

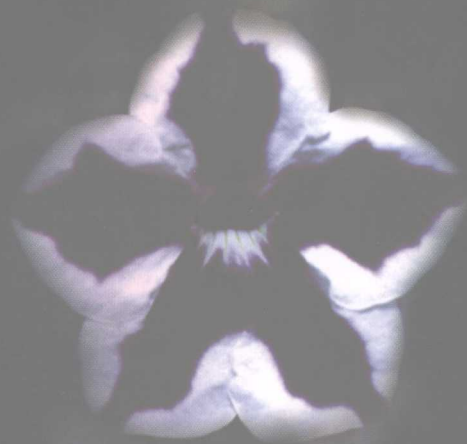
回顾与展望丛书(影印版)



RNA SILENCING

# RNA 沉默

Esra Galun



科学出版社  
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北京

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## 内 容 简 介

本书聚焦于真核发育与分子遗传学中新出现的重要问题——各种小 RNA 对基因表达的控制。人们已经在这个令人激动的领域中做了很多工作,本书是唯一一本由单个作者独立完成的全面介绍 RNA 沉默的著作。

Fire 和助手们 1998 年完成了双链小 RNA 在线虫基因表达中的作用的相关研究,这项开创性工作被认为是 RNA 沉默研究的开端。本书介绍了此前相关领域的历史背景,描述了多种动植物如原生动物、简单后生动物、昆虫、非哺乳类脊椎动物和哺乳动物中的 RNA 沉默。每种生物中的 RNA 沉默,在提供实验结果的同时,还介绍了相关背景知识,并伴有丰富的图解。书中附录是 Eithan Galun 所著《基因治疗》的相关内容。附录中内容关乎 RNA 沉默在未来的可能应用,可以作为有志于探索这种可能性的研究人员的一份指南。

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## Preface

וַיֹּאמֶר הַמֶּלֶךְ קְחוּ-לִי חֶרֶב  
וַיָּרֶן אֶת הַיָּלֶד הַחַי לְשֵׁנָיו

*"And the king said, bring me a sword ...  
and divide the living child in two"*

(King Solomon's trial, Hebrew Bible, Kings I, Chap. 3.)

Τόδε περί ἀμάξης ἐμυθεύετο, ὅστις λύσειε τοῦ ζυγοῦ τῆς ἀμάξης τὸν δεσμόν, τοῦτον χρῆναι ἄρξαι τῆς Ἀσίας.... Ἀλέξανδρος δέ... παίσας τὸ ξίφει διέκοψε τὸν δεσμόν...

*"This was the myth about the coach, that whoever would undo the knot on its yoke, he would become the ruler of Asia. Alexander then drew his sword and cut the knot."*

(The myth of the Gordian Knot and Alexander the Great in *Alexandrou Anabasis Book B*, by Arrianos.)

\*\*\*\*\*

In the above two mottos the **Sword** was instrumental in resolving intriguing problems. In the trial of King Solomon, the wise King revealed who the mother of the living child was. In the cutting of the Gordian Knot, Alexander quickly resolved an ancient problem and marched to his conquests.

How are these mottos related to RNA Silencing? Scholars of RNA silencing also used a **sword** although they termed it *Dicer* and the cutting, *Dicing*. Most RNA silencings are initiated with *Dicing* double-stranded RNA by the *Dicer*. We shall see that these scholars followed the wisdom of King Solomon and made ample use of *Dicing* to study major problems in genetics, in the regulation of gene expression and in the development of eukaryotes.

Scientific revolutions may be sensed in advance by scholars with an exceptional intuition but the *site* where such revolutions take place is frequently a complete surprise. Not all major revolutions in science took place in main towns. Who would have predicted that the revolution in cosmology, initiated by Copernicus, would take place in a tiny town as Frauenburg (then in Poland, thereafter in Eastern Prussia and now called Frombork in Poland)? In genetics, the first revolution, initiated by Gregor Mendel, happened in a modest Augustinian monastery near Brunn, in Moravia. A further revolution, initiated by Barbara McClintock, took place in a very tranquil surrounding: the Cold Spring Harbor Laboratory of the Carnegie Institution of Washington. This latter revolution heralded the *Transposable Elements* and was the subject of my previous book (Galun, 2003). Another department of the Carnegie Institution of Washington (in Baltimore, MD) was the site of a further genetic revolution: the awareness of the profound role of small dsRNA sequences in gene expression, defense against invaders, remodeling chromatin and regulation of development in eukaryotic organisms.

For brevity I shall adhere to the term *RNA Silencing* as the name of this book. But there are several types of RNA sequences that affect gene expression (e.g. small interfering RNA, microRNA). As in the cases of the revolutions initiated by Mendel and McClintock, the *RNA silencing* was also preceded by information that by retrospect could lead to novel molecular-genetic understanding. But the "birth" of this field of endeavor can be traced to one specific publication by Mello and Fire (Fire *et al.*, 1998) that heralded the silencing of RNA.

Although *RNA silencing* is a young field of investigation it has already solved several genetic/biological riddles and has started to be instrumental to further genetic and developmental studies as well



as to lead to practical applications as gene therapy. As in my previous books, *Transgenic Plants* (Galun and Breiman, 1997), *Manufacture of Medical and Health Products by Transgenic Plants* (Galun and Galun, 2001) and the aforementioned book *Transposable Elements* (Galun, 2003), this book is targeted to a wide range of readers. Therefore, some basic subjects that are prerequisites for the understanding of *RNA silencing* will be discussed briefly as a kind of introduction to the main theme of this book. While most of this book is intended for the novice in this field, it also includes an Appendix that deals with the use of gene silencing for gene therapy. This Appendix was added for the benefit of those who are considering using this approach in their investigations. Consequently, the Appendix is written in a manner that will render it useful for such an endeavor.

While writing this book, I have received generous help from many people and I am very grateful for this help. Professor Dan Segal of the Tel Aviv University invested a great deal of efforts to correct my mistakes in the drafts of the chapters on the nematodes and on *Drosophila* and mosquitoes. My wife, Professor Margalith Galun, read parts of the manuscript and made useful remarks. My son, Professor Eithan Galun, not only wrote the Appendix of this book, but also read and corrected parts of the book that deal with the medical aspects.

My gratitude goes also to several colleagues in the Department of Plant Sciences of the Weizmann Institute of Science: Dr Yuval Eshed, Professor Robert Fluhr, Professor Gad Galili and Professor Avraham Levy who provided useful remarks on specific chapters. I owe thanks to Professor Shulamit Michaeli of Bar-Ilan University who read and corrected Chap. 6. I also thank Dr Izhak Bentwich of the Rosetta-Genomics company in Nes-Ziona for reading Chap. 10 and to Professor Aglaia Athannassiadou of the Medical School of the University of Patras, Greece, for the Greek text of the *Myth of the Gordian Knot*.

I appreciate very much the work of the Secretary of our Department, Mrs Renee Grunebaum, who typed and retyped the manuscript and handled the hundreds of references almost daily during many months and with endless patience and for being able to bear with me during my moody moments. Thanks are due to the Graphic Arts

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I acknowledge the outstanding collaboration of the Publisher, Dr K.K. Phua, Chairman of the World Scientific Publishing Co. (WSPC) in Singapore, and Ms Magdalene Ng, senior editor at WSPC, who was instrumental in rendering my draft into a real book.

I acknowledge the permissions to use figures and tables that were granted by the respective publishers. I provided the source below each table or figure and the full citations are listed in the references. These citations should mean that I am grateful for the consents of the respective publishers to use their copyright material in this book.

To Dr Nillie Weinstein I endow my final and very special gratitude. She encouraged me to commence this book and provided continuous support during my writing period. I benefited immensely from her wisdom and knowledge especially but not only in philosophical issues. Dr Weinstein took an active part in the integration of these issues into the book and carefully reviewed the appropriate phrasings. For the above and for additional contributions, she became an inseparable constituent of my endeavor.



## Acronyms and Abbreviations

<b>AGO</b>	ARGONAUTE
<b>CP</b>	viral coat protein
<b>DCL</b>	dicer-like (enzymes)
<b>GFP</b>	green fluorescent protein
<b>GUS</b>	$\beta$ -glucuronidase
<b>HR</b>	hypersensitive response
<b>miR...</b>	genes coding for miRNAs
<b>miRNA</b>	microRNA
<b>ORF</b>	open reading frame
<b>PAZ</b>	PIWI, argonaute & zwiille (a protein interaction domain)
<b>PCR</b>	polymerase chain reaction
<b>PKR</b>	protein kinase, dsRNA dependent
<b>PSTV</b>	potato spindle tuber viroid
<b>PTGS</b>	post transcriptional gene silencing
<b>PVK</b>	potato virus X
<b>PVY</b>	potato virus Y
<b>RdDM</b>	RNA-directed DNA methylation
<b>RdRP</b>	RNA-dependent RNA polymerase
<b>RISC</b>	RNA-induced silencing complex
<b>RT</b>	reverse transcription
<b>TE</b>	transposable element
<b>TEV</b>	tobacco etch virus
<b>TGMV</b>	tomato golden mosaic virus
<b>TGS</b>	transcriptional gene silencing
<b>TMV</b>	tobacco mosaic virus
<b>TncRNA</b>	3'-untranslated region (of transcripts)
<b>VIGS</b>	virus induced gene silencing



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Acronyms and Abbreviations

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# Introduction

## Philosophical Contemplations

The motto of my previous book, *Transposable Elements — Guide to the Perplexed and the Novice* (Galun, 2003), was a quote from Heraclitus of Ephesus (500 BC): “Many fail to grasp what they have seen and cannot judge what they have learned, although they tell themselves they know”. In the preface of the above mentioned book I then reiterated the wisdom of several old masters, stating that only the combination of keen observations with rationalization and logic thinking will lead to meaningful new knowledge. This old wisdom is also relevant to the emerging field of RNA Silencing. I shall present a brief history of RNA Silencing below. We shall see that an important step in the awareness of RNA silencing was the study by Mello and Fire (Fire *et al.*, 1998) on the impact of short RNA fragments on the nematode *Caenorhabditis elegans*. These investigators found that if they introduced *C. elegans* into fragments of a single-stranded mRNA or the antisense of this mRNA, there was a specific genetic interference; but when they used the double-stranded RNA (dsRNA), composed of sense and antisense mRNA, the interference was several-fold greater. Albeit, further studies showed that the single-stranded RNA (ssRNA) effects were *artifacts*. The preparations of the ssRNAs were contaminated by dsRNA and it was only the latter RNA which caused the interference. The verification of the effect of the dsRNA provided the accelerated endeavor of RNA silencing. One additional example to support the notion that you believe what you see can be misleading is from my own research that led to the publication of an artifact. In collaboration with the laboratory professor Nathan Sharon (also of the Weizmann Institute of Science) to study the impact of a lectin on fungal growth,

I observed that the lectin wheat-germ-agglutinin (WGA) caused the explosion of the hyphal tips of *Trichoderma*, arresting the growth of the fungal hyphae. We published it in the journal *Nature* (Mirelman *et al.*, 1975) and this publication was then amply cited... but later it was found that because we used "home-made" WGA, this WGA contained traces of chitinase. Why was there a contamination of chitinase in our WGA? Now we know. The Sharon laboratory devised an efficient method to isolate and purify WGA. A wheat-germ homogenate was passed through an affinity column that contained oligo N-acetyl glucosamine. The WGA was bound to the oligomer and subsequently washed out of the column. But the same oligomer also retained the trace amounts of chitinase in the wheat-germ homogenate so that this chitinase was washed out with the WGA. These chitinase traces caused the antifungal effect.

Let us briefly carry the question of the difference between what the investigator "sees" and what the "real" nature is, to the philosophical level. Since the classical Greek philosophical period (i.e. 500–300 BC) there is debate whether or not humans are capable of perceiving the real nature. Could it be that by using his senses man perceives only an *illusion* of the real nature? If the latter is true, is there a way by which the real nature can be perceived by human rationalism? Note that during this classical period, there were only observations; experimentation was not applied yet. Plato illustrated this enigma by his famous *Analogy of the Cave* (The Republic, book VII, Plato, and see the Epilogue in Galun, 2003). Maimonides (1135–1204) claimed, on the basis of logical arguments, that there must be forces (or laws) of nature that humans cannot comprehend but these laws do exist. In his *Guide to the Perplexed* (Part I, Chap. 73) Maimonides claimed that it is agreed that the earth is ball-shaped (a globe) and that there are people on the opposite side of the globe. These people are also standing on the globe but with their heads away from us; still they do not fall "down". Hence, there must be a force (law of nature) that keeps them from falling. Maimonides claimed that the people at the other side (the lower side opposite us) should fall only by our imagination; if we consider the situation by logical thinking we shall recognize that the latter people were also standing on earth, from their point of view. Maimonides did not mention gravity. Well, with respect to this example of Maimonides,

Isaac Newton (1642–1727) revealed the Laws of Universal Gravitation. These laws could explain the enigma of Maimonides. They were since amended although the real nature of gravitation is still enigmatic. Shall we ever reveal and understand the real nature? Immanuel Kant (1724–1804) added a decisive contribution to the question of apparent versus real nature. In brief he claimed that there is a real nature (world), but humans cannot perceive this noumenon by direct cognition. Humans are only able to perceive the real world when rational recognition is added to direct observations. In a way Kant argued what Heraclitus of Ephesus and Maimonides claimed 2300 years and 500 years earlier, respectively. Kurt Gödel (1906–1978) who was born in Brünne, the Moravian town near which Gregor Mendel made his revolutionary genetic discoveries, developed an important mathematical-logic theorem. He stated that certain branches of mathematics are based, in part, on propositions that are not provable *within* the system itself, although they may be proven by means of logical systems outside of (pure) mathematics. Does this lead to the philosophical claim that a full understanding of the real nature of a system can be achieved only if one is positioned *outside* of the system? Hence, for our deliberation: outside of the known world. If this claim is correct a full understanding of the world will never be achieved by humans. Does this mean we should give up? The answer is negative. Being aware of the logical possibility that a certain goal cannot be achieved should not deter us from trying. The *way* and the *effort* to achieve a goal could be more rewarding than *reaching* the goal. This was clearly presented by Albert Camus (1913–1960) in his book, *Le Mythe de Sisyphe*.

Not equipped with adequate philosophical background to contribute convincing arguments in favor or against the claim that humans can comprehend the nature of the *real* world, I shall thus leave this question open.

There is a claim, expressed especially by mathematicians and physicists that esthetically beautiful solutions to problems are commonly the correct solutions. This was the notion of Watson and Crick when they derived an elegant solution to the double helix structure of DNA: the elegant solution must be the correct one. Let us now turn our attention to biology. We shall assume that a certain biological phenomenon is being investigated and performed from several angles and in a step-wise manner. When all the results are



combined, the phenomenon appears clarified; moreover, the now clarified phenomenon is a sound basis for further investigations of related phenomena, until an esthetic, elegant and beautiful general-picture of biological phenomenon is reached. Do we mind if this picture may not represent the "real" nature in the philosophical sense? Obviously, a step-wise endeavor to understand biological phenomena, and in general terms any phenomenon in nature, is rewarding by itself (i.e. Sisyphus of Camus). For the scholars themselves this reward would suffice. Baruch Spinoza (1632–1677) went even further and in his *Ethics* claimed that the revelation of the nature of Nature is a source of joy and happiness. Albeit, this may be a sufficient reward to satisfy the investigator, it could pose a problem with respect to the recognition of the investigators' achievement by his peers. The Nobel committees for prizes in the sciences have an additional requirement. These committees usually wait with the endowment of prizes until the discoveries find applications in further studies. This waiting could take many years. As prizes are awarded only to living people, longevity is of advantage to potential Nobel awardees.

We shall see that components of the RNA silencing systems, such as *micro RNA*, may affect the differentiation of Eukaryotic organisms and thus be decisive for the final shape of their organs. Here is a pit-fall that is commonly overlooked by naïve biologists. When the latter are told about the gene that causes additional petals in a flower or additional fingers in a hand, they may assume that these genes contain all the information to shape a flower or a hand, respectively. This is obviously wrong. We still have no notion as to how, from the *linear* information that is stored in the DNA, a three-dimensional structure can emerge. Well, with a few exceptions. The genes in some bacterial phages are now known and their expression leads to proteins that interact to produce the final shape of the respective phage. Also, the genes that code for the histone components are known and the derived structure of the nucleosome is now understood at the molecular level. We are therefore beginning to understand, at the chemical/molecular level, how euchromatin, in which the nucleosomes are separated, is converted to heterochromatin in which there is a dense packaging of nucleosomes. But these examples are the exception; we still do not know how the final structure of organs (e.g. of a specific petal)



is derived. True enough, it is obviously structured by orchestration of cell division, cell enlargement and arrest of cell division, at very specific locations in the organ. But for that to happen, there must be a coordination based on sensing the space in which this orchestration of differentiation takes place. We shall see below that there are beginnings to the understanding the structuring of animal and plant organs and that this understanding is assisted by changes in gene-expression. RNA silencing mechanisms are involved in these changes and therefore the basic questions of control of differentiation are relevant to the theme of this book. For correct differentiation a given cell should either stay idle or divide and/or enlarge. For that the cell should have a correct orientation of its location in "space" — relative to the other cells in the specific organ. We can carry our question further. When we look from a certain distance, at a sycamore tree and a pine tree, the silhouettes of each of these trees are very different and typical to each of them. How do the cells in these trees know where and how to divide and enlarge to reach the same general shape of the tree all the time? Do the individual cells attain a perception of space? If they indeed have a perception of space, their perception could be fundamentally different from ours. Here we return to Immanuel Kant who claimed, already in the 18th century, that humans have an inherited and intuitive sense of Euclidian geometry and perceive space accordingly. We now know that there are other geometries. Could it be that cells of animals and plants use different space-orientations from those used by the intuitive human mind? All the above contemplations are intended to draw the attention of the reader to the rather complicated issue of differentiation. A comprehensive and beautiful analysis of the problem of differentiation was presented in the book of Enrico Coen (1999).

### **A Short History of RNA Silencing**

History is commonly meant to start with written records. Take, for example, the *history* of the Middle East started with the first writings that were found in Uruk (South-Eastern Mesopotamia) and in Memphis (Egypt). Both were dated to about 3000 BC. Anything that happened before this time is considered *Pre-history*. We shall adopt the same approach to RNA silencing, meaning that the history of RNA

silencing started with the first written reports on this phenomenon. Still, there are differences. First, in the early reports on what we now term RNA silencing, the term Gene silencing was used. Albeit, Gene silencing included not only the phenomenon of RNA silencing. This will be clarified below. Another difference is that for the pre-history of RNA silencing there are still living witnesses (as the author of this book) who remember this period, while for the common pre-history of countries and nations, our "witnesses" are merely archeological artifacts.

### ***Remarks on the "pre-history" of gene (RNA) silencing***

Summarizing "unrecorded" records is ambiguous. Still, the following remarks and information are relevant to our deliberations. The first remark is that these "unrecorded-records" concerned primarily plants (in this context — actually angiosperms). Investigators dealing with disease resistance of plants and especially with viroid and viral diseases were faced with a situation in which plants that were infected with a mild pathogen (i.e. a mild virus) showed various degrees of resistance to a second infection by the same or a similar pathogen. In a few cases such cross protections were reported in reviewed publications or in lectures in scientific meetings (see: Niblett *et al.*, 1978; Sherwood, 1987; Lomonossoff, 1995). During this period, that is, *pre-historic* with respect to RNA silencing, there were numerous written records on cross protection. In several of these records the authors even furnished suggestions for the mechanisms of these protections, mentioning the involvement of proteins and/or RNA. Albeit, the specific role of dsRNA was not put forward until the mid 1990s. It is noteworthy that such protections were not always reproducible but they did appear "real" and horticulturalists swiftly utilized this phenomenon. Take, for example, certain *Citrus* cultivars were infected with a mild virus (that occasionally caused some stunting) to immunize the trees against virulent viruses. Whole orchards were thus immunized for a double purpose: to reduce the size of the trees, for easier management of the orchard and to prevent the severe virus diseases.

In later investigations on viral cross-protection in plants, the involvement of dsRNA came to light. These latter investigations will be discussed in some detail in this book.

Another case of *pre-historical* silencing concerns transposable elements (TEs) (see: Galun, 2003). Since the pioneering studies of Barbara McClintock on “controlling” TEs it became evident that the maize transposons can undergo a reversible change from active to inactive (silent) elements. Note that the two first transposable element systems, *Ac/Ds* and *Spm* were established already in 1948 and 1953, respectively (*Ac/Ds* by McClintock and *Spm* independently by McClintock and Peterson; see Galun, 2003). In some cases McClintock even revealed cycled changes from active to inactive phases of the transposable elements. This was observed in the *Suppressor-mutator* (*Spm*) elements as well as in the *Activator* (*Ac*) element of the *Ac/Ds*, and later also in the *Mu* transposable elements of maize. These changes that led to active and to silenced phases of *Ac* and *Spm* were revealed by genetic studies but the molecular meaning of silencing the activity of a TE was not known for 30 years after the discovery of these elements. Only after molecular studies identified genes for transposases in the TEs, the silencing of the respective transcripts could be followed. A detailed account on this subject, with emphasis on the *Spm* transposable elements, was provided by Fedoroff (1995).

Efficient genetic transformation of plants was achieved by the use of *Agrobacterium*-mediated transformation and properly engineered plasmids. This happened in the early 1980s (see: Galun and Breiman, 1997, for a detailed review). Very hectic activity of plant-genetic transformation was then initiated in many laboratories. All those engaged in these transformation activities witnessed the same general phenomenon: the expression of the transgene in the transformed plants was very variable even among the plants that resulted from the same transformation experiment. Moreover, when the transgene was inserted into a plant genome, in more than a single site, the expression of the transgene was frequently *lower* than when introduced only into one site. This silencing of the transgenes had an epigenetic character, commonly carried to the next sexual generations. Most of this silencing was not reported. The investigators were interested in the highest expression of the transgene. So they just focused on one or a few transgenic plants for their further research and ignored the transgenic plants that had a low expression of the transgene. There could be various reasons for the low expression of transgenes, such as position