Kyriacos Felekkis
Konstantinos Voskarides *Editors*

Genomic Elements in Health, Disease and Evolution

Junk DNA

Springer

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Preface

Although the term "junk" DNA was used since the early 1960s, the term's origin was attributed to Susumo Ohno who officially used the term to describe pseudogenes' sequences resulted from gene duplication and subsequent mutagenesis events. Since then, the term was widely used to describe any non-coding sequence of the genome. Today, "junk" DNA refers to any genomic sequence that does not play a functional role in the organism. The use of the term was accompanied by various unanswered questions: Why do we have so much "junk" DNA in our genome? Do these non-coding sequences have functional significance? The discovery of novel genomic elements in the recent years was a step forward in an attempt to address these issues. It appears that the percentage of the non-functional DNA is being significantly reduced as more and more functions are attributed to those non-coding regions of the genome. Despite the continuous shrinkage of the non-functional portion of the genome, it is believed that a significant part of the genome is indeed non-functional.

In this book, we attempt to provide a thorough review of various non-coding genomic elements and discuss in depth their role in health, disease and evolution. We begin our exploration with non-coding RNA molecules, miRNAs, piRNAs, LncRNAs and transposable elements as these moieties dominate the scientific literature in the last 10 years. We proceed with the discussion of copy number variation regions, mini- and micro-satellites, and proximal and distal elements of the genome. The last section of this book focuses on the review of well-known non-coding regions of the genome, introns, centromeres and telomeres, but enriched with newly discovered functions. As the vast amount of data in regard to these elements is attributed to a great degree to the growing technology in the field of biomedicine, the last chapter of this book discusses the latest development in the field of Next Generation Sequence and the potential applications of this technology in the study of non-coding regions of the genome.

The original structure of this book was greatly shaped by many conversations with colleagues in Cyprus and abroad. We are indebted to all the authors contributing to this publication for their in-depth review of the subject and their excellent writing. We must also thank all the scientists whose work is included in this book.

Special thanks to our respective institutions and colleagues for their support and critical interventions. The driving force of our inspiration is equivocally our students who provide us with the strength and willingness to sit down and write. Last but not least, we are indebted to our families and friends for their continuous support and encouragement.

Nicosia, Cyprus

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Contents

1 **MiRNAs' Function and Role in Evolution: Under the View of Genomic Enhancement Phenomena** 1
Konstantinos Voskarides and Kyriacos Felekkis

2 **MicroRNAs in Disease** 17
Gregory Papagregoriou

3 **piRNAs-Transposon Silencing and Germ Line Development** 47
Catherine Demoliou

4 **Long Non-coding RNA** 83
Monika Gullerova

5 **Unveiling Transposable Elements Function to Enrich Knowledge for Human Physiology and Disease Pathogenesis** 109
Ioannis S. Vizirianakis, Elsa P. Amanatiadou, and Sotirios S. Tezias

6 **Copy Number Variation in Human Health, Disease and Evolution** 129
Carolina Sismani, Costas Koufaris, and Konstantinos Voskarides

7 **Mini- and Micro-Satellite Markers in Health, Disease and Evolution** 155
Vasiliki A. Galani, Sofia Markoula, Leandros Lazaros,
Paris Ladias, and Ioannis Georgiou

8 **Intron Biology, Focusing on Group II Introns, the Ancestors of Spliceosomal Introns** 195
María Dolores Molina-Sánchez, Rafael Nisa-Martínez,
Fernando M. García-Rodriguez, Francisco Martínez-Abarca,
and Nicolás Toro

9 **Centromeres in Health, Disease and Evolution** 221
Thian Thian Beh and Paul Kalitsis

10 Structure and Functions of Telomeres in Organismal Homeostasis and Disease 247
Penelope Kroustallaki and Sarantis Gagos

11 Proximal Regulatory Elements with Emphasis on CpG Rich Regions 285
Pavlos Fanis

12 Genomic Analysis Through High-Throughput Sequencing 297
Michalis Hadjithomas

Index 313

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Chapter 1
MiRNAs' Function and Role in Evolution: Under the View of Genomic Enhancement Phenomena

Konstantinos Voskarides and Kyriacos Felekkis

Introduction: Biogenesis, Nomenclature and the Principal Role of miRNAs

MiRNAs are short, single-stranded RNA molecules approximately 21–23 nucleotides in length (mature miRNAs), which usually have a uridine at their 5′ end and they are partially complementary to one or more messenger RNA (mRNA) molecules. Although they cover only a small part of the genome, their role in gene expression regulation is considered to be of high significance. MiRNAs were discovered by Victor Ambros, Rosalind Lee and Rhonda Feinbaum in 1993 during a study of the gene *lin-14* in *C. elegans* development [1]. They found that LIN-14 protein abundance was regulated by a short RNA product encoded by the *lin-4* gene, which was partially complementary to many regions of the *lin-14* 3′ UTR. This complementarity was sufficient to inhibit the translation of the *lin-14* mRNA and necessary for the worm development. A huge amount of data has been accumulated since then for miRNAs and many more are yet to be discovered in the near future.

Due to this great and continuous flow of data, a specific nomenclature system has been created. The prefix "mir" is followed by a dash and a number, the latter often indicating order of naming. For example, mir-123 was named and likely discovered prior to mir-456. The uncapitalized "mir-" refers to the pre-miRNA, while a capitalized "miR-" refers to the mature form. MiRNAs with nearly identical sequences bar one or

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two nucleotides are annotated with an additional lower case letter. For example, miR-123a would be closely related to miR-123b. Pre-miRNAs that lead to 100 % identical mature miRNAs but that are located at different locations in the genome are indicated with an additional dash-number suffix. For example, the pre-miRNAs hsa-mir-194-1 and hsa-mir-194-2 lead to an identical mature miRNA (hsa-miR-194) but are located in different regions of the genome. Species of origin is usually designated with a three-letter prefix, e.g., hsa-miR-123 is a human (*Homo sapiens*) miRNA and oar-miR-123 is a sheep (*Ovis aries*) miRNA. When relative expression levels are known, an asterisk following the name indicates a miRNA that is expressed at low levels relative to the miRNA in the opposite arm of a hairpin. For example, miR-123 and miR-123* would share a pre-miRNA hairpin, but more miR-123 would be found in the cell.

But what is known about the "molecular play" of miRNAs? Each miRNA is thought to regulate multiple genes. Since hundreds of miRNA genes are predicted to be present in higher eukaryotes, the potential regulatory circuitry afforded by miRNAs is vast [2, 3]. Acting at the post-transcriptional level, these molecules may regulate the expression of more than 30 % of all mammalian protein-coding genes [4]. Several research groups have verified that miRNAs may act as key regulators of processes as diverse as embryonic development, cell proliferation, cell growth, tissue differentiation and apoptosis. The mature miRNA can bind even with partial complementarity to the target mRNA (typically on 3′ UTR), downregulating the translation of the mRNA. The mature miRNA mainly acts by targeting a miRNA recognition element (MRE) on the mRNA's 3′ UTR and binds on it through a Watson–Crick base-pairing manner. MiRNA target recognition properties depend on its 'seed region'. Recognition binding sequences are short, usually 6–8 nt [5–8]. The expression of a large number of the predicted human miRNA genes has been confirmed, but many predicted miRNA targets remain to be identified and verified (reviewed in Chap. 2).

Most of the miRNA genes are located in "spacing" DNA, the DNA that is found between different genes. A percentage of 40 % of miRNA genes may lie in the introns of protein and non-protein coding genes or even in exons of long non protein-coding transcripts [9]. These are usually, though not exclusively, found in a sense orientation [10, 11] and are usually regulated together with their host genes [9, 12, 13]. A number of miRNA genes have a common promoter, including 42–48 % of all polycistronic miRNAs (found in the same genetic region), containing multiple discrete loops from which mature miRNAs are processed [14, 15]. This does not necessarily mean the mature miRNAs of a family will have similar structure and functions. The promoters mentioned have some similarities in their motifs to promoters of other genes transcribed by RNA polymerase II, such as protein coding genes [14, 16]. In brief, animal miRNAs are transcribed by RNA polymerase II and processed by the microprocessor protein complex (DGCR8/Drosha) into precursor stem-loop miRNAs in the nucleus. The cleavage of a pri-miRNA by microprocessor begins with DGCR8 recognizing the ssRNA–dsRNA junction typical of a pri-miRNA. Drosha is approaching its substrate through interaction with DGCR8 and cleaves the stem of a pri-miRNA ~11 nt away from the two single stranded segments (~22 nts away from the loop) (summarized by Felekkis et al. [17]). Drosha removes the double-stranded stem from the remainder of the pri-miRNA by cleaving proximal and distal of the stem, generating a pre-miRNA that has a 5′-monophosphate and a 3′-2-nt overhang (Fig. 1.1).

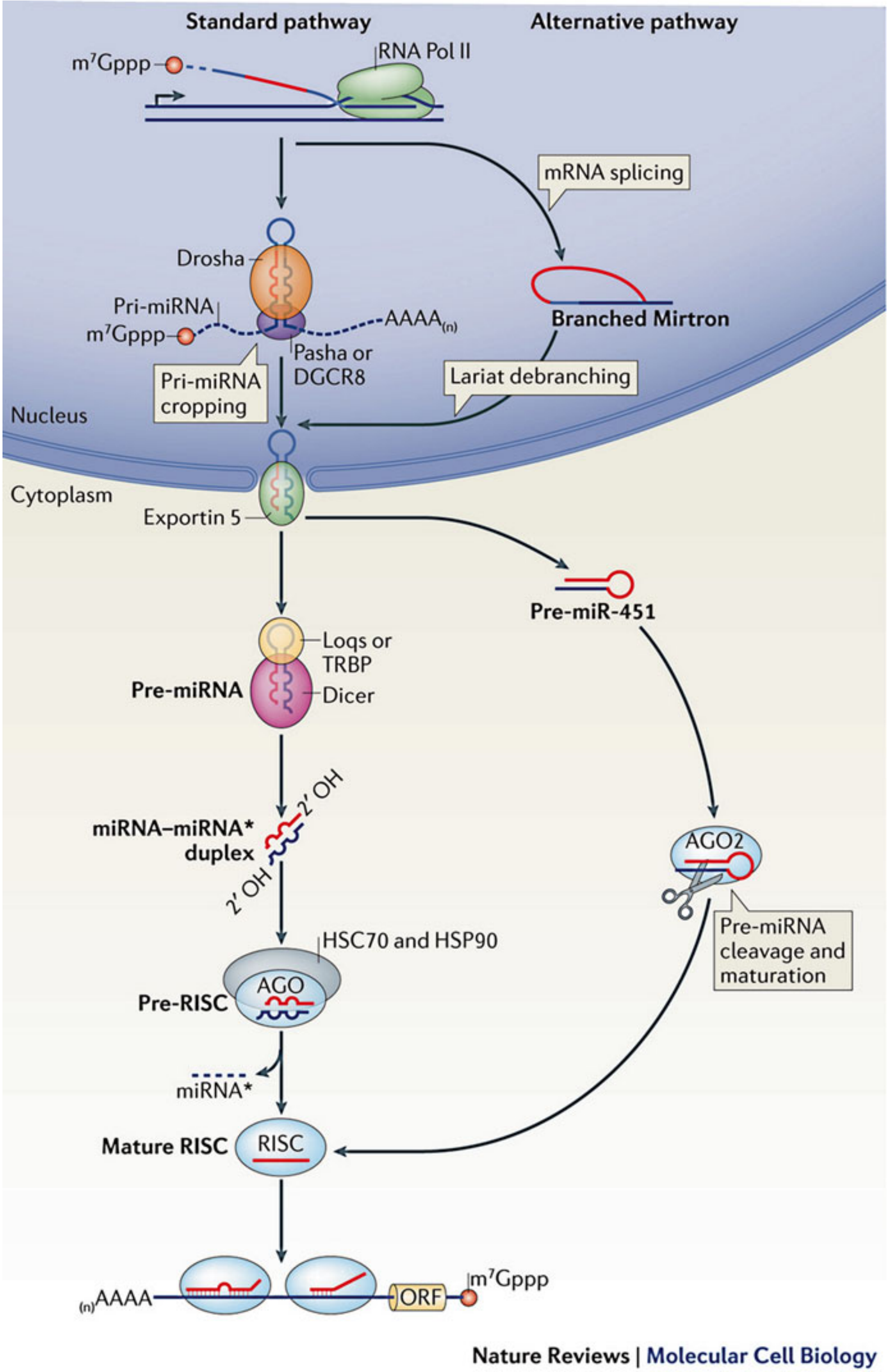

Fig. 1.1 Several layers of regulation control miRNA gene biogenesis in animals: transcription activation, splicing, recognition by Drosha, post-processing, RNA editing, sub-cellular localization, nuclear export, and hairpin arm selection. From Ameres SL, Zamore PD. Diversifying microRNA sequence and function. Nat Rev Mol Cell Biol. 2013 Aug; 14(8):475–488. Reprinted with permission from Nature Publishing Group

Precursor miRNAs (pre-miRNAs) are exported into the cytoplasm by Exportin-5 and are cleaved by Dicer to produce mature miRNAs. After cleavage, the miRNA duplex is unwound by an unidentified RNA helicase and the mature miRNA strand binds to an Argonaute (Ago) protein into an RNPcomplex (RSIC) (Fig. 1.1). The other miRNA strand is degraded. A primary determinant as to which of the two strands of a miRNA duplex will be loaded on Ago proteins is the inherent thermodynamic asymmetry of the miRNA duplex. The RNA strand whose 5′- end is less stably bound to the opposite strand will be loaded to Ago proteins and form the mature miRNA [17].

Mature miRNAs recognize their respective target mRNAs and mediate post-transcriptional repression of their targets through translational repression, deadenylation, or enhanced mRNA decay. The procedure is similar in plants, with the difference that plants have no Drosha homolog. The plant Dicer homolog, DICER-LIKE1 (DCL1), orchestrates both processing events within the nucleus, typically resulting in an 21-nucleotide mature miRNA/miRNA passenger strand duplex with two-nucleotide 39 overhangs [18–20].

MiRNA Genes Evolution

Chromosomal Organization of miRNA Genes

An interesting observation is that miRNA gene number per chromosome correlates with the protein-coding gene density. This indicates that integration and/or maintenance of miRNA genes roughly follows protein-coding genes. However, *Homo Sapiens* chromosomes 14, 19, and X are exceptionally enriched for miRNA genes, something that may be related with the evolutionary history of those chromosomes.

As previously mentioned miRNAs that are found in genetic clusters are called polycistronic and tend to have a common promoter. It is obvious that those miRNAs were formed though repeated genomic duplication events caused by the unequal recombination—the not perfect alignment of homologous chromosomes during meiosis—a very frequent phenomenon in chromosomal regions with genetic repeats. The end result of such event will be the gain or loss of a single copy.

Plausibly, employment of an already existing functional promoter by new miRNA genes is an efficient way to express new miRNAs, eliminating the need for de novo establishment of promoter-enhancer sequences upstream of the miRNA gene. The subsequent result of this phenomenon is miRNAs (in regions up to ~50 kb) that tend to be co-expressed [12, 15]. Amplification of an ancestral miRNA inside a cluster is not only significant for “de novo” birth of novel miRNAs but possibly contributes to the effective dosage of a given expressed miRNA homolog [11, 15].

Old and Young miRNA Genes

Conserved miRNA sequences among species, are considered as the oldest ones. Newly derived miRNAs are the non-conserved miRNAs and are derived through duplication events and subsequent nucleotide substitutions [21]. Frequency of duplication of derived miRNAs may be different in plants and animals since there is evidence that hairpin structure is the main source of new miRNAs in *Drosophila* species instead of duplication events that dominate in miRNAs' birth in plants [22, 23]. Nozawa et al. [22] found evidence that miRNAs are initially controlled by neutral evolution but once miRNA genes acquired their functions, they appear to evolve very slowly, maintaining essentially the same structures for a long time. This suggests that once a miRNA gains a function it undergoes extreme purifying selection. On the other hand, miRNAs which are more recently derived (and thus presumably non-functional) are frequently lost (Fig. 1.2).

A lot of interest has been displayed on functional differences among old and young miRNAs. Comparative genomics suggests that ancient miRNAs have on average two fold more targets than newly generated ones [24]. On the other hand, new miRNAs genes were shown to have lower expression compared with the old ones [25]. Expression regulation of intronic miRNAs (intragenic) is of considerable interest. Do they follow the expression pattern of their host gene? A recent publication provides evidence that expression of intragenic miRNAs differs between young and old miRNAs. Young intragenic miRNAs display lower levels of co-expression with host genes than old ones [26]. This interesting co-evolutionary relation requires further investigation.

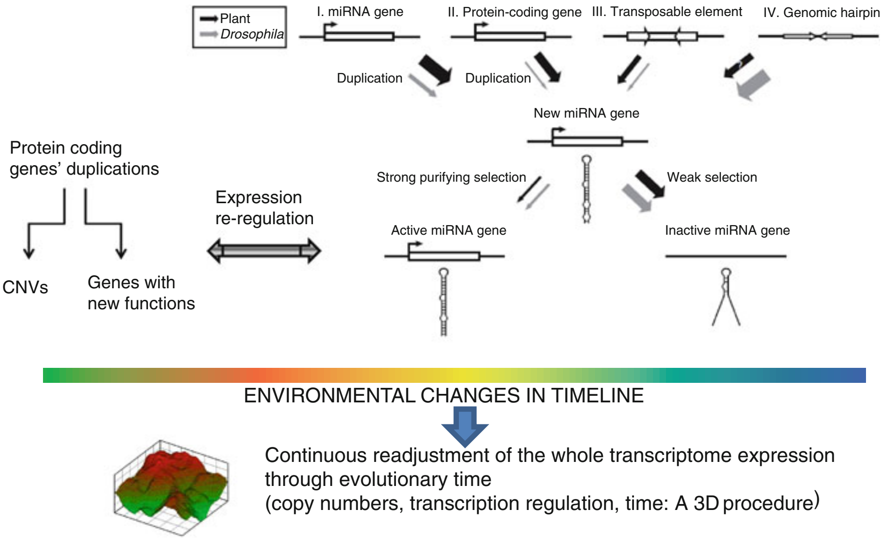
Plant
Drosophila
I. miRNA gene
II. Protein-coding gene
III. Transposable element
IV. Genomic hairpin
Duplication
Duplication
New miRNA gene
Protein coding genes' duplications
CNVs
Genes with new functions
Expression re-regulation
Strong purifying selection
Weak selection
Active miRNA gene
Inactive miRNA gene
ENVIRONMENTAL CHANGES IN TIMELINE
Continuous readjustment of the whole transcriptome expression through evolutionary time
(copy numbers, transcription regulation, time: A 3D procedure)

Fig. 1.2 Model of miRNAs as regulators of duplication phenomena in evolution. From Nozawa M, Miura S, Nei M (2012) Origins and evolution of miRNA genes in plant species. Genome Biol Evol 4:230–239. With permission from Oxford University Press

Without a doubt, the main question is "when did the first miRNAs appeared and why?" A way to answer this question is to identify the old (ancient) miRNAs and study their functions. Comparative sequence data indicate that the oldest known animal miRNA is miR-100. Related to this miRNA the miR-125 and let-7 that were initially active in neurosecretory cells located around the mouth. Other sets of ancient miRNAs were first present in locomotor ciliated cells, specific brain centers, or, more broadly, one of four major organ systems: central nervous system, sensory tissue, musculature and gut. These findings reveal that miRNA evolution and the establishment of tissue identities were closely coupled in bilaterian evolution [27].

But what about plants? Combined with numerous data sets from high-throughput sequencing experiments, eight miRNA families have been identified in the common ancestor of all embryophytes. The MIR396 family was present in the common ancestor of all tracheophytes (vascular plants), while the MIR397 and MIR398 families were acquired in the common ancestor of all spermatophytes. A high proportion of species-specific or non-conserved miRNA genes were also observed in various plant species. This demonstrates that many plant miRNAs have been raised during speciation processes (for more information see the detailed review of Cuperus et al. [28]).

Genomic Duplications, Repetitive Regions and Transposons (TEs) as Potential Sources of miRNA Genes

A duplication can be segmental (from a few nucleotides to several thousand kilobases—"tandem" is a similar term used for exactly the same and connected genetic repeats) or may cover the whole genome (an event also called polyploidization). Segmental duplication (or small-scale duplication) and polyploidization correspond to distinct evolutionary processes with widely different impacts. Another type of frequent duplications in genomes is the inverted duplications. These are segmental and inversely repeated genetic regions.

Just as with protein-encoding genes, a major route for new miRNA genes evolution is genomic duplications. Segmental or inverted duplications can be a source of new miRNA genes. There is substantial evidence that many of the miRNA families originated through multiple genomic duplication events or through repetitive genetic elements [2, 21, 29–33]. Several molecular mechanisms can determine the future of such new miRNA genes. Sub-functions or completely new functions can be "born". We encourage the readers who are interested in all the evolutionary processes regarding miRNAs to read the detailed review "The evolution and functional diversification of animal miRNA genes" by Liu et al. [34].

Tandem duplications can result in paralogous miRNA sequences that are located on the same transcript and organized as tandem paralog clusters [31, 35]. In a recent study, evidence is given that repetitive elements contribute to the de novo origin of miRNAs in mammalian genomes and that large segmental duplication events accelerate the expansion of miRNA families, including those derived from repetitive sequences [33]. The latter ones are considered as the younger miRNA genes, being also the less conserved among species. Sun et al. [36] found similar evidence in plants, underlining that differences exist between the miRNAs that are found in repetitive elements and those that are not. The former tend to have longer hairpin precursor, lower G-C content in hairpin precursor sequences and lower minimum free energy.

Based on the observation that some miRNA precursors had extended similarity beyond miRNA sequences with target genes, Allen et al. [37] proposed the inverted duplication model. Under this hypothesis, new miRNA genes are generated from inverted duplication events happened on one of their target genes by forming two adjacent gene segments in either convergent or divergent orientation. Recent observations showing that a large proportion of miRNA genes are included in transposable elements (TEs) or pseudogenes, urged scientists to believe that inverted duplications are closely related with TEs (see next paragraph) or pseudogenes. Zhang et al. [38] further confirmed that the inverted duplication model in plants is happening via TEs or pseudogenes, showing also that inverted duplications give rise to miRNAs much more frequently that segmental duplications.

Data regarding the evolution of miRNAs from TEs are significant and continuously accumulating. Smalheiser and Torvik [39] reported 11 instances of presumably TE-derived mammalian miRNAs. Later studies by Piriyapongsa and Jordan [40] and Piriyapongsa et al. [41] identified that 12 % of miRNAs in their study data set overlap with TEs in the human genome. These miRNAs reside within TE copies of all four major TE classes including short interspersed repetitive elements (SINEs), long interspersed repetitive elements (LINEs), long terminal repeats (LTRs), and DNA transposons, suggesting that the formation of novel miRNAs from these elements has occurred multiple times during the human genome evolution. Devor [42] demonstrated seven marsupial-specific miRNAs that possibly evolved from marsupial-specific TEs. Recently, strong evidence was published for similar procedures in plants [32, 36, 43]. The very detailed bioinformatic analysis by Li et al. [32] gave results supporting the notion that TEs in gene rich regions in plants can form foldbacks in non-coding part of transcripts that may eventually evolve into miRNA genes or be integrated into protein coding sequences to form potential targets in a "temperate" manner. A similar work by Sun et al. [36] in four plant species confirmed that a significant number of miRNAs in plants derived from TEs. In addition, Lenhert et al. [44] found evidence in mice that lineage-specific retrotransposons have played an important role in the birth of new miRNA genes during evolution.

Dahary et al. [45] published more evidence on TEs and miRNAs evolution, giving also data for the first time in regards to the relationship of CpG islands (CG repeats) with miRNAs. They found that 300 bp upstream and downstream of the miRNA gene, the observed-to-expected ratio of CpG is significantly higher than the genome average. Further analysis identified 65 human miRNAs that overlap CpG islands (59 of these are fully contained within CpGs) and that none of them were TE associated. The authors believe that the association between miRNAs and CpG islands raises two possible scenarios. Either CpG-rich regions serve as genomic material for miRNAs to emerge or that miRNAs are preserving these regions from their natural decay by methylation and deamination.

MiRNAs Target Sites' Evolution

Co-evolution of miRNA Genes and Their Targets

As mentioned before, a single miRNA appears to control many protein-coding genes. But are the miRNA's target seed sites conserved among different species? Hertel et al. [31] recently reported that human, *Drosophila melanogaster*, and *Caenorhabditis elegans* share 20 common miRNA genes in their genomes. However, bioinformatic analysis suggested that only five miRNA genes share the same target genes among these species [46]. Additionally, as mentioned above, ancient miRNAs have on average twofold more targets than newly generated ones [24]. This finding indicates that most of the miRNAs have experienced gains and losses of their target genes during evolution.

Miura et al. [47] investigated the target sites of miRNAs in Hox genes (the genes that control the body segmentation of metazoan embryos), in 12 *Drosophila* species and in *Daphnia*. Phylogenetic analysis of target sites in Abd-A, Ubx, and Antp Hox genes showed that the old target sites, which existed before the divergence of the 12 Drosophila species, have been well maintained in most species under purifying selection. By contrast, new target sites, which were generated during Drosophila evolution, were often lost in some species and mostly located in non-conserved regions of the 3′ UTRs. These results indicate that these regions can be a potential source for new target sites and in this way creating targets in multiple genes for each miRNA in animals.

More complex factors may contribute to the dual evolution of miRNAs and their targets. The recent and very interesting study of Chen et al. [48] showed that the number of miRNA types that regulate a gene is the strongest indicator of proteins' and genes' evolution. They also divided proteins into low and high intrinsically disordered proteins, finding this way differences in their evolution rates. Additionally, they found that phosphorylated proteins tend to have a higher level of miRNA regulation in their genes and that the number of phosphorylation sites of a protein is correlated with the level of miRNA regulation in low intrinsically disordered proteins. Many different scenarios can explain these results, demonstrating the evolutionary complexity of miRNAs genesis.

Duplications as a Major Evolutionary Pressure for miRNAs' Target Sites Emergence and Their Genomic Distribution

A crucial matter in miRNAs evolution is which factors determine the type and the number of miRNAs' targets in 3′ UTRs. Very recently, it was demonstrated that genomic duplications events constitute the main evolutionary factor for this process. Firstly, Ha et al. [49] proved that small RNAs produced during interspecific mating or polyploidization serve as a buffer against the genomic shock in interspecific hybrids and allopolyploids. The authors came to this conclusion after studying allotetraploids coming from *A. thaliana* and *A. arenosa*, identifying adoptive alterations of the miRNAs and siRNAs levels in comparison with the parental species. Abrouk et al. [50] found evidence that the above mechanism may be a standard procedure in plants after euploidy, especially in euploidy events that are involved in evolutionary speciation. They further suggested that miRNAs may be implicated in genes participating in stress responses pathways, which are essential for plant adaptation and useful for crop variety innovation.

On the other hand, whole genome duplication events are rare in animals and in other phylogenetic clades. Despite this, genetic dosage effects of smaller scale are present in such species. Importantly, Lehnert et al. [51] showed that sense Alu repeat sequences are enriched for miRNA target sites. Even more noteworthy, Li et al. [52] and D'Antonio and Ciccrelli [53] found that miRNA targets are significantly enriched for paralogs genes. Characteristically, Li et al. [52] mention that their results suggest that "miRNA-mediated regulation plays an important role in the regulatory circuits involving duplicated genes including adjusting imbalanced dosage effects of gene duplicates, and possibly creating a mechanism for genetic buffering". On the other hand, D'Antonio and Ciccrelli [53] found that this fine tuning is more significant for "ohnologs", i.e. the paralogs genes that came through vertebrate-specific whole genome duplication events. A more complicate analysis by Fernandez and Chen [54] revealed that human paralogs of poorly packed proteins (categorized so according to special structural criteria) are more likely to be targeted by miRNAs, thus underscoring a means to buffer dosage imbalance effects arising from gene duplication.

Our team provided further evidence for this "genomic duplication" hypothesis. By performing *in silico* whole genome analysis, we demonstrate that both the number of miRNAs that target genes found in Copy Number Variations (CNVs) regions as well as the number of miRNA-binding sites are significantly higher than those of genes found in non-CNV regions [55]. In addition, by examining the miRNA–CNV genes interactions in eight different species we demonstrated that there appears to be an evolutionary dependence on gene expression regulation by miRNAs [56]. There is significant indication that a number of genes located within CNVs have increased (or sometimes reduced) expression level [57–60]. This suggests that miRNAs may have acted as equilibrators of gene expression during evolution in an attempt to regulate aberrant gene expression and to increase the tolerance to genome plasticity. Our results were further confirmed by Woodwark and Bateman [61]. These data raise the possibility that miRNAs may have been created under evolutionary pressure, as a mechanism for increasing the tolerance to genome plasticity.

One specific example that shows the role of miRNAs in tuning the gene dosage of paralogs is represented by atrophins, a phylogenetically conserved family of transcriptional regulators that appeared in metazoans (Atro) and duplicated in vertebrates (ATN1 and Rere). The dosage of the fly atrophin gene Atro is under the tight control of miR-8 [62]. The lack of miR-8 produces Atro overexpression and results in elevated apoptosis in the brain, behavioral defects and severe defects in animal survival [62, 63]. Additionally, reduced Atro expression causes impaired survival, indicating that the fine-tuning dosage of this gene is crucial for its activity [62].

A rarer phenomenon is the targeting of miRNAs on coding sequences. Recent data suggest that in such cases, target sites arise from highly repeated sequences inside the ORFs. The same researchers showed that such sequence repeats largely arise through duplications and occur particularly frequently within families of paralogous C2H2 zinc-finger genes [64].

Adjustment of Expression Levels' Variability According Current Environmental Frame

Wu et al. [65] summarize a number of studies to support the idea that miRNAs are vital molecules for natural selection that proceeds through canalizing evolution. Canalization refers to the process by which phenotypes are macro-evolutionary stabilized within species. The existence and identity of canalizing genes have thus been an important, but controversial topic. For example, Rutherford and Lindquist [66] showed that hsp90 gene may be one of those genes, since its deletion in *Drosophila* can result in many abnormalities, in contrast to "cryptic" genetic variations that are found on it and do not cause any observable phenotype. Hsp90 is an important chaperone, regulating the folding of many proteins. In the same way, miRNAs affect the expression of many genes of the genome.

Despite the evidence for the importance of gene regulation by miRNAs, the typical magnitude of observed repression by miRNAs is relatively small [65, 67, 68]. Wu et al. [65] cite a number of previous studies to show that miRNAs can be functionally classified in two categories. The first category sets the mean of the expression level of the target genes (referred to as expression tuning) and the second one reduces their variance (expression buffering, or homeostasis). On the other hand, some miRNAs' deletions seem not to be "significant" at the organism level. Additionally, decay of some miRNAs genes is considered to be accomplished fast, maybe within millions of years only. Of course, a controversial observation is the conservation over species of a number of miRNA genes and their targets. All these advocate to "canalizing" phenotypes, regulated by miRNAs.

Despite the attractiveness of Wu's ideas [65], we believe that the real situation for miRNAs' evolution is slightly different. "Canalizing" evolution is of course a very important evolutionary phenomenon, since this way significant phenotypic frameworks are secured over the danger of drastic evolutionary changes (like new mutations' emergence, founder effects, genetic drift, occasional directional or adaptive evolution etc.). But in case of miRNAs, we have a kind of a very "flexible" and "adjustable" canalizing evolution. A whole network of gene regulation has been invented by nature in a way that genome can adapt very easily in current environmental changes; but of course without any drastic changes of the main phenotypes. Adding to this equation the phenomenon of genomic duplications, we have then an even larger network, increasing even more the genomic plasticity and "evolvability". In this way, maybe the majority of population members can adopt effectively under an environment change. Alternatively, conventional evolution knowledge demands the elimination of a big part of the population by natural selection, resulting to the survival of few of the best adopted individuals. Such catastrophic evolutionary procedures are highly energy consuming and can slow down evolution for just some relatively mild environmental changes. Sometimes, the only thing needed is just a fine tuning re-regulation of the expression network and this can be achieved effectively by the miRNAs'.

Going back again to genomic duplications, we can suggest that that genomic duplications are the "fuels" and miRNAs are the "regulators" of the genomic engine. An interesting question is what came first, duplications or miRNAs? Gu et al. [69] found evidence that support the evolutionary scenario that gene/genome duplications in the early stage of vertebrates expand the protein-encoding genes and miRNAs simultaneously. The fact is that this is a very complex co-regulation. Adding to this the parameter of evolutionary time, then we can actually have a three dimensional evolutionary process (Fig. 1.2).

Additionally, we must have in mind that environment is not only the one outside the organism boundaries but it is also the one outside the cell boundaries (usually termed as "internal environment"). Under this framework we can examine the results of Mukherji et al. [70] where they found that repression through miRNAs varies a lot among different individual cells. Additionally, they found that a miRNA can behave both as a switch, in the target expression regime below the threshold, and as a fine-tuner, in the sensitive transition between the threshold and the minimal repression regime at high mRNA levels. So, do miRNAs finally regulate their function at the cellular level and not at the organismic level? We previously commented on duplication events as significant factors for miRNAs' evolution. Interestingly, it is now well established that somatically derived CNVs may be an important factor of genetic differentiation among same type of cells. In this concept, evolution in cellular level micro-environments can be the reason of different proteomic profiles that are found in similar cell types and can explain the results of Mukherji et al. [70] and those of other studies. This hypothesis is more plausible in rapidly proliferating cells, like bone marrow stem cells and cancer cells. Selection can act more efficiently through a big number of cell cycles and very possibly new miRNA genes or target sites can emerge or decay this way. Future studies are needed and especially "wet lab" experiments to test these theoretical assumptions.

Conclusions

Nobody can doubt that miRNAs are significant buffering tools that can modulate problems associated with drastic changes in gene expression. It would not be an exaggeration to suggest that miRNAs could have emerged under the pressure of genomic duplications, in order to control "genomic boundaries" and "genomic expression". It is also possible that miRNAs emerged multiple times through evolution (there are some data supporting this hypothesis in animal and plants), for the above reason. What is certain is that since they emerged, they are continuously gaining significant cellular, developmental and evolutionary roles. On the other hand, it would be a unreasonable to believe that this is the whole story in gene expression regulation. We believe that we still have a lot to learn regarding gene regulation and the associated mechanisms.

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Chapter 2
MicroRNAs in Disease

Gregory Papagregoriou

The Study of MicroRNAs

Overview

MicroRNAs or miRNAs are a newly described class of short non-coding RNA molecules with the distinctive role of fine-tuning the expression of mRNAs in the living cells of all organisms [1]. Their targets are usually mRNA molecules that bear specific miRNA recognition sites on their 3′ UTRs, 5′ UTRs or coding regions; miRNAs bind onto their target sites in a Watson–Crick base pairing manner and eliminate mRNA translation (Fig. 2.1a) [2]. In humans, such post-transcriptional regulation of gene expression cannot be overlooked as more than half of all genes have evolutionary conserved miRNA target sites [3]. Consequently, miRNAs are prime regulators of all kinds of physiological cellular processes; hence faulty regulation of mRNA expression can lead to disease.

Target Prediction Algorithms

More than a handful of algorithms are available online and are elegantly designed to deliver predictions for miRNA binding sites on the 3′ UTR of protein-coding mRNAs, or to predict target sites for any miRNA sequence. Their numbers keep growing in order to serve the emerging demands of the scientific community for customization and credibility of results. Search variables usually include the

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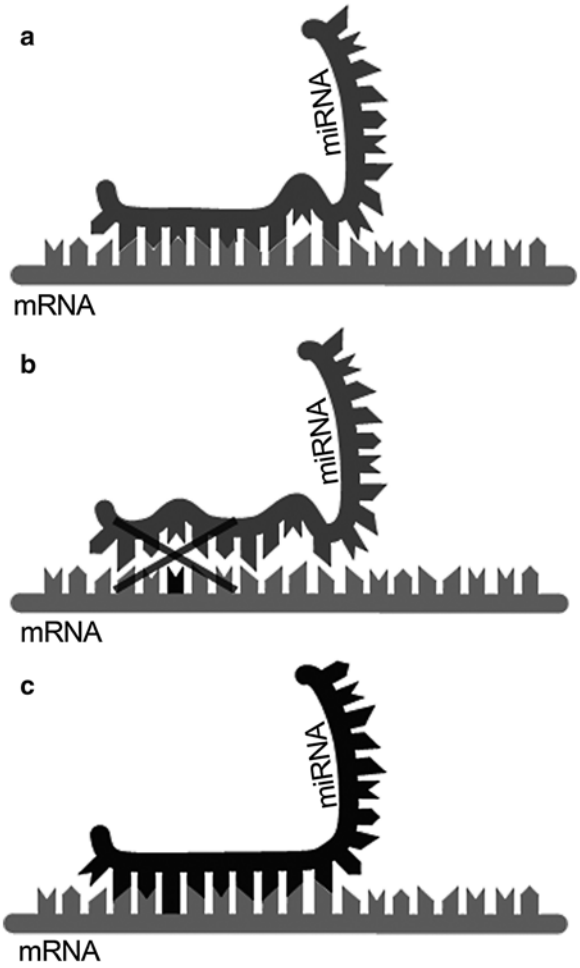

Fig. 2.1 Polymorphisms on miRNA target sequences (miRSNPs). Normal binding of miRNAs (**a**) can be diminished when target nucleotides corresponding to the miRNAs seed region are altered (**b**). In some cases, miRSNPs serve as gain-of-function mutations, when they create new target sites for different miRNAs (**c**)

seed-region length and miRNA/mRNA species, while each algorithm allows for further customization of search criteria depending on the prediction approach or even a cutoff p-value in correspondence to the statistical processing followed in each case. One must always have in mind the philosophy of predicting a miRNA target site on the 3′ UTR of an mRNA or elsewhere: a certain algorithm follows precise and predetermined criteria to predict such sites, which some are based solely on Watson–Crick complementarity between the miRNA and its target sites and other encompass more complicated approaches such as a learning algorithm or free energy values, therefore hits returned are destined to include false-positive results. Consequently, not all prediction results are valid and there is always a need for using a filtering approach in an effort to isolate the most useful and in a sense true miRNA–mRNA pairs. Filtering strategies can revolve around a wide or a narrow spectrum of criteria that are predominantly "*making sense*"; hence, both the miRNA and its potential mRNA targets should be expressed in the same tissue for example, or they are encountered in the same pathway or developmental stage. Moreover, all algorithms are enforced with a powerful statistical evaluation of prediction results, which in most cases can itself filter out correct pairs. In some studies, researchers repeat prediction analyses using a number of available algorithms and align prediction results to pick up only the pairs predicted by the most of them.

All algorithms predict target sites on 3′ UTR mRNA sequences, while some of them expand their predictions to include the gene's coding sequence, its 5′ UTR, sequences located upstream the transcription start points and even on the mitochondrial genome [4, 5]. The miRWalk algorithm works by "walking" on the gene sequence in a window of seven or more nucleotides and a miRNA is predicted to target a specific mRNA based only on seed region complementarity [4]. TargetScan on the other hand, is searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA, while it "ranks" a predicted miRNA target based on site length, type, context and accessibility [6]. The miRanda algorithm considers the miRNA sequence as input and searches a sequence dataset for potential target regions and successful predictions are made based on the alignment score and the minimum free energy of the miRNA bound to the potential target sequence [7]. The miRDB algorithm uses a completely different approach, as it is based on an SVM algorithm which is trained by a wiki database, in which users are able to input sequences of validated miRNA/mRNA couples [8], while the RNA22 algorithm uses a reverse approach as it first examines gene sequences for putative miRNA binding sites and then identifies a miRNA that could target an identified 3′ UTR site [9]. Predictions can be easily made by visiting the appropriate web location of each algorithm and placing a query about your miRNA or mRNA of choice. Results will be returned instantly and will depict the target site, its length, and a statistical score describing the likelihood this interaction is true based on the parameters on which each algorithm operates. The validity of prediction algorithms has been the number of many studies, all indicating an increased rate of false-positive results emerging from predictions, therefore a good bioinformatics analysis and filtering of predicted targets should be then supported by functional experiments [10].

Validation of miRNA Targets

As prediction algorithms can be a starting point in miRNA target discovery, direct interaction between a miRNA and an mRNA can only be valid when at least proven in vitro. Luciferase reporter constructs have been widely used as a straight-forward solution in studying direct binding of a miRNA on its target sequence. Albeit a useful and reliable tool, luciferase reporter constructs can only serve in examining one particular miRNA–mRNA target site and can be laborious at times. Target sites are introduced into the 3′-untranslated ending of the luciferase gene and plasmids are transfected into cell lines together with miRNA mimics or inhibitors (also called antagomirs). miRNA-analogous oligonucleotides are commercially available, relatively low in price and ready to use in cell cultures in various types; “mimics” that share the same sequence as a mature miRNA, “inhibitors” that are used to silence a specific miRNA, or “target site protectors” that prohibit miRNA binding onto its target sequence. Commercially available mimics and inhibitors are frequently chemically modified to LNAs (Locked Nucleic Acids) by the insertion of a 2′O-5′C Methyl-bridge, which helps the RNA oligonucleotide to keep an open conformation and to be thermally more stable than conventional oligos. (The reader is encouraged to see [11] and references within for a good presentation of currently used techniques in miRNA research and their limitations).

A good miRNA–mRNA couple can significantly reduce luciferase expression levels and in the same time a target site mutation, preferably at the nucleotides corresponding to the miRNA’s seed region, can abolish miRNA binding ability and therefore increase luciferase expression; this is a useful approach when investigating the effects of SNPs occurring at miRNA target sites.

As miRNAs are considered as post-transcriptional regulators, further investigation of their properties can include the determination of the protein levels of target mRNAs. This can be achieved by performing protein assays, i.e. western blots, amino acid stable isotope labelling or proteomics, after the overexpressing or knocking-down miRNAs in vitro. It has been found that specific miRNAs can regulate a restricted number of proteins, while changes in protein levels can sometimes be subtle [12]. Such effects are somehow expected, as protein levels are determined by a number of factors including mRNA transcription rate or protein degradation.

Identifying miRNAs in Tissues, Bodily Fluids and Exosomes

Isolation of miRNA species can be achieved by using readily available kits in the market developed by various companies. Specific applications require specific kits usually depending on the starting material, for example isolation of an enriched miRNA fraction from urine samples, serum or formalin-fixed paraffin-embedded (FFPE) tissues can be performed using appropriate commercial kit protocols. Alternatively, the TRIzol reagent can be used, although a biased loss of small RNAs with low GC content when processing a small number of cells with TRIzol has been reported [13]. Exosomes are small, 30–150 nm sized vesicles secreted by cells that contain miRNAs among other molecules; the isolation of miRNAs from exosomes can be achieved either by the use of Total exosome isolation reagents or by ultracentrifugation in sucrose gradients [14, 15]. Quantitation and quality assessment of enriched miRNA fractions can be performed using a microfluidics-based platform or an equivalent electrophoresis system, rather than a standard spectrophotometer due to their small size and reduced abundance compared to total RNA.

MicroRNAs can be detected using a number of methods, such as northern blots, real-time PCR or miRNA-specific probe hybridization. Although having limited sensitivity, northern blots are widely used for the identification of specific miRNAs and while their workflow is relatively simple, they require heavy optimization. A sample is let to run on an electrophoresis gel, which is consequently transferred to a porous membrane and miRNA-specific probes are let to hybridize onto targets while emitting fluorescence or radioactivity [16]. Relative quantification of cell-extracted miRNAs can be easily performed by real-time quantitative PCR (qRT-PCR). Following extraction, reverse transcription of miRNA species is performed in a two-step procedure: miRNA molecules are extended (3′ end) and single strand synthesis is completed with a universal primer [17, 18]. With the use of appropriate primers, miRNA sequences are detected in qRT-PCR and can be quantified by being compared to a number of small RNAs used as reference. Alternatively, reverse transcription can be performed using a stem-loop approach and miRNAs can be accurately detected via highly specific TaqMan probes [19]. In samples originating from exosomes or biofluids, quantitative analysis of miRNA expression can be difficult, as there is usually a lack of a stably-expressed small RNA to be used as a reference. For this purpose, a synthetic miRNA is usually spiked-in at a predetermined concentration to assist in downstream analyses [20–22].

In fresh or preserved tissue sections, miRNAs can be easily detected by in situ hybridization (ISH), where labelled probes with complementary sequences to target miRNAs are left to hybridize and reveal miRNA localization and differential expression [23]. Despite its wide use, ISH requires optimization and can sometimes be a laborious process. Nevertheless, when the probe affinity for its target is high, ISH can be used as a semiquantitative method of determining miRNA abundance in tissues and single cells. High resolution analysis of miRNA expression at single-cell level can be also performed by pairing fluorescent ISH with flow cytometry (flow-FISH), a technique able to give additional useful information on mRNAs or proteins of interest simultaneously [24, 25].

High-Throughput Methods in miRNA Research

The special nature of miRNA molecules makes their study a cumbersome matter; miRNAs are quite small in size and, unlike mRNAs, they do not share any common sequence features that ease their simultaneous isolation [26]. Tissue and cell miRNA profiling or disease biomarker discovery can be performed with the use of high-throughput methods such as commercially available microarray platforms or next-generation sequencing (NGS) of isolated miRNA fractions [27, 28]. Both techniques are considered acceptable, albeit approaching miRNA identification in a different manner. Microarrays identify fluorescently labelled miRNA cDNAs as they hybridize in complementary glass-immobilized probes, while NGS detects miRNAs by sequencing them; hence through NGS previously unidentified or novel miRNAs can be identified, while miRNA chip microarrays work with a predetermined range of miRNAs depending on the chip of choice. Differential expression of miRNAs on the other hand can be efficiently performed by both techniques, while small-scale study of specific miRNAs can be also performed using qRT-PCR [29]. Results are quite simple to read after they are further validated with qRT-PCR: certain miRNAs are expected to be either up- or down- regulated in a pathogenic tissue or cell type compared to controls, thus giving a sense of a pattern to characterize a disease.

Nevertheless, the elucidation of the exact biological meaning of any findings can at times be problematical and unfortunately in studies available, further functional investigation of findings is rarely performed.

Furthermore, microRNA targets can be also detected using high-throughput methods. Integration of high-throughput sequencing methods and protein immunoprecipitation, led to HITS-CLIP (High-throughput sequencing of RNA isolated with crosslinking immunoprecipitation) a robust method established by Chi et al. [10], which is exploited for the simultaneous isolation of miRNA–mRNA couples.

Antibodies raised against AGO are used for the immunoprecipitation of RNA-binding protein complexes from 254 nm UV-crosslinked samples, and at the same time the mRNAs bound on the miRNAs which these complexes accompany. An improved version of HITS-CLIP was developed by Hafner et al. [30] in an attempt to overcome technical limitations emerging from inefficient UV-crosslinking, named as Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP). In PAR-CLIP, cells are treated prior to crosslinking with 4-thiuridine, which is incorporated into targeted mRNAs to facilitate the precise binding position of the riboprotein complex by detecting thymidine to cytidine transitions. By PAR-CLIP, crosslinking efficiency was severely enhanced by irradiating cells with UV light and RNA recovery was dramatically improved. Moreover, Cross-linking ligation and sequencing of hybrids, or CLASH is a recently developed technique used to map miRNA–mRNA interactions by NGS [31]. In CLASH, cells that keep a stable expression a tagged AGO1 protein (PTG-AGO) are UV irradiated and lysed. PTH-AGO is then purified and samples are treated with RNAses that trim RNA–RNA duplexes, which are in turn ligated together and form chimeric miRNA–mRNA molecules that are eventually sequenced with NGS.

MicroRNAs Triggering Diseases

miRNA-Related Mutations as the Primary Cause of a Disease

A *miRSNP* (Fig. 2.1) can either be a single nucleotide change affecting the target region of a miRNA or its sequence at maturity. Such SNPs can effectively eliminate or weaken the binding of a miRNA to its target mRNA 3′ UTR (Fig. 2.1b) site and/or create a new binding site for a different miRNA (Fig. 2.1c); thus miRNAs can be considered as both primary or secondary players in disease development. In both cases, the protein levels of a targeted mRNA can be altered; at times, such changes can be phenotypically evident. Mutations in genes coding for miRNAs are considered as being quite rare; up to date only a small number of publications report such mutations that run in families in a Mendelian manner. Following the one-to-many mode of action, miRNAs with mutated seed regions lose the ability to target the range of mRNAs they usually aim at but inevitably gain novel targets. In some cases, mutations in the seed or other vital miRNA gene regions affect the abundance of the mature miRNA.

The frequency of SNPs in miRNA genes was thoroughly investigated by Gong et al. in 2011, who gathered all known SNPs from dbSNP v.132 that fell into such sequences. Notably, only 757 polymorphisms (SNPs and indels) out of 30 million in total were found to be located in 440 pre-miRNA regions, with 50 of them to be positioned in the seed regions of 41 miRNAs [32]. Such small numbers imply that there is a great possibility of a seed region sequence change to be a disease causing mutation rather than have no effect. Moreover, Gong et al. report that evolutionary conserved miRNAs and clustered miRNA genes tend to have less SNPs, a fact that can be attributed to the functional importance of certain miRNAs. The potential role for each SNP was calculated and recorded in the miRNASNP database (www.bioguo.org/miRNASNP).

The first work reporting seed region mutations was by Mencia et al., who identified two variants in miR-96, at positions 4 (+13G>A) and 5 (+14C>A) of its seed region, in a Spanish family with autosomal dominant non-syndromic hearing loss (ADNSHL) [33]. These mutations are responsible for the defected action of miR-96, as it fails to target and regulate five genes expressed in the inner ear; *AQP5*, *CELSR2*, *MYRIP*, *ODF2* and *RYK*. MiR-96 is expressed together with miR-182 and miR-183 as a multicistronic transcript in the mouse retina as well as in the inner ear. However, miR-96 seed region mutation carriers did not have an ocular phenotype, hence miR-96 is thought to target genes expressed in the ear rather than in the retina. In a different study, 882 ADNSHL patients from Italy were screened for miR-96 seed region mutations [34]. Interestingly, a novel mutation that segregated with the disease in one family was successfully identified, but was located outside the mature miRNA sequence. Being part of the pre-miRNA hairpin sequence, this mutation was found to effectively alter both mature miR-96 and miR-96* passenger miRNA biogenesis. Furthermore, Dorn et al. in 2012 described a similar mutation at the 3′ end (u17c) of miR-499 sequence that fell outside its seed region [35]. This mutation was identified while investigating a cohort of 2.606 individuals in search of genetic factors contributing in cardiomyopathy. By using luciferase reporter constructs and a mouse model, the authors identified a series of mRNAs that escaped miR-499 regulation possibly due to the c17 mutation.

Mutations in the miR-184 seed region have also been reported. MiR-184 was found to be abundantly expressed in the corneal and lens epithelia [36]. A single mutation at the fourth seed region nucleotide (c.57 C>U) of miR-184 was found to be associated with autosomal dominant familial keratoconus with early-onset anterior polar cataract in a Northern Irish family [37]. This mutation was identified after deep sequencing of a genomic locus indicated by linkage analysis in three generations of the family. The same mutation was also identified in a Spanish family with early onset cataract paired with various ocular abnormalities, while it has also been found in patients with EDICT syndrome (endothelial dystrophy, iris hypoplasia, congenital cataract and stromal thinning) [38, 39]. A different study identified two other mutations (+8C>A and +3A>G) in miR-184 in patients with isolated keratoconus that significantly repressed the expression of miR-184 [40]. Nevertheless, the exact mechanism explaining the pathogenesis of miR-184 seed region mutations remains elusive.

Mutations in miRNA Genes or Target Sites Contributing to Disease

Currently, there has been great interest in the discovery and functional characterization of miRSNPs located both on miRNA genes and miRNA target sites, as being contributors to a pathological phenotype. Such polymorphisms can act as phenotype modifiers by improving or exacerbating disease manifestation, or contributing to the risk of developing primary or secondary clinical states. Validated and predicted miRSNPs in human and mouse genes are recorded into four databases, Patrocles, dbSMR, PolymiRTS and MiRSNP [41–44]. Published data implicate miRSNPs in diseases in more ways than one; they can have a significant contribution to the pathogenesis of a disease, they can modify well characterized phenotypes of patients bearing a single mutation in a specific gene, they can regulate drug responses, or they can have no effect at all. Whatever the case may be, miRSNPs cannot be overlooked by modern geneticists. The role of miRSNPs will be analyzed below using some good examples from the current bibliography.

In diseases with monogenic inheritance, miRSNPs can have a significant effect. Evidence for such mechanism was shown for miR-24 when a point mutation that altered its binding to *SLITRK1* gene was identified in patients with Tourette syndrome [45]. Similarly, point mutations on *REEP1* which is a candidate gene for hereditary spastic paraplegia were found on the binding sites of two miRNAs (miR-140 and miR-691) [46, 47].

The role of miRSNPs as phenotype modifiers was demonstrated in CFHR5 nephropathy, where all patients found as far share an identical duplication of exons 2 and 3 in the *CFHR5* gene [48]. Although related, certain patients are clinically distinguishable with a portion of them rapidly progressing to mid-life end-stage renal failure requiring renal transplantation, and the rest having only some episodes of microscopic or macroscopic hematuria but with uncompromised renal function [49]. During the progression of the disease a genetic trigger channels the clinical fate of each patient towards a certain direction. The SNP rs13385 located on the 3′ UTR of the *HBEGF* gene and the target region of miR-1207-5p was reported to be associated with the severity of CFHR5 nephropathy in these patients, as the T-allele can eliminate the miRNA binding onto its target sequence [50].

Complex genetic traits are often the result of a joint action among a number of genetic and environmental factors that construct phenotypes, which are usually highly variable among patients. Impressively, a number of examples are available demonstrating the implication of the same common miRSNP in a number of different multifactorial phenotypes. For example, a miRSNP spotted on the miR-146a precursor (rs2910164 G>C) has been recently associated with susceptibility to leprosy [51]. When macrophage-like THP-1 cells were infected with live or irradiated strains of *Mycobacterium leprae*, live bacteria induced the expression of miR-146a, thus suggesting a pivotal role for this miRNA in disease progression. Consequently, C-allele carriers demonstrated a higher expression of miR-146a in nerves compared to patients with non-leprous neuropathies and this result was directly correlated with low levels of TNF recorded as a failure of the immune system to be effectively regulated. Impressively, the same SNP was also found to be associated with other multifactorial phenotypes, such as ischemic stroke, colorectal cancer survival [52], control of cell apoptosis, migration and growth in non-small cell lung cancer cells [53], or Hirschprung disease by eliminating *ROBO1* expression levels [54]. A different common SNP (rs11614913-C>T) on miR-162a2, was found to be related with increased susceptibility to esophageal squamous cell carcinomas [55], to the development of cardiovascular disease in patients with type 2 diabetes [56], but not Parkinson's disease [57]. Both SNPs on miR-146a and miR-162a2 are considered to be common polymorphisms, with Minor Allele Frequencies (MAF) 0.38 and 0.39 respectively, and together with rs71428439 (A/G, MAF 0.15) on miR-149, rs3746444 (A/G, MAF 0.18) on miR-499, rs895819 (T/C, MAF 0.36) on miR-27a stem loop, rs4938723 (T/C, MAF 0.31) on the Pri-miR-34b/c promoter form a team of miRSNPs that have been weakly or strongly associated with all kinds of diseases. However, only a small number of published works extend their findings in characterizing a functional relationship between the miRSNPs and relevant target genes to bridge gaps between genotypes and clinical phenotypes.

In a large number of publications, the role of miRSNPs in cancer susceptibility has been thoroughly evaluated [58]. Unfortunately, the functional characterization of such polymorphisms, as well as their comprehensive association to specific clinical features, is not the norm in most of them. Nevertheless, miRSNPs are proven to have an emerging role in cancer prognosis. A polymorphism on the 3′ UTR of *IGF-1R* gene limited the binding of miR-515-5p and increased the risk of developing breast cancer in subjects with *BRCA1* mutation [59]. In addition, increased susceptibility to breast cancer was attributed to two more SNPs identified on *TGFB1* and *XRCC1* which alter their expression levels as the target site of miR-187 and miR-183 is respectively interrupted [60]. In a Chinese lung cancer cohort, a SNP on the 3′ UTR of *CD133* was found to be significantly associated with a decreased risk in developing the disease, evidently by enhancing the binding of miR-135a/b to reduce CD133 levels [61].

Non-canonical miRNA Targeting Properties in Disease

As previously mentioned, miRNAs recognize and bind target sequences on the 3′ UTR of mRNAs waiting to be translated. In some rare cases, miRNAs were found to target mRNAs in other regions as well: the coding sequence and the 5′ UTR. Non-canonical miRNA targeting has been established by CLASH experiments, with 60 % of miRNAs to bind onto mRNAs with an irregular manner; mismatched nucleotides in the seed region, non-seed targeting and some of them binding to 5′ UTRs [31]. In addition, miRNAs were found to have similar efficiently in binding onto 3′ UTRs as they have when targeting 5′ UTRs in vitro to regulate the expression of mRNAs [62]. In some instances, miRNAs acting on the 5′ UTR of target mRNAs induce rather than repress their translation [63, 64]. Contrastingly, coding sequence targets are thought to be recognized by miRNAs less effectively compared to 3′ UTR target sites [65].

Akhtar et al. [66] presented evidence using luciferase reporter constructs that miR-602 and miR-608 target the sonic hedgehog (SHH) mRNA not on its 3′ UTR but on predicted target sites of the coding region. In osteoarthritis, *SHH* expression levels are elevated and eventually promote cartilage degradation. A potential mechanism explaining SHH upregulation involves the induction of SHH expression indirectly by IL-1β, through the direct suppression on the expression of miR-602 and miR-608 that repress SHH expression levels. In another study, the p53 inactivator MDM4 was found to have a miR-34a target site on its last 11th exon [67]. Moreover, a previously reported polymorphism rs79824231 located on this target site appeared to have the ability to disrupt miR-34a binding.

Functional miRNA target sites on the 5′ UTR are rarely found. Recently, miR-103a-3p was found to target and suppress the cancer related gene GPRC5A through two target sites on the gene's 5′ UTR in pancreatic cells [68]. Kim et al. [69] demonstrated a relationship between miR-34 family members, induced by p53, and the Axin2 mRNA in colorectal cancer. *Axin2* bears functional miR-34 target sites in both 5′ and 3′ UTRs that presumably act as "sponges" to negatively modulate miR-34 levels in cancer cells, which present elevated Axin2 and low miR-34 levels. Furthermore, a good example of non-canonical 5′ UTR miRNA binding is the ability of miR-122, a liver specific miRNA, to bind onto two 5′ UTR target sites of the Hepatitis C virus (HCV) genome and protects the UTR from host nucleolytic degradation to eventually promote its autonomous replication [70].

Emerging Pharmacogenomics Due to miRNA-Linked Genetic Variation

As expected, genomic variation related to miRNAs has also been implicated in patient response to administrated pharmaceutical therapy. In addition, certain drugs were found to formulate unique responses by interfering with miRNA expression levels. Such microRNAs have been described by a number of researchers and are frequently teamed under the term "Pharmaco-miRs". One can assume that mutated miRNA target sites on mRNAs engaged in pathways related to drug metabolism or absorption, can inevitably implicate miRNAs in drug responses as well [71]. In 2007 Mishra et al. presented evidence that miRSNPs can actually regulate drug responses. A polymorphism at the 3′ UTR of the dihydrofolate reductase (*DHFR*) gene, which was previously associated with upregulation of DFHR expression, was found to interrupt the conserved target site of miR-24 and caused the reported overexpression of DHFR [72]. As a result, elevated DHFR led to methotrexate resistance, a widely used chemotherapeutic agent. Polymorphisms in miRNA 3′ UTR target sites and/or miRNA genes have been associated with resistance to chemotherapy in patients suffering from various subtypes of cancer. The CREAM (Chemotherapy ResistancE-Associated MiRSNP) repository lists 150 such SNPs predicted to interfere with 1164 chemotherapy response compounds [73]. In breast cancer patients with estrogen receptor alpha expression, the acquired resistance to tamoxifen treatment has been associated with an elevated expression of miR-519a, which in turn targets a number of tumor-suppressor mRNAs to decrease the life expectancy of patients [74].

MicroRNAs in Developing Disease

Over the last few years many studies were designed to capture the effects of miRNA action in complex disease entities. Multifactorial diseases not only depend on the genetic load of an individual but are assisted towards overcoming a triggering threshold by lifestyle as well. MicroRNAs are considered as key regulators in disease development as they are implicated in all kinds of cellular processes and most importantly cell and tissue development and differentiation. Hence, the elucidation of miRNA signatures in such diseases, is postulated to unveil basic and advanced understanding of their pathology and progression and at the same time assist the development of tailored therapies per different patient. For this purpose, DICER knockout animal models, in vitro studies and expression assays have been recruited, as well as next generation high-throughput technologies.

MicroRNA signature of complex disease is a trending topic in scientific literature. Biomarkers are molecular signatures of a pathological state and can either be substances or molecules, which can be detected and measured in an objective way. MiRNAs have the specifications of being ideal and powerful biomarkers for non-invasive assays, as they can be measured in more than one ways, are abundantly expressed in all tissues and bodily fluids, are stable molecules and belong to a diverse and multitudinous family of non-coding RNAs.

miRNAs and Cancer

MicroRNA implication in cancer has been extensively studied in the past years and they have been found to be differentially expressed in a wide spectrum of malignant states. Being characterized as being both tumorigenic or tumor suppressive, miRNAs associated with cancer have been named as *Oncomirs* [75]; however, this characterization is only valid when a given miRNA is targeting an oncogene or a tumor suppressor gene. Deregulated miRNA expression under variable circumstances can alter their targeting potential against an mRNA, which in turn is associated with a specific type of cancer. In general, induction and progression of cancer is the orchestrated interaction and balance between tumor enhancers and suppressors and miRNAs seem to play a pivotal role in cancer development as more than half of miRNA encoding genes are found in genomic cancer hot-spots or at fragile chromosomal regions associated with translocations [76]. Tumor progression is marked by abnormal changes in cellular function and metabolism that lead to uncontrollable proliferation, resistance to apoptosis, escape from tumor suppressor action, induction of angiogenesis and eventually, invasion and metastasis; inevitably, miRNAs were found to be involved in all stages [77].

The first study implicating miRNAs in cancer by Calin et al. in 2002 demonstrated the involvement of miR-15a and miR-16-1 in B cell chronic lymphocytic leukemia. Their expression was found to be compromised by frequently observed deletions of the genes that encode for them in the 13q14 locus, which in turn is associated with the disease [78]. The miR-15a/miR-16 cluster of miRNA genes, encodes for miRNAs that are thought to be tumor suppressors under physiological states, by targeting BCL2 among other well described oncogenes [79]. The complexity of miRNA involvement in cancerous states has been widely observed. In some cases a specific miRNA has been given the properties of an oncomiR, while the same miRNA was found to act suppressively in other types of cancer. A good example is miR-125b; its levels drop in thyroid, ovarian and oral squamous cell carcinomas to halt cell proliferation and interfere with the progression of the cell-cycle, while in prostate cancer it was found to inhibit p53-dependent apoptosis of cancerous cells [80]. The p53 transcription factor is a direct regulator of miR-34a and miR-34b/c, while these miRNAs target and regulate p53 expression [81]. In chronic lymphocytic leukemia, miR-34a, miR-34b/c and *DAPK1* were found to be epigenetically inactivated by hypermethylation of their promoter to disrupt the tumor suppressive p53 pathway [82]. Hypermethylation of miR-34b/c is also considered as a potential diagnostic factor in Stage I non-small cell lung carcinoma [83]. In breast tumors, downregulation of miR-34a was significantly associated with metastasis [84].

In certain types of cancer miRNAs have a proven diagnostic and prognostic value, which has become of great significance in clinical practice. MicroRNA expression is evidently fluctuating in cancerous cells; affected tissues are distinguished by their expression potential of miRNAs. MiRNAs can be easily and efficiently isolated from formalin-fixed paraffin-embedded tissues, which is the starting material in most cases. Identification of miRNAs in biofluids, such as blood serum, saliva or urine, is also supporting the need of establishing non-invasive diagnostic and prognostic tests, as well as tumor classification tests.

The prognostic value of miRNAs has been investigated in many types of cancer, such as lung cancer, liver cancer, melanoma or prostate cancer. Inevitably, a number of specific miRNAs are recurrently found to be elevated or diminished in tissues or cell types studied. This fact can be explained by the potential role such miRNAs have in cancer development and their role as biomarkers cannot be overlooked. For example, in prostate cancer, miR-141 was found to be considerably elevated in the serum of patients with prostate cancer compared to controls and is considered as a prognostic marker with a 100 % specificity [85]. The same miRNA was also found to be increased in patients with ovarian cancer, while in metastatic colon cancer it was correlated with the levels of the carcinoembryogenic antigen (CEA) and poor prognosis [86, 87].

In a cohort of colon carcinoma patients, miR-21 was found to have a higher expression in adenomas as well as in patients with advance malignancy classification stage tumors. Survival of patients in the same study was also correlated with high miR-21 expression, as well as their response to therapy [88]. MiR-21 has also been found to be overly expressed in tumorous tissue from both breast and lungs [89, 90]. It is considered as a very important oncomiR and apoptosis suppressor and the therapeutic value of its inhibition was examined in breast cancer cells and mice with positive results [91]. In non-small cell lung carcinoma let-7 is not only considered as a primal tumor suppressor but also as a putative therapeutic agent. Poor prognosis of lung cancer in patients was directly correlated with compromised let-7 expression [92].

miRNAs and Diabetes

Diabetes mellitus (DM) is a complex disease affecting 347 million people of all ages worldwide. It is mainly characterized by elevated blood glucose levels and can be found in two forms depending on insulin availability or usage; pancreatic β-cells fail to produce insulin in Type 1 (T1D) patients, while Type 2 (T2D) patients are insulin resistant [93]. The role of miRNAs in diabetes starts with the control over pancreatic islet β-cell proliferation and function. In islet-specific Dicer1 mice knockouts, β-cells were completely absent and animals die soon after birth at P3 [94]. Beta-cell Dicer1 knockdown mice presented with a dramatic reduction in insulin production rates in isolated β-cells compared to wild-type animals [95]. Differentiation of insulin producing cells is a process mediated by a cross-talk between Neurogenin3 produced by endocrine progenitor cells and Hes1. Pancreatectomized mice presented a failure in β-cell regeneration from pancreatic pro-endocrine cells and defected post-transcriptional regulation of Neurogenin3 protein expression by miRNAs was found to be the cause; miR-15a, miR-15b, miR-16 and miR-195 were found to be highly expressed in treated mice and have predicted target sites on the Neurogenin3 transcript [96]. Beta-cell development and function is thought to be guarded specifically by miR-375. In vitro studies demonstrated an increase of insulin secretion after glucose stimulation in cells lacking miR-375, while miR-375 knockout mice presented with fasting hyperglycemia at the 12th week of life and β-cell mass was reduced responding to limited levels of proliferation [97, 98]. Using luciferase reporter constructs, miR-375 was found to have a conserved functional target site on the 3′ UTR of 3′-phosphoinositide-dependent protein kinase-1 (*PDK1*), an actively involved protein in insulin signaling and β-cell response in insulin demand through the phosphatidyloinositol 3 kinase (PI3-K) pathway [99]. Islet β-cell function is also regulated by miR-7a, which is regarded as a negative regulator of insulin granule exocytosis. In β-cell miR-7a2 knockout mice, insulin secretion was increased in response to high glucose levels, suggesting a higher tolerance to glucose [100].

Pancreatic β-cells sense glucose levels and respond by releasing insulin. Prolonged exposure of the pancreatic β-cell line MIN6 to high glucose levels initially induced miR-15a levels and eventually reduced them, in accordance with insulin production levels [101]. MiR-15a regulates insulin synthesis in an indirect manner, by targeting the mRNA of the uncoupling protein-2 gene (*UCP-2*), which codes for an important protein that monitors ATP generation triggered by glucose.

Insulin protein stability requires the polypyrimidine tract binding protein (PTB), which is in turn targeted by miR-133a. In glucose-treated human islets, miR-133 was found to be elevated and PTB biosynthesis was effectively reduced accompanied by a reduction in insulin synthesis as well [102]. In addition, insulin release is also regulated by miRNAs, with miR-124a targeting directly the exocytosis regulator Ras-related protein Rab27A, miR-96 and miR-9 increasing the expression levels of the Rab GTPase effector granuphilin, and miR-34a targeting the vesicle-associated membrane protein 2 (VAMP2) [103–105].

Tissue resistance to insulin leads to T2D and a number of studies demonstrate the involvement of miRNAs in this poorly understood process. A number of signaling pathways were found to be regulated by specific miRNAs in the liver, the adipose tissue and skeletal muscle, which uptake blood glucose in response to stimulation by endogenous or administrated insulin. Adipocyte development is facilitated by miR-143. Inhibition of miR-143 in pre-adipocytes reduced triglyceride accumulation by 75 %, while halted the expression of important genes such as *GLUT4*, *aP2*, *HSL* and *PPAR-γ2* [106]. The influence of miR-143 on adipogenesis was recently found to have a stage-specific role during their development, possibly by the direct regulation of MAP2K5 and consequently the MAPK signaling pathway [107]. In addition, insulin resistance in adipocytes is thought to be regulated by miR-320 via the direct regulation of the p85 PI3-K subunit, which in turn modulates the phosphorylation levels of Akt and Glut4 to assist downstream signaling pathways [108]. In obesity models, the pattern of miRNA expression in developing adipocytes appears to be paradoxically inversed [109].

The use of miRNAs as biomarkers in DM is also currently examined. In T1D non-obese mice, elevated miR-375 levels preceded the onset of diabetes by 2 weeks suggesting the use of this miRNA as a valid biomarker to indicate β-cell death and initiation of diabetes [110]. In T2D, a comprehensive evaluation of miRNAs in the plasma of patients, revealed the downregulation of miR-21, miR-24, miR-15a, miR-125, miR-191, miR-197, miR-223, miR-320 and miR-486 and the upregulation of miR-28-3p compared to healthy controls [111]. MiR-21 in particular, was found to be significantly upregulated in diabetic mice and correlated with the development of microalbuminuria and renal fibrosis and inflammation; soon after knocking down miR-21 in the same animals, renal symptoms ameliorated thus suggesting a potential role for miR-21 as a therapeutic agent for diabetic nephropathy [112]. Ethnic origin of T2D patients also appeared to play a role in circulating miRNA signatures. For instance, miR-144 was found to be significantly associated with T2D in Swedish patients, but not Iraqis, while miR-24 and miR-29b appeared to be consistently marking the disease in both populations [113].

miRNAs and Neurodegeneration

In neurodegenerative disorders, the physiological function or structure of neurons is gradually compromised leading to degeneration and inevitably cell death. Patients in most cases present with motor and/or cognitive decline depending on the impairment of specific brain regions, while clinicopathological symptoms occasionally overlap among different disease entities. Defected development of the central nervous system has been a common finding in Dicer knock-out animals, suggesting a pivotal role for miRNAs in neuronal differentiation and brain cortex size, while neurogenesis was found to be induced by a number of miRNAs, such as miR-9, miR-24, miR-125b and miR-128 [114, 115].

In sporadic Parkinson's disease (PD), mutations in *LRRK2* are considered as causative factors. LRRK2 protein levels are thought to be effectively regulated by miR-205 through a conserved binding site, while patients with sporadic PD demonstrated significantly reduced levels of miR-205, resulting in elevated LRRK2 levels and neurite outgrowth [116]. The role of LRRK2 in disease pathogenesis is still unclear, although its function as a kinase and GTPase implicates this protein in a number of molecular pathways possibly implicated with PD [117]. Nevertheless, in a drosophila model mutated *LRRK2* was found to interact with microRNA biogenesis through an RNA-independent association with the RISC complex [118]. Additionally, miR-133b was found to be reduced in the substantia nigra of PD patients and regulates the maturation and function of midbrain dopaminergic neurons possibly via Pitx3, which in turn modulates the expression level of miR-133b in a proposed negative feedback loop [119]. More genes involved in PD are also targeted by miRNAs, such as α-synuclein which was found to be regulated post-transcriptionally by miR-7 and miR-153 [120].

In developing Alzheimer's Disease (AD), specific miRNAs were found to regulate relevant genes. The β-amyloid precursor protein (APP) was found to be targeted by miR-106a and miR-502c [121], the tau protein is bound and regulated by miR-34a [122], and miR-98 targets IGF-1 [123]. Furthermore, exons 7 and 8 of APP are abnormally spliced in post-mitotic neurons of dicer conditionally knocked-out mice and miR-124 was found to be responsible for this effect assisted by its target gene *PTBP1* [124]. It appears that the abundance of miR-124 in neurons is concomitant with the occurrence of the neuronal APP isoform which lacks exons 7 and 8; hence, its absence promotes AD through the accumulation of non-neuronal APP. Furthermore, miR-9 was also found to be decreased in response to amyloid beta (Aβ) accumulation in primary neurons and is a direct regulator of *BACE1* expression, which in turn regulates APP cleavage [125, 126]. BACE1 mRNA is also targeted by miR-29 and miR-107, with the latter found to be downregulated in both AD and PD patients [119, 127, 128]. Moreover, in AD neuronal aging is promoted by an increase in miR-34 levels prior to the accumulation of Aβ in mice, possibly through its target gene *Bcl-2* [129].

The role of miR-146a in neurodegeneration has been explored by a number of studies. This miRNA is upregulated in brain regions affected in AD, while it is also induced by IL-1β, TNFα and Aβ42 peptides which in turn are pro-inflammatory cytokines triggering AD (reviewed in [130]). The same miRNA was also found to mark prion induced neurodegeneration [131]. The expression of miR-146a was found to be mainly induced by NF-κB in Toll/IL-1 receptors and represses the release of chemokines and IL-8 via a negative regulation of IL-1β as a part of a feedback loop to eventually regulate innate immune responses [132].

Identification of solid biomarkers in neurodegenerative disorders is still under great consideration by the scientific community. Examples of biomarkers in AD include miR-125b that has been proposed as a circulating biomarker with 68.3 % specificity and 80.8 % sensitivity when patients are compared to healthy controls [133]. Moreover, miR-384 which targets APP and BACE-1 was isolated from the cerebrospinal fluid of patients with mild cognitive impairment and Alzheimer's type dementia and was found to be significantly lower compared to controls [134]. In a recent meta-analysis of eight studies in search of the diagnostic validity of biomarkers in neurodegeneration, authors found contrasting results between different studies and suggest the usage of assays taking into account multiple miRNAs instead of a single species assay [135]. In other disorders such as multiple sclerosis, miR-21, miR-142-3p, miR-146a/b, miR-155 and miR-326 were found to be elevated in mononuclear blood cells and white matter brain regions in MS patients (reviewed in [136]). Also, in amyotrophic lateral sclerosis mice and patients the muscle-enriched miR-206 was found to be elevated in circulation and is proposed as a potential disease prognostic marker [137].

miRNAs and Cardiovascular Disease

The cardiovascular disease (CVD) group consists of a number of highly prevalent disorders that, together with cancer, are the leading causes of death in the western world [138]. Coronary artery disease presenting with acute myocardial infarction (MI), as well as essential hypertension, cardiac hypertrophy and atherosclerosis are the main categories of CVDs. Cardiac hypertrophy and failure are responses triggered by stress factors such as MI or hypertension, which in turn alter the hemodynamic environment and lead heart cells to undergo reprogramming in order to adapt. Consequently, heart cells regress to an expression potential that resembles a fetal type and cardiac cell specifically expressed miRNAs are thought to play a pivotal role in this procedure [139]. Dicer inactivation in cardiac cells of mice caused progressive dilated cardiomyopathy, accompanied by heart failure and eventually death after birth, while impaired expression of the dicer endonuclease was also identified in patients with dilated cardiomyopathy [140].

In the developing heart, miR-1, miR-133a/b and miR-208 are considered as basic players for regulating the expression of genes in cardiac muscles and therefore the heart's growth and function [141]. It has been demonstrated that at the onset of pressure-overload cardiac hypertrophy, miR-1 expression is repressed to cause downstream global changes in gene expression [142]. In Dahl hypertensive rats, silencing of miR-208a in the heart, prevented cardiac remodeling and myosin switching while increased rat survival [143].

MicroRNAs contribute in the development and stabilization of atherosclerotic plaques, with miR-92a to modulate plaque angiogenesis in mice [144], miR-21 to be stress-induced in the endothelium and modulates apoptosis [145], and miR-155 to regulate pro-inflammatory macrophages, act repressively on Bcl6 and enhance plaque formation [146], among others. In addition, miR-155, miR-145 and miR-126 are thought as potential candidates for atherosclerosis treatment [147]. Other factors conferring risk in atherosclerosis development, such as the high levels of low-density lipoprotein (LDL) and low levels of the high-density lipoprotein are regulated by miRNAs, with miR-122 to be responsible for increasing LDL levels and miR-33 upregulation to be associated with low HDL levels [148, 149]. Elevated LDL due to miR-122 upregulation is used as a control feature in miR-122 administrated antagomir therapy against Hepatitis C virus [148]. In an atherosclerotic mouse model, administration of miR-33 antagomirs increased HDL levels and plaque size appeared to be reduced and their stability increased [150].

In mice undergoing acute MI, members of the miR-29 family were found to be downregulated in the fibrotic heart region being adjacent to the infarct and to regulate the expression of fibrotic genes such as *COL1A1*, *COL1A2*, *COL3A1* and *FBN1* [151]. Cardiac fibrosis due to miR-29 might consequently lead to hypertension and it has been shown that adjustment of aerobic training habits in rats individuals helped in increasing miR-29c levels [152]. Moreover, miR-15 family members are found to be regulated in infarcted heart regions responding to ischemia-reperfusion injury in MI mice and pig models [153]. Therapeutic administration of miR-15 antagomirs, succeeded in sequestrating miR-15 tissue levels and reduced infarct size, while induced tissue remodeling.

In essential hypertension, human cytomegalovirus (HCMV)-encoded miRNA, hvmn-miR-UL112 was found to be differentially expressed between hypertensive patients and controls and it direct regulator of interferon regulatory factor 1 (IRF-1) mRNA, which in turn upregulates angiotensin II type3 receptor to deregulate blood pressure [154]. In pulmonary arterial hypertension (PAH), miR-204 was found to be significantly downregulated in vitro as a response to the aberrant expression of STAT3, which normally induces the transcription of miR-204 host gene [155]. Intratracheal delivery of miR-204 mimics in PAH affected rats presented with reduced arterial pulmonary pressure. Additionally, pulmonary vascular remodeling due to hypoxia in PAH has been associated with elevated miR-21 in mice [156].

The use of miRNAs as biomarkers in CVDs has also been extensively explored (reviewed in [157]). Myocardial damage is characterized by a release of miR-208b and miR-499 into circulation and the prognostic value of both miRNAs is under study [158]. In addition, the use of miR-133a as a biomarker for cardiomyocyte death in CVD patients has also been established [159]. Circulating levels of let-7b, miR-30a and miR-195 were identified in patients with developing MI and found to have up to 90 % sensitivity and 90 % specificity in discriminating patients from controls [160].

miRNAs and Renal Disease

Kidney development and growth is dependent on miRNAs. In mouse podocytes, the highly differentiated epithelial cells of the glomerulus, Dicer inactivation depleted foot processes and induced apoptosis, while animals developed albuminuria followed by glomerular sclerosis, tubulo-interstitial fibrosis, an abnormal appearance of the glomerular basement membrane, mesangial expansion, acute renal disease progression and eventually died after 6–8 weeks [161–163]. Impressively, proteins involved in podocyte function, such as nephrin and podocin, were found to be significantly decreased, while the major transcription factor that drives podocyte differentiation WT1 was found to be unaffected; hence miRNAs are believed to have limited influence in triggering podocyte differentiation but are essential in preserving podocyte function [163]. Like Dicer knockouts, clinical features of end-stage renal disease (ESRD) were recorded in Drosha-ablated mouse podocytes and animals presented with collapsing glomerulopathy, thus suggesting a pivotal role for miRNAs in podocyte function [164]. Microarray experiments revealed the specific enrichment of miR-192, miR-194, miR-204, miR-215 and miR-216 in the kidney, with miR-192 having a proven role in diabetic nephropathy (DN) [165, 166].

Diabetic patients developing nephropathy present proteinuria, thickening of the glomerular basement membrane (GBM), expansion of the mesangium and accumulation of the extracellular matrix, where laminin, fibronectin and collagens type I and IV fail to preserve GBM's normal structure [167]. In diabetic mice, miR-192 was found to be elevated in their glomeruli [168]. This miRNA can regulate E-box repressors expression, which in turn regulate *Col1a1* and *Col1a2* gene expression through TGF-β, leading to their accumulation. In addition, hyperglycemia seems to induce the abnormally high expression of miR-377 in mesangial cells, causing the targeted PAK1 and mnSOD mRNA downregulation and finally enhanced production of fibronectin [169]. Furthermore, in early diabetic nephropathy miRNA-21 was found to have reduced levels in db/db mice, while its over-expression paused mesangial cell proliferation and decreased the albumin excretion rate [170]. In podocytes cultured in high glucose levels, as well as in diabetic mice manifesting proteinuria, miR-195 expression was found to be elevated [171]. Furthermore, in Hypertensive Nephrosclerosis (HN), a disease where hypertension leads to arterial sclerosis which in turn causes glomerular sclerosis and hypertrophy, atrophy of the tubules and interstitial fibrosis, miR-200a and b, miR-141, miR-429, miR-205 and miR-192 were found in abundance and their levels correlated with the presence of proteinuria [172].

A frequent clinicopathological finding in glomerular disease is focal segmental glomerulosclerosis (FSGS), and urine miR-196a, miR-30a-5p and miR-490 were found to characterize patients with active FSGS compared to patients with FSGS in remission [173]. In mesangial glomerulonephritis, miR-21 and miR-124 were found to be upregulated in WKY rats [174]. Lupus nephritis (LN) manifests with mesangial glomerulonephritis, and in LN patients, miR-146a of glomerular origin was also found to be upregulated in both B6.MRLc1 mice and humans [175, 176]. In patients with IgA nephropathy, miR-200c was found to be downregulated with its levels of expression correlating with proteinuria, while miR-192, miR-141, miR-205 were upregulated, with miR-192 demonstrating an association with glomerulosclerosis and a decrease in glomerular filtration rate [177]. Furthermore, in a group of ESRD patients having received a kidney transplant, serum miR-181a, miR-483-5p and miR-557 were differentially expressed during the first 7 days following transplantation surgery, hence acting as factors predicting graft rejection [178].

In polycystic kidney disease (PKD), miRNAs were found to be important players in disease pathogenesis. Cyst development mechanisms in PKD are still poorly understood [179]. MiR-15a expression levels were found to be downregulated in both autosomal dominant and recessive PKD, which subsequently caused the upregulation of its target Cdc25A, a cell cycle regulator [180]. In a PKD rat model, differential expression of miRNAs demonstrated an increased expression of miR-21 and downregulation of miR-31, miR-164a and miR-125 [181]. Furthermore, the overexpression of the oncogenic miRNA cluster miR-17-92 in mice was found to be directly regulated with cyst growth, while its inactivation in a PKD mouse model delayed cyst development, as *Pkd1*, *Pkd2* and *Hnf-1b* gene transcripts have functional binding sites for both miR-17 and miR-92a [182].

MicroRNAs as Therapeutic Agents

The use of artificial miRNA molecules as therapeutic agents is currently under progressive development. Such molecules are modified oligonucleotides that can either sequestrate a specific miRNA to enhance the expression of its targets (antagomirs), or boost its levels to target disease promoting mRNAs (mimics). Based on their one-to-many way of function miRNA-like molecules can be used to treat diseases having more than one pathways affected, but on the other hand limit the specificity of a putative therapeutic approach [183]. Hence, good therapeutic candidates can be miRNAs that have a well-documented way of action and are ideally tissue specific. Moreover, drug delivery can be problematic and challenging in some cases (reviewed in [184]).

As mentioned earlier, in a work by Joplin et al. [70] miR-122 was found to target two specific sites at the 5′ UTR of the HCV genome to stabilize and protect it from nucleolytic degradation, thus assisting in hepatitis C propagation [185]. HCV infection is the primary cause of liver disease and affects more than 170 million people worldwide; hence the development of effectual therapeutic approaches is more than necessary and the exploitation of miR-122 properties over HCV will hopefully give rise to the first approved miRNA-related drug [186]. To develop this *miravirsen* drug, a series of anti-miR-122 oligonucleotides targeting the miRNA's 5′ end sequence were tested in order to block its seed region, and finally a highly stable sequence specific 15-mer LNA-modified oligo (SPC3649) demonstrated strong inhibitory effects on miR-122 function in hepatocytes of both mice and African green monkeys, even at low concentrations [148, 187]. When this miravirsen was administrated in HCV infected chimpanzees, they presented a significant reduction in traceable HCV, while HCV levels continued to drop even 2 weeks after the drug administration was concluded, thus rendering the miravirsen a more comprehensive therapeutic agent compared to other approaches [188]. Currently, this miravirsen is in Phase 2a clinical trials with very encouraging results. Clinical trials exhibit a dose-dependent and persistent reduction of HCV levels, while no side-effects were recorded [189]. Impressively, 14 weeks after concluding the drug administration four out of nine patients that received a high dose (7 mg/kg) of miravirsen, the viral RNA was undetectable, depicting a lack of resistance of the virus against the drug.

Besides miR-122, a small number of other miRNAs are also thought as potential therapeutic candidates. For example, miR-92a was found to control angiogenesis and its inhibition through an antagomir in mouse models presenting with limb ischemia and myocardial infarction, accelerated the recovery of affected tissues and promoted angiogenesis as it naturally targets relevant genes [144]. Additionally, the members of miR-15 family are found to be implicated in cell survival and regulate cell cycle progression and are upregulated in infarcted regions of mice and pigs in response to injury caused by ischemia [153]. Administration of modified LNAs in animals sequestrated the expression levels of miR-15 family members and increased the viability of cardiomyocytes after hypoxia, while cardiac function was enhanced.

Another important miRNA drug under development is MRX34, a double stranded RNA molecule that mimics miR-34 and is currently in Phase 1 clinical trials. As mentioned above, miR-34 family expression is promoted by p53 and is found to be downregulated in various types of cancer, as it is considered an important tumor suppressor (reviewed in [183]). The miR-34 family consists of three miRNAs that share the same seed-region, namely miR-34a, b and c, with miR-34a to be the most abundant. Tumor suppression in vivo was succeeded in animals with xenografts that promoted prostate cancer [190], lymphoma [191], non-small cell lung cancer [192] and others, which had miR-34 delivered intravenously or directly with intratumoral injections. Phase 1 trials of miR-34 replacement are ongoing and started with a debate on the drug delivery approach to be used. After exploring a number of available solutions, an ionizable liposome (NOV340—SMARTICLES) was selected to carry the miR-34 oligonucleotide based on its use in mouse models, the miRNA bio-distribution and the vehicle's safety [193]. When this liposome is released in biofluids with neutral pH it gains a slightly anionic character that prevents it from having non-specific interactions with negatively charged cellular membranes, while it becomes cationic in tumor regions where the environment has lower pH and becomes active. This specific vehicle is preferably delivered to the liver, thus liver cancer was proposed as the disease model in this case.

Conclusions

Without doubt, miRNA involvement in diseases is a trending matter in the literature. From causing a disease to modifying complex genotypes, miRNAs seem to be implicated in every aspect of a cell's effort to develop, differentiate, and lead a healthy living or program its death. Although a number of different approaches both in vitro and in vivo have been developed as a response to the growing needs of the scientific community to study miRNAs, the need to discover robust prognostic and diagnostic miRNA markers is essential. In addition, miRNA bioinformatics have been greatly used as the starting point in deciphering miRNA–mRNA target pairings and have become more and more efficient through time. Hopefully the development of novel high-throughput technique in the years to come will facilitate the comprehensive functional characterization of miRNAs in disease; the knowledge emerging from endless lists of differentially expressed miRNAs in cells or tissues is of little help in understanding their true biological meaning.

Conclusively, miRNAs have what it takes to become the next generation of therapeutic agents. Taking miRNA research from the bench to the bedside, it is indeed exciting that the first two miRNA drugs, miravirsen and miR-34 replacement, are already in clinical trials. What remains is the development of more drugs and why not, tailored therapies for patients.

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Chapter 3
piRNAs-Transposon Silencing and Germ Line Development

Catherine Demoliou

Introduction

DNA in eukaryotic cells is packaged into two forms of chromatin: euchromatin, and heterochromatin. Euchromatin is rich in actively transcribed genes. Heterochromatin is rich instead, in repeated, non-coding sequences representing multiple copies of ‘selfish’ or “parasitic” intergenic genetic elements that are epigenetically repressed [1–3]. These elements, called transposons or transposable elements (TEs), are able to mobilize “jump” within the genome, and to multiply during their transposition. TEs represent 45 % of the human and primate genomes. They are mainly scattered between and within genes in mammals including humans, and at constitutive heterochromatin pericentromeric and subtelomeric regions in plants and in *Drosophila* fruit flies. There are two major TE classes. Class I includes the autonomous retrotransposons, which either have long interspersed nuclear elements (LINEs), or no long terminal repeats (non-LTRs). Both types replicate in a “copy-and-paste” manner. That is, a TE is transcribed first to messenger RNA (mRNA), which associates with self-encoding, reverse transcriptases and endonucleases in the cytoplasm, and then it is transported back into the nucleus. There, the TE-RNA is reverse transcribed to DNA and integrated into a new site in the host genome. Subtypes of LTRs, the non-autonomous LTRs, contain short interspersed elements (SINEs), which replicate using the activities of an endonuclease and of a reverse transcriptase encoded either within the LINE-1 elements (L1), or within other TEs. In contrast, the Class II TEs are of the DNA-type. They transpose via a DNA “cut-and-paste” mechanism using the “target capture” action of a transposase, which is often encoded within the DNA sequence of the TE.

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TEs have contributed to genomic changes that had an impact on the evolution of eukaryotes, and are responsible for intra-population genetic variations. Recent evidence shows that through evolution, TEs, once thought to be only parasites, have been recruited by genomes to defend TE invasions. TE transpositions, however, continue to be a thread, since TEs have not stopped replicating and mobilizing independently within the genome. Mutations caused by TE transpositions may result in the deregulation of transcription and translation of genes required for normal cell function. Furthermore, TE transpositions can be the cause for diseases including cancer. *De novo* transpositions of TEs are linked to at least 65 known human diseases. Most importantly, TE transpositions during germ line development can be the cause of developmental, hereditary and fertility disorders [1–6].

The control of gene expression in eukaryotic organisms is by gene silencing at DNA level, involving chromatin remodelling that is regulated via DNA methylation and epigenetic modifications [4–6]. In addition, eukaryotes have evolved highly efficient and specialized systems, which detect, guide, and repress the expression of genes and especially of active TEs. Such systems recruit non-coding RNAs to regulate TE-expression via transcriptional gene silencing (TGS), or posttranscriptional gene silencing (PTGS). TGS systems operate in the nucleus and can induce epigenetic modifications guided by long or short antisense non-coding RNAs. PTGS systems inactivate or degrade targeted mRNA or TEs. They operate mainly in the cytoplasm where they are guided by short non-coding RNAs. These small RNAs associate with effector proteins to form the catalytic core of the RNA-induced silencing complex (RISC) [7–9]. In addition, as we will see later on, there are other partner proteins, which contribute to the assembly of a functional RISC. The degradation of targeted mRNA via RISC, is called RNA interference (RNAi), and it was first observed in plants [10, 11].

The small RNAs used for TGS or PTGS in metazoan include several classes: the micro RNAs (miRNAs), which regulate gene expression; the small interfering RNAs (siRNAs), which regulate gene expression and TE transpositions, and the small non-coding P-element induced RNAs (piRNAs), which repress TE expression and mobilization specifically in germ line cells during gametogenesis (Fig. 3.1). piRNAs (24–32 nt long) exert their effect by binding to the P-element induced wimpy testis (PIWI) subfamily of proteins of the Argonaute family. The smaller miRNAs and siRNAs (20–23 nt long), exert their effects by binding instead to the AGO subfamily members of the Argonaute family [14–17].

This chapter will concentrate on the biological roles of piRNAs and the PIWI proteins, which are expressed in germ cells and form the catalytic core of piRISC. Genetic studies on PIWI proteins using animal models and the isolation and sequencing of piRNAs have aided in building up a coherent picture of piRISC functions during gametogenesis [12, 18–25]. This picture, although not complete, shows the evolution of interrelationships between piRNAs and TEs expressed during embryogenesis and postnatal germ cell development. Germ cells use TEs in *cis*, as the source of small RNA regulatory sequences (e.g. piRNAs) to guide the degradation of TEs or mRNA. TEs in *trans*, appear to provide instead RNA regulatory sequences for modulating gene transcription/translation (e.g. DNA methylation, RNA inactivation by deadenylation etc.) [1–3, 22–25].

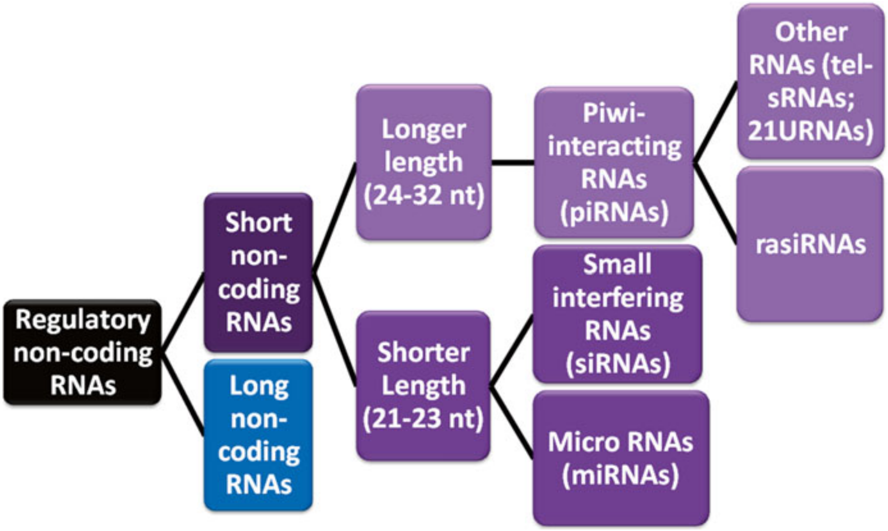

Regulatory non-coding RNAs
Short non-coding RNAs
Long non-coding RNAs
Longer length (24-32 nt)
Shorter Length (21-23 nt)
Piwi-interacting RNAs (piRNAs)
Small interfering RNAs (siRNAs)
Micro RNAs (miRNAs)
Other RNAs (tel-sRNAs; 21URNAs)
rasiRNAs

Fig. 3.1 Classes of regulatory non-coding RNAs and subsets of piRNAs. Long non-coding RNAs refer to the antisense RNAs involved in epigenetic functions [7, 8]. piRNA subsets: repeat-associated-siRNAs (rasiRNAs) identified in organisms like *Drosophila* and Zebrafish [12]; mouse heterochromatin associated pi-like small RNAs (tel-sRNAs) [13]; 21U RNAs, small RNAs expressed in the *C. Elegans* germ line [14]

Germ Cells and Embryonic Development

Mammalian germ cells arise relatively late during embryo development from a small population of extra-embryonic mesoderm cells of the epiblast. The cells develop into primordial germ cells (PGCs) that migrate and colonize the genital ridges that will form the gonads. Sex specification of PGCs in foetal testes, is defined by paternally inherited determinants expressed in the early development of the zygote. In the ovaries, it is defined by maternally inherited determinants present in the germ plasma of the oocytes. During ovary development, the PGCs first proliferate via mitosis, and then differentiate into germline stem cells (GSCs), known as secondary oocytes (Fig. 3.2). Subsequently, GSCs enter meiosis I and arrest in prophase. The completion of meiosis I occurs only upon onset of sexual maturation and ovulation when again they arrest in metaphase of meiosis II. At this stage, the oocytes build-up special cytoplasmic granules, the so-called germ plasma that is required to support the embryo for the next generation of PGCs. In addition, the presence of GSCs drives the development of support somatic (follicle) cells. In mice, the mature oocytes remain transcription-silent until fertilization occurs. Fertilization signals the oocyte meiosis to resume, and the highly differentiated oocytes finally transform into totipotent embryos [26].

Male mammalian PGCs, in contrast, undergo only mitotic proliferation during the stages of embryonic development (Fig. 3.2). They then arrest in G1 phase (spermatogonia), and differentiate to GSCs in the male gonad. Mitotic proliferation and

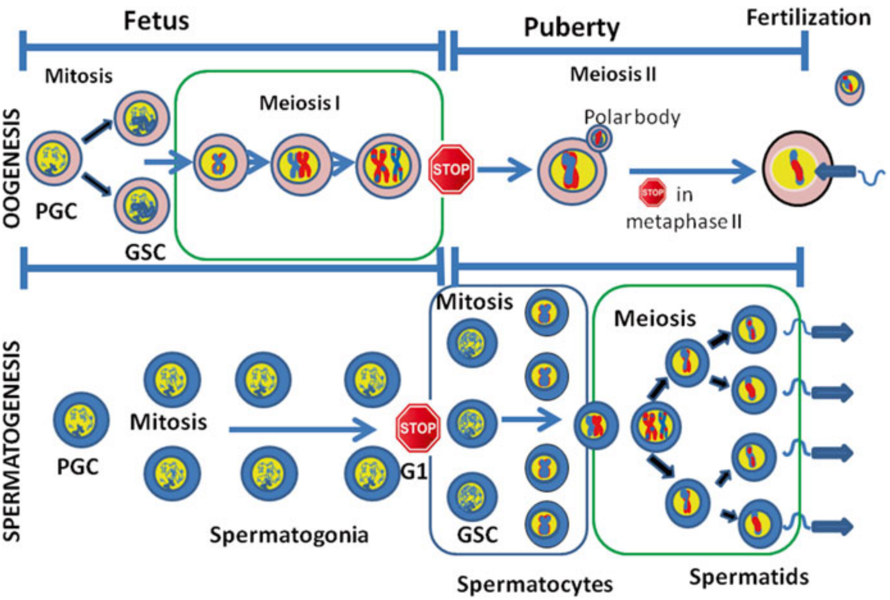


Fig. 3.2 Developmental germ cell transitions during gametogenesis. A schematic diagram showing mammalian female and male gametogenesis in the fetus, and adulthood [26]. In ovaries the number of primary oocytes is determined during foetal development. There is no further proliferation after birth; meiosis starts with sexual maturation, and is completed only upon fertilization. Male germ cells continue to proliferate upon sexual maturation to form spermatocytes that undergo meiosis to form haploid spermatids; *PGS* primordial germ cell, *GSC* germ stem cells

differentiation of spermatogonia into spermatocytes resume in the testes, only upon sexual maturation. Adult spermatocytes undergo meiosis to form each, four haploid spermatids that lose their cytoplasm as they develop into mature spermatozoa (sperm) able to fertilize an oocyte [26].

Mitotic proliferations of PGCs and transition into GSCs, prenatally in females, and during juvenile development in males, are characterized by a genome-wide DNA CpG demethylation by DNA methyltransferase (DNMT) enzyme(s). These processes require the expression of pluripotent stem cell markers (i.e. Oct4, Vasa, Fragilis and Nanog) [27], as well as several developmental factors, which define whether the cells will differentiate (specification) or continue to proliferate as pluripotent GSCs [28]. DNA demethylation is required in order to erase inherited imprints from both parents, and to ensure the pluripotency of the GSCs. It is also required to enable genomic reprogramming and resetting of imprinting for germ cell specification [6, 26]. It is this period of DNA demethylation that TEs can be particularly active due to the relaxation of epigenetic control and to the increased expression of transcription factors [2].

The maintenance of the GSC phenotype and GSCs proliferation involve extensive changes in histone modifications and RNA-driven gene silencing processes, which are specific to the germ line. Signals and molecules that commit germ cells to undergo meiosis are provided by somatic support cells. Meiotic progression events (i.e. crossing over, meiotic silencing of unpaired chromatin, imprinting), are also guided by epigenetic transitions that involve the re-establishment of DNA methylation patterns (*de novo* DNA methylation) as well as the post-translational modification of major histones [27–30].

The RNA-driven gene silencing processes are closely associated with the maternally inherited piRNAs and with the PIWI-dependent biogenesis of regulatory piRNAs from RNA-precursor transcripts [21]. During the window period of DNA demethylation and during germ cell mitotic self-renewal and meiosis, the piRNAs (inherited and newly generated), bind PIWI to form piRISC for targeted TGS or PTGS [21, 31–37]. These processes require the support of Tudor-domain-containing proteins known as Tudor domain-related proteins (TDRDs), which form the cytoplasmic scaffold for piRNA loading and processing and for piRISC mobilization and function [38–49]. In addition, a functional piRNA-PIWI pathway is also required for ovarian somatic cell-support during germ cell development in the zygote [34, 50]. The PIWI protein expression and the piRNAs signatures identified in diverse species such as *Drosophila*, zebra fish and mammals, indicate that this pathway has been conserved through evolution [51].

Other short RNAi pathways, i.e. the miRNA pathway and the siRNAs-pathway, are also important for the proper development of germ cells. Recent findings suggest that PTGS during the very early developmental stages of the embryo, between oocyte fertilization and blastocyst implantation, may involve RNAi processes that are guided by temporal transitions of endogenous small RNAs, from retrotransposon-derived siRNAs/piRNAs or zygote synthesized miRNAs [52, 53] (Fig. 3.3).

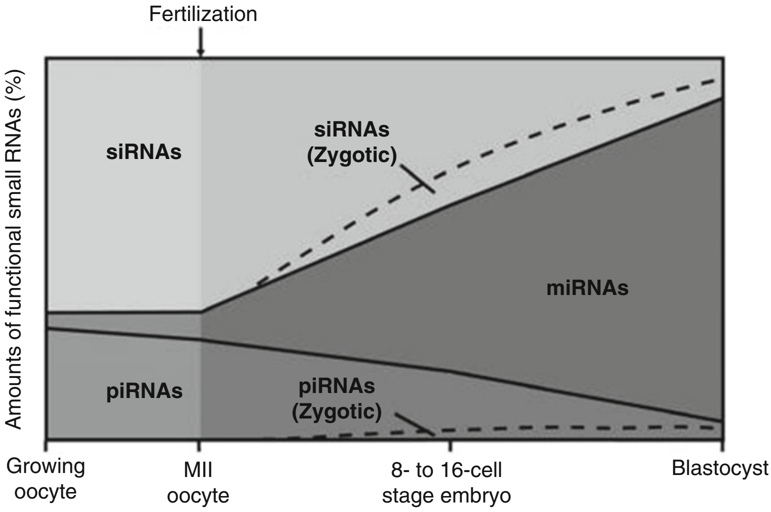


Fig. 3.3 Proposed transition of functional small RNAs during mammalian oogenesis and early embryogenesis. The schematic diagram is not drawn to scale. *Dotted lines* represent the putative expression of zygotic siRNAs and piRNAs. Reprinted from Ohnishi Y, et al. Small RNA class transition from siRNA/piRNA to miRNA during pre-implantation mouse development. Nucleic Acids Res. 2010; 38(15):5141–5151 (Open Access) [52]

The PIWI Proteins

Most of the information about the function of PIWI proteins comes primarily from studies in *Drosophila* and mice. The *Drosophila* PIWI proteins Aubergine (Aub), Piwi and Argonaute-3 (Ago3) comprise the PIWI subfamily of the highly conserved Argonaute family. The other subfamily, referred to as AGO, includes the Ago1 and Ago2 proteins that bind miRNA and siRNA, respectively [40, 54–56]. AGO proteins are ubiquitously expressed and have been found in almost all eukaryotes. Metazoans have representatives of both Argonaute subfamilies. Fungi, green algae and plants encode exclusively AGO-like proteins whereas amoebas encode exclusively PIWI-like proteins, placing *piwi* as the oldest ancestor gene and first in the line of evolution of the Argonaute clan [12, 14–17, 46, 51].

In *Drosophila*, the Ago3 and Aub proteins are expressed primarily in the cytoplasm of male and female germ cells and in the cytoplasm of female somatic nurse cells. In contrast, Piwi is a nuclear protein and it is expressed in both, germline and ovarian somatic cells [54]. PIWI proteins are required for the biogenesis of *Drosophila* piRNAs and for the piRISC-mediated degradation of TEs in germ cells, as well as for TGS in somatic and in germ cells [21]. These activities are interlinked and essential for germ line maintenance, proliferation and differentiation that are required to ensure fertility. Piwi and Aub expression is required for ensuring both male and female fertility whereas Ago3 expressions seems to be more important in female fertility [40, 57]. Mutations in PIWI proteins result in the over expression and mobilization of retrotransposons in male flies. This results in DNA damage and germ cell apoptosis that causes sterility since GSC maintenance, proliferation and embryonic axis specifications are defective [20–22, 24, 25, 32–34, 50, 54–56].

The Mouse PIWI Family

The mouse Miwi (*Piwil1*), Mili (*Piwil2*) and Miwi2 (*Piwil4*) proteins, share significant homology with their *Drosophila* and human counterparts. Their expression in different, overlapping periods during gametogenesis, is specific to the male germ line (Fig. 3.4). Mili is expressed at E12.5 of embryonic stage, and is present throughout gametogenesis to the round post-meiotic spermatid stage; Miwi2 is transiently expressed at E15.5 of embryonic stage until shortly after birth [41], and Miwi is expressed from the pachytene meiotic stage to post-meiotic round spermatid stage in the adult testis [42, 55, 58]. Mili binds piRNAs of 24–28 nt long, whereas Miwi2 and Miwi bind piRNAs that are a little longer (27–32 nt) [36, 58]. Studies have shown that Mili plays a role in TE control, GSC maintenance and differentiation; Miwi2 in TE control and genocyte proliferation, and Miwi in meiosis during spermatogenesis [20–23]. Their differential role has also been suggested from evidence that shows binding (Miwi2, Mili) to prenatal piRNAs with nucleotide sequences complementary to TEs and binding (Miwi, Mili) to TE-derived postnatal piRNA or to non-coding intergenic and genic piRNAs during spermatogenesis (pre and post meiosis) [35, 47, 48].

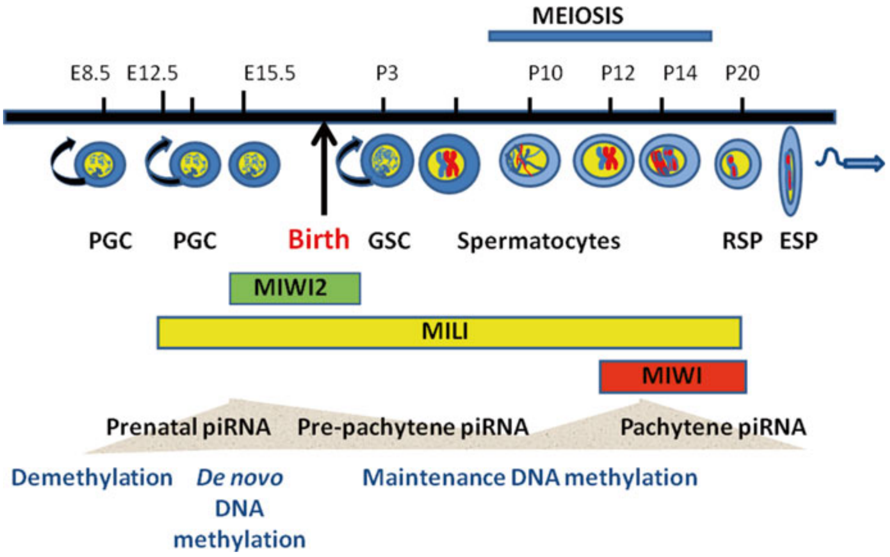


Fig. 3.4 PIWI protein and piRNA expression during mammalian spermatogenesis. A schematic representation of mouse spermatogenesis on a time coordinate showing the periods during which Mili, Miwi2, Miwi and piRNA are expressed and epigenetic reprogramming takes place (i.e. DNA demethylation/methylation) [2]. *PGC* primordial germ cells, *GSC* germ stem cells, *RSP* round spermatids, *ESP* elongated spermatids. *Bent arrows* indicate cell self-renewal

Several studies using PIWI mutant/deficient mice have provided an inside of PIWI protein functions. Prenatal Miwi2 and Mili expressions have been associated with piRNA-guided *de novo* DNA methylation that represses TE expression as well as with the piRNA-targeted cleavage of TE-RNA [20–23, 58–62]. If Miwi2 or Mili proteins are not expressed in prenatal germ cells, the genomic regions of Line1 and intracisternal A-particle (IAP) type transposons (non-LTR) are hypomethylated. As a result, TE mRNA levels are up-regulated causing spermatogenic stem cell arrest at leptotene (Miwi2) or zygotene/early pachytene (Mili) stages that results in germ cell apoptosis and sterility [45, 47, 48, 58]. Since Mili and Miwi2-deficient mice have impaired prenatal piRNA production, Mili and Miwi2 are considered to be involved in piRNA biogenesis [63]. Deficiency in Miwi, which also results in sterility, affects adult germ cells instead, by arresting spermatogenesis and thus preventing spermiogenesis [35, 64]. Furthermore, ribonucleoprotein-associated mRNA that is required for GSC renewal and for spermiogenesis are stabilized by Mili and Miwi, respectively [59, 63, 65].

In contrast to the male germ line, female mouse germ cells express only Mili. Studies with female knockout mice for Mili and/or proteins that associate with piRISC, have shown that female fertility is not affected. These sex-specific differences in mice suggest that the expression of PIWI members and/or associated proteins may be redundant for mammalian oogenesis. Mammalian oogonia, unlike germ cells, proliferate and undergo meiosis only during gestation (Fig. 3.2), and they may have an adequate amount of maternally inherited piRNAs for controlling TE expression [21, 35, 41, 45]. Alternatively, transposon control in mammalian oocytes, may involve other non-coding RNAs, like siRNAs, or proteins that block TE integration and/or provide innate immunity against retroviruses [52, 53, 66–68].

The specific requirements for PIWI function and the mechanisms of TE silencing observed in the male germ line may be unique to mammals and the result of the evolution of the mammalian germ cell development system. In other vertebrates (i.e. *Drosophila* flies, aphids and zebra fish), and in distant organisms (i.e. sea urchin), both, male and female gametogenesis require the contributions of all PIWI members for normal cell specification, maintenance and germ cell meiosis [69]. In *Drosophila*, for example, maternally inherited piRNAs may contribute to female PGCs formation and GSCs specification during early embryogenesis. However, unlike mammals, GSC self-renewal divisions in *Drosophila*, requires a functional Piwi that resides in the nurse and somatic cells of the ovary [34, 50, 55, 70, 71].

The Human PIWI Family

All known members of the AGO family have been identified in the human genome [72]. PIWI proteins are expressed mainly in human testes and consist of the Piwil1/Hiwi, Piwil4/Hiwi2, Piwil3/Hiwi3 and the Piwil2/Hili proteins with homologues in other mammals and vertebrates. The *PIWL1, PIWIL4, PIWIL3* and *PIWIL2* genes are on chromosomes 12, 11, 22 and 8, respectively. The three AGO genes (*AGO1, AGO3, and AGO4*) are closely clustered on chromosome 1 suggesting a common evolution from concurrent gene duplications originating from the *AGO2* gene (of a more ancestral origin) found on chromosome 8 [73]. In normal human testes, Hiwi (the Miwi protein homologue), is specifically expressed in spermatocytes and round spermatids during spermatogenesis, and its over-expression is associated with seminoma tumours [74]. Hiwi2/Miwi2, the only protein member expressed in human somatic cells ubiquitously, may play a role in chromatin remodelling [75].

The Endonuclease Activity of the PIWI Proteins

The members of the PIWI subfamily, like those of the AGO subfamily, are characterized by the conserved PAZ (Piwi-Argonaute-Zwille) domain, the PIWI sequence domains and a middle (MID) sequence domain (Fig. 3.5). The PAZ and PIWI-MID domains are responsible for facilitating the formation of the double stranded RNA complex (i.e. small RNA bound to single stranded target mRNA), required for the cleavage of target RNA. The MID-domain recognizes and binds specifically the characteristic 5′ end phosphate of Uridine (1U-bias) of piRNA-precursor molecules or piRNAs generated products, and ensures the correct orientation of the bound piRNA by anchoring it. The PAZ domain at the N-terminal region, forms a pocket that binds the 3′ overhang of the piRNA. Cleavage of mRNA occurs at the PIWI

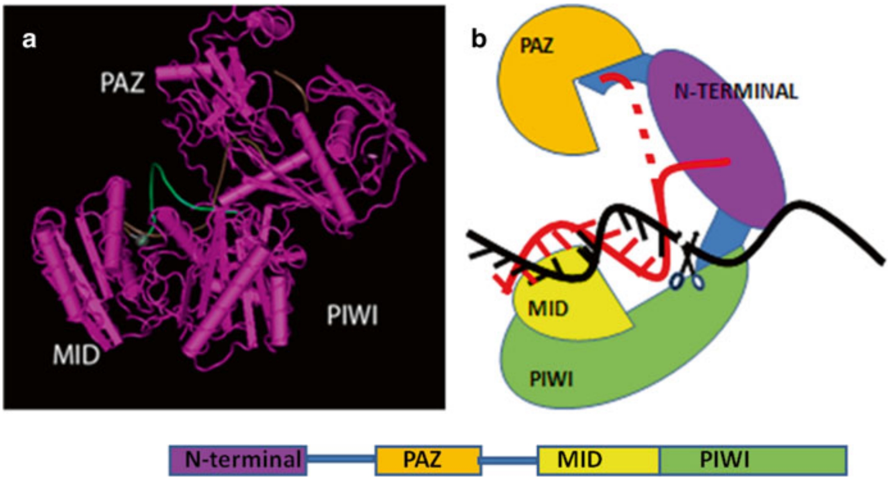


Fig. 3.5 Protein structure and a putative model of PIWI slicer activity based on Ago structure. (**a**) The crystal structure of *Thermus thermophilus* Ago, showing the PAZ, MID and PIWI domains (PDB identity 3F73; created using Cn3D). (**b**) A putative model of PIWI-bound piRNA (*red*) forming a double strand with target-RNA (*black*) at the catalytic pocket of PIWI. The 5′ end of piRNA is stabilized by the MID domain. The *solid* and *dotted lines* at the 3′ end of piRNA denote a PIWI conformational change after base pairing that is considered to trigger PIWI cleavage of target RNA [76]

domain, which is at the C terminal region and contains the catalytic DDH triad composed of two aspartic acid residues (D) and one histidine (H). This catalytic site has an RNAse H-like "slicer" endonuclease activity responsible for the dsRNA guided hydrolysis of target mRNA bound to piRNA (Fig. 3.5) [76–79].

The endogenous PIWI-slicer activity of some PIWI proteins is probably responsible for both, the biogenesis of regulatory mature piRNAs and the piRNA-targeted TE degradation [35, 37, 63–67, 79]. The first phase of the piRNA-PIWI pathway involves the pre-cleavage of very long RNA transcripts to fragments of various lengths (primary piRNA-precursors), to which PIWI proteins may bind via the pre-bound piRNAs. The intermediates formed are then cleaved into smaller fragments by the PIWI slicer activity, trimmed to size by a 3′ to 5′ exonuclease (i.e. Ago2, which co-localizes with Ago3 and Miwi in the chromatoid body of male germ cells), and subsequently 2′-O-methylated 3′ by the HEN1 methyl transferase to form the mature and stable "primary piRNAs" with regulatory function(s) [20, 32, 63, 65, 75–82]. The 5′ to 3′ processing of primary piRNA-precursors is considered to be undertaken by an AGO subfamily member [20, 81, 82]. However, in vivo studies using the method of cross-linking prior to immunoprecipitation (HITS-CLIP method) of PIWI-piRNA complexes, have suggested instead, that long primary piRNA-precursors are processed to mature piRNAs via a 5′ to 3′ endonuclease activity of PIWI proteins (Mili, Miwi) without being guided by pre-bound piRNA [65]. Furthermore, studies with mouse Miwi-catalytic mutants of the DDH triad and with Miwi deficient mice have indicated that the biogenesis of primary piRNA-precursors and primary piRNAs is independent of PIWI-slicer activity [63].

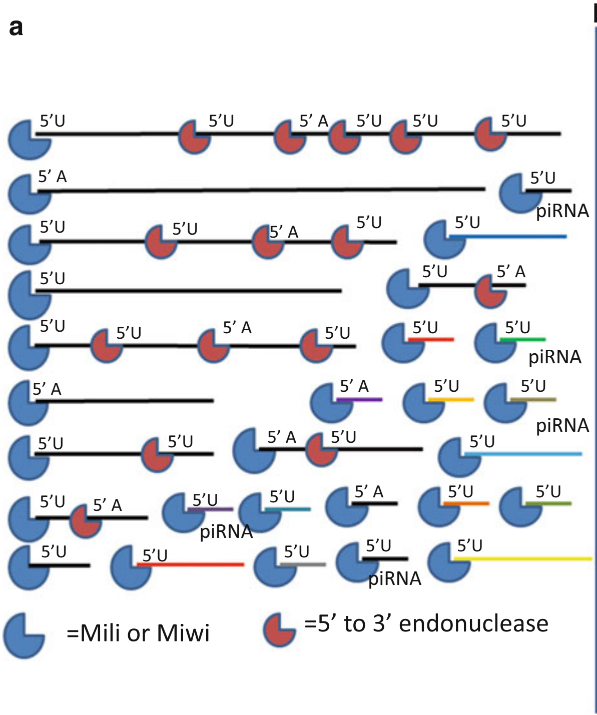


Fig. 3.6 A schematic representation of a putative mechanism for primary biogenesis of piRNAs. (**a**) PIWI (*blue packman*) binds non-coding RNA through the 5′ end U and promotes its 5′ to 3′ cleavage by an endonuclease (*red packman*). The fragments generated continue to be loaded onto PIWI to be further cleaved and/or as they get smaller to be finally trimmed to the right piRNA size that bind PIWI with high affinity and gets 2′-O-methylated at the 3′ end to mature piRNAs with diverse sequences (shortest lines in different colours). (**b**) Changes in the 3D structure of a random sequence RNA fragment as it is cleaved by a 5′ to 3′ endonuclease while its 5′ end is bound to PIWI. *Parallel lines* show base pairing

Based on current evidence an alternative hypothesis could be that Ago2 or another endonuclease may cleave very long mRNA transcripts that are bound with low affinity to PIWI proteins via their 5′U/A ends (Fig. 3.6a). After each round of endonuclease cleavage in a 5′ to 3′ direction, the 5′U end fragments generated will have unique secondary or 3D structures and upon binding to PIWI may now provide different new sites for endonuclease cleavage (Fig. 3.6b). Depending on their size, the PIWI-bound fragments would continue either to be further cleaved by the endonuclease, or trimmed by a 3′ to 5′ exonuclease. Those with the right size to bind PIWI with higher affinity (i.e. by 3′ binding to the PAZ domain) may then form a stable piRNA-PIWI complex, which can be methylated by HEN1 methyltransferase to produce mature piRNAs having primarily U at the 5′-end but diverse sequences (Fig. 3.6a). On the basis of such a model, Mili and Miwi, may participate and speed up primary piRNA biogenesis by stabilizing the 5′ end of single stranded piRNA precursors without using their endogenous slicer activity [65]. This slicer activity, in fact, could be required only for secondary processing as suggested from studies on LINE1 transposon silencing during spermatogenesis [63, 83]. Nuclear Miwi2 is most likely to be involved instead in TGS, by regulating DNA methylation of target transposon loci [58–61]. As mentioned above, the cooperation of several other proteins, especially of TDRDs, is needed for the loading of piRNAs intermediates into Piwi as well as for acting as chaperons (i.e. heat shock protein 90, HSP90, homologues) for piRNAs mobilization and the regulation of TE and/or transcription of precursor piRNA [33, 49, 66, 84–89].

The N-terminal region of PIWI proteins, rich in R-G or R-A amino acids, is the site for post transcriptional methylations by symmetrical arginine methyltransferase (SAM) enzymes that are important in specification and maintenance of germ cells. The symmetrically dimethylated (sDMS) status of the N-terminal of PIWIs (Fig. 3.7), may determine the interactions with components involved either in piRNA biogenesis or in macromolecular assembly required for piRISC mobilization and function [90–92]. The stable association of the mature primary piRNA

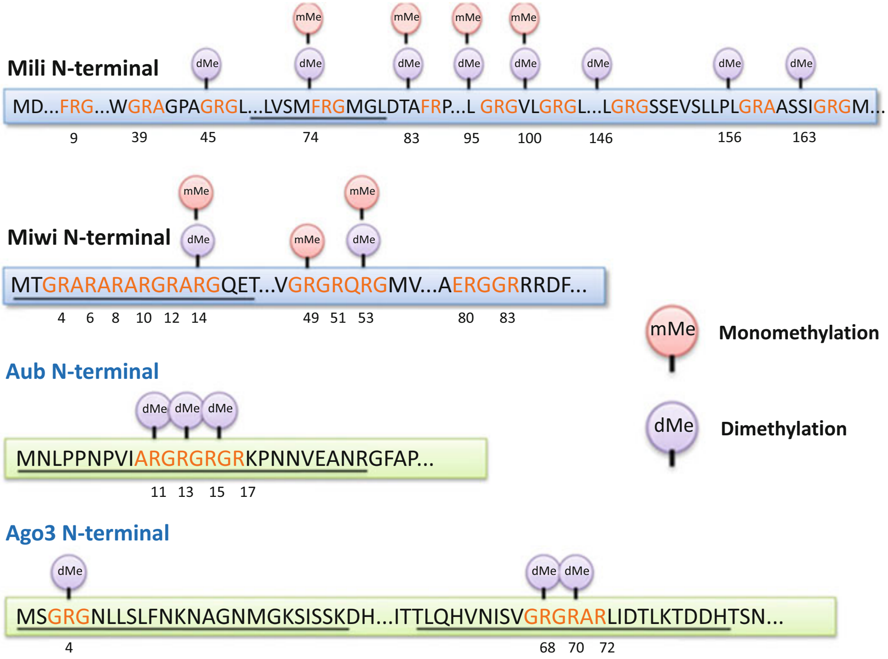
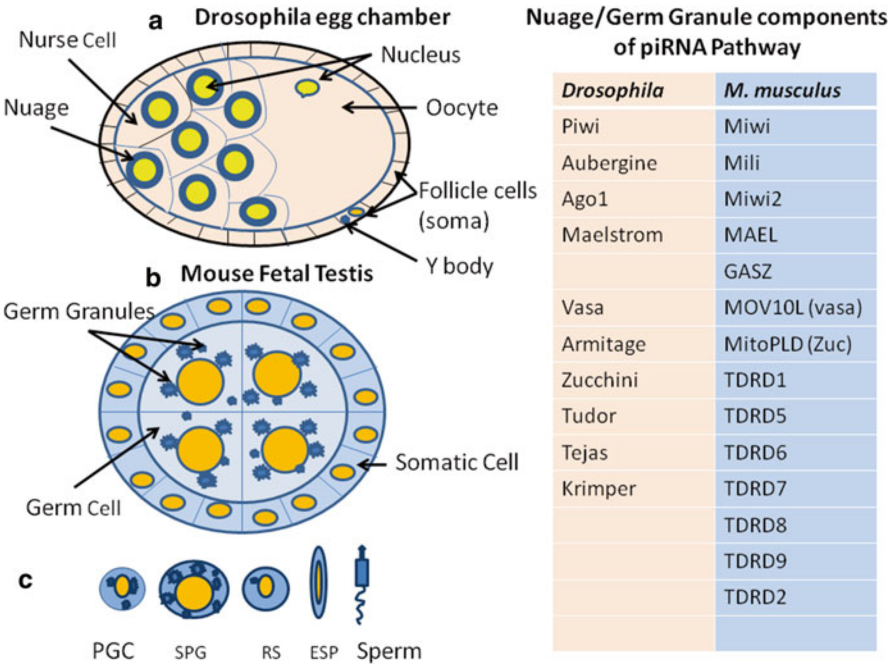

Mili N-terminal
mMe
dMe
MD...FRG...WGRAGPAGRGL...LVSMFRGMGLDTAFRP...L GRGVLGRGL...LGRGSSEVSLLPLGRAASSIGRGM...
9 39 45 74 83 95 100 146 156 163
Miwi N-terminal
MTGRARARARGRARGQET...VGRGRQRGMV...AERGGRRRDF...
4 6 8 10 12 14 49 51 53 80 83
Aub N-terminal
MNLPPNPVIARGRGRGRKPNNVEANRGFAP...
11 13 15 17
Ago3 N-terminal
MSGRGNLLSLFNKNAGNMGKSISSKDH...ITTLQHVNISVGRGRARLIDTLKTDDHTSN...
4 68 70 72
mMe Monomethylation
dMe Dimethylation

Fig. 3.7 Arginine methylation status of PIWI proteins. The N-terminal sequences of mouse PIWI (Mili and Miwi) and fly PIWI (Aub and Ago3) are shown with putative sDMA motifs (in *pink*). Identified methylation sites (dimethylation [dMe] or monomethylation [mMe]) are shown above the relevant arginine, with the residue numbers below. *Underlined* sequences indicate the synthetic peptides used in studies for pull-down assays. Reprinted from Siomi MC, Mannen T, Siomi H. How does the royal family of Tudor rule the PIWI-interacting RNA pathway? Genes Dev. 2010; 24:636–646. Review. (Open Access)

with Piwi may induce a conformation change at the N-terminal of Piwi, which dictates the methylation pattern of PIWI by the symmetric arginine dimethylase enzyme (dPRMT5). This pattern in turn, may define which TDRD protein binds to PIWI to modulate and/or guide piRISC function(s) specified by the signature of the bound piRNA (i.e. PIWI localization, piRNA biogenesis, mobilization to the nucleus for TE silencing, and/or the regulation of gene transcription/translation) [37, 55, 85, 86, 92–103].

During *Drosophila* embryogenesis, piRISC-associate proteins co-localize with PIWI proteins in the oocyte germ plasma and in cell specific cytoplasmic granules (nuage), in nurse and ovarian somatic cells (Fig. 3.8). The precise molecular function of these proteins and the mechanisms that ensure binding specificities are not as yet, known. However, loss of TDRD protein function results in the expressions of TEs, impaired gametogenesis, meiosis arrest and sterility. In mammalian cells, like in *Drosophila*, a larger number of such associate TDRDs, co-localize with PIWI in similar germ and somatic cell granules (Fig. 3.8). Their pattern of expression is highly correlated with PIWI expression, and it appears that distinct combinations



Nuage/Germ Granule components of piRNA Pathway

Drosophila	*M. musculus*
Piwi	Miwi
Aubergine	Mili
Ago1	Miwi2
Maelstrom	MAEL
	GASZ
Vasa	MOV10L (vasa)
Armitage	MitoPLD (Zuc)
Zucchini	TDRD1
Tudor	TDRD5
Tejas	TDRD6
Krimper	TDRD7
	TDRD8
	TDRD9
	TDRD2

Fig. 3.8 Schematic representation of (**a**) *Drosophila* ovarian egg chamber (**b**) mouse fetal testis and (**c**) mouse germ cells. Tudor (TUD), tudor-domain containing proteins (TDRD), developmental factors and PIWI proteins that accumulate to form macromolecular assemblies in germ granules (*blue*) and in the nuage (*blue*) are listed in the Table for *Drosophila* and mouse. Adapted from Chen C, Nott TJ, Jin J, Pawson T. Deciphering arginine methylation: Tudor tells the tale. Nat Rev Mol Cell Biol 2011; 12:629–642 with permission from Nature Publishing Group

of TDRDs-PIWI assemblies characterize transposon silencing and spermiogenesis. Targeted deletions of TDRDs do not affect germ cell viability but they do affect the maintenance of GSCs and male fertility [49, 100–103]. There are also a number of other developmental markers, which co-localize with PIWI proteins in the cytoplasmic granules and are just as important for GSC development and spermatogenesis (Fig. 3.8). The PIWI associations with the various proteins including TDRDs in cytoplasmic granules, appear to be conserved through evolution [21, 90, 91, 94–96, 101].

There is limited knowledge at present as to how the expression of PIWI proteins is regulated during gametogenesis. Hou et al. [104] have shown that the expression of *Miwi* from mid pachytene spermatocyte stage to round spermatid stage, is controlled by developmental transcription factors through a DNA methylation-dependent mechanism involving epigenetic modifications at the putative PIWI promoter region. Regulation of PIWI protein expression by developmental transcription factors would be in agreement with the fact that PIWI proteins are highly expressed only in germ line cells during gametogenesis.

PIWI Evolution

The RNAi system of defence against retrotransposons is very ancient. A plausible direct evolutionary connection between prokaryotes and the RNAi system in eukaryotes, is supported by the evidence for the existence of a prokaryotic immune system that uses as guides RNA or DNA molecules and homologues of the AGO family to degrade nucleic acids of invading elements [105]. Furthermore, organisms from far back in evolution, such as sponges, and organisms near the evolutionary basis of metazoans, express *piwi*-like genes in somatic stem cells that give rise to the next generation, in support of a PIWI ancestral origin and its ancient role in gametogenesis [106–110].

The diversification of the AGO gene family into the AGO and PIWI families in contemporary animals including humans, is considered to have been established since the origin of metazoan, (i.e. more than 600 million years ago), and it must have played an important role in the evolution of multicellularity [15, 73, 105–108]. Phylogenetic analysis shows that after branching out, both, the AGO and the PIWI proteins have undergone a marked degree of expansion. Both must have evolved following vertebrate-specific duplication events, which were independent and lineage-specific (Fig. 3.9), in agreement with the reported absence of pairwise orthologies between the PIWI members of *Drosophila* and mice. The *Drosophila* PIWI proteins (Piwi, Aub and Ago3), for example, are related structurally more to each other rather than to the mouse PIWI family members (Mili, Miwi and Miwi2) [105]. In terms of the evolution of PIWI structure, the PAZ domains of Piwi-like and of Dicer-like proteins appear to cluster together, whereas the PAZ domain of the AGO members appears to have evolved separately. This suggests that the multidomain formation in AGO members may have occurred after an *ago-piwi* gene duplication with AGO inheriting the PAZ domain from an ancestral *piwi* gene [15, 105, 108, 111].

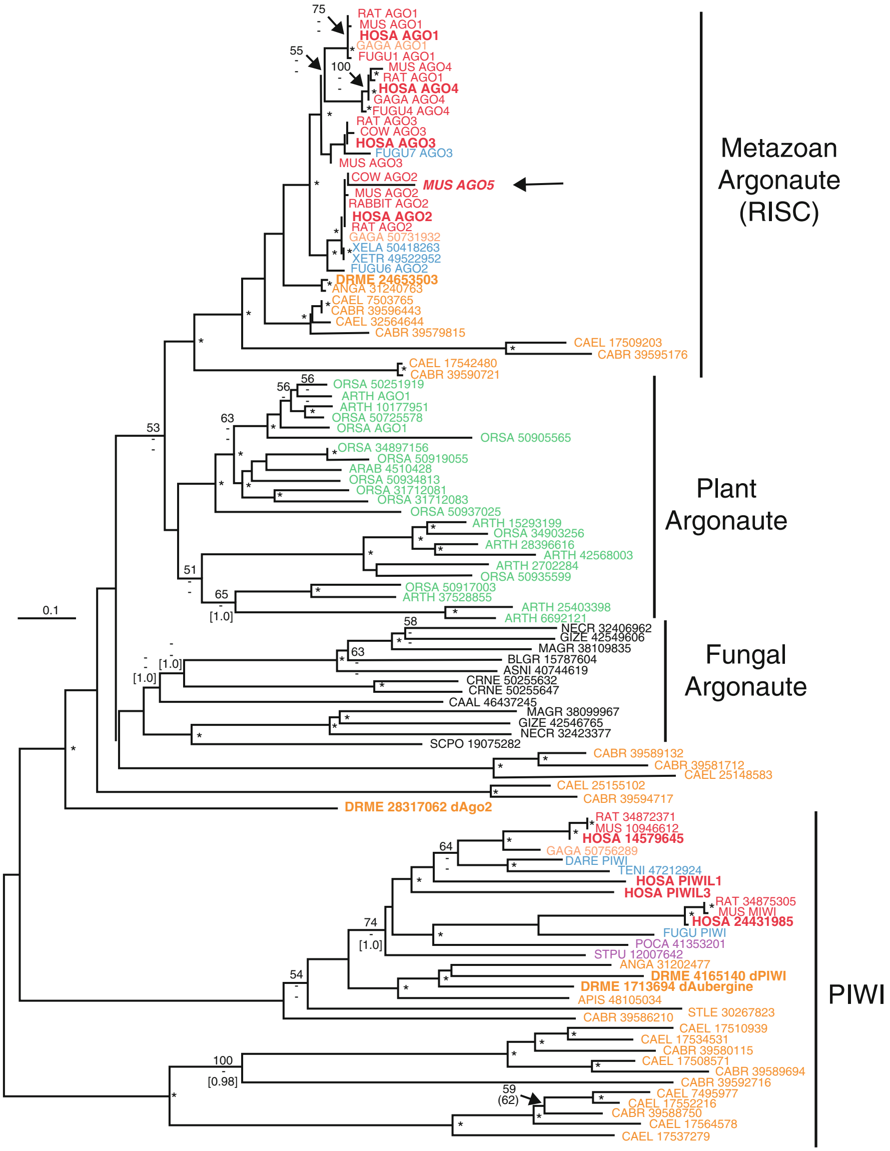


Fig. 3.9 Neighbor-joining phylogenetic tree of Argonaute/PIWI protein family. Major organism groups (with colours) are mammals (*red*), birds (*light red*), cold-blooded vertebrates (*deep blue*), urochordates (*light blue*), deutrostome invertebrates (*purple*), protostome invertebrates (*orange*), plants (*green*), fungi (*black*), and protists (*light purple*). The tree is unrooted. *Asterisks* ("*") indicate those nodes supported 60 % or greater occurrence and >0.90 probability in square parentheses. Nodes with one or two values less than 50 % have *dashes* ("–") while values less than 50 % are unmarked. *Scale bar* represents 0.1 expected amino acid residue substitutions per site. Human and Drosophila PIWI/Ago proteins are in larger font. The branch leading to a putative, but unlikely, fifth Argonaute gene homolog in mouse, mAgo5, is labeled with a large *arrow*. Other branches are labeled by a four letter species identifier (the first two letters from the genus and species names) and the GenBank accession number). From Murphy D, Dancis B, Brown JR. The evolution of core proteins involved in microRNA biogenesis. BMC Evol Biol. 2008; 8:92 (Open Access) [73]

The independent and lineage-specific evolution of the Argonaute family of genes may have resulted from the specific cell-line diversification in protein expression profiles, which could have evolved independently in each species because of specific developmental needs, i.e. the role of *Drosophila* Piwi in ovarian somatic cells vs. Aub and Ago3 involvement in TE cleavage in germ cells [50, 109–112]. The expansion of AGO and PIWI homologues and their functional specialization after gene duplication events, appears to have been well conserved during evolution, and can be observed even in distantly related organisms (Fig. 3.9) [73, 113, 114]. A representative example is the pea aphid, which expresses eight copies of *piwi* and two copies of *ago3* genes that have evolved separately in the germ and somatic cells of the organism, respectively, thus providing a greater plasticity for its sexual and asexual reproductive cycles [71]. Studies on the *Drosophila* piRISC assembly proteins have indicated that evolutionary selection dynamics would have favoured codon bias in the effort to combat TE abundance and invasions. That is, genes of the piRNA machinery with greater translational and possibly functional efficiency may have been selected through evolution in order to cope as best as possible with TE expansions [113].

Location of piRNA Biogenesis, and piRNA Origin and Evolution

Location of piRNA Biogenesis

Experimental evidence indicates that *Drosophila* piRNA biogenesis and the loading of mature piRNA onto PIWI proteins destined for nuclear function(s), occur in the germ plasma and in distinct cytoplasmic granules, proximal to the nucleus of germ cells. Similar granules make up the nuage structures around the nucleus of nurse oocyte cells, and the Yb-bodies of somatic ovarian cells [20, 115]. All of these granules are associated with active transcription, PGC specification and maintenance, and with germ cell differentiation and maturation. Granule formations require the hierarchical recruitment of components, such as developmental factors, TDRD proteins, and other components, a number of which form the macromolecular assembly for piRISC function(s) as, previously discussed. TDRD protein expression is gender and cell specific, and TDRD mutations affect negatively piRNA biogenesis, loading of mature piRNA to PIWI proteins and TE repression, which result in the abnormal development of germ cells and sterility [49, 69, 94, 98, 99].

Cytoplasmic granules similar to those observed in *Drosophila*, also exist in mammals. In the mouse male embryonic germ cells, Mili and Miwi2 are localized in two different granules (P-bodies). In the adult mouse testes, the P-body components form the inter-mitochondrial cement area seeing in spermatocytes, and later on, the chromatoid body seeing in round spermatids. The P-bodies appear to be the key sites for piRNA biogenesis, and to interact with each other functionally and physically during the critical developmental stage of *de novo* DNA methylation. They contain either Mili (called pi-bodies), or Miwi2 (called piP-bodies), and different TDRD proteins and other associate molecules required for the piRISC assembly structure, and for its localization and function(s) during gametogenesis. Disruption of the Mili pi-bodies affects negatively the integrity of piP-bodies but not *vice versa*, suggesting that Miwi2 is downstream of Mili regarding cytoplasmic granule communications. However, disruption of both types of P-bodies causes down regulation of piRNA production, accumulation of TEs and reduction in *de novo* DNA methylation, which result in germ cell loss and sterility [38, 39, 90, 91, 116]. Like in *Drosophila*, the integrity of P-bodies depends on the expression of TDRD and other proteins. Miwi is present in the whole of the cytoplasm in spermatocytes, and it concentrates in the chromatoid bodies in the round spermatids [81]. Mili and/or Miwi mutations, in combination with deficiencies in specific TDRDs, cause reductions in piRNA levels in mouse male germ cells, and increase retrotransposon transcription (LINE and LTR). These effects are associated with DNA hypomethylation, developmental germ cell defects and sterility [49, 85, 87, 88, 101–105].

Genomic Origin of piRNAs and Processing Selection

Deep sequencing technology has greatly aided in the characterization of the size and sequence of individual piRNAs [117–119]. It has also led to the development of algorithms to predict piRNA sequences and their genomic origin [119–122]. *Drosophila* piRNAs map mainly within intergenic pericentromeric and telomeric repetitive sequences including TEs [33, 34]. The majority of mammalian piRNAs map at intergenic, intronic and exonic sites, the functional significance of which is relatively unknown. Furthermore, although in *Drosophila* and Zebra fish most piRNAs have antisense homologies with transposon transcripts from all major classes of TEs, in mice only 17–20 % of piRNAs have any correspondence to known repetitive TEs and to retrotransposon coding regions. The remaining mammalian piRNAs (about 80%) correspond to unique non-coding sequences that represent the majority of “junk DNA” and may have important regulatory roles as new functions of “junk” DNA are being discovered [31–33, 43–47].

Sequence comparisons of piRNAs isolated from *in vitro* and *in vivo* studies, have shown that the hundreds of thousands of piRNAs are highly variable, and they are processed from long single stranded RNA precursors encoded in large tandem arrays of chromosomes, called clusters, ranging from 20 to 100 kb [47, 119, 120]. piRNA precursors may originate from one strand (+/–), called “uni-strand” or “monodirectional” clusters, or from both strands, called “dual strand” clusters. In some cases, in mice and in flies, the two halves of piRNA precursor may map to both genomic strands but in opposite orientations originating from “bidirectional clusters”, due to transcription from a centrally located promoter (0.1–1 kb long) [119].

In addition to the variability in the sequences of the piRNAs, there are differences in the frequency with which piRNAs map to specific cluster regions of chromosomes. piRNA densities of clusters can range from 40 to 4000 [120–124]. It has been debated as to whether the higher abundance of some piRNAs (i.e. those derived from intergenic non-coding sequences and from 3′ untranslated regions (3′ UTRs)), represent a programmed biogenesis of piRNAs with regulatory functions, or instead, local levels due to differences in translation efficiency, sequence motifs or stability. Robine et al. [124] argue that the 3′ UTR-derived piRNA production is, most likely, selected actively for *trans* regulatory functions since in *Drosophila* and mouse gonads, the piRNAs come from distinct piRNA-precursor categories. In terms of evolution, the co-transcription of piRNA clusters within protein coding gene sequences may have provided a greater range of regulatory piRNAs for a more efficient response to cell-specific requirements during the various stages of gametogenesis.

Master loci in *Drosophila*, express putative regulatory transcripts as substrates for the production of piRNAs that target protein mRNA coded in transposons. Examples include: the locus of the suppressor of the stellate gene (*su*(*ste*)), which prevents the over expression of stellate protein that crystallizes in spermatocytes and causes sterility [109, 125]; the *flamenco* locus involved in the repression of LTR retrotransposons of the *gypsy* family (*gypsy*, *ZAM* and *Idefix*), and the *3R-TAS* locus responsible for the silencing of the *P* element involved in female hybrid dysgenesis [16, 17, 21, 126]. Control of transposon transcription via piRISC in *Drosophila*, may have evolved after the transposition of a TE into a piRNA cluster. This event would have provided an evolutionary advantage to the fly by enabling it to discriminate mobile transposons from transcripts of endogenous genes, and eliminate them [50, 109, 111–114, 124].

The few studies on the mechanism of regulation of piRNA transcription from clusters in *Drosophila*, have indicated that uni-strand cluster transcription involves Polymerase II that requires a transcription start site and histone methyltransferase (H3K4me2) activity [127]. Transcription of dual-strand piRNA clusters in germ and somatic cells of the gonads, require instead the presence of double stranded DNA for the binding of specific epigenetic and transcription factors (i.e. HP1a, H3K9methyl-3, Cuff, Del). Binding is regulated by the heterochromatin Rhino, which acts as a licensing factor by distinguishing piRNA loci for cluster expression [127–129].

Taken together, it would appear that in *Drosophila*, the piRNA-PIWI pathway may regulate piRNA cluster expression or TGS by modifying chromatin status at specific genomic targets, via a piRISC-recruitment of epigenetic and transcription factors [125–130]. Heterochromatin binding of piRISC may involve direct piRNA binding to DNA, whereas euchromatin binding may involve binding to nascent RNA transcripts of 100–800 bp long, needed for the recruitment of histone methyltransferase and other proteins [130]. An H3K9 dependent vs. independent mechanism and the piRNA-Piwi complex bound to specific transcription factor(s) (i.e. the gametocyte-specific factor 1), have been shown to be involved in differentiating piRNA-precursor-transcription from piRNA-Piwi-repression of TE transcription [21, 130]. Specific RNA binding proteins may also be involved in the tagging and movement of cluster-derived RNA to the cytoplasm for the delivery of signals as to which PIWI protein may bind and process the precursor piRNA.

Comparisons on the similarities between rat and mouse genome, suggest that in mammals, most piRNA clusters may have originated via ectopic recombination and insertion of long sequences at regions flanked by chromosome-specific repetitive elements [117, 123]. Since in mice, there are no master loci as in *Drosophila*, potential regulatory sites for piRNA-cluster expression may most likely be interspersed in the genome and regulated by chromatin remodelling in a manner similar to that seen in *Drosophila*. Alternatively, expression of developmentally regulated mRNA transcripts may provide the substrates for the generation of piRNAs in response to specific need for TGS and/or PTGS. Present evidence indicates that the expression of specific RNA clusters during mouse spemiogenesis is regulated by zinc-type-transcription factors and the MYB-related protein A [131, 132].

piRNA Cluster Evolution

Comparisons of orthologous regions between rat, mouse and human genomes using the sequences of 140 known rodent piRNA clusters, have identified 37 of these to be of an ancestral origin and conserve in all three species. These clusters overlap protein-coding genes by spanning several exons and introns in most cases. The remaining 103 clusters are contained within intergenic regions and do not overlap protein-coding genes. Forty three of the one hundred and three clusters appear to have an ancestral origin, the remaining 60 appear to have evolved recently with most (44 clusters) having been acquired after rodent–primate divergence without a single cluster loss. Rapid expansion occurred before the rat-mouse split through recombination and long sequence insertions, primarily within genomic regions that were not preserved after cluster acquisition. In terms of large scale evolution, the rate of cluster expansion appears to exceed the highest known rates for any of the families of mammalians genes. On a small scale, however, the evolutionary rates of piRNAs are similar to those observed for other mammalian sequences. On given evidence, it has been suggested that piRNA cluster expansion is driven by positive selection, possibly as the result of the interplay between invading TEs and the transposition of existing TEs into clusters. Mammals in response to such evolutionary pressures may have selected to enhance the piRNA repertoire for silencing TEs [117, 125].

The mammalian piRNA clusters are by majority syntenic (i.e. their presence and chromosomal organization are conserved in the mouse, rat and human) [31–33, 43–47]. Positive selection and conservation of the piRNA cluster location, however, does not result in piRNA sequence conservation between closely related species and even between individual animals of the same species, as demonstrated from the sequencing of piRNAs isolated from the *Xenopous tropicalis* oocytes [133]. piRNAs are highly diverse due to the irregular manner of their biogenesis from different regions of cluster transcripts, and from clusters transcribed from one strand in preference to the other (strand asymmetry) [37, 51]. A quasi-random sub-saturation processing from common precursors has been suggested to explain the diversity in piRNAs and the low frequency of piRNAs with overlapping sequences [119]. Studies using computer simulations for TE transposition in *Drosophila*, and comparisons of sequences of a number of RNAi defence genes (including those of the piRISC) and of genes of the immune system, have suggested that intra-population and inter-species variations in transpositions, may contribute to the generation of individual piRNA sequences. Non-coding sequences are less conserved, and the generation of piRNAs from randomly cleaved long primary piRNA-precursors may further contribute to piRNA sequence diversity, as previously discussed. Based on the evidence available, it has been suggested that the piRNA defence system against transposons is most likely a dynamic one, and operates in a manner analogous to that of the immune defense system [113, 114, 134, 135]. A strong negative selection at the sequence level of piRNAs has only been observed in human African populations, in agreement with the findings that Africans have much higher rates of TE insertions than other populations [136, 137].

The Classes of piRNAs

The sequence signatures of piRNAs isolated from *Drosophila* and mice, have led to the proposal of two mechanistically different pathways for piRNAs biogenesis *in vivo* [31, 34, 43, 55, 65]: (a) The primary pathway that generates primary piRNA-precursors with U at the 5′ end, from long mRNA transcripts that are processed to mature piRNAs, as previously discussed. The biological significance of this in guiding/regulating piRISC or TGS is not known, at present. (b) The secondary pathway, also called the ping-pong amplification pathway, is assumed to use primary piRNAs to trigger the production of secondary piRNAs that drive TE-RNA degradation. Both pathways are active in the germline, whereas only the primary pathway is involved in piRNA biogenesis in gonad somatic cells [138].

piRNA isolation and sequencing have revealed some features that are shared between the diverse mature piRNAs species and are used to identify their origin and to understand the mechanism(s) of mature piRNA biogenesis [18, 21, 31–34, 43, 47]:

1. The mammalian piRNAs and *Drosophila* piRNAs/rasiRNAs as well as the pi-like-RNAs in other somatic cells/tissues [118], are larger (24–32 nt long) than miRNAs and siRNAs.
2. Mature piRNAs and primary piRNA-precursors carry a phosphate group at their 5′ end, indicative of being generated from a dsRNA precursor intermediate that is processed by an endonuclease.
3. Mature piRNAs are 2′-O-methylated at their 3′ end for greater stability [77, 79].
4. Mature piRNAs derived from piRNA-precursors via primary processing, show a very strong preference for U at the 5′ end and no nucleotide bias at position 10. Those derived via secondary processing show instead, a bias for Adenine (A) at position 10 and no 5′ end bias.
5. Antisense piRNAs derived from RNA transcripts containing TE repeats may share higher complementarity with sense piRNAs at the 5′ end [21, 31–34, 43–47].

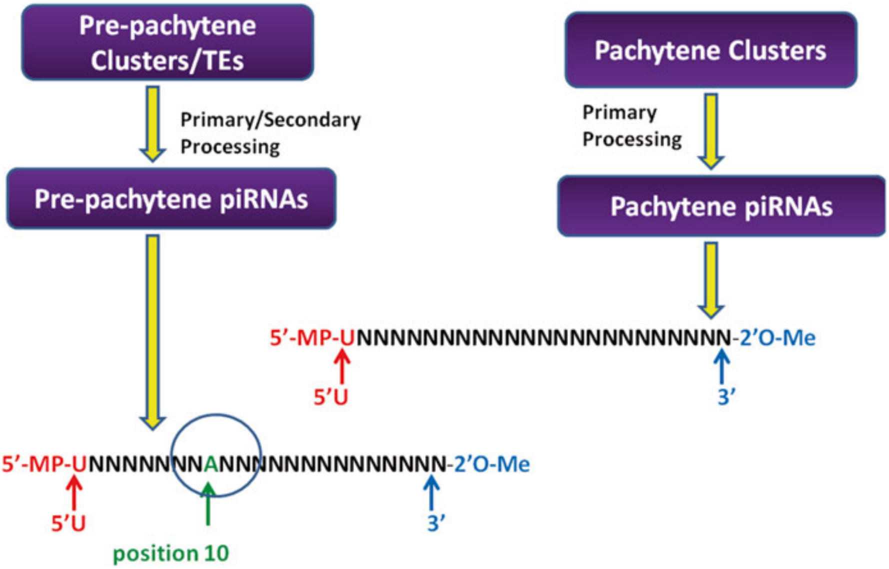

Pre-pachytene Clusters/TEs
Primary/Secondary Processing
Pre-pachytene piRNAs
Pachytene Clusters
Primary Processing
Pachytene piRNAs
5′-MP-UNNNNNNNNNNNNNNNNNNNNNNNNN-2′O-Me
5′U
3′
5′-MP-UNNNNNNNNANNNNNNNNNNNNNNN-2′O-Me
5′U
position 10
3′

Fig. 3.10 The major classes of mammalian piRNAs. *A* Adenine, *U* Uracil, *N* any ribonucleotide (Adenine, Uracil, Cytosine or Guanine)

Because there are no other known secondary structures motifs to distinguish piRNAs (i.e. hairpin-like structures as in miRNAs and siRNAs), their classification in mammals, is based on the above features, origin of piRNA cluster/strand and the stage of gametogenesis during which piRNAs are most abundant. The two major and distinct populations identified are the pre-pachytene and pachytene classes of piRNAs (Fig. 3.10).

The Pre-pachytene Class of piRNAs

This class of mammalian piRNAs, originate in a set of clusters (referred to as pre-pachytene piRNA clusters), and match those of *Drosophila* piRNAs originating in transposon master loci. These are "dual-strand clusters", rich in repetitive elements. The mouse pre-pachytene clusters are distinct, more numerous, and smaller in genomic coverage than the clusters of the pachytene class. Pre-pachytene piRNAs expressed in male growth-arrested gonocytes during embryonic development, are detected up to the mature spermatid-stage in adulthood. The majority show a sense bias: they contain A at position 10 (Fig. 3.10), and their first ten nucleotides are often complementary to piRNAs derived from antisense precursors. Pre-pachytene piRNAs are considered, therefore, to be processed from RNA transcripts via the ping-pong amplification pathway (secondary processing) [34, 50, 55, 61, 68, 69, 86].

Pre-pachytene piRNAs associate primarily with Miwi2 (80 %) and Mili, in mouse gonocytes, and with Mili and Miwi after birth and during adulthood. Their presence is related primarily to the *de novo* methylation of DNA and the silencing of TE via piRISC [55, 58–65]. Reduction in pre-pachytene piRNAs due to loss of Mili or Miwi activity, results in retrotransposon expression and male sterility, as previously discussed [63, 64, 83]. The piRNA-like small RNA (pilRNA) identified in hematopoietic cells (B-cells, T-cells, NK-cells), and in somatic cells from various tissues of other animals including flies, mice and rhesus macaque monkeys, resemble more the pre-pachytene class of piRNAs, and are considered to be involved in TE and/or endogenous retrovirus suppression [118].

The Pachytene Class of piRNAs

The mammalian pachytene class of piRNAs [34, 44, 47, 64, 65, 139, 140], are found in abundance (>90 %), in testicular germ cells during the pachytene stage, when spermatocytes enter meiosis to become spermatids. Pachytene piRNAs persist until the haploid round spermatid stage after which they disappear gradually during sperm differentiation. The majority (70 %), map to highly conserved monodirectional ("uni-strand") piRNA clusters, which are transcribed into long precursors ranging in length, from several to >100 kb. These clusters, are distributed over most chromosomes, mainly at intergenic regions but also at protein coding-genic regions (exons and introns) or 3′ UTRs, and they show no repeats or evidence for a transposon origin [44, 64, 124]. Pachytene piRNAs show primarily a 5′ end U bias, no bias for A at position 10, and no significant complementarily to each other (Fig. 3.10). They are considered therefore, to be the products of primary processing. As mentioned above, the functional significance of these piRNAs is unknown. They associate primarily with Miwi but also with Mili [64, 65, 83, 139]. It has been suggested that pachytene piRNAs may represent the degradation of end products of mRNA, which are destined for clearance during the final differentiation of spermatids into sperm [65].

Drosophila Classes of piRNAs

piRNAs from *Drosophila* germ line and nurse cells, have been mapped to discrete clusters transcribed from loci that contain defective transposons, or from master loci that contain active transposons [21, 25]. Most piRNA clusters are "dual-strand clusters" with antisense bias. piRNAs generated from sense precursor transcripts show no 5′ end U, have A at position 10 (typically Ago3-bound piRNAs), and are complementary to the first ten nucleotides of piRNAs generated from antisense precursor transcripts (Aub/Piwi-bound piRNAs). The majority of piRNAs in *Drosophila* germ line cells, therefore, are the products of TE cleavage, via secondary processing. Mature piRNAs of germ cells originating in clusters that do not contain TE sequences or in 3′ UTRs, are thought to be the products of primary processing by Piwi instead.

In ovarian somatic cells of *Drosophila*, which express only Piwi, piRNAs are mainly the products of primary processing that takes place in cytoplasmic granules, called the Yb bodies [112, 115]. These piRNAs show a bias for U at position 1. They originate mainly in the antisense strand of "uni-strand" clusters, and are destined for maternal transmission acting as *trans*-silencers of TE transposition during the early stages of embryogenesis [21, 25, 50, 57, 68, 138, 141, 142]. A few piRNAs derived from select master loci may have specific functions that are required for somatic cell maintenance [124]. Overall, piRNAs in *Drosophila,* can be categorized into (a) the germ line piRNAs (resembling the pre-pachytene class of mammalian piRNAs), which are the products of secondary processing, and (b) the ovarian somatic piRNAs (resembling the pachytene class of mammalian piRNAs), which are the products of primary processing [21, 50, 112].

Genomic studies in sea anemones and sponges that diverged before the emergence of bilateral animals (e.g. human, flies and worm), have provided evidence that both types of piRNA may have existed since the origin of metazoan [105, 109].

PIWI Role in piRNA Biogenesis

Primary piRNA Biogenesis

The existence of an operational primary piRNA pathway was originally proposed from studies on *Drosophila* ovarian somatic cell, which express only Piwi [57, 138, 141–143]. Processing of primary piRNA-precursors from clusters (>200 kb long) requires the Piwi association with ribosomal components in the cytoplasm and/or cytoplasmic granules, to produce piRNA from both genomic strands [124, 138, 141]. piRNA-precursors are loaded onto piRNA-bound Piwi (ovarian somatic cells), and/or piRNA-bound Aub (nurse and germ cells), with the help of cytoplasmic granule proteins (the nuclease Zuc and the putative helicases Armi and Yb), to be tailor-processed from the 3′ end into mature piRNAs of a size that is determined by the footprint of bound piRNA [57, 84, 109, 112, 138]. Mature piRNAs are finally loaded onto Piwi, which enters the nucleus for downstream target recognition and silencing. In germ line cells, Yb is not expressed and therefore, only Armi and Zuc are considered to be involved in the biogenesis of primary piRNAs destined to initiate the secondary pathway [138, 141]. The *armi* and *zuc* gene homologues are conserved in mice, and in the absence of the *armi* germ line orthologue, spermatogenesis is blocked and both Mili and Miwi2 lack bound piRNA [144].

The direct role of Piwi/Miwi2 in primary piRNA biogenesis is not clear. Mature piRNAs can still be produced even when *Drosophila* Piwi or mouse Miwi2 are mutated. This means that primary piRNA production in the germ line is carried out either by sequence independent endonucleases, or by an AGO member as mentioned above [60, 83]. In line with these observations, the localization of Piwi/Miwi2 in the nucleus and the distinct sizes of bound piRNAs in *Drosophila* and mice, suggested that the main roles of Piwi/Miwi2 may be in TGS guided by the loaded piRNAs, rather than in piRNA biogenesis per se [115]. Recent studies in *Drosophila* with Piwi deficient ovarian germ or somatic cells [21, 60], or with cultured ovarian somatic cells [21], or from inserting ectopic piRNA targets in the fly genome [130], have shown that the Piwi is guided by piRNA to euchromatin DNA targets, which are silenced via the Piwi recruitment of epigenetic factors that convert DNA to chromatin. A role in piRNA biogenesis to silence transcription of a locus may still be required for complete TE silencing, and may involve the chaperoning and export to the cytoplasmic sites of Piwi bound to nascent transcripts by other proteins [145].

Data sequences of mammalian pachytene piRNAs isolated from *in vivo* and *in vitro* studies, indicate that the majority of piRNAs (70–80 %) that bind Mili and Miwi, have signatures of primary piRNA biogenesis in evidence that this pathway is operating in mammals [18, 32, 44, 65, 96]. Since both of these proteins associate also with piRNAs that bear the signatures of secondary processing, it is possible that *in vivo*, the PIWI-symmetric dimethylation status defined by the bound piRNA may dictate (in association with the other components of the piRISC assembly), whether Miwi and Mili are involved in primary or secondary piRNA biogenesis. Primary piRNAs generated from mRNA transcripts from various species, appear to be evolutionarily more conserved and may be involved in gene regulation at more than one level of transcription and translation [64, 124, 140].

It has been argued whether the long piRNA-precursor molecules may have a biological function in directing DNA methylation and/or TGS during mitosis/meiosis. Recent analysis of the correlations between DNA methylation and the density of piRNAs at piRNA coding regions, in human and mouse chromosomes, has indicated a preferential methylation close to such regions up to 16 Mb long, suggesting that long precursor piRNA-PIWI complexes may epigenetically control the expression of non-coding regions, in a manner similar to X-chromosome silencing [146].

The Ping-Pong Circular Model

The putative ping-pong mechanism is assumed to enable piRNA amplification and transposons silencing to occur simultaneously during active transposon expression, which increase the efficiency of TE degradation via the production of antisense piRNAs. The mechanism is considered to be driven by the maternally inherited mature piRNAs during early embryogenesis in *Drosophila* germ cells, and possibly in mammalian oocytes. It involves the catalysis of 5′ cleavage of TE-RNA strands of opposite orientation. This is via reciprocal cycles of slicer activity of Aub/Piwi (bound to antisense piRNAs) and Ago3 (bound to sense piRNAs), which amplify the pool of complementary sense and antisense secondary piRNAs that feed into the cycle. The piRNAs that associate with Aub or Ago3, often overlap at their 5′ end by

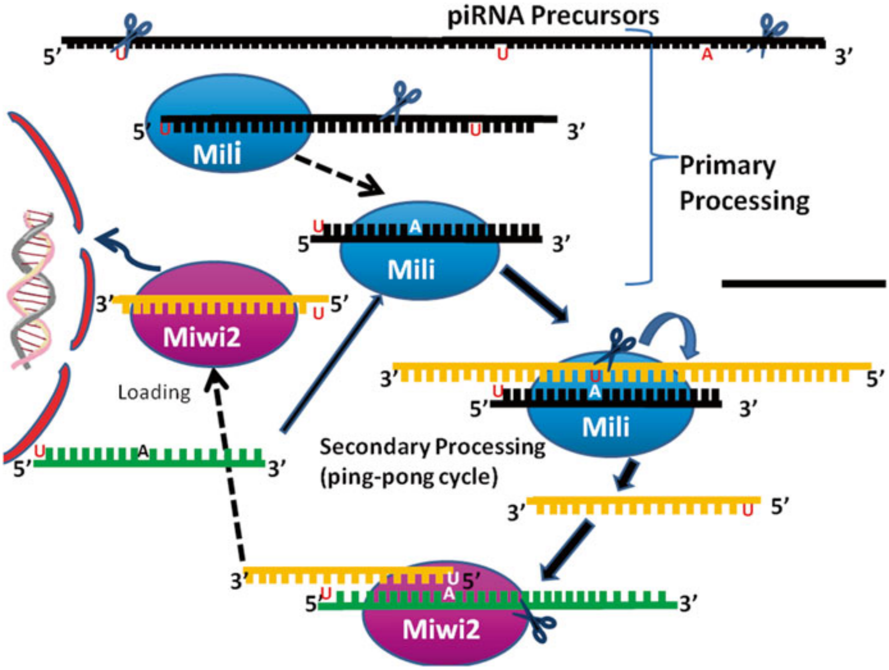


Fig. 3.11 The ping-pong model for piRNA biogenesis in mammals. Sense piRNAs derived from primary processing are used as guides by Mili to cleave antisense TE transcripts. The cleaved antisense transcripts serve in turn as guides for Miwi2 to cleave sense RNA thus fuelling an amplification cycle in which the 5′ ends of piRNAs are defined by PIWI cleavage. The 3′ ends are assumed to be shortened by an endonuclease and/or exonuclease and subsequently 2′-O-Methylated. Regulatory piRNAs may also be loaded onto to Miwi2 (*long dotted arrow*) for nuclear transport and piRISC function in TGS

ten nucleotides, which enables the transfer of the 5′-sliced precursor between the ping-pong partners that assemble on the surface of other proteins (i.e. vasa, TDRD proteins) [34, 64, 73, 138].

Based on the piRNA signatures generated, mice, unlike *Drosophila*, are assumed to use mature piRNAs derived from sense piRNA-precursors, in order to prime antisense piRNA production for TE-RNA degradation (Fig. 3.11). In foetal gonocytes, Mili associates primarily with sense piRNAs that have A at position 10, and Miwi2 associates with antisense piRNA with 5′ U, indicative of ping-pong signatures [19, 32, 34, 46, 51, 55]. However, studies with catalytically inactive Mili- and with Miwi2-DDH mutants, have shown that secondary piRNA biogenesis (pre-pachytene piRNA), and normal germ cell development depend on Mili and not on Miwi2 slicer-activity. Furthermore, in terms of piRNA levels, Mili appears to compensate for Miwi2 when the latter is not expressed [140]. piRNA analyses from *in vitro* (Mili) and from *in vivo* studies using Miwi- and Mili-DDH mouse mutants [64, 83, 103, 124], have shown that Miwi and Mili can catalyze secondary piRNA production (pre-pachytene piRNA), via an intra-amplification mechanism using complementary piRNAs as guides. As previously discussed, since the expression of Miwi2 but not its slicer-activity is required for *de novo* DNA methylation and normal gametogenesis, the role of Miwi2 may be limited to piRISC transcriptional and post-transcriptional functions [45, 83]. Alternatively, Miwi2 (like Mili and Miwi) may also promote primary piRNA biogenesis via the stabilization of mRNAs so that the mRNA can be cleaved by other endonucleases.

piRNA signatures for the function of the two pathways have been obtained from many animal species, including planarias, flies, zebra fish, frog, silkworm and mice, suggesting that they are conserved through evolution for the defence against invading TEs [33, 46, 51, 61, 66, 133, 147, 148]. Genetic studies on the evolutionary origin of the *Drosophila* somatic piRNA clusters and PIWI proteins, support the notion that the primary piRNA-Piwi pathway in these cells, evolved as a counter defence to the colonization of a follicular niche/soma by specific TEs, which tried to avoid germ line piRNA surveillance. Since mutations in genes that act only in the secondary pathway, result in the collapse of the entire piRNA pool, the role of the ping-pong mechanism could also be to ensure that all primary piRNA samples are used [23, 57, 110, 117, 134, 138, 141].

The Biological Significance of the piRNA-PIWI Pathway

Role in Human Diseases

A large number of investigations have provided evidence that the piRNA-PIWI pathway in metazoan has been associated with transcriptional and post transcriptional repression of TEs, and possibly of other genetic elements that are involved in the regulation of gametogenesis (from germ cell specification to GSC maintenance and proliferation, meiosis and to spermiogenesis). The biological significance of this pathway in human sperm development, and any relationship to idiopathic male sterility, however, have hardly been investigated. There has been one study in a Chinese population, on genetic variations in PIWI *vs.* spermiogenic failure. The study identified an SNP in the 3′ untranslated region of *HIWI2* associated with increased risk of oligozoospermia, and a non-synonymous SNP in *HIWI3* that was associated with reduced risk of oligozoospermia [149]. Some other studies have shown that the loss of the *HIWI* locus correlates with testicular atrophy and with the lack of development of proper secondary sexual characteristics [150, 151].

The effect of the *piwi* gene on germline development, has been related to the gonad somatic cell functions [40]. In lower organisms, it appears to play a role in genome rearrangement (Ciliates), synaptic plasticity and in associative memory development (Aplysia), as well as in whole-body regeneration (Colonial Ascidians) [132]. Maternal germline piRNA in *Drosophila,* has been implicated in the programming of somatic cells, and both maternal and zygotic Piwi proteins appear to be required for the establishment of heterochromatin and the suppression of phenotypic variations through epigenetic mechanisms. In addition, Piwi has been shown to have a role as an epigenetic activator [132]. A role of Piwi in somatic tissue differentiation, has been recently supported from studies on the time expression of piRNA during rat liver differentiation [152].

piRNA-PIWI It has been considered, therefore, that the "stem-like" proliferative phenotype of cancer cells could be related to the expression of components (i.e. PIWI proteins) involved in germ cell maintenance and self-renewal. Indeed investigations on PIWI re-expression in a cancer context, in humans, have shown that the Hiwi and Hili proteins and specific piRNAs (e.g. piRNA 651), are differentially expressed in precancerous and cancer cells depending on the cell type of the tumor (i.e. testicular (seminomas) and ovarian cancer, prostate, breast, gastrointestinal and endometrial cancers) [13, 22, 28, 104, 153–161].

Studies using ovarian tumours have shown that the high expression of *HIWI2* is associated with the silencing of genes that regulate apoptosis (i.e. Stat3/Bcl-X(L); p14ARF/p53), and with the expression of stem cell markers (e.g. *c-KIT*) thus correlating PIWI expression with the growth of cancer cells [13, 159–161]. The oncogen-like function of Hiwi2 was attributed to a Hiwi2-induced global DNA-hypermethylation of repetitive elements, which caused the silencing of tumour suppressor genes like the *CDKIs* [160]. Furthermore, Hiwi-mediated tumorigenesis was reversed by a methyltransferase inhibitor suggesting a connection between Hiwi2 and DNA methyltransferase [159].

Although investigations at the molecular level on the role of the piRNA-PIWI pathway in cancer are limited, the available studies suggest that if components of the piRNA-PIWI path are expressed in an aberrant way, they may contribute instead to the progression of cancer. In support, recent evidence has shown that the ectopic expression of piRNA pathway-associated genes contribute to the development of brain malignancies in *Drosophila* [168]. Furthermore, recent evidence has shown that promoter CpG island hypermethylation and inhibition of Piwil1, Piwil2, Piwil 4 and TDRD1 protein expression are related to reduced piRNA biogenesis and LINE-1 repetitive sequence hypomethylation, in primary seminoma and nonseminoma testicular tumors. Based on these, it is possible that the epigenetic inactivation of the PIWI-family of proteins could indeed contribute to male infertility and at least to the infertility-associated testicular cancer [162]. There are still many questions regarding the role of the PIWI proteins in cancer epigenetics. As matters stand at present, [153–161], the piRNA levels and the expression of specific piRNAs have the potential to serve as cell markers for cancer diagnosis, and/or to be used as treatment for altering the DNA methylation patterns to silence cancer related genes.

Conclusions

TEs have several opportunities to transpose themselves during gametogenesis, especially during periods when gene silencing and gene activation are taking place simultaneously (i.e. mitosis and meiosis of germ cells). The degradation of TEs transcripts via the direct catalytic activity of piRISC, and the repression of TE expression via piRISC-mediated epigenetic modifications, are two important functions that sexually reproducing organisms have evolved for defending TE invasions during gametogenesis. These functions have been well conserved and have evolved in mammals for optimal responses. Optimization may have driven the evolution of the spatiotemporal expression of PIWI proteins during male gametogenesis and the co-operation of multiple protein partners to facilitate piRISC activities in an orchestrated manner. This co-operation involves the receipt of messages (transcription of coding and non-coding mRNAs) during male gamete development and maturation, and the sorting and processing of these messages at specific cytoplasmic granules near the nucleus by the PIWI proteins and their associate partners. The generation of a large and highly diverse number of piRNAs that represent TE-RNA degradation products or serve as guides to degrade TEs, ensure feedback responses for piRISC and TGS so that the integrity of the genome is maintained [99]. Although the biological significance of piRNAs generated from non-coding intergenic DNA regions without repeats remains obscure at present, their number and diversity suggest that they may have functions beyond those of defending transposon mobilization. Based on studies in *Drosophila* and mice, such additional functions during gametogenesis include:

1. the degradation of mRNA (maternal) via recruitment of mRNA modifying enzymes [163];
2. the stabilization of coding mRNA via PIWI binding [31, 59, 63, 65] and,
3. the piRNA-PIWI-recruitment of transposable elements required for the assembly of the telomere protein complex to prevent telomere fusion [70, 164].

The piRNA-PIWI pathway may also have roles outside of the germ line as suggested by the evidence of the co-expression of PIWI-members and piRNAs in somatic stem cells of various tissues (including brain) of *Drosophila*, mouse and rhesus macaque [118] and in human brain [165]. Such roles are supported by the observe relationship between a specific piRNA and the repression of expression of the human melatonin receptor 1A gene [166], and the evidence for a piRNA regulatory role in the transcriptional regulation of plasticity-related genes involved in memory functions of the central nervous system of snails [167].

Compared with other small RNA systems, the piRNA-PIWI pathway is still not well understood, however. Many questions need to be answered with regard to the regulation/mechanisms of piRNA cluster transcription and primary piRNA biogenesis, the biological significance of the diverse piRNAs and the relationships of PIWI proteins with the various partners in cytoplasmic granules. The mechanisms and components that piRISC uses for the transcriptional/translational regulation of TEs and of other genes required for gametogenesis are been revealed slowly, and there are still a lot of questions.

Studies on the piRNA sequence and piRNA cluster evolution are consistent with the fast rates of evolution of the PIWI proteins in agreement with their interdependent roles in genome defence against transposons. The abundance and redundancy of mature piRNAs generated (with high sequence diversity), may also be part of the evolutionary strategy of organisms for coping with the fast rates of the ever expanding transposons. Whether different evolutionary pressures apply to piRNAs used for the regulation of transcription/translation of other genetic elements remain to be seen.

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Chapter 4
Long Non-coding RNA

Monika Gullerova

Introduction

Rapid developments in sequencing technologies during past decade revealed that protein coding genes represent only 2 % of the human genome [1]. This came across as a surprise considering that genomes of the other organisms (from yeast to *Caenorhabditis elegans*) are quite dense. Is the rest of the human genome merely "junk" DNA? This question was partially answered by experimental approaches like high-throughput sequencing or whole genome high density tailing arrays. Now it is known that this "junk" DNA is transcribed throughout mammalian genomes and because it lacks protein coding capacity it is referred to as long non-coding RNA (lncRNA) [2–7].

Non-coding transcripts have similar structure to messenger or coding RNA (mRNA): they are transcribed by RNA polymerase II (Pol II), they possess a cap and a polyA tail and they can be even spliced. However their function remains enigmatic [8]. Non-coding RNAs can be also divided into two groups based on their length: short and long non-coding RNAs (ncRNAs) or based on their primary function: structural and regulatory ncRNA (Fig. 4.1). Unlike mRNA and structural ncRNAs, most of lncRNAs are localized in nucleus [7].

Prior to the sequencing era, some lncRNA were discovered using old-fashioned gene cloning methods. Initially they were thought to be coding RNAs, however, deeper analyses revealed a lack of open reading frames (ORF). Furthermore they were thought to be random in nature more so than stable elements in genome. This opinion changed when FANTOM consortium analyzed over 60,000 full length cDNAs and identified over 11,000 lncRNAs in mouse [9]. Interestingly, a large portion of identified lncRNAs

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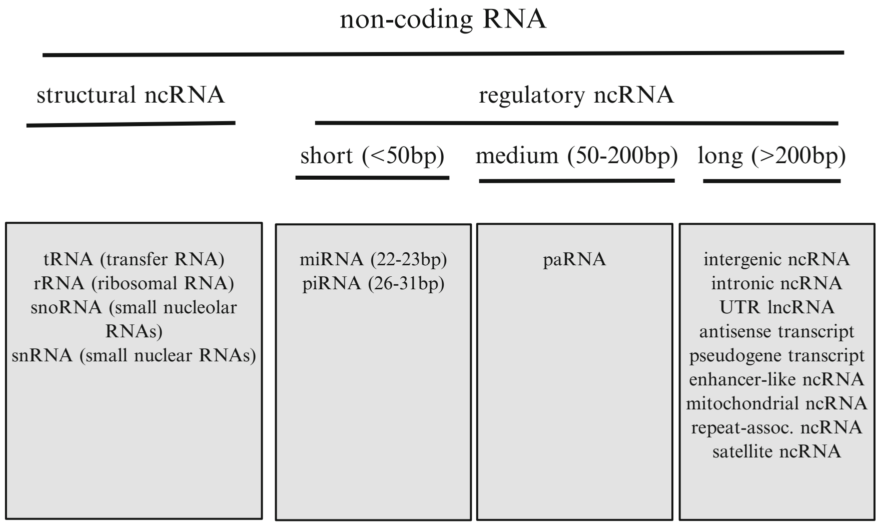

Fig. 4.1 Summary of various types of non-coding RNAs

is transcribed in antisense orientation to protein-coding genes, thus are referred to as natural antisense transcripts (NATs). Another study extended these findings by identifying NATs in human genome [10]. Interestingly many cancer associated genes, particularly tumor suppressor genes have long antisense ncRNA.

More recently, it was shown that intergenic regions also express thousands of long non-coding RNAs, named large intervening non-coding RNAs (lincRNAs). These transcripts were discovered by analyses of active chromatin marks (H3K4 acetylation and H3K36 trimethylation) genome-wide and eliminating those regions corresponding to protein coding genes and microRNAs. This approach was followed by extended analyses using RNA-seq experiments [4–6, 11–13]. Up to date, there are more than 8000 lincRNAs identified. More than half one them are confirmed lincRNAs; they can be localized in nucleus, cytoplasm or both and they are multi-exonic, capped and polyadenylated.

A major interest now lies in functional analysis of lncRNA. The fact that they are not evolutionary conserved, even between related species could indicate that most of them are non functional and may represent just transcriptional noise. To date, only 200 lncRNAs have been studied functionally with variable outcome, although many of them show at least functional evidence in vitro. Only a few lncRNAs have been studied in animal models, suggesting that they are not essential for viability. For example, mouse homologue of HOTAIR is poorly conserved in sequence and its deletion, along with the deletion of the HoxC cluster, has only a little effect in vivo, neither on the expression pattern or transcription efficiency, nor on the amount of K27me3 coverage of different Hoxd target genes [14]. On the other hand it is possible that the lack of one particular lncRNA can substituted by another one.

Overall, it is certain that mammalian genomes, including humans, produce thousands of lncRNAs. Due to the complexity and variability of lncRNAs it may take several years to understand and clarify their functional identity. It is very likely that some lncRNA are involved in a range of biological processes. In this chapter, I will discuss a number of important topics regarding lncRNAs: their origin, function, and role in disease.

Origins and Genome Localization of lncRNA

Low degree of lncRNA conservation among species suggests their different evolutionary design to protein coding genes. The limited phylogenetic range of lncRNA could be also explained by specific existence, but rapid declination within particular lineages [15]. A first possible scenario for lncRNA emergence is metamorphosis of protein-coding gene into non-coding RNA sequence. A protein-coding gene may under go mutations such as a frame shift that disrupts its open reading frame while maintaining the expression of the RNA transcript. For example, *Xist* gene encodes an lncRNA that is crucial for X chromosome inactivation. Recently, it's been shown that several exons and the promoter of *Xist* are derived from the protein-coding gene *Lnx3* that has acquired frame shift mutations during early mammalian evolution [16, 17]. It is possible that such a metamorphosis involved two steps: initial degeneration of the original sequence, followed by subsequent emergence of residual exons into newly formed Xist gene. Possibly these events occurred concurrently (Fig. 4.2a). A nother possibility includes chromosomal rearrangement, when two separate sequences are joined and together create an expressed non-coding sequence (Fig. 4.2b). One such example comes from the observation that a dog testis-derived non-coding RNA has arisen only recently following a lineage specific change. Also duplications in a non-coding RNA sequence could cause repeats, increasing the length of the transcript. Rare examples of duplicated lncRNA include Neat2 (mouse nuclear enriched abundant transcript) and mouse testis-derived lncRNA that are separate paralogous to non-exonic sequences elsewhere in the

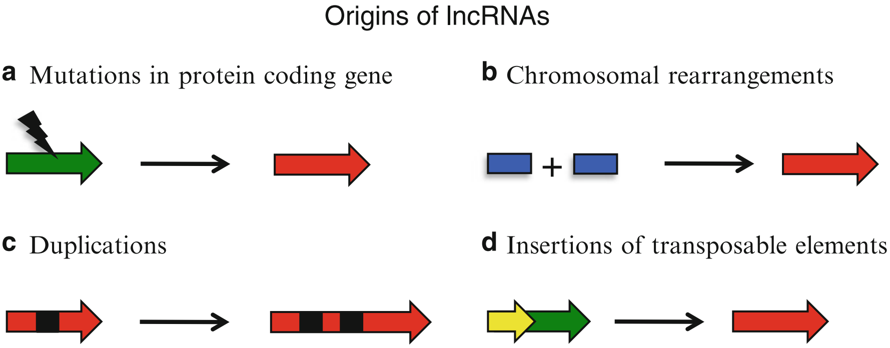
Origins of lncRNAs
a Mutations in protein coding gene
b Chromosomal rearrangements
+
c Duplications
d Insertions of transposable elements

Fig. 4.2 Origins of lncRNAs. *Block arrows* represent genes. *Black* regions in *block arrows* are introns. (**a**) Mutations in protein coding gene. (**b**) Chromosomal rearrangements. (**c**) Duplications. (**d**) Insertions of transposable elements

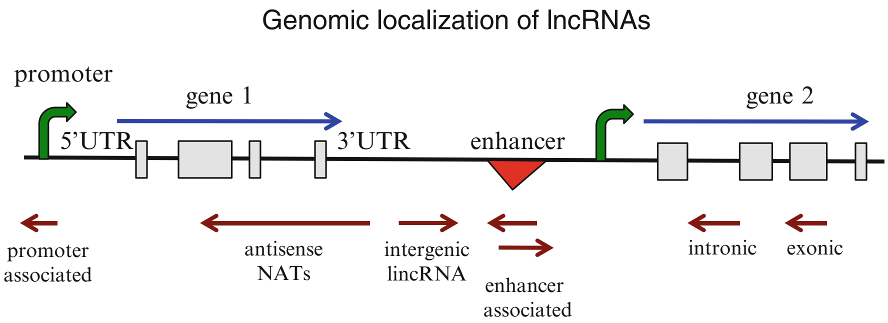

Fig. 4.3 Genomic localizations of lncRNAs. Schematic diagram illustrating organization of lncRNAs associated with protein-coding genes. *Arrows* pointing towards right represent sense transcription, *arrows* pointing towards left correspond to antisense transcription

genome [18]. Local, tandem duplication might also lead to generation of repeats increasing the size of lncRNA (Fig. 4.2c). Insertions of transposable elements may also emerge as lncRNA. For example, BC1 and BC200 (brain cytoplasmic RNA1 and 200-nucleotide) ncRNAs derive from two separate transposons, in the rodent and anthropoid lineages. Despite their lack of common origin, BC1 and BC200 are involved in similar roles in translational regulation. Furthermore, a transposable element containing a transcriptional start site can be inserted into the genome to create a functional, but noncoding RNA sequence [19] (Fig. 4.2d).

In respect of protein-coding genes, lncRNA can overlap with a gene as well as being associated with the gene's promoter region. They can be transcribed from intragenic sequence, exonic or intron, or from intergenic region (Fig. 4.3). In general, lncRNA are expressed at low levels and their expression varies with location, time, development and physiological stimuli.

Natural Antisense Transcripts (NATs)

Strand specific sequencing methods, like RNA-seq, reveal complex overlapping transcription with many lncRNA being transcribed from complementary DNA strands of protein coding genes. These are referred to as antisense transcripts (AS). AS can arise from novel transcription start sites as well as from bi-directional promoters, or through transcriptional read-through. In yeast, AS do not occur randomly, but have been linked to sexual differentiation or stress response genes [20–22] as well as genes with higher variability in expression. Furthermore, certain AS transcript pairs are conserved across several yeast species.

Recently genome-wide transcriptome studies reveal that natural AS transcripts (NATs) frequently occur across mammalian genomes [23, 24]. The sizes and features of NATs can be variable. They can be classified based on their transcriptional start site, splicing, capping and polyadenylation. With respect to transcriptional start sites, NATs can be divided into three types: (1) overlapping, (2) intronic and (3) intergenic [25, 26]. Sequence of overlapping NATs partly overlaps with sense mRNA sequence with preference for the 3′-untranslated region (3′ UTR). Due to sequence complementarity, these regions can mutually interact by complete or partial hybridization. Thus NATs may function to regulate the expression of the overlapping sense mRNA [27–29].

Long Intergenic ncRNAs (lincRNAs)

Mammalian genomes encode >1000 long intergenic noncoding (linc)RNAs that are clearly conserved across mammals and are potentially functional. These lincRNAs have been implicated in diverse biological processes, including cell-cycle regulation, immune surveillance, and embryonic stem cell pluripotency. However, the mechanism by which these lincRNAs function remains elusive. To date, there are approximately 3300 of human lincRNAs identified by analyzing chromatin-state maps in various human cell types. It is known that one of the well-characterized lincRNA HOTAIR binds the polycomb repressive complex (PRC2). Remarkably, approximately 20 % of lincRNAs expressed in various cell types are bound by PRC2. Other lincRNA interact with different chromatin-modifying complexes. Furthermore depletion of certain lincRNAs associated with PRC2 leads to changes in gene expression, causing the up-regulation of genes that are normally silenced by PRC2. Therefore it is suggested that some lincRNAs guide chromatin-modifying complexes to specific genomic loci to regulate gene expression [2, 5].

Promoter Associated ncRNAs (CUTs, PROMPTs)

Several genome-wide studies have revealed the unanticipated property of RNA polymerase II (Pol II) to initiate transcription in promoter regions in both directions. Such a bi-directional transcription results in so called cryptic unstable transcripts (CUTs) in budding yeast [30–32]. CUTs are Pol II-dependent transcripts produced from promoters in opposite direction to the coding gene, which are degraded by the nuclear exosome shortly after their synthesis. Another type of transcript derived from promoter regions are stable unannotated transcripts (SUTs), which are not processed by exosome [32]. Bidirectional transcription is not only limited to yeast species, but extends to higher eukaryotes too. Studies using exosome depletion in human fibroblasts have revealed lncRNAs, which correspond to upstream regions of protein coding genes. Such promoter-upstream transcripts are referred to as PROMPTs [33]. Furthermore, RNA-seq analysis from mouse embryonic stem cells has shown many promoter associated transcripts, which are transcribed in non-random, divergent orientation [34]. Another sequencing approach employed global run on sequencing (GRO-seq) to indentify nascent RNAs in human fibroblasts. This study revealed that almost 80 % of the active promoters display bidirectional transcriptional activity [35]. It is now known that bidirectional transcription is a widespread phenomenon, which is conserved across species. There are suggestions that this bidirectional transcription may be a type of gene expression regulation that promotes an open chromatin structure at promoters by recruiting positive or negative transcriptional regulators.

Enhancer Associated ncRNAs (eRNAs)

Enhancers act to regulate expression of protein-coding genes from a distance in an orientation independent manner. A recent study, which analysed genome wide location of the enhancer binding protein CBP, showed over 12,000 positive loci in mouse neurons. Further transcriptional analysis also confirmed that Pol II is present at 25 % of those loci. Enhancer associated transcripts were identified as lncRNAs (termed eRNAs) produced in both directions and their expression does correlate with mRNA synthesis from nearby gene [36]. There is a possibility that eRNAs may be directly involved in enhancer function. eRNAs may facilitate recruitment of enhancer-associated proteins or enhance chromatin looping to provide contact between the enhancer and the promoter of a particular regulated gene [37].

Repeat Associated ncRNAs

Retrotransposons are genetic elements that can amplify themselves within a genome. They are ubiquitous components of the DNA of many eukaryotic organisms, and are particularly abundant in plants, where they are often a principal component of nuclear DNA. In mammals, almost half the genome (45–48 %) comprises retrotransposons, which possess extensive transcriptional activity. FANTOM 4 project has revealed that in human and mouse genomes, retrotransposons are expressed in a tissue specific manner. They are located close to promoter regions of protein-coding genes, suggesting that they may play a role in controlling alternative promoters or in the post-transcriptional regulation of gene expression [38].

Pseudogenes are another type of repetitive elements that can be transcribed into lncRNA. These can regulate protein-coding genes through competition for regulatory miRNA binding [39, 40].

Biogenesis, Processing and Structure of lncRNAs

Biogenesis of lncRNA is very similar to mRNA. LncRNAs are produced from many regions across the genome by transcribing Pol II. Only some lncRNAs have been shown to be products of Pol III. The majority of lncRNAs are 5′ capped, polyadenylated and spliced. LncRNA can be divided into two categories based on their orientation: they can be encoded on positive or negative DNA strand (sense or antisense orientation).

Many lncRNA transcripts are not end products, but face to further processing into a final functional form. The presence of sense lncRNA, which contain exons from mRNA sequences and intronic lncRNA that are derived entirely from intronic sequence, has lead to the hypothesis that many lncRNA transcripts are unprocessed pre-mRNAs prior to splicing and that intronic lncRNAs are by-products of this processing step. However, this is not the case for all sense and intronic lncRNAs since the expression patterns of some of these transcripts are not the same as their associated protein-coding gene [41]. Another hypothesis suggests that some lncRNA sequences are precursors to short miRNAs. An example of this is the lncRNA *H19* which encodes the miRNA *miR-675* [4]. Based on this evidence, post-transcriptional processing may occur with many lncRNA transcripts, but until more of them are functionally defined this question remains open.

Secondary structure formation is an important consideration in lncRNAs because they are able to interact with proteins or genomic DNA via these structures. Recently, models used for prediction of secondary structure have redefined the question of lncRNA evolution by looking at sequence conservation or compensatory mutations that would maintain secondary structure motifs [42]. However, approaches that predict RNA secondary structures with high precision have yet to be developed. Therefore, the number of structured ncRNAs remains to be determined, but is expected in intergenic, intronic and UTR regions and lacking in exon sequence.

Mechanism of Action

Despite the fact that only a fraction of all identified lncRNAs has been examined experimentally, an emerging paradigm suggests that they are implicated in many biological contexts. To date, lncRNAs have been implicated in regulation of gene expression, guidance of chromatin-modifying complexes, X chromosome inactivation, genomic imprinting, nuclear compartmentalization, nuclear-cytoplasmic trafficking, RNA splicing and translational control [43–46].

Regulation of Chromatin Structure

LncRNAs have been implicated in epigenetic gene regulation. Recent studies propose two basic models for lncRNA action at the chromatin level:

1. epigenetic silencing in *cis*, where lncRNA transcripts coat gene clusters and silence their expression by making them inaccessible to transcription machinery. These lncRNAs can also recruit chromatin remodeling proteins to epigenetically mark the region for heritable gene silencing;
2. epigenetic silencing in *trans*: lncRNAs can interact with chromatin modifying proteins to epigenetically silence genes at another locus (Fig. 4.4).

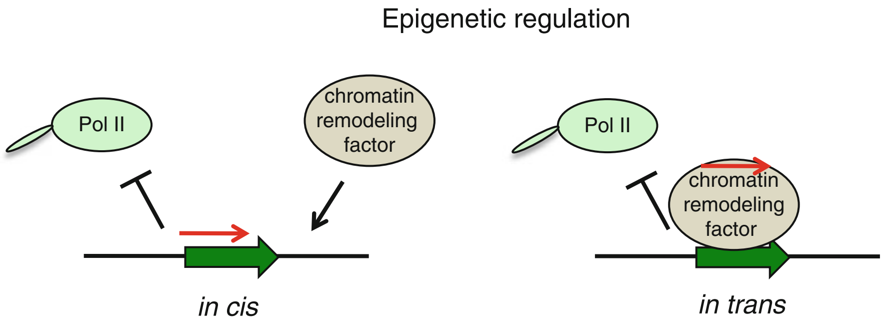

Fig. 4.4 Epigenetic regulation of gene expression by lncRNAs. *Block arrow* represents protein-coding gene, *red arrow* depicts lncRNA

Epigenetic Regulation in *cis*

One of the well studies lncRNAs to date is Xist, which is crucial for X chromosome inactivation in female somatic cells. It has been discovered in 1991 and despite of enormous effort, the exact mechanism of Xist-mediated X chromosome inactivation is still not fully understood. It is accepted that Xist is associated as an RNA compartment with the inactivated X chromosome [47]. The coating the chromatin that is silenced provides the first model for how lncRNA might function in stable epigenetic gene silencing in *cis*. Xist establishes a specialized, Pol II free, region, into which most of the X chromosome becomes localized during inactivation [48]. It should be noted that Xist coating of chromatin is stable even during metaphase, suggesting a form of epigenetic memory for the inactive X chromosome to remain silent over many cell divisions [49].

RepA, a small repeat region within Xist, is transcribed from both X chromosomes along with Tsix lncRNA (antisense partner of Xist) [50]. Tsix prevents RepA binding to either X chromosomes until post-cellular differentiation, when RepA in association with the chromatin-modifying complex PRC2 (polycomb repressive complex 2) binds to one of the two X chromosomes at the so called inactivation center [50]. Full-length Xist, produced from the X chromosome, destined to be inactivated, also binds to PRC2 and leads to the spreading of X inactivation from the center to the entire X chromosome in *cis*. Active chromatin status of the other X chromosome is protected by Tsix, which blocks transcription of Xist. It is not well understood, what prevents Xist from escaping the inactive X chromosome and acting on the active X chromosome in *trans* or how 20 % of X chromosome genes escape inactivation in human females [51, 52]. Once inactivation is established, X chromosome is condensed into facultative heterochromatin and forms a round body at the nuclear periphery [53]. The inactive chromosome possesses repressive chromatin marks and DNA methylation at CpG islands [52, 54, 55].

Genomic imprinting is another epigenetic phenomenon that utilizes lncRNA [56]. Imprinted genes play an important role in mammalian development and therefore their expression has to be tightly regulated [57]. Interestingly, many imprinted gene loci express lncRNAs that play a crucial role in regulating the expression of neighboring imprinted coding genes in *cis* [58]. One such lncRNA involved in genomic imprinting in *cis* is *Air*, which is mono-allelically expressed from the paternal allele. *Air* is known to bind to G9a histone methyltransferase and associate with chromatin to participate on silencing of three imprinted genes: Slc22a3, Slc22a2 and Igf2r. Loss of *Air* leads to bi-allelic expression of Slc22a3 and loss of G9a recruitment to imprinted genes. It has been suggested that Air acts to guide G9a to chromatin at the Slc22a3 promoter [59].

Epigenetic Regulation in *trans*

In contrast, to previous examples, a long intervening lncRNA, HOTAIR, regulates human genes expression in *trans* on a genome-wide scale by associating with chromatin modifying complexes such as polycomb repressive complex (PRC2), LSD1 and CoREST/REST [5, 60–62]. It has been shown that 5′ domain of HOTAIR binds PCR2, whereas a 3′ domain of HOTAIR binds LSD1/CoREST complexes. This way HOTAIR guides PCR2 and LSD1/CoREST to their endogenous targets. Consequently, PRC2 methylates histone H3 lysine 27, whilst LSD1/CoREST demethylates histone H3 at lysine 4. This collectively leads to the loss of active histone marks (H3K4 dimethylation) and the gain of a repressive histone marks (H3K27 trimethylation) at the target loci [62].

Gene Regulation Through lncRNA Transcription

Transcription of lncRNA itself can act as both, a positive (activation) or negative (repression) regulator of gene expression (Fig. 4.5), affecting expression of neighboring genes.

Activation: the act of lncRNA transcription can help to open the chromatin structure of a genetic locus to permit access of transcription machinery to neighboring protein-coding genes. In *fission yeast*, transcription of lncRNAs *UAS1* and *UAS2* have been shown to activate the expression of the *fbp1* gene by this mechanism. Pol II transcribes several species of ncRNAs at the *fbp1* locus during transcriptional activation. The chromatin is progressively converted to an open configuration, which is coupled to translocation of Pol II through the upstream region of the *fbp1* transcriptional start site. It has been shown that transcription through the promoter region is required to make DNA sequence accessible to transcriptional activators and to Pol II [63]. Similar example of gene transcription regulation have been observed within β-globin locus [64].

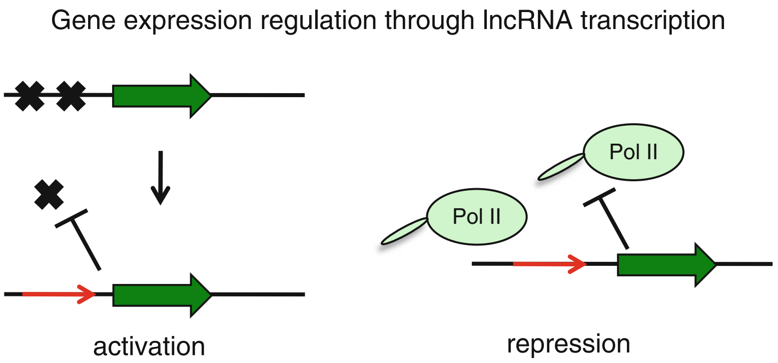

Fig. 4.5 Gene expression regulation through lncRNA transcription. *Block arrows* are protein-coding genes, *red arrows* are lncRNAs. *Block crosses* depict negative transcription factor

Repression: transcription of lncRNAs near protein-coding loci can also act as a negative regulator. The presence of the transcription machinery on the lncRNA gene locus can physically prevent transcription machinery from binding to the protein-coding gene. In *budding yeast*, transcription of the lncRNA *SRG1* inhibits transcription of the overlapping *SER3* gene. This repression occurs by a transcription-interference mechanism in which *SRG1* transcription across the *SER3* promoter interferes with the binding of activators [65]. Such a transcriptional interference process may represent a widespread function for lncRNAs. There seems to be a strong conservation of their promoter regions in contrast to weaker conservation of their transcripts, which is consistent with the act of transcription itself having a greater biological impact than the transcript sequence [7, 66].

Transcriptional Regulation

Protein coding gene expression is tightly regulated process, which involves direct interactions of proteins with other proteins or DNA. Another aspect of the regulation of gene expression comes from an additional layer of complexity consisting of dynamic interactions between RNA, DNA or proteins. Transcription of lncRNAs can regulate the expression of neighboring genes (*in cis* regulation) or can also target distant transcriptional activators or repressors (*in trans* regulation) (Fig. 4.6).

Transcriptional Regulation in *cis*

If lncRNA sequence overlaps through complementarity with the binding site of a transcription factor, the lncRNA transcript can hybridize to this site and so prevent a transcription factor from binding. One such an example is a lncRNA that is transcribed from a minor promoter upstream of the human dihudrofolate reductase

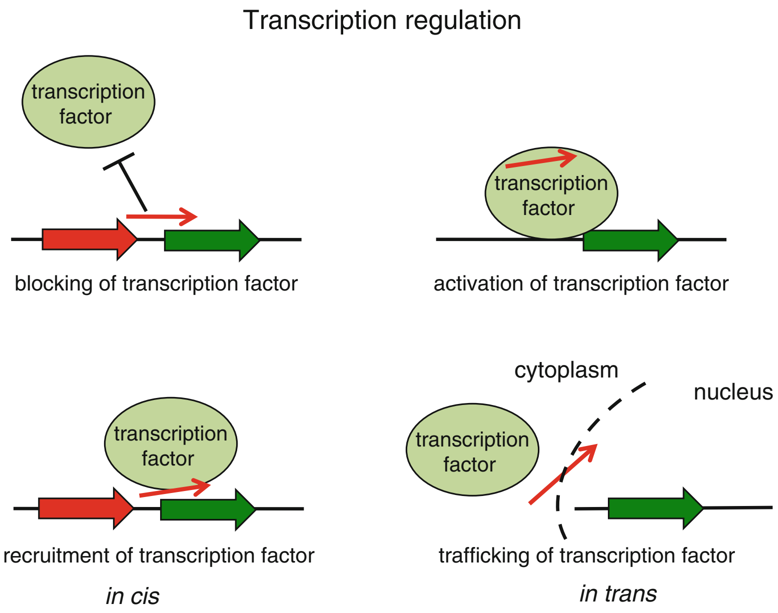
Transcription regulation
transcription factor
blocking of transcription factor
transcription factor
activation of transcription factor
cytoplasm
nucleus
transcription factor
recruitment of transcription factor
transcription factor
trafficking of transcription factor
in cis
in trans

Fig. 4.6 Types of transcriptional regulation by lncRNAs. *Block arrows* are protein-coding genes, *red arrows* are lncRNAs. *Dashed line* depicts nuclear membrane

DHFR gene. Transcription of this full length lncRNA is thought to repress transcription from the major *DHFR* promoter [67] in an RNA dependent manner. The lncRNA binds to the major *DHFR* promoter and the general transcription factor IIB and leads to dissociation of the pre-initiation complex. It is proposed that the single-stranded lncRNA hybridizes to double-stranded DNA in the promoter region to form a triplex structure. Such a structure is predicted to be most concentrated around human promoters [68], but it is unclear whether this is a common mechanism for lncRNA transcriptional repression.

Another type of transcriptional gene regulation by lncRNA is the recruitment of transcription factors. When a lncRNA sequence is located near to transcription factor binding site, the lncRNA transcript may enhance the binding of the transcription factor to promoter region. An example of this type of regulation is the lncRNA called *Evf2*, which regulates two homeodomain genes, *Dlx5* and *Dlx6*, involved in neuronal differentiation, migration and limb pattering [69]. Single-stranded *Evf2* forms a complex with *Dlx2*, another homeodomain protein. This *Evf2-Dlx2* complex activates *Dlx5/6* enhancer, by a yet unknown mechanism.

Transcriptional Regulation in *trans*

Another way that lncRNA may regulate transcription is through their affect on transcription factors trafficking in the cell. In particular, lncRNA can either enhance transcription factor access to DNA binding sites or prevent it, as in the case of the lncRNA *NRON*. This lncRNA prevents the transcription factor *NFAT* (nuclear factor of activated T cells) from entering the nucleus by directly interacting with importin-beta 1, one of the nuclear-cytoplasm transport factors [70]. The *NRON* gene contains three exons and can be alternatively spliced, producing variant transcripts ranging in size from 0.8 to 3.7 kb. Depletion of *NRON* leads to increased levels and activity of *NFAT* in nucleus. Interestingly *NRON*'*s* predicted secondary structure is rich in stem loops, which is conserved between diverse vertebrates and requires further study.

LncRNA can bind to accessory proteins to activate them allosterically, or induce their oligomerization and activation. One such lncRNA is *HSR1* (heat shock RNA-1), which together with an eukaryotic translation-elongation factor 1A, stimulates trimerization of heat-shock factor 1 (*HSF1*) [71]. Trimeric *HSF1* activates heat-shock proteins by binding to their promoters. Formation of *HSR1-HSF1* is induced by heat shock and knockdown of *HSR1* causes cells to become thermo-sensitive. This suggests that HSR1 may be a part of cellular thermo-sensing machinery, resembling a similar mechanism in bacteria.

Post-transcriptional Processing

In addition to all of the above transcriptional mechanisms, many lncRNAs are also involved in post-transcriptional processing of protein-coding mRNAs, including regulation of splicing, editing, transport, translation, and degradation of their corresponding mRNA transcripts.

Natural antisense transcripts (NATs) are typical example of lncRNAs that act to regulate mRNA dynamics. Unlike NATs associated with imprinting genes such as Tsix, Air or HOTAIR, which induce epigenetic changes in chromatin and lead to gene silencing, other NATs can form RNA duplexes to mask key *cis* regulatory elements. This can lead to an alternative splicing pattern of overlapping gene transcripts. For example, the Zeb2/Sip1 NAT is complementary to the 5′ splice site of an intron of the zinc finger Hox mRNA Zeb2, which is involved in epithelial-mesenchymal transition (EMT). Zeb2 NAT is expressed upon EMT and masks the splice site, so blocking spliceosome function. This causes the translation machinery to recognize and bind to an internal ribosome entry site (IRES) in the retained intron resulting in more efficient Zeb2 translation (Fig. 4.7) [72].

More recently, a NAT specific for tyrosine kinase containing immunoglobulin and epidermal growth factor homology domain-1 (Tie-1) was identified in zebrafish, mouse and human. The tie-1 NAT specifically binds tie-1 mRNA in vivo, forming an RNA–RNA duplex. This leads to down-regulation of the Tie-1 protein with consequent specific defects in cellular endothelial contact junctions [73].

In contrast, the expression of beta-secretase-1 (BACE-1) NAT increases stability of BACE-1 mRNA and leads to high production of Abeta (amyloid-beta) 1-42 through a post-transcriptional feed-forward mechanism [74, 75]. In this way BACE-1 NAT acts a positive regulator of Abeta 1-42 through stabilization of BACE-1 mRNA.

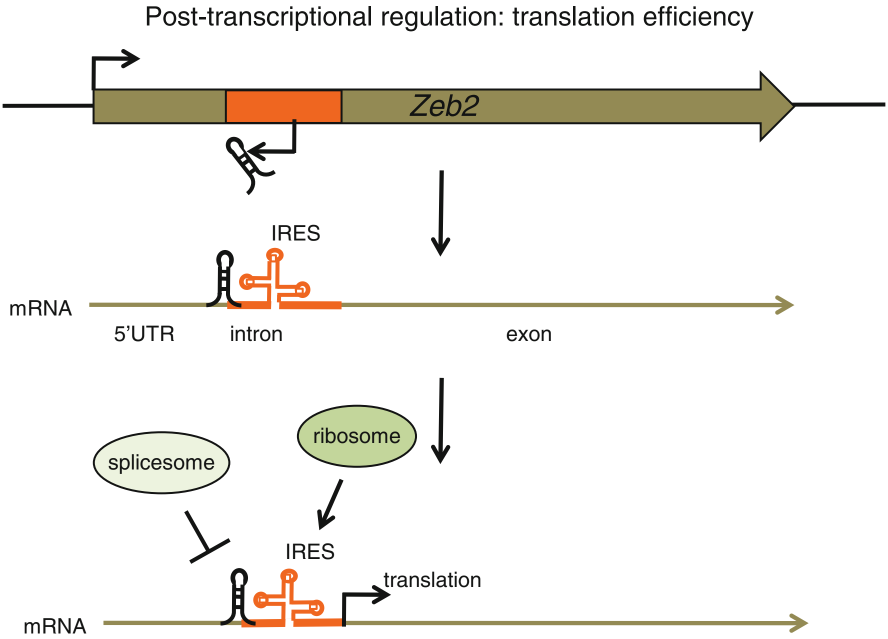

Fig. 4.7 Post-transcriptional regulation by lncRNA. *Brown block arrow* is Zeb2 gene. Intron is colored in *orange*. Hairpins correspond to miRNAs

Alternative splicing is a well-known mechanism of pre-mRNA processing in higher eukaryotes. The serine/arginine (SR) splicing factors regulate cell type specific alternative splicing in a concentration and phosphorylation-dependent manner. How levels of active SR proteins are regulated is not well understood. Recent studies on the long nuclear-retained regulatory RNA (nrRNA) called MALAT (metastasis-associated lung carcinoma transcript 1) implicated its role in alternative splicing [76, 77]. MALAT1 (also known as NEAT2) a 7 kb RNA is localized in nuclear speckles, where it interacts with SR splicing factor, SRSF1 and affects the distribution of other splicing factors. Depletion of MALAT1 changes the alternative splicing profile of multiple endogenous pre-mRNAs. More importantly, MALAT1 regulates the phosphorylation status of SR proteins, thereby regulating pre-mRNA processing via modulation of active SR proteins levels.

Furthermore, there is growing evidence showing that transcripts produced from pseudogenes play an important role in regulating mRNA stability of the gene paralogue. For example, transcripts from the tumor suppressor pseudogene of *PTEN* (*PTENP1*) and oncogenic *KRAS* (*KRASP*) regulate levels of their gene counterparts, *PTEN* and *KRAS* [39, 78]. It is biologically relevant to keep the right dosage of *PTEN* in the cell. A number of miRNA and pseudogene transcripts are also directly involved in *PTEN* dosage regulation at a post-transcriptional level. *PTEN* and *PTENP1* 3′ UTRs are highly conserved. *PTENP1* RNA, which is also referred to as competing endogenous RNA (ceRNA), binds to common miRNA preventing their

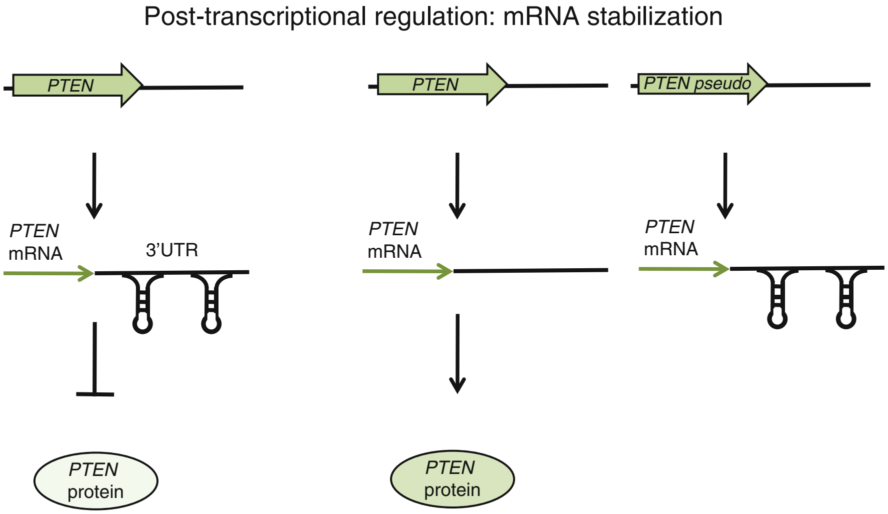

Fig. 4.8 Post-transcriptional regulation by lncRNA. *Block arrows* depict protein coding gene PTEN. Hairpins represent miRNAs

binding to miRNA response element in 3′ UTR of *PTEN*. This competitive binding of *PTENP1* to miRNA results in increased levels of *PTEN* RNA and consequently *PTEN* protein (Fig. 4.8) [40, 79, 80].

Additionally a similar mechanism has been shown for *KRAS* mRNA, which is increased by expression of ceRNAs of *KRASP*. Interestingly, some protein-coding genes, such as *ZEB2*, *VAPA* and *CNOT6L* can also act as ceRNAs.

The initial studies describing links between imprinting and X chromosome inactivation were based on discovery of the *H19* and *Xist* RNAs. The *H19* gene encodes a 2.3 kb lncRNA that is expressed exclusively from the maternal allele and is spliced, polyadenylated and exported into cytoplasm where it accumulates [81]. *H19* cause imprinting of its counterpart protein-coding gene, the insulin-like growth factor 2, *IGF2*. Recent studies revealed that *H19* is host to an exonic miRNA, *miR-675*, which is also imprinted and maternally expressed. *H19* and *miR675* are conserved across mammalian species, suggesting that both are selected [82]. It is proposed that *H19* might act through the nonsense mediated RNA decay pathway. Indeed, a key component of this pathway has been shown to regulate the levels of *H19* RNA during embryonic stem cell differentiation [83].

lncRNA and Disease

Recent and rapid progress in lncRNA research reveals a growing body of evidence that lncRNA play an important role in variety of normal physiological processes. Consequently their mis-regulated expression contributes to numerous diseases, including cancer.

lncRNA and Cancer

New technological approaches, such as genome-wide studies, RIP-RNA sequencing, gene expression screens, region-targeted assays and gene knock-down/knock-out experiments all contribute to the determination of lncRNA function in pathogenesis. Accumulating data show that lncRNAs are indeed involved in carcinogenesis, invasion and metastasis. Based on their function, lncRNAs can be divided into two major categories: oncogenic and tumor suppressor classes.

Oncogenic lncRNAs

Some lncRNAs, referred to as oncogenic transcripts, can regulate cellular pathways that lead to oncogenesis. Recent studies identify more and more of onco-lncRNAs such as *KRASP*, *HULC*, *HOTAIR*, *MALAT1*/*NEAT1*, *p15AS*, *ANRIL*, *H19*, *SRA1*, *p21NAT* or *RICTOR*. Some lncRNAs can act as oncogenic as well as tumor suppressor transcripts, depending on cellular context.

The lncRNA, referred to as cancer metastasis-associated lung adenocarcinoma transcript, *MALAT1*, was identified in non-small-cell lung cancer [84]. *MALAT1* is abundant and plays a key role in cell proliferation, migration and invasion. It localizes predominantly in nuclear speckles in a transcription dependent manner to regulate mRNAs post-transcriptional processing such as alternative splicing [76, 85]. *MALAT1* is also up-regulated in other types of cancer, including breast, prostate, liver and colon [84, 86–90]. Furthermore, higher expression of *MALAT1* is associated with metastatic tumors where it is correlated with poor prognosis. Recent studies demonstrate that *MALAT1* is involved in cell mobility at it targets genes, required for cell migration, in order to regulate their gene expression at both a transcriptional and post-transcriptional level. However, the underlying mechanism of *MALAT1* in tumor metastatic process remains unclear.

A genome-wide study unveiled associations of multiple genetic variants in a large "gene-desert" region of chromosome 8q24 with susceptibility to prostate cancer (PC). Re-sequencing approaches helped to identify a 13 kb long intron-less lncRNA, termed *PRNCR1* (prostate cancer non-coding RNA1) [91]. Depletion of *PRNCR1* attenuated the viability of PC cells and the *trans*-activation activity of the androgen receptor. Therefore, it has been proposed that *PRNCR1* is involved in prostate carcinogenesis through androgen receptor activity. These findings provide a novel insight into understanding the pathogenesis of genetic factors for prostate cancer.

The lincRNA *HOTAIR* is expressed in many posterior and distal sites during evolution and is highly conserved in vertebrates [60]. *HOTAIR* is over-expressed in metastatic breast cancer and correlates with poor prognosis [61]. De-regulation of *HOTAIR* represses the expression of a subset of cell-to-cell interaction promoting genes, including *JAM2*, *PCDH10*, *PCDHB5* and *EPHA1*. Furthermore, the interaction of *HOTAIR* and *PRC2*, which leads to increased H3K27 trimethylation and silencing of metastasis suppressor genes, is responsible for *HOTAIR* mediated tumor cell invasion and subsequent metastasis. Additionally, increased levels of *HOTAIR* were detected in hepatocellular carcinoma (HCC), suggesting that *HOTAIR* could be a candidate biomarker for tumor recurrence prediction. Depletion of *HOTAIR* in liver cancer cells results in reduced cell viability and sensitized apoptosis induced by *TNF-alpha* [92]. These studies indicate that lincRNAs have active roles in modulating the cancer epigenome and could be an important predictor for cancer outcome as well as novel targets for cancer therapy.

Loss of imprinting (LOI) is involved in a number of human hereditary diseases and cancers [93]. Disruption in the expression of imprinted genes such as *H19*, *p57*, *IGF2* and *KvLQT1*, results in almost 80 % of Beckwith–Wiedemann syndrome (BWS). About 5–10 % of BWS patients are predisposed to a number of childhood tumors. *Kcnq1ot1* is an imprinted antisense lncRNA, which is about 60 kb long and possesses a silencing domain at its 5′ end [94]. *Kcnq1ot1* transcript is associated with multiple chromosomal rearrangements in BWS. Its abnormal expression was observed in 50 % of BWS patients and 53 % of colorectal cancers [95–97]. Loss of *Kcnq1ot1* imprinting is accompanied by loss of methylation of the control element, a CpG-island called *KvDMR1*. *KvDMR1* contains the promoter for the paternally expressed *Kcnq1ot1*. Disruption of this promoter abolishes *Kcnq1ot1* transcripts leading to activation of neighboring genes such as tumor repressor *CDKN1C* [98]. These data suggest that abnormal expression of *Kcnq1ot1* contributes to carcinogenesis.

Recent studies report consistent differences in the expression of sense and antisense transcripts between normal and neoplastic cells. A group of genes that generate NATs in normal, but not cancer cells are involved in essential metabolic processes. Altered ratio of sense and antisense transcription contributes to tumorigenesis and cancer progression [99–103]. For example, leukemic cells express higher amounts of antisense p15 NATs and smaller amounts of its partner p15 sense mRNA than normal lymphocytes. Many NAT lncRNAs may have relevance to the cancer genes, including p21, p53, E-cadherin or myc [104]. Thus, it is proposed that tumorigenic NATs are a trigger for heterochromatin formation and DNA methylation in tumor suppressor silencing.

Tumor Suppressor lncRNAs

Some lncRNAs are found to function as tumor suppressors, resembling some protein-coding genes. This group of lncRNAs includes *MEG3*, *GAS5*, *lincRNA-p21*, *PTENP1*, *TERRA*, *CCND1* and *TUG1*.

MEG3 is a lncRNA transcript of a maternally imprinted gene, which is expressed in normal human cells. Loss of *MEG3* was found in meningiomas and adenomas of gonadotroph origins [105, 106]. *MEG3* is a positive regulator of the tumor suppressor gene, p53. Ectopic expression of *MEG3* up-regulates p53 protein levels and dramatically induces p53 transcription. Furthermore, *MEG3* selectively enhances p53 binding to its target promoter, such as *GDF15*. Expression of *MEG3* is able to inhibit cell proliferation in the absence of p53. All these data suggest that lncRNA *MEG3* functions as a tumor suppressor in both a p53-dependent and p53-independent manner [107, 108].

LincRNA-p21 is another example of a tumor suppressor lncRNA, whose expression is directly induced by the p53-signaling pathway. *LincRNA-p21* is required for global repression of genes that interfere with p53 function to regulate cellular apoptosis. This occurs through physical interaction with RNA-binding protein hnRNP-K leading to its localization on gene promoters, which are thought to be repressed in a p53-dependent manner [109].

GAS5 (growth arrest-specific 5) is tumor suppressor lncRNA, which regulates normal growth in lymphocytes. Depletion of *GAS5* inhibits apoptosis and maintains rapid cell cycling, which indicates that its expression is necessary for normal growth arrest. *GAS5* regulates the expression of a critical group of genes with tumor suppressive functions. Additionally, several snoRNAs are transcribed solely from *GAS5* introns. Under starvation, *GAS5* directly interacts with the DNA binding domain of glucocorticoid receptor (GR), leading to inhibition of GR binding to its target gene promoters. Such repression is not limited only to GR, but applies also to other members of the nuclear receptor family. Interestingly, *GAS5* is significantly downregulated in breast cancer cells [110–112].

Cis-acting lncRNA, *CCND*, originates from the promoter of the *CCND1* gene encoding cyclin D1 protein. Upon induction, *CCND* lncRNA transcript is tethered to the *CCND1* promoter and so inhibits *CCND1* expression. Cyclin D1 is frequently over-expressed in human tumors. Therefore, it is proposed that *CCND1* transcript functions as a tumor suppressor to repress tumorigenesis [113].

Expression of the telomere-related lncRNA, *TERRA*, is highly dependent on development, nuclear reprogramming, telomere length, cellular stresses and chromatin structure. Many abnormal telomere phenotypes in aging and cancer cells are linked to mis-regulated expression of *TERRA*. Low levels of *TERRA* have been observed in the tumor-derived and in vitro immortalized cell lines. It has been proposed that *TERRA*-regulated telomere length plays an important role in tumor development [114–117].

lncRNA and Other Diseases

LncRNAs in the context of their cellular function can also be involved in diseases other than cancer.

Patients with *SCA8* have a trinucleotide expansion in an lncRNA called ataxin 8, which is antisense to the *KLHL1* gene. Involvement of this type of mutation in disease progression was confirmed in mouse model transgenic mice with this repeat expansion displaying a progressive neurological phenotype similar to human *SCA8* [118].

An inherited form of alpha-thalassaemia is caused by the translocation of an antisense lncRNA to a neighboring region of the alpha-globin gene (*HBA2*). Induction of this lncRNA results in epigenetic silencing of *HBA2* leading to anemia [119].

The expression of the antisense transcript to *BACE1* gene, as a response to cell stress, leads to progression of the well-studied Alzheimer's disease [74, 120].

Also, psoriasis-associated RNA induced by stress, called *PRINS*, is up-regulated in skin cells of patients with psoriasis. It acts through down-regulation of *G1P3*, gene encoding a protein with anti-apoptotic effect in keratinocytes leading to psoriasis progression [121].

A study using a single-nucleotide polymorphism marker identified a lncRNA called *MIAT* (myocardial infarction-associated transcript) on chromosome 22 in patients with myocardial infarction [122]. Furthermore, genome-wide analysis identified a region encompassing a lncRNA, *ANRIL*, which is linked to coronary artery disease [123, 124].

Overall, identified lncRNAs play a clear role in pathology of various diseases. It remains to be determined what is their specific function and how they are associated with human pathology.

lncRNA as Biomarkers

To date, although our understanding on how lncRNAs cause disease is far from complete, certain features of lncRNAs make them ideal candidates for therapeutic intervention. For example, only a minority of lncRNAs are unstable. LncRNA half-lives vary over a wide range, comparable to that of mRNAs. Combining half-lives with comprehensive lncRNA annotations hundreds of unstable (half-life < 2 h) intergenic, *cis*-antisense, and intronic lncRNAs, as well as lncRNAs showing extreme stability (half-life > 16 h) were identified. Intergenic and *cis*-antisense RNAs are more stable than those derived from introns [125].

LncRNA expression is elevated in several types of cancers, including human prostate cancer, renal cell carcinomas, breast and ovarian cancer, as well as human lung cancer, suggesting that lncRNAs may become a promising biomarker in disease diagnostics. For example, the prostate specific lncRNA, *DD3*, shows higher specificity than serum prostate-specific antigen (PSA), suggesting that is could be developed into highly specific biomarker [126]. HCC-associated lncRNA, *HULC*, is also upregulated in the blood of hepatocarcinomas, implying its potential use in diagnosis of this type of cancer [127]. Expression of *HOX* specific antisense RNA, *HOTAIR*, is increased in breast tumor cells, suggesting that it may become a powerful predictor of patient outcome such as metastasis and death. *SNORD*-host RNA, *Zfas1* is an antisense transcript of the protein-coding gene *Znfx1*. *Zfas1* is highly expressed in mammary glands and it's obviously down-regulated in breast cancer cells, suggesting its potential for diagnosis of breast cancer [128].

LncRNAs-based biomarkers could also be developed for diseases other than cancer. For example, noncoding transcript for beta-secretase-1 (*BACE1*), which regulates *BACE1* mRNA and protein production, is upregulated in Alzheimer's disease and thus could be exploited as a biomarker [129]. ANRIL lncRNA, is expressed in tissues and cells affected by atherosclerosis, which makes it a potential biomarker for coronary artery disease [130].

Overall it is clear that lncRNAs possess a significant potential for development of new approaches in diagnostics and therapy.

lncRNA and Stem Cell Development

Cellular reprogramming demonstrates the plasticity of cell fates. LncRNAs, whose expressions are linked to pluripotency, are direct targets of key transcription factors [131]. One such a lncRNA (*RoR*) modulates cellular reprogramming, which has been identified by loss-of function and gain-of function approaches. This provided first evidence for the critical function of a lncRNA in the derivation of pluripotent stem cells. LncRNAs also help to regulate development by physically interacting with proteins to coordinate gene expression in embryonic stem cells (ESCs) [132]. This is contrary to the dogma that proteins alone are the key regulators of this process. LncRNA determine the fate of ESCs by keeping them in their unspecialized state or by directing them along a pathway to cellular differentiation.

lncRNA and Immunity

Whole-transcriptome analysis has shown that lncRNAs are associated with diverse biological processes in different tissues and are also involved in the host response to viral infection and innate immunity [6]. Also a recent study revealed altered expression of lncRNA during CD8+ T cell differentiation upon antigen recognition [133]. Likewise, eight mRNA-like lncRNAs were differentially expressed in virus-infected birds [134]. Whole-transcriptome analysis of severe acute respiratory syndrome in coronavirus-infected lung samples shows that there is a widespread differential regulation of lncRNAs in response to viral infection [135]. All of this suggests that lncRNAs are involved in regulating the host response in virus-infected cells, including innate immunity.

Concluding Remarks

The discovery of lncRNAs has changed our view of the complexity of the mammalian transcriptome. LncRNAs are becoming widely recognized as key regulators of protein-coding gene expression and so provide an additional layer of transcriptional control. To date, lncRNAs have been shown to be involved in many different stages of gene expression regulation. This diversity in function suggests that lncRNAs will ultimately be found to participate at all levels of transcriptional control, from nuclear localization of transcription factors to transcriptional termination. Several lncRNAs have been implicated in the mediation of chromatin structure. Enabling the accessibility of the genome to Pol II and its associated factors is the most efficient way to activate or repress transcription. LncRNAs also function in X chromosome inactivation and genomic imprinting through chromatin remodeling.

Future discoveries may struggle to identify additional transcriptional regulatory lncRNA that share a function with known lncRNAs, because different RNAs can have similar functions even though they lack detectable sequence similarity.

Real challenges lie in determining the biological significance of lncRNAs-protein interaction. Scientists have to clearly demonstrate biological roles of particular lncRNAs and relate them to their associated transcriptional units. It is peculiar that lncRNAs are not evolutionary conserved, they are expressed in very low levels and their knock-out don't show a clear phenotype. Therefore, their biological significance remains an open topic for a further analysis.

The de-regulation of lncRNA expression in the context of cell pathology represents a new layer of complexity in the molecular architecture of human disease. Several lines of evidence have suggested that even small-scale mutations can affect lncRNA structure and function. Future studies need to elucidate the mechanism by which disease-causing mutations in lncRNA functional motifs can affect its regulatory domains and thereby contribute to disease pathology.

Future research of lncRNA may lead to discoveries of their biological functions and ultimately propose new RNA-based targets for the prevention and treatment of human disease.

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Chapter 5
Unveiling Transposable Elements Function to Enrich Knowledge for Human Physiology and Disease Pathogenesis

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Introduction

Transposable elements (TEs) are DNA sequences that have the ability to move from one chromosomal location to another through their integration into the genome at different sites. TEs have been found in virtually all eukaryotic organisms, covering 3–80 % of their genomes and considerably influencing evolutionary history [1, 2]. In humans, TEs have been estimated to occupy approximately 45 % of genomic sequence, a proportion being one of the highest among mammals. However, as it has been proposed, it is possible that this impressive number represents an underestimation, since sequences of ancient TEs may have deteriorated beyond recognition [3].

TEs can be grouped in two major classes [4]. Class II elements or DNA transposons account for roughly 3 % of the genome and move by a cut and paste mechanism through an element encoded transposase. There is no indication of their activity to date which is believed to have subsided 37 million years ago [5, 6]. Class I elements or retrotransposons move by a copy and paste mechanism through an RNA intermediate which is reverse transcribed and then inserted at a new site in the host genome. This process is known as retrotransposition. Retrotransposons can be further categorized in two major subclasses: long terminal repeat retrotransposons (LTR) and non-LTR retrotransposons. LTR retroelements include endogenous retroviruses (ERVs) and consist 8 % of the human genome. Although inactive in humans for millions of years their activity is significant in rodent germline [7]. The LTR retrotransposon subclass has a mode of retrotransposition very similar to retroviruses. Non-LTR retroelements include long interspersed nuclear elements (LINEs), short interspersed elements (SINEs) such as Alu elements and hybrid SINE-R/VNTR

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(variable number of tandem repeat)/Alu elements (SVAs) which are all evidently capable of retrotransposition in the human genome. LINEs are termed autonomous, since they encode the proteins required for their mobilisation. All the other elements are non-autonomous, since they encode no proteins and parasitize LINE machinery in order to achieve their genome mobilization.

The LINE-1 (L1) family is represented by more than 500,000 copies in the human genome comprising an estimated 17 % [8]. Most of these copies are inactive due to accumulated mutations and only 80–100 elements are functional and capable of retrotransposition in human [9]. In contrast, several thousand L1 elements are still active in mice. A full length L1 is 6 kb long and includes a 5′-untranslated region (5′-UTR), two open reading frames (ORF1 and ORF2), a 3′-UTR and a poly(A) tail. The 5′-UTR serves as a RNA-polymerase II promoter while the polyA signal in the 3′-UTR is weak and often read through during transcription allowing 3′-flanking sequences to be co-transcribed along L1 sequence. ORF1 and ORF2 encode proteins with RNA-chaperone and endonuclease/ reverse transcriptase activity respectively. Both of the proteins are required for retrotransposition which takes place through a mechanism called “target-site primed reverse transcription” (TPRT). The synthesized RNA transcript from L1 element transcription is first exported to the cytoplasm in order that ORF1 and ORF2 are produced and then re-enters the nucleus and gets reverse-transcribed directly at the site of integration [10]. ORF1 and ORF2 exhibit a *cis* preference to the RNA they were translated from but also act *in trans* to promote the mobilisation of the non-autonomous retroelements such as Alus [11, 12] and SVAs [13, 14].

More than 1,000,000 copies of Alu elements have been identified so far, a fact that renders them the most abundant repeats in the human genome occupying an estimated 11 %. Alu elements are derived from 7SL RNA, a functional component of the signal recognition particle. B1 SINE elements in mice most closely resemble Alus and also derived from 7SL RNA. Alus are 300 bp long and transcribed by RNA polymerase III. They end in a polyA tail of variable length which has been proved to be critical for retrotransposition as is also the integrity of the internal RNA polymerase III promoter [15]. In addition, it has been demonstrated that Alus require only ORF2 of L1 for their mobilization since expression of L1 elements with mutant ORF1 in ex vivo assays allows Alu mobilization [12]. Alu elements exhibit the highest retrotransposition rate per live births (1/21) among the currently mobilizing elements in humans [16]. Interestingly, Alu sequences are highly polymorphic with respect to their presence or absence among individuals.

SVA elements are intermediate in size (~2 kb) and much fewer than the other retrotransposons such as L1 and Alu (2700 copies, 0.2 % of human genome), since they are hominid-specific and originated less than 25 million years ago [17]. SVAs have a composite structure and are generally believed to be transcribed by RNA polymerase II. They include a hexamer repeat region, an Alu like region, a variable number of tandem repeat (VNTR) region, a short interspersed element of retroviral origin (SINE-R) and a polyA tail. Their mode of mobilization relies on the L1 enzymatic machinery as is also the case with Alu elements [18].

Transposable Elements: Innate Role and Function Controversy

Mobile elements and their impressive prevalence in genomes across species has been the subject of intense scientific debate since their discovery by McClintock [19]. An initially underestimated discovery that was faced with skepticism waited for years for validation and finally led to the appreciation of its significance and a Nobel Prize Award. During this course, various experimental efforts have been successfully carried out, while a lot of light has been shed on the structure, distribution and mode of activity of TEs leaving though their true reason of existence still elusive. Barbara McClintock, whose maize breeding experiments provided the first detailed descriptions of TEs, proposed that the genome is a dynamic entity subject to alteration and rearrangement and also referred to TEs as controlling units suggesting they may serve important regulatory roles.

A lot of efforts describing TE functional characteristics have tried to attribute a central more specific role to their presence and mobilization, ranging from control of embryonic development to the well described contribution in producing genetic variability and promoting the evolutionary process. In certain species such as insects, TEs play a role in fundamental cellular processes like the maintenance and structural integrity of DNA during cell division. In fruit fly and silk worm, a type of transposon is suggested to move bits of DNA to the end of the chromosomes to prevent the loss of telomeres following chromosomal replication [20, 21]. Since TE structure, distribution and mode of activity seems to be variable across species their presence might be accompanied by discrete and differential key roles in each case. Most certainly, and based on knowledge accumulated thus far, the term "junk DNA" is presently at least not representative.

The increasing availability of vast amount of genomic information from multiple species combined with the advancements happening in computational techniques for comparative studies has greatly promoted research concerning the impact of TEs on the evolutionary process. Several examples demonstrate clearly that TEs have served as an important creative force in the evolution of genomes crucially affecting variation of genome size, composition and structure among species. Indeed, it is TE presence that defines the differences in genome size of higher organisms compared to prokaryotes, although the number of existed gene structures increases also [1]. Interestingly, even among mammals there is a correlation of genome size with the TE genomic content. Moreover, the types and families of TEs in vertebrate genomes differ greatly, defined in part by the rate of activity and elimination of ancestral TEs, while inter-individual variation within the same species is also present [5]. Such differences can be actually used as markers in conducting phylogenetic analysis. Apart from the structural genetic diversity that results from TE activity, the impact of the new insertion events is considerable, since genome functionality can be affected in various ways, further driving biodiversity and genome evolution [4].

Mechanisms Through Which TE Activity Affects Gene Expression and Function

The most obvious way in which a retrotransposition event can affect the host genome integrity is insertional mutagenesis. TEs can be inserted into or near genes remodeling the local genomic region and can do so often accompanied by 3′ transduction phenomena. In this case, DNA sequences downstream of a L1 element for example, can be transcribed by RNA polymerase II and co-mobilize due to the presence of a weak L1 termination and polyA signal [22]. The rate at which adjacent genomic regions can be transduced along L1 insertion has been computationally estimated at one every five L1 retrotransposition events [23].

The impact of the insertion event can be detrimental to the host, regardless of whether it involves integration into intron or exon sequences of a gene. Insertion in exon regions can cause the creation of new chimeric mRNA and proteins providing that the integration results in a tolerable phenotype. Insertion in the intron region of genes has been reported to result in mis-splicing, Alu exonization or exon skipping, as well as in reduced mRNA levels due to inefficiency of the RNA polymerase II to transcribe through the transposable element [24, 25]. Another phenomenon observed and associated to the insertion of a mobile element is the deletion of an adjacent genomic sequence. Insertion-mediated deletions caused by Alu and L1 have been confirmed by comparative genomic studies and two mechanisms, TPRT-dependent insertion mediated deletion and endonuclease-independent insertion have been identified that can mediate such deletions of host DNA [26].

In addition, instead of disrupting gene function insertions may alter the expression pattern of nearby genes interfering this way with gene regulation. It has been estimated that TE-derived sequences are contained in the coding region of 4 % of human genes and in 25 % of human promoters [27]. The introduction of functional both splice and polyA sites which can lead to aberrant processing of RNA transcripts, or the introduction of promoter sequences and regulatory regions can lead to changes of gene expression profiles with a potential impact on the host behavior [28–31]. Interestingly, it has been shown that 7–10 % of transcription factor binding sites that have been experimentally characterized derive from repetitive sequences including simple sequence repeats and TEs [32]. The first report of interference with transcriptional control of a gene involves a L1 element residing in the apolipoprotein(a) transcriptional control region (ACR). It was demonstrated in vitro that the L1 element when linked in either orientation to the apolipoprotein(a) minimal promoter can confer a tenfold increase in transcriptional activity [33]. On the other hand, an Alu element at the distal part of the human BRCA2 promoter contains a 221 base-pair silencer region. This region has been found to negatively regulate BRCA2 gene expression in breast cell lines in a tissue specific manner [34].

Genome integrity is directly compromised by the induction of double-strand breaks (DSBs) which are related to L1 ORF2 endonuclease activity. Expression of a transiently transfected L1 element in HeLa cells led to a at least tenfold greater induction of DSBs than the rate of L1 integration under the same transfection conditions and the damage was confirmed to be specific to the L1 endonuclease activity [35]. It is difficult to assess the significance this process might hold in vivo since L1 induced DSBs cannot be distinguished by the ones that arise through different mechanisms. On the other hand, inaccurate repair of DSB DNA lesions can lead to random mutations that further compromise genomic integrity [36, 37].

TEs can also affect host genome long after the integration process is completed. Genetic instability can result through non-allelic homologous recombination (NAHR), which it consists the main disease-causing mechanism for Alu elements. In fact, these post-insertional rearrangements pose a far greater risk to the integrity of the genome than the initial insertion event. Homologous recombination is a fundamental biological mechanism and is highly conserved along species. Normally, programmed homologous recombination occurs once during meiosis. However, the presence of abundant and highly homologous sequences in relatively close proximity, such as Alu elements, increases the potential for mutagenic NAHR. This process can result in genomic deletions, duplications, chromosomal inversions, interchromosomal translocations and NAHR events have been implicated in a number of human genetic diseases as they occur at appreciable rates [26, 38].

Another way in which TE activity can perturb normal gene regulation is by altering the epigenetic state at the site of integration. The recruitment of heterochromatin inducing factors that promote DNA methylation and subsequent histone deacetylation may influence the expression of genes that lie in the vicinity of the integration site, apart from suppressing TE element transcription. It has been reported that in humans, Alu elements are methylated in a more focused manner than LTR and L1 elements which provoke larger chromatin modifying effects [38, 39]. In accordance to the above, it has been suggested that L1 elements may play a role in X-inactivation in mammals. The strong presence of L1 elements in the X-chromosome has been proposed to lead to silencing of the intervening sequences through heterochromatization. Therefore LINE elements may act as modifiers of the epigenetic state of the mammalian genome [40].

Mechanisms Involved in the Regulation of TE Activity Within the Cells

TE mobilization has been clearly demonstrated to affect genome stability and function. Under this perspective, it is not surprising that the hosts have evolved several different mechanisms to limit and regulate TE activity (Fig. 5.1). In particular, it has been suggested through comprehensive analysis of the rate of expansion of L1 families that the most advanced mammalian species have the highest ability to restrict L1 expression [41]. These suppressive mechanisms can act on different stages of the TE amplification cycle, both on the transcriptional, as well as on post-transcriptional level. Of major importance, epigenetic regulation and the RNA interference (RNAi) pathway have also been adopted to limit the negative effects of retrotransposition.

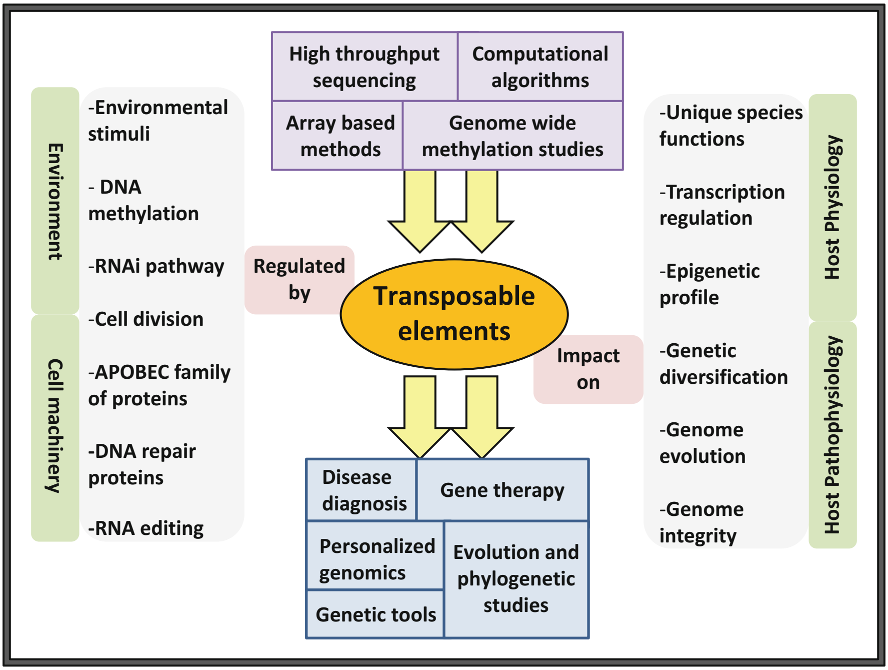

Fig. 5.1 Depiction of principal mechanisms that regulate transposable element activity and the variable ways it can affect host physiology and pathophysiology. In addition, current advancements in the field of DNA sequencing and computational analysis are expected to promote the exploitation of knowledge accumulated and lead to positive outcomes. Many of the concepts presented here are further elaborated on inside the text. *APOBEC* Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like; *RNAi* RNA interference pathway

DNA methylation is the best established mechanism that may influence heterochromatin formation. In mammals, DNA methylation occurs in cytosines embedded in CpG islands. TE promoters are normally transcriptionally-silent due to increased DNA methylation patterns and formation of repressive chromatin states. Actually, methylation measurements of L1 promoters indicate that they range from 20 to 100 % methylated [42]. Most Alus are highly methylated and consequently repressed in differentiated cells (but not in male germ-line cells) [43, 44]. In addition, SVA elements are found in extensively methylated states in most human tissues [45]. SVAs contain numerous CpGs in the VNTR domain and have been suggested to act as species-specific CpG islands [46]. It is noteworthy, that DNA methylation has been proposed to have evolved for the specific purpose of suppressing TE activity [47]. Apart from immediate transcriptional repression, DNA methylation can result in permanent inactivation by C→T deamination [48].

Mutations in genes that belong to the DNA methylation machinery invariably enhance TE transcription and result in reduced viability and fertility. Most representatively, mouse embryos that lack Dnmt1, a DNA methyltransferase, reactivate intracisternal A particles (IAPs), endogenous retroviral sequences representing an important class of TEs, and die before mid-gestation [49, 50]. Dnmt3L knock-out mice exhibit loss of methylation of L1 and LTR elements and a corresponding increase in the levels of expression of their RNA transcripts resulting in failure of the male germline [51]. The same situation exists for proteins that indirectly contribute to the execution of the DNA methylation pathway. Mutation of Lsh, a chromatin remodeling protein that makes DNA accessible to Dnmts, leads to hypomethylation and increase in transcription of IAPs as well as postnatal lethality in mouse embryonic cells [52].

Demethylation of CpG rich promoters is expected to result in enhancement of their activity and increased expression of the TEs. This holds true for several cancer cell lines where the hypomethylation status in these tumor cells has been correlated to increased L1 transcription [53, 54]. This situation is also depicted in the case of chronic myeloid leukemia (CML) where the hypomethylation of L1 elements has been implicated with disease progression [55]. One should bear in mind that demethylation of potent TE promoters has possible implications beyond the activation of retrotransposition. This way, transcription factor balance may be disturbed or transcriptional activation may expand to include genes in the vicinity of demethylated promoters [56]. Activation of the antisense promoter that is located in the 5′ UTR of L1 elements is another possible concern. Interestingly, treatment with azacytidine and decitabine, two hypomethylating agents, has been shown to alter the expression of c-Met oncogene in colon carcinoma and myeloid leukemia cells. The cause of this effect is demethylation of an antisense promoter located in a L1 element in the second intron of the c-Met gene [57]. Histone acetylation on the other hand and its impact on suppressing TE activity is less straightforward and understood so far. It has been reported though that in some cell types after the insertion of engineered L1s the surrounding chromatin is rapidly deacetylated [58].

Of equal importance, the RNAi pathway is presented as a powerful mechanism to control TE activity. RNAi involves small non-coding RNAs of different classes that guide the RNA-induced silencing complex to degrade target transcripts through homology based recognition. The effector complex is made-up of PIWI/Argonaute proteins with RNase H-like activity that can slice single-stranded nucleic acids. The PIWI/Argonaute protein family is subdivided into AGO (Argonaute) families which can bind to small interfering RNAs (siRNAs), microRNAs (miRNAs) in many tissues and PIWI families which can bind to the germ cell specific Piwi-interacting RNAs (piRNAs). Along with DNA methylation, these discrete mechanisms seem to overlap instead of only acting independently in favor of the host genome defense. It has been proposed that miRNAs may direct methylation at the L1 promoter this way maintaining L1 DNA methylation [59]. In addition, mutant male mice for either Mili or Miwi2, two of the PIWI proteins, display loss of DNA methylation at retrotransposon loci and an increase in retrotransposon mRNA expression leading to small testes and sterility [60, 61]. The piRNA pathway appears to hold a dual role in male germline restricting TE activity both by post-transcriptional degradation and by transcriptional silencing through DNA methylation [48, 61, 62].

The female germline differs greatly in that Mili or Miwi2 mutations, even when combined, do not lead to activation of TEs and remain fertile [48]. Alternative mechanisms including the siRNA pathway control TE expression in this case. Although siRNAs were thought to arise primarily from exogenous sources it seems that endogenously produced siRNAs (endo-siRNAs) play a role in restraining TE mobilization. Profiling of the total small RNA population in mouse oocytes uncovered a broad class of endo-siRNAs that was previously unrecognized. This class derived from retroelements including LINE, SINE and LTR retrotransposons and it was suggested that retrotransposons are suppressed through the RNAi pathway in mouse oocytes [63]. siRNAs containing L1, IAP and Mouse Transcript (MT) sequences are produced in oocytes though it has been proposed that they mainly target genes bearing TE repeats in their 3′ UTR region instead of TEs themselves [64]. The control of transposon activity by endo-siRNAs is a well characterized mechanism in *Drosophila* both in somatic tissues and in the germline [65].

The host response and capacity of regulating TE activity is of course multifactorial and reflects the need for accurate tuning and preservation of a critical balance. Epigenetic regulation as well as the RNAi pathway emerged as valuable controlling mechanisms to this end. Several other mechanisms also facilitate the process of TE restraining. To mention a few RNA editing, DNA repair proteins and the APOBEC family of proteins have been shown to modulate TE activity (reviewed in [66]).

Transposable Element Functions in Physiology and Human Pathophysiology

The fact that TE activity can have implications on human disease has been broadly demonstrated and viewed with great interest [24, 26, 67–69]. This way, the ultimate effect of a new transposition event can be neutral, beneficial or harmful to the host, with the propensity of the last to be more easily detected and further examined. Despite the noted effect on creating genetic instability and contributing to human disease pathogenesis, there are numerous examples described in which TEs have had a positive impact on the host physiology resulting occasionally in a favorable phenotype. As an example, a L1-induced transposition event of the cyclophylin gene into the TRIM5 gene is the causative reason owl-monkeys exhibit resistance to human immunodeficiency virus (HIV) 1 infection [70]. Additionally, an Alu insertion polymorphism gene has been associated with protection from dry/atrophic form of age-related macular degeneration [71]. In certain cases host genomes have managed to even exploit TE-encoded proteins in order to support useful functions. This seems to be the case with RAG-1 protein, which is involved in the V(D)J recombination process during antibody production and probably emerged from a DNA transposase [72].

On the other hand, several intricate ways in which TE activity has affected human pathophysiology have been also well documented. The list of human diseases attributed to transposable element mobilization is ever increasing since the initial discovery of a L1 insertion in exon 14 of the factor VIII gene in two unrelated patients with hemophilia A [73]. Approximately a hundred retroelements insertions that cause a wide range of diseases have been recorded accounting for 1/250 (0.4 %) of all disease-causing mutations in human [69]. Numerous examples of all currently mobilizing elements in the human genome (L1, Alu and SVA) exist where de novo insertion led to manifestation of disease. L1 insertions have been reported to result in β-thalassemia, colon cancer and most recently to neurofibromatosis (NF) type I disorder (also known as von Recklinghausen disease) [74]. Alu insertions have in many cases disrupted genes residing on the X chromosome resulting this way in a wide range of human disease (see review article [8]). Interestingly, L1, Alu and SVA elements exhibit a different retrotransposition rate per live births (1/212 for L1, 1/21 for Alu and 1/916 for SVA) that so far coincides with the number of disease-causing mutations attributed to each type of TE (25 for L1, 60 for Alu and 7 for SVA) [8, 16]. In general, the calculated number of retrotransposition events causing monogenic (single-gene involvement) disease has been recently estimated at 500/year [8]. Advancements in whole-genome scale approaches such as high throughput sequencing or novel microarray-based methods are expected to contribute to better accuracy in measurements and thus more insight in the investigation of TE role in human structural variation and disease pathogenesis at the molecular level.

Exploitation of TE Activity for Therapeutic Applications

As an interesting twist to their well-established role in human pathogenesis, TEs have emerged as useful tools for insertional mutagenesis, germline *trans*-genesis, functional genomics applications, and, most importantly, for gene therapy efforts (reviewed in [66]) (Fig. 5.1). In fact, the latest generation in transposon technology has been stratified in a stressful attempt to develop non-viral gene delivery approaches, thus circumventing the problems faced with traditional viral vectors and overcoming the weaknesses that most non-viral vectors exhibit when it comes to gene transduction experimentation studies.

Effective gene therapy involves the process of transducing the gene into the target cells efficiently, succeeding long term expression and also avoiding possible secondary effects such as immune reactions or transformed cell growth. Insertional mutagenesis resulting from the use of integrating viral vectors is a major setback and most non-viral vectors are unable to achieve high and stable expression of the therapeutic gene, highlighting the need for safe and efficient alternatives [75].

DNA transposons replicate through a “cut and paste” mechanism getting excised from a locus and subsequently integrated into another by the transposase protein they encode. The transposase can also act *in trans* on practically any DNA fragment that is flanked by the terminal repeat sequences present at each end of the transposon. This really attractive property led to the development of alternative gene delivery systems in which the DNA sequence of interest can be placed between the transposon terminal repeats and the transposase is supplied in the form of an expression plasmid or mRNA synthesized in vitro.

The fact that DNA transposons are inactive in vertebrates was overcome by genetic engineering in order that these elements obtain the capacity to transpose in mammalian tissues. The transposons Sleeping Beauty (SB), reconstructed from a Tc1/*mariner*-type element, and Piggyback (PB) originating from the cabbage looper *Trichoplusia ni*, demonstrate transposition activity in a wide variety of vertebrate cell lines and species including humans [76]. In a further effort to promote human gene therapy applications, SB100X, a novel hyperactive SB transposase was recently developed through mutations and modifications of the originally reconstructed SB enzyme. The result was a up to 100 times enhancement of its transpositional activity in HeLa cells [77]. In fact, the efficiency in *trans*-gene delivery reaches those of viral vectors and this robustness is extremely useful in applications such as the transfection of primary and other hard to transfect cell types. The SB100X system was able to support 35–50 % stable gene transfer in $CD34^+$ cells enriched in hematopoietic stem or progenitor cells. The gene-marked $CD34^+$ cell population was further transplanted to immunodeficient mice and resulted in long-term engraftment and hematopoietic reconstitution [77].

The interest in applying TE-based systems for effective gene delivery is constantly increasing. These efforts are trying to approach the treatment of several conditions as is junctional epidermolysis bullosa [78], type 1 tyrosinemia [79], hemophilia A and B [80, 81]. Most importantly, the “DNA Advisory Committee” approved in 2008 the first human gene therapy clinical trial that is based on the use of transposons [82]. The aim is to genetically modify T cells with the use of SB transposons in an attempt to treat patients with $CD19^+$ B-lymphoid malignancies. T cells will be co-transfected with a transposon encoding a chimeric antigen receptor (CAR) to enable T cells to recognize lineage-specific tumor antigen, such as CD19, along with a construct expressing an early generation hyperactive SB transposase. The outcome and promise of this effort is eagerly anticipated.

Erythroid Maturation Program of MEL Cells: Lessons Learned from the Blockade of Differentiation by Methylation Inhibitors and the Activation of TEs

Previous work from our laboratory has indicated that the induction of haemoglobin synthesis and terminal erythroid maturation of murine erythroleukemia (MEL) cells in vitro is associated with changes in methylation of RNA species, as well as with alterations in the intracellular concentration of intermediates involved in the active methylation cycle [83–85]. Such a conclusion has gained further support by the fact that N^6-methyladenosine (N^6mAdo) has inhibited commitment of MEL cells to terminal maturation through its intracellular conversion into S-N^6-methyladenosylhomocysteine (N^6-SAH), an active intermediate that affects methylation of RNA and DNA [86]. It has been interesting then to observe that MEL cells exposed to chemical inducers and simultaneously treated with methylation inhibitors (DNA/RNA methylation blockers) although they express the β^{major}

globin gene, however, they: (a) exhibit a blockade to terminal erythroid maturation and (b) produce relatively short polyA$^-$ (non-polyA tail) RNA transcripts accumulated in the cytoplasm that share significant structural homology with 3′-end downstream β^{major} globin gene DNA sequences including the B1 retrotransposon element located within this region (designated as B1-559; see Fig. 5.2) [85, 87, 88]. It is noteworthy to emphasize that B1 repeat element has been found to be located in a DNA region where several consensus binding sequences exist for the erythroid-specific transcription factors GATA-1, AP1/NF-E2 and EKLF. Recently moving toward elucidating the potential functionality of B1-559, we have published data showing that this locus has been: (1) a candidate region encoding the core structure of some of the cloned non-polyA tail RNA species accumulated mainly in the cytoplasm of MEL cells treated with methylation inhibitors; and (2) capable to recruit transcription factors and drive the expression of luciferase gene in cooperation with DNase I hypersensitive site 2 (HS2) derived from locus control region (LCR) of human β-globin gene cluster [89, 90]. Such a direction has been further supported by earlier reports indicated the involvement of Alu-like repetitive sequences in the coordinated post-transcriptional control of gene expression [91], while non-globin DNA sequences located in large distance from the Gγ-globin and other globin genes were found to be potentially homologous to an RNA polymerase III template [92].

In our case, the fact that the B1-559 DNA fragment exhibited potential promoter-like activity in cooperation with HS2 tend to suggest that the identified three consensus sequences for GATA-1, AP1/NF-E2 and EKLF located within this fragment (see Fig. 5.2a) may contribute to transcriptional activation mechanisms. It has been proposed, based on these data, that B1-559 exerts a potential transcriptional role in this part of the genome and thus may affect the expression pattern of β^{major} gene during development, as shown in Fig. 5.2b, by cooperating with enhancer sequences previously known to serve major roles [93–95]. To this end, recent findings implicate the involvement of B1 repeat family in complex regulatory elements in the control of gene expression in the mouse genome. In particular, insertion of a B1 in the 3′-untranslated region of the rabbit β-globin gene generated a construct that conferred transcriptional regulation of this recombinant gene upon its transfection into the cells [91]. Such transcriptional activation of B1-559 also coincides with data implicating B1 retrotransposon elements in the recruitment of transcription factors like PAX6 at discrete binding sites throughout the mouse genome [96]. Moreover, a novel abundant NF-κB-binding site residing in specialized Alu-repetitive elements having the potential for long range transcription regulation has been recently identified by genome analysis [97]. Similar observations were also shown for L1s. P53 DNA binding sites have been detected within L1 which are involved in the regulation of their expression in the mouse genome [98]. Indeed, the transcriptional activation of L1 elements appears to be closely related with hypomethylation of their promoter region, whereas such activity of L1 has been recently shown to contribute to the progression and clinical behavior of chronic myeloid leukemia (CML) [55]. Overall, such functions of TEs to regulate gene activation or silencing represent processes of great interest that are under scrutiny [55, 99]. To this regard, an additional diverse role of TEs in the mouse and human genome was

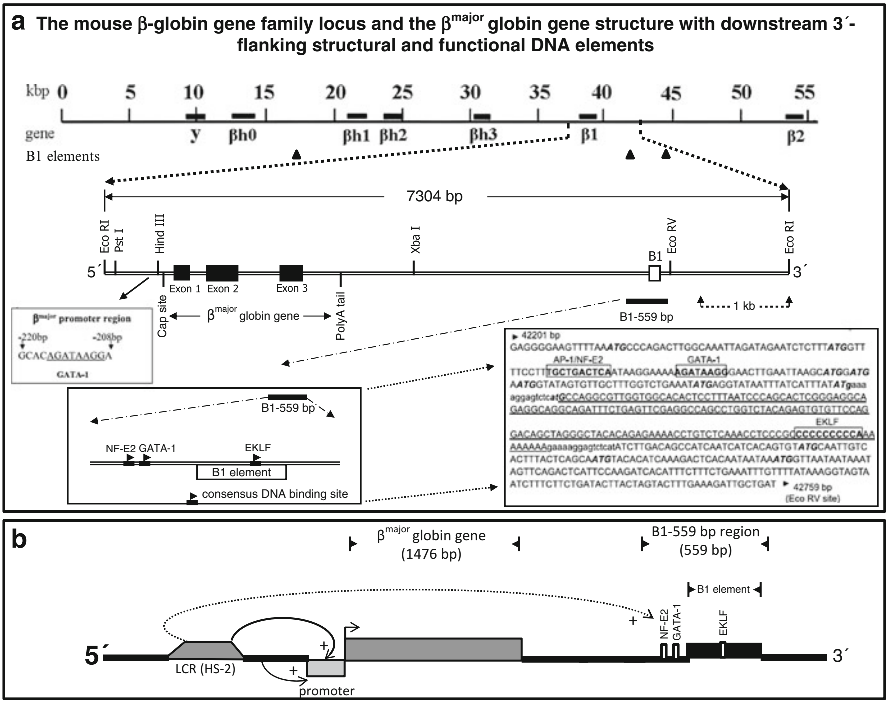
a The mouse β-globin gene family locus and the βmajor globin gene structure with downstream 3´-flanking structural and functional DNA elements
kbp 0 5 10 15 20 25 30 35 40 45 50 55
gene y βh0 βh1 βh2 βh3 β1 β2
B1 elements
7304 bp
Eco RI
Pst I
Hind III
Xba I
Eco RV
Eco RI
B1
5´
3´
Exon 1
Exon 2
Exon 3
Cap site
βmajor globin gene
PolyA tail
B1-559 bp
1 kb
βmajor promoter region
-220bp -208bp
GCACAGATAAGGA
GATA-1
B1-559 bp
NF-E2 GATA-1
EKLF
B1 element
consensus DNA binding site
42201 bp
GAGGGGAAGTTTTAAATGCCCAGACTTGGCAAATTAGATAGAATCTCTTTATGGTT
AP-1/NF-E2
GATA-1
TTCCTTTGCTGACTCAATAAGGAAAAAGATAAGGGAACTTGAATTAAGCATGGATG
AATGGTATAGTGTTGCTTTGGTCTGAAATATGAGGTATAATTTATCATTTATATgaaa
aggagtctcatGCCAGGCGTTGGTGGCACACTCCTTTAATCCCAGCACTCGGGAGGCA
GAGGCAGGCAGATTTCTGAGTTCGAGGCCAGCCTGGTCTACAGAGTGTGTTCCAG
EKLF
GACAGCTAGGGCTACACAGAGAAACCTGTCTCAAACCTCCCGCCCCCCCCCCAAA
AAAAAAgaaaaggagtctcatATCTTGACAGCCATCAATCATCACAGTGTATGCAATTGTC
ACTTTACTCAGCAATGTACACATCAAAGACTCACAATAATAAATGGTTAATAATAAAT
AGTTCAGACTCATTCCAAGATCACATTTCTTTCTGAAATTTGTTTTATAAAGGTAGTA
ATCTTTCTTCTGATACTTACTAGTACTTTGAAAGATTGCTGAT
42759 bp
(Eco RV site)
b
βmajor globin gene
(1476 bp)
B1-559 bp region
(559 bp)
B1 element
+
NF-E2
GATA-1
EKLF
5´
3´
+
+
LCR (HS-2)
promoter

Fig. 5.2 Diagrammatic outline of mouse β-globin gene family locus along with unique structural and functional DNA sequences located within the 3′-flanking sequences of β^{major} globin gene with potential cooperativity to distal enhancer elements to affect transcription. *Panel A*: The depiction of the DNA locus of mouse β-globin gene family is shown at the top along with the B1 repetitive elements identified (shown as *solid triangles*). Moreover, the structural analysis of sequences located at the 3′-flanking region of β^{major} globin gene is indicated with the presence of several consensus sequences for the binding of the NF-E2, GATA-1 and EKLF transcription factors. In addition, this area has several ATG initiation codons (shown in *bold* and *italics*) and a B1 element whose sequence is *underlined*. Finally, the GATA-1 consensus sequence known to exist within the promoter region of β^{major} globin is also illustrated. For more details see [87, 108]. *Panel B*: The influence of close and distant DNA elements on the expression of β^{major} globin gene is presented where besides the known positive cooperative effect of LCR sequences with promoter sequences is well known, it is proposed also a cooperation between the B1 element containing region (B1-559), with LCR to affect β^{major} globin gene transcription based on recent data published from our laboratory shown in [89]. For more details see [89, 90]. (This figure is based on data published from our laboratory and others as shown in [87, 89, 90, 108])

further outlined. In particular, it has been documented that the presence of Alu elements within the 3′-UTR region of genes may lead to their targeted silencing [100]. Furthermore, the creation of two isoforms of rodent natural killer (NK) cell-activating receptor NKG2D gene seems to be driven by a B1 retrotransposon insertion leading to gene regulatory change and its final functional diversification [101]. And consistent with these results, novel transcripts of the human neuronal apoptosis inhibitory protein (NAIP) gene has been shown to arise from transcription start sites that initiate within an Alu retrotransposon element generating RNA transcripts that may have novel functions intracellularly [102].

The impact of a potential cooperation between enhancer (HS2) and B1-559, as proposed in Fig. 5.2b, is very interesting and remains to be further proved. However, the possibility that HS2 distant elements and DNA domains like B1-559 exert a regulatory effect on β^{major} globin gene expression via a "trans-regulated circuit" in hematopoietic cells upon development and under the influence of DNA methylation state is quite challenging. Alternatively, TEs may serve to complex transcriptional switches in eukaryotic systems. One such example applies to the activation of interferon-beta (IFN-β) transcription which has been uncovered to be a highly ordered process beginning with the delivery of NF-kB to the IFN-β enhancer through a process involving stochastic interchromosomal interactions between the IFN-β enhancer and specialized Alu elements [103, 104]. In any case, however, all the above mentioned examples support the hypothesis that TEs in the mammalian genomes might be considered as modulatory elements in transcriptional regulatory circuits (TRCs) ensuring coordinated expression of genes organized in transcriptional units like globin genes and many others.

New Concepts Regarding the Usefulness of "Junk DNA" Toward the Clinical Exploitation of Personal Genome Variations

A few years ago, in the first comprehensive whole-genome analysis of mobile elements-related structural variants of an individual, it has been demonstrated that TEs play an important role in generating interindividual structural variability by estimating the Alu, L1 and SVA retrotransposition rates to be one in 21 births, 212 births, and 916 births, respectively [16]. Now, it is a fortune that very recently a vast amount of functional data from the "ENCODE Project Consortium" (The Encyclopedia of DNA Elements; ENCODE) have been released related to the regulation and function of human and other complex genomes (see at: http://genome.ucsc.edu/ENCODE/ and www.nature.com/encode). It is interesting to note that these data provide new insights into genetic variability patterns seen in individuals and populations especially in terms of 'junk DNA" structure by providing evidence that ~80 % of the human genome serves some function [105–107]. As it has been shown, many previously clinically-validated DNA variants are located outside of the exome and within or very close to intergenic regions and other non-coding functional DNA elements. As a consequence, by also including in genome-wide association studies the variations seen in "junk DNA" could provide new ways on how to more efficiently achieve the clinical translation of genomic information to link specific genetic polymorphisms with disease etiology and progression profiles. Such new genetic information impinge on the regulation of complex mechanisms involved in human genome function which in turn may contribute to molecular pathophysiology mechanisms. To this end, the "junk DNA" functionality in DNA replication, transcription and translation machineries seems to contribute in interindividual variability, more than previously believed, and this in turn may affect the way by which the clinical exploitation of the generated knowledge could be enhanced. By including such information in clinical genomic studies, it is expected to enrich and strengthen our translational medicine capabilities and achieve major benefits in therapeutics for all patients worldwide.

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Chapter 6
Copy Number Variation in Human Health, Disease and Evolution

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Introduction

The most widespread type of variation in the human genome is Single Nucleotide Polymorphisms (SNPs). Therefore until recently the basis of studying genome variability and disease pathogenicity was mainly focused on SNPs. However, the advancement of genome-wide technologies that allow comprehensive screening of the entire genome has enabled the identification of another important abundant form of genetic variation in the human genome that of structural variation. These studies have also shown that the extent of structural variation is much greater than was previously anticipated [1].

Structural Variation (SV) is a collective term for a group of genomic alterations that change the structure but not the sequence of the genome. SVs include quantitative changes such as Copy Number Variations (deletions and duplications), positional changes such as translocations and changes in terms of sequence orientation such as inversions. Copy Number Variations (CNVs) represent a large category of structural variation and have been defined as a segment of DNA that is larger than 1 kb and present at a variable copy number in comparison to a reference genome [2].

CNVs can be generally divided into two major categories based on their frequency. The first category includes CNVs which are common in the general population with an overall frequency higher than 1 %. These copy number changes are also

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referred as CNPs (Copy Number Polymorphism). Common CNVs (CNPs) are usually small, typically they are less than 10 kb and can have no phenotypic effect on an individual or they might be associated with susceptibility to complex genetic diseases such as psoriasis and Crohn's disease [3, 4].

The second category involves CNVs that are rare in the general population. These CNVs are typically much larger in size than the common CNVs and consequently have a much larger risk of involving dosage-sensitive genes resulting in a phenotypic effect. The pathological conditions caused by genomic rearrangements (deletions/duplications, inversions, insertions and translocations) are collectively defined as genomic disorders [5].

CNV are found in the genomes of all individuals, are wide-spread across the genome, and include both inherited and de novo CNVs [6]. CNVs are usually stable and can potentially be inherited. As the importance of the duplications and deletions that result in these variants is becoming apparent, cataloging them and assessing their frequencies is now an important goal. A continuously updated summary of CNVs can be found in The Database of Genomic Variants (http://projects.tcag.ca/variation/). In addition the Database of Chromosomal Imbalance and Phenotype in Humans using Ensembl Resources (DECIPHER; https://decipher.sanger.ac.uk/information) is cataloguing clinically relevant CNVs. Furthermore new genetic disorders/syndromes caused by CNVs are also catalogued in the Online Mendelian Inheritance in Man (OMIM, www.omim.org) database.

Mechanisms of CNV Formation

Three major types of mechanisms have been proposed for the formation of CNVs in the human genome namely, (a) Homologous Recombination Mechanism (HRM) with the major mechanism being Non-Allelic Homologous Recombination (NAHR), (b) Non-homologous recombination mechanism (NHRM) with the major mechanism being Non-homologous End joining (NHEJ) and (c) replication based mechanisms (RBM) with the major mechanism being Fork Stalling and Template Switching/mediated break-induced replication FoSTeS/MMBIR. HRM are mostly associated with recurrent CNVs with recurrent breakpoints and are found in regions of extensive homology (1–5 kb). Non-recurrent rare CNVs occur at regions with very limited homology, microhomology (2–15 bp) or even no homology at all and are mostly formed by NHRM and RBM. These mechanisms occur both in germ cells and somatic cells, where the rearrangements can be associated with genomic disorders and cancer, respectively.

Non-allelic Homologous Recombination (NAHR)

NAHR is the predominant molecular genetic mechanism responsible for recurrent genomic rearrangements, those that share a common size and show clustering of breakpoints. NAHR is mostly mediated by low-copy repeats (LCR)s also known as segmental duplications (SD) which are genomic fragments of high sequence identity (>95 %), usually 10–500 kb in size, which account for ~5 % of the human genome [7].

Meiotic recombination between non-allelic LCRs in direct orientation on the same chromosome results in deletions or reciprocal duplications of the genomic region located between them. The rearrangement breakpoints of NAHR tend to cluster within LCR, termed recombination hotspots.

Non-homologous End Joining (NHEJ)

Some simple non-recurrent rearrangements can occur via Non-homologous end joining (NHEJ) [8]. NHEJ is one of the two major mechanisms employed by the cell to repair double-stranded breaks and has been described in several organisms, from bacteria to mammals. Unlike NAHR, NHEJ does not require substrates with extended homology.

NHEJ involves four steps: detection of double-stranded DNA break, molecular bridging of both broken DNA ends, modification of the ends to make them compatible and ligatable, and the final ligation step.

Microhomology Mediated Replication Error Mechanisms

Recent studies of genomic disorders have shown that complex non-recurrent structural rearrangements, can be explained by replication-based human genomic rearrangement mechanisms such as FoSTeS/MMBIR (fork stalling and template switching/microhomology-mediated break-induced replication) [9, 10]. In these models, the DNA replication fork stalls or collapses, the lagging strand disengages from the original template and anneals to another replication fork in physical proximity, utilizing microhomology at the 3 Ά end, “priming” or reinitiating DNA synthesis [11].

Evolution of CNV

CNVs and Purifying Selection

Generally, CNVs seems to preferentially locate outside of genes and highly conserved elements. It is also quite logical that significantly lower proportion of deletions than duplications overlaps with disease-related genes and more generally with protein coding regions. But are we sure that this large pool of CNVs found in “junk” genetic regions are evolutionarily and phenotypically neutral? No, since these regions are usually understudied in genetic and genomic research. Even in the case of functionally neutral CNVs, this is a quite big pool of amplified DNA that it can potentially gain a role after sequential mutation and/or after change of environmental conditions. We must always have in mind that evolution is a very dynamic procedure and genetic regions that are currently under negative selection can have adaptive benefit/s under a different environment.

Nguyen et al. [12] were the first to support that purifying (negative) selection is the main evolutionary force on CNV regions, despite the fact that the same group gave evidence for the opposite (that positive selection directs CNVs) 2 years before [13]. They finally concluded that positive selection is the exception and not the rule. After separating available by then CNVs in four groups and analyzing each group under an evolutionary concept they concluded in very interesting results. In brief, their data supported a model of reduced purifying selection (Hill-Robertson interference) within copy number variable regions that are enriched in nonessential genes, allowing both the fixation of slightly deleterious substitutions and increased drift of CNV alleles [12]. At the same sense, Schuster-Böckler et al. [14], by analyzing all the available CNV human population database records they found that dosage-sensitive genes are under-represented in CNV regions. They concluded that this is a strong indication for action of negative selection on human CNV regions.

Berglund et al. [15], found evidence for negative selection acting on CNVs in dogs. By analyzing CNV regions in many canine breeds, they noticed that 98 % of them are observed in multiple breeds. CNVs that predicted to disrupt gene function were significantly less common than expected by chance. Their data supported the fact that purifying selection is a major factor acting on structural variation and shaping so the dog genome, concluding from this that many CNVs are "unwarranted" by evolution, even if found in "junk" DNA. These sequence features may have driven genome instability and chromosomal rearrangements throughout canine evolution [15]. In the same line of thinking, recent work by another group support the hypothesis that ohnologs (paralogous genes that have originated by whole-genome duplication) are overrepresented in pathogenic copy number mutations possibly because they include critical dosage-sensitive elements of the genome [16].

Zhou et al. [17] established a number of second-chromosome substitution lines in *Drosophila melanogaster* in order to uncover CNV characteristics when these are in homozygous state. They found that more than 70 % of the dosage-sensitive CNVs are recessive with undetectable effects on transcription in heterozygotes, this being supporting for negative selection effect. Gazave et al. [18] performed comparative genomic hybridizations in four primate species populations in order to reveal CNV frequencies and hotspots. They showed that CNVs fate has been possibly determined by selective pressures in different lineages, this resembling a kind of genomic divergent evolution. Evidence for purifying selection was stronger in gorilla CNVs overlapping genes, while positive selection appeared to have driven the final frequencies of structural variants in the orangutan lineage. In contrast, chimpanzees and bonobos present high levels of common structural polymorphism, which is indicative of relaxed purifying selection.

Gene Duplication and Positive Selection

Very early after CNVs discovery, evidence was found for positive selection acting on these genomic elements, within the modern human populations [13]. Those researchers supported that they found evidence for positive selection by analyzing a set of 627 human CNV regions, based on the fact that those CNVs were enriched in genes, particularly genes implicated in secretion and sensing of the environment.

Poptsova et al. [19] performed pathway enrichment analysis for genes found in CNVs in a collection of individuals with Caucasian, Asian or Yoruban descent, combining high-resolution array and sequencing data. The analysis suggested possible examples of positive selection, in pathways like NF-kB and MAPK signaling, and Alu/L1 retrotransposition factors. The authors believe that their results show that constitutional CNVs may modulate subtle pathway changes through specific pathway enzymes, which may become fixed in some populations [19].

Positive selection may result in pleiotropic effects for the genes in specific CNV regions. An interesting example is the 16p11.2 chromosomal region. Duplications or deletions at the chromosomal locus 16p11.2 have been implicated in microcephaly, autism [20], schizophrenia [21], epilepsy, and other neuropsychiatric disorders, as also in anorexia, underweight and obesity phenotypes [22]. Data show that genetic balance is sensitive at this region. As a result of this, energy balance, brain structure and IQ levels [23] can be easily disturbed by different kind of re-arrangements. Recently, animal model studies showed that the neurological phenotypes at 16p11.2 can be attributed on *KCTD13* gene dosage [24]. It would be interesting if evolutionary studies could be performed for this gene in order to understand better its selection and its role. Human lineage specific traits, like intelligence, may be related with *KCTD13* evolutionary history.

Below we will analyze the three major evolutionary fates of CNVs in genomes and consequently in populations, based on widely accepted models (Fig. 6.1). For a more detailed analysis on these models please read the review of Hahn [25]. Such genetic evolutionary phenomena are difficult to be directly observed since they are happening in the depth of macro-evolutionary time. Despite this, pieces of evidence are always there. Range of genetic diversity is something that can be directly measured and be compared within different species. Sudmant et al. [26] compared the diversity and rates of copy number and single nucleotide variation across the hominid phylogeny. They found a correlation between duplications and single nucleotide diversity, believing that this recapitulates greatly the phylogeny of apes. Duplications are redundant compared with deletions by 2.8-fold. The load of segregating duplications remains significantly higher in bonobos, Western chimpanzees, and Sumatran orangutans-populations that have experienced recent genetic bottlenecks ($P = 0.0014$, 0.02, and 0.0088, respectively). The authors conclude that demographic effects, such as bottlenecks, have contributed to larger and more gene-rich segments being deleted in the chimpanzee lineage and that this effect may have contributed to episodic bursts in CNV during hominid evolution.

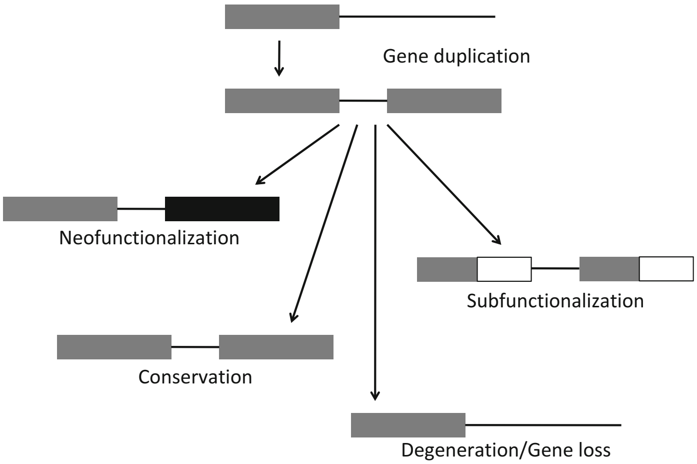

Fig. 6.1 The evolutionary fates of duplicate genes (see text for details)

Neofunctionalization

After the gene duplication, some of the new copy numbers may gain a novel function since they gradually accumulate mutations. This process is called neofunctionalization (Fig. 6.1). Of course, alternatively, if evolutionarily necessary, the new copies can preserve the function of the "maternal" copy for thousands of years (Fig. 6.1). New copies may serve as "back-ups" for this gene function or contribute to increased dosage, if this is required. It is believed that new duplication copies are often free from selective pressure. Thus duplicate genes accumulate mutations faster than a functional single-copy gene. This is why that some scientists believe that this process is the end stage for all subfunctionalized genes.

One characteristic example is the evolution of the antifreeze protein in the Antarctic zoarcid fish. In this case, type III antifreeze protein gene diverged from a paralogous copy of sialic acid synthase (SAS) gene. The ancestral SAS gene was found to have both sialic acid synthase and additional ice-binding properties, this witnessing that neofunctionalization happened. It is obvious here, that after duplication, one of the paralogs began to accumulate mutations that lead to the replacement of the SAS domains of the gene and allowing this way for the antifreeze property to arise. This specialization allows Antarctic zoarcid fish to survive in extreme low temperatures of the Antarctic Sea [27].

Subfunctionalization

Another possible fate for duplicate genes is that both copies are equally free to accumulate degenerative mutations, so long as any defects are complemented by the other copy. This leads to a phenomenon often termed as neutral "subfunctionalization" or DDC (duplication-degeneration-complementation) in which the functionality of the original gene is distributed among the two copies. Neither gene can be lost, as both can perform non-redundant functions, but ultimately neither is able to achieve novel functionality.

A usefulness of the subfunctionalization process is when a gene specializes among different tissues, developmental stages, or environmental conditions, so there is a need of many differently specialized copies. Isozymes are a good example of this phenomenon. These are encoded from paralogs that catalyze the same biochemical reaction, but have evolved a "catalytic freedom", catalyzing a spectrum of substrates in a variety of cell types. Human hemoglobin is an example of a subfunctionalization process, despite this is not a typical enzyme. The ancestral gene copy is undoubtedly a version of the beta globin gene. Through the subfunctionalization, new versions of the gene derived, like the alpha globin genes. Today, functional hemoglobin molecules are dimers of a number of available chains. For an analytical review on evolution of mammalian globin genes please read Storz et al. [28].

One other good example of segregation avoidance occurs in the acetylcholinesterase (AChE1) locus of the common mosquito, Culex pipiens. An allele of this gene has evolved to confer resistance to organophosphate pesticides. It has been found in heterozygote form as a separate duplicated locus in multiple mosquito populations (Hahn [25] and references therein).

Loss

Sometimes, genomic copy numbers can lead to deleterious increased gene expression such as Rett-like syndrome and Pelizaeus–Merzbacher disease [29]. Such pathogenic mutations are likely to be lost from the population and it is very unlikely to be established in the population and gain new functions. However, it is widely known that most duplications are in fact not damaging or beneficial. These neutral sequences can be increased or lost in the population randomly by genetic drift, this being an evolutionary process in molecular level that Kimura proposed for first time in 1962 (neutral evolution).

The Examples of the *DUF1220* and the Amylase Genes

DUF1220 domains' duplications are of great evolutionary interest in human speciation. DUF1220 protein domains have been duplicated many times in the human lineage probably exhibiting the most extreme human lineage–specific copy number increase of any protein coding region in the human genome [30, 31]. The majority of DUF1220 sequences are located at 1q21.1. Copy numbers at the chromosomal region 1q21 have been recently associated with neurological disorders and human cognitive functions as well. Neurological disorders that have been associated with this region are microcephaly, autism and schizophrenia. Teams of Dumas and Sikela showed that copy numbers of DUF1220 are highly expanded in humans, reduced in African great apes, further reduced in orangutan and Old World monkeys, represent only a single-copy in non-primate mammals, and are absent in non-mammalian species. Examination of expression in brain showed that neuron-specific DUF1220 signals are present in the cortical layers of the hippocampus and they are also abundant in neurons within the neocortex. Davis et al. [32] showed a positive correlation between the DUF1220 copy numbers and the human cognitive abilities in two independent populations of European descent, this being the first replicated association with these traits. The actual cognitive traits measured were the total IQ and mathematical aptitude scores. It is obvious through these and other studies, that expansion of DUF1220 copy numbers follow the expansion of the human brain size and the development of the higher cognitive functions in apes.

The α-amylase genes are located in a cluster on the chromosome that includes salivary amylase genes (*AMY1*), two pancreatic α-amylase genes (*AMY2A* and *AMY2B*) and a related pseudogene. The *AMY1* genes show extensive copy number variation which is directly proportional to the salivary α-amylase content in saliva. Perry et al. [33] published an exceptional paper examining the increase in copies of the salivary amylase gene in humans throughout evolutionary history. Perry and colleagues found that *AMY1* copy numbers started to increase 120,000 years ago, something that was catalytic for adaptation of humans to starch consumption. A most recent study found very strong evidence that *AMY1* copy number are associated with increased Body Mass Index (BMI) [change in BMI per estimated copy $=-0.15$ (0.02) kg/m^2; P $=6.93\times10^{-10}$] and obesity risk [odds ratio (OR) per estimated copy $=1.19$, 95 %, CI $=1.13-1.26$; P $=1.46\times10^{-10}$]. This is one of the many evolutionary examples where we observe a change of gene function after a sudden environmental change. Our genome has been evolved under very different environmental conditions than today and evolutionary time was not adequate to adopt in food plethora of present times. This is part of the answer why today we have an extreme increasing of diseases like obesity and diabetes.

Co-evolution of CNVs with MicroRNAs

Evolution of genomic elements must be always considered in relation with other genomic elements, especially the ones having a special regulatory role. This subchapter summarizes recent findings about co-evolution of CNVs with microRNAs, that it is discussed in more detail in Chap. 1.

Existing data show that tandem duplications can result in paralogous microRNA sequences that are located on the same transcript and are organized as tandem paralog clusters [34, 35]. In a recent study, evidence was found that repetitive elements contribute to creation of new microRNAs in mammals and that large segmental duplication events accelerate the expansion of microRNA families, including those derived from repetitive sequences [36]. The latter ones are considered as the younger microRNA genes, being also the less conserved. Similar evidence was found in plants where microRNAs that are found in repetitive elements tend to have longer hairpin precursor, lower G-C content in hairpin precursor sequences and lower minimum free energy [37].

An interesting model for the deriving of new microRNA genes has been proposed by Allen et al. [38], called the inverted duplication model. Under this hypothesis, new microRNA genes are generated from inverted duplication events happened on one of their target genes by forming two adjacent gene segments in either convergent or divergent orientation. Recent observations showed that many microRNA genes are found in transposable elements and pseudogenes. This was considered as an indication that these microRNAs have been derived through inverted duplications. Zhang et al. [35] confirmed further the inverted duplication model in plants, this happening via TEs or pseudogenes, showing also that inverted duplications give rise to microRNAs much more frequently that segmental duplications.

A crucial matter in microRNAs evolution is what factors determine the type and the number of microRNAs' targets in 3′ UTRs. Ha et al. [39] proved that small RNAs produced during interspecific mating or polyploidization serve as a buffer against the genomic shock in interspecific hybrids and allopolyploids. The authors came to this conclusion after studying allotetraploids coming from *A. thaliana* and *A. arenosa*, identifying adoptive alterations of the microRNAs and siRNAs levels in comparison with the parental species. Abrouk et al. [40] found evidence that the above mechanism may be a standard procedure in plants after euploidy, especially in euploidy events that are involved in evolutionary speciation.

But since whole genome duplications are very rare to animal species, research has been performed for duplications of smaller scale in such species. Li et al. [41] and D'Antonio and Ciccrelli [42] found that microRNA targets are significantly enriched for paralogs genes. Characteristically, Li et al. [41] mention that their results suggest that "microRNA-mediated regulation plays an important role in the regulatory circuits involving duplicated genes including adjusting imbalanced dosage effects of gene duplicates, and possibly creating a mechanism for genetic buffering". A more complicate analysis by Fernandez and Chen [43] revealed that human paralogs of poorly packed proteins (categorized so according special structural criteria) are more likely to be targeted by microRNAs, thus underscoring a means to buffer dosage imbalance effects arising from gene duplication.

Recently, our team proceeded to analysis of available data of public genetic databases and we found evidence that miRNAs and Copy Number Variations must have co-evolved and interacted in a way to maintain the balance of the dosage sensitive genes. Our findings raised the possibility that miRNAs may have been created under evolutionary pressure, as a mechanism for increasing the tolerance to genome plasticity [44, 45]. Our results were further confirmed by Woodwark and Bateman [46]. There is significant indication that a number of genes located within CNVs have altered expression level [9, 47–49]. This suggests further that microRNAs may have acted as equilibrators of gene expression during evolution in an attempt to regulate aberrant gene expression and to increase the tolerance to genome plasticity.

Methodologies for Detecting CNVs

The discovery of CNVs has been accelerated in the last years due to the advances in array-based methods and more recently Next Generation Sequencing (NGS) that allow comprehensive screening of the entire genome. In addition several other methodologies have been implemented that target defined loci such as Fluorescence In situ Hybridization (FISH), Quantitative Polymerase Chain Reaction (Q-PCR) or other probe based methods like Multiplex Ligation Probe Amplification (MLPA) and Multiplex Amplifiable Probe Hybridization (MAPH).

Whole Genome Approaches

Array-Based Methods

Two different array-based approaches are currently used for the detection and analysis of CNVs. The first approach is based on array-Comparative Genomic Hybridization (array-CGH) using differentially labelled reference and test samples which are co-hybridized on an array slide containing mapped DNA sequences which are spaced across the whole genome. This method was initially described in 1997 but has tremendously evolved over the years [50]. Today the most widely used DNA sequences for mapping DNA are BAC clones or short oligonucleotides [51, 52].

In array-CGH, the relative fluorescent intensities of two DNA samples (tagged with two different fluorophores respectively) hybridized to glass attached probes is measured. If there is more fluorescent signal from the sample under investigation relative to the control, then there is a gain of that specific genomic region, and if there is less signal relative to the control, then there is a loss of that DNA region. Gains and losses of clones are detected as a ratio that is plotted against the annotated genomic position (Fig. 6.2).

The resolution of array-CGH is based on the density, size and genomic distance of the probes. Currently commercially available arrays contain up to one million oligonucleotide probes (Agilent technologies, www.agilent.com).

The second approach utilizes the existing SNP (Single Nucleotide Polymorphism) genotyping platforms [53]. Although both approaches are quite similar there is a fundamental difference between the two approaches, as SNP arrays were initially designed for genotype and later validated for CNV detection. The difference between the two platforms are in the type of hybridization, comparative hybridization, comparing it to a reference sample versus single one colour hybridization following a subsequent comparison with a set of reference values from controls. In addition, genotyping arrays provide information on SNP genotypes. The resolution of the SNP array is based on the number of SNPs on the array. Currently commercially available SNP arrays contain well over one million SNPs.

In the last few years, SNP-CGH "hybrid arrays" combining properties from both platforms have been developed (CGH-SNP arrays) and include probes in regions with known copy number variation that do not contain SNPs (Affymetrix, www.affymetrix.com and Illumina, www.illumina.com).

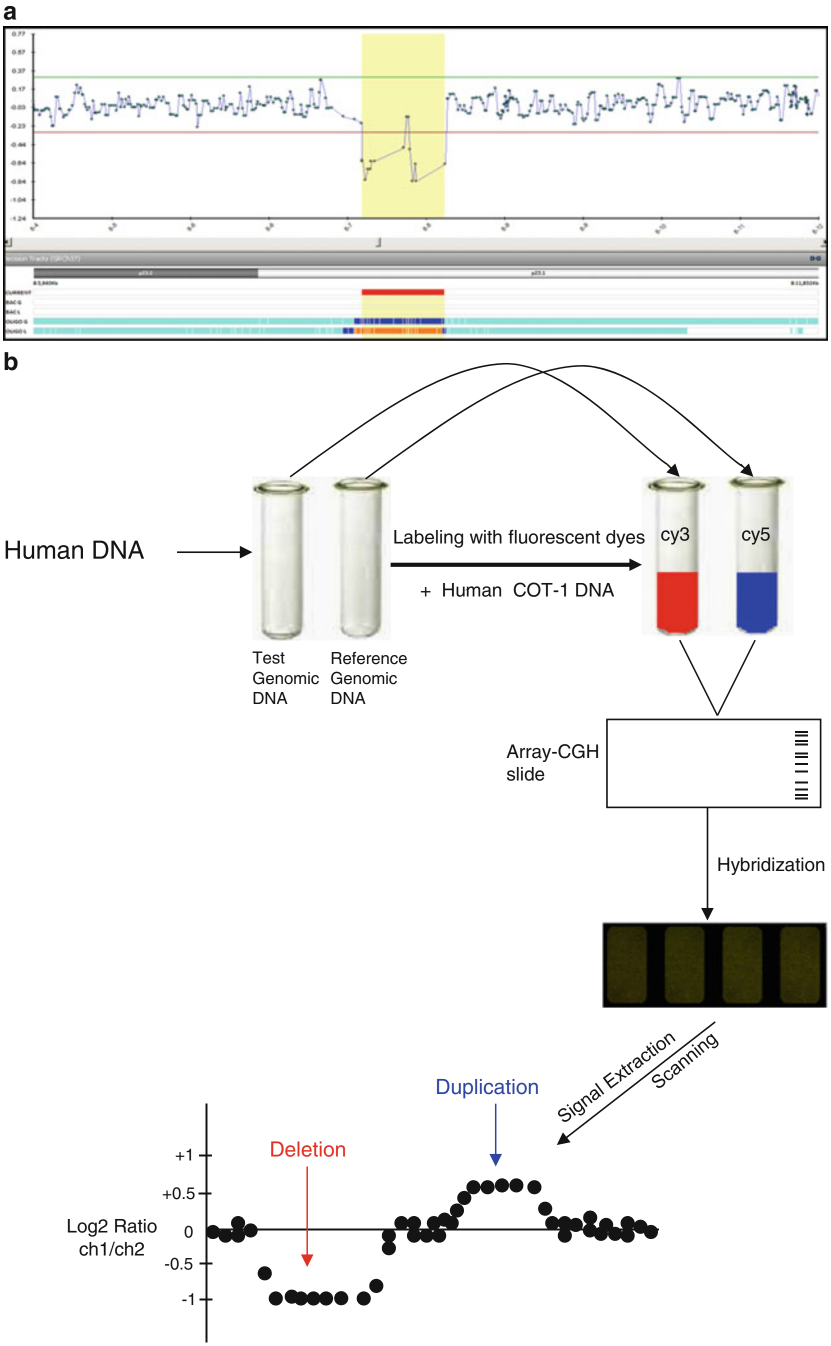

Fig. 6.2 (**a**) Illustration of a deletion (*highlighted*) of approximately 0.8 Mb in size on the short arm of chromosome 8 (q-arm) at chromosomal band 8p23.1 [(location: 7242978–8079890) using build GRCh37 (hg19)]. The current deletion lies within a highly polymorphic region as it is indicated shown by the *dark blue* for duplication and *orange* for deletion. The colors represent the recurrence of the same aberration regarding to our local database. (**b**) Array-CGH methodology for the identification of CNV

Next Generation Sequencing (NGS)

Traditionally, array-CGH is the golden standard for genome-wide method of choice detection of CNVs. However, as resolution of array-CGH is relatively low, higher than 10 kb, even for the ultrahigh resolution platforms of one million probes, smaller CNVs are still difficult to detect. In the last years, NGS, a high throughput sequencing technology has revolutionized the field of genetics [54]. Whole genome NGS has now emerged as a very promising tool for genome-wide detection of CNVs at much higher resolution than array-CGH high resolution (<10 kb) [55]. In addition, NGS has the benefit to detect any type of variation even down to the base pair level [56]. In NGS sequence assemblies from test and reference samples are compared computationally and differences in sequences, copy number and orientation are annotated. However as statistical approaches of NGS are still very limited in relation to CNV detection, further studies are needed prior to its routine implementation for the detection of CNVs.

Targeted/Locus Specific Approach

Fluorescence In Situ Hybridization (FISH)

FISH is a very well established method with a wide range of applications in cytogenetics. FISH is a targeted locus specific approach to detect CNVs. FISH employs fluorescently labeled probes that are hybridized on metaphase chromosomes from the test samples and hybridization patterns (absence/presence, duplication of signal) are compared to reference samples. However the low resolution (>100 kb) as well as the targeted nature of the method does not allow FISH to be implemented on large scale genome wide screenings.

Q-PCR

Real Time quantitative PCR (RT-PCR or Q-PCR) is another targeted strategy for the detection of CNVs. The basis of Q-PCR is that the rate of amplification of a region is proportionate to the number of template copies [57, 58]. Amplification (fluorescence signal representing the target) is then measured in real time during exponential phase and compared to a control region of known copy number, allowing the detection of copy number of the target region.

Probe Based Multiplex Assays

Multiplex Ligation-dependent Probe Amplification (MLPA) and Multiplex Amplifiable Probe Hybridization (MAPH) are able to detect abnormal copy numbers based on quantification of probes specifically designed for the regions of interest [59, 60]. Both methods are based on multiplex PCR methodology using fluorescently labeled universal primers and rely on probe amplification of different size probes rather than amplification of the genomic test DNA. Both methods can detect abnormal copy numbers for up to 50 regions simultaneously and hybridization ratios are compared with control regions. The main difference between the two methods is that MLPA is performed in solution and the probes are designed in two parts and are dependent on the presence of the targeted region for ligation and creation of a contiguous probe to be amplified. In MAPH the denatured test DNA is bound on a nylon membrane and then hybridized with a probe set which are subsequently amplified.

Again, the targeted nature of both methods do not allow implementation on large scale genome wide screening.

Effect of CNV on Human Variation and Disease

Mechanisms by Which CNV Affect Cell or Organism Phenotype

The most widely accepted mechanism by which CNV, whether residing in coding or "junk" DNA, can affect the phenotype of cells or organisms is by altering the expression levels of dosage-sensitive genes. For the majority of human genes modest increases or decreases in expression levels caused by CNV deletions or duplications will have minimal or no observable phenotypic effects. However, certain classes of genes are known to be particularly sensitive to dosage levels changes. The best established categories of dosage sensitive genes are those coding for structural proteins or members of protein complexes [61]. Theoretical and experimental data supports that modest changes in the dosage levels of such genes can disrupt protein–protein interactions and assembly of molecular complexes, thus impairing their biological functions. Similarly, modest changes in copy number of rate-limiting enzymes in metabolic pathways can also affect the output and function of metabolic and signalling pathways.

A straightforward mechanism by which CNV can affect gene dosage is by altering the number of gene copies in the genome. Studies have demonstrated that CNV deletions or duplications of protein-coding genes are in general positively correlated to changes in gene expression [48, 62]. Additionally, CNV located in non-coding regions of the DNA are also known to be able to affect expression of genes. One way this can be achieved is through the disruption of *cis* and *trans*-regulatory sequences (such as enhancers, insulators, and promoters), thus affecting the expression of genes that are under the regulation of these elements. It has also been determined that large CNV can affect the expression of neighbouring genes outside the affected region, as well as in genomic regions further away [63, 64]. Changes in the copy number of non-coding regulatory RNA genes (such as microRNA and long non-coding RNA) can also disrupt the physiological regulation of their target genes. For example CNV amplifications of miRNA will result in lower expression of their target genes, while conversely CNV deletions of the same miRNA gene will result in their increased expression.

Dramatic over-expression of individual genes can also result in prominent phenotypic effects which are not observable after modest changes in gene expression. For example the dramatic amplification of oncogenes such as tyrosine kinases, growth factor receptors, and cell growth genes—often to hundreds of copies per genome—can be a major driver of the process of carcinogenesis. Highly over-expressed proteins can also engage in promiscuous off-target molecular interactions, affecting phenotypes that are irrelevant to the normal functioning of the protein [65].

Complete loss-of-function can occur when CNV deletions unmask a recessive mutation or epigenetic aberration affecting the second allele. The complete loss-of-function of genes with non-redundant biological functions due to the combination of a CNV deletion with genetic or epigenetic lesion on the second allele can adversely affect the fitness of the organism. The aberration affecting the second allele can be another CNV, a point mutation or dysfunctional epigenetic regulation. For example, the coexistence of CNV deletions with point mutations affecting NRXN1 or CNTNAP2 genes has been found in patients with Pitt-Hopkins-like syndrome [66]. A single CNV deletion can also result in loss-of-function of genes if located in the X chromosome in males or when one allele is silenced by parental imprinting mechanisms.

Researchers have also suggested mechanisms by which large-scale CNV can influence organism fitness irrespective of the effects on the individual genes included within the affected genomic regions. A first proposed mechanism is that the simultaneous over-expression of a large number of proteins can act to overwhelm protein control mechanisms and proteasomal degradation, potentially impacting essential cellular functions [67]. A second proposed mechanism is that the large scale amplification of genomic regions containing repetitive elements acting as a drain on the cellular methyl pool, thus promoting DNA hypomethylation and associated genomic instability [68].

Contribution of CNV to Non-pathological Individual Variation

On average CNV affect around four million bases per genome, which is similar to the number of bases that differ between individuals due to single nucleotide polymorphisms (SNP) [69]. Consequently, CNV could be important contributors to the "normal", non-pathogenic phenotypic variability between humans. At present much less attention has been placed on CNV underlying non-pathogenic human traits, although some interesting links have been reported. One interesting case discussed previously relates to the salivary amylase gene ("The Examples of the *DUF1220* and the Amylase Genes" section above). In regards to polygenic complex human traits, studies have indicated that CNV are involved in the observed variability in intelligence [70, 71], height [72, 73], and even musical aptitude [74], although these remain highly debated.

Interpretation of the Pathogenic Significance of CNV

The demarcation of benign from pathogenic CNV is a challenging, but also an essential step for the accurate interpretation of clinical and research findings. Criteria used for the interpretation of CNV include its inheritance pattern, frequency in the population, genomic size of the CNV, and gene content. Classical evolutionary theory predicts that purifying selection acts to remove inherited CNV that are detrimental to the organism from the gene pool (see section "Evolution of CNV" for details). Indeed, CNV disrupting protein-coding genes are depleted amongst high frequency deletions [69], indicating the action of purifying selection. It can therefore be predicted based on evolutionary theory that an inverse association should exist between the penetrance of a given CNV and its frequency in the population. It is expected that common CNV are benign or have very low to moderate penetrance and contribute only modestly to disease risk, while pathogenic CNV of high penetrance are present at lower frequencies in the population. Determining whether a CNV found in a patient was of de novo origin or is inherited from a healthy parent and investigating the presence of the variant in databases of patient cohorts (e.g. DECIPHER decipher.sanger.ac.uk) or healthy controls (e.g. DGV dgv.tcag.ca) are important steps in CNV evaluation, especially for severe phenotypes.

CNV in Intellectual Disability/Developmental Delay and Microdeletion/Microduplication Syndromes

Among the earliest and best established pathogenic phenotypes linked to CNV are intellectual disability and developmental delay. It had been established for a long time by traditional karyotyping that chromosomal abnormalities are associated with intellectual disability and development delay. Over the past decade large case-control studies indicate that 15–20 % of ID and developmental delay cases are caused by CNV that were too small to be detected by traditional karyotyping [75, 76]. Screening for pathogenic CNV is now routinely used for clinical diagnosis of ID and developmental delay.

Microdeletions and microduplications have been recognized as a causative genetic factor of syndromic intellectual disability for many decades now. Typical examples include Prader–Willi (OMIM# 176270) and Angelman (OMIM# 105830) syndromes (15q11-q13 deletion), Williams–Beuren syndrome (7q11.23 deletion, OMIM# 194050), Smith–Magenis syndrome (deletion of 17p11.2, OMIM# 182290) and others. Prior to the implementation of genome wide array-CGH and the establishment of publically available databases, the identification of these syndromes was based on genotype-phenotype approach where a series of patients with similar recognizable clinical features were investigated and the genetic cause of the syndrome was subsequently discovered. However many syndromes have a wide range of clinical features, variability in expression and penetrance which hampered this genotype-phenotype approach. Implementation of high-resolution genome wide array-based approaches, in the last decade, has led to the identification of continuously growing number of recurrent microdeletion and microduplication syndromes caused by submicroscopic CNVs such as the 1p36 microdeletion syndrome, the 17q21.31 microdeletion syndrome and many others [77, 78]. Whole genome array-CGH investigation allows the identification of a similar genomic aberration in patients prior to common clinical presentation delineation. Today more than hundred new microdeletion/microduplication syndromes have been identified [79] using both approaches.

As most recurrent genomic rearrangements are mediated by sequences such as segmental duplications [80] and low copy repeats and are caused by NAHR it was expected that the implementation of array-CGH would lead to the identification of the reciprocal duplication of most of the previously identified recurrent microdeletion syndromes. Indeed, much reciprocal duplication was identified such as the 7q11.23 microduplication syndrome and the 22q11.2 microduplication syndrome and many others [81–83]. However the reciprocal duplication were identified for only a fraction of the microdeletion deletion syndromes mostly due to the fact that microduplications generally result in milder phenotypes or sometimes in no phenotype and consequently can escape detection.

Furthermore, the collection of clinical and genetic information in databases such as DECIPHER and other free databases has been crucial for discriminating between patients with rare aberrations and those with new microdeletion/duplication syndromes.

CNV in Psychiatric Diseases

It is now established that pathogenic CNV are important genetic contributors to the incidence of major psychiatric diseases such as autism, schizophrenia, and bipolar disorder. Particularly strong evidence supports causal associations between rare and de novo CNV and psychiatric diseases, with a number of studies reporting an enrichment of these CNV types in psychiatric patients compared to controls [84]. Beyond their enrichment in psychiatric patients, evidence that CNV are implicated in these diseases has also been gathered from animal models. One example is the display of autism-related behaviour in mice knockout for Cntnap2, a neuronal transmembrane protein implicated in autism [85]. In animal models CNV have also been demonstrated to cause similar anatomical abnormalities as observed in humans, for example 16p11.2 affecting brain growth (for details see “Gene Duplication and Positive Selection” section above) [20].

Ultimately, the CNV disruption of susceptibility genes will in some manner interfere with the physiological development of the brain. The strong links between CNV and psychiatric diseases possibly reflect the inherent sensitivity of neurodevelopmental pathways and neuronal structures involved in learning and memory to abnormal gene dosage. In accordance with this a significant number of established susceptibility genes affected by CNV are synaptic scaffolding proteins or regulators of the levels of synaptic proteins [86]. A significant confounding factor for interpreting the role of CNV in psychiatric diseases is their variable expressivity and incomplete penetrance [84]. Almost all CNV associated with psychiatric disorders are present at lower frequencies in neurotypical controls, while individual CNV are often associated with a number of psychiatric diseases. This phenotypic variability can be probably attributed to the interaction of the CNV with environmental factors and with other genetic factors in order to generate the pathological phenotype. A second issue is that recurrent CNV in psychiatric diseases are often very large, making it difficult to locate the pathogenic genes. An important challenge for researchers is going to be to elucidate how CNV cause the neurobiological abnormalities that eventually lead to the development of psychiatric diseases.

CNV in Cancer

CNV, of both inherited and de novo origin, have been associated with an increased disposition to cancer, prognosis of the disease, and response to therapies. Rare inherited CNV have been detected in nearly half the known cancer predisposition genes, potentially contributing to the incidence of human cancers [87, 88]. However, highly penetrant inherited CNV are believed to account for only a small fraction of familial cancers, which themselves constitute a minority of clinical cancer cases. Consequently, it is considered probable that CNV of low or moderate penetrance are the main contributors to hereditary cancer predisposition associated with CNV [88]. The identification of CNV of low or moderate penetrance associated with increased predisposition to cancer is a challenging task, requiring very large cohorts. Nevertheless, in recent years studies have reported a number of common CNV to be associated with increased disposition to various cancers, for example CNV in 20p13 [89], 2p24.3 [90], and CNV-67048 [91]. An interesting case of an inherited CNV deletion within a gene desert that was shown to be associated with a 1.3 odds ratio of pancreatic cancer is CNVR2966.1 at 6q13 [92]. The proposed mechanism by which this inherited CNV affects cancer risk is its ability to directly interact with the upstream sequence of *CDKN2B* and affect the expression of this known cancer gene. It is expected that efforts to identify inherited CNV associated with increased cancer risk will be an area of intensive research in the coming years.

The accumulation of somatic mutations in the form of aneuploidies, point mutations, and CNV is an essential aspect of carcinogenesis and neoplastic progression. Studies have demonstrated that somatic CNV are especially prominent across cancer genomes [93]. The high rate of de novo CNV formation in tumor tissue is in accordance with the genomic instability and high mutation rate that characterises cancer cells. Recently the largest study examining the patterns of somatic CNV across various cancer types has been published [94]. In this study the authors examined the copy number profiles of 4943 primary cancer specimens across 11 cancer types. The authors report patterns of somatic CNV that were common in the various cancers examined. One observation was the tendency for CNV to be longer and more frequent near the telomeres compared to CNV located internally, indicating that these are formed by different mechanisms. By analysing all cancer lineages this study identified 70 recurrently amplified and 70 recurrently deleted genomic regions, however only a minority of those regions contained known cancer genes. Another observation was that large duplications of genome (such as whole genome duplication) are associated with subsequent increases in somatic CNV within those regions. It is established that CNV disruptions of cancer genes contribute to tumorigenesis. However, a large number of somatic CNV detected in tumors are expected to be "passenger" mutations that arise during cancer evolution but do not contribute to the malignancy. The demarcation of driver from passenger CNV in tumors is a highly challenging task. The strongest case for somatic CNV contributing to cancer development can be made for those that recur at appreciable frequencies, indicating that they contribute to cancer development. However, recurrent CNV could also be explained in certain cases due to their location in fragile sites or due to absence of negative selection against them in evolving cancer genomes.

The classic Knudson's "two-hit" model considers that cancer progression requires the disruption of both alleles of tumor-suppressor genes. According to this model, a CNV deletion, either of germline or somatic origin, resulting in the loss-of-function of one copy of a tumor-suppressor gene is only adverse when the second functioning allele is also disrupted. The disruption of the second allele can be another CNV deletion, a point mutation or epigenetic dysregulation. Germline CNV deletions of tumor suppressor genes can increase the predisposition against cancer, as only a single mutational event that affects the second allele is required to complete the loss-of-function of the protein. Somatic CNV deletions of tumor suppressor genes such as Tp53 and retinoblastoma have been long established as important mutational events in the multi-step process of carcinogenesis, again requiring the disruption of second allele to allow cancer progression. Recently an alternative model to the "two-hit" model has been proposed to explain the recurrent focal regions of hemizygosity observed in cancer which do not contain known tumor-suppressor genes [95]. According to this model the synergistic haploinsufficiency of genes involved in repressing cell proliferation result in a proliferative advantage to cancer cells.

Unlike tumor-suppressor genes, oncogenes drive carcinogenesis through their increased activity. Somatic amplification of gene copy number of oncogenes has long being accepted to be an important carcinogenic mechanism. Classic examples include the amplification of Myc in Burkett's lymphoma and N-Myc in neuroblastoma. More recent studies have highlighted that CNV affecting regulatory elements can also drive oncogenic expression. A recent example of this is the finding of recurrent structural variants in medulloblastomas placing the *GLI1* family genes close to active enhancers, increasing their expression, which contributes to cancer progression [96]. Although less established than CNV affecting tumor suppressor genes, inherited CNV affecting oncogenes are also implicated in cancer. For example individuals with higher copy number of the g.CNV-30450 CNV located in the promoter region of the MAPKAPK2 oncogene resulting in greater expression of the protein, had greater incidence and worse prognosis of lung cancer [97].

Genomic instability is often observed in cancer. The increased rate of formation of new mutations is believed to be a driver of tumorigenesis by causing new carcinogenic mutations. Disruption of genes involved in DNA repair and maintenance can also lead to a greater mutation rate and susceptibility to cancer development. Subset of rare CNV associated with cancer, affect genes with genomic stability and DNA repair such as BRCA1, MSH2, and HRPT2 [88]. At least one example of a common CNV associated with greater mutation rates has been recently reported, affecting the cytidine deaminase APOBEC3B [98], with perhaps many more remaining to be identified.

CNV and Xenobiotics Metabolism

Humans possess highly specialised enzymatic machinery involved in the biochemical modification and excretion of substances that are of external origin. Inherited CNV affecting the number of copies of xenobiotics enzymes were amongst the first to be recognised and have been linked to individual susceptibility to environmental agents and to pharmaceuticals. These inherited CNV are also of particular interest due to their high degree of variability between individuals and ethnic populations.

Evidence supports that CNV affecting metabolic enzymes involved in the detoxification and removal of environmental carcinogens are associated with cancer incidence. The most extensively studied are CNV deletion polymorphisms affecting the *GSTM1* gene, which codes for an phase II metabolism enzyme involved in the glutathione-mediated reduction of electrophilic chemicals, which has been linked by molecular epidemiology studies to cancer susceptibility [99]. Another interesting case involved CNV affecting *CYP2A6*, an enzyme with a crucial function in nicotine metabolism and clearance. The number of copies of *CYP2A6* varies between ethnicities and has been associated with smoking behaviour and tobacco-related diseases [100].

CNV also affect the efficacy and side-effects associated with pharmaceutical treatments. *SULT1A1* is an enzyme catalyzing the sulphate conjugation of a wide variety of drugs. Deletions and duplications of this gene (ranging from one to five copies) are correlated with the hepatic activity of this enzyme, supporting the functional effects of CNV affecting this gene. Again, considerable inherited variability in copy number exists between populations, with 26 % of Caucasians vs. 63 % of African-Americans having three or more copies of *SULT1A1* [101]. Another important potential link is an association between the *CYP2D6* genotype and response to tamoxifen in postmenopausal women, although this is currently a matter of debate [102].

Other Diseases Reported to Be Affected by CNV

Beyond the more established contribution of CNV to intellectual disability, psychiatric diseases, and cancer, studies have indicated the contribution of CNV to a wide range of other human diseases. Examples include CNV linked to risk of osteoporosis [103], early-onset obesity [104], atherosclerosis [105], and Alzheimer's disease [106]. These will also be areas of intensive research in the future.

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Chapter 7
Mini- and Micro-Satellite Markers in Health, Disease and Evolution

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Abbreviations

CNS	Central nervous system
CNVs	Copy number variations
DSBs	Double strand breaks
FSHD	Facioscapulohumeral muscular dystrophy

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G4	G-quadruplex
HD or HTT	Huntington disease
HERVs	Human endogenous retroviruses
HNPCC	Hereditary non-polyposis colorectal cancer
HVR	Hypervariable
LINEs	Long interspersed elements
LP-BER	Long-patch base excision repair
LTRs	Long repetitive sequences
MD	Myotonic dystrophy
MMR	Mismatch repair
MSI	Microsatellite instability
NAHR	Non-allelic homologous recombination
Nt	Nucleotides
ORFs	Open reading frames
PABPs	Poly(A) binding proteins
SBMA	Spinobulbar muscular atrophy or Kennedy disease
SCA	Spinocerebellar ataxia
SCA8	Spinocerebellar ataxia type 8
SINEs	Short interspersed elements
SN-BER	Single nucleotide-base excision repair
SNPs	Single nucleotide polymorphisms
SSRs	Simple sequence repeats or STRs: short tandem repeats
SVAs	SINE-VNTR-ALUs
TEs	Transposable elements
TNR	Trinucleotide repeat
TREDs	Trinucleotide repeat expansion disorders
TRs	Tandem repeats
VNTRs	Variable number tandem repeats

Introduction

DNA repeats are common in eukaryotic and in prokaryotic organisms classified into two main categories. The first category is represented by the interspersed repeats, so-called because they are consisted of repeated units which contain distinct remnants of transposons [1, 2]. Interspersed repeats are quantitatively the most abundant sequences of repeated motifs, occupying approximately 45 % in humans and 33 % in mice, explaining to a certain extent the degree of variation in genome size between different organisms [2]. The second category includes the tandem repeats (TRs) that occur in low complexity DNA when a steady pattern of one, two or many more nucleotides is repeated and the repetitions are not only sequential but also directly adjacent to each other [1]. Because TRs were initially identified as satellite bands in density-gradient centrifugal separations of genomic DNA, the name satellite DNA has been widely used in reference to TRs, although there are marked differences between satellites and TRs. TRs are further stratified in the (a) microsatellites or Short Tandem repeats (STR) the (b) minisatellites or Variable Number Tandem Repeats (VNTRs) and (c) the recently identified megasatellites [3]. Tandem repeats constitute about 8 % of the human genome while only microsatellites comprise approximately 3 % of the sequenced human genome [4]. The sequence CAGCAGCAGCAG, for example, is a short TR, where the repeated unit is the tri-nucleotide CAG sequence, and in this example is repeated four times.

In tandem repetitive DNA sequences in the human genome, in particular micro-satellites and minisatellites, differ from the other repetitive DNAs such as, satellite DNA, telomeres, transposable and retrotransposable sequences and high copy number genes in many ways [5, 6]. In contrast to satellite DNA, as for example alpha satellite DNA, which is found in heterochromatic regions of the chromosomes, minisatellites and microsatellites are generally found in euchromatin [7]. Telomeres, although by definition have a repeat length similar to microsatellites, are unlike to TRs constituted of invariable repetitive sequences of (TTAGGG)n, that are specific to the chromosome tips and responsible for protection from chromosome fusion and rearrangement [8–10]. In contrast also to transposable and retrotransposable sequences, the TRs do not propagate themselves in the genome by a cut and paste or a copy and paste mechanism, but they rather originate from endless cycles of DNA recombination, replication and repair due to their contribution and participation in all these processes [11].

Unlike also to high copy number genes, that are, in a sense, repetitive complex sequences, the TRs are not transcribed autonomously, but they may harbor regulatory elements or extend in exons or introns and interfere with protein synthesis [12].

Tandem Repeats (Micro-, Mini- and Mega-Satellite) Sequence Definitions

TRs are classified according to their size in repeats with units less than nine nucleotides (nt) in length, which are known as microsatellites, or also as simple sequence repeats (SSRs), or short tandem repeats (STRs), and those with units of 10 nt or greater in length that are known as minisatellites [1]. It has been suggested that TRs with enormously long units, greater than 135 nt, comprise a separate class of repeats termed megasatellites [13]. They can be further classified into perfect or imperfect repeats (also called exact or approximate repeats) depending on whether they are precise copies or differ by ≥1 bp due to mismatch mutations, insertions or deletions. Microsatellites are the most prevalent and are almost perfect repeats [3, 14].

Since there was no direct association between the content in DNA repeats of a living organism with its evolutionary history, the repeats were initially regarded as having no biological effect. For this reason they were referred to as non-functional junk or selfish DNA during the first decades in the advent of DNA research [15–17].

Microsatellite DNA

STRs are in general less frequent than SNPS. According to "The 1000 Genomes Project Consortium 2012" there are approximately 700,000 STR loci identified in individual genomes tested at the first phase of the 1000 genome Project. In contrast 3.8 million SNPs were identified in 270 individuals and catalogued by the International HapMap Consortium, by October 2007. Consequently in an approximated frequency of 1SNP per 1000 genome bases, STRs are estimated to less than 1 per 5000 bases.

Microsatellite DNA is formed of much smaller units than any other satellite, with only **1** to 9 bp and they can be found in both coding and non-coding regions. The vast majority of microsatellites have 2 bp repeats the so called dinucleotide repeats, being most frequently (CA)n, followed by (AT)n, (GA)n and (GC)n, with the last combination of repeat being rare [14] or 3 bp repeats consisting either a codon, as for example most frequently (CAG), coding for glutamine, or other triplets without coding properties [14, 18].

There is evidence that the regional variation in microsatellite frequency cannot be explained by their base composition alone [19] and that the density of the microsatellite is almost twice higher at the ends of the arms of chromosomes in the human and mouse genomes [20]. Microsatellites are also frequently found in proximity to interspersed repetitive elements, such as the short interspersed nuclear elements (SINEs) and the long interspersed nuclear elements (LINEs). For example, human ALU repeats are often accompanied by structures resembling to microsatellites at their 3′ ends, that have probably evolved by the insertion of poly(A) tails of reversed transcribed messages that follow retrotransposable elements at their insertion in a new position in the genome [21]. In addition, mononucleotide poly(A) arrays or (A)n and other conformations of A-rich microsatellites predominate at ALU insertions and this is considered as potential evidence for the association of microsatellites with poly(A) tails [14].

More than one million microsatellite loci are dispersed in the human genome. This number includes a significant group of microsatellites interrupted by other sequences and a group that is uninterrupted monomorphic [14]. In addition, mononucleotide repeats, particularly A and T repeats, are encountered in half a million loci, while the number of pentanucleotide repeat loci is only a few thousand and the higher the repeat sequence is, the more rare the microsatellite becomes. It is probable that larger repeats are less common because microsatellites have arisen with the processes of the so called "DNA slippage", "polymerase slippage", or "slipped strand mispairing" [22, 23]. When the new strand is synthesized from the template strand during replication of the microsatellite sequence, it will sometimes pair with another part of the repeat sequence. If the template strand is looped out then a contraction of the microsatellite will result. If the nascent strand loop is integrated, then expansion of the microsatellite will occur [24, 25]. In addition, the recombination events, such as unequal crossing over and gene conversion, can also cause the contractions and expansions of TR sequences. It has been widely discussed and accepted that replication is the most common cause of instability in microsatellites, but recent studies provide evidence that recombination may also cause microsatellite instability [26].

Microsatellite repetitions are found in the order of 10 to 100 times and are therefore the most useful DNA markers for fingerprinting, population, recombination and evolution studies but also for direct and indirect diagnosis in the clinical practice that require polymorphic markers [14, 27–31].

Minisatellite DNA

Minisatellite DNA is consisted of repeated units of 10–400 bp long with an average of about 20 bp. In general the repeat units cover a stretch of approximately 1–5 Kb, with 20–50 repetitions. Specifically, in humans, 90 % of minisatellites sequences are located to subtelomeric regions [32] and are hypervariable stretches of DNAs [33]. The basic repeat unit may vary in length from 10 to >50 nucleotides, with mutation rates ranging from 0.5 to >20 % per generation. They include some of the most variable loci in the human genome and often referred to as **V**ariable **N**umber **T**andem **R**epeats (**VNTR**s), which is probably the most familiar terminology in the majority of the manuscripts. This terminology also highlights the fingerprinting properties of the minisatellites in proximity to genes or in dispersed clusters in euchromatic regions of the chromosomal DNA [27–29]. Minisatellites have been frequently used as genetic markers in linkage analysis studies [34] and also in population studies [35]. They have been considered as regulatory regions of gene expression or as parts of genuine open reading frames [36]. Finally, minisatellites as also microsatellites, are associated with chromosome fragile sites and are found in the vicinity of a number of recurrent translocation breakpoints [37].

Minisatellites have also been reported to act as “hot spots” for homologous recombination [38]. The huge expansions in minisatellite sequences are a result of unequal crossover or of unequal sister chromatid exchange. Thus, these genetic mechanisms probably account for the extreme variability that is often seen between individuals at these loci [39].

Megasatellite DNA

Megasatellite DNA is part of the satellite DNA families, with extremely large tandem repetitive sequences that cannot be classified as minisatellites. They are called either macro- or megasatellite DNA [40–44]. One characteristic example of a megasatellite DNA is the RS447 which is found on human 4p16.1 to consist of 20–100 copies with a 4746 bp unit sequence, containing an open reading frame of 1590 bp encoding an intronless functional deubiquitinating enzyme (USP17) gene [44–46]. The RS447 megasatellite itself contains the functional expression unit of USP17 that is expressed in human cells [46]. While USP17 is ubiquitously expressed in human tissues, presents with a unique expression pattern in the human brain, with its complementary strand transcribed as an antisense transcript that potentially regulates the level of USP17 expression [46]. The high-level expression of USP17 antisense RNA in brain may suppress expression of USP17 and stimulate ubiquitin-dependent protein degradation. Strikingly the copy number of RS447 is hypervariable (HVR) and highly polymorphic between individuals, as well as among mammalian species [44], but the unit sequences of RS447 are extremely homologous within species [47]. In conclusion, the unstable nature of RS447 megasatellite DNA leads to its hypervariability and may contribute to the structural dynamics of this repetitive DNA in the genome.

In addition, the megasatellite repeat DXZ4, localized at Xq23-24, has 50–100 copies of a CpG-rich 3-kb monomer. DXZ4 is found in constitutive heterochromatin on the active X chromosome characterized by a highly structured pattern of H3K9me3 nucleosomes. In contrast, an amount of DXZ4 is found in euchromatin on the inactive X chromosome characterized by the modified histones H3K4me2 and H3K9Ac. It has been put forward, that this megasatellite repeat is implicated in a novel function involving X chromosome inactivation [48].

Tandem Repeats Function in the Genome

Satellites have a spectrum of functions in the genome and were recently recognized as key players in evolution, methylation, regulation and stabilization. Tandem repeats in the coding sequence may cause the generation of toxic or malfunctioning proteins and the non-coding repeats may result in chromosome fragility, in the silencing of the genes in which they are located, in the regulation of transcription and translation, and in the sequestering of proteins that are associated with splicing and cell architecture [49].

Tandem repeats are not uniformly represented in all mammalian genes with an average representation to about 17 % of the genes, but their unique property is that they are often unstable or hypervariable in mammals. Flows in DNA replication and repair are constantly introducing changes in the repeated sequences and in the number of repeat units and result in TR mutations. The frequency of TR mutations depends on the number of repeat units, the repeat consistency and the length of the repeat unit. The mutation rates of TRs are also caused by environmental factors and the higher transcription and replication rates that may lead to increased instability [50].

The longer and the more consistent a repeat region is, the more unstable it becomes and the mutation rates are often 10–100,000 times higher than the average mutation rates in other parts of the genome [25, 51–53]. Tandem repeat polymorphisms are emerging as a third major class of genetic mutation, with counterparts the single nucleotide polymorphisms (SNPs) and the copy number variations (CNVs). Moreover, tandem repeat variability is by definition an important genetic change by itself, but also influences certain changes to concomitant epigenetic marks due to the high instability and the complete reversibility [2].

The most prominent example of instability and versatility of TRs is that most humans have 30 CGG•CGG repeats in the 5′ UTR of their FMR1 gene [54–56], while population studies in Caucasians indicate that ~1 in 246–468 females have 55–200 repeats while ~1 in 3717–8918 males have 200 to >1000 repeats in the FMR1 gene due to instability induced through maternal meiosis [57]. Thus, the TRs in the genomes are polymorphic, with some individuals or families having some tandem repeat regions that are significantly longer than those recorded in the general population. In addition, these larger repeat lengths are not biologically neutral. Indeed, FMR1 alleles with 55–200 CGG•CCG repeats, in the premutation range, are related to neurodegeneration [58] and ovarian insufficiency [59, 60]. While extended alleles with >200 repeats, in the full mutation range, are related to intellectual disability and autistic symptoms [61]. The FMR1 gene is not unique as regards significant repeat length polymorphism and also in the fact that is related to disease pathogenesis. To date, >15 disorders have been identified in humans as the results of the presence of large expansion-prone DNA tandem repeats in their corresponding genes [49].

Many genetic disorders, mostly neurodegenerative and late onset, are caused by dynamic mutations, concerning almost exclusively trinucleotide repeat (TNR) expansions. The aberrant expansion of TNRs and consequently their instability within specific genes in germ line cells and somatic cells are the causes of these genetic disorders [62]. While many repeats are located within non-coding regions, TNRs also occur often within coding and regulatory regions [53, 63]. Several neurodegenerative diseases are associated to variation of specific repeat areas located within coding regions, but variation of repeats located in introns and untranslated regions of genes can also lead to various disorders [62].

In general the repeat expansion diseases that are analyzed separately in the text of this chapter can be stratified into two subclasses:

- Those with TNRs located in an exon coding for polyglutamine tracts
- Those with TNRs in non-coding, regulatory or coding but non-translated sequences [64–66].

Both coding and non-coding TNR repeats can have significant effects on cell processes [62]. TNRs are highly polymorphic and most importantly above a threshold, in average of 30–50 repeat units, the repeats transcend the afforded length and undergo transition to the pathogenic unstable repeat range. TNRs that are located in different parts of the genes, including the 3′ and the 5′ UTRs, the exons and the introns, are related to various disorders with the common feature of repeat instability over a given threshold [67].

Repeat instability can speed up the disease progression, depending on the expansion, the age at onset of the parents and the gender of the carrier parent. The degree of variation of the repeat sequence over time is associated with tissue or cell-type [67]. It has been observed that in most of the CAG/CTG disorders, repeat instability usually affects the brain, except the cerebellum, which presents with reduced repeat instability [67]. Moreover, the fact that the somatic CAG instability is usually greater in the Central Nervous System (CNS) and, more specifically, in the neurons, indicates the implication of inappropriate mismatch repair rather than DNA replication processes as presented in this chapter in the DNA repair and instability section [68].

Variable tandem repeats that are located in promoters of the human genome or coding sequences can act as mediators for rapid phenotypic changes. The frequent contraction or expansion of the repeat tract leads to quantitative and progressive changes in gene expression or function [69]. Thus, TR sequences in the promoters are involved in gene expression variation, suggesting that such sequences confer an important regulatory role due to genetic variation and can accelerate the evolution of gene expression. This is because the inherent instability of tandem repeats in promoters may lead to altered levels of transcription, generated by the polymorphism that allows rapid divergence [69].

On the other hand, while some variable TRs can influence gene expression by modifying the transcription factor binding sites or by spacing between promoter elements, many variable TRs may control promoter activity by modifying the structure of the chromatin itself [2].

In higher vertebrates, TRs also mediate evolvability in organismal morphology and these unstable repeats may give evolutionary flexibility to organ and/or body shape [53].

Microsatellite Functions

The exceptional variability of microsatellite sequences has permitted microsatellites to be utilized in various applications such as genetic mapping, forensic science, conservation, population and evolutionary studies.

Some microsatellites may serve as regulatory elements of gene expression due to their considerable density in promoters, together with their ability to function as structural elements [70, 71]. Promoter related microsatellites are frequently G/C rich, and many are found within or near the 5′ UTR, CpG islands, and G-quadruplex (G4) structures [72].

The highly conserved microsatellites are abundant in the promoter regions of diverse mammalian genes, many of which seem to regulate cell and tissue growth and development [73]. The promoter function is induced by the variation of promoter microsatellites, which leads to a variation of phenotypes. This variation may be beneficial or disease causing [74, 75]. Nevertheless dynamic increases in the repeat lengths of microsatellites found within promoters can at times distort the normal phenotypes and produce abnormal ones [2]. It is well known that the expansion of microsatellites in protein coding or 5′ untranslated regions causes Huntington's disease and fragile-X syndrome respectively [2].

Promoter microsatellites also have the ability to create various DNA secondary structures, with varying consequences in the regulation of gene expression and the chromatin conformation [76]. Indeed, microsatellites with the motif AC/GT can form ZDNA, which is a left-handed spin double helix [77], and microsatellites that consist of the motif AG/CT can form H-DNA a DNA triplex structure [78–80]. Another DNA secondary structure is the G-quadruplex (G4) [81]. The G4 secondary structure plays an important role in gene regulation [82, 83] and can be highly conserved in mammals [83], particularly in promoter regions [82, 84]. These particular DNA structures can either regulate transcription by modulating RNA polymerase activity [85, 86] or by affecting RNA folding when present in 5′ UTR [87, 88].

G/C rich motifs that include the so called CpG dinucleotides are candidate sites for epigenetic modifications, due to the abundance of cytosines. Each one of the total G/C containing microsatellites, with the exception of the unusual mononucleotide motif C/G, includes CpG dinucleotide targets of epigenetic modification [89].

It has been observed that changes in repeat number of these CpG islands in microsatellites would change the number of methylation sites. In addition, changes in microsatellite length may influence the structural potential, which is important because G4 creation appears to limit the methylation at CpG dinucleotides [90]. Thus, the longer the microsatellites are, the higher the probability to have increased structural potential, and may in turn meddle in methylation [91].

Moreover, microsatellites composed of the motif AC/GT can have significant effects, on human phenotypes, when they acquire instability. The promoters of neural development genes include an impressive number of conserved microsatellites [73, 92]. Changes in AC/GT length have been shown to regulate gene expression [93], and the AC/GT length variation is associated with human phenotypes that depend on the regulation of genes harboring AC/GT [72]. It has been also suggested that the microsatellite motif can also influence RNA structure, due to the identification of certain strand-specific biases [92].

A remarkable fact is that the human chromosomes have very low numbers of A repeats and also absence of ACG repeats. This phenomenon could be explained by various arguments:

- These microsatellites play no structural role in the genome and if they occur by chance, they may not be able to maintain during evolution.
- They may offer strong signals for methylation of CG sequences.
- They are probably involved in DNA transcription and replication, possibly through the formation of unusual DNA structures [94].
- Slippage of DNA strands which is essential for the repeats to grow in length [31] may not be possible with some microsatellite sequences and the secondary structures they form.
- The insertion of repeat sequences in the range of 24–40 nucleotides in either nucleosome cores or inter-nucleosomal DNA, affects chromosome structure and cannot be maintained [95]. An observation that underlies the above arguments comes from Figs. 7.1 and 7.2, where our bioinformatic approach clearly demonstrates that the repeat frequencies of certain di- and tri-nucleotides are substantially different throughout the genome and that they cannot be all maintained in equal repeat lengths depending on their nucleotide composition.

Thus, the abundance of microsatellites varies strongly for each sequence motif. Microsatellites with different repeated motifs may be associated with the determination of chromosome structure. Some chromosome structures have also been associated with a higher percentage of AT nucleotides, such as cohesin binding sequence [96] or scaffold attached regions [97]. In conclusion, changes in microsatellite sequence repeat copy number occur mainly in somatic cells [51]. Mutagenesis rate for microsatellite DNA is expected to be in the range of 0.1–0.2 % [51, 98, 99], although a higher mutation rate has also been reported to reach as high as 1.5 % at the human DXS981 locus [100]. In contrast to the above, triplet repeats, that are implicated in several inherited neuromuscular degenerative diseases, can be increased in either male or female germinal cells [101], despite their stability in the majority of the somatic cells with the exception of brain and CNS cells [67].

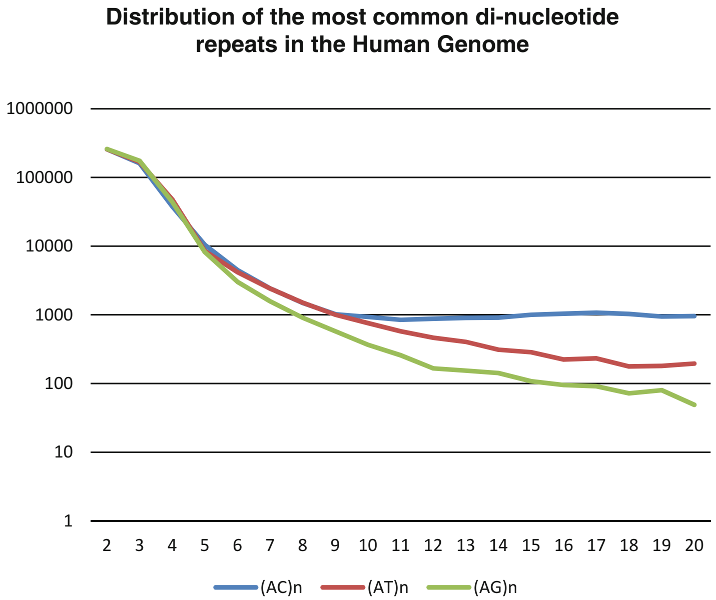

Fig. 7.1 Distribution of most common di-nucleotide repeats in human genome (hg19). The occurrence of dinucleotide sequence lengths are depending on the nucleotide pair variation as demonstrated in this graph concerning the distribution of the most common dinucleotides in the genome. The differences in the distributions support the literature as concerns microsatellite sequence length divergence. The figure clearly shows that the repeat sequence lengths of dinucleotides are different throughout the genome and that microsatellites cannot be all maintained in equal repeat lengths. The vertical axis represents the frequency of the repeats, while the horizontal, the number of the repeats in each particular sequence found in the genome

Minisatellite Functions

Minisatellites are associated with the regulation of genes by various processes. Certain minisatellites are incorporated into open reading frames, some of which may be, polymorphic in the human population [36]. Other minisatellites are binding sites for proteins with a spectrum of functional properties, while minisatellites located in the 5′ region of genes contribute to the regulation of transcription [102]. Certain minisatellites located within introns interfere with splicing, creating multiple splice donor sites [103]. Minisatellites at imprinted loci have been linked to the maintenance of the imprint control [104, 105]. Minisatellites have also been suggested in the literature as intermediate structures in the initiation process of chromosome pairing in eukaryotic genomes [11, 106]. Minisatellites may also compose chromosome fragile sites, identified through induction of replication stress in cell cultures [37]. They have also been found in the locality of a number of repeated translocation breakpoints and in the control recombination site in immunoglobulin heavy chain genes [107].

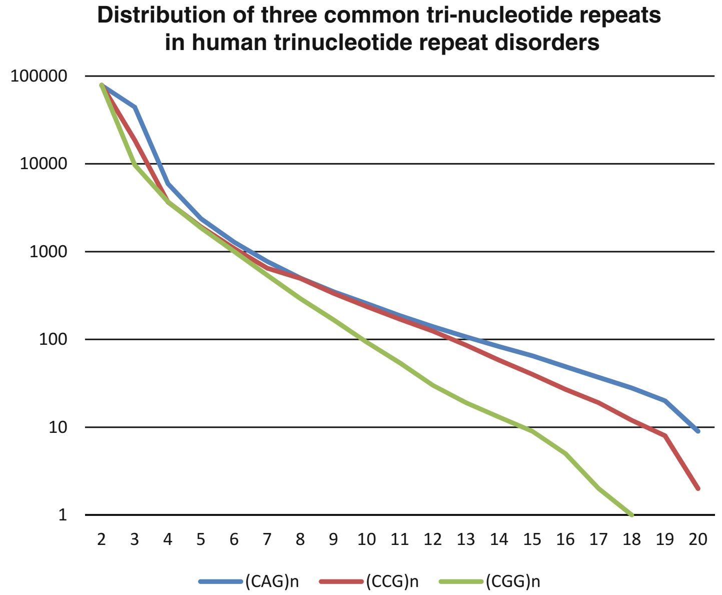

Fig. 7.2 Distribution of most common tri-nucleotide repeats in human genome (hg19). The distribution of three trinucleotide repeats involved in polyglutamine (PolyQ) and non-polyglutamine trinucleotide repeat expansion disorders shows also in this figure a marked microsatellite repeat length divergence. The figure clearly demonstrates that the repeat sequence lengths of certain trinucleotides are substantially different throughout the genome and that microsatellites cannot be all maintained in equal repeat lengths, depending on the three nucleotide combinations (CAG)n in PolyQ disorders, (CCG)nin FRAXE and (CGG)nin FRAXA. The vertical axis represents the frequency of the repeats, while the horizontal, the number of the repeats in each particular sequence found in the genome

In addition, one subcategory of minisatellites includes the highly polymorphic arrays of short tandem repeats with no known function that are used as powerful DNA markers, referred to as variable number tandem repeats (VNTRs). These sequences usually contain 1–5 kb of DNA with repeated units of 15–100 nucleotides [34, 39]. Several minisatellites across the genome share enough sequence homology to be amplified and consequently analyzed by a single primer pair and one probe, yielding efficient DNA fingerprints. Such is a 10–15 bp core sequence of myoglobin minisatellites which includes an invariant core sequence (GGGCAGGANG) among several polymorphic VNTR loci [33, 108].

In conclusion, minisatellites are the most variable loci in the mammalian and human genomes and are therefore excellent molecular markers for studying TR variability and instability. Simple intra-allelic rearrangements are the result of low levels of somatic instability, while complex gene conversions are due to the high frequency of germline instability, which is almost certainly occurring at meiosis. Increased level of instability in microsatellites and minisatellites coincides as a prominent concurrent phenomenon in many human cancer cells [109, 110], atherosclerotic plaques [111] and cells derived from irradiated animals [112, 113].

Telomere Function

Satellites by definition, contain also a particular class of long TRs, located at the tips of all chromosomes which are known as telomeres. Telomeric DNA has 10–15 kb of hexanucleotide repeats (mostly TTAGGG) and is added to the telomeres of all chromosomes by the enzyme telomerase. Such DNA is most certainly functional because it protects the ends of chromosomes from degradation and provides a way for the complete replication of telomeric sequences. It also partially regulates the pairing and the orientation of chromosomes during cell division. Functional aspects that apply to the micro- and mini-satellites, as regards their dynamic behavior, apply also to the telomeres. Telomeric DNA sequences belong, according to the sequence definition given before in this chapter, to microsatellites but some authors regard them as yet another subgroup of minisatellites. The telomeric sequences have 10–15 kb of hexanucleotide repeats, most usually TTAGGG in the human genome, at the tips of the chromosomes. A dedicated enzyme telomerase is occupied solely with ensuring that the complete replication of the chromosome is achieved by adding these sequences perpetually to the telomeres. Telomeres of somatic cells are generally shorter than in germ cells that are designed to live and replicate longer. In humans, it has been suggested that telomeric loss is associated with ageing and tumorigenesis and this is also a landmark being tested in research involving senescence [114].

Megasatellite Functions

The macro- or megasatellite DNA copy number may have an influence on gene expression and the clinical phenotype and may sometimes cause genetic disease [115]. In facioscapulohumeral muscular dystrophy (FSHD), a lower copy number of 3.3-kb tandem repeat D4Z4, is strongly associated with both lower expression levels of adjacent genes and disease severity [42]. The copy number and the methylation status in RS447 DNA may affect both the chromatin structure and the expression of genes in the immediate neighboring region [116, 117].

Moreover, many neurodegenerative diseases, such as Parkinson's disease, are associated with insufficiency of the ubiquitin-proteasome system [118]. Thus, the expression level of the USP17 gene, which may be associated with RS447 copy number and methylation status, may also affect the activity of USP17 deubiquitinating enzyme and cause dysfunction of the ubiquitin-proteasome system, which in turn may alter or even cause the disease [47].

Tandem Repeats Evolutional Drive and Recombination

The scientific community has previously considered regions of tandem repeat DNA as "junk" DNA without any significant mutagenic or selective function in the human genome. However, recent extensive research showed evidence that these DNA fragments have critical functions in the human genome going under natural selection through evolution. Tandem repeats are evolutionary pertinent due to their powerful mutagenic nature. They are mutating in a rate between 10^{-3} and 10^{-6} per cellular division and three phenomena are correlated with TR's evolutionary instability: replication slippage, repair and recombination. During replication, errors occur in result of slip-strand mispairing. Some of them are corrected by exonucleolytic proofreading but many escape this repairing process e.g. by creating secondary structures that escape DNA repairing mechanism. During recombination events, errors occur through unequal crossing-over or by gene conversion that can change TR's repeat number. Interaction between replication slippage, repair and recombination could also lead to mutations as in the case of many neurological diseases, where recombination repair through gene conversion is believed to be responsible for trinucleotide expansions [119].

Strong evidence suggests that tandem repeats are not only randomly distributed along human genome but that their base composition, length and position are conserved and modulated in strict terms through evolution. The establishment of tandem repeats in the ORFs of 17 % of human genes indicates their prevalence over time and their evolutionary impact [2]. An interesting finding by Sawaya et al. [72] made clear that microsatellites can function as regulators of gene expression. This is justified by the high density of microsatellites in promoters which are highly conserved, combined with their ability to function as structural elements. Promoter microsatellites are significantly associated with CpG islands, G4 quadruplexes and untranslated regulatory regions. Variation within these regions could be either evolutionary beneficial to genes that are selectively transcribed or potentially harmful as regards expression of diseases associated genes. Furthermore, Riley and Krieger [120] made an interesting observation for some dinucleotide repeats and their flanking sequences. The findings of their study were informative of the nature and history of tandem repeats and more specifically of the dinucleotide repeats. They found 252 human genes containing $(AC)_{n,}(GT)_{n,}$ $(AG)_{n,}$ or $(CT)_n$ repeats in their UTRs of which 22 had conserved upstream flanking sequences by comparing two different species (Homo sapiens and marsupial species). More interestingly, 18 from these 22 genes were proven to have critical functions in the mammalian embryonic nervous system and an additional one was responsible for kidney's podocyte cells development, cells that have many similarities to neurons. As long as these structures are important for gene transcription, translation, chromatin organization, recombination and DNA replication, natural selection seems to act in a non-random way as regards to TRs size expansions/contractions or base alteration.

Tandem Repeats in Homologous and Non-homologous Recombination

Recombination in all genomes drives evolution and permits meiotic and mitotic changes that accumulate overtime. The gradual accumulation of genetic alterations over a long period of time in reproductive or somatic cells would result in a large-scale reformulation and reconstruction of the human genome. Recombination is clustered in regions of the genome called "hotspots" that undergo recombination in higher rates than average and contribute to genome diversity. Hotspots are distributed in many locations of the human genome as a result of the non-random DSBs (double strand breaks) that initiate recombination events. Regions enriched for these hotspots include telomeres with their flanking sequences, as well as other regions containing repetitive sequences and sequences enriched in GC content. The major conserved regulator protein of recombination hotspots is PR domain zinc finger protein 9, encoded by the PRDM9 gene. PRDM9 is a meiosis-specific histone methyltransferase with a tandem repeat zinc-finger domain encoded by a minisatellite sequence [121]. This specific protein is considered to trigger chromatin remodeling via histone 3 lysine 4 methylation allowing SPO11 to create DSBs so as to initiate recombination [122]. The bizarre in this case is that PRDM9's binding affinity depends on variations in the minisatellite itself enhancing an interesting but yet speculative theory that PRDM9 is driving its own evolution [123]. The fact that PRDM9 regulates about 40 % of human hotspots [124] combined with the discovery of a new hotspot in human genome that is essentially a tandem repeat of a hypervariable minisatellite, with a recombinative potential up to 13.5 times over average [38], declares that some minisatellites are major regulators of human hotspots and recombination.

On the other hand, several reports declare an association between microsatellite polymorphisms recombination rates in the human genome but no one has answered yet the question whether the recombination is mutagenic to microsatellites or microsatellites act as recombination signals. Although microsatellites were considered as genetic markers that have no contribution in phenotypic variations in the past, an interesting finding though by Biet et al. [125] showed significant association of dinucleotide repeats with recombination enzymes in yeast, bacteria and humans. More specific, a common denominator affecting the binding of the recombination enzymes scRad51, hsRad51, ecRecA to oligonucleotide dinucleotide repeats (GT, CA, CT, GA, GC, AT) is the formation of secondary structures such as Z-DNA in ssDNA that is conserved through evolution. As regards to their binding preference, oligonucleotides with CT, GT, CA repeats have stronger binding affinity than GA, AT, CG repeats. As one would expect, di-nucleotide repeats according to Biet, are positively correlated with recombination rates. But, according to Guo et al. [126], the correlation between dinucleotide repeats and recombination rates seem not to be significant compared to motifs of other lengths such us tri-, tetra- or mono-nucleotide repeats. More specifically, dinucleotide microsatellites with different motifs consisting of either A or T lead to increased recombination rates compared to dinucleotide microsatellites with motifs consisting of both A & T together, lead to decreased recombination rates in the human genome. Tri-nucleotide repeats on the other hand show the strongest direct effect on recombination rates in the human genome. That can be explained by the fact that gene densities are remarkably linked with higher recombination rates [127] and are abundant of trinucleotide repeats [128]. But, the question remains whether do microsatellites themselves act as recombination signals. It is commonly known that microsatellites are associated with meiotic hotspots in the MHC loci, as well as in regions with high recombination rates on human chromosome 22 [129], but this is a more complex and multifactorial phenomenon. According to Myers et al. [130], recombination is clustered in "hotspot", regions that have much more recombination potential than the genome average, where certain microsatellite motifs are overrepresented. So the question is why these certain motifs are overrepresented in hotspots. In addition it is unclear whether the majority of hotspots contain microsatellites. An interesting report showed that the major factor which results in converting microsatellite motifs into recombination signals is their base composition affecting the stability of the DNA helix [131]. Furthermore, an interesting report showed flanking sequences have undergone the same evolutionary drive as microsatellites themselves and act as regulators regarding their microsatellite's sequence variation and their motif length [132]. The theory that certain sequence motifs, neighboring to or inside the microsatellites, drive microsatellite and genome diversity through evolution, gains more and more support [132].

Tandem Repeats in Replicative Recombination

Almost 45 % of the human genome is composed of DNA segments related to transposable elements (TEs) which represent truncated of full length transposon sequences within genomes commonly known also as interspersed repeats. Approximately 90 % of these DNA sequences are related to retrotransposable elements, but only a minority of those is active and can exhibit new transposition events. TEs are divided in two major categories: (1) the DNA transposons and (2) the retrotransposons. DNA transposons represent nearly 3 % of the human genome and are characterized by their ability to "cut" themselves from a genomic locus and migrate to another genomic locus (cut and paste). Although DNA transposons lost their ability to propagate themselves in the human genome during evolution, they had highly active role before 38 million years. On the other hand, the retrotransposons are characterized by their ability to copy themselves through RNA to DNA reverse transcription (copy and paste). The outcome of this procedure is the increase of retrotransposon copies in the human genome over time. The retrotransposons are subdivided in two categories, focusing on the presence or absence of long repetitive sequences (LTRs). Human endogenous retroviruses (HERVs) are the so called LTR elements constituting approximately 9 % of the human genome. Most of the HERVs were introduced in the genome before 25 million years and their activity is quite limited to humans. In contrast, the majority of TEs in the human genome is the outcome of the activity of non-LTR elements including LINEs, ALUs, and SVAs which represent nearly 1/3 of the human genome [133]. Data indicate that certain LTR and non-LTR elements have still an active role in human genome, so it is of outmost importance to define the relationship between tandem and interspersed repeats.

Increasing evidence show a strong association between non-LTR elements and microsatellite sequences. More specific, microsatellite sequences can arise during retrotransposition of non-LTR elements, mainly LINE1 and ALU subfamilies, into the genome. Two specific studies showed that 36 % of mono-, di-, tri-, tetranucleotide microsatellites were "born" during the integration of LINEs and ALUs into three primate genomes (human, chimpanzee, orangutan) and that as high as 25 % of microsatellite "births" and 24 % of microsatellite "deaths" occur within LINE1 and ALU sequences during retrotransposition [134]. A common feature of these two major families of non-LTR elements is a poly(A) tail at their 3′ end of their sequence which gives them the unique ability to mobilize [135]. This poly(A) tail is a crucial component of the retrotransposition process and its length strongly influences the dynamics of these major retroelement families [136]. In LINES, a poly(A) RNA tail is normally formed during the transcription process as a 3′ end of LINE mRNA. Contrary to LINEs, only active ALUs have the poly(A) RNA tail which is recognized by LINE ORF1 protein and therefore undergo LINE1 mediated retrotransposition [137]. Poly(A)RNA tails are the binding sites of PABPs (Poly(A) binding protein) creating a critical ribonucleoprotein complex between LINE1 proteins and LINE/ALU RNAs. Several reports demonstrated that the length of these poly(A) tails is positively correlated with LINEs and ALUs ability to integrate into genomes [138, 139]. Not only the overrepresentation of poly(A) repeats in human genome is justified by poly(A) tails of both LINEs and ALUs [128] but also di- and tetra-microsatellite repeats derived from poly(A) tails [21, 140]. Furthermore, the abundance of non-LTR elements in human genome made the perfect environment for the conversion of CAA to NAA, a mechanism responsible for the generation of a large number of A-rich trinucleotide repeats in human genome. More than 60 % of these repeats are located within ALU poly(A) tail sequences [141].

Another interesting non-autonomous non-LTR retrotransposon, that is currently active via LINE1 retrotransposition machinery, is SVA (SINE-VNTR-ALU). SVAs, which are the newest class of retroelements, also members of the family of non-autonomous retrotransposons, have a special feature that makes them unique. Being the only active composite retrotransposons, each component of an SVA is derived either from a retrotransposon or from a repeat sequence, characterizing SVAs as a repeat of repeats. A typical SVA is consisted from the 5′ prime end to the 3′ prime, of a hexameric CCCTCT repeat, followed by a sequence homologous to antisense ALU sequences, a variable number of tandem minisatellite repeats (VNTR domain), and finally a sequence homologous to HERVK-10 endogenous retrovirus, followed by a polyadenylation (polyA) sequence [142]. SVA's core sequence is a VNTR (Variable Number Tandem Repeat) but its functional role is not yet understood, although its length has increased through evolution. SVAs are highly polymorphic, and therefore each repeat domain is capable of misaligning with another locus containing similar SVA element or another repeat sequence, leading to NAHR (non-homologous recombination) [143]. In conclusion, older and younger retroelements are associated with satellite DNA sequences and have a potentially synergistic natural history in the genome. In particular the youngest human specific retroelements, SVAs, have incorporated in their complex genome sequence, parts from microsatellites, minisatellites and other older retroelements.

Repeat Instability Through Meiosis and Repair

Since the discovery of triplet repeat expansions molecular and structural studies explored the hypothesis, that intermediate unstable secondary structures were the culprits of unstable expansions [144–146]. The length of the repeat and the probability of a given sequence to form a secondary structure determine, to a certain point, the likelihood of repeat expansions in polyglutamine or non-polyglutamine disorders [147–149].

Although expansions can arise in both germ cells and somatic cells, anticipation in inheritance of trinucleotide repeat disorders is associated with repeat instability in the germ cells [150]. Furthermore expansions are found in dividing as well as in non-dividing cells meaning that dynamic mutations occur either during DNA replication or during DNA repair [145].

Triplet Repeat Instability at Meiosis

Male meiosis is rather more permissive to trinucleotide expansion studies than female meiosis and oocyte studies. This is due to the very high numbers of spermatozoa in the ejaculate and also to the subsequent high rates of replication cycles during spermatogenesis. A detailed study to identify the particular gametogenesis stage during male germ cell development, at which instability takes place, would elucidate the mechanisms of trinucleotide expansions. Therefore gene expression involved in replication, repair and recombination may answer questions related to the effects of male germ cell mitosis, meiosis or maturation on instability [26]. In the pre-meiotic stage, expansions can occur during mitotic replication and repair, while in the post-meiotic stage expansions can be influenced by meiotic recombinations. Studies of the mismatch repair protein genes in both humans and mice have shown that both MSH2 and MSH3 have expression variability with the first being highly expressed in mitotic spermatogonia, while MSH3 increases expression to peak values at the meiotic spermatocyte stage [151]. Consequently these results may indicate that abnormal repeat expansions may arise as early as at the stage of replicating spermatogonia. In Huntington Disease (HD), which is the most frequent disorder in the group of polyglutamine (CAG)n repeat expansion disorders, elegant studies using modern technology have reported intriguing results. Expansion products were found in almost equal rates in both pre- and post-meiotic cells but the proportion of large expansions in post-meiotic spermatids and spermatozoa was significantly higher [152]. The above indicate that male germ cells expansion in HD can occur before and potentially during or after meiosis [150].

Repeat instability mechanisms during gametogenesis in HD may have similarities to other repeat expansion loci causing instability disorders, but have also marked differences that underline dissimilarities in molecular pathology and genetic counseling. Studies of families with similar (but not CAG) repeat expansion disorders, as is Myotonic Dystrophy (DM1) and Spinocerebellar ataxia type 8 (SCA8), caused by the unstable (CTG)n, have shown repeat expansion bias from the maternal side in contrast to the paternal. In addition, FMR1 gene (CGG)n repeat expansions are invariably maternal and arise in the oocyte, as mothers rather than fathers are responsible for the unstable transmission. FMR1 paternal instability transmission does not take place as (CGG)n stretches contract in FRAXA males to premutation range lengths [153]. Contractions or deletions of the expanded alleles may also be identified in male SCA8 patients in analogy to FRAXA patients [154].

As regards transmission in male HD patients who have a higher risk for sperm cell HTT gene DNA expansions, there could be measures to reduce the (CAG)n instability anticipation. Pearson suggests early pubertal semen storage, to be used for future assisted reproduction, as he postulates from the literature that at the onset of puberty the late onset HD carriers have lower chances to harbor expansions compared to their semen samples later in life [150].

Triplet Instability in Somatic Tissues

The findings in reproductive tissues and germ cells are not necessarily analogous to those of somatic tissues, which undergo completely unrelated to germ cells processes. Differences exist even between tissues of patients or between different trinucleotide repeat expansion disorders. For example different somatic tissue cells from 12 HD patients were found to display repeat mosaicism as in their germ cells [155]. Surprisingly the greatest level of polyglutamine coding (CAG)n mosaicism in HD was detected in both brain and sperm. On top of that finding, affected brain regions by obvious neuropathological lesions were having the highest repeat mosaicism [155].

In a dual experimental approach, both DM patients' fibroblasts were extracted and cultured at progressing ages and DM transgenic mice tissues were studied. The results were intriguing due to the fact that replication progression was associated with the expansions and may affect tissue and age specific repeat instability [156].

In conclusion although germ cells and somatic cells may poses unrelated replication processes, repeat instability is a tissue specific phenomenon that is probably observed predominantly in affected tissues and the germ cells which are known to have varying replication rates but also high plasticity of their DNA [157, 158].

Triplet Repeat Instability Due to Defects in DNA Repair

As presented above CAG instability is more prominent from all somatic cells in the brain neurons of the CNS, implicating mechanisms independent of the replication process [68]. Sound hypothesis and adequate experimentation have suggested and consequently shown that triplet repeats form different secondary DNA structures, depending on their sequence [159]. The secondary DNA specific conformation predetermines stability or instability, explaining why CAG repeats forming predominantly random coils are unstable, while CTGs which tend to form hairpins are stable [160]. Trinucleotide repeat length can also influence stability by generating multiple conformations, raising thus the complexity of the DNA structures [161]. Transcription of the repeats leads to the formation of hybrid DNA–RNA structures in addition to the DNA–DNA structures that may arise in nuclear chromatin and affect the stability of the initial conformation of the repeat secondary structure [162]. Finally, other important nuclear processes may influence the primary and secondary repeat structures, such as methylation, chromatin remodeling and transcription and have an important effect on stability [146].

In mice it has been shown that repeat instability is reduced as a direct consequence of mismatch repair genes inactivation [163, 164]. The same is true for the long-patch base excision repair and the single nucleotide excision repair systems [165, 166].

Base excision repair (BER) is a dedicated DNA repair pathway occupied with the elimination of DNA base flows. Oxidative damage due to 8-oxoguanine (8-oxoG) is the most common DNA lesion [167], that BER is destined to correct by a series of enzymes. The enzymes remove the unmatched DNA base (glycosylase), cleave the abasic site, (Ape1 endonuclease) and repair the either single base defect or the multibase stretch defect (DNA polymerase β-Polβ and flap endonuclease 1-Fen1) [62, 168].

BER protein significance in TNR instability has been studied in repeat instability models of yeast and mice suggesting that replication errors together with BER may contribute to instability. Although there is no clear view which of the two, replication or repair, is more crucial for instability, the disruption of Lig1 and PCNA interaction increased instability due to replication errors in yeast [169]. Lig1 protein deficiency on the other hand did not result in instability, in contrast to Lig1 overexpression that led to repair dependent instability [170]. Similarly, in Lig1 mutant DM mice, a maternal bias with shift to increased contractions and decreased expansions was found, indicating the interplay between replication and repair in instability [171]. Furthermore, in HD mice with *Neil* deficiency somatic and germline instability was reduced, including the brain tissue. Neil1 is a DNA glycosylase acting on pyrimidine-derived lesions, that can also remove duplex and single-strand DNA lesions and 8-oxoG lesions in both somatic and germ line tissues [172]. Finally, in a fragile X premutation model the DNA oxidizing molecule, potassium bromate, exacerbated repeat instability in the germline driving the gene towards the repeat expansion, indicating repair of oxidative DNA lesions as related to the instability of (CGG)n repeats [173].

Age increases oxidative damage on DNA in certain tissues related to repeat instability, as in the germ cells and the brain. Also accessibility of repair proteins to hairpin secondary conformations of (CAG)n and (CTG)n is potentially reduced due to inappropriate recognition [174]. On top, hairpin conformations of (CAG)n and (CTG)n repeats are considered as hot spots for DNA oxidative damage [175]. Consequently, reduced accessibility and increased susceptibility of repeat secondary structures to DNA damage may facilitate accumulation of oxidative lesions at (CAG)nor (CTG)n repeats or even deteriorate to generate unstable expansions [62].

Satellite Repeats in Human Disorders

Satellite repeats, either mini-or micro-satellites with short or long repeated sequences of DNA, are usually prone to acquire repeat instabilities due to errors that occur during the repair process that follows replication. Replication errors, that affect repeat instability, may happen in germ cells at meiosis or in somatic cells at mitosis with defects in recognition and repair of replication errors.

The two major types of satellite repeat involvement in human inherited or acquired disorders are given below:

- Micro- and mini-satellite instability disorders that relate to impaired DNA mismatch repair during replication in somatic cells. Instability disorders are present in cancer cells as the result of the nuclear proofreading machinery failure to correct mismatches that spontaneously arise in fast replicating nuclei. Instability is present in coding, as well as in non-coding microsatellites with potential functional oncogenic effects, either on the chromatin conformation or on the gene expression. Such failures exist (1) due to predisposition owing to inherited defects of the mismatch repair genes (2) due to acquired inability of the cell nucleus to sustain high fidelity replication of the DNA and correct distribution of the genetic material to the daughter cells. Defects in recognition and repair of replication errors are most frequently found in tumors with inactivating mutations on mismatch repair (MMR) genes. Such inactivating mutations are found in disorders inherited from the germ line, such as in human non-polyposis colorectal cancers or in sporadic colorectal, endometrial and gastric cancers. Inactivating MMR gene alterations result in instability of both mini- and micro-satellite repeats in cancer cells and can be used as diagnostic or prognostic markers for these types of cancer.
- Repeat instabilities at meiosis are either contractions or expansions, but the later are those related to human disorders known as the trinucleotide repeat expansion disorders. Triplet repeat expansion disorders, which are common human genetic defects, are characterized by abnormal expansion of a triplet repeat stretch adjacent to a functional gene during meiosis, resulting in an abnormal repeat number interfering with expression or regulation of this gene. The most common repeat forms in expansion disorders are the trinucleotides CAG, CGG, CTG and GAA.

Micro- and Mini-Satellite Instability Disorders

Predisposing germ line mutations, responsible for recognition and repair failures in hereditary non-polyposis colorectal cancer (HNPCC), are more frequently those affecting the MLH1 and MSH2 genes. Both genes belong to classes of mismatch repair homologous genes present in prokaryotes and eukaryotes.

More than 30 different genes have been found to harbor mutations at coding repeat sequences in sporadic cancers with microsatellite instability [176]. Among them are genes directly related to mismatch DNA repair as MLH3, MSH3, MSH6 and PMS2.

Almost all the above mismatch repair genes (MLH1, MSH2, MSH3, MSH6 and PMS2) have been tested for microsatellite mutational biases in different species (yeast, mouse and human) and have been found to favor either deletions or insertions [177].

Cancers may also destabilize minisatellites in patients with MMR gene inherited defects manifested as colorectal cancers, specifically in those patients known to have microsatellite instability (MSI). Furthermore there is evidence that DNA instability may be detected in minisatellites even in cases with a MMR gene mutation, where MSI has not been observed [178].

Repeat Expansion or Trinucleotide Repeat Instability Disorders

Trinucleotide repeat expansion disorders (TREDs) are a defined group of genetic disorders affecting the neural and the neuromuscular system. In a total of more than 15 disorders, (CAG)n and (CTG)n repeat expansions are the most frequent, encountered in the majority of these disorders. The repeats causing the TREDs are polymorphic in the general population with a normal higher repeat range of approximately 30–50 repeats. Loss of function or gain of function is related to the specific gene location of the expansion which is either at the 5′ or 3′ UTR or within introns or exons. Unstable repeats are pathogenic and result in dynamic mutations that can expand further in the germline and somatic tissues and also from one generation to the next [179]. The deterioration of the TREDs from a normal or permutation status to a full mutation is described in medical genetics as anticipation, meaning the clinical presentation of the early onset disorder in the future generations with more pronounced symptoms [26].

There are many important features in each particular TRED, of which the noteworthy are: (1) expansions are more frequent than contractions, (2) germ cells may have the same pathogenic expansions as selective tissue cells, (3) expansions tend to be increased in the affected tissue cells and in particular to the brain cells, (4) repeat sequence tracts in affected somatic tissues seem to increase with age [157, 180].

At least five different trinucleotide sequences are involved in repeat expansion disorders, CGG, CCG, CAG, GAA and CTG, with a frequent range from over 21 tandem repetitions to more than 250 and an excessive range in some particular disorders, as in the FRAX syndrome, with more than 10^3 repeats in full mutations. Maternal meiosis is the common cause of triplet repeat expansions, but contractions may also occur [181].

Various genes in mammalian species and in humans contain repetitive triplets CAG or CAA coding for glutamine. The universal code for glutamine (CAG) is found in more than half of the repeat expansion disorders in humans. Due to the translation of these expansions in the affected cells a deleterious polyglutamine tract is formed leading to polyglutamine or alternatively termed polyQ disorders. The cells may not effectively get rid of proteins with excessively long polyglutamine tracts that eventually can accumulate in nerve cells over time and damage their cytoplasm. For this reason, the stratification of the repeat expansion disorders is facilitated by the simple discrimination between polyglutamine and non-polyglutamine disorders.

Polyglutamine Disorders (CAG)N

There is a significant association of the polyglutamine tract length with a tendency for early onset appearance of the polyglutamine (polyQ) disorders. The most frequent polyglutamine disorder is Huntington chorea, followed by SBMA (Spinobulbar Muscular Atrophy or Kennedy disease) and the Spinocerebellar ataxias (SCA1, SCA2, SCA3, SCA6, SCA7 & SCA17) (Table 7.1).

Non-polyglutamine Disorders

The most frequent disorders in this group are Fragile X syndrome (FRAXA), Myotonic Dystrophy (DM) and Friedreich's ataxia (FRDA), followed by other less frequent disorders, as FRAXE (Fragile XE mental retardation) and Spinocerebellar ataxias (SCA8 & SCA12) (Table 7.2).

Fragile X syndrome (FRAXA) is the most frequent and prominent disorder among the non-polyglutamine disorders, owing its name to the association of a fragile site on the X chromosome, with mental retardation in karyotyped mentally retarded patients. Although the triplet CGG in fragile X syndrome gene FMR1 codes for arginine, the gene expansion is located at the 5′ prime untranslated region of the gene and therefore affects directly on the regulation of the protein FMRP expressed. The long stretches of >55 repeats of CGG and particularly those of full mutations affect mostly the methylation of this region creating more CpG island targets for methylation. Abnormal methylation interacts with all aspects of the molecular function of the FMR1 gene including regulation, expression and replication.

Contribution of Microsatellite and Minisatellite DNAs to Medical Genetics

Microsatellite and minisatellite DNA variations have played a crucial role in the development of medical genetics. Their use in linkage analysis studies revealed the association of various genes with hundreds of hereditary diseases, helped in the chromosomal abnormality screening and in the mapping of genetic loci susceptible to tumors. Many micro- and mini-satellites found to be functional promoter polymorphisms are acting as common genetic risk factors for disorders, like diabetes, or pathogenic mutations (Huntington Disease, Fragile X Syndrome, Myotonic Dystrophy, Spinocerebellar Ataxias). Finally, microsatellites and minisatellites, constituting a powerful tool for the genome identification, the parentage confirmation and the sexual assault examinations, favored significantly the forensic science. The contribution of these DNA variations in medical genetics is described in the following pages.

Table 7.1 Polyglutamine disorders (PolyQ)

Disorder	Gene/product category	Chromosome	(CAG)n normal range	(CAG)n expanded range	Inheritance	GCid
Huntington Chorea (**HD**)	HTT/Protein binding	4p16.3	6–35	36–250	**AD Late Onset**	**GC04P003076**
Spinobulbar Muscular Atrophy (**SBMA**)	AR/DNA binding	Xq12	9–36	38–62	**X-Linked**	**GC0XP066763**
Spinocerebellar Ataxia, Type 1 (**SCA1**)	SCA1/Protein binding	6p23	6–35	49–88	**AD**	**GC06M016299**
Spinocerebellar Ataxia Type 2 (**SCA2**)	SCA2/RNA binding	12q24.1	14–32	33–77	**AD**	**GC12M111890**
Spinocerebellar Ataxia Type 3 (**SCA3**)	SCA3/Protein binding	14q21	12–40	55–86	**AD**	**GC14M092524**
Spinocerebellar Ataxia Type 6 (**SCA6**)	SCA6/Protein binding	19p13	4–18	21–30	**AD**	**GC19M013317**
Spinocerebellar Ataxia Type 7 (**SCA7**)	SCA7/Protein binding	3p21.1	7–17	38–120	**AD**	**GC03P063825**
Spinocerebellar Ataxia Type 17 (**SCA17**)	SCA17/DNA binding	6q27	25–42	47–63	**AD**	**GC06P170863**
Dentatorubropallidoluysian atrophy (**DRPLA**)	ATN1/Protein binding	12p13.31	6–35	48–93	**AD**	**GC12P007033**

1. *AD* Autosomal Dominant, *AR* Autosomal Recessive, *GCid* GeneCards Identifiers
2. Data collected from www.GeneCards.org

Table 7.2 Non-polyglutamine (non-PolyQ) disorders

Disorder	Gene/product category	Chromosome	Repeat normal range	Expanded repeat range	Triplet gene position	Inheritance	GCid
Fragile X Syndrome (**FRAXA**)	FMR1/RNA binding	Xq27.3	6–53	55–200 Premutation >230 Full Mutation	**CGG 5′ UTR**	**X-Linked**	**GC0XP146993**
Myotonic Dystrophy (**DM**)	DMPK/Protein binding	19q13.3	5–37	>50	**CTG Non-coding**	**AD**	**GC19M046272**
Friedreich's Ataxia (**FRDA**)	FXN/Protein binding	9q21.11	7–34	>100	**GAA Intron**	**AR**	**GC09P071650**
Fragile XE Mental Retardation (**FRAXE**)	FMR2/RNA binding	Xq28	6–35	>200	**CCG 5′ UTR**	**X-Linked**	**GC0XP147582**
Spinocerebellar Ataxia Type 8 (**SCA8**)	SCA8/Protein binding	13q21	16–37	110–250	**CTG is Transcribed to mRNA as CUG repeat with toxicity**	**AD**, **Reduced Penetrance**	**GC13U900338**

1. *AD* Autosomal Dominant, *AR* Autosomal Recessive, *GCid* GeneCards Identifiers
2. Data collected from www.GeneCards.org

Microsatellite DNAs and Medical Genetics

Microsatellite markers constitute powerful tools for testing genetic loci associated with common diseases. For example, Huntington's disease which as described previously, is caused by an expansion of a CAG repeat encoding a polyglutamine stretch in the huntingtin protein, the size of which is inversely associated with the age at onset of Huntington's disease [182]. Similarly, Fragile X syndrome, caused by an expansion of a CGG repeat in the fragile X gene, exceeds a threshold length at which the protein produced by the gene cannot be detected [183]. STRs have also been used for the prenatal diagnosis of Down syndrome using amniotic cells [184], the detection of hemophilia A and B [185, 186], the diagnosis of prostate adenocarcinoma in transrectal prostate biopsy specimens [187], the detection of Duchenne muscular dystrophy [188] and the duplication screening in Charcot–Marie-Tooth patients [189].

The requirement of very small amount of DNA, pure or even degraded to some extent, for the analysis of microsatellite markers as well as their adaption to high-throughput systems favored their use in linkage analysis genetics. With the development of the second generation genetic linkage maps of human chromosomes, the responsible genes and the respective mutations for over 2000 genetic disorders were identified. Microsatellite markers have played an important role in the linkage analysis of recessive diseases with very low incidence, in which only a very small number of patients can be collected [190]. In cases of patients, whose ancestors lived in the same area for a long period, only few patients are sufficient to map a gene associated with a genetic disease. The responsible genes for Fukuyama-type congenital muscular dystrophy, an autosomal recessive, severe muscular dystrophy associated with brain anomalies [191], and for the Gelatinous drop-like corneal dystrophy, an autosomal recessive disorder characterized by severe corneal amyloidosis leading to blindness [192], were isolated using microsatellite markers. Finally, the analysis of STRs in the Xq11–Xq13 interval can provide a simple and rapid scan of the genetic loci associated with prostate carcinoma predisposition in large populations [193].

STRs, due to their high heterozygosity, are very useful in paternity and forensic testing. The genotyping of autosomal, Y-chromosomal and mitochondrial STRs has increased the ability to solve problems relevant to paternity or relevant to relatedness on the maternal or the paternal lineage [194–196]. On the other hand, forensic genotyping, providing reliable evidence for sentencing the offenders and exonerating the innocent suspects, has great impact on the society. The use of STR analyses offered a doubtless identification of an individual implicated in violent crimes such as murders or sexual assaults and the identification of remains of missing persons or victims of mass disasters [197–199].

Furthermore, the analysis of autosomal, Y-chromosomal and mitochondrial microsatellites has been widely used for the identification of "founder effect" phenomena [200, 201], which are caused when a small group of individuals cuts out from a larger population and establishes a new population. These populations are usually characterized by increased frequencies of certain genetic diseases, such as Tay–Sachs disease in Ashkenazi Jews [202] and asthma in Hutterites [203].

Consequently, the study of founder populations is very useful for identifying which genes are involved in a genetic disease and which is the genetic profile of the disorder. STRs constitute a valuable tool for the above analysis offering a fine-mapping of the disease genes.

DNA typing is necessary for the engraftment documentation after allogeneic bone marrow transplantation or allogeneic peripheral blood stem cell transplantation [204]. The serial chimerism analysis of STRs offers a reliable and simple screening for the detection of relapse and the identification of patients with progressive mixed chimerism [205]. In case of chronic myeloid leukemia, although the common STR analysis is an appropriate screening test for patients in the post-transplant setting, the use of leukemia STR specific sensitive molecular assay is necessary in patients exhibiting mixed hematopoietic chimerism [206]. Finally, STR analysis is very useful in leukemia patients with graft failure after a bone marrow transplant and in need of a second transplant [207].

Minisatellite DNAs and Medical Genetics

The minisatellite DNAs are highly polymorphic sequences with high heterozygosity in given populations [34], frequently encountered in the literature as variable number of tandem repeats (VNTRs).

Alec Jeffreys, who discovered the technique of genetic fingerprinting in 1984, showed that minisatellites constitute the most variable markers of human genome, exhibiting this variation in the numbers of repeat units or “stutters”. The hypervariation of VNTRs has been utilized for the discrimination of individual genomes in criminal forensic studies and for parentage testing [208]. The use of VNTRs in such cases offered a reliable ‘DNA fingerprinting’, unique for any individual, and a powerful tool for criminal justice [209]. However, allele databanks for every population are essential so as to estimate the probability of a particular allele combination [210]. The analysis of highly variable VNTRs led to a trustworthy paternity testing and eliminated the non-paternity, which constituted a common source of bias in the estimation of mutation rates when they were obtained from family data with discordance of parental and offspring genotypes [211].

The use of VNTRs has also contributed significantly in the detection of total or partial chromosomal losses by the disappearance of a paternal or maternal allele on Southern blot analysis. Specifically, loss of VNTR alleles was revealed in 40 % of colorectal carcinomas from constitutionally heterozygous patients at the chromosome 17p loci, suggesting that hemi- or homo-zygosity of 17p alleles plays a role in the development of these tumors [212]. Furthermore, the detection of genetic material loss from the short arm of chromosome 17, evidenced by a VNTR allele loss, has shown that the increased p53 mRNA expression is involved in breast tumor biology [213]. Similar partial chromosomal deletions, detected with VNTR analysis, were associated with gastric carcinoma [214], neuroblastoma [215], non-Hodgkin lymphoma [216], head and neck cancer [217].

VNTRs have also played a role in the study of tumor suppressor genes, most of which were characterized by inactivation of both alleles in tumors. The use of a p53 cDNA probe and two VNTR probes on chromosome 17 has shown that the allelic loss of p53 may play an important role in the development and progression of ovarian carcinomas [218]. Furthermore, rare alleles of the H-ras VNTR, located downstream of the H-ras oncogene, have been associated with breast cancer and they have been proposed as informative markers for the breast cancer risk [219]. The use of four intragenic VNTRs as probes has shown that the loss of heterozygosity of the Rb gene is correlated with pRb protein expression and p53 alteration in human esophageal cancer [220]. Finally, a VNTR located at the intron 16 of the human retinoblastoma (RB1) gene is associated with tumor presence in retinoblastoma [221].

Many VNTRs, constituting functional promoter polymorphisms, act as genetic risk factors or pathogenic mutations for various diseases. The evaluation of a minisatellite marker expansion in the promoter of the CST6 gene has been found to be informative for the recessive myoclonus epilepsy (EPM1) onset [222], while the expansion of a VNTR upstream of the insulin gene, which regulates the insulin expression, is indicative of the polycystic ovary syndrome [223]. The alleles of another insulin gene VNTR regulatory polymorphism (26–63 repeats) predispose to type 1 diabetes in a recessive inheritance mode or are dominantly protective (140 to more than 200 repeats) [224]. Furthermore, a VNTR polymorphism of the P-selectin glycoprotein ligand-1 is a significant determinant of thrombotic predisposition in patients with antiphospholipid syndrome [225], whereas a VNTR polymorphism in the intron 2 of the interleukin-1 receptor antagonist gene is informative for the age at onset of neuropsychiatric symptoms in Wilson's disease [226].

VNTRs have also been used in prenatal diagnosis for the confirmation of parentage and the elimination of maternal contamination of chorionic villus or amniotic cell samples [227, 228]. Additionally, a VNTR identified in the human phenylalanine hydroxylase (PAH) gene has been used for the prenatal diagnosis of classical phenylketonuria [229], while prenatal diagnosis of hemophilia A can be achieved using an intragenic marker (BCL1) and an extra-genic VNTR (DXS52) [230]. Finally, VNTR genetic markers have been used in clinics to detect chimerism after bone marrow transplantation [231, 232].

Novel Methods for the Analysis of Micro- and Minisatellite DNAs

Taking into account the above, we can assume that micro- and minisatellite DNAs constitute useful genetic markers in coding and regulatory regions of the human genome. However, the role of many STRs and VNTRs remains unclear due to the technical difficulties of tandem repeat sequencing. Specifically, the next generation sequencing is probably not suitable for the analysis of genomic regions with tandem repeats because of reads with short lengths. For this reason, new methods for the analysis of micro- and minisatellite markers have been developed [233, 234].

The only limitation of these methods is their ability to genotype only repeats with short unit length and low copy numbers. Recently, a new method based on targeted enrichment for tandem repeats followed by sequencing has been developed [235], permitting a better assessment of repeat variability between individuals. The use of this method has revealed a high degree of variability in STRs and VNTRs, even between direct relatives, and the occurrence of de novo mutations. The development of such techniques will give the opportunity for new genome-wide association studies that will testify the linkage of tandem repeats with inherited or multifactorial disorders.

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Chapter 8
Intron Biology, Focusing on Group II Introns, the Ancestors of Spliceosomal Introns

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Characteristics of Group II Introns

Group II introns are mobile metalloribozymes that self-splice from precursor RNA to generate excised intron lariat RNA forms, which invade new DNA genomic locations by reverse splicing. These retroelements also encode a reverse transcriptase that stabilizes the RNA structure for forward and reverse splicing and finally converts the inserted intron RNA back to DNA. For these reasons, group II introns initially identified in the mitochondrial and chloroplast genomes of lower eukaryotes and plants, and subsequently found in bacteria and archaea are thought to be the ancestors of nuclear spliceosomal introns and non-long terminal repeat (non-LTR) retrotransposons [1–4]. Recently identified structural and functional similarities between group II introns and spliceosomal nuclear RNAs have suggested that group II introns may have played an important role at the very start of eukaryote evolution. It is now thought that their invasion of pre-eukaryotic genomes and their proliferation in those genomes may have driven the evolutionary separation of nucleus and cytoplasm [5].

Typically, group II introns consist of a conserved RNA structure organized into six domains. Domain V is the most conserved of these domains and is considered to be an

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essential part of the catalytic core (Fig. 8.1) [6, 7]. In mobile introns, a protein (the intron-encoded protein) is commonly encoded by domain IV, which contains a specific subdomain (subdomain DIVa) responsible for facilitating the canonical interaction generating the ribonucleoprotein (RNP) particles involved in the invasion of new DNA targets [8]. The IEP has two conserved domains: an N-terminal RT domain and a maturase/thumb domain (also known as the X-domain). Some IEPs also have a C-terminal DNA-binding (D) region followed by a DNA endonuclease (En) domain (Fig. 8.1).

Group II Intron Ribozyme Sequences

Group II intron ribozymes are characterized by a conserved secondary structure, which varies in size from 100 nt up to about 3000 nt [9]. The first model was established on the basis of a phylogenetic data comparison, looking for potential base pairings that had been preserved by evolution despite primary sequence divergence [6, 10]. The only sequences of group II intron ribozymes that are strongly conserved are the intron boundaries (GUGYG at the 5′ exon junction and AY at the 3′ junction), which resemble those of spliceosomal introns (GU…AG) and few nucleotides dispersed throughout the rest of the structure [11].

Group II intron ribozymes are organized into six domains, DI–DVI, radiating from a central core (Fig. 8.1). They form a structure consisting of a set of double helices resulting from Watson-Crick and Crick wobble base-pairing [12]. The six domains fold into a catalytically active tertiary structure with the assistance of a series of conserved motifs involved in long-range tertiary interactions surrounding a catalytic four-metal-ion center [13]. Some interactions involve Watson-Crick base pairs (α-α′, β-β′, γ-γ′, δ-δ′, ε-ε′, IBS1-EBS1, IBS2-EBS2 and IBS3-EBS3), whereas other are tetraloop-receptor interactions of known geometries (ζ-ζ′, η-η′ and θ-θ′), or other types of less well defined non-Watson-Crick interactions (λ-λ′, κ-κ′ and μ-μ′) [14, 15]. Two of the six domains are essential for catalysis: the largest domain (DI), and DV. Recent crystallographic studies have revealed that the shape of the RNA molecule is dictated by a set of tertiary interaction networks within domains I and III that creates the scaffold responsible for binding and activating catalytic domain V [16]. DI is essential for exon recognition in forward and reverse splicing reactions. DV contains the catalytic triad, AGC at its base, the G residue being invariant and critical for splicing. Another important catalytic motif is the AC bulge of DV. Tertiary contacts between conserved nucleotides in the linker region J2/3 and the fifth nucleotide of the intron (λ position) have been described and reported to bring together these nucleotides to form a metal ion-binding platform directly involved in catalysis [10, 17–20]. Domain VI (DVI) contains a highly conserved bulged adenosine residue that acts as the branch point for lariat formation during splicing [7]. DVI also takes part in the long-range η-η′ interaction underlying the organization of the terminal loop of DVI and an internal helix of DII, which has been reported to be important for transesterification at the 3′-splice site [9].

Group II introns can be classified into three structural subclasses, according to the recognition of their flanking exons. Subclass IIA and IIB introns, which display

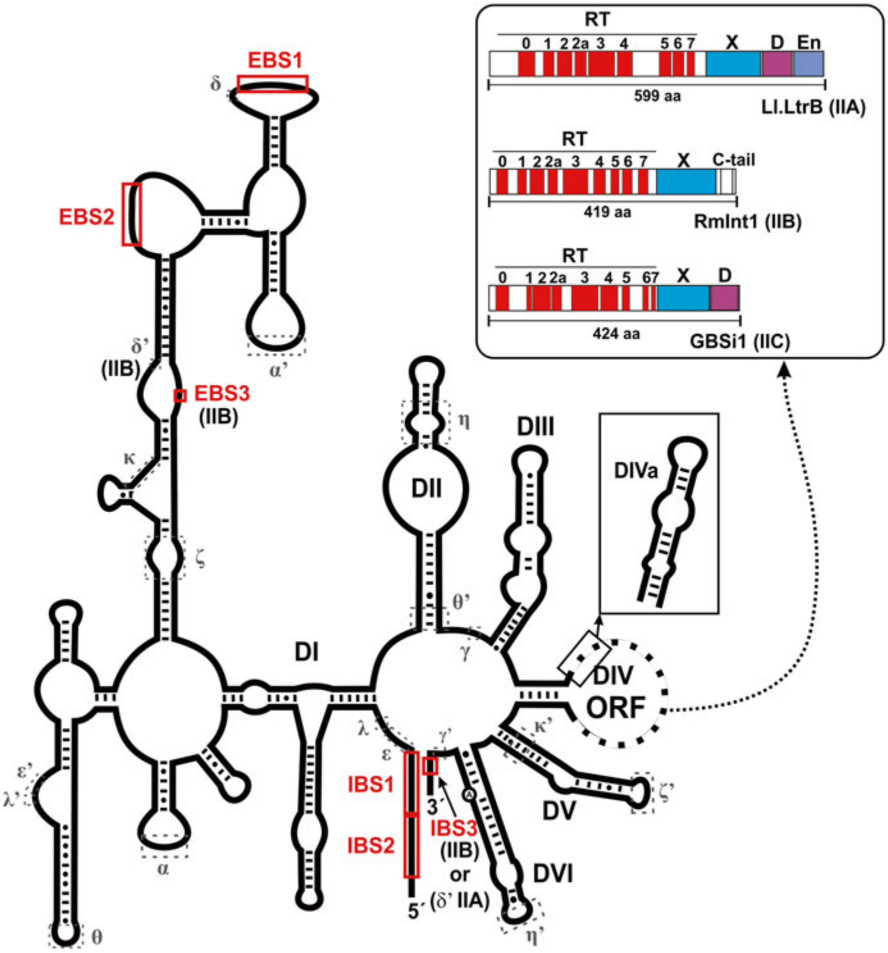

Fig. 8.1 Secondary structure of group II intron RNAs and domain structure of the intron-encoded proteins (IEP). Structure of a representative ribozyme (not to scale): domains (DI–DVI), the EBS/IBS elements, the bulged adenosine within DVI, and the sequences involved in the tertiary interactions (Greek letters) occurring during splicing, as described in the text. Major differences between group IIA and IIB introns are indicated in *brackets*. Note that group IIC introns lack the EBS2 element. The loop of DIV, which encodes the IEP, is depicted by *dashed lines*, with a box showing the location and structure of DIVa, a high-affinity binding site for the IEP. Diagrams (*drawn to scale*) of the Ll.LtrB (IIA), RmInt1 (IIB) and GBSi1 (IIC) IEPs encoded within intron DIV are boxed on the right, with the predicted reverse transcriptase (RT), maturase (X), variable DNA-binding region (D) and conserved DNA endonuclease (En) domains indicated. The C-tail denotes a C-terminal extension of 20 amino-acid residues conserved in the RmInt1 IEP and involved in maturase activity and DNA target recognition. The numbers above the RT domain identify conserved amino-acid sequence blocks characteristic of RTs

strong DNA target specificity, recognize their two exons via three base-pair interactions (IBS1/EBS1 and IBS2/EBS2 for the 5′ exon and δ-δ′ (IIA) or IBS3/EBS3 (IIB) for the 3′ exon), whereas subclass IIC introns use only two of these interactions (IBS1/EBS1 and IBS3/IBS3) and require a stem-loop structure in ssDNA that is generally derived from a transcription terminator by an as yet unknown recognition mechanism [21]. Additional subclasses have also been defined on the basis of specific structural differences: A1, A2, B1, B2 and B3 [10, 22].

Group II Intron-Encoded Proteins

The IEP component is a multifunctional protein containing a reverse transcriptase (RT) domain with subdomains conserved across other RT families (subdomains 0, 1, 2, 2a, 3, 4, 5, 6, 7) [8, 23]. Downstream from the RT domain is domain X, which functions as the thumb domain of the RT, and has a sequence conserved among group II introns but not between group II introns and other types of RTs [4, 24]. Domain X is immediately followed by a DNA-binding domain (D), defined on the basis of its function, but displaying no sequence conservation. Finally, many group II IEPs have an endonuclease domain (En) at their C-terminus that is required for the retromobility of these introns (Fig. 8.1). However, most bacterial IEPs have no En domain. Instead, they have a C-terminal portion that constitutes a distinctive, characteristic signature of this ORF class and has probably been conserved throughout evolution. This region of the protein is a group-specific functionally important protein region participating in both maturase function and intron mobility [25]. Recent studies on the consensus amino-acid sequences of the maturase and C-terminal domains have expanded our knowledge of subclasses of intron ORFs with no recognizable D/En region [26].

The classification and phylogenetic analysis of group II introns on the basis of their IEPs have resulted in the definition of several main groups: A, B, C, D, E, F, CL1 (chloroplast-like 1), CL2 (chloroplast-like 2) and ML (mitochondrion-like) [4, 26, 27], although additional types of intron ORF have recently been identified [26]. The introns of classes A, C, D, E and F and the newly identified *g1* introns encode proteins with no En domain [26]. The En domain seems to have been acquired only once in recent phylogenetic lineages of group II introns, by the common ancestor of classes B, CL and ML [26]. Intron RNA structures are generally congruent with ORF phylogeny [27].

Group II Intron Splicing

Group II introns have developed a series of splicing mechanisms to ensure their removal from the pre-mRNA and, hence, their survival in the host genome [28]. Organellar group II introns mostly interrupt essential genes, whereas prokaryotic group II introns are found in mobile genetic elements or intergenic regions [29]. Some group II introns can self-splice in vitro [30], but excision in vivo is generally assisted by protein factors that facilitate either ribozyme folding or the splicing reaction itself [31].

Excision Mechanisms of Group II Introns

Several excision mechanisms have been described for group II introns, generating different intron splicing products and ligated exons [15, 32]. These mechanisms generally co-exist within the same host [33–36], but some group II introns have been reported to display excision exclusively via a specific pathway [37–39]. Major advances have been made towards understanding the mechanism and kinetics of group II intron splicing in studies based on self-splicing assays, in which the intron precursor is incubated in non-physiological conditions (high temperatures and salt concentrations) in vitro [30].

Group II intron catalysis generally involves two sequential transesterification reactions (Fig. 8.2a). Mg^{2+} ions are involved in ensuring the correct folding of the catalytic core of the intron ribozyme and they also orchestrate the rearrangement of the single active site between the first and second splicing reactions [19, 40, 41]. The branching reaction is the most common excision pathway among group II introns [15, 42, 43] (Fig. 8.2a [1]). The 2′-OH of a bulged adenosine located in ribozyme domain VI initiates a nucleophilic attack on the phosphate bond at the intron-5′ exon junction, generating an intron intermediate in which the adenosine is covalently linked to the first intron nucleotide. In a second step, the free 3′-OH of the 5′ exon triggers a nucleophilic attack on the 3′ splice site, leading to exon ligation and intron lariat release. Alternatively, the first transesterification reaction can be initiated by a hydroxyl group from a water molecule, generating a linear excised intron and ligated exons [44–46] (Fig. 8.2a [2]). This excision pathway has classically been associated with group II introns that have lost the bulged adenosine [37–39]. Both steps in the branching reaction are reversible [47], but the first reaction of the hydrolytic pathway seems to be irreversible [33, 48]. The most recently discovered intron excision mechanism mediates the release of the intron as a true circle [35, 36, 49–51] (Fig. 8.2a [3]). The mechanism underlying this reaction remains unknown, but it has been suggested that the 3′-OH of a free 5′ exon could attack the 3′ splice site, releasing the 5′ exon-intron linear intermediate and linked exons. The hydroxyl group at the end of the intron thus reacts with the first intron residue and forms the circular intron. The maturase activity of the IEP modulates the balance between intron lariats and circles in vivo [52]. It has recently been suggested that the splicing of wheat mitochondrial group II introns under cold stress plays a regulatory role [53]. Introns with a conventional branchpoint structure display classical lariat-type splicing regardless of germination temperature. By contrast, the excision of non-conventional introns shifts from a predominantly hydrolytic pathway at room temperature to the production of circular molecules in the cold.

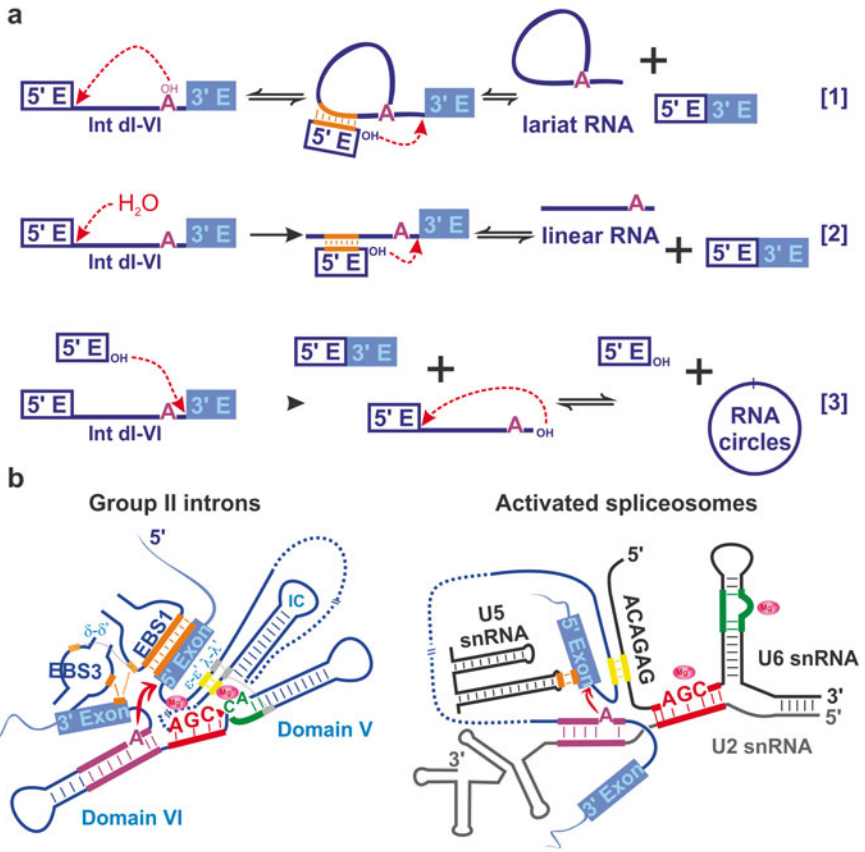

Fig. 8.2 Mechanisms of group II intron excision. (**a**) Intron lariat, linear and circular molecules may be generated, depending on the excision pathway: branching [1], hydrolysis [2] and circle formation [3], respectively. The intron RNA sequence is indicated by a *dark blue line*, and the exon sequences are represented by an *empty blue box* (5′ exon) or a *light blue box* (3′ exon). *Orange bars* correspond to the EBSs/IBSs interaction. The bulged adenosine in intron domain VI is represented as an *A*. *Dotted red lines* indicate the nucleophilic attacks occurring during each step of the reaction. (**b**) Structural similarities between the catalytic core of group II introns and activated spliceosomes. *Dark blue lines* represent the intron RNA, whereas *light blue boxes* and lines correspond to exon sequences (*dotted lines* indicating connecting regions of variable length omitted from the diagram for simplification). snRNA segments are shown in *black* or *dark gray*, and *pink spheres* represent Mg^{2+} ions. The catalytic triad is shown in *red*; the unpaired CA region in intron domain V and the equivalent segment in the U6 snRNA are shown in *green* and the bulged adenosine (A) region is shown in *violet*. The nucleophilic attack of the bulged adenosine on the 5′ exon-intron junction is indicated by a *red arrow. Orange bars* correspond to the EBSs/IBSs interaction. Other long-range contacts are identified by *yellow* and *light gray bars*

The 5′ and 3′ splice sites are recognized principally by base pairing. The EBS1-IBS1 interaction is crucial to 5′ splice site recognition during the splicing reaction. By contrast, the EBS2-IBS2 duplex is entirely dispensable for catalysis and splice site fidelity [36, 54]. Indeed, group IIC introns naturally lack the EBS2-IBS2 interaction, instead requiring the recognition of an upstream transcription terminator stem-loop [21]. Similarly, recognition of the 3′ splice site involves base pairing between the first nucleotide of the 3′ exon and the nucleotide preceding the EBS1 sequence (δ) in group IIA introns, or the EBS3 residue located in the coordination loop in group IIB and IIC introns [55, 56]. In addition to the long-range interactions underlying the catalytic conformation, tertiary contacts and structural constraints determine the efficiency and fidelity of identification, for both splice sites [11, 36, 57] (Fig. 8.2b, left panel).

Degeneration is common in organellar group II introns, for both RNA structures and ORF motifs. Thus, the intron ribozyme may be encoded in two or more pieces located at different positions in the genome, with disruptions often observed within dIV [58]. The intron and the flanking exons are transcribed separately, in a manner similar to that for *cis*-splicing introns, making the ligation of two distant exons possible. *Trans*-splicing is generally reported in higher plants, and this process requires the assistance of host-encoded protein factors [59]. A more dramatic process, intron fragmentation, has been observed in the chloroplast genome of *Euglena gracilis*, in which 155 small group II intron fragments are found [60].

Alternative splicing reactions have also been reported for bacterial and organellar group II introns [61–63]. These reactions occur at low frequency and result from misrecognition of the 3′ or 5′ splice site, inducing ORF truncations or small insertions. Alternative excision was initially thought to result in unproductive processing, but a recent study has revealed that this constitutive regulated process involving an unknown mechanism generates four functional surface-layer protein isoforms in the human pathogen *Clostridium tetani* [64].

Proteins Assisting Intron Excision

The efficiency of group II intron splicing in vivo is dependent on two groups of proteins [8]: those encoded by intron domain IV (IEPs), which are involved in *cis-splicing* reactions and found mostly in bacteria, and a group of host-encoded proteins with diverse functions, mediating the *trans*-splicing of organellar group II introns in yeasts, algae and higher plants [31, 65]. IEPs promote the splicing of group II introns in the maturase domain. They are highly specific splicing factors, playing little or no role in the excision of any intron other than the intron that encodes them [66–68]. They are usually expressed in *cis*, but they can promote the splicing of genomic copies of ORF-less introns [68, 69].

The organellar introns have diverged considerably from their bacterial ancestors, through a decrease in the number of maturase-encoding genes. A few intron-encoded ORFs are found in the mitochondria of lower eukaryotes (i.e., *Marchantia polymorpha*), but only one organelle-encoded protein has been reported to assist in the splicing of about 20 group II introns in vascular plants. This protein is called MatR in mitochondria, and MatK in chloroplasts [70, 71]. Moreover, a series of maturase-related proteins (nMat1a, nMat1b, nMat2a, and nMat2b) have been identified in the nucleus of angiosperms. After translation, these proteins are imported into mitochondria and chloroplasts to mediate the excision of group II introns [72–74]. An extensive search of the genomic sequences of recently sequenced green algae and land-plant mitochondria identified a number of new genes potentially encoding maturases, the role of which in the maturation of group II introns remains to be elucidated [75].

A plethora of nuclear-encoded proteins from various families, with diverse functions, has been reported to contribute to the correct folding and excision of organellar group II introns [9, 31, 65, 76]. One of the most numerous and well characterized groups of proteins identified is the DEAD-box proteins, which act as ATP-dependent RNA chaperones, ensuring the correct folding of the ribozyme or resolving inactive kinetic traps and then triggering productive RNA folding (the yeast factor MSS116, CYT-19 in *N. crassa*; Ded1 in *S. cerevisiae*; SrmB in *E. coli*; PMH2 in *A. thaliana*) [77–80]. Only a small number of these proteins (MSS116, CYT-19 and Ded1) can stimulate group II intron splicing in vitro in near-physiological conditions, suggesting that additional cofactors must be required to trigger splicing in vivo. Genetic and biochemical data have shown that organellar group II intron RNAs and multiple protein factors form functional high-molecular weight, spliceosome-like complexes [81, 82] (Fig. 8.2b).

Group II Intron Mobility

In addition to acting as catalytic RNAs, some group II introns are also target-specific mobile genetic elements (recently reviewed in [8, 83]). Mobile group II introns can insert site-specifically into a DNA sequence identical to the splice site (homing), at a frequency of up to 100 % [84–86], or more randomly, at low frequency, into ectopic sites (transposition) [87–89]. These mechanisms occur through an RNA intermediate and are referred to as retrohoming and retrotransposition, respectively [90, 91]. Both events occur via full reverse splicing, mediated by a ribonucleoprotein particle (RNP) formed by the association of the IEP with DIVa and the catalytic core regions of the excised intron RNA [92]. The IEP is essential for maintenance of the active intron RNA structure, to ensure that the intron can reverse splice into the DNA target site. Group II intron mobility mechanisms were first studied for the yeast mtDNA introns aI1 and aI2 [93–96], and have since been investigated in bacteria [66, 90, 97]. The main difference between yeast and bacterial mobile introns is that one or both exons may accompany the intron (co-conversion of flanking exons) in yeast, but not in bacteria [98].

The retrohoming of group II introns is highly site-specific, because a ≈20–25-bp DNA target sequence is recognized by the RNP via domain D or other regions. The IEP recognizes the upstream (positions −23 to −1) and downstream (positions +4 to +9) exon DNA sequences (Fig. 8.3a). Thirteen nucleotides of the DNA target are recognized by base pairing between the intron RNA and exon sequences, through EBS2/IBS2, EBS1/IBS1 and either δ-δ′ (IIA introns) or EBS3/IBS3 (IIB, IIC introns) interactions (Fig. 8.3b; [12, 32, 99, 100]). The essential role of each of the

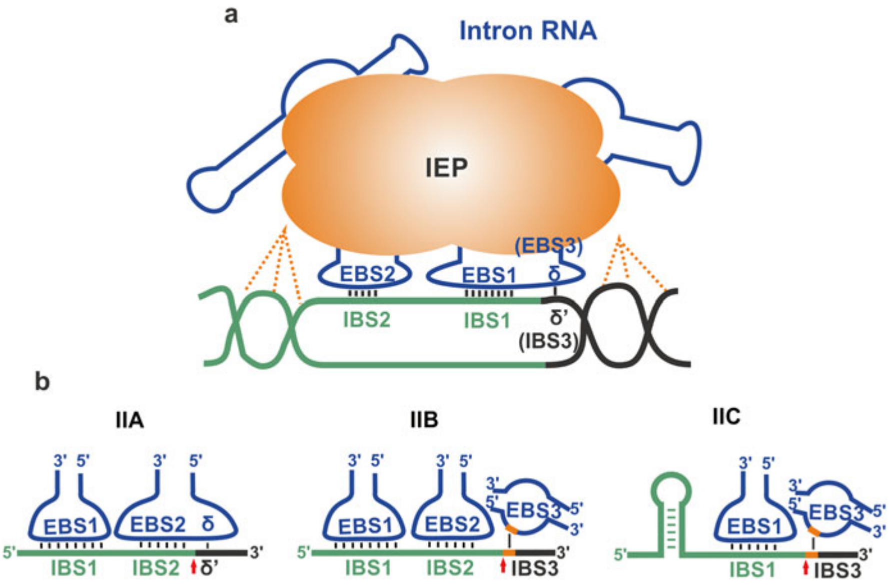

Fig. 8.3 DNA target site recognition: (**a**) RNP complexes recognize the target site on double- or single-stranded DNA primarily through EBS-IBS pairing (and by δ–δ′ interactions in subgroup IIA), whereas the IEP also binds specifically to key nucleotide residues in distal 5′ and 3′ exon regions indicated by *dotted lines* in the diagram. (**b**) Comparison between the base-pairing interactions used by group IIA, IIB and IIC introns for DNA target site recognition. *EBS* exon binding site, *IBS* intron binding site

base-pairing interactions has been demonstrated by mutating the DNA target site and observing the inhibition of reverse splicing in vitro or of intron mobility in vivo. These mutations can be rescued by compensatory mutations in the intron RNA. DNA target specificity is mostly controlled by the intron RNA, as the IEP can recognize only a few nucleotide positions. Initial recognition appears to involve interactions in the major groove between the IEP and key bases in the distal region of the 5′ exon, in the chain into which the intron subsequently reverse splices [101]. These interactions, enhanced by contact between the phosphate backbone and the IEP, involve unwinding of the DNA, allowing the reverse splicing of the intron RNA by pairing with IBS and/or δ sequences. DNA target sites have been defined experimentally for the yeast introns *coxI*-I1 [102] and *coxI*-I2 [103], the *L. lactis* Ll.LtrB intron [99], the *S. meliloti* RmInt1 intron [104], the *B. halodurans B.h.*I1 intron [21], the *E. coli E.c.*I5 intron [105], the *T. elongatus T.e.*I4h intron [106] and the *Enterobacter cloacae* group IIC *E.cl.*GOC intron [107]. Group IIA, IIB and IIC introns differ in terms of DNA target site recognition, and these differences affect design and performance in the biotechnological context (Fig. 8.3b).

Group II Intron Mobility Pathways

After reverse splicing of the RNA into the DNA target site, at least three different mechanisms may complete the mobility of yeast group II introns [96, 108]. One of these mechanisms involves a minor pathway through which a small proportion of the intron mobility events occur in natural conditions without the coconversion of exons, probably through the synthesis of a full-length intron cDNA, which is joined by DNA repair. A second RT-independent pathway (≈40 %), in which intron integration occurs by homologous recombination of both the 5′ and 3′ exon sequences, involves the repair of the nicks generated by RNPs in the DNA target, by the double-strand break reaction (DSBR) mechanism (Fig. 8.4c). Finally, the major pathway (≈60 %) entails the coconversion of the 5′ exon only, and involves cDNA synthesis by target-primed reverse transcription (TPRT) and the integration of the intron by homologous recombination (DSBR) (Fig. 8.4a, b).

The bacterial group II intron mobility pathway was first described for the *L. lactis* Ll.LtrB IIA intron, in studies involving in vivo plasmid-based genetic assays in both *L. lactis* and *E. coli* [66, 90]. Retrohoming was subsequently characterized by analyzing the biochemical characteristics of RNPs reconstituted from the purified IEP (LtrA) and in vivo excised lariat RNA [67, 109]. In vivo, the RNPs bind the DNA nonspecifically and then scan for the accurate target site by facilitated diffusion [109]. Ll.LtrB RNPs recognize a relatively long target region through three sequence motifs in DI of the RNA (EBS1, EBS2 and δ), and they base pair to complementary sequences in the DNA target site (IBS1, IBS2 and δ′) and through interactions of the IEP with nucleotides located in positions −25 to +9 of the insertion site (Fig. 8.3a) [83, 99, 100, 110, 111]. Once the target has been recognized, retrohoming occurs by TPRT (Fig. 8.4d). The RNA cleaves the sense strand of the double-stranded DNA at the exon junctions, and the intron RNA integrates into the target site. At the same time, LtrA cleaves the antisense strand at position +9, through its En activity. The 3′ end of the antisense strand is used by the RT domain of the IEP for the reverse transcription of the inserted RNA intron. The resulting cDNA is then integrated into the host DNA by homologous recombination-independent repair mechanisms [90].

Some mobile group IIB introns have an IEP with no En domain. Their IEPs have RT activity, and their RNPs can mediate reverse splicing into double- or single-stranded DNA substrates but cannot carry out site-specific second-strand cleavage; they therefore require a variant of the TPRT retrohoming pathway (Fig. 8.4e) [91, 105, 112, 113]. Most En$^-$ group II introns are inserted into the strand used as a template for synthesis of the lagging strand during replication [114]. They can therefore insert into single-stranded DNA only when the replication fork has overtaken the insertion site. The IEP thus uses the nascent lagging strand to prime reverse transcription [91]. Other mechanisms have also been suggested for the initiation of the cDNA synthesis, including random nicks in the antisense strand (*Schizosaccharomyces pombe cob*-I1 intron, [115]), or de novo initiation (RT encoded by the Mauriceville

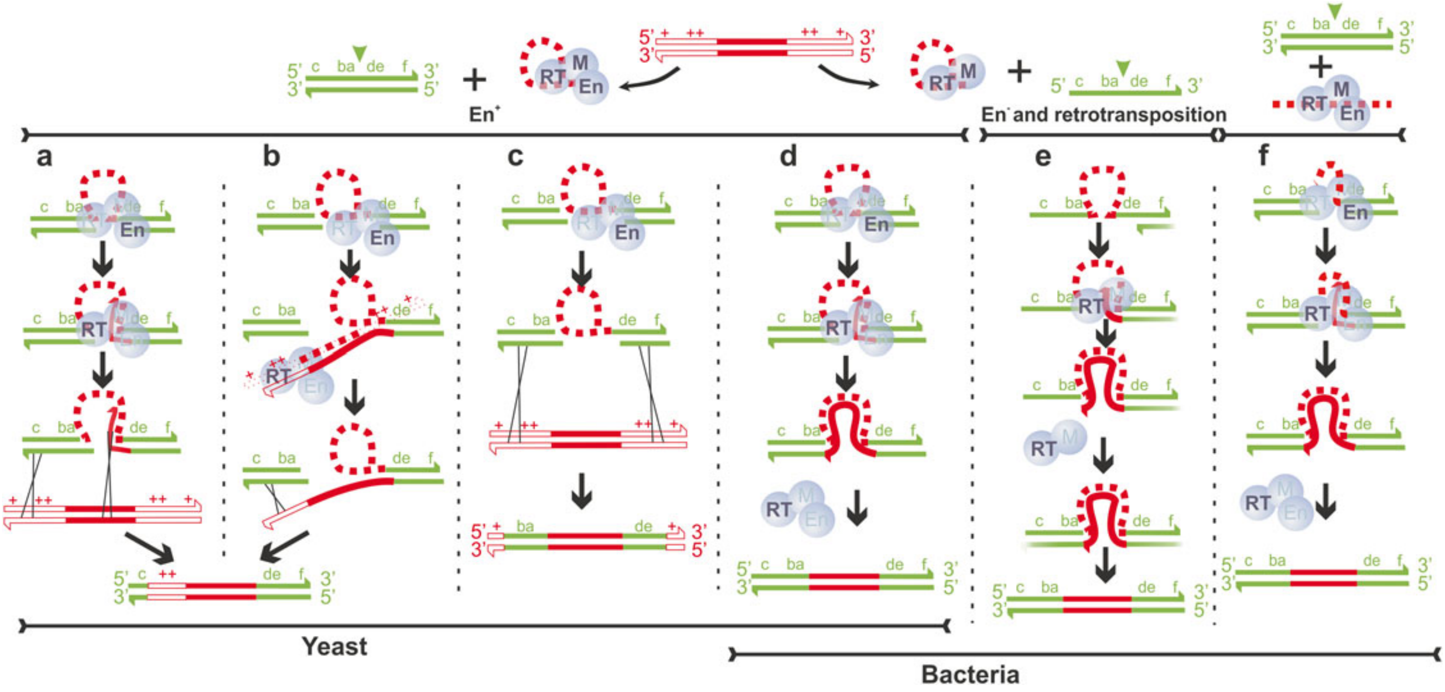

Fig. 8.4 Group II intron mobility pathways: Mechanisms *a*, *b* and *c* have been described only in yeast; all these mechanisms are dependent on homologous recombination. *a* and *b* are the major retrohoming pathways in yeast, whereas *c* is the minor pathway and is RT-independent. *d* is the major pathway in Ll.ltrB and a minor pathway in yeasts. This mechanism is independent of homologous recombination. *e* is the retrohoming pathway for introns lacking the En domain and the mechanism associated with retrotransposition. *f* is the retrohoming pathway for linear group II introns

plasmid in *Neurospora*, [116]). The best studied IIB-like intron is RmInt1 [69, 91, 112], which recognizes a DNA target site extending 20 nt into the 5′ exon and 5 nt into the 3′ exon. Target recognition occurs primarily by base-pairing between the EBS1, EBS2 and EBS3 of the intron RNA and the corresponding IBS sequences in the DNA target. The RmInt1 RT recognizes two critical nucleotide residues, possibly with the contribution of additional sequences [104, 112].

Unlike group IIA and IIB introns, group IIC introns have limited specificity due to the recognition of short IBS1 and IBS3 sequences (Fig. 8.3b). Moreover, the IBS2/EBS2 pairing seems to be replaced by the recognition of a palindromic Rho-independent transcription terminator motif or phage attachment site (*attC* sites), through an as yet unidentified mechanism [21, 37, 116–118]. Group IIC intron-encoded proteins also lack the En domain, but retain both domain Z (DNA binding) and domain X (maturase activity) [107]. Introns of this kind are found after non-identical terminators, inserted into the top or bottom strand, with a leading or inverse orientation, respectively [12, 107]. The integration of these introns resembles that of IIB introns, as it occurs through reverse splicing into single-stranded DNA at the replication fork or transcription bubble, with the nascent lagging strand preferentially used to prime reverse transcription of the intron.

It was thought that linear introns could not undergo reverse splicing [119–121], but recent studies have shown that yeast and bacterial linear group II introns reverse splice efficiently (Fig. 8.4f) [122–124]. The retrohoming of linear Ll.LtrB intron was demonstrated in eukaryotes, by the microinjection of RNPs into *Xenopus laevis* oocyte nuclei or *Drosophila melanogaster* embryos [123, 124]. The linear RNA undergoes the first reverse splicing reaction, becoming attached to the 3′ exon but not to the 5′ exon. The IEP then reverse transcribes the RNA, and the cDNA is ligated to the 5′ exon by the non-homologous end-joining (NHEJ) factor Lig 4 and the DNA repair polymerase θ (polQ). Other DNA ligases and polymerases can also perform this function, but at lower efficiency [124]. This mechanism may also mediate the retrohoming of linear RNAs, not only in eukaryotes, but also in many prokaryotes with homologous NHEJ machinery [8, 125].

Group II introns can also retrotranspose to ectopic DNA target sites, albeit at low frequency (10^{-4}–10^{-5}) [88, 114, 126–128]. The pattern of spread of Ll.LtrB within the *L. lactis* genome is consistent with intron retrotransposition into double- or single-stranded DNA through a homologous recombination-independent mechanism [114], similar to that described for the mitochondrial and bacterial RmInt1 introns [32]. In *L. lactis*, the retrotransposition of the Ll.ltrB intron is biased towards reverse splicing into transiently single-stranded DNA, with priming by the nascent lagging strand (Fig. 8.4e). By contrast, the retrotransposition of Ll.LtrB in *E. coli* is characterized by the preferential use of double-stranded DNA targets, with or without En cleavage of the opposite strand [129], indicating a role of the host cell, in addition to the intron, in pathway selection [130].

Host Factors Influencing the Retrohoming Pathway of Group II Introns

Mobile group II introns are genetic elements with specific molecular characteristics favoring their retention and spread in the genome. However, their mobility depends on the genetic background of the host, and retrohoming is dependent on the completion of cell functions [111]. The replication machinery of the cell is required in the early stages, but the host repair machinery is essential during late stages of retrohoming. The first experiments performed with the group II intron Ll.LtrB from *L. lactis* in the heterologous host *E. coli* [131] led to the formulation of a model of retrohoming involving host factors that either increased or decreased the efficiency of mobility. Thus, exonucleases (Recj, MutD, and PolI) cutting the ends of the DNA, RNases (RNase H) degrading the RNA template after cDNA synthesis, DNA and repair polymerase complexes (PolII, PolIII, PolIV and PolV) ensuring correct synthesis of the second DNA chain and DNA ligases all facilitate intron mobility. By contrast, degradative enzymes may decrease retrohoming levels. For example, RNase I and E, may eliminate the intron RNA, and exonuclease III (XthA) may degrade the newly synthesized cDNA or top strand in the upstream exon. Further studies revealed that some enzymes from the degradosome (RNase E) may affect retrohoming levels, depending on the physiological status of the cell [132]. It was subsequently shown that Ll.LtrB mobility was influenced by cell interactions and responses to cellular or environmental stresses, through global regulators [133–136]. One recent study [137] confirmed previous findings and revealed, through genetic and biochemical analyses, a possible role for replication restart proteins in the retrohoming mechanism.

Use of Group II Introns in Biotechnology

Group II introns have a number of characteristics that render them suitable for use as biotechnological tools: (1) they integrate into their DNA targets highly efficiently, in a homologous recombination-independent manner; (2) they can mobilize foreign DNA inserted within the intron; (3) minimal host functions, in the form of common cellular DNA repair mechanisms, are required for intron integration and (4) group II introns recognize the target DNA mostly through base pairing with the intron RNA. This last characteristic makes it possible to change intron specificity simply by changing the EBS/δ sequences. Currently, Ll.LtrB [138] a group IIA intron from *Lactoccocus lactis*, and the group IIB introns EcI5 [105] from *Escherichia coli* and RmInt1 from *Sinorhizobium meliloti* [139] are used as biotechnological tools. A chimeric intron based on the TeI3c ribozyme and the TeI4c IEP from *Thermosynechococcus elongatus* have also been used for gene targeting in thermophilic bacteria [140].

Introns were initially modified for the recognition of new target sites by identifying target sites matching the requirements of the IEP and then modifying the EBS/δ sequences to ensure base pairing with the new IBS/δ′ sequences. The retargeted introns are known as targetrons. Several algorithms have been developed for the retargeting of the Ll.LtrB [138], EcI5 [105] and RmInt1 [141] introns. These algorithms are based on the observed nucleotides frequencies obtained in invasion experiments using both randomized EBSs/δ-intron donor libraries and randomized IBSs/δ′-intron target site libraries. The algorithm scores a DNA sequence across a sliding window with 1 bp increments. The length of the sliding window depends on the intron: 45 bp for Ll.LtrB, 36 bp for EcI5 and 25 bp for RmInt1. A score is assigned to the potential target sites identified, with higher scores associated with a greater probability of a high invasion frequency. For each algorithm, a threshold value has been defined, above which the retargeted intron insertion frequency is high enough for the identification of intron insertion into the selected new target site in a simply assay, such as colony PCR. Once the best potential target site has been identified, the EBSs/δ sequences of the introns are modified to ensure base pairing with the new target site and are inserted into the intron donor plasmid, in which the IBSs/δ′ regions of the flanking exons are also modified to provide complementarity with the modified EBS/δ regions, for efficient RNA splicing. Intron donor plasmids also contain the IEP sequence, together with the corresponding intron from a position outside the DIV of the ribozyme (ΔORF) and downstream from the intron RNA. This conformation has been shown to be more efficient for retrohoming than the wild-type conformation with the IEP within DIV of the intron RNA [69, 105, 110]. Different promoters have been used for expression of the targetron and the associated IEP: constitutive promoters, such as the Km promoter used for the RmInt1 targetron [69], the T7 promoter recognized by the T7 RNA polymerase used in the expression of EcI5 and Ll.LtrB targetrons in *E. coli*, inducible promoters, such as the m-toluic acid-inducible promoter or tac promoter [142, 143] and endogenous promoters from the bacterial strain in which the targetron is used [144–146].

Intron integration can be detected by colony PCR or through the use of a selectable marker such as an antibiotic resistance gene. A retrotransposition-activated marker (RAM) has been developed for this purpose [145, 147]. The RAM cassette is based on a selectable marker with its own promoter inserted in reverse orientation into group II intron domain IV of the intron RNA. The marker is interrupted by the td group I intron in the forward orientation. The selectable marker is thus expressed only if retrohoming occurs. Subsequent modifications, with the selectable marker flanked by FRT sites recognized by the site-specific recombinase Flp, made it possible to remove the marker gene and led to the adaptation of the system for multiple gene disruptions.

Retargeted introns have been used in various species of the genera *Agrobacterium* [142], *Azospirillum* [148], *Bacillus* [149], *Clostridium* [145], *Ehrlichia* [150], *Escherichia* [105], *Francisella* [146], *Lactococcus* [151], *Listeria* [152], *Paenibacillus* [153], *Pasteurella* [154], *Proteus* [155], *Pseudomonas* [142], *Ralstonia* [143], *Salmonella* [111], *Shewanella* [156], *Shigella* [111], *Sinorhizobium* [139], *Sodalis* [157], *Staphylococcus* [144], *Vibrio* [158], and *Yersinia* [159]. In bacteria, targetrons are used primarily to obtain knockout mutants. In *Clostridium*, a genus in which transformation is difficult, retargeted intron derivatives of Ll.LtrB, known as ClosTron [145], have proved useful in several studies of the biology of the various species. When retargeted ΔORF introns insert into the sense strand, a conditional disruption is obtained as splicing can take place if the IEP is expressed, even *in trans*. However, intron targeting to the antisense strand leads to an unconditional mutation.

It is also possible to use targetrons to deliver foreign DNA into specific sequences [151, 160, 161]. The cargo gene is transported into the deleted region of DIV. For Ll.LtrB, fragments of less than 100 bp in length have a slight effect on intron insertion, but mobility efficiency is greatly reduced by fragments of more than 1 kb [162]. The secondary structure of the cargo sequences also affects intron mobility [156].

A method for bacterial genome editing using both targetrons and the Cre/lox system has recently been used [156]. This system has been used for insertions of 12 kb and deletions of up to 120 kb in *E. coli* and *S. aureus*, inversions in *E. coli* and *Bacillus subtilis* and one-step cut-and-paste manipulations for the translocation of 120 kb of genomic sequence to a site 1.5 Mb away.

Group II introns (Ll.LtrB) have also been used in eukaryotic cells [163]. This approach is less well developed in eukaryotes than in prokaryotes and several hurdles have yet to be overcome. The principal problem concerns the concentration of Mg^{2+}, which is below that required for the movement of Ll.LtrB in eukaryotic cells. Furthermore, the chromatinization of cellular DNA strongly inhibits intron integration. In eukaryotic cells, group II introns are microinjected into the cell nucleus as in vitro reconstituted ribonucleoproteins (RNPs). *Xenopus laevis* oocytes, and embryos of *Drosophila melanogaster* and zebrafish have been used for this purpose. RNPs have been reconstituted with both lariat and linear RNAs. In addition to the RNPs, a mixture of 500 mM Mg^{2+} and 17–20 mM each of dATP, dCTP, dGTP and dTTP is also injected into the nucleus, to optimize intron insertion. The RNPs and Mg^{2+} must be injected separately, because RNPs precipitate at this Mg^{2+} concentration. In these conditions, lariat RNPs injected into the *X. laevis* oocyte nuclei can both insert into an injected plasmid target at high frequency and stimulate DNA integration by homologous recombination, by producing target-site double-strand breaks. In *D. melanogaster* embryos, intron integration into the *yellow* gene has been achieved with introns retargeted against this gene. More knowledge is required about the behaviour of group II introns in eukaryotic cells, for the development of tools for use in eukaryotes.

Evolutionary Aspects of Bacterial Group II Introns

Group II introns display structural, functional and mechanistic similarities to eukaryotic pre-mRNA nuclear introns [164–168]. Nuclear pre-messenger RNA introns [11] and non-long terminal repeat retrotransposons may have evolved from mobile group II introns [169]. It has been suggested [2, 168, 170] that, at an early stage in the evolution of eukaryotes, the ancestral group II intron structure was split into the non-catalytic spliceosomal introns and the catalytically active RNA component of the spliceosome. This transition was accompanied by the degradation of the reverse transcriptase ORF. Maturases may have persisted in plants, during evolution, through the acquisition of a targeting signal enabling them to function within the organelles, to support the splicing of organellar group II introns [75, 171]. The evolution of eukaryotic cell organization may also have been a defensive response to the deleterious effect of group II intron proliferation in the host genome [172, 173]. Nevertheless, a recent report [174] suggests that the compartmentalization of eukaryotic cells into nucleus and cytoplasm does not prevent group II intron invasion of the host genome, although it may control proliferation of the intron, through transient or stable nucleolar sequestration. Strikingly, when the IEP loses its maturase activity, the protein becomes localized in nuclear speckles, domains of the nucleus enriched in pre-mRNA splicing factors [175], including small nuclear ribonucleoproteins (snRNPs) and serine-arginine (SR) proteins located in the interchromatin regions of the nucleoplasm. This is consistent with the hypothesis that eukaryotic spliceosomal introns may have evolved from group II introns.

Bacterial group II introns are tending to evolve towards an inactive form by fragmentation, with the loss of the 3′ terminus, including the IEP [176, 177]. The significance of fragmented introns within a particular genome remains unclear. It has recently been suggested that, as for transposable elements (TEs), the dispersal and dynamics of group II intron spread within a bacterial genome follows a selection-driven extinction model, predicting the removal of highly colonized genomes from the population by purifying selection [178]. Only 25 % of the bacterial genomes sequenced to date [8] harbor recognizable group II introns. This suggests that these introns did not act as a major force with a broad effect in the promotion of evolutionary change, but caution is required in the interpretation of these observations, because the 5′ end of fragmented intron sequences lacking the encoded ORF is unlikely to have been detected in sequenced bacterial genomes.

It is generally accepted that the "selfish" features of mobile elements underlie their acquisition and maintenance in bacterial genomes, but these elements may also be beneficial to the host. In bacteria, group II introns are thought to be tolerated to some extent because they self-splice and preferentially home to sites outside key functional genes, generally within intergenic regions or other mobile genetic elements [179]. Other studies have suggested that group II introns are beneficial to the host because they control other potentially harmful mobile genetic elements [180], and contribute to the generation of diversity and the remodeling of genomes in times of stress [135]. These features may decrease negative effects on the host organism, resulting in the maintenance of these retroelements for longer periods in bacterial populations. It also seems likely that the gradual eradication of group II introns by the host during evolution would not result in the complete elimination of intron sequences, with some intron fragments remaining and continuing to evolve in the genome. It thus remains possible that these fragments provide sequence variation on which selection can act, leading to their persistence and continuing evolution in the genomes of some bacterial lineages [181].

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Chapter 9
Centromeres in Health, Disease and Evolution

Thian Thian Beh and Paul Kalitsis

Abbreviations

ANIFAD	Associated network instability, and facial anomalies division
ALP-WDLPS	Atypical lipomas and well-differentiated liposarcomas
AML	Acute myeloid leukemia
ATLL	Adult T-cell leukemia/lymphoma
CCAN	Constitutive centromere-associated network
CENP	Centromere protein
CGI	CpG island
CIN	Chromosomal instability
CLL	Chronic lymphocytic leukemia
CPC	Chromosome passenger complex
FISH	Fluorescence in situ hybridization
HOR	Higher order repeat
ICF	Immunodeficiency, centromeric instability, and facial anomalies
MVA	Mosaic variegated aneuploidy
PCD	Premature centromere division
PCS	Premature chromatid separation
ROB	Robertsonian translocation
SAC	Spindle assembly checkpoint
TE	Transposable element

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Introduction

The term centromere (*kentron*, center; *meros*, part), initially coined by Waldeyer in 1903 for the neck of sperm, was reinterpreted by Darlington in 1936 as the centric constriction on metaphase chromosomes to which spindle fibers attach during cell division [1]. Centromeres were cytologically distinguished by their constricted morphological appearance and with C-banding which is a Giemsa staining procedure that preferentially stains the heterochromatin regions [2]. Now, fluorescence in situ hybridization (FISH) probes against centromeric DNA of specific chromosome and antibodies against centromere proteins are commonly used for the localization of centromeric regions [3–5].

The human centromere is a region on the chromosome consisting of an underlying alpha satellite repetitive DNA sequence that winds around nucleosomes containing centromere protein (CENP)-A, a histone H3 variant. Hence, CENP-A is the epigenetic mark of a centromere and is one of the 17 proteins forming the constitutive centromere-associated network (CCAN) which is crucial in marking and maintaining the active centromere throughout the cell cycle [6, 7]. The kinetochore, on the other hand, is important in providing an interface for spindle microtubule binding, stabilizing correct attachments and participating in the spindle assembly checkpoint (SAC), as well as the movement of sister chromatids towards opposite poles during anaphase [8].

Together, the function of the centromere and kinetochore is to ensure high fidelity of chromosome segregation during cell division because an erroneous chromosome segregation can lead to cell arrest or cell death, or more dangerously, chromosomal instability (CIN) and aneuploidy in the daughter cells. CIN, the rate of karyotypic change resulting in anomalous organization and/or number of chromosomes, has been reported as one of the key features in cancer cells and was postulated to precede aneuploidy. Aneuploidy, however, is the karyotypic state depicted by abnormal number of chromosomes and has long been associated with carcinogenesis and birth disorders. The first suggestion of a possible link between aneuploidy and cancer was in the monograph published by Boveri in 1914 [9–11].

Health

The main functional role of the centromere is to ensure that replicated chromosomes are distributed equally to daughter cells during cell division. These functions can be divided into the following classes: (1) Genetic/epigenetic marking or identity of the locus along a specified region of each chromosome, (2) SAC and correct attachment of microtubules, (3) sister chromatid cohesion and release, (4) movement of chromosomes to opposing poles and (5) cytokinesis where a group of transient proteins mark the site for the final separation of the daughter cells.

Centromere Structure

The centromere is comprised of three main zones (Fig. 9.1); (1) the cohesion zone that holds the two replicated sister chromatids together until the onset of anaphase, (2) the DNA interface where proteins directly interact with centromere DNA to mark and maintain the centromere site between cell divisions, and (3) a platform for the capture of spindle microtubules—this zone is commonly referred to as the kinetochore.

Centromere DNA

Human centromere DNA is composed of a tandemly repeated AT-rich monomer of 171 bp commonly known as alpha satellite [12]. This repeat is organized into higher order repeats (HORs) ranging in size from 2 to 35 monomers, which are then organized into further tandem arrays spanning (250 kb to 3 Mb) (Fig. 9.2) [13]. One feature of alpha satellite HORs is that they have chromosome specificity [4]. Differences in the primary sequence of each HOR monomer repeat give rise to its unique chromosome specificity. This difference allows researchers and diagnostic scientists to use techniques such as FISH to identify single chromosomes such as the X or the Y. However, not all chromosomes can be distinguished by a single alpha

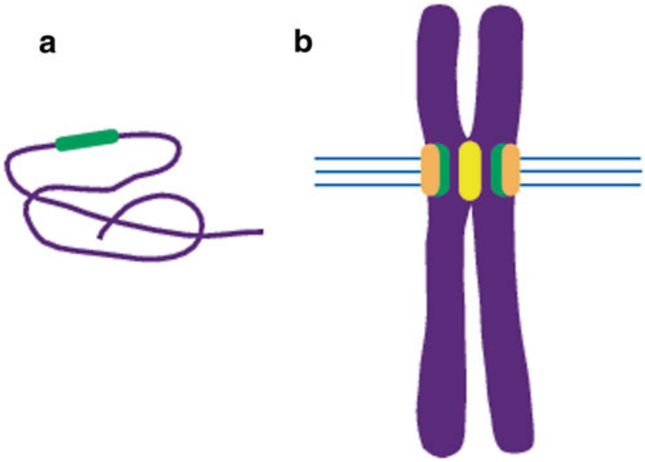

Fig. 9.1 Centromere structure during interphase and metaphase. (**a**) The centromere locus is marked by a group of proteins (*green*) known as the CCAN complex which are found at the same chromosomal site throughout the cell cycle. (b) After DNA replication, the chromatin (*purple*) condenses to form the mature metaphase chromosome attached to spindle microtubules (*blue*). It is during this stage that the centromere attracts other proteins involved in microtubule spindle attachment (*orange*) and sister centromere cohesion (*yellow*)

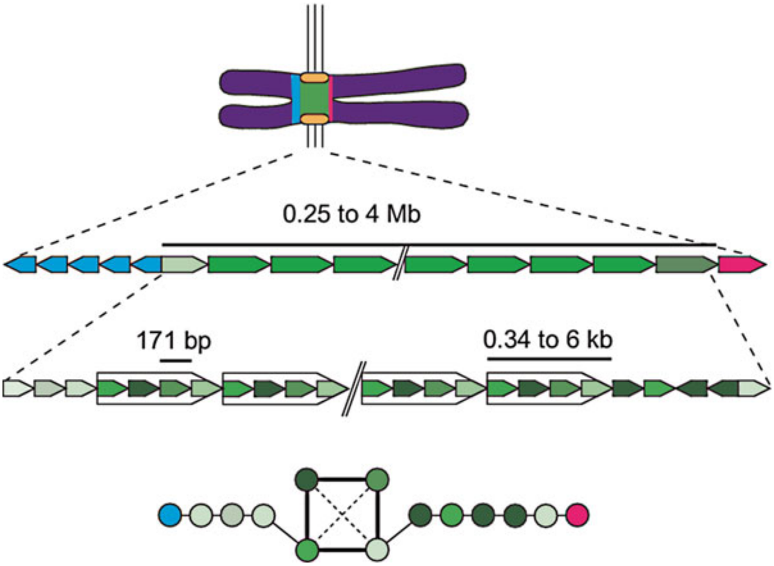

Fig. 9.2 Schematic illustrating the genomic organization of human centromeres. A condensed metaphase chromosome showing centromeric alpha satellite (*green*) and pericentric DNA families (*blue* and *pink*). Note that the repetitive pericentromeric DNA is composed of different sized monomer sequences that are not similar to alpha satellite. The fundamental repeating unit of alpha satellite is an AT-rich 171 bp unit. This is organized into higher order repeats (HORs) that have a high level of sequence identity. Individual centromeres can be distinguished by their HOR type which varies in length and sequence structure. Furthermore, the overall length of the alpha satellite domain is highly polymorphic between individuals. An alternative way to view the organization of centromeric DNA is to break it up into monomer units shown as *colored circles*. The sequential order of each unit is linked with a line and the HOR monomer units are linked with thicker lines which represent multiple HORs

satellite HOR class. This is nicely illustrated in the acrocentric chromosomes, 13, 14, 15, 21 and 22. HORs from these chromosomes share a high level of sequence homology, where single chromosomes cannot be differentiated by hybridization techniques such as FISH or Southern blot.

Alpha satellite DNA also contains a conserved 17-bp motif known as the CENP-B box. This sequence is present in varying frequencies in alpha satellite HORs, ranging from 50 to 0 % in chromosomes 21 and Y, respectively [14]. The CENP-B protein binds to the CENP-B box and is thought to be important for the de novo assembly of the centromere. The formation and stability of artificial chromosomes in the laboratory is dependent on CENP-B-box rich DNA [15]. Paradoxically, once the chromosome is in the cell it does not need the CENP-B protein for full centromere function, as shown by several lines of evidence—(1) knockouts of the CENP-B gene in mouse exhibit full centromere function, develop normally and are fertile, (2) natural centromeres such as in the Y chromosome do not contain the CENP-B box, and (3) human neocentromeres form on DNA that has no alpha satellite or CENP-B box motifs [14, 16–19].

Alpha Satellite DNA Mapping and Sequencing

Alpha satellite DNA was one of the first sequences to be identified and sequenced, however when one examines the centromeric regions of the human reference genome it is quite apparent that they contain megabase-size gaps due to difficulties with contig assembly. While high-throughput genome sequencing has led to a revolution in rapidly identifying the molecular defects behind many human disorders, centromeres remain incomplete due to the short read length of the current parallel sequencing technologies, satellite DNA regions again suffer from poor assembly. Recently, novel computational methods involving unit monomer analysis has provided new ways in analyzing these regions. By grouping similar monomer units together predictions can be made that reveal the overall array length for haploid centromeres such as in the X and Y chromosomes can be made (Fig. 9.2) [20].

CENP-A and Alpha Satellite: Centromeric Chromatin

The centromere-specific histone H3 variant CENP-A (described in "Centromere Proteins" section below) is normally present within subsections of the HOR region of alpha satellite DNA. This has been shown with elegant anti-CENP-A and alpha satellite FISH experiments on extended chromatin fibers [21]. This subdomain structure of CENP-A and alpha satellite is considered to play a role in the three dimensional assembly of the mature mitotic centromere, since alpha satellite DNA is present within the inner (pairing) and outer (microtubule binding) regions of the centromere (Fig. 9.1).

Eviction of the Invaders

Unlike centromeres of multi-cellular eukaryotes, human centromeres are mostly made up of one class of DNA, alpha satellite DNA. It is rare to find the presence of LINE and SINE transposable elements (TEs) within the HOR array. What is the possible mechanism that keeps the intruders at bay? Detailed sequence map analysis at the border regions of alpha satellite has shown that the age of TE insertion decreases as one goes from outer non-alpha satellite DNA to the inner higher order alpha arrays [22]. This suggests that TEs are rapidly pushed away from the HOR region to the periphery. A simple mechanism that would explain this would be unequal crossing over between homologous chromosomes or sister chromatids, which also contributes to the evolution of centromere DNA.

Centromere Proteins

Classes of Centromere/Kinetochore Proteins

The centromere/kinetochore complex can be broadly classified into two main groups based on structure and function with respect to chromosome segregation. The centromere locus needs to be identified and maintained in one region per chromosome, this memory between cell cycles is maintained by a group of proteins that are present at the centromere throughout the cell cycle (Fig. 9.1). The second group falls into the active process of preparing and executing chromosome segregation. These proteins are present at the centromere/kinetochore in a transient manner beginning after DNA replication to the completion of telophase.

To date, over 100 proteins have been shown to locate to the centromere/kinetochore at some stage during the cell cycle. For the purpose of this chapter we are only including proteins that have multiple lines of evidence such as antibody and epitope fusion localization. Some proteins have been misclassified because of artifact signals from antibody staining experiments.

The first set of human centromere proteins discovered were identified using auto-immune sera from patients with scleroderma disease [5]. Protein immunoblotting uncovered three common antigens, named CENP-A, -B and -C in ascending molecular weight order [23]. Serendipitously, these three proteins bind to the centromere DNA and form the foundation platform onto which other centromere and kinetochore proteins assemble the mature, functional structure.

Centromere Function

Epigenetic Marking

Most eukaryotic centromeres are characterized by long tracts of repeat DNA, either satellite or transposable elements. Furthermore, this DNA was often specific to the centromeric locus, for example alpha satellite in humans. One popular hypothesis regarding the interaction between centromere DNA and protein was that the protein had specific DNA-binding affinity, such as the CENP-B protein binding to the CENP-B box motif in alpha satellite [14]. However, immuno-fluorescence analysis of variant chromosomes such as dicentrics or neocentromeres (described in “Disease” section below) showed that some centromere proteins were only present at functionally active centromeres whether alpha satellite DNA was present or absent, and other proteins were present at both active and inactive centromeres [19, 24]. This line of evidence showed that centromeres had genetic and epigenetic characteristics unlike their telomere counterparts which are strictly genetic.

CENP-A: The Primary Mark

CENP-A is a histone H3 variant that is only found at active centromeres [25]. It replaces both units of histone H3 of the histone octamer which provides the centromeric epigenetic mark and a chromatin platform onto which the constitutive centromere-associated network (CCAN) of proteins bind to [6] (see Table 9.1). Further evidence to support the foundation role of CENP-A is shown in gene knockout/knockdown studies which result in the loss of downstream centromere proteins and the absence of a functional kinetochore [26].

Spindle Assembly Checkpoint (SAC)

After chromosomes have replicated and condensed, they then are captured by the mitotic spindle via the interaction with the kinetochore. The chromatid pairs then shuffle between spindle poles to ensure that each sister centromere has attached to spindle microtubules emanating from one pole and thus achieving bi-orientated attachment. Once all chromosomes have acquired correct attachment and equal tension, the chromatids are then ready to segregate to opposite poles. The cell is able to detect the tension and signal for the beginning of anaphase. A group of proteins that are essential for the correct attachment of chromosomes were identified through elegant genetic screens in budding yeast [27, 28]. These spindle assembly checkpoint (SAC) proteins are conserved in humans and mutations elevate the rate of chromosome segregation errors and have a role in cancer predisposition (see "Disease" section).

Sister Centromere Cohesion

After DNA replication, sister chromatids need to be held together to prevent them from prematurely separating, which can result in mis-segregation. A conserved protein complex, known as cohesin, holds the sister chromatids together until the early stages of mitosis when cohesin is progressively removed from the arms and remains at the centromere region until the onset of anaphase. A protector protein, Shugoshin, binds to the centromeric pool of cohesin and thus prevents its premature removal [29]. So the last chromosomal region to be held together before anaphase is the inner centromere domain. In addition to mitosis, cohesin also plays an important role during meiosis I when homologous chromosomes are held together at the centromere by a meiotic-specific cohesin complex. It is hypothesized that weakening of this complex due to aging may contribute to higher rates of chromosomal non-disjunction in women of advanced maternal age (see "Disease" section).

Table 9.1 Centromere and kinetochore proteins organized into functional classes

Centromere protein complex	Proteins	Function
Constitutive Centromere-Associated Network (CCAN)	CENPA, CENPC, CENPH, CENPI, CENPK, CENPL, CENPM, CENPN, CENPO, CENPP, CENPQ, CENPR, CENPS, CENPT, CENPU, CENPW, CENPX	CCAN complex plays a central role in establishing a foundation for mitotic-specific kinetochore proteins.
MIS18 complex	MIS18A, MIS18B, KNL2, PLK1, HJURP	Licensing and loading of CENPA to centromeric chromatin.
KMN network	MIS12, KNL1, DSN1, PMF1, NSL1, NDC80, NUF2, SPC24, SPC25	Outer kinetochore complex required for correct chromosome alignment, mitotic checkpoint signalling and attachment of the kinetochore to microtubules.
RZZ complex	ROD, ZWILCH, ZW10, ZWINT	Mitotic checkpoint role, prevents cells from prematurely exiting mitosis
SKA complex	SKA1, SKA2, SKA3	Microtubule-binding complex required for correct chromosome segregation
Mitotic Checkpoint Complex	BUB1, BUB1B, MAD2L1, CDC20, BUB3, MAD1L1, TTK, AURKB	Ensures all chromosomes have bi-orient attachments to the mitotic spindle, corrects mis-alignment errors and signals onset of anaphase
Chromosome Passenger Complex	INCENP, SURVIVIN, AURKB, BOREALIN	Key regulating complex of mitosis, corrects chromosome mis-alignments, required for chromatin-induced microtubule stabilisation and marks the spindle midzone during anaphase.
Centromere Cohesion	CBX5, CBX1, CBX3, SGOL1, SGOL2, REC8	Holds sister centromeres together around pericentric heterochromatin, protects from premature chromatid separation.
Microtubule-Binding Proteins	CENPE, CENPF, CLASP1, CLASP2, CLIP1, DYNEIN, DYNACTIN, EB1, KIF18A, MCAK, PINX1	Microtubule motor and tracking proteins essential for movement and alignment of chromosomes during mitosis.

Most proteins in this list have been shown to localize to the centromere at some stage during the cell cycle and have a functional role in chromosome segregation. Many other proteins have been found at the centromere but have not been included in this table because they either do not have a clear role in chromosome segregation or their centromeric localization is secondary to their primary function

Chromosome Movement

One of the key roles of the kinetochore is to capture the spindle microtubules, align the chromosomes to the midzone and then move them to the opposite poles. Affinity biochemical experiments from yeast have shown that the budding yeast centromere comprises of one super-complex that binds to one microtubule. Humans contain around 20 microtubules per kinetochore attachment, thus there are multiple subunits that act together in concert [30]. Once each sister centromere is captured to the microtubules they then go through a pushing and pulling action between spindle poles to establish equal tension. This movement is partly triggered by motor, microtubule binding and checkpoint proteins. A protein complex at the heart of this process is the KMN network (Table 9.1). Again, like other complexes, it is conserved in a multitude of eukaryotic organisms and plays an essential role in chromosome segregation. Components of this complex are transiently present at the centromere and form a link between the centromeric chromatin and the outer kinetochore.

Cytokinesis

As described above, centromere cohesion plays an important role in holding the sister chromatids together until the beginning of anaphase. Additional roles include tension sensing and chromosome alignment or error correction. The complex of proteins at the heart of this region is the Chromosome Passenger Complex (CPC) [31]. This includes the four subunits, Aurora B kinase, INCENP, SURVIVIN and BOREALIN. Furthermore, the CPC has an additional role once chromosomes begin to move to opposite poles. They are left behind at the spindle midzone thus marking this region as the site of cellular/cytoplasmic constriction and eventual cleavage of the membranes and spindle microtubules to release the two daughter cells. Any defects in this later stage of mitosis can lead to cells with multiple copies of the genome (polyploidy) and are thought to be involved in tumour progression.

Disease

Structural abnormalities implicating the centromeric DNA, namely the presence of more than one centromere, repositioning of the centromere to a non-centromeric DNA site, prematurely separated centromeres, mutations and aberrant expression of centromere-associated kinetochore proteins, anomalous methylation and altered transcription of alpha satellite, as well as pericentric regions have all been associated with human diseases.

Chromosome Structural Abnormalities

Dicentric Chromosomes

Robertsonian translocations (ROBs) are the most common constitutional structural rearrangements in humans, observed at a rate of one in every thousand live births. ROBs involve whole-arm exchanges between two of the five non-homologous human acrocentric chromosomes (13, 14, 15, 21 and 22), giving rise to a karyotypically metacentric chromosome [32]. Carriers of balanced ROBs are generally normal but with increased risk of infertility due to conception of non-viable fetuses and also with elevated chance of having offspring with Down syndrome.

The other commonly reported constitutional dicentric chromosomes are the isodicentric X chromosomes especially idic(X)(p11) which could occur as both mosaic or non-mosaic. Idic(X)(p11) cases account for about 18 % of Turner syndrome patients, amounting to an incidence rate of approximately 1 in 14,000 females. Other dicentric X chromosomes might include rearranged derivatives of X chromosomes or isodicentrics that have breakpoints at sites other than Xp11 [33].

A rarer non-homologous, non-ROBs had also been reported to give rise to constitutional dicentric chromosomes. Thus far, only 27 cases were reported since the 1970s. Most cases (23/27) involved an acrocentric chromosome and 15/19 of cytogenetically distinguishable heterodicentric chromosomes had only one primary constriction whereby 12/15 of the inactivated centromere being the acrocentric centromeres. This is probably due to the relative stability of the dicentric formed as p-arm deletion of acrocentric chromosomes is not embryonic lethal and the centromeres of acrocentrics have higher tendency to become inactivated [34, 35].

Constitutional dicentric chromosomes are stably transmitted through cell divisions because one of the two centromeres is either inactivated via epigenetic mechanisms or deleted partially or fully (Fig. 9.3) [36, 37]. An inactivated centromere is positive for CENP-B but negative for the essential proteins, CENP-A, -C and -E and hence, is distinguishable from functionally active centromeres [38]. Stability of a dicentric chromosome with two functional centromeres could also be achieved through close proximity of the centromeres—an intercentromeric distance of less than 12 Mb as seen on isodicentric X chromosomes [39].

In malignancies, dicentric chromosomes are generally an outcome of telomere fusion events due to telomere instability of cancer cells as observed in giant cell tumor of the bone, meningioma, chronic lymphocytic leukemia (CLL), pancreatic cancer and osteosarcoma [40, 41]. However, most dicentric chromosomes in hematological malignancies arise from reciprocal translocation that produces a dicentric chromosome and an acentric chromosomal fragment which might be lost in subsequent mitoses. Thus far, the mechanism of centromere inactivation in malignancies has not been well studied. Investigations into the dicentric chromosomes of acute myeloid leukemia (AML) and myelodysplastic syndromes indicated that a repertoire of strategies namely functional (epigenetic) inactivation, intercentromeric deletion, inversion to reduce intercentromeric distance, and partial or full centromere excision were deployed to produce a more stable chromosome [41].

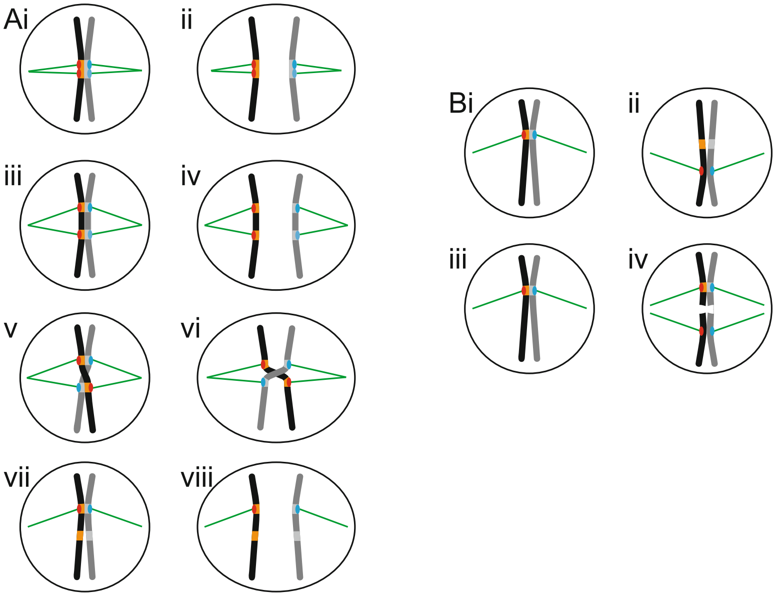

Fig. 9.3 Epigenetic status of the centromere in abnormal chromosomes. Replicated sister chromatids (*black* and *grey*) are shown aligned and attached to microtubules. The satellite-rich centromere DNA (*orange* and *light grey* shaded boxes) mark the centromere locus. Functionally active centromeres build a mature kinetochore (*red* and *blue ovals*) which capture spindle microtubules and move chromatids to opposite poles. (*Ai* and *ii*) Functional dicentric chromosomes with closely spaced centromeres act in unison to correctly segregate the chromatids. (*Aiii* and *iv*) Dicentric chromosomes with centromeres spaced further apart can also segregate correctly but (*Av* and *vi*) sister chromatids can twist between the two centromeres resulting in single chromatids attached to both poles which causes possible breakage of the chromosome. (*Avii* and *viii*) Epigenetic inactivation of one of the centromeres (loss of the kinetochore) resolves the conflict between the two active centromeres and thus chromosomes can correctly segregate. Neocentromeres form on non-alpha satellite DNA, often in euchromatic regions. (*B*) Two possible mechanisms of neocentromere formation, (*Bi* and *ii*) repositioning of the centromere to a new region along the chromosome. The old centromere is subsequently inactivated. (*Biii* and *iv*) Another mechanism shows a breakage and the formation of an acentric fragment. This chromosomal fragment is rescued by the formation of a kinetochore but the underlying alpha satellite DNA is absent

Neocentromeres

Neocentromere is the term coined for an ectopic centromere which forms in a region of the chromosome outside the repetitive alpha satellite DNA [19]. It binds all known centromere proteins except CENP-B and functions similarly to the native centromere [42] although the level of CENP-A incorporation [43], cohesion [44, 45] and error correction by Aurora B [46] appear to be lowered. Neocentromeres have been found in euchromatic sites and the formation of neocentromeres does not seem to correlate with reduced expression of the genes in those regions [47].

The first report of a constitutional human neocentromere in 1993 was from cytogenetic screening of a 4 year-old patient who was presented with delayed speech development [19]. Subsequent discoveries were made in patients with a wide spectrum of clinical presentations including facial dysmorphism and growth retardation in younger patients to infertility and high proportion of miscarriages in adult patients [47]. In children, several cancer types including retinoblastoma [48], Wilms tumor [49], cystic hygroma [50] and hemangioma [51] were reported as co-morbidities with the other developmental disorders.

In addition, neocentromeres have also been specifically associated with a few cancers thus far, namely AML, atypical lipomas and well-differentiated liposarcomas (ALP-WDLPS), lung sarcomatoid carcinoma and T-cell non-Hodgkin lymphoma [52]. The presence of neocentromeres on either a supernumerary ring or a long marker chromosome, both derived from the long arm of chromosome 12, is a defining characteristic of ALP-WDLPS of borderline malignancy [53]. These chromosomes have amplification of the 12q14-15 region containing oncogenes that include *MDM2* and *CDK4* [54]. However, the same amplified region is also found in other more aggressive liposarcomas but on chromosome 12 with alpha satellites suggesting that the neocentromere formed was to stabilize the complex rearranged acentric chromosome containing amplified 12q14-15 which might confer selective advantage within the tumor microenvironment besides highlighting the difference between neocentromere and the native centromere with alpha satellites [47].

Premature Centromere Division

Premature centromere division (PCD; OMIM #212790) is a cytogenetically detectable trait where the X chromosome appears to have no discernible centromere resulting in a rod-shaped X chromosome. The frequency of lymphocytes showing PCD and DNA damage increases as we age but for sporadic Alzheimer's disease patients, the increased frequency was even more significant when compared to their age-matched controls. In addition, PCD was shown to be consistently more prominent in females than males and was thought to be the cause of chromosomal instability resulting in tissue mosaicism and neuronal cell death in Alzheimer's disease [55].

PCD is also found in older females who experience significantly higher chance of spontaneous abortion and bearing children with trisomies especially trisomy 21. In females, the immature oocytes arrest in prophase I and only proceed with meiosis upon hormonal stimulation during the period after puberty until menopause. Hence, the chiasmata between homologous chromosomes and cohesion of the sister chromatid arms in prophase I as well as the subsequent centromere cohesion between the sister chromatids in meiosis II have to be properly maintained by the cohesin complexes for many years before these oocytes are released and potentially fertilized [56]. This long period of arrest led to the postulation of an age-dependent 'cohesin fatigue' being a contributing factor to the much higher aneuploidy rate of oocytes in older women [57, 58].

Centromere Protein Genes

CENP-A and HJURP in Cancer

Studies performed in colorectal, testicular, liver, breast and lung cancers were reported to have elevated expression of CENP-A while separate studies in lung, breast and brain cancers had reported on overexpression of the CENP-A chaperone, HJURP [59, 60]. CENP-A and HJURP could potentially be used as prognostic markers for certain groups of cancer. CENP-A has been demonstrated to correlate positively with pathological grade and negatively with survival prognosis in lung adenocarcinoma [61], epithelial ovarian cancer [62] and estrogen-receptor positive breast cancers that were not treated with systemic therapy [63]. HJURP has shown a similar pattern of correlation with astrocytomas, the most common type of adult brain cancer [59]. In combination, upregulation of both CENP-A and HJURP at their mRNA levels were found to be associated with decreased survival in breast cancer patients [64].

BUB1B, ESCO2, CASC5 and CENP-E in Developmental Disorders

Mosaic variegated aneuploidy syndrome (MVA; OMIM #257300) is a collective term for the cytogenetic characteristic where mosaic aneuploidies are commonly observed with clinical features namely microcephaly, mental retardation and growth retardation. In a subset of MVA patients, premature chromatid separation (PCS; OMIM #176430) was evident [65]. PCS is another cytogenetic description for a spectrum of diseases, in which a significant percentage of the mitotic lymphocytes appear to have separated centromeres and splayed chromatids. This is in contrast to the metaphase chromosome of normal, colchicine or colcemid treated cells where two sister chromatids are linked at the centromere region [66].

The SAC gene, *BUB1B* was not only the first gene found to be associated with MVA but also the first mitotic SAC gene where its allelic mutations in the germline were linked to a human disease [67]. Monoallelic *BUB1B* mutations appeared to give rise to the most severe phenotype including high occurrence of PCS, cataracts, Dandy–Walker syndrome and cancer. Biallelic *BUB1B* mutations yielded moderate phenotype while MVA without *BUB1B* mutations rarely had PCS and showed no signs of cataracts, Dandy–Walker syndrome and cancer [68]. Hence, many have postulated that other mitotic SAC genes might have important role in instigating the remaining forms of MVA.

The other cytogenetically observed trait around the centromere is heterochromatin repulsion which is most noticeable on chromosomes with large tracts of heterochromatin namely chromosomes 1, 9 and 16 [69]. This affects most metaphase chromosomes of patients with Roberts syndrome (RBS; OMIM #268300) and the milder SC phocomelia syndrome (SC; OMIM #269000). The causative gene for both of these syndromes was found to be Establishment of Cohesion 1 Homologue 2 (*ESCO2*) and these syndromes can be regarded as a spectrum depending on the variants of the mutated *ESCO2*. Clinical features of these patients include growth retardation, mental retardation and the presence of craniofacial abnormalities with microcephaly being the most common besides several others including hypertelorism, hypoplastic nasal alae and malar hypoplasia. The presence of cleft lip and palate was associated with the severity of limbs malformations while corneal opacities correlated with mental retardation and cardiac defects [70].

CASC5 or *KNL1* mutations were reported to cause autosomal recessive primary microcephaly (MCPH; OMIM #251200). CASC5 is a member of the conserved KMN (KNL1/MIS12 complex/NDC80 complex) network of proteins within a kinetochore that links the chromosome to the microtubules. CASC5 which localizes to the kinetochore from G2 til late anaphase is also part of the SAC machinery as it is known to bind to BUB1B [71]. Compound heterozygous variants of *CENPE* had recently been described in two siblings with microcephalic osteodysplastic primordial dwarfism (MOPD2; OMIM #210720) which was a disease previously reported to be linked to mutated centrosome-associated protein, pericentrin (PCNT) [72]. CENP-E is a dimeric kinesin-like motor protein which was shown to be important for the stability of binding between kinetochore and the dynamic microtubules, while PCNT is essential in the formation of microtubule arrays at the centrosome [73]. This suggests that the overlapping phenotype for both *CENPE* and *PCNT* mutations might be spindle-related [72].

Other Centromere Protein Genes in Cancers

Kinetochore protein genes that are crucial for the normal function of the centromere have been reported to be mutated or differentially expressed in various cancers. Mutations in *BUB1* were implicated in colorectal, adult T-cell leukemia/lymphoma (ATLL), lung and pancreatic cancers while mutations in *BUB1B* had been reported in more cancer types including colorectal cancer, MVA, ATLL, glioblastomas, Wilms tumor and B-cell lymphoma [74].

In addition to mutation, the level of expression for SAC proteins appears to be important in tumorigenesis. *BUB1*, *BUB1B* and *BUB3* were reported to be unregulated in gastric cancer [75]. However, in pediatric glioblastoma, expression of *BUB1* and *BUB1B* were upregulated whereas *BUB3* was downregulated [76]. In clear cell renal carcinomas investigated for the expression of their SAC genes, *BUB1*, *BUB1B* and *MAD2L1* (MAD2 mitotic arrest deficient-like 1) were found to be overexpressed while *MAD1* had decreased expression [77].

Epigenetics

Epigenetics is the study of the changes in gene expression or protein function that are not due to alterations in the DNA sequence of the gene, but are heritable through cell division. Such changes could occur at, (1) the genome structural level involving

DNA methylation, histone modifications, nucleosome positioning, and histone variants, (2) the RNA level which includes RNA splicing and RNA interference, and (3) the protein level in cases of prion formation where the 'infectious' proteins are able to induce conformational change of native proteins rendering them 'infectious' as well as dysfunctional [78, 79].

DNA Methylation

Cytosine residues of the non-CpG islands (non-CGIs) or else referred to as the global CpG dinucleotides within intronic and intergenic regions, especially transposable elements and simple repeat sequences, are mostly methylated in somatic tissues as opposed to unmethylated cytosines in the CGIs that are known to coincide with gene promoters or regulatory regions [80].

Abnormal DNA methylation had been reported in immunodeficiency, centromeric instability, and facial anomalies (ICF) syndrome. ICF is a rare autosomal recessive disease which is currently categorized into three groups namely ICF1 (OMIM #242860) with mutations found in the DNA methyltransferase 3B (*DNMT3B*) gene, ICF2 (OMIM #614069) with mutations in zinc finger- and BTB domain-containing 24 (*ZBTB24*) gene, and the final group with currently unknown molecular etiology provisionally designated ICFX [81]. Although all three groups exhibit hypomethylation of satellites 2 and 3 which are part of the constitutive heterochromatin, ICF2 and ICFX, however, show additional hypomethylation at the alpha satellite [82]. In the heterochromatic region that exhibit reduced DNA methylation from an average level of 80 % in normal cells to 30 % in ICF cells, some heterochromatic genes were shown to have escaped silencing compared to the control, although each patient appeared to have his own signature of heterochromatic genes that escaped silencing across different chromosomes [83].

Wilms tumor is the most common renal tumor in children under 5 years of age, accounting for 90 % of the total pediatric renal cancer cases and contributing to approximately 7 % of all pediatric malignancies [84]. Hypomethylation of alpha satellite on chromosomes 1 and 10 was observed in Wilms tumor patient samples but it was not correlated with aneuploidy. To a lesser extent and frequency, satellite 2 was also hypomethylated on these chromosomes [85]. These studies into ICF and Wilms tumors indeed pose an interesting question about the mechanisms that lead to the differences in their hypomethylation profiles.

In cancer as well as aged cells, global hypomethylation and concomitant increase in the methylation of promoters have been observed and were thought to contribute to genomic instability and gene silencing respectively. Furthermore, global non-CGIs could be further subcategorized and studied. Hypomethylation of Alu, LINE-1 and alpha satellite in CLL patients were examined and alpha satellite hypomethylation was suggested to be a potential negative prognostic marker for CLL [86]. Separately, in a study performed in ovarian epithelial tumors, satellite 2 hypomethylation on chromosomes 1 and 16 was strongly correlated with both genome-wide hypomethylation and the degree of tumor malignancy. Extensive hypomethylation of chromosome 1 alpha satellite was also observed in larger proportion of carcinomas compared to the more benign forms [80]. Therefore, it appears that the level of DNA methylation at centromeric satellite sequences is a useful biomarker in the study and diagnosis of cancers.

Pericentric and Centromeric Transcription and Histone Modifications

Expression of pericentric and centromeric transcripts has been found to be altered in senescent cells and human cancer samples when compared to normal tissues, reflecting the global epigenetic deregulation [87]. This could partly be facilitated by the altered DNA methylation in these regions. In addition to global DNA methylation changes, the global histone marks have also been found to be altered. The loss of both H4K20me3, the pericentric constitutive heterochromatin mark, and H3K27me3, the facultative heterochromatin mark, were reported in lung cancer cells when examined with the non-tumor cells [88].

Upregulation of pericentric satellite 3 was also observed in a Hutchinson-Gilford progeria syndrome (HGPS) patient. HGPS (OMIM #176670) is a disease of rapid aging due to the expression of mutant Lamin A, a developmentally regulated gene. However, the expression of alpha satellite was unaltered, suggesting that the expression of pericentric and centromeric sequences are controlled by different mechanisms. The upregulation of satellite 3 was accompanied by the loss of H3K27me3 and pericentric constitutive heterochromatin mark H3K9me3 but by the increase of another constitutive heterochromatin mark, H4K20me3 [89]. Hence, thus far, the relationships between the expression of repetitive satellites and both DNA methylation as well as histone modifications remain to be clarified.

Although the cases aforementioned were characterized by upregulation of centromeric and/or pericentromeric sequences, the right transcriptional balance between sense and antisense strand of both pericentric and centromeric sequences appears to be crucial for the proper formation and function of a centromere [90, 91].

Evolution

Primate Centromere DNA

Alpha satellite DNA is a relatively conserved centromeric repeat family. It is found in great apes, old world monkeys and new world monkeys, which span approximately 43 million years of evolution since the last common ancestor. In great apes it is organized into HOR structures, however in more distant species, it is mainly found in divergent monomeric forms. One proposed hypothesis is that HOR structure arose after the divergence of the great apes from the rest of the primate species

[92]. Interestingly, HOR structures are not necessarily restricted to certain species or particular chromosomes, for example the centromere array of the mouse Y chromosome contains a HOR but the autosomes and X chromosome only have the monomeric form [93].

As described above, alpha satellite is found in many primate species across 43 million years of evolution. This appears to be rather a long time when compared to other mammalian centromere satellites such as the mouse minor satellite DNA. The monomer repeat unit is 120 bp and is only found in a subset of species in the *Mus* genus spanning about 5–7 million years [94]. The higher rate of centromere DNA evolution in the mouse may be related to a much shorter generation time which increases the chance of the centromere array to rearrange and diverge during meiotic recombination. Even though minor and alpha satellite DNAs are quite diverged, they do share some features such as a high AT content and the conservation of the CENP-B box motif.

The Rapidly Evolving Y Centromere

The human Y chromosome exists in a haploid state in males, and offers a unique perspective into the evolution of centromere DNA within a species since there is no homologous counterpart of this region for meiotic recombination to occur. Like most other Y chromosome sequences that do not recombine with a homologue, the centromere DNA has undergone a rapid rate of sequence divergence. Some of the features that mark the Y centromere as separate from other human centromeres include; a diverged alpha satellite monomer, absence of the CENP-B box motif, diverged HOR and a significantly smaller overall length [13, 14]. Evidence for the rapid divergence in the Y alpha satellite sequence is nicely illustrated in the analysis of the HOR in humans and chimpanzees. The HOR of the X and 17 alpha satellite exhibits a conserved co-linearity of the HOR, whereas the Y alpha satellite has completely lost this conserved structure and the length of the HOR between humans and chimpanzees is also different [93].

The functional consequences of a rapidly diverging and smaller Y centromere may be responsible for the Y chromosome's partial instability during division of aging cells [95, 96]. Measurement of the CENP-A protein on Y centromeres shows that it contains around half the amount when compared to the autosomes and X centromeres [43]. This lower amount is consistent with less alpha satellite DNA present at Y centromeres. Y alpha satellite DNA ranges in length from 250 to 1500 kb, in contrast to the X centromere which is megabases in size, ranging from 1300 to 3700 kb [13]. On the extreme end of low amounts of alpha satellite, neocentromeres are formed on non-alpha satellite regions of the genome and are found to contain even lower amounts of CENP-A than the Y centromere [43] (see "Disease" section).

Adaptive Evolution of Foundation Centromere Proteins

It has been hypothesized that rapidly evolving centromere DNA can expand and create larger centromeres that can bind more spindle microtubules [97]. Other evolutionary mechanisms can increase the size of a centromere, such as translocations of acrocentric chromosomes in humans to generate metacentric (Robertsonian) chromosomes with two adjacent centromeres [98]. These chromosomes have a higher chance of being inherited during the asymmetric cell divisions of female meiosis where the egg spindle pole releases more microtubules to capture the bigger centromere than the polar body pole. To prevent a complete runaway of chromosomes with larger and larger centromeres the cell counters this expansion by epigenetic means, through the adaptive evolution of centromere chromatin proteins such as CENP-A and CENP-C [99]. Evidence for this hypothesis is now accumulating in many species groups, such as flies, plants and primates that show these two proteins are under adaptive evolution [100–102]. In contrast, when similar sequence analysis across the primate group was performed, it did not show any evidence for adaptive selection for the non-essential CENP-B protein, even though this protein directly binds to the alpha satellite DNA [102].

ZNF397, an Evolutionary New Centromere Protein

Many centromere proteins are conserved in eukaryotic species, ranging from the single-celled budding yeast to humans. In some instances in evolution, new proteins appear via a variety of molecular mechanisms. One example of this is zinc finger protein 397 (ZNF397), which presumably arose from a gene duplication event after the separation of placental and marsupial mammals. We had previously identified ZNF397 using anti-centromere antibodies from a patient with autoimmune disease [103]. Interestingly, this protein has a unique cell cycle localization pattern where it is present from the end of telophase through to early prophase. Knockout experiments in mouse showed that the protein is not essential for chromosome segregation. One attractive hypothesis is that the protein has acquired centromere targeting activity but it is yet to be directly involved in full kinetochore function.

Karyotype Evolution and Meiotic Drive: Robertsonian Translocations

Mendel's law of segregation implies that the two homologous chromosomes in a parent segregate at meiosis into the gametes to ensure the offspring acquire only one copy of each chromosome from each parent, thereby maintaining the proper chromosome number in sexually reproducing organisms. This law assumes that the process of segregation occurs in a random and non-biased manner. However, in humans, the most common ROBs namely der(13q14q) and der(14q21q) arise mainly during oogenesis but not spermatogenesis [32]. It was postulated that the chromosomal rearrangements of ROBs cause functional heterozygosity at the centromere of homologous chromosomes leading to the differential interactions with the meiotic spindle. This then contributes to preferential segregation of the rearranged chromosomes into functional meiotic product instead of the polar bodies [104]. These ROB chromosomes in male carriers are not subjected to the same meiotic drive owing to the process of spermatogenesis where polar bodies do not form, hence, do not render the opportunity for preferential segregation.

Neocentromeres and Evolutionary New Centromeres

One of the hallmarks of speciation at the genetic level is the divergence of karyotypes between two newly-formed species. This separation can often produce a reproductive barrier between the two groups which then further accelerates the rate of evolution. Chromosomal rearrangements including, translocations, inversions, deletions and duplications, are a driving force in the emergence of new karyotype configurations. As a consequence of genomic rearrangements, the centromere can also change position to either rescue an acentric chromosomal fragment or compensate for a partially deleted (inactivated) centromere, as has been observed in de novo clinical cytogenetic cases (Fig. 9.3) (see “Disease” section). Changes in centromere position in closely related species led to the concept of Evolutionary New Centromeres (ENCs) [105]. ENCs were initially thought to have arisen due to the physical repositioning of an extant centromere. This hypothesis has been replaced by the ENC hypothesis because of more accurate genome and cytogenetic mapping. So the evolutionary timeline of the formation of an ENC is as follows: (1) chromosomal rearrangement, (2) neocentromere formation and (3) accumulation of satellite DNA at the neocentromeric locus [106].

A good example that illustrates this progress from neocentromere to an ENC is in the orangutan. Early cytogenetic analyses of orangutan centromeres using alpha satellite FISH showed that at least one chromosome was devoid of alpha satellite DNA [107]. It wasn’t until high-throughput sequencing and CENP-A pulldown technologies that definitively revealed that chromosome 12 contained a neocentromere but had not yet acquired any alpha satellite DNA sequences [108]. The ENC chromosome 12 is present in 2 species of orangutan, *Pongo abelii* (Sumatran) and *Pongo pygmaeus* (Bornean) which shared a common ancestor between 0.4 and 1 million years ago. This shows that ENCs can take a long time to acquire satellite sequences. Interestingly, the progenitor chromosome 12 with the alphoid centromere still exists together with the ENC form in the two orangutan species.

Conclusions

In the last few decades we have made rapid progress in the discovery of most of the genomic and protein elements that make up a functional centromere. Human centromere DNAs have been identified and mapped to each chromosome. Current sequencing methods have made some in-roads towards completing the genome map of these repeat-rich regions. Furthermore, novel computational methods have allowed the interrogation of high-throughput genome sequencing results from individuals, however, gaps still remain. The next breakthrough in long-read single-molecule sequencing will allow these gaps to be closed and analyzed centromere-by-centromere. Insights will be made in the rate of evolution across populations and within families. This will enable researchers to further understand the contribution of variation and mutation on centromere dysfunction in human chromosome instability disorders.

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Chapter 10
Structure and Functions of Telomeres in Organismal Homeostasis and Disease

Penelope Kroustallaki and Sarantis Gagos

Telomeres Co-evolved with Linear Chromosomes

From the origins of life, the ancestral circular prokaryotic genome developed a complex biological machinery committed to maintain DNA integrity that ensures cellular homeostasis and the fidelity of the transmission of precise genetic information through cell generations [1–3]. In both prokaryotes and eukaryotes, a highly efficient repair system, evolved to sense and restore DNA base pair alterations or miss-matches, as well as any discontinuity of the genome, such as single or double strand breaks of the DNA helix [4, 5]. DNA repair is a highly dynamic biological process that implies the congression of several types of specialized factors at the site of the DNA lesion, and in parallel, interacts with the course of the progression of the cell cycle affecting dramatically the overall cellular fate [6, 7]. Activation of the DNA repair machinery is operated by a series of post-translational modifications of numerous protein substrates that are proficient to sense the site of the damage, they are capable to slow down the cell cycle as long as the damage is repaired, and they are even destined to kill the cell if the damage is unrepairable [8].

In eukaryotes, the circular ancestral genome evolved into multiple linear chromosomal entities that in many species can be visualized by light microscopy in cytological preparations stained by nucleophilic dyes [9, 10]. The adaptivity of the ancestral linear chromosomes was challenged by two very important biological constrains that literally shaped life as we know it: (a) The so-called, "chromosome end-replication problem" proposed independently by Watson and Olovnikov during the 1970s [11–13] and (b) the putative activation of DNA damage responses at the

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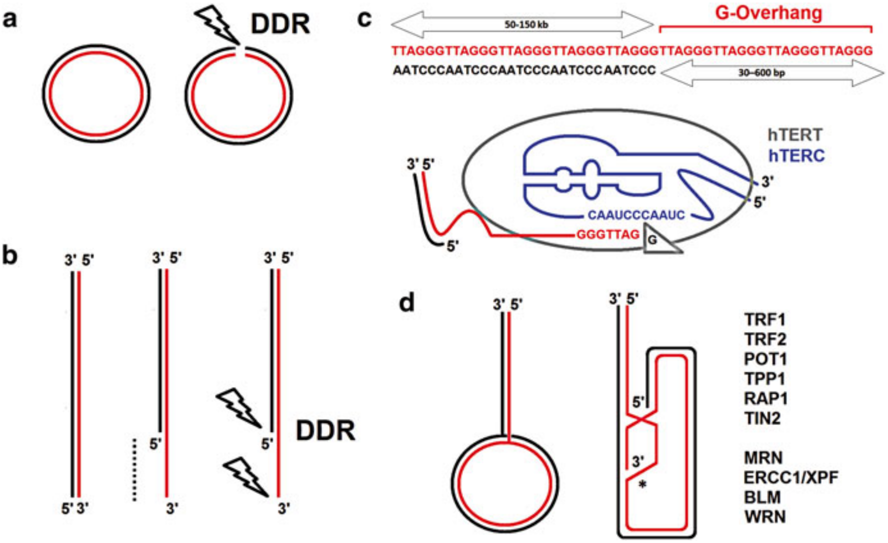

Fig. 10.1 The two major telomeric constrains and nature's solutions to the chromosome terminal problem: Any interruption of the integrity of the prokaryotic circular DNA molecule in the form of DSB, activates DNA damage responses (DDR) that may lead to cell cycle arrest, or even to cell death (**a**). The evolution of circular chromosomes to linear DNA molecules was challenged by the universal insufficiency of the DNA replication machinery to replicate completely the 5′-end of the linear DNA molecule. This process shortens the chromosome by each cell division and can jeopardize genome integrity and organismal homeostasis. In addition, the termini of linear DNA molecules can be perceived as DNA lesions, they can activate local DDR and can be degraded by nucleases (**b**). The partially dispensable repetitive, G-rich, primary structure of eukaryotic telomeres and the development of telomere replenishment mechanisms, such as the ribozyme telomerase, resolved the end-replication problem. Like most telomerases, human telomerase reverse transcriptase (hTERT) assembles with its RNA component (hTERC), anchors itself to the telomere, and neo-synthesizes G-rich telomeric repeats according to its single-stranded complementary RNA template (**c**). To resolve the second telomeric constrain, the ends of linear chromosomes evolved secondary structures that mimic the circular ancestral DNA and can hide the single-stranded telomeric G-overhang into a telomeric loop (T-loop). The T-loop is formed by the invasion of the G-overhang into double stranded telomeric repeats that is mediated by a three-stranded DNA displacement loop (D-loop) (*asterisk*). The formation of T-loops is facilitated by shelterin components and telomere interacting factors (**d**)

linear chromosome's "blunt" ends [14, 15] or the catastrophic activity of DNA exonucleases at the exposed chromosome termini [16] (Fig. 10.1a, b).

The "chromosome end-replication problem" stems from the inert insufficiency of the semiconservative DNA replication machinery that is incapable to fully polymerize the lagging DNA strand and eventually trims down a terminal part from the 5′ end of the linear chromosome every time a DNA molecule replicates itself and the cell divides [17]. The second telomeric constrain, entailed the immediate evolution of a specialized terminal chromosomal structure that hides the discontinuity of the DNA helix from the DDR machinery and in parallel, protects chromosome blunt ends from nucleolytic degradation [18–20].

The specialized entities that comprise linear chromosome ends, were termed "telomeres" from the Greek words "τέλος" (-end) and "–μέρος" (part) by the Nobel laureate geneticist Herman Muller in 1938 [21]. The role of telomeres in chromosome protection and overall genome integrity was recognized during the 1930s by another Nobel laureate, Barbara McClintock who performed a series of studies in irradiated maize (*Zea mays*) cells and described the ongoing mutagenic phenomenon of Breakage/Fusion/Bridge cycles (B/F/B cycles) [22, 23]. Dividing cells undergoing subsequent rounds of terminal chromatid, or chromosome breakage, followed by fusion and anaphase bridging, display multiple structural chromosome anomalies such as dicentric (or poly-centric) and ring chromosomes [23, 24]. The B/F/B cycles are capable to generate chromosomal translocations, inversions or duplications and can lead to losses of large genomic segments or even whole chromosomes [25–28]. Today, it is well established that B/F/B cycles are illegitimate outcomes of Non-Homologous End Joining (NHEJ) or Homologous Recombination (HR) DNA repair pathways and play an important role in the ongoing chromosomal instability that is considered a hallmark of cancer [29, 30].

The Primary Nucleotide Structure of Telomeres and Mechanisms of Telomere Length Maintenance

The molecular era of telomere research begins in 1978, when E. Blackburn and J. Gall, described that the terminal structures of the bulky extrachromosomal rDNA molecules of *Tetrahymena*, are composed by tandemly repeated hexanucleotide sequences [31]. In the early 1980s, J. Szostak and E. Blackburn, showed that protozoan telomeric function from *Tetrahymena*, could be transferred to yeast chromosomes of *Saccharomyces cerevisiae* [32]. Driven by these findings, E. Blackburn, and C. Greider, described for the first time, that eukaryotic telomeres consist of tandemly repeated G- and C-rich complementary DNA sequences and set the basis to resolve the end-replication problem [33]. Their subsequent studies revealed that in *Tetrahymena*, the G-rich telomeric DNA strand can be neo-synthesized by an RNA-dependent, reverse transcriptase, termed "telomerase" [33, 34]. These discoveries immediately raised the interest of the broad scientific community that very soon called telomeres the "thread of life" and telomerase the "fountain of youth". The important contributions of E. Blackburn, J. Szostak, and C. Greider to science were recognized by the 2009 Nobel Prize in Physiology or Medicine.

In the past three decades, our understanding about the structure and the function of telomeres has significantly improved through the efforts of a relatively small, but vigorously thriving research community: In 1989, Morin [35] showed that the primary structure of human telomeres is composed by tandem repeats of the complementary hexa-nucleotides TTAGGG/AATCCC (G-rich and C-rich strands). The same group documented human telomerase activity, while J. Meyne used Fluorescent in Situ Hybridization (FISH) to visualize telomeres at chromosome termini, showing in parallel that the TTAGGG repeats are highly conserved between mammals [35, 36]. In fact, from anthozoans (corals) to humans, different species share the same TTAGGG repeat at their chromosome termini indicating that telomere homeostasis is operated by highly conserved biological pathways [20, 37–39].

The primary nucleotide structure of mammalian telomeres is composed of long stretches of double-stranded TTAGGG/AATCCC hexa-nucleotide repeats that vary in size between different organisms and species [36, 40]. The double telomeric strand terminates in a relatively short, single-stranded, TTAGGG-rich overhang that protrudes out of the 3′-end of each linear DNA molecule [41]. The length of the G-overhang ranges between 30 and 600 nucleotides [36, 41, 42]. The average length of double-stranded mammalian telomeres varies between chromosomes of the same cell and amongst species, from 5–20 kb in humans, to 50–150 kb in several rodents [36, 43, 44]. The telomerase holoenzyme, acts as a reverse transcriptase to polymerize the 3′-overhang of the telomeric sequence using as template a specialized nuclear non-coding RNA that contains sequence complementarity with the G-rich telomeric repeat [34] (Fig. 10.1c).

Studies in *Caenorhabditis elegans*, revealed that double stranded telomeres can also terminate in 5′-end single stranded C-rich overhangs [45]. Similar to the worms, G1/S phase arrested or terminally differentiated mammalian cells, display frequent C-overhangs [46]. Telomeric C-rich overhangs are less well studied than G-overhangs and may play a role in biological processes involving homologous telomeric recombination [46].

Replicative Senescence and Ageing

Normal human cells *in culture* have a limited proliferative life-span, often termed the “Hayflick limit”. After 50–100 population doublings (PDs), most of the cultured cells enter a static phase termed “senescence”. Senescent cells stop to divide, undergo “crisis” and eventually they die [47, 48]. The term “replicative senescence”, was introduced by Greider and Harley in 1990, who elaborated the “telomeric hypothesis of aging”, based on their observations in human diploid cells that progressively lost their telomeres after consequent population doublings (PDs) [49]. According to this theory, most dividing somatic cells do not possess an active mechanism of telomere maintenance; hence, anytime a cell replicates its genome, a portion of the telomeres will be lost and cannot be replenished [11, 13, 49]. Thus gradual telomeric shortening must be associated with cellular, tissue and organismal ageing. Indeed, several studies have shown that there is a positive correlation with telomere length of somatic tissues and life-span [50, 51]. Furthermore, cells from individuals with premature aging syndromes show reduced telomeric length and proliferative capacity *in culture* [52, 53]. However, there is a marked deviation of telomere length between individuals belonging in the same age group, indicating genetic and multifactorial effects on telomere metabolism [54–57].

In longed-lived mammals and humans, the gradual telomeric loss and the loss of replicative capacity act as barriers to carcinogenesis [58, 59]. On the contrary, many short-lived animals such as laboratory mice, have significantly longer telomeres than humans, and do not display replicative aging [44, 60, 61]. There is now substantial evidence that in short lived mammals, like rodents, cellular senescence does not act as an oncosuppressor pathway [62–64]. Primary normal diploid mouse cell cultures undergo spontaneous transformation and become polyploid, upon a limited number of PDs, whereas normal human cells are constrained by the Hayflick limit [64, 65]. Ectopic expression of telomerase activity in cultured, diploid, human retinal pigment epithelial cells is capable to confer immortalization and bypasses the Hayflick barrier [66].

Biogenesis of Telomerases

Although telomerase activity can be reconstituted in vitro just by the combined presence of the reverse transcriptase (TERT) and its RNA template (TERC), the different telomerase holoenzymes between species, are composed from several protein components [67, 68]. These molecules take part in biogenesis, assembly, targeting and regulation of telomerases. The molecular cloning of the telomerase holoenzyme constituents, in various experimental in vivo models and in human cells, enabled the investigation and manipulation of the biological processes that regulate telomere length [60, 67, 69, 70]. In *S. cerevisiae*, the telomerase ribonucleoprotein (RNP) is composed of the three protein subunits, Est1, Est2 and Est3 [71–73]. Depletion of any one of these proteins in vivo, is associated with a yeast phenotype described as “Ever Shorter Telomeres” and leads to massive cell death through replicative senescence [74, 75].

Based on sequence similarity Reichenbach et al., and Snow et al. (2003) identified the Suppressor of Morphogenesis in Genitalia (SMG), hEST1A(SMG6), hEST1B(SMG5) and hEST1C(SMG7) proteins as human homologues of the yeast Est genes. Despite the weak sequence homology, hEST1A and hEST1B were proven to be part of the active human telomerase holoenzyme [76, 77]. The conserved moiety of hEST1C revealed a 14-3-3 phosphoserine binding motif, but no association with telomerase RNP [78]. In yeast and humans, telomerase is considered to act as a multimer (dimer in humans) [79–81] while in protozoa like Tetrahymena, acts as a monomer [82, 83]. Telomerase activity is regulated by negative feed-back mechanisms. In *Saccharomyces Cerevisiae* the telomerase dependent telomere elongation was found increased in chromosomes carrying shorter telomeres [84].

Telomerase RNAs have been identified in several protozoa, yeast and vertebrate species including humans [85]. Despite the extensive phylogenetic divergence in primary and secondary structures [86] the RNA region that bears the template of all telomerases, appears to be always single-stranded and is capable to associate with the active reverse transcriptase site of TERT [86] (Fig. 10.1c). In humans, precursor hTR molecules, are transcribed by RNA polymerase II and immediately associate with a protein heterotrimer composed of Dyskerin, NHP2, and NOP10, that is bound to the RNP assembly chaperone NAF1 [87, 88]. The process is facilitated by two helicases, Pontin (TIP49, TIP49a, or RUVBL1) and Reptin (TIP48, TIP49b, or RUVBL2), and by the Dyskerin chaperone SHQ1 [89–92].

Cajal bodies are subnuclear compartments that accumulate RNP particles involved in splicing, ribosome biogenesis and telomere maintenance [93]. After co-transcriptional assembly with the Dyskerin heterotrimer and RNA processing, hTERC is directed towards the nucleoli and then to Cajal bodies [94–96]. The helicases Pontin and Reptin, bind to Dyskerin and TERT independently and facilitate the assembly of the telomerase RNP [95]. The mature telomerase RNP concentrates in Cajal bodies by the aid of TCAB1/WDR79 (Telomerase Cajal Body protein 1) and then it is rooted to the telomeres [16, 95, 97, 98]. The two subunits of the Ku heterodimer implicated in Non-Homologous End Joining (NHEJ) DNA repair, associate directly to the telomerase RNA component to facilitate telomere neosynthesis [99]. Telomerase holoenzyme recognizes the telomere, and binds its substrate DNA, through sequence complementarity encoded by its RNA template [95]. The di-hexa-nucleotide RNA template (CAAUCCCAAUCC) of hTERC catalyzes the addition of a single nucleotide at the time, to the 3′ end of the telomeric G-overhang [100] (Fig. 10.1c). Neo-synthesis of the next telomeric nucleotide, requires a slight translocation of the active site along the template (type I translocation) [33, 95, 101]. If during this process telomerase remains associated to the telomere, the holoenzyme is considered to present nucleotide processivity [102]. In vitro DNA synthesis by most telomerases is Nucleotide Processive [103]. When the final telomeric nucleotide of the template is synthesized, a realignment of the catalytic site to the beginning of the RNA di-hexa-nucleotide is required, to allow a new round of reverse transcription (translocation II). If telomerase remains bound to the telomere during translocation II, is considered to display Repeat Processivity [33, 101]. Different species exert variable degrees of relative repeat addition processivity of their telomerases [35, 101, 104–106]. In cultured human cancer cell lines, the pharmaceutical disruption of repeat addition processivity inhibits telomerase activity, inducing telomere shortening and replicative senescence [107]. Evaluation of telomerase processivity can be achieved using the original protocol applied for the detection of telomerase activity by Greider and Blackburn [33]. The method takes advantage of the enzymatic activity of telomerase that is capable to elongate terminal repeats in the presence of radio-labelled dNTPs [108], the products of the reaction are visualized as DNA ladders by gel electrophoresis [33].

Measuring Telomere Length

The classical procedure to measure the length of repetitive telomeric sequences is based on extensive digestion of genomic DNA with frequent cutting restriction enzymes, that do not break down telomeric and subtelomeric regions (Terminal Restriction Fragment assay) [49, 109–111]. Size resolution of sub-terminal and terminal restriction fragments is achieved via agarose gel electrophoresis. Telomeric DNA is visualized as smears by Southern blotting or in-gel hybridization, using probes specific for C-or G-rich telomeric repeats, hence it presents extensive length variation between cells and chromosomes [57, 111]. The TRF assay is considered to be the most accurate method to measure the length of telomeric repeats in epidemiological studies [112]. Currently there are several methods to measure the length of telomeric repeats in biological samples, clinical specimens, cell nuclei or even individual chromosome ends [113]. Telomeric in situ Quantitative-FISH or Telomeric Flow-FISH are commonly used in both research and clinical settings [114–119]. These two protocols are based on FISH of telomere specific fluorescent PNA (peptide nucleic acid analog) probes that stoichiometrically bind to terminal DNA repeats and can quantify telomeric length in situ, based on the intensity of the emitted fluorescence signals [120–124].

The arsenal of telomere length quantification protocols has been expanded by the introduction of PCR-based methods such as Single Telomere Length Analysis (STELA) [54, 125] and of MM-Q-PCR that utilizes Real-Time PCR technology [126]. STELA is a DNA ligation-based method that uses primers designed for specific subtelomeric sequences, and can accurately measure telomere length of specific chromosome ends [125]. STELA can be modified to evaluate extremely short telomeres [127, 128]. MM-Q-PCR normalizes the amplification rate of telomeric DNA to a single gene, to validate the overall amount of telomeres in a given biological specimen [126]. MM-Q-PCR requires small amounts of DNA and is extensively applied in large scale population studies [126, 129, 130].

Telomeres and Cancer

In 1990, de Lange et al. [131] showed that primary tumors display shorter telomeres than their adjacent normal tissues and proposed the “telomeric theory of cancer”. De Lange’s hypothesis was supported by the findings of Counter et al. [132], who revealed that telomerase is activated in ovarian tumor cells, but not in stromal nonmalignant cells, suggesting that telomerase activity might be linked to continuous proliferation of cancer cells. The studies on telomerase activity in neoplasia boost up from 1994, when J.W. Shay and W.E. Wright, presented the Telomeric Repeat Amplification Protocol (TRAP) assay to evaluate telomerase activity with high sensitivity. In Kim et al. (1994), the two researchers and their colleagues, used the TRAP technology to demonstrate for the first time, that the majority of human tumors activate telomerase [133, 134]. The TRAP assay has been modified through the years and became quantitative by Q-RT-PCR [108], it’s general principle though, remains the same: In protein extracts, an oligonucleotide with the TTAGGG repeat is incubated with unlabeled nucleotides. Then the products of the reaction are PCR amplified by the addition of a G-rich complementary primer that bears a short tract of non-telomeric DNA at the 5′ end to prevent repetitive sequence miss-alignment [133].

An alternative mechanism of telomere elongation (Alternative Lengthening of Telomeres-ALT) that is mediated by homologous recombination of telomeric repeats was discovered in 1993 in yeast mutants deficient for telomerase activity [74]. Three years later, the group of R. Reddel, presented for the first time, that a small proportion of human immortalized or cancer cell lines do not express telomerase activity, but they utilize an alternative pathway of telomere lengthening (ALT-pathway) [135]. In cancer or virally immortalized human cell lines, the ALT pathway is associated with a “loose” heterochromatin structure at telomeres and centromeres [136, 137], and is characterized by increased homologous telomeric recombination, extreme deviation of telomere length from very short, to as long as 50–60 kb, frequent presence of the so called ALT associated PML bodies, and extensive chromosomal instability [136, 138–145]. In addition, cells utilizing the ALT pathway display abundant extrachromosomal, C-rich, telomeric repeats (C-circles) and increased rates of telomeric sister chromatid exchanges (T-SCEs) [146] (Fig. 10.2c). The ALT-associated PML bodies (APBs) are ALT-characteristic, sub-nuclear compartments that contain Promyelocytic Leukemia body protein (PML), telomeric DNA, telomere associated factors and proteins involved in DDR (i.e. RAD51, RPA1, 53BP1 and the MRN complex (MRE11, RAD50, NBS1) [147–152]. APBs are enriched in G2/M [153, 154] and may play a role in telomere recombination by tethering together chromosome ends and by promoting heterologous telomeric interactions [139, 141, 155]. The orphan nuclear receptors of the NR2C/F classes (TR2, TR4, COUP-TF1, COUP-TF2 and EAR2), which belong to the nuclear hormone receptor (NHR) family of transcription factors, are frequently found at the telomeres of ALT cells and may favor the telomere–telomere recombination necessary for ALT maintenance [156]. There is now increasing evidence that embryonic and somatic stem cells display both known mechanisms of telomere maintenance [157–159].

Mouse Models

Telomerase activity knock-out mice have normal development [160]. Reduced fertility and degenerative defects in highly proliferating tissues emerge only after three generations [160, 161]. The phenotype is more pronounced in the sixth generation, with severe congenital malformations, male sterility and an increase in the incidence of spontaneous lymphomas and carcinomas [160, 161]. In addition, tumor cells from late generation double knockout mice, null for mTERC and p53, showed elevated frequencies of chromosome fusions, anaphase bridges, and nonreciprocal translocations [162]. Conversely, telomerase over-expression in aging transgenic mice was associated with spontaneous emergence of epidermal tumors, mammary and lung carcinomas or lymphomas [163–167]. Hence, both telomerase depletion and overexpression, can lead to carcinogenesis in mice. Lack of telomerase activity generates genomic instability and promotes tumorigenesis [161, 162], while excess of telomerase facilitates neoplastic transformation since most tumors depend on telomerase to maintain continuous cellular proliferation [30, 133].

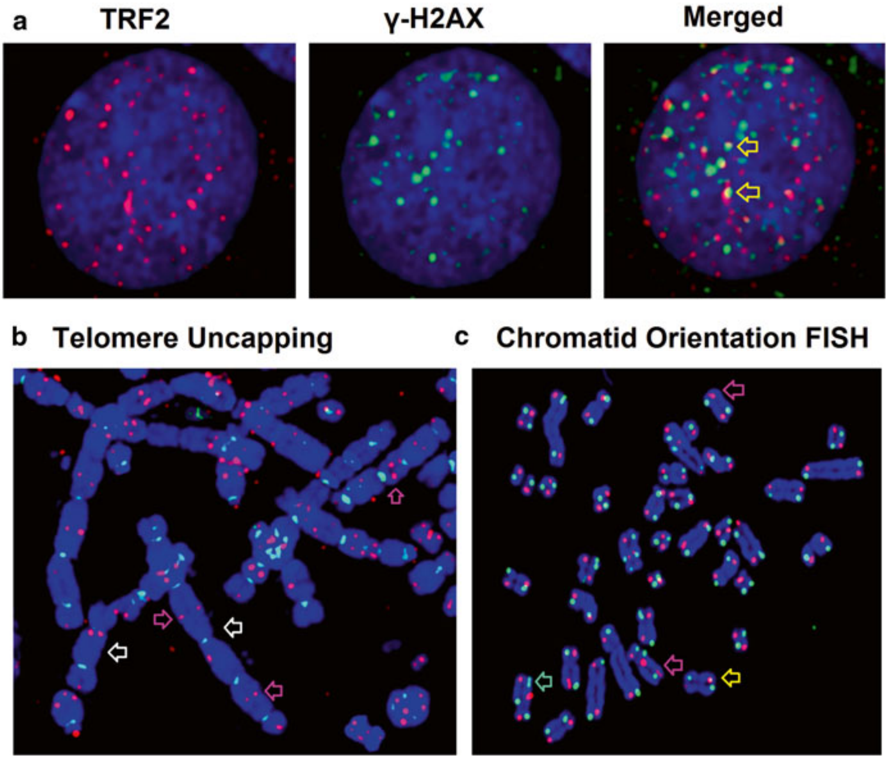

Fig. 10.2 Telomere dysfunction induced foci (TIFs) (*arrows*) in a cell nucleus from an immortalized human cell line (VA-13) 24 h after exposure to gamma-irradiation. Immuno-Fluorescence microscopy reveals co-localizations (*yellow spots*) of the telomere specific protein TRF2 (shelterin component) with the phosphorylated histone H2A (γ-H2AX) that is a marker of DNA damage responses. TRF2 is labelled with Alexa-568 (*red*), γ-H2AX with Alexa-488 (*green*). DAPI (4′,6-diamidino-2-phenylindole) is *blue*, (630×) (**a**). A partial metaphase spread from human BJ fibroblasts depleted for TRF2 and counter-stained with DAPI (630×). Fluorescence In Situ Hybridization (FISH) with probes specific for all human centromeres (labelled green with FITC) and for TTAGGG telomeric repeats (labelled red with Rhodamine), display multi-centromeric chromosomes generated by extensive Non-Homologous End Joining (NHEJ) of uncapped chromosome termini (*white arrows*). Junction points maintain visible telomeric repeats (*red arrows*) (**b**). Strand specific telomeric Chromatid Orientation FISH in pig iPS cells [366] demonstrates Telomeric Sister Chromatid Exchanges (T-SCE) (*yellow arrows*) and induction of fragile telomeres (FT) in both the G-and C-rich telomere strands (*red* and *green arrows*). Peptide Nucleic Acid analog (PNA) probes specific for TTAGGG (green = FITC) and AATCCC repeats (red = Rhodamine), DAPI is *blue* (×630). T-SCEs have been associated with increased telomeric recombinogenicity in the ALT pathway and extreme telomere dysfunction when telomeres are critically shortened [367, 368]. FTs have been connected to increased telomeric replication stress [211]

The first attempt to extend longevity in genetically engineered mice through telomere manipulation was presented by the group of M. Blasco, in 2008, who showed that overexpression of mTERT in cancer resistant Sp53/Sp16/SARF mice, can delay aging [168]. In 2011, Jaskelioff et al. [169] proved that in prematurely aged mTERT deficient mice, reconstitution of telomerase activity can revert a number of age-related phenotypes caused by extreme telomere shortening. Prompted by these findings, in 2012 de Jesus and associates examined the effects of adenoviraly-mediated increase at the levels of TERT, in naturally aged laboratory mice. Interestingly the life-span of these animals was significantly increased with no effects in cancer susceptibility. Therefore, at the organismal level, telomerase acts as a longevity gene by preventing premature telomere attrition [170]. Understanding the complex mechanisms of telomere length regulation, will provide in the near future the means to ameliorate the biologic consequences of replicative ageing and in parallel to protect tissues and organs from neoplasia.

The High Order Structure of Telomeres and Nature's Solution to the Second Basic Telomeric Constrain

The description of the repetitive nature of the telomeric DNA sequences and the biological pathways of telomere elongation, effectively resolved the first telomeric constrain set by the end replication problem [34]. Nature's solution to the second telomeric limitation, required a complex structural organization of the telomeric territories, specialized to efficiently protect chromosome termini from being perceived as targets of DNA damage responses or enzymatic degradation [19, 171]. The peculiar telomere protective structure is shaped both by the unique properties of primary telomeric DNA sequences, and by a dynamic interaction of telomeric repeats with telomere associating nuclear factors [15, 20, 171]. Albeit some sequence dissimilarities between species, most eukaryotic telomeres are rich in Guanines [39] this unique property provides to the telomeric repeats the ability to fold into non-canonical secondary four-stranded DNA structures formed by Guanine-quartets, known as G-quadruplexes [20, 172–174]. These unusual DNA conformations are much more stable than the double-stranded DNA and have to be resolved to permit telomere neosynthesis [172, 175]. Unresolved G-quadruplex formations would probably inflict a structural barrier to DNA replication and could be a potential source of genomic instability [20, 175]. G-quadruplexes inhibit telomerase activity and if stabilized by a chemical compound, can act synergistically with Camptothecin or PARP-1 inhibitors to suppress tumor growth in mice [176, 177].

The budding yeast Cell division control protein 13 (Cdc13) and the Repressor-activator protein 1 (Rap1), were between the first telomere associating proteins to be discovered [178]. In *S. cerevisiae*, Cdc13 acts as a single stranded telomeric DNA binding protein that controls telomere elongation by telomerase, whereas Rap1 is responsible for the formation of a multi-protein terminal chromosome cap, termed the "Telosome" [179–181]. The Telosome is composed by the silent information regulator proteins Sir2, Sir3, Sir4 and the telomere-length controllers Rif1 and Rif2 [178, 181–183]. In *S. cerevisiae*, Rap1–Rif1 complexes are negative regulators of telomere length [184–187]. In fission yeast *S. pombe*, Rap1 and Rif1 bind to double-stranded telomeric DNA, interact with the telomere repeat-binding protein Taz1, and regulate telomere length and status of telomeric heterochromatin [20, 188].

Rif1 is highly expressed in mouse embryonic stem (ES) cells and germ cells [189, 190]. The primary function of mammalian Rif1 appears to be involved in DNA-damage [191–193]. However, human RIF1 localizes to dysfunctional telomeres and to telomeric DNA clusters in ALT cells [192], whereas mouse Rif1 maintains telomere length homeostasis of embryonic stem cells (ESCs) by mediating sub-terminal heterochromatin silencing [194].

The protective telomeric structure in mammals is conferred by the assembly of the shelterin complex that caps and shelters chromosome ends from being processed as DNA Double Strand Breaks (DSBs) [171]. Human shelterin is composed of three TTAGGG binding subunits (TRF1, TRF2, and POT1) and three interconnecting molecules (TIN2, TPP1, and RAP1) [171]. Multiple modules of the six shelterin proteins bind to double stranded telomeric repeats via the TRF1 and TRF2 Myb domains, while POT1 associates with single stranded telomeric repeats via its Oligonucleotide/Oligosaccharide-Binding (OB) folds [195, 196] (Fig. 10.3a).

Electron microscopy on purified telomeres of diverse origins, revealed that telomeres do not end as linear DNA molecules, but they form lasso-like structures, that were termed T-loops [197]. It is now well established that the T-loop is formed by the invasion of single-stranded G-overhang into double stranded telomeric repeats, to form a three-stranded DNA displacement loop (D-loop), that renders the 3′ end biochemically inaccessible to DNA repair sensors and nucleases [171, 198] (Fig. 10.1d). T-loops can reach several kb in size, whereas the size of the D-loop is limited by the size of the G-overhang [197, 199].

de Lange [200] proposed that the T-loop structure is the most ancestral element of telomere protection. T-loops preceded protein capping and the emergence of telomerase. The formation of the early T-loops, utilized the complex recombination-dependent, replication (RDR) machinery that pre-existed chromosome linearization as a part of the circular DNA repair system [200, 201].

Human shelterin interacts with several components of the recombinatorial DNA repair machinery such as the MRN complex (Mre11/Rad50/Nbs1) to shape and maintain the T-loop [171, 202, 203]. The DNA helicases RTEL1 (regulator of telomere elongation helicase 1), and the Werner syndrome helicase (WRN) resolve T-loops to enable telomere replication or terminal DNA repair [204–206]. Doksani et al. [207] used stochastic optical reconstruction microscopy (STORM) to show that in mouse chromosomes, TRF2 is the only required shelterin component for biogenesis and/or maintenance of T-loops. Depletion of the other mouse shelterin factors such as TRF1, Rap1, or the POT1 proteins (POT1a and POT1b) did not affect the T-loops.

Several combinations of proteins with similarities to the human shelterin components are found in other species [20]. Mouse shelterin is considered to be composed by TRF1, TRF2, Rap1, and two human POT1 homologues POT1a and POT1b [208–210]. Rap1 is the single human shelterin homologue found at budding yeast telomeres, while Ciliate telomeres contain POT1 and TPP1 [20].

From yeast to humans, the independent or combined depletion of Shelterin or Telosome components from the telomeres, has been related to terminal dysfunction, increased rates of telomeric DNA replication stress, aberrant patterns of telomere sister chromatid exchanges (T-SCEs) and activation of telomeric DNA damage responses [14, 20, 210, 211] (Fig. 10.2c). Mammalian shelterin-free telomeres elicit ATM/ATR (ataxia telangiectasia mutated/ataxia telangiectasia and Rad3 related) DNA damage responses and are processed either by canonical or alternative Non Homologous End Joining (NHEJ) or by homologous recombination (HR) [212].

Telomere dysfunction-induced foci (TIF) are nuclear structures formed by the accumulation of DDR factors, such as γ-H2AX or 53BP1, at telomeres rendered dysfunctional by critical DNA repeat shortening, or by depletion of telomere protective factors [213, 214] (Fig. 10.2a). In the absence of 53BP1, shelterin deprived telomeric repeats immediately become targets for nucleolytic degradation [212].

Shelterin components interact with a plethora of DDR proteins, and nuclear factors implicated in telomere metabolism or the perpetuation of mitotic fidelity (Fig. 10.1d). Several of these molecules such as ATM, ATR, ERCC1/XPF, DNA-PK, BRCA1, BRCA2, PARP-2, TANK1 and TANK2 have been implicated in premature aging syndromes, in hereditary cancer predisposition and in sporadic tumors [18, 215–218] others have DNA binding domains such as HMBOX1 and the COUP nuclear orphan receptors [156].

In addition to chromosome end capping and protection, the shelterin components play important regulatory roles in telomere replenishment and homeostasis [20, 161, 171]. Longer telomeres bind more TRF1 and TRF2 factors and elicit a negative feedback for telomerase activity [219]. Human POT1 and its homologue TEBP from *Tetrahymena*, have been shown to be capable to regulate the formation of terminal G-quadruplexes and to control telomere accessibility by telomerase [220–223] (Fig. 10.3a). Loss of Rap1 induces telomere recombination [224] while TRF2 interacts directly with the DDR machinery through ATM [225], and acts together with Ku70/Ku80 to suppress homologous telomeric crossovers [226].

The Heterochromatic Higher Order of Telomeres and "The Telomere Position Effect"

Beyond the formation of protective, telomere specific nucleoprotein structures, the integrity and functionality of eukaryotic chromosomes depend also on large scale epigenetic modifications that increase the architectural and functional complexity of the linear DNA termini [20, 227]. With the exception of lower eukaryotes, in most species telomeric DNA is organized in unusually spaced, tightly packed nucleosomes [181, 228]. Telomeric nucleosomes display higher mobility compared to the nucleosomes structured on average genomic sequences [229, 230]. The peculiar characteristics of the primary telomeric DNA sequences seem to represent a crucial determinant for chromatin organization both in terms of nucleosomal positioning and spacing [20].

Dipterans like *Anopheles* and *Drosophila melanogaster* have evolved unique biological ways for terminal chromosome organization and capping, as well as for telomere length maintenance [231, 232]. In contrast to most organisms studied,

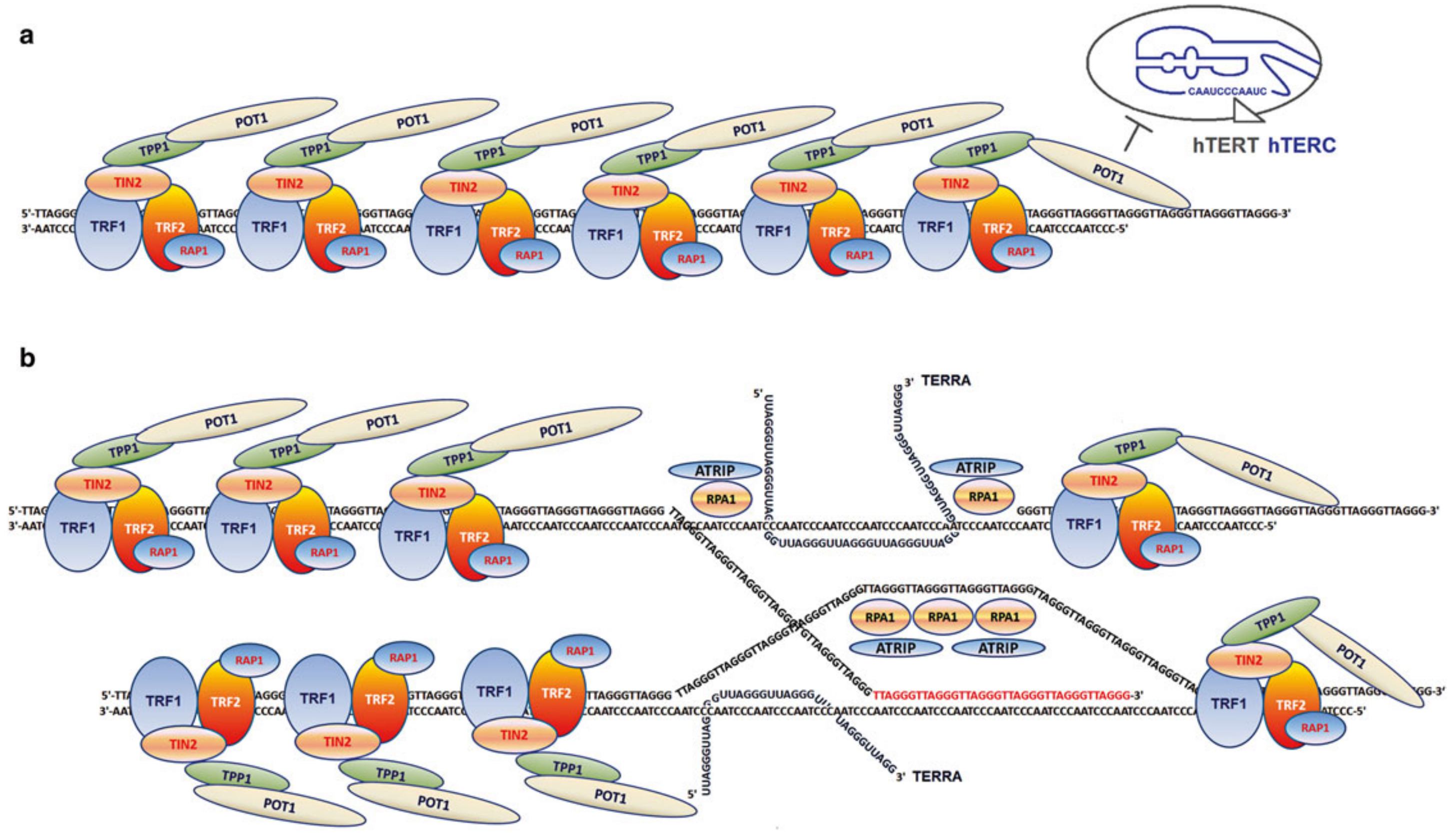

Fig. 10.3 Schematic representation of shelterin binding to human chromosome termini and a hypothetical model for Alternative lengthening of telomeres: Multiple modules of the six known components of human shelterin bind along the whole length of double stranded telomeric repeats via TRF1 and TRF2. POT1 associates with single stranded telomeric DNA of the G-overhang and plays a negative regulatory role in telomere replenishment by telomerase. Longer telomeres bind more TRF1 and TRF2 and elicit a negative feedback for telomerase activity (**a**). A putative Break-Induced-Replication (BIR) model for telomerase-independent telomere elongation: This process generates large segments of single-stranded telomeric DNA that may be protected by single-stranded DNA proteins such as RPA1 and ATRIP and can form DNA–RNA hybrids with TERRAs (*blue*). Neo-synthesized telomeric repeats are depicted in *red color* (**b**)

dipterans do not use telomerase to elongate their telomeres [231, 232]. Telomeres in *Drosophila* are maintained by homologous recombination and gene conversion of the telomere-specific LTR repetitive retro-transposable DNA elements *HeT-A* [231, 233, 234]. Notably the Het-A elements of Drosophila contain sequences that allow the formation of G-quartets in vitro [235]. Fly chromosome termini are capped and protected by the assembly of the "terminin" protein complex [236]. Terminin is composed from the heterochromatin protein 1 (HP1), the HP1/ORC-associated protein HOAP, and the gene products of modigliani (Moi), Ver (Verrocchio), and HipHop [236–240]. The dependence of *Drosophila* chromosome capping on the protein HP1, which is also a major component of mammalian heterochromatin regulation, and the well-established, heterochromatic state of mammalian telomeres suggests a major role of chromatin regulation in the metabolism of the telomeres of linear chromosomes [137, 238].

The heteropyknotic, highly heterochromatic nature of telomeres of several mammalian species was recognized at the early years of chromosome labelling research (1960–1970s) when B. Dutrillaux, T.C. Hsu, J.M. Scheres, J. Lejeune and other pioneer cytogeneticists discovered the C- and T-Banding technologies that were efficiently staining centromeric and telomeric heterochromatin in interphase nuclei and metaphase chromosomes [9, 241–246].

In close proximity to the telomeres, eukaryotic chromosomes display additional structural territories that are termed "sub-telomeres" [247–249]. The sub-terminal DNA regions are located immediately adjacent to telomeric repeats and are comprised of different types of repetitive genomic elements [250]. In humans, subtelomeres contain canonical and degenerate telomeric repeats, and they are highly polymorphic because they frequently undergo large segmental duplications [249].

In contrast to yeast and dipterans, mammalian telomeres and subtelomeric regions, accumulate repressive histone modifications and display extensive hypermethylation of subtelomeric DNA [20, 227, 250, 251]. Mouse subtelomeres are enriched for the H3K9m3 heterochromatin mark, mediated by the Suv39h1 and Suv39h2 histone methyl-transferases [137]. In mice, Rif1 maintains H3K9me3 levels at subtelomeric regions through the negative modulation of the expression of Zscan4 [194]. In humans, DNA methylation accumulates at highly repetitive chromosome territories such as centromeres, pericentric regions and subtelomeres and is considered to act as a suppressor of illegitimate recombination [252–254]. Mammalian telomeres display trimethylated lysines in histones H3 and H4, extensive histone hypoacetylation, accumulation of HP1 and hypermethylation of subtelomeric CpG islands [227, 255]. The heterochromatic state of telomeres is believed to play important roles in the organization of nuclear architecture; it may also contribute to interphase chromatin interactions and the regulation of the mechanisms of telomere replenishment [227, 256].

Telomeres not only constitute targets of epigenetic modifications but they also act as epigenetic agents per se, via a mechanism that is capable to spread heterochromatic silencing to nearby euchromatin [257, 258]. The so called Telomere Position Effect (TPE) regulates the transcriptional activity of genes adjacent to telomere ends, by repressing their expression [259, 260]. TPE is extending in a continuously decreasing fashion from the telomeres to the centromeric chromosomal regions [261]. Telomere position effects have been identified in a variety of lower organisms such as *Schizosaccharomyces pompe*, *Trypanosoma brucei*, *Plasmodium falciparum*, as well as in plants, mice and humans [255, 259, 262–264].

The first organism in which TPE was described was *Drosophila melanogaster* [265–267]. However the TPE phenomenon has been thoroughly investigated and best understood in *Saccharomyces cerevisiae* [266–268]. More than 50 proteins are implicated in *S. Cerevisiae* TPE, with the Ku heterodimer (yKu70p and yKu80p), the Sir-complex (Sir2p, Sir3p and Sir4p) and the C-terminal domain of Rap15 being essential TPE components, whereas in *S. Pompe*, TPE depends on Taz1p, spRap1 (homolog of Rap1p) and Swi6 (ortholog of HP1) [185, 188, 255, 257, 269–271].

Little is known about the molecular mechanism of TPE in Homo sapiens. The histone deacetylase, SIRT6 is considered essential for maintenance of TPE in human cells [272]. Furthermore, the heterochromatin protein HP1, the chromatin remodeling factor SAL1 and the shelterin components TRF1 and TIN2 are thought to be key players of the human TPE processes [273, 274]. In humans, TPE was first systematically studied in HeLa cells, in which an exogenous reporter gene was stochastically incorporated into the genome. Clones with the exogenous gene inserted adjacent to telomeres, presented tenfold decreased expression, in contrast to clones carrying the reporter in random genomic positions, with the phenomenon being telomere length and heterochromatin formation dependent. In this context, hTERT overexpression that results in telomere elongation, led to further decrease of the adjacent transgene's expression, while overexpression of TRF1 or treatment with the histone deacetylation inhibitor Trichostatin A, restored expression [259, 275].

The biological machinery that regulates the expression of subtelomeric genes may be implicated in normal human ageing and nosology and especially in age related diseases when telomeres are substantially shorter [259]. A rare myopathy termed Facio-Scapulo-Humeral Dystrophy (FSHD) is currently the only human disorder directly associated to TPE. The leading causative factor of FSHD, is the DUX4 homeobox protein, expressed by a gene located adjacent to the subtelomeric D4Z4 tandem repeat array, within chromosome band 4q35 [276]. Normal FSHD alleles carry 11–110 copies of the D4Z4 repeat, that are acting as a barrier of DUX4 expression, whereas pathogenic alleles have only 1–10 tandem repeats and allow the expression of DUX4 [258, 276–281]. Stadler et al. [258] have shown that the expression of DUX4 can be further up-regulated in FSHD myoblasts and myotubes with short telomeres.

TERRAs: Novel Partners of Telomere Homeostasis

In 2007, Azzalin et al. [282] brought down one longstanding dogma of molecular biology: In contrast to the "good-chromatin" euchromatin, that is "unpacked" and capable to be transcribed into coding or non-coding RNAs, highly heterochromatic regions such as the mammalian centromeres and subtelomeres, were generally considered transcriptionally silent. The groups of J. Lingner in 2007, and M. Blasco (2008) showed that throughout eukaryotes, subtelomeric regions and telomeres are transcribed into variable sized, long non-coding telomeric repeat-containing RNAs that were termed TERRAs [282–285]. From anthozoans to humans, TERRAs consist of subtelomeric sequences and multiple tracts of the hexa-nucleotide repeat (5′-UUAGGG-3′) [283]. TERRA molecules are considered to be much shorter than their available C-rich telomeric DNA template and were shown to display heterogeneous lengths between species and chromosomes, ranging from 100 bp, up to more than 9 kb [178, 282, 285–288].

It is very possible that the eukaryote telomeric transcriptome will be expanded with several types of telomeric non coding RNA transcripts: In addition to TERRA, *Schizosaccharomyces pombe* telomeres express several types of telomeric transcripts named ARIA, ARRET and anti-ARRET [289, 290]. ARIAs were also found in plants [291]. They are mainly comprised by C-rich complementary RNA sequences that use the G-rich telomeric DNA as template, suggesting that in parallel to subtelomeric regions, canonical telomeric DNA repeats can serve as transcription start-sites for RNA polymerases [289, 290]. ARRET display sequence complementarity with proximal subtelomeric regions and lack canonical telomere repeats [289]. The anti-ARRETs are transcribed by the antiparallel strand of the ARRET template hence they are complementary to ARRET [283, 289].

Up to date, TERRA is the most well studied species of the telomere transcriptome [283]. Chromatin immunoprecipitation (ChIP) experiments revealed that from fungi to humans, TERRAs are neosynthesized by the DNA-dependent RNA polymerase II (RNAPII) that produces G-rich, TERRA molecules, using the C-rich telomeric DNA strand as a template [282, 285]. A proportion of TERRAs is modified at their 3′-end, by the addition of a polyadenylation tag [292, 293]. In human cells polyadenylated TERRAs fractionate within the nucleoplasmic fraction and do not associate with chromatin [293]. It is estimated that around 7 % of human TERRA is polyadenylated [292]. The larger fraction of human TERRAs is non-polyadenylated and found associated with telomeric heterochromatin [293].

Human CpG dinucleotide-containing TERRA promoters were found in at least half of the highly heterochromatic human subtelomeres [282]. TERRA promoters are active during G1 and G2 phases of the cell cycle and silenced during the S-phase [293]. Subtelomeric DNA methylation and other subterminal epigenetic modifications are direct modulators of TERRA metabolism. Combined depletion of the DNA methyltransferases DNMT1 and DNMT3b that control heterochromatin state of the subterminal CpG islands of human chromosomes, led to up-regulation of TERRA transcription in diploid and cancer cell lines [294]. The shelterin component TRF2 is considered a negative controller of TERRAs. Telomere de-protection induced by TRF2 knockdown, leads to overproduction of TERRA in human fibroblasts and HeLa cells [283, 295, 296]. On the contrary, Deng et al. [297] have shown that the chromatin organizing factor CTCF and the cohesin subunit Rad21 bind to subtelomeric regions and promote TERRA transcription in human cell lines. In budding yeast, Rif1 and Rif2 block TERRA transcription at all telomeres whereas the proteins of the Sir-family are involved in TERRA repression only at the subset of telomeres carrying the conserved yeast subtelomere structures termed Y'-elements [283, 298].

The formation of DNA/RNA hybrids named R-loops is a rare natural outcome of transcription, caused by invasion of the DNA double strand, by nascent RNA transcripts. R-loops have been considered causative factors of genome fragility and can induce repressive chromatin marks over mammalian gene terminators [299]. Co-transcriptional base-complementarity may promote the formation of DNA/RNA hybrids at telomeres [300, 301]. G-rich telomeric DNA/RNA hybrids are capable to form G-quadruplexes and thus they can inhibit telomerase accessibility to telomeres [283].

In yeast, the poly(A)-polymerase Pap1p polyadenylates and stabilizes TERRAs whereas overexpression of the 5′-3′ exonuclease Rat1p, is capable to fully degrade TERRAs from the nucleus [302]. Yeast defective for Rat1p, accumulate TERRAs and display some degree of telomere shortening, suggesting that telomere-associated TERRAs may directly affect the mechanisms of telomere replenishment [283]. Telomere attrition in Rat1 deficient cells was rescued by overexpression of RNaseH, indicating that telomere metabolism is affected by the formation of telomeric RNA/DNA hybrids [284]. In human cell lines utilizing the alternative lengthening of telomeres (ALT-pathway) depletion of RNaseH1 caused TERRA-telomeric hybrid accumulation, exposure of single-stranded telomeric DNA, increased levels of the single strand DNA binding protein RPA and abrupt telomere length excision, whereas overexpression of RNaseH1 suppressed ALT telomeric recombination and led to telomere shortening [300]. Analogous changes at the levels of RNaseH1 in telomerase positive cells did not demonstrate any of the above phenotypes suggesting a specific role for TERRA in alternative lengthening of telomeres [300].

TERRA association with human telomeres is regulated by proteins known to participate in the biological processes of mRNA decay (NMD), such as the RNA/DNA helicase and ATPase UPF1, the RNA endonuclease hEST1A/SMG6, and the protein kinase SMG1 [282, 283]. Depletion of UPF1 in telomerase positive HeLa cells affected replication of the leading telomere strand causing telomeric fragility [303]. UPF1-depleted cells accumulate telomeric R-loops because the C-rich telomeric strand acts as the template for both TERRA and leading-strand DNA replication [303].

Telomeropathies

The first premature aging disorder linked to impaired telomere metabolism was Dyskeratosis Congenita (DC), an inherited bone marrow failure condition, first described in 1906 by Zinsser [304]. DC is a rare, childhood onset disease, estimated to occur with a frequency of 1, in one million individuals, with death occurring at an average age of 16 [305]. The three distinctive clinical characteristics of DC are skin pigmentation abnormalities, nail dystrophy and mucosal leukoplakia [306, 307]. These phenotypes are present in almost 80–90 % of diagnosed cases [307].

Organ failure is a typical outcome of DC patients, with bone marrow in 80 % of the cases being the first tissue to be affected, leading to aplastic anemia [306, 308]. In addition to bone marrow failure, pulmonary fibrosis and cancer, are the main fatal complications of the disease [306]. The most frequent malignancies are myelodysplastic syndromes (MDS), head and neck cancers (squamous carcinomas), and acute myeloid leukemias (AML) [115, 309]. The clinical phenotype of DC is expanding continuously with a plethora of disease manifestations such as immune deficiency, cardiomyopathy, liver cirrhosis, mental retardation, epiphora (excessive tears in the eyes), dental loss, osteoporosis, premature hair loss/greying and deafness [15, 19].

DC patients, present abnormally short telomeres as compared to individuals belonging in the same chronological age group [114, 310]. The premature telomere attrition is considered to be the underlying cause behind most of the pathological features of DC signifying this disease as a "Telomeropathy" [311–314]. This is supported by the fact that all major mutations linked to DC, involved genes implicated in telomere maintenance: About 50 % of DC patients have a mutation in one of the three main components of the telomerase holoenzyme complex, Dyskerin, TERC and TERT [315]. The DKC1 gene, that encodes Dyskerin protein, is located at the X chromosome [316]. Mutations of DKC1 cause a recessive, X-linked form of DC characterized by a severe phenotype [316]. An autosomal dominant milder form of DC, results from defects of the gene responsible for TERC, whereas mutations in the reverse transcriptase component TERT, have been linked to autosomal dominant and autosomal recessive inheritance [311, 314, 317, 318]. Many TERT mutations affect severely the activity of telomerase [311, 319–321]. However, in some cases the phenotype appears relatively milder therefore they are thought to behave more as risk polymorphisms, than as determinants of disease [312] (Table 10.1).

Homozygous mutations of the NOP10 and NHP2 components of telomerase holoenzyme are considered responsible for an autosomal recessive form of DC [322, 323]. Approximately 11 % of DC cases result from mutations in the TINF2 gene, encoding the Shelterin component TIN2 [171, 324]. TIN-2 telomeropathy is linked to a severe clinical phenotype and transmitted in an autosomal dominant mode [325]. While five out of six DC genes result in impaired telomerase activity, TIN2 mutations are thought to jeopardize either the protection of telomeres or the accessibility of telomere ends by telomerase, leading to more pronounced telomere attrition [312]. Most TINF2 mutation patients, display exceptionally short telomeres from a much earlier age than the patients bearing any other mutant DC gene [312].

Dyskeratosis congenita is considered a highly heterogeneous disorder with its genetic cause and phenotype overlapping significantly with various syndromes such as Hoyeraal-Hreidersson (HHS), Coats-Plus, and Revesz syndrome. HHS particularly, is believed to be a severe DC form and the phenotype diverges by the addition of cerebellar hypoplasia, microcephaly and intrauterine growth retardation [326]. Similarly to HHS, Revesz syndrome is also extremely rare and is presenting with HHS symptoms and bilateral exudative retinopathy [327]. Patients of those severe telomeropathies exert extremely short telomeres and a higher mortality rate than

Table 10.1 Telomeropoathies and implicated genes

Genes involved	Process affected	Role in telomere metabolism	Diseases	References
TERC	Telomerase activity	Telomere length maintenance	AD-DC, Sporadic & Familial IPF, AA, MDS	[315, 318, 320, 321, 336–338, 344–346]
TERT	Telomerase activity	Telomere length maintenance	AD-DC, Sporadic & Familial IPF, AA, MDS	[311, 314, 315, 317, 320, 321, 336–338, 344–346]
DKC1	Telomerase activity	Telomere length maintenance	XR DC, HHS	[315, 316]
CTC1	Telomere capping/CST complex	Telomere protection	Coats-Plus syndrome	[328, 329]
TINF2	Shelterin	Telomere regulation	AD-DC, HHS, Revesz syndrome	[171, 312, 324, 325]
NOP10	Telomerase activity	Telomere length maintenance	AR-DC	[323]
NHP2	Telomerase activity	Telomere length maintenance	AR-DC	[322]
TCAB1	Telomerase activity	Telomerase assembly	AR-DC	[350]
FANCD2	Telomere maintenance	Telomere regulation	FA	[347, 348]
RECQL4	D-loop resolution	Telomere regulation	RT	[349]
NBN	MRN complex	Telomere length maintenance	NBS	[53, 148, 350]
ATM	Telomeric DDR?	Telomere regulation	AT	[351–353]
BLM	DNA unwinding?	Telomere regulation	BS	[359]
WRN	D-loop resolution	Telomere regulation	WS	[358]
DNMT3B	Heterochromatinization	Subtelomere/Telomere regulation	ICF	[360–364]

AD/AR/XR-DC autosomal dominant/autosomal recessive/X-linked recessive, dyskeratosis congenita, *IPF* idiopathic pulmonary fibrosis, *AA* aplastic Anemia, *MDS* myelodysplastic syndromes, *HHS* Hoyeraal-Hreidersson syndrome, *FA* Fanconi Anemia, *RT* Rothmund Thomson, *NBS* Nijmegen Breakage syndrome, *AT* ataxia telangiectasia, *BS* bloom syndrome, *WS* Werner syndrome, *ICF* immunodeficiency centromere instability and facial anomalies syndrome, *DDR* DNA damage response classic DC cases. Coats-Plus syndrome has been linked to CTC1 mutations and patients exhibit exudative retinopathy and intracranial calcifications, features that are also observed in HHS and Revesz syndromes [328, 329]. Due to the small number of HHS, Revesz and Coats-Plus syndrome patients and their overlapping clinical findings, it is extremely difficult to determine guidelines distinguishing the three disorders [330].

The most common adulthood onset manifestation of impaired telomere maintenance is the idiopathic pulmonary fibrosis (IPF) which is a fatal progressive lung disease that typically presents in the fifth decade with the incidence increasing with advanced age [50, 331–333]. IPF is characterized as sporadic and only 0.5–2.2 % of the cases present familial inheritance [334, 335]. Mutations in TERT and TERC telomerase subunits have been identified in about 1–3 % of sporadic and 8–15 % of familial IPF cases [321, 336–338]. The pattern of inheritance in most families is autosomal dominant and consistent with incomplete penetrance and haploinsufficiency of telomerase [335, 339–342]. Furthermore, 37 % of familial and 25 % of sporadic cases present telomere length shorter than the 1/10 of the general population, suggesting an association with impaired telomere replenishment. This phenomenon may be explained by the underlying telomerase mutations and/or the effects of environmental causes such as smoking, which is a common characteristic of at least 50 % of IPF patients [342]. Because telomerase is expressed only in mitotically active cells, IPF may result, partly, from the senescence or loss of a stem cell population in lungs, capable to respond to continual injuries over time. Due to the haploinsufficiency of telomerase, the clinical phenotype arises only after adequate time has elapsed and thus the cells have conducted enough division rounds to present critically short telomeres [321, 343]. Mutations in TERT and TERC have also been found in bone morrow failure disorders other than DC, such as the myelodysplastic syndromes (MDS) and aplastic anemia (AA) [320, 344–346]. Another adult-onset disorder presenting telomerase mutations is familial liver cirrhosis, which is a known complication of dyskeratosis congenita [116, 311].

Dysfunctional or prematurely shortened telomeres have been observed in several premature ageing or cancer pre-disposing syndromes caused by mutations in genes encoding proteins that are key players of DNA damage responses and are also implicated in telomere metabolism [347]. Telomere dysfunction foci (TIFs) and short telomeres have been found in lymphocytes from patients with Fanconi anemia (FA) [348]. A subset of FA patients, carry mutations in FANCD2, a protein that interacts with telomeric DNA and regulates the levels of the shelterin component TRF1 [347]. Mutations in the RECQL4 helicase that resolves telomeric D-loops, are the causal factors of the Rothmund–Thomson progeroid syndrome (RT), a disease with some DC clinical characteristics that is accompanied by increased TIF frequencies and excessive telomeric instability [349]. The Nijmegen chromosome breakage syndrome occurs due to mutations of the Nibrin (NBN) gene that encodes a member of the MRN (MRE11/RAD50/NBN) complex [53]. The MRN multimer is involved in the mechanisms regulating telomere length maintenance [148, 350]. Cells from patients with Nijmegen syndrome show decreased telomere length and poor proliferation rates [53].

Ataxia telangiectasia (AT) is characterized by neurological deterioration, immunodeficiency, spontaneous chromosomal instability, hypersensitivity to ionizing radiation, predisposition to leukemias and lymphomas and premature ageing [351]. In yeast, the Ataxia telangiectasia mutated (Atm) homolog, Tel1, is necessary for normal telomere length regulation [352]. Lymphocytes of AT patients exert accelerated telomere shortening and frequent terminal chromosome fusions [351, 353].

Two other rare genetic cancer predisposition diseases Werner's syndrome (WS), and Bloom's syndrome (BS), are related to premature aging [354]. WS and BS are caused by loss of function of the RecQ helicases WRN and BLM, respectively [355, 356]. Both diseases are characterized by replication errors, hyper-DNA recombination and chromosomal instability [355, 357]. Mutations in the WRN gene were associated with insufficient replication of the G-rich telomeric strand [358]. BLM also contributes to chromosome-end maintenance through its genome-wide activity in resolving difficult-to-replicate regions such as telomeres. BLM-deficient normal human fibroblast cells display elevated frequencies of telomere dysfunction [359].

The Immunodeficiency, Centromeric region instability and Facial anomalies syndrome (ICF) is a rare condition caused by mutations in the DNA methylotransferase gene DNMT3B [360]. Cells from ICF patient's display telomeric abnormalities and reduced subtelomeric methylation [361–364]. Remarkably, ICF cells are characterized by abnormally high levels of TERRA transcripts suggesting the first link between alterations in the telomere transcriptome and human nosology [365].

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Chapter 11
Proximal Regulatory Elements with Emphasis on CpG Rich Regions

Pavlos Fanis

Introduction

All cells of a complex multicellular organism contain the same genome but carry out different biological processes such as proliferation, differentiation, cell survival and apoptosis. The execution of these diverse functions is characterized by differentially expression of the genes in the different cell types of the organism. The mammalian genome encodes 30,000–40,000 genes which are transcribed in the proper spatial and temporal patterns. The regulation of the genes can be controlled at many phases such as transcription initiation, elongation and termination. Moreover, the control of the transcription can be applied at other levels including RNA processing, export from the nucleus to the cytoplasm, mRNA translation and mRNA and protein degradation. To better understand the mechanisms that are responsible for the distinct gene expression patterns we need to have better knowledge of the transcriptional regulatory elements influencing the transcription. In the eukaryotic genome there are various types of transcriptional regulatory elements that are involved in the control of gene expression. Here, we discuss the general features of eukaryotic transcription focusing on the GC rich regions and their roles in health and disease. We give an outline of the eukaryotic transcription initiation process and then we focus at the structural, function and mechanistic function of GC rich regions in health and disease as well as methods currently used to identify transcription regulatory elements in the human genome.

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Eukaryotic Transcription

The initiation is the first step in transcription where the highest regulation is occurring. The process begins when the RNA polymerase (RNA pol) binds to template DNA strand and begins the production of the complementary RNA. In eukaryotes there are five RNA polymerases, RNA pol I that transcribes a set of genes that encode the ribosomal RNAs (rRNAs), RNA pol III that is responsible for the transcription of genes that encode the transfer RNA (tRNA), the 5S RNA and some small RNAs and RNA pol II (will be highlighted in this chapter) that transcribes the majority of genes that encode the mRNA which serve as template for the production of protein molecules [1–3]. Finally, in plants there are the RNA pol IV and the RNA pol V that synthesize small interfering RNA (siRNA) and RNAs involved in siRNA-directed heterochromatin formation, respectively [4, 5].

Protein-coding genes are controlled by *cis*-acting regulatory DNA elements and by *trans*-acting remodeler, mediator complexes and transcription factors that recognize the *cis*-acting regulatory DNA elements. The *cis*-acting regulatory elements can be divided into the distinct proximal regulatory elements (promoter) and distal regulatory elements (Fig. 11.1).

The basic transcription machinery is composed by the RNA pol II enzyme, the general transcription factors such as TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH and the mediator complex [6]. TFIID general transcription factor interact with the core promoter and participate in the recruitment of the pre-initiation complex (PIC) [7]. TFIID is a multi-subunit protein complex containing the TATA-box binding protein (TBP) and the TBP-associated factors (TAFIIs). After the TFIID binds to the promoter the general transcription factors, RNA pol II and the mediator complex are recruited in a highly ordered fashion to form the PIC. When formation of PIC at the promoter is complete, transcription proceeds through a series of steps before the fully establishment of the transcription elongation RNA pol II complex [8].

The formation of PIC in the core promoter of a gene it serves to accurately initiate transcription. Transcriptional activity can be further activated through changes in the activities of transcription factors (co-activators) [9]. Transcription factors are usually DNA-binding proteins that affect the transcription of a gene positively or negatively by binding to DNA regulatory elements or by interactions with other proteins [10]. The recognition sites of transcription factors are specific and usually located upstream of the core promoter. The precise sequence of a transcription factor binding site can be important for the binding strength of a transcription factor, thus can have an impact in the levels of gene expression [11]. Transcription factors often work in groups or complexes that allow various levels of transcriptional control [11] and are classified in different categories according to their DNA-binding domains such us basic leucine zipper (bZIP), basic helix-loop-helix (bHLH), zinc finger, homeodomain and nuclear hormone factors [12].

Transcription factors bind to DNA and mark a gene for activation or repression through interactions with co-activators and co-repressors. Co-activators and co-repressors bind to transcription factors and function by recruiting other proteins

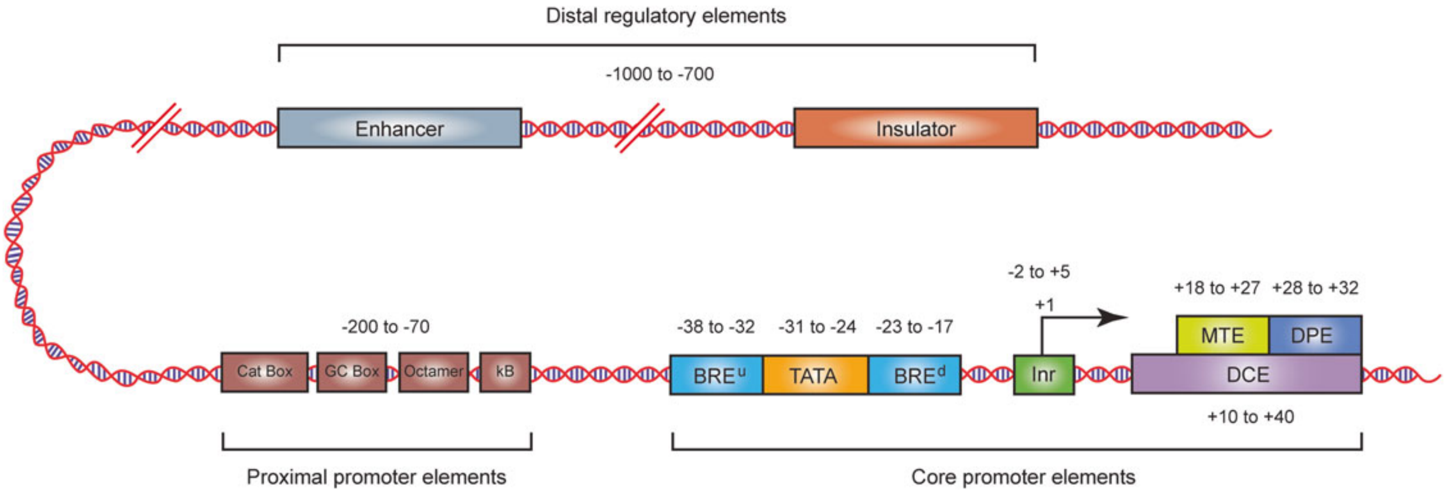

Fig. 11.1 Gene regulatory elements. Illustration of a typical eukaryotic gene regulatory region with clusters of core promoter elements, proximal promoter elements and distal regulatory elements. The promoter is composed of core promoter and proximal promoter elements. The core promoter elements may include the TATA box, the Initiator Element (Inr), the TFIIB-Recognition Element (BRE), the Downstream Promoter Element (DPE), the Motif Ten Element (MTE) and the Downstream Core Element (DCE). The Proximal promoter elements may include the CAAT box (Cat Box), the GC Box, the Octamer and the NF-kB (kB) elements. The locations of the elements are indicated relative to the transcription start site (+1)

with enzymatic activities that alter chromatin structure [13]. Chromatin compact state often makes gene promoters inaccessible to transcriptional machinery and/or transcription factors. Thus, co-activators including histone modification and ATP-dependent remodeling protein complexes alter the structure of chromatin by making it accessible to the binding of transcription factors [14]. The opposing effects on chromatin structure, by making it inaccessible to transcription factors, have the co-repressors.

Transcription in more than half of human genes is initiated from genomic regions with increase content of G and C nucleotides referred to as CpG islands.

GC Rich Regions

DNA methylation is carried out by the transfer of a methyl group at the 5 position of the pyrimidine ring of cytosine to form the methylcytosine and is observed in most of the organisms but the rate of methylation differs strongly, some species like yeast luck DNA methylation [15]. As the embryonic stem cell (ES) undergoes differentiation into different tissues, DNA methylation changes the expression of genes [16]. Methylation of DNA at the 5 position of cytosine has the specific effect of reducing gene expression. In somatic cells, DNA methylation usually occurs in a CpG dinucleotide context. CpG are DNA regions where a cytosine occurs next to a guanine. CpG means “Cytosine-phosphate-Guanine”. The CpG is used to distinguish the linear sequence from the G-C base pairing. Enzymes that add the methyl group to the cytosine are called DNA methyltransferases (DNMTs) [17].

The frequency of CpG dinucleotides in human genome is ~1 %, lower than would be expected due to random chance. It is proposed that the CpG deficiency is due to increase susceptibility of methylcytosines to deaminate to thymidine [18]. In mammals CpG islands are regions in the genome with a high content of CpG sites. CpG islands are typically 300–3000 bp in length and generally associated and found in or closed to ~40 % of promoter regions of mammalian genes [19, 20]. The CpG sites of the CpG island of promoters are unmethylated if the genes are expressed. Methylation of CpG sites in the promoter of a gene may inhibit gene expression [21]. The regulation of these promoters involves proteins which specifically bind at non-methylated CpGs and have an effect at the modification status of CpG island chromatin [22]. CpG islands typically exist at/or near transcription start site of genes, especially of housekeeping genes (genes that expressed in all cell types) and a significant fraction of the brain or the neutrally expressed genes [23, 24]. In humans, approximately 70 % of the gene promoters are associated with CpG islands making this the most common promoter type [25]. A large group of CpG islands are in distant regions from the transcription start site but show existence of promoter activity [20, 26].

Genes transcribed by RNA polymerase II can be divided in two different classes according to the CpG density across theirs 5′ ends. In the first class the CpG density is the same as the genome average (1 every 100 nucleotides). In this class there are genes that are expressed in a limited number of cell types. In the second class, the 5′ end of the genes is surrounded by CpG islands [27]. The association of CpG islands with the upstream regions of many genes can be used to predict promoters and/or genes in the mammalian genome [28]. As mentioned before, CpGs at the CpG islands of active genes remain not methylated. The features of non-methylation and the high density of G+C are accompanied by distinctive chromatin organization. The CpG islands show the properties of the active chromatin, such as hyperacetylation of histones H3 and H4 and nuclease enhance sensitivity at nucleosome-free regions [21, 29, 30].

CpG Islands and Methylation

CpG islands were first identified by digestion of mouse genomic DNA with a methyl-CpG specific restriction enzyme. A part of the DNA was consist of very highly fragmented DNA and was found to be composed of clusters of non-methylated CpG sites, the CpG islands [19, 45]. In addition, computational prediction and sequencing techniques identify approximately 27,000 CpG islands [43].

The characteristic clustering of CpG sites is because of their immunity against de novo methylation by DNA methyltransferases (DNMTs) during the earliest stages of mammalian development. A reason for this might be the binding of transcription factors that prohibit DNMT association at CpG island sequences [41].

The majority of CpG islands are hypomethylated but, as mentioned before, a small percentage is methylated during development. Some of these examples are shown to play a role in X-inactivation and genomic imprinting [46, 47]. Hypermethylation of CpG island promoters result in transcriptional repression. Promoters with relatively low CpG content were found to be more often hypermethylated [48]. Moreover, sites of CpG island methylation frequently found to genomic regions distal to promoters [49].

Transcription in de novo methylation CpG islands (in tumor cells or in cell lines) is strongly repressed. This event does not occur in physiological conditions in the organism except for the CpG islands of the imprinted genes [31, 32]. Repression of the transcription in the methylated DNA is mediated by proteins such as MeCP and MBD family proteins that bind specifically methylated CpGs and recruit histone deacetylases and transcriptional corepressors [33, 34]. DNA methylation in CpG-poor promoters correlates with their level of expression. Many examples suggest that although DNA methylation affects gene expression it is unlikely to play a general role as a transcriptional regulator. For example, demethylation of the promoter of the aminotransferase gene in rats does not lead to its activation in cells where it was previously methylated and inactive despite the presence of proteins that bind to the promoter [35]. DNA methylation is essential for proper mammalian development as shown by the embryonic lethality caused by disruption of the methyltransferase genes *Dnmt1*, *Dnmt3α* or *Dnmt3b* in mouse [36, 37].

CpG islands remain free of methylation in the heavily methylated genome. One possibility is the protection by the transcription factors that bind and provide less accessibility to DNA methyltransferases [41]. It is still unclear the existence of CpG islands because ~40 % of all human promoters are CpG-poor and can operate without CpG islands. An example is the erythroid specific α-and β-globin genes that are expressed to produce the haemoglobins. The α-globin gene is associated with a CpG island but the β-globin gene is not. The mouse α-and β-globin genes both are not associated with a CpG island [42]. This difference between human and mouse CpG islands can be found in approximately 20 % of human CpG island promoters compared to the mouse orthologues [23, 43] suggesting that some human CpG islands obtain a CpG island or some mouse promoters lost a CpG island during evolution [42]. CpG islands can be lost when they de novo methylated in the germ line and replaced through deamination by TpG instead of CpG but this cannot explained how they appear in the first place. One possibility is that CpG islands might have emerged as a genome footprint in the chromosomes by the replication initiation event [44]. Comparative foot-printing analyses across human and mouse CpG islands have revealed that the protein–DNA interaction pattern varies among the two species. Despite the high degree of conservation of their coding sequences this observation suggest that regulatory regions are more permissive to changes than coding regions [41].

Role of CpG Islands in Bidirectional Transcription and DNA Replivatio

An interesting feature of CpG island promoters is the elevated prevalence of bidirectional transcription [38]. The reason for this might be the high frequency of transcription factors bound to them and the presence of elements that are responsible for activating bidirectional transcription in vitro. This kind of organization allows the coordinate regulation of the two genes [39]. Some chromosomal replication origins have been mapped close to gene promoters in human cells. Moreover, it is also shown that transcription factors stimulate replication in many organisms. These evidences suggested that CpG islands might serve not only as promoters but also as replication origins because of the high density occupancy of transcription factors and the open chromatin organization. An example is the binding of the origin recognition complex (ORC) to the CpG islands [40].

Genomic Approaches for Identifying Regulatory Elements

There are many experimental and computational approaches for the identification or prediction of proximal regulatory elements in the genomes of eukaryotes.

Functional Assays

One of the most efficient ways for identifying and examining the regulatory activity of a DNA element is the use of a reporter gene assay. In such assay the DNA region of interest is cloned into a plasmid containing a reporter gene that can be easily measure [such as green fluorescent protein (GFP), luciferase, β-galactosidase, chloramphenicol acetyltransferase (CAT)]. The resulting construct is then introduced into cells or organism of interest and the reporter gene expression is measured. The nature of the plasmid construct depends on the regulatory elements to be identified. For example if the element to be tested is for promoter activity, is placed immediately upstream of the reporter gene. Once the element is identified further studies, such as deletions or creation of mutations, can be performed for more accurate characterization of the element. There are limitations of using such functional assays for identification of proximal regulatory elements. First, transfection assays mostly are performed in immortalized cell lines that are not representing the natural occurring environments. Secondly, an upstream regulatory element, in reality, might be used only in limited content such as specific tissue, developmental stage or specific environmental responses that differs from the cell culture that is selected for the assay. Transgenic assays by injecting the construct into embryos of animal models and follow the expression of the reporter gene through development can be done to overcome this limitation. Transgenic assays have also their limitation as they can reveal the specific expression pattern in the early developmental stages as they are sometimes instable because of embryonic cell multiplication [50].

Identification of Proximal Regulatory Elements on a Genome-Wide Scale

A technique for identification of transcription factor binding sites (TFBSs) on a genome-wide scale is the DNase I hypersensitive site (HS) mapping in which nucleosome-free regions are easily digested by the DNase I enzyme. These open chromatin regions are functionally related to transcriptional activity due to the binding of transcription factors [51, 52]. DNase I hypersensitive technique can be combined with high throughput sequencing or chip to provide a genome view of DNase I HS in specific cells at a specific developmental stage [53, 54].

Another powerful technique for determination of genomic sequences that are bound by a specific protein in vivo is the chromatin immunoprecipitation (ChIP). In this technique the protein(s) of interest are crosslinked temporarily with the associated chromatin in the living cells which then are lysed and the DNA–protein complexes are sheared by sonication at the desired size. The crosslinked DNA fragments that are associated with the protein(s) of interest are immunoprecipitated by a protein specific antibody and the associated DNA regions and their sequences can be determined by microarrays (ChIP-chip), by high throughput sequencing (ChIP-seq) or ChIP-exo, an extension of ChIP-seq to increase the resolution of TF bound sites [55]. Limitations of ChIP assays are that specific antibodies must be created for each DNA binding factor of interest. Moreover, ChIP assays cannot distinguish between different isoforms of a specific transcription factor.

Computational Approaches Identifying Proximal Regulatory Elements

Ninety eight percent of the human genome consist of non-coding DNA, thus is likely to contain regulatory regions [56]. Identifying a promoter of a specific gene can be difficult as core promoters are found far from the first exon because of the existence of the 5′ untranslated region (UTR) and/or introns. Furthermore, not all the promoters contain the core promoter elements thus the identification can be a challenge [57]. For prediction and identification of such regulatory regions, computational approaches are very helpful. Such approaches can look for common sequences to all known promoters and they search the genome to identify new regions with such sequences. These methods can be used alone or in combination with the existence of a CpG island or the presence of a possible first exon. Application of such approaches in genome-wide scale is limited because of lacking specificity and sensitivity. The reason of this is due to the fact that these computational programs rely on the amount and quality of the available data that they use for finding new regulatory regions/elements. Moreover, these approaches can identify proximal promoter elements that are already identified.

Identification of New Upstream Regulatory Elements

In TFBSs prediction programs a given sequence is scanned for known transcription factor sequence motifs that are experimentally identified. Examples of such databases are the TRANSFAC [58] and the JASPAR [59]. In these databases potential TFBSs are predicted, which in many cases the number of sites are large with many false positives. The reason for this is, in part, the quality of the data that are used to build the databases. Furthermore, these databases are not fully complete because not all of the DNA binding factors have been identified and some of the known DNA binding factors are not thoroughly defined. Another approach for identification of novel TFBSs is the examination for common sequence motifs in the upstream region of genes that are co-expressed. Known algorithms that use this approach are the MEME and AlignACE. Comparative genomic approaches can be used for prediction/identification of TFBSs. With these approaches TFBSs assumed to be conserved across evolution and DNA sequences from species separated by large evolutionary distances are compared. Sequences that are conserved are candidates to be functional TFBSs. Example of programs that perform such analyses are the PhastCons [60] and the Footprinter [61]. The limitation with these approaches is not all the TFBSs are common among species due to the fact that the same factor may bind to sequence variants of the TFBSs or a specific regulatory element is not conserved among species without affecting the expression of a gene. In addition, some regulatory elements important for human development and disease can be found only in humans. New analytical approaches in comparative genomics are required for detection of weak conserved TFBSs by increasing, for example, the number of species that the genome sequencing information is accessible.

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Chapter 12
Genomic Analysis Through High-Throughput Sequencing

Michalis Hadjithomas

Introduction

The advent of high-throughput sequencing in the early years of the millennium has disrupted the way research is being conducted in the biological sciences across the board, from human biology to microbial ecology. High-throughput sequencing was primarily developed to produce massive amounts of sequences in order to reconstruct the large genomes of higher-organisms, especially humans. However, it was quickly realized that massively parallel sequencing could be used to probe and answer questions that were previously outside the reach of scientific methods. This has led to the development of a multitude of high-throughput sequencing applications including exome sequencing (i.e. targeted re-sequencing of an organism's exons), long range chromatin interaction analysis, ribosome profiling, and many more, some of which we will discuss in this chapter.

The development of applications of high-throughput sequencing was paralleled by an explosion of technology development, which has caused the per-base cost of sequencing to drop dramatically, faster than was expected based on Moore's law, which predicts that the output of a technology doubles—and therefore its cost halves—every 2 years (Fig. 12.1) [1]. The natural consequence of this drop in costs is twofold; experiments can be done in a grander scale and smaller labs (thus more researchers) are able to apply this technology to their own research.

The purpose of this chapter is to give a high level overview of the most popular sequencing technologies in the market at the time of writing, and also introduce the reader to some of the major applications that can be used in the study of genomic

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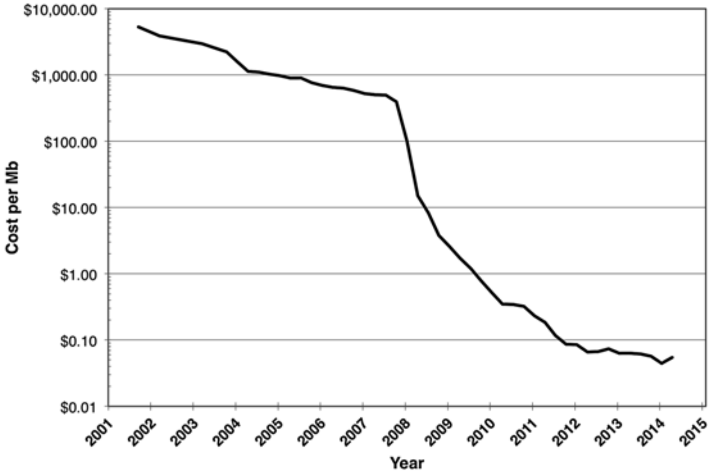

Fig. 12.1 The declining cost of DNA sequencing (data from NHGRI [1])

elements that play a role in human health. However, before starting the discussion, it is useful to define some of the terminologies that are often used when talking about high-throughput sequencing:

amplicon	An amplicon is the DNA fragment that is the product of PCR amplification of a target sequence.
contig	Most sequencing platforms produce short reads that are then assembled by computer algorithms to form longer sequences. A contig is a unit of the longest possible contiguous sequence that can be assembled by a specific algorithm using the available overlapping reads.
scaffold	A scaffold is a sequence that is composed of one or more contigs, which have been paired through the use of paired-end sequencing, or through the use of other experimental evidence, such as optical mapping.
read trimming	The computational operation to remove adapter sequences from reads after sequencing.
read	High-throughput sequencing is the result of the massive parallelization of individual sequencing reactions that are performed simultaneously. A "read" is the sequence output of each reaction.
polony	Polony derives from the term "polymerase colony" which describes a cluster of immobilized, clonally amplified DNA molecules.
paired-end tags	A paired-end tag (PET) is a fragment of DNA that results from the ligation of two sequences that may be on the opposite ends of a contiguous sequence or may be on different molecules but close in 3D space.
multiplexing	The combination of libraries coming from different samples in one sequencing reaction.
barcode	A short unique sequence of DNA added during library construction to allow for post-sequencing separation of reads coming from different sources/samples.

(continued)

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library	A collection of adapter ligated DNA fragments to be used as a template for sequencing.
coverage	Sometimes the term “coverage” is used inter-changeably with the term “depth of coverage”. However, when coverage is reported in percentages, it refers to the fraction of sequence recovered given a known length of input DNA. For example, if an amplicon is 1000 bases long, but only 900 bases are sequenced, the coverage is reported to be 90 %.
depth of coverage	The number of times that each nucleotide base is sequenced. The average depth of coverage can be calculated using Eq. (12.1) (“Overview and Costs in Applications” section below).

Technologies

There are three major players in the current market of high-throughput sequencers that are discussed in this section: Illumina/Solexa, Life Technologies’ Ion Torrent and Pacific Bioscience’s PacBio RSII system. Each technology has its strengths and weaknesses, which are summarized in Table 12.1. Naturally, the decision of which technology to acquire or use will depend on the intended application. A fourth emerging technology is not discussed here but is mentioned in the last section of the chapter, which discusses the future of this field.

A common characteristic of all the technologies discussed in this chapter is that they require a step of library construction before the sequencing run can be performed. The purpose of this step is to attach adapters to the ends of DNA molecules. For the Illumina and Ion Torrent platforms, these adapters are used for the clonal amplification of the library inserts in order to increase the detectable signal during sequencing. The sensitivity of the PacBio system is high enough that clonal amplification is not needed. Additionally, all these platforms need the adapters for sequencing initiation.

Illumina/Solexa

Illumina technology has the highest share of the high-throughput sequencing market as it is the oldest of the most popular technologies and also because its high sequencing output made it an attractive technology to acquire in the early days of high-throughput sequencing. Similarly to the other technology platforms discussed here, Illumina uses a Sequencing By Synthesis approach to elucidate the sequence of a DNA molecule.

The sequencing reactions in all Illumina platforms are performed on a flow cell, which may be divided from 1 to 8 lanes depending on the instrument being used. Attached to the flow cell are special oligonucleotides that are complimentary to the adapters used in the library preparation and are used to capture the single stranded DNA fragments. Using these complimentary adapter sequences, these fragments are clonally amplified clusters of DNA through bridge-PCR. During this process, the extended DNA forms a bridge with a near-by immobilized complimentary adapter,

Table 12.1 Summary of major sequencing technologies

Manufacturer	Version	Throughput	Read size	Strengths	Weaknesses
Illumina	MiSeq NextSeq 500 HiSeq	Up to 15 GB Up to 120 GB Up to 1 TB	Up to 300 bp Up to 150 bp Up to 250 bp	High throughput Established methodologies	Shorter read sizes
LifeTechnologies	PGM Proton	Up to 2 GB Up to 10 GB	Up to 400 bp Up to 200 bp	Less expensive technology to acquire Shortest run times	Errors associated with homopolymers
Pacific Biosciences	PacBio RS	Up to 1 GB	Up to 40 kbp reported	Long reads Direct detection of DNA modifications	Expensive Lower throughput

which now serves as the primer for the next round of synthesis. At the end of the clonal amplification reaction, one strand of DNA is removed from each template through the use of a restriction site on the flow cell bound adapter. The end result is a collection of single-stranded polonies that serve as the template for DNA polymerization.

Ion Torrent Semi-conductor Sequencing

The technology used by Life Technologies' IonTorrent platform is similar in many ways to the now defunct 454 pyrosequencing platform in that it uses emulsion-PCR (emPCR) for sample amplification and also in that only one type of nucleotide is provided during each flow cycle of the sequence run. IonTorrent semiconductor sequencing is unique in that it is the first mainstream platform that does not use light as the readout signal, but instead it detects the proton ions released by the addition of each nucleotide. The intensity of the signal is directly related to the number of bases incorporated at each cycle. A major advantage of this technique is that, since there is no need for image acquisition, sequencing run times can be as low as 2.3 h depending on the chip used. One drawback to this approach is that in cases where the same base is repeated multiple times (homopolymer) the signal output is high, which makes it challenging to accurately estimate the size of the repeat between homopolymers of similar size. For example, a homopolymer of nine cytosines may have a signal similar in intensity to that of a homopolymer of ten cytosines. This leads to a higher rate of false insertions or deletions in homopolymers.

There are two instruments offered by IonTorrent with different output capabilities, the Personal Genome Machine (PGM) and Proton. The PGM chip with the highest output (Ion318) produces up to 2 Gb of sequence per run, while the Proton can produce up to 10 Gb per run with the IonP1 chip. Life Technologies has announced recently that they will be releasing the Proton-II chip which will increase the output of the Proton to around 32 Gb which will enough to sequence a human genome to a depth of coverage of 10×, while maintaining the short sequencing run time. Additionally, Life Technologies is developing an alternative method to emPCR, which relies on isothermal template amplification [2]. The application of this method promises to greatly reduce template preparation time, without the need for a dedicated instrument, thus also reducing costs.

Pacific Biosciences SMRT

Pacific Biosciences (PacBio) with its single molecule real-time (SMRT) technology is the most recent player to emerge in the high-throughput sequencing field. The PacBio SMRT method relies on zero-mode waveguide (ZMW) wells, each containing a single polymerase enzyme, DNA template, sequencing primer and fluorescently labeled nucleotides. The fluorescent signal associated with each nucleotide incorporation to the growing DNA strand is recorded in real time [3]. This process takes about 2 days and it produces up to 5 Gb of sequence.

The PacBIO SMRT technology has three major strengths over the other sequencing methods discussed. First, it is the only technology, so far, that uses single molecule detection, which means it does not rely on PCR amplification that may introduce biases and errors. Secondly, it produces reads that are considerably longer that what Illumina and IonTorrent currently produce, with read lengths over 30,000 bases reported. These longer reads are extremely important when studying genomic regions with a high number of repetitive sequences. Additionally, longer reads allow for unambiguous mRNA isoform resolution since assembly of transcripts, and the artifacts associated with this process, is not necessary. Lastly, since DNA chain extension is observed in real time this method is able to capture polymerase kinetics. The rate with which polymerase incorporates nucleotides differs between modified and unmodified nucleotides. Consequently, the variation in the kinetics of nucleotide incorporation during DNA synthesis can be used to infer modifications in the DNA template and thus provide a direct way to studying base modifications [4].

Applications

Overview and Costs

In this section, major applications of high-throughput sequencing technologies will be discussed in some detail. However, this is by no means a complete collection of applications, since the number of applications is limited only by the number of methods that one can use to isolate DNA or RNA, and also because new applications will surely have emerged by the time this book is published. Software tools necessary for data analysis vary based on the application. Some commonly used and publicly available tools are summarized in Table 12.2, while other tools are specific to the sequencing technology and are provided by the manufacturer.

The general workflow of a high-throughput sequencing experiment is similar between most technologies in the market. The first step of the experiment, which in essence defines the application, is the isolation and selection of the nucleic acid material to be studied. These methodologies will be discussed briefly in each subsection. In the case of RNA based approaches, the RNA is reverse transcribed to DNA. Additionally, amplification of the isolated DNA is required for the Illumina and IonTorrent technologies. The resulting DNA molecules are then ligated to proprietary and technology specific adapters in the second step of library construction. The last step is the sequencing of the DNA libraries.

The cost of sequencing highly depends on different elements of the experimental design, in addition to the technology used. The first consideration is the depth of coverage of sequencing required, because this affects the amount of data (or output) that needs to be produced. The depth of coverage can be calculated using:

$$Depth\ of\ Coverage = \frac{O}{I} = \frac{R \times L}{I} \tag{12.1}$$

Table 12.2 Commonly used tools

	Name	Reference
Read Trimmers	Cutadapt	[42]
	PRINSEQ	[43]
	Skewer	[44]
	Trimmomatic	[45]
Genome assemblers	Celera	[46]
	Newbler	[47]
	SOAPdenovo2	[48]
	Velvet	[49]
	ALLPATHS	[50]
Transcriptomics	Tophat (Spliced alignment)	[51]
	Cufflinks (Assembler)	[52]
	PASA	[53]
	Rnnotator	[54]
	Trinity	[55]
Short read mappers	Bowtie	[56]
	BWA	[57]
	SOAP2	[58]

where O is the unassembled sequencing output, which can also be described as the number of reads (R) multiplied by the average length of each read (L), and I is either the estimated length of the input DNA (e.g. the size of a genome) or the sum of the length of the assembled contigs. Using this equation and having an estimate of the length of the DNA to be sequenced, a researcher may estimate the amount of sequencing needed to achieve the desired depth of coverage, which may be as high as the 1000× coverage required for confident identification of somatic mutations.

Often, the minimum output of a sequencing run may be orders of magnitude higher than the output needed for a sample, especially if the sample is comprised of only a limited number elements, as in the case of small RNAs. In these cases, the researcher may combine samples from multiple sources or experiments in one sequencing run by multiplexing. This is achieved through the addition of short unique DNA tags (barcodes) to each sample during library construction. The samples can then be separated computationally during post-sequencing analysis.

While multiple samples can be combined in one sequencing run, each sample will still need to go through the step of library construction. This raises the costs of sequencing experiments considerably since the economies of scale do not apply. Additionally, the DNA adapters and formulations necessary for library construction often are proprietary, which makes it difficult to find lower cost alternatives.

DNA Based Applications

Targeted Re-sequencing

Targeted re-sequencing is usually associated with sequencing the amplicons generated by the PCR amplification of exons. This can be performed on all predicted exons (i.e. the exome) or on a subset of these exons. This approach can easily be applied to the study of the non-genic genomic elements discussed in this book by simply designing custom PCR primers targeting areas of interest. Care needs to be taken when designing primers for targeted re-sequencing in order to avoid non-specific binding of oligo-nucleotides. In the case where a high number of loci are being targeted, potentially cross reacting primers maybe partitioned into separate primer mixes in order to be used in different PCR reactions.

An advantage of using this approach is that the length of the fragments to be sequenced are known in advance and therefore the step of fragment size selection can be avoided for established protocols. Additionally, knowing the input sequences simplifies downstream bioinformatic analyses.

Epigenomics

Epigenomics refers to the field that studies the global changes of gene expression that do not result from the change in DNA sequence, but from chemical modifications of either the DNA or the histones associated with DNA [5]. Epigenomic applications, therefore, depend on which of these two kinds of modifications one intends to study.

Chip-seq is used for the study of histone modifications. Chromatin, i.e. the complex of DNA and proteins, is crosslinked to form stable complexes, and then enzymatic treatment or physical shearing is used to break the chromatin into smaller pieces. DNA fragments bound to histones are selected using antibodies that target specific post-translational modifications. These fragments are sequenced and are then mapped to a reference genome to create a map of the particular modifications across the genome [6].

The methylation of DNA can be studied using a variety of approaches. The more straightforward approach is to use the Pacific Biosciences SMRT technology, which does not use PCR amplification and therefore preserves DNA modifications. The sequencing signal for this technology is the rate of base incorporation by polymerase during chain elongation. This rate depends on the chemical structure of the base being incorporated and therefore, also differs for chemically modified bases such as methylated adenines [7] and cytosines [8]. Additionally, this approach has the benefit that it does not require the existence of a reference sequence.

A more indirect way of studying DNA methylation of cytosines is using bisulfite sequencing. Bisulfite converts cytosine to uracil in treated DNA; however, methylated cytosines are protected from this conversion [9]. PCR amplification of bisulfite treated DNA will therefore result in the unmethylated cytosines to be converted to thymines while methylated cytosines will remain cytosines. The reads produced after a high-throughput sequencing run can then be mapped and compared to a reference genome to find which cytosines remained cytosines after bisulfite treatment, and were therefore methylated [10, 11].

Long-Distance Genomic Interactions

An innovative application of massively parallel high-throughput sequencing is the study of long distance chromatin interactions that occur between genomic elements. There are several variations of methods for studying the three-dimensional structure of chromatin in the nucleus, such as Circularized Chromosome Conformation Capture (4C) [12], Carbon-Copy Chromosome Conformation Capture (5C) [13], Hi-C [14], ChIA-PET [15] and others. All of these techniques are variations of the Chromosome Conformation Capture (3C) method in which chromatin is cross-linked, sheared (either enzymatically or physically) and then ligated. The result of this approach is that DNA fragments that are close in three-dimensional space but maybe far apart in sequence space or even on a different chromosome are linked together. Sequencing the ligated fragments, therefore, allows for studying the spatial organization of chromatin and how it differs between healthy and disease states, e.g. in healthy vs cancer cells [16].

Metagenomics

The term "metagenomics" describes the application of high-throughput sequencing in the study of microbial communities. It has traditionally been employed in the study of environmental samples, such as those gathered from soil or water, or samples collected from symbiotic environments, e.g. the root systems of plants. Metagenomics has more recently gained considerable traction in the study of human health. As a result of the colonization of intestinal and air-accessible surfaces on humans by bacteria and fungi, there are more non-human cells in the human body than human cells. It was therefore not a surprise that the composition of these microbial populations has a direct effect on human health. Some of the most impressive examples include the observation that the health of the gut microbiome is associated with diseases like obesity [17] and diabetes [18], or that the method of delivery at birth (viz. natural vs Caesarian) influences the health of the baby by changing the skin microbiome [19]. These studies, in addition to many others, employed extensive use of high-throughput sequencing to study the microbial communities inhabiting a human biome.

There are two common approaches to studying microbial communities using high-throughput sequencing. The first one targets the 16S ribosomal RNA genes (rRNA) of microbes using targeted PCR amplification. This approach gives an overview of the taxonomic composition of a microbiome. The main benefit of this approach is that it requires less sequencing throughput and the data analysis is relatively straight forward. However, preferential amplification of 16S rRNA genes may lead to over-representation of some bacterial groups [20]. Additionally, sequencing and assembly errors may lead to the formation of hard to identify chimeras [21]. The second approach, shotgun metagenomic sequencing, does not focus on specific genetic elements, but instead it is based on the genomic analysis of the total DNA extracted from microbial communities. Although the analysis of these data is more complex compared to 16S rRNA data, shotgun metagenomic sequencing is a much richer source of information. Besides revealing the composition of a microbiome, shotgun metagenomic sequencing can be used to study the functional properties of these organisms and to investigate the effect that the microbiome's secondary metabolism may have on the host [22]. Additionally, full reconstruction of microbial genomes is possible using these data [23]. Since shotgun metagenomic sequencing is unbiased, it can also be useful in the discovery of new bacterial groups and viruses [24].

RNA Based Applications

Small Non-coding RNA (miRNA) and Long Non-coding RNA (lncRNA) Sequencing

Non-coding RNAs play important roles in modulating the regulation of gene expression in all major cellular functions and, as such, they have been implicated in many diseases ranging from cancer to cardiac failure [25–27]. A major obstacle in studying ncRNAs with high-throughput sequencing methods stems from the difficulty in extraction and isolation of high quality samples at satisfactory yields, especially in the case of miRNAs (~22 bp in length). Up to 1 μg of miRNA can be isolated from cell lines and fresh tissue, but in the latter case the sample is heterogeneous [28]. Homogeneous samples from tissues can be retrieved through laser capture microdissection, which, however, drastically reduces the yield to less than 10 ng. Plasma and urine samples also have similarly low yields, which hinders accurate mRNA quantification and subsequent analyses. This problem is further exacerbated by the fact that miRNAs usually represent a small fraction of a cell's total RNA and the absence of a "hook" sequence that could be used for the targeted amplification or selection of these molecules.

Once an ncRNA sample has been attained, high-throughput sequencing can be used for the discovery of new ncRNA species [29] that could be used both to study their function but also as biomarkers for disease [30]. Additionally, using immuneprecipitation techniques (see below) the interactions of ncRNAs with other macromolecules can be further investigated.

RNA Interactions

Several methods exist to probe the interaction between RNA and proteins. Similarly to ChIP approaches, these methods involve the crosslinking of RNA to bound proteins, which are then selected using specific antibodies. Some of the most commonly used techniques are CLIP-seq [31], individual-nucleotide resolution Cross-Linking and ImmunoPrecipitation (iCLIP) [32] and Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP) [33]. These techniques enable the characterization and study of post-transcriptional control and the mechanisms through which mis-regulation may lead to disease [34]. For example, CLIP-Seq has been used to study the binding patterns of the exon junction complex, which plays a major role in the post-transcriptional fate of mRNA [35]. Another study determined the transcriptome-wide binding sites and preferences of RNA-binding proteins (RBPs) and microRNA-containing ribonucleoprotein complexes (miRNPs) [33]. Understanding these processes will help elucidate the link between genetic variations and disease, especially in the cases of synonymous mutations or non-genic mutations that disrupt the mechanisms of post-transcriptional gene regulation.

Ribosome Profiling

The number of copies of an mRNA transcript present in a cell generally correlates with the level of its encoded protein being produced. However, this is not always the case as the translation of specific mRNAs may be attenuated. Ribosome profiling identifies which mRNAs are actively being translated, by using an approach similar that used for studying RNA–protein interactions [36]. Ribosomes and mRNA are crosslinked and then the overhanging strands of mRNA are digested by ribonucleases. The protected RNA is released, reverse transcribed to DNA that is then sequenced and mapped back to the original genome.

Besides providing a global view of all active protein expression in the cell, ribosome profiling also provides valuable information regarding translation elongation rates, and initiation and pause sites. Additionally, ribosome profiling allows for the discovery of elements that encode small proteins that may be missed by conventional methods. Lastly, the role of upstream open reading frames in the regulation of gene expression can be studied using this approach.

TAIL-seq

A very recently developed application for the study of gene expression regulation through the control of mRNA stability is TAIL-seq [37]. This method specifically targets the 3′ of mRNA molecules. Briefly, as in other RNA methods, total RNA is rRNA-depleted, tagged with a biotinylated adaptor at the 3′ end and then partially digested. The resulting fragment is pulled down using streptavidin, modified with a 5′ adapter, gel-purified, reverse transcribed and sequenced.

Although normally the length estimation of long homopolymers—in this case poly(A) and poly(T)—through sequencing is challenging, the authors of this study noticed that there was a correlation between the fluorescence signal intensity and the location of the end of a poly(T) homopolymer. Using this observation and statistical methods, they were able to accurately calculate the genome-wide poly(A) length distribution of mRNA tails in HeLA and NIH 3T3 in addition to observing pervasive uridylation and guanylation of poly(A) tails [37].

Future and Challenges

Sequencing technologies and protocols are constantly being improved as all of the companies try to remain competitive. New chemistries that extend read length and improve quality are being introduced regularly, whereas there is a push to simplify workflows as much as possible to make this technology accessible to any research or clinical lab, irrespective of its size. Illumina has recently announced that they will release model HiSeq X Ten in a bid to be the first technology to break the $1000 human genome barrier. The HiSeq X Ten system is in reality 10 HiSeq X instruments bundled together for a cost expected to exceed 10 million dollars. The major technological improvement in this new system is that the flow cells now come with ordered ‘nanowells’ where the template oligos are attached, as opposed to the random placement in the earlier systems. Additionally, Illumina is introducing a medium sized sequencer, NextSeq to occupy the space between MiSeq and HiSeq currently filled by Ion Torrent’s Proton sequencer.

Ion Torrent is also expected to release improved chemistries and protocols. The Ion Rapid Isothermal Amplification Chemistry is being developed to simplify and speed up library template preparation, which is now performed by the Ion Touch system. Additionally, Ion Torrent will release Ion Hi-Q™ Sequencing Chemistry, which is expected to reduce insertion and deletions errors by 90 %.

A lot of excitement has been created by the announcement of Oxford Nanopore Technologies’ intention to release two new sequencing systems, MinION and GridION, that use an entirely novel approach to sequencing. A biological nanopore is set in a membrane layer and allows electrical current to pass through. When an electrically charged particle, e.g. a nucleic acid polymer, passes through the pore it disrupts the electrical current. This disruption of current can be measured. Moreover, the intensity of signal change varies according to which base is passing through the nanopore, thus providing the means for DNA sequencing. The promise of this technology is the reduction both in sequencing costs but also the size of the sequencing instrument. For example, Oxford Nanopore’s MinION is a disposable device that is not much bigger than a pack of chewing gum and that connects directly to the USB port of a computer. The performance of MinION device sequencing is currently being evaluated through a community access program.

There has been considerable effort in recent years to develop the methodologies for applying genomic and transcriptomic techniques at the single cell level. The greatest challenge to this single cell sequencing is in amplifying the whole genome or transcriptome without introducing errors while avoiding contamination from other cells. Currently, the prevailing approaches to whole genome amplification are Multiple Displacement Amplification (MDA) [38] and Polymerase Chain Reaction (PCR). Both approaches have biases and may also lead to the introduction of artifacts; therefore, the decision of which method to use depends on the type of genetic elements and sequence variations to be studied [39]. Despite these hurdles, single cell sequencing will provide scientists with the capability to study the genomes and transcriptomes of healthy and diseased cells from the same individual over time, in addition to providing insights in the genomic heterogeneity of individuals. Lastly, a natural application of single cell genomics is in the field of pre-implantation genetic diagnosis [40] in addition to non-invasive pre-natal testing using isolated fetal cells from maternal blood [41].

With the continuing increase of sequencing throughput, the decrease in sequencing costs and the development of new technologies and methods there are two major hurdles for the adoption of high-throughput sequencing technologies by research labs. First is the availability of properly trained bioinformaticians who are able to analyze the large data sets produced by high-throughput sequencing. The second limitation is the cost associated with securely storing and managing these data, in addition to the cost of the high capacity computing capabilities needed to process and analyze the data. Despite these limitations, the benefits of acquiring high-throughput sequencing capabilities outweigh the associated effort and costs, as this opens new and unprecedented avenues towards understanding the role of non-genic elements in human health.

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Index

A

Adaptation, 9, 136, 208
Alternative lengthening of telomeres (ALTs), 254, 255, 257, 263
Aneuploidy, 145, 222, 232, 233, 235
Antagomirs, 19, 33, 35, 36

B

Bioinformatic, 7, 8, 19, 36, 163, 304
Biomarkers, 21, 27, 28, 30, 32, 33, 98, 100, 236, 306
Birth disorder, 222

C

Cancers, 11, 25–29, 32, 36, 48, 72, 84, 96–100, 115, 117, 130, 145–148, 165, 174, 175, 180, 181, 222, 227, 230, 232–236, 249, 252–255, 258, 262, 264, 266, 267, 305, 306
Centromere, 222–240, 254, 255, 260, 261, 265
Chromatin, 47, 48, 51, 54, 63, 64, 69, 84, 87–91, 94, 99, 101, 113–115, 162, 166–168, 172, 174, 223, 225, 227–229, 238, 258, 260–263, 288–291, 297, 304
Chromosome instability, 240
Chromosomes, 4, 54, 62–64, 67, 69, 85, 89, 90, 96, 97, 100, 101, 111, 113, 117, 131, 132, 136, 139, 140, 142, 157–160, 163, 164, 166, 168, 176, 179–181, 222–240, 247–251, 253–262, 264, 266, 267, 290, 305
Copy number variations (CNVs), 9, 11, 129–148, 160
CpG islands, 7, 72, 90, 114, 162, 167, 176, 235, 260, 262, 288–290, 292

D

Disease, 17, 21–25, 27–32, 34–36, 48, 85, 96, 98–100, 102, 113, 115, 116, 121, 129–131, 135, 136, 143–145, 147, 148, 161–163, 166, 167, 176, 179, 181, 226, 229, 232–236, 238, 261, 263, 264, 266, 267, 285, 293, 305, 306
DNA methylation, 48, 51, 53, 57, 61, 69, 71, 72, 90, 98, 113–115, 121, 235, 236, 260, 262, 288, 289, 304
DNA repair, 116, 147, 161, 167, 171, 173, 174, 204, 206, 207, 247, 249, 252, 257

E

Epigenetic, 28, 47–51, 53, 59, 63, 69, 72, 73, 89–91, 94, 99, 113, 142, 146, 160, 162, 222, 227, 230, 236, 238, 258, 260, 262
Epigenetic regulation, 90–91, 113, 116, 142
Epigenomics, 304
Evolution, 4–10, 59–65, 131–137, 143, 236–239

G

Gametogenesis, 48, 50, 52, 54, 58, 59, 62, 63, 66, 71–73, 171
Gene dosage, 9, 133, 141, 144
Gene expression, 1, 9, 11, 17, 32, 34, 48, 87–92, 97, 101, 112, 119, 121, 135, 137, 141, 142, 159, 161–163, 166, 167, 171, 174, 234, 285, 286, 288, 289, 291, 304, 306, 307

K. Felekkis, K. Voskarides (eds.), *Genomic Elements in Health, Disease and Evolution: Junk DNA*, DOI 10.1007/978-1-4939-3070-8

Gene expression regulation, 1, 9, 12, 88, 101, 307
Genome, 1, 2, 6, 7, 9–11, 19, 26, 35, 47, 48, 50, 54, 64, 69, 71, 73, 83, 84, 86–88, 91, 97, 100, 101, 109–113, 115, 117, 119, 121, 129–132, 135–138, 140–143, 146, 156–161, 163–170, 176, 180, 181, 198, 201, 206, 207, 209, 210, 225, 229, 234, 235, 237, 239, 240, 247–250, 261, 263, 267, 285, 288, 289, 291–293, 301, 303, 304, 307, 308
Genome-wide, 50, 84, 86–88, 91, 97, 100, 121, 129, 140, 141, 143, 182, 235, 267, 291, 292, 307
Germ cells, 48–55, 57–59, 61, 67–72, 115, 130, 166, 171–173, 175, 257
Group II introns, 195, 196, 198, 199, 202, 205–207, 209

H
Heterochromatic, 258–261
Heterochromatin, 47, 49, 63, 71, 90, 98, 113, 114, 160, 222, 228, 233, 235, 236, 254, 256, 260–262, 286
Heterochromatization, 113
High-throughput sequencing, 22, 297, 298
Human disease, 48, 71–72, 102, 116, 117, 148, 173–174, 229, 233, 250, 261
Human physiology and disease pathogenesis, 109–122

I
Immune-precipitation, 306
Immuno-precipitation, 22, 55, 262, 291, 306
Intellectual disability, 143, 148, 161
Interactome, (AU: Found only in the reference)
Inverted duplications, 6, 7, 136, 137

K
Kinetochore, 222, 223, 226–229, 231, 234, 238

L
Linkage, 23, 159, 176, 179, 182
Long non-coding RNA, 83, 84, 141

M
Maturases, 210
Medical genetics, 175, 176
Megasatellites, 156, 157, 159, 160, 166
Metagenomics, 305
MicroRNAs, 3, 22, 26, 31, 60, 84, 115, 136, 137, 141, 306
Microsatellites, 157, 158, 162–165, 168, 169, 174, 175, 179
Minisatellites, 156, 157, 159, 164–166, 168, 170, 175, 176, 180, 181
miRNA genes, 2–8, 10, 11, 22–24, 26, 28, 142
miRNA targets, 2, 9, 17–20, 24–26
miRSNPs, 18, 24–26
Mobile genetic elements, 198, 202, 210

N
Natural selection, 10, 11, 166, 167
Next-generation sequencing, 21
Non-pathogenic, 142

O
Oncogenes, 28, 142, 146, 232
Oncogenesis, 97

P
Paternity testing, 180
p-element induced wimpy testis proteins (PIWI), 48, 51–61, 63, 68–73, 115
piRNAs, 48, 49, 51–57, 61–73, 115
Promoters, 2, 86–88, 93, 94, 99, 112, 114, 115, 141, 161–163, 167, 208, 235, 262, 287–290, 292
Proximal regulatory elements, 286, 290, 291

R
Repeat expansion disorders, 165, 171, 172, 174, 175
Repeat instability, 161, 171–173
Repetitive DNA, 157, 159, 222
Retroelements, 109, 110, 116, 117, 170, 195, 210
Retrohoming, 202, 204–208
Reverse transcriptase (RTs), 47, 110, 140, 195–198, 204, 205, 210, 248–251, 253, 264–266
Ribozymes, 196, 197, 199, 201, 202, 207, 208, 248
RNA, 1–4, 17, 20–22, 31, 36, 47, 48, 50, 51, 53–57, 59, 62–67, 69, 70, 73, 83–87, 89, 90, 92–97, 99, 100, 102, 109, 110, 112–116, 118, 121, 141, 159, 162, 163, 169, 170, 172, 177, 178, 195, 196, 198, 200–202, 204, 206–209, 235, 248–252, 259, 262, 263, 285, 286, 288, 302, 305–307

RNA polymerases, 2, 83, 87, 110, 112, 119, 162, 208, 251, 262, 286, 288
RNA-seq, 84–87, 97, 200, 262

S
Satellite DNA, 156, 157, 159, 170, 224–226, 231, 236–239
Segmental duplications (SD), 6, 7, 131, 136, 137, 144, 260
Self-catalytic RNA
Shelterin, 257, 258, 264, 265
Splicing, 3, 31, 83, 86, 88, 89, 94–97, 112, 160, 164, 195–204, 206, 208–210, 235, 252
Syndrome, 23, 24, 98, 101, 130, 135, 142–144, 162, 175, 176, 179, 181, 230, 233, 235, 236, 250, 257, 258, 264–267

T
Tandem repeats (TRs), 156–170
Targetrons, 208, 209
Telomerases, 251–252, 254, 265
Telomere position effects (TPE), 260, 261
Telomeres, 157, 166, 247–263, 265, 266
Telomeric repeat-containing RNA (TERRA), 98, 99, 262, 263, 267
Telomeropathy, 263–267
Transcription, 2–4, 9, 17, 19–21, 28, 29, 33, 34, 48–50, 57–59, 61–64, 69, 71, 73, 84, 86–98, 101, 110, 112–115, 119–122, 132, 160–164, 167, 169, 170, 172, 198, 200, 204, 206, 229, 236, 252, 254, 260, 262, 263, 285–292, 306, 308
Transcriptomes, 5, 86, 101, 262, 267, 303, 306, 308
Transposable elements, 7, 47, 73, 85, 86, 112, 114, 116, 137, 169, 210, 225, 226, 235
Transposons, 7, 47, 53, 54, 57, 59, 62, 63, 65–67, 69, 73, 86, 109, 111, 116–118, 156, 169
Transposon silencing, 57, 59

U
Unveiling transposable elements function, 109–122

V
Variable number tandem repeats (VNTRs), 109, 110, 114, 156, 159, 165, 170, 180, 181