The influence of miRNAs on variation of gene expression during T-cell development

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Declaration

I, Rory Blevins, declare that this thesis is my own work and that any work performed by others has been acknowledged in the text and properly referenced.
Abstract

MicroRNAs (miRNAs) are small non-coding RNAs which regulate gene expression post-transcriptionally, by binding to specific mRNAs. It has been suggested that miRNAs have a role in "canalising" development and reducing variability in gene expression.

Using conditional deletions of the miRNA biogenesis enzyme Dicer in mice, I investigated the effect of miRNA depletion on gene expression during T-cell development. I used flow cytometry to obtain the distribution of proteins on a single cell basis. Proteins encoded by miRNA-regulated transcripts showed a Dicer-dependent increase in both mean protein expression and cell-cell variation. In particular, the genes Sca-1 and Cd44 show increased expression and cell-cell variation in Dicer-deleted double positive thymocytes, and Cd69 showed increased expression and cell-cell variation in Dicer-deleted thymocytes activated by stimulation of the T-cell receptor. Using fluorescent reporter constructs, the effect of the 3' UTR of each mRNA on reporter expression was investigated to find miRNA binding sites. I identified binding sites for the miR-181, miR-130 and miR-20 miRNA families in the Cd69 3’ untranslated region (UTR). To further investigate how these miRNAs might regulate the expression of Cd69, I investigated how expression of miRNAs changed on T-cell activation: observing that the miR-181 family is downregulated after activation in thymocytes, and the miR-20 family is upregulated after activation in both thymocytes and mature peripheral T-cells. I used both miRNA inhibitors and deletion of specific miRNA families to confirm that the miR-181 and miR-20 families both regulate expression of Cd69 during thymocyte activation. Finally, I looked at theoretical models of how miRNAs might regulate biological noise, and found that the feedforward loop motif can reduce noise compared to an unregulated gene under conditions of moderate miRNA repression. These results show that depletion of miRNAs can result in increased cell-cell variation in developmentally regulated thymocyte genes.
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<tr>
<td>Ago</td>
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<td>FFL</td>
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1

Introduction

Development in multicellular organisms is a remarkably precise and robust process, with even the simplest of multicellular organisms requiring the co-ordination of the expression of thousands of genes across many different cell types. During development, cells develop from pluripotent progenitor cells to become progressively more specialised and spatially organised into tissues and organs. In 1957 Conrad Waddington suggested that the increasing specialisation and commitment of cells during development could be visualised as a marble rolling down a slope from the highest point, with different ridges and valleys separating different developmental pathways, a metaphor he termed the “epigenetic landscape”. Crucial to this view of development is the idea of “canalisation”: that mechanisms exist which separate these pathways and keep them distinct, preventing abnormal gene expression and development[1]. While many of the pathways involved in differentiation have been, or are being, elucidated, the mechanisms behind canalisation remain relatively unknown. How do organisms cope with perturbations to developmental pathways, and ensure that development is robust in the face of internal and external challenges? Canalisation can occur at several levels: for example, by the silencing of non-lineage-specific genes in a particular cell type through the expression of a transcriptional repressor, or by changes in chromatin structure. In particular, as the tools and techniques required to look at cell biology in a quantitative manner have developed, it has emerged that the stochastic nature of biochemical reactions can lead to significant deviation from optimal patterns of gene expression. This has in turn lead to investigation into
1. INTRODUCTION

the mechanisms which control this “biological noise”.

1.1 Biological noise

Biochemical events within the cell are caused by chemical reactions between one or more molecules. Because a chemical reaction is dependent on the reactants meeting with the appropriate energies, each reaction is essentially a random discrete event, with a probability dependent on a number of factors, principally the concentration of reactants in the system. For many chemical processes, such as diffusion, the number of molecules and reactions involved is large enough that the random effect of single reactions can be considered to “average out”, and the variation in the system is negligible. As the number of reacting molecules gets smaller, however, the significance of stochastic effects in a system increases. These stochastic effects present a particular issue in biological systems, as such systems often involve relatively few reacting molecules: for instance, in transcription and translation there are usually only one or two copies of a particular gene, and often less than 100 mRNA molecules produced from a specific gene. This problem is compounded by the interconnected nature of biological networks: for example, a relatively small deviation from normal gene expression may be amplified by downstream mechanisms. How organisms regulate these variations in the output of biochemical reactions, and prevent them causing potentially damaging problems, is still a poorly understood area of cell biology.

Biochemical noise is commonly observed as variation in gene expression between otherwise similar cells. The first observations of such cell-cell variation were made in bacteria, where variable responses to antibiotic treatment or bacteriophage infection were observed. More recent experiments have used experimental techniques such as fluorescent reporter proteins, which allow the expression of particular genes to be observed at the single cell level with either fluorescence microscopy or flow cytometry. This has allowed the variation of gene expression to be observed across populations and some basic principles of noise to be determined.

Quantifying biological noise requires a reliable measure of cell-cell variation in gene expression: several different statistical measures have been used, depend-
1.1 Biological noise

ing on the systems investigated and the methods used. The simplest, and most common measure of population variation is the standard deviation, alternatively known as $\sigma$, a mathematical measure of population spread calculated as:

$$
\sigma = \sqrt{\frac{1}{n-1} \sum_{i=1}^{n} (x_i - \mu)^2}
$$

Where $n$ is the number of samples, $\mu$ is the population mean and $x_1 \ldots x_n$ are the individual expression values. Standard deviation is a statistical measure used in many fields other than biology. However, there is evidence that in biological systems the standard deviation may change proportionally with the mean expression$^6$. To investigate systems where both a change in cell-cell variation and a change in mean levels of gene expression is seen, a measure of noise is needed that is unaffected by change in the mean level of gene expression. To achieve this, another commonly used measure of noise is the coefficient of variation (CV), calculated as:

$$
\frac{\sigma}{\mu}
$$

This normalises the standard deviation by the mean level of expression. There are several other possible measures of variation, most significantly the noise strength, also known as the Fano factor, calculated as:

$$
\frac{\sigma^2}{\mu}
$$

The noise strength is often used in theoretical studies of noise. This is because Poisson processes, a class of random processes often used to simulate the occurrence of random chemical reactions (as well as other random processes, such as radioactive decay), have a noise strength of 1, making the noise strength a convenient measure of noise relative to a simple random process$^7$.

A number of theoretical studies have looked at biological systems from a mathematical viewpoint, modelling biological systems as deterministic (noise-free) or stochastic (containing a random component) differential equations, which
1. INTRODUCTION

can be solved to obtain testable hypotheses about how biological systems might behave. Where systems are too analytically complex to solve directly, simulations of biological systems have been developed to analyse how they might behave. These studies often use some version of the Gillespie algorithm, a computational simulation method in which a system is represented by the levels of each of the reagents and products: individual chemical reactions are modelled as exponentially distributed random events, with the time to a reaction having the cumulative distribution function

\[ F(t) = 1 - e^{-\lambda t} \]

Where \( F(t) \) is the probability of the event occurring within time \( t \). The constant \( \lambda \) is the key parameter determining the expected time until an event occurs; this constant may depend on the concentration of one of more reagents: in biological reactions with one limiting component, \( \lambda \) is often defined as

\[ \lambda = k[\text{reagent}] \]

Where \( k \) is the rate constant for that reaction, and \([\text{reagent}]\) is the concentration of the reagent. More complex reactions may depend on the levels of many reagents, or respond to changes in concentration of reagents in a non-linear manner. In the Gillespie algorithm, the time to a reaction is randomly generated from an exponential distribution given the concentration of reagents. The levels of reagents are altered to reflect the reaction that has occurred, and another chemical reaction is simulated: this is repeated until the required length of time has been simulated. By averaging the behaviour of many iterations of a simulations, characteristics of the system such as the mean or standard deviation of the concentrations of reagents can be calculated. Simulations are particularly important for looking at biological noise, which cannot be modelled with deterministic differential equations and which is often too mathematically complex to allow a direct solution using stochastic differential equations.

Noise in gene expression varies between genes, and can be broken down into two main categories depending on where the noise originated: extrinsic and
Biological noise

1.1 Biological noise

Extrinsic noise is due to sources that are external to the expression of that particular gene, such as variation of other pathway components (such as upstream transcription factors), or of global factors (such as the cell cycle, or the availability of ATP). Intrinsic noise is inherent to the process of gene expression, and can be further subdivided into components such as transcriptional and translational noise. The relative magnitude of these different categories of noise depends on the gene and organism observed: early work on noise was in prokaryotes such as E.coli and B.subtilis, and suggested that most intrinsic noise in gene expression was due to “translational bursting”, where the relatively small number of mRNAs produced in the cell at any time results in bursts of translation as one mRNA is produced, translated multiple times, and then degraded, although changes in the rate of transcription still contribute to the noise.

In eukaryotes such as S.cerevisiae, patterns of intrinsic noise are considerably different: instead of translation being the major source of noise, transcriptional bursting is the major source of intrinsic noise in gene expression. In transcriptional bursting, rather than transcribing genes at a fixed rate that is changed by the binding of particular transcription factors or repressors, transcription occurs in bursts. A given promoter can be either in a high-expression state, where it will produce mRNAs at a high rate, or a low expression rate, where mRNA production is negligible. The reasons for these transitions have yet to be experimentally elucidated, but as transcriptional bursting is mainly found in eukaryotes, it has been suggested that the bursting is due to changes in chromatin structure or binding of eukaryote-specific components of the transcriptional machinery. Experimental measurement of this bursting effect in mammalian cells with fluorescent reporters or mRNA fluorescent in situ hybridisation (FISH) show that it represents a significant proportion of intrinsic noise in mammalian cells, although this varies between genes.

Variation in gene expression is generally considered a negative factor in development, potentially leading to deleterious over- or under-expression of genes. For example, it has been suggested that accumulation of non-genetic heterogeneity may lead to tumourogenic phenotypes such as increased cell proliferation. Stochastic gene expression may also enhance cancer survival by generating a more diverse population which is better able to respond to challenges from a
changing environment: in some human cell lines, variation of gene expression has been shown to increase the diversity of responses to challenges such as pro-apoptotic ligands. However in some contexts variation in gene expression may be advantageous: several systems have been described which rely on stochastic expression of particular genes to make decisions between different cell fates. Typically, a small stochastic difference in the expression of a particular gene between cells is amplified by positive feedback mechanisms to determine what fate a cell adopts. One of the most well-characterised gene regulatory networks, the decision between lysis and lysogeny in lambda phage, is determined stochastically by the relative abundance of the fluctuating CII and Cro proteins. In Drosophila, the stochastic expression of the spineless gene determines which of two fates a particular ommatidia will adopt. Cell fate decisions in mammals may rely on similar mechanisms: one recent study on noise in haematopoietic progenitors found that fluctuations in expression of haematopoietic marker Sca-1 could bias populations towards either the erythroid or myeloid lineages, although subsequent dissection of this system suggests that stochastic expression of many additional genes are involved. Similarly, studies of reprogramming in induced pluripotent cells have found that reprogramming by expression of Oct4, Sox2, Klf4, and c-Myc is a randomly determined process.

The mechanisms by which noise is regulated within the cell remain mostly unknown: this is partly due to the complexity of systematically analysing or stochastically modelling gene networks to investigate noise. One important simplifying concept is that genes are found as part of “network motifs”: small sub-networks which occur in many different parts of a genetic network (Figure 1.1). In particular, the negative feedback loop has been suggested as a mechanisms to reduce noise, and to prevent noise being propagated and amplified during development (Figure 1.1a). The negative feedback loop, where one downstream component of the motif produces a product which negatively regulates an upstream component, is probably the most well known network motif, and has been predicted to reduce variation in both in silico simulations and artificially constructed gene networks in vitro. A second network motif, known as the feedforward loop (FFL), has been suggested as another mechanism to reduce noise. An FFL consists of an upstream regulator which regulates a downstream target by two
1.2 MicroRNAs

different routes: a direct regulation, and by regulating an intermediate target that in turn also regulates the downstream target. FFLs can be broadly divided into coherent FFLs (Figure 1.1b), where both the direct interaction between the upstream regulator and the downstream target and the indirect interaction through the intermediate regulator have the same effect (up- or down-regulation), and incoherent FFLs (Figure 1.1b), where the direct and indirect interactions have the opposite effects (i.e. if the direct interaction increases expression, the indirect interaction decreases expression, or vice versa). The FFL is more complex than a negative feedback loop, but it is thought that some configurations may reduce noise in systems by a number of mechanisms. Coherent FFLs may act to reduce noise by adding redundancy to a system, for example by repressing inappropriate gene expression through both direct and indirect regulation. Incoherent FFLs may play a number of roles, depending on the dynamics of the components: one suggestion is that FFLs reduce noise by buffering downstream targets from noise in the upstream regulator. For example, a transient variation in the level of a transcription factor would produce a similar variation in its downstream targets, leading to the propagation of noise. If the transcription factor is part of an incoherent FFL, then this transient variation would be countered by an opposite variation produced through the indirect pathway of the FFL, resulting in reduced noise in the FFL targets. Computer simulations of some configurations of FFLs have supported this idea, showing that noise can be reduced by commonly found FFL configurations, as well as possessing other useful characteristics such as improved signal responses. While these network motifs can be found in many gene regulatory networks, it has been suggested that the class of small non-coding RNAs known as MicroRNAs (miRNAs) may have a particularly important role in reducing cell-cell variation and increasing the robustness of genetic networks to biochemical noise.

1.2 MicroRNAs

First identified in C.elegans, miRNAs are small (20-22 nt) non-coding RNAs that repress specific target mRNAs and play a role in a large number of biological processes, including cell differentiation, cell cycle regulation and developmental
Figure 1.1: Common transcriptional motifs - (a) Example negative feedback loop (b) Coherent FFL configurations (c) Incoherent FFL configurations. Repressors can be transcriptional repressors or miRNAs. Activators can be transcription factors or other enhancers of gene expression.
patterning, as well as in a number of diseases, such as cancer. Approximately 500 miRNAs have been identified so far in mice, generally from sequencing of small RNAs. MicroRNAs are members of the family of small RNAs which also includes short interfering RNAs (siRNAs) and Piwi-associated RNAs (piRNAs). Similarly to miRNAs, siRNAs are also 22nt RNAs which are processed by Dicer and act in complex with members of the Ago family. Unlike miRNAs, siRNAs are formed from double-stranded RNA molecules (dsRNA) which are a product of transcription or exogenous to the cell (for example, from viral infection), and generally bind to mRNA sites with exact complementarity and direct endonucleolytic cleavage of their target miRNA. The mechanisms of piRNA biogenesis and function are less well understood. They tend to be 20-32 nt in length, and are processed in a Dicer-independent manner, and are generally found only in germ line cells, where they are thought to act to repress transposable elements.

The role of many miRNAs remains unknown, however, and the effect of individual miRNA knockouts is often phenotypically small, although as a class of regulators they are essential for mammalian development. Because of this, it has been suggested that they may play a role in maintaining the robustness of gene expression networks to external perturbations and fluctuations in gene expression.

1.2.1 Biogenesis

1.2.1.1 Transcription

MicroRNA genes can be found in intergenic regions, transcribed by a separate promoter. Alternatively, miRNAs may be encoded in the introns of protein-coding or non-coding transcripts, where they may use their own promoter or the promoter of the host gene. In both intronic and non-intronic sites, miRNAs may be found either alone or as part of a polycistronic cluster of several miRNAs. Identification of miRNA promoters shows that they are usually transcribed by RNA polymerase II (or, less commonly, RNA polymerase III), and that miRNA promoters share many features and characteristics with mRNA promoters, such as TATA and BRE elements. Once transcribed, most miRNA transcripts are capped and polyadenylated in a similar manner to protein-coding mRNAs, and
Figure 1.2: Transcription, processing and action of microRNAs - Figure showing simplified pathways of miRNA biogenesis and action.
1.2 MicroRNAs

contain one or more 70 nt stem-loop structures containing a mature miRNA sequence; this stage of processing is known as the primary miRNA (pri-miRNA).

1.2.1 Nuclear processing

Following transcription, the majority of pri-miRNAs are processed by a nuclear complex known as the Microprocessor complex. In mice and humans, this complex contains the RNAse III Drosha and the RNA-binding protein DGCR8, both of which are required for miRNA processing. Drosha contains two RNAse domains, A and B, which respectively cleave the 3' and 5' strands of the stem-loop to release the stem-loop from the pri-miRNA. The resulting 70nt RNA stem-loop is known as a precursor-miRNA (pre-miRNA). Following Drosha processing, the pre-miRNA is exported from the nucleus by exportin-5 and Ran-GTP.

1.2.1.3 Cytoplasmic processing

In the cytoplasm, the pre-miRNA is further processed into the mature miRNA by the RNAse III Dicer in complex with TRBP and, in humans, PACT. Dicer cleaves the pre-miRNA hairpin, by binding to the pre-miRNA with a PAZ domain and cleaving the RNA with an RNAase III domain, (facilitated by a helicase domain). This produces a 21 nt dsRNA with a 2 nt 3' unpaired overhang and a 5' phosphate, from which one of the strands is then degraded to leave the mature miRNA. Which of the two pre-miRNA strands becomes the mature miRNA is generally determined by the thermodynamic stability of the 5' end of each strand: the strand with the less stable 5' end becomes the mature miRNA, and the remaining strand is normally degraded. In some cases, both strands of the pre-miRNA can become mature miRNAs.

Following miRNA processing, TRBP recruits a member of the Argonaute (Ago) family of proteins and the mature miRNA is incorporated into the RNA Induced Silencing Complex (RISC). The Argonaute family of proteins is the key effector of miRNA-mediated mRNA repression. Argonaute family members, of which there are four members in humans and five members in mice, are composed of three main domains: the PAZ, PIWI and MID domains, which each play a role in miRNA binding and action. Structural studies show that the PAZ do-
main binds the 3' overhang of miRNAs while the PIWI and MID domains bind the 5' end of the miRNA\[^{50}\]. In some members of the Ago family, particularly Ago2 in humans, the PIWI domain is catalytically active, and capable of acting as an RNAse by cleaving targets bound to it, but this is not generally the case in miRNA-mediated repression\[^{51}\]. The role of the different members of the Ago family in the RISC remains unclear, but it is thought that in humans, they all contribute to miRNA-mediated repression\[^{51,52}\], although there is some evidence that different members of the Ago family may be associated with different mechanisms of mRNA degradation\[^{53}\], or have differing levels of repressive efficacy\[^{54,55}\].

As well as Dicer, TRBP, and the Ago family, other, less-well characterised proteins have also been identified as part of the RISC, including a number of helicases and RNA-binding proteins\[^{56-58}\]. The mature miRNA-incorporating RISC (miRISC) can then target mRNAs for repression.

### 1.2.2 MicroRNA targeting

The miRISC targets specific mRNAs which contain a binding site for that miRNA family, principally determined by complementarity to the seed sequence of the miRNA. The seed sequence is a 7 nt sequence found at positions 2-8 of the 5’ end of the miRNA, which is common to all members of a particular miRNA family\[^{59}\]. While complementarity to a miRNA seed sequence is an important determinant of miRNA binding affinity, it is not sufficient or necessary for miRNA action, and several other features can enhance miRNA binding. Computational prediction of miRNA binding sites using seed sequence complementarity often has to use additional features, such as conservation or increased AU content in the rest of the miRNA binding site, to detect potential sites with accuracy\[^{60,61}\]. Another commonly found feature of miRNA binding, which distinguishes miRNAs from siRNAs, is the lack of complementarity around bases 10-11 of the short RNA. MicroRNA binding sites are most commonly found in the 3’ untranslated region (UTR) of mRNAs, but have also been found in the 5’ UTR or in coding regions\[^{62,63}\]. Computational prediction of miRNA binding sites in humans suggests that many thousands of mRNAs, or up to 60% of all genes, may contain miRNA binding sites\[^{64,65}\]. However, despite this growing understanding of the determi-
nants of miRNA binding, computational target prediction still has a high level of negative predictions: proteomic studies of miR-233-depleted cells showed that while Targetscan is the most effective algorithm for prediction of miR-233 binding sites, only 33% of predicted sites were more responsive than transcripts with no predicted sites. Interestingly, recent analysis of data from miRNAs and mRNAs bound to Ago and pulled down by crosslinked immuno-precipitation has identified an additional binding mode for miRNA targets characterised by a bulged G nucleotide at position 5-6 of the miRNA binding site. This mechanism may account for around 15% of miRNA binding, suggesting that exact seed sequence complementarity may be a less important determinant of miRNA binding than previously thought.

1.2.3 Mechanisms of miRNA action

The repressive effect of miRNA targeting is well documented, but the mechanism by which it occurs remains controversial. In plant miRNA and in mammalian siRNA pathways, the RISC complex works by cleaving the targeted transcripts between bases 10 and 11 of the siRNA binding site, resulting in the endonucleolytic cleavage and degradation of the transcript, but this mechanism is rare in mammalian miRNAs. Several possible modes of action have been suggested, including sequestration of targeted transcripts away from the translational machinery in P-bodies or stress granules, mRNA degradation triggered by removal of mRNA associated proteins or the mRNA poly-A tail, and inhibition of translational initiation or of translational elongation. Experimental evidence exists for each of these mechanisms: it seems likely that in reality the miRISC works at multiple stages of gene expression.

1.2.3.1 Translational inhibition

Early miRNA experiments suggested that repression by miRNAs was entirely due to inhibition of translation, and that target mRNA levels were unchanged. While repression of translation is no longer thought to be the exclusive mechanism of miRNA-mediated repression, translational repression remains a controversial area of research, particularly the stage of translation at which inhibition
occurs: conflicting evidence exists that inhibition occurs during both the initiation and elongation steps.

The idea that miRNAs repress translation in a post-initiation step was first suggested when it was observed that, in *C. elegans*, miRNAs and miRNA targets localised with polysomes in sucrose gradient fractionation, suggesting that targeted mRNAs remained associated with ribosomes while repressed. However, subsequent studies have found conflicting results, with some showing that miRNAs and their targets localise with polysomes (and dissociate when treated with inhibitors of translation, such as puromycin), while others show that miRNA repression results in a shift in targeted mRNAs from the polysome fraction into lighter fractions, consistent with inhibition of translational initiation. The reasons for this apparent contradiction remain unclear, but one possibility is that moderate miRNA repression does not prevent all translation despite targeting by the RISC, resulting in the complex being associated with polysomes.

Supporting the idea that translational repression occurs at the initiation stage of translation, several studies have found that the m7-G-cap and poly-A tail found on mRNAs are required for miRNA repression of reporter genes, suggesting that miRNA action interferes with the proteins that bind to these structures and initiate translation, such as the cap-binding translation initiating factor eIF4F. Cap-independent translation, such as from an IRES or using tethered initiation factors, was unaffected by miRNA repression, which supports this idea. However, other studies have shown that miRNA inhibition of translation from IRES-containing transcripts can occur, and there is no clear way to reconcile these contradictory observations. One potential, but still controversial, solution is that miRNA regulation may depend on the promoter driving transcription of the target: this was proposed in a study which showed that transcripts from the TK promoter localised with polysomes, while the same transcript produced from the SV40 promoter did not. If miRNA repression of translation can occur post-initiation, a clear mechanism has yet to emerge. One suggested mechanism was the recruitment of proteases that co-translationally degrade the nascent polypeptide. However, targeting polypeptides to the ER, which should render them inaccessible to cytoplasmic proteases, has no effect on miRNA repression. An alternative theory is that miRNA targeting produces a reduction in translation.
rates resulting in an increased probability of ribosome drop-off \[^{76}\], but no clear evidence has emerged supporting either mechanism. There is still considerable controversy over the mechanisms of translational repression: strong evidence exists that repression occurs at the initiation stage, through the disruption of the assembly of the ribosomal initiation complex, but this does not explain a significant number of contradictory studies which suggest that translation is inhibited after inhibition by an unknown mechanism.

1.2.3.2 mRNA degradation

While translational inhibition was originally thought to be the only source of miRNA repression, considerable evidence has emerged, from both microarray profiling and studies of individual miRNAs, that miRNAs also cause degradation of target mRNAs \[^{54,84,85}\]. Studies in a number of systems suggest that this degradation is mediated by removal of the poly-A tail and subsequent digestion by RNA exonucleases \[^{86,87}\]. In particular, miRNA-mediated degradation has been linked to proteins commonly found in structures known as P-bodies (or GW-bodies), which have been associated with many proteins involved in mRNA degradation, such as the endonuclease XRN1, as well as decapping and deacylding enzymes \[^{88}\]. The protein GW182, which is localised to P-bodies, has been found to interact with and localise with Ago proteins. Silencing of GW182 reduces repression of miRNA targeted proteins, and disrupts P-bodies \[^{89,91}\], and tethering of GW182 to mRNAs results in their repression, even in the absence of Ago proteins, suggesting that GW182 operates downstream of Ago proteins \[^{92}\]. Argonaute proteins, miRNAs and repressed mRNAs have all been found to localise to P-bodies \[^{78}\]. GW182 may promote degradation by recruiting the deadenylation complex CCR4:NOT1 and the decapping complexes DCP1:DCP2 or RCK/p54 \[^{92,93}\].

While miRNA-directed transcript degradation is now an accepted component of miRNA-mediated repression, which factors determine the degree of either mRNA degradation or translational inhibition are still poorly understood: proteomic studies have shown that most miRNA targets are repressed at both the protein and mRNA level \[^{66,67}\]. Recent studies have also shown that translational
1. INTRODUCTION

inhibition occurs before mRNA-degradation: For example, studies of the impact of miR-430 on transcript localisation in zebrafish show that the level of ribosome-associated transcripts drops before the level of transcripts, indicating that translational inhibition occurs before mRNA degradation.74

1.2.3.3 mRNA sequestration

While the Ago-GW182 interaction was thought to be primarily associated with miRNA degradation, interactions with GW182 also localise RISCs and targeted mRNAs to P-bodies,78 suggesting that there may also be a repressive effect from physically isolating transcripts from the ribosomal machinery. Other studies have shown that miRNA repression may be reversible under certain conditions.9596 Investigation of the miRNA target CAT-1 showed that induction of the stress response resulted in the release of CAT-1 mRNA from P-bodies, suggesting that not all miRNAs in the P-bodies are degraded.29 This suggests another mechanism of miRNA repression: mRNA transcripts could be reversibly sequestered in P-bodies, away from the ribosomal machinery, to prevent protein synthesis. However, profiling studies of P-body mRNA populations in glucose-starved yeast have shown that only a small number of transcripts are reversibly sequestered, with the majority being degraded in P-bodies, suggesting that the majority of miRNA-targeted transcripts sequestered to P-bodies are degraded.97 Furthermore, other studies have shown that P-bodies, unlike Ago and GW182, are not required for miRNA-mediated repression: disruption of P-bodies by depletion of P-body components such as Lsm1 do not affect repression.93

Stress granules are cytoplasmic structures formed in cells under stress conditions or conditions of translational inhibition.98 As well as enrichment in P-bodies, studies have also shown enrichment of Ago proteins in stress-granules in a miRNA-dependent manner.99 However, it is not clear if this is a pathway for repression or an artefact of mRNAs, and their associated proteins (such as the miRISC), localising to stress granules under stress conditions.

It is not yet clear which of these mechanisms of repression is most common: high-throughput proteomic studies have shown that miRNA targeting results in repression of both the protein and mRNA, suggesting that in most cases more
1.2 MicroRNAs

than one of these mechanisms actually occurs. Furthermore, it is possible that these translational inhibition and mRNA degradation are actually coupled, so that translational repression may feed into mechanisms of mRNA degradation, for example by recruiting proteins that result in mRNA degradation.

1.2.3.4 Non-canonical miRNA pathways

As well as the classic “canonical” pathways for miRNA biogenesis and action, a large number of less common mechanisms of biogenesis, processing and action have been observed for specific miRNAs. For example, while the majority of miRNAs are processed into pre-miRNAs by Drosha, there are Drosha-independent modes of processing, such as the miRtron pathway, where a miRNA-containing intron is processed into a pre-miRNA by the splicing machinery in a Drosha-independent manner.

As well as repressing translation of proteins and degrading mRNAs, there is some suggestion that miRNAs may have a role in post-transcriptional gene silencing: this is a well-documented function of miRNAs in plants, but evidence for it in animals remains controversial: one recent study in fibroblasts showed that AGO proteins can localise to the nucleus and associate with heterochromatin, and that transcription from reporters containing miRNA-binding sites was reduced in the presence of miRNAs, but evidence for miRNA-mediated silencing remain rare.

Finally, there is some evidence that miRNAs may also have a role in the up-regulation of specific transcripts, possibly by the recruitment of upregulating proteins or by competing with other repressive factors. These effects seem to occur at specific stages of the cell cycle and in conditions of cell-cycle arrest, which may explain why these effects had been previously overlooked.

1.2.3.5 Modulators of miRNA action

Many miRNAs are also regulated post-transcriptionally by specific proteins: for example, the protein Lin28 has been found to block the processing of members of the let-7 family of miRNAs in embryonic cells, resulting in reduced levels of mature miRNAs. A number of mechanisms for this action have been proposed.
including inhibition of processing of let-7 members by Drosha in the nucleus and inhibition of Dicer processing in the cytoplasm, either directly or by uridylation of the 3’ end of the pre-miRNA in the cytoplasm\cite{108,110}. Conversely, the SMAD family of proteins can promote processing of miR-21 in response to TGF-β or BMP signalling by binding to p68, a helicase which can form part of the microprocessor complex\cite{111}. Individual studies and high throughput sequencing have also found that many miRNAs are post-transcriptionally edited, replacing adenosine residues with inosine residues: the effect of this modification is poorly understood, but may trigger inhibition of Dicer activity or degradation of modified miRNAs\cite{31,112}. Some proteins may directly repress miRNA action: the mRNA-binding protein HuR has been found to relieve repression of the \textit{CAT-1} mRNA by miR-122, potentially by changing mRNA folding to render the miR-122 binding site inaccessible\cite{79}. This wide range of miRNA-modulating proteins adds another layer of regulatory complexity to miRNA-mRNA interactions.

One particularly interesting area of research has been the role of miRNAs as a medium for intracellular signalling. Recent research has shown the miRNAs can allow cross-talk between particular RNA transcripts, so that if one transcript containing a number of miRNA binding sites is expressed, this depletes the available “pool” of those miRNAs available to bind other transcripts, reducing the repressive effect of those miRNAs\cite{113}.

Finally, miRNAs may not in fact function in the cell in which they are transcribed: a number of studies have detected the presence of functional miRNAs in vesicles, and shown that miRNAs may be transported to other cell types to repress mRNAs, although this function remains controversial. For example, fluorescently tagged miRNAs have been found to transfer from B-cells to T-cells on cell contact\cite{114}, and miR-containing exosomes have been identified from mast cells\cite{115}.

### 1.2.4 MicroRNA function

While the mechanisms by which miRNA are produced and act are now well-characterised, the role of many miRNAs remains unclear. Depletion of all mature miRNAs by constitutive deletion of \textit{Dicer} is lethal in mice, resulting in deple-
Deletion of the *C. elegans* Dicer ortholog *dcr-1* renders *C. elegans* sterile, preventing maternal inheritance of *dcr-1* by additional knockdown of maternal *dcr-1* transcripts results in lethality. Deletion of other essential components of the miRNA biogenesis pathway have similar effects: deletion of *Drosophila* TRBP homolog *loquacious* results in depletion of germ-line cells and sterility. Similarly, deletion of *DCGR8* in mice results in early embryonic lethality, although *DCGR8* and *Dicer* mutant phenotypes show some differences, potentially because they process slightly different subsets of miRNAs. To investigate the role of *Dicer* in mouse tissues, conditional *Dicer* knockouts have been developed, which show a variety of developmental abnormalities: for example, in limb development, *Dicer* deletion under the control of the limb mesoderm specific promoter *prx1* results in delayed development and a smaller limb bud, but no defects in patterning or tissue specification; in lung development, *Dicer* deletion under the control of the *shh* promoter results in fewer epithelial pouches and increased cell death.

Investigation of the function of individual miRNAs generally show less dramatic effects. While miRNAs are predicted to target a large proportion of the genome, the function of many of these interactions remains to be determined. The first microRNAs discovered, such as the let-7 family, were identified by forward genetic screens, linking mutated miRNAs with specific phenotypes and giving clear evidence of the primary roles of a specific miRNA. Relatively few miRNAs have been identified in this fashion, however, with most being identified by sequencing experiments. Proteomic and microarray studies have shown that knock-out of specific miRNAs results in relatively small changes in the expression of both mRNA and protein of a large number of genes, but phenotypic effects from specific miRNA knock-out are observed comparatively infrequently: in *C. elegans*, individual mutations of 95% of known miRNAs resulted in no significant phenotypic changes.
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1.2.4.1 MicroRNAs in development

MicroRNAs were originally discovered as regulators of development in *C. elegans* and many more instances of miRNA regulation in development of *C. elegans* and other model organisms have since been discovered. In mammals, miRNAs play a number of important roles right from the start of development: *Dicer*-deleted ES cells are viable, but show proliferation defects and fail to successfully differentiate\[^{123}\]. These problems are thought to be due to depletion of the miR-290 family, which has been shown to regulate regulators of the cell cycle, causing proliferation defects, and *de novo* methyl transferases, disrupting mechanisms of differentiation\[^{124,125}\]. Many MiRNAs are also found later in development: for example, miR-196 has been found to regulate expression of Hox genes during mouse hindlimb development\[^{126}\] and many other instances of miRNA regulation in mammals and other organisms have been observed\[^{122,127,128}\]. However, only a small fraction of predicted miRNA-mRNA interactions during development have been investigated: the majority have not been experimentally validated and the significance and effects of these predicted interactions, if any, are unknown.

Some conclusions about the roles of miRNAs can be drawn from looking at the expression patterns of miRNAs and their targets: for example, a number of tissue-specific miRNAs have been identified, including miR-1 in muscles and miR-124 in neural tissue\[^{129}\].

Given these features of miRNA expression, a number of (not necessarily contradictory) different hypotheses have been suggested for the general role of miRNAs as a class, to explain the lack of strong interactions that have been observed.

Firstly, it has been suggested that miRNAs “tune” gene expression, adjusting gene expression by relatively small amounts to an optimum value, integrating the signal from many miRNAs through multiple miRNA binding sites on a given mRNA. Some papers have described this function of miRNAs a “rheostat” function, which is an analogy with the rheostat in electrical circuits, which allows the resistance of the circuit to be varied: similarly, miRNAs allow the degree of repression in a genetic network to be varied. Several papers have categorised miRNA-mRNA into “tuning” and “switch” interactions, depending on
whether the response of the target to miRNA regulation is a small adjustment or a large response (typically downregulation of the target to functionally negligible levels). An example of a tuning interaction would be the regulation of senseless by miR-9a in Drosophila. The decision to adopt the sensory organ precursor (SOP) lineage in Drosophila is made by a combination of positive feedback between proneural genes and lateral inhibition through the Notch-Delta pathways. In particular, the transcription factor Senseless has a key role in this system: it is upregulated by proneural transcription factors, and in turn increases the expression of proneural genes, forming a positive feedback loop, as well as increasing the expression of Delta, which works to inhibit proneural expression in neighbouring cells by activation of the Notch receptor. While the initial inputs to the system are stochastic, the combination of positive feedback and lateral inhibition means that only a fixed proportion of cells can become SOP precursors. In this system, miR-9a represses Senseless expression and sets a threshold of Senseless expression required for full activation of the positive feedback loops, essentially tuning Senseless expression to produce the required number of sensory organs. miR-9a-deleted flies show increased numbers of sensory neurons.

However, this categorisation is a considerable over-simplification: much repression by miRNAs cannot be clearly categorised into tuning or switch relationships, and falls somewhere in between the two categories: causing significant changes in gene expression, but not completely repressing genes. This categorisation also fails to take into account the dynamic natures of gene regulation: during development, gene expression is rarely static and it is not clear how miRNAs tune a dynamically regulated gene.

Secondly, miRNAs may act to repress inappropriate gene expression: many miRNA targets are found downregulated in tissues where the targeting miRNA is expressed, leading to suggestions that the major role of miRNAs is to downregulate lineage-inappropriate gene expression by repressing expression of inappropriately transcribed mRNAs, or to sharpen developmental transitions by repressing mRNAs remaining following transcriptional downregulation during a developmental change. This idea was initially supported by the identification of predicted miRNA targets that were downregulated as their targeting miRNA was expressed, and that miRNA targets were often expressed in adjacent tissues.
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to those expressing targeting miRNAs\textsuperscript{132}. However, it is not clear from available
data whether the downregulation seen in miRNA targets when expressed in the
same tissue is a consequence of miRNA regulation or due to transcriptional reg-
ulation, and other studies have found that many miRNA regulatory interactions
are upregulated at the same time as their targets\textsuperscript{135}.

Finally, a third theory synthesises these ideas and incorporates concepts from
systems biology: miRNAs may act to increase the robustness of gene networks,
both to external challenges such as temperature change and to internal challenges
such as biological noise. This hypothesis combines the idea that miRNAs work to
downregulate inappropriately expressed genes, as well as the idea that miRNAs
can tune gene expression to appropriate levels, but also incorporates a new idea:
that miRNAs can also function as part of larger network motifs to reduce bio-
ological noise.

1.2.4.2 MicroRNAs and noise

With a few notable exceptions, experimental deletion or depletion of specific
miRNAs often have relatively limited phenotypic effects. One hypothesis to
explain this, and to explain the general role of miRNAs has been that they act
to buffer transcriptional networks against perturbations, including biological
noise\textsuperscript{28,136}. Because mature miRNAs are produced by less complex mechanisms
than proteins, it is thought that they may respond faster to transcriptional upreg-
ulation, and therefore make superior regulators in network motifs such as nega-
tive feedback loops. The short length of miRNA sequences and miRNA binding
sites also mean that miRNA-mRNA interactions need fewer mutations to form
than protein-DNA interactions, and are therefore more likely to appear during
evolution.

In particular, it has been suggested that miRNAs function as part of FFL net-
work motifs (Figure 1.1). In a miRNA-containing FFL, an upstream transcrip-
tional factor will regulate the transcription of both a miRNA and a gene which
is regulated by that miRNA. Like other FFLs, MicroRNA-containing FFLs can be
divided into two categories: coherent FFLs, where the miRNA and other com-
ponents produce the same effect on gene expression, or incoherent FFLs, where
the miRNA and other components oppose each other. It was initially suggested from predictions in *Drosophila* that the majority of FFLs were coherent loops involved in reinforcing developmental boundaries. However, more recent studies have shown that miRNA containing FFLs of both types are overrepresented in the human genome, compared to other transcriptional motifs. Both forms of FFL have been hypothesised to increase robustness in gene expression, but through different mechanisms. It is easy to see that coherent FFLs containing miRNAs could reduce noise in gene expression by increasing repression of inappropriately expressed genes, effectively reinforcing changes in gene expression. Because miRNAs target mRNAs, they can target remaining transcripts after transcription has ceased, sharpening developmental transitions. The role of incoherent FFLs is less clear, as the effect of incoherent regulation depends on the dynamics of miRNA-mRNA regulation, but several models of miRNA action have suggested that they may also act to increase the robustness of gene expression. The simplest model, suggested by Hornstein and Shomrom, is that regulation by miRNAs allows an increased rate of transcription to compensate for translational repression. As "bursting" in transcription is the principal source of noise in gene expression, and increasing transcription reduces the noise from "bursting", miRNA regulation allows transcriptional noise to be reduced while maintaining the same rate of gene expression. An alternative suggestion is that miRNAs act to buffer the expression of genes against extrinsic noise from upstream factors such as transcription factors: for example, in a simple incoherent FFL, transient increases in the expression of the upstream transcription factor will result in an increase of transcription of both a targeted gene, and also a miRNA; the expression of the miRNA will repress translation of the downstream target, cancelling out the effect of the transient increase in the original transcription factor. Several, more mathematical models have also been suggested, using either direct analytical solution of simplified models of the basic interactions in miRNA regulation (transcription, translation etc) or computational simulations which simulate many iterations of the system and calculate the average expression and other statistics such as standard deviation. A number of these computational studies have looked at miRNA-based FFLs, and have shown that miRNA-based FFLs can reduce variation in gene expression, both compared to unregulated genes, genes
regulated by miRNAs that are not part of an FFL, and genes regulated by protein FFLs and that miRNA-based regulation is more effective at reducing noise than transcription factor based regulation, primarily because protein translation requires more processing steps (i.e., translation) which result in additional noise in the system \textsuperscript{138}. Other simulations have also found that regulation of gene expression at the mRNA level is more effective at reducing noise, and that miRNA-based is more effective at filtering upstream noise than protein-based gene regulation \textsuperscript{139,140}.

However, most of the investigations into the role of miRNA in controlling gene expression noise have remained \textit{in silico}, and remain sensitive to the choice of model architecture and of reaction parameters: experimental evidence for the role of miRNAs in regulating variation in gene expression remains limited: many coherent and incoherent FFLs have been identified, but relatively few have then been linked directly to robustness or reduced variation in gene expression.

In \textit{Drosophila}, it has been shown that knockout of miR-7, which forms part of a complex network and regulates the \textit{enhancer of split} transcription factor, coupled with fluctuating temperature results in defects in sensory organ development that are not present under stable temperature conditions, suggesting that miR-7 increases developmental robustness to temperature variation \textsuperscript{141}.

1.2.4.3 MicroRNAs in disease

MicroRNAs have been implicated in a number of diseases. Supporting the idea that miRNAs are involved in increasing the robustness of gene expression, only two Mendelian genetic conditions associated with mutations in miRNAs are known, but miRNAs have been found in many diseases associated with stress and gene disregulation, particularly cancer \textsuperscript{142}.

The two known miRNA-associated mendelian genetic conditions are nonsyndromic autosomal dominant progressive hearing loss, caused by mutations in the seed sequence of miR-96 \textsuperscript{143}, and Feingold syndrome, which is caused mainly by mutations in the \textit{MYCN} gene, but in a minority of cases is caused by mutations in the miR-17-92 cluster \textsuperscript{144}. A large number of different miRNAs have been associated with cancer in different cell types. For example, overexpression of miR-155
1.3 T-lymphocytes

T-lymphocytes, so called because they develop in the thymus, are one of the principal components of the adaptive immune system. Because of their experimental accessibility and importance to human disease, T-lymphocytes are one of the best understood mammalian developmental systems, with many of the developmental stages, signalling pathways and transcriptional programmes well characterised. This makes T-cell development an ideal experimental system for studies of developmental principles. Mature T-cell populations consist of two main cell types, T helper (T\textsubscript{H}) and T cytotoxic (T\textsubscript{C}) cells, experimentally distinguished by the expression of either CD4 or CD8 cell surface proteins. A smaller population of cells expressing the \(\gamma\delta\)-TCR variant also exists, forming a separate lineage. Each of these populations has a distinct role in the immune system. T-helper cells secrete cytokines which can trigger responses in neighbouring cells, while cytotoxic T-cells bind to foreign cells and trigger a number of responses which act to kill targeted cells. T-helper cells can be further subdivided into a number of subsets, including Th-1, Th-2, T-regulatory, and Th-17 lineages, characterised by the secretion of different subsets of cytokines. Th1 cells secrete interferon \(\gamma\) (IFN-\(\gamma\)) and tumour necrosis factor \(\beta\) (TNF-\(\beta\)), Th2 cells secrete a number of interleukins (IL-4,-5,-10 and -13) while Th-17 are characterised by production of IL-17\(\textsuperscript{152}\). T-regulatory cells have a role in suppressing the immune response,
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through mechanisms such as the secretion of immunosuppressive cytokines IL-10 and TGF-β, and expression of immunosuppressive surface molecules such as CTLA-4\textsuperscript{153}. While many different T-cell subtypes have been identified and characterised, the T-cell system also shows considerable plasticity, with a number of cases of T-cells switching subtype or showing altered cytokine secretion in response to particular stimuli; for example, Th-2 cells have been shown to switch to Th-1 cells under certain conditions\textsuperscript{154,155}.

1.3.1 T-cell activation

Both T\textsubscript{H} and T\textsubscript{C} cells bind to antigens displayed as part of the major histocompatibility complex (MHC) through the T-cell Receptor (TCR). T\textsubscript{H} cells bind to class II MHCs, expressed by antigen presenting cells (APCs) such as B-cells, dendritic cells, and macrophages, while T\textsubscript{C} cells bind to class I MHC, which are expressed on the majority of cells. Binding of the MHC to the TCR in complex with CD3 (along with costimulation of the CD28 receptor) triggers signalling pathways that cause a program of gene expression, cytokine secretion and differentiation that is known as T-cell activation.

The TCR is a heterodimer composed of either α and β chains or γ and δ chains. The two variants have different binding characteristics and functions: αβ TCR expressing cells are better understood, and bind to MHC-antigen to trigger canonical T\textsubscript{H} or T\textsubscript{C} responses. γδ-expressing T-cells are a separate lineage (with no T\textsubscript{H}/T\textsubscript{C} subsets) whose function is less well understood: some γδ T-cells can bind to native antigen without requiring MHC presentation and the γδ TCR shows limited variability compared to the αβ TCR\textsuperscript{156}.

T-cell activation requires stimulation through successful binding of the TCR to a suitable MHC-antigen complex, as well as costimulation of CD28 by members of the B7 family. Engagement of the TCR allows phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) on the cytoplasmic domains of TCR-associated CD3 complex by members of the Src tyrosine kinase family such as Lck or Fyn. The mechanisms by which this is triggered are likely to be a combination of conformational change in CD3 polypeptides allowing access to ITAMS that were previously blocked by the plasma membrane\textsuperscript{157}, and aggregation of
multiple TCR receptors (and coreceptors) to allow transphosphorylation of CD3 chains, and exclusion of inhibitory phosphatases. Lck kinase binds to the co-receptors CD4 and CD8 and, as well as phosphorylating CD3 ITAMs, also acts to recruit CD4/8 coreceptors by binding to phosphorylated tyrosine residues via SH2 domains. The interaction of TCR and other proteins with the MHC proteins and costimulatory ligands is sometimes referred to as the formation of the “immunological synapse”, by analogy to neural synapses. The immunological synapse is a complex structure, involving a large number of receptors, scaffolding and signalling proteins. Key among them are the members of the Syc family, ZAP70 and Syk, which bind via SH2 domains on CD3 phosphorylation and are in turn phosphorylated, recruiting two more key adaptor proteins, LAT and SLP-76. These proteins activate a number of different intracellular signalling pathways, as well as triggering cytoskeletal rearrangements and changes in external cell signalling through proteins such as integrins. Three main intracellular signalling pathways are triggered by T-cell activation, as briefly summarised below. LAT engages PLCγ, which hydrolyses phosphatidylinositol (4,5) biphosphate (PIP2) into diacyl-glycerol (DAG) and inositol 1,4,5 trisphosphate (IP3). IP3 triggers calcium influx from the endoplasmic reticulum, triggering signalling through calcium-dependent pathways such as the NFAT transcription factor. DAG activates the Ras-ERK signalling pathway, triggering a chain of kinases which results in the activation of the transcription factor AP-1. DAG also activates PKCθ, which allows the assembly of a membrane-bound complex of proteins including CARMA1, BCL-10 and MALT1, known as the CBM complex which can activate the NF-κB signalling pathway by activation of IKK, which phosphorylates IκB, releasing NF-κB (Figure 1.3). As well as direct binding of the TCR, binding to other co-receptors is also required for full T-cell activation: without CD28 binding, TCR engagement produces a response known as “anergy” where the cells become unresponsive to further TCR stimulus. CD28 binds to members of the B7 family of ligands, which are highly expressed on activated APCs, and contributes to the activation of the PI3K signalling pathway, as well as upregulation of other T-cell activation pathways. TCR and coreceptor pathways show considerable crosstalk and interaction, allowing modulation of the immune response depending on what combinations of signals the TCR and other
coreceptors have received: for example, expression of only the Ca$^{+}$-induced transcription factor NFAT acts to induce genes associated with anergy, but activation of both NFAT and the AP-1 transcription factor allows expression of genes associated with full activation$^{167,168}$. T-cell activation must be highly specific to allow detection of a relatively small number of antigen-presenting cells displaying pathogen-specific protein/MHC against a background of many cells displaying self-peptide/MHC. To achieve this the signalling pathways of T-cell activation involve a network of interactions to assure correct activation. Key among them is “kinetic proofreading”, where for a ligand to achieve full T-cell activation, it must bind to the receptor for long enough to recruit a number of cofactors and to overcome a number of repressive mechanisms, such as the phosphatase SHP-1, which dephosphorylates a number of signalling components, such as Lck$^{169}$.

Full T-cell receptor signalling results in immediate activation of a number of transcription factors, including NFAT NF-$\kappa$B, c-Myc and c-Fos and c-Jun (which are together also known as AP-1). These in turn produce a number of responses, including upregulation of cell surface markers CD69 and CD25, expression of IL-2 and rapid cell proliferation, as well as differentiation into effector or memory subtypes. T-cell activation responses are rapidly induced: activation of transcription factors takes place within 30 minutes of TCR binding, and expression of early activation markers such as CD25 is detected within 2 hours$^{152}$.

CD69 is a homodimeric cell surface glycoprotein, which is an important marker of T-cell activation, and is strongly upregulated in T-cells immediately after activation$^{170}$. This response is thought to be driven mainly by the ERK pathway, as expression of a dominantly-negative Ras allele reduced CD69 expression following activation$^{171}$. CD69 is also expressed on a wide variety of other cells in the haematopoietic system, including platelets, natural killer (NK) cells and neutrophils$^{172}$. Little is known about the biological function of CD69: studies have shown that it has roles in TGF-$\beta$ signalling, T-cell emigration and regulation of the sphingosine-1-phosphate-receptor 1, but no ligand for it has yet been identified$^{173,174}$.
Figure 1.3: T-cell activation signalling - Simplified diagram of major components and pathways of T-cell activation
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1.3.2 T-cell development

All types of T-cell originate from the thymus, which was first identified as a key component of the immune system in 1961, when it was found that removal of the thymus immediately after birth resulted in increased mortality from infection and a reduction in the number of lymphocytes177. The development of $\alpha\beta$ T-cells from haematopoietic precursor cells into mature, functional components of the adaptive immune system is a complex, multi-stage process which includes the process of TCR rearrangement and selection. TCR rearrangement is the process by which the loci containing the two chains of the TCR, ($\alpha$ and $\beta$) are rearranged by the RAG1/2 recombinase. The resulting diversity of different receptors undergoes selection to eliminate receptors which cannot bind MHC-antigen complexes or which have too high an affinity for MHC presenting self antigen.

All TCR polypeptides are made up of variable and constant domains in a similar manner to immunoglobins. During T-cell development, they undergo rearrangement of variable (V), diversity (D) and joining (J) gene segments to produce a complete chain containing one of each segment in a similar manner to immunoglobins. The mouse $\alpha$ TCR chain has around 150 V, and 50 J segments, while the $\beta$ chain has around 20 V, 2 D and 10 J segments: successful rearrangement joins one of each of the segments (V and J in $\alpha$, V, D and J in $\beta$) to express a complete variable domain178. T-cell rearrangement is catalysed by the RAG-1/2 enzyme, which binds to 7- and 9- nt recognition sequences which flank the V, D and J segments. The RAG recombinase then excises a segment and the double-strand-breaks that remain are repaired by components of the DNA repair pathways. Additional diversity is generated by the imperfect cleavage and joining of segments, which may introduce additional nucleotides at segment junctions179. This rearrangement process allows the generation of considerable combinatorial diversity in TCR sequence across the thymocyte population, which is crucial for the processes of adaptive immunity. The structure of the TCR genes is such that a successful rearrangement of the $\alpha$ chain deletes the constant domain of the $\delta$ chain, preventing co-expression of both receptors.

T-cell development begins when lymphoid precursors migrate from the bone marrow to the thymus. As the immature T-cells mature, rearrange and express
the TCR, and undergo selection, they go through a series of developmental stages characterised by the expression of several cell surface protein markers, principally CD4 and CD8, but substages of development are characterised by other markers. Initially, new entrants to the thymus do not express CD4 and CD8 and are known as double negative (DN) cells. These cells are further divided into four subsets of cells, distinguished by expression of CD25, CD44 and c-kit. Initially cells are known as DN1, and express CD44 and c-kit: after entry to the thymus, DN1 cells proliferate and develop into DN2 cells, which also express CD25. At the DN2 stage, cells start to undergo rearrangement of the TCR β, δ and γ chains. As thymocytes transition to the DN3 stage, a small percentage of cells diverges to become γδ T-cells, while the remaining cells downregulate c-kit and CD44 and become DN3 cells. At the DN3 stage, proliferation is reduced and the TCR β chain is expressed, forming the pre-T-cell receptor with the pre-Tα and CD3. Formation of functional pre-TCR results in signalling that causes the cell to stop β-chain rearrangement, allows TCR α chain rearrangement and triggers differentiation to the CD4<sup>+</sup>CD8<sup>+</sup> double positive (DP) stage of development. At
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the DP stage, both CD4 and CD8 are expressed, and the rearrangement and expression of the TCR $\alpha$ chain occurs\[152].

Following expression of a complete TCR $\alpha\beta$ chain, thymocytes undergo thymic selection, a process where they interact with cells in the thymic epithelium to select for cells which express suitable TCRs to function as mature T-cells. If TCR rearrangement does not produce functional TCR, a thymocyte may go through several rounds of TCR $\alpha$ chain rearrangement and expression before either successfully expressing a functional TCR or dying of neglect. The mechanisms which control T-cell selection are complex, depending on the interaction of rearranged TCR and the thymic environment.

During TCR selection cells undergo both positive and negative selection. In positive selection, only cells which express a TCR capable of binding sufficiently to MHC in the thymus survive to differentiate into single positive thymocytes. Individual T-cells that survive selection and successfully engage MHC class I or II will become CD8$^+$ or CD4$^+$ SP cells respectively. Mice incapable of producing class I or class II MHC cannot produce SP CD8$^+$ or SP CD4$^+$ thymocytes respectively\[180,181]. Positive selection eliminates thymocytes which do not express a functional TCR, or express a TCR with insufficiently strong binding to MHC; this can be $\sim$90% of the thymocyte population\[182]. Key to the survival of thymocytes is the orphan nuclear receptor ROR$\gamma$, and members of the $\beta$-catenin signalling pathway (such as TCF-1), which regulate expression of the anti-apoptotic factor Bcl-xL. Knockout of ROR$\gamma$ or TCF-1 results in increased levels of apoptosis in DP thymocytes and reduced levels of Bcl-xL\[183,184]. The mechanisms by which successfully selected TCRs recognise antigen/MHC as opposed to the self-peptide/MHC which they are exposed to in the thymus is still not clearly understood, although there is some evidence that TCR binding during selection is relatively promiscuous during TCR selection, and TCR binding in mature T-cells is more selective\[185].

In negative selection, cells that bind too strongly to self-MHC undergo apoptosis, eliminating around half of the remaining, positively selected thymocytes. This process was observed in thymocytes expressing the V$\beta$17a TCR segment: V$\beta$17a$^+$ cells were not present in the mature thymocyte population of mice expressing the class II MHC molecule IE, but were present in immature T-cells,
indicating that some mechanism was eliminating those cells in a TCR-binding dependent manner\textsuperscript{186}. The mechanisms of positive selection remain unclear, and several important questions remain, such as the role of co-receptors and the mechanisms by which positively selecting TCR engagement is differentiated from negatively selecting TCR engagement. It is thought that induction of negative selection from TCR engagement occurs through a process of kinetic proofreading, where not only is TCR engagement required, but ligands must remain bound for sufficient time to allow full activation of the TCR pathways: this allows cells to distinguish between a high concentration of low-avidity ligands and a low concentration of high-avidity ligands\textsuperscript{169}. Another key question in negative selection is whether binding of a co-receptor such as CD28 is required. Knockout of CD28 and other coreceptors such as CD43 has little effect, although stimulation of these receptors with antibodies results in increased apoptosis\textsuperscript{187}.

Following successful TCR rearrangement and selection, remaining DP cells further differentiate, first into CD4\textsuperscript{+}CD8\textsuperscript{lo} intermediates, then into CD4\textsuperscript{+}CD8\textsuperscript{−} or CD4\textsuperscript{−}CD8\textsuperscript{+} single positive (SP) T-cells, depending on which MHC class they are capable of binding. The mechanisms by which TCR binding determines lineage choice remains controversial, but it is known that CD4\textsuperscript{+} precursors upregulate the transcription factors ThPok and cKrox\textsuperscript{188,189}, while CD8\textsuperscript{+} cells upregulate the RUNX family of proteins. These factors drive the silencing of CD8 and CD4 respectively, and differentiation into mature T-cells\textsuperscript{190}. These cells then migrate out of the thymus into the circulation, where they become mature naive T-cells, and circulate between the blood and lymph systems.

### 1.3.3 MicroRNAs in T-cell development

MicroRNAs have been found to play a role at several stages of T-cell development: studies using conditional \textit{Dicer}-deleted mouse strains have observed the effect of depleting miRNAs on thymic development, and found that deletion of \textit{Dicer} at the DN stage of development results in a significantly reduced thymic population (~tenfold fewer cells) mainly due to reduced survival of \(\alpha\beta\) lineage T-cells. Despite this, surviving cells appear to undergo selection and lineage commitment to CD4\textsuperscript{+} and CD8\textsuperscript{+} lineages normally\textsuperscript{191}. 
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As well as investigations of the effect of Dicer deletions in T-cell development, a number of studies have looked at the role of individual miRNAs in regulating gene expression at particular stages of T-cell development. These have identified a number of miRNAs which play a role in T-cell development and function. These miRNAs include the miR-181 family, the miR-17-92 cluster and homologs, miR-155 and miR-150.

1.3.3.1 The miR-181 family

One particularly well-characterised miR family in thymocyte development is the miR-181 family, whose role in the immune system was first investigated when it was found that overexpression of miR-181 in haematopoietic progenitor cells produced a shift in lymphocyte towards the B-cell lineage. This family, which is made of miR-181 a, b, c and d, is expressed in three clusters: miR-181ab-1, miR-181ab-2 and miR-181cd. Mir-181a and b have been found to be highly expressed at the CD4+ CD8+ DP stage of thymocyte development, where they have been shown to regulate a number of different targets, including the cell surface marker CD69, anti-apoptotic protein Bcl-2, and the TCRα chain. miR-181a has also been shown to alter the sensitivity of thymocytes to TCR stimulation, potentially by downregulating the phosphatases SHP2, PTPN2, DUSP5 and DUSP6, which dephosphorylate components of the activation pathway, including Lck, ZAP70 and ERK 1/2. This effect partially explains the increased sensitivity to TCR activation that is observed in immature thymocytes.

1.3.3.2 The miR-17-92 cluster

One of the first polycistronic miRNA transcripts identified, the miR-17-92 cluster is made up of six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92), and also has two additional homologous clusters: miR-106a-363 and miR-106b-25. These clusters express many different miRNAs from four different families (miR-17, miR-18, miR-19, and miR-25) and have been highly conserved during vertebrate evolution. The mir-17-92 cluster is ubiquitously expressed during lymphocyte development, but is downregulated on maturation. Deletion of the miR-17-92 cluster is lethal, resulting in death shortly after birth.
1.4 Aims of this investigation

The principal aim of this study is to investigate the potential role of miRNAs in regulating cell-cell variation. To this end, focusing on the well-characterised
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developmental system of the thymus, we use conditional Dicer knockout mice to deplete miRNAs and single-cell methods to quantify the changes in gene expression and variation that result. To further characterise the system, we use a combination of methods, including fluorescent reporter constructs, individual miRNA knockdown and miRNA profiling by RT-qPCR, to identify specific miRNAs which may regulate noise and attempt to dissect the mechanisms by which they act.
2 Materials & methods

2.1 Mice

Animal work performed in accordance with the Animals (Scientific Procedures) Act, under project license 70/6845. LckCre $\text{Dicer}^{\Delta/\Delta}$, cd4Cre $\text{Dicer}^{\Delta/\Delta}$ and $\text{Dicer}^{\text{lox}/\text{lox}}$ mice generously provided by B. Cobb. C57Bl/6 mice and C57Bl/6 Thy1.1$^+$ mice provided by L. Bruno. Mir-181ab-1 knockout mice were generously provided by Chang-Zheng Chen, and experiments using these mice were performed in Stanford, USA under local animal regulations.

2.2 Cell culture

2.2.1 Primary cell culture

Unless otherwise stated, cell culture reagents were supplied by Gibco Invitrogen. Primary mouse thymocytes, peripheral T-cells and thymic reaggregates were cultured at 37 °C in 5% CO$_2$ in T-cell media: Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 10% foetal calf serum, penicillin (100 U/ml)/streptomycin (100 µg/ml), 2 mM L-Glutamine and 50 µM 2-β mercaptoethanol.

To create primary whole thymus and peripheral T-cell cultures, thymi or peripheral lymphoid organs (axillary, brachial, inguinal, cervical and mesenteric
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lymph nodes) were dissociated in 10 ml T-cell media using a 2 ml syringe plunger to pass cells through a 70 µm cell strainer. Subsequently, cells were centrifuged at 240 g for 5 minutes and cell pellets were resuspended in 10 ml T-cell media. Cells were counted using a haemocytometer and resuspended at an appropriate density for the required experiments.

2.2.2 Cell lines

293T cells (provided by L. Bruno) for retroviral production were grown in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% foetal calf serum, penicillin (100 U/ml)/streptomycin (100 µg/ml), 2 mM L-Glutamine and grown at 37 °C in 5% CO₂. Cells were detached from the plate surface by pipetting and split 1/10 every 2-3 days, to maintain an average cell density of 10⁶ cells/ml.

2.2.3 Fetal thymic organ culture

Thymic stromal cells prepared by removing thymi from 14-16 day C57Bl/6 embryos, separating thymi into individual thymic lobes using forceps, and incubating for 5-7 days on 0.8 µm nuleopore filters floating on T-cell medium (described above) supplemented with 1.35 µM 2-deoxyguanosine (dGuo) to eliminate the resident thymocytes. Following incubation, lobes were washed with PBS, pelleted by centrifugation at 240 g for 5 minutes, and then incubated for 30 min in 5 ml 0.05% trypsin-EDTA. Following trypsinisation, 2 ml FCS was added to neutralise the trypsin, and the cells were then resuspended in 2 ml media and fully dissociated by repeated pipetting. The resulting stromal cells were mixed with experimental primary thymocytes in a ratio of 1:4 to respectively. The aggregated cell mix was pelleted and the supernatant removed to produce a cell slurry. The mix was pipetted onto a 0.8 µm nuleopore filter floating on T-cell medium and incubated for 24 hours before disaggregation (using a 30G syringe), cell surface staining, and analysis.
2.2 Cell culture

2.2.4 T-cell activation

For thymocyte and peripheral T-cell activation with plate-bound anti-TCR antibody, Nunclon Delta flat-bottom plates were coated with anti-TCR antibody at a concentration of 500 ng/ml in PBS unless otherwise stated, either overnight at 4 °C or for four hours at 37 °C. These plates were then washed twice with PBS (without Ca²⁺ or Mg²⁺) before cells were added. T-cells were added to the plates at a density of 10⁶ cells/ml unless otherwise stated, and were co-activated with anti-CD28 antibody at a concentration of 2 µg/ml. Unless otherwise stated, cells were then incubated for 18 hours at 37 °C in 5% CO₂.

For retroviral infection, peripheral T-cells were activated with mouse CD3/CD28 coated dynabeads (Invitrogen). 25 µl beads per 10⁶ cells were washed 3 times with T-cell media and resuspended in 100 µl per 10⁶ cells. Peripheral cells were suspended at a density of 10⁶ cells/ml and added to 24 well Nunclon plates, 1 ml per well. 100 µl washed beads were added to each well. Cells incubated for 18 hours at 37 °C in 5% CO₂.

2.2.5 Retrovirus production and infection

293T cells were transfected with retroviral plasmids and packaging plasmids pCL-Eco by CaCl₂ precipitation. Briefly, DNA precipitated onto cells by addition of 500 µl fresh CaCl₂ (0.4 M concentration, dissolved from solid CaCl₂ fresh for each experiment and sterilised by passing through a 0.45 µm syringe filter), containing 4 µg of the packaging plasmid and 4 µg of the experimental plasmid, to 500 µl HEBS (HEPES buffered saline): pH 7.05 buffer (280 mM KCl, 10 mM KCl, 1.5 mM Na₂HPO₄.2H₂O, 2 mM glucose, 50 mM HEPES free acid). This mix is added dropwise to 30% confluent 293T cells in 9 ml of 293T medium. 12 and 24 hours after transfection, media was removed and replaced with 10 ml fresh 293T media. At 36, 48 and 60 hours post-transfection, virus-containing media was removed, stored at 4 °C, and replaced with fresh 293T media.

Thymocytes and peripheral T-cells were transduced with retrovirus by spin infection. Cells, in 24-well plates at a density of 10⁶ cells/ml, were spun for 5 min at 240 g, media was removed and replaced with 1 ml viral supernatant per well. Cells were centrifuged for 90 minutes at 900 g at 30 °C. After centrifugation,
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Cells were incubated at 37 °C for 2 hours, then centrifuged to pellet cells (5 min at 240 g and room temperature (RT)), viral supernatant was removed, and cells were resuspended in T-cell media. After infection, peripheral T-cells were incubated at 37 °C for 48 hours, while thymocytes were used to create fetal thymic organ cultures as described above.

2.2.6 Transfection of miRNA inhibitors

Both hairpin inhibitors (Dharmacon) and tiny LNA inhibitors (8 nt LNA oligonucleotides complementary to the seed sequences of the miR-20, miR-181 and miR-130 families purchased from Exiqon) were transfected into T-cells using Amaxa nucleofector kits according to manufacturer’s protocols. Briefly, 5 million cells were resuspended in 100 µl nucleofector transfection solution, inhibitors were added at the required concentrations (0.5 µM or 10 µM for Dharmacon hairpin inhibitors and tiny LNA inhibitors, respectively). Cells were electroporated using nucleofector programme X-01 using an Amaxa 3 machine and immediately resuspended in pre-warmed 1.5 ml Amaxa T-cell Nucleofector medium. After 2 hours, cells were resuspended in T-cell media.

2.3 Flow Cytometry

2.3.1 Cell surface staining

Cells were stained using a number of fluorophore conjugated antibodies, as described in the results, diluted as described in table 2.1. To stain cells, cells were pelleted by centrifugation (320 g, 5 min, RT) and washed with FACS Buffer (PBS from Gibco, supplemented with 5% FCS). Cells were then resuspended at a concentration of $4 \times 10^6$ cells/ml (for analysis) or $10 \times 10^6$ cells/ml for sorting in FACS buffer containing staining antibodies and stained for 30 min at 4 °C. After staining, cells were washed with FACS Buffer and analysed or sorted as required.
2.3.2 Intracellular staining

Intracellular staining for phosphorylated ERK performed by 10 min fixation with 2% paraformaldehyde in RPMI medium at room temperature, followed by 1 hour on ice in 90% methanol, added dropwise. All subsequent steps took place at 4 °C. Following fixation, cells were rehydrated in FACS buffer containing phosSTOP phosphatase inhibitors (Roche Applied Sciences, made up according to manufacturer’s instructions) for 20 min. Cells were washed with FACS buffer, then stained for 45 min in FACS buffer with rabbit anti-ppERK antibody (as well as additional fluorescently conjugated antibodies for cell surface markers), and phosSTOP. Cells were washed in FACS buffer, then incubated with secondary FITC-conjugated goat anti-rabbit antibody in FACS buffer with phoStop (See Table 2.1 for full antibody details). Cells washed with FACS buffer and analysed.

2.3.3 Acquisition and analysis

Cells from both surface and intracellular staining were analysed with BD LSR II or FACScalibur flow cytometers, or sorted with a BD FACSaria II or FACSaria III cell sorter. Forward scatter (FSC) and side scatter (SSC) profiles were used to identify live cells and doublet discrimination was performed using FSC-W/FSC-A gating on LSR II and FACSaria machines. Fluorescence emission overlap between fluorophores was compensated by calibration of compensation parameters with single-stained controls. Flow cytometry data was analysed using FlowJo (Treestar). Further analysis for estimation of experimental noise was performed using R²13 and total least squares regression analysis of reporter construct expression was performed using MATLAB (The MathWorks, Inc).

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2.4.1 Primer design

For RT-qPCR primers, if validated primers were available for a given mRNA transcript from Primerbank (http://pga.mgh.harvard.edu/primerbank/), then these were used; otherwise, primers were designed to amplify ~150 nt exonic regions.
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of targeted transcripts using the Primer3 algorithm (http://frodo.wi.mit.edu/) on default settings, with the exception of a minimum required primer length of 20 nt. All primers were tested using a serial dilution curve to ensure linear response and primer amplification efficiency greater than 1.8.

2.4.2 Cloning

Dual Fluorescence (DF) constructs were created from pMSCVpuro plasmids from Clontech. MonomericCherry (mCherry) sequence was generously provided by N. Navaratnam and was amplified using primers containing HindIII and ClaI restriction sites. EnhancedGFP (eGFP) was cloned from MGIP plasmid provided by B. Cobb, using primers with a BgIII restriction site and a BamHI-Xho1-EcoR1 polylinker. 3’ UTR fragments were cloned from lymphocyte cDNA generated by RT-PCR using SuperscriptIII (Invitrogen) following manufacturer’s instructions, amplified using primers containing EcoR1 and Xho1 restriction sites. Site directed mutagenesis (SDM) of the Cd69 3’ UTR carried out by two rounds of PCR, first using the Cd69 3’ UTR primers and internal primers containing the mutation to generate two partial fragments each containing the mutated site at one end, then using a second round of PCR with the CD69 3’ UTR primers to produce a full-length fragment containing the mutation. All PCR reactions used Phusion DNA polymerase (New England Biolabs) in a 50 µl reaction volume made up according to manufacturer’s instructions, using the following PCR programme: 98 °C for 30 s, then 35 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 30s, with a final step of 72 °C for 10 minutes. Following PCR, all fragments were gel purified, digested with appropriate restriction enzymes (New England Biolabs) using manufacturer’s buffers and protocols and run on a low melting point agarose gel. Vectors were dephosphorylated by addition of 1 µl calf intestinal phosphatase (New England Biolabs) for 20 min at the end of restriction enzyme treatment. Vectors were cut out of the gel and purified with a Qiagen gel purification kit. Inserts were cut out of the gel and melted at 70 °C. 5 µl purified vector and 10 µl melted insert were ligated using T4 DNA ligase (New England Biolabs). Ligated product was transformed into competent DH5α E.coli by KCM transformation: 10 µl ligation mix added to 70 µl water and 20 µl KCM buffer (0.5 M KCl, 0.15 M
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CaCl₂, 0.25 M MgCl₂), 100 µl competent DH5α cells added. Cells cooled on ice for 10 min, then incubated at RT for 10 min. 500 µl LB broth (Sigma-Aldrich) (containing 50 µg/µl ampicillin (Sigma-Aldrich)) added, and cells were incubated at 37 °C for 30 min and then 50 ul of mix was plated onto LB-agar plates (1.5% (w/v) bacto agar (Merk) in LB broth (supplemented with ampicillin as above)), and incubated overnight at 37 °C. Colonies were picked into 5ml LB broth (supplemented with ampicillin), incubated overnight at 37 °C and DNA minipreps of the colonies were purified using Qiagen QiaPrep kits, screened by restriction enzyme digestion and sequenced by the MRC CSC Genomics Core Facility to ensure correct insertion.

Figure 2.1: Dual fluorescence reporter vector - Map of Dual Fluorescence reporter vector, showing important sequences and significant restriction sites
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2.4.3 RNA extraction

Whole RNA fractions were extracted using RNA-Bee isolation reagent (AmsBio), according to manufacturers protocols. Small RNAs were isolated using MirVana miRNA isolation kit, following manufacturer’s protocol, isolating separate small and large RNA fractions, and eluting in 50 µl RNAse-free water. RNA yield was measured using a Nanodrop machine (Thermo Scientific).

2.4.4 Real-Time PCR

Any residual DNA was digested with Turbo DNAse (Ambion), and RNA was reverse transcribed with SuperscriptIII (Invitrogen) following manufacturer’s instructions. Real-time PCR analysis of mRNA levels was carried out on a Chromo4 DNAengine machine running Opticon 3 software. Template cDNA was diluted × 10 and 2 µl of diluted template was added to 10 µl iQ SYBR Green Supermix (Biorad) and 0.6 µl primers at 10 µM concentration in a total reaction volume of 20 µl. The PCR program used was 95 °C for 3 minutes, then 40 cycles of 94 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s. All experiments were conducted in duplicate, with a blank control for each primer pair. For each experiment, a melting curve between 70 °C and 95 °C was run to ensure consistent annealing. Transcript levels were calculated by the ∆ΔCT method and normalised to expression of Hprt. All primers are listed in table 2.2.

For RT-qPCR of small RNAs, small RNA samples were reverse transcribed using Taqman miRNA-specific primers and RT kits (all from Applied Biosystems). 3 µl template RNA was added to 6.16 µl RNA-free water, 1 µl Multiscribe reverse transcriptase, 1.5 µl 10 × Multiscribe buffer, 0.15 µl 100 mM dNTPs, 0.19 µl RNAse inhibitors and 3 µl miRNA-specific primers. Mix was incubated at 16 °C for 30 min, 42 °C for 30 min, and 85 °C for 5 min. For qPCR, 1.35 µl template cDNA was added to 10 µl Taqman mastermix, 1 µl miRNA-specific probe and 7.65 µl water in a total reaction volume of 20 µl. The PCR programme used was 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All experiments were conducted in triplicate, with a blank control of each probe. Relative levels of miRNAs calculated using the ∆ΔCT method, normalising to the geometric average of housekeeping small nucleolar RNAs snoRNA-135 and
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snoRNA-202. For copy number calculations, standards of known concentration were also run alongside experimental samples, and for each experiment, a regression line was plotted to allow conversion of CT values into copy numbers for specific miRNAs.

2.4.5 Western blotting

One million cells from each sample were lysed by briefly vortexing and incubating for 5 minutes at 95 °C in 100 µl sample buffer (0.05 M Tris pH 6.8, 2% Sodium dodecyl sulfate (SDS), 20% glycerol, 0.001% bromophenol blue and 5% beta-mercaptoethanol) and loaded on an SDS-polyacrylamide gel, consisting of a 10% separating gel (10% acrylamide, 0.4 M Tris pH 8.8, 0.1% SDS, 0.05% ammonium persulfate (APS), 0.1% N, N', N'-tetramethylethylenediamine (TEMED)) and a 4% stacking gel (4% acrylamide, 0.125 M Tris pH 6.8, 0.1% SDS, 0.0667% APS, 0.12% TEMED) along with Benchmark prestained protein ladder (Invitrogen) for molecular weight reference. Gels were run in running buffer (25 mM tris base, 192 mM glycine, 0.1% SDS) on a Bio-Rad minigel system for 1 hour at 30 mA.

Following successful electrophoresis, gels were transferred to Milipore Immobilon-FL PVDF membrane. Membranes were pre-treated for 30 s in 100% methanol, 5 minutes in distilled water, and 5 minutes in transfer buffer (48 mM Tris base, 39 mM Glycine, 0.037% SDS, 20% methanol), then assembled with the separating portion of the gel between two layers of Whatmann paper in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio Rad). The cell was run for 75 minutes at 140 mA.

Membranes were blocked with 5% bovine serum albumin (Sigma-Aldrich) dissolved in wash buffer (150 mM NaCl, 10 mM tris base) for 30 min, then stained overnight at 4 °C with anti-ppERK antibody (see table 2.1) dissolved in wash buffer supplemented with 5% bovine serum albumin. After primary staining, membrane was washed three times with wash buffer and secondary staining was performed for 30 min with rabbit anti-goat antibody (see table 2.1) conjugated to Alexa Fluor 680. Following staining, membrane was washed three times with wash buffer and imaged on an Odyssey 2 luminometer. After ppERK staining, cells were stained for 2 hours with anti-lamin B (see table 2.1) as a loading control,
washed 3 times with wash buffer, secondary stained for 30 minutes with donkey anti-goat antibody (see table 2.1) conjugated with Alexa Fluor 680, washed three times with wash buffer and imaged on an Odyssey 2 luminometer (LI-COR Biosciences).

2.5 Computational simulation

Simulations of transcriptional networks were carried out using the Gillespie exact stochastic simulation algorithm\(^8\), programmed and analysed using R\(^{213}\). See Appendix 1 for example code. Simulation times were determined by running test simulations and observing the time required to reach an approximate steady state: subsequent simulations were run for at least double this time to ensure the systems are close to a steady state when observations were recorded.
2.5 Computational simulation

<table>
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<th>Host</th>
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Table 2.1: Antibodies
# 2. MATERIALS & METHODS

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Table 2.2: Cloning primers
2.5 Computational simulation

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Table 2.3: RT-qPCR primers
2. MATERIALS & METHODS
3

**Dicer** deletion results in increased cell-cell variation in specific genes

3.1 *Cd44* and *Sca-1* show developmentally regulated expression

Previous experimental analysis has shown that miRNAs play important roles in T-cell development\textsuperscript{[91,93,214]}. In particular, microarray comparison of transcript levels between wild-type and **Dicer**-deleted DP thymocytes showed significant changes, particularly upregulation, in the expression of many genes, suggesting a large number of transcripts are regulated by miRNAs. Bioinformatic comparison of these upregulated transcripts with developmental gene expression profiling data found that upregulated transcripts were enriched in genes which are differentially expressed during thymocyte development, and that these genes were enriched for 3’ UTR sequences containing binding sites for a number of miRNAs, particularly the miR-181 and miR-17 families, suggesting that miRNA-mediated regulation plays a significant role in regulating the expression of these transcripts\textsuperscript{[215]}. From these data, we chose a number of genes to further investigate at the single cell level, based on availability of reliable antibodies for quantitative flow cytometry. In particular, we looked at the expression of cell surface proteins CD44, a protein involved in cell-cell interactions and cell migration, and Sca-1 (also known as LY6A), a marker of stem-cell and haematopoietic development.
3. DICER DELETION RESULTS IN INCREASED CELL-CELL VARIATION IN SPECIFIC GENES

(see Introduction). We validated that these genes show differential regulation during thymocyte development by sorting cell populations from thymii and peripheral lymphoid organs from wild-type (WT) C57Bl/6 mice into DN, CD4⁺ CD8⁺ DP, CD4⁺ SP, peripheral CD4⁺ and peripheral CD8⁺ stages of T-cell development, and quantifying mRNA expression by RT-qPCR. Both our genes of interest show an increase in gene expression as cells mature from the DP to the CD4⁺ SP stage of T-cell development (Figure 3.1).

![Graph showing mRNA expression of Cd44, Sca-1 and Cd69 during T-cell development](image)

**Figure 3.1: Cd44, Sca-1 and Cd69 show differential expression patterns during T-cell development** - Expression of Cd44, Sca-1 and Cd69 determined by RT-qPCR in CD4⁻ CD8⁻ DN, CD4⁺ CD8⁺ DP, CD4⁺ SP and peripheral CD4⁺ and CD8⁺ cells sorted by flow cytometry. Expression was normalised to Hprt and scaled so that the maximum expression of each gene was 1. Error bars show standard error calculated from 3 biological replicates.
3.2 CD44 and Sca-1 protein expression is deregulated in Dicer-depleted thymocytes

To investigate the role of miRNAs in the regulation of the robustness of developmental pathways in T-cells, we used Dicer\textsuperscript{lox/lox} mice\cite{1} containing LoxP sites flanking the RNAse III domain of Dicer, which is required for mature miRNA production. To deplete miRNAs at specific stages of development, these mice were crossed with strains expressing the Cre recombinase under the control of a developmental-stage specific promoter. In particular, lckCre Dicer\textsuperscript{lox/lox} mice which have both the lox-flanked Dicer allele and Cre under the control of the Lck promoter, which is expressed at the DN stage of thymocyte development, showed a depletion of ~90% of mature miRNAs in DP thymocytes\cite{1}. Similarly, to look at later stages of T-cell development, Cre expressed under the control of the CD4 promoter can deplete miRNAs after the DP stage of development\cite{2}.

To look at potential deregulation of transcripts, we analysed expression of CD44 and Sca-1 in TCR\textsuperscript{low} CD4\textsuperscript{+} CD8\textsuperscript{+} DP thymocytes by flow cytometry, which allows the observation of protein expression in single cells across a population. Gating for the large population of TCR\textsuperscript{low} DP thymocytes allowed us to analyse the large, homogenous population of pre-selection DP thymocytes, to ensure that expression of our genes of interest was not affected by thymic selection. Comparison of histograms of CD44 and Sca-1 expression in populations of Dicer\textsuperscript{lox/lox} and lckCre Dicer\textsuperscript{Δ/Δ} cells shows a clear increase in protein expression compared with other cell surface markers such as LY6C (Figure 3.2).

Mean expression data from these histograms was calculated and pooled, showing a median increase in Sca-1 expression of 90% and a median increase of CD44 expression of 50% in miRNA-depleted cells, consistent with miRNA-dependent regulation of these transcripts (Figure 3.3) expression of the control LY6C showed an increase in expression of less than 4%.
3. *DICER DELETION RESULTS IN INCREASED CELL-CELL VARIATION IN SPECIFIC GENES*

**Figure 3.2:** Sca-1 and CD44 proteins show changes in the distribution of protein expression in populations of *Dicer*-deleted thymocytes - (a) Typical flow cytometry histogram showing gating for TCR\textsuperscript{low} populations. (b-d) Typical flow cytometry expression histograms of (b) Sca-1, (c) CD44 and (d) LY6C in *Dicer\textsuperscript{lox/lox}* (black) and lckCre *Dicer\textsuperscript{Δ/Δ}* (red) TCR\textsuperscript{low} DP mouse thymocytes. Histograms are normalised to the total size of each population. Mean and CV of each population inset in plot.
3.2 CD44 and Sca-1 protein expression is deregulated in Dicer-depleted thymocytes

Figure 3.3: Sca-1 and CD44 proteins show increased mean expression in Dicer-depleted thymocytes - Box-and-Whiskers plots showing the distribution of mean protein expression of (a) Sca-1, (b) CD44 and (c) LY6C in Dicer<sup>lox/lox</sup> (black) and lckCre Dicer<sup>Δ/Δ</sup> (red) CD4<sup>+</sup> CD8<sup>+</sup> DP TCR<sup>low</sup> thymocytes, analysed by flow cytometry. Data are normalised to mean expression of each protein in Dicer<sup>lox/lox</sup> cells. Figures show pooled data from between 10 and 30 biological replicates, incorporating some data from M. Merkenschlager.
3. DICER DELETION RESULTS IN INCREASED CELL-CELL VARIATION IN SPECIFIC GENES

3.3 Correlation of protein and mRNA levels

Although RT-qPCR-based measures of mRNA levels can only measure the average mRNA level over a population, we could check whether mRNA levels correlated with protein levels as recorded by flow cytometry by sorting DP thymocytes by expression of CD44 or Sca-1 into populations expressing high or low levels of these proteins. Levels of mRNA in these populations were assayed by RT-qPCR and were found to be consistent with the results obtained through flow cytometry: cells expressing high levels of protein also express higher levels of mRNA than cells expressing low levels of protein, and miRNA-depleted cells in both populations express higher levels of mRNA, consistent with relief of miRNA-mediated repression (Figure 3.4).

3.4 CD44 and Sca-1 show increased cell-cell variation in Dicer-deleted thymocytes

Interestingly, as well as an increase in mean levels of gene expression, flow cytometry histograms of Sca-1 and CD44 showed a noticeable increase in the width of the flow cytometry peaks (Figure 3.2). This suggests that deletion of Dicer results in an increase in cell-cell variation. For accurate investigation of the effect of miRNAs on gene expression and on cell-cell variation in gene expression, we needed to be able to quantify protein expression at a single-cell level to enable us to get an accurate snapshot of the distribution of protein expression across a population of cells, and calculate from this population data a measure of cell-cell variation. Flow cytometry observation of protein expression across a population has the required single-cell resolution, but to quantify variation in protein expression several different measures could be used: the simplest, and most common measure of population variation is the standard deviation, but other possible measures of variation are the coefficient of variation (CV) or the noise strength (see Introduction).

The coefficient of variation was used, as, under the circumstances of miRNA depletion, it is the most stringent measure of an increase in noise: i.e. if an in-
3.4 CD44 and Sca-1 show increased cell-cell variation in Dicer-deleted thymocytes

Sca-1 low expression  Sca-1 high expression

CD44 high expression  CD44 low expression

Cd44 mRNA expression

Figure 3.4: Sca-1 and Cd44 mRNA and protein levels show similar responses to Dicer-depletion - mRNA levels of (a) Sca-1 and (b) Cd44 mRNA determined by RT-qPCR in Dicer\textsuperscript{lox/lox} (black) and IckCre Dicer\textsuperscript{Δ/Δ} (red) thymocytes sorted by flow cytometry for CD4\(^+\) CD8\(^+\) DP TCR\textsuperscript{low} populations expressing high and low levels of Sca-1 or CD44, respectively. Lower flow cytometry histograms show typical protein expression and gating, normalised by the size of each population. Transcript expression levels normalised to Hprt and shown relative to the Dicer\textsuperscript{lox/lox} cells low protein expression sample for each protein. Error bars indicate standard error from three independent replicates.
3. DICER DELETION RESULTS IN INCREASED CELL-CELL VARIATION IN SPECIFIC GENES

crease in the CV is observed at the same time as an increase in mean protein expression (which would be expected in knockdown of a miRNA which both represses a gene, and regulates its variation), both the standard deviation and the noise strength will also have increased. The reverse is not true: if an increase in the standard deviation or the noise strength is observed at the same time as an increase in the mean expression of a protein, this could be because of a change in variation due to miRNA regulation, or it could be because the standard deviation increases proportionally with the mean expression. Thus, under our experimental protocols, an increase in both mean expression and CV would unambiguously indicate an increase in cell-cell variation by any of these measures of noise.

Figure 3.5: Sca-1 and CD44 proteins show increased CV in Dicer-depleted thymocytes - Box-and-Whiskers plots showing the distribution of the CV of (a) Sca-1, (b) CD44 and (c) LY6C in Dicer\textsuperscript{lox/lox} (black) and lckCre Dicer\textsuperscript{Δ/Δ} (red) CD4\textsuperscript{+} CD8\textsuperscript{+} DP TCR\textsuperscript{low} thymocytes, analysed by flow cytometry. Data are normalised to the CV of gene expression in Dicer\textsuperscript{lox/lox} cells. Figures show pooled data from between 10 and 30 biological replicates, incorporating some data from M. Merkenschlager
3.5 Estimation of experimental noise

Calculating the CV of Sca-1 and CD44 protein expression across populations of DP thymocytes, a clear increase in CV relative to wild-type thymocytes can be observed. In CD44 this shows a median increase of 30% over the CV of wild-type populations; in Sca-1 expression, a median increase of 15% is observed; a median increase of 4% is seen in the LY6C control (Figure 3.5).

3.5 Estimation of experimental noise

For these measurements of CV in CD44 and Sca-1 to be an accurate measures of biological cell-cell variation, we must also estimate the experimental noise ie. the variation attributable to the experimental techniques, such as variations in fluorescence measurements. We followed a similar procedure to that used in a recent study\cite{216} and stained DP thymocytes for CD8\(\alpha\) and CD8\(\beta\) chains, which are expressed at a 1:1 stoichiometry. By measuring the CV in the ratio between the two subchains, an upper bound for the experimental noise can be obtained, as the measured noise will be a combination of biological variation in the CD8\(\alpha\):CD8\(\beta\) ratio, which is low, and experimental noise from factors such as autofluorescence, incomplete antibody binding and measurement error in the flow cytometer. In our experiments, this was approximately 20% (Figure 3.6). This compares with measurements of CVs in our experimental systems, which are typically around 30-60%. Dicer\(^{\Delta/\Delta}\) and lckCre Dicer\(^{\Delta/\Delta}\) cells show similar estimates of experimental noise, as would be expected if the noise in our estimates are largely due to non-biological sources.

3.6 Depletion of miRNAs results in increased CD69 expression and variation in activated thymocytes

Another candidate miRNA-regulated protein was also investigated, CD69: a cell-surface marker known primarily as a marker of early T-cell activation. Little is known about CD69 function in T-cells, but it is thought to have a role in cell signalling and cell migration\cite{172}. CD69 has been identified as a deregulated gene in Dicer\(^{\Delta/\Delta}\) thymocytes, and previous studies have also identified it as a miRNA
DICER DELETION RESULTS IN INCREASED CELL-CELL VARIATION IN SPECIFIC GENES

Figure 3.6: Estimation of experimental noise - Typical flow cytometry plots of DP Thymocytes from a) Dicer\textsuperscript{lox/lox} and b) lckCre Dicer\textsuperscript{Δ/Δ} cells costained for CD8α and CD8β chains. c) Histogram of CD8α/CD8β ratio in Dicer\textsuperscript{lox/lox} (black) and lckCre Dicer\textsuperscript{Δ/Δ} DP thymocytes, normalised by the size of each population. Experimental noise estimated by calculating the CV of this ratio.

CV of Dicer\textsuperscript{lox/lox} cells: 17.6%
CV of lckCre Dicer\textsuperscript{Δ/Δ} cells: 19.2%
target of the miR-181 family of miRNAs\textsuperscript{193}. CD69 is particularly interesting, because, in addition to a low level of basal expression during thymocyte development, CD69 expression is upregulated shortly after T-cell activation. For example, activation of thymocytes with increasing concentrations of plate-bound anti-TCR antibody, coupled with costimulation by soluble anti-CD28 antibody, results in an increasing proportion of activated cells marked by high CD25 and CD69 expression (Figure 3.7a). CD69 upregulation also shows a bimodal "all-or-nothing" response, being expressed at either low levels in unactivated cells or high levels in activated cells, with very low levels of intermediate levels of expression (Figure 3.7b). CD69 is both a miRNA target and highly regulated during T-cell activation, making it a potential candidate for involvement in a miRNA-containing FFL during activation.

To look at CD69 expression during T-cell activation, we created whole-thymocyte primary cultures, and activated them with varying concentrations of plate-bound anti-TCR antibody, costimulating with soluble anti-CD28 (2 µg/ul) for 18 hours. While this system is somewhat artificial compared to the thymic environment, it is highly consistent, showing a saturation curve with maximum stimulation using 500 ng/ml anti-TCR antibody, and reduces the possibility of additional experimental noise caused by changes in the activatory stimulus. Activated cells were identified by gating for CD25\textsuperscript{+} CD69\textsuperscript{+} cells. Deletion of Dicer results in a ∼50% increase in mean levels of CD69 expression, relative to wild-type Dicer\textsuperscript{lox/lox} cells, in CD4\textsuperscript{+} SP cells after activation with a range of concentrations of anti-TCR antibody (Figure 3.7c). As with CD44 and Sca-1, we also looked at changes in CD69 CV in lckCre Dicer\textsuperscript{Δ/Δ} relative to Dicer\textsuperscript{lox/lox} cells: CD69 CV shows a increases of ∼20% in lckCre Dicer\textsuperscript{Δ/Δ} cells (Figure 3.7c). To further investigate this, we looked at DP and CD4\textsuperscript{+} SP thymocytes and CD4\textsuperscript{+} peripheral T-cells after 18 hours of activation with 500 ng/ml anti-TCR antibody. Similarly to CD44 and Sca-1, flow cytometry histograms in activated (CD25\textsuperscript{+} CD69\textsuperscript{+}) lckCre Dicer\textsuperscript{Δ/Δ} thymocytes showed both higher expression and a broader histogram peak, consistent with miRNA regulation of both CD69 mean expression and cell-cell variation in CD69 levels (Figure 3.8). Interestingly, this Dicer-dependent regulation of CD69 noise appears to occur only at specific stages of development: unlike in thymocytes, CD69 expression in activated peripheral CD4\textsuperscript{+} T-cells shows
3. **DICER DELETION RESULTS IN INCREASED CELL-CELL VARIATION IN SPECIFIC GENES**

![Figure 3.7](image)

**Figure 3.7: Activation of CD4^+ SP thymocytes by anti-TCR antibody** - (a) Typical CD69 and CD25 density plots after 18 hours’ activation of CD4^+ thymocytes with anti-TCR antibody at the stated concentrations. Cells also costimulated with anti-CD28 antibody at 2 µg/ml. Percentages indicate percentage of activated (CD25^+ CD69^+) CD4^+ cells. (b) Representative histograms showing effect of 18 hours’ activation with increasing concentrations of plate-bound anti-TCR antibody on CD69 CV in CD4^+ SP thymocytes. Cells also costimulated with anti-CD28 antibody at 2µg/ml. (c-d) Effect of increasing concentrations of plate-bound anti-TCR antibody on (c) mean CD69 levels and (d) CV of CD69 expression in activated (CD25^+ CD69^+) CD4^+ Dicer^lox/lox^ (black) and lckCre Dicer^{Δ/Δ} (red) cells after 18 hours’ activation. Mean and CV values normalised to 500 ng/ml Dicer^lox/lox^ mean and CV respectively. Cells also costimulated with anti-CD28 antibody at 2µg/ml. Error bars show standard error from four experiments.
3.6 Depletion of miRNAs results in increased CD69 expression and variation in activated thymocytes

![Diagram showing normalised cell count vs CD69 expression for DP and CD4+ SP peripheral CD4+ T-cells for Dicer Δ/Δ and Dicer lox/lox cells.]

**Figure 3.8: Expression pattern of CD69 in activated thymocytes** - Typical histograms of CD69 expression in activated (a) DP (b) CD4+ SP and (c) peripheral CD4+ cells. Dicer lox/lox (black) and IckCre Dicer Δ/Δ cells activated with anti-TCR (500 ng/ml) and costimulated with anti-CD28 (2 µl/ml) for 18 hours and gated for activated CD25+ CD69+ cells.

A clear increase in expression, but no increase in cell-cell variation.

To quantify these changes in expression, we pooled mean CD69 expression levels and CVs of CD69 from multiple experiments. Analysis of the mean level of CD69 expression in activated DP thymocytes, CD4+ SP thymocytes and peripheral CD4+ T-cells showed median increases of 56%, 53% and 40%, respectively, in CD69 expression in Dicer Δ/Δ cells over Dicer lox/lox cells (Figure 3.9a-c), supporting our observations of a strong increase in CD69 expression at all three stages of T-cell development investigated. Pooling data on the CVs of CD69 expression in activated thymocytes showed increases in CD69 CV of 56% and 30% in activated DP and CD4+ SP thymocytes respectively, but a small decrease in CD69 CV in activated CD4+ SP peripheral T-cells (Figure 3.9d-f). This suggests that while CD69 expression is regulated by miRNAs at all stages of development that were analysed, the changes in CD69 noise we observed appear only in immature thymocytes.

To confirm that changes in CD69 expression were due to the depletion of miRNAs, a number of important controls were required: firstly, we checked that
3. DICER DELETION RESULTS IN INCREASED CELL-CELL VARIATION IN SPECIFIC GENES

Figure 3.9: MicroRNA-depleted thymocytes show increased CD69 mean expression and CV on activation - (a-c) Box-and-whiskers plots showing change in mean CD69 expression in activated (with 500 ng/ml anti-TCR and 2 µg/ml anti-CD28 antibodies) lckCre DicerΔ/Δ CD25+ T-cells (red) from (a) DP cells, (b) CD4+ SP cells and (c) CD4+ peripheral T-cells, relative to Dicerlox/lox T-cells (black). Mean CD69 expression is normalised to mean expression in Dicerlox/lox cells. (d-f) Box-and-whiskers plots showing change in CV of CD69 expression in activated lckCre DicerΔ/Δ CD25+ T-cells (red) from (a) DP cells, (b) CD4+ SP cells and (c) CD4+ peripheral T-cells, relative to Dicerlox/lox T-cells (black). CD69 CVs are normalised to mean CVs in Dicerlox/lox cells. Plots show pooled data from 46 biological replicates in thymocytes and 12 biological replicates in peripheral T-cells. Plots incorporate some data from M. Merkenschlager.
3.6 Depletion of miRNAs results in increased CD69 expression and variation in activated thymocytes

the expression of the Cre recombinase is not causing abnormal gene expression and additional noise by repeating the activation experiments using lckCre thymocytes as controls, instead of Dicer\textsuperscript{lox/lox} cells.

Comparison of activated lckCre and lckCre Dicer\textsuperscript{Δ/Δ} thymocytes showed similar patterns in mean expression and CV of CD69. DP thymocytes show a median increase of \sim 2.5-fold in mean CD69 expression, and a median increase of 40% in CD69 CV. CD4\textsuperscript{+} SP thymocytes show a median increase of 40% in mean CD69 expression and an increase of 24% in CD69 CV (Figure 3.10). These results indicate that the Dicer-dependent increases in mean and CV of CD69 expression observed in activated cells are not a product of lckCre expression alone.

It has been previously reported that lckCre Dicer\textsuperscript{Δ/Δ} thymocytes are more susceptible to cell death\textsuperscript{191,215}. To check that changes in variation in gene expression observed in Dicer-depleted cells were not due to selective death of lckCre Dicer\textsuperscript{Δ/Δ} thymocytes during activation, we mixed lckCre Dicer\textsuperscript{Δ/Δ} cells and wild-type C57 BL/6 Thy1.1\textsuperscript{+} cells in culture, and activated them for 18 hours with anti-TCR and anti-CD28 antibodies. After activation, cells were stained and analysed using flow cytometry, using staining for Thy1.1 to distinguish the two populations, and detect changes in the relative ratios of the C57BL/6 and lckCre Dicer\textsuperscript{Δ/Δ} cells on activation, allowing the detection of any significant increase in cell death in Dicer\textsuperscript{Δ/Δ} cells. Analysis of these ratios showed no significant change in the relative numbers of WT and Dicer\textsuperscript{Δ/Δ} cells, suggesting that activation of thymocytes does not result in selection for a particular population of cells (Figure 3.11).

Deletion of the Dicer RNAse III domain does not completely deplete cells of mature miRNAs, typically reducing miRNA expression to 10% of previous levels\textsuperscript{191}. It is possible that observed populations of Dicer-deleted thymocytes may actually contain a small population of cells which express WT levels of miRNAs. If this is the case, and if WT and miRNA-depleted thymocytes express CD69 at different levels, our observations of miRNA-depleted cells could actually be observations of the addition of two populations (a large Dicer-deleted population, and a residual population of WT cells), which have the same CV but different levels of mean expression, and the addition of these two populations might result in a combined population with a larger CV. To check that the increase in CV ob-
3. DICER DELETION RESULTS IN INCREASED CELL-CELL VARIATION IN SPECIFIC GENES

Figure 3.10: Expression of Cre recombinase is not responsible for changes in CD69 expression - (a) Relative mean expression of CD69 in activated (with 500 ng/ml anti-TCR and 2 µg/ml anti-CD28 antibodies) DP thymocytes (b) Relative CV of CD69 expression in activated DP thymocytes (c) Mean expression of CD69 in activated lckCre and lckCre DicerΔ/Δ CD4+ thymocytes (d) Relative CV of CD69 expression in activated lckCre and lckCre DicerΔ/Δ CD4+ thymocytes. All results normalised to the mean of the lckCre measurements. Plots show pooled results from 10 replicates. Error bars show minimum and maximum values.
3.6 Depletion of miRNAs results in increased CD69 expression and variation in activated thymocytes

Figure 3.11: Thymocyte activation does not select for or against IckCre Dicer$\Delta/\Delta$ thymocytes - Thymocytes from Thy1.1$^+$ and IckCre Dicer$\Delta/\Delta$ mice mixed at a 1:1 ratio and activated with anti-TCR (500 ng/ml) and anti-CD28 antibodies for 18 hours. After activation proportion of Thy1.1$^+$ and IckCre Dicer$\Delta/\Delta$ cells determined by gating CD4$^+$ CD8$^+$ DP and CD4$^+$ SP populations on Thy1.1 expression. Graphs show percentages of (Thy1.1$^+$) and IckCre Dicer$\Delta/\Delta$ at each stage of development. Error bars show standard error from three independent experiments.
3. **DICER DELETION RESULTS IN INCREASED CELL-CELL VARIATION IN SPECIFIC GENES**

...serve is not due to a residual population of miRNA expressing wild-type cells, in silico experiments were performed to mix Dicer\textsuperscript{loxp/loxp} and lckCre Dicer\textsuperscript{Δ/Δ} CD4\textsuperscript{+} SP thymocyte populations, activated for 18 hours with anti-TCR and CD28 antibodies, recorded from earlier experiments, and to measure the effect on the CV of CD69 expression observed. A varying percentage of Dicer\textsuperscript{loxp/loxp} cells were added to a fixed population of lckCre Dicer\textsuperscript{Δ/Δ} cells, and the CV of the resulting mix was calculated, and averaged across data from 10 different experiments. Addition of a population of Dicer\textsuperscript{loxp/loxp} cells results in at most an increase in CV of 1%, compared to a typical increase in CV of 30% in activated CD4\textsuperscript{+} SP thymocytes (Figure 3.12a).

However, our data from lckCre Dicer\textsuperscript{Δ/Δ} cells may already contain a small population of miRNA-expressing cells, which could mask any increase in CV that would be produced by computational addition of more miRNA-expressing cells. To address this issue, cells were computationally removed from an experimentally recorded population of Dicer-depleted cells, following the distribution of Dicer\textsuperscript{loxp/loxp} cells, and the effect on CD69 CV was observed. Subtracting populations resulted in a decrease in CV, which would be consistent with a hidden population of miRNA-expressing cells increasing the observed CV; however, the changes in CV observed were substantially smaller than those seen experimentally: to produce a 5% increase in CV, the hidden miRNA-expressing population would have to comprise over 40% of the total cells, which would not produce the 90% reduction in miRNA levels previously observed (Figure 3.12b). Neither adding nor removing WT cells from our miRNA-depleted cells affected the CV in the manner seen experimentally: these results show that increases in CD69 CV observed are not readily accounted for by residual Dicer-expressing cells.

Finally, we investigated to what extent deletion of Dicer affects the upstream pathways of T-cell activation, in particular, the ERK pathway, which is one of the principal drivers of activation-induced CD69 expression\cite{171} and could contain upstream miRNA targets, which might produce changes in CD69 expression in Dicer-deleted cells. To quantify ERK pathway activation at the single-cell level we fixed Dicer\textsuperscript{loxp/loxp} and lckCre Dicer\textsuperscript{Δ/Δ} whole thymus populations after 3 hours' activation, and stained cells using antibodies specific for phosphorylated ERK1/2. Analysis of activated CD4+ SP activated (CD25+) cells showed no in-
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Figure 3.12: Effect of computationally adding or removing $Dicer^{\Delta/\Delta}$ thymocytes to a population of lckCre $Dicer^{\Delta/\Delta}$ thymocytes - (a) Change in CV produced by computational addition of a population of $Dicer^{\Delta/\Delta}$ cells to a lckCre $Dicer^{\Delta/\Delta}$ population. Using CD69 expression data from lckCre $Dicer^{\Delta/\Delta}$ and $Dicer^{\Delta/\Delta}$ CD4+ SP thymocytes activated as in 3.9, the graph shows how the total population CV varies as a percentage of $Dicer^{\Delta/\Delta}$ cells in the population is increased. (b) Change in CV produced by computational subtraction of a population of $Dicer^{\Delta/\Delta}$ cells from a lckCre $Dicer^{\Delta/\Delta}$ population. Using CD69 expression data from lckCre lckCre $Dicer^{\Delta/\Delta}$ and $Dicer^{\Delta/\Delta}$ CD4+ SP thymocytes activated as in 3.9, the graph shows how the total population CV varies as a percentage of $Dicer^{\Delta/\Delta}$ cells subtracted from the population is increased. Error bars in both graphs show standard error from simulations using 10 different sets of results.
increase in either mean ppERK levels or the CV of ppERK (Figure 3.13a and b). DP cells could not be quantified by this method, as they show insufficient CD25 expression after three hours activation for reliable analysis of the activated cell population. To investigate ERK phosphorylation in activated DP cells, we also sorted populations of DP and CD4+ SP Dicerlox/lox and lckCre DicerΔ/Δ cells and activated for 3 hours with plate-bound anti-TCR and anti-CD28 antibodies. These cells were lysed and were assayed for ppERK by western blotting. As with the flow cytometry data, no increase of ERK phosphorylation was seen (Figure 3.13c and d), suggesting that Dicer-dependent changes in components of the ERK pathway are not responsible for the observed changes in CD69 expression.

In conclusion, we have identified a number of genes which show increased cell-cell variation, as measured by the CV, in Dicer-deleted T-cells. In particular, the cell surface markers CD44 and Sca-1 show increased expression in miRNA-depleted thymocytes, consistent with their regulation by miRNAs. They also show increased cell-cell variation, suggesting that miRNAs may play a role in regulating the variation in levels of specific genes during development. We also looked at regulation of CD69 during T-cell activation, and found that following activation, Dicer-dependent increases in mean CD69 expression are recorded at all stages of development. In thymocytes, this increase in mean expression is also accompanied by an increase in cell-cell variation in Dicer-deleted cells. However, this increase in variation is not seen in peripheral T-cells, suggesting that the mechanism is developmentally regulated. Further investigation has shown that these changes in gene expression and noise are not due to residual miRNA expression in Dicer-deleted cells, or by increased cell death of Dicer-deleted cells during activation. Investigation of changes in the upstream ERK pathway showed no increase in ERK phosphorylation which could explain the increase in CD69 expression and noise.
3.6 Depletion of miRNAs results in increased CD69 expression and variation in activated thymocytes

![Flow cytometry plot](image)

Figure 3.13: Effect of Dicer Deletion on ppERK signalling - (a) Typical flow cytometry plot of ERK phosphorylation in activated Dicer_{lox/lox} (black) and lckCre Dicer_{Δ/Δ} (red)CD25^{+} CD4^{+} SP thymocytes and unactivated Dicer_{lox/lox} CD4^{+} SP thymocytes (dashed line). (b-c) Relative change in (b) mean level or (c) CV of ERK phosphorylation measured by flow cytometry in Dicer_{lox/lox} (black) and lckCre Dicer_{Δ/Δ} (red) CD4^{+} SP thymocytes after 3 hours activation with anti-TCR and anti-cd28 antibodies. Box-and-whiskers plot summarises results from 8 replicates. (d) Western blot for ppERK and lamin B in activated and unactivated WT and Dicer-deleted thymocytes. (e) Quantified ppERK levels, normalised to lamin B. Error bars show standard error from four separate experiments.
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4

MicroRNA regulation of gene expression noise

4.1 Dicer-dependent regulation of Cd69, Cd44 and Sca-1 3’ UTRs

Identifying genes which show increased variation and gene expression on deletion of Dicer is not sufficient to show that they are miRNA targets: while it is possible that the changes in gene expression observed are due to depletion of a specific miRNA targeting that gene, they could also be due to other effects of Dicer deletion, such as regulation of the gene under investigation by another miRNA target.

To determine whether the genes identified were direct targets of microRNAs, and that the changes in expression and variation we observed were not a downstream effect from changes in the expression of another gene regulated by miRNAs, we investigated whether each gene was a direct miRNA target. We used the TargetScan algorithm\(^5^4\) to obtain computational predictions of which miRNAs were likely to target Cd69, Cd44 and Sca-1 transcripts, based on complementarity to miRNA seed sequences and evolutionary conservation. The 842 nt Cd69 3’ UTR was found to contain three potential miRNA binding sites, for the miRNA families miR-181, miR-130 and miR-17-20, with binding sites for the seed sequences starting at positions 255, 354 and 391 respectively. The 2990 nt Cd44 3’ UTR con-
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tains a single predicted binding site for the miR-21 family, starting at position 573 within the 3' UTR. There were no conserved predicted miRNA binding sites in the 385 nt Sca-1 3’ UTR (Figure 4.1a). All three 3’ UTRs contained many poorly conserved miRNA binding sites: these are less likely to be functional sites, as evolutionary conservation is a predictor of functional miRNA binding sites.

To test whether these mRNAs were targeted by miRNAs, we developed a dual fluorescence (DF) retroviral reporter construct containing two fluorescent reporter proteins, eGFP and mCherry, under the control of the separate LTR and PGK promoters, as well as a cloning site in the 3’ UTR of the eGFP transcript. In a similar manner to luciferase reporter constructs, 3’ UTRs of interest can be cloned into this site, and the effect of the 3’ UTR on expression of GFP relative to mCherry can be recorded. This approach has several advantages over traditional approaches based on luciferase vectors, including the ability to directly measure reporter expression by flow cytometry, making it easier to directly measure the effect of endogenous miRNAs on construct expression in sorted primary cell populations.

We cloned the 3’ UTRs of Cd69 and Sca-1 (excluding the polyA signal from each 3’ UTR) and bases 1-891 (containing the predicted miR-21 binding site, as the entire 3’ UTR of Cd44 was too long to clone into a single construct) of the Cd44 3’ UTR into the fluorescent reporter construct (Figure 4.2a). For successful retroviral infection, a population of dividing cells is required. To achieve this in peripheral cells, cells were activated with CD3/CD28-coated beads for 24 hours to drive cell division, and infected with retrovirus containing the reporter construct. After a further 24 hours, expression of eGFP and mCherry could be directly quantified by flow cytometry, with an infected mCherry+ eGFP+ population of ~ 30-40% of T-cells (Figure 4.2a).

This allowed miRNA-dependent regulation to be measured by comparing the relative expression of eGFP in WT and miRNA-depleted cells. To model the relationship between GFP and mCherry, orthogonal linear regression, a variation of linear regression that assumes errors in both variables, was used, with the relative level of eGFP to mCherry calculated as the gradient of the fitted line. These ratios of eGFP expression to cherry expression are normalised to the eGFP/mCherry ratio of the empty vector, to quantify the change in eGFP expression in experi-
4.1 Dicer-dependent regulation of Cd69, Cd44 and Sca-1 3’ UTRs

Figure 4.1: Dual fluorescence reporter construct - (a) Schematic of dual fluorescence reporter construct and inserted 3’ UTRs, showing Targetscan-predicted miRNA binding sites (b) Typical density plot showing mCherry and eGFP expression of the empty vector in CD4+ peripheral T-cells, and representative gating for mCherry+ eGFP+ cells. Percentages indicate proportion of cells in each quadrant. (c) Representative log-log dot plot of gated mCherry+ eGFP+ T-cells expressing the construct containing the Cd69 3’ UTR in Dicer\textsuperscript{lox/lox} and lckCre Dicer\textsuperscript{Δ/Δ} cells, showing fitted lines used to calculate increase in eGFP expression.
Figure 4.2: Dual fluorescence reporter construct shows Dicer-dependent downregulation of Cd69 expression in peripheral T-cells - (a) Changes in eGFP expression relative to empty DF vector in Dicer<sup>lox/lox</sup> (black) and lckCre Dicer<sup>Δ/Δ</sup> (red) CD4<sup>+</sup> peripheral T-cells (b) Changes in GFP relative to empty DF vector in Dicer<sup>lox/lox</sup> (black) and lckCre Dicer<sup>Δ/Δ</sup> (red) CD8<sup>+</sup> peripheral T-cells. All cells activated with CD3/CD28 beads for 24 hours to allow efficient infection. Error bars in both graphs indicate standard error from four experiments.
4.1 Dicer-dependent regulation of Cd69, Cd44 and Sca-1 3’ UTRs

mental vectors compared to the empty vector. By comparing eGFP expression from Dicer\textsuperscript{lox/lox} and Dicer\Delta/\Delta cells the level of miRNA-dependent repression can also be observed.

In peripheral cells, only the Cd69 3’ UTR showed miRNA dependent regulation: peripheral CD4\textsuperscript{+} Dicer\textsuperscript{lox/lox} cells infected with the DF vector containing the Cd69 3’ UTR showed a ~40% decrease in eGFP expression relative to the empty vector; in CD4Cre Dicer\Delta/\Delta cells a ~7.5-fold increase in GFP expression is seen instead, indicating that as well as repressive miRNA-binding sites, the Cd69 3’ UTR may contain sequences that enhance expression. Neither the Sca-1 3’ UTR nor the Cd44 3’ UTR fragment showed significant Dicer-dependent increases in eGFP expression, and insertion of the Cd44 3’ UTR section actually showed considerable upregulation of eGFP expression in both WT and Dicer-deleted cells, suggesting that the Cd44 section contains sequences which enhance gene expression in a miRNA-independent manner (Figure 4.2a). Similar results were seen in CD8\textsuperscript{+} T-cells: although insertion of the Cd69 3’ UTR produced no downregulation in eGFP expression relative to the empty DF vector, deletion of Dicer produces a ~9-fold increase in eGFP expression, consistent with miRNA-mediated repression of eGFP by the Cd69 3’ UTR. Again, neither the Sca-1 3’ UTR nor the Cd44 3’ UTR fragment shows significant Dicer-dependent upregulation (Figure 4.2b).

This experiment was repeated in thymocytes; to successfully infect the reporters into thymocytes, they need to be cultured for several days as part of a fetal thymic organ culture (FTOC) system, in which fetal thymic lobes are incubated in media supplemented with deoxyguanosine to eliminate native thymocytes. The remaining stromal cells are mixed with thymocytes infected with the DF vector (Figure 4.3a). The stromal cells provide support for immature DN thymocytes to divide, become infected with the vector and mature into DP and SP thymocytes. Because Dicer\Delta/\Delta thymocytes show increased cell mortality, they could not be cultured this way, so all results in thymocytes are from wild-type C57Bl/6 mice. As with CD4\textsuperscript{+} peripheral T-cells, only the Cd69 3’ UTR produced any downregulation in GFP expression: this was observed in DP, and both CD4\textsuperscript{+} and CD8\textsuperscript{+} SP cells, producing reductions of 75%, 55% and 72% in GFP expression in DP, CD4\textsuperscript{+} SP and CD8\textsuperscript{+} cells relative to the empty vector. Expression of constructs
Figure 4.3: Expression of reporter constructs using fetal thymic organ cultures -
(a) Schematic of thymic organ culture system. (b) Typical flow cytometry plot from DP thymocytes infected with empty reporter vector in fetal thymic organ culture. Percentages show relative frequency of each quadrant.
4.1 *Dicer*-dependent regulation of *Cd69*, *Cd44* and *Sca-1* 3' UTRs

Figure 4.4: *Cd69* 3' UTR represses reporter construct expression in thymocytes - Expression of DF reporter construct containing *Cd69*, *Sca-1* and *Cd44* 3' UTRs in (a) Double positive (b) CD4+ Single positive and (c) CD8+ SP thymocytes. Plots show GFP expression relative to empty DF vector. Error bars indicate standard error from between 4 and 7 experiments.
containing the SCA1 and CD44 3’ UTRs showed an increase in eGFP expression, indicating that they may contain sequences which enhance expression (Figure 4.4). As miRNA-deficient cells cannot be infected using FTOC due to high cell death, miRNA-dependent repression cannot be directly observed in thymocytes. Because their 3’ UTRs do not produce any repression of eGFP expression in WT cells, it is unclear whether Cd44 and Sca-1 are direct targets of miRNAs in thymocytes, but given their lack of Dicer-dependent regulation in peripheral T-cells, we focused on further investigating the regulation of the Cd69 3’ UTR by miRNAs.

4.2 Cd69 is regulated by multiple microRNAs

In order to identify repressive elements in the Cd69 3’ UTR we cloned a number of truncations of the 842 bp Cd69 3’ UTR into the DF vector and expressed them in thymocytes. In both DP and CD4+ SP thymocytes, segments of the Cd69 3’ UTR which contained bases 200-430 showed roughly equal levels of repression. However, the 3’ UTR truncation containing only bases 410-842 showed ∼12-fold upregulation of eGFP expression (Figure 4.5). This suggests that bases 200-430 of the 3’ UTR (which contain all three predicted miRNA binding sites) contain the principal repressive sequences in the 3’ UTR.

To further dissect the regulation of Cd69 by miRNAs, we mutated each of the predicted binding sites in the Cd69 3’ UTR, randomising the seed sequence at each predicted binding site, and observed the effect this had on reporter expression in T-cells at all three stages of development. If the site is in fact a miRNA binding site, this should result in increased eGFP expression relative to the complete 3’ UTR. In peripheral T-cells, mutating each of the predicted miRNA binding sites showed the Dicer-dependent effect of each predicted site on eGFP expression. Mutating the predicted miR-181 family binding site in the Cd69 3’ UTR produced no change in eGFP expression compared to the unmutated Cd69 3’ UTR vector, but mutating the miR-130 and miR-20 family binding sites resulted in upregulation of ∼2- and ∼5-fold, respectively, relative to the complete Cd69 3’ UTR (Figure 4.6a). These actually increased eGFP expression over the basal level of expression from the empty DF vector, suggesting that as well as repressive miRNA binding sites, there are elements of the Cd69 3’ UTR which can enhance expression of
4.2 *Cd69* is regulated by multiple microRNAs

**Figure 4.5:** Repressive regions of the *Cd69* 3' UTR fall in the 0-410 region -

(a) Schematic of truncations made to the *Cd69* 3' UTR, showing location of predicted miRNA binding sites. (b-c) Effect of truncation of *Cd69* 3' UTR: eGFP expression (relative to the empty DF vector) from (b) DP and (c) CD4+ SP thymocytes infected with DF reporter construct containing full-length *Cd69* 3' UTR (bases 0-842) and truncated versions (bases 0-430, 0-630, 200-842 and 410-842) Error bars show standard error from two experiments.
eGFP. Deletion of Dicer resulted in a ~10-fold upregulation of eGFP expression in all Cd69 3’ UTR-containing constructs, abolishing any difference in expression due to miRNA binding site mutations. Similar results were seen in CD8+ peripheral T-cells: mutation of the miR-130 and miR-20 binding sites produces Dicer-dependent increases in eGFP expression of ~2- and ~7-fold respectively (Figure 4.6b). These patterns of expression are consistent with the miR-130 and miR-20 families regulating Cd69 expression in both types of peripheral T-cells. It does not appear that the miR-181 family site has a repressive effect in peripheral T-cells.

In thymocytes, mutation of any of the three sites produces similar results in all three cell types investigated. In DP thymocytes, mutation of the miR-181, miR-130 and miR-20 binding sites results in a decrease of repression from the Cd69 3’ UTR by 35%, 45% and 80% respectively, consistent with targeting of the 3’ UTR by endogenous members of each family of miRNAs in thymocytes. These effects are also additive: multiple mutations of the different binding sites result in increased expression of eGFP: mutation of all three predicted sites results in a ~ 7-fold increase in eGFP expression. Similar results are also observed in both CD4+ and CD8+ SP thymocytes (Figure 4.7b and c). These results show that the Cd69 3’ UTR is regulated by all three predicted miRNA binding sites in immature thymocytes.

4.3 Changes in miRNA expression in response to T-cell activation

If miRNAs form part of genetic regulatory networks which reduce noise, we need to identify the mechanisms by which they are co-regulated with other genes, and whether they function as part of motifs such FFLs or negative feedback loops. One possibility for the regulation of CD69 is that miRNAs are also upregulated during activation. For example, computational prediction with the miR-gen database of predicted miRNA-regulating transcription factors suggested that the transcription factor AP-1, a target of the ERK signalling pathway, might target promoters of both miR-181a and Cd69. To test whether miR-181 or other miRNAs might be upregulated by T-cell activation we sorted populations of DP,
4.3 Changes in miRNA expression in response to T-cell activation

Figure 4.6: Mutation of predicted miR-130 and miR-20 family binding sites reduces Dicer-dependent repression of the Cd69 3' UTR in mature T-cells - (a-b) Changes in GFP expression in Dicer<sup>lox/lox</sup> (black) and lckCre Dicer<sup>Δ/Δ</sup> (red) (a) CD4<sup>+</sup> and (b) CD8<sup>+</sup> peripheral cells activated and infected with DF constructs containing the Cd69 3' UTR with the indicated mutations made to predicted miRNA binding sites. All expression values shown relative to empty DF vector and scaled to unmutated Cd69 3' UTR. Error bars show standard error calculated from between 4 and 14 different experiments, depending on mutation.
Figure 4.7: Mutation of predicted miR-181, miR-130 and miR-20 family binding sites reduces repression of the *Cd69* 3′ UTR in thymocytes - Fold-change in GFP expression in (a) DP thymocytes (b) CD4+ SP thymocytes (c) CD8+ SP thymocytes relative to empty DF vector of DF vector containing the *Cd69* 3′ UTR, with indicated miRNA family binding sites mutated. All expression values shown relative to empty DF vector and scaled to unmutated *Cd69* 3′ UTR. Error bars show standard error calculated from between 4 and 14 different experiments, depending on mutation.
4.3 Changes in miRNA expression in response to T-cell activation

CD4+ SP thymocytes and CD4+ peripheral cells from WT C57Bl/6 mice for activated (CD25+ CD69+) cells after 18 hours’ activation with anti-TCR antibody (500 ng/ml, costimulated with 2 µg/ml anti-CD28 antibody) and profiled Cd69-targeting miRNA families for changes in expression by RT-qPCR (Figure 4.8). These data show two main results in thymocytes: a downregulation in expression of miR-181 family members, and an upregulation in members of the miR-20 family, particularly miR-17 and miR-20a. In peripheral T-cells, upregulation of the miR-20 family, particularly miR-17, remains, but the level of miR-181 family expression in unactivated peripheral T-cells is reduced compared with thymocytes, and little downregulation is observed. The levels of the miR-130/301 family remain low in both activated and unactivated cells.

However, analysis of Cd69 mRNA levels after T-cell activation shows that Cd69 levels rise rapidly after activation, peaking after 3-6 hours, and then decline to original levels. If Cd69 is regulated by a miRNA which has the same patterns of expression, this suggests that miRNA levels could rise along with CD69, and similarly decline, so that co-regulation of Cd69 and a targeting miRNA might occur, but not be detectable 18 hours after activation. To address this possibility, I also activated CD4+ SP thymocytes and mature CD4+ T-cells for 3 hours, and investigated changes in miRNA levels. After 3 hours’ activation, markers of activation such as CD69 and CD25 are not yet sufficiently expressed to allow sorting of a population into activated and unactivated cells by their expression. Instead, to profile miRNA expression in cells after 3 hours’ activation, thymocytes and peripheral T-cells were sorted for CD4+ single positive cells, of which ∼70% will become activated upon stimulation. These sorted CD4+ cells were then activated with anti-TCR antibody for three hours, and this population of cells, which mainly consists of activated cells, were lysed and profiled for miRNA expression with RT-qPCR (Figure 4.9). After 3 hours’ activation, we found little change in the levels of any miRNA profiled, suggesting that the upregulation of Cd69 expression after activation is not mirrored in any of the miRNAs predicted to target the Cd69 3’ UTR.

T-cell activation in thymocytes also requires a significant alteration in cell morphology, including a significant increase in cell size. Changes in cell volume could also affect expression of housekeeping genes such as snoRNAs used to normalise
Figure 4.8: Changes in miRNA levels after 18 hours’ activation - (a) WT Thymocytes and (b) peripheral T-cells activated for 18 hours with 500 ng/ml anti-TCR antibody, then sorted by flow cytometry into DP, CD4^+ SP and CD4^+ populations. MicroRNA levels in activated cells and unactivated controls then measured by RT-qPCR. Expression levels normalised to snoRNAs 135 and 202. Error bars show standard error from three experiments.
miRNA quantification by RT-qPCR. To check that this was not masking changes in miRNA expression, we established copy-number calibration curves for miR-181a and miR-20a to investigate whether changes in copy number between activated and unactivated cells was significantly different. As with earlier profiling, CD4+ SP thymocytes from WT C57Bl/6 mice were sorted and activated for 3 hours with anti-TCR and anti-CD28 antibodies. Activated and unactivated control cells were assayed for expression of miR-181a and miR-20a, along with a calibration curve to allow direct calculation of copy number for both miRNAs. Copy numbers showed no significant change in miRNA numbers after activation, supporting our original findings that miRNA levels do not change significantly after 3 hours of T-cell activation.

4.4 miR-181a expression is developmentally regulated

Next we considered whether changes in miRNA expression might be triggered by developmental changes, rather than activation. Previous work has shown that miR-181a is developmentally regulated, with reduced expression as thymocytes mature. We theorised that miR-181a might be continuously reduced in expression as cells mature, and that this might work to reduce cell-cell variation by counteracting other developmental changes, such as changes in the sensitivity of the TCR to stimuli, producing more consistent expression of CD69 across a population of cells of differing maturity. We subdivided DP and CD4+ SP populations by maturity by sorting for expression of TCRα, which is expressed as DP cells mature, and for CD24, which is downregulated as CD4+ SP cells mature. Copy numbers of miR-181 in each population were determined by RT-qPCR. By doing this, we can see a continuous reduction of miR-181a expression as T-cells mature (Figure 4.10).
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Figure 4.9: Changes in miRNA levels after 3 hours’ activation - (a) Thymocytes and (b) peripheral T-cells sorted into CD4$^+$ populations and activated (with 500 ng/ml anti-TCR and 2 $\mu$g/ml anti-CD28 antibodies) for 3 hours. MicroRNA levels in activated cells and unactivated controls then measured by RT-qPCR. Expression levels normalised to snoRNAs 135 and 202. (c) Copy numbers of miR-181a and miR-20a in T-cells activated for 3 hours. Error bars show standard error from three experiments.
4.4 miR-181a expression is developmentally regulated

Figure 4.10: Changes in miR-181a expression over thymocyte development - Thymocytes sorted into developmental substages and miR-181a copy number per cell assayed by RT-qPCR. Error bars indicate standard error from three separate experiments.
4.5 Effect of inhibition of specific miRNAs on CD69 expression

To verify that each of the miRNAs identified as regulators of CD69 had a role in regulating the changes in CD69 expression we had observed, we tried to knock down expression of specific miRNAs (miR-181a, miR-20a and miR-130b) by transfection of hairpin miRNA inhibitors into fetal thymocytes in an FTOC system. After 24 hours, cultures were resuspended and thymocytes were activated for 18 hours with anti-TCR and anti-CD28 antibodies. These activated cells were analysed for CD69 expression and cell surface markers by flow cytometry. Inhibition of both miR-181a and miR-20a miRNAs produced an increase in CD69 expression in activated DP thymocytes, of 12% and 7% respectively (Figure 4.11a). Similar results were observed in CD4+ SP thymocytes, but the effect of miR-181a inhibition was smaller (~ 7%) and the effect of inhibition of miR-20a was slightly larger (~ 10%) (Figure 4.11b). No significant effect was seen from attempts to knockdown miR-130b in either cell type. We also looked at the effect of individual miRNA inhibition on the CV of CD69 expression in activated cells: in both DP and CD4+ SP thymocytes, no increase in CV was observed after inhibition of any of the individual miRNAs tested (Figure 4.11c and d). The relatively small effect of knockdown of miR-20a, which is predicted to be the strongest repressor of the \textit{Cd69} 3’ UTR from mutating the predicted miRNA binding sites in the reporter constructs, may be due to redundancy effects in miRNA families, as miR-20a has many co-expressed family members.

To reconcile these results with our reporter constructs, we also repeated these experiments using tiny locked nucleic acid (LNA) inhibitors, short 8-nt sequences of LNA, a modified polynucleotide which shows superior stability and binding compared to RNA, and, as they only target the seed sequence of a miRNA family, are capable of targeting an entire family of miRNAs. As with the hairpin inhibitors, thymocytes were transfected with LNAs using a fetal thymic organ culture system, incubated for 24 hours to allow knockdown of miRNAs, and then activated for 18 hours with 500 ng/ml anti-TCR antibody and anti-CD28 antibody.

Inhibition of the miR-181 and miR-130 families showed no increase in CD69
**4.5 Effect of inhibition of specific miRNAs on CD69 expression**

(a-b) Change in mean CD69 expression in activated (a) DP and (b) CD4$^+$ SP thymocytes on transfection of hairpin inhibitors to miR-181a, miR-130b or miR-20a, relative to control hairpin. (c-d) Change in CV of CD69 expression in (c) DP and (d) CD4$^+$ SP thymocytes on transfection of hairpin inhibitors to miR-181a, miR-130b or miR-20a, relative to control hairpin. Error bars indicate standard error calculated from three biological replicates. * indicates $p \leq 0.05$. ** indicates $p \leq 0.01$. P-statistics determined by two-tailed Student's T-test.
4. MICRORNA REGULATION OF GENE EXPRESSION NOISE

Figure 4.12: Effect of transfection of tiny LNA miRNA inhibitors into thymocytes

(a-b) Change in mean CD69 expression in activated (a) DP and (b) CD4⁺ SP thymocytes on transfection of LNA inhibitors to miR-181a, miR-130b or miR-20a, relative to control LNA. (c-d) Change in CV of CD69 expression in (c) DP and (d) CD4⁺ SP thymocytes on transfection of hairpin inhibitors to miR-181a, miR-130b or miR-20a, relative to control LNA. Error bars indicate standard error calculated from three biological replicates.
4.6 Deletion of miR-181ab cluster increases CD69 expression on activation

expression, while inhibition of the miR-20 family showed an upregulation of ~35% in activated DP thymocytes, and ~30% in CD4+ thymocytes. Similar increases in expression were observed when all three LNAs were co-transfected into thymocytes together. The upregulation of mean CD69 expression when the miR-20 family is inhibited is consistent with the broader specificity of LNAs allowing targeting of the whole family. Analysis of changes in CD69 CV showed no upregulation in CV following inhibition by any of the LNAs (Figure 4.12c and d).

4.6 Deletion of miR-181ab cluster increases CD69 expression on activation

Given apparently contradictory results on the effect of inhibiting the miR-181 family in thymocytes, to further investigate the role of the miR-181 family in regulating CD69, we obtained mice from the Chen lab which contained a mutation in the miR-181ab-1 loci, which expresses the majority of the miR-181 family members. Thymocytes and peripheral T-cells from wild-type C57Bl/6 mice, miR-181ab-1-/- homozygous and miR-181ab-1-/+ heterozygous mice were activated for 18 hours with anti-TCR (500 ng/ml) and anti-CD28 (2 µg/ml) antibodies, and the expression of CD69 in activated cells was analysed by flow cytometry.

In activated T-cells at all stages of development, a clear increase in CD69 expression of ~20% was seen in miR-181a/b-deleted cells compared to wild-type, consistent with direct miR-181 regulation of CD69 expression. However, no increase in CD69 CV was seen in miR-181a/b-depleted cells (Figure 4.13).

4.7 In silico experiments show possible models of noise regulation

To look further at how miRNAs might regulate biological noise as part of regulatory motifs, we carried out a number of simulations of simple FFLs, using the Gillespie exact stochastic simulation method, building on models described in
4. MICRORNA REGULATION OF GENE EXPRESSION NOISE

Figure 4.13: Knockout of miR-181ab expression increases CD69 expression - (a-c) Mean or (d-f) CV of CD69 expression in activated CD25+ CD69+ T-cells at from miR-181ab-1 -/-, miR-181ab-1 +/- and WT mice at indicated levels of development. CD69 expression measured by flow cytometry after 18 hours’ activation with 500 ng/ml anti-TCR antibody and 2 µg/ml anti-cd28. Data for each condition from at least 5 mice.
4.7 In silico experiments show possible models of noise regulation

recent theoretical studies (See Introduction)\textsuperscript{138}. For regulation by transcription factors, TF regulation is modelled as a Hill function, in which the rate constant of transcription responds sigmoidally to increasing concentrations of transcription factor:

\[ k = \frac{k_b [TF]^c}{h^c + [TF]^c} \]

Where \( k \) is the rate constant, \( k_b \) is the base rate of transcription, \([TF]\) is the concentration of transcription factor, \( h \) is the dissociation coefficient, which determines the strength of the response to the TF and \( c \) is the Hill coefficient, which determines how sharp the response to increasing concentration of TF is.

As miRNAs may act through multiple mechanisms, we modelled two different mechanisms of miRNA action, to determine how different mechanisms might affect the control of noise. First, we modelled miRNAs as purely repressive of translation, modelling their action as a reduction in the translation rate of mRNAs, dependent on the concentration of miRNAs according to a Hill equation:

\[ k = \frac{k_b}{1 + \left( \frac{[miRNA]}{h} \right)^c} \]

Where \( k \) is the resulting rate constant, \( k_b \) is the base rate of translation, \([miRNA]\) is the miRNA concentration, \( h \) is the dissociation constant, and \( c \) is the Hill coefficient. Rates of other reactions, such as mRNA or protein degradation, were modelled as proportional to the concentration of their limiting factor multiplied by a rate constant (for full system specifications, see Appendix 1). Using values for the rate constants derived from previous models\textsuperscript{138}, we simulated this system, and a miRNA-free system in which a transcription factor regulates only an output protein, to obtain steady-state values for simulated protein expression. By comparing a system with miRNA regulation to the same system without, we can see that a miRNA-containing FFL is clearly capable of reducing noise compared to a circuit with no miRNA regulation (Figure 4.14c). Comparing circuits with and without miRNA regulation showed a relative increase in mean output protein expression of 50% and a increase in relative CV of 42\%, similar to the changes seen in our experimental systems.
4. MICRONRNA REGULATION OF GENE EXPRESSION NOISE

Figure 4.14: *In silico* experiments show miRNAs can regulate noise in theoretical models of regulatory motifs - (a) Schematic of miRNA FFL model in which miRNAs inhibit translation (b) Schematic of miRNA FFL model in which miRNAs bind to mRNAs and enhance degradation of the mRNA (c) Density plot of 10,000 simulations of gene regulation with (black) and without (red) miRNA regulation. Parameters are: rate of TF transcription 0.06, rate of TF and output mRNA degradation 0.006, rate of TF translation 0.04, rate of TF and output protein degradation 0.002, base rate of miRNA transcription 0.5, dissociation constant for TF regulation of miRNA and output mRNA transcription 200, rate of miRNA degradation 0.006, rate of output mRNA transcription 0.8, base rate of output translation 0.04, miRNA dissociation constant 60, and all Hill coefficients, 2. (d) Density plot of 10,000 simulations of gene regulation with (black) and without (red) miRNA regulation. Rate constants are the same as in the first model, but with no translational repression: the rate of output protein translation is 0.04, and with a number of additional rate constants: the rate constant of miRNA-mRNA complex formation and dissociation is 0.0001, while the rate constant for mRNA degradation in complex is 0.02.
4.7 In silico experiments show possible models of noise regulation

Secondly, we developed a stochastic model based on deterministic models recently developed to look at thresholding in miRNA repression, in which miRNAs bind to mRNAs to form a miRNA-mRNA complex, from which mRNAs cannot be translated and which can either degrade or release the mRNA (Figure 4.14b). Simulating this system produced a similar, though more modest regulation of noise: comparing the circuit with miRNA regulation to the circuit without showed an increase in mean output expression of 70% and an increase in CV of 23% (Figure 4.14d). The smaller noise regulation may be due to the additional variability introduced by modelling the additional steps of random miRNA-mRNA binding.

Varying the parameters associated with production and binding of the transcription factor produced a number of different responses, depending on which constants were altered. These responses could be divided into a number of different categories.

Changing rate constants associated with the production and action of the transcription factor, such as the rate of transcription or translation, showed that noise reduction occurs only at intermediate values of the rate constants: if too little transcription factor is produced, no downstream transcription occurs, conversely, if sufficient TF is produced to saturate the system and produce maximum transcription and noise from the transcription factor is not transmitted to the output protein, and miRNA regulation has little effect on noise (Figure 4.15a and b).

Altering the rate of miRNA decay in both systems also showed that maximal regulation of variation was achieved at intermediate levels of repression: if miRNA decay is slow then levels of miRNA are high, resulting in strong repression of target mRNAs, but there is no control of noise, while if miRNA decay is fast miRNAs decay too fast to have significant effect, and no repression occurs. Optimal repression of noise occurs with a repression of between 20-60% of unregulated protein levels (Figure 4.15c). Similar results are obtained by varying the miRNA dissociation constant in the model of translational repression by miRNAs.

Changing rate constants that affect the base level of the output protein, such as the base rate of output transcription, or the base rate of output translation in the translational inhibition model, has little effect on the relative levels of protein
Figure 4.15: Changing rate constants in models shows noise regulation strongest at intermediate levels of repression by miRNAs - (a-d) The effect of changing rates constants in both miRNA models. All other constants are unchanged from previous figure, while plots show the relative effect on mean and CV of output expression by changing (a) the rate of transcription factor translation, (b) the rate of transcription factor degradation, (c) the miRNA degradation rate and (d) the base rate of output protein transcription. Blue line indicates the miRNA-mRNA binding model, while the red line indicates the results in the translational inhibition model. (e-f) Effect of changing parameters of the miRNA-mRNA binding model. Effect of changing (e) the rate of miRNA binding to mRNA and (f) the rate of mRNA degradation from the miRNA-mRNA complex. 200 simulations run per condition.
expression, but produces an initial increase in relative CVs, before reaching a stable maximum level of noise regulation (Figure 4.15d).

Modifying the rates of miRNA-mRNA binding and miRNA-mediated mRNA degradation in the mRNA degradation model produces some interesting results: increasing the miRNA binding rate results in a linear increase in repression of protein output, and, as seen before, produces maximal noise repression at intermediate levels of repression (Figure 4.15e). Conversely, altering the rate of degradation of mRNA from the miRNA-mRNA complex results in no change in mean repression, but alters the strength of the regulation of noise observed (Figure 4.15f). This is particularly interesting as it represents a potential mechanism by which the noise might be regulated independently from the mean level of expression, although this mechanism is dependent on miRNA-mRNA binding being an accurate model of miRNA action.

Despite these changes, it seems that the miRNA-dependent regulation of noise is remarkably robust: under conditions of moderate repression (such as repression of 20-50% of gene expression by the miRNA), FFLs seem to be effective at reducing noise in downstream genes under a wide variety of different parameters. This reduction is typically fairly modest, typically around 20-30%. These effects also seem to be robust to changes in potential mechanisms of miRNA action: despite very different models of miRNA effects, both models show a reduction in noise under most conditions.

In conclusion, we have investigated the direct regulation of Cd44, Sca-1 and Cd69 by miRNAs in thymocytes, and have found miRNA-dependent inhibition of the Cd69 3’ UTR during T-cell development. In particular, we have found predicted binding sites for three families of miRNAs, miR-181, miR-130 and miR-20, which alleviate repression when mutated. We profiled changes in miRNA expression on activation in T-cells to determine whether miRNAs that targeted Cd69 were also upregulated on activation, and observed that members of the miR-181 family, which are highly expressed in thymocytes but not in peripheral T-cells, are downregulated on activation and members of the miR-20 family are upregulated in activation in both thymocytes and peripheral T-cells. Members of the miR-130 family are expressed at low levels in both activated and unactivated cells. To determine whether developmental regulation of miR-181 might change as T-cells...
mature, we profiled miR-181a expression across subsets of thymocytes of different maturities, to confirm that miR-181 expression shows a decline as cells mature. We verified the miRNA regulation of CD69 with a combination of specific miRNA deletions and miRNA inhibition experiments, which verified that knockdown of miR-181 and miR-20 resulted in an increase in the expression of CD69 in activated thymocytes. However, no increase in cell variation was observed from the knockdown of any single miRNA. To investigate theoretical mechanisms by which miRNAs might reduce variation, we used computational models to show that simple models of the FFL, using biologically plausible parameters, can produce similar changes in variation to those seen in our experimental systems. By varying the model parameters, we have also seen that miRNA regulation in these models produce a reduction in variation under a wide variety of parameters.
5

Discussion

5.1 Overview

MicroRNAs are small non-coding RNAs which post-transcriptionally repress target mRNAs and have previously been shown to have a number of important roles in many aspects of animal development. In particular, it has been suggested that one of the functions of miRNAs is to enhance the robustness of gene regulatory networks to perturbations, including biological noise. Computational simulations have shown that miRNAs may function to reduce noise as part of network motifs such as feedforward loops\textsuperscript{138}, and some studies have shown that deletion of miRNAs results in reduced robustness to environmental fluctuations during development\textsuperscript{141} but no study has yet shown a direct link between miRNAs and regulation of biological noise. Previous work in our lab has shown that miRNAs, particularly members of the miR-181 and miR-20 families, target many genes which are differentially expressed during T-cell development\textsuperscript{215}. To investigate the role of miRNAs in the regulation of biological noise during development, we identified a number of genes which showed increased cell-cell variation in Dicer-deleted thymocytes. To confirm that these genes were direct miRNA targets, we identified miRNA binding sites in these genes using a fluorescent reporter construct, and validated them using miRNA knockdown experiments, looking in particular at the marker of activation CD69. Finally, we investigated how CD69 and the miRNAs which target it might fit into gene regulatory networks which
would act to reduce cell-cell variation.

5.2 Role of Dicer in regulating cell-cell variation

Building on previous work from the lab, which showed that a number of genes were upregulated in Dicer-deleted thymocytes compared to wild-type thymocytes, we aimed to extend this analysis using single-cell methods to allow us to investigate the effect of miRNAs on biological noise during development. To assess the effect of miRNAs on biological noise, we first identified a number of cell surface genes which showed developmental regulation, and validated this by RT-qPCR. To observe the effect of miRNAs on the expression of these genes, we used conditional Dicer-deleted mice to deplete miRNAs at specific stages of T-cell development, initially looking at gene expression in DP thymocytes using flow cytometry, which records protein expression at the single-cell level, allowing the observation of the distribution of protein expression across a population, and the quantification of variation as the CV.

We found that two genes, cd44 and Sca-1, showed an increase in the mean level of protein expression in Dicer-depleted cells, consistent with regulation by miRNAs. Quantification of cell-cell variation in protein expression by the CV of CD44 and Sca-1 protein expression by flow cytometry in DP thymocytes showed increased variation in Dicer-deleted cells. Sorting cells into populations expressing high and low levels of protein allowed us to observe how closely mRNA expression related to protein expression observed by flow cytometry: the higher levels of mRNA expression seen in the high protein expression groups confirm that mRNA and protein levels are linked. These genes also showed an increase in mRNA levels in Dicer-deleted cells, indicating that if these genes are directly repressed by miRNAs, repression occurs through mRNA degradation, not exclusively through inhibition of translation. Estimation of the measurement noise from flow cytometry by comparing relative fluorescence of CD8α and β chains, which are expressed at a 1:1 ratio, gave an upper bound of <20%, which compares well with experimentally observed levels of variation, which typically show CVs in the range of 30-100%. No difference in the estimates of experimental noise was observed when comparing WT Dicer^lox/lox and Dicer-deleted IckCre Dicer^Δ/Δ.
5.2 Role of Dicer in regulating cell-cell variation

cells, consistent with deletion of Dicer having no effect on the experimental noise, as opposed to the biological noise. This represents the first attempt to assess the effect of miRNAs on cell-cell variation in mammalian cells.

We also investigated the expression of CD69, a cell surface marker that has been previously identified as a miRNA target in T-cells. CD69 is generally expressed at low levels in thymocytes, but on activation of T-cells by engagement of the TCR and co-receptors, it is rapidly upregulated. The expression of CD69 in the population forms a bimodal distribution, with a separable population of activated CD69+ cells. CD69 has been previously identified as a target of the miR-181 family, a family of miRNAs highly expressed in thymocytes\[193\], which suggested it as a possible candidate for forming part of an incoherent FFL, which could act to regulate noise. In activated T-cells at the DP, CD4+ SP and CD4+ peripheral T-cell stages of development, we have seen an increase in the mean level of CD69 expression in Dicer\[Δ/Δ\] cells activated with saturating levels of anti-TCR antibody. An increase in the CV of CD69 expression in Dicer-deleted thymocytes is also seen. Interestingly, however, this change in CD69 noise is not observed in peripheral T-cells, suggesting the miRNAs act to regulate noise in a developmentally stage-specific manner. Whether this is due to regulation by a miRNA specific to immature thymocytes, or because miRNAs act to control noise from a source specific to immature thymocytes, such as the wider range of TCR specificities expressed in thymocytes during thymic selection, remains to be seen, and represents a possible avenue for future studies. Further experiments showed that this change in variation was not due to other effects from the deletion of Dicer, such as residual miRNA-expressing cells, or by selective death of thymocytes during activations. To confirm that the increase in CD69 CV was not due to changes in the level of T-cell activation, we carried out flow cytometry and western blotting for phosphorylated ERK protein, a key part of the T-cell activation signalling pathway. These found no change in the mean level of phosphorylated ERK in DP and CD4+ SP thymocytes, and no change in the CV of ERK phosphorylation in CD4+ SP thymocytes. This result does not agree with a recent study which found that inhibition of miR-181a resulted in decreased levels of ERK phosphorylation in activated thymocytes, which the authors attributed to changes in the expression of protein tyrosine phosphatases such as DUSP5, DUSP6, SHP-2 and
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PTPN22[154] Previous work in our lab, however, found no changes in the level of these phosphatases in Dicer-deleted thymocytes[215]. It is not clear what causes these different results, but it may be due to differing experimental protocols or to compensatory effects from the depletion of other miRNAs in Dicer-deleted cells. While these results do not eliminate the possibility of changes in other pathways of T-cell activation, such as the NF-κB pathway, knockout of the RAS GTPase has identified the ERK pathway as the principal pathway of CD69 upregulation[171].

5.3 Role of miRNAs in regulating Cd69, Cd44 and Sca-1

While the increase in cell-cell variation in Dicer-deleted thymocytes is a strong indicator that miRNAs have a role in regulating variation, it does not exclude the possibility that the increase in cell-cell variation is due to deregulation of other miRNA targets, or to deregulation of other Dicer-dependent pathways. To confirm whether the Dicer-dependent change in cell-cell variation is due to direct miRNA targeting of Sca-1, Cd44 or Cd69, we developed a retroviral reporter construct containing two fluorescent reporter proteins, which could be used to quantify the impact of inserted 3′ UTRs on reporter expression. This can be used to determine the effect of endogenous 3′ UTR binding factors, such as miRNAs, on reporter expression. Inserting the 3′ UTRs of Cd44, Sca-1 and Cd69 into the reporter construct and comparing expression from these reporter constructs in WT and Dicer-deleted peripheral T-cells showed that the Sca-1 and Cd44 3′ UTRs show no significant Dicer-dependent repression, suggesting that these genes are not direct miRNA targets in mature T-cells. Successful retroviral infection of thymocytes requires support of developing thymocytes using FTOC. Dicer-deficient thymocytes cannot be retrovirally infected using FTOC due to their increased cell death, so the effect of miRNA depletion on repression cannot be directly assessed in thymocytes, but insertion of the Sca-1 3′ UTR shows no repression of eGFP expression relative to the empty vector, and insertion of the Cd44 3′ UTR produces an increase in eGFP expression, suggesting that the effect of these 3′ UTRs is neutral or enhancing on expression. Comparison of the expression of
5.3 Role of miRNAs in regulating Cd69, Cd44 and Sca-1

reporter constructs containing the Cd69 3’ UTR in Dicer-deleted cells, however, showed repression of eGFP expression relative to the empty vector in both peripheral T-cells and thymocytes, and Dicer-deletion in peripheral cells showed a significant upregulation in Dicer-deleted cells. To further dissect the role of elements of the Cd69 3’UTR in miRNA-dependent repression we cloned a series of truncations and mutations of the Cd69 3’ UTR into our reporter construct. Expression of truncated versions of the Cd69 3’ UTR in thymocytes showed broadly similar expression in constructs containing bases 210-400 of the UTR; however, constructs containing only the 410-842 region showed a tenfold increase in eGFP expression, showing that the majority of repressive elements are in the 210-400 region. This corresponds to the region which contains all three predicted miRNA binding sites. To validate the predicted binding sites, each was individually mutated and the effect on eGFP expression in thymocytes and peripheral T-cells was observed. In peripheral cells, mutation of the miR-20 and miR-130 sites resulted in a reduction of Dicer-dependent repression, but mutation of the miR-181 site showed no effect. In thymocytes, mutation of all three miRNA-binding sites resulted in upregulation of eGFP expression consistent with miRNA-mediated repression through all three binding sites. Combinatorial mutations of the binding sites showed a roughly additive effect, with mutation of all three binding sites resulting in an upregulation of around tenfold, which is comparable to the miR-dependent upregulation seen when comparing the expression of eGFP from the DF vector containing the Cd69 3’ UTR in peripheral WT and Dicer-deleted cells. This suggests that these three sites are the major cause of the repressive effect of the Cd69 3’ UTR in wild-type cells. The most likely reason for the observed decline in repression mediated by the miR-181 site is the previously observed reduction in levels of miR-181 family members as thymocytes mature to become peripheral T-cells\textsuperscript{60}. 

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5.4 MicroRNAs as part of developmental regulatory networks

For miRNAs to have a role in regulating biological noise during development, they must form part of the regulatory networks which control T-cell development and activation. As well as regulating the expression of transcripts such as Cd69, they must in turn be regulated by other transcription factors, in such a way as to reduce the noise in the developmental system. The simplest of these possible mechanisms are basic network motifs such as negative feedback loops or feedforward loops. To try and identify the mechanisms by which noise might be controlled, we investigated how the miRNAs which target Cd69 might be regulated themselves. Having shown that the Cd69 3’ UTR contains a number of repressive miRNA binding sites, we looked at how these miRNAs were regulated in thymocytes, in particular whether any miRNAs were upregulated following T-cell activation, at both 3 hours and 18 hours post-activation. This found two main effects: firstly, in thymocytes, where miR-181a and b are expressed at higher levels, activation causes a downregulation in miR-181 a/b expression. This is not seen in peripheral T-cells, as miR-181 family members are expressed at lower levels. Secondly, members of the miR-20 family, particularly miR-17 and miR-20, are upregulated following activation. Members of the miR-130/301 family were expressed at low levels in both activated and unactivated cells. Of the potential CD69-targeting miRNAs, only the miR-17 family shows expression that would be consistent with co-regulation during activation, although it is expressed in both thymocytes and mature T-cells, whereas the regulation of noise we have observed occurs only in thymocytes.

Building on our finding that the miRNA-dependent changes in noise we have observed are specific to immature thymocytes, and not mature T-cells, we looked further at the miR-181 family, which has been previously documented to be highly expressed in DP thymocytes, and then decline in expression as cells mature. Members of the miR-181 family have been previously associated with changes in thymocyte sensitivity to activation, which declines significantly as thymocytes mature, and it was suggested that co-regulation of the miR-181 family at the same time as the sensitivity of the TCR changes during development might
result in stabilisation of genes such as CD69 across a population of developing cells. However, our finding that miR-181a/b1 deletion results in no increase in CD69 CV on activation shows that miR-181 is not the primary miRNA involved in the regulation of CD69 noise.

We attempted to confirm miRNA targeting of all three miRNAs in thymocytes by knockdown of each miRNA. Using hairpin inhibitors to knock down both miR-181a and miR-20a showed significant upregulation of CD69 expression in activated thymocytes. However, no change in CD69 expression was seen when miR-130 was inhibited, and the change in CD69 expression observed from miR-20a inhibition was smaller than would have been expected, given the upregulation in eGFP from the reporter constructs seen when the miR-20 family binding site is mutated: this may be due to the high level of redundancy in the miR-20 family. Hairpin inhibitors are specific to one particular miRNA and may not knock down other family members, resulting in incomplete knockdown. To address this issue further, we attempted to inhibit miRNAs with tiny-LNA inhibitors, a recently developed technique which uses LNAs complementary to the seed sequence of a miRNA family to inhibit the entire family. Transfection of miR-20 family LNAs into thymocytes produced a significantly larger increase in CD69 expression, which is consistent with the broader targeting of tiny LNAs producing stronger downregulation of the miR-20 family. However, no increase in expression was observed on transfection of tiny LNAs against either the miR-130 or miR-181 family. In the case of miR-181, inhibition by hairpin inhibitors and LNAs show conflicting results: this may be because of different binding affinities. It is not clear why miR-130 shows no increase in CD69 expression when inhibited by either hairpin or tiny-LNA inhibitors. Profiling of miRNA levels in thymocytes showed low levels of miR-130b and miR-301a, which suggests that these miRNAs may not be responsible for repression of CD69, despite the effect of mutating the predicted miR-130 family binding site on expression of the reporter constructs. It may be that the predicted miR-130 family binding site is targeted by another miRNA, such as miR-19 which has poorly conserved binding sites located close to or overlapping with the mutated predicted miR-130 binding site.

In all three miRNAs, inhibition with either hairpins or tiny-LNAs produced no increase in noise. Investigation of changes in cell-cell variation requires that
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no additional noise is introduced into the system without appropriate controls to allow the isolation of changes in noise due to changes of experimental variables. Transfection of thymocytes with inhibitors in FTOC is an complicated procedure which make it difficult to reproduce the conditions of adult miRNA-depleted thy-
mocytes, in particular, introducing additional variation both through the FTOC protocols and by transfection of varying levels of inhibitor or control into indi-
vidual cells. To confirm the role of the miR-181 family in regulating CD69, and resolve the contradictory results obtained using hairpins and tiny-LNAs, we ob-
tained mice with the miR-181ab-1 locus deleted, and observed the level of CD69 in activated thymocytes and peripheral T-cells compared to wild-type controls. This confirmed the results obtained using the reporter constructs and the hair-
pin inhibitors, showing that the miR-181 family targets CD69. Interestingly, in the miR-181ab-1-deleted mice, an increase in CD69 expression was also seen in peripheral T-cells, despite no change in expression of our Cd69 3’ UTR reporters being seen in peripheral T-cells when the predicted miR-181 binding site was mu-
tated. This suggests that constitutive knockout of miR-181ab-1 may lead to develop-
mental changes in the mechanisms of CD69 expression that result in increased expression of CD69 on activation even after miR-181 expression has been down-
regulated. As with inhibition of miR-181 in thymocytes, no increase in CD69 CV was observed in miR-181ab-1-deleted cells. This suggests that miR-181 regula-
tion of CD69 is not the primary mechanism responsible for the increase in CD69 CV seen in activated thymocytes.

Although our experiments confirm that CD69 is a direct miRNA target, the question of how miRNAs may regulate noise needs further investigation: al-
though depletion of the miR-181 and miR-20 families, by deletion or LNA in-
hibition, showed increases in gene expression in activated thymocytes, unlike in Dicer-deleted thymocytes, no increase in noise was seen. Noise remains an experimen
tally complex area to study, due to the requirement that experiments that investigate mechanisms of noise must not introduce additional noise into the sys-
tem. This is particularly problematic in transcriptional circuits, as the response of the circuit to a given perturbation cannot be intuitively predicted in the same way as, for example, the response in mean levels of a particular mRNA to increasing levels of a transcriptional activator can be. Our results show that CD69 shows in-
5.4 MicroRNAs as part of developmental regulatory networks

creased noise in Dicer-depleted thymocytes, and that this Dicer-dependent noise regulation is developmentally regulated, as it is not seen in mature T-cells. Experiments using fluorescent reporter constructs, miRNA inhibitors and miRNA-knockout mice have shown that Cd69 is a direct miRNA target. However, it is not clear how exactly the depletion of small RNAs caused by Dicer-depletion results in the increases in cell-cell variation we have seen.

Finally, we used computational simulations to investigate potential theoretical mechanisms by which miRNAs might regulate cell-cell variation. Although we could not determine exact estimates for all the rate constants required to accurately specify a model, simulating simple miRNA-containing FFLs with biologically plausible rate constants shows that such circuits can produce comparable changes in mean protein expression and protein CV to our observations in experimental systems. Interestingly, the regulation of variation appeared in both our models of miRNA action, despite considerable differences in the two systems. We also noted that in our miRNA-mRNA binding model, the relative levels of mean expression and cell-cell variation could be independently altered by varying different parameters of miRNA action. In both systems, moderate regulation of biological noise occurred under a wide range of parameters, suggesting that noise regulation is a robust property of the miRNA-containing FFL. While this model shows that miRNA regulation might be capable of regulating cell-cell variation, it is important to remember that the modelled system is a steady-state system, where the various components have reached an equilibrium. In reality, particularly in the case of the rapid induction of CD69 expression, this is unlikely to be the case.

These results suggest two possible scenarios for the Dicer-dependent regulation of CD69 noise. Firstly, CD69 could be regulated directly by a miRNA such as miR-17 or miR-20a, which is upregulated on T-cell activation at the same time as CD69 and could act in an incoherent FFL to reduce cell-cell variation (Figure 5.1b). In the case of the miR-20 family, this circuit must principally act to reduce noise from a source specific to thymocytes, but which is not present in mature T-cells. Secondly, some other upstream, Dicer-dependent mechanism such as the miRNA regulation of components of the T-cell activation pathway, or other factors involved in T-cell maturation, could regulate CD69 cell-cell variation, which
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Figure 5.1: Potential mechanisms for noise regulation of Cd69 - (a) CD69 expression is regulated as part of an incoherent feed-forward loop in which T-cell activation upregulates both the miR-20 family and CD69. (b) CD69 expression is regulated by some other, upstream Dicer-dependent mechanism which is independent of the direct miRNA regulation we have identified results in an increase in CD69 variation in Dicer-deleted cells independent of the direct regulation of CD69 by miRNAs (Figure 5.1b).

5.5 Future directions

These results suggest a number of future experiments. To continue to investigate the regulation of Cd69 expression by miRNAs, further work on the effect of deleting other Cd69-targeting miRNAs would allow better understanding of the mechanisms by which Dicer-deletion results in an increase in noise. For example, mice containing deletions of the miR-17-92 cluster have been generated, and it would be interesting to investigate how deletion of the complete 17-92 cluster compares with deletion of the miR-181ab cluster.

While my experiments show that specific genes show increased variation in Dicer-deleted cells, the phenotypic effect of this regulation remains to be seen. The functions of CD69, Sca-1 and CD44 are all still poorly understood, but a number of ligands and binding partners have been identified. For example, future experiments could look at the effect of the increase in CD44 noise observed in
5.5 Future directions

*DicER*-deleted cells on the response of cells to hyaluronic acid or other ligands of CD44. Variation in levels of Sca-1 has already been shown to affect lineage choice in haematopoietic progenitor cells, and to be associated with different levels of transcription factors such as GATA1 or PU. It would be interesting to investigate whether similar effects can be observed in thymocytes. While no ligand has yet been identified for CD69, future experiments could investigate the effect of increased variation on the expression of S1P, a binding partner of CD69, or on thymic migration, which CD69 has been shown to influence.

The control of CD69 noise during activation only in immature thymocytes is particularly interesting, as it may reflect a broader mechanism of control that may extend to other miRNA-regulated genes during selection: it would also be interesting to investigate whether the increase in CD69 noise in activated thymocytes is due in part to thymic selection. T-cell selection and activation is already under precise control from a number of pathways: for example, the CD8 coreceptor has been found to tune T-cell responsiveness. As CD69 expression is upregulated by TCR-mediated activation, it would be interesting to see how much of the noise seen in the thymus is due to varying TCR sensitivities, and whether this noise regulation occurs in other genes induced by T-cell activation. This could be investigated through the use of TCR-restricted mice such as OT1 or AND TCR transgenic mice in Rag knockouts.

Further work on modelling the action of miRNAs would be useful: in particular, while a number of theoretical studies have simulated particular transcription motifs or networks, relatively little work has been done to directly quantify important constants which determine miRNA dynamics, such as the rates of miRNA production and decay. With accurate measurements of these various constants, different models of miRNA action can be simulated and tested against experimental data more accurately, allowing the construction of better models and the identification from these models of areas of miRNA action in need of further investigation.

Future experiments may also expand into different experimental systems: while T-cell development is a well-characterised system, the dynamics of T-cell development make experiments such as efficient miRNA knockdowns experimentally complicated, and may introduce additional variability into experiments.
5. DISCUSSION

Future experiments could investigate the role of miRNAs in regulating variation in other systems. For example, several miRNAs are known to be involved in embryonic stem cell development, and there are a number of documented ES cell miRNAs involved in regulation of the ES cell state.

Our results have shown that deletion of Dicer, an essential component of the miRNA biogenesis pathway, results in an increase in both the mean level of expression and also the cell-cell variation in expression of particular proteins, including highly expressed proteins such as CD69 in activated T-cells. Reporter construct studies have identified CD69 as the target of a number of miRNAs including the miR-181, miR-130 and miR-20 families. Regulation of CD69 by miR-181 and miR-20 family members was confirmed by miRNA inhibition and deletion experiments. Further work is needed to characterise the regulation of CD69 by miRNAs, and the mechanisms by which deletion of Dicer results in increases in the cell-cell variation of specific genes.
References


REFERENCES


REFERENCES


REFERENCES


transfer of mRNAs and microRNAs is a novel mechanism of genetic regulation and promotes tumour biology.


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REFERENCES


Appendix 1

6.1 Basic Gillespie simulation function

Electronic copies of simulation code can be found at: https://github.com/RoryBlevins/PhD-Thesis

# Gillespie simulation function

gillespied=function (N, T=100, dt=1, ...)
{
  tt=0
  n=T/%/%dt
  x=N$M
  S=t(N$Post-N$Pre)
  u=nrow(S)
  v=ncol(S)
  xmat=matrix(0,ncol=u,nrow=n)
  i=1
  target=0
  repeat {
    h=N$h(x, th, ...)
    h0=sum(h)
    if (h0<1e-10)
      tt=1e99
    else
      tt=tt+rexp(1,h0)
    while (tt>=target) {

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\[
\begin{align*}
&x_{\text{mat}[i,j]} = x \\
&i = i + 1 \\
&\text{target} = \text{target} + dt \\
&\text{if} \ (i > n) \\
&\quad \text{return} (\text{ts} (x_{\text{mat}}, \text{start} = 0, \text{deltat} = dt)) \\
&\} \\
&j = \text{sample} (v, 1, \text{prob} = h) \\
&x = x + S[i,j] \\
&\} \\
&\}
\]

6.2 Simulation of miRNA FFL with translational inhibition

# test of miRNA-containing FFL

FFL=list()  
# starting values
FFL$M=c(
100,100,100,100,100
)

# Reaction matrices
FFL$Pre=matrix(c(
0,0,0,0,0,
1,0,0,0,0,
1,0,0,0,0,
0,1,0,0,0,
0,0,0,0,0,
0,0,1,0,0,
0,0,0,0,0,
0,0,0,1,0,
0,0,0,0,1,
),nrow=5,byrow=TRUE)

FFL$Post=matrix(c(  
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6.2 Simulation of miRNA FFL with translational inhibition

1,0,0,0,0, 
0,0,0,0,0, 
1,1,0,0,0, 
0,0,0,0,0, 
0,0,1,0,0, 
0,0,0,0,0, 
0,0,0,1,0, 
0,0,0,0,0, 
0,0,0,1,1, 
0,0,0,0,0
), ncol=5, byrow=TRUE)

# rate constant calculation
FFL$h=function(x, th)
{
  return(c(
    th[1], # TF transcription
    th[2]*x[1], # TF mRNA degradation
    th[3]*x[1], # TF translation
    th[4]*x[2], # TF protein degradation
    th[8]*x[3], # miRNA degradation
    th[2]*x[4], # output mRNA degradation
    (th[10]*x[4])/(1+(x[3]/th[11])^th[7]), # output translation
    th[4]*x[5] # output degradation
  ))
}

# test of miRNA-independent circuit

TF=list()
# starting values
TF$M=c(
  100, 100, 100, 100, 100,
)

# Reaction matrices
6. APPENDIX 1

```r
TF$Pre=matrix(c(
  0,0,0,0,0,
  1,0,0,0,0,
  1,0,0,0,0,
  0,1,0,0,0,
  0,0,0,0,0,
  0,0,0,1,0,
  0,0,0,1,0,
  0,0,0,0,1
 ),ncol=5,byrow=TRUE)

TF$Post=matrix(c(
  1,0,0,0,0,
  0,0,0,0,0,
  1,1,0,0,0,
  0,0,0,0,0,
  0,0,0,1,0,
  0,0,0,0,0,
  0,0,0,1,1,
  0,0,0,0,0
 ),ncol=5,byrow=TRUE)

# rate constant calculation
TF$h=function(x,th)
{
  return(c(
    th[1], # TF transcription
    th[2]*x[1], # TF mRNA degradation
    th[3]*x[1], # TF translation
    th[4]*x[2], # TF protein degradation
    th[2]*x[4], # output mRNA degradation
    th[10]*x[4], # output translation
    th[4]*x[5] # output degradation
  ))
}
```

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6.2 Simulation of miRNA FFL with translational inhibition

# Timecourse data

T <- 5000
dt <- 10

measuretime <- 4000/dt

# number of simulations
n <- 200

# rate constants
th <- c(
  0.06,  # transcription rate
  0.006,  # TF mRNA degradation rate
  0.04,  # TF translation rate
  0.002,  # TF protein degradation rate
  0.5,  # base miRNA transcription rate
  200,  # miRNA dissociation coefficient
  2,  # hill coefficient for miRNA
  0.006,  # miRNA degradation rate
  0.8,  # base output transcription rate
  200,  # miRNA dissociation coefficient
  2,  # hill coefficient for miRNA
  0.006,  # output mRNA degradation rate
  0.04,  # base translation rate
  60,  # output translation repression
  2,  # hill coefficient for translation
  0.002  # output protein degradation
)

# number of simulations
n <- 10000

FFLresults <- 0
TFresults <- 0

for (ii in (1:n)){

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```r
FFLoutput <- gillespie(FFL, T, dt)
TFoutput <- gillespie(TF, T, dt)

if (ii%%10==0){
  print(ii)
}

FFLresults <- rbind(FFLresults, FFLoutput[measuretime,])
TFresults <- rbind(TFresults, TFoutput[measuretime,])

TFresults <- TFresults[-1,]
FFLresults <- FFLresults[-1,]

TFMean <- mean(TFresults[,5])
FFLMean <- mean(FFLresults[,5])

TFSD <- sd(TFresults[,5])
FFLSD <- sd(FFLresults[,5])

RelativeMean <- TFMean/FFLMean

FFLCV <- FFLSD/FFLMean
TFCV <- TFSD/TFMean

RelativeCV <- TFCV/FFLCV

RelativeMean
RelativeCV
```
6.3 Simulation of miRNA FFL with miRNA-mRNA binding

# test of miRNA-containing FFL

FFL=list()
# starting values
FFL$M=c(
  100, # TF mRNA
  100, # TF protein
  100,# miRNA
  100,# Output mRNA
  100,# Output protein
  0 # miRNA-protein complex
)

# Reaction matrices
FFL$Pre=matrix(c(
  0,0,0,0,0,0,# TF transcription
  1,0,0,0,0,0,# TF mRNA degradation
  1,0,0,0,0,0,# TF translation
  0,0,0,0,0,0,# TF protein degradation
  0,0,0,1,0,0,# miRNA-transcription
  0,0,0,1,0,0,# miRNA-output mRNA binding
  0,0,0,0,1,0,# miRNA-output mRNA dissociation
  0,0,0,0,1,0,# miRNA-output mRNA degradation of mRNA
  0,0,0,0,0,0,# Output transcription
  0,0,0,0,0,0,# Output mRNA degradation
  0,0,0,0,0,0,# Output translation
  0,0,0,0,0,0,# Output protein degradation
),ncol=6,byrow=TRUE)

FFL$Post=matrix(c(
  1,0,0,0,0,0,# TF transcription
  0,0,0,0,0,0,# TF mRNA degradation
  1,1,0,0,0,0,# TF translation
  0,0,0,0,0,0,# TF protein degradation
),ncol=6,byrow=TRUE)
### APPENDIX 1

```
0,0,1,0,0,0,# miRNA transcription
0,0,0,0,0,0,# miRNA degradation
0,0,0,0,0,1,# miRNA-output mRNA binding
0,0,1,1,0,0,# miRNA-output mRNA dissociation
0,0,1,0,0,0,# miRNA-output mRNA degradation of mRNA
0,0,0,1,0,0,# Output transcription
0,0,0,0,0,0,# Output mRNA degradation
0,0,0,1,1,0,# Output translation
0,0,0,0,0,0 # Output protein degradation
),ncol=6,byrow=TRUE)

#rate constant calculation
FFL$h=function(x,th)
{
  return(c(
   th[1], #TF transcription
   th[2]*x[1],#TF mRNA degradation
   th[3]*x[1],#TF translation
   th[4]*x[2],#TF protein degradation
   th[8]*x[3],#miRNA degradation
   th[9]*x[3]*x[4],# miRNA-output mRNA binding
   th[10]*x[6],# miRNA-output mRNA dissociation
   th[11]*x[6],# miRNA-output mRNA degradation of mRNA
   th[15]*x[4],#output mRNA degradation
   th[16]*x[4],#output translation
   th[17]*x[5] #output degradation
))
}

# test of miRNA-independent circuit
TF=list()
#starting values
```
6.3 Simulation of miRNA FFL with miRNA-mRNA binding

TF$M=c(
100, # TF mRNA
100, # TF protein
100, # miRNA
100, # Output mRNA
100, # Output protein
0 # miRNA-protein complex
)

#Reaction matrices
TF$Pre=matrix(c(
0,0,0,0,0,0,# TF transcription
1,0,0,0,0,0,# TF mRNA degradation
1,0,0,0,0,0,# TF translation
0,1,0,0,0,0,# TF protein degradation
0,0,0,0,0,0,# Output transcription
0,0,0,1,0,0,# Output mRNA degradation
0,0,0,1,0,0,# Output translation
0,0,0,0,1,0 # Output protein degradation
),ncol=6,byrow=TRUE)

TF$Post=matrix(c(
1,0,0,0,0,0,# TF transcription
0,0,0,0,0,0,# TF mRNA degradation
1,1,0,0,0,0,# TF translation
0,0,0,0,0,0,# TF protein degradation
0,0,0,1,0,0,# Output transcription
0,0,0,0,1,0,# Output mRNA degradation
0,0,0,1,1,0,# Output translation
0,0,0,0,0,0 # Output protein degradation
),ncol=6,byrow=TRUE)

#rate constant calculation
TF$h=function(x,th)
{
  return(c(
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th[1], #TF transcription
th[2]*x[1], #TF mRNA degradation
th[3]*x[1], #TF translation
th[4]*x[2], #TF protein degradation
th[15]*x[4], #output mRNA degradation
th[16]*x[4], #output translation
th[17]*x[5] #output degradation
)}

#Timecourse data

T<- 5000
dt<- 10
measuretime <- 4000/dt

#rate constants
th=c(
0.06,   # transcription rate
0.006,  # TF mRNA degradation rate
0.04,   # TF translation rate
0.002,  # TF protein degradation rate
0.5,    # base miRNA transcription rate
200,    # miRNA dissociation coefficient
2,      # hill coefficient for miRNA
0.006,  # miRNA degradation rate
0.0001, # miRNA complex formation rate
0.0001, # miRNA-mRNA dissociation rate
0.02,   # miRNA-mRNA degradation rate
0.8,    # base output transcription rate
200,    # mRNA dissociation coefficient
2,      # hill coefficient for transcription
0.006,  # output mRNA degradation rate
0.04,   # base translation rate
0.002   # output protein degradation
)
6.3 Simulation of miRNA FFL with miRNA-mRNA binding

```r
#number of simulations
n <- 10000

FFLresults <- 0
TFresults <- 0

for (ii in (1:n)){
  FFLOutput <- gillespied(FFL, T, dt)
  TFOutput <- gillespied(TF, T, dt)

  if (ii%%10==0){
    print(ii)
  }
}

FFLresults <- rbind(FFLresults, FFLOutput[measuretime,])
TFresults <- rbind(TFresults, TFOutput[measuretime,])

TFresults <- TFresults[-1,]
FFLresults <- FFLresults[-1,]

TFMean <- mean(TFresults[,5])
FFLMean <- mean(FFLresults[,5])

TFSD <- sd(TFresults[,5])
FFLSD <- sd(FFLresults[,5])

RelativeMean <- TFMean/FFLMean
FFLCV <- FFLSD/FFLMean
TFCV <- TFSD/TFMean
RelativeCV <- TFCV/FFLCV
```

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RelativeMean

RelativeCV