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Canalization of development by microRNAs

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Animal development is an extremely robust process resulting in stereotyped outcomes. Canalization is a design principle wherein developmental pathways are stabilized to increase phenotypic reproducibility. Recent revelations into microRNA (miRNA) function suggest that miRNAs act as key players in canalizing genetic programs. We suggest that miRNA interactions with the network of protein-coding genes evolved to buffer stochastic perturbations and thereby confer robustness to developmental genetic programs.

Living systems maintain phenotypic stability in the face of perturbations arising from environmental changes and less-then-perfect gene regulation. Throughout evolution, there has been a pervasive selection for systems that are able to endure stochastic perturbations and reproduce stereotyped phenotypic outcomes. Canalization is the term coined to describe evolved robustness that decreases interindividual variability (see **Box 1** for a discussion of terminology).

'Canalization' can be visualized as the process of formation of virtual 'canals' in which developmental programs flow. The deeper the walls of these canals, the smaller the chances for the programs to deviate from the optimum[1–4]. Canalization is thought to be mediated by the buffering activity of specific genes; however, with the one notable exception of *hsp90* (refs. 5,6), there have been no successful attempts at explaining the molecular framework underlying canalization.

Loss of miRNA function rarely results in a dramatic phenotype

miRNAs are abundant gene-regulatory molecules, occupying between 1–5% of the genes in any given animal genome. Through binding of a minimal-recognition 'seed' sequence, miRNAs repress the expression of protein-coding mRNAs ('targets'), providing a previously unappreciated regulatory mechanism for gene expression[7]. Comparative phylogenetic studies uncovered conserved miRNA-binding sequences in more then one-third of all genes, suggesting that miRNA regulation may be relevant to a large portion of cellular processes (for recent predictions, see refs. 8–13).

miRNA function was first discovered through forward genetic screens, an approach that favors bold phenotypes[14–20]. miRNAs that were uncovered this way seem to have exceptional avidity to their targets: for example, the 3′ untranslated region of *lin-14* holds seven binding sites for the miRNA *lin-4* (refs. 14,15). However, only a small fraction of all messenger RNAs have more then a single miRNA binding site. Even more striking, as few as 2% of targets have more then two such binding sites, and only 0.02% of targets have as many as the seven exemplified by *lin-14* (refs. 8,10). Thus, the small set of miRNAs identified by virtue of clear loss-of-function phenotype is probably characterized by atypically avid miRNA-target relationships.

The global function of miRNA activity can be evaluated through inactivation of the RNase III–related enzyme Dicer, which is needed for generating functional miRNA from the pre-miRNA hairpin precursor. With one exception[21], genetic inactivation of Dicer in vertebrates does not severely affect differentiation and patterning[22–26]. Bearing in mind that hundreds of regulatory molecules are inactivated, and thousands of target genes are predicted to be derepressed, the observed effects are surprisingly subtle.

An miRNA network

One way to conceptualize the roles of the miRNAs is in the context of an integrated network emerging from summation of the interactions of miRNAs and targets. This network is highly wired and is biased toward certain cellular processes while omitting others[10]. At the molecular level, each miRNA molecule may target hundreds of mRNAs, and some targets are combinatorially affected by multiple miRNAs[8–10,27,28]. The wiring of most miRNA-target pairs depends on minimally complementary 'seed' matches[29] that are experimentally insufficient to confer overt repression[10,30]. Therefore, the network edges are tenuous, with the consequence that a traditional experimental setup will not be able to capture many facets of its function.

Network motifs for miRNAs

The wiring of genetic networks contains repeatedly used motifs such as the feed-forward loop (FFL). FFLs are composed of two input channels, one of which regulates the other; both channels jointly regulate the expression of a third component (the target gene)[31]. miRNA can also be intuitively viewed along this train of thinking (for example, see ref. 32), within at least two types of FFLs (**Fig. 1**). In the first type of FFL (a 'coherent' FFL; type 3; ref. 31) the miRNA is induced, directly or indirectly, by genes that repress the transcription of the target in the same cell where the miRNA is expressed. This enforces mutually exclusive domains for the target and its cognate miRNA[10,33,34]. The logic of this circuit is 'coherent' in that the posttranscriptional repression by the miRNA is synergistic with transcriptional inhibition of the same target. This is a failsafe mechanism that enhances the fidelity of the genetic program by safeguarding against 'leaky' mRNA in cells were the target genes are already repressed.

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BOX 1 TERMINOLOGY OF CANALIZATION

The terms genetic buffering, robustness and canalization are used to describe facets of phenotypic stability. Canalization was originally coined to describe the phenomenon of discrete developmental outputs[1]; however, in its long history, this definition was later expanded to include the ability of systems to withstand genetic or environmental perturbations. In the literature, the reader can often find the jargon used in a liberal way, such that genetic buffering, robustness and canalization are effectively used as synonyms (for recent examples, see refs. 4,64–66). However, evolutionary nomenclature distinguishes these terms as follows:

- Genetic buffering refers to inherited mechanisms that keep a trait constant and hence decrease the variance about the mean[3].
- Canalization is genetic buffering that has evolved under natural selection in order to stabilize the phenotype and decreases its variability[3].
- Robustness is the invariance of phenotype in the face of perturbations. Therefore, mechanisms such as genetic buffering (a cause) should underlie robustness (a result). Canalization, on the other hand, refers to evolved robustness under stabilizing selection.

At the root of the hypothesis presented here, we suggest that miRNA regulation satisfies the *bona fide* definition of canalization: that miRNA regulatory networks evolved under natural selection in order to stabilize the phenotype and decrease the variability of specific traits. Further, we predict that miRNAs may provide a molecular explanation for 'classic' canalized traits, including Rendel's study of scutellar bristle number[2,67] and Waddington's example of the Bithorax phenocopy[53]. Nonetheless, for purpose of flow and simplicity we use 'canalization' and related terms synonymously in accordance with refs. 4,64–66 such that 'canalizing activity' is actually 'genetic buffering' that evolved under stabilizing selection to decrease phenotypic variability.

How does canalization contribute to evolvability?

Canalized traits have an increased capacity to absorb mutational variance. This suggests that although a restricted range of phenotypic variations is demonstrated in canalized traits, they may actually hide relatively large genetic variations. These are dubbed 'cryptic genetic variations'[68], because in the canalized state, these genetic variations do not affect the phenotype and thus are not subject to natural selection. However, upon loss of canalization, these genetic variations are uncovered—one example being *hsp90* (refs. 5,6). Therefore, canalization potentially contributes not only to developmental robustness but also to evolutionary innovations[54,55,66,68].

The second network motif is the 'incoherent' FFL (type 1; ref. 31; **Fig. 1**), in which the same pathway that induces the expression of the target induces the miRNA. Coexpression of both components is 'incoherent' in the sense that two seemingly opposing processes are executed together. Plausibly, the miRNA is important in fine-tuning the expression level of the target gene[35]. One such example is that of c-myc, which induces the expression of *miR-17-5p* and *miR-20a*, along with the induction of the joint target, *E2F1*, which is repressed by the same miRNAs[36]. Specific examples of miRNA-target coexpression and global analyses suggest that these motifs are frequently used in the architecture of the network[13,37–41].

Viewing miRNAs in this 'systems' context leads to the realization that one of their important roles may be in the canalization of the genetic network. First, the dimension of added connectivity, *per se*, provides robustness to genetic networks[42,43]. Such connectivity is affected both by the linking of multiple targets through common regulation by individual miRNAs and by the feedback between targets and the miRNAs themselves[30]. More importantly, because of the nature of the coherent and incoherent FFLs, miRNAs contribute to the robustness of the genetic network in at least two additional specific ways.

miRNAs repress 'leaky' transcripts

First, miRNAs confer robustness to developmental genetic programs when an miRNA and its target set are expressed in mutually exclusive domains, as has been well documented in many cases[10,33]. In this setting, miRNAs repress targets that are of low abundance in the tissue where the miRNA is expressed[44]. For example, muscle-specific *miR-1* and neuronal *miR-124* repress the expression of 'leaky' transcripts that should be expressed in neighboring tissues but not in the cells where the miRNA is expressed[10]. The levels of the respective miRNA are very high, and the levels of the target transcript are virtually zero. Similarly, Hox cluster–related miRNAs *miR-196* and *miR-iab-4-5p* are expressed in nonoverlapping domains with their respective targets, *Hoxb8* and *Ubx*. *miR-196* enhances the fidelity of the vertebrate Hox program by cleaning up any unwanted *Hoxb8* transcript inappropriately transcribed in the hindlimb, and *miR-iab-4-5p* is likely to serve a similar aim in the developing *Drosophila melanogaster* embryo[33,34,45] (**Fig. 1**). For such a relationship to be possible, the miRNA and the target must be reciprocally affected by the milieu of transcription factors inside the cell. The many cases in which miRNAs are expressed in high levels, but in which their targets are undetectable, imply that miRNAs and targets evolved reciprocal *cis* binding sites for the regulation of transcription. Therefore, the interaction of miRNA and target with the transcriptional milieu can be schematized in accordance with the coherent FFL (**Fig. 1**).

Differentiation of cells during development is achieved through changes in the milieu of transcription factors. For example, when neuronal tissue differentiates from ectoderm, its expression program is rewired. This includes tissue-specific miRNAs, which are expressed to canalize the neuronal and non-neuronal fates. Neurons highly express *miR-124*, whereas other ectodermal derivatives express *miR-9a* and *miR-279*. In parallel, the 3′ UTR of ectodermal genes enrich for *miR-124* sites, whereas genes expressed in neuronal tissue enrich for *miR-9a* and *miR-279* sites[10] (**Fig. 2**). By repressing genes of sibling tissues, tissue-specific miRNAs decrease the chances of fate ambiguity. In zebrafish, *miR-430* is involved in the maternal-to-zygotic transition. The temporal reciprocity of this miRNA with many of its targets[41] implies that the miRNA is wired into multiple coherent FFLs in which the transcriptional activation of *miR-430* in the zygote facilitates the clearance of (transcriptionally suppressed) maternal mRNAs. Intriguingly, lack of *miR-430* does not arrest the embryo in a maternal state, suggesting that although *miR-430* sharpens developmental transition and hence confers robustness to the zygotic program, it does not act like a genetic switch.

miRNAs buffer genetic noise

Unlike the previous situation, in which the miRNA is typically expressed in much higher levels than the target, a second class of genetic buffering by miRNAs emerges in cases where the miRNA and the target

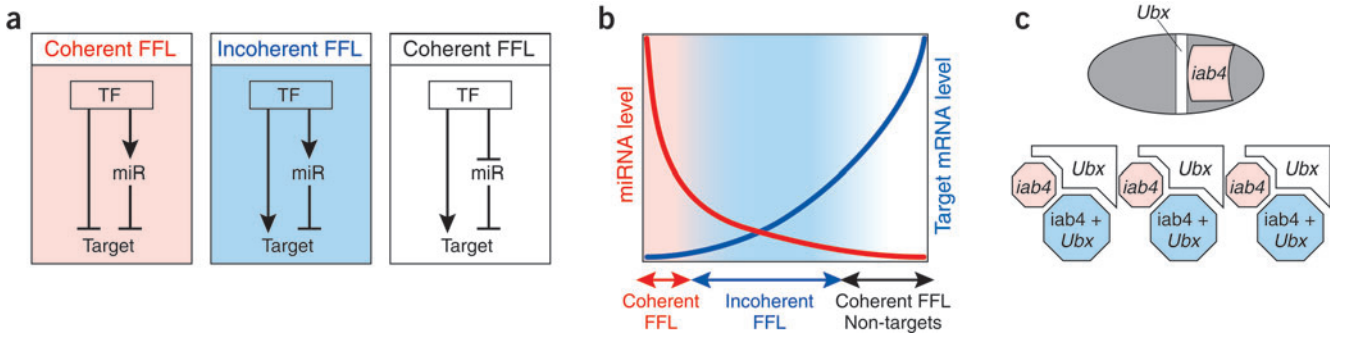

Figure 1 The architecture of the miRNA-target network. (**a**) The logics of the miRNA interaction with target genes can be viewed in the context of feed-forward loops (FFLs)[31]. In the coherent FFL on the left (type 3, pink), the cellular milieu of transcription factors (TF) generates a transcriptional channel to repress a downstream target gene. Simultaneously, the transcription of an miRNA creates a safeguarding post-transcriptional channel that further represses leaky mRNA. Reciprocally, transcription factors may activate target transcription and repress miRNA production (type 4, right, in white). Alternatively, an incoherent FFL results from coactivation of miRNA and target transcription (center, blue). (**b**) When wired into a coherent FFL, a mutually exclusive expression pattern emerges for the miRNA and its target in which miRNA levels (red line) are high, and target levels (blue line) approach zero (pink background; color-coded in accordance with the FFLs in **a**) or vice versa (white background). However, when coexpressed (center, blue background), the miRNA is likely to have a role in reducing the noise during target expression. (**c**) In the early *D. melanogaster* embryo (top), domains of mutually exclusive expression of *miR-iab-4-5p* (*iab4*, pink) and its target, the homeobox transcription factor *Ubx* (white) are observed. Additionally, after gastrulation, a third expression domain type can be visualized (bottom) in which *miR-iab-4-5p* and *Ubx* are coexpressed (blue). Colors correspond to colors used in FFLs in **a** and **b**. Schematic drawing of *D. melanogaster* embryo in **c** is loosely based on stained *D. melanogaster* embryos in ref. 34.

are coexpressed in intermediate levels[13,40,41]. A likely role of the miRNA in such situations is to buffer fluctuations in the target expression at a posttranscriptional point. Why is this necessary? The activation of promoters has a marked stochastic component, which results in random fluctuations of transcript number. This genetic 'noise' may perturb the nominal function of the genetic program and thus be detrimental to phenotype. Noise-buffering mechanisms exist in order to confer robustness to genetic pathways (reviewed in refs. 46,47).

Transcription rate correlates inversely with noise[48–50]. Thus, when a gene is expressed at very low levels, stochastic changes in the activation of its promoter are more prominent. Additionally, regardless of the transcription rate, noise increases linearly with translation rate, so that strongly translated genes are implicated in particularly noisy processes[51,52]. Taken together, robustness is gained in pathways in which frequent transcription is followed by inefficient translation[46]. In our view, intermediate miRNA concentrations and low target avidity (the result of a single 'seed' binding site) intentionally allow for target protein synthesis but place a burden on this process, which most likely provides a means to buffer stochastic fluctuations in mRNA levels. Coexpression reflects similar response to cellular transcriptional cues, so we expect to find noise reduction performed by miRNAs embedded in incoherent FFLs (**Fig. 1**).

We suggest that the reason one does not necessarily observe a striking phenotype in the absence of miRNAs is because they often serve a different purpose from primary gene regulation.

miRNAs buffer genetic noise of coexpressed targets within the incoherent FFL architecture and curb leaky transcripts of supposedly 'shut-off' promoters, in a coherent FFL. It is worthwhile to note that dampening the stochastic firing of a shut-off promoter can be articulated as a private case of noise reduction. This is because the apparent readout value of such a shut-off genetic circuit should be zero, and thus any erroneous transcription event is 'noisy'.

Our hypothesis implies that the miRNA-target circuitry evolved under stabilizing selection to enhance robustness. Furthermore, we predict that miRNA FFLs are instrumental in reducing phenotypic variability in the population, thereby fulfilling the requirement to view miRNAs as canalizing genes (**Box 1**).

The buffering effect of the miRNAs decreases the penalty paid for mistakes in gene regulation. Mild genetic variations affecting either the expression levels of transcription factors or *cis* binding sites in promoters will have no consequences. On an evolutionary scale, this enables mutations (cryptic genetic variations, **Box 1**) to silently accumulate without being subjected to selective forces and thus may contribute to evolvability[53–55].

Clues of canalization by miRNAs

A recent knockout study of *D. melanogaster miR-1* fits nicely with the view of miRNA-based canalization. *miR-1* is a muscle-specific miRNA, conserved in sequence and expression pattern throughout *bilateria*. Intriguingly, the loss of *miR-1* does not affect muscle differentiation in *D. melanogaster*. The musculature comes to a phenotypic crisis only in

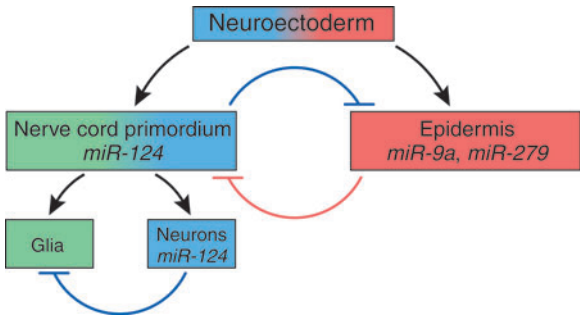

Figure 2 miRNAs may sharpen developmental transitions. Mutual exclusion of miRNAs and respective target sets was nicely characterized in *D. melanogaster* for the developing neuroectoderm[10]. Two derivates of the neuroectoderm, the nerve cord primordium and the epidermis, express miRNAs that target genes of the alternative fate. Thus, *miR-124* is expressed within the nerve cord primordium (green-blue), whereas epidermal cells express *miR-9a* and *miR-279* (pink). Further differentiation into neurons makes use of *miR-124* to represses the sibling glial fate (blue).

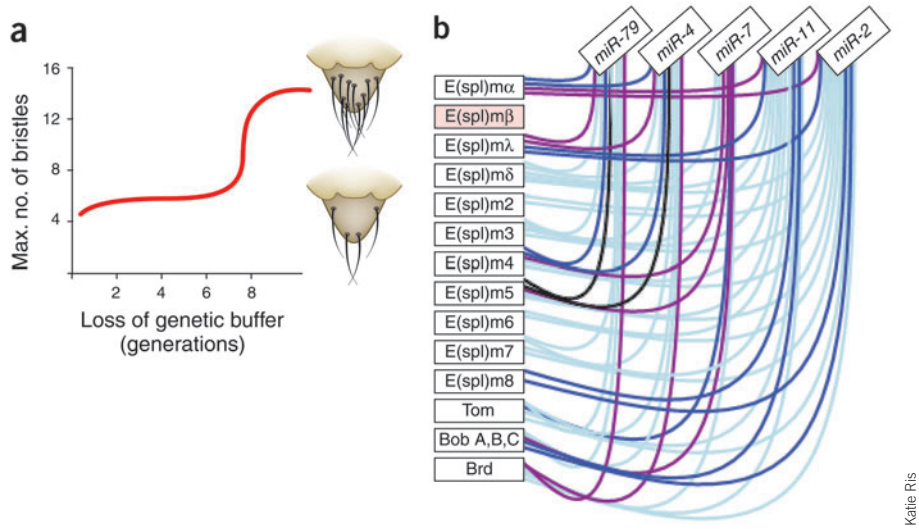

Figure 3 miRNAs are suggested to canalize the pathway controlling scutellar bristle number. (**a**) Selection for decanalization of the scutellar bristle phenotype results in increased maximal bristle number and increased variance[2]. At about eight generations under selection, a critical point is reached in which the genetic buffer breaks down, thereby decanalizing the pathway controlling bristle number. At right, a scheme of wild-type (lower) and hypernumerous (upper) scutellar bristle phenotype in adult *D. melanogaster* is shown. (**b**) A dense network of miRNA interactions with the *E[spl]-C* and *Brd-C* mRNAs[59,60] conceivably has a major role in providing robustness to the pathway that sets the number of scutellar bristles in the proneural field. Lines are color-coded according to number of miRNA binding sites: one, light blue; two, navy blue; three, purple; or four, black.

the stressful 'rapid growth' phase[56]. This observation is interpreted as loss of robustness of the muscle phenotype[57]. *miR-1* is expendable for the program of muscle differentiation, but in its absence, the phenotype is not canalized and is frequently forced away from the optimum under growth stress and possibly by other insults.

A second example that correlates the presence of miRNA regulation with canalization can be observed in wild-type *D. melanogaster*, in which the number of scutellar bristles almost never varies from fly to fly. Multiple studies of bristle formation within the proneural field suggest that a buffering mechanism canalizes the phenotype[2]. Under experimental selection, this buffering capacity can be lost, underscoring the genetic source of the canalizing activity. After a few generations of selection, the number of bristles starts increasing linearly with additional insult, and so does the variance about the mean bristle number (**Fig. 3a**). Mathematical modeling of this network suggests that it is highly robust to changes in effective gene dosage and to changes in its topology, thanks to the canalization of a circuit comprising the *E[spl]-C* genes[58]. Intriguingly, *E[spl]-C* genes are directly regulated by multiple miRNAs[59–61] (**Fig. 3b**). It will be interesting to see if, indeed, the *E[spl]-C* miRNA network has a role in maintaining low variability, and if loss of miRNA function correlates with the pathway's decanalization.

A third classic example of canalization that can be understood in the light of recent reports on miRNA biology concerns the homeotic genes, which are responsible for defining the identity of the various body segments along the anterior posterior axis[62]. The expression of one of these homeotic genes, *Ultrabithorax* (*Ubx*), sets the fate of the haltere, a balancing organ that develops instead of a second set of wings in true flies. In wild-type *D. melanogaster*, homeotic abnormalities such as transformation of a haltere into a wing are almost nonexistent. This low phenotypic variability relies on the strong genetic canalization of *Ubx*. Furthermore, experimental selection for loss of *Ubx* canalization often results in a classic haltere-to-wing transformation[53,62]. The mechanisms that canalize the *Ubx* phenotype are unknown, but genetic evidence suggests dependence, at least partially, upon sequences in the *Ubx* 3′ UTR[63]. Strikingly, this correlates well with the presence of binding sites in the 3′ UTR of *Ubx* for *miR-iab-4-5p*, a Hox cluster miRNA[12]. Further, the misexpression of *miR-iab-4-5p* in the haltere disc phenocopies the 50-year-old observation of decanalized *Ubx*[34,53]. The patterns of miRNA and mRNA expression might provide clues to the mechanism by which the miRNAs confer robustness to *Ubx*. In the early embryo, mutually exclusive expression suggests that *miR-iab-4-5p* is directing the cleavage of leaky *Ubx* mRNA in its domain, whereas later in embryonic development some domains coexpress *miR-iab-4-5p* and *Ubx*, implying function as a noise reduction module[34] (**Fig. 1c**).

Conclusions

Classic studies of canalized traits, such as the setting of scutellar bristle number and the prevention of the *Ubx* phenotype, are converging with our new understanding of miRNA biology to enable a new view of canalization. miRNAs generate a network that confers robustness to genetic pathways in at least two ways: first, miRNAs repress 'leaky' target mRNAs in tissues where they would be disruptive or deleterious. This is accomplished by high levels of miRNA expression in cells where targets are not transcribed, using the logics of a coherent FFL. At the same time, posttranscriptional control of genetic noise is carried out by miRNAs that are coexpressed with their targets, such that the miRNA and target are wired into an 'incoherent' FFL circuit. Thus, the miRNA network provides a molecular framework that may explain many aspects of the 65-year-old 'canalization' principle.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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