The roles of microRNAs in mouse development

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Abstract | Hundreds of microRNAs (miRNAs) are expressed in distinct spatial and temporal patterns during embryonic and postnatal mouse development. The loss of all miRNAs through the deletion of critical miRNA biogenesis factors results in early lethality. The function of each miRNA stems from their cumulative negative regulation of multiple mRNA targets expressed in a particular cell type. During development, miRNAs often coordinate the timing and direction of cell fate transitions. In adults, miRNAs frequently contribute to organismal fitness through homeostatic roles in physiology. Here, we review how the recent dissection of miRNA-knockout phenotypes in mice as well as advances related to their targets, dosage, and interactions have collectively informed our understanding of the roles of miRNAs in mammalian development and adaptive responses.

Seed sequence
Nucleotides 2–7 of microRNAs, which largely determine the target binding repertoire of microRNAs.

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MicroRNAs (miRNAs) are short, non-coding RNAs that function by destabilizing and/or suppressing the translation of their target RNAs. Mature ~22-nucleotide miRNAs are derived from longer primary transcripts (pri-miRNAs) transcribed by RNA polymerase II (REF.[1]). pri-miRNAs are cleaved in the nucleus into pre-miRNAs by the microprocessor complex, which consists of DGCR8 and DROSHA[1]. Pre-miRNAs are then exported to the cytoplasm, where DICER cleaves the characteristic stem–loop structure at its base and apex to produce a linearized double-stranded RNA. One strand of the resulting double-stranded RNA is then loaded onto an Argonaute (AGO) protein to form the RNA-induced silencing complex (RISC)[1].

miRNAs pair with target mRNAs based predominantly on a six to eight base sequence that is complementary to the miRNA-defining seed sequence. Robust target sites are most common in the 3′ untranslated region (UTR) of transcripts, although some may be found in open reading frames[2]. The binding relationship of miRNAs with potential target RNAs is affected by various local factors, such as the surrounding A+U content and structural accessibility of the target site[2]. Due to the prevalence of 6–8mers complementary to miRNA seed sequences in transcripts, each miRNA can post-transcriptionally regulate many targets. Unlike plant miRNAs, metazoan miRNAs rarely slice their targets[3]. Instead, metazoan RISC acts through the recruitment of effectors, including deadenylases, decapping enzymes, and exonucleases, resulting in the destabilization and translation inhibition of target RNAs[3,4]. The identity of targets and the magnitude of their suppression ultimately define the function of each miRNA.

The essential role of miRNAs in metazoan development is evidenced by the striking phenotypes associated with the ablation of critical miRNA biogenesis factors[5–11]. For example, zygotic deletion of the biogenesis factor *dicer1* in zebrafish results in growth arrest following organogenesis 8 days after fertilization and lethality after 2 weeks[7]. Maternal and zygotic deletion of *dicer1* show earlier phenotypes, including developmental delay as early as 24 hours and grossly apparent morphogenesis defects by 36 hours, although axial formation remains intact[8]. When both maternal and zygotic *Dgcr8* or *Dicer1* are disrupted in mice, pre-implantation development, including formation of the earlier embryonic lineages, proceeds normally, before a perigastrulation requirement for miRNAs leads to lethality[12,13]. The earliest role of miRNAs in mouse development is extraembryonic, promoting self-renewal of trophectoderm as well as patterning of visceral endoderm[13]. Indeed, when extraembryonic *Dicer1* is intact and selectively deleted in the embryo, the developmental defects are far less severe than complete knockouts[13].

The necessity of individual miRNAs for metazoan development has been less clear. Initial genome-wide phenotypic studies on miRNAs were performed in the nematode *Caenorhabditis elegans*. Surprisingly few miRNAs showed phenotypes, leading to the concept that most individual miRNAs are redundant or have minor roles[14,15]. This view persisted after the initial evaluation of miRNA-knockout phenotypes in mice[16]. However, recent findings stemming from refined genetics and phenotyping paint a very different picture and justify revisiting this concept. For example, roughly 80% of miRNA knockouts in the fruit fly *Drosophila*

melanogaster have obvious phenotypes[3,17], and phenotypes have been ascribed to the majority of conserved miRNA knockouts in mice[3]. This includes dozens of individual miRNAs that are essential for viability (FIG. 1a). The essential miRNAs include some conserved from the bilaterian ancestor and others that are conserved only from the last common ancestor of mammals and bony fish (Supplementary Table 1). Essential miRNAs are required at various developmental stages, including ten at birth, when the postnatal requirements of miRNAs, such as miR-218 in respiration, become apparent[18].

This Review extends the narrative of miRNA function in multicellular evolution and mammalian development and physiology by incorporating recent phenotypes of mouse miRNA knockouts. These reports clarify the developmental stages when miRNAs function, expand the repertoire of miRNA functions in coordinating the timing and transit between cell types, and reveal many new miRNA roles in regulating physiology. These studies also illuminate the importance of functional miRNA targets, miRNA dosage and interactions between miRNAs as well as the impact of cellular context on miRNA targets.

miRNAs in multicellular evolution

The concordance between increased miRNA and cellular diversity across species suggests that miRNAs may have contributed to the evolution of new cell types during speciation. miRNAs are strikingly absent in the genomes of most unicellular organisms[19,20] but generally increase in number along with animal cellular diversity, with notable expansions between cnidarians and bilaterians, and again between bilaterians and vertebrates[21–25].

Several observations are consistent with the hypothesis that miRNAs contribute to the evolution of multicellularity. miRNAs preferentially target rapidly evolving genes as opposed to more ancient genes with housekeeping functions[26]. Their expression patterns are consistent with roles in cell fate decisions and specialized cellular functions, as exemplified by ~68% of miRNAs conserved across vertebrates exhibiting tissue-specific expression during zebrafish development[27,28]. Similarly in mice and humans, many miRNAs show tissue-specific dominant expression such as *mir-122* in hepatocytes, *mir-1* in muscle and *mir-9* and *mir-124* in the central nervous system[28,29]. This distribution stems, in part, from the fact that more than half of all miRNAs are processed from introns of genes with tissue-restricted expression profiles[30]. The concordance between particular miRNAs and cellular lineages suggests a tight relationship between miRNA expression and lineage specification. The transcriptional control model posits that lineage-specific expression of miRNAs is an integral part of their evolution; from the pool of novel miRNAs that are initially tolerated, miRNAs evolve adaptive roles through purifying selection that depletes deleterious target sites as well as selection for increased abundance and refined spatial expression[31]. A corollary of this model is that many conserved miRNAs have lineage-specific or lineage-defining roles.

Now that the phenotypes associated with disruption of many individual miRNAs have been characterized during mouse embryogenesis, it is apparent that the onset of miRNA functions actually coincides with cellular diversification (FIG. 1b, left panel). Collectively, miRNAs conserved from the bilaterian ancestor seem to play earlier roles in development than miRNAs conserved from only the last common ancestor of mammals and bony fish (FIG. 1b, right panel). The later developmental roles among miRNAs selected later in evolution may reflect accumulation of fewer regulatory interactions but also fit a model whereby miRNAs contribute to speciation, in part, by regulating lineage diversification. Connecting the genomic evolution of miRNAs to lineage differences across species will help elucidate this relationship, including whether divergence in miRNA target relationships and/or the regulatory elements controlling miRNA expression are central to lineage evolution.

miRNAs coordinate developmental timing

Probably the best characterized role for miRNAs is their role in regulating developmental timing. The founding miRNA, *lin-4*, was famously discovered during the characterization of a heterochronic *C. elegans* phenotype[32]. In the absence of *lin-4*, the invariant sequence of *C. elegans* divisions is altered such that repetition rather than progression to the stage-appropriate division event occurs[33]. Hence, *lin-4* is a heterochronic mutant in that it is required to align the timing of cell fate decisions in several lineages with the rest of the organism. The subsequent discovery that another miRNA, *let-7*, acted in the same pathway — later in development and downstream of *lin-4* — reinforced the central role of miRNAs in developmental timing[34].

Heterochronic miRNAs have also been uncovered in mammals. One of the earliest hints came from the evaluation of *mir-196*, which is located in the HOX cluster[35]. The HOX cluster is organized in a collinear fashion where more anterior genes are expressed earlier and more posterior genes are expressed later. This timing is regulated in part by the negative feedback of posterior genes on anterior genes[36]. *mir-196* exemplifies this phenomenon in that it is posterior and expressed late, feeding back on anterior genes and refining their spatial and temporal expression[36,37]. However, the limb develops normally in the absence of *mir-196* (REF.[38]), suggesting redundancy with other control mechanisms. Therefore, *mir-196* behaves like a heterochronic miRNA molecularly, but its disruption alone does not manifest itself in a heterochronic phenotype.

Other miRNAs showed hints of not only suppressing previously expressed mRNAs during development but also regulating carefully timed cell fate transitions. For example, miR-124, the most abundant miRNA in the central nervous system, contributes to the timely onset of neurogenesis in the embryonic spinal cord via suppression of *Ctdsp1* (REFS[29,39]). In the adult central nervous system, miR-124 promotes transition of subventricular zone-derived transit-amplifying neural progenitors to postmitotic neuroblasts by targeting *Sox9*. Precursors are maintained when miR-124 is antagonized, whereas ectopic miR-124 causes premature neurogenesis[40].

Recent miRNA knockouts in mice have cemented the role of miRNAs in control of developmental timing

Purifying selection
The selective removal of deleterious alleles by natural selection.

Developmental timing
The schedule of events during development of unperturbed organisms.

Heterochronic
Unsynchronized events relative to the expected schedule.

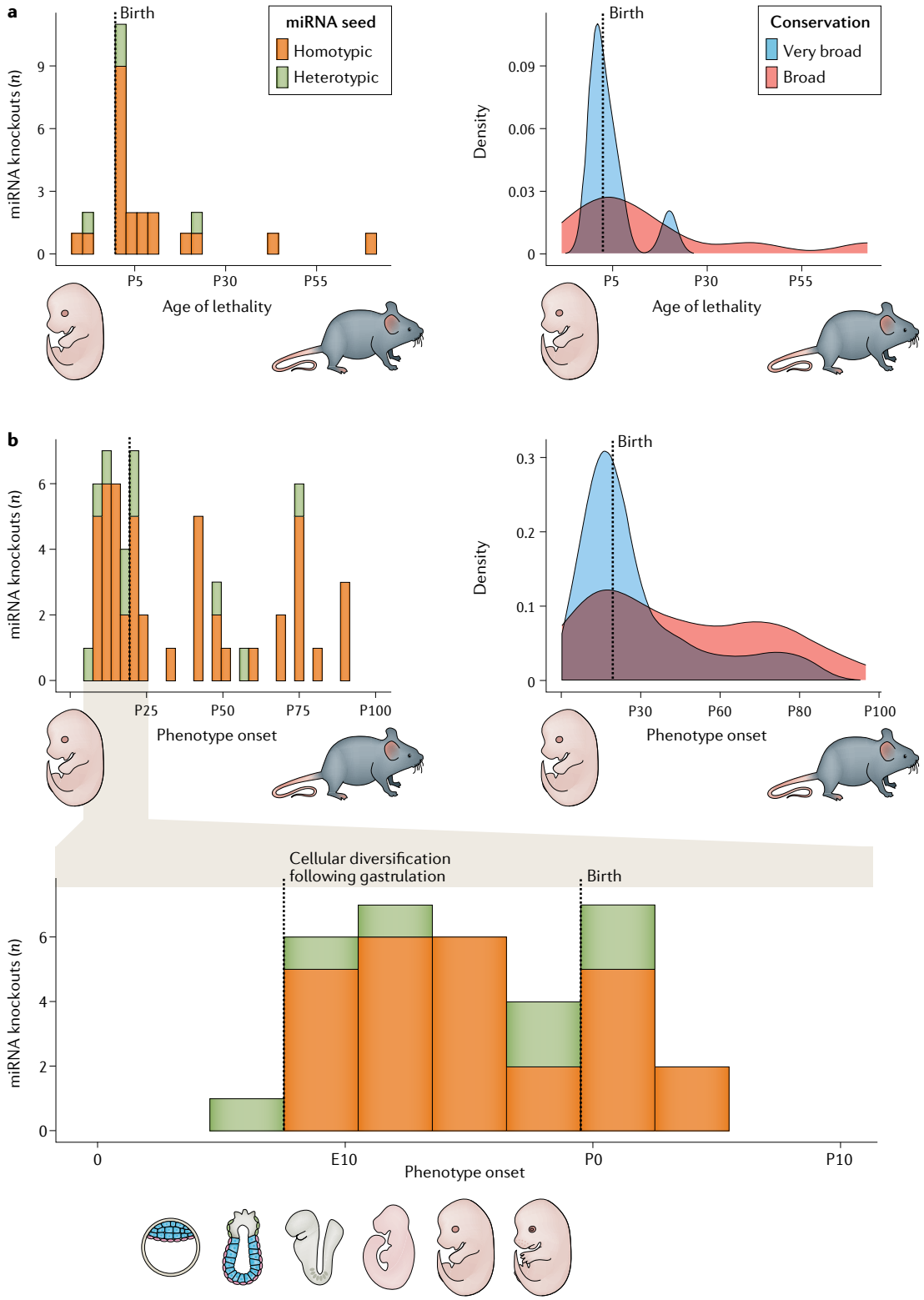
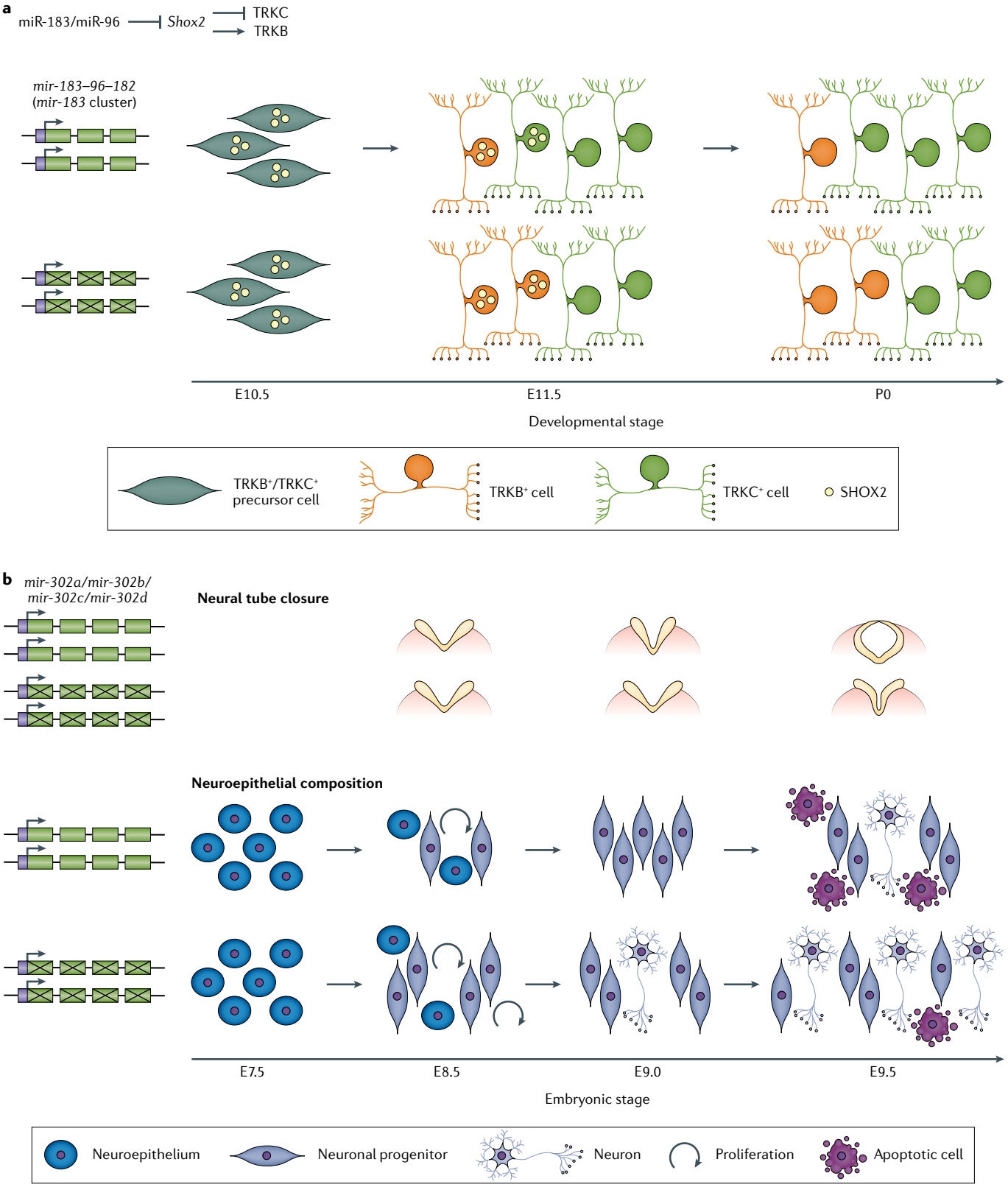


Fig. 1 | **The developmental stage of miRNA functions. a** | The stage of mouse development when the absence of essential microRNAs (miRNAs) is lethal (see Supplementary Table 1 for source data and references). The stage of lethality was defined as the midpoint of lethality in the cohort. Age includes embryogenesis and days postnatally. 'Heterotypic miRNA seed' refers to deletion of a miRNA cluster containing distinct seed families. 'Homotypic miRNA seed' refers to deletion of one or more miRNAs that share the same seed and hence are members of the same miRNA family. **b** | The developmental onset of phenotypes associated with miRNA disruption (see Supplementary Table 2 for source data and references). For miRNAs with multiple phenotypes, the earliest phenotype is depicted for each miRNA. The phenotypic onset was defined as the midpoint when the phenotype was evident in the cohort. The bottom graph represents an expansion of the upper graph to highlight the number of phenotypic knockouts reported during embryonic and perinatal development. E, embryonic day; P, postnatal day.



in mammals. For example, *mir-183–96–182* is a heterochronic miRNA cluster that regulates the composition of the mechanosensitive neuron populations[41]. During development, touch-sensitive neurons arise from a common progenitor pool, with specification of TRKB$^+$ neurons preceding that of TRKC$^+$ neurons. Disruption of the *mir-183–96–182* cluster causes overspecification of early TRKB$^+$ neurons at the expense of TRKC$^+$ neurons[41]. This heterochronic phenotype is caused by perdurance of the miR-183/miR-96 target *Shox2*, whose protein product specifies TRKB$^+$ neurons[41] (FIG. 2a). Similarly, *mir-302* is required to promote the appropriate

miRNA cluster
Multiple microRNAs (miRNAs) that are physically adjacent in the genome.

◀ Fig. 2 | **miRNAs regulate developmental timing. a** | miR-183 balances the production of touch neuronal subtypes. Relief of miR-183-mediated suppression of *Shox2* expression coordinates the transition of TRKB$^+$ and TRKC$^+$ mechanosensitive neurons being specified from precursors[41]. **b** | miR-302 coordinates the timing of neuronal differentiation during development. In the absence of miR-302, neuronal differentiation is precocious[42]. E, embryonic day; P, postnatal day.

neuronal maturation schedule[42] (FIG. 2b). In the absence of *mir-302*, the neuroepithelium prematurely differentiates into neural precursors and then postmitotic neurons. The overexpression of the miR-302 target *Fgf15* is sufficient to cause precocious neuronal differentiation[42]. Loss of *mir-302* also diminishes the proliferative capacity of cardiomyocytes, potentially by regulating the developmental timing of their differentiation[43]. *mir-290~295*, a physically separate miRNA cluster that, except for miR-293, shares a common seed sequence with miR-302, also has a heterochronic phenotype in that trophoblast progenitor cells deficient in miR-290~295 differentiate prematurely[44]. Understanding how heterochronic mammalian miRNAs are integrated into transcriptional networks to control developmental timing and how such networks differ across species is of ongoing importance.

miRNAs regulate cell fate

In addition to developmental timing, miRNAs play roles in directing and maintaining cell fates. A clear example of a miRNA directing cell fate choice again comes from *C. elegans*, where the miRNA *lsy-6* regulates the choice between the alternative taste receptor neurons 'ASE left' (ASEL) and 'ASE right' (ASER)[45]. These two neurons arise from a hybrid precursor state with the potential to form either fate before resolution into neurons with distinct sets of chemoreceptors that discriminate specific environmental inputs[45]. In *lsy-6* mutants, ASEL markers are lost and ASER markers are gained[45].

miRNAs also regulate cell fate choices in mice. For example, miR-133a targets *Prdm16* mRNA, which encodes a master regulator of brown adipocyte development[46]. By doing so, it promotes a white adipocyte programme of lipid storage over a brown fat programme of lipid consumption and heat production. Like many other miRNAs, miR-133a regulates cell fate by silencing an alternative cell fate programme. Consolidation of neuronal fate is also miRNA-dependent, requiring both miR-9 and miR-124 (REF.[47]). miR-9 and miR-124 jointly repress BAF53A (also known as ACTL6A) to mediate its replacement in the BAF complex by BAF45A[47]. When the exchange between BAF53A and BAF45A is disrupted by mutagenesis of the miR-9/miR-124 target sites in the *Baf53a* 3′ UTR, activity-dependent dendritic outgrowth is impaired[47].

Other miRNAs coordinate the balance of self-renewal and differentiation. miR-205 is essential for the expansion of skin stem cells[48]. miR-205-deficient pups die as neonates with severe deficits in epidermal and hair follicle growth[48]. Rather than expanding, the hair follicle skin stem cells in miR-205-deficient neonates become quiescent prematurely[48]. miR-205 promotes expansion of hair follicle skin stem cells by suppressing several inhibitors of the PI3K–AKT pathway; indeed, a constitutively active AKT mutant rescues stem cell colony formation in *mir-205* mutants[48]. Similarly, miR-29a maintains the self-renewal and proliferative capacity of haematopoietic stem cells in adult mice[49]. In the absence of miR-29a, global mRNA expression in haematopoietic stem cells resembles that of committed progenitors, consistent with a failure of *mir-29a*$^{-/-}$ haematopoietic stem cells to self-renew[49]. Removing one copy of *Dnmt3a*, a putative miR-29a target, rescues haematopoietic reconstitution in *mir-29a*$^{-/-}$ mutants[49].

miR-34 and miR-449, which share seed sequences and thus are members of a common miRNA family, also regulate the decision between self-renewal and differentiation. Combined knockouts of *mir-34* and *mir-449* result in postnatal death, infertility and respiratory dysfunction due to deficient ciliogenesis[50]. Characterization of the reduced brain size of these mice revealed that the miR-34/miR-449 family regulates self-renewal and cell fate specification. During cortical development, the onset of neurogenesis involves a transition from symmetric to asymmetric cell divisions. Neuroepithelium expands by symmetric divisions before it generates radial glial (RG) cells; RG cells then produce neurons through asymmetric divisions. The spindle orientation of RG cells affects the cell fate outcome of these divisions: when the spindle of RG cells is parallel to the ventricular plate, their division directly yields a neuron and an RG cell, whereas offset orientations yield intermediate progenitor cells that divide again symmetrically to yield two neurons. In miR-34/miR-449-deficient embryos, reduced spindle angles result in excess symmetric expansion of RG cells at the expense of intermediate progenitors and consequently neurons[51] (FIG. 3). These examples likely provide a very small sample of miRNA roles in cell fate. Given the increased resolution of cell fates offered by recent advances in single-cell profiling and phenotypic analysis, the diversity of such roles is certain to increase[52].

miRNAs in physiology and homeostasis

miRNA functions extend from roles in development to mammalian physiology. For example, *mir-200b* and *mir-429* are required to coordinate the surge in luteinizing hormone from the pituitary, which in turn regulates female fertility[53]. The anovulation and infertility of miR-200b/miR-429-deficient female mice is rescued by superovulation induced with ectopic hormones[53]. *Zeb1* is a computationally predicted target of miR-220b/miR-429 and is a transcriptional repressor whose expression increases in the pituitary in the absence of both *mir-200b* and *mir-429* (REF.[53]). Electrophoretic mobility shift assays support the binding of ZEB1 to three elements upstream of the β-subunit of luteinizing hormone (*Lhb*)[53]. Functionally, both the decreased release of luteinizing hormone following knockdown of ZEB1 in the gonadotroph LβT2 cell line and recapitulation of the anovulatory phenotype in mice following transgenic expression of ZEB1 in the pituitary support the relevance of *Zeb1* as a *mir-200b*/*mir-429* target[53].

Another example of miRNAs regulating cell physiology is miR-128. Disruption of the *mir-128-2* locus results in fatal epilepsy that can be rescued with anticonvulsants[54]. Dopamine D1 receptor-expressing

miRNA family
MicroRNAs (miRNAs) encoded by distinct genomic loci with common seed sequences.

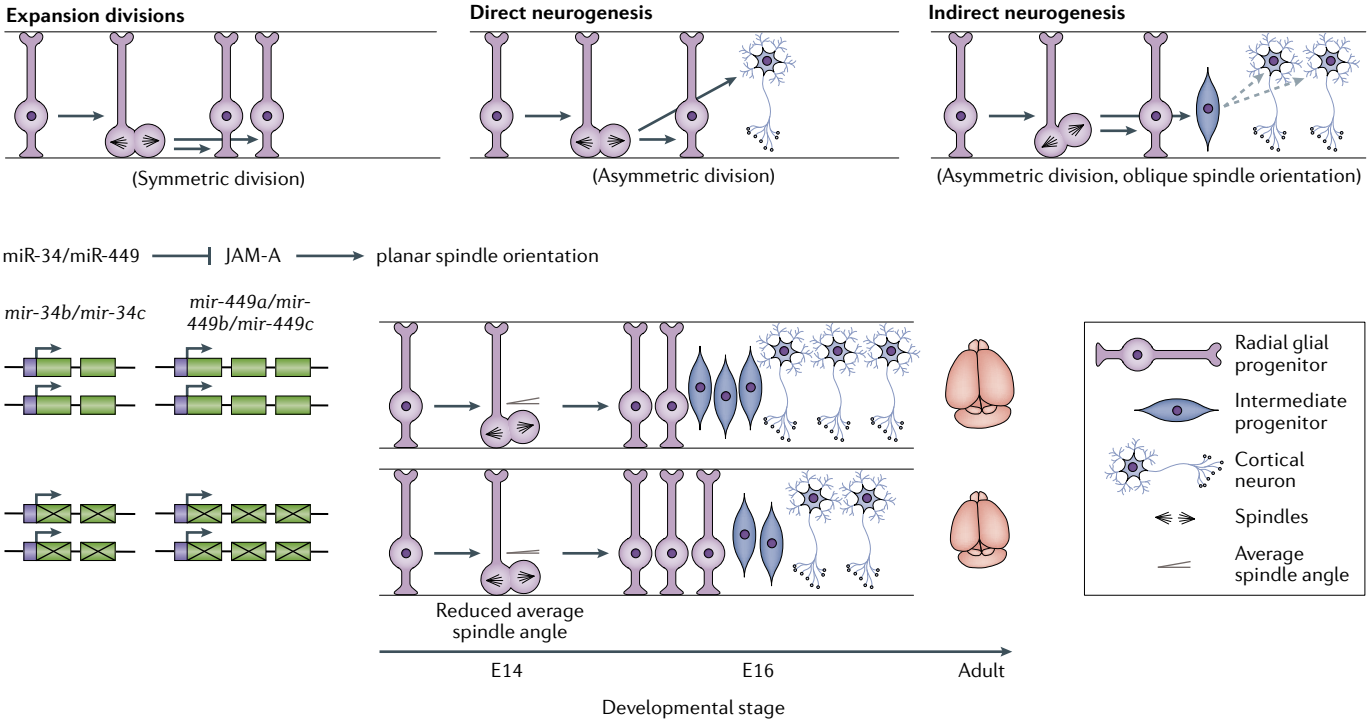


Fig. 3 | **miRNAs coordinate cell fate decisions.** miR-34 and miR-449 regulate spindle orientation of radial glial cells, which affects the cell fate of their progeny. When *mir-34* and *mir-449* are absent, relatively planar divisions occur (with respect to the ventricular plate), resulting in excessive symmetric expansion of radial glial cells instead of intermediate progenitors and their neuronal progeny[51]. E, embryonic day; JAM-A, junction adhesion molecule-A.

neurons deficient in miR-128 exhibit increased functional dendritic spines and overexcitation[54]. miR-128 targets a variety of neurotransmitter transporters and ion channels that in excess are believed to contribute to the phenotype[54].

In addition to homeostatic physiological controls, miRNAs regulate responses to environmental perturbations that are not immediately apparent in the controlled environmental conditions of the typical vivarium. An early indication that miRNAs conferred developmental robustness during environmental fluctuations came from interrogation of *mir-7* in *D. melanogaster*. miR-7 is integrated in feedforward and feedback loops that stabilize signalling cascades in photoreceptors, proprioceptor organs and olfactory organs[55]. miR-7 acts in these loops to limit the spontaneous induction or reversion of cell fate transitions[55]. Strikingly, loss of *mir-7* has little or no effect on sensory organ development under standard conditions but leads to numerous defects in cell fate specification when environmental temperature fluctuates during development[55].

While screens for miRNAs required in specific stress responses are impractical in mice, candidate approaches are revealing a broad range of miRNAs that regulate stress or environmental responses. This includes a protective role for miR-451 against oxidative stress in erythroblasts[56] (FIG. 4a). miR-451 acts, in part, by targeting transcripts encoding 14-3-3ζ, a protein that sequesters the antioxidant transcription factor FOXO3 in the cytoplasm (REF.[56]). In another example, miR-208 and miR-214 play protective roles in cardiac stress. Knockout of the cardiac-specific *mir-208* results in grossly normal mice with a slight reduction in heart contractility at 2 months of age[57]. However, unlike wild-type mice, in which the heart remodels in response to increased afterload, miR-208-deficient mice show virtually no hypertrophy in response to the same stress[57]. In the case of *mir-214*, the protective effect during ischaemia–reperfusion injury illustrates a different adaptive stress response. Following myocardial infarction, cardiomyocyte death and reduced cardiac function can occur when intracellular Ca^{2+} concentration increases abnormally during reperfusion. By repressing the Ca^{2+} influx regulator *Ncx1* (also known as *Slc8a1*) and other downstream effectors of Ca^{2+} signalling, miR-214 limits cardiomyocyte apoptosis and the associated loss in cardiac contractility during reperfusion injury[58].

miRNAs also coordinate pathogen responses, another form of environmental stress. For example, while *mir-155* is not grossly required for myeloid or lymphoid development[59], it plays various roles in adaptive immune responses. *mir-155* promotes selection of B cell clones with somatic mutations encoding high affinity antibodies in germinal centre B cells following immunization[60] (FIG. 4b), is required in dendritic cells for efficient T cell activation[59], ensures balanced production of type 1 and type 2 T helper cells, and modulates the immune response by coordinating lymphocyte cytokine production[59,60]. A different miRNA, *mir-210*, coordinates IL-17-producing T helper cell (T_H17 cell) responses that occur in the airways and intestine[61] (FIG. 4c). T_H17 cells play an important role in eradication of extracellular

bacteria and fungi and have been associated with tissue inflammation and autoimmune disease[62]. Hypoxia and T cell receptor activation synergistically promote *mir-210* expression, integrating T cell activation in response to antigen recognition with the hypoxic stress of the inflammatory sites in which it occurs[61]. Following activation, miR-210 negatively feeds back on *Hif1a*, which specifies T_H17 cells, to limit specification of T_H17 cells[61]. Together, these examples show how miRNAs regulate a variety of responses to stress and environmental perturbations.

miRNAs regulating physiology and homeostasis are expected to encounter different selective pressure between organisms. For example, *mir-7* buffers *D. melanogaster* development through temperature fluctuations that do not occur in endotherms. *mir-7* is highly conserved from annelids to humans but its role buffering the development of sensory organs seems to be a role acquired in insects, involving an enhancer of its expression and critical target sites not found in vertebrates[55]. Studying whether such repurposing of miRNAs to regulate responses to variable stresses is common should be an interesting future pursuit.

Influence of cell type on miRNA targets

As miRNAs are predominantly expressed in specific tissues, dissecting the function of individual miRNAs in individual cell types has been instrumental to elucidating their function. The cellular context is also a critical dimension for miRNA function. Some miRNAs have common functions across cell types; for example, miR-34/miR-449 regulate cilia maturation in part through the target *Cp110* (also known as *Ccp110*) in various multiciliated cells, including sperm, and epithelium lining the trachea and fallopian tube[50]. However, in other casess, for example, miR-155 in the immune system[59,60], miRNAs can have very different functions between cell types, in part due to changing targets[63].

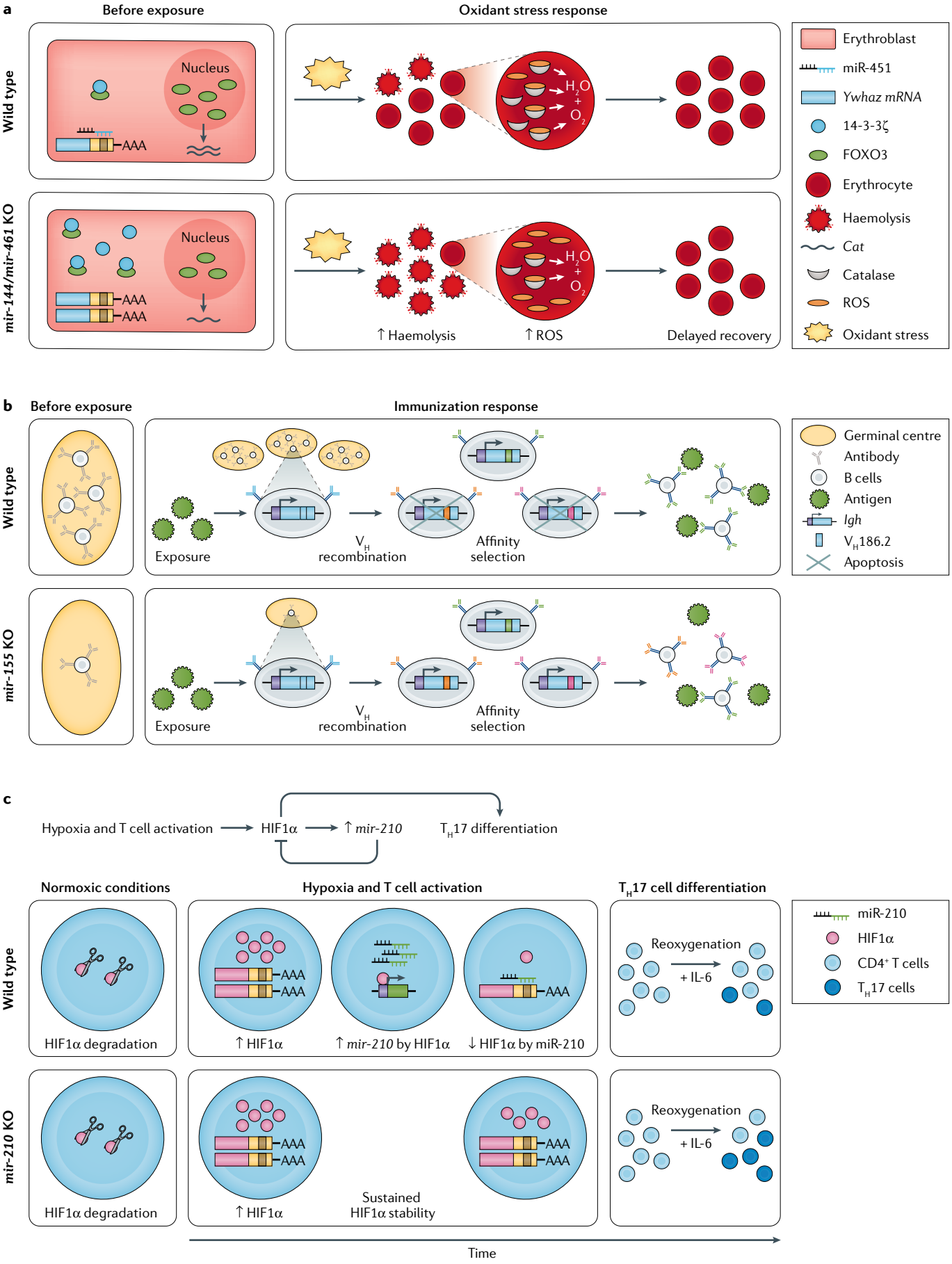
The 90 broadly conserved miRNA families are predicted to target upwards of 60% of all coding genes, with each miRNA family targeting on average 400 mRNAs, representing virtually all gene ontologies[64]. Therefore, the number of possible pairwise miRNA–mRNA interactions in any one context is vast, with many mRNAs potentially being targeted by more than one miRNA family[64]. However, many targets predicted by evolutionary analyses or crosslinking immunoprecipitation (CLIP) binding assays do not exhibit suppression. In conservation-based approaches, false-positive miRNA targets can arise from regions conserved for reasons other than miRNA targeting; the number of such false positives can be estimated, although defining the identity of false positives remains a challenge for computational predictions[64]. Similarly, both false positives and false negatives arise in CLIP. An analysis of the miR-144/miR-451 family during erythropoiesis provides a telling example: of the 2,212 miR-144/miR-451 binding sites defined by high-throughput sequencing of RNA isolated by CLIP, only 53 of the corresponding transcripts were stabilized in the absence of miR-144/miR-451 (REF.[65]). The discrepancy between miRNA binding and target mRNA destabilization may partially be explained by cases where translational repression occurs in the absence of target destabilization[66]. Hence, resolving the identity of a miRNA target network in a particular context from its potential target repertoire is an ongoing challenge.

Sufficient miRNA abundance and target expression are accepted prerequisites for suppression of a target by a miRNA, but whether specific miRNA–target relationships are altered by factors that differ between cell types is debated. One parameter that plays a clear role in cell specificity is alternative polyadenylation (APA). Following transfection of different cell types with the same miRNAs, appreciably altered targets were observed between HeLa, HEK293 and Huh7 cells[67]. These differences mostly disappeared when APA was taken into account, suggesting that polyadenylation sites are a major component of the cellular context influencing miRNA–target relationships[67] (FIG. 5a). Moreover, computationally predicted targets also generalize well across other cell types after APA has been accounted for[2], suggesting that other aspects of cellular context have small effects on miRNA targeting.

By contrast, studies using AGO2-associated photoactivated ribonucleotide-enhanced CLIP and RNAimmunoprecipitation (RIP) to compare miRNA targets in B cells at different stages of maturation and activation suggest that context-dependent differences in miRNA-based targeting are widespread and cannot simply be explained by APA[68]. The enrichment of specific motifs flanking the context-dependent miRNA target sites suggest RNA-binding proteins (RBPs) may be responsible for the differences between these maturing and activated B cells[68] (FIG. 5b). Comparison of *mir-155* targets defined by differential individual nucleotide-resolution CLIP and evaluated by RNA sequencing in four immune cell types also support differences in the target binding and suppression across cell types (FIG. 5c), leading again to the suggestion that RBPs might be responsible for context-based differences in targeting[63]. Individual examples of RBPs disrupting miRNA–target relationships have been reported, such as ELAV2 binding to attenuate miR-9-mediated repression of *Foxg1* in the ventricular zone at embryonic day 16.5[69]. The extensive interplay, both cooperative and competitive, between miRNAs and RBPs has been reviewed elsewhere[70,71]. These sometimes conflicting data suggest there remains much to be learned about what regulates differences in effective targeting across various cellular contexts, including during development.

miRNA abundance and phenotype

The mild or absent phenotypes resulting from deletion of some individual miRNAs suggest the possibility of redundancy with other miRNAs that suppress common targets or were compensated by other components of the network in which they act. One cause of perceived redundancy is the existence of miRNA families in which multiple individual miRNAs share a common seed sequence and hence have largely overlapping targets. More than half of expressed miRNAs are part of a family[72]. Family members are often co-expressed, in part due to extensive genomic clustering and expression


a
Before exposure
Oxidant stress response
Wild type
mir-144/mir-461 KO
Nucleus
AAA
H_2O + O_2
↑ Haemolysis
↑ ROS
Delayed recovery
Erythroblast
miR-451
Ywhaz mRNA
14-3-3ζ
FOXO3
Erythrocyte
Haemolysis
Cat
Catalase
ROS
Oxidant stress
b
Before exposure
Immunization response
Wild type
mir-155 KO
Exposure
V_H recombination
Affinity selection
Germinal centre
Antibody
B cells
Antigen
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V_H186.2
Apoptosis
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Hypoxia and T cell activation
HIF1α
↑ mir-210
T_H17 differentiation
Normoxic conditions
Hypoxia and T cell activation
T_H17 cell differentiation
Wild type
mir-210 KO
HIF1α degradation
↑ HIF1α
↑ mir-210 by HIF1α
↓ HIF1α by miR-210
Reoxygenation
+ IL-6
Sustained HIF1α stability
Time
miR-210
HIF1α
CD4+ T cells
T_H17 cells

◀ Fig. 4 | **miRNAs buffer against environmental perturbations. a** | miR-451 protects against oxidant stress in erythrocytes by coordinating nuclear translocation of the antioxidant transcription factor FOXO3 in erythroblasts[56]. miR-451 targets *Ywhaz*, which encodes 14-3-3ζ, in erythroblasts[56]. 14-3-3ζ limits accumulation of the antioxidant transcription factor FOXO3 in the nucleus. **b** | Following immunization, miR-155 promotes the selection of somatic hypermutation events in the variable V_H186 segment of the immunoglobulin heavy chain (*Igh*) encoding high affinity antibodies in germinal centres[60]. In miR-155-deficient animals, the fraction of germinal centre B cells was reduced. miR-155 is upregulated when B cells are stimulated. When miR-155-deficient animals are immunized, they generate only ~20% of the antigen-specific antibody titre that wild-type animals do. Stimulated *mir-155*$^{-/-}$ B cells proliferate normally and exhibit immunoglobulin heavy chain (IgH) recombination rates comparable to those of wild-type cells but selection of high-affinity clones is compromised. miR-155 likely contributes to selection of cells with high-affinity antibodies through regulation of cytokine production. **c** | miR-210 limits specification of IL-17-producing T helper cells (T_H17 cells) in response to T cell activation and hypoxia[61]. Tumours and sites of inflammation where antigens activate T cells are both hypoxic. T cell receptor activation and hypoxia synergistically promote expression of *Hif1a* as well as *mir-210*, which is dependent on hypoxia-inducible factor 1α (HIF1α). HIF1α promotes specification of T_H17 cells, whereas miR-210 negatively feeds back on *Hif1a* mRNA to limit T_H17 cell specification. KO, knockout; ROS, reactive oxygen species.

as polycistronic pri-miRNAs[73–75]. The copy number and regulatory elements driving expression of family members at distinct genomic loci dictate their overall expression[72,76]. Thus, the loss of a single family member may have a relatively small effect on the overall expression of that family.

The severity, and in some cases nature, of phenotypes resulting from miRNA disruption correlate with the amplitude of altered miRNA abundance. Disruption of one miRNA allele illustrates dose dependency of a miRNA. Although such haploinsufficient miRNAs are rare, miR-96 offers one such example, as loss of a single allele of *mir-96* is associated with hearing loss[77] (FIG. 6A). Deletion of a subset of miRNA family members further illustrates the relationship between the magnitude of miRNA depletion and function. For example, deletion of *mir-128-2*, which is responsible for 80% of total miR-128 expression, but not of *mir-128-1* results in juvenile hyperactivity and fatal early-onset epilepsy related to more excitable D1 receptor-expressing neurons and a 20% increase in the numbers of dendritic spines[54] (FIG. 6Ba). Disruption of the two independent *mir-219* loci has a similarly graded phenotype. Deletion of *mir-219-1*, which results in a roughly 20% reduction in overall miR-219 abundance, is viable[78], deletion of *mir-219-2* and the resulting 65% reduction in overall miR-219 abundance leads to adult tremors, whereas deletion of both is lethal at birth[78] (FIG. 6Bb). In general, the phenotypes associated with the loss of individual family members may underestimate their role due to compensation from other family members.

In some cases, the partial deletion of a seed family is entirely masked by the presence of the intact members. For example, disruption of either the *mir-34b–34c* cluster or the *mir-449a–449b–449c* cluster, which produce miRNAs that share a common seed sequence, does not cause a discernible phenotype[79,80]. This lack of phenotype may reflect a compensatory upregulation of one locus when the other is disrupted in the brain, and, to a lesser extent, in the testis[81]. Conversely knockout of both *mir-34* and *mir-449* is perinatal lethal in 46% of animals, with greater severity in males[81]. All of the viable animals missing both *mir-34* and *mir-449* are runted, are sterile due to defective spermatogenesis and have stunted brain growth due to inappropriate cell fate decisions[81] (FIG. 6C). This case illustrates how compensatory upregulation of a related miRNA locus can rescue the loss of other family members.

While disruption of miRNA sequences results in increased target abundance, the overexpression of miRNAs can result in greater target suppression and associated phenotypes. An important illustration of this dichotomy is seen with *mir-137* variants. Individuals with heterozygous microdeletions encompassing *MIR137* typically exhibit autism spectrum disorder[82,83]. In mice, homozygous deletion of *mir-137* is lethal[84], with mice surviving until postnatal day 21, whereas heterozygous disruption of *mir-137* results in synaptic overgrowth[85] (FIG. 6D). In the heterozygous disruption, the increased spine density, dendritic growth and complexity all suggest synaptic pruning deficits[85]. These synaptic changes are associated with repetitive behaviours, decreased social behaviour and impaired learning and memory, paralleling autism spectrum disorder in humans[85]. By contrast, an allele leading to increased abundance of miR-137 is associated with schizophrenia[86]. Induced human neurons harbouring four homozygous non-coding SNPs adjacent to *mir-137* that are associated with schizophrenia exhibit increased miR-137 levels specifically in neurons and show impaired presynaptic vesicle release[87]. Hence, miRNA-regulated networks can be sensitive to both decreased and increased miRNA dosage.

These data show that considering effects of mutations on overall miRNA levels across a family is important when one is considering the likely contribution of a miRNA-related genetic variant to a disease phenotype. Disease-associated SNPs have been found in mature miRNAs, pri-miRNAs, pre-miRNAs and miRNA promoters, all of which can influence the abundance of the functional miRNA[88]. Weighted genomic approaches that consider miRNA families collectively and weight variants proportionally to the contribution each locus makes to the overall miRNA pool should provide a better understanding of their roles in disease.

miRNA abundance and target suppression

miRNA target suppression depends on the abundance of both miRNAs and targets. In addition to copy number, transcription and processing, miRNA levels are influenced by miRNA stability. In general, miRNAs are thought to be highly stable. For example, miR-122 has an estimated half-life of ~10 days, resulting in an estimated 120,000 molecules per hepatocyte[89,90]. miRNA stability is regulated by a number of factors both intrinsic and extrinsic to the miRNA. Intrinsic factors include sequences within the miRNA itself. For example, the seed sequence and bases at positions m01–m04 at the 3′ end of some *mir-16* family members are destabilizing, allowing the rapid alleviation of the *mir-16* block of the G1 to S transition as cells exit G0 (REF.[91]). Similarly, other miRNAs enriched in retinal neurons accumulate to higher levels in response to light as a result of default rapid turnover of many retinal miRNAs and their increased transcription in light-adapted retinas[92]. Extrinsic factors

Target suppression
Destabilization, translational inhibition or cleavage of RNAs bound by microRNAs.

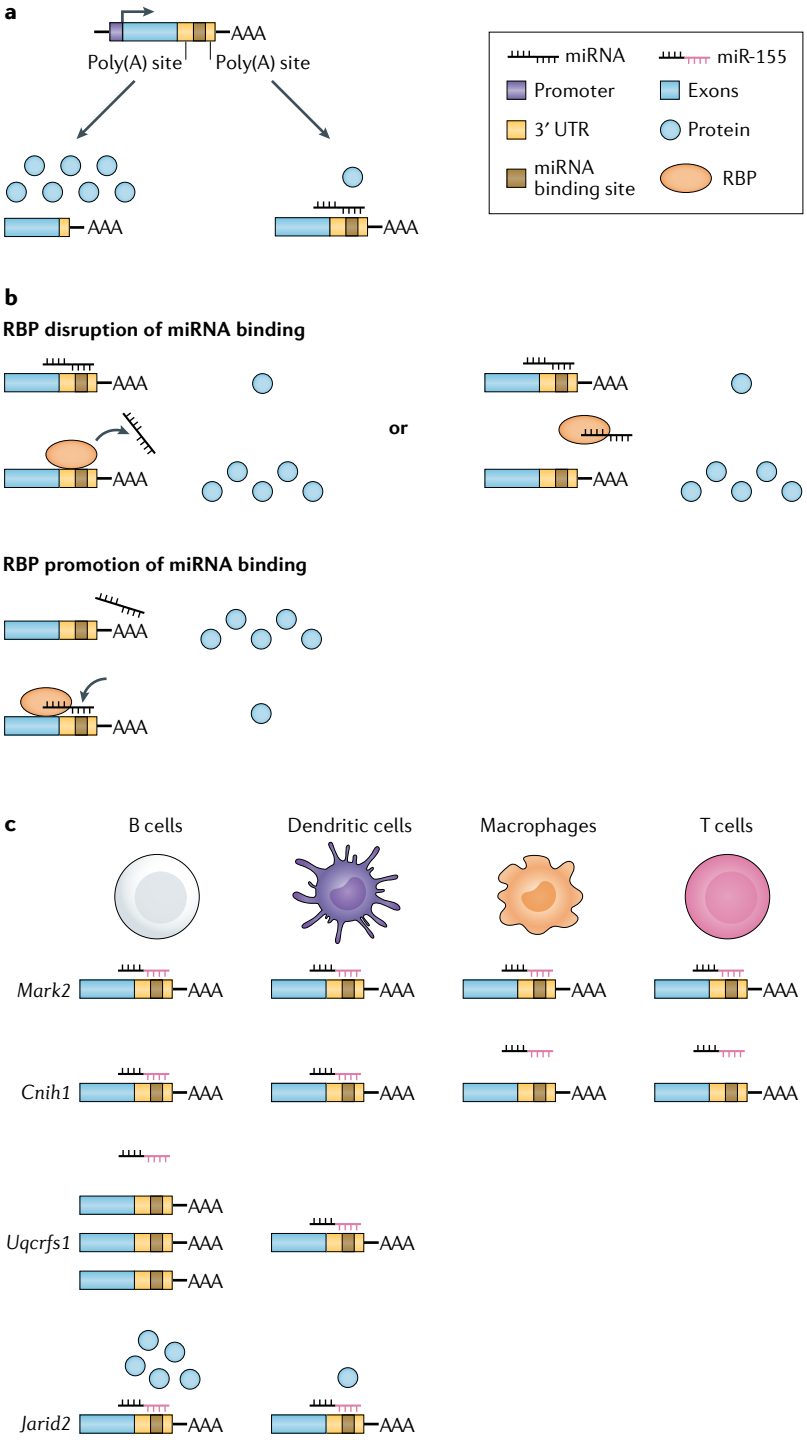

Fig. 5 | **miRNA targeting is affected by cellular context.** **a** | Alternative polyadenylation (poly(A)) sites alter the repertoire of potential target sites by changing length of the 3′ untranslated region (UTR). **b** | RNA-binding proteins (RBPs) can modify microRNA (miRNA)–target relationships, by either inhibiting or promoting miRNA function. **c** | The targets of miR-155 differ between four immune cell types[63]. *Mark2* mRNA is universally bound by miR-155 in B cells, dendritic cells, macrophages and T cells. By contrast, among these four immune cell types *Cnih1* is bound only in B cells and dendritic cells. miR-155 preferentially binds and promotes degradation of *Uqcrfs1* mRNA in dendritic cells relative to B cells. miR-155 binds *Jarid2* in both B cells and dendritic cells but preferentially represses JARID2 expression in dendritic cells.

include RBPs, such as LIN28, which leads to the destabilization of let-7 miRNAs (reviewed in REF.[3]). miRNAs can also be destabilized by target interactions. Highly complementary targets can displace miRNAs from AGO2, thus negating the stability conferred by AGO2 occupancy[93]. Seedless non-canonical targets also destabilize miRNAs through displacement from AGO2 as well as 3′-end destabilization that partially occurs while miRNAs are loaded on AGO2 (REF.[94]). Target mRNAs also direct the degradation of complementary miRNAs[95]. RNA capable of inducing such 'target RNA-directed miRNA degradation' (TDMD) in duplex with a miRNA cannot be physically accommodated by the human AGO2, causing a bend in the RNA–miRNA duplex that makes the 3′ end of the miRNA accessible for enzymatic attack[96]. TDMD occurs independently of target degradation, such that a single target molecule can sequentially mediate degradation of multiple miRNA molecules[95]. Hence, the production and degradation dynamics of individual miRNAs differ considerably.

Recent studies investigating a neuronal circuit of non-coding RNAs in the brain have illuminated a new mechanism regulating miRNA abundance[97,98]. The conserved long non-coding RNA *Cyrano* (also known as *Oip5os1*) promotes degradation of mature miR-7 that is dependent on a conserved internal sequence complementary to miR-7 (REF.[98]). In turn, miR-7 downregulates another non-coding RNA, *Cdr1as* (also known as *Cdr1os*). *Cdr1as* is a circular RNA that is conserved in mammals, has at least 70 miR-7 sites within it and dampens neuronal activity[97,99]. Disruption of *Cyrano* results in a tenfold decrease in Cdr1as levels, which is miR-7 dependent[98]. How miR-7, *Cyrano* and *Cdr1as* control neuronal activity remains an active area of investigation, but the working model is that they regulate sensorimotor gating and presynaptic transmission, perhaps through spatial or temporally localized target suppression in neuronal synapses[97,98] (FIG. 7). Indeed, there is emerging evidence that the activity of several miRNAs is subcellularly localized[100,101]. How the dynamics of mRNA/miRNA abundance, as well as subcellular distribution, affect targeting remain exciting areas of active investigation.

Cooperativity between miRNAs

miRNAs function cooperatively. The cumulative role of miRNAs has been studied in many cell types by deletion of biogenesis factors, but the contribution of each seed family to global miRNA-knockout phenotypes is less clear. The 90 mouse miRNA families conserved from the last common ancestor of mammals and bony fish each have hundreds of targets, which means that many transcripts are regulated by multiple miRNAs[64]. As miRNAs act to suppress targets, multiple miRNAs regulating the same target gene are inherently cooperative; such heterotypic regulation is proposed to confer additional regulatory specificity[102]. Typically, multiple miRNAs repress common targets in an additive fashion but tend to exhibit synergistic targeting when they bind 8–40 bases apart[103]. The existence of co-regulation of

Synergistic targeting
Target suppression that exceeds the additive suppression of multiple microRNAs.

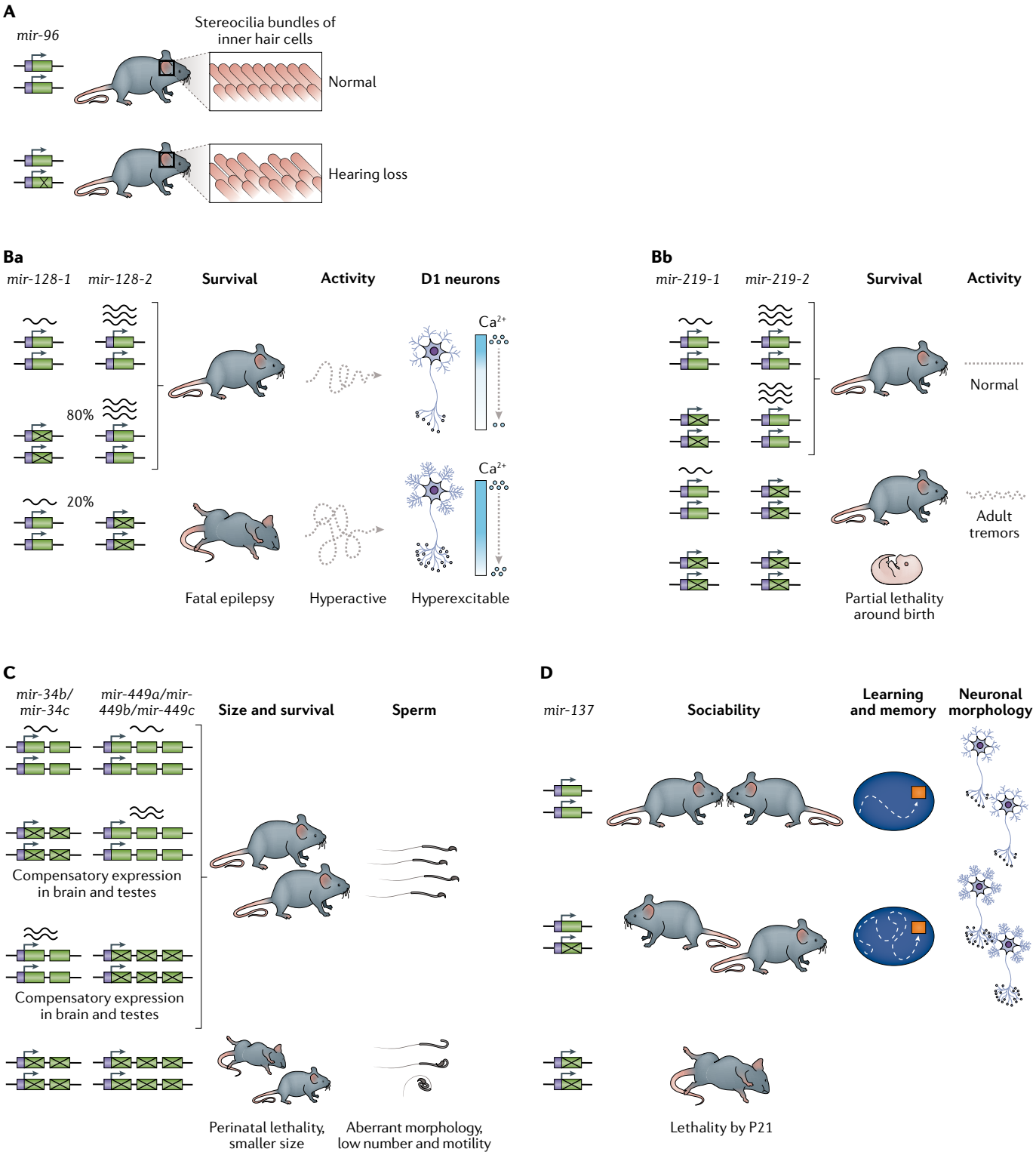

Fig. 6 | **MicroRNA phenotypes can be dose-dependent. A** | miR-96 is haploinsufficient. Loss of a single *mir-96* allele results in hearing loss[77]. **B** | The phenotypes stemming from disruption of miR-128/miR-219 seed family members at separate genomic loci correlate with dosage. Mice survive disruption of *mir-128-1* that results in a 20% reduction in the miR-128 pool . In contrast, disruption of *mir-128-2* depletes the pool of miR-128 by 80% and leads to fatal epilepsy[54] (part **Ba**). Similarly, disruption of *mir-219-2*, which produces the majority of the miR-219 pool, results in adult tremors, whereas disruption of *mir-219-1* does not[78] (part **Bb**). **C** | Seed family members of miR-34/miR-449 compensate for reciprocal loss. When either *mir-449a/mir-449b/mir-449c* or *mir-34b/mir-34c* is disrupted in the brain or testes, compensatory expression from the remaining intact locus occurs[81]. **D** | Phenotype severity relates to the number of mutated *mir-137* alleles. Synaptic overgrowth occurs when one allele of *mir-137* is disrupted[85]. Deletion of both alleles is more severe, resulting in lethality by postnatal day 21 (P21)[85]. D1 neurons, dopamine D1 receptor-expressing neurons.

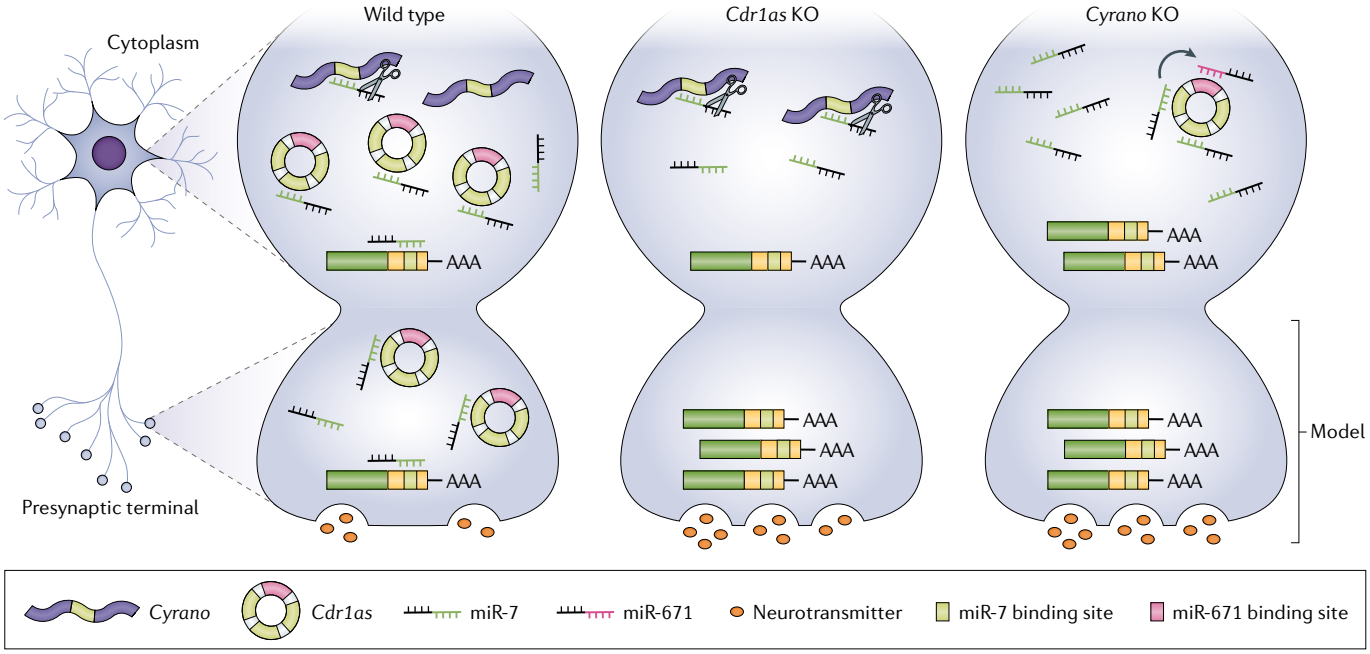

Fig. 7 | **Regulatory network of neuronal non-coding regulatory RNAs.** Neuronal activity is regulated through a network of non-coding RNAs, including long non-coding RNA *Cyrano*, the circular RNA *Cdr1as* and microRNAs miR-7 and miR-671. The schematic depicts cytoplasmic relationships as well as a working model of how network disruptions impact synapses. *Cdr1as* is a circular RNA with at least 70 miR-7 sites that dampens neuronal activity[97,99]. miR-7 downregulates *Cdr1as*, while the long non-coding RNA *Cyrano* promotes miR-7 degradation through a conserved site[98]. When *Cyrano* is disrupted, miR-7 accumulates and a concomitant decrease in *Cdr1as* expression results in increased neuronal activity[98].

common targets by miRNAs from distinct families raises the question of how such co-regulation was selected through evolution.

Common roles of clustered miRNAs. Whether clustered miRNAs with distinct seed sequences co-evolve on the basis of common functions remains an active discussion. Rtl1 antisense transcript (*Rtl1*as) illustrates one example supporting co-evolution of miRNAs in a heterotypic cluster. *Rtl1*as encodes a heterotypic miRNA cluster that is maternally expressed as it resides in an imprinted locus. The miRNAs processed from *Rtl1*as target the paternally expressed *Rtl1* transcript to which they are antisense. The deletion of six heterotypic miRNAs in *Rtl1*as leads to placentomegaly associated with expansion of the inner spaces of the fetal capillaries[104]. Singular deletion of *mir-127*, the most abundant miRNA in the *Rtl1*as cluster, results in a less severe placentomegaly, suggesting the cumulative suppression by the cluster tunes *Rtl1* dosage[105] (FIG. 8a). Conversely, paternal deletion of *Rtl1* resulted in growth retardation of the fetus and placenta[104]. The inverse phenotypes stemming from maternally and paternally contributed deletions illustrate an unusual arrangement where the transcript antisense to a heterotypic cluster of miRNAs is the primary target. miRNAs with distinct seeds at separate genomic locations can also collaborate, illustrated by miR-219 and miR-338, which coordinately promote myelination, potentially through regulation of common targets[78].

Because polycistronic miRNAs are co-expressed and their regulation is under common selective pressure, miRNAs with different seed sequences in the same cluster might be expected to cooperate more than miRNAs that do not share *cis*-regulatory elements. This expectation is sufficiently entrenched that targets are often prioritized on the basis of the presence of target sites corresponding to distinct miRNAs from the same cluster. For example, *Shox2* was prioritized as a *mir-183–96–182* cluster target regulating specification of mechanosensitive neurons on the basis of it being a heterotypic target, containing both miR-183 and miR-96 target sites[41]. Since up to half of all miRNAs are clustered in mammalian genomes, enriched co-targeting by miRNAs with different seeds from the same cluster might be another source of compensation between miRNAs.

The additive suppression by distinct miRNAs in some heterotypic clusters is required. There are three copies of the bicistronic transcript encoding miR-1 and miR-133, with '1' and '2' transcribed in heart and skeletal muscle and a third transcribed in somites[106]. Deletion of *mir-1-1*/*mir-1-2* and *mir-133-1*/*mir-133-2* results in a more severe phenotype than deletion of either alone. Specifically, deletion of *mir-1-1*/*mir-1-2* results in ventricular chamber dilation and death before postnatal day 17, and disruption of *mir-133a-1*/*mir-133a-2* causes lethal septal defects in nearly half of embryos or neonates[107,108]. By contrast, deletion of both *mir-1-1*/*mir-1-2 and mir-133-1*/*mir-133-2* results in impaired circulation and heart beating with lethality by embryonic day 11.5[106] (FIG. 8b).

Another classic heterotypic miRNA cluster is the *mir-17~92* cluster. In a genetic tour de force, Han et al. generated an allelic series to functionally dissect the contribution of individual miRNAs as well as subsets

Heterotypic cluster
A microRNA cluster that encodes microRNAs from more than one seed family.

Cooperative targeting
Distinct microRNAs additively suppressing a common target.

of miRNAs[109]. No singular miRNA deletion resulted in lethality[109]. The cardiac defects and lung hypoplasia thought to cause lethality resulted only when *mir-17, mir-18* and *mir-92* were removed, illustrating functional cooperation of the different seeds. Similarly, the shorter mesophalanx attributed to deletion of the *mir-17* family was exacerbated by deletions of *mir-18* and *mir-92* (FIG. 8c). Intriguingly, expression profiling of the allelic series supported extensive cooperation between the heterotypic miRNA on the basis of the considerable overlap in their targets[109]. These findings suggest co-evolution of the targets and distinct miRNAs in this cluster.

Common targets of clustered miRNAs. Whether cooperative regulation of targets is a general feature of clustered heterotypic miRNAs is debated. Parallels to operons where distinct genes encoding related functions are produced in a common transcript inspired analyses to test whether targeting by clustered heterotypic miRNAs was cooperative[110]. Target prediction, expression following transfection of distinct miRNAs from the *mir-17~92* cluster and lower expression of mRNAs co-targeted by multiple clustered heterotypic miRNAs rather than separate heterotypic miRNAs all supported cooperation of clustered heterotypic miRNAs[110]. However, whether cooperative targeting is more common among clustered heterotypic miRNAs than expected by chance across the genome hinges on whether the *mir-183–96–182* cluster is classified as heterotypic. The seed sequence of miR-182 differs from the other two miRNAs in this cluster, miR-183 and miR-96, by only one base at position 8; therefore, their targets are expected to overlap. Only when the *mir-183–96–182* cluster is classified as heterotypic, a questionable classification, does the coadaptation model

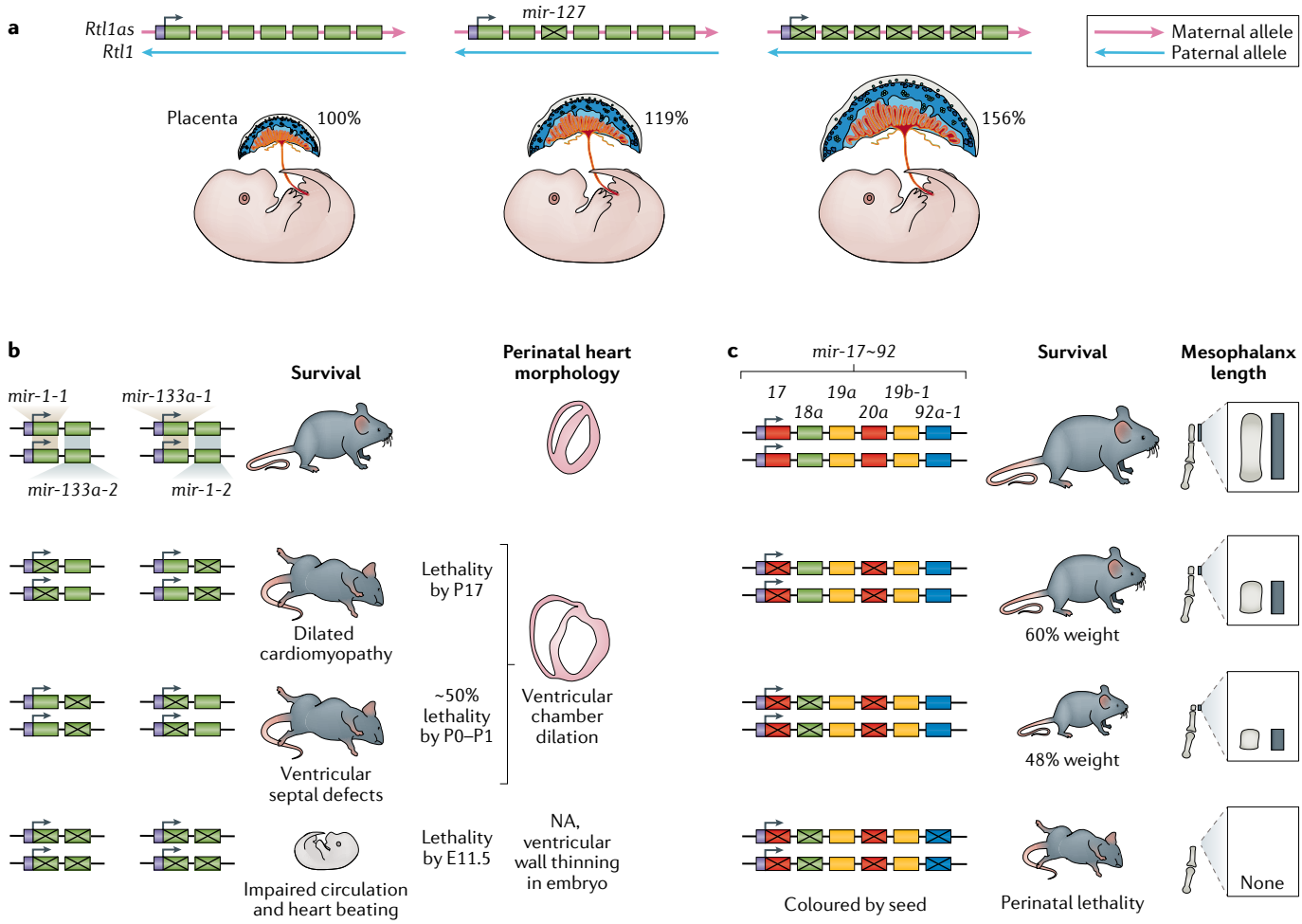

Fig. 8 | **miRNAs with distinct seed sequences cooperate during development. a** | The severity of placentomegaly correlates with the dosage of seed family microRNAs (miRNAs) produced from the *Rtl1* antisense transcript (*Rtl1*as). Deletion of *mir-127* alone results in 19% increase in placental size, whereas deletion of six of the seven miRNAs in the transcript results in a 56% increase[104,105]. **b** | *mir-1* and *mir-133* are expressed in a bicistronic transcript in cardiac, skeletal and somitic muscle. miR-1 and miR-133 have distinct seed sequences but cooperatively regulate cardiogenesis. Loss of all miR-1 isoforms results in dilated cardiomyopathy with postnatal lethality, loss of all miR-133 isoforms results in ventricular septal defects with perinatal lethality, and the loss of both miR-133 and miR-1 isoforms results in circulatory defects with early midgestation lethality[106–108]. **c** | miRNAs with four distinct seed sequences are expressed from the *mir-17~92* cluster. In the schematic, miRNAs with common seeds are depicted in the same colour. These miRNAs interact genetically, yielding a more severe phenotype when simultaneously disrupted than would be expected from phenotypes of the individual miRNA knockouts (KOs)[109]. Cooperation between the heterotypic *mir-17~92* cluster is also supported by enrichment in the overlap of the targets. These phenotypes include diminished weight, reduced mesophalanx length, and lethality[109]. E, embryonic day, NA, not applicable; P, postnatal day.

have genome-wide support[111]. There are also suggestions of cooperative regulation by clustered miRNAs of separate targets in the same pathway[112], but whether this is an enriched feature of heterotypic clusters in the genome is an even more challenging consideration to assess. A systematic dissection of the functional impact of heterotypic clusters on targets and phenotypes is needed to fully understand the importance of cooperative targeting.

Emerging concepts and future directions

Advances in miRNA phenotyping. Technical advances will facilitate further resolution of miRNA functions. In cases where global miRNA loss results in clear phenotypes, the reintroduction or overexpression of individual miRNAs can efficiently uncover their roles in cellular processes[51,113,114]. miRNA-focused CRISPR–Cas9 libraries offer a modern approach to implement loss-of-function miRNA screens[115]. CRISPR–Cas9 approaches include targeting the miRNA itself, typically the seed sequence, or alternatively mutating surrounding sequences required for their biogenesis[116]. Given the high efficiencies of CRISPR–Cas9, it has been increasingly practical to make multiple deletions, including entire extended miRNA families[117]. It is also possible to take a reverse approach such as miRNA sponges, where ectopic target decoys are expressed to pair with miRNAs thereby reducing the pool available to suppress endogenous targets[118]. Such an approach can be particularly useful in interrogation of large miRNA families. For example, lentiviral sponges against miR-15a supported loss of miR-15a as a prostate cancer driver[119]. However, sponges typically result in only partial suppression[118]. While these techniques allow the more efficient interrogation of miRNAs, the subtle and/or hidden effects frequently associated with miRNAs will still require careful phenotyping driven by well-developed hypotheses.

Resolution of miRNA target sets. Understanding how a miRNA results in a phenotype requires the identification and interrogation of its targets. Uncovering physiological targets can be a difficult task. However, several recent advances should help elucidate miRNA–target relationships. As most mammalian miRNAs act through target destabilization, measuring changes in mRNA stability in response to miRNA perturbation provides a more direct and more sensitive measure of miRNA effects than simply measuring steady-state mRNA levels[65,120]. Another major advance is the incorporation of biochemically measured target affinities of several miRNAs within predictive models of all miRNA target binding affinities[121]. Notably, some miRNAs almost entirely bind canonical targets, whereas other miRNAs bind non-canonical targets with affinities similar to those for canonical targets[121]. If common features distinguish miRNAs with largely non-canonical targets, defining them will clarify the subset of miRNAs where biochemical affinities are most useful. These collective advances will help resolve the RNAs repressed by miRNAs across diverse cell types.

Elucidating the impact that post-transcriptional modifications of miRNAs and their targets have on miRNA–target binding relationships will also help refine target networks. miRNA modifications including uridylation and RNA editing have been shown to alter target repertoires, so each of these may influence targeting between cellular contexts as well[122,123]. On the target side, adenosine methylation on mRNAs that overlaps AGO-binding sites may alter miRNA target binding, in part through altering polyadenylation sites[124]. Characterizing these modifications should help resolve differences in miRNA–target relationships across cell types.

miRNA target contribution to phenotypes. The full target cohort contributing to many miRNA functions is unknown. The dogma of miRNAs targeting hundreds of transcripts is supported by target conservation and some expression profiles of miRNA-depleted samples. Compound-binding site knockouts to discern the set of targets required to phenocopy miRNA-knockouts would be the ideal approach, but remains technically challenging. Therefore, rather than empirical testing, selective genetic disruption of miRNA–target interactions based on strong hypotheses is likely the best current approach. The repression of BAF53A by miR-9 and miR-124, which is critical to activity-dependent dendritic outgrowth, was partially characterized by target site mutagenesis[47]. In another illustration, deletion of the miR-322 binding site in *Mek1* supported direct stabilization of *Mek1* by miR-322, counter to expectations of miRNA target destabilization[125]. Furthermore, studies selectively disrupting miRNA–target interactions in specific cell types would clarify the relevant targets in a particular context, as the positive selection of miRNA–target pairs may be based on roles in different cell types or during specific environmental perturbations.

Co-profiling miRNA and mRNA in single cells. Joint profiles of miRNA and mRNA expression from the same cells could help illuminate the dynamics of their relationship. Advances in single-cell RNA sequencing have provided unprecedented scale and resolution within populations to identify new cell types and help define the regulatory changes responsible for the transitions between cell types. Single-cell co-profiling of miRNAs with mRNAs will improve predictions and tests of the contribution that miRNAs make in cell state transitions, including trajectories of differentiations and stress responses. However, single-cell miRNA sequencing has lagged behind single-cell mRNA sequencing. In an early success, thousands of unique small RNAs were recovered per single cell by adapting and amplifying RNA with 5′-phosphate and 3′-hydroxy groups[126]. These high-resolution profiles efficiently separated cell types; since size selection was not used, the approach is scalable from the dozens of cells captured in the initial application[126]. Subsequently, miRNA and mRNA were simultaneously captured from 312 cells in the developing human cortex by inclusion of primers specific for mature miRNA species during the reverse transcription by the Fluidigm C1 (REF.[127]). This approach enabled the identification of dynamic miRNA–mRNA functional molecules contributing to cortical diversity. Another promising approach

is co-profiling by splitting cells in half and profiling miRNAs from one half and mRNAs from the other half of the pair[128]. The impressive fidelity of this approach was demonstrated by comparing either miRNA or mRNA profiles from two halves of the same cell: these mirrored samples showed impressive concordance, with correlation coefficients of 0.93 (REF.[128]). The high-resolution profiles from this 'half-cell' approach were sufficient to show anticorrelation between the most abundant miRNA in K562 cells, miR-92a, and its targets[128]. Moreover, the heterogeneity between single cells was used to predict a regulatory relationship, that AKT suppression activated *mir-146b* in K562 cells[128]. Scaling miRNA–mRNA co-profiles to larger populations has immense promise to dissect the function of miRNA during development and in physiology; when combined with miRNA disruptions, co-profiling would increase the resolution of phenotypes within populations, across stages of development and between different populations.

Conclusions

miRNAs are essential for the timing and execution of differentiation events during development, as well as the physiology of various cell types in mature organisms. At least 30 miRNAs are essential for normal mouse embryogenesis; other miRNAs are required for adult viability. However, the roles of most miRNAs remain unknown. Compensation by miRNA families or distinct miRNAs with common targets can mask the extent of a particular miRNA's function and complicate analyses. Dissecting the downstream targets of miRNAs adds additional complexity to dissecting their function as each miRNA has multiple targets and these targets can differs across contexts and can be influenced by RNA modification and RBPs. These often intimidating hurdles to tackling miRNA biology are subsiding with improving technologies to manipulate miRNA function and to phenotype the consequences of such manipulations.

Data availability

The authors declare that data supporting the figures in this study are available in the article and the Supplementary Information. The full source data for FIG. 1 are provided in Supplementary Tables 1 and 2.

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