ABSTRACT

Quantitative analysis of cell fate decisions

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Organismal development depends upon countless cell decisions to adopt particular fates at the appropriate time and place. These decisions are executed by systems of biochemical reactions called regulatory networks. Elucidating the general principles underlying the structure and function of these networks is vital to understanding all developmental processes, as well as the diseases that arise when they fail.

Prior studies of regulatory networks, and the decisions they implement, have heavily relied upon qualitative analysis of experimental data. It has since become clear that quantitative strategies are needed to unravel the complexities of systems-level behavior. The research enclosed in this dissertation therefore combines chemical engineering, computer science, statistics, and experimental data to quantitatively explore how regulatory networks reliably coordinate cell fate decisions.

The findings are consolidated into three distinct chapters. The first two are anchored to a common model system of the *Drosophila* larval eye. They deploy an assortment of novel computational tools, mathematical models, and statistical methods to derive meaningful insight from experimental measurements of the processes that govern cell fate decisions during retinal patterning. The final chapter introduces a mathematical modeling framework

to lead the development of an exciting new hypothesis; auxiliary negative regulators enable development to proceed more quickly by mitigating erroneous cell fate decisions when cells are rapidly metabolizing.

Beyond their insights into the mechanics of cell fate decisions, these efforts have spawned several computational tools that may prove valuable to the broader community. All of these resources have been made freely available (see Appendix C), with the hope that their continued development will contribute toward a more quantitative future for developmental biology.

CHAPTER 1

Introduction

The natural world presents a stunning variety of multicellular organisms. We tend to distinguish them by their physiological traits. After all, we recognize penguins by their unusual stature, black and white fur, long beak, and impressive ability to wobble around on ice. These stereotyped features are a culmination of many complex cellular processes, collectively known as *development*.

Development begins with a single cell, with subsequent growth and division events driving progression toward adulthood. Cells acquire increasingly specific roles and functions as growth proceeds, ultimately giving rise to stereotyped adult morphologies. This process, known as lineage restriction, demands that each cell decides to pursue the correct fate at the appropriate time and place [1].

Cell fate decisions are remarkably robust, collectively yielding consistent phenotypes amidst the vast array of conditions encountered in natural environments. They are so reliable that we often take them for granted. After all, it is hard to imagine a scenario in which we might mistake another human for a penguin. However, they can and do make mistakes with dire consequences for human health [2–4]. Researchers therefore continue to study these complex processes in the hope that they might one day be able to control their behavior; either to exploit them in novel biotechnologies, or rectify the diseases that arise when they fail. Research efforts are predominantly motivated by two fundamental questions. First, how do cells make decisions? Second, how do they make them reliably?

This dissertation addresses subtle aspects of both questions by combining chemical engineering, computer vision, and statistics to extract meaningful insight from experimental data. The remaining sections of this chapter serve to prime the reader with the context needed to situate the presented findings within the broader literature.

1.1. Molecular origins of cell fate decisions

Early experiments in the common fruit fly demonstrated that developmental processes are encoded in the genome [5, 6]. Genetics were therefore believed to provide a predefined road map for a the journey from embryo to adulthood, inspiring researchers throughout the twentieth century to probe the roles of individual genes in coordinating adult phenotypes [7–9]. Most of their efforts embraced a common philosophy; break something and see what happens. In *Drosophila*, the genes themselves bare the legacy of this approach, as they are predominantly named after the phenotypes that emerge in their absence. Perturbing *eyeless* or *wingless* may now seem rudimentary, but these types of genetic perturbations were vital to the discovery of genetic components and architectures that dictate cell fate decisions in all organisms [9–11]. They revealed that some genes confer pleiotropic functions across several stages of development, while others are limited to a single context [10, 12, 13]. They also showed that some genes are only essential for proper development when others are absent, making them unnecessary under normal conditions [14–16]. Moreover, *Drosophila* continues to be a prominent model system for studying developmental processes today, owing to its conveniently short life cycle, wealth of prior knowledge, and deep library of available genetic machinery [17–19].

Among these tools, gene-specific reporters augment traditional genetic perturbations by providing localized readouts of transcript and protein abundance. Researchers can now break something and see what happens to specific components of the developmental program.

Reporters have proven particularly useful for monitoring the activities of transcription factors; proteins that bind the promoter region of other genes in order to modulate their expression. Multiple transcription factors may interact with each other, allowing for the assembly of gene regulatory networks (GRN) that integrate upstream signaling cues to elicit specific changes in gene expression [20, 21].

GRNs thus arm cells with a chemical mechanism to orchestrate cell fate decisions in space and time. A prominent example occurs during the during the earliest stages of *Drosophila* embryogenesis, where spatial morphogen gradients drive variegated expression of the Gap genes [22, 23]. The expressed proteins trigger subsequent developmental events in a concentration-dependent manner, inducing localized cascades of GRN activity that ultimately give rise to distinct morphological segments [24]. The embryonic landscape of Gap gene expression thereby defines a template for later stages of patterning. Developmental success is contingent upon GRNs tightly controlling the spatial precision of the template over time [25]. Thus, segment polarity definition exemplifies a broader role of GRNs; they confer positional information to inform downstream cell fate decisions [26].

The resolution of positional information is thought to be refined over time. This assertion is in part based on experimental studies of retinal patterning in the *Drosophila* eye, a setting with an enduring experimental legacy that continues to garner attention [11, 17]. It remains popular in part because the temporal history of cell fate decisions is visibly encoded in the eye imaginal disc. During the third larval instar, a wave of differentiation steadily progresses across a disordered pool of multipotent cells [27, 28]. Differentiating cells propagate this morphogenetic furrow (MF) by relaying extracellular cues downstream. Decades of experiments have steadily revealed how cells interpret the signals and commit to particular fates [29]. Fate-specific reporters have shown that R8 photoreceptor neurons are recruited from pools of multipotent cells at the leading edge of the furrow [30]. The first

cell to differentiate uses paracrine signaling to inhibit differentiation among its neighbors, resulting in a repeated pattern of regularly-spaced R8 cells that gives rise to the crystalline lattice structure of the adult eye [31]. The process dynamically transforms a disordered collection of cells into an ordered template for subsequent stages of patterning. In other words, it refines the spatial resolution of the developing eye field.

Experiments suggest cell decisions to commit to an R8 fate are non-deterministic. Differentiation is driven by the expression of Atonal, a transcription factor initially induced in all cells along the leading edge of the furrow [30, 32, 33]. Stochastic differences in cells reception of signaling cues yields variation in Atonal levels and, consequently, cells propensities to adopt an R8 fate [34, 35]. The eventual R8 cell therefore does not appear to be predetermined. Equivalent mechanisms have been shown to control many other cell fate decisions, including sensory bristle specification and the choice between epidermal and neural fates in the neuro-ectoderm of *Drosophila* [36, 37].

These examples expose another important feature of GRNs: their output is non-deterministic. Dual-reporter experiments in *E. coli* have elegantly shown that variation may be attributed to intrinsic thermal fluctuations at the molecular scale, as well as extrinsic variation in cell state [38]. Both types of noise have since been shown to manifest in the population-wide penetrance of complex organismal phenotypes [39–42]. Some developmental systems appear to leverage noise, using GRNs to amplify and reinforce stochastic fluctuations in order to limit signal responses to a randomly chosen subset of cells [34, 36, 37]. Indeed, Pel'aez et al. recently speculated that a similar mechanism may explain why subsequent R cell fate transitions coincide with rapid increases in transcription factor expression heterogeneity [43].

These experiments and others like them form our contemporary systems-level view of development, in which complex networks of regulatory interactions guide cells toward the

appropriate fates by refining their stochastic behavior over time. However, it is unknown precisely how cell fates are resolved from the dynamic activities of GRNs. It is also unclear how cells integrate stochastic inputs to execute reliable cell fate decisions. These ambiguities persist despite the wealth of experimental data generated throughout the past century. New approaches are therefore needed to unravel the complexities of GRNs and their execution of cell fate decisions.

1.2. The power of quantitative analysis

Experimental perturbations continue to supply some of the most potent tools in the arsenal available to researchers, but it has become clear that quantitative and systematic analysis frameworks are needed to tease apart the complex interactions that govern cell fate decisions [44, 45]. Moreover, attempts to either rectify faulty decision mechanisms or engineer new ones, such as for cancer treatment or the generation of induced pluripotent stem cells, demand predictive models backed by quantitative data [4].

The focus has therefore gradually shifted from break something and *see* what happens to break something and *measure* what happens. The transition is supported by simultaneous advances in the resolution with which we can quantify GRN activity during development. Early approaches assayed the aggregate transcript or protein content of entire tissues. Strategies have since diverged in three separate directions, each of which sharpens the focus to single-cell resolution while prioritizing a different dimension of measurement. First, high-throughput transcriptomics strategies emphasize the breadth of genes surveyed. Second, flow cytometric approaches stress measurement precision. In both cases, cells identities may be crudely inferred from lineage-specific barcodes or biomarkers [46], but the precise spatial identify of each cell is discarded. In contrast, methods based on quantitative microscopy

prioritize spatial resolution because cells are measured in their native context. These techniques generally entail imaging gene-specific fluorescent reporters before using software to detect individual cells and quantify their fluorescence levels. They are particularly well-suited to probing the intricacies of GRNs subject to signaling from adjacent cells, and have consequently become a preferred option for studying tissue-scale patterning. In terms of microscopy, the state of the art has steadily progressed from static ex vivo imaging of fixed tissues to in situ recording of single-cell expression dynamics [47]. Computational methods to support quantitative image analysis have enjoyed similar progress [48]. Combined, these advances allow researchers to collect the data needed to conduct rigorous analyses and make testable predictions [49]. That is, they promote quantification.

Quantification has completely redefined the precision of GRN analysis. The enhanced resolution has inspired exciting new research into the specific mechanisms of information transmission and processing that underlie cell fate decisions in a broad variety of model systems [43, 50–52]. These pursuits have also emboldened researchers to pause and reconsider canonical wisdom. Petkova et al. recently combined quantitative dynamic measurements and information theory to show that gap gene expression provides sufficient spatial context to uniquely define the positions of all cells [52]. The authors argued that downstream GRNs likely decode these signals with near-optimal efficiency, implying that many cell fates could, at least in principle, be determined during the earliest stages of *Drosophila* embryogenesis. The findings imply that the resolution of positional information does not necessarily need to be refined as development proceeds. Similarly, cell fates need not be spatially resolved over time, prompting closer scrutiny of the extent to which cell fate decisions during later stages of development are truly non-deterministic. In causing the field to revisit these tenets of established dogma, the study exemplifies the power of quantification to produce novel insight.

Despite its revelatory potential, biologists have not universally embraced quantification, instead continuing to rely on visual analysis of imaging data. Resistance may partially come down to the scarcity of computational proficiency in experimental labs. Collaborations help alleviate this bottleneck, but they are often plagued by long turnaround times as researchers attempt to juggle many unrelated commitments. User-friendly analysis software would benefit experimentalists caught in this predicament by eliminating the need for computational proficiency altogether. Automated analysis frameworks have already played a similar role in many other subdisciplines of biology [53–65]. Indeed, without the support of automated alignment software, next-generation sequencing would be inaccessible to all but a few labs with extensive programming and statistical modeling experience. Similar platforms are available to support quantification of microscopy data [48, 66], but comparatively few are tailored to address the intricacies of specific model systems and experimental pipelines [67]. Further development of context-specific quantification platforms is therefore needed in order to lower the barrier to adoption of data-driven analysis.

1.3. Mathematical modeling of cell fate decisions

Mathematical models have reinvigorated the study of cell fate decisions in a broad variety of contexts. Most modeling efforts fall into one of two categories; those that use a data-driven approach to recapitulate molecular mechanism, and those that provide a sparse representation of systems-level behavior.

The first class of models strive to parameterize specific biomolecular interactions by fitting a model directly to data. They typically describe the time-evolution of transcripts and proteins using systems of coupled ordinary differential equations (ODEs) reminiscent of those familiar to chemical engineers and ecologists. Despite the illusion of mechanistic detail, these models still deploy a healthy dose of abstraction. None of the commonly used rate

represent true elementary reactions, instead opting for empirical representations such as linear degradation and cooperative binding kinetics. Nevertheless, many novel GRN behaviors and functions have been elegantly proposed and tested in this manner [40, 68, 69].

The second class of models forego molecular detail in favor of a coarse-grained representation of a particular phenomenon. These approaches provide a powerful means to identify, characterize, and predict behaviors that span a broad variety of model systems and developmental contexts. Among the common modeling frameworks, control theory has proven particularly fertile for generating and testing hypotheses related to GRN dynamics. Bacterial chemotaxis offers a compelling example in which molecular models were supplanted by a simple integral control framework [68, 70–72]. Analogous strategies have drawn inspiration from several disciplines to discover numerous novel functions of GRNs [42, 73–77].

Coarse-grained models are particularly well suites to studying how cell fates are resolved from spatiotemporal signaling cues. These problems would otherwise be intractable due to the many complex transport processes that shuttle signaling molecules between neighboring cells. Lubensky et al. developed a relatively simple reaction-diffusion approach to model pattern formation in the larval eye. They showed that inductive signaling cues could drive cell-autonomous positive feedback to account for the emergence of a hexagonal lattice of R8 cells, as well as an otherwise inexplicable striped pattern observed in some mutants [78]. Gavish et al. used an even simpler model to show that an additional inhibitory signal is required to stabilize retinal patterning against minor fluctuations in cells spatial arrangement. The authors then used an experimental technique known as quantitative mosaic analysis to identify the unknown diffusible inhibitor [35].

Mathematical models have also shone light on the regulatory interactions that implement cell fate decisions within individual cells. One study explored how cells generate all-or-none responses to morphogen gradients in the *Drosophila* ventral ectoderm [79]. An

ultrasensitive response mechanism was proposed to dictate the expression of Yan, a transcriptional repressor known to impede cell fate transitions [80–82]. A later study proposed that Yan plays a different role in the larval eye, instead forming a bi-stable switch through reciprocal antagonism with a transcriptional activator named Pointed (Pnt) [83]. The authors used a psuedo-molecular model to demonstrate that the decision to adopt an R cell fate could be triggered by an irreversible transition between two stable states; one characterized by high Yan and low Pnt expression, and the other by low Yan and high Pnt expression. Both transcription factors are known to be subject to several seemingly redundant sources of negative feedback. The model rationalized the purpose of these inhibitors by postulating that they enforce bi-stability of the two distinct states. Transitions between the states are triggered by an increase in Pnt levels. Shwartz et al. extended the model to include autoregulatory interactions that help flip the switch by converting transient inductive signals into sustained Pnt expression [84].

Soon thereafter, the advent of a Pnt-specific fluorescenct reporter curiously revealed that Yan and Pnt are co-expressed in several developmental contexts [85]. Peláez et al. then published quantitative measurements indicating Yan exhibits mono-stable expression dynamics in the larval eye [43]. These two studies fundamentally contradict the existing model, prompting renewed interest in both Yan and Pnt. Namely, how do they cooperate to mediate cell fate decisions? How do they do so reliably? And why are they subject to so much negative feedback?

1.4. Roles for negative feedback in GRNs

Theory supports many potential uses for negative feedback in developmental GRNs [86]. Perhaps the most obvious application is the maintenance of cell states — that is, rejecting exogenous disturbances and driving cells toward a desired set point [71, 87, 88].

Negative feedback has also been shown to improve information transmission by linearizing input-output relationships and expanding dynamic range in several developmental signaling cascades [40, 69, 89–91]. Yi et al. used a FRET-based reporter of the Gα-subunit to demonstrate dose-response alignment of G-protein signaling activity at the highest level of the yeast mating response pathway [91]. Yu et al. later quantified pFus3 activity to show that negative feedback further increases signal fidelity throughout much of the downstream pathway [69]. These functions mimic critical roles for negative feedback in human-engineered systems [92].

Negative feedback is also vital to the reliable execution of cell fate decisions. Rahimi et al. showed that Wnt-mediated negative feedback enhances the precision of morphogen gradients in the ventral domain of the *Drosophila* embryo [74]. Paulsen et al. showed that the fidelity of BMP4 signaling in the *Xenopus* embryo is improved by concomitant expression of a repressor that suppresses transduction of extrinsic noise. Eliminating the repressor leads to increased variation of pathway outputs, as well as the cell fate decisions they control [40]. These studies provide direct experimental evidence that negative feedback can suppress phenotypic variation.

Experiments in *Drosophila* indicate that short non-coding transcripts, called microRNAs, confer a similar function by buffering developmental processes against both environmental and genetic variability [93–96]. Li et al. studied the effect of perturbing miR-7 activity during sensory organ development, and found that the microRNA stabilizes both gene expression and fate commitment decisions against fluctuating environmental conditions [95]. Cassidy et al. used directional selection to quantify the heritability of bristle formation defects in miR-9a mutants, revealing that microRNAs can also suppress the influence of intrinsic genetic variation [94]. The same group later showed that miR-9 suppresses the penetrance of genetic variants deleterious to viability at elevated temperatures [93]. Negative

feedback mediated by microRNAs has therefore been shown to directly affect the evolutionary fitness of adult organisms by promoting robustness against varying environmental conditions. Precisely how this effect is achieved remains unknown.

1.5. Evolutionary drivers of robust cell fate decisions

Studies of developmental robustness are often traced back to C.H. Waddingtons claim that there is scarcely a mutant that is comparable in constancy with the wild type [97]. His comment reflects the consistency of adult phenotypes amidst modest levels of genetic and environmental variation, which gives way to variation when severe perturbations are introduced [98–101]. Robustness has since been attributed to individual genes and their products [102–104], as well as the systems-level architectures that they comprise [40, 93–95, 104–106]. A handful of conserved regulatory motifs, and the strategies they implement, are now understood to pervade most developmental processes [86, 87, 107, 108]. Robustness has thus come to be accepted as a fundamental organizational principle underlying the evolution of all biological systems [109, 110]. That is, selection for genotypes that confer robustness is widely assumed to shape the evolution of gene regulatory network topologies.

The specific evolutionary drivers for increased robustness of GRNs remain unclear [111]. Citing phenomenological examples, Waddington attributed robustness to evolutionary selection for optimal traits. More recently, Siegal et al. argued that developmental robustness can arise without stabilizing selection for any particular phenotype [112]. They used a gene interaction network model to demonstrate that robustness is an emergent property of complex networks subject only to selection on the basis of their own functional stability. The same authors used in silico evolution to show that most genes in developmental networks buffer phenotypic variation, indicating that robustness may be an emergent byproduct of GRN architecture [113]. Computational studies have also questioned the notion that global GRN

properties are a consequence of adaptive evolution, instead suggesting that local properties of genetic circuits could drive the emergence of macroscopic features, such as those that ensure robust development [114, 115].

When a gene or its products are absent or fail to perform their functions, the simplest contingency is to have others ready to compensate. It consequently seems intuitive that cells could incorporate functional redundancy to guarantee reliable cell fate decisions [107, 116]. One computational study showed that genetic redundancy is particularly evolutionarily stable in developmental systems. By simulating functionally overlapping pairs of genes, the authors demonstrated that increasing the probability with which each gene independently fails to carry out its function places increasing selection pressure upon the pair as a whole [117]. They reasoned that the resultant increase in evolutionary stability explains the prevalence of redundancy in developmental systems, where failure rates are high and erroneous cell fate decisions yield deleterious phenotypes. Their suggestion is consistent with extensive documentation of functional redundancy in developmental systems [109].

Redundancy could arise through gene duplication or convergent evolution. A. Wagner argued against the former. He analyzed gene sequence and expression data in yeast to show that the phenotypic severity of loss-of-function mutations does not correlate with the availability of functionally equivalent genes. He concluded that functional redundancy is not the primary source of robustness against genetic variation, instead favoring epistatic interactions between otherwise unrelated gene products [118]. Combined, these studies suggest the prevalence of functional redundancy in GRNs may be driven by as-of-yet unknown forces.

CHAPTER 5

Conclusions

The preceding chapters are unified by their pursuit of a common question: how do the structure and function of gene regulatory networks control cell fate decisions and yield emergent properties at the organismal scale? Each chapter combined quantitative data and mathematical analysis to address subtle aspects of this question in a unique way. The following sections provide an introspective summary of how each chapters findings, and the lessons learned therein, impact the present understanding and future research directions of quantitative, developmental, and systems biology.

5.1. Implications for developmental and systems biology

Chapter 2 introduced a computational framework for automated analysis of genetic mosaics; a class of experiments commonly used to probe cell fate decisions *in situ* [19, 135]. The framework combines computer vision and statistics to automate the labor-intensive steps of a quantitative work-flow, enabling automated and systematic comparison of cells subject to control and perturbation conditions in an otherwise equivalent background.

Quantitative mosaic analysis is not new, nor is it uncommon in high profile publications [35, 136, 139]. Yet, these studies deploy an irreproducible mix of ad hoc implementations and costly commercial software. Worse still, qualitative analysis pervades less prominent corners of the literature. Contributing a fully automated framework to the open-source ecosystem will make quantitative mosaic analysis equally accessible to the entire community. A unified framework will also dramatically simplify the reproduction of existing analyses. Chapter 2

therefore advances the quantification of developmental biology by adding potential for rigor and reproducibility where they are currently lacking.

Chapter 3 explored a novel cell fate decision mechanism underlying photoreceptor specification in the *Drosophila* larval eye. Computer vision techniques were used to extract quantitative measurements of Pnt and Yan dynamics from a wealth of confocal microscopy data. Statistical analysis revealed that differentiation is driven by dynamic changes in the ratio between Pnt and Yan, and is agnostic to changes in their absolute concentrations as long as the ratio remains constant. The data therefore provide the first direct evidence that cell fate decisions can be triggered by changes in the relative abundance of separate transcription factors. This finding rebukes the canonical model of photoreceptor specification [83]. More importantly, it adds a new dimension to our understanding of how multiple transcription factors cooperatively control cell fate decisions, with broad implications for many developmental contexts within and beyond *Drosophila*.

The ratiometric sensing mechanism identified in Chapter 3 adds to a growing body of evidence that cells are able to sense relative changes in the abundance of GRN components [50, 187]. These discoveries are exciting because relative sensing could potentially isolate cell fate decisions from environmental sources of variation. This is because environmental fluctuations would likely manifest as correlated extrinsic noise that affects all GRN components in a similar manner, causing absolute but not relative concentrations to vary between cells. Relative sensing might then increase fitness in variable environments.

Regulatory interactions may provide additional layers of stability. Dual-reporter experiments have shown that regulation buffers cell-by-cell differences in yeast gene expression to enhance the precision of decisions to commit to a mating response phenotype [42]. Indeed,

Chapter 4 also showed that the microRNA miR-7 buffers Yan expression levels, and by extension R cell fate decisions, against varying biosynthesis capacity. Perhaps future experiments could address how Pnt levels are affected before and after IPC ablation in $yan^{\Delta miR\text{-}7}$ mutants.

These observations reflect a central theme of this dissertation; the structure and function of developmental GRNs dictate the robustness of cell fate decisions to environmental variation. Chapter 4 directly embraced this sentiment. It proposed a new theory to explain why the regulatory networks that coordinate cell fate decisions often contain many seemingly redundant repressors acting upon the same target genes. The theory posits that auxiliary negative regulators mitigate erroneous cell fate decisions when cells are rapidly metabolizing, and implies that auxiliary repressors may help GRNs adapt their behavior to environmentally driven variation in cell metabolism. The theory is supported by a diverse collection of experiments in which repressor loss-of-function phenotypes were reversed when biosynthesis rates were slowed. A quantitative modeling framework was used to explore the mechanistic origin of this effect, and theoretically demonstrated that auxiliary repressors could avert erroneous decisions by expanding cells capacity to buffer excess protein expression. Quantitative measurements of transcription factor activity confirmed the hypothesized mechanism in vivo.

Chapter 1 introduced the idea that developmental GRNs guide organisms through a tortuous journey from embryo to adulthood. The journey is not a sprint. Rather, cells must carefully navigate the many twists and turns of developmental programs; rapidly synthesizing GRN components when and where they are needed, then degrading them with equal urgency. Protein synthesis and degradation machineries supply the engine and brakes needed to negotiate these obstacles (Fig. 5.1A). Individuals stand to benefit from completing the journey quickly because they are generally vulnerable until adulthood, with little means to defend themselves against predation and other dangers. They could naively swap out the engine for something more potent, but adding power escalates risk, particularly around sharp

corners (Fig. 5.1B). Simultaneously upgrading the brakes is a winning strategy (Fig. 5.1C). Analogously, evolution could accelerate development by expanding biosynthesis capacity. The resultant boost in protein expression would strain each cell's ability to localize distinct GRN activities in time, causing increased risk of erroneous fate decisions that lead to the emergence of deleterious phenotypes. However, by selecting for auxiliary repressors, cells could tolerate faster biosynthesis rates without compromising the quality of fate decisions. The data presented in Chapter 4 confirm this hypothesis by illustrating the inverse perspective. Repressors were shown to be dispensable when metabolism was slow, much in the way that brakes aren't needed to round corners at low speed.

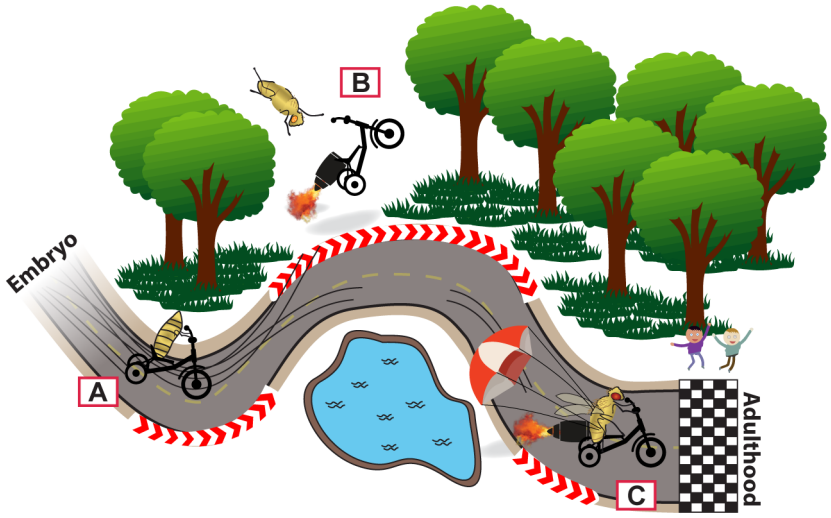

Figure 5.1. The race from embryo to adulthood. Individuals must navigate the many twists and turns of the developmental program to reach the finish line. (A) Slow and steady loses the race. (B) Adding power improves acceleration, but increases the risk of failure. (C) Adding power along with better brakes is a winning strategy. The analogous experimental conditions are (A) slow metabolism without auxiliary repression, (B) fast metabolism without auxiliary repression, and (C) fast metabolism with auxiliary repression. Evolution suggests this wildtype configuration consistently wins the race.

This line of reasoning implies that a novel evolutionary driving force may shape the structure and function of developmental gene regulatory networks. Shorter generational times confer a selective advantage beyond reducing individuals exposure during infancy. They facilitate rapid exploration of the phenotypic landscape, enabling fast adaptation to variable

environments. GRNs should therefore be expected to incorporate any topological features that allow development to proceed more quickly. The abundance of seemingly redundant regulation found in developmental GRNs certainly appears to support this hypothesis, and thus reinforce our contemporary understanding that robustness is a fundamental organizational principle underlying the evolution of biological systems [109, 110].

The findings also contribute to an emerging view that cells capacity to rapidly adjust protein homeostasis directly affects organismal fitness and health [298–300]. The assertion is backed by convincing experimental evidence. Burnaevskiy et al. used a dual-reporter scheme in *C. elegans* to show that cellular differences in the abundance of protein synthesis machinery manifest in the population-wide penetrance of adult phenotypes. The authors went on to speculate that longevity might be similarly be affected [300]. Tollerson et al. showed that Elongation factor P alleviates a translational bottleneck caused by ribosomal queuing in *E. coli*, facilitating adaption to environmentally-driven increases in cell metabolism [299]. Chapter 4 contributes unique evidence that the accuracy of cell fate decisions depends upon cells ability to dynamically balance the push and pull of protein synthesis and degradation.

5.2. Avenues for further exploration

This dissertation prompts several exciting new directions for future research. This section begins with a survey of those that merit further attention, before elaborating on some preliminary analyses to guide prospective efforts.

Chapter 3 proposed that R cell fate commitment in the larval eye is driven by dynamic changes in the relative abundance of Pnt and Yan. Experimental evidence was limited to correlative observations because intrinsic regulation precluded direct manipulation of the Pnt-to-Yan ratio by varying gene dosage. Future studies could rigorously confirm the hypothesis by applying the same gene dosage perturbations in a genetic background that lacks the

complete set of regulatory interactions needed to stabilize the Pnt-to-Yan ratio. Disrupting the ratio control mechanism would first require characterizing its biomolecular implementation. Computational simulations could explore the space of plausible GRN topologies, then leverage insight derived from existing experimental data to distill a manageable number of options for experimental validation. Establishing an unambiguous picture of R cell fate commitment might then allow for a complete model of retinal patterning dynamics, as is an ongoing mission among computational biologists [35, 78].

Chapter 3 used an equilibrium modeling framework to explore the effect of *cis*-regulatory interactions between Yan monomers on the equilibrium binding occupancy of promoters regulated by Pnt and Yan. The approach was inspired by the work of Hope et al., who used an equivalent model, limited to a single binding component, to explore how the *cis*-regulatory syntax of target genes modulates transcriptional output [220]. The multi-species implementation introduced in Chapter 3 was comparatively underutilized. Future studies could ask many questions related to how *cis*-regulatory syntax modulates the transcriptional output of genes regulated by two or more polymerizing transcription factors. How do the number and arrangement of individual binding sites modulate transcriptional output? What about the spacing or distribution of high and low affinity sites? What if anti-cooperative or steric interactions are included? Does the number of unique binding species matter? All of these questions could readily be explored using the open-source platform developed to support this dissertation (see Appendix C).

The theory developed in Chapter 4 posits that auxiliary repressors stabilize cell fate decisions against environmental variation in cells capacity to synthesize and degrade proteins. This assertion was backed by both experiments and computational analysis showing that repressors were dispensable when either ATP turnover or translation capacity were reduced. It is well known that many mutant phenotypes are also suppressed in animals raised at

low temperatures. Future studies could ask whether reduced temperature imparts similar effects on the GRN dynamics that govern cell fate decisions. From a modeling perspective, the primary challenge would be deciding precisely how to incorporate the relative effects of temperature on the protein synthesis and degradation machineries. The analogous decisions were comparatively obvious for *ILP2-GAL4 UAS-Rpr* and *RP* mutants, in which protein synthesis is disproportionately affected. In principle, experimental efforts to quantify the dependence of protein synthesis and degradation rates on temperature could prove fruitful.

Alternatively, researchers could computationally survey the landscape of plausible relationships between proteostasis and the environment to identify conditions under which auxiliary repressors are dispensable. For instance, consider the simplest possible model of protein expression dynamics (Fig. 5.2A):

$$\frac{dP}{dt} = kI - \gamma P \tag{5.1}$$

where P and I are the protein and stimulus levels, and k and γ are the synthesis and degradation rate parameters. Repressors may be implemented as proportional feedback, as they were in Chapter 4:

$$\frac{dP}{dt} = kI - \gamma P - \eta P \tag{5.2}$$

where η is the feedback strength. The frequency of developmental errors induced by losing the repressor is readily evaluated using the same procedure described in Chapter 4 (Fig. 5.2B). Rather than hard-coding an explicit dependence of k, γ, and η on environmental conditions, each parameter can be scaled by a latent dimension λ that reflects the environmental state

of the cell:

$$k \propto \lambda^{\nu_k}$$
$$\gamma \propto \lambda^{\nu_\gamma} \tag{5.3}$$
$$\eta \propto \lambda^{\nu_\eta}$$

where ν_k, ν_γ, and ν_η define the respective sensitivities of synthesis, degradation, and feedback strength to environmental conditions. Consider an example in which the environmental state of the cell is halved relative to some reference condition, i.e. $\lambda = \lambda_0/2$. For $\nu_{i \in k,\gamma,\eta} = 1$, parameter i is also halved. If $0 < \nu_i < 1$ or $\nu_i > 1$, parameter i exhibits sub- or super-linear dependence on the environment, respectively. Finally, $\nu_i < 0$ implies that parameter i should actually increase when λ is halved.

The robustness checks presented in Section 4.9 surveyed a handful of discrete values for the model parameters analogous to ν_k, ν_γ, and ν_η. The scaling assumptions used to represent conditions of reduced energy metabolism throughout Chapter 4 were loosely equivalent to $(\nu_k, \nu_\gamma, \nu_\eta) = (1, 0, 2)$. Applying the same assumptions to the simplified model recapitulates a core result of Chapter 4; error frequency is dependent upon the environmental state of the cell (Fig. 5.2C,D).

Combined, equations 5.2 and 5.3 facilitate continuous enumeration of the phase diagram spanned by ν_k, ν_γ, and ν_η in order to identify the range of scaling assumptions under which auxiliary repressors may be dispensable. Performing this exercise revealed that induced error frequencies are highest when the strength of the removed repressor is more sensitive to the environment than the intrinsic rate of protein turnover (Fig. 5.3A, region IV). The same is true of error frequency suppression, indicating that highly sensitive repressors have the highest propensity to become dispensable (Fig. 5.3B, region IV). This observation is consistent with intuition, as environmental conditions that limit the influence of a repressor would also be expected to mitigate the impact of its removal.

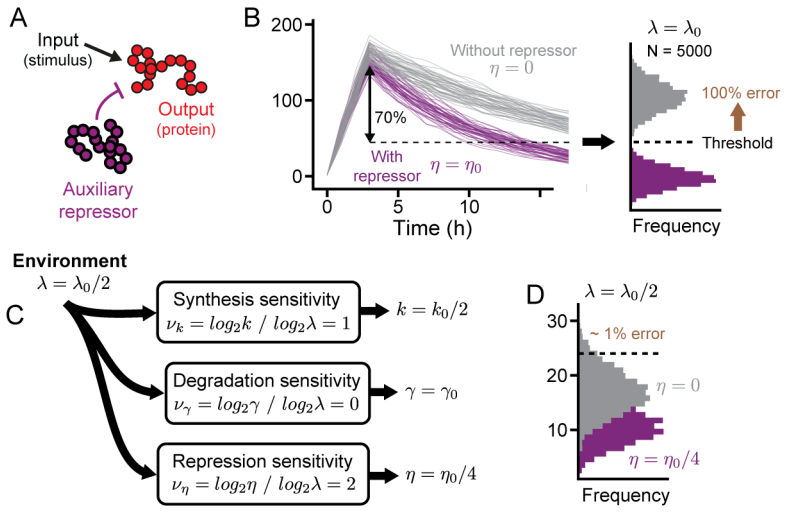

Figure 5.2. Simplified framework for modeling the loss of an auxiliary repressor. (A) Schematic representation of protein expression in response to a transient input. Output is subject to regulation by a single auxiliary repressor. (B) Simulated emergence of developmental errors. Simulations may be performed with (purple) or without (grey) the auxiliary repressor. Lines reflect a random sample of 5000 trajectories. The two sets of trajectories are compared when 99% of trajectories simulated with the repressor cross a threshold value (dashed line). Without the auxiliary repressor, very few trajectories successfully cross the threshold. (C) Graphic representation of the relation between environmental conditions and the rate parameters that dictate protein synthesis, degradation, and repressor strength. (D) Error frequency is dramatically suppressed by a change in environmental conditions.

This preliminary analysis could guide the design of future experiments that survey the effects of temperature on mutations that compromise repressor function. For example, experiments could quantify ν_k, ν_γ, and ν_η for a particular cell-fate determinant by measuring steady-state protein levels in both wildtype and repressor loss-of-function mutants across a range of temperatures. They could then use the model to generate testable predictions for alternate temperatures.

Chapter 4 also raises the question of whether any other features of GRNs are dispensable under certain environmental conditions. For instance, how about promoters? Preliminary analysis may again provide some insight to guide future work. Consider another scenario in which an auxiliary promoter is added to the simple model given by Equation 5.1:

$$\frac{dP}{dt} = kI + k_{aux}I - \gamma P \tag{5.4}$$

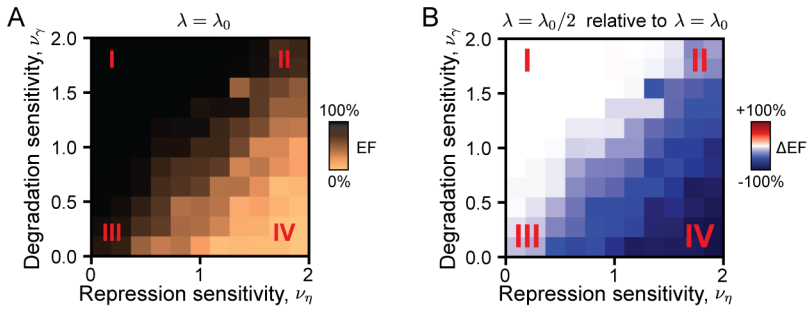

Figure 5.3. Suppression depends on parameter sensitivity to the environment. Phase diagrams span a range of protein degradation and repressor strength sensitivities to environmental conditions. Heatmaps show (A) error frequency induced by the loss of an auxiliary repressor when $\lambda = \lambda_0$ and (B) change in error frequency when $\lambda = \lambda_0/2$. All simulations use $k = 1$, $\gamma = 0.001$, $\eta = 0.001$, and $\lambda_0 = 1$. Dark blue indicates strong error frequency suppression. Red numerals label quadrants. The example shown in Fig. 5.2 is taken from quadrant IV.

where k_{aux} is the rate constant for synthesis driven by the auxiliary promoter. The relative influence of the auxiliary promoter is given by its strength relative to the primary promoter, i.e. $log_2(k_{aux}/k)$. The respective sensitivities of both promoters and degradation to environmental conditions are again parameterized in terms of λ:

$$\begin{aligned} k &\propto \lambda^{\nu_k} \\ k_{aux} &\propto \lambda^{\nu_{k-aux}} \\ \gamma &\propto \lambda^{\nu_\gamma} \end{aligned} \tag{5.5}$$

The simulation procedure described in 4 is readily modified to evaluate the frequency of developmental errors induced by removing the auxiliary promoter, i.e. by setting $k_{aux} = 0$. Intuition suggests promoter loss should cause a decrease in output protein levels. Error frequency is therefore redefined to reflect the extent to which protein is *under-expressed* when the auxiliary promoter is removed. The metric is evaluated by computing the fraction of trajectories simulated with a single promoter that fail to reach the lower bound of trajectories simulated with both promoters. Surveying a range of promoter strengths and relative influences reveals that error frequencies are most severe when strong and influential auxiliary promoters are removed (Fig. 5.4A). Figure 5.4B shows the subsequent change in error

frequencies when $(\nu_k, \nu_{k-aux}, \nu_\gamma) = (1, 1, 1)$ and $\lambda = \lambda_0/2$. The phase diagram is punctuated by a diagonal band in which error frequencies are suppressed. Below this band, suppression is minimal because the auxiliary promoter is inconsequential to normal protein expression dynamics (Fig. 5.4A,B, region I). Above it, removing the auxiliary promoter imparts a severe perturbation that cannot be recovered by the proposed change in environmental conditions (Fig. 5.4A,B, region II). Similar zones arise when the equivalent procedure is performed using the model of auxiliary repressor loss defined by equation 5.2 (Fig. 5.5). Here, the influence of the auxiliary repressor is defined relative to the intrinsic degradation rate, i.e. $log_2(\eta/\gamma)$.

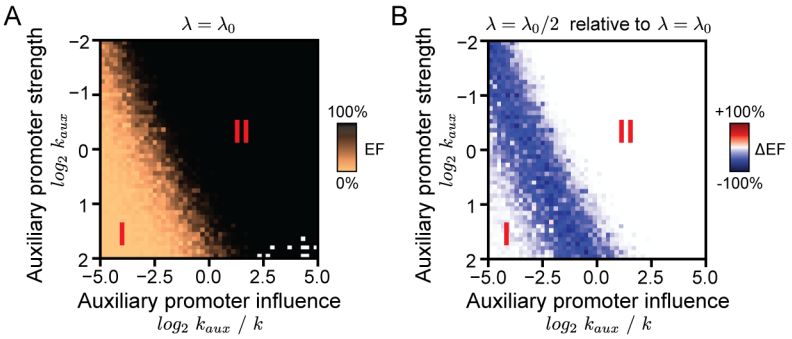

Figure 5.4. Phase diagram of auxiliary promoter loss. Phase diagrams span a range of auxiliary promoter strengths and influences. Heatmaps show (A) error frequency induced by the loss of the auxiliary promoter when $\lambda = \lambda_0$ and (B) change in error frequency when $\lambda = \lambda_0/2$. All simulations use $\gamma = 0.001$ and $\lambda_0 = 1$. Dark blue indicates strong error frequency suppression. Perturbations targeting promoters in region I are inconsequential. Those targeting promoters in region II are too severe to be recovered. Blue band is the Goldilocks zone.

The bands observed in Figures 5.4B and 5.5B indicate the existence of a Goldilocks zone in which perturbations are strong enough to be felt but weak enough to be recoverable. In other words, there exists a finite and continuous range of conditions in which error frequencies can be suppressed by slowing biosynthesis. Given appropriate knowledge of promoter and repressor strengths, a model could inform the selection of experimental perturbation targets that fall within the range. Such an approach would also require quantification of ν_i and λ for a given model system.

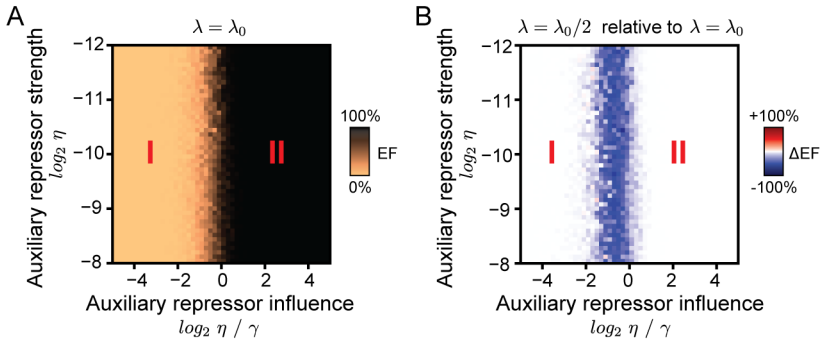

Figure 5.5. Phase diagram of auxiliary repressor loss. Phase diagrams span a range of auxiliary repressor strengths and influences. Heatmaps show (A) error frequency induced by the loss of the auxiliary repressor when $\lambda = \lambda_0$ and (B) change in error frequency when $\lambda = \lambda_0/2$. All simulations use $k = 1$ and $\lambda_0 = 1$. Dark blue indicates strong error frequency suppression. Perturbations targeting repressors in region I are inconsequential. Those targeting repressors in region II are too severe to be recovered. Blue band is the Goldilocks zone.

The value of the models defined by equations 5.2 and 5.4 lies in their simplicity. Because each strictly employs linear kinetics, the stochastic dynamics are analytically tractable via a closed system of moment equations [301]. Even the more complex models presented in Chapter 4 are approximately solvable, courtesy of modern moment closure and finite state projection techniques [302, 303]. Future theoretical efforts could be directed toward the development of a complete analytical framework for studying the interplay of GRN dynamics, cell fate decisions, and the environment. An analytical platform would vastly reduce the computational burden imposed by the numerical methods used to conduct all of the analysis presented in this section and throughout Chapter 4. It would also expedite fitting the model to experimental data in order to generate testable predictions.

5.3. Perspective on quantitative biology

This dissertation surveyed developmental cell fate decisions through a quantitative lens. It used numbers and equations to derive nuanced understanding of processes that are notoriously difficult to characterize. In many cases, doing so required forcible reconciliation

with time-honored traditions in biological analysis. Two major obstacles encountered along the way merit further discussion.

First, models should fit the data; not the other way around. While it may be possible to coax data into a conventional framework, tailoring one appropriate for the task at hand will generally return more meaningful insight. Mathematical flexibility was a key strength of this dissertation. Each chapter leveraged a customized modeling framework to elicit deeper meaning out of experimental data than would have been possible using conventional techniques.

Chapter 2 deployed a Bayesian statistical framework to infer cell genotypes from an image of clonal marker expression. Biological intuition suggests it should have enforced detection of three distinct components strictly delimited by clonal marker level. Instead, the model was designed to tolerate an arbitrary number of components that also considered spatial context, which were later aggregated into the three anticipated genotypes. This empirical formulation buffered uncertainties imparted by expression heterogeneity and imaging noise to improve annotation performance overall.

Chapter 3 used an equilibrium binding model to explore how *cis*-regulatory interactions between Yan monomers bias the transcriptional output of genes simultaneously regulated by Pnt. Conventional wisdom suggests a competitive binding model using the Hill-Langmuir equation would be appropriate [304]. Instead, a statistical mechanical approach was adapted from earlier work by Hope et al. [220]. Contrary to the empirical strategies used in other chapters, this model substantially *increased* the resolution of analysis. Doing so provided detailed mechanistic insight into the intricacies of polymerizing transcription factors, which would have otherwise been inaccessible (see Fig. 3.5C).

Chapter 4 sought to model the dynamic output of GRNs controlling a broad spectrum of cell fate decisions. The conventional first step would have been to identify and consolidate all known regulatory interactions in each system. Such an endeavor was certainly possible for Yan-mediated control of retinal patterning, as the pertinent interactions have been studied for decades [27, 82, 85, 95, 223] and in some cases quantified [43]. Chapter 4 instead drew inspiration from control theory to develop a model strictly concerned with the two salient features of pulsatile dynamics; the magnitude of induction and timescale of decay. The model jettisoned the molecular details of protein expression and regulation in each system, instead favoring a coarse-grained depiction of output dynamics. The reductionist approach was vital to simultaneously depicting the behavior of a diverse assortment of cell fate decisions.

Chapter 4 was also forced to circumvent the second major obstacle: The resolution of analysis should match the resolution of the data. Biological networks are complex, often far more complex than we can intuitively comprehend. Their emergent behavior arises from the collective interactions of numerous components, many of which are often unknown. These uncertainties make it particularly dangerous to think of a GRN as the sum of its parts. Similarly, predictions are all but meaningless when made by aggregating disparate interactions that were characterized in isolation.

Despite slowly coming under scrutiny, these practices remain tragically common. They are perhaps most strongly embodied in the abundance of cartoons that purport to depict systems level-behavior with a compendium of qualitative regulatory interactions. Figure 3.11A provides a modest example. This cartoon is harmless in isolation, but problems arise when unsuspecting viewers ascribe physical meaning to the various interactions. After all, there is no unified standard to define what an arrow, or "edge," means. A single edge might actually represent an entire sub-network of complex nonlinear processes. Intermediate steps within an edge might even interact with other intermediates in other edges drawn elsewhere

in the diagram. Cartoons are thus rife with ambiguity that hinders the communication of otherwise outstanding research. For instance, a basic attempt to simulate the system shown in Figure 3.11A will reveal that the illustrated "regulatory loop" cannot maintain a constant Pnt-to-Yan ratio in response to varying *pnt* or *yan* gene dosage (data not shown). Yet, Figure 3.11A was necessary because these depictions are so deeply ingrained in the biological literature that the representation in Figure 3.11B would likely be misconstrued without it.

This ambiguity starkly contrasts the standardized descriptive languages commonly used in engineering [44]. To be fair, most human-engineered systems do not suffer the same extent of uncertainty as their biological counterparts. Notable exceptions arise in electrical, chemical, and biomolecular engineering. Autonomous vehicles, advanced robotics, process plants, and synthetic biological circuits must all contend with disturbances whose origin and character may be unpredictable or unknown. Fortunately, a viable solution to this challenge already exists. Control theory emphasizes an empirical representation of systems-level dynamics that is easily matched to the resolution of available information [282]. In many cases, the specific details of a system's internal dynamics may remain unknown. Sensors need only monitor the minimum set of variables necessary to ensure that a given process is controllable. Often, this simply entails monitoring systems-level output and taking corrective action when it deviates from a desired set point.

Control theory offers a particularly compelling perspective for rationalizing the behavior of developmental GRNs. Most are inherently dynamic, contain numerous unknown components and interactions, and ultimately coordinate a manageable number of outputs. This rationale inspired the conceptual model of ratiometric control used to interpret the R cell fate decision analyzed in Chapter 3 (see Fig. 3.11B). After all, Pnt and Yan are transiently expressed, subject to extensive uncharacterized regulation, and appear to mediate R cell fate transitions through a single observable output; the Pnt-to-Yan ratio. Similar reasoning

inspired the modeling framework used throughout Chapter 4. The breadth of experiments pointed toward a dynamic phenomenon agnostic to the molecular detail of repressors and their targets. Furthermore, among all systems surveyed, only Yan and Sens expression were measurable. The resolution of analysis was therefore matched to the resolution of the data by modeling the representative dynamics of a generic protein, subject to feedback at each of the three levels that were experimentally surveyed (see Fig. 4.6). Combined, these approaches reflect a growing trend of interpreting biological robustness from a control perspective [92].

As biology and engineering converge on common interests, it is important to reconcile the strengths of both disciplines. Biology contributes a wealth of prior knowledge and experimental techniques that frequently bewilder even the most seasoned engineers. Engineering contributes quantitatively rigorous frameworks to systematically analyze, interpret, predict, and design complex systems. The promise of successful integration prompts continued dialogue to resolve any growing pains and advance the field of quantitative biology as a whole.