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# Preface



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# List of Abbreviations and Symbols

<b>TFR1</b>	transferrin receptor 1 . . . . .	3
<b>GSH</b>	glutathione . . . . .	4
<b>ROS</b>	reactive oxygen species . . . . .	1
<b>GPX4</b>	glutathione peroxidase 4 . . . . .	4
<b>ONT</b>	Oxford Nanopore Technology	
<b>RCD</b>	regulated cell death . . . . .	3
<b>PUFA</b>	poly-unsaturated fatty acid . . . . .	3
<b>5mC</b>	5-methylcytosine . . . . .	6



# Chapter 1

## Abstract

Cancer cells exhibit a characteristic ability to evade programmed cell death, a feature critical for their uncontrolled proliferation. An emerging strategy in cancer therapy involves targeting alternative forms of cell death, distinct from apoptosis, as evidenced by recent studies (Tait, Ichim, and Green, 2014). Non-apoptotic cell death mechanisms, including necroptosis and pyroptosis, offer promising avenues to circumvent apoptosis resistance in tumors, thus enhancing the efficacy of anti-cancer therapies (Ye et al., 2018).

One such non-apoptotic pathway, ferroptosis, reliant on iron and reactive oxygen species (ROS), represents a novel approach in cancer treatment. However, inherent resistance to ferroptosis induction poses a challenge in its therapeutic application (Zou and Schreiber, 2020). Detecting drug resistance before treatment initiation could significantly impact treatment outcomes (Volm and Efferth, 2015).

Epigenetic modifications, such as DNA methylation, RNA methylation, and non-coding RNAs, have emerged as key regulators of cellular processes. Notably, DNA methylation alterations in genes associated with ferroptosis, including GPX4, FSP1, and NRF2, influence their expression levels, potentially modulating ferroptosis susceptibility (X. Wang et al., 2023).

Bisulfite genomic sequencing is widely utilized for DNA methylation detection, despite its limitations. Challenges such as incomplete bisulfite conversion or assay failures may compromise data accuracy (Li and Tollefsbol, 2011; Heiss and Just,

2018).

Recent advancements in sequencing technologies, such as nanopore sequencing, offer improved accuracy and resolution compared to traditional methods. Nanopore episequencing, in particular, shows promise in refining genetic associations and identifying novel loci previously undetected by microarray platforms (Gombert et al., 2023; Flynn et al., 2022).

In this project, we will aim to develop a machine learning model which, based on a nanopore episequencing profile, will be able to predict the likelihood of ferroptosis resistance for a given tumor strain. The model's capacity to generalize to other (un-trained) strains will be tested. Lastly, we will identify specific methylation changes brought about by ferroptosis-inducing drugs by sequencing sensitized strains.



# Chapter 2

## Introduction

### 2.1 The Occurrence of Ferroptosis

The exploration of ferroptosis has unveiled a multitude of cellular factors and potential regulatory pathways. At its core, it is an intracellular, non-apoptotic, iron-dependent form of regulated cell death (RCD) that is distinct from apoptosis, necrosis, and autophagy (Yan et al., 2021). As the name suggests, this phenomenon is principally driven by iron molecules absorbed by cells from the surrounding extracellular environment. One such receptor is transferrin receptor 1 (TFR1), which mediates endocytosis of diferric transferrin (S. Wang et al., 2019). This intracellular iron is often transported and stored in storage proteins such as ferritin, aiding in maintaining iron homeostasis and metabolism (Knovich et al., 2009).

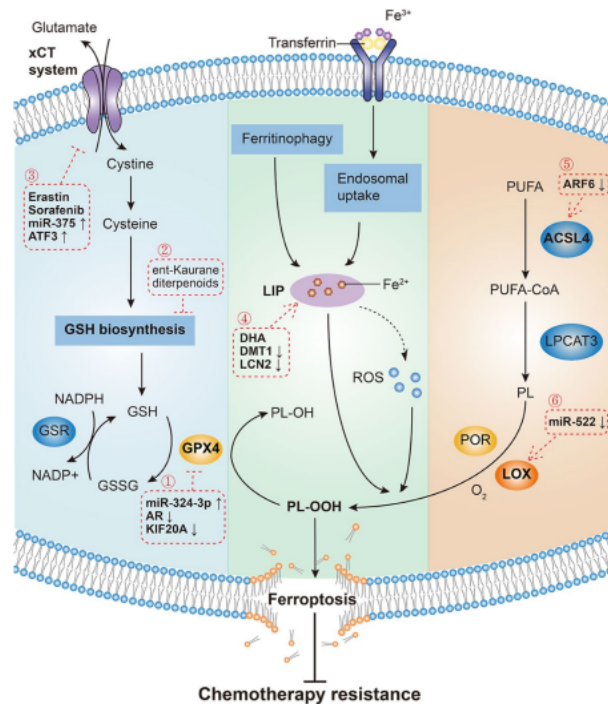
Labile iron, however, which refers to loosely bound or chelatable iron, becomes a critical player in the ferroptotic process. Labile iron participates in the Fenton reaction, leading to the generation of ROS, particularly hydroxyl radicals (Cabantchik, 2014). ROS, in turn, contributes to oxidative stress within the cell.

Poly-unsaturated fatty acids (PUFAs) in cellular membranes are highly susceptible to oxidative damage by ROS (Ayala, Muñoz, and Argüelles, 2014). Lipid peroxides are formed as a result of the oxidation of these fatty acids. Accumulation of lipid peroxides disrupts the integrity of cellular membranes, particularly the lipid bilayer (Gaschler and Stockwell, 2017). Membrane damage leads to the loss of compart-

mentalization and function within the cell, including the mitochondrion (Ademowo et al., 2017).

Healthy mammalian cells stave off this threat in a number of ways. The primary cellular mechanism of protection against oxytosis/ferroptosis is mediated by glutathione peroxidase 4 (GPX4), a glutathione-dependent hydroperoxidase that converts lipid peroxides into non-toxic lipid alcohols (Xie et al., 2023). glutathione (GSH) itself, which is produced from cysteine, is able to neutralize ROS present within the cell directly, playing the role of antioxidant (Averill-Bates, 2023).

Even when GPX4 functionality is uncompromised, research has shown that the mitochondrion plays a substantial role in ferroptosis regulation and induction, being the major compartment for cellular iron metabolism (Liu et al., 2023). Mitochondrial shrinkage and rupture are also common observations when a cell undergoes ferroptosis, since this organel is enveloped in a membrane (Gao et al., 2019).



**Figure 2.1:** Figure depicting the various regulatory pathways associated with ferroptosis (Zhang et al., 2022a).

## 2.2 The Prospect of Ferroptosis in Cancer Therapy

Cancer is the second leading cause of death worldwide, behind cardiovascular disease (H. Wang et al., 2016). However, improvements in cancer survival rates have occurred due to the increasing use of precision medicine or immunotherapy.

The prevalence of acquired resistance to targeted therapy underscores the urgent need for effective strategies to combat drug resistance. Recent findings highlight the significant role of ferroptosis in cancer therapy, disrupting target molecules and influencing cancer progression. Furthermore, studies have indicated that leveraging ferroptosis may offer a promising avenue to overcome resistance to targeted therapy (Zhang et al., 2022b).

Ferroptosis is commonly acknowledged as a tumor suppressor mechanism. Research has demonstrated that the inactivation of the p53 tumor suppression pathway, implicated in the genesis of most human cancers, is correlated with the suppression of ferroptosis (Jiang et al., 2015). Activation of p53 obstructs cystine uptake via the cystine/glutamate antiporter, thereby reducing intracellular GSH levels and shielding tumor cells from ferroptosis.

The synthesis and validation of ferroptosis inducers through various *in vivo* experiments underscore their potential as a viable anticancer therapy for clinical use pending extensive population validation. Consequently, numerous investigations have delved into the development of diverse inducers to trigger ferroptosis, examining its efficacy in various cancer treatment modalities including chemotherapy, immunotherapy, radiotherapy, nanotherapy, SDT, and PDT.

Tumor drug resistance arises from a multitude of mechanisms, with the disruption of redox homeostasis emerging as a pivotal factor. Tumor cells bolster their resilience against oxidative stress by curtailing their own production of acsROS, fostering the development of acquired drug resistance (Yang et al., 2015).

## 2.3 DNA Methylation and its Role in Cancer

Recent advancements in next-generation sequencing techniques applied to cancer genomes have unveiled a prevalent pattern of mutations recurring in diverse epigenetic modulators. These modulators encompass mediators of DNA methylation, covalent histone modifiers, and genes encoding subunits of chromatin remodelers (You and Jones, 2012). The aberrant functioning of these pivotal epigenetic regulators leads to the dysregulation of gene expression and is implicated in a myriad of malignancies, spanning various cancer types (Hanahan and Weinberg, 2011).

## 2.4 DNA Methylation Detection Techniques

Bisulfite genomic sequencing stands as the benchmark technology for detecting DNA methylation due to its ability to offer qualitative, quantitative, and highly efficient insights into identifying 5-methylcytosine at single base-pair resolution. Initially pioneered by Frommer et al., 1992, this method capitalizes on the discovery that the amination reactions of cytosine and 5-methylcytosine (5mC) yield markedly distinct outcomes following treatment with sodium bisulfite.

In this context, cytosines present in single-stranded DNA undergo conversion into uracil residues, which are subsequently recognized as thymine during PCR amplification and sequencing. However, 5mCs remain unaffected by this conversion, retaining their cytosine identity and enabling the distinction between methylated and unmethylated cytosines (Li and Tollefsbol, 2011).

Following bisulfite treatment, a subsequent PCR step becomes imperative to ascertain the methylation status at specific loci, employing methylation-specific primers. The actual methylation status can then be determined through either direct sequencing of PCR products (for the detection of average methylation status) or subcloning sequencing (for the detection of the distribution of methylation patterns at the single-molecule level) (Li and Tollefsbol, 2011).

Despite being the current gold standard, bisulfite sequencing is not without its flaws. Understanding bisulfite-treated DNA data requires careful consideration

of two types of conversion errors: failed conversion and inappropriate conversion. Failed conversion, extensively studied, arises when an unmethylated cytosine fails to deaminate, resulting in its appearance as methylated in sequencing data. In mammalian somatic cells, where 5-methylcytosine primarily occurs at CpG cytosines, the frequency of failed conversion is indicated by the proportion of non-CpG cytosines appearing as cytosines in sequence data (Bird, 1992). Ignoring failed conversion in data analysis can inflate methylation density estimates and impede the determination of DNA methyltransferase sequence motif preferences.

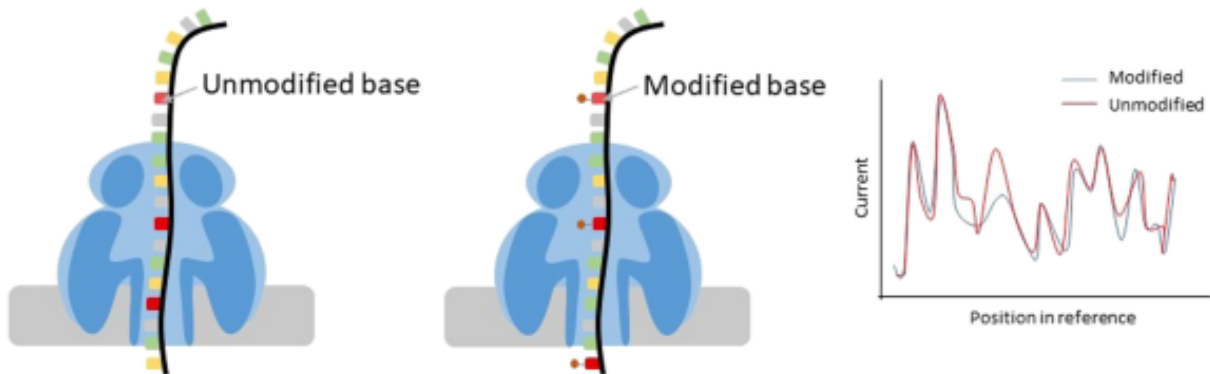
Inappropriate conversion, the second type of error, occurs when a methylated cytosine undergoes deamination, yielding thymine (Shiraishi and Hayatsu, 2004). Similar to uracils resulting from cytosine deamination, thymine pairs with adenine during PCR. Consequently, 5-methylcytosines subjected to inappropriate conversion are misinterpreted as unmethylated. Failure to account for inappropriate conversion leads to underestimation of genomic methylation densities. However, if the frequency of inappropriate conversion is known, it can be integrated as a parameter in data analysis. Therefore, information on both failed and inappropriate conversion frequencies is crucial for drawing meaningful insights from detailed DNA methylation patterns.

In addressing the limitations of bisulfite sequencing, new techniques have emerged despite its status as the gold standard for detecting methylation marks. These limitations encompass the aforementioned confounding effects of bisulfite treatment on DNA methylation, alongside the challenge of distinguishing between DNA methylation and hydroxylation (López-Catalina et al., 2024). In light of these considerations, we advocate for the utilization of Nanopore sequencing as a promising alternative for identifying methylation marks.

Nanopore sequencing is a next-generation sequencing technology that enables the real-time analysis of DNA or RNA molecules as they pass through a nanoscale pore. This technique is based on the principle that as a DNA strand translocates through a nanopore, changes in the electrical current can be measured and used to deduce the sequence of the DNA molecule Y. Wang et al., 2021.

The raw electrical signal is translated into DNA sequence information through a

process called basecalling. DNA methylation involves the addition of a methyl group to a cytosine base, commonly referred to as a 5-methylcytosine. Nanopore sequencing can detect these modifications as changes in the electrical current during translocation using specialized algorithms and basecallers (Fig. 2.2).



**Figure 2.2:** Direct base modification detection with ONT.

One of the key advantages of nanopore sequencing is its ability to generate long reads, providing information about entire genomic regions in a single continuous sequence. Short-read sequencing offers a cost-effective and accurate solution, bolstered by a diverse array of analysis tools and pipelines (Heather and Chain, 2016). However, the vast range of lengths spanning natural nucleic acid polymers, extending over eight orders of magnitude, presents a challenge when sequencing them in short amplified fragments, hindering the reconstruction and quantification of the original molecules.

Long reads, on the other hand, hold the potential to enhance de novo assembly, ensure mapping accuracy, facilitate transcript isoform identification, and enable the detection of structural variants. Moreover, the sequencing of native molecules, both DNA and RNA, using long reads, eliminates amplification bias while retaining base modifications (Depledge et al., 2019). These capabilities, coupled with ongoing advancements in accuracy, throughput, and cost reduction, are establishing long-read sequencing as a viable option for a wide spectrum of genomic applications in both model and non-model organisms (Yuan et al., 2017).

Adaptive sampling is a feature that helps optimize the sequencing process by adjusting data collection parameters in real-time based on the characteristics of the DNA being sequenced. The system monitors various parameters such as the qual-

ity of the signal, the speed at which the DNA is translocating through the nanopore, and other relevant metrics (Martin et al., 2022). The software requires a user to upload a file of whitelisted reference sequences, and the system can be set to either deplete or enrich for these on a specified set of channels.

In order to achieve this, the software basecalls the first few hundred bases of each read and compares it with the target reference sequences. Matching or unmatching sequences are rejected, depending on whether the software is set to enrich or deplete. By using this, coverage of our genes of interest is enriched, greatly reducing noise.





# **Chapter 3**

## **Aims**

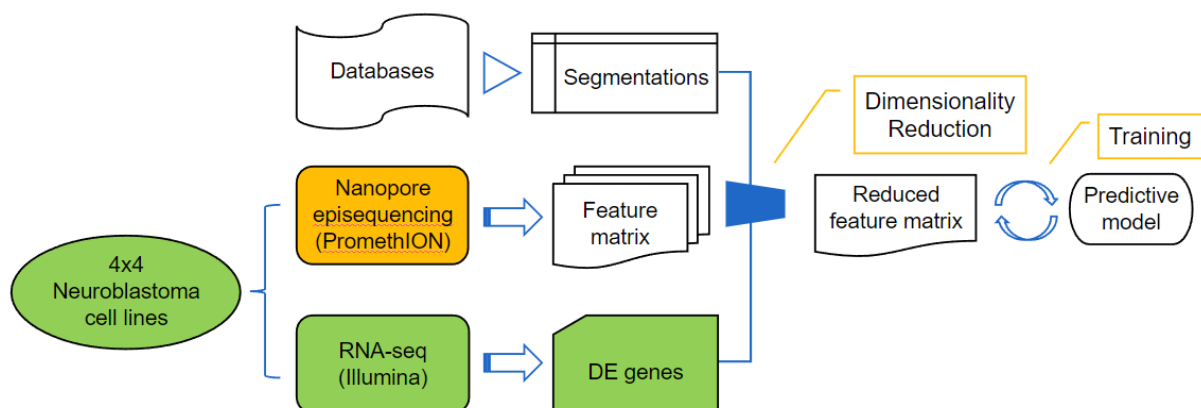


# Chapter 4

## Methods

### 4.1 Introduction

Firstly, differential gene expression analysis was performed. Then, feature reduction of the base-level modification information obtained from ONT episequencing was carried out. Finally, we trained a predictive model using the reduced feature matrix.



**Figure 4.1:** Overview workflow.

## 4.2 Samples

To start off, expression data from 4 cell lines was gathered, namely 2 resistant and 2 sensitive neuroblastoma cell lines in quadruplicate (16 samples in total). These cell lines were characterized in terms of sensitivity towards ferroptosis beforehand experimentally. Furthermore, neuroblastoma is highly iron addicted which makes it exceptionally vulnerable to ferroptosis (Floros et al., 2021). Metadata for each cell line can be found in Table 4.1.

Cell Line	Tumor Type	Ferroptosis Sensitivity	Gender	Age (months)
IMR-32	Neuroblastoma	Sensitive	Male	13
SH-EP	Neuroblastoma	Sensitive	Female	48
SH-SY5Y	Neuroblastoma	Resistant	Female	48
SK-N-BE(2)-C	Neuroblastoma	Resistant	Male	26

**Table 4.1:** Cell Line Metadata

## 4.3 Gene Whitelist

Genes thought to be associated with ferroptosis were compiled into a custom gene whitelist. The full list can be found in Appendix X.

## 4.4 RNA-seq

## 4.5 ONT-seq

For the episequencing, a PromethION nanopore sequencing device based at OHMX.bio was used, containing 24 channels per flow cell. PromethION offers a significantly higher number of channels compared to other ONT platforms like MinION and GridION. This high channel count enhances the platform's capacity for parallel sequencing.

From the whitelist, a BED file was constructed. Additionally, each gene was extended by 15kB, after which overlapping genes were joined together.

Library prep involved following protocol SQK-NBD114.24, describing how to carry out native barcoding of genomic DNA (gDNA) using the Native Barcoding Kit 24 V14. Raw signal data in pod5 format was basecalled with a SUP model and 5(h)mC calling in CG context using GPU hardware on a GridION unit installed at OHMX.bio, and then subsequently demultiplexed. The basecalling model was set to fast, with the intent of making the decision of whether to reverse the voltage of the pore as fast as possible. The resulting unmapped bam files were then aligned against a reference genome, and finally sorted and indexed using SAMtools (Danecek et al., 2021).

## **4.6 Differential Gene Expression Analysis**

## **4.7 Feature Reduction**

Since the episequencing returns many thousands of individual detected CpGs by locus, the number of features in our model will be staggering if used in its raw form. The first reduction of features will be actualized by bundling CpG sites into regions (e.g. CpG islands). A second dimensionality reduction will also be performed based on drivers derived from expression data, to remove sites or regions without genes that are differentially expressed by sensitivity.



# **Chapter 5**

## **Results**





# **Chapter 6**

## **Discussion**



# **Appendix A**

## **Appendix**



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