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Preface

Contents

Preface	v
1 Abstract	1
2 Introduction	3
2.1 The Occurrence of Ferroptosis	3
2.2 The Prospect of Ferroptosis in Cancer Therapy	7
2.3 DNA Methylation and its Role in Cancer	8
2.4 DNA Methylation Detection Techniques	10
3 Aims	15
4 Methods	17
4.1 Introduction	17
4.2 Samples	17
4.3 Gene Whitelist	18
4.4 RNA-seq	18
4.5 ONT-seq	18
4.6 Differential Gene Expression Analysis	19
4.7 Feature Engineering	19
4.8 Feature Reduction	19

5 Results	21
5.1 Differential Gene Expression Analysis	21
5.2 Feature Engineering	21
5.3 Feature Reduction	21
6 Discussion	25
A Appendix	27

List of Figures

2.1	Lipid Peroxidation Mechanism. (1) Initiation begins when R (an reactive oxygen species (ROS)) abstracts a hydrogen from the highlighted region. The carbon-centered lipid radical is stabilized by molecular rearrangement, forming a conjugated diene. (2) In propagation, lipid radicals rapidly react with oxygen to form a lipid peroxy radical. (3) Propagation continues with the lipid peroxy radical abstracting a hydrogen from another lipid molecule. This generates another unsaturated lipid radical as well as a lipid hydroperoxide. (4) Termination of the radical reaction occurs when antioxidants donate a hydrogen atom to the lipid peroxy radical species, forming nonradical products. However, termination is not possible in the absence of radical species. Therefore, propagation will continue until there is a radical sink. Figure created using BioRender and redrawn from Ayala, Muñoz, and Argüelles, 2014	5
2.2	Overview of the various regulatory pathways associated with ferroptosis (C. Zhang et al., 2022a).	6
2.3	Direct base modification detection with Oxford Nanopore Technology (ONT).	12
4.1	Overview workflow.	17
5.1	Volcano Plot	21
5.2	MA-Plot	22

5.3	PCA	22
5.4	Pval dist	23

List of Tables

4.1 Cell Line Metadata	18
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List of Abbreviations and Symbols

5caC 5-carboxylcytosine	9
5fC 5-formylcytosine	9
5hmC 5-hydroxymethylcytosine	9
5mC 5-methylcytosine	9
CGI CpG island	9
CoQ10 ubiquinone	6
CoQ10H2 ubiquinol	6
CpG cytosine-guanine	8
DNMT DNA methyltransferase	8
FSP1 Ferroptosis Suppressor Protein 1	5
gDNA genomic DNA	18
GPX4 glutathione peroxidase 4	4
GSH glutathione	4
I-Cys2 I-cystine	5
I-CysH I-cysteine	4
I-Glu I-glutamate	5
ONT Oxford Nanopore Technology	ix
PCR poly-chain reaction	10

PDT	photodynamic therapy	8
PUFA	poly-unsaturated fatty acid	4
RCD	regulated cell death	3
ROS	reactive oxygen species	ix
SAM	S-adenosyl-L-methionine	8
SDT	sonodynamic therapy	8
SxC-	System xc-	5
TET	ten-eleven translocation	9
TFR1	transferrin receptor 1	3
TDG	thymine DNA glycosylase	9

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 (J. Ye et al., 2018).

One such non-apoptotic pathway, ferroptosis, reliant on iron and 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

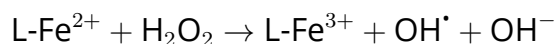
2.1 The Occurrence of Ferroptosis

The exploration of ferroptosis has unveiled a multitude of cellular factors and potential regulatory pathways. At its core, it is defined as 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. An important aspect of iron's function is its ability to undergo cyclic oxidation and reduction, participating in a wide array of metabolic processes, playing crucial roles in oxygen transport, DNA synthesis, and electron transport (Lieu et al., 2001). However, this redox activity can generate free radicals and other strongly oxidizing species, capable of causing a wide range of biological injury. Among these unwanted activities, labile iron participates in what's known as the Fenton reaction.

The Fenton reaction, originating from observations by Fenton in the 1880s, involves

iron and hydrogen peroxide to generate a strong oxidant. It has been extensively studied as a source of hydroxyl radicals and an initiator of biological damage (Im-lay, 2003). In its simplest form, when iron is complexed to ligand L, it can be represented as:



As a result of this reaction, excessive concentrations of labile iron leads to the generation of ROS, particularly hydroxyl radicals (Cabantchik, 2014). ROS, in turn, contribute to oxidative stress within the cell, resulting in oxidative damage to vital macromolecules such as DNA, proteins, and lipids (L. A. Pham-Huy, He, and C. Pham-Huy, 2008).

Poly-unsaturated fatty acids (PUFAs), core components of 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. Lipid peroxidation undergoes three distinct phases: initiation, propagation, and termination (Yin, Xu, and Porter, 2011). Once initiated, lipid peroxidation sets off a cascade of chain reactions that persist until termination products are generated, which only occurs upon the intervention of an antioxidant molecule. A summary of the lipid peroxidation mechanism can be found on Fig. 2.1.

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 compartmentalization and function within the cell, including the mitochondrion (Ademowo et al., 2017). Eventually, the cell membrane will rupture, resulting in cell death.

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 l-cysteine (l-CysH), is able to neutralize ROS present within the cell directly, playing the role of antioxidant (Averill-Bates, 2023). For an overview

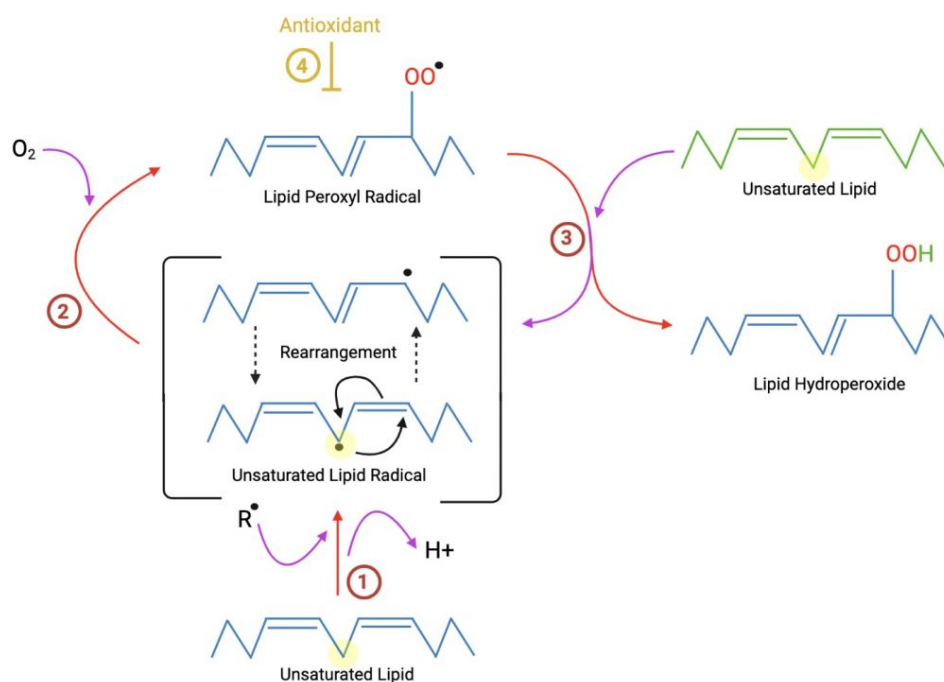


Figure 2.1: Lipid Peroxidation Mechanism. (1) Initiation begins when R (an ROS) abstracts a hydrogen from the highlighted region. The carbon-centered lipid radical is stabilized by molecular rearrangement, forming a conjugated diene. (2) In propagation, lipid radicals rapidly react with oxygen to form a lipid peroxy radical. (3) Propagation continues with the lipid peroxy radical abstracting a hydrogen from another lipid molecule. This generates another unsaturated lipid radical as well as a lipid hydroperoxide. (4) Termination of the radical reaction occurs when antioxidants donate a hydrogen atom to the lipid peroxy radical species, forming nonradical products. However, termination is not possible in the absence of radical species. Therefore, propagation will continue until there is a radical sink. Figure created using BioRender and redrawn from Ayala, Muñoz, and Argüelles, 2014

of the various co-dependent pathways and effects classically associated with ferroptosis, see Fig. 2.2.

System xc⁻ (Sx⁻C⁻), an amino acid antiporter, primarily facilitates the exchange of extracellular l-cystine (l-Cys₂) and intracellular l-glutamate (l-Glu) across the cellular plasma membrane. Studies initially emphasized the transport of l-Cys₂ influx, as in numerous cells, this process acts as a rate-limiting step for supplying intracellular l-CysH essential for GSH synthesis (Bridges, Natale, and Patel, 2012).

The Xc⁻/GSH/GPX4 axis functions as a GSH-dependent defense mechanism against ferroptosis, representing the most crucial and well-studied antioxidant system for resisting this form of cell death (Y. Ye et al., 2022). However, in certain cell types or lines, inhibiting this system alone isn't sufficient to trigger ferroptosis, suggesting the existence of alternative pathways (Doll, Freitas, et al., 2019). One such mechanism involves the GSH-independent enzyme Ferroptosis Suppressor Protein

1 (FSP1), which operates alongside GPX4, serving as a key regulator of ferroptosis (M. Yang et al., 2022). Positioned at the plasma membrane with myristoylation, FSP1 utilizes NADH/NADPH as a cofactor to reduce ubiquinol (CoQ10) to its active form, ubiquinol (CoQ10H₂). While CoQ10 mainly resides within the inner mitochondrial membrane, CoQ10H₂ acts as a lipophilic antioxidant, scavenging free radicals to prevent lipid peroxidation.

Beyond these key proteins and enzymes, it has been proven that the mitochondrion plays a substantial role in ferroptosis regulation and induction, being the major compartment for cellular iron metabolism (Liu et al., 2023). Ferroptosis is characterized by significant morphological alterations in mitochondria such as fragmentation and cristae enlargement (Doll, Proneth, et al., 2017), is closely linked with cellular mechanisms. Interestingly, certain potent ferroptosis inhibitors demonstrate a remarkable specificity towards mitochondria (Krainz et al., 2016). Mitochondrial shrinkage and rupture are also common terminal observations when a cell undergoes ferroptosis, since this organelle is enveloped in a membrane (Gao et al., 2019).

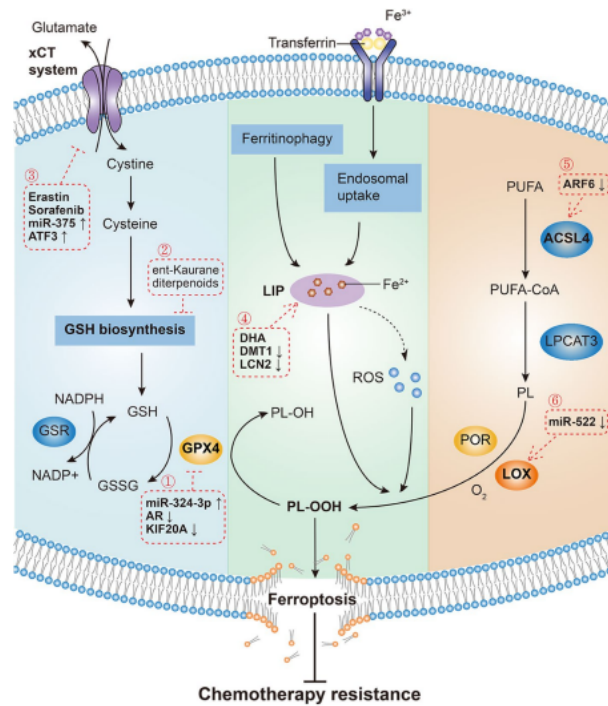


Figure 2.2: Overview of the various regulatory pathways associated with ferroptosis (C. Zhang et al., 2022a).

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). Ongoing global research efforts have led to notable enhancements in cancer survival rates over the years. Despite these significant advancements in cancer treatment, including surgery, radiation therapy, chemotherapy, hormone therapy, targeted therapy, and immunotherapy (Debela et al., 2021), resistance to these therapies remains a prevalent challenge in clinical practice (Bukowski, Kciuk, and Kontek, 2020).

The phenomenon of drug resistance, where diseases develop tolerance to pharmaceutical treatments, initially emerged with antibiotic-resistant bacteria. However, this concept extends beyond bacteria to encompass various diseases, including cancer. While some resistance mechanisms are disease-specific, others, like drug efflux, occur across microbes and drug-resistant cancers, indicating evolutionary conservation. Despite initial susceptibility to chemotherapy, many cancers eventually develop resistance through mechanisms such as DNA mutations and metabolic changes, which hinder drug inhibition and promote degradation (Housman et al., 2014).

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 (C. Zhang et al., 2022b). Typically in cancer, the apoptotic pathway faces inhibition through various mechanisms, such as the overexpression of anti-apoptotic proteins and the under-expression of pro-apoptotic proteins. These alterations often lead to intrinsic resistance to chemotherapy, a prevalent anticancer therapy. It is primarily due to ferroptosis' profile as a non-apoptotic form of RCD that makes it an incredibly promising avenue for new therapy strategies, allowing it to circumvent many tumors' natural resilience to apoptotic induction.

In the field, ferroptosis is indeed 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 natural 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, sonodynamic therapy (SDT), and photodynamic therapy (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 ROS, fostering the development of acquired drug resistance (C. Yang et al., 2015). Being able to predict which tumors are more inclined than others to respond well to ferroptosis induction would aid significantly in lowering the bar for targeted research and treatment.

2.3 DNA Methylation and its Role in Cancer

In mammalian cells, the process of DNA methylation predominantly involves the addition of a methyl group to the carbon-5 atom of cytosine within cytosine-guanine (CpG) dinucleotides. This biochemical reaction is facilitated by three main enzymes known as DNA methyltransferases (DNMTs), including DNMT1, DNMT3A, DNMT3B (Jin and Robertson, 2013). Essential for mammalian development, these enzymes facilitate the transfer of a methyl group from the universal methyl donor, S-adenosyl-L-methionine (SAM), to the 5-position of cytosine residues in DNA (Robertson, 2005). Following DNA replication, the newly synthesized DNA strand lacks methylation. DNMT1 predominantly methylates the unmethylated strand of hemimethylated

DNA, with DNMT3a/3b assisting in ensuring efficient propagation of DNA methylation, especially at sites overlooked by DNMT1 (Jin and Robertson, 2013).

While DNMTs are responsible for adding methyl groups, the ten-eleven translocation (ten-eleven translocation (TET)) family of dioxygenases has emerged as key players in DNA demethylation. Through successive enzymatic processes, TET enzymes can oxidize 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), and further to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Pastor, Aravind, and Rao, 2013). This oxidation of 5mC contributes to the gradual loss of DNA methylation during cell replication. Additionally, the oxidized intermediates can be converted back to cytosine through iterative oxidation followed by base excision repair facilitated by thymine DNA glycosylase (TDG) (Kohli and Y. Zhang, 2013). Together with DNMTs, these enzymes orchestrate the dynamic regulation of DNA methylation.

Methylated cytosine residues are prone to spontaneous deamination, leading to the conversion of cytosine to thymine, a transition that is often poorly repaired. Consequently, a significant portion of familial mutations and single-nucleotide polymorphisms associated with diseases are found within methylated CpG sites. Moreover, CpG residues within gene bodies or coding regions in somatic cells frequently serve as mutational hotspots, exemplified by inactivating C to T transitions in the tumor suppressor gene p53 (Peter A Jones and Baylin, 2002).

This phenomenon also results in a diminished presence of the CpG palindrome throughout the human genome, except within regions identified as CpG islands (CGIs). CGIs, originally characterized by Gardiner-Garden and Frommer, 1987, are defined as 200-bp DNA segments with a 50% C + G content and an observed CpG frequency exceeding the expected value of 0.6. While most CpGs undergo methylation, those within CGIs typically remain unmethylated (Meissner et al., 2008). CGIs are predominantly located within approximately half of all gene promoters (Mikkelsen et al., 2007). Conversely, non-CGI promoters are usually methylated and transcriptionally silent. These genes are more likely to exhibit tissue-specific expression, resulting in only a fraction of non-CGI promoters remaining unmethylated and accessible to transcription factors in each tissue type (Eckhardt et al.,

2006).

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 Peter A. 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 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 poly-chain reaction (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 sub-cloning 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. 2.3).

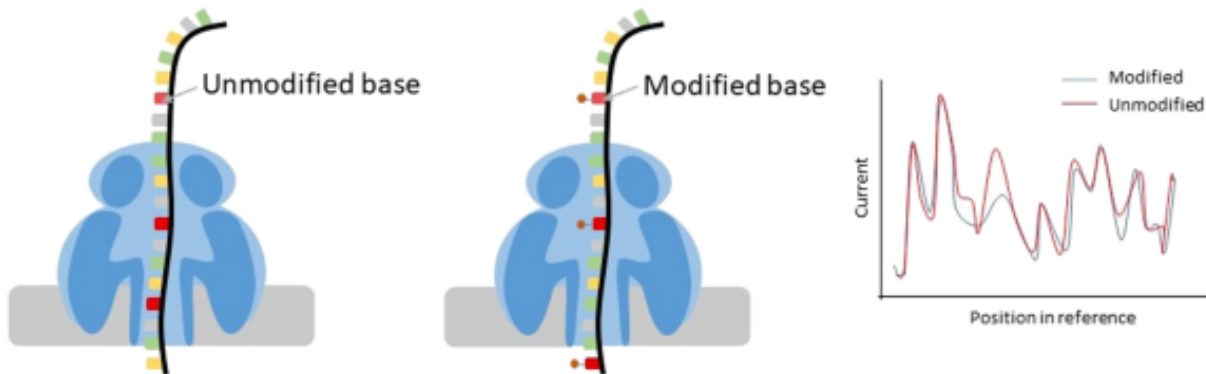


Figure 2.3: Direct base modification detection with ONT.

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.

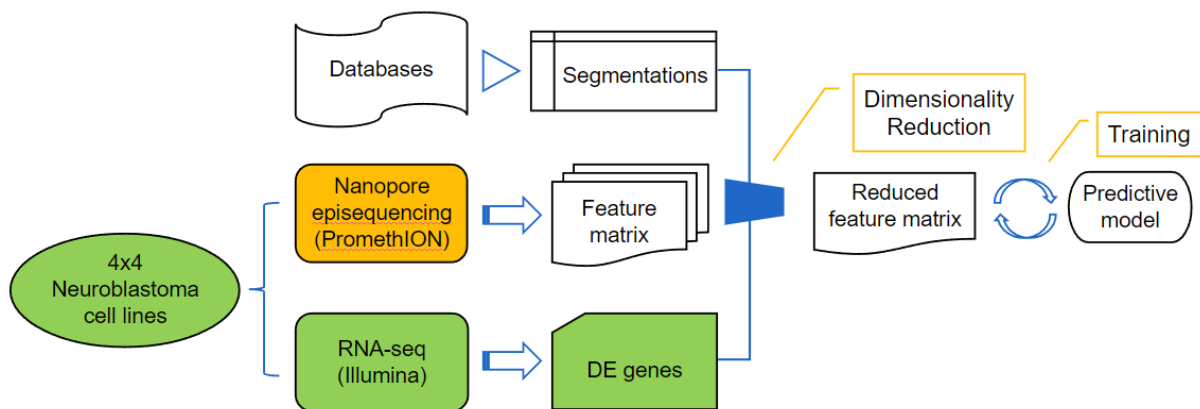


Figure 4.1: Overview workflow.

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 Engineering

4.8 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

5.1 Differential Gene Expression Analysis

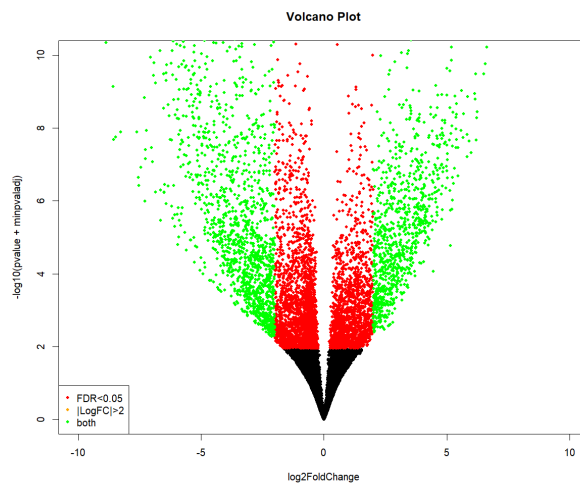


Figure 5.1: Volcano Plot

5.2 Feature Engineering

5.3 Feature Reduction

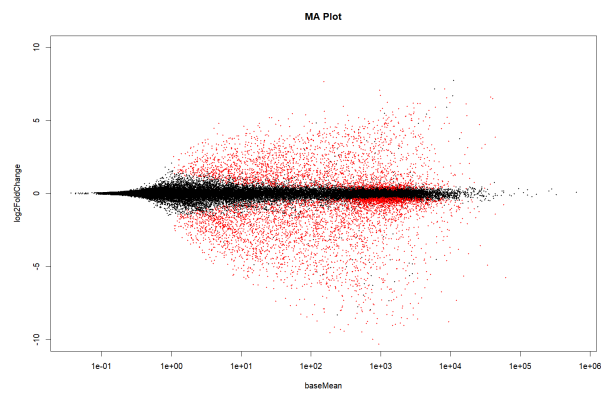


Figure 5.2: MA-Plot

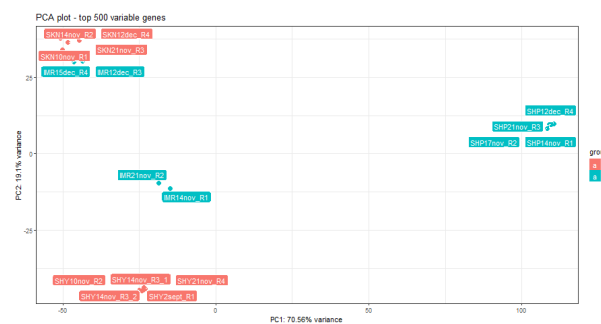


Figure 5.3: PCA

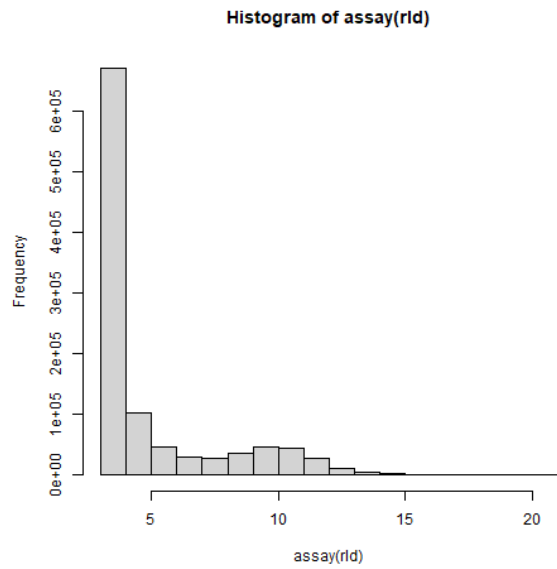


Figure 5.4: Pval dist

Chapter 6

Discussion

Appendix A

Appendix

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