided, open-source small molecule series bind to distinct TBXT pockets with low micromolar affinity[2].

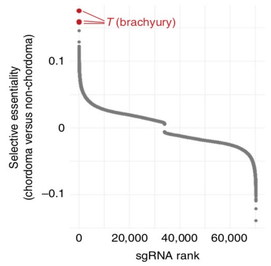


**Figure 1.** TBXT promotes chordoma cell proliferation. A genome-wide CRISPR screen identified *TBXT* (*T*, Brachyury) as the most selectively essential gene in chordoma[1].

### Crystal structure of TBXT bound to DNA identified new binding pockets

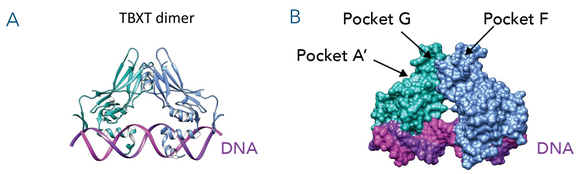


**Figure 2.** Structure of the TBXT DNA-binding domain (DBD) bound to DNA[3]. (A) Ribbon diagram of the TBXT DBD dimer in contact with DNA. (B) Surface rendering of the DBD shows three shallow pockets that are targeted by the open-source small molecules.

## OPPORTUNITIES FOR DRUGGING TBXT

### Leveraging AI for TBXT Drug Development

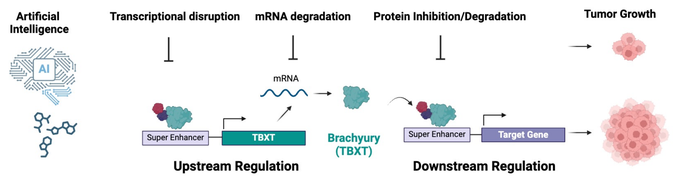


**Figure 3.** Targeting TBXT with different therapeutic modalities. Leveraging the power of emerging AI, we aim to target *TBXT* transcription, translation, or protein function using antisense oligonucleotides, rSMs, functional inhibitors and/or degraders to repress TBXT function and inhibit tumor growth.

## BUILDING A PRECLINICAL ASSAY PIPELINE

### Purified full-length TBXT binds to the TBXT response element in vitro

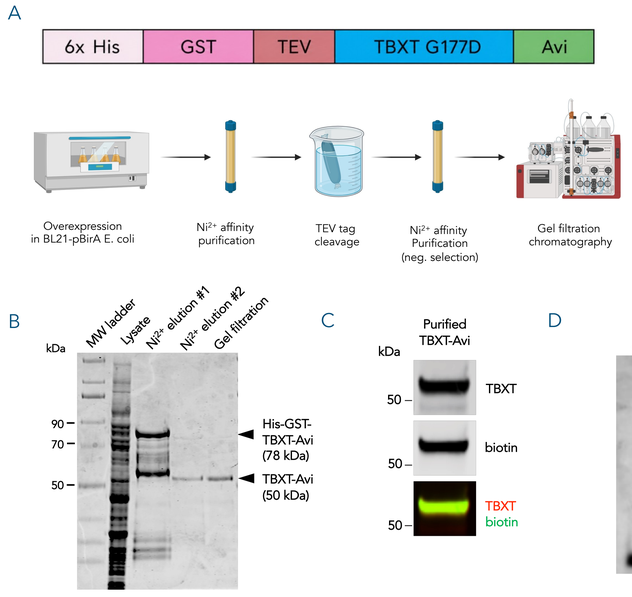


**Figure 4. Workflow for TBXT purification and functional assessment by a DNA-binding assay.** (A) Schematic of recombinant TBXT expression in *E.coli* and purification by nickel-affinity and size-exclusion chromatography. (B) Coomassie-stained protein gel analyzing different steps of the purification process. (C) Purified TBXT-Avi was immunoblotted for TBXT and probed with Streptavidin conjugated to an infrared dye. (D) Increasing concentrations of purified TBXT were incubated with 5 nM of annealed DNA oligos containing the TBXT response element, resolved by gel electrophoresis under native conditions, and stained with SYBR green.

### High-throughput SPR identifies small molecule hits and measures steady-state affinity

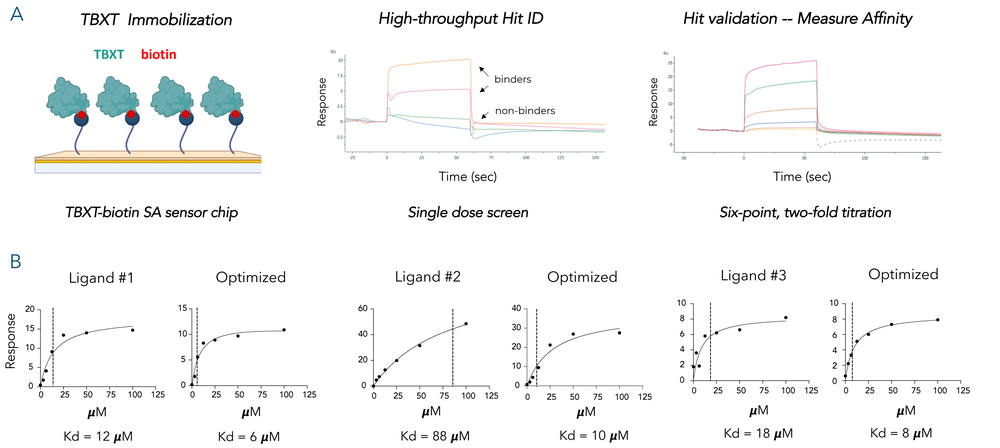


**Figure 5.** High-throughput TBXT SPR and the initial optimization of TBXT ligands. (A) Biotinylated TBXT is immobilized on a streptavidin-coated sensor chip in a Biacore 8K SPR system. High-throughput Hit Identification using a single dose can distinguish between binders and non-binders. Hits are validated using a six-point, two-fold titration to measure their steady-state affinity. (B) Three TBXT ligands were optimized by a single round of medicinal chemistry and show higher TBXT affinity. Binding curves show ligand concentration vs response.

### Development and validation of a TBXT reporter assay

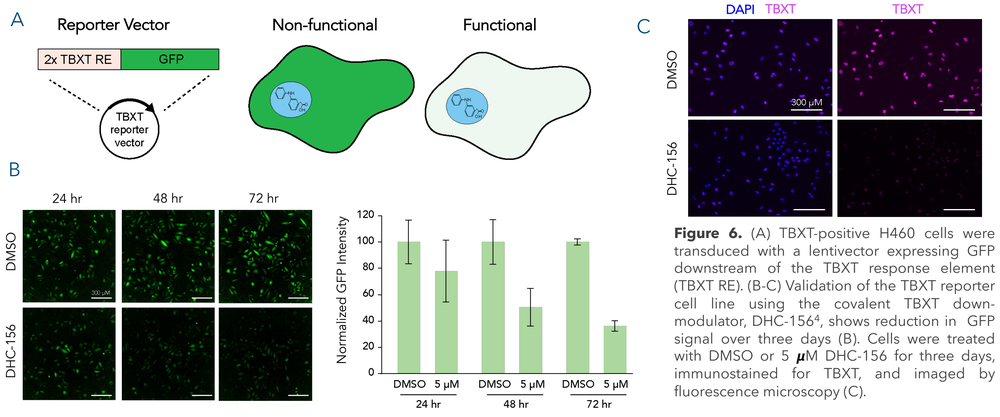


**Figure 6.** (A) TBXT-positive H460 cells were transduced with a lentivector expressing GFP downstream of the TBXT response element (TBXT RE). (B-C) Validation of the TBXT reporter cell line using the covalent TBXT down-modulator, DHC-156[4], shows reduction in GFP signal over three days (B). Cells were treated with DMSO or 5 μM DHC-156 for three days, immunostained for TBXT, and imaged by fluorescence microscopy (C).

## BUILDING A PRECLINICAL ASSAY PIPELINE

### *TBXT* knockdown reduces the expression of its target genes

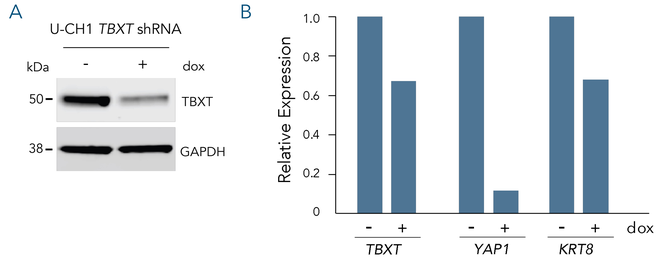


**Figure 7.** TBXT knockdown in U-CH1 cells reduces the expression of its gene targets. (A) Whole cell lysates from doxycycline-inducible U-CH1 *TBXT* shRNA cells treated with or without doxycycline for six days and immunoblotted for TBXT and GAPDH. (C) U-CH1 *TBXT* shRNA cells were treated with or without doxycycline for six days and the expression of its targets *TBXT*, *YAP1*, and *KRT8* were assessed by RT-PCR.

### Assessing the role of TBXT in other solid tumors

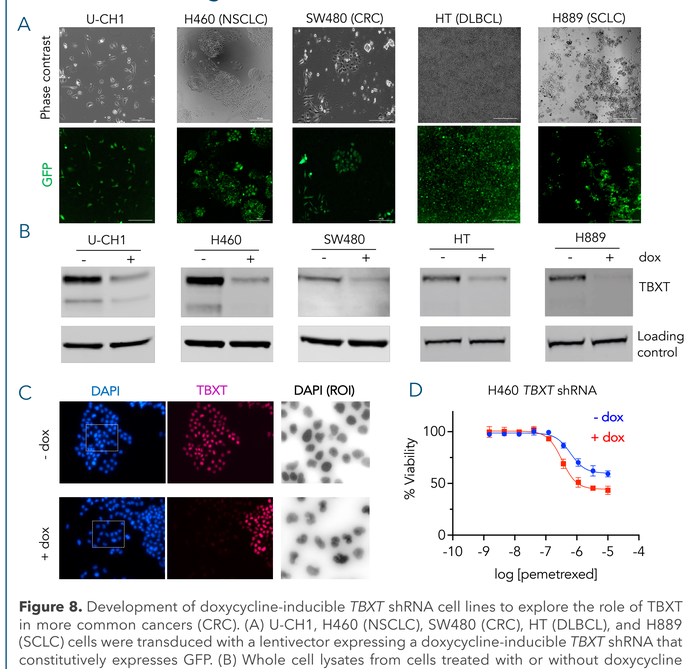


**Figure 8.** Development of doxycycline-inducible *TBXT* shRNA cell lines to explore the role of TBXT in more common cancers (CRC). (A) U-CH1, H460 (NSCLC), SW480 (CRC), HT (DLBCL), and H889 (SCLC) cells were transduced with a lentivector expressing a doxycycline-inducible *TBXT* shRNA that constitutively expresses GFP. (B) Whole cell lysates from cells treated with or without doxycycline were immunoblotted for TBXT and GAPDH or KU80. (C) TBXT knockdown in H460 cells alters nuclear morphology. H460 *TBXT* shRNA cells were cultured with or without doxycycline for four days, fixed and immunostained for TBXT and DAPI, and imaged by fluorescence microscopy. (D) H460 *TBXT* shRNA cells were cultured with or without doxycycline for seven days and treated with a titration of pemetrexed for three days. Cell viability was measured with CellTiter-Glo.

## KEY FINDINGS

- Small-molecule TBXT ligands have been discovered and are being optimized for therapeutic development.
- The CF labs biochemical and cell-based assay capabilities catalyze TBXT-targeting drug discovery for chordoma and other TBXT-positive tumors.
- These capabilities are supporting the TBXT Challenge (tbxtchallenge.org), a global prize competition designed to identify and advance small-molecule binders targeting TBXT.
- We are also developing TBXT-HiBit and other assays to enable the discovery of TBXT degraders.
- Preliminary data suggest that TBXT inhibition may sensitize non-chordoma tumor cells to standard-of-care therapy. Ongoing studies will elucidate TBXT's role in tumor growth and resistance to therapy.

## REFERENCES


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