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Davide Cacchiarelli, Daniele Santoni & Irene Bozzoni

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Point of View

microRNAs as prime players in a combinatorial view of evolution

Davide Cacchiarelli, Daniele Santoni and Irene Bozzoni*

Institute Pasteur Cenci-Bolognetti; Department of Genetics and Molecular Biology and I.B.P.M.; University of Rome "La Sapienza"; Rome, Italy

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microRNA-mediated gene regulation allows the establishment of complex circuitries acting in many different phases of development and differentiation. MicroRNA genes and their target sites are under Darwinian selection and the idea that microRNAs can act as prime players in determining species identity has been recently speculated. By studying the 3'-untranslated regions (UTRs) of orthologous neuronal genes, we found that, at variance with house-keeping genes, the density of miRNA target sites involved in complex cell networks increased from invertebrates to human, paralleling the increase in species complexity. This suggests that genes contributing to complex cellular functions had a selective advantage for acquiring and potentiate miRNA-mediated regulation.

Gene duplication, exon shuffling and alternative splicing, events that allow organisms to acquire new functions and on which selection can eventually operate, have provided powerful means to increase coding complexity and drive genetic evolution in eukaryotes.[1]

However, such mechanisms are not sufficient to justify the huge evolutionary gap between the different eukaryotic classes (invertebrates, fishes, amphibia, reptiles, birds and mammals). In fact, the diversity among eukaryotes cannot be explained neither by the sheer number of genes (the number of *D. melanogaster* genes is only half of the human ones), nor by the number of transcripts (the alternative splicing rate is quantitatively similar for all considered eukaryotes).[2] Furthermore, a fraction of human alternative splicing transcripts does not lead to functional products.[3] Therefore, it has been suggested that evolutionary jumps might be due to the combinatorial ability to use the same basic elements to produce new functional entities and to finely tune gene expression in response to increasing varieties of stimuli.

miRNAs are good candidates to be placed in this network as possible players in the "evolutionary override" towards more complex functions. They are tiny non-coding RNAs, highly conserved among distantly related species, whose function in metazoa is to control RNA stability and translation through incomplete base paring with the target sequences in the 3'UTR of messenger RNAs.[4]

*Correspondence to: Irene Bozzoni; University of Rome; LA SAPIENZA; P.le A. Moro 5; Rome 00185 Italy; Tel.: +39.06.4991.2202; Fax: +39.06.4991.2500; Email: irene.bozzoni@uniroma1.it

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Because individual miRNAs can regulate hundreds of different targets, their mode of action provides a way to pleiotropically control gene expression without affecting transcription. It can be derived that mutations in 3'UTR sequences can introduce or eliminate miRNA target sites and confer new negative or positive regulations to a specific mRNA. This action, along with miRNA complexity, estimated to comprise 1–5% of animal genes,[5] can amplify the process, with the potential to deeply influence phenotypic complexity and diversity along animal phylogeny.

A significant example useful to explain how miRNAs are responsible for deep changeovers during evolution, is the species-specific expression of 20 miRNA families that are essential for the correct organogenesis of all higher bilateria, but absent in lower metazoa.[6] Accordingly, recent findings suggest that in human a large set of miRNA is primate-specific.[7]

The miRNA regulatory system was shown to have a strong impact on how genes have acquired or lost miRNA target sequences: in fact, in *D. melanogaster*, ubiquitously expressed house-keeping genes, such as those coding for proteins involved in ribosome biogenesis (r-proteins), escape miRNA targeting by exclusion of target sites and by having short 3'UTRs.[8] On the contrary, transcripts involved in complex circuitries, such as neurogenesis, were shown to be more susceptible to miRNA regulation, and to have longer 3'UTRs.[8]

In order to study whether this difference might have evolutionary significance on how different classes of genes evolved their way of undergoing different levels of regulation, we extended this analysis to the same two classes of transcripts in distantly related organisms.

Initially, analyzing Gene Ontology collection data obtained from A. Stark and S. Cohen,[8] we filtered out 364 *D. melanogaster* neurogenesis (GO:0007399) and 128 ribosomal protein (GO:0005840) genes looking for orthologs conserved in human quering ENSEMBL database. We obtained a subset of neuronal and r-protein mRNAs conserved between the two species (see Suppl. Materials).

Figure 1A shows the comparison of 3'UTR lengths for both types of transcripts where it appears that both in *H. sapiens* and in *D. melanogaster*, house-keeping genes tend to have much shorter 3'UTRs than neuronal genes as already revealed in *D. melanogaster*.[8] The predicted 3'UTR sequences were then used to query miRBase program[9] in order to obtain the number of miRNAs targeting each transcript. We obtained a miRNA target site average density value for the two species for both neuronal and r-protein transcripts (Fig. 1B). To validate our computational approach through the miRBase algorithm, we compared the analysis performed on our *D. melanogaster* transcript subsets with previous 3'UTR length

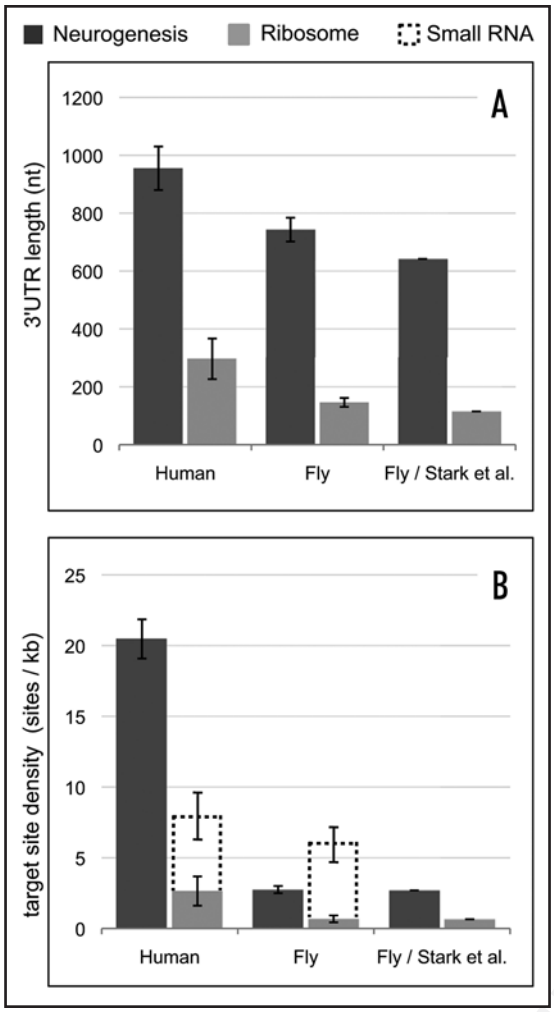

Figure 1. Estimation of 3'UTRs length and miRNA target site density of orthologous neurogenesis and ribosomal protein transcripts in *H. sapiens* and *D. melanogaster*. (A) shows the 3'UTR length average for for 162 orthologous neuronal transcripts (black bars) and for 100 orthologous ribosomal genes (grey bars). The histograms Fly/Stark are taken from Stark et al.[8] (B) shows the miRNA target density average for neuronal (black bars), ribosomal (grey bars) and sncRNA transcription and processing (dashed bars) proteins. An unpaired t-test performed with the ensemble of the mammalian neurogenesis transcripts against each one of the ensemble of the other animals indicated that the reported differences are statistically significant ($p < 0.001$). Species abbreviations: Human, *Homo sapiens*; Fly, *Drosophila melanogaster*.

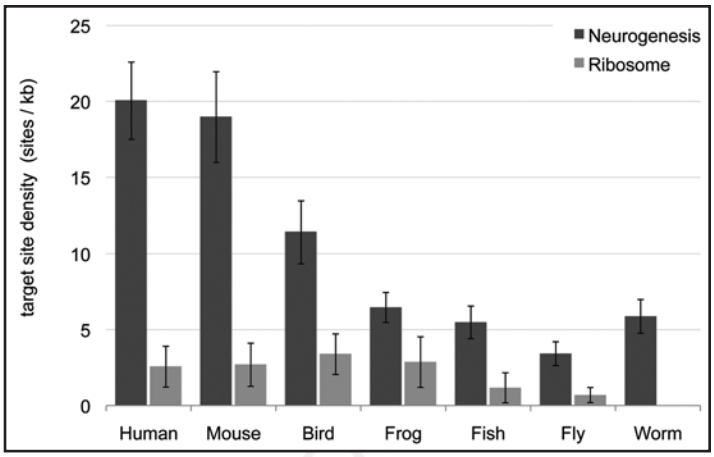

Figure 2. Estimation of miRNA target site density in the 3'UTRs of 65 orthologous neurogenesis and 24 ribosomal protein transcripts from seven different eukaryotes. The number of target sites obtained for each transcript was normalized for its 3'UTR length obtaining a miRNA density value. For each species, the miRNA target site density average for the neuronal transcripts is indicated by black bars, while for the ribosomal genes, is shown by grey bars. No data on the 3'UTR length of r-protein transcripts from *C. elegans* are shown since they are reported to be only few nucleotides. An unpaired t-test performed with the ensemble of the mammalian neurogenesis transcripts against each one of the ensemble of the other animals indicated that the reported differences are statistically significant ($p < 0.001$). Species abbreviations: Human, *Homo sapiens*; Mouse, *Mus musculus*; Bird, *Gallus gallus*; Frog, *Xenopus tropicalis*; Fish, *Danio rerio*; Fly, *Drosophila melanogaster*; Worm, *Caenorhabditis elegans*.

and miRNA density analysis performed by Stark et al. The output obtained is very similar in the two cases (compare histograms Fly and Fly/Stark et al., in Fig. 1B).

The results indicate a great increase (eight-fold) in miRNA target sites in human neuronal genes with respect to the insect orthologs. As an additional housekeeping gene control we analized the genes involved in the transcription and processing of small non coding RNAs, such as tRNA, snoRNA and snRNA (see Suppl. Materials). The results indicate that there is no increase in miRNA target site density from Drosophila to human also for this type of housekeeping genes (Fig. 1B).

The increase in number of miR target sites in human neuronal transcripts with respect to fly cannot be simply accounted by the increase in miRNA complexity: in fact, while the number of miRNAs increases by a factor of 4 (152 in fly and 678 in human), the miRNA density in neuronal transcripts increases by eight-fold.

The analysis was then extended to seven eukaryotic organisms of different clades: five vertebrates, a fly and a nematode. Figure 2 shows that the miRNA target density for neuronal transcripts linearly increases along the evolutionary scale, reaching a maximum in mammals. On the contrary, the miRNA target site density in the 3'UTRs of r-protein transcripts remained similar in all animals. These findings indicate that transcripts specifically expressed during neurogenesis had a selective evolutionary advantage in acquiring miRNA target sites, supporting the notion that miRNA-dependent regulatory circuitries act as "molecular driving forces" for increasing functional complexity; this becomes more evident if considering that most of the neurogenesis transcripts encode for transcription factors. By extension, it can be speculated that the microRNA gain-of-function activity could be the key element introduced during animal evolution to allow the evolutionary jump from invertebrates to vertebrates.

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Note

Supplementary materials can be found at:
www.landesbioscience.com/supplement/
CacchiarelliRNA5-3-Sup.pdf

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