4D Nucleome-Guided Cellular Reprogramming

by

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DEDICATION

It is with deep gratitude that I dedicate this dissertation to

My parents, Dr. Glenn Averil Dotson and Mrs. Karen Olivia Dotson, who instilled in me the importance of faith and perseverance, without which I could not have completed this journey;

My brother, Gabriel Alexander Dotson, who has always been there for me with uplifting words and a listening ear;

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“Write the vision and engrave it plainly on [clay] tablets so that the one who reads it will run. For the vision is yet for the appointed [future] time. It hurries toward the goal [of fulfillment]; it will not fail.” (Habakkuk 2:2-3)
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LIST OF ACRONYMS

1D  One-dimensional
3D  Three-dimensional
4D  Four-dimensional
4DN 4D nucleome
ATAC-seq  Assay for transposase-accessible chromatin using sequencing
ChIP-seq  Chromatin immunoprecipitation sequencing
CRC  Colorectal cancer
FISH  Fluorescence in situ hybridization
GVHD  Graft-versus-host disease
Hi-C  Genome-wide chromosome conformation capture
kb  kilobase
Mb  Megabase
MR  Master regulator
NGS  Next-generation sequencing
RNA-seq  RNA sequencing
TAD  Topologically associating domain
TF  Transcription factor
scRNA-seq  Single-cell RNA sequencing
ST  Spatial transcriptomics
The 4D Nucleome (4DN) refers to the dynamical relationship between chromatin architecture and gene expression in the nucleus that gives rise to cellular phenotype. Our ability to steer cellular phenotype hinges on our ability to understand and precisely characterize a dynamic cellular state. The 4DN framework can be instrumental in helping to realize such reprogramming potential [288, 198]. In this dissertation, I demonstrate how 4DN data can be used to evaluate the impact of perturbations to a cell, examining how TF silencing sensitizes colorectal cancer cells to treatment and assessing how altering genome structure could be a viable therapeutic strategy harnessed for directly modulating in vivo disease processes. I then showcase how recent experimental innovations have allowed us to refine the 4DN framework. Profiling spatially-resolved gene expression in tissue, I characterize the disruption of adult adipose tissue morphogenesis during obesity progression. Finally, I offer a computational framework to analyze multi-way chromatin interactions and reveal organization principles of the genome that govern how transcription factors regulate the formation of transcription clusters. The culmination of this work has allowed us to move beyond the traditional scope of 4DN and consider insights from spatial networks and complex higher-order genome organization. Together, these advances can enable us to develop reprogramming regimes that reach target states with higher fidelity through exploring endogenous nucleome modifications, considering the anatomical context of cells, and ensuring recapitulation of a target cell’s conformational state.
CHAPTER 1

Introduction

1.1 Research Overview

Cellular reprogramming refers to the transformation of one cell type into another cell type, whether directly or passing through a pluripotent state. This is most commonly achieved through the overexpression of externally introduced transcription factors (TFs) though can also be achieved by introducing chemical compounds to cells, RNAi-mediated TF silencing, or CRISPR/Cas9-mediated activation or silencing. Regardless of the approach, understanding the state of the source and target cell is integral to the successful realization of cellular transformations. While gene regulatory networks and gene expression profiles have proven sufficient in guiding the discovery of TF sets that can enable direct or pluripotent reprogramming, reprogramming efforts could further benefit from additional genomic measurements that take into consideration genome conformation and nuclear organization. Towards this end, the 4D Nucleome (4DN), which captures the phenotype-driving dynamical relationship between chromatin architecture and gene expression in the nucleus, offers a robust means of elucidating defining features of a cell [284, 61].

With the right set of TFs, it is largely guaranteed that a target cell type’s transcriptional program will be successfully recovered. Maintaining that established state, however, remains a formidable challenge in the field. Low cell fate conversion efficiencies owing to the presence of TFs that maintain the starting cell’s gene regulatory network, lineage-specific repressors, inaccessible chromatin, and more, contribute to cell types reverting back to their initial state or stalling in an intermediate state. Recent attempts to overcome this barrier have considered the role of epigenetic factors like DNA methylation and histone modifications, as certain chromatin features can interfere with reprogramming [21]. Structural information and tissue dynamics are often neglected in reprogramming studies but could have implications towards improving reprogramming efficiency as well.

In this dissertation, I outline my contributions towards furthering the field of cellular reprogramming through insights from 4DN analyses. This includes the analysis of novel 4DN datasets and the development of novel methods to perform this analysis. The remainder of this research
overview will summarize the work presented in each chapter and following sections will provide a background of relevant themes introduced in these chapters.

Chapter II, “Cellular Reprogramming: Mathematics Meets Medicine” provides an overview of computational approaches that have guided characterization of cell identity and prediction of transitions between cell identities to facilitate TF-mediated cellular reprogramming. Cellular reprogramming has far-reaching therapeutic potential with the promise of generating new cells to repair tissues throughout the body. Data-guided mathematics present a valuable intersection through which to assess and improve reprogramming outcomes. Computational approaches incorporating perspectives ranging from synthetic biology to information and control theory have made considerable advancements towards fine-tuning the prediction of optimal sets of factors and conditions that will ensure efficient cell conversion.

Chapter III, “Partial Reprogramming of Colorectal Cancer Cells Towards Treatment Sensitivity”, examines how TF silencing partially reprograms colorectal cancer (CRC) cells to a treatment-sensitive state. In CRC, aberrant Wnt signaling leads to constitutive activity of the Wnt genetic program including several genes belonging to the T cell factor (TCF) family. The overexpression of TCF4, one of the main downstream effectors of the Wnt signaling network, in CRC cells has previously been implicated in mediating resistance to chemoradiotherapy [162]. In this work, we used RNAi-mediated silencing to knockdown TCF7L2, the gene encoding TCF4, in CRC cells and collected paired bulk RNA-seq and Hi-C data over a 72 hour time series to understand the changes in genome structure and function that facilitate the development of this treatment-sensitive state. We used properties of linear algebra and spectral graph theory to find that the regulation of the epithelial-to-mesenchymal transition (EMT) and E2F networks were most impacted by TCF4 silencing. Evaluating the dynamics of these two networks revealed additional TFs with the potential to further interfere with the transcriptional program of CRC cells. This work emphasizes how TF silencing can transform the integrity of a cancer cell and drive it towards a destabilized state for better intervention and that further disturbance towards a senescent or apoptotic state could be achieved by combinatorially targeting TFs critical to relevant pathways.

Chapter IV, “Rearrangement of T Cell Genome Architecture Regulates GVHD”, explores the genome architecture of mature T cells and evaluates the effect of disrupting genome architecture on the regulation of mature T cell function. Mature T cells are critical to immunopathologies like graft-versus-host disease (GVHD) and we set out to understand how such a disease state would respond in the absence of a key regulator of genome architecture. We again employ principles from spectral graph theory to extract changes in genome structure and function from population-level Hi-C and RNA-seq data of mature T cells in the presence and absence of the cohesin release factor, WAPL, in vivo at baseline and following non-antigen and antigen stimulation. We observed dysregulation of cell cycling regions of the genome that were accompanied by a reduction in GVHD
in the absence of WAPL. Void of a temporal dimension, this study does not fully embody the 4DN, but nonetheless identifies coupling between genome structure and function that characterizes a clinically-relevant disease state. The clinical implications of WAPL regulating \textit{in vivo} immune responses mediated by T cells in this work inspire us to consider what could be possible if we couple TF-mediated cellular reprogramming with direct alterations to genome architecture.

Chapter V, “Obesity Disrupts Innate-Adaptive Immune Network Patterning in Adipose Tissue”, describes the shifting adipose tissue landscape from a lean state to obesity. Immune cells and inflammation contribute to adipose tissue dysfunction, but these changes are difficult to profile outside of their native tissue context. In this study, we combined spatial transcriptomics (ST) with single cell RNA-sequencing (scRNA-seq) across a time course of diet-induced obesity in mice to capture adipose tissue immune cell niches. Through integration of these data, we identified dominant cell type signatures preserved in their anatomical context, quantified spatial gene expression patterns, performed cell type network analysis, and identified changes in ligand-receptor signaling. We further adapted Alan Turing’s mathematical theory of morphogenesis \cite{354, 270} to summarize tissue dynamics, revealing disrupted multicellular tissue function between lean and obese adipose tissue. This work introduces an extension to the 4DN framework, wherein features of genome function are preserved in their spatial context, a critical layer of information when studying disease. This work also highlights how communication between cells is crucial to the tissue microenvironment and impresses the need to view cellular reprogramming through a multicellular lens that could ultimately allow us to reprogram entire tissues.

Chapter VI, “Deciphering Multi-way Interactions in the Human Genome”, proposes a computational framework for analyzing higher-order chromatin organization. We highlight the recently-developed Pore-C approach \cite{355}, a derivative of Hi-C, that leverages long-read sequencing to map genome-wide multi-way contacts as opposed to only pairwise contacts. The long-range chromatin interactions directly captured through this technique allow us to define clusters of genomic loci that colocalize for more efficient transcription, which we term “transcription clusters”. Combining multi-way contact data collected from B lymphocytes, adult fibroblasts, and neonatal fibroblasts with chromatin accessibility, gene expression, and transcription factor binding data, we provide a comprehensive summary of genome-wide transcription clusters, cell-type specific architectural signatures, and the transcription factors that likely regulate these clusters. Our hypergraph representation of these multidimensional relationships allow us to compare cell-type specific chromatin architectures using hypergraph entropy and hypergraph-based similarity measures. This work constitutes a significant advancement for the 4DN framework, as multi-way chromatin interactions can uncover more complex and nuanced features of genome structure. Moreover, we gain insight into how TFs support cell type-specific conformational states, demonstrating a role that chromatin organization plays in transcriptional regulation not often appreciated in cellular reprogramming
Together, the work presented in this dissertation provide a refined 4DN framework for better understanding the dynamics of genome organization within and between cells, with applications for cellular reprogramming. Insights from this work will guide future research with implications in cancer treatment, tissue regeneration, wound healing, and personalized medicine.

1.2 The 4D Nucleome

The genome is more than a linear sequence of base pairs. Meticulous arrangement of the genome in three-dimensional (3D) nuclear space dictates regulatory relationships that drive gene function, as the spatial proximity produced by chromatin folding enables interactions between regulatory elements, like enhancers, and target genes that are non-adjacent along the linear genome sequence. Consequently, genome structure (chromatin folding) and genome function (gene activity) are inextricably linked. Moreover, this relationship is dynamically changing over time, introducing the fourth dimension at play in the 4D nucleome (4DN).

There are several classes of data that can capture 4DN properties, most notably emerging from imaging, modeling, and next-generation sequencing (NGS) [89, 214, 278]. Early efforts to characterize the 4DN leveraged fluorescence in situ hybridization (FISH), revealing placement of chromosomes relative to the center of the nucleus, periphery of the nucleus, or other nuclear landmarks and enabling measurement of physical distance between genomic loci [394, 35, 343]. Though limited in throughput and coverage compared to genomics-based approaches, the development of oligopaints [25], multiplexed FISH [241], and high-throughput FISH [108] have helped overcome some of those limitations. Theoretical and data-driven modeling [146] have further filled knowledge gaps, leveraging insights from the laws of polymer physics to describe constraints on possible chromatin configurations [219], adapting mathematical methods like multidimensional scaling (MDS) to predict chromatin folding and reconstruct 3D genome structures [347, 185], and testing hypotheses to propose molecular mechanisms like loop extrusion [238, 111].

Still, some of the most prominent 4DN studies have turned to genomics to characterize genome structure and function at the molecular level. Chromosome conformation capture (3C)-based approaches have been at the forefront of these studies. 3C involves cross-linking, digesting, then ligating genomic loci in close spatial proximity followed by sequencing to delineate pairwise chromatin contact frequencies [91]. Hi-C, a high-throughput, genome-wide derivative of 3C that captures pairwise chromatin interactions between all genomic loci [190] has led to remarkable insights pertaining to the hierarchical organization of DNA into chromosome territories [269, 77, 107], compartmentalization into active and inactive chromatin regions [190], and preferential contact within local domains [274, 94]. Other -omics approaches capturing one-dimensional (1D) features
like gene expression (RNA-seq), chromatin accessibility (ATAC-seq), and epigenetic marks (ChIP-seq) supplement these findings and are typically integrated with 3C-based data to provide a more comprehensive view of synergistic behavior in the cell. Sequencing-based approaches for determining higher-order chromatin interactions like GAM [22], SPRITE [263], and ChIA-Drop [405] have also garnered attention in recent years for more reliably characterizing long-range interactions involving multiple loci and uncovering associated functional roles.

The field of 4DN analysis is continually expanding and evolving. The last decade has ushered in single-cell methods to replace population-level approaches [234, 324, 271] and seen inclusion of haplotype and karyotype information to characterize allele-specific genome organization [194] and enable elucidation of cancer-specific genome organization [306], respectively, among other advances. The following work presents additional extensions, introducing analytical frameworks incorporating two new data types compatible with the notion of 4DN - spatial transcriptomics, capturing position-specific gene expression [323], and Pore-C, capturing multi-way chromatin interactions [355]. The power of the 4DN framework lies in our ability to integrate all these data modalities to paint a fuller picture of the inner-workings of the cell, offering an ideal platform for further studying cellular control.

1.3 Cancer Reprogramming

As with TF-mediated somatic cell reprogramming, cancer cells can be directly reprogrammed to another differentiated state or reprogrammed to a pluripotent state. For cancer cells, that pluripotent state is referred to as a cancer stem cell (CSC), which like iPSCs possess the ability to self-renew and specialize [149]. Yamanaka factor-driven reprogramming of cancer cells has led to considerable success in stripping cancer cells of their tumorigenicity, malignancy, and methylation signatures, yielding a cellular state with a benign phenotype capable of differentiating into normal lineages [357, 222]. Lineage-specific TFs have demonstrated comparable success as well [402, 216, 141]. In spite of such promising outcomes, as with normal cells, reprogramming efficiency in cancer cells remains low. Even more difficult to reconcile is the genomic variability from cancer cell to cancer cell in comparison to the largely fixed genetic profile among normal cells of the same lineage, rendering a ’one size fits all’ reprogramming regime infeasible.

While the transcriptional landscape of a cancer cell is variable, mutational profiles reveal that there exist core modules of master regulators (MRs) consistently found across diverse cancer contexts that regulate cancer hallmarks (i.e. apoptosis, G2M checkpoint, inflammatory response) [47, 255]. It is also believed that tumor-specific MRs operate within these tumor-independent modules to maintain a dysregulated homeostatic state, making both classes of MRs attractive therapeutic targets. Pharmacological intervention has proven promising in this area as well, as the
advent of differentiation therapy demonstrated that pharmacological agents could be used to drive cancer cells to more mature cell states, disrupting the cancer phenotype by compromising their ability to self-renew [85].

It is understood that the structural landscape of cancer cells is an important factor in defining cancer cell identity [286, 404]. Marked by chromosomal translocations that enable oncogene activation, finding reprogramming factors that alter cancer-promoting spatial organization is imperative as well. Ultimately, cancer cell reprogramming requires precision in characterizing cell state and once obtained can give rise to an array of reprogramming possibilities including a state of senescence (indefinite growth arrest), apoptosis (cell death), or simply a destabilized state that can more feasibly be attenuated.

1.4 Genome Structure and Immunity

Immune cells help maintain tissue homeostasis by clearing pathogens and malignant cells. When their transcriptional program is compromised, immune cells can contribute to a number of pathologies including cancer, immunodeficiency disorder, and autoimmune disease. Immune cells undergo a myriad of processes to reach terminal differentiation and activation, many of which rely on timely genomic rearrangements to mediate regulatory interactions. This relationship between genome organization and immune response has been established most extensively within adaptive immune cells, comprised of B cells and T cells. Studies reveal a highly dynamic topological landscape that is actively responding to cues that help to gatekeep developmental stage-specific phenotypes and prevent premature maturation that can adversely alter immune response.

In particular, chromatin remodeling drives antigen receptor loci recombination, better known as V(D)J rearrangement, a process that occurs in B cells and T cells [14, 158, 364]. FISH and 3C-based analyses have attributed this genomic shuffling to compartment and domain-level spatial organization [84, 224]. Gene segments of a given immunoglobulin (Ig) or T cell receptor (Tcr) locus are held in distinct sub-domains of a common TAD until at a specific stage of differentiation, compartment switching releases key lineage-commitment TF loci (like PAX5 in B cells or BCL11B in T cells) from inactive regions, activating their expression and allowing their protein products to be recruited to the locus. These TFs in conjunction with more universal structural proteins (like CTCF and YY1) then initiate a process called "locus contraction" in which the elements of sub-domains are brought into close spatial proximity to recombine [155]. Similar to cancer cells, there is substantial diversity among immune cells, owing to the several stages of development and vast array of gene segment combinations during recombination, estimated at more than $10^{11}$ [233], that encode antigen-specific receptors and thus sub-classes of antibody-producing immune cells.

Genome structure dynamics continue to play a critical role after maturation of immune cells.
to facilitate proper activation. CD4+ T cells, for example, can further differentiate into one of several T helper cell subtypes upon activation of particular transcriptional signatures that are dependent in part on the repositioning of chromatin that brings key TF loci and cytokine-encoding loci together [317, 123, 137]. Additionally, beyond antigen-receptor rearrangement and mature lymphocyte activation, genome structure has also been implicated in guiding clonal expansion, somatic hypermutation, and allelic exclusion [293, 57, 136].

Overall, TFs play a central role in regulating immune response through genome structure. Moreover, key lineage-specific TFs have been demonstrated to drive immune cell commitment, in a manner independent of transcription [301, 159]. Such observations go to show that genome structure is essential to shaping cell identity and provide a proof of principle that lineage-specific TFs associated with these conformational dynamics are viable reprogramming targets. Some models suggest that such TFs inevitably carry properties of global genomic organization, namely the ability to maintain the epigenome and transcriptional program specific to a cell [237].

1.5 Tissue Reprogramming

Tissues are composed of diverse and differentially localized cells that form distinct patterns throughout the tissue. This heterogeneous cellular composition and crosstalk at the molecular level across the tissue give rise to the emergence of tissue function. Understanding the spatial context of a cell, including its precise position in the tissue, relationship with surrounding cells, and its tissue-specific properties is critical to the development of effective and timely therapeutic interventions.

Such spatial tissue profiling has already seen strides in mapping the trajectory of developing tissue [11], elucidating the tissue architecture of cancers [223, 156, 186], and exploring the spatial dynamics of other pathologies [210]. This is not to suggest that a core transcriptional program conserved across tissue types and even disease contexts is not integral to therapeutic discovery, but that recent technologies like spatial transcriptomics [323, 273] have uncovered new layers of information that enable us to detect more nuanced dynamics. These technologies have the potential to guide cellular reprogramming towards engineering tissue or restoring cellular function through in situ treatments with higher fidelity.

Computational frameworks for studying and integrating these data with other data modalities further invites the application of rigorous mathematical principles like tensor and network theory to deconvolute the nested network that is tissue comprised of cells driven by their respective nuclear networks, as well as Alan Turing’s mathematical theory of tissue morphogenesis, modeling tissue function in terms of a linear system of equations that capture the production and diffusion of morphogens within and between cells, respectively [353, 270]. Ultimately, the translational suc-
cess of tissue reprogramming requires precise biomanufacturing, employed to mimic aspects of the native tissue microenvironment from inter-cellular behavior down to the construction of the extra-cellular matrix (ECM) and 3D tissue architecture [43]. This has been achieved with recent success through 3D bioprinting [183, 1, 407, 199, 310] which involves positioning cells and biomolecules on a tissue scaffold and incorporating biological structural components to give rise to functioning multicellular tissue. Beyond regenerative tissue therapy, using cellular reprogramming to artificially construct tissue also has implications in \textit{in vitro} modeling and drug screening [43, 6].
CHAPTER 2

Cellular Reprogramming: Mathematics Meets Medicine

This chapter is based on a paper by Gabrielle A. Dotson, Charles W. Ryan, Can Chen, Lindsey A. Muir, and Indika Rajapakse [101].

2.1 Abstract

Generating needed cell types using cellular reprogramming is a promising strategy for restoring tissue function in injury or disease. A common method for reprogramming is addition of one or more transcription factors that confer a new function or identity. Advancements in transcription factor selection and delivery have culminated in successful grafting of autologous reprogrammed cells, an early demonstration of their clinical utility. Though cellular reprogramming has been successful in a number of settings, identification of appropriate transcription factors for a particular transformation has been challenging. Computational methods enable more sophisticated prediction of relevant transcription factors for reprogramming by leveraging gene expression data of initial and target cell types, and are built on mathematical frameworks ranging from information theory to control theory. This review highlights the utility and impact of these mathematical frameworks in the field of cellular reprogramming.

2.2 Introduction

In 1989, pioneering work by Hal Weintraub demonstrated conversion of human skin cells into muscle cells through overexpression of a single transcription factor, MYOD1. In 2007, Shinya Yamanaka reprogrammed human skin cells into induced pluripotent stem cells (iPSCs) using four transcription factors, a discovery that would earn him a Nobel Prize in 2012. These remarkable findings demonstrated that the genome is a system that can be controlled via an external input of
transcription factors. Following these experimental discoveries, engineering, statistical and mathematical methods predicting candidate transcription factors for cellular reprogramming began to emerge.

In this review, we show how experimental methodologies for cellular reprogramming and mathematics have come together to enhance our understanding and control of cell fate conversions. We start with a comprehensive overview of key milestones in cellular reprogramming before touching on the translational impact in the field thus far. We then offer a mathematical perspective of cellular reprogramming, covering the use of mathematical principles to describe cellular states and their dynamics. Additionally, we explore how we can harness mathematics to improve the specificity and efficacy of cellular reprogramming by summarizing existing computational frameworks designed to facilitate and validate reprogramming methodologies. Finally, we discuss future applications and implications of engaging mathematics in the domain of cellular control.

2.3 Background

The premise of cellular reprogramming arose following the discovery of DNA as the “genetic code”, with all cells in an organism possessing the same sequences of DNA. From this point forward, biologists sought to understand how cells with the same underlying code take on different phenotypes. From neurons and cardiomyocytes to dermal epithelia, cells with identical DNA somehow differentiate into subsets of cells with complementary functions in a beautifully coordinated process. These specialized cells coalesce to form tissues with emergent functions, which in turn form organs and ultimately organisms. While this process is tightly controlled with awe-inspiring fidelity, errors do occur leading to a wide spectrum of human pathology. Errors along the developmental process, either due to germline mutations or environmental exposures, may lead to developmental malformations with high morbidity and mortality. In addition, tumorigenesis is often considered the de-differentiation of a cell, wherein a cell loses its identity and fails to conform to its intended function [390]. Finally, tissues begin to fail with age as the epigenetic factors that define cell identity wane, rendering a need for restorative cellular reprogramming techniques [167, 160]. For these reasons and more, it is necessary to develop our understanding of the underlying mechanisms of cell fate decision making, and to use that knowledge to improve methods for control of cellular differentiation.

2.3.1 Cellular Differentiation

Typically, cells are envisioned as having a fixed identity capable of performing specialized functions. Indeed, fully differentiated cells show remarkable functional stability over a range of
physiological conditions. However, cells do not start off this way but rather develop from precursor cells called stem cells. The earliest stem cells, embryonic stem cells (ESCs), form following conception of a zygote and are totipotent, meaning they can give rise to all cell types. These totipotent cells proliferate, and some of their progeny take on specialized identities through a process called differentiation. As a cell differentiates, the spectrum of possible progeny cells that it can give rise to narrows and it begins to express only those genes needed for its subsequent role. As a zygote develops, gradients of cell signals across the principle axes of the organism lead to differential expression of transcription factors, which modify the expression levels of genes in such a way as to give rise to distinct phenotypes, thus differentiating cells from one another (Figure 2.1A). Over time, this difference in gene expression caused by transcription factor availability is thought to be reinforced by epigenetic markers, which package the DNA depending on the needs of the cell. Actively expressed genes are left relatively unpacked (euchromatin) while silenced genes are tightly stowed away (heterochromatin) [7].

The mechanism by which cells suppress unused genes as they differentiate and specialize their function remains an open question, though biologists have proposed theories since the mid-twentieth century. A popular model of unidirectional differentiation was postulated by Conrad Waddington in 1957 suggesting that stem cells can be thought of as a high-energy state in which all genes are active [367]. As cells differentiate, genes unnecessary for the cell’s subsequent role were thought to be silenced as the cell enters a lower-energy stable state, analogous to a ball rolling down a hill. While an insightful model for its time, this analogy of a ball rolling down a hill implies a unidirectional fatalistic process. Cellular reprogramming challenges this notion of cell state permanence, and allows a cell’s fate to be redirected. This requires us to level the hierarchical epigenetic landscape [176] envisioned by Waddington and instead adopt a flattened landscape in which cells are free to shift from one cell potency and state to another, under the right conditions (Figure 2.1B-C). That is, one or more core genetic elements may be manipulated in a way that forces a cell to take on a new form and function.

Typically, this cellular transformation is facilitated by an exogenous stimulus like the addition or removal of one or more transcription factors. Transcription factors (TFs) are DNA-binding proteins that modulate how often a gene is transcribed into messenger RNA (mRNA) for protein production. Of the roughly 20,000 known proteins in the genome, around 1,500 are known to function as TFs [144]. Therefore, focusing on TFs as target inputs significantly reduces the dimensionality of the problem. Notably, the exogenous expression of only one to three TFs is often sufficient to achieve the acquisition of specialized lineages from other cell types [339].
Figure 2.1: Cellular Differentiation and Reprogramming. (A) Cellular differentiation in developmental biology. During normal development, concentration gradients of morphogens (red spheres) lead to differing levels of transcription factor (dimeric green spheres) activation in a distance-dependent manner. This leads to differing transcription profiles of cells as a function of spatial location, allowing for body patterning. (B,C) Re-imagining of Waddington’s epigenetic landscape. Waddington’s original model (B) best describes the process of cellular differentiation and presents an intuitive illustration of non-specialized cell types traversing down peaks and settling in valleys once specialized. In a re-imagined version of Waddington’s landscape (C), the essence of cellular reprogramming is captured, where a cell is unimpeded by the gravity of a hierarchical model and can flow between any potency, germ layer, and cell state. The common center of the concentric circles represents the totipotent state while the subsequent inverted circles represent decreasing levels of cellular potency moving outwards. Here, direct reprogramming is analogous to ‘direct conversion’ or ‘transdifferentiation’, and refers to a change in cell fate that does not incorporate a pluripotent or progenitor state.

2.3.2 Cellular Reprogramming

This notion of cellular reprogramming is far from a theoretical whim. Rather, it has been established over the last four decades in several contexts. Reprogramming has been achieved by introducing critical TFs to susceptible cell types and by transferring the nucleus of a somatic cell into an oocyte to “rejuvenate” the nucleus into a totipotent zygote.
Epigenetics
The study of heritable changes in gene activity caused by mechanisms other than changes in the underlying DNA sequence. These can include biochemical modifications of genomic DNA or histones, the proteins that help package genomic DNA.

Differentiation
The unidirectional progression of a cell towards a specialized cell type.

Reprogramming
The directed transformation of one cell type into a less or differently specialized cell type, typically mediated by exogenous TF expression.

Cell Potency
The potential for a cell to develop into a more specialized cell type. The more lineages the cell can give rise to, the higher the potency and the closer it is to a stem cell-like state.

Reprogramming Factor
One or more transcription factors capable of driving a cell from one state to another when exogenously overexpressed.

Gene Regulatory Network
Cell-type specific set of interactions between genes, governed by the regulatory influence they exert on each other.

Table 2.1: Glossary of Terms

2.3.2.1 Transcription Factor Mediated Reprogramming

In 1989, Harold Weintraub et al. demonstrated that non-muscle cells could be driven to express muscle-specific genes by MYOD1 activation [378]. This was the first success in directly reprogramming one differentiated cell to another and demonstrated that TFs such as MYOD1 possess the capability to override expression programs. This inspired others to search for additional TFs capable of driving differentiation. In 2006, Shinya Yamanaka’s group redefined what was possible when they demonstrated that murine adult fibroblasts could return to a pluripotent state following lentiviral transduction of just four TFs: Oct3/4, Sox2, Klf4, and c-Myc [341]. These factors would be coined the “Yamanaka factors” and Yamanaka would be awarded the Nobel Prize in Physiology or Medicine in 2012 for this groundbreaking discovery. Not only were these cells transcriptionally similar to embryonic stem cells, but they successfully differentiated into all three germ layers - endoderm, ectoderm, and mesoderm - when introduced into murine blastocysts, indicating true pluripotency. The following year, the Yamanaka factors were again used to convert human fibroblasts to iPSCs whose differentiation to neurons and cardiomyocytes could be subsequently induced, and that could form teratomas with all germ layers present when injected into nude mice [340]. These results indicate that cell identity remains malleable through adulthood, and when the correct TFs are applied, cells may be driven towards identities that meet experimental or therapeutic needs.
2.3.2.2 Somatic Cell Nuclear Transfer

Another route to reprogram cells is by transferring the nucleus of one cell type into the cytoplasm of another. The cell-signaling molecules in the recipient cytoplasm, including TFs, act to reprogram the donor nucleus to exhibit an expression profile more reminiscent of the recipient cell. The oocyte is an ideal recipient cell - its large cytoplasmic volume buffers it against the donor signaling molecules, as it contains sufficient quantities of TFs to overpower those contained in the donor nucleus. In addition, the oocyte’s role in generating the embryo and resetting the sperm nucleus suggests that it contains factors necessary for nuclear reprogramming to a totipotent state. These ideas became reality when in 1952, it was demonstrated that a nucleus from a blastula cell may be introduced into an enucleated frog egg to give rise to a normal embryo [38]. Following this discovery, John Gurdon demonstrated that even nuclei of intestinal epithelial cells were capable of giving rise to an entire embryo when implanted into an oocyte [126]. In 1996, the first mammalian clone was produced by transferring sheep mammary nuclei into an enucleated oocyte [366]. Finally, in 2013, the first hESC cell line was produced using somatic cell nuclear transfer techniques [338].

These nuclear transfer experiments highlight a potential application to regenerative medicine, where a nucleus derived from a patient skin cell could be introduced into an oocyte for reprogramming into embryonic stem cells, which may in turn be differentiated into cell types of clinical interest. Since the generated cells would contain DNA of the graft recipient, this would provide an avenue for autologous tissue grafting without concern for rejection.

2.3.2.3 Translational Success

Recently, cellular reprogramming has made the journey from bench to bedside as efficiency and safety have improved. The therapeutic potential of autologous grafts derived from reprogrammed cells is broad. Easily accessible yet malleable cells such as dermal fibroblasts, for example, may be reprogrammed to various cell types for a range of downstream medical applications (Figure 2.2). One such example is the application of reprogrammed cells to treat age-related macular degeneration. As described in a 2017 report, retinal pigment epithelium (RPE) cells were derived from fibroblasts induced with non-integrating episomal vectors carrying TFs to treat a 77-year old patient with wet macular degeneration [209]. A skin sample was taken and reprogrammed to an iPSC state, and subsequently differentiated into RPE cells. These reprogrammed cells exhibited DNA methylation and gene expression patterns consistent with RPE cells. Additionally, genome sequencing indicated that no mutagenesis had occurred during the process, mitigating the possibility for oncogenesis. Since these cells are autologous and therefore carry the DNA of the patient, there is no risk of graft rejection upon re-introduction. The reprogrammed cells were surgically grafted
Figure 2.2: Medical Applications of Cellular Reprogramming. Dermal fibroblasts can be acquired via a minimally invasive punch biopsy, and subsequently differentiated into a desired cell type via the addition of select TFs. Finally, autologous cultured cells of the desired type can be reintroduced into the patient without concern for graft rejection.

into the patient’s retina and found to have engrafted successfully after one year without evidence of vision loss. This early success demonstrates the clinical potential of cellular reprogramming using non-integrating TF delivery methods, as an autologous graft was generated from reprogrammed cells without introducing potentially harmful mutations.

Clinically-relevant reprogramming successes in laboratory settings have presented exciting potential as well. For example, critical steps have been made towards autologous hematopoietic stem cell therapies for post-chemotherapy leukemia patients, as murine committed lymphoid and myeloid progenitors [283] as well as human fibroblasts [314] were converted into hemogenic cells. This success extends to the field of nerve regeneration therapy, as fibroblasts were reprogrammed
into glutaminergic neurons [393]. Yet another clinically-relevant success is the differentiation of human fibroblasts to cardiomyocytes [50, 110], as the ability to cultivate autologous cardiomyocytes may allow replacement therapy following myocardial injury in the future. Finally, with type-I and late-stage type-II diabetes mellitus characterized by the loss of insulin-producing beta-islet cells, success in generating autologous beta cells has curative potential. The feasibility of this solution was recently demonstrated with the conversion of pancreatic acinar cells to beta-islet cells [55] as well as the conversion of fibroblasts to beta-islet-like cells via directed differentiation through endodermal intermediates [409]. Taken together, these results indicate that an easily accessible and malleable cell type such as the fibroblast may be converted into a range of clinically applicable cell types for autologous grafting, potentially challenging the permanence of diseases ranging from macular degeneration to diabetes mellitus.

2.4 Mathematics of Cellular Reprogramming

Cellular reprogramming is a very calculated process involving inputs, transition states, and outputs - a control theory problem at its core. Long before control theory concepts would be implemented in cellular reprogramming studies, however, mathematicians were already conceptualizing how biological systems could be systematically controlled [200]. From observing state-dependent network entropy during differentiation [267] to modeling cells as complex networks subject to perturbations that can modulate the system’s equilibrium and placement in an n-dimensional state space[75], it became clear that mathematics could be exploited to optimize cellular reprogramming.

2.4.1 Cellular States

During cellular reprogramming, cells encounter a start state and end state, occupying one or more transition states in between. These states are most commonly described in terms of their gene expression profiles or gene regulatory networks [46, 104, 264, 92, 288]. Derived from RNA-sequencing data, gene expression can be represented as a vector of \( n \) non-negative values, where \( n \) is the roughly 20,000 genes in the human genome. A single expression vector represents a state-dependent snapshot of a genome’s transcriptional landscape in time. Collecting data as the cell evolves can then give rise to a sequence of expression profiles reflecting discrete points along a reprogramming trajectory. While gene expression has provided sufficient cellular state representation thus far, additional measurements such as those from ChIP-seq, DNase-seq, or chromosome conformation data can contribute to a more comprehensive definition of cellular states and may be considered in future studies [348].
Entropy plays an important role in cellular dynamics and offers a quantitative measure of a cell’s differentiation status (i.e., a cell’s placement within the Waddington landscape). One frame of thought is that the specialization of cells minimizes entropy and thus controllability [267, 268]. The closer a cell is to a pluripotent state, the higher its entropy - as there exists a wider availability of signaling pathways that can be activated and conformations the cell can adopt (more uncertainty) in the lesser committed state [206, 76]. We can glean local and global entropy from data on gene expression and protein networks [16]. Local entropy indicates the susceptibility of specific signaling pathways in the cell to perturbation and is derived simply from the Shannon entropy for a particular gene or protein. Global entropy indicates how specialized the cell is and can be captured by finding the average of those local entropies.

2.4.2 Cellular State Perturbations

Perturbations push cells to overcome the epigenetic barriers that consign them to a single differentiated state [207]. In the context of cellular reprogramming, these perturbations are often the external introduction of one or more TFs that evoke changes in transcriptional activation and inhibition throughout the cell’s gene regulatory network. The internal response to these external perturbations, however, is not fully deterministic. That is, perturbations may drive the cell towards the vicinity of a target state (i.e., a basin of attraction) with the expectation that the cell will converge to the intended target state (attractor) in a stochastic manner.

In their work on perturbation patterns and network topology, Marc Santolini and Albert-László Barabási demonstrate how topological models can offer substantial insight into accurately predicting the influence of perturbations on biological networks [298]. Santolini and Barabási explain that the effects of external stimuli do not diffuse throughout the genome uniformly. Rather, perturbation spread is confounded by interaction properties like directedness, modularity, or whether an interaction is activating or repressive. As such, every node in a biological network will correspond to a differential equation with parameters encompassing these properties. Network topologies can then be captured from a set of differential equations by constructing the Jacobian matrix, a matrix of partial derivatives that describes the impact of node \( i \) on the activity of node \( j \) and that quantifies direct interactions between all pairwise nodes in the network. An adjacency matrix, describing the influence of the network, can subsequently be derived as \( A = \text{sign}(J^\top(x^*)) \), where the \( \text{sign} \) function represents the directionality of the edges between nodes and is applied element-wise, \( J^\top \) is the transpose of the Jacobian, and \( x^* \) is a vector of magnitudes (i.e. gene expression) representing the steady state of the system. To account for indirect interactions in a network, Santolini and Barabási also describe a sensitivity matrix, \( S \), where each element is the full derivative between two nodes.
The relationship between the sensitivity and Jacobian matrices becomes:

\[ S = (I - J)^{-1} D (1/(I - J)^{-1}) , \]

where, \( I \) is the identity matrix, \( D \) is the diagonal operator, and the / operator denotes element-wise division. By modifying (perturbing) the differential equation of nodes of interest (TFs) and analytically deriving the sensitivity matrix, we get a sense of how that node’s influence propagates throughout the network. Taken together, this predicative framework facilitates evaluation of how cells might react to a perturbation, providing an avenue for which to test the influence of candidate reprogramming factors.

### 2.5 Computational Tools

The earliest cellular reprogramming studies incorporated a systematic “guess-and-check” method for identifying TFs that would be necessary and sufficient for reprogramming in their experimental system. The four Yamanaka factors were narrowed down from an initial list of 24 candidate reprogramming factors that were selected based on their properties and suspected involvement in promoting and maintaining an embryonic stem cell-like state. It took several trials of transducing different permutations of these factors to finally converge upon the four factors capable
of inducing pluripotency in differentiated cells. Several computational frameworks for streamlined data-guided prediction of reprogramming factors have since been developed (Table 2.2), demonstrating that we can bypass the “guess-and-check” method of validating TFs, which tends to be time consuming and cost prohibitive.

2.5.1 CellNet

The successful realization of a target cell identity following reprogramming can be assessed by establishing transcriptional similarity using gene expression profiling and hierarchical clustering analyses. This validation approach, however, fails to capture functional dissimilarities that may exist between the reprogrammed cell and its naturally occurring counterpart. A more quantitative and rigorous system is therefore required to verify the integrity of reprogrammed cells. To this end, in 2014, Patrick Cahan and co-author Samantha Morris et al. introduced CellNet, a computational tool employing network biology to assess and improve the fidelity of reprogrammed cells [46]. In a companion paper, Morris and Cahan put CellNet to the test with B cell to macrophage and fibroblast to hepatocyte-like cell conversions, experimentally verifying that original reprogramming schemes could not achieve full conversion but that CellNet-refined schemes could [227].

As described in the original publication, CellNet first generates a training dataset containing cell- and tissue- (C/T) specific GRNs derived from a large array of publicly sourced microarray datasets. The CellNet algorithm has since been extended for compatibility with RNA-sequencing data [265]. Subsequently, based on input gene expression data from a reprogramming experiment, CellNet classifies the query cell by its most likely C/T-specific GRN and evaluates how close it is to the target phenotype’s GRN. If the two GRNs deviate significantly, CellNet proposes TFs that could further drive the reprogrammed cell to better mimic its target identity. Therefore, CellNet's measure of reprogramming success is the extent to which a reprogrammed cell has established its target state’s GRN. To evaluate the reprogrammed cell’s GRN in comparison to the target cell type’s GRN, the CellNet algorithm computes a GRN status score as follows:

$$\text{RGS} = \sum_{i=1}^{n} \text{Zscore}(\text{gene}_i)_{\text{C/T}} \times (\text{gene weight}_i)_{\text{GRN}},$$

where, $n$ is the number of genes in the reprogrammed cell’s GRN, the z-score of gene $i$ is derived from the distribution of expression values for gene $i$ in the C/T-specific training data, and the gene weight is the expression of gene $i$ as determined from the reprogrammed cell’s expression profile. If the reprogrammed and target GRNs are not equivalent, CellNet then investigates how transcriptional regulators (TRs) of the target GRN can push the reprogrammed cell to fully convert.
This is determined by computing a network influence score (NIS) for TRs (i.e., TFs) in the GRN:

\[
\text{NIS}(\text{TR}) = \sum_{i=1}^{n} (\text{Zscore}(\text{target}_i)_{C/T} \times \text{weight}_{\text{target}_i}) + n \times \text{Zscore}(\text{TR})_{C/T} \times \text{weight}_{\text{TR}},
\]

where, \( n \) is the number of genes in the target C/T-specific GRN, the z-score of the target is derived from the distribution of expression values for gene \( i \) in the training data, \( \text{weight}_{\text{target}_i} \) is the mean expression value of gene \( i \) in the training data, the z-score of the given TR is based on the distribution of expression values for the TR in the training data, and \( \text{weight}_{\text{TR}} \) is the mean expression value of the TR in the training data.

With CellNet, Cahan et al. uncover variations in reprogrammed cells’ regulatory networks compared to the target state thereby highlighting inherent limitations in specificity that accompany current reprogramming methods. This technique is unique in that it seeks to address both how well a reprogrammed cell recapitulates its target and how the administered combination of TFs can be revised to better recapitulate that target - what Cahan later designates the ‘assessment’ and ‘improvement’ problems, respectively [45].

2.5.2 D’Alessio et al. Method

In contrast to CellNet, a later method for identifying reprogramming factors described by D’Alessio et al. in 2015 incorporates only the expression of TFs instead of genome-wide expression data in its prediction algorithm. [104]. Specifically, TFs are ranked by their specificity and uniqueness, where a given TF is highly ranked and ideal for reprogramming if it is highly expressed in the query (i.e., target cell type) dataset and not expressed in cell types comprising the background dataset. To identify such TFs, D’Alessio et al. define the distribution of a TF’s expression level across cell types, such that the expression of a TF in a query cell type may be compared to the expression of that TF in all other cell types. The cell type specificity of each TF is evaluated using an entropy-based score adapted from previously described applications of Jensen-Shannon (JS) divergence for describing tissue-specific gene expression [349] and lincRNA expression [42]. Specifically, a distance metric describing the difference between a query expression pattern and the ideal expression pattern is obtained by taking the square root of the JS divergence as follows:

\[
\text{JS}_{\text{dist}}(\mathbf{e}, \mathbf{e}^I) = \left[ H\left( \frac{\mathbf{e} + \mathbf{e}^I}{2} \right) - \frac{H(\mathbf{e}) + H(\mathbf{e}^I)}{2} \right]^{1/2},
\]

where, \( \mathbf{e} \) is an \( n \)-dimensional vector, \( \mathbf{e} = [e_1, e_2, \ldots, e_n] \), consisting of the expression-derived abundance density of a specific TF across \( n \) cell types. Vector \( \mathbf{e}^I \) represents the ideal distribution in the form of a binary \( n \)-dimensional vector where \( e_i^I = 1 \) if \( i \) = query cell type and 0 for all
other cell types. Lastly, $H(\cdot)$ represents the Shannon entropy. TFs with cross-cell type expression distributions closely aligned to the ideal distribution, (i.e., expressed in the query cell type but not in other cell types) will yield JS distances close to zero. D’Alessio et al. identified the top ten TFs with expression profiles most similar to the ideal expression profile for nearly 200 cell type and tissue pairs using this approach. Experimental validation of TFs predicted to define RPE cells demonstrated successful reprogramming from fibroblasts into RPE cells. This success lends credence to the identification of reprogramming factors based on the influence that TFs have on the identity of a desired cell type, as determined by the level of unique and relative expression.

2.5.3 Mogrify

Similar to CellNet, Mogrify leverages regulatory networks to predict TFs for direct conversion of cells, selecting TFs that exert regulatory influence on genes that identify most with the target cell type [264]. Using publicly available gene expression data, the Mogrify algorithm starts by accumulating TFs that are differentially expressed between the target cell type and a background set of non-target cell types. A tree-based approach is employed to ensure that the background set is not saturated with cell types that are too highly or distantly related to the target cell type. Selected TFs are subsequently ranked based on the combination of their differential expression (scored as the product between the log-transformed fold change and adjusted p-value) and local network influence based on protein-DNA and protein-protein interaction data. This network influence is derived as follows:

$$N_x = \sum_{r \in V_x} G_r \times \frac{1}{L_r} \times \frac{1}{O_r},$$

where, $r \in V_x$ are genes ($r$) that are nodes ($V_x$) in the local subnetwork of TF $x$, $G_r$ is the aforementioned differential expression-based score for gene $r$, $L_r$ is the number of edges between gene $r$ and TF $x$ in the local subnetwork, and $O_r$ is the out-degree of gene $r$’s parent node in the network. This score is determined for the network of known gene protein product interactions and separately for the network of known TF-DNA interactions. With the described distance-based and connectivity-based weightings, Mogrify carefully selects for TFs with high influence and regulatory specificity. The subsequent combination of gene and network scores yields a set of TFs that are highly ranked if they are non-ubiquitous with a high fold change and low p-value. To further prune the list, TFs that regulate over 98% of the same genes are deemed redundant and the higher ranked TF preserved. Additionally, TFs in the ranked list that are already highly expressed in the starting cell type are removed from consideration.

While Mogrify’s systematic identification of reprogramming factors is only as good as the fidelity of publicly available gene expression data and breadth of known TF binding interactions, it
offers robust predictive power in mediating direct cell conversion, having recovered 84% of previously discovered conversion factor cocktails and validated two novel conversions at its time of publication. Mogrify-enabled predictions for TF-mediated conversions between cell types represented in the FANTOM5 gene expression atlas have been compiled into an online directory (http://mogrify.net/), providing a convenient one-stop shop for a wide range of direct reprogramming objectives [250]. Additionally, since its inception, Mogrify has undergone its own transformation from technology to corporation, now leading the way in commercialized direct human cell conversion and revolutionizing cell therapy.

2.5.4 Del Vecchio et al. Method

The natural dynamics of a cell’s GRN are not always amenable to preset overexpression of TFs [228, 302, 120, 388]. Del Vecchio et al. address this uncertainty in reprogramming success by employing mathematical modeling to facilitate the design of a genetic feedback controller, independent of GRN dynamics, that iteratively adjusts TF input concentrations based on the difference between current and target state TF concentrations [92]. In this method, a cellular GRN (x) with n TFs, \( x = [x_1, \ldots, x_n] \) is modeled as a set of ordinary differential equations represented as:

\[
\frac{dx_i}{dt} = H_i(x) - \gamma_i x_i + u_i \text{ for } i \in \{1, \ldots, n\},
\]

where \( H_i(x) \) is the Hill function capturing the GRN regulation of TF \( x_i \), \( \gamma_i \) is the constant decay rate of TF \( x_i \), and \( u_i \) is a non-negative scalar indicating the input perturbation corresponding to TF \( x_i \). In contrast to open loop control which takes a constant or time-varying TF concentration as input, the authors employ closed loop feedback control which updates the input concentration for a system in real time. Since the feedback control is dependent on the error between the most current concentration, \( x_i \), and the target concentration, \( x_i^* \), the \( u_i \) term in the former equation can be replaced with the expression \( G_i(x_i^* - x_i) \), where \( G_i \) is a large positive constant representing gain that renders \( H_i \) and \( \gamma_i x_i \) negligible. The resulting form describes the simultaneous production and degradation of TF \( x_i \) mRNA that govern the behavior of Del Vecchio et al.’s novel synthetic genetic controller circuit. Operation of this circuit is facilitated by two inducers, one acting on a synthetic copy of gene \( x_i \) and the other on siRNA complementary to both the endogenous and synthetic mRNA, where the inducer concentrations are informed by \( x_i^* \). Implemented for each TF in the GRN, or a predetermined subset, the circuit steers the concentration of a given TF \( x_i \) and its mRNA, \( m_i \), towards \( x_i^* \) and \( m_i^* \) by inducing the concurrent production of the synthetic TF and degradation of the endogenous TF. Once the desired concentration is achieved, the inducers are set to zero to stop the synthetic controller and the endogenous system takes over TF production to maintain the stability of the reprogrammed state.
At its time of publication, the proposed approach had been simulated on a two-node model of the pluripotency network but not yet demonstrated on a real cellular GRN. The authors suggested, however, that the application of their controller may ensure more success in iPSC reprogramming as pluripotent states can be difficult to attain with preset TF overexpression due to the potential for the TF to dominate the network’s behavior. Though highly theoretical at this time, this work offers a promising and well thought out synthetic biology approach for fine-tuning cellular reprogramming.

2.5.5 Data-Guided Control (DGC)

Coupling gene expression and TF binding data with control theory, Ronquist et al.’s universal algorithm for cellular reprogramming offers a slightly more mathematically rigorous framework than its predecessors for data-guided prediction of reprogramming factors, or data-guided control (DGC) [288]. In addition to identifying candidate reprogramming factors, Ronquist et al. also present an optimization method for identifying the ideal time during the cell cycle for addition of TFs. The foundation of this algorithm is the following linear, control theory difference equation:

\[ x_{k+1} = A_k x_k + B u_k \]

where \( x_k \in \mathbb{R}^N \) is a gene expression vector representing the cell state at time \( k \). To ease the computational burden, the authors reduce the dimensions of this vector from \( \sim 20,000 \) to \( \sim 2,000 \) (\( \mathbb{R}^{\tilde{N}} \)) by summing the expression levels of genes that are in close proximity to one another, or more specifically, that occupy the same cell-type invariant topological domains. \( A_k \in \mathbb{R}^{\tilde{N}\times \tilde{N}} \) is the transition state matrix representing time-varying changes to the cellular state at time \( k \). \( B \in \mathbb{R}^{\tilde{N}\times M} \) is the input matrix indicating interactions between genomic domains (\( \tilde{N} \)) and TFs (\( M \)). Elements of the matrix are weighted by the magnitude of regulatory influence, determined from publicly available TF binding site data, and signed according to whether a given TF activates or represses the genes in each domain. Finally, \( u_k \in \mathbb{R}^M \) is a binarized input vector, with nonzero elements indicating which TF(s) to introduce at time \( k \). An overview of this framework is reproduced in Figure 2.4.

Once the solution to the difference equation is obtained, TFs are subsequently scored by exe-
Figure 2.4: Data-Guided Control Overview. (A) Summary of control equation variables. (B) Each box represents a topological domain containing several genes. The blue connections represent the edges of the network and are determined from time series RNA-seq data. The small green plots at each node represent the expression of each domain changing over time. The red arrows indicate additional regulation imposed by exogenous TFs. (C) Conceptual illustration of determining TFs to push a cell state from one basin to another. Figure reproduced with permission from [288].

Cutting the following optimization problem for all possible input signals:

$$\text{minimize}_{u} \| x_T - z_F(u) \|$$

subject to

$$u_{m,k}, \quad k = 1, 2, \ldots, F$$

$$u_{m,k}, \quad \text{if } m \notin \hat{p}$$

$$u_{m,k+1} \geq u_{m,k}$$

where, the objective is to minimize the distance between the final state, $z_F$, and target state, $x_T$, with $z_F(u)$ denoting that the final state is dependent on the input signal, $u$. We can evaluate this distance as the Euclidean norm ($\| \cdot \|$) of the difference between the gene expression levels at each state. The first constraint addresses the restriction that TFs only be added to the cell and not removed. The second constraint requires all elements of input signal $u_k$ to be zero if they do not correspond to the subset of TFs ($\hat{p}$) selected to drive the system. Lastly, the third constraint indicates that select TFs can be added consecutively or added sequentially, but that once added, the TFs must continue to be exogenously expressed until the final time point. Notably, Ronquist et al. demonstrate that TF scores are dependent on the time of input, with some TFs showing preference for addition towards the beginning of the cell cycle and others towards the end. Overall, Ronquist et al. present a convincing case for incorporating time-varying data into TF prediction frameworks and considering the time of TF input to a system. This approach mimics the natural course of differentiation and may increase the efficiency of reprogramming to achieve yields sufficient for tissue grafting, though experimental validation is needed.
<table>
<thead>
<tr>
<th>Method</th>
<th>Input</th>
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<th>Approach</th>
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<tr>
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<td>Assessment of original reprogramming scheme and proposal for refined reprogramming scheme</td>
<td>Scores TFs based on GRN status and network influence</td>
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<td>Set of candidate TFs for conversion to target cell type</td>
<td>Ranks TFs based on influence over differentially expressed genes in the target cell type</td>
<td>Comparison to previously confirmed conversion cocktails and novel conversions were experimentally validated</td>
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<tr>
<td>Del Vecchio et al. Method</td>
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<td>Time-series gene expression and TF binding data</td>
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<td>Comparison to previously confirmed conversion cocktails</td>
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</tbody>
</table>

1 Data necessary to facilitate prediction of reprogramming factors. Input of initial and target cell types is assumed.

Table 2.2: Summary of Computational Tools for Reprogramming

2.6 Experimental Realization

With the guidance of predictive models such as those mentioned above, we can narrow down the list of candidate TFs to quantities achievable with modern high-throughput methods. Rather than considering any combination of the approximate 1,500 TFs in the human genome, the problem is reduced to only those with favorable predictions. Now that the problem is of a more manageable size, experimental validation is critical.

2.6.1 Transcription Factor Delivery

In order to experimentally achieve reprogramming, it is necessary to determine a method of adding TFs to cells. TFs are proteins coded by genes, and thus could be introduced to the cell directly or via DNA or mRNA coding for that protein (Figure 2.5).
Figure 2.5: Transcription Factor Delivery. (A) Transduction of a TF-encoding gene by a DNA integrating virus such as a lentivirus. The virus first binds to the host membrane and fuses its viral envelope with the eukaryotic phospholipid membrane to enter the cell. Nucleic acids are released in the cytosol, where RNA-dependent DNA polymerase creates double stranded DNA. Viral DNA is integrated into the host genome. Host cells then express virally-delivered genes, which are translated into functional proteins. (B) Introduction of mRNA coding for a TF by a non-integrating virus such as a Sendai virus. As above, the virus docks, fuses with the cell membrane, and releases nucleic acids into the cytosol. RNA-dependent RNA polymerase then generates positive sense RNA, which is translated to functional protein. (C) Delivery of modified mRNA via a lipid nanoparticle. First, the lipid particle-embeded peptides bind target cell receptors and trigger receptor-mediated endocytosis. Next, the endosome is acidified by $\text{H}^+$ pumps. The acidified endosome and lipid nanoparticle are destabilized, releasing mRNA into the cytosol. Free mRNA is translated to functional protein. (D) Bacterial type-III secretion system as a TF delivery mechanism. First, a bacterial DNA plasmid is expressed and protein is produced. Next, the bacterial T3SS delivers the protein to the eukaryotic cell through a molecular needle.
2.6.1.1 Viral Vectors

Perhaps the most common modality by which biologists introduce genes into cells is lentiviral transduction. Lentiviruses are a class of retroviruses commonly used in experimental procedures. Retroviruses, such as the lentivirus, are made up of an RNA-based genome, RNA-dependent DNA polymerase (reverse transcriptase), and DNA integrase surrounded by a protein capsid and a phospholipid envelope. These viruses function by fusing with the membrane of cells and releasing their RNA into the cytoplasm, which is reverse transcribed into DNA and integrated into the host genome [295]. These viruses serve as a useful tool, as one can package a gene of interest into their RNA genome and use these viruses to integrate that gene into the genome of a cell in culture. In addition, antibiotic selection genes and fluorescent proteins can be added to aid in selecting cells that have taken up and readily express the viral genome. Despite these advantages, insertional mutagenesis may confound experimental results and increase the risk of tumorigenesis in therapeutic applications [356]. To circumvent this risk, non-integrating viruses such as the Sendai virus can be used to temporarily express exogenous TFs and initialize reprogramming processes within the cell. While this solves one problem, both retroviruses and non-integrating viruses trigger host cell innate immune responses as viral RNA is sensed by pattern recognition receptors (PRRs). This leads to a wide range of cell signaling events and transcriptional changes that alter cell behavior, potentially interfering with experimental and therapeutic applications [296].

2.6.1.2 Modified mRNA

An alternative to viral vectors is direct application of TF-encoding mRNA packaged in lipid nanoparticles (LNPs). These LNPs are taken up via receptor-mediated endocytosis, and nucleic acids are released into the cytoplasm as acidification of the endosome leads to dissolution of the lipid nano-particle and endosome [277]. An advantage of liposomal delivery of synthetic mRNA molecules over viral transduction is the avoidance of the host cell immune response, as synthetic mRNAs can be designed to mimic host mRNA and avoid PRR binding [191]. In addition, similar to non-integrating viruses, modified mRNA does not cause changes to the host genome and therefore is not associated with risk of mutagenesis of oncogenes or tumor suppressor genes. Much progress over the last decade has led to efficient protocols to generate iPSCs from differentiated cells by TF introduction via modified mRNA [374]. Nanoparticle-delivery of mRNA has also been explored as a COVID-19 vaccine candidate in human trials, highlighting the safety and stability of LNPs in vivo [153].
2.6.1.3 Bacterial Type-III Secretion System

A disadvantage of mRNA-based gene introduction is the lag time between initiation of translation and accumulation of functional protein due to the time it takes for a cell to translate delivered mRNA molecules into proteins of interest. In addition, the dependence of the rate of translation on cellular properties may further confound experiments, as the number of available ribosomes and tRNA substrate is not universal [290]. As suggested in the Del Vecchio et al. and Ronquist et al. methods above, cellular reprogramming experiments may benefit from introducing high concentrations of TFs at a precise point in time which presents a challenge for mRNA-delivery methods considering the time needed for translation. An emerging technique to introduce proteins directly into cells takes advantage of the bacterial type-III secretion system (T3SS). The bacterial T3SS functions as a molecular needle, puncturing the membrane of eukaryotic cells and injecting proteins that carry an N-terminal secretion signal. Originally a mechanism of toxin delivery by pathogens such as Salmonella, Shigella, and Yersinia species, this system has been commandeered for use in various peptide delivery experiments. This system has several advantages over nucleic acid-based approaches. First, the work of protein production is outsourced to bacterial cells, which generate and deliver the protein of interest to the eukaryotic cell at a rate that is independent of recipient cell state. In addition, the host response to foreign nucleic acids is avoided, as proteins are delivered directly without a nucleic acid intermediate. Finally, no changes to the genome are made, mitigating the risk of mutagenesis seen with lentiviral delivery methods. This system therefore has the potential to deliver TFs to cells without permanent genetic aberrations while minimizing confounding cell behavior caused by foreign nucleic acids.

Bacterial T3SS delivery methods have recently been employed in several contexts with promising results. A T3SS expressing strain of Pseudomonas aeruginosa successfully delivered MYOD to mouse embryonic fibroblasts (MEFs) to reprogram MEFs to myotubes [28], and facilitated differentiation of human ESCs and iPSCs into cardiomyocytes by sequential addition of five TFs [157]. In addition to the aforementioned advantages, the versatility of bacteria allows for engineering of the system. Recently, optogentic interaction control switches were added to the Yersinia enterocolitica T3SS such that protein injection occurred in a light-dependent manner, further increasing the ability to control the delivery of peptides with respect to space and time [192]. The T3SS therefore represents a novel path forward in TF delivery for cellular reprogramming, both experimentally and therapeutically.
2.7 Future Directions

2.7.1 Biology

Incredible progress has been made in the development of cellular reprogramming regimes that give rise to a broad spectrum of cell types across all germ layers. However, overcoming low cell fate conversion efficiencies is a primary hurdle facing the future of cellular reprogramming [13, 122, 140]. While direct reprogramming reduces some of the common side-effects of iPSC reprogramming like mutagenesis and tumorigenesis, full conversion to an intended cell identity is still not guaranteed. It is common for reprogramming perturbations to drive cells to a stable yet hybrid state where some of the starting cell’s transcriptional program is retained and the target identity is only partially acquired. Such failures to fully convert have been attributed to the presence of TFs that maintain the starting cell’s gene regulatory network, lineage-specific repressors, and inaccessible chromatin among other factors. More rigorous reprogramming regimens involving the silencing of endogenous genes via CRISPR/Cas9 [370], endogenous gene activation using CRISPR/Cas9-based methods [189, 143], and cocktails of chemical compounds with or without TFs [51, 114, 135] have already shown considerable success in combating these roadblocks. Recent achievements in direct reprogramming spanning these and other conversion methods are summarized in Table 2.3. Further improvements in determining effective combinations of source cell types, reprogramming mechanisms, delivery methods, and cultivation conditions will be necessary to achieve consistent large-scale production of reprogrammed cells.

Stable cultivation and expansion of reprogrammed cells remains a challenge [64], limiting sufficient quantities of cells to reconstitute tissues and organs. Once methods of reprogrammed cell proliferation are improved, it is theoretically possible to regenerate organs derived from a patient’s somatic cells for autologous transplantation [399]. Since these organs would be recipient-derived, there would be little concern for transplant rejection. Moreover, lifelong immunosuppression, which is currently a significant source of mortality among transplant recipients, would no longer be necessary.

2.7.2 Mathematics

Looking forward, a vast arena of mathematical principles with the potential to further sufficient understanding and facilitation of cellular reprogramming still remains. The high dimensional nature of cellular states, for example, invites an opportunity to examine cellular data in a tensor state space [59]. Tensors are multidimensional arrays generalized from vectors and matrices, and have wide applications in many domains such as social sciences, biology, applied mechanics, machine learning and signal processing [70, 202, 373, 380]. Classical linear control systems, as used in Ron-
<table>
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<tr>
<th>Starting Cell Fate</th>
<th>Target Cell Fate</th>
<th>Reprogramming Factors</th>
<th>Delivery Method</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>Dermal fibroblasts</td>
<td>Adipocyte-like cells</td>
<td>PPAR-γ2</td>
<td>Lentivirus</td>
<td>[62]</td>
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<tr>
<td>Dermal fibroblasts</td>
<td>Endothelial progenitor cells</td>
<td>ETV2</td>
<td>mRNA transfection</td>
<td>[363]</td>
</tr>
<tr>
<td>Embryonic fibroblasts</td>
<td>Antigen-presenting dendritic cells</td>
<td>PU.1, IRF8, and BATF3</td>
<td>Lentivirus</td>
<td>[289]</td>
</tr>
<tr>
<td>Glioblastoma cells</td>
<td>Neuronal cells</td>
<td>forskolin, ISX9, CHIR99021, I-BET 151, and DAPT</td>
<td>Small molecule cocktail treatment</td>
<td>[182]</td>
</tr>
<tr>
<td>Embryonic fibroblasts</td>
<td>Hepatocytes</td>
<td>GATA4, FOXA2, HHEX, HNF4A, HNF6A, MYC, and P53-siRNA</td>
<td>Lentivirus</td>
<td>[387]</td>
</tr>
<tr>
<td>Bone marrow-derived cells, Fibroblasts, and Keratinocytes</td>
<td>Neural precursor cells</td>
<td>MSI1, NGN2, and MBD2</td>
<td>Plasmid transfection</td>
<td>[3]</td>
</tr>
<tr>
<td>Dermal fibroblasts</td>
<td>Cardiomyocyte-like cells</td>
<td>GATA4, MEF2C, and TBX5</td>
<td>Nanoparticles</td>
<td>[164]</td>
</tr>
<tr>
<td>Foreskin fibroblasts</td>
<td>Cardiac progenitor cells</td>
<td>GATA4, HAND2, MEF2C, TBX5, and MEIS1</td>
<td>CRRISPR-cas9 based endogenous gene activation</td>
<td>[371]</td>
</tr>
</tbody>
</table>

Table 2.3: Recent Successes in Direct Reprogramming

quist et al.’s work, often fail to fully capture the dynamics of cellular reprogramming because state vectors only represent gene expression, neglecting structural information. Chen et al. generalized the classical systems notion of controllability into multilinear systems in which the states, inputs, and outputs are preserved as tensors [59]. Multilinear control systems can significantly relieve the difficulty of describing genome-wide structure and gene expression simultaneously, and will be beneficial in analyzing the dynamics of cellular reprogramming more comprehensively. Further, Chen et al. exploited the notion of hypergraphs in modeling the network dynamics of cellular reprogramming [58]. A hypergraph is a generalization of a graph in which its edges can join any number of nodes. The notion of transcription factories supports the existence of multiway interactions involving multiple genomic loci [72], which implies that the human genome configuration can be more accurately captured by hypergraphs. Chen et al. developed the notions of entropy and controllability for hypergraphs, which will be potentially advantageous in investigating network dynamics of cellular reprogramming (e.g. detecting cell identities transition points and identifying minimum TF inputs) [58, 60]. Ultimately, cellular reprogramming would be required to account for nonlinearity or nonlinear control in the multiway dynamical system representation and analysis framework, and is an important direction for future research.
2.7.3 Medicine

Reprogrammed cells have many translational applications, from their direct use in replacement therapy to use as experimental models of disease and pharmacologic screens [320]. Many human diseases are characterized by the loss of a functioning tissue, such as the loss of hematopoietic stem cells in aplastic anemia or insulin-secreting beta-islet cells in diabetes mellitus. Reprogramming methods to generate cells lost in such disease states have been described and are rapidly maturing in the laboratory setting, such as those to produce hematopoietic stem cells, neurons, cardiomyocytes and pancreatic beta-islet cells [312]. Once refined, their translation to clinical trials will pave the way for curative approaches to chronic diseases. Early clinical successes, such as the successful autologous graft of RPE cells, described above, indicate that autologous grafts using reprogrammed cells are safe and teeming with potential.

Moreover, reprogrammed human cells have the potential to make superior models of disease compared to animal models. A patient-specific disease model may be created by generating iPSCs from skin fibroblasts and differentiating them into the cell type relevant to the patient’s condition. This approach allows the experimentalist to shed light on the mechanisms of pathogenesis, as cells of patients with susceptibility to disease may be closely observed from their developmental infancy to their diseased state. Such iPSC-derived models have already been developed for several conditions, from microcephaly [178] to autism spectrum disorder [213]. While a useful tool in several settings, iPSCs may be limited in their ability to model age-related disease such as neurodegenerative conditions, as epigenetic rejuvenation caused by transit through pluripotent states may reverse aspects of pathogenesis. Direct reprogramming techniques have been employed to circumvent this, as this approach avoids widespread reversal of age-related epigenetic changes [350, 184]. Using these techniques, reprogrammed cell lines may be generated from patients to study the mechanisms and pharmacologic susceptibilities of their disease, enabling better understanding of the connections between patient genotype and pathologic phenotype [67].

2.8 Conclusion

This review sought to examine advancements in cellular reprogramming and computational methods that contribute to them. Many promising approaches in cellular reprogramming are under development, though discovery of new TF recipes to efficiently convert source cells to target cells remains limiting. Mathematically-based approaches such as those outlined here may facilitate their systematic discovery.

Moreover, convergent ideas in the areas of biology, mathematics and medicine point to hybrid (concurrent TF addition and removal), time-dependent, and concentration-dependent reprogram-
ming regimes as viable next steps to improve reprogramming methods. These refinements could offer increased control over cell identity of normal and abnormal cells, and their \textit{in vivo} regenerative potential.
PARTIAL REPROGRAMMING OF COLORECTAL CANCER CELLS
TOWARDS TREATMENT SENSITIVITY

This chapter is motivated by a paper by Markus A. Brown, Gabrielle A. Dotson, Scott Ronquist, Georg Emons, Indika Rajapakse, and Thomas Ried [39].

3.1 Abstract

Canonical Wnt signaling is crucial for intestinal homeostasis as TCF4, the major Wnt signaling effector in the intestines, is required for stem cell maintenance. The capability of TCF4 to maintain the stem cell phenotype is contingent upon \( \beta \)-catenin, a potent transcriptional activator, which interacts with histone acetyltransferases and chromatin remodeling complexes. We used RNAi to explore the influence of TCF4 on chromatin structure (Hi-C) and gene expression (RNA sequencing) across a 72-hour time series in colon cancer. We found that TCF4 reduction results in a disproportionate up-regulation of gene expression, including a powerful induction of \( SOX2 \). Integration of RNA sequencing and Hi-C data revealed a TAD boundary loss, which occurred concomitantly with the over-expression of a cluster of \( CEACAM \) genes on chromosome 19. We identified EMT and E2F as the two most deregulated pathways upon TCF4 depletion and \( LUM, TMPO, \) and \( AURKA \) as highly influential genes in these networks using measures of centrality. Results from gene expression, chromatin structure, and centrality analyses were integrated to generate a list of candidate transcription factors crucial for colon cancer cell homeostasis. The top ranked factor was c-JUN, an oncoprotein known to interact with TCF4 and \( \beta \)-catenin, confirming the usefulness of this approach.
3.2 Introduction

The Wnt signaling transcription factor, TCF4, is crucial for homeostasis of the mammalian intestine [170]. Loss of TCF4, in either embryonic or adult mice, results in ablation of the proliferative compartment of the intestine [362]. The capability of TCF4 to drive a crypt progenitor phenotype and maintain intestinal homeostasis is contingent upon its binding partner. When Wnt signaling is active, cytoplasmic β-catenin migrates to the nucleus where it binds TCF4, resulting in target gene expression. β-catenin is a potent transcriptional activator which influences the surrounding chromatin by recruiting histone acetyltransferases, chromatin remodeling factors, and RNA polymerase associated factors [2, 133, 18, 229]. Therefore, the binding of a βcat/TCF4 complex significantly enhances the recruitment of the cellular machinery necessary for transcription, thereby driving a crypt progenitor phenotype [360].

Conversely, when TCF4 is bound by the TLE family of transcriptional repressors, target gene expression is repressed. Despite the presence of TCF4 throughout the intestine, active Wnt signaling is sequestered to the base of the colonic crypts, thereby limiting βcat/TCF4 complex formation, and the resulting crypt progenitor phenotype, to cells of the crypt base [19, 300, 360].

In colorectal cancer, mutations in the Wnt signaling pathway, primarily in APC, result in constitutive Wnt signaling activity [168, 291]. High levels of nuclear β-catenin result in the constitutive expression of Wnt target genes, such as MYC and CCND1 [225, 171, 132, 345]. Given that epigenetic modifications and chromatin remodeling have been reported to occur prior to the expression of Wnt target genes, the high levels of nuclear β-catenin in colorectal cancer likely influence the surrounding chromatin structure [18, 253].

It has become evident that understanding chromatin structure lends insight into the regulation of gene expression [190, 94]. Aspects of chromatin structure include the accessibility of genomic loci to the transcriptional machinery as well as the 3-dimensional configuration of the chromatin, which may facilitate cooperativity between genomic regions sharing a particular 3-dimensional space. Division of the genome into euchromatin and heterochromatin domains, referred to as A and B compartments, respectively, reflects the interaction between chromatin structure and function at the chromosomal level [190]. Chromosome folding brings distant sites along the linear genome in close spatial proximity, forming topologically associating domains (TADs), which are insulated regions of the genome sharing epigenetic modifications, gene expression, and replication timing [94, 274, 251]. Given the influence of chromatin structure on gene expression, the dissection of a cellular response to a stimulus requires an understanding of both structural and functional dynamics [266, 284].

Herein, we explored the dynamical influence of silencing TCF7L2, the gene encoding TCF4, on chromatin structure and gene expression across a 72-hour time series in the colon cancer cell line,
SW480. Silencing of TCF7L2 not only allows us to investigate the changes which occur as a result of silencing a major transcription factor, it also represents a clinically relevant model as TCF7L2 expression correlates with resistance to chemoradiotherapy (CRT), a treatment modality in rectal cancer [119, 162, 80]. For the time series, an inverse RNAi transfection protocol was designed and optimized, which significantly reduced confounding factors typically found in time series data. A 4DN approach to data analysis was used, which integrated structural (Hi-C) and functional (RNA sequencing) data and allowed us to identify influential genes in the most dynamically changing networks 3.1. We then identified candidate reprogramming factors, to be perturbed alongside, or in lieu of, TCF7L2 to debilitate the colorectal cancer cell.

3.3 Results

3.3.1 Gene Expression is Disproportionately Up-Regulated Across the Time Series

The time series data was generated by sequential addition of a small, interfering RNA (siRNA) targeting the 3' UTR of TCF7L2 at equally spaced time points (0, 24, 48, and 72 hours) according to a modified siRNA transfection protocol (Figure S1A in Brown et al. [39], see Methods). The modified protocol was designed to mitigate varying cell cycle distributions, which occur naturally due to varying growth times and are a major confounding factor in extended (>48 hours) time series data (Figure S1C and D in Brown et al. [39]). The modified protocol resulted in 70% of the cells in the G1 phase of the cell cycle at each time point (Figure S1B in Brown et al. [39]).

To assess the efficacy of siRNA-mediated TCF7L2 silencing, the abundance of TCF7L2 transcripts was determined using quantitative PCR (qPCR). Transcript levels decreased progressively across the time series resulting in a 2-fold decrease by 72 hours (Figure 3.1B). The amount of TCF4, the protein product of TCF7L2, was determined using a Western Blot. TCF4 protein levels decreased marginally after 24 hours, 35% after 48 hours and 70% after 72 hours (Figure 3.1C). Principal component analysis (PCA) of the RNA sequencing data demonstrated a directed change in the global gene expression program, with biological replicates clustering together (Figure 3.1D). The number of differentially regulated genes which exhibited both a statistically significant ($p < 0.05$) and a 4-fold change in expression increased dramatically across the time series (Figure 3.1E). However, the balance was positively skewed with nearly twice the number of genes up-regulated as down-regulated. Considering that SW480 cells have constitutive Wnt signaling activity, and therefore persistent transcription through $\beta$-cat/TCF4 complexes, the general increase in gene expression following the reduction in TCF4 is unexpected.

To determine the biological relevance of the disproportionate up-regulation of gene expres-
sion, the expression profiles of individual genes with known biological roles were assessed. The expression of prominent Wnt signaling target genes, such as MYC and CCND1, decreased consistently across the time series, similar to that of TCF7L2 [132, 376, 342]. It has been previously reported that loss of β-cat/TCF4-mediated transcription results in the up-regulation of genes associated with intestinal differentiation [360]. We confirm this observation as genes associated with differentiation, such as MUC2 and LGALS4, were significantly up-regulated following TCF7L2 silencing (p < 0.05) (Figure S2A in Brown et al. [39]).

3.3.2 SOX2 Up-Regulation is a Consequence of TCF7L2 Silencing

We identified a powerful (log₂FC ~3.55) up-regulation of SOX2, which began after 24 hours and continued to increase until 72 hours post-transfection, which we found was dose-dependent in relation to the levels of TCF7L2 (Figure 3.1F, Figure S2B in Brown et al. [39]). This may represent direct repression of SOX2 by TCF4, as TCF4 has been previously reported to bind the SOX2 promoter in colorectal cancer [131]. To determine the influence of nuclear β-catenin on this interaction, we performed a Western Blot (Figure 3.1G). Levels of active β-catenin did not fluctuate over the time series, indicating that the interaction between TCF4 and SOX2 is independent of β-catenin, in this system, supporting our direct repression hypothesis. Given the potency of the SOX2 up-regulation, we also investigated whether a concomitant shift in nuclear structure occurred at the SOX2 locus. SOX2 resides at a TAD boundary in SW480 cells, however the boundary remained unchanged across the time series, indicating that nuclear structure was not a contributing factor in the up-regulation of SOX2 (Figure S3A in Brown et al. [39]). The observation that TCF7L2 silencing results in the over-expression of SOX2 suggests that TCF7L2 may enforce a transcriptional module which potently represses SOX2 under physiological conditions.

3.3.3 Genome-wide A/B Compartments are Maintained Over Time

Hi-C was performed to capture genome-wide changes in chromosome structure across the time series to determine the influence of TCF4 on chromatin organization. TCF7L2 silencing resulted in a significant, genome-wide increase in both the number and strength of pairwise chromatin interactions, as evidenced by matrix subtraction between 0 and 72 hour Hi-C contact maps (Figure 3.2A). The contact maps demonstrate a higher interaction frequency at 72 hours, while the algebraic difference between the two contact maps reveals that the overwhelming direction of change is an increase in chromatin interaction from 0 to 72 hours. Out of all possible 1Mb-scale chromatin interactions, SW480 cells at 0 hours harbored 74% of those interactions, whereas by 72 hours the proportion of interactions had risen to 87%, demonstrating that the chromatin became more compact over time.
Figure 3.1: Conceptual Approach and Gene Expression Dynamics Following TCF7L2 Silencing. (A) Schematic summary of the experimental approach. We sought to explore how silencing TCF7L2 impacted the colorectal cancer gene network in terms of chromatin structure and gene expression. (B) qPCR demonstrated progressive silencing of TCF7L2 over time with a 75% reduction in TCF7L2 transcript levels after 72 hours. Each dot represents a biological replicate, with three replicates plotted per time point (n = 3). The green line represents the mean, while the green-shaded ribbon represents the standard deviation. (C) Western blot analysis demonstrated a 70% decrease in TCF4 protein abundance by the last time point with β-actin as loading control. (D) PCA of gene expression (TPM) over the time course, with time points differentiated by color (n = 2). (E) Volcano plots show differential gene expression genome-wide with thresholds set at log2FC = 2, and p = 0.5 (equivalent to 1.3 on the –log10P scale). Genes in red are significantly down-regulated and undergo a > 4-fold decrease in expression, while those in green are significantly up-regulated and experience a > 4-fold increase in expression. The number of genes which fall within these regions are shown. (F) Expression profile of SOX2, which increases dramatically across the time series. The dots correspond to each biological replicate (n = 2), the shaded ribbon represents the standard deviation. (G) Western Blot for active β-catenin over time with β-actin as loading control. No noticeable change in active β-catenin protein levels occurs during the time series.
To further assess changes in genome-wide chromatin organization, the genome was assigned to A/B compartments using a spectral graph theory approach, which calculates the Fiedler number for each 100kb bin (Figure S3B in Brown et al. [39][63]. The sign of the Fiedler number, positive or negative, indicates within which compartment the bin resides. An A/B compartment switch occurred in ~4% of the genome at any given point during the time series, which encompassed 1,305 genes. K-means clustering identified eight distinct switching patterns in which a specific genomic locus switched compartments unidirectionally (A-to-B or B-to-A) or bidirectionally (a permutation of A-to-B-to-A-to-B) over time (Figure 3.2B). Approximately 52% of the regions demonstrating an A/B compartment switch underwent a single switch, while the remaining 48% underwent multiple switching events. Despite the switching events, the overall proportion of A/B compartments genome-wide remained relatively unchanged with most loci residing in the same chromatin state across the time series (52% A and 48% B)(Table S2 in Brown et al. [39]). Furthermore, ~1.3% of the genes within the A/B switching regions were significantly differentially expressed following the compartment switch.

3.3.4 TAD Boundary Loss in the CEACAM Gene Cluster

Local partitioning of chromatin into TADs provided insight into the coupling of structure and function. Genes occupying the same TADs have been shown to be co-expressed, likely by accessing the same cluster of transcriptional machinery as a result of their spatial proximity [94, 74]. The number of TADs genome-wide fluctuated by 5% over the time series, while the number of common TADs shifted by 7% (Table S3 and S4 in Brown et al. [39]). To gain insight regarding which factors may be involved in the TAD domain switching, we used publicly available CTCF and TCF4 ChIP-seq data sets from the HCT116 colorectal cancer cell line (see Methods, Table S5 and S6 in Brown et al. [39]). We observed that the binding locations of CTCF in HCT116 matched 75% of the TAD boundaries of SW480 while the binding locations of TCF4 matched 41% of the TAD boundaries. However, while TAD boundary binding by CTCF was relatively consistent across chromosomes, the binding of TCF4 fluctuated, with chromosome 19 showing the highest levels of TCF4 binding.

On chromosome 19, a ~10Mb span of the genome houses a group of CEACAM family genes, which are involved in colorectal cancer progression and metastasis [23]. At the initial time point, CEACAM5, CEACAM6, and CEACAM7 were located within the same TAD, while CEACAM1 and CEACAM8, were located in an adjacent, smaller TAD (Figure 3.2C). Changes in expression of the two CEACAM gene clusters after 24 hours were negligible. However, at the 48 hour time point, the boundary separating the two TADs was lost, joining CEACAM5, CEACAM6, and CEACAM7 into a larger TAD with CEACAM1 and CEACAM8. Combination of the TAD domains as well as
Figure 3.2: Changes in Global and Local Hi-C Partitioning Over Time. (A) Algebraic difference between genome-wide Hi-C contact maps at 0 and 72 hours demonstrates an overall increase in contact frequency over time. (B) Global partitioning (A/B compartmentalization) dynamics revealed that 1,091 100-kb genomic bins switched compartments at one or more time points genome-wide. The Fiedler number (see Methods) of each loci are k-means clustered into eight groups (denoted to the left) exhibiting different switching dynamics. The number of genomic bins in each cluster is specified on the right. A 95% change in Fiedler number from one time point to another was set as a threshold to remove inherent noise.
a significant increase in expression for both CEACAM groups occurred seemingly simultaneously at 48 hours. Gene expression for both groups continued to increase following the TAD boundary loss (Figure 3.2C). The increase in gene expression likely represents increased accessibility to the transcriptional machinery for both gene clusters. Silencing of TCF7L2 in multiple colon cancer cell lines demonstrates that the up-regulation of CEACAM1 is present in both APC mutated (SW480, DLD1) as well as APC wild-type (HCT116, LS174T) colon cancer cell lines (Figure 3.2D). The up-regulation of CEACAM1 is however weaker in COLO201, which is derived from a metastatic site.

### 3.3.5 TAD Boundaries of the CEACAM Gene Cluster Show Enrichment for the SP1, KLF4, ZFX, and MZF1 Transcriptions Factors

To identify which transcription factors may be mediating this TAD partitioning loss, a transcription factor enrichment analysis was performed using oPOSSUM-3 (Figure 3.2E) [175]. Over-representation of transcription factor binding sites (TFBS) in the DNA sequences of the 64 genes occupying the differentially conformed region of chromosome 19 were investigated. The top four identified transcription factors were SP1, KLF4, ZFX, and MZF1 (Figure 3.2F). The top hit, SP1, is found ubiquitously and is involved in chromatin remodeling. KLF4 is involved in embryonic development and binds a GC-rich motif highly similar to SP1. ZFX plays a role in self-renewal of hematopoietic stem cells and MZF1 has been associated in inflammatory bowel disease. To further explore the role of SP1, and TCF4, in mediating this TAD boundary change, we used publicly available SP1 and TCF4 ChIP-seq data sets (see Methods) from the HCT116 colorectal cancer cell line. We found that both SP1 and TCF4 bind the site of the TAD boundary observed in SW480 and
it is therefore feasible that they are responsible for the repartitioning of this domain (Figure 3.2C).

### 3.3.6 Coordinated Pathway Responses Suggest a Loss in Cell Cycle Progression and DNA Synthesis Capabilities

To assess the dynamics of TCF4 loss on pathway behavior, fast Gene Set Enrichment Analysis (fgsea) was performed using the 50 Hallmark gene sets and the \( \log_2 \) expression values from each time point to generate a normalized enrichment score (NES) [328, 308]. The NESs from fgsea were compared and the 20 gene sets (10 up-regulated and 10 down-regulated) with the greatest change by the last time point were plotted (Figure 3.3A). Strong decreases in expression for both MYC Target gene sets as well as the E2F Targets and G2M Checkpoint gene sets were observed. All four of these gene sets are involved in growth and the regulation of cell cycle progression. The E2F pathway is also involved in DNA synthesis, thereby linking DNA synthesis to cell cycle progression [148, 281].

Interestingly, the Notch, Cholesterol homeostasis, Estrogen Response, and MTORC1 signaling pathways all demonstrated a similar NES profile over time, suggesting coordinated function. Several articles identify interplay between these pathways, supporting this hypothesis [56, 34]. The EMT, Apoptosis, and Interferon Gamma pathways demonstrated a coordinated network of up-regulated pathways. The most dynamically up-regulated and down-regulated gene sets upon TCF7L2 silencing were EMT and E2F, respectively.

### 3.3.7 Pathway Level Structure-Function Relationships

We then sought to compare structure-function relationships in these pathways, utilizing the Frobenius norm, which describes the space of a vector (gene expression) or matrix (chromatin interaction frequencies) [326] (Figure 3.3B). We find that, generally, the pathways which demonstrate more drastic changes in expression also occupy a more open conformation. For instance, strongly down-regulated E2F Targets, MYC Targets V1, V2, and Unfolded Protein Response pathway genes reside in a more open conformation than do the moderately down-regulated genes in the MTORC1, Notch, G2M Checkpoint, and Estrogen Response Early pathways. This remains largely true for the up-regulated pathways with the Coagulation, Apical Junction, and KRAS Signaling pathway genes residing in a more open conformation than the Allograft Rejection, Interferon Alpha Response, Apoptosis, and Interferon Gamma Response pathway genes.

However, the exceptions are the EMT, Cholesterol Homeostasis, and DNA Repair pathways. The EMT pathway undergoes the most dramatic shift in expression, yet is in the most constricted conformation, while the Cholesterol Homeostasis and DNA Repair pathways have the least change in expression yet still reside in one of the most open conformations.
Figure 3.3: Pathway Level Gene Expression and Structural Dynamics. (A) Fast Gene Set Enrichment Analysis (fgsea) was performed using the Hallmark Gene Sets and log\textsubscript{2}FC for each time point to generate a normalized enrichment score (NES). The NESs of the ten most up- and down-regulated pathways were then plotted as a heatmap to show pathway level gene expression over time. The NES at the initial time point is set to 0. (B) The montage of plots reflect the change in Frobenius norm of pathway-specific contact frequency data for each time point and pathway of interest. Norm values are min-max normalized across all pathways represented. Each pathway is represented as four points, with each point corresponding to a time point, with higher values indicating a more open conformation. The E2F Targets, MYC Targets V1 and V2, Coagulation, and KRAS Signaling pathways all demonstrate high degrees of openness.

3.3.8 EMT and E2F Signaling Genes are Highly Connected in SW480 Cells

The two most dynamic gene sets, EMT and E2F, were then probed to gauge the connection between these networks in three-dimensional space. A Hi-C-derived, synthetic 5C contact map was generated to highlight the inter-loci interaction frequencies among EMT and E2F signaling genes. The contact map is comprised of 1Mb bins containing genes in either of the pathways (see Methods). Compared to a synthetic 5C contact map composed of randomly sampled, gene-containing genomic bins, we observe that the EMT and E2F signaling genes contact map is denser, suggesting that these genes are more closely interacting than other genes in the genome (Figure 3.4A). Permutation testing reveals that these genes are indeed more inter-connected than genes outside either network ($p < 0.001$), with a Fiedler number that surpasses those of 1,000 sets of randomly sampled genes (Figure S4A-B in Brown et al. [39]).

To understand the evolving behavior of individual genes in the EMT, E2F, and Wnt signaling
networks, a gene-level, structure-function analysis was performed. Each gene was examined at 5kb resolution and was assigned a 35kb-length window – 5kb overlapping the transcription start site and 15kb flanking either side of that 5kb region – creating a seven by seven sub-matrix for each gene. Several network-based approaches have previously been used to characterize behaviors in dynamically changing genomes [305, 198]. We apply one such approach - a derivative of Von Neumann Entropy (VNE) - to measure local chromatin organization of individual gene regions [65]. Higher VNE values indicate that the number of conformations available to the gene and its immediate neighborhood are higher, indicating that chromatin is more accessible. We computed VNE on the EMT, E2F, and Wnt signaling gene sub-matrices and plotted them as a function of gene expression, creating a phase portrait (Figure 3.4B).

The phase portraits show the gene-level trajectory of chromatin accessibility and expression for the ten most dynamic genes, as determined by the area of their ellipse. Overlap between the gene ellipses demonstrates a homogeneous concerted pathway response in the EMT and E2F gene sets, indicating that the genes, in their respective pathways, are in a similar chromatin environment and demonstrate coordinated changes in expression. The WNT gene network undergoes a greater diversity in its response, indicative of varying chromatin environments and diverse transcriptional regulation at the individual gene loci. Genes with different expression patterns, yet whose phase portraits still overlap, are indicative of regulation at the transcriptional level (such as transcription factor binding), rather than at the chromatin level. For example, $CCNY$ and $LEF1$ are present in similar chromatin environments, as indicated by their overlapping phase portraits, yet show opposite functional patterns, indicating that their regulation occurs at the transcriptional level. Overall, we observe that the fitted ellipses change dynamically in terms of structure and function, confirming that $TCF7L2$ silencing influences chromatin structure.

### 3.3.9 Network Interactions are Preserved as Genes Move Spatially Closer Over Time

To further explore chromatin topology, we performed network centrality analysis on inter-loci interaction data from our synthetic 5C contact maps. Network centrality describes the influence or importance of nodes in a network, which yields insight into how the network relays information [33, 240, 198]. We calculated four measures of centrality: betweenness, closeness, degree, and eigenvector. Betweenness centrality identifies nodes which lie along the shortest path between other nodes, closeness centrality is determined by the average farness to all other nodes, degree centrality captures the number of edges connected to a node, and eigenvector centrality determines the influence of a node based on the influence of the neighboring nodes.

Roughly 57% of the EMT and E2F network genes underwent a significant change in between-
Figure 3.4: Centrality Analyses and Gene Level Structure-Function Relationships. (A) Gene network-level synthetic 5C contact maps were generated by extracting bins corresponding to genes in the EMT and E2F signaling networks from a 1Mb adjacency matrix and stitched together (top) to create a synthetic contact map (right). Synthetic 5C contact map for randomly sampled gene bins (left) shown for comparison. (B) Structure versus function phase portrait for the EMT, E2F, and WNT signaling pathway genes. Min-Max normalized Von Neumann Entropy (see Methods) and gene expression represent structure and function, respectively. The ten most dynamic genes (those whose fitted ellipse have the largest areas) in each network are shown. Genes that decrease in expression over time are labelled in blue and genes that increase in expression are labelled in orange. Phase portraits reveal the extent to which chromatin structure and gene expression are coupled. (C) Closeness centrality increases over time, indicating that the network becomes increasingly compact. An increase in betweenness centrality is also observed over time which reaches a maximum at 48 hours. Peaks in betweenness centrality plot identify bins (genes) which significantly regulate the network. The Random 5C Assembly demonstrates a progressive increase in closeness centrality, reflective of the general increased contact frequencies over time.
ness centrality, the majority of which experienced a decrease in their betweenness centrality score (Figure 3.4C). LUM, TMPO, and AURKA had the highest betweenness centrality scores at any given time point, suggesting that they are highly influential in their respective networks. Since silencing of TCF7L2 resulted in decreased betweenness centrality scores for these genes, they represent the most likely nodes by which TCF4 may influence the EMT and E2F networks.

Closeness centrality scores provided insight into the evolving proximity of the network genes. We observe that closeness centrality increased with time for all loci with either of the latter two time points (48 and 72 hours) having the highest scores (Figure 3.4C). This accompanies the previous observation that pairwise chromatin interactions increase over time, i.e., chromatin organization is becoming denser. Since a higher closeness centrality score indicates that a node is relatively closer to all other nodes in the network, this trend suggests that the genes become more organized with time. We observe that the eigenvector and degree centralities of our network genes fluctuate very little with time - meaning the same gene interactions with a similar magnitude of connectivity occur at each time point (Figure S4C in Brown et al. [39]).

3.3.10 4DN Analysis Provides Insight Into TF-Driven Controllability of CRC Cells

Our findings demonstrate that silencing TCF7L2 is sufficient to mediate structural and functional changes that influence the behavior of colorectal cancer cells. We suspect that there are a number of reprogramming factors that, when silenced alongside TCF7L2, could further destabilize the colorectal cancer cell. Genes that have significant nodal influence represent ideal candidate reprogramming factors, as a result of their ability to destabilize and promote wide-spread changes in the network. Betweenness centrality is one measure of nodal influence and is particularly characteristic of cross-talk control. We find that in the colorectal cancer network, 187 1Mb bins containing 215 genes from the EMT and E2F signaling gene networks harbored a significant change in betweenness centrality from 0 to 72 hours. These genes were ranked according to their magnitude of change in betweenness centrality, with higher magnitudes corresponding to a higher ranking. The genes were then ranked separately by their percent change in gene expression and Frobenius norm between 0 and 72 hours to incorporate aspects of gene function and structure. The average of the three rankings for each gene was computed and the genes ordered accordingly, with lower rankings indicating a gene with the most controllability potential. Considering that transcription factors regulate a network of genes, we summarize the rankings and dynamics for the most likely transcription factor-encoding genes from our candidate gene list in Table 3.1. The highest ranked candidate was c-JUN, an oncogenic subunit of the AP-1 transcription factor, whose activity is augmented in many cancer types, and has been shown to interact with TCF4 and β-catenin to form a
ternary, transcriptionally-competent complex [236].

<table>
<thead>
<tr>
<th>Gene</th>
<th>Network</th>
<th>Gene Expression (Fold Change)</th>
<th>Structure (Frobenius Norm)</th>
<th>Betweenness Centrality (Fold Change)</th>
<th>Biological Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>JUN</td>
<td>EMT</td>
<td>0.56</td>
<td>87.11</td>
<td>-4.29</td>
<td>Stabilizes β-cat/TCF4 complexes, decreases MYC degradation</td>
</tr>
<tr>
<td>SNAI2</td>
<td>EMT</td>
<td>-1.35</td>
<td>81.57</td>
<td>2.81</td>
<td>Promotes invasion and metastasis in EMT</td>
</tr>
<tr>
<td>TP53</td>
<td>E2F</td>
<td>-0.74</td>
<td>66.56</td>
<td>2.45</td>
<td>Crucial tumor suppressor involved in regulating DNA repair/apoptosis</td>
</tr>
<tr>
<td>MXD3</td>
<td>E2F</td>
<td>-0.10</td>
<td>78.36</td>
<td>-4.21</td>
<td>Suppresses MYC dependent cell transformation</td>
</tr>
<tr>
<td>MSX1</td>
<td>EMT</td>
<td>0.12</td>
<td>85.91</td>
<td>-3.35</td>
<td>Transcriptional repressor in limb-pattern formation</td>
</tr>
<tr>
<td>MYBL2</td>
<td>E2F</td>
<td>-0.68</td>
<td>149.56</td>
<td>-2.27</td>
<td>Regulates cell survival, proliferation, and differentiation</td>
</tr>
<tr>
<td>PRRX1</td>
<td>EMT</td>
<td>-1.36</td>
<td>59.32</td>
<td>3.00</td>
<td>Regulator of muscle creatine kinase</td>
</tr>
<tr>
<td>E2F8</td>
<td>E2F</td>
<td>-0.23</td>
<td>131.82</td>
<td>-1.29</td>
<td>Regulates progression from G1 to S phase</td>
</tr>
<tr>
<td>DNMT1</td>
<td>E2F</td>
<td>-0.12</td>
<td>92.32</td>
<td>1.02</td>
<td>May silence tumor suppressor genes by methylation</td>
</tr>
</tbody>
</table>

Table 3.1: Ranked Candidate Reprogramming Factors. A total of 215 genes from the EMT and E2F signaling networks were ranked according to the magnitude of their change in gene expression, frobenius norm (structure), and betweenness centrality, between the 0 and 72 hour time points, irrespective of direction. The list of candidate factors was then filtered by removing genes which are not well characterized as transcription factors as well as those whose motif binding information was unavailable.

### 3.4 Discussion

#### 3.4.1 TCF4 as a Transcriptional Repressor

Colorectal cancers exhibit constitutively active Wnt signaling, most commonly due to mutations in APC [168]. The over-expression of Wnt target genes is driven by β-catenin/TCF4 complexes, as TCF4 is the major Wnt signaling transcription factor in colorectal cancer [171]. Due to the central role of Wnt signaling in colorectal tumorigenesis as well as the capability of β-catenin to modulate the chromatin environment, we explored the dynamical influence of TCF4 on chromatin structure and gene expression in the SW480 colorectal cancer cell line.

**TCF7L2** silencing resulted in a progressive deregulation of the transcriptome with the most dynamic changes occurring at the last time point. Surprisingly, nearly twice the number of genes were up-regulated upon **TCF7L2** silencing. While difficult to distinguish between direct and indirect effects, these results hint at a repressive role for TCF4. In support of this, naturally occurring dominant-negative variants of TCF4, have been described [358, 44]. The gene **SOX2** demonstrated a powerful increase in expression, which occurred progressively over the time series. We reason that this induction is a result of decreased repression by dominant negative TCF4 isoforms, as TCF4 has been shown to bind the **SOX2** promoter [131]. Additionally, this interaction occurs independently of fluctuating β-catenin levels, in line with repression by a dominant-negative isoform. Furthermore, interactions between canonical Wnt and **SOX2** have been described in various cell types. In lung epithelia, an antagonizing role between canonical Wnt signaling and **SOX2** has been described in which activation of Wnt signaling led to the loss of **SOX2** and interfered with the devel-
opment of the bronchiolar epithelium [130]. In gastric tumorigenesis, SOX2 functioned as a tumor suppressor which antagonized Wnt-driven adenomas [299]. These two examples demonstrate a mutual, antagonizing relationship between SOX2 and canonical Wnt signaling, which influences the development of normal tissue (lung) as well as adenomas (stomach). Here, we observe an antagonizing role between TCF4 and SOX2 in colorectal cancer. Taken together, these results suggest that TCF4 maintains a transcriptional module for repressing SOX2, which may be necessary for generating and/or maintaining the intestinal lineage.

3.4.2 TCF4 Influences Local Genome Organization

Time series Hi-C experiments demonstrated that TCF4 influences chromatin organization both globally and locally. Silencing of TCF7L2 led to an increase in pairwise chromatin interactions, demonstrating an overall compaction of the chromatin across the genome. This may be due to a decrease in β-cat/TCF4 binding, which would lead to decreased β-catenin-mediated chromatin acetylation. The high levels of nuclear β-catenin present in colorectal cancer may be sufficient for mediating a genome-scale chromatin effect. However, the compaction was not sufficient to cause a decrease in gene expression.

Approximately 4% of the genome underwent an A/B compartment switch at any given time point. The silencing of a single transcription factor leading to compartment switching in 4% of the genome is comparable to over-expressing four transcription factors which influence ~20% of the genome [322]. Despite changes in A/B compartment switching, the total number of bins which resided in the A/B compartments remained stable over time (52% A, 48% B). This supports the existence of a genome-wide ‘balance’ of open and closed chromatin domains. However, the A/B compartment balance in normal cells of the mouse is different, at 40% A and 60% B [322]. This may be a reflection of the difference in species or could be an indication that, since cancer cells have enlarged nuclei, they may be able to support a larger percentage of the genome in the A compartment, and therefore support a more diverse transcriptome. However, an increased nuclear volume could merely reflect the increased DNA content of cancer cells. Another explanation for the relatively stable A/B compartment balance is a result of noise within the data. Since a single time series was performed for Hi-C, we are unable to exclude this possibility.

TAD analysis demonstrated that the number of TAD domains fluctuated by ~5% over time. Incorporating CTCF ChIP-seq data from HCT116 demonstrated that the majority (80%) of the TAD boundaries are likely bound by CTCF, whereas 41% are likely to be bound by TCF4. On chromosome 19, a TAD domain change was coordinated with an increase in gene expression in the CEACAM family genes. The most dramatic change in the expression of these genes occurred concomitantly as the TAD boundary loss, hinting at a causal link between the two. Following the
TAD domain coalescence, the expression of both *CEACAM* gene groups continued to increase, likely by accessing the same transcriptional machinery, as they are part of the same gene family. Incorporation of ChIP-seq data for TCF4 and SP1, the transcription factor with the highest degree of enrichment in the switching region, suggested that TCF4 and SP1 could feasibly be responsible for the TAD domain change as peaks for both transcription factors were present near the initial TAD boundary. Given the current understanding of structure-function relationships, we interpret the dynamics of the event thus: *TCF7L2* silencing results in the decreased activity of a factor, such as TCF4 itself, which then influences the activity of SP1, which results in loss of the TAD boundary. Boundary loss increases the probability that the two *CEACAM* groups will come in close contact, thereby allowing them to coordinate recruitment of the transcriptional machinery.

### 3.4.3 Structure-Function Relationships in SW480

From fgsea, we observed down-regulation of the MYC, E2F, and the G2/M gene sets, all which mediate growth and cell cycle progression. In addition to regulating growth, genes in the E2F pathway are also responsible for DNA synthesis [148, 281]. Additionally, despite showing the greatest decrease in pathway gene expression, E2F genes are still expressed to a higher degree than genes in EMT, the most up-regulated gene set (Figure 3.4A - Gene Expression). Previous evidence has demonstrated that TCF4 expression corresponds with resistance to CRT and that loss of TCF4 increases sensitivity [119, 162, 37]. Furthermore, it has been suggested that the mutation rate in intestinal stem cells is relatively equal to the mutation rate in liver stem cells, despite the higher proliferative rate of the former [31, 118]. We hypothesize that high levels of DNA synthesis/DNA proofreading activity, mediated by E2F, could maintain DNA sequence integrity in the face of continued stem cell proliferation and, in colorectal cancers, this same DNA program would defend against CRT. Loss of TCF4 results in cell cycle arrest, ensuring that cells with hampered DNA repair capabilities do not propagate in the stem cell crypt.

Investigation of structure and function relationships in the top 20 most dynamic pathways demonstrated that pathways whose genes resided in a more open conformation also demonstrated more dramatic changes in expression. Pathways which demonstrated less dramatic changes in expression also resided in chromatin environments which were more constricted. This trend was observed in 85% of the most dynamically acting pathways. According to these results, we find that the conformation of the chromatin is not a determinant of gene expression, but rather, functions as a filter. Genes which reside in open conformations are easily accessed by the transcriptional machinery and thus experience a full response, while genes in a more constricted environment have less exposure and therefore undergo less dynamic changes in expression. The general decrease in chromatin accessibility across the time points is a result of the genome-wide chromatin com-
paction which occurred as a result of TCF7L2 silencing. In the remaining 15% of the pathways, specifically EMT, Cholesterol Homeostasis, and DNA Repair, the connection between expression and structure was not pronounced. Despite showing the most dynamic change in expression, the EMT pathway genes resided in the most constricted conformation out of the top 20 pathways. Conversely, the Cholesterol and DNA Repair pathway genes resided in the most accessible conformation yet underwent less dynamic changes in expression than other pathways. Therefore, in some instances, chromatin conformation is not a determinant of gene expression.

3.4.4 Candidate Reprogramming Factors for Colorectal Cancer

Centrality analysis allowed us to determine the most influential genes in their respective networks. The genes with the highest betweenness centrality were LUM, TMPO, and AURKA, identifying these genes as the most useful targets for propagating a stimulus through their respective pathways (EMT or E2F). We subsequently identified a list of factors which can be used in concert with loss of TCF4 to profoundly perturb the colorectal cancer cell. The top ranked candidate reprogramming factor was c-JUN, an oncoprotein which forms part of the AP-1 transcription factor. Previous work has shown that c-JUN forms a complex with TCF4 and β-catenin, which stabilizes β-catenin, and drives expression of the JUN promoter, initiating a self-stimulating feedback loop [212, 113]. Abrogation of the c-JUN, TCF4, β-catenin complex led to reduced tumor number and size, as well as prolonged lifespan [236]. High levels of c-JUN and Wnt signaling activity resulted in increased resistance to cisplatin whereas abrogation of c-JUN or Wnt signaling activity increased the sensitivity of ovarian cancer cells to treatment.

3.5 Conclusion

In conclusion, we find that TCF7L2 influences both structure and function in the SW480 colon cancer cell line. Loss of TCF4 results in a potent up-regulation of SOX2, which in a saturated WNT environment, is not dependent upon β-catenin, hinting at a repressive role for TCF4. When TCF4 is present, chromatin resides in a more open conformation genome-wide, which is likely mediated by βcat/TCF4 complexes. At the local chromatin level, the expression of a group of CEACAM genes situated at a TAD boundary was greatly increased upon TAD boundary loss, a change which is likely mediated by TCF4 or SP1. The expression of pathway specific genes corresponded to the accessibility of those genes, suggesting that chromatin conformation acts as a filter to attenuate the potency of gene expression regulation. The two most dynamic pathways following TCF7L2 silencing – EMT and E2F – interact in 3-dimensional space and the most dynamic pathway genes inhabit overlapping structure-function space. Incorporation of chromatin structure, gene expres-
sion, and centrality data allowed us to generate a list of transcription factors which would be most effective at perturbing the colorectal cancer cell alongside TCF4. Our top ranked transcription factor, c-JUN, is known to bind TCF4 and β-catenin, thereby confirming the validity of network connectivity analysis in identifying relevant biological interactions.

3.6 Materials and Methods

3.6.1 Cell Culture

The SW480, DLD1, COLO201, HCT116, and LS174T colon cancer cell lines were obtained from the American Type Culture Collection. SW480, DLD1, and COLO201 cells were grown in RPMI-1640 growth medium (Thermo Fisher; 11875093), HCT116 cells were grown in McCoy’s 5A (Modified) medium (Thermo Fisher; 16600082), and LS174T cells were grown in Minimum Essential medium (Thermo Fisher; 11095080). All growth media were supplemented to 10% FBS (Thermo Fisher; 10082147) and 2mM L-glutamine (Thermo Fisher; 25030149). Cells were incubated at 37°C in 5% CO2. No antibiotics were used. Proper cell line identity was confirmed using STR profiling and the absence of mycoplasma contamination was confirmed routinely using PCR (Genlantis; MY01050).

3.6.2 siRNA Inverted Transfections

Cells were seeded in a 6-well tissue culture plate (Corning; 353046) at a density of 150,000 cells per well. The wells contained 2mL of RPMI-1640 growth medium. Silencer Select siRNAs (Thermo Fisher), Negative Control No.1 (4390843) and siTCF7L2-s13880 (4392420), were delivered to the cells using the lipofectamine RNAiMAX reagent (Thermo Fisher; 13778150) according to the manufacturer’s instructions. This resulted in the addition of 7.5µL of RNAiMAX and 25pmol of siRNA per well of the 6-well plate. The siRNA:lipofectamine complexes were not removed during the time course. The time series was constructed thus: 0 and 72 hour time points were transfected with siNEG and siTCF7L2, respectively, 24 hours after seeding. The 48 hour time point was transfected, with siTCF7L2, 24 hours after the 0 and 72 hour time points. The 24 hour time point was transfected, with siTCF7L2, 24 hours after the 48 hour time point. The cells were harvested 24 hours later, resulting in exposure to siTCF7L2 for 72, 48, and 24 hours. The 0 hour time point spent 0 hours in siTCF7L2 and serves as the transfection control. This procedure is illustrated in Figure S1 in Brown et al.[39].
3.6.3 Quantitative PCR

RNA was extracted from at least three independent inverted transfections using the RNeasy Mini Kit and on-column DNase treatment (Qiagen; 74104, 79254), according to the manufacturer’s instructions. RNA concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher). Synthesis of cDNA was performed using the Verso cDNA Synthesis kit (Thermo Fisher; AB1453B) based on the manufacturer’s instructions. The optional RT Enhancer was used and equal amounts of both the Anchored oligo dT and Random Hexamers were added. Quantitative PCR was performed for TCF7L2, SOX2, CEACAM1, and YWHAZ on an ABI PRISM 7000 sequence detection system (Applied Biosystems) using Power SYBR Green PCR Master Mix (Thermo Fisher; 4367659), according to the manufacturer’s instructions. Primer sequences can be found in Table S7 [39]. The thermocycling protocol consisted of a 10 minute hold at 95°C followed by 40 two-step cycles consisting of 15 seconds at 95°C and 1 minute at 60°C. Fold change was determined using the YWHAZ reference gene and the $2^{(-\Delta\Delta CT)}$ method.

3.6.4 Western Blot

Protein was extracted using a 1% NP-40 Lysis Buffer solution containing a protease inhibitor cocktail (Millipore; 11836153001). To extract protein, 350µL of the 1% NP-40 solution was added to each well of a 6-well plate and cells were scraped into the buffer using a cell scraper and transferred to a 1.5mL eppendorf tube. The mixture was incubated on ice for 30 minutes and spun at 14,000 rpm for 30 minutes at 4°C. The supernatant was transferred to a new tube. Protein was prepared for quantification using the Pierce BCA Protein Assay Kit (Thermo Fisher; 23225), according to the manufacturer’s instructions, and quantified on a SpectraMax M2e microplate reader (Molecular Devices). Nuclear protein samples (20µg) were electrophoresed using a 4-12% Bis-Tris gel (Thermo Fisher; NP0322BOX) in a XCell SureLock Mini-Cell (Thermo Fisher; EI0002) with MOPS Running Buffer (Thermo Fisher; NP0001). Proteins were transferred into a PVDF membrane (Millipore; IPVH00010) using Transfer Buffer (Thermo Fisher; NP00061) and an XCell II Blot Module. Membranes were incubated with antibodies targeting TCF4 (Origene; TA333645), active β-catenin (Millipore; 05-665), or β-actin (CST; 4970S). An HRP-secondary antibody (CST; 7074S, 7076S) was applied and detected using Pierce ECL Western Blotting Substrate (Thermo Scientific; 32209). Blots were imaged in an Azure c600 Gel Imaging Station (Azure Biosystems).

3.6.5 RNA Sequencing and Data Processing

RNA was extracted from three independent inverted transfections using the RNeasy Mini Kit and on-column DNAse treatment (Qiagen; 74104, 79254), according to the manufacturer’s instruc-
tions. RNA concentration was determined using a NanoDrop 1000 spectrophotometer (Thermo Fisher). RNA integrity was determined using the 4200 TapeStation (Agilent). RNA samples with an RNA integrity number (RIN) of 9 or higher were used for library preparation. Libraries were prepared using the TruSeq Stranded Total RNA Library Prep Gold kit (Illumina; 20020598). Ribosomal RNA (rRNA) was removed using biotinylated, target-specific oligos combined with Ribozero rRNA removal beads. The RNA was then fragmented and the cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers, followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. The resulting double-strand cDNA was used as input for Illumina library preparation with end-repair, adapter ligation, and high-fidelity PCR. The final purified product was quantified by qPCR. Samples were sequenced on a HiSeq2500 using Illumina TruSeq v4 chemistry generating 125 base pair, paired-end reads. RNA sequencing data was processed using the CCBR Pipeliner Version 3.0. Briefly, FASTQC was used to assess sequencing quality and Cutadapt was used to remove adapter sequences and perform quality trimming, respectively [9, 215]. Kraken, KronaTools, and FastQ Screen were used to assess microbial contamination [384, 245]. Our samples were confirmed to be free of microbial contamination. STAR (two-pass) was used to align reads to the hg19 reference genome [95]. Picard, Preseq, SAMtools, and RSeQC were used to assess alignment quality [82, 188, 372]. Gene expression was quantified using RSEM [187]. Transcripts per million (TPM) were computed to normalize gene expression values. Differential gene expression was performed using limma [180, 316]. Volcano plots were generated using the EnhancedVolcano package [30].

### 3.6.6 Gene Set Enrichment Analysis

Gene Set Enrichment Analysis was performed using R and the fgsea package, available through Bioconductor [328, 308]. Differentially expressed genes were pre-ranked according to their log2FC values. Gene set enrichment was calculated using the fgsea command and the Hallmark gene sets available from MSigDB (https://www.gsea-msigdb.org/gsea/msigdb/collections.jsp). The minSize and maxSize parameters were set to 15 and 500, respectively. The heatmap was generated using the ComplexHeatmap package [124].

### 3.6.7 Hi-C Sequencing

The in situ Hi-C protocols from Rao et al. [19] were adapted with slight modifications. For each Hi-C library, approximately 3 x 10^6 cells were incubated in 250µl of ice-cold Hi-C lysis buffer (10mM Tris–HCl pH 8.0, 10mM NaCl, 0.2% Igepal CA630) with 50µl of a protease inhibitor cocktail on ice for 30 minutes and washed with 250µl lysis buffer. The nuclei were pelleted by centrifugation at 2,500g for 5 minutes at 4°C, re-suspended in 50µl of 0.5% sodium dodecyl sulfate.
(SDS) and incubated at 62°C for 10 minutes. Afterwards, 145µl of water and 25µl of 10% Triton X-100 were added and incubated at 37°C for 15 minutes.

Chromatin was digested with 200 units of MboI (NEB) overnight at 37°C with rotation. Chromatin end overhangs were filled in and marked with biotin-14-dATP (Thermo Fisher Scientific) by adding the following components to the reaction: 37.5µl of 0.4mM biotin-14-dATP (Life Technologies), 1.5µl of 10mM dCTP, 1.5µl of 10mM dGTP, 1.5µl of 10mM dTTP, and 8µl of 5U/µl DNA Polymerase I, Large (Klenow) Fragment (NEB). The marked chromatin ends were ligated by adding 900µl of ligation master mix consisting of 663µl of water, 120µl of 10X NEB T4 DNA ligase buffer (NEB), 100µl of 10% Triton X-100, 12µl of 10mg/ml BSA, 5µl of 400U/µl T4 DNA Ligase (NEB), and incubated at room temperature for 4 hours.

Chromatin reverse crosslinking was performed by adding 50µl of 20mg/ml proteinase K (NEB) and 120µl of 10% SDS and incubated at 55°C for 30 minutes, adding 130µl of 5M sodium chloride and incubated at 68°C overnight. DNA was precipitated with ethanol, washed with 70% ethanol, and dissolved in 105µl of 10 mM Tris–HCl, pH 8. DNA was sheared on a Covaris S2 sonicator. Biotinylated DNA fragments were pulled with the MyOne Streptavidin C1 beads (Life Technologies). To repair the ends of sheared DNA and remove biotin from unligated ends, DNA-bound beads were resuspended in 100µl of mix containing 82µl of 1X NEB T4 DNA ligase buffer with 10mM ATP (NEB), 10µl of 10 (2.5mM each) 25mM dNTP mix, 5µl of 10U/µl NEB T4 PNK (NEB), 4µl of 3U/µl NEB T4 DNA polymerase (NEB), and 1µl of 5U/µl NEB DNA polymerase I, Large (Klenow) Fragment (NEB).

After end-repair, dATP attachment was carried out in 100µl of mix consisting of 90µl of 1X NEBuffer 2, 5µl of 10mM dATP, 5µl of 5U/µl NEB Klenow exo minus (NEB), and incubated at 37°C for 30 minutes. The beads were then cleaned for Illumina sequencing adaptor ligation which was done in a mix containing 50µl of 1X T4 ligase buffer, 3µl T4 DNA ligases (NEB), and 2µl of a 15µM Illumina indexed adapter at room temperature for 1 hour. DNA was dissociated from the bead by heating at 98°C for 10 minutes, separated on a magnet, and transferred to a clean tube.

Final amplification of the library was carried out in multiple PCR reactions using Illumina PCR primers. The reactions were performed on a 25µl scale consisting of 25ng of DNA, 2µl of 2.5mM dNTPs, 0.35µl of 10µM each primer, 2.5µl of 10X PfuUltra buffer, and 0.5µl of PfuUltra II Fusion DNA polymerase (Agilent). The PCR cycle conditions were set to 98°C for 30 seconds as the denaturing step, followed by 16 cycles of 98°C 10 seconds, 65°C for 30 seconds, 72°C for 30 seconds, then with an extension step at 72°C for 7 minutes.

After PCR amplification, the products from the same library were pooled and fragments ranging in sized of 300–500 bp were selected with AMPure XP beads (Beckman Coulter). The sized selected libraries were sequenced to produce paired-end reads on the Illumina HiSeq 2500 platform with the V4 for 125 cycles. Quality control metrics of the Hi-C data is given in Table S1 of Brown.
et al. [39].

3.6.8 Hi-C Matrix Generation

Paired end reads were processed using the juicer pipeline with default parameters [103]. Reads were mapped to reference genome hg19, with “-s” (site parameter) MboI. Reads with MAPQ > 30 were kept for further analysis. Data was extracted and input to MATLAB using the Juicebox tools command “dump”. Knight-Ruiz (KR) normalization was applied to all matrices, observed over expected (O/E) matrices were used for A/B compartmentalization and identification of topologically associating domains (TADs). Rows and columns for which more than 10% of entries had zeros were removed from the matrix.

3.6.9 A/B Compartmentalization

Hi-C data was partitioned into A/B compartments according to the sign of the chromatin accessibility metric termed the Fiedler vector (the eigenvector corresponding to the second smallest eigenvalue of the normalized Laplacian matrix). Positive vector values were assigned to the ‘A’ compartment representing euchromatic loci and negative values were assigned to the ‘B’ compartment representing heterochromatic loci. The magnitude of the Fiedler values indicate the level of openness or closedness of the chromatin at a given loci. Hi-C data was at 100kb resolution.

3.6.10 TAD Calling

Topologically associating domains (TADs) were designated using spectral identification as described in [63]. Briefly, the Fiedler vector is calculated for a normalized Hi-C adjacency matrix and initial TADs are organized according to neighboring regions whose Fiedler values have the same sign. The initial TAD structure is repartitioned if, for a given domain, the Fiedler number falls below a user-specified threshold ($\lambda_{thr}$). This ensures that TADs are not overly large and repartitions the domains until the Fiedler number is larger than the threshold or until the TAD reaches the smallest allowable TAD size (default is 3). This process is performed iteratively for a set of chromosome-specific Hi-C contact maps. For our analysis, $\lambda_{thr}$ was chosen to ensure a median TAD size of 900kb, as the expected median TAD size in mammalian genomes is 880kb (rounded to 900kb since bins are in intervals of 100kb) [32]. $\lambda_{thr}$ was chosen individually for each chromosome to ensure each TAD clustering set would have the same approximate median TAD size. Hi-C data was at 100kb resolution.
3.6.11 Transcription Factor Enrichment Analysis

Transcription factor enrichment analysis was performed using oPOSSUM-3 software [175]. oPOSSUM-3 tests for over-representation of transcription factor binding sites (TFBS) in the DNA sequences of a set of genes, using Z-score and Fisher exact probability to determine significance. For our analysis, 64 genes from a differentially conformed region (CEACAM region) of chromosome 19 were submitted as query and compared against a background set of genes in the oPOSSUM-3 database. Both sets of genes were compared to the JASPAR CORE database of preferential TFBS, representing 719 vertebrate TFBS, and the rate of TFBS occurrence for each set of genes was measured [163]. The following analysis parameters were used: 8 bit minimum profile specificity, 0.40 conservation cutoff, 85% matrix score threshold, and a 5kb flanking length.

3.6.12 ChIP Sequencing Analysis

To explore key TF binding patterns throughout the genome and specifically at TAD boundaries, we obtained publicly available SP1, CTCF, and TCF4 ChIP-seq data from the HCT116 colorectal cancer cell line. We accessed the ChIP-seq data from GEO using the accession numbers GSM1010902 (SP1), GSM1010903 (CTCF), and GSM782123 (TCF4). Two replicates were available for SP1 and CTCF, hence replicate profiles were averaged together. Profiles were then binned at 100kb resolution to be compatible with our RNA-seq and Hi-C data. The percentage of nonzero CTCF and TCF4 peak intensities that coincided with TAD boundaries called on the Hi-C data are reported in Tables S5 and S6 of Brown et al. [39]. Additionally, because TFEA analysis revealed that SP1 is the most highly enriched TF near the CEACAM loci on chromosome 19, we evaluated SP1 peak intensities at these loci. To evaluate the influence of TCF4 binding on the CEACAM loci, we aligned the TCF4 ChIP-seq profile to the region as well. Because ChIP-seq data for SP1 and TCF4 were acquired from different experiments, direct comparison of their binding behavior at the CEACAM loci was facilitated by performing min-max normalization on each profile.

3.6.13 Synthetic 5C Map

We constructed a synthetic 5C contact map derived from a genome-wide 1Mb Hi-C contact map for genomic regions containing genes in the EMT and E2F gene networks. This was done by locating the genomic bins corresponding to the network genes, extracting the inter-chromosomal and network-specific intra-chromosomal interaction frequencies for those bins, and stitching them together in genomic order. Our working set of network genes was sourced from the KEGG database and included 189 EMT genes and 189 E2F genes distributed across all chromosomes in the genome. Some of the 378 total genes occupy the same 1Mb genomic bins resulting in a 333
by 333 1Mb-resolution synthetic 5C adjacency matrix.

3.6.14 Permutation Test

To determine whether the EMT and E2F gene networks are more closely interacting than other regions of the genome, we performed a permutation test with 1000 randomly sampled sets of 333 1Mb genomic bins across the genome. The Fiedler number for each set was computed and the distribution of numbers was plotted with the observed Fiedler number corresponding to the combined EMT and E2F gene networks. Our null hypothesis is that the EMT/E2F gene networks and random gene sets do not differ and our significance level is 0.1.

3.6.15 Von Neumann Entropy

Entropy is a measure of “disorder” in a given system – the higher the entropy, the greater the disorder. In the context of genome structure, the higher the entropy, the more conformations available to the system [258]. If the distant ends of a genomic region, e.g., a gene, interact to form a loop, there are fewer conformations available to the gene and thus the entropy of that genomic region is reduced. In this way, entropy can be considered a signature for chromatin conformation. We compute the Von Neumann Entropy (VNE) - multivariate entropy - using the following equation:

$$H = -\sum_{i=1}^{n} \lambda_i \ln \lambda_i.$$  

Here, $H$ takes on positive values with larger values indicating higher chromatin accessibility.

3.6.16 Data Availability

The RNA sequencing and Hi-C datasets generated in this study have been deposited to the Gene Expression Omnibus (GEO) and can be accessed via their accession numbers, GSE151934 and GSE151970, respectively.
CHAPTER 4

Rearrangement of T Cell Genome Architecture Regulates GVHD

This chapter is based on a paper by Yaping Sun, Gabrielle A. Dotson, Lindsey A. Muir, Scott Ronquist, Katherine Oravec-Wilson, Daniel Peltier, Keisuke Seike, Lu Li, Walter Meixner, Indika Rajapakse, and Pavan Reddy [330].

4.1 Abstract

The cohesin complex modulates gene expression and cellular functions by shaping three-dimensional (3D) organization of chromatin. WAPL, cohesin’s DNA release factor, regulates 3D chromatin architecture. The 3D genome structure and its relevance to mature T cell functions \textit{in vivo} is not well understood. We show that \textit{in vivo} lymphopenic expansion, and allo-antigen driven proliferation, alters the 3D structure and function of the genome in mature T cells. Conditional deletion of \textit{Wap} in T cells reduced long-range genomic interactions, altered chromatin A/B compartments and interactions within topologically associating domains (TADs) of the chromatin in T cells at baseline. Comparison of chromatin structure in normal and WAPL-deficient T cells after lymphopenic and allo-antigen driven stimulation revealed reduced loop extensions with changes in cell cycling genes. WAPL-mediated changes in 3D architecture of chromatin regulated activation, cycling and proliferation of T cells \textit{in vitro} and \textit{in vivo}. Finally, WAPL-deficient T cells demonstrated reduced severity of graft-versus-host disease (GVHD) following experimental allogeneic hematopoietic stem cell transplantation. These data collectively characterize 3D genomic architecture of T cells \textit{in vivo} and demonstrate biological and clinical implications for its disruption by cohesin release factor WAPL.
4.2 Introduction

The three-dimensional (3D) architecture of the genome includes coiling of genomic DNA around histone proteins to form the chromatin fiber, which folds into higher-order structures such as loops, domains, compartments, and chromosomes [32, 107, 220, 267, 266]. High resolution chromatin conformation capture experiments reveal that the 3D spatial architecture of the chromatin at various scales is conserved, reproducible at the cellular level, and regulates gene expression [77, 78, 90, 91]. It is increasingly appreciated that higher spatial organization can have specific alterations during mammalian development and in some pathologies including cancers and infections [107, 368]. However, whether a priori disruption of this 3D organization alters in vivo cellular functions and disease processes remains poorly understood.

The multi-unit cohesin ring complex plays a critical role in 3D genomic organization and in cell division. It consists of SMC1/3, SCC1 (RAD21), and STAG subunits that are loaded by the SCC2/SCC4 complex onto genomic DNA and establish the cohesin ring structure [79, 235, 259, 280]. Cohesin dependency has been demonstrated by depletion of various cohesin units [259]. Cohesin release from chromatin is driven by WAPL, which opens an exit site at the interface of the SMC3/SCC1 subunits of the cohesin ring [127, 129, 313]. Prior studies have elegantly demonstrated that the absence of WAPL reduces cohesin turnover, alters chromatid loop extensions, and leads to defects in interphase chromosome organization [41, 127, 129, 139, 313, 344].

WAPL is essential during mammalian embryonic development [344]. However, the role of WAPL-dependent chromatin alterations on in vivo functions after embryonal development or in mature T cells is not known. The 3D chromatin landscape has been recently described in T cell development, in T cell lines and following in vitro stimulation [142, 150, 220, 275, 285, 391, 40, 24]. It remains unclear however whether the specific changes in the 3D chromatin landscape influence or emerge from T cell development. Furthermore, the functional chromatin landscape in mature T cells and their alterations following in vivo activation remain unknown. The role of cohesin ring formation in mature T cell development, its function in vivo, and its disruption by the absence of WAPL in mature T cell immunity are also not fully understood.

Mature T cells can cause graft-versus-host disease (GVHD) following allogeneic hematopoietic cell transplantation (HCT), a potentially curative therapy against many hematological malignant and non-malignant diseases [29, 385, 398]. GVHD has precluded widespread utilization of this effective therapy. The chromatin landscape of allogeneic T cells, and whether the disruption of this landscape can regulate the severity of T cell-mediated GVHD remains unexplored.

Herein, we utilized genome-wide chromosome conformation capture (Hi-C) and RNA-sequencing to describe the 3D chromatin architecture of mature T cells in vivo, specifically at baseline (unstimulated, naive, pre-transplant) and following non-antigen stimulated lymphopenia.
induced proliferation (syngeneic) and allo-antigen driven (allogeneic) stimulation [208] (Figure S1 in Sun et al. [330]). We evaluated 3D chromatin architecture at the chromosome, TAD, and sub-TAD levels to capture both global and local trends of T cell genome structure (Figure 4.1A). We generated T cell-specific WAPL-deficient mice and demonstrated that WAPL regulates the 3D chromatin structure, function, and in vivo biological response of T cells such as GVHD. These data collectively demonstrate that a priori disruption of chromatin structure regulates mature T cell function.

4.3 Results

4.3.1 Characterization of mature naïve T cell genome architecture following in vivo stimulation

We first determined the genome architecture of mature naïve T cells at baseline and following in vivo lymphopenic and antigen-driven stimulation. To mimic clinically-relevant in vivo stimulation, we compared naïve T cells before and after experimental syngeneic and allogeneic transplantation. To this end, we utilized MHC-disparate B6 into a BALB/c model of transplantation [276]. CD62L+ naive donor T cells were harvested from the splenocytes of B6 donors and transplanted into congenic syngeneic B6 or allogeneic BALB/c recipients (see Methods). Recipient animals were sacrificed seven days after transplantation and their splenic T cells were isolated using congenic markers and analyzed for genomic architecture. While we generated Hi-C contact maps to profile genome-wide chromatin interactions [274] for harvested T cells, variability in the number of mappable Hi-C reads among unstimulated and stimulated T cell settings (Table S1 in Sun et al. [330]) precluded us from reliably making direct comparisons between the genome architecture of naïve, syngeneic, and allogeneic T cells. From the RNA-seq data, however, we did observe significant differences in gene expression ($|\log_{2}FC| \geq 1$, $p \leq 0.001$) before and after in vivo stimulation (Figure S2 in Sun et al. [330]).

4.3.2 Generation of T cell conditional WAPL-deficient mice

Because T cell function changed following stimulation, we next sought to evaluate which conformational features in the genome are critical for T cell function. Cohesin promotes chromatin looping while WAPL is important for the release of cohesin from chromatin. WAPL is essential for embryonal development and its deficiency has been shown to cause defects in chromatin structure [344]. Specifically, WAPL has been shown to restrict chromosome loop extension [41, 129, 128, 139]. In addition, we previously demonstrated that mir142 regulates mature T cell
proliferation, targets WAPL expression in T cells and that it may modulate T cell activation [333]. Therefore, we determined whether WAPL regulates 3D chromatin architecture and the function of mature T cells.

Because WAPL is critical for embryonal development, we generated a T cell conditional Wapl knock-out (KO) mouse using CRISPR-Cas9 and CD4-CRE systems [272, 337]. The Wapl locus on chromosome 14q has 18 exons and one non-coding exon (Figure S3A in Sun et al. [330]). We designed two sgRNAs specific to exon 2 of the Wapl gene to generate a double strand break in exon 2 (Figure 1A and Figure S3B in Sun et al. [330]). Our first line of mice carried an insert of two sgRNAs targeting exon 2 in Wapl (Figure 1A and Figure S3C in Sun et al. [330]). The second and third lines carried Rosa26-floxed STOP-Cas9 knock-in on B6J (The Jackson Laboratory, Stock No:026175) or were CD4-CRE transgenic mice (The Jackson Laboratory, Stock No: 017336) (Figure S3C in Sun et al. [330]). Triple crosses were screened for sgRNA insert showing the positive 457 bp band (sgRNA-Wapl) (Figure 1B in Sun et al. [330]), CRE positive as a 100 bp band, loxP-SV40pA x3-loxP-CAS9-GFP (LSL) cleavage activity demonstrating 1,123 bp for WT LSL, and a 285 bp band for cleaved LSL after CRE recombination processing. The conditional KO mice developed normally. The Wapl KO T cells were verified for GFP expression (Figure 1B in Sun et al. [330]). The higher levels of LSL cleavage and GFP expression assured successful depletion of WAPL (Figure 1B, lane 4 in Sun et al. [330]). We further confirmed WAPL protein depletion through Western blotting (Figure 1C) in T cells isolated from multiple knockout pups. Finally, to further confirm efficient deletion, we performed RNA-seq on the T cells sorted from these mice and compared them with littermate WT T cells, which demonstrated efficient loss of exon 2 in the Wapl gene (Figure 1D in Sun et al. [330]).

4.3.3 WAPL regulates T cell genome architecture

Because WAPL is known to regulate genome architecture and genome architecture entrains transcription [174, 344, 386], we next evaluated the impact of WAPL deficiency on genome architecture and gene expression of unstimulated naïve T cells. To analyze whole genome architecture (structure) and gene expression (function), we integrated Hi-C and RNA-seq data (see Methods). We studied changes in genome structure and function at the chromosome, TAD, and sub-TAD levels (Figure 4.1A). RNA-seq was performed on T cells harvested from naïve B6 and those harvested on day+7 from transplanted syngeneic and allogeneic B6 recipients (see Methods).

We evaluated the statistical dissimilarity between wildtype (WT) and knockout (KO) Hi-C contact matrices at the chromosome level in each setting by employing the Larntz-Perlman (LP) procedure (see Methods) which revealed regions critically impacted by Wapl knockout. WT and KO matrices were significantly dissimilar across all chromosomes (p < .001). Though statistically
different, WT and KO chromosomes in unstimulated naïve T cells, except for chromosomes 16 and 19, had a lesser number of differential regions when compared to WT and KO chromosomes in syngeneic or allogeneic T cells (Figure 4.1B and Table S2). This conservation of structure is consistent with the notion that unstimulated naïve T cells are in a quiescent-like state and therefore less impacted by the loss of WAPL. Further, differential regions in the naïve setting appear more concentrated between ends of chromosomes – suggesting that long-range interactions may be critical to maintaining structural integrity in unstimulated T cells.

We highlight regions in the 99th percentile of significant changes across chromosomes 1, 2, 7, and 11 in Figure 4.1B, due to their abundance of differential expression and compartmentalization (Figures S4 and S5 in Sun et al. [330]). Across all naïve chromosomes, 15-25% of genes within these LP-dissimilar regions are significantly differentially expressed ($p < 0.05$) compared to 8.5-13% across LP-dissimilar regions of syngeneic chromosomes and 3-7% across LP-dissimilar regions of allogeneic chromosomes. Interestingly, there are differential regions in common between the syngeneic and allogeneic settings that are not detected in the naïve setting (red arrows in Figure 4.1B), indicating stimulation-specific genome architecture.

We then bi-partitioned chromosomes into individual stretches of accessible (active) and inaccessible (inactive) chromatin, termed A (euchromatin) and B (heterochromatin) compartments, respectively [190]. We demarcated these regions using the signed values of the Fiedler vector which measures underlying chromatin accessibility [61]. The positive values of the Fiedler vector reflect compartment A and negative values reflect compartment B. We observed A/B compartment switch events mediated by the loss of WAPL in all settings (Figure S5 in Sun et al. [330]). Across all naïve chromosomes, 184 genomic bins (100kb-length) containing 217 genes in the unstimulated naïve KO T cells occupied a different compartment when compared to WT T cells (Figure S5 in Sun et al. [330]). These switch events demonstrated a bias from compartment B to compartment A (70.7% of switch events) (Figure S5 in Sun et al. [330]).

In the context of lymphopenic stimulation (syngeneic), KO T cells exhibited 303 switch events involving 375 genes when compared to WT T cells. In the allogeneic context, KO T cells demonstrated 413 switch events involving 485 genes when compared to WT T cells (Figure S5 in Sun et al. [330]). The switch bias was once again in the direction of compartment B to compartment A in syngeneic T cells (60.4% of switch events) and in allogeneic T cells (53.8% of switch events). Additionally, allogeneic T cells had more switch events per chromosome than unstimulated naïve and syngeneic T cells (Figure S5 in Sun et al. [330]). Interestingly, switched compartment loci tended to congregate towards the ends of chromosomes rather than in the middle or spread evenly throughout. No switch events were observed among contiguous regions of the genome in any of these settings. Overall, 14%, 68%, and 70% of switch events in naïve, syngeneic, and allogeneic T cells, respectively, occurred within the previously identified LP-dissimilar regions. While there are
coordinated changes in expression and chromatin accessibility genome-wide and most notably at LP-dissimilar regions, chromatin compartmentalization largely remained stable between WT and KO T cells (Table S2 in Sun et al. [330]).

4.3.4 WAPL impacts internal structure of TADs and local gene transcription

Stability in global compartment organization throughout the genome does not preclude changes to local genome organization. Chromatin preferentially interacts within locally-distributed and insulated regions called topologically associating domains (TADs) that regulate transcription [94]. Thus, we next analyzed the impact of WAPL depletion in TADs. One highly supported mechanism of TAD formation is loop extrusion [111] mediated by the ring-shaped cohesin complex and WAPL, which enables TAD dynamics by promoting cohesin turnover [129]. Specifically, genes residing in the same TAD experience coordinated regulation and expression [94, 242, 311, 181] while changes to TAD boundaries due to altered CTCF binding influence anomalous gene-enhancer interactions [109, 205]. We therefore utilized spectral graph theory to identify the positional boundaries that define TADs [63]. TADs have been widely suggested to be highly conserved across cell types and conditions in mammalian genomes [94, 242, 274], so we pooled our Hi-C data across settings to enable higher resolution binning of the data and reliable detection of TAD boundaries within chromosomes (see Methods).

TAD analysis revealed the emergence of a unique feature in the context of syngeneic and allogeneic T cells in the absence of WAPL - the appearance of ‘corner peaks’. Corner peaks are the enrichment of interaction frequency at domain boundaries seen at the bottom left and top right corners of TADs [274]. To evaluate differences in corner peak signal between WT and KO T cells, we established a local neighborhood around the corners of each TAD and determined the average number of observed contacts in each neighborhood (Figure 4.2A). On aggregate, we observed that corner peaks became more pronounced upon WAPL depletion in syngeneic and allogeneic T cells (Figure 4.2B and Figure S6 in Sun et al. [330]), consistent with the known activity of WAPL to limit loop extension [128, 129]. Further, several studies have demonstrated that corner peaks are associated with a longer residence time of the cohesin complex at TAD borders [129, 304, 336]. We did not, however, observe this rise in corner peak abundance in the KO unstimulated naïve T cells, but rather a decrease (Figure 4.2B and Figure S6 in Sun et al. [330]), suggesting that the development of corner peaks in stimulated T cells might be a consequence of their proliferation following activation.

When compared against a background set of interactions – surrounding interactions occurring at a comparable distance to opposing TAD boundaries - we found that the rise in corner peak
Figure 4.1: Genome-wide Effects of WAPL Knockout in Unstimulated Naïve T Cells and After Syngeneic/Allogeneic Transplantation. (A) Illustration of hierarchical Hi-C analysis workflow.
Figure 4.1: (B) Chromosome-level features and contact maps for chromosomes 1 and 2 from wild-type and Wapl knockout T cells. Gene expression (top track) is shown as a vector of log2(TPM) values binned at 100kb resolution and chromatin accessibility (bottom track) is shown as signed values from the Fiedler vector of the Hi-C contact map where positive values (red) denote A compartments and negative values (blue) denote B compartments. ICE and O/E normalized Hi-C contact maps are shown at 100-kb resolution and log-scale. Matrix dissimilarity between WT and KO conditions was detected by the Larntz-Perlman procedure (see Methods) and genomic regions with dissimilarity in the 99th percentile are shaded in green. These areas indicate the largest perturbations to the chromatin architecture upon Wapl knockout.

Signal from WT to KO in syngeneic and allogeneic T cells was statistically significant in a large proportion of TADs (p < 0.01, Table S3 in Sun et al. [330]). We next determined whether there was any functional significance to the observed corner peak dynamics. Syngeneic T cells harbored the most significantly differentially expressed genes (DEGs) (|log_{2}FC| ≥ 1, p ≤ 0.01) between WT and KO out of all settings (297 in naïve, 566 in syngeneic, and 46 in allogeneic) and had a particularly high concentration of DEGs on chromosome 11 (58 DEGs, Figure S4 in Sun et al. [330]). Given this functional dissimilarity, we further investigated intra-TAD interactions on Chromosome 11 at 50kb resolution (Figure 4.2C) and present an earlier iteration of this analysis on Chromosome 7 at a lower 100kb resolution in Figure S6 (in Sun et al. [330]).

We were particularly interested in identifying regions where differential expression overlapped with increases in corner peak signal. We noted statistically significant corner peak increases in 3 naïve, 47 syngeneic, and 50 allogeneic TADs upon WAPL deletion across Chromosome 11, respectively. Of the TADs exhibiting these corner peak increases, two contained DEGs in naïve T cells, 10 in syngeneic T cells, and three in allogeneic T cells. We highlighted two such regions on Chromosome 11 (Figure 4.2C, bottom left and right). The first region we observed ranged from position 105.5Mb to 107.2 Mb on Chromosome 11 and encompassed a TAD exhibiting a significant corner peak signal increase most notably in the syngeneic and allogeneic contexts. Furthermore, this TAD contains the gene, Wipi1, which exhibited a 2.8-fold increase in expression from WT to KO in syngeneic cells yet a less significant 0.8-fold increase in expression from WT to KO in both naïve and allogeneic T cells. Another stand-out TAD within region 109.4Mb to 111.1Mb on Chromosome 11 demonstrated differential expression of Cdc42ep4 in syngeneic T cells with a 1.5-fold increase in expression upon WAPL deletion. Overall, Chromosome 11 contained 25 DEGs in naïve T cells, 58 DEGs in syngeneic T cells, and 4 DEGs in allogeneic T cells dispersed across several TADs.
Figure 4.2: Comparison of Internal TAD Organization Between WT and KO T Cells. (A) Schematic of TADs illustrating how ‘corner peaks’ – interactions between opposite TAD boundaries – were characterized in terms of their local neighborhoods.
Figure 4.2: The nonzero mean of absolute read counts in each local corner peak neighborhood (CPN) was used to define the strength of TAD boundary interactions. (B) Change in corner peak signal from WT (blue axis) to KO (orange axis) for each TAD (individual gray lines) on Chromosome 11. The average change in corner peak signal between the two conditions is plotted as a solid black line in each panel to capture the overall trend across settings. TADs and their corner peak signals are represented at 50kb resolution. (C) (Top center) Chromosome 11 observed contact map pooled across samples at 50kb resolution. (Bottom left) TAD region of Chromosome 11 extending from 105.5 Mb to 107.2 Mb capturing corner peak increase from WT to KO most notably in the syngeneic and allogeneic settings; differential expression of gene Wipi1 occurs in the centermost TAD in this region. (Bottom right) TAD region of Chromosome 11 extending from 109.4 Mb to 111.1 Mb capturing corner peak increase from WT to KO; differential expression of gene Cdc42ep4 occurs in the centermost TAD in this region. (D) Heatmap of differentially expressed cell cycle genes residing on Chromosome 11.

Impact of WAPL on the Cell Cycle Gene Network

WAPL is critical for sister chromatid cohesion and loop extrusion dynamics. These processes correlate with cellular proliferation and cell cycling, and because we observed notable changes in corner peaks of Chromosomes 7 and 11 only in the context of syngeneic and allogeneic settings in the absence of WAPL, we next explored changes in cell cycle genes and the TADs that these genes reside in. One gene-rich region in Chromosome 7, extending between positions 60Mb and 70Mb on the chromosome, exhibited considerable intra-TAD reorganization in the KO T cells when compared to WT unstimulated (Figure S7D in Sun et al. [330]), syngeneic (Figure S7E in Sun et al. [330]), and allogeneic (Figure S7F in Sun et al. [330]) T cells. This region contains cell cycle genes [96, 147]: Fanci, Prc1, and Blm. These cell cycle genes (Fanci, Prc1, and Blm) were not significantly differentially expressed, as they did not meet the cutoff of $|\log_2 FC| \geq 1$. However, there were seven non-cell cycling genes (Mesp2, Arpin, Fes, Homer2, Saxo2, Cemip, and Arnt2) directly upstream and downstream of the cell cycle genes in this region that were significantly differentially expressed. These data suggest that in the unstimulated KO T cells, these three cell cycle genes on Chromosome 7 were not differentially expressed despite the changes in intra-TAD interactions when compared to WT T cells.

Similar to the unstimulated context, in the syngeneic T cells, there was a differential expression of non-cell cycle genes upstream and downstream of cell cycle genes in this region (Agbl1, Isg20, Can, Hapln3, Ribp1, Mesp2, Anpep, Fes, Scl28a1, Homer2, Adamsl3, and Tmc3). In allogeneic T cells, non-cell cycle genes (Agbl1, Can, Rhcg, and Adamsl3) once again were differentially expressed. Thus, in the absence of WAPL, the highlighted region of Chromosome 7 containing the three cell cycle genes did not dynamically change but demonstrated significant changes in the expression ($|\log_2 FC| \geq 1$, $p \leq 0.05$) and internal structural rearrangement of genes in their vicinity. No other cell cycle genes on Chromosome 7 demonstrated differential expression.
Chromosome 11 contained 13 differentially expressed cell cycle genes (Figure 4.2D). Of these 13, only one gene (Gas2l1) was significantly differentially expressed between naïve WT and KO T cells, two genes (Myh10 and Sphk1) between syngeneic WT and KO T cells, and none in the allogeneic setting. We did not however observe an overlap between the differential expression of these genes and TADs with altered internal TAD structure. While WAPL depletion did not disrupt the cell cycling transcriptional program within unstimulated and stimulated contexts much on Chromosome 11, the expression of these genes was quite different between contexts (Figure 4.2D).

While we did not see a change in the three cell cycle genes noted above in the analysis of Chromosome 7 nor significant coupling of cell cycle differential expression and corner peak dynamics on Chromosome 11, WAPL is known to regulate cell cycling so we next explored the entire breadth of cell cycle genes throughout the genome. To this end, we extracted and stitched together 141 Hi-C genomic bins that corresponded to a curated set of 170 cell cycle genes genome-wide, generating a Hi-C-derived 5C contact matrix (see Methods). 5C, like Hi-C, is a derivative of the original chromosome conformation capture technique [91], and is useful in identifying interactions among select genomic regions that bear relationship to one another [98]. In unstimulated naïve T cells, we observed that the connectivity of the cell cycle network decreased in the absence of WAPL, as determined by the element-wise Pearson correlations moving toward zero (Figure 4.3A). In syngeneic and allogeneic T cells, however, connectivity appeared to strengthen in the absence of WAPL, as demonstrated by the correlation tending towards ± 1 (Figure 4.3A).

The connectivity (structure) of cell cycle genes changed between the WT and KO T cells in all settings, but the maximal change was noted in the syngeneic setting (Figure 4.3B). However, changes in the structure of the cell cycle network did not trend with the function (expression). The expression (function) of the cell cycle gene network changed the most between WT and KO T cells in the context of allogeneic setting (Figure 4.3B). The genome-wide structural analysis of the cell cycle gene network highlighted two subgroups of highly connected genes (shown in purple and green boxes in Figure 4.3A). One of these subgroups negatively correlated with most of the network (Figure 4.3A, purple box) while the other demonstrated positive correlation with most of the network (Figure 4.3A, green box) in all three settings. The measure of inter-connectivity within these two subgroups, in terms of degree centrality, is shown in Figure 4.3C. Ultimately, while gene expression of cell cycle genes Fanci, Prc1, and Blm, did not change in the absence of WAPL, other genes in the cell cycle network did change significantly (Table S4 in Sun et al. [330]). Five cell cycle genes were up-regulated in the absence of WAPL in unstimulated naïve T cells. By contrast, 12 cell cycle genes were down-regulated and 4 up-regulated between WT and KO T cells in the syngeneic context. In the context of allo-antigen stimulation, only one gene was down-regulated.
Figure 4.3: Cell Cycle Gene Network Across T Cells. (A) Hi-C-derived 5C contact maps representing the cell cycle gene network. Rows and columns correspond to genomic bins across all chromosomes that contain cell cycle genes. Maps are shown at 1-Mb resolution as a Pearson correlation of normalized (observed/expected) contacts. The purple and green boxes highlight subgroups of interest that are assessed in C. The purple subgroup is comprised of 10 loci (1Mb length) ranging non-contiguously from 18 Mb to 197 Mb and containing 10 cell cycle genes. The cell cycle gene loci in WT and KO T cells in each subgroup is comprised of 12 loci (1Mb length) ranging non-contiguously from 1,489 Mb to 1,606 Mb and containing 16 cell cycle genes. (B) Structural and functional differences between all cell cycle gene loci in WT and KO T cells in each setting. Difference is measured as the Frobenius norm of the difference between WT and KO data (see Methods). The left y-axis (blue) reflects the Frobenius norm of the difference between WT and KO Hi-C matrices (measure of structural change) in each setting and the right y-axis (orange) reflects the Frobenius norm of the difference between WT and KO gene expression vectors (measure of functional change) in each setting. (C) Degree centrality (i.e. the row sum of a matrix) of subgroups of interest.
4.3.5 WAPL-induced changes in genome structure alter T cell gene expression

We next determined whether the changes in the chromatin architecture related to WAPL deficiency in T cells affected genome function. Three-dimensional genome structural changes have been suggested to affect T cell development [307]. Whether a priori disruption of the cohesin ring affected T cell function, however, is not clear. The transgenic mice with conditional Wapl KO in T cells displayed normal birth and growth rates and generated enough mature naïve T cells. To better analyze the developmental impact of WAPL deficiency on T cell development, we next analyzed the thymii from 8–10-week-old WT and Wapl KO littermates. The thymocytes from the Wapl KO animals showed significant changes when compared to the WT littermate controls. Specifically, they showed reduction in the total number of thymocytes, double positive (DP), and CD8 SP thymocytes (Figure 5A and Figure S8A in Sun et al. [330]).

We next analyzed the secondary lymphoid organ (spleens) for peripheral T cell subsets. Wapl KO littermates demonstrated lower numbers of total T cells in the spleen (Figure 5B in Sun et al. [330]) and lower numbers of CD3+CD4+ and CD3+CD8+ T cells (Figure S8B and C in Sun et al. [330]) when compared to WT littermates. During the β-selection checkpoint in the thymus, the β chain of the T cell receptor rearranged by the thymocytes must retain the structural properties allowing it to be presented on the surface of the thymocytes. We therefore screened for TCRβ+ CD4+ and CD8+T cells in the spleen and found that the Wapl KO mice demonstrated lower numbers of TCRβ+ CD4+ and CD8+ T cells in the spleen when compared to WT littermates (Figure S8D and E in Sun et al. [330]). The splenocytes from the KO animals also demonstrated lower numbers of CD3+CD69+, CD4+CD69+, CD8+CD69+ and CD4+CD25+ T cells (Figure S8A-D in Sun et al. [330]).

Because WAPL deficiency altered chromatin architecture, we next analyzed whether these structural changes were associated with changes in gene expression and proliferation, at baseline and following in vivo stimulation. To this end, we first determined whether Wapl expression itself changed in T cells following stimulation. Consistent with our previous observation, we observed significant upregulation of WAPL protein in T cells upon co-culture with allogeneic DCs for 60 hours (Figure 5C in Sun et al. [330]) [331, 332]. To determine the impact of this structure-function relationship, we performed gene set enrichment analysis (GSEA) for the cell cycle gene network highlighted in our earlier Hi-C-derived 5C analyses (Figure 4.3). We found that cell cycle genes were differentially regulated between WT and WAPL-deficient T cells (Figure 5D in Sun et al. [330]). We verified results with real-time quantitative PCR. Gas2l1, a gene induced upon growth arrest [121], and Mcm3 that increases as cells progress from G0 into the G1/S phase and regulates the cell cycle [352], were significantly upregulated in WAPL-deficient T cells compared
to WT T cells (Figure 5E and F in Sun et al. [330]). In contrast, Sphk1, a gene that plays a key role in TNF-a signaling and the canonical NF-κB activation pathway important in inflammatory, apoptotic, and immune processes [8], and Myh10, a gene required for completion of cell division during cytokinesis [165, 325] were both downregulated in WAPL-deficient T cells (Figure 5G and H in Sun et al. [330]). These observations demonstrated that changes in genome structure related to WAPL deficiency in T cells promote differential gene expression that suppresses apoptosis, cell proliferation and cell cycle progress.

To further investigate the impact of WAPL deficiency on T cell cellular responses, we stimulated WAPL-deficient and WT T cells in vitro, either with allo-antigen stimulation in mixed lymphocytes reaction (MLR) by co-culturing T cells with allogeneic DCs for four days or with CD3/CD28Ab for two or three days and pulsed with H3-TdR. The WT T cells demonstrated significantly greater H3-TdR incorporation when compared with Wapl KO T cells suggesting that WAPL deficiency caused reduced proliferation (Figure 5I and Figure S9E in Sun et al. [330]). T cell proliferation was directly assessed in vitro using a dye dilution assay. Specifically, we utilized CellTrace™FarRed dilution to avoid the interference with GFP fluorescence in Wapl KO T cells. The Wapl KO T cells demonstrated reduced dye dilutions when compared to WT T cells upon stimulation by either allogeneic DCs or CD3/CD28ab (Figure 5J and Figure S8F in Sun et al. [330]). It is possible that cell death from apoptosis could contribute to the reduction in T cell expansion related to WAPL deficiency. We therefore also determined apoptosis after stimulation of both WT and Wapl KO T cells. The WAPL-deficient T cells also showed significantly decreased apoptosis when compared to littermate WT T cells (Figure 5K in Sun et al. [330]). These data demonstrate that the absence of WAPL altered T cell proliferation and apoptosis following in vitro stimulation.

The interaction between cohesin and WAPL plays an essential role in maintaining chromosome structure and separation of sister chromatids during mitosis and cell proliferation [256]. With the coordinated changes in expression of cell cycling genes and genomic architecture in the absence of WAPL, and the reduction in proliferation, we next examined cellular mechanisms underlying the reduced proliferative capacity of WAPL-deficient T cells following in vitro and in vivo stimulation. Specifically, we explored the hypothesis that altered gene expression of cell cycling genes from the change in genomic architecture related to WAPL deficiency leads to a reduction in cell cycling. To assess kinetic cell cycling we utilized flow cytometry analyses of DNA content with FxCycle™FarRed Stain to avoid interference from the GFP fluorescence in Wapl KO T cells. Purified WT or Wapl KO T cells were stimulated with CD3/CD28T cell activator dynabeads and analyzed for 2C and >2C populations. Wapl KO T cells demonstrated significantly lower 2C percentages but higher >2C percentages when compared with WT T cells at several time points after CD3/CD28 stimulation (Figure 5L and Figure S10A in Sun et al. [330]). We next analyzed whether the effect on cell cycling was observed in vivo. To this end, we once again utilized the allogeneic
bone marrow transplantation (BMT) model system, where the host splenocytes were harvested and analyzed for WT or Wapl KO donor T cells as above. As shown in Figure 5M and Figure S9B (in Sun et al. [330]), the in vivo stimulation of WT and Wapl KO T cells demonstrated similar differences as from in vitro stimulation. These data indicated that Wapl KO T cells had cell cycle deficiency, which impairs T cell proliferation and alters T cell function in vitro and in vivo, consistent with cell cycle gene analyses by Hi-C derived 5C (Figure 5 in Sun et al. [330]), GSEA, and qPCR analyses (Figure 5D-H in Sun et al. [330]).

4.3.6 WAPL deficiency in T cells improves survival after allogeneic BMT

Mature T cells in the allogeneic donor T cells are the principal mediators of GVHD, a major cause of mortality after allogeneic BMT. Because WAPL regulated mature T cell responses following in vitro and in vivo allo-antigen stimulation, we next determined whether this has a clinical impact on GVHD severity following experimental allo-BMT. To this end, we once again utilized the MHC mismatched B6 (H2) → BALB/c (H2d) mouse model where the congenic B6 animals served as the syngeneic controls. BALB/c recipient mice were lethally irradiated (800 cGy total body irradiation, split dose) and transplanted with T cell-depleted WT BM from B6 donors along with purified mature T cells from the spleen of either WT or Wapl KO B6 animals[332]. The recipient animals were monitored for survival and GVHD severity as described in Methods.

We first determined whether WAPL expression changed after allo-BMT. Consistent with the in vitro allo-antigen stimulation shown in Figure 5C (in Sun et al. [330]), WT cells demonstrated higher expression of WAPL protein in donor T cells harvested from recipient spleens 7 days after allogeneic BMT when compared to syngeneic controls (Figure 6A in Sun et al. [330]). Survival analysis demonstrated that all the syngeneic animals survived, but the allogeneic animals that received WT T cells died with signs of severe clinical GVHD (Figure 6B in Sun et al. [330]). In contrast, allogeneic animals that received WAPL-deficient T cells showed significantly improved survival (53% versus 17%; p < 0.01) (Figure 6B in Sun et al. [330]) and reduced clinical severity of GVHD (p < 0.01) (Figure 6C in Sun et al. [330]). We confirmed the reduction in GVHD with detailed histopathological analyses of GVHD target organs including the liver, gastrointestinal (GI) tract, and skin. As shown in Figure 6D (in Sun et al. [330]), allogeneic animals that received T cells from WAPL-deficient donors had significantly reduced histopathological GVHD in the liver (p < 0.01), GI tract (SI and LI) (p < 0.05) and skin (p < 0.05) on day +21 after BMT. Consistent with reduced mortality, the recipients of allogeneic Wapl KO T cells demonstrated reduced serum levels of proinflammatory cytokines such as IFN-γ and TNF-α when compared with WT T cell recipients (Figure 6E and F in Sun et al. [330]).

The allogeneic animals that received WT or KO T cells demonstrated >98% donor engraftment
on day 21, ruling out mixed chimerism or engraftment as a cause for reduction in GVHD. Furthermore, consistent with above results, Wapl KO T cells showed significantly reduced expansion when compared with WT donor T cells (Figure 6G in Sun et al. [330]) in the recipient spleens harvested 7 days after BMT, suggesting that the reduction in GVHD was a consequence of reduced expansion of allo-antigen stimulated T cells. Consistent with this, the allogeneic Wapl KO T cells demonstrated fewer absolute numbers of CD3+CD69+ cells (Figure 6H in Sun et al. [330]) and regulatory T cells (despite a higher percent) when compared to WT T cells (Figure 6I and J in Sun et al. [330]).

4.4 Discussion

The cohesin complex and its regulators, such as WAPL, are critical determinants of 3D genome architecture, which regulates replication, repair, and transcriptional processes [79, 220, 235, 280]. Given the paucity of data on the genomic organization of mature T cells and its impact on T cell function in vivo, we describe the genome architecture of mature T cells following in vivo lymphopenic and allogeneic stimulation.

Prior to the availability of Hi-C methods, structural features of the T cell genome following in vitro stimulation were explored in a seminal paper by Kim et al. [166]. Since then, other studies have built on it with the advent of Hi-C techniques following in vitro stimulation of T cells with anti-CD3/CD28 [391, 40, 24]. We now expand on those studies by exploring the changes following in vivo homeostatic and antigen-driven stimulation. Previously, chromosome 6 in differentiating T cells was explored in its entirety, though global spatial characterization was limited to chromosome territories [166]. Here, we profile the entire genome architecture of mature T cells following in vivo activation, including local structures like TADs. We further connect those changes to genome-wide functional changes in gene expression and T cell responses. Hu et al. demonstrated a key role for transcription factor BCL11B in the development of T cells and associated genome structure changes [142], but did not characterize the relevance of structural integrity to mature T cell functions. Our data focus on the genomic landscape of mature T cells. We define a mechanistic role for genomic architecture in the regulation of gene expression, cellular function, and biological responses in vivo that impact clinically-relevant disease states such as GVHD. We find that differences in 3D genome architecture are a consequence of the cellular state of activation, and that its disruption, by altering the function of the cohesin complex, contributes to the regulation of mature T cell functions in response to allogeneic-stimulation.

We explored the mechanistic relevance of genome structure to function (gene expression) and to the cellular functions and biological impact of mature T cells by deleting WAPL, a key regulator of genome structure. WAPL deletion led to reduction of long-range interactions in the baseline
unstimulated state of naïve T cells. However, following lymphopenic (syngeneic) and antigen (allogeneic) activation, there was a greater amplification of longer-range interactions following WAPL deletion. This is consistent with previous reports on WAPL-deficient cell lines [129, 197] and with the notion of extruded DNA loops beyond CTCF barriers [5]. These data suggest that WAPL alters genomic architecture, at baseline and after activation of T cells.

The in vivo role for WAPL-mediated regulation of chromatin structure is crucial for embryonal development [344]. We now extend these studies and demonstrate that WAPL also regulates in vivo immune responses mediated by T cells. T cell-specific WAPL-deficient animals developed normally despite the T cells showing genomic structural changes at homeostasis. Upon stimulation, WAPL-deficient T cells demonstrated mitosis defects and more axial structures during interphase suggesting that they exit mitosis with less intact cohesin. However, it is important to note that the absence of WAPL neither caused a complete loss of development of T cells nor a total shutdown of mature T cell proliferation. The T cells developed and proliferated in the absence of WAPL, albeit at a much lower level, the reasons for which remain unclear. One possible explanation is that separation of chromatids during mitosis in T cells may be independent, or only partially dependent, on WAPL, based on their strength and duration of stimulation/activation [244, 319, 395]. This notion is consistent with the observation that several cohesinopathies in humans are caused by mutations in various components of cohesin complex and yet do not appear to cause T cell defects [259, 280, 315]. Future studies may determine the role of WAPL and disruption of cohesin in thymopoiesis and in regulation of T cells in secondary lymphoid organs.

The structural changes in our study reflect a polyclonal response from a combination of alloreactive/lymphopenia induced proliferating cells, and the non-proliferating mature T cells. The T cells therefore might be in various stages of early/mid G1, S, G2, M phases. Thus, the genome contact frequency and associated structural changes are reflective of the T cells in these various stages after stimulation. T cells from naïve, syngeneic, and allogeneic settings demonstrated significant changes in internal TAD structure, which were consistent despite the polyclonal nature of the T cell subsets. Nonetheless, while the genome architectures were significantly different, whether these are the direct cause or a consequence of proliferation differences cannot be definitively ascertained. Furthermore, whether antigen-specific T cell responses vary based on the type of antigen cannot be determined from our study. However, our assessment of polyclonal responses is akin to biologically and clinically relevant situations such as allogeneic transplantation.

Our data collectively demonstrate for the first time, to our knowledge, that altering genomic structure a priori, at baseline, regulates T cell gene and cellular functions. Though possible that WAPL could regulate gene expression independent of genome structure, it is widely held that form precedes function and that genome structure and function are coordinated [52, 266]. Mechanistically, the data show that WAPL alters chromatin architecture at cell cycling genes and therefor
links genome structure and function. Nonetheless, validation studies to confirm the exact mechanistic role of WAPL in this context will need to be explored. Additionally, while we only validate select cell cycling genes concomitant with changes in genome structure in this study, it is possible that other cell cycling genes might be involved in the proliferative defects we observe and future studies will need to validate these in a systematic manner as well. Future studies may also benefit from integrating single-cell Hi-C and single-cell RNA-seq to refine observations, as features at the TAD and sub-TAD levels observed in this study via bulk Hi-C are a population average and could be variable and distinct between various T cell subsets that develop and differentiate after stimulation. Regardless, our data indicate that disruption of 3D chromatin architecture by WAPL may directly regulate gene expression and cellular function of T cells in a physiologically and clinically relevant disease context.

Importantly, we introduce a simple yet robust approach for evaluating TADs and their internal structure. Due to the experimental limitations of performing Hi-C in an in vivo setting we obtained a lower number of cells and thus mappable reads than what Hi-C performed on cell lines or in an in vitro setting would typically yield. Consequently, to reliably investigate TAD and sub-TAD level features, we pooled data across all our samples allowing us to bin our data at a higher resolution. From this pooled matrix, we were able to determine TAD boundaries and subsequently superimpose them onto each sample’s raw Hi-C contact map. Informed by this common TAD backbone, we were able to perform targeted analysis of the internal TAD structure in each sample and evaluate how sensitive the backbone and its internal organization are to perturbations across the different settings.

There are no reported cases of isolated germline WAPL mutations in humans [116, 259, 280, 315]. This is likely because of its critical role during embryonic development. However, somatic mutations in WAPL have been linked to epithelial carcinogenesis [368]. Our study demonstrates that WAPL deficiency in T cells did not cause a profound defect in development of T cells, nor cause T cell malignancy. Thus, WAPL may play a nuanced role in different cell subtypes, depending on their developmental stage, context, and stimulation. Future studies will need to carefully assess the biological implications of WAPL deficiency on T cell subsets and other immune cells. Because WAPL-deficient T cells demonstrated a reduction in GVHD, it is tempting to speculate whether targeting it uniquely in T cells might be a novel strategy to mitigate immunopathologies such as GVHD, allograft rejection, or autoimmunity. Such a strategy may be feasible with emerging gene editing strategies for adoptive transfer of T cells, however, the viability of the strategy to delete WAPL clinically will need significant further investigation. At a broader level, our data provide a proof of concept for the notion that targeting 3D genomic architecture may be a therapeutic strategy that can be potentially harnessed for directly modulating in vivo disease processes.
4.5 Materials and Methods

4.5.1 Mice

B6 (H2b, CD4.1 and CD45.2), BALB/c (H2d, CD45.1 and CD45.2), Rosa26-floxed STOP-Cas9 knockin (B6J) and CD4CRE (B6) mice were purchased from The Jackson Laboratory and the National Cancer Institute. The ages of the mice used for experiments ranged between 8 and 12 weeks. Mice were housed in sterilized microisolator cages and received filtered water and normal chow.

4.5.2 Generating conditional Wapl KO T cells

We designed 2 sgRNAs targeting exon 2 of the Wapl locus which are localized in mouse Chromosome 14 qB (Figure S3 in Sun et al. [330]) [272]. Three lines of mice with B6 background were used to generate conditional Wapl KO mice in T cells. The first line was generated to carry CRISPR-sgRNAs targeting insert to identify exon 2 in Wapl (Figure 1A in Sun et al. [330]). The second and third lines are Rosa26-floxed STOP-Cas9-GFP knockin on B6J (The Jackson Laboratory, Stock No:026175) and CD4-CRE transgenic mice (The Jackson Laboratory, Stock No: 017336). Tail DNA was screened for a positive sgRNAs-Wapl insert, positive CRE, and a stop signal in loxP-SV40pA x3-loxP cleavage or deletion. The potential Wapl KO T cells were sorted for positive GFP (Figure 1B in Sun et al. [330]). To confirm the success of conditional in KO in T cells, T cells were processed for SDS-PAGE and detected with anti-Wapl antibody. Additionally, total RNAs were isolated from Wapl KO and WT T cells and processed for RNA-seq.

4.5.3 DC isolation and purification

Dendritic cells (DC) were isolated from splenocytes either from WT B6 or BALB/c mice. Single-cell suspensions were prepared according to the manufacturer’s instruction then subjected to CD11c microbead (MACS) staining and positive selection using LS column (Miltenyi Biotec). The purity of enriched CD11c+ DC preparation was 85.6-90%.

4.5.4 T cell isolation and purification, and mixed lymphocyte reaction (MLR)

WT and Wapl KO B6 T cells were isolated by negative selection (>95% purity) (Pan T Cell Isolation Kit II; Miltenyi Biotec). T cells were co-cultured with B6 WT or BALB/c DCs at a ratio of 40:1 (T cells versus DCs 3 × 105:7.5 × 103) for 96 hours using 96-well flat-bottomed plates
(Falcon Labware), or stimulated with Dynabeads T cell activator CD3/CD28 (25 ul/106/ml) for 2 or 3 days respectively. Proliferation was determined by incubating the cells with H3-thymidine (1 Ci/well [0.037 MBq]) for the last 20 or 6 hours, respectively. H3-thymidine incorporation in T cells was counted on a 1205 Betaplate reader (Wallac, Turku, Finland).

4.5.5 BMT and systemic analyses of GVHD

Bone marrow transplantations (BMTs) were performed as described previously [332]. The donor T cells (WT B6 or WAPL deficiency) were isolated from spleens and purified by negative selection (using the Pan T cell Isolation Kit II; Miltenyi Biotec). Bone marrow cells from tibia and fibula were harvested and TCD (T cell deletion) BM cells were isolated with positive deletion using anti-CD90.2 microbeads and LS column (Miltenyi Biotec). The recipient BALB/c mice received an 800-cGy total body irradiation on day -1 (split dose) and T cells (1 × 106, either B6 WT or WAPL deficiency T cells, and TCD BM cells (5 × 106, from WT B6 mice) were injected intravenously into the recipients on day 0. The syngeneic B6 control mice received a 1000-cGy total body irradiation on day -1. T cells (2 × 106, isolated from B6 WT mice) and TCD BM cells (5 × 106 from B6 WT mice) were injected intravenously into the recipients on day 0. Mice were housed in sterilized microisolator cages and received normal chow and autoclaved hyperchlorinated drinking water for the first 3 weeks after BMT.

4.5.6 Immunoblotting

T cell lysates were extracted as previously described [332], and 50 to 100 µg of protein extract was separated in SDS-PAGE and transferred onto a PVDF membrane (GE Healthcare). The membrane was blocked with 5% nonfat milk for 30 minutes and then incubated overnight at 4°C with the following Abs in 5% nonfat milk: anti-Wapl rabbit polyclonal Ab (1:500 in nonfat milk, Proteintech Cat 16370-1-AP), anti–β-actin mouse mAb (1:1,000 in 5% nonfat milk; Abcam, catalog ab8226). After washing 3 times with TBST for 5 minutes, the blot was incubated with specific HRP-labeled secondary Ab, washed again with TBST, and signals generated and visualized using the Enhanced Chemiluminescence Kit (Thermo Scientific, Cat 32106).

4.5.7 Study approval

Study approval. All animal studies were reviewed and approved by the University Committee on Use and Care of Animals of the University of Michigan, based on University Laboratory Animal Medicine guidelines.
4.5.8 GVHD and pathology scoring

Survival was monitored daily. The degree of systemic GVHD was assessed by a standard scoring system with four criteria scores: percentage of weight change, posture, activity, fur texture, and skin integrity, and subsequently graded from 0 to 2 for each criterion (maximum index = 10) [332]. Acute GVHD was also assessed by histopathologic analysis of the ileum and the ascending colon, liver, and ear skin. Specimens were harvested from animals on day 21 after BMT, then processed and stained with hematoxylin and eosin. Coded slides were examined systematically in a blind manner by using a semi-quantitative scoring system to assess the following abnormalities known to be associated with GVHD, small intestine: villous blunting, crypt regeneration, loss of enterocyte brush border, luminal sloughing of cellular debris, crypt cell apoptosis, outright crypt destruction, and lamina propria lymphocytic infiltrate; colon: crypt regeneration, surface colonocytes, colonocyte vacuolization, surface colonocyte attenuation, crypt cell apoptosis, outright crypt destruction, and lamina propria lymphocytic infiltrate; liver: portal tract expansion, neutrophil infiltrate, mononuclear cell infiltrate, nuclear pleomorphism, intraluminal epithelial cells, endothelialitis, hepatocellular damage, acidophilic bodies, mitotic figures, neutrophil accumulations, macrophage aggregates, macrocytosis; skin: apoptosis in epidermal basal layer or lower malpighian layer or outer root sheath of hair follicle or acrosyringium, lichenoid inflammation, vacuolar change, lymphocytic satellitosis. The scoring system denoted 0 as normal, 0.5 as focal and rare, 1.0 as focal and mild, 2.0 as diffuse and mild, 3.0 as diffuse and moderate, and 4.0 as diffuse and severe. Scores were summed together to provide a total score for each specimen [332].

4.5.9 RNA-seq library generation and data processing

Naive WT and Wapl KO T cells, or WT and Wapl KO T cells isolated from syngeneic or allogeneic BMT on day 7 were first purified as described previously [332]. All dead cells were excluded by sorting for far-red fluorescent reactive dye (Invitrogen, Cat. L10120). Then, WT T cells were sorted for PE-CD3 and APC-CD45.2 while Wapl KO T cells were sorted for APC-CD3, PE-CD45.2 and positive GFP. Each sample contained pooled T cells from 3-4 mice, and samples in each group were biologically triplicated. RNA was isolated using DNA/RNA mini Kit (Qiangen Cat 80204) by following the RNA isolation procedures. Sequencing was performed by the University of Michigan (UM) DNA Sequencing Core, using the Illumina Hi-Seq 4000 platform, paired-end, 50 cycles and Ribosomal Reduction library prep.

We obtained read files from the UM Sequencing Core and concatenated those into a single FASTQ file for each sample. We checked the quality of the raw read data for each sample using FastQC (version 0.11.3) to identify features of the data that may indicate quality problems (e.g., low quality scores, overrepresented sequences, inappropriate GC content). We used the Tuxedo
Suite software package for alignment, differential expression analysis, and post-analysis diagnostics. Briefly, we aligned reads to the reference genome (GRCm38) using TopHat (version 2.0.13) and Bowtie2 (version 2.2.1). We used default parameter settings for alignment, with the exception of: “--b2-very-sensitive” telling the software to spend extra time searching for valid alignments. We used FastQC for a second round of quality control (post-alignment), to ensure that only high-quality data would be input to expression quantitation and differential expression analysis. We used Cufflinks/CuffDiff (version 2.2.1) for expression quantitation, normalization, and differential expression analysis, using GRCm38.fa as the reference genome sequence. For this analysis, we used parameter settings: “--multi-read-correct” to adjust expression calculations for reads that map in more than one locus, as well as “--compatible-hits-norm” and “--upper-quartile–norm” for normalization of expression values. We generated diagnostic plots using the CummeRbund R package. We used locally developed scripts to format and annotate the differential expression data output from CuffDiff. Briefly, we identified genes and transcripts as being differentially expressed based on FDR \(\leq 0.05\), and fold change \(\geq \pm 1.5\). We annotated genes with NCBI Entrez GeneIDs and text descriptions. RNA-seq reads in bam format were mapped to the most recent mouse genome (mm10) using the Integrative Genomics Viewer (IGV). The RNA-seq data reported here can be found in the Gene Expression Omnibus (GEO) database with the series accession ID GSE134975.

### 4.5.10 Generation of Hi-C libraries for sequencing

The in situ Hi-C protocols from Rao et al. [274] were adapted with slight modifications. For each Hi-C library, approximately 1 x 10^6 cells were incubated in 250 \(\mu\)l of ice-cold Hi-C lysis buffer (10mM Tris-HCl pH8.0, 10mM NaCl, 0.2% Igepal CA630) with 50\(\mu\)l of protease inhibitors (Sigma) on ice for 30 minutes and washed with 250 \(\mu\)l lysis buffer. The nuclei were pelleted by centrifugation at 2500xg for 5 minutes at 4°C, re-suspended in 50 \(\mu\)l of 0.5% sodium dodecyl sulfate (SDS) and incubated at 62°C for 10 minutes. Afterwards 145 \(\mu\)l of water and 25 \(\mu\)l of 10% Triton X-100 (Sigma) were added and incubated at 37°C for 15 minutes.

Chromatin was digested with 200 units of restriction enzyme MboI (NEB) overnight at 37°C with rotation. Chromatin end overhangs were filled in and marked with biotin-14-dATP (Thermo Fisher Scientific) by adding the following components to the reaction: 37.5 \(\mu\)l of 0.4mM biotin-14-dATP (Life Technologies), 1.5 \(\mu\)l of 10mM dCTP, 1.5 \(\mu\)l of 10mM dGTP, 1.5 \(\mu\)l of 10mM dTTP, and 8 \(\mu\)l of 5U/\(\mu\)l DNA Polymerase I, Large (Klenow) Fragment (NEB). The marked chromatin ends were ligated by adding 900 \(\mu\)l of ligation master mix consisting of 663 \(\mu\)l of water, 120 \(\mu\)l of 10X NEB T4 DNA ligase buffer (NEB), 100 \(\mu\)l of 10% Triton X-100, 12 \(\mu\)l of 10mg/ml BSA, 5 \(\mu\)l of 400 U/\(\mu\)l T4 DNA Ligase (NEB), and incubated at room temperature for 4 hours.

Chromatin de-crosslinking was performed by adding 50 \(\mu\)l of 20mg/ml proteinase K (NEB)
and 120 µl of 10% SDS and incubated at 55°C for 30 minutes, adding 130 µl of 5M sodium chloride and incubate at 68°C overnight. DNA was precipitated with ethanol, washed with 70% ethanol, and dissolved in 105 µl of 10 mM Tris-HCl, pH 8. DNA was sheared on a Covaris S2 sonicator. Biotinylated DNA fragments were pulled with the MyOne Streptavidin C1 beads (Life Technologies). To repair the ends of sheared DNA and remove biotin from unligated ends, DNA-bound beads were re-suspended in 100 µl of mix containing 82 µl of 1X NEB T4 DNA ligase buffer with 10mM ATP (NEB), 10 µl of 10 (2.5mM each) 25mM dNTP mix, 5 µl of 10U/µl NEB T4 PNK (NEB), 4 µl of 3U/µl NEB T4 DNA polymerase (NEB), and 1 µl of 5U/µl NEB DNA polymerase I, Large (Klenow) Fragment (NEB).

After end-repair, dATP attachment was carried out in 100 µl of reaction solution, consisting of 90 µl of 1X NEBuffer 2, 5 µl of 10mM dATP, and 5 µl of 5U/µl NEB Klenow exo minus (NEB). The reaction was incubated at 37°C for 30 minutes. The beads were then cleaned for Illumina sequencing adaptor ligation which was done in a mix containing 50 µl of 1X T4 ligase buffer, 3 µl T4 DNA ligases (NEB), and 2 µl of a 15 µM Illumina indexed adapter at room temperature for 1 hour. DNA was dissociated from the bead by heating at 98°C for 10 min, separated on a magnet, and transferred to a clean tube.

Final amplification of the library was carried out in multiple PCRs using Illumina PCR primers. The reactions were performed in 25 µl scale consisting of 25 ng of DNA, 2 µl of 2.5mM dNTPs, 0.35 µl of 10 µM each primer, 2.5 µl of 10X PfuUltra buffer, PfuUltra II Fusion DNA polymerase (Agilent). The PCR cycle conditions were set to 98°C for 30 seconds as the denaturing step, followed by 14 cycles of 98°C 10 seconds, 65°C for 30 seconds, 72°C for 30 seconds, then with an extension step at 72°C for 7 minutes.

After PCR amplification, the products from the same library were pooled and fragments ranging in size from 300-500 bp were selected with AMPure XP beads (Beckman Coulter). The size-selected libraries were sequenced to produce paired-end Hi-C reads on the Illumina HiSeq 2500 platform with the V4 of 125 cycles.

4.5.11 Hi-C data processing

We generated the Hi-C matrices using Juicer [103]. Juicer uses BWA-mem to align each paired-end read separately. It then determines which reads can be mapped uniquely and keeps unambiguously mapped read pairs. Each read is assigned to a “fragment”, determined by the restriction enzyme cut sites and paired reads that map to the same fragment are removed. The Juicer pipeline creates a binary data file (namely, the “.hic” file), which contains Hi-C contacts. The .hic file is imported into MATLAB-compatible variables via our in-house MATLAB toolbox, 4DNvestigator [193]. Centromeric and telomeric regions were removed in the process. Then, Hi-C data were
subsequently normalized and binned at 50kb and 100kb resolution. ICE [145] and “observed over expected” (O/E) normalized contact maps at 100kb resolution were used for chromosome-level analysis of A/B compartments. Observed contact maps at 50kb resolution were used for detection and analysis of TADs. Hi-C data for this study are available through the NCBI BioProject database (accession number: PRJNA608895).

4.5.12 Integration of Hi-C and RNA-seq data

To integrate our Hi-C and RNA-seq datasets, we first binned measurements at the same resolution. Accordingly, each element in the binned RNA-seq expression vector corresponds to a row (or column) of the Hi-C contact matrix that captures the same set of genomic loci. Genomic bins spanning a given region contain measurements reflecting the sum of its parts, where expression values for genes sharing the same bin are summed and contact frequencies for loci occupying the same bin are summed. This allows us to directly compare the transcriptional and conformational behavior across regions of the genome.

4.5.13 Frobenius Norm

The Frobenius norm describes the size or volume of a matrix [326]. As such, the Frobenius norm can be used as a measure of variance or variability in data [102]. For matrix $A$ of dimension $m$, that is $A \in \mathbb{R}^{m \times n}$, the mathematical definition of the Frobenius norm is as follows:

$$\|A\|_F = \sqrt{\sum_{i=1}^{m} \sum_{j=1}^{n} a_{ij}^2},$$

(4.1)

where $a_{ij}$ is an element of the data matrix $A$. Equivalently this expression can be written as,

$$\|A\|_F^2 = \text{trace}(A^TA),$$

(4.2)

where $\text{trace}$ is the sum of the diagonal elements of a matrix [105]. When comparing two sets of data, the notation can is modified as follows:

$$\|A - B\|_F^2 = \text{trace}((A - B)^T(A - B)),$$

(4.3)

where $A$ and $B$ are matrices of the same dimensions. The Frobenius norm of a vector is equivalent to the Euclidean norm.
4.5.14 Larntz-Perlman Procedure

The Larntz-Perlman (LP) procedure is a method designed to test the equivalence of correlation matrices [179]. We applied the LP procedure as described in the 4DNvestigator Toolbox [193] to compare Hi-C contact matrices between WT and Wapl KO conditions. Briefly, correlation matrices are derived from data by taking the pairwise linear correlation coefficient between each pair of columns in the Hi-C contact matrices. Then, a null hypothesis is defined from corresponding population correlation matrices. We then compute a Fisher z-transformation on the correlation matrices and calculate a test statistic for the chi-squared distribution to determine whether or not the null hypothesis (that Hi-C matrices are equivalent) should be rejected with a corresponding p-value.

4.5.15 A/B Compartmentalization

Chromatin can either take on a condensed heterochromatic form or a looser euchromatic form. We use the Fiedler vector – the eigenvector corresponding to the second smallest eigenvalue (Fiedler number) of the normalized Laplacian matrix – to describe this feature mathematically which allows us to bi-partition the data into signed compartments – “A” (positive values) corresponding to euchromatin and “B” (negative values) corresponding to heterochromatin. The Fiedler vector and first principal component (PC1) vector are mathematically equivalent. However, unlike the PC1 vector, the Fiedler vector is accompanied by its corresponding Fiedler number which allows us to assign a magnitude of connectivity to a network. We characterize compartment switch events by identifying genomics bins with differing sign and magnitude above the 90th percentile of vector values between settings.

4.5.16 Identification of TADs

Topologically associating domains (TADs) were identified using spectral clustering as described in chen et al. [63]. This technique calculates the Fiedler vector for a normalized Hi-C adjacency matrix and initially organizes neighboring regions whose Fiedler vector values have the same sign into shared domains. This initial TAD structure is repartitioned if for a given domain, the Fiedler number is less than a user-defined threshold ($\lambda_{thr}$) to ensure that the TADs represent well-connected regions and are not too large. Resulting TADs are iteratively repartitioned until their respective Fiedler numbers are larger than $\lambda_{thr}$ or until the smallest allowable TAD size (default is 300kb) is reached. We perform this procedure for chromosome-level Hi-C contact maps pooled across samples at 50kb resolution. For our analysis, thr was chosen to ensure a median TAD size of 900kb, as the expected median TAD size in mammalian genomes is 880kb (rounded to 900kb
for our data since bins are in intervals of 50kb) [94, 32]. A specific $\lambda_{thr}$ was chosen for each chromosome and sample to ensure each TAD clustering set would have the same approximate median TAD size.

**4.5.17 Quantifying internal TAD organization**

The internal structure of TADs is comprised of transient interactions over varying lengths that form chromatin ‘loops’. In the absence of WAPL, loop length increases, enriching contact frequency between either boundary of a given TAD [129]. Visually, we identify these interactions by the notable density of contacts present in the top right or bottom left corners of TADs. We quantify the strength of these interactions by designating a local neighborhood with a window size of 150kb by 150kb centered around the corner of each TAD. We then find the nonzero average of observed contacts in each window. TADs that are less than or equal to the window size are ignored (corner peak signal set to 0). This is performed for every TAD across each setting and chromosome. To evaluate the statistical significance of a given change in corner peak signal between WT and KO cells, we compared each corner peak signal to a background set of interactions. This background set is made up of contacts in the region between the diagonals encompassing the corner peak neighborhoods. We evaluate the p-value of each corner peak signal based on the proportion of background interactions with a signal less than the corner peak signal. As such, we determined that if a corner peak has a higher signal in the KO cells compared to WT cells and is significantly higher than interactions at comparable distances ($p < 0.01$), then the corner peak enrichment is statistically significant.

**4.5.18 Hi-C derived 5C contact map generation**

We constructed a synthetic 5C contact map derived from a genome-wide 1Mb Hi-C contact map for genomic regions containing genes in the cell cycle gene networks. This was done by locating the genomic bins corresponding to cell cycle genes, extracting the inter-chromosomal and cell cycle loci-specific intra-chromosomal interaction frequencies for those bins, and stitching them together in genomic order. Our working set of cell cycle genes was sourced from the KEGG database and included 170 genes distributed across all chromosomes in the genome. Some of the genes occupy the same 1Mb genomic bins resulting in a 141 by 141 dimension adjacency matrix at 1Mb-resolution.
4.5.19 Cell proliferation assay

CellTrace™Far-Red was utilized for our cell proliferation assay. Purified T cells were labeled with far-red at a final concentration of 1 µmol/L according to the manufacturer’s instruction (Molecular Probes, C34564). Far-red-labeled T cells were cultured similar to MLR for allogeneic reaction or Dynabeads T cell activator CD3/CD28 stimulation for up to 7 days. Then, collected cells were examined with flow cytometry, gated for CD3 (APC) positive, and Far-red dilution was determined.

4.5.20 FxCycle™Far-Red Stain for DNA content measurement

For in vitro experiments, WT or Wapl KO T cells were treated with Dynabeads T cell activator CD3/CD28 stimulation for up to 4 days. For in vivo experiments, transferred WT and Wapl KO T cells were isolated and purified from spleens of BALB/c recipient mice on day 7 after allogeneic BMT. The T cells were fixed with 10% formaldehyde for 30 mins, washed 3 times with PBS and the sample cell concentration was adjusted at 1 × 106 cells/mL. FxCycle™Far-red stain (200 nM) (Molecular Probes, F10348) and 5 µL of RNase A (20 mg/mL) (Roche Cat. 70294823) were added to flow cytometry samples and continued for incubation at room temperature for 30 minutes and protected from light. Samples were analyzed with flow cytometer (AttuneNxT) without washing using 633nm excitation and emission collected in a 660 bandpass. The DNA contents were determined as 2C and >2C by fluorescence intensities.

4.5.21 ELISA

Concentrations of TNF-α and IFN-γ in sera on day 21 after allogeneic BMT were measured with specific anti-mouse ELISA kits from BD Biosciences. Assays were performed per the manufacturer’s protocol and read at 450nm in a microplate reader (Bio-Rad). The concentrations were calculated from triplicate samples as mean ± SEM.

4.5.22 FACS

Single-cell suspensions of spleens and thymii were prepared as previously described [332]. Briefly, to analyze surface phenotype, purified T cells and thymocytes from B6 WT, Wapl KO deficiency mice, or transplanted animals, were washed with FACS wash buffer (2% bovine serum albumin [BSA] in phosphate-buffered saline [PBS]), pre-incubated with the rat anti-mouse FcR mAb 2.4G2 for 15 minutes at 4°C to block nonspecific FcR binding of labeled antibodies, then resuspended in FACS wash buffer and stained with conjugated monoclonal antibodies purchased from BD Biosciences (San Jose, CA): allophycocyanin (APC)-conjugated monoclonal antibodies
(MoAbs) to CD4, CD8, CD3, CD45.2, CD45.1, CD25 and CD69; phycoerythrin (PE)-conjugated MoAbs to CD3, CD4, CD8, CD25, and TCRβ; allophycocyanin (APC)-conjugated MoAbs to CD3, CD4, and PerCP/Cy5.5-conjugated MoAbs to CD3, CD4 and CD8 were purchased from eBioscience (SanDiego, CA). Next, cells were analyzed using an AttuneNxT flow cytometer. For intra-cellular staining, cells were stained for CD4 and CD25 antibodies as above, then fixed with IC Fixation Buffer (Biolegend, Cat. No.420801), incubated 20-60 minutes at room temperature, followed by continued addition of 2 mL of 1X Permeabilization Buffer (Biolegend Cat. No. 421002) and centrifugation at 400-600 x g for 5 minutes at room temperature. Cell pellets were resuspended in 100 µL of 1X permeabilization buffer and PE-conjugated FoxP3 antibody (eBioscience, SanDiego, CA, Cat. 126403) was added at 0.5 µg/million cells/100 µl and incubated for 30 minutes at room temperature. Stained cells were resuspended in an appropriate volume of Flow Cytometry Staining Buffer for flow cytometry analyses. Apoptotic cells were detected by PE-Annexin V staining.
CHAPTER 5

Obesity Disrupts Innate-Adaptive Immune Network Patterning in Adipose Tissue

This chapter is based on a paper by Gabrielle A. Dotson, Indika Rajapakse, and Lindsey A. Muir [100] (under review).

5.1 Abstract

Obesity drives significant changes in adipose tissue that precede development of tissue and systemic insulin resistance. Immune cell infiltration and inflammation are known contributors to these changes but there is limited understanding of their spatial context tissue-wide. We sought to identify spatial patterning in epididymal adipose tissue immune cells in a time course of diet-induced obesity in mice. Using spatial transcriptomics and single-cell RNA-sequencing, we identified dominant cell type signatures preserved in their anatomical context, quantified gene expression patterns at spots throughout adipose tissue, performed cell type network analysis, and investigated ligand-receptor colocalization. Our data support increased innate immune cells, including macrophages, monocytes, and innate lymphoid cells with tissue-wide interspersion, and dampened adaptive immune cell signatures with obesity. Network analysis identified increased heterogeneity in all major immune cell types, consistent with increased subtypes. To capture tissue dynamics at obesity onset, we draw on mathematical principles from linear algebra and spectral graph theory and provide a framework for better understanding cell cooperation toward emergence of multicellular tissue function. The culmination of these analyses revealed a widespread paradigm shift in ligand-receptor activity with near-exclusive macrophage-macrophage or monocyte-macrophage interactions at crown-like structures across adipose tissue during obesity.
5.2 Introduction

The global increase in obesity raises significant concern about the development of cardiovascular and metabolic disease, increased risk of adverse events in viral infection, and economic and health care costs [221, 81]. Changes in white adipose tissue (WAT) immune cell types and polarization occur in obesity and are associated with metabolic dysfunction in mice and humans [204, 203, 231, 230].

Population and single-cell transcriptomics enable low-bias characterization of these obesity-related changes in WAT immune cells [154, 365, 230]. However, the molecular events triggering tissue dysfunction remain poorly understood in their anatomical context, which is lost with tissue digestion. Tissue immunostaining captures anatomical context but is limited to a small number of concurrent markers that may not fully distinguish cells with similar surface proteins. In adipose tissue, macrophages (ATMs) and dendritic cells (ATDCs) have been challenging to distinguish, yet have unique participation in tissue homeostasis and development of insulin resistance [294, 69, 261]. Thus low-bias capture of immune cells and subtypes within a tissue landscape will be critical for identifying functional distinctions relevant to disease.

Spatial transcriptomics (ST) captures signatures of gene expression across tissue sections, which in studies of amyotrophic lateral sclerosis (ALS) led to discovery of new anatomical subregions and spatiotemporal patterns underlying tissue dysfunction [211, 11, 66, 369, 86]. ST was recently used to profile different adipocyte subtypes with distinct responses to insulin stimulation in human adipose tissue [15]. Here, we mapped murine adipose tissue and immune cell populations in obesity using a combination of spatial transcriptomics and single cell RNA-sequencing. Over a time course of diet-induced obesity and development of the pre-diabetic state, one of our primary goals was to capture dynamics of early immune cell infiltration as tissue dysfunction appears. We present the tissue landscape as a network of cells and interrogate spatial patterning and cell-cell communication in early obesity. Our analyses capture early monocyte infiltration, progressive dominance of monocyte and macrophage crosstalk, and dampening of adaptive immune cell signatures. Furthermore, ligand-receptor analyses show putative monocyte-macrophage interactions in pre-crown-like anatomical neighborhoods by 8 weeks of high-fat diet feeding, implicating monocyte signaling in development of crown-like structures in a role distinct from their differentiation into ATMs.
Figure 5.1: Diet-Induced Obesity and Adipose Tissue Remodeling. (A) Time course for mice fed a 60% high-fat diet (HFD) for 8 weeks (8w) or 14 weeks (14w), versus normal diet (ND) controls. Measurements included weight gain (B), final body weight (C), epididymal adipose tissue (eWAT) weight and eWAT as a percentage of body weight (D), and glucose tolerance test data showing area under the curve (AUC) across time points (E) and glucose measurements for cohorts one week prior to endpoint tissue collection (F). (G) Frequency distribution and average adipocyte size in eWAT of ND, 8w, and 14w cohorts. (H) Representative images from H&E staining of eWAT and liver. (I) Representative maps showing spatially preserved gene expression in eWAT. Each spot is 55 µm diameter location of transcript capture from a tissue section, colored corresponding to the expression level (log₂-transformed) of select obesity-related genes. (J) Gene expression changes
Figure 5.1: in HFD-fed mice compared to ND mice corresponding to the genes shown in part (I). Upper graph, log$_2$(fold change) in the quantity of spots in HFD cohorts; Lower graph, log$_2$(fold change) in average tissue-wide expression of a gene in HFD cohorts.

5.3 Results

5.3.1 Spatial Analysis of Adipose Tissue Across Early Obesity

We evaluated tissue and metabolic function in our diet-induced obesity model using mice fed a normal diet (ND), a high-fat diet for 8 weeks (8w), and a high-fat diet for 14 weeks (14w) (Figure 5.1A). As expected, mice fed a high-fat diet (HFD) gained body and epididymal white adipose tissue (eWAT) weight (Figure 5.1B-D). Glucose tolerance tests (GTT) showed increased area under the curve (AUC) starting at one week of HFD feeding, with the highest AUC and variability at intermediate time points (Figure 5.1E,F). Adipocyte sizing showed increased frequency of large adipocytes and greater mean adipocyte size in the 8w and 14w cohorts (Figure 5.1G,H). These data are consistent with pre-diabetes of this HFD-fed model, showing early insulin resistance during HFD feeding and a period of variable insulin resistance that aligns with dynamic restructuring of adipose tissue.

Changes in SVCs in murine diet-induced obesity include immune cell infiltration and activation that progressively disrupts homeostatic mechanisms [226, 231, 261, 260]. To capture this changing tissue landscape, we performed spatiotemporal molecular profiling, which can uncover key patterns in breakdown of tissue function [11, 66]. Here, we spatially profiled genome-wide transcripts across eWAT sections from ND and HFD-fed mice using the Visium (10X Genomics) spatial transcriptomics (ST) platform [323, 15]. We initially evaluated expected immune cell and obesity-related gene expression, and found increased pan-immune cell *Ptprc* (*CD45*), myeloid-associated *CD68*, recruited ATM marker *Itgax* (*CD11c*), resident ATM marker *Mrc1*, and *Trem2*, which is expressed in lipid-associated ATMs and has proposed functions in protective lipid homeostasis [154, 195, 309] (Figure 5.1I,J). *Adipoq* progressively decreased as expected in the HFD-fed conditions.
Figure 5.2: Workflow of Spatial Transcriptomics (ST) and Single-Cell RNA-Sequencing (scRNA-seq) Data Analyses. Epididymal white adipose tissue was collected from mice in a time course of high fat diet feeding. scRNA-seq data were obtained from within-cohort pooled samples, and ST data were collected from a section of fresh frozen adipose tissue from each diet condition.
Figure 5.2: scRNA-seq data were then pooled across diet conditions, clustered (Algorithm 2), and cell populations were annotated using a data-driven approach (see Methods). Existing literature and databases were used to query highly-scored markers and call cell types for each cluster. ST data were evaluated for distribution of obesity marker genes. scRNA-seq and ST datasets were integrated by mapping cell types to the adipose tissue sections, performed by finding the overlap between identified cell type signatures (scRNA-seq) and tissue spot expression profiles (ST). Given the size of each tissue spot, more than one cell type signature was likely to be found at each spot. Consequently, every cell type was assigned at each tissue spot based on the proportion of its signature expressed at the spot, generating layers of tissue spot assignments with decreasing likelihood, a process we refer to as spot deconvolution (Algorithm 3). Once tissue spots were annotated, patterns in the intra-cell type spatial tissue network were characterized. Finally, ligand-receptor signaling was classified and colocalization and cell-cell interaction trends characterized (Algorithm 4).

5.3.2 Breakdown of Adipose Tissue Immune Cell Networks in Early Obesity

Multiple nuclei can contribute transcripts to each spatial data spot, therefore in parallel we performed single-cell RNA-sequencing (scRNA-seq) in SVCs to clarify changes in immune cell types and facilitate signature identification in spatial data. SVCs were isolated from fat pads contralateral to the fat pads used for spatial data and enriched for CD45+ immune cells. We identified cell types in scRNA-seq data using spectral clustering and analysis of signature genes for each cluster, considering expression and uniqueness (Algorithm 2, Table S1 in Dotson et al. [100], Methods and Materials). Cell type signatures were further verified by alignment with ImmGen profiles (Figure S1 in Dotson et al. [100]). To identify cell types present at spots, we integrated scRNA-seq and ST datasets, determining overlap between a cell type signature and tissue spot gene expression (Figure 5.2, Algorithm 3, Methods and Materials).

Spectral clustering and data-guided cell type identification yielded six broad immune cell types: monocytes, T cells, ATMs, ATDCs, natural killer cells (NKCs), and B cells (Figure 5.3A, Figure S1 in Dotson et al. [100]). Changes in the cellular composition of adipose tissue in lean versus obese mice were largely driven by T cells, B cells, and ATMs, where the proportion of single cells classified as T cell and B cell decreased with obesity (17% to 5% in T cells and 22% to 3% in B cells), while the proportion classified as ATMs sharply increased (43% to 76%). These findings are consistent with prior scRNA-seq studies of immune cell populations in diet-controlled mouse models [154, 377], where the same panel of immune cells has been observed and where monocytes and ATMs have been the predominant populations present.

We next established cell type localization patterns in lean and obese adipose tissue. Cell types were assigned to tissue spots hierarchically based on the proportion of their signature expressed at the spot. In this way, the dominant cell type at a spot is the one with the highest number of its
representative genes expressed. Dominant cell types across all spots make up the dominant layer, or Layer 1. Layer 2 is assigned as the cell type with the second highest number of signature genes expressed, and the remaining cell types are assigned subsequent layers in the same manner. The outcome of this assignment is a series of layers representing a decreasing likelihood of a given cell type’s presence at a particular spot (Figure S2 in Dotson et al. [100]). We refer to this process as spot deconvolution and the resulting tissue assignment layers as 'deconvoluted layers' (Algorithm 3, Figure 5.2, Methods and Materials). Cell type localization in the dominant layer over time was consistent with trends in cellular composition from the scRNA-seq data, suggesting decreasing T cells and B cells and increasing ATMs in the HFD settings (Figure 5.3B).

5.3.3 Spatial Network Patterning Highlights Activation of Innate and Dampening of Adaptive Immune Cell Signatures

Inflammation is a key factor in development of adipose tissue dysfunction and insulin resistance in obesity. Cells of the adaptive and innate immune system both contribute to inflammatory processes in adipose tissue, including T cells, B cells, ATMs, ATDCs, and innate lymphoid cells (ILCs) [217]. This network of cells, and their subtypes, modulate inflammation through cytokine and chemokine secretion and cell-cell interactions, reducing anti-inflammatory signaling and promoting proinflammatory cell types and processes over time. To broadly compare patterns of adaptive and innate immune cell networks in obesity, each tissue spot in the spatial data was classed as adaptive (T cell, B cell), innate (monocyte, NKCs, ATM, or ATDC), or mixed type based on the first two tissue assignment layers from Algorithm 3. We found that spots containing only adaptive immune cells consistently decreased with obesity, from 21% to 6% (3.5 fold change), while spots containing only innate immune cells increased with obesity, from 1% to 9% (9 fold change) by 14w (Figure 5.3C), consistent with known shifts in cellularity with HFD feeding [231]. Among total spots, 75%-85% had mixed type signatures, with the higher frequencies found in obesity. Overall, these data suggest a stronger influence of adipose tissue innate immune cells than adaptive immune cells with obesity, through increased quantity and frequency, strong spatial interspersion, and increased potential for cross-talk with the adaptive immune system (Figure 5.3C).
Figure 5.3: Immune Cell Type Identification and Localization in Adipose Tissue. (A) Cellular composition. UMAP projections of scRNA-seq data over time, stratified by cell type. Gray points represent the UMAP projection from pooled clustering of all three samples and colored points represent sample-specific single cells. (B) Spatial tissue patterning. (Top) H&E staining of adipose tissue sections. (Bottom) Zoomed view of annotated ST-derived tissue landscapes. Cell type assignments represent the dominant spot assignments after performing spot deconvolution (Algorithm 3).
5.3.4 Turing-Inspired Analysis Reveals Increased Interconnectivity Among Monocytes and Macrophages

To further assess how immune cells contribute to shifting multicellular tissue function, we described intra- and inter-cell type relationships at the cellular level and identified transcriptional diversity within cell type populations at the genome level. For modeling this tissue morphogenesis,
we introduce the notion of the Turing system. Briefly, the Turing system considers the coordinated
effect that between-cell and within-cell dynamics have on emerging tissue function (Supplemen-
tary Notes)[270]. Here, we characterized between-cell dynamics in terms of correlation between
position-specific gene expression profiles weighted by Euclidean distance from ST data and within-
cell dynamics in terms of correlation between single-cell gene expression profiles from scRNA-seq
data (Methods and Materials). We then captured emerging tissue function in terms of individual
cell type contributions, by computing the trade-off between cell and genome connectivity for each
immune cell type, derived from between-cell and within-cell dynamics, respectively (Figure S3 in
Dotson et al. [100], Methods and Materials). Our results were consistent with increased contribu-
tions from macrophage and monocytes to the changing adipose tissue landscape in obesity (Figure
5.3D). The individual contributions of T cells and B cells to emerging tissue function decreased
with obesity, consistent with localization trends described in Figure 5.3B.
To determine the contribution of pairs of cell types to tissue function in a potentially coordi-
nated manner – emergence of multicellular tissue function – we normalized the emergence of local
tissue function (cell and genome connectivity trade-off derived from merged data across two cell
types) by the emergence of global tissue function (cell and genome connectivity trade-off derived
from merged data across all cell types). While the majority of cell type inter-connectivities demon-
strated decreased or stable coupling with obesity (Figure S4 in Dotson et al. [100]), the inter-cell
type contributions between monocyte-macrophage and monocyte-dendritic cell increased in obe-
sity (Figure 5.3D). In addition, macrophage inter-connectivities were high overall, particularly with
other innate immune cell types (Figure S4 in Dotson et al. [100]), while the B-T cell axis had one
of the lowest inter-cell type connectivities, decreasing over time (Figure 5.3D).
The increased monocyte-macrophage and monocyte-dendritic cell inter-cell type relationships
in HFD feeding may reflect signatures related to monocyte differentiation [392] or crosstalk be-
tween these cells. We further explored the distribution of key myeloid cell markers, and found
growing colocalization of common monocyte (Traf1, Ccr2, Il1b, Napsa, Plac8, S100a9, Msrb1,
Ifitm6, Ms4a4c, Cd209a) and macrophage (Adgre1, Ctsk, Lyve1, Cd209f, Mertk, Ccl8, Trem2,
Cd63, Mmp12, Gpnmb, Rhoc, Ctsd, Mfge8) markers across the tissue (Figure 5.3E), indicating
a potential role for undifferentiated monocytes in cooperation with macrophages in obesity. In
contrast, little to no overlap between B cell and T cell marker colocalization was found 5.3E),
consistent with the low and decreasing B cell-T cell inter-connectivity with obesity.
We next evaluated transcriptional correlation between monocytes and macrophages grew over
time as an indicator of a lineage relationship. The correlation between monocyte and macrophage
expression profiles increased non-randomly from $R = 0.25$ to $R = 0.30$ (p < 0.01) (Figure 5.3F).
While this supports monocyte to macrophage differentiation, the magnitude of the correlation was
low, potentially due to increased diversity of subtypes in these populations. Overall, these data sup-
port the presence of monocyte-macrophage crosstalk as well as ongoing differentiation of monocytes.

5.3.5 Increased Heterogeneity of Signatures Within Immune Cells Captures Phenotype Shifts Across Adipose Tissue

Obesity promotes shifts in immune cell polarization and the appearance of subtypes that affect tissue function. In mice, proportional increases in ATMs relative to tissue weight and patterns of proliferation and apoptosis during weight gain and loss suggest active mechanisms of homeostatic signaling [231] that may eventually be overridden by chronic obesity [396]. We hypothesized that evaluation of immune cell networks in our time course data would provide insight into phenotype shifts that disrupt adipose tissue function.

To better quantify these shifts, we constructed spatial cell networks for each major cell type, where nodes were the tissue spots assigned to the cell type based on the dominant layer and edges between nodes represented a correlation based on transcriptional signature. Surprisingly, cell type network edges decreased universally in obesity, indicating loss of transcriptional correlation among cells of the same type (Figure 5.4A). This was concurrent with increased heterogeneity in expression of cell type-specific signature genes. Loss of spatial patterning and increased heterogeneity support the emergence of immune cell subtypes in obesity that are not present in the lean state. Macrophages in particular show many phenotypic states [329, 389, 294]. Upon clustering macrophage-classified single cells into two sub-clusters, only one of the two clusters was represented in the normal diet setting while both were represented in the two high-fat diet settings (Figure S5 in Dotson et al. [100]). The emergent cluster in the HFD settings was significantly enriched in Cd9 (log2FC of 11.8), which has previously been observed in lipid-laden macrophages in obesity [138, 154, 287]. In contrast to the observed macrophage subpopulations, T cell, B cell, and NKC sub-clusters were less distinct with fewer differentially expressed genes.

5.3.6 Monocyte and Macrophage Ligand-Receptor Pairs Dominantly Colocalize in Obese Adipose Tissue

To better understand specific interactions that might mediate shifted spatial patterning in obesity, we evaluated colocalized expression of paired ligands and receptors over time. We first captured ligand and receptor expression in adaptive and innate classes, characterizing four interaction states: (1) adaptive ligand and adaptive receptor, (2) innate ligand and innate receptor, (3) adaptive ligand and innate receptor, and (4) innate ligand and adaptive receptor (Materials and Methods). For this study, we determined transcript colocalization of known ligand-receptor pairs as a proxy
for cell-cell communication given the restricted area of each tissue spot.

Interrogating known ligand-receptor (LR) pairs from the literature, we observed an increase in colocalization across the tissue over time, with both the number of colocalized spots and the number of LR pairs at each colocalized spot increasing (Figure 5.4B). From the scRNA-seq data, we found the proportions of adaptive single cells expressing colocalized pairs’ ligand and receptor, respectively, and the proportions of innate single cells expressing colocalized pairs’ ligand and receptor, respectively (Materials and Methods). This allowed us to infer whether signaling interactions sourced from adaptive, innate, or an interplay of both contributed to the changes we saw between lean and obese adipose tissue. We defined a score for each state by taking the mean proportion between all pairwise combinations of adaptive- or innate-sourced ligands and receptors (Materials and Methods). This score represents the likelihood that a LR pair exhibits signaling activity within (or between) the particular class(es) of cell types. Then, for each colocalized LR pair, we identified which of the four interaction states exhibited the highest score and found that the percentage of ligand-receptor pairs dominantly involved in adaptive ligand-adaptive receptor, adaptive ligand-innate receptor, and innate ligand-adaptive receptor signalling decreased during obesity progression while the percentage of pairs dominantly involved in innate ligand-innate receptor signaling increased (Figure 5.4C).

We performed the same steps to explore the contribution of different cell types to specific ligand-receptor interactions in lean versus obese adipose tissue (Algorithm 4, Figure 5.2). Interestingly, we found that there was a preference for ligands and receptors interacting from different cell types rather than the same cell type, though decreasingly so with obesity (Figure 5.4D). Interactions where the ligand cell type and/or the receptor cell type was a macrophage scored highly among most LR pairs compared to other cell types (Figure 5.4E). Global interaction states – derived by taking the average of interaction state scores across all colocalized LR pairs – revealed a diverse panel of cell-cell interactions involving LR signaling in ND that shifted towards a growing preference for LR signaling between macrophage-macrophage and monocyte-macrophage by 8w (Figure 5.4F).
Figure 5.4: Intra-Cell Type Spatial Patterning. (A) Network nodes represent transcriptionally correlated tissue spots assigned to the cell type in the dominant tissue assignment layer. Color intensity of network nodes represents the number of cell type-specific signature genes expressed at the spot. The color bar reflects the maximum number of signature genes (out of 50) expressed at any given spot across all three tissue settings. Network edges represent high correlation (correlation coefficient ≥ 0.85) between cell type-specific gene expression profiles at two spots.

B

Ligand-Receptor Colocalization

ND (n = 240) 8 weeks (n = 552) 14 weeks (n = 577)

C

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D

Proportion

ND 8w 14w

E

Local Macrophage Interactions

ND

F

Preferential Cell-Cell Interactions

Receptor Cell Type

Ligand Cell Type

Interaction Score

Mon T Mac DC NKC B Mon T Mac DC NKC B Mon T Mac DC NKC B

ND 8 weeks 14 weeks
Figure 5.4: (B) Distribution of colocalized ligand-receptor (LR) pairs. Color intensity denotes the number of LR pairs colocalized at the tissue spot (normalized by the total number of tissue spots). (C) Percent of LR pairs that preferentially interact between adaptive and/or innate cell types. (D) Proportion of ligand-receptor interactions between single cells of the same cell type (intra-cell type) versus single cells of different cell types (inter-cell type). (E) Range of interaction scores across all colocalized LR pairs in all macrophage-involved cell-cell pairs. Each LR pair’s interaction score between cell types is evaluated by computing the mean between the proportion of single cells of one cell type expressing the ligand and the proportion of single cells of another cell type expressing the receptor. (F) Number of LR pairs that preferentially interact between two cell types. Frequencies are normalized by the total number of colocalized LR pairs in the sample. Preferential interaction was determined by evaluating individual LR interaction scores based on the mean proportion of single cells in a cell type expressing the ligand and the mean proportion of single cells in a cell type expressing the receptor (described in Algorithm 4).

5.3.7 Pre-Crown-Like Structure Neighborhoods Appear in Early Obesity

Crown-like structures (CLS) appear in adipose tissue in chronic obesity, where macrophages accumulate around dead or dying adipocytes (Figure 5.5A). We used Itgax and Trem2, which are involved in CLS formation [195, 151], and Cd9, a marker of lipid-laden macrophages, to identify CLSs across the 8w and 14w tissue sections, verifying the known trend for their emergence in response to HFD feeding (Figure 5.5B). Since CLSs do not form in lean adipose tissue, we screened for Cd9 on its own in ND tissue to identify tissue spots that could serve as a baseline comparison. We then demarcated neighborhoods around each CLS signature-expressing tissue spot to capture surrounding cells involved in CLS dynamics and explore features that could reveal key attributes of CLS dynamics during obesity (Figure 5.5C, Materials and Methods).

We first determined whether CLS neighborhoods exhibited differential expression. We compared expression pooled across all ND baseline neighborhoods with expression pooled within each individual CLS neighborhood at 14w, finding that 939 genes were uniquely (compared to control neighborhoods, see Materials and Methods) and significantly differentially expressed (\(|\log_2 FC| \geq 1.5, p < 0.01\)). Of those, 24 genes were differentially expressed in at least 20% of CLS neighborhoods (Figure 5.5D). Moreover, all differentially expressed genes were up-regulated, with Trem2, Cd9, and Itgax among the most frequently occurring DEGs across CLS neighborhoods, as expected. In addition, we found several genes related to tumor progression (Soat1, Hk3, Syngr1) [282, 262], which has been associated with CLS formation.

We then evaluated how LR colocalization trends with CLS emergence. We found that the increase in CLS numbers with obesity was concordant with an increased proportion of LR colocalization within CLS neighborhoods (Figure 5.5E). Specifically, LR colocalization at CLS neighborhoods increased from 18% in ND (17% in control neighborhoods) to 58% at 14w (30% in control
neighborhoods). These colocalized LRs exhibited a variety of dynamical patterns, becoming more enriched or depleted across CLSs over time and interacting between different cell types (Materials and Methods). An overwhelming majority of LR pairs demonstrated an overall increase in the proportion of CLS neighborhoods they colocalized from the lean to obese states (Figure 5.5F). Most of these were LR pairs that increased from ND to 8w and again from 8w to 14w (54%). Interestingly, there was a considerable proportion (20%) of LR pairs whose involvement at CLS neighborhoods increased from ND to 8w then decreased from 8w to 14w. We previously observed this similar “amplification-attenuation” trend among the intra-cell type tissue contributions where both natural killer cells and monocytes demonstrated increased connectivity within their respective cell type networks followed by weakened connectivity (Figure 5.3). This trend led us to speculate about a pre-CLS state in our intermediate HFD feeding time point.

To elucidate cell types responsible for driving dynamic signaling at CLS neighborhoods, we revisited cell types found to dominantly express each LR pair from our earlier analysis in Figure 5.4. Monocytes and dendritic cells dominantly express a large proportion of LR pairs at baseline neighborhoods in the increase-increase group in the ND setting. By 14w, macrophage accounted for the majority of ligands (60.7%) secreted and picked up by receptors (77%) in the CLS niche. We further examined specific LR pairs from the increase-increase group, screening for LR pairs that appeared in CLS neighborhoods only after HFD feeding.

We identified 12 LR pairs that colocalized at no baseline neighborhoods in lean adipose tissue then emerged prominently at these sites in obese adipose tissue (Table S2 in Dotson et al. [100]). Of the remaining LR pairs, some involved a ligand and/or receptor with a previously characterized role in obesity, like $C3$, linked to lipid metabolism and contribution to inflammation in adipose tissue during obesity [17]. $C3$ was a recurrent, dominantly monocyte-expressed ligand in this group, with an average percent increase in colocalization with receptors at CLS neighborhoods of 54.8% from ND to 14w. $C3$ was associated with receptors $Itgax$, $Itgb2$, $C3ar1$, and $Lrp1$, which were expressed by a mix of innate cell types in the data at 8w. At 14w, $C3$ only colocalized with receptors associated with macrophages. Several LR pairs exhibited similar behavior.
Figure 5.5: Emergence of Crown-Like Structures. (A) H&E staining of the 14w adipose tissue section showing a characteristic crown-like structure (CLS). (B) Identification of CLSs in the ST data. In HFD settings, tissue spots were classified as CLSs if they expressed Cd9 with either Itgax or Trem2. As CLSs are not found in lean adipose tissue, only the more general lipid-laden macrophage marker, Cd9, was queried in the ND setting. (C) Illustration of CLS neighborhood demarcation. Spots within two outdegrees of the CLS-classified spot (landmark) were defined as the CLS neighborhood. Following analyses evaluated different features relative to these neighborhoods. (D) Differentially expressed genes ($|\log_{2}\text{FC}| \geq 1.5$, $p < 0.01$) found in at least 20% of CLS neighborhoods. Color intensity indicates fold change between ND and 14w. Red values indicate an increase in expression (positive FC) and blue indicates a decrease in expression (negative FC).
Figure 5.5: (E) LR pairs in CLS neighborhoods. Red neighborhoods correspond to CLSs while blue neighborhoods correspond to a control population containing housekeeping gene Ppia. The transparency of the filled-in neighborhoods indicates the number of colocalized LR pairs found in the neighborhood, with more opaque shading corresponding to a higher number of colocalized pairs. For visual clarity, neighborhoods are shown to an outdegree of 1, but analyses were performed on neighborhoods with an outdegree of 2. (F) Patterns of LR colocalization dynamics at CLS neighborhoods. The majority of LRs increased in colocalization at CLSs from ND to 8w and 8w to 14w (orange-orange block). The second most frequent pattern observed was LRs that increased in colocalization at CLSs from ND to 8w, then decreased from 8w to 14w (orange-light blue block).

5.4 Discussion

Inflammation is a prominent feature of obese adipose tissue that contributes to development of insulin resistance. Here, investigation of immune cell signatures in their spatial context highlights the tissue-wide scale of innate and myeloid cell population expansion. While scRNA-seq data improved inference of cell identity, exploring sequencing-based spatial transcriptomic platforms with higher resolution will be valuable [68].

A positive cooperative relationship is consistent with monocyte infiltration and differentiation into macrophages in obesity, and is further supported by the increase in monocyte contribution to tissue function shown in Figure 5.3D. Our spatial patterning analysis showed increased heterogeneity for all immune cell types. This finding is consistent with adoption of additional polarization states or phenotypes in immune cells in obesity, for example in Cd9-enriched macrophages in obesity [138, 154, 287]. Emerging subtypes in other immune cells were less identifiable, possibly due to lower potential for different functional states in those cell types. Known shifts in subtypes include increased Cd8+ T effector and Cd4+ T H1 cells and decreased regulatory T cells in obesity [279, 261, 260].

Additionally, while increased IgG+ B cells have been observed in obese murine visceral adipose tissue [382], the only differentially expressed gene in our B cell sub-clustering was the immunoglobulin gene, Ighg3, which was enriched in the high-fat diet settings (log2FC of 1).

Ligands and receptors expressed in innate cells were preferentially colocalized across the obese tissue compared to ligands and receptors expressed in adaptive cells or a combination of innate and adaptive cells. These interactions were predominantly from monocytes and ATMs, consistent with their increased quantity in obesity. Our adaptation of Turing’s system for within-cell and between-cell dynamics similarly showed an increasing contribution of monocytes and ATMs to tissue function in obesity, alongside a decreasing contribution of adaptive immune cells, NK cells,
Finally, differential and ligand-receptor expression at mapped crown-like structures revealed a shift toward monocyte-driven signaling to lipid-associated ATMs in short-term high-fat diet feeding, prior to architectural formation of crown-like structures. These data support a model in which monocytes, recruited from circulation, both differentiate into lipid-associated macrophages and participate in cell-cell signaling via specific obesity-activated ligands. Notably, this pattern was present after 8w of HFD feeding, which precedes observable CLS architecture.

Future work will stain crown-like structure biomarkers to confirm their presence, though colocalization of CLS-forming markers in addition to properties such as increased gene signatures associated with tumor-infiltrating cell types provide initial confidence towards their emergence in our samples. Additionally, while we cannot say with certainty that the cell type interactions reported account for the observed ligand-receptor activity at these tissue landmarks, or rule out the involvement of adipocytes and other non-immune cell types, our data capture the dominant immune cell types participating in colocalized ligand-receptor expression during HFD feeding. High numbers of CLSs have been linked to more severe clinical outcomes of obesity and associated disorders like cancers [232, 152, 53, 172]. Further assessment and validation of the trends we observed in this study could help uncover mechanisms of CLS initiation and therapeutic targets in early obesity.

This study provides proof-of-concept for predicting the trajectory of tissue states in disease. Our model of disrupted tissue morphogenesis in disease could be generalized to other tissues to define a disease index that distinguishes healthy from disrupted tissue states predictive of metabolic dysfunction or other diseases. Relevant to obesity are liver and intestinal tissue, which have defined tissue changes in gene expression in obesity [54, 88, 87]. Integrated analysis of spatial and single-cell transcriptomics in these tissues could allow us to establish a consistent inter-tissue index in obesity.

Some obesity-related changes in adipose tissue immune cells persist even in weight loss [396], highlighting the need to better understand mechanisms that promote adipose tissue dysfunction. These mechanisms could also tie obesity to the risk for other conditions, including cardiovascular disease and cancer [48, 112], with one study finding that metabolically activated macrophages in mammary adipose tissue promotes triple-negative breast cancer [346]. Future studies using time course spatial transcriptomics could be particularly powerful in understanding tissue dynamics and the evolution of cell-cell communication in adipose tissue.
Figure 5.6: Mechanism of Crown-Like Structure Emergence. As obesity progresses, we observe increasing co-localization and signaling between monocytes and macrophage, supporting a mechanism for CLS formation in which monocytes, recruited from circulation, both differentiate into lipid-associated macrophage and cooperate with existing lipid associated macrophage through ligand-receptor signaling. Remarkably, this pattern is present in 8w tissue, preceding the formation of these structures. As macrophage accumulate at formed CLSs in 14w tissue, monocytes persist in their communication with macrophage.
5.5 Materials and Methods

5.5.1 Animals

C57BL/6J mice were used for these experiments (Jackson Laboratories 000664). Male mice were fed ad libitum a control normal chow diet (ND; 13.4% fat, 5L0D LabDiet) or high-fat diet (HFD; 60% calories from fat, Research Diets D12492) for the indicated amount of time starting at 9 weeks old. Animals were housed in a specific pathogen-free facility with a 12 h light/12 h dark cycle and given free access to food and water except for withdrawal of food for temporary fasting associated with glucose tolerance tests. All mouse procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan (Animal Welfare Assurance Number D16-00072 (A3114-01), #PRO00008583), and care was taken to minimize suffering adhering to the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals.

5.5.2 Glucose Tolerance Tests

For glucose tolerance tests (GTT), starting four hours into the light cycle, mice were fasted with ad libitum access to water for six hours in clean cages. A 100 mg/mL D-glucose (Sigma G7021) solution was prepared in sterile --/- DPBS and injected at 0.7 g/kg of body weight. Area under the curve (AUC) calculations were performed using the log trapezoidal method.

5.5.3 Stromal Cell Isolation

Stromal vascular cells (SVCs) were collected from adipose tissues as in [231]. After cardiac perfusion, adipose tissues were collected, minced finely to 3-5 mm pieces, and added to ice cold HBSS+Ca/Mg. Up to 1.5 g of tissue per sample was digested in 10 ml of 1 mg/mL collagenase II (Sigma C68850) in HBSS+Ca/Mg at 37°C for 45 minutes with vigorous shaking. Digests were filtered through buffer-soaked 100 micron cell strainers and centrifuged at 300 x g at 4C to pellet SVCs.

5.5.4 Immune Cell Enrichment and Single-Cell RNA-Sequencing

SVCs were enriched for CD45+ immune cells using Biolegend MojoSort Mouse CD45 Nanobeads (Biolegend 480027), following the manufacturer’s protocol. Briefly, SVC pellets were resuspended in 1 mL MojoSort Buffer, pooling the four samples from each cohort into a single respective cohort tube (ND, 8w, 14w), then filtered through a 70 micron cell strainer and placed in 5 mL polypropylene tubes. After addition of nanobeads, samples were sequentially processed for
magnetic separation. To increase purity, three magnetic separations in total were performed on the labeled fractions. Final cell suspensions were filtered through 40 micron pipette tip filters. Cell viability was >80% with <15% aggregation.

5.5.5 Spatial Transcriptomics Tissue Preparation

Within 30 minutes of cardiac perfusion, adipose tissues were pre-soaked in ice cold O.C.T. Compound (VWR 25608-930) and placed in biopsy cryomolds (VWR 25608-922) with fresh O.C.T., rapidly frozen by immersion in liquid nitrogen-cooled isopentane, and kept on dry ice or at -80°C until sectioning. Fresh tissue sections were cut at 10 µm after 20 minute equilibration in a cryochamber set to -26°C or below with specimen arm at -40°C. Samples were placed on a 10X Genomics/Visium Spatial Gene Expression slide and processed by the University of Michigan Advanced Genomics Core according to the manufacturer’s protocol.

5.5.6 Single-Cell RNA-Sequencing Data Processing

Raw single-cell RNA-sequencing data were processed using the 10X Genomics CellRanger (version 4.0.0) pipeline and resulting feature-barcode matrices were loaded into MATLAB for further processing using an in-house pipeline. Single cells (barcodes) with fewer than 500 expressed genes were filtered from the data matrix leaving 1,231 cells in the ND setting, 6,011 cells in the HFD8 setting, and 6,258 cells in the HFD14 setting. Genes (features) were filtered out according to the same criteria used to filter genes in the spatial transcriptomics data. The median number of genes expressed per cell for each setting was: 1,707, 1,644, and 1583, respectively. Additionally, filtered matrices were normalized in the same way as the spatial transcriptomics data matrices.

5.5.7 Spatial Transcriptomics Data Processing

Raw sequencing data were processed using the 10X Genomics SpaceRanger (version 1.0.0) pipeline with mouse reference GRCm38, and resulting feature-barcode matrices were loaded into MATLAB for further processing using an in-house pipeline. Matrices contain absolute transcript counts, reported as unique molecular identifier (UMI) counts, pertaining to each feature (rows) and tissue-associated barcode (columns). Informed by the 10X Genomics Loupe Browser visualization of barcoded tissue spots overlaying tissue histology images, we filtered out barcoded spots that overlapped visible holes in the tissue and therefore had no tissue material for gene expression to be detected at. This resulted in no barcoded spots being discarded from the ND setting, 1,458 being discarded from the HFD8 setting, and 490 from the HFD14 setting. After filtering, there were 2,035 , 1,936, and 1,504 viable spots remaining in each setting, respectively. Genes that
were detected at no barcoded tissue spots (indicated by a row sum of 0) were filtered from the feature-barcode matrices. Additionally, we filtered out mitochondrial and ribosomal genes as well as genes known to be linked to mapping errors (Malat, Lars2, Kcnq10t1, and Gm42418) [361]. Our working features list contained 19,941 total genes. The median number of expressed genes per tissue spot for each setting was: 91, 105, and 173, respectively. Finally, we normalized each feature-barcode matrix by dividing by a scaling factor - computed as the median UMI counts per barcode divided by the total UMI counts per barcode - then log-transforming the matrix.

5.5.8 Integration of Spatial and Single-Cell Transcriptomics Data

The cellular composition of each tissue section was characterized by mapping expression profiles from scRNA-seq data to their paired spatial landscape. To achieve this, we first aggregated our scRNA-seq data matrices, mean-centered and dimensionally reduced the resulting matrix to the first 20 singular vectors, then performed spectral clustering using Euclidean distance as the metric for generating the similarity graph. Informed by known cell types found in adipose tissue from literature, we selected for 15 clusters (k = 15). We then employed a data-guided cell type identification approach where we scored markers for each cluster based on ubiquity (percent of cells in the cluster expressing each gene), cluster-averaged expression (average expression of each gene among all cells in the cluster), and uniqueness (see 'Marker Uniqueness' subsection). For each cluster, the top 50 genes with the highest cumulative score that were also expressed across all three dietary settings and expressed in the spatial transcriptomics data, were selected as cluster-specific signatures. We used prior literature and the CellMarker database [403] to query these signatures and call cell types for each cluster. We further narrowed our set of clusters by removing those indicative of fibroblast contamination, removing those of unknown lineage whose signatures contained non-essential genes, and merging clusters with redundant cell type classifications, leaving us with six total clusters. We validated our cell type annotations using Immgen and found that they aligned with Immgen signatures [134]. To annotate our spatial transcriptomics tissue sections, we mapped cell types identified from the scRNA-seq data to tissue spots, based on the proportion of signature genes expressed at each spot (see 'Spot Deconvolution' subsection).

5.5.8.1 Marker Uniqueness

To evaluate the uniqueness of potential cell type markers to a cluster, we found the ratio of the total number of clusters overexpressing a given gene (fold change ≥ 2) to the number of times that gene is overexpressed by the cluster of interest. The resulting value increases a marker’s score proportional to how frequently we see the gene expressed highly in one cluster compared to all other clusters, but offsets the score if the gene is highly expressed in multiple clusters. Ultimately,
non-unique genes rank low with this approach. We found that this approach is similar to the Term Frequency-Inverse Document Frequency (TF-IDF) technique commonly applied in machine learning contexts to determine the importance of a word within a document or set of documents [297]. For proof-of-concept, we adapted the TF-IDF technique for marker ranking by treating documents as clusters and words as genes, where term frequency corresponded to the number of times a given gene is overexpressed by the cluster of interest and inverse document frequency to the number of clusters overexpressing the gene. In doing so, we yielded similar results (data not shown).

5.5.8.2 Spot Deconvolution

Given the thickness of tissue sections and diameter of tissue spots, it is likely that two or more cell identities will colocalize at the same tissue spot. To deconvolute each spot into their possible cellular components, we start by defining a dominant tissue layer wherein a cell type is assigned to a spot if at least five of the cell type-specific signature genes are expressed at the spot and a higher proportion of its signature is expressed at the spot than any other cell type. We define subsequent tissue layers by assigning remaining cell types to each tissue spot following the same criteria as long as the cell type has not already been assigned to the spot in a previous layer. Additionally, we impose a tiebreak condition for when two or more cell types express the same number of genes at a spot. When this happens, the tied cell types are assigned according to highest to lowest cumulative expression of their expressed signature genes. With this approach, each cell type has an equal likelihood of being assigned to a layer, giving us a total number of layers equivalent to the number of cell types identified in the tissue.

5.5.9 Modeling Tissue Function

While our spatial transcriptomics data is not single-cell resolution, we are able to assign a dominant cell type to each tissue spot based on the expression of cell type-specific signatures. We can then treat each tissue spot as an individual cell in our model of emerging tissue function. While we also described non-dominant layers of cell type assignments at each spot, we focus our model on the dominant cell type assignment. Our model draws on two subnetworks of the overall tissue network - the cell-cell network and within-cell genome network. Taken together, we can represent each cell type’s individual contribution to tissue function and define an index describing the tissue’s state in terms of cellular and genome connectivity.
5.5.9.1 Cellular Connectivity

Spatial transcriptomics captures the expression profiles of cells tissue-wide in Euclidean space, allowing us to observe patterns of between-cell connectivity across the tissue. We start by characterizing *intra*-cell type relationships within individual cell populations across the tissue (emergence of tissue function) by finding the transcriptome-wide correlation between tissue spots assigned to a given cell type. We then weight each pairwise correlation coefficient by the Euclidean distance between their corresponding tissue spots, generating an adjacency matrix. We next perform eigenvector decomposition on the normalized Laplacian of the adjacency matrix to obtain the Fiedler number, representing the connectivity of the network of transcriptionally similar cells across the tissue – cell connectivity. The cell type-specific Fiedler number is then divided by the number of tissue spots assigned to the cell type to normalize for the variability in network sizes across the tissue.

To capture *inter*-cell type contributions to tissue function (emergence of *multicellular* tissue function) we consider a network of networks where each cell type is a subnetwork within the larger tissue network. We repeat our previous steps, this time generating an adjacency matrix based on the correlation in expression between tissue spots from two cell types instead of one. We do this for every pair of cell types represented in our tissue samples to demonstrate how cell type coupling contributes to tissue function.

5.5.9.2 Genome Connectivity

From single-cell RNA-seq, we compare the diversity in gene expression profiles within a given cell type to capture within-cell genome connectivity across the tissue. Similar to our computation for cellular connectivity, we find the transcriptome-wide correlation between single cells classified as a given cell type. We then perform eigenvector decomposition on the normalized Laplacian of this correlation matrix to obtain the Fiedler number which we use to define genome connectivity. Again, we normalize the Fiedler number, this time dividing by the number of single cells assigned to the cell type.

5.5.10 Ligand-Receptor Analysis

Known ligand-receptor pairs in mice were taken from a compendium of pairs reported throughout the literature compiled by the Lewis Lab at UCSD. [10]. For each ligand-receptor pair and timepoint, we scanned the tissue space to identify tissue spots where both the ligand and receptor of the pair colocalize. We define colocalization as the spatial overlap or co-expression of the ligand and receptor (UMI count > 0) belonging to a known pair at the same x-,y- coordinate (tissue spot) in the spatial transcriptomics data. Paired ligands and receptors that colocalized at at least one
tissue spot were further queried for cell-type specific expression at the single-cell level, where the independent expression of the ligand and receptor in adaptive and innate immunity cells was evaluated. The proportions of single cells annotated as: (1) adaptive immunity cell types that expressed the ligand, (2) adaptive immunity cell types expressing the receptor, (3) innate immunity cell types expressing the ligand, and (4) innate immunity cell types expressing the receptor were captured in a 2-by-2 contingency table. The UMI count cutoff for ligand or receptor expression was 3 and pairs where the ligand and/or receptor were not expressed in more than 1% of cells in at least one cell type were discarded. Based on these proportions, we developed an interaction score between the immunity classes for each LR pair where we took the mean of the ligand immunity class proportion and a receptor immunity class proportion, allowing scores to represent the likelihood that the LR pair interacts between those two immune cell classes. We generated local state matrix to reflect these scores, where for a given ligand-receptor pair the intra-interaction between the ligand and receptor in adaptive cells (mean of proportions 1 and 2), the intra-interaction between the ligand and receptor in innate cells (mean of proportions 2 and 3), the inter-interaction between the ligand in adaptive and receptor in innate cells (mean of proportions 1 and 4), and the inter-interaction between the ligand in innate cells and receptor in adaptive cells (mean of proportions 3 and 2) were captured. For each LR pair, we could then determine which class had the highest score, which we interpret as the preferential immunity class pairing exhibiting that LR pair’s signaling. Finally, a global state matrix summarizing these intra- and inter-interactions among all colocalized ligand-receptor pairs was derived by taking the element-wise mean across the local state tables. Local and global state matrices were derived for individual cell types as well, as described in Algorithm 4.

5.5.11 Tissue Landmark Analysis

The tissue landmark of focus for this study was crown-like structures (CLSs). To identify tissue positions associated with this tissue niche, we queried each barcoded spot for expression of the macrophage marker \textit{Cd9} in addition to either \textit{Itgax} or \textit{Trem2}, two markers known to be highly expressed at CLS sites. This selection criteria was applied to our two HFD settings. CLSs are a hallmark of obesity and are not present in lean adipose tissue, so we screened tissue positions in the ND setting using only \textit{Cd9} for a baseline comparison. CLSs involve multiple cells (a ring of macrophages surrounding an adipocyte) and because adipocytes are expanded in size in obese tissue, activity related to these structures span multiple tissue spots. To capture all relevant signals at and around the CLS positions, we defined landmark neighborhoods, centered around each identified CLS that encompass tissue spots within two outdegrees from the center. We also defined control neighborhoods, centered around spots expressing hallmark gene \textit{Ppia}. Control neighborhoods that overlapped with CLS neighborhoods were removed from analysis. We then assessed
features at CLS neighborhoods and used control neighborhoods as a background distribution of tissue spots to gauge the significance of observed trends.

For neighborhood differential expression analysis, expression profiles at spots across all neighborhoods in the ND setting were first pooled together by taking the average expression across spots. Differential expression was performed between pooled expression in ND and each individual CLS neighborhood in the 14w setting, where average expression across spots within a neighborhood were used. The same analysis was performed for ND and 14w control neighborhoods. Genes were assessed for how many CLS neighborhoods they were significantly differentially expressed in ($|\log_2\text{FC}| \geq 1.5, p < 0.01$). Overlaps with DEGs in control neighborhoods were filtered.

We evaluated ligand-receptor (LR) signaling at CLS neighborhoods by evaluating the proportion of neighborhood spots colocalized by an LR pair. Only LR pairs found to be colocalized in all three settings were used in this analysis (132 pairs). We evaluated the same proportion for control neighborhoods and compared the average proportion across both sets of neighborhoods for each setting. We also determined how the colocalization of each LR pair at CLS neighborhoods changed over time, relative to the control neighborhoods, grouping LRs based on their pattern of change from ND to 8w and 8w to 14w. For example, LR pairs whose colocalization at CLS neighborhoods (percent of neighborhoods where the LR pair was co-expressed in at least one spot) consistently increased over time were grouped together while those whose colocalization increased from ND to 8w then decreased from 8w to 14w were grouped together. Within each group, LR pairs were then ranked in descending order according to their cumulative change over time and those whose rate of colocalization at CLS neighborhoods were not at least 10% different than at control neighborhoods were further disregarded. Individual LR pairs across groups were further evaluated based on observed trends for biological relevance.
CHAPTER 6

Deciphering Multi-way Interactions in the Human Genome

This chapter is based on a paper by Gabrielle A. Dotson, Stephen Lindsly, Can Chen, Anthony Cicalo, Sam Dilworth, Charles Ryan, Sivakumar Jeyarajan, Walter Meixner, Nicholas Beckloff, Amit Surana, Max Wicha, Lindsey A. Muir, and Indika Rajapakse [99] (under review).

6.1 Abstract

Chromatin architecture, a key regulator of gene expression, can be inferred using chromatin contact data from genome-wide chromosome conformation capture, or Hi-C. However, classical Hi-C does not preserve multi-way contacts. Here, we use long sequencing reads to map genome-wide multi-way contacts and investigate higher order chromatin organization in the human genome. We use hypergraph theory for data representation and analysis, and quantify higher order structures in neonatal fibroblasts, biopsied adult fibroblasts, and B lymphocytes. By integrating multi-way contacts with chromatin accessibility, gene expression, and transcription factor binding, we introduce a data-driven method to identify cell type-specific transcription clusters. We provide transcription factor-mediated functional building blocks for cell identity that serve as a global signature for cell types.

6.2 Introduction

Structural features of the genome are integral to the regulation of gene expression and corresponding generation of cellular phenotypes [190, 220, 61]. Aspects of genome structure have been inferred by studying genomic regions that are in close physical proximity. Chromosome conformation capture (3C)-based methods capture these interactions (contacts) through chemical fixation,
digestion of DNA, and proximity ligation, followed by sequencing of ligated DNA to identify genomic regions that are in contact. A variety of cell types have now been characterized using Hi-C, a genome-wide 3C-based method, adding substantially to our understanding of genome architecture. However, limitations on read length during sequencing lead to over-representation of simple interactions, predominantly pairwise. Identification of more complex, higher-order interactions can help us build a more complete set of principles of genome architecture.

Multi-way contacts have been identified using targeted 3C-based methods [12, 243, 249], through inference from pairwise contacts [196], and on occasion using classical Hi-C [83]. Ligation-free approaches, such as GAM and SPRITE, have recently enabled large scale capture of multi-way interactions [22, 263, 405], though comparisons of different methods find under- and over-representation of higher order contacts in the absence of proximity ligation [93, 161].

A recent extension of Hi-C preserves multi-way interactions and uses sequencing of long reads (e.g. Pore-C) [93] to unambiguously identify sets of contacts among multiple loci. Multi-contact 4C sequencing (MC-4C) also uses long-read sequencing to capture contact complexity [5], however, it was designed to capture local topology for individual genes and regulatory regions and does not generate multi-way contacts genomewide. While direct capture of multi-way contacts can clarify higher order structures in the genome, new frameworks are needed to address unique analysis and representation challenges posed by the multi-way data.

To address this gap, we generated Pore-C data from neonatal and biopsied adult fibroblasts and collected publicly available Pore-C data for B lymphocytes [93] and constructed hypergraphs to represent the multidimensional relationships of multi-way contacts among loci. Hypergraphs are similar to graphs, but hypergraphs contain hyperedges instead of edges. Hyperedges can connect any number of nodes at once, while edges can only connect two nodes [26, 60, 58]. Prior work on neural networks highlights the utility of hypergraph representation learning to denoise and analyze existing multi-way contact data and to predict de novo multi-way contacts [401]. Here, we use incidence matrix-based representation and analysis of multi-way chromatin structure directly captured by Pore-C data, which is mathematically simple and computationally efficient, and yet can provide new insights into genome architecture.

In our hypergraph framework, nodes are genomic loci and hyperedges are multi-way contacts among loci. In our incidence matrices, rows are genomic loci and columns are individual hyperedges. This representation enabled quantitative measurements of chromatin architecture through hypergraph entropy and the comparison of different cell types through hypergraph similarity measures. In addition, we integrated Pore-C with other data modalities to discover biologically relevant multi-way interactions, which we term transcription clusters. The cell-type specific transcription clusters we identified support a role in maintaining cell identity, consistent with prior work on transcriptional hubs or factories [247, 248, 303, 408, 321]. Furthermore, the formation of transcription
clusters in the nucleus is consistent with small world phenomena in networked systems [375, 36].

We use the following definitions. **Entropy**: a measure of structural order in the genome. **Hyperedge**: an extension of edges where each hyperedge can contain any number of nodes (multi-way contact). **Hypergraph**: an extension of graphs containing multiple hyperedges. **Hypergraph motifs**: an extension of network motifs that describe connectivity patterns of 3-way, 4-way, . . . , n-way hyperedges. **Incidence matrix**: a representation for hypergraphs where rows are nodes and columns are hyperedges. **Transcription cluster**: a group of genomic loci that colocalize for efficient gene transcription.

### 6.3 Results

#### 6.3.1 Capturing Multi-way Contacts

We conducted Pore-C experiments using adult human dermal fibroblasts obtained from a skin biopsy and neonatal human dermal fibroblasts, and obtained additional publicly available Pore-C data from B lymphocytes [93]. The experimental protocol for Pore-C is similar to Hi-C, including cross-linking, restriction digestion, and ligation of adjacent ends followed by sequencing (Figure 6.1A). Alignment of Pore-C long reads to the genome enables fragment identification and classification of multi-way contacts (Figure 6.1B).

Hypergraphs represent multi-way contacts, where individual hyperedges contain at least two loci (Figure 6.1C, left). Hypergraphs provide a simple and concise way to depict multi-way contacts and allow for abstract representations of genome structure. Computationally, we represent multi-way contacts as incidence matrices (Figure 6.1C, right). For Hi-C data, adjacency matrices are useful for assembly of pairwise genomic contacts. However, since rows and columns represent individual loci, adjacency matrices cannot be used for multi-way contacts in Pore-C data. In contrast, incidence matrices permit more than two loci per contact and provide a clear visualization of multi-way contacts. Multi-way contacts can also be decomposed into pairwise contacts, similar to those in Hi-C, by extracting all pairwise combinations of loci (Figure 6.1D).
Figure 6.1: Pore-C Experimental and Data Workflow. (A) The Pore-C experimental protocol, which captures pairwise and multi-way contacts (Materials and Methods). (B) Representation of multi-way contacts at different resolutions (top). Incidence matrix visualizations of a representative example from Chromosome 8 in adult human fibroblasts at each resolution (bottom). The numbers in the left columns represent the location of each genomic locus present in a multi-way contact, where values are either the chromosome base-pair position (read-level) or the bin into which the locus was placed (binning at 100 kb, 1 Mb, or 25 Mb). (C) Hypergraph representation of Pore-C contacts (left) and an incidence matrix (right) of four multi-way contacts within (yellow-to-yellow) and between (yellow-to-purple) chromosomes. Contacts correspond to examples from A. The numbers in the left column represent genomic bins in which a locus resides. Each vertical line represents a multi-way contact, with nodes at participating genomic loci. (D) Multi-way contacts can be decomposed into pairwise contacts. Decomposed multi-way contacts can be represented using graphs (left) or incidence matrices (middle), which when decomposed are interchangeable with traditional Hi-C contact matrices (right). Contacts correspond to examples from A and C. (E) Flowchart overview of the computational framework. Descriptions of file type formats (red text) are in Table S1 in Dotson et al. [99].
6.3.2 Decomposing Multi-way Contacts

From our Pore-C experiments using adult human dermal fibroblasts, neonatal human dermal fibroblasts, and additional publicly available Pore-C data from B lymphocytes, we constructed hypergraphs at multiple resolutions (read level, 100 kb, 1 Mb, and 25 Mb) [93]. We first analyzed individual chromosomes at 100 kb resolution by decomposing multi-way contacts into their pairwise contacts. Decomposing Pore-C data into pairwise contacts provides more information than Hi-C, as each Pore-C read can contain many pairwise contacts [93]. It also allows us to identify topologically associated domains (TADs) using established methods (Materials and Methods) [94, 274, 63]. We demonstrate identification of TAD boundaries from decomposed multi-way contacts and show intra- and inter-TAD relationships using multi-way contacts (Figures 6.2, S1 in Dotson et al. [99]). The loci that frequently participate in these multi-way contacts give rise to the block-like pattern of chromatin interactions often seen in Hi-C data.

6.3.3 Chromosomes as Hypergraphs

To gain a better understanding of genome structure with multi-way contacts, we constructed hypergraphs for entire chromosomes at 1 Mb resolution. We show an incidence matrix of Chromosome 22 as an example in Figure 6.3A, and in Figure 6.3B, we visualize the distribution of 1 Mb contacts at multiple orders (2-way contacts, 3-way contacts, etc.) on Chromosomes 22. Figure 6.3C highlights the most common intra-chromosomal multi-way contacts on Chromosome 22 using multi-way contact “motifs”, which we use as a simplified way to show hyperedges. Figure 6.3D shows how multi-way contacts at lower resolutions (25 Mb, 1 Mb) are composed of many multi-way contacts at higher resolutions (100 kb, read level), and Figure 6.3E visualizes the multi-way contacts contained in Figure 6.3D as a hypergraph.

We also identified multi-way contacts that contain loci from multiple chromosomes. These inter-chromosomal multi-way contacts can be seen at 1 Mb resolution in Figure 6.3F and in 25 Mb resolution for both adult fibroblasts and B lymphocytes in Figure 6.4A and 6.4B, respectively. Figure 6.4 gives a summary of the entire genome’s multi-way contacts, by showing the most common intra- and inter-chromosomal multi-way contacts across all chromosomes. We highlight examples of multi-way contacts with loci that are contained within a single chromosome (“intra only”), spread across unique chromosomes (“inter only”), and a mix of both within and between chromosomes (“intra and inter”). We note that many of the “inter only” contacts observed in Figure 6.4 may also have intra-chromosomal contacts when viewed at a higher resolution, similar to Figure 6.3D. Finally, we found the most common inter-chromosomal multi-way contacts across all chromosomes, which we summarize with five example chromosomes in Figure 6.5 using multi-way contact motifs. These multi-way contacts between distant genomic loci may offer insights.
Figure 6.2: Local Organization of the Genome. (A) Incidence matrix visualization of a region in Chromosome 22 from adult fibroblasts (V1-V4). The numbers in the left column represent genomic loci at 100 kb resolution, vertical lines represent multi-way contacts, where nodes indicate the corresponding locus’ participation in this contact. The blue and yellow regions represent two TADs, T1 and T2. The six contacts, denoted by the labels i-vi, are used as examples to show intra- and inter-TAD contacts in B, C, and D. (B) Hyperedge and read-level visualizations of the multi-way contacts i-vi from the incidence matrix in A. Blue and yellow shaded areas (bottom) indicate which TAD each locus corresponds to.
Figure 6.2: (C) A hypergraph is constructed using the hyperedges from B (multi-way contacts i-vi from A). The hypergraph is decomposed into its pairwise contacts in order to be represented as a graph. (D) Contact frequency matrices were constructed by separating all multi-way contacts within this region of Chromosome 22 into their pairwise combinations. TADs were computed from the pairwise contacts using the methods from [63]. Example multi-way contacts i-vi are superimposed onto the contact frequency matrices. Multi-way contacts in this figure were determined at 100 kb resolution after noise reduction, originally derived from read-level multi-way contacts (Materials and Methods).

into the higher-order structural patterning of the genome and its relationship with transcriptional regulation.

Figure 6.3: Patterning of Intra- and Inter-chromosomal Contacts. (A) Incidence matrix visualization of Chromosome 22 in adult fibroblasts. The numbers in the left column represent genomic loci at 1 Mb resolution. Each vertical line represents a multi-way contact, in which the nodes indicate the corresponding locus’ participation in this contact. (B) Frequencies of Pore-C contacts in Chromosome 22. Bars are colored according to the order of contact. Blue, green, orange, and red correspond to 2-way, 3-way, 4-way, and 5-way contacts. (C) The most common 2-way, 3-way, 4-way, and 5-way intra-chromosome contacts within Chromosome 22 are represented as motifs, color-coded similarly to B. (D) Zoomed in incidence matrix visualization in 100 kb resolution shows the multi-way contacts between three 1 Mb loci: L19 (blue), L21 (yellow), and L22 (red). An example 100 kb resolution multi-way contact is zoomed to read-level resolution. (E) Hypergraph representation of the 100 kb multi-way contacts from D. Blue, yellow, and red labels correspond to loci L19, L21, and L22, respectively.
Figure 6.3: (F) Incidence matrix visualization of the inter-chromosomal multi-way contacts between Chromosome 20 (orange) and Chromosome 22 (green) in 1 Mb resolution. Within this figure, all data are from one adult fibroblast sequencing run (V2) and multi-way contacts were determined after noise reduction at 1 Mb or 100 kb resolution accordingly (Materials and Methods).

Figure 6.4: Genome-wide Patterning of Multi-way Contacts. Incidence matrix visualization of the top 10 most common multi-way contacts per chromosome. Matrices are constructed at 25 Mb resolution for both adult fibroblasts (top, V1-V4) and B lymphocytes (bottom). Specifically, 5 intra-chromosomal and 5 inter-chromosomal multi-way contacts were identified for each chromosome with no repeated contacts. If 5 unique intra-chromosomal multi-way contacts are not possible in a chromosome, they are supplemented with additional inter-chromosomal contacts. Vertical lines represent multi-way contacts, nodes indicate the corresponding locus’ participation in a multi-way contact, and color-coded rows delineate chromosomes. Highlighted boxes indicate example intra-chromosomal contacts (red), inter-chromosomal contacts (magenta), and combinations of intra- and inter-chromosomal contacts (blue). Examples for each type of contact are shown in the top right corner.
Figure 6.4: Multi-way contacts of specific regions are compared between cell types by connecting highlighted boxes with black dashed lines, emphasizing similarities and differences between adult fibroblasts and B lymphocytes. Normalized degree of loci participating in the top 10 most common multi-way contacts for each chromosome in adult fibroblast and B lymphocytes are shown on the left. Red dashed lines indicate the mean degree for adult fibroblasts and B lymphocytes (top and bottom, respectively). Genomic loci that do not participate in the top 10 most common multi-way contacts for adult fibroblasts or B lymphocytes were removed from their respective incidence matrices and degree plots. Multi-way contacts were determined at 25 Mb resolution after noise reduction (Materials and Methods).

6.3.4 Transcription Clusters

We use the following definitions: **Transcription cluster**: a group of genomic loci that colocalize for efficient gene transcription. **Master regulator**: a self-regulating transcription factor that binds its gene analog. **Specialized transcription cluster**: a transcription cluster where at least one master regulator binds. **Self-sustaining transcription cluster**: a transcription cluster where a TF binds and its gene analog is expressed.

Genes are transcribed in short sporadic bursts in areas with high concentrations of transcriptional machinery [335, 73, 74], including transcriptionally engaged polymerase and accumulated transcription factors (TFs). Colocalization of multiple genomic loci in these areas may facilitate more efficient transcription, which is supported by studies using fluorescence in situ hybridization (FISH) that show colocalization during active transcription [247]. Simulations also provide evidence that genomic loci that are bound by common transcription factors can self-assemble into clusters, forming structural patterns commonly observed in Hi-C data [74]. We refer to these instances of highly concentrated areas of transcriptional machinery and genomic loci as transcription clusters. The colocalization of multiple genomic loci naturally leads to multi-way contacts, but these interactions cannot be fully captured from the pairwise contacts of Hi-C. Multi-way contacts derived from Pore-C reads can detect interactions between many genomic loci, and are well suited for identifying potential transcription clusters (Figure 6.6).
Figure 6.5: Inter-chromosomal Interactions. The most common 2-way, 3-way, 4-way, and 5-way inter-chromosome combinations for each chromosome are represented using motifs from adult fibroblasts (top), neonatal fibroblasts (center), and B lymphocytes (bottom). Rows represent the combinations of 2-way, 3-way, 4-way, and 5-way inter-chromosomal interactions, and columns are the chromosomes. Inter-chromosomal combinations are determined using 25 Mb resolution multi-way contacts after noise reduction (*Materials and Methods*) and are normalized by chromosome length. Here we only consider unique chromosome instances (i.e. multiple loci in a single chromosome are ignored).
Figure 6.6: Data-driven Identification of Transcription Clusters. (A) Blue shaded area: A 5 kb region before and after each locus in a Pore-C read (region between red dashed lines) is queried for chromatin accessibility and RNA Pol II binding (ATAC-seq and ChIP-seq, respectively). Multi-way contacts between accessible loci that have $\geq 1$ instance of RNA Pol II binding are indicative of potential transcription clusters. Gray shaded area: Gene expression (RNA-seq, $E_1$ for gene 1 and $E_2$ for gene 2, respectively) and transcription factor binding sites (TF$\_1$ and TF$\_2$) are integrated to determine potential coexpression and coregulation within multi-way contacts with multiple genes. Transcription factor binding sites are queried $\pm 5$ kb from the gene’s transcription start site (Materials and Methods). Genes are colored based on the overlapping Pore-C locus, and the extended horizontal line from each gene represents the 5 kb flanking region used to query transcription factor binding sites. (B) Pipeline for extracting transcription clusters (Supplementary Materials). (C) Schematic representation of a transcription cluster.

As actively transcribed regions are accessible and transcription is carried out by locally-bound RNA Pol II [169], we require all loci in a candidate transcription cluster to be accessible, evidenced by ATAC-seq, and at least one of those loci to contain an RNA Pol II ChIP-seq signature. Using these criteria, we identified 12,364, 16,080, and 16,527 potential transcription clusters from neonatal fibroblasts, adult fibroblasts, and B lymphocytes, respectively (Table 6.1, Materials and Methods). The majority of these clusters involved at least one expressed gene (94.2% in neonatal fibroblasts, 95.0% in adult fibroblasts, 90.5% in B lymphocytes) as well as at least two expressed...
 genes (69.6% in neonatal fibroblasts, 71.9% in adult fibroblasts, 58.7% in B lymphocytes). While investigating the colocalization of expressed genes in transcription clusters, we found that over half of clusters containing multiple expressed genes had common transcription factors based on binding motifs in fibroblasts (61.9% in neonatal fibroblasts, 65.2% in adult fibroblasts) and that over half of these common transcription factors were master regulators (55.9% in neonatal fibroblasts, 63.4% in adult fibroblasts). These proportions were slightly lower in B lymphocytes where we observed that 50.0% of clusters containing multiple expressed genes had common transcription factors while 46.8% of these common transcription factors were master regulators. Example transcription clusters derived from 3-way, 4-way, and 5-way contacts in fibroblasts and B lymphocytes are shown in Figure 6.7. Transcription clusters contained at least two expressed genes with at least one common transcription factor binding motif.
Figure 6.7: Example Transcription Clusters. Six examples of transcription clusters are shown for neonatal fibroblasts (left), adult fibroblasts (center), and B lymphocytes (right) as multi-way contacts (hypergraph motifs). Black labels indicate genes and chromosomes (bold). Red labels correspond to transcription factors shared between the majority of genes within the transcription cluster. For three-way contacts (green motifs), we highlight the transcription clusters’ biological analog (blue-shaded box), showing how fragments of chromatin fold and congregate at a common transcription cluster (grey sphere). Each node (black dot) of the hyperedge and its denoted chromosome and gene in the hypergraph motif corresponds to a single chromatin fragment, colored according to chromosome, in the biological analog. Thus, a three-way hyperedge is depicted by three chromatin fragments in close spatial proximity. Multi-way contacts used for adult and neonatal fibroblasts include all experiments (V1-V4). Examples were selected from the subset of multi-way contacts summarized in the “Clusters with Common TFs” column of Table 6.1.

We tested the criteria for potential transcription clusters for statistical significance (Materials and Methods). That is, we tested whether the identified transcription clusters are more likely to include genes, and if these genes were more likely to share common transcription factors, than random multi-way contacts. We found that the identified transcription clusters were significantly more likely to include $\geq 1$ gene and $\geq 2$ genes than random multi-way contacts ($p < 0.01$). In addition, transcription clusters containing $\geq 2$ genes were significantly more likely to have transcription factors and master regulators in common ($p < 0.01$). After testing all orders of multi-way transcription clusters together, we also tested the 3-way, 4-way, 5-way, and 6-way (or more) cases individually. We found that all cases were statistically significant ($p < 0.01$) except for clusters
with common transcription factors or master regulators in the 6-way (or more) case for both fibroblasts and B lymphocytes. We hypothesize that these cases were not statistically significant due to the large number of loci, naturally leading to an increased overlap with genes. This increases the likelihood that at least two genes will have common transcription factors or master regulators. Over half of the transcription clusters where the majority of genes contained common transcription factors also contained at least one enhancer in adult fibroblasts (98.2%) and B lymphocytes (87.9%), further suggesting regulatory function within these multi-way contacts [49, 318] (Table S2 in Dotson et al. [99]). In contrast, only 11.6% of transcription clusters in neonatal fibroblasts exhibited the same properties, which may be a factor of the significantly sparser enhancer annotation available for this cell type compared to the others from the EnhancerAtlas 2.0 database.

To understand how our transcription clusters aligned with factors that have known involvement in chromatin architecture and transcriptional regulation, we evaluated CCCTC-binding factor (CTCF) for binding using ChIP-seq data. CTCF specifically mediates chromatin looping and TAD boundary insulation [257, 246] and binds generously throughout the genome [274]. We found significantly higher CTCF binding in our identified transcription clusters compared to multi-way contacts that were not classified as transcription clusters (Figure S3 in Dotson et al. [99]). In adult and neonatal fibroblasts, CTCF binding was nearly two-fold greater in transcription clusters compared to randomly selected multi-way contacts (80% vs. 45% and 81% vs. 47%). In B lymphocytes, CTCF binding was present at 82% of transcription clusters compared to 59% of random multi-way contacts. We additionally investigated cohesin for its colocalization with CTCF and involvement in regulation of chromatin architecture, such as chromatin loop extrusion [379, 252, 4, 129]. In particular, the cohesin subunits RAD21 and SMC3 have been previously linked to CTCF-mediated transcriptional regulation [274]. ChIP-seq data showed preferential binding of RAD21 and SMC3 at transcription clusters compared to random multi-way contacts in adult fibroblasts (79% vs. 42% for RAD21, 71% vs. 37% for SMC3, p < 0.01) and B lymphocytes (76% vs. 55% for RAD21, 79% vs. 53% for SMC3, p < 0.01) (Figure S3 in Dotson et al. [99]). Together these data suggest that the identified transcription clusters are important sites of transcriptional regulation, and support a model in which CTCF and cohesin actively mediate multi-way interactions.

We next sought to determine which TFs might be involved in cell type-specific regulation in transcription clusters. For each cell type, we ranked expressed TFs by frequency of binding sites across transcription clusters. Among TFs with the most frequent binding sites, 39% were shared across all three cell types, compared to 72% between adult and neonatal fibroblasts (Table S6 in Dotson et al. [99]). Fibroblast and B lymphocyte TF binding sites had less overlap, at 52% (adult) and 45% (neonatal), than binding sites between fibroblasts, supporting cell type-specific regulation of transcription cluster subsets. Of 18 TFs whose binding sites were unique to the transcription clusters of neonatal fibroblasts, the most frequently occurring was RARB, found at

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10.2% of clusters, while in adult fibroblasts, binding sites for ZNF667 were the most frequent among 14 TFs at 6.7% of transcription clusters. In B lymphocytes, binding sites for TFEC were the most frequent among 161 TFs at 7.6% of transcription clusters. Prior studies support the cell type-specific roles of these uniquely-binding TFs in fibroblasts and B lymphocytes [351, 292].

Given the role of TFs in coordinating transcription among clusters of genes [303, 400], we hypothesized that TF loci might feature in a subset of transcription clusters. To investigate this question, we looked for the binding motif and encoding gene locus for a given TF within the same transcription cluster, defining this class as a self-sustaining transcription cluster (Figure 6.8A-B). We identified nine, eight, and thirteen self-sustaining transcription clusters in adult fibroblasts, neonatal fibroblasts, and B lymphocytes, respectively (Table S7 in Dotson et al. [99]). In adult fibroblasts, we observed that the binding motif for FOXO3, a master regulator, exists at a 4-way transcription cluster expressing the FOXO3 gene. The neonatal fibroblast and B lymphocyte datasets had a self-sustaining transcription cluster in common where STAT3 had a binding motif and the STAT3 gene was expressed. While self-sustaining transcription clusters demonstrate the capacity for a TF to regulate itself, not every TF co-occupying a transcription cluster with its gene analog is classified as a master regulator (Table S7 in Dotson et al. [99]). Therefore, we further stratify these clusters into self-sustaining transcription clusters where the TF is a master regulator and thus binds its gene analog (stronger coupling) and self-sustaining transcription clusters where the TF binds in the cluster but not at its gene analog (weaker coupling). We propose that these strongly-coupled self-sustaining transcription clusters are ‘core’ transcription clusters that serve as transcriptional signatures for a cell type. It also follows that strongly-coupled self-sustaining transcription clusters are specialized transcription clusters (Figure S4 in Dotson et al. [99]). We then considered two classes of analog-independent transcription clusters - where either a TF and its gene analog occupy different clusters (Figure 6.8C) or a TF occupies a cluster, but its gene analog occupies no cluster (Figure 6.8D). Since both the TF and gene analog belong to transcription clusters in Figure 6.8C, they are coupled, though lesser so than either class of self-sustaining transcription clusters. In contrast, Figure 6.8D represents an architecturally uncoupled state - 23.3%, 25.7%, and 40.1% of TF gene analogs in adult fibroblasts, neonatal fibroblasts, and B lymphocytes, respectively, were not expressed in any transcription cluster. Lastly, we binned all multi-way contact loci involved in self-sustaining transcription clusters at 100 kb resolution and plotted the interaction frequencies of their decomposed pairwise components (Figure 6.8E).
Figure 6.8: Classes of Transcription Clusters. In a self-sustaining transcription cluster, a TF and the gene encoding that TF are both present. The inter- and intra-chromosomal examples in (A) and (B), respectively, illustrate this phenomenon where in (A) we see the TF of interest (orange triangle) circulating at the cluster, its binding motif present on the chromatin (orange portion), and its corresponding gene expressed (orange rectangle on Chromosome 6). The gray shapes represent additional TFs with binding motifs (gray portion of chromatin) at the cluster. Black rectangles on Chromosomes 3, 9, and 19 represent additional genes present in the cluster. (C) An analog-independent class of transcription clusters where we observe a TF (red square) bind at a transcription cluster (red cluster) and its corresponding gene expressed in a separate transcription cluster (grey cluster), yet not in the same cluster. (D) An analog-independent class of transcription clusters where we observe a TF (green circle) bind at a transcription cluster (green cluster) and its corresponding gene expressed but not within a transcription cluster. (E) Genome-wide cell type-specific self-sustaining transcription clusters extracted from multi-way contact data and decomposed into Hi-C contact matrices at 100 kb resolution. Contact frequencies are log-transformed for better visualization. Frequencies along the diagonal indicate interaction between two or more unique multi-way loci that fall within the same 100 kb bin. Axis labels are non-contiguous 100 kb bin coordinates in chromosomal order. Multi-way contacts that make up the self-sustaining transcription clusters are superimposed. Multi-way contacts with green-colored loci represent ‘core’ transcription clusters - transcription clusters containing a master regulator and its gene analog. An example read-level contact map for the inter-chromosomal FOXO3 self-sustaining transcription cluster is denoted by the orange highlighted box in the adult fibroblast contact matrix and a read-level contact map for the intra-chromosomal ZNF320 self-sustaining transcription cluster is denoted by the blue highlighted box. Values along the left axis of these read-level contact matrices are base-pair positions of the contacting loci in the genome.
<table>
<thead>
<tr>
<th>Order</th>
<th>Multi-way Contacts</th>
<th>Transcription Clusters</th>
<th>Clusters with $\geq 1$ Gene</th>
<th>Clusters with $\geq 2$ Genes</th>
<th>Clusters with Common TFs</th>
<th>Clusters with Common MRs</th>
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Table 6.1: Summary of Multi-way Contacts and Transcription Clusters. Multi-way contacts from B lymphocytes (white rows), neonatal fibroblasts (light gray rows), and adult fibroblasts (dark gray rows, V1-V4) are listed after different filtering criteria. Multi-way contacts are considered to be potential transcription clusters if all loci within the multi-way contact are accessible and at least one locus binds RNA Pol II. These multi-way contacts are then queried for nearby expressed genes. If a transcription cluster candidate has at least two expressed genes, we determine whether the majority of these genes have common transcription factors (TFs) through binding motifs. If only two expressed genes are contained within a transcription cluster candidate, we require both genes to have common TFs. From the set of transcription clusters with common TFs, we calculate how many clusters have at least one common master regulator (MR) (Algorithm 3 in Dotson et al. [99]).
Algorithm 1: Multi-way Contact Analysis

1: **Input:** Aligned Pore-C data (A), RNA-seq (R: gene expression), RNA Pol II (P: ChIP-seq), ATAC-seq (C: chromatin accessibility), transcription factor binding motifs (B)
2: **for** each set of Pore-C data \( A_i \in A \) **do**
3: Construct incidence matrix \( H_l \) using Algorithm 2 in Dotson *et al.* [99]
4: Identify transcription clusters \( T_{lp}, T_{lc}, \) and \( T_{ls} \) using Algorithm 3 in Dotson *et al.* [99]
5: Calculate entropy \( S_l \) using Algorithm 4 in Dotson *et al.* [99]
6: **end for**
7: Compute hypergraph distance \( d_{ij} \) between pairs \( H_i \) and \( H_j \) with \( p \geq 1 \) using Algorithm 5 in Dotson *et al.* [99]
8: Calculate the statistical significance \( \alpha_{ij} \) for hypergraph distance \( d_{ij} \) using the permutation test in Algorithm 6 in Dotson *et al.* [99].
9: **Return:** Hypergraph incidence matrices \( H_l \in \mathbb{R}^{n \times m} \), hypergraph entropy \( S_l \), potential transcription clusters \( T_{lp} \), transcription clusters \( T_{lc} \), specialized transcription clusters \( T_{ls} \), and hypergraph distance matrix \( [d_{ij}] \) with statistical significance \( \alpha_{ij} \).

6.4 Discussion

In this work, we introduce a hypergraph framework to study higher-order genome organization from Pore-C long-read sequence data. We demonstrate that higher-order genome architecture can be precisely represented and analyzed using hypergraph theory. Using direct capture of multi-way contacts, we identified transcription clusters with physical proximity and coordinated gene expression. Our framework thus enables study of explicit structure-function relationships that are observed directly from data, without needing to infer multi-way contacts. In engineering and social systems, hypergraph representation of data has revealed higher-order organization principles efficiently [26, 60, 58, 334]. Our work here extends the application of hypergraphs, demonstrating a natural way to represent and analyze genome organization across scales.

Exploring long-range, inter-chromosomal interactions genome-wide offers the opportunity to establish fundamental principles of genome organization. Unbiased capture and study of multi-way contacts can help identify biologically important assemblies that affect transcription, such as transcription clusters [74, 267]. This approach can also connect genome organization principles to the study of transcription factors and how they govern cell type-specific network architecture, which resembles small world phenomena [36, 327]. Our results support the idea of cell type-specific formation of transcription clusters that serve as a basis for efficient navigation of information within the nucleus, and thereby reflect a signature of small world architecture. Analogous to the behavior
of short-path information propagation in social networks, we posit that transcription clusters act as decentralized nodes, or critical architectures relevant to cell identity. Future work to explore these phenomena systematically will undoubtedly help us understand cell type-specific organization principles. Another exciting direction will be to investigate time series multi-way interactions during cellular transitions such as differentiation and cell reprogramming, with single cell observations. Furthermore, we imagine that multi-way chromatin structure together with spatial transcriptomics will guide us to uncover formation principles in tissue patterning and organogenesis [273, 125].

6.5 Materials and Methods

6.5.1 Cell Culture

Primary human adult dermal fibroblasts were obtained from a donor and were maintained in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1X Glutamax (Thermo Fisher Scientific Cat no. 35050061) and 1X nonessential amino acid (Thermo Fisher Scientific Cat no. 11140050).

6.5.2 Cross-linking

Protocols for cross-linking were based on Ulahannan et al. [93]. 2.5 million cells were washed three times in chilled 1X phosphate buffered saline (PBS) in a 50 centrifuge tube, pelleted by centrifugation at 500 x g for 5 min at 4 between each wash. Cells were resuspended in 10 room temperature 1X PBS 1% formaldehyde (Fisher Scientific Cat no. BP531-500) by gently pipetting with a wide bore tip, then incubated at room temperature for 10 min. To quench the cross-linking reaction 527 of 2.5 M glycine was added to achieve a final concentration of 1% w/v or 125 mM in 10.5. Cells were incubated for 5 min at room temperature followed by 10 min on ice. The cross-linked cells were pelleted by centrifugation at 500 x g for 5 min at 4.

6.5.3 Restriction Enzyme Digest

The cell pellet was resuspended in 500 of cold permeabilization buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.2% IGEPAL CA-630, 100 of protease inhibitor cock-tail Roche Cat no. 11836170001) and placed on ice for 15 min. One tablet of protease inhibitor cocktail was dissolved in 1 ml nuclease free water and 100 from that was added to a 500 permeabilization buffer. Cells were centrifuged at 500 x g for 10 min at 4 after which the supernatant was aspirated and
replaced with 200 of chilled 1.5X New England Biolabs (NEB) cutsmart buffer. Cells were cen-
trifuged again at 500 x g for 10 min at 4, then aspirated and re-suspended in 300 of chilled 1.5X
NEB cutsmart buffer. To denature the chromatin, 33.5 of 1% w/v sodium dodecyl sulfate (SDS,
Invitrogen Cat no. 15553-035) was added to the cell suspension and incubated for exactly 10 min
at 65 with gentle agitation then placed on ice immediately afterwards. To quench the SDS, 37.5 of
10% v/v Triton X-100 (Sigma Aldrich Cat no. T8787-250) was added for a final concentration of
1%, followed by incubation for 10 min on ice. Permeabilized cells were then digested with a final
concentration of 1 U/ of NlaIII (NEB-R0125L) and brought to volume with nuclease-free water
to achieve a final 1X digestion reaction buffer in 450. Cells were then mixed by gentle inversion.
Cell suspensions were incubated in a thermomixer at 37 for 18 hours with periodic rotation.

6.5.4 Proximity Ligation and Reverse Cross-linking

NlaIII restriction digestion was heat inactivated at 65 for 20 min. Proximity ligation was set up
at room temperature with the addition of the following reagents: 100 of 10X T4 DNA ligase buffer
(NEB), 10 of 10 mg/mL BSA and 50 of T4 Ligase (NEB M0202L) in a total volume of 1000 with
nuclease-free water. The ligation was cooled to 16 and incubated for 6 hours with gentle rotation.

6.5.5 Protein Degradation and DNA Purification

To reverse cross-link, proximity ligated sample was treated with 100 Proteinase K (NEB
P8107S-800U/ml), 100 10% SDS (Invitrogen Cat no. 15553-035) and 500 20% v/v Tween-20
(Sigma Aldrich Cat no. P1379) in a total volume of 2000 with nuclease-free water. The mixture
was incubated in a thermal block at 56 for 18 hours. In order to purify DNA, the sample was trans-
ferred to a 15 centrifuge tube, rinsing the original tube with a further 200 of nuclease-free water to
collect any residual sample, bringing the total sample volume to 2.2. DNA was then purified from
the sample using a standard phenol chloroform extraction and ethanol precipitation.

6.5.6 Nanopore Sequencing

Purified DNA was Solid Phase Reversible Immobilization (SPRI) size selected before library
preparation with a bead ratio of 0.48X for fragments > 1.5 kb. The > 1.5 kb products were pre-
pared for sequencing using the protocol provided by Oxford Nanopore Technologies. In brief, 1
of genomic DNA input was used to generate a sequencing library according to the protocol pro-
vided for the SQK-LSK109 kit (Oxford Nanopore Technologies, Oxford Science Park, UK, version
GDE.9063_v109_revU_14Aug2019). After the DNA repair, end prep, and adapter ligation steps,
SPRI select bead suspension (Cat No. B23318, Beckman Coulter Life Sciences, Indianapolis, IN,
USA) was used to remove short fragments and free adapters. A bead ratio of 1X was used for DNA repair and end prep while a bead ratio of 0.4X was used for the adapter ligation step. Qubit dsDNA assay (ThermoFisher Scientific, Waltham, MA, USA) was used to quantify DNA and ∼300-400 ng of DNA library was loaded onto a GridION flow cell (version R9, Flo-MIN 106D). For adult fibroblasts, 4 sequencing runs were conducted generating a total of 6.25 million reads (referred to as V1-V4). For neonatal fibroblasts, 4 sequencing runs were conducted generating a total of 11.85 million reads.

6.5.7 Sequence Processing.

Reads which passed Q-score filtering (--min_qscore 7, 4.56 million reads) after base calling on the Oxford Nanopore GridION (Guppy, version 4.0.11) were used as input for the Pore-C-Snakemake pipeline (https://github.com/nanoporetech/Pore-C-Snakemake, commit 6b2f762). The pipeline maps multi-way contacts to a reference genome and stores the hyperedge data in a variety of formats. The reference genome used for mapping was GRCh38.p13 (https://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.39/). Each of the four sequencing runs were assigned a sequencing run label and then concatenated. The combined pipeline outputs were used as standard inputs for all downstream analysis.

6.5.8 Hypergraphs

A hypergraph is a generalization of a graph. Hypergraphs are composed of hyperedges, which can join any number of nodes [27]. Mathematically, a hypergraph is a pair such that \( G = \{ V, E \} \) where \( V \) is the node set and \( E \) is the hyperedge set. Each hyperedge in \( E \) is a subset of \( V \). Examples of hypergraphs include email communication networks, co-authorship networks, film actor/actress networks, and protein-protein interaction networks. For genomic networks, traditional graph-based methods fail to capture contacts that contain more than two genomic loci once, which results in a loss of higher order structural information. Hypergraphs can capture higher order connectivity patterns and represent multidimensional relationships unambiguously in genomic networks [26, 383]. In hypergraphs obtained from Pore-C data, we defined nodes as genomic loci at a particular resolution (e.g. read level, 100 kb, 1 Mb, or 25 Mb bins), and hyperedges as contacts among genomic loci. We switch between these different resolutions both for computational efficiency and visual clarity. Most higher order contacts are unique in Pore-C data at high resolution (read level or 100 kb), so for these data we considered unweighted hypergraphs (i.e. ignore the frequency of contacts). For lower resolutions (1 Mb or 25 Mb), we considered edge weights (frequency of contacts) to find the most common intra- and inter-chromosomal contacts.
6.5.9 Hypergraph Filtering

We performed an additional filtering step while constructing genomic hypergraphs. We first decomposed each multi-way contact into its pairwise combinations at a particular resolution. From these pairwise contacts, we counted the number of times a contact was detected for each pair of loci, and identified the highest frequency locus pairs. Pairwise contacts were kept if they occurred above a certain threshold number, which was set empirically at the 85th percentile of the most frequently occurring locus pairs. For example, in fibroblast data binned at 1 Mb resolution, a locus pair with six detected contacts corresponded to the 85th percentile. Thus all pairs of loci with fewer than six detected contacts were not considered, which increases confidence in the validity of identified multi-way contacts.

6.5.10 Incidence Matrices

An incidence matrix of the genomic hypergraph was an $n$-by-$m$ matrix containing values zero and one. The row size $n$ was the total number of genomic loci, and the column size $m$ was the total number of unique Pore-C contacts (including self-contacts, pairwise contacts, and higher order contacts). Nonzero elements in a column of the incidence matrix indicate genomic loci contained in the corresponding Pore-C contact. Thus, the number of nonzero elements (or column sum) gives the order of the Pore-C contact. The incidence matrix of the genomic hypergraph can be visualized via PAOHvis [359]. In PAOHvis, genomic loci are parallel horizontal bars, while Pore-C contacts are vertical lines that connect multiple loci (see Figures 6.1, 6.2, 6.3, and 6.4). Beyond visualization, incidence matrices play a significant role in the mathematical analysis of hypergraphs.

6.5.11 Data-driven Identification of Transcription Clusters

We used Pore-C data in conjunction with multiple other data sources to identify potential transcription clusters (Figure 6.6). Each locus in a Pore-C read, or multi-way contact, was queried for chromatin accessibility and RNA Pol II binding (ATAC-seq and ChIP-seq peaks, respectively). Multi-way contacts were considered to be potential transcription clusters if all loci within the multi-way contact were accessible and at least one locus had binding of RNA Pol II. The loci in potential transcription clusters were then queried for nearby expressed genes. A 5 kb flanking region was added upstream and downstream of each locus when querying for chromatin accessibility, RNA Pol II binding, and nearby genes [239]. Gene expression (RNA-seq) and transcription factor binding site data were then integrated to determine coexpression and coregulation of genes in potential transcription clusters. If a potential transcription cluster candidate had at least two genes
present, and these genes had common transcription factors based on binding motifs in the cluster, the potential transcription cluster was determined to be a real transcription cluster. From the set of transcription clusters with common transcription factors, we calculated how many clusters were regulated by at least one master regulator, a transcription factor that also regulates its own gene, and classified these as specialized transcription clusters (Figure 6.6).

6.5.12 Transcription Factor Binding Motifs

Transcription factor binding site motifs were obtained from “The Human Transcription Factors” database [177]. FIMO (https://meme-suite.org/meme/tools/fimo) was used to scan for motifs within ± 5 kb of genes’ transcription start sites. The results were converted to a 22,083 × 1,007 MATLAB table, where rows were genes, columns were transcription factors, and entries were the number of binding sites for a particular transcription factor and gene. The table was then filtered to only include entries with three or more binding sites in downstream computations. This threshold was determined empirically and is adjustable in the MATLAB code.

6.5.13 Identifying Self-Sustaining Transcription Clusters

From identified transcription clusters (Table 6.1), we obtained a subset containing TF-encoding genes specific to each cell type, yielding 79, 54, and 144 transcription clusters from the adult fibroblast, neonatal fibroblast, and B lymphocyte data, respectively. We then classified these clusters as self-sustaining if the TF binding motif corresponding to the expressed TF-encoding gene was also at the cluster. We further determined whether the self-sustaining TFs were master regulators based on protein-DNA interaction data. Results are summarized in Figure 6.8 and Table S7.

6.5.14 Public Data Sources

Pore-C data for B lymphocytes were downloaded from Ulahannan et al. [93]. ATAC-seq and ChIP-seq data were obtained from the Encyclopedia of DNA Elements (ENCODE) to assess chromatin accessibility and RNA Pol II binding, respectively. These data were compared to read-level Pore-C contacts to determine whether colocalizing loci belong to accessible regions of chromatin and had RNA Pol II binding for both fibroblasts and B lymphocytes. RNA-seq data were also obtained from ENCODE to ensure that genes within potential transcription clusters were expressed in their respective cell types. Additionally, ChIP-seq data for CTCF, RAD21, and SMC3 binding were obtained from ENCODE to evaluate binding preference at transcription clusters. A summary of these data sources can be found in Table 6.2.
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<th>Cell Type</th>
<th>Data Description and Source</th>
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<td>Human adult primary dermal fibroblasts were derived from a donor skin biopsy</td>
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<tr>
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<td>BJ</td>
<td>Human foreskin fibroblasts from BJ cell line (Cat no. CRL-2522, ATCC, Manassas, VA)</td>
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<td>B lymphocyte Pore-C data obtained from Ulahannan et al. [93]</td>
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<tr>
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<td>Foreskin fibroblast primary gene expression data on BJ cell line</td>
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<td>GM12878</td>
<td>B lymphocyte enhancer location data from EnhancerAtlas 2.0 [115]</td>
</tr>
</tbody>
</table>

Table 6.2: Data Sources. Data obtained from ENCODE unless otherwise specified [71].

### 6.5.15 Hypergraph Entropy

Network entropy is often used to measure the connectivity and regularity of a network [58, 254, 218]. We defined a new notion of hypergraph entropy to quantify the organization of chromatin structure from Pore-C data. Denote the incidence matrix of the genomic hypergraph as \( H \). The Laplacian matrix is then an \( n \)-by-\( n \) matrix (\( n \) is the total number of genomic loci in the hypergraph), which can be computed by

\[
L = D - HE^{-1}H^T \in \mathbb{R}^{n \times n},
\]

where \( D \in \mathbb{R}^{n \times n} \) is a diagonal matrix containing the degrees of nodes along its diagonal, and \( E \in \mathbb{R}^{n \times m} \) is a diagonal matrix containing the orders of hyperedges along its diagonal. Inspired by von Neumann graph entropy (which utilizes the distribution of the eigenvalues from the graph
Laplacian matrix), we define the hypergraph entropy as

$$\text{Hypergraph Entropy} = - \sum_{i=1}^{n} \bar{\lambda}_i \ln \bar{\lambda}_i,$$

where $\bar{\lambda}_i$ are the normalized eigenvalues of $L$ such that $\sum_{i=1}^{n} \bar{\lambda}_i = 1$, and the convention $0 \ln 0 = 0$ is used. In mathematics, eigenvalues can quantitatively represent different features of a matrix [326]. Biologically, genomic regions with high entropy are likely associated with high proportions of euchromatin (i.e. less organized folding patterns), as euchromatin is more structurally permissive than heterochromatin [206, 268, 193].

We computed the entropy of intra-chromosomal genomic hypergraphs for both fibroblasts and B lymphocytes as shown in Table S8 in Dotson et al. [99]. It is expected that larger chromosomes have larger hypergraph entropy because more potential genomic interactions occur in the large chromosomes. However, there are still subtle differences between the fibroblast and B lymphocyte chromosomes, indicating differences in their genome structure. In order to better quantify the structural properties of chromosomes and compare between cell types, it may be useful to introduce normalizations to hypergraph entropy in the future.

### 6.5.16 Hypergraph Distance

Comparing graphs is a ubiquitous task in data analysis and machine learning [97, 97, 106, 381]. In order to quantify difference between two genomic hypergraphs $G_1$ and $G_2$ at different scales, we propose using several hypergraph distance or similarity measures. These measures are based on the conversion of a hypergraph into a graph representation, see [334] for details. Denote the incidence matrices of two genomic hypergraphs by $H_1 \in \mathbb{R}^{n \times m_1}$ and $H_2 \in \mathbb{R}^{n \times m_2}$, respectively. For $i = 1, 2$, construct the adjacency matrices $A_i$ and normalized Laplacian matrices $\tilde{L}_i$:

$$A_i = H_i E_i^{-1} H_i^\top, \quad \tilde{L}_i = I - D_i^{-\frac{1}{2}} H_i E_i^{-1} H_i^\top D_i^{-\frac{1}{2}} \in \mathbb{R}^{n \times n},$$

respectively, where $I \in \mathbb{R}^{n \times n}$ is the identity matrix, $E_i \in \mathbb{R}^{m_i \times m_i}$ is a diagonal matrix containing the orders of hyperedges along its diagonal, and $D_i \in \mathbb{R}^{n \times n}$ is a diagonal matrix containing the degrees of nodes along its diagonal [406]. The degree of a node is equal to the number of hyperedges that contain that node. Given these adjacency and normalized Laplacian matrices, we use following three distance measures in our application to determine differences in the two genomic hypergraphs at both local and global scales:

- **Hamming Distance:** measures local similarity and is based on absolute values of difference
between the two adjacency matrices, i.e.,

\[ D_H(G_1, G_2) = \frac{1}{n^2} \sum_{j=1}^{n} \sum_{k=1}^{n} |A_{1,jk} - A_{2,jk}|, \]

where, the notation \(A_{i,jk}\) implies \(jk\)-th entry of the matrix \(A_i\).

• **Spectral Distance:** measures global similarity and is based on the \(p\)-norm for difference between ordered set of eigenvalues of the two Laplacians, i.e.,

\[ D_\lambda(G_1, G_2) = \frac{1}{n} \left( \sum_{j=1}^{n} |\lambda_{1,j} - \lambda_{2,j}|^p \right)^{1/p}, \quad (6.4) \]

where \(\lambda_{i,j}\) is the \(j\)th eigenvalue of \(\tilde{L}_i\) for \(i = 1, 2\), and \(p \geq 1\). In our analysis, we choose \(p = 2\).

• **DeltaCon Distance:** measures both local and global similarity, and is based on the fast belief propagation method of measuring node affinities using the matrix [173],

\[ S_i = \left( I + \epsilon^2 D_i^\alpha - \epsilon A_i \right)^{-1}, \]

where, \(0 < \epsilon \ll 1\) is small constant capturing the influence between neighboring nodes, and \(D_i^\alpha\) is the \(n \times n\) diagonal matrix with the diagonal entries \(D_i^\alpha_{jj} = \sum_{k=1}^{n} A_{i,jk}\). DeltaCon then compares the two matrices \(S_1\) and \(S_2\) via the Matusita difference as the measure,

\[ D_\Delta(G_1, G_2) = \frac{1}{n^2} \left( \sum_{j=1}^{n} \sum_{k=1}^{n} \left( \frac{1}{2} S_{1,jk} - \frac{1}{2} S_{2,jk} \right)^2 \right)^{1/2}. \quad (6.5) \]

where we have added a normalization factor \(\frac{1}{n^2}\). In our analysis, we found results too insensitive to the choice of \(\epsilon\), and report the results for \(\epsilon = 10^{-3}\).

Further details on the properties of these different distances can be found in Supplemental Notes.

We computed hypergraph distance between genome-wide hypergraphs derived from adult fibroblasts, neonatal fibroblasts, and B lymphocytes using the Hamming, spectral, and DeltaCon distances described above and examined distances statistically through a permutation test. Figure S2A1-3 in Dotson et al. [99] demonstrates that the adult fibroblast and B lymphocyte hypergraphs are significantly different at the chromosome level, especially along Chromosome 21, in stark contrast to the distance between adult and neonatal fibroblasts. Additionally, we computed the same distance measures at the genome level, incorporating inter-chromosomal data, and found that the
genomic hypergraphs between fibroblasts and B lymphocytes were significantly different, with a p-value of 0 compared to an observed insignificant difference between adult and neonatal fibroblasts (p-value of 1) (Figure S2B in Dotson et al. [99]).

6.5.17 Statistical Significance via Permutation Test

In order to assess the statistical significance of the transcription cluster candidates we determined using our criteria (Figure 6.6), we used a permutation test which builds the shape of the null hypothesis (i.e. the random background distribution) by resampling the observed data over $N$ trials. We randomly selected $n$ 3rd, 4th, 5th, and 6th or more order multi-way contacts from our Pore-C data, where $n$ was based on the number of transcription cluster candidates we determined for each order. For example, we randomly selected $n = 11,261$ multi-way contacts from the set of 3rd order multi-way contacts in fibroblasts (Table 6.1). For each trial, we determined how many of these randomly sampled “transcription clusters” match our remaining criteria: transcription clusters with $\geq 1$ gene, $\geq 2$ genes, common TFs, and common MRs. The background distribution for each of the criteria was then constructed from these values. The proportion of values in the background distributions that was greater than their counterparts from the data-derived transcription cluster candidates yielded the $p$-value. This analysis was based on the assumption that transcription clusters will be more likely to contain genes and that those genes are more likely to have common transcription factors than random multi-way contacts. For this analysis, we chose $N = 1,000$ trials.

Similarly, we used a permutation test to determine the significance of the measured distances between two hypergraphs. Suppose that we are comparing two hypergraphs $G_1$ and $G_2$. We first randomly generate $N$ hypergraphs $\{R_i\}_{i=1}^N$ that are similar to $G_1$ (“similar” means similar number of node degree and hyperedge size distribution). The background distribution therefore can be constructed by measuring the hypergraph distances between $G_1$ and $R_i$ for $i = 1, 2, \ldots, N$. The proportion of distances that was greater than the distance between $G_1$ and $G_2$ in this background distribution yielded the p-value. For this analysis, we again chose $N = 1,000$ trials. See Supplemental Notes in Dotson et al. [99] for details.
CHAPTER 7

Concluding Remarks

Cellular reprogramming has seen exciting clinical success in the 21st century. Our growing understanding of cell identity has translated into age-related macular degeneration [397], Parkinson’s disease [20], and myocardial injury [201] treatment potential. The commercial success and scalability of cellular reprogramming solutions in recent years has even made direct-to-consumer cell therapies a tangible option. Persisting low conversion efficiencies and discrepancies between reprogrammed cells and their native counterparts, however, challenge us to consider what we still do not know about cell identity and lineage commitment decision-making. In Chapter 2, we touch on some of those challenges and cover computational approaches designed to predict how cells will respond to perturbation and better facilitate reprogramming success. We highlight how integration of different data modalities has enabled more creativity in predicting reprogramming regimes, from factoring in time of perturbation and implementing feedback control to adjust transcription factor input concentrations real-time, to designing hybrid schemes that both introduce and inhibit transcription factors in a system and more. Fortunately, the advent of next-generation sequencing nearly 20 years ago and the rapidly-growing development of new techniques like spatial transcriptomics and Pore-C that are more sensitive to the nuances of cellular and genome dynamics, allow us to better elucidate cell phenotype from the dynamical relationship between genome structure and function. With such informative data at our fingertips, our next step is to let this refined 4DN framework guide cellular reprogramming solutions into an era of higher efficiency rates and ultimately tissues biomanufactured from reprogrammed cells.

A uniform regime for transforming cancer cells into normal cells remains elusive in the field of cellular reprogramming due to robustness and clonal diversity. In Chapter 3, we highlight that cellular reprogramming need not be limited to inducing a new lineage’s transcriptional program, but that merely attenuating the transcriptional program of a cancer cell can open the door to several more feasible and effective outcomes like acquisition of a treatment-sensitive, senescent, or apoptotic state. We used 4DN analysis to demonstrate that silencing the transcription factor, TCF4, in colorectal cancer cells progressively disrupted pathways promoting colon cancer cell homeostasis, a perturbation sufficient for restoring the cells’ sensitivity to chemoradiotherapy. In this work,
we characterized specific changes in chromatin partitioning at key loci that were coordinated with functional changes and appeared to contribute to the observed treatment sensitization phenotype. Our application of network centrality analysis of the local genome architecture further recovered TF-encoding genes previously implicated in co-regulating tumor progression, demonstrating the utility of the 4DN framework in extracting clinically-relevant reprogramming factors.

Coordinated changes in genome structure and function have been described in several studies and architectural proteins have been shown to drive key lineage-commitment events in cells, yet genome structure continues to be an unappreciated target for cell therapy. In Chapter 4, we perform 4DN analysis on Hi-C and RNA-seq data collected in vivo from T cells at baseline and in two stimulated, proliferative states before and following knockout of the cohesin-related WAPL protein, necessary for proper chromatin looping. We found that the absence of WAPL correlated with universal defects to internal TAD structure that were coordinated with differential expression in relevant cell cycling genes. Importantly, the loss of WAPL was also accompanied by a reduction in GVHD severity, suggesting that disruption of key regulators of genome structure can invoke desirable clinical outcomes. Further investigation will need to be performed to ensure that such a perturbation would also drive changes in the transcriptional program that are compatible with the target state.

Current gold-standard functional analyses capture gene expression in cells from dissociated or homogenized tissue, removing molecular measurements from their spatially-aware context. In Chapter 5, we enter the emerging field of spatial transcriptomics to interrogate the morphing cellular and genomic landscape of adipose tissue during obesity progression. We combined spatial transcriptomics with single-cell RNA-seq in lean and diet-induced obese mice over time to reveal extensive disruption of innate-adaptive immune response. Dynamically changing ligand-receptor signaling at key tissue landmarks showcased a decisive shift from adaptive immune cell-driven interactions to innate immune-cell driven interactions, highlighting patterns of associated position-specific activity. These data allowed us to profile cross-tissue heterogeneity and represent a valuable extension to the 4DN framework. Moreover, knowledge of gene expression spatial constraints could better aid in selection of factors for reprogramming and inform placement of reprogrammed cells for tissue biomanufacturing. Advances in refining tissue spot resolution to the single-cell level will be needed to make more precise observations about the intricate cell-cell network in the tissue moving forward. Additionally, if chromosome conformation technologies rise to the challenge of preserving spatial context, the promise of spatially-resolved 4DN analysis could soon be on the horizon.

Since its introduction nearly two decades ago, chromosome conformation capture and its many derivatives like Hi-C have taken the genomics world by storm, revealing important regulatory relationships, characterizing hierarchical genome organization, and uncovering properties of key
architectural proteins. While informative, it has become clear in recent years that pairwise chromatin interactions only tell part of the genome organization story. Several new technologies have since emerged facilitating the capture or recovery of simultaneously-occurring chromatin interactions involving more than two loci in the vicinity of one another. In Chapter 6, we introduce a framework for processing and analyzing these interactions captured using Pore-C sequencing. The foundation of our proposed framework is hypergraph representation of multi-way contacts, allowing us to use sophisticated quantitative measurements to describe entropy and similarity among these complex structures. We further integrated Pore-C contacts with additional data modalities to define transcription clusters where transcription factors and functionally-related loci converge in three-dimensional space to undergo efficient transcription. This approach allows us to elucidate key regulatory relationships occurring at an inter-chromosomal scale that were previously undetected through pairwise conformation capture assays. Further, the cell type-specific multi-way interactions that we are able to classify have the potential to guide prediction of reprogramming factors that regulate the conformational properties of specific cell lineages. Adapting this framework to existing single-cell Pore-C data and further validating the existence of multi-way chromatin interactions using imaging remain critical next steps before clinical application of this framework.
Supplemental Materials

Supplementary materials, including figures, tables, and additional notes can be found at [39] for Chapter 3, [330] for Chapter 4, [100] and [99] for Chapter 6.

A.1 Chapter 5 Algorithms

**Algorithm 2: Clustering and Visualization**

<table>
<thead>
<tr>
<th>Input:</th>
<th>Data matrix $X_{m \times n} = (x_1, \ldots, x_n) \in \mathbb{R}^{m \times n}$ where $m$ rows are genes and $n$ columns are cells.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Output:</td>
<td>Cell clusters and a low dimensional projection</td>
</tr>
<tr>
<td>1</td>
<td>Compute the sample mean $\mu_n$ and the centered matrix $X_c = X - \mu_n 1^T$ where 1 is a vector of ones</td>
</tr>
<tr>
<td>2</td>
<td>Compute the SVD of $X_c = U \Sigma V^T$</td>
</tr>
<tr>
<td>3</td>
<td>Construct $P_{n \times r} = [v_1 \quad v_2 \quad \ldots \quad v_r]$ where each column in $P$ is a right singular vector of $X_c$. Here $r$ can be chosen using the optimal hard threshold [117] on $X_c$</td>
</tr>
<tr>
<td>4</td>
<td>Construct a similarity matrix $A_{n \times n}$ from $P$ by determining the distance between each row. The choice of distance measure depends on the data type and user preference. Examples include Gaussian similarity, Euclidean distance, Manhattan distance (city block distance), Kullbeck-Liebler divergence, and correlation</td>
</tr>
<tr>
<td>5</td>
<td>Perform clustering: spectral or modularity clustering on $A$ with $k$ clusters. $k$ can be chosen using domain knowledge or by testing multiple values of $k$ and evaluating the best performance. Note: $k$ may be $\leq r$</td>
</tr>
<tr>
<td>6</td>
<td>Visualization: t-SNE or UMAP to reduce the dimensions of $P$ and visualize data colored according to clusters</td>
</tr>
</tbody>
</table>
Algorithm 3: Cell Type Localization and Spot Deconvolution

**Input:** scRNA-seq feature-barcode matrices $\mathbf{F}(s) \in \mathbb{R}^{m \times n}$ where $m$ rows are genes and $n$ columns are cells; ST feature-barcode matrices $\mathbf{T}(s) \in \mathbb{R}^{m \times n}$ where $m$ rows are genes and $n$ columns are tissue spots

**Output:** Tissue spot cell type assignments

1. Perform spectral clustering on $\mathbf{F}(s)$ aggregated across all timepoints $s$
2. Rank $m$ genes for each cell type (cluster) $k$. Genes are ranked by ubiquity, cluster-averaged expression, and uniqueness (see Methods and Materials)
3. Construct cell type-specific signatures $\mathbf{S}(k) \in \mathbb{R}^{50 \times 1}$, containing highly ranked genes common across timepoints
4. Query expression of cell type-specific signature genes at tissue spots by finding $\mathbf{T}(s) \subset \mathbf{S}(k)$
5. Assign to each tissue spot the cell type expressing the most and at minimum 5 of its signature genes
6. Deconvolute tissue spots: repeat step 5 until an attempt to assign each cell type to every tissue spot has been made. Each successive assignment represents a deconvoluted tissue layer.

Algorithm 4: Ligand-Receptor Colocalization Dynamics

**Input:** Table of ligand-receptor pairs $\mathbf{L}$, from literature

**Output:** Global state matrix, $\mathbf{S} \in \mathbb{R}^{n \times n}$

1. **ST Data:** For each ligand-receptor pair $p$ in $\mathbf{L}$, assign colocalization score:

   $$c = \begin{cases} 
   1 & \text{if } l > 0 \text{ and } r > 0 \\
   0 & \text{otherwise}
   \end{cases}$$

   where $l$ and $r$ are UMI counts for the ligand and receptor in $p$, respectively, at a given tissue spot

2. **scRNA-seq Data:** If $p$ has a colocalization score of 1 at least once across the tissue, construct contingency table $\mathbf{C}_{2 \times n}$, where $C_{11}$ and $C_{21}$ are the proportions of single cells classified as 'cell type 1' with $l$ and $r > 3$, respectively, $C_{12}$ and $C_{22}$ are the proportions of single cells classified as 'cell type 2' with $l$ and $r > 3$, respectively, and so on up to 'cell type $n$' ($C_{1n}$ and $C_{2n}$)

3. Construct local state matrix for $\mathbf{S}(p) \in \mathbb{R}^{n \times n}$, by finding the mean between all pairwise combinations of ligand-specific ($C_{11}, C_{12}, \ldots, C_{1n}$) and receptor-specific ($C_{21}, C_{22}, \ldots, C_{2n}$) proportions in $\mathbf{C}$

4. After performing steps 1-3 for all $p$ in $\mathbf{L}$, define global state matrix, $\mathbf{S} \in \mathbb{R}^{n \times n}$, by computing the element-wise mean across all $\mathbf{S}(p) \in \mathbb{R}^{n \times n}$: $\sum_{p=1}^{m} \mathbf{S}(p)/m$, where $m$ is the number of ligand-receptor pairs, $p$


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