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MicroRNAs and their roles in developmental canalization

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Abstract

Robustness is a fundamental property of biological systems. The type of robustness that ensures uniform phenotypic outcomes in the face of variation during an organism's development is called canalization. Here, we discuss the roles that microRNAs play in providing canalization to animal development, citing recent theoretical and experimental advances. MicroRNAs repress protein expression, and they do this in ways that create thresholds in expression and provide adaptation to regulatory networks. Numerous examples have now been described where the developmental impact of environmental variation is suppressed by individual microRNAs. A recent paper has found that the impact of genomic variation between individuals is similarly suppressed by a microRNA operating in a developmental network. Thus, genetic variability is held in check, which is potentially important for both animal evolution and manifestation of disease.

Organisms are naturally subject to fluctuating environments, and yet their morphological development is generally robust to such challenges. Indeed, robustness is a universal emergent property of living systems. The inverse relationship between developmental robustness and morphological variation in natural populations has long been remarked upon [1], and Waddington coined the word canalization to describe the process [2]. The stronger the canalization of development, the less phenotypic variation exists among individuals in a population. Hence, the impact of environmental variation on a population's phenotypic variation becomes suppressed.

Canalization also decouples the effects of genomic variation on development, which geneticists have long described by the incomplete penetrance and expressivity of morphological phenotypes [3]. This genome-suppressing property of canalization has implications for evolutionary mechanisms. In one sense, canalization should inhibit evolvability since it suppresses the phenotypic variation that selection acts upon (Fig. 1A). However, if a phenotype is robust to the effects of genome variation, then this variation can accumulate without affecting the phenotype. When canalization becomes impaired, then this genomic diversity suddenly expresses phenotypic variability, which can be subject to selection, thus potentially accelerating evolvability. There are two routes to impairing canalization. One is to disable by mutation the molecular mechanisms that generate

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robustness. The other is to overwhelm the canalization apparatus by environmental stress. It has been speculated that under times of sudden environmental change in Earth's history, impaired canalization caused pre-existing genotypes to rapidly switch from being neutral to being potentially adaptive [1].

The extent of overlap between canalization of environmental and genomic variation has been a subject of intense debate [1,4,5]. Waddington himself explored the interactions between environment and the genome in a series of artificial selection experiments [6,7]. Under controlled environmental stress, developing *Drosophila* expressed novel phenotypes that could be selected upon and enriched over multiple generations. Other experiments aimed at analyzing canalization have relied upon selection of phenotypes that were uncovered by gene mutation [3]. Indeed, it is striking how many regulatory gene mutations not only alter a trait's phenotype but also enhance the variation in phenotype, a hallmark of impaired canalization [8]. From a systems perspective, it might argue that disruption of key nodes in a developmental network can impair both the robustness and performance of the network (Fig. 1B).

The importance of protein chaperones for canalization of development has been demonstrated [9,10]. Deliberate weakening of HSP90 activity causes an extensive unmasking of phenotypic variation, some of which can be selected upon and therefore has an underlying genetic component. It is intuitive that protein chaperones might fulfill such a function since they assist in the folding/unfolding of proteins and assembly/disassembly of protein complexes. These processes are dependent upon probabilistic events, and chaperones provide greater deterministic behavior. Hence, genomic or environmental variation that affect such probabilistic events would be suppressed by chaperones.

It also seems intuitive that some regulators of gene expression might be capable of suppressing phenotypic variation. Here, we explore the mounting evidence that small non-coding RNAs have this property. Non-coding RNAs are not the sole regulatory arbiters of canalization; extensive though circumstantial evidence has found that transcription factors can suppress phenotypic variation [8,11]. However, we refer the reader to other reviews that discuss these factors [12,13].

MicroRNAs

MicroRNAs (miRNAs) are gene regulators that inhibit the protein output of their targets [14,15]. These noncoding RNAs are transcribed from genes in the genome following the same rules as protein-coding genes. After a nuclear processing step, the pre-miRNA products are translocated to the cytoplasm. There, Dicer processes them to create duplex miRNAs of approximately 21 bp length. The duplexes are short-lived since they are rapidly unwound when they associate with members of the Ago protein family. The miRNA-bound Ago proteins seek out and hybridize with complementary sequences, called target sites, found within particular mRNA transcripts. Target sites are typically found in the 3’UTR of mRNAs [16,17]. Almost all cellular mRNAs contain miRNA target sites, and the majority of these contain target sites for multiple miRNAs.

The key feature of target site recognition involves Watson-Crick base pairing of miRNA nucleotides 2–8 with mRNA, representing the seed region [14]. Base pairing mismatches are usually found at miRNA nucleotide 10, thus preventing Ago-catalyzed cleavage of the mRNA strand, and instead promoting repression of mRNA translation. Ago-miRNA interactions with target transcripts often lead to transcript destabilization as well [18,19]. Importantly, protein output from most miRNA targets is modestly repressed by miRNAs [15]. The widespread scope but low magnitude of miRNA-mediated regulation indicates that miRNAs primarily tune protein output from the genome.

A growing body of evidence suggests that miRNAs can stabilize processes that control development. Some miRNA genes, when mutated, lead to increased variance of quantitative traits because of perturbed development [20-23]. This reduced phenotypic robustness would suggest that canalization has been compromised in these situations. Indeed, synthetic approaches have shown that protein expression is buffered from transcription noise by miRNAs [24].

Canalization of the environment

The ever changing environment forces organisms to deal with fluctuating conditions every day. Laboratory animals are probably the only ones living the utopia of a non-stressful life, and that is one reason why environmental robustness studies are not more common. Another challenge is to accurately simulate natural fluctuations in the laboratory since we know little about the conditions to which model species are adapted to. A few variables are obviously important for many animals to develop normally: temperature, food, crowding, hydration. Most canalization studies have relied upon varying one or more of these conditions. There is an extensive literature detailing how animal development compensates for environmental perturbation (e.g. [25]). However, the challenge has been to define the molecular mechanisms that mediate such compensation. We describe the potential roles that miRNAs and other small RNAs might play.

Several studies have shown that non-optimal environmental conditions can modulate the abundance of miRNAs. For example, miRNA genes are differentially expressed when crowding or nutrient restriction induce dauer development in *Caenorhabditis elegans* [26], and light levels can regulate miRNA abundance in the mouse retina [27]. Stress can also increase target transcript abundance, titrating away free miRNAs [28] , and stress can affect Ago miRNA silencing activity [29,30].

Some miRNA mutant phenotypes appear only when certain external stresses are present. The *Drosophila* miR-7 gene is one such example [31]. Although miR-7 acts within two gene regulatory networks that control differentiation of sensory neurons, loss of miR-7 has negligible impact on their differentiation under uniform temperature conditions. However, when individuals are subjected to modest temperature fluctuation during development, miR-7 becomes essential for normal gene expression and robust neuronal differentiation. In other situations, miRNA mutant phenotypes become enhanced by environmental conditions. Loss of miR-8 causes pigmentation defects that are strongly enhanced when *Drosophila* are raised at elevated temperatures [32].

In *C. elegans*, the nuclear receptor DAF-12 promotes diapause and formation of dauer larvae when conditions are unfavorable due to starvation, crowding or high temperature. Hochbaum et al. [33] showed that development of *daf-12* mutants was hypersensitive to temperature fluctuations. Interestingly, DAF-12 regulates transcription of genes that encode miRNAs and their protein cofactors, and knockdown of these transcription targets causes much greater variability in dauer development. This study suggests that canalization depends upon the collaboration between transcriptional and post-transcriptional mechanisms.

It is even possible that other noncoding RNAs such as siRNAs can participate in developmental canalization [34]. When an early *Drosophila* embryo is subjected to temperatures that vary with position (its anterior domain is incubated at a different temperature from its posterior domain), then non-synchronous development ensues. However, at a defined stage, the faster half slows down and allows the slower half to catch up [34]. This compensation is sufficient to allow the embryo to survive and produce an adult fly. Mutation of either the Dicer or Ago gene specific for the siRNA pathway causes such embryos to fail to compensate [34]. Under uniform temperatures, the mutant embryos develop normally and are fully viable. Thus, even siRNAs can participate in environmental canalization.

Canalization of the genome

Canalization suppresses the ability of genomic variation to contribute to phenotypic variance. The heritability of phenotypic variance is used to identify and quantify the genomic contribution. Heritability can be measured in many ways, the most famous being human twin studies [35]. Artificial selection has also been used to estimate heritability, but in this case the heritability is narrow-sense or realized. It reflects the additive effects of genomic variation on phenotypic variance.

Cassidy et al. [22] performed a controlled selection experiment to measure realized heritability for a developmental trait in *Drosophila*. Patterning of the adult dorsal thorax with sensory bristles is highly precise; the scutellum invariably has four bristles per individual. Impaired transcription of two proneural genes causes bristle number to both decrease on average and become much more variable [36]. A sizable proportion of this phenotypic variability has a genetic component, as estimated by heritability measurements [37]. Hence, the developmental mechanism becomes less canalized due to proneural gene error. Cassidy et al. wanted to see if impairing miR-9a further crippled canalization of the genome. This miRNA was chosen because it targets a key proneural gene called *senseless* (*sens*), and its loss results in more variable bristle number phenotypes [21]. Selection experiments showed that miR-9a heterozygosity was sufficient to increase heritability two- to three-fold [22]. In other words, copy number reduction weakened canalization of the genome. A similar effect on canalization was seen if the binding sites for miR-9a in the 3’ UTR of *sens* transcripts were mutated. These findings suggest that a single miRNA-target interaction can help canalize genomic diversity from affecting a developmental outcome.

The authors then went on to ask what regions of the genome were normally being canalized. They performed DNA sequencing of pooled genomes from populations before and after selection. Fewer than 99.8% of identified SNPs showed signs of responding to selection when miR-9a was intact, but the number of responding SNPs jumped two-fold when miR-9a copy number was reduced. These additional SNPs were clustered in a handful of loci across the *Drosophila* autosomes. It suggests that miR-9a reduction enabled pre-existing genomic variants within the population to affect bristle development more effectively. This result fulfills a key prediction of the canalization model.

How might miRNAs contribute to canalization?

There are at least two different ways that miRNAs could provide robustness to gene expression, resulting in developmental canalization. One is the “weak-buffer” mechanism. A simple miRNA-target interaction can create a threshold in the relationship between mRNA and protein outputs from the target gene [38]. Below the mRNA threshold, protein output is silenced because free miRNA is in excess over target transcript. Above the threshold, protein output becomes highly sensitive to transcription because the target transcript is in excess over free miRNA. This means that protein output is insensitive to fluctuations in transcript concentration when transcription is low and gene expression can potentially exhibit the greatest noise. The interaction between miR-9a and *sens* has all of the developmental hallmarks of this weak-buffer mechanism [21,22].

Another way robustness can be generated is by adaptation. Biological systems frequently adapt to a change in stimuli, returning at steady-state to the same output they started from. Adaptation can be achieved if a network contains negative feedback between key components [39]. Another motif called the incoherent feedforward loop can accomplish the same outcome [40]. In this motif, an upstream factor regulates output from a downstream gene by two routes; one route is direct and the other route is through an intermediate factor. Each leg opposes the other, with one leg increasing and the other leg decreasing downstream gene output. A majority of mammalian miRNAs are computationally predicted to function in feedfoward motifs [41], and incoherent regulatory relationships involving mammalian miRNAs are not infrequent [18]. Strikingly, miR-7 functions in at least one incoherent feedforward loop that regulates target protein expression in the *Drosophila* eye [31].

Conclusions

Robustness is an essential feature of living systems and yet its mechanisms are poorly understood. We can see how miRNA-mediated gene regulation provides robust development to model organisms that are challenged with environmental or genomic variation. Important aims for the future will be to discover: the scope of this functionality within the miRNA phylogenetic family, the molecular mechanisms by which it is achieved, and the impact it has had on evolution. Another exciting direction will be to explore the impact on human disease, which is frequently caused by environmental and genetic stresses.

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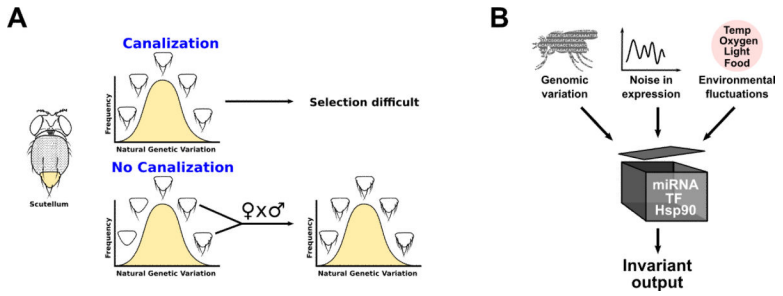

Figure 1.

A) Canalization of a developmental trait against natural genetic variation. The example shown is the development of mechanosensory bristles on the *Drosophila* scutellum, which are invariably four in number per individual. If this trait is canalized, then genetic variation has no impact on phenotypic variation, making selection of novel heritable traits difficult. If canalization is reduced, then the same natural variation induces phenotypic diversity. This allows selection of heritable phenotypes such as five or more bristles per individual. **B) Developmental robustness.** Animal development compensates for genomic, stochastic and environmental perturbations. Diverse mechanisms are thought to suppress phenotypic variation, including microRNAs, transcription factors, and chaperones.

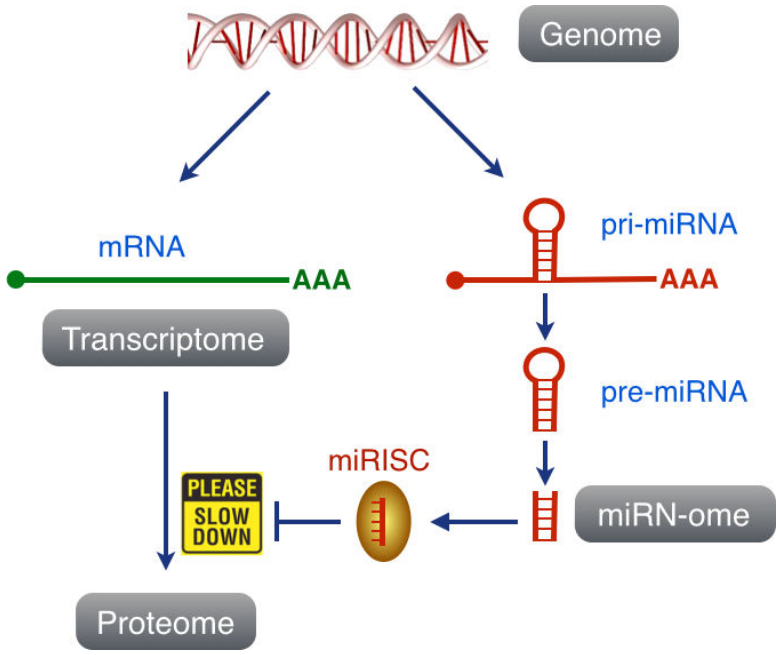

Figure 2.

Production of proteins within the proteome is modestly attenuated by microRNAs that associate with target mRNAs within the transcriptome. Both mRNAs and pri-miRNAs are transcribed by RNA polymerase II, and capped and polyadenylated.

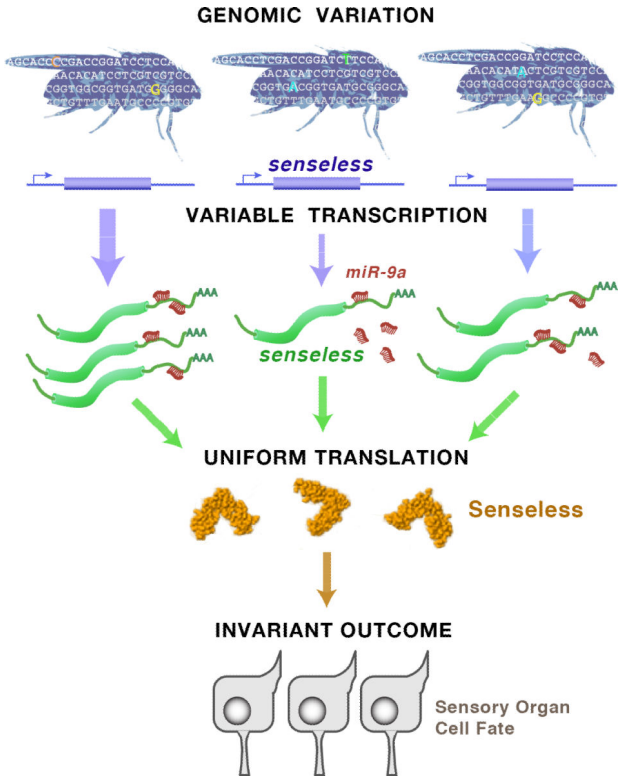

Figure 3.

Cells of *Drosophila* that potentially form sensory organs transcribe senseless mRNA. This occurs through a complex gene regulatory network. Individual flies bear abundant genomic diversity, some of which affects the level of senseless mRNA. Thus, senseless mRNA abundance varies between individuals. The microRNA miR-9a represses production of Senseless protein by associating with senseless mRNA, normalizing Senseless protein levels between individuals. This ensures a reliable outcome in choice of four scutellar sensory cells, which does not vary between individuals.

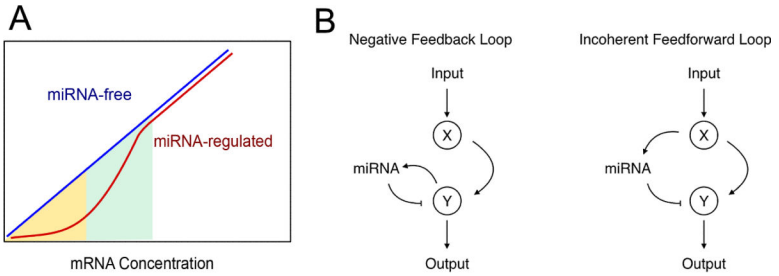

Figure 4.

A) A weak-buffer mechanism for gene regulation by a miRNA. Nonlinear protein output from gene transcripts under the regulation of a miRNA will generate a threshold (light blue), below which protein output is insensitive to fluctuations in transcript concentration (yellow). Above the threshold, output is fully responsive to transcript concentration. Such thresholding when coupled to feedback can create switch-like behavior in regulatory networks. **B) Network adaptation**. In a network with negative feedback between miRNA and target, repression is proportional to output strength, making the network adapt to variation in input. In a network with an incoherent feed-forward loop with a miRNA, a similar adaptation of output to input variation is also possible.