New RNA playgrounds : non-coding RNAs and RNA-binding proteins control cellular processes

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Chapter 1:

General Introduction and outline of this thesis

Cancer

A rough estimation puts the number of cells making up a human body to about 10^{14}, or one hundred thousand billion cells. These cells all stem from only one ancestor, the fertilized egg, or zygote, which carries in its DNA, folded neatly into chromosomes, all information necessary to build that body. With hundreds of different cell types, creating many different tissues all having to be developed and maintained it is not hard to imagine that sometimes errors arise. All cells in a multicellular organism are altruistic, they exist to support the germ cells (these will form the gametes, or sperm and eggs), which will give rise to the next generation. A fundamental difference between a normal cell and a cancer cell is that the latter loses its altruistic nature; cancer cells display selfish behaviour in that they don't listen to signals (to stop proliferation) from their neighbours any more, compete for nutrients and invade into new tissues disregarding their social rules and destroying them while reproducing.

Cancer cells often resemble their normal counterparts but differ through the presence of a series of genetic alterations, or mutations, that deregulate several regulatory circuits that govern normal cell proliferation and homeostasis. In their landmark paper Hanahan and Weinberg described six essential alterations cellular physiology that most, if not all, cancers share: insensitivity to anti-growth signals, self-sufficiency in growth signals, limitless replicative potential, evasion of programmed cell death (apoptosis), sustained angiogenesis (blood vessel formation), and tissue invasion and metastasis (Hanahan and Weinberg, 2000). These traits individually may apply to different cancers to different degrees. One additional characteristic that cancer cells have is the instability of their genomes, or their increased mutational capacity. This characteristic enables tumor cells to reach the six above-mentioned traits by mutating genes coding for components of the signalling pathways involved in these traits. If not for this characteristic, cancer would be a very rare disease because our genome maintenance and repair systems have a very high fidelity. It is possible to recapitulate these traits in *in vitro* experimental systems using normal human cells, these can be transformed into cancer cells by introduction of only a few genes (Hahn et al., 1999).

Mutated cancer genes generally come in three flavors; oncogenes, tumor-suppressor genes, and stability genes. These mutations are best explained by analogies that Bert Vogelstein used (Vogelstein and Kinzler, 2004). A mutation in an oncogene is analogous to a stuck accelerator in an automobile; the car still moves forward even when the driver removes his foot from it. An example of such a gene is is Ras, activating mutations in this gene are found in approximately 30% of human cancers, it provides the cell with an intrinsic growth signal (Duursma and Agami, 2003). A mutation in a tumor-suppressor gene is analogous to a dysfunctional brake in an automobile; the car doesn't stop even when the driver attempts to engage it. The best known example of a tumor suppressor is p53, this is the most frequently mutated gene in cancer (Oren, 2003). The p53 gene encodes a protein whose function is to inhibit cell growth and stimulate cell death when induced by cellular stress. Another example relevant for this thesis is the tumor suppressor $p27^{KIP1}$, this protein is an inhibitor of the cell cycle and is often expressed to lower levels in human cancers (Chu et al., 2008). Noteworthy is that inactivating mutations of p27 are rarely observed in human cancer, other mechanisms, including microRNA

mediated downregulation are involved (le Sage et al., 2007). In the analogy to autos, stability genes represent the mechanics and a defective stability gene is akin to an inept mechanic. The breast cancer susceptibility gene BRCA1 is a well known example of this class of genes, it is involved in DNA repair (Gudmundsdottir and Ashworth, 2006).

Outline of this thesis

Described above are some basic molecular principles in cancer biology. In my thesis I will describe several players in signalling pathways that are in some way connected to these basic principles of cancer. Sometimes this connection is not so clear, that is because in cancer research we often stumble upon interesting biological phenomena that provide a deeper insight into the inner workings of cells. Eventually, this greater understanding will hopefully lead to new insights to treatment of diseases, including cancer. In **chapter 4** I have investigated the role of hTR, the RNA component of telomerase (an enzyme involved in the acquired capability of cancer cells to reach a limitless replicative potential) in the response to DNA damage. This response, in turn, is connected to the characteristic of cancer cells of genome instability, in this case through a reduced potential of cancer cells to respond to DNA damage (Kedde et al., 2006). The analysis of the function of Dead End 1, described in **chapter 3** (and reviewed in **chapter 2** (Kedde and Agami, 2008)), may be important for our understanding of cancers deriving from the germ line but is quite likely more generally applicable (Kedde et al., 2007). This chapter also reveals a glimpse of a layer of gene regulation previously underappreciated; that is noncoding RNA regulation. In **chapter 5** a novelty in such an RNA regulation mechanism is described, namely the targeting of a relatively new class of RNAs, microRNAs (miRNAs), to the coding region of the DNMT3b transcript (Duursma et al., 2008). What all studies described in this thesis have in common is the regulation of cellular processes by noncoding RNA molecules, functions that just a decade ago would almost exclusively be ascribed to proteins. Therefore, I have tentatively dubbed these new playgrounds for RNA in this thesis. With the recently acquired deeper knowledge about our genome, it is beginning to be appreciated that RNA plays a major role in regulation of cellular processes, much bigger than previously anticipated (Mattick, 2007).

I will give a short introduction on some relevant topics for this thesis below. The DNA damage response is closely related to cancer as said above, some background on this topic is relevant for **chapter 4**, where I describe a modulator of this response. This modulator is an RNA molecule that was identified about two decades ago, as a cofactor for the telomerase enzyme (Greider and Blackburn, 1987; Greider and Blackburn, 1989). For several years, this was one of the few examples of an RNA molecule having a regulatory role, it was thought to merely aid the telomerase enzyme in doing its job. The notion that only a few RNAs would act as cofactors for some specific enzymes stems from those and earlier times. That is why I have classified human telomerase RNA (hTR) as a “classical” RNA. The other chapters (**chapters 2, 3, and 5**) in this thesis are related to miRNAs. These molecules were identified in the early nineties, but they came only very recently under great attention when it became clear that this is a large class of new genes, capable of regulating a big part of our genome. I will introduce miRNAs below, and they will be further discussed in **chapter 2**, in the context of Dead end, a protein

we identified as a modulator of certain miRNAs.

DNA Damage, the ATR side of the story

Our genome is under constant attack by ultraviolet (UV) and ionizing radiation (IR), chemicals from our enviroment such as from food and cigarette smoke, and from metabolites produced by our own cells, such as H_2O_2. Such DNA damage is detrimental to us, therefore elaborate mechanisms have evolved to deal with this damage. Understanding the DNA damage response is very important for cancer biology because DNA damage not only causes cancer by introducing mutations in our genes, it is also used to treat cancer in the form of irradiation and many chemotherapeutics, and it is the cause of many side effects of treatments (Kastan and Bartek, 2004). DNA damage triggers processes in the cell that lead either to damage repair and safe resumption of the cellular life cycle, or to programmed cell death (apoptosis), depending on the amount and type of damage. There are several types of DNA damage, and different protein complexes exist for recognition and repair of these lesions.

The DNA damage response in cells is organised by a network of pathways that contain sensor proteins, mediators, transducers, and effectors (Zhou and Elledge, 2000). Central to nearly all DNA damage responses are the transducer phospho-inositide kinase-related protein kinases ATM (ataxia telangectasia mutated) and ATR (ATM-Rad3-related). In response to DNA damage, these kinases are immediately activated by interactions with the sensor complexes and the damaged DNA and phosphorylate downstream targets that eventually orchestrate the response of the cells. This can be halting the cell cycle and activating repair proteins, permanently stopping the cell cycle (also referred to as senescence) if the damage is more extensive, or activating the apoptosis pathway, thereby killing the cell (Bartek and Lukas, 2007; Zhou and Elledge, 2000). ATM is mainly activated in response to double stranded DNA breaks, the most toxic type of damage to cells, if unresolved, the cell will die. ATR responds primarily to replication stress and single stranded DNA (ssDNA) gaps, it also responds to double stranded DNA breaks albeit with slower kinetics. ATR and its binding partner ATRIP bind to replication protein A (RPA), which coats ssDNA as soon as it is formed by a replication block, resolvement of ssDNA breaks or other damage (Byun et al., 2005; Cortez et al., 2001; Zou and Elledge, 2003). Other proteins, like the Rad17 and 9-1-1 complex are also loaded on the DNA which give rise to a docking platform for DNA repair proteins, mediators and transducers (Harper and Elledge, 2007). This complex further interacts with a protein called TopBP1, which then fully activates ATR kinase activity (Kumagai et al., 2006; Mordes et al., 2008). At this point, a myriad of proteins is recruited to the site of damage and a so-called DNA damage focus forms, it is thought that these foci function as a surrogate for DNA damage recruiting proteins that orchestrate the response to solve the problem. An important player in this process is the histone variant H2AX, which becomes phosphorylated on serine residue 139 (then dubbed γH2AX) by ATM, ATR, and DNA PK (Rogakou et al., 1998). Recently, DNA damage foci have have been detected in early stage tumors, and linked to replication stress induced by activation of oncogenes (Bartkova et al., 2005; Di Micco et al., 2006; Gorgoulis et al., 2005). These data show that DNA damage is an early event in tumorigenesis,

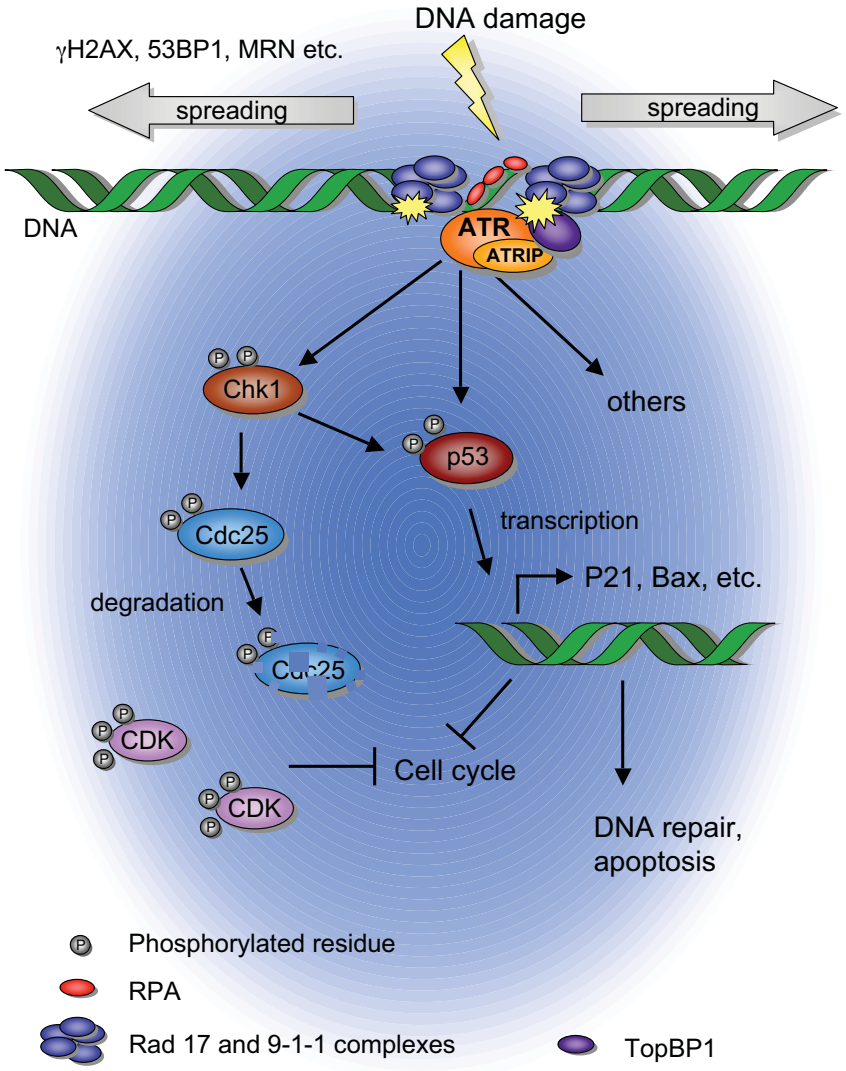

Figure 1. The ATR mediated DNA damage response. Multiple DNA damage insults activate ATR, such as replication stress, ssDNA gaps and UV-induced adducts. Sensors (the 9-1-1 complex and Rad17) are then recruited to the site of damage and ATR is activated. A DNA damage focus forms by spreading of H2AX phosphorylation and association of the MRN complexes and proteins such as 53BP1. Chk1 and p53 are amongst ATRs most important targets, they are activated by phosphorylation, TopBP1 is required for activation of Chk1, whereas p53 is directly activated by the ATR-atrip dimer. Chk1 immediately halts the cell cycle by phosphorylating the Cdc25 proteins, thereby targeting them for degradation, it can also phosphorylate p53. P53 activates transcription of genes that sustain the cell cycle arrest (such as p21) and activate DNA repair or apoptosis. See text for details.

however, to become cancerous, cells have additional safeguards to overcome, such as senescence, which was shown to be a barrier to tumorigenesis *in vivo* (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005; Serrano et al., 1997).

If we now go more downstream in the DNA damage response, towards the effectors/transducers, we find proteins that regulate DNA repair, senscence, splicing, chromatin remodelling, apoptosis, and, important for this thesis, the cell cycle. This is the reproductive cycle of cells, from replicating their DNA, followed by the division of the nuclei and the partitioning of the cytoplasm to yield two daughter cells. In the G1 phase (gap1), cells make decisions regarding whether to differentiate, self-renew, or die, on the bases of complex signals from their surroundings and from within the cells (see below). In S phase (synthesis of DNA), cells copy their genomes in order to divide it between their daughters in M phase (for mitosis), but not before everything is checked in G2 phase. Regulation of the cell cycle is orchestrated by oscillations in the activities of cyclin dependent kinases (Cdks). Inhibition of Cdks is achieved by tyrosine phosphorylations by proteins such as Wee1 and binding of Cdk inhibitor proteins, such as p21 and $p27^{KIP1}$, whereas activation is achieved by cyclins and de-phosphorylation by proteins such as Cdc25. Chk1 is an important ATR downstream target kinase that is involved in directly halting the cell cycle through stimulating the degradation of Cdc25 proteins (Liu et al., 2000; Mailand et al., 2000; Molinari et al., 2000; Sorensen et al., 2003). ATR activates Chk1 by phosphorylation on two serine residues (S317 and S345) (Zhao and Piwnica-Worms, 2001). Chk1, like ATR, is an essential gene, disruption causes embryonic lethality in mice and loss of viability of knockout cells, this shows that the ATR pathway is required for normal replication (Brown and Baltimore, 2003; Takai et al., 2000). Chk1 also phosphorylates, and thereby activates, the p53 tumor suppressor, which is involved in the maintenance of the cell cycle arrest (Shieh et al., 2000).

The p53 protein is a direct target of ATR as well, ATR phosphorylates serine 15 (Tibbetts et al., 1999). The p53 protein sits at the junction of an extremely complex network of cellular pathways responding to inputs such as oncogene activation, DNA damage, mitotic impairment or oxidative stress. In response to these stresses p53 is activated in a specific manner by post-translational modifications, these stabilize the protein, leading to greater concentrations, as well as enhance its DNA binding capacity, activating its transcriptional activity. It then orchestrates diverse outputs such as DNA repair, cell cycle arrest, senescence, or apoptosis. A few examples of p53 target genes are GADD45a, which is involved in DNA repair, p21, that initiates cell cycle arrest, and BAX, is a factor that stimulates apoptosis (Riley et al., 2008). P53 is mutated in approximately half of all cancers, in many other cancers components of the p53 pathway are mutated, underscoring the vital importance of this protein (Oren, 2003).

The `classical` RNA world, the tale of hTR

The distinction between `new` and `classical` RNAs is just made here to emphasize the difference in thinking about RNA before the early nineties and nowadays, I will explain this here. Our thinking about gene regulation and organization has been shaped in large part by the description of the lac operon in bacteria by Jacob and Monod in 1961

(Jacob and Monod, 1961). Their model of gene structure and regulation is highly linear, one stretch of DNA codes for only one function, either regulatory or protein coding. Although they already suggested that RNA could also have a regulatory role as the repressor of the lac operon, this factor was later found to be a protein. Potential regulatory roles for RNA were dismissed for years to come and the gene models remained protein-centric. After the discovery of splicing (genes are interrupted, rather than coding for protein from beginning to end) in 1977, the intronic RNAs were considered to be unimportant, merely byproducts of the splicing process (Berget et al., 1977; Chow et al., 1977). It was, however, appreciated that RNA plays an important role in the translation process of mRNA to proteins. The ribosome was found to consist, in large part, of RNA, the translation process depends on transfer-RNAs, and for splicing, several snRNAs were identified that guide the process. The discovery in the 1980's by Thomas Cech, that non protein coding RNAs can also have catalytic functions (rybozymes) showed that RNA is a lot more versatile than previously thought and started the debate whether an "RNA world" is the initiator of cellular life (Zaug and Cech, 1986).

Only about one percent of our genome codes for proteins, does that make the rest of the genome "junk" DNA? In the advent of the human genome project (with the first draft published in the year 2000) and the development of better techniques to analyse the expression of the genome, scientists found that over 90% of our genome is transcribed (Cawley et al., 2004; Cheng et al., 2005; Kapranov et al., 2007a). These and other studies have shown that there are hundrends of thousands of ncRNAs expressed in cells, and in the last few years several of those have been shown to have specific regulatory capacities (Mattick, 2007; Prasanth and Spector, 2007). Many of the ncRNAs are conserved, have specific spatial and temporal expression patterns and several are involved in disease (Amaral et al., 2008; Mercer et al., 2008; Perez et al., 2008). Thousands of proteins are capable of binding RNA but for only a few, some of their targets are known. Together with the discoveries of miRNAs (see below) and several other small RNA species, these data suggest that the roles of ncRNAs have been underestimated and we are just scratching the surface of this `new` RNA world.

Human telomerase RNA (hTR) was identified in 1987 as the essential cofactor for the telomerase protein, providing the template to elongate telomeres (Greider and Blackburn, 1987; Greider and Blackburn, 1989). The only functionally important part of the molecule was considered to be the template region (6 nucleotides of the 451 in total) and the rest of the molecule was thought to merely aid in the processing and binding to the telomerase protein, this is why I classify it as a`classical` RNA (Chen et al., 2000; Feng et al., 1995). In chapter 4, however, I describe the identification of an unexpected, telomerase independent, role of hTR in DNA damage regulation.

Telomerase is the complex of hTR with the telomerase enzyme (hTERT) and a protein called dyskerin, that can extend the ends of chromosomes, called telomeres (Cohen et al., 2007). This complex is only active in germ cells, certain stem cells, and is re-activated in most (>85%) cancers (Kim et al., 1994). Telomeres are specialised structures consisting of telomeric DNA repeats (5'-TTAGGG-3') and associated proteins at the ends of the chromosomes that protect them from activating a DNA damage response and from the loss of genomic information. Because the DNA in higher eukaryotes

is linear, normal cells that don't express telomerase activity, lose a bit of the end of their chromosomes each cell division. This is due to the inability of DNA polymerases to synthesize the ends of linear stretches of DNA (they need an RNA primer and only work in one direction). Obviously, this problem, also called the end replication problem, will eventually lead to loss of genetic material, and such a dsDNA `break` would elicit a DNA damage response (Longhese, 2008). In fact, this is the reason that cells have a limited replicative lifespan, they reach the so-called Hayflick limit after a certain number of doublings, and go into replicative senescence (permanent withdrawal from the replicative cycle) (Harley et al., 1990; Hayflick and Moorhead, 1961). This is a barrier that cancer cells have to overcome to reach their limitless replicative potential (see above). To overcome this barrier cancer cells either increase hTERT and hTR levels or use a recombination based way of extending telomeric repeats (ALT, for Alternative Lengthening of Telomeres) (Bryan et al., 1997; Bryan et al., 1995; Kim et al., 1994).

hTR is a ubiquitously expressed RNA, whereas hTERT is only expressed in stem cells and cancer cells, this suggests that hTR may have a seperable role from telomere lengthening. This was also suspected from in vitro data from cell lines, mouse models and in fact, even from human tumor samples but never investigated. It was shown that hTR may also play a role in initiation of tumorigenicity for it was shown that its upregulation is an early event, even in mice where telomere length is not an issue at this stage, because telomeres are suficiently long (Blasco et al., 1996; Broccoli et al., 1996). Very convincing data for this point comes from several studies that investigated human tumors and show that hTR, and not hTERT levels or not even telomerase activity is correlated to tumor stage, these studies include cervical carcinoma, lung cancer, leukemia, head and neck cancer, renal cell carcinoma, esophageal adenocarcinoma, childhood neuroblastic tumors, as well as breast carcinomas and several other cancers (Brown et al., 1997; Cao et al., 2008; Dome et al., 2005; Maitra et al., 1999; Morales et al., 1998; Rushing et al., 1997; Soder et al., 1997; Yashima et al., 1997; Yashima et al., 1998). In chapter 4 I will describe that we have shown that increased levels of hTR, as found in these tumors, can dampen the ATR mediated DNA damage response and thereby fuel genomic instability as shown by the induction of fragile sites. hTR levels are controlled by several, not fully understood, transcriptional and post-transcriptional mechanisms (Cairney and Keith, 2008). We have shown that also ATR activation by UV irradiation can cause an upregulation of hTR that provides a negative feedbackloop to ATR (Kedde et al., 2006). It is conceivable that a similar mechanism upregulates hTR in response to replication stress in early lesions to counteract ATR activity, however, this remains to be tested.

The `new` RNA world, microRNAs lead the way

Only very recently a whole new world of RNAs has been revealed, starting at the characterization of the *lin-4* locus, which produces small, noncoding RNA, regulating developmental timing in C. elegans (Lee et al., 1993). This small RNA was found to contain complementary sequences to the 3' untranslated region (UTR) of the lin-14 mRNA and inhibit its translation by an RNA-RNA interaction. Several years later Fire, Mello and coworkers found that dsRNAs, when fed to elegans worms, are cleaved into small ~22nt RNAs that trigger gene silencing

of complementary endogenous targets, a process they dubbed RNA interference (RNAi) (Fire et al., 1998). The existence of similar interference processes in all higher eukaryotes, including humans, was identified soon and RNAi started to be generally used as a tool to inhibit gene expression and thereby systematically investigate the genome (Brummelkamp and Bernards, 2003; Brummelkamp et al., 2002). From zebrafish to man, these systems were exploited beacause of the ease of using dsRNA (for Drosophila and C. elegans), or short hairpin or siRNAs (human and mouse cells) to inhibit gene expression. However, scientists knew that a similar mechanism must be used for an endogenous pathway of gene silencing. This was becoming apparent when scores of miRNAs were discovered (~1000 are predicted) that are encoded in genes in our genome and miRNAs were

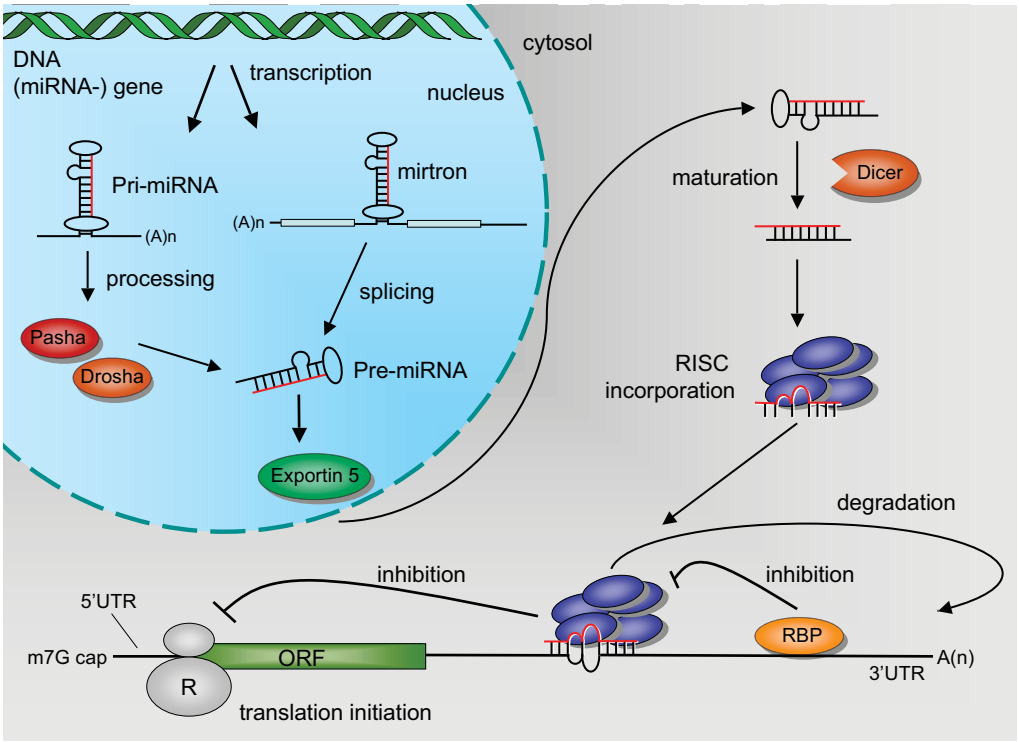

Figure 2. The miRNA pathway. Most miRNAs are transcribed by RNA polymerase II as long RNAs (Pri-miRNAs) that are converted to ~70 nt long pre-miRNAs by Drosha and Pasha (Lee et al., 2003). Mirtrons are a new class of miRNAs that can bypass this step, they are generated through splicing (Ruby et al., 2007). The pre-miRNAs are then exported to the cytoplasm by Exportin 5, converted to ~22 nt mature miRNAs by Dicer and one strand of the duplex is incorporated into the RNA induced silencing complex (RISC)(Gregory et al., 2005; Maniataki and Mourelatos, 2005). In animals, miRNAs utilize a seed sequence at their 5' end (nt 2-8) to associate with 3'UTR regions of mRNAs to suppress gene expression by inhibiting translation that occasionally is associated with mRNA decay (Bagga et al., 2005; Lim et al., 2005; Pillai et al., 2005). In **Chapter 5**, we show that in some cases miRNAs can also bind to targets in their coding region and repress their expression. Repressed mRNAs, miRNAs, and most proteins constituting the miRNA-RISC complex (miRNP) are enriched in cytoplasmic processing bodies called P-bodies (Liu et al., 2005). Some RNA binding proteins (RBPs), like Dead end, can inhibit miRNA function. R is the ribosome, ORF is open reading frame of the mRNA.

predicted to regulate over a third of our genome (Aravin and Tuschl, 2005; Lagos-Quintana et al., 2001; Lagos-Quintana et al., 2003; Lagos-Quintana et al., 2002; Lau et al., 2001; Lee and Ambros, 2001; Lewis et al., 2005). MiRNAs have now been shown to paticipate in a wide variety of cellular processes and the vast majority of miRNAs show tissue or developmental stage-specific expression (Lagos-Quintana et al., 2002; Lim et al., 2005; Wienholds et al., 2005). Importantly, aberrant expression or activity of miRNAs can lead to disease, such as cancer (Filipowicz et al., 2008; Kloosterman and Plasterk, 2006).

A typical literature search for miRNA targets now yields approximately 1000 results, starting at the first identification by Lee and Ambros (Lee and Ambros, 2001). More and more miRNAs and miRNA targets are being discovered. Recently a whole new class of conserved miRNAs were discovered in introns, dubbed mirtrons, that bypass processing by Drosha (Berezikov et al., 2007; Ruby et al., 2007). Adding to this complexity of post transcriptional regulation of mRNAs by miRNAs are diverse ways of regulation of miRNAs themselves by transcription, editing, processing, and subcellular localisation as well as modulation of miRNA function by proteins such as HuR and Dead end 1 (also described in the next chapter and chapter 3)(Bhattacharyya et al., 2006; Filipowicz et al., 2008; Kedde and Agami, 2008; Kedde et al., 2007). Intriguingly, it has now been reported that in response to proliferation cues, certain cells decrease their 3'UTR length by switching to alternatve poly adenylation sites in order to avoid miRNA regulation (Sandberg et al., 2008).

Not long after the discovery of miRNAs our protein centered view of the genome was challenged by the findings that transcription is widespread and highly interleaved (Kapranov et al., 2007b; Mattick, 2007; Prasanth and Spector, 2007). In these findings lies a whole new RNA world, waiting to be discovered. The notion that functional ncRNAs have a huge size range, from ~22bp for miRNAs to ~18kb for XIST (X-inactive specific transcript) and ~108kb for AIR (antisense IGF2R RNA) and that ncRNAs far outnumber protein coding RNAs highlight their importance. The fact that ncRNAs are already being identified as diagnostic markers for cancer and other diseases shows that there is not just very interesting biology to learn here but also potential benefit for patients (Amaral et al., 2008; Calin et al., 2007; Kloosterman and Plasterk, 2006; Kumar et al., 2007; Perez et al., 2008). In light of this, miRNA silencing by LNA oligonucleotides has already been reported in non-human primates, paving the way for miRNA targeting therapeutics in the future (Elmen et al., 2008).

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