Multi-Omics Characterization of PTEN in Chordoma

By

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Preface and Acknowledgments

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Abstract *Multi-Omics Characterization of PTEN in Chordoma*

by Golara Malaki, ScM candidate, Brown University, May 2026.

Background: Derived from remnant notochordal tissue, chordoma is a rare, slow-growing tumor. With the standard of care being resection and radiation, there are limited treatment options, and many patients have recurrent chordoma. Many patients with chordoma have reduced expression or loss of PTEN [Phosphatase and Tensin Homolog], a tumor suppressor that counteracts the activity of PI3K [PI3 Kinase], inactivating the signaling lipid PIP3 [Phosphatidylinositol (3,4,5)-trisphosphate] by dephosphorylating it to PIP2 [Phosphatidylinositol 4,5-bisphosphate], acting as a negative regulator of the PI3K-Akt pathway, which is one of the most mutated pathways in cancer. However, the expression status of PTEN is not yet known across chordoma cell line models and archival tissue samples from Brown's biobank. **Methods:** We assessed PTEN mRNA expression using complementary assays: RNAscope (spatial ISH) and NanoString (probe-based counting) in archival FFPE chordoma tissue, and by RT-qPCR (amplification-based quantification) in cell line models. In addition, we used differential expression analysis techniques on publicly available RNA-seq datasets. **Results:** We found a trend of greater PTEN expression in primary chordoma relative to recurrent in both patient samples and cell lines that showed high variability. These findings suggest that the loss of PTEN expression is not universal in all chordoma cell models. **Conclusions:** Suggesting heterogeneity of PTEN expression within and between chordoma tumors, next is to assess possible mechanisms of reduced PTEN expression. Our current studies include (1) Sanger sequencing to evaluate disrupted coding of PTEN exons that are important regulatory sites and (2) RNA-seq data analysis within our own datasets to determine the role of non-coding RNA in PTEN regulation. Ultimately, these approaches will support understanding of whether epigenetic

mechanisms contribute to the reduced expression of PTEN, with the regulation of PTEN as a potential therapeutic target for chordoma.

Chapter 1: Introduction

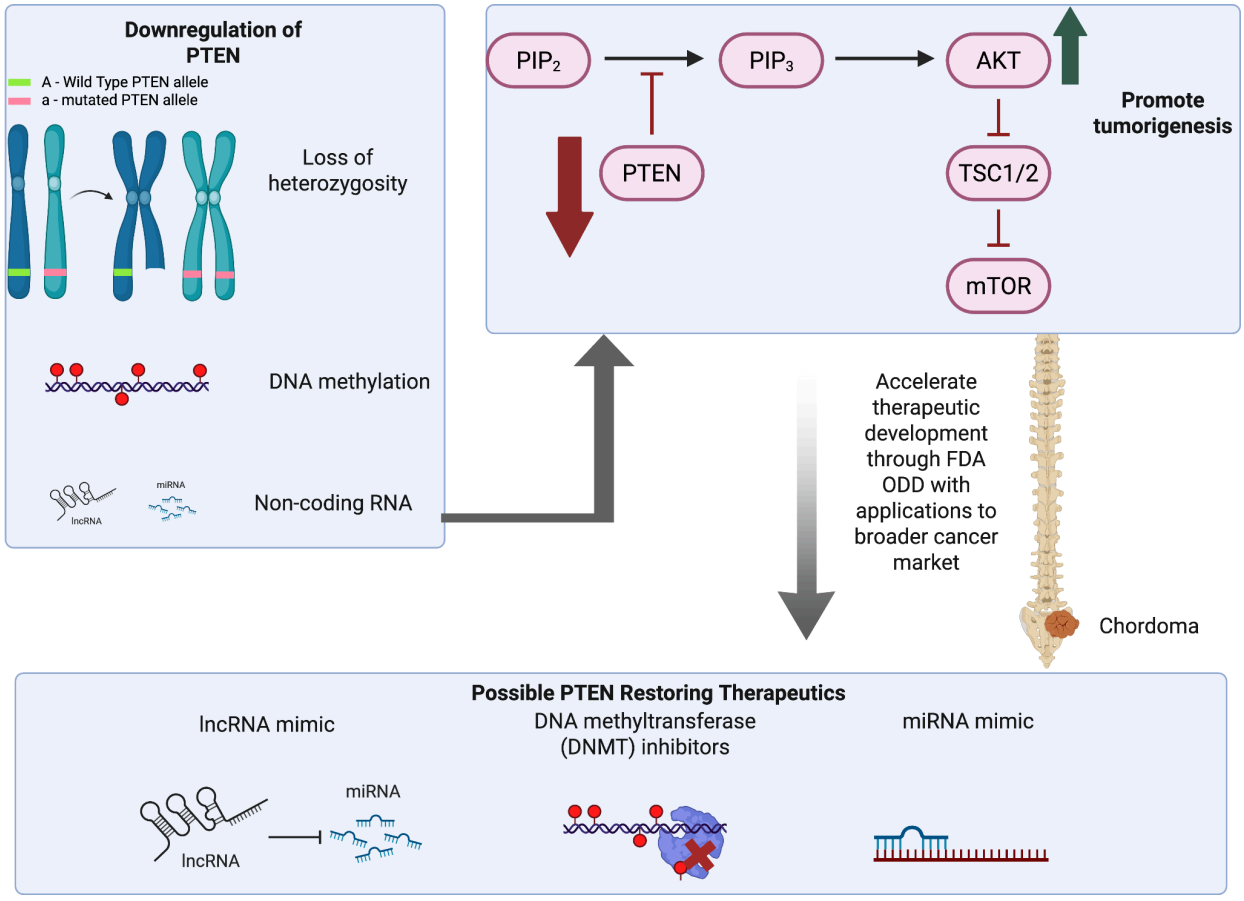


Figure 1: *How epigenetic factors can be targeted in chordoma and why chordoma is a disease model of interest to accelerate the development of novel therapeutics with broader oncological applications*. Downregulation of PTEN expression can occur through different mechanisms, such as loss of heterozygosity coupled to a genetic mutation, DNA methylation, and non-coding RNA. The figure then shows the molecular mechanism of how loss of PTEN promotes oncogenesis. Through removing a phosphate from PIP3, PTEN acts as a tumor suppressor. When PTEN is not present, PIP3 still has the phosphate binding that promotes downstream oncogenic signaling. Through removing phosphate from PIP3 to PIP2, PTEN acts as a tumor suppressor. When PTEN is not present, PIP3 still has phosphate, which promotes downstream oncogenic signaling such as mTOR (Abadi et al. 2021). Through developing therapeutics that target PTEN downregulating

mechanisms within chordoma, which could provide a proof of concept that has applications for malignancies beyond chordoma. A few potential therapeutics are those that target non-coding RNAs (e.g., lncRNA, miRNA) or methylation. Image created with BioRender.

Due to chordoma's slow growth, many patients eventually develop locoregional relapse (Wedekind et al., 2021; Schroeder et al., 2024) with few treatment options outside of the standard of care, which is en bloc resection and radiation (Wedekind et al., 2021; Jeys et al., 2007; Boriani et al., 2006; Fourney et al., 2005; Fuchs et al., 2005; Gokaslan et al., 2016; Moojen et al., 2011; Yamamoto & Tsuchiya, 2019; Schroeder et al., 2024; Schroeder et al., 2024; Lockney et al., 2017; Frezza et al., 2019; Catton et al., 1996). As such, new therapeutic strategies are needed for chordoma. As illustrated in Figure 1, the PTEN (Phosphatase and Tensin homolog) is a key tumor suppressor that removes a phosphate from PIP3 [Phosphatidylinositol (3,4,5)-trisphosphate] to be dephosphorylated to PIP2 [Phosphatidylinositol 4,5-bisphosphate], reducing stimulation of downstream oncogenic pathways. The PI3K-mTOR-Akt pathway, of which PTEN is an inhibitor, is one of the most mutated pathways in cancer, and activation of the pathway is usually increased through amplification of PI3K and Akt or PTEN loss (Manning & Toker, 2017; Hoxhaj & Manning, 2020). While PTEN's relevance is well known for carcinomas, it is still unclear whether a targeted therapy for PTEN is effective in a slow-growing tumor such as chordoma. As such, this gap in understanding the role of PTEN in chordoma progression is critical to investigate. Others who have characterized epigenetic dysregulation of PTEN in chordoma did not find that transcriptional silencing of PTEN was associated with whether the promoter region of the PTEN gene was methylated (Yu & Li, 2015). What has yet to be investigated are alternative methods of transcriptional silencing of PTEN that can contribute to

malignant behavior and therapy resistance, such as epigenetic regulation of gene expression through non-coding RNAs (ncRNAs). Within this thesis, we explore how PTEN expression is altered within chordoma and the possible mechanisms of dysregulation; we propose that instead of relying on anatomical site alone, molecular stratification in chordoma can guide treatment selection, trial eligibility, and follow-up.

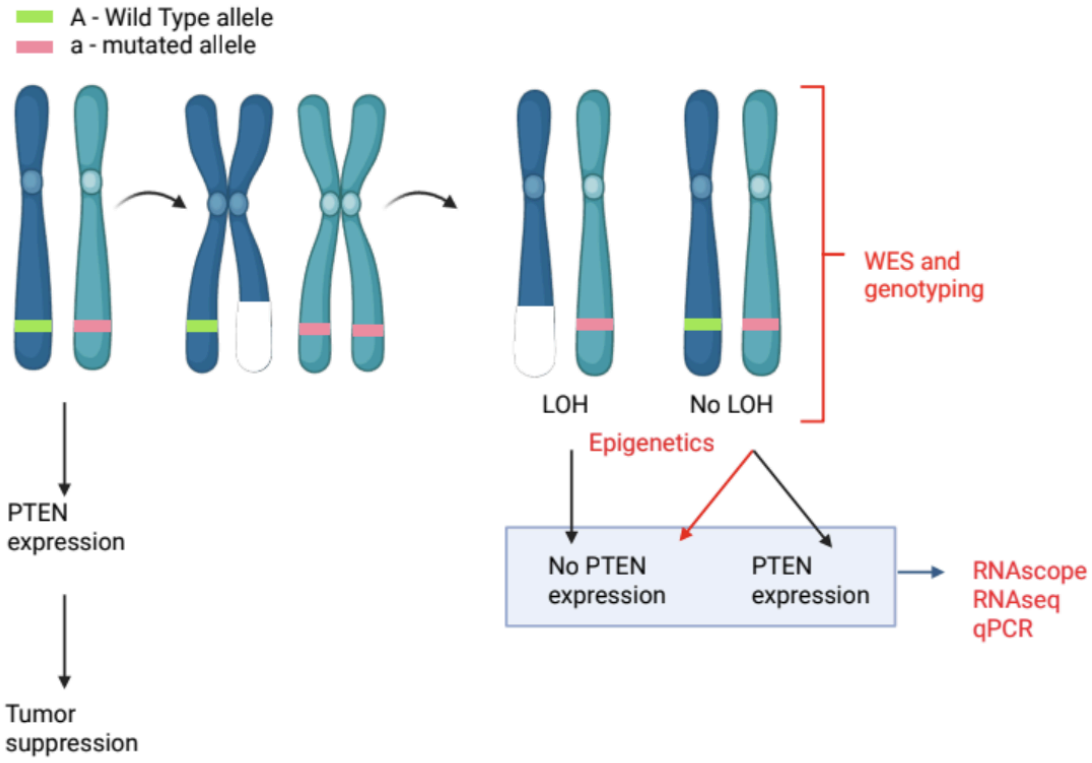


Figure 2: *PTEN loss of heterozygosity (LOH) and overview of how to profile PTEN expression.* During cell proliferation in chordoma, the loss of a wild-type allele during mitosis is called LOH. Typically, the other chromosome expresses wild-type PTEN, keeping its expression and function, and it loses it when the other allele is mutated. However, oftentimes, PTEN is lost even though there is a wild-type gene, which would not explain the phenotype. Therefore, we hypothesize

that there has to be another mechanism, such as epigenetic silencing. Our lab is interested in profiling PTEN expression within chordoma to understand the nature of PTEN downregulation or loss. Whole-exome sequencing would provide insights into whether or not there is LOH. Genotyping would show the presence of mutations. qPCR would confirm the presence of PTEN transcripts. RNAscope would profile the spatial expression of PTEN transcripts. RNA-seq would both profile PTEN transcript expression and the presence of noncoding RNA that regulates PTEN. Image created with BioRender.

PTEN expression in Chordoma

Association between PTEN and Chordoma

PTEN is expressed within healthy cells due to its role as a tumor suppressor, with reduced PTEN expression observed in numerous malignancies (e.g., bladder, glioblastoma, prostate cancer), including chordomas (Ashrafizadeh et al., 2020; Carpenter et al., 2025; Abadi et al., 2021; Pagani et al., 2023; Tian et al., 2022; Teleanu et al., 2025; Zeinali et al., 2025; Seeling et al., 2023; Yang et al., 2020; Michmerhuizen et al., 2020). In ~13% (Shalaby et al., 2011) to 28% (Seeling et al., 2023) of patients with chordoma have loss of PTEN expression. In genetics, Loss of Heterozygosity (LOH) refers to the loss of a copy of an allele, so it doesn't have the insurance of the other allele. If PTEN LOH is coupled with a genetic mutation in the other allele, this loss of PTEN function is associated with increased proliferation during tumorigenesis (Potapova & Gorbsky, 2017). LOH at 10q23.31 has been observed in up to 80% of cases of sporadic chordoma, and this ablates PTEN, resulting in genetic complete loss of function (Le et al., 2011; Choy et al., 2014), which is also illustrated in Figure 2. Further studies have implicated PTEN and its downstream mTOR signaling pathway in clivus invasion of chordoma, and also that

PTEN is a prognostic factor for local invasion and recurrence in sacral chordoma (Wu et al., 2015; Chen et al., 2014). Although Alholle et al. did not find direct differential regulation of PTEN between the various groups, the article found dysregulation of mTOR pathways within chordoma, which is closely regulated by PTEN (Alholle et al., 2015). In particular, immunohistochemistry revealed that reduced PTEN and increased mTOR were associated with increased local invasion of chordoma and reduced disease-free survival time (Chen et al., 2014). However, there have been observed cases where LOH does not couple with a genetic mutation in the conserved allele, and still, there's PTEN loss. In that case, epigenetic modifications, such as methylation or non-coding RNAs, can mask the wild-type allele. While PTEN methylation has not been extensively studied in chordoma due to its rarity, insights from other tumor types may offer significant potential to advance the understanding of this important mechanism and inform future chordoma therapeutic development (Moreno et al., 2023) (Figure 2).

Epigenetics of PTEN in other cancers

Within Ghafouri-Fard et al.'s review, multiple reports underscored the role of microRNA (miRNA) in regulating PTEN expression in various cancers. For example, miR-19a downregulates PTEN expression across various conditions and cancers (Ghafouri-Fard et al., 2021). In breast and lung cancer, Abadi et al. review the impact of miRNA regulation of PTEN expression through the binding of UTR on mRNA to alter PTEN translation. Previous work has demonstrated that potential antitumor compounds baicalein, propofol, and curcumin alter miRNA expression and result in increased PTEN expression, inhibiting breast and lung cancer progression (Abadi et al. 2021). For example, curcumin has been shown to suppress cancer growth in breast cancer MCF-7 cell lines (Wang et al., 2017). Additionally, long non-coding

RNAs (LncRNAs) such as PTENP1 promote PTEN expression by promoting miRNAs that upregulate PTEN (e.g., miRNA-20a) and downregulate miRNAs that downregulate PTEN (e.g., miRNA-19b) (Abadi et al. 2021). Also, miRNAs have been implicated in regulating PTEN during the initiation and progression of malignancies such as prostate (Bergez-Hernández et al., 2024) and colorectal cancer (Herichová, 2025). Further investigation of miRNA expression could help elucidate mechanisms of PTEN regulation and help discover mechanisms of treatment resistance and new therapeutic targets.

Role of molecular profiling in treatment planning

The application of -omics within clinical settings is personalized medicine, which is the process of customizing treatment based on the molecular and genetic profile of both the patient and their disease (Chai et al., 2019). Currently, personalized medicine is more common for the treatment of somatic tumors to attempt to deliver more effective cancer treatment, though personalized medicine has yet to make an impact in most tumor types (Shortliffe et al., 2021; Martinez Moreno et al., 2023). Large-scale -omic data benefits personalized therapeutic strategies to provide a robust reference and a targetable molecular vulnerability (Shortliffe et al., 2021). These large-scale uses of -omic data especially have the potential to improve outcomes in rare cancers and cancers with various molecular subtypes, particularly when the subtypes have different and potentially targetable pathologic driver mutations. The role of biomarkers within precision medicine can vary, serving as either a diagnostic or therapeutic target. This subsection discusses how molecular profiling can impact treatment planning and aid in patient stratification.

Though already routinely employed in the context of research, there is a push towards integration of large-scale -omic data (specifically genomic and transcriptomic) within clinical applications (Shortliffe et al., 2021; Martinez Moreno et al., 2023). To determine the relevance of eight new chordoma cell lines for biomarker discovery, von Witzleben et al.'s article characterized genomic, mRNA, and protein expression while confirming generalizability with 43 chordoma samples. This demonstrates a process using genomic, transcriptomic, and proteomic data to determine new biomarkers for chordoma (von Witzleben et al., 2015). For transcriptomic profiling, Bell et al. used whole-transcriptome shotgun sequencing for 14 FFPE skull base chordoma samples, which demonstrates the process of validating biomarker candidates with RNA data (Bell et al., 2016). Khan et al. employ proteomics of four chordoma cell lines and found the presence of the surface protein LA2R1, which they argue could be a therapeutic target (Khan et al., 2024). Tatman et al. determined kinase activation of different subtypes based on proteomic analysis of mass spectrometry data. Not only does the article have implications for understanding the mechanism of disease, but it also has implications in drug discovery since the article proposes kinases of interest (Tatman et al., 2017).

Singh et al performed pathway analysis for various chordoma cell lines following different treatments to identify activation of pathways (e.g., death receptor and apoptotic) in different treatment groups. So genomic data not only helps discover drug targets but also assists in the assessment of therapeutics to improve current treatments (Singh et al., 2021). Zolotovskaia et al. discussed the oncobox system used to calculate pathway instability due to how the biomarker potential of cancer mutations is enhanced by applications of molecular pathway-based approaches. Mutation drug score values were also used to rank drug capacity to interfere with

mutated molecular pathways (Zolotovskaia et al., 2020). Passeri et al. correlated clinical data (e.g., histological, radiological) with molecular data (e.g., RNA sequencing, targeted next-generation sequencing, whole-exome sequencing) to identify markers to be targeted by radiation-based therapies (Passeri et al., 2023). Bosotti et al. used genomic and mRNA profiling of novel cell lines to provide the foundation for evaluating kinase activation, which can be used to develop pharmacological targets (Bosotti et al., 2017). With participants already enrolled in a phase 1 trial for sarcoma (18.1%, which included ultrarare sarcomas that included chordoma), Moyers et al. performed sarcoma-matched biomarker analysis of genomic data to better determine whether the therapeutic targeted the intended biomarker (Moyers et al., 2023). The findings discussed are exemplified by a review discussing how metabolomic and genomic data uncover mechanisms of glioma pathogenesis and how microenvironment and tumor genotypes interact (Bi et al., 2020). This is relevant since it discusses an example of how -omic data translates into proposed targets that can benefit patients clinically. This can provide an example of types of analysis that can be done for chordoma to develop targeted therapeutics that address interaction with the microenvironment, which is already of interest within immunotherapeutics.

How molecular characterization aids in patient stratification

These articles create the context for how biomarker targets are discovered through the analysis of DNA (genomic) and RNA (transcriptomic) data within chordoma. Within Zhang et al.'s article, multi-omic data from 187 skull base chordoma cases identified an immune cold subtype linked to chromosome 9p/10q loss. This demonstrates the use of genomic data, along with other forms of -omic data, in order to determine immune subtypes and develop treatments for those resistant to current standard treatments (Zhang et al., 2024). Sa et al.'s article discussed how whole-exome

sequencing (profiling exon expression) in combination with other omic techniques was used to find novel gene fusions, mutations, germline SNPs of the T gene, and various chromosomal aberrations (Sa et al., 2017). Bai et al.'s article determined genetic alterations with high concordance between the genomic profiles of paired primary and recurrence/metastasis skull base samples to show the relevance of certain genes for the initiation of chordoma development, providing a possible diagnostic biomarker generalizable to various skull base chordomas (Bai et al., 2021). Gröschel et al. used genome and whole-exome data from patient-derived chordoma tumors from a precision oncology program to characterize genomic patterns and homologous recombination-related alterations (Gröschel et al., 2019). Li et al. investigated differential genetic expression between clival and sacral chordoma samples. Additional databases propose protein-protein interaction networks, which model possible altered signaling pathways and gene targets (Li et al., 2019). In another study, Bell et al. determined the transcriptome of spine and skull base chordoma to determine differential expression (LMX1A was particular to skull base chordoma and SALL3 to spine chordoma) and overall T (Bell et al., 2018). Park et al determine the clinical significance of various levels of stroma richness within chordoma based on radiologic, histologic, and transcriptomic data. Use of transcriptomic analysis to determine how stroma abundance may inform aggressiveness of chordoma (Park et al., 2022).

These articles create the context for how biomarker targets are discovered through the analysis of protein (proteomic) and epigenetic (epigenomic) data within chordoma. Yin et al. combined histopathological with clinical data to classify into molecular subtypes (bone microenvironment-dominant, mesenchymal-derived, and mesenchymal-to-epithelial transition-mediated pattern) (Yin et al., 2024). Chen et al. differentiated protein expression

between primary and recurrent chordoma samples to process prognostic biomarkers (Chen et al., 2015). Wu et al. demonstrate the use of proteomic analysis to determine differences in pathways relevant to different subtypes of chordoma based on bone invasiveness (Wu et al., 2015). Zhang et al.'s article, as discussed in the genomics selection, combined genomic and proteomic data to be used to find immune subtypes (Zhang et al., 2024).

For epigenomic profiling, Alholle et al. used epigenomic techniques to analyze differential methylation between various subgroups of chordoma. Based on HumanMethylation450 BeadChip array analysis, the article was able to see differences in hypermethylation and hypomethylation of probes. This gives insights into possible epigenetic targets for chordoma (Alholle et al., 2015). Bai et al. profiled long non-coding RNA (lncRNA) and mRNA profiles of 12 chordoma samples with variations in dural penetration to determine different expressions of non-coding RNA. An lncRNA and mRNA co-expression network was constructed to understand how non-coding RNA may regulate differing expression that leads to differences in dural penetration (Bai et al., 2020).

Therapeutic Relevance of Stratification

Once one determines the biomarkers of interest through -omic data, the next step is how these biomarkers inform therapeutics. Xu et al. had genomic data used to determine differential expression with the addition of TNF-alpha treatment. With additional analysis to construct protein-protein interaction networks, researchers can better understand how therapeutics alter disease mechanisms (Xu et al., 2020). Bell et al. developed targets based on profiling the differing RNA-seq expression of spine and skull base chordoma (Bell et al., 2018). Liang et al.

proposed applications of multi-omics in precision medicine to identify therapeutic targets, due to the possibility of informing treatment plans for various chordoma subtypes based on genomic and transcriptomic data-informed determination of sensitivity to therapeutics (Liang et al., 2018). A phase II single-arm, open-label trial by Teleanu et al. stratified participants into possible non-responder phenotypes based on CDK4/6/pRB IHC. While they did not see a correlation between outcomes and responder phenotypes, they followed with genomic and transcriptomic profiling to explore possible therapeutic targets and resistance mechanisms to CDK4/6 inhibitors (Teleanu et al., 2025). Yin et al. used histopathology, clinical, and imagological features to classify molecular subtypes (bone microenvironment-dominant, mesenchymal-derived, and mesenchymal-to-epithelial transition-mediated patterns) and proposed informing therapeutic strategies based on subtyping to improve treatment outcomes (Yin et al., 2024). Zhang et al. developed a new therapeutic for those resistant to standard treatments by understanding immune subtypes based on proteogenomic data (Zhang et al., 2024). Ultimately, instead of relying on anatomical site alone, molecular stratification in chordoma can guide treatment selection, trial eligibility, and follow-up.

Potential Role of PTEN in Chordoma

A Framework for Novel Chordoma Therapeutics

Loss of heterozygosity (LOH) at PTEN is frequent in chordoma, which suggests that reducing PTEN expression is relevant to its pathogenesis. There is well-documented evidence that reduced PTEN expression is a risk factor for developing various cancers, including chordoma. These miRNAs are relevant because they provide insights into post-transcriptional inhibition of PTEN

expression, which can explain how PTEN expression is reduced or absent even when both copies of the allele are present.

Though PTEN epigenetic modifications, such as methylation and miRNA modification, are established as important in tumorigenesis in other cancers, their mechanisms remain largely unexamined in chordoma (Li et al., 2018). As summarized above, PTEN is altered in chordoma, and epigenetic regulators of other biomarkers have been targeted in chordoma, and epigenetic regulators of PTEN have been targeted in other cancers. If the expression of PTEN can be significantly altered by targeting miRNA that regulates PTEN in breast and lung cancer, we are interested in seeing if these methods of determining miRNA regulation of PTEN can translate into the treatment of chordoma. Long non-coding RNAs (lncRNAs) have been shown to sequester miRNA, which in turn regulates PTEN expression (Li et al., 2018; Hashemi et al., 2022). We are interested in whether lncRNAs impact PTEN regulation in chordoma through the regulation of miRNA, as seen in lung and breast cancer (Abadi et al., 2021; Seeling et al., 2023). As mentioned prior, PTENP1 would sequester and miRNA (e.g., miRNA-19) (Li et al., 2018), which restores PTEN's function as a tumor suppressor (Abadi et al., 2021). These lncRNA and circRNA can be used to shape the target miRNA activity that regulates PTEN expression and lead to increased PTEN expression, which represents potential therapeutic targets (Hashemi et al., 2022).

Hence, we are interested in investigating targetable epigenetic changes of PTEN in chordoma. These non-coding RNAs and epigenetic regulators of PTEN in chordoma can serve as both therapeutic targets and deliverable therapeutics. We have established possible dysregulation of

PTEN in chordoma, and that previous therapeutics have been proposed around targeting epigenetic regulation of other targets in chordoma, so now we must explore possible epigenetic factors that can be employed to regulate PTEN within chordoma. Though miRNA regulation of PTEN in chordoma has not been directly investigated, Bai et al. profiled long non-coding RNAs (lncRNAs) and mRNA to determine an lncRNA-mRNA co-expression network within various patient-derived chordoma samples and found that lncRNAs participated in the regulation of the PTEN-PI3K-Akt signaling pathway (Bai et al., 2020). This co-expression network suggests that modulating these lncRNA-mRNA interactions could restore PTEN's function as a tumor suppressor, and could be developed into new therapeutics as part of a precision medicine strategy for patients with chordoma.

Clinical Opportunity: The Case for Chordoma and Beyond

A therapy that possibly restores PTEN function could help patients with chordoma. We are interested in determining whether targeting a regulator of PTEN would achieve the desired effect of increasing PTEN expression.SiRNA has relevance for understanding how loss of biomarkers (e.g., the surface protein PLA2R1) (Khan et al., 2024) impacts chordoma growth. So if we were to test whether the use of non-coding RNA regulating PTEN could slow the growth of chordoma, we want to explore miRNA. MiRNA stability relative to siRNA would allow for translatability as a therapeutic for chordoma. Ultimately, we are interested in profiling miRNAs that regulate PTEN in chordoma, possible regulators of those target miRNAs, and whether miRNAs can be directly delivered to disrupt chordoma growth through upregulating PTEN or whether there are more stable therapeutics that can be delivered to regulate those target miRNAs.

While PTEN is a driver in many common cancers, as a rare (orphan) disease, chordoma is a favorable disease to investigate PTEN (a driver in many common cancers) therapeutics because of the FDA's Orphan Drug Designation (ODD) approval pathway for orphan diseases (Stacchiotti et al., 2012). Through incentives that reduce financial costs and logistical barriers, the ODD regulatory pathway makes an "orphan-first" strategy/ framework appealing (U.S. Food and Drug Administration, 2026). This orphan first strategy within chordoma is exemplified by the development of Bavarian Nordic's BN-Brachyury Vaccine, a phase 2 clinical trial in October 2018 and January 2022. With the FDA's grant of Orphan Drug Designation (ODD), researchers were able to develop a vaccine targeting the brachyury protein within a Phase 2 clinical trial between October 2018 and January 2022 with 29 participants (Chordoma Foundation, 2018; Cote et al., 2024). The FDA recognized that conventional large-scale trials are difficult for the population of chordoma patients due to their rarity. Through the ODD pathway, developers have benefits such as securing seven years of market exclusivity after approval, tax credits for clinical trials, and a waiver from user fees (Commissioner, 2024). Outside of Chodoma, brachyury has applications for many solid tumor types with overexpression of brachyury, such as lung, breast, ovarian, chordoma, prostate, colorectal, and pancreatic adenocarcinoma (ClinicalTrials.gov, 2021b), hence why eligibility for the MVA-Brachyury-TRICOM trial included those with "metastatic or unresectable locally advanced malignant solid tumors" (ClinicalTrials.gov, 2021b). So these chordoma clinical trials can provide a proof of concept for treatments with broader tumor applications. Therefore, investigating PTEN in the context of an orphan disease like chordoma provides the signal that PTEN targeting therapies are biologically relevant to innovate therapeutics that can eventually benefit the broader cancer population.

More broadly, the biology applies to other cancers, as the mechanism has been seen in other, more common cancers. Yin & Ren reviewed novel target and anti-PD-1/PD-L1 blockade therapies within bone sarcomas. A limitation of the study is that chordoma was included as a subtype of sarcomas despite not being derived from bone tissue. PD-1 ligation targets two main pathways, PTEN-PI3K-Akt and RAS-MEK-ERK, so there is a connection between immunotherapies and PTEN regulation (Yin & Ren, 2024). Xu et al. targeted genes within the PTEN-PI3K-Akt signaling pathway while studying the effects of TNF-alpha treatment on chordoma (Xu et al., 2020), which further demonstrates the importance of altering PTEN and regulators of PTEN when developing treatments for chordoma.

Profiling PTEN expression and mechanism of PTEN loss in chordoma does not just have implications for the 300 US patients with chordoma each year (National Cancer Institute, 2024), but also can impact those with other tumor types through possible shared mechanisms of PTEN regulation. Understanding the mechanism of epigenetic regulation of PTEN provides a foundation for the development of possible therapeutics that address these reversible targets to upregulate and restore PTEN's tumor-suppressing function within both rare and common cancers. Through characterizing PTEN expression in primary and recurrent chordoma cell lines and archive FFPE samples from patients with chordoma, we could identify possible mechanisms of PTEN loss, stratify patient populations based on PTEN expression profiles, and develop therapeutic targets. Within this thesis, our two main aims are to (1) characterize PTEN expression in cell lines and archival samples (whether or not each cell line expresses PTEN, to what degree the archival tissue expresses PTEN, and pilot spatial characterization of PTEN

expression in chordoma tissue) and (2) explore the possible mechanism of PTEN loss within chordoma.

Chapter 2: Methods

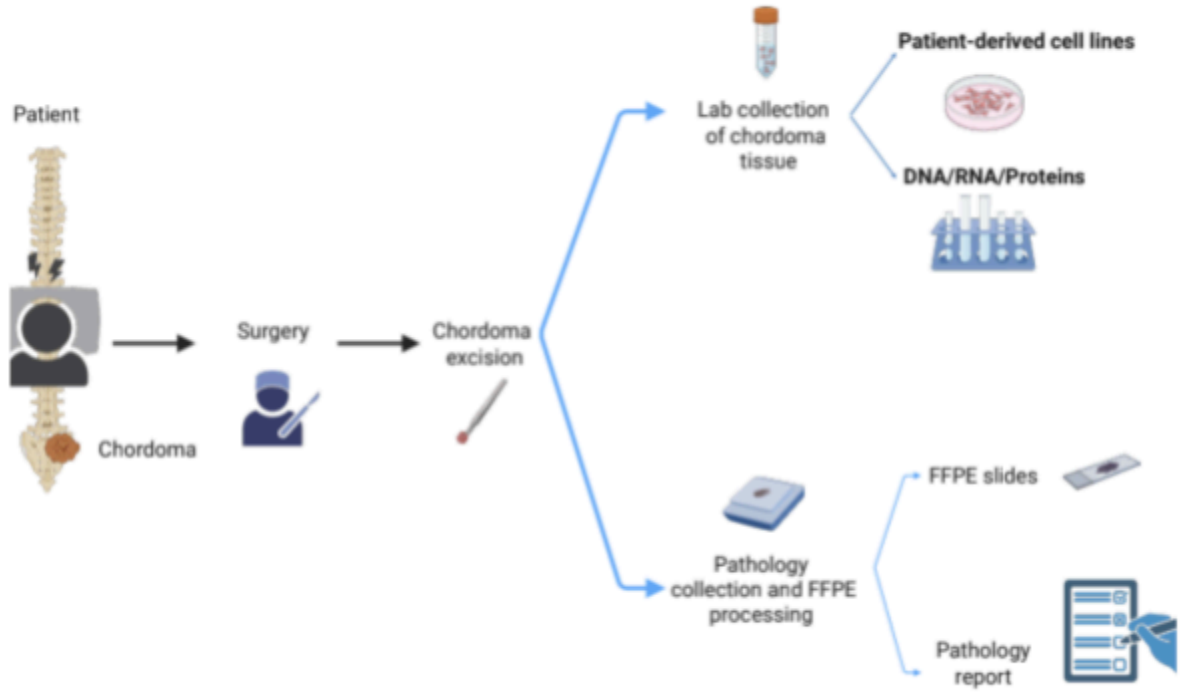


Figure 3: *Biobanking methods and how intraoperatively collected patient-derived cell lines are generated.* After the tumor is removed during surgery, Pathology collects and processes the tissue. Formalin-fixed paraffin-embedded (FFPE) tissue is used for clinical reports, as well as archived for research purposes. Fresh tissue is also collected by the lab, where the sample is profiled for various biomarkers and developed into patient-derived cell lines. Image created with BioRender.

2.1 Cell Culture Models

The cell lines cultivated and studied were human embryonic kidney (HEK), recurrent sacrococcygeal chordoma (UCH1, MUG-Chor1), notochordal (UCH1N), mouse-explanted

UCH1N (NSG1) cells, primary sacral chordoma (UCH12, UCH7, and JHC7), and intraoperatively collected patient-derived (Figure 3) primary sacral chordoma (ZG1, ZG7, and ZG8) cells. For HEK, ZG1, ZG7, ZG8, and JHC7, media preparation was 500 mL of DMEM, 50 mL of Fetal Bovine Serum (FBS) (10%), and 5 mL of Penicillin Streptomycin (1%). For UCH1N, media preparation was 500 mL of MEM-Alpha, 50 mL of Fetal Bovine Serum (FBS) (10%), and 5 mL of Penicillin - Streptomycin (1%). For UCH1, media preparation was 400 mL Iscove's Modified Dulbecco's Medium (IMDM) + 100 mL RPMI 1640, 50 mL of Fetal Bovine Serum (FBS) (10%), 5 mL of 200mM L-glutamine (1%), and 5 mL of Penicillin - Streptomycin (1%). For UCH12, media preparation was 400 mL Iscove's Modified Dulbecco's Medium (IMDM) + 100 mL RPMI 1640, 50 mL of Fetal Bovine Serum (FBS) (10%), 5 mL 200mM L-glutamine, (1%), 5 mL ITS-G, and 5 mL of Penicillin - Streptomycin (1%). For UCH7, media preparation was 400 mL Iscove's Modified Dulbecco's Medium (IMDM) + 100 mL RPMI 1640, 50 mL of Fetal Bovine Serum (FBS) (10%), 5 mL 200mM L-glutamine (1%), and 5 mL of Penicillin - Streptomycin (1%). For MUG-Chor1, media preparation was 400 mL Iscove's Modified Dulbecco's Medium (IMDM) + 100 mL RPMI 1640, 50 mL of Fetal Bovine Serum (FBS) (10%), 5 mL of 200mM L-glutamine (1%), and 5 mL of Penicillin - Streptomycin (1%). For the intraoperatively collected patient-derived primary sacral chordoma (ZG1, ZG7, and ZG8) cells, the chordoma resected is first delivered to pathology to approve the distribution of the biopsy sample. With tissue sent to be processed by clinical and research teams, as well as archived on FFPE slides that were stored within pathology, a portion is used to develop patient-derived cell lines, where cells are enzymatically dissociated in an incubator-shaker at 30rpm/ 37°C in trypsin. Then, the cells are isolated and plated. The cell line is expanded through passaging, with cryovials, supernatant, and flash-frozen cell pellets collected between passages.

The RNA extracted from patient-derived cell lines is then characterised for various chordoma and stemness-related markers through quantitative PCR. To encourage attachment of cells, the bottoms of the T-75 flasks were incubated at 37°C in 5% CO2 and pre-coated with collagen. Once the flask was about 70% confluent, the cells were passaged, with weekly media changes in between.

2.2: FFPE Tissue Collection and Process

We used 8 FFPE tumor samples archived by Pathology from chordoma surgeries at Rhode Island Hospital (Figure 3) obtained through IRB-approved protocols (IRB#1750392, #862559). The samples were chosen using a morphology marker to ensure there was a distinction between where the sample was tumor, stromal, or immune compartment. Samples 1, 3, 6, 7, and 8 are primary chordoma. Samples 2, 9, 10, 11, and 12 are recurrent chordoma samples.

2.3: RNA Extraction

For cell culture, pellets were generated during passaging in which cells are flash-frozen in liquid nitrogen and stored in -80°C. The RNA was extracted using TRIzol Reagent (Invitrogen) and PureLink RNA Mini Kit (ThermoFisher Scientific) reactions. For extraction, these flash-frozen pellets first undergo phase separation in which chloroform is added before centrifuging to isolate the aqueous RNA-containing layer. Ethanol is then added to this RNA-containing layer before loading into PureLink spin columns. In order to remove contaminants (e.g., DNA, proteins), buffer washes are done to the columns before eluting the RNA with RNase-free water. Phenol: chloroform extraction and ethanol precipitation further purified the eluted RNA sample before washing and resuspending the RNA pellet with RNase-free water.

For FFPE tissue, the RNA was extracted using a deparaffinization and proteolytic digestion-based protocol (Qiagen RNeasy Mini Kit). 10µm sections were scraped from 3-5 scrolls before being deparaffinized with deparaffinization solutions from the Qiagen RNeasy Mini Kit and heat incubation before storing in -80°C. The deparaffinized tissue scrolls undergo protein digestion and RNA crosslink reversal through proteinase K and heated incubations, respectively. The lysate is then centrifuged before DNAase I is added to degrade genomic DNA. Within a spin column, the sample is loaded and mixed with ethanol to enhance RNA binding to the silica membrane. The column underwent washes before eluting the RNA with RNase-free water.

The final concentration was measured either with a QuBit fluorometer or a NanoDrop spectrophotometer. These RNA samples are stored at -80°C for applications, such as RT-qPCR and Nanostring.

2.4: RT-qPCR (Reverse Transcription-Quantitative Polymerase Chain Reaction)

Using the manufacturer's protocol, RNA extracted from cell lines and chordoma FFPE samples was converted to complementary DNA (cDNA) using Invitrogen™ SuperScript™ III First-Strand Synthesis System (Fisher Scientific) through reverse-transcription. In order to determine the presence of PTEN transcripts in RNA from FFPE samples and cell lines, we used qPCR with Green Master Mix (Applied Biosystems). To characterize chordoma cell lines (UCH1: P10, P11, P12, P15, P16, P17, P18; UCH12: P9; MUG-Chor1: P6, P7; UCH7: P2; JHC7: P9, P14, P31; ZG1: P9, P14, P15, P16, P17; ZG7: P3, P4, P5; ZG8: P4, P5, P6, P7),

notochordal cell lines (UCH1N: P6, P10, P27, P32; NSG1: P3, P4, P5, P6; NSG1-S: P3, P4, P5, P6), HEK cell line (P26), and 8 FFPE samples, triplicates were performed for PTEN. As well as GAPDH and beta-actin, which were used as housekeeping genes as a control to normalize data. Negative controls included no primer, no sample, and no primer and no sample. The Cq (amplification cycles) were normalized as relative expression to housekeeping genes. Expression was compared between primary and current to determine if PTEN is differentially expressed.

2.5: NanoString nCounter Profiling

The Nanostring PanCancer Progression panel for transcriptomic profiling of transcripts from RNA extracted from 8 FFPE tumor samples. To determine if samples were suitable for Nanostring, NanoDrop spectrophotometry was used to assess where the A260/A280 ratios were between 1.8 and 2.1. The fragment analyzer within Brown University Core Facilities and Small RNA analysis Kit (Agilent Bioanalyzer) confirmed the quantity and quality of RNA before profiling with the NanoString nCounter system. Counts were normalized to housekeeping genes, and housekeeping genes with counts <20 were excluded from that step. Following the Nanostring guidelines, the PTEN expression threshold (32 counts) was determined by the mean of the negative controls with two standard deviations.

2.6: RNAscope In Situ Hybridization

Biobank primary FFPE tissue was collected, with consecutive serial sections to control for tumor microenvironment variance and tissue architecture. To spatially visualize transcripts for PTEN, the RNAscope assay then 2.5 HD Protocol were administered by ACDBio. In order to optimize the pretreatment condition, different incubation times were used for epitope retrieval and

protease digestion steps. First, the FFPE slides are baked and deparaffinized. Then, to reduce background noise and block endogenous peroxidase activity, RNAscope® Hydrogen Peroxide was applied. Then, the epitope retrieval step was performed. During formalin fixation, there's cross-linking of protein that reduces access to targets, so epitope retrieval reverses these cross-links. The RNAscope® Protease Plus was applied to permeabilize the sample to allow optimized binding of probes to mRNA targets. Then, for the RNAscope® 2.5 Assay, "double-Z" oligo probes were used to bind to epitopes of interest. For PTEN, the PTEN target probe was used. For the negative control, the dapB probe is meant to target the dihydrodipicolinate reductase gene of Bacillus subtilis, which should not have a signal in human tissue. For the positive control, use the Hs-PPIB probe, which is meant to target peptidylprolyl isomerase B, which is a housekeeping gene expressed across various human tissues. Then, to amplify the signal from mRNA, amplification reagents were added to hybridize to the pre-amplifier binding site Z probe. DAB chromogenic staining was added to generate a precipitate that is imaged with a bright-field microscope.

2.7 Bioinformatics

We reanalyzed previously published data (Baluszek et al. 2023) accessed through PedCBioPortal. There are 32 skull-based chordoma samples and 4 nucleus pulposus (control disk tissue) samples in the dataset. Within the dataset (chdm_gse230168), the three main files used were RNA counts (data_mrna_expression_continuous_rna_seq_V2_mrna.txt), attributes of samples (Data_clinical_sample_attributes.txt), and attributes of patients (Data_clinical_patient_attributes.txt).

The DESeq2 package was used for differential expression analysis, which normalizes and shrinks log2 fold changes to improve the reliability of results used to study differential expression between chordoma and healthy control samples (Love et al. 2014). For pre-processing, dataframes were combined into a matrix. First, data was cleaned and reformatted (e.g., getting rid of unnecessary columns) before combining dataframes (e.g., cancer type was added to the sample dataframe from the patient data frame). The DESeqDataSetFromMatrix command to set up a matrix with RNA counts, which was combined with attributes of age, sex, and cancer type (chordoma or nucleus pulposus) per sample. For data visualization purposes and to account for higher variance in lowly expressed genes, counts were VST normalized, and the lfcShrink function was used for log2 fold change shrinkage. The ashr type was used due to flexibility for the setup of contrasts (Stephens et al, 2016). Quality control steps, including exploratory data analysis and visualization, were done on normalized count data and differential sequencing statistics. Sample-to-sample heat maps were used to visualize grouping between samples. The plot counts were constructed to look at PTEN counts relative to known regulators (e.g., PTENP1, PTENP1-AS) to see the spread of chordoma data. Volcano plots were constructed to visualize fold changes of expression of genes in chordoma relative to nucleus pulposus, with PTEN, regulators of PTEN, and a few downstream of PTEN labelled. Heat maps (Gu et al., 2021) were used to visualize high and low expression of specific genes within and between samples. GSEA analysis was used to determine what categories of genes were differentially expressed. If unable to find the name of the gene, we checked Genecards (https://www.genecards.org/genecarna) if the gene went by another name. The details of the R code are accessible on GitHub (https://github.com/chordoma/PTEN-DESeq2).

2.8 Statistical Analysis

GraphPad Prism was used to perform statistical analysis and data visualization due to the software's ease of use to generate high-quality data visualizations and built-in statistical tools. R was used to perform statistical analysis and visualization related to bioinformatic packages due to its ability to generate figures such as volcano plots. Nanostring and RT-qPCR results were considered significant at p-value < 0.05, and were analyzed using Mann-Whitney tests. Bioinformatics results were considered significant at Benjamini-Hochberg corrected p-value (padj) < 0.05 to correct for multiple hypotheses, since the p-value might be falsely significant due to 30413 non-zero genes being profiled. Mann-Whitney tests were used since PTEN expression between samples did not have a normal distribution, and we are interested in whether recurrent cell lines have higher or lower expression relative to primary cell lines. The regression analysis was done with RNA-seq counts results due to the continuous nature of PTEN expression. Rather than comparing expression of PTEN regulators between arbitrary low versus high PTEN expression level groups, the scatter plot and regression analysis allow studying the relationship at various degrees of PTEN and PTEN regulator expression. Most tests were used to determine if there was a relationship between PTEN expression and regulators in the bioinformatics analysis, as well as whether there was a significant difference in PTEN expression between primary and recurrent chordoma cell lines.

Chapter 3: Results

3.1 PTEN mRNA Levels in Notochordal Cell Lines and Chordoma Cell Lines

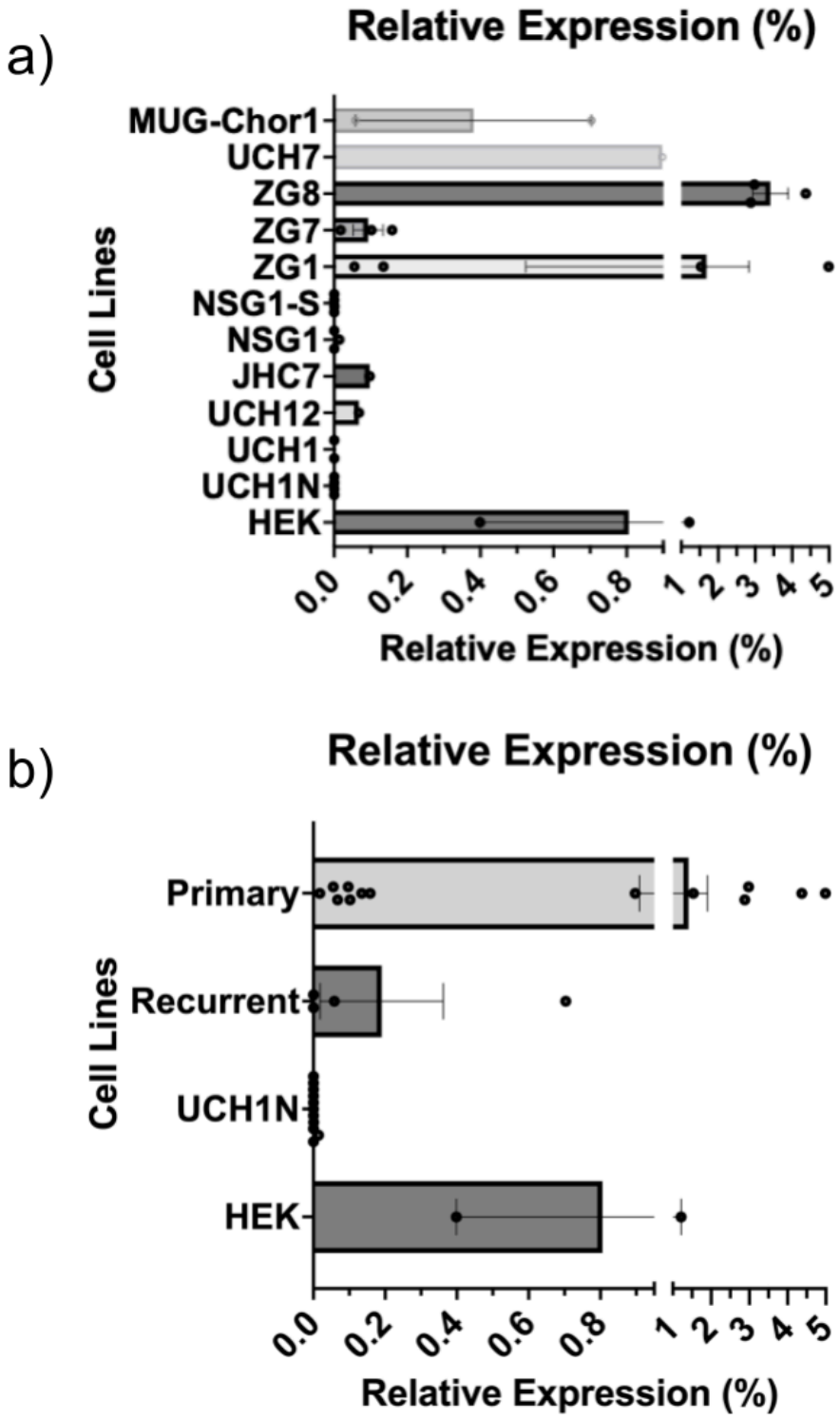


Figure 4: *PTEN mRNA qPCR results across chordoma cell lines.* a) SEM error bars with the relative expression of each cell line. b) SEM error bars with cell line groups by primary and recurrent. UCH1N and HEK cells are present as controls.

In order to study PTEN loss, we first have to identify which of our cell lines do not express PTEN. Using qPCR, Cq (amplification cycles) were measured to study the presence or absence of PTEN mRNA transcription to characterize PTEN expression. Beta-actin and GAPDH were housekeeping genes that served as controls. PTEN primers were used to study the presence of PTEN transcripts. We hypothesized that recurrent chordoma would have a loss of PTEN expression due to previous characterization of LOH of PTEN in recurrent chordoma samples (Ptaszyński et al., 2009; Lee et al., 2015; Michmerhuizen et al., 2020; Rinner et al., 2012). The housekeeping gene controls were used to normalize expression as relative expression. The levels of relative PTEN transcript correspond to the bar graphs in Figure 4. Expression of PTEN mRNA was undetectable within recurrent sacrococcygeal chordoma (UCH1), notochordal (UCH1N), and mouse-explanted UCH1N (NSG1) cells. However, PTEN mRNA was detectable within recurrent sacrococcygeal chordoma (MUG-Chor1), primary sacral chordoma (UCH12, UCH7, and JHC7), and intraoperatively collected patient-derived primary sacral chordoma (ZG1, ZG7, and ZG8) cells (Figure 4a). There was no significant difference between the primary and recurrent cell lines (Mann-Whitney test, $p=0.0597$); however, the one cell line with PTEN loss was recurrent (Figure 4b). There were two recurrent cell lines with MUG-Chor1 expressing PTEN and UCH1 not expressing PTEN. Since cell lines without PTEN expression are recurrent, we do not think PTEN presence or absence is predictive of primary versus recurrent.

The loss of PTEN in UCH1 might be indicative of the cell line and the patient rather than the cells being derived from recurrent chordoma. These findings suggest that the loss of PTEN expression is not universal in all chordoma cell models. This opens the possibility of shared compensatory mechanisms for cell lines that exhibit loss of PTEN mRNA expression.

3.2 Characterization of PTEN in Patient FFPE Samples using Probe-Based Assay

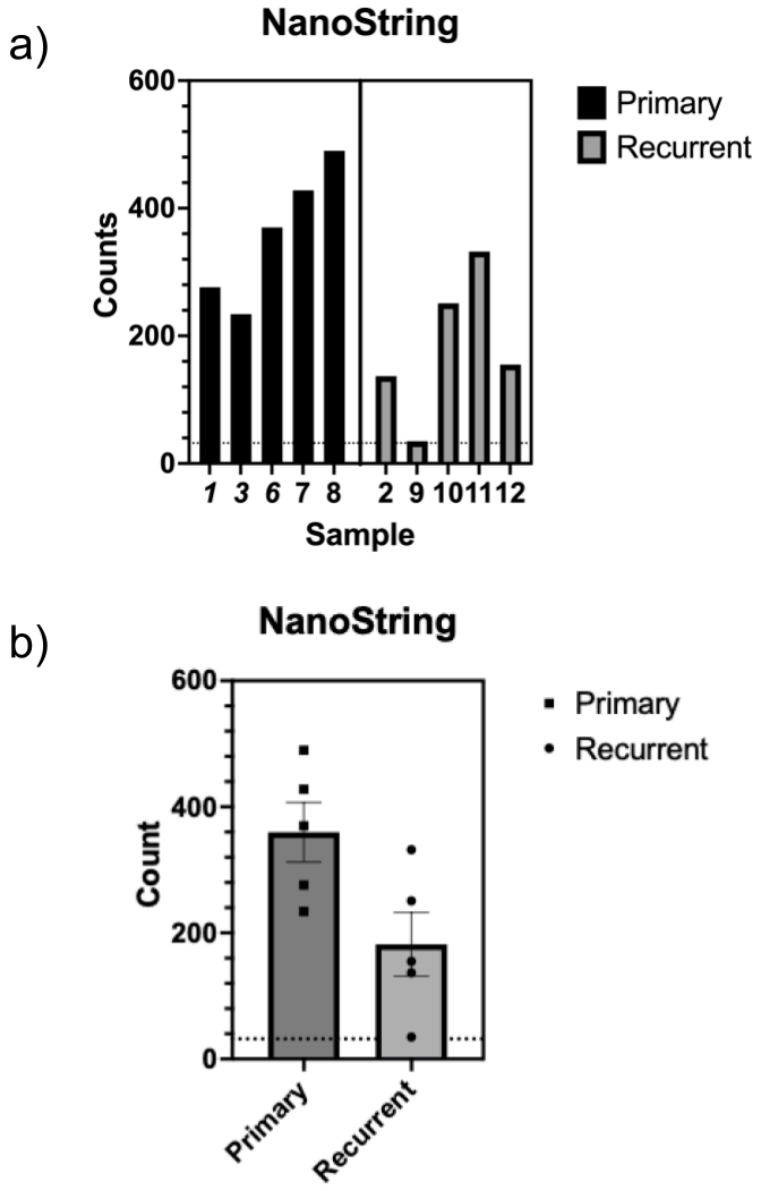


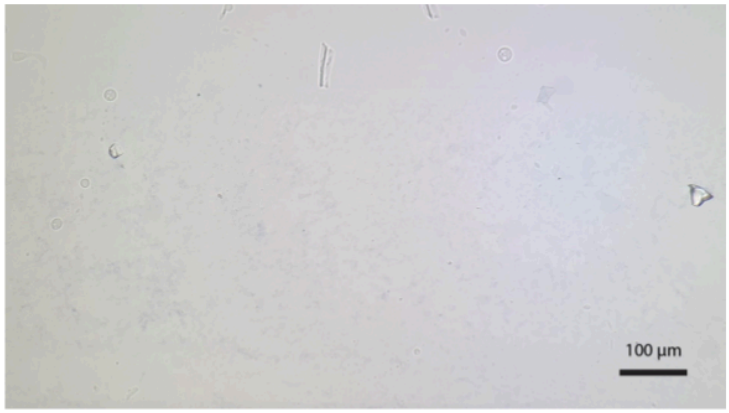
Figure 5: *PTEN expression was assessed using NanoString profiling.* a) Bar graph of nanostring counts per sample with dashed line representing count 32, which is the threshold for PTEN being considered expressed. b) Bar graph with SEM comparison of nanostring counts between primary and recurrent chordoma.

Using Nanostring, a probe-based assay, mRNA counts were measured to study not only the presence or absence of PTEN mRNA transcription, but also the quantity of transcripts in order to characterize the degree of PTEN expression. Additionally, probe-based assays like Nanostring allow for targeting of degraded RNA (e.g., degradation during FFPE tissue processing), whereas amplification methods such as qPCR require greater RNA integrity. We hypothesized that the primary would have a greater degree of expression of PTEN than recurrent, due to previously stated how LOH for PTEN has been characterized. The levels of PTEN transcript counts correspond to the bar graph in Figure 5. The graphs show that all samples express PTEN. Low PTEN expression may reflect tumor heterogeneity, with subsets of non-neoplastic or infiltrating cells potentially contributing to detectable PTEN transcripts despite loss in chordoma cells. Figure 5b shows a nonsignificant trend where PTEN is less expressed in recurrent relative to primary (Mann-Whitney test, $p=0.0556$). This is similar to the trend suggested in the cell culture data. Though in our nanostring data, there is reduced PTEN in chordoma, but not a loss of expression. A possible explanation is that the PTEN detected was not from chordoma cells, but rather from neighboring stroma and immune cells in the FFPE tissue slide. A way to address this possible overestimation of PTEN expression due to samples including a mix of chordoma and healthy cells is to spatially characterize PTEN expression. Since Nanostring lacked spatial

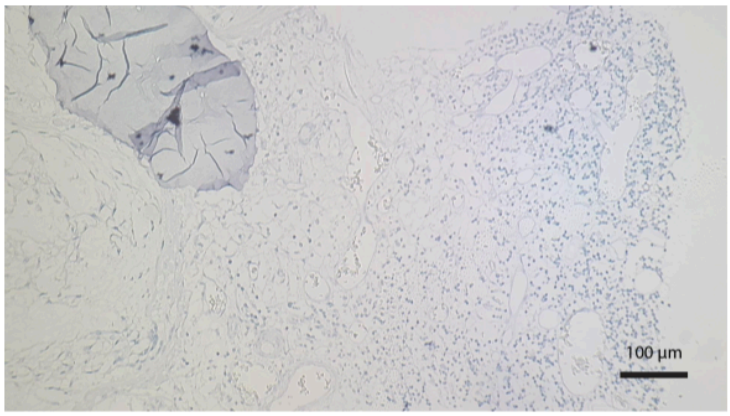
understanding of why all expressed PTEN at different degrees, the next step of interest was to pilot RNAscope, a spatial transcriptomics technique, for characterizing PTEN transcripts in chordoma spatially.

3.3 Optimizing Spatial Transcriptomics Profiling of PTEN in Chordoma

a)



b)



c)

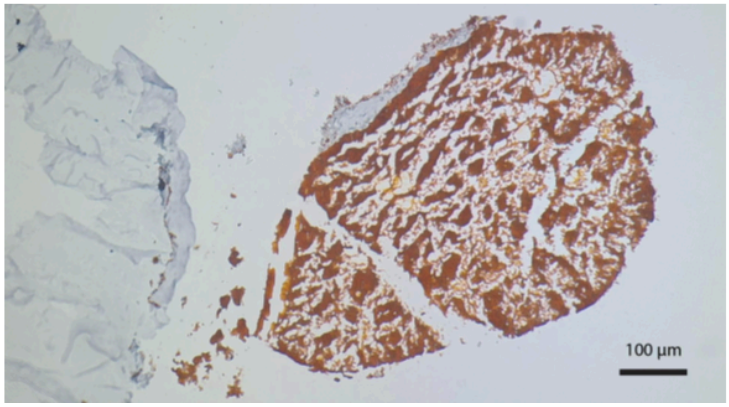


Figure 6: *RNAscope analysis of PTEN expression in intervertebral disc tissue and in a patient with primary chordoma.* (a) Positive control probe in chordoma tissue. (b) Negative control probe in chordoma tissue. (c) PTEN probe in chordoma tissue.

RNAscope, a spatial transcriptomics method, was piloted to determine whether PTEN transcripts can be visualized in primary FFPE archive chordoma tissue. We hypothesize that within the positive control, which is an Hs-PPIB (target peptidylprolyl isomerase B) probe targeting expression of a housekeeping gene, will be expressed in most tissues. We hypothesize that for negative control, the dapB probe is meant to target the dihydrodipicolinate reductase gene of Bacillus subtilis, and should not have a signal in human tissue. We expect staining with the PTEN probe due to the expected expression of PTEN in the sample. The sample used for RNAscope corresponds with Nanostring analysis, where the sample had a PTEN count of 381. We saw that the positive control did not have staining, which was likely related to the sample preparation process, so further work can be done to troubleshoot permeability and antigen retrieval (Figure 6a). We saw there was no staining in the negative control, which is expected (Figure 6b). This verifies that there is no false positive, non-targeting probe binding (e.g., non-targeting probe binding even without a target, amplifying the background). In the sample treated with the PTEN probe, we saw varied staining of the tissue (Figure 6c). This shows that there is heterogeneity in tissue within the chordoma samples that have expression. More PTEN staining was observed on edges, so that might mean there is a permeability difference in the

center relative to edges, which means further protocol optimization is recommended.

3.4 Bioinformatics Profiling of Chordoma RNA-Seq to Characterize Transcriptomic Expression of PTEN and Regulatory LncRNAs

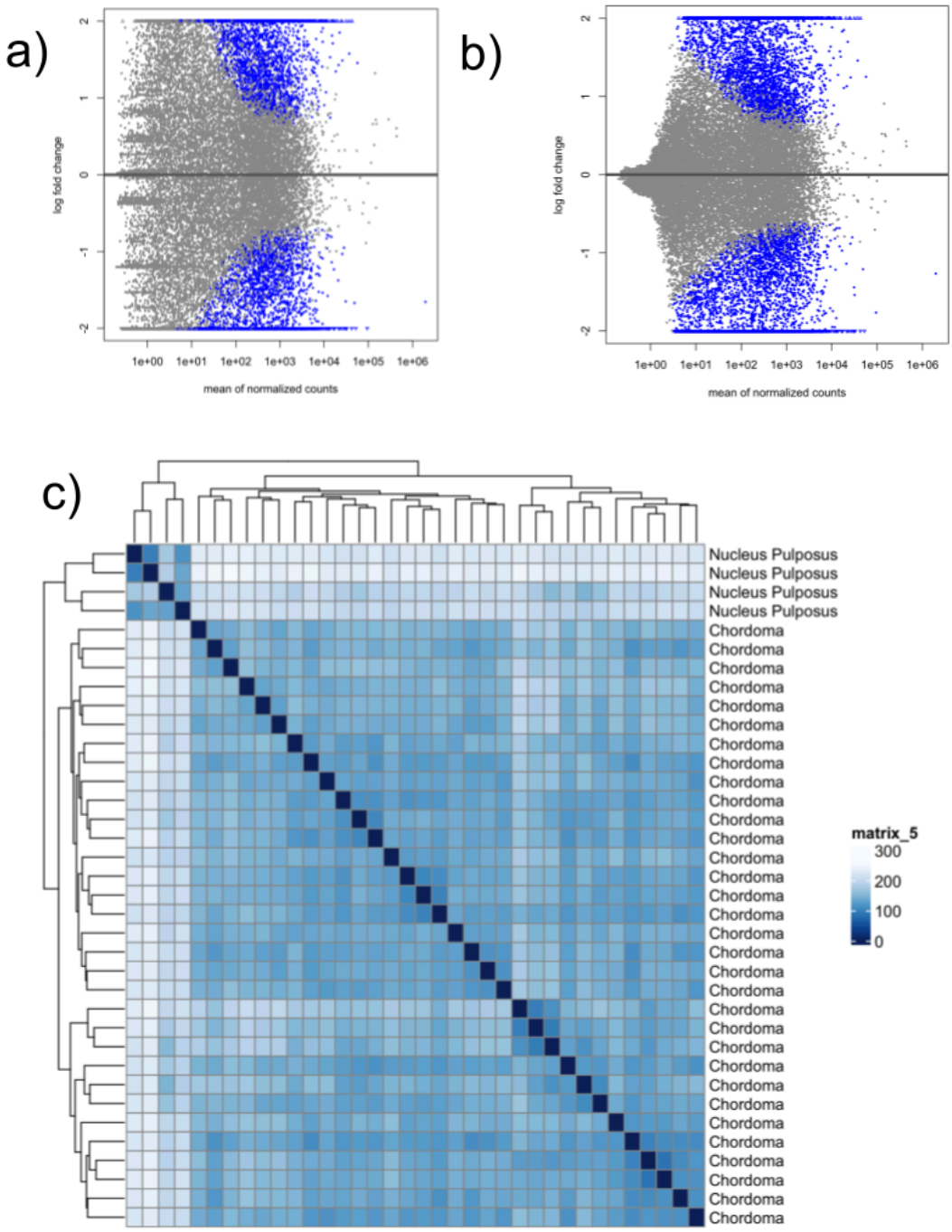


Figure 7: *Visualizing normalization and clustering of data.* a) MA plot (mean normalized counts versus log fold change) before the lfcShrink function with blue points showing significance at alpha = 0.1. b) MA plot (mean normalized counts versus log fold change) after the lfcShrink function with blue points showing significance at alpha = 0.1. c) Sample-sample heatmap showing gene expression for nucleus pulposus and chordoma samples.

As we sort through regulatory channels to allow analysis of our own RNA-seq data, we reanalyzed previously published data (Baluszek et al. 2023) to look for differentially expressed genes in chordoma relative to healthy disk tissue. The published data was generated through Baluszek et al., conducting RNA-seq using Illumina from fresh frozen biopsy tissue from 32 patient skull-based chordomas and 4 healthy nucleus pulposus (disk tissue) as a control. The cell lines and FFPE tissue sample from our own data focused on sacral chordoma, so we were interested in skull-based chordomas data to see if there is still heterogeneity of PTEN expression. First, to assess the quality of the published raw counts data and visualize normalization of data, we constructed MA plots that show the relationship between mean normalized counts and fold change to assess bias within the data. We also constructed sample-sample heatmaps that show sample gene counts to assess sample clustering in order to visualize clustering of samples based on subtypes. For the MA plots of the unnormalized (unshrunk) and normalized (shrunk) data, we hypothesize that the normalization process will help with clustering data, particularly smaller values. For the MA plot, the y-axis is the change between conditions (chordoma versus nucleus pulposus), and the x-axis is counts. The top half of the plot represents upregulation, and the

bottom half of the plot represents downregulation. This helps show why data was normalized with lfcShrink (shrink log2 fold changes) to help account for higher variance in lowly expressed genes, where log2 fold change (MMSE) was shown between chordoma vs nucleus pulposus (control) data. The y-axis is the change between conditions (chordoma versus nucleus pulposus), and the x-axis is the counts. The blue represents significance in change with alpha = 0.1. For the unshrunk data, there are large, nonsignificant logfold2changes, especially at low count values. The unshrunk data is noisy in that the data is not clustered around the logfold2change line (Figure 7a). This is likely due to the heterogeneity between chordoma samples that can be discussed later in the sample-sample heatmap. The MA plot for shrunk helps visualize how the shrinkage estimator reduces noise in the data, which is relevant for situations where the counts are low (Figure 7b). Overall, for shrunk vs unshrunk, the data for smaller values seems to cluster more in shrunk. For the sample-sample heat map, we expect to see clustering based on type or condition (chordoma vs nucleus pulposus). We found clustering of healthy tissue and chordoma, with potential subclustering within chordoma (Figure 7c). The sample-to-sample heat map shows that chordoma samples are heterogeneous.

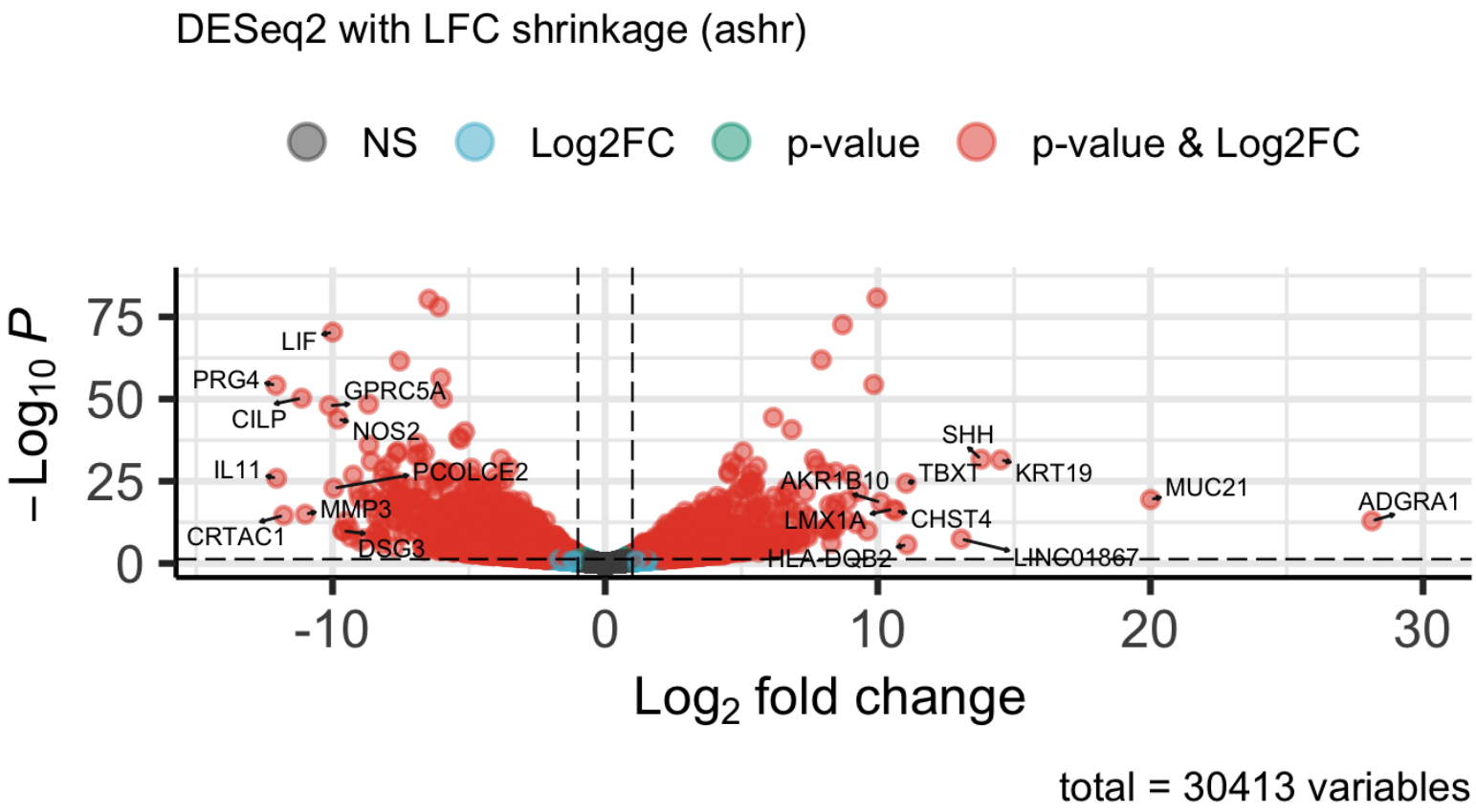

DESeq2 with LFC shrinkage (ashr)
NS
Log2FC
p-value
p-value & Log2FC
−Log10 P
75
50
25
0
LIF
PRG4
GPRC5A
CILP
NOS2
IL11
PCOLCE2
MMP3
CRTAC1
DSG3
SHH
TBXT
KRT19
AKR1B10
MUC21
ADGRA1
LMX1A
CHST4
HLA-DQB2
LINC01867
-10
0
10
20
30
Log2 fold change
total = 30413 variables

Figure 8: *Volcano plot to visualize differential expression between chordoma and nucleus pulposus*. Log2fold change such that a positive log2fold change means the gene is more expressed in chordoma, and a negative log2fold change means the gene is more expressed in nucleus pulposus. The points annotated are the top 50 differentially expressed genes between chordoma and nucleus pulposus. The red dots mean the adjusted p-value < 0.05.

In order to visualize the differential expression of chordoma with healthy disk tissue, we used a volcano plot as a general overview of the differential expression of PTEN and genes related to PTEN expression relative to other genes. The x-axis shows fold change, where left (negative) is when a gene is more expressed in the healthy (nucleus pulposus) samples, while right (positive) is when a gene is more expressed in the chordoma samples. The y-axis is the significance of the level of differential expression. Of the 30413 nonzero total read counts, 3199 (11%) were significantly (padj < 0.05) upregulated in chordoma and 3067 (10%) were significantly downregulated in chordoma (Figure 8). We found that PTEN was less expressed in chordoma relative to healthy disk tissue (log2FoldChange = -0.70, padj = 0.14). Exploratory data analysis and visualization were used to investigate how PTEN and related genes are expressed in chordoma relative to healthy tissue.

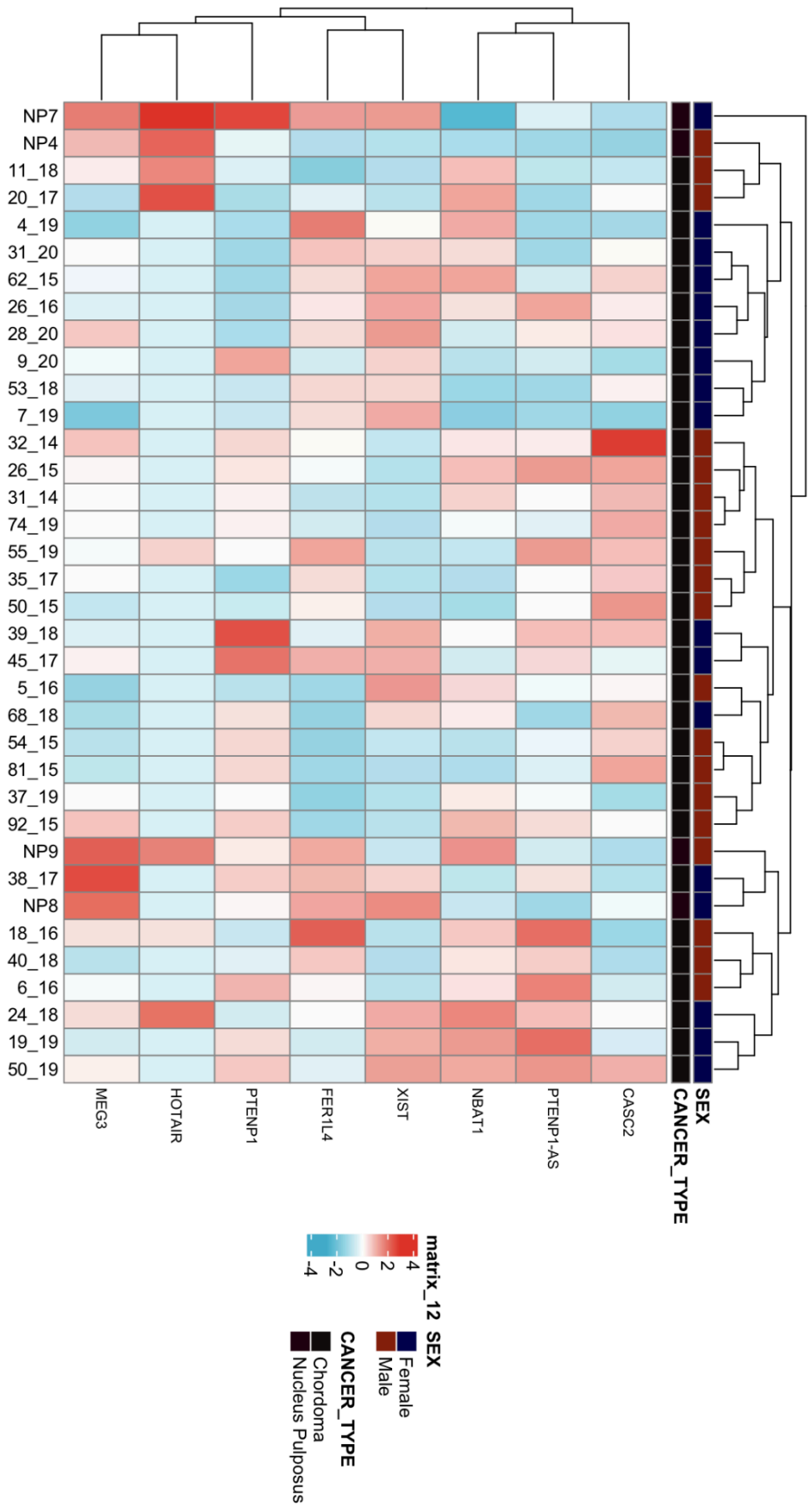
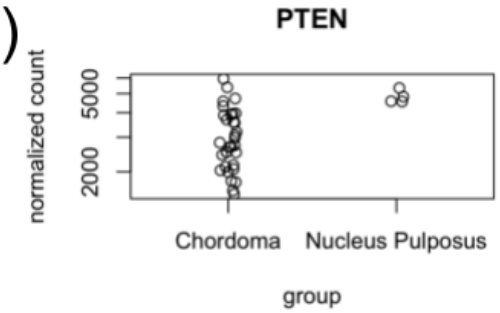

NP7
NP4
11_18
20_17
4_19
31_20
62_15
26_16
28_20
9_20
53_18
7_19
32_14
26_15
31_14
74_19
55_19
35_17
50_15
39_18
45_17
5_16
68_18
54_15
81_15
37_19
92_15
NP9
38_17
NP8
18_16
40_18
6_16
24_18
19_19
50_19
MEG3
HOTAIR
PTENP1
FER1L4
XIST
NBAT1
PTENP1-AS
CASC2
SEX
CANCER_TYPE
matrix_12
-4
-2
0
2
4
SEX
Female
Male
CANCER_TYPE
Chordoma
Nucleus Pulposus

Figure 9: *Heatmap of lncRNA that regulates PTEN across samples of scaled counts.* Scaled counts per sample of the following PTEN-regulating lncRNAs: PTENP1, PTENP1-AS, CASC2, FER1L4, NBAT1, XIST, MEG3, and HOTAIR (Li et al., 2018).

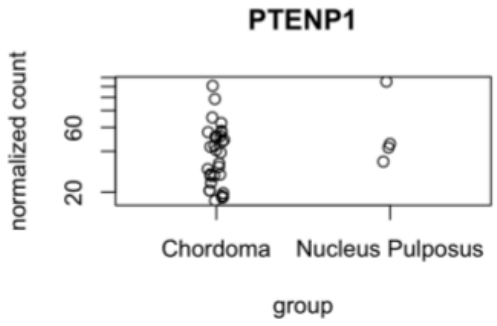
Other researchers have related PTEN expression with deficiency in other tumor suppressors (Seeling et al., 2023) or associated metabolic pathways (e.g., constitutive activation of Akt signaling) (Han et al., 2009). If there is a point mutation that brings on the loss of PTEN expression, the regulation of PTEN by noncoding RNA would not be relevant to restore PTEN expression. If there is no point mutation, regulation through noncoding RNA might be interesting since these noncoding regulators can inhibit PTEN without a genetic mutation. Hence, our interest in characterizing the expression of lncRNAs in chordoma that regulate PTEN in other cancers (Li et al., 2018). For lncRNAs that upregulate PTEN (CASC2, FER1L4, NBAT1, XIST, MEG3, PTENP1), if the sample has low PTEN counts, we expect these genes to be lowly expressed. The caveat being that expression of mRNA transcripts does not necessarily mean PTEN is functionally expressed. For lncRNAs that downregulate PTEN (PTENP1-AS, HOTAIR), if the sample has low PTEN, we expect these genes to be highly expressed. For lncRNAs that upregulate PTEN, we saw less expression within chordoma relative to disk tissue for FER1L4 (log2FoldChange = -1.8, padj = 0.0056), MEG3 (log2FoldChange = -6.0, padj = 2.9E-17), and PTENP1 (log2FoldChange = -0.39, padj = 0.57). However, we also saw greater expression of CASC2 (log2FoldChange = 0.68, padj = 0.13), NBAT1 (log2FoldChange = 1.1 , padj = 0.10), and XIST (log2FoldChange = 0.57, padj = 0.47). For lncRNAs that downregulate PTEN, we saw greater expression within chordoma relative to disk tissue for PTENP1-AS (log2FoldChange = 2.0, padj = 0.057). However, we saw greater expression within chordoma

relative to disk tissue for HOTAIR (log2FoldChange = -0.22, padj = NA due to too low counts) (Appendix Table 1). The heatmap helps visualize the variation between samples (Figure 9). Ultimately, these non-coding RNA are regulate PTEN in other cancers, so the unexpected relationship between PTEN and these genes could be a function of differences in cancer type.

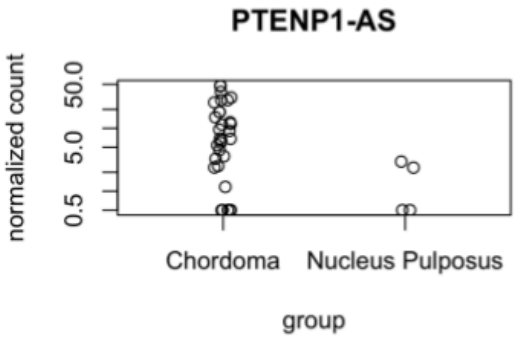
a)


PTEN
normalized count
5000
2000
Chordoma
Nucleus Pulposus
group

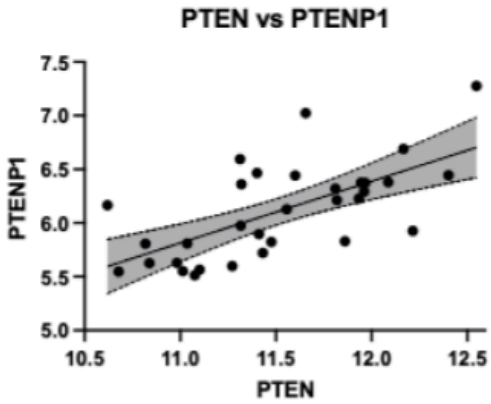
b)


PTENP1
normalized count
60
20
Chordoma
Nucleus Pulposus
group

c)


PTENP1-AS
normalized count
50.0
5.0
0.5
Chordoma
Nucleus Pulposus
group

d)


PTEN vs PTENP1
PTENP1
7.5
7.0
6.5
6.0
5.5
5.0
10.5
11.0
11.5
12.0
12.5
PTEN

e)

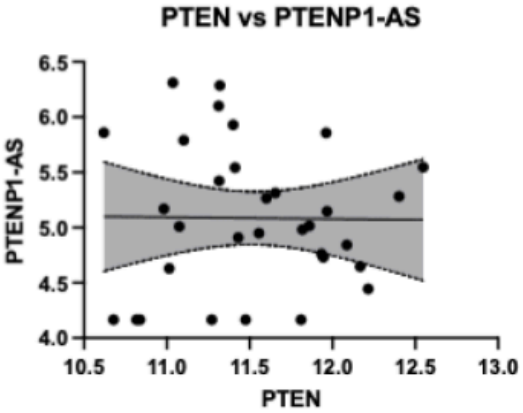

PTEN vs PTENP1-AS
PTENP1-AS
6.5
6.0
5.5
5.0
4.5
4.0
10.5
11.0
11.5
12.0
12.5
13.0
PTEN

Figure 10: *Relationship between PTEN and PTEN regulators PTENP1 and PTENP1-AS.* a) PTEN counts plotted for chordoma and nucleus pulposus samples. b) PTENP1 counts plotted for chordoma and nucleus pulposus samples. c) PTENP1-AS counts plotted for chordoma and nucleus pulposus samples. d) Scatter plot correlating PTEN and PTENP1 counts within chordoma samples. The regression line is bordered with a 95% confidence interval with r^2= 0.43, y-intercept = -0.5061, and slope = 0.5745. e) Scatter plot correlating PTEN and PTENP1-AS counts within chordoma samples. The regression line is bordered with a 95% confidence interval with r^2= 0.00014, y-intercept = 5.264, and slope = -0.01545.

We next wanted to understand the relationship of PTEN to these lncRNAs that regulate PTEN expression. Since PTENP1 upregulates PTEN expression, we expect greater PTEN expression to correlate with greater PTENP1 expression in chordoma. Since PTENP1-AS downregulates PTEN expression, we expect greater PTEN expression to correlate with less PTENP1-AS expression in chordoma. We saw that PTEN expression was varied within the chordoma, with high counts in the nucleus pulposus (Figure 10a). We saw that PTENP1 expression was varied within chordoma, with moderate to high counts in nucleus pulposus (Figure 10b). We saw that PTENP1-AS expression was varied within chordoma, with moderate to low counts in nucleus pulposus (Figure 10c). The varied expression of PTEN and PTEN regulators shows the heterogeneity of PTEN expression. For PTENP1, PTEN is moderately positively correlated (r^2= 0.43) within chordoma (Figure 10d). For PTENP1-AS, PTEN expression does not seem to be correlated (r^2= 0.00014) within chordoma (Figure 10e). Though these lncRNAs have minor effects on their own, we propose that through targeting these noncoding RNA regulators, the tumor suppressor effects of PTEN could be restored through these fine-tuning mechanisms.

Further studies are necessary to confirm whether targeting these lncRNAs in chordoma can restore PTEN function.

Chapter 4: Discussion and Conclusions

4.1 Stratify PTEN Expression between Primary and Recurrent

Findings: We found a possible trend of differential PTEN expression between primary and recurrent tumors in Nanostring and RT-qPCR results. Characterized cell lines can be the basis for further research on PTEN in relation to other biomarkers or therapeutics. This aligns with previous research that found that LOH occurs for PTEN in the recurrent chordoma. The limitation is that most of the samples were sacral, so there was no comparison of the difference between the locational expression of PTEN. *Limitations:* Though UCH1N (notochordal cells from which chordoma is derived) was meant as a PTEN control, we saw a lack of PTEN expression. A possible explanation is that UCH1 (the recurrent cell line that we found to have PTEN loss) is the parent cell line of UCH1N. UCH1N is a sub-section of stem cells within UCH1, so even though the cell line is a model of notochordal cells, it does not regain PTEN function. Others have found that UCH1N and UCH1 share similar properties, such as the same mutation in p53, another tumor suppressor gene (Kato et al., 2011). So though UCH1N is notochordal-like, the cell line may not fully represent notochordal tissue that would be found directly from a human. The main constraint of non-cell line notochordal tissue as a healthy control is that fresh notochordal tissue is often not available as a control, so we could explore using other similar tissue, as we have done with disk tissue. An additional limitation is that due to the tissue processing process, the RNA integrity in FFPE samples tends to be insufficient for assays that rely on high RNA quality (e.g., RT-qPCR). Since RT-qPCR relies on reverse transcriptase, the quality has to be such that the enzyme can bind and convert RNA to cDNA. So, within our own experiments, we were unable to. A possible solution is to use fresh frozen patient

samples rather than FFPE archival tissue. The solution we chose was to explore methods, such as probe-based assays (both spatial and non-spatial), that are less sensitive to RNA degradation. Also, even though the published data that was used for the RNA-seq analysis had 31% recurrent, the primary and recurrent samples were pooled together, so they could not be separated. In the future, we could reach out to Baluszek et al. to ask which samples were recurrent and which were primary to investigate whether there's stratification of PTEN expression in skull-based chordoma. *Importance:* If we can stratify primary and recurrent based on PTEN expression, there are implications to understanding the possible reason why recurrent is more treatment resistant than primary. Alternatively, rather than stratification, PTEN could be lost in primary, which leads to loss of PTEN expression in or after recurrence. If there is overlap in PTEN and therapeutic targets for recurrent chordoma, it could be interesting to explore if targeting PTEN loss would improve the efficacy of combined therapeutics by restoring PTEN's cancer protective effects. PTEN therapy can be done in combination with other treatments to restore the tumor suppressor in recurrent chordoma and possibly improve the efficiency of other treatments by restoring these cancer protective mechanisms. *Future studies:* Since there was heterogeneity in PTEN expression in nanostring between samples, spatially understanding how primary and recurrent differ could give insights into the nature of the trend of less PTEN expression in recurrent relative to primary. For example, what is happening is that the concentration of PTEN is lost in recurrent chordoma, or rather, there is diffuse PTEN expression but low PTEN expression throughout the tissue. Additionally, characterizing differential PTEN expression in relation to markers such as Brachyury could suggest possible interactions between PTEN and other biomarkers of chordoma, similar to the Seelin et al. (2023) study that characterized P16

and PTEN within the same chordoma cell lines to determine whether there was a relationship between PTEN and P16 expression.

4.2: Importance of Comprehensive PTEN Transcriptomics Profiling in Understanding Heterogeneity Within and Between Chordoma Samples

Findings: In qPCR, we were able to determine whether or not there is the presence of PTEN. With Nanostring, we were able to get counts to determine the degree to which PTEN is expressed and able to see heterogeneity between samples. In the Nanostring data, even though they all expressed PTEN, some expression PTEN with counts multiple folds greater than other samples. Within the RNA-seq data, there was variation in how much PTEN was expressed between chordoma samples. Within the RNAscope, the expression of PTEN was seen in specific clusters rather than uniformly throughout the tissue. *Limitations:* The qPCR worked for cell culture, but not for the archive FFPE tissue, so we couldn't directly compare how qPCR and nanostring characterized the same tissue. Additionally, the RNA-seq data come from a published dataset, so the results do not directly relate to the sample studied with qPCR, nanostring, or RNAscope. Vice versa, the probe-based assay (Nanostring) and spatial transcriptomics (RNAscope) used the archived FFPE tissue, but cell culture was not characterized using Nanostring or RNAscope. Additionally, RNAscope was a pilot to optimize the protocol within the chordoma tissue. Since we saw differential staining on the edges, that implies further troubleshooting of methods is required before the method can be used to compare the spatial transcriptomic profile samples with other assays and between samples. *Importance:* Different types of results can be put together to give a comprehensive understanding of what occurs for PTEN expression, beyond whether there is or is not expression. Given these findings, it would be

worth revisiting these results that characterize PTEN in whether there is or is not expression to understand whether those samples studied with probe-based assays would find that there might be PTEN expression, but not to the same degree. The importance of understanding PTEN expression beyond yes or no expression, but also spatially, allows us to understand whether low expression level is there due to low expression across most of the tissue, or rather, there are clusters of high expression with mostly no expression. Based on whether the situation is that there are many cells with low PTEN or if there is a mix of cells with PTEN expression and those without PTEN, those without PTEN expression would still be targets for PTEN expression, even if there are cells with PTEN expression present in the tissue. *Future studies:* Once RNAscope's protocol is troubleshooted (e.g., straining most prominently at edges, which is likely due to further optimization needed in the permeabilization step), the method can be used to spatial transcriptomically characterize the tissue, which was characterized by NanoString. We are interested in understanding whether the heterogeneity in PTEN expression is due to variation in expression between cells or if there is varied expression within cells. By investigating spatial heterogeneity in PTEN, we can see whether there are areas where PTEN expression is concentrated or if there is no spatial heterogeneity. Also, by investigating how to recover RNA quality for RT-qPCR of RNA from FFPE tissue, we could see how RT-qPCR (a tool cheaper than nanostring) characterization of the patient samples would be of interest. Additionally, differential expression of RNA-seq and exome data to determine differences and similarities in differential expression between genetic and transcriptional data. For genes differentially expressed between DNA and RNA data, do STRING analysis to determine whether/ how these genes relate to PTEN. Another possible assay to investigate heterogeneity of expression is single-cell RNA sequencing to profile heterogeneity of PTEN expression between various cells in the tumor.

4.3: Possible Paths for Developing PTEN Therapeutics in Chordoma

Findings: Within other cancers, researchers have found targetable noncoding RNA that regulates PTEN expression. We also looked at how lncRNA related to PTEN regulation is expressed and expected similar relationships. However, since PTEN was expressed variably between samples, there didn't seem to be a clear relationship between PTEN and these regulators. Though there was heterogeneity of PTEN regulators, there were general trends for the presence of certain regulators relative to PTEN (e.g., PTENP1). However, though we expected a certain relationship between PTEN and regulators, the relationships/ correlations were not as clear as expected. Limitations: Though there were 30413 non-zero genes characterized, there was a limited sample size of 32 chordoma samples, with 4 nucleus pulposus samples. Additionally, the dataset is public, so there is no control over how the data is prepared. Chordoma is derived from remnant notochordal tissue, and though notochordal tissue can be within the nucleus pulposus, the nucleus pulposus has non-notochordal tissue. However, isolated notochordal tissue is difficult to procure as a control. The location of the chordoma was also not clear in the dataset for each sample. Additionally, for PTENP1-AS, there are alpha and beta subtypes that regulate PTEN differently. PTENP1-AS alpha downregulates PTEN while PTENP1-AS beta upregulates PTEN (Li et al., 2018). Our results show almost a flat horizontal line when correlating PTEN expression with PTENP1-AS, which could possibly be due to a mixing of the beta and alpha subtypes. *Importance:* By characterizing PTEN and genes that are related, there are clinical implications for the treatment of chordoma. For example, if there is a clear relationship between the presence of PTEN and a regulator of PTEN, that regulator could be a possible therapeutic target. There are also diagnostic implications where a chip test can be obtained from the sample

to see the presence of noncoding RNA that downregulates PTEN or the absence of noncoding RNA that upregulates PTEN. *Future Studies:* Though looking through the regulators we did, future researchers can either look at these markers in a different way or look for other biomarkers related to PTEN regulation. Additionally, rather than using published datasets, we can, in the future, use RNA-sequencing data from our own samples. That would allow for confirmatory assays to be done based on findings in the same samples that were sequenced. Additionally, relating nanostring or RNA-sequencing data with other molecular sequencing data, such as whole exome sequencing, to relate exome expression to RNA expression to determine whether the reduced RNA expression is from a genetic mutation or regulation of the gene's expression. For example, to understand how LOH impacts transcripts, allele frequency (which can determine LOH) can be correlated with NanoString RNA counts.

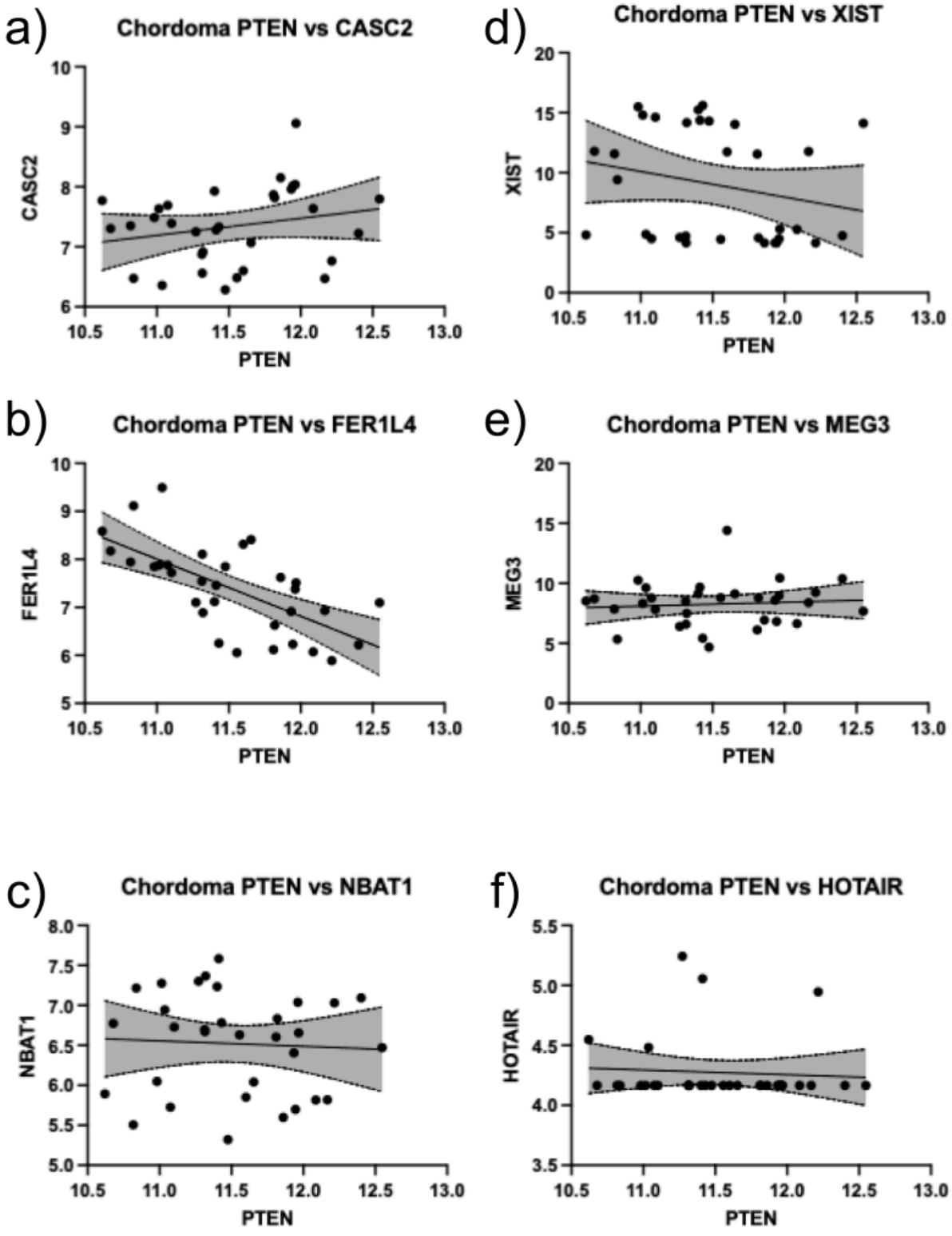
4.4: Conclusion

We profiled PTEN expression in various cell lines and patient samples, as well as beginning the process of studying the mechanism of PTEN loss in the hope of creating a foundation to find new biomarkers for chordoma, as well as new therapeutic targets. This thesis studies the expression of PTEN in chordoma with future implications for understanding epigenetic regulation of PTEN when the cause of PTEN loss is not a genetic mutation. Finally, this research reinforced the importance of stratifying patients and finding new treatments for rare cancers such as chordoma.

Appendix

Gene	BaseMean	Log2FoldChange	LfcSE	P-Value	Padj
PTENP1	43.91	-0.39	0.59	0.39	0.573
MEG3	1776.63	-6.0	0.71	2.3E-19	2.9E-17
XIST	11925.33	0.57	0.90	0.29	0.47
NBAT1	66.06	1.1	0.71	0.034	0.10
FER1L4	182.20	-1.8	0.70	0.00088	0.0056
CASC2	136.87	0.68	0.39	0.049	0.13
PTENP1-AS	10.88	2.0	1.6	0.016	0.057
HOTAIR	1.49	-0.22	1.6	0.53	NA
PTEN	3275.69	-0.71	0.41	0.053	0.14

Appendix Table 1: *DESeq2 results for PTEN and lncRNA PTEN regulators (CASC2, FER1L4, NBAT1, XIST, MEG3, and HOTAIR).* The columns consist of the base mean, log2foldchange, lfcSE, p-value, and Benjamini-Hochberg corrected p-values (padj). The full list of differentially expressed transcripts between chordoma and nucleus pulposus samples can be found in the following link (https://docs.google.com/spreadsheets/d/1KUzNPsiu-I7Pa2j1ZjifKavmamR3_wNVixiMmwBt9Kg/edit?usp=sharing).


a)
Chordoma PTEN vs CASC2
CASC2
PTEN
b)
Chordoma PTEN vs FER1L4
FER1L4
PTEN
c)
Chordoma PTEN vs NBAT1
NBAT1
PTEN
d)
Chordoma PTEN vs XIST
XIST
PTEN
e)
Chordoma PTEN vs MEG3
MEG3
PTEN
f)
Chordoma PTEN vs HOTAIR
HOTAIR
PTEN

Appendix Figure 1: *Relationship between PTEN and PTEN regulators CASC2, FER1L4, NBAT1, XIST, MEG3, and HOTAIR.* a) Scatter plot correlating PTEN and CASC2 counts within chordoma samples. The regression line is bordered with a 95% confidence interval with r^2= 0.051, y-intercept = 4.1, and slope = 0.29. b) Scatter plot correlating PTEN and FER1L4 counts within chordoma samples. The regression line is bordered with a 95% confidence interval with r^2 = 0.43, y-intercept = 21, and slope = -1.2. c) Scatter plot correlating PTEN and NBAT1 counts within chordoma samples. The regression line is bordered with a 95% confidence interval with r^2= 0.0030, y-intercept = 7.3, and slope = -0.068. d) Scatter plot correlating PTEN and XIST counts within chordoma samples. The regression line is bordered with a 95% confidence interval with r^2= 0.054, y-intercept = 33, and slope = -2.1. e) Scatter plot correlating PTEN and MEG3 counts within chordoma samples. The regression line is bordered with a 95% confidence interval with r^2= 0.0076, y-intercept = 4.6, and slope = 0.32. f) Scatter plot correlating PTEN and HOTAIR counts within chordoma samples. The regression line is bordered with a 95% confidence interval with r^2= 0.0052, y-intercept = 4.7, and slope = -0.040.

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