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

Kedde, M.

Citation

Kedde, M. (2009, January 22). *New RNA playgrounds : non-coding RNAs and RNA-binding proteins control cellular processes*. Retrieved from https://hdl.handle.net/1887/13414

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/13414

Note: To cite this publication please use the final published version (if applicable).

New RNA playgrounds

non-coding RNAs and
RNA-binding proteins control cellular processes

Martijn Kedde

New RNA playgrounds

non-coding RNAs and regulatory
RNA-binding proteins control cellular processes

proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,
volgens het besluit van het College voor Promoties
te verdedigen op donderdag 22 januari 2009
klokke 13.45 uur

door

Martijn Kedde

geboren te Ede

in 1979

Promotiecommissie

Promotor:	Prof. Dr. J.J. Neefjes
Co-Promotor:	Dr. R. Agami
	(Nederlands Kanker Instituut, Amsterdam)
Referent:	Prof. Dr. R.H. Medema
	(Universiteit Utrecht)
Overige Leden:	Prof. Dr. R. Bernards
	(Universiteit Utrecht)
	Dr. R.F. Ketting
	(Universiteit Utrecht)
	Prof. Dr. T.N.M. Schumacher
	Prof. Dr. L.H.F. Mullenders
	Dr. A.G. Jochemsen

© 2008, M. Kedde

Cover: ‘Dagrecreatie: Spelende kinderen in een speeltuin’ 18 augustus 1953, herkomst: stadsarchief Amsterdam.

The research in this thesis was performed at the department of Tumor Biology at the Netherlands Cancer Institute (NKI) and supported by the Dutch Cancer Society (KWF) and a European Young Investigator award (EURYI) to Reuven Agami. The printing of this thesis was supported by the Dutch Cancer Society (KWF).

Printed by: Wöhrmann Print Service, Zutphen

Contents

Chapter 1: General introduction and outline of this thesis 7

Chapter 2: Interplay between microRNAs and RNA-binding proteins determines developmental processes 25
Cell Cycle, 2008

Chapter 3: RNA-Binding Protein Dnd1 Inhibits MicroRNA Access to Target mRNA 39
Cell, 2007

Chapter 4: Telomerase-independent regulation of ATR by human telomerase RNA 65
Journal of Biological Chemistry, 2006

Chapter 5: miR-148 targets human DNMT3b protein coding region 83
RNA, 2008

Chapter 6: General discussion 91

Nederlandse samenvatting 101

Curriculum vitae 105

List of publications 107

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,

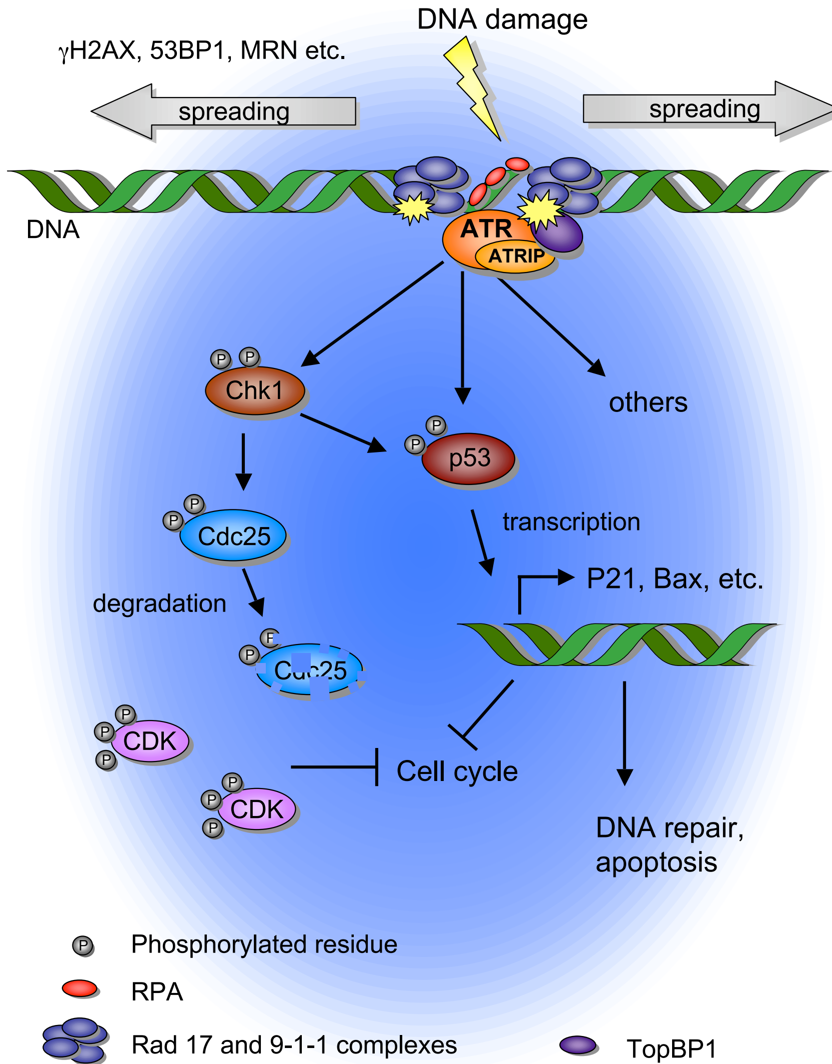


Figure 1. The ATR mediated DNA damage response. Multiple DNA damage insults activate ATR, such as replication stress, ssDNA gaps and UV-induced adducts. Sensors (the 9-1-1 complex and Rad17) are then recruited to the site of damage and ATR is activated. A DNA damage focus forms by spreading of H2AX phosphorylation 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

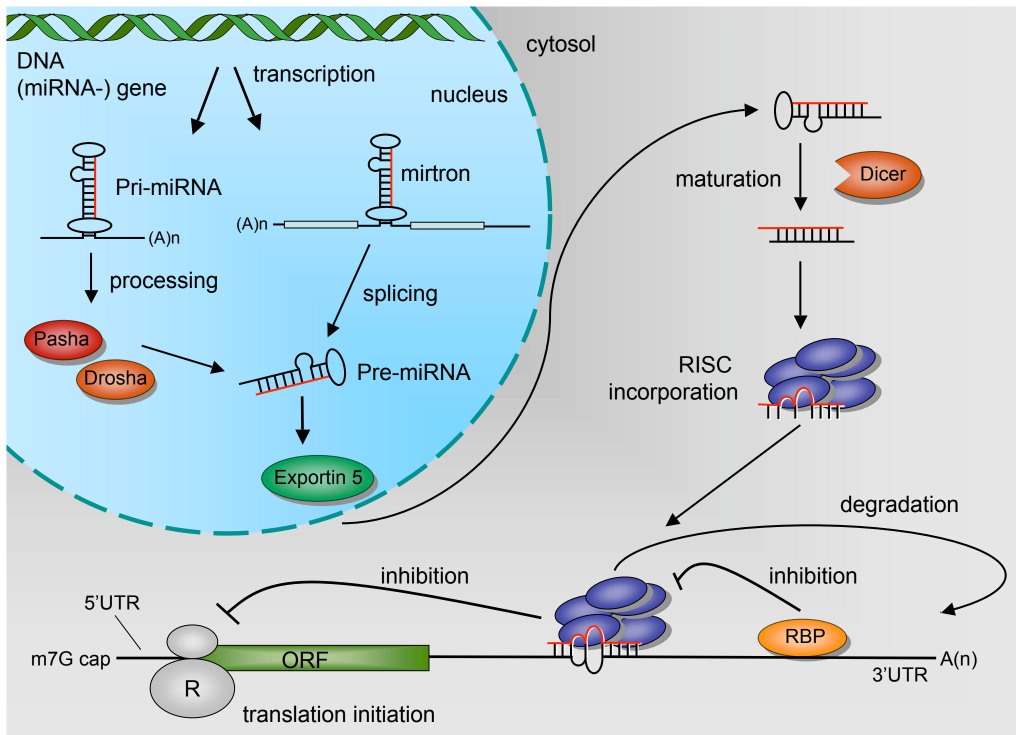


Figure 2. The miRNA pathway. Most miRNAs are transcribed by RNA polymerase II as long RNAs (Pri-miRNAs) that are converted to ~70 nt long pre-miRNAs by Drosha and Pasha (Lee et al., 2003). Mirtrons are a new class of miRNAs that can bypass this step, they are generated through splicing (Ruby et al., 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).

References

Amaral, P. P., Dinger, M. E., Mercer, T. R., and Mattick, J. S. (2008). The eukaryotic genome as an RNA machine. Science *319*, 1787-1789.

Aravin, A., and Tuschl, T. (2005). Identification and characterization of small RNAs involved in RNA silencing. FEBS Lett *579*, 5830-5840.

Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R., and Pasquinelli, A. E. (2005). Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell *122*, 553-563.

Bartek, J., and Lukas, J. (2007). DNA damage checkpoints: from initiation to recovery or adaptation. Curr Opin Cell Biol *19*, 238-245.

Bartkova, J., Horejsi, Z., Koed, K., Kramer, A., Tort, F., Zieger, K., Guldberg, P., Sehested, M., Nesland, J. M., Lukas, C., *et al.* (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature *434*, 864-870.

Berezikov, E., Chung, W. J., Willis, J., Cuppen, E., and Lai, E. C. (2007). Mammalian mirtron genes. Mol Cell *28*, 328-336.

Berget, S. M., Moore, C., and Sharp, P. A. (1977). Spliced segments at the 5' terminus of adenovirus 2 late mRNA. Proc Natl Acad Sci U S A *74*, 3171-3175.

Bhattacharyya, S. N., Habermacher, R., Martine, U., Closs, E. I., and Filipowicz, W. (2006). Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell *125*, 1111-1124.

Blasco, M. A., Rizen, M., Greider, C. W., and Hanahan, D. (1996). Differential regulation of telomerase activity and telomerase RNA during multi-stage tumorigenesis. Nat Genet *12*, 200-204.

Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A. H., Schlegelberger, B., Stein, H., Dorken, B., Jenuwein, T., and Schmitt, C. A. (2005). Oncogene-induced senescence as an initial barrier in lymphoma development. Nature *436*, 660-665.

Broccoli, D., Godley, L. A., Donehower, L. A., Varmus, H. E., and de Lange, T. (1996). Telomerase activation in mouse mammary tumors: lack of detectable telomere shortening and evidence for regulation of telomerase RNA with cell proliferation. Mol Cell Biol *16*, 3765-3772.

Brown, D. F., Gazdar, A. F., White, C. L., 3rd, Yashima, K., Shay, J. W., and Rushing, E. J. (1997). Human telomerase RNA expression and MIB-1 (Ki-67) proliferation index distinguish hemangioblastomas from metastatic renal cell carcinomas. J Neuropathol Exp Neurol *56*, 1349-1355.

Brown, E. J., and Baltimore, D. (2003). Essential and dispensable roles of ATR in cell cycle arrest and genome maintenance. Genes Dev *17*, 615-628.

Brummelkamp, T. R., and Bernards, R. (2003). New tools for functional mammalian cancer genetics. Nat Rev Cancer *3*, 781-789.

Brummelkamp, T. R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science *296*, 550-553.

Bryan, T. M., Englezou, A., Dalla-Pozza, L., Dunham, M. A., and Reddel, R. R. (1997). Evidence for an alternative mechanism for maintaining telomere length in human tumors and tumor-derived cell lines. Nat Med *3*, 1271-1274.

Bryan, T. M., Englezou, A., Gupta, J., Bacchetti, S., and Reddel, R. R. (1995). Telomere elongation in immortal human cells without detectable telomerase activity. Embo J *14*, 4240-4248.

Byun, T. S., Pacek, M., Yee, M. C., Walter, J. C., and Cimprich, K. A. (2005). Functional uncoupling of MCM helicase and DNA polymerase activities activates the ATR-dependent checkpoint. Genes Dev *19*, 1040-1052.

Cairney, C. J., and Keith, W. N. (2008). Telomerase redefined: integrated regulation of hTR and hTERT for telomere

maintenance and telomerase activity. Biochimie *90*, 13-23.

Calin, G. A., Liu, C. G., Ferracin, M., Hyslop, T., Spizzo, R., Sevignani, C., Fabbri, M., Cimmino, A., Lee, E. J., Wojcik, S. E.*, et al.* (2007). Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas. Cancer Cell *12*, 215-229.

Cao, Y., Bryan, T. M., and Reddel, R. R. (2008). Increased copy number of the TERT and TERC telomerase subunit genes in cancer cells. Cancer Sci *99*, 1092-1099.

Cawley, S., Bekiranov, S., Ng, H. H., Kapranov, P., Sekinger, E. A., Kampa, D., Piccolboni, A., Sementchenko, V., Cheng, J., Williams, A. J.*, et al.* (2004). Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. Cell *116*, 499-509.

Chen, J. L., Blasco, M. A., and Greider, C. W. (2000). Secondary structure of vertebrate telomerase RNA. Cell *100*, 503-514.

Chen, Z., Trotman, L. C., Shaffer, D., Lin, H. K., Dotan, Z. A., Niki, M., Koutcher, J. A., Scher, H. I., Ludwig, T., Gerald, W.*, et al.* (2005). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. Nature *436*, 725-730.

Cheng, J., Kapranov, P., Drenkow, J., Dike, S., Brubaker, S., Patel, S., Long, J., Stern, D., Tammana, H., Helt, G.*, et al.* (2005). Transcriptional maps of 10 human chromosomes at 5-nucleotide resolution. Science *308*, 1149-1154.

Chow, L. T., Roberts, J. M., Lewis, J. B., and Broker, T. R. (1977). A map of cytoplasmic RNA transcripts from lytic adenovirus type 2, determined by electron microscopy of RNA:DNA hybrids. Cell *11*, 819-836.

Chu, I. M., Hengst, L., and Slingerland, J. M. (2008). The Cdk inhibitor p27 in human cancer: prognostic potential and relevance to anticancer therapy. Nat Rev Cancer *8*, 253-267.

Cohen, S. B., Graham, M. E., Lovrecz, G. O., Bache, N., Robinson, P. J., and Reddel, R. R. (2007). Protein composition of catalytically active human telomerase from immortal cells. Science *315*, 1850-1853.

Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A. J., Barradas, M., Benguria, A., Zaballos, A., Flores, J. M., Barbacid, M.*, et al.* (2005). Tumour biology: senescence in premalignant tumours. Nature *436*, 642.

Cortez, D., Guntuku, S., Qin, J., and Elledge, S. J. (2001). ATR and ATRIP: partners in checkpoint signaling. Science *294*, 1713-1716.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., Schurra, C., Garre, M., Nuciforo, P. G., Bensimon, A.*, et al.* (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature *444*, 638-642.

Dome, J. S., Bockhold, C. A., Li, S. M., Baker, S. D., Green, D. M., Perlman, E. J., Hill, D. A., and Breslow, N. E. (2005). High telomerase RNA expression level is an adverse prognostic factor for favorable-histology Wilms' tumor. J Clin Oncol *23*, 9138-9145.

Duursma, A. M., and Agami, R. (2003). Ras interference as cancer therapy. Semin Cancer Biol *13*, 267-273.

Duursma, A. M., Kedde, M., Schrier, M., le Sage, C., and Agami, R. (2008). miR-148 targets human DNMT3b protein coding region. Rna *14*, 872-877.

Elmen, J., Lindow, M., Schutz, S., Lawrence, M., Petri, A., Obad, S., Lindholm, M., Hedtjarn, M., Hansen, H. F., Berger, U.*, et al.* (2008). LNA-mediated microRNA silencing in non-human primates. Nature *452*, 896-899.

Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A., Chiu, C. P., Adams, R. R., Chang, E., Allsopp, R. C., Yu, J., and et al. (1995). The RNA component of human telomerase. Science *269*, 1236-1241.

Filipowicz, W., Bhattacharyya, S. N., and Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet *9*, 102-114.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.

Gorgoulis, V. G., Vassiliou, L. V., Karakaidos, P., Zacharatos, P., Kotsinas, A., Liloglou, T., Venere, M., Ditullio, R. A., Jr., Kastrinakis, N. G., Levy, B.*, et al.* (2005). Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. Nature *434*, 907-913.

Gregory, R. I., Chendrimada, T. P., Cooch, N., and Shiekhattar, R. (2005). Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell *123*, 631-640.

Greider, C. W., and Blackburn, E. H. (1987). The telomere terminal transferase of Tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. Cell *51*, 887-898.

Greider, C. W., and Blackburn, E. H. (1989). A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. Nature *337*, 331-337.

Gudmundsdottir, K., and Ashworth, A. (2006). The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. Oncogene *25*, 5864-5874.

Hahn, W. C., Counter, C. M., Lundberg, A. S., Beijersbergen, R. L., Brooks, M. W., and Weinberg, R. A. (1999). Creation of human tumour cells with defined genetic elements. Nature *400*, 464-468.

Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. Cell *100*, 57-70.

Harley, C. B., Futcher, A. B., and Greider, C. W. (1990). Telomeres shorten during ageing of human fibroblasts. Nature *345*, 458-460.

Harper, J. W., and Elledge, S. J. (2007). The DNA damage response: ten years after. Mol Cell *28*, 739-745.

Hayflick, L., and Moorhead, P. S. (1961). The serial cultivation of human diploid cell strains. Exp Cell Res *25*, 585-621.

Jacob, F., and Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. J Mol Biol *3*, 318-356.

Kapranov, P., Cheng, J., Dike, S., Nix, D. A., Duttagupta, R., Willingham, A. T., Stadler, P. F., Hertel, J., Hackermuller, J., Hofacker, I. L., *et al.* (2007a). RNA maps reveal new RNA classes and a possible function for pervasive transcription. Science *316*, 1484-1488.

Kapranov, P., Willingham, A. T., and Gingeras, T. R. (2007b). Genome-wide transcription and the implications for genomic organization. Nat Rev Genet *8*, 413-423.

Kastan, M. B., and Bartek, J. (2004). Cell-cycle checkpoints and cancer. Nature *432*, 316-323.

Kedde, M., and Agami, R. (2008). Interplay between microRNAs and RNA-binding proteins determines developmental processes. Cell Cycle *7*, 899-903.

Kedde, M., le Sage, C., Duursma, A., Zlotorynski, E., van Leeuwen, B., Nijkamp, W., Beijersbergen, R., and Agami, R. (2006). Telomerase-independent regulation of ATR by human telomerase RNA. J Biol Chem *281*, 40503-40514.

Kedde, M., Strasser, M. J., Boldajipour, B., Vrielink, J. A., Slanchev, K., le Sage, C., Nagel, R., Voorhoeve, P. M., van Duijse, J., Orom, U. A., *et al.* (2007). RNA-Binding Protein Dnd1 Inhibits MicroRNA Access to Target mRNA. Cell *131*, 1273-1286.

Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. (1994). Specific association of human telomerase activity with immortal cells and cancer. Science *266*, 2011-2015.

Kloosterman, W. P., and Plasterk, R. H. (2006). The diverse functions of microRNAs in animal development and disease. Dev Cell *11*, 441-450.

Kumagai, A., Lee, J., Yoo, H. Y., and Dunphy, W. G. (2006). TopBP1 activates the ATR-ATRIP complex. Cell *124*, 943-955.

Kumar, M. S., Lu, J., Mercer, K. L., Golub, T. R., and Jacks, T. (2007). Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat Genet *39*, 673-677.

Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. Science *294*, 853-858.

Lagos-Quintana, M., Rauhut, R., Meyer, J., Borkhardt, A., and Tuschl, T. (2003). New microRNAs from mouse and human. Rna *9*, 175-179.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., and Tuschl, T. (2002). Identification of tissue-specific microRNAs from mouse. Curr Biol *12*, 735-739.

Lau, N. C., Lim, L. P., Weinstein, E. G., and Bartel, D. P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science *294*, 858-862.

le Sage, C., Nagel, R., Egan, D. A., Schrier, M., Mesman, E., Mangiola, A., Anile, C., Maira, G., Mercatelli, N., Ciafre, S. A., *et al.* (2007). Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. Embo J *26*, 3699-3708.

Lee, R. C., and Ambros, V. (2001).

An extensive class of small RNAs in Caenorhabditis elegans. Science *294*, 862-864.

Lee, R. C., Feinbaum, R. L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843-854.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., and Kim, V. N. (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature *425*, 415-419.

Lewis, B. P., Burge, C. B., and Bartel, D. P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell *120*, 15-20.

Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., Bartel, D. P., Linsley, P. S., and Johnson, J. M. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature *433*, 769-773.

Liu, J., Valencia-Sanchez, M. A., Hannon, G. J., and Parker, R. (2005). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol *7*, 719-723.

Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., *et al.* (2000). Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev *14*, 1448-1459.

Longhese, M. P. (2008). DNA damage response at functional and dysfunctional telomeres. Genes Dev *22*, 125-140.

Mailand, N., Falck, J., Lukas, C., Syljuasen, R. G., Welcker, M., Bartek, J., and Lukas, J. (2000). Rapid destruction of human Cdc25A in response to DNA damage. Science *288*, 1425-1429.

Maitra, A., Yashima, K., Rathi, A., Timmons, C. F., Rogers, B. B., Shay, J. W., and Gazdar, A. F. (1999). The RNA component of telomerase as a marker of biologic potential and clinical outcome in childhood neuroblastic tumors. Cancer *85*, 741-749.

Maniataki, E., and Mourelatos, Z. (2005). A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes Dev *19*, 2979-2990.

Mattick, J. S. (2007). A new paradigm for developmental biology. J Exp Biol *210*, 1526-1547.

Mercer, T. R., Dinger, M. E., Sunkin, S. M., Mehler, M. F., and Mattick, J. S. (2008). Specific expression of long noncoding RNAs in the mouse brain. Proc Natl Acad Sci U S A *105*, 716-721.

Michaloglou, C., Vredeveld, L. C., Soengas, M. S., Denoyelle, C., Kuilman, T., van der Horst, C. M., Majoor, D. M., Shay, J. W., Mooi, W. J., and Peeper, D. S. (2005). BRAFE600-associated senescence-like cell cycle arrest of human naevi. Nature *436*, 720-724.

Molinari, M., Mercurio, C., Dominguez, J., Goubin, F., and Draetta, G. F. (2000). Human Cdc25 A inactivation in response to S phase inhibition and its role in preventing premature mitosis. EMBO Rep *1*, 71-79.

Morales, C. P., Lee, E. L., and Shay, J. W. (1998). In situ hybridization for the

detection of telomerase RNA in the progression from Barrett's esophagus to esophageal adenocarcinoma. Cancer *83*, 652-659.

Mordes, D. A., Glick, G. G., Zhao, R., and Cortez, D. (2008). TopBP1 activates ATR through ATRIP and a PIKK regulatory domain. Genes Dev *22*, 1478-1489.

Oren, M. (2003). Decision making by p53: life, death and cancer. Cell Death Differ *10*, 431-442.

Perez, D. S., Hoage, T. R., Pritchett, J. R., Ducharme-Smith, A. L., Halling, M. L., Ganapathiraju, S. C., Streng, P. S., and Smith, D. I. (2008). Long, abundantly expressed non-coding transcripts are altered in cancer. Hum Mol Genet *17*, 642-655.

Pillai, R. S., Bhattacharyya, S. N., Artus, C. G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E., and Filipowicz, W. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science *309*, 1573-1576.

Prasanth, K. V., and Spector, D. L. (2007). Eukaryotic regulatory RNAs: an answer to the 'genome complexity' conundrum. Genes Dev *21*, 11-42.

Riley, T., Sontag, E., Chen, P., and Levine, A. (2008). Transcriptional control of human p53-regulated genes. Nat Rev Mol Cell Biol *9*, 402-412.

Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S., and Bonner, W. M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. J Biol Chem *273*, 5858-5868.

Ruby, J. G., Jan, C. H., and Bartel, D. P. (2007). Intronic microRNA precursors that bypass Drosha processing. Nature *448*, 83-86.

Rushing, E. J., Yashima, K., Brown, D. F., White, C. L., 3rd, Shay, J. W., Risser, R. C., and Gazdar, A. F. (1997). Expression of telomerase RNA component correlates with the MIB-1 proliferation index in ependymomas. J Neuropathol Exp Neurol *56*, 1142-1146.

Sandberg, R., Neilson, J. R., Sarma, A., Sharp, P. A., and Burge, C. B. (2008). Proliferating cells express mRNAs with shortened 3' untranslated regions and fewer microRNA target sites. Science *320*, 1643-1647.

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell *88*, 593-602.

Shieh, S. Y., Ahn, J., Tamai, K., Taya, Y., and Prives, C. (2000). The human homologs of checkpoint kinases Chk1 and Cds1 (Chk2) phosphorylate p53 at multiple DNA damage-inducible sites. Genes Dev *14*, 289-300.

Soder, A. I., Hoare, S. F., Muir, S., Going, J. J., Parkinson, E. K., and Keith, W. N. (1997). Amplification, increased dosage and in situ expression of the telomerase RNA gene in human cancer. Oncogene *14*, 1013-1021.

Sorensen, C. S., Syljuasen, R. G., Falck, J., Schroeder, T., Ronnstrand, L., Khanna, K. K., Zhou, B. B., Bartek, J., and Lukas, J. (2003). Chk1 regulates the S phase checkpoint by coupling the physiological turnover and ionizing radiation-induced accelerated proteolysis of Cdc25A. Cancer Cell *3*, 247-258.

Takai, H., Tominaga, K., Motoyama, N., Minamishima, Y. A., Nagahama, H., Tsukiyama, T., Ikeda, K., Nakayama, K., Nakanishi, M., and Nakayama, K. (2000). Aberrant cell cycle checkpoint function and early embryonic death in Chk1(-/-) mice. Genes Dev *14*, 1439-1447.

Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. (1999). A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev *13*, 152-157.

Vogelstein, B., and Kinzler, K. W. (2004). Cancer genes and the pathways they control. Nat Med *10*, 789-799.

Wienholds, E., Kloosterman, W. P., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., Horvitz, H. R., Kauppinen, S., and Plasterk, R. H. (2005). MicroRNA expression in zebrafish embryonic development. Science *309*, 310-311.

Yashima, K., Litzky, L. A., Kaiser, L., Rogers, T., Lam, S., Wistuba, II, Milchgrub, S., Srivastava, S., Piatyszek, M. A., Shay, J. W., and Gazdar, A. F. (1997). Telomerase expression in respiratory epithelium during the multistage pathogenesis of lung carcinomas. Cancer Res *57*, 2373-2377.

Yashima, K., Milchgrub, S., Gollahon, L. S., Maitra, A., Saboorian, M. H., Shay, J. W., and Gazdar, A. F. (1998). Telomerase enzyme activity and RNA expression during the multistage pathogenesis of breast carcinoma. Clin Cancer Res *4*, 229-234.

Zaug, A. J., and Cech, T. R. (1986). The intervening sequence RNA of Tetrahymena is an enzyme. Science *231*, 470-475.

Zhao, H., and Piwnica-Worms, H. (2001). ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol Cell Biol *21*, 4129-4139.

Zhou, B. B., and Elledge, S. J. (2000). The DNA damage response: putting checkpoints in perspective. Nature *408*, 433-439.

Zou, L., and Elledge, S. J. (2003). Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. Science *300*, 1542-1548.

Chapter 2:

Interplay between microRNAs and RNA-binding proteins determines developmental processes

Germ cells reveal a complex RNA world

Cell Cycle 7:7, 899-903; 1 April 2008

Chapter 2

Interplay between microRNAs and RNA-binding proteins determines developmental processes

Germ cells reveal a complex RNA world

Martijn Kedde and Reuven Agami

MicroRNAs (miRNAs) are genes involved in normal development and cancer. They inhibit gene expression by associating with 3'-Untranslated regions (3'UTRs) of messenger RNAs (mRNAs), and are thought to regulate a large proportion of protein coding genes. However, it is becoming apparent that miRNA activity is not necessarily always determined by its expression in the cell. MiRNA activity can be affected by RNA-binding proteins (RBPs). For example, the RNA-binding protein HuR associates with the 3'UTR of the CAT1 mRNA after stress, counteracting the effect of miR-122.[1] Second, we found that the expression of an RNA binding protein called Dead end (Dnd1) prohibits the function of several miRNAs by blocking the accessibility of target mRNAs.[2] Dnd1 function is essential for proper development of primordial germ cells (PGCs) in zebrafish and mammals, indicating a crucial role for RBP/miRNA interplay on 3'UTRs of mRNAs in developmental decisions. In this review we discuss the interplay between RBPs and miRNAs in the context of germ cells and review current observations implicating RBPs in miRNA function.

Germ Cell Development

One of the first steps occurring in a developing embryo is the formation of the germ line, a necessity for species continuity. To remain totipotent (capable to form an entire organism), the germ cells have to preserve the naive state of their genomes and inhibit the activation of somatic genes. The mechanisms behind the specification, migration and further development of germ cells are being elucidated in various organisms and appear to be remarkably similar.[3,4] The understanding of the molecular mechanisms governing germ cell development is likely to provide insights to genome protection, to analogous cell differentiation processes in somatic stem cells, and to germ cell diseases, such as testicular and ovarian cancers.

The so-called primordial germ cells (PGCs) are set apart from somatic cells early in development (in some organisms PGCs are the first cell lineage to be determined) at a location distinct from the gonad, and start migrating toward the future gonad. After the PGCs arrive in the gonad they start proliferating and differentiate into germ-line stem cells capable of forming the gametes. The migration of this small group of PGCs (40–45 in mice and zebrafish) is a remarkable feat of these cells, requiring guidance by attractive and repulsive cues from somatic tissues.[5] The involved chemokines and receptors, such as Sdf-1 and Cxcr4, are conserved throughout evolution.[6-9] In zebrafish, the PGCs are specified in 4 different locations in the developing embryo, then they follow six different migration steps and practically all arrive in the gonad.[5] The RNA-binding protein (RBP) Dead end (Dnd1) is essential for PGC motility in zebrafish, as cells depleted of Dnd1 do not polarize, form psuedopodia, and

subsequently die by apoptosis.[10] The requirement of Dnd1 for PGC survival appears to be conserved. Mice with a naturally occurring truncation mutation in the *Dnd1* gene (the Ter mutation) lose most PGCs. However, still few Dnd1-mutant PGCs end up in the gonad, showing that the migration capacity of PGCs, in general, is not affected.[11] In most cases, the control of PGCs survival is intimately linked with migration, because PGCs that end up in the wrong place are usually quickly eliminated.[12] In some cases though, PGCs end up in extragonadal sites and escape elimination. It is thought that these cells can give rise to pediatric germ-line tumors.[13,14] The identification of Dnd1 targets and their function would therefore be important to determine the molecular mechanisms involved in cell migration, at least in zebrafish, germ cell survival, and germ-line cancers.

The Germ Plasm: RNA Regulation Determines Germ Cell Fate

One of the most distinctive features of PGCs is the appearance of electron-dense structures associated with mitochondria that contain RNA and proteins called either germ plasm, nuage or chromatoid bodies.[4,15] In mammals, PGCs do not have an apparent germ plasm, rather; a mammalian counterpart of germ plasm with a similar overall structure and homologous components is present in mouse haploid male germ cells. Nonetheless, germ plasm components, such as Dnd1 and Vasa, are required for proper PGC function also in mammals.[11,16,17]

As shown in zebrafish embryos by transplantation experiments, the germ plasm itself is indispensable for germ cell establishment.[18] It appears that germ plasm is a place in the cytoplasm where certain mRNA species are stored and repressed, awaiting activating signals for expression. Studies from a variety of different organisms have indicated that the primary role of germ plasm is the handling of RNA. This includes storage and regulation of mRNA translation as well as regulation of some non-coding RNAs. Many different RBPs, such as Vasa, Dnd1, Argonaute, PIWI, Tudor, and SM, are localized to the germ plasm, together with several RNAs, including the *nanos*, *tdrd7*, and *dead end* mRNAs and also micro- and Piwi-associated-small RNAs.[4,10,15,19-22]

Why do germ cells rely so much on RNA for their survival? Seydoux and Braun have put forward the interesting hypothesis that germ cells rely on RNA-based program of posttranscriptional regulation to keep their genome in a ‘totipotent’ state.[4,19] This stems from the notion that germ cell nuclei present extensive chromatin remodeling activity to establish unique chromatin while transcription is widely repressed.[23] Germ plasm would be the physical manifestation of this RNA-based program. Dnd1, a protein that is localized to the germ plasm, is associated with actively translated *nanos*, and *tdrd7* mRNAs, that are also located in the germ plasm.[2,10,21] These data suggest that germ plasm is a site of active translation.

An alternative explanation may be that the translationally active Dnd1-mRNA complexes are localized elsewhere, where they are inaccessible to translational repressors (miRNPs, also shown to be in the germ plasm, see below).[2] However, these locations have not been detected thus far. As our current knowledge solely depends on in situ mRNA hybridization experiments and RNA reporters coding for fluorescent proteins carrying a 3’UTR of genes of interest, colocalisation of endogenous Dnd1 protein with translationally active target mRNAs needs to be examined.[2,10,21,24]

MicroRNAs, the Germ Cell Perspective

It has been recently suggested that the cytoplasmic processing body (P-body), the site where repression of mRNA translation is thought to occur, might be the somatic counterpart of the germ plasm.[22] These structures are similar and share several constituents such as translationally-repressed mRNAs, factors involved in inhibition of translation, miRNAs, and most proteins constituting the miRNA-RISC complex (miRNP, see below). In line with this, cytoplasmic P-bodies lack ribosomes and translation initiation factors and are known to be sites of translational repression and mRNA decay.[25,26]

MicroRNAs are ~22-nucleotides (nt) RNAs widely expressed in metazoans.[27,28] These small RNAs are negative regulators of gene expression capable of defining and altering cell fate. Around 500–1000 miRNAs are estimated to be present in the mammalian genome, and a large proportion of human protein-coding genes are thought to be under the regulation of one or more miRNAs.[29,30] Analogous to transcription factors, miRNAs regulate mRNAs in a combinatorial fashion and single miRNAs can repress the translation of many mRNAs. A growing body of evidence suggests that miRNAs participate in the regulation of a large variety of cellular processes and that the vast majority of miRNAs show tissue or developmental stage specific expression.[31-33] Importantly, aberrant expression or activity of miRNAs can lead to disease.[28,34,35] Most miRNAs are transcribed by RNA polymerase II as long RNAs that are converted to ~70 nt long pre-miRNAs by Drosha.[36] 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).[37,38] 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.[26,32,39] Translation is thought to be inhibited at the initiation step, by competition of Ago proteins with translation initiation factors eIF6 and eIF4e.[40-42] Last, MVH (Mouse Vasa Homolog) was found to interact with Dicer in germ plasm and therefore has been proposed to be the germ cell-specific helicase for the miRNA pathway.[22] Some critical components of the miRNA pathway, such as Dicer, and likely also some miRNAs, are essential for germ cell maintenance and put miRNAs forward as important players in germ cell biology.[24,25,43-46]

The fact that miRNAs are present in germ plasm together with most proteins constituting the miRNA-RISC complex, suggests germ plasm being a repressive hub for mRNAs. However, most peculiarly, it was recently documented that two miRNA targets of miR-430 in zebrafish are not repressed in PGCs, while miR-430 is present.[24] MiR-430 is a family of several miRNAs that are required for clearance of maternal transcripts in the developing embryo.[4,47] Mishima and colleagues have suggested that specific regions in the 3’UTR of the *nanos1* and *tdrd7* genes counteract miRNA-mediated repression in the germ-line cells. Therefore, we hypothesized that germ cells contain specific factors that bind mRNAs and counteract miRNA function. Using a genetic screening approach, we identified the RNA-binding protein Dead end as a factor involved in this phenomenon in both zebrafish PGCs and human germ cells.[2] By binding to U-rich mRNA regions (URRs), Dnd1 suppressed miR-430 function, at least in part through blocking miRNA accessibility.

The human microRNA homologues of miR-430 in zebrafish are miR-373, -372, -520, -302 and -93 (here termed miR-373 family). We have shown that the miR-373 family acts as potential oncogenes in human germ cells, presumably by facilitating rapid proliferation.[48] Moreover, expression of this miRNA family is associated with enhanced cellular migration, invasion and metastasis.[49] Since inhibition of Dnd1 expression in human testicular germ cell tumor cell lines resulted in the loss of miR-373 family targets, it is possible that a balance between miR-373 family function and Dnd1 exists in germ cells and increased level of miR-373 family overcomes Dnd1 function, resulting in target inhibition and accelerated proliferation. This notion is supported by findings made in mice. A truncating mutation in Dnd1 in the background of the 129-mouse strain (Ter mice), but not other mouse strains, results in testicular tumor formation. These tumors arise from the few germ cells that able to survive and further develop in the absence of Dnd1.[11] Interestingly, miRNA-107 was identified as one susceptibility gene in the 129 Ter strain that can allow germ cell tumor growth in the absence of Dnd1.[50] This may point for a connection between Dnd1, miRNAs and testicular germ cell cancer. However, no mutations were observed in Dnd1 in human testicular germ cell tumors.[51] Further experiments are required to examine the role of Dnd1 in other tumor types such as brain tumors, where Dnd1 is occasionally expressed.

From these data, it is clear that miRNAs and Dnd1 are closely tied together in germ cells. However, the extent of this interplay is yet to be determined. For instance, we do not know how many miRNA targets Dnd1 regulates and also the exact impact of germ cell miRNAs on gene expression is not clear. On top of that, we do not know whether the sole function of Dnd1 is the repression of miRNA activity or perhaps there are other functions that have been concealed in the experiments thus far. It will be interesting to determine in the near future whether blocking miRNA activity is the most important function of Dnd1 during development of germ cells.

piRNAs, Germ Plasm Reveals more Essential RNAs

Another novel class of small RNAs exclusively expressed in the germline was recently identified in several organisms including Drosophila, zebrafish, and mouse.[52] These are 24–30 nucleotide RNAs that are generated by a Dicer independent mechanism and interact with the Piwi class of Argonaute proteins, therefore named piRNAs.[52-54] The Piwi protein was identified a decade ago in Drosophila as a germ plasm factor essential for the regulation of germline stem cells.[55] In the past two years, several independent laboratories identified piRNAs to be crucial for germ line development of several organisms.[53,54,56-61] In general, piRNAs are thought to silence selfish DNA elements and maintain germ line DNA integrity.How exactly piRNAs are generated is not completely understood. It was shown that in Drosophila ovaries, most piRNAs appear to be derived from a discrete number of sites enriched in transposon sequences, the most abundant piRNAs derive from the antisense strand of retrotransposons.[62,63] Piwi and Aubergine were shown to bind piRNAs that are mostly antisense to transposons, whereas Ago3 appears to bind predominantly sense piRNAs, these proteins can cleave their target RNAs. A ‘ping-pong’ model for piRNA production was proposed where sense and antisense piRNA populations are amplified in a loop wherein each piRNA-directed cleavage event generates the 5’ end of a new

piRNA.[62,63] However, what determines their specific production in germ cells awaits further investigation. Moreover, whether and how they control gene transcription, translation, and genome organization is not well understood.

One interesting phenotype observed in flies and mice that are mutated in the piRNA pathway is the emergence of DNA damage.[64-66] This phenotype can only in part be explained by activation of transposons in the mouse and fly genomes. Several studies have indicated that there is no evidence of transposon activation when DNA damage is detected after piRNA depletion.[52] Also, in mice there is less evidence that piRNAs target transposons directly. Is there a direct role for piRNAs in DNA repair or suppression of DNA damage pathways? This is an intriguing possibility. It was previously shown that RNA may function to regulate the activity of checkpoint proteins. For example, in human cells, the telomerase template RNA (hTR), can suppress the ATR kinase.[67]

Can Dnd1 also affect the piRNA pathway? Thusfar, we have not identified piRNAs as targets of Dnd1, but the link between piRNAs and the miRNA pathway, and their presence in germ plasm may suggest that these pathways influence each other. Germ cells that lack Dicer, which is crucial for the generation of miRNAs, develop normally, whereas Dnd1-depleted germ cells die.[10,68] Mutations in the piRNA pathway, as Dnd1 depletion, causes germ cells to go into apoptosis, perhaps indicating a connection between the two. It remains to be seen whether Dnd1 and piRNAs functionally interact.

Emerging Modes of miRNA Regulation

The interplay between Dnd1 and miRNAs is one example of modulation of gene expression by RNA-binding proteins (RBPs). Other groups have also recently found RBPs to modulate miRNA activity.[1,69-71] Two of these groups showed that miRNA-repressed mRNAs can be relieved from repression by synaptic stimulation of neuronal cells.[69,70] When treated with BDNF, cultured rat neurons partially relieve Limk1 mRNA repression by miRNA-134.[70] The mechanism of miRNA derepression in this system thus far remains unknown. Ashraf and colleagues showed that external stimulation of Drosophila olfactory neurons induces degradation of the Armitage protein, which is required for miRNP assembly.[69] As a result, the translation of the protein kinase CaMKII mRNA, which is controlled by miRNAs, is enhanced. The fact that many RBPs, including Dnd1, are specifically expressed in brain suggests that miRNA reversibility is a more general mode of regulation.[11,72] In light of these studies, it is conceivable that also Dnd1 plays a role in brain function, although specific brain defects have not been observed in Ter mice.[11,72] To this end, it is also worth noting that inactivation of Dnd1 in mice leads to partial embryonic lethality, this is likely due to a role of Dnd1 in some critical, yet unknown, organ systems of the developing embryo.[72]

The group of Witold Filipowicz showed that HuR (ELAV1), an AU-rich element (ARE) binding protein, relieves CAT-1 mRNA from miR-122-mediated repression, a process that involves binding of HuR to the 3'-UTR of CAT-1 mRNA.[1] The CAT-1 mRNA localizes to P-bodies in a human hepatocarcinoma cell line in a miRNA-122 dependent manner. Upon stress, the CAT-1 mRNA relocalizes from P-bodies to the cytoplasm, where it associates with polysomes and becomes translationally active. This stress-induced effect is dependent on the translocation of HuR from the nucleus to the cytoplasm.

The mechanisms underlying HuR translocation are still poorly understood. Also, it is unknown whether HuR binding promotes the dissociation of miRNPs from the target mRNA, as Dnd1 appears to do, or just prevents them from repressing their targets. Whether Dnd1 also shuttles mRNA targets from P-bodies or germ plasm to active translation sites in the cytoplasm, is yet to be investigated. However, as Dnd1 in the systems we studied is localized in the germ plasm, apparently together with at least one of its targets, *nanos* RNA, it seems likely that Dnd1 does not translocate its targets from these bodies. It is worth to note that mouse Dnd1 appears to be either nuclear, or cytosolic, depending on the cell type.[72] We also reported significant amounts of the Dnd1 protein in the nucleus, when overexpressed in both MCF7 and HEK293 cells.[2] Whether this is an overexpression artifact, or reflects subcellular shuttling of Dnd1, remains to be investigated.

Other types of regulation of the miRNA pathway have also been observed. For instance, two studies showed recently that certain miRNAs, such as let-7 and miR-138, are not efficiently processed in all tissues and cell types in the mouse, whereas their precursors are present in these tissues.[73,74] Interestingly, the group of Scott Hammond present data supporting a role for such mechanisms of miRNA-downregulation in cancer and embryonic stem cells.[74] Furthermore, certain miRNAs are modified by RNA editing, a process that can lead to changes in miRNA stability or alter their target selection.[75-77] As expected, there are also examples of transcriptional regulation and epigenetic silencing of miRNAs.[78,79] To add to this list of possibilities, a case of activation of translation is now also described for a miRNA.[80] This study shows that AGO2 acts, together with the FMRP-related protein FXR1, as an activator of translation when binding to the 3'-UTR of tumor necrosis factor-α mRNA in serum-starved human cells.

Concluding Remarks

The discovery of miRNAs has shown us that 3'UTRs, analogous to promoters, are a widely used playground for modulators of gene expression. The discovery that RNA-binding proteins can modulate miRNA activity reveals an increasing dynamic regulation of gene expression that will —no doubt— impact many cellular processes. We have recently demonstrated that the interplay between miRNAs and one such RBP, Dnd1, is important for the maintenance of the primordial germ cells.[2] We show that the function of Dnd1 is conserved throughout evolution from zebrafish to humans and its mechanism involves counteracting miRNA binding to target mRNAs. However, several questions emerge. For instance; what are the (critical) targets of Dnd1? What are the protein partners of Dnd1? What is the sub-cellular localization of Dnd1 target mRNAs? Does Dnd1 interact with the miRNP directly? Does it influence miRNP activity? Is Dnd1 function restricted to primordial germ cells? Does Dnd1 function contribute to prevention of testicular germ cell cancer? These, and other questions will have to be answered in the future to get a clear picture of what Dnd1, and other RBPs do, and how a healthy and productive germ line is maintained.

Acknowledgements

We thank all members of the Agami Lab for discussions. This work was supported by grants from the Dutch Cancer Society (KWF) and the European young investigator award (EURYI).

References

1. Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell 2006; 125:1111-24.

2. Kedde M, Strasser MJ, Boldajipour B, Vrielink JA, Slanchev K, le Sage C, Nagel R, Voorhoeve PM, van Duijse J, Orom UA, Lund AH, Perrakis A, Raz E, Agami R. RNA-Binding Protein Dnd1 Inhibits MicroRNA Access to Target mRNA. Cell 2007; 131:1273-86.

3. Raz E. Primordial germ-cell development: the zebrafish perspective. Nat Rev Genet 2003; 4:690-700.

4. Seydoux G, Braun RE. Pathway to totipotency: lessons from germ cells. Cell 2006; 127:891-904.

5. Weidinger G, Wolke U, Koprunner M, Klinger M, Raz E. Identification of tissues and patterning events required for distinct steps in early migration of zebrafish primordial germ cells. Development 1999; 126:5295-307.

6. Ara T, Nakamura Y, Egawa T, Sugiyama T, Abe K, Kishimoto T, Matsui Y, Nagasawa T. Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1). Proc Natl Acad Sci USA 2003; 100:5319-23.

7. Doitsidou M, Reichman-Fried M, Stebler J, Koprunner M, Dorries J, Meyer D, Esguerra CV, Leung T, Raz E. Guidance of primordial germ cell migration by the chemokine SDF-1. Cell 2002; 111:647-59.

8. Knaut H, Werz C, Geisler R, Nusslein-Volhard C. A zebrafish homologue of the chemokine receptor Cxcr4 is a germ-cell guidance receptor. Nature 2003; 421:279-82.

9. Molyneaux KA, Zinszner H, Kunwar PS, Schaible K, Stebler J, Sunshine MJ, O'Brien W, Raz E, Littman D, Wylie C, Lehmann R. The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. Development 2003; 130:4279-86.

10. Weidinger G, Stebler J, Slanchev K, Dumstrei K, Wise C, Lovell-Badge R, Thisse C, Thisse B, Raz E. dead end, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. Curr Biol 2003; 13:1429-34.

11. Youngren KK, Coveney D, Peng X, Bhattacharya C, Schmidt LS, Nickerson ML, Lamb BT, Deng JM, Behringer RR, Capel B, Rubin EM, Nadeau JH, Matin A. The Ter mutation in the dead end gene causes germ cell loss and testicular germ cell tumours. Nature 2005; 435:360-4.

12. Boldajipour B, Raz E. What is left behind—quality control in germ cell migration. Sci STKE 2007; 2007:pe16.

13. Gobel U, Schneider DT, Calaminus G, Haas RJ, Schmidt P, Harms D. Germ-cell tumors in childhood and adolescence. GPOH MAKEI and the MAHO study groups. Ann Oncol 2000; 11:263-71.

14. Schneider DT, Schuster AE, Fritsch MK, Hu J, Olson T, Lauer S, Gobel U, Perlman EJ. Multipoint imprinting analysis indicates a common precursor cell for gonadal and nongonadal pediatric germ cell tumors. Cancer Res 2001; 61:7268-76.

15. Kotaja N, Sassone-Corsi P. The chromatoid body: a germ-cell-specific RNA-processing centre. Nat Rev Mol Cell Biol 2007; 8:85-90.

16. Tanaka SS, Toyooka Y, Akasu R, Katoh-Fukui Y, Nakahara Y, Suzuki R, Yokoyama M, Noce T. The mouse homolog of Drosophila Vasa is required for the development of male germ cells. Genes Dev 2000; 14:841-53.

17. Fujiwara Y, Komiya T, Kawabata H, Sato M, Fujimoto H, Furusawa M, Noce T. Isolation of a DEAD-family protein gene that encodes a murine homolog of Drosophila vasa and its specific expression in germ cell lineage. Proc Natl Acad Sci USA 1994; 91:12258-62.

18. Hashimoto Y, Maegawa S, Nagai T, Yamaha E, Suzuki H, Yasuda K, Inoue K. Localized maternal factors are required for zebrafish germ cell formation. Dev Biol 2004; 268:152-61.

19. Vasudevan S, Seli E, Steitz JA. Metazoan oocyte and early embryo development program: a progression through translation regulatory cascades. Genes Dev 2006; 20:138-46.

20. Yoon C, Kawakami K, Hopkins N. Zebrafish vasa homologue RNA is localized to the cleavage planes of 2- and 4-cell-stage embryos and is expressed in the primordial germ cells. Development 1997; 124:3157-65.

21. Koprunner M, Thisse C, Thisse B, Raz E. A zebrafish nanos-related gene is essential for the development of primordial germ cells. Genes Dev 2001; 15:2877-85.

22. Kotaja N, Bhattacharyya SN, Jaskiewicz L, Kimmins S, Parvinen M, Filipowicz W, Sassone-Corsi P. The chromatoid body of male germ cells: similarity with processing bodies and presence of Dicer and microRNA pathway components. Proc Natl Acad Sci USA 2006; 103:2647-52.

23. Blackwell TK. Germ cells: finding programs of mass repression. Curr Biol 2004; 14:229-30.

24. Mishima Y, Giraldez AJ, Takeda Y, Fujiwara T, Sakamoto H, Schier AF, Inoue K. Differential regulation of germline mRNAs in soma and germ cells by zebrafish miR-430. Curr Biol 2006; 16:2135-42.

25. Liu J, Valencia-Sanchez MA, Hannon GJ, Parker R. MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol 2005; 7:719-23.

26. Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, Cougot N, Basyuk E, Bertrand E, Filipowicz W. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science 2005; 309:1573-6.

27. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993; 75:843-54.

28. Pillai RS, Bhattacharyya SN, Filipowicz W. Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol 2007; 17:118-26.

29. Aravin A, Tuschl T. Identification and characterization of small RNAs involved in RNA silencing. FEBS Lett 2005; 579:5830-40.

30. Lewis BP, Burge CB, Bartel DP.

Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 2005; 120:15-20.

31. Lagos Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. Curr Biol 2002; 12:735-9.

32. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, Bartel DP, Linsley PS, Johnson JM. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 2005; 433:769-73.

33. Wienholds E, Kloosterman WP, Miska E, Alvarez-Saavedra E, Berezikov E, de Bruijn E, Horvitz HR, Kauppinen S, Plasterk RH. MicroRNA expression in zebrafish embryonic development. Science 2005; 309:310-1.

34. Kloosterman WP, Plasterk RH. The diverse functions of microRNAs in animal development and disease. Dev Cell 2006; 11:441-50.

35. Voorhoeve PM, Agami R. Classifying microRNAs in cancer: the good, the bad and the ugly. Biochim Biophys Acta 2007; 1775:274-82.

36. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Radmark O, Kim S, Kim VN. The nuclear RNase III Drosha initiates microRNA processing. Nature 2003; 425:415-9.

37. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell 2005; 123:631-40.

38. Maniataki E, Mourelatos Z. A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes Dev 2005; 19:2979-90.

39. Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R, Pasquinelli AE. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell 2005; 122:553-63.

40. Chendrimada TP, Finn KJ, Ji X, Baillat D, Gregory RI, Liebhaber SA, Pasquinelli AE, Shiekhattar R. MicroRNA silencing through RISC recruitment of eIF6. Nature 2007; 447:823-8.

41. Kiriakidou M, Tan GS, Lamprinaki S, De Planell-Saguer M, Nelson PT, Mourelatos Z. An mRNA m7G cap binding-like motif within human Ago2 represses translation. Cell 2007; 129:1141-51.

42. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008; 9:102-14.

43. Forstemann K, Tomari Y, Du T, Vagin VV, Denli AM, Bratu DP, Klattenhoff C, Theurkauf WE, Zamore PD. Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. PLoS Biol 2005; 3:236.

44. Hatfield SD, Shcherbata HR, Fischer KA, Nakahara K, Carthew RW, Ruohola-Baker H. Stem cell division is regulated by the microRNA pathway. Nature 2005; 435:974-8.

45. Knight SW, Bass BL. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. Science 2001; 293:2269-71.

46. Murchison EP, Stein P, Xuan Z, Pan H, Zhang MQ, Schultz RM, Hannon GJ. Critical roles for Dicer in the female germline. Genes Dev 2007; 21:682-93.

47. Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, Inoue K, Enright AJ, Schier AF. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science2006; 312:75-9.

48. Voorhoeve PM, le Sage C, Schrier M, Gillis AJ, Stoop H, Nagel R, Liu YP, van Duijse J, Drost J, Griekspoor A, Zlotorynski E, Yabuta N, De Vita G, Nojima H, Looijenga LH, Agami R. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell 2006; 124:1169-81.

49. Huang Q, Gumireddy K, Schrier M, le Sage C, Nagel R, Nair S, Egan DA, Li A, Huang G, Klein Szanto AJ, Gimotty PA, Katsaros D, Coukos G, Zhang L, Pure E, Agami R. The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis. Nat Cell Biol 2008.

50. Zhu R, Ji Y, Xiao L, Matin A. Testicular germ cell tumor susceptibility genes from the consomic 129.MOLF-Chr19 mouse strain. Mamm Genome 2007.

51. Linger R, Dudakia D, Huddart R, Tucker K, Friedlander M, Phillips KA, Hogg D, Jewett MA, Lohynska R, Daugaard G, Richard S, Chompret A, Stoppa-Lyonnet D, Bonaiti-Pellie C, Heidenreich A, Albers P, Olah E, Geczi L, Bodrogi I, Daly PA, Guilford P, Fossa SD, Heimdal K, Tjulandin SA, Liubchenko L, Stoll H, Weber W, Einhorn L, McMaster M, Korde L, Greene MH, Nathanson KL, Cortessis V, Easton DF, Bishop DT, Stratton MR, Rapley EA. Analysis of the DND1 gene in men with sporadic and familial testicular germ cell tumors. Genes Chromosomes Cancer 2008; 47:247-52.

52. Klattenhoff C, Theurkauf W. Biogenesis and germline functions of piRNAs. Development 2008; 135:3-9.

53. Houwing S, Kamminga LM, Berezikov E, Cronembold D, Girard A, van den Elst H, Filippov DV, Blaser H, Raz E, Moens CB, Plasterk RH, Hannon GJ, Draper BW, Ketting RF. A role for Piwi and piRNAs in germ cell maintenance and transposon silencing in Zebrafish. Cell 2007; 129:69-82.

54. Vagin VV, Sigova A, Li C, Seitz H, Gvozdev V, Zamore PD. A distinct small RNA pathway silences selfish genetic elements in the germline. Science 2006; 313:320-4.

55. Lin H, Spradling AC. A novel group of pumilio mutations affects the asymmetric division of germline stem cells in the Drosophila ovary. Development 1997; 124:2463-76.

56. Aravin A, Gaidatzis D, Pfeffer S, Lagos-Quintana M, Landgraf P, Iovino N, Morris P, Brownstein MJ, Kuramochi-Miyagawa S, Nakano T, Chien M, Russo JJ, Ju J, Sheridan R, Sander C, Zavolan M, Tuschl T. A novel class of small RNAs bind to MILI protein in mouse testes. Nature 2006; 442:203-7.

57. Girard A, Sachidanandam R, Hannon GJ, Carmell MA. A germline-specific class of small RNAs binds mammalian Piwi proteins. Nature 2006; 442:199-202.

58. Grivna ST, Beyret E, Wang Z, Lin H. A novel class of small RNAs in mouse spermatogenic cells. Genes Dev 2006; 20:1709-14.

59. Grivna ST, Pyhtila B, Lin H. MIWI associates with translational machinery and PIWIinteracting RNAs (piRNAs) in regulating spermatogenesis. Proc Natl Acad Sci USA 2006; 103:13415-20.

60. Lau NC, Seto AG, Kim J, Kuramochi-Miyagawa S, Nakano T, Bartel DP, Kingston RE. Characterization of the piRNA complex from rat testes. Science 2006; 313:363-7.

61. Watanabe T, Takeda A, Tsukiyama T, Mise K, Okuno T, Sasaki H, Minami N, Imai H. Identification and characterization of two novel classes of small RNAs in the mouse germline: retrotransposon-derived siRNAs in oocytes and germline small RNAs in testes. Genes Dev 2006; 20:1732-43.

62. Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, Hannon GJ. Discrete small RNA-generating loci as master regulators of transposon activity in Drosophila. Cell 2007; 128:1089-103.

63. Gunawardane LS, Saito K, Nishida KM, Miyoshi K, Kawamura Y, Nagami T, Siomi H, Siomi MC. A slicer-mediated mechanism for repeat-associated siRNA 5’ end formation in Drosophila. Science 2007; 315:1587-90.

64. Carmell MA, Girard A, van de Kant HJ, Bourc’his D, Bestor TH, de Rooij DG, Hannon GJ. MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. Dev Cell 2007; 12:503-14.

65. Chen Y, Pane A, Schupbach T. Cutoff and aubergine mutations result in retrotransposon upregulation and checkpoint activation in Drosophila. Curr Biol 2007; 17:637-42.

66. Klattenhoff C, Bratu DP, McGinnis-Schultz N, Koppetsch BS, Cook HA, Theurkauf WE. Drosophila rasiRNA pathway mutations disrupt embryonic axis specification through activation of an ATR/Chk2 DNA damage response. Dev Cell 2007; 12:45-55.

67. Kedde M, le Sage C, Duursma A, Zlotorynski E, van Leeuwen B, Nijkamp W, Beijersbergen R, Agami R. Telomerase-independent regulation of ATR by human telomerase RNA. J Biol Chem 2006; 281:40503-14.

68. Giraldez AJ, Cinalli RM, Glasner ME, Enright AJ, Thomson JM, Baskerville S, Hammond SM, Bartel DP, Schier AF. MicroRNAs regulate brain morphogenesis in zebrafish. Science 2005; 308:833-8.

69. Ashraf SI, McLoon AL, Sclarsic SM, Kunes S. Synaptic protein synthesis associated with memory is regulated by the RISC pathway in Drosophila. Cell 2006; 124:191-205.

70. Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, Greenberg ME. A brain-specific microRNA regulates dendritic spine development. Nature 2006; 439:283-9.

71. Huang J, Liang Z, Yang B, Tian H, Ma J, Zhang H. Derepression of microRNA-mediated protein translation inhibition by apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) and its family members. J Biol Chem 2007; 282:33632-40.

72. Bhattacharya C, Aggarwal S, Zhu R, Kumar M, Zhao M, Meistrich ML, Matin A. The mouse dead-end gene isoform alpha is necessary for germ cell and embryonic viability. Biochem Biophys Res Commun

2007; 355:194-9.

73. Obernosterer G, Leuschner PJ, Alenius M, Martinez J. Post-transcriptional regulation of microRNA expression. Rna 2006; 12:1161-7.

74. Thomson JM, Newman M, Parker JS, Morin-Kensicki EM, Wright T, Hammond SM. Extensive post-transcriptional regulation of microRNAs and its implications for cancer. Genes Dev 2006; 20:2202-7.

75. Kawahara Y, Zinshteyn B, Sethupathy P, Iizasa H, Hatzigeorgiou AG, Nishikura K. Redirection of silencing targets by adenosine-to-inosine editing of miRNAs. Science 2007; 315:1137-40.

76. Knight SW, Bass BL. The role of RNA editing by ADARs in RNAi. Mol Cell 2002; 10:809-17.

77. Yang W, Chendrimada TP, Wang Q, Higuchi M, Seeburg PH, Shiekhattar R, Nishikura K. Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. Nat Struct Mol Biol 2006; 13:13-21.

78. Fazi F, Racanicchi S, Zardo G, Starnes LM, Mancini M, Travaglini L, Diverio D, Ammatuna E, Cimino G, Lo-Coco F, Grignani F, Nervi C. Epigenetic silencing of the myelopoiesis regulator microRNA-223 by the AML1/ETO oncoprotein. Cancer Cell 2007; 12:457-66.

79. O'Hara A, Vahrson W, Dittmer DP. Gene alteration, pre- and mature micro RNA changes contribute to the miRNA signature of Primary Effusion Lymphoma (PEL). Blood 2007.

80. Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can upregulate translation. Science 2007; 318:1931-4.

Chapter 3:

RNA-Binding Protein Dnd1 Inhibits MicroRNA Access to Target mRNA

Cell; 131 (7):1273-86, Dec 28 2007

DOI 10.1016/j.cell.2007.11.034

RNA-Binding Protein Dnd1 Inhibits MicroRNA Access to Target mRNA

Martijn Kedde,[1] Markus J. Strasser,[2] Bijan Boldajipour,[2] Joachim A.F. Oude Vrielink,[1] Krasimir Slanchev,[2,5] Carlos le Sage,[1] Remco Nagel,[1] P. Mathijs Voorhoeve,[1] Josyanne van Duijse,[1] Ulf Andersson Ørom,[3] Anders H. Lund,[3] Anastassis Perrakis,[4] Erez Raz,[2,*] and Reuven Agami[1,*]
[1]The Netherlands Cancer Institute, Division of Tumor Biology, Plesmanlaan 121, 1066CX, Amsterdam, The Netherlands
[2]Max-Planck-Institute for Biophysical Chemistry, Germ Cell Development, Am Fassberg 11, 37070 Goettingen, and Institute for Cell Biology, ZMBE, Center for Molecular Biology of Inflammation, University of Münster, Münster 48149, Germany
[3]Biotech Research and Innovation Centre, University of Copenhagen, Ole Maaløes Vej 5, 2200N, Copenhagen, Denmark
[4]The Netherlands Cancer Institute, Division of Molecular Carcinogenesis, Plesmanlaan 121, 1066CX, Amsterdam, The Netherlands
[5]Present address: Max-Planck-Institute for Immunology, Stuebeweg 51, 79108 Freiburg, Germany.
*Correspondence: erezraz@uni-muenster.de (E.R.), r.agami@nki.nl (R.A.)
DOI 10.1016/j.cell.2007.11.034

SUMMARY

MicroRNAs (miRNAs) are inhibitors of gene expression capable of controlling processes in normal development and cancer. In mammals, miRNAs use a seed sequence of 6–8 nucleotides (nt) to associate with 3′ untranslated regions (3′UTRs) of mRNAs and inhibit their expression. Intriguingly, occasionally not only the miRNA-targeting site but also sequences in its vicinity are highly conserved throughout evolution. We therefore hypothesized that conserved regions in mRNAs may serve as docking platforms for modulators of miRNA activity. Here we demonstrate that the expression of dead end 1 (Dnd1), an evolutionary conserved RNA-binding protein (RBP), counteracts the function of several miRNAs in human cells and in primordial germ cells of zebrafish by binding mRNAs and prohibiting miRNAs from associating with their target sites. These effects of Dnd1 are mediated through uridine-rich regions present in the miRNA-targeted mRNAs. Thus, our data unravel a novel role of Dnd1 in protecting certain mRNAs from miRNA-mediated repression.

INTRODUCTION

MicroRNAs (miRNAs) constitute a family of ∼22 nucleotide (nt) RNAs widely expressed in metazoans (Lee et al., 1993; Pillai et al., 2007). These regulators of gene expression are capable of defining and altering cell fate. Recent estimations suggest the existence of 500–1000 miRNAs per genome and that a large proportion of human protein-coding genes are under the regulation of one or more miRNAs (Aravin and Tuschl, 2005; Lewis et al., 2005). Evidence suggests that miRNAs participate in the regulation of a large variety of cellular processes and that 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 (Kloosterman and Plasterk, 2006; Pillai et al., 2007; Voorhoeve and Agami, 2006).

Most miRNAs are transcribed by RNA polymerase II as long RNAs that are converted to ∼70-nt-long pre-miRNAs by Drosha (Lee et al., 2003). 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). Repressed mRNAs, miRNAs, and most proteins constituting the miRNA-RISC complex (miRNP) are enriched in cytoplasmic processing bodies called P bodies. P bodies are known to be sites of translational repression and mRNA decay. They are enriched in factors involved in inhibition of translation and lack ribosomes and translation-initiation factors (Liu et al., 2005; Pillai et al., 2005).

Importantly, it is becoming increasingly clear that during their biogenesis, the activity of miRNAs is subjected to intense regulation. For example, the nuclear processing of certain miRNAs (e.g., let-7 in ES cells) can be repressed by a yet unknown mechanism (Thomson et al., 2006). Furthermore, certain miRNAs are modified by RNA editing, a process that can lead to changes in miRNA stability or alter their target selection (Kawahara et al., 2007; Knight and Bass, 2002; Yang et al., 2006). Additionally, two recent reports indicated that miRNA-mediated repression in neurons is reversible. Treatment of cultured rat neurons with brain-derived neurotrophic factor leads to partial relief of Limk1 mRNA repression by miRNA-134 (Schratt

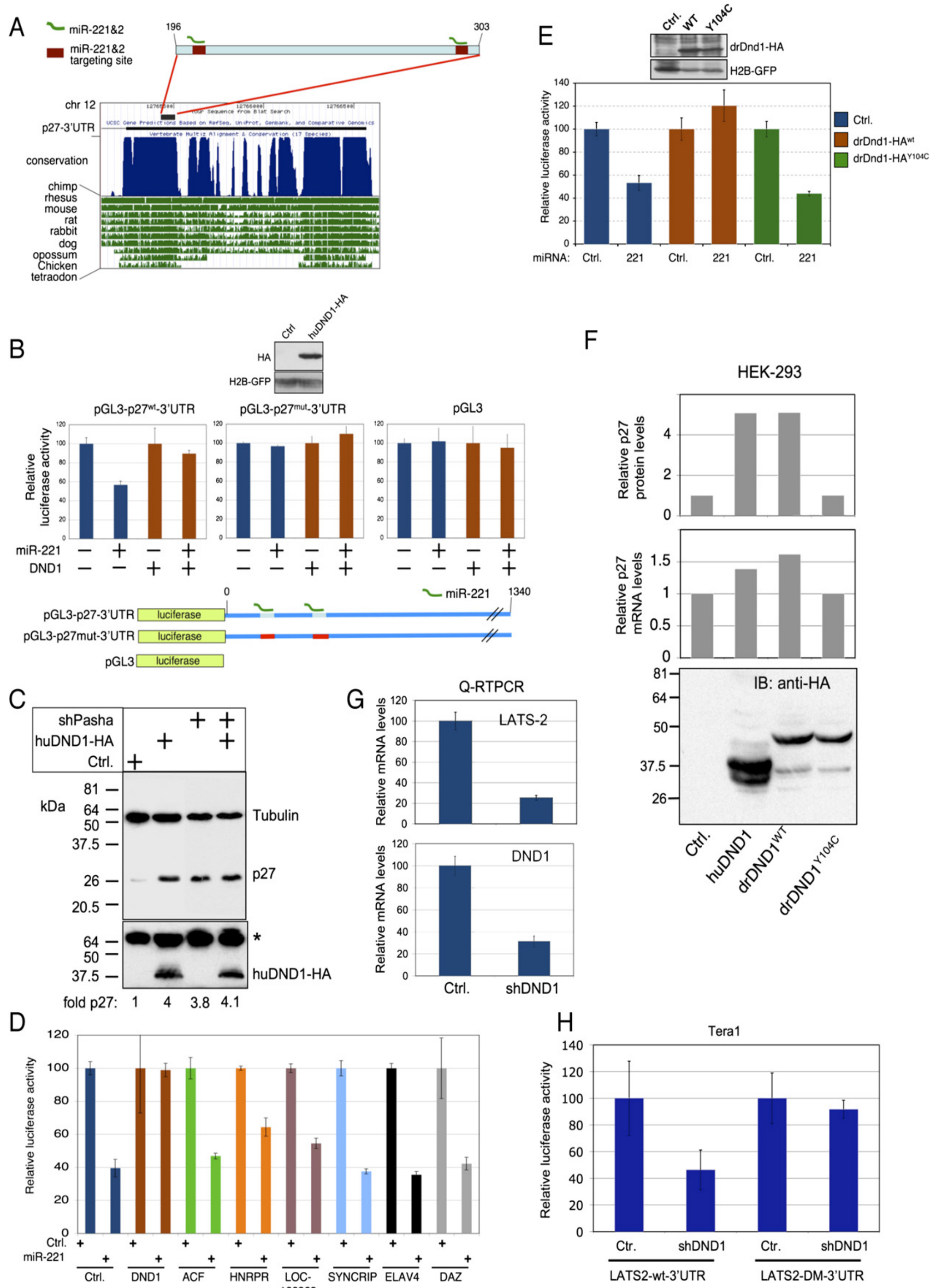


Figure 1. Dnd1 Counteracts the Inhibition of p27 Expression by miR-221

(A) Conservation analysis of p27-3′UTR from human to fish (from Kent et al. [2002]). The positions of the two target sequences of miR-221 are marked.

et al., 2006). This process possibly involves the mTOR pathway, although the mechanism of miRNA derepression remains unknown. Second, external stimulation of *Drosophila* olfactory neurons induces degradation of the Armitage protein, which is required for miRNP assembly (Ashraf et al., 2006). As a result, the translation of the protein kinase CaMKII mRNA, which is controlled by miRNAs, is enhanced. Last, the repression of CAT-1 mRNA by miR-122 in hepatocarcinoma cells is relieved in stress conditions by binding of HuR, an AU-rich element-binding protein, to a region at the end of the 3′UTR of CAT-1 (Bhattacharyya et al., 2006).

In addition, evidence in zebrafish suggests that, a subset of miR-430 targets is protected from repression in germ cells (Mishima et al., 2006). In particular, miR-430-mediated repression of nanos1 and TDRD7 is not effective in germ cells. As specific regions in the 3′UTR of these genes counteract their repression in germline cells, it is reasonable to assume that germ cell-specific factors bind and counteract miR-430 function in these cells. Germ cells are known to contain structures referred to as germ plasm that resemble P bodies; these contain an array of RNA-binding proteins (RBPs) giving these cells unique control over translation and the stability of mRNA and proteins (Kotaja et al., 2006; Kotaja and Sassone-Corsi, 2007). Together, these observations indicate that the miRNA pathway can be controlled at different levels, from stability, processing, sequence identity, and binding to target mRNAs. Here we show that the RBP dead end 1 (Dnd1) prohibits miRNA-dependent inhibition of gene expression in human cells and in zebrafish primordial germ cells.

RESULTS

Dnd1 Relieves miRNA Repression in Human Germline Cells

Using functional genetic-screening approaches, we have recently identified the miRNA-mRNA interactions between p27 and miR-221 and between LATS2 and miR-372 as promoting cancer (le Sage et al., 2007; Voorhoeve et al., 2006). Both p27 and LATS2 harbor at their 3′UTR two nearby evolutionary conserved target sequences for miRNA-221 or -372, respectively, that are required and sufficient for miRNA function (Figures 1A, S1A, and S1B). Interestingly, in both cases the conservation was not restricted to the miRNA-targeting sequences, but rather included the whole region in between the two miRNA-targeting sites. This observation suggests that other factors (proteins or RNA) could associate with these regions to influence miRNA/mRNA function and/or interaction. To investigate the possibility that RBPs influence the activity of the miRNA pathway, we examined the effect of expression of several RBPs on miR-221-mediated repression of p27. We utilized a reporter assay with the wild-type 3′UTR of p27 coupled to luciferase and measured miRNA-induced repression. We found that human dead end 1 (Dnd1), a protein whose function is required for germ cell survival and migration in zebrafish (Weidinger et al., 2003), affects miRNA activity. Specifically, cotransfection of Dnd1 diminished miR-221-mediated inhibition of luciferase-p27-3′UTR expression (Figure 1B). Dnd1 had no effect on expression from either a construct encoding luciferase-p27 3′UTR that is mutated at the two miR-221 sites or on an empty luciferase reporter vector, suggesting that the increase in luciferase expression was not caused by a general effect on transcription or translation efficiency but rather was specific to miRNA-repressed translation. Similar results were obtained with LATS2/miR-372 and connexin-43/miR-1 and -206 (Figure S1C). To further test whether Dnd1 activity depends on miRNA function we inactivated miRNA synthesis with an shRNA vector targeting Pasha, a component specific to the miRNA pathway (Gregory et al., 2004). Figure S1D demonstrates the inhibitory effect of shPasha on miR-221 processing. In HEK293 cells, which endogenously express p27 and miR-221 (Figure 2C), introduction of Dnd1 or inhibition of Pasha, elevated endogenous p27 protein levels to a comparable extent (~4-fold, Figure 1C). Interestingly, cointroduction of Dnd1 and shPasha did not cause further elevation in p27 levels, further supporting the idea that Dnd1 activity depends on miRNA function.

Dnd1 contains a conserved RNA-binding domain that bears high similarity to that of apobec complementation factor (ACF), LOC166863, Syncrip, hnRNPR, ELAV4, and DAZ. To test whether the effect of Dnd1 on miRNA activity is specific and mediated through its RNA-binding domain, we used expression plasmids of Dnd1 homologs and related genes and found neither to significantly inhibit

(B) Expression vectors for miR-221 and human Dnd1 (huDnd1) were cotransfected with the indicated luciferase constructs. Relative luciferase activity is the ratio between *firefly* luciferase and *renilla* control luciferase, adjusted to 100%. An immunostaining with anti-HA antibody demonstrates the expression of huDnd1 while H2B-GFP was used to control transfection efficiency. The results are represented as means and SD from three independent experiments.

(C) HEK293T cells were transfected with the indicated constructs and whole-cell lysates were immunostained with anti-Tubulin, p27, and HA antibodies. p27 protein level was analyzed using Tina 2.0 software (Raytest, Sheffield, UK).

(D and E) Similar to (B), only that several RBPs, as well as the zebrafish Dnd1 homolog (drDnd1) and a mutant in the RNA-binding domain ($drDnd1^{Y104C}$), were cotransfected together with pGL3-p27-3′UTR and *renilla* luciferase control.

(F) HEK293T cells were transfected with the indicated constructs and subjected to RPA with probes to detect p27 mRNA and control cyclophilin and to immunoblot analysis using p27 and control Tubulin antibodies. Quantification of protein levels was performed using Tina 2.0 software (Raytest; Sheffield, UK).

(G) Tera1 cells were transfected with shDnd1 and subjected to quantitative RT-PCR analysis for LATS2, Dnd1, and GAPDH control. The results are represented as means and SD from three independent experiments.

(H) Similar to (B), Tera1 cells were transfected with the indicated constructs.

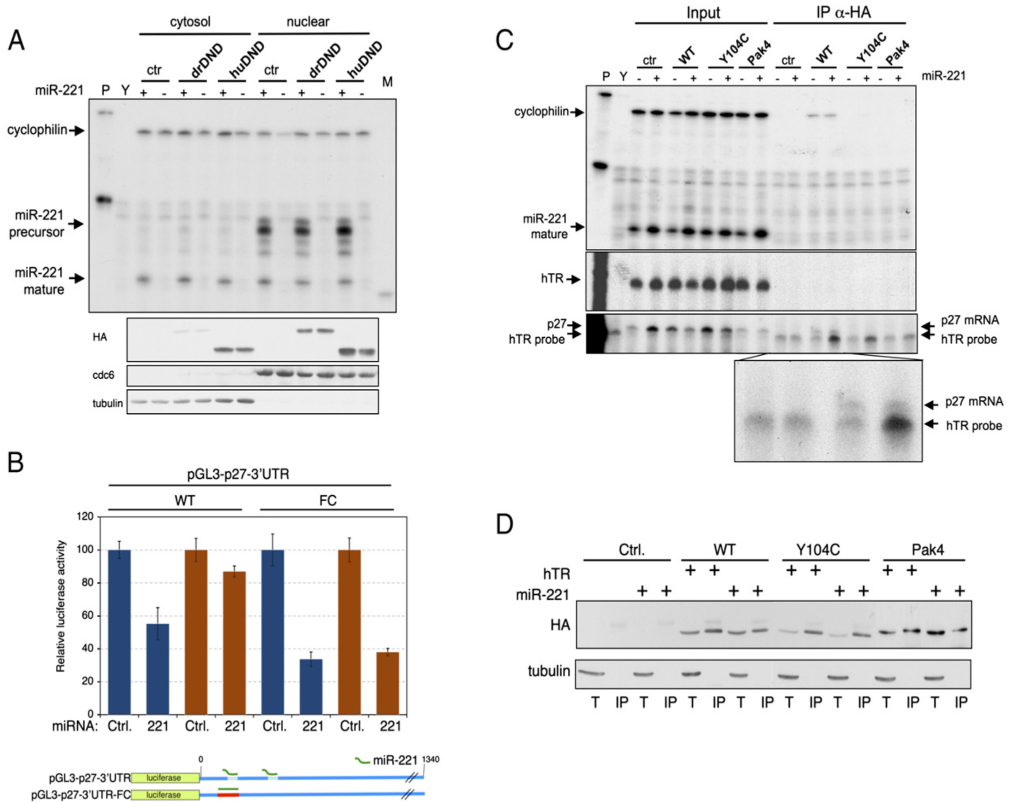


Figure 2. Dnd1 Preferentially Associates with mRNAs

(A) MCF-7 cells were transfected with the indicated constructs and subjected to subcellular fractionation to separate nuclear from cytoplasmic material, and both protein and RNA were extracted. RPA was performed with probes to detect miR-221 and cyclophilin. Immunoblot analysis was performed with HA antibodies to demonstrate expression of Dnd1 and to detect Cdc6 and Tubulin, to test the purity of fractionation. P is probes alone, Y is yeast total RNA.

(B) Expression vectors for miR-221 and human Dnd1 (huDnd1) were cotransfected with the indicated luciferase constructs and proceeded as in Figure 1B. Blue and brown bars represent control and Dnd1 vectors, respectively.

(C) HEK293 cells were cotransfected with the indicated expression constructs and subjected to IP with anti-HA antibodies. RNA was extracted from 80% of each IP and 5% from each input and subjected to RPA analysis to detect p27 and control cyclophilin mRNAs, human telomerase RNA (hTR), and miR-221. Below, a blow up of p27 signal in control and wild-type IP samples is shown for clarity.

(D) The same amount of extracts from input and IP (marked T and IP, respectively) was used for immunoblot analysis with anti-HA and control anti-Tubulin antibodies.

miR-221-mediated suppression of luciferase-p27 3′UTR (Figure 1D, expression controls shown in Figure S1E). In contrast, repression of miR-221 activity was observed also when the zebrafish and mouse homologs of Dnd1 were introduced (Figure 1E and data not shown). Interestingly, transfection of a zebrafish Dnd1 mutated at a single conserved residue within its RNA-binding domain (Y104C, a mutant that cannot rescue loss of germ cells when Dnd1 is depleted in zebrafish embryos; K.S and E.R., unpublished data) hampered Dnd1's ability to inhibit miR-221 function (Figure 1E). Also substituting the RNA-binding domain of Dnd1 for that of ACF resulted in loss of Dnd1 function (Figure S1F). Collectively, these results suggest that inhibiting miRNA function is specific to Dnd1 and indicate that for counteracting miRNA activity, Dnd1 requires an intact RNA-binding domain.

Suppression of gene expression by miRNAs is exerted by translational inhibition that occasionally is associated with mRNA decay (Bagga et al., 2005; Lim et al., 2005; Pillai et al., 2005). To test the possible effect of Dnd1 on these pathways, we examined the endogenous p27 RNA and protein levels in HEK293 cells (expressing endogenous p27 and miR-221and -222, Figure 2C). Figure 1F shows that transfection of either human or wild-type zebrafish Dnd1, but not the Y104C mutant, caused elevation in p27 mRNA (up to 1.5-fold) and protein (up to 5-fold), indicating that Dnd1 can potently block endogenous miR-221 and -222-mediated mRNA decay and translation inhibition.

In all our experiments so far we examined the function of human Dnd1 by ectopic introduction. To study endogenous Dnd1 function, we first tested by quantitative RT-PCR several human cancer cell lines for the expression of Dnd1 and found the teratoma Tera1 cell line to be positive. Interestingly, Tera1 cells express high levels of the miR-372 family, which we have previously shown to target LATS2 through two sites at its 3′UTR (Figure S1B). We therefore examined endogenous LATS2 expression in Tera1 cells following suppression of Dnd1 expression by an effective shRNA vector (Figure S1G). As Dnd1 affected both miRNA-mediated translation inhibition and mRNA stability (Figure 1F), we used quantitative RT-PCR and found a marked reduction in LATS2 mRNA levels associated with the inhibition of endogenous Dnd1 expression (Figure 1G), suggesting that Dnd1 protects LATS2 expression. To directly measure the effect of endogenous Dnd1 on the activity of endogenous miR-372 family, we used sensor molecules containing the luciferase gene under the control of either wild-type LATS2-3′UTR or a mutant in the 372 target sites (le Sage et al., 2007; Voorhoeve et al., 2006). Figure 1H shows that transfection of shDnd1, but not a control vector, reduced the expression of a cotransfected luciferase LATS2 3′UTR reporter gene only when the miR-372 targeting sites were present. Altogether, these results indicate that endogenous Dnd1 in Tera1 cells protects the expression of endogenous LATS2 from being targeted by the miR-372 family.

Preferential Association of Dnd1 with mRNAs

Since Dnd1 requires an intact RNA-binding domain to counteract miRNA function, it is conceivable that Dnd1 interferes with either the expression, the processing to mature miRNA, or the subcellular localization of miR-221. To examine the effects of Dnd1 on miR-221 biogenesis, MCF-7 cells were cotransfected with vectors encoding either human or zebrafish Dnd1 and miR-221 or control miRNA, and subsequently subjected to subcellular fractionation, RNA isolation, and to an RNase protection assay (RPA) to detect cyclophilin control RNA and miR-221 precursor and mature forms. As shown in Figure 2A, the expression level, efficiency of processing, or subcellular localization of mature miR-221 were not altered by Dnd1. Western blot analysis with anti-HA confirmed the expression of human and zebrafish proteins, whereas nuclear Cdc6 and cytoplasmic Tubulin verified cellular fractionation (Figure 2A, bottom).

An alternative explanation that could account for Dnd1 activity is that the protein binds miRNAs and inhibits their function. To test this option we converted one miR-221-targeting site in p27 3′UTR to an RNAi site (named FC, a *f*ull *c*omplementary region to miR-221) and mutated the seed of the second site (Figure 2B). In this way, p27-3′UTR FC was solely subjected to RNAi-mediated degradation by miR-221. In cotransfection assays, the presence of Dnd1 did not reduce miR-221 activity toward p27-3′UTR FC, suggesting that Dnd1 was unable to counteract the RNAi pathway. These results are in line with the assumption that Dnd1 counteracts miR-221 activity either at the level of RISC-incorporated-mature miR-221 or downstream, rather than by steric hindrance of miR-221.

Next, we checked whether Dnd1 interacts with mature miR-221 or with p27 3′UTR. We transfected HEK293 cells with HA-tagged Dnd1, Dnd1^{Y104C}, and as controls HA-tagged PAK4 and empty vector. We also cotransfected an expression vector for miR-221 to enhance the possible association with Dnd1. Subsequently, we subjected whole-cell extracts to immunoprecipitation (IP) using anti-HA antibodies, extracted RNA, and performed RPAs to detect the interacting RNAs. This analysis revealed clear binding of Dnd1 to endogenous p27 and cyclophilin mRNAs but not to hTR, a nontranslated-RNA coding for human telomerase RNA (Figure 2C) (Kedde et al., 2006). The interaction of Dnd1 with p27 and cyclophilin required its intact RNA-binding capacity, as no apparent interaction was observed with the Dnd1^{Y104C} mutant. Furthermore, no specific association was detected with miR-221, even when it was cotransfected with Dnd1. Immunoblot analysis with HA antibody confirmed the expression and equal immunoprecipitation of Dnd1 and PAK4 (Figure 2D). Altogether, these results demonstrate that Dnd1 binds mRNAs but not miRNAs to block miRNA activity.

Dnd1 Alleviates miR-430 Repression of Nanos1 and TDRD7 in Primordial Germ Cells of Zebrafish

We next examined the function of Dnd1 in zebrafish to question whether the relief of mRNA-mediated repression described above is part of the in vivo function of Dnd1 in the context of a developing organism. Whereas Dnd1 has been shown to be essential for germline development in zebrafish and mouse, the actual molecular mechanisms by which it exerts its function are unknown (Weidinger et al., 2003; Youngren et al., 2005). We followed the expression changes of three germline specific genes: nanos1, TDRD7, and Vasa. The specific expression of nanos1 and TDRD7 in the PGCs is considered to be the result of miR-430-dependent inhibition in somatic cells, while Vasa regulation is miR-430 independent (Mishima et al., 2006). Indeed, mutating the single miR-430 site in the 3′UTR of either nanos1 or TDRD7 results in ubiquitous expression. However, while PGCs allow nanos1 and TDRD7 expression, they also express miR-430, suggesting that miR-430-induced repression of these genes is suppressed in PGCs (Mishima et al., 2006).

To study Dnd1 function, we knocked down the translation of the gene in zebrafish embryos using morpholino antisense oligonucleotides. RNA was extracted from these embryos, and the endogenous levels of the germline-specific genes (nanos1, TDRD7, and Vasa) were compared to Odc1, a ubiquitously expressed gene. In line with our hypothesis, the inhibition of endogenous Dnd1 caused a marked reduction in endogenous nanos1 and TDRD7 mRNA levels, but not Vasa (Figures 3A and S2A). Then, we cloned the 3′UTRs of nanos1, TDRD7, and control Vasa downstream of fluorescence marker genes, to determine whether the Dnd1 effect is mediated through their 3′UTR. Similar to the endogenous expression pattern,

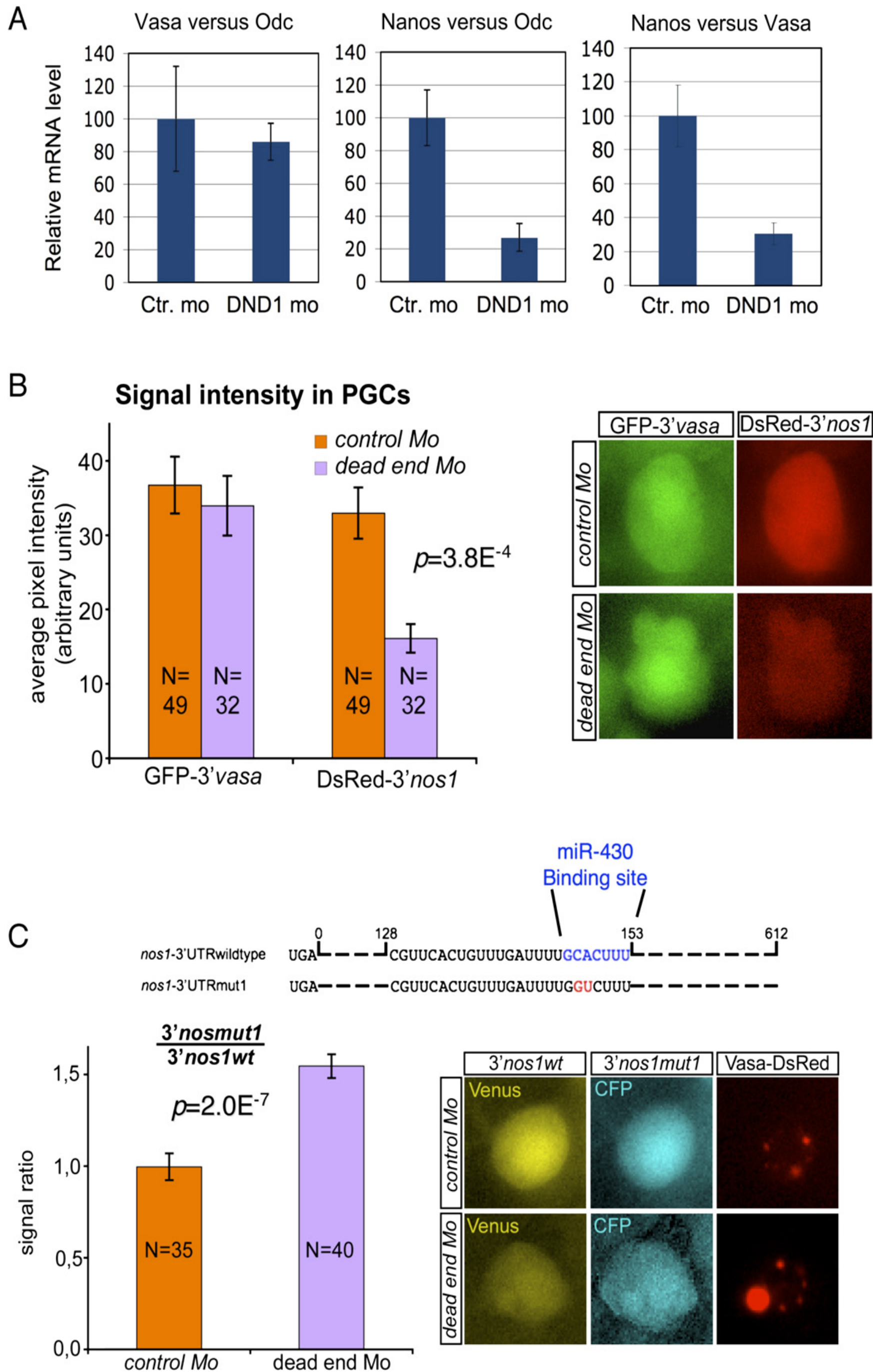


Figure 3. Zebrafish Dnd1 (drDnd1) Counteracts Inhibition of Nanos by miR-430

(A) One-cell-stage zebrafish embryos were injected with Dnd1 morpholino or control morpholino. RNA was extracted and subjected to quantitative RT-PCR analysis to compare endogenous levels of *nanos1* and *vasa* to *odc*, and *nanos* to *vasa*.

(B) One-cell-stage zebrafish embryos were coinjected with *DsRed-nos1*-3′UTR and *gfp-vasa*-3′UTR together with dead end morpholino or control morpholino.

the injection of RNA, encoding a fluorescent marker gene fused to the 3′UTR of *nanos1*, TDRD7, or Vasa, into zebrafish embryos leads to preferential fluorescence in PGCs (Koprunner et al. [2001] and data not shown). Upon Dnd1 knockdown, quantitative pixel-intensity analysis shows a clear and significant reduction in red fluorescent signal (DsRed-nos1 3′UTR or DsRed-TDRD7 3′UTR), but not green (coinjected GFP-vasa 3′UTR) in PGCs (Figures 3B and S2B), indicating that Dnd1 affects gene expression through the 3′UTR of nanos1 and TDRD7. Next, we examined whether the reduction in nanos1 and TDRD7 following loss of Dnd1 depends on the ability of miR-430 to interact with these genes. Both nanos1 and TDRD7 harbor one miR430-targeting site (Figures 3C and S2C). We therefore compared the expression of marker genes fused to a wild-type or a mutated miR-430-targeting site in *nos1* 3′UTR and TDRD7 3′UTR. Quantitative pixel-intensity analysis showed that the reduction in fluorescence resulting from knocking down Dnd1 function was effectively suppressed by mutating the miR-430-targeting site (Figures 3C and S2C). Altogether, our data suggest that Dnd1 functions to relieve miR-430-mediated nanos1 and TDRD7 inhibition in zebrafish PGCs.

U-Rich Regions Mediate Dnd1 Binding and Function

Dnd1 contains two single-strand RNA recognition motifs, spanning approximately residues 60–131 and 140–213. Our data suggest that these domains bind mRNAs at specific sites. A BLASTP search of the Dnd1 sequence against the database of known structures (the PDB) revealed several significant homologs (E values of 10^{-5} and 10^{-3}) and allowed straightforward homology-based modeling using the SWISS-MODEL server (Schwede et al., 2003). Superimposing the two homology-modeled domains in the structure of the similar *Drosophila* sex-lethal protein bound to uridine-rich single-stranded RNA indicated clearly that Dnd1 has all the hallmarks necessary to bind U-rich single-stranded RNA (Handa et al., 1999). We therefore reasoned that Dnd1 might also bind U-rich regions (URRs). Interestingly, two URRs are found in between the two miR-221-binding sites in p27 3′UTR (Figure 4A, marked purple). To test whether the region in between the two miRNA sites is sufficient to confer Dnd1 activity, we cloned it into a luciferase reporter vector (p27 3′UTR$^{(196–300)}$). Figure 4B shows that this minimal region is sufficient to allow full repression by miR-221 and derepression by Dnd1. To more specifically address the role of the URRs, we generated two mutants: mutant 1, in which the two URRs were replaced by non-URRs, and mutant 2, where adjacent sequences were similarly mutated as control. Importantly, mutating the URRs, but not the adjacent sites, conferred resistance to the Dnd1 effect (Figure 4C). No significant change in the repression activity of miR-221 was noted in mutant 2 (data not shown), indicating that these mutations did not affect miR-221-mediated repression of the p27 3′UTR. To further investigate the role of each URR, three more mutants were produced where either URR 1 or 2 were mutated (Figure 4A). Both mutants supported full Dnd1 activity (Figures 4C and 4D), suggesting that Dnd1 requires at least one URR adjacent to the miR-221 sites to function.

Next, we examined whether the URRs serve as docking sites for Dnd1. We immunoprecipitated (IP) HA-tagged Dnd1, Dnd1$^{(Y104C)}$, or control proteins from HEK293 cells and incubated them with RNA purified from MCF-7 cells expressing control vector, wild-type, or mutant 1 luciferase-p27 3′UTR$^{(196–300)}$. As detected by RPA on the bound material, a clear and significant enrichment of luciferase-p27 3′UTR$^{(196–300)}$ was observed when wild-type Dnd1-HA was used for the pull down assay (Figure 4E). In contrast, no significant pull down of luciferase was observed when the URRs of luciferase-p27 3′UTR were mutated (mutant 1) or with Dnd1$^{(Y104C)}$. The specificity of the binding as well as the equal efficiency of pull-down was demonstrated by absence of binding between Dnd1 and endogenous hTR, while equal precipitation of endogenous cyclophilin was seen in all extracts (Figure 4E). Although we cannot rule out that mutations in URRs have effects on the binding of other RBPs, our results suggest that Dnd1 binds URRs and thereby mediates suppression of miRNAs.

To test the in vivo relevance of URRs to Dnd1 function, we mutated URRs found within the zebrafish *nos1* and *TDRD7* 3′UTRs and tested the effect of these mutations on gene expression. Figure 5A shows that mutating one URR (mut3, downstream of miR-430 site) of *nos1* 3′UTR reduced its expression. Similar results were obtained with *TDRD7* 3′UTR (Figure S2D). To test whether the effect of URR mutation was dependent on miRNA function, we introduced the mutation of the URR in the background of the miR-430 target-site mutant. Figure 5B shows that mut3 did not reduce gene expression when miR-430 target site was mutated, suggesting that also in this case Dnd1 effect is miRNA dependent.

Mechanism of Dnd1 Function

Our results point to a model by which Dnd1 positively regulates gene expression by prohibiting miRNA-mediated gene suppression. To test this model we examined the direct interaction of miR-221 with its target p27 in human HEK293 cells, in the presence or absence of human Dnd1. We designed a synthetic RNA duplex mimicking miR-221 where the sense oligo was tagged with a 3′-biotin group for efficient pull-down using streptavidin beads. As control, we used a seed mutated, biotin-tagged, miR-221

(C) One-cell-stage zebrafish embryos were coinjected with RNA containing the *venus* open-reading frame fused to the wild-type *nanos*1 3′UTR (*3′nos1wt*), RNA containing the *cfp* open-reading frame fused to the miR-430-binding site mutated *nanos*1 3′UTR (*cfp-3′nos1mut1*) and vasa-dsRed (for labeling the germinal granule for easier identification of germ cells) together with dead end morpholino or control morpholino. Error bars depict the standard error of the mean (SEM); the p value was calculated using t test.

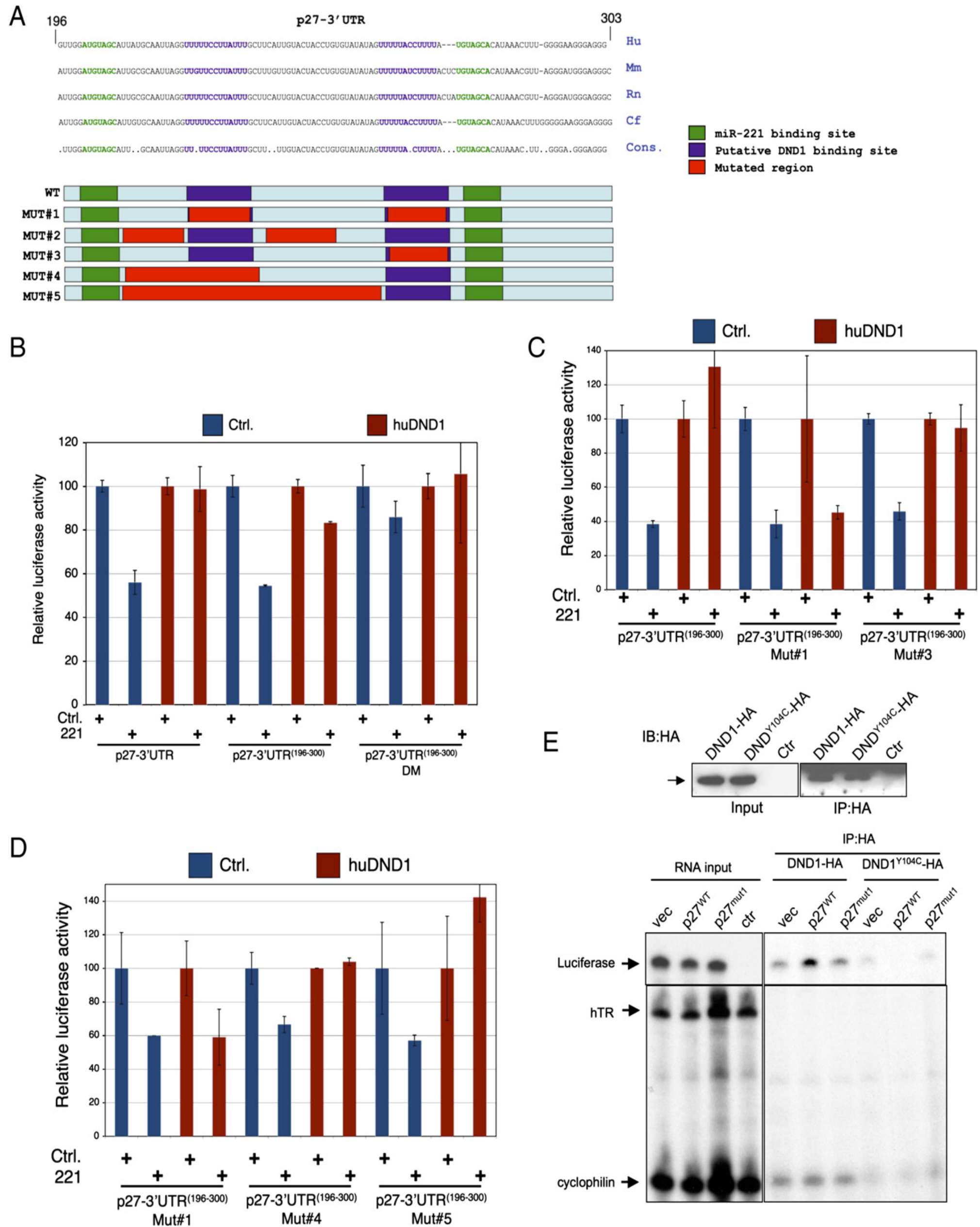


Figure 4. Binding of Dnd1 to URRs Mediates Derepression of p27 Inhibition by miR-221

(A) A diagram aligning the sequence area in p27-3′UTR containing the two miR-221 target regions. In red are regions mutated in the indicated constructs.

(B–D) Luciferase reporter assay were performed as in Figure 1B using the indicated pGL3-p27-3′UTR constructs and the expression vectors for huDnd1 and miR-221. The results are represented as means and SD from three independent experiments.

(E) HEK293 cells were transfected with the indicated Dnd1-HA expressing vectors, and Dnd1 was IPed using anti-HA antibody. Immunoblot analysis shows the expression and IP of Dnd1. MCF-7 cells were transfected with luciferase-p27-3′UTR constructs; RNA was extracted after 48 hr and equally divided between the IPs. Beads were incubated with the RNA for 45 min at 6°C, washed, and subjected to RPA using probes to detect luciferase and cyclophilin mRNAs, and the noncoding human telomerase RNA (hTR).

duplex. The activity of these tagged molecules toward p27 3′UTR was verified in a luciferase assay (Figure S3) and Dnd1 toward p27 in western blot (Figure 6A, left). As HEK293T cells express miR-221, exogenous introduction of miR-221 results in only a slight effect on p27 expression, while introduction of human Dnd1 raises p27 levels (see also Figure 1C). Subsequently, we examined whole-cell extracts and pull-downs from the same cell populations using RPA and detected a specific association between wild-type miR-221 and endogenous p27 mRNA (Figure 6A, middle and right panels). No specific interaction was observed with hTR, our negative control. Most intriguingly, introduction of human Dnd1 completely abolished the interaction of miR-221 with p27 mRNA, indicating that Dnd1 inhibits miR-221 accessibility. Collectively, our results indicate that Dnd1 counteracts miRNA function by binding URRs in 3′UTRs of mRNAs and reducing their affinity to miRNAs (Figure 6B). However, we cannot exclude at this point that additional functions, such as counteracting RISC activity or subcellular sequestration, may contribute to Dnd1 activity.

DISCUSSION

In this study we provide evidence that primordial germ cells possess factors such as Dnd1 that protect the expression of several genes from repression by miRNAs, as exemplified by miR-430. The expression of at least some miR-430 RNA targets, such as *nanos1* and *TDRD7*, is allowed in primordial germ cells in the presence of miR-430. Similarly, the expression of LATS2, a target gene for the miR-372-family, is dependent on the expression of Dnd1 in Tera1, a human cell line derived from a germ-cell tumor that contains high levels of the miR-372 family. Our findings provide an explanation for this phenomenon. We suggest that Dnd1 suppresses miR-430 and miR-372-family function toward several of its mRNA targets by binding to URRs that are located within these mRNAs. Our results pinpoint the mechanism by which Dnd1 exerts its function. Binding of Dnd1 to mRNAs prohibits miRNA interaction.

Dnd1 in Germ-Cell Development

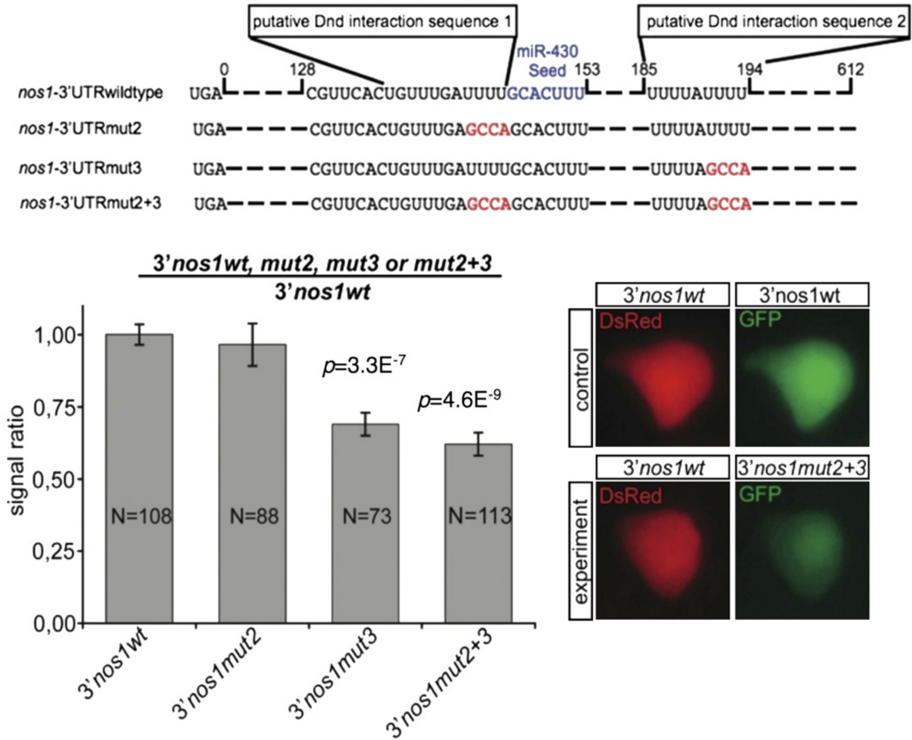
Both in zebrafish and in mouse, Dnd1 is essential for germ-cell survival, whereas in the 129-mouse background it induces testicular germ-cell tumors (TGCTs) arising from the few germ cells that develop in the absence of Dnd1 (Weidinger et al., 2003; Youngren et al., 2005). These tumors resemble human testicular germ-cell tumors, which are the most common cancers affecting young men (Oosterhuis and Looijenga, 2005; Youngren et al., 2005). It remains to be established which mutation(s) from the 129 strain synergize with the Dnd1 mutation to cause the development of TGCTs. However, recently three protein-coding genes, from which two are RBPs and one miRNA, were identified to be candidate disease genes from the 129 strain (Zhu et al., 2007). Additionally, a recent finding in *C. elegans* showed that disruption of the germ plasm by loss of two genes involved in RNA biology in these animals can also lead to the development of similar tumors (Ciosk et al., 2006). Our results demonstrate that loss of Dnd1 enhances miRNA repression of some genes that are essential for primordial germ-cell development (Koprunner et al., 2001) (among them *nanos1* and *TDRD7*) and predict that this mechanism can be responsible for defects in germ-cell survival or for tumor formation. Whether protection of mRNAs from miRNAs is the only function of Dnd1 in germ cells remains to be investigated. Nevertheless, our results show that counteracting or balancing miRNA function is important during development, at least to maintain viable and functional germ lines in zebrafish and mouse.

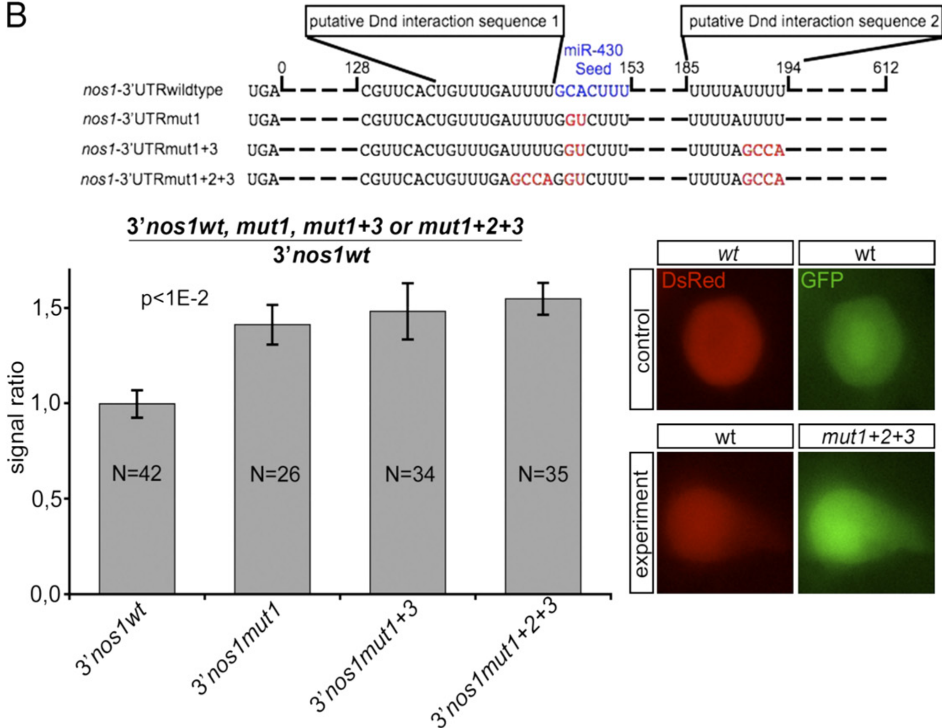
Dnd1 and Other RBPs

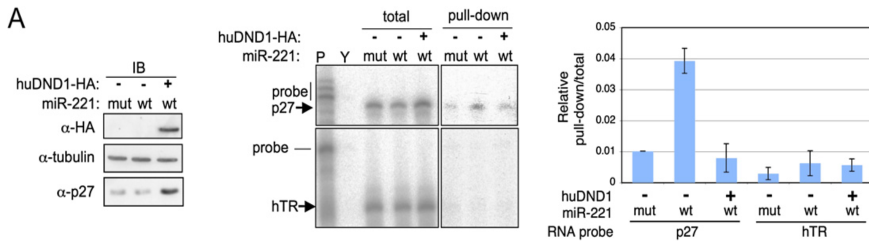
Recent publications have implicated mechanisms that counteract the activity of miRNAs on specific mRNAs (Ashraf et al., 2006; Bhattacharyya et al., 2006; Schratt et al., 2006). Together with these studies, our data unveil a dynamic regulation of miRNA suppression on the 3′UTRs of target mRNAs. The most relevant work showed that HuR (ELAV1), an AU-rich element (ARE) binding protein, relieves CAT-1 mRNA from miR-122-mediated repression, a process that involves binding of HuR to the 3′UTR of CAT-1 mRNA (Bhattacharyya et al., 2006). The mechanism of HuR action is yet unknown. Here, we identified Dnd1, another RBP, whose activity is comparable to HuR. However, our results highlight several differences between Dnd1 and HuR. While Dnd1 depends on URRs to relieve the miRNA repression, HuR depends on AU-rich elements. Second, Dnd1 seems to have a broad effect, as we have strong evidence showing that the repression of p27 by miR-221, LATS2 by miR-372, connexin-43 by miR-1 and -206 (Anderson et al., 2006), and Nanos and TDRD7 by miR-430 in zebrafish PGCs, are all being antagonized by Dnd1; for now HuR seems to have a more restricted function, suppressing CAT-1 expression. Last, the expression of HuR is induced following stress in liver cells, whereas Dnd1 expression is restricted to primordial germ cells and certain neuronal tissues (Youngren et al., 2005). By binding to mRNA, Dnd1 prevents miRNA-mediated repression. Whether this is a general mechanism applicable to HuR, or other RBPs, remains to be seen.

Counteracting miRNAs and siRNAs

Our work shows that Dnd1 activity can counteract gene silencing induced by miRNAs but not by siRNA-mediated RNA interference (RNAi). When one miR-221-targeting site in p27 3′UTR was converted to a full miR-221 complementary sequence (Figure 2B) as well as when a fully complementary shRNA was used to target the 3′UTR of LATS2 (Figure S4), Dnd1 function was lost. Since Dnd1 associates with mRNA and not with miRNAs, we can envision two possibilities that could provide an explanation. (1) Dnd1 binding could change the RNA structure to be unfavorable for miRNA binding by, for example, enforcing


A
putative Dnd interaction sequence 1
putative Dnd interaction sequence 2
miR-430
Seed
0
128
153
185
194
612
nos1-3'UTRwildtype UGA CGUUCACUGUUUGAUUUUGCACUUU UUUUAUUUU
nos1-3'UTRmut2 UGA CGUUCACUGUUUGAGCCAGCACUUU UUUUAUUUU
nos1-3'UTRmut3 UGA CGUUCACUGUUUGAUUUUGCACUUU UUUUAGCCA
nos1-3'UTRmut2+3 UGA CGUUCACUGUUUGAGCCAGCACUUU UUUUAGCCA
3'nos1wt, mut2, mut3 or mut2+3
3'nos1wt
signal ratio
1,00
0,75
0,50
0,25
0,00
p=3.3E-7
p=4.6E-9
N=108
N=88
N=73
N=113
3'nos1wt
3'nos1mut2
3'nos1mut3
3'nos1mut2+3
control
experiment
3'nos1wt
3'nos1wt
3'nos1wt
3'nos1mut2+3
DsRed
GFP
DsRed
GFP


B
putative Dnd interaction sequence 1
putative Dnd interaction sequence 2
miR-430
Seed
0
128
153
185
194
612
nos1-3'UTRwildtype UGA CGUUCACUGUUUGAUUUUGCACUUU UUUUAUUUU
nos1-3'UTRmut1 UGA CGUUCACUGUUUGAUUUUGGUCUUU UUUUAUUUU
nos1-3'UTRmut1+3 UGA CGUUCACUGUUUGAUUUUGGUCUUU UUUUAGCCA
nos1-3'UTRmut1+2+3 UGA CGUUCACUGUUUGAGCCAGGUCUUU UUUUAGCCA
3'nos1wt, mut1, mut1+3 or mut1+2+3
3'nos1wt
signal ratio
1,5
1,0
0,5
0,0
p<1E-2
N=42
N=26
N=34
N=35
3'nos1wt
3'nos1mut1
3'nos1mut1+3
3'nos1mut1+2+3
control
experiment
wt
wt
wt
mut1+2+3
DsRed
GFP



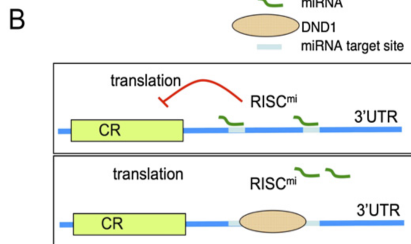


Figure 6. Mechanism of Dnd1 Function

(A) HEK293T cells (endogenously expressing miR-221 and p27) were transfected as indicated. Cells were harvested 48 hr later and subjected to pull-down assay with streptavidin beads. Pull-down and whole-cell extracts were analyzed by immunoblot and RPA. Note, because HEK293T cells endogenously express miR-221, only a moderate reduction in p27 level was observed upon introduction of miR-221 oligos, while Dnd1 expression induced p27 expression. The results are represented as means and SD from three independent experiments.

(B) Schematic model depicting the mechanism of Dnd1 action. The miRNA-RISC loaded with miRNAs targeting a 3′UTR inhibits its translation (upper panel). By binding to URRs in the 3′UTR, Dnd1 prevents miRNAs from binding to and inhibiting translation, thereby prohibiting miRNA function (lower panel). CR is coding region.

a secondary structure that hides the sequence required for miRNA-seed recognition. As siRNAs do not entirely depend on seed sequences and their association with mRNAs is tighter, their binding to 3′UTRs could therefore be less affected. (2) Dnd1 could localize mRNAs to locations in the cell that are not accessible to miRNA-mediated silencing but still mRNA degradation by an RNAi-mediated mechanism is possible.

3′UTRs as Binding Platforms for Regulating miRNAs and RBPs

Our results strongly suggest that the 3′UTRs of at least some mRNAs are binding platforms for both miRNAs that repress translation and RBPs that regulate this repression. In particular, RBPs can restore gene expression in the presence of inhibitory miRNAs. This mode of regulation has several consequences. First, as it affects protein synthesis and mRNA stability rather than controlling the expression of miRNAs in the cells, it is potentially a very rapid mechanism. Second, relief of repression can be exerted simultaneously on only a subset of the miRNA-targeted mRNAs, thereby giving modularity to miRNA function. Third, it adds robustness to expression patterns as different RBPs can in principle relieve the repression of different sets of mRNAs, even if these are regulated by the same miRNA. Fourth, this mechanism allows differential gene regulation in different tissues while keeping the expression of both miRNAs and mRNAs constant in the cell. One implication of this mode of regulation is that the presence of a miRNA and its target in the same tissue does not necessarily result in repression, therefore allowing coexpression of both miRNA and target mRNA. However, how broad Dnd1 function is: how many mRNAs and how many miRNAs are affected by Dnd1 remains to be

Figure 5. URRs Are Required for Dnd1 to Efficiently Repress miR-430-Mediated Nanos Inhibition

(A) RNA containing the *DsRed* open-reading frame fused to the wild-type *nanos*1 3′UTR (*3′nos1wt*) was coinjected into one-cell-stage zebrafish embryos together with RNA containing the *gfp* open-reading frame fused to different versions of the *nanos*1 3′UTR. The different *nanos*1 3′UTRs that were used are shown above; mutations are marked in red. The ratio between the signal intensity provided by GFP whose open-reading frame was fused to either one of the *nanos* UTRs was divided by that originating from DsRed that was fused to the wild-type *nanos* RNA UTR. Representative single cells are shown in the right panels.

(B) An experimental setting similar to that described in (A) was used to examine the function of *nanos* UTR containing a combination of mutations in the miR-430 and putative Dnd-binding sites. The different *nanos*1 3′UTRs that were used are shown above; mutations are marked in red. The ratio between the signal intensity provided dividing the signal intensity of GFP by that of DsRed whose open-reading frame was fused to the wild-type *nanos* RNA UTR. Representative single cells are shown in the right panels. Error bars depict the standard error of the mean (SEM); p value was calculated using Ttest.

elucidated. Our results indicate that several mRNAs and miRNAs are potentially regulated by Dnd1. Genome-wide proteome and RNome analysis comparing normal cells to cells lacking Dnd1 may address these issues in the future.

EXPERIMENTAL PROCEDURES

Constructs and Antibodies

miR-Vec constructs were described before (Voorhoeve et al., 2006), and Dnd1 open-reading frames were cloned as described (Weidinger et al., 2003) into a pCS2-based CMV expression vector to contain a double carboxy-terminal HA tag. Dnd1 homologs ACF (IOH52413.1), HNRPR (IRALp962F134.1), LOC-166863 (IRAKp961 H0534.1), and SYNCRIP (IRATp970H1055D) were similarly cloned into this vector (sequences obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH; Berlin, Germany). The PAK4-HA expression vector was described (Wells et al., 2002). The 3′UTRs of p27 and connexin-43 were PCR amplified from genomic DNA and cloned into pGL3 (Promega) downstream of the luciferase gene; constructs bearing the LATS2 3′UTR were described (Voorhoeve et al., 2006). The mutations in pGL3-p27mut-3′UTR were cloned by PCR to contain the following sequences from the original 3′UTR from nt 183–282: GCCTCTAAAAGCGTTGG***GGATCCC***ATTATGCAATTAGGTTTTTCCTTATTTGCTTCATTGTACTACCTGTGTATATAGTTTTTACCTTTT***GGATCCC***AC. In italics are the BamHI sites that substituted the two seed sequences. The other mutants were similarly cloned to contain the following sequences from nt 183–282; miRNA seeds are shown in bold and mutations in italics. FC: GC*gaaacccagcagacaatgtagct*TTATGCAATTAGGTTTTTCCTTATTTGCTTCATTGTACTACCTGTGTATATAGTTTTTACCTTTT***GGATCCC***AC; MUT1: GCCTCTAAAAGCGTTGG**ATGTAGC**ATTATGCAATTAGGT*gctcatggc*TTTGCTTCATTGTACTACCTGTGTATATAGT*gctcatggc*TT**ATGTAGC**AC; MUT2: GCCTCTAAAAGCGTTGG**ATGTAGC**ATT*gctcatggc*GGTTTTTCCTTATTTGCTTCATTGTACTACCT*gctcatggc*GTTTTTACCTTTT**ATGTAGC**AC; MUT3: GCCTCTAAAAGCGTTGG**ATGTAGC**ATTATGCAATTAGGTTTTTCCTTATTTGCTTCATTGTACTACCTGTGTATATAGT*gctcatggc*TT**ATGTAGC**AC; MUT4: GCCTCTAAAAGCGTTGG**ATGTAGC**AT*gtgattgcgatgcgctcatggcgg*TGCTTCATTGTACTACCTGTGTATATAGTTTTTACCTTTT**ATGTAGC**AC; MUT5: GCCTCTAAAAGCGTTGG**ATGTAGC**AT*gtgattgcgatgcgctcatggcgggtgattgcgatgcgctcatggcg*tatagttttttacctttt**atgtagc**ac. Constructs for RPA detection of hTR and cyclophilin were described (Kedde et al., 2006), antisense probes for detection of p27 (nt 340–577) and firefly luciferase (nucleotides 0–156) were generated by PCR, a T7 promoter sequence was included for labeling. shRNA for pasha was described before (Gregory et al., 2004); the shDnd1 sequence is GCAGCGACTTCGCCAGCAG—this was cloned in pSUPER. All constructs were sequence verified.

Antibodies used were CDK4 (C-22), HA Y-11 (sc805), Cdc6 180.2 (sc9964) from Santa Cruz Biotechnology, Tubulin (YL1/2 ECACC), and rabbit GFP.

Cell Culture, Transfections, and Dual Luciferase-Activity Analysis

MCF7 and HEK293 cells were cultured in DMEM and Tera1 in McCoy's 5A supplemented with 10% heat-inactivated fetal calf serum in 5% CO_2 at 37°C. For protein-expression analysis and immunoprecipitation HEK293 cells were transiently transfected using calcium-phosphate precipitation.

MCF7- and Tera1 cells were transfected using PEI (Polysciences, Inc.) or Fugene (Roche), respectively, for luciferase analysis with 10 ng of reporter, 5 ng of renilla control plasmid, and 250 ng of either miR-Vec or miR-Vec control, and 250 ng of either miR-Vec control or expression plasmid for Dnd1 or homologs. Dual luciferase-activity assays were performed 48 hr after transfection according to the manufacturer's directions (Promega). The results are represented as means and standard deviation (SD) from three independent experiments.

Immunoprecipitation, Western Blotting, miRNA Pull-Down, and RNase Protection Assays

Dnd1 was IPed from extracts of transfected HEK293 cells using GammaBind G Sepharose (GE Healthcare). Extracts were made and beads were washed with lysis buffer (125 mM NaCl, 50 mM HEPES (pH 7.5), 0,1% Nonidet P-40, 0.5% Tween-20, 10 mM $MgCl_2$, and protease inhibitor mixture [Roche Applied Science]). Interaction studies were performed with total RNA from transfected MCF7 cells extracted with Trizol reagent (Invitrogen) and split over IP samples in 50 μl lysis buffer suplemented with RNase-OUT (Invitrogen). Reactions were carried out for 45 min in an orbital shaker placed at 6°C; thereafter, beads were washed and RNA was extracted to be subjected to RPA.

For western blot analysis, extracts were separated on 10% SDS-PAGE gels and transferred to Immobilon-P membranes (Milipore). Western blots were developed with Supersignal (Pierce) or ECL (Amersham Biosciences) and exposed to film (Kodak). Cellular fractionation was performed on MCF7 cells with NE-PER kits from Pierce, according to manufacturer's instructions.

Pull-downs with miRNAs were performed with miRNA oligos where the sense strand contains a biotin group at its 3′ end (Dharmacon) in the mutant miRNA the seed (AGC UAC AUU) was mutated to AG*G* *AUC C*UU. Cells were transfected with FuGENE (Roche) (150 nM miRNA and 10 μg of control or Dnd1 vector) and after 48 hr lysed in 20 mM Tris with pH 7.5, 200 mM NaCl, 2.5 mM $MgCl_2$, 0.05% NP40, 60U RNaseOUT/ml (invitrogen), 1 mM DTT, and protease inhibitors (Roche). miRNAs were pulled down with streptavidin sepharose beads (GE healthcare) preblocked with yeast tRNA (Invitrogen) and RNase free BSA, (Ambion), and washed four times with lysis buffer. RNA was extracted and subjected to RPA.

RPAs for Luciferase, p27, cyclophilin, and hTR were performed using the HybSpeed RPA and MAXIscript kits from Ambion as described (Kedde et al., 2006). For miR-221 we used mirVana kits (Ambion) according to the manufacturer's instructions and the primer GCAACAGCTACATTGTCTGCTGGGTTTCAGGCTcctgtctc. We used 2 μg of total RNA (∼2% of input) and half of IP samples per reaction.

Quantitative RT-PCR Analysis

RNA was extracted using Trizol reagent (Invitrogen), and cDNA (from 3 μg RNA) was synthesized with superscript III and primed with oligo-dT according to manufacturer's instructions (Invitogen). Primers for zebrafish qPCR were nanos1 For/Rev: AGACTGAGGCCGTGTACACCTCTCACTACT / GAGCAGTAGTTCTTGTCCACCATCG, ODC For/Rev: ACACTATGACGGCTTGCACCG / CCCACTGACTGCACGATCTGG, vasa1 For/Rev: CCTGCTGCCTATCCTACAGC / CAGGTCCCGTATGCAAACTT, TDRD7 For/Rev: TCTACCCAGCGGAAGCTTTA / CTGGTGTCCCACTGGTCTTT. Primers for human Dnd1 were For/Rev: CTCCACAGGCACCCTGAATG / GGTGCCATAGGTCCCTGTCC; other primers were as described (Voorhoeve et al., 2006). Analyses were carried out using SYBR Green PCR master mix (Applied Biosystems) and Chromo 4 system (BioRad Laboratories).

Zebrafish Strain and Fish Maintenance

Zebrafish (*Danio rerio*) of the AB genetic background were maintained, raised, and staged as previously described (Kimmel et al., 1995; Westerfield, 1995).

Morpholino Knockdown of Dnd1

The dead end morpholino antisense oligonucleotide (dnd MO, 5′-GCTGGGCATCCATGTCTCCGACCAT-3′) and the standard control MO were obtained from Genetools, Philomath, OR. Six hundred picograms were injected into one-cell stage embryos to efficiently knock down Dnd1 function (Weidinger et al., 2003).

RNA Expression Constructs

Capped sense RNA was synthesized using the mMessageMachine kit (Ambion) and microinjected into one-cell stage embryos. The following constructs were used: pSP64T-*mgfp-vasa*-3′UTR (GFP-3′*vasa* in Figure 3A) (Wolke et al., 2002), pSP64T-*mgfp-nos1*-3′UTR (Koprunner et al., 2001), pSP64T-*vasa-dsRedEx-nos1*-3′UTR (Ds-Red-3′*nos1* in Figure 3B), pSP64T-*dsRedEx-nos1*-3′UTR, T3-*venus-nos1*-3′UTR (3′*nos1wt* in Figures 3C and 5). To obtain a mutation in the miR-430-binding site pSP64T-*ecfp-nos1*-3′UTR was amplified using primers (GTCTTTTTGTGTGTGTGTAT and CAAAATCAAACAGTGAACGC) resulting in pSP64T-*ecfp-nos1*-3′UTRmut1 (3′*nos1mut1* in Figure 3C). To obtain a mutation in the putative Dnd interacting sequence 1 pSP64T-*mgfp-nos1*-3′UTR was amplified using primers (CAGCA CTTTTTGTGTGTGTGTATA and GCTCAAACAGTGAACGCACACAT) resulting in pSP64T-*mgfp-nos1*-3′UTRmut2 (3′*nos1mut2* in Figure 5). To obtain a mutation in the putative Dnd-interacting sequence 2 pSP64T-*mgfp-nos1*-3′UTR was amplified using primers (CAGTGTGC ACTGGTGTTGTGTT and GCTAAAACACAGCAAACACACACA) resulting in pSP64T-*mgfp-nos1*-3′UTRmut3 (3′*nos1mut3* in Figure 5). To obtain a double mutation in both putative Dnd-interacting sequences pSP64T-*mgfp-nos1*-3′UTRmut2 was amplified using primers (CAGTG TGCACTGGTGTTGTGTT and GCTAAAACACAGCAAACACACACA) resulting in pSP64T-*mgfp-nos1*-3′UTRmut2+3 (3′*nos1mut2+3* in Figure 5). To obtain a double mutation in the putative Dnd-interacting sequence 2 and in the miR-430-binding site, pSP64T-*mgfp-nos1*-3′UTRmut1 was amplified using primers (CAGTGTGCACTGGTGT TGTGTT and GCTAAAACACAGCAAACACACACA) resulting in pSP64T-*mgfp-nos1*-3′UTRmut1+3 (3′*nos1mut1+3* in Figure 5). To obtain a triple mutation in both putative Dnd-interacting sequences and in the miR-430-binding site, pSP64T-*mgfp-nos1*-3′UTRmut2+3 was amplified using primers (GTCTTTTTGTGTGTGTGTAT and ACCT GGCTCAAACAGTGAACGC) resulting in pSP64T-*mgfp-nos1*-3′UTR mut1+2+3 (3′*nos1mut1+2+3* in Figure 5). To obtain pSP64T-*ds RedEx-TDRD7*-3′UTR (3′*TDRD7* in Figures S2B and S2C), pSP64T-*m gfp-TDRD7*-3′UTR (3′*TDRD7wt* in Figure S2D) and pSP6T-*eyfp-TDRD7*-3′UTR the 3′UTR of TDRD7 (EF643554) was amplified using primers (AAACTCGAGTACTCTCAGAACTGCACTTTC and AAATCT AGATAATACAACAAAACCTGAACACC) and cloned into corresponding vectors. To obtain a mutation in the miR-430- binding site pSP64T-*mgfp-TDRD7*-3′UTR was amplified with primer (TCTTTG GTTTGTTTTGCTGTGTTT/CCAAAATCAAAAAGTACAAACAATG) and subloned into an ecfp-containing vector resulting in pSP64T-*ecfp-TDRD7*-3′UTRmut1 (3′*TDRD7mut1* in Figure S2). To obtain a mutation in the putative Dnd-interacting sequence 2 pSP64T-*mgfp-TDRD7*-3′U TR was amplified using primers (CAGCACTTTGGTTTGTTTGCT and GCTCAAAAAGTACAAACAATGC) resulting in pSP64T-*mgfp-TDRD 7*-3′UTRmut2 (3′*TDRD7mut1* in Figure S2).

Fluorescence Microscopy and Imaging of Live Cells

Images were obtained using a Zeiss Axioplan2 microscope controlled by the Metamorph software (Universal Imaging). Average pixel intensity in germ cells was measured and subtracted from the background signal using the ImageJ software. Error bars represent the SEM. The p values were calculated using t test.

Supplemental Data

Supplemental Data include four figures and can be found with this article online at http://www.cell.com/cgi/content/full/131/7/1273/DC1/.

ACKNOWLEDGMENTS

We thank all members of the Agami Lab for help and discussions. We thank Dieuwke Engelsma and Coenraad Kuijl for providing help and reagents. This work was supported by grants from the Max-Planck Society and the DFG to E.R. and the Dutch Cancer Society (KWF, NKI 2003-2963) and European Young Investigator (EURYI) grants to R.A.

Received: June 18, 2007
Revised: November 7, 2007
Accepted: November 28, 2007
Published online: December 20, 2007

REFERENCES

Anderson, C., Catoe, H., and Werner, R. (2006). MIR-206 regulates connexin43 expression during skeletal muscle development. Nucleic Acids Res. *34*, 5863–5871.

Aravin, A., and Tuschl, T. (2005). Identification and characterization of small RNAs involved in RNA silencing. FEBS Lett. *579*, 5830–5840.

Ashraf, S.I., McLoon, A.L., Sclarsic, S.M., and Kunes, S. (2006). Synaptic protein synthesis associated with memory is regulated by the RISC pathway in Drosophila. Cell *124*, 191–205.

Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R., and Pasquinelli, A.E. (2005). Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell *122*, 553–563.

Bhattacharyya, S.N., Habermacher, R., Martine, U., Closs, E.I., and Filipowicz, W. (2006). Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell *125*, 1111–1124.

Ciosk, R., DePalma, M., and Priess, J.R. (2006). Translational regulators maintain totipotency in the Caenorhabditis elegans germline. Science *311*, 851–853.

Gregory, R.I., Yan, K.P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., and Shiekhattar, R. (2004). The Microprocessor complex mediates the genesis of microRNAs. Nature *432*, 235–240.

Gregory, R.I., Chendrimada, T.P., Cooch, N., and Shiekhattar, R. (2005). Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell *123*, 631–640.

Handa, N., Nureki, O., Kurimoto, K., Kim, I., Sakamoto, H., Shimura, Y., Muto, Y., and Yokoyama, S. (1999). Structural basis for recognition of the tra mRNA precursor by the Sex-lethal protein. Nature *398*, 579–585.

Kawahara, Y., Zinshteyn, B., Sethupathy, P., Iizasa, H., Hatzigeorgiou, A.G., and Nishikura, K. (2007). Redirection of silencing targets by adenosine-to-inosine editing of miRNAs. Science *315*, 1137–1140.

Kedde, M., le Sage, C., Duursma, A., Zlotorynski, E., van Leeuwen, B., Nijkamp, W., Beijersbergen, R., and Agami, R. (2006). Telomerase-independent regulation of ATR by human telomerase RNA. J. Biol. Chem. *281*, 40503–40514.

Kent, W.J., Sugnet, C.W., Furey, T.S., Roskin, K.M., Pringle, T.H., Zahler, A.M., and Haussler, D. (2002). The human genome browser at UCSC. Genome Res. *12*, 996–1006.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. (1995). Stages of embryonic development of the zebrafish. Dev. Dyn. *203*, 253–310.

Kloosterman, W.P., and Plasterk, R.H. (2006). The diverse functions of microRNAs in animal development and disease. Dev. Cell *11*, 441–450.

Knight, S.W., and Bass, B.L. (2002). The role of RNA editing by ADARs in RNAi. Mol. Cell *10*, 809–817.

Koprunner, M., Thisse, C., Thisse, B., and Raz, E. (2001). A zebrafish nanos-related gene is essential for the development of primordial germ cells. Genes Dev. *15*, 2877–2885.

Kotaja, N., and Sassone-Corsi, P. (2007). The chromatoid body: a germ-cell-specific RNA-processing centre. Nat. Rev. Mol. Cell Biol. *8*, 85–90.

Kotaja, N., Bhattacharyya, S.N., Jaskiewicz, L., Kimmins, S., Parvinen, M., Filipowicz, W., and Sassone-Corsi, P. (2006). The chromatoid body

of male germ cells: similarity with processing bodies and presence of Dicer and microRNA pathway components. Proc. Natl. Acad. Sci. USA *103*, 2647–2652.

Lagos-Quintana, M., Rauhut, R., Yalcin, A., Meyer, J., Lendeckel, W., and Tuschl, T. (2002). Identification of tissue-specific microRNAs from mouse. Curr. Biol. *12*, 735–739.

le Sage, C., Nagel, R., Egan, D.A., Schrier, M., Mesman, E., Mangiola, A., Anile, C., Maira, G., Mercatelli, N., Ciafre, S.A., et al. (2007). Regulation of the p27(Kip1) tumor suppressor by miR-221 and miR-222 promotes cancer cell proliferation. EMBO J. *26*, 3699–3708.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843–854.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S., and Kim, V.N. (2003). The nuclear RNase III Drosha initiates microRNA processing. Nature *425*, 415–419.

Lewis, B.P., Burge, C.B., and Bartel, D.P. (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell *120*, 15–20.

Lim, L.P., Lau, N.C., Garrett-Engele, P., Grimson, A., Schelter, J.M., Castle, J., Bartel, D.P., Linsley, P.S., and Johnson, J.M. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature *433*, 769–773.

Liu, J., Valencia-Sanchez, M.A., Hannon, G.J., and Parker, R. (2005). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat. Cell Biol. *7*, 719–723.

Maniataki, E., and Mourelatos, Z. (2005). A human, ATP-independent, RISC assembly machine fueled by pre-miRNA. Genes Dev. *19*, 2979–2990.

Mishima, Y., Giraldez, A.J., Takeda, Y., Fujiwara, T., Sakamoto, H., Schier, A.F., and Inoue, K. (2006). Differential regulation of germline mRNAs in soma and germ cells by zebrafish miR-430. Curr. Biol. *16*, 2135–2142.

Oosterhuis, J.W., and Looijenga, L.H. (2005). Testicular germ-cell tumours in a broader perspective. Nat. Rev. Cancer *5*, 210–222.

Pillai, R.S., Bhattacharyya, S.N., Artus, C.G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E., and Filipowicz, W. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science *309*, 1573–1576.

Pillai, R.S., Bhattacharyya, S.N., and Filipowicz, W. (2007). Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol. *17*, 118–126.

Schratt, G.M., Tuebing, F., Nigh, E.A., Kane, C.G., Sabatini, M.E., Kiebler, M., and Greenberg, M.E. (2006). A brain-specific microRNA regulates dendritic spine development. Nature *439*, 283–289.

Schwede, T., Kopp, J., Guex, N., and Peitsch, M.C. (2003). SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res. *31*, 3381–3385.

Thomson, J.M., Newman, M., Parker, J.S., Morin-Kensicki, E.M., Wright, T., and Hammond, S.M. (2006). Extensive post-transcriptional regulation of microRNAs and its implications for cancer. Genes Dev. *20*, 2202–2207.

Voorhoeve, P.M., and Agami, R. (2006). Classifying microRNAs in cancer: The good, the bad and the ugly (. Biochim. Biophys. Acta *1775*, 274–282.

Voorhoeve, P.M., le Sage, C., Schrier, M., Gillis, A.J., Stoop, H., Nagel, R., Liu, Y.P., van Duijse, J., Drost, J., Griekspoor, A., et al. (2006). A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell *124*, 1169–1181.

Weidinger, G., Stebler, J., Slanchev, K., Dumstrei, K., Wise, C., Lovell-Badge, R., Thisse, C., Thisse, B., and Raz, E. (2003). dead end, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. Curr. Biol. *13*, 1429–1434.

Wells, C.M., Abo, A., and Ridley, A.J. (2002). PAK4 is activated via PI3K in HGF-stimulated epithelial cells. J. Cell Sci. *115*, 3947–3956.

Westerfield, M. (1995). The Zebrafish Book (Oregon: University of Oregon Press).

Wienholds, E., Kloosterman, W.P., Miska, E., Alvarez-Saavedra, E., Berezikov, E., de Bruijn, E., Horvitz, H.R., Kauppinen, S., and Plasterk, R.H. (2005). MicroRNA expression in zebrafish embryonic development. Science *309*, 310–311.

Wolke, U., Weidinger, G., Koprunner, M., and Raz, E. (2002). Multiple levels of posttranscriptional control lead to germ line-specific gene expression in the zebrafish. Curr. Biol. *12*, 289–294.

Yang, W., Chendrimada, T.P., Wang, Q., Higuchi, M., Seeburg, P.H., Shiekhattar, R., and Nishikura, K. (2006). Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. Nat. Struct. Mol. Biol. *13*, 13–21.

Youngren, K.K., Coveney, D., Peng, X., Bhattacharya, C., Schmidt, L.S., Nickerson, M.L., Lamb, B.T., Deng, J.M., Behringer, R.R., Capel, B., et al. (2005). The Ter mutation in the dead end gene causes germ cell loss and testicular germ cell tumours. Nature *435*, 360–364.

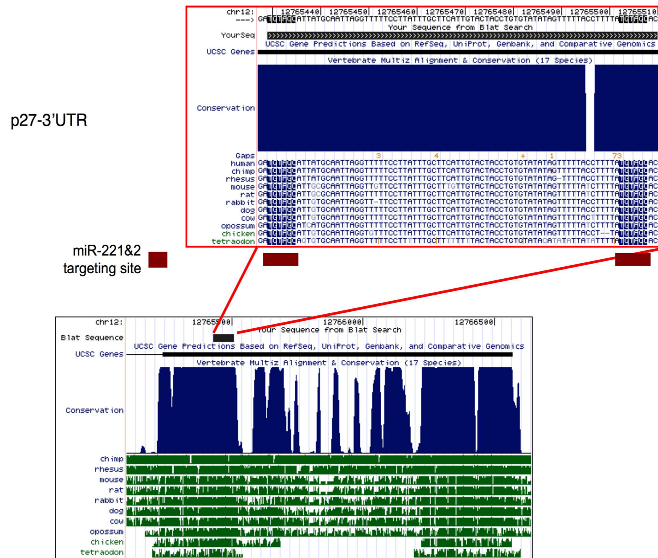
Zhu, R., Ji, Y., Xiao, L., and Matin, A. (2007). Testicular germ cell tumor susceptibility genes from the consomic 129.MOLF-Chr19 mouse strain (. Mamm. Genome *18*, 584–595.

Supplemental Data

RNA-Binding Protein DND1 Inhibits MicroRNA Access to Target mRNA

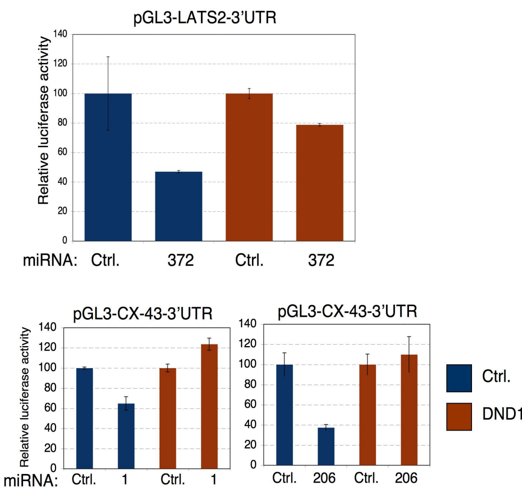
Martijn Kedde, Markus J. Strasser, Bijan Boldajipour, Joachim A.F. Oude Vrielink, Krasimir Slanchev, Carlos le Sage, Remco Nagel, P. Mathijs Voorhoeve, Josyanne van Duijse, Ulf Andersson Ørom, Anders H. Lund, Anastassis Perrakis, Erez Raz, and Reuven Agami

Supplementary Figure 1A

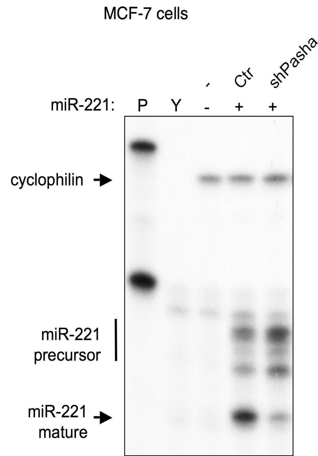


Supplementary Figure 1B

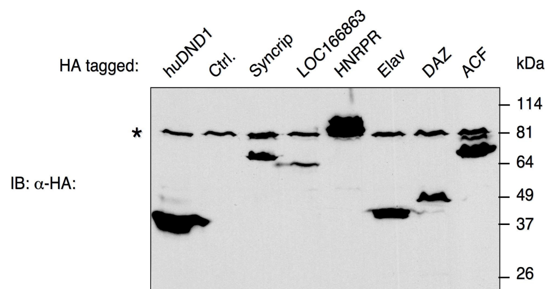
Supplementary Figure 1C



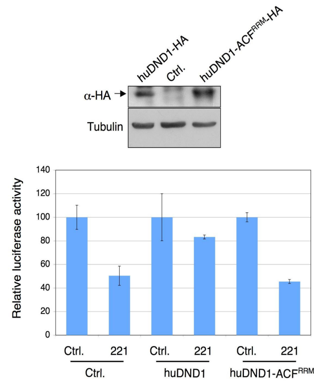
Supplementary Figure 1D



Supplementary Figure 1E



Supplementary Figure 1F



Supplementary Figure 1G

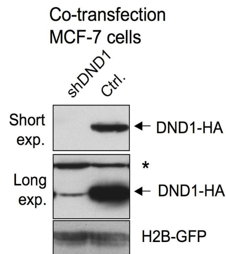
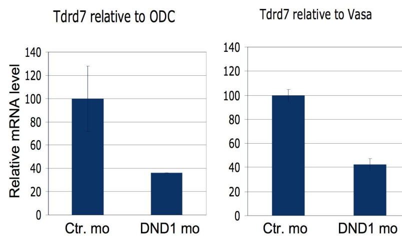


Figure S1. DND1 specifically relieves miRNA-mediated repression. (A) Evolutionary conservation of the human p27-3'UTR. miR-221 targeting sequences are marked brown and

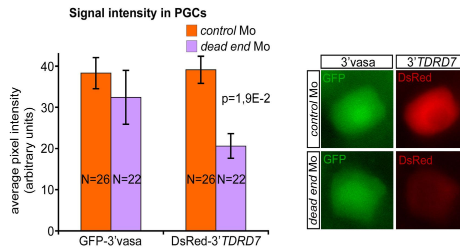
blue. (B) Evolutionary conservation of the human LATS2-3'UTR. miR-372 targeting sequences are marked brown and blue. (C) *firefly*-luciferase-LATS2-3'UTR and *firefly*-luciferase-connexin-43 (CX43)-3'UTR constructs were cotransfected with miR-372, and miR-1 and 206 vectors, respectively. Experiments were performed and analyzed exactly as described in Figure 1. The results are represented as means and SD from three independent experiments. (D) MCF7 cells were transfected with pSUPER control or pS-Pasha, RNA was isolated 48 hours later and analysed by RPA as described for Figure 2A. (E) HEK-293 cells were transfected with the indicated HA-tagged constructs. Whole cell extract was made 72 hours after transfection and subjected to immunoblot analysis with HA antibody. (F) *firefly*-luciferase-p27-3'UTR and indicated constructs were cotransfected. Experiments were performed and analyzed exactly as described in Figure 1. The results are represented as means and SD from three independent experiments. Immunoblot was performed with anti-HA and tubulin antibodies to confirm expression. (G) MCF7 cells were transfected with indicated constructs. Whole cell extract was made after 48 hours and subjected to immunoblot analysis with HA- and GFP-antibodies.

Supplementary Figure 2A

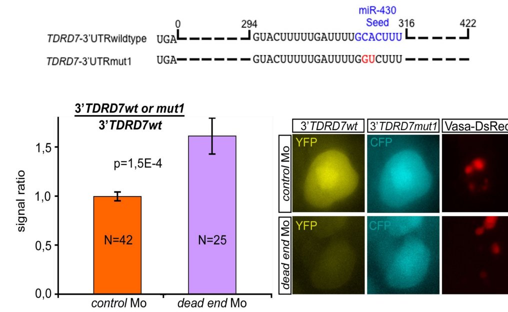


Supplementary Figure 2

B



C



Supplementary Figure 2

D

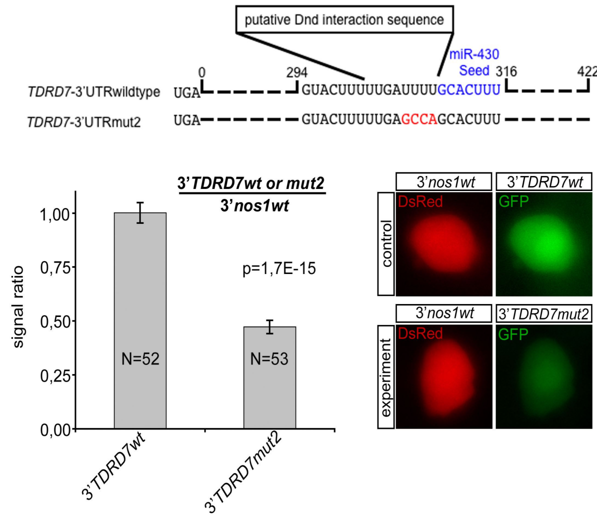


Figure S2. Zebrafish DND1 (drDND1) counteracts inhibition of *TDRD7* by miR-430 through binding to adjacent URRs. (A) One-cell-stage zebrafish embryos were injected with dead end morpholino or control morpholino. RNA was extracted and subjected to Q-RTPCR analysis to compare endogenous levels of TDRD7 and Vasa to Odc, and TDRD7 to Vasa. (B) One-cell-stage zebrafish embryos were co-injected with *DsRed-TDRD7*-3'UTR (3'*TDRD7*) and *gfp-vasa*-3'UTR (3'*vasa*) together with dead end morpholino or control morpholino. (C) One-cell-stage zebrafish embryos were co-injected with RNA containing the *yfp* open reading frame fused to the wild-type *TDRD7* 3' UTR (*3'TDRD7wt*), RNA containing the *cfp* open reading frame fused to the miR-430 binding site mutated *TDRD7* 3' UTR (*cfp-3'TDRD7mut1*) and vasa-dsRed (for labelling the germinal granule for easier identification of germ cells) together with dead end morpholino or control morpholino. (D) RNA containing the *DsRed* open reading frame fused to the wild-type *nanos*-1 3' UTR (*3'nos1wt*) was co-injected into one-cell-stage zebrafish embryos together with RNA containing the *gfp* open reading frame fused to different versions of the *TDRD7*-1 3' UTR. The different *TDRD7* 3' UTR that were used were: wild-type *TDRD7* 3'UTR (*3'TDRD7wt*) or *TDRD7* 3'UTR where the putative DND1 interaction sequence was mutated (*3'TDRD7mut2*). Error bars depict the standard error of the mean (SEM), p-value was calculated using Ttest.

Supplementary Figure 3

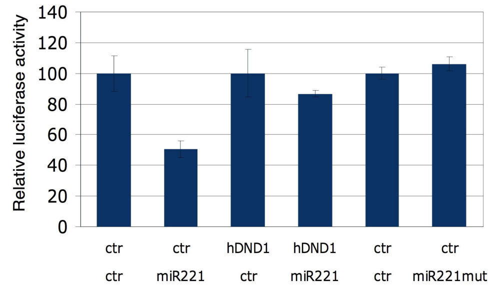


Figure S3. Synthetic biotin-tagged 221 miRNAs are functional and DND1 inhibits their suppression of the p27-3'UTR. MCF7 cells were transfected with 150 nM of the indicated miRNAs, control vector and/or DND1 and cotransfected with *firefly*-luciferase-p27-3'UTR. Subsequently, experiments were performed and analysed exactly as described in figure 1.

Supplementary Figure 4

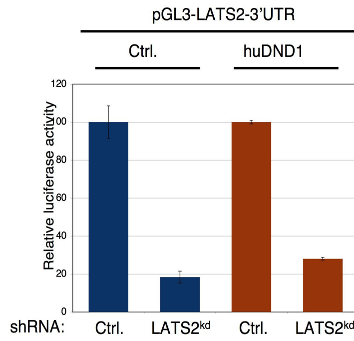


Figure S4. DND1 is unable to counteract RNAi mediated LATS2 suppression. An shRNA construct designed to target LATS2-3'UTR in the second miRNA-targeting region was used to suppress the expression of a firefly-luciferase-LATS2-3'UTR construct. The experiment was performed and analyzed exactly as described in Figure 1.

Chapter 4:

Telomerase-independent regulation of ATR by human telomerase RNA

J Biol Chem. 2006 Dec 29;281(52):40503-14

DOI 10.1074/jbc.M607676200

Telomerase-independent Regulation of ATR by Human Telomerase RNA*[S]◆

Received for publication, August 11, 2006, and in revised form, November 6, 2006 Published, JBC Papers in Press, November 10, 2006, DOI 10.1074/jbc.M607676200

Martijn Kedde, Carlos le Sage, Anja Duursma, Eitan Zlotorynski, Bart van Leeuwen, Wouter Nijkamp, Roderick Beijersbergen, and Reuven Agami[1]

From the Division of Tumor Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066CX Amsterdam, The Netherlands

The human telomerase RNA (hTR), together with the telomerase reverse transcriptase, hTERT, constitute the core components of telomerase that is essential for telomere maintenance. While hTR is ubiquitously expressed, hTERT is normally restricted to germ cells and certain stem cells, but both are often deregulated during tumorigenesis. Here, we investigated the effects of changes in hTR cellular levels. Surprisingly, while inhibition of hTR expression triggers a rapid, telomerase-independent, growth arrest associated with p53 and CHK1 activation, its increased expression neutralizes activation of these pathways in response to genotoxic stress. These hTR effects are mediated through ATR and are sufficiently strong to impair ATR-mediated DNA-damage checkpoint responses. Furthermore, in response to low UV radiation, which activates ATR, endogenous hTR levels increase irrespective of telomerase status. Thus, we uncovered a novel, telomerase-independent, function of hTR that restrains ATR activity and participates in the recovery of cells from UV radiation.

Most human somatic cells have a limited replicative lifespan when propagated *in vitro* which is due to their inability to maintain chromosome ends, the telomeres (1, 2). Telomeres are specialized DNA-protein structures that preserve the integrity of the ends of the chromosomes and the stability of the genome (2). However, with each cell division telomeres are shortened and once one or more erode to a certain critical point, the shortening induces a DNA damage checkpoint, thereby posing a barrier to continued cell growth and, therefore, to cancer (3–8). Cells that escape replicative senescence by inactivating critical cell cycle checkpoint genes, such as p53, continue to erode their telomeres, eventually reaching a point that is called crisis (9). The only way to escape crisis is to ensure that telomeres are maintained. Most cells do this by reactivating telomerase, but alternative mechanisms (ALT),[2] involving recombination, are also observed (10–12).

Telomerase, or TERT, is a ribonucleoprotein that copies a short RNA template into telomeric DNA, thereby maintaining eukaryotic chromosome ends and preventing replicative senescence (13, 14). Telomerase activity is low or absent in normal human cells but is high during development in certain stem cells, germ cells, and 80–90% of human cancers and immortalized human cell lines (10, 11, 15, 16). In fact, telomerase expression is sufficient for immortalization of primary human cells, rendering them insensitive to replicative senescence and crisis (17).

In humans, the core components of the telomerase complex are the protein catalytic subunit hTERT and the telomerase RNA subunit hTR (14, 18). hTR is transcribed by RNA polymerase II and is 3′ processed to generate a 451-nucleotide-long mature transcript. Its secondary structure is very well conserved with telomerase RNAs from several vertebrate species, indicating an important role for RNA structure in telomerase function (19). Several proteins have been described to bind to hTR and these are involved in hTR stability, maturation, accumulation, and functional assembly of the telomerase ribonucleoprotein complex (2). Certain mutations or deletions in hTR lead to a rare skin and bone marrow failure syndrome called dyskeratosis congenita, which is believed to be caused by defective telomere maintenance in stem cells (20, 21). As expression of hTR has been found to be essential for telomere maintenance in human disease and also for telomere length maintenance in mouse models, it is expected to be up-regulated in cancer cells (22). Indeed, several studies have shown that hTR up-regulation is an early event in tumorigenesis and that hTR levels correlate better to tumor grade than telomerase activity or hTERT expression (23–31). Up-regulation of hTR was also found to be an early event in mouse models of tumorigenesis. Here, telomerase RNA levels did not parallel the amount of telomerase activity detected (32, 33). These studies show that even in tumors that lacked telomerase activity, telomerase RNA was up-regulated. Therefore, hTR may have functions that are sep-

* This work was supported by grants from the Dutch Cancer Society (Koningin Wilhelmina Fonds) (to M. K. and R. A.) and by a EURYI award (to R. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

◆ This article was selected as a Paper of the Week.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S10.

[1] To whom correspondence should be addressed: Division of Tumor Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066CX, Amsterdam, The Netherlands. Tel.: 31-20-512-2079; Fax: 31-20-512-2029; E-mail: r.agami@nki.nl

[2] The abbreviations used are: ALT, alternative lengthening of telomeres; TERT, telomerase reverse transcriptase; hTR, human telomerase RNA; mTR, mouse telomerase RNA; ATM, ataxia-telangiectasia-mutated protein; ATR, ataxia-telangiectasia and Rad3-related protein; PIKK, phosphatidylinositol 3-kinase-like protein kinase; PBS, phosphate-buffered saline; CMV, cytomegalovirus; TAP, tandem affinity purification; GST, glutathione *S*-transferase; RT, reverse transcriptase; TRAP, telomeric repeat amplification protocol; TEV, tobacco etch virus; snoRNA, small nucleolar RNA; Gy, gray; RPA, RNase protection assay; siRNA, small interfering RNA; shRNA, short hairpin RNA; IR, ionizing radiation.

arable from its role in telomerase activity (32). Contrasting these observations are mice in which mouse telomerase RNA (mTR) was deleted from the germ line. These mice are viable for six generations until telomeres have completely eroded (34). However, the first generation of these mice, which still have long telomeres, have less skin tumors than wild-type mice following skin chemical carcinogenesis, indicating for some telomerase-independent effects of mTR (35). This argues that most mTR function is dependent on telomerase. However, as germ line gene knock-out may allow for compensating events to occur while acute inhibition in somatic cells may not (36), inhibition of telomerase RNA by RNA interference in cancer cell lines may expose its telomerase independent functions.

Telomeres are intimately linked with DNA damage responses. Dysfunctional telomeres are recognized as damaged DNA and directly associate with many DNA damage response proteins (37). Moreover, the main cellular transducers of DNA damage, ATM and, to a lesser extent, ATR, have been shown to play an important role in telomere homeostasis (38, 39). ATM and ATR are phosphatidylinositol 3-kinase-like protein kinases (PIKKs) that coordinate the repair, cell cycle checkpoint, and apoptotic responses to DNA damage (40). Loss of ATM causes telomere decapping and shortening in every organism investigated thus far, and TRF2, a telomeric DNA binding protein, has been shown to bind and inhibit ATM activation (37, 41). Probing a telomeric function for ATR in mammalian cells has not been possible since ATR is essential for cell viability (42). Interestingly, a recent report has shown that in *Arabidopsis* ATR is required for maintenance of telomeric DNA (43). Thus, PIKKs function in telomere homeostasis and telomeres with their telomere-associated factors influence PIKKs activity.

Telomerase and telomeres are attractive targets for cancer therapies and have been extensively explored to this end (2). Inhibition of telomerase by antisense strategies or a dominant negative hTERT protein leads to the expected telomere shortening, although the growth inhibition induced by this mechanism requires a long lag period due to the number of cell divisions required for telomeres to become substantially shortened to induce growth arrest (44, 45). Recently, some studies have reported rapid cytotoxic responses of cancer cell lines in response to low levels of hTERT and hTR. A novel telomere-independent growth inhibitory response pathway was proposed (46, 47). Here, we investigated the effects exerted by hTR on the cellular growth and checkpoint controls and uncovered a novel, telomerase-independent, function of hTR to counterbalance the activity of endogenous ATR.

EXPERIMENTAL PROCEDURES

Materials and Antibodies—UV radiation was performed with a Stratalinker (Stratagene), and ionizing irradiation was performed with a 2 × 415-Ci^{137}Cs source. Prior to UV radiation, medium was reserved, and cells were washed with phosphate buffered saline (PBS). Antibodies used in this study were directed against cdk4 (C-22), p53 (DO1) (Santa Cruz Biotechnology), phospho-Ser-15 p53, phospho-Ser-317 Chk1, phospho-T68 Chk2 (Cell Signaling), FLAG (M2, Sigma), γ-H2AX (Ser-139, Upstate Biotechnology), and ATR (ab2905, Abcam).

Constructs—hTR knockdown constructs were cloned in pSuper, pRetroSuper(pRS)-Hygro (48), and pRS-GFP (49). The sequences used were as follows: hTR^{kd}#1, GTCTAACCCTA-ACTGAGAAGG; hTR^{kd}2, CCGTTCATTCTAGAGCAAAC; hTR^{kd}3, GAGTTGGGCTCTGTCAGCC. The CMV-hTR expression construct was cloned by PCR in pcDNA3.1 vector (Invitrogen) to contain 149 nucleotides downstream of the full-length hTR for proper processing; H/ACA box snoRNA constructs were also cloned into this vector. pRS-p53kd and pRS-Rb were described previously (49). ATR^{kd} sequences used were as follows: #1, GACGGTGTGCTCATGCGGC; #2, CCT-GATGGAGTGGCCGGAG; we used a combination of the two constructs cloned in pSuper. We cloned the ATR cDNA from the pBJ5.1-Flag-ATR (a kind gift from Professor Steve Jackson) vector into the pZome1N vector (Cellzome) downstream of the TAP tag (50). The GST-p53 (amino acids 1–101) construct was cloned by PCR into pGEX-1N (Amrad). pTRI-GAPDH and -cyclophilin constructs for the RNase protection assay (RPA) were from Ambion, and the hTR RPA vector was cloned by PCR into pTRI (Ambion) from nucleotides 105–370 in reverse orientation.

Cell Culture, Retroviral Transduction, and Transfection—All cells described here were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum in 5% CO_2 at 37 °C. BJ cells were transduced at population doubling 35. The generation of BJ-tert cells has been described previously (49); GM847 cells were a kind gift from Professor Batsheva Kerem. MCF7, U2OS, and TIG3 cell lines expressing the ecotropic receptor were infected with ecotropic retroviral supernatants as described previously (48) to generate polyclonal pools of cells. Except for kinase assays, transfection was done by electroporation as described previously (51). For kinase assays, HEK293, U2OS, and MCF7 cells were transiently transfected using calcium phosphate precipitation.

Western Blotting, Cell Cycle Profile Analysis, and Competitive Growth Assays—For Western blot analysis, whole-cell extracts were prepared and separated on 10% SDS-PAGE gels, and ATR immunoblots were separated on 5% SDS-PAGE gels and transferred to Immobilon-P membranes (Milipore). Western blots were developed with Supersignal (Pierce), and densitometric quantitation of Western blots was performed with Aida 3.40 software (Raytek, Sheffield, UK). Cell cycle profile analysis was performed as described before by Duursma *et al.* (52). For competitive growth assays, cells were infected with pRS-GFP-hTR^{kd} or pRS-GFP retrovirus and allowed to recover for 4 days; the initial percentage of GFP-positive cells varied between 15 and 50%. The cells were analyzed by flow cytometry with the CellQuest program (BD Bioscience).

Telomerase Activity Assays, Quantitative RT-PCR, and RNase Protection Assays—MCF7 extracts were assayed for telomerase activity using a PCR-based telomeric repeat amplification protocol (TRAP) assay (53). For RT-PCR, cDNA was transcribed using SuperSCRIPT III (Invitrogen) with random hexamers following the manufacturer's instructions. Quantitative PCR was performed with a SYBR Green master mix (Applied Biosystems), and the samples were amplified and analyzed by an ABI-prism 7000 sequence detection system (Applied Biosystems). Primers for OAS1 (2′5′-oligoadenylate

synthetase) were described before (54), and C_t values were normalized for β-actin. RPAs were performed using the HybSpeed RPA and MAXIscript kits from Ambion according to manufacturer's instructions. We used 5– 8 μg of RNA per reaction. *In vitro* transcription of pTRI-hTR yielded an RNA of 300 nucleotides, 265 nucleotides of which are complementary to hTR.

Immunoprecipitation Kinase Assays—To determine ATR activity, TAP-ATR was immunoprecipitated from extracts of transfected HEK293, MCF7, or U2OS cells using rabbit IgG-Sepharose (Sigma). Prior to immunoprecipitation, cells were UV irradiated with either 80 or 20 J/m^2 or mock treated. Beads were washed three times with ELB lysis buffer (125 mM NaCl, 50 mM Hepes, pH 7.5, 0.1% Nonidet P-40, 0.5% Tween 20, 0.5 mM NaOV, 2 mM β-glycerophosphate, and protease inhibitor mixture (Roche Applied Science)) and ATR was cleaved from the beads by addition of recombinant TEV protease (Invitrogen) for 2 h at 8 °C according to manufacturer's instructions. The substrate, GST-p53-(1–101) bound to glutathione beads (Amersham Biosciences), was washed three times with kinase buffer (20 mM HEPES (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol, 0.5 mM NaOV, and 2 mM β-glycerophosphate). Reactions were carried out in a 50-μl volume of kinase buffer with cleaved ATR, 10 μg of GST-p53-(1–101), 10 μCi of [γ-^{32}P]ATP (500 mCi/mmol, Amersham Biosciences) at 30 °C for 15 min. In samples with RNase A, 0.5 μg was added to the kinase reaction prior to the addition of the substrate; we added 1 μg of *in vitro* transcribed hTR or H/ACA snoRNA to the indicated samples, which was produced from linearized CMV-hTR/snoRNA plasmid with a MAXIscript *in vitro* transcription kit (Ambion) according to manufacturer's instructions. The beads with GST-p53-(1–101) were then extensively washed and subjected to 10% SDS-PAGE, and the gel was dried, stained with Coomassie Blue, and exposed to a phospho imager screen for quantitation on a Basreader 3000 (Fuji) with Aida 3.40 software (Raytek, Sheffield, UK).

Immunofluorescence, Mitotic Entry, and Fragile Site Assays—MCF7 cells were transfected by electroporation (>90% efficiency) with the indicated constructs. Cells were washed with PBS, fixed, permeabilized in 4% formaldehyde and 0.2% Triton X-100, and washed with PBS containing 0.05% saponin. Slides were blocked with 10% normal goat serum in PBS with 0.05% saponin. Cells were stained with antibody directed against phosphorylated H2AX; fluorescein isothiocyanate-conjugated goat anti-mouse antibodies were used as secondary antibodies. Images were recorded with a Leica TCS SP2-AOBS (Leica Microsystems, Heidelberg, Germany) confocal system. For counting mitotic entry, after 72 h, transfected MCF7 cells were treated with 5 Gy ionizing radiation (IR) or mock treated and incubated with nocodazole (0.25 μg/ml) for 24 h. Cells were fixed with 3.7% formaldehyde, permeabilized for 5 min with 0.1% Triton X-100 (Sigma) and stained with Hoechst. Mitotic cells were scored double blind with a Zeiss RS III microscope. For each sample, 600 cells were counted. For fragile site assays, cells were grown on coverslips, and common fragile sites were induced by growing the cells in M-199 medium in the presence of 0.4 μM aphidicolin and 0.5% ethanol, for 24 h prior to the fixation of chromosomes by standard procedures. Images were obtained with a Zeiss Axiovert 100 TV inverted microscope controlled by SmartCapture2 software. For each sample, 400 chromosomes were counted double blind.

RESULTS

Inhibition of hTR Expression Induces a Rapid Cellular Growth Arrest—To inhibit hTR expression we designed shRNA constructs to target sequences in different regions of the hTR molecule. We aimed our constructs to three distinct single-stranded (loops) regions of the hTR molecule, as predicted by its very strong and conserved secondary structure (19) (Fig. 1*A*). To assess the efficacy of inhibition of hTR expression, we transfected MCF7 breast carcinoma cells with the shRNA constructs or a vector control. We used RPA with RNA probes partially complementary to hTR or cyclophilin control to assess hTR RNA levels in cell extracts (Fig. 1*B*). Quantitative analysis revealed that hTR^{kd}#1 and hTR^{kd}#2 reduced the levels of endogenous hTR to 19 and 67%, respectively, whereas no hTR reduction was seen by hTR^{kd}#3. U2OS osteosarcoma cells, which lack expression of hTR and hTERT but maintain their telomeres by ALT (55, 56), were used as a control. Indeed, no hTR expression was observed in U2OS cells, which could be re-expressed through transfection of a CMV-hTR plasmid (Fig. 1*B*). We also verified the extent of hTR knockdown in nuclear and cytoplasmic extracts to exclude knockdown of hTR only in cytoplasmic fractions (supplemental Figs. S1 and S2). We found that hTR resides mainly in the nucleus, and upon expression of hTR^{kd}#1, hTR levels in both nuclear and cytoplasmic fractions were dramatically decreased. To assess the effects of inhibition of hTR on telomerase activity we performed a TRAP assay with extracts of MCF7 cells transfected with the hTR^{kd} constructs or control plasmid. As expected we found almost no telomerase activity in hTR^{kd}#1-transfected cells, and hTR^{kd}#2 gave a moderate reduction, whereas hTR^{kd}#3 did not inhibit telomerase activity.

To investigate the effect of hTR knockdown on cell growth, we transfected MCF7 cells with the three hTR^{kd} constructs and analyzed them 2 days later by flow cytometry. Surprisingly, the introduction of hTR^{kd}#1 shRNA vector induced a cell cycle arrest at both G_1 and G_2 (Fig. 1*D*), whereas the hTR^{kd}#2 arrest was reduced, and hTR^{kd}#3 had no effect (data not shown). To verify this result we tested additional hTR-targeting siRNAs with RPA and flow cytometric analysis in MCF7 cells (supplemental Figs. S3 and S4). Transfection of these siRNAs suppressed the expression of hTR and elicited a comparable rapid growth arrest as hTR^{kd}#1. Consistent with these results, the long term survival of hTR^{kd}#1-transfected MCF7 cells (Fig. 1*E*) and virally transduced hTR^{kd}#1-MCF7 cells (Fig. 1*F*) was markedly impaired. The observed inhibition of proliferation by hTR^{kd}#1 was not a consequence of non-relevant toxicity as this vector induced no interferon response (Fig. 1*G*). Furthermore, no anti-proliferative response was seen in U2OS cells, as these express no hTR (Fig. 1*B*). Both by flow cytometry as well as by competitive growth assays, hTR^{kd}#1 was not toxic to U2OS cells but was highly toxic in MCF7 cells (Fig. 1, *H* and *I*). hTR^{kd}#2 inhibited growth of MCF7 cells to intermediate levels, reflecting its capacity to knockdown hTR. These results are further strengthened by the use of additional siRNA reagents targeting hTR (supplemental Figs. S2 and S3). This shows that

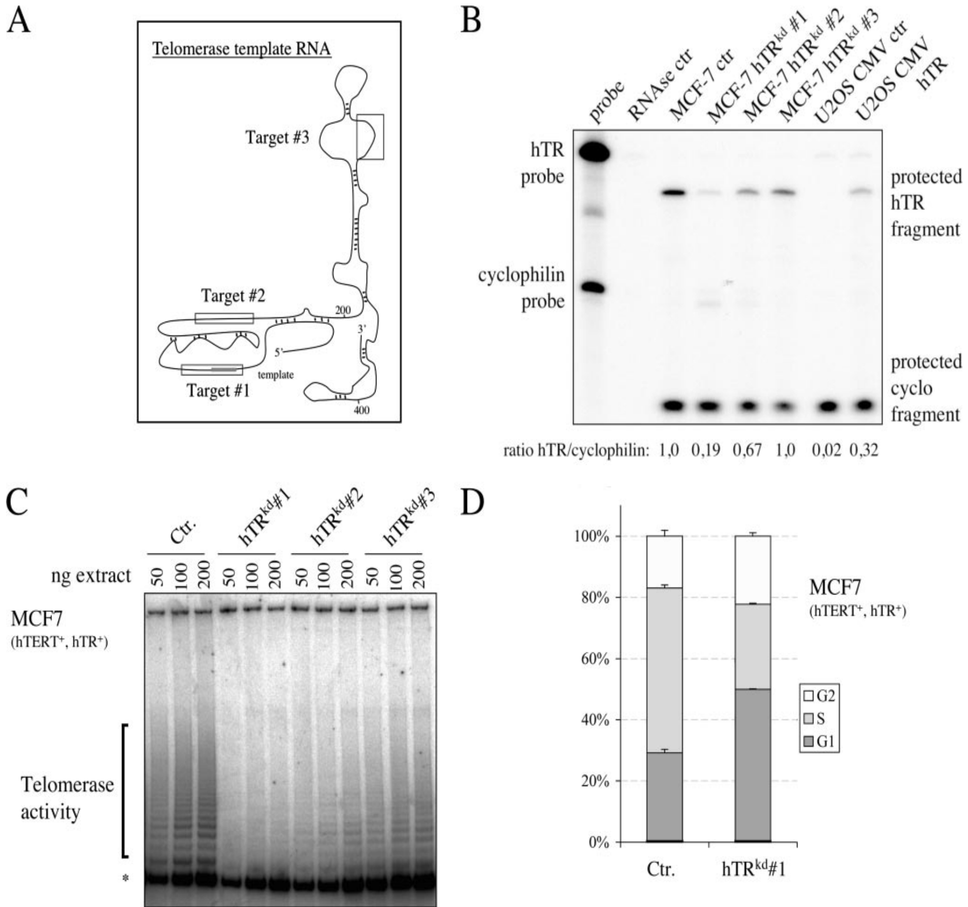


FIGURE 1. **Reduction of telomerase activity and rapid growth inhibition by reduction of hTR expression.** *A*, schematic representation of the hTR RNA. *Boxed* regions correspond to targeted sequences by shRNAs 1, 2, and 3, and the template sequence is shown in a *closed box*. *B*, RPA was used to detect the levels of hTR and the control cyclophilin. The full-length probes and protected fragments are indicated, and 10% of the input probe was loaded on the gel. Quantification was performed by densitometry. *C*, a TRAP assay was performed to detect telomerase activity in extracts of MCF7 cells transfected with the indicated constructs. *D*, MCF7 cells were transfected with hTR^{kd}#1 or vector constructs and subjected to flow cytometric analysis. The percentages of cells in G_1, S, and G_2 phases are shown. S.D. is from three independent experiments. *E*, a colony growth assay of MCF7 cells transfected with the hTR knockdown constructs or a vector control. Cells were selected with hygromycin for 10 days and stained with Coomassie Blue. *F*, colony growth assay of virally transduced MCF7 cells. *G*, MCF7 cells were transfected with indicated constructs, and RNA was extracted after 3 days. OAS1 mRNA levels were measured by quantitative RT-PCR, shown relative to β-actin mRNA. S.D. is from three independent experiments. *H*, flow cytometry is the same as in *D* only that U2OS cells were used. *I*, competitive growth assay the U2OS and MCF7 cells were transduced with indicated pRS-GFP constructs. Fluorescence was monitored by flow cytometry at the indicated timepoints after transduction. *J*, competitive growth assay performed on transduced primary BJ fibroblasts as in *I*. *ctr*, control.

the growth inhibition triggered by the knockdown of hTR in MCF7 cells is dependent on the level of knockdown of hTR. The same anti-proliferative effect of hTR^{kd}#1 was also obtained in other human cell lines, such as HaCaT immortalized keratinocytes, HeLa cervical carcinoma cells, and T47D mammary carcinoma cells (supplemental Fig. S5). Altogether, these results show that inhibition of hTR expression by shRNA elicits a rapid anti-proliferative response in human cells.

Due to the continuous rapid proliferation of cancer cell lines and emergence of critically short telomeres, the inhibition of hTR expression may induce a stress response. To examine this, we studied hTR inhibition in young primary human cells (passage 35) that express hTR and contain sufficiently long telomeres to maintain proliferation for 30–40 additional passages. Also in these cells, suppression of hTR inhibited cell growth depending on the level of hTR knockdown (Fig. 1*J*). Altogether, our results indicate that inhibition of hTR expression induces a cell cycle arrest, which is not caused by critically short telomeres.

Cell Cycle Arrest Induced by Inhibition of hTR Expression Requires p53 and CHK1—To elucidate the response of cells to reduced hTR levels we monitored p53 levels, a tumor suppressor and a major transducer of cell cycle arrest in response to oncogenic and genotoxic stresses. We transfected MCF7 cells with $p53^{kd}$ (57) and hTR^{kd} or control constructs and found p53 protein levels to be three times higher in the hTR^{kd}#1-transfected cells compared with controls (Fig. 2*A*). This suggests that the growth arrest observed in hTR^{kd} cells involves p53 activa-

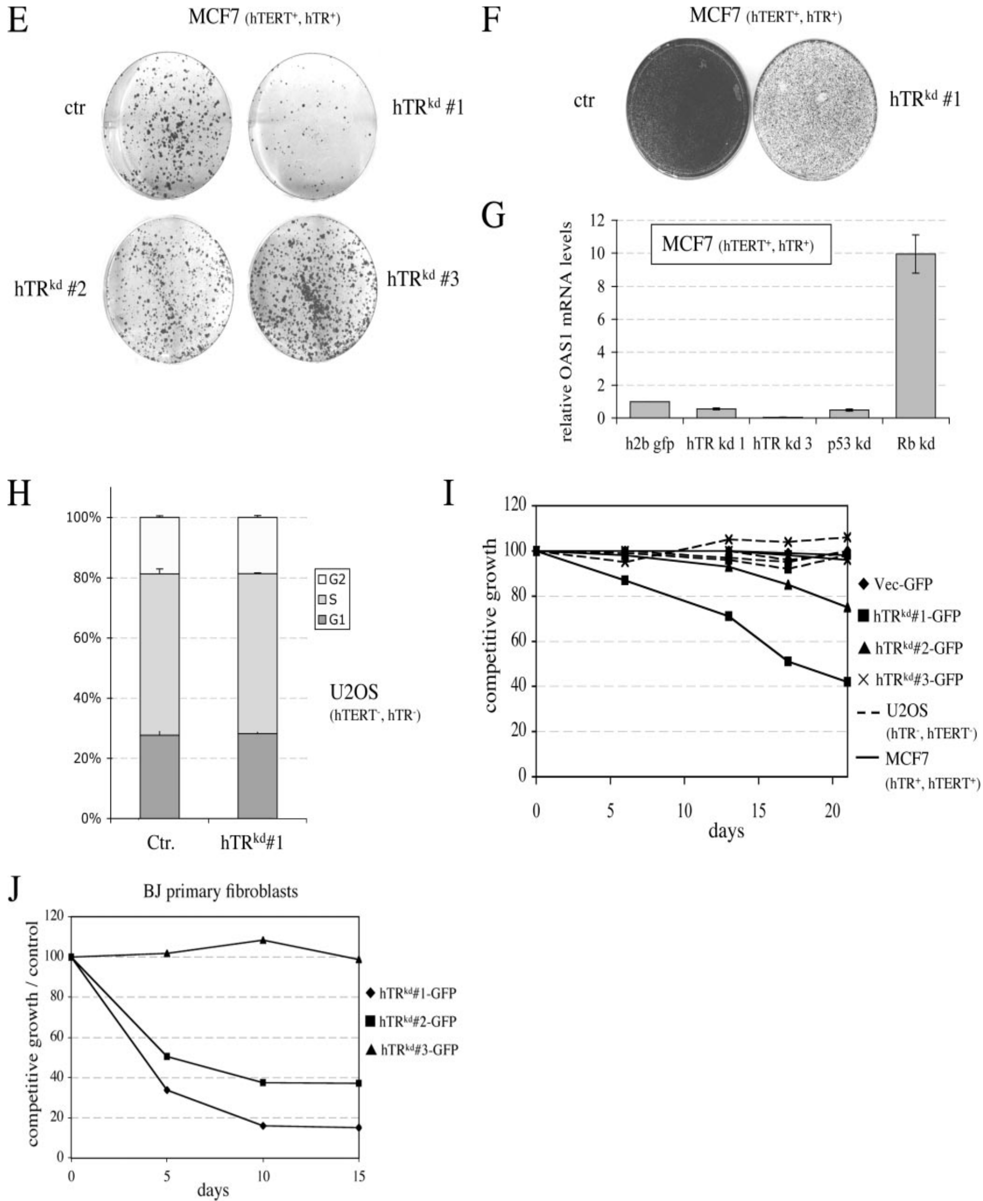


FIGURE 1—*continued*

tion. To test this directly, we performed competitive growth assays using MCF7-p53^{kd} and MCF7 control cells that were virally transduced with the GFP-hTR^{kd}#1 or GFP control vector. Fig. 2*B* shows that loss of p53 expression, which completely abrogates a DNA damage response (57), only partially rescued the arrest induced by loss of hTR. A similar result was obtained in TIG3 primary fibroblasts (supplemental Fig. S6).

Activation of p53 can be a result of activation of the kinases ATM and ATR, which in turn activate CHK2 and CHK1, respectively (40). To examine whether the cell cycle arrest induced by the loss of hTR depends on the combination of p53 and CHK1, we performed a GFP competition assay with BJ and BJ-p53^{kd} cells in the presence of the CHK1 inhibitor UCN-01 or vehicle as control (58, 59). Fig. 2*C* shows that hTR^{kd}#1-induced toxicity was almost completely abrogated when both p53 expression and CHK1 activity were inhibited. Similar results were obtained in MCF7 cells (data not shown). These results indicate that loss of hTR activates both p53 and CHK1 to elicit a rapid cell cycle arrest, indicating the involvement of ATR in this process.

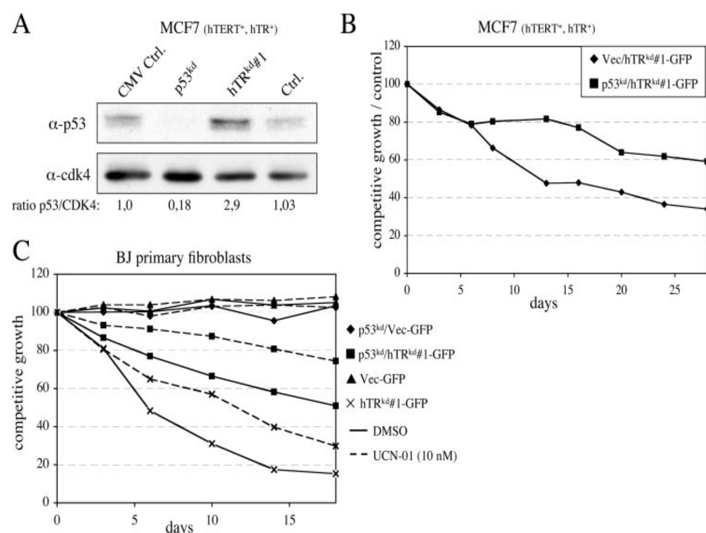


FIGURE 2. **Chk1 and p53 are required for induction of the hTR^{kd}-mediated cell cycle arrest.** *A*, MCF7 cells were transfected with indicated constructs and subjected to immunoblot analysis to detect p53 and the control CDK4. Band intensity was calculated by densitometry. *B*, MCF7 cells were transduced with $p53^{kd}$ or control vector, drug-selected, and transduced with pRS-GFP control and hTR^{kd}#1-GFP. Competitive growth assays were performed as described in the legend to Fig. 1*I*. *C*, competitive growth assays with BJ cells as in *B*. Cells were either treated with UCN-01 (10 nM, *dashed lines*) or with vehicle (dimethyl sulfoxide (*DMSO*), *solid lines*).

hTR Inhibits ATR Activity—Our results suggest an inverse correlation between hTR levels and ATR activity. Reduction in hTR levels induces p53 and CHK1, two main substrates of ATR. To examine this further, we asked whether the presence of hTR inhibits ATR activity. ATR is activated by DNA damage assaults such as UV radiation, thereby inducing the phosphorylation of $p53^{S15}$ and $CHK1^{S317}$, respectively (60–62). To test the role of hTR in this process we used U2OS cells, which express no hTR, and ectopically expressed hTR (Fig. 1*A*). Importantly, the levels of expressed hTR in U2OS cells were lower than the endogenous levels observed in MCF7 cells and could not complement for telomerase activity due to the lack of hTERT expression in these cells (data not shown). We irradiated hTR expressing and control cells with UV and followed the phosphorylation kinetics of $CHK1^{S317}$and $p53^{S15}$ in time. Interestingly, upon radiation, the phosphorylations of both $CHK1^{S317}$ and $p53^{S15}$ were severely attenuated in U2OS cells expressing hTR (Fig. 3*A*). In addition, p53 stability was not increased upon radiation in hTR-expressing cells as compared with control cells.

Since U2OS cells express neither hTR nor hTERT, it seems that hTR inhibits ATR in an hTERT independent manner. To examine this issue we aimed at testing the effects of hTR knockdown in a telomerase negative cell line. Therefore, we used GM847 human fibroblasts; these cells elongate telomeres by the ALT mechanism and express hTR but not hTERT (12). We transfected cells with the hTR^{kd}#1 construct, treated the cells with UV radiation (3 J/m^2), and monitored phosphorylation of $CHK1^{S317}$ and $p53^{S15}$ using immunoblot analysis. We found that reduction in endogenous hTR levels triggered $CHK1^{S317}$ and $p53^{S15}$ phosphorylation, as well as p53 stabilization, indicating ATR activation (Fig. 3*B*, time 0′). To exclude off target effects of hTR knockdown, we tested additional siRNAs that inhibit hTR expression. We found also those to elicit a rapid cell cycle arrest and to trigger $CHK1^{S317}$ and $p53^{S15}$ phosphorylation and p53 stabilization (supplemental Figs. S3, S4, and S7). This strongly suggests that inhibition of hTR causes the reduction in cell proliferation capacity and activation of DNA damage checkpoints. Interestingly, hTR depletion activated the DNA damage response to almost full extent as treating hTR^{kd} cells with UV resulted in only a slight activation of p53 and CHK1 compared with control cells (3B). Altogether, these results suggest that endogenous levels of hTR are functioning to balance ATR activity, a function that is telomerase-independent.

To study the effects of hTR on ATR kinase activity in more detail, we used an ATR construct with a TAP (tandem affinity purification) tag containing a TEV protease cleavage site (50). We transfected HEK293 cells with TAP-ATR or TAP control, purified them from extracts by immunoprecipitation using IgG beads (Fig. 3*C*, *lanes B*), and released using TEV protease (*lanes S*). We studied effects of hTR on ATR-kinase activity using GST-coupled p53 (amino acids 1–101) as its substrate. Fig. 3*D* shows that our purified ATR could phosphorylate p53, as expected (*lane ctrl*). When hTR is incubated with ATR a clear and potent decrease in ATR activity is observed (*lane hTR*). As control we used a truncated hTR RNA encompassing the first 211 nucleotides of hTR (*lane hTR 211*). This truncated hTR inhibited ATR activity *in vitro* to a lesser extent than the full-length hTR, indicating for the specificity of the hTR effect. Additionally, since hTR contains a H/ACA box (2, 19), we tested related H/ACA snoRNAs for their ability to influence ATR kinase activity. We found that these RNAs hardly affect ATR kinase activity, indicating that the inhibition of ATR by hTR is specific (supplemental Fig. S8). As HEK293 cells express hTR, we reasoned that addition of RNase should increase ATR activity. Indeed, when RNase was added to ATR, or to ATR and hTR, kinase activity was relatively increased. These results indicate that ATR kinase activity is inhibited by hTR *in vitro*.

To further examine the effects of hTR on ATR kinase activity we performed kinase assays with TAP-ATR in U2OS cells that lack hTR expression. Cells were transfected with TAP-ATR or TAP-control plasmid and cotransfected with CMV-hTR or control plasmids. Three days after transfection, cells were either left untreated or UV irradiated to activate ATR, and subsequently ATR was purified and subjected to a kinase assay. Fig. 3*E* shows that exogenous ATR is active in mock treated U2OS

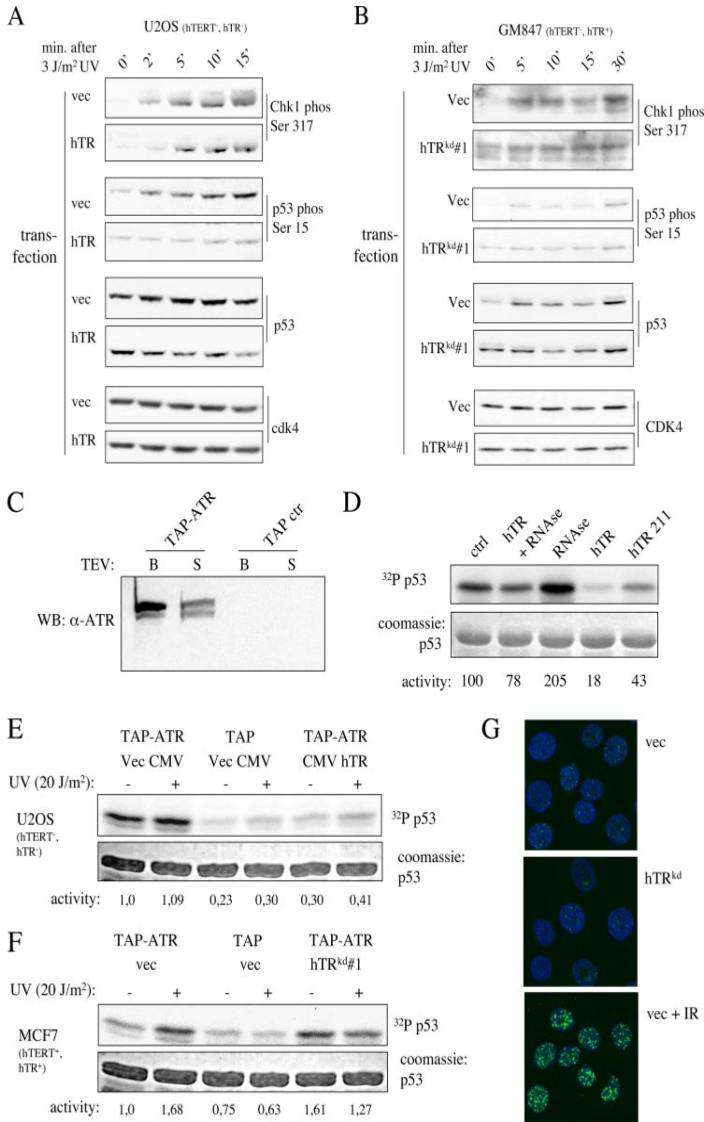


FIGURE 3. **hTR inhibits ATR kinase activity.** *A*, U2OS cells were transfected with the hTR^{kd} or control, irradiated after 3 days with 3 J/m^2 UV, and harvested at the indicated time points. Whole cell extracts were immunoblotted to detect $CHK1^{S317}$ phosphorylation, $p53^{S15}$ phosphorylation, p53, and CDK4 as a loading control. *B*, GM847 fibroblasts were transfected with hTR^{kd} or control and treated as in *A*. *C*, HEK293 cells were transfected with TAP-ctr or TAP-ATR and immunoprecipitated with IgG. TAP-ATR was cleaved with TEV protease, and beads (*B*) and supernatant (*S*) were immunoblotted to detect ATR. *WB*, Western blot. *D*, kinase assay performed with immunoprecipitation and TEV cleaved TAP-ATR from HEK293 cells with GST-p53 (residues 1–101) as a substrate. Cleaved ATR was split, and hTR, RNase, and mock were added prior to kinase reaction. Samples were separated by 10% SDS-PAGE and stained with Coomassie Blue to detect GST-p53 protein, and autoradiography was performed to detect kinase activity. Band intensities were measured by densitometry. *E*, U2OS cells were transfected with the indicated constructs and kinase assays were performed as in *B*. *F*, MCF7 cells were transfected with the indicated constructs, and kinase assays were performed as in *B*. *G*, immunofluorescence images of MCF7 cells transfected with hTR^{kd}#1, vector, or control irradiated cells (5 Gy IR), stained with γ-H2AX antibody. Nuclei are stained with DAPI (*blue*). Pictures were made with ×200 magnification. *vec*, vector.

cells and is slightly activated when cells are irradiated (*lanes 1* and *2*). However, when hTR is coexpressed with TAP-ATR, ATR activity is markedly decreased, almost to the levels of the control transfection (*lanes 5* and *6*). Next, we performed a similar experiment using MCF7 cells that, unlike U2OS cells, express hTR. Fig. 3*F* shows ATR activation upon UV radiation (*lanes 1–4*). However, reduction in hTR levels induced ATR activity already in the untreated cells (*lane 5*), which was not further activated by UV at this time point after treatment (*lane 6*).

As shown above, ATR signaling is activated when hTR is depleted. Our results so far suggest that the effect of hTR on ATR activity is direct. ATR is normally activated by DNA damage, upon which it activates p53 and CHK1 but also phosphorylates histone H2AX (40). Phosphorylated H2AX (γ-H2AX) colocalizes with other factors, such as p53BP1, MRE11, and SMC1, to foci marking sites of DNA damage, thereby facilitating the assembly of checkpoint and DNA repair factors (40). Such foci are also found at dysfunctional telomeres (5, 37). To test whether hTR depletion acts directly through ATR or indirectly via induction of DNA damage, we stained hTR^{kd} cells for γ-H2AX foci. MCF7 cells were transfected with hTR^{kd} or vector control or irradiated as positive control. While IR generated γ-H2AX foci, no increase in foci formation above control was observed in cells expressing hTR^{kd} shRNA, hTR^{kd} siRNA (data not shown) (Fig. 3*G*). A similar result was obtained previously with 53BP1 foci in LOX cells (46). Thus, we found no evidence of DNA damage in cells depleted of hTR supporting the notion that the effects of hTR on ATR are direct. Altogether, our results show that the expression of hTR influences ATR kinase activity *in vivo*.

Partial Abrogation of the G_2/M Checkpoint and Enhanced Appearance of Fragile Sites by hTR Expression—ATR has been shown to be involved in the radiation-induced G_2/M phase checkpoint in eukaryotic cells as it prevents mitotic entry of cells mainly in the late phase of the response to IR and cooperates with ATM in the early phase of the response (42). Since our results show that hTR inhibits ATR, we studied

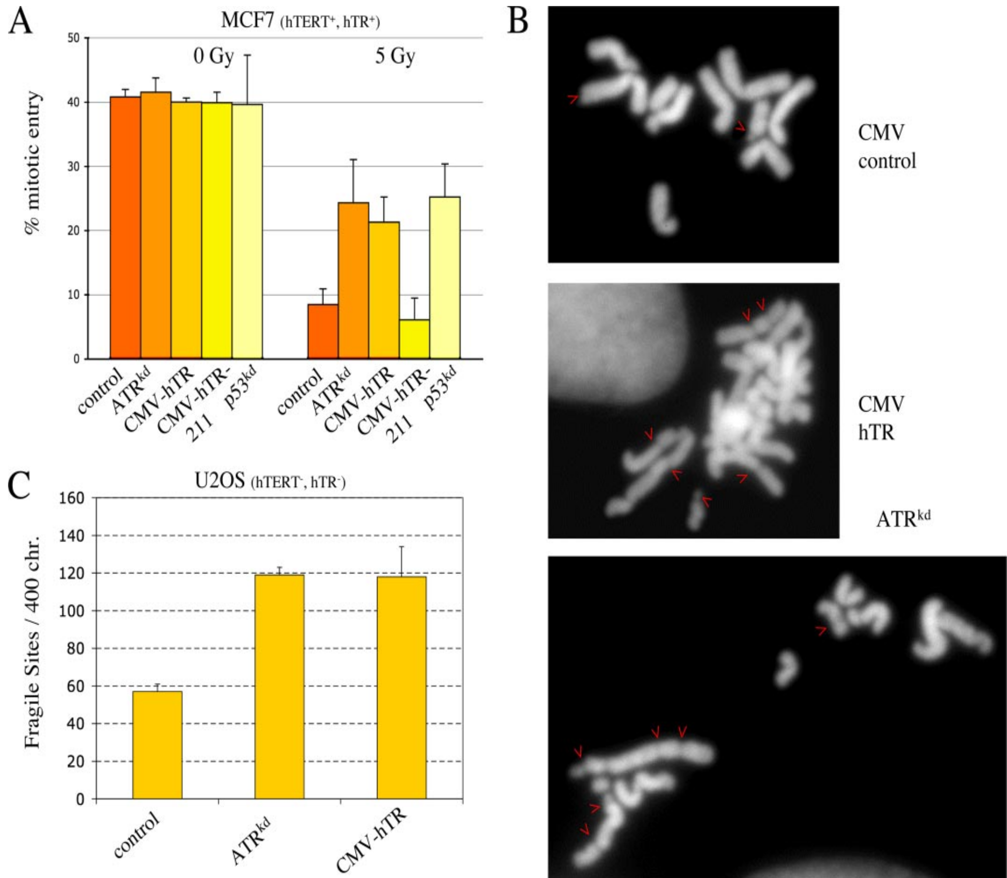


FIGURE 4. **hTR partially abrogates the G_2/M checkpoint and enhances the expression of fragile sites.** *A*, MCF7 cells were transfected with the indicated constructs irradiated with 5 Gy IR or mock treated 3 days later and incubated with nocodazole. Twenty-our hours later, cells were fixed and stained with Hoechst, and mitotic entry was scored. S.D. from three independent experiments is shown. *B*, the expression of fragile sites in U2OS cells transfected with the indicated constructs. Pictures were made with ×1000 magnification. *C*, quantification of the expression of fragile sites in U2OS cells as shown in Fig. 4*B*. The number of fragile sites expressed in 400 chromosomes is shown; data are representative of three independent experiments.

the effects of hTR expression on the G_2/M checkpoint. We monitored mitotic entry of MCF7 cells in response to IR as a measure for the number of cells able to arrest in G_2/M. We transfected MCF7 cells with CMV-hTR and as controls ATR^{kd}, p53^{kd}, or CMV-control constructs (for validation of the ATR^{kd} constructs, see supplemental Fig. S9). Three days later we irradiated cells with 5 Gy IR and treated the cells with nocodazole to inhibit progression of the cell cycle in mitosis. Cells were then fixed, stained with Hoechst and mitotic cells were counted (Fig. 4*A*). While we found no difference in the accumulation of mitotic cells in unirradiated controls, hTR overexpression, ATR^{kd} and p53^{kd}, showed a partial override of the G_2/M arrest. In contrast, cells expressing a truncated form of hTR (hTR211) behaved as control cells. Notably, hTR levels are only moderately increased in MCF7 cells upon expression of CMV-hTR (a 35% increase, supplemental Fig. S10). Altogether, these results indicate that an increase in hTR levels impairs ATR-mediated DNA damage responses in a manner similar to those induced by loss of ATR.

To further investigate whether hTR expression perturbs ATR function, we studied fragile sites stability in U2OS cells. Common fragile sites are specific chromosomal loci that appear as constrictions, gaps, or breaks on metaphase chromosomes from cells that have been exposed to partial inhibition of DNA replication (63). Addition of low levels of the DNA polymerase inhibitor aphidicolin to cells in culture has been shown to induce fragile sites. ATR recognizes stalled and collapsed DNA replication forks and activates their repair and the restarting of replication. Fragile sites are believed to be expressed when stalled forks escape the ATR replication surveillance (64). We transfected U2OS cells with CMV-hTR, ATR^{kd}, or CMV-ctrl, and treated the cells for 24 h with aphidicolin. Chromosomes were fixed, stained, and dropped onto slides to count chromosomes and fragile sites. Upon inhibition of replication, we found fragile sites instability in control cells, which was exacerbated by either loss of ATR or expression of hTR (Fig. 4*B*). Quantification of the frequency of fragile sites expression showed a 2-fold increase in the number of fragile sites in the hTR express-

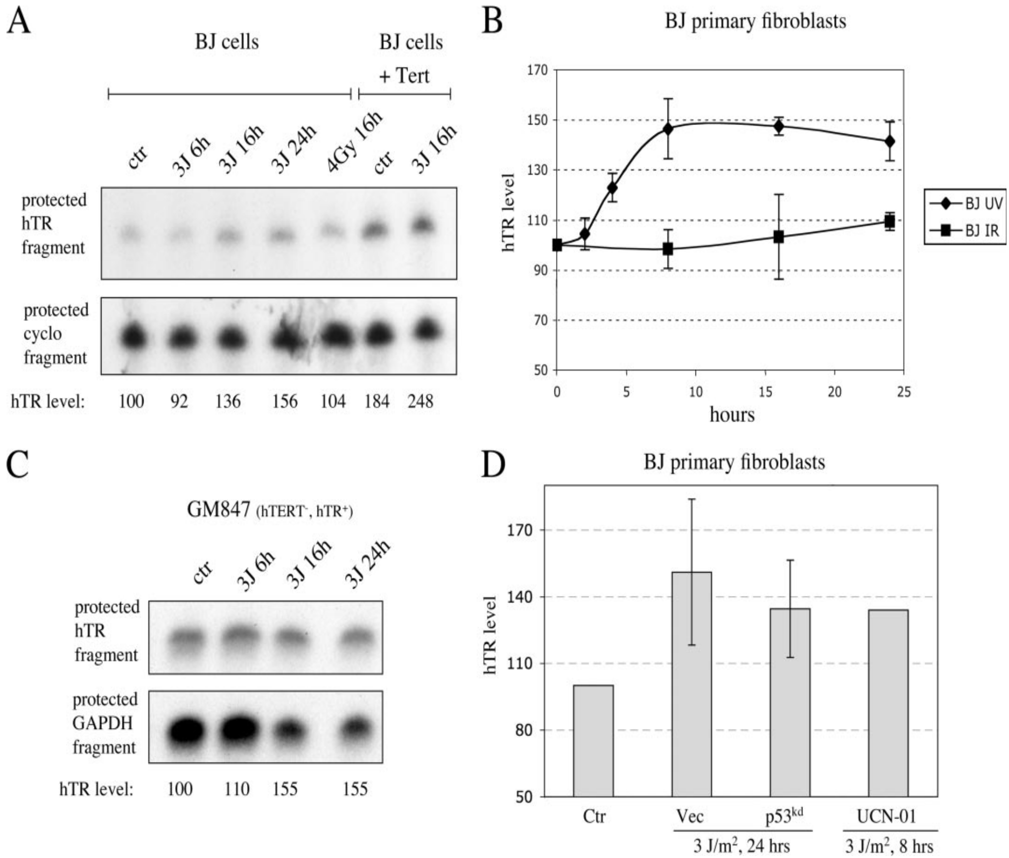


FIGURE 5. **Up-regulation of hTR levels following UV radiation.** *A*, RPA performed on extracts from BJ cells and BJ cells immortalized with hTERT irradiated with 3 J/m^2 UV or 4 Gy ionizing radiation. Cells were harvested after irradiation at the indicated time points, and quantification was performed by densitometry. *B*, quantification of RPAs on extracts of BJ cells irradiated with either 3 J/m^2 UV or 4Gy ionizing radiation. Samples were harvested at indicated time points after irradiation. S.D. is from three independent experiments; band intensities were quantified by densitometry. *C*, RPA performed on extracts of GM847 cells irradiated with 3 J/m^2 UV as in *A*. *D*, quantification of RPAs on extracts of the indicated cells irradiated with 3 J/m^2 UV. UCN-01 was added to a final concentration of 100 nM 1 h prior to irradiation. S.D. is from three independent experiments. *Ctr*, control; *Vec*, vector.

ing cells as well as in the ATR^{kd} cells (Fig. 4*C*). As maintaining low levels of fragile site expression are a prime function of ATR, our results indicate that hTR inhibits ATR activity. Altogether, these results show that hTR inhibits ATR activity to a level sufficient to impair different ATR-mediated DNA-damage checkpoint responses, implying a novel function for hTR.

hTR Up-regulation following UV Radiation—We next set out to elucidate the function of the inhibitory effect of hTR on ATR. To this end, we investigated whether stimuli that activate ATR also influence the expression of hTR. We treated primary BJ fibroblasts and BJ cells overexpressing hTERT (BJ-ET) with 3 J/m^2 UV light and extracted RNA at several time points after radiation. Subsequently, we subjected these RNA extracts to RPA with an hTR probe and cyclophilin or GAPDH as controls. Fig. 5*A* shows that following UV radiation, but not IR (4 Gy), hTR levels increase in parent BJ cells. In BJ-ET cells, as published previously (30), higher levels of hTR are detected in untreated cells. Still, a clear up-regulation of hTR levels following UV treatment is detected (Fig. 5*A*). The increase in hTR level following UV radiation was not due to a cell cycle arrest of the cells, as hTR levels did not increase in IR-treated cells (Fig. 5, *A* and *B*).

Next, we characterized the response of hTR to UV. First, we examined dependence on hTERT by using GM847 cells that express hTR but not hTERT. Also in these cells hTR was up-regulated in response to UV radiation (Fig. 5*C*), indicating that the up-regulation of hTR is independent of hTERT. Second, we investigated whether the up-regulation of hTR is dependent upon p53 or CHK1 activity by using BJ-p53^{kd} cells and cells treated with UCN-01. In these experiments, hTR was still up-regulated in response to UV radiation when compared with control time points, indicating that p53 and CHK1 are not involved in the up-regulation of hTR in response to UV radiation (Fig. 5*D*). Collectively, we uncovered that hTR is specifically up-regulated in response to low levels of UV radiation. This up-regulation is not observed in response to ionizing radiation and is independent of p53, CHK1, and hTERT.

DISCUSSION

We have identified a causative genetic interaction between the hTR and the checkpoint kinase ATR. Ectopic expression of hTR inhibits ATR, while reduction in hTR levels stimulates ATR activity. This interaction is independent of telomerase activity and telomere length as it was observed in cells lacking hTERT and in young primary human cells with telomeres long enough to allow proliferation for at least 20 to 30 passages. Interestingly, the effect of hTR on ATR activity was strong enough to influence cellular pathways. Inhibition of hTR expression elicited a p53/CHK1-dependent cell cycle arrest in the absence of apparent DNA damage, while increased expression of hTR caused defects in ATR-dependent checkpoints, such as override of the G_2/M arrest in response to DNA damage and the enhanced induction of fragile sites. These effects are specific since the hTR knockdowns do not elicit any interferon response and phenotypically depend on the endogenous expression of hTR, and the effects were observed with mild (less than 50% above endogenous) overexpression of hTR. Notably, such a mild increase in hTR expression is also observed when cells are UV irradiated, indicating that such an increase is sufficient to impair ATR dependent checkpoints (Fig. 5*B*). An important observation is that both the inhibition of ATR activity by hTR and the up-regulation of hTR by UV damage are independent of hTERT. Activation of ATR in response to UV is very rapid, occurring within minutes (Fig. 3, *A* and *B*), whereas the increase in hTR levels takes several hours and is comparable with the increase seen in MCF7 cells following hTR overexpression resulting in checkpoint suppression (Fig. 5*B* and supplemental Fig. S10). These kinetics are consistent with the idea that cell cycle inhibition has to be rapid while the recovery takes several hours, depending on the extent of damage. Based on these findings, our results imply a model where up-regulation of hTR in response to UV constitutes a feedback loop bringing down ATR activity to reinitiate cell cycle progression (Fig. 6).

Our results show that reduction in endogenous hTR levels leads to ATR activation without the induction of apparent DNA damage. These results prompted us to investigate whether ATR and hTR interact directly. Interestingly, homologues of ATR in yeast and *Arabidopsis* are involved in telomere metabolism and in yeast Mec1p (ATR homolog) associates with telomeres (38, 43, 65, 66). We were unable to demonstrate a direct interaction of ATR and hTR. Thus, our further experiments should elucidate by which mechanism hTR influences ATR activity. ATR was previously shown to be involved in signaling pathways activated by replication arrest and DNA damage assault in cells in S and G2 phases (42, 62, 67). Common fragile sites are known to be susceptible hotspots to chromosomal breakage, rearrangement, and deletion and have been implicated in the genomic instability frequently observed in cancer (63). ATR is involved in maintaining the stability of fragile sites, as its inhibition leads to an increase in the expression of fragile sites (64). Our findings demonstrate that hTR expression induces the appearance of fragile sites to an extent similar to ATR^{kd}, thus suggesting that an increase in hTR levels can lead to genomic instability. Supporting this conclusion is our observation that an increase in hTR levels weakens the G_2/M arrest, a checkpoint controlled also by ATR. These results also suggest that hTR expression can lead to reduced fidelity of the ATR-dependent checkpoints and therefore to increased genomic instability.

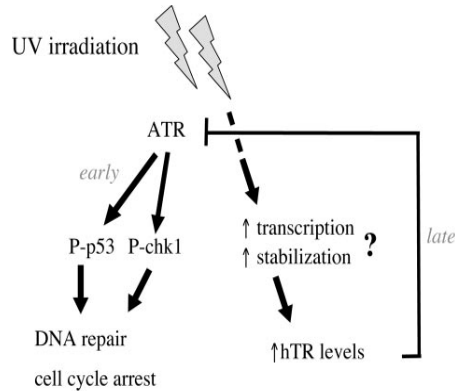


FIGURE 6. **Model for the hTR-mediated negative feedback loop on ATR activity in response to UV.** Low levels of UV radiation activate the ATR kinase early in the UV response, which phosphorylates downstream targets, among which are p53 and Chk1, leading to a cell cycle arrest and induction of DNA repair. Independently, hTR levels are increased by a yet unknown mechanism. These increased hTR levels inhibit ATR at a later stage.

Recently, the group of Elizabeth Blackburn has shown that reduction in hTR levels in cancer cells elicits a rapid anti-proliferative response (46). However, when comparing hTR knockdown in HCT116 cells with p53-null HCT116 cells they conclude that the growth inhibitory response is p53 independent. Our results, on the other hand, indicate a partial dependence on p53 function. One obvious reason for this discrepancy can be that Li *et al.* (46) based their conclusion on results obtained with two HCT116 cell lines with very different growth rates, the p53-null cell line grows an order of magnitude slower than the wild type cell line, making a comparison between growth inhibitions very difficult. In contrast, we used in our study primary human BJ and TIG3 fibroblasts as well as MCF7 breast carcinoma cells and directly compared them with corresponding knockdown p53 cells. Furthermore, our results clearly indicate that the regulation of ATR by hTR is sufficient to affect cellular pathways.

Our results seem to contrast the findings that $mTR^{-/-}$ mice have no obvious phenotype in the first generations (34). The fact that first generation $mTR^{-/-}$ mice are less prone to developing skin tumors upon chemical carcinogenesis can be explained by mild activation of some DNA damage checkpoints induced by loss of mTR (35). Whether this effect is mediated through ATR remains to be elucidated. Additionally, there are several differences between mouse and human telomere homeostasis that complicate extending findings from mouse models to the human setting. First, murine cells have extremely long and hypervariable telomeres, and telomerase activity is detectable in most somatic tissues (68, 69). Second, although telomerase is activated and mTR is up-regulated *in vivo* in several mouse tumor models, it appears not to be required for growth during the cell divisions necessary for tumor formation, suggesting that mTR and/or telomerase have additional functions to telomere extension (32–34). In addition, telomere dysfunc-

tion in mice appears to be solely dependent on p53, whereas in human cells the pRB pathway is also activated (70, 71). Third, while RNA interference causes a fast reduction in hTR levels, knock-out of telomerase RNA in germ line cells may allow for compensation events to occur (36). Thus, our results may indicate for either a difference in telomerase RNA biology between mice and men or to differences in methods used. Further experiments with murine cells are required to establish whether mTR also regulates ATR.

Last, our results may provide an explanation why in the vast majority of somatic human cells, hTR is ubiquitously expressed, whereas both hTERT and telomerase activity are mostly absent. hTR regulates DNA damage pathways in a telomerase and hTERT-independent manner. Our results, thus, can explain observations suggesting that hTR plays a role in the initiation of tumorigenicity and that it is frequently up-regulated in human cancer cell lines (23–33).

Acknowledgments—We thank all members of the Agami laboratory for stimulating discussions and Steve Jackson and Batsheva Kerem for reagents.

REFERENCES

1. Hayflick, L., and Moorhead, P. S. (1961) *Exp. Cell Res.* **25,** 585–621
2. Cong, Y. S., Wright, W. E., and Shay, J. W. (2002) *Microbiol. Mol. Biol. Rev.* **66,** 407–425, table of contents
3. Harley, C. B., Futcher, A. B., and Greider, C. W. (1990) *Nature* **345,** 458–460
4. Hemann, M. T., Strong, M. A., Hao, L. Y., and Greider, C. W. (2001) *Cell* **107,** 67–77
5. d'Adda di Fagagna, F., Reaper, P. M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N. P., and Jackson, S. P. (2003) *Nature* **426,** 194–198
6. Stewart, S. A., Ben-Porath, I., Carey, V. J., O'Connor, B. F., Hahn, W. C., and Weinberg, R. A. (2003) *Nat. Genet.* **33,** 492–496
7. Takai, H., Smogorzewska, A., and de Lange, T. (2003) *Curr. Biol.* **13,** 1549–1556
8. Herbig, U., Jobling, W. A., Chen, B. P., Chen, D. J., and Sedivy, J. M. (2004) *Mol. Cell* **14,** 501–513
9. Shay, J. W., Pereira-Smith, O. M., and Wright, W. E. (1991) *Exp. Cell Res.* **196,** 33–39
10. Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. (1994) *Science* **266,** 2011–2015
11. Shay, J. W., and Bacchetti, S. (1997) *Eur. J. Cancer* **33,** 787–791
12. Bryan, T. M., Englezou, A., Gupta, J., Bacchetti, S., and Reddel, R. R. (1995) *EMBO J.* **14,** 4240–4248
13. Greider, C. W., and Blackburn, E. H. (1985) *Cell* **43**, 405–413
14. Lingner, J., Hughes, T. R., Shevchenko, A., Mann, M., Lundblad, V., and Cech, T. R. (1997) *Science* **276,** 561–567
15. Masutomi, K., Yu, E. Y., Khurts, S., Ben-Porath, I., Currier, J. L., Metz, G. B., Brooks, M. W., Kaneko, S., Murakami, S., DeCaprio, J. A., Weinberg, R. A., Stewart, S. A., and Hahn, W. C. (2003) *Cell* **114,** 241–253
16. Wright, W. E., Piatyszek, M. A., Rainey, W. E., Byrd, W., and Shay, J. W. (1996) *Dev. Genet.* **18,** 173–179
17. Counter, C. M., Avilion, A. A., LeFeuvre, C. E., Stewart, N. G., Greider, C. W., Harley, C. B., and Bacchetti, S. (1992) *EMBO J.* **11,** 1921–1929
18. Feng, J., Funk, W. D., Wang, S. S., Weinrich, S. L., Avilion, A. A., Chiu, C. P., Adams, R. R., Chang, E., Allsopp, R. C., Yu, J., Le, S., West, W. D., Harley, C. B., Andrews, W. H., Greider, C. W., and Villeponteau, B. (1995) *Science* **269,** 1236–1241
19. Chen, J. L., Blasco, M. A., and Greider, C. W. (2000) *Cell* **100,** 503–514
20. Mitchell, J. R., Wood, E., and Collins, K. (1999) *Nature* **402,** 551–555
21. Vulliamy, T., Marrone, A., Goldman, F., Dearlove, A., Bessler, M., Mason, P. J., and Dokal, I. (2001) *Nature* **413,** 432–435
22. Chiang, Y. J., Hemann, M. T., Hathcock, K. S., Tessarollo, L., Feigenbaum, L., Hahn, W. C., and Hodes, R. J. (2004) *Mol. Cell. Biol.* **24,** 7024–7031
23. Soder, A. I., Hoare, S. F., Muir, S., Going, J. J., Parkinson, E. K., and Keith, W. N. (1997) *Oncogene* **14,** 1013–1021
24. Brown, D. F., Gazdar, A. F., White, C. L., 3rd, Yashima, K., Shay, J. W., and Rushing, E. J. (1997) *J. Neuropathol. Exp. Neurol.* **56,** 1349–1355
25. Rushing, E. J., Yashima, K., Brown, D. F., White, C. L., III, Shay, J. W., Risser, R. C., and Gazdar, A. F. (1997) *J. Neuropathol. Exp. Neurol.* **56,** 1142–1146
26. Morales, C. P., Lee, E. L., and Shay, J. W. (1998) *Cancer* **83,** 652–659
27. Maitra, A., Yashima, K., Rathi, A., Timmons, C. F., Rogers, B. B., Shay, J. W., and Gazdar, A. F. (1999) *Cancer* **85,** 741–749
28. Yashima, K., Litzky, L. A., Kaiser, L., Rogers, T., Lam, S., Wistuba, I. I., Milchgrub, S., Srivastava, S., Piatyszek, M. A., Shay, J. W., and Gazdar, A. F. (1997) *Cancer Res.* **57,** 2373–2377
29. Yashima, K., Milchgrub, S., Gollahon, L. S., Maitra, A., Saboorian, M. H., Shay, J. W., and Gazdar, A. F. (1998) *Clin. Cancer Res.* **4,** 229–234
30. Yi, X., Tesmer, V. M., Savre-Train, I., Shay, J. W., and Wright, W. E. (1999) *Mol. Cell. Biol.* **19,** 3989–3997
31. Dome, J. S., Bockhold, C. A., Li, S. M., Baker, S. D., Green, D. M., Perlman, E. J., Hill, D. A., and Breslow, N. E. (2005) *J. Clin. Oncol.* **23,** 9138–9145
32. Blasco, M. A., Rizen, M., Greider, C. W., and Hanahan, D. (1996) *Nat. Genet.* **12,** 200–204
33. Broccoli, D., Godley, L. A., Donehower, L. A., Varmus, H. E., and de Lange, T. (1996) *Mol. Cell. Biol.* **16,** 3765–3772
34. Blasco, M. A., Lee, H. W., Hande, M. P., Samper, E., Lansdorp, P. M., DePinho, R. A., and Greider, C. W. (1997) *Cell* **91,** 25–34
35. Gonzalez-Suarez, E., Samper, E., Flores, J. M., and Blasco, M. A. (2000) *Nat. Genet.* **26,** 114–117
36. Sage, J., Miller, A. L., Perez-Mancera, P. A., Wysocki, J. M., and Jacks, T. (2003) *Nature* **424,** 223–228
37. d'Adda di Fagagna, F., Teo, S.-H., and Jackson, S. P. (2004) *Genes Dev.* **18,** 1781–1799
38. Takata, H., Kanoh, Y., Gunge, N., Shirahige, K., and Matsuura, A. (2004) *Mol. Cell* **14,** 515–522
39. Verdun, R. E., Crabbe, L., Haggblom, C., and Karlseder, J. (2005) *Mol. Cell* **20,** 551–561
40. Sancar, A., Lindsey-Boltz, L. A., Unsal-Kacmaz, K., and Linn, S. (2004) *Annu. Rev. Biochem.* **73,** 39–85
41. Karlseder, J., Hoke, K., Mirzoeva, O. K., Bakkenist, C., Kastan, M. B., Petrini, J. H., and de Lange, T. (2004) *PLoS Biol.* **2,** E240
42. Brown, E. J., and Baltimore, D. (2003) *Genes Dev.* **17,** 615–628
43. Vespa, L., Couvillion, M., Spangler, E., and Shippen, D. E. (2005) *Genes Dev.* **19,** 2111–2115
44. Hahn, W. C., Stewart, S. A., Brooks, M. W., York, S. G., Eaton, E., Kurachi, A., Beijersbergen, R. L., Knoll, J. H., Meyerson, M., and Weinberg, R. A. (1999) *Nat. Med.* **5,** 1164–1170
45. Herbert, B., Pitts, A. E., Baker, S. I., Hamilton, S. E., Wright, W. E., Shay, J. W., and Corey, D. R. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14276–14281
46. Li, S., Crothers, J., Haqq, C. M., and Blackburn, E. H. (2005) *J. Biol. Chem.* **280,** 23709–23717
47. Li, S., Rosenberg, J. E., Donjacour, A. A., Botchkina, I. L., Hom, Y. K., Cunha, G. R., and Blackburn, E. H. (2004) *Cancer Res.* **64,** 4833–4840
48. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) *Cancer Cell* **2,** 243–247
49. Voorhoeve, P. M., and Agami, R. (2003) *Cancer Cell* **4,** 311–319
50. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M., and Seraphin, B. (1999) *Nat. Biotechnol.* **17,** 1030–1032
51. Agami, R., and Bernards, R. (2000) *Cell* **102,** 55–66
52. Duursma, A., and Agami, R. (2005) *Mol. Cell. Biol.* **25,** 6937–6947
53. Kim, N. W., and Wu, F. (1997) *Nucleic Acids Res.* **25,** 2595–2597
54. Scherr, M., Battmer, K., Schultheis, B., Ganser, A., and Eder, M. (2005) *Gene Ther.* **12,** 12–21
55. Bryan, T. M., Englezou, A., Dalla-Pozza, L., Dunham, M. A., and Reddel,

R. R. (1997) *Nat. Med.* **3,** 1271–1274
56. Scheel, C., Schaefer, K. L., Jauch, A., Keller, M., Wai, D., Brinkschmidt, C., van Valen, F., Boecker, W., Dockhorn-Dworniczak, B., and Poremba, C. (2001) *Oncogene* **20,** 3835–3844
57. Brummelkamp, T. R., Bernards, R., and Agami, R. (2002) *Science* **296,** 550–553
58. Busby, E. C., Leistritz, D. F., Abraham, R. T., Karnitz, L. M., and Sarkaria, J. N. (2000) *Cancer Res.* **60,** 2108–2112
59. Graves, P. R., Yu, L., Schwarz, J. K., Gales, J., Sausville, E. A., O'Connor, P. M., and Piwnica-Worms, H. (2000) *J. Biol. Chem.* **275,** 5600–5605
60. Tibbetts, R. S., Brumbaugh, K. M., Williams, J. M., Sarkaria, J. N., Cliby, W. A., Shieh, S. Y., Taya, Y., Prives, C., and Abraham, R. T. (1999) *Genes Dev.* **13,** 152–157
61. Zhao, H., and Piwnica-Worms, H. (2001) *Mol. Cell. Biol.* **21,** 4129–4139
62. Heffernan, T. P., Simpson, D. A., Frank, A. R., Heinloth, A. N., Paules, R. S., Cordeiro-Stone, M., and Kaufmann, W. K. (2002) *Mol. Cell. Biol.* **22,** 8552–8561
63. Schwartz, M., Zlotorynski, E., and Kerem, B. (2006) *Cancer Lett.* **232,** 13–26
64. Casper, A. M., Nghiem, P., Arlt, M. F., and Glover, T. W. (2002) *Cell* **111,** 779–789
65. Ritchie, K. B., Mallory, J. C., and Petes, T. D. (1999) *Mol. Cell. Biol.* **19,** 6065–6075
66. Takata, H., Tanaka, Y., and Matsuura, A. (2005) *Mol. Cell* **17,** 573–583
67. Shechter, D., Costanzo, V., and Gautier, J. (2004) *Nat. Cell Biol.* **6,** 648–655
68. Kipling, D., and Cooke, H. J. (1990) *Nature* **347,** 400–402
69. Prowse, K. R., and Greider, C. W. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 4818–4822
70. Smogorzewska, A., and de Lange, T. (2002) *EMBO J.* **21,** 4338–4348
71. Chin, L., Artandi, S. E., Shen, Q., Tam, A., Lee, S. L., Gottlieb, G. J., Greider, C. W., and DePinho, R. A. (1999) *Cell* **97,** 527–538

Legends to supplementary Figures

Fig. S1. hTR knockdown in MCF7 cytoplasmic and nuclear extracts. RPA performed on equal amounts of cytosolic and nuclear extracts from MCF7 cells transfected with either pS-hTR^{kd}#1 or control pS (t= total lysate; c= cytoplasmic extract; n=nuclear extract). Cells were harvested 4 days after transfection cellular fractionation was performed using NE-PER kit (Pierce) according to manufacturer's instructions.

FIG. S2. A control immunoblot performed with 50 ug of cytosolic and nuclear protein extracts obtained from the same cell populations described in S1.

Fig. S3. Multiple siRNAs inhibit hTR expression. RPA performed on extracts from MCF7 cells transfected with pS, hTR^{kd}#1, and three different siRNAs targeting the hTR regions indicated (the siRNAs targeting nucleotides 44-62 and 165-184 of hTR were the same as used by Li *et al*. (ref. 46)). Cells were harvested 4 days after transfection. Quantification was performed by densitometry.

Fig. S4. Inhibition of hTR expression triggers rapid growth inhibition. MCF7 cells were transfected with hTR^{kd}#1, vector constructs and indicated siRNAs and subjected to flow cytometric analysis. The percentages of cells in G1-, S- and G2-phases are shown. SD is from 3 independent experiments.

Fig. S5. Rapid growth inhibition by inhibition of hTR expression. Competitive growth assay of Hacat, HeLa, and T47D cell lines. Cells were transduced hTR^{kd}#1GFP construct and fluorescence was monitored by flow cytometry at the indicated timepoints after transduction.

Fig. S6. Induction of the cell cycle arrest mediated by hTR^{kd} in TIG3 fibroblasts is partially dependent on p53. TIG3 cells were transduced with p53^{kd} or control vector, drug selected and subsequently transduced with hTR^{kd}#1-GFP, or pRS-GFP control. Competitive growth assays were performed as described in Fig. S4.

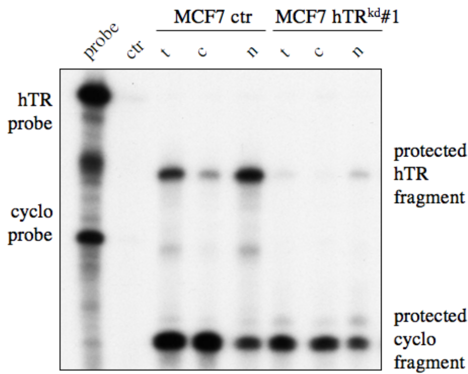
Fig. S7. Inhibition of hTR expression triggers activation of ATR downstream targets. GM847 fibroblasts were transfected with hTR^{kd}#1, control pS and the indicated siRNAs. Whole cell extracts were immunoblotted to detect CHK1^{S317} phosphorylation, p53^{S15} phosphorylation, p53, and CDK4 as a loading control.

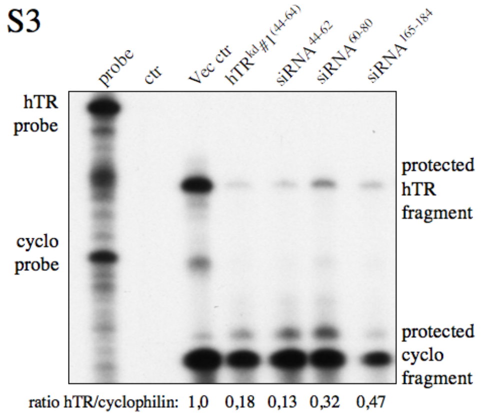
Fig. S8. Inhibition of ATR kinase activity is specific for hTR. Kinase assay performed with IP and TEV cleaved TAP-ATR from HEK293 cells with GST-p53 (residues 1-101) as a substrate. Cleaved ATR was split and mock, hTR, hTR 211, and indicated H/ACA SnoRNAs were added prior to kinase reaction. Samples were separated by 10% SDS-PAGE and stained with coomassie blue to detect GST-p53 protein and autoradiography was performed to detect kinase activity. Band intensities were measured by densitometry.

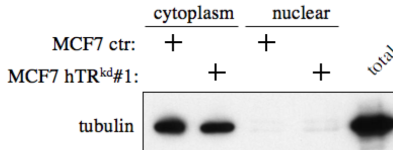
Fig. S9. Validation of ATR^{kd} constructs. Indicated ATR^{kd} constructs were cotransfected with Flag-ATR and Flag-DGK as internal control. Anti-Flag immunoprecipitation was performed 3 days after transfection and IP's were immunoblotted for Flag.

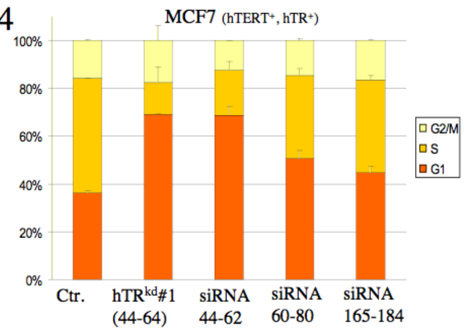
Fig. S10. hTR overexpression in MCF7 cells. RPA performed on extracts from MCF7 cells transfected with CMV-hTR, or CMV-control constructs, cells were harvested 4 days after transfection. Quantification was performed by densitometry.

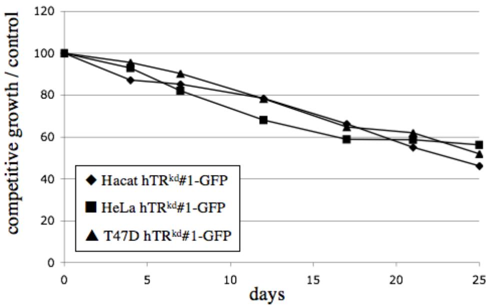
ATR regulation by hTR

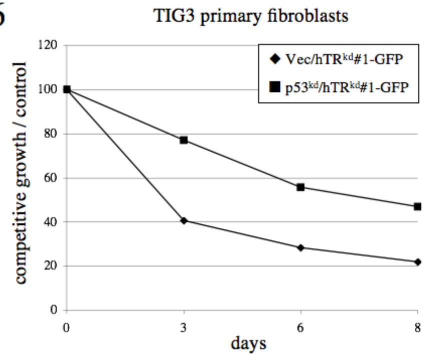


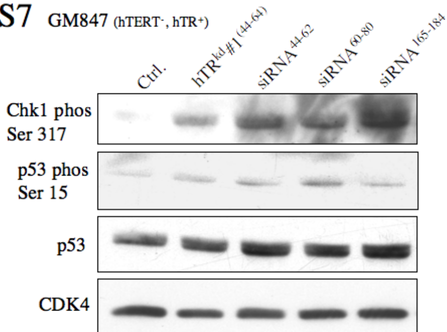


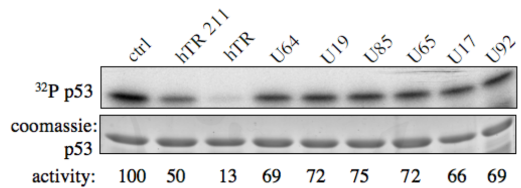


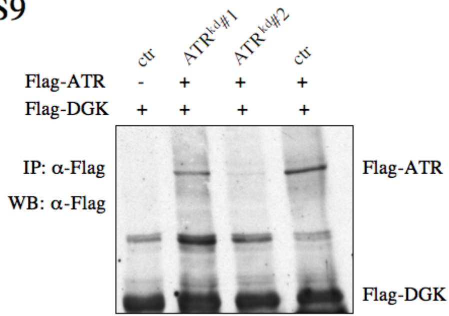


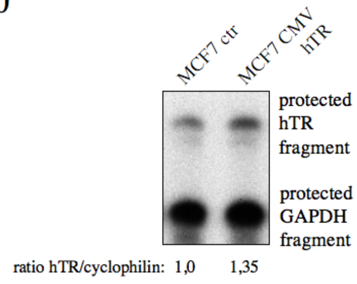









S9
ctr
ATRkd#1
ATRkd#2
ctr
Flag-ATR - + + +
Flag-DGK + + + +
IP: α-Flag
WB: α-Flag
Flag-ATR
Flag-DGK


S10
MCF7 ctr
MCF7 CMV hTR
protected hTR fragment
protected GAPDH fragment
ratio hTR/cyclophilin: 1,0 1,35

Chapter 5:

miR-148 targets human DNMT3b protein coding region

RNA, 2008 RNA, May 1; 14 (5), 2008

DOI:10.1261/rna.972008

Chapter 5

miR-148 targets human DNMT3b protein coding region

ANJA M. DUURSMA, MARTIJN KEDDE, MARIETTE SCHRIER, CARLOS LE SAGE, and REUVEN AGAMI

Division of Tumor Biology, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands

ABSTRACT

MicroRNAs (miRNAs) are small noncoding RNA molecules of 20–24 nucleotides that regulate gene expression. In animals, miRNAs form imperfect interactions with sequences in the 3′ Untranslated region (3′UTR) of mRNAs, causing translational inhibition and mRNA decay. In contrast, plant miRNAs mostly associate with protein coding regions. Here we show that human miR-148 represses DNA methyltransferase 3b (*Dnmt3b*) gene expression through a region in its coding sequence. This region is evolutionary conserved and present in the Dnmt3b splice variants Dnmt3b1, Dnmt3b2, and Dnmt3b4, but not in the abundantly expressed Dnmt3b3. Whereas overexpression of miR-148 results in decreased DNMT3b1 expression, short-hairpin RNA-mediated miR-148 repression leads to an increase in DNMT3b1 expression. Interestingly, mutating the putative miR-148 target site in *Dnmt3b1* abolishes regulation by miR-148. Moreover, endogenous Dnmt3b3 mRNA, which lacks the putative miR-148 target site, is resistant to miR-148-mediated regulation. Thus, our results demonstrate that the coding sequence of Dnmt3b mediates regulation by the miR-148 family. More generally, we provide evidence that coding regions of human genes can be targeted by miRNAs, and that such a mechanism might play a role in determining the relative abundance of different splice variants.

Keywords: miRNA; coding region; DNMT3b; splice variants

INTRODUCTION

MiRNAs are small noncoding RNAs that have been identified as post-transciptional regulators of mRNA expression in many multicellular organisms such as plants, insects, and mammals. In animals, miRNAs have been shown to inhibit mRNA translation and to decrease mRNA stability by binding sequences in the 3′UTR (Wu et al. 2006; Standart and Jackson 2007). Perfect binding of nucelotides 2–7, the miRNA "seed," to the target mRNA is considered to play a key role in target recognition (Doench and Sharp 2004; Lewis et al. 2005). Binding of other miRNA nucleotides has been suggested to be involved in mRNA repression as well, but in this instance no perfect complementarity is required (Grimson et al. 2007).

In plants, most described miRNAs bind the protein coding sequence (CDS) of their target mRNAs with very high-sequence complementarity, and this induces translational repression or RNA degradation in a way similar to RNA interference (Llave et al. 2002; Rhoades et al. 2002; Chen 2004). The existence of miRNA-binding sites in animal CDS has been predicted by computational approaches. By analyzing mRNAs for conserved complementarity to the miRNA seed sequence, about a thousand target sites were predicted to occur in vertebrate CDSs (Lewis et al. 2005) and a similar approach in flies predicted sites in the CDSs as well (Stark et al. 2007). Furthermore, by combining computational approaches and human mRNA expression data, effective miRNA sites were detected in human CDS (Grimson et al. 2007). In an experimental assay, let-7-mediated repression was observed in zebrafish embryos when a let-7 target site was fused to the GFP CDS (Kloosterman et al. 2004). Furthermore, a miRNA pull-down of mRNAs and subsequent validation of predicted CDS sites of these mRNAs in reporter assays showed that fly miRNAs can target CDSs (Easow et al. 2007). Yet, to our knowledge, no functional miRNA-binding sites have been described in mammalian coding regions.

DNA methylation is an epigenetic modification that is involved in gene silencing, chromatin remodeling, and genome stability (Jones and Baylin 2007). In mammals, DNA methyltransferases attach methyl groups to cytosine residues of long stretches of CpG dinucleotides (CpG islands) that occur in repetitive sequences or in gene promoter regions (Gal-Yam et al. 2008). DNMT3a and DNMT3b have been identified as "de novo" methyltransferase, which

Reprint requests to: Reuven Agami, Division of Tumor Biology, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands; e-mail: r.agami@nki.nl; fax: 31-20-5122029.

Article published online ahead of print. Article and publication date are at http://www.rnajournal.org/cgi/doi/10.1261/rna.972008.

methylate DNA during early development and gametogenesis (Okano et al. 1999). Inactivation of *Dnmt3b* results in embryonic lethality and *Dnmt3a* knock-out mice die shortly after birth (Okano et al. 1999). Although DNMT3a and DNMT3b have overlapping functions in imprinting of genes, DNMT3b was shown to be essential for methylation of centromeric minor satellite repeats (Okano et al. 1999). Furthermore, mutations in the human *Dnmt3b* gene have been linked to the ICF (Immunodeficiency, Centromere instability and Facial anomalies) syndrome (Xu et al. 1999). Lymphocytes from these patients display hypomethylation of centromeric repeat sequences, chromatin decondensation, and genomic instability (Jeanpierre et al. 1993; Tuck-Muller et al. 2000). In cancer, single nucleotide polymorphisms in the *Dnmt3b* promoter that were hypothesized to result in elevated Dnmt3b expression have been correlated with an increased risk of lung cancer (Shen et al. 2002; Lee et al. 2005). Interestingly, a recent study showed direct regulation of the Dnmt3a and Dnmt3b 3′UTRs by miR-29 and reduced expression of miR-29 correlated inversely with increased expression of these Dnmts in lung cancer tissue (Fabbri et al. 2007).

Dnmt3b has several splice variants, of which Dnmt3b1 and Dnmt3b3 are expressed most abundantly (Robertson et al. 1999). DNMT3b1 and DNMT3b2 have been shown to be catalytically active (Okano et al. 1998), whereas the role of DNMT3b3 in DNA methylation is not fully understood. Murine DNMT3b3 is catalytically inactive both in vitro (Aoki et al. 2001) and in vivo (Chen et al. 2003); however, the DNA methyltransferase activity of human DNMT3b3 depended on which substrate was chosen (Soejima et al. 2003; Chen et al. 2005). This discrepancy might reflect a change in target preference since Dnmt3b3, but also Dnmt3b4, lack a motif that could be responsible for target recognition (Kumar et al. 1994). Interestingly, high expression of splice variant Dnmt3b4 has been associated with DNA hypomethylation at pericentromeric satellite regions (Saito et al. 2002). This could be a result of dominant-negative regulation or an example of changed target preference. The latter could result in altered gene expression, and this was indeed observed by microarray analysis in cells that exogenously expressed Dnmt3b7, a splice variant that was found highly expressed in cancer cells (Ostler et al. 2007). Another possibility would be that DNMT3b3 stimulates DNMT3b1 catalytic activity in a similar way to the DNMT3b-related protein DNMT3L, which also lacks methyltransferase activity (Chen et al. 2005).

Here, we describe our finding that human miR-148 regulates DNMT3b expression through interaction with a conserved site in its protein coding sequence. Interestingly, the target site is absent in the abundantly expressed Dnmt3b3 splice variant, making it resistant to miRNA-mediated regulation. Thus, the expression of miR-148 changes the relative abundance of DNMT3b splice variants.

RESULTS AND DISCUSSION

In plants, most miRNAs regulate mRNA expression by interacting with highly homologous CDS (Rhoades et al. 2002). We identified a site in the *Dnmt3b* CDS (nucleotides 2384–2411) that is highly homologous to the miR-148 family (Fig. 1A). This region has the potential to interact with all 22 nucleotides (nt) of miR-148a and miR-148b (leaving only one gap) and therefore resembles miRNA target interactions observed in plants. Importantly, the target site (referred to as site #1) is conserved in rhesus, mouse, rat, dog, horse, and armadillo *Dnmt3b1*, *Dnmt3b2*, and *Dnmt3b4*, suggesting that this sequence is retained through evolution (Fig. 1A). However, this particular sequence is not present in the *Dnmt3b3* splice variant (Fig. 1B; data not shown). Another site (#2) that contains complementarity to the miR-148 family is situated between nucleotides 1424 and 1439 (determined using targetscanS and RNA22) (Fig. 1B). Although it has a good match of the miRNA seed sequence (nucleotides 2–7), which is thought to specify miRNA targets in 3′UTRs (Doench and Sharp 2004; Lewis et al. 2005), extensive complementarity such as observed with site #1 is not seen.

Next, we designed experiments to study the regulation of Dnmt3b by miR-148. First, we used miR-Vec-148a, a miRNA-expression vector for ectopic expression of miR-148a (Voorhoeve et al. 2006). Using an RNase protection

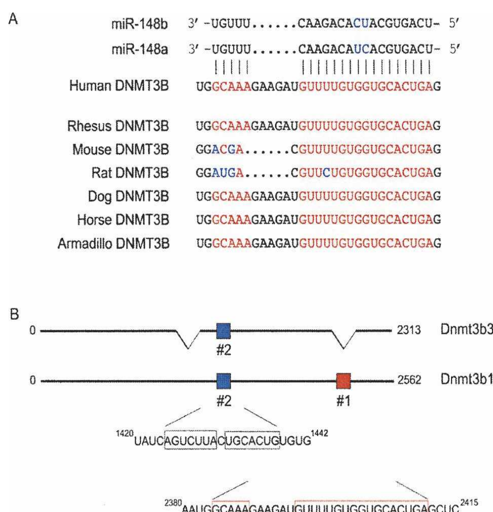


FIGURE 1. Schematic representation of putative miR-148 sites in the *Dnmt3b1* coding region. (*A*) miR-148a and miR-148b are highly complementary to nucleotides 2382–2412 of human Dnmt3b. This potential miR-148 target site is conserved in rhesus, mouse, rat, dog, horse, and armadillo. This site is referred to as target site #1. (*B*) Schematic representation of two possible miR-148 target sites in the *Dnmt3b* coding region.

assay (RPA) for miR-148 in HeLa cells, which were previously shown to be negative for miR-148 (Barad et al. 2004), transient and stable introduction of miR-Vec-148a led to high expression of this miRNA (Fig. 2A). Second, we determined whether we could identify cell lines that endogenously express miR-148 by quantitative real-time (qRT) PCR specific for mature miR-148a. We observed high miR-148 expression in the 2102EP testicular germ-cell line, 293T cells, Jurkat T cells, but not in HeLa, MCF-7 cells, and diploid BJ fibroblasts that were immortalized with telomerase (Fig. 2B). Notably, the high expression observed in the transient transfected miR-148a HeLa cells was very similar to the endogenous expression of miR-148a found in Jurkat cells (Fig. 2B). In contrast to miR-148a, we were unable to detect endogenous expression miR-148b (which was originally cloned from rat) in human cell lines (data not shown). Third, we cotransfected miR-Vec-148a with an expression vector containing the coding region for *Dnmt3b1* downstream of the green fluorescent protein (*GFP-Dnmt3b1*) and performed immunoblotting analysis for GFP. Notably, Dnmt3b was cloned in-frame with GFP, which was verified by sequencing. Furthermore, the translational fusion product was detected at the appropriate size by Immunoblotting analysis (data not shown). We observed a marked reduction of 60% of GFP-DNMT3b1 expression, but not of cotransfected H2B-GFP, in cells expressing miR-148 (Fig. 2C). As expected from the sequence similarity between miR-148a and miR-148b, a similar reduction of GFP-DNMT3b1 expression in cells expressing miR-148b was observed (Fig. 2D). These results suggest that the presence of miR-148 represses the expression of DNMT3b1.

To determine whether the observed reduction in DNMT3b1 expression by miR-148 is dependent on the putative miR-148 target sites, we mutated these regions to significantly reduce recognition by the miRNAs. Importantly, mutating these sites did not change the frame of the fusion proteins, as was verified by sequencing and immunoblotting analysis (data not shown). Transient cotransfection experiments revealed that mutating the highly homologous target site #1 almost completely abrogated miR-148-mediated regulation (Fig. 2E). However, a mutant of target site #2, which has much less complementarity to miR-148, still retained sensitivity to miR-148. This suggests that miR-148 targets Dnmt3b1 through a highly complementary sequence in the Dnmt3b1 coding region.

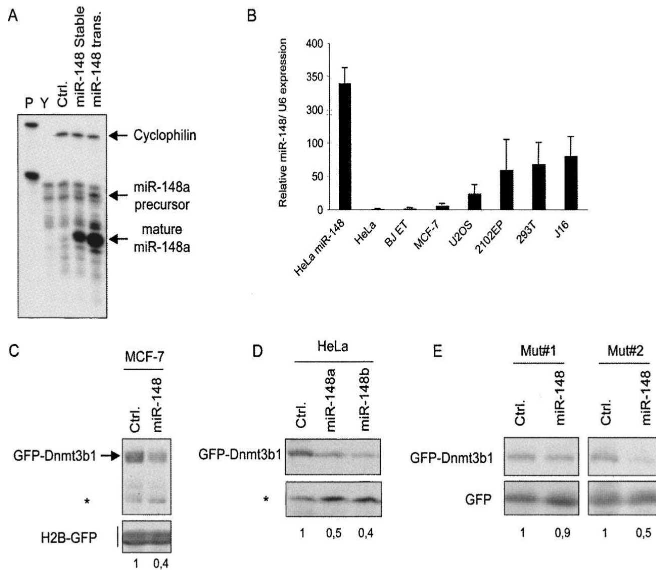


FIGURE 2. miR-148 regulates exogenous DNMT3b protein expression through interaction with its protein coding region. (*A*) RPA was used to detect miR-148 levels in HeLa cells that were transfected with miR-148, a control miRNA (Ctrl) or in a stable polyclonal pool of miR-148a expressing cells. The protected fragments are indicated. Lane P shows the probes for cyclophilin and miR-148a. In lane Y, yeast RNA was used as control. (*B*) qRT-PCR to detect miR-148a expression was performed using the indicated cell lines. Error bars represent standard deviation. (*C*) MCF-7 cells were cotransfected with either miR-Vec-148a or a miR-Vec control, together with GFP-Dnmt3b and H2B-GFP. Whole-cell extracts were subjected to immunoblot analysis to detect GFP. H2B-GFP was used to demonstrate equal transfection efficiencies. Asterisk marks a nonspecific band and shows equal loading. (*D,E*) As described in *C*. HeLa cells were transfected with the indicated constructs.

The results above imply that the Dnmt3b3 splice variant, which lacks miR-148 target site #1, is resistant to miR-148-mediated regulation. To examine this issue, we designed primers for qRT-PCR that distinguish endogenous Dnmt3b3 from Dnmt3b1 and other variants that express the targeted exon such as Dnmt3b2 and Dnmt3b4 mRNA (Fig. 3A). This analysis revealed that stable expression of miR-148a in HeLa cells resulted in small but significant reduction ($P < 0.001$) in Dnmt3b1 mRNA levels of 25%, while no reduction in Dnmt3b3 mRNA levels was observed (Fig. 3A). Thus, the expression of miR-148a affects the mRNA stability of Dnmt3b1, but not Dnmt3b3, leading to a relative increase in the abundance of Dnmt3b3 compared with Dnmt3b1 and all other Dnmt3b splice variants that express the targeted site.

DNMT3b protein is highly expressed in undifferentiated embryonic stem cells, whereas its expression level is much reduced in somatic cells (Okano et al. 1998). To study the effect of miR-148a on endogenous DNMT3b protein expression, we attempted to detect it in several tumor cell lines using immunoblot analysis. In our hands, only 2102EP testicular germ-cell tumor cells

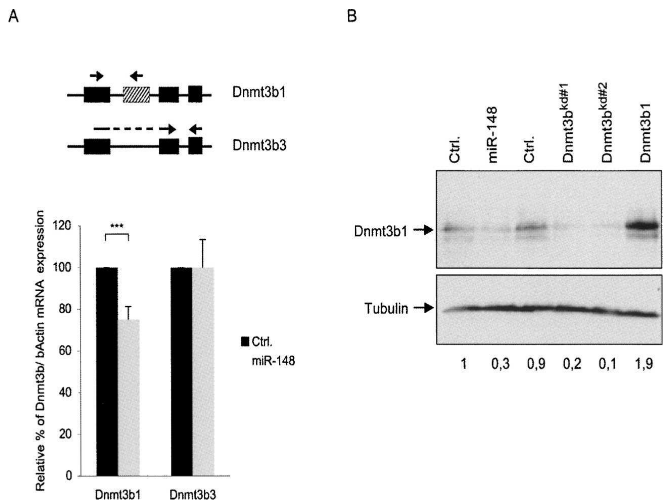


FIGURE 3. miR-148 reduces endogenous Dnmt3b1 mRNA and protein level. (*A*) qRT-PCR of either miR-Vec-148 or control (Ctrl.) transfected HeLa cells. Specific primers were used to detect Dnmt3b1 and Dnmt3b3 as shown schematically. Error bars represent standard deviation, $n = 4$ and (***) $P < 0.001$. (*B*) 2102EP cells were electroporated with the indicated constructs and subjected to immunoblot analysis for DNMT3b and TUBULIN.

expressed endogenous DNMT3b in sufficient amounts to allow detection by Western blot (data not shown). Furthermore, these cells express moderate levels of miR-148a (Fig. 2B). To assess whether increasing the miR-148 level would result in a reduced endogenous DNMT3b1 protein level, we electroporated the miR-148 expression vector into 2102EP cells and subjected them to immunoblotting analysis with a specific anti-Dnmt3b antibody. In line with the results above, overexpression of miR-148a reduced DNMT3b1 protein levels up to 70% compared with two different miRNA control constructs (Fig. 3B). We used two short-hairpin RNA (shRNA) constructs targeting Dnmt3b and an expression vector for *Dnmt3b1* to control for the specificity of the Dnmt3b antibody (Fig. 3B).

Finally, we asked whether inhibition of miR-148a activity would elevate DNMT3b1 expression. We designed two shRNA constructs targeting the miR-148 precursor RNA. Electroporation of 21202EP cells with these constructs showed that one construct, miR-148$^{kd\#2}$, was functional in reducing endogenous miR-148 levels as determined by RPA analysis (Fig. 4A). Western blot analysis of cells electroporated with miR-148$^{kd\#2}$ indeed revealed an increase in the DNMT3b1 protein levels (Fig. 4B). We therefore conclude that endogenous DNMT3b1 expression is controlled by miR-148a in 2102EP cells.

Our data reveal a novel function of miRNA-mediated regulation. miRNA targeting of human coding regions in addition to 3′UTRs not only extends the range of potential target sites, but also allows for splice variant-specific regulation. Significantly, different DNMT3b splice variants might play a role in regulating DNMT3b activity or target-site preference. This could be an additional mechanism to the regulation of splice variant abundance by alternative splicing. Our results rule out the possibility that alternative splicing is induced by miR-148 targeting, since miR-148 reduced expression of exogenous DNMT3b-GFP in a site-specific manner.

It is interesting that only the high-affinity miR-148-target site in Dnmt3b1 was affected by miR-148. In the future, it will be important to determine whether miRNA binding to CDS requires more complementarity with the target than interactions with 3′UTRs. Plant miRNAs interact with high-sequence complementarity with their target CDS eliciting, in most of the cases, an RNA interference-mediated cleavage of the target mRNA (Llave et al. 2002; Rhoades et al. 2002). Although we observed reduction of Dnmt3b mRNA in the presence of miR-148, the reduction was less prominent than the observed reduction on protein levels. This suggests that miR-148 induces both translational repression and mRNA degradation of Dnmt3b1. Further experiments should approach this point.

The mechanism by which miRNAs mediate repression is not completely understood. Whereas some evidence exists for interference with initiation of translation, others suggest that miRNAs prevent the "closed loop" mRNA configuration induced by interaction of poly(A)-binding proteins

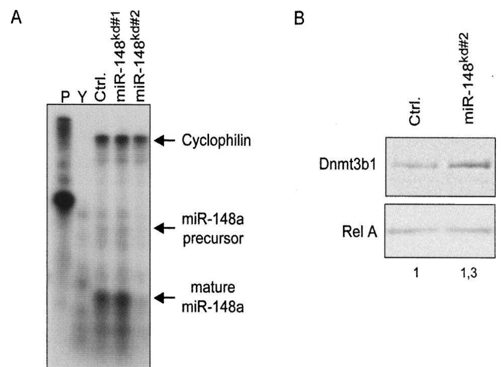


FIGURE 4. Suppression of endogenous miR-148 increases DNMT3b1 expression. (*A*) miR-148a level was detected by RPA as described in Figure 2A. (*B*) 2102EP cells were electroporated with either control (Ctrl.) or miR-148$^{kd\#2}$ constructs and subjected to immunoblot analysis with Dnmt3b and Rel A antibodies.

with initiation factors at the 5′ cap (Standart and Jackson 2007). The latter enhances translation efficiency. miR-148 targets Dnmt3b1 ∼150 nt before the stop codon in the CDS, which is near the 3′UTR and the poly(A) tail. Therefore, the location of the miR-148 target site might allow for miRNA-mediated repression in a similar fashion as 3′UTR binding miRNAs. On the other hand, experiments with miRNA target sites cloned in the 5′UTR of a luciferase construct showed miRNA-mediated repression (Lytle et al. 2007), suggesting that repression might be independent of the target site location. The exact mechanism through which miR-148 targets Dnmt3b1 remains to be explored.

In conclusion, we present evidence for a functional interaction of human miR-148 with the Dnmt3b1 CDS. It would be interesting to see whether miRNA-mediated regulation of protein coding regions turn out to be a common theme in miRNA targeting of mammalian mRNAs or the exception that confirms the rule.

MATERIALS AND METHODS

Constructs and antibodies

Dnmt3b expression constructs were cloned by PCR in pEGFP-C2 (Clontech). *Dnmt3b* mutant constucts were generated by site-directed mutagenesis using PCR, and constructs were verified by DNA sequence analysis. Mutant#1 ^{1429}TACTGCACTG^{1438} was replaced by TTCTCGTCA. Mutant#2 ^{2398}TTGTGGTGCACT^{2409} was replaced by TTAGCCGCGACC. All miRNAs were expressed from a retroviral miR-Vec vector as described before (Voorhoeve et al. 2006). We used pSuper short-hairpin constructs (Brummelkamp et al. 2002) targeting Dnmt3b and miR-148: Dnmt3b^{kd}#1 AGATGACGGATGCCTAGAG Dnmt3b^{kd}#2 (AGGTAGGAAAGTACGTCGC) miR-148^{kd}#1 ACTCTGAGTATGATAGAAG miR-148^{kd}#2 GTCAGTGCACTACAGAACT. Antibodies against Dnmt3b (H-230, Santa Cruz), Tubulin (YL1/2, ECACC), and NFkB p65 (C-20 Santa Cruz) and GFP (a gift from J. Neefjes) were used.

Cell culture and transfection

Hela, 2102EP, 293T, MCF-7, U2OS, BJ ET primary fibroblasts (expressing the ecotrophic receptor and human telomerase) were grown in Dulbecco's Modified Eagle Medium and Jurkat cells in Iscove's Modified Dulbecco's Medium suplemented with 10% fetal bovine serum. Hela cells were transfected with Fugene (Roche) using the manufacturer's protocol. 2102 EP cells were transfected by electroporation as described (Agami and Bernards 2000). A Gene Pulser Xcell Electroporation System (Bio-Rad) was used at 120 volts, 10× 1.5 msec burst duration and 1 sec interval.

Immunoblotting

For Western blot analysis, whole-cell extracts were separated at 6% SDS-PAGE minigels and transferred to polyvinylidene difluoride membranes (Millipore). Western blots were developed using enhanced chemiluminescence (Amersham Biosciences). Densitometric quantification of Western blot assays was done by using Tina (version 2.09f) software.

RNA isolation, RPA, and real-time RT-PCR

RNA was isolated from cells using Trizol (Invitrogen). RNase protection assays (RPAs) were performed using the mirVana miRNA probe construction and detection kits (Ambion) according to the manufacturers' protocol. A total of 5 μg of RNA was used per assay and a ATAGAAGTCAGTGCACTACAGAACTTTGTCTCCCTGTCTC primer was used to make a miR-148a probe. Quantification was performed by densitometry.

cDNA was prepared from 1.5 μg of RNA using random hexamer primers (Superscript III first-strand synthesis system for RT-PCR, Invitrogen). Quantitative RT-PCR was performed with a standard two-step amplification protocol of a MiniOpticon System (Bio-Rad) apparatus using a SYBRgreen PCR master mix (Applied Biosystem) and specific primers:

Dnmt3b1 forward GCCGTTCTTCTGGATGTTTGAG;
Dnmt3b1 reverse ATCCTATTGTATTCCAAGCAGTCC;
Dnmt3b3 forward ATCTCACGGTTCCTGGAGTG;
Dnmt3b3 reverse AAGCCAAAGATCCTGTTCATCC;
β Actin forward CCTGGCACCCAGCACAAT; and
β Actin reverse GGGCCGGACTCGTCATACT.

The mirVana qRT-PCR miRNA detection kit (Ambion) was used to detect miRNA expression by quantitative RT-PCR. Specific miR-148a and U6 RT and PCR primers were used (Ambion) and 25 ng of RNA was used per reaction.

ACKNOWLEDGMENTS

We thank all members of the Agami laboratory for stimulating discussions. This work was supported by the Dutch Cancer Society (KWF) and the European Young Investigator award (EURYI) to R.A.

Received December 21, 2007; accepted February 6, 2008.

REFERENCES

Agami, R. and Bernards, R. 2000. Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. *Cell* **102:** 55–66.

Aoki, A., Suetake, I., Miyagawa, J., Fujio, T., Chijiwa, T., Sasaki, H., and Tajima, S. 2001. Enzymatic properties of de novo-type mouse DNA (cytosine-5) methyltransferases. *Nucleic Acids Res.* **29:** 3506–3512. doi: 10.1093/nar/29.17.3506.

Barad, O., Meiri, E., Avniel, A., Aharonov, R., Barzilai, A., Bentwich, I., Einav, U., Gilad, S., Hurban, P., Karov, Y., et al. 2004. MicroRNA expression detected by oligonucleotide microarrays: System establishment and expression profiling in human tissues. *Genome Res.* **14:** 2486–2494.

Brummelkamp, T.R., Bernards, R., and Agami, R. 2002. A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296:** 550–553.

Chen, X. 2004. A microRNA as a translational repressor of APETALA2 in *Arabidopsis* flower development. *Science* **303:** 2022–2025.

Chen, T., Ueda, Y., Dodge, J.E., Wang, Z., and Li, E. 2003. Establishment and maintenance of genomic methylation patterns

in mouse embryonic stem cells by Dnmt3a and Dnmt3b. *Mol. Cell. Biol.* **23:** 5594–5605.

Chen, Z.X., Mann, J.R., Hsieh, C.L., Riggs, A.D., and Chedin, F. 2005. Physical and functional interactions between the human DNMT3L protein and members of the de novo methyltransferase family. *J. Cell. Biochem.* **95:** 902–917.

Doench, J.G. and Sharp, P.A. 2004. Specificity of microRNA target selection in translational repression. *Genes & Dev.* **18:** 504–511.

Easow, G., Teleman, A.A., and Cohen, S.M. 2007. Isolation of microRNA targets by miRNP immunopurification. *RNA* **13:** 1198–1204.

Fabbri, M., Garzon, R., Cimmino, A., Liu, Z., Zanesi, N., Callegari, E., Liu, S., Alder, H., Costinean, S., Fernandez-Cymering, C., et al. 2007. MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B. *Proc. Natl. Acad. Sci.* **104:** 15805–15810.

Gal-Yam, E.N., Saito, Y., Egger, G., and Jones, P.A. 2008. Cancer epigenetics: Modifications, screening, and therapy. *Annu. Rev. Med.* **59:** 267–280.

Grimson, A., Farh, K.K., Johnston, W.K., Garrett-Engele, P., Lim, L.P., and Bartel, D.P. 2007. MicroRNA targeting specificity in mammals: Determinants beyond seed pairing. *Mol. Cell* **27:** 91–105.

Jeanpierre, M., Turleau, C., Aurias, A., Prieur, M., Ledeist, F., Fischer, A., and Viegas-Pequignot, E. 1993. An embryonic-like methylation pattern of classical satellite DNA is observed in ICF syndrome. *Hum. Mol. Genet.* **2:** 731–735.

Jones, P.A. and Baylin, S.B. 2007. The epigenomics of cancer. *Cell* **128:** 683–692.

Kloosterman, W.P., Wienholds, E., Ketting, R.F., and Plasterk, R.H. 2004. Substrate requirements for let-7 function in the developing zebrafish embryo. *Nucleic Acids Res.* **32:** 6284–6291.

Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Posfai, J., Roberts, R.J., and Wilson, G.G. 1994. The DNA (cytosine-5) methyltransferases. *Nucleic Acids Res.* **22:** 1–10. doi: 10.1093/nar/22.1.1.

Lee, S.J., Jeon, H.S., Jang, J.S., Park, S.H., Lee, G.Y., Lee, B.H., Kim, C.H., Kang, Y.M., Lee, W.K., Kam, S., et al. 2005. DNMT3B polymorphisms and risk of primary lung cancer. *Carcinogenesis* **26:** 403–409.

Lewis, B.P., Burge, C.B., and Bartel, D.P. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120:** 15–20.

Llave, C., Xie, Z., Kasschau, K.D., and Carrington, J.C. 2002. Cleavage of Scarecrow-like mRNA targets directed by a class of *Arabidopsis* miRNA. *Science* **297:** 2053–2056.

Lytle, J.R., Yario, T.A., and Steitz, J.A. 2007. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5′ UTR as in the 3′ UTR. *Proc. Natl. Acad. Sci.* **104:** 9667–9672.

Okano, M., Xie, S., and Li, E. 1998. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat. Genet.* **19:** 219–220.

Okano, M., Bell, D.W., Haber, D.A., and Li, E. 1999. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* **99:** 247–257.

Ostler, K.R., Davis, E.M., Payne, S.L., Gosalia, B.B., Exposito-Cespedes, J., Le Beau, M.M., and Godley, L.A. 2007. Cancer cells express aberrant DNMT3B transcripts encoding truncated proteins. *Oncogene* **26:** 5553–5563.

Rhoades, M.W., Reinhart, B.J., Lim, L.P., Burge, C.B., Bartel, B., and Bartel, D.P. 2002. Prediction of plant microRNA targets. *Cell* **110:** 513–520.

Robertson, K.D., Uzvolgyi, E., Liang, G., Talmadge, C., Sumegi, J., Gonzales, F.A., and Jones, P.A. 1999. The human DNA methyltransferases (DNMTs) 1, 3a and 3b: Coordinate mRNA expression in normal tissues and overexpression in tumors. *Nucleic Acids Res.* **27:** 2291–2298. doi: 10.1093/nar/27.11.2291.

Saito, Y., Kanai, Y., Sakamoto, M., Saito, H., Ishii, H., and Hirohashi, S. 2002. Overexpression of a splice variant of DNA methyltransferase 3b, DNMT3b4, associated with DNA hypomethylation on pericentromeric satellite regions during human hepatocarcinogenesis. *Proc. Natl. Acad. Sci.* **99:** 10060–10065.

Shen, H., Wang, L., Spitz, M.R., Hong, W.K., Mao, L., and Wei, Q. 2002. A novel polymorphism in human cytosine DNA-methyltransferase-3B promoter is associated with an increased risk of lung cancer. *Cancer Res.* **62:** 4992–4995.

Soejima, K., Fang, W., and Rollins, B.J. 2003. DNA methyltransferase 3b contributes to oncogenic transformation induced by SV40T antigen and activated Ras. *Oncogene* **22:** 4723–4733.

Standart, N. and Jackson, R.J. 2007. MicroRNAs repress translation of m7Gppp-capped target mRNAs in vitro by inhibiting initiation and promoting deadenylation. *Genes & Dev.* **21:** 1975–1982.

Stark, A., Lin, M.F., Kheradpour, P., Pedersen, J.S., Parts, L., Carlson, J.W., Crosby, M.A., Rasmussen, M.D., Roy, S., Deoras, A.N., et al. 2007. Discovery of functional elements in 12 *Drosophila* genomes using evolutionary signatures. *Nature* **450:** 219–232.

Tuck-Muller, C.M., Narayan, A., Tsien, F., Smeets, D.F., Sawyer, J., Fiala, E.S., Sohn, O.S., and Ehrlich, M. 2000. DNA hypomethylation and unusual chromosome instability in cell lines from ICF syndrome patients. *Cytogenet. Cell Genet.* **89:** 121–128.

Voorhoeve, P.M., le Sage, C., Schrier, M., Gillis, A.J., Stoop, H., Nagel, R., Liu, Y.P., van Duijse, J., Drost, J., Griekspoor, A., et al. 2006. A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. *Cell* **124:** 1169–1181.

Wu, L., Fan, J., and Belasco, J.G. 2006. MicroRNAs direct rapid deadenylation of mRNA. *Proc. Natl. Acad. Sci.* **103:** 4034–4039.

Xu, G.L., Bestor, T.H., Bourc'his, D., Hsieh, C.L., Tommerup, N., Bugge, M., Hulten, M., Qu, X., Russo, J.J., and Viegas-Pequignot, E. 1999. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. *Nature* **402:** 187–191.

‘We’re still confused, but on a higher level.’
Enrico Fermi

Chapter 6:

General discussion

General discussion

Our protein-centered view of cellular processes is being challenged by findings that non-coding RNAs regulate development and homeostasis. Apart from a few examples of RNA posessing enzymatic activity, such as ribozymes, miRNAs are now known to regulate translation of many, if not most, proteins. The regulation of protein activity, such as described in chapter 4 for hTR, is also becoming a common theme in RNA regulation. Ever more such mechanisms are being discovered, leading to the realisation that much of our genome actually codes for regulatory RNAs of some sort (Amaral et al., 2008; Mattick, 2007). These non-coding RNAs are bound by RNA binding proteins, and other RNAs, eventually guiding them to their targets. One such mechanism is described in chapter 3 for DND1. We are just beginning to understand some parts of these huge regulatory networks that we know are important for proper development and normal homeostasis of cells.

In chapter 3, I have described the regulatory role of the RNA binding protein Dead end 1 in the development of germ cells, as we found in zebrafish model systems and human cell lines. By binding to mRNAs, DND1 inhibits miRNA activity towards certain targets that are important in the development of germ cells. It does so by inhibiting access of these miRNAs to their target messenger RNAs through binding to specific sequences. In chapter 5 we revealed a novel mode of miRNA mediated regulation. We identified a functional miRNA target sequence in the coding region of DNA methyl transferase 3b, a mode of miRNA regulation thus far only described in plants. Chapter 4 describes the elucidation of a novel function of the RNA component of telomerase, hTR. HTR levels were found to balance the activity of the ATR checkpoint kinase, independent of telomerase. Elevated hTR levels, as often found in tumor cells, inhibit the checkpoint kinase ATR, whereas a decrease in hTR levels induces ATR activity. Decreased ATR activity has been shown to induce fragile sites which fuel genomic instability and, thereby, cancer.

hTR, an oncogene fueling cancer without telomerase?

We have shown a genetic interaction between ATR and hTR and confirmed this in several tumor- and primary cell lines. An obvious question is whether this observed interaction is a direct one. We have addressed this question in several ways, from direct binding of labelled hTR to ATR, electrophoretic mobility shift assays (EMSA), and direct precipitation of ATR from cells, detecting hTR by rt-pcr/southern/qPCR. Our (unpublished) data suggests that ATR does have a (direct) binding capacity towards RNA and a certain specificity for hTR. Since ATR is a very large protein (2644 amino acids, ~310 kDa), such analysis is difficult to perform in a quantitative and controlled way. For this reason I also constructed GST-tagged, overlapping, parts of the ATR protein and tested those for interaction in *in vitro* binding assays. Strikingly, we found interaction with hTR for the carboxy-terminal kinase domain and one other, more amino-terminal part of ATR and not for 5 other GST-tagged partial ATR proteins. The kinase domains of several other proteins were also tested and found not to bind RNA. Unfortunately, mutational analysis and competition studies were not conclusive in pinpointing the residues of both ATR and hTR necessary for this interaction. Therefore, at this point, we conclude that ATR seems to bind RNA and has an increased affinity for hTR. It is

possible that ATR reacts more generally to RNA or possibly binds to the newly identified telomeric RNA transcripts, or TERRA (Azzalin et al., 2007; Schoeftner and Blasco, 2008). It has recently been shown that ATR activity is at least partially kept in check at telomeres by the telomeric protein POT1 (Denchi and de Lange, 2007). Whether this inhibition is direct, or possibly through other factors, such as hTR, remains unknown. More sensitive techniques and different approaches will hopefully solve this issue in the future.

The gene that codes for hTR, TERC, is frequently amplificated in human cancers, leading to higher levels of hTR (Cao et al., 2008). Also, hTR levels are regulated by transcription factors SP1 and NF-Y, and pRB at the transcriptional level. Additionally, the MAPK and JNK signalling patways appear to regulate hTR expression (Cairney and Keith, 2008). The levels of hTR can also be regulated by altering its stability as shown in Figure 5A in chapter 4. This shows the increase in hTR levels upon expression of hTERT in BJ cells, which has previously been shown to be caused by an increase in stability (Yi et al., 1999). These examples show that hTR levels are tightly regulated in cells (in most cells in the absence of hTERT) implying another, telomerase-independent role for hTR. As discussed before, hTR levels are increased both in mouse models and human cancers and correlate better to tumor grade than hTERT levels or telomerase activity in some cases (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). The tumorigenic effects of hTERT overexpression rely on the expression of hTR, and there are a few examples providing evidence that overexpression of hTERT in the absence of hTR has anti-tumorigenic effects (Cayuela et al., 2005; Gonzalez-Suarez et al., 2000).

Several oncogenic viruses have been shown to have an effect on telomerase activity and the Marek's disease viruse even encodes the telomerase RNA (Fragnet et al., 2003; Trapp et al., 2006). Marek's disease virus causes fatal lymphomagenesis in chickens, it contains two copies of the chicken telomerase RNA. These are thought to be picked up from the chicken genome during the virus's evolution, as is the case for other known viral oncogenes such as *myc*, *abl* and *src*. Interestingly, the viral telomerase RNA was shown to be essential for Marek's disease virus induced lymphomagenesis. *In vitro* studies with telomerase RNA overexpressing cell lines showed that these cells displayed several characteristics of transformation (Trapp et al., 2006). So, at least in the chicken, telomerase RNA can act as a classical oncogene. A few studies in human cells also suggest that an increase in hTR levels can cause clonal overgrowth of cells. Whether hTR can transform human cells, and therefore act as an oncogene, remains to be investigated.

Novel miRNA regulatory networks, who targets who?

The miRNA field has been developing very fast and its focus has shifted from identifying targets to real implementation of miRNAs in cellular networks, where miRNAs are one level of regulation that is in its turn regulated by other factors. In chapter 3 I have shown that Dead end 1 is such an additional factor regulating the outcomes of miRNA expression. At present, the details of this interaction and other described examples in the literature remain unclear, it is also not fully understood how miRNAs themselves work (Bhattacharyya et al., 2006; Filipowicz et

al., 2008; Neumuller et al., 2008).
The rules governing binding of miRNAs to their target mRNA are still very much unclear, although some new techniques involving proteomics are quickly moving this field ahead (Baek et al., 2008; Selbach et al., 2008). 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; Filipowicz et al., 2008; Lim et al., 2005; Pillai et al., 2005). Prediction algorythms are widely being used but these still give a lot of false hits (le Sage et al., 2007). One of the pitfalls of these methods is the sole use of 3'-UTR regions for predicting miRNA regulation of mRNAs. As was shown in an experimental zebrafish assay, miRNAs can regulate messengers when their target site is located in the coding region (Kloosterman et al., 2004). As we have shown in chapter 5, miRNA 148/152 is capable of regulating its target DNMT3b by binding the coding region of its mRNA. It may be possible that in the case of CDS targeting different rules apply, for the interaction we found has considerable higher sequence complementarity, as is also the case for plants (Rhoades et al., 2002). Since the target site we identified for the miR-148/152 family is only present in specific DNMT3b splice variants, this observation might hint towards a whole novel concept of regulation of splice variant abundance for miRNAs.
DND1 shares an overall amino acid identity of 34% with apobec complementation factor (ACF) (Youngren et al., 2005). This protein is the essential RNA-binding cofactor of apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 1 (APOBEC1), these proteins together comprise the editosome witch converts specific cytidines to uridines in the apolipoprotein B transcript and other mRNAs. Recently, DND1 was found to interact with APOBEC3, a factor that is known to hypermutate cDNAs of retroviruses in human cells, thereby inhibiting viral replication of, for instance HIV (Bhattacharya et al., 2008). APOBEC3G has recently been described to derepress miRNA function on artificial reporter constructs in human tumor cell lines (Huang et al., 2007). It is unclear whether this function of APOBEC3G is important for endogenous translational regulation nor whether it is dependent on DND1 in germ cells. It will be interesting to test whether DND1 effects are dependent on APOBEC3G, or any other APOBEC factors, whether they target the same, or different transcripts, and whether they act synergystically or antagonistically. It is tempting to speculate for a function of DND1 or any of the APOBEC proteins in the repression of viral replication in germ cells by inhibition of the miRNA pathway since many viruses have been described to make use of miRNAs (Gottwein and Cullen, 2008). These are new questions that we will follow up in our search for the mechanism of DND1 function.
When DND1 is lost from primordial germ cells in zebrafish and mice, the germ cells die (Weidinger et al., 2003; Youngren et al., 2005). When we knocked down DND1 in human testicular germ cell tumor lines, this phenotype was not immediately clear, although we did not perform growth nor apoptosis assays. It is possible that these tumor cells have become refractory for DND1 knockdown because they have compensated for DND1 function by overexpressing miRNAs, their targets, or both. Previously, our group described that in fact miR-372 is highly expressed in many testicular germ cell tumors (Voorhoeve et al., 2006). A recent study in TGCT tumor samples found no evidence for mutations of DND1 (Linger et al., 2008). To establish a direct link between DND1 and germ cell

tumors, we need to look at a correlation between DND1 levels and miRNAs in tumor samples, as also discussed by René Ketting (Ketting, 2007).
We need to know whether DND1 effects are indeed mediated through miRNAs and which are the target mRNAs of DND1. Therefore we are in the process of identifying the consensus sequence for DND1 and rescue experiments in zebrafish mutants for Dicer are underway.

In conclusion, the chapters presented here show surprising new interactions between non-coding RNAs and various essential cellular pathways. Counterbalancing the function of ATR may be a function of hTR that facilitates tumorigenesis. The protective function of DND1 towards certain miRNA targets may be important for the inhibition of tumorigenic miRNAs. These studies show that there are certainly more new RNA playgrounds to discover.

References

Amaral, P. P., Dinger, M. E., Mercer, T. R., and Mattick, J. S. (2008). The eukaryotic genome as an RNA machine. Science *319*, 1787-1789.

Azzalin, C. M., Reichenbach, P., Khoriauli, L., Giulotto, E., and Lingner, J. (2007). Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. Science *318*, 798-801.

Baek, D., Villen, J., Shin, C., Camargo, F. D., Gygi, S. P., and Bartel, D. P. (2008). The impact of microRNAs on protein output. Nature *455*, 64-71.

Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R., and Pasquinelli, A. E. (2005). Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell *122*, 553-563.

Bhattacharya, C., Aggarwal, S., Kumar, M., Ali, A., and Matin, A. (2008). Mouse apolipoprotein B editing complex 3 (APOBEC3) is expressed in germ cells and interacts with dead-end (DND1). PLoS ONE *3*, e2315.

Bhattacharyya, S. N., Habermacher, R., Martine, U., Closs, E. I., and Filipowicz, W. (2006). Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell *125*, 1111-1124.

Brown, D. F., Gazdar, A. F., White, C. L., 3rd, Yashima, K., Shay, J. W., and Rushing, E. J. (1997). Human telomerase RNA expression and MIB-1 (Ki-67) proliferation index distinguish hemangioblastomas from metastatic renal cell carcinomas. J Neuropathol Exp Neurol *56*, 1349-1355.

Cairney, C. J., and Keith, W. N. (2008). Telomerase redefined: integrated regulation of hTR and hTERT for telomere maintenance and telomerase activity. Biochimie *90*, 13-23.

Cao, Y., Bryan, T. M., and Reddel, R. R. (2008). Increased copy number of the TERT and TERC telomerase subunit genes in cancer cells. Cancer Sci *99*, 1092-1099.

Cayuela, M. L., Flores, J. M., and Blasco, M. A. (2005). The telomerase RNA component Terc is required for the tumour-promoting effects of Tert overexpression. EMBO Rep *6*, 268-274.

Denchi, E. L., and de Lange, T. (2007). Protection of telomeres through independent control of ATM and ATR by TRF2 and POT1. Nature *448*, 1068-

1071.

Dome, J. S., Bockhold, C. A., Li, S. M., Baker, S. D., Green, D. M., Perlman, E. J., Hill, D. A., and Breslow, N. E. (2005). High telomerase RNA expression level is an adverse prognostic factor for favorable-histology Wilms' tumor. J Clin Oncol *23*, 9138-9145.

Filipowicz, W., Bhattacharyya, S. N., and Sonenberg, N. (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet *9*, 102-114.

Fragnet, L., Blasco, M. A., Klapper, W., and Rasschaert, D. (2003). The RNA subunit of telomerase is encoded by Marek's disease virus. J Virol *77*, 5985-5996.

Gonzalez-Suarez, E., Samper, E., Flores, J. M., and Blasco, M. A. (2000). Telomerase-deficient mice with short telomeres are resistant to skin tumorigenesis. Nat Genet *26*, 114-117.

Gottwein, E., and Cullen, B. R. (2008). Viral and cellular microRNAs as determinants of viral pathogenesis and immunity. Cell Host Microbe *3*, 375-387.

Huang, J., Liang, Z., Yang, B., Tian, H., Ma, J., and Zhang, H. (2007). Derepression of microRNA-mediated protein translation inhibition by apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) and its family members. J Biol Chem *282*, 33632-33640.

Ketting, R. F. (2007). A Dead End for MicroRNAs. Cell *131*, 1226-1227.

Kloosterman, W. P., Wienholds, E., Ketting, R. F., and Plasterk, R. H. (2004). Substrate requirements for let-7 function in the developing zebrafish embryo. Nucleic Acids Res *32*, 6284-6291.

le Sage, C., Nagel, R., and Agami, R. (2007). Diverse ways to control p27Kip1 function: miRNAs come into play. Cell Cycle *6*, 2742-2749.

Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., Bartel, D. P., Linsley, P. S., and Johnson, J. M. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature *433*, 769-773.

Linger, R., Dudakia, D., Huddart, R., Tucker, K., Friedlander, M., Phillips, K. A., Hogg, D., Jewett, M. A., Lohynska, R., Daugaard, G.*, et al.* (2008). Analysis of the DND1 gene in men with sporadic and familial testicular germ cell tumors. Genes Chromosomes Cancer *47*, 247-252.

Maitra, A., Yashima, K., Rathi, A., Timmons, C. F., Rogers, B. B., Shay, J. W., and Gazdar, A. F. (1999). The RNA component of telomerase as a marker of biologic potential and clinical outcome in childhood neuroblastic tumors. Cancer *85*, 741-749.

Mattick, J. S. (2007). A new paradigm for developmental biology. J Exp Biol *210*, 1526-1547.

Morales, C. P., Lee, E. L., and Shay, J. W. (1998). In situ hybridization for the detection of telomerase RNA in the progression from Barrett's esophagus to esophageal adenocarcinoma. Cancer *83*, 652-659.

Neumuller, R. A., Betschinger, J., Fischer, A., Bushati, N., Poernbacher,

I., Mechtler, K., Cohen, S. M., and Knoblich, J. A. (2008). Mei-P26 regulates microRNAs and cell growth in the Drosophila ovarian stem cell lineage. Nature *454*, 241-245.

Pillai, R. S., Bhattacharyya, S. N., Artus, C. G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E., and Filipowicz, W. (2005). Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science *309*, 1573-1576.

Rhoades, M. W., Reinhart, B. J., Lim, L. P., Burge, C. B., Bartel, B., and Bartel, D. P. (2002). Prediction of plant microRNA targets. Cell *110*, 513-520.

Rushing, E. J., Yashima, K., Brown, D. F., White, C. L., 3rd, Shay, J. W., Risser, R. C., and Gazdar, A. F. (1997). Expression of telomerase RNA component correlates with the MIB-1 proliferation index in ependymomas. J Neuropathol Exp Neurol *56*, 1142-1146.

Schoeftner, S., and Blasco, M. A. (2008). Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. Nat Cell Biol *10*, 228-236.

Selbach, M., Schwanhausser, B., Thierfelder, N., Fang, Z., Khanin, R., and Rajewsky, N. (2008). Widespread changes in protein synthesis induced by microRNAs. Nature *455*, 58-63.

Soder, A. I., Hoare, S. F., Muir, S., Going, J. J., Parkinson, E. K., and Keith, W. N. (1997). Amplification, increased dosage and in situ expression of the telomerase RNA gene in human cancer. Oncogene *14*, 1013-1021.

Trapp, S., Parcells, M. S., Kamil, J. P., Schumacher, D., Tischer, B. K., Kumar, P. M., Nair, V. K., and Osterrieder, N. (2006). A virus-encoded telomerase RNA promotes malignant T cell lymphomagenesis. J Exp Med *203*, 1307-1317.

Voorhoeve, P. M., le Sage, C., Schrier, M., Gillis, A. J., Stoop, H., Nagel, R., Liu, Y. P., van Duijse, J., Drost, J., Griekspoor, A., *et al.* (2006). A genetic screen implicates miRNA-372 and miRNA-373 as oncogenes in testicular germ cell tumors. Cell *124*, 1169-1181.

Weidinger, G., Stebler, J., Slanchev, K., Dumstrei, K., Wise, C., Lovell-Badge, R., Thisse, C., Thisse, B., and Raz, E. (2003). dead end, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival. Curr Biol *13*, 1429-1434.

Yashima, K., Litzky, L. A., Kaiser, L., Rogers, T., Lam, S., Wistuba, II, Milchgrub, S., Srivastava, S., Piatyszek, M. A., Shay, J. W., and Gazdar, A. F. (1997). Telomerase expression in respiratory epithelium during the multistage pathogenesis of lung carcinomas. Cancer Res *57*, 2373-2377.

Yashima, K., Milchgrub, S., Gollahon, L. S., Maitra, A., Saboorian, M. H., Shay, J. W., and Gazdar, A. F. (1998). Telomerase enzyme activity and RNA expression during the multistage pathogenesis of breast carcinoma. Clin Cancer Res *4*, 229-234.

Yi, X., Tesmer, V. M., Savre-Train, I., Shay, J. W., and Wright, W. E. (1999). Both transcriptional and posttranscriptional mechanisms regulate human telomerase template RNA levels. Mol Cell Biol *19*, 3989-3997.

Youngren, K. K., Coveney, D., Peng,

X., Bhattacharya, C., Schmidt, L. S., Nickerson, M. L., Lamb, B. T., Deng, J. M., Behringer, R. R., Capel, B., *et al.* (2005). The Ter mutation in the dead end gene causes germ cell loss and testicular germ cell tumours. Nature *435*, 360-364.

Nederlandse Samenvatting

Het vormen en in stand houden van een menselijk lichaam, bestaande uit grofweg honderdduizendmiljard cellen, uit één enkele bevruchte eicel, of zygote, is een zeer gecompliceerde taak. Hiervoor is een nauwe samenwerking tussen honderden verschillende celtypen en weefsels, en een strenge controle op celdeling nodig. Het is dan ook niet moeilijk voor te stellen dat er in de ontwikkeling of het onderhoud wel eens iets mis gaat. Zulke fouten worden meestal door het lichaam hersteld, maar soms leiden ze tot ziekten, zoals kanker.

Eigenlijk zijn alle cellen in een multicellulair organisme altruïstisch, zij hebben een ondersteunende functie voor de geslachtscellen, die de volgende generatie gaan vormen. Eén van de fundamentele verschillen tussen normale cellen en kankercellen is dat kankercellen egoïstisch zijn. Kankercellen luisteren niet meer naar signalen van hun buren om te stoppen met delen, ze wedijveren voor voedingsstoffen met omliggende cellen en zaaien uit naar andere weefsels zonder zich aan de daar heersende sociale regels te houden en vernietigen deze weefsels vaak terwijl ze zich voortplanten.

In de algemene introductie (**Hoofdstuk 1**) staat kort beschreven welke fysiologische veranderingen de meeste kankercellen hebben ondergaan. In de meeste gevallen liggen genetische veranderingen, ofwel mutaties, hieraan ten grondslag. In het laboratorium is het bijvoorbeeld mogelijk om normale cellen, na introductie van een beperkt aantal factoren (meestal in kankercellen gemuteerde genen) die in kankercellen gedereguleerd zijn, te veranderen in kankercellen. Met behulp van dit soort modellen kun je vaststellen in welke mate veranderingen die men in kankercellen vindt een bijdrage leveren aan het onstaan of voortbestaan van deze cellen. Op die manier zijn bijvoorbeeld ook factoren te vinden die wellicht te remmen zijn met medicijnen, om zo de kanker te bestrijden.

In dit boekje wordt een aantal factoren beschreven die in meer of mindere mate een bijdrage leveren aan intracellulaire moleculaire signalerings-cascades die belangrijk zijn voor het ontstaan of de handhaving van kanker. In sommige gevallen is deze connectie niet direct evident. Dit komt doordat we in het onderzoek vaak interessante fenomenen tegenkomen die ons een dieper inzicht geven in hoe cellen werken. Uiteindelijk zal zo’n groter inzicht hopelijk ook leiden tot nieuwe inzichten voor de behandeling van kanker en andere ziekten.

Hoofdstuk 4 beschrijft de studie die we hebben gedaan naar de rol van hTR, de RNA-component van telomerase, in de respons van cellen op DNA-schade (beknopt uitgelegd in **hoofdstuk 1**). Telomerase is een eiwit-RNA-complex dat de uiteinden van chromosomen (het erfelijk materiaal, met de blauwdruk van de werking van onze cellen) stabiliseert en kan verlengen. In vrijwel alle normale cellen in ons lichaam worden de uiteinden van chromosomen (de telomeren) korter, een proces dat na een beperkt aantal celdelingen leidt tot het stoppen van de celcyclus. Dit is als het ware een natuurlijke klok, of rem op de voortplantingscapaciteit van cellen. Kankercellen hebben deze rem in alle gevallen gesaboteerd om te kunnen blijven delen, meestal door meer aan te maken (overexpressie) van de individuele componenten van het telomerasecomplex, hTR en het telomerase eiwit. Een interessant en weinig bestudeerd fenomeen is dat veel tumoren al in een vroeg stadium de RNA-component hTR tot overexpressie brengen, terwijl telomerase en de activiteit van het complex afwezig of laag zijn.

Ook hebben vrijwel alle normale cellen in ons lichaam hTR, en niet telomerase. Door de hoeveelheid aan hTR in zowel normale als tumorcellen te reduceren met behulp van de RNA-interferentietechniek (RNAi) en met behulp van een aantal andere proeven hebben wij ontdekt dat hTR een telomerase-onafhankelijke rol in de DNA-schaderespons heeft. Een grotere hoeveelheid aan hTR is aanwezig in kankercellen en wordt in normale cellen aangemaakt als respons op UV-bestraling (een specifieke vorm van DNA-schade), dit leidt tot een onderdrukking van de respons op DNA-schade. Dit mechanisme zet deze respons in normale cellen uiteindelijk weer uit en werkt de ongebreidelde groei van de kankercellen in de hand doordat ze schade aan hun DNA negeren.

De analyse van de functie van het eiwit Dead End 1, beschreven in **hoofdstuk 3** kan ons iets leren over het ontstaan van kiemceltumoren, maar de lessen die we hier hebben geleerd zijn ook veel algemener toepasbaar. In deze studie hebben we een nieuwe en complexe manier van genregulatie aan het licht gebracht, die in het geval van Dead End 1 essentieel is voor de ontwikkeling van de kiemcellen. Dit zijn de cellen die uiteindelijk sperma en oöcyten gaan vormen en dus aan de basis staan van de volgende generatie.
Dead End werkt op het niveau van boodschapper-RNA's. Dit zijn kopieën van de genen die in het DNA (in de celkern) gecodeerd zijn en die worden vertaald in eiwitten (in het cytoplasma). Om de aanmaak van eiwitten te reguleren kun je bijvoorbeeld de productie van boodschapper-RNA's (transcriptie) remmen of de vertaling naar eiwitten (translatie) blokkeren. MicroRNA's zijn korte stukjes RNA die worden gecodeerd door genen in het DNA die de translatie van boodschapper-RNA's remmen. Dit doen ze door te binden aan plaatsen in de boodschapper-RNA's die gedeeltelijk complementair zijn. Een eiwitcomplex (RISC, voor RNA induced silencing complex) zorgt er dan voor dat de productie van het eiwit geremd word. MicroRNA's coderen, net als het in **hoofdstuk 4** beschreven hTR-RNA-molecuul, niet voor de productie van een eiwit, maar functioneren als RNA-molecuul. Vandaar de naam niet-coderend RNA (non-coding RNA). MicroRNA's zijn pas kortgeleden ontdekt, er zijn er grofweg 1000 van in ons genoom en er is voorspeld dat zij meer dan de helft van alle genen reguleren. Ons lab en andere labs hebben laten zien dat enkele microRNA's als tumor-suppressors of oncogenen kunnen werken. Hoe microRNA's precies werken en voornamelijk hoe zij worden gereguleerd is nog grotendeels onduidelijk. Onze studie naar Dead End laat zien dat microRNA-activiteit kan worden gereguleerd door RNA-bindende eiwitten zoals Dead End 1, zonder daarbij de hoeveelheid microRNA's te veranderen.
Wij hebben laten zien dat het Dead End-eiwit bindt aan uridinerijke plaatsen in een aantal boodschapper-RNA's waar het ervoor zorgt dat microRNA's niet kunnen binden. Op deze manier zorgt Dead End 1 dat onderdrukking door microRNA's wordt opgeheven en dat de gebonden boodschapper-RNA's worden vertaald in eiwitten. De microRNA's blijven dus beschikbaar in de cel om andere boodschapper-RNA's, die geen plaatsen hebben waar Dead End kan binden, te onderdrukken. **Hoofdstuk 3** beschrijft onze studie naar de functie van Dead End, terwijl **hoofdstuk 2** deze studie in de literatuur over microRNA's en kiemcellen plaatst en de achtergrond en implicaties van onze vindingen beschrijft.

Tot nu toe hebben alle geïdentificeerde humane boodschapper-RNA's die door microRNA's worden gereguleerd hun bindingsplaatsen voor deze moleculen aan een van de uiteinden van deze RNA's die niet worden getransleerd (de 3' UTR, of untranslated region). In **hoofdstuk 5** beschrijven we dat de microRNA 148/152 familie in het coderende deel van de DNMT3b-boodschapper-RNA bindt. Een van de (splice-)varianten van DNMT3b heeft deze microRNA bindingsplaats niet, met als gevolg dat deze variant ook niet gereguleerd wordt door microRNA 148/152. Deze vinding breidt de mogelijkheden voor microRNA-doelen drastisch uit, daar tot nu toe alleen in de 3'UTR's werd gekeken naar potentiële bindingsplaatsen.

De rode draad die door dit proefschrift getiteld "New RNA playgrounds, non-coding RNAs and RNA-binding proteins control cellular processes" loopt, is non-coderend RNA. De ontrafelde functies van de verschillende non-coderende RNA's die hier beschreven staan laten zien dat het speelveld (de "playground") van niet-coderende RNA's steeds maar weer groter blijkt dan eerder werd aangenomen. In de afgelopen paar jaar zijn verscheidene microRNA's en andere non-coderende RNA's gevonden die een rol spelen in het ontstaan van kanker en andere ziekten. Naar verwachting zullen er in de komende jaren nog veel meer worden gevonden. Doordat de activiteit van RNA's te beïnvloeden is door middel van binding van vergelijkbare moleculen die gemakkelijk te produceren zijn en een hoge mate van specificiteit hebben zijn RNA moleculen wellicht goede therapeutische doelen. Preklinische testen hebben al uitgewezen dat het mogelijk is om de activiteit van microRNA's te remmen in diermodellen. Het behandelen van ziekten met behulp van middelen die op RNA moleculen werken (en niet zoals conventionele medicijnen op eiwitten) vergt nog veel onderzoek, maar zal uiteindelijk realiteit worden.

Curriculum vitae

Op Goede Vrijdag 13 april 1979 werd Martijn Kedde geboren te Ede. Hij behaalde in 1997 zijn VWO-diploma aan het Marnix College, tevens te Ede. Na bijna een jaar op wereldreis te zijn geweest begon hij in 1998 aan zijn studie Medische Biologie aan de Universiteit van Amsterdam.
Zijn fascinatie voor onderzoek naar kanker kreeg een eerste impuls tijdens zijn eerste stage, op de afdeling Experimentele Hepatologie in het Academisch Medisch Centrum in Amsterdam. Hier deed hij onderzoek naar adenovirale gentherapie voor de behandeling van alvleesklierkanker. Hierna belandde hij voor zijn tweede stage op het Nederlands Kanker Instituut in de groep van Prof. Dr. Jannie Borst. Hier nam hij deel aan een project over apoptoseregulatie door verschillende BH3-eiwitten onder begeleiding van Dr. Arlette B. Werner. Het moleculaire en biochemische werk dat hij hier deed sprak hem zo aan dat hij op het NKI wilde blijven. In 2003 begon hij in de groep van Dr. Reuven Agami aan zijn promotieonderzoek wat uiteindelijk heeft geleid tot dit boekje.
Na zijn promotie zal hij nog enige tijd in de groep van Dr. Agami blijven om een aantal uitdagende projecten voort te zetten.

List of Publications

Kedde M, le Sage C, Duursma A, Zlotorynski E, van Leeuwen B, Nijkamp W, Beijersbergen R, Agami R.
Telomerase-independent regulation of ATR by human telomerase RNA.
J Biol Chem. 2006 Dec 29;281(52):40503-14.

Kedde M, Strasser MJ, Boldajipour B, Vrielink JA, Slanchev K, le Sage C, Nagel R, Voorhoeve PM, van Duijse J, Ørom UA, Lund AH, Perrakis A, Raz E, Agami R
RNA-binding protein Dnd1 inhibits microRNA access to target mRNA.
Cell. 2007 Dec 28;131(7):1273-86

Martijn Kedde and Reuven Agami
Interplay between microRNAs and RNA-binding proteins determines developmental processes.
Cell Cycle, (7), 7, 1 April 2008

Anja M. Duursma, **Martijn Kedde**, Mariette Schrier, Carlos le Sage and Reuven Agami
miR-148 targets human DNMT3b protein coding region.
RNA, 2008 RNA, May 1; 14 (5), 2008