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MicroRNA Identity and Function in Astrocyte Differentiation

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MicroRNA Identity and Function in Astrocyte Differentiation

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For my parents, Jayashree and Saseendran Shenoy

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Official business

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MicroRNA Identity and Function in Astrocyte Differentiation

Archana Shenoy

Abstract

Astrocytes are a dominant cell type in the central nervous system (CNS), intimately associated with neuronal synapses and CNS function. Studies in recent years have yielded insight into the remarkable morphological and functional diversity of astrocytes. However, mechanisms underlying the specification and differentiation of glial-specified precursors (GPCs) into astrocytes during development are not fully understood. In Chapter 2&3, I present work to address the hypothesis that miRNAs target key regulatory genes and pathways to promote terminal astrocyte differentiation, using a conditional Dgcr8 knockout model in embryonic stem cell–derived GPCs. In Chapter 4, I show that the function of Dgcr8 is largely limited to its known roles in canonical miRNA biogenesis, supporting its use in studying miRNA function. In GPCs, the loss of Dgcr8 and subsequent loss of miRNAs prevents upregulation of astrocyte markers and activation of JAK-STAT signaling during differentiation. Using a screening approach, I discovered that two miRNA families expressed in GPCs and astrocytes, let-7 and miR-125, rescue the upregulation of GFAP during differentiation but not activation of the JAK-STAT pathways. However, forced activation of the JAK-STAT pathway is sufficient to rescue

the differentiation phenotype, suggesting that let-7 and miR-125 may regulate the pathway at downstream steps. Microarray and bioinformatics analysis following add back of the two miRNAs revealed direct and indirect targets. While individual knockdown of targets is insufficient to recapitulate the effect of let-7 and miR-125, at least one let-7/miR-125 target, Plagl2, inhibits astrocyte differentiation when overexpressed in wild-type cells. Taken together, these observations strongly suggest that the coordination of multiple miRNAs effects on multiple targets is necessary to promote the cell fate transition that leads to generation of astrocytes.

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Thesis Summary

Nervous system development hinges upon a set of cell fate transitions that allows for sequential generation of three major cell types – neurons, astrocytes and oligodendrocytes. Astrocytes and oligodendrocytes, which arise in late embryonal and early postnatal stages, are collectively referred to as glia. In mammals, both neurons and glia are derived from a common neuroepithelial progenitor that is sequentially restricted to a neural and then a glial fate during development, through key transcriptional and epigenetic changes. Mechanisms underlying the terminal differentiation of neurogenic progenitors have been studied extensively. However, relative to neuronal differentiation, a complete understanding of the drivers of astroglial differentiation is lacking. This thesis investigates one regulatory component, microRNA-mediated modulation of the transcriptome, which is necessary for proper differentiation of glial restricted progenitors (GPCs) into astrocytes.

MicroRNAs (miRNAs), a class of regulatory small RNAs, have emerged in the last decade as important regulators of cell fate transitions. Each cell type is characterized by the dominant expression of a few miRNA families (Marson et al., 2008). A single miRNA simultaneously modulates hundreds of mRNA transcripts, a characteristic that enables miRNAs to participate in

cellular transitions that require large-scale transcriptomic changes. Studies of global miRNA loss have shown that miRNAs are required for proper development of the neural lineage (De Pietri Tonelli et al., 2008; Dugas et al., 2010; Zhao et al., 2010b). The miRNAs expressed and the mRNAs they target to promote differentiation of lineage-restricted precursors into neurons and oligodendrocytes have been extensively studied (Conaco et al., 2006; Zhao et al., 2009; Dugas et al., 2010; Zhao et al., 2010b). However, due to the lack of markers to isolate or target astrocyte progenitors, the function of miRNAs in astrocyte differentiation is not well studied. I hypothesized that miRNAs target key regulatory pathways to promote terminal differentiation of glial progenitors into astrocytes.

Here, I describe derivation and use of a homogenous ESC-derived glial precursor population that specifically undergoes astrocyte differentiation under conditions that promote astrogliogenesis *in vivo*. In order to study the function of individual miRNAs and identify their targets, I have used a miRNA-deficient model, generated by knocking out a key miRNA biogenesis protein, Dgcr8. In **Chapter 2**, I characterize the phenotype of Dgcr8 knockout GPCs, and identify the miRNAs that promote astrocyte differentiation. In **Chapter 3**, I elucidate the mechanisms underlying miRNA-mediated regulation of astrocyte differentiation. In **Chapter 4** of my thesis, I show that the

ability of Dgcr8 to direct cleavage of hairpin substrates is restricted to miRNA biogenesis and autoregulation.

Chapter 1: microRNA biology of lineage-committed progenitors

Introduction

The highly specialized, functionally diverse cell types in mammals are derived through an orchestrated unfolding of a developmental program following fertilization. Early development is marked by the rapid expansion of pluripotent cell populations in the blastocyst and epiblast. Following this expansion, pluripotent cells begin differentiating into multipotent cells of the three germ layers, ectoderm, mesoderm and endoderm. These cells undergo patterning and further differentiation into lineage-restricted stem/progenitor cells that undergo several cycles of self-renewal, further differentiation and migration before exiting the cell cycle to give rise to their terminally differentiated progeny. The path from specification of stem/progenitor cells to their final post-mitotic progeny is marked by large transcriptomic changes including upregulation of a new gene expression program and downregulation of the stem/progenitor program that allow a cell to eventually become functionally and morphologically specialized. During this time, some progenitor cells are also set aside in many tissues and become adult stem cells.

The balance between proliferation, apoptosis and differentiation of lineage-committed stem/progenitor cells is essential for proper development. In recent years, a class of non-coding small RNAs called microRNAs (miRNAs) has come to the fore as a regulator of proliferation and cell fate transitions. miRNAs are small, 21 nucleotide RNAs that bind target mRNAs via base pairing and lead to translational repression and mRNA degradation. MiRNAs simultaneously down regulate hundreds of mRNAs and thus, are primed to regulate and promote transitions that involve silencing of a given cellular state (Lim et al., 2005). Recent studies have also shown that miRNAs are integrated into the feedback and feed forward loops with important transcription factors, thus providing robustness to gene expression programs (Tsang et al., 2007).

The loss of miRNA biogenesis proteins leads to an early embryonic lethal phenotype of miRNA-deficient mice (Bernstein et al., 2003) (Wang et al., 2007a). In the developing blastocyst, three stem cell populations embryonic stem cell, trophoblast stem cell and extraembryonic endoderm stem cells (ES, TS and XEN) can be derived from the three main cellular compartments – inner cell mass, trophectoderm and primitive endoderm respectively. Analysis of the effects of global miRNA loss in these cells has shown that miRNAs are required for the proliferation of these compartments (Wang et al., 2007a; Spruce et al., 2010). Since each cell can express hundreds of miRNAs, a multitude of studies have focused on identifying functionally important miRNAs and

their targets. The most detailed analyses of miRNA function have been in pluripotent ES cells (Wang et al., 2008; Melton et al., 2010; Wang et al., 2013b). The ease of the culture system and viability of knockout cells makes the ES cells amenable to detailed functional characterization of expressed miRNAs and their targets. These early studies established that the highly expressed miR-290/302 family of miRNAs functions in processes central to ES cell identity. The miR-290 family miRNAs maintain the unique cell cycle structure of ESCs, upregulate gene networks necessary for pluripotency and suppress the expression of differentiation-promoting miRNAs (Wang et al., 2008; Melton et al., 2010). Thus, miRNA function is necessary to maintain the unique characteristics of ES cells.

ES cells mark a specialized developmental stage in which all cells of the embryo are pluripotent. As development progresses and patterning occurs, pluripotent cells first differentiate into the three germ layers. Cells in these layers then give rise to all tissue stem/progenitor cells. In comparison to ES cells, somatic stem/progenitor cells often have an elongated cell cycle due to an extended G1 phase and restricted lineage during differentiation. While more difficult to study than ES cells, a multitude of studies have revealed the identity and function of miRNAs in numerous lineages. In this review, we will examine how miRNAs maintain cellular identity in somatic stem/progenitor cells and promote the transition to their final terminally differentiated

state. We will also examine commonalities and differences in miRNA functions across various lineages.

What are the fate choices of somatic stem/progenitor cells?

Proliferation: Development of organs systems in mammals is dependent upon the specification and differentiation of stem/progenitor cells that arise from the three germ layers formed in the epiblast. Following specification, stem/progenitor cells proliferate and expand. Transcription factors and signaling pathways associated with the "stemness" program are highly expressed at this stage and promote movement through the cell cycle by impinging on cell cycle regulators (Li et al., 2008; Lee et al., 2013; Mi et al., 2013).

Differentiation: During organogenesis, differentiation of stem/progenitor cells is typically a multistep process that ultimately ends in terminal differentiation. A lineage-committed stem cell population often differentiates into multiple restricted progenitors, either sequentially or concomitantly. This is best exemplified in the hematopoietic system where multipotent hematopoietic stem cells (HSCs), differentiate into the more restricted progenitors of the lymphoid and myeloid lineage, which in turn differentiate into unipotent progenitors (Orkin, 2000). In the nervous system, this process occurs sequentially to generate the two main cell types

– neurons and glia. Stem cells in the neuroepithelium first give rise to neurogenic progenitors during embryogenesis and gliogenic progenitors in late embryogenesis and early postnatal stages (Rowitch and Kriegstein, 2010). A proper balance of cell fate choices made by common progenitors is essential for generating a diversity of cell types and proper function of a given organ.

Following production of fate-restricted progenitors, these cells terminally differentiate, giving rise to functionally specialized cell types. While proliferation requires precise control of pathways controlling movement through the various phases of cell cycle and ultimately cell number, proper differentiation requires a coordinated shutdown of the "stemness" program of the progenitors, exit of the cell cycle and activation of genes required for functional and morphological specialization of the mature cell. With a few exceptions, exit from cell cycle is inseparably linked with functional specialization of a terminally differentiated cell (Buttitta and Edgar, 2007).

Apoptosis: Proliferation and differentiation of stem/progenitor populations is balanced with apoptosis to maintain precise control of cell numbers. The role of apoptosis in development has been studied extensively and is described in a number of reviews (Jacobson et al., 1997). The

significance of apoptosis in progenitor cell populations is seen in phenotypic analyses of neuronal development in mice lacking pro-apoptotic genes such as Bax and Bak or Apaf1 (Yoshida et al., 1998; Lindsten et al., 2000). Deletion of these genes leads to reduced apoptosis in developing structures enriched in rapidly expanding progenitors such as the neuroepithelium. This results in an overgrowth of brain structures and a neural tube closure defect(Cecconi, 2004).

Thus, during development, progenitor cell self-renewal, apoptosis, cell fate commitment and terminal differentiation are tightly controlled to allow for the emergence of functionally specialized cells of the adult organism.

How does the global loss of miRNAs influence these decisions?

Profiling studies of miRNAs using microarray and small RNA sequencing have established that a single cell can express hundreds of miRNAs to varying levels, and miRNAs often have overlapping targets (Lim et al., 2005). Therefore, in order to study the roles that miRNAs play during development, lineage-specific Cre-based deletions of the miRNA biogenesis proteins Dicer, Dgcr8 and Drosha have been used. Due to the fact that these proteins are predominantly involved in miRNA biogenesis, their loss often results in similar phenotypes (Yi et al., 2009; Bezman et al., 2010).

However, there has been a lot of interest in determining miRNA biogenesis-independent roles of these proteins. Dicer has known roles outside of the maturation of canonical miRNAs. For example, in mouse ES cells, Dicer processes other subclasses of miRNAs including mirtrons and short hairpin RNAs as well as endogenous siRNAs (Babiarz et al., 2008). Similarly, Dicer processes endogenous siRNAs in mouse oocytes (Tam et al., 2008; Watanabe et al., 2008).

Consistent with these additional roles of Dicer, Dgcr8 knockout (KO) ES cells have less severe phenotypes than Dicer knockout ES cells. However, the Dgcr8-Drosha complex, called the microprocessor, was recently shown to have an additional role in directly destabilizing a mRNA target(Han et al., 2009). Specifically, it can cleave hairpins in the 5′UTR and coding region of the Dgcr8 mRNA, which in turns destabilizes the mature transcript. This negative feedback loop on Dgcr8 suggests the importance of tight homeostatic control of the microprocessor in normal cellular function. The finding that the microprocessor can directly influence *Dgcr8* mRNA levels raises the possibility that this mechanism may affect many other mRNAs. In **Chapter 2** of this thesis, I describe work showing that the dominant functions of Dgcr8 are miRNA biogenesis and auto-regulation. There is limited evidence for cleavage of mRNAs by Dgcr8, establishing the validity of using the Dgcr8 KO model to study miRNA biology.

In every tissue-type and lineage examined, global miRNA loss leads to defects in tissue morphogenesis (**Table 1-1**). Thus, miRNAs are likely indispensable for proper development of all tissues. An interesting question raised by these observations is the variation in miRNA function across lineages. For instance, the let-7 family of miRNAs, which is expressed in differentiated tissues, targets G1-S phase activators to extend G1 during early ES cell differentiation (Wang et al., 2013b). Is this a continued function of let-7 across all lineages or has it been co-opted for lineage-specific functions? While these questions are not fully answered, here we summarize the knowledge gained from such studies regarding miRNA function in somatic stem/progenitor cells, and show that global miRNA function varies across lineages, with some recurrent themes.

Proliferation: Studies of miRNA loss in somatic stem/progenitor cells suggest that unlike in early development, miRNAs do not have obligatory roles in promoting proliferation. In fact, stem/progenitor compartments in multiple lineage-specific Dicer and Dgcr8 knockout mice do not exhibit proliferation defects, suggesting that unlike in the earliest stem cells, miRNAs are not widely necessary for expansion of somatic stem/progenitor cell populations. For instance, the loss of Dicer in neural crest cells and nephrogenic progenitors does not affect proliferation as

assayed by incorporation of BrdU or Ki67 staining (Zehir et al., 2010; Nagalakshmi et al., 2011). Some have suggested that miRNA function may be most important during the cell fate transitions that give rjkljklise to their differentiated progeny rather than in the expansion of stem/progenitor cells (De Pietri Tonelli et al., 2008). Alternatively, compensatory mechanisms may exist in some cells to overcome the loss of miRNAs.

However, a few studies do show that miRNAs, in a context-dependent manner, can promote proliferation. Cell types in which Dicer loss leads to a reduction in proliferative capacity include neuronal progenitors, hair follicle cells and naïve T cells (Andl et al., 2006; McLoughlin et al., 2012). In some cases, such as in adult hematopoietic stem cells, the loss of Dicer leads to increased proliferation, which the authors hypothesized to be compensatory to increased apoptosis (Guo et al., 2010). However, a recent study has implicated the miRNA let-7 in suppressing the cell cycle in adult HSCs and contributing to the maintenance of the stem cell pool, suggesting that the increased proliferation may be a direct effect of miRNA loss (Copley et al., 2013). Thus, in specific lineages, miRNAs play important roles in promoting proliferation of progenitor populations. Studies of specific miRNAs will eventually reveal whether the lack of proliferation defects seen upon global loss of miRNAs in some progenitor populations reflects compensatory mechanisms rather than an absence of miRNA function.

Differentiation: The ability to modulate hundreds of mRNAs perfectly situates miRNAs to promote many aspects of differentiation simultaneously. The necessity of miRNAs for proper differentiation of many lineage-committed stem/progenitor cells is highlighted by the striking phenotypes of miRNA-deficient mutants (O'Rourke et al., 2007; De Pietri Tonelli et al., 2008). During lineage commitment of tissue stem cells, miRNAs in several instances play important roles in ensuring proper ratios of various cell fates. The loss of Dicer in HSCs for instance skews differentiation towards the myeloid lineage (Buza-Vidas et al., 2012). The loss of Dicer in developing T cells skews differentiation towards the IFNgamma producing subset at the expense of the IL-4 producing subset (Muljo et al., 2005). In the nervous system and during development of cells of the retina, it has been shown that the loss of Dicer can inhibit fate specification of later progenitors, which can be rescued by overexpression of specific miRNAs (Georgi and Reh, 2010; La Torre et al., 2013).

Following multiple differentiation events, multipotent stem/progenitor cells eventually give rise to unipotent progenitors that must undergo terminal differentiation. Terminal differentiation of committed progenitors is often defective in the absence of miRNAs, as evidenced by a loss or reduction of cells expressing markers of terminal differentiation. For example, the loss of Dicer

in neural stem/progenitor cells blocks upregulation of both neuronal and glial markers during terminal differentiation (Andersson et al., 2010). Interestingly, some progenitor populations such as the neural crest lineage do not exhibit a dependence on miRNAs for proper terminal differentiation (Zehir et al., 2010). Taken together, these studies further suggest that miRNAs are variably required for differentiation across different cell populations.

Apoptosis: The loss of miRNAs most commonly leads to increased apoptosis in stem/progenitor populations. Increased apoptosis following Dicer/Dgcr8 loss has been reported in many lineages including limb, muscle, neural, neural crest, hematopoietic, hair follicle, nephrogenic and ureteric epithelium cells (O'Rourke et al., 2007; De Pietri Tonelli et al., 2008; Yi et al., 2008; Guo et al., 2010; Zehir et al., 2010; Nagalakshmi et al., 2011). There are a few, very uncommon examples of tissues unaffected by Dicer loss, such as the developing epidermis (Andl et al., 2006). In contrast to somatic progenitors, the loss of miRNAs in pluripotent ES cells leads to a number of proliferation and differentiation phenotypes in the absence of appreciable cell death, except when stressed by serum starvation or a DNA damaging agent (Wang et al., 2007a). Interestingly, during early development, apoptosis is low in the pre-implantation blastocyst, the source of ES cells, and starts increasing post-implantation, coincident with hypersensitivity to

DNA damage (Heyer et al., 2000). In the nervous system, it has been reported that aneuploid cells preferentially undergo programmed cell death (Peterson et al., 2012).

It is tempting to speculate that miRNAs, which are involved in critical processes in early embryonic stem cells such as maintenance of their unique cell cycle structure, may play an additional role in somatic stem/progenitor cells, i.e for example as a buffer suppressing the increased cell death propensity. Alternatively, miRNAs may function in a greater number of cellular processes in progenitor cells. In the case of global miRNA loss, this may lead to greater proteomic perturbances caused by miRNA loss. It is unclear as to what extent the apoptosis-inhibiting and differentiation-inducing functions of miRNAs are related, especially in the cases where apoptosis occurs during differentiation. In a limited number of settings, inhibiting apoptosis has led to discoveries about additional roles of miRNAs during differentiation (Koralov et al., 2008).

Summary: Studies of global miRNA loss have uncovered dominant functions of miRNAs during embryogenesis. miRNAs, while modulating a combination of proliferation, survival and differentiation in every lineage, do not play identical functions in every lineage. On a molecular level, further studies are needed to determine the extent to which the various functions of

miRNAs are related. For instance, increased proliferation in the absence of miRNAs could be linked to decreased cell cycle exit during differentiation. Studies of global miRNA loss are limited in their ability to provide mechanistic insight into miRNA function during cell state transitions. Furthermore, it has been suggested in some cases that the robustness of the signaling and transcriptional networks during cell fate transitions can produce subtle phenotypes seen in some lineages following miRNA loss (Michon et al., 2010). More targeted studies have been performed to delineate the targets and identity of miRNAs functional in development and will be described in the following sections.

How are functional miRNAs identified?

In ES cells, the complete loss of canonical miRNAs leads to defects in the cell cycle as well as an inability to silence self-renewal and stemness during differentiation (Wang et al., 2007b). Since these miRNA-deficient cells continue to self-renew indefinitely, the role of individual miRNAs can be evaluated in add-back experiments where single or combinations of miRNAs are transfected back in. An unbiased screening approach for all miRNAs has uncovered miRNAs that at least partially rescue both phenotypes (Wang et al., 2008). Notably, the miRNAs with the largest effects on cell cycle were the most dominantly expressed miRNAs in ES cells – the miR-

294/302 family. This family of miRNAs also had the strongest effect in promoting de-differentiation of fibroblasts to iPS cells in a screen(Judson et al., 2013).

Due to the fact that somatic stem/progenitors cells are not always amenable to easy isolation, purification and culture, studies to identify miRNAs with important roles in somatic cell fate choices have not used unbiased screening approaches. Instead, global profiling studies have identified lineage-specific miRNAs that are also often differentially expressed during lineage differentiation (Lagos-Quintana et al., 2002; Sempere et al., 2004). These findings have driven the hypothesis that miRNAs upregulated during differentiation likely assist in silencing the progenitor transcriptional program and promoting differentiation. Over the last few years, functional approaches have supported this hypothesis for a number of candidate miRNAs such as miR-9/miR-124 in neuronal differentiation, miR-219/miR-338 in oligodendrocyte differentiation, miR-1/206/133 in muscle differentiation, and miR-203/205 in epidermal differentiation (Lagos-Quintana et al., 2002; Lena et al., 2008; Yi et al., 2008; 2009; Dugas et al., 2010; Zhao et al., 2010b).

An underexplored possibility is that miRNAs expressed prior to differentiation may prime somatic progenitor cells for differentiation. One study recently showed that miR-200, which is

expressed in neural progenitor cells, may prime them for differentiation by maintaining adequate levels of the self-renewal TFs Sox2 and E2F3 (Peng et al., 2012). It remains to be seen how widespread is such a role for miRNAs.

What are the targets of the lineage differentiation miRNAs and how do they cooperate?

The candidate studies of miRNAs in various developmental transitions have provided enormous insight into commonalities in miRNA function across cell types during terminal differentiation. In the following sections, we will provide examples of how specific miRNAs in progenitor cells are integrated into the central gene networks that control cell fate transitions and cellular identity focusing on four model lineages: myogenesis, hematopoiesis, epidermal development and neural development. We will examine how miRNA expression and function is regulated as well as the cellular processes and genes targeted by the miRNAs. We will show that miRNAs are poised to exert an effect at many steps of cell fate choices during development such as survival, terminal differentiation and downstream specification of oligopotent progenitors.

We will illustrate many common themes that emerge from the plethora of miRNA studies. First, miRNA expression in many lineages is transcriptionally regulated by epigenetic mechanisms and transcription factors classically associated with a given cell lineage. Second, mature miRNA

levels can be regulated post-transcriptionally by regulating their biogenesis and/or stability. The classical example of such regulation is the Lin28-let7 miRNA interaction. Lin28 has been shown to repress the precursor miRNAs of the let-7 family by uridylating and preventing Dicer cleavage, thus preventing production of the mature miRNAs (Heo et al., 2008; Viswanathan et al., 2008). Third, the function of mature miRNAs can be regulated by direct interactions with RNA-binding proteins or indirectly by modulation of miRNA target availability and expression. For example, alternative polyadenylation results in differential lengths of 3'UTRs and since miRNAs function by binding to cognate elements in the 3'UTR, it has long been hypothesized that miRNA activity may be modulated by 3'UTR length. Finally, key targets in a given cell type can be co-targeted by several expressed miRNAs, thus ensuring functional redundancy. In this section, we will illustrate recurrent and emergent themes of miRNA biology in organogenesis.

Myogenesis: During development, the three types of muscle – skeletal, cardiac and smooth- are derived from separate populations in the mesoderm (Margaret Buckingham, 2003). In this section, we will focus on studies in skeletal myogenesis. Multipotent cells in the mesoderm undergo fate restriction and produce progenitors of muscle, myoblasts, as well as quiescent muscle stem cells. Myoblasts proliferate, differentiate and then fuse to form muscle fibers. *In vivo* studies in muscle development are often complemented with *in vitro* approaches using a

mouse myoblast cell line C2C12, derived through serial passaging of myoblasts after injury. C2C12 cells can be maintained in culture and induced to terminally differentiate into myofibers.

Regulation of miRNA levels: The extensive miRNA studies during myogenesis have led to the most detailed understanding of how lineage-specific miRNAs are integrated within the regulatory transcription and epigenetic networks of a specific cell type. The presence of enhancer elements for myogenic TFs in miRNA promoters as well as the locations of some miRNA loci within introns of myogenic genes leads to their highly regulated expression. For instance, two of the three myogenic miRNAs, miR-1 and miR-133 are increased during the differentiation of both skeletal and smooth muscle (Zhao et al., 2005). The myogenic transcription factors that are known drivers of skeletal muscle specification and differentiation, such as MyoD and myogenin, bind to and activate the promoters of these miRNAs whereas cardiac muscle-specific factors bind and activate these loci in cardiac muscle (Zhao et al., 2005; Sweetman et al., 2008). This has been reviewed in detail previously (Sokol, 2012). Studies have also shown that miRNA expression is also controlled by epigenetic mechanisms. In C2C12 cells, the myogenic microRNA miR-29 is epigenetically silenced by its target Rybp (Zhou et al., 2012). During differentiation, Rybp levels are reduced and miR-29 levels reciprocally increase. Together, these

studies show that miRNA expression is transcriptionally regulated and integrated into the feedback loops that stabilize muscle cell states.

miRNA function: Numerous studies over the past decade have established that miRNAs regulate myoblast differentiation and fusion. Studies in myogenesis provide excellent examples of how the combinatorial regulation of miRNAs can promote both cell cycle exit and upregulation of a gene expression program during differentiation. For instance, Pax3, a transcription factor that promotes proliferation and is downregulated during muscle differentiation, is a target of the myogenic miR-206(Boutet et al., 2012). miR-206 continues to play similar roles during the differentiation of satellite cells postnatally. During differentiation, miR-206 along with miR-1 are highly upregulated and target Pax7, a transcription factor highly expressed in satellite cells(Chen et al., 2010). In contrast to miR-206, miR-221/222 miRNAs are expressed in myoblasts and down regulated during differentiation(Cardinali et al., 2009). Profiling data has shown that in myoblasts, miR-221/222 target the cell cycle inhibitor p27 and promote cell cycle progression. Thus, the inhibition of Pax3 and the upregulation of p27 likely contribute to cell cycle exit during differentiation of myoblasts. During differentiation, miR-214 targets Ezh2, the H3K27 methyltransferase in PRC2, which leads to de-repression and expression of at least 2 muscle-specific genes, muscle creatine kinase (MCK) and myosin(Juan et al., 2009). Therefore,

the combined action of miRNAs upregulated during myoblast differentiation leads to both cell cycle exit and activation of genes associated with terminal differentiation.

Regulation of miRNA activity: The co-targeting of Pax7 by miR-1 and miR-206 is likely one of numerous examples of functional synergy between myogenic miRNAs. Recent studies have shown that muscle miRNA function can also be modulated by alternative polyadenylation (APA) and RNA-binding protein (RBP) interactions. The expression of miR-206 and its target Pax3 are mutually exclusive in a majority of skeletal muscle quiescent stem cells (QSCs), the adult stem cells. However, QSCs in a subset (limb and diaphragm) of muscles as well as myogenic progenitors during development exhibit high expression of both Pax3 and miR-206. Boutet et al have shown that the majority of Pax3 in these cells is a short isoform lacking the miR-206 site, which allows Pax3 mRNA to avoid targeting(Boutet et al., 2012). This brings up the interesting possibility that miR-206 expression has been co-opted for alternate targets specifically in these muscle stem cells. Furthermore, a recent study showed that the binding sites of the RBP HuR and miR-1192 overlap on the differentiation-promoting cytokine Hmgb2 in vitro in C2C12 cells(Dormoy-Raclet et al., 2013). Presence of HuR blocks the ability of the miRNA to inhibit Hmgb2, thus preserving its function during terminal differentiation of myoblasts during development and in injury settings.

Hematopoiesis: During development, definitive hematopoietic stem cells (HSCs) are first found in the fetal liver which fetal HSCs undergo rapid expansion and differentiation into progenitors of the lymphoid or myeloid lineage (reviewed in (Orkin, 2000)). Lymphoid progenitors further differentiate to give rise to progenitors of B cells, T cells and natural killer cells whereas myeloid cells go on to give rise to platelets, red blood cells, granulocytes and macrophages. Later in development, the site of hematopoiesis shifts from the liver to the bone marrow. In the adult, HSCs are largely quiescent and have greatly reduced proliferation. How miRNAs regulate various steps of adult hematopoietic self-renewal and differentiation has been extensively studied and reviewed(Ute Bissels, 2012).

Regulation of miRNA levels: Similar to examples in the muscle lineage, miRNAs that are necessary for proper development of the hematopoietic lineage are regulated both transcriptionally and post-transcriptionally. For example, the let-7 miRNA family known for inhibiting cell cycle progression is highly expressed in adults HSCs but silenced post-transcriptionally in fetal HSCs by the RBP Lin28(Copley et al., 2013). In contrast, during erythropoiesis, miR-23 is transcriptionally activated by the key regulator GATA-1, and promotes terminal differentiation of erythroid progenitors(Zhu et al., 2013).

miRNA function: Studies of miRNA function in hematopoiesis highlight how miRNAs can directly regulate apoptotic genes in progenitors and during differentiation. For example, the loss of Dicer in HSCs was shown to lead to reduced numbers of HSCs, which could be rescued with addition of miR-125, which is highly expressed in HSCs. The pro-apoptotic gene Bak1 was shown to be a direct target of miR-125 and the loss of Bak1 rescues the reduction in HSCs seen with Dicer loss(Guo et al., 2010). Further down the lineage during HSC differentiation, apoptosis in the pro- to pre- B cell transition is regulated by miR-17-92 regulation of the pro-apoptotic gene Bim. Loss of Dicer during this transition led to upregulation of Bim, a target of the highly expressed miR-17-92 cluster, and increased cell death(Koralov et al., 2008). Knocking out Bim reversed the cell death phenotype.

The hematopoietic system, due to the diversity of cell types generated from a single pool of stem cells, requires that cell fate choices be highly regulated. Many studies have identified individual miRNAs that can function in cell fate choices of oligopotent progenitors. For example, miR-9 has been shown to function in the cell fate choice of committed progenitors capable of undergoing myelopoiesis or lymphopoiesis. The expression of miR-9 promotes myelopoiesis and

inhibit lymphopoiesis(Senyuk et al., 2013). Similarly, the expression of miR-223 promotes granulopoiesis relative to erythropoiesis of the common erythroid progenitor(Fazi et al., 2005).

Skin development: The epidermis and hair follicles, which make up mammalian skin, are derived from a common epithelial progenitor during embryonic development(Fuchs and Raghavan, 2002). The mammalian epidermis is a stratified tissue composed of progenitor cells in the innermost basal layer, which undergo terminal differentiation and travel to the surface. The hair follicle develops when epithelial cells form placodes that differentiate into hair follicle stem cells, which in turn undergo further differentiation.

Regulation of miRNA expression: Despite being derived from a common progenitor, miRNA expression profiles differ in epidermal and hair follicle cells. Each cell type is enriched in specific subsets of miRNAs such as miR-200a, miR-141 and miR-429 in the epidermis and miR-199a in the hair follicle(Yi et al., 2006). The specific expression of various miRNAs suggests transcriptional regulation of these loci. The transcription factors and/or epigenetic factors responsible however remain to be determined.

miRNA function: The identity and function of some miRNAs important for hair follicle development have been studied in detail using *in vivo* models. The expansion of epidermal and hair follicle stem cells is regulated in part by miR-125 and miR-205, as shown by separate studies in mice overexpressing miR-125 and mice deficient for miR-205 specifically in hair follicle cells(Zhang et al., 2011; Wang et al., 2013a). During epidermal differentiation however, the level of another miRNA, miR-203, rises dramatically and remains highly expressed in terminally differentiated cells (Jackson et al., 2013). Ectopic expression of miR-203 in primary keratinocytes causes them to exit the cell cycle and prevents colony formation(Lena et al., 2008). It is unclear if the exit from cell cycle leads to activation of terminal differentiation markers. Jackson et al recently explored the role of multiple targets in effecting the phenotypes seen upon miR-203 overexpression(Jackson et al., 2013). For instance, in cells overexpressing miR-203, the forced expression of Skp2 but not p63, Msi2 or Vav3 blocked the ability of miR-203 to decrease cells in the S-phase. However, all 4 targets were able to partially rescue the loss of colony formation induced by miR-203 overexpression.

Neurogenesis: The three main cell types of the nervous system – neurons, astrocytes and oligodendrocytes – are derived from progenitors in the neuroepithelium, which is first specified at e8.0 (reviewed in (Rowitch and Kriegstein, 2010)). Neuroepithelial cells expand through cell

division and give rise to radial glial cells (RGCs) with neurogenic competence. For a large part of embryogenesis, RGCs differentiate into neurons that migrate outwards to form the cortex. During the end of embryogenesis and in early postnatal stages, RGCs acquire gliogenic competence, giving rise to astrocytes and oligodendrocytes. In the neural lineage, miRNAs involved in neuronal differentiation have been studied most extensively. The role of miRNAs in the glial lineage is less clear and limited to a small number of studies examining miRNAs in oligodendrocyte differentiation.

Regulation of miRNA levels: Recent studies have shown that the oligodendrocyte-enriched miRNA miR-338 is regulated by the binding of Sox10 upstream of a promoter for *Aatk*, its host gene(Gokey et al., 2012). Similarly, the two miRNAs classically associated with neurogenesis, miR-9 and miR-124, are upregulated during the progenitor to neuron transition. The expression of miR-9 is regulated by a feedback loop involving its target, the self-renewal promoting factor TLX. TLX is highly expressed in progenitors and represses miR-9(Zhao et al., 2009). Prior to neuronal differentiation, there is some evidence that miR-124 is suppressed by its target REST, a protein known to silence neuronal genes (Conaco et al., 2006) .

miRNA function: The conditional loss of Dicer in neurogenic compartments, using several different Cre mouse lines, results in increased apoptosis and striking defects in neuronal differentiation(De Pietri Tonelli et al., 2008; Kawase-Koga et al., 2009; Andersson et al., 2010). Profiling of neural stem cells derived from Dicer knockout brains shows misregulation of apoptotic and survival genes(Kawase-Koga et al., 2009). Unlike the hematopoietic lineages, however, the apoptosis has not been linked to direct targeting of apoptotic genes by expressed miRNAs. Thus, one possibility is that the apoptosis is a secondary effect of the transcriptomic and proteomic dysregulation that occurs in the absence of miRNAs.

Studies of miRNA biology in the nervous system illustrate how the combinatorial action of distinct miRNAs expressed in a cell can concomitantly drive cell cycle exit and activate a new gene expression program during differentiation. For instance, during development, the miRNA let7 has been shown to promote cell cycle exit by targeting the G1-S inhibitor Cyclin D1(Zhao et al., 2010a). The differentiation of neuronal progenitors is marked by upregulation of neuronal-specific genes. This process occurs partly via epigenetic mechanisms, i.e the switching of a subunit of a chromatin-remodeling complex. Baf53a is required for neural progenitor proliferation but when downregulated during differentiation, is replaced by a neuronal Baf subunit that drives expression of neuron-specific genes(Lessard et al., 2007). Yoo et al have

shown that miR-9* and miR-124 target the Baf53a subunit of the chromatin remodeling complex(Yoo et al., 2009). In addition, both let-7 and miR-9 target TLX and miR-124 targets the anti-neural factors REST/SCP1, further promoting activation of the neuronal gene expression program during differentiation. Therefore, as in myogenesis, miRNAs expressed during neurogenesis target cell cycle inhibitors and epigenetic complexes to promote terminal differentiation.

During oligodendrocyte differentiation, miR-219 and miR-338 increase 10-100 fold and target the transcription factors Sox6 and Hes5 that have been associated with progenitor self-renewal and "stemness" (Dugas et al., 2010; Zhao et al., 2010b). The identity and function of microRNAs important for astrocyte differentiation are poorly understood. In **Chapter 3&4**, I show work that addresses the identity and targets of miRNAs that promote astrocyte differentiation.

Regulation of miRNA function: Mature neurogenic miRNAs are extensively regulated, perhaps highlighting the requirement for rapid functional activation during the early stages of terminal differentiation. The activity of let-7 is regulated by TRIM-NHL, a protein that binds to let-7 containing RISC complexes and potentiates miRNA activity(Schwamborn et al., 2009). TRIM-NHL is preferentially expressed in the differentiating daughter cell following asymmetric cell

division of neurogenic progenitors. Potentiation of let-7 activity by TRIM-NHL promotes neurogenesis. miRNA functional outcome is also regulated by co-targeting such as miR-9 and miR-124 targeting Baf-53a and REST/SCP1 thus acting in sum to inhibit their expression.

Conclusion: Taken together, studies of miRNA biology during development show how miRNAs constitute an essential part of the gene networks that drive proliferation, viability and differentiation. Studies examining effects of global miRNA loss in various lineages show that miRNA effects on proliferation, apoptosis and differentiation are often separable. While miRNA activity is crucial for the development of every lineage examined, the specific cellular functions vary.

Table 1-1, Lineage specific miRNA knockouts

Lineage/Organ	Defect	Reference
Limb	Morphogenesis defect, Apoptosis	(Harfe et al., 2005)
Skin	Hair follicle development defects, Normal epidermis development	(Andl et al., 2006; Yi et al., 2006)
Pancreas	Increased apoptosis, Beta-cell differentiation defect	(Lynn et al., 2007)
Skeletal Muscle	Increased apoptosis, myofiber morphogenesis	(O'Rourke et al., 2007)
B cells	Increased apoptosis	(Koralov et al., 2008)
Neurons	Increased apoptosis, defective neuronal differentiation	(De Pietri Tonelli et al., 2008)
Osteoblasts	Differentiation defect	(Gaur et al., 2010)
Chondrocytes	Differentiation defect	(Kobayashi et al., 2008)
Adipose tissue	Imapired differentiation of white adipose tissue but not brown adipose	(Mudhasani et al., 2011)
Kidney	Increased apoptosis, defects in morphogenesis of nephrogenic and uretogenic compartments	(Nagalakshmi et al., 2011)
Hematopoiesis	Increased apoptosis, Differentiation skewed towards myeloid lineage	(Guo et al., 2010)
Oligodendrocytes	Differentiation defect	(Dugas et al., 2010; Zhao et al., 2010b)
Neural crest	Increased apoptosis	(Zehir et al., 2010)
Tooth	Mild morphogenesis defect	(Michon et al., 2010)

Chapter 2: MicroRNAs are required for proper terminal differentiation of GPCs

Summary

Astrocytes, a dominant cell type in the central nervous system, have recently emerged as critical regulators of learning, memory and neurogenesis. While the number of studies of astrocyte functions is growing, molecular mechanisms underlying the generation of astrocytes following glial specification remain largely unknown. miRNAs are a class of small non-coding RNAs that are important regulators of cell fate transitions throughout development. Each miRNA targets hundreds of mRNAs via base pairing in a seed region of the miRNA. Identifying miRNAs important for cell fate transitions, in turn, has led to the identification of genes and pathways important for that transition. In this study, we set out to determine the role of miRNAs in regulating astrocyte differentiation. We used a directed differentiation approach to generate Rosa26CreER::Dgcr8$^{flox/\Delta}$ glial precursor cells (GPCs) from embryonic stem cells. These GPCs can be maintained in a self-renewing state and differentiate into functional astrocytes upon exposure to proper cues. We find that tamoxifen-induced deletion of Dgcr8 and subsequent loss of miRNAs leads to separable survival and differentiation defects. To identify miRNAs that play a role in this transition, we performed a large-scale screen and identified two families of

miRNAs expressed in GPCs and astrocytes that robustly rescue the differentiation phenotype in Dgcr8$^{\Delta/\Delta}$ GPCs. Identifying the functional targets of these miRNAs during this cell fate transition will provide novel insights into glial biology and serve as a paradigm for the role of miRNAs in terminal differentiation of somatic progenitors.

Introduction

Astrocytes are a dominant cell type in the central nervous system (CNS), intimately associated with neuronal synapses and CNS function(Zhang and Barres, 2010). Traditionally, neurons were viewed as the core functional units of the nervous system while glia were thought to be support cells for neurons. Therefore, mechanisms and related markers underlying neuronal differentiation have been better studied than glial differentiation. However in recent years, studies of glia have started to reveal a similar morphological and functional diversity of astrocytes. Evolutionary studies show that glia originated in close apposition to neurons, and that the ratio of astrocytes:neurons has been increasing(Verkhratsky and Butt, 2013). Recently, astrocytes have been shown to be necessary for synapse elimination in both the developing and adult CNS (Chung et al., 2013). Mice transplanted with human astrocytes exhibit improved performance in a number of behavioral tests associated with learning and memory, suggesting the functional importance of this cell type(Han et al., 2013). Astrocytes have recently also been suggested to be

lymphatic substitutes in the CNS and their misregulation is associated with aggressive malignancies such as glioblastoma multiforme (Xie et al., 2013). While the number of studies examining the functions of mature astrocytes is growing, very little is known regarding the molecular mechanisms underlying the specification and differentiation of glial precursors into astrocytes during development.

It is known that both neurons and glia arise from a common stem cell pool in the neuroepithelium. During embryogenesis, neuroepithelial stem cells give rise to radial glial cells, which in turn give rise largely to neurogenic progenitors and subsequently neurons. At the end of embryogenesis, the wave of neurogenesis subsides and a wave of gliogenesis begins and continues through early postnatal stages(Anon, 2010). It is unclear whether radial glial cells at this stage first give rise to glial-restricted precursors, which in turn differentiate into astrocytes or whether radial glial cells themselves terminally differentiate into astrocytes.

miRNAs are small non-coding RNAs that are important regulators of cell fate transitions throughout development. The expression of miRNAs in a given cell type helps define and maintain its unique transcriptome, as evidenced by the transcriptomic changes seen with global loss of miRNAs or addition of new miRNAs (Lim et al., 2005; Sinkkonen et al., 2008). Indeed,

recent studies have shown that the addition of cell type-specific miRNAs is sufficient to drive a differentiated cell to transdifferentiate into the miRNA-enriched cell (Anokye-Danso et al., 2011; Yoo et al., 2011). This suggests that miRNAs have evolved to modulate functional gene networks in various cell types. Thus, miRNAs are increasingly being used to identify important molecular pathways via identification of their targets.

In the neural lineage, the function of miRNAs has been largely studied in neuronal and oligodendrocyte differentiation
(Krichevsky et al., 2009; Zhao et al., 2009; Dugas et al., 2010; Zhao et al., 2010b). Distinct sets of miRNAs, miR-9/124 in neurons and miR-219/338 in oligodendrocytes, are highly upregulated during differentiation of progenitors and drive differentiation by inhibiting mRNAs important for maintenance of the progenitor states. The paucity of information about astrocyte miRNAs and their role in differentiation can be attributed to the lack of a lineage-specific marker to isolate pure astrocyte precursor cells from their neuronal or oligodendrocyte counterparts. A number of *in vivo* studies have shown that miRNAs are required for neuronal fate-restricted precursors to achieve glial competence. In the retina, the specific miRNAs that promote glial specification following retinal neurogenesis have been described (La Torre et al., 2013). In the spinal cord, deletion of Dicer in a region that normally gives rise to astrocytes led to an inhibition of GFAP

expression(Zheng et al., 2010b). While these studies suggest that miRNAs are required for the specification of glia during mid to late gestation, it is unclear whether they are necessary for terminal astrocyte differentiation of the glial-specified precursors.

Furthermore, among the studies examining miRNA function during astrocyte differentiation, there is not a great consensus on the phenotypes seen with miRNA loss in neural precursors. Kawase-koga et al recently reported increased cell death both during establishment and differentiation of multipotent Dicer KO stem cells derived from brains of Emx1-Cre mice (Kawase-Koga et al., 2009). However, in differentiation conditions of mitogen withdrawal, surviving cells were reported to express markers of the neural and glial lineage. In contrast, Andersson et al recently reported that multipotent Dicer KO NSCs fail to survive in conditions that specifically promote neuronal differentiation, but survive without expressing glial markers in conditions promoting astrocytic differentiation(Andersson et al., 2010).

In this study, I sought to establish a homogenous differentiation system to determine the identity and function of miRNAs important for astrocyte differentiation. To fully elucidate miRNA identity, targets and functions during astrocyte differentiation, we used a directed differentiation approach to derive a pure population of glial-restricted progenitor cells from mouse embryonic

stem cells (ESCs). The directed differentiation protocol was modified from previously published studies(Okabe et al., 1996; Anon, 1999). A related method for astroglial differentiation of human ES cells has previously been shown to generate astrocytes that are functional when transplanted *in vivo*(Krencik et al., 2011). Using a conditional Dgcr8 deletion system, we are then able to generate GPCs lacking all miRNAs and study the function of individual miRNAs in astrocyte differentiation.

Results

Generating ESC-derived GPCs and astrocytes

In order to study astrogliogenesis, glial-restricted precursors were derived from ES cells using a previously established neural differentiation protocol(Okabe et al., 1996). Briefly, embryoid bodies were plated in selection media to promote neural differentiation and then passaged into N2 media with growth factors. The growth factor bFGF was added for the first two passages and both bFGF and EGF were added for later passages. Neural precursors produced using this protocol could be induced to differentiate with 1% FBS and withdrawal of growth factors. Similar to the developmental timeline *in vivo* where neuronal specification precedes glial specification, the progenitors produced in the first 2-3 passages were capable of differentiating into β**III**-tubulin-expressing neurons. (**Figure 2-1A**). While the heterogeneity in early passages

likely reflects heterogeneity in differentiation kinetics across plated EBs, the culture as a whole progressively became fate-restricted such that by P4, progenitor cells largely differentiated into astrocytes. Modifications of this protocol and use of the growth factor Pdgf have previously been shown to promote oligodendrocyte fate (Brüstle and McKay, 1999).

Once established, GPCs could be maintained in proliferative conditions for several passages and gave rise largely to cells expressing GFAP when induced to differentiate with FBS/B27 or BMP-4 in growth factor withdrawal conditions (**Figure 2-1B**). To compare *in vitro*-derived and *in vivo* astrocytes, we determined levels of astrocyte-enriched transcripts in the profiling data of ESC-derived astrocytes **(Figure 2-1C**). This analysis showed that ESC-derived astrocytes express many mRNAs enriched in astrocytes isolated *in vivo,* including GFAP, Aqp4, Slc39a12, Aldh1l1 (Fthfd) and Aldoc. Furthermore, the average expression of astrocyte transcripts was higher than other transcripts (**Figure 2-1D**). To establish that these represented *in vivo* astrocytes, cells labeled with GFP were transplanted into neonatal cortex following 24 hours of differentiation. The majority of surviving cells expressed GFAP and morphologically resembled *in vivo* astrocytes for 6 weeks following transplantation (**Figure 2-1E**). This set of experiments suggests that while ESC-derived astrocytes may not be identical to the mature astrocytes found in

postnatal brain, they bear enough similarities such that the ESC system can be used to provide insights into the cell fate transitions that lead to astrogliogenesis.

In order to determine the changes in miRNA populations during differentiation, we sequenced small RNA populations in ESC-derived GPCs and astrocytes using the Illumina platform (**Figure 2-1F**). Using miRNA RT-qPCR, we evaluated the levels of the highest expressed miRNAs in ESC-derived GPCs, astrocytes and *in vivo* astrocytes FACS-purified from Aldhl1-GFP mice at e18.5 (**Figure 2-1G**). Preliminary analysis suggests that the levels of the 5 miRNAs examined were similar in ESC-derived astrocytes and *in vivo*-purified astrocytes.

Strategy for generating conditional Dgcr8$^{\Delta/\Delta}$ GPCs

Dgcr8 knockout ES cells exhibit significant differentiation defects, and therefore cannot be differentiated into GPCs. Therefore, previous efforts in the lab led to the generation of Dgcr8$^{flox/\Delta}$:: R26 CreER condition ES cells(Wang et al., 2007a). Wild-type ES cells targeted with Rosa26-CreER were then targeted twice to generate Dgcr8 delta/flox alleles. The directed differentiation approach described in the previous section was used to derive GPCs from Dgcr8$^{flox/\Delta}$::R26-CreER ES cells. Cells expressing Rosa26-CreER alone were used as controls.

Dgcr8$^{\Delta/\Delta}$ GPCs exhibit increased apoptosis and fail to differentiate normally

To determine phenotypes upon miRNA depletion, cells were treated with tamoxifen for 24-48 hours. Dgcr8 and subsequent miRNA loss was confirmed by qPCR 7-9 days following tamoxifen treatment (**Figure 2-2A,B**). Microarray analysis showed that transcripts that are de-repressed following microRNA loss are enriched for seed sequences of the major miRNA families expressed in GPCs (**Figure 2-2C**). As reported previously in *in vivo* derived multipotent NSCs, Dgcr8$^{\Delta/\Delta}$ GPCs exhibited an increased dependence on growth factors for survival(Andersson et al., 2010). Dgcr8$^{\Delta/\Delta}$ cells grown for 48 hours without supplementing with EGF and FGF exhibited increased apoptosis as assayed by CC3 staining (**Figure 2-2D**). Consistent with previous reports, the apoptosis could be reduced by daily supplementation of growth factors. During differentiation, which requires removal of growth factors, apoptosis was often increased. (**Data not shown**)

When induced to differentiate with FBS/B27 for 48 hours, most Dgcr8$^{\Delta/\Delta}$ GPCs failed to upregulate GFAP (**Figure 2-2E**). Approximately 3-8% of cells do upregulate GFAP, which may reflect the ability of these cells to overcome miRNA loss. Alternatively, it may reflect incomplete loop out of Dgcr8 or kinetics of miRNA loss in a population. To determine whether other differentiation markers are affected by loss of Dgcr8, we performed qPCR on control and

Dgcr8$^{\Delta/\Delta}$ cells following 48 hours of differentiation (**Figure 2-2F**). In addition to GFAP, Aqp4 was also reduced during differentiation in Dgcr8$^{\Delta/\Delta}$ GPCs. Slc39a12 and Clusterin levels were variable and not reproducibly changed. This is similar to the phenotype reported in Dicer KO cortical neural stem cells cultured from a Nestin-Cre:Dicer$^{flox/flox}$ mouse brain(Andersson et al., 2010). In Dicer KO NSCs, microarray analysis showed that the defect in upregulation of GFAP reflects the inability to upregulate a part of the differentiation program including GFAP and Aqp4.

The JAK-STAT pathway is classically associated with astrocyte differentiation(He et al., 2005). In previous studies, STAT3 phosphorylation increased coincident with astrocyte differentiation of cortical neural stem cells and inhibition of STAT3 with a dominant negative form inhibited astrocyte differentiation. In ChIP studies, it has been shown that STAT3 forms a complex with SMAD and p300 and binds to the GFAP promoter to activate transcription{Nakashima:1999cz}. To determine whether the JAK-STAT signaling pathway was disrupted upon the loss of miRNAs, we examined STAT3 levels in control and Dgcr8$^{\Delta/\Delta}$ GPCs over a differentiation time course. When exposed to FBS/B27 or BMP-4, control cells exhibited a striking and sustained increase in phosphorylation of STAT3 at tyrosine residue 705. Dgcr8$^{\Delta/\Delta}$ GPCs, however, showed greatly attenuated levels of STAT3 phosphorylation in response to differentiation cues (**Figure 2-2G**).

Total levels of STAT3 protein were unchanged in Dgcr8$^{\Delta/\Delta}$ cells relative to control or during differentiation.

The apoptosis and differentiation phenotypes in Dgcr8$^{\Delta/\Delta}$ GPCs are separable

Since Dgcr8$^{\Delta/\Delta}$ cells exhibit both and apoptosis and differentiation defect, it is possible that they are coupled. That is, cells undergoing apoptosis could be competent to differentiate, especially during differentiation when growth factors are withdrawn. Alternatively, the variability in confluence due to the variable apoptosis may contribute to differentiation delays. To address this possibility, we set out to decouple the apoptosis and differentiation phenotypes by inhibiting apoptosis in Dgcr8$^{\Delta/\Delta}$ cells. We took a genetic approach by creating a conditional apoptosis-resistant, miRNA-deficient ES cell line. Bax and Bak are canonical pro-apoptotic genes that when knocked out prevent apoptosis in developing neural progenitors. Neural progenitors derived from Bax/Bak double KO mice differentiate normally into neurons and astrocytes while exhibiting reduced apoptosis(Lindsten et al., 2003). To this end, we engineered Bax$^{f/f}$ Bak$^{-/-}$ Dgcr8$^{\Delta/flox}$::Rosa26-CreER ES cells. **(Figure 2-3A)** The targeting of Dgcr8 and Rosa26-CreER was performed as described in the previous section into Bax$^{f/f}$ Bak$^{-/-}$ ES cells derived from mice.

Tamoxifen treatment of GPCs generated from $Bax^{f/f}$ $Bak^{-/-}$ $Dgcr8^{\Delta/flox}$::Rosa26-CreER cells resulted in cells that were Bax/Bak/Dgcr8 triple KO (TKO). The loss of the conditional Bax and Dgcr8 alleles was confirmed by qRT-PCR. Bax/Bak/Dgcr8 TKO cells exhibited a striking reduction in apoptosis but continued to exhibit a striking differentiation phenotype (**Figure 2-3B&C**).

Identification of GPC miRNAs that rescue differentiation in $Dgcr8^{\Delta/\Delta}$ GPCs

In order to identify miRNAs that could rescue differentiation in $Dgcr8^{\Delta/\Delta}$ GPCs, we carried out an unbiased miRNA mimic-based screen of all mouse miRNAs (Schematic in **Figure 2-4A**). $Dgcr8^{\Delta/\Delta}$ GPCs were plated in 96 well format and transfected with one miRNA mimic per well. Mock transfection was used as negative control. Three days following transfection, FBS/B27 differentiation media was added for 48 hours. Cells were processed for immunofluorescence to detect GFAP and the In Cell Analyzer was used for automated imaging and analysis to determine percentage of GFAP-positive cells per well. SSMD scores were calculated using the mock-transfected wells as negative references.

To determine screen-positive hits, the following criteria were used: (i) SSMD score > 2.5, (ii) Presence of multiple members of miRNA family, (iii) Expressed in astrocytes and (iii) Confirmation with independently synthesized mimics. Criteria i - iii led to the identification of 4 miRNA families – let-7, miR-125, miR-30 and miR-7. However, upon testing with independently synthesized mimics, miR-7 was found to have mild effects and miR-30 had moderate and highly variable effects on rescuing differentiation (**data not shown**). However, let-7 and miR-125 miRNAs consistently and robustly rescued differentiation of Dgcr8$^{\Delta/\Delta}$ GPCs, and were therefore chosen for follow up (**Figure 2-4C**). Additionally, since miR-9 and miR-181 are highly expressed in GPCs, we tested their capacity to rescue differentiation. Transfection of miR-9, miR-181 and miR-30 into Dgcr8$^{\Delta/\Delta}$ GPCs did not rescue differentiation whereas let-7 and miR-125, included as positive controls, did (**Figure 2-4E**). Analysis of sequencing data showed that showed that the let-7 and miR-125 miRNAs are highly expressed in GPCs and do not change significantly during early differentiation (**Figure 2-1E&F**), suggesting that these miRNAs function by priming fate-committed GPCs for differentiation.

Discussion

Our findings using a Dgcr8$^{\Delta/\Delta}$ ESC-derived astrocyte differentiation model confirm and extend previous findings regarding the role of miRNAs in astrocyte differentiation using a Dicer

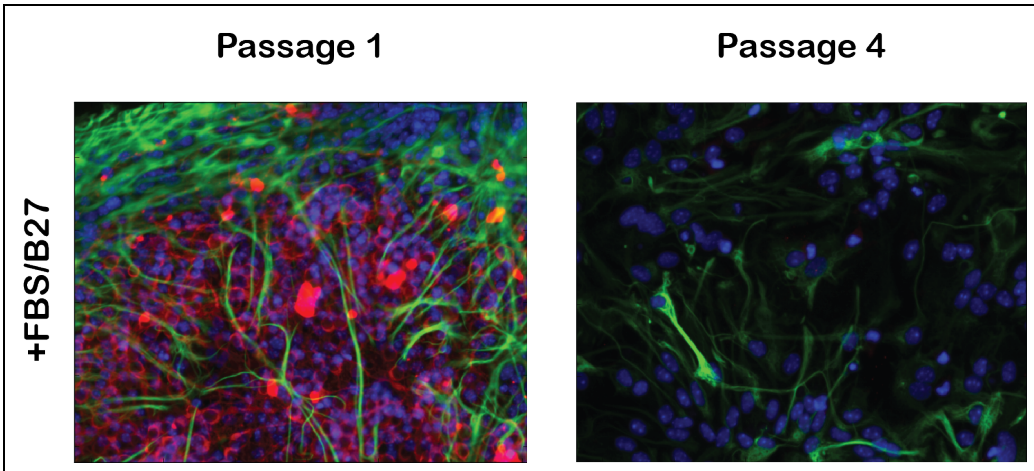
knockout model(Andersson et al., 2010). In our study we show that in the absence of miRNAs, GPCs are deficient in activation of GFAP, a canonical astrocyte marker, as well as Aqp4, an astrocyte marker of functional importance. The above-mentioned study of Dicer KO cells has identified additional astrocyte markers that are not upregulated to the same extent upon loss of miRNAs. These will likely be candidates for confirmation in Dgcr8$^{\Delta/\Delta}$ cells. In addition to a defect in upregulation of markers, GPCs exhibit an attenuated activation of the JAK-STAT pathway that has been classically associated with astrogliogenesis.

Microarray analysis of Dgcr8$^{\Delta/\Delta}$ GPCs showed that upregulated mRNAs are enriched for a motif complementary to the seed sequence of two highly expressed miRNAs, let-7 and miR-9. Such an analysis has previously been limited to the discovery of the miR-290 family motif in mRNAs upregulated in Dicer knockout ESCs(Sinkkonen et al., 2008). There are many possible reasons for the lack of enrichment of other miRNA motifs. For instance, RNA binding proteins may potentiate the activity of let-7 and miR-9. In neurogenic progenitors undergoing differentiation, TRIM32 binding to the RISC complex potentiates the activity of a subset of expressed miRNAs including let-7(Schwamborn et al., 2009). Similarly in ESCs, Trim71 potentiates the activity of the highly expressed miR-290 family of miRNAs(Chang et al., 2012).

To determine the identity of miRNAs that play a role in differentiation, we performed an unbiased screen adding back single miRNA mimics and assayed for the rescue of GFAP expression. This represents the first unbiased miRNA screen performed in a somatic stem/progenitor cell. As in ES cells, the most robust differentiation-promoting effects are caused by two miRNA families highly expressed in GPCs and astrocytes – let-7 and miR-125. Let-7 and miR-125 are the first two miRNAs identified in *C.elegans* in a screen for heterochronic mutations(Reinhart et al., 2000). In the mouse, these miRNAs are upregulated and highly expressed in many lineages following the differentiation of the pluripotent epiblast in mid-embryogenesis. Two members of these families, let-7i and miR-125b are co-expressed on the same primary transcript and have been proposed in bioinformatics analyses to co-target mRNAs(Tsang et al., 2010).

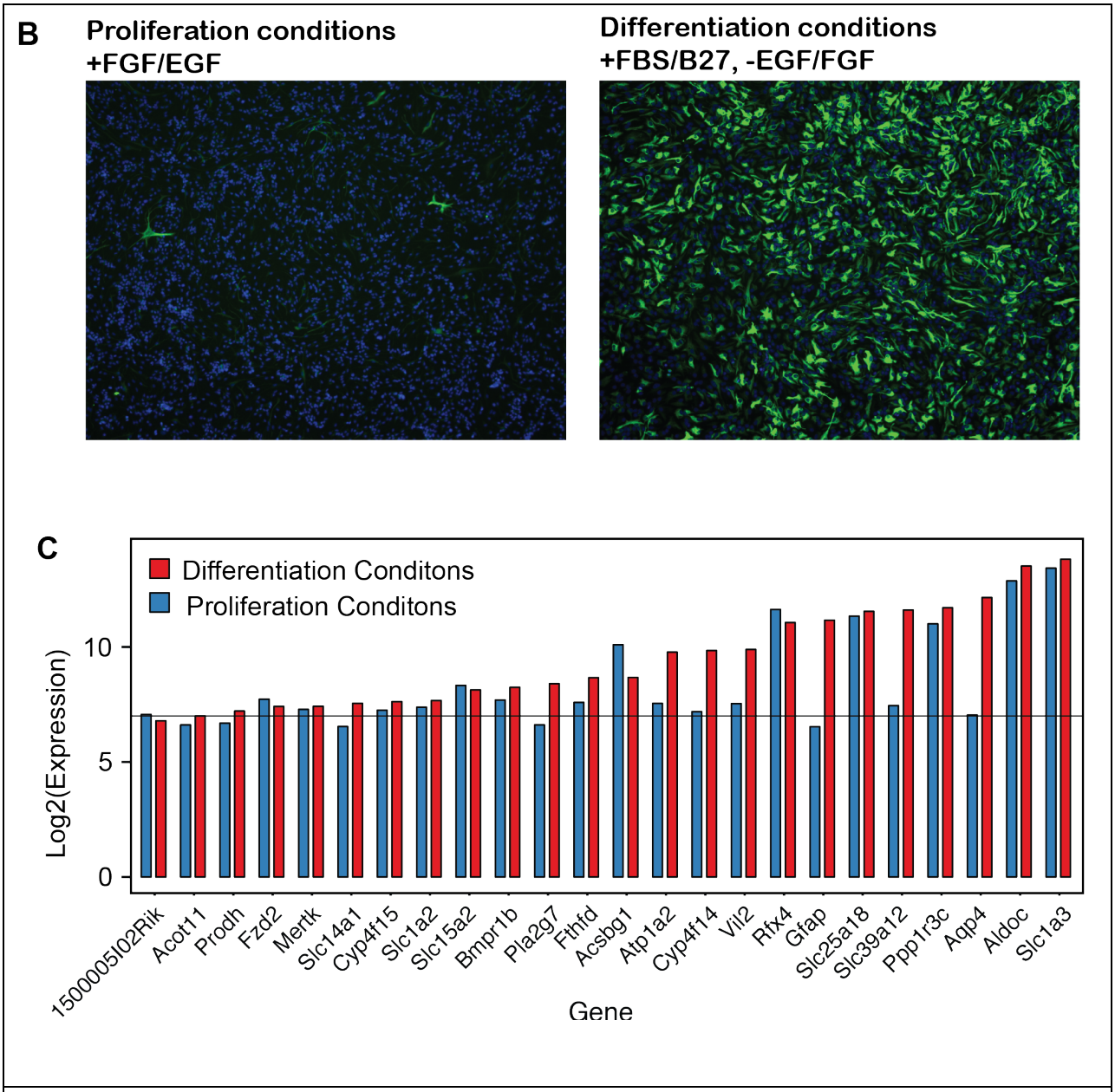
The fact that let-7 and miR-125 are expressed in GPCs and not strikingly upregulated in astrocytes during early differentiation stages suggests that these miRNAs may be priming GPCs for differentiation cues. Indeed, the expression of let-7 and miR-125 is insufficient to rescue differentiation in the absence of cues such as FBS/B27 or BMP-4. In **Chapter 4**, I explore the mechanisms and targets underlying let-7/miR-125-mediated rescue of differentiation in Dgcr8$^{\Delta/\Delta}$ GPCs.

Figure 2-1A, Differentiation of ESC-derived neural progenitors



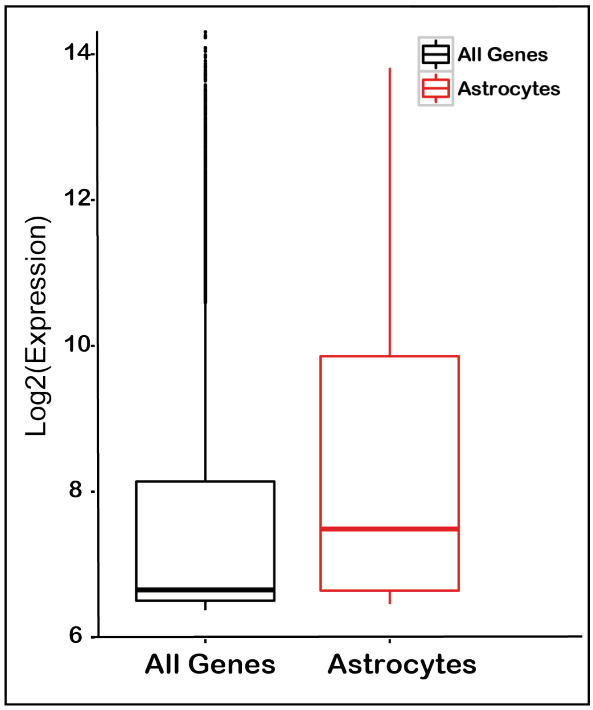
Differentiation of ESC-derived neural progenitors for 5 days results in both neurons and astrocytes in early passages (Passage 1) and in largely astrocytes in later passages (Passage 4).
Green – GFAP, Red- Tuj1, Blue - DAPI

Figure 2-1, B-C, Characterization of ESC-derived astrocytes



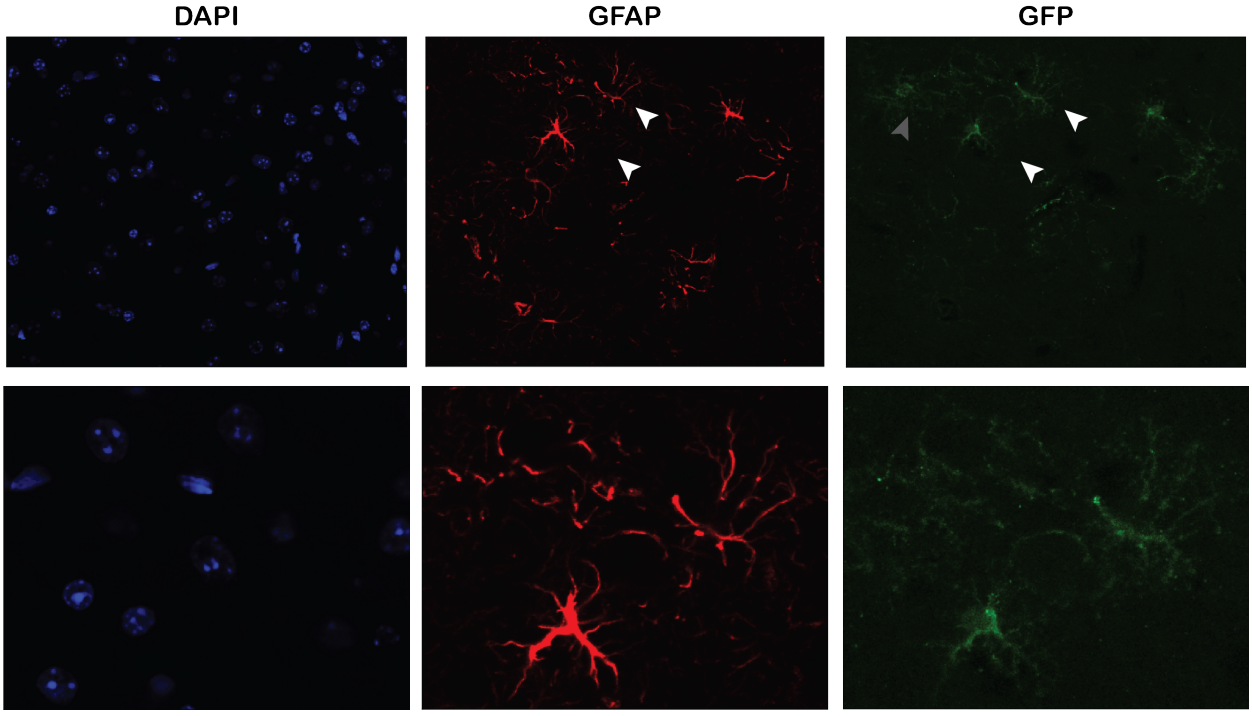
B) GFAP expression in ES-derived GPCs (in proliferation conditions) and following 48 hours of differentiation **Green – GFAP, Blue - DAPI** C) Expression of *in vivo* astrocyte markers in ES-derived astrocytes. Data are expressed as average of triplicate Illumina microarray samples.

Figure 2-1, D, Astrocyte genes are highly expressed in ES-derived astrocytes



Box plots showing distribution of expression levels of astrocyte mRNAs (as shown in Figure 2-1B) relative to all mRNAs

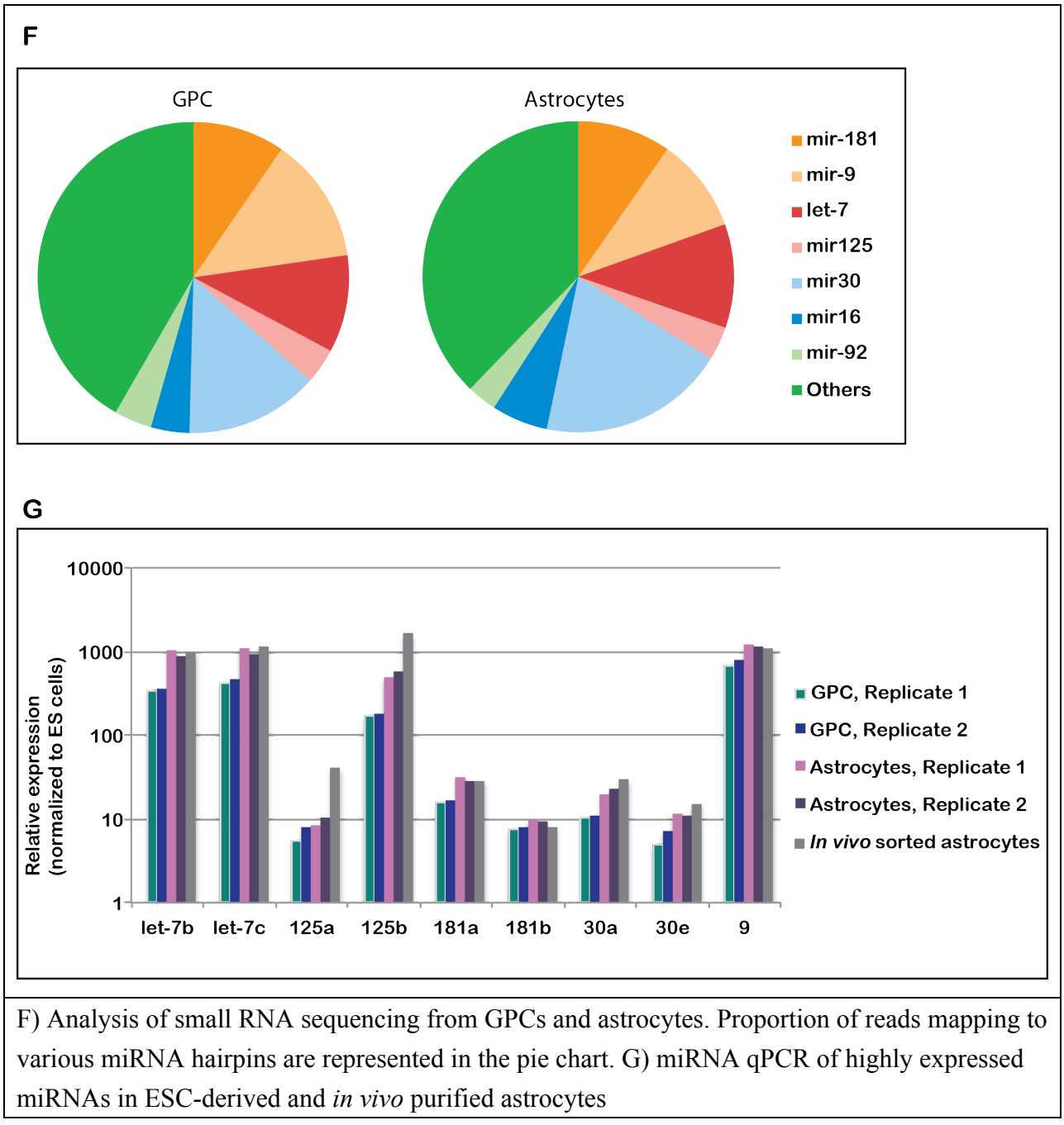
Figure 2-1E, ESC-derived astrocyte transplantation



Transplantation and survival of ESC-derived astrocytes into neonatal cortex

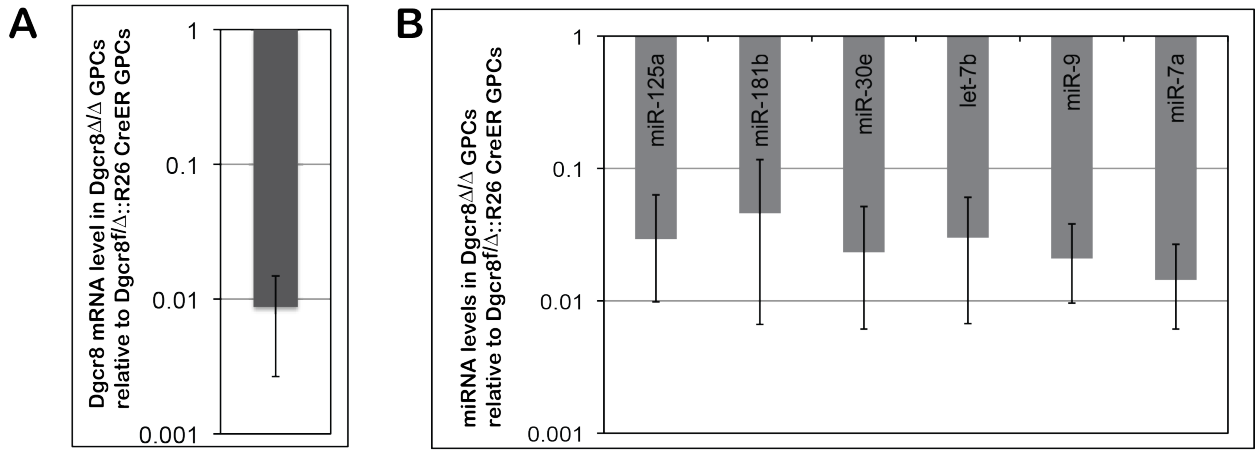
ESC derived GPCs expressing GFP were differentiated for approximately 24 hours and then transplanted into neonatal cortex. Mouse brains were analyzed 1 month following transplants and GFP and GFAP positive cells were assayed by immunofluorescence (n=2). Magnified view of GFP+ GFAP+ cells (**white arrows**) is shown in the bottom panel. Grey arrow highlights a cell that is GFP+ and GFAP- . **Red – GFAP, Green - GFP, Blue - DAPI**

Figure 2-1, F-G, miRNA expression profiling in GPCs and astrocytes



F) Analysis of small RNA sequencing from GPCs and astrocytes. Proportion of reads mapping to various miRNA hairpins are represented in the pie chart. G) miRNA qPCR of highly expressed miRNAs in ESC-derived and *in vivo* purified astrocytes

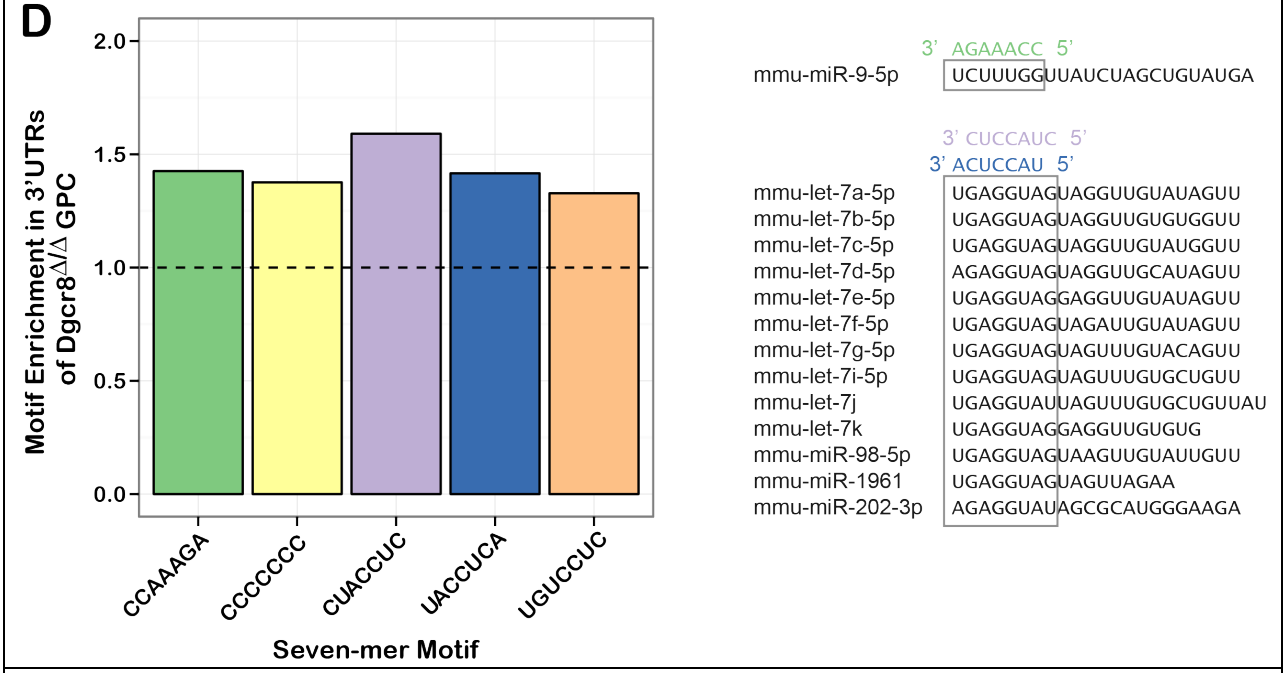
Figure 2-2, A-B, Efficient deletion of Dgcr8 and miRNAs



Tamoxifen treatment induces efficient deletion of Dgcr8 and miRNA loss in Dgcr8$^{f/\Delta}$ **GPCs leads to increased apoptosis in GPCs**

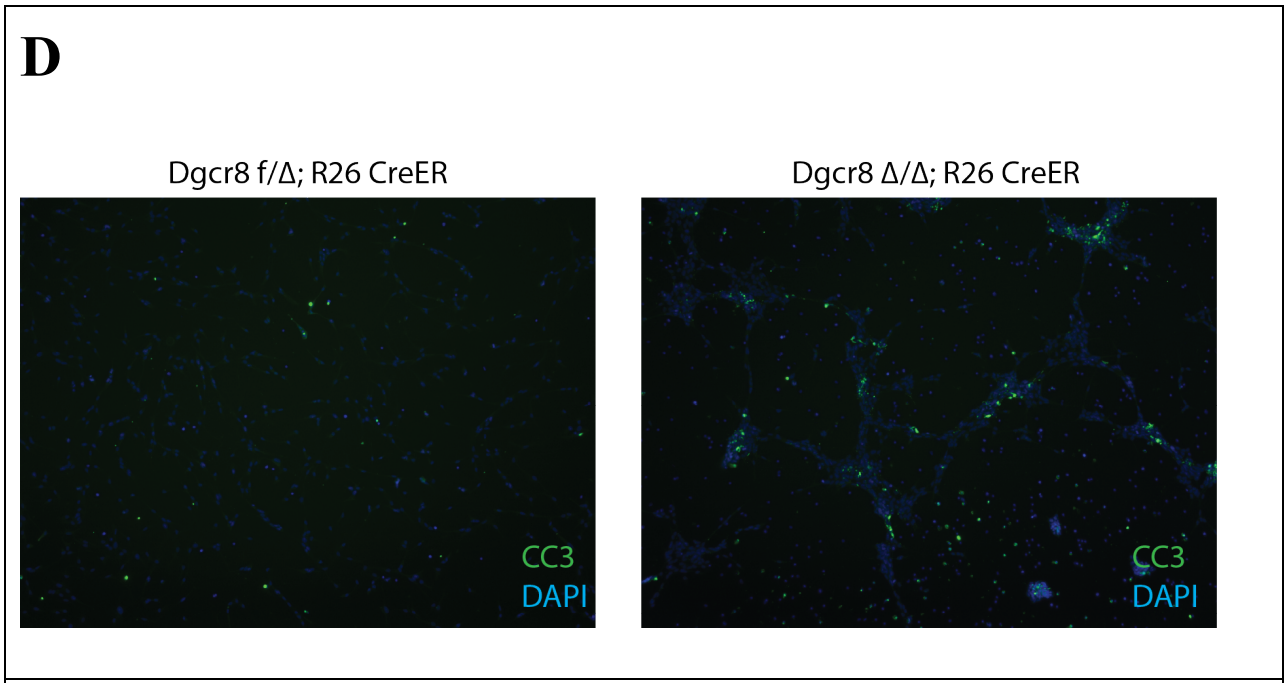
Quantitative RT-PCR for Dgcr8 Exon 3 (A) and highly expressed miRNAs (B) following tamoxifen treatment in GPCs (n=3). All values are represented relative to levels in Dgcr8$^{f/\Delta}$ and normalized to actin.

Figure 2-2, C, Let-7 and miR-9 seed sites are enriched in 3'UTRs of Dgcr8$^{\Delta/\Delta}$ GPCs



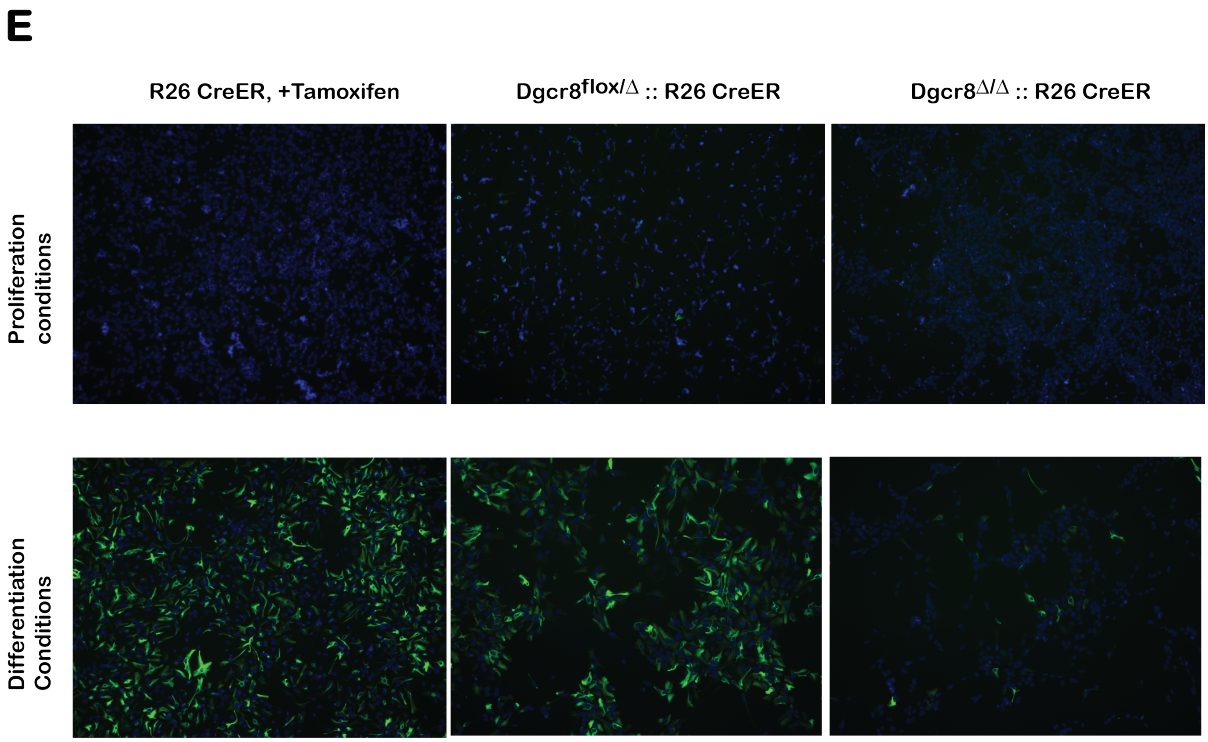
Unbiased 7-mer motif enrichment was performed using 3'UTR sequences. Enrichment was calculated between upregulated and unchanged transcripts in Dgcr8$^{\Delta/\Delta}$ GPCs relative to control, and was normalized for total length of UTR sequence in each set of transcripts. Motifs passing a FDR cutoff of 0.05 using a Benjamini-Hochberg procedure are shown here.

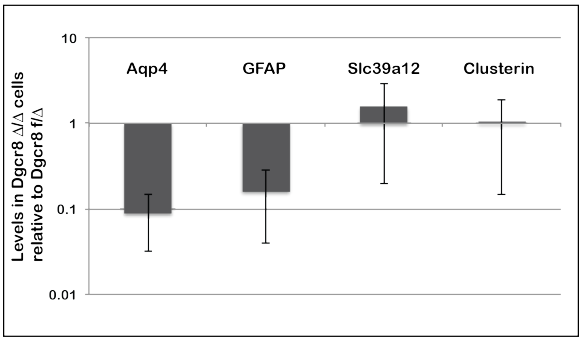
Figure 2-2, D, Dgcr8 loss leads to increased apoptosis in GPCs



Cleaved caspase-3 staining in Dgcr8$^{f/\Delta}$ and Dgcr8$^{\Delta/\Delta}$ GPCs 48 hours following addition of EGF and FGF, **Green – Cleaved Caspase 3, Blue - DAPI**

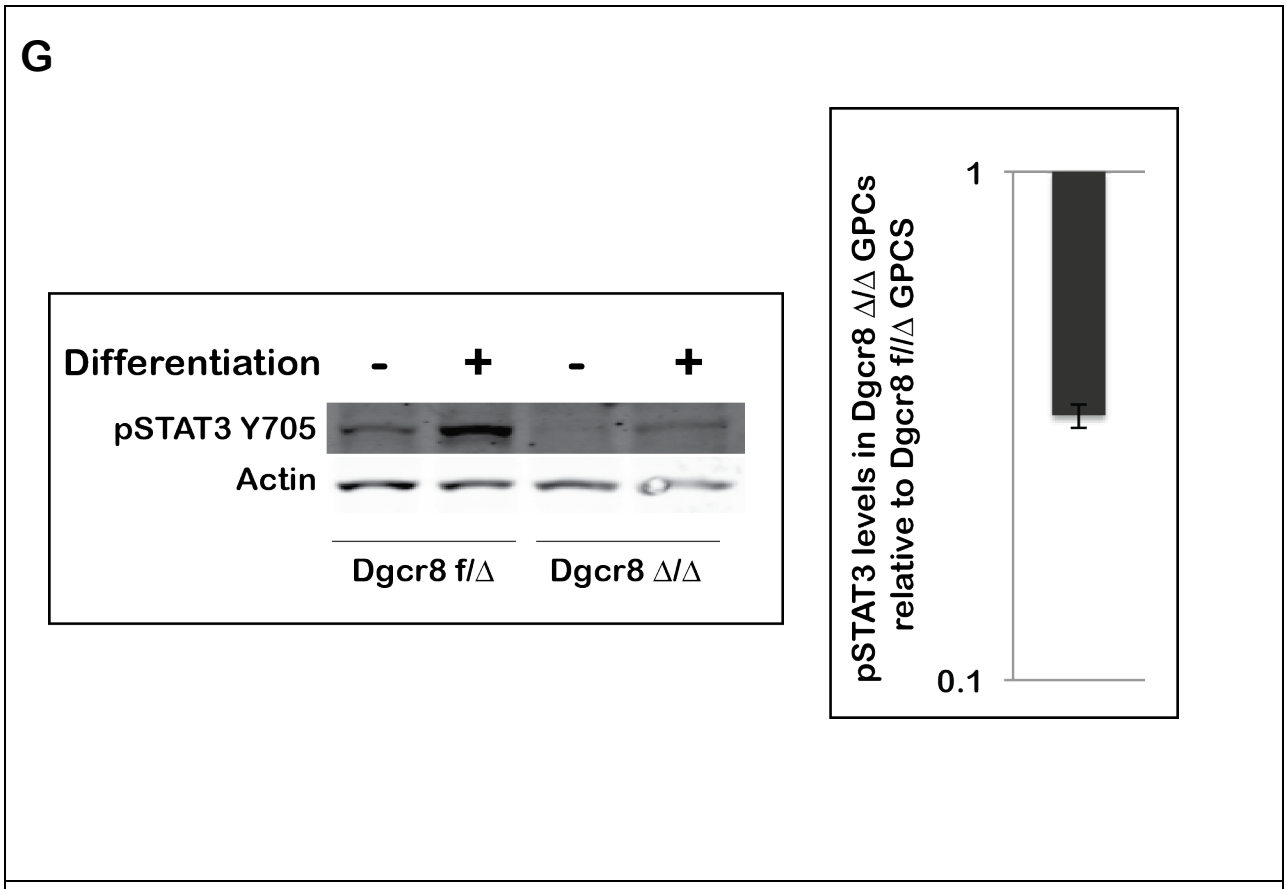
Figure 2-2, E&F, Dgcr8 loss leads to differentiation defects of GPCs





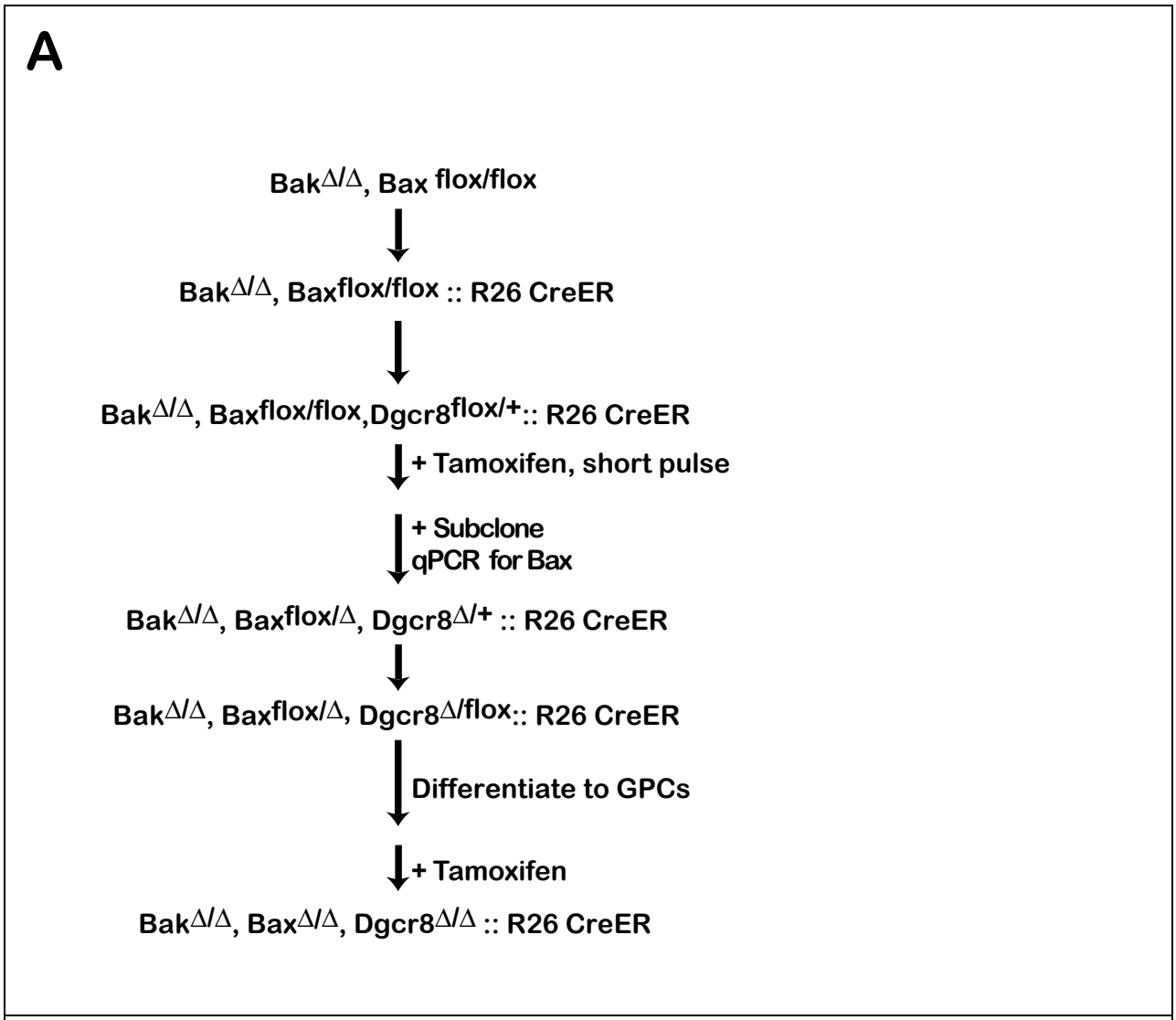
E) Control (R26 CreER +Tamoxifen and Dgcr8 f/Δ) and Dgcr8 Δ /Δ cells were grown in proliferation conditions and then induced to differentiate. GFAP levels pre- and post-differentiation were assayed by immunofluorescence (n=4). **Green – GFAP, Blue - DAPI**
F) Quantitative RT-PCR was performed on cells cultured as described in D for the astrocyte markers GFAP, Aqp4, Slc39a12 and Clusterin. Data are represented as mean +/- range (n=2).

Figure 2-2, G, JAK-STAT signaling during differentiation is attenuated in Dgcr8$^{\Delta/\Delta}$ GPCs



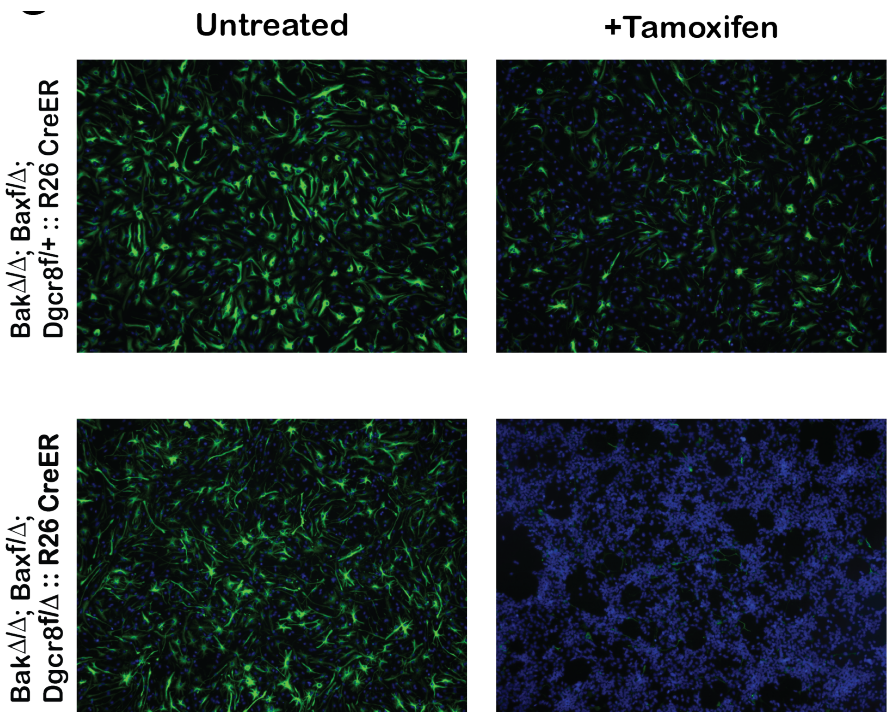
The activation of JAK-STAT during GPC differentiation was assayed by western blot for levels of phospho-STAT3 Y705 in control and Dgcr8$^{\Delta/\Delta}$ GPCs pre- and 6 hours post-differentiation. Actin was used as a loading control. Quantitation is shown on the right (n=2), error bars represent the range of the two experiments.

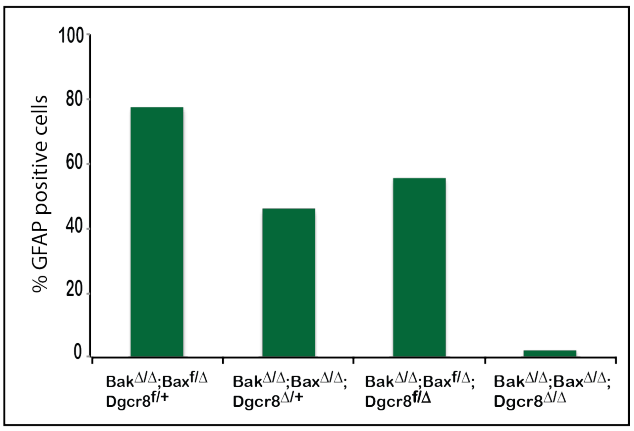
Figure 2-3, A, Targeting schematic



Schematic of targeting to generate Bax/Bak/Dgcr8 triple knockout GPCs

Figure 2-3, B&C, Differentiation defect in Bax/Bak/Dgcr8 triple knockout GPCs





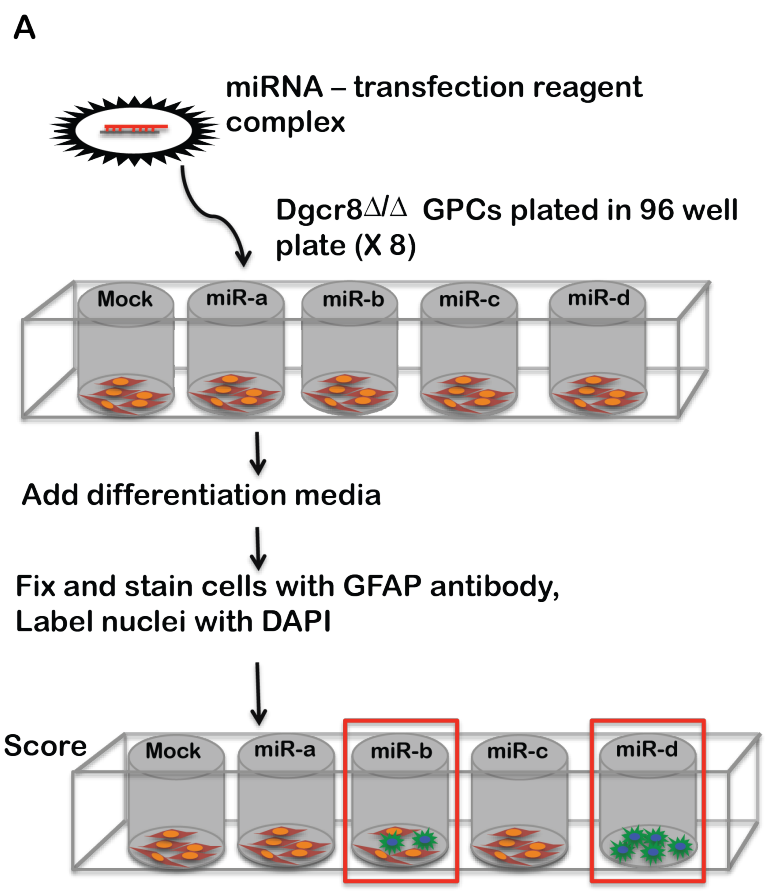
Inhibition of apoptosis does not rescue differentiation defects in Dgcr8 knockout GPCs

B) GFAP levels in differentiated control and Bax/Bak/Dgcr8 Δ/Δ GPCs were assayed by

immunofluorescence. **Green – GFAP, Blue - DAPI**

C) Quantitation of GFAP staining in **G** (n=1)

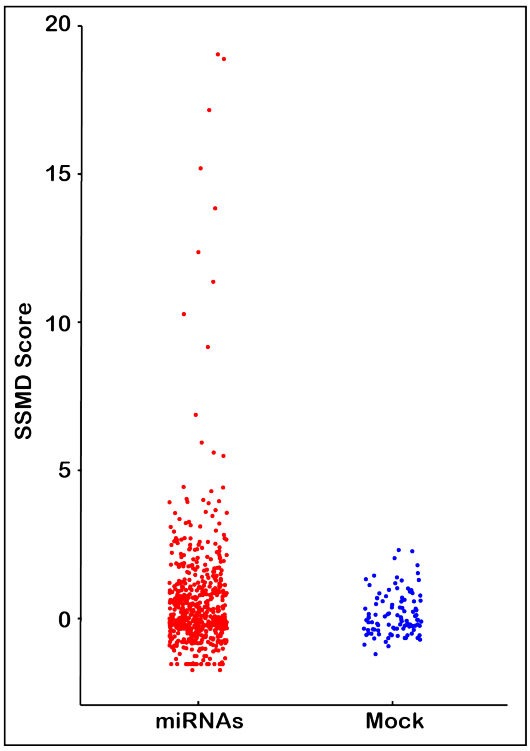
Figure 2-4, A, Screen schematic



Schematic of screen to identify miRNAs that can rescue differentiation defect of Dgcr8$^{\Delta/\Delta}$ GPCs

Figure 2-4, B, Results of miRNA screen

B

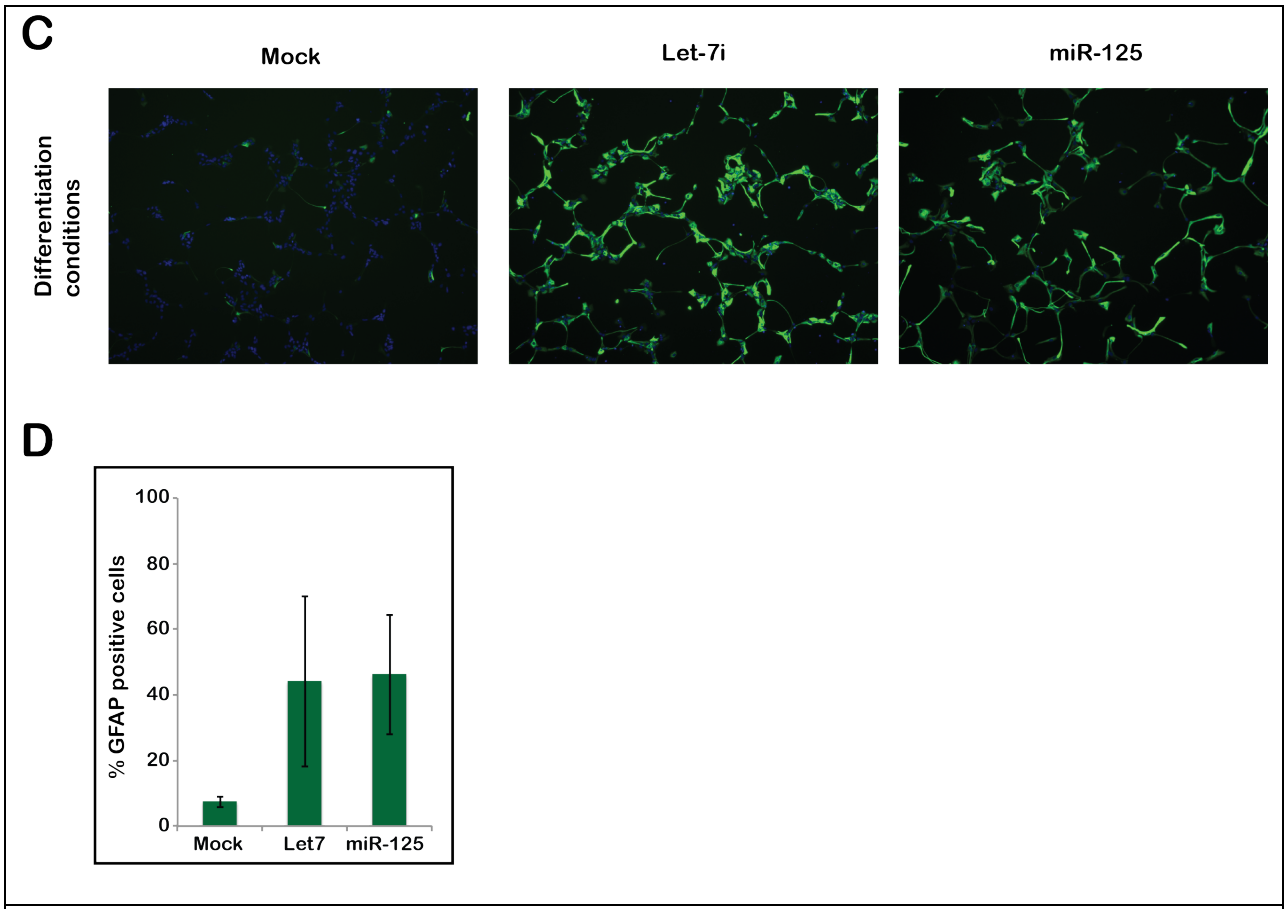


SSMD Score	miRNA	Sequence
19	let-7a	ugagguaguagguuguauaguu
18.9	let-7f	ugagguaguagauuguauaguu
9.16	let-7i	ugagguaguaguuugugcuguu
3.9	let-7a	ugagguaguagguuguauaguu
2.8	let-7d	agagguaguagguugcauaguu
3.9	let-7g	ugagguaguaguuuguacaguu
Score	miRNA	Sequence
12.4	miR-125b	ucccugagacccuaacuuguga
2.96	miR-125a	ucccugagacccuuuaaccuguga

Results of miRNA screen performed as described in Figure 3-3A

SSMD score for each well was calculated using % GFAP positive cells in each well relative to mock treated wells used as negative reference. Let-7 and miR-125 were chosen for follow up and have high SSMD score for several family members. The seed sequence for each miRNA is highlighted in red.

Figure 2-4, C&D, Independent confirmation of screen results

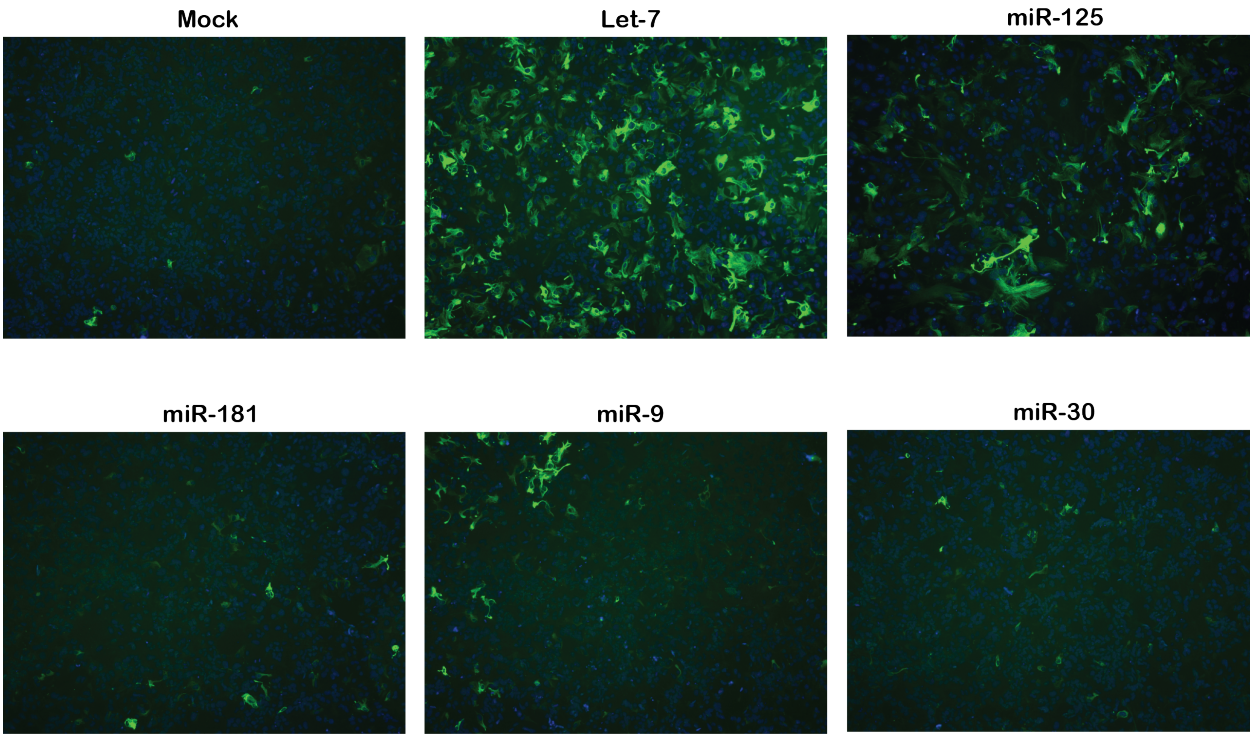


Confirmation of screen results with independently synthesized miRNA mimics

Dgcr8$^{\Delta/\Delta}$ GPCs were transfected with Mock, Let-7 or 125 for 72 hours and induced to differentiate. GFAP levels were assayed 48 hours later by immunofluorescence. **Green – GFAP, Blue - DAPI**

D) Quantitation of GFAP staining (n=4), P-values were calculated using the Mann-whitney rank test, $p<0.05$

Figure 2-4, E, Introduction of other highly expressed GPC miRNAs



Other highly expressed GPC miRNAs do not rescue differentiation defect in Dgcr8$^{\Delta/\Delta}$ GPCs

Dgcr8$^{\Delta/\Delta}$ GPCs were transfected with the miRNAs indicated for 72 hours and induced to differentiate for 48 hours. GFAP was assayed using immunofluorescence (n=3).

Green – GFAP, Blue - DAPI

Chapter 3: Mechanisms underlying miRNA-mediated rescue of astrocyte differentiation

Summary

In the absence of miRNAs, Dgcr8$^{\Delta/\Delta}$ GPCs exhibit survival defects and are unable to undergo proper astrocyte differentiation. In experiments described in the previous chapter, I discovered that the let-7 and miR-125 families of miRNAs are able to rescue the astrocyte differentiation defect. Here we identify hundreds of direct and indirect targets downregulated by these two miRNAs in GPCs. In support of their function as differentiation-promoting miRNAs, their targets show enrichment for a gene set previously identified as being highly expressed in glioblastoma multiforme and enriched in glioma stem cells. We further show that one let-7/miR-125 target Plagl2, inhibits wild-type astrocyte differentiation when overexpressed. Furthermore, we show that forced activation of the JAK-STAT pathway partially rescues the differentiation defect. However, let-7 and miR-125 do not appreciably rescue JAK-STAT activation but require JAK signaling to rescue differentiation. Thus, we propose that an alternate GPC miRNA may be inhibiting a repressor of the JAK-STAT pathway, while let-7 and miR-125 may be acting downstream of the JAK-STAT pathway to promote the signaling. This work provides insight

into the mechanisms underlying miRNA-mediated rescue of the differentiation defect in Dgcr8$^{\Delta/\Delta}$ GPCs.

Introduction

In Chapter 3, I used a screening assay to identify two miRNA families that promote astrocyte differentiation likely via priming GPCs. miRNA function is dictated by their ability to downregulate mRNA targets. Thus, to identify the mechanism of miRNA function, it is important to identify downstream function targets and the pathways they may modulate. In this Chapter, I investigate the pathways and targets that ultimately drive the effects of the let-7 and miR-125 family of miRNAs.

Results

Functional analysis of miRNA target pathways

It has been previously suggested in other developmental settings that miRNAs converge on signaling pathways to promote or inhibit a cell fate transition (Judson et al., 2013). Based on the observations described in preceding sections the JAK-STAT pathway is attenuated in Dgcr8 KO GPCs, we set out to determine whether other pathways may be misregulated with global miRNA loss and whether let-7 and miR-125 function by rescuing these pathways. We first performed a

targeted small molecule screen to identify signaling pathways that may be important to promote astrocyte differentiation. Inhibitors and/or activators of the JAK/STAT (JAKi), TGFβ (SB145321), Wnt (IWP-2, ChIR99021) and ERK (PD0325901) pathways were added during differentiation of wild-type cells and GFAP expression was examined 24 hours post-differentiation (**Figure 3-1A**). The results of this experiment showed that Chir99021, a Wnt pathway activator and a JAK inhibitor (JAKi) strongly inhibited GFAP expression during differentiation. The inhibition of the MEK/ERK pathway (PD0325901) and Wnt pathway (IWP-2) did not appreciably reduce GFAP expression.

To study whether Wnt signaling may be activated in Dgcr8$^{\Delta/\Delta}$ GPCs , we first examined levels of β -catenin, the downstream signal transducer of Wnt signaling. The binding of Wnt ligands to their receptor normally leads to inhibition of β -catenin phosphorylation and its translocation into the nucleus. We compared levels of total and phosphorylated β -catenin in control and Dgcr8$^{\Delta/\Delta}$ GPCs and found that they were largely unchanged (**Figure 3-1B**), suggesting that misregulation of the Wnt pathway may not contribute to the inhibition of astrocyte differentiation in Dgcr8$^{\Delta/\Delta}$ GPCs. It remains to be explored whether there are changes in Wnt signaling during early differentiation that are affected following Dgcr8 loss.

Since the JAK-STAT pathway is known to play important roles in astrocyte differentiation and is attenuated in Dgcr8$^{\Delta/\Delta}$ cells, we hypothesized that activation of this pathway may alleviate the inhibition of differentiation. Indeed, exogenous addition of LIF during differentiation increased the number of cells expressing GFAP in Dgcr8$^{\Delta/\Delta}$ cells (**Figure 3-1C**), suggesting that activation of JAK-STAT pathway at least partially rescues differentiation.

We next asked whether the miRNAs function via rescue of JAK-STAT pathway activation to promote differentiation in Dgcr8$^{\Delta/\Delta}$ GPCs. We collected protein samples at various time points during differentiation following the addition of let-7 and mir-125 and assayed for phospho-STAT3 at tyrosine residue 705. Addition of let7 and/or 125 did not appreciably rescue STAT3 phosphorylation in Dgcr8$^{\Delta/\Delta}$ cells over any time point examined (**data not shown**). However, the JAK-STAT pathway is required for let-7 and miR-125 induced differentiation, as addition of JAK inhibitor blocked the ability of miRNAs to rescue differentiation in Dgcr8$^{\Delta/\Delta}$ cells (**Figure 3-1D**). Taken together, this set of experiments suggests that let-7 and miR-125 do not regulate the activation of the JAK-STAT pathways during astrocyte differentiation. However, since LIF rescues the Dgcr8 KO phenotype, we propose that let-7 and miR-125 may function partially by targeting an mRNA or pathway that may inhibit the ability of STAT3 to activate downstream transcription. Further experiments are necessary to address this possibility.

Identification of mRNA targets of let-7 and miR-125

miRNAs exert their function by binding a complementary 6-8 nucleotide seed sequence in mRNAs and leading to their degradation. In order to identify direct and indirect targets of let-7 and miR-125, we performed microarray analysis 24 hours following transfection of $Dgcr8^{\Delta/\Delta}$ GPCs with let-7 and miR-125. Passage matched $Dgcr8^{flox/\Delta}$ GPCs were used as control. This analysis showed that miR-125 and let-7 regulate hundreds of transcripts (**Figure 3-2A**). Seed enrichment analysis revealed that mRNAs down regulated upon addition of let-7 and miR-125 were enriched for let-7 and miR-125 sites in their 3'UTRs and ORFs (**Figure 3-2B**). To determine whether let-7 and miR-125 are rescuing the knockout phenotype at the level of the transcriptome, I asked whether mRNAs up regulated and down regulated by the miRNAs were decreased and increased respectively in $Dgcr8^{\Delta/\Delta}$ GPCs relative to wild-type cells (**Figure 3-2C**). This analysis showed that the addition of let-7 and miR-125 rescues transcriptional misregulation of mRNAs normally regulated by miRNAs.

Functional relevance of miRNA target genes

To identify targets of let-7 and 125 that may be functionally relevant for their differentiation-rescuing function, we performed a siRNA screen. Since let-7 and miR-125 act by

downregulating target mRNAs, we hypothesized that knocking down individual targets of let-7 and 125 may recapitulate the differentiation-promoting effects of these miRNAs in Dgcr8$^{\Delta/\Delta}$ GPCs. Furthermore, we hypothesized that let-7 and miR-125 may be acting partially through common targets to effect differentiation. Thus, the mRNA to target with siRNAs were chosen based on the following criteria: (**i**) Down-regulated upon addition of let-7, (**ii**) Downregulated upon addition of miR-125, (**iii**) Contains a 3'UTR seed match for let-7 or 125 and (**iv**) Upregulated in Dgcr8$^{\Delta/\Delta}$ cells relative to control. This resulted in a list of approximately 65 mRNAs. I performed an siRNA screen to determine whether downregulation of these mRNAs rescued differentiation in Dgcr8$^{\Delta/\Delta}$ GPCs (**Figure 3-3A**). Knockdown of individual mRNAs was insufficient to rescue differentiation, as assayed by GFAP expression. This result suggests that miRNAs rescue differentiation by their combined effects on multiple mRNAs. Alternatively, the criteria used to select the subset of targets to test in the siRNA screen may have been too restrictive.

Since knockdown of a single target may not be sufficient to rescue differentiation, we then asked the reciprocal question; whether overexpression of individual targets could block differentiation of WT GPCs. We cloned 8 of the 70 mRNAs used in the siRNA screen into constructs co-expressing mCherry to aid in identification of transfected cells. Luciferase overexpression was

performed as a negative control. Preliminary analysis shows that one gene, Plagl2, robustly inhibited the differentiation of WT GPCs when overexpressed while luciferase and Ezh2 did not (**Figure 3-3B**). In a preliminary experiment, another let-7/miR-125 target Igf2bp2, also inhibited wild-type differentiation though these experiments remain to be reproduced (**data not shown**). Plagl2 is up regulated 4.3 fold in $Dgcr8^{\Delta/\Delta}$ GPCs relative to control. It is significantly down regulated upon addition of let-7 and miR-125 relative to mock transfected $Dgcr8^{\Delta/\Delta}$ cells (Fold changes: let7/mock: 0.58±0.16, 125/Mock:0.77±0.09).

In a previous study, Plagl2 was identified as a gene that is amplified in gliomas (Zheng et al., 2010a). Overexpression of Plagl2 in p53-/- neural stem cells inhibited astrocyte differentiation, partly via modulation of the Wnt pathway. However, since the Wnt pathway is likely not misregulated in our system, Plagl2 is likely acting via other targets to inhibit differentiation. Plagl2 is predicted to be a direct target of both let-7 and miR-125, based on seed sequence analysis of its 3'UTR. Luciferase experiments will be performed to confirm targeting of these sites by let-7 and miR-125. I propose to test additional targets of let-7 and miR-125 in the overexpression assay to discover other inhibitors of astrocyte differentiation.

Significance: Let-7 and miR-125 targets are enriched for a glioma signature

Gliomas are a class of brain tumors named for their cell of origin, which is widely believed to be glia. The most benign of these are called astrocytoma, characterized by their expression of markers associated with differentiated astrocytes. The most malignant and advanced glioma subtype is called glioblastoma multiforme (GBM). Since malignancy is associated with more stem cell like characteristics and less differentiation, we sought to investigate whether let-7 and miR-125 targets are more highly expressed with increasing malignancy. A previous study identified a 30 gene signature of genes with higher expression in glioma stem cells relative to normal stem cells, as well as increasing expression with increasing cancer severity(Sandberg et al., 2013). Overlapping the 30 gene signature with the let-7/miR-125 target data set resulted in 17 genes expressed/detected in our microarrays. Enrichment analysis showed that the genes in the signature were highly enriched in the gene set down regulated by addition of let-7 or mir-125, and were not significantly enriched in the gene set up regulated by the miRNAs (**Figure 3-4**).

Discussion

The experiments performed in this study were designed to find the genes and pathways targeted by let-7 and miR-125 to promote astrocyte differentiation. It has been shown in other cell fate contexts that miRNAs converge on targets situated in signaling pathways important for a specific

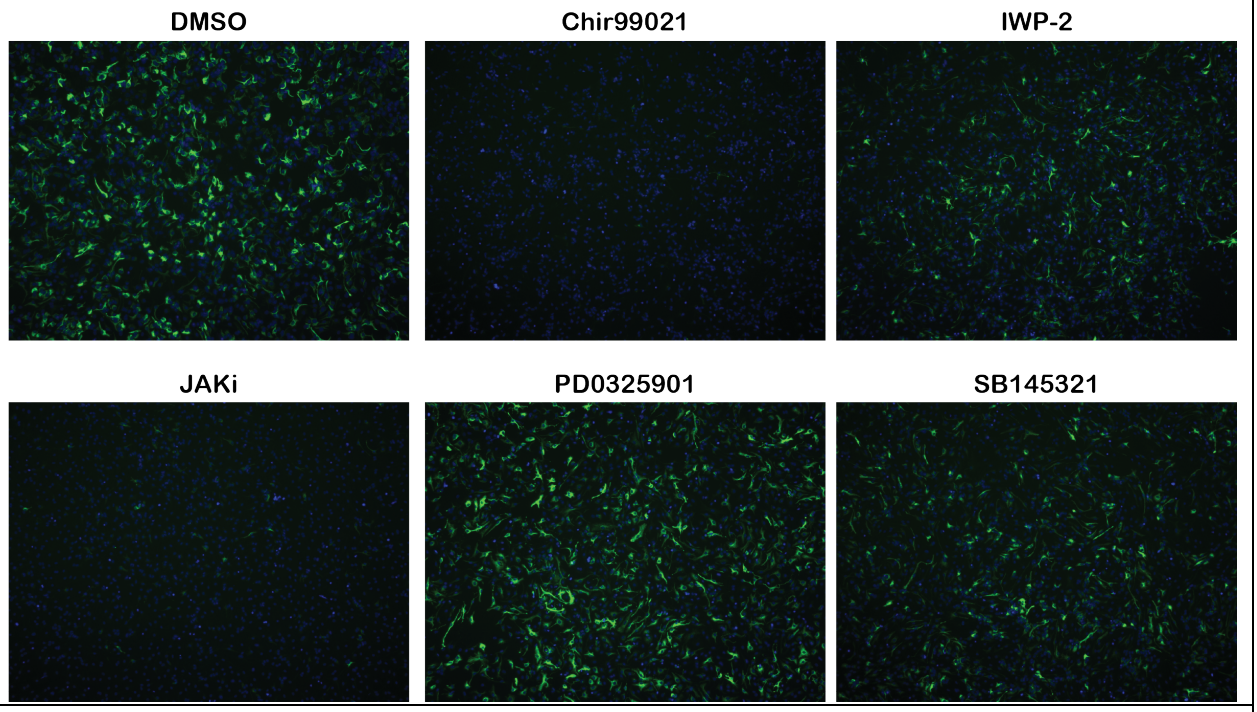
cellular process(Judson et al., 2013). In a limited screen of small molecules, the inhibition of the JAK-STAT pathway and activation of Wnt pathway were sufficient to inhibit differentiation of wild-type cells. Upon examination of these pathways in Dgcr8$^{\Delta/\Delta}$ GPCs, we discovered that while the Wnt pathway is largely unaffected, the activation of the JAK-STAT pathway during differentiation is inhibited. However, barring technical limitations of transfections, we were unable to find evidence for activation of the JAK-STAT pathways by let-7 and miR-125. Forced activation of the pathway with addition of LIF was sufficient to rescue the phenotype and inhibition of JAK blocked the ability of let-7 and miR-125 miRNAs to rescue differentiation. Together, these results suggest that other GPC miRNAs may be inhibiting a repressor of the JAK-STAT pathway. Let-7 and miR-125 may be acting downstream of phospho-STAT3 to provide robustness to the signaling pathway, perhaps by regulating chromatin or binding site accessibility. Thus, in Dgcr8$^{\Delta/\Delta}$ cells, the addition of let-7 and miR-125 may be sufficient to allow the reduced amount of pSTAT3 to activate downstream target genes such as GFAP. Indeed, a number of studies have suggested that competing factors may block accessibility of STAT3 to its cognate promote elements during astrocyte differentiation(Cheng et al., 2011).

We used microarray analysis to discover the direct mRNA targets of let-7 and miR-125. The addition of let-7 and miR-125 into Dgcr8$^{\Delta/\Delta}$ cells leads to the up- and downregulation of

hundreds of mRNAs, a subset of which is overlapping between the two miRNAs. Individual siRNA-based knockdown of approximately 70 targets in Dgcr8$^{\Delta/\Delta}$ GPCs was insufficient to appreciably rescue differentiation as assayed by GFAP levels. However, preliminary experiments show that the overexpression of one of these, Plagl2, is sufficient to inhibit differentiation in wild-type GPCs. We cannot exclude the possibility that some siRNAs are rescuing a part of the differentiation program that does not include GFAP. Our findings suggest that the concerted action of let-7 and miR-125 on multiple targets is required to prime GPCs for differentiation. This is consistent with previous studies, such as in ES cells, where miRNAs downregulate multiple inhibitors of G1-S progression to regulate the cell cycle but siRNA knockdown of single inhibitors has no effect(Wang et al., 2008).

It has been suggested that miRNAs have evolved to target gene networks that have coordinated function. In addition to discovering miRNA function in astrogliogenesis, this study provides a number of interesting candidates that may be promoting a progenitor state and inhibiting astrocyte differentiation. This is supported by analysis showing that in the setting of a glial cancer such as glioma, genes that increase with severity of the cancer are enriched in the set of let-7 and miR-125 targets (**Figure 3-5**).

Figure 3-1, A, Small molecule screen for regulators of astrocyte differentiation

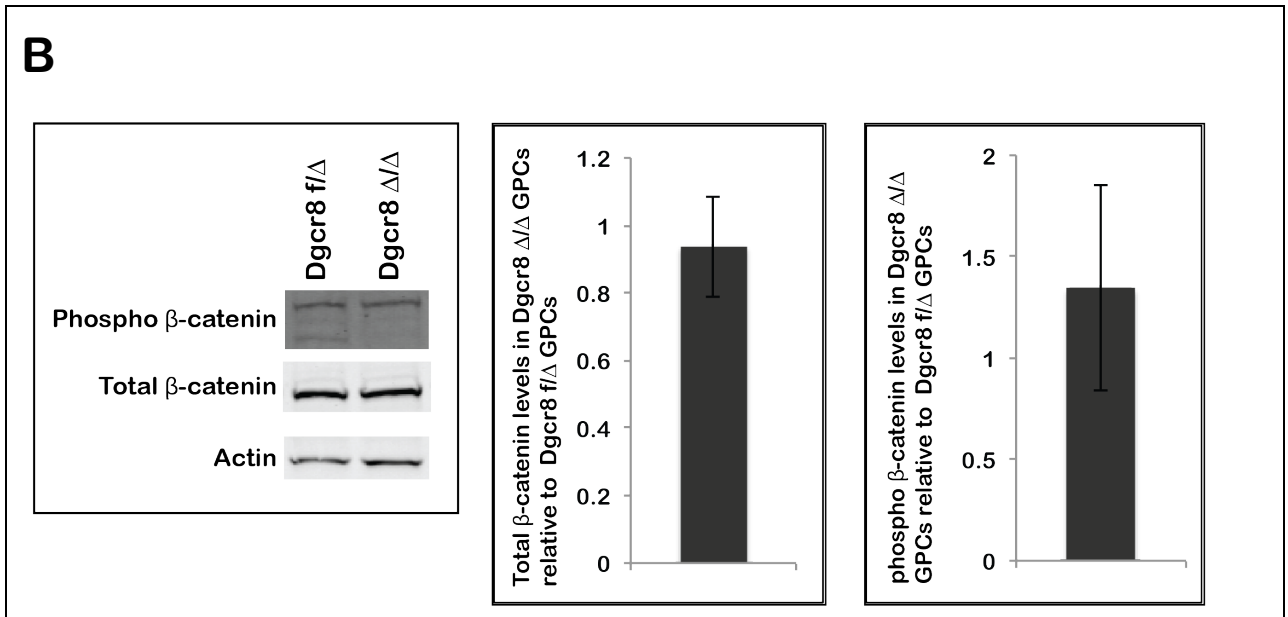


Small molecules targeting JAK and activating Wnt inhibit astrocyte differentiation

Dgcr8 f/d GPCs were incubated in differentiation conditions with the listed compounds for 24 hours. GFAP expression was assayed by immunofluorescence.

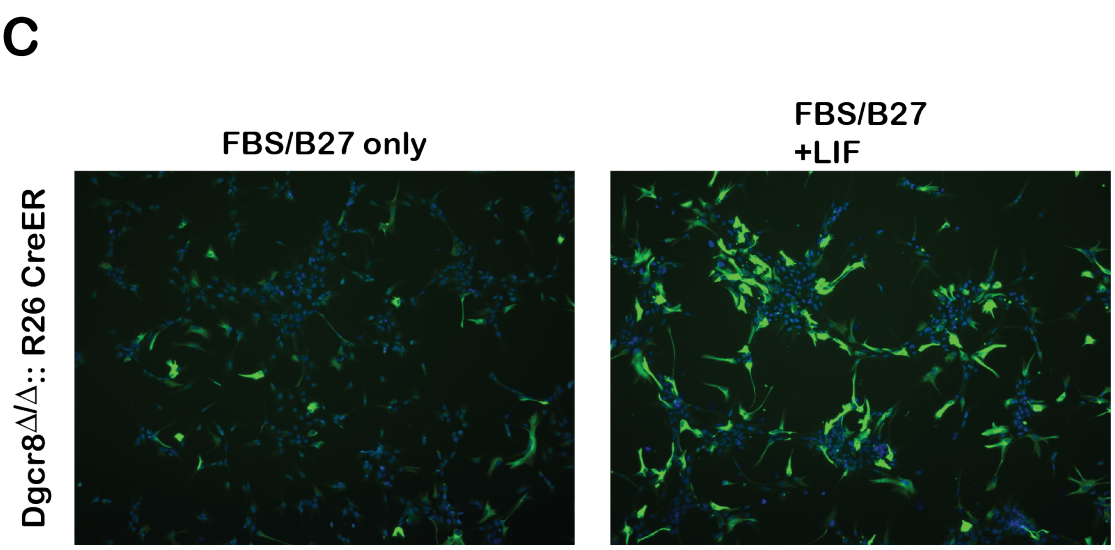
Green – GFAP, Blue - DAPI

Figure 3-1, B, Wnt signaling is not disrupted in Dgcr8$^{\Delta/\Delta}$ GPCs



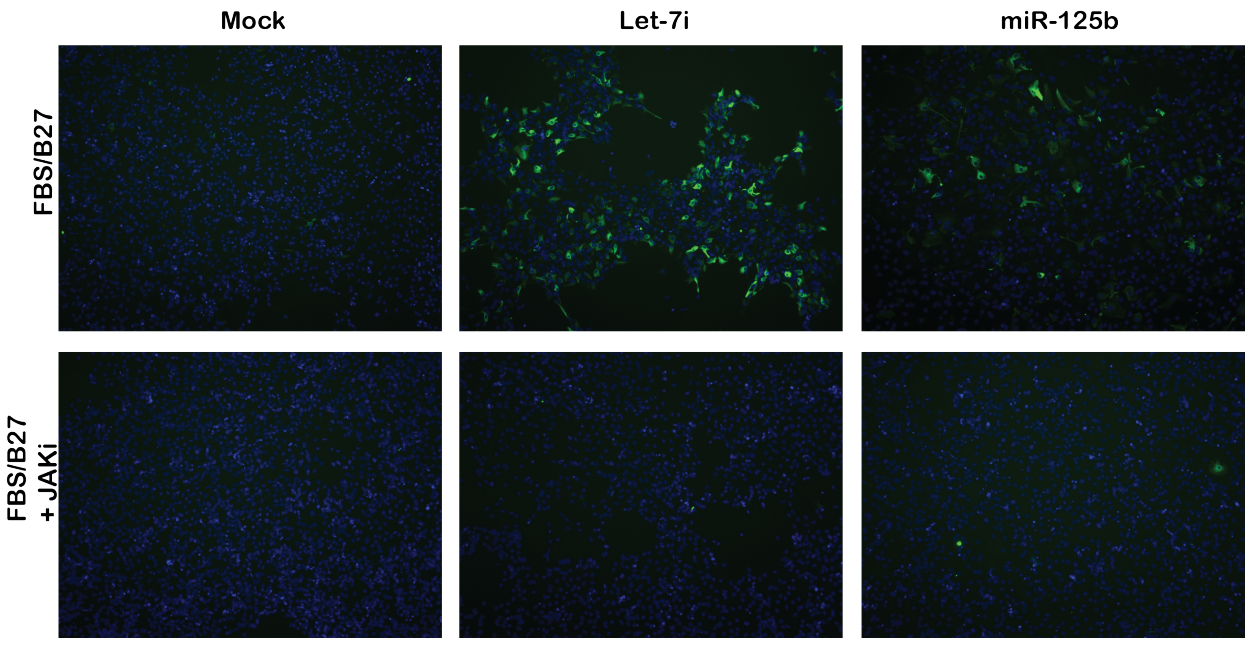
Total and phospho β-catenin levels were assayed by western blot and quantified using Image J. Actin was used as a loading control. All quantification data is normalized to actin and to levels in Dgcr8 f/d GPCs (n=4).

Figure 3-1, C, Activation of JAK-STAT pathway rescues Dgcr8$^{\Delta/\Delta}$ GPC differentiation



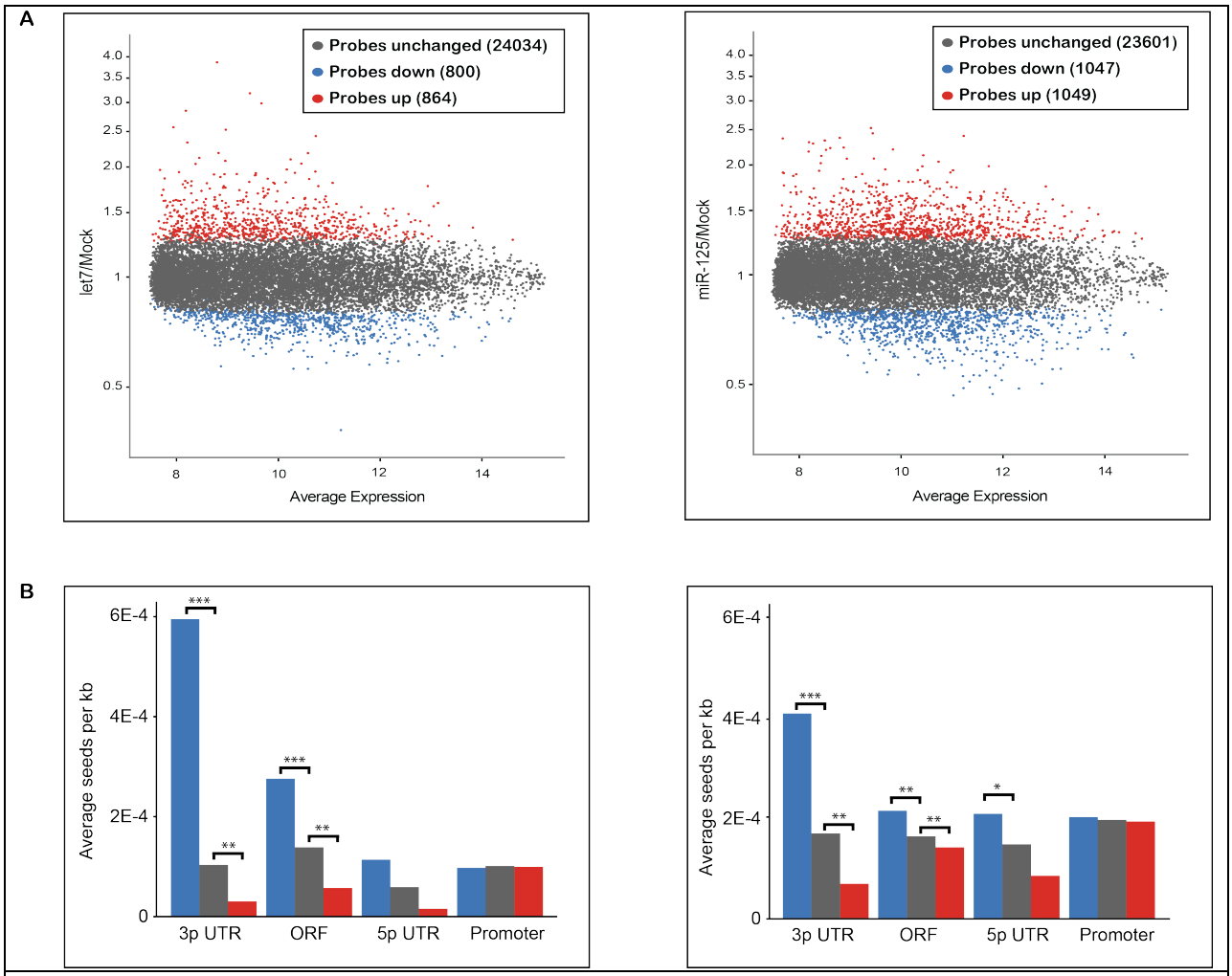
Differentiation media was added to Dgcr8$^{\Delta/\Delta}$ GPCs with or without LIF for 48 hours. GFAP levels were assayed by immunofluorescence. Representative image is shown (n=3).
Green – GFAP, Blue - DAPI

Figure 3-1, D, JAK-STAT signaling is necessary for miRNA-rescue of GPC differentiation in Dgcr8$^{\Delta/\Delta}$ GPCs



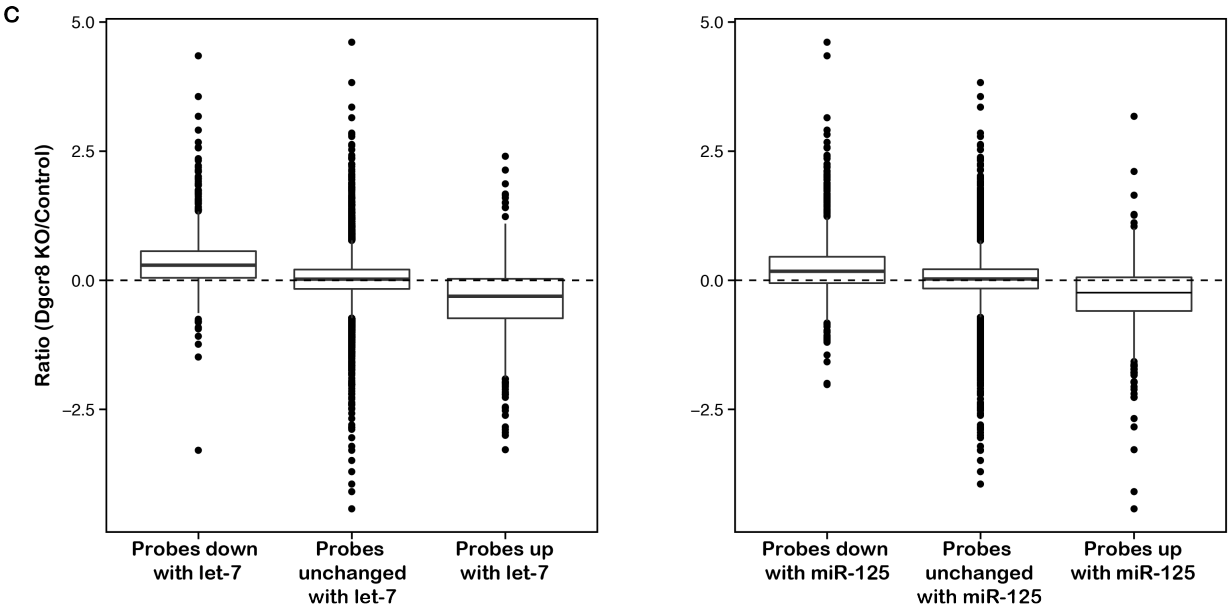
Differentiation media was added to mock-, let-7- or miR-125 transfected Dgcr8$^{\Delta/\Delta}$ GPCs with or without JAK inhibitor for 48 hours. GFAP levels were assayed by immunofluorescence.
Green – GFAP, Blue - DAPI

Figure 3-2, A&B, Identification of targets of let-7 and miR-125



Let-7 and miR-125 suppress hundreds of transcripts by binding their ORF and/or 3'UTR
(A) Microarray analysis following introduction of let-7i and miR-125 in Dgcr8$^{\Delta/\Delta}$ GPCs. Upregulated transcripts are shown in red, downregulated transcripts in blue (Adjusted P value < 0.05). (b) Analysis of seed matches in the promoter, 5´UTR, ORF, and 3´UTR of downregulated and upregulated transcripts. Data are presented as the mean number of seeds matches per kb of sequence for the listed groups of altered genes described in A. P-values calculated by the Wilcoxon Rank Sum Test and Bonferroni corrected are shown for p < 0.01 (*** represents p value < 1e-40, ** represents p value < 1.5e-8 and * represents p value < 1e-3)

Figure 3-2, C, let-7 and miR-125 regulate genes that change upon loss of Dgcr8 in GPCs



Presented are boxplots showing levels in Dgcr8$^{\Delta/\Delta}$ GPCs of genes upregulated, unchanged and downregulated upon addition of let-7 and miR-125.

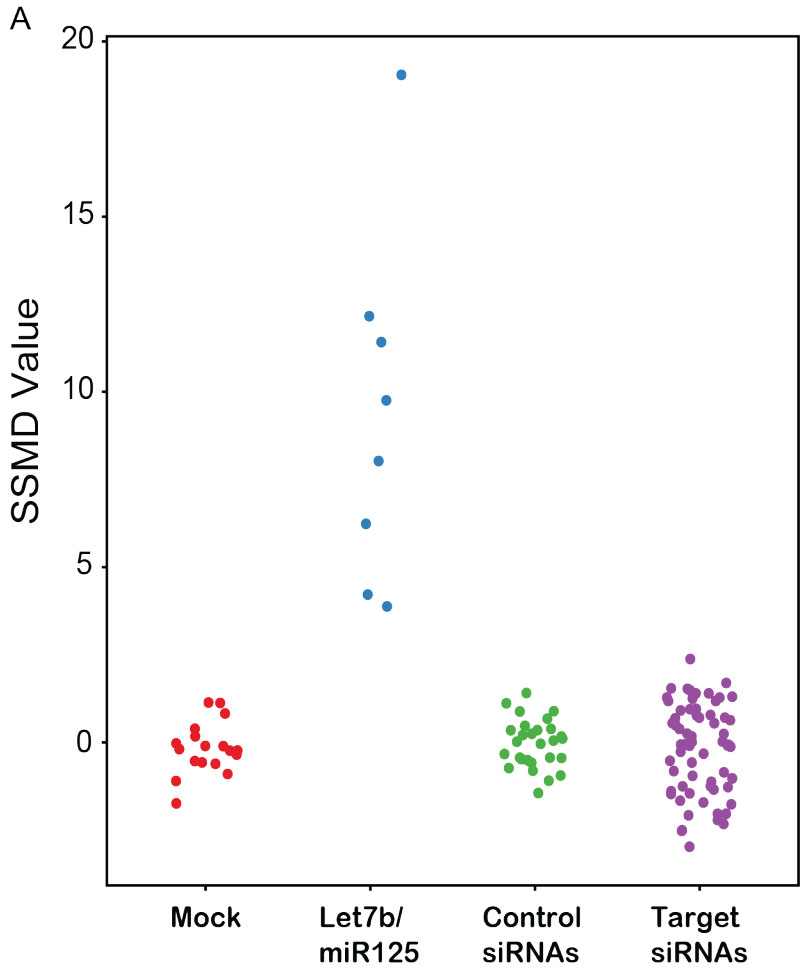
Table 3-1, List of genes used in siRNA screen

GeneName	Let7 3'UTR matches	miR125 3'UTR matches	Fold change in expression (Dgcr8 KO/Control)	Fold change in expression (let7/Mock)	Fold change in expression (miR125/Mock)
Nap1l1	2	0	1.54	0.56	0.79
Mycl1	1	0	1.53	0.58	0.72
Plagl2	1	2	4.30	0.58	0.78
Hic2	5	4	2.68	0.61	0.67
Smarcad1	2	0	1.62	0.61	0.70
Punc	2	0	20.38	0.61	0.50
Tmem2	1	0	4.00	0.62	0.53
Plk3	0	1	2.38	0.63	0.79
Prtg	2	2	2.18	0.63	0.54
Tgfbr1	2	1	3.63	0.64	0.77
Mmp11	1	1	1.26	0.65	0.66
Igf2bp3	1	1	1.95	0.65	0.47
Igf2bp2	2	3	7.51	0.65	0.55
Cbx2	1	2	2.10	0.66	0.70
L3mbtl2	0	1	1.23	0.67	0.60
Nr6a1	8	3	2.27	0.67	0.69
Pbx2	2	0	1.96	0.68	0.78
Espl1	1	0	1.28	0.69	0.74
Ciapin1	0	1	1.32	0.69	0.79
Cdc25a	1	0	1.89	0.69	0.79
Rbm38	1	1	1.31	0.69	0.76
Zfp41	1	3	1.32	0.70	0.58
Fign	2	0	2.07	0.70	0.71
Cep120	2	0	1.74	0.70	0.79
Dusp1	1	0	3.58	0.70	0.80
Clp1	1	0	1.19	0.71	0.78
Smug1	2	1	1.28	0.71	0.74
Lmnb2	0	2	1.26	0.71	0.80

Wdr77	1	2	1.31	0.71	0.80
Nuak2	1	0	3.17	0.72	0.67
Piga	1	0	1.41	0.72	0.76
Golm1	1	1	2.88	0.73	0.63
Arid3a	4	7	1.40	0.73	0.67
Pgrmc1	1	0	1.36	0.73	0.75
Hs2st1	1	1	1.44	0.74	0.70
Tmem167	1	0	1.17	0.74	0.77
Zswim4	1	1	1.22	0.74	0.67
Narg1l	1	0	1.58	0.75	0.81
Stx3	2	1	2.46	0.75	0.70
Mknk2	0	2	1.83	0.76	0.61
Map3k3	1	0	2.92	0.76	0.76
Ezh2	1	0	1.48	0.76	0.76
Ddx19b	2	4	1.21	0.77	0.68
Ghr	1	1	1.11	0.77	0.66
E2f2	1	3	1.18	0.77	0.65
Ube2j1	1	1	1.38	0.77	0.66
Tgm2	1	0	1.14	0.78	0.69
Brip1	0	1	1.52	0.78	0.68
Arid3b	3	3	2.32	0.78	0.74
Rnf44	1	3	1.97	0.78	0.67
Clcc1	1	0	1.19	0.78	0.76
Uhrf2	1	0	1.23	0.78	0.79
Glt8d3	2	2	1.66	0.79	0.71
Hdlbp	1	2	1.37	0.79	0.73
Cdca5	1	0	1.18	0.79	0.74
Fanci	1	0	1.14	0.79	0.78
Gpr23	0	1	1.23	0.80	0.58
Epha3	1	0	1.32	0.80	0.60
Slco3a1	1	1	1.40	0.80	0.72
Tspan14	0	1	1.20	0.80	0.61
Arcn1	0	1	1.13	0.81	0.76
Hspa5	0	1	1.15	0.81	0.79
Tmem169	0	1	1.30	0.81	0.75

Zbtb9	0	2	1.51	0.84	0.65

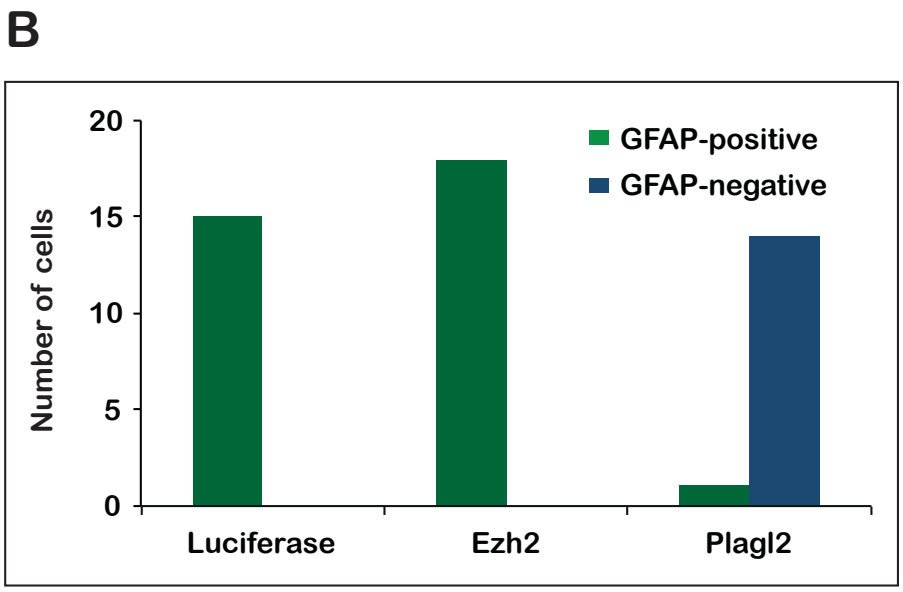
Figure 3-3, A, siRNA screen of let-7 and miR-125 targets



siRNA-based knockdown of individual targets of let-7 and miR-125 does not rescue differentiation of Dgcr8$^{\Delta/\Delta}$ GPCs

A small-scale screen (n=1) was performed using 70 targets of let-7b/125. SSMD was calculated using control non-targeting siRNAs as negative reference. Let-7b and miR-125 were included as positive controls. Mock transfection and Control siRNAs were used as negative controls.

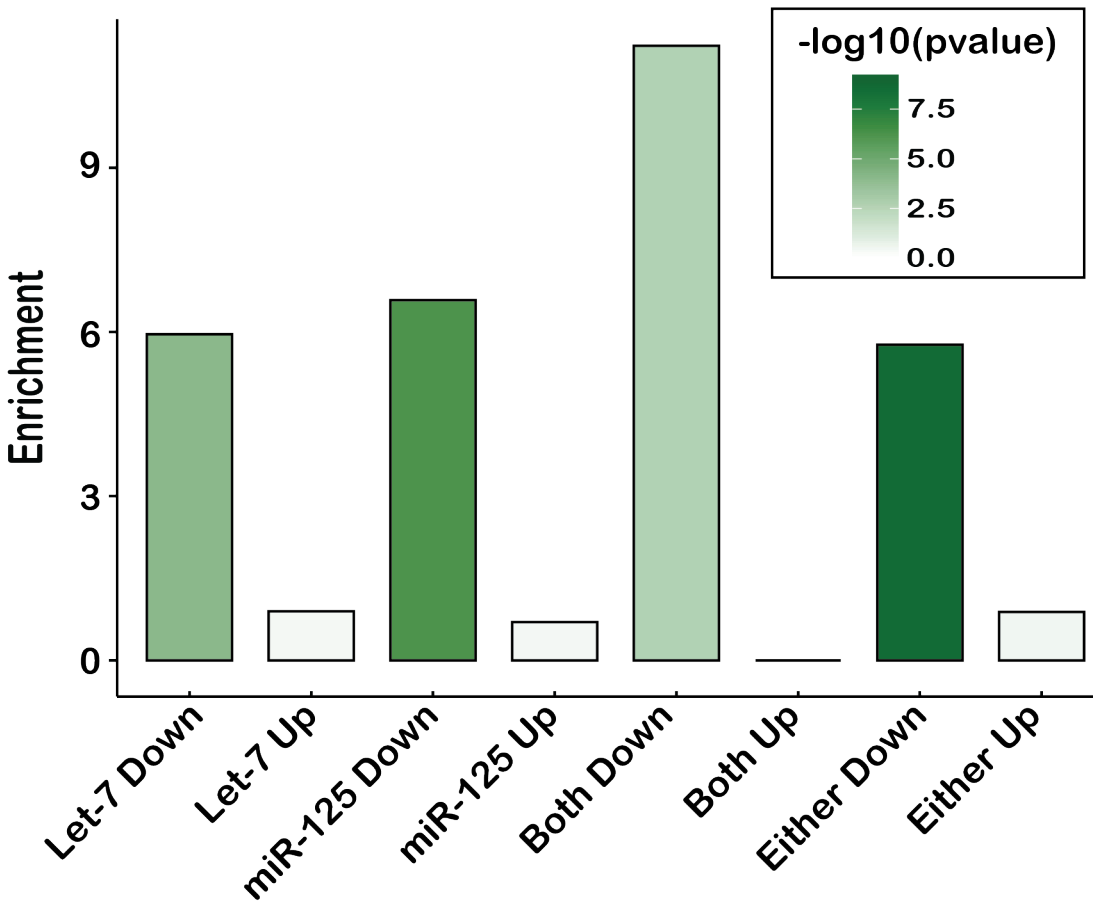
Figure 3-3, B, Overexpression of let-7 and miR-125 targets



Overexpression of the let-7/miR-125 target Plagl2 but not Ezh2 inhibits wild-type differentiation

Constructs expressing mCherry with the gene of interest as indicated on the graph were nucleofected into GPCs. Differentiation was induced 24 hours post-nucleofection and fixed for GFAP immunofluorescence 24 hours later. mCherry-positive cells were identified in each overexpression condition and their GFAP expression was scored (n=1). Approximately 15-20 cells were counted in each overexpression condition.

Figure 3-4, Overlap of glioma signature and miRNA target data sets

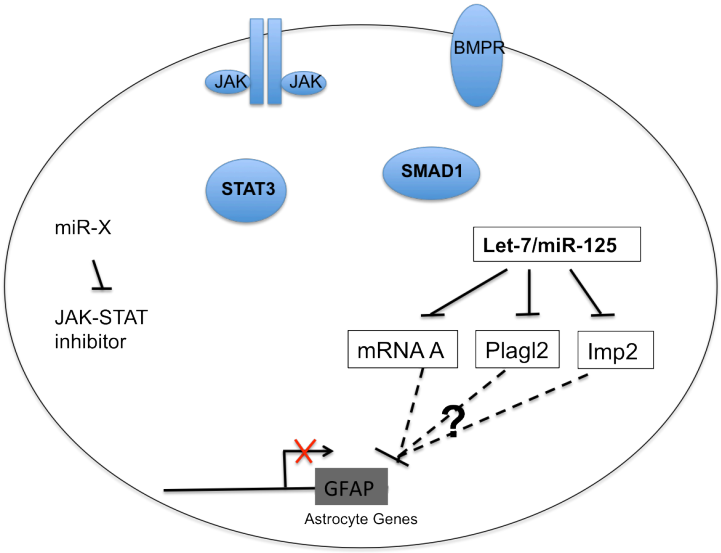


Glioma signature is enriched in mRNAs downregulated by let-7 and miR-125

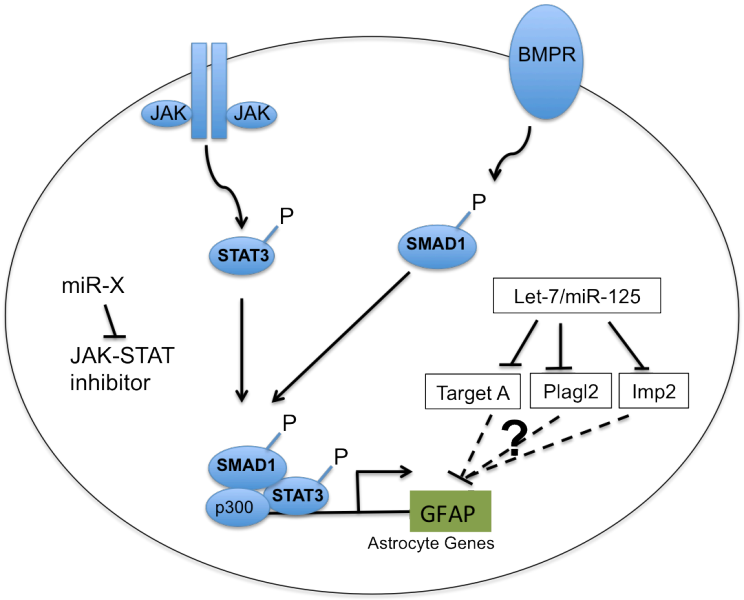
The glioma signature identified by Sandberg et al was overlapped with gene sets regulated by let-7 and miR-125 in Dgcr8 knockout GPCs(Sandberg et al., 2013). Enrichment results are shown as a ratio of % of glioma genes in each group (as labeled on the X-axis) relative to % of glioma genes in the unchanged gene set.

Figure 3-5, Model

Progenitor State



Differentiation



Model for miRNA function in promoting astrocyte differentiation

Chapter 4: Genomic analysis suggests that mRNA destabilization by the Microprocessor is specialized for the auto-regulation of Dgcr8

Summary

The microprocessor, containing the RNA binding protein Dgcr8 and RNase III enzyme Drosha, is responsible for processing primary microRNAs to precursor microRNAs. The microprocessor regulates its own levels by cleaving hairpins in the 5′UTR and coding region of the Dgcr8 mRNA, thereby destabilizing the mature transcript. To determine whether the microprocessor has a broader role in directly regulating other coding mRNA levels, we integrated results from expression profiling and ultra high-throughput deep sequencing of small RNAs. Expression analysis of mRNAs in wild-type, Dgcr8 knockout, and Dicer knockout mouse embryonic stem (ES) cells uncovered mRNAs that were specifically upregulated in the Dgcr8 null background. A number of these transcripts had evolutionarily conserved predicted hairpin targets for the microprocessor. However, analysis of deep sequencing data of 18 to 200nt small RNAs in mouse ES, HeLa, and HepG2 indicates that exonic sequence reads that map in a pattern consistent with microprocessor activity are unique to Dgcr8. We conclude that the microprocessor's role in

directly destabilizing coding mRNAs is likely specifically targeted to Dgcr8 itself, suggesting a specialized cellular mechanism for gene auto-regulation.

Introduction

MicroRNA maturation involves two processing steps(Babiarz and Blelloch, 2009). First, a long primary miRNA (pri-miRNA) is cleaved by the Microprocessor, containing the RNA binding protein Dgcr8 and the RNAseIII enzyme Drosha, to produce a 60–75 nucleotide hairpin precursor miRNA (pre-miRNA) in the nucleus(Lee et al., 2003; Denli et al., 2004; Gregory et al., 2004; Han et al., 2009; Landthaler et al., n.d.). The pre-miRNA is translocated to the cytoplasm where it is cleaved to a miRNA duplex (~19–25 nt in length) by the RNAseIII enzyme Dicer (Bernstein et al., 2001). A single strand of the duplex enters the RNA-induced silencing complex (RISC) with the help of another RNA binding protein, TRBP (Chendrimada et al., 2005; Haase et al., 2005). Dicer has roles outside of the maturation of canonical miRNAs. For example, in mouse ES cells, Dicer processes other subclasses of miRNAs including mirtrons and short hairpin RNAs as well as endogenous siRNAs (Babiarz et al., 2008). Similarly, Dicer processes endogenous siRNAs in mouse oocytes(Tam et al., 2008; Watanabe et al., 2008). Consistent with these additional roles of Dicer, Dgcr8 knockout (KO) ES cells have less severe phenotypes than Dicer knockout ES cells (Wang et al., 2007a).

The Microprocessor was recently shown to have an additional role in directly destabilizing a mRNA target. Specifically, it can cleave hairpins in the 5′UTR and coding region of the Dgcr8 mRNA, which in turns destabilizes the mature transcript(Pedersen et al., 2006; Han et al., 2009; Triboulet et al., 2009). This negative feedback loop on Dgcr8 suggests the importance of tight homeostatic control of the Microprocessor in normal cellular function. The finding that the Microprocessor can directly influence *Dgcr8* mRNA levels raises the possibility that this mechanism may affect many other mRNAs.

To further test whether there is a broader role of the Microprocessor in the direct regulation of mRNAs, we evaluated the mRNA and small non-coding RNA profiles of wild-type, Dgcr8 KO and Dicer KO cells as well as a recently published data set of small RNAs less than 200 nucleotides from human Hela and HepG2 cell lines (Fejes-Toth et al., 2009). While many mRNAs were differentially expressed between Dgcr8 and Dicer KO ES cells, there was no evidence for Microprocessor based processing of these mRNAs, with the striking exception of Dgcr8 itself. Similarly, analysis of the Hela and HepG2 data sets identified many sequence reads from the Dgcr8 hairpins showing a pattern consistent with Microprocessor activity, but none from any other predicted hairpins within spliced mRNAs. These findings suggest that the

Microprocessor's role in directly regulating mRNA levels is specific to auto-regulation of Dgcr8, highlighting the importance of this negative feedback regulation of Microprocessor levels.

Results

Microarray analysis identifies mRNAs upregulated specifically in Dgcr8-/- ES cells relative to Dicer -/- ES cells

mRNAs regulated by a direct microprocessor cleavage mechanism should be upregulated in cells deficient for the microprocessor, but not in Dicer deficient cells. Therefore, we evaluated coding mRNA profiling data from wild-type, Dgcr8 KO and Dicer KO mouse ES cells. Normalized mRNA levels in Dgcr8 KO and Dicer KO cells were compared to wild-type ES cells (**Figure 4-1**). Most mRNAs that were upregulated or downregulated were similarly altered in both mutants. However, similar to previous studies (Tam et al., 2008; Triboulet et al., 2009), we found multiple mRNAs whose expression were specifically altered in cells that lacked Dgcr8. Using a false discovery rate of 5%, there were 778 transcripts there were upregulated in Dgcr8 KO cells relative to both wild-type and Dicer KO. There were 843 transcripts that were downregulated.

Evaluation of predicted hairpins in mRNAs upregulated in Dgcr8 -/- ES cells

If genes specifically upregulated in Dgcr8 KO cells are normally cleaved by the microprocessor, there should be hairpin substrates for the complex within these mRNAs. Therefore, we searched

for evolutionary conserved hairpins within these mRNAs using predictions generated by the EvoFold algorithm(Wang et al., 2007a). The 5'UTR hairpin in Dgcr8 was first identified by this method. EvoFold predictions are grouped based on their location in CDS, 5´UTR, 3´UTR, intron and intergenic regions. We determined mouse genome coordinates for EvoFold hairpins in CDS, 5´UTR and 3´UTR regions (see Methods), mapped them to the coding mRNA database, and compared the relative expression levels of all positive hits in Dgcr8 KO, Dicer KO, and wild-type ES cells (**Figure 4-1**). A total of 824 out of 23805 (3.5 %) coding mRNAs contained predicted hairpins. Of these 824, 43 mRNAs were specifically upregulated in Dgcr8 KO cells, while 24 mRNAs were specifically downregulated in the Dgcr8 KO cells. Therefore, there was a subset of genes specifically upregulated in Dgcr8 KO cells that contain predicted hairpins and hence could be direct targets of the microprocessor.

If hairpins within the Dgcr8 KO- upregulated gene set are indeed cleaved by the microprocessor, we hypothesized that there would be Dgcr8-dependent small RNAs that map to these hairpins. Therefore, we evaluated ultra-high throughput deep sequencing data representing small RNAs ranging from 18-32 nucleotides from the wild-type, Dgcr8 KO and Dicer KO ES cells. As expected, multiple sequence reads mapped to the EvoFold predicted 5´UTR and coding region hairpins of Dgcr8 mRNA in WT cells **(Figure 4-2A)**. None of the reads mapping to the coding

region hairpin were found in either Dgcr8 or Dicer KO libraries confirming their Dgcr8- and Dicer-dependence (**Figure 4-2B**). Interestingly, two sequence reads mapping to the 5´UTR hairpin were found in the Dicer KO library (**Figure 4-2A**). One of these reads mapped just 5' to the hairpin. Such Dgcr8-dependent, Dicer-independent reads have been previously observed at miRNA loci in Drosophila and mouse small RNA sequencing studies and appear to be a 5' remnant of Drosha cleavage that is further degraded by an unknown 5'-3' exonuclease(Bernstein et al., 2001; Pedersen et al., 2006). The remaining read that was uncovered in the Dicer KO library had a 5' end that did not map to the 5' or 3' end of the hairpin suggesting that it was a degradation product of the full length hairpin. Analysis of all EvoFold-predicted hairpins in the Dgcr8 KO-upregulated set of coding mRNAs failed to identify a single other hairpin with corresponding small RNAs.

Small RNA-sequencing based analysis of potential cleavage sites in mRNAs upregulated in Dgcr8 -/- ES cells

Analysis of only EvoFold predicted loci could miss poorly conserved hairpins. Therefore, to extend the analysis, sequencing reads from WT ES cells were mapped to all exons of the transcripts whose expression was altered in Dgcr8 KO versus WT and Dicer KO cells. 12 out of the 778 Dgcr8 KO- upregulated transcripts and 15 out of the 844 downregulated transcripts had

at least 5 small RNA reads that overlapped with their exons (**Figure 4-1**). As microprocessor activity is predicted to destabilize the mRNAs, we looked more closely at the 8 transcripts upregulated in Dgcr8 KO cells. The small RNAs that mapped within exonic regions of these annotated transcripts fell into two groups based on their distribution. Four had multiple small RNAs with a similar 5' or 3' end, consistent with specific endonuclease cleavage **(Figure 4-3A and Figure 4-3C-E)**. The remaining five had small RNAs mapping across the exon without shared 5' or 3' ends consistent with degradation **(Figure 4-3B and Figure 4-3F-H)**. Most importantly, all of these small RNAs were present in the Dgcr8 null background **(Figure 4-3A-I)**. Hence, they are not products of Microprocessor cleavage.

A small number of annotated miRNAs map to exonic regions of coding genes (~37 in mice)(Fejes-Toth et al., 2009). Therefore, analogous to Dgcr8, the host genes for these miRNAs might be expected to be downregulated by microprocessor-induced cleavage. Upon examination of the exonic miRNAs, we found only 10 to fully lie within annotated exons **(Table 4-1)**. We were able to find small RNA reads to three of these exonic miRNAs (mmu-miR-21, mmu-miR-671, mmu-miR-147)**.** However, the mRNA levels of the host genes of these three miRNAs were not altered in the Dgcr8 and Dicer KO ES cells. Therefore, production of these miRNAs does not appear to influence the overall levels of the annotated host mRNAs. Together, these detailed

analyses of both mRNA expression profiling and small RNA sequencing data from ES cells failed to uncover any genes other than Dgcr8 that are directly destabilized by the microprocessor.

Analysis of 200nt sequencing data from HeLa and HepG2 cells

It is possible that 18-32 nucleotide small RNA sequencing missed microprocessor-cleaved exonic hairpins that are sequestered and/or are not processed by Dicer. Microprocessor miRNAs are typically 60-75 nucleotides in length. Therefore, to directly identify these hairpins, we analyzed ultra high-throughput sequencing data sequence sets produced from small RNAs less than 200 nucleotides in length from Hela and HepG2 cells(Fejes-Toth et al., 2009). The forty small RNA libraries generated in the study were derived from whole cell, cytoplasmic and nuclear fractions, as well as from cells following enzymatic treatments that enrich for either mono-, di-, tri-phosphate modified or 5' capped RNAs. Sequence reads from all forty libraries were mapped to exonic EvoFold hairpins. The largest number of hits, 184, mapped to the Dgcr8 5´UTR hairpin and 4 mapped to the coding region hairpin (**Figure 4-4A**). Most of these reads had a uniform 5' end consistent with microprocessor cleavage. There was an additional read just 5' to the hairpin, a likely remnant of the microprocessor cleavage, similar to that seen in the ES cell small RNA libraries (**Figure 4-2A**). A large number (166 out of 184) of the 5' UTR reads were derived from nuclear libraries, consistent with previous work showing that the cleaved

5'UTR hairpin is confined to the nuclear fraction(Han et al., 2009). When mapping reads from the libraries to known pre-miRNA hairpins, many reads extend beyond the known mature miRNA into the loop region of the hairpin **(Figure 4-4B)**, thereby confirming that these libraries contain hairpin products of the Microprocessor cleavage. These findings show that the analysis of the Hela and HepG2 small RNA data sets should identify other hairpins that are cleaved by the microprocessor even if they are not further processed.

In order to identify any other potential mRNA substrates, we next mapped the HeLa and HepG2 datasets to all UTR and CDS EvoFold loci. There were 106 additional EvoFold hairpins containing overlapping small RNAs, although the number of reads mapping to any one of these hairpins was much less than seen for Dgcr8 **(Table 4-2)**. Only four of these hairpins had at least 5 sequence reads. Furthermore, none of the small RNA reads in these hairpins mapped in a manner consistent with microprocessor cleavage. That is, they had heterogeneous 5' and 3'ends and/or the ends went beyond the extremes of the hairpins (**Figure 4-5,A-D**). For example, the second highest-ranking hairpin, which mapped to the gene RPS3, had 14 reads. However, unlike the reads mapping to the Dgcr8 hairpins, they did not have a defined 5' end, but instead mapped across the locus, more consistent with RNA degradation than microprocessor cleavage.

Therefore, analysis of small RNAs less than 200 nucleotides failed to identify any Evofold loci within exons other than Dgcr8 that are cleaved in a microprocessor-like fashion.

Again, limiting the analysis to Evofold predicted hairpins would miss non-conserved hairpins. Therefore, we mapped small RNAs from HeLa and HepG2 libraries to exons of transcripts upregulated over 2-fold with siRNA-mediated knockdown of both Drosha and Dgcr8 relative to siGFP. Expression information was extracted from recently published microarray data in HeLa cells (see Methods) (Han et al., 2009). As expected, Dgcr8, which was upregulated in the Drosha knockdown sample, had 188 small RNAs mapping to the first exon. Upon examining protein-coding genes upregulated in both Drosha and Dgcr8 knockdown samples, 31 transcripts had >= 10 small RNAs mapping to at least one exon **(**45 exons total**, Table 4-3)**. Notably, 15 out of the 31 were genes that encode ribosomal protein subunits, which are highly abundant in cells. Out of the 31, 11 transcripts had small RNA reads distributed over the exon, as would be expected for degradation products. The remaining 20 transcripts had small RNA reads clustering in small window(s) within exons. However, further examination of the regions in these 20 transcripts using RNAfold did not reveal the presence of any good hairpin structures, in contrast to the Dgcr8 small RNA mapping-regions.

Discussion

Our findings show a focused role for the Microprocessor in destabilizing coding mRNAs by the direct cleavage and destabilization of spliced transcripts. Indeed, we only find evidence for the cleavage and destabilizaton of Dgcr8. Similar to previous reports, our mRNA profiling analysis of wild-type, Dgcr8, and Dicer deficient cells identifies many mRNAs that are specifically upregulated with the loss of Dgcr8(Kadener et al., 2009). The presence of such mRNAs would be consistent with Microprocessor regulation of coding mRNAs through direct cleavage and may be a broadly used mechanism of mRNA regulation. However, closer analysis of these mRNAs and evaluation of ultra-high throughput deep sequencing for small RNAs either in the 18–32 or <200 nucleotide range failed to identify any additional mRNAs that are regulated by such a mechanism. In this study, we examined data from cell lines representing three different tissues: ES (inner cell mass of the blastocyst), Hela (kidney), and HepG2 (liver). In all deep sequencing datasets examined, we find numerous reads to the Dgcr8 hairpins but are unable to find a single additional similar candidate, suggesting that any additional examples would be extremely rare. We cannot exclude the possibility that rare examples of Microprocessor-mediated destabilization of mRNAs may be found in specific cellular contexts or at levels too low to be identified using current deep sequencing technology.

Indeed, since our publication, there have been a number of follow up papers addressing the question of potential microprocessor cleavage of mRNAs. Another paper has suggested that one mRNA Neurogenin2 may undergo cleavage by the microprocessor during neuronal differentiation as a means of downregulating its expression (Knuckles et al., 2012). Chong et al have also suggested that in the DN3 subset of T cells but not in CD4+ or Tregs, there may be some examples of mRNA cleavage by the microprocessor(Chong et al., 2010). The Hannon lab recently performed deep sequencing to identify cleavage sites in mRNAs in wild-type and Drosha KO ES cells and identified approximately 70 potential mRNAs that may be subjected to Drosha-mediated cleavage (Karginov et al., 2010). However, these mRNAs are not differentially up regulated in Dgcr8 versus Dicer knockout ES cells in microarray profiling data (**Figure 4-6**). This may suggest that Drosha has Dgcr8-independent functions. Further studies are necessary to confirm and characterize the loci identified as potential targets of the microprocessor in DN3 and mES cells.

One locus shown by Karginov et al to have increased expression in Drosha KO cells with a clear mRNA cleavage signature is Rcan3, a previously annotated miRNA that overlaps an exon-intron junction of Rcan3. Interestingly, in our studies, Rcan3 is upregulated in both Dicer and Dgcr8 KO cells, suggesting that the cleavage of the hairpin may not significantly affect mRNA levels.

It has been suggested recently that at least in cases of miRNAs that overlap exon-intron junctions, alternative splicing mechanisms may allow the mRNA and miRNA to be co-expressed(Melamed et al., 2013).

In other cases of exonic miRNAs, the absence of Dgcr8-dependent upregulation of the host transcripts is worth noting (**Table 4-1)**. A number of these annotated exonic miRNAs were not present in our small RNA libraries even though the host gene is clearly expressed. This finding may be the result of mis-annotation of these sequences as miRNAs or that processing of the hairpins is somehow suppressed in ES cells. One example of an annotated exonic miRNA that is present in large numbers in ES cells is miR-21. Its host gene, Tmem49, is not upregulated in Dgcr8 or Dicer knockout ES cells. Possible explanations include: 1) there are alternative transcripts responsible for miR-21 production either from an alternative promoter or an alternative splicing event or 2) only a small subset of the Tmem49 transcripts is processed by the Microprocessor to produce the pre-miR-21 hairpin.

Taken together, this body of work suggests that while limited exceptions may exist, microprocessor activity is largely specific to canonical microRNA biogenesis and autoregulation.

The Hornstein lab showed recently through computational modeling and experiments that Dgcr8 levels are finely tuned to levels of primary miRNAs (Barad et al., 2012).

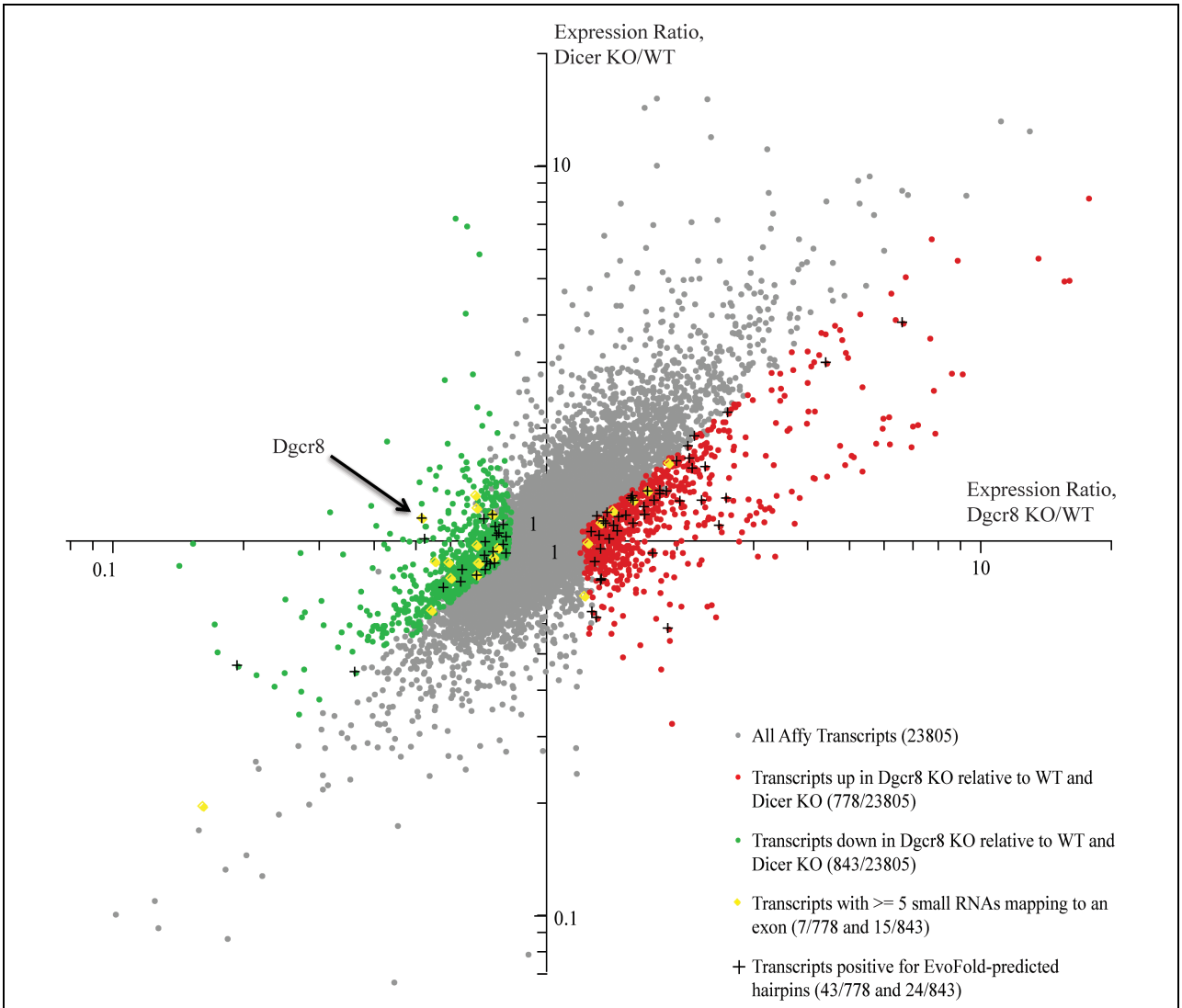
These studies however raise an unanswered question - that is, how do hairpins in the transcriptome escape microprocessor cleavage? Secondary structure, which includes hairpins, is an inherent component of RNA that often has regulatory implications(Pedersen et al., 2006). One answer to how the microprocessor achieves specificity may be that it is specifically recruited to its target regions. In fact, it has been suggested that at least for intronic miRNAs, the microprocessor may be recruited by the spliceosome (Dye et al., 2006). However, HITS-CLIP data shows that the microprocessor associates with a large number of regions, suggesting that it may be "scanning" the genome for potential targets (Macias et al., 2012). Recent studies have uncovered additional sequence determinants downstream of the hairpin site that promote microprocessor cleavage (Auyeung et al., 2013). Further studies are needed to examine how additional alternate functions of Dgcr8 suggested by some may impact the transcriptome.

A very specific role for the Microprocessor in destabilizing Dgcr8 and hence providing a negative feedback on Microprocessor levels itself suggests that homeostatic control of microRNA processing is central to normal cellular physiology. This is consistent with recent

findings showing that much regulation is occurring at the level of Microprocessor activity. For example hnRNAP, Lin28, and KSRP have been suggested to regulate Microprocessor activity on specific miRNAs (Michlewski et al., 2008; Newman et al., 2008; Viswanathan et al., 2008; Trabucchi et al., 2009). Furthermore, SMAD signaling alters the processing of pri- to pre-miR-21 (Davis et al., 2008). A carefully controlled balance between the levels of the Microprocessor and these regulators are likely important for proper physiologic function.

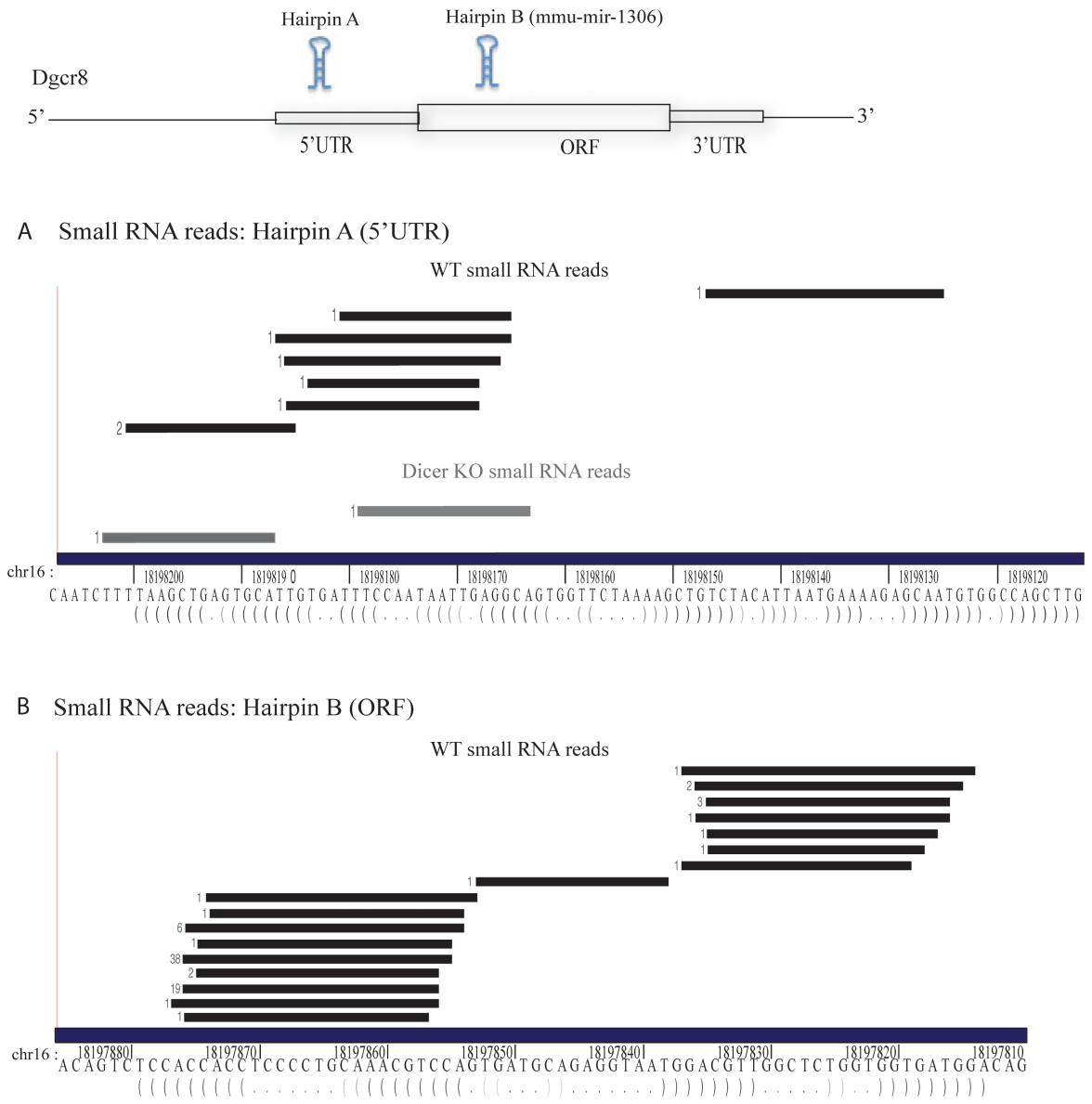
Dgcr8 levels are differentially regulated during development and in cancers. Interestingly, some cancers have decreased, while other cancers have increased levels of Dgcr8 (Ambs et al., 2008; Merritt et al., 2008). Similarly, Dicer levels and/or activity appear to be altered in cancers (Chiosea et al., 2006; Ambs et al., 2008; Merritt et al., 2008; Melo et al., 2009). A direct role for changes in processing activity in cancer is supported by a mouse model of lung cancer where heterozygous loss of Dicer promotes tumor progression (Kumar et al., 2007). Together, these findings suggest that the biogenesis of miRNAs is not simply a passive process, but rather a tightly controlled one. Therefore, it will be important to determine in greater detail how the level and the activity of the biogenesis machinery influence the molecular constitution of cells.

Figure 4-1, Microarray analysis of Dgcr8/Dicer KO ESCs



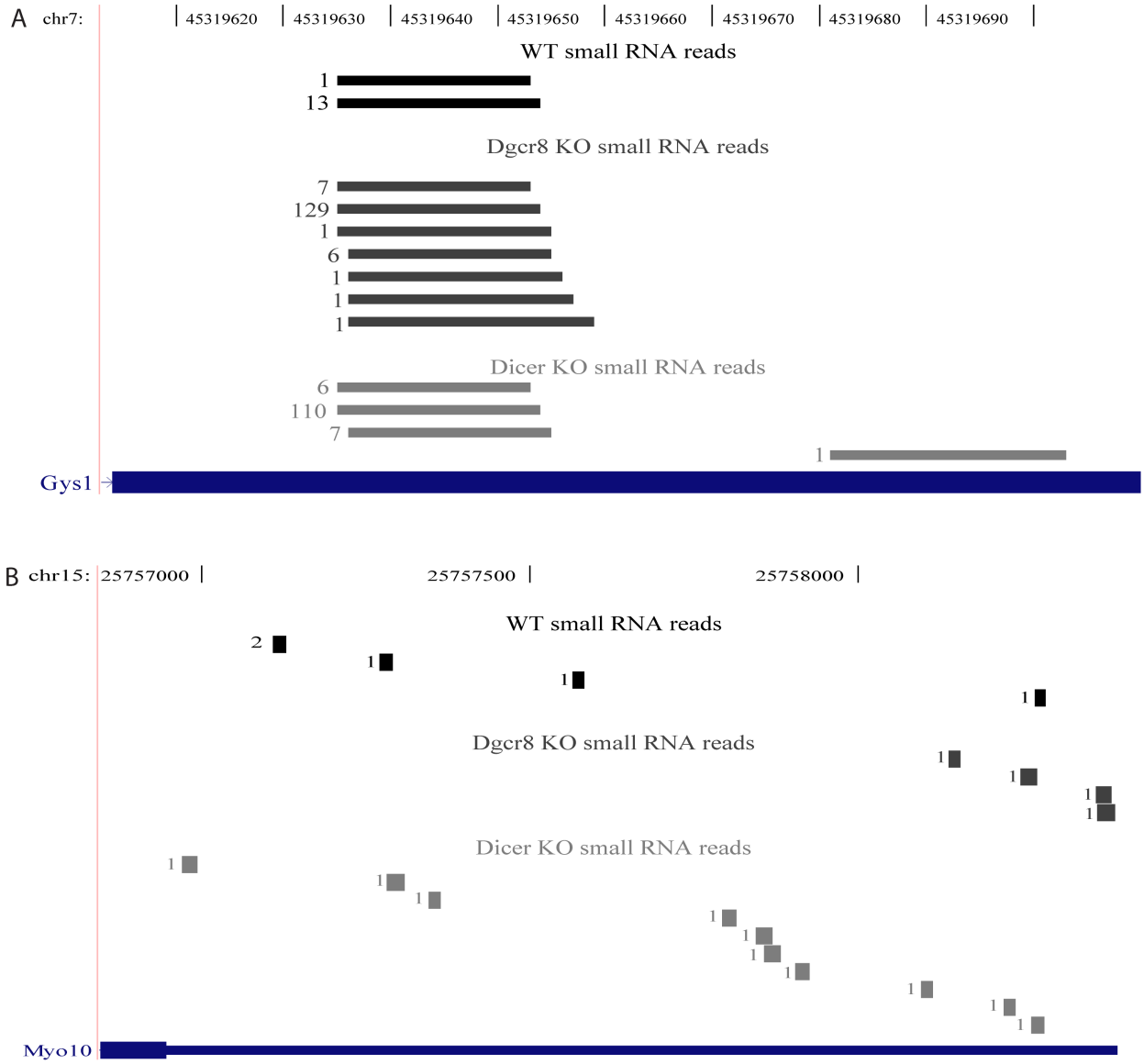
Transcripts differentially regulated in Dgcr8 KO relative to WT and Dicer KO ES cells. The sets of genes differentially up- and down- regulated in Dgcr8 KO relative to Dicer KO and WT ES cells were determined based on a cutoff of FDR <5%. Data are represented as a mean of 3 biological replicates of WT, Dgcr8 KO and Dicer KO arrays. Transcripts positive for EvoFold hairpin predictions and transcripts with 5 or more small RNAs mapping to their exons are shown (see legend). Arrow points to Dgcr8 expression levels, which, as expected, is down in Dgcr8 KO (exon 3 deletion results in premature termination codon and, hence, non-sense mediated RNA decay [Rebbapragada I, Lykke-Andersen J (2009) Execution of nonsense].

Figure 4-2, Read distribution across Dgcr8 exonic hairpins



The distribution of reads across hairpins in the first exon of Dgcr8 in mES cells.
The location of each small RNA read relative to the exon is represented by a grey bar and was generated using the custom tracks feature on the UCSC genome browser. For each RNA species, the number of reads that were obtained with that sequence is indicated at the left. The predicted secondary structure is represented below the genomic sequence. Genomic coordinates are based on UCSC Known Genes annotations (mm8) (A) 5′UTR hairpin (B) CDS hairpin. Small RNA reads in WT cells are represented by black bars and small RNA reads in Dicer KO cells are represented by a grey bar.

Figure 4-3, A-B, Representative examples of read distribution in exons with >5 reads in WT cells

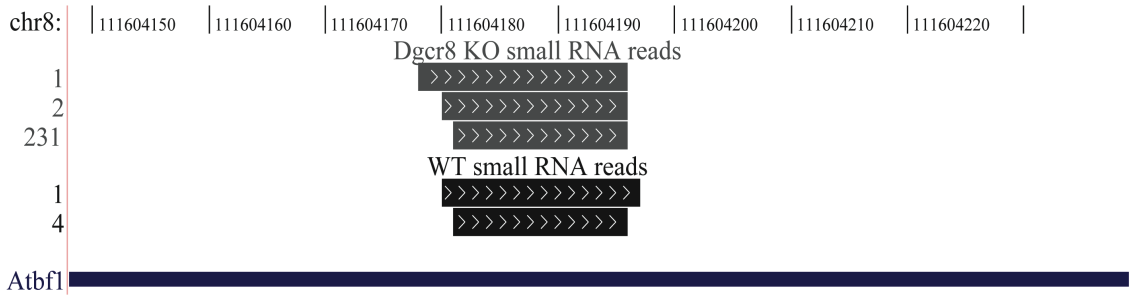


The location of unique small RNA reads from WT (black bars), Dgcr8 KO (dark grey bars) and Dicer KO (grey bars) are represented. For each RNA species, the number of reads that were obtained with that sequence is indicated at the left. Genomic coordinates are based on UCSC Known Genes annotations (mm8) (A) Example showing reads that are localized to a small window consistent with Microprocessor cleavage but are not Dgcr8-dependent (B) Reads are distributed across the range of the exon and most likely represent degradation products.

Figure 4-3, C, Read distribution across Hn1 exon

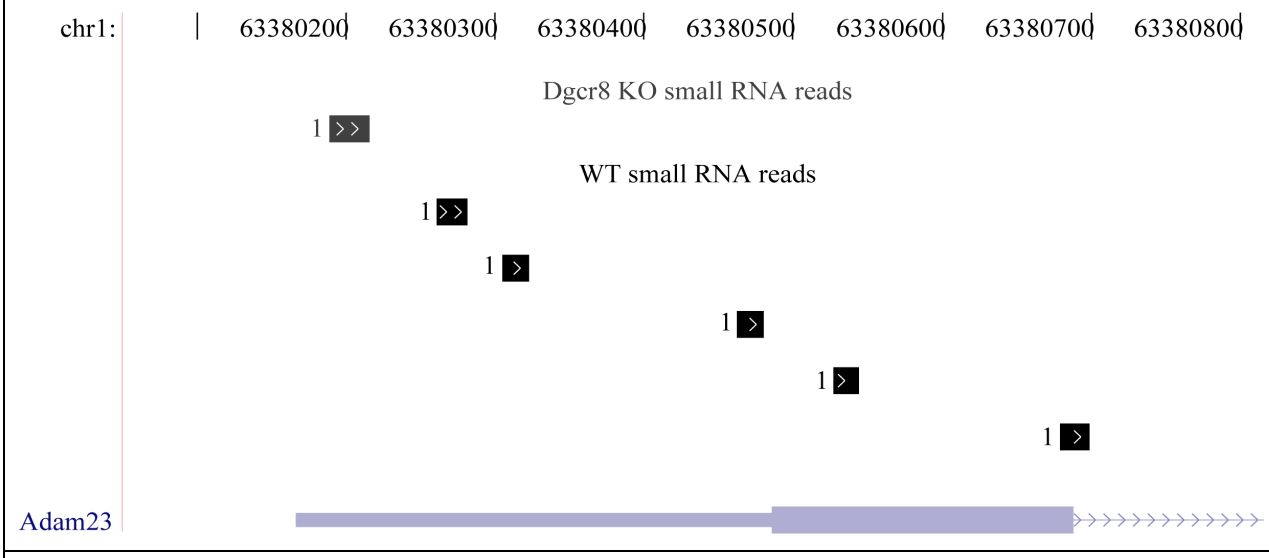
Distribution of small RNA reads from Dgcr8 KO and WT libraries across the Hn1 exon. Small RNA locations are presented as in Figure 4-2 (WT reads = black bars, Dgcr8 KO reads = grey bars). Genomic coordinates are based on UCSC Known Genes annotations (mm8).

Figure 4-3, D, Read distribution across Atbf1 exon



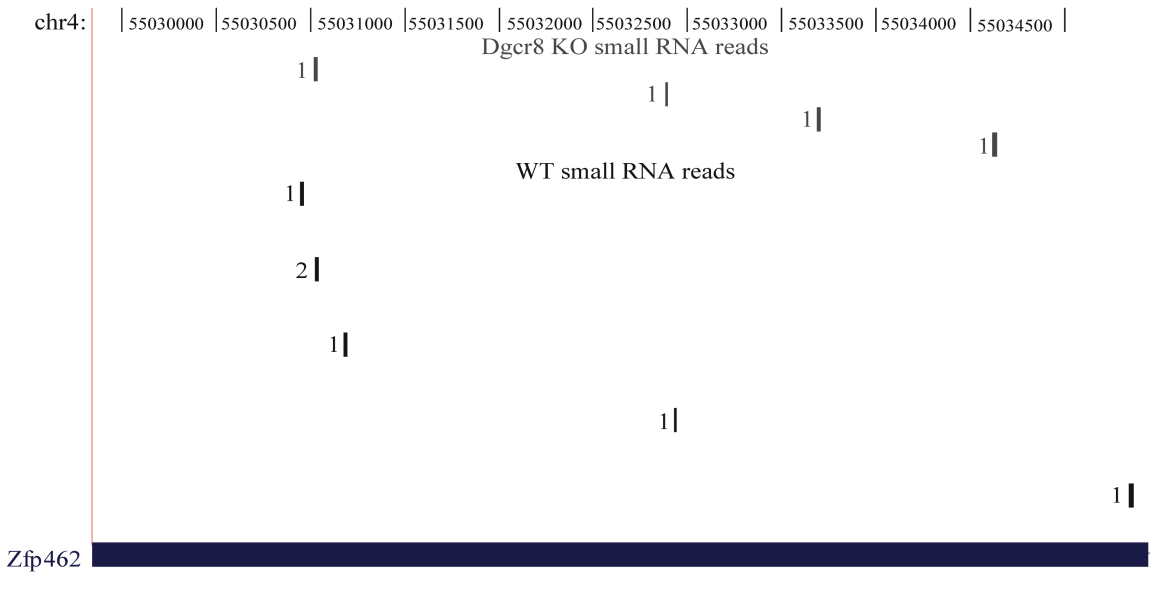
Distribution of small RNA reads from Dgcr8 KO and WT libraries across the Atbf1 exon.

Figure 4-3, E, Read distribution across Adam23 exon



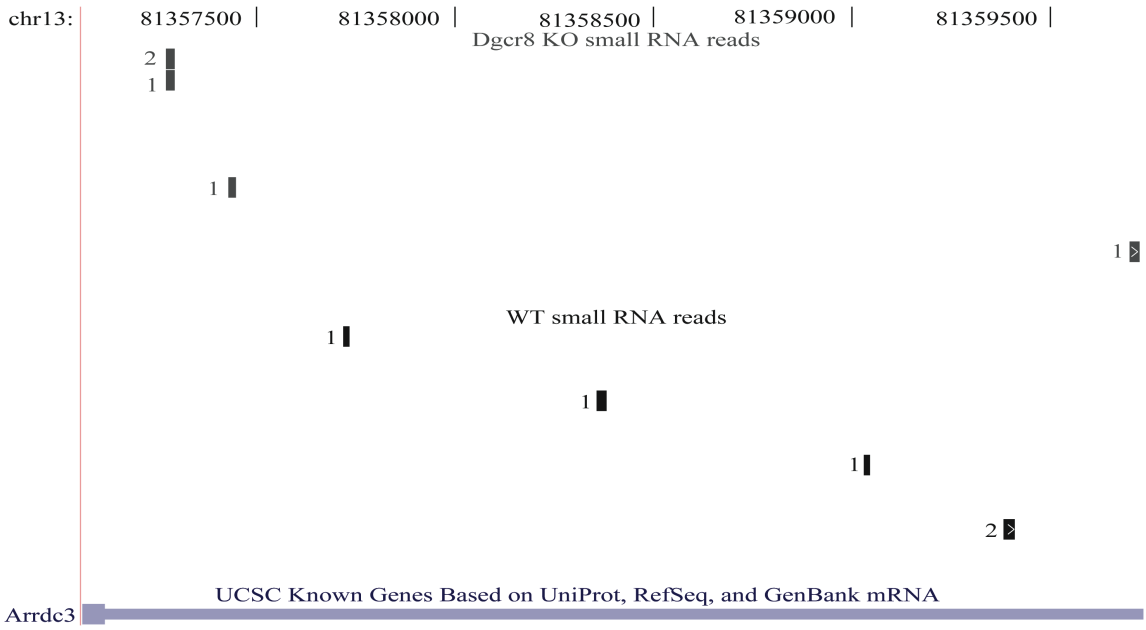
Distribution of small RNA reads from Dgcr8 KO and WT libraries across the Adam23 exon.

Figure 4-3, F, Read distribution across Zfp462 exon



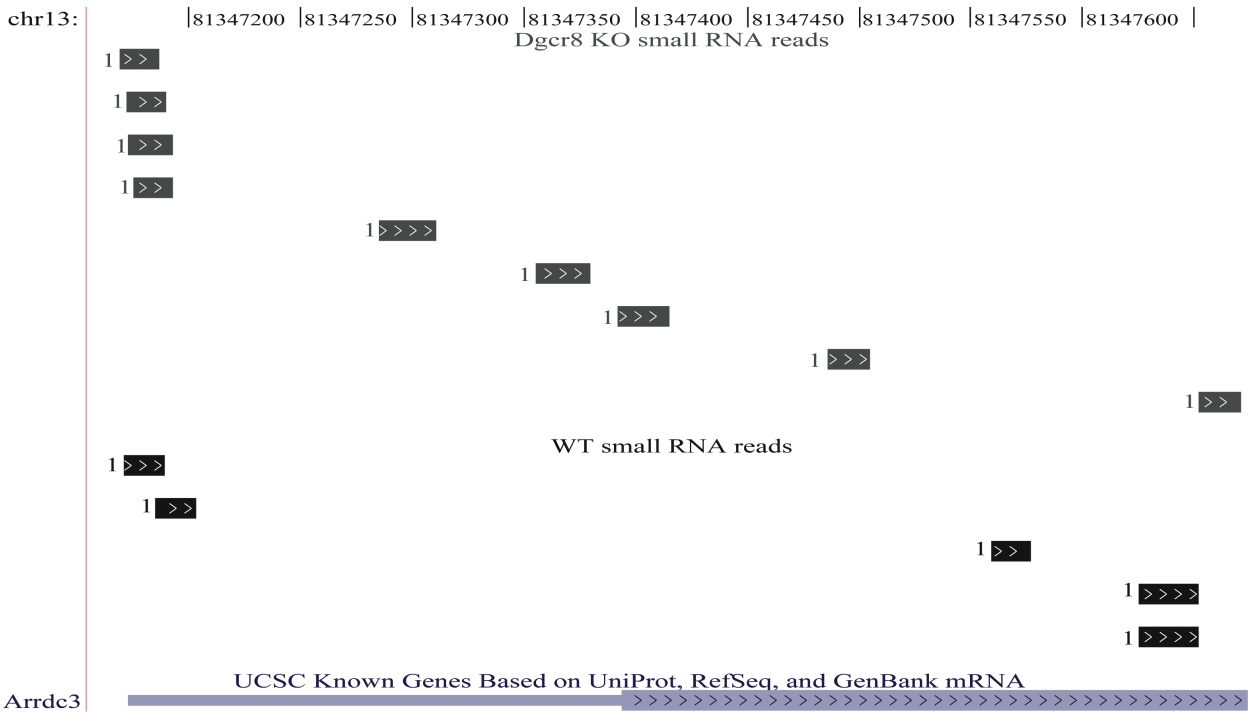
Distribution of small RNA reads from Dgcr8 KO and WT libraries across the Zfp462 exon.

Figure 4-3, G, Read distribution across Arrdc3 exon



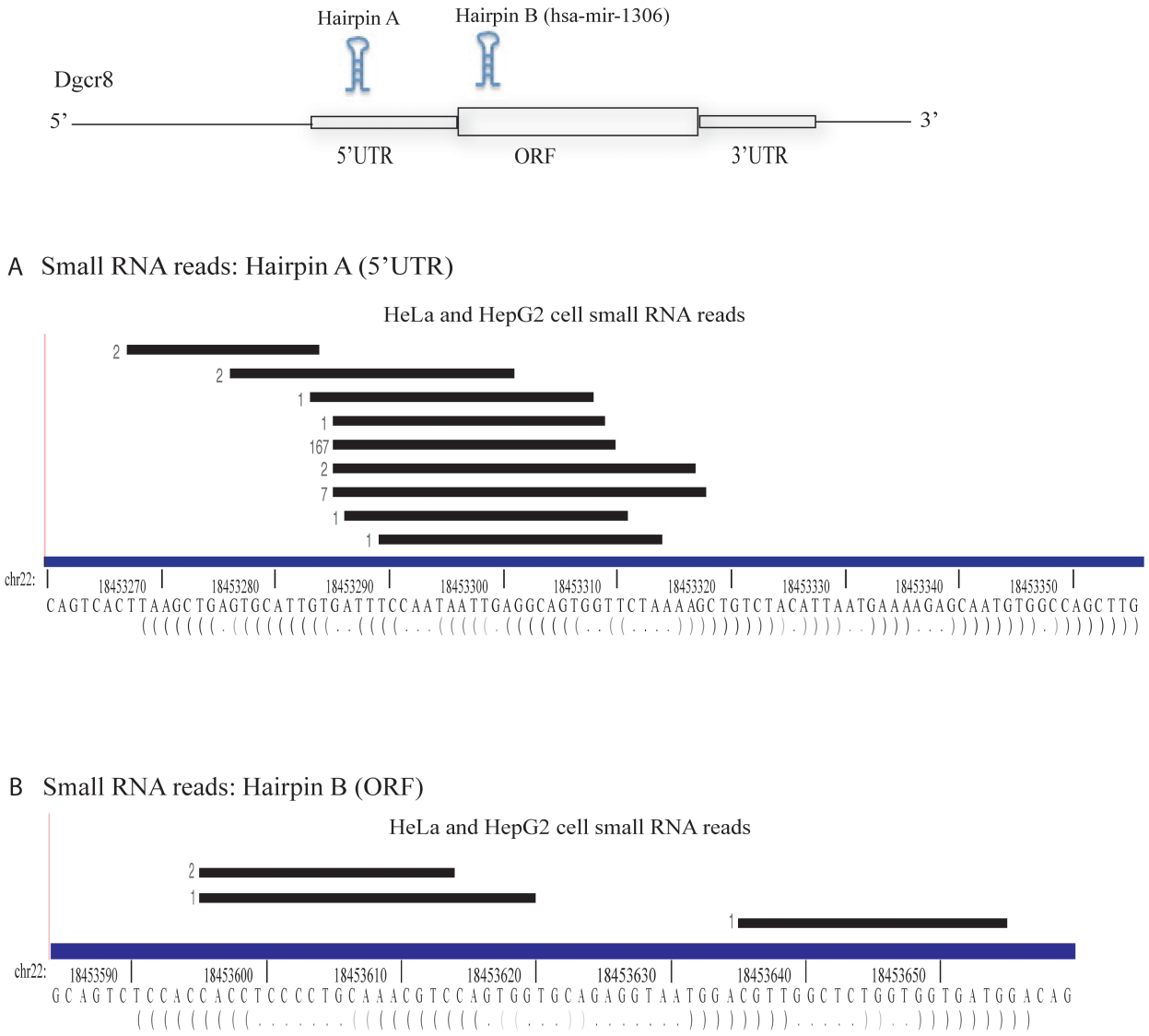
Distribution of small RNA reads from Dgcr8 KO and WT libraries across the Arrdc3 exon.

Figure 4-3, H, Read distribution across another Arrdc3 exon



Distribution of small RNA reads from Dgcr8 KO and WT libraries across the Arrdc3 exon.

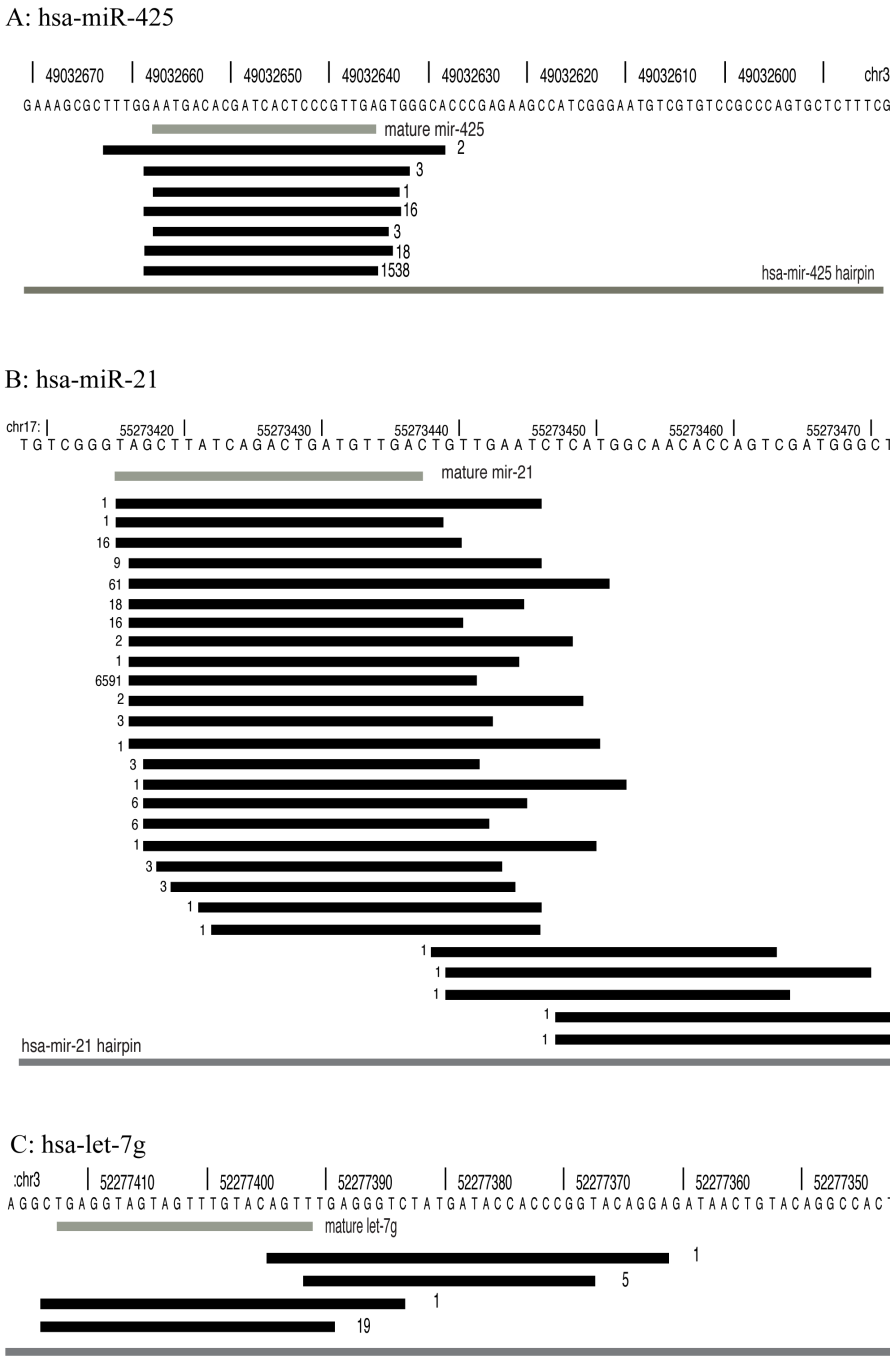
Figure 4-4, A-B, Read distribution across Dgcr8 exon in <200nt small RNA sequencing data from HeLa and HepG2 cells.



Read distribution across hairpins in the first exon of Dgcr8 in <200nt small RNA sequencing data from HeLa and HepG2 cells.

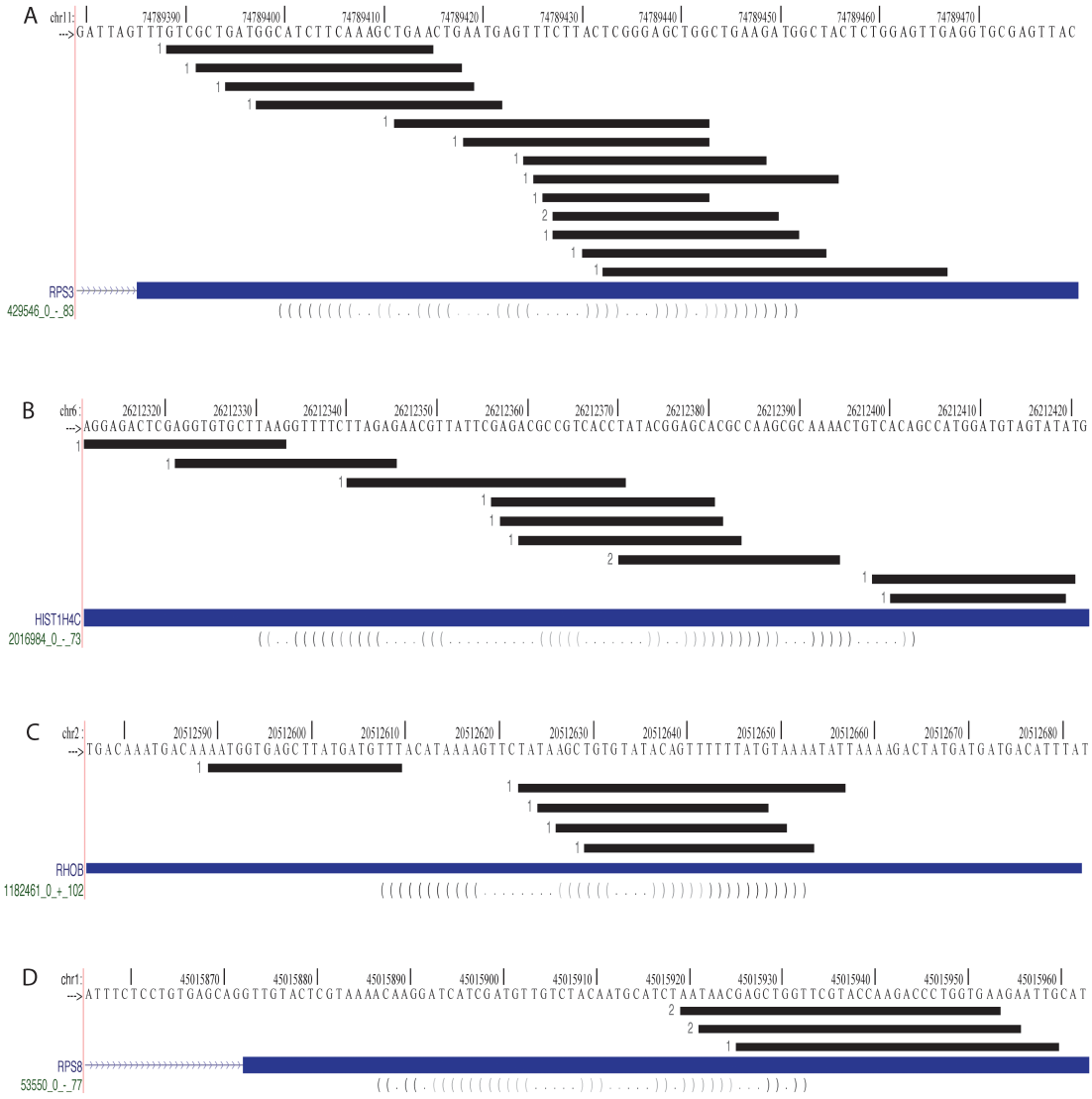
Small RNA locations are presented as in Figure 2. Genomic coordinates are based on UCSC Known Genes annotations (hg18) (A) 5′ UTR hairpin (B) CDS hairpin.

Figure 4-4, C, Read distribution across pre-miRNA hairpins



Distribution of 25 to 36 nt sequence reads from HeLa and HepG2 cell <200nt libraries across pre-miRNA hairpins. Locations are presented as in Figure 4-2. Genomic coordinates are based on mirbase annotations.

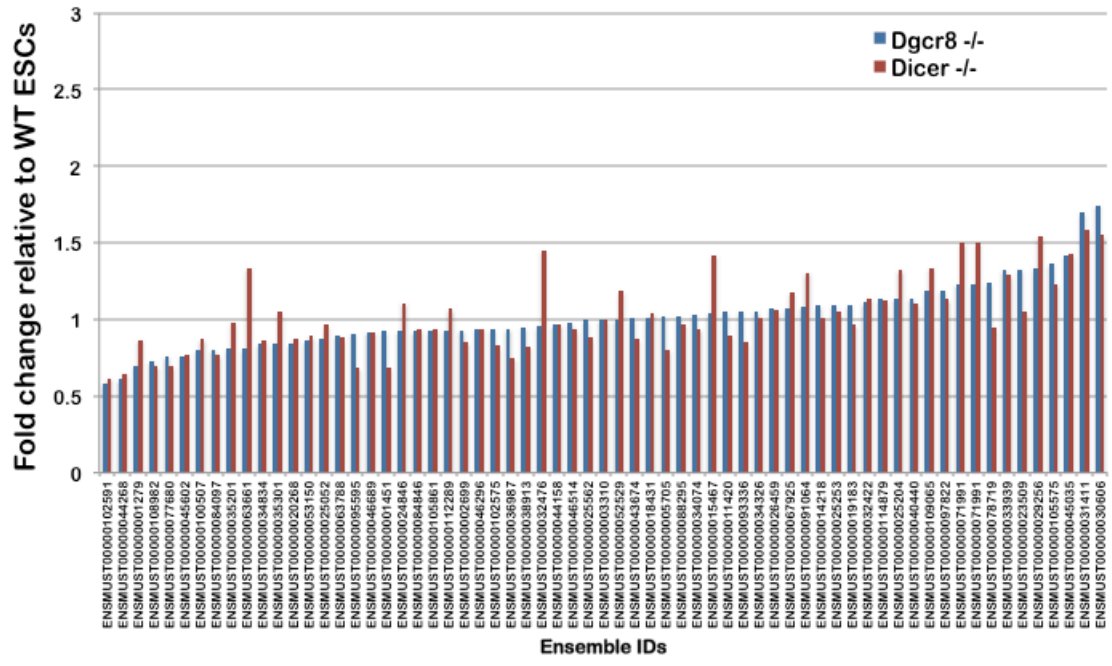
Figure 4-5, Read distribution across hairpins with >5 reads



Read distribution across hairpins positive for >5 small RNA reads in HeLa cell <200 nt small RNA sequencing data.

Small RNA locations are presented as in Figure 2. Genomic coordinates are based on UCSC Known Genes annotations (hg18) Reads across hairpins in (A) RPS3 (B) HIST1H4C (C) RHOB and (D) RPS8.

Figure 4-6, Expression levels of putative Drosha mRNA targets in Dgcr8 KO ESCs



Fold change of mRNAs identified as putative Drosha targets by Karginov et al in Dgcr8 KO and Dicer KO ESCs relative to WT.

Table 4-1, Exonic miRNAs and host gene expression

MicroRNA ID	# of reads	Host Gene	Location in host genes	Host Gene Expression	Dgcr8 KO/WT	Dicer KO/WT
mmu-mir-21	60267	Tmem49	3'UTR	612.2	1.13	1.43
mmu-mir-671	120	2010209O12Rik*	CDS	122.8	1.02	0.96
mmu-mir-147	58	AA467197*	3'UTR	53.6	1.68	14.3
mmu-mir-546	-	Ctdsp2	3'UTR (in one transcript variant)	176	1.28	1.39
mmu-mir-718	-	Irak1	5'UTR	299.9	1.03	1.42
mmu-mir-678	-	Prmt2	3'UTR	203.3	0.75	0.98
mmu-mir-686	-	Psmb5	CDS	1028.6	1.11	0.99
mmu-mir-705	-	Rab11fip5	3'UTR	97.7	2.37	2.86
mmu-mir-632	-	Zfp207	5'UTR	1334.9	0.92	0.89
mmu-mir-124-1	-	AK044442	ORF-3'UTR	no signal on array		
mmu-mir-1306	81	Dgcr8	CDS	889	0.5	1
*low expression value						

Table 4-2, Hairpin reads in HeLa and HepG2 <200nt sequencing

Supplementary Table 2: EvoFold-predicted hairpins with small RNA reads in HeLa and HepG2 cell <200nt small RNA sequencing

Chr#	Hairpin Start	Hairpin Stop	Hairpin ID:Location	Gene Name	Known Genes ID	No. of Small RNA reads
chr22	18453268	18453356	1479323_0:5UTR	DGCR8	NM_022720	184
chr11	74789399	74789452	429546_0:CDS	RPS3	NM_001005	14
chr6	26212330	26212403	2016984_0:CDS	HIST1H4C	NM_003542	10
chr1	45015886	45015933	53550_0:CDS	RPS8	NM_001012	5
chr2	20512607	20512653	1182461_0:3UTR	RHOB	NM_004040	5
chr17	53436424	53436497	1019832.3_0:3UTR	SFRS1	NM_006924	4
chr22	18453590	18453657	1479328_0:CDS	DGCR8	NM_022720	4
chr20	33057917	33057982	1421035_0:3UTR	TRPC4AP	NM_015638	3
chr3	137350991	137351043	1643066.1_0:3UTR	MSL2	NM_018133	3
chr3	102887389	102887458	1607591_0:CDS	RPL24	NM_000986	3
chr5	167877551	167877629	1979614_0:CDS	RARS	NM_002887	3
chr5	148866812	148866872	1958110_0:CDS	CSNK1A1	NM_001892	3
chr6	53241416	53241475	2046168_1:3UTR	ELOVL5	NM_021814	3
chr7	26198400	26198556	2161326.13_0:3UTR	HNRNPA2B1	NM_031243	3
chr10	124913985	124914040	343631_2:3UTR	BUB3	NM_004725	2
chr12	103037222	103037275	584906_1:3UTR	NFYB	NM_006166	2
chr13	106007999	106008067	676980.4_0:3UTR	ARGLU1	FLJ99982	2
chr16	65790233	65790286	922007_0:3UTR	E2F4	NM_001950	2
chr2	8741816	8741882	1172612_0:3UTR	ID2	NM_002166	2
chr22	38040663	38040702	1500570_0:CDS	RPL3	NM_001033853	2
chr3	187117940	187118001	1685896_1:3UTR	SFRS10	NM_004593	2
chr4	78195399	78195452	1750622_0:CDS	CCNI	NM_006835	2
chr4	78136635	78136705	1750519_0:CDS	SEPT11	NM_018243	2
chr6	86378408	86378463	2067792.0_1:3UTR	SYNCRIP		2
chr6	139735162	139735224	2113402.0_0:3UTR	CITED2	NM_006079	2
chr6	114289612	114289669	2092155.7_3:3UTR	MARCKS	NM_002356	2
chr8	117944557	117944616	2361273_0:CDS	RAD21	NM_006265	2
chr9	99457812	99457869	2449075_0:CDS	NCBP1	NM_002486	2
chr9	74159201	74159294	2430400_0:3UTR	ZFAND5	NM_001102421	2
chr1	243084066	243084124	229491.12_0:3UTR	HNRNPU	NM_004501	1
chr1	232809350	232809395	221878_0:3UTR	IRF2BP2	NM_001077397	1
chr1	51510204	51510258	60867_1:3UTR	RNF11	NM_014372	1
chr10	32597510	32597592	257724.7_0:3UTR	EPC1		1
chr10	11247494	11247559	240330_0:CDS	CUGBP2	NM_001025077	1
chr10	98302726	98302798	310472_0:CDS	TM9SF3	NM_020123	1
chr10	22072232	22072308	249307_0:3UTR	MLLT10	NM_001009569	1
chr11	47246698	47246750	402198_0:CDS	NR1H3	NM_001130102	1
chr11	129611894	129611953	484461_0:CDS	ZBTB44	NM_014155	1
chr11	64419073	64419190	416135_0:CDS	ATG2A	NM_015104	1
chr11	72225593	72225668	426170_0:3UTR	FCHSD2	NM_014824	1
chr12	3019582	3019671	493566_0:CDS	TEAD4	NM_003213	1
chr12	120754863	120754912	602307_0:3UTR	SETD1B	NM_015048	1
chr12	69022305	69022354	555352_0:CDS	CNOT2	NM_014515	1
chr12	55319296	55319349	542360_0:CDS	ATP5B	NM_001686	1
chr12	52680150	52680219	538040.11_0:5UTR	HOXC9	NM_006897	1
chr13	96843966	96844010	669051_0:3UTR	MBNL2	NM_207304	1
chr14	68324131	68324223	732514.0_0:CDS	ZFP36L1	NM_004926	1
chr14	50171787	50171844	711037_1:CDS	SAV1	NM_021818	1
chr14	80716343	80716432	748739_0:CDS	GTF2A1	NM_015859	1
chr14	30717104	30717153	694359_0:CDS	HECTD1	NM_015382	1
chr14	101463726	101463769	767519_0:3UTR	PPP2R5C	NM_178586	1
chr15	39058437	39058494	791188_0:3UTR	INO80	NM_017553	1
chr15	70663268	70663391	829045_0:3UTR	ARIH1	NM_005744	1
chr17	50754616	50754697	1015958_1:3UTR	HLF	NM_002126	1
chr17	27345057	27345102	980920_0:CDS	SUZ12	NM_015355	1
chr17	4121473	4121576	954158_0:3UTR	UBE2G1	NM_003342	1
chr17	1194583	1194628	949763.0_0:3UTR	YWHAE	NM_006761	1
chr17	55096942	55097005	1022181_0:CDS	CLTC	NM_004859	1
chr17	1528575	1528632	950417_0:CDS	PRPF8	NM_006445	1
chr17	53437376	53437428	1019834.0_0:3UTR	SFRS1	NM_006924	1
chr18	9765279	9765346	1057523_0:CDS	RAB31	NM_006868	1
chr18	9944459	9944501	1057673.2_0:3UTR	VAPA	NM_194434	1
chr19	39410723	39410799	1141337_0:3UTR	LSM14A	NM_015578	1
chr19	40248000	40248070	1142068_0:CDS	HPN	NM_002151	1
chr19	12640343	12640394	1123545_0:CDS	C19orf56	NM_016145	1
chr19	2222435	2222503	1112386_0:CDS	OAZ1	NM_004152	1
chr19	4312632	4312746	1114581_0:CDS	SH3GL1	NM_003025	1
chr19	60864244	60864306	1165919.0_0:CDS	U2AF2	NM_001012478	1
chr2	61559205	61559251	1225558.4_0:3UTR	XPO1	NM_003400	1
chr2	61572686	61572759	1225580_1:CDS	XPO1	NM_003400	1
chr2	199844632	199844693	1343743_0:3UTR	SATB2	NM_015265	1
chr2	197974720	197974767	1341653_0:CDS	SF3B1	NM_012433	1
chr2	42651992	42651144	1206602_0:CDS	MTA3	NM_020744	1
chr20	42970489	42970563	1433726_0:3UTR	YWHAB	NM_003404	1
chr20	59984537	59984597	1449212_0:3UTR	TAF4	NM_003185	1
chr20	59983691	59983751	1449210_0:3UTR	TAF4	NM_003185	1
chr3	153664969	153665095	1657971.20_0:3UTR	MBNL1	NM_207295	1
chr3	145193834	145193882	1651150_0:3UTR	C3orf58	NM_173552	1
chr3	157744860	157744917	1660658_0:CDS	SSR3	NM_007107	1
chr3	151822879	151822934	1656317_0:CDS	SELT	NM_016275	1
chr3	57889874	57889924	1568930_0:3UTR	SLMAP	NM_007159	1
chr3	133740467	133740538	1638631_0:3UTR	DNAJC13	NM_015268	1
chr3	153664471	153664529	1657972.14_0:3UTR	MBNL1	NM_207296	1
chr4	1195251	1195312	1697760_0:3UTR	CTBP1	NM_001328	1
chr4	4915471	4915569	1701068_0:CDS	MSX1	NM_002448	1
chr5	14763093	14763137	1845619_0:3UTR	ANKH	NM_054027	1
chr5	137869828	137869886	1942750_2:3UTR	ETF1	NM_004730	1
chr5	71525503	71525547	1885506.0_2:CDS	MAP1B	NM_005909	1
chr5	72182837	72182908	1886106_0:CDS	TNPO1	NM_153188	1
chr5	137869981	137870072	1942750_0:3UTR	ETF1	NM_004730	1
chr5	158528454	158528540	1969423_0:CDS	RNF145	NM_144726	1
chr5	54678611	54678679	1870325_0:CDS	SKIV2L2	NM_015360	1
chr6	149761927	149762012	2122718_0:CDS	MAP3K7IP2	NM_015093	1
chr6	64481769	64481866	2051557_0:3UTR	PHF3	NM_015153	1
chr6	43861520	43861561	2037351_0:3UTR	VEGFA	NM_003376	1
chr6	119270582	119270660	2096110_0:3UTR	ASF1A	NM_014034	1
chr6	43862099	43862185	2037354_0:3UTR	VEGFA	NM_001025368	1
chr6	26324515	26324562	2017108_1:CDS	HIST1H2BG	NM_003518	1
chr7	44891282	44891369	2180237.6_0:CDS	PURB	NM_033224	1
chr7	129262016	129262063	2247448_0:CDS	UBE2H	NM_182697	1
chr7	75794055	75794112	2196908_2:3UTR	YWHAG	NM_012479	1
chr7	151462946	151463021	2269434_1:3UTR	MLL3	NM_170606	1
chr8	117740330	117740395	2361069.10_0:CDS	EIF3H	NM_003756	1
chr9	125559838	125559908	2477802_0:CDS	DENND1A	NM_024820	1
chr9	130387946	130388000	2485026_0:CDS	SPTAN1	NM_003127	1

Note - Hairpin 201129_0:CDS (chr1: 207672116 - 207672203, GeneID:LOC642587) and hairpin 797937_0:3UTR (chr15:43512536-43512620, GeneID: C15orf48) were excluded from further analysis because they overlap unvalidated/spurious ORFs that are likely intergenic primary miRNA transcripts..

Table 4-3, Small RNA-mapping exons of transcripts upregulated in siDrosha and siDgcr8 in HeLa cells

Chr#	Exon Start	Exon Stop	RefSeq ID	Strand	No. of small RNA reads	Pattern of read distribution	Gene name	miRNA-like fold prediction
chr9	19370187	19370235	NM_001010	-	681	Distributed over exon	RPS6	-
chr15	67532212	67532413	NM_001003	+	475	Cluster around start site	RPLP1	-
chr11	74788209	74788269	NM_001005	+	280	Cluster in 5'UTR	RPS3	-
chr12	119123268	119123397	NM_001002	-	83	Cluster at splice site	RPLP0	-
chr4	74520796	74520928	NM_001134	+	78	Cluster at start site/whole exon	AFP	-
chr17	8068863	8072362	NM_025099	-	75	Clusters in 3'UTR	C17orf68	-
chr19	59396537	59396568	NM_001013	+	75	Cluster at start site/whole exon	RPS9	-
chr6	34501786	34501854	NM_001014	-	66	Cluster in 5'UTR	RPS10	-
chr1	93070181	93070262	NM_000969	+	53	Cluster near start/5'UTR	RPL5	-
chr4	74533915	74534048	NM_001134	+	52	Distributed over exon	AFP	-
chr11	799935	800039	NM_001004	+	47	Cluster in 5'UTR	RPLP2	-
chr20	60395515	60395587	NM_001024	+	47	Cluster at end of exon	RPS21	-
chr12	119120739	119120925	NM_001002	-	42	Cluster at splice site	RPLP0	-
chr21	34209901	34210028	NM_001697	-	41	Cluster around 5'UTR/ORF start	ATP5O	-
chr4	155703581	155703734	NM_005141	+	39	Clustered at start site	FGB	-
chr5	81609894	81609991	NM_001025	-	36	Cluster around ORF start/splice site	RPS23	-
chr4	74532042	74532257	NM_001134	+	32	Distributed over exon	AFP	-
chr4	74529573	74529703	NM_001134	+	31	Distributed over exon	AFP	-
chr4	74525182	74525394	NM_001134	+	27	Distributed over exon	AFP	-
chr4	74527927	74528025	NM_001134	+	25	Distributed over exon	AFP	-
chr11	74789385	74789516	NM_001005	+	24	Distributed over exon	RPS3	-
chr9	110817235	110822606	NM_001099734	-	21	Distributed over 3'UTR	C9orf5	-
chr8	62575669	62578622	NM_004318	-	20	Distributed over 3'UTR of one transcript variant	ASPH	-
chr2	10186811	10188997	NM_001034	+	19	Distributed over 3'UTR	RRM2	-
chr19	54691445	54691540	NM_001015	+	17	Cluster around ORF start/splice site	RPS11	-
chr4	74522754	74522887	NM_001134	+	17	Distributed over exon	AFP	-
chr2	69325860	69329961	NM_032208	+	16	Distributed over 3'UTR	ANTXR1	-
chr3	23933642	23933668	NM_002948	+	16	Distributed over exon	RPL15	-
chr1	6182208	6182266	NM_000983	-	15	Cluster in 5'UTR/ORF start	RPL22	-
chr1	93071689	93071805	NM_000969	+	15	Clusters in exon	RPL5	-
chr19	1389362	1389427	NM_001018	+	14	Cluster around ORF start/5' UTR	RPS15	-
chr20	60396293	60396365	NM_001024	+	14	Cluster around ORF start/splice site	RPS21	-
chr3	23935690	23937336	NM_002948	+	14	Distributed over end of transcript	RPL15	-
chr9	19366253	19366386	NM_001010	-	14	Distributed over exon	RPS6	-
chr11	35207251	35210525	NM_001001392	+	13	Distributed over 3'UTR	CD44	-
chr2	177803278	177804982	NM_006164	-	13	Distributed over end of transcript	NFE2L2	-
chr14	94148466	94148537	NM_001085	+	12	Cluster in 5'UTR	SERPINA3	-
chr2	3600727	3600815	NM_001011	+	12	Cluster in 5'UTR	RPS7	-
chr20	30285936	30290128	NM_015352	+	12	Distributed over 3'UTR	POFUT2	-
chr4	74526876	74527009	NM_001134	+	11	Clustered around exon start	AFP	-
chr7	138896855	138908683	NM_022740	-	11	Distributed over 3'UTR	HIPK2	-
chr8	62699648	62701393	NM_032468	-	11	Distributed over 3'UTR of one transcript variant	ASPH	-
chr2	20311933	20314958	NM_015317	-	10	Distributed over 3'UTR	PUM2	-
chr22	40195074	40195132	NM_001098	+	10	Cluster around start site	ACO2	-
chr4	9632001	9632212	NM_020041	-	10	Cluster around start site	SLC2A9	-

Note - Exon at Chr17:1563746-1564058 was excluded from this analysis because it appears to be an unvalidated/spurious exon annotation of likely an intergenic miRNA host gene (hsa-mir-22).

Conclusions

The work presented in this thesis focuses on two separate questions. In Chapter 2&3, I present work to address the hypothesis that miRNAs target key regulatory genes and pathways to promote terminal astrocyte differentiation. I used a Dgcr8 knockout model to study the global roles of miRNAs and discovered that the loss of miRNAs prevents upregulation of astrocyte markers and activation of JAK-STAT signaling during differentiation. Using a screening approach, I discovered that two miRNA families, let-7 and miR-125, rescue the upregulation of GFAP during differentiation but not activation of the JAK-STAT pathways. However, forced activation of the JAK-STAT pathway is sufficient to rescue the differentiation phenotype, suggesting that let-7 and miR-125 may regulate the pathway at downstream steps. These steps could include regulation of chromatin or binding site accessibility or expression of co-factors.

Microarray and bioinformatics analysis following addback of the two miRNAs in GPCs revealed direct and indirect targets. While individual knockdown of targets is insufficient to recapitulate the effect of let-7 and miR-125, at least one let-7/miR-125 target, Plagl2, inhibits astrocyte differentiation when overexpressed in wild-type cells. Future analysis will likely reveal many more targets that inhibit differentiation when overexpressed. Taken together, these observations

strongly suggest that the coordination of multiple miRNAs effects on multiple targets is necessary to promote the cell fate transition that leads to generation of astrocytes.

Work presented in Chapter 4 addresses the hypothesis that a key component of the miRNA biogenesis pathway, the Dgcr8/Drosha complex, recognizes and cleaves hairpins in many mRNAs in addition to miRNAs. Using microarray and deep sequencing data collected from a number of ES cell lines deficient for Dgcr8 and Dicer, I have shown that Dgcr8 function is largely limited to canonical miRNA biogenesis and autoregulation. This supports the use of Dgcr8 knockout as a good model for studying the effects of global miRNA loss.

Implications for astrocyte development

While the number of studies on astrocyte function and glial specification are growing, there is still a paucity of information regarding the mechanisms that drive glial precursor cells to undergo terminal astrocyte differentiation. It has been suggested previously that miRNAs have evolved to target important gene networks and provide robustness to gene regulation. Thus, discovering miRNA targets may provide insights into mRNAs that regulate a given cell state. In the case of astrocyte differentiation, the targets of expressed miRNAs such as let-7/125 and miR-9 may

provide insights into the regulation of differentiation following glial specification. Furthermore, candidates derived from this study may warrant more rigorous in vivo analysis.

Implications for miRNA biology

This study provides several insights into miRNA biology of somatic stem/progenitor cells. First, this study provides an example of miRNAs priming a progenitor cells for differentiation. In a number of other settings, as reviewed in Chapter 1, miRNAs associated with differentiation normally are lowly expressed in progenitors and increase during differentiation. The miRNAs that rescue astrocyte differentiation, let-7 and miR-125, are present in GPCs and not significantly up regulated in early stages of differentiation. An analysis of relevant signaling pathways suggests that GPC miRNAs may be creating a permissive environment for STAT3 to be activated in response to differentiation signals, perhaps by inhibiting a repressor. These miRNAs may also be regulating inhibitors of cell cycle progression and promoting cell cycle exit in response to differentiation cues.

Second, this study suggests the intriguing possibility that broadly expressed miRNAs such as let-7 and miR-125 may have maintained expression but have varied function across lineages. For instance, miR-125 is highly expressed in skin stem cells but in contrast to astrocytes, it has

recently been shown to promote stemness when overexpressed. The two miRNA families highly expressed in GPCs that are represented among genes upregulated upon miRNA loss, miR-9 and let-7, are expressed in NPCs and have both been implicated in promotion of neurogenesis. At least one important miR-9 and let-7 target in NPCs, TLX, is not expressed in GPCs. It is tempting to speculate that in the context of astrocyte differentiation, miR-9 and let-7 may have been co-opted for down regulating astrocyte-specific gene networks. Alternatively, they may largely play similar roles during neurogenesis and gliogenesis with the exception of a minority of targets. In future studies, it will be interesting to compare differential targets of these miRNAs in NPCs and GPCs.

Implications for cancer biology

The let-7 and miR-125 families of miRNAs have been classically associated with differentiation since they are upregulated following the loss of pluripotency during development. Since malignant cancers are often less differentiated, many studies have examined the expression and effects of let-7 in tumor cells(Schultz et al., 2008). The levels of let-7 are reduced in cell lines established from glioblastoma cells, and addition of let-7 limits the size of tumors that arise from transplantation of cell(Lee et al., 2010). In this study, we show that let-7 and miR-125 targets are enriched for a gene subset that exhibits increased expression in glioma stem cells and in higher

grades of glioblastoma. An exciting future direction for these studies would be to expand this analysis to all mRNAs highly expressed in GBMs and determine a subset that could be simultaneously targeted for therapy.

Methods

Methods: Chapter 2&3

Cell culture and GPC derivation

ES cell culture has been previously described(Wang et al., 2007a). Briefly, ES cells were maintained in media supplemented daily with 1000 units/ml LIF. For neural differentiation, the protocol was adapted from a previously described method(Okabe et al., 1996). Briefly, ES cells were dissociated and grown as embryoid bodies culture in media without LIF. EBs were plated in ITSF media as described by Okabe et al for 5-8 days and then dissociated into N2 media supplemented with 10 ng/ml FGF on to laminin coated plates. Following 1-2 passages, 10 ng/ml EGF was added to the media along with FGF to stimulate glial specification. For differentiation, cells were growth to 70-80% confluency unless described otherwise. Growth factors were withdrawn and N2 media supplemented with 1% FBS and B27 was added for 24-48 hours.

Cells were treated with 1 uM tamoxifen for 16-24 hours and 2 days later, treated with 200 nM tamoxifen for 16-24 hours to stimulate efficient Cre-lox mediated recombination. To minimize cell death following loss of Dgcr8, cells were supplemented daily with growth factors until inducing differentiation.

For experiments with inhibitors, concentrations were used at concentration ranges as previously described(Tesar et al., 2007; Buehr et al., 2008; Berge et al., 2011). JAKi was used at 1 uM.

Transfections

miRNA mimics were ordered from Dharmacon, resuspended in sterile H2O and transfected using the DharmaFECT3 reagent at a final concentration of 50-100 nM. Media was changed 24 hours later and differentiation was induced 72 hours following transfection. For the miRNA target knockdown screen, Dharmacon siGenome smartpool siRNAs were used at a final concentration of 50 nM.

Plasmid overexpression

For overexpression of miRNA target genes, cDNAs were cloned into a vector driven by EF1alpha and contained a T2A-mCherry element downstream of the cloned insert. Nucleofection was performed using the Glial cell kit with the 'A033' program to express the plasmid in GPCs. Differentiation was induced 24 hours following nucleofection and cells were fixed 24 hours later and processed for immunofluorescence.

Immunofluorescence

For immunofluorescence, cells were fixed in 4% PFA for 10-15 mins, permeabilized with PBS containing 0.2% triton-X100 and blocked in PBS containing BSA and goat serum. Primary and secondary antibody incubation was done in blocking solution and cells were counterstained with DAPI. GFAP antibody was from Dako cytomation and used at a dilution of 1:500.

Microarray analysis

RNA was extracted from cells following lysis with Trizol and processed for Illumina bead chip arrays at the UCLA genome core facility. All data were quantile normalized using the Bead Array R package. As one set of Mock-Let-7 arrays were performed at a separate time, differential expression analysis was performed using the RankProd R package. Seed enrichment analysis was performed as previously described using custom python scripts and R code(Melton et al., 2010).

Small RNA sequencing and analysis

Total RNA was extracted using Trizol Reagent (Invitrogen). Small RNAs were cloned and multiplexed using the Illumina Tru-Seq kit protocol and in house reagents. 3' adapter was adenylated prior to small RNA cloning as described previously(Babiarz et al., 2008). All

adapters and PCR primers were purified on a 22% urea-acrylamide gel prior to use. Libraries were diluted to 10 nM and sequenced on a HiSeq 2000 at UCSF.

Small RNA sequencing data was processed using custom perl scripts and mapped to miRNAs using Bowtie. Sequences were trimmed of the 3′ adapter by requiring 8 nt of perfect match to the adapter sequence. Sequences were mapped to the mouse pre-miRNA hairpins in miRBase v22.0 using bowtie. All reads perfectly mapping no more than to 5 hairpins were included in counts. Read counts for each miRNA were summarized and reported.

Methods: Chapter 4

Solexa sequencing data for Dgcr8 KO, Dicer KO and WT cells were previously published(Wang et al., 2008). Information about exonic miRNAs and host genes was extracted from the CoGemir database(Maselli et al., 2008).

Microarray analysis

Microarray experiments on the wild-type, Dgcr8 KO, and Dicer KO cells were performed by the Gladstone Genomic Core Facility using the Affymetrix 1.0 mouse gene ST arrays with 3 biological replicates per genotype (wild-type (v6.5), *Dgcr8* knockout, *Dicer* knockout ES cells).

Dgcr8 and Dicer knockout ES cell derivation and culture has been previously described (Wang et al., 2007a; Babiarz et al., 2008). Protocol used for preparation of RNA and hybridization for microarray has been previously described(Wang et al., 2008). Array data was normalized using the robust multi-array average (RMA) algorithm. Normalized data has been deposited at GEO (#GSE16923). Genes upregulated and downregulated in Dgcr8 KO relative to WT and Dicer KO were determined by FDR analysis using the SAM software package from Stanford. (http://www-stat.stanford.edu/~tibs/SAM/). Specifically, two sets of genes were determined: 1) Genes upregulated in Dgcr8 KO relative to Dicer KO and 2) Genes upregulated in Dgcr8 KO relative to wild-type. Overlapping transcripts between these two sets of genes were assigned to the group upregulated in Dgcr8 KO relative to both Dicer KO and WT. Genes downregulated in Dgcr8 KO relative to Dicer and WT were determined using the same approach. For the analysis of overlap between small RNAs or predicted hairpins and protein coding mRNAs, we excluded Affy transcripts annotated only as miRNAs, transcripts mapping to the mitochondrial genome, chromosome Y and transcripts missing gene ID annotations.

HeLa cell microarray data was previously published(Han et al., 2009). siGFP, siDrosha and siDgcr8 expression data was averaged for 24 and 48 hr timepoints for each Affy ID, which resulted in 4 biological samples/gene. AffyIDs upregulated at least 2 fold (n = 1195) in both siDrosha and siDgcr8 relative to siGFP were analyzed further.

Mapping small RNA reads to exons

Small RNA reads from the Solexa sequencing dataset were first mapped to the genome (mouse, version mm8) using Eland. Uniquely mapping small RNA reads were mapped to exons by examining overlap between genomic coordinates of a small RNA read and each exon. Any small RNA overlapping with beginning and end of an exon as well as lying within in an exon was included as a positive hit. Exon information was determined using annotations from the UCSC Known Genes and Ensemble databases (mouse, version mm8) and all transcripts were collapsed to match to Affy ID annotations(Anon, 2008).

For analysis of data from HeLa and HepG2 cells, small RNAs from all libraries were first mapped to the genome (hg18) using Eland. Sequence length of HeLa cell libraries ranged from 15 to 26 nt. Sequence length of HepG2 cell libraries ranged from 15 to 36 nt. Genomic coordinates of the small RNAs were then mapped to exons of transcripts upregulated with siDrosha and siDgcr8 relative to siGFP. Exon information was determined using RefSeq annotations, which were matched to Affy IDs. The positive hits were further filtered manually of snoRNAs. The remaining exons were ranked based on the number of small RNA reads and exons containing >10 small RNAs were analyzed further using custom tracks at the UCSC genome browser. For exons with small RNA reads localized to a small window, sequences

surrounding the small RNA reads were extracted and fold predictions were generated using RNAfold.

Mapping Small RNA reads to miRNA hairpins

Genomic locations of miRNA hairpins were extracted from miRBase and converted to the hg18 assembly using the liftover tool. Genomic coordinates of small RNA sequences (25 to 36 nt) from HeLa and HepG2 cells were mapped to miRNA hairpin locations.

EvoFold Analysis

Lists of long CDS and 5′UTR hairpins and their location in the human genome (mapping based on May 2004 release) were downloaded from the EvoFold database (available online at:http://www.cbse.ucsc.edu/~jsp/EvoFold/) [16]. The genomic coordinates were converted to the mouse genome (version mm8) using the LiftOver tool at the UCSC genome browser. Predicted hairpins were then mapped to mouse exons from UCSC known genes and Ensemble data sets and matched to the corresponding Affy IDs. Small RNAs were mapped to the hairpins using genomic coordinates using the same approach used when mapping small RNAs to exons.

For analysis of data from HeLa and HepG2 cells, EvoFold UTR and CDS hairpin coordinates were converted to this version using the Liftover tool at the UCSC genome browser. Small RNAs were directly mapped to the hairpins as described earlier.

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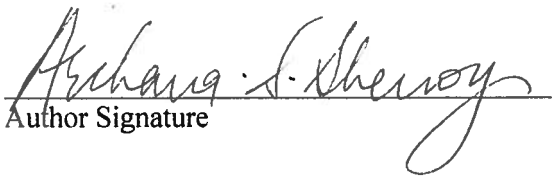

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