# TARGETED APPROACHES REVEAL EPIGENETIC REGULATION OF LYSOSOMAL EXOCYTOSIS CONTRIBUTING DRUG SENSITIVITY AND TRANSCRIPTIONAL ACTIVATION

By

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*to Dudu, Fiko, and Ismo..*

*to my grandfather, Hasan Ali Güven..*

*to my uncle, Hasan Bezirgan..*

# ABSTRACT

**Targeted Approaches Reveal Epigenetic Regulation of Lysosomal Exocytosis Contributing Drug Sensitivity and Transcriptional Activation**

The limited effectiveness of chemotherapy in many solid tumors often stems from intrinsic cellular mechanisms that reduce intracellular drug accumulation. One such mechanism is lysosomal sequestration followed by exocytosis, wherein chemotherapeutic agents are trapped within acidic vesicles and expelled from the cell, thereby diminishing cytotoxic efficacy. Although this process is increasingly recognized as a contributor to treatment failure, its upstream regulatory control has remained poorly defined. In particular, the role of chromatin-modifying enzymes in governing lysosomal dynamics has not been systematically investigated.

This thesis explores the hypothesis that lysosomal exocytosis is subject to epigenetic regulation and that pharmacologic inhibition of specific chromatin-modifying enzymes may suppress vesicle-mediated drug clearance. A focused epidrug library comprising 175 small-molecule inhibitors was conducted to identify compounds that influence lysosomal exocytosis, vesicle accumulation, and cisplatin sensitization in cancer cells. Two inhibitors targeting Type I protein arginine methyltransferases (PRMTs) emerged as potent hits, significantly enhancing cisplatin cytotoxicity without causing baseline toxicity. These inhibitors reduced lysosomal exocytosis and increased lysosomal content independently of canonical transcription factors governing lysosomal biogenesis. Transcriptomic profiling following PRMT inhibition revealed suppression of chromatin maintenance and DNA repair pathways, alongside induction of oxidative stress and apoptosis-related genes. Gene set enrichment analysis further demonstrated enrichment of lysosome-related pathways, including vesicle-mediated transport and regulation of lysosomal enzymes, providing additional support for the impact of PRMT inhibition on lysosomal dynamics. Integration with public ChIP-seq datasets indicated that many differentially expressed genes are direct PRMT targets and the promoter occupancy of PRMTs to these target genes are further verified in ChIP-qPCR experiments. All of these findings were validated across multiple cell lines and extended to other lysosomally sequestered drugs, highlighting broader therapeutic relevance. Moreover, analysis of patient-derived transcriptomes demonstrated that high PRMT1

and PRMT6 expression correlates with poor chemotherapy response, reinforcing the clinical significance of epigenetic control over lysosomal exocytosis.

To complement this insight, the second part of the thesis investigates whether epigenetic modifiers can also regulate the transcription of genes involved in lysosome-mediated metal homeostasis and drug resistance. A CRISPR/Cas9-mediated knock-in model was developed by inserting a firefly luciferase cassette into exon 1 of one ATP7B allele, generating a reporter cell line that preserves the endogenous chromatin environment. Screening the same epidrug library in reporter assay identified several histone deacetylase (HDAC) inhibitors as potent inducers of ATP7B transcription. These effects were validated through promoter-reporter assays, RT-qPCR, and chromatin immunoprecipitation, which showed a specific enrichment of H3K18 acetylation mark over H3K9 and H3K27 acetylation at the ATP7B promoter following HDAC inhibition. Importantly, this transcriptional activation was conserved across hepatic and non-hepatic cell types and occurred independently of ROS accumulation.

Together, these studies uncover chromatin-level mechanisms that regulate both lysosomal drug efflux and the expression of a clinically relevant copper transporter. By identifying epigenetic targets that influence drug retention and gene activation, this work provides a conceptual and experimental framework for improving therapeutic strategies in cancer and metal storage disorders.

**Keywords:** Epigenetic Regulation, Lysosomal Exocytosis, Epidrug Library Screening, Type I PRMTs, Cisplatin, Knock-in Reporter System, ATP7B

# ÖZETÇE


**Hedefe Yönelik Yaklaşımlar ile Lizozomal Ekzositozun Epigenetik Düzenlenmesinin İlaç Duyarlılığı Ve Transkripsiyonel Aktivasyon Üzerindeki Etkilerinin İncelenmesi**

Birçok solid tümörde kemoterapinin sınırlı etkinliği, sıklıkla hücre içi ilaç birikimini azaltan hücresel mekanizmalardan kaynaklanır. Bu mekanizmalardan biri, lizozomal hapsolma ve bunu takip eden ekzositozdur; bu süreçte kemoterapötik ajanlar asidik veziküller içinde hapsedilir ve hücre dışına atılarak sitotoksik etkinlikleri sınırlandırılır. Bu mekanizma tedavi başarısızlığına sebebiyet veren bir süreç olarak giderek daha fazla tanınsa da, söz konusu olayı düzenleyen mekanistik regülasyonlar hâlâ yeterince aydınlatılamamıştır. Özellikle, kromatin düzenleyici enzimlerin lizozomal dinamikleri nasıl kontrol ettiği sistematik olarak araştırılmamıştır.

Bu tez, lizozomal ekzositozun epigenetik bir düzenlemeye tabi olabileceği ve belirli kromatin düzenleyici enzimlerin farmakolojik inhibisyonunun vezikül aracılı ilaç uzaklaştırımını baskılayabileceği hipotezini araştırmaktadır. Bu amaçla, 175 küçük molekül inhibitör içeren hedefe yönelik bir epigenetik ilaç (epi-ilaç) kütüphanesi taranmış ve lizozomal ekzositoz, vezikül birikimi ve sisplatin duyarlılığı üzerinde etkili bileşikler belirlenmiştir. Tip I protein arjinin metiltransferazlarını (PRMT'ler) hedefleyen iki inhibitör güçlü adaylar olarak öne çıkmış ve kendileri bazal toksisite oluşturmaksızın sisplatin sitotoksisitesini anlamlı düzeyde artırmıştır. Bu inhibitörler, lizozomal ekzositozu azaltmış ve Hücre içindeki lizozomal içeriği artırmıştır; üstelik bu etkiler lizozomal biyogenezden sorumlu kanonik regülasyon ve buna ait transkripsiyon faktörlerinden bağımsız olarak gerçekleşmiştir. PRMT inhibisyonunu takiben yapılan transkriptom analizleri, kromatin bakımı ve DNA onarım yollarının baskılandığını, oksidatif stres ve apoptoz ile ilişkili genlerin ise arttığını göstermiştir. Gen seti zenginleştirme analizleri, PRMT inhibisyonunun lizozomal dinamikler üzerindeki etkisini destekler nitelikte, vezikül aracılı taşıma ve lizozomal enzimlerin düzenlenmesi gibi lizozomla ilişkili yolların zenginleştiğini de ortaya koymuştur. Çevrimiçi erişilebilir ChIP-seq verileriyle yapılan entegrasyon, farklı şekilde eksprese edilen genlerin birçoğunun doğrudan PRMT hedefi olduğunu göstermiş ve bu genlerin promotör bölgelerinde PRMT bağlanmasının varlığı ChIP-qPCR deneyleriyle doğrulanmıştır. Tüm bu bulgular birden fazla hücre hattında doğrulanmış ve lizozomal olarak hapsedilen diğer ilaçlara da genellenerek terapötik etkinliğin daha geniş bir bağlamda değerlendirilebileceğini göstermiştir. Ayrıca, hastalara ait transkriptom analizleri, yüksek PRMT1 ve PRMT6 ekspresyonunun zayıf kemoterapi yanıtıyla ilişkili olduğunu göstermiş ve lizozomal ekzositozun epigenetik kontrolünün klinik önemini desteklemiştir.

Bu bulgulara tamamlayıcı olarak, tezin ikinci bölümünde epigenetik düzenleyicilerin, lizozom aracılı metal homeostazı ve ilaç direnciyle ilişkili genlerin transkripsiyonunu da düzenleyip düzenleyemeyeceği araştırılmıştır. Bu amaçla, ATP7B genine alel spesifik "firefly luciferase" ekspresyon kaseti eklenerek CRISPR/Cas9 ile düzenlenmiş bir "knock-in" model geliştirilmiş ve böylece endojen kromatin ortamını koruyan bir raporlayıcı hücre hattı elde edilmiştir. Aynı epigenetik ilaç kütüphanesinin bu rapor sisteminde taranması sonucunda, bazı histon deasetilaz (HDAC) inhibitörlerinin ATP7B transkripsiyonunu güçlü şekilde indüklediği belirlenmiştir. Bu etkiler, promotör-raporlayıcı analizleri, RT-qPCR ve kromatin immünopresipitasyonu ile doğrulanmış; HDAC inhibisyonunu takiben ATP7B promotöründe H3K9 ve H3K27 asetilasyonuna kıyasla özellikle H3K18 asetilasyonunun arttığı gösterilmiştir. Önemli olarak, bu transkripsiyonel aktivasyon hem hepatik hem de hepatik olmayan hücre türlerinde korunmuş ve reaktif oksijen türleri (ROS) birikiminden bağımsız gerçekleşmiştir.

Bu çalışmalar birlikte ele alındığında, hem lizozomal ilaç dışa atımını hem de klinik açıdan önemli bir bakır taşıyıcısının ekspresyonunu kontrol eden kromatin düzeyindeki mekanizmaları ortaya koymaktadır. İlaç tutulumunu ve gen aktivasyonunu etkileyen epigenetik hedeflerin tanımlanması yoluyla, bu tez kanser ve metal birikim hastalıklarında terapötik stratejilerin geliştirilmesine yönelik kavramsal ve deneysel bir çerçeve sunmaktadır.

**Keywords:** Epigenetik Düzenleme, Lizozomal Ekzositoz, Epigenetik İlaç Kütüphanesi Taraması, Tip I PRMT'ler (Protein Arjinin Metiltransferazlar), Sisplatin, Knock-in Raporlayıcı Sistem, ATP7B

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# ABBREVIATIONS

**3′UTR;** 3′ Untranslated Region

**5-FU;** 5-Fluorouracil

**ABC;** ATP-Binding Cassette

**ADMA;** Asymmetric Dimethylarginine

**ATAC-seq;** Assay for Transposase-Accessible Chromatin using sequencing

**BET;** Bromodomain and Extra-Terminal Domain

**Cas9;** CRISPR-associated protein 9

**CETCh-seq;** CRISPR Epitope Tagging Chromatin Immunoprecipitation Sequencing

**ChIP-seq;** Chromatin Immunoprecipitation Sequencing

**chr;** Chromosome

**CLEAR;** Coordinated Lysosomal Expression and Regulation

**CRISPR;** Clustered Regularly Interspaced Short Palindromic Repeats

**CRPC;** Castration-Resistant Prostate Cancer

**Cu;** Copper

**$CuCl_2$;** Copper Chloride

**CSC;** Cancer Stem Cell

**dCas9;** Dead Cas9

**DNMT;** DNA Methyltransferase

**DTP;** Drug-Tolerant Persister

**ECM;** Extracellular Matrix

**EMT;** Epithelial-to-Mesenchymal Transition

**Epidrug;** Epigenetic Modifier Drug

**ER;** Endoplasmic Reticulum

**FDA;** Food and Drug Administration

**FLAG;** Short Peptide Tag DYKDDDDK

**GFP;** Green Fluorescent Protein

**GSH;** Glutathione

**GST;** Glutathione S-Transferase

**HDR;** Homology-Directed Repair

**HDAC;** Histone Deacetylase

**HAT;** Histone Acetyltransferase

**HR;** Homologous Recombination

**IRES;** Internal Ribosome Entry Site

**lncRNA;** Long Non-Coding RNA

**MDR1;** Multidrug Resistance 1 (gene encoding P-glycoprotein)

**miRNA;** MicroRNA

**MiT/TFE;** Microphthalmia/Transcription Factor E family

**MMA;** Monomethylarginine

**MNK;** Menkes Disease

**MRE;** Metal-Responsive Element

**MT;** Metallothionein

**MTF1;** Metal-Responsive Transcription Factor 1

**NSCLC;** Non-Small Cell Lung Cancer

**P-gp;** P-glycoprotein

**P2A;** Porcine teschovirus-1 2A peptide

**PDAC;** Pancreatic Ductal Adenocarcinoma

**PRMT;** Protein Arginine Methyltransferase

**RT-qPCR;** Reverse Transcription quantitative Polymerase Chain Reaction

**ROS;** Reactive Oxygen Species

**SAM;** S-adenosylmethionine

**SASP;** Senescence-Associated Secretory Phenotype

**SDMA;** Symmetric Dimethylarginine

**SCLC;** Small-Cell Lung Cancer

**sgRNA;** Single Guide RNA

**SNARE;** Soluble NSF Attachment Protein Receptor

**T2A;** Thosea asigna virus 2A peptide

**TFEB;** Transcription Factor EB

**TME;** Tumor Microenvironment

**TSS;** Transcriptional Start Site

**UPR;** Unfolded Protein Response

**V-ATPase;** Vacuolar $H^+$-ATPase

**WD;** Wilson's Disease

*Chapter 1*

# TARGETING EPIGENETIC MODULATORS OF LYSOSOMAL EXOCYTOSIS TO ENHANCE CHEMOTHERAPEUTIC EFFICACY IN CANCER

## SUMMARY

Chemotherapy remains a cornerstone of cancer treatment, yet its effectiveness is often limited by cellular mechanisms that reduce drug accumulation and activity. One such mechanism is lysosomal sequestration followed by exocytosis, in which chemotherapeutic agents are compartmentalized within acidic vesicles and actively expelled from the cell. This process decreases intracellular drug levels and can restrict the efficacy of widely used agents, including platinum-based compounds. Although lysosomal exocytosis has been linked to reduced treatment effectiveness, the upstream regulatory pathways controlling this process remain poorly defined. In particular, the contribution of epigenetic regulators to lysosomal function has not been systematically explored.

In this study, we hypothesized that lysosomal exocytosis is under epigenetic control and that pharmacological inhibition of specific chromatin-modifying enzymes could suppress this process and improve chemotherapeutic efficacy. To test this, we screened a 175-compound epigenetic drug library for effects on lysosomal exocytosis, lysosomal accumulation, and cisplatin sensitization in cancer cells. Two inhibitors of Type I protein arginine methyltransferases (PRMTs), MS023 and GSK3368715, emerged as potent hits. These compounds reduced lysosomal exocytosis, increased intracellular lysosome content, and significantly enhanced the cytotoxicity of cisplatin, without inducing baseline toxicity. These effects occurred independently of canonical lysosomal biogenesis gene expression.

Transcriptomic profiling following MS023 treatment revealed broad gene expression changes, including repression of chromatin maintenance and DNA repair genes, alongside upregulation of pathways involved in vesicle trafficking, oxidative stress, and apoptosis. Integration with public ChIP-seq datasets confirmed that many differentially

expressed genes are direct targets of PRMTs, supporting an epigenetic mechanism of lysosomal modulation. Key transcriptional changes were validated in two additional cancer cell lines and with both PRMT inhibitors. The compounds also sensitized cells to other lysosomally sequestered agents, indicating broader therapeutic potential. Finally, analysis of clinical gene expression datasets showed that high PRMT1 and PRMT6 expression correlates with no response to therapy regimes, reinforcing the clinical relevance of our findings.

In conclusion, this study identifies lysosomal exocytosis as an epigenetically regulated process that limits the effectiveness of chemotherapy. By uncovering a novel role for Type I PRMTs in controlling lysosomal dynamics, the findings establish a mechanistic basis for combining epigenetic inhibitors with standard chemotherapeutics. This work lays the foundation for future strategies aimed at improving treatment outcomes by targeting vesicle-mediated drug clearance through chromatin-level intervention.

**Keywords:** Lysosomal Exocytosis, Epidrug Library Screening, Lysosomal Biogenesis, Type I PRMT, Cisplatin

*Chapter 1*

*Section 1*

# 1. INTRODUCTION

## 1.1. Chemotherapy Resistance and Tolerance in Solid Tumors: Mechanisms of Tumor Adaptation

Chemotherapy remains one of the central strategies in managing solid tumors, providing initial tumor regression and extending survival in many cancer types. Despite its benefits, one of its most significant limitations is the development of resistance, a phenomenon that frequently results in treatment failure and disease relapse (Chabner & Roberts, 2005; Holohan et al., 2013). This resistance may be intrinsic, where cancer cells possess pre-existing traits that render them less susceptible to drugs, or acquired, developing over the course of treatment as tumors adapt to the selective pressure of chemotherapy (Vasan et al., 2019). These adaptive changes, which emerge in response to the cytotoxic environment imposed by drugs, are central to the persistence and progression of malignancies. In this context, some cells evade cell death not by permanent genetic resistance but through reversible survival strategies, a phenomenon referred to as chemotherapy tolerance (De Conti et al., 2021; Sharma et al., 2010). Understanding how cancer cells develop both resistance and tolerance is essential to improving the long-term efficacy of chemotherapeutic regimens.

Intrinsic resistance is present before therapy begins, often due to genetic mutations or cellular phenotypes that diminish drug effectiveness (Gunnarsson et al., 2020; Khan et al., 2024; Masud et al., 2023). Some cancer cells may already express high levels of drug efflux transporters, altered drug targets, or possess superior DNA repair capabilities, making them inherently less sensitive to chemotherapeutic agents. In contrast, acquired resistance emerges through clonal selection. When chemotherapy eliminates sensitive cells, resistant clones—whether pre-existing or newly adapted—survive and repopulate the tumor, often with enhanced fitness and drug tolerance (Mahgoub et al., 2024; Mansoori et al., 2017). This evolutionary process is facilitated by intratumoral heterogeneity, a defining characteristic of most solid tumors. Cancer cells within the same tumor may differ in genotype, gene expression, metabolic profile, or proliferative

behavior, enabling diverse survival strategies under therapeutic stress (Eslami et al., 2024; Khan et al., 2024).

In solid tumors such as non-small cell lung cancer (NSCLC), prostate cancer, and pancreatic ductal adenocarcinoma (PDAC), heterogeneity-driven resistance is frequently observed. In NSCLC, platinum-based drugs can kill the majority of cells, but subpopulations with enhanced DNA repair or impaired apoptotic machinery often survive and contribute to recurrence (Cao et al., 2024; Espinet et al., 2022; Evan et al., 2022; Gutierrez et al., 2021; Sullivan et al., 2014). Similarly, in small-cell lung cancer (SCLC), patients typically respond well to initial chemotherapy, yet relapse with refractory disease is nearly universal, suggesting the persistence and outgrowth of resistant clones (Hamilton et al., 2024; Maleki et al., 2024). In prostate tumors, androgen-dependency varies among cell populations, influencing their chemotherapy responses. PDAC, with its complex microenvironment and poor drug delivery, harbors tumor cells in niches that are inherently less accessible to drugs like gemcitabine, supporting both intrinsic and acquired resistance (Khan et al., 2024; Kneppers et al., 2022; Liang et al., 2017).

Multiple mechanisms contribute to the ability of cancer cells to evade chemotherapy. A common strategy involves reducing intracellular drug accumulation. Cancer cells may do this by increasing expression of ATP-binding cassette (ABC) efflux pumps, which actively transport a wide range of drugs out of the cell (Choi & Yu, 2014; Fan et al., 2023; Pan & Lee, 2025). P-glycoprotein (P-gp), encoded by the MDR1 gene, is a prototypical efflux pump that confers multidrug resistance. Its upregulation has been observed in drug-resistant prostate cancer cell lines and is associated with decreased sensitivity to docetaxel (Lima et al., 2021; Sarwar et al., 2024). In pancreatic cancer, efflux pumps are implicated in the poor intracellular retention of gemcitabine and other agents, contributing to PDAC's resistance profile (Jia & Xie, 2015; Rudin et al., 2011; Yang et al., 2024). NSCLC cells also exploit this mechanism to escape the effects of various chemotherapeutic drugs (Tian et al., 2023; Ughachukwu & Unekwe, 2012). In parallel, some cells reduce drug uptake by downregulating transporter proteins essential for drug entry. For instance, the nucleoside transporter hENT1 is required for gemcitabine uptake in PDAC cells. Its downregulation hinders drug influx, decreasing drug efficacy (Beutel & Halbrook, 2023; Tavano et al., 2014). These adaptations can emerge during therapy,

allowing minor subpopulations with efficient drug efflux or impaired uptake to dominate the post-treatment tumor landscape.

Another level of adaptation involves alterations in drug metabolism. Some chemotherapeutic agents require intracellular activation to exert cytotoxic effects. If tumor cells acquire defects in activating enzymes or upregulate drug-detoxifying pathways, drug efficacy is diminished. For example, gemcitabine must be phosphorylated by deoxycytidine kinase (dCK) to become active. PDAC cells resistant to gemcitabine often display reduced dCK expression or activity (Beutel & Halbrook, 2023; Saiki et al., 2012). Additionally, these cells may overexpress catabolic enzymes such as cytidine deaminase, which degrade gemcitabine before it becomes toxic (Bjanes et al., 2020). Tumor cells can also modify or amplify drug targets to evade inhibition. Methotrexate resistance, for instance, is linked to increased levels or gene amplification of dihydrofolate reductase (DHFR), the drug's target enzyme (Goker et al., 1995; Morales et al., 2009). Similarly, resistance to 5-fluorouracil (5-FU) often involves elevated thymidylate synthase activity, bypassing the drug's inhibitory effects (Peters et al., 2002; Showalter et al., 2008). In tumors treated with platinum-based drugs, enhanced expression of glutathione and conjugation enzymes detoxifies reactive intermediates, a mechanism prominent in lung and ovarian cancers (Nunes & Serpa, 2018; Sawers et al., 2014; Sharma et al., 1993).

Direct modifications of drug targets provide another pathway to resistance. Alterations in target structure or expression can impede drug binding, reducing efficacy. This is evident in tumors resistant to taxanes, which target microtubules. Resistance may arise through mutations in β-tubulin or increased expression of class III β-tubulin, an isoform with reduced drug affinity (Stengel et al., 2010). In prostate cancer, silencing class III β-tubulin can restore sensitivity to docetaxel, emphasizing its role in resistance (Terry et al., 2009). Similarly, resistance to topoisomerase inhibitors like doxorubicin or etoposide is often associated with mutations or reduced expression of topoisomerase II (Burgess et al., 2008; Ganapathi & Ganapathi, 2013). For platinum agents, increased DNA repair capacity effectively alters the target environment by reversing platinum-induced DNA damage, limiting drug action (Stefanou et al., 2021; Wong-Brown et al., 2020). Amplification of drug targets, as seen in methotrexate-resistant cells with DHFR gene

duplication, further illustrates this resistance strategy (Goker et al., 1995; Morales et al., 2009).

A prominent mechanism in many resistant tumors is the upregulation of DNA repair pathways. Chemotherapeutic agents that damage DNA rely on the inability of cancer cells to repair that damage. However, if cells restore or enhance repair mechanisms, they survive. In NSCLC, high ERCC1 expression correlates with cisplatin resistance by accelerating the removal of DNA adducts (Bellmunt et al., 2007; Olaussen et al., 2006). Restoration of homologous recombination, such as through BRCA1/2 reversion mutations, enables repair of double-strand breaks, conferring resistance to platinum and PARP inhibitors (Waks et al., 2020; Walmsley et al., 2024). Tumor cells may also tolerate DNA damage without undergoing apoptosis by using translesion synthesis or fork protection mechanisms (Andersen et al., 2008; Zafar & Eoff, 2017). In this setting, stress response pathways like ATR and CHK1 help stabilize replication forks, allowing continued proliferation despite genotoxic stress (Fernandez et al., 2025; Gralewska et al., 2020). Some resistant cells evade death simply by disabling damage checkpoints, continuing to divide despite accumulating DNA lesions. For example, NSCLC cells with disrupted G2/M checkpoints can bypass the normal arrest triggered by cisplatin, continuing to cycle with damaged DNA (Kryczka et al., 2021; Sarin et al., 2017).

Resistance is also conferred through impaired apoptotic signaling. Many chemotherapies induce cell death through DNA damage, which normally activates intrinsic apoptosis via the p53 pathway. However, p53 is mutated in over half of all cancers, compromising its ability to initiate apoptosis (Hientz et al., 2017; Huang et al., 2019). In prostate cancer, cell lines lacking p53 often display altered sensitivity to chemotherapy; while some may be more prone to mitotic catastrophe, others survive by bypassing apoptosis altogether (Ianzini et al., 2006; Ofner et al., 2025). Anti-apoptotic proteins such as BCL-2, BCL-xL, and MCL-1 are frequently upregulated in resistant tumors, tipping the balance away from programmed cell death (Choudhary et al., 2015; Punnoose et al., 2016). In CRPC, elevated BCL-xL expression is associated with resistance to docetaxel, and targeting it can restore drug sensitivity (Parrondo et al., 2013; Westaby et al., 2024). Similarly, loss-of-function mutations in pro-apoptotic proteins like BAX further reduce the likelihood of drug-induced apoptosis (Moujalled et al., 2023; S. Wang et al., 2024). Additionally, cancer cells may activate survival signaling pathways

in response to treatment. NF-κB, a transcription factor regulating anti-apoptotic genes, is commonly activated in chemoresistant tumors (Godwin et al., 2013; Q. Guo et al., 2024). In prostate cancer models, continuous NF-κB signaling blunts docetaxel efficacy, and its inhibition resensitizes cells to therapy (Magadoux et al., 2014; Thomas-Jardin et al., 2020). The JAK/STAT3 pathway, often triggered by IL-6, is another survival axis that shields cancer cells from chemotherapy-induced apoptosis, particularly in NSCLC (Huang et al., 2022; Li et al., 2015).

Stress response mechanisms such as autophagy also contribute to drug tolerance. Autophagy allows cancer cells to recycle damaged organelles and proteins, providing energy and mitigating stress induced by therapy (Sui et al., 2013). Chemotherapeutics like cisplatin, 5-FU, and doxorubicin are known to induce autophagy, which often supports cell survival under treatment (Ahmadi-Dehlaghi et al., 2023; He et al., 2018; Sumkhemthong et al., 2021). In PDAC, increased autophagy correlates with gemcitabine resistance, and inhibiting autophagy enhances drug sensitivity (Marchand et al., 2023). Another protective mechanism is the unfolded protein response (UPR), activated in response to ER stress caused by misfolded proteins during chemotherapy. Cells that successfully manage UPR survive by restoring protein homeostasis, reducing global protein synthesis, and increasing chaperone expression (Li et al., 2011; Madden et al., 2019). Chemotherapy-induced senescence, a state of reversible cell cycle arrest, also plays a role in tolerance. Senescent cells survive in a dormant-like state during therapy, later re-entering the cycle or secreting cytokines that influence neighboring cells—a phenotype known as senescence-associated secretory phenotype (SASP) (Bajtai et al., 2025; Banerjee et al., 2021).

Phenotypic plasticity enables tumor cells to transition into more drug-tolerant states. The epithelial-to-mesenchymal transition (EMT) is one such process, where epithelial cancer cells acquire mesenchymal features, including motility, invasiveness, and resistance (Dart, 2023; Nurwidya et al., 2012). EMT-like states are associated with reduced proliferation, enhanced DNA repair, and increased expression of efflux pumps and anti-apoptotic proteins (Wani et al., 2025; Xu et al., 2022). This transition often parallels the emergence of cancer stem cell (CSC) traits. CSCs are a minority population within tumors with self-renewal capacity and intrinsic resistance to chemotherapy due to slow division rates and robust defense mechanisms (Li et al., 2021; Zhou et al., 2021). In

prostate and pancreatic cancers, CSC-like cells survive treatment and drive tumor recurrence (Banerjee et al., 2023; Gzil et al., 2019). Notably, non-stem cells can acquire stem-like features through EMT or stress responses, highlighting the reversible and plastic nature of resistance. A related phenomenon is the emergence of drug-tolerant persister (DTP) cells—rare, non-genetically resistant cells that survive high drug doses through reversible epigenetic and metabolic changes (He et al., 2024; Nie & Hu, 2024). These cells can later repopulate tumors, acting as a bridge to permanent resistance.

The tumor microenvironment (TME) plays a pivotal role in chemotherapy resistance. Composed of stromal cells, immune cells, blood vessels, and the extracellular matrix (ECM), the TME can shield cancer cells from therapeutic insults (Wu et al., 2021; Yuan et al., 2023). In PDAC, a dense stroma and dysfunctional vasculature create physical barriers to drug penetration, leaving regions of the tumor underdosed (Jiang et al., 2020). Hypoxic niches within tumors further reduce drug efficacy by promoting survival pathways via HIF-1 activation, shifting metabolism to glycolysis, and suppressing apoptosis (Ozcan, 2023). Stromal components like cancer-associated fibroblasts (CAFs) secrete cytokines such as IL-6 and HGF, activating survival pathways like JAK/STAT3 and MET, which enhance drug resistance (Cao et al., 2025; Kittirat et al., 2022). Immune components, such as tumor-associated macrophages, contribute to resistance by producing growth factors and remodeling enzymes, while suppressing cytotoxic immune responses (Chen et al., 2023). Tumor cells can also communicate resistance via extracellular vesicles, transferring resistance-related proteins and microRNAs to nearby cells (Fontana et al., 2021). Moreover, certain niches in the body, like bone marrow or liver, may support dormant cancer cells that escape drug action due to slow cycling and niche-derived signals (Basu et al., 2022; Gao et al., 2017).

In conclusion, chemotherapy resistance in solid tumors arises from a wide array of interlinked mechanisms. These include drug efflux, altered metabolism, target modification, enhanced repair, impaired apoptosis, stress responses, phenotypic switching, and microenvironmental protection. These adaptations, whether genetic or epigenetic, stable or transient, underscore the profound resilience of cancer. Importantly, many of these mechanisms coexist in resistant tumors, making single-agent therapies ineffective. As research continues to unveil the complexity of tumor adaptation, integrated therapeutic approaches that target both cancer cells and their supportive

environments hold the greatest promise for overcoming resistance and improving patient outcomes.

## 1.2. Cellular Resistance Mechanisms Against Platinum-Based Chemotherapeutic Agents

Platinum-based chemotherapeutic agents - particularly cisplatin, carboplatin, and oxaliplatin - are key components of treatment regimens for numerous solid tumors, including those of the ovary, lung, bladder, head and neck, testis, and gastrointestinal tract. These drugs exert their antitumor effects mainly through the formation of DNA adducts and crosslinks, triggering replication arrest and activating cell death programs. Despite their broad clinical utility, resistance to platinum compounds is a major challenge that limits durable treatment outcomes. Tumor cells can possess intrinsic resistance from the outset or acquire it during therapy through a complex interplay of genetic, epigenetic, metabolic, and microenvironmental adaptations (Galluzzi et al., 2012). The sections below summarize the principal cellular mechanisms through which cancer cells escape platinum-induced cytotoxicity.

### *1.2.1. Genetic Determinants of Resistance*

Genetic alterations are foundational to many platinum resistance phenotypes. One of the most significant determinants is the status of the TP53 gene, which encodes the p53 protein—a key regulator of the DNA damage response. p53 promotes cell cycle arrest and apoptosis in response to DNA lesions caused by platinum drugs. Tumors harboring TP53 mutations often show poor responses to platinum therapy, as dysfunctional p53 fails to initiate apoptosis, allowing damaged cells to survive and proliferate (Koberle & Schoch, 2021; Yang et al., 2022). In contrast, tumors with wild-type p53, such as testicular germ cell tumors, tend to be highly responsive to cisplatin, in part due to robust p53-mediated apoptosis (Cavallo et al., 2013).

Resistance can also evolve through secondary mutations. A striking example is found in BRCA1/2-mutated tumors that initially respond to platinum owing to deficient homologous recombination (HR). Over time, some of these tumors acquire “reversion mutations” that restore BRCA function, reinstating HR and thereby undermining the synthetic lethality exploited by platinum drugs (Norquist et al., 2011; Sakai et al., 2008).

These reversions are frequently detected in relapsed, platinum-resistant ovarian cancers but are rare in platinum-sensitive disease (Collet et al., 2024).

Beyond TP53 and BRCA, amplifications of oncogenes such as CCNE1 (cyclin E1) have been linked to platinum resistance. CCNE1 amplification accelerates S-phase entry and disrupts DNA damage checkpoints, reducing the effectiveness of platinum-induced arrest (Brown et al., 2023; Yang et al., 2022). Conversely, loss of tumor suppressors like RB1 can increase sensitivity, highlighting how the balance of cell cycle regulators influences platinum responses (Witkiewicz et al., 2018). Additionally, loss-of-function mutations in mismatch repair genes such as MLH1 or MSH2 can eliminate the DNA damage signaling necessary for apoptosis induction, further contributing to resistance (Hassen et al., 2016). Functional screens have also identified other modulators such as CHD4, whose loss enables resistance via translesion synthesis, bypassing the need for BRCA-mediated repair (Guillemette et al., 2015).

### *1.2.2. Epigenetic Reprogramming and Non-Genomic Adaptations*

Beyond irreversible genetic alterations, cancer cells utilize epigenetic plasticity to modulate gene expression in response to chemotherapy. One key mechanism is promoter hypermethylation, which silences genes essential for platinum sensitivity. For example, methylation-induced silencing of MLH1 in colorectal and ovarian cancers results in mismatch repair deficiency and platinum resistance (Martin et al., 2008). Similarly, loss of IGFBP-3 expression in resistant lung cancer cells has been traced to promoter methylation, and re-expression of IGFBP-3 restores cisplatin sensitivity (Ibanez de Caceres et al., 2010).

MicroRNAs (miRNAs) and long non-coding RNAs (lncRNAs) also regulate resistance. Loss of tumor-suppressive miRNAs such as miR-214 and miR-100 enhances pro-survival signaling through targets like PTEN, promoting resistance (Liu et al., 2015). In contrast, overexpression of miR-21 downregulates pro-apoptotic regulators and promotes resistance (Buscaglia & Li, 2011). LncRNAs such as H19 and ROR support survival by increasing glutathione synthesis or inhibiting p53 signaling, respectively (Zhang et al., 2013; Zheng et al., 2016). These regulatory RNAs can also affect DNA methylation and histone modifications, contributing to a heritable but reversible resistance phenotype (Zheng et al., 2016).

Histone-modifying enzymes are involved as well. Overexpression of histone deacetylases (HDACs), particularly HDAC6, contributes to resistance by repressing pro-apoptotic genes (Z. Wang et al., 2016). Inhibition of HDACs or DNA methyltransferases has shown promise in re-sensitizing resistant tumors, leveraging the reversibility of these epigenetic changes (Namdar et al., 2010).

### *1.2.3. Metabolic Rewiring and Detoxification Pathways*

Platinum compounds generate oxidative stress and react with nucleophilic biomolecules. Tumor cells counteract this by increasing intracellular thiol levels—particularly glutathione (GSH)—and upregulating glutathione S-transferases (GSTs) that catalyze the conjugation of platinum to GSH, neutralizing its reactivity. In resistant cells, over 60% of intracellular platinum can be inactivated by GSH conjugation within hours of exposure. Inhibiting GSH synthesis (e.g., with buthionine sulfoximine) can reverse resistance, though systemic toxicity limits clinical utility (Allocati et al., 2018).

Metallothioneins (MTs), cysteine-rich proteins that bind metal ions, also sequester platinum, reducing its nuclear availability. Their expression is elevated in resistant tumors and correlates with poor outcomes (Khan et al., 2021; Rodrigo et al., 2021). Knockdown of MTs restores cisplatin sensitivity, and their expression may be epigenetically regulated via miRNAs (Shi et al., 2021; Wang et al., 2023). Oxidative stress defenses are also enhanced. Resistant cells often upregulate glutathione reductase, glutathione peroxidase, and enzymes in the pentose phosphate pathway to increase NADPH, sustaining redox homeostasis (Lu, 2013). In hypoxic environments, stabilization of HIF-1α drives metabolic shifts that reduce ROS production and increase resistance (Kim & Dang, 2006).

### *1.2.4. Transporter-Mediated Modulation of Platinum Accumulation*

Reduced intracellular accumulation of platinum is a cornerstone of resistance. This may occur via downregulation of influx transporters or upregulation of efflux pumps. Organic cation transporters (OCT1, OCT2) and copper transporter CTR1 are key mediators of platinum import. Loss or mislocalization of these proteins, often due to epigenetic silencing or drug-induced degradation, lowers drug uptake (Cong et al., 2021; Liu et al., 2016). For example, reduced OCT2 expression in oxaliplatin-resistant colorectal cancer impairs drug accumulation (Zhou et al., 2020). CTR1 is another critical

uptake channel. Exposure to cisplatin can trigger its degradation, thereby limiting further drug influx. Modulating CTR1 with copper-chelating agents like tetrathiomolybdate has been explored as a way to enhance cisplatin uptake (Ishida et al., 2002; Kilari et al., 2016).

Efflux transporters such as MRP2 (ABCC2) and MRP4 (ABCC4) export platinum–GSH conjugates, decreasing cytosolic platinum levels. High MRP2 expression correlates with cisplatin resistance in lung and ovarian cancers (Guminski et al., 2006; Huang et al., 2021). The copper efflux pumps ATP7A and ATP7B also play important roles by sequestering and transporting platinum into vesicles for exocytosis. High ATP7B expression predicts poor response in colorectal and ovarian cancer patients (Huang et al., 2021; Petruzzelli et al., 2022). These transporters are regulated by miRNAs such as miR-495 and miR-133a, suggesting another layer of control (L. Wang et al., 2024). In addition to membrane pumps, cancer cells may expel platinum via exosomes, transferring drugs out of the cell and potentially spreading resistance traits to neighboring cells (Kumar et al., 2024; L. Wang et al., 2024).

### *1.2.5. DNA Damage Recognition and Repair Pathways*

Once inside the cell, platinum drugs form intrastrand and interstrand crosslinks. The effectiveness of therapy depends on whether the cell can repair or tolerate these lesions. Nucleotide excision repair (NER) is responsible for removing intrastrand adducts, and elevated ERCC1-XPF activity correlates with poor response to platinum therapy (Poklar et al., 1996). ERCC1 expression levels have prognostic value in lung and gastric cancers, and experimental knockdown sensitizes cells to cisplatin (He et al., 2020; Liu et al., 2013). Interstrand crosslinks are more lethal and are primarily processed by the Fanconi anemia (FA) pathway and homologous recombination (HR). HR-deficient tumors, such as those with BRCA mutations, are highly platinum-sensitive. However, resistance often arises via reversion mutations or compensation by alternative repair proteins (Yao et al., 2015). Proteins like WWOX and USP13 also regulate HR; their loss impairs repair foci formation and confers resistance (Y. Li et al., 2017; Park et al., 2022).

Mismatch repair (MMR) proteins detect DNA distortions and signal apoptosis. Loss of MMR components (e.g., MLH1, MSH2) allows cells to tolerate platinum lesions without activating cell death, contributing to resistance (Kweekel et al., 2005; Mehmood et al., 2014). Notably, oxaliplatin's efficacy is less dependent on MMR status than

cisplatin's, possibly due to differences in adduct processing (Mehmood et al., 2014). Translesion synthesis (TLS), mediated by error-prone polymerases, enables replication through platinum adducts and allows cells to avoid replication fork collapse. This tolerance mechanism can sustain cell viability at the cost of increased mutagenesis (Arianna & Korzhnev, 2024; Patel et al., 2021).

### *1.2.6. Disruption of Apoptotic Signaling and Survival Enhancement*

Even in the presence of DNA damage, apoptosis must be properly activated to eliminate damaged cells. Platinum-resistant tumors frequently exhibit suppression of apoptosis through multiple routes. Overexpression of anti-apoptotic proteins such as BCL-2, BCL-XL, and MCL-1, and downregulation of pro-apoptotic effectors like BAX, PUMA, and NOXA, are hallmarks of resistant phenotypes (Beale et al., 2000; Yuan et al., 2020; Yuan et al., 2011). Elevated levels of inhibitors of apoptosis proteins (IAPs), including survivin and XIAP, also block caspase activation (Chawla-Sarkar et al., 2004). Survival signaling pathways such as PI3K/AKT, MAPK/ERK, and NF-κB are frequently activated, suppressing apoptosis and enhancing cell viability. AKT can inactivate BAD and stabilize MDM2, which inhibits p53 (Heavey et al., 2014). NF-κB promotes transcription of survival genes and is often induced in response to platinum (Godwin et al., 2013; Heavey et al., 2014). These pathways can be targeted pharmacologically to restore drug sensitivity.

In many tumors, p53 mutation or degradation renders the apoptotic response ineffective. Even with wild-type p53, its downstream targets may be epigenetically silenced, or p53 may be functionally inactivated by MDM2 overexpression (Haronikova et al., 2021; Tovar et al., 2006). Small-molecule inhibitors of MDM2, such as nutlin-3, aim to restore p53 activity but depend on intact p53 (Gu et al., 2008; Tovar et al., 2006). Non-coding RNAs also regulate apoptosis. miR-21, miR-214, and lncRNAs such as ROR and MALAT1 modulate the expression of apoptotic regulators and contribute to resistance (Lei et al., 2022; Yang et al., 2013). These RNAs fine-tune survival programs and may represent targets for therapy.

Autophagy, a process that recycles damaged cellular components, also contributes to resistance. In most contexts, it acts as a protective response against platinum-induced

stress. Inhibiting autophagy can sensitize resistant cells, supporting the notion that it functions as a survival mechanism in this setting (Lei et al., 2022).

### *1.2.7. Tumor Microenvironment and Stromal Contributions*

The tumor microenvironment (TME) significantly influences chemotherapy response. Poor vascularization and high interstitial pressure reduce drug delivery, creating gradients of sublethal platinum exposure across the tumor mass (Weniger et al., 2018). Fibrotic stroma and extracellular matrix components impede diffusion, especially in tumors like pancreatic cancer (Carvalho et al., 2021; Tanaka et al., 2023; Weniger et al., 2018). Hypoxia within the TME stabilizes HIF-1α, which promotes glycolysis, reduces ROS, and upregulates anti-apoptotic proteins—all contributing to platinum resistance (19689405). HIF-1 also enhances the expression of efflux transporters and supports stemness features, further protecting cells (Belisario et al., 2020; Yong et al., 2022). Cancer-associated fibroblasts (CAFs) secrete cytokines such as IL-6 and HGF that activate STAT3 and MET pathways in tumor cells, promoting resistance (Guo et al., 2023; Shintani et al., 2016). They also provide cysteine and glutathione precursors, enhancing cellular detoxification of platinum (Zhu, Fang, et al., 2024). CAFs themselves are resistant to platinum and may assist in post-treatment tumor recovery. Tumor-associated macrophages (TAMs), especially those of the M2 phenotype, secrete TGF-β and IL-10, promoting epithelial-to-mesenchymal transition (EMT), stemness, and immune suppression (Y. Chen et al., 2018; Linares et al., 2023). Exosomal transfer of miRNAs from macrophages to tumor cells can further entrench resistance (Kwon et al., 2020).

### *1.2.8. Therapeutic Implications of Platinum-Based Chemotherapy Resistance*

Resistance to platinum-based chemotherapy is not a single event but a multifactorial process shaped by genetic instability, dynamic epigenetic changes, adaptive metabolic responses, defective transport, proficient DNA repair, survival signaling, and the influence of the tumor microenvironment. These resistance mechanisms are often concurrent within the same tumor, creating redundancy and complicating therapeutic efforts (Apelian et al., 2025; Lheureux et al., 2019).

Advances in high-throughput sequencing and functional genomics have illuminated these diverse resistance nodes. Current therapeutic strategies increasingly focus on combination therapies that simultaneously target multiple pathways—such as pairing platinum with PARP inhibitors in BRCA-mutant tumors or ATR inhibitors in repair-proficient cancers (32560564). Epigenetic drugs, metabolic modulators, and inhibitors of survival signaling are also under investigation to overcome resistance (Soung & Chung, 2023).

Ultimately, overcoming platinum resistance requires a personalized approach informed by the tumor's molecular profile. Integrating resistance biomarkers into treatment planning and disrupting both intrinsic and extrinsic survival pathways hold the key to enhancing the long-term efficacy of platinum-based therapies (McMullen et al., 2020).

### 1.3. Lysosomal Sequestration and Exocytosis as Emerging Mediators of Chemotherapy Tolerance

Chemotherapy tolerance refers to the ability of cancer cells to endure and survive chemotherapeutic treatment that is otherwise lethal to sensitive counterparts. This adaptive capability, which may be inherent to certain subpopulations or acquired during exposure to drugs, constitutes one of the most significant hurdles to long-term therapeutic success in oncology (Basile & Aplin, 2012; Sharma et al., 2010). While well-characterized resistance mechanisms such as enhanced drug efflux, target mutation, DNA repair enhancement, metabolic detoxification, and apoptotic evasion have dominated the landscape of cancer pharmacology, recent studies have highlighted the lysosomal system as a critical and underappreciated contributor to drug tolerance (Geisslinger et al., 2020; Hussein et al., 2021; Zhitomirsky & Assaraf, 2016). Historically considered the cellular waste disposal machinery, lysosomes have emerged as active players in multidrug resistance by functioning as drug storage compartments and conduits for drug expulsion, especially in the context of chemotherapeutic agents that are hydrophobic and weakly basic in nature (Hussein et al., 2021; Zhitomirsky & Assaraf, 2017). This lysosome-centered model of drug detoxification offers an alternative and complementary resistance strategy in many cancer types and drug classes.

Lysosomes are acidic, membrane-bound organelles rich in hydrolytic enzymes, maintained in their functional state by the vacuolar $H^+$-ATPase (V-ATPase) that sustains their low internal pH. The acidic luminal environment allows efficient degradation of macromolecules and organelles, supporting critical functions in autophagy, metabolism, and intracellular trafficking. In cancer cells, lysosomes are often altered both structurally and functionally, with an increase in their number, volume, and enzymatic activity serving as an adaptive trait that supports survival under stress (Zhitomirsky & Assaraf, 2016). This transformation of the lysosomal system has been increasingly linked to its ability to sequester drugs away from their intracellular targets. Many widely used chemotherapeutic agents, including anthracyclines and certain tyrosine kinase inhibitors, fall into the category of weak base compounds. These drugs are uncharged under neutral or basic conditions, allowing passive diffusion across cellular membranes. However, once they reach the acidic interior of the lysosome, they become protonated and charged, effectively trapping them within the lysosomal lumen due to their inability to freely diffuse back across the membrane – a process termed ion trapping (Chen et al., 2020; Trybus et al., 2023). The net result is a pharmacological sink: drug molecules accumulate in the lysosome, depleting their concentrations in the cytoplasm and nucleus, where their therapeutic targets are located.

This ion trapping mechanism has been shown to correlate with decreased drug efficacy and increased tolerance in numerous cancer models. For instance, studies have demonstrated that the higher the intracellular accumulation of doxorubicin within lysosomes, the less cytotoxic it becomes, with cancer cells exhibiting greater survival under identical dosing conditions (Guo et al., 2016; Zhitomirsky & Assaraf, 2016). In this regard, lysosomes act not merely as passive receptacles but as active barriers that limit drug access to its critical molecular targets. Moreover, the expansion of the lysosomal compartment itself can be induced by drug exposure, suggesting a feedforward protective loop where the stress of chemotherapy leads to enhanced lysosome biogenesis, further boosting the cell's sequestration capacity (Zhitomirsky & Assaraf, 2015, 2016). Central to this phenomenon is the activity of V-ATPase, the proton pump that maintains lysosomal acidity. Inhibiting this pump neutralizes the lysosomal pH, reducing protonation of weak base drugs and allowing their diffusion back into the cytosol, where they can engage their targets and exert cytotoxic effects (Dey et al., 2024; Stransky et al., 2016; Zhai & El Hiani, 2020). Experimental interventions using pharmacologic inhibitors

like bafilomycin A or genetic silencing of V-ATPase subunits have confirmed this dependency, showing restored drug sensitivity in previously resistant cancer cells (Chow et al., 1985; Kulshrestha et al., 2019; Whitton et al., 2018). Clinically, tumors with high expression of V-ATPase components, such as ovarian carcinomas expressing elevated V0a2 subunits, have been associated with poor response to platinum-based chemotherapy (Kulshrestha et al., 2020).

In addition to passive ion trapping, active transport systems contribute to lysosomal drug sequestration. Among the most notable is P-glycoprotein (P-gp), an ATP-dependent efflux pump traditionally known for expelling drugs at the plasma membrane. Intriguingly, in drug-resistant cancer cells, P-gp has been localized to lysosomal membranes, where it actively transports substrates like doxorubicin into the lysosomal lumen rather than out of the cell (Seebacher et al., 2016; Yamagishi et al., 2013). This subcellular relocalization effectively creates an intracellular drug sink that reinforces chemoresistance. The involvement of copper-transporting ATPases, such as ATP7A and ATP7B, adds another layer to this system. These transporters, normally involved in metal ion homeostasis, have been shown to sequester cisplatin and related agents into vesicular compartments, including lysosomes, thereby reducing nuclear platinum accumulation and contributing to platinum resistance (Kalayda et al., 2008; Y. Q. Li et al., 2018). Their overexpression correlates with diminished therapeutic response and is reversible through targeted inhibition, further supporting their role in lysosome-mediated drug tolerance.

The endomembrane trafficking system within cells supports this process by routing drug-loaded vesicles to the lysosome. Drugs internalized through endocytosis or taken up into autophagosomes can be delivered to lysosomes via vesicular fusion events, enabling their sequestration. In some cases, metal–protein complexes formed after drug exposure are processed through autophagic pathways and end up in the lysosome for degradation or storage (Ju et al., 2020; Muro, 2018). Such trafficking patterns extend the protective function of the lysosome beyond ion trapping and encompass a range of drug–macromolecule interactions that would otherwise be lethal if they reached the DNA or vital enzymes. The spatial dynamics of lysosomes also matter. Drug-induced repositioning of lysosomes from the perinuclear region toward the cell periphery is a key preparatory step for exocytosis – the process through which lysosomal contents are

expelled from the cell entirely (Eriksson & Ollinger, 2024; Halaby, 2019; Hussein et al., 2021; Zhitomirsky & Assaraf, 2017).

Lysosomal exocytosis allows the final disposal of sequestered drugs. In this two-step mechanism, lysosomes first migrate toward the plasma membrane via microtubule-mediated transport, and then fuse with the membrane in a calcium-dependent manner, releasing their contents – including entrapped drugs – into the extracellular environment (Zhitomirsky & Assaraf, 2017). This vesicle fusion process is facilitated by SNARE proteins and is tightly regulated by cytosolic calcium signals. It has been clearly demonstrated that weak base drugs like doxorubicin accumulate in lysosomes and are subsequently discharged from the cell via exocytosis, an event accompanied by the extracellular release of lysosomal hydrolases such as cathepsin D, confirming the complete evacuation of lysosomal cargo (Halaby, 2019; Zhitomirsky & Assaraf, 2017). From a survival standpoint, this two-step sequence – drug sequestration followed by exocytosis – offers cancer cells a highly efficient mechanism to escape chemotherapy-induced cytotoxicity.

A central regulator of lysosomal biogenesis and trafficking is the transcription factor TFEB. Under resting conditions, TFEB is phosphorylated by mTORC1 and retained in the cytoplasm. However, under stress – such as drug-induced lysosomal alkalinization – TFEB is dephosphorylated via calcineurin activation and translocates to the nucleus, where it drives expression of genes involved in lysosome formation, autophagy, and trafficking (Roczniak-Ferguson et al., 2012; Zhitomirsky et al., 2018). Drug accumulation in lysosomes can trigger this lysosomal stress response by buffering protons and slightly elevating lysosomal pH, activating TFEB through calcium signaling. Once active, TFEB increases lysosome production and promotes their movement toward the plasma membrane, enhancing both sequestration capacity and exocytosis (Medina et al., 2015; Zhitomirsky et al., 2018). This dual regulation has been described as a coordinated cellular effort to detoxify the intracellular environment. In this context, lysosomal exocytosis is not merely a mechanical process, but a transcriptionally reinforced program driven by stress sensors and responsive gene networks.

The cumulative effect of these lysosome-centered mechanisms is a profound cellular capacity to tolerate chemotherapeutic insult. By partitioning drugs away from their targets

and ejecting them from the cell altogether, lysosomes enable cancer cells to withstand drug concentrations that would otherwise trigger apoptosis or senescence (Halaby, 2019; Zhitomirsky & Assaraf, 2016). This form of tolerance has been observed across multiple malignancies, including breast cancer, neuroblastoma, leukemia, ovarian cancer, and lung cancer. In many of these models, lysosomal expansion, V-ATPase upregulation, and increased lysosomal drug accumulation are common features of drug-resistant cells (Kulshrestha et al., 2019; You et al., 2009). For instance, neuroblastoma cells exposed to chronic cisplatin develop enlarged lysosomes and become less sensitive to the drug, but can be resensitized with lysosomal inhibitors (Hauser et al., 2019). Similarly, small-cell lung cancer cells that resist apoptosis via Bcl-2 overexpression can be pushed into cell death when V-ATPase is inhibited, suggesting that lysosomal pH regulation is critical to their survival (Shu et al., 2016). In some cases, stromal or endothelial cells can also engage in lysosomal sequestration of anti-angiogenic drugs, limiting the efficacy of vascular-targeted therapies – a phenomenon reversible with agents like chloroquine or bafilomycin (Chandra et al., 2020).

Therapeutically, the recognition of lysosomes as key players in chemoresistance has prompted efforts to target this pathway. Lysosomotropic agents such as chloroquine and hydroxychloroquine disrupt lysosomal acidification, releasing trapped drugs back into the cytosol. Direct V-ATPase inhibitors like bafilomycin A and novel small molecules aim to achieve a similar outcome by collapsing the proton gradient essential for drug trapping. Early clinical trials combining chloroquine with chemotherapy have produced mixed results, with some indications of benefit in disease stabilization (Amaravadi et al., 2019). The heterogeneity in outcomes may reflect the complexity of timing, dosing, and tumor-specific factors. More advanced strategies include modifying drug structures to reduce lysosomal trapping potential or developing therapies that selectively induce lysosomal membrane permeabilization, leading to cell death (Kallunki et al., 2013). The underlying premise of these approaches is that lysosomes, once seen as passive storage units, are now therapeutic targets in their own right.

In conclusion, lysosomal sequestration and exocytosis represent a vital axis of chemotherapy tolerance in cancer. Through ion trapping, active transport, vesicular trafficking, and regulated exocytosis, lysosomes enable cancer cells to reduce intracellular drug exposure and survive otherwise lethal treatments. This resistance

mechanism operates across cancer types and drug classes and often coexists with other tolerance strategies, reflecting the multifaceted nature of chemoresistance. As research continues to illuminate the molecular regulators of lysosome-mediated drug handling, there is growing optimism that interventions targeting this system will restore drug sensitivity in resistant cancers. The therapeutic future may well include combinations that dismantle lysosomal defenses, allowing chemotherapeutics to exert their full cytotoxic potential and improving outcomes for patients with drug-tolerant malignancies.

### 1.4. Epigenetic Control of Lysosomal Function and Drug Response: An Underexplored Axis

Epigenetic regulation encompasses heritable and reversible changes in gene expression that do not alter the underlying DNA sequence but nonetheless have profound biological consequences. These regulatory mechanisms shape the transcriptional landscape of a cell through covalent modifications to DNA and histone proteins, as well as through the action of non-coding RNAs (Chen & Xue, 2016; Zhang et al., 2020). Together, they form an adaptable system that determines chromatin accessibility and gene expression potential. In the context of cancer, epigenetic reprogramming is a pervasive hallmark, enabling tumor cells to toggle between transcriptional states that support proliferation, immune evasion, metabolic rewiring, and, notably, treatment resistance (Asano, 2020; Janson & Winqvist, 2011; Recillas-Targa, 2022; Verdikt & Allard, 2021). These adaptations are not static; they evolve in response to environmental and therapeutic pressures, contributing to a flexible state of tumor plasticity. Among the most well-characterized epigenetic modifications are DNA methylation, histone acetylation and methylation, and regulatory RNA networks (Portela & Esteller, 2010). Each of these components operates within a finely tuned system that governs when, where, and how genes are expressed. Methylation of cytosine residues within CpG islands, especially in gene promoter regions, is commonly associated with transcriptional silencing (Meng et al., 2015). This is frequently observed in cancer, where hypermethylation of tumor suppressor gene promoters can effectively shut down critical pathways involved in cell death, DNA repair, or immune surveillance (Laird & Jaenisch, 1996). Histone acetylation, conversely, is generally associated with transcriptional activation by promoting a relaxed chromatin state that permits access to transcriptional machinery (Turner, 2000). Enzymes such as histone acetyltransferases (HATs) and histone

deacetylases (HDACs) maintain a dynamic equilibrium in chromatin architecture, with HDACs promoting a more condensed and repressive chromatin configuration (Shen et al., 2015). Histone methylation can have activating or repressive effects depending on the specific residue and degree of modification. For example, trimethylation at lysine 4 of histone H3 (H3K4me3) correlates with active gene transcription, while trimethylation at lysine 27 (H3K27me3) leads to repression (Miller & Grant, 2013; Zaib et al., 2022).

In parallel, non-coding RNAs such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) provide additional layers of gene regulation. MicroRNAs typically bind to the 3' untranslated regions of target mRNAs, resulting in translational inhibition or mRNA degradation (Saliminejad et al., 2019). Long non-coding RNAs function through diverse mechanisms, including scaffolding of chromatin modifiers, modulation of transcription factor binding, and sequestration of miRNAs (Herman et al., 2022). These epigenetic processes are essential in determining the cellular response to environmental cues and are particularly significant in cancer cells, which rely on transcriptional plasticity to adapt to chemotherapeutic stress.

One of the most intriguing and increasingly relevant areas of epigenetic regulation in cancer biology involves the lysosomal system. While lysosomes were originally characterized as cellular waste disposal organelles, they are now recognized as central hubs for metabolic control, signaling integration, and drug handling (Abou Sawan et al., 2020). Their function is critical not only in autophagy and macromolecule degradation but also in chemotherapy response, particularly through mechanisms of drug sequestration and exocytosis (Zhitomirsky & Assaraf, 2016). Lysosomes in cancer cells are frequently found to be altered in number, size, composition, and behavior (Hamalisto & Jaattela, 2016). These adaptations are not incidental—they are part of a coordinated survival response to the metabolic and genotoxic stresses imposed by tumor progression and drug exposure (Tang et al., 2020).

A central regulator of lysosomal gene expression is the transcription factor TFEB. Belonging to the MiT/TFE family, TFEB governs the expression of genes within the CLEAR (Coordinated Lysosomal Expression and Regulation) network (Franco-Juarez et al., 2022). These genes encode lysosomal hydrolases, membrane proteins, and autophagy machinery (Roczniak-Ferguson et al., 2012). Furthermore, under nutrient-rich conditions,

TFEB is kept in an inactive, phosphorylated state in the cytoplasm due to mTORC1 activity. Upon starvation, oxidative stress, or lysosomal dysfunction, TFEB becomes dephosphorylated, translocates to the nucleus, and initiates a broad transcriptional program that expands the cell's degradative and recycling capabilities (Franco-Juarez et al., 2022; Napolitano et al., 2018). This lysosome-focused adaptation is critical for cell survival under stress conditions and is now known to be epigenetically regulated at multiple levels.

For instance, Kim et al. (2024) discovered an epigenetic switch that governs TFEB activation based on nutrient availability. Under nutrient-replete conditions, the transcription factor USF2 cooperates with HDAC1 to bind CLEAR motifs, resulting in histone deacetylation (specifically loss of H3K27ac) and chromatin condensation at lysosomal gene promoters. This represses transcription of lysosomal genes when they are not needed. Upon nutrient deprivation, stress-induced phosphorylation reduces USF2's DNA-binding ability, allowing TFEB to displace USF2 and initiate transcription. Without HDAC1-mediated repression, TFEB binding promotes local histone acetylation, chromatin opening, and robust gene activation (Kim et al., 2024). This mechanism illustrates how transcription factor accessibility is governed by epigenetic cues, and how environmental signals such as nutrient levels are transduced into chromatin-based gene expression programs. Importantly, this system is not limited to physiological adaptation. In cancers, constitutive activation or dysregulation of TFEB—whether through genomic rearrangements, altered post-translational modifications, or chromatin derepression—can enhance lysosomal biogenesis and support tumor cell survival and drug tolerance (Kim et al., 2021; Y. Li et al., 2020; Zhu et al., 2021).

In addition to regulating gene expression at the chromatin level, epigenetic enzymes can directly modify TFEB itself. Acetylation of TFEB by HDAC inhibitors has been shown to enhance its transcriptional activity. Specific lysine residues such as K91, K103, K116, and K430 become hyperacetylated upon HDAC inhibition, resulting in increased expression of lysosomal and autophagy-related genes (Zhang et al., 2018). Conversely, acetylation by GCN5 at different lysines (K274/K279) impairs TFEB's DNA-binding activity and suppresses lysosomal gene expression (Wang et al., 2020). These observations underscore the complexity of TFEB regulation and show that epigenetic enzymes influence not only chromatin state but also the activity of master transcriptional

regulators themselves. Furthermore, these modifications have functional consequences in cancer cells. In settings where TFEB is hyperactivated due to HDAC inhibition, the resulting increase in autophagy and lysosomal activity can lead to either enhanced survival or, in some cases, lysosome-driven cell death, depending on cellular context and drug combinations.

Metabolic pathways also intersect with epigenetic regulation of lysosomal genes. The production of acetyl-CoA, a substrate for histone acetylation, is critical for the activation of lysosomal transcription. ACSS2, a nuclear-localized enzyme that generates acetyl-CoA from acetate, has been shown to bind TFEB at lysosomal gene loci and facilitate localized histone acetylation, promoting transcriptional activation (X. Li, X. Qian, et al., 2017; X. Li, W. Yu, et al., 2017). In summary, this mechanism links cellular metabolism to chromatin structure and lysosomal function, allowing tumor cells to integrate nutrient availability into gene regulation. This is especially relevant in metabolically demanding tumors such as glioblastoma, where enhanced lysosomal activity supports survival under hypoxic and nutrient-depleted conditions.

The influence of oncogenic signaling on lysosomal regulation is another emerging theme. MYC, a well-known oncogene and transcriptional amplifier, has been shown to suppress lysosomal gene expression by displacing TFEB and potentially recruiting repressive complexes to CLEAR elements. In acute myeloid leukemia, this leads to decreased TFEB activity and alters the lysosomal-autophagy landscape of the tumor, contributing to resistance to metabolic and chemotherapeutic stress (Annunziata et al., 2019). These findings support a model in which epigenetic regulation of lysosomal function is dynamically shaped by the oncogenic context of the tumor.

Non-coding RNAs further refine this regulatory network. MicroRNAs such as miR-30a inhibit autophagy by targeting genes like BECN1 and ATG5, which are essential for autophagosome formation. Downregulation of these genes impairs the autophagic process and can sensitize cancer cells to chemotherapy (B. B. Li et al., 2020; Zhang et al., 2017). In contrast, other miRNAs may promote autophagy by targeting negative regulators, creating a context-dependent spectrum of effects. Long non-coding RNAs exert similar control. Some act in the nucleus to recruit chromatin modifiers like PRC2 to lysosomal gene promoters, enforcing transcriptional silencing via H3K27

trimethylation (Balas et al., 2021). Others act in the cytoplasm to sequester miRNAs that suppress autophagy-related genes. For instance, PVT1 lncRNA can sponge miR-20a, thereby stabilizing ULK1 expression and enhancing autophagy, which contributes to drug tolerance in gastric cancer (R. Li et al., 2022). These non-coding RNAs function as epigenetic modulators, altering chromatin states and transcript stability in ways that reshape lysosomal and autophagy responses.

Beyond gene expression, epigenetic enzymes also regulate the trafficking and exocytosis of lysosomes—processes critical for cellular adaptation to chemotherapeutic stress. A key player in this regard is HDAC10, a cytoplasmic deacetylase that promotes lysosomal exocytosis. In neuroblastoma cells, HDAC10 activity supports the movement of drug-loaded lysosomes to the plasma membrane, facilitating their fusion and expulsion of chemotherapeutic agents like doxorubicin (Ridinger et al., 2018). Inhibition of HDAC10 results in the intracellular retention of drug-laden lysosomes, increased nuclear drug accumulation, and enhanced DNA damage, thereby sensitizing the cells to chemotherapy. Mechanistically, HDAC10 may regulate the acetylation status of cytoskeletal or trafficking proteins required for lysosome mobility and fusion. Related deacetylases like HDAC6 and SIRT2, known to target α-tubulin and motor proteins, have also been implicated in regulating vesicle dynamics and autophagy (Inoue et al., 2014; Passaro et al., 2021). Although these functions extend beyond the canonical scope of chromatin regulation, they highlight the broader role of epigenetic enzymes in modulating stress response systems, including organelle positioning and secretion.

The ability of cancer cells to resist therapy often depends on the emergence of drug-tolerant subpopulations. These persister cells exhibit profound transcriptional and metabolic shifts, including increased lysosomal and autophagic activity (De Conti et al., 2021; Ohata et al., 2023; Punzi et al., 2025). Critically, these states are not driven by fixed genetic mutations but are maintained by reversible epigenetic changes. One landmark study demonstrated that the histone demethylase KDM5A is essential for establishing and maintaining a drug-tolerant phenotype. By removing activating H3K4me3 marks, KDM5A induces a chromatin state conducive to survival in the presence of targeted therapies (Hou et al., 2012). These cells also rely on stress-response pathways such as autophagy and lysosomal biogenesis, suggesting that the epigenetic regulation of these systems is central to tolerance. Inhibiting KDM5A or disrupting the autophagy-lysosome

axis can eliminate persister cells and prevent relapse, providing a compelling rationale for therapeutic targeting of these pathways.

Evidence from multiple cancer types supports the centrality of epigenetically regulated lysosomal activity in shaping treatment outcomes. In hepatocellular carcinoma, SIRT1 and SIRT6 deacetylate autophagy proteins, enhancing autophagic flux and resistance to doxorubicin (Han et al., 2019; Tang et al., 2024). In lung cancer, the level of TFEB expression correlates with chemotherapy response, with hyperactive TFEB contributing to drug sequestration and resistance (Akman et al., 2024). These observations point to a consistent theme: epigenetic plasticity underpins the regulation of lysosomal function, which in turn modulates how cancer cells survive therapy.

To summarize everything aforementioned, therapeutic strategies aimed at disrupting this axis are now being tested. HDAC inhibitors, DNA methyltransferase inhibitors, and bromodomain inhibitors have been shown to sensitize cancer cells to chemotherapy by altering lysosomal gene expression or impairing autophagy. Combinations of epigenetic drugs with chemotherapy or targeted therapies have yielded promising results in preclinical models and early-phase clinical trials. Inhibiting autophagy or lysosomal acidification with agents like chloroquine or bafilomycin has also demonstrated efficacy in reversing drug tolerance. However, the success of these strategies likely depends on the epigenetic context of the tumor, including chromatin accessibility at lysosomal gene loci and the activity of key regulators like TFEB or HDAC10. Thus, understanding the epigenetic landscape that governs lysosomal behavior is crucial for designing effective, personalized interventions.

Despite these advances, the specific role of epigenetic regulation in controlling lysosomal exocytosis—the final step in the detoxification and drug expulsion process—remains poorly understood. While lysosomal gene expression and autophagy have received increasing attention in cancer epigenetics, the regulation of lysosomal trafficking and fusion with the plasma membrane has been largely overlooked. Current knowledge is limited to a few examples, such as the role of HDAC10 in facilitating lysosomal exocytosis in neuroblastoma cells. This gap in the literature represents a significant blind spot in our understanding of how cancer cells expel chemotherapeutic agents and survive treatment. Our study addresses this knowledge deficit by

systematically investigating how epigenetic regulators influence lysosomal exocytosis and how this, in turn, affects drug retention and chemotherapy response. We propose that the modulation of lysosomal exocytosis through epigenetic mechanisms represents a critical, yet underexplored, axis of chemotherapy tolerance. By targeting this pathway, it may be possible to prevent drug efflux, increase intracellular drug accumulation, and overcome resistance in tumors that rely on lysosome-based defense strategies. Unraveling the epigenetic control of lysosomal exocytosis offers a new frontier in cancer therapy, one that bridges molecular epigenetics with cellular trafficking to restore drug sensitivity and improve treatment efficacy.

## 1.5. Protein Arginine Methyltransferases (PRMTs) in Chromatin Regulation and Cancer Progression

Protein arginine methyltransferases (PRMTs) are a family of evolutionarily conserved enzymes that mediate the methylation of arginine residues on target proteins. This type of post-translational modification plays a vital role in regulating chromatin structure, transcription, RNA processing, DNA repair, and multiple signaling pathways (Wolf, 2009). The discovery of arginine methylation on histones positioned PRMTs as significant players in the epigenetic regulation of gene expression (Hwang et al., 2021). All PRMTs use S-adenosylmethionine (SAM) as a methyl donor and share a conserved structural motif that facilitates SAM binding. The guanidino group in arginine provides two nitrogen atoms that can be methylated, allowing for the addition of one or two methyl groups. Depending on the site and pattern of methylation, three forms can be generated: monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) (Fulton et al., 2019; Hwang et al., 2021).

Accordingly, PRMTs are divided into three types based on their catalytic activity. Type I PRMTs—including PRMT1, PRMT2, PRMT3, PRMT4 (also known as CARM1), PRMT6, and PRMT8—produce ADMA. Type II enzymes, including PRMT5 and PRMT9, catalyze the formation of SDMA. PRMT7 is the only known Type III PRMT, generating MMA exclusively (Gao et al., 2024). Among these, PRMT1 is considered the dominant enzyme, responsible for the majority of total cellular arginine methylation, with estimates suggesting it accounts for up to 90% of global activity (Fulton et al., 2019). While arginine methylation does not alter the net positive charge of the amino acid, it significantly influences hydrogen bonding potential, which in turn can impact protein-

protein and protein-DNA interactions (Hwang et al., 2021). Importantly, these modifications are generally stable, and several PRMTs are essential for embryonic development, as demonstrated by knockout studies in mice showing embryonic or perinatal lethality (Lee et al., 2012; Lin et al., 2013; Ma et al., 2023; Wang et al., 2021). This foundational knowledge frames PRMTs as core regulators of gene expression and chromatin biology.

One of the most prominent functions of PRMTs lies in the epigenetic regulation of gene expression through modification of histone tails. Arginine methylation constitutes a critical layer of the histone code, shaping how chromatin is structured, and which genes are accessible for transcription (Wolf, 2009). These methyl marks can either activate or repress gene expression depending on the context and the specific site and symmetry of methylation. For instance, PRMT1-mediated asymmetric dimethylation of histone H4 at arginine 3 (H4R3me2a) is generally associated with active transcription and is thought to facilitate histone acetylation in nearby residues, creating a more relaxed chromatin configuration (Beacon et al., 2020; Jahan & Davie, 2015). Conversely, PRMT5 catalyzes symmetric dimethylation at the same residue (H4R3me2s), which often leads to transcriptional repression by recruiting repressor complexes such as the polycomb-like complex (Liu et al., 2020; Pal et al., 2004; Zhu & Rui, 2019). This example underscores the functional opposition between PRMT family members even at the same arginine residue.

The influence of PRMTs extends to other histone sites as well. PRMT4/CARM1 dimethylates histone H3 at arginine 17 and 26 (H3R17me2a and H3R26me2a), modifications that are generally linked to gene activation in promoter and enhancer regions (Wu et al., 2012; Wu & Xu, 2012). In contrast, PRMT6 introduces H3R2me2a, a repressive mark that interferes with MLL complex recruitment, thereby inhibiting H3K4 trimethylation and preventing transcriptional activation (Guccione et al., 2007). Interestingly, PRMT5 can also methylate H3R2 symmetrically (H3R2me2s), which in some contexts has been associated with enhanced transcription via promotion of H3K4 trimethylation (Yuan et al., 2012). These nuanced outcomes illustrate the complexity of interpreting methylation marks, which are highly context dependent. In general, asymmetric dimethylation tends to support transcriptional activation, while symmetric

dimethylation is more often associated with repression, although there are notable exceptions.

PRMTs also regulate transcription through their activity on non-histone substrates, including transcription factors and chromatin-associated cofactors. These proteins frequently contain glycine–arginine-rich (GAR or RGG) motifs that serve as prime methylation sites for PRMTs. Such modifications can influence a factor's DNA-binding ability, protein interactions, or subcellular localization. For example, PRMT4 methylates CBP/p300, a transcriptional coactivator, at arginine 742, enhancing its interaction with nuclear hormone receptors such as the estrogen receptor (Chevillard-Briet et al., 2002; Xu et al., 2001). PRMT1 and PRMT5 methylate SPT5, a transcription elongation factor, which stabilizes its interaction with RNA polymerase II, facilitating transcriptional elongation (Kwak et al., 2003). Additionally, PRMT1 modifies E2F1, a key cell cycle regulator, to promote activation of G1/S genes, while PRMT5 similarly targets E2F1 and p53, altering their transcriptional activity in ways that often favor cell survival and proliferation over apoptosis (Barczak et al., 2020; Zheng et al., 2013).

Chromatin remodeling complexes are also functionally modulated by PRMT activity. PRMT5 interacts with the SWI/SNF remodeling complex and deposits repressive histone marks such as H4R3me2s and H3R8me2s at target promoters, including those of tumor suppressor genes like ST7 and NM23, effectively silencing them in cancer cells (Pal et al., 2004). Conversely, PRMT4 methylates the SWI/SNF subunit BAF155 at arginine 1604, which alters the targeting and function of the complex and has been associated with increased cell migration and invasion in breast cancer (Wang et al., 2014). These examples demonstrate how PRMTs influence not only the composition of chromatin but also the dynamics of nucleosome positioning and chromatin accessibility, thereby playing a multifaceted role in transcriptional control.

Each member of the PRMT family contributes uniquely to cellular physiology and pathology. PRMT1 is ubiquitously expressed and essential for the establishment of transcriptionally active chromatin via H4R3me2a methylation. It serves as a coactivator for several transcription factors, modulates estrogen-responsive genes through coactivator methylation, and is critical for DNA damage repair processes (Brobbey et al., 2022). PRMT1 methylates MRE11, a core component of the MRN complex involved in

DNA double-strand break repair by homologous recombination, as well as 53BP1, a DNA repair mediator important for non-homologous end joining (NHEJ) (Boisvert, Dery, et al., 2005; Boisvert, Rhie, et al., 2005). These modifications enhance recruitment and function of repair proteins at damage sites, and PRMT1 loss leads to defects in DNA repair and increased genomic instability (Giuliani et al., 2021; Walton et al., 2024).

PRMT5, as the main Type II enzyme, functions largely as a transcriptional repressor through the deposition of symmetric methylation marks, although it can also support gene activation in certain settings (Tarighat et al., 2016). PRMT5's repressive activity is well-documented in tumor suppressor gene silencing via chromatin remodeling complexes. At the same time, PRMT5 contributes to mRNA splicing through methylation of spliceosomal proteins such as Sm proteins and SRSF1, thereby impacting gene expression post-transcriptionally (Radzisheuskaya et al., 2019). Its influence extends to transcription factor stability as well, for instance by methylating E2F1 and KLF4 to control their degradation or functional output (Barczak et al., 2020; Hu et al., 2015). In the DNA damage response, PRMT5 plays a critical role in checkpoint activation and repair pathway choice by methylating RAD9, RUVBL1, and 53BP1 (He et al., 2011; Hwang et al., 2020; Zheng et al., 2013).

PRMT4/CARM1 is especially known for its coactivator role in transcriptional complexes. It targets histone H3 (H3R17 and H3R26) and facilitates gene activation in multiple contexts, including hormone response elements and cell cycle regulatory genes (Xie et al., 2024). In cancer cells, PRMT4 methylates chromatin remodeling proteins such as BAF155 and transcriptional coactivators like CBP/p300, enhancing oncogenic gene expression and, in some settings, recruiting DNA damage response proteins like BRCA1 to regulatory regions (Chevillard-Briet et al., 2002; Lee et al., 2011; Wang et al., 2014). Its methylation of metabolic enzymes like PKM2 has also been implicated in reprogramming metabolism to favor tumor growth (Liu et al., 2017).

Additional PRMTs contribute in more specialized ways. PRMT6 suppresses tumor suppressor genes like p21 and p16 through H3R2me2a methylation, and it also influences DNA methylation by modulating UHRF1 recruitment (Veland et al., 2017). PRMT7, the only Type III enzyme, supports epithelial-mesenchymal transition (EMT) and metastasis through cooperation with transcription factors like YY1 and HDAC3 to silence E-

cadherin (Yao et al., 2014). PRMT2 has dual roles, sometimes acting as a repressor and at other times promoting oncogenic transcription, depending on the cellular context (Dong et al., 2018; J. Li et al., 2022; T. Liu et al., 2024). PRMT9 has emerging links to splicing and cancer-related transcript regulation but remains less characterized overall (Jiang et al., 2018; Shen et al., 2024; Yang et al., 2015).

Dysregulation of PRMTs has been widely observed in cancer, where it contributes to both tumor initiation and progression (Hamamoto & Nakamura, 2016; Li et al., 2024; Yoshimatsu et al., 2011). PRMT1 is frequently upregulated in breast, lung, colorectal, and pancreatic cancers, where it activates proliferative and invasive pathways through methylation of oncogenic regulators such as EZH2, Twist1, and GLI1 (Avasarala et al., 2015; Z. Li et al., 2020; Y. Wang et al., 2016). It has also been implicated in therapy resistance, notably in colorectal cancer where it methylates EGFR and promotes resistance to anti-EGFR therapies like cetuximab (Liao et al., 2015; Yao et al., 2021). PRMT5 is similarly overexpressed across cancer types, with links to repression of microRNAs, activation of growth signaling pathways, and inhibition of apoptosis through p53 and E2F1 methylation (Scoumanne et al., 2009; Wu et al., 2023). PRMT5 is also synthetically lethal in MTAP-deleted cancers, making it an attractive therapeutic target (Fedoriw et al., 2019; Mavrakis et al., 2016).

CARM1 promotes proliferation and metastasis in breast cancer and is involved in reprogramming tumor metabolism. Its inhibition has shown therapeutic potential in lymphomas with specific coactivator mutations (Veazey et al., 2020). PRMT6 and PRMT7 are both associated with tumor aggressiveness and EMT signatures in various cancer types, while PRMT2 and PRMT9 have more context-specific roles that are still being elucidated (Wu et al., 2023).

The cumulative evidence from these studies positions PRMTs as central hubs in epigenetic reprogramming, transcriptional regulation, and genome maintenance—all of which are critical processes for cancer cell survival and therapeutic resistance. Their ability to methylate histone and non-histone substrates alike makes PRMTs particularly versatile and influential in controlling cellular phenotype. Not surprisingly, several PRMT inhibitors are now in clinical trials, targeting either specific PRMT isoforms or multiple family members simultaneously, with the aim of sensitizing tumors to

conventional therapies or suppressing aggressive tumor behavior (Dong et al., 2022; X. Li et al., 2019; Rugo et al., 2020). Continued research on the specific roles and regulation of each PRMT will further clarify their value as biomarkers and therapeutic targets across cancer types.

## 1.6. Rationale for Targeting Epigenetic Regulators to Reprogram Lysosomal Dynamics

The development of chemoresistance remains one of the most formidable barriers to effective cancer therapy. While multiple mechanisms underlie a tumor's ability to resist cytotoxic agents, increasing attention has been drawn to lysosomal sequestration and exocytosis as critical contributors to drug tolerance. Lysosomes, once seen merely as degradative compartments, are now recognized as dynamic organelles with complex roles in nutrient sensing, stress adaptation, and cellular clearance. In cancer cells, these vesicular structures have acquired functions that actively facilitate resistance to chemotherapeutic agents by serving as storage and export sites for cytotoxic compounds. Drugs that become trapped within acidic lysosomal lumens may be effectively neutralized and eventually expelled through lysosomal exocytosis, thereby preventing their interaction with intracellular targets and undermining treatment efficacy (Eriksson & Ollinger, 2024; Hrabeta et al., 2020).

This lysosome-centered mechanism of drug resistance presents an additional layer of cellular defense, complementing more classical resistance strategies such as enhanced DNA repair, efflux pump activation, or metabolic detoxification. Among the agents particularly prone to lysosomal sequestration are weak base chemotherapeutics, including anthracyclines, vinca alkaloids, and certain tyrosine kinase inhibitors, all of which tend to accumulate in acidic vesicular environments through ion-trapping mechanisms (Dey et al., 2024; Zhitomirsky & Assaraf, 2015). Once sequestered, these drugs may remain in the lysosome indefinitely or be expelled via lysosomal exocytosis—a regulated process involving lysosomal trafficking to the plasma membrane, fusion, and the extracellular release of contents (Hrabeta et al., 2020; Skoupa et al., 2020). Notably, this drug-clearing function is not a passive phenomenon but a tightly orchestrated response, governed by calcium signaling, cytoskeletal dynamics, and membrane fusion machinery (Skoupa et al., 2020; Zhitomirsky & Assaraf, 2017). The capacity of cancer cells to upregulate this

vesicular clearance system represents a significant and underappreciated form of therapy adaptation.

Despite the growing recognition of lysosomal exocytosis as a mediator of chemoresistance, the molecular regulators governing this pathway remain incompletely understood. Current knowledge suggests that its execution involves a range of proteins including SNAREs, Rab GTPases, tethering complexes, cytoskeletal adaptors, and calcium channels, which coordinate vesicle movement and membrane fusion (Tancini et al., 2020; Trojani et al., 2024). Transcriptional regulators such as TFEB have been implicated in promoting lysosomal biogenesis and function under stress, while changes in lysosomal positioning and exocytic readiness appear responsive to intracellular signaling and metabolic state (Chen et al., 2024; Franco-Juarez et al., 2022). Yet, relatively little is known about how this machinery is epigenetically controlled.

Epigenetic regulation—which includes DNA methylation, histone modifications, and non-coding RNA-mediated gene silencing—serves as a powerful modulator of gene expression programs in both normal and diseased states. In cancer, epigenetic aberrations frequently promote survival, metabolic adaptation, and therapeutic evasion by reshaping transcriptional networks without altering the genetic code. Given the plasticity of epigenetic states and their responsiveness to environmental signals, it is possible that cancer cells use epigenetic tools to fine-tune lysosomal activity in response to therapy-induced stress (Chae et al., 2023; Yu et al., 2023). Indeed, various studies have shown that components of the lysosome-autophagy network can be epigenetically modulated. Histone deacetylases (HDACs), for instance, have been linked to transcriptional repression of autophagy genes through chromatin compaction, while some sirtuins regulate lysosomal biogenesis and function through post-translational modification of key transcription factors and trafficking proteins (Budayeva & Cristea, 2016; Luo et al., 2024; Oh et al., 2008).

A notable example is the epigenetic control of TFEB, a master regulator of lysosomal gene expression. TFEB activity can be modulated not only by nutrient signaling via mTORC1 but also through acetylation, which affects its DNA-binding capacity and transcriptional output (T. Li et al., 2022; Zhang et al., 2018). Epigenetic enzymes such as HDACs and histone acetyltransferases (HATs) influence TFEB's access to gene

promoters, thereby governing lysosomal gene expression in response to cellular cues. Additionally, chromatin-associated factors like USF2, in partnership with HDAC1, have been shown to repress the expression of lysosomal and autophagy genes by reducing local histone acetylation, a repression that can be lifted during stress to allow TFEB-driven transcription (d'Azzo & Annunziata, 2020; Kim et al., 2024). These findings suggest that lysosomal programs are not hardwired but dynamically regulated through chromatin remodeling mechanisms.

Beyond TFEB, the regulation of lysosomal trafficking and exocytosis may also be subject to epigenetic control. For example, HDAC10, a cytoplasmic deacetylase, has been shown to influence lysosomal positioning and exocytic activity by modulating cytoskeletal elements that support lysosome mobility (Oehme et al., 2013; Ridinger et al., 2018). Inhibition or depletion of HDAC10 alters lysosomal distribution within the cell and impairs drug efflux, pointing to a mechanistic link between acetylation states and lysosomal export function. This expands the scope of epigenetic regulation from gene transcription to include control over organelle trafficking and functional output. Similarly, sirtuins such as SIRT2, which deacetylate cytoskeletal proteins, may affect the movement of lysosomes toward the plasma membrane, although this area remains underexplored (Budayeva & Cristea, 2016).

The intersection of epigenetic modulation and lysosomal exocytosis represents a relatively novel and understudied axis in cancer biology. While considerable research has explored how epigenetic dysregulation contributes to global gene expression changes and therapy resistance, few studies have examined its role in shaping vesicle dynamics and intracellular trafficking. This gap in knowledge is particularly striking given that lysosome-mediated drug resistance has been observed across multiple cancer types and with diverse chemotherapeutic agents. If epigenetic regulators influence the machinery that governs lysosomal exocytosis, then manipulating these regulators could offer a strategy to disrupt vesicle-based drug clearance and restore intracellular drug accumulation (Settembre et al., 2013).

Targeting epigenetic enzymes offers unique advantages as a therapeutic strategy. Epigenetic states are reversible, unlike genetic mutations, making them amenable to pharmacologic intervention. Several epigenetic inhibitors—commonly termed

"epidrugs"—have been developed to target specific chromatin modifiers, including DNA methyltransferases, HDACs, bromodomain-containing proteins, and protein arginine methyltransferases (PRMTs). Some of these agents are already FDA-approved for hematological malignancies, and an expanding array is under clinical investigation for solid tumors (Ganesan et al., 2019; Sahafnejad et al., 2023). By altering chromatin accessibility and transcription factor activity, these drugs can reprogram cancer cell behavior, sensitize tumors to chemotherapy, and potentially disrupt resistance mechanisms rooted in the tumor epigenome (Kronfol et al., 2017; Yu et al., 2024).

Among the epigenetic targets of growing interest are the PRMTs, a family of enzymes that catalyze the methylation of arginine residues on histone and non-histone proteins. PRMTs contribute to transcriptional regulation, DNA damage response, RNA splicing, and metabolic signaling—processes integral to cancer progression and therapy resistance (Gao et al., 2024; Zhu, Xia, et al., 2024). Dysregulation of PRMTs, particularly PRMT1 and PRMT5, has been implicated in the silencing of tumor suppressor genes, enhancement of DNA repair, and promotion of survival pathways in diverse cancer types (Kim & Ronai, 2020; Martinez et al., 2024). These enzymes also modify components of the cytoskeleton and vesicle trafficking networks, suggesting that their influence may extend into regulating lysosomal positioning and exocytic potential (Albrecht et al., 2015; Qualmann & Kessels, 2021). The methylation status of such substrates could impact lysosomal motility, fusion competence, or cargo release, providing a mechanistic bridge between epigenetic signaling and vesicular function.

Despite these compelling connections, the epigenetic control of lysosomal exocytosis remains largely undefined. While isolated examples—such as HDAC10's role in drug efflux or TFEB's transcriptional control under chromatin regulation—point toward epigenetic involvement, a comprehensive understanding of how chromatin modifiers shape lysosomal export programs is lacking. This knowledge gap is significant, as it may obscure novel therapeutic opportunities. If cancer cells depend on epigenetically tuned lysosomal systems to evade drug toxicity, then disrupting these epigenetic settings could undermine the vesicular defense network and re-sensitize cells to treatment. Importantly, such a strategy would not rely on targeting a single transporter or vesicle protein but could instead recalibrate the entire lysosomal landscape by modulating upstream regulatory nodes.

The rationale for investigating this interface—between epigenetics and lysosomal exocytosis—therefore rests on both biological plausibility and therapeutic potential. Epigenetic plasticity equips cancer cells with adaptive flexibility under stress, while lysosomal exocytosis functions as a critical escape mechanism under chemotherapeutic pressure. These two systems, though previously studied in isolation, likely converge in cancer cells to form a coordinated resistance program. Uncovering how chromatin regulators influence lysosomal trafficking and drug clearance could provide a blueprint for combination therapies that pair cytotoxic agents with epigenetic drugs to overcome tolerance. Moreover, identifying the epigenetic determinants of lysosomal function might offer new biomarkers for predicting chemotherapy response or for selecting patients likely to benefit from epidrug-based regimens.

In conclusion, while lysosome-mediated drug sequestration and exocytosis represent well-documented mechanisms of chemoresistance, their epigenetic regulation remains poorly understood. Clarifying how chromatin modifiers control lysosomal behavior may reveal previously unrecognized pathways of resistance and unveil novel strategies to restore drug efficacy in cancer. This conceptual framework supports the need to explore epigenetic regulators as key modulators of lysosomal dynamics—particularly exocytosis—in the context of therapeutic resistance. Bridging this gap holds promise for developing innovative, mechanism-based interventions to reprogram cellular drug handling and improve cancer treatment outcomes.

### 1.7. Objectives and Scope of the Study

The emergence of drug resistance remains one of the greatest challenges in modern oncology. While genetic mutations have long been considered the primary drivers of therapy failure, increasing attention has turned to non-genetic mechanisms—particularly those involving lysosomal drug sequestration and exocytosis—as central contributors to chemotherapy tolerance. Lysosomes, beyond their degradative role, function as dynamic mediators of cellular stress response, capable of isolating and expelling chemotherapeutic agents from the intracellular environment. This capacity, especially in the context of platinum-based compounds such as cisplatin, creates a protective barrier against cytotoxic damage and enables cancer cells to persist through treatment. Notably, the acidic pH of lysosomes promotes drug trapping, and subsequent lysosomal exocytosis removes these agents from the cell, reducing their therapeutic efficacy.

Despite growing evidence implicating lysosomal exocytosis in chemoresistance, the regulatory framework that governs this process—particularly at the chromatin level—remains insufficiently explored. Epigenetic regulation is known to orchestrate transcriptional responses to stress, control vesicle trafficking machinery, and modify key regulatory proteins through histone and non-histone methylation or acetylation. While some studies have linked epigenetic enzymes such as HDAC10, SIRT1, and histone demethylases to aspects of lysosomal function and autophagy, a systematic understanding of how lysosomal exocytosis is epigenetically regulated is lacking. This gap is critical, given the emerging view of lysosomes as central nodes of drug resistance and survival signaling in cancer.

This study aims to investigate whether targeting epigenetic regulators can reprogram lysosomal dynamics—specifically lysosomal exocytosis—to overcome chemotherapy resistance. The primary objective is to identify epigenetic modulators that interfere with lysosome-mediated drug expulsion and thereby increase the intracellular retention and cytotoxicity of chemotherapeutic agents. By focusing on a mechanistic link between epigenetic control and lysosomal behavior, the study seeks to expand the current understanding of non-genetic drug resistance and explore new therapeutic avenues for enhancing treatment efficacy in cancer.

To this end, the study leverages an unbiased pharmacological approach using a diverse panel of epigenetic inhibitor compounds. It examines how these agents affect lysosomal exocytosis, lysosomal gene expression, and drug sensitivity profiles in cancer cells. Furthermore, transcriptomic analyses are employed to dissect downstream regulatory networks involved in lysosomal trafficking, with a particular interest in identifying novel transcriptional or post-transcriptional mediators of vesicle function. The investigation also explores connections between epigenetic regulators and canonical lysosomal transcription factors, such as TFEB and MITF, while accounting for chromatin remodeling enzymes that may influence lysosomal gene expression or vesicle mobilization.

The broader scope of the study encompasses multiple layers of investigation—from small-molecule screening and gene expression analysis to mechanistic interpretation of lysosomal pathways—all oriented around a central hypothesis: that epigenetic

modulation of lysosomal dynamics represents a therapeutically actionable strategy to combat drug tolerance. In bridging the fields of chromatin regulation and lysosomal biology, this work proposes a new conceptual framework for understanding and disrupting vesicle-based chemotherapy resistance.

Ultimately, the study aspires to fill a critical gap in cancer biology by uncovering how lysosomal exocytosis is epigenetically regulated, and by providing mechanistic insight into how this regulation influences drug response. The findings have the potential to inform new combinatorial strategies for enhancing the efficacy of conventional therapies, particularly in tumors where lysosomal adaptation plays a key role in survival. By targeting the epigenetic architecture that enables lysosomal escape, this study aims to advance the field toward more effective and durable cancer treatments.

*Chapter 1*

*Section 2*

# 2. MATERIALS and METHODS

## 2.1. Cell Culture

Du145 (ATCC, HTB-81) and H1299 (ATCC, CRL-5803) cells were cultured in RPMI-1640 medium (Gibco, 11875093) supplemented with 10% fetal bovine serum (FBS; Biowest, S1810) and 1% penicillin-streptomycin (Pen/Strep; Biowest, L0022) at 37°C in a humidified incubator with 5% $CO_2$. Panc1 (ATCC, CRL-1469) and HEK293T (ATCC, CRL-3216) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; Gibco, 11965118) with the same FBS and Pen/Strep supplementation and environmental conditions. All other cell lines were cultured according to the standard ATCC-recommended media and protocols. Cells were routinely passaged or refreshed every 3–4 days using 0.05% Trypsin-EDTA (Gibco, 25300-054), depending on confluency.

## 2.2. Epigenetic Modifier Drug (Epidrug) Library Screening

A library of 175 small-molecule epigenetic inhibitors (Cayman, 11076) was used for screening on Du145, Panc1, and H1299 cell lines (detailed library content information given in **Table 1.1**). For combination cell viability assays, $1\times10^3$ cells were seeded into 96-well plates and allowed to adhere overnight. The following day, cells were pre-treated (primed) with individual epidrugs at a final concentration of 5 μM for 72 hours. After priming, cells were treated with a combination of the respective epidrug and cisplatin at defined concentrations for an additional 72 hours. Cell viability was subsequently assessed using the Sulforhodamine B (SRB) assay, as detailed below.

For functional screening of lysosomal phenotypes, Du145 cells were seeded in either 12-well plates ($2\times10^5$ cells/well) for β-hexosaminidase (β-Hex) lysosomal exocytosis assays or in 96-well plates ($4\times10^3$ cells/well) for LysoTracker Green (LTG) staining. Cells were treated with epidrugs (5 μM) for 72 hours, after which β-Hex or LTG-based assays were performed according to their respective protocols described in the following sections.

**Table 1.1.** Cayman Epigenetic Modifier Drug Library content.

| Cayman Item No. | Epidrug Name |
|---|---|
| 10496 | Sulforaphane |
| 10875 | UNC669 |
| 10443 | SB939 |
| 10444 | PCI 34051 |
| 11876 | L-α-Hydroxyglutaric Acid (sodium salt) |
| 10523 | Sirtinol |
| 13856 | 5-Nitroso-8-quinolinol |
| 10549 | C646 |
| 14088 | JNJ-26481585 (hydrochloride) |
| 10566 | Garcinol |
| 14314 | Plumbagin |
| 10572 | Scriptaid |
| 10574 | Suberohydroxamic Acid |
| 10575 | Apicidin |
| 14969 | LMK 235 |
| 10582 | UNC0321 (trifluoroacetate salt) |
| 15200 | HDAC6 Inhibitor |
| 10599 | Cl-Amidine (hydrochloride) |
| 15487 | SP-2509 |
| 10641 | JGB1741 |
| 16081 | A-366 |
| 10676 | I-BET762 |
| 10734 | UNC0638 |
| 16095 | OICR-9429 |
| 16173 | EPZ004777 (formate) |
| 16174 | EPZ6438 |
| 16272 | PBIT |
| 10975 | Zebularine |
| 11012 | Delphinidin (chloride) |
| 11045 | ITF 2357 |
| 16365 | Octyl-α-hydroxyglutarate |
| 16400 | UNC0379 |
| 11091 | Methylstat (hydrate) |
| 16615 | BVT 948 |
| 16918 | BG45 |
| 17123 | UMB-32 |
| 11138 | 2,4-Pyridinedicarboxylic Acid (hydrate) |
| 11155 | PFI-1 |
| 11164 | 5-Azacytidine |
| 11165 | SGI-1027 |
| 11166 | Decitabine |
| 11181 | I-BET151 |
| 11187 | (+)-JQ1 |
| 17285 | EPZ015666 |
| 17385 | BI-2536 |
| 17448 | BAZ2-ICR |
| 11323 | Sodium 4-Phenylbutyrate |
| 17471 | OG-L002 |
| 11572 | IOX1 |
| 11620 | MI-2 (hydrochloride) |
| 17472 | ML-324 |
| 11690 | Gemcitabine |
| 17488 | GSK484 (hydrochloride) |
| 17553 | Resminostat (hydrochloride) |
| 17662 | NI-57 |
| 12033 | Daminozide |
| 12054 | GSK-J1 (sodium salt) |
| 17663 | PFI-4 |
| 12073 | GSK-J4 (hydrochloride) |

| Cayman Item No. | Epidrug Name |
|---|---|
| 13631 | UNC0224 |
| 19136 | ORY-1001 |
| 19160 | EPZ020411 |
| 13828 | 3-Deazaneplanocin A |
| 13829 | Sinefungin |
| 13870 | Pyroxamide |
| 13944 | N-Oxalylglycine |
| 19403 | GSK2879552 |
| 13965 | AMI-1 (sodium salt) |
| 13966 | EPZ005687 |
| 13967 | SGC0946 |
| 13968 | UNC1215 |
| 19551 | Cyproheptadine (hydrochloride hydrate) |
| 14094 | GSK343 |
| 14119 | Bromosporine |
| 14120 | GSK2801 |
| 14407 | SIRT1/2 Inhibitor IV |
| 14468 | I-CBP112 (hydrochloride) |
| 14469 | SGC-CBP30 |
| 14604 | UNC0642 |
| 14621 | UNC1999 |
| 14678 | (R)-PFI-2 (hydrochloride) |
| 15066 | HPOB |
| 19828 | CPTH6 (hydrobromide) |
| 15267 | PFI-3 |
| 15338 | JIB-04 |
| 15403 | CAY10683 |
| 15415 | GSK126 |
| 15479 | CPI-203 |
| 15774 | 6-Thioguanine |
| 19835 | HAT Inhibitor II |
| 19956 | CeMMEC1 |
| 15947 | OTX015 |
| 20059 | PCI 24781 |
| 20209 | BML-278 |
| 20224 | CeMMEC13 |
| 16175 | EPZ5676 |
| 16265 | MC 1568 |
| 20541 | 4'-bromo-Resveratrol |
| 16374 | α-Hydroxyglutaric Acid (sodium salt) |
| 20864 | AZD 5153 |
| 16424 | RVX-208 |
| 20893 | Todralazine (hydrochloride) |
| 16427 | LAQ824 |
| 21057 | HDAC3 Inhibitor |
| 16439 | GSK-LSD1 (hydrochloride) |
| 21159 | CXD101 |
| 16917 | RGFP966 |
| 16919 | BRD73954 |
| 21234 | NCH-51 |
| 21273 | UF010 |
| 21568 | RGFP109 |
| 89730 | Trichostatin A |
| 89740 | CAY10398 |
| 9001839 | RSC-133 |
| 10005019 | BML-210 |
| 21601 | C-7280948 |
| 10009797 | CAY10591 |
| 22031 | EED226 |

| Cayman Item No. | Epidrug Name |
|---|---|
| 10009929 | SAHA |
| 10010494 | Tranylcypromine (hydrochloride) |
| 22404 | CAY10722 |
| 22942 | PAOA |
| 23242 | TMP-195 |
| 26068 | Bufexamac |
| 11706 | BAPTA |
| 21601 | C-7280948 |
| 34886 | GSK3368715 |
| 21879 | SGC2085 |
| 17718 | TC-E 5003 |
| 19160 | EPZ020411 |
| S6414 | Apilimod |
| Unused | Unused (DMSO) |
| 11085 | UNC0646 |
| 13124 | BIX01294 (hydrochloride hydrate) |
| 16614 | Ryuvidine |
| 17017 | SGC707 |
| 20425 | Vacuolin-1 |
| 11796 | Wedelolactone |
| 13148 | Lysine-specific Demethylase Inhibitor (1C) |
| 13212 | Pimelic Diphenylamide 106 |
| 13686 | Chidamide |
| 16874 | Nexturastat A |
| 20985 | GSK6853 |
| 9002910 | (+)-JQ1 (free acid) |
| 21273 | UF010 |
| 10004974 | Ionomycin |
| 10007965 | FK-506 |
| 17692 | Tasquinimod |
| 12084 | CI-994 |
| 12086 | CPTH2 (hydrochloride) |
| 17699 | MM-102 |
| 17718 | TC-E 5003 |
| 12095 | Butyrolactone 3 |
| 17778 | L002 |
| 13085 | Tenovin-1 |
| 13086 | Tenovin-6 (hydrochloride) |
| 17897 | BI-9564 |
| 18124 | RN-1 (hydrochloride) |
| 13144 | Anacardic Acid |
| 13145 | AGK2 |
| 13146 | CAY10603 |
| 13168 | Splitomicin |
| 13172 | CBHA |
| 18287 | Mocetinostat |
| 13176 | Oxamflatin |
| 13178 | Salermide |
| 18317 | A-196 |
| 18348 | MS049 (hydrochloride) |
| 18354 | GSK591 |
| 13280 | Panobinostat |
| 13284 | MS-275 |
| 18361 | MS023 (hydrochloride) |
| 13302 | RG108 |
| 13373 | 2',3',5'-triacetyl-5-Azacytidine |
| 19093 | PRT4165 |

### 2.3. β-Hex Lysosomal Exocytosis Assay

Lysosomal exocytosis was assessed by measuring β-hexosaminidase (β-Hex) activity using a physiological buffer composed of 10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 2 g/L glucose. Du145, Panc1, or H1299 cells were seeded at a density of $2\times10^5$ cells per well in 12-well plates. After 24 hours, cells were gently washed once with regular buffer and incubated for 1 hour in

either basal (buffer only) or stimulated (buffer supplemented with $CuCl_2$; Sigma, 222011) conditions.

Following incubation, the supernatant was collected and reacted with 3 mM 4-nitrophenyl-N-acetyl-β-D-glucosaminide (Sigma, N9376) for 1 hour at 37 °C. The enzymatic reaction was terminated using borate buffer, and absorbance was measured at 405 nm. For quantifying total β-Hex content, cells were lysed with 1% Triton X-100 in PBS, and lysates were subjected to the same colorimetric reaction. Enzymatic activity was calculated based on the formation of 4-nitrophenol and calibrated using a standard curve prepared with known concentrations of 4-nitrophenol (Sigma, N7660) in 0.1 M citrate buffer.

The percentage of secreted β-Hex was calculated using the formula:
β-Hex secreted (% of total) = [$A_{405}$ (supernatant)] / [$A_{405}$ (supernatant + lysate)] × 100

**2.4. LysoTracker Green (LTG) Staining Assay**

To evaluate lysosomal content following epidrug treatment, LysoTracker Green (LTG) staining was conducted. Du145, Panc1, and H1299 cells were seeded in 96-well plates at a density of $4\times10^3$ cells per well. The next day, cells were treated with the indicated compounds. At the experimental endpoint, cells were washed once with PBS (Biowest, L0615), and culture medium containing 100 nM LysoTracker Green (Thermo, L7526) was added. Cells were incubated at 37 °C for 1 hour in the presence of the dye. After incubation, cells were washed five times with PBS to remove excess dye, and fluorescence was measured using a Synergy H1 Hybrid Reader (BioTek).

Following fluorescence reading, cells were lysed with 25 µL of 0.2% SDS, and total protein content was quantified using the BCA Protein Assay Kit (Thermo, 23225). Fluorescence values were blank-subtracted and normalized to total protein concentration (µg), yielding RFU/µg protein as the final readout.

For flow cytometry-based analysis, cells were stained with 100 nM LysoTracker Green under the same conditions. After the 1-hour incubation, cells were washed twice with PBS, detached using 0.25% Trypsin-EDTA, and resuspended in PBS containing 2% FBS to preserve viability. Samples were analyzed on a Beckman Coulter CytoFLEX flow cytometer, with a minimum of 10,000 events recorded per sample. Median

fluorescence intensity (MFI) was calculated for each sample and normalized to control values. Population shifts in fluorescence intensity were visualized using FlowJo software (v10.10.0).

### 2.5. Sulforhodamine B (SRB) Cell Viability Assay

Cells were seeded in 96-well plates at the indicated density prior to drug treatment. Following incubation with compounds, at the termination time-point, cells were fixed with 10% (w/v) trichloroacetic acid (TCA; Sigma, T6399) for 1 hour at 4 °C. After fixation, wells were washed thoroughly with deionized water and allowed to air-dry at room temperature. Cells were then stained with 50 µL of 0.4% (w/v) Sulforhodamine B (SRB; Santa Cruz, sc-253615) for 30 minutes. Excess dye was removed by washing with 1% acetic acid, and plates were left to dry completely. Bound SRB dye was solubilized with 150 µL of 10 mM Tris base solution (Sigma, T1503), and absorbance was measured at 564 nm using a Synergy H1 Hybrid microplate reader (BioTek). Cell viability was calculated using the formula:

% Viability = 100 × (Sample Abs - Blank) / (Non-treated Control Abs - Blank)

### 2.6. Crystal Violet Colony Formation Assay

Cells were seeded at a density of 0.5–2×10³ cells per well in 12-well plates and allowed to adhere overnight. The following day, treatments were applied, and the culture medium was refreshed every 72 hours thereafter. Colonies were allowed to form over a period of 1–3 weeks, depending on the cell line and experimental conditions.

At the endpoint, culture medium was aspirated and wells were gently washed with PBS (Biowest, L0616). Cells were fixed with cold methanol for 15–20 minutes at –20 °C, followed by two PBS washes. Fixed cells were stained with 0.05% (w/v) crystal violet solution for 30–60 minutes, washed with deionized water, and air-dried. Stained colonies were visualized and quantified using ImageJ software, and the percentage of colony formation was calculated relative to controls.

### 2.7. Calculation of Combination Index (CI)

Synergistic interactions between drug combinations were assessed using the Combenefit software. Dose–response curves were generated from SRB cell viability assays for each drug alone and in combination. The Combenefit platform calculated

combination index (CI) values based on the Bliss independence model, using the formula:

$$\text{Effect } (a + b) = E(a) + E(b) – E(a)\times E(b)$$

Interpretation of synergy or antagonism followed the Combenefit scoring criteria: positive scores indicate synergy, while negative scores reflect antagonism. Increasing absolute values signify stronger interaction effects and are visually represented by intensified color gradients in the surface plots, which illustrate both cell viability and synergy profiles across combined dose matrices.

Synergy scoring analysis shown in the following figures were performed by PhD student Neslihan Yuksel-Catal; Figure 1.9, 1.21B, 1.22B, 1.23B, 1.24B, 1.25B, and 1.26B.

### 2.8. Western Blotting

Cells were lysed using RIPA buffer (EcoTech, RIPA-100) supplemented with PMSF (Merck, 10837091001) and PhosSTOP phosphatase inhibitor cocktail (Merck, 4906845001), following the manufacturer's guidelines. Protein concentrations were quantified using the BCA Protein Assay Kit (Thermo Fisher, 23225). Equal amounts of protein were denatured at 95 °C for 10 minutes in Laemmli buffer (Bio-Rad, 1610747) containing DTT (Sigma, D0632), and resolved on Mini-PROTEAN TGX Stain-Free SDS-PAGE gels (Bio-Rad, 4568086).

Proteins were transferred onto PVDF membranes (Bio-Rad, 1620177), which were subsequently blocked with 5% BSA (Sigma, A3733) and incubated overnight at 4 °C with primary antibodies: anti-aDMA (1:5000; CST, 13522), anti-sDMA (1:2500; CST, 13222), anti-PRMT1 (1:1000; Abclonal, A1055), anti-PRMT6 (1:1000; Abclonal, A5085), anti-FLAG (1:2000; Thermo, F3165), and anti-GAPDH (1:5000; Abcam, AB9485).

After washing with TBS-T (EcoTech, TBST2005), membranes were incubated with HRP-conjugated secondary antibody (Abcam, ab205718) for 1.5 hours at room temperature. Protein bands were visualized using the Immobilon Forte Western HRP substrate (EMD Millipore, WBLUF0500) and imaged using the Licor Odyssey FC Imaging System.

### 2.9. Real-Time Quantitative PCR (RT-qPCR)

Total RNA was extracted using the NucleoSpin RNA II Kit (Zymo, R1051) according to the manufacturer's instructions. For cDNA synthesis, 1 µg of total RNA was reverse transcribed using the M-MLV Reverse Transcriptase kit (Invitrogen, 28025-013) following the supplied protocol.

Quantitative PCR was carried out using the LightCycler 480 SYBR Green I Master Mix (QIAGEN, 1076717) and gene-specific primers (listed in **Table 1.2**). Each 20 µL reaction contained SYBR Green master mix, cDNA template, and primers. Amplification was performed on a LightCycler 480 Instrument II (Roche) using the following cycling conditions: initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 72 °C for 1 minute.

Expression levels of target genes were normalized to GAPDH as the internal control, and relative expression changes were calculated using the 2^(-ΔΔCt) method. Amplification specificity was confirmed through melting curve analysis and, where needed, validated by agarose gel electrophoresis. Statistical comparisons between experimental groups were performed using Student's t-test, based on at least three independent biological replicates.

**Table 1.2.** Sequence information of primers used in the RT-qPCR experiments.

| | Fwd Primer (5'-3') | Rev Primer (5'-3') |
|---|---|---|
| ABCA1 | ACAAGATGCTGAGGGCTGATG | CCAGTTTCTCCCTTGGTAGGC |
| ABCA2 | ACACCTCTGGTTCTACTCACGG | CCGACAATGTCTGCACCAGTGA |
| ABCA3 | CTTGACAGTCGCAGAGCACCTT | CTCCGTGAGTTCCACTTGTCCT |
| ALPG (ALPPL2) | TACTCCATACCTGGGATTTCCG | TTCTCCTCCTCAACTGGGAT |
| ATOX1 | TCTCTCGGGTCCTCAATAAGC | AAGCAGAGTGTCCATGCTGTG |
| ATP6V0D1 | ACAAGACGCTGGAGGACCGATT | ACGATGTTGCGACACTCCTGCT |
| ATP6V1A | GAAACTTCTGGTGTGTCTGT | CCATAATGCCAGGACCAAG |
| ATP7A | TTTGGAACTTGTTGTGAGGGG | AGGGCCACGGAGCAGTATAG |
| ATP7B | GTGGGCAATGACACCACTTT | TGGGTGCCTTTGACATCTGA |
| BCHE | TCCATAGTGAAACGGTGGGC | AGGCCAGCTTGTGCTATTGT |
| CALHM5 | GCAAGGGTAAGCCCAAAGAGTG | CGCTGAACAAATCAGGCACCATC |
| CARF | GAGATTATTCCAGCAACGCTTCA | ATTTTGGGCTGAAATGAGGAGAGTG |
| CAVIN2 | GCGGTCAAAGAGCGCATGGATA | AAACACGCTGGCAGGGATCTCA |
| CD38 | CAGCACTTTTGGGAGTGTGG | GATCCTGGCATAAGTCTCTGG |
| CITED2 | TGCCGCCCAATGTCATAGACAC | CAGCTCCTTGATGCGGTCCAAA |
| CLIC3 (CLI3) | CATCCTGCTCTATGACAGCGAC | GGTGTTGGACTCCCTGTAACGA |
| CPA4 | GGGGACATGAGGTGGATACTG | CCTCAAAACTTGGTCCCCAAAAA |
| CTR1 | TGCGTAAGTCACAAGTCAGCA | AGGTGAGGAAAGCTCAGCATC |
| CTSB | GCTTCGATGCACGGGAACAATG | CATTGGTGTGGATGCAGATCCG |
| CTSD | GCAAACTGCTGGACATCGCTTG | GCCATAGTGGATGTCAAACGAGG |
| DDB2 | TTGTGGGCCGATACCCAGAT | AAGCGAACTGATGCCAGAAGA |
| DMGDH | GGCTGAAACGAAGACTGGTCTG | GCTTCCAGATGTCGTGTTGCCA |
| E2F5 | CCATTCAGGCACCTTCTGGTAC | AGCAGCACATGGATAGGTCCTG |
| EGR1 | AGCAGCACCTTCAACCCTCAGG | GAGTGGTTTGGCTGGGGTAACT |
| FAXDC2 | ACTGTGGCTACCACCTTCCCTT | GGTCTGCTTGAACATGGTGTCAG |
| FBXO36 | TGGTCACCCGGTCTCAGGTAAT | GGGTTTCTTTTGCTTCTCCAGGT |
| GAPDH | CTGACTTCAACAGCGACACC | GTTGTCATACCAGGAAATGAGC |
| GLIPR1L2 | TTTGCCAGACGAGGAGGACGTA | CCATGCTCTAGCAGTCCGTGAT |
| GOLGA7B | CGGATTTTACGCAGAGGCTGAG | CATTCTGCTCCTGGATGTAGCG |
| HTRA1 | CAGACGTGATCTCAGGAGCGTA | TCGCTGACATCATTGGCGGAGA |
| HTRA3 | CAAGAAGTCGGACATTGCCACC | CGTTGTCACTGTGTTCTGTAGGG |
| LAMP1 | CGTGTCACGAAGGCGTTTTCAG | CTGTTCTCGTCCAGCAGACACT |
| LAMP2 | GGCAATGATACTTGTCTGCTGGC | GTAGAGCAGTGTGAGAACGGCA |
| LRP11 | GTCAGTGGACATGAAGGTGCC | GGAAGGTGTAGGTTCCCTCCT |

| | Fwd Primer (5'-3') | Rev Primer (5'-3') |
|---|---|---|
| MCOLN1 | CGGACTGCTATACCTTCAGCGT | GGTGCTTACACTCCTGGATGTG |
| MGMT | CCTGGCTGAATGCCTATTTCCAC | GCAGCTTCCATAACACCTGTCTG |
| MITF | GGCTTGATGGATCCTGCTTTGC | GAAGGTTGGCTGGACAGGAGTT |
| MRE11 | TGCCCAGGAAAATGAAGTGGA | CAGGCCGATCACCCATACAA |
| MSH5 | CGAACCAAGGAGCTGGATGCAT | GGAGGCAAGGTCCAATACTCGG |
| OPN3 | ATGGTCACCTGGTCACTCCAAC | GAGGCACAGAAGCTGCAAAAGG |
| PGM5 | CTGATGGACTCAGGACGTTGCA | ATGGAGAGCCAGACCAAGACAG |
| PPT1 | GGCGTACTCCAAAGTTGTTCAGG | CTGCCAAGAAGATGCTGTGGTTG |
| PRMT1 | CCTCACTTACCGCAACTCCA | CTCGATCCCGATGACCTTGC |
| PRMT3 | TGTCAGAACCTGCTCGTCAT | TCTCGGTAGCTTTCTGTTCGT |
| PRMT6 | AAGATGTCGCAGCCCAAGAA | GGACCGAAACGTCCGAGTAG |
| PRMT8 | AGCGAGTGGATGGGCTACT | CTGCCCGGTCTGGAAACATAA |
| PRMT9 | CAGGACCTTGCAGACTACTGG | AGGTTAGCGAGGGCACTACA |
| PTPRB | TCTTCCCGACAAGTGGTTGTGG | AGCCAGGAAACGCTGAGGTAGT |
| PTPRR | GTAACCGATTCATTGAGCACCTAC | GCTGTCTTCCTGCCAAACCATC |
| RRAD | CGAGAGCGTTTACAAGGTGCTG | ATGGAGCGATCATAGGTGTGCC |
| SECTM1 | GACAAGTCACGCTGGAGGTTTC | CACCTGTACCAGGCGAACATGA |
| SERPINB7 | CGATGCCAAAGTGGAGCGAGTT | CAGCAGATGAGCTTATGCCACC |
| SERPINE1 | GCCGCCTCTTCCACAAATCA | GGCAGTTCCAGGATGTCGTA |
| SLAMF7 | ACTGTGGAAATACCGAAAAAGATGG | ATAGCCTTGGTGTGTCTGGC |
| SLC1A1 | CGAAAGAACCCTTTCCGATTTGC | GAAGGTGACAGGCAGTGTTGCT |
| SLC6A3 | CCTCAACGACACTTTTGGGACC | AGTAGAGCAGCACGATGACCAG |
| TFEB | CCTGGAGATGACCAACAAGCAG | TAGGCAGCTCCTGCTTCACCAC |
| TM4SF1 | ATGCCTCCGAAAACCACCTC | TGGCAGGAGCATCAGCA |
| TMEM107 | CGGAGCATTCTGGGAAGAAGT | TAACGCGCTGGTATGAGCAA |
| TMEM116 | TCGTTTGCTGTCCTTCCTCTC | TGTATTGCAGGAAGAACCGAGG |
| TMEM205 | GCAAATGTGGGTGACCTTCGTC | GCAGAGGTTGATGAAGGCACAG |
| TMEM240 | CCGATTCCACAACTACATCCTCC | CGGGATCACGTAGTGGATATGG |
| TMEM255B | CATCGTGGACGGCGTATTTGCA | ACAGGTGACCTCTGTCTGGTAG |
| TMEM40 | TTTCGTCCTCCTGTGCTTTGCC | GTCCGAAGTAGATGCCAACGGT |
| TPP1 | GGTGGCTTCAGCAATGTGTTCC | GAAGTAACTGGATGGTGGCAGG |
| TRPM6 | ACCCTTCTTTCAGGCTACCG | GGAGTGTCTCTGGTGCTTGT |
| VAMP7 | CGGTTCAAGAGCACAGACAGCA | ATCCACTTGGGCTTGAGTCTCC |

### 2.10. Cloning PRMT1 and PRMT6 into pLJC2 Lentiviral Overexpression Vector

To generate FLAG-tagged PRMT1 and PRMT6 overexpression constructs for use in FLAG-based ChIP-qPCR assays, the coding sequences of PRMT1 and PRMT6 were amplified from commercially available plasmids (DNASU: #HsCD00950899 for PRMT1 and #HsCD00962806 for PRMT6) using gene-specific primers designed for subcloning (listed in **Table 1.3**). PCR amplification was performed with the Phusion High-Fidelity DNA Polymerase Kit (NEB, E0553L) according to the manufacturer's instructions.

Amplified PCR products were digested with PacI (NEB, R0547) and NotI (NEB, R0189) to generate cohesive ends. Simultaneously, the destination vector pLJC2-GPT2-3XFLAG (Addgene, #163447) was digested with the same enzymes to excise the GPT2 insert and linearize the vector backbone. Digested products were resolved on a 1%

agarose gel, and the appropriate DNA fragments were gel-extracted and purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel, #740609.50).

Purified PRMT1 and PRMT6 inserts were ligated into the digested pLJC2-3XFLAG backbone using T4 DNA Ligase (Thermo Fisher Scientific, EL0013) following the recommended protocol. Ligation mixtures were transformed into E. coli Stbl3 chemically competent cells, and colonies were selected on LB agar plates containing the appropriate antibiotic. Positive clones were screened, and plasmid DNA was isolated using the NucleoSpin Plasmid EasyPure Kit (Macherey-Nagel, #740588.50). Sequence-verified constructs were subsequently used for lentiviral production and downstream transduction experiments.

**Table 1.3.** Sequence information of primers used in PRMT1 and PRMT6 cloning into pLJC2 lentiviral overexpression vector.

| | |
|---|---|
| **PRMT1-PacI_Fwd** | atatatTTAATTAAATGGCGGCAGCCGAGGCCGC |
| **PRMT1-NotI_Rev** | atatatatGCGGCCGCGCGCATCCGGTAGTCGG |
| **PRMT6-PacI_Fwd** | gcgcgcgcTTAATTAAATGTCTCAGCCTAAGA |
| **PRMT6-NotI_Rev** | atatatGCGGCCGCATCCTCCATGGCGAAGTCTTT |

### 2.11. Generation of PRMT1/6-FLAG Overexpressing Cells

Lentiviral particles encoding FLAG-tagged PRMT1 or PRMT6 were produced using early-passage HEK293T cells. Cells were seeded at a density of $3.5 \times 10^6$ per 100 mm dish and cultured overnight. The next day, the culture medium was replaced with antibiotic-free medium prior to transfection. For virus production, a transfection mixture was prepared by combining 2.25 µg of psPAX2 (Addgene, #12260), 250 ng of VSVG (Addgene, #8454), and 2.5 µg of the plasmid encoding PRMT1 or PRMT6 in 200 µL of Opti-MEM (Thermo Fisher, #31985062). Separately, 15 µL of polyethyleneimine (PEI) (Sigma, #408727) was diluted in another 200 µL of Opti-MEM. The two solutions were mixed, incubated at room temperature for 25 minutes to allow complex formation, vortexed briefly, and then added dropwise to the HEK293T cells. After 18–24 hours, the medium was replaced with fresh complete medium.

Conditioned medium containing viral particles was collected at 48 and 72 hours post-transfection and filtered through a 0.45 µm PVDF membrane. To concentrate the virus, the filtered supernatant was mixed with PEG8000 (Sigma, #25322) and incubated

overnight at 4 °C, followed by centrifugation at 3,000 × g for 30 minutes. The viral pellet was resuspended in PBS, aliquoted, and stored at –80 °C until use. For transduction, Du145, Panc1, and H1299 cells were seeded at a density of $5 \times 10^4$ cells per well in 6-well plates. Then, 30 µL of concentrated virus was added to each well along with 8 µg/mL protamine sulfate. After 24 hours, the medium was replaced with fresh complete medium. At 48 hours post-transduction, selection was initiated using 2 µg/mL puromycin, with media refreshed every 48 hours until all non-transduced control cells were eliminated.

### 2.12. Chromatin Immunoprecipitation qPCR (ChIP-qPCR)

PRMT1/6-FLAG overexpressing Du145, Panc1, and H1299 cells were harvested at a density of $3 \times 10^6$ cells per sample. Crosslinking was performed with 1% formaldehyde (Sigma Aldrich, #818708) at room temperature for 10 minutes, and the reaction was quenched by the addition of glycine to a final concentration of 125 mM (Sigma Aldrich, #G7126) for 5 minutes. Cells were centrifuged at 1,000 × g for 5 minutes at 4 °C, washed twice with cold PBS, and resuspended in ChIP Lysis Buffer (50 mM HEPES, 1 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1% SDS, 1 mM PMSF, and protease inhibitor cocktail). Samples were kept on ice for 15 minutes. Chromatin was sheared to 100–500 bp fragments using the Bioruptor Plus sonicator (Diagenode), and the lysates were centrifuged at 10,000 × g for 10 minutes at 4 °C. The resulting supernatant was collected and pre-cleared by incubation with pre-washed Protein G Dynabeads® (Thermo Fisher, #88848) for 30 minutes at 4 °C.

Antibody–bead complexes were prepared by incubating anti-FLAG antibody (Thermo, #F3165) or mouse IgG isotype control (Sigma Aldrich, #10400C) with magnetic beads in PBST for 1 hour at room temperature. These complexes were added to the pre-cleared chromatin and incubated overnight at 4 °C. Immunoprecipitates were then washed twice (5 minutes each) with ChIP Lysis Buffer, followed by successive washes with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 140 mM NaCl, pH 8.1), high-salt buffer (same as low-salt buffer but with 500 mM NaCl), LiCl-containing buffer (0.25 M LiCl, 1% IGEPAL-CA 630, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and finally Tris-EDTA (TE) buffer.

Chromatin was eluted using a buffer containing 1% SDS (Bio-Rad, #1610418) and 0.1 M $NaHCO_3$ (Merck, #S6014). Reverse crosslinking was performed at 37 °C for 30 minutes with RNase A (0.3 mg/mL; Thermo Fisher, #12091039) and 5 M NaCl (Merck, #S9888), followed by incubation with proteinase K (0.3 mg/mL; Thermo Fisher, #EO0492) at 65 °C for 1 hour. DNA was purified using the ChIP DNA Clean & Concentrator Kit (Zymo Research, #D5205). Primers for ChIP-qPCR were designed using the UCSC Genome Browser and Ensembl Genome Browser, and their sequences are listed in **Table 1.4**. Quantitative PCR was performed on a LightCycler 480 instrument (Roche), and enrichment was calculated using the % input method.

**Table 1.4.** Sequence information of primers used in the ChIP-qPCR experiments.

| | |
|---|---|
| **ChIP_STX4_Fwd** | TGAGAACGGCGTCCCAGAGA |
| **ChIP_STX4_Rev** | CCAAACTCGTGGACCGCAGA |
| **ChIP_CACNG4_Fwd** | CCGGCGATTTCTGGTTGATTGG |
| **ChIP_CACNG4_Rev** | GGCTGGTTGAAAAGCCAGGG |
| **ChIP_CHMP3_Fwd** | GCGCAGAATCGAGTGAGTGG |
| **ChIP_CHMP3_Rev** | AGCACCGAATCCGTGGTCTC |
| **ChIP_HYAL3_Fwd** | ACAGCTTGGAAACGGTCCGA |
| **ChIP_HYAL3_Rev** | AGTCTAAGGGCGGCTGCTTC |
| **ChIP_MGMT_Fwd** | GGGGTAGTTTGATCCCTTCGGT |
| **ChIP_MGMT_Rev** | GAGAGACCACTCTGCAGGTCT |
| **ChIP_ZCWPW1_Fwd** | GCGCCTAGCACCTTCACCTA |
| **ChIP_ZCWPW1_Rev** | GTTCCGCCCATCGCCTTTAG |
| **ChIP_GON7_Fwd** | CATCCGTTTGTCGGAAGTCGC |
| **ChIP_GON7_Rev** | CGAGTGCCAGGAACCAATGAC |
| **ChIP_FLCN_Fwd** | CGCTGCCCGACAATTACCCT |
| **ChIP_FLCN_Rev** | GTGCGGTCTGAACCATTCGG |
| **ChIP_NEU1_Fwd** | CGCGCTTCCCGGACTCTAAT |
| **ChIP_NEU1_Rev** | AAGAGGGCCAATCGGAAGGG |
| **ChIP_ABCA3_Fwd** | GGTGGCCTCTGACAGGAATGAC |
| **ChIP_ABCA3_Rev** | ATGGAGAAACGGCCAAAGTCG |

### 2.13. RNA Sequencing and Analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Library preparation, poly-A mRNA enrichment, and next-generation sequencing were outsourced to BGI Group as a contract research service. Sequencing was performed on the BGIseq platform, generating ~20 million paired-end reads (150 bp) per sample. Raw sequencing data were stored and processed using the Koç University High-Performance Computing Cluster (KUACC). Initial quality assessment of the FASTQ files was conducted using FastQC, which provided metrics on base quality scores, sequence content, and potential adapter contamination. In cases

where low-quality bases or adapter sequences were detected, TrimGalore was used for quality trimming.

High-quality reads were aligned to the human reference genome (NCBI GRCh38) using HiSAT2. Post-alignment quality control was performed with RSeQC and Picard Tools to identify and remove mitochondrial reads, mis-spliced transcripts, and PCR duplicates. This ensured high-confidence transcript quantification for downstream analysis. Gene-level read counts were generated using featureCounts from the Subread package. Differential gene expression analysis was conducted with the DESeq2 package in R. Genes with statistically significant differential expression were further annotated using the biomaRt database. To interpret the functional relevance of the results, Gene Set Enrichment Analysis (GSEA) was performed, identifying enriched pathways and gene networks associated with the differentially expressed gene sets. The RNA-seq dataset has been deposited in the Gene Expression Omnibus (GEO) under the accession number GSE277490.

### 2.14. Statistical Analysis

All data analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc.). The specific statistical tests applied for each experiment are detailed in the corresponding figure legends. Information regarding sample sizes, representation of error bars, and significance levels (p-values denoted by asterisks) is likewise provided within the figure legends for clarity and reproducibility.

*Chapter 1*

*Section 3*

# 3. RESULTS

## 3.1. Cell line screening identifies Du145 as a suitable model and reveals an inverse correlation between lysosomal exocytosis and lysosomal volume

Lysosomal exocytosis has been increasingly recognized as a cellular adaptation to therapeutic stress, particularly in the context of chemotherapeutic drug sequestration and clearance. Given this, the functional characterization of lysosomal exocytosis in cancer models requires a quantitative and reproducible assay system, as well as appropriate cell lines capable of supporting this lysosomal function under both basal and stimulated conditions. In this study, we employed the β-Hexosaminidase (β-Hex) secretion assay as a quantitative surrogate for lysosomal exocytosis. To select an optimal cell line model for downstream functional assays and drug screening, we systematically screened a panel of 19 cancer cell lines to assess their basal exocytosis levels and inducibility following copper chloride ($CuCl_2$) stimulation, a well-established approach to enhance lysosomal exocytosis by promoting lysosomal fusion with the plasma membrane.

The schematic overview of the β-Hexosaminidase (β-Hex) secretion assay is presented in **Figure 1.1**. This assay measures the activity of β-Hex, a soluble lysosomal enzyme, that is released into the extracellular medium when lysosomes undergo exocytosis. Because β-Hex is predominantly localized within lysosomes, its presence in the supernatant provides an indirect but reliable readout of lysosomal exocytosis. The amount of secreted β-Hex correlates with the degree of lysosome–plasma membrane fusion, allowing for quantitative comparison of exocytosis rates across different cell lines or treatment conditions.

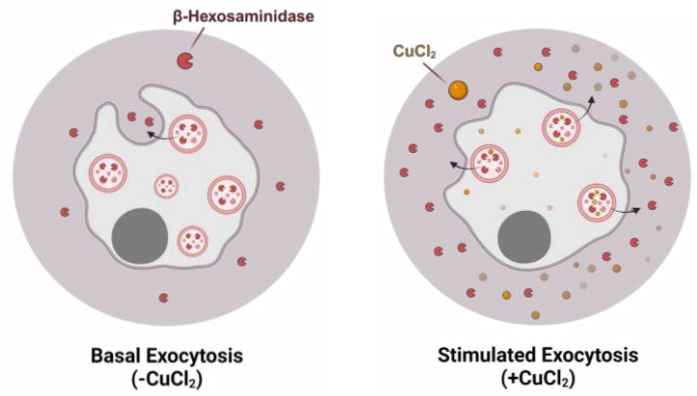



**Figure 1.1. The β-hexosaminidase assay enables quantification of basal and stimulated lysosomal exocytosis.** This schematic illustrates the experimental workflow for measuring lysosomal exocytosis under basal and $CuCl_2$-stimulated conditions by assessing β-hexosaminidase secretion. (Created with BioRender.com)

Through initial optimization, the β-Hex assay demonstrated reliable sensitivity and specificity for secreted lysosomal enzyme activity. We next evaluated β-Hex secretion profiles in the panel of cell lines. Among the 19 tested lines, Du145 prostate cancer cells emerged as the most robust model, exhibiting both substantial basal exocytosis and a pronounced increase in secretion upon $CuCl_2$ stimulation (**Figure 1.2**). Panc1, MIA-PaCa-2, H1299, and U373 cells also demonstrated measurable activity and were retained as secondary models for confirmatory experiments. The other cell lines either exhibited suboptimal basal activity or failed to respond to $CuCl_2$ induction, making them less suitable for downstream screening applications.

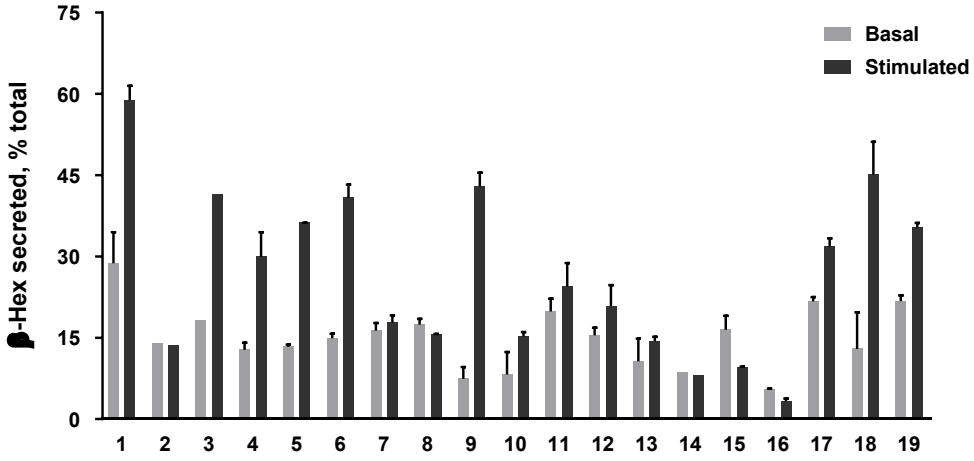



**Figure 1.2. Basal and stimulated lysosomal exocytosis levels vary across cancer cell lines.** β-hexosaminidase secretion was quantified under basal and $CuCl_2$-stimulated conditions in 19 cancer cell lines. The results are expressed as a percentage of total enzyme activity to reflect lysosomal exocytosis capacity. Each numbered data point represents an individual cell line as follows: **1.** MIA-PaCa-2 (PAAD), **2.** HeLa (CESC),

**3.** U2OS (SARC), **4.** U373 (GBM), **5.** PC3 (PRAD), **6.** Du145 (PRAD), **7.** 22Rv1 (PRAD), **8.** LNCaP (PRAD), **9.** H1299 (LUAD), **10.** A549 (LUAD), **11.** HepG2 (LIHC), **12.** Huh7 (LIHC), **13.** MCF-7 (BRCA), **14.** MDA-MB-231 (BRCA), **15.** A2780 (OV), **16.** Kuramochi (HGSOC), **17.** OSCC40 (HNSC), **18.** Panc1 (PAAD), **19.** RPE-1.

To investigate the relationship between lysosomal exocytosis and total lysosomal volume, we performed correlation analyses based on data from LysoTracker Green (LTG) staining. We hypothesized that suppression of exocytosis would result in lysosomal retention and thus increased lysosomal volume. Indeed, our quantitative analysis revealed an inverse relationship between total lysosomal volume and β-Hex secretion. Under basal conditions, this relationship approached significance ($R^2 = 0.39$, $p = 0.052$), and under $CuCl_2$-stimulated conditions, the correlation reached statistical significance ($R^2 = 0.52$, $p = 0.019$) (**Figure 1.3**). These results suggest that lysosomal content and exocytic activity are tightly linked across cell types and further support the use of LTG fluorescence as an indirect measure of exocytosis blockade.

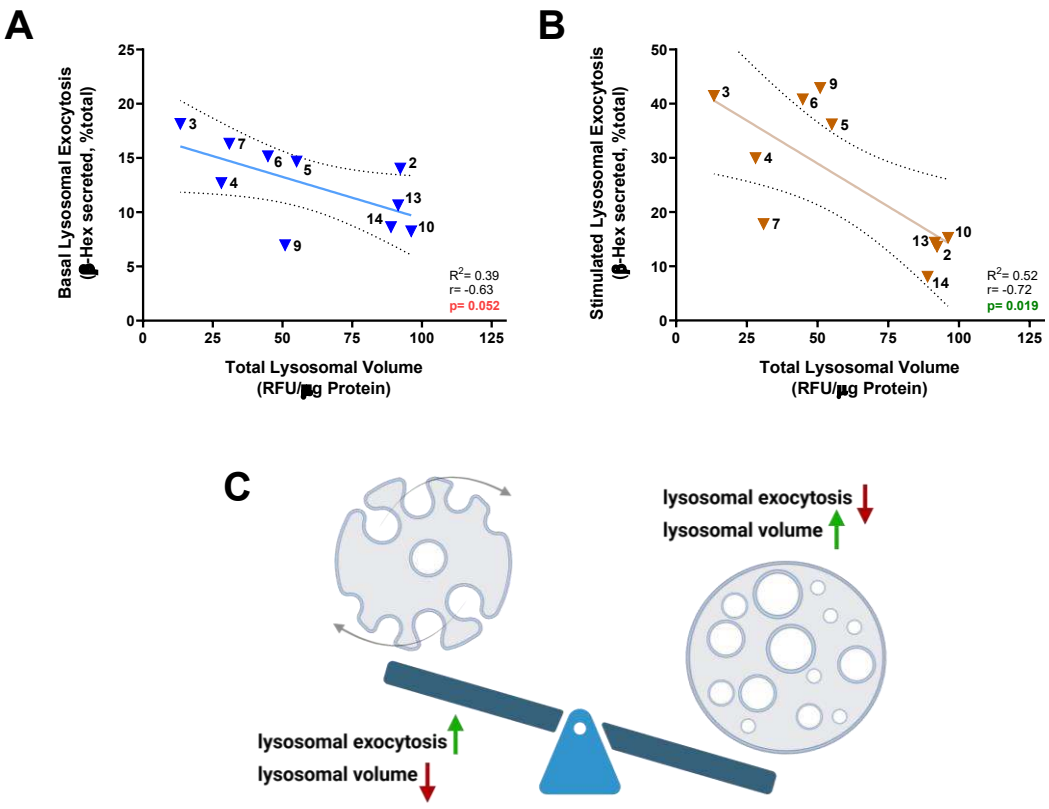



**Figure 1.3. Lysosomal volume and exocytosis exhibit an inverse correlation across cancer cell lines.** Correlation between total lysosomal volume (measured via LysoTracker fluorescence) and exocytosis under (A) basal condition and (B) $CuCl_2$-stimulated condition. Each numbered data point represents an individual cell line as follows: **1.** MIA-PaCa-2 (PAAD), **2.** HeLa (CESC), **3.** U2OS (SARC), **4.** U373 (GBM),

**5.** PC3 (PRAD), **6.** Du145 (PRAD), **7.** 22Rv1 (PRAD), **8.** LNCaP (PRAD), **9.** H1299 (LUAD), **10.** A549 (LUAD), **11.** HepG2 (LIHC), **12.** Huh7 (LIHC), **13.** MCF-7 (BRCA), **14.** MDA-MB-231 (BRCA), **15.** A2780 (OV), **16.** Kuramochi (HGSOC), **17.** OSCC40 (HNSC), **18.** Panc1 (PAAD), **19.** RPE-1. Pearson's r, $R^2$, and p-values are indicated on the plots. (C) Illustration showing the inverse correlation between total lysosomal volume and lysosomal exocytosis.

Taken together, these data provided a rational basis for selecting Du145 cells as a primary model for lysosomal function assays and drug screening. The validated β-Hex assay and complementary LTG-based analysis established a robust platform for monitoring changes in lysosomal dynamics in response to pharmacological perturbations.

### 3.2. Epigenetic drug screening identifies Type I PRMT inhibitors as dual modulators of lysosomal function and cisplatin sensitivity

To identify epigenetic modulators that influence lysosomal dynamics and sensitize cancer cells to chemotherapy, we conducted a focused drug screen using a curated library of 175 small-molecule inhibitors targeting various chromatin-modifying enzymes. These compounds included inhibitors of histone acetyltransferases, methyltransferases, deacetylases, DNA methyltransferases, and bromodomain-containing readers, many of which are in clinical trials or already FDA-approved for various diseases (Ghasemi, 2020; Gladkova et al., 2023; Zhanqiang et al., 2023). The screen was designed to capture compounds that **(1)** enhance cisplatin cytotoxicity, **(2)** suppress lysosomal exocytosis, and **(3)** increase lysosomal content—three phenotypes hypothesized to intersect via lysosomal reprogramming.

Du145 cells were chosen for primary screening due to their robust and inducible lysosomal exocytosis capacity, as established in preliminary optimization experiments. All epidrugs were applied at 5 µM for 72 hours to allow transcriptional priming while minimizing off-target toxicity. The viability screen involved a sequential treatment strategy, where Du145 cells were first exposed to each epidrug for 72 hours, followed by co-treatment with a sublethal dose of cisplatin. This design enabled the detection of sensitization effects without masking them with high baseline cytotoxicity. As shown in **Figure 1.4**, each pair of green and red dots represents the viability effect of a single epidrug alone (**green**) and in combination with cisplatin (**red**). While the majority of epidrugs showed little to no impact, a distinct subset selectively enhanced cisplatin cytotoxicity, emerging as candidate synergy hits for further evaluation.

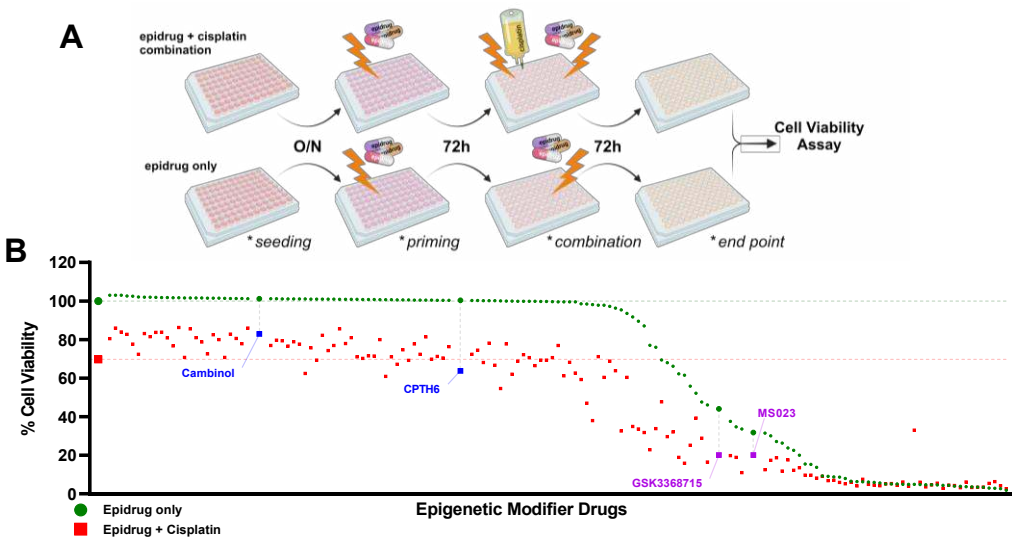



**Figure 1.4. High-content screening identifies epidrugs that sensitize Du145 cells to cisplatin treatment. (A)** Schematic representation of the screening procedure used to identify epidrugs synergizing with cisplatin in a viability assay. Du145 cells were seeded on 96-well plates and pre-treated (primed) with epidrugs (5 µM) for 72 h, followed by combined treatment with epidrugs (5 µM) and cisplatin (5 µM) for an additional 72 h (created with BioRender.com). **(B)** Scatter plot representing cell viability results from the epidrug library screening, with each dot indicating an individual epidrug. Drugs were classified based on their ability to synergize with cisplatin (red) compared to epidrug-only treatments (green). Key compounds are indicated in blue and purple.

In parallel, we assessed the impact of each epidrug on lysosomal exocytosis using the β-Hexosaminidase secretion assay. Measurements were taken under both basal and $CuCl_2$-stimulated conditions to capture inducible exocytic capacity. We reasoned that while cells may tolerate modest suppression of exocytosis at baseline, a significant reduction in stimulated exocytosis would point to compromised lysosomal function. The results revealed that several epidrugs specifically reduced β-Hex release under stimulated conditions, without severely affecting basal exocytosis. Among these, MS023 and GSK3368715 stood out as consistent inhibitors of lysosomal exocytosis (**Figure 1.5B**). In the third library screening, LysoTracker Green staining revealed that these two inhibitors also increased total lysosomal content, consistent with intracellular lysosome accumulation due to impaired exocytic clearance (**Figure 1.5C**). The dual effect of suppressed exocytosis and expanded lysosomal volume strongly suggested a lysosome-targeted phenotype.

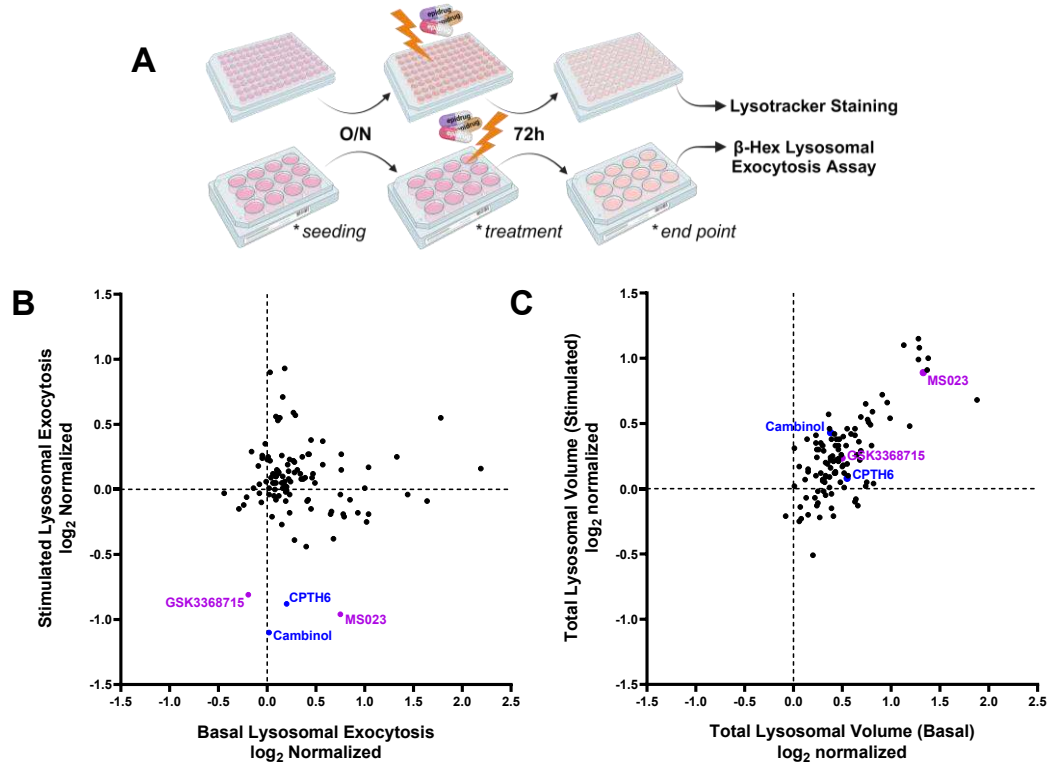



**Figure 1.5. Epidrug treatment suppresses lysosomal exocytosis and increases lysosomal volume. (A)** Experimental design for measuring lysosomal exocytosis and volume after 72-hour epidrug exposure. **(B)** Screening results showing the effects of epidrugs (5 µM, 72 h) on lysosomal exocytosis activity under basal and stimulated conditions, quantified as extracellular and intracellular β-hexosaminidase activity and represented in log2-normalized values. **(C)** Lysosomal volume changes upon epidrug treatment (5 µM, 72 h), measured by Lysotracker staining. Total lysosomal volume was quantified at basal and stimulated conditions, and values are normalized to the untreated control.

To determine whether these effects were cell line-specific, we repeated the viability screen in two additional cancer models: Panc1 and H1299. As shown in **Figures 1.6 and 1.7**, both MS023 and GSK3368715 replicated their synergy with cisplatin in these independent lines, validating the robustness and reproducibility of the phenotype. These results indicated that the observed sensitization was not restricted to a single cellular background and could potentially reflect a conserved mechanism across epithelial tumor types.

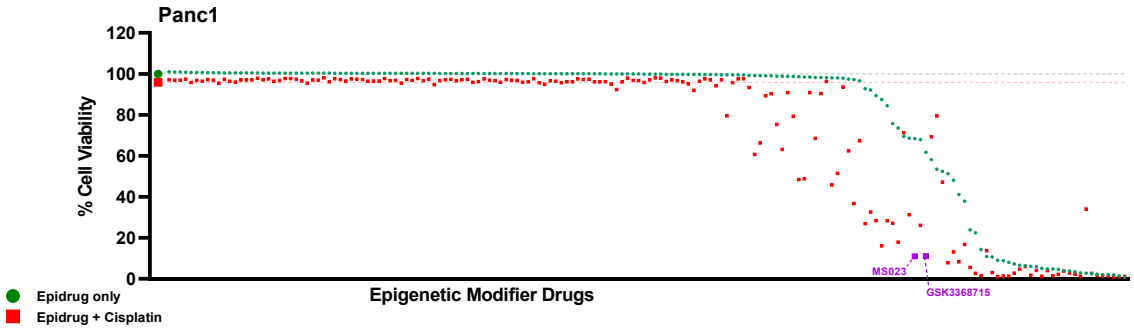



**Figure 1.6. Several epidrugs enhance cisplatin cytotoxicity in Panc1 cells.** Panc1 cells were primed with 5 µM epidrugs for 72 hours, followed by co-treatment of epidrugs with 5 µM cisplatin for an additional 72 hours. Cell viability was assessed via SRB assay. Dashed lines represent the viability thresholds for untreated and cisplatin-only conditions. MS023 and GSK3368715 are highlighted as synergistic compounds.

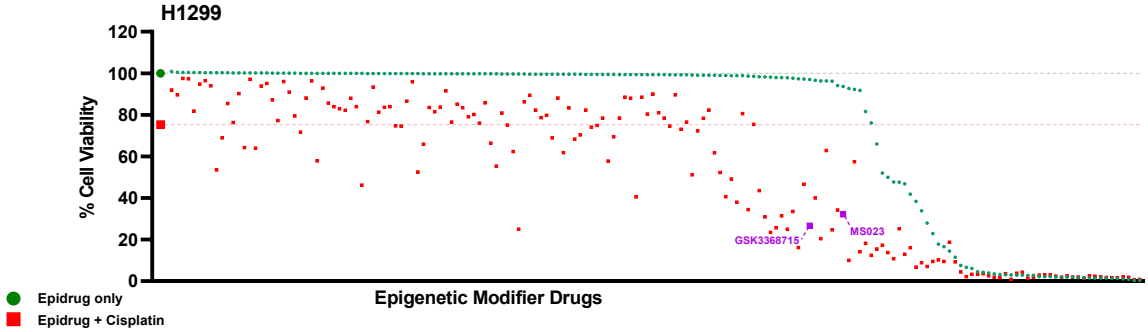



**Figure 1.7. Type I PRMT inhibitors synergize with cisplatin in H1299 cells.** H1299 cells were pre-treated with 5 µM epidrugs for 72 hours, then exposed to 25 µM cisplatin as co-treatment with epidrugs. Viability was measured using the SRB assay. MS023 and GSK3368715 displayed synergistic effects and are highlighted in the plot.

In summary, this multiparametric screen identified MS023 and GSK3368715 as the only compounds that consistently sensitized cells to cisplatin and disrupted lysosomal homeostasis. Their reproducible effects across models and assays established them as promising candidates for mechanistic dissection in subsequent experiments.

### 3.3. Type I PRMT inhibitors modulate lysosomal dynamics and enhance chemotherapy response through a non-cytotoxic, transcriptionally primed manner

Following the identification of MS023 and GSK3368715 as dual-acting epidrugs that suppress lysosomal exocytosis and enhance cisplatin sensitivity, we next sought to validate and mechanistically characterize their effects across multiple cancer cell models. These two compounds consistently emerged as hits in both the lysosomal and viability screens in Du145 cells, and were among the few that affected both phenotypes

simultaneously. As shown in the summary map of the screening outcomes (**Figure 1.8**), MS023 and GSK3368715 clustered within the subset of epidrugs that both decreased lysosomal exocytosis and enhanced cisplatin cytotoxicity. These dual properties set them apart from other compounds, which typically affected only one of the phenotypes or had inconsistent effects across conditions.

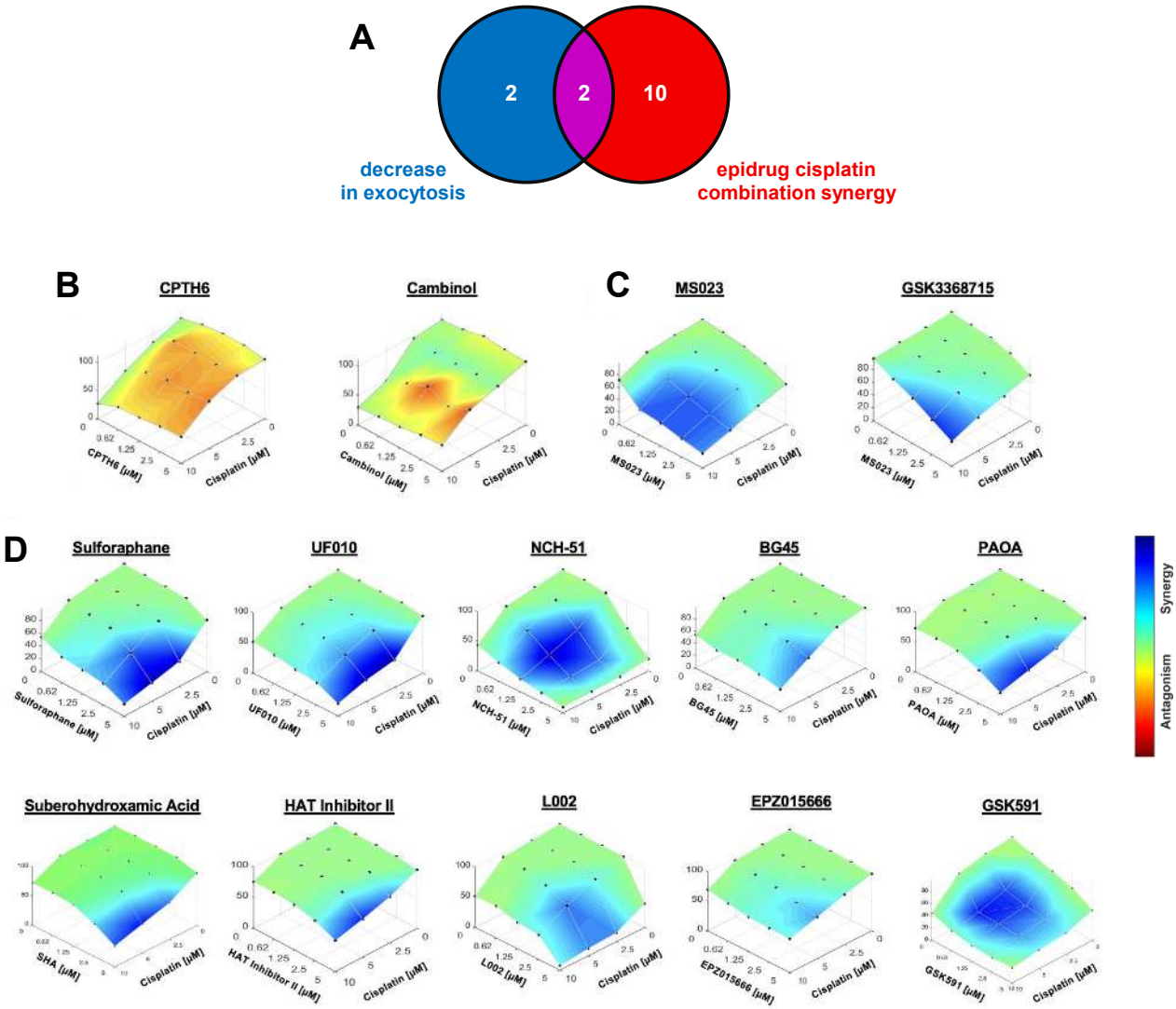



**Figure 1.8. Epidrugs are functionally categorized by their effects on lysosomal exocytosis and cisplatin synergy. (A)** Venn diagram summarizing the results of the epidrug library screening performed on Du145 cells, identifying epidrugs that inhibit lysosomal exocytosis and/or show synergistic effects with cisplatin. Surface plots represent combined drug response (epidrug + cisplatin) in viability assays: **(B)** two epidrugs (CPTH6, Cambinol) that decreased lysosomal exocytosis but did not synergize with cisplatin, **(C)** two epidrugs (MS023, GSK3368715) that both inhibited exocytosis and exhibited synergy with cisplatin, and **(D)** ten epidrugs that synergized with cisplatin without affecting lysosomal exocytosis. Color gradients indicate the intensity of synergy

and antagonism, while cell viabilities are shown on the y-axis for combined doses of two drugs.

To further quantify the synergy observed in the initial combination screens, we performed BLISS synergy scoring based on the cell viability surface plots for MS023 and GSK3368715 combined with cisplatin. Both compounds demonstrated strong and reproducible synergy across a range of concentrations, confirming their potential as epigenetic sensitizers (**Figure 1.9**). These findings motivated us to evaluate whether their effects on lysosomal function and drug synergy could be reproduced in other cell lines and whether the observed phenotypes resulted from transcriptional reprogramming rather than acute enzymatic inhibition.

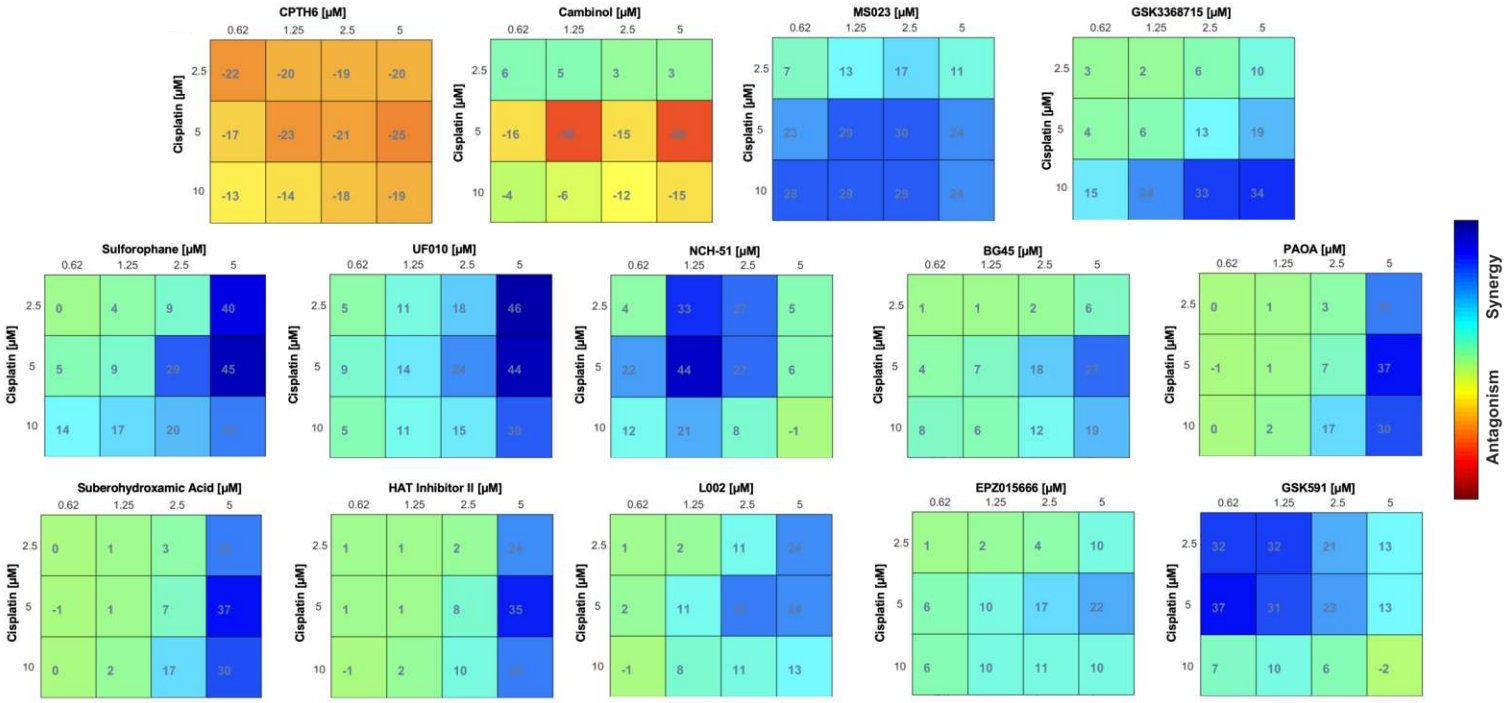



**Figure 1.9. BLISS synergy analysis confirms cooperative effects of epidrug–cisplatin combinations.** A synergy score matrix was generated using the Combenefit software. Viability data from Du145 cells treated with selected epidrug–cisplatin combinations were analyzed to identify additive, synergistic, or antagonistic interactions.

We first assessed lysosomal exocytosis by measuring β-Hex secretion under basal and $CuCl_2$-stimulated conditions after 72-hour treatment with MS023 and GSK3368715. The results revealed a consistent and significant suppression of stimulated β-Hex release across Du145, Panc1, and H1299 cells (**Figure 1.10**). These findings confirmed that Type I PRMT inhibition leads to reduced lysosomal exocytosis, independent of cell line origin. To distinguish whether this reduction in exocytosis resulted from direct, acute effects of

the inhibitors or from prolonged transcriptional priming, we treated Du145 cells with MS023 and GSK3368715 for just 1 hour and assessed β-Hex secretion. This short-term exposure did not result in any measurable reduction in exocytosis, indicating that the inhibitors require prolonged treatment to exert their functional effects (**Figure 1.11**).

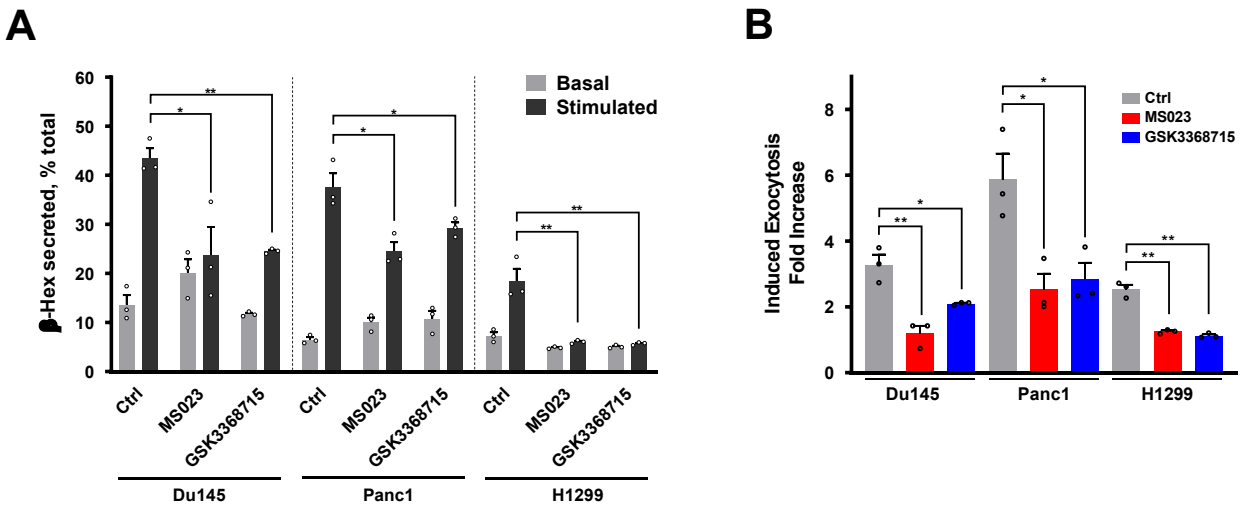



**Figure 1.10. Type I PRMT inhibitors suppress $CuCl_2$-stimulated lysosomal exocytosis. (A)** β-hexosaminidase secretion was measured in Du145, Panc1, and H1299 cells under basal and $CuCl_2$-stimulated conditions following treatment with MS023 or GSK3368715. **(B)** Fold induction of exocytosis was calculated by dividing stimulated by basal secretion, demonstrating a marked reduction after PRMT inhibitor exposure (*p<.05, **p<.01, n.s. not-significant, Student's t-test).

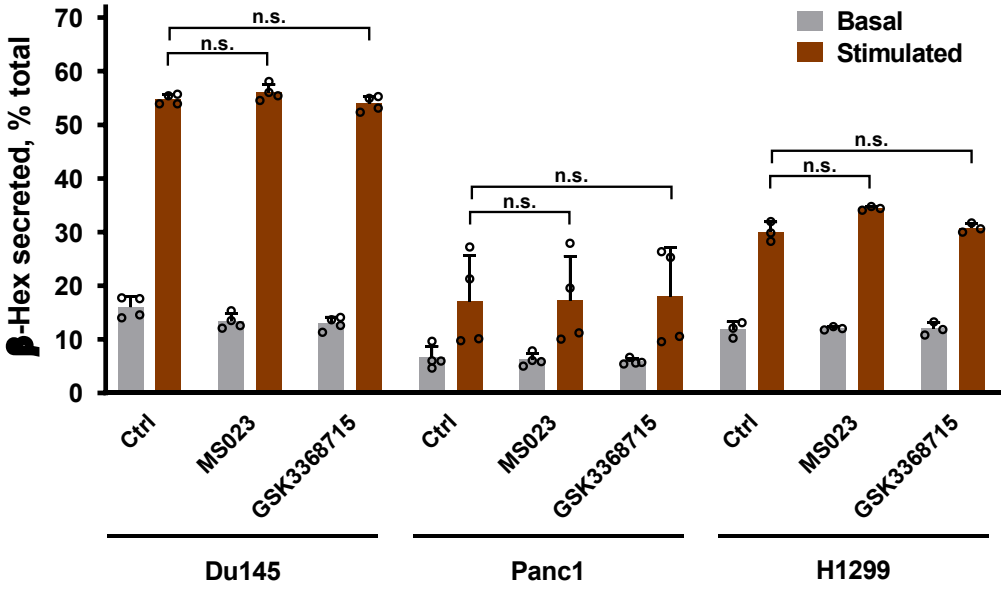



**Figure 1.11. Brief exposure to PRMT inhibitors does not inhibit lysosomal exocytosis.** Du145, Panc1, and H1299 cells were treated with MS023 or GSK3368715 for 1 hour, then subjected to β-hexosaminidase exocytosis assays. No significant

reduction in exocytosis was observed (*p<.05, **p<.01, n.s. not-significant, Student's t-test).

We next analyzed total lysosomal volume using LysoTracker Green (LTG) fluorescence intensity as a surrogate marker for acidic vesicle accumulation. In all three cell lines, treatment with MS023 or GSK3368715 led to a marked increase in LTG signal, indicating an accumulation of lysosomes in the cytoplasm (**Figure 1.12**). These changes were further validated by flow cytometry, which showed a clear rightward shift in LTG fluorescence distribution in treated cells compared to controls, confirming the expansion of the lysosomal compartment (**Figure 1.13**). The coordinated reduction in exocytosis and increase in lysosomal volume strongly supported a functional block in lysosomal trafficking in all 3 cell lines used in the study.

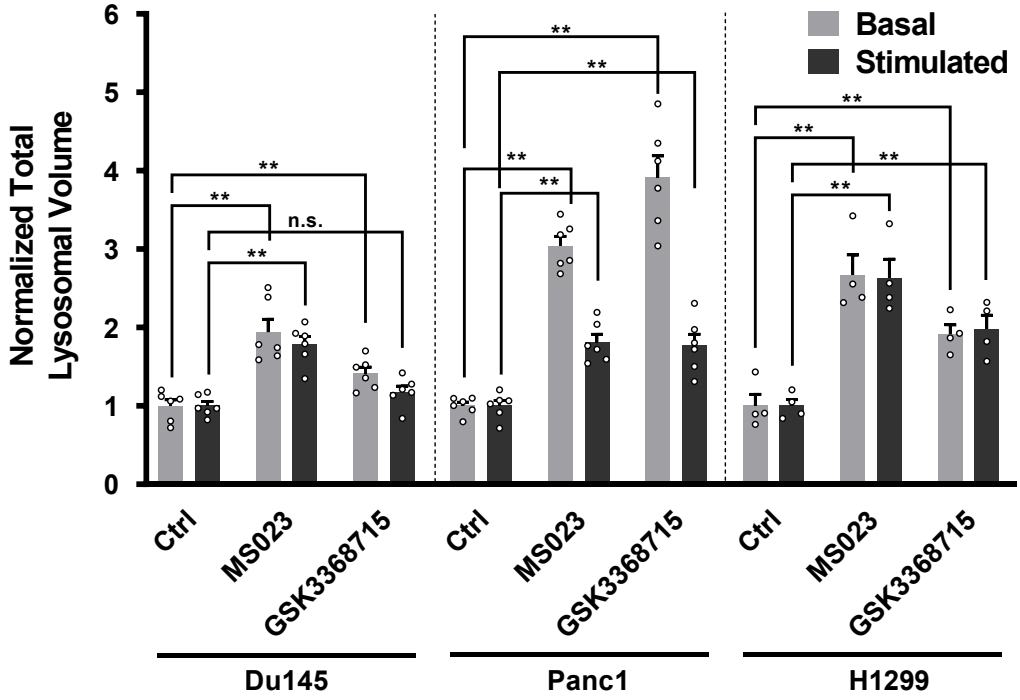



**Figure 1.12. MS023 and GSK3368715 increase lysosomal volume in cancer cells.** Du145 and Panc1 cells treated with PRMT inhibitors (5 uM, 72 h) showed increased total lysosomal content, as determined by LysoTracker fluorescence intensity (*p<.05, **p<.01, n.s. not-significant, Student's t-test).

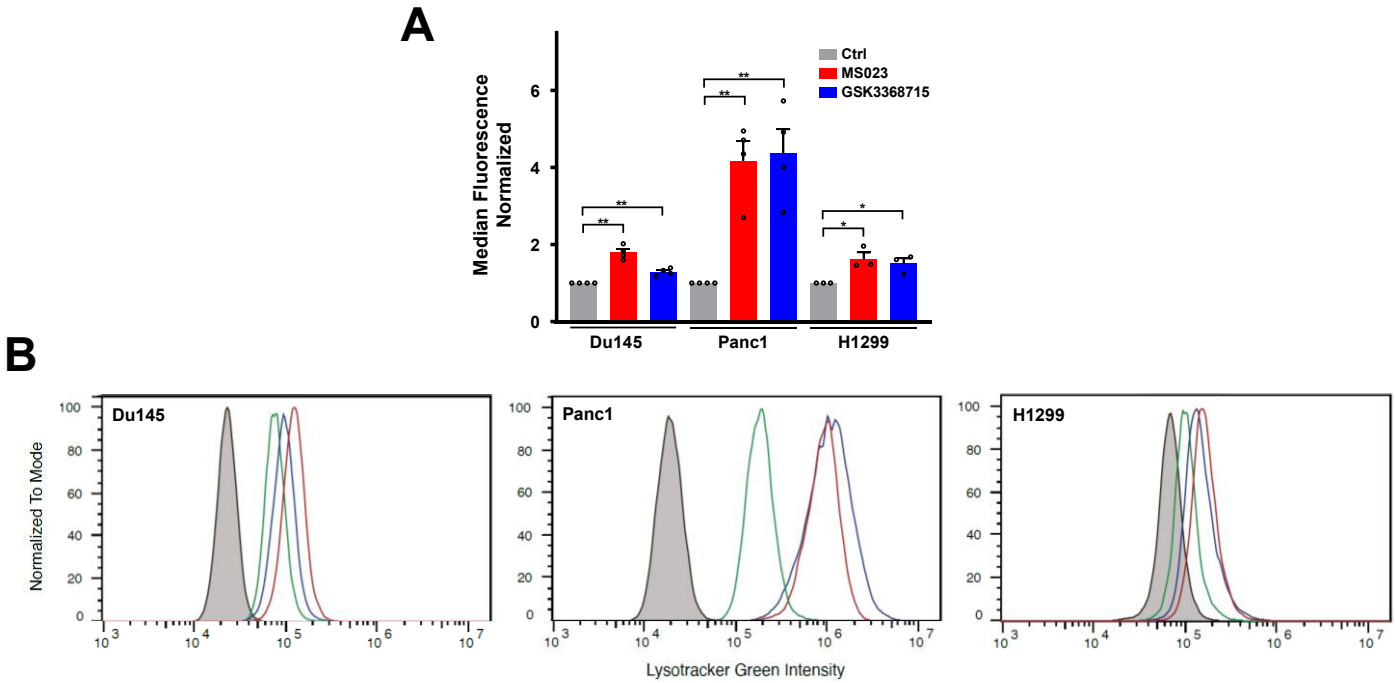



**Figure 1.13. Flow cytometry confirms increase in intracellular lysosomal content after PRMT inhibitor treatment. (A)** Median fluorescence intensity (MFI) shifts indicate increased lysosomal content in Du145, Panc1, and H1299 cells after 72-hour treatment with MS023 or GSK3368715. **(B)** Representative histograms illustrate rightward shifts in lysosomal staining intensity (*p<.05, **p<.01, n.s. not-significant, Student's t-test).

Given that lysosomal expansion and dysfunction can occur as downstream consequences of cytotoxic stress (Lakpa et al., 2021; Zhang et al., 2023), we next evaluated whether these PRMT inhibitors were toxic on their own at the doses used for lysosomal assays. A 72-hour viability assay revealed that neither MS023 nor GSK3368715 caused significant cell death across Du145, Panc1, or H1299 at concentrations up to 40 μM (**Figures 1.14** and **1.15**). These findings validated our use of 5 μM as a non-toxic dose for phenotypic screening and mechanistic experiments, confirming that the observed changes in lysosomal function were not due to cytotoxicity.

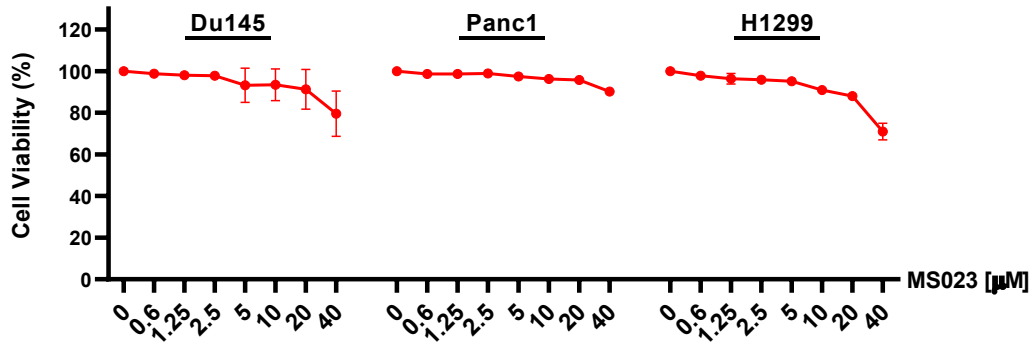



**Figure 1.14. MS023 shows minimal cytotoxicity at high doses.** Du145, Panc1, and H1299 cells were exposed to increasing concentrations of MS023 for 72 hours. SRB assay results confirmed low toxicity even at doses around 40 µM, supporting the idea of using inhibitors at 5 µM is sublethal.

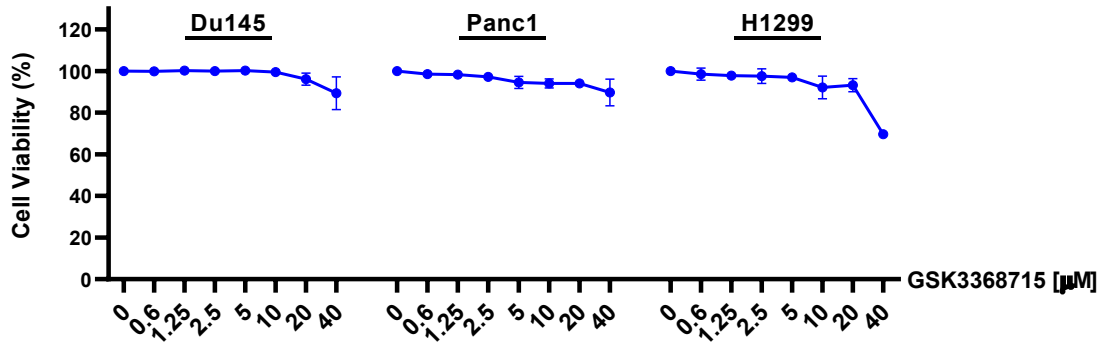



**Figure 1.15. GSK3368715 exhibits low cytotoxicity at high doses.** Cell viability of Du145, Panc1, and H1299 cells remained above cytotoxic thresholds after 72-hour exposure to increasing doses of GSK3368715. These findings confirm its utility at the 5 µM concentration used in functional experiments.

A schematic overview of the PRMT family was generated to contextualize the mechanistic role of PRMTs in cellular signaling (**Figure 1.16**). Protein arginine methyltransferases (PRMTs) are categorized into three types based on the methylation marks they generate: Type I enzymes (e.g., PRMT1, PRMT3, PRMT4, PRMT6, PRMT8) catalyze asymmetric dimethylarginine (aDMA); Type II enzymes (e.g., PRMT5, PRMT9) generate symmetric dimethylarginine (sDMA); and Type III, represented solely by PRMT7, produces monomethylarginine (MMA). These enzymes act on histone and non-histone substrates, regulating chromatin structure, transcriptional activity, RNA splicing, and DNA repair. Among them, PRMT1 is the dominant Type I enzyme responsible for the majority of aDMA in mammalian cells, while PRMT6 has overlapping but distinct substrate specificity and functional targets. Dysregulated PRMT activity has

been implicated in cancer progression and resistance to therapy (Gao et al., 2024; Hwang et al., 2021; Zhu, Xia, et al., 2024). Figure 16 provides a conceptual illustration of PRMT subtypes, their enzymatic functions, and cellular consequences of their methylation activity.

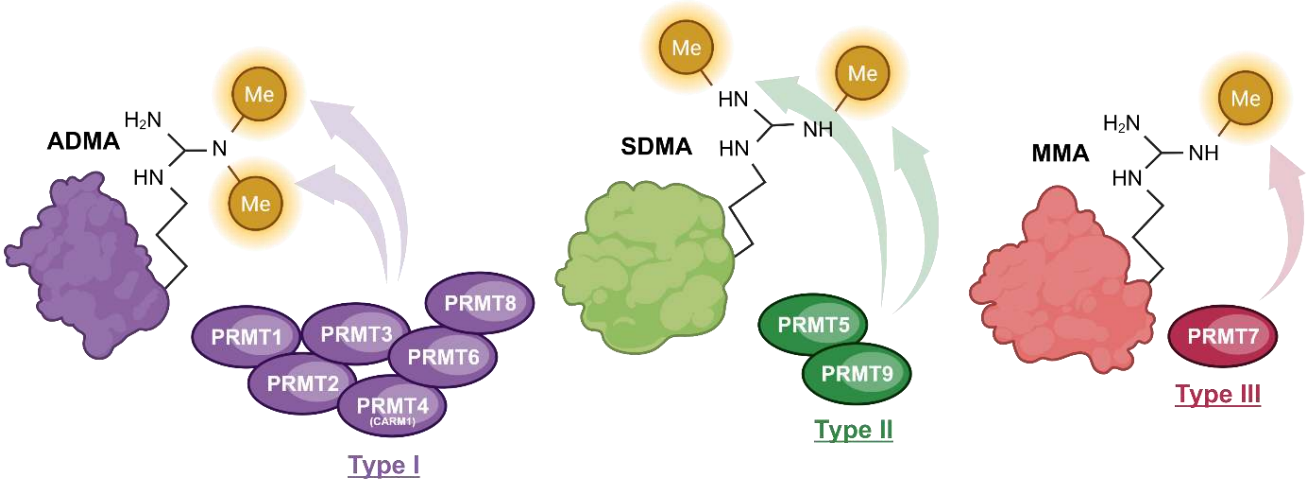
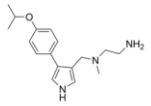
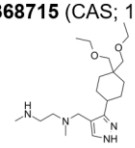



**Figure 1.16. PRMTs modulate the faith of target proteins through distinct methylation patterns.** Illustration showing the arginine methylation patterns acting on target proteins by different PRMT epigenetic modifiers (Created using Biorender.com).

To pharmacologically inhibit Type I PRMTs, we employed two structurally distinct small-molecule inhibitors, MS023 and GSK3368715, both of which exhibit high potency against PRMT1 and PRMT6, enzymes prominently implicated in transcriptional regulation and epigenetic control. MS023 is a Type I PRMT inhibitor with nanomolar-range $IC_{50}$ values against PRMT1 (30 nM), PRMT6 (4 nM), and PRMT8 (5 nM), while also retaining activity against PRMT3 and PRMT4/CARM1. In comparison, GSK3368715 shows an even higher selectivity for PRMT1 (3.1 nM) and PRMT8 (1.7 nM), with slightly reduced potency against PRMT6 (5.7 nM) and limited activity toward PRMT4/CARM1 (1148 nM) (**Table 1.5**). The use of two structurally different inhibitors with overlapping target specificity allowed us to validate that the observed cellular effects were attributable to Type I PRMT inhibition rather than off-target activity. This complementary pharmacological approach enhanced the robustness of our findings and increased confidence in attributing the downstream phenotypes to selective suppression of PRMT1 and PRMT6.

**Table 1.5.** Information about type I PRMT inhibitors and their inhibition IC50 values on target PRMTs

| | | | | | |
|---|---|---|---|---|---|
| **MS023** (CAS; 1831110-54-3)  | **Inhibition $IC_{50}$** | | | | |
| | PRMT1 | PRMT3 | PRMT4/CARM1 | PRMT6 | PRMT8 |
| | 30 nM | 119 nM | 83 nM | 4 nM | 5 nM |
| **GSK3368715** (CAS; 1629013-22-4)  | **Inhibition $IC_{50}$** | | | | |
| | PRMT1 | PRMT3 | PRMT4/CARM1 | PRMT6 | PRMT8 |
| | 3.1 nM | 48 nM | 1148 nM | 5.7 nM | 1.7 nM |

To test whether the observed phenotypes reflected a loss of PRMT enzymatic function rather than protein downregulation, we examined PRMT1 and PRMT6 expression levels by Western blot following 72-hour treatment with MS023 or GSK3368715. Protein levels of both enzymes remained stable, indicating that the drugs do not induce PRMT degradation. However, immunoblotting for global asymmetric dimethyl arginine (aDMA) and symmetric dimethyl arginine (sDMA) revealed a significant reduction in aDMA and a compensatory increase in sDMA, consistent with effective inhibition of Type I PRMT activity (**Figure 1.17**). These results confirm that the effects of MS023 and GSK3368715 on lysosomal function and drug sensitization are due to post-translational inhibition of PRMT enzymatic activity, not changes in protein abundance.

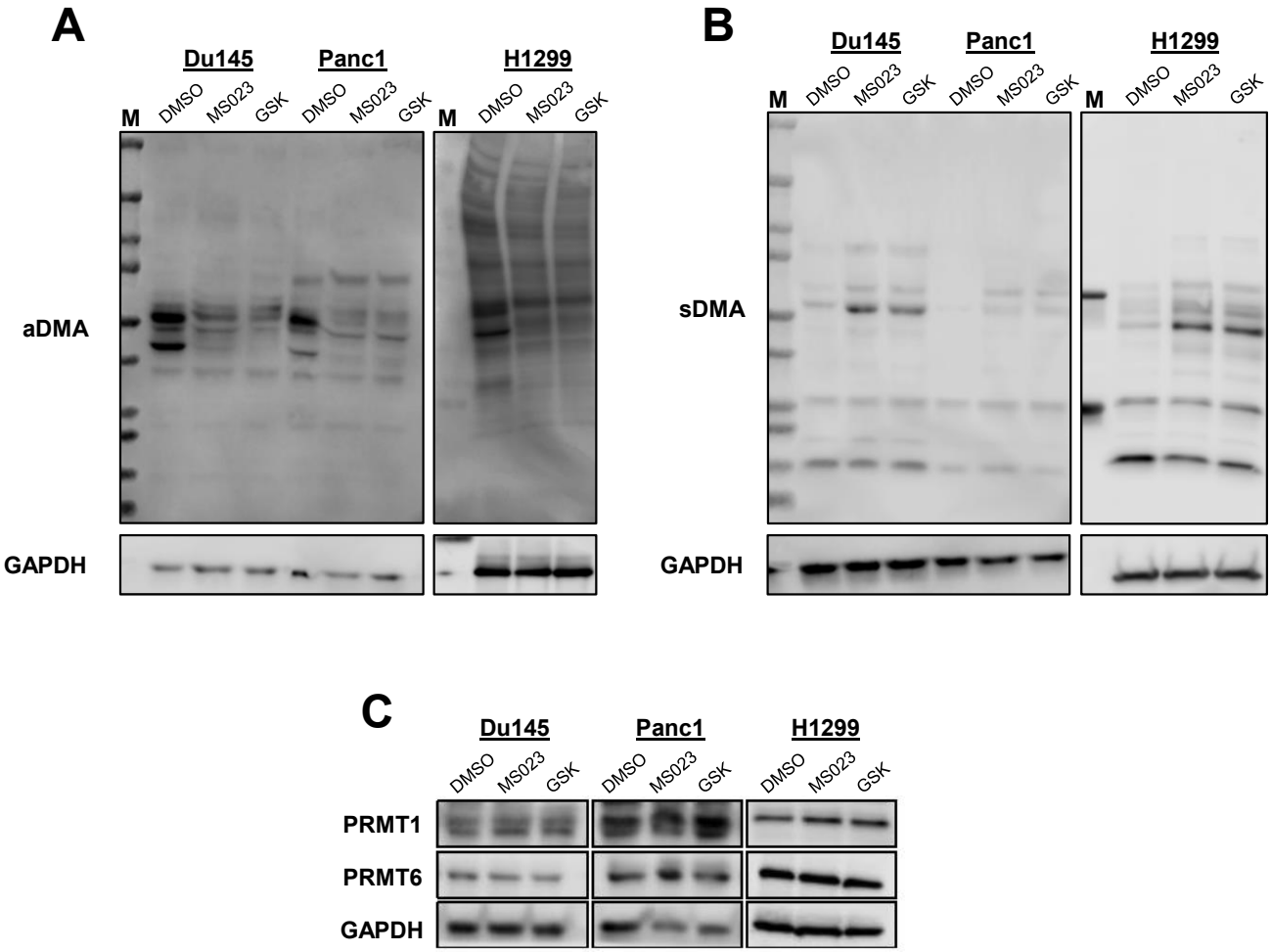



**Figure 1.17. PRMT inhibitors reduce asymmetric dimethylation without altering PRMT protein levels.** Western blot analysis shows **(A)** decreased levels of asymmetric dimethylarginine (aDMA) and **(B)** a compensatory increase in symmetric dimethylarginine (sDMA) modifications act on global cellular protein targets of type I PRMTs after treatment with MS023 or GSK3368715 on Du145, Panc1, and H1299 cells. **(C)** PRMT1 and PRMT6 protein levels remained unchanged post-treatment, indicating an inhibition specifically in catalytic properties of type I PRMTs without degradation at the protein level (this figure was generated by PhD student Neslihan Yuksel-Catal).

We then examined whether these lysosomal alterations were linked to changes in the expression of genes that regulate lysosomal biogenesis and trafficking. RT-qPCR analysis was performed on a panel of canonical lysosomal genes and master transcription factors, including TFEB and MiTF. Surprisingly, PRMT inhibitor treatment did not result in consistent or significant upregulation of lysosomal genes across Du145 (**Figure 1.18**), Panc1 (**Figure 1.19**), or H1299 (**Figure 1.20**) cells. These findings suggested that the lysosomal defects induced by MS023 and GSK3368715 were not mediated by transcriptional activation of lysosomal gene programs and likely occurred through

alternative regulatory pathways independent of TFEB and MiTF. These RT-qPCR findings prompted us to perform RNA-seq analysis in order to uncover broader transcriptomic alterations that may underlie the observed changes in lysosomal dynamics, particularly those not accounted for by canonical lysosomal gene regulation.

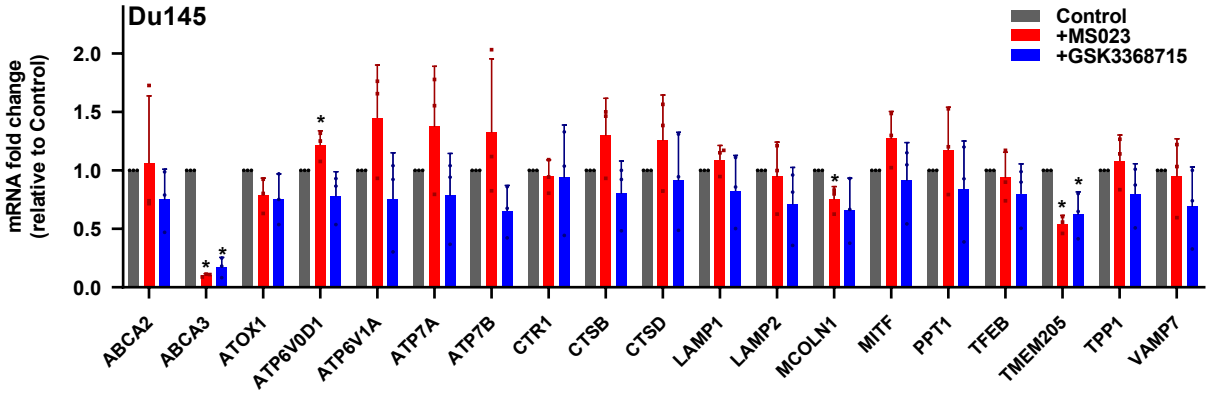



**Figure 1.18. PRMT inhibitors do not broadly affect lysosomal gene expression in Du145 cells.** RT-qPCR results show no significant transcriptional changes in lysosomal biogenesis genes in Du145 cells treated with MS023 or GSK3368715 (5 µM, 72 h) (*p<.05, n.s. not-significant, Student's t-test).

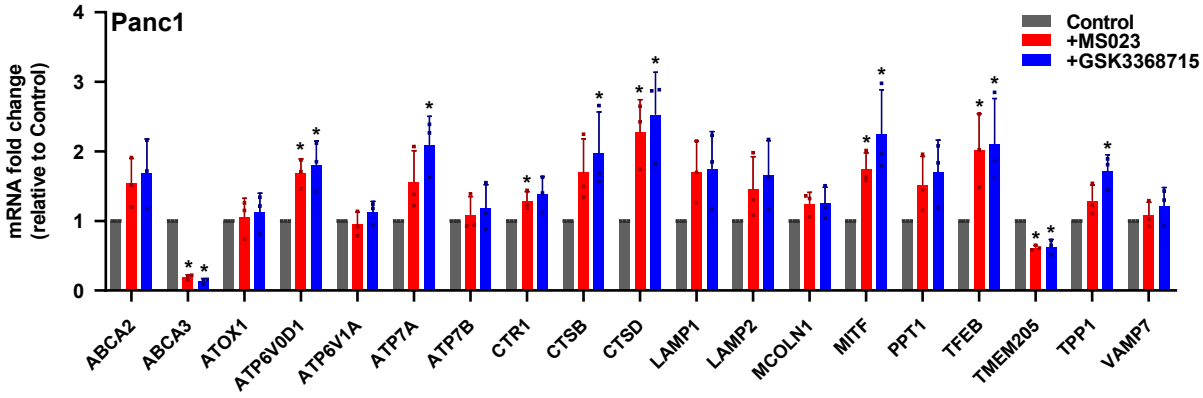



**Figure 1. 19. Type I PRMT inhibition fluctuates the expression of several lysosomal genes in Panc1 cells.** RT-qPCR analysis of Panc1 cells treated with MS023 or GSK3368715 (5 µM, 72 h) revealed minimal changes in the expression of lysosomal genes, suggesting that lysosomal biogenesis was not globally altered (*p<.05, n.s. not-significant, Student's t-test).

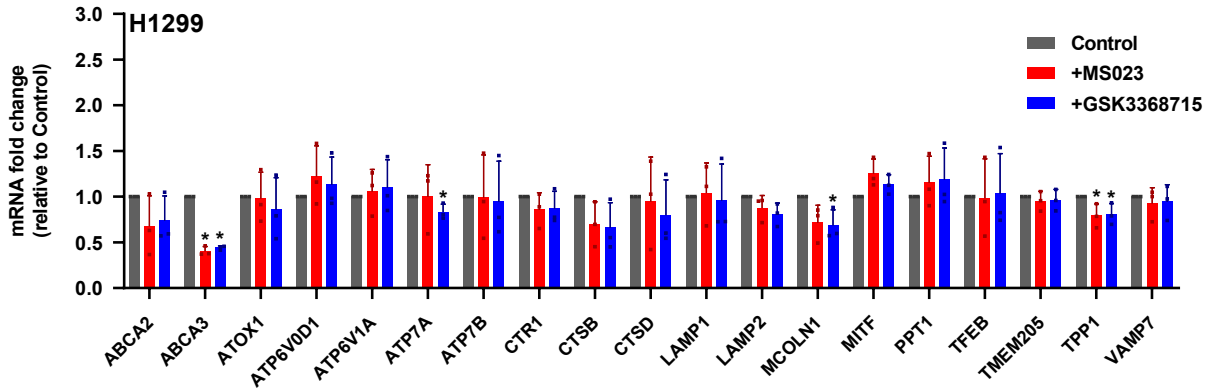



**Figure 1.20. PRMT inhibitors do not disrupt lysosomal gene transcription in H1299 cells.** Analysis of H1299 cells treated with MS023 or GSK3368715 revealed no widespread alterations in lysosomal gene expression, supporting the specificity of PRMT inhibition toward functional pathways such as exocytosis rather than biosynthesis (*p<.05, n.s. not-significant, Student's t-test).

To investigate whether the effects of MS023 and GSK3368715 could generalize to other chemotherapeutic agents beyond cisplatin, we conducted combinatorial viability screens in Du145, Panc1, and H1299 cells using sunitinib, doxorubicin, carboplatin, and oxaliplatin. These agents are also known to be susceptible to lysosomal sequestration (Azijli et al., 2015; Circu et al., 2017; Merlos Rodrigo et al., 2019; Toriyama et al., 2021). Across all three cell lines, the combination of MS023 or GSK3368715 with each chemotherapeutic agent resulted in consistent reductions in viability, further validating the synergistic potential of these epidrugs beyond cisplatin (**Figures 1.21, 1.22, and 1.23**).

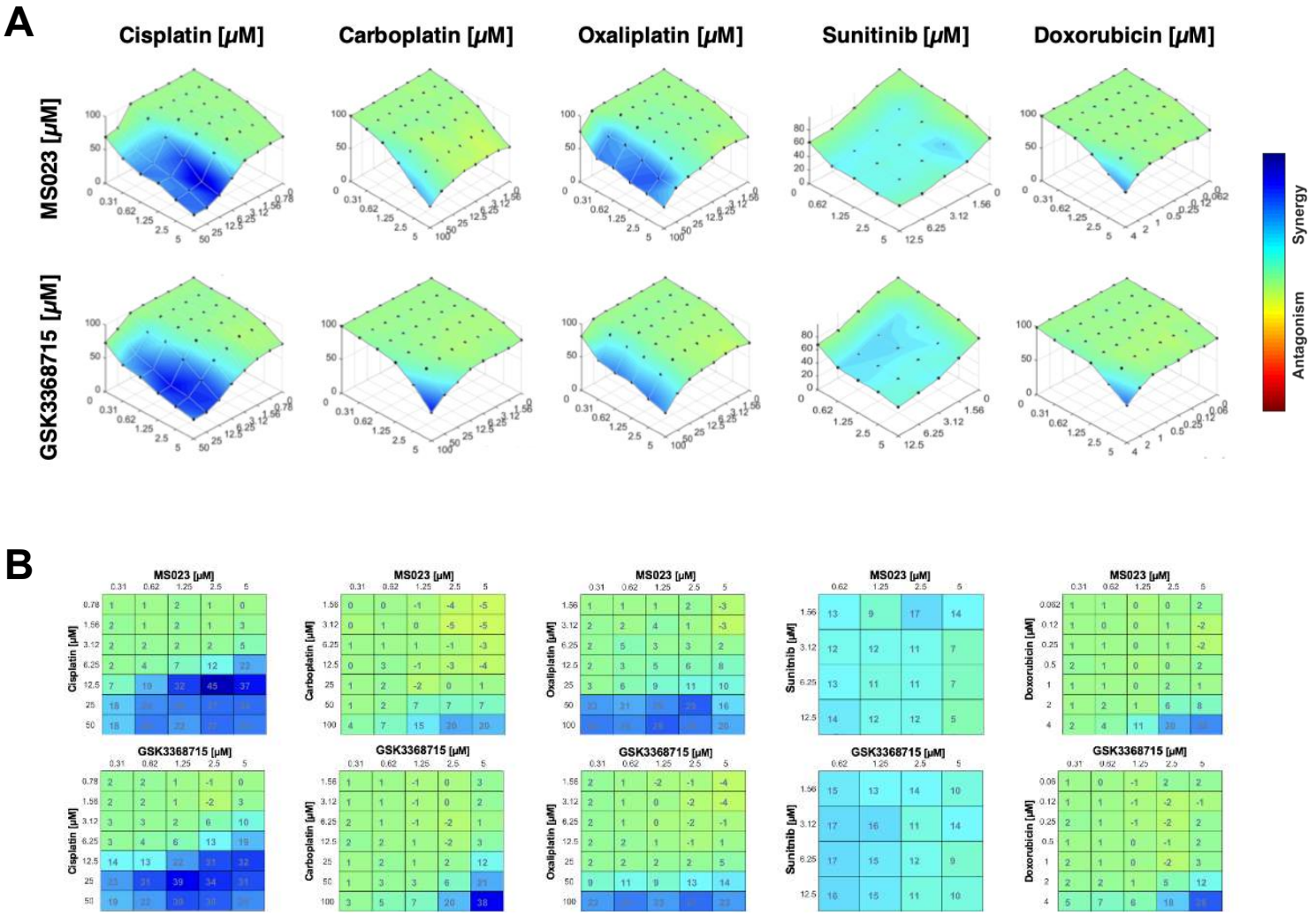



**Figure 1.21. Type I PRMT inhibitors synergize with multiple chemotherapeutics in Du145 cells. (A)** Surface plots illustrate the cell viability (y-axis) and synergistic interactions between type I PRMT inhibitors (MS023 and GSK3368715) and various chemotherapeutics (cisplatin, carboplatin, oxaliplatin, sunitinib, and doxorubicin) in Du145 cells. Cells were first primed with epidrugs (MS023 or GSK3368715) for 72 h, followed by combined treatment with the indicated drugs at specified concentrations for an additional 72 h. Cell viability was assessed, and synergy analysis was performed using the Bliss model via Combenefit software, where the deepest shade of blue indicates the highest level of synergy. **(B)** Cell viability assay results from combination treatments of the Type I PRMT inhibitors MS023 and GSK3368715 with chemotherapeutic agents in Du145 cells were analyzed using the Combenefit software employing the BLISS model. Synergy status of each drug combinations is indicated in the synergy score matrix.

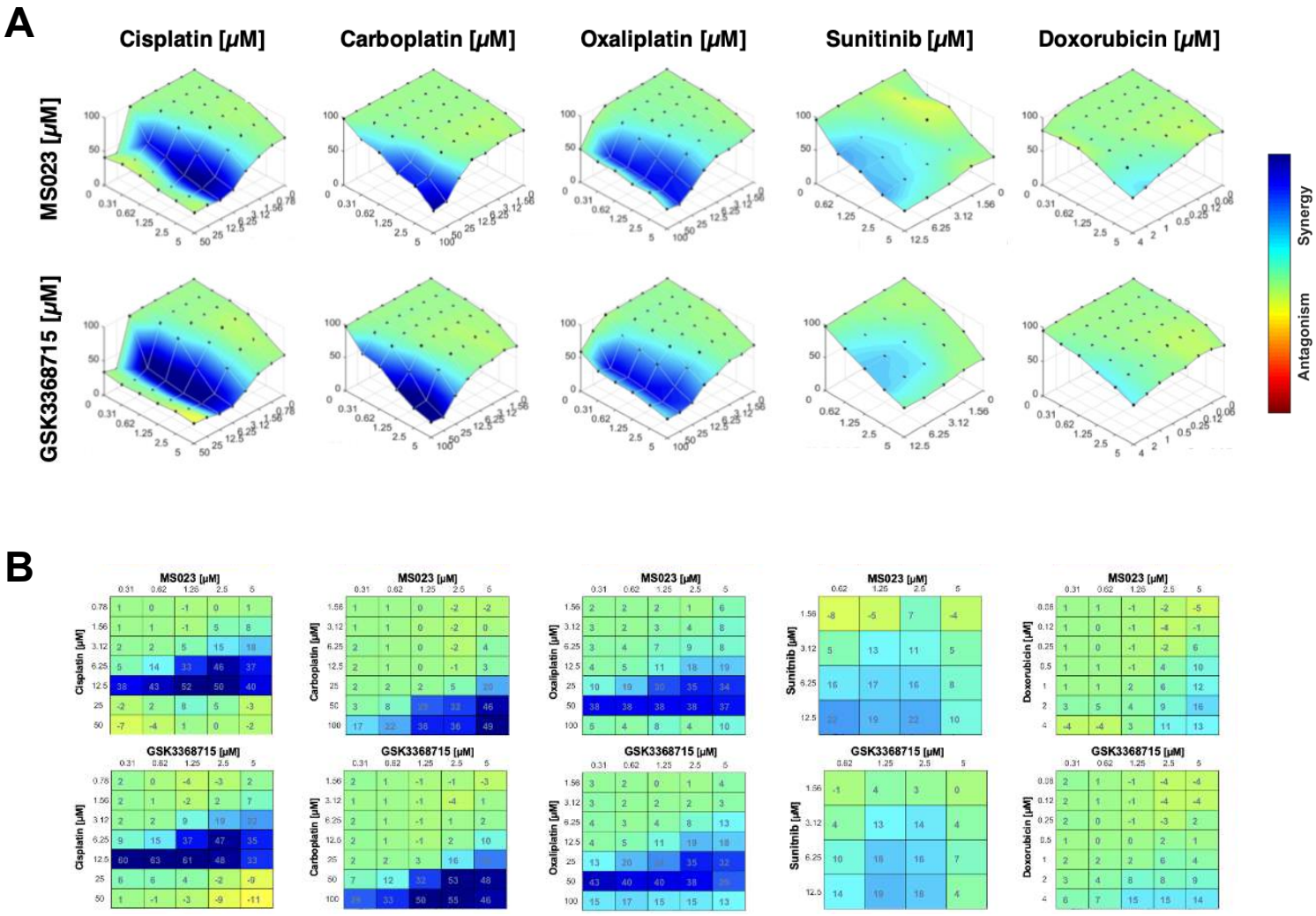



**Figure 1.22. MS023 and GSK3368715 enhance selected chemotherapy drug response in Panc1 cells. (A)** Surface plots illustrate the cell viability (y-axis) and synergistic interactions between type I PRMT inhibitors (MS023 and GSK3368715) and various chemotherapeutics (cisplatin, carboplatin, oxaliplatin, sunitinib, and doxorubicin) in Panc1 cells. Cells were first primed with epidrugs (MS023 or GSK3368715) for 72 h, followed by combined treatment with the indicated drugs at specified concentrations for an additional 72 h. Cell viability was assessed, and synergy analysis was performed using the Bliss model via Combenefit software, where the deepest shade of blue indicates the highest level of synergy. **(B)** Cell viability assay results from combination treatments of the Type I PRMT inhibitors MS023 and GSK3368715 with chemotherapeutic agents in Panc1 cells were analyzed using the Combenefit software employing the BLISS model. Synergy status of each drug combinations is indicated in the synergy score matrix.

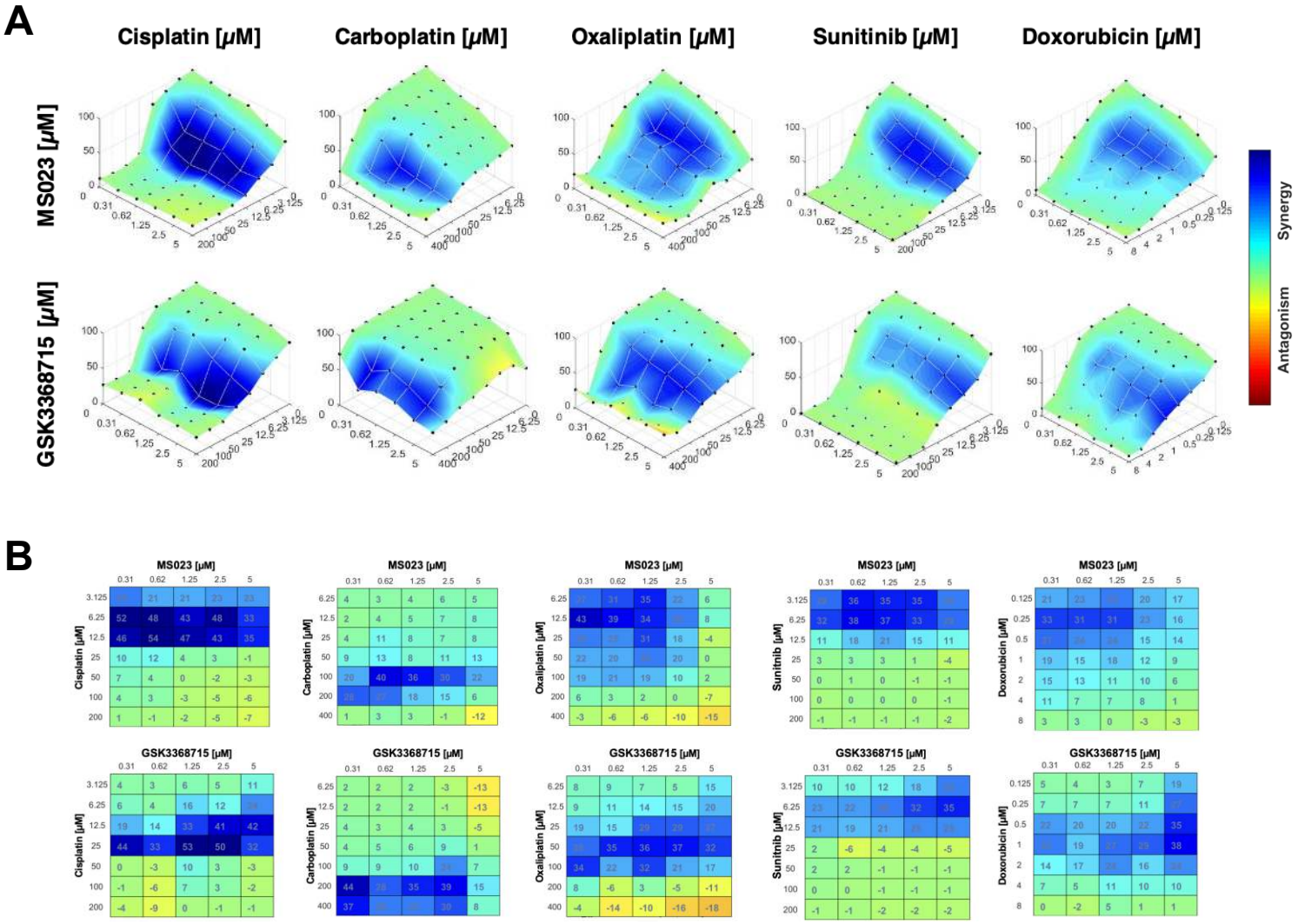



**Figure 1.23. Type I PRMT inhibition sensitizes H1299 cells to certain chemotherapeutic agents. (A)** Surface plots illustrate the cell viability (y-axis) and synergistic interactions between type I PRMT inhibitors (MS023 and GSK3368715) and various chemotherapeutics (cisplatin, carboplatin, oxaliplatin, sunitinib, and doxorubicin) in H1299 cells. Cells were first primed with epidrugs (MS023 or GSK3368715) for 72 h, followed by combined treatment with the indicated drugs at specified concentrations for an additional 72 h. Cell viability was assessed, and synergy analysis was performed using the Bliss model via Combenefit software, where the deepest shade of blue indicates the highest level of synergy. **(B)** Cell viability assay results from combination treatments of the Type I PRMT inhibitors MS023 and GSK3368715 with chemotherapeutic agents in H1299 cells were analyzed using the Combenefit software employing the BLISS model. Synergy status of each drug combinations is indicated in the synergy score matrix.

Interestingly, when cells were not pretreated with the PRMT inhibitors and were instead exposed to both drugs simultaneously, no significant synergy was observed. This lack of effect confirmed that transcriptional priming via prolonged epigenetic

reprogramming was necessary to achieve drug sensitization (**Figures 1.24, 1.25, and 1.26**).

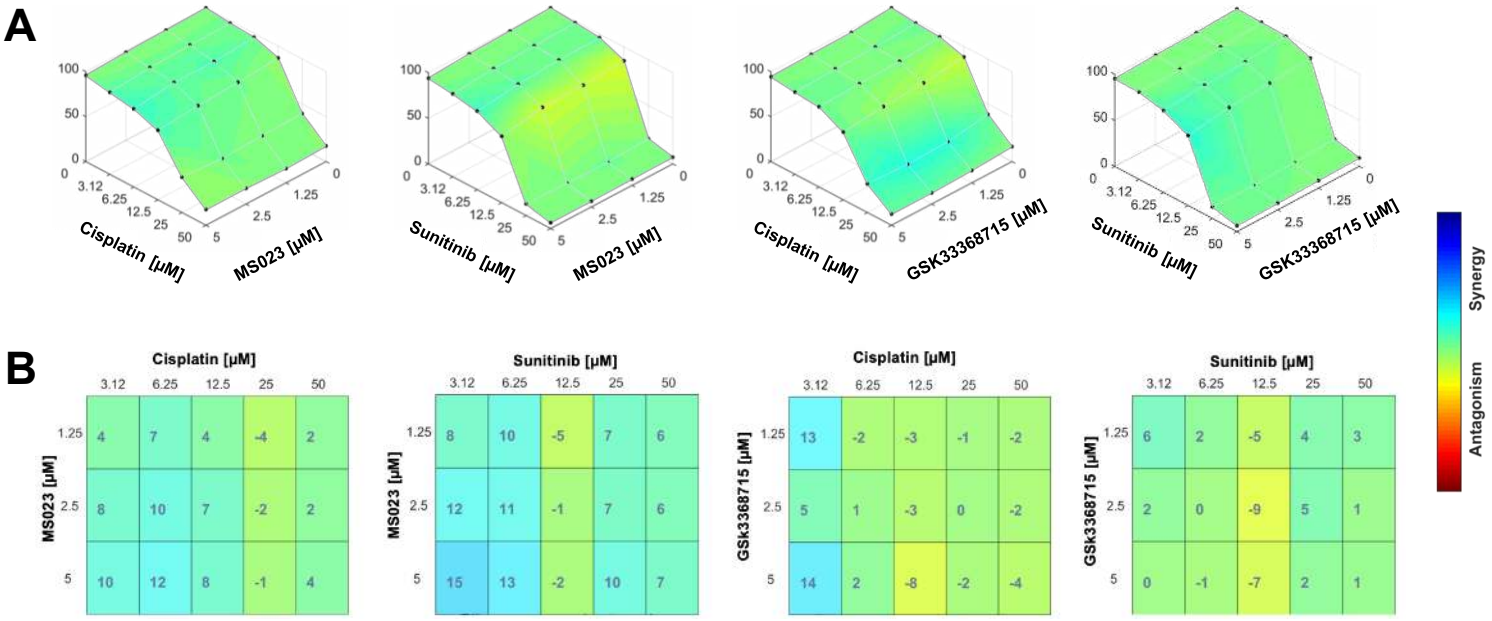



**Figure 1.24. Priming cells with epidrugs is essential for observed synergy in Du145 cells.** Epidrugs were only co-treated with cisplatin or sunitinib for 72 h at indicated concentrations without priming on Du145 cells. **(A)** Epidrug and cisplatin/sunitinib combination doses were shown at the horizontal axis, whereas % cell viability values were plotted at the vertical axis. **(B)** Calculated synergy scores using BLISS scoring method are indicated below as a matrix.

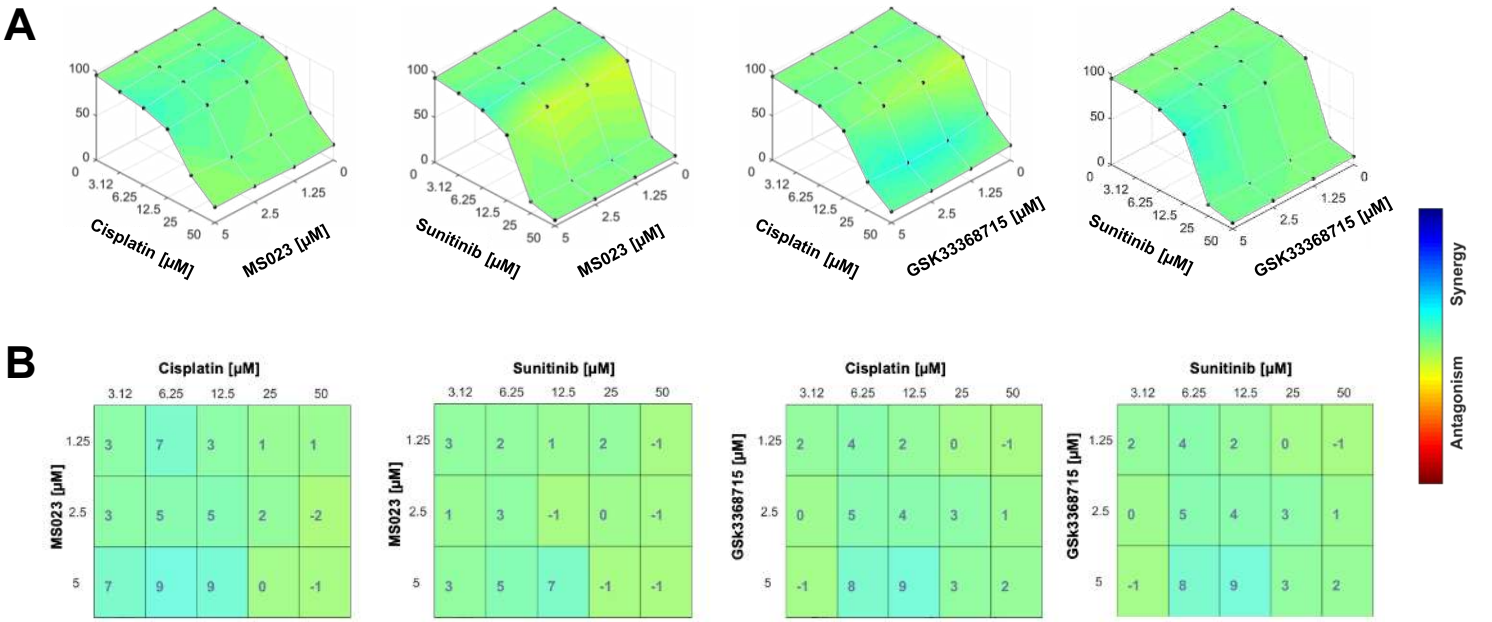



**Figure 1.25. Lack of priming abolishes synergy observed between Type I PRMT inhibitors and chemotherapeutic agents in Panc1 cells.** Epidrugs were only co-treated

with cisplatin or sunitinib for 72 h at indicated concentrations without priming on Panc1 cells. **(A)** Epidrug and cisplatin/sunitinib combination doses were shown at the horizontal axis, whereas % cell viability values were plotted at the vertical axis. **(B)** Calculated synergy scores using BLISS scoring method are indicated below as a matrix.

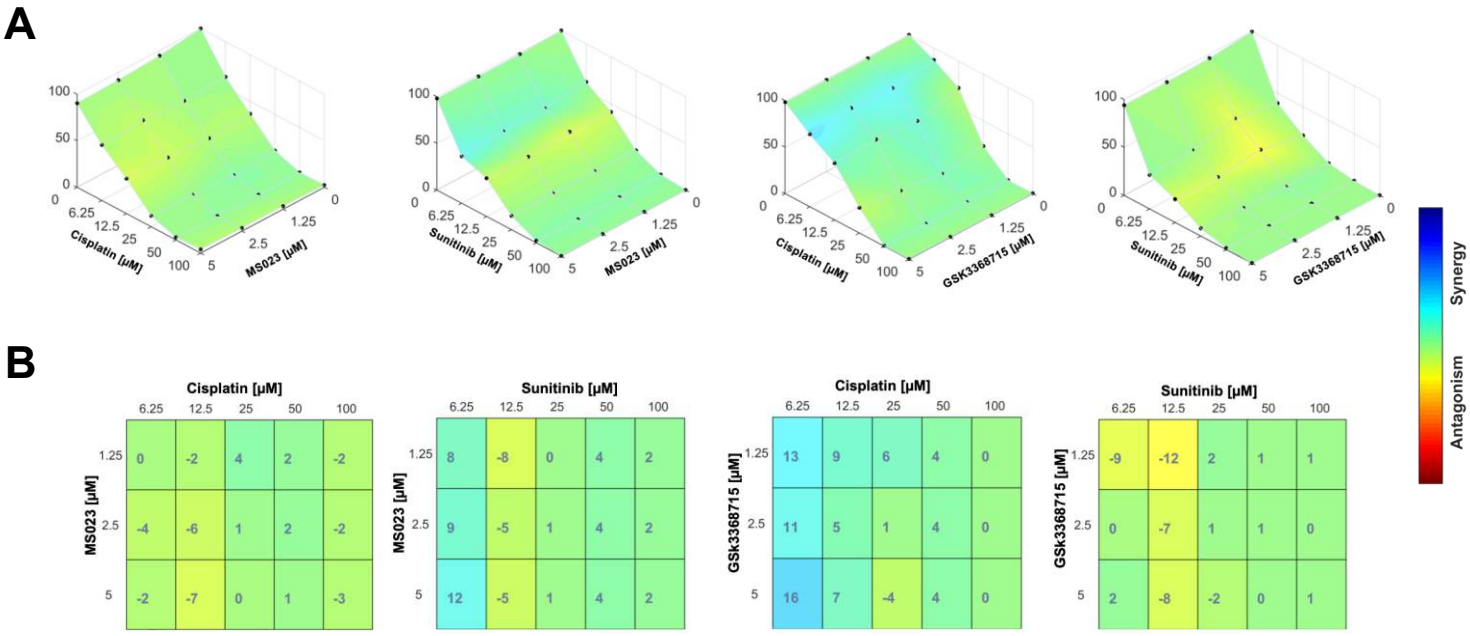



**Figure 1.26. H1299 cells fail to respond synergistically to unprimed epidrug and chemotherapy agent combinations.** Epidrugs were only co-treated with cisplatin or sunitinib for 72 h at indicated concentrations without priming on H1299 cells. **(A)** Epidrug and cisplatin/sunitinib combination doses were shown at the horizontal axis, whereas % cell viability values were plotted at the vertical axis. **(B)** Calculated synergy scores using BLISS scoring method are indicated below as a matrix.

Finally, we assessed the long-term consequences of combinatorial treatment using a colony formation assay in Du145 and Panc1 cells. Co-treatment with MS023 or GSK3368715 and chemotherapeutic agents markedly reduced the ability of cells to form colonies, demonstrating that the observed synergy translated into durable cytostatic and cytotoxic outcomes (**Figure 1.27**). Colony formation assays were not performed on H1299 cells due to their inherently poor clonogenic capacity, which limits the interpretability of long-term survival assays in this model.

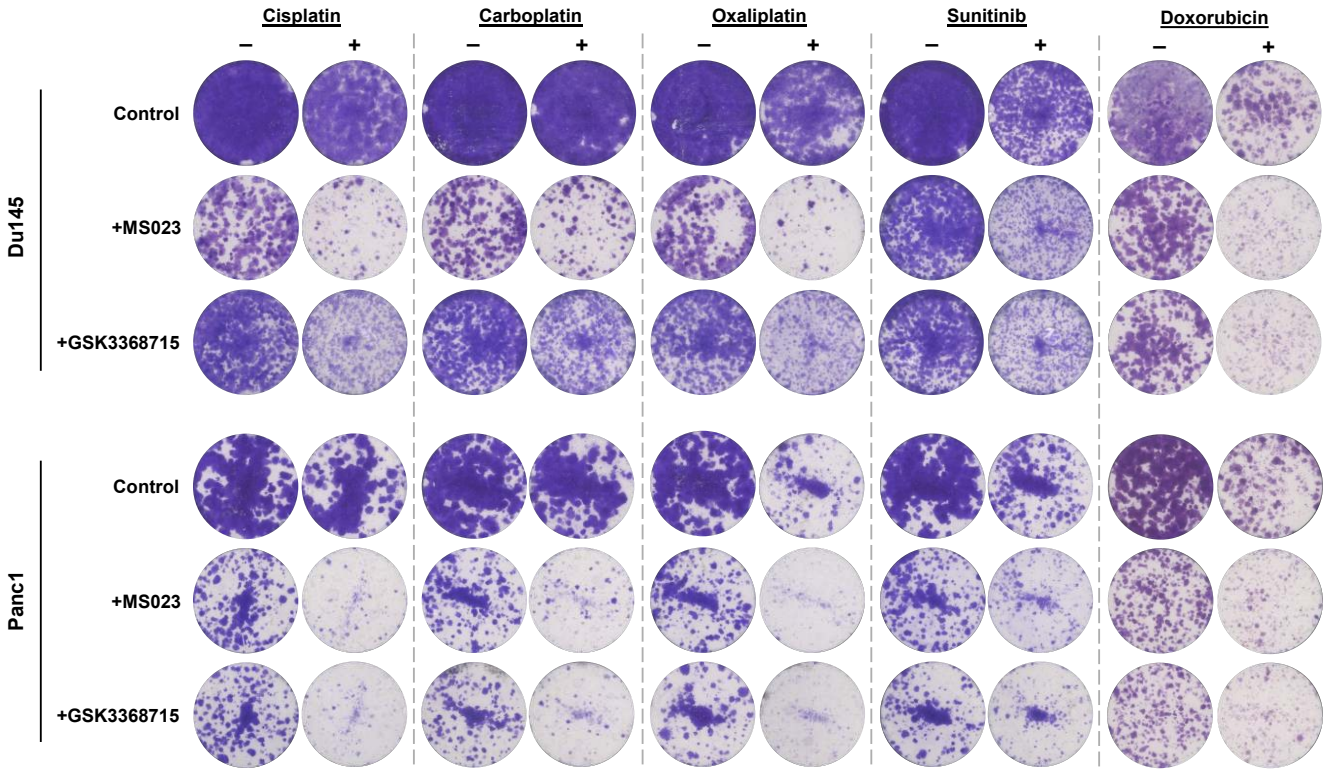



**Figure 1.27. Combination treatments reduce clonogenic survival in Du145 and Panc1 cells.** Colony formation assay performed on Du145 and Panc1 cells confirmed the synergistic effect through inhibition of colony forming capacity of cells after the combination of each epidrug with chemotherapeutic agents. Du145 cells and Panc1 cells were primed with epidrugs MS023 and GSK3368715 for 72 h, then combination treatments were carried out with epidrugs and indicated chemotherapeutic agents for 72h.

Taken together, these findings demonstrate that MS023 and GSK3368715, two selective inhibitors of Type I PRMTs, disrupt lysosomal homeostasis by reducing exocytosis and promoting the accumulation of acidic vesicles in cancer cells. These effects were consistent across multiple cell lines and occurred independently of cytotoxicity, requiring prolonged treatment to manifest—supporting a model in which transcriptional reprogramming rather than acute enzymatic inhibition underlies the observed phenotypes. Importantly, neither compound induced significant changes in canonical lysosomal gene expression, suggesting that PRMT-mediated regulation of lysosomal dynamics likely proceeds through non-canonical pathways. In functional assays, both compounds enhanced the cytotoxic effects of several chemotherapeutic agents, with the strongest synergy observed when cells were pretreated for 72 hours, underscoring the necessity of epigenetic priming. The suppression of colony formation in Du145 and Panc1 cells further validated the long-term impact of this combination treatment. Altogether, these results support a central role for Type I PRMTs in regulating

lysosome-driven drug tolerant phenotype and highlight the therapeutic potential of targeting Type I PRMTs to overcome drug tolerance in solid tumors.

### 3.4. Transcriptomic profiling through RNA-seq analysis reveals PRMT-dependent regulation of vesicular, apoptotic, and epigenetic programs

To gain deeper mechanistic insight into how Type I PRMT inhibition reshapes cellular state and lysosomal dynamics, we performed transcriptome-wide RNA-seq analysis in Du145 cells treated with MS023 for 72 hours. This unbiased approach was designed to capture global changes in gene expression that might underlie the observed disruption of lysosomal exocytosis and synergistic chemosensitization.

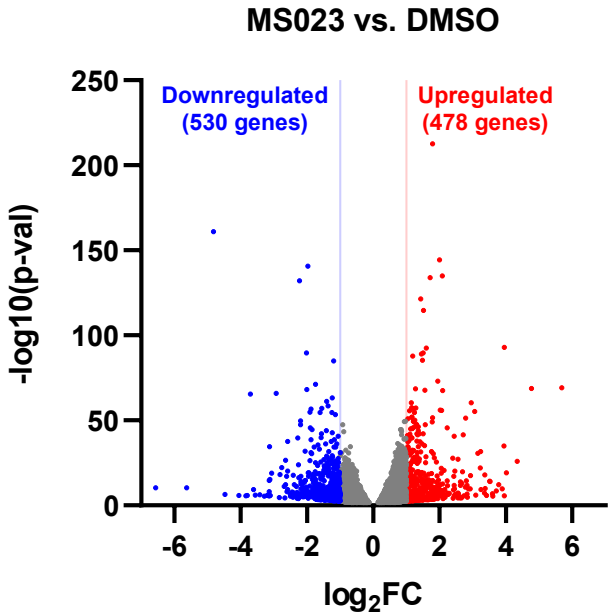



**Figure 1.28. MS023 induces global transcriptomic changes in Du145 cells.** A volcano plot displays differentially expressed genes (DEGs) following MS023 treatment in Du145 cells, with significant up- and downregulated genes highlighted based on $|\log_2 FC| > 1$ and p-val < 0.05.

The overall transcriptional impact of MS023 is visualized as a volcano plot in Figure 28, where significantly upregulated and downregulated genes are displayed according to fold change and statistical significance. To confirm the reproducibility of the dataset, we selected a panel of top upregulated and downregulated genes based purely on rank-order differential expression, without prior knowledge of their functional roles. RT-qPCR analysis validated the majority of these targets, supporting the integrity of the RNA-seq

dataset (**Figures 1.29** and **1.30**). These validated genes were not selected for pathway relevance, but served as internal benchmarks to confirm expression-level perturbations.

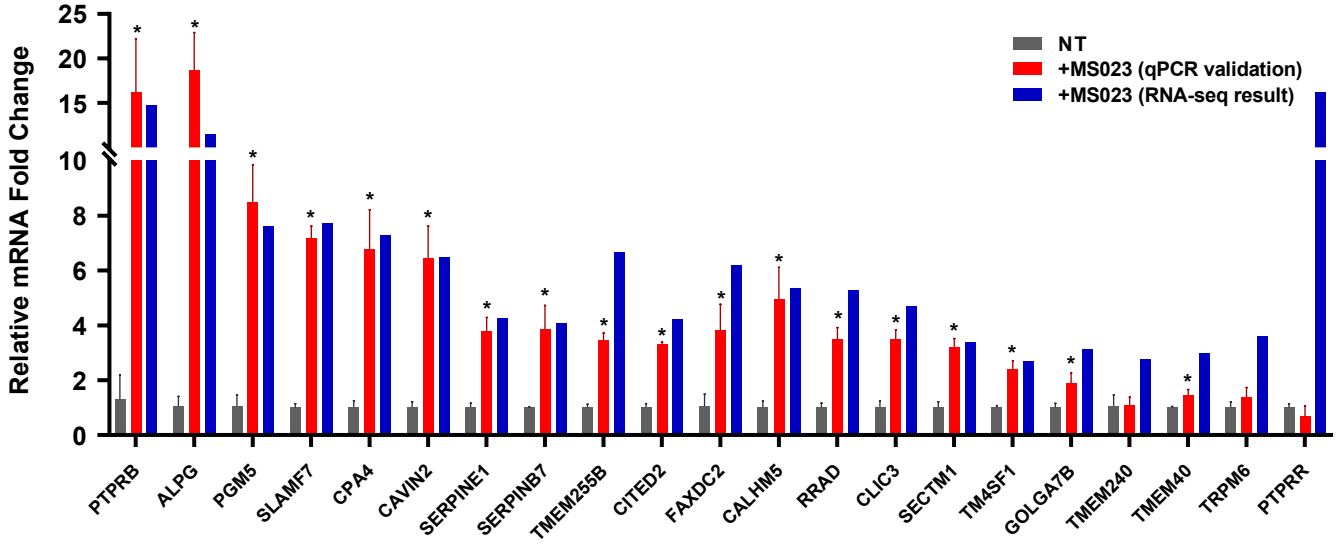



**Figure 1.29. Validation of mRNA expression change of top selected up-regulated genes.** RT-qPCR validation of RNA-seq results confirmed up-regulation of selected genes following MS023 treatment in Du145 cells (*p<.05, n.s. not-significant, Student's t-test).

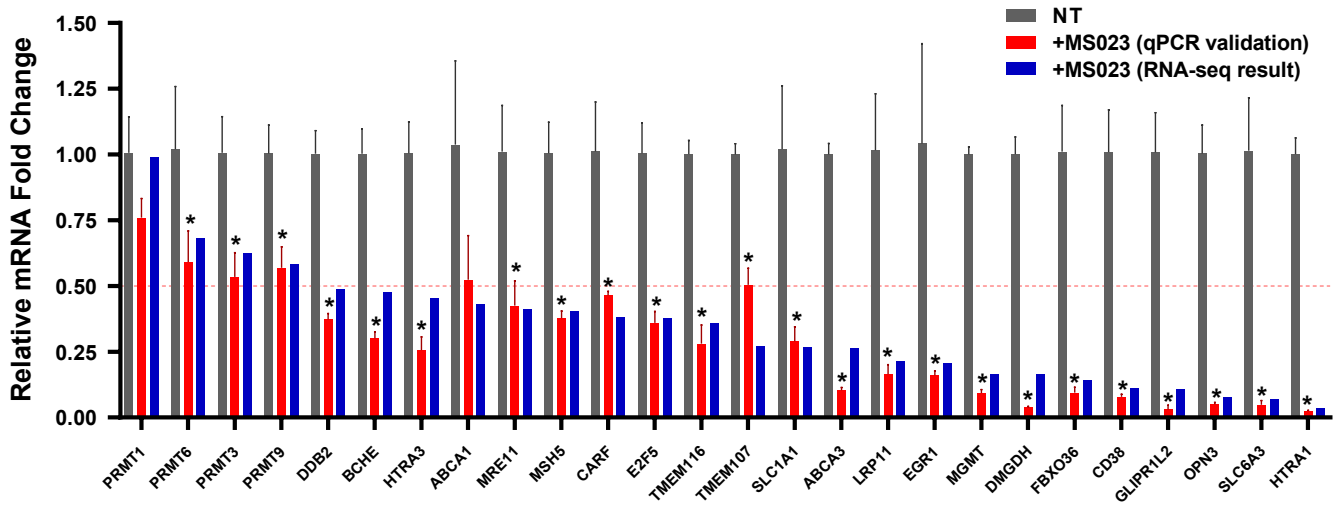



**Figure 1.30. Validation of mRNA expression change of top selected down-regulated genes.** RT-qPCR validation of RNA-seq results confirmed down-regulation of selected genes following MS023 treatment in Du145 cells (*p<.05, n.s. not-significant, Student's t-test).

To interpret these transcriptional changes at the systems level, we conducted Gene Ontology (GO)-based gene set enrichment analysis (GSEA). GO Biological Process enrichment revealed that MS023 treatment resulted in the positive enrichment of gene

sets associated with cell adhesion, positive regulation of cell killing, reactive oxygen species (ROS) metabolism, negative regulation of proteolysis, and extrinsic apoptotic signaling (**Figure 1.31**). These findings are consistent with the activation of apoptotic and oxidative stress responses, which align with the enhanced chemotherapy sensitivity we observed in earlier functional assays. Notably, additional positively enriched terms related to vesicle-mediated transport and negative regulation of catalytic activity suggest remodeling of intracellular trafficking and proteolytic balance — pathways functionally tied to lysosomal dynamics.

Conversely, negatively enriched GO Biological Process categories predominantly mapped to canonical PRMT-linked functions, including DNA repair, chromosome organization, methylation, DNA recombination, and mismatch repair. This pattern strongly supports the notion that MS023-induced PRMT inhibition disrupts chromatin integrity and epigenetic control networks, consistent with known PRMT1/6 roles in transcriptional regulation and genome maintenance.

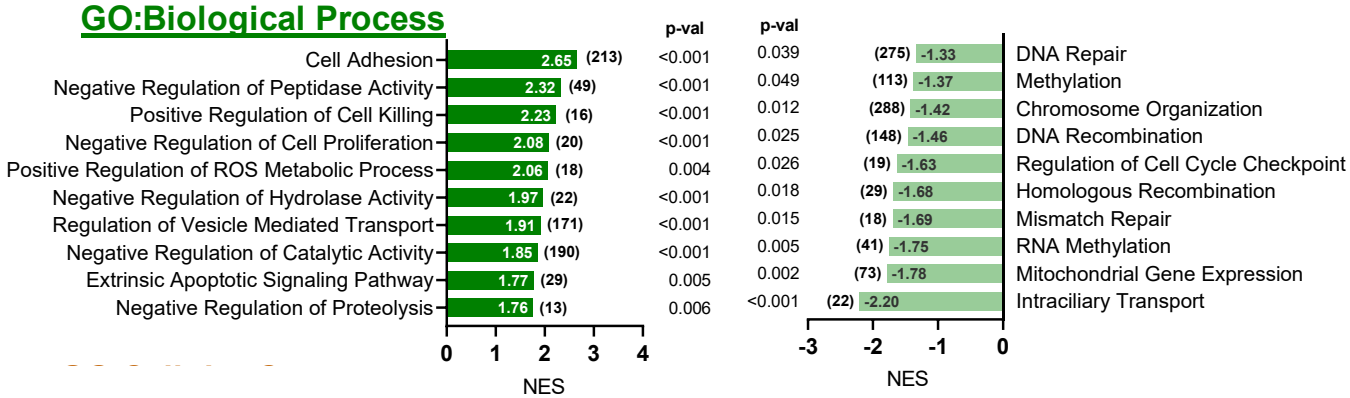



**Figure 1.31. PRMT inhibition reshapes biological pathways linked to lysosomal and metabolic regulation.** Gene Set Enrichment Analysis (GSEA) revealed significantly enriched (either positively or negatively) Gene Ontology Biological Process (c5.go.bp.v2023.1.Hs.symbols.gmt) categories in MS023-treated Du145 cells, including vesicular transport, metabolic regulation, and protein localization. NES scores of each enriched pathways are shown within bars, whereas numbers in parenthesis indicate number of genes whose expression is altered in the pathway.

GO Cellular Component analysis further reinforced the dual themes of lysosomal reprogramming and epigenetic collapse. Positively enriched terms included transport vesicle membrane, clathrin-coated vesicle membrane, autophagosome, endolysosome, and vacuole — all structural hallmarks of vesicular trafficking and lysosome biology (**Figure 1.32**). Meanwhile, negatively enriched categories involved ribosomal subunits,

nucleosomes, mitochondrial matrix, and chromatin-related complexes, indicating suppressed translation and nuclear organization upon PRMT inhibition.

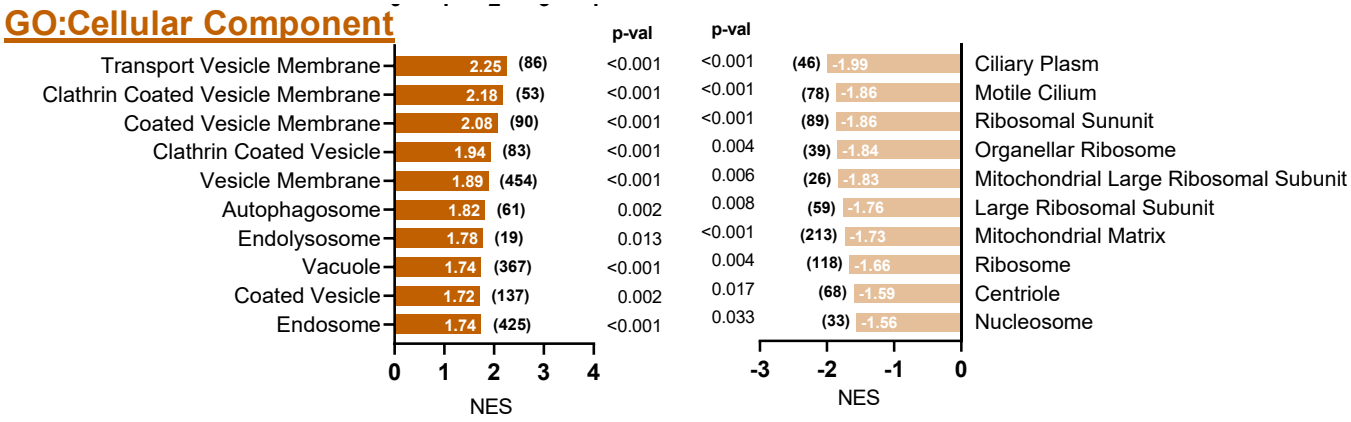



**Figure 1.32. MS023 alters expression of genes pathway enrichments associated with lysosomal compartments.** Gene Set Enrichment Analysis (GSEA) revealed significantly enriched (either positively or negatively) Gene Ontology Cellular Component (c5.go.cc.v2023.1.Hs.symbols.gmt) categories in MS023-treated Du145 cells, including pathways associated with lysosomal compartments, vesicular trafficking. NES scores of each enriched pathways are shown within bars, whereas numbers in parenthesis indicate number of genes whose expression is altered in the pathway.

Furthermore, GO Molecular Function GSEA revealed positive enrichment of functions related to protease binding, endopeptidase inhibition, lipase activity, and enzyme regulator activity (**Figure 1.33**). These molecular shifts likely reflect the observed accumulation of undegraded lysosomal cargo. At the same time, the negative enrichment of terms such as RNA methyltransferase activity, chromatin binding, DNA-binding transcription factor activity, and catalytic activity on nucleic acids confirms that PRMT activity is central to maintaining transcriptional and post-transcriptional regulatory circuits.

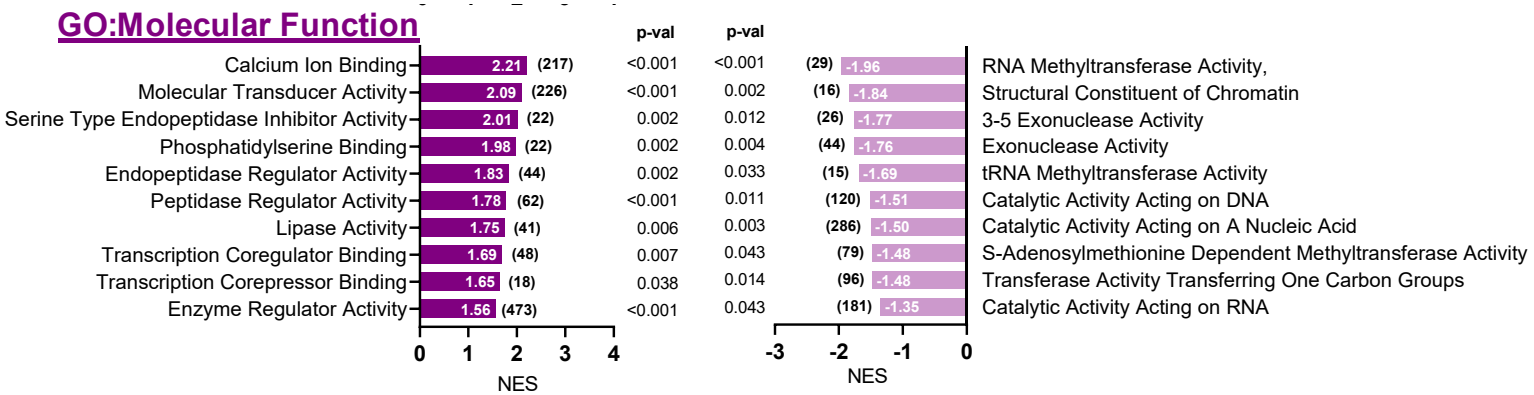



**Figure 1.33. Gene Set Enrichment Analysis (GSEA) identifies enriched pathways related to molecular function upon MS023 treatment in Du145 cells.** Gene Set

Enrichment Analysis (GSEA) revealed significantly enriched (either positively or negatively) Gene Ontology Molecular Function (c5.go.mf.v2023.1.Hs.symbols.gmt) categories in MS023-treated Du145 cells, including pathways associated with hydrolase activity, catalytic binding, and transporter activity. NES scores of each enriched pathways are shown within bars, whereas numbers in parenthesis indicate number of genes whose expression is altered in the pathway.

Given the diversity of pathways uncovered, we aimed to extract a more functionally focused subset of DEGs relevant to the core hypothesis of the study. To this end, we constructed by manually annotating DEGs into six thematic categories: lysosomal exocytosis, lysosome-related genes, vesicle sequestration-associated genes, methylation regulators, DNA repair genes, and RNA processing factors. These categories were defined based on both literature and enrichment data from Figures 30–32 (**Table 1.6** lists each of the gene sets found in the combined gene sets). The rationale behind this approach was to stratify DEGs into biologically interpretable modules that reflect the dual axis of PRMT function: epigenetic regulation and vesicular trafficking (**Figure 1.34**). This focused classification allowed us to highlight candidate genes likely to contribute to the effects observed in our screen. Moreover, these combined gene sets were subsequently used in cross-analysis with public ChIP-seq datasets to prioritize putative direct transcriptional targets of Type I PRMTs, which are discussed in the following results.

**Table 1.6.** The list of gene sets being used to curate combined gene sets for RNA-seq analysis

| |
|---|
| **Exocytosis-related Gene Sets**<br>GO:0006887, GO:0045955, GO:2000301, GO:0045956, GO:0045055, GO:0017158, GO:0017157, GO:2000300, GO:0016079 |
| **Lysosome-related Gene Sets**<br>GO:0008333, GO:0032510, GO:0090160, GO:0006622, GO:1905671, GO:0062196, GO:0150031, GO:0004563, GO:0015929, GO:0007042, GO:0097212, GO:1905146, GO:0007041, GO:0035751, GO:1905165, GO:0033299, GO:0098574, GO:0043202, GO:0097401, GO:0007035, HP:0004356 |
| **Sequestering-related Gene Sets**<br>GO:0051238, GO:0140487, GO:0140313, GO:0140311 |
| **Methyltranferase-related Gene Sets**<br>GO:0035242, GO:0008276, GO:0008169, GO:0034708, GO:0035097, GO:0140939 |
| **DNA Repair-related Gene Sets**<br>GO:0006281, GO:0006302, GO:0036297, GO:0006289, GO:1990391, WP4946, WP4753, R-HSA-5693532, R-HSA-73894, R-HSA-5696398, hsa03420, Pubmed 26771021, Pubmed 17891185 |
| **RNA Processing-related Gene Sets**<br>GO:0008380, GO:0001510, GO:0009451, GO:0003723 |

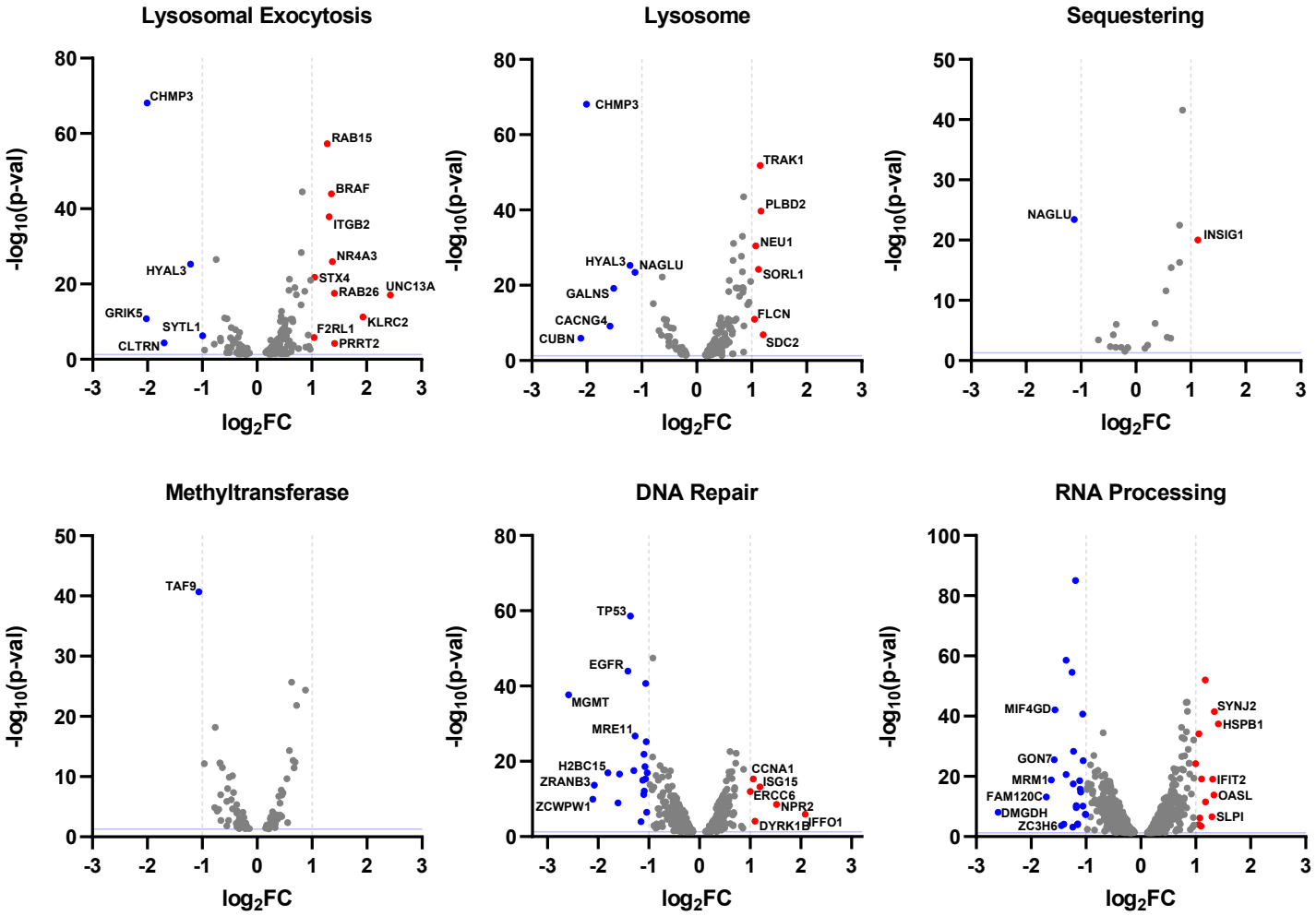



**Figure 1.34. MS023 modulates the expression of genes involved in lysosomal activities and PRMT-targeted cellular processes.** Gene sets from the GSEA Molecular Signature Database were manually selected and categorized into six distinct "combined gene sets" based on their functional relevance (details given in Table 3): lysosomal exocytosis-related gene sets, lysosome-related gene sets, sequestration-related gene sets, methyltransferase-related gene sets, DNA repair-related gene sets, and RNA processing-related gene sets. The differentially expressed genes within each of these combined gene sets are marked, and volcano plots were generated, with grey vertical lines indicating the $|\log_2 FC| > 1$ cutoff and the blue horizontal line representing p-val<0.05.

Together, this RNA-seq and GSEA-based analysis shows that PRMT inhibition leads to broad transcriptomic changes, downregulating DNA repair, chromatin organization, and methylation-related pathways, while upregulating genes linked to lysosomal stress, vesicle accumulation, and apoptotic signaling. These shifts offer a mechanistic explanation for the enhanced chemotherapy response observed earlier. By organizing the most significantly altered genes into functional categories related to lysosomal activity, vesicular transport, epigenetic regulation, and genome maintenance, we identified the

core pathways affected by PRMT inhibition. The enrichment analysis further supported this view, revealing consistent suppression of repair programs alongside activation of vesicle-related and stress-induced processes. Altogether, these results suggest that PRMT1/6 inhibition disrupts both nuclear and vesicular homeostasis, creating a vulnerable cellular state that enhances sensitivity to anticancer treatments.

### 3.5. Combined RNA-seq and ChIP-seq analysis uncovers PRMT1/6-mediated epigenetic control of target genes

To further understand the mechanistic basis by which PRMT1 and PRMT6 regulate lysosomal function and transcriptional programs downstream of MS023 treatment, we performed an integrative analysis combining our RNA-seq results with publicly available ChIP-seq datasets. Specifically, we sought to determine whether differentially expressed genes (DEGs) from our six combined gene sets (introduced in **Figure 1.34**) overlapped with known PRMT1/4/6 promoter-binding profiles, thereby identifying putative direct transcriptional targets of these methyltransferases.

**Figure 1.35** provides an overview of the analytical strategy. ChIP-seq datasets were retrieved using the ChIP-Atlas platform, which aggregates uniformly processed ChIP-seq data from diverse studies and multiple cell types. Among Type I PRMTs, we identified publicly available datasets for PRMT1, PRMT4 (CARM1), and PRMT6. Notably, this was advantageous because our top two epigenetic hit compounds—MS023 and GSK3368715—are known to potently inhibit PRMT1 and PRMT6, making them mechanistically relevant to the observed transcriptional effects. The genomic peak data were filtered to include only those located within regulatory and promoter-proximal regions (−5 kb to +100 bp relative to the transcription start site) and were then intersected with DEGs from the six curated RNA-seq gene sets: exocytosis, lysosomal genes, vesicular sequestration, methylation, DNA repair, and RNA processing. This filtering process allowed us to narrow down a subset of genes that were not only transcriptionally altered by MS023 treatment, but also showed PRMT1/4/6 occupancy in promoter regions across multiple datasets. Detailed metadata and study sources for these ChIP-seq datasets are listed in **Table 1.7**.

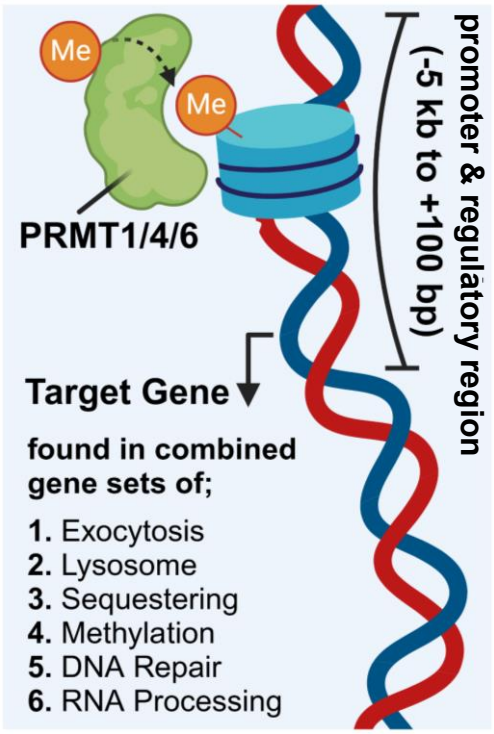



**Figure 1.35. Integrative analysis of RNA-seq and ChIP-seq data identifies PRMT occupancy at DEG promoters.** A schematic summarizes the analytical pipeline used to map PRMT1, PRMT4, and PRMT6 binding to regulatory and promoter regions (−5 kb to +100 bp relative to transcription start sites) of DEGs using publicly available ChIP-Atlas datasets. (Created with BioRender.com).

**Table 1.7.** Interpretation of online available ChIP-seq Data retrieved from ChIP-Atlas.

| Target Gene Name | Combined Gene Set Number 1.Exocytosis 2. Lysosome 3. Sequestering 4. Methylation 5. DNA Repair 6. RNA Processing | RNA-seq Fold Change | ChIP-Atlas ChIP-seq Data (Cell Type/Cell Line, Enrichment Peak Score, ChIP-seq Data ID) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | PRMT1 | | | | | PRMT4/CARM1 | | PRMT6 |
| AGO2 | 6 | 2.15 | Keratinocytes, 227.0, SRX3642946 | Keratinocytes, 308.0, SRX3642947 | - | - | - | Kasumi-1, 249.0, SRX15946676 | Kasumi-1, 296.0, SRX15946675 | - |
| APLF | 5 | 0.48 | Keratinocytes, 146.0, SRX3642947 | Keratinocytes, 93.0, SRX3642946 | - | - | - | Kasumi-1, 136.0, SRX15946675 | - | - |
| ARC | 6 | 2.08 | - | - | - | - | - | - | - | - |
| ASCC1 | 5, 6 | 0.48 | Keratinocytes, 90.0, SRX3642947 | - | - | - | - | - | - | - |
| BCLAF1 | 6 | 0.47 | Keratinocytes, 112.0, SRX3642946 | Keratinocytes, 104.0, SRX3642947 | - | - | - | - | - | - |
| BRAF | 1 | 2.56 | - | - | - | - | - | - | - | - |
| C7orf50 | 6 | 0.43 | Keratinocytes, 101.0, SRX3642947 | - | - | - | - | - | - | - |
| CACNG4 | 2 | 0.33 | Keratinocytes, 476.0, SRX3642946 | Keratinocytes, 424.0, SRX3642947 | Keratinocytes, 100.0, SRX3642948 | - | - | MCF-7, 265.0, SRX5185601 | - | - |
| CCNA1 | 5 | 2.08 | Keratinocytes, 114.0, SRX3642946 | - | - | - | - | - | - | - |
| CELF5 | 6 | 2.14 | - | - | - | - | - | - | - | - |
| CHMP3 | 1, 2 | 0.25 | Keratinocytes, 149.0, SRX3642946 | Keratinocytes, 168.0, SRX3642947 | Keratinocytes, 253.0, SRX3642946 | Keratinocytes, 302.0, SRX3642947 | - | Kasumi-1, 228.0, SRX15946675 | - | - |
| CLTRN | 1 | 0.31 | - | - | - | - | - | - | - | - |
| CUBN | 2 | 0.23 | Keratinocytes, 160.0, SRX3642946 | - | - | - | - | - | - | - |
| DDB2 | 5 | 0.49 | Keratinocytes, 133.0, SRX3642947 | Keratinocytes, 102.0, SRX3642947 | - | - | - | - | - | - |
| DHFR2 | 6 | 0.5 | Keratinocytes, 72.0, SRX3642947 | - | - | - | - | - | - | - |
| DMGDH | 6 | 0.16 | - | - | - | - | - | - | - | - |
| DUS4L | 6 | 0.42 | Keratinocytes, 109.0, SRX3642946 | Keratinocytes, 81.0, SRX3642946 | Keratinocytes, 130.0, SRX3642947 | - | - | Kasumi-1, 89.0, SRX15946675 | - | - |
| DYRK1B | 5 | 2.13 | Keratinocytes, 83.0, SRX3642947 | - | - | - | - | MCF-7, 151.0, SRX5185600 | MCF-7, 244.0, SRX5185601 | - |
| EGFR | 5 | 0.38 | Keratinocytes, 158.0, SRX3642946 | Keratinocytes, 166.0, SRX3642947 | - | - | - | - | - | - |
| ERCC6 | 5 | 2 | Keratinocytes, 351.0, SRX3642947 | Keratinocytes, 408.0, SRX3642946 | - | - | - | - | - | - |
| ERCC8 | 5 | 0.46 | - | - | - | - | - | Kasumi-1, 71.0, SRX15946675 | - | - |
| F2RL1 | 1 | 2.06 | Keratinocytes, 151.0, SRX3642947 | Keratinocytes, 119.0, SRX3642946 | - | - | - | - | - | - |
| FAM120C | 6 | 0.3 | - | - | - | - | - | - | - | - |
| FASN | 6 | 2.25 | Keratinocytes, 139.0, SRX3642947 | - | - | - | - | - | - | - |
| FLCN | 2 | 2.07 | Keratinocytes, 179.0, SRX3642947 | Keratinocytes, 125.0, SRX3642946 | - | - | - | - | - | - |
| GALNS | 2 | 0.35 | - | - | - | - | - | - | - | - |
| GEN1 | 5 | 0.47 | Keratinocytes, 64.0, SRX3642946 | - | - | - | - | Kasumi-1, 138.0, SRX15946675 | - | - |
| GON7 | 6 | 0.33 | Keratinocytes, 145.0, SRX3642946 | Keratinocytes, 137.0, SRX3642947 | - | - | - | Kasumi-1, 249.0, SRX15946676 | Kasumi-1, 255.0, SRX15946675 | - |
| GRB7 | 6 | 2.1 | Keratinocytes, 117.0, SRX3642946 | Keratinocytes, 114.0, SRX3642947 | Keratinocytes, 193.0, SRX3642947 | Keratinocytes, 121.0, SRX3642946 | - | - | - | - |
| GRIK5 | 1 | 0.25 | - | - | - | - | - | - | - | - |
| H1-0 | 6 | 0.44 | - | - | - | - | - | - | - | - |
| H2AJ | 5 | 0.48 | - | - | - | - | - | - | - | - |
| H2BC15 | 5 | 0.29 | Keratinocytes, 99.0, SRX3642947 | Keratinocytes, 135.0, SRX3642946 | - | - | - | - | - | - |
| HLA-A | 6 | 1.99 | - | - | - | - | - | - | - | - |
| HSPB1 | 6 | 2.66 | Keratinocytes, 186.0, SRX3642946 | Keratinocytes, 235.0, SRX3642947 | - | - | - | - | - | - |
| HYAL3 | 1, 2 | 0.43 | Keratinocytes, 261.0, SRX3642947 | Keratinocytes, 148.0, SRX3642946 | - | - | - | - | - | MDA-MB-231, 98.0, SRX17024137 |
| IFFO1 | 5 | 4.22 | - | - | - | - | - | - | - | - |
| IFIT1 | 6 | 2.26 | - | - | - | - | - | - | - | - |
| IFIT2 | 6 | 2.47 | - | - | - | - | - | - | - | - |
| INSIG1 | 3 | 2.18 | - | - | - | - | - | MCF-7, 102.0, SRX5185601 | - | - |
| ISG15 | 5 | 2.28 | Keratinocytes, 186.0, SRX3642947 | Keratinocytes, 101.0, SRX3642946 | - | - | - | - | - | - |
| ITGB2 | 1 | 2.48 | Keratinocytes, 283.0, SRX3642947 | Keratinocytes, 172.0, SRX3642946 | - | - | - | - | - | MDA-MB-231, 132.0, SRX17024137 |
| JAKMIP1 | 6 | 0.45 | - | - | - | - | - | - | - | - |
| KCTD12 | 6 | 0.48 | Keratinocytes, 120.0, SRX3642946 | Keratinocytes, 158.0, SRX3642946 | Keratinocytes, 105.0, SRX3642947 | Keratinocytes, 133.0, SRX3642947 | - | - | - | - |
| KLRC2 | 1 | 3.81 | - | - | - | - | - | - | - | - |
| KRT18 | 6 | 2.07 | Keratinocytes, 146.0, SRX3642946 | - | - | - | - | - | - | - |
| MCMDC2 | 5 | 0.33 | - | - | - | - | - | - | - | - |
| MGMT | 5 | 0.17 | Keratinocytes, 205.0, SRX3642947 | - | - | - | - | - | - | - |
| MIF4GD | 6 | 0.34 | - | - | - | - | - | - | - | - |
| MRE11 | 5 | 0.41 | - | - | - | - | - | - | - | - |
| MRM1 | 6 | 0.32 | - | - | - | - | - | - | - | - |
| MSH5 | 5 | 0.41 | - | - | - | - | - | - | - | - |
| NAGLU | 2, 3 | 0.46 | Keratinocytes, 164.0, SRX3642946 | Keratinocytes, 183.0, SRX3642947 | - | - | - | - | - | - |
| NEU1 | 2 | 2.1 | Keratinocytes, 166.0, SRX3642947 | Keratinocytes, 85.0, SRX3642947 | - | - | - | - | - | - |
| NPR2 | 5 | 2.86 | Keratinocytes, 105.0, SRX3642947 | - | - | - | - | - | - | - |
| NR0B1 | 6 | 0.44 | - | - | - | - | - | - | - | - |
| NR4A3 | 1 | 2.59 | - | - | - | - | - | - | - | - |
| OASL | 6 | 2.51 | Keratinocytes, 110.0, SRX3642946 | - | - | - | - | - | - | - |
| OOEP | 5, 6 | 0.45 | - | - | - | - | - | - | - | - |
| PCDHGA9 | 6 | 10.18 | - | - | - | - | - | - | - | - |
| PCID2 | 6 | 0.42 | Keratinocytes, 107.0, SRX3642947 | Keratinocytes, 164.0, SRX3642946 | - | - | - | - | - | - |
| PIWIL4 | 6 | 0.38 | - | - | - | - | - | - | - | - |
| PLBD2 | 2 | 2.24 | Keratinocytes, 105.0, SRX3642947 | Keratinocytes, 79.0, SRX3642946 | - | - | - | - | - | - |
| PRIMPOL | 5 | 0.46 | - | - | - | - | - | - | - | - |
| PRRT2 | 1 | 2.67 | - | - | - | - | - | - | - | - |
| RAB15 | 1 | 2.42 | - | - | - | - | - | - | - | - |
| RAB26 | 1 | 2.65 | Keratinocytes, 125.0, SRX3642946 | Keratinocytes, 167.0, SRX3642947 | - | - | - | - | - | - |
| RAD54B | 5 | 0.47 | - | - | - | - | - | - | - | - |
| RBM44 | 6 | 0.42 | Keratinocytes, 146.0, SRX3642946 | - | - | - | - | - | - | - |
| REXO5 | 6 | 0.49 | Keratinocytes, 143.0, SRX3642946 | - | - | - | - | Kasumi-1, 175.0, SRX15946675 | Kasumi-1, 120.0, SRX15946676 | - |
| SDC2 | 2 | 2.31 | Keratinocytes, 81.0, SRX3642947 | - | - | - | - | - | - | - |
| SEPSECS | 6 | 0.46 | Keratinocytes, 469.0, SRX3642947 | Keratinocytes, 437.0, SRX3642946 | - | - | - | - | - | - |
| SLPI | 6 | 2.45 | - | - | - | - | - | - | - | - |
| SLX1B | 5 | 0.33 | Keratinocytes, 181.0, SRX3642946 | Keratinocytes, 208.0, SRX3642947 | - | - | - | Kasumi-1, 139.0, SRX15946676 | Kasumi-1, 181.0, SRX15946675 | - |
| SORL1 | 2 | 2.18 | Keratinocytes, 200.0, SRX3642947 | Keratinocytes, 166.0, SRX3642947 | Keratinocytes, 190.0, SRX3642946 | - | - | - | - | - |
| SRBD1 | 6 | 0.46 | - | - | - | - | - | Kasumi-1, 190.0, SRX15946675 | Kasumi-1, 210.0, SRX15946676 | - |
| STX4 | 1 | 2.07 | Keratinocytes, 120.0, SRX3642946 | Keratinocytes, 189.0, SRX3642947 | - | - | - | - | - | - |
| SYNJ2 | 6 | 2.53 | Keratinocytes, 79.0, SRX3642946 | - | - | - | - | - | - | - |
| TAF9 | 4, 5, 6 | 0.48 | Keratinocytes, 207.0, SRX3642946 | Keratinocytes, 171.0, SRX3642947 | - | - | - | - | - | - |
| TP53 | 5, 6 | 0.39 | Keratinocytes, 119.0, SRX3642947 | Keratinocytes, 195.0, SRX3642946 | Keratinocytes, 113.0, SRX3642947 | Keratinocytes, 167.0, SRX3642946 | Keratinocytes, 162.0, SRX3642947 | - | - | - |
| TRAK1 | 2 | 2.23 | - | - | - | - | - | - | - | - |
| TRMT10A | 6 | 0.44 | Keratinocytes, 109.0, SRX3642947 | Keratinocytes, 112.0, SRX3642946 | Keratinocytes, 174.0, SRX3642946 | Keratinocytes, 76.0, SRX3642948 | - | Kasumi-1, 178.0, SRX15946675 | - | - |
| TST | 6 | 0.39 | - | - | - | - | - | - | - | - |
| UFL1 | 5 | 0.47 | Keratinocytes, 193.0, SRX3642947 | Keratinocytes, 180.0, SRX3642946 | Keratinocytes, 97.0, SRX3642947 | - | - | Kasumi-1, 106.0, SRX15946675 | - | - |
| UNC13A | 1 | 5.39 | - | - | - | - | - | - | - | - |
| ZC3H6 | 6 | 0.37 | Keratinocytes, 203.0, SRX3642947 | Keratinocytes, 83.0, SRX3642946 | - | - | - | Kasumi-1, 160.0, SRX15946675 | - | - |
| ZCWPW1 | 5 | 0.23 | Keratinocytes, 120.0, SRX3642947 | Keratinocytes, 153.0, SRX3642946 | Keratinocytes, 233.0, SRX3642947 | Keratinocytes, 84.0, SRX3642946 | - | Kasumi-1, 111.0, SRX15946675 | - | - |
| ZRANB3 | 5 | 0.24 | Keratinocytes, 104.0, SRX3642947 | - | - | - | - | - | - | - |

To visualize the outcome of this integrative analysis, **Figure 1.36** presents a comprehensive summary of 89 genes that were differentially expressed upon MS023 treatment and belonged to one of the six combined gene sets curated earlier. These genes were filtered based on a $\log_2$ fold change threshold ($|\log_2 FC| > 1$), and subsequently assessed for PRMT occupancy at their promoter regions using publicly available ChIP-seq data. The bar plots in Figure 36 show the direction and magnitude of expression change for each gene, while accompanying bubble plots display average ChIP-seq peak

scores and the number of independent studies reporting PRMT1, PRMT4, or PRMT6 enrichment at the corresponding promoter regions. Each gene is annotated with its combined gene set category (1 through 6), representing lysosomal exocytosis, lysosomal function, sequestration, methylation, DNA repair, or RNA processing. This consolidated view clearly illustrates that a substantial proportion of MS023-responsive genes are also directly associated with PRMT promoter occupancy, supporting the hypothesis that Type I PRMTs play a key transcriptional role in regulating these functional networks.

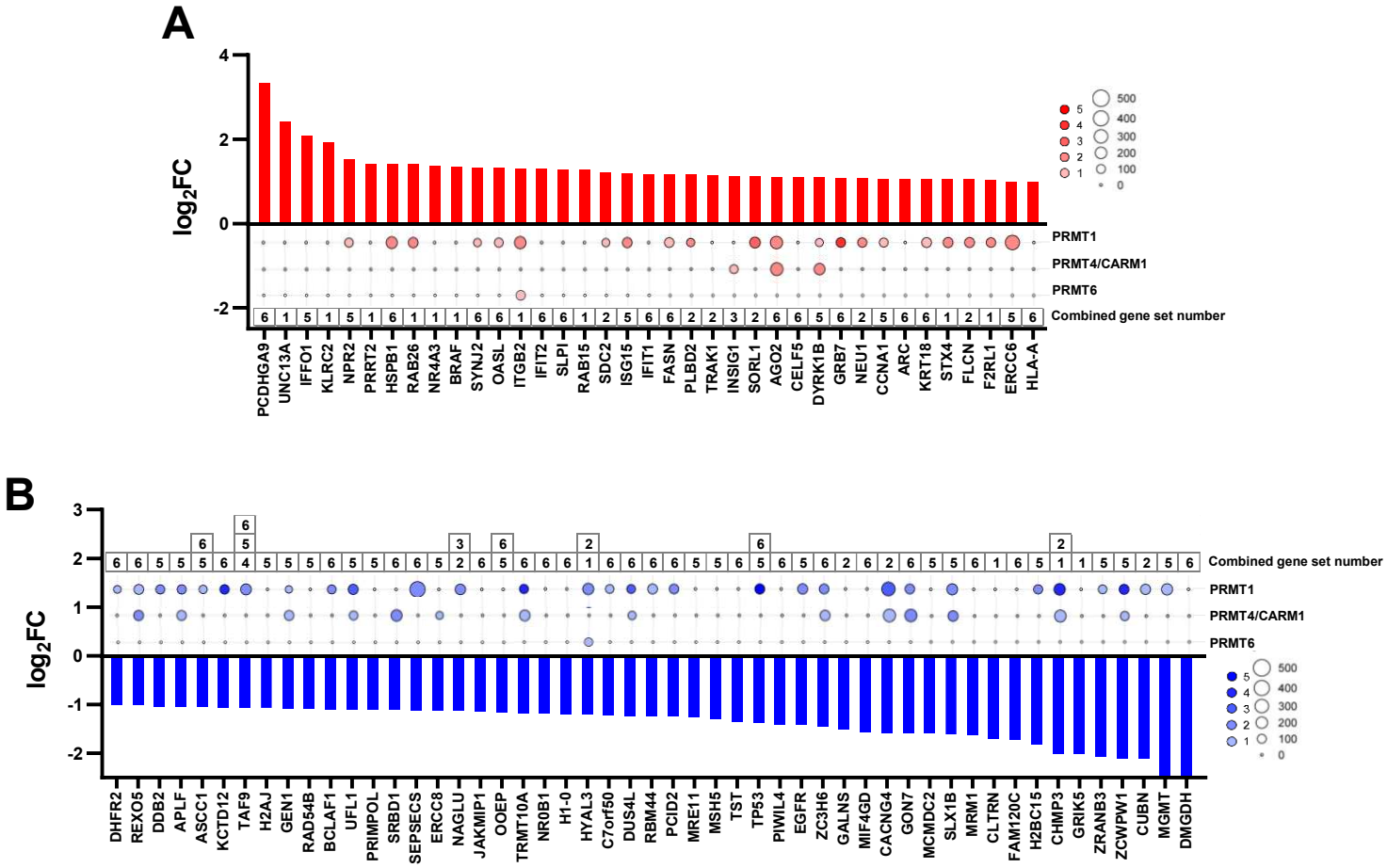



**Figure 1.36. PRMTs occupy promoters of functionally regulated genes following MS023 treatment. (A)** Up-regulated and **(B)** down-regulated DEGs identified by our RNA-seq following MS023 treatment in Du145 cells were categorized based on their associated combined gene sets: **1.** Exocytosis, **2.** Lysosome, **3.** Sequestering, **4.** Methylation, **5.** DNA Repair, and **6.** RNA Processing. Bar plots indicate logFC expression values, and bubble plots represent PRMT occupancy at promoter regions. Bubble size corresponds to average ChIP-seq enrichment scores, and bubble color intensity reflects the number of ChIP-seq datasets confirming the occupancy peaks.

To experimentally validate whether PRMT1 and PRMT6 indeed localize to the promoter regions of selected RNA-seq/ChIP-seq overlapping genes, we next generated stable overexpression cell lines expressing FLAG-tagged PRMT1 or PRMT6. To achieve engineered lines, the coding regions of PRMT1 and PRMT6 were cloned into a pLJC2-3xFLAG lentiviral vector. Lentiviral particles were generated via transfection of HEK293T cells and used to infect Du145, Panc1, and H1299 cells. Western blot analysis confirmed robust overexpression in all three models, as indicated by FLAG signal and elevated levels of endogenous PRMT1 and PRMT6 protein (**Figure 1.37**). These engineered lines were used in downstream ChIP-qPCR experiments.

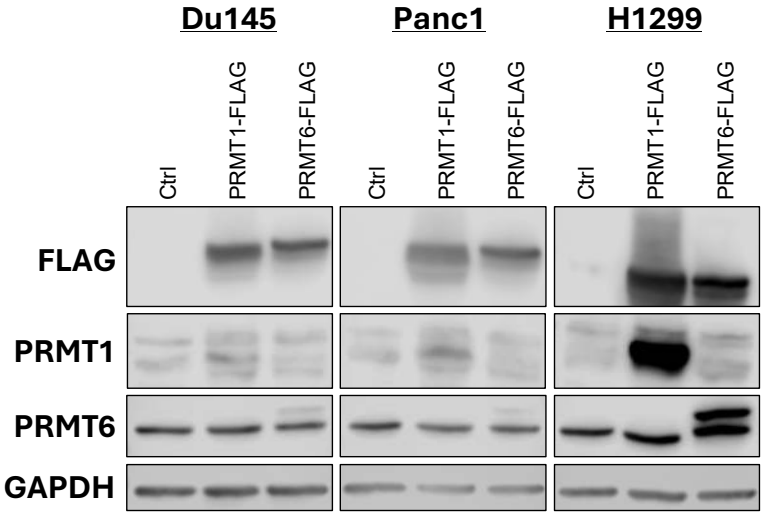



**Figure 1.37. Western blot confirms FLAG-tagged PRMT1 and PRMT6 expression in overexpression models.** PRMT1 and PRMT6 constructs were cloned into pLJC2-3xFLAG lentiviral overexpression backbone and stable overexpression was validated via western blotting experiments using anti-FLAG (expected molecular weight; 42 kDa), anti-PRMT1 (molecular weight; 42 kDa), and anti-PRMT6 (molecular weight; 42 kDa) antibodies in Du145, Panc1, and H1299 cells. GAPDH (molecular weight; 37 kDa) was used as a loading control (this figure was generated by PhD student Beyza Tutunculer).

Although our integrative ChIP-seq screen identified 89 candidate genes with potential PRMT1/6 promoter occupancy, we selected 10 representative targets to validate through ChIP-qPCR: ABCA3, CACNG4, CHMP3, FLCN, GON7, HYAL3, MGMT, NEU1, STX4, and ZCWPW1. These genes were chosen based on their presence in combined gene sets and biological relevance, with most representing lysosomal or exocytosis-related functions and a few reflecting DNA repair, RNA processing, or methylation. In PRMT1-FLAG and PRMT6-FLAG overexpressing Du145 cells, ChIP-qPCR analysis confirmed promoter enrichment at multiple target genes (**Figure 1.38**). These findings

were validated in Panc1 (**Figure 1.39**) and H1299 (**Figure 1.40**) cells, confirming that PRMT occupancy at key regulatory gene loci is conserved across models.

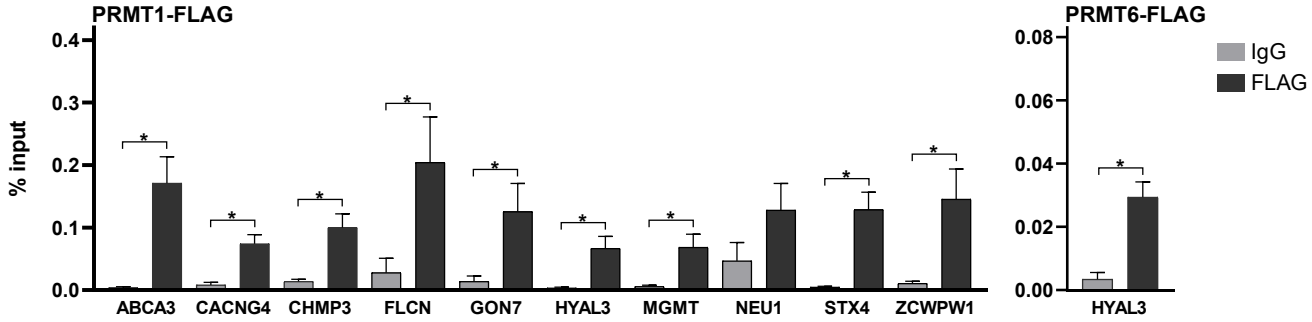



**Figure 1.38. ChIP-qPCR confirms direct binding of PRMT1 and PRMT6 at the promoter of target genes in Du145 cells.** ChIP-qPCR in Du145 cells with FLAG-tagged PRMT1 or PRMT6 revealed significant enrichment at promoters of key DEGs, supporting their direct regulatory roles. Du145 cells expressing PRMT6-FLAG or PRMT1-FLAG were used to measure promoter occupancy. Error bars indicate standard error of the mean (SEM) from three independent biological replicates done in duplicate (*p<0.05, Mann–Whitney U test).

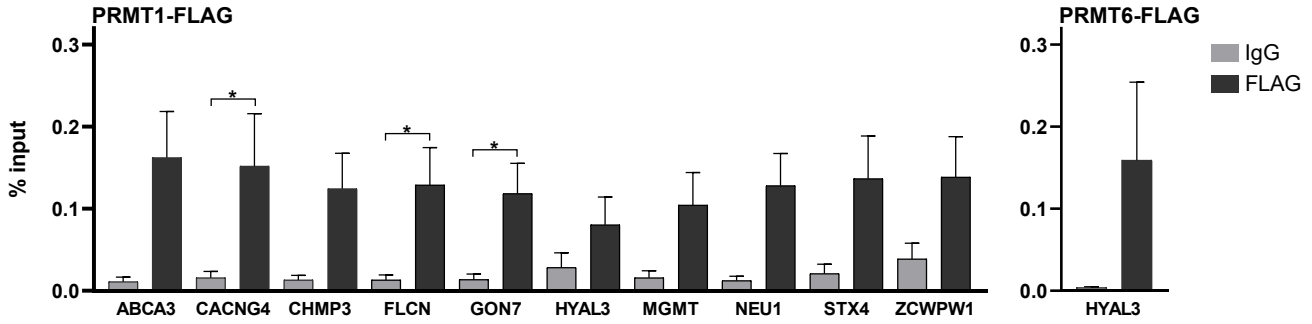



**Figure 1.39. ChIP-qPCR analysis confirm that promoter regions of selected genes found in combined gene sets are occupied by PRMT1 and PRMT6 in Panc1 cells.** ChIP-qPCR in Panc1 cells with FLAG-tagged PRMT1 or PRMT6 revealed significant enrichment at promoters of key DEGs, supporting their direct regulatory roles. Panc1 cells expressing PRMT6-FLAG or PRMT1-FLAG were used to measure promoter occupancy. Error bars indicate standard error of the mean (SEM) from three independent biological replicates done in duplicate (*p<0.05, Mann–Whitney U test).

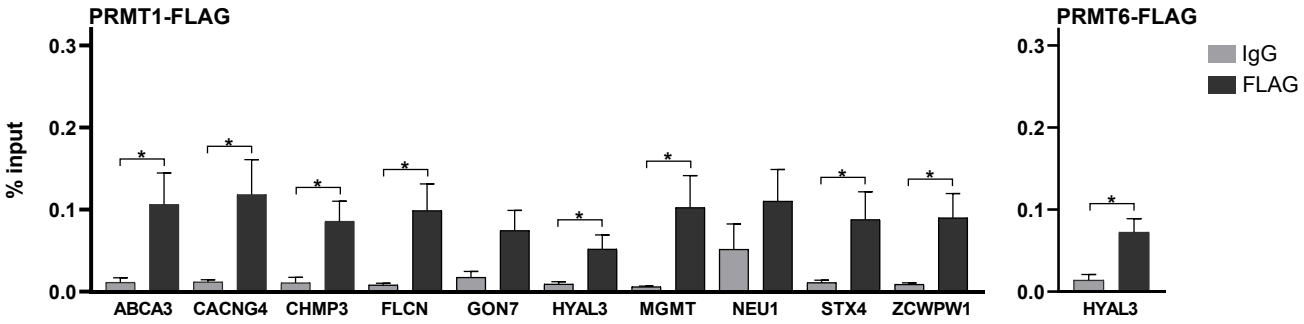



**Figure 1.40. ChIP-qPCR analysis confirm that promoter regions of selected genes found in combined gene sets are occupied by PRMT1 and PRMT6 in H1299 cells.** ChIP-qPCR in H1299 cells with FLAG-tagged PRMT1 or PRMT6 revealed significant enrichment at promoters of key DEGs, supporting their direct regulatory roles. H1299 cells expressing PRMT6-FLAG or PRMT1-FLAG were used to measure promoter occupancy. Error bars indicate standard error of the mean (SEM) from three independent biological replicates done in duplicate (*$p<0.05$, Mann–Whitney U test).

Collectively, these findings provide compelling evidence that PRMT1 and PRMT6 directly bind and transcriptionally regulate a defined set of genes involved in lysosomal function, exocytosis, and chromatin-based genome maintenance. By intersecting RNA-seq-derived DEGs with ChIP-seq promoter occupancy data, we were able to refine our candidate list to a biologically meaningful subset of 89 genes with both transcriptional responsiveness and PRMT promoter binding. Among these, a panel of 10 representative genes spanning lysosomal biogenesis, membrane trafficking, DNA repair, RNA processing, and methylation pathways were selected for targeted validation by ChIP-qPCR across three cancer cell models. The consistent enrichment of PRMT1 and PRMT6 at the promoter regions of these genes in Du145, Panc1, and H1299 cells confirms that their regulation is not incidental or cell line–specific, but likely represents a conserved epigenetic mechanism. These genes—many of which are implicated in lysosomal degradation, vesicle docking, and genome surveillance—likely contribute to the functional phenotypes we observed in earlier assays, including lysosomal accumulation and enhanced chemotherapy efficacy. These results establish a direct mechanistic link between PRMT1/6-mediated epigenetic control and the transcriptional regulation of gene networks governing lysosomal dynamics, exocytosis, and DNA repair, thereby reinforcing the rationale for targeting PRMTs to disrupt resistance mechanisms in cancer.

### 3.6. Patient-derived gene expression data links PRMT1/6 overexpression to poor chemotherapy response

To evaluate the potential clinical relevance of our findings, we next examined publicly available chemotherapy response datasets using the CTR-DB platform. Specifically, we investigated whether expression levels of PRMT1 and PRMT6 correlate with patient responses to chemotherapeutic agents that are known to undergo lysosomal sequestration—a process that may contribute to treatment resistance and is functionally linked to lysosomal dynamics regulated by PRMT1/6 in our study. Out of hundreds of datasets available, we prioritized a curated subset in which the primary treatment regimens involved carboplatin, imatinib, sorafenib, or bevacizumab—all of which are reported to undergo lysosomal sequestration (Burger et al., 2015; Chapuy et al., 2009; Colombo et al., 2014; Karpinska et al., 2023).

**Figure 1.41** presents gene expression comparisons for PRMT1 across five independent studies involving diverse cancer types and treatment contexts. In each case, PRMT1 expression levels were significantly higher in non-responders compared to responders, with positive $\log_2$ fold changes and p-values below significance thresholds. For example, in head and neck cancer patients treated with carboplatin + paclitaxel, PRMT1 expression was markedly elevated in non-responders (LogFC = 1.1; AUC = 0.88). Similar trends were observed in cohorts treated with imatinib (leukemia, chronic myeloid leukemia), sorafenib (NSCLC, hepatocellular carcinoma), and in every case, non-response was associated with higher PRMT1 expression.

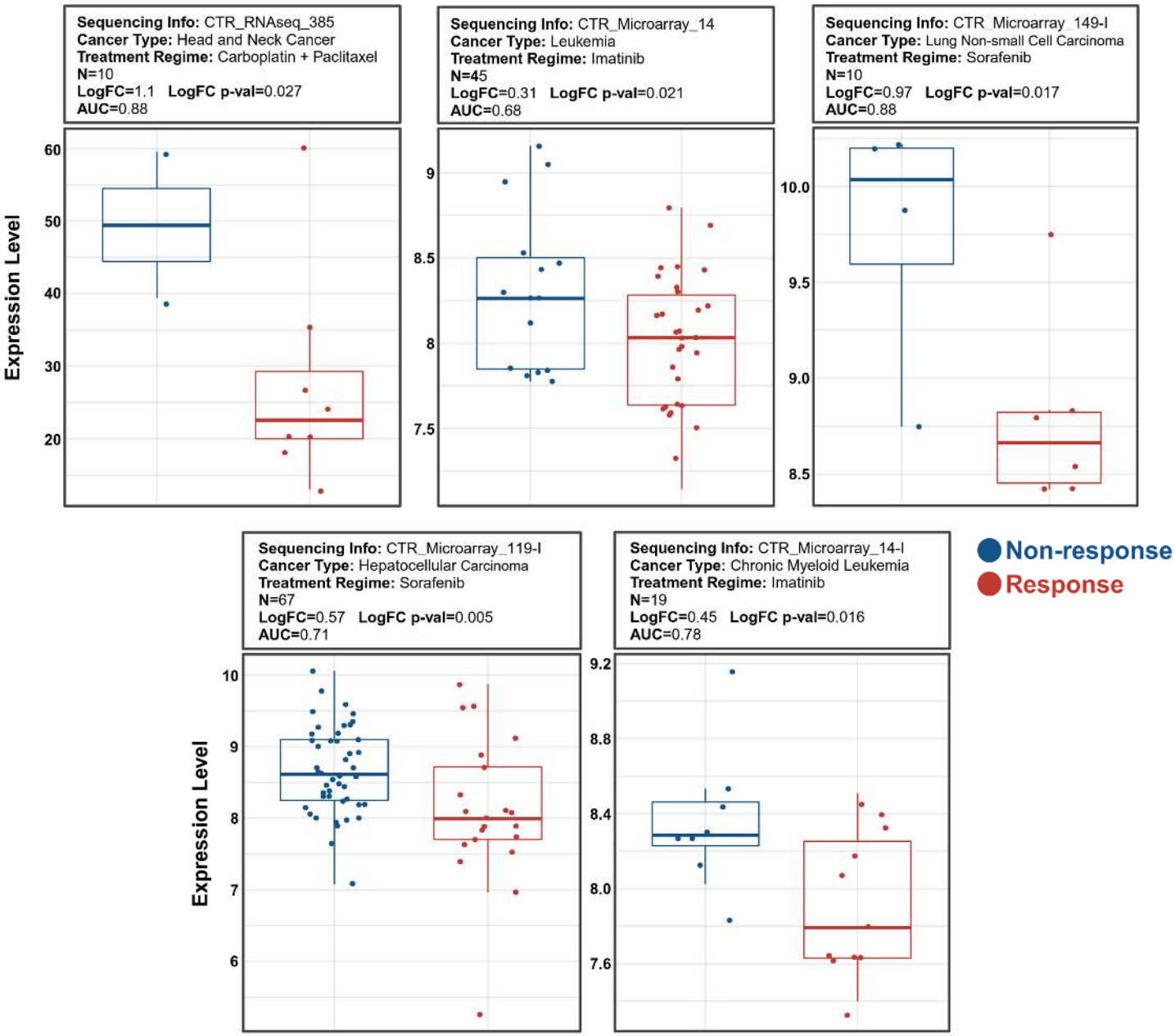



**Figure 1.41. High PRMT1 expression correlates with no response to chemotherapy regime.** Expression levels of PRMT1 varies in patients classified as responsive (red) or non-responsive (blue) to various chemotherapy regimens. Patient data were retrieved from the Cancer Treatment Response Gene Signature Database (CTR-DB). The specific cancer types, chemotherapy regimens, sample numbers (N), fold-change values (LogFC), statistical significance (p-values), and area under the curve (AUC) values are indicated for each comparison.

**Figure 1.42** shows a parallel analysis for PRMT6, again revealing consistent expression elevation in non-responders across two additional datasets. In glioblastoma patients treated with bevacizumab + irinotecan and colorectal cancer patients treated with bevacizumab, PRMT6 levels were significantly higher in those who failed to respond to

therapy (LogFC = 1.60 and 0.73, AUC = 0.82 and 0.89, respectively). These results align with our earlier in vitro findings showing that PRMT1 and PRMT6 modulate lysosomal dynamics and impact drug sensitivity through epigenetic regulation of trafficking-related genes.

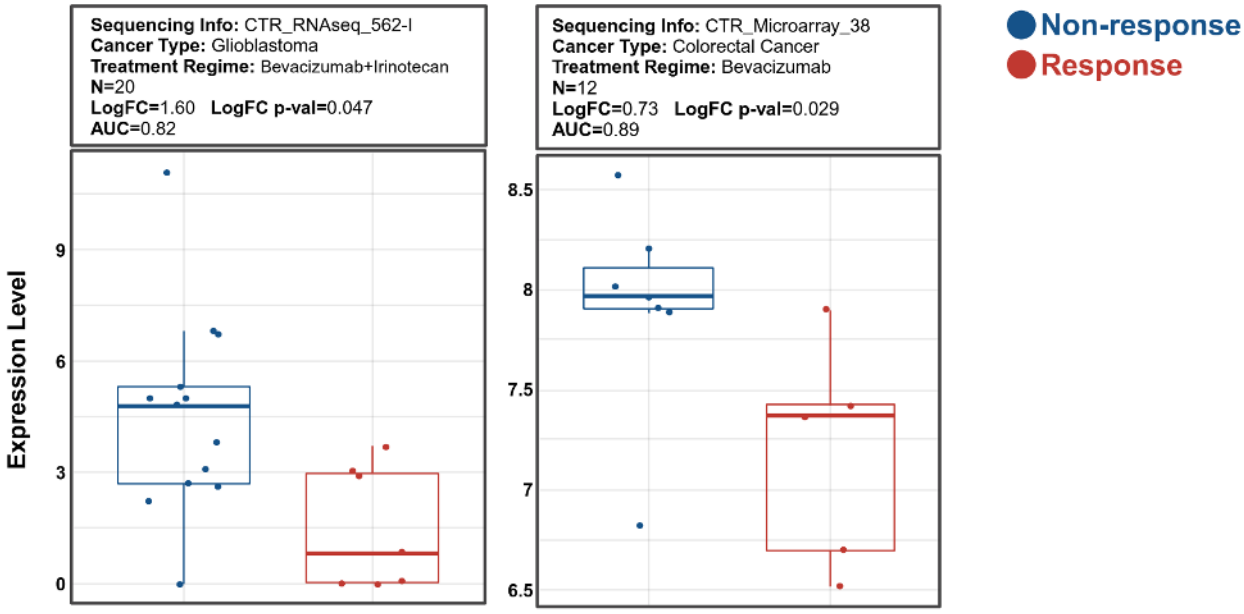



**Figure 1.42. High PRMT6 expression levels correlate with patient response to chemotherapy treatment.** Box plots illustrate differences in PRMT6 expression levels between two groups, indicating higher PRMT6 expression in non-responsive patients. Patient data were retrieved from the Cancer Treatment Response Gene Signature Database (CTR-DB). The specific cancer types, chemotherapy regimens, sample numbers (N), fold-change values (LogFC), statistical significance (p-values), and area under the curve (AUC) values are indicated for each comparison.

Together, this cross-analysis of patient-derived expression profiles and drug response data provides compelling support that elevated expression of PRMT1 and PRMT6 is consistently associated with poor response to chemotherapeutics known to undergo lysosomal sequestration. This trend, observed across diverse cancer types and treatment regimens, reinforces the idea that PRMT1/6 activity may influence lysosomal trafficking and thereby alter drug sensitivity in clinical contexts. However, it is important to emphasize that these observations, while reproducible, do not independently establish PRMT1 or PRMT6 as predictive determinants of treatment response. The complexity of chemotherapy response likely involves a broad spectrum of molecular alterations beyond PRMT overexpression. Nevertheless, the recurrence of elevated PRMT1/6 expression across multiple independent studies suggests that these enzymes may exert a distinct and

measurable impact on therapeutic outcomes in a subset of patients. These results underscore the potential clinical relevance of PRMT-regulated lysosomal dynamics and warrant further mechanistic and translational investigation in future studies.

*Chapter 1*

*Section 4*

# 4. DISCUSSION

Lysosomes, long viewed as mere degradative compartments of the cell, have in recent years emerged as dynamic organelles with crucial roles in nutrient sensing, membrane repair, cell survival, and intercellular communication. One of the most striking manifestations of lysosomal activity beyond catabolism is lysosomal exocytosis—a calcium-dependent process whereby lysosomes fuse with the plasma membrane, releasing their contents into the extracellular space and simultaneously contributing to membrane remodeling. This process, initially recognized in specialized secretory cells, has now been observed in a broad range of cancer cell types, where it appears to play a pivotal role in tumor adaptation and therapy evasion.

In the context of cancer, lysosomal exocytosis contributes to chemotherapy inefficacy by enabling the sequestration and removal of cytotoxic agents. Upon exposure to chemotherapeutic drugs, particularly hydrophobic weak bases and platinum-based compounds, cancer cells often increase lysosomal biogenesis and trap the drugs within acidic vesicles. These drug-loaded lysosomes are then shuttled toward the cell periphery and expelled via exocytosis, reducing intracellular drug concentrations and limiting therapeutic efficacy.

While lysosomal sequestration and exocytosis have been implicated in chemotherapy failure, the regulatory mechanisms underlying these processes—particularly at the level of chromatin and epigenetic control—remain poorly defined. Most current studies focus on canonical transcriptional pathways or vesicle trafficking machinery, leaving a significant gap in understanding of how lysosomal dynamics are transcriptionally and epigenetically programmed in cancer cells. Given the increasing success of epigenetic drugs (epidrugs) in modulating gene expression without altering DNA sequence, it is plausible that certain epidrugs might target lysosomal functions either directly or indirectly, with therapeutic consequences. Yet, this hypothesis has not been systematically tested in the context of lysosomal exocytosis or biogenesis.

This thesis was built upon the premise that modulating lysosomal flux through epigenetic means could offer a new strategy to overcome chemotherapy resistance. By targeting the upstream regulatory landscape that controls lysosomal behavior, we aimed to uncover novel druggable axes that re-sensitize cancer cells to conventional therapies. To this end, a multidisciplinary platform was developed that combines high-content functional screening, quantitative imaging, and transcriptomic profiling, enabling the identification of epidrugs capable of disrupting lysosomal dynamics in a mechanistically informative manner. The broader vision of this study is not only to catalog such compounds but also to understand how they exert their effects at the molecular level, ultimately informing rational combination strategies with chemotherapeutics like cisplatin.

The hypothesis of this project is two-step. **First**, it is proposed that the epigenetic drug library contains compounds that can modulate lysosomal exocytosis and biogenesis either through chromatin-based regulation or through other mechanisms. These modulatory compounds can be identified through robust, high-throughput functional screening approaches across multiple cancer cell models. Upon identifying effective modulators, downstream transcriptomic analyses will allow us to determine whether these compounds exert their influence through epigenetic regulation of lysosomal gene networks. Such findings would provide mechanistic insight into the chromatin-level control of lysosomal flux and potentially identify key components of lysosomal biogenesis and exocytosis pathways under epigenetic control. We further hypothesize that drug tolerant phenotype, especially to platinum-based compounds, may be associated with concurrent transcriptional shifts and lysosomal remodeling, and that interrupting this axis may exhibit sensitivity.

**Second**, we hypothesize that epidrugs that inhibit lysosomal flux can be leveraged to enhance the therapeutic efficacy of frontline chemotherapy agents. Since lysosomal trafficking and exocytosis are key contributors to drug detoxification and tolerance, pharmacological disruption of these processes may synergize with standard-of-care drugs by increasing their intracellular accumulation, on-site mehanism of action, and apoptotic potential. Thus, such epidrugs could serve as effective combination partners or adjuvants. In addition, genes or proteins whose expression or stability is significantly altered by

these compounds may themselves serve as targets for therapeutic intervention, providing additional layers of translational relevance.

To identify a relevant cancer model in which lysosomal exocytosis contributes to drug tolerance, an initial screening was performed across a panel of solid tumor cell lines. This survey revealed a marked heterogeneity in lysosomal volume and exocytosis activity among different lines. Based on its robust lysosomal signal and moderate exocytosis capacity, Du145 prostate cancer cells were selected as the primary model for downstream assays. In addition of Du145, other cell lines including Panc1, MIA-PaCa-2, H1299, and U373 cells also demonstrated measurable activity and were retained as secondary models for confirmatory experiments. Importantly, an inverse correlation emerged between the abundance of acidic vesicles and the level of lysosomal exocytosis. Cells with high lysosomal volume tended to exhibit reduced exocytosis, whereas those with lower vesicle load demonstrated greater extracellular β-hexosaminidase release. This observation suggested that lysosomal abundance alone does not dictate flux; rather, the regulation of lysosomal trafficking and membrane fusion likely contributes to the functional output of the lysosomal compartment. Beyond these pathways, vesicle turnover can be shaped by multiple factors, including the rate at which lysosomes fuse with autophagosomes, the speed at which late endosomes mature into fully functional lysosomes, and the recycling of vesicular membranes back into the endosomal network (Ju et al., 2020; Amaravadi et al., 2019; Passaro et al., 2021). Such processes may replenish or deplete the acidic vesicle pool independently of exocytic activity. Additional layers of regulation are provided by nutrient-sensing pathways, calcium signaling cascades, and cytoskeletal transport systems, all of which can influence the readiness of lysosomes to fuse with the plasma membrane without necessarily changing their overall abundance. In certain stress conditions or adaptive cellular states, it is also possible for lysosomal content and exocytosis to rise in parallel, particularly when biogenesis and fusion are both upregulated. Taken together, these considerations indicate that the relationship between lysosomal abundance and exocytosis should be viewed within the context of a broader, highly integrated vesicular trafficking network, rather than as a simple, direct inverse correlation.

Having established a suitable model, a focused screen using an epigenetic compound library was conducted to uncover modulators of lysosomal exocytosis. Among 175

agents, several compounds reduced lysosomal exocytosis without inducing cytotoxicity, thereby pointing to a functional inhibition of lysosomal trafficking rather than nonspecific cell damage. Notably, two inhibitors - targeting epigenetic regulators of type I PRMTs - emerged as particularly potent: they suppressed exocytosis, induced intracellular lysosomal accumulation, and simultaneously enhanced the cytotoxic response to cisplatin. The enhancement was not due to their own cytotoxicity, as both agents had minimal impact on viability even at the highest doses administered. Instead, their presence appeared to sensitize cells to platinum-based chemotherapy, implying a mechanistic synergy rather than additive toxicity. Colony formation assays confirmed that this sensitization translated into long-term proliferative disadvantage, supporting the idea that lysosomal inhibition can augment chemotherapy efficacy.

Furthermore, to investigate whether the accumulation of acidic vesicles induced by these compounds stemmed from enhanced biogenesis or impaired clearance, lysosomal content was assessed using LysoTracker staining. The treated cells consistently showed increased total cellular lysosomal content, yet the expression of master transcriptional regulators of lysosomal function—TFEB and MITF—remained unchanged at the mRNA level. Similarly, canonical lysosomal genes did not exhibit a uniform pattern of alteration in expression. This disconnect between lysosomal accumulation and lysosomal gene expression suggested that the observed phenotype was not transcriptionally driven through TFEB/MITF pathways. Instead, the inhibitors appeared to act through a non-canonical mechanism that enhanced lysosomal buildup independently of traditional lysosomal biogenesis programs. This uncoupling provided the first indication that lysosomal dynamics could be modulated post-transcriptionally or epigenetically without activating classical transcriptional regulators.

Although TFEB and MITF transcript levels remained unchanged, these transcription factors are predominantly regulated through post-translational modification cycles (e.g. phosphorylation–dephosphorylationT) that determine their subcellular localization and functional activity. In their phosphorylated state, TFEB retains within the cytoplasm, whereas dephosphorylation promotes nuclear translocation and the initiation of lysosomal and autophagy gene expression programs (Chen et. al., 2024; Franco-Juarez et. al., 2022; Medina et. al., 2015). In this study, the activation status and nuclear localization of TFEB and MiTF were not examined, leaving open the possibility that Type

I PRMT inhibition could influence these factors through post-translational modifications rather than through transcriptional regulation. Given the central role of such modifications in lysosomal gene regulation, future investigations should assess the phosphorylation state and intracellular localization of these transcription factors under inhibitor treatment. Such analyses could help determine whether PRMT-mediated epigenetic changes intersect with cytoplasmic signaling pathways to fine-tune lysosomal dynamics, providing a potential mechanistic link between chromatin-level modulation and vesicular trafficking control.

To gain a comprehensive understanding of how these compounds exerted their effects, RNA sequencing was performed following treatment. Differential gene expression analysis revealed broad transcriptomic changes, encompassing both upregulated and downregulated gene sets. Genes involved in vesicle-mediated transport, reactive oxygen species metabolism, and extrinsic apoptotic signaling were positively enriched, while those associated with DNA repair, methylation, and chromatin organization were significantly repressed. These patterns implied a dual action of the compounds: on one hand, priming cells for cell death and oxidative stress responses; on the other, suppressing epigenetic maintenance and DNA repair programs that often underpin therapy resistance. Thus, the inhibitors appeared to reshape the transcriptomic landscape in a way that simultaneously sensitized cells to external stressors and undermined their internal resilience mechanisms. To probe the epigenetic basis of these transcriptional changes, ChIP-seq data for the epigenetic regulators targeted by the compounds were integrated with the RNA-seq results. This analysis confirmed that many of the differentially expressed genes - particularly those involved in lysosomal exocytosis, lysosomes, sequestration, DNA repair, methylation, and RNA processing - were directly bound by the epigenetic regulators investigated PRMT1 and PRMT6. These findings provided mechanistic validation that the inhibitors did not only cause downstream effects but actively reprogrammed gene expression through direct epigenetic targeting. Importantly, this regulation extended beyond classical lysosomal genes, encompassing broader transcriptional programs that collectively orchestrate lysosomal behavior and cellular responses to given chemotherapeutics.

To assess the translational relevance of our mechanistic findings, publicly available gene expression datasets from the Cancer Treatment Response Gene Signature Database

(CTR-DB) were analyzed. These datasets covered diverse cancer types and treatment regimens that involved chemotherapeutic agents known to undergo lysosomal sequestration, including carboplatin, imatinib, sorafenib, and bevacizumab. Across multiple independent cohorts, higher expression of the epigenetic regulators identified in this study was consistently associated with poor treatment response. Specifically, non-responder groups exhibited elevated baseline expression compared to responders, as observed in head and neck, colorectal, hepatocellular, glioblastoma, and leukemia cohorts. These consistent trends—reflected in significant fold-change values and high area-under-curve scores—suggest that the regulators implicated in lysosomal and epigenetic remodeling may influence clinical drug sensitivity in settings where lysosomal sequestration is known to occur. While these associations do not alone establish predictive biomarker status, they highlight a convergent relationship between elevated expression of the candidate regulators and resistance to lysosome-targeted chemotherapies in patients, thus reinforcing their clinical and mechanistic relevance.

The findings presented thus far reveal that specific epigenetic regulators exert a measurable impact on lysosomal exocytosis and intracellular lysosome accumulation, ultimately altering the cellular response to chemotherapeutic agents. Through systematic screening, functional assays, and transcriptional profiling, a subset of chromatin-modifying enzymes emerged as central modulators of these processes. This convergence prompted a deeper inquiry into whether such enzymes have previously been implicated in the regulation of lysosomal biology. To address this, it is essential to consider the existing literature on protein arginine methyltransferases, particularly in relation to their established functions in chromatin dynamics and their potential, yet largely unexplored, roles in lysosomal regulation within cancer cells.

A growing body of literature points to important roles for protein arginine methyltransferases (PRMTs) in regulating lysosome-related processes, including autophagic flux, vesicle trafficking, and lysosomal biogenesis. Despite these advances, no prior studies have shown that PRMTs directly regulate lysosomal exocytosis. The current study is the first to demonstrate that pharmacological inhibition of Type I PRMTs suppresses lysosomal exocytosis in cancer cells. This finding introduces a new conceptual link between PRMT activity and the final step of the lysosomal lifecycle—fusion with

the plasma membrane and the release of lysosomal contents outside the cell—an area that had not been explored in the context of arginine methylation.

Of the Type I PRMTs, PRMT1 has been the most thoroughly investigated in relation to lysosomal function. A mechanistic study of canonical Wnt signaling revealed that PRMT1 methylates G3BP1, a stress granule-associated RNA-binding protein. This modification is necessary for the internalization and sorting of Wnt co-receptors into multivesicular bodies (MVBs), which are then delivered to lysosomes through microautophagy (Rhoades et al., 2019). In response to Wnt stimulation, arginine-methylated proteins rapidly accumulate in LAMP1-positive compartments, and PRMT1 activity is required for their efficient sequestration. Loss of PRMT1 or expression of a non-methylatable G3BP1 mutant disrupted the incorporation of signaling proteins into intraluminal vesicles and impaired downstream β-catenin activation. Although that study did not evaluate lysosomal exocytosis, it clearly showed that PRMT1 controls how cargo is sorted and delivered to lysosomes, which may influence the content and behavior of lysosomes, including their capacity to undergo secretion. In addition, PRMT1 is known to methylate structural proteins like desmoplakin, which are involved in cytoskeletal organization. Since lysosome positioning and movement rely on cytoskeletal tracks, these modifications may indirectly affect lysosomal trafficking and fusion dynamics (Gross et al., 2016). While a direct role in lysosomal secretion had not been established previously, these findings highlight PRMT1 as a regulator of lysosomal trafficking and degradation—both important components of lysosomal dynamics.

CARM1 (PRMT4), another Type I PRMT, plays a key role in controlling the expression of autophagy and lysosome-related genes, especially under metabolic stress. In starved cells, CARM1 methylates the ATPase Pontin, promoting its interaction with the Tip60 acetyltransferase and the transcription factor FOXO3a (Deng et al., 2022). This complex binds to enhancers of autophagy genes such as LC3 and ATG5, increasing their transcription by enhancing chromatin accessibility and histone acetylation. CARM1 has also been reported to interact with TFEB, the master regulator of lysosomal biogenesis, during fasting, particularly in muscle tissue (Wang et al., 2018). These studies show that CARM1 increases lysosomal capacity at the transcriptional level by promoting the expression of genes involved in autophagy and lysosome formation. However, none of these works examined whether CARM1 plays a role in lysosomal fusion with the plasma

membrane or the release of lysosomal contents. While the current data confirm CARM1's importance in regulating the growth of the lysosomal network, this study is the first to suggest that inhibiting CARM1-containing PRMT activity may also affect lysosomal exocytosis, expanding our understanding of its functional scope.

PRMT6, another nuclear Type I methyltransferase, has been linked to lysosomal function through its role in autophagy regulation. In hepatocellular carcinoma cells, loss of PRMT6 was found to promote autophagy, especially under stress conditions like hypoxia or chemotherapy exposure (Puri & Singhvi, 2020). This effect occurs through PRMT6-mediated methylation of the co-chaperone BAG5. When BAG5 is methylated, it destabilizes HSC70, a chaperone that supports autophagy. In the absence of PRMT6, BAG5 remains unmethylated, allowing HSC70 to persist and drive autophagic activity. While the study did not assess lysosomal trafficking or secretion directly, the increase in autophagic flux suggests altered lysosomal function, including changes in their enzyme content and membrane composition—factors that can influence exocytic behavior. These findings position PRMT6 as a negative regulator of autophagy, acting through a post-translational mechanism that affects lysosome-dependent degradation. Yet, like PRMT1 and CARM1, there was no evidence before this study that PRMT6 could control lysosomal exocytosis. Our findings now raise the possibility that PRMT6 may also influence downstream lysosomal release events.

PRMT8 is a unique Type I PRMT with distinct structural and functional features. It is membrane-bound due to an N-terminal myristoylation motif and is mainly expressed in the brain. PRMT8 exhibits both arginine methyltransferase activity and phospholipase-like lipid-modifying activity (Jochmans et al., 2019; Melendi et al., 2020). While it has not been studied in the context of lysosomes, its enzymatic functions suggest it may play a role in regulating vesicle fusion and membrane behavior. PRMT8 generates phosphatidic acid (PA), a signaling lipid that promotes membrane curvature and fusion—two processes essential for vesicle dynamics. In neurons, PRMT8 is involved in dendritic branching and synapse maturation, both of which rely on actin remodeling and membrane plasticity. Since lysosomal exocytosis requires actin-based positioning and fusion-competent membrane domains, it is plausible that PRMT8 could affect these pathways, even though there is no experimental data yet to support such a link. As a membrane-

localized PRMT with dual enzymatic activity, PRMT8 may influence organelle interactions at the cell periphery, and this possibility warrants further study.

Although this study focuses on Type I PRMTs, it is important to briefly consider other PRMT family members. PRMT5 (Type II) and PRMT7 (Type III) have also been associated with autophagy and lysosomal gene expression. PRMT5 catalyzes symmetric dimethylation and has been shown to regulate autophagy and stress granule formation through the methylation of autophagy regulators and transcriptional machinery (Behmanesh et al., 2019). PRMT7 has been linked to TFEB activation in oxidative stress conditions, suggesting it may contribute to lysosomal gene transcription in response to stress (Lorenzoni et al., 2017). These findings indicate that arginine methylation across different PRMT subclasses contributes to lysosomal homeostasis. However, the inhibitors used in the present study selectively target Type I PRMTs, and the observed suppression of lysosomal exocytosis can be attributed to inhibition of this specific subclass. To date, there are no studies showing that either PRMT5 or PRMT7 affects lysosomal secretion. Thus, while other PRMTs help expand our understanding of methylation in lysosomal biology, the exocytotic block observed here likely results from loss of Type I PRMT activity.

Collectively, the literature paints a picture in which PRMTs influence multiple steps of lysosomal function through both transcriptional and post-translational mechanisms. These include control of autophagy initiation, regulation of cargo delivery to lysosomes, and modulation of lysosomal gene expression. However, one critical step in the lysosomal lifecycle—exocytosis—has remained largely unexplored. There are no prior reports of PRMTs affecting lysosomal fusion with the plasma membrane or secretion of lysosomal contents. Common assays used to study this process, such as LAMP1 surface expression or live imaging of lysosome–plasma membrane fusion, have not been applied in PRMT-focused studies. This leaves a gap in understanding how methylation-based signaling might govern lysosomal export.

This study fills a critical gap in the literature by providing the first evidence that inhibition of Type I PRMTs suppresses lysosomal exocytosis. This expands the known functional scope of arginine methylation, suggesting that PRMTs may regulate not only intracellular trafficking and degradation, but also the capacity of lysosomes to fuse with

the plasma membrane and release their contents. Although the specific substrates involved remain to be identified, existing literature offers plausible mechanisms. PRMTs may directly methylate trafficking components such as small GTPases, SNARE regulators, or tethering factors essential for lysosome–plasma membrane fusion. Alternatively, they may influence cytoskeletal adaptors and motors that control lysosomal positioning and calcium-dependent fusion readiness. PRMTs may also exert indirect effects through broader transcriptional and structural remodeling. For example, CARM1-driven expression of autophagy and lysosome genes could affect the number or maturity of lysosomes poised for exocytosis, while PRMT6-mediated suppression of autophagy may impact lysosomal content or membrane properties. Even PRMT8, though not studied in this context, could influence lysosomal membrane dynamics through its roles in lipid metabolism and actin remodeling. Thus, PRMTs may shape lysosomal exocytosis both through direct modification of trafficking machinery and through upstream regulatory pathways that affect lysosome structure and mobility.

While no direct evidence currently exists for Type I PRMT-mediated methylation of cytosolic proteins involved in vesicular transport and lysosomal exocytosis, it remains plausible that such modifications occur and contribute to the phenotype observed in this study. The absence of methylation on a critical trafficking protein could impair lysosomal positioning, docking, or fusion capacity, thereby reducing exocytic output. Such an effect could, in turn, help explain the observed synergy between Type I PRMT inhibitors and chemotherapeutic agents prone to lysosomal sequestration, as impaired exocytosis would limit drug efflux and increase intracellular retention. In this context, methylation-sensitive components of the trafficking machinery—whether small GTPases, actin–microtubule linkers, or calcium-sensing fusion proteins—represent attractive candidates for future investigation. Detailed proteomic analyses aimed at identifying PRMT-dependent methylation events in the cytosolic trafficking network will be required to validate these hypotheses and clarify whether direct post-translational modification of exocytic proteins represents a mechanistic link between PRMT activity and lysosomal drug handling.

The therapeutic implications are significant. Lysosomal exocytosis contributes to drug resistance and tumor invasion by enabling cancer cells to expel cytotoxic agents and remodel their extracellular environment. The ability of PRMT inhibitors to suppress this

process—without impairing overall lysosome biogenesis—suggests a novel epigenetic strategy to trap chemotherapeutic agents inside tumor cells. This dual function of PRMTs, linking gene regulation to vesicle export, reveals a previously unrecognized regulatory axis in cancer biology. By identifying PRMTs as modulators of lysosomal secretion, this study introduces a new therapeutic entry point for reprogramming lysosomal behavior to overcome drug resistance. To summarize the literature knowledge and implying them to current ongoing work, while previous work has linked PRMTs to autophagy, gene expression, and lysosomal trafficking, their role in lysosomal exocytosis had not been explored. This study establishes a novel connection between Type I PRMT activity and the terminal secretory function of lysosomes, uncovering a new dimension in the epigenetic regulation of vesicle biology.

While the present study provides a systematic and mechanistically grounded investigation of epigenetic control over lysosomal exocytosis and chemotherapy response, several aspects need further exploration to deepen the biological insights and broaden the translational implications. First, although lysosomal exocytosis was functionally evaluated through enzymatic release and intracellular vesicle accumulation, the molecular machinery orchestrating lysosome–plasma membrane fusion remains poorly defined. Future studies integrating imaging-based platforms or fusion-specific surface markers would allow real-time visualization of lysosomal dynamics and help delineate the precise cellular mechanisms disrupted by epigenetic inhibitors.

Transcriptomic profiling in this study was strategically focused on Du145 cells treated with a single inhibitor to ensure depth and feasibility. While the top gene expression changes were independently validated in additional models and with a second compound, a more comprehensive analysis across multiple cell lines and epigenetic perturbations would better resolve shared versus context-specific transcriptional programs. Expanding the RNA-seq platform to include other models, such as Panc1 and H1299, could reveal conserved axes of epigenetic regulation underlying lysosomal remodeling in diverse cellular environments.

In parallel, the integration of publicly available ChIP-seq datasets with in-house RNA-seq results offered a cost-effective and informative strategy to probe the regulatory architecture governing the observed transcriptional shifts. This approach provided

evidence that several genes altered by epigenetic treatment are directly bound by the implicated regulators, supporting the link between chromatin control and functional output. Nonetheless, generating matched ChIP-seq or ChIP-qPCR data in the same drug-treated conditions would enable the study of dynamic changes in chromatin occupancy and establish a more direct mechanistic relationship between epigenetic inhibition and target gene regulation.

Further clarification of the epigenetic impact of the compounds could also be achieved through direct profiling of histone methylation marks. Given that the inhibitors used in this study target Type I protein arginine methyltransferases, follow-up experiments assessing site-specific changes in methylation—such as H4R3me2a or H3R2me2a—would offer valuable insight into how these enzymes shape chromatin landscapes in relation to lysosomal control.

In terms of translational relevance, the association between high expression of PRMT1/6 and poor response to lysosomally sequestered therapies across multiple cancer cohorts provides an encouraging starting point. Yet, these findings are currently associative. Future work in patient-derived models or prospective clinical datasets will be necessary to determine whether these epigenetic factors can serve as predictive biomarkers of treatment response or therapeutic targets in combination regimens.

In order to determine whether the cellular effects observed upon pharmacological inhibition of Type I PRMTs could be reproduced through genetic means, we performed shRNA-mediated knockdown of PRMT1 and PRMT6. These two enzymes were selected based on their known potency as targets of MS023 and GSK3368715, with both compounds exhibiting high inhibitory efficacy against PRMT1 and PRMT6. While PRMT8 was also identified as a potential target based on reported IC50 values, its expression was undetectable by qPCR in the Du145, Panc1, and H1299 cell lines used in this study, suggesting that it is either absent or expressed at levels too low to be functionally relevant. Upon stable knockdown of PRMT1 or PRMT6, we evaluated lysosomal exocytosis to assess whether the inhibitory phenotype seen with drug treatment could be genetically recapitulated. However, neither PRMT1 nor PRMT6 knockdown led to a reduction in lysosomal exocytosis compared to control shGFP-expressing cells (data not shown).

The lack of phenotypic overlap between pharmacological inhibition and genetic knockdown may be attributed to several factors. First, it is possible that residual PRMT activity remains after shRNA-mediated knockdown, and that this partial depletion is not sufficient to disrupt lysosomal exocytosis. In contrast, small-molecule inhibitors such as MS023 and GSK3368715 may achieve a more complete and acute inhibition of enzymatic function. Second, chemical inhibitors often target multiple Type I PRMTs simultaneously, which may produce a broader epigenetic effect than silencing a single gene. Therefore, the observed reduction in exocytosis with MS023 and GSK3368715 may require the concurrent inhibition of several PRMT family members. Lastly, the possibility of adaptive or compensatory mechanisms in the context of long-term PRMT knockdown cannot be ruled out. Cells may re-establish exocytosis dynamics through redundant pathways or transcriptional feedback that are not triggered under acute drug exposure. These differences highlight the complexity of functionally validating epigenetic drug targets and suggest that phenotypic responses may depend on the mode and extent of PRMT inhibition. Further studies using CRISPR-based knockouts or combinatorial knockdown approaches will be necessary to more precisely define the genetic contributions of PRMT1, PRMT6, and other Type I PRMTs in regulating lysosomal exocytosis.

Lastly, several transcriptional targets of the epigenetic inhibitors were identified as differentially expressed and ChIP-enriched, but their individual functional roles in lysosomal trafficking and drug response were not tested directly. Genetic perturbation studies using CRISPR interference, knockout, or overexpression systems will be important for clarifying which targets act as effectors of the lysosomal phenotype and contribute to chemotherapy sensitization.

Together, these directions chart a path for expanding the current study into a broader research program. By addressing these open questions through mechanistic, epigenomic, and translational extensions, future investigations will be well positioned to define the full therapeutic potential of targeting chromatin regulators to overcome lysosome-mediated chemotherapy tolerance.

*Chapter 1*

*Section 5*

# 5. CONCLUSION

Chemotherapy tolerance remains a central challenge in the treatment of solid tumors, often preceding the development of stable drug resistance. Among the emerging mechanisms that support this tolerant state, lysosomal sequestration and exocytosis have garnered increasing attention. These processes enable cancer cells to compartmentalize and actively expel chemotherapeutic agents, thereby lowering intracellular drug concentrations and evading cytotoxic effects. While the functional impact of lysosomal exocytosis has been acknowledged, its upstream regulatory mechanisms - particularly those governed by epigenetic control - have remained largely unexplored.

This thesis was built on the hypothesis that chromatin-modifying enzymes could modulate lysosomal dynamics and, in doing so, influence drug response. By systematically screening a library of epigenetic compounds, we identified regulators capable of suppressing lysosomal exocytosis and enhancing chemotherapy sensitivity in cancer cells. Transcriptomic analysis revealed that these compounds not only disrupted lysosomal flux but also reprogrammed gene expression in pathways related to apoptosis, vesicle transport, and chromatin maintenance.

Importantly, our findings uncovered a regulatory axis in which protein arginine methyltransferases (PRMTs) - previously studied for their roles in gene expression, DNA repair, and RNA processing - emerged as modulators of lysosomal behavior. This regulatory function had not been previously attributed to PRMTs, and its discovery represents a conceptual advance in the understanding of lysosome-driven chemotherapy tolerance. Integrative analysis further linked the transcriptional effects of PRMT inhibition to known binding targets, supporting a mechanistic relationship between epigenetic modulation and lysosomal function.

To assess the translational relevance of these findings, we analyzed clinical gene expression datasets and observed that high expression levels of the identified regulators correlated with poor response to lysosomally sequestered chemotherapies. While these

associations are preliminary and correlative, they support the relevance of PRMT-driven lysosomal control in patient outcomes.

In parallel, this study established a practical experimental workflow - combining phenotypic screening, transcriptomics, and data integration - that can serve as a model for future efforts to uncover mechanistic drivers of drug tolerance. This workflow is summarized in **Figure 1.43**, which provides a visual overview of the study's design and key outcomes.

In summary, this study establishes a mechanistic framework for understanding how epigenetic regulators influence lysosomal behavior in cancer cells and modulate their response to chemotherapy. By integrating high-throughput functional screening with transcriptomic profiling and regulatory analysis, the work identifies lysosomal exocytosis as an epigenetically tunable process that contributes to chemotherapeutic tolerance. Notably, beyond reaffirming the well-characterized roles of protein arginine methyltransferases in DNA repair, RNA processing, and chromatin remodeling, this study reveals their previously unrecognized regulatory impact on lysosomal dynamics, lysosomal exocytosis in particular. To our knowledge, this is the first demonstration that Type I PRMTs directly influence lysosomal exocytosis, a process increasingly understood to underlie the drug-tolerant phenotype in cancer.

These findings offer a conceptual advance in linking chromatin-level regulation to vesicular trafficking and drug handling within tumor cells. More importantly, they open the possibility of incorporating epigenetic modulators as rational adjuvants to enhance chemotherapy efficacy in cancers. By uncovering a non-canonical axis of lysosomal control and mapping its epigenetic underpinnings, this thesis lays the groundwork for the development of targeted combination strategies aimed at overcoming drug tolerance and improving clinical outcomes.

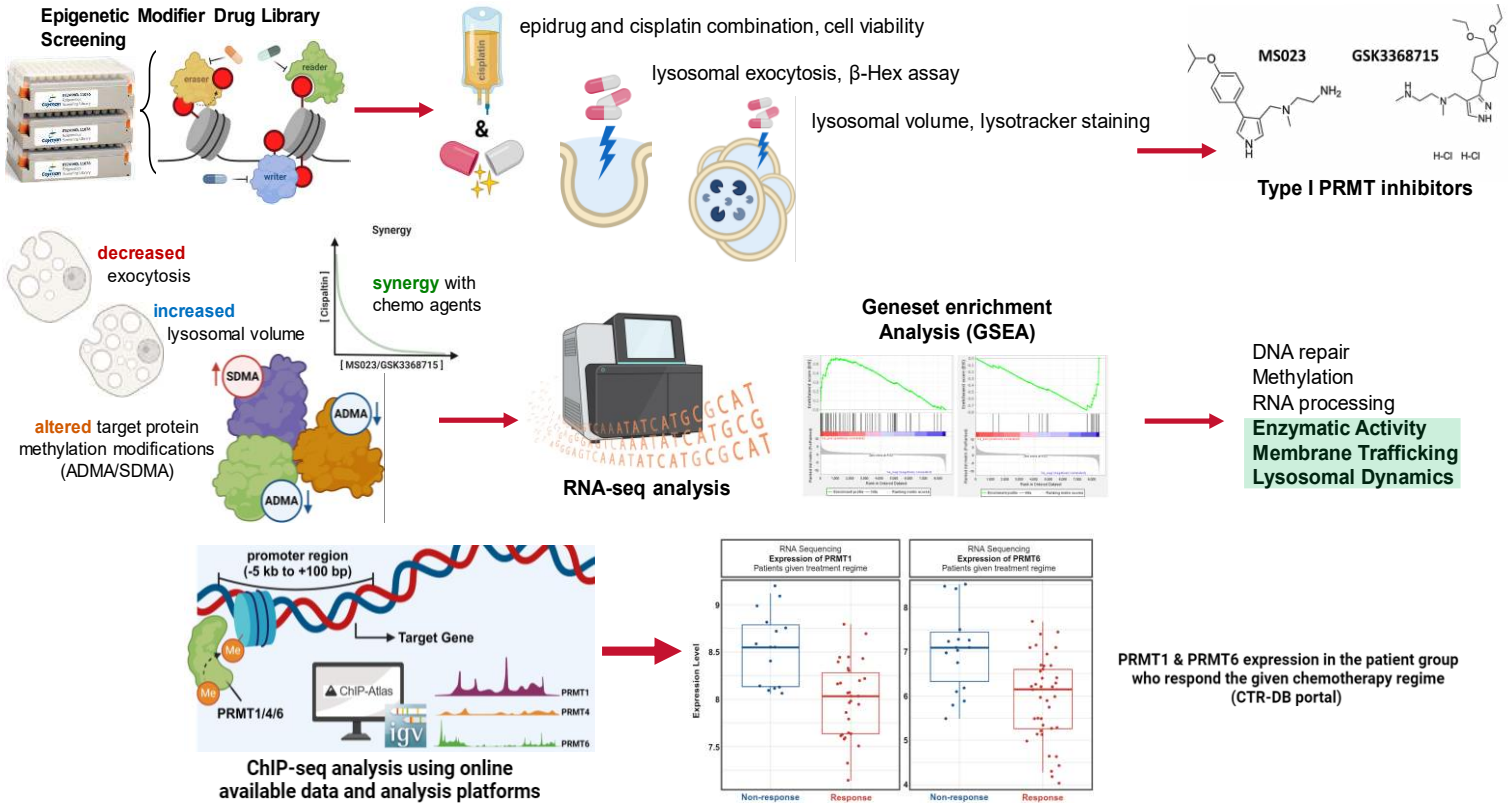



**Figure 1.43. Graphical abstract of the experimental flow.** The first chapter of this thesis investigates how targeting the epigenetic regulation of lysosomal exocytosis using epidrugs influences cellular function, with a focus on the downstream biological consequences of this inhibition. Experimental flow includes **(1)** use of epigenetic modifier drug (epidrug) library in **(2)** drug screening experiments for three different screening setups, **(3)** identification of hit epidrugs among hundreds of epidrugs in the library, **(4)** assessment of phenotypic changes in lysosomal dynamics and trafficking, investigation of changes in arginine methylation marks (ADMA, SDMA) and synergy between hit epidrugs with chemotherapeutic agents, **(5)** transcriptomic profiling using RNA sequencing to identify differentially expressed genes, **(6)** GSEA enrichment analysis and further investigation of enriched pathways, **(7)** ChIP-seq analysis using publicly available platforms (e.g., ChIP-Atlas) to identify target genes of PRMT1, PRMT4, and PRMT6 and integration of RNA-seq and ChIP-seq data, and **(8)** evaluation of PRMT1 and PRMT6 expression in clinical samples (via CTR-DB) to correlate expression with chemotherapy response.

*Chapter 2*

# EPIGENETIC MODIFIER DRUG LIBRARY SCREENING USING A KNOCK-IN REPORTER MODEL IDENTIFIES HDAC INHIBITORS AS ENHANCERS OF ATP7B EXPRESSION THROUGH SELECTIVE PROMOTER ACETYLATION

## SUMMARY

ATP7B is a copper-transporting ATPase that plays a vital role in maintaining copper homeostasis. Its expression must be tightly controlled: reduced ATP7B activity leads to Wilson's disease (WD), while elevated ATP7B in tumors contributes to resistance against platinum-based chemotherapy. While metal-sensing transcription factors involved in ATP7B regulation are known, the role of epigenetic mechanisms has not been systematically explored. This study aimed to identify chromatin-level regulators of ATP7B transcription and evaluate their mechanism of action.

To achieve this, a CRISPR/Cas9-based knock-in model was developed by inserting a firefly luciferase cassette into exon 1 of a single ATP7B allele in HEK293T cells. This monoallelic reporter preserved the gene's natural regulatory context, enabling quantitative assessment of promoter activity. An epigenetic modifier drug library of ~150 small molecules was screened at non-toxic doses to identify compounds that modulate ATP7B transcription. Hits were validated using endogenous ATP7B mRNA measurements, promoter reporter assays, ROS analysis, ChIP-qPCR, and cross-validation in Huh7 and Du145 cell lines.

Histone deacetylase (HDAC) inhibitors emerged as the most promising class of compounds that consistently increased both luciferase activity and native ATP7B expression. Five HDAC inhibitors—Chidamide, MS-275, KD5170, Pyroxamide, and PAOA—induced robust and dose-dependent transcriptional activation. Promoter reporter studies stated that 1 kb promoter fragment of ATP7B was sufficient to mediate this elevated response. Mechanistically, HDAC inhibition led to selective enrichment of H3K18ac, but not H3K9ac or H3K27ac, at the ATP7B promoter, pointing to a localized chromatin relaxation that facilitates transcription. Furthermore, this effect of increased

gene expression by epidrugs was not mediated by ROS elevation and were conserved across hepatic and non-hepatic cell types.

Overall, this work provides the first experimental evidence that ATP7B expression is regulated by a reversible chromatin regulatory mechanism. HDAC inhibitors relieve promoter repression by increasing H3K18 acetylation, establishing a functional link between chromatin state and gene expression. This knock-in model offers a valuable tool for studying epigenetic control at native gene loci and suggests potential therapeutic strategies for both Wilson's disease and chemotherapy resistance by targeting ATP7B transcription.

**Keywords:** ATP7B, Epidrug Library, Epigenetic Regulation, Knock-in Reporter System, HDAC Inhibition

*Chapter 2*

*Section 1*

# 1. INTRODUCTION

## 1.1. Copper Homeostasis and ATP7B Function in Health and Disease

Copper is an essential trace element required for a wide array of fundamental biochemical processes. It serves as a catalytic cofactor for enzymes involved in mitochondrial respiration, antioxidant defense, neurotransmitter synthesis, iron mobilization, and connective tissue formation (Chen et al., 2022; Linder & Hazegh-Azam, 1996; Linz & Lutsenko, 2007). For instance, cytochrome c oxidase, a key component of the electron transport chain, and Cu/Zn-superoxide dismutase, a major antioxidant enzyme, both rely on copper for proper function. Despite its biological necessity, copper is a redox-active metal that can generate harmful reactive oxygen species (ROS) when present in excess, leading to oxidative damage of proteins, lipids, and DNA (Chen et al., 2022). As such, copper must be maintained within a narrow concentration range through tight homeostatic regulation at both the systemic and cellular levels (Maung et al., 2021; Shim & Harris, 2003).

Systemic copper homeostasis begins with dietary absorption, which occurs primarily in the duodenum and upper small intestine. Copper is initially present as $Cu^{2+}$ and must be reduced to $Cu^{+}$ by metalloreductases such as STEAP and DCYTB to be imported into enterocytes via the high-affinity copper transporter CTR1 (SLC31A1) (Chen et al., 2022; Zhou & Gitschier, 1997). Once inside enterocytes, copper is either used locally or exported across the basolateral membrane into the portal circulation by ATP7A, a P-type ATPase expressed in intestinal epithelial cells (Chen et al., 2022; Shim & Harris, 2003). In circulation, copper binds to carrier proteins such as albumin, transcuprein, and amino acids and is delivered to the liver, the central organ of copper storage, distribution, and excretion (Chen et al., 2022).

Within hepatocytes, copper uptake is again mediated by CTR1. Once internalized, copper is escorted by chaperone proteins to specific intracellular destinations: ATOX1 delivers copper to ATP7B at the trans-Golgi network, COX17 targets mitochondria, and CCS delivers copper to cytosolic superoxide dismutase (Chen et al., 2022). ATP7B, the

liver-enriched copper-transporting ATPase, plays a dual role: under physiological copper levels, it facilitates the metallation of apoceruloplasmin in the Golgi, producing functional holo-ceruloplasmin that is secreted into plasma (Shim & Harris, 2003). When hepatic copper is elevated, ATP7B relocalizes to vesicles near the bile canalicular membrane, where it actively transports excess copper into bile for excretion (Bartee & Lutsenko, 2007; Chen et al., 2022). This bile-mediated excretion is the main pathway by which the body eliminates copper, with approximately 1 mg/day removed under normal dietary conditions.

ATP7B and its homolog ATP7A are the two primary P-type ATPases responsible for maintaining cellular copper balance (Bull & Cox, 1994; Linz & Lutsenko, 2007). While ATP7A is broadly expressed in tissues such as the brain, placenta, and intestinal mucosa and is essential for copper delivery to peripheral organs, ATP7B expression is highly enriched in the liver and plays a protective role by preventing copper overload (Chen et al., 2022). These transporters are not only similar in structure but also share a copper-responsive trafficking behavior, shifting from the trans-Golgi network to the plasma membrane or endolysosomal compartments in response to elevated intracellular copper levels.

The physiological importance of ATP7B is underscored by its causative role in Wilson's disease (WD), an autosomal recessive disorder characterized by impaired biliary copper excretion due to loss-of-function mutations in ATP7B (Bull et al., 1993; Czlonkowska et al., 2018). In the absence of functional ATP7B, copper accumulates in hepatocytes, leading to oxidative stress, mitochondrial damage, and hepatocellular injury (Chen et al., 2022; Huster, 2014). As hepatic copper-binding capacity is exceeded, excess copper is released into the bloodstream and deposited in extrahepatic organs, including the brain, kidneys, and corneas. Clinically, WD presents with hepatic dysfunction, neuropsychiatric symptoms, and characteristic Kayser–Fleischer rings in the cornea (Walshe, 2007). Biochemically, patients often show low serum ceruloplasmin and markedly elevated hepatic copper levels.

In contrast, mutations in ATP7A lead to Menkes disease, an X-linked recessive disorder of systemic copper deficiency (Tumer & Moller, 2010). ATP7A dysfunction results in copper entrapment within intestinal enterocytes, preventing copper distribution

to the brain and peripheral tissues. Affected infants suffer from severe neurodevelopmental delay, connective tissue abnormalities, seizures, and failure to thrive. The proximity of Menkes Disease (MNK) and WD highlights the dual toxicity of copper dysregulation: ATP7A deficiency causes copper starvation, while ATP7B deficiency leads to copper overload, each with devastating clinical outcomes (Czlonkowska et al., 2018; Shim & Harris, 2003; Tumer & Moller, 2010).

Beyond inherited disorders, ATP7B has also been implicated in acquired disease states, particularly in oncology. Elevated ATP7B expression has been observed in various cancers and is strongly associated with resistance to platinum-based chemotherapy agents such as cisplatin (Dmitriev, 2011; Komatsu et al., 2000; Mangala et al., 2009). Mechanistically, ATP7B appears to bind and export platinum drugs, reducing their nuclear accumulation and thereby limiting DNA damage and cytotoxicity. Experimental overexpression of ATP7B in carcinoma cells increases cisplatin resistance, while knockdown of ATP7B restores drug sensitivity. Clinically, high tumor ATP7B levels have correlated with poor response to platinum chemotherapy, suggesting that tumors may hijack copper transport pathways as a defense mechanism against cytotoxic agents (Dmitriev, 2011; Komatsu et al., 2000). These findings position ATP7B as not only a gatekeeper of copper homeostasis but also a critical modulator of chemotherapy efficacy in cancer.

Given these physiological and pathological roles, the regulation of ATP7B expression and activity is of central importance. Cells must produce enough ATP7B to manage copper export and cuproenzyme biosynthesis yet avoid overactivity that may compromise drug responses or disturb copper balance (Chen et al., 2022). Regulation occurs at multiple levels: transcriptional, post-transcriptional, and post-translational. Relocalization of ATP7B in response to copper levels is rapid and reversible, while transcriptional control may be governed by nutritional cues, hormonal signals, and possibly epigenetic modifications (Medici & LaSalle, 2019). Dysregulation of ATP7B—whether through genetic mutation, altered expression, or epigenetic silencing—can result in copper mismanagement with wide-ranging clinical consequences. Thus, understanding the nuanced regulation of ATP7B is a critical frontier in both copper biology and therapeutic modulation.

### 1.2. Transcriptional Control of ATP7B: Role of Copper and MTF1

The expression of ATP7B is tightly regulated in response to fluctuations in intracellular copper levels, reflecting the need to finely balance copper utilization and detoxification. One of the principal regulatory mechanisms involves a set of conserved cis-elements in the ATP7B promoter known as metal-responsive elements (MREs), which mediate copper-inducible gene transcription through the action of metal-sensing transcription factors. Among these, the most well-established regulator is metal-responsive transcription factor 1 (MTF1), a zinc-finger protein that translocates to the nucleus and binds MRE motifs under conditions of elevated metal availability (Fitisemanu & Padilla-Benavides, 2024).

Within the ATP7B promoter, a specific MRE known as MREe has been experimentally validated as a direct MTF1 binding site. Electrophoretic mobility shift assays (EMSAs) and reporter gene experiments have confirmed that MTF1 engagement at MREe significantly enhances ATP7B promoter activity, thereby driving transcriptional upregulation (Stalke et al., 2020). This response is physiologically relevant: in hepatocytes, rising copper levels trigger nuclear accumulation of MTF1, which in turn binds to MREe and activates ATP7B transcription (H. I. Chen et al., 2018). The functional consequence of this interaction has been demonstrated in a WD patient harboring a homozygous ATP7B promoter mutation (chr13:g.52,586,149T>C) that disrupts the MTF1-binding site. This patient exhibited a significantly blunted transcriptional response to copper, underscoring the critical role of the MTF1–MRE axis in copper-dependent ATP7B regulation.

Structurally, MREs are characterized by a core consensus motif (5′-TGCRCNC) followed by a GC-rich stretch, which confers binding specificity for MTF1 (Fitisemanu & Padilla-Benavides, 2024). While zinc is the canonical metal that activates MTF1, mammalian MTF1 also harbors a C-terminal cysteine-rich domain that responds to copper. Copper binding to this domain facilitates MTF1's nuclear localization and DNA-binding activity, allowing it to act as a copper-sensitive transcriptional switch for genes like ATP7B. This system ensures that ATP7B transcription is promptly increased under conditions of metal stress, enabling the cell to mobilize copper export machinery and prevent toxic accumulation.

In addition to MTF1, recent studies have identified β-catenin and TCF4, key components of the canonical Wnt signaling pathway, as additional transcriptional regulators of ATP7B. In cancer models, the β-catenin/TCF4 complex has been shown to bind directly to the ATP7B promoter and promote its transcription, suggesting that ATP7B may be subject to modulation by broader signaling cascades beyond metal sensing (Y. T. Liu et al., 2024). However, the physiological relevance of this pathway in hepatic copper homeostasis remains to be fully defined.

In contrast, other general metal-responsive transcription factors such as Sp1 and NF-κB—both of which are activated by copper or oxidative stress and regulate other metal-handling genes—have not been shown to directly regulate ATP7B. While Sp1 plays a role in the transcriptional control of CTR1 and ATP7A, no functional Sp1-binding sites have been identified in the ATP7B promoter (Fitisemanu & Padilla-Benavides, 2024). Similarly, despite NF-κB's sensitivity to copper-induced stress, there is no evidence for direct NF-κB regulation of ATP7B (McElwee et al., 2009).

Furthermore, HNF4α, a liver-enriched transcription factor involved in regulating hepatocyte-specific genes, is known to be suppressed by copper (Song & Freedman, 2011). Although this raises the possibility that copper may influence hepatic transcriptional networks more broadly, a direct regulatory link between HNF4α and ATP7B has yet to be demonstrated. Taken together, these observations highlight the MTF1–MRE pathway as the primary and most robust mechanism for copper-induced ATP7B expression, with emerging contributions from the β-catenin/TCF4 axis.

While the above findings have been firmly established in human cells, the extent to which these regulatory elements and mechanisms are conserved in other species, such as mouse or rat, remains an open question. Nonetheless, the evidence to date supports a model in which ATP7B transcription is selectively activated in response to intracellular copper accumulation via MTF1 binding to specific MREs in its promoter. This mechanism allows hepatocytes to dynamically adjust their copper-export capacity to maintain systemic metal balance and prevent toxicity (H. I. Chen et al., 2018; Stalke et al., 2020).

### 1.3. Hypomorphic Mutations and Promoter Variants Causing Wilson's Disease

WD is an autosomal recessive disorder caused by pathogenic variants in the ATP7B gene, which encodes a copper-transporting P-type ATPase essential for biliary copper excretion. Most WD mutations lead to complete loss of ATP7B function, but a significant proportion of identified variants are hypomorphic in nature—meaning they reduce but do not eliminate gene expression or protein activity (Weiss & Schilsky, 1993). These hypomorphic alleles represent an important subset of disease-causing mutations because they can result in milder or delayed disease onset, and they reveal the threshold sensitivity of copper homeostasis to changes in ATP7B dosage and function (de Bie et al., 2007; Fitisemanu & Padilla-Benavides, 2024; Nolan et al., 2021).

One of the clearest examples of a transcriptionally hypomorphic mutation is a 15-base pair deletion (c.–441_–427del) in the ATP7B promoter. This deletion is a founder mutation in the Sardinian population and is the most common WD allele in that region, present on approximately 92% of WD chromosomes associated with the major Sardinian haplotype (Loudianos et al., 1999). Functional assays have shown that this deletion removes critical transcription factor binding sites, leading to a ~75% reduction in ATP7B promoter activity and expression (Cullen et al., 2003). Remarkably, this reduction in mRNA output—despite an otherwise intact coding sequence—is sufficient to cause clinically manifest WD, indicating that substantial loss of ATP7B transcription alone can induce copper accumulation and disease symptoms.

Other promoter variants further support this concept. Two point mutations located at –215A>T and –133A>C in the proximal ATP7B promoter were identified in unrelated Asian patients with WD but were absent in healthy controls. Luciferase reporter assays demonstrated that these mutations reduce ATP7B promoter activity by ~50%, confirming their role in attenuating gene transcription (Hoflich et al., 2021). Additionally, a single nucleotide variant (SNV) at position –676T>C was found to disrupt a functional metal-responsive transcription factor 1 (MTF1) binding site. In HepG2 cells, this mutation impaired copper-induced transcriptional activation of ATP7B, reducing expression by approximately one-third (H. I. Chen et al., 2018). These findings highlight that the ATP7B promoter contains multiple regulatory elements, and their disruption by inherited variants can lead to reduced gene expression, even in the absence of coding defects.

In addition to promoter mutations, several coding region variants also behave as hypomorphic alleles. The most common WD mutation among individuals of European descent is p.His1069Gln (c.3207C>A), a missense variant that causes partial misfolding and retention of ATP7B in the endoplasmic reticulum (ER), yet allows residual copper transport activity (Huster et al., 2012). Patients homozygous for this variant typically present with neurological symptoms at a later age (around 21 years), often exhibiting higher ceruloplasmin levels and milder hepatic manifestations compared to individuals with more severe mutations (Mukherjee et al., 2014).

Another illustrative example is the c.1934T>G (p.Met645Arg) variant, frequently observed in Spanish and Latin American populations. While the amino acid substitution itself has minimal impact on copper transport function, the mutation primarily causes a splicing defect—leading to exon 6 skipping in approximately 70% of transcripts. The resulting frame-shifted mRNA is degraded or produces nonfunctional protein, but importantly, ~30% of transcripts are normally spliced and generate functional ATP7B (Merico et al., 2020). This “leaky” splicing mechanism results in a partial expression phenotype, classifying the variant as hypomorphic.

Other missense variants such as p.Met769Val and p.Arg778Leu have also demonstrated retained—but impaired—ATP7B function in biochemical assays (Huster et al., 2012). In many of these cases, the mechanism of dysfunction involves abnormal intracellular trafficking. For instance, H1069Q and similar mutants fail to reach the trans-Golgi or lysosomal compartments necessary for copper excretion, instead accumulating in the ER, which limits their physiological function despite preserved catalytic potential (Huster et al., 2012).

Emerging data also suggest that alternative splicing and isoform switching might provide limited compensation in some patients (Zhou et al., 2025). One case report described a patient with a homozygous coding deletion who increased expression of an alternative ATP7B isoform lacking exon 12, which retained ~80% of normal function and was associated with a milder disease phenotype (Hoflich et al., 2021). These findings underscore the complexity of ATP7B regulation at the transcriptional, post-transcriptional, and post-translational levels.

Clinically, the presence of at least one hypomorphic allele can shift the disease course toward later onset or milder symptoms, though genotype–phenotype correlations in WD remain variable (Mukherjee et al., 2014). Notably, even modest reductions in ATP7B expression—down to 25–30% of normal—can be sufficient to cause symptomatic disease when present on both alleles. This dose-sensitive effect supports the idea that restoring partial ATP7B function may be therapeutically meaningful in WD. It also highlights the utility of in vitro models that can sensitively report on promoter activity or copper transport capacity, aiding in both the classification of novel variants and the discovery of compounds that modulate ATP7B expression.

In summary, hypomorphic ATP7B mutations—including promoter deletions, transcription factor motif-disrupting SNVs, and missense or splicing variants with residual function—contribute significantly to the clinical and mechanistic landscape of WD. These mutations reveal that ATP7B dysfunction exists on a spectrum, and even partial deficits in expression or activity can disrupt copper homeostasis and lead to disease (Huster et al., 2012; Merico et al., 2020).

### 1.4. CRISPR-Based Knock-In Reporter Models for Studying Transcriptional Regulation

Transcriptional reporters have long been employed as tools to assess gene regulation, particularly in high-throughput screening settings. Classical reporter constructs typically rely on plasmids in which a minimal promoter drives the expression of a reporter gene such as luciferase or GFP. While useful, these systems are often limited in their ability to capture the full complexity of endogenous gene regulation, primarily because they exist outside the native chromatin environment and do not incorporate distal regulatory elements such as enhancers, silencers, or insulators (Rojas-Fernandez et al., 2015). In vivo, promoters are rarely regulated in isolation; rather, they are embedded within extended regulatory landscapes, and their transcriptional output is tightly modulated by dynamic chromatin states and three-dimensional genome architecture (Rojas-Fernandez et al., 2015). Consequently, reporter assays using episomal plasmids may fail to reflect how a gene is truly regulated within its genomic context.

CRISPR/Cas9-mediated knock-in reporter systems offer a powerful solution to this limitation by enabling the integration of reporter genes directly into the endogenous locus

of interest. In these systems, the reporter is inserted at a precise genomic position—usually at or near the transcriptional start site (TSS) of the target gene—so that it is controlled by the native promoter, regulatory elements, and chromatin context (Rojas-Fernandez et al., 2015). As a result, the reporter's expression reflects the real-time transcriptional activity of the gene under physiological or experimental conditions. This genome-integrated approach allows the reporter to respond to native transcription factors, chromatin modifications, and long-range enhancer interactions, thereby yielding a more accurate and biologically relevant readout of gene regulation.

The technical implementation of CRISPR knock-in reporters generally involves the use of Cas9 nuclease to introduce a targeted double-strand break at the desired locus, guided by a single-guide RNA (sgRNA) complementary to the target sequence. Homology-directed repair (HDR) is typically used to insert the reporter gene, which is flanked by homology arms matching the genomic target site. The donor cassette often includes luciferase or GFP sequences, along with a self-cleaving peptide (such as T2A or P2A) or an internal ribosome entry site (IRES), enabling bicistronic expression of both the endogenous gene and the reporter (He et al., 2016). For example, in a study by Rojas-Fernandez et al., a luciferase-IRES-GFP cassette was inserted at the start codon of the endogenous PAI-1 gene, allowing the reporter output to faithfully mirror TGFβ-induced expression of the gene (Rojas-Fernandez et al., 2015). This model outperformed traditional plasmid reporters, which could not fully recapitulate the endogenous response. In certain contexts, non-homologous end joining (NHEJ)-based strategies such as HITI (homology-independent targeted integration) may also be employed, especially when HDR is inefficient (He et al., 2016).

Depending on the experimental goals, reporters may be inserted at endogenous loci (e.g., under the control of the native promoter) or at so-called "safe harbor" sites such as the AAVS1 locus for synthetic constructs. While the latter enables consistent expression of designed transcriptional cassettes, only the former captures native regulatory features and is therefore more suitable for studying gene-specific regulation (X. Guo et al., 2024; Z. Li et al., 2018). For example, knock-in reporters have been developed at endogenous loci such as SREBP1, BMAL1, and PGRN, enabling researchers to directly monitor transcriptional activity in response to stimuli, small molecules, or epigenetic modifiers (Y. Li et al., 2019; Z. Li et al., 2018; Sun et al., 2022). In each case, luminescence or

fluorescence output correlated with endogenous mRNA levels, validating the fidelity of these models for real-time transcriptional readouts.

These systems have also been applied in diverse fields, including cancer biology, metabolism, immunology, and chronobiology. In a notable example, Li et al. developed a luciferase knock-in model under the control of the PGRN promoter in HEK293 cells, demonstrating that luciferase expression tracked endogenous gene activity upon stimulation with phorbol ester (PMA) or CRISPR activation systems (Y. Li et al., 2019). Similarly, in U2OS osteosarcoma cells, luciferase was inserted into the BMAL1 clock gene locus to study circadian regulation, yielding a reporter line sensitive to circadian disruptors and epigenetic perturbations (Sun et al., 2022). Importantly, these models enable not only descriptive studies but also mechanistic dissection of regulatory pathways. For instance, CRISPR knock-in of GFP at the CXCL9 locus allowed genome-wide screens to identify novel modulators of chemokine expression relevant to tumor immunology (House et al., 2023).

In addition to transcriptional readouts, CRISPR knock-in can facilitate epigenetic investigations. Because the reporter is embedded within native chromatin, it reflects changes in DNA methylation, histone modification, and transcription factor binding. The CETCh-seq method exemplifies this principle: Savic et al. inserted FLAG tags at endogenous transcription factor loci in HepG2 and MCF7 cells, enabling ChIP-seq of tagged factors without antibody limitations while preserving physiological expression patterns (Savic et al., 2015). Similarly, gene-trap approaches using knock-in reporters have been proposed for studying chromatin modifiers or enhancer activity in situ (Asakawa & Kawakami, 2009; Song & Cui, 2013; Trinh le & Fraser, 2013).

Collectively, CRISPR-based knock-in reporters have transformed the study of transcriptional regulation by allowing researchers to monitor gene expression within its full regulatory context. Compared to transient systems, these models offer superior accuracy, reproducibility, and compatibility with screening applications. They are particularly valuable for investigating genes whose regulation depends on epigenetic cues or distal enhancer interactions—an increasingly important consideration in cancer biology and functional genomics. As the field continues to expand, these knock-in platforms are expected to play a central role in the discovery of transcriptional and

epigenetic regulators, as well as in high-throughput screening for therapeutic compounds (Z. Li et al., 2018; Rojas-Fernandez et al., 2015; Sun et al., 2022).

### 1.5. Epigenetic Mechanisms in Gene Regulation and the Potential for Therapeutic Modulation

Epigenetic regulation encompasses heritable yet reversible modifications that influence gene expression without altering the DNA sequence itself. These mechanisms govern chromatin structure, modulate DNA accessibility, and ultimately determine whether specific genes are transcriptionally active or silent (Dawson & Kouzarides, 2012; Fritz et al., 2022; Kanherkar et al., 2014). In mammalian cells, the epigenetic landscape is shaped by interdependent processes, including DNA methylation, histone modifications, ATP-dependent chromatin remodeling, and non-coding RNA-mediated regulation. Together, these processes orchestrate developmental programs, sustain tissue-specific gene expression, and maintain genome stability (Fritz et al., 2022; Marei, 2025). Dysregulation of these epigenetic pathways is implicated in various pathological states such as cancer, neurodevelopmental syndromes, and metabolic disorders (Crispo et al., 2019; Jakovcevski & Akbarian, 2012; Marei, 2025).

DNA methylation, primarily at CpG dinucleotides, is a well-established epigenetic modification that typically represses gene expression. This process is catalyzed by DNA methyltransferases (DNMTs), with DNMT1 maintaining existing methylation patterns during DNA replication, and DNMT3A/B establishing new methylation marks during development. DNA methylation can repress transcription by recruiting methyl-binding proteins or by physically obstructing transcription factor access to DNA (ref). Aberrant methylation patterns are a hallmark of many cancers—hypermethylation of promoter CpG islands can silence tumor suppressor genes, while global hypomethylation may destabilize the genome and activate oncogenes (Baylin, 2005; Moore et al., 2013). Disrupted DNA methylation also underlies imprinting disorders, such as Beckwith–Wiedemann and Angelman syndromes, as well as neurodevelopmental dysfunctions (Moore et al., 2013; Paulsen & Ferguson-Smith, 2001).

Histone modifications are covalent post-translational modifications of histone tails that influence chromatin dynamics and gene expression. Acetylation of lysine residues, catalyzed by histone acetyltransferases (HATs), neutralizes the positive charge of

histones, weakening their interaction with DNA and promoting a more relaxed chromatin conformation conducive to transcription. In contrast, histone deacetylases (HDACs) remove acetyl groups, leading to chromatin compaction and transcriptional repression (Li & Seto, 2016). These enzymes also regulate non-histone substrates involved in cell cycle control and apoptosis, further broadening their functional relevance. Dysregulated HDAC activity has been linked to tumorigenesis through inappropriate silencing of tumor suppressor genes (Li & Seto, 2016).

Histone methylation represents another key regulatory layer, with diverse functional consequences depending on the residue modified and the degree of methylation. For instance, H3K4 trimethylation (H3K4me3) is generally associated with active promoters, whereas H3K27 trimethylation (H3K27me3), catalyzed by the Polycomb Repressive Complex 2 (PRC2) via EZH2, is linked to gene silencing (Wan & Chang, 2010). Aberrant histone methylation can drive disease; gain-of-function mutations in EZH2 in lymphomas, for example, result in excessive repression of tumor suppressors. Therapeutic agents such as tazemetostat target EZH2 to reverse this silencing and restore transcriptional activity (Julia & Salles, 2021; Morin et al., 2021).

ATP-dependent chromatin remodeling complexes, including SWI/SNF (BAF), ISWI, CHD, and INO80 families, use the energy from ATP hydrolysis to reposition or evict nucleosomes, thereby regulating access to DNA. These complexes collaborate with histone modifiers to dynamically shape enhancer and promoter architecture in response to environmental and developmental cues (Hao et al., 2025; Hota & Bruneau, 2016). Loss-of-function mutations in SWI/SNF subunits are frequent in many cancers, highlighting their role in maintaining cell identity and chromatin integrity (Hao et al., 2025).

Non-coding RNAs (ncRNAs), including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), regulate gene expression at multiple levels. miRNAs typically bind to complementary sequences in mRNAs to inhibit translation or promote degradation, while lncRNAs often act as scaffolds or guides for chromatin-modifying complexes. For instance, HOTAIR—a well-characterized lncRNA—binds to PRC2 and redirects it to the HOXD locus, leading to altered chromatin states and misregulation of gene expression

(Wan & Chang, 2010). Dysregulation of ncRNAs has been associated with diverse human diseases due to their widespread role in gene regulatory networks (Patil et al., 2014).

Given the central role of these epigenetic mechanisms in governing cell fate and function, they have emerged as compelling therapeutic targets. A growing class of "epidrugs" has been developed to modulate chromatin-modifying enzymes and restore appropriate transcriptional programs. For example, HDAC inhibitors such as vorinostat and romidepsin have been FDA-approved for treating cutaneous T-cell lymphoma, while belinostat and panobinostat have shown efficacy in peripheral T-cell lymphoma and multiple myeloma, respectively (Laubach et al., 2015; Lee et al., 2015; Mann et al., 2007; Prince et al., 2013). These agents promote histone acetylation and reactivation of silenced genes. DNA methylation inhibitors like azacitidine and decitabine reverse aberrant methylation by inhibiting DNMTs, thereby reactivating tumor suppressor gene expression in hematologic malignancies (Baylin, 2005; Kaminskas et al., 2005; Kim et al., 2022). EZH2 inhibitors, such as tazemetostat, have been approved for epithelioid sarcoma and are being explored in lymphomas (Horvath et al., 1990), while BET inhibitors (e.g., JQ1) disrupt acetyl-lysine readers like BRD4 to inhibit oncogenic transcriptional programs (Xu & Vakoc, 2017).

Importantly, the therapeutic potential of epidrugs extends beyond oncology. HDAC inhibitors are under investigation for neurodegenerative diseases, cardiac pathologies, and immune disorders, based on their ability to reprogram dysfunctioning transcriptional networks (Ganai et al., 2016; Sanchez-Fernandez et al., 2024; Shukla & Tekwani, 2020). Still, challenges such as achieving cell-type specificity and minimizing off-target effects remain central to the clinical development of epigenetic therapies (Didonna & Opal, 2015; Feehley et al., 2023; Moran et al., 2023).

Recent advances in epigenomic technologies, including ChIP-seq, ATAC-seq, and whole-genome bisulfite sequencing, have deepened our understanding of chromatin landscapes. Coupled with CRISPR-based epigenetic editing platforms—such as dCas9 fused to histone acetyltransferases or DNA demethylases—these tools now enable targeted manipulation of epigenetic marks at specific loci, offering unprecedented precision in modulating gene expression (Pulecio et al., 2017; Xu et al., 2016; Zhang et al., 2024).

In summary, epigenetic mechanisms provide a dynamic and reversible framework through which cells interpret environmental and developmental cues. As our mechanistic insights expand, and as tools for targeted chromatin modulation improve, the possibility of reprogramming gene expression to treat previously intractable diseases—including those involving ATP7B dysregulation—has become increasingly tangible.

### 1.6. Epigenetic Regulation of ATP7B: Current Gaps and Preliminary Evidence

Despite the well-established role of ATP7B in hepatic copper homeostasis and its clinical relevance in WD and platinum-based chemotherapy resistance, its epigenetic regulation remains surprisingly underexplored. While ATP7B transcription is known to respond to metal availability through transcription factors such as MTF1, much less is known about the chromatin-level and post-transcriptional mechanisms that might modulate its expression under physiological or pathological conditions.

To date, the most direct epigenetic regulators identified for ATP7B are non-coding RNAs. MicroRNA-mediated regulation is supported by multiple lines of evidence. For instance, miR-139 has been shown to directly bind the 3′ untranslated region (3′UTR) of ATP7B, leading to reduced protein levels and increased sensitivity to cisplatin in ovarian cancer cells (Xiao et al., 2018). Similarly, miR-133a targets ATP7B mRNA and its overexpression decreases ATP7B protein levels, resensitizing cisplatin-resistant cancer cells to the drug (X. Wang et al., 2016). These findings suggest that post-transcriptional repression via microRNAs represents a bona fide layer of ATP7B regulation with functional consequences in disease.

In addition to RNA-mediated mechanisms, histone deacetylase (HDAC) inhibitors have been reported to influence ATP7B expression or splicing. For example, sodium 4-phenylbutyrate (4-PBA), a weak HDAC inhibitor and chemical chaperone, increased the steady-state levels of several misfolded ATP7B variants in cellular models of WD (van den Berghe et al., 2009). Plant-derived HDAC inhibitors such as p-coumaric and caffeic acid have also been shown to regulate ATP7B splicing indirectly by downregulating hnRNP A1, a known splicing factor, thereby promoting exclusion of exon 12 from ATP7B transcripts (Lin et al., 2015). These studies provide mechanistic links between epigenetic modifiers and RNA-level regulation of ATP7B.

Despite these isolated findings, no studies to date have systematically examined whether DNA methylation or histone modifications directly influence ATP7B transcription. There is no published evidence on ATP7B promoter CpG methylation or histone modification patterns at this locus in mammalian cells (Medici & LaSalle, 2019). The involvement of canonical chromatin regulators—such as PRC2-mediated H3K27 methylation or H3K9 acetylation—in ATP7B regulation remains unexplored. Furthermore, no public ChIP-seq or ATAC-seq datasets have interrogated ATP7B's regulatory landscape in liver, cancer, or chemoresistant contexts (Medici & LaSalle, 2019).

Indirect support for epigenetic modulation of ATP7B comes from genome-wide methylation studies in WD models and patients. In the Atp7b^tx-j mouse model, fetal liver methylomes displayed hundreds of differentially methylated regions (DMRs) compared to wild-type controls. Notably, maternal dietary supplementation with choline, a methyl donor, partially normalized these methylation differences, suggesting that copper metabolism can influence the hepatic epigenome (Mordaunt et al., 2018). However, these changes did not map specifically to the Atp7b promoter.

In human WD livers, methylome profiling revealed widespread hyper- and hypomethylation across numerous regions, including liver-specific enhancers and promoters bound by key hepatic transcription factors such as HNF4A and FOXA1/2 (Mordaunt et al., 2019). A subset of these WD-associated methylation signatures was also detectable in peripheral blood, and some patterns distinguished patients with hepatic-predominant versus neurologic-predominant phenotypes (Mordaunt et al., 2019). While these findings underscore the systemic epigenetic consequences of ATP7B dysfunction, they do not establish whether ATP7B itself is epigenetically regulated.

The current literature also lacks evidence for alternative layers of epigenetic control, such as ATP7B regulation via long noncoding RNAs, m^6A RNA methylation, or higher-order chromatin looping. One review has explicitly noted that the intersection between ATP7B genetic variation and epigenetic changes in shaping WD phenotypes remains unexplored (Medici & LaSalle, 2019).

Given the increasing recognition of ATP7B's involvement in cisplatin resistance and other stress-response pathways, elucidating its epigenetic regulation represents an

important knowledge gap. Emerging technologies—such as CRISPR-based epigenome editing, locus-specific ChIP-seq, and targeted methylome analysis—could now be employed to directly interrogate ATP7B's promoter activity, chromatin accessibility, and regulatory factor occupancy under various cellular states (Medici & LaSalle, 2019; Mordaunt et al., 2019). Identifying how these layers interact to modulate ATP7B may offer new avenues for therapeutic intervention, particularly in diseases where ATP7B expression is aberrantly suppressed.

### 1.7. Rationale and Objectives of This Study

ATP7B, a copper-transporting P-type ATPase, has emerged as a critical gatekeeper of intracellular metal balance, with profound implications for both inherited metabolic disease and chemotherapy resistance. As reviewed in the preceding sections, ATP7B plays a central role in hepatic copper detoxification through its ability to sequester copper into the trans-Golgi network for cuproenzyme synthesis and, under copper overload conditions, to traffic toward vesicular compartments near the canalicular membrane for biliary excretion. This trafficking and redistribution behavior are tightly regulated by intracellular copper levels and mediated by transcriptional mechanisms such as MTF1-dependent activation of the ATP7B promoter. In WD, loss-of-function mutations in ATP7B result in hepatocellular copper accumulation, leading to oxidative damage and multi-organ dysfunction. Conversely, ATP7B overexpression in various malignancies, particularly solid tumors, has been strongly associated with resistance to platinum-based chemotherapeutics, including cisplatin.

This duality underlines the paradoxical—or "double-edged sword"—nature of ATP7B expression. In hepatic physiology, its activity is protective, shielding cells from the pro-oxidant stress of copper overload. Yet in cancer, the same molecular machinery is hijacked to export platinum compounds, diminishing nuclear drug accumulation, impairing DNA damage, and enabling tumor cell survival. Hence, ATP7B's expression level must be precisely tuned to its cellular context: insufficient expression leads to copper toxicity and disease, while excessive expression supports chemoresistance. This narrow therapeutic window demands a deeper understanding of the molecular mechanisms that govern ATP7B expression across physiological and pathological states.

Despite substantial progress in characterizing ATP7B's protein function, intracellular trafficking, and metal-induced transcriptional activation, our understanding of its epigenetic regulation remains surprisingly incomplete. Sections 1.2 and 1.3 have highlighted well-defined genetic and transcription factor–driven pathways, including MTF1 binding to conserved metal response elements and the transcriptional consequences of promoter-disrupting variants. Similarly, recent reports have demonstrated that specific microRNAs—such as miR-133a and miR-139—can suppress ATP7B post-transcriptionally in cancer cells, modulating their cisplatin sensitivity. However, these findings represent only fragments of a larger, poorly charted regulatory landscape. Key questions remain: Does ATP7B expression respond to histone acetylation or methylation? Can chromatin accessibility at its promoter be dynamically modified? Are there histone modifiers, reader complexes, or epigenetic drug targets that could enhance or silence ATP7B expression in a controlled fashion?

The clinical importance of these questions is underscored by emerging evidence that ATP7B dysregulation contributes to both hereditary disease and acquired drug resistance. In WD, hypomorphic mutations—particularly promoter deletions and splicing-disrupting variants—cause partial ATP7B loss, yielding phenotypic diversity that cannot be explained solely by coding sequence defects. In oncology, high ATP7B expression levels often correlate with poor cisplatin response, and experimental overexpression or silencing of ATP7B can significantly modulate drug sensitivity. This indicates that not only genetic variation but also gene dosage and transcriptional control are critical to ATP7B's function. Yet, systematic investigations into how ATP7B is epigenetically regulated, especially in human disease models, are virtually absent.

Adding to this knowledge gap is the absence of robust cellular tools capable of reporting ATP7B transcriptional activity in a chromatin-sensitive, locus-specific manner. Conventional promoter-reporter plasmids are insufficient, as they fail to replicate the endogenous chromatin state or incorporate regulatory elements such as enhancers and insulators. CRISPR-Cas9–based knock-in strategies now offer a transformative opportunity to create reporter models where transcriptional readouts are embedded within the native gene locus. By integrating a luciferase cassette directly into the ATP7B locus, one can achieve physiologically relevant transcriptional monitoring, allowing for high-throughput screening of epigenetic modulators in a context-sensitive fashion.

The present study seeks to fill this critical gap by systematically investigating the epigenetic regulation of ATP7B using a genome-integrated luciferase knock-in reporter model. By targeting the endogenous ATP7B locus in hepatic or cancer cell lines, the study aims to create a platform in which transcriptional activity can be quantitatively monitored in response to both physiological stimuli and pharmacological perturbations. This model preserves the native promoter, chromatin structure, and potential long-range regulatory elements—features essential for capturing real regulatory behavior. The generated reporter line will serve as a powerful tool to interrogate ATP7B's transcriptional dynamics under various conditions, including metal exposure, transcription factor manipulation, and epigenetic drug treatment.

Using this knock-in model, we further aim to screen a focused epigenetic compound library consisting of small-molecule inhibitors targeting DNA methyltransferases (DNMTs), histone deacetylases (HDACs), bromodomain proteins (BETs), and other chromatin modifiers. The objective is to identify compounds capable of enhancing ATP7B promoter activity, as indicated by increased luciferase signal. Such hits would represent potential tools for transcriptional reactivation of ATP7B in conditions where its expression is pathologically suppressed—such as in hypomorphic WD alleles. Conversely, in oncology models, the same system can be adapted to test compounds that repress ATP7B expression, potentially resensitizing tumor cells to cisplatin.

Hit compounds emerging from the screen will be further validated by RT-qPCR to confirm induction of endogenous ATP7B transcripts, and in select cases, ChIP-qPCR assays will be employed to assess histone modification patterns (e.g., H3K9ac, H3K27ac) at the ATP7B promoter. This integrative approach will allow us to mechanistically dissect how chromatin-level modifications influence ATP7B transcription, and whether these changes correlate with altered drug sensitivity.

Collectively, the objectives of this study are threefold: **(1)** to generate a CRISPR knock-in luciferase reporter model that faithfully reports ATP7B promoter activity within its native chromatin environment. **(2)** to perform a functional screen of an epigenetic drug library to identify compounds that either upregulate or downregulate ATP7B expression. **(3)** to mechanistically validate epigenetic regulators of ATP7B expression, focusing on

histone acetylation and chromatin accessibility, and to explore their relevance to therapeutic modulation in contexts such as WD and cisplatin resistance.

The expected outcomes of this study include the identification of actionable epigenetic modifiers that can fine-tune ATP7B transcription in a gene- and context-specific manner. For WD, this could offer a pathway to partially restore ATP7B expression in cases with residual function, improving copper excretion capacity and clinical outcomes. For cancer therapy, it opens the door to ATP7B repression strategies aimed at sensitizing drug-resistant tumors to platinum agents. More broadly, the study aims to establish a new paradigm for exploring epigenetic regulation of clinically relevant transporters using genome-integrated reporter systems.

In conclusion, this work is grounded in a mechanistic appreciation of ATP7B's dual roles in health and disease, and it is driven by an unmet need to understand—and ultimately manipulate—its transcriptional control. By bridging functional genomics, epigenetic pharmacology, and gene regulation, this study endeavors to uncover a new layer of ATP7B biology that may unlock novel therapeutic strategies for both inherited and acquired disorders.

*Chapter 2*

*Section 2*

# 2. MATERIALS & METHODS

## 2.1. Cell Culture

HEK293T and Huh7 cell lines were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM), while Du145 cells were maintained in RPMI-1640 medium. All media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin to support optimal cell growth and prevent bacterial contamination. Cultures were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$.

## 2.2. Generation of fLuc Knock-in Reporter Cell Line Model

To establish a luciferase knock-in reporter system, HEK293T cells were seeded into six-well plates at a density of $3.5 \times 10^5$ cells per well, achieving approximately 70% confluence by the following day. Cells were then co-transfected with two plasmids: a donor construct carrying the fLuc-puromycin cassette and a CRISPR-Cas9 plasmid encoding the ATP7B-specific sgRNA (pSp-Cas9-ATP7B). For each well, 1.25 µg of each plasmid was delivered using Lipofectamine 3000 (Thermo Fisher, L3000015) in accordance with the manufacturer's protocol. All knock-in procedures were conducted under sterile conditions using antibiotic-free media during transfection to maximize cell viability and transfection efficiency. Following transfection, puromycin selection (2 µg/mL; Gibco, A1113803) was applied for 48 hours to enrich for transfected cells. GFP-positive cells were isolated by single-cell sorting into 96-well plates using the SONY SH800S cell sorter.

After single-cell sorting, colonies were cultured until they reached adequate confluence, after which luciferase activity was screened using a luminescence assay. Colonies with detectable luminescence were selected and expanded for further validation. Genomic DNA was extracted from these clones, and integration of the luciferase cassette into the ATP7B locus was confirmed by PCR using primer pairs flanking the upstream, downstream, and knock-in insertion regions. Clones positive for targeted integration were then used for downstream assays. Plasmid constructs were sequence-verified prior to transfection to ensure sgRNA accuracy and donor integrity. Transfection efficiency and

initial editing rates were monitored using GFP reporter expression as a proxy prior to selection.

### 2.3. Validation of Knock-in via Genomic DNA PCR and Sequencing

To verify the successful integration of the luciferase-puromycin (fLuc-puro) knock-in cassette at the ATP7B genomic locus, genomic DNA was extracted from both knock-in HEK293T clones and non-edited control cells. DNA isolation was performed using the Genomic DNA Isolation Kit (Macherey-Nagel, 740952.50) according to the manufacturer's instructions. Four distinct primer pairs were designed to assess specific regions surrounding and within the insertion site (primer sequences listed in **Table 2.1**).

**Table 2.1.** List of primer sequences used in knock-in confirmation PCR experiments

| | Fwd Primer (5'-3') | Rev Primer (5'-3') |
|---|---|---|
| **Confirmation 1** | AGCAGAAGAAAACGCGGTAA | CTCGAAGTACTCGGCGTAGG |
| **Confirmation 2** | GCAACCTCCCCTTCTACGAG | ACCCCTCCCAACATTTCTTC |
| **Confirmation 3** | TGGCTCCTGAGAAAGCAAGC | GCAGAGGTTGCACTGAATTAGAC |
| **Confirmation 4** | GCTAAGGTGGTGGACTTGGA | AGTTCTTGCAGCTCGGTGAC |

For validation of the 5′ junction (upstream), PCR was conducted using a forward primer targeting the endogenous ATP7B promoter and a reverse primer located within the knock-in cassette (1F–1R). The 3′ junction (downstream) was assessed using a forward primer within the knock-in cassette and a reverse primer situated in ATP7B exon 1, downstream of the integration site (2F–2R). A third primer pair (3F–3R) was used to amplify the unmodified promoter-to-exon 1 region, serving as an indicator of wild-type alleles. Additionally, a fourth primer set (4F–4R) was used to amplify the entire inserted knock-in fragment to evaluate integration integrity.

PCR reactions were carried out using Phusion DNA Polymerase (New England Biolabs, M0530S) under optimized annealing and cycling conditions. Amplicons were resolved on a 1% agarose gel stained with ethidium bromide and visualized under UV light. Clones were considered knock-in positive if they displayed clear bands of approximately 1.5 kb with the upstream, downstream, and full knock-in primer pairs, while showing no amplification with the 3F–3R primers. In contrast, wild-type HEK293T cells only yielded a product with the 3F–3R pair. PCR products from validated clones were further purified and subjected to Sanger sequencing to confirm precise genomic integration of the knock-in construct.

### 2.4. Epigenetic Modifier Drug (Epidrug) Library Screening

Screening for epigenetic modulators of ATP7B expression was performed using a commercially available epidrug library (Cayman Chemical, #11076), comprising approximately 150 small-molecule compounds. HEK293T cells harboring the ATP7B fLuc knock-in reporter (HEK-fLuc-Ki) were seeded at a density of $4 \times 10^3$ cells per well in 96-well plates. The following day, cells were treated with each compound at a fixed concentration of 5 μM and incubated for 72 hours.

Following treatment, 30 μg of D-luciferin substrate (GoldBio, #LUCK-1G) was added to each well, and luminescence signals were measured using a plate reader set to a gain of 75. To normalize luminescence intensity to cell content, cells were lysed directly in the wells using 25 μL of 0.2% SDS (Bio-Rad, #1610418). Total protein content per well was subsequently quantified using the BCA Protein Assay Kit (Thermo Scientific, #2322). The relative luciferase activity was expressed as a luminescence signal normalized to protein concentration.

### 2.5. Real-Time Quantitative PCR (RT-qPCR)

Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel), following the manufacturer's protocol. For each sample, 500 ng of RNA was reverse-transcribed into complementary DNA (cDNA) using the M-MLV Reverse Transcriptase cDNA synthesis kit (Invitrogen). The resulting cDNA was used as a template for real-time quantitative PCR, which was performed with the LightCycler 480 SYBR Green I Master Mix (Roche) on a LightCycler 480 Instrument II system (Roche). Gene expression levels were quantified by normalizing each target gene's expression to that of the housekeeping gene GAPDH. Amplification specificity was confirmed by melting curve analysis following each run.

### 2.6. Western Blotting

Cells were lysed using RIPA buffer (EcoTech, RIPA-100) supplemented with phenylmethylsulfonyl fluoride (PMSF; Merck, 10837091001) and PhosSTOP phosphatase inhibitor cocktail (Merck, 4906845001), as per the manufacturer's instructions. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, 2322). Equal amounts of protein samples were denatured by

heating at 95°C for 10 minutes in Laemmli buffer (Bio-Rad, 1610747) containing dithiothreitol (DTT; Sigma, D0632).

Proteins were separated by SDS-PAGE using Mini-PROTEAN TGX Stain-Free Gels (Bio-Rad, 4568086) and transferred onto PVDF membranes (Bio-Rad, 1620177). Membranes were blocked in 5% bovine serum albumin (BSA; Sigma, A3733) and incubated overnight at 4°C with primary antibodies: anti-H3K9Ac (1:1000; Abclonal, A21107), anti-H3K18Ac (1:1000; Abclonal, A20735), anti-H3K27Ac (1:1000; Abclonal, A7253), and anti-total H3 (1:1000; Abcam, ab18521). After washing with TBS-T, membranes were incubated for 1.5 hours at room temperature with HRP-conjugated secondary antibodies (Abcam, ab205718). Chemiluminescent detection was carried out using the Immobilon Forte Western HRP substrate (EMD Millipore, WBLUF0500), and signals were visualized with the Odyssey FC Imaging System (LI-COR Biosciences).

### 2.7. Chromatin Immunoprecipitation quantitative PCR (ChIP-qPCR)

HEK293T cells were treated with the indicated epidrugs for 24 hours prior to chromatin preparation. Crosslinking was performed by adding 1% formaldehyde (Sigma-Aldrich, 818708) directly to the culture medium for 10 minutes at room temperature. The reaction was quenched by adding glycine (final concentration: 125 mM; Sigma-Aldrich, G7126) and incubating for 5 minutes. Cells were collected by centrifugation at 1000 × g for 5 minutes at 4°C, washed twice with cold PBS, and resuspended in ChIP lysis buffer (50 mM HEPES, 1 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 1% SDS, 1 mM PMSF, and protease inhibitor cocktail). Samples were incubated on ice for 10 minutes, followed by sonication using a Bioruptor Plus sonicator (Diagenode) to shear chromatin into fragments of approximately 100–500 bp. After centrifugation at 10,000 × g for 10 minutes at 4°C, the supernatant containing sheared chromatin was collected. Pre-clearing was performed by incubating the chromatin with pre-washed Dynabeads® Protein A magnetic beads (Thermo Fisher, 10001D) for 30 minutes at 4°C to minimize nonspecific binding.

For immunoprecipitation, beads were pre-incubated with one of the following antibodies for 1 hour at room temperature in PBST: anti-H3K9Ac (Abclonal, A21107), anti-H3K18Ac (Abclonal, A20735), anti-H3K27Ac (Abclonal, A7253), or non-specific

IgG control (Sigma-Aldrich, PP64B). Antibody-conjugated beads were then added to the pre-cleared chromatin and incubated overnight at 4°C. After incubation, bead–chromatin complexes were sequentially washed with the following buffers: ChIP lysis buffer, low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, 140 mM NaCl, pH 8.1), high-salt wash buffer (identical composition with 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% IGEPAL-CA 630, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and twice with TE buffer.

DNA was eluted from the beads using an elution buffer containing 1% SDS and 0.1 M $NaHCO_3$. Reverse crosslinking was performed by treating the eluates with RNase A (0.3 mg/mL; Thermo Fisher, 12091039) and 5 M NaCl (Merck, S9888) at 37°C for 30 minutes, followed by incubation with proteinase K (0.3 mg/mL; Thermo Fisher, EO0492) at 65°C for 1 hour. DNA was purified using the ChIP DNA Clean & Concentrator Kit (Zymo Research, D5205). Primers targeting regions of interest were designed using the UCSC Genome Browser and Ensembl Genome Browser. Primer sequences are listed in **Table 2.2**. Quantitative PCR (qPCR) was performed using the LightCycler 480 system (Roche), and enrichment was calculated using the percent input or fold enrichment method.

**Table 2.2.** Primer sequences used in ChIP-qPCR experiments

| | Fwd Primer (5'-3') | Rev Primer (5'-3') |
|---|---|---|
| **Promoter Region 1** | ACTAGTCTACGCCTCGGCA | CGGGCCAATTGTGCGTTAC |
| **Promoter Region 2** | ACAATTGGCCCGTGGGG | CCCCGAACTCAGGAAACGCT |

### 2.8. Cell Viability Assay

To assess cell viability following drug treatment, cells were seeded into 96-well plates at the appropriate density and allowed to adhere. After the indicated treatment periods, cells were fixed with 10% (w/v) trichloroacetic acid (TCA; Sigma, T6399) for 1 hour at 4°C. Wells were subsequently washed with deionized water and allowed to air dry at room temperature. Fixed cells were stained with 50 µL of 0.4% (w/v) sulforhodamine B (SRB) dye (Santa Cruz, sc-253615) for 30 minutes. Excess dye was removed by washing with 1% acetic acid, and the plates were air-dried completely. The bound dye was solubilized in 150 µL of 10 mM Tris base solution (Sigma, T1503), and absorbance was measured at 564 nm using a Synergy H1 Hybrid microplate reader (BioTek).

Cell viability was calculated using the formula:

% Viability = [100 × (Sample Abs – Blank) / (Non-treated Control Abs – Blank)].

### 2.9. Determination of Oxidative Stress via Reactive Oxygen Species (ROS) Activity Assay

Intracellular reactive oxygen species (ROS) levels were quantified using the Muse® Oxidative Stress Kit (Luminex, MCH100111) in combination with the Muse® Cell Analyzer. Cells treated with compounds or left untreated were harvested and resuspended in PBS containing 1% FBS, adjusted to a final concentration of approximately 100–500 cells/µL. For staining, 190 µL of the cell suspension was mixed with 10 µL of the Muse® Oxidative Stress Reagent working solution and incubated at 37°C for 30 minutes in the dark. Immediately following incubation, samples were analyzed using the Muse® Cell Analyzer. The system distinguishes ROS-positive and ROS-negative populations based on the oxidative conversion of the probe into a fluorescent product, allowing quantification of oxidative stress levels within the cell population.

### 2.10. Promoter Luciferase Reporter Assay

To evaluate the transcriptional activity of the ATP7B promoter, various lengths of the promoter region (up to 1000 bp upstream of the transcription start site) were amplified from HEK293T genomic DNA using Phusion High-Fidelity DNA Polymerase. The PCR products were digested with SmaI (NEB, R0141) and BglII (NEB, R0144) and cloned into the pMDR1-1202 luciferase vector (Addgene #37627), in which the MDR1 gene had been removed. Cloning was performed using T4 DNA Ligase (NEB, M0202), and the resulting constructs were sequence-verified prior to transfection.

HEK293T cells were seeded onto poly-L-lysine–coated 96-well plates at a density of $1.5 \times 10^4$ cells per well. Twenty-four hours later, cells were transfected with the promoter-luciferase constructs using Lipofectamine 3000 (Thermo, L3000015) following the manufacturer's instructions. After 18 to 24 hours, the medium was replaced, and luminescence measurements were taken 48 hours post-transfection. For luminescence detection, 30 µg of D-luciferin (GoldBio, LUCK-1G) was added directly to the wells, incubated for 1 minute to allow cellular uptake, and luminescence was measured using a Synergy H1 Hybrid reader (BioTek). After measurement, the medium was removed, and cells were lysed in 25 µL of 0.2% SDS solution for 10 minutes at room temperature. Total

protein content per well was quantified using the Pierce BCA Protein Assay Kit (Thermo Scientific, 23225). Luminescence signals were normalized to total protein concentration and expressed as relative luminescence units per microgram of protein.

For HDAC inhibitor experiments, the same transfection protocol was followed. At 24 hours post-transfection, the medium was replaced with a fresh medium containing 5 μM of selected inhibitors (MS275, Chidamide, KD5170, PAOA, and Pyroxamide). Luminescence was recorded 72 hours after transfection, and values were normalized to total protein content as described above.

### 2.11. Statistical Analysis

All statistical analyses were conducted using GraphPad Prism version 8 (GraphPad Software, Inc.). The statistical methods applied to individual experiments are specified in the respective figure legends. Details regarding the number of biological replicates, the type of error bars used, and the threshold for statistical significance (indicated by asterisks) are also provided within each figure legend to ensure transparency and reproducibility.

*Chapter 2*

*Section 3*

# 3. RESULTS

## 3.1. Generation of a CRISPR-Based Luciferase Knock-In Reporter Model for Measuring ATP7B Promoter Activity

To investigate the transcriptional regulation of ATP7B within its endogenous chromatin context, we established a luciferase knock-in reporter system in HEK293T cells using CRISPR-Cas9-mediated genome editing. Unlike conventional plasmid-based promoter assays, which often fail to recapitulate native chromatin architecture and regulatory cues, this knock-in model enables real-time monitoring of ATP7B promoter activity within its physiological genomic environment.

The design strategy involved the targeted insertion of a firefly luciferase (*fLuc*) coding sequence into the first exon of ATP7B, positioned immediately downstream of its native promoter but upstream of the translation initiation codon (**Figure 2.1A**). This configuration preserves all upstream cis-regulatory elements while replacing the coding sequence of ATP7B with that of the luciferase reporter, thereby ensuring transcriptional fidelity while preventing protein translation. The approach was intended to functionally disrupt the gene without compromising regulatory inputs, allowing precise measurement of promoter activity.

To implement this strategy, a single guide RNA (sgRNA) targeting the first exon of ATP7B was co-delivered with a donor plasmid containing the fLuc cassette flanked by homology arms. Following transfection and selection, we screened for successful integration using genomic PCR with four independent primer sets designed to span the targeted locus (**Figure 2.1B**). In clones #5, #6, and #8, amplicons corresponding to the upstream (1F–1R), downstream (2F–2R), and complete knock-in (4F–4R) junctions were detected, confirming accurate insertion of the fLuc cassette. Importantly, the wild-type promoter-exon 1 amplicon (3F–3R) was absent in these clones, indicating successful editing of at least one ATP7B allele (**Figure 2.1C**). As expected, wild-type HEK293T cells exhibited only the 3F–3R product, without any knock-in-specific bands.

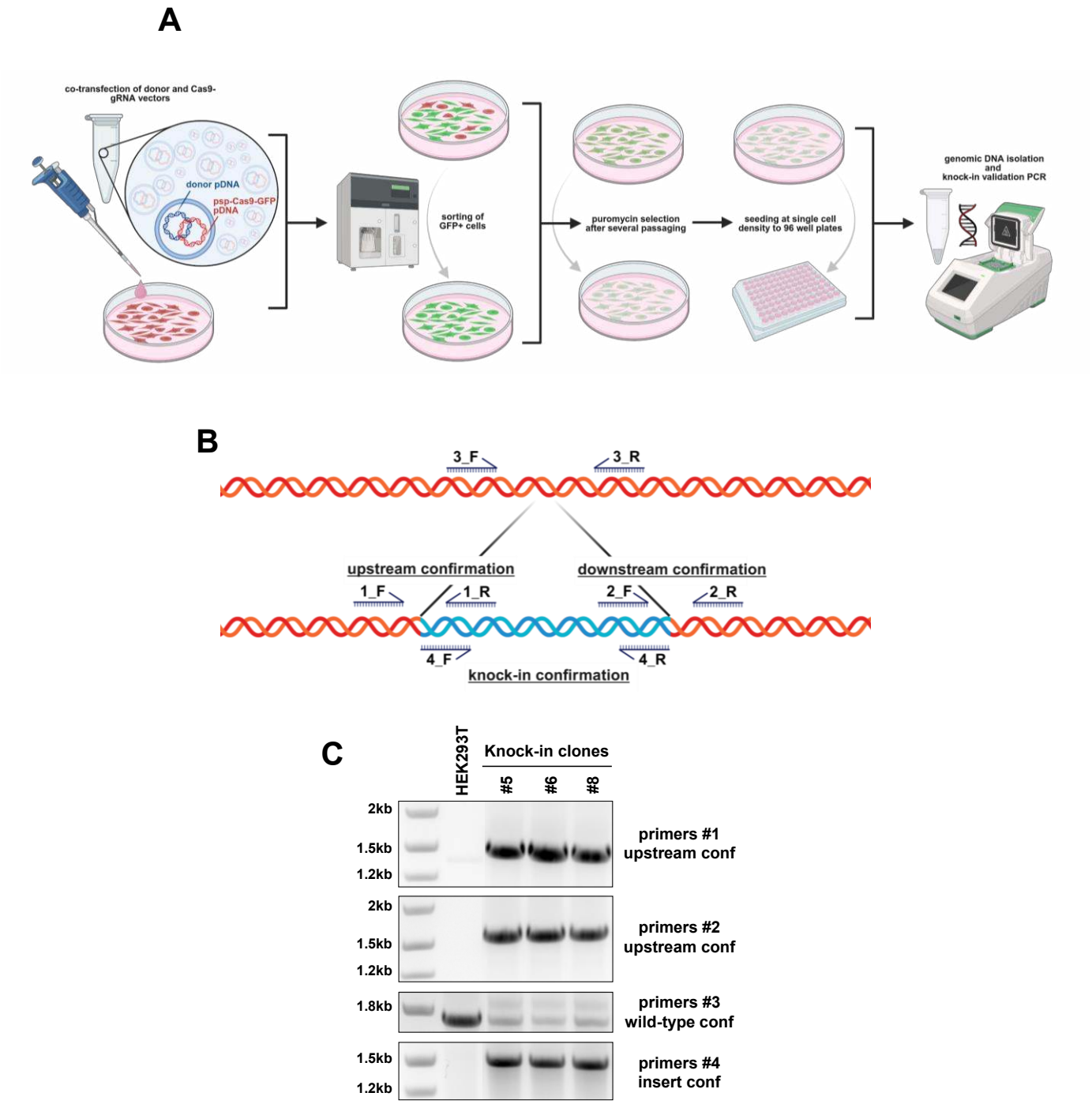



**Figure 2.1. Luciferase reporter is successfully integrated under the ATP7B promoter via CRISPR/Cas9-mediated knock-in. (A)** Schematic representation of the CRISPR-Cas9 knock-in strategy used to integrate the firefly luciferase (fLuc) reporter gene under the ATP7B promoter. The knock-in replaces the ATP7B coding region while maintaining the promoter activity. **(B)** Diagram of the ATP7B genomic locus before (wild-type) and after (knock-in) successful integration of the luciferase gene. Primer locations for genomic PCR validation are indicated: 1F-1R (upstream knock-in confirmation), 2F-2R (downstream knock-in confirmation), 3F-3R (wild-type ATP7B promoter-exon region), and 4F-4R (entire knock-in region). **(C)** Genomic PCR results

confirming knock-in integration. HEK293T control cells show no amplification, while ATP7B-prom-fluc knock-in colonies (#5, #6, and #8) show amplified products for upstream, downstream, and knock-in regions (approximately 1.5 kb).

To determine whether the knock-in event disrupted ATP7B expression, we examined endogenous ATP7B protein levels by Western blotting. All three knock-in clones showed a partial reduction in ATP7B levels compared to parental cells, suggesting monoallelic disruption. This preserved the expression of ATP7B from the non-edited allele, thereby avoiding complete functional ablation and potential compensatory effects (**Figure 2.2**).

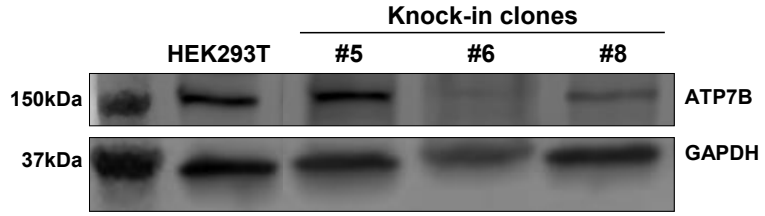



**Figure 2.2. Introduced *fLuc* knock-in results in only a partial depletion of endogenous ATP7B protein expression.** Western blot analysis of ATP7B (molecular weight; 165 kDa) protein expression in HEK293T control cells and ATP7B-prom-fluc knock-in clones (#5, #6, and #8). GAPDH (molecular weight; 37 kDa) is used as a loading control.

We next assessed whether luciferase activity in the knock-in cells faithfully reflected ATP7B promoter activity. Luminescence measurements demonstrated robust reporter expression in clones #5, #6, and #8, whereas no signal was detected in unedited HEK293T cells (**Figure 2.3**). These data confirm that the inserted fLuc cassette is transcriptionally driven by the endogenous ATP7B promoter and is functionally competent for use as a live-cell readout of promoter activity.

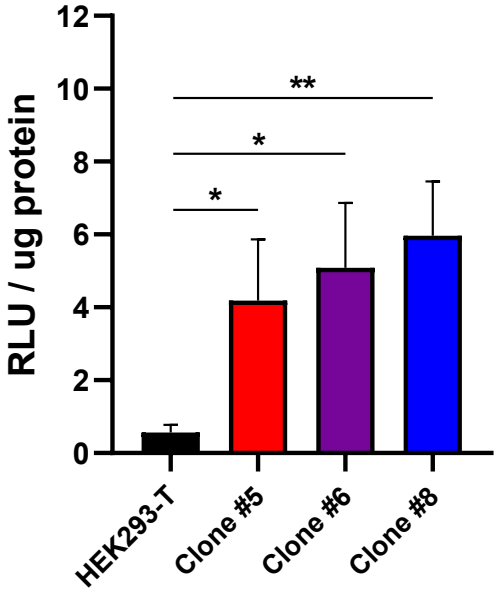



**Figure 2.3. Luciferase activity is robustly induced in knock-in clones, confirming functional promoter targeting.** Normalized relative luminescence (RLU/µg protein) measurements of HEK293T control cells and ATP7B-prom-fluc knock-in clones (#5, #6, and #8). Knock-in colonies exhibit significant luminescence, confirming successful luciferase integration under the ATP7B promoter. Error bars represent the standard deviation of at least three biological replicates (*p<.05, student's t-test)

To evaluate the regulatory responsiveness of the knock-in system, we tested its sensitivity to modulation by known transcriptional regulators and modulators of ATP7B. Metal Regulatory Transcription Factor 1 (MTF1) is a well-characterized copper-responsive factor that activates ATP7B transcription through binding to metal-responsive elements (MREs) in the promoter region. Overexpression of MTF1, and exposure of cells to $CuCl_2$ led to increased mRNA expression levels of ATP7B in HEK293T cells (**Figure 2.4**).

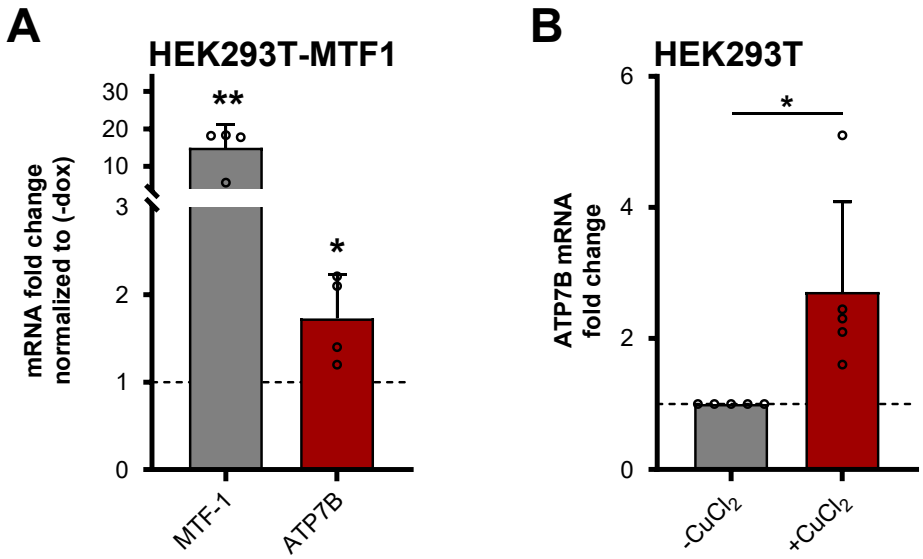



**Figure 2.4. Both MTF1 overexpression and copper overload increase ATP7B mRNA expression.** qPCR analysis of MTF1 and ATP7B mRNA levels under two separate conditions: **(A)** doxycycline-inducible MTF1 overexpression in engineered HEK293T cells (HEK293T-MTF1) (2 µg/mL doxycycline for 72 hours to induce overexpression), and **(B)** copper-induced response in HEK293T cells (100 µM $CuCl_2$ for 72 hours). MTF1 overexpression and $CuCl_2$ treatment each elevate ATP7B transcript levels, confirming their independent capacity to transcriptionally upregulate ATP7B. Data are presented as mean ± SD from at least three biological replicates (*p<.05, **p<.01, student's t-test) (Ayca Acar, MSc contributed to these experiments).

Collectively, these findings validate the successful generation of a CRISPR/Cas9-based ATP7B-luciferase knock-in reporter system that preserves endogenous promoter elements and provides a dynamic, chromatin-integrated platform for studying transcriptional regulation. The cell line used to generate the model faithfully responds to physiologically relevant transcriptional inputs. As such, this engineered system serves as a foundational tool for all subsequent experiments aimed at dissecting the epigenetic control and transcriptional activation of ATP7B expression in this study.

### 3.2. Epigenetic Drug Screening Identifies HDAC Inhibitors as Transcriptional Activators of ATP7B

To uncover epigenetic regulators capable of modulating ATP7B transcription, we conducted a targeted chemical screen using our CRISPR-engineered HEK293T knock-in reporter model. This approach leveraged a chemically diverse library of approximately 150 epigenetic compounds, each with known selectivity toward chromatin-modifying enzymes or chromatin-associated reader proteins. The library included inhibitors

targeting histone deacetylases (HDACs), histone methyltransferases and demethylases, DNA methyltransferases, as well as bromodomain-containing proteins (BET family), among others. By monitoring luciferase expression from the ATP7B promoter, we aimed to identify druggable regulators capable of altering transcriptional output from the endogenous locus.

Each compound was administered to three independently validated ATP7B-prom-fLuc knock-in clones (#5, #6, and #8) at a final concentration of 5 µM for a duration of 72 hours. This fixed-dose strategy was selected to enable high-throughput screening while minimizing the risk of cytotoxic artifacts and maintaining assay consistency. Luminescence output was normalized to total protein content in each well to account for variability in cell density or viability. Any compound that reduced total protein to less than 25% of the untreated (NT) control was excluded from further analysis, in order to eliminate non-specific transcriptional effects arising from cell death or metabolic stress.

We acknowledge that screening at a single micromolar concentration may have limited the detection of highly potent compounds with low nanomolar activity or narrow therapeutic windows. Nevertheless, this widely adopted approach allowed us to efficiently detect early transcriptional responses and prioritize high-confidence candidates for validation.

The screen revealed a clear enrichment of transcriptional activators among HDAC inhibitors, several of which induced a reproducible increase in luminescence across all three knock-in clones (**Figure 2.5**).

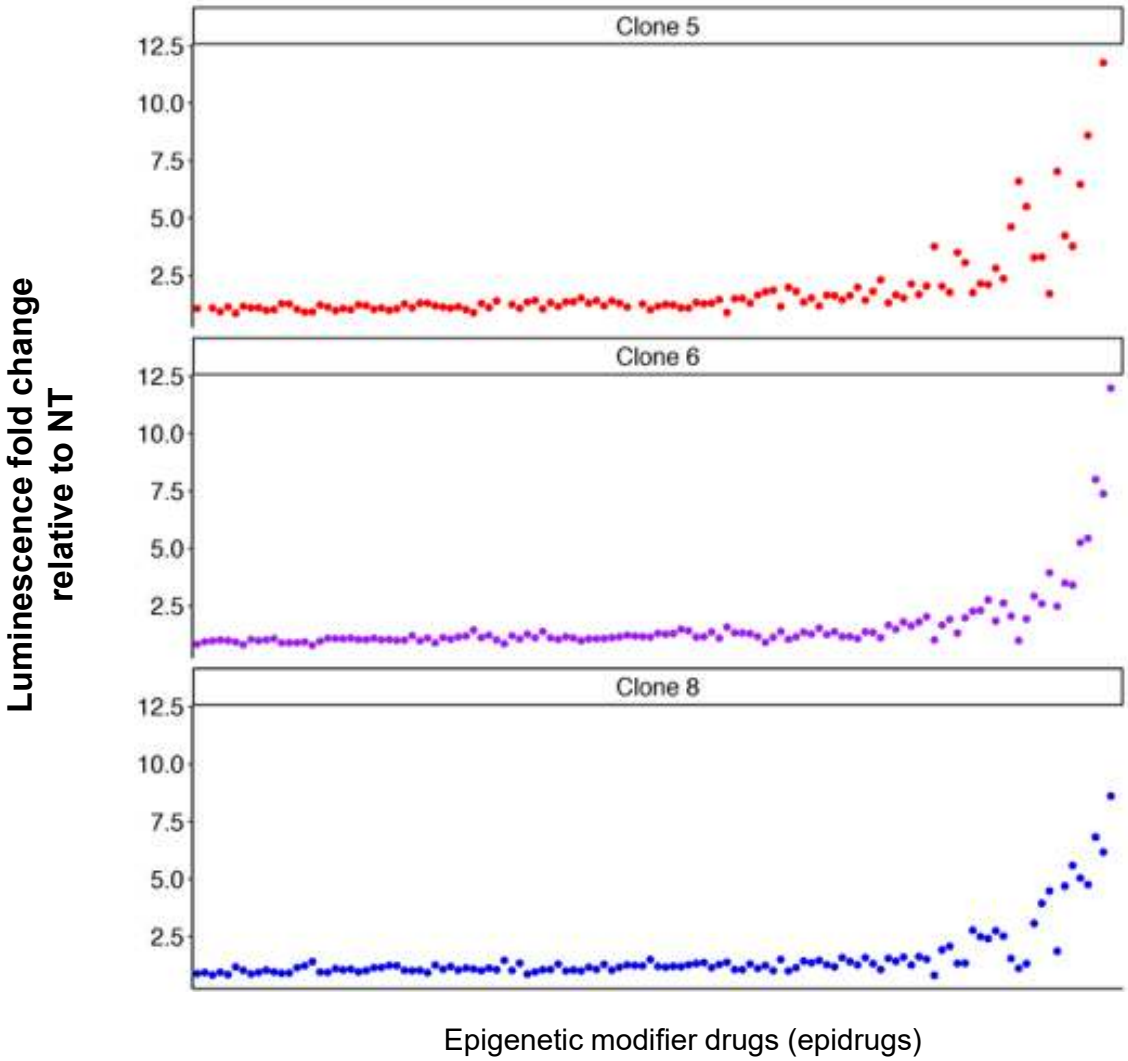



**Figure 2.5. High-throughput screening of an epigenetic drug library identifies HDAC inhibitors as top inducers of ATP7B promoter activity.** High-throughput screening results of ~150 epigenetic drugs tested on ATP7B-prom-fLuc knock-in clones (#5, #6, and #8), with luminescence signal normalized to non-treated control. The x-axis represents the individual epidrugs, ordered by increasing luminescence activity (RLU/µg protein), with epidrugs that significantly reduce cell viability (<25% of total protein of non-treated control) excluded from the analysis. Non-knock-in HEK293T cells show no significant luminescence, confirming specificity to knock-in clones.

Compounds such as Chidamide, MS-275, and KD5170 consistently elevated luciferase expression, indicating activation of the ATP7B promoter. Interestingly, certain BET inhibitors—namely JQ1, I-BET151, and CPI-203—also appeared to enhance reporter activity in the primary screen. However, subsequent validation revealed that these compounds did not induce endogenous ATP7B mRNA expression and were therefore considered false positives.

To systematically nominate the most promising compounds, we applied a multi-tiered selection strategy based on luminescence magnitude, chemical class representation, and consistency across clones. A total of 15 HDAC inhibitors were identified as primary hits, all exhibiting robust and reproducible activation of the reporter. From this pool, five HDAC inhibitors—Chidamide, KD5170, MS-275, Pyroxamide, and PAOA—were selected for further validation. These candidates span a range of structural scaffolds and inhibitory profiles, encompassing both class I-selective and pan-HDAC targeting mechanisms.

To confirm whether these compounds also regulate ATP7B at the native genomic locus, we treated wild-type HEK293T cells with the five shortlisted compounds, alongside UF010 as an additional HDAC inhibitor. Endogenous ATP7B transcript levels were assessed by qPCR following 72 hours of treatment. All six HDAC inhibitors led to a ≥2-fold increase in ATP7B mRNA levels relative to the NT control (**Figure 2.6**), consistent with their activity in the reporter assay. This transcriptional upregulation provides strong evidence that HDAC inhibition relieves chromatin-mediated repression at the ATP7B promoter. In contrast, BET inhibitors did not produce any measurable increase in ATP7B transcripts, supporting their reclassification as assay artifacts rather than true regulators.

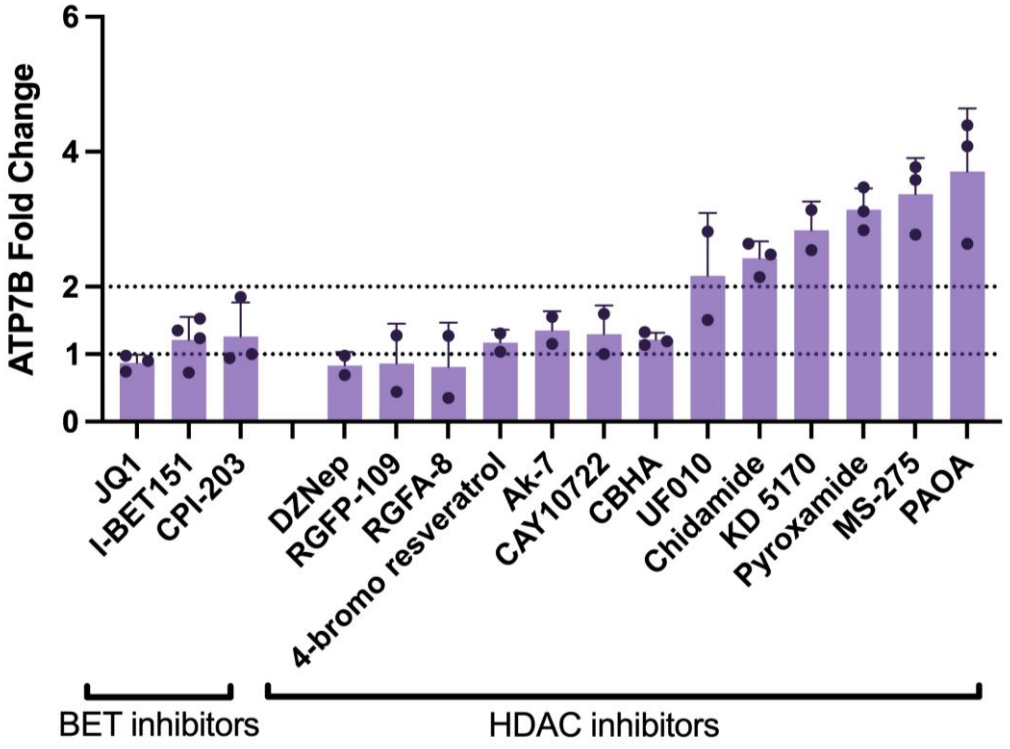


**Figure 2.6. qPCR validation confirms upregulation of ATP7B transcript levels by selected epidrugs.** qPCR validation of ATP7B mRNA expression in HEK293T cells treated with selected hit epidrugs, mainly HDAC inhibitors producing the highest fold increase in ATP7B expression relative to non-treated control. BET inhibitors showed increased luminescence in the epidrug library screen are also included in qPCR experiments. Data are presented as mean ± SD from at least two biological replicates (Ayca Acar, MSc contributed to these experiments).

To further characterize the potency and dose responsiveness of the selected HDAC inhibitors, we performed a titration experiment in the knock-in clones. Cells were treated with increasing concentrations of each compound (0.3–5 µM), and luciferase activity was quantified after 72 hours. All five compounds elicited a clear dose-dependent increase in luminescence, indicating progressive derepression of ATP7B promoter activity with escalating HDAC inhibition (**Figure 2.7**). Notably, Chidamide and MS-275 exhibited the steepest and most dynamic induction profiles, suggesting higher efficacy in promoting transcriptional activation through epigenetic remodeling.

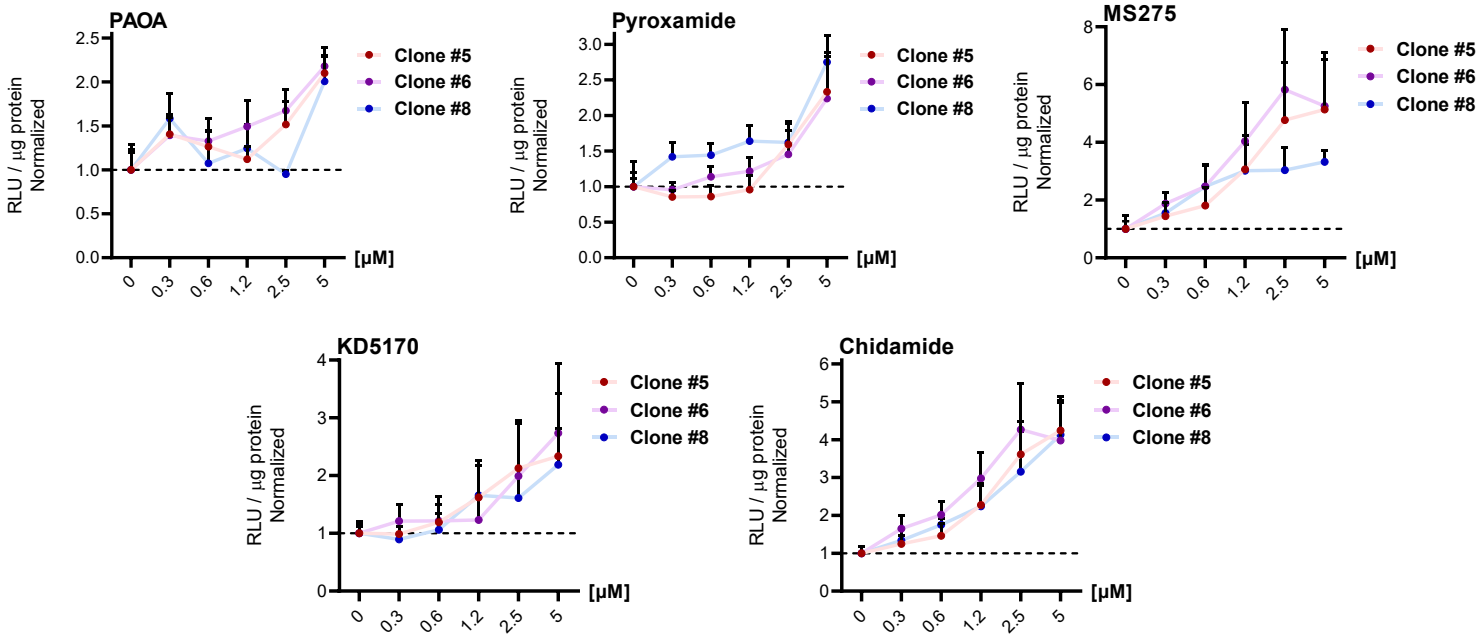



**Figure 2.7. Top HDAC inhibitors induce increased luminescence activity in a dose-dependent manner on knock-in clones.** Dose-response luminescence data for the top five selected HDAC inhibitors (Chidamide, KD5170, MS-275, Pyroxamide, PAOA) tested on ATP7B-prom-fLuc knock-in colonies (#5, #6, and #8). Data demonstrate a dose-dependent increase in luminescence, confirming the efficacy of these drugs in modulating ATP7B promoter activity. Data are presented as mean ± SEM from three biological replicates.

Together, these findings validate the luciferase knock-in platform as a robust and physiologically relevant tool for functional screening of epigenetic regulators. The high concordance between luminescence output, endogenous transcript induction, and dose-response behavior strongly supports the specificity of HDAC inhibitors as activators of ATP7B transcription. This screen not only identifies HDAC inhibition as a promising epigenetic axis for modulating ATP7B expression but also establishes a scalable experimental framework for dissecting gene regulation at endogenous loci.

### 3.3. HDAC Inhibitors Upregulate ATP7B via Promoter-Directed and Chromatin-Dependent Mechanisms Across Cell Types

To assess the broader applicability of HDAC inhibitor–induced ATP7B transcriptional activation beyond the HEK293T background, we extended our investigation to include additional cellular contexts, including cancer-derived lines. As an initial step, we aimed to validate the transcriptional competence of a defined promoter fragment of ATP7B in a plasmid-based assay. For this purpose, we cloned a 1-kilobase region upstream of the ATP7B transcription start site (–1000 to +1 bp) into a luciferase reporter plasmid and transiently transfected it into HEK293T cells. This construct was compared to vectors containing either a strong viral promoter (CMV), the promoter of the multidrug resistance gene MDR1, or an empty vector lacking any promoter sequence (**Figure 2.8A**).

As expected, the CMV and MDR1 promoter constructs yielded high levels of luminescence, while the empty vector produced only background signal. Importantly, the ATP7B-1kb promoter construct also generated a robust luminescence signal, exceeding background by more than 30-fold, although lower than that of the stronger control promoters (**Figure 2.8B**). These results confirmed that the 1-kb upstream segment of ATP7B contains transcriptionally active elements and is functionally suitable for downstream regulatory analyses.

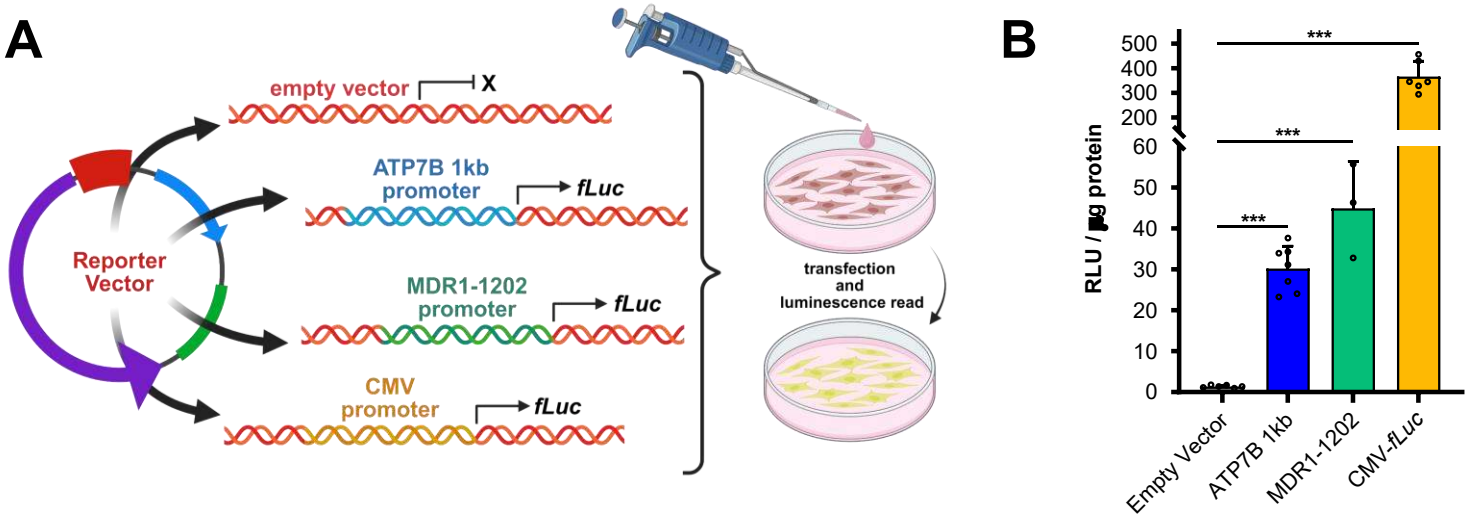



**Figure 2.8. ATP7B-1kb promoter construct drives specific luciferase activity in transient reporter assays. (A)** Schematic representation of the various promoter luciferase reporter constructs and experimental setup. **(B)** Comparison of luminescence activity (RLU/µg protein) in HEK293T cells transfected with luciferase reporter constructs driven by the ATP7B-1kb promoter, MDR1 promoter, or CMV promoter. The ATP7B-1kb promoter construct (–1000 bp to +1 bp) shows significant luminescence compared to the empty vector, demonstrating its functionality. Data are presented as mean ± SD from at least three biological replicates (*p<.05, **p<.01, ****p<0.001, student's t-test) (Experiments in panel B were performed by Batuhan Altay, MSc).

Building on prior evidence that MTF1 positively regulates ATP7B expression through its interaction with metal-responsive elements (MREs) within the promoter, we next assessed whether the ATP7B-1kb construct is sensitive to MTF1-mediated regulation. CRISPR-Cas9-mediated knockout of MTF1 in HEK293T cells led to a marked decrease in luminescence from the ATP7B-1kb construct (**Figure 2.9**), indicating that the reporter faithfully captures MTF1-dependent transcriptional regulation and retains key cis-regulatory motifs.

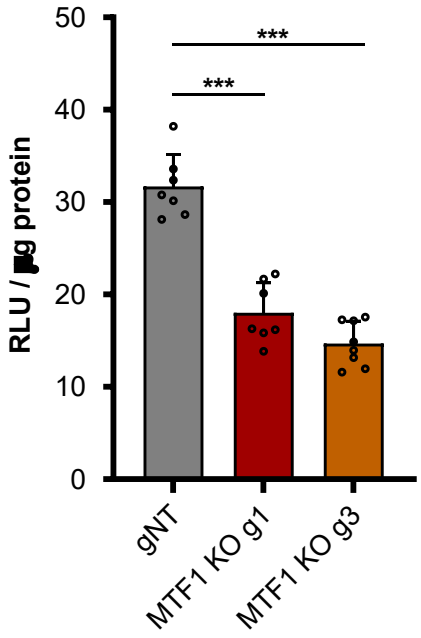



**Figure 2.9. MTF1 knockout reduces ATP7B-1kb promoter-driven luciferase activity.** Effect of MTF1 transcription factor knockout (KO) on ATP7B-1kb promoter luciferase activity in HEK293T cells. Cells were transfected with the ATP7B-1kb promoter luciferase reporter construct after MTF1 knock-out using two different gRNAs (g1 and g3). MTF1 KO significantly reduces luminescence compared to control HEK293T cells, supporting MTF1's role in regulating ATP7B expression through its interaction with the promoter region. Data are presented as mean ± SD from three biological replicates (*p<.05, student's t-test) (These experiments were performed by Batuhan Altay, MSc).

To test whether HDAC inhibitors act through promoter-proximal chromatin modulation, we treated HEK293T cells transfected with the ATP7B-1kb reporter construct using five of the top-performing HDAC inhibitors (Chidamide, KD5170, MS-275, PAOA, and Pyroxamide). All compounds somehow managed to induce a significant increase in luminescence relative to untreated controls (**Figure 2.10**), consistent with the earlier findings in the knock-in model. These results indicate that the transcriptional upregulation of ATP7B is, at least in part, mediated by chromatin-level alterations at the proximal promoter region, rather than by distal regulatory elements or secondary cellular pathways.

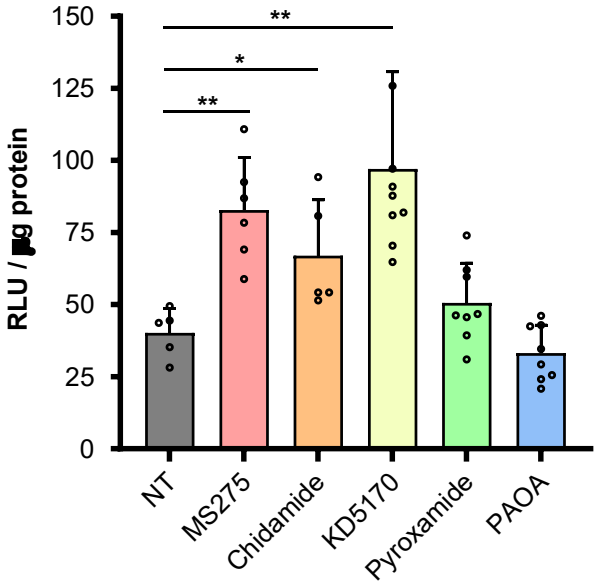



**Figure 2.10. Epidrug treatment modulates ATP7B-1kb promoter activity in transient luciferase assays.** In order to quantify the modulation of ATP7B-1kb promoter luciferase activity by epidrugs, HEK293T cells transfected with the ATP7B-1kb promoter luciferase reporter construct were treated with the indicated epidrugs (5 µM, 72 h), followed by luminescence measurements (These experiments were performed by Batuhan Altay, MSc).

Given that certain cellular stresses, including oxidative stress, have been previously associated with the induction of ATP7B expression along with stress response genes, we explored whether HDAC inhibitors increase the oxidative stress and it might contribute to the observed increase in ATP7B expression (Hossain et al., 2024; Roelofsen et al., 2000; Spee et al., 2005). To this end, we measured intracellular reactive oxygen species (ROS) levels in HEK293T cells treated with each of the four HDAC inhibitors, in the presence or absence of the antioxidant N-acetyl cysteine (NAC). Flow cytometric analysis revealed that only Pyroxamide treatment resulted in a notable increase in ROS-positive cells, which was fully reversed by NAC co-treatment (**Figure 2.11**). The other three compounds—Chidamide, MS-275, and PAOA—did not elicit significant ROS accumulation.

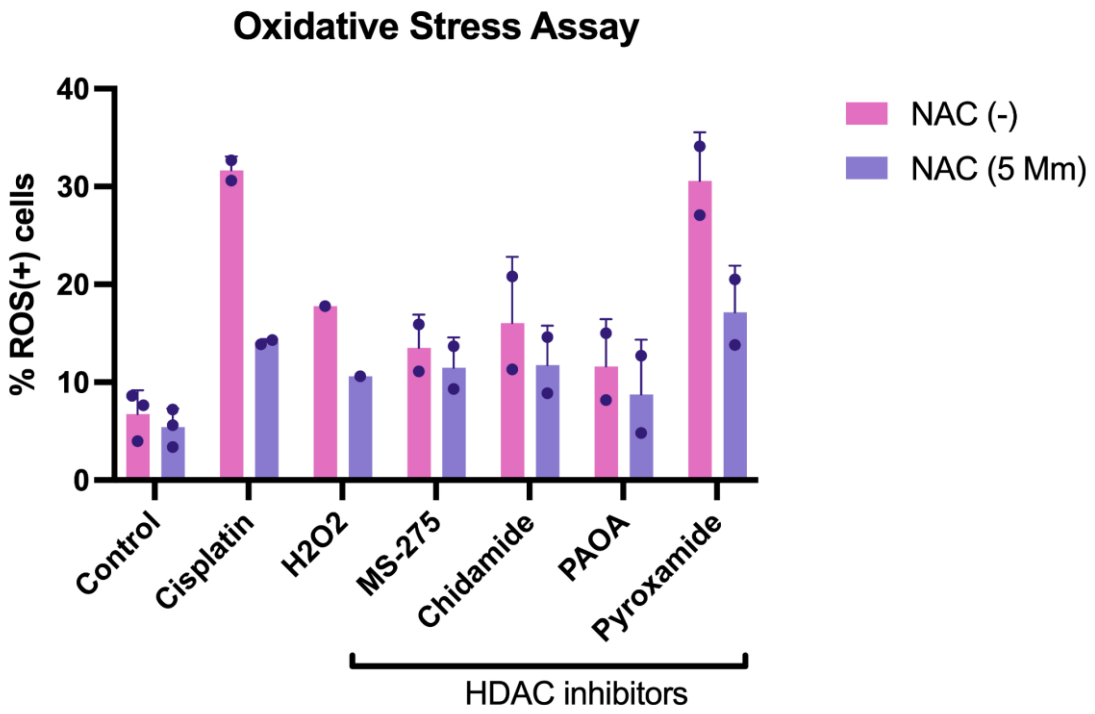



**Figure 2.11. HDAC inhibitors induce moderate ROS accumulation.** Percentage of reactive oxygen species (ROS)-positive cells under various treatment conditions in the presence or absence of the ROS scavenger N-acetylcysteine (NAC, 5 mM). Cells were treated with cisplatin, $H_2O_2$ (positive control for oxidative stress), and HDAC inhibitors MS-275, Chidamide, PAOA, and Pyroxamide (5 µM, 72 h). Cisplatin and $H_2O_2$ induce significant ROS levels compared to control cells, while HDAC inhibitors moderately increase ROS, not as much as cisplatin and $H_2O_2$. NAC treatment reduces ROS levels across all conditions, confirming its antioxidant activity and reversibility of increase in ROS levels. Data are presented as mean ± SD from two biological replicates (This figure was generated by Ayca Acar, MSc).

Importantly, qPCR analysis demonstrated that ATP7B transcript levels remained elevated across all treatment groups, including those co-treated with NAC (**Figure 2.12**). These data suggest that although ROS production may occur as an off-target effect of some HDAC inhibitors, it is not required for ATP7B induction, thereby reinforcing the epigenetic specificity of the response.

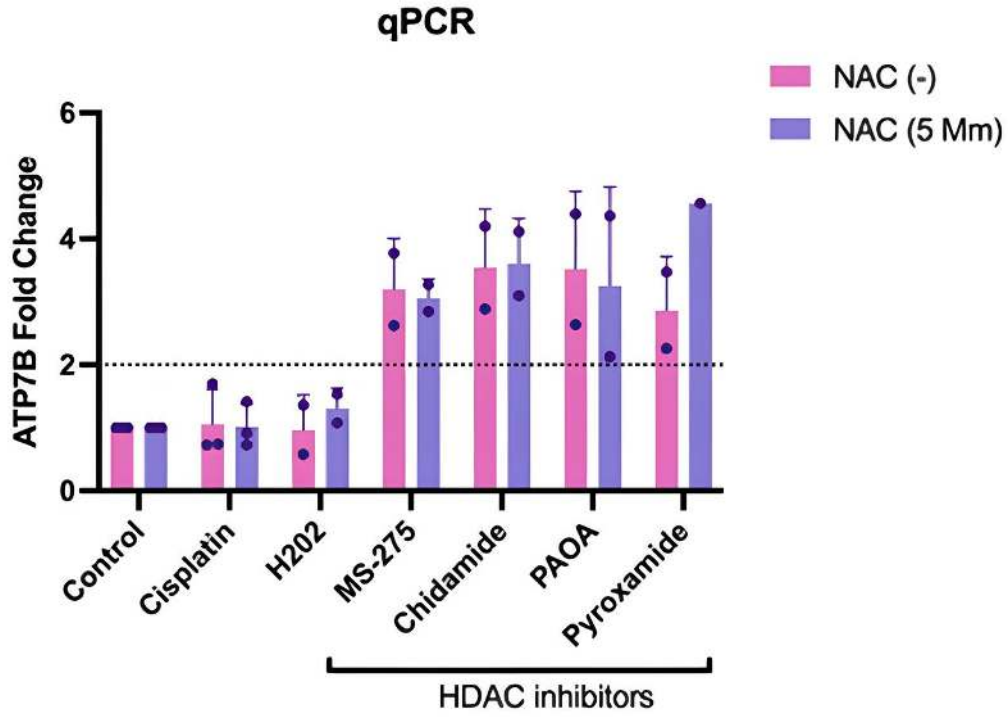



**Figure 2.12. HDAC inhibitor-induced ATP7B upregulation occurs independently of ROS levels.** ATP7B mRNA expression gets increased in cells treated under the same conditions as the ROS activity experiment. HDAC inhibitors significantly elevate ATP7B expression compared to control cells, regardless of NAC treatment. Cisplatin and $H_2O_2$ do not considerably alter ATP7B expression. The lack of an NAC effect on HDAC inhibitor-induced ATP7B expression suggests that ATP7B transcriptional activation happens independently of accumulated ROS levels in cells. Data are presented as mean ± SD from two biological replicates (This figure was generated by Ayca Acar, MSc).

To evaluate whether this regulatory mechanism extends to cancer cells of different tissue origins, we treated two representative cancer cell lines—Huh7 (hepatocellular carcinoma) and Du145 (prostate carcinoma)—with the five HDAC inhibitors identified in the initial screen (Chidamide, KD5170, MS-275, Pyroxamide, and PAOA). In both cell lines, qPCR analysis revealed a significant increase in ATP7B mRNA levels following treatment with each compound, although the magnitude of response varied by cell type and inhibitor (**Figure 2.13**). These findings indicate that the transcriptional activation of ATP7B by HDAC inhibitors is not confined to HEK293T cells but likely reflects a generalizable mechanism of epigenetic derepression that is conserved across diverse cellular contexts.

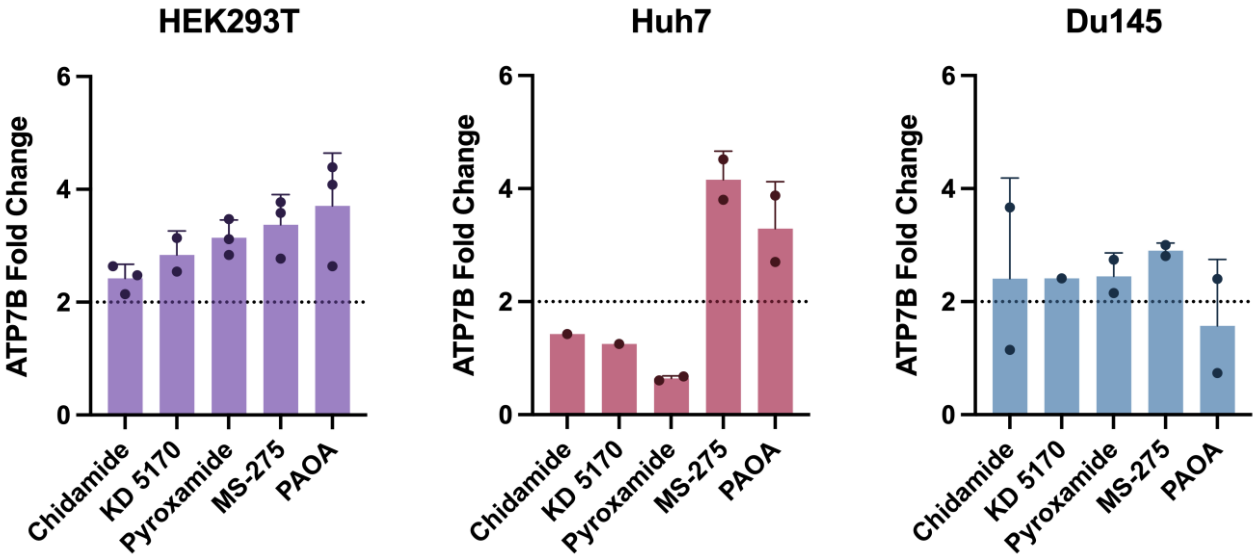



**Figure 2.13. HDAC inhibitors induce ATP7B mRNA expression in a cell line-dependent manner.** ATP7B mRNA fold change following treatment with HDAC inhibitors (Chidamide, KD5170, Pyroxamide, MS-275, and PAOA) in HEK293T, Huh7, and Du145 cell lines. Data are presented as mean ± SD, each biological replicate was shown as individual dot (*p<.05, student's t-test) (Ayca Acar, MSc contributed to these experiments).

Finally, to further validate the specificity of HDAC-mediated regulation, we revisited the activity of BET inhibitors in these cancer cell lines. Treatment of Huh7 and Du145 cells with JQ1, I-BET151, or CPI-203 failed to elicit any significant increase in ATP7B mRNA expression (**Figure 2.14**), thereby reaffirming their classification as non-functional hits in the initial screening assay. Collectively, these findings underscore the specificity of HDAC inhibitors in promoting ATP7B transcription and highlight their potential utility as epigenetic modulators of gene expression in both hepatic and non-hepatic malignancies.

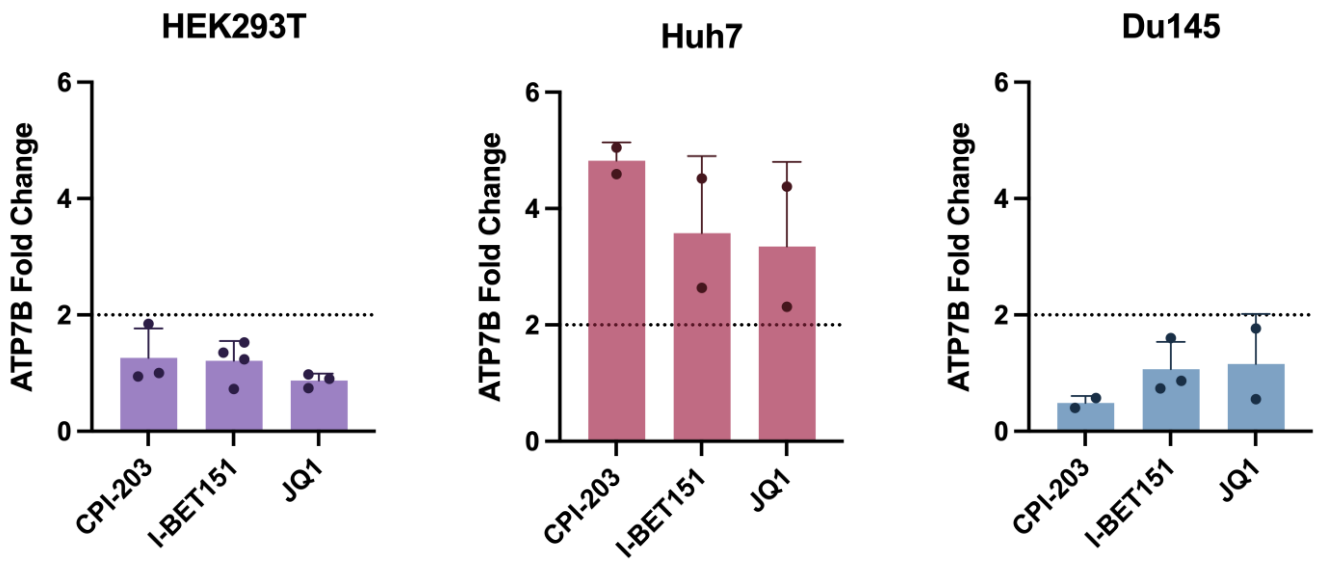



**Figure 2.14. BET inhibitors variably affect ATP7B expression across different cell lines.** ATP7B mRNA fold change following treatment with BET inhibitors (CPI-203, I-BET151, and JQ1) in HEK293T, Huh7, and Du145 cell lines. Data are presented as mean ± SD, each biological replicate was shown as individual dot (*p<.05, student's t-test) (Ayca Acar, MSc contributed to these experiments).

### 3.4. ATP7B Transcriptional Activation by HDAC Inhibitors Is Mediated by Promoter-Specific Histone Acetylation

While our screening approach identified HDAC inhibitors as potent inducers of ATP7B expression, the underlying chromatin-based mechanisms driving this transcriptional activation remained to be elucidated. Given that HDAC inhibitors function by blocking histone deacetylase activity—resulting in hyperacetylation of histone tails and thereby loosening chromatin structure—we hypothesized that these compounds enhance ATP7B expression by directly modulating acetylation patterns at its promoter. Such epigenetic remodeling may facilitate transcription factor recruitment, improve chromatin accessibility, or relieve repressive chromatin states, ultimately leading to enhanced transcriptional output.

To test this hypothesis, we first examined global changes in histone acetylation levels in HEK293T cells following treatment with four of the most effective HDAC inhibitors identified in our screen: Chidamide, MS-275, PAOA, and Pyroxamide. Cells were exposed to each compound for 24 hours, and levels of three key acetylation marks—H3K9ac, H3K18ac, and H3K27ac—were assessed by Western blotting (**Figure 2.15**). These specific marks were selected based on their established roles in transcriptional activation and enhancer-promoter communication. All four compounds led to increased

acetylation across these histone residues, confirming their chromatin-modifying activity. Notably, Chidamide and MS-275 induced the most robust global acetylation, particularly at H3K9ac and H3K27ac, suggesting that these inhibitors possess stronger or more sustained HDAC-blocking activity at the tested concentrations.

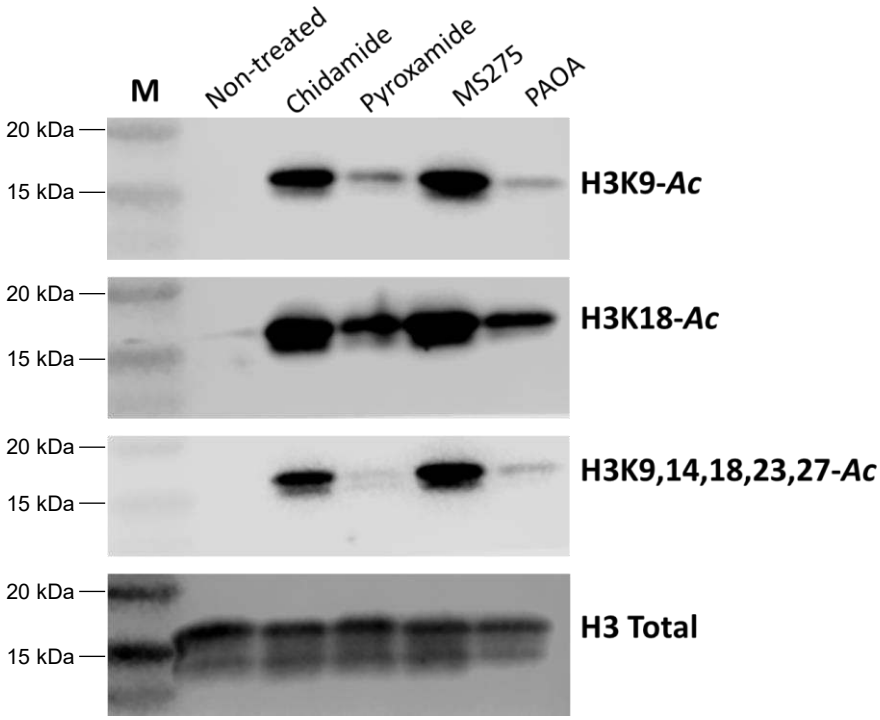



**Figure 2.15. HDAC inhibitors globally increase histone H3 acetylation at multiple lysine residues.** Western blot analysis of global histone acetylation levels following treatment with the HDAC inhibitors MS-275 and Chidamide. Acetylation marks H3K9ac, H3K18ac, and H3K27ac are shown. Total H3 antibody was used as a control. Molecular weight of all target proteins is about 17 kDa. Both MS-275 and Chidamide increase the levels of these acetylation marks, confirming their inhibitory effect on HDAC activity and the associated increase in histone acetylation.

While global acetylation provides initial confirmation of HDAC inhibition, it does not necessarily imply promoter-specific changes that could account for the observed induction of ATP7B. To resolve this, we performed chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) to directly assess acetylation changes at the endogenous ATP7B promoter. We selected Chidamide and MS-275 for these experiments based on their superior transcriptional potency and global histone acetylation effects. HEK293T cells were treated for 24 hours with either compound, followed by ChIP using antibodies specific to H3K9ac, H3K18ac, and H3K27ac. Two independent primer sets were used to interrogate distinct regions within the ATP7B promoter.

The results revealed a non-uniform and selectively enriched pattern of histone acetylations at the promoter (**Figure 2.16**). H3K18ac levels were markedly elevated at both promoter regions upon treatment with either Chidamide or MS-275. This finding is notable, as H3K18 acetylation is frequently associated with active transcription start sites and has been linked to enhanced binding of basal transcription machinery. In contrast, a significant decrease in H3K9ac and H3K27ac levels was observed at the ATP7B promoter following Chidamide treatment, and to a lesser extent with MS-275. This reduction, while seemingly paradoxical in the context of global acetylation increases, may reflect redistribution of these marks away from the ATP7B locus or selective histone turnover at transcriptionally engaged regions. Alternatively, it may suggest that promoter activation is not uniformly dependent on all acetylation marks, but instead driven by specific epigenetic signatures such as H3K18ac.

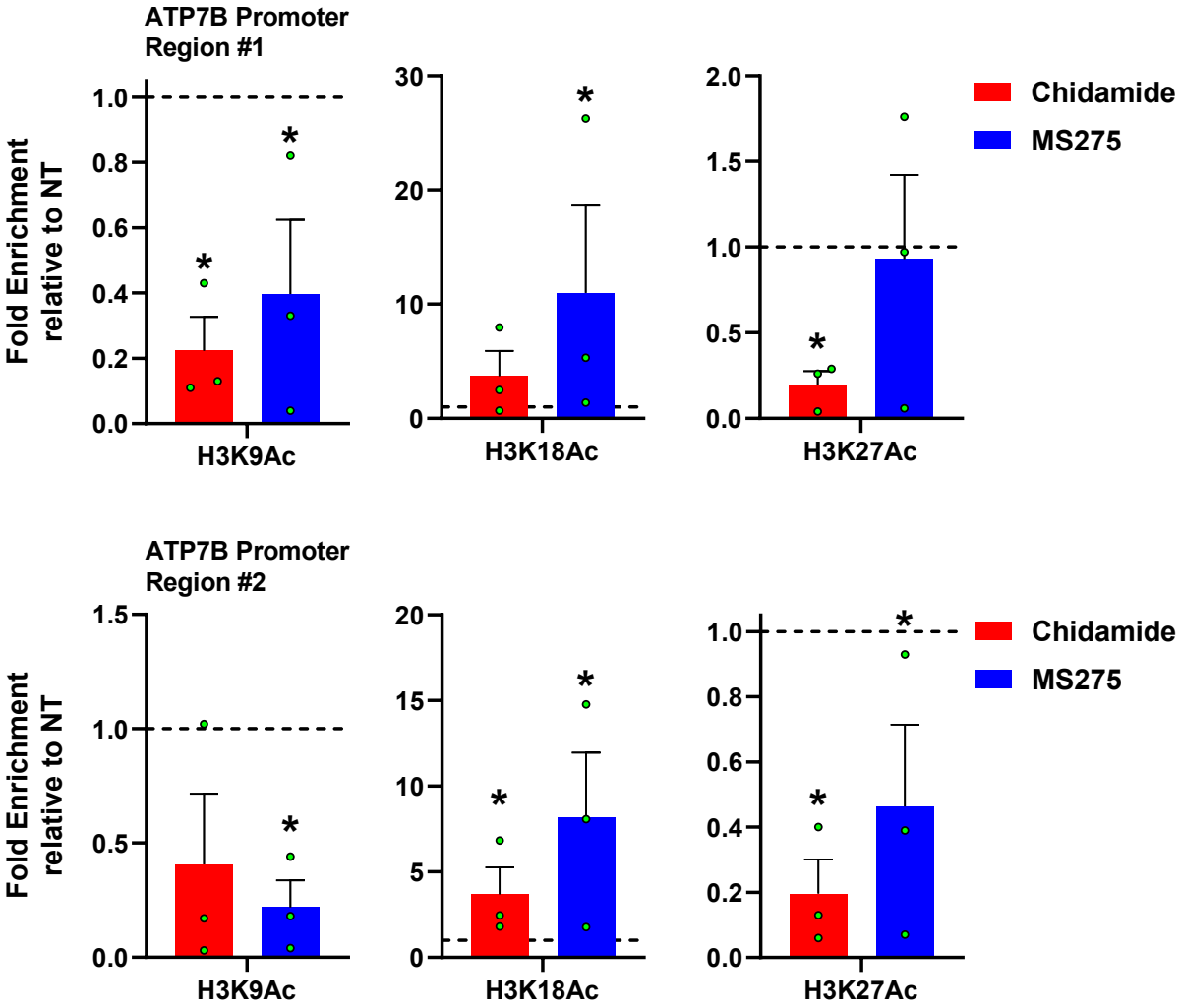


**Figure 2.16. HDAC inhibitors enhance histone H3 acetylation at K18 specific residue at the ATP7B promoter region.** ChIP-qPCR analysis showing the enrichment of acetylation marks H3K9ac, H3K18ac, and H3K27ac at the ATP7B promoter region following treatment with MS-275 and Chidamide. The increased H3K18 acetylation levels suggest that these HDAC inhibitors enhance ATP7B transcription through specific promoter acetylation. Data are presented as mean ± SEM from three biological replicates (*p<.05, one-tailed Mann Whitney-U test).

The observation that HDAC inhibitor treatment induces a distinct and localized enrichment of H3K18ac at the ATP7B promoter provides a mechanistic link between chromatin remodeling and transcriptional activation. This supports a model in which HDAC inhibition promotes a permissive chromatin state at the ATP7B promoter, likely facilitating access by transcriptional activators such as MTF1 or general transcription factors. The differential pattern of histone acetylation also underscores the specificity of epigenetic changes induced by HDAC inhibitors, which do not uniformly affect all promoter regions or histone residues, but rather generate targeted remodeling landscapes that are functionally coupled to gene activation.

Taken together, these findings establish that HDAC inhibitors increase ATP7B expression not merely through indirect signaling cascades or stress responses, but via direct epigenetic modulation of its promoter region. The selective enrichment of H3K18ac highlights this histone modification as a potential key regulator of ATP7B transcription and raises the possibility of exploiting acetylation-specific reader proteins or enhancer elements for future mechanistic dissection. These results provide critical mechanistic validation of the initial screening findings and affirm the chromatin-level basis of ATP7B transcriptional control by HDAC-targeting epidrugs.

*Chapter 2*

*Section 4*

# 4. DISCUSSION

Gene-expression programmes are governed by two inter-dependent layers. Sequence-specific transcription factors bind DNA to initiate or repress transcription, while epigenetic mechanisms—including DNA methylation, histone-tail modifications, ATP-dependent nucleosome remodelling and regulatory non-coding RNAs—alter chromatin structure to fine-tune promoter accessibility (Dawson & Kouzarides, 2012; Fritz et al., 2022). Because most of these marks are reversible, small molecule "epidrugs" that reset inappropriate chromatin states have already reached clinical practice (Mann et al., 2007). Nevertheless, for many clinically relevant loci the specific epigenetic switches that set transcriptional output remain undefined.

A prominent example is ATP7B, the copper-transporting P-type ATPase whose reduced activity causes WD and whose over-expression supports platinum-drug resistance in several tumour types (Komatsu et al., 2000). ATP7B expression is extremely dosage-sensitive: deletion of only fifteen promoter bases or a single-nucleotide change that disrupts the metal-responsive transcription-factor MTF1 lowers mRNA by roughly 75 %, precipitating WD (H. I. Chen et al., 2018; Loudianos et al., 1999); conversely, elevated ATP7B in tumours promotes cisplatin efflux and diminishes DNA damage. These observations highlight a therapeutic paradox: hepatic insufficiency must be corrected, whereas oncogenic over-expression should be curtailed.

Despite its medical importance, the chromatin landscape surrounding ATP7B is largely unexplored. Published datasets provide almost no information on histone marks, DNA methylation or chromatin accessibility at this locus in human tissue, and only isolated reports describe indirect regulation by microRNAs or weak histone-deacetylase inhibitors. Consequently, the mechanisms that keep the promoter open or closed under physiological and pathological conditions remain to be mapped. Closing this gap is essential: epigenetic activation could raise residual ATP7B levels in hypomorphic WD alleles, while transient repression might re-sensitise platinum-resistant tumours during chemotherapy cycles.

To interrogate ATP7B transcription within its native chromatin, this thesis employs a CRISPR/Cas9-mediated genomic reporter. A firefly luciferase cassette was inserted into exon 1 of one ATP7B allele in HEK293T cells, leaving upstream regulatory elements, distant enhancers and higher-order chromatin architecture intact (Rojas-Fernandez et al., 2015). Because only one allele is edited, basal copper handling is preserved; meanwhile, the integrated reporter provides a quantitative, real-time read-out of endogenous promoter activity.

The experimental workflow was structured around four sequential objectives; **(i) Establishment of a chromatin-integrated reporter line;** ATP7B–luciferase knock-in was generated and validated at the genomic, transcriptional and protein levels. **(ii) Systematic screening of an epigenetic-modifier library;** approximately 150 compounds targeting DNA- and histone-modifying enzymes, chromatin readers and remodelers were tested at a single, non-toxic concentration. Luciferase output normalised to total protein identified compounds that altered transcription. **(iii) Molecular validation of lead compounds;** endogenous ATP7B mRNA was measured to confirm reporter fidelity, and locus-specific chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) was used to determine whether histone-mark changes at the promoter accompanied transcriptional shifts. **(iv) Contextual assessment across cell types;** selected hits were examined in hepatic (Huh7) and non-hepatic (Du145) cancer lines to evaluate applicability across disease settings.

The screening phase had no preconceived bias toward any particular epigenetic class; histone deacetylase (HDAC) inhibitors emerged as leading activators only after unbiased library interrogation. This sequence underscores the value of a broad discovery approach and cautions against assuming a single epigenetic axis a priori.

Collectively, the study tests two linked hypotheses: **(i)** that ATP7B transcription can be modulated—upward or downward—by chemical alteration of chromatin state; and **(ii)** that a locus-integrated reporter enables rapid, robust detection of such modulation. By illuminating previously unknown epigenetic control points and identifying tractable compounds, the work lays a foundation for precision strategies aimed at restoring ATP7B function in WD or attenuating it in chemoresistant tumours.

In order to answer these questions in well-structured experimental setup, to determine whether ATP7B transcription can be modulated by chromatin regulators, we adopted a stepwise experimental strategy, with each stage designed to answer a specific mechanistic question and to build toward an integrated understanding of ATP7B epigenetic control.

We began by creating a chromatin-integrated reporter system capable of accurately reflecting ATP7B promoter activity in its native context. Using CRISPR/Cas9, we inserted a firefly luciferase cassette into exon 1 of a single ATP7B allele in HEK293T cells. PCR analysis confirmed precise editing across both the 5′ and 3′ junctions, and loss of the wild-type amplicon confirmed monoallelic integration. Western blotting showed a partial reduction in ATP7B protein, which was consistent with disruption of only one allele. Importantly, luciferase signal was absent in the parental cell line but robust in the knock-in clones, and this signal increased further upon overexpression of MTF1 or the addition of extracellular copper. These results validated that the engineered reporter line preserved native transcriptional regulation and provided a reliable and scalable readout suitable for chemical screening.

To explore which chromatin regulators might influence ATP7B transcription, we next conducted an unbiased screen of approximately 150 small molecules. These compounds targeted a broad range of epigenetic regulators, including writers, erasers, and readers. After 72 hours of treatment at a subtoxic dose, luciferase activity was normalized to total protein content. The screen revealed a consistent increase in reporter signal in response to several histone deacetylase (HDAC) inhibitors, including both class I-selective and pan-HDAC compounds. These results were reproducible across three independent knock-in clones. Although some bromodomain (BET) inhibitors also appeared to increase luciferase activity, they did not affect endogenous ATP7B mRNA levels and were therefore excluded from further analysis. These data indicated that the loss of HDAC activity is a strong inducer of ATP7B transcription, while other epigenetic targets showed little or no influence under the screen conditions tested.

To verify that reporter activation reflected true upregulation of the native gene, we selected the six most active HDAC inhibitors from the screen and tested their effects in wild-type HEK293T cells. All compounds increased endogenous ATP7B mRNA levels by at least twofold, with Chidamide and MS-275 showing the strongest responses and the

steepest dose–response curves. Notably, these effects were achieved without significant cytotoxicity across the concentration range tested. This confirmed that luciferase activity from the knock-in model accurately tracked transcriptional changes at the native locus and that HDAC inhibition produced scalable activation of ATP7B.

To identify the regulatory sequences involved, we investigated whether the proximal ATP7B promoter was sufficient to mediate the effects of HDAC inhibition. A 1 kb promoter fragment was cloned upstream of a luciferase reporter in a plasmid vector and tested in transient assays. This fragment drove strong basal expression, and deletion of MTF1 significantly reduced its activity, confirming that key regulatory elements were preserved. When cells were treated with HDAC inhibitors, luciferase output from the plasmid construct increased, indicating that the proximal promoter is a direct target of HDAC-mediated repression. These findings suggest that the essential response elements lie within the first kilobase upstream of the transcription start site and that HDAC inhibitors exert their effect locally at this region.

Since events related to oxidative stress are known to generate reactive oxygen species (ROS), which can independently alter ATP7B and stress-related gene expression, we examined whether HDAC inhibition via epidrugs are contributed to ROS induction and subsequently affecting the ATP7B expression (in an off-target manner). Of the compounds tested, only Pyroxamide led to detectable increases in ROS levels. However, co-treatment with the antioxidant N-acetyl-cysteine neutralized ROS without dampening ATP7B mRNA upregulation. Moreover, the other HDAC inhibitors did not elevate ROS levels at all. These results confirmed that the observed transcriptional activation of ATP7B was not an indirect consequence of oxidative stress but rather a direct outcome of HDAC inhibition.

Given the physiological and clinical importance of ATP7B in both hepatic function and chemotherapy resistance, we next assessed whether HDAC-induced ATP7B activation was generalizable across cell types. Five leading HDAC inhibitors were tested in two additional cell lines: Huh7, a hepatoma model, and Du145, a prostate cancer model. In both lines, ATP7B mRNA levels increased following treatment, although the magnitude of induction varied by cell type. As in HEK293T cells, BET inhibitors showed no effect. These findings established that the HDAC–ATP7B regulatory axis is conserved

across different cellular backgrounds, supporting its relevance in both hepatic and non-hepatic disease contexts.

Finally, to uncover the underlying mechanism of transcriptional activation, we examined histone acetylation patterns globally and at the ATP7B promoter. Western blotting of bulk histones after 24 hours of HDAC inhibitor treatment revealed global increases in H3K9ac, H3K18ac, and H3K27ac, with Chidamide and MS-275 producing the most pronounced effects. Chromatin immunoprecipitation focused on the ATP7B promoter showed a localized enrichment of H3K18ac, but not H3K9ac or H3K27ac, across two promoter regions. This pointed to H3K18ac as the key histone mark associated with transcriptional activation at the ATP7B locus in response to HDAC inhibition.

Altogether, these findings establish that chromatin regulation plays a direct and significant role in controlling ATP7B transcription. Our engineered CRISPR knock-in system served as a faithful reporter of promoter activity and revealed that HDAC inhibitors—among a wide array of tested compounds—are strong and specific activators of ATP7B expression. This effect is dose-dependent, does not rely on ROS signaling, and is preserved across multiple cell types. Mechanistically, HDAC inhibition leads to local enrichment of H3K18ac at the ATP7B promoter, linking chromatin relaxation directly to transcriptional activation. These insights provide a chromatin-based explanation for how epigenetic drugs can modulate ATP7B expression and offer a foundation for developing targeted therapies in disorders of copper metabolism and drug resistance.

Epigenetic targets found in this study, histone deacetylases (HDACs), are enzymes that remove acetyl groups from histones and other proteins, which generally condenses chromatin and suppresses gene transcription. In studies of WD, class IIa HDACs (HDAC4 and HDAC5) were found to be strongly downregulated in the liver. For example, Sarode et al. showed that in the Atp7b^tx-j^ mouse model (which accumulates copper like WD), HDAC4/5 protein levels were about 5–10 fold lower than in controls, and this was accompanied by large increases in histone H3 acetylation (including H3K9ac and H3K27ac) (Sarode et al., 2021). This HDAC4/5 loss was linked to changes in liver gene expression: genes involved in energy metabolism were misregulated, and key transcription factors PPARα and PPARγ were upregulated in the copper-loaded mice. Notably, dietary or chemical treatments that reduced copper or boosted methyl-donor

availability (choline or penicillamine) partly restored HDAC4/5 levels and normalized H3 acetylation. These findings indicate that in an intact mammalian system HDAC4/5 (class IIa) can be modulated by copper status and in turn influence expression of metabolic genes (though ATP7B itself was not directly measured) (Sarode et al., 2021). In related work, human HepG2 liver cells exposed to high copper showed reduced HDAC5, and lowering copper levels restored HDAC5 in a dose-dependent way (Dev & Hamilton, 2021). Thus far, class I HDACs (e.g. HDAC1/2/3) or class III sirtuins have not been specifically linked to ATP7B transcription. It is worth noting that global HDAC inhibition often does not simply turn on all genes: for example, HDAC3 loss in liver upregulates fat genes but pharmacologic inhibition of HDAC3 did not always de-repress its targets (Sun et al., 2013). In summary, class II HDACs clearly affect the hepatic chromatin environment and can regulate many liver genes (such as nuclear receptors) under copper overload, but there is no report yet of a direct HDAC binding to the ATP7B promoter in human cells.

Indirect mechanisms linking HDACs to ATP7B expression have also been described. HDAC inhibitors are known to alter microRNA and splicing factor levels, which in turn can impact ATP7B transcripts. For example, HDAC inhibitors can modulate noncoding RNAs, including miRNAs (Liu et al., 2025). One study noted that miR-133b has a site complementary to the 3′-UTR of ATP7B mRNA and that low miR-133b correlates with higher ATP7B activity (Karpenko et al., 2023). In Parkinson's patients, reduced miR-133b was linked to higher ceruloplasmin (copper protein) and this miRNA was predicted to repress ATP7B. Because HDAC inhibitors can influence miRNA profiles, this suggests HDACs might indirectly affect ATP7B by regulating miR-133b or similar RNAs (Karpenko et al., 2023; Liu et al., 2025). Another example of indirect regulation comes from splicing: Lin et al. showed that treating cells with p-coumaric acid (a natural HDAC inhibitor) lowered the splicing factor hnRNP A1, which in turn increased skipping of ATP7B exon 12 (Lin et al., 2015). Thus the HDAC inhibitor did not change ATP7B transcription directly, but by downregulating a splicing regulator it produced an alternative, truncated ATP7B mRNA (these experiments were done in CHO cells, a hamster kidney line, but illustrate a plausible indirect route). In other systems, HDACs intersect with transcription factors that control ATP7B: for instance, the Wnt/β-catenin pathway has been shown to bind the ATP7B promoter via TCF4 and activate its transcription, and HDACs often partner with or oppose such factors in gene regulation,

although direct HDAC involvement in that context has not yet been reported (Y. T. Liu et al., 2024).

In summary, direct evidence for HDACs binding the ATP7B promoter is lacking. However, copper overload clearly downregulates class IIa HDACs (HDAC4/5) in liver, leading to broad changes in histone acetylation and gene expression. HDAC activity also indirectly affects ATP7B levels by influencing miRNAs (such as miR-133b) and splicing regulators (hnRNP A1) that target ATP7B transcripts. The role of class I and III HDACs in ATP7B regulation remains unclear from current literature. Overall, HDACs can shape the epigenetic landscape of cells in ways that impact ATP7B expression, but detailed mechanisms -especially in humans- need further study.

Our knock-in reporter and accompanying epigenetic screen constitute a strong proof-of-concept that chromatin enzymes can be systematically interrogated at native gene loci. The model integrates seamlessly with high-throughput workflows, delivers a quantitative read-out, and preserves essential cis-elements absent from plasmid systems. This framework now invites several aspects of exploration as future directions; **1. Isoform-specific HDAC mapping:** Class I HDACs were well represented among active compounds, yet class IIa enzymes are known to shuttle between nucleus and cytoplasm in response to stress. CRISPR affinity-tagging of individual HDACs followed by CUT&RUN could reveal whether distinct isoforms occupy the ATP7B promoter under basal or copper-loaded conditions. **2. Epigenome editing:** dCas9-p300 or dCas9-HDAC fusion proteins targeted to the ATP7B promoter would test whether artificial acetylation or deacetylation suffices to modulate transcription, thereby verifying causality. **3. Clinical translation:** Patient-derived hepatocytes or iPSC-based WD models could be engineered with the same reporter cassette to evaluate HDACi-mediated rescue of hypomorphic alleles. Parallel work in platinum-resistant tumor organoids might clarify whether selective HDAC activation or repression alters chemotherapy response. **4. Chromatin readers:** Because H3K18ac enrichment correlates with transcriptional gain, identifying bromodomain proteins that “read” this mark at ATP7B could yield alternative drug targets that modulate expression with greater specificity than broad-spectrum HDAC inhibitors.

**As per limitations**, several conditions highlight our conclusions. **First**, the work relies primarily on a monoallelic HEK293T model; although the transcriptional activation was validated in Huh7 and Du145 cells, the chromatin landscape in differentiated hepatocytes or in vivo liver tissue may differ. **Second**, the screen used a single 5 μM dose and 72 h window; highly potent or slow-acting compounds might have been missed. **Third**, our ChIP-qPCR interrogated only three acetyl marks; a comprehensive CUT&Tag survey could uncover additional modifications relevant to transcriptional control. **Finally**, luciferase insertion disrupts one ATP7B allele, precluding assessment of long-term copper transport function in the edited cells. Addressing these limitations will strengthen the physiological relevance of our findings and pave the way toward therapeutic applications.

In summary, this chapter of the thesis establishes, for the first time, a direct mechanistic link between histone deacetylase activity and the transcriptional output of ATP7B - a gene at the crossroads of copper metabolism, WD pathology, and platinum-drug resistance in cancer. By harnessing a CRISPR/Cas9-mediated, chromatin-integrated reporter, we revealed that HDAC inhibitors relieve promoter repression, deposit an activating H3K18ac signature, and boost ATP7B expression across disparate cell types. These insights expand the catalogue of epigenetic circuits governing metal-homeostasis genes and spotlight HDACs as tunable modulators of ATP7B dosage.

From a clinical standpoint, selective HDAC inhibition could offer a two-pronged therapeutic strategy: partial transcriptional rescue of hypomorphic ATP7B alleles in WD, and conversely, rational attenuation of ATP7B in platinum-resistant tumors to restore drug efficacy. Beyond ATP7B, the experimental blueprint presented here exemplifies how genome-integrated reporters can unlock chromatin-level regulation of any medically relevant transporter or enzyme.

To sum up, the study elevates epigenetic control from a speculative layer of ATP7B biology to a tractable, druggable reality. By illuminating the chromatin logic that adjusts copper export, we take a decisive step toward precision interventions that recalibrate metal balance and improve therapeutic outcomes in metabolic and oncologic diseases alike.

*Chapter 2*

*Section 5*

# 5. CONCLUSION

Maintaining copper homeostasis is essential for cell survival and physiological function. While insufficient copper can disrupt enzymatic activity, its accumulation is toxic and leads to severe cellular damage. The ATP7B protein, a copper-transporting P-type ATPase, is a critical regulator of this balance. Loss-of-function mutations in ATP7B cause WD, a rare disorder marked by hepatic and neurological copper overload. Conversely, high ATP7B expression has been linked to resistance against platinum-based chemotherapy in certain cancer types. Although the genetic and transcriptional regulation of ATP7B has been well studied—particularly through metal-responsive elements like MTF1—the role of chromatin-level mechanisms remained largely undefined. Chapter 2 of this thesis addressed this gap by investigating whether ATP7B transcription is regulated by epigenetic mechanisms, with a focus on histone deacetylases (HDACs), and whether such regulation could be therapeutically modulated.

To achieve this goal, a CRISPR/Cas9-mediated knock-in model was developed, where a firefly luciferase cassette was inserted into exon 1 of a single ATP7B allele in HEK293T cells. This genome-integrated reporter maintained the native upstream regulatory elements, allowing luciferase expression to directly reflect transcriptional activity at the endogenous ATP7B promoter. Using this model, a chemical screen of approximately 150 epigenetic compounds was conducted, covering a broad range of histone and DNA-modifying enzymes. All compounds were tested at subtoxic concentrations over a 72-hour period. Key findings from the screen were validated by measuring endogenous ATP7B mRNA levels, evaluating promoter responsiveness in a plasmid-based reporter assay, mapping histone acetylation patterns by ChIP-qPCR, and confirming reproducibility in two additional cell lines: Huh7 and Du145.

Among all tested compounds, HDAC inhibitors emerged as the only group that robustly increased both luciferase output and native ATP7B transcription. Five inhibitors—Chidamide, MS-275, KD5170, Pyroxamide, and PAOA—showed clear, dose-dependent effects. A one-kilobase region upstream of the ATP7B transcription start site was found sufficient to mediate this activation, suggesting that the necessary

regulatory elements lie within this proximal promoter. Although global acetylation of several histone marks increased upon HDAC inhibition, only H3K18ac showed specific enrichment at the ATP7B promoter, pointing to a local chromatin change linked to transcriptional activation. This effect was not dependent on oxidative stress and was observed in both hepatic and non-hepatic cell lines, indicating a broadly conserved regulatory mechanism.

These findings establish that ATP7B is not only regulated by classical DNA-binding transcription factors but also by reversible chromatin states. Under baseline conditions, HDACs appear to repress ATP7B by limiting histone acetylation at its promoter. Inhibition of HDAC activity allows for acetylation at H3K18, a modification known to promote open chromatin and transcriptional accessibility. This marks the first direct evidence that HDAC-mediated deacetylation controls ATP7B promoter activity in mammalian cells. In addition to providing mechanistic insight, the study introduces a powerful experimental tool—a genome-integrated, functional reporter system—that enables quantitative and high-throughput interrogation of chromatin regulation at endogenous loci.

From a translational standpoint, these findings suggest dual therapeutic possibilities. In WD, many patients carry hypomorphic ATP7B alleles that retain partial function but are expressed at insufficient levels. Selective HDAC inhibition might restore ATP7B transcription to near-physiological levels, offering a pharmacological alternative to gene therapy. Conversely, in cancers where ATP7B overexpression contributes to platinum resistance, carefully modulated HDAC inhibition or targeted chromatin editing might suppress expression and resensitize cells to chemotherapy. These directions warrant further validation in patient-derived models and in vivo systems.

Despite the strength of the findings, several limitations must be acknowledged. The screen was conducted at a single concentration and time point, potentially missing drugs with delayed or more subtle effects. The ChIP analysis focused on only three histone marks; additional chromatin features, such as DNA methylation or nucleosome positioning, may also influence ATP7B regulation. Furthermore, the monoallelic reporter model does not allow for direct assessment of ATP7B's copper-transport function, as one allele remains unmodified. Future studies could benefit from broader chromatin mapping,

compound titration across longer time courses, and the development of biallelic or conditional reporter systems in hepatic models.

In conclusion, this study reveals that ATP7B transcription is directly controlled by HDAC-mediated chromatin remodeling and identifies H3K18ac as a key histone mark associated with its activation. The CRISPR knock-in reporter system proved to be a reliable and scalable platform to study epigenetic regulation in a chromatin-competent context. The work not only advances the fundamental understanding of ATP7B biology but also opens new therapeutic avenues for diseases linked to its dysregulation. By demonstrating that chromatin modifiers can fine-tune ATP7B expression, this chapter positions epigenetic control as a promising, druggable layer in the regulation of copper metabolism and chemoresistance.

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