Identification and Analysis of Dicer Associated Proteins in Human Cells

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1. Summary

Dicer is a multidomain RNase III like enzyme involved in the initial steps of RNA interference (RNAi) and microRNA (miRNA) pathways, two mechanisms of posttranscriptional gene silencing conserved throughout most eukaryotic species. Dicer has been shown to be necessary and sufficient to cut long dsRNA and miRNA precursors into small (21-25 nt) RNAs. In metazoa, the small RNA products of Dicer are further incorporated into a multiprotein RNA induced silencing complex (RISC), which target mRNAs in a sequence specific manner to induce mRNA cleavage (guided by siRNAs) or inhibition of translation (guided by miRNAs).

We aimed to identify proteins interacting with human Dicer. Specific anti-Dicer antibodies were used to immunoprecipitate Dicer from HEK293 and HeLa cells and co-immunoprecipitating proteins were analyzed by mass spectrometry. Proteins identified to specifically co-purify with Dicer fall into different categories: (1) known components of RNA silencing pathways, such as Argonaute proteins; (2) translation factors; (3) RNA helicases; (4) RNA binding proteins, and others.

Among the proteins characterized by mass spectrometry we identified TRBP [HIV-1 transactivating response (TAR) RNA-binding protein], as a protein containing three dsRNA binding domains (dsRBD). We found that this protein interacts with human Dicer *in vivo* and *in vitro,* and that this interaction involves Dicer's N-terminal helicase domain and TRBP's third dsRBD. TRBP has previously been assigned several different functions, including inhibition of the interferon (IFN)-induced dsRNA-regulated protein kinase PKR, modulation of

HIV-1 gene expression through its association with TAR, and control of cell growth. To analyze TRBP's function in miRNA and siRNA pathways, we generated stable TRBP knock down cell lines. Using a Luciferase reporter system we showed that TRBP is necessary for efficient silencing mediated by endogenous miRNAs. Dicer cleavage activity tested *in vitro* using a pre-miRNA substrate was impaired in TRBP knock down extracts. However, endogenous miRNA levels were not significantly changed upon knock down of TRBP. We also found that RNAi against an endogenous gene, induced by transfection of siRNAs, was impaired in TRBP knock down cell lines. Taken together our observations show that TRBP interacts with Dicer, and suggest that, besides its impact on Dicer cleavage activity *in vitro*, TRBP functions mainly downstream of Dicer cleavage in miRNA and siRNA pathways. Our data provide support to the notion that large RNaseIII-type Drosha and Dicer nucleases work in conjunction with small dsRBD protein partners. They also raise the possibility of a cross talk between RNA silencing and the IFN/PKR pathways in normal and virus-infected cells.

To further characterize the Dicer/TRBP complex, we generated anti-TRBP antibodies, and analyzed TRBP immunoprecipitates by mass spectrometry. Data obtained from peptide sequencing of Dicer and TRBP immunopurifications were compared, and proteins identified in both preparations, among them the E3 Ubiquitin ligase Ro52, were considered for further analyses as likely components of a Dicer/TRBP complex. Ro52, also known as Sjoegren Syndrome Antigen 1 (SSA-1) or Tripartite motif protein 21 (TRIM21) was first identified as one of the

major autoantigens in Sjoegren Syndrome and Systemic Lupus Erythematosus (SLE), two severe human autoimmune diseases. Recently, it was demonstrated that Ro52 can act as a RING dependent E3 Ubiquitin ligase, and the E3 ligase activity of Ro52 was shown to be required for S-phase progression in mammalian cells.

We found that Ro52 associates with the Dicer/TRBP complex, and interacts with TRBP *in vitro*. The presence of an E3 Ubiquitin ligase in the Dicer/TRBP complex raised the possibility that components of the RNA silencing complexes are post-translationally modified by Ubiquitin. Our data indicate that TRBP can be covalently tagged by Ubiquitin. It appears that TRBP is monoubiquitinated, rather than polyubiquitinated and degraded by the proteasome. Future work will be required to establish the function of ubiquitination of TRBP, and the role of Ro52 in RNA silencing pathways.

2. Introduction

2.1. RNA silencing by small interfering (si)RNAs and micro (mi)RNAs

Over the past ten years, different classes of small RNAs (20- to 30-nt in length) have been discovered that regulate gene expression in eukaryotes by different mechanisms. This introduction will give an overview about RNA silencing and components involved, focusing on post-transcriptional gene silencing (PTGS) in mammals.

2.1.1. The discovery of RNA silencing –a brief historical overview

In 1990, the attempt of generating deep purple petunia flowers by the introduction of a transgene resulted in a predominantly white flower phenotype. This phenomenon was termed “co-suppression”, since introduction of a transgene promoted silencing of the homologous endogenous locus. RNA was suggested to be involved in this mechanism (Napoli et al., 1990; van der Krol et al., 1990). In *Caenorhabditis elegans*, the unexpected observation that introduction of both sense and antisense RNA could trigger gene silencing paved the way to the identification of dsRNA as the trigger of sequence specific mRNA destruction (Fire et al., 1998). During the following years it has been shown by three different groups that the initial dsRNA trigger becomes converted into small 21- to 25-nt RNAs, named small interfering RNAs (siRNAs), that guide ribonucleoprotein complexes (RNPs) for sequence specific mRNA destruction (Hamilton and Baulcombe, 1999; Hammond et al., 2000; Zamore et al., 2000). At the same time, small non-coding RNAs, named small temporary RNAs (stRNAs),

were discovered as products of heterochronic genes affecting the transitions between different stages in the life cycle of C*. elegans* by regulating the expression of protein-coding genes. In 1993, Ambros and co-workers described lin-4 as the first stRNA and proposed that lin-4 regulates lin-14 and lin-28 mRNAs by imperfect basepairing to their 3' UTRs (Lee et al., 1993). In 2000, a second stRNA, let-7, was discovered in worms (Pasquinelli et al., 2000; Reinhart et al., 2000; Slack et al., 2000). While no obvious orthologues of lin-4 have been found in other organisms, let-7 and its target mRNA lin-41 are conserved throughout metazoans. Lin-4 and let-7 served as founding members of an evolutionarily conserved class of small non-coding RNAs (ncRNAs) that have later been termed micro-RNAs (miRNAs) ((Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Lee et al., 1993; Llave et al., 2002; Park et al., 2002); for review see: (Banerjee and Slack, 2002)).

SiRNA and miRNA silencing pathways were discovered independently and no formal connection between these two phenomena has been made until the discovery of Dicers, the RNase III-like enzymes processing long double-stranded RNA (dsRNA) into 21- to 25-nt siRNAs, and longer, highly structured miRNA precursors (pre-miRNAs) into mature miRNAs.

2.1.2. siRNA- and miRNA-mediated post-transcriptional gene silencing (PTGS)

SiRNAs and miRNAs are 21- to 22-nt small RNAs that are defined by their biogenesis. SiRNAs arise from long dsRNA, whereas miRNA precursors are

imperfect RNA hairpin structures. During RNAi, long dsRNA, which can originate from viral replication, antisense transcription or transfection, is processed by the RNase III-like enzyme Dicer into 21- to 22nt siRNA duplexes. Only one strand of the siRNA duplex, the guide strand, is selectively incorporated into an RNA induced silencing complex (RISC), and guides cleavage of fully complementary target RNAs (Zamore et al., 2000). Cleavage of the target RNA requires the RNase H activity of Argonaute (Ago) proteins that are the core components of RISC. In this reaction, the 5' end of the guide strand sets the ruler for target RNA cleavage, which occurs opposite nt 10 and 11, measured from the 5' end of the siRNA (for review see (Rana, 2007)). Endogenous siRNAs, discovered in various organisms, fall into different classes. In plants, trans-acting siRNAs (tasiRNAs) correspond to sense and antisense siRNAs originating from a limited number of non-coding RNAs. Their formation requires components of the miRNA machinery (for review see: (Meins Jr et al., 2005; Vaucheret, 2005; Willmann and Poethig, 2005)). In *Tetrahymena thermophila,* small scan RNAs (scnRNAs) have been suggested to scan DNA sequences for homology thereby regulating genome rearrangement (for review see: (Mochizuki and Gorovsky, 2004)). In Schizos*accharomyces pombe* and *Arabidopsis thaliana* specific classes of siRNAs are involved in transcriptional gene silencing (for review see: (Carrington, 2005; Vazquez, 2006)). In mammals, no endogenous siRNAs have been discovered so far, but exogenous siRNAs are capable of target RNA cleavage, demonstrating that the mechanism of the siRNA-mediated mRNA cleavage is evolutionarily conserved. The siRNA-mediated target RNA degradation is

nowadays widely exploited as a biotechnological tool to knock down genes by expression of long dsRNA or miRNA-like precursor RNAs in a cell or an organism, or by transfection of siRNAs. In addition, siRNA mediated knock down of specific mRNAs is under evaluation as a therapeutic tool for clinical use.

In miRNA-mediated gene silencing, primary-miRNAs (pri-miRNAs) are transcribed from endogenous genes by RNA polymerase II (Pol II) ((Cai et al., 2004; Lee et al., 2004a)), and in some cases by Pol III (Borchert et al., 2006), as long precursor RNAs that form imperfect stem loop structures. Pri-miRNAs are processed into mature miRNAs by the consecutive action of two RNase III-like enzymes. The nuclear RNase III, Drosha, processes pri-miRNAs into ~70-nt long pre-miRNAs that are exported into the cytoplasm by Exportin5 (Exp5), a Ran-GTP dependent nucleo-cytoplasmic cargo transporter (Bohnsack et al., 2004; Lee et al., 2003; Lund et al., 2004; Yi et al., 2003). In the cytoplasm, pre-miRNAs are processed into mature ~22-nt miRNA duplexes by Dicer. One strand of the miRNA duplex, the mature miRNA, is incorporated into a miRNP/miRISC. Recent reports suggest that miRNA processing is tightly regulated post-transcriptionally at the steps of Drosha and Dicer processing. Some pri-miRNAs are highly expressed in early mouse development, but no corresponding pre-miRNAs or mature miRNAs are detected until later stages in development, suggesting that miRNA biogenesis can be regulation at a Drosha processing step (Thomson et al., 2006). Also regulation at the Dicer processing level has been observed (Ambros et al., 2003; Obernosterer et al., 2006). In animals, most miRNAs, unlike siRNAs, bind their target mRNAs within the 3'UTR with imperfect

complementarity, and either promote translational inhibition or degradation of their target mRNAs (for review see: (Filipowicz et al., 2005)). Foremost, the 5' end of the miRNA, e.g. nt 2 to 8, shows almost perfect complementarity to its target site, and was termed a "seed region". Functional miRNA:mRNA interactions require continuous Watson-Crick base pairing within the seed region, and contain different bulges and mismatches including those at positions 10/11 of the miRNA to prevent RISC mediated mRNA cleavage. The degree of repression largely depends on the stability of the interaction between the miRNA seed region and its target mRNA. In addition, a perfect Watson-Crick base-pairing is required within the seed region, since G-U base pairing has been shown to decrease miRNA function to a great extent. Interaction between the 3' of the miRNA and its target mRNA is of less functional importance, but can influence the efficiency of miRNA-mediated repression (Brennecke et al., 2005; Doench and Sharp, 2004). Studies conducted with artificially designed miRNA binding sites suggest that multiple miRNA binding sites within the 3'UTR of a single target-mRNA are necessary for efficient repression (Doench et al., 2003). However, in some cases only one miRNA binding site is sufficient to inhibit the target mRNAs. In this context, sequences flanking the miRNA binding site have been suggested to influence the biological read out of the miRNA:mRNA interaction (Didiano and Hobert, 2006). Interestingly, experimental tethering of Ago proteins to the 3'UTR of a reporter gene mimics miRNA-mediated translational inhibition (Pillai et al., 2004). This suggests that miRNAs serve as guides to recruit Ago proteins and associated factors to specific mRNAs.

Our current knowledge does not result in a unified model for the mechanism of miRNA-mediated repression. Different models were proposed, supporting the idea of translational repression either at the level of initiation or at a post-initiation level. In addition, there is evidence for miRNA-induced mRNA degradation that occurs independently of Agos' RNase H activity. However, for most of the cases tested the reduction of protein levels is much greater than it could be explained by the reduction of mRNA levels, which argues for a combined effect of miRNPs on translation and mRNA stability. Differences in the outcome of these studies might result from different experimental approaches (e.g. use of different reporter constructs), but might also reflect different naturally occurring modes of the miRNA-mediated post-transcriptional regulation, which might depend on additional regulatory elements within the mRNA or differences in the miRISC composition.

Evidence supporting translational repression at early steps of translational initiation was provided by several authors (Bhattacharyya et al., 2006; Humphreys et al., 2005; Pillai et al., 2005; Wang et al., 2006a). Analysis of exogenous reporter constructs as well as endogenous miRNA targets in human cell lines, and extensive analysis of the requirement for a m^7G-cap suggested that miRNAs affect early steps of translational initiation, possibly involving m^7G-cap recognition. In support of this model Mourelatos' laboratory demonstrated recently that Argonaute proteins directly interact with the m^7G-cap, and that this interaction is required for translational repression (Chendrimada et al., 2007; Kiriakidou et al., 2007; Thermann and Hentze, 2007). Mechanisms for miRNA-

mediated silencing involving translational repression at post-initiation steps were suggested by early studies of the let-7 and lin-4 target mRNAs in *C. elegens* (Olsen and Ambros, 1999; Seggerson et al., 2002), and in mammalian cells (Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006). In mammalian cells, co-sedimentation of miRNAs and their target mRNAs with elongation-compentent polyribosomes led to the suggestion of premature translational termination and enhanced ribosomal drop-off (Petersen et al., 2006), and of co-translational protein degradation (Nottrott et al., 2006) as possible mechanisms for miRNA-mediated repression. Experiments addressing the importance of poly(A)-tails showed that poly(A) is not essential but affects the efficiency of miRNA-mediated repression of *in vitro* transcribed mRNA, which was transfected into human cells (Humphreys et al., 2005; Pillai et al., 2005). In addition, destabilization of the target mRNA, most likely due to enhanced de-adenylation, was observed in *C. elegans, Drosophila* and mammals (Bagga et al., 2005; Krutzfeldt et al., 2005; Lim et al., 2005; Olsen and Ambros, 1999; Rehwinkel et al., 2006; Schmitter et al., 2006; Seggerson et al., 2002).

The mRNAs that are regulated by miRNAs contain generally long 3'UTRs (Stark et al., 2005), and are likely to contain additional regulatory motifs. Bhattacharya et al. (Bhattacharyya et al., 2006) showed that the presence of AU-rich binding motifs (AREs) within the 3'UTR of the cationic amino acid transporter 1 (CAT-1) mRNA can counteract miRNA-mediated translational repression when the motifs are bound by the ARE binding protein HuR, which re-localizes from the

nucleus to the cytoplasm under stress conditions. This example showed that miRNA-mediated translational repression is a reversible process.

Translationally inactive miRNP-mRNA complexes are re-localizing to discrete cytoplasmic foci termed processing bodies (P-bodies) that contain various factors involved in translational repression and decay (for review see: (Eulalio et al., 2007; Pillai et al., 2007)). P-bodies are conserved from yeast to mammals. In 1997, Bashkirov et al. showed that XRN1, the main cytoplasmic 5' to 3' exoribonuclease in eukaryotic cells, is "highly enriched in discrete, prominent cytoplasmic foci" in mammalian cells (Bashkirov et al., 1997). Components of the decapping complex, the decapping enzymes Dcp1 and Dcp2 and associated co-factors localize to XRN1-bodies in yeast and mammals (Cougot et al., 2004; Ingelfinger et al., 2002; Lykke-Andersen, 2002; Sheth and Parker, 2003). The human autoantigen GW182 localizes to descrete cytoplasmic structures termed GW-bodies (Eystathioy et al., 2002). GW182 is a cytoplasmic phospho-protein that contains glycine and tryptophan repeats, and is recognized by the autoimmune-serum of patients suffering from motor and sensory neuropathy. GW-bodies, Xrn1-bodies, and P-bodies were discovered independently, but were later shown to be identical structures. In addition, many proteins with an established role in mRNA decay, such as deadenylases (like Ccr4: Not1), decapping factors and co-activators (Ge-1, Rap55 and others), translational repressors like Rck/p54, eIF4E-T and Pat1p, and components of miRNA silencing (Argonautes, miRNAs) reside in P-bodies. Ribosomes and translation initiation factors, with the exception of eIF4E, have not been found in P-bodies,

and the work of different laboratories established an inverse correlation between polysome association and P-body localization of mRNAs (Bhattacharyya et al., 2006; Brengues et al., 2005; Coller and Parker, 2005; Pillai et al., 2005; Teixeira et al., 2005). P-bodies are highly dynamic aggregates, representing sites of mRNA storage and decay. Their integrity, size and number depend on the constant supply of translationally inactive mRNAs. GW182 is one of the components that is essential for P-body integrity, since its depletion leads to P-body loss. In addition, GW182 interacts, either directly or indirectly with Ago proteins in *Drosophila* and humans, and is required for miRNA-mediated post-transcriptional gene silencing (for review see: (Eulalio et al., 2007; Pillai et al., 2007)).

Stress granules (SGs) are cytoplasmic structures induced by different types of stress in mammalian cells, and are frequently intimately associated with P-bodies. In contrast to P-bodies, SGs contain 40S ribosomal subunits and some translation initiation factors, and are devoid of Dcp1 and Dcp2 and GW182, suggesting that these two cytoplasmic RNA-protein aggregates are functionally distinct. Despite these differences, dynamic movement of mRNAs and protein components between these structures under stress conditions has been suggested (Anderson and Kedersha, 2006; Kedersha et al., 2005; Kedersha et al., 1999; Kimball et al., 2003; Wilczynska et al., 2005). In addition, it has been propose that SGs might also be involved in miRNA mediated repression (Leung et al., 2006; Leung and Sharp, 2006).

2.1.3. miRNAs and their target mRNAs

Hundreds of miRNAs have been identified by Tuschl's, Bartel's, Ambros' and other groups, who extensively cloned and sequenced endogenous small RNAs in the range of ~20- to 25-nt from worms, flies, and mammals. Different features were experimentally shown to be indicative for the identity of a miRNA: (1) 5' phosphates and 3' 2-nt overhangs, the characteristic features of an RNase III cleavage, (2) origin from transcripts that can fold into hairpin structures, (3) temporally as well as spatially regulated expression, and (4), in many cases, evolutionary conservation. (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Additional miRNAs have been cloned from RISC preparations (Dostie et al., 2003; Mourelatos et al., 2002), or polyribosomal fractions (Kim et al., 2004). In parallel, bioinformatic approaches to identify miRNAs have been developed over the last years and are constantly refined by including the features of experimentally verified miRNAs (Lim et al., 2003a; Lim et al., 2003b; Reinhart et al., 2002). The miRNA Registry contains an up-to-date collection of experimentally validated and computationally predicted miRNAs (Griffiths-Jones, 2004; Griffiths-Jones, 2006; Griffiths-Jones et al., 2006); http://microrna.sanger.ac.uk). Currently, there are 541 human, 135 worm, 85 *Drosophila*, and 202 *Arabidopsis* miRNAs listed in the miRNA registry (release 9.1, February 2007 [(Griffiths-Jones et al., 2006)]). According to bioinformatics predictions there exist approximately one thousand human miRNAs, thus accounting for ~3% of all human genes (for commentary see: (Taganov et al.,

2007)). However, some predictions put the number of human miRNAs even at ten thousands (Miranda et al., 2006).

The understanding of the functions and the biological impact of miRNAs greatly relies on the identification of target mRNAs. In plants, most miRNAs baise-pair to mRNAs-targets with nearly perfect complementarity, resulting in target mRNA cleavage. This makes target mRNA predictions relatively straightforward in plants. In other organisms, however, imperfect base pairing of miRNAs to their target mRNAs make predictions much more difficult. In the latter case computational predictions rely on rules extrapolated from experimentally validated miRNA:mRNA interactions. Different prediction algorithms, for example Targetscan (www.targetscan.org; (Lewis et al., 2005; Lewis et al., 2003)), have been developed and are constantly revised. Approximately 200 mRNAs are predicted to be targeted by a single miRNA in mammals, suggesting that ~30% of the mammalian genes are regulated by miRNAs (Lewis et al., 2005). This high number, however, assumes co-expression of a given miRNA and its target mRNAs within the same cell, and therefore might be overestimated. Cohen and colleagues reported that some genes tend to avoid miRNA regulation by harboring short 3'UTRs (Stark et al., 2005). Further, they showed that miRNAs and their target mRNAs are generally not co-expressed in the same tissue or cell. Their analyses suggest that the mRNAs that are co-expressed with a certain miRNA have significantly lower incidence of binding sites for this particular miRNA. According to the model proposed by Cohen and colleagues, miRNAs confer fidelity and robustness to developmental processes rather than acting as

developmental switches. Developmental robustness is crucial during evolution, and indeed several miRNAs are highly evolutionarily conserved. Giraldez et al. (Giraldez et al., 2006), identified a miRNA, miR-430, that accelerates the decay of hundreds of maternal mRNAs during the onset of zygotic transcription. Their data suggest that single miRNAs can regulate hundreds of mRNAs to facilitate developmental transitions, rather than being absolutely required. However, other miRNAs might act very differently, as either developmental switches or factors "fine-tuning" translational efficiency and mRNA stability. Further studies of miRNAs and their target mRNAs will be necessary to shed light on a general function and importance of miRNA-mediated regulation.

2.2. The initiator phase of RNA silencing: siRNA and miRNA biogenesis

RNase-III like enzymes: Drosha and Dicers

Dicers, the RNase III-like enzymes processing long dsRNA and pre-miRNA into 20- to 25-bp small RNAs harboring 5' phosphates and 3' 2-nt overhangs, the signature of RNase III processing, were discovered in *Drosophila, C. elegans* and human cells in 2001 (Bernstein et al., 2001; Billy et al., 2001; Boutla et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). RNase III was originally discovered as a dsRNA specific endonuclease in *Escherichia coli.* In eukaryotes, RNase III enzymes function in pre-rRNAs processing, maturation of specific mRNAs, and processing of several small nucleolar RNAs (snoRNAs) and small nuclear RNAs (snRNAs) (Chanfreau et al., 1998a; Chanfreau et al., 1998b; Kufel et al., 1999; Qu et al., 1999; Seipelt et al., 1999) for review see: (Nicholson, 2003)). RNase III-like enzymes, were

originally classified into 3 groups based on differences in their domain architecture (Blaszczyk et al., 2001). Class I encompasses eubacterial RNase III enzymes and the yeast ortholog Rnt1. These enzymes contain one catalytic domain and a dsRNA-binding domain (dsRBD). Class II enzymes, Droshas, and class III enzymes, Dicers, are large multidomain proteins. In addition to two RNase III catalytic domains Drosha enzymes contain a single dsRBD and proline-rich and arginine/serine-rich N-termini. Like Droshas, Dicers contain two RNase III domains, and can contain additional domains comprising a ATPase/helicase domain, DUF283 (domain of unknown function), PAZ (Piwi/Argonaute/Zwille), and a dsRBD. DUF283 resembles a divergent dsRNA-binding domain (Dlakic, 2006). While Dicers are found in most eukaryotes, with *Saccharomyces cerevisiae* being an exception, Drosha is conserved only among metazoans. Jaskiewicz et al. (2007, in press), proposed a new classification that devides RNase III enzymes into only two classes, thereby distinguishing enzymes with one or two catalytic domains. Class I comprises enzymes with a single RNase III domain, and includes bacterial and fungal proteins, that function as homodimers. Class II encompasses Drosha and Dicer enzymes that contain two RNase III domains, and variable additional domains, and function as intramolecular pseudodimers. This new classification is based on the identification of “primitive” Dicers in unicellular eucaryotes leading to the notion that the complexity of the domain arcitecture is no longer a distingushing feature of Drosha and Dicer proteins (Macrae et al., 2006; Shi et al., 2006). It is also worth noting that in plants Dcl-1 conducts both RNase III mediated cleavage

steps, conversion of pri-miRNA to pre-miRNA and cleavage of pre-miRNA to mature miRNA, in the initiation phase of miRNA silencing (Kurihara and Watanabe, 2004; Papp et al., 2003).

The number of Dicer proteins varies from one in vertebrates to four in plants. The four plant Dicer like proteins (Dcls) have distinct functions. Dcl-1 processes long primary miRNA transcripts (pri-miRNAs) into pre-miRNA and, in a consecutive step, pre-miRNAs into mature miRNAs. Dcl-2 functions in the anti-viral defense pathway by cleaving viral dsRNA into siRNAs. Dcl-3 generates siRNAs that promote transcriptional gene silencing (TGS), and Dcl-4 generates trans-acting siRNAs (tasi-RNAs) that originate from non-coding RNAs and target degradation of mRNAs (for review see: (Meins Jr et al., 2005; Vaucheret, 2005; Willmann and Poethig, 2005)). Drosophila contains two Dicer enzymes, Dicer-1 and Dicer-2. Dicer-1 is responsible for miRNA maturation from pre-miRNAs, and Dicer-2 functions in the RNAi pathway by processing long dsRNA into siRNAs. Interestingly, although Drosophila Dicers are specialized in processing of different substrates, their functional separation is not absolute, since both enzymes are required for siRNA-mediated target mRNA cleavage. On the other hand, Dicer-1 but not Dicer-2 is essential for miRNA mediated translational repression (Lee et al., 2004b). Lee et al. suggest that Dicer-1 and Dicer-2 act at different steps of siRISC assembly. The genomes of *Caenorhabditis elegans* and mammals encode only one Dicer, processing both long dsRNA and pre-miRNAs (for review see: (Carmell and Hannon, 2004; Hammond, 2005). A schematic representation of RNase III enzymes is shown in Fig. 2.

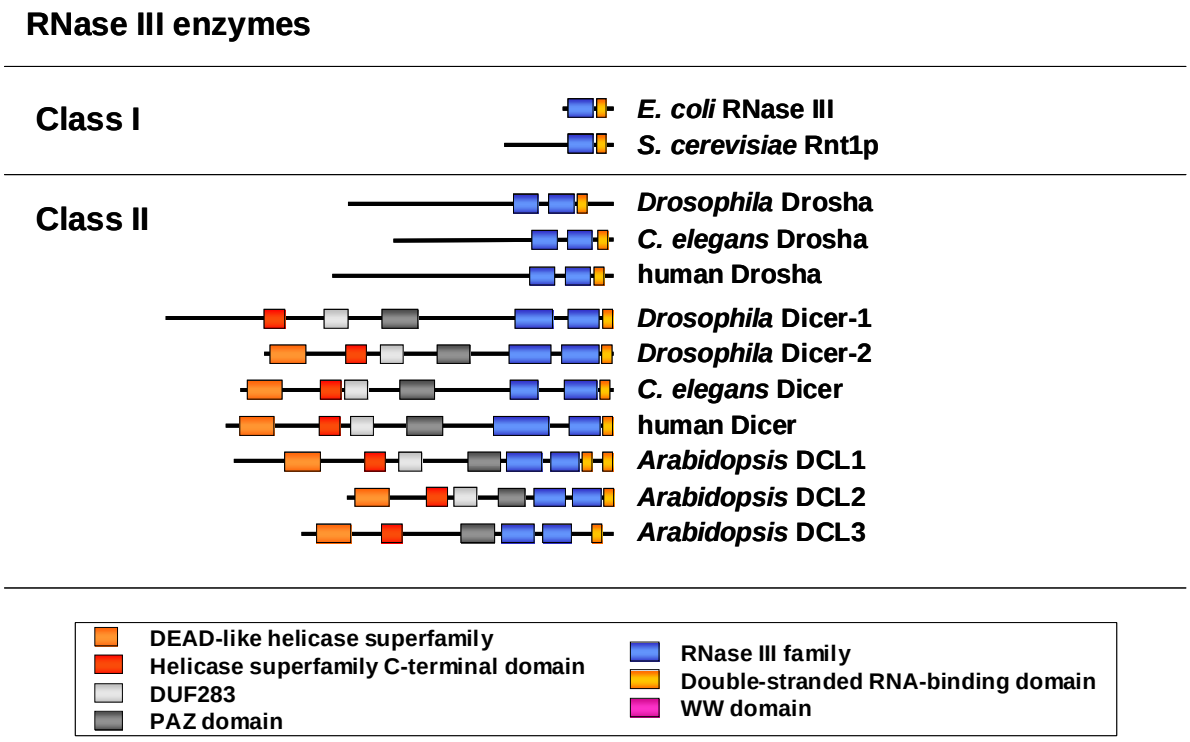

Figure 2. A schematic representation of the domain architecture of RNase III enzymes.

The mechanism of the RNase III mediated dsRNA cleavage, initially proposed by Blaszczyk et al. (Blaszczyk et al., 2001) as based on the crystal structure of the bacterial RNase III, was revised by Zhang et al. (2004). Biochemical characterization of bacterial RNase III and human Dicer revealed a common mechanism of action for RNase III-like enzymes. It has been shown that these enzymes contain a single dsRNA processing center for the cleavage of two nearby phosphodiester bonds on the opposite strands of dsRNA, producing small dsRNA fragments with characteristic 2-nt 3' overhangs, and containing 3' OH and 5' phosphate termini. While bacterial RNase III functions as a homodimer, human Dicer forms an intramolecular pseudo-dimer that exhibits independent catalytic

activities of the two RNase III domains. In 2006, the crystal structure of *Giardia intestinalis* Dicer was solved and confirmed that Dicer acts as an intramolecular pseudo-dimer (Macrae et al., 2006). *G. intestinalis* encodes one Dicer that harbors a PAZ domain and two RNase III domains, but lacks a N-terminal helicase domain and a dsRBD. The crystal structure, like the biochemical characterization by Zhang et al., (2004), indicates that the free end of the dsRNA harboring the 2-nt 3' overhang is bound by the PAZ domain. In addition, (Macrae et al., 2006) suggest that the α-helical region connecting PAZ with the RNase III domains functions as a "ruler" determining the length of the excised dsRNA fragment. Insights from the structure also provide explanation for the preference of Dicers to cleave dsRNA substrates starting from the end of the dsRNA as earlier demonstrated by Zhang et al., (2002).

Knock out of the *Dicer* gene in mice is early embryonic lethal. *Dicer*-null embryos die at E7.5 suggesting a role of Dicer during early mammalian development. Analysis of *Dicer*-null embryonic stem (ES) cells suggested a requirement for Dicer in stem cell maintenance (Bernstein et al., 2003). The *Dicer* knock out mouse ES cells displays severe defects in differentiation, and in epigenetic silencing of centromeric repeats (Kanellopoulou et al., 2005). *Dicer* deficient oocytes arrest in meiosis I and display defects in chromosomal segregation (Murchison et al., 2007; Tang et al., 2007). Interestingly, disruption of *DGCR8 (DiGeorge syndrome critical region gene-8)*, a dsRNA binding protein necessary for Drosha-mediated pri-miRNA processing, shows less severe phenotypes than disruption of *Dicer*. *DGCR8* deficient ES cells are able to

differentiate to a certain extent although they do not fully down regulate pluripotency markers. These data are consistent with a role of miRNA silencing in stem cell maintenance, but also indicate an additional miRNA-independent function for Dicer in early development (Wang et al., 2007). In zebrfish, *dicer* mutants show severe defects in morphogenesis and die on day five of development. Interestingly, injection of one single miRNA, miR-430, could rescue the early morphogenesis defects. MiR-430 miRNAs are expressed at the onset of zygotic transcription and accelerate the decay of hundreds of maternal mRNAs thereby facilitating the developmental transition (Giraldez et al., 2005; Giraldez et al., 2006).

2.3. The effector phase of RNA silencing: the RNA induced silencing complex (RISC)

2.3.1. Argonaute proteins: the heart of RISC

The siRNA directed mRNA cleavage activity has been reported to reside in complexes with a wide range of apparent molecular weight, including ~160 kD (Martinez et al., 2002), ~200-kD (Nykanen et al., 2001), ~ 500-kD (Hammond et al., 2001; Hutvagner and Zamore, 2002; Mourelatos et al., 2002) and 80S complexes (Pham et al., 2004). Argonaute (Ago) proteins were always found in RISC preparations from different organisms and deserve particular interest. They are highly basic proteins with a molecular weight of ~100-kD, and are also called PPD proteins because of the presence of a PAZ and a PIWI domain. PAZ (PIWI/ Argonaute/ Zwille) domains, which are also present in Dicers, contain an OB-fold, which suggests a role in nucleic acid binding. The PAZ recognizes a single

stranded 3' protruding end of the RNA duplex in a sequence independent manner (Lingel et al., 2003; Ma et al., 2004; Song et al., 2003; Yan et al., 2003). Interestingly, the structures of PIWI domains of Agos from *Pyrococcus furiosus* and *Achaeoglobus fulgidus* were determined by crystallography, and were shown to resemble the structure of RNase H (Parker et al., 2004; Song et al., 2004; Yuan et al., 2005). *Escherichia coli* RNase H1 cleaves the RNA strand in DNA-RNA hybrid by a divalent metal ions dependent mechanism (Keck et al., 1998). RNase H-like domains are also found in retroviral integrases and transposases that catalyze two consecutive reactions, donor-end processing and nucleotidyl transfer, resulting in strand transfer (for review see: (Haren et al., 1999)). Human Ago2 has been shown to harbor RNase H activity, an observation supported by mutation analysis of its catalytic residues, and recombinant Ago2 can recapitulate RISC cleavage activity *in vitro*, providing the ultimate proof that RISC cleavage activity resides within the Ago PIWI domain (Liu et al., 2004; Rivas et al., 2005). RISC complexes containing catalytically active Agos have been shown to cleave the phosphodiester bond of the target mRNA opposite nts 10 and 11 of the siRNA (Liu et al., 2004; Meister et al., 2004). Taken together these studies demonstrate that the endonuclease activity of RISC, which was previously named "Slicer", resides in Ago proteins themselves. Ago/PPD proteins are evolutionarily highly conserved and have been implicated in RNA silencing, developmental regulation, and stem cell fate determination in several organisms. *A. thaliana* encodes ten Ago proteins, *Drosophila* encodes five, *C. elegans* harbors at least 27, and mammals contain seven or eight members. Ago/PPD

proteins are also found in some prokaryotes, but their function in these organisms remains unclear.

The Argonaute protein family can be divided into two subfamilies, those resembling *Arabidopsis* AGO1, and those related to *Drosophila* PIWI (for review see: (Carmell et al., 2002). In contrast to the Ago subfamily that is expressed in most tissues tested, the expression of PIWI subfamily members is restricted to the germline, and to some stem cells (for review, see (Kim, 2006)). Mammalian genomes encode four Ago proteins (Ago1-4) and three or four PIWI subfamily-members (PIWI, Hili/Mili, Hiwi/Miwi, Hiwi2/Miwi2). The first mammalian Argonaute protein identified, eIF2C, was characterized and its cDNA cloned from rabbit reticulocyte lysate, and was originally found in a protein fraction that enhances translation (Zou et al., 1998). Human Ago1 was identified as a Golgi- and Endoplasmic reticulum (ER) associated protein with an apparent MW of 95kD (GERp95) (Cikaluk et al., 1999). Ago proteins have been shown to genetically and biochemically interact with siRNA- and miRNA-pathways (Grishok et al., 2001; Hutvagner et al., 2001; Hutvagner and Zamore, 2002; Ketting et al., 2001; Knight and Bass, 2001; Mourelatos et al., 2002). Argonaute proteins associate with Dicers. *Drosophila* Argonautes co-immunoprecipitate with Dicer (Hammond and Hannon, Science 2001; Caudy and Hammond, GD 2002), and human Ago2 directly interacts with Dicer, involving Dicers RNase III domain and the PIWI box of the PIWI domain of Ago2 (Tahbaz et al., 2004). The interaction between Dicer and Argonaute proteins represents a physical link between the initiator and effector phase of RNA silencing.

Members of the PIWI subfamily function in germ and stem cell fate decisions. *Drosophila* PIWI proteins are expressed in male and female germline, and associate with a specific class of small RNAs termed PIWI associated RNAs (piRNAs), most of which correspond to repeat associated small RNAs (rasiRNAs) in *Drosophila*. PIWI associated small RNAs were cloned and characterized in different organisms (Aravin et al., 2006; Aravin et al., 2003; Girard et al., 2006; Houwing et al., 2007; Lau et al., 2006; Saito et al., 2006; Vagin et al., 2006). They represent a new class of small RNAs that is distinct from siRNAs and miRNAs by its biochemical properties and biogenesis. piRNAs derive from long single stranded genomic transcripts with little predicted secondary structures. PiRNA accumulation in *Drosophila* is independent of key components of siRNA and miRNA pathways. Both *Droshophila* Dicers, R2D2, and Loquacious are dispensable for piRNA biogenesis, but Spindle-E and Armitage, two RNA helicases also implicated in RNAi and miRNA silencing, are required. In contrast to mammalian miRNAs, but similar to miRNAs and siRNAs in plants (Li et al., 2005; Yang et al., 2006; Yu et al., 2005), piRNAs are modified by 2'-O-methylation at their 3' termini (Kirino and Mourelatos, 2007; Ohara et al., 2007). In plants, the methyltransferase HEN1 modifies 3' termini of miRNA and siRNA duplexes. In mammals and *Drosophila*, the RNA-methyltransferase responsible for piRNA modifications has not yet been identified. Siomi's and Hannon's laboratories suggested a mechanism for piRNA biogenesis that requires the RNase H activity of PIWI proteins for the generation of the 5' end of piRNAs.

How 3' termini of piRNA are generated remains unknown (Brennecke et al., 2007; Gunawardane et al., 2007).

Recently, another phylogenetic analysis of Argonaute proteins identified a third clade. These group 3 Argonaute proteins are only found in worms and contain mainly Argonaute proteins that lack RNase H activity (Tolia and Joshua-Tor, 2007). The *C. elegans* Argonaute proteins act sequentially in RNA silencing and only mutation of multiple Argonaute proteins, including group 3 Argonautes, result in a loss of germline and somatic RNAi in worms (Yigit et al., 2006).

2.3.2. RISC: protein composition and assembly

RISC was originally identified by biochemical fractionation of siRNA guided mRNA cleavage activity from *Drosophila* S2 cells (Hammond et al., 2001). Besides Argonautes, several other proteins were identified to reside in RISC. The *C. elegans* Argonaute protein, RDE-1 (RNAi deficiency), exists in a complex with Dicer, the small dsRNA binding protein RDE-4, the RNA dependent RNA polymerase RDE-9, and the Dicer-related DExH box helicases Drh-1 and Dhr-2 (Tabara et al., 2002). Human Ago2 associates with the RNA helicase Gemin3, and Gemin4, components of the SMN complex (Mourelatos et al., 2002). In *Drosophila* Schneider (S2) cells, two putative RNA-binding proteins, the Drosophila homolog of the fragile X mental retardation protein (FMRP), dFXR, and VIG (Vasa intronic gene), and the nuclease Tudor-SN copurify with RISC activity (Caudy et al., 2003; Caudy et al., 2002; Jin et al., 2004a; Jin et al., 2004b). *Drosophila* FXR resides in a complex with Ago2, Dicer, the ribosomal proteins L5 and L11, together with 5S rRNA and the homolog of p68 RNA

helicase (Dmp68) (Ishizuka et al., 2002). Knock down analyzes in S2 cells revealed that Dmp68 but not FRM1 is required for efficient RNA silencing.

In addition to p68, further RNA helicases have been implicated in RNA silencing in different organisms. SDE3 encodes a DExD-box RNA helicase that is required for PTGS in *Arabidopsis* (Dalmay et al., 2001). Its Drosophila homolog, Armitage (Armi), is required for translation repression of oskar mRNA and embryonic axis specification. Armi mutant flies exhibit female sterility and disruption of RNAi and miRNA silencing in oocytes. Armi functions in early RISC assembly (Cook et al., 2004; Tomari et al., 2004a). Its human homolog, MOV10, was also shown to be a component of RISC, and to be required for RNA silencing (Meister et al., 2005). RCK/p54/DDX-6, an evolutionarily highly conserved DEAD-box RNA helicase, associates with Argonaute proteins and is required for the miRNA-mediated silencing (Chu and Rana, 2006). Observations that besides Dicer and Argonautes also RNA helicases, dsRNA binding proteins and other factors associate with RISC suggest the existence of RISC complexes of different sizes, composition, and possibly function.

In plants, fungi and worms, RNA-dependent RNA polymerases (RdRPs) are required for RNA mediated gene silencing. According to the current model, a small RNA processed by Dicer serves as a primer for the RdRP reaction. The RdRP synthesizes a complementary RNA strand thereby converting the single stranded RNA into a dsRNA, which in turn acts as a substrate for Dicer cleavage and results in a highly efficient amplification loop (Cogoni and Macino, 1999; Dalmay et al., 2000; Mourrain et al., 2000; Nishikura, 2001; Sijen et al., 2001).

No RdRP homologs have been identified in flies or mammals so far. Studies on end requirements of siRNA duplexes showed that free 3'-OH, which would be expected to be required for a RdRP dependent amplification mechanism, is not required for RNAi in these organisms, which makes the existence of an RDRP dependent amplification mechanism in flies and mammals unlikely (Chiu and Rana, 2002; Schwarz et al., 2002). However, it was recently described that RdRP can function in a primer-independent way (Zhou et al., 2006).

The mechanisms of siRISC-assembly was studied by Zamore's and Sontheimer's laboratories in *Drosophila*. Their data show that *Drosophila* siRISC complexes assemble in a stepwise manner. Tomari et al. (2004) refer to these complexes as complex A through C. Pham et al. (2004) use the terms R1, R2, and R3/holo-RISC. Native PAGE analysis demonstrated that siRNAs introduced into *Drosophila* embryo extracts assemble via defined intermediates into a "holo-RISC". The first complex, R1, binds siRNAs and comprises Dicer2 and R2D2, a small dsRNA binding protein. Complex A, also known as RISC-loading complex (RLC), differs from R1, since its assembly requires ATP and it seems to contain additional protein factors. R1/A assemble into "holo-RISC" via a transient intermediate complex (R2/B). Holo-RISC formation is an ATP-dependent process that is accompanied by siRNA unwinding. In *Drosophila* embryo extracts, RISC activity resides within a "holo-RISC" with a sedimentation coefficient of 80S (Pham et al., 2004; Tomari et al., 2004a). Unwinding of the siRNA duplex is facilitated by Ago2 mediated cleavage of the "passenger" strand (Matranga et al., 2005; Rand et al., 2005).

Zamore and colleagues addressed the intriguing issue of the siRNA guide-strand selection and passenger strand degradation. They demonstrated that *Drosophila* Dicer-2 and its dsRNA binding protein partner R2D2 (Liu et al., 2003) sense thermodynamic asymmetry of the siRNA duplex. R2D2 binds the end with the stronger dsRNA character while Dicer binds the thermodynamically less stable end of the duplex (Tomari et al., 2004b). The siRNA strand that has its 5' terminus at the end bound by Dicer is subsequently incorporated into siRISC as the guide strand. The other strand of the siRNA duplex, the passenger strand, which has its 5' terminus at the end bound by R2D2, is degraded. Degradation of the passeger strand can involve its cleavage by dAgo2, but also occurs in the absence of dAgo2 although less efficiently (Matranga et al., 2005).

In human cell extracts, Pellino et al. (2005) identified a complex containing Dicer and siRNA duplexes that might resemble an intermediate in the holo-RISC assembly, similar to the complexes formed during *Drosophila* siRISC assembly. The human complex is slightly larger than Dicer alone, which suggests the presence of an aditional factor, and its formation is significantly enhanced by ATP (Pellino et al., 2005).

Far less is known about the assembly of miRISC. Maniataki and Mourelatos (2005) showed that human Ago2 co-immunoprecipitates, also containing also Dicer and its dsRNA binding partner, TRBP, process pre-miRNAs into mature miRNAs *in vitro*. They also asymmetrically incorporate the functional miRNA guide strand, and finally cleave a model target RNA containing a site perfectly complementary to the miRNA. The human RISC assembly with pre-miRNAs and

the target cleavage are ATP-independent. Interestingly these experiments also showed that RISC cleavage activity dissociates from Dicer after pre-miRNA processing (Maniataki and Mourelatos, 2005). Shiekhattar and colleges also demonstrated that miRNA biogenesis and RISC cleavage as coupled consecutive events. This coupling appears to confer increased efficiency of miRNA incorporation into RISC and target RNA cleavage. The use of a pre-miRNA as a substrate for RISC assembly resulted in up to ten times more efficient cleavage of target mRNA when compared with the RISC assembled with siRNA-like miRNA duplexes. Rather surprisingly, no requirement for ATP hydrolysis was observed at any step, including pre-miRNA processing, RISC assembly, and target RNA cleavage (Gregory et al., 2005). A trimeric complex consiting sole of Dicer, Ago2 and TRBP has been suggested to confine a minimal system for pre-miRNA processing and RNA-target cleavage (Gregory et al., 2005). However experiments presented in this work have been conducted with immunoprecipitates and not with recombinant proteins, which does not exclude the presence of additional protein components.

A schematic representation of the miRNA- and the siRNA silencing pathways in mammals is shown in Fig. 2.

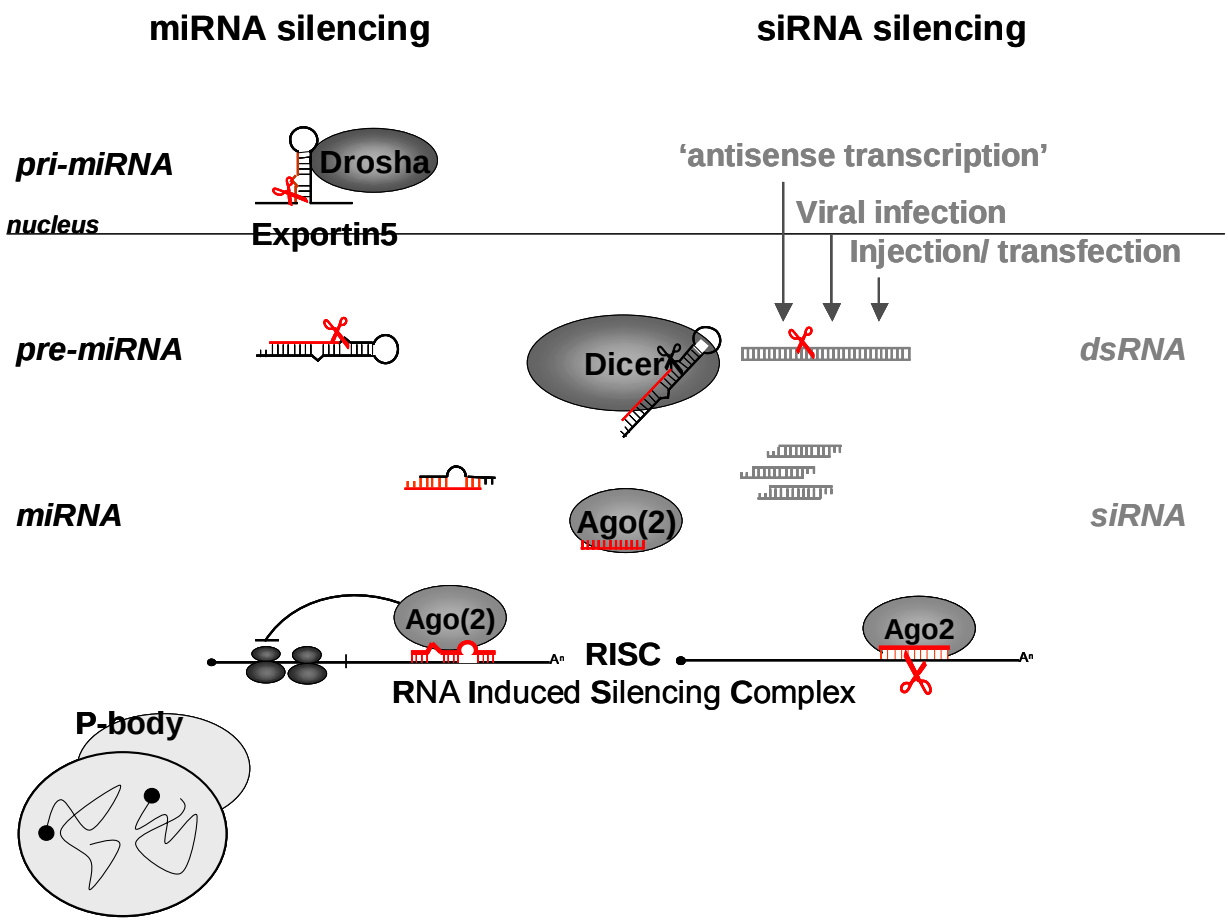

Figure 2. miRNA and siRNA silencing pathways in mammals. In miRNA silencing, depicted on the left hand side, the nuclear class II RNase III enzyme, Drosha, processes pri-miRNAs into pre-miRNAs. Pre-miRNAs are further exported to the cytoplasm by an Exportin 5 dependent mechanism. In the cytoplasm pre-miRNAs are processed into mature miRNAs by Dicer. Consecutively, the miRNA guide strand is selected and incorporated into a RISC complex. Argoanute proteins are the main protein components of RISC. MiRNAs within RISC interact with the 3' UTRs of target-mRNAs with imperfect complementarity, and lead to the inhibition of translation. The inhibited mRNA bound by RISC is translocated to P-bodies, and either stored in a translationally inactive state or subjected to degradation. Stored, translationallly inactive mRNAs can exit P-bodies and can resume translation under certain conditions. The siRNA pathway is outlined on the right hand side. SiRNAs are defined by their biogenesis from long perfectly double

stranded RNA precursors. These dsRNAs possibly arise from antisense transcription, viral infection or transfection/injection, and can be processed by Dicer into siRNAs. These siRNAs are further incorporated into a RISC harboring an RNaseH cleavage competent Ago. siRNAs within RISC target mRNAs with perfect complementarity, and lead to mRNA cleavage by Slicer-activity and consecutive mRNA degradation. While miRNA silencing is known to occur in mammals, siRNAs derived from long dsRNA have not been detected so far (therefore depicted in grey). However, the mammalian system is capable of miRNA cleavage by Ago2 when provided with exogenous siRNAs.

3. Results

3.1. Identification of proteins associated with Dicer in human cells

In 2001, human Dicer (hDicer) was identified as a key-enzyme of siRNA and miRNA biogenesis in mammalian cells (Bernstein et al., 2001; Billy et al., 2001). Although the biochemistry and biology of Dicer have been intensively studied in mammals, little was known about proteins associated with Dicer, and their function in RNA silencing. The aim of this project was to identify proteins associated with hDicer, and to characterize their function in the RNA silencing pathways.

With the aim to purify hDicer complexes, we constructed a fusion of hDicer with the Tandem Affinity Purification (TAP) tag. Fig. 3A shows a schematic representation of the TAP-tag and the purification procedure. The TAP-tag consists of a Protein A domain that tightly binds IgG, and a calmodulin binding peptide (CBP), separated from the Protein A domain by a Tabacco Etch Virus (TEV) protease cleavage site. The TAP method allows purification of protein complexes under native conditions. This purification scheme was initially developed in yeast and later adapted for various organisms ((Rigaut et al., 1999); for review see: (Puig et al., 2001)). A TAP-tagged Dicer (TAP-Dicer) expression plasmid, in which transcription of the TAP-Dicer mRNA is driven by a tetracycline (tet) inducible promoter, was randomly integrated into the genome of HEK293-TReX cells, and stable, clonal cell lines were generated. HEK293-TReX cells stably express the tet-repressor. In the un-induced state, the tet-repressor binds to the tetracycline operator region of the inducible promoters and inhibits their

transcription. Treatment of the cells with tetracycline induces transcription from the tetracycline inducible promoter. After induction of the TAP-Dicer expression with tetracycline for 48, the recombinant protein was purified from cytoplasmic extracts, as shown in Fig. 3B. Extracts from cells expressing the TAP-tag only were used as a negative control. Cytoplasmic cell lysates of the TAP-Dicer cell line and the control are shown on the left hand side. After tandem affinity purification, TAP-Dicer was eluted from calmodulin beads using EGTA (shown on the right). The differences in the molecular weight (MW) of TAP-Dicer in the cytoplasmic extract and after elution result from the cleavage of the Protein A domain by the TEV protease.

In 2003, Forler et al. modified the TAP strategy by combining the expression of a TAP-tagged human protein of interest with simultaneous knock down of the *Drosophila* homolog in *Drosohpila* S2 cells, to reduce competition for the TAP-tagged protein from the endogenous protein (Forler et al., 2003). This combination of RNAi and TAP was termed “iTAP”. We adapted this strategy for human cell lines by expressing TAP-Dicer together with a short hairpin to knock down endogenous Dicer in HEK293 cells. Since the short hairpin targets the coding sequence (cds) of Dicer, we introduced two silent point mutations in the region targeted by the hairpin to avoid degradation of the mRNA encoding the TAP-Dicer construct (iTAP-Dicer), represented in Fig. 3C. The iTAP-Dicer was stably transfected into the Dicer knock down cell line (293/Dicer kd) in the HEK293-TReX background (Schmitter et al., 2006) (Fig. 3C and D). The iTAP-Dicer and the short hairpin targeting Dicer were under the control of a

tetracycline (tet) inducible protmoter. Upon treatment with tetracycline, expression of the short hairpin knocking down endogenous Dicer and expression of the iTAP-Dicer was induced. Fig. 3D shows the analysis of cell extracts from the Dicer knock down cell line, control cells, and the iTAP-Dicer cell line before and after tetracycline induction. Induction of the short hairpin construct knocked down endogenous Dicer protein levels (Fig. 3D, lanes 1 and 2). Tetracyline induction of the iTAP-Dicer cell line resulted in a knock down of endogenous Dicer and simultaneous expression of the resistant iTAP-Dicer construct (Fig. 3D, lanes 5 and 6).

To test the functionality of the iTAP-Dicer protein, we performed Dicer processing assays using either cytoplasmic extracts from iTAP-Dicer cell lines or purified iTAP-Dicer (Fig. 3E). An in vitro transcribed and purified pre-let7 RNA served as a substrate for Dicer cleavage. Extracts from Dicer knock down cells lack Dicer cleavage activity, as expected (Fig. 3E, lane 1). Cells expressing a non-targeting hairpin were used as a control (Schmitter et al., 2006). Expression of a control hairpin did not affect Dicer cleavage activity compared to the parental cell line (Fig. 3E, lanes 2 and 3). Expression of iTAP-Dicer in Dicer knock down cells (iTAP-Dicer cell line) partially restored Dicer cleavage activity (Fig. 3E, lane 4), and iTAP-Dicer maintained its enzymatic activity after tandem affinity purification (Fig. 3E, lane 5).

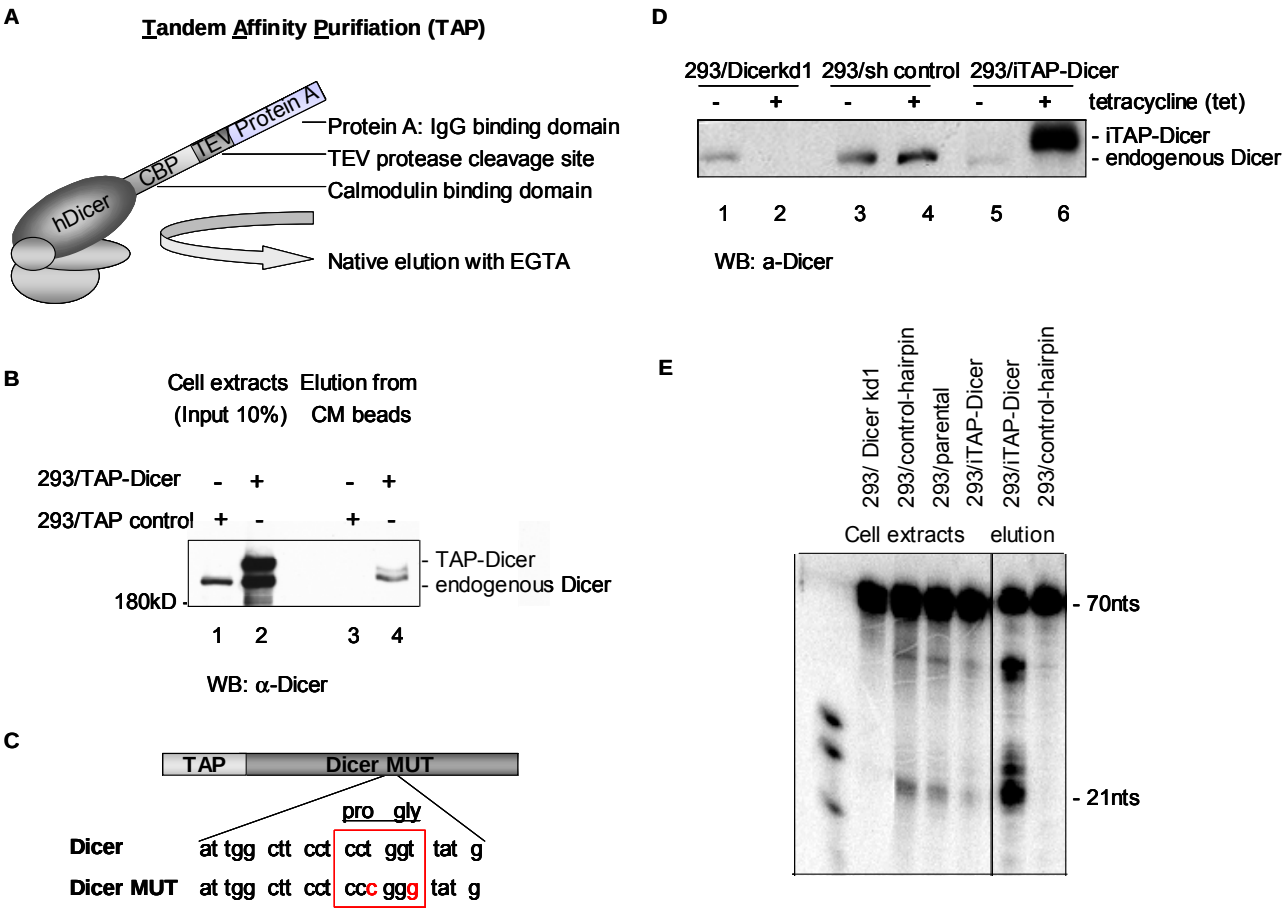

Figure 3. Tandem affinity purification of TAP-tagged human Dicer. (**A**) A schematic representation of the TAP strategy according to Seraphin et al. (**B**) TAP-Dicer was stably expressed in HEK293/TRex cells and purified according to Seraphin et al. Cell extracts, and elutions from calmodulin (CM) beads by EGTA were analyzed by western blotting. (**C**) Two silent point mutations were introduced in Dicers cds of the TAP-Dicer construct to prevent degradation of its mRNA by the α-Dicer shRNAs. (**D**) Stable cells lines expressing a short hairpin to knock down Dicer and the iTAP-Dicer constructs, both expressed from tetracycline inducible promoters, were generated and tested for knock down of endogenous Dicer and expression of iTAP-Dicer by western blotting. Lanes 1 and 2 show that a cell line expressing the α-Dicer shRNA. Upon tetracycline inductions, Dicer is efficiently knocked down as demonstrated by western blotting. Lanes 3 and 4 respresent cells expressing a non-targeting control shRNA. Lanes 5 and 6 show an iTAP-Dicer cell line. Upon induction with tetracycline, endogenous Dicer is knocked down and iTAP-Dicer is expressed. (**E**) Enzymatic activity of iTAP-Dicer was tested by *in vitro* processing

assays using pre-let7 RNA as a substrate, and either cytoplasmic extracts or tandem affinity purified iTAP-Dicer as an activity source. Production of 21nt mature miRNAs was monitored by autoradiography on a 15% PAGE.

As shown in Figure 3B, tandem affinity purification of TAP-Dicer was not very efficient. More detailed analysis revealed, that the TEV cleavage step using a commercially available recombinant protease was the first limiting step. Different temperatures and duration of the TEV cleavage reaction were tested, but could not much improve the yield of the soluble cleaved protein. Additional steps in the purification could be optimized: (1) growing cells in liquid cell cultures and not as adherent cells on plates could facilitate the growth of larger amounts of cells; (2) different preparations of the TEV protease should be tested; (3) changes in the buffer composition for elution of the cleaved protein after TEV could minimize unspecific rebinding to the igG beads; (4) inefficient elution from calmodulin beads by chelating calcium, could be avoided when native elution of the protein complex is not required, as for mass spectrometry, by eluting using more stringent conditions (e.g. SDS).

In parallel to the TAP approach, monoclonal α-Dicer antibodies, generated in our laboratory were optimized for immuno-affinity purification of endogenous Dicer. Advantages and disadvantages of these two approaches are listed in Table 1.

Approach	Pros	Cons
Tandem Affinity Purification (TAP)	- Increased purity of the purified complex due to the two-step purification scheme. - Native purification conditions	- The protein-tag might influence the functions and interactions of the tagged protein. - Overexpression of the tagged protein might reduce its integration into functional endogenous protein complexes due to limiting amounts of interacting partners. - This two-step purification required large amounts of starting material.
Immunoprecipitation	- independent of cloning and ectopic gene expression - allows purification of endogenous proteins	- dependent on the availability of good antibodies - cross-reactivity of the antibody

Table 1. Strengths and weaknesses of affinity approaches for the retrieval of protein complexes.

Dicer immunopurifications from HEK293 and HeLa cell extracts were repeated several times. Immunoprecipitates were separated by 1D or 2D SDS-polyacrylamide gel electrophoresis (PAGE), and analyzed by liquid chromatography tandem mass spectrometry (LC-MSMS). An example of an analyzed 2D-PAGE analysis is shown in Haase et al., 2005 (chapter 3.2.). Representative coomassie-stained 1D-PAGE analyses are shown in Fig. 4. Dicer was immunopurified from HEK293 (Fig. 4A) or HeLa (Fig. 4B) cytoplasmic extract using the combination of three monoclonal α-Dicer antibodies cross-linked to Protein G Sepharose beads (lanes 2). Monoclonal α-myc antibodies were used as an isotype control (lanes 1). Proteins are visualized by coomassie stain. Lanes corresponding to α-Dicer and to control immunoprecipitates were fragmented and all protein containing gel fragments were subjected to LC-MSMS analysis. Proteins that were identified in the Dicer immunoprecipitates and were absent from the control purification are indicated on the right hand side. Additional information about the identification of proteins in α-Dicer immunoprecipitates is documented in the appendix section.

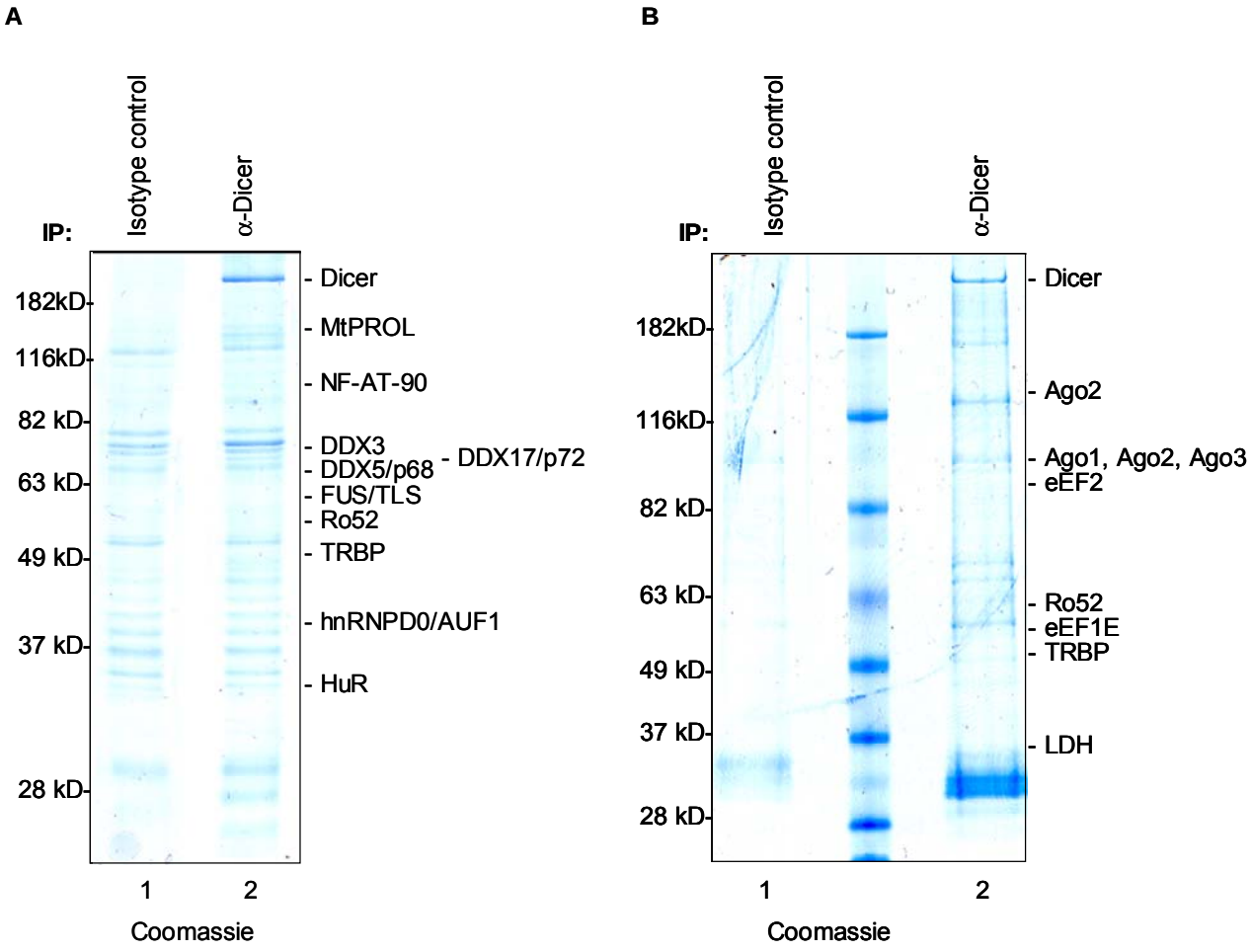

Figure 4. Analysis of Dicer immunoprecipitates. Dicer was immuno-purified from (**A**) HEK293 and (**B**) HeLa cytoplasmic extracts using α-Dicer monoclonal antibodies cross-linked to IgG-Sepharose beads. An α-Myc isotype antibody was used as a negative control. The immunoprecipitates were separated by SDS-PAGE, and proteins were visualized by coomassie stain. Individual lanes representing Dicer immunoprecipitates and the control precipitation were entirely fragmented and protein containing gel fragments were digested with trypsin and analyzed by LC-MSMS. Some of the proteins that were identified in Dicer immunoprecipitates and absent from the control precipitation are indicated on the left hand side. Further LC-MSMS data are provided in the appendix section.

Proteins identified to specifically co-purify with Dicer fall into different categories: (1) known components of RNA silencing pathways, (2) translation factors, (3) translational regulators, (4) RNA helicases, (5) RNA binding proteins, and others. Proteins that were reproducibly found in independent α-Dicer immunoprecipitates are summarized in Table 2.

Analysis of six (1-6) analyses of independent α-Dicer immunoprecipitates								
Functional group	**Protein name**	**Accession number**	**1**	**2**	**3**	**4**	**5**	**6**
Argonaute proteins	Ago1/ eIF2c1	Q9UL18						X
	Ago2/ eIF2c2	Q9UKV8			X			X
	Ago3// eIF2c3	Q9H9G7						X
RNA helicases	DDX3/ HLP2	O00571			X		X	
	DDX5/ p68	P17844			X		X	
	DDX17/ p72	Q92841				X	X	
RNA binding proteins	TRBP	Q9BRY2	X		X	X	X	X
	Oncogene FUS/ TLS	P35637			X		X	
	Ewing Sarcoma Breakpoint Region 1 (EWSR1)/ EWS	Q01844	X		X			
	Nucleolysin TIAR/ TAI-1-related protein	Q01085			X			
	HnRNP A/B;APOBEC-1-binding protein 1; ABBP-1	Q99729			X			
Translational regulators	Nascent polypeptide-associated complex subunit alpha (NACA)	Q13765	X					
	hnRNP D0/ AUF1	Q1413			X		X	
	Interleukin enhancer-binding factor 3 (ILF3), Nuclear factor of activated T-cells 9 kD (NF-AT-9)	Q1296			X		X	
	FBP2 (FUSE binding protein 2)/ KSRP (KH type splicing regulator)	Q92945	X		X			
	ELAV-like protein 1 (Hu-antigen R) (HuR)	Q15717	X		X	X	X	
Translation factors	Eukaryotic translation initiation factor 3 subunit 9; Prt1 homolog	P55884	X					
	Elongation Factor 2	P13639						X
	Elongation factor 1-alpha 1	P6814			X	X		X
RBCC/ TRIM protein	Ro52/SS-A/TRIM21	P19474				X	X	X
	Sec24c protein transport protein	P53992	X					
DNA-dependent RNA polymerase	DNA-directed RNA polymerase, mitochondrial (MtRPOL)	O411			X	X	X	
Actin binding, profilin binding	Developmentally-regulated brain protein (Drebrin)	Q16643	X	X				
Actin binding/ cytoskeletal protein	Epithelial protein lost in neoplasm (EPLIN)	Q9UHB6	X	X				

Table 2. Proteins reproducibly identified in Dicer immunoprecipitates. Six independent experiments were performed and analyzed by LC-MSMS: α-Dicer mmunoprecipitates from HEK293 extracts (1-5), and from HeLa extracts (6). Proteins identified by LC-MSMS specifically in Dicer immunoprecipitates and not in control samples are listed. Ribosomal proteins and heat shock proteins are excluded from this list.

3.2. TRBP, a Regulator of Cellular PKR and HIV-1 Virus Expression, Interacts with Dicer and Functions in RNA Silencing (Haase et al., 2005)

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TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing

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Dicer is a key enzyme involved in RNA interference (RNAi) and microRNA (miRNA) pathways. It is required for biogenesis of miRNAs and small interfering RNAs (siRNAs), and also has a role in the effector steps of RNA silencing. Apart from Argonautes, no proteins are known to associate with Dicer in mammalian cells. In this work, we describe the identification of TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein) as a protein partner of human Dicer. We show that TRBP is required for optimal RNA silencing mediated by siRNAs and endogenous miRNAs, and that it facilitates cleavage of pre-miRNA *in vitro*. TRBP had previously been assigned several functions, including inhibition of the interferon-induced double-stranded RNA-regulated protein kinase PKR and modulation of HIV-1 gene expression by association with TAR. The TRBP–Dicer interaction shown raises interesting questions about the potential interplay between RNAi and interferon–PKR pathways.

Keywords: Dicer; TRBP; PKR; RNA interference; microRNA

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INTRODUCTION

RNA interference (RNAi)-mediated and microRNA (miRNA)-mediated reactions have emerged recently as important pathways regulating eukaryotic gene expression at various levels. Specificity of these processes is dependent on 20- to 25-nt small interfering RNAs (siRNAs) and miRNAs, acting as guides that recognize sequences of target nucleic acids. To perform their effector function, siRNAs and miRNAs are incorporated into ribonucleoprotein (RNP) complexes, referred to as si- or mi-RISCs (acting post-transcriptionally) or RITS (acting at the chromatin level). Dozens of different proteins have been identified as either essential or regulatory factors for RNAi and miRNA reactions (Tomari & Zamore, 2005). Moreover, it has become increasingly apparent that both pathways intersect with several other cellular processes, such as chromosome segregation (Matzke & Birchler, 2005), RNA editing (Scadden, 2005) and nonsense-mediated degradation (Domeier *et al*, 2000; Kim *et al*, 2005).

MicroRNAs are generated from the genome-encoded precursor hairpins by the sequential action of two ribonuclease (RNase) III-type nucleases, Drosha and Dicer. Dicer is also responsible for the excision of siRNAs from double-stranded (ds)RNA (Kim, 2005; Tomari & Zamore, 2005). However, Dicer is not confined to miRNA and siRNA biogenesis. Each of the two *Drosophila* Dicers, Dcr1 and Dcr2, also seems to be essential for the effector step of RNAi (Tomari & Zamore, 2005). Dcr2, which functions primarily in RNAi, heterodimerizes with a dsRNA-binding domain (dsRBD) protein R2D2 (Liu *et al*, 2003). The resulting complex senses the differential stability of the ends of the siRNA duplex and determines which siRNA strand will enter the RISC (Tomari *et al*, 2004). Recent studies have shown that Drosha and Dcr1, the *Drosophila* Dicer specializing in miRNA biogenesis, function in complexes with dsRBD protein partners DGCR8/Pasha (reviewed by Tomari & Zamore, 2005) and Loquacious (Loqs; Förstemann *et al*, 2005; Saito *et al*, 2005), respectively.

In contrast to *Drosophila*, mammals and *Caenorhabditis elegans* express only a single Dicer protein. Like most other Dicers, mammalian enzymes are large, ~200 kDa, proteins containing ATPase/RNA helicase, DUF283, PAZ domains, two catalytic RNase III domains and a carboxy-terminal dsRBD. Although the biological importance and biochemistry of mammalian Dicer have been intensively studied, little is known about its protein partners. The only proteins known to interact directly with Dicer in mammals are members of the Argonaute (Ago) family, which represent siRNA/miRNA-associating core components

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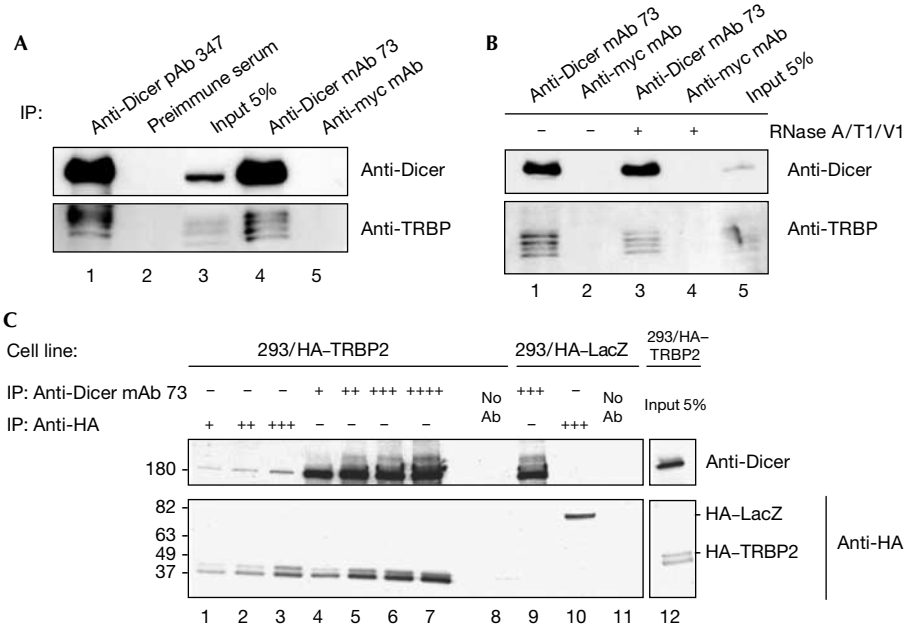

Fig 1 | TRBP and Dicer co-immunoprecipitation. (**A**,**B**) Anti-Dicer antibodies (Abs) pull down endogenous TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein) in extracts of human embryonic kidney (HEK)293 cells, and co-immunoprecipitation of Dicer and TRBP is not sensitive to ribonuclease (RNase) treatment (**B**). mAb, monoclonal antibody; pAb, polyclonal antibody. (**C**) Dicer–TRBP interaction studied with a HEK293 cell line expressing haemagglutinin (HA)-tagged TRBP2. A HEK293/HA–LacZ cell line was used as a control. The identity of the Abs, some at increasing concentrations (+ through ++++), used for immunoprecipitation (IP) is indicated at the top of the panels. Abs used for western blots are indicated on the right.

of RISCs (reviewed by Tomari & Zamore, 2005). The interaction, involving the RNase III domain of Dicer and a PIWI domain of Argonautes (Tahbaz *et al*, 2004), is probably central to the handover of the products of Dicer catalysis (siRNAs and miRNAs) to Argonautes during RISC assembly.

In this work, we describe the identification of TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein; Gatignol *et al*, 1991) as a dsRBD protein partner of human Dicer, and show that TRBP is required for optimal RNA silencing mediated by siRNAs and endogenous miRNAs. TRBP has previously been assigned several functions, including inhibition of the interferon (IFN)-induced dsRNA-regulated protein kinase PKR (Daher *et al*, 2001), modulation of HIV-1 gene expression through its association with TAR (Dorin *et al*, 2003) and control of cell growth (Benkirane *et al*, 1997; Lee *et al*, 2004). A mouse TRBP homologue, Prbp, was shown to function as a translational regulator during spermatogenesis, and mice that have this deletion, in addition to being male sterile, usually died at the time of weaning (Zhong *et al*, 1999). The TRBP–Dicer interaction established in this work raises the possibility of crosstalk between RNAi and IFN–PKR pathways in normal and virus-infected cells.

RESULTS AND DISCUSSION

Dicer and TRBP interact *in vivo* and *in vitro*

We raised monoclonal antibodies (mAbs) against human Dicer (supplementary Fig S1 online). The mAbs 33, 73 and 83, which effectively immunoprecipitate Dicer from extracts of different cultured cells (data not shown), were used to identify proteins associated with Dicer in human embryonic kidney (HEK)293 cells. Proteins retained with either mAbs 33/73/83 or anti-Myc mAb, used as a control, were separated using two-dimensional gel electrophoresis, and spots enriched in Dicer immunoprecipitates were processed for mass spectrometry analysis. One protein reproducibly co-purified with Dicer was identified as TRBP, a protein containing three dsRBDs (supplementary Fig S1 online). Members of the Argonautes family were also among the selected proteins, as were some others, but the reproducibility and significance of their interactions with Dicer were not further investigated (data not shown).

To validate the Dicer–TRBP interaction, we performed co-immunoprecipitation experiments using either extracts from human HEK293 cells or purified proteins. Two anti-Dicer antibodies (Abs), mAb 73 and polyclonal Ab 347, but not the control mAb isotypic with mAb 73, immunoprecipitated endogenous TRBP that was present in HEK293 cells, as shown by western blotting with anti-TRBP Abs (Fig 1A; several forms of TRBP, with apparent molecular masses of 43–46 kDa, are expressed in human cells (see below)). Treatment with RNases digesting both double- and single-stranded RNAs did not eliminate the association (Fig 1B), indicating that the interaction is not mediated by RNA. As immunoprecipitating anti-TRBP Abs were not available, we generated a stable HEK293 cell line, HEK293/HA–TRPB2, expressing the haemagglutinin (HA)-tagged version of the best-studied isoform of TRBP, TRBP2. Co-immunoprecipitation experiments performed with the HEK293/HA–TRPB2 extract and either anti-HA or anti-Dicer Abs showed that each Ab was able to pull down the partner protein (Fig 1C). Further indication that

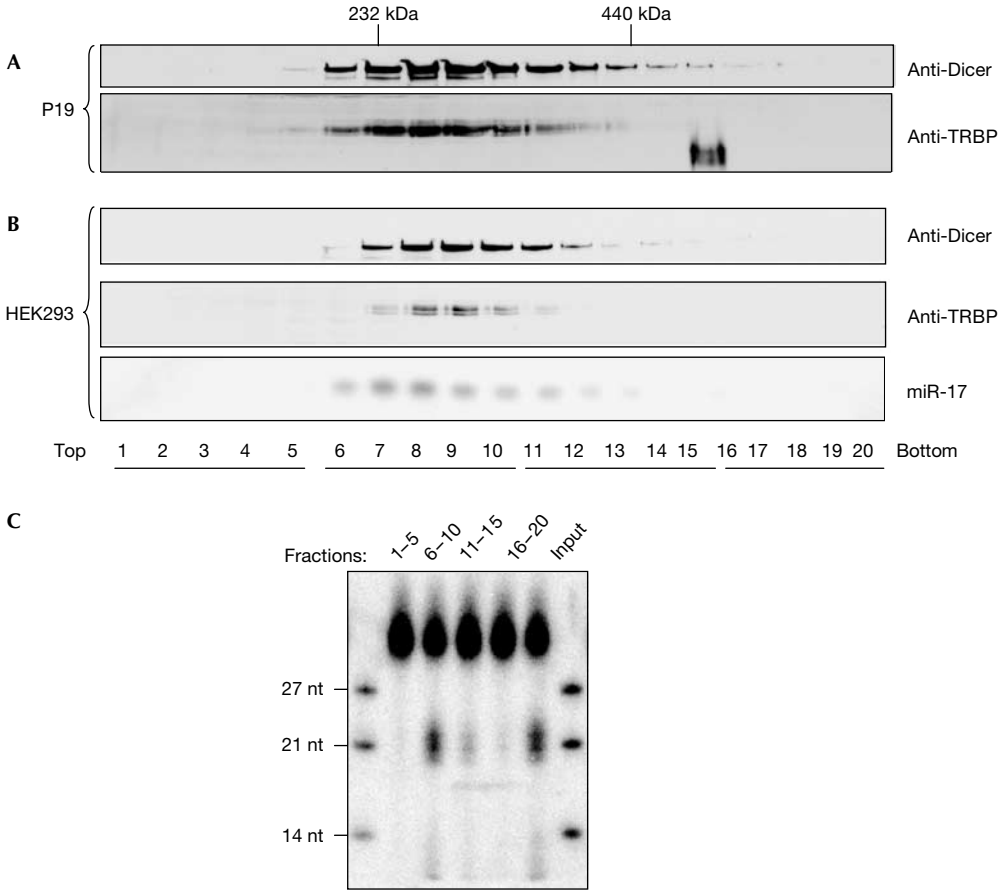

Fig 2 | TRBP and Dicer co-sedimentation on glycerol gradients. (**A**,**B**) Sedimentation of cytoplasmic extracts from P19 cells (**A**) and human embryonic kidney (HEK)293 cells (**B**). Gradient fractions were analysed for Dicer and TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein) by western blotting and for miR-17 by northern blotting. (**C**) Activity of fractions analysed in (**B**), pooled as indicated (top of the panel), in processing 30-bp double-stranded RNA into small interfering RNA.

TRBP and Dicer form part of the same complex was provided by gradient sedimentation experiments. Analysis of cytoplasmic extracts prepared from either human HEK293 or mouse teratoma P19 cells showed that Dicer and TRBP, or their mouse counterparts, co-sediment in a region corresponding to a molecular mass of ~250 kDa (Fig 2A,B). Notably, miR-17, an abundant miRNA in HEK293 cells, was also enriched in this region, as was the activity of processing a 30-bp dsRNA to siRNA (Fig 2B,C). Taken together, the data indicate that Dicer and TRBP interact with each other in mammalian cells.

To find out whether the Dicer–TRBP interaction was direct, we purified both proteins, as recombinant fusions with His_6 and a maltose-binding protein (MBP) from insect cells and *Escherichia coli*, respectively (Fig 3A). The proteins were incubated together and applied either to Protein G–Sepharose beads coated with different Abs or to amylose beads. Dicer mAb 73, but not control anti-HA mAb, effectively pulled down TRBP2. Likewise, TRBP2 retained on amylose beads pulled down Dicer (Fig 3B). The low efficiency of the latter pull-down could be the result of a sterical hindrance caused by the MBP tag or owing to the propensity of TRBP to form homodimers (see below). To eliminate the possibility that proteins co-purifying with either Dicer or MBP–TRBP2 preparations are involved in binding, we studied the Dicer–TRBP interaction in the yeast two-hybrid (2H) assay (Fig 3C). As the budding yeast does not encode TRBP or Dicer homologues, any interaction detected in this system would probably result from direct binding. Plasmids encoding full-length TRBP2, or different regions thereof, fused to the Gal4 DNA-binding domain, and a plasmid encoding Dicer appended to the Gal4 activation domain, as well as several control plasmids, were transformed into yeast. We detected interactions between Dicer and TRBP2 and all its mutants encompassing amino acids 228–366. This region of TRBP includes the dsRBD C domain, suggesting that this domain mediates the interaction (see Conclusions). We also detected TRBP2 interacting with itself, which was consistent with its ability to homodimerize (Daher *et al*, 2001). Taken together, our results indicate that Dicer and TRBP interact directly with each other.

The 45 kDa TRBP2 consists of three dsRBDs. Another previously described TRBP isoform, TRBP1, differs from TRBP2 at the amino terminus (Bannwarth *et al*, 2001; see Fig 3D). By complementary DNA cloning and by inspecting EST databases, we identified another TRBP splice variant, potentially encoding a TRBP3 isoform, which would miss the C-terminal dsRBD (Fig 3D). Interestingly, one of the three isoforms of Loqs, the probable

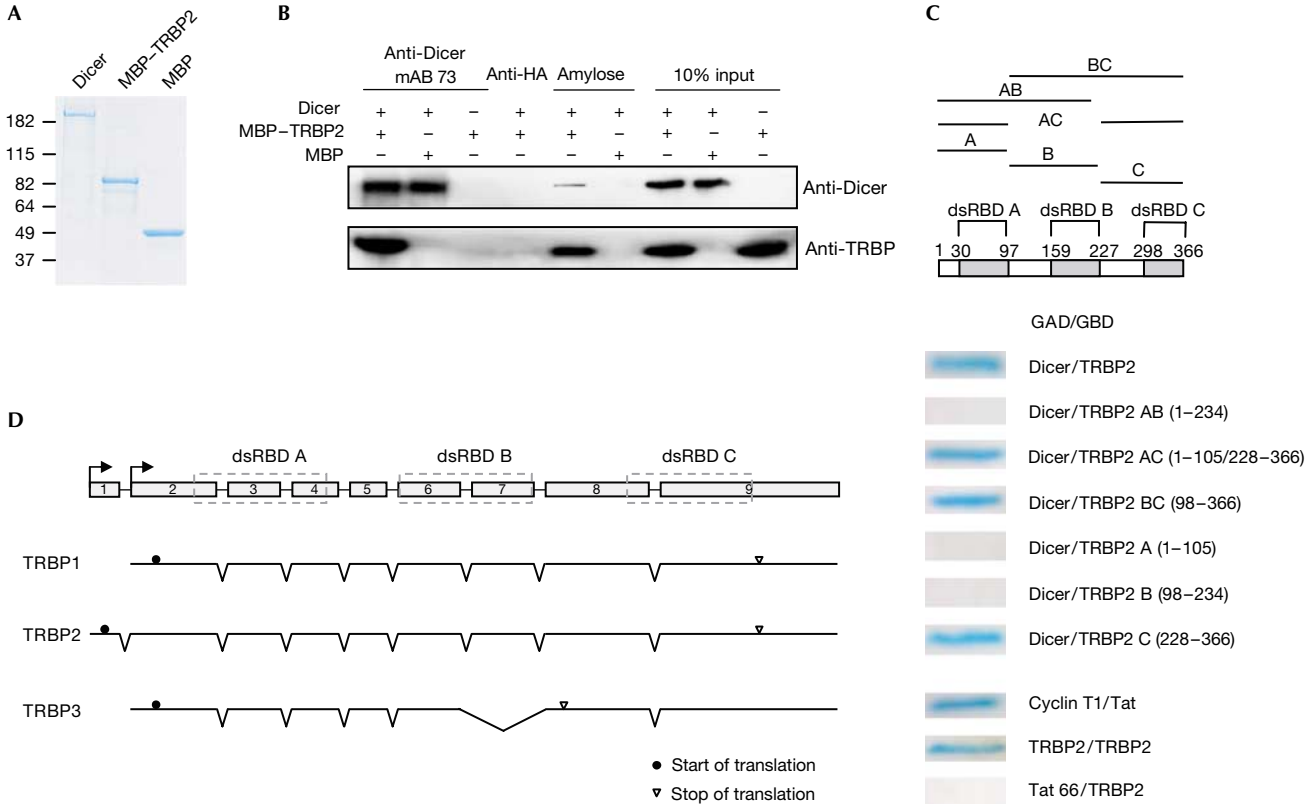

Fig 3 | Interaction of TRBP with Dicer studied with purified proteins (**A**,**B**) and in the yeast two-hybrid system (**C**), and schematic representation of different TRBP transcripts expressed in human cells (**D**). (**A**) SDS–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of indicated purified recombinant proteins. MBP, maltose-binding protein. (**B**) Purified TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein) and Dicer interact with each other. Added proteins, antibodies (Abs) and amylose beads are indicated at the top. Abs used for western blotting are shown on the right. HA, haemagglutinin; mAb, monoclonal antibody. (**C**) Dicer and TRBP interact in a yeast two-hybrid (2H) assay through the carboxy-terminal domain of TRBP. Top: scheme of TRBP2 and its fragments included in 2H constructs. Double-stranded RNA-binding domains (dsRBDs) are shown as grey boxes. Bottom: β-galactosidase staining diagnostic of the interaction between proteins expressed as fusions with either Gal4 DNA activation domain in pGADGH (GAD) or Gal4 DNA binding domain in pGBT9 (GBD). Cyclin T1/Tat and TRBP2/TRBP2 are positive controls. Tat/TRBP2 is a negative control. (**D**) Scheme of the TRBP gene and its encoded transcripts. Intron positions and exon regions encoding three dsRBDs are indicated. Two alternative transcription starts, and translation initiation and stop codons, are indicated by arrows, circles and triangles, respectively. Skipping of exon 7 in TRBP3 causes translation to terminate in exon 8.

Drosophila homologue of TRBP, is also devoid of the C-terminal dsRBD (Förstemann *et al*, 2005). The biological function of individual TRBP variants remains unknown. The alignment of TRBP2 with dsRBD Dicer protein partners from other species and with a TRBP-related mammalian protein PACT is shown in supplementary Fig S3A online. High sequence conservation of the C-terminal dsRBD in Loqs, TRBP2 and PACT (supplementary Fig S3B online) suggests that this domain has a function distinct from two other dsRBDs (see below).

TRBP is required for miRNA and siRNA silencing

To assess the potential role of TRBP in RNAi and miRNA pathways, we generated stable HEK293T-REx cell lines, in which the expression of TRBP is knocked down by RNAi. In cell lines 293/TRBPkd1 and 293/TRBPkd2, the expression of stably integrated short hairpins targeting different regions in TRBP messenger RNA is controlled by a pol III promoter with tetracycline (Tet) operator sites. Real-time PCR and western analyses indicated that TRBP expression was about fivefold lower at both mRNA and protein levels. In either cell line, partial decrease of the protein had already occurred in the absence of Tet induction, indicating some leakiness of the system (Fig 4A; supplementary Fig S2 online). TRBP depletion had no appreciable effect on cell growth (data not shown).

We compared the miRNA-precursor-processing activity of extracts prepared from different cell lines (Fig 4B). Extracts from either TRBPkd cell line processed pre-let-7 RNA less efficiently than extracts from control cells. The decrease in activity was not due to destabilization of Dicer, as its level was similar in control and TRBPkd cells (Fig 5B). Despite extracts from TRBPkd cells being deficient in pre-miRNA processing, steady levels of several miRNAs in these cells were not significantly different from control HEK293 cells, and there was no apparent accumulation of pre-miRNAs (Fig 4C; data not shown). Notably, as in the case of TRBP,

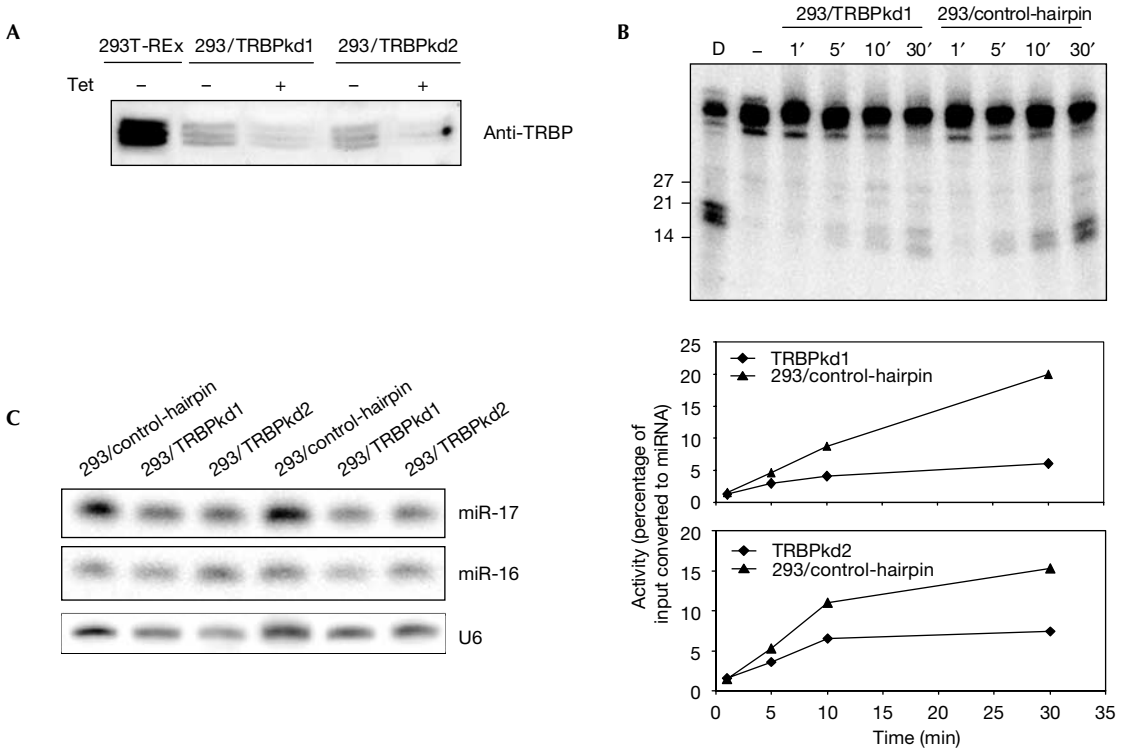

Fig 4 | Depletion of TRBP affects pre-microRNA processing *in vitro* but has no effect on accumulation of mature microRNAs *in vivo*. (**A**) Levels of TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein) in 293/TRBPkd1/2 and human embryonic kidney (HEK)293T-REx control cell lines after treatment with tetracycline (Tet) for 72 h as indicated. Anti-TRBP antibody was used for western blotting. (**B,C**) Effect of TRBP knockdown on processing of pre-let-7 RNA *in vitro* (**B**) and on accumulation of mature microRNAs (miRNAs) *in vivo* (**C**). (**B**) Processing of pre-let-7 RNA was assayed in extracts from 293/TRBPkd1, 293/TRBPkd2 and 293/control-hairpin cell lines. Phosphorimage of 293/TRBPkd1 (upper panel) and quantification of data for both TRBPkd cell lines (bottom panels) are shown. (**C**) Total RNA isolated from TRBPkd cell lines, from two independent cultures and control cell lines was analysed by northern blot using probes specific for indicated RNAs. Quantification of data from four independent northern blots showed no significant differences in miR-16 and miR-17 levels between TRBPkd and control cultures. A representative experiment is shown. Similar results were obtained for miR-19B and let-7 miRNAs (data not shown).

depletion of Loqs in *Drosophila* S2 cells had no principal effect on mature miRNA levels although extracts of Loqs knockdown cells were deficient in pre-miRNA processing. However, in contrast to TRBP, depletion of Loqs resulted in accumulation of pre-miRNAs in S2 cells (Förstemann *et al*, 2005; Saito *et al*, 2005).

We used the miRNP-mediated mRNA-reporter-cleavage assay to find out whether depletion of TRBP had an effect on the activity of endogenous miRNPs in HEK293 cells. TRBPkd and control cells were transiently transfected with constructs expressing either control *Renilla* luciferase (RL) reporter mRNA or a reporter harbouring the site perfectly complementary to miR-17 in its 3′ untranslated region. In control cells, insertion of the miR-17 site repressed RL expression by ~80%. However, in TRBPkd cells, the miRNA-mediated inhibition was about threefold less pronounced (Fig 5A), indicating that TRBP is required for either the assembly or functioning of miRNPs.

To investigate whether TRBP has a role in the RNAi reaction mediated by exogenous siRNA, we determined the efficiency of the lamin A/C RNAi in TRBPkd and various control cells. The siRNA treatment had a strong effect on lamin A/C level in parental HEK293T-REx cells or cells stably expressing a control hairpin. However, lamin A/C depletion was largely abolished in TRBPkd cells. Similar suppression of the lamin A/C knockdown was observed in a HEK293 cell line in which Ago2 was depleted by expression of a specific hairpin (Fig 5B).

Taken together, our data indicate that TRBP is primarily required for the assembly and/or functioning of si- or mi-RISCs in mammalian cells, but it may also facilitate the cleavage of pre-miRNAs by Dicer. The apparent discrepancy between the effect of TRBP knockdown on pre-miRNA processing in cells and cell extracts is readily explained by incomplete depletion of the protein, allowing for the manifestation of processing deficiency *in vitro* but not *in vivo*. It is worth noting that, as in the case of *Drosophila* Loqs (Förstemann *et al*, 2005; Saito *et al*, 2005) but in contrast to R2D2 (Liu *et al*, 2003), depletion of TRBP did not lead to appreciable destabilization of Dicer (Fig 5B).

CONCLUSIONS

Our findings that mammalian Dicer forms a complex with a dsRBD protein TRBP add support to the idea that large RNase III-type Drosha and Dicer nucleases generally require dsRBD protein partners for their function. *Drosophila* R2D2 and Loqs are two *Drosophila* dsRBD proteins that work in conjunction with Dcr2 and Dcr1, acting in RNAi and miRNA pathways, respectively

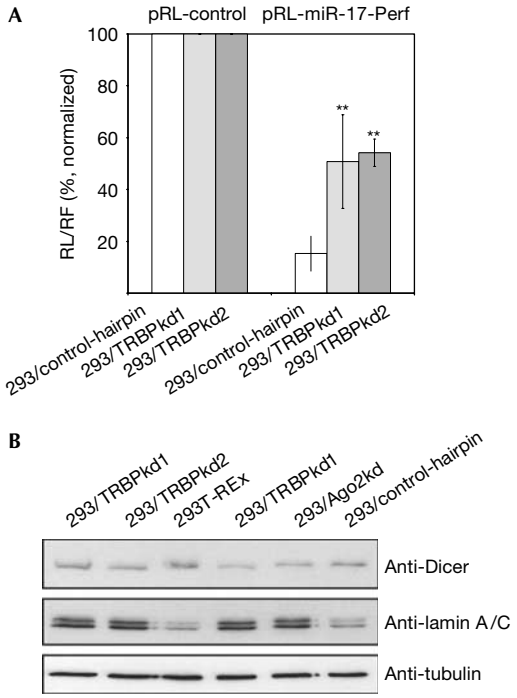

Fig 5 | Effect of TRBP depletion on RNA silencing. Depletion of TRBP (human immunodeficiency virus (HIV-1) transactivating response (TAR) RNA-binding protein) decreases the efficiency of RNA interference mediated by the endogenous microRNA miR-17 (**A**) and by transfected anti-lamin A/C small interfering RNA (**B**). Cell lines used for analysis are indicated. In (**A**), activities of *Renilla* luciferase (RL)-miR-17-Perf reporter in every cell line are expressed in relation to activities of RL-control reporter (set as 100%). Values are means ± s.d. of four transfections (**$P < 0.01$). Similar results were obtained in several independent experiments. Antibodies used for western blotting in (**B**) are indicated on the right.

(Liu *et al*, 2003; Förstemann *et al*, 2005; Saito *et al*, 2005). The R2D2–Dcr2 association is required for asymmetric loading of siRNAs into RISC (Tomari *et al*, 2004), whereas Loqs and Dcr1 are essential for efficient pre-miRNA processing, and also participate in gene silencing that is triggered by artificial dsRNA hairpins and endogenous *Supressor of Stellate* repeats (Förstemann *et al*, 2005; Saito *et al*, 2005). The observation that TRBP is required for efficient cleavage of pre-miRNA *in vitro* and for the function of RISC programmed with either endogenous miRNA or transfected siRNA in cells indicates that TRBP combines at least some functions that are performed separately by R2D2 and Loqs in *Drosophila*. However, it should be noted that TRBP is structurally more related to Loqs than to R2D2 (supplementary Fig S3 online; Förstemann *et al*, 2005). We investigated, using both cell extracts and recombinant proteins, whether Dicer and TRBP are involved in sensing the thermodynamic stability of the 5′ ends of the siRNA strands in the same way as Dcr2 and R2D2. These experiments, using 5-iodo-U-modified siRNAs, have not produced conclusive results (L.J., unpublished results).

After the submission of this work, another work describing the TRBP–Dicer partnership has been reported (Chendrimada *et al*, 2005). Two observations described in the Chendrimada *et al* paper are in disagreement with our results. First, they found that depletion of TRBP resulted in a decrease of steady-state levels of miRNAs, whereas in our analysis the miRNA content was not significantly changed. Second, in contrast to our findings, Chendrimada *et al* report that knockdown of TRBP causes destabilization of Dicer, and vice versa. However, we note that the analysis of TRBP and Dicer levels was carried out not with total extracts from cells depleted in either protein but with Argonaute (Ago2) immunoprecipitates. Hence, it is possible that depletion of Dicer or TRBP affects the ability of the partner protein to form a complex with Ago2 rather than destabilizing the protein. This interpretation would be consistent with a relatively mild phenotype of mice with a knockout of Prbp, the mouse homologue of TRBP (Zhong *et al*, 1999), contrasting with the lethal phenotype of the Dicer knockout (Bernstein *et al*, 2003). It would also support our observations that the efficient depletion of Dicer in HEK293 cell lines has no principal effect on the level of TRBP (A.H., K. Tang & W.F., unpublished results).

Another three-dsRBD protein, PACT, 42% identical to TRBP, is expressed in mammals. In contrast to TRBP, which inhibits PKR, PACT (or its mouse homologue Rax) has a stimulatory effect on PKR (Gupta *et al*, 2003; Bennett *et al*, 2004, and references therein). The effects of TRBP and PACT on PKR activity are mediated by the C-terminal dsRBDs, which are devoid of detectable dsRNA-binding properties (Gupta *et al*, 2003, and references therein). In addition to effects on PKR, the C-terminal domains of PACT and TRBP can mediate heterodimerization of both proteins and also homodimerization of PACT (Hitti *et al*, 2004; G. Laraki & A.G., unpublished results). The apparent involvement of the C-terminal TRBP region in association with Dicer (Fig 3C) raises the possibility that RNAi/miRNA and PKR pathways are subject to reciprocal regulation by a rather complex network of protein–protein interactions. As both RNAi and IFN–PKR pathways have a role in antiviral responses, communication between them would not be surprising. In the future, it would be interesting to find out whether Dicer also associates with PACT, and how these protein interactions affect RNA silencing and other defence pathways in normal and virus-infected cells. The latter question is particularly interesting in the light of a recent report that the HIV-1 TAR-binding protein Tat functions as an RNAi suppressor, possibly compromising the activity of Dicer (Bennasser *et al*, 2005).

METHODS

Co-immunoprecipitations. Anti-Dicer mAbs 33, 73 and 83 and control mAbs were crosslinked to Protein G–Sepharose 4 Fast Flow (Amersham Biosciences, Little Chalfont, UK) and used to purify Dicer complexes from HEK293 cytoplasmic extracts (S10). Co-immunoprecipitates were washed five times with lysis buffer (20 mM Tris–HCl, pH 7.5, 300 mM NaCl, 0.5% NP-40, 2.5 mM $MgCl_2$) and analysed by liquid chromatography tandem mass spectrometry (LC-MSMS) and western blot.

293/TRBPkd1, 293/TRBPkd2 and 293/control-hairpin cell lines. Plasmids pTER-TRBPsh1, pTER-TRBPsh2 and pTER-control-hairpin were co-transfected with a puromycin resistance plasmid into HEK293T-REx cells (Invitrogen, Carlsbad, CA, USA) to generate stable cell lines.

Other procedures. Detailed methods can be found in the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing

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SUPPLEMENTARY MATERIAL

MATERIALS AND METHODS

cDNA cloning and construction of plasmids

Cloning of TRBP cDNAs. TRBP2 cDNA was cloned by reverse transcription (RT) from HEK293 total RNA using GAGGACGGATCCTGGGCTGGCTGAGG as a primer and Superscript II Reverse Transcriptase kit (Invitrogen). The cDNA generated was used as a template for PCR using CCTAGCTCGTCGGCTGTGTATTGG as a sense primer and GTACCAGAGCTGCGCAGATACATGAGC as an antisense primer. PCR products were subcloned into PCRII-TOPO (Invitrogen) and sequenced. Among these clones cDNAs encoding TRBP2, TRBP1 and a new splice variant, referred to as TRBP3, were found. TRBP3 cDNA is missing exon 7, which changes the reading frame of the following exons and results in a translational stop signal within exon 8 (Fig 3D). Database searches revealed human ESTs gi|45695934, gi|46281253, gi|24041628, gi|12601190, gi|19091656 supporting the existence of such a splicing variant.

Cloning of pT-REx-DEST30-HA-TRBP2. Sequences encoding a hemagglutinin (HA) tag flanked by XhoI and SalI sites, and a NotI site, were introduced upstream and downstream of the coding region of TRBP2 by PCR, respectively. HA-TRBP2 was cloned into modified pT-REx-DEST30 (Invitrogen), using SalI and NotI restriction sites introduced into this plasmid.

Renilla luciferase (RL) reporter constructs, pRL-miR-17-Perf and pRL-control. pRL-miR-17-Perf is a derivative of pRL-control (obtained from R. Pillai of this laboratory). pRL-control expresses humanized RL, originating from phRLTK (Promega), under the control of a CMV promoter. To generate pRL-miR-17-Perf, oligonucleotides CTAGAACTACCTGCACTGTAAGCACTTTGC and TCGAGCAAAGTGCTTACAGTGCAGGTAGTT were annealed and ligated into XbaI and NotI sites within pRL-Con to create a perfect complementary site for miR-17-5p (referred to hereafter as miR-17) within the 3'UTR of RL (underlined is the sequence complementary to miR-17).

Constructs expressing anti-TRBP and control short hairpins (sh). Plasmids pTER-TRBPsh1 and pTER-TRBPsh2 were generated by cloning annealed synthetic 63-mer oligonucleotides into BglII and HindII sites within pTER (a kind gift of M. van de Wetering (van de Wetering et al., 2003)). Oligonucleotides GATCCCGCCTGGATGGTCTTCGAAATTCAAGAGATTTCGAAGACCATCCAGG CTTTTTGGAAA and AGCTTTTCCAAAAA GCCTGGATGGTCTTCGAAATCTCTTGAATTTCGAAGACCATCCAGGCGG were

used to generate pTER-TRBPsh1. pTER-TRBPsh2 was cloned using oligonucleotides GATCCCGGATTCTCTACGAAATTCATTCAAGAGATGAATTTCGTAGAGAATCC TTTTTGGAAA and AGCTTTTCCAAAAAGGATTCTCTACGAAATTCATCTCTTGAATGAATTTCGTA GAGAATCCGG. pTER-control-hairpin, cloned in an analogous way using GATCCCATTCTCCGAACGTGTCACGTTCAAGAGACGTGACACGTTCGGAGAA TTTTTTGGAAA and AGCTTTTCCAAAAAATTCTCCGAACGTGTCACGTCTCTTGAACGTGACACGTT CGGAGAATG oligonucleotides was kindly provided by K. Tang of this laboratory. Sense and antisense siRNA sequences within the hairpins are underlined.

Plasmids for two hybrid assays. pGADGH-CyclinT1, -TRBP, and -Tat and pGBT9-TRBP, -TRBP fragments, and -Tat have been described (Dorin et al., 2002; Battisti et al., 2003). To construct pGADGH-Dicer, the SalI-NotI (Klenow filled) fragment of pBluescript-Dicer-His_6 was inserted into SalI-XhoI (filled in) sites of pGADGH. The frame was restored by amplifying the SalI-DraIII fragment by PCR using CACGCGTCGACCATGAAAAGCCCTGCTTTGCATGGC as sense primer and GGTCAAGTGAGGCAGGTGAG as antisense primer to insert an additional C between the SalI site and the ATG. This SalI-DraIII fragment was inserted in the previous vector to obtain pGADGH-Dicer.

Other plasmids. Plasmids encoding Dicer-His_6, MBP-TRBP2, and MBP were described previously (Zhang et al., 2002; Dorin et al., 2003). pCIneo-HA-LacZ was kindly

provided by R. Pillai of this laboratory. pCMV-FL, encoding firefly luciferase (FL) was previously described (Pillai et al., 2004).

Cell culture and preparation of stable cell lines

Cell culture. HEK293 human embryonic kidney cells and P19 mouse teratoma cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with L-glutamine (2mM) and 10% heat inactivated fetal calf serum (FCS). HEK293-T-REx (Invitrogen) cells were grown similarly but in the presence of blasticidin (Invitrogen), according to the manufactures protocol.

293/HA-TRBP2, 293/HA-LacZ, 293/TRBPkd1, 293/TRBPkd2, 293/Ago2kd and 293/control-hairpin cell lines. To generate stably transfected tetracycline (Tet) inducible cell lines, HEK293-T-REx cells were co-transfected with a pBABE-puro (Clontech), encoding a puromycin resistance marker, and pT-REx-DEST30-HA-TRBP2, pTER-TRBPsh1 or pTER-TRBPsh2. Cells were grown in the presence of puromycin and blasticidin according to the manufacturers protocol to select stably transfected clones. Single clones were selected to generate monoclonal cell lines. 293/Ago2kd, expressing a short hairpin targeting human Ago2 was constructed similarly and kindly provided by D. Schmitter of this laboratory. Following treatment with Tet, expression of Ago2 was reduced approximately 80% in this cell line. To generate 293/HA-LacZ and 293/control-hairpin cells, pCIneo-HA-LacZ and pTER-control-hairpin, respectively, were co-transfected with pBABE-puro into HEK293-T-REx cells and a pool of stable transformants was selected.

SiRNA transfection. siCONTROL Lamin A/C siRNA (Dharmacon) was transfected into 293/TRBPkd, 293/Ago2kd and 293/control-hairpin cells using Lipofectamine 2000 (Invitrogen) at a final concentration of 20 nM according to the manufactures protocol. Cells were harvested 72 h after transfection. Whole cell extracts were separated by SDS-polyacrylamide gel electophoresis (SDS-PAGE) and analyzed by western blotting for lamin A/C, Dicer and α-tubulin.

Antibodies

Generation of anti-Dicer mAbs. A recombinant human Dicer His_6-tagged at the C-terminus was purified as described by Zhang et al., 2002. Final eluate was dialysed against immunization buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM $MgCl_2$, 20% glycerol). Immunization of mice and isolation of hybridoma followed standard procedures (Harlow and Lane, 1998). Initial immunization (53 μg of Dicer per injection/per mouse) was followed by three boosts (25 μg each). Splenocytes were prepared, fused with myeloma (P3-X63-Ag8.653) cells and selected in HAT-medium. Hybridomas were subcloned and screened by ELISA and western blotting. Positive clones were selected and expanded in IMEM medium containing 10% IgG reduced FCS. mAbs were purified using protein G-sepharose (Amersham) and concentrated in PBS buffer with 50% glycerol. To map Dicer regions recognized by individual mAbs they were tested by western blotting using *Escherichia coli* cell extracts overexpressing deletion fragments of Dicer. mAbs 33 and 73 recognize the C-terminal part of Dicer

containing two RNaseIII domains and dsRBD. mAb 83 recognizes the central domain of Dicer encompassing the DUF283 and PAZ domains (data not shown).

Other antibodies. Following Abs were used for western analysis: anti-Dicer Ab347 (Billy et al., 2001), anti-TRBP672 (Duarte et al., 2000), anti-HA High Affinity (3F10) (Roche), anti-Lamin A/C (H-110) (Santa Cruz Biotechnology), and anti-α-tubulin (DM1A) mAb (Labvision/Neomarkers). For western analysis all antibodies except anti-α-tubulin were used at 1:1000 dilution; anti-α-tubulin was used at 1: 3000 dilution. Following Abs, in addition to anti-Dicer mAbs, were used for immunoprecipitations: anti-Dicer Ab347 (Billy et al., 2001), anti-HA (12CA5) mAb, anti-Myc (9E10) mAb. All co-immunoprecipitations were performed as described for anti-Dicer mAbs.

Two dimensional PAGE analysis and liquid chromatography tandem mass spectrometry (LC-MSMS) analysis

For LC-MSMS analyses, immunoprecipitates were analysed by one dimensional (1D)-PAGE gels, which were stained with a Colloidal Blue staining kit (Invitrogen). For two dimensional (2D)-PAGE analysis followed by LC-MSMS, immunoprecipitates were resuspended in 7M urea, 2M thiourea, 4% CHAPS, 1% DTT, 0.3% Pharmalytes 3-10. After isoelectric focusing on Immobile DryStrips (pI 4.0-7, linear) (Amersham Bioscience) using IPGphor, proteins were separated by 10% SDS PAGE. The gels were stained using Silver Quest silver staining kit (Invitrogen) and analyzed by Proteome Weaver (Definiens). Protein containing gel fragments, were digested with trypsin according to Schrimpf et al. (2001) and analyzed by LC-MSMS (LCQ Deca XP, Thermo

Finnigan). Proteins were identified using Turbo Sequest and MASCOT, searching SwissProt database restricted to human proteins.

Northern blotting and real time PCR

miRNA northern blots. Oligonucleotide probes complementary to miR-17 CTAGAACTACCTGCACTGTAAGCACTTTGC and miR-16 CTAGAACTACCTGCACTGTAAGCACTTTGC (miRNA-specific sequences are underlined), were 5′-end-labeled, using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP. U6 snRNA was quantified using CGTTCCAATTTTAGTATATGTGCTGCCGAAGCGAGCAC oligonucleotide probe to normalize signals for equal loading. Total cellular RNA was isolated using TRIZOL reagent (Invitrogen), separated by 12% Urea-PAGE and transferred to Hybond N+ membranes (Amersham). Prehybridisation and hybridization solutions contained 2x SSPE, 5x Denhardt's solution, 0.1% SDS and 20% formamide. After prehybridisation for 3 h, hybridization with specific labeled antisense oligonucleotides was performed at 37 °C for at least 5 h. Membranes were washed 5 times with 2x SSPE, 0.2% SDS. Quantifications were done using Storm 860 PhosphorImager and ImageQuant (Molecular Dynamics). To determine miRNA abundance in 293/TRBPkd cell lines, two independent RNA preparations have been analyzed in quadruplicates with different probes and P-values have been calculated (T-test: 1-tailed; equal variance).

Real Time PCR. Knock down and control cells were induced with 2 μg/ml Tet for 1 day. Total RNA was isolated using TRIZOL reagent. Reverse transcription was performed with random hexamers and oligo-dT primers using Taq Man Reverse transcription

Reagents (Roche). For detection of mRNAs, Hs00366328_m1 and GAPDH Hs99999905_m1 Taq Man Gene Expression Assays (Applied Biosystems) were used in combination with Taq Man universal PCR Mix. ABI Prism 7000 (Applied Biosystems) was used for analysis and quantification. TRBP mRNA levels were normalized to GAPDH mRNA levels.

Recombinant proteins and *in vitro* interaction

Protein expression and purification. Recombinant Dicer was purified as described by Zhang et al., (2002). MBP-TRBP2 and MBP were expressed in *Escherichia coli* BL21(DE3) cells and purified as described by Dorin et al., (2002). Purity of the proteins was analysed by 8% SDS-PAGE. Protein concentration was determined by a Bradford method.

In vitro protein interaction. Purified recombinant proteins were mixed in buffer containing 30 mM Tris-HCl, pH7.5, 1 mM $MgCl_2$, 150 mM NaCl, 0.2% NP-40 and 10% glycerol and incubated at 25 °C for 1 h. The reaction was split in two and to one half anti-Dicer antibody was added, incubated at 25 °C for 30 min and applied to protein G-sepharose (Amersham). Similar incubation with anti-HA antibody served as a control. To the second half, amylose beads (New England Biolabs) were added. Samples were incubated for additional 1 h at 25 °C. Reactions were spun down, washed 4 times with binding buffer and eluted in a SDS-PAGE sample buffer. Eluates were resolved by 8% SDS-PAGE and analysed by western blotting.

Preparation of RNA Substrates and RNA processing assays

Preparation of RNA substrates. The internally ^{32}P-labeled 30-bp dsRNA containing 2-nt 3' protruding ends was prepared essentially as described before (Zhang et al., 2002). RNAs were synthesised by the T7 polymerase *in vitro* transcription, using the Ambion T7 MaxiScript transcription kit and [α-^{32}P]UTP. After transcription, samples were treated with DNase I, extracted with phenol, and RNA purified by denaturating 8% PAGE. Following dephosphorylation by calf intestine phosphatase (CIP), RNAs were 5′-end-phosphorylated, using T4 polynucleotide kinase and ATP. Complementary RNA strands were annealed at 95 °C for 3 min in 20 mM NaCl, transferred to 75 °C, and then slowly cooled down to 20 °C. The internally ^{32}P-labeled pre-let-7 RNA bearing authentic 5' and 3' termini was prepared as described (Kolb et al., 2005; Zhang et al., 2004) Prior to use, pre-let-7 RNA was dissolved in water and renaturated by incubation at 90 °C for 1 min, followed by incubation at 25 °C for 15 min in 30 mM Tris-HCl, pH 6.8 containing 50 mM NaCl, 2 mM $MgCl_2$ and 10% glycerol.

RNA Processing Assays. For preparation of S10 cell extracts used in pre-let7 RNA processing, HEK293 cells were lysed in buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.25% NP40 and 1.5 mM $MgCl_2$, and centrifuged at 10,000 x g for 10 min at 4 °C. Protein concentration in the extracts was adjusted to 5 mg/ml using the lysis buffer. Processing assays (50 µl) were carried out as previously described (Zhang et al. 2004, 2002). 3-5 fmol of ^{32}P-labeled substrate was incubated with a cytoplasmic S10 extract (final concentration 2.5 mg/ml) in buffer containing 20 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 75 mM NaCl and 10% glycerol at 37 °C. RNA was extracted with

phenol/chloroform and analyzed by 10% denaturing PAGE. Quantification was done using the Storm 860 PhosphorImager.

Gradient sedimentation, luciferase reporter assays and yeast two hybrid analysis

Glycerol gradients. S10 extracts, prepared as described above, were separated on 10-30% glycerol gradients by centrifugation at 36,000 rpm for 20 h in a Beckman SW41 Ti rotor at 4 °C, according to Zhang et al., (2004). Fractions were collected and processed for western analysis with specific anti-Dicer and anti-TRBP antibodies. For northern blot analysis gradient fractions were extracted with phenol and chloroform, precipitated with sodium acetate and ethanol, separated by 12% Urea-PAGE, and transferred to Hybond N+ membranes for hybridization with specific oligonucleotide probes. For Dicer cleavage activity assays, Dicer was immunoprecipitated from pooled gradient fractions using anti-Dicer mAb 73 cross-linked to Protein G-Sepharose beads. Immunoprecipitates were washed with 20 mM Tris-HCl, pH 7.5, 300 mM NaCl, 0.5% NP40, 2.5 mM $MgCl_2$ and resuspended in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5 mM $MgCl_2$. Dicer cleavage activity was measured using internally ^{32}P-labeled 30-bp dsRNA containing 2-nt 3' protruding ends, as described above. Catalase and ferritin (HMW Gel Filtration Calibration kit, Amersham Bioscience) were sedimented on parallel gradients as molecular mass markers.

Luciferase reporter assays. Stable cell lines were induced for 3 days with 2 μg/ml Tet and transfected in 24-well plates with indicated RL constructs and pCMV-FL using Lipofectamine PLUS reagent (Invitrogen). 5 ng of pRL-miR-17 or 5 ng of pRL-control

together with 20 ng of pCMV-FL were co-transfected into one 24-well-plate. pRL-control, lacking the binding site for miRNA, was used to measure RL expression from plasmids devoid of miRNA-binding sites. Cells were lysed 48 h after transfection and luciferase activities were measured using the Dual-Luciferase Reporter System (Promega) as recommended in the manufactures instructions. All RL activities were normalized to FL activites to correct for transfection efficiency (RL/FL). Four transfections were performed per each individual experiment. P-values were calculated (T-test: one-tailed; equal variance).

Yeast two-hybrid assay. Yeast expression plasmids were introduced into the yeast reporter strain SFY526. The double transformants were selected and screened for β-galactosidase activity as described by Daher et al., (2001).

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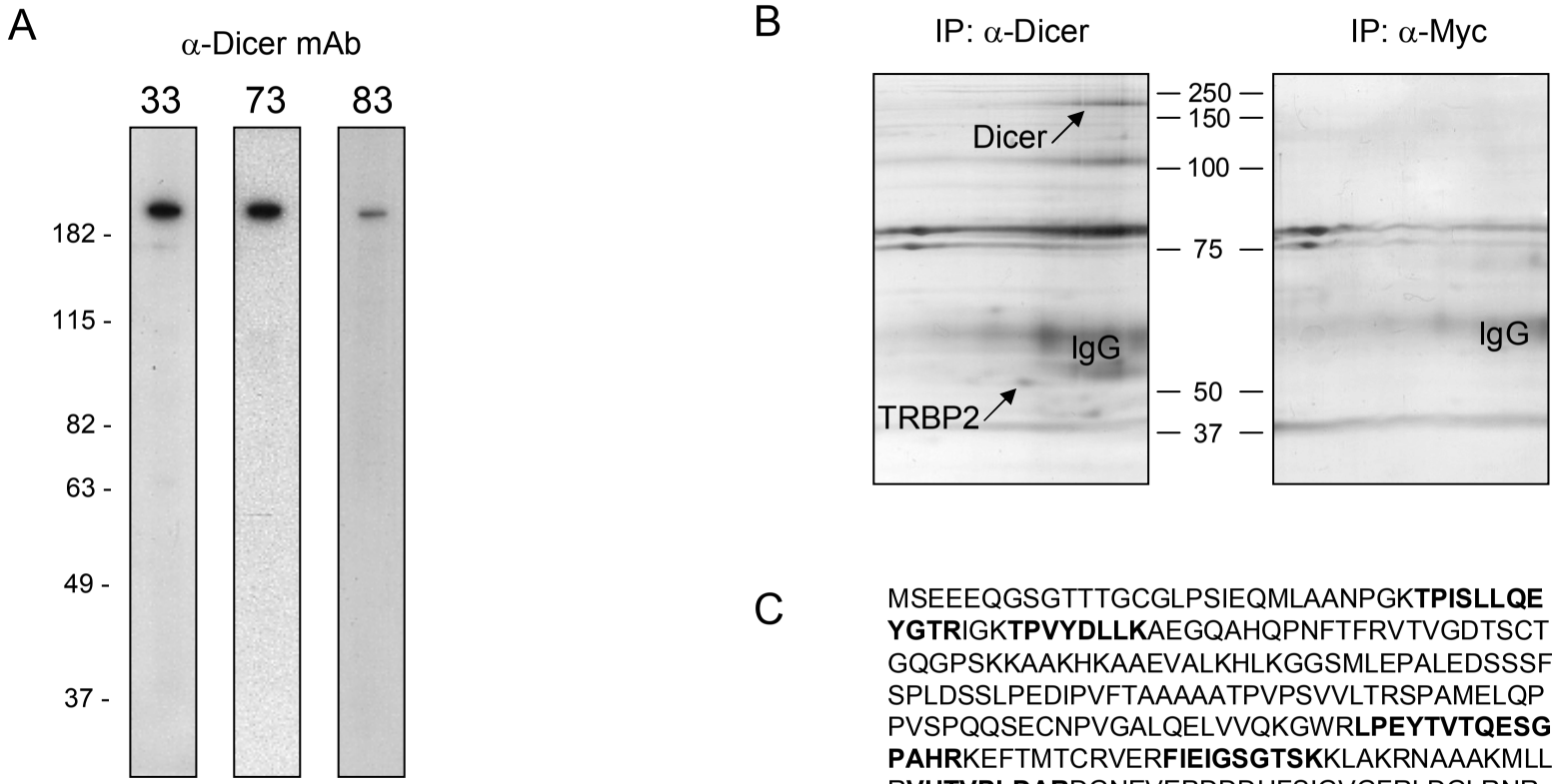
Supplementary figure legends

Fig S1. Specificity of anti-Dicer mAbs (A) and identification of TRBP in Dicer immunoprecipitates by LC-MSMS analysis (B and C). (**A**) anti-Dicer mAbs 33, 73 and 83 recognize Dicer in HeLa cell S10 extracts. Hybridoma supernatants were used at 1:10 dilution for western blotting. (**B**) Silver staining of two-dimensional PAGE demonstrating that TRBP is specifically co-immunoprecipitated by anti-Dicer mAb (left upper panel) but not control anti-Myc mAb (right upper panel). Spots yielding Dicer and TRBP peptides as well as IgG spots are indicated. (**C**) Sequence of TRBP2 (Swiss-Prot Q9BRY2) with TRBP peptides from three independent LC-MSMS experiments shown in bold.

Fig S2. Levels of TRBP mRNA in HEK293 TRBP knock down and 293T-REx control cell lines. RNA levels were determined by real time PCR. Values of the bars are means of two experiments, with the range indicated.

Fig S3. Sequence alignment (**A**) and phylogenetic tree (**B**) of dsRBDs of human TRBP2 and related proteins. PRBP is mouse homolog of TRBP2. The two proteins are 93% identical and 94% similar. In (A) dsRDB regions are indicated by black bars. Abbreviations used: hs, *Homo sapiens*; mm, *Mus musculus*; dm, *Drosophila melanogaster*; ce, *Caenorhabditis elegans*. Alignment and phylogenetic tree were prepared using ClustalW (http://www.ebi.ac.uk/clustalw/).

Fig. S1 Haase et al.



C

MSEEEQGSGTTTGCGLPSIEQMLAANPGK**TPISLLQE**
YGTRIGK**TPVYDLLK**AEGQAHQPNFTFRVTVGDTSCT
GQGPSKKAAKHKAAEVALKHLKGGSMLEPALEDSSSF
SPLDSSLPEDIPVFTAAAAATPVPSVVLTRSPAMELQP
PVSPQQSECNPVGALQELVVQKGWR**LPEYTVTQESG**
PAHRKEFTMTCRVER**FIEIGSGTSK**KLAKRNAAAKMLL
R**VHTVPLDAR**DGNEVEPDDDHFSIGVGFRLDGLRNR
GPGCTWDSLRNSVGEKILSLRSCSLGSLGALGPACC
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Fig. S2 Haase et al.

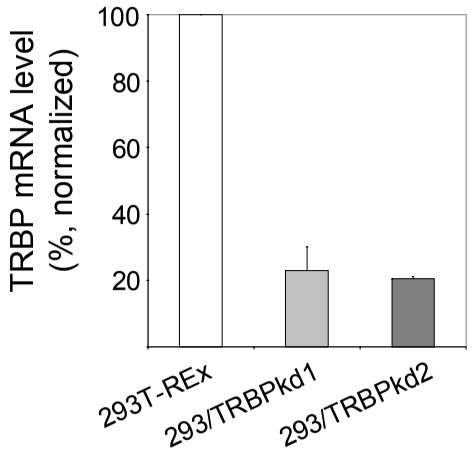

Fig. S3
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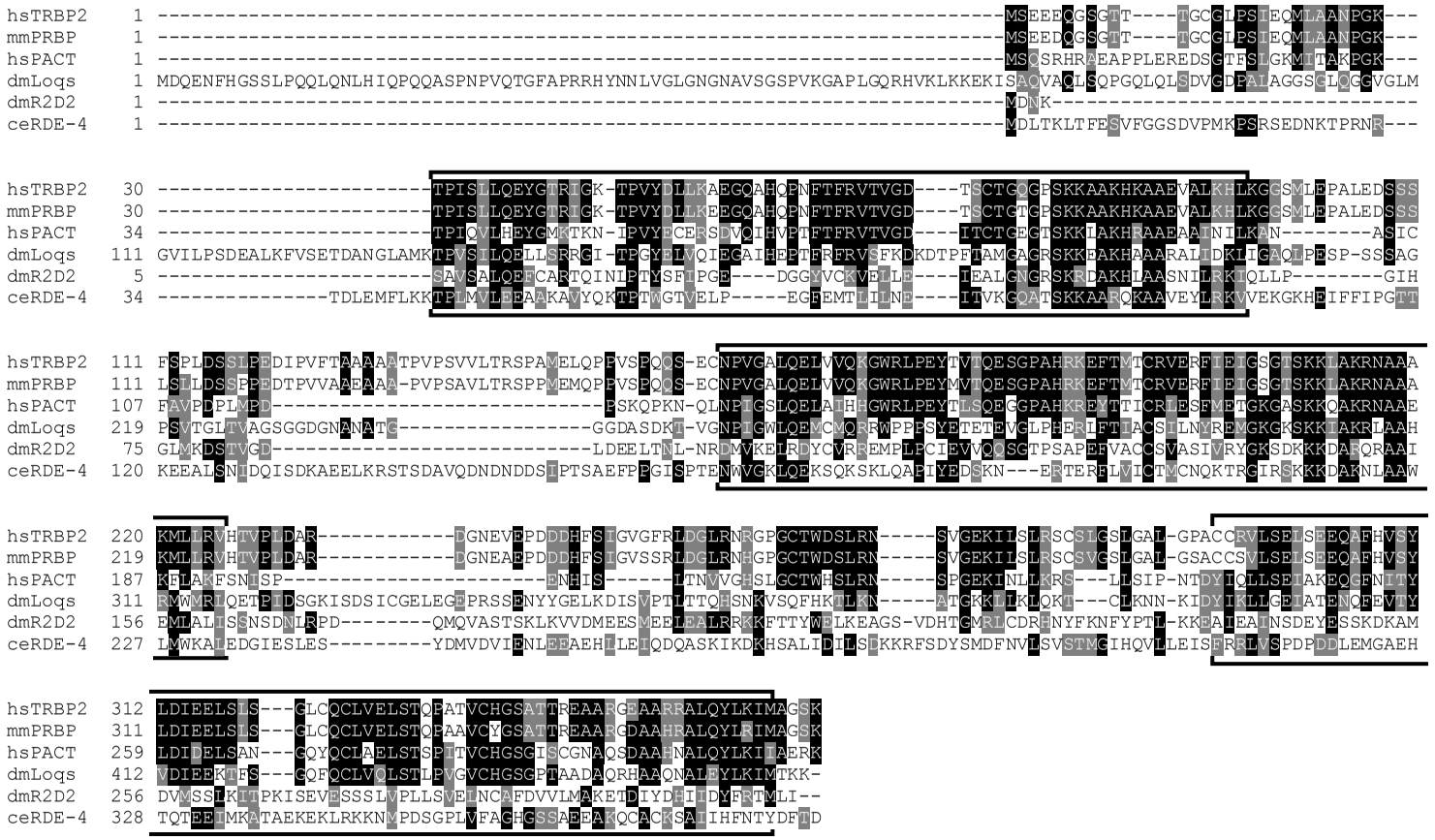
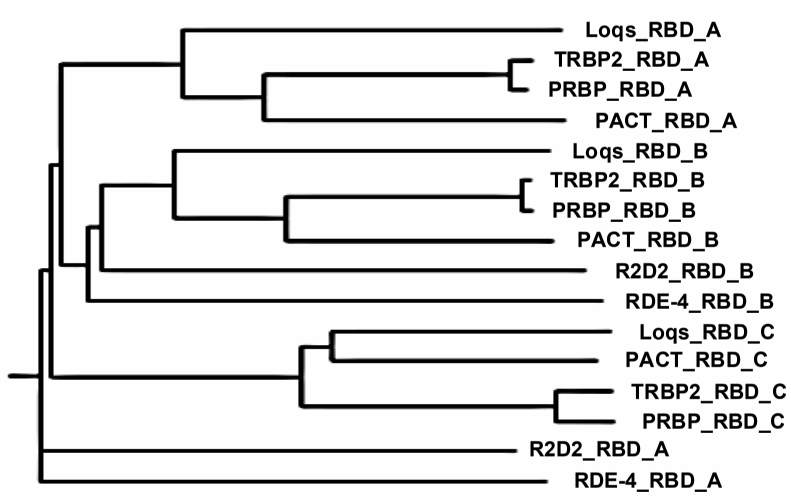
A



B



3.3. Additional characterization of TRBP and the Dicer/TRBP complex

Our work, presented in chapter 3.2., and work from other laboratories resulted in the characterization of TRBP as an interaction partner of Dicer, and as a positive regulator of miRNA and siRNA meditated silencing in human cells (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006b). To further characterize the Dicer/TRBP interaction, we identified the domain within Dicer necessary for binding TRBP, and analyzed the dsRNA binding properties of the Dicer/TRBP complex *in vitro.*

The Dicer N-terminal helicase domain is required for the interaction with TRBP. After having shown that Dicer directly interacts with TRBP *in vitro* (see chapter 3.2.), we investigated which domain of Dicer is required for its interaction with TRBP. To this end we expressed Dicer deletion constructs comprising Helicase/PAZ, PAZ, or RNase III/dsRBD domains of Dicer as N-terminal GST-fusion proteins (Tahbaz et al., 2004) in HEK293 cells together with Myc-tagged TRBP2. A schematic representation of truncated Dicer contructs is shown in Fig. 5A. Dicer mutants lacking the N-terminal helicase domain were unable to co-immunoprecipitate Myc-TRBP2, as shown by western blotting (Fig. 5B, lower panel). These data suggest that Dicer's N-terminus comprising two highly conserved helicase motifs, whose functional involvement in RNA silencing remains unclear, is critical for the interaction with TRBP. Our data are in agreement with work from NV Kim's laboratory, showing that Dicer's large N-terminal helicase domain is required for its interaction with TRBP (Lee et al., 2006b).

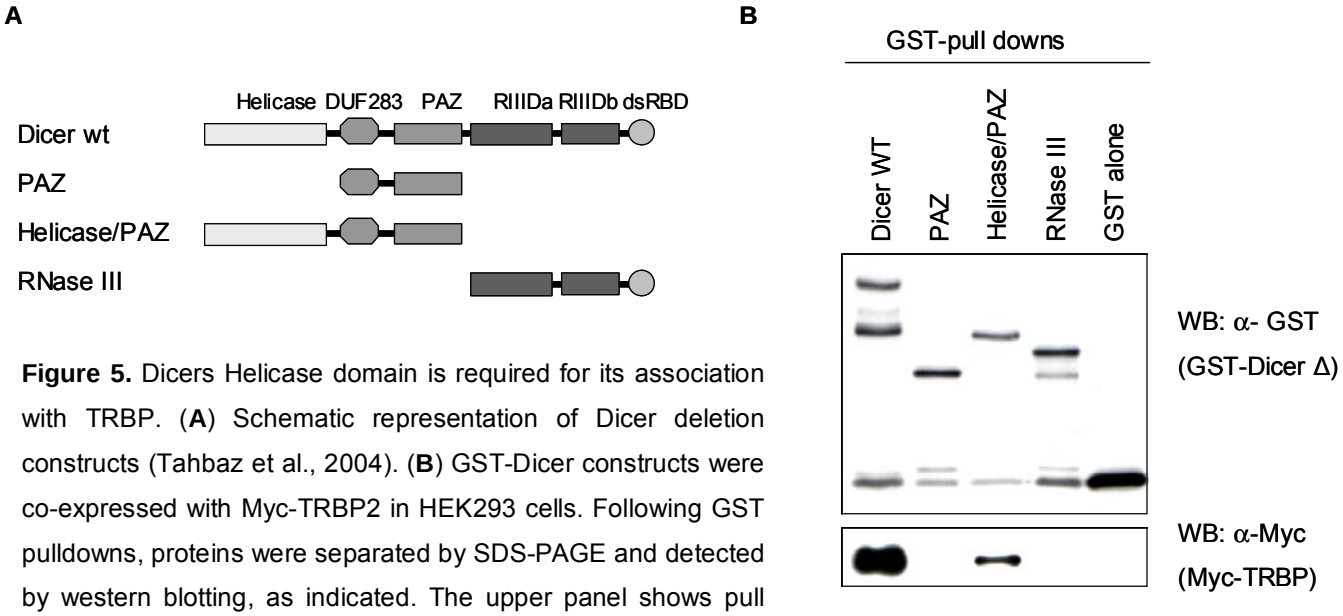


Figure 5. Dicers Helicase domain is required for its association with TRBP. (**A**) Schematic representation of Dicer deletion constructs (Tahbaz et al., 2004). (**B**) GST-Dicer constructs were co-expressed with Myc-TRBP2 in HEK293 cells. Following GST pulldowns, proteins were separated by SDS-PAGE and detected by western blotting, as indicated. The upper panel shows pull down of the different GST-Dicer contructs. The lower panel shows co-purification of Myc-TRBP2.

The Dicer/TRBP complex and TRBP alone bind dsRNAs *in vitro*. To characterize the nucleic acid binding properties of the Dicer/ TRBP complex, we performed electromobility shift assays (EMSA) with purified recombinant proteins using dsRNAs and a pre-miRNA as substrates. Dicer-His_6 and MBP-TRBP2 were purified from insect cells and *Escherichia coli*, respectively. MBP alone was used as a control. ^{32}P-labeled RNA was incubated with the recombinant proteins, and protein/RNA complexes were resolved by native polyacrylamide gel electrophoresis and visualized by autoradiography. 21-bp, and 30-bp long dsRNAs contained 3' 2nt-overhangs, thus mimicking siRNAs or Dicer cleavage products. Pre-let7 mimics a Droha cleavage product. Incubation of Dicer with dsRNA substrates resulted in the disappearance of free dsRNA, but did not show

a defined band of slower mobility (Fig. 6 A and B, lanes 1), whereas binding of TRBP to dsRNA was visualized as defined bands (lanes 2). Binding of the Dicer/TRBP complex to different dsRNA substrates resulted in a supershift compared to TRBP alone (lanes 4).

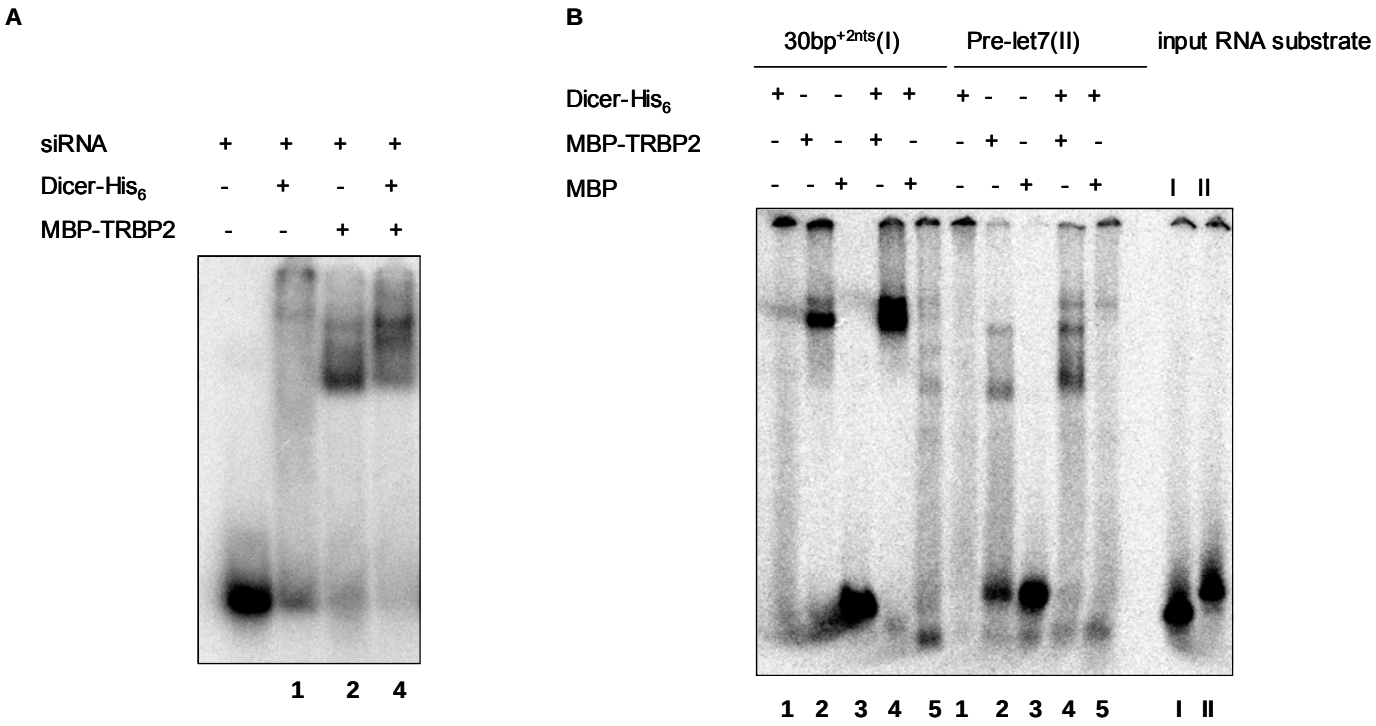


Figure 6. dsRNA binding of Dicer/TRBP. Binding of recombinant Dicer-His_6 and MBP-TRBP2 to short RNA duplexes with 3' 2nt-overhangs (**A** and **B**) and a pre-miRNA (**B**) was monitored by EMSA by 4% native PAGE. RNAs were labeled with ^{32}P, and RNPs were visualized by autoradiography.

In vitro interaction studies using Dicer-His_6 and MBP-TRBP2, presented is chapter 3.2., and RNA binding studies have been performed using the same buffer conditions. Taken together, these data provide evidence that Dicer and

TRBP interact with each other, and form a complex with dsRNA and pre-miRNA *in vitro*.

Characterization of α-TRBP antibodies. To further investigate TRBP's function in RNA silencing, we raised polyclonal peptide antibodies against TRBP. Two immunogenic peptides were chosen that reside outside the conserved dsRBDs and are common to all predicted isoforms of TRBP (Fig. 7A). α-TRBP antibodies were purified by affinity chromatography on beads containing immobilized peptides, and tested for their ability to specifically recognize TRBP in western blotting, immunoprecipitation, and immunoflourescence (Fig. 7). α-TRBP antibody #1 (Ab#1) (Fig. 7B, upper panel) and antibody #2 (Ab#2) (Fig. 7B, lower panel) specifically recognized overexpressed HA-TRBP2 and endogenous TRBP by western blotting. Cell extracts from TRBP knock down cell lines (293/TRBPkd1 and 293/TRBPkd2) were used as a negative control, and an HA-TRBP2 expressing cell line (293/HA-TRBP2) as a positive control. α-TRBP Ab#1 and two independent preparations of α-TRBP Ab#2 (prep1 and prep2) were used to immunoprecipitate HA-TRBP2 from 293/HA-TRBP2 cytoplasmic extracts (Fig. 6C). Both α-TRBP antibodies immunoprecipitate HA-TRBP2 (Fig. 7C, lanes 1-3), while an α-FLAG isotype antibody, used as a negative control, does not (Fig. 7C, lane 4). Cells expressing HA-LacZ (293/HA-LacZ) were used as a negative control. Neither antibody enriches HA-LacZ in this assay (Fig. 7C, lanes 5-8). Fig. 7D shows that α-TRBP Ab#1 enriched HA-TRBP2, from 293/HA-TRBP2 cell extracts by immunoprecipitation in a dose-dependent manner (lanes 1-3, middle

and lower panel), and co-immunoprecipitated endogenous Dicer (upper panel). An immunoprecipitation using an α-HA antibody was performed as a positive control (lane 4). An α-FLAG isotype antibody served as a negative control (lane 5).

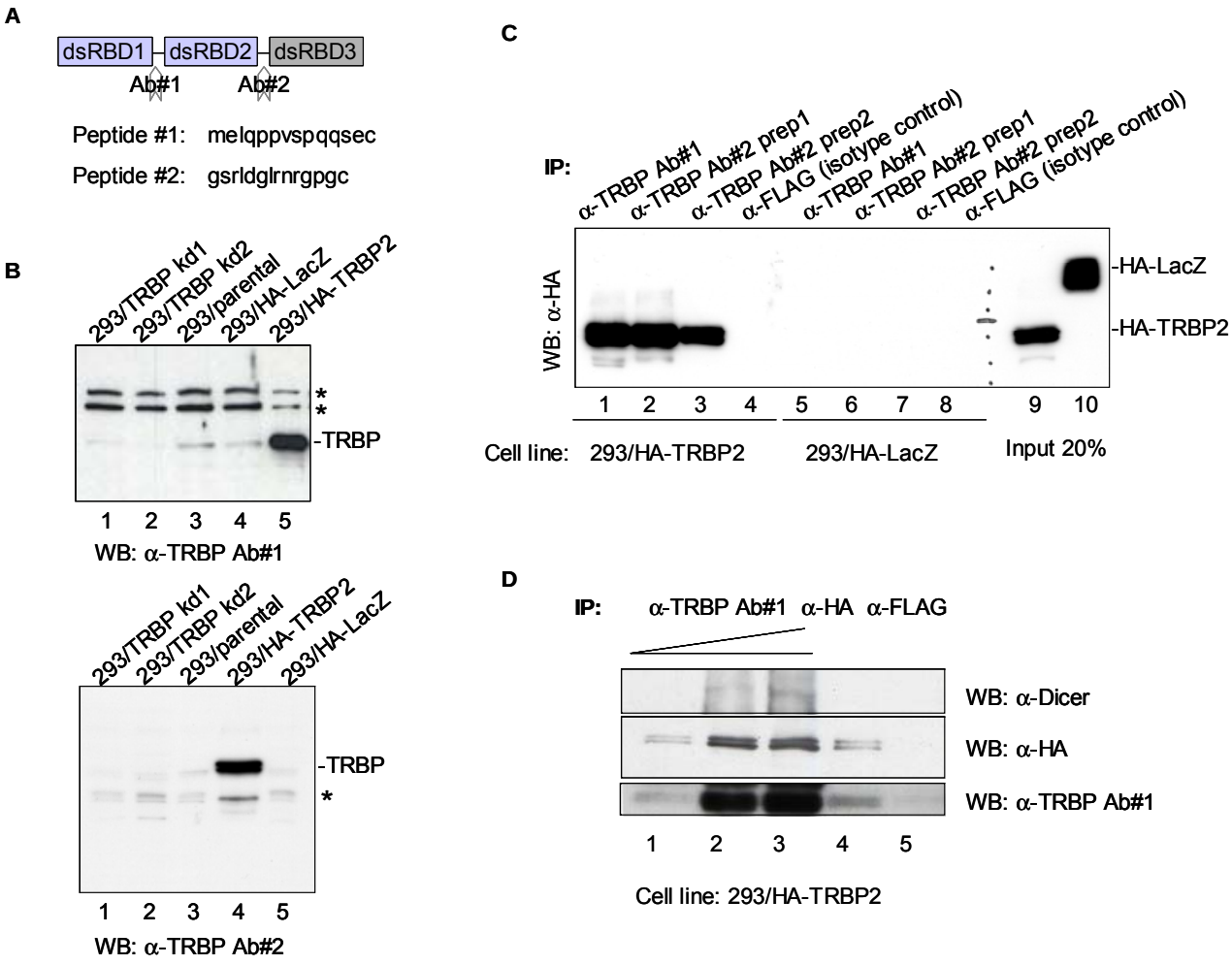


Figure 7. Characterization of rabbit polyclonal α-TRBP antibodies (**A**) The sequences of the immunogenic peptides and their localization within TRBP2 are depicted. (**B**) α-TRBP antibody (Ab) #1 (upper panel) and Ab #2 (lower panel) were tested for their ability to recognize overexpressed HA-TRBP2 and endogenous TRBP from HEK293 cells by western blotting. Cell extracts from TRBP knock down cell lines (293/TRBP kd1 and 2), and cells expressing HA-LacZ (293/HA-LacZ) were used as controls. α-TRBP Ab#1 recognizes overexpressed HA-TRBP2 as

well as endogenous TRBP (upper panel), but showed two major contaminating bands that were unrelated to TRBP, because they are not decreased upon knock down of TRBP. α-TRBP Ab#2 recognized overexpressed HA-TRBP2, but gave only very weak signals for endogenous TRBP (lower panel). Signals recognized by α-TRBP Ab#1 and #2 that are unrelated to TRBP are indicated by asterisks (*). (**C**) α-TRBP Ab#1 and two independent purifications of α-TRBP Ab#2 enriched HA-TRBP2 from HEK293 cells stably expressing HA-TRBP2 (293/HA-TRBP2), but did not enrich stably expressed HA-LacZ (293/HA-LacZ) by immunoprecipitation (lanes 4-7). An α-FLAG polyclonal antibody was used as an isotype control (lanes 4 and 8). 20% of the input cell extract is show in lanes 9 and 10. HA-TRBP2 and HA-LacZ were visualized by western blotting using an α-HA antibody. (**D**) Different concentrations of α-TRBP Ab#1 immunoprecipitated HA-TRBP2 in a dose-dependent manner from 293/HA-TRBP2 cell extracts (lanes 1-3). An α-HA antibody was used as a positive control, and α-FLAG antibody as a negative control for the immunoprecipitation. Co-immunoprecipitation with Dicer was detected using the α-Dicer 349 antibody in western blotting (upper panel). HA-TRBP2 and endogenous TRBP were visualized by western blotting using either α-TRBP Ab#1 (lower panel) or an α-HA antibody (middle panel).

In immunoflourescence analysis of HeLa cells (Fig. 8), the α-TRBP Ab#1 visualized nuclear and cytoplasmic staining of TRBP, consistent with its established localization (right columns). As a positive control, cells were transfected with Myc-TRBP2 and GFP-Ago2, and Myc-TRBP2 was detected with an α-myc antibody (left column). DAPI staining was used to visualize cell nuclei. Since Ago2 localizes to P-bodies, and TRBP was shown to associate with Ago2 (Chendrimada et al., 2005), we wanted to know, if TRBP co-localizes with Ago2 in P-bodies. GFP-Ago2 and HA-Ago2 constructs were expressed and detected to visualize P-bodies. Single color frames and merged images are shown, and antibodies used for the immunofluorescence analysis are indicated. GFP-Ago2 was detected by GFP-fluorescence (right columns). In these experiments we did not detect co-localization in P-bodies of over-expressed (left column) or endogenous TRBP (right columns) with HA-Ago2 or GFP-Ago2, respectively.

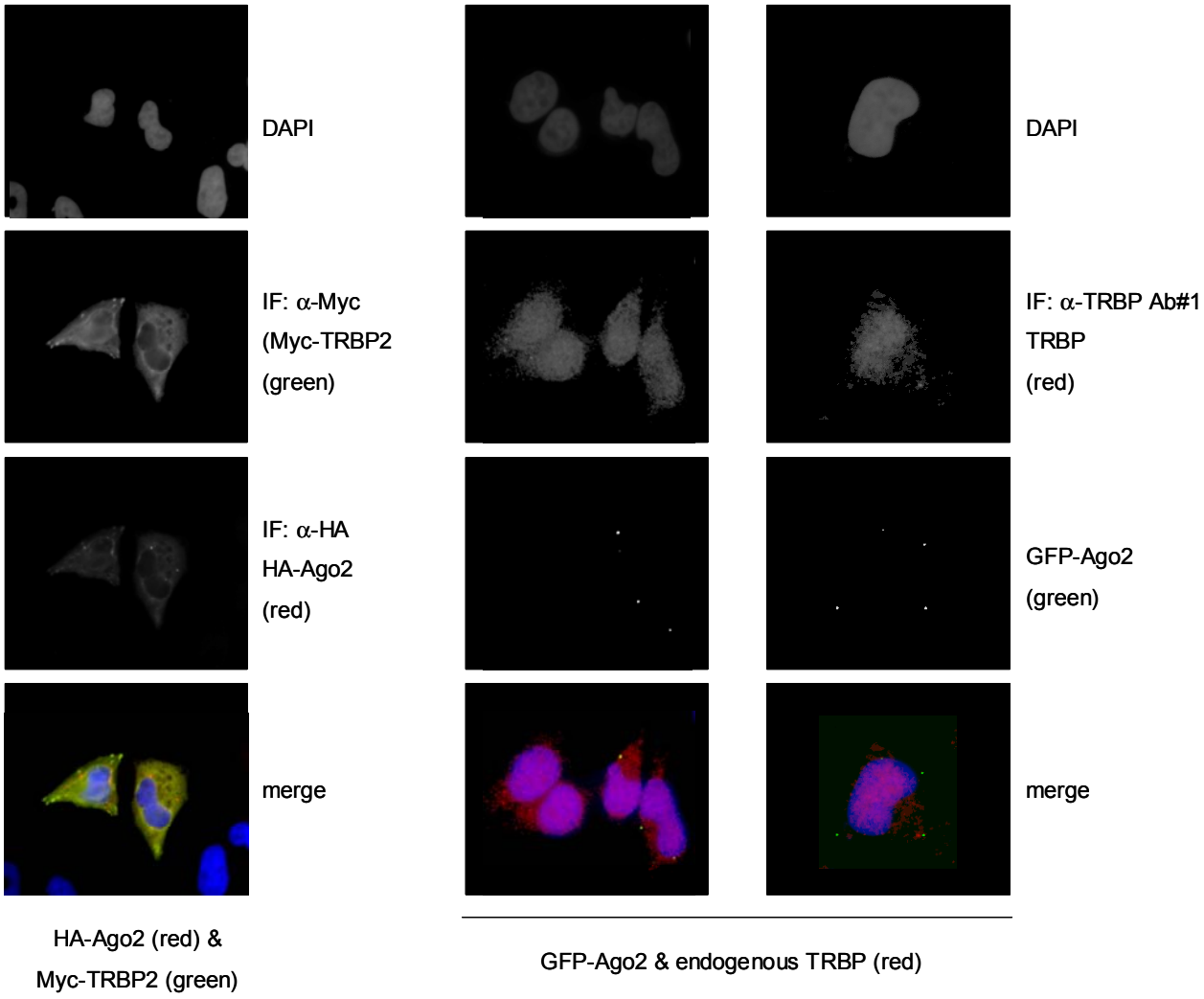


Figure 8. Immunofluorescence localization analysis of TRBP and Ago2. Single frames are shown in the upper three rows. Merged pictures are shown in the lowest row. HeLa cells were transiently transfected with HA-Ago2 and Myc-TRBP2 (left column). HA-Ago2 was detected using an α-HA antibody and a secondary antibody linked to Alexa-594 fluorochrom, and Myc-TRBP2 was detected by α-Myc 9E10 and a secondary antibody linked to Alexa-488 (left row). Endogenous TRBP was detected using α-TRBP Ab#1 and secondary antibody linked to Alexa-594, and GFP-Ago2 was detected by autofluorescence (right columns). Cell nuclei were visualized by DAPI stain (top row).

Identification of proteins associated with TRBP. To further characterize the Dicer/TRBP complex in respect to its protein composition, we immunopurified endogenous TRBP from HeLa cytoplasmic extracts, and analyzed co-purifying proteins by LC-MSMS, in an approach analogous to the Dicer complex purifications described above. Data obtained from this analysis were compared to data obtained from peptide sequencing of Dicer co-immunoprecipitates, and proteins found in Dicer as well as TRBP immunpurifications were considered for further investigation. Endogenous TRBP was immunoprecipitated from HeLa cytoplasmic cell extracts using affinity purified α-TRBP antibodies. An α-HA isotype antibody was used as a negative control. Fig. 9B shows western blot analysis of α-TRBP, α-Dicer, and α-Ago2 immunoprecipitates. Our data indicate that the majority of cytoplasmic TRBP resides in a complex with Dicer, and vice versa, while Ago2 represents only a minor component of this complex, or is only associated with a fraction of the complex (Fig. 9B lanes 1 and 2).

Fig. 9A shows a coomassie blue staining of α-TRBP immunoprecipitates separated by SDS-PAGE. Bands were cut out from lanes, representing TRBP- and control immunoprecipitates, and were subjected to LC-MSMS analysis after tryptic digestion. (The complete data set of proteins identified in this LC-MSMS experiment is shown in chapter 6.2.) Interestingly, TRBP immunoprecipitates showed to major bands in coomassie stain, corresponding to the molecular weight of TRBP and Dicer (indicated in Fig. 9A). The presence of TRBP and Dicer in these bands was verified by mass spectrometry.

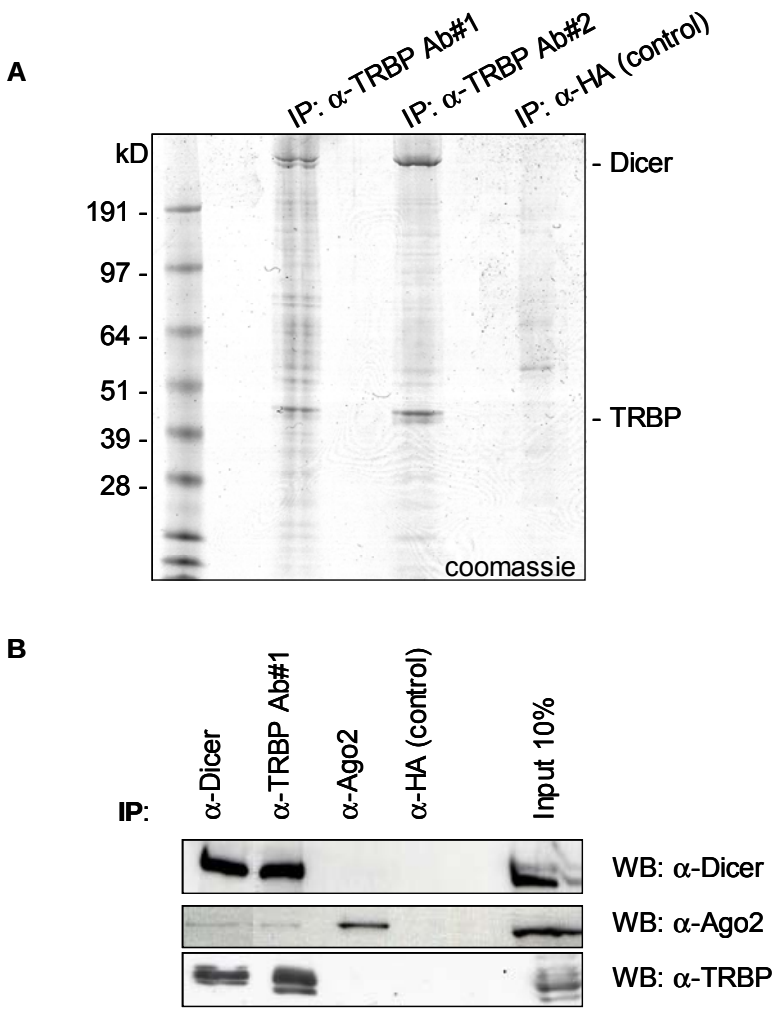


Figure 9. Analyzes of α-TRBP immunoprecipitates. (**A**) α-TRBP was immunoprecipitated from HeLa cell extracts using α-TRBP Ab#1 and #2. An α-HA isotype antibody was used as negative control. Immunoprecipitates were separated by SDS-PAGE and stained with coomassie. Bands enriched in TRBP-immunoprecipitates were analyzed by LC-MSMS. (**B**) α-Dicer, α-TRBP and α-Ago2 immunoprecipitates were analyzed by western blotting for Dicer, Ago2 and TRBP, as indicated.

Since TRBP is an integral component of a ~350kD Dicer complex (chapter 3.2.), data obtained from peptide sequencing of Dicer and TRBP immuno-purifications were compared, and proteins identified in both preparations were

considered for further analysis as likely components of a Dicer/TRBP complex. Proteins identified to specifically co-purify with Dicer and TRBP represent factors involved in: (1) RNA silencing, (2) translation, (3) translational regulation; or represent (4) RNA helicases, (5) RNA binding proteins, and other proteins (summarized in Table 3). The E3 Ubiquitin ligase, Ro52/TRIM21/SSA-1, identified in α-Dicer and α-TRBP immunoprecipitates, was chosen for further investigations (chapter 3.4.).

			IP: α-Dicer						α-TRBP	
Protein name	Accession number	Function	1	2	3	4	5	6	7	8
Dicer	Q9UPY3	RNA silencing	X	X	X	X	X	X	X	X
TRBP	Q9BRY2	RNA silencing	X		X	X	X	X	X	X
Nascent polypeptide-associated complex subunit alpha (NACA)	Q13765	Translational regulation	X						X	X
Ro52/SS-A/TRIM21	P19474	RBCC/ TRIM protein				X	X	X		X
hnRNP D/ AUF1	Q1413	Translational regulation			X		X			X
Interleukin enhancer-binding factor 3 (ILF3), Nuclear factor of activated T-cells 9 kDa (NF-AT-9)	Q1296	Translational regulation, dsRNA binding			X		X		X	
Oncogene FUS/ TLS	P35637	RNA binding			X		X		X	
Eukaryotic translation initiation factor 3 subunit 9; Prt1 homolog	P55884	Translation initiation	X						X	
Elongation Factor 2	P13639	Translation elongation						X	X	
Elongation factor 1-alpha 1	P6814	Translation elongation			X	X		X	X	
DNA-directed RNA polymerase, mitochondrial (MtRPOL)	O411	DNA-dependent RNA polymerase			X	X	X		X	
Developmentally-regulated brain protein (Drebrin)	Q16643	Actin binding, profilin binding	X	X					X	X
Epithelial protein lost in neoplasm (EPLIN)	Q9UHB6	Actin binding/ cytoskeletal protein	X	X						X
Peroxiredoxin, Thioredoxin peroxidase 2	Q683	redox regulation			X				X	X

Table 3. Comparison of in dependent LC-MSMS analyses of Dicer and TRBP immunoprecipitates. Data from the analyses of six independent α-Dicer immunoprecipitatations are summarized in lanes 1-6, and are compared to LC-MSMS analyses of α-TRBP immunoprecipitates using either α-TRBP Ab# 1 (lane 7) or α-TRBP Ab# 2 (lane 8).

Immunoprecipitations were either performed using HEK293 extracts (lanes 1-5) or HeLa cell extracts (lanes 6-8). Proteins found in Dicer as well as in TRBP immunoprecipitates are shown.

3.4. A potential role of an E3 ubiqutin ligase and of a ubiquitination in RNA silencing.

3.4.1. Ro52 associates with Dicer/ TRBP, and interacts with TRBP in vitro.

Ro52 also known as Sjoegren Syndrome Antigen 1 (SSA-1) or Tripartite motif protein 21 (TRIM21) was first identified as one of the major autoanitgens in Sjoegren Syndrome and Systemic Lupus Erythematosus (SLE), two severe human autoimmune diseases (Chan et al., 1991). Although anti-Ro52 autoantibodies have been used in clinical diagnostics for a long time, the function of Ro52 remained uncharacterized until three groups showed recently that Ro52 acts as a RING dependent E3 Ubiquitin ligase (Espinosa et al., 2006; Sabile et al., 2006; Wada and Kamitani, 2006a; Wada and Kamitani, 2006b; Wada et al., 2006). Ro52 contains a RING finger motif, a B-box, and a Coiled-Coiled domain, the domain signature of RBCC proteins, also termed TRIpartite Motif (TRIM) protein family. TRIM proteins have been implicated in various cellular processes, such as cell proliferation, differentiation, development, oncogenesis, apoptosis and antiviral defense (for review see: (Meroni and Diez-Roux, 2005; Nisole et al., 2005; Reymond et al., 2001; Saurin et al., 1996)). Espinosa et al. showed that Ro52 protein levels are increased in peripheral blood mononuclear cells (PBMCs) of patients with Sjoegren syndrome. In their cell culture system, overexpression of Ro52, but not of a mutant lacking the RING-domain, leads to

decreased proliferation and increased cell death, indicating a function for Ro52 as an E3 Ubiquitin ligase in these processes (Espinosa et al., 2006). Sabile et al. identified Ro52 as a protein associated with Skp2 within a SCF^{Skp2} complex, which directs ubiquitination and subsequent degradation of the cyclin-dependent kinase inhibitor p27. p27 inhibits the activity of cyclin E-CDK2 complexes thereby blocking S-phase progression. E3 ligase activity of Ro52 was shown to be required for S-phase progression in mammalian cells (Sabile et al., 2006).

The association of Ro52 with Dicer and TRBP, initially identified by mass spectrometry, was confirmed by western blot analysis of immunoprecipitates (Fig. 10A and 10B). Ro52 specifically co-immunoprecipitated with Dicer and TRBP (lanes 2-4), and was also present in α-Ago2 immunoprecipitates, though at lower amounts (lane 5) (Fig. 10A, middle panel). To further confirm the association of Ro52 with TRBP, we transiently expressed HA-Ro52 and Myc-TRBP2 in HeLa cells, and analyzed α-HA immunoprecipitates for the presence of Myc-TRBP2 by western blotting (Fig. 10B). Lane 2 shows that Myc-TRBP2 specifically co-purified with HA-Ro52 and was not bound by the α-HA antibody or the Sepharose beads in the absence of HA-Ro52 (lane 3). Having demonstrated the association of Ro52 with the Dicer/TRBP complex, we wanted to know if Ro52 directly interacts with one of these two proteins or with Ago2. To answer this question, we performed *in vitro* interaction studies using recombinant MBP-Ro52 and tagged Dicer, TRBP2 and Ago2 proteins prepared by *in vitro* transcription and translation (Fig. 10C). In vitro translated proteins were labeled with [^{35}S]-methionine and detected by autoradiography after separation on an

SDS-PAGE (Fig. 10C, right panel). MBP-Ro52 was incubated with the different *in vitro* translated proteins, the samples were purified on amylose, and proteins co-purifying with MBP-Ro52 were visualized by autoradiography after SDS-PAGE. FLAG-β-TrCP2, a known interaction partner of Ro52 (Sabile et al., 2006), was used as a positive control. HA-LacZ was used as a negative control. FLAG-TRBP2, but neither FLAG-Dicer nor HA-Ago2 was found to interact with Ro52 in this assay (Fig. 10C, three left panels). These *in vitro* interaction studies were performed in collaboration with W. Krek's laboratory, and represent experiments performed by Andrea Meyer.

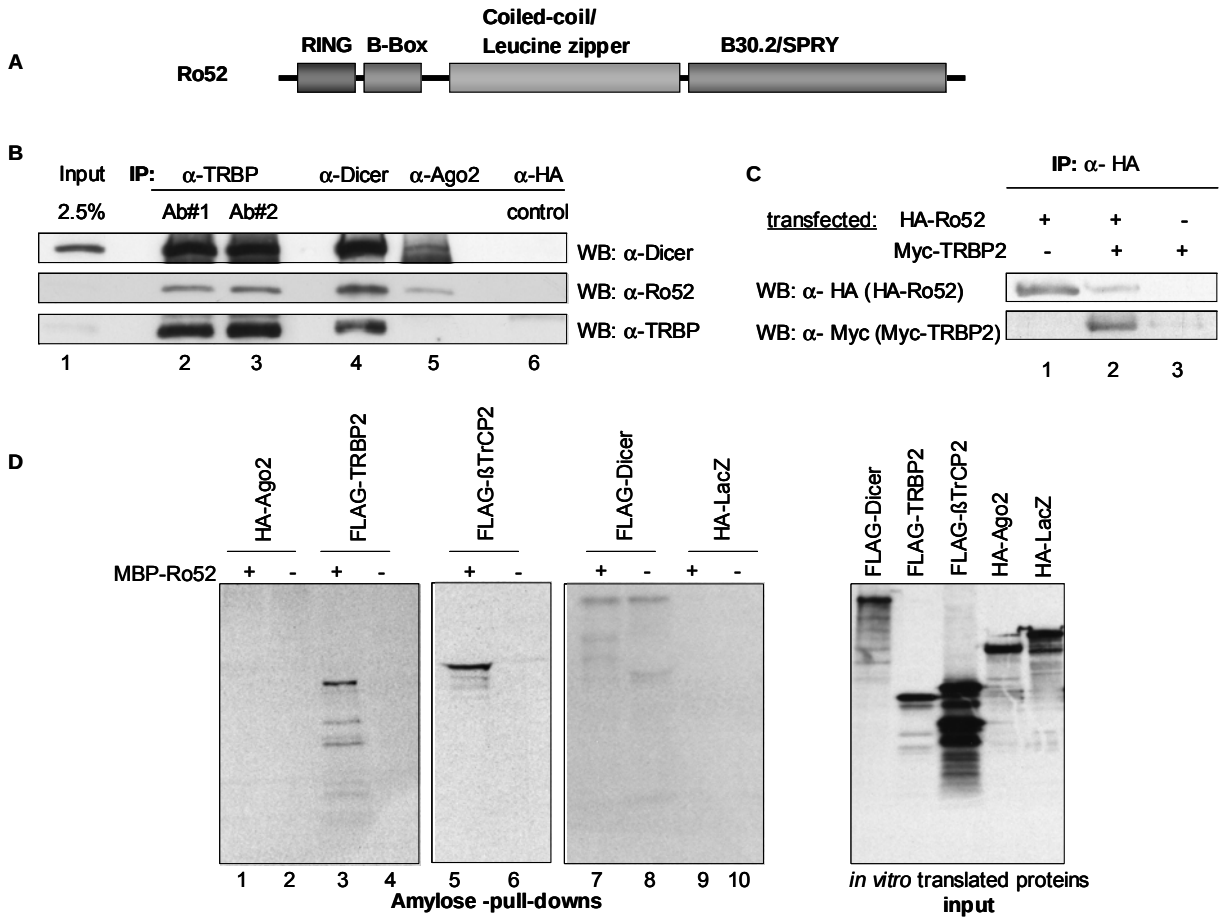


Figure 10. Ro52 associates with Dicer and TRBP, and interacts with TRBP *in vitro*. (**A**) A schematic representation of the domain architecture of Ro52. Ro52 harbors an N-terminal RING-finger, a B-box, a central coiled-coil/ leucine zipper domain, and a C-terminal B30.2/SPRY domain. (**B**) α-TRBP, α-Dicer, α-Ago2, and control immunoprecipitates were analyzed for the presence of Ro52 by western blotting. (**C**) HA-Ro52 and Myc-TRBP2 were co-transfected into HeLa cells. α-HA antibodies were used to immunopurify HA-Ro52 and Myc-TRBP2 was detected by western blotting. (**D**) FLAG-Dicer, FLAG-TRBP2 and HA-Ago2 were *in vitro* transcribed and translated in the presence of [^{35}S] Methionine and tested for *in vitro* interaction with recombinant MBP-Ro52 by pull downs using Amylose beads. FLAG-β-TrCP2 and HA-LacZ were used as positive and negative controls respectively. Interacting proteins were visualized by autoradiography.

Since Ro52 was shown to act as a RING-dependent E3 Ubiquitin ligase (Sabile et al., 2006), and TRBP was shown to undergo ubiquitination in highly confluent human cells (Lee et al., 2006a), we further investigated the ubiquitination status of TRBP in our experimental system (chapter 3.4.2.).

3.4.2. TRBP can be covalently tagged by Ubiquitin.

Functional diversity of an organism's proteome can be greatly expanded by posttranslational modifications including phosphorylation, acetylation, methylation, and ubiquitination (also termed ubiquitylation). In the case of ubiquitination, an entire polypeptide, Ubiquitin, is covalently attached to a target protein by isopeptide linkage of the C-terminus of Ubiquitin to the ε-amino group of a lysine in the substrate protein. Linkage of Ubiquitin to a target protein requires the consecutive action of three enzymes, an E1 Ubiquitin activation enzyme (E1), an E2 Ubiquitin conjugation enzyme (E2) and an E3 Ubiquitin ligase (E3). The mammalian genome encodes only one E1 enzyme, 20-30 E2 enzymes and hundreds of E3 Ubiquitin ligases, which function alone or in multidomain complexes as substrate recognition modules. There are two main types of E3 Ubiquitin ligases, the RING (Really Important New Gene) class and the HECT (Homologous to the E6-AP Carboxyl Terminus) class. Ubiquitination can be reversed by deubiquitinating enzymes (DUBs). DUBs like E3s show a high degree of substrate specificity and turn Ubiquitin into a highly dynamic post-translational modifier. The attachment of Ubiquitin to a target protein comes in

different flavors, the linkage of one molecule, i.e. monoubiquitination, or attachment of polymers of different topology. These different modes of Ubiquitin attachment result in different readouts and contribute to great functional diversity of the Ubiquitin system. Monoubiquitination has been functionally implicated in endocytosis, transcriptional regulation and nuclear-cytoplasmic transport (Haglund et al., 2003). Polyubiquitination represents an assembly of multiple ubiquitin chains, wherein one Ubiquitin moiety is linked to the target protein and further Ubiquitins are linked to one of the seven lysine (K) residues within Ubiquitin itself. These Ubiquitin chain formations are named by indicating the branching lysine within Ubiquitin. K48 branching is the best-characterized form of polyubiquitination, and is recognized by the proteasome, a multiprotein complex that catalyzes degradation of K48 Ubiquitin tagged proteins (Ciechanover, 1998; Ciechanover and Schwartz, 1998; Hershko and Ciechanover, 1998). K63 linked Ubiquitin chains are involved in cellular processes such as DNA repair, endocytosis and signal transduction (Chan and Hill, 2001). Ubiquitin chains branching at other residues as well as mixed chains have been observed *in vivo* and *in vitro* but little is known about the downstream biochemical mechanisms. Ubiquitination, like other posttranslational modifications, is recognized by specific Ubiquitin receptors, or Ubiquitin binding proteins (UBPs), that translate the Ubiquitination mark into a functional signal. Different Ubquitin binding domains (UBDs) have been identified that recognize specific flavors of ubiquitination. UBDs have been identified in several proteins with divers functions, indicating

that ubiquitination and Ubiquitin recognition is involved in many different cellular processes.

Posttranslational regulation of protein components in RNA silencing has not been investigated so far. Our recent finding that the E3-Ligase, Ro52, interacts with TRBP and associates with a TRBP/ Dicer complex, and data from Lee et al. suggesting that TRBP is regulated by proteasomal degradation (Lee et al., 2006a), prompted us to investigate the ubiquitination status of TRBP. To investigate if TRBP can be ubiquitiated in our experimental system, we co-transfected FLAG-TRBP2 and His_6-Myc-Ubiquitin into HeLa and HEK293 cells. The His-tag fused to Ubiquitin allows its purification under denaturing condition, thereby co-purifying only proteins that are covalently attached to the His_6-tagged-Ubiquitin. His_6-Myc-Ubiquitin, retained on Nikel-agarose beads under denaturing conditions, co-precipitated FLAG-TRBP2 (Fig. 11A: lane 6; and Inga Loedige unpublished data). Interestingly, ubiquitinated FLAG-TRBP2 appeared as a single band on western blot with a higher molecular weight than expected for the unmodified protein (compare lanes 6 and 3), but not as multiple high molecular bands as expected for e.g. K48-polyUbiquitination. In addition, we want to note that only a minor fraction of FLAG-TRBP2 is covalently linked to His_6-Myc-Ubiquitin, as shown by comparison with the input cell extract (lane 3). To test if the ubiquitination of TRBP2 involves branching of Ubiquitin chains at K48 of Ubiquitin, we performed denaturing pull down experiments using His_6-Myc-Ubiquitin or a His_6-Myc-Ubiquitin mutant, in which Lysine 48 (K58) was mutated to Arginine (R) (His_6-Myc-Ubiquitin K48R). HEK293 cells were transfected with

combinations of FLAG-TRBP, His_6-Myc-Ubiquitin, and His_6-Myc-Ubiquitin K48R, as indicated. 48 hours after transfection, the cells were lysed and the His-tagged constructs were pulled down using Nikel-agarose beads under denaturing conditions (Fig. 11B). Co-purification of FLAG-TRBP2 was visualized by western blotting using an α-FLAG antibody. His_6-Myc-Ubiquitin, as well as His_6-Myc-Ubiquitin K48R pulled down FLAG-TRBP2 (Fig.11B, lanes 4 and 5). These data indicated that the attachment of a Ubiquitin-moiety to FLAG-TRBP2 does not involve branching at K48 of Ubiquitin. K48-polyubiquitination is known to target proteins for proeasomal degradation. To obtain further evidence that the modification of TRBP does not involve K48-polyubiquitination, we compared pull downs from cell extracts prepared from either control cells or cells treated with the proteasome inhibitor MG132 (Fig.11C). Lanes 3 and 4 show that His_6-Myc-Ubiquitin is linked to FLAG-TRBP2 in a similar manner with and without inhibition of the proteasome by MG132. K48-polyubiquitination is known to appear as a smear of very high molecular weight, which is not seen for TRBP in our experiments. To summarize, our experiments show that TRBP can be covalently modified by Ubiquitin. Modified TRBP appears with a molecular weight that might correspond to a mono-ubiquitinated form, or to the attachment of short Ubiquitin-chains. No poly-ubiquitination, such as K48 polyubiquitination, has been observed.

In addition, we treated HeLa cells with cycloheximide (CHX) or MG132 for three hours and analyzed protein-levels of endogenous Dicer, Ago2 and TRBP by western blotting (Fig. 11D). Upon MG132 treatment, endogenous

protein levels of Dicer, Ago2 and TRBP did not increase significantly as it would be expected for proteins regulated by fast proteasomal turn-over (Fig.11D, lanes 3 and 4). Cycloheximide (CHX) inhibits eucaryotic translation by interfering with the peptidyl transferase activity of the 60S ribosomal subunit, thus blocking translational elongation. Lanes 4 and 6 indicate that none of the analyzed proteins showed any significant decay within three hours of inhibition of translation. Taken together our data suggest that TRBP can be ubiquitinated, but that ubiquitination of TRBP does not correspond to K48-polyubiquitination and does not induce proteasomal degradation. Ubiquitination of TRBP might rather correspond to mono-or poly-ubiquitination involved in signaling or protein-protein interactions. In future work, we want to address the role of post-translational protein modifications in RNA silencing. We want to investigate the potential function of the ubiquitination of TRBP, and the role of Ro52 as an E3 Ubiquitin ligase, possibly involvement of the ubiquitination of TRBP, in the context of RNA silencing.

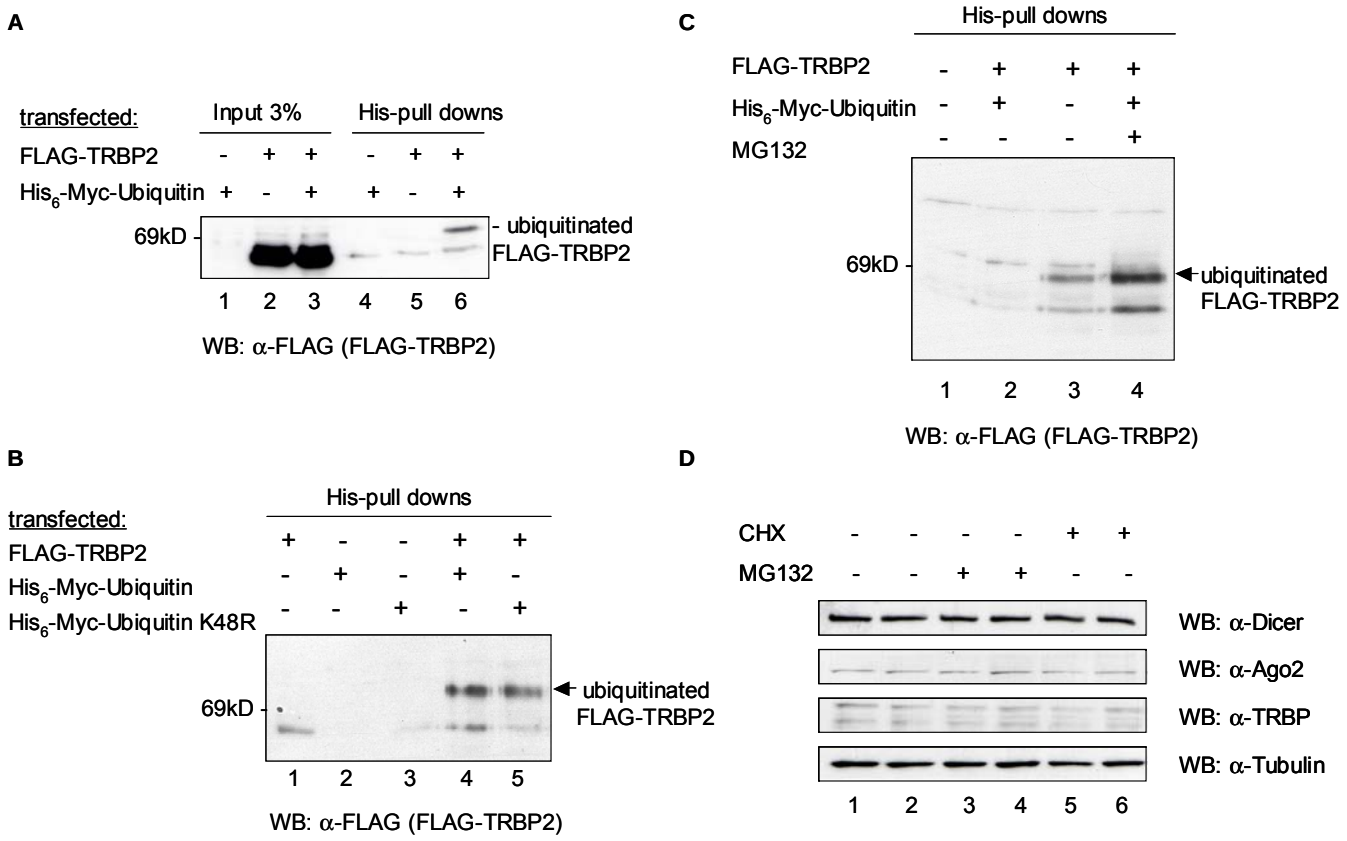


Figure 11. Ubiquitination of TRBP, and protein half-lives. (**A-C**) His_6-Myc-Ubiquitin or His_6-Myc-Ubiquitin K48R, and FLAG-TRBP2 were co-transfected into HeLa cells. After pull down under denaturing conditions via the His_6-tag, FLAG-TRBP2 was detected by western blotting. (**A**) Lanes 1-3 show FLAG-TRBP2 expression in the input cell extracts. Lanes 4-6 show FLAG-TRBP2 linked to His_6-Myc-Ubiquitin after retention on Ni^{++}-agarose beads. A fraction of FLAG-TRBP is specifically linked to His_6-Myc-Ubiquitin, and appears as a protein of higher molecular weight than unmodified FLAG-TRBP2 (lane 6). (**B**) Mutation of lysine (K) 48 within His_6-Myc-Ubiquitin to Arginin (R) (His_6-Myc-Ubiquitin K48R) does not impair the modification of FLAG-TRBP2 (lane 5) compared to His_6-Myc-Ubiquitin. (**C**) Treatment of the transfected cells with the proteasome inhibitor MG132 does not change the appearance of FLAG-TRBP2 linked to His_6-Myc-Ubiquitin (lane 4). (**D**) HeLa cells were treated with the cycloheximide (CHX) (10 µg/ml), inhibiting translation, or MG132 (10 µM), inhibiting proteasomal degradation, for three hours. Endogenous proteins were detected by western blotting as indicated. Detection of tubulin is used as a normalization control.

4. Discussion

4.1. Proteins associated with Dicer.

Dicer and Argonaute proteins represent two key components of RNA silencing pathways in different organisms. Therefore the identification of proteins associated with Dicer and Argonautes is crucial for our understanding of these pathways. Work presented in this thesis identified proteins associated with human Dicer by the analyses of α-Dicer immunoprecipitates by LC-MSMS.

Another study conducted by Mello and co-workers identified proteins associated with *C. elegans* Dicer-1 by mass spectrometry (Duchaine et al., 2006). *C. elegans*, like mammals, encodes only one Dicer, Dicer-1, which serves the processing of different small RNAs. Mello and co-workers identified several known components of small RNA silencing pathways as well as additional proteins associated with Dicer-1 (Duchaine et al., 2006). Proteins that were found by them to associate with Dicer are involved in the processing of three different classes of small RNAs: (1) endogenous siRNAs, (2) exogenous siRNAs, and (3) miRNAs. The Argonaute protein, RDE-1, the small dsRNA binding protein, RDE-4, and the Dicer-related RNA helicases DRH1 and DRH2 co-purified with Dicer-1 in the screen as previously described (Tabara et al., 2002). In addition, Duchaine et al. found a third paralogue of DRH1 and DHR2, DHR3 associated with Dicer-1. Another Dicer-1 associated protein, *Pir-1* (tm1496) encodes an RNA phosphatase that functions in processing of RNA-dependent RNA polymerase (RdRP) dependent secondary siRNAs. Of interest for our work, Mello and co-

workers found the RING-finger B box coiled coiled (RBCC) protein Lin-41, that was previously shown to be required for translational repression of LIN-29 in the hypodermis (Slack et al., 2000) as a protein associated with Dicer-1. Functionally, Lin-41 was however found not to be essential for RNAi or miRNA mediated silencing. It has been previously reported that two enhancers of RNAi (eri), eri-1 and the RdRP rrf-3, associate with Dicer-1 (Kennedy et al., 2004; Simmer et al., 2002). These two proteins, and two newly identified eri-proteins, eri-3 and eri-5, were found to associate with Dicer-1 by Duchaine et al. (2006). Interestingly, proteins associated with Dicer-1 include positive and negative regulators of RNA silencing pathways. Mello and colleagues suggest a model in which specific interacting proteins direct Dicer-1s activity towards competing small RNA mediated gene-silencing pathways (Duchaine et al., 2006).

Our analysis of proteins associated with human Dicer identified many proteins that fall into several different groups comprising (1) Argonaute proteins, (2) RNA helicases, (3) translational regulators, (4) RNA binding proteins, and others (described in chapter 3.1.). The number and the diversity of proteins identified to associate with Dicer in *C. elegans* and human cell lines might reflect the complexity and dynamics of Dicer complexes in RNA silencing *in vivo*. Different Dicer complexes might, for example, reflect different steps in holo-RISC assembly as it was proposed for siRISC formation in *Drosophila* (Pham et al., 2004; Tomari et al., 2004a), but might also result from dynamic transient association of different regulatory factors.

Differences between our proteomic analysis and data presented for *C. elegans* Dicer-1 complexes (Duchaine et al., 2006) might result from differences in the RNA silencing pathways in these organisms. Well established differences of RNA silencing pathways in different organisms, including mammals, *C. elegans, Drosophila*, and plants strongly suggest the requirement for specific protein factors. While in mammals the miRNA pathway is thought to be predominant, endogenous siRNA pathways that require additional protein factors are operating in *Drosophila*, *C. elegans*, and plants. In addition, RdRPs were characterized in *C. elegans* and plants. These enzymes allow amplification of the initial small RNA trigger, and this additional step in RNA silencing may require specific protein factors, and possibly specialized Dicer complexes to process the newly synthesized dsRNA. No RdRPs are encoded in the genomes of *Drosophila* and mammals. In contrast to these differences, RNA helicases have been implicated in RNA silencing pathways in many different organisms. Consistently, several RNA helicases were found in our screens, but their precise molecular function remains to be established. The two highly related DEAD-box RNA helicases p68 (DDX-5) and p72 (DDX-17) were identified in our LC-MSMS analysis to be associated with Dicer. The Drosophila homolog of p68, Dmp68 was shown to associate with RISC, and to be required for RNAi (Ishizuka et al., 2002). Further p68 and p72 were found to associate with Drosha in mammals (Gregory et al., 2004), and p68 and p72 are required for pri-miRNA processing (Fukuda et al., 2007). Other RNA helicases have been implicated to function at various steps of RNA silencing in different organisms. The DExD-box RNA

helicase SDE3 is required for PTGS in Arabidopsis (Dalmay et al., 2001). Its *Drosophila* homolog Armitage was shown to be involved in RISC maturation (Tomari et al., 2004a), and the mammalian RNA helicase MOV10/Armitage is required for miRNA mediated cleavage of a reporter mRNA (Meister et al., 2005). Another RNA helicase, RCK/p54, which is a P-body component, is required for miRNA mediated translational repression (Chu and Rana, 2006), and recently RNA helicase A was shown to be involved in RISC loading in mammals (Robb and Rana, 2007). In addition, holo-RISC formation was shown to be an ATP-dependent process that is accompanied by siRNA unwinding in Drosophila (Pham et al., 2004; Tomari et al., 2004a). However, a minimal RISC complex consisting of Dicer, TRBP and Ago2 that couples pre-miRNA processing and target-RNA cleavage in an ATP-independent manner has been proposed in mammals (Gregory et al., 2005; Maniataki and Mourelatos, 2005). Gregory et al., (2005) hypothesize that shifting the equilibrium from RNA-RNA interaction to RNA interaction with the PAZ domain of Ago2 might generate the energy required for unwinding of the miRNA duplex. Taken together, there is no unified opinion at the moment about the involvement of RNA helicases and ATP-dependent unwinding of siRNA and miRNA duplexes during RISC assembly. Further studies will be necessary to characterize the function of RNA helicases at different steps of RNA silencing in more detail.

4.2. RNase III enzymes and their dsRNA-binding partners.

DsRNA binding proteins have been found to associate with Dicers in different organisms, and have been studied extensively. We and others identified TRBP as a dsRNA binding partner of human Dicer (Chendrimada et al., 2005; Haase et al., 2005). The first part of our work, presented in this thesis, focused on the biochemical characterization of Dicer's interaction with TRBP, and the analysis of the function of TRBP in RNA silencing. Over the last few years it has become apparent that RNase III-like enzymes of class II in higher eucaryotes function in conjunction with small dsRNA binding partners. Functional analysis revealed that these dsRNA binding proteins function in different RNA silencing pathways, and at different steps within the pathways (Table 4).

RNase III enzyme	**associated dsRBD protein**	organism	**Function of dsRBD proteins in RNA silencing**	**reference**
Dicer-1	RDE-4	C. elegans	- Required for the initial step of RNAi - not required for miRNA processing or development	(Tabara et al., 2002)
Dicer-2	R2D2	*Drosophila*	- required for RNAi but not for the miRNA pathway - required for Dicer protein stability - required for siRNA binding of the Dicer-2/R2D2 heterodimer, but not for Dicer-2s cleavage activity *in vitro* - the Dicer-2/R2D2 heterodimer senses thermodynamic properties of the siRNA duplex and decides determines the guide versus the passenger strand	(Liu et al., 2003; Tomari et al., 2004b)
Dicer-1	Loqus/R3D1	*Drosophila*	- required for miRNA silencing and development - required for efficiency and specificity of substrate processing by Dicer-1	(Forstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005; Tomari et al., 2004b)
Dicer	TRBP	mammals	- required for miRNA and siRNA silencing - required for efficient Dicer processing *in vitro*	(Chendrimada et al., 2005; Haase et al., 2005)
Dicer	PACT/PRKRA	mammals	- required for efficient miRNA accumulation	(Lee et al., 2006b)
DCL1	HYL1	plants	- required for pri-miRNA processing	(Hiraguri et al., 2005)
Drosha	Pasha	*Drosophila*	- required for pri-miRNA processing	(Landthaler et al., 2004)
Drosha	DGCR8	mammals	- required for pri-miRNA processing	(Gregory et al., 2004; Han et al., 2004a)

Table 4. Class II RNase III enzymes and their dsRNA binding partners

The first dsRNA binding protein discovered in RNA silencing, RDE-4, was a partner of Dicer-1 in *C. elegans*. RDE-4 is required for efficient dsRNA processing by Dicer-1. RDE-4 mutants are defective in RNAi, but do not exhibit developmental phenotypes, which argues against its involvement in miRNA mediated silencing (Parrish and Fire, 2001; Tabara et al., 1999; Tabara et al., 2002). In *Drosophila* and mammals, Drosha associates and functions in pri-miRNA processing in concert with a dsRNA binding protein known as

Pasha/DGCR8, encoded in the DiGeorge syndrome critical region 8 (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004a; Han et al., 2006; Landthaler et al., 2004; Wang et al., 2007; Yeom et al., 2006). In plants, DCL1 is required for pri-miRNA maturation and associates with the dsRBP HYL1 (Han et al., 2004b; Hiraguri et al., 2005; Song et al., 2007; Vazquez et al., 2004).

Different functions of individual dsRBD proteins might reflect general differences in the pathways in different organisms. In mammals, TRBP interacts with Dicer and is necessary for efficient siRNA and miRNA silencing (Chendrimada et al., 2005; Haase et al., 2005; Lee et al., 2006b). Although no endogenous siRNAs have been discovered to date in mammalian cells, mammals maintained their ability to conduct RNAi induced by exogenous dsRNA or siRNAs. The biological relevance of this functional conservation remains, however, elusive. In contrast to this dual function of Dicer and TRBP in mammals, miRNA and siRNA silencing pathways are more separated in other organisms. In *Drosophila*, Dicer-1 processes pre-miRNAs to mature miRNAs, and works in conjunction with the small dsRBP R3D1/Loquacious (Loqs) (Forstemann et al., 2005; Jiang et al., 2005; Saito et al., 2005). Loqs, like TRBP, is required for efficient miRNA silencing, and despite its positive effect on Dicer cleavage activity it does not influence the steady state levels of miRNAs *in vivo*. On the other hand, R2D2, the dsRNA binding partner of *Drosophila* Dicer-2 that is involved in RNAi, functionally differs from Loqs and TRBP (Liu et al., 2003; Tomari et al., 2004b). R2D2, unlike TRBP and Loqs, is required for the stability of the Dicer-2 protein, and the Dicer-2/R2D2 heterodimer senses thermodynamic

asymmetry of the siRNA duplex thereby determining, which strand is further into RISC and which strand is degraded. There is no evidence so far, that Loqs or TRBP have similar functions in asymmetry decision for the miRNA or siRNA duplexes.

Interestingly, Dicers in different organisms have generally been attributed a single dsRNA binding partner, whereas human Dicer functions in association with two different dsRBPs: TRBP and its paralogue PACT/PRKRA (protein kinase, interferon-inducible dsRNA dependent activator) (Lee et al., 2006b). Both, TRBP and PACT appear to act as positive regulators of RNA silencing, but while knock down of TRBP strongly impairs RNA silencing efficiency, as demonstrated by reporter assays, the knock down of PACT does not have this effect. On the other hand, PACT, in contrast to TRBP, is required for efficient miRNA accumulation *in vivo*. This finding raises an interesting general question: What is the correlation between miRNA abundance and its biological function?

Both TRBP and PACT can interact with helicase domain or Dicer via their third dsRBD, suggesting that Dicer might not be capable to interact with both at the same time, and some additional factors would have to determine which of these proteins associates with Dicer (Haase et al., 2005; Lee et al., 2006b). Human Dicer, like Dicers in other organisms, might therefore employ only one dsRBP, but unlike others can choose between TRBP and PACT.

We want to note that PACT was not identified in our analyses of α-Dicer immunoprecipitates. It might be that our analyses were not exhaustive enough to identify proteins present at low level in the immunoprecipitates. We could also

speculate that Dicer interacts with either TRBP or PACT in different cells or under different physiological conditions. We showed that endogenous Dicer and TRBP associate with each other in HEK293 and HeLa cells (chapter 3.2.), whereas experiments showing the association of Dicer and PACT were done with over-expressed proteins (Lee et al., 2006b). Over-expression of either PACT or TRBP changes the ratio of PACT and TRBP available for interacting with Dicer, and might thereby interfere with Dicer's natural preference for one or the other binding partner.

Concerning Dicers in other organisms, it would be interesting to find out whether they could also associates with different dsRBPs, and to investigate functional differences of such possible interactions.

4.3. RNAi and innate immunity: Is there a link in mammals?

TRBP and PACT, the two dsRNA binding partners of human Dicer, are particularly interesting because of their known function in innate immune defense. Innate immunity is a phylogenetically ancient mechanism used by metazoans to defend themselves against invading pathogens like bacteria, fungi and viruses. The evolutionarly conserved RNAi response to exogenous dsRNA is thought to have evolved as a form of innate immunity against viruses and other foreign genetic material. In fact, one of the first indications of the involvement of RNA silencing pathways in innate immunity, was the discovery of siRNAs derived from viral dsRNA in plants (Hamilton and Baulcombe, 1999). Currently, there is evidence that invertebrates, nematodes and insects use RNAi as an antiviral

mechanism (Galiana-Arnoux et al., 2006; Schott et al., 2005; van Rij et al., 2006; Wang et al., 2006b; Wilkins et al., 2005). In contrast to the situation in plants and invertebrates, there is no such evidence for a role of RNAi as an antiviral defense mechanism in vertebrates, which have evolved other means of innate immune defense, in particular the interferon (IFN) system. Non-sequence specific defense mechanisms against foreign nucleic acids, in particular the dsRNA induced IFN response may have overtaken this function of RNAi in vertebrates. One of the major effectors of the IFN response is the interferon inducible protein kinase R (PKR). Upon dsRNA binding, PKR is activated by autophosphorylation and phosphorylates eIF2α, what in turn leads to a general inhibition of translation and results in apoptosis ((Meurs et al., 1990); for review see: (Cole, 2007; Garcia et al., 2006; Taylor et al., 2005; Williams, 2001)). Another pathway that is induced by dsRNA leads to the activation of RNase L by 2'-5' oligoadenylates. Activated RNase L promotes cleavage of viral as well as cellular RNAs (for review see: (Clemens, 2005)). Both TRBP and PACT can directly interact with PKR. While binding of TRBP inhibits PKR, PACT functions as an activator of PKR (Gupta et al., 2003). Despite their similarity in domain architecture, and protein and RNA binding properties, these two dsRBPs are functionally very different. In addition to their potential to interact with PKR and Dicer, TRBP and PACT can also homo- and heterodimerize (Kok et al., 2007; Lee et al., 2006b). What determines the preference of TRBP and PACT to associate with each other or with one of their potential binding partners, and how these interactions are regulated and which additional factors are involved, is not understood. It also remains largely

unknown, if Dicer or other components of RNA silencing pathways play a role in innate immune defense in mammals.

Data shown in this thesis and data by Lee et al., (2006b) demonstrated that the N-terminal RNA helicase domain is required for Dicer's interaction with TRBP. Interestingly, three RNA helicases containing domains related in sequence to the helicase domain of Dicer act as cytoplasmic sensors of viral dsRNA in mammals ((for review see (Meylan and Tschopp, 2006)). Two of them, the retinoic-acid-inducible gene 1 (RIG-1/DDX58) and the melanoma-differentiation-associated gene 5 (MDA5/Helicard), harbor two Caspase recruitment domains (CARD) in addition to the RNA helicase domain. Upon dsRNA binding, RIG-1 and MDA5 interact with Cardif (CARD adapter inducing interferon-beta) also known as MAVS (Mitochondrial antiviral-signaling protein)/IPS-1 (Interferon-beta promoter stimulator protein 1) or VISA (Virus-induced-signaling adapter), which in turn activates NF-KB and IRF3, resulting in a type I IFN response. The third Dicer related RNA helicase, LGP2, is devoid of CARDs, and was recently suggested to act as a regulator of the IFN response following RNA virus infection (Saito et al., 2007; Venkataraman et al., 2007). In *C. elegans,* the Dicer related helicases DRH-1, -2, and -3 were shown to associate with Dicer and function in RNA silencing (Duchaine et al., 2006). The mammalian Dicer related RNA helicases RIG-1, MDA5 and LGP2 function in viral defense. In mammals, the RNA helicase domain of Dicer interacts with TRBP and PACT. These observations raise several questions: (1) Are Dicer related RNA helicases also involved in RNA silencing in mammals?, (2) Does Dicer's

recognition of dsRNA, in particular viral dsRNA, involve its helicase domain?; (3) Do TRBP and PACT associate with Dicer related helicases and function in cytoplasmic dsRNA recognition, thereby connecting RNA silencing, PKR response, and RIG-1/MDA5/LGP2-mediated defense?; (4) Do these different pathways compete for the interaction with TRBP, or PACT, or both of them? The potential relationship between RNA silencing and innate immunity pathways in mammals, as mentioned above, remains to be elucidated.

We want to note that neither RIG-1 nor MDA5 or LGP2 were identified in our LC-MSMS analysis, which might result from non-exhaustive sequencing of peptides, but might also reflect the low abundance of these helicases in non-infected cells or cell not treated with IFN inducers.

In HIV infected cells, TRBP binds the HIV-1 trans-activation response (TAR) RNA, and enhances HIV-1 expression and replication. The TAR hairpin also acts as a binding site for the Tat protein and this interaction stimulates the activity of the long terminal repeat promoter (Kulinski et al., 2003). In contrast to TRBP, Tat is encoded by the viral genome. Interestingly, Tat was suggested to act as an RNA silencing supressor by inhibiting cleavage activity of Dicer (Bennasser et al., 2005). In addition, sequestration of TRBP by TAR was suggested to inhibit RNA siencing in virus infected cells (Bennasser et al., 2006). On the other hand, miRNAs derived from the viral genome were suggested to function in the regulation of HIV-1 gene expression (Weinberg and Morris, 2006). These latter data would argue for HIV using the RNA silencing machinery in its favor, whereas the expression of TAT and the sequenstration of TRBP were shown to

inhibit RNA silencing. If the RNA silencing pathway is benefecial for the virus, or if these pathways contrubute to antiviral defense mechanisms of the host cell, or if both machanisms compete during viral infections remains to be elucidated.

4.4. Ubiquitin as a regulatory component of RNA silencing?

Besides TRBP, we identified another component of the Dicer/TRBP complex. Ro52/TRIM21 is associated with the Dicer/TRBP complex *in vivo* and interacts with TRBP *in vitro*. The presence of the E3 Ubiquitin ligase Ro52 in Dicer/TRBP complexes raised a possibility that components of RNA silencing complexes are post-translationally modified by Ubiquitin and prompted us to investigate the function of ubiquitination in RNA silencing. Interestingly, proteasomal degradation of the RNA helicase Armitage, a RISC assembly factor (Cook et al., 2004; Tomari et al., 2004a), was observed during synaptic stimulation of protein synthesis associated with learning in *Drosophila* olfactory neurons (Ashraf, 2006). In addition, proteasomal degradation of TRBP was described in human cell lines growing at high cell density (Lee et al., 2006a). Besides these two observations, posttranslational modifications have not been studied in the context of RNA silencing so far. Since Ro52 directly interacts with TRBP, we investigated the ubiquitination status of TRBP. Our data indicated that TRBP can be ubiquitinated. However, no evidence was obtained in support of poly-ubiquitination and proteasomal degradation of TRBP. Our data indicated that TRBP, in addition to stimulating Dicer cleavage activity, is also involved in RISC assembly, perhaps at the step of RISC loading (Haase et al., 2005). Possibly, ubiquitination of TRBP functions in the holo-RISC assembly.

E3-Ubiquitin ligases exist both as monomeric proteins and within multiprotein complexes that interact specifically with a substrate protein to promote its ubiquitination. Ro52 was shown to be part of a SCF^{Skp2} complex that targets p27 for poly-ubiquitination and proteasomal degradation (Sabile et al., 2006). We showed that Ro52 interacts with TRBP2 *in vitro*, and that TRBP can be ubqiutinated (chapter 3.4.). Since Ro52 can interact with TRBP, it is possible that Ro52 functions as an E3 ligase in the ubiquitination of TRBP. Further work is however required to verify this.

One can also speculate about a possible role of ubiquitination in the assembly of P-bodies. To date the biochemical nature of P-bodies remains poorly understood. Recently, the small ubiquitin like modifier (SUMO) has been linked to PML (promyelotic leukemia) nuclear body-formation (PML-NBs). Sumoylation and noncovalent interactions with SUMO through SUMO binding motifs were found to mediate the assembly of PML-NBs and the dynamic distribution of proteins between PML-NBs and the nucleoplasm. Interestingly, a ubiquitin interaction domain (UBA) was predicted within one of the major P-body components, GW182, in *Drosophila* and mammals (Behm-Ansmant et al., 2006; Eystathioy et al., 2002). If ubiquitination and ubiquitin-recognition indeed contribute to the integrity of P-bodies remains to be elucidated.

5. Conclusions and future perspectives

Our analysis of proteins interacting with human Dicer led us to the identification of TRBP as a dsRNA binding partner of Dicer. The characterization of TRBP as a Dicer interacting protein and its function in RNA silencing contribute to the general notion that arose during the last years, that class II RNase III enzymes work in conjunction with dsRNA binding partners in different organisms. Our ongoing work on the characterization of the E3-ubiquitin ligase Ro52 as a component of the Dicer/TRBP complex, as well as observations that TRBP is ubiquitinated but not subjected to proteasomal degradation raised the possibility that RNA silencing is regulated at the post-translational level.

The validation and characterization of additional proteins identified in our Dicer- and TRBP- immunopurifications should lead towards a better understanding how specificity and efficiency of RNA silencing is controlled in mammals. The investigation of post-translational modifications of the Dicer complex components argues for an additional level of complexity of RNA silencing pathways. In addition, our identification of TRBP as a partner of Dicer contributed to the emerging idea that RNA silencing and innate immunity pathways might be connected in mammals, as already shown for other organisms. How these pathways cross-talk with each other, and what confers their specificity for different RNA substrates remains to be elucidated. Fig. 12 illustrates our data in the context of the known pathways to emphasize the possible cross-talk of different dsRNA responding pathways, and the complexity of the formation and regulation of different RNPs in RNA silencing.

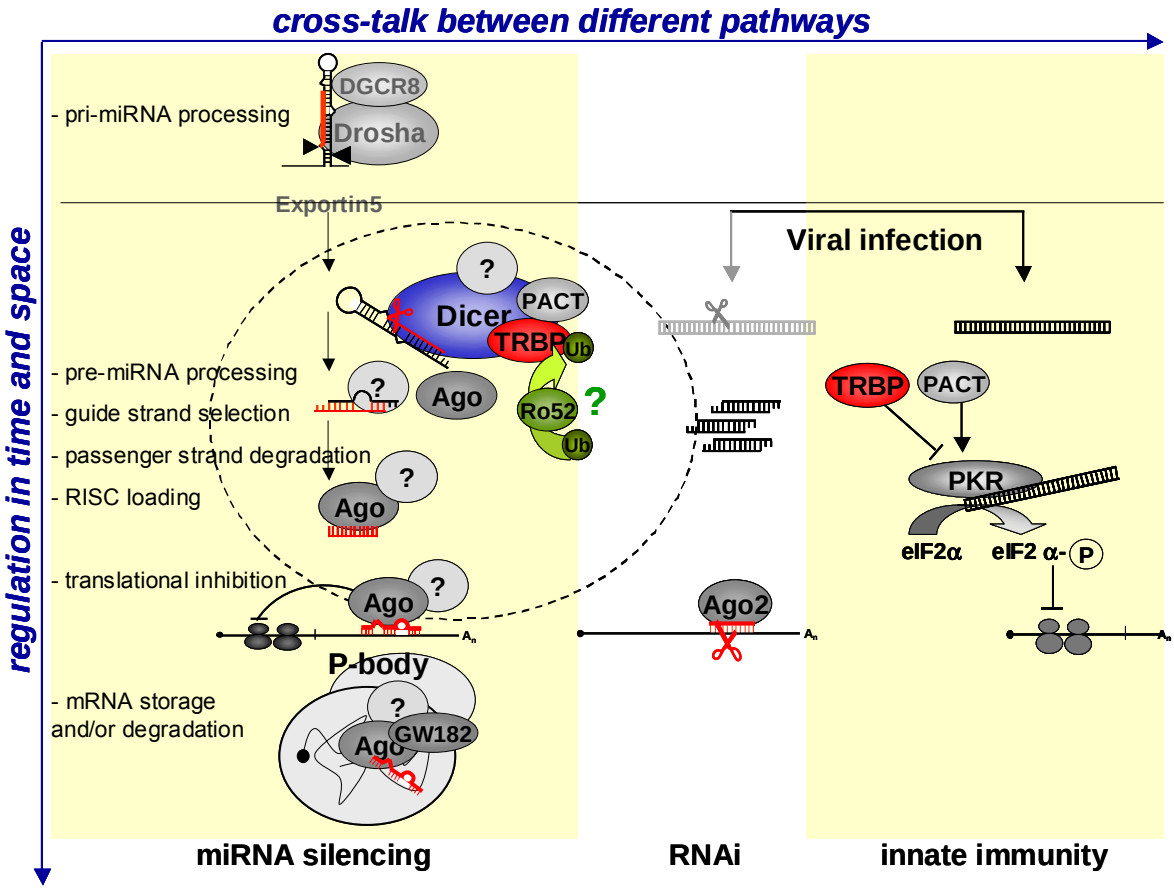


Figure 12. **Cross-talk between different dsRNA responding pathways.** Our data are illustrated in the context of the known pathways to emphasize (1) the possible crosstalk between RNA silencing and antiviral defense pathways, (2) the different dimensions of the RNA silencing pathways comprising timing and localization during pre-RNA processing, RISC formation, and target RNA silencing, (3) a regulatory layer leading to a possible high degree of efficiency, specificity and flexibility, and (4) the possible complexity or RNP formation during the initiator phase of RNA silencing, where the pre-miRNA is processed, asymmetry of the miRNA duplex is determined that leads to the degradation of the passenger strand and to the incorporation of the guide strand into RISC.

5. Materials and Methods - Experimental procedures

Cloning of N-TAP-Dicer and TAP-control plasmids, and generation of stable cell lines. Plasmids containing a TAP-tag for mammalian expression were kindly provided by Henry R Parker. The N-terminal TAP-tag was PCR-amplified from pcDNA4/TO N-TAP using primers #1 and #2, and inserted upstream of and in frame with Dicer cds within pETR1A (Zhang et al., 2002) by directional cloning. The pENTR1A-Dicer was digested with AflII and SalI. The N-TAP-PCR fragment was cut with AflII and XhoI, and ligated into AflII – SalI within pENTR1A-Dicer. Ligation of the XhoI site to the SalI site resulted in destruction of either site and creation of a Taq1 restriction site, which was used to screen for positive clones by restriction digest. For the TAP-control construct, a STOP-codon was added to the N-TAP by PCR using primers #1 and #3, and cloned into pENTR1A. The Gateway technology (Invitrogen) allowed rapid site specific cloning of these constructs into the mammalian expression vector pTREx-Dest-30. This destination vector harbors a CMV promoter under the control of two tetracycline (tet) operator sites, and a G418 resistance gene, enabling the stable selection of transfected mammalian cells. For cloning of the TAP-Dicer construct resistant to the Dicer targeting hairpin, a part of Dicers cds comprising the site targeted by the short hairpin construct was PCR-amplified using primers #4 and #5, and subcloned into pCRII-TOPO (Invitrogen). Silent mutations were introduced into this fragment by site directed mutagenesis (Stratagene: site directed mutagenesis kit) using primers #6 and #7. Mutagenesis of the site within Dicer

cds targeted by the short hairpin introduced a new SmaI/XmaI restriction site that was used for screening for positive transformants. The mutated construct was reintroduced into the context of full length TAP-Dicer by directional cloning into pENTR1A TAP-Dicer using EcoRV and BamHI restriction sites, and cloned into pTREx-Dest-30 using the gateway technology.

Primers:

Primer #	Restriction sites	Sequence 5' → 3'
P1 fwd	AflII	CGGACTCTAGCGTTTAAACTTAAGGCCATGAAAGCTGATGCGC
P2 rev	XhoI	CGCCTCGAGTCCGCCATAATCAAGTGCCCCGGAGG
P3 rev	NotI	TTTGCGGCCGCTTTATTCGCCATAATCAGGTGCCC
P4 fwd	EcoRV	CGTTATCATTCCAAGATATCGCAATTTTGATCAGC
P5 rev	BamH1	CATCTTCTTCAACAGCTTTGCTAGGATCCAGATAGC
P6 fwd	SmaI/XmaI	CCTGTGAATTGGCTTCCCGGGTATGTA
P7 rev	SmaI/XmaI	CTACATACCCGGGAGGAAGCCAATTCACAGG

For the generation of stable cell lines, the TAP-Dicer, iTAP-Dicer or the TAP-control plamids were transfected into HEK293-TREx cell lines, stably expressing the tet-repressor (Invitrogen), or into a HEK293-TREx cell line harboring the pTER-anti-Dicer short hairpin construct (293/Dicer kd), respectively. The 293/Dicer kd cell line was generated by Kaifu Tang in our laboratory (Schmitter et al., 2006). Cells were transfected using Lipofectamine PLUS (Invitrogen). 24 h after transfection the cells were splitted 1:10, and selection with G418 using a final concentration of 200μg/ml was started. After

antibiotic selection for three weeks, stable transformants were picked, and expanded to give rise to stable clonal cell lines. After induction of the cell lines for 48 h with 10 μg/ml tet, expression of the TAP-contruct was tested by western blotting. A typical example of a western blot screening of TAP-Dicer expressing clones is shown in Fig. S4.

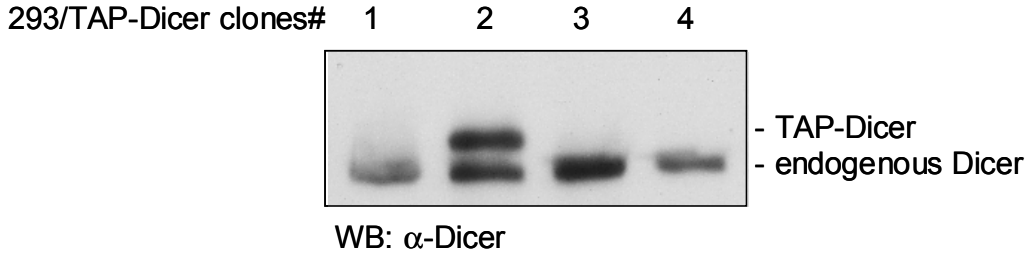


Figure S4. The TAP-Dicer construct was stably transfected into HEK293-TREX cells. After antibiotic selection, independent cell clones were picked and expanded. Stable clonal transformants were treated with tetracycline for 48 h. Cell lysates were subjected to SDS-PAGE, and Dicer was detected by western blotting. While clones # 1, 3 and 4 do not show expression of the transgene, clone #2 shows expression of a higher molecular weight version of Dicer corresponding to TAP-Dicer in addition to endogenous Dicer, as indicated.

Tandem Affinity Purification (TAP). TAP-Dicer or iTAP-Dicer cell lines were treated with 2 μg/ml tetracycline for 48 h to induce expression of the integrated contructs. Cells were trypsinized, washed with PBS and lysed in lysis buffer containing 20 mM TRIS.Cl pH 7.5, 150-300 mM NaCl, 0.5 mM NP40 and 3 mM $MgCl_2$, and complete protease inhibitors (Roche). Cells extracts were centrifuged at 10000xg for 15 min at 4°C, and supernatants were incubated with equilibrated IgG sepharose (Amersham) for 1-3 h in lysis buffer with rotation. After three washes with lysis buffer and two washes with TEV cleavage buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% NP40, 0.5 mM EDTA, 1 mM DTT),

for equilibration, the beads were resuspended in TEV cleavage buffer containing 100 Units of the TEV protease (Invitrogen) per ml of TEV cleavage buffer, and rotated at 16 – 24°C for 3-12 h. The supernatant was collected and buffer conditions were adjusted to final concentrations of 10 mM Tris.HCl pH 8.0, 150 M NaCl, 1 mM Magnesium acetate, 2 mM Calcium acetate, 0.1% NP40, 10 mM β-mercaptoethaol, and 1 mM imidazole corresponding to a Calmodulin binding buffer. Binding to Calmodulin beads (Stratagene) was performed in Calmodulin binding buffer for 1-12 h at 4°C. After three washed with calmodulin binding buffer, elution of the bound proteins was perfomed using a calmodulin elution buffer (10 mM Tris.HCl pH 8.0, 150 mM NaCl, 1 mM magnesium acetate, 20 mM EGTA, 0.1% NP40, 10 mM β-mercaptoethaol, and 1 mM imidazole). An example of purifications according to these experimental conditions is shown in Fig. S5. TAP-tagged proteins were purified from 293/iTAP-Dicer and 293/TAP-control cell lines. iTAP-Dicer was detected by western blotting using the α-Dicer 347 antibody. Lanes 1 and 2 show 1% of the input cell extracts. Lanes 3 and 4 represent 1% of the flow through (FT) fraction after binding to IgG sepharose. Lane 3 indicates that iTAP-Dicer is efficiently retained on the IgG sepharose, since it is absent from the flow through. ITAP Dicer could not be detected in the eluate after TEV cleavage (lane 5). Lanes 7 and 8 show 50% aliquots of the proteins eluted from Calmodulin (CM) beads. Lanes 9 and 10 show the material not eluted from CM beads. This example illustrates that less than 1% of the input protein can be eluted from CM beads after TAP. Most of the recombinant protein seems to be lost during or after the TEV cleavage step.

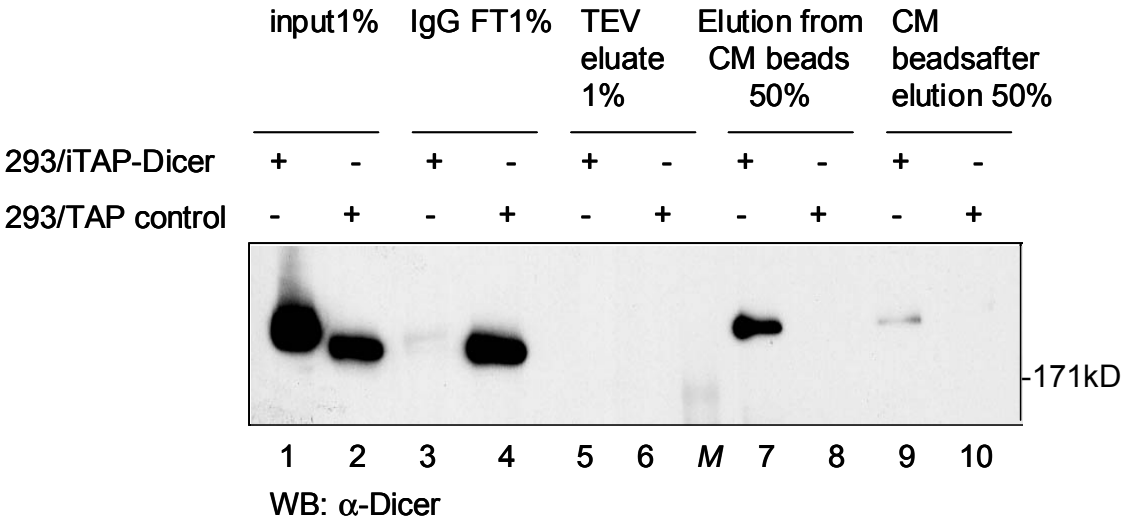


Figure S5. TAP-tagged proteins were purified from 293/iTAP-Dicer and 293/TAP-control cell lines. 1% of the input cell extracts was loaded in lanes 1 and 2. Lanes 3 and 4 represent 1% of the flow through (FT) fraction after binding to IgG sepharose. Lanes 5 and 6 represent a fraction of the TEV eluate after cleavage. Lanes 7 and 8 show iTAP-Dicer eluted from calmodulin (CM) beads. After elution from CM beads, CM beads were incubated with SDS containing Laemmli sample buffer, and the material recovered is shown in lanes 9 and 10. *M* indicates the protein marker.

Purification of monoclonal α-Dicer antibodies and optimization of α-Dicer immunoprecipitations for LC-MSMS analysis. Growth conditions for the hybridoma cells expressing α-Dicer monoclonal antibodies (mAb) and the purification of the antibodies are described in chapter 3.2. It is worth noting that efficient purification of α-Dicer antibodies from the hybridoma supernatants by their ability to bind Protein G was only achieved by growing the hybridoma cells with ultra-low IgG FCS (GIBCO). The use of standard FCS led to the purification of high amounts of bovine IgGs, present in the serum, and did not allow the quantification of the purified α-Dicer antibodies. Purified α-Dicer antibodies were separated by SDS-PAGE and visualized by silver staining according to (Shevchenko et al., 1996) (Fig. 6A). A BSA protein standard was used for quantification of the purified antibodies. The purified α-Dicer antibodies were used to immunoprecipitate endogenous Dicer from P19 (Fig 6B, upper panel) and HEK293 (Fig 6B, lower panel) cytoplasmic extracts. Cell extracts accounting for ~1 mg of total protein were incubated with ~1 μg of purified antibodies, as indicated, for 3 h. The antibody-complexes were captured by incubation with Protein G Sepharose for 1 h. After three washes, the immunoprecipitates were eluted with Laemmli sample buffer and separated by SDS-PAGE. Dicer was detected by western blotting as indicated. Cell lysis and all subsequent steps of the immunoprecipitation were performed in a buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP40, 2 mM $MgCl_2$, 50 mM NaF and complete protease inhibitors (Roche)

The immunoprecipitation procedure was optimized for LC-MSMS analysis considering several parameters: (1) A combination of three monoclonal antibodies (α-Dicer mAb #33/73/83) was used to ensure multiple possible paratope-epitope interactions. (2) The antibodies were cross-linked to Protein G sepharose beads using dimethyl pimelimidate-HCl (DMP) (SIGMA), described below, to enable elution of the immunoprecipitates without co-elution of the antibodies. (3) Immunoprecipitations using the α-Dicer antibodies cross-linked to Protein G sepharose were performed at different buffer conditions, to evaluate efficiency of the immunoprecipitation as a function of stringency of the purification (concerning hydrophilic and hydrophobic interactions.) Therefore, different concentrations of NaCl and NP40 were tested. 20 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$ and protease inhibitors were not changed. Fig. S6C shows one experiment testing salt concentrations ranging from 0.15 to 0.5 M NaCl, as indicated. 0.5% NP40 was used in these experiments. Lanes 1-4, representing α-Dicer immunoprecipitates, show that Dicer is efficiently immunoprecipitates at 0.15 and 0.3 M NaCl. Increasing the NaCl concentration further results in a reduction in Immunoprecipitation efficiency. (4) Before incubation with the immunoprecipitating antibody, the cell extracts were precleared by incubating them with Protein A or G sepharose (or any appropriate matrix used for the immunoprecipitation) for 1 h, which decreased unspecific binding of proteins from the extracts to the beads during immunopurification. All steps of the immunoprecipitation are performed at 4°C and in the presence of protease inhibitors. Fig. S6D shows one representative preparation of α-Dicer and control

immunoprecipitates for 2D-PAGE and consecutive LC-MSMS analysis. α-Dicer antibodies #33, 73 and 83 were pooled in a 1:1:1 molar ratio and cross-linked to Protein G Sepharose beads. Dicer was immunoprecipitated from HEK293 cytoplasmic extracts that were precleared with Protein G sepharose before. Lanes 1, 6 and 7 represent input of the precleared cytoplasmic extract. The immunoprecipitated material was eluted from the beads using a buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, and 1% DTT. The elutions were precipitated according to (Wessel and Flugge, 1984), and were resuspended in 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.3% Pharmalytes 3-10. Precipitation of the eluted protein was performed to decrease co-eluted ions, since isoelectric focusing is very sensitive to ions. 90% of the immunoprecipitates was separated by 2D-PAGE, as described in chapter 3.2., and 10% of the material was analyzed by western blot (lanes 2 and 3). After elution of the precipitated proteins from the beads, the material remaining on the beads was extracts with Laemmli sample buffer and analyzed by western blotting for elution efficiency (lanes 4 and 5).

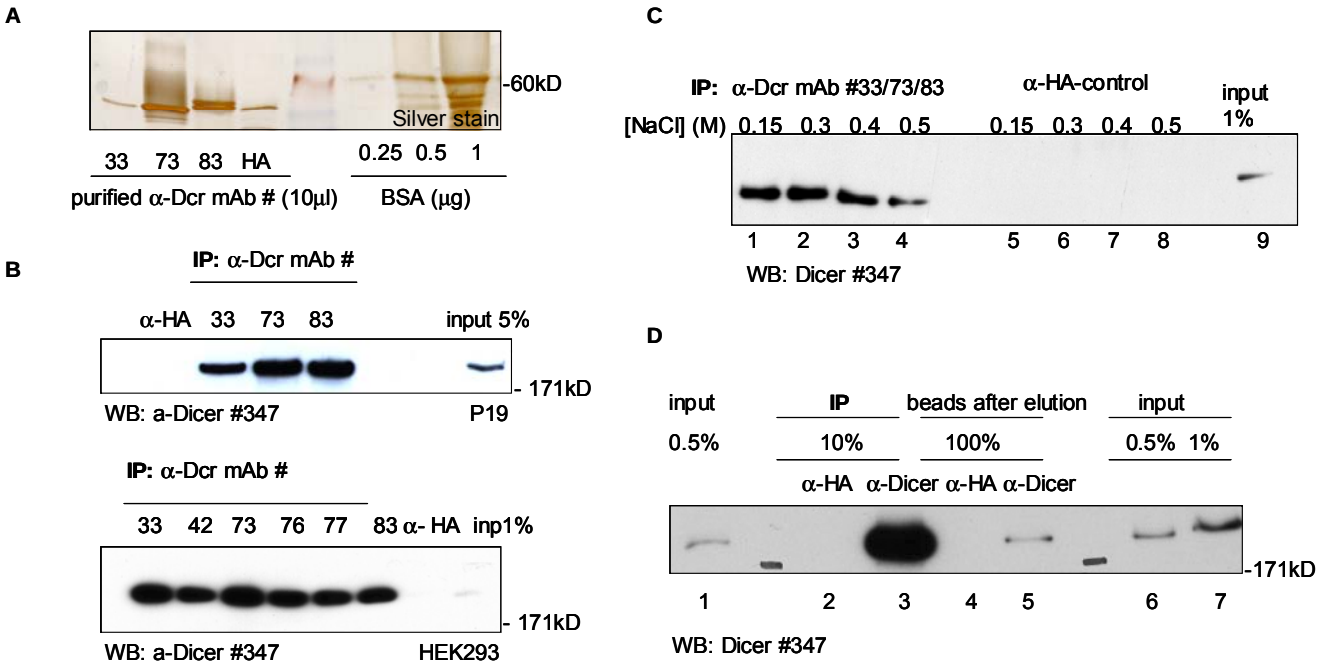


Figure S6. Optimization steps for the analysis of α-Dicer immunoprecipitates by LC-MSMS. (**A**) Monoclonal α-Dicer antibodies were purified from hybridoma supernatants using Protein G sepharose. The purified antibodies were separated by SDS-PAGE and visualized by silver staining. Different concentrations of BSA were used to determine the approximate protein concentrations of the purified antibodies. (**B**) Different α-Dicer antibodies were used to immunoprecipitate Dicer from mouse P19 (upper panel) and human HEK293 (lower panel) cell extracts. An α-HA antibody was used as an isotype control. (**C**) α-Dicer antibodies #33, 73 and 83 were cross-linked to Protein G sepharose and used for immunoprecipitations. Dicer was immunoprecipitated from HEK293 extracts using buffers with different salt concentrations, as indicated. (**D**) Dicer was immunoprecipitated from HEK293 cell extracts. 10% of the immunoprecipitates were analyzed by western blotting for the efficiency of immunoprecipitation and elution of the immunoprecipitates.

Dimethyl pimelimidate-HCl (DMP) cross-link of antibodies to protein A or protein G sepharose. DMP was purchased from SIGMA. The antibodies are bound to the Protein A or G Sepharose in binding buffer (100 mM $NaHPO_4$ pH 8.0) for 1-3 h. After two washes with 50 mM $NaHPO_4$, pH 9.0 and one wash with 200 mM $NaHPO_4$, pH 8.0, cross-linking is performed for 1 h using DMP at a final concentration of 5.2 mg/ml in 200 mM $NaHPO_4$ pH 9.0. (The pH of the cross-linking step is critical and has to be carefully readjusted after addition of the freshly prepared DMP solution.) The cross-linking reaction is stopped by 2 short washed with 0.2 M ethanolamine, pH 8.0 followed by 1h incubation in the same buffer. The cross-linked antibody-beads are further washed with PBS. One very brief wash with 20 mM glycine pH 2.3-2.5 ensures the removal of non-cross linked antibodies. The cross-linked material can be stored in PBS with 0.02% NaN_3 at 4°C for up to two weeks. Before use for immunoprecipitations, the antibody-beads are washed with PBS and equilibrated in the immunoprecipitation buffer. All steps in this procedure are performed on a rotating wheel at room temperature.

α-TRBP antibodies and immunoprecipitations. The α-TRBP antibodies were generated by EUROGENTEC. Peptides for the immunization of rabbits were chosen according to their presence in all TRBP splicing variants, their specificity for TRBP to avoid cross-reactivity with other dsRBD containing proteins, and their immunogenic potency. The peptide #1 melqppvspqqsec and the peptide #2

gsrldglrnrgpgc were coupled through the C-terminal cysteine residues for immunization. Both peptides were injected together into two rabbits.

α-TRPB antibodies were purified from rabbit serum by affinity purification using their cognate peptide cross-linked to Sepharose beads. After elution of the antibodies from the peptide-columns using 20 mM glycine, pH 2.3, the pH was immediately re-adjusted to pH 7.5. The eluates were concentrated using Centricon tubes (Millipore) with a cut off of 10 kD. Concentrated antibodies are stored in PBS with 50% glycerol at –20°C.

For western blotting, α-TRBP Ab#1 is used at a 1:1000 to 1:5000 dilution, α-TRBP Ab#2 is used at a 1:1000 dilution in PBS containing 5% BSA, 0.1% Tween20 and 0.02% NaN_3. For immunofluorescence, α-TRBP Ab#1 is used at 1:300 to 1:500 dilution. Immunoprecipitations using α-TRBP antibodies were performed in a way similar to α-Dicer immunoprecipitates. HeLa cytoplasmic extracts served as a source of material in this experiment. For immunoprecipitations, the purified α-TRBP antibodies or control antibodies were cross-linked to Protein A Sepharose (Amersham Biosciences, Little Chalfont, UK) using DMP (as described for the cross-link of α-Dicer antibodies described above). HeLa cells were lysed in immunoprecipitation buffer (20 mM Tris–HCl, pH 7.5, 300 mM NaCl, 0.5% NP-40, 2.5 mM MgCl2), and centrifuged two times for 10 min at 10,000xg. The supernatants, corresponding to the soluble cytoplasmic fraction, were pre-cleared with protein A sepharose for one h. Antibodies cross-linked to beads were incubated with the pre-cleared extracts for three h. After five washes with immunoprecipitation buffer, the

immunoprecipitates were separated by SDS-PAGE and visualized using Coomassie-blue staining (Invitrogen). Protein containing gel fragments, were digested with trypsin according to Schrimpf et al. (2001) and analyzed by LC-MSMS (LCQ Deca XP, Thermo 7 Finnigan). Proteins were identified using Turbo Sequest and MASCOT, searching SwissProt database restricted to human proteins. Alternatively, immunoprecipitates were analyzed by western blotting.

Denaturating His_6-Myc-Ubiquitin-pull downs. His_6-Myc-Ubiquitin and FLAG-TRBP2 were transfected into HeLa or HEK293 cells using Lipofectamine 2000 (Invitrogen). 48 h after transfection the cell were lysed in lysis buffer [50 mM Na_2PO4, 8 M Urea, 300 mM NaCl, 0.5% Triton-X 100) at pH 8.0 supplemented with protease inhibitors (complete, Roche)]. The lysates were sonicated for three times 30 sec and unsoluble material was pelleted by centrifugation at 13,000xg for 20 min. The supernatants were incubated with pre-equilibrated NiNTA agarose (Invitrogen) and rotated for 2 h. After binding, two washes in lysis buffer followed by 3 washes in wash buffer (50 mM Na_2PO4, 8 M Urea, 300 mM NaCl, 0.5% Triton-X 100, 20 mM imidazole, pH 6.5) were performed. The precipitated proteins were eluted in elution buffer at pH 6.5 (50 mM Na_2PO4, 8 M Urea, 300 mM NaCl, 0.5% Triton-X 100, 200 mM imidazole, 2% SDS). All steps were performed at room temperature. The eluted material was separated by SDS PAGE and analyzed by western blotting.

Preparation of RNA substrates and analysis of Dicer, TRBP, and Dicer/TRBP RNP formation by native PAGE. The internally ^{32}P-labeled 30bp+2 dsRNA and the pre-let7 RNA were prepared as described before (Kolb et al., 2005; Zhang et al., 2002; Zhang et al., 2004). The RNAs were synthesized by the T7 polymerase in vitro transcription, using the Ambion T7 MaxiScript transcription kit and [α-^{32}P]UTP. After transcription, RNA was purified by denaturing 8% PAGE. The 5'-end-labeling of siRNA, using T4 polynucleotide kinase and either [γ-^{32}P]ATP or cold ATP, was carried out as described by Sambrook et al., (1989). Complementary RNA strands were annealed at 95 °C for 3 min in 20 mM NaCl, transferred to 75 °C, and then slowly cooled down to 20 °C.

10-μl reactions containing ~500 μMoles of each protein, and 5 fMoles of ^{32}P-labeled RNA were incubated for 90 min at 4°C in buffer containing 30 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 150 mM NaCl, 0.2% NP-40 and 10% glycerol. Complexes were analyzed on 4% native polyacrylamide gels with acrylamide/bis-acrylamide ratio on 19:1. Gels were electrophorezed in 1xTBE buffer at 4 °C, dried and quantified by PhosphoImager.

Immunoflourescence analysis. HeLa cells were transfected with the indicated plasmids using Lipofectamine 2000. 24 h after transfection, the cells were splitted onto cover slips. 48 h after transfection, the cells were washed with PBS and fixed using 2% paraformaldehyde (PFA) and 0,05% Tween20 in PBS pH 7.5 for 5 min at room temperature (RT), followed by a 100% methanol shock at –20°C for one minute. After blocking with 10% horse serum in PBS supplemented with

0.05% Tween20 for 10 min at RT, cells were incubated with the first antibody in the blocking solution for one h at RT. The α-TRBP Ab#1 was used in a 1: 500 dilution, and α-Myc 9E10 antibodies were diluted 1: 10. After three washes in wash buffer (PBS supplemented with 2.5% horse serum and 0.05% Tween20), secondary antibodies coupled to flourochroms as indicated were diluted 1:500 in blocking buffer, and binding was performed at RT for 1 h in darkness. After three washing steps with wash buffer, the cells were mounted using Prolong Gold (Invitrogen) supplemented with DAPI. Cells were analyzed at a Widefield SIMPLE Axiovert 200 microscope using the Metamorph software.

Cloning of FLAG-TRBP2 and FLAG-Dicer, and HA-Ro52 and His_6-Myc-Ubiquitin. FLAG-TRBP was cloned by restriction digestion of p-TRExDest30-HA-TRBP2 (described in chapter 4) with SalI and NotI. The resulting fragment containing TRBP2 cds was gel-purified and ligated into SalI and NotI sites of pCIneo-FLAG, generated by Michael Doyle in our laboratory. FLAG-Dicer was kindly provided by Michael Doyle.

His_6-Myc-Ubiquitin was a kind gift of D. Everett. HA-Ro52 in described by Sabile et al., (2006).

6. Appendix

6.1. Analysis of α-Dicer immunoprecipitates by LC-MSMS: dataset of two experiments

Accession	HEK293 control IP	HEK293 Dicer CoIP	HeLa control IP	HeLa Dicer CoIP	Uniprot	Description
DICER_HUMAN	0	8	0	22	Q9UPY3	Endoribonuclease Dicer (EC 3.1.26.-) (Helicase with RNase motif) (Helicase-MOI),
TRBP2_HUMAN	0	4	0	2	Q15633	TAR RNA-binding protein 2 (Trans-activation responsive RNA-binding protein)
ROA2_HUMAN	3	4	0	0	P22626	Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2 / hnRNP B1)
GRP75_HUMAN	1	2	1	2	P38646	Stress-70 protein, mitochondrial precursor (75 kDa glucose regulated protein) (GRP 75) (Pe
TBB2_HUMAN	0	2	1	1	P07437	Tubulin beta-2 chain (OK/SW-cl.56),
RPOM_HUMAN	0	2	0	0	O00411	DNA-directed RNA polymerase, mitochondrial precursor (EC 2.7.7.6) (MtRPOL),
Q6SPF2_HUMAN	1	2	0	0	Q6SPF2	CArG binding factor
HNRPC_HUMAN	1	2	0	0	P07910	Heterogeneous nuclear ribonucleoproteins C1/C2 (hnRNP C1 / hnRNP C2)
HNRPD_HUMAN	2	2	0	0	Q14103	Heterogeneous nuclear ribonucleoprotein D0 (hnRNP D0) (AU-rich element RNA-binding protein,
HNRH3_HUMAN	3	2	0	0	P31942	Heterogeneous nuclear ribonucleoprotein H3 (hnRNP H3) (hnRNP 2H9),
EF1A1_HUMAN	0	1	1	3	P68104	Elongation factor 1-alpha 1 (EF-1-alpha-1) (Elongation factor 1 A-1) (eEF1A-1) (Elongation,
RO52_HUMAN	0	1	0	2	P19474	52 kDa Ro protein (Sjogren syndrome type A antigen) (SS-A) (Ro(SS-A)) (52 kDa ribonucleopr
HSP71_HUMAN	0	1	2	2	P08107	Heat shock 70 kDa protein 1 (HSP70.1) (HSP70-1/HSP70-2),
HSP7C_HUMAN	1	1	1	2	P11142	Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8)
TBA6_HUMAN	0	1	1	1	Q9BQE3	Tubulin alpha-6 chain (Alpha-tubulin 6),
GRP78_HUMAN	1	1	0	1	P11021	78 kDa glucose-regulated protein precursor (GRP 78) (Immunoglobulin heavy chain binding pr
TBBX_HUMAN	1	1	0	1	P68371	Tubulin beta-? chain (Tubulin beta-2 chain),

RS4X_HUMAN	0	1	0	0	P62701	40S ribosomal protein S4, X isoform (Single copy abundant mRNA protein) (SCR10),
RLA0_HUMAN	0	1	0	0	P05388	60S acidic ribosomal protein P0 (L10E)
RL7_HUMAN	0	1	0	0	P18124	60S ribosomal protein L7
Q6ICD6_HUMAN	0	1	0	0	Q6ICD6	DDX17 protein,(Q92841) Probable RNA-dependent helicase p72 (DEAD-box protein p72) (DEAD-box protein 17)
ELAV1_HUMAN	0	1	0	0	Q15717	ELAV-like protein 1 (Hu-antigen R) (HuR),
HNRH2_HUMAN	0	1	0	0	P55795	Heterogeneous nuclear ribonucleoprotein H
HNRPQ_HUMAN	0	1	0	0	O60506	Heterogeneous nuclear ribonucleoprotein Q (hnRNP Q) (hnRNP-Q) (Synaptotagmin binding, cyto
Q5RI17_HUMAN	0	1	0	0	Q5RI17	Heterogeneous nuclear ribonucleoprotein U (Scaffold attachment factor A) (Fragment),
Q8NCA5_HUMAN	0	1	0	0	Q8NCA5	Hypothetical protein FLJ90386,
Q5SQ64_HUMAN	0	1	0	0	Q5SQ64	Lymphocyte antigen 6 complex, locus G6D,
DDX3X_HUMAN	1	1	0	0	O00571	DEAD-box protein 3, X-chromosomal (Helicase-like protein 2) (HLP2) (DEAD-box, X isoform)
ROA3_HUMAN	1	1	0	0	P51991	Heterogeneous nuclear ribonucleoprotein A3 (hnRNP A3)
HNRPU_HUMAN	1	1	0	0	Q00839	Heterogenous nuclear ribonucleoprotein U (hnRNP U) (Scaffold attachment factor A) (SAF-A)
ILF2_HUMAN	1	1	0	0	Q12905	Interleukin enhancer-binding factor 2 (Nuclear factor of activated T-cells 45 kDa) (PRO306
ILF3_HUMAN	1	1	0	0	Q12906	Interleukin enhancer-binding factor 3 (Nuclear factor of activated T-cells 90 kDa) (NF-AT-,
PABP1_HUMAN	1	1	0	0	P11940	Polyadenylate-binding protein 1 (Poly(A)-binding protein 1) (PABP 1)
DDX5_HUMAN	1	1	0	0	P17844	Probable RNA-dependent helicase p68 (DEAD-box protein p68) (DEAD-box protein 5)
FUS_HUMAN	4	1	0	0	P35637	RNA-binding protein FUS (Oncogene FUS) (Oncogene TLS) (Translocated in liposarcoma protein,

I2C2_HUMAN	0	0	0	10	Q9UKV8	Eukaryotic translation initiation factor 2C 2 (eIF2C 2) (eIF-2C 2),
Q9BRY2_HUMAN	0	0	0	4	Q9BRY2	Tar (HIV-1) RNA binding protein 2 (TAR (HIV) RNA binding protein 2)
ACTB_HUMAN	0	0	1	2	P60709	Actin, cytoplasmic 1 (Beta-actin)
I2C3_HUMAN	0	0	0	2	Q9H9G7	Eukaryotic translation initiation factor 2C 3 (eIF2C 3) (eIF-2C 3) (Argonaute 3)
HS90A_HUMAN	0	0	0	2	P07900	Heat shock protein HSP 90-alpha (HSP 86),
Q5CAQ7_HUMAN	0	0	0	2	Q5CAQ7	Heat shock protein HSP 90-alpha 2
HS90B_HUMAN	0	0	1	2	P08238	Heat shock protein HSP 90-beta (HSP 84) (HSP 90),
Q53G55_HUMAN	0	0	0	2	Q53G55	TNF receptor-associated protein 1 variant (Fragment)
YBOX1_HUMAN	1	0	0	2	P67809	Nuclease sensitive element binding protein 1 (Y-box binding protein-1) (Y-box transcriptio,
Q96HG5_HUMAN	0	0	0	1	Q96HG5	Actin, beta (Fragment)
ENOA_HUMAN	0	0	0	1	P06733	Alpha enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (Non-neural enolase) (NNE)
Q27I68_HUMAN	0	0	0	1	Q27I68	Alpha tubulin
PYR1_HUMAN	0	0	0	1	P27708	CAD protein [Includes
EF1A2_HUMAN	0	0	0	1	Q05639	Elongation factor 1-alpha 2 (EF-1-alpha-2) (Elongation factor 1 A-2) (eEF1A-2) (Statin S1)
Q58J86_HUMAN	0	0	0	1	Q58J86	Elongation factor 2
EF2_HUMAN	0	0	0	1	P13639	Elongation factor 2 (EF-2)
Q53FT9_HUMAN	0	0	0	1	Q53FT9	Enolase 1 variant (Fragment)
I2C1_HUMAN	0	0	0	1	Q9UL18	Eukaryotic translation initiation factor 2C 1 (eIF2C 1) (eIF-2C 1) (Putative RNA-binding p
Q5TA58_HUMAN	0	0	0	1	Q5TA58	Eukaryotic translation initiation factor 2C, 1
Q5JV32_HUMAN	0	0	0	1	Q5JV32	Eukaryotic translation initiation factor 2C, 3
Q5VXF0_HUMAN	0	0	0	1	Q5VXF0	Eukaryotic translation initiation factor 2C, 4
Q4LE83_HUMAN	0	0	0	1	Q4LE83	FASN variant protein (Fragment)

Q6P4U5_HUMAN	0	0	0	1	Q6P4U5	Fatty acid synthase
ALDOA_HUMAN	0	0	0	1	P04075	Fructose-bisphosphate aldolase A (EC 4.1.2.13) (Muscle-type aldolase) (Lung cancer antigen
Q53X65_HUMAN	0	0	0	1	Q53X65	GAPD protein (Fragment)
GBLP_HUMAN	0	0	0	1	P63244	Guanine nucleotide-binding protein subunit beta 2-like 1 (Guanine nucleotide-binding prote
HS70L_HUMAN	0	0	0	1	P34931	Heat shock 70 kDa protein 1L (Heat shock 70 kDa protein 1-like) (Heat shock 70 kDa protein
HSP76_HUMAN	0	0	0	1	P17066	Heat shock 70 kDa protein 6 (Heat shock 70 kDa protein B
Q59EJ3_HUMAN	0	0	0	1	Q59EJ3	Heat shock 70kDa protein 1A variant (Fragment)
Q53GZ6_HUMAN	0	0	0	1	Q53GZ6	Heat shock 70kDa protein 8 isoform 1 variant (Fragment)
Q1HB43_HUMAN	0	0	0	1	Q1HB43	Heat shock 70kDa protein 9B (Mortalin-2)
LDHA_HUMAN	0	0	1	1	P00338	L-lactate dehydrogenase A chain (EC 1.1.1.27) (LDH-A) (LDH muscle subunit) (LDH-M),
LDHB_HUMAN	0	0	1	1	P07195	L-lactate dehydrogenase B chain (EC 1.1.1.27) (LDH-B) (LDH heart subunit) (LDH-H)
Q6LC01_HUMAN	0	0	0	1	Q6LC01	MRNA encoding beta-tubulin. (from clone D-beta-1) (Fragment)
Q9H4U6_HUMAN	0	0	0	1	Q9H4U6	OTTHUMP00000030683
Q5J7W1_HUMAN	0	0	0	1	Q5J7W1	Phosphoglycerate kinase (EC 2.7.2.3)
PGK1_HUMAN	0	0	0	1	P00558	Phosphoglycerate kinase 1 (EC 2.7.2.3) (Primer recognition protein 2) (PRP 2) (OK/SW-cl.11
KPYM_HUMAN	0	0	1	1	P14618	Pyruvate kinase, isozymes M1/M2 (EC 2.7.1.40) (Pyruvate kinase muscle isozyme) (Cytosolic ,
Q53HW2_HUMAN	0	0	0	1	Q53HW2	Ribosomal protein P0 variant (Fragment)
Q6NXQ8_HUMAN	0	0	0	1	Q6NXQ8	Ribosomal protein SA
METK2_HUMAN	0	0	0	1	P31153	S-adenosylmethionine synthetase isoform type-2 (EC 2.5.1.6) (Methionine adenosyltransferas
TKT_HUMAN	0	0	1	1	P29401	Transketolase (EC 2.2.1.1) (TK),

Q53EM5_HUMAN	0	0	0	1	Q53EM5	Transketolase variant (Fragment)
TBA1_HUMAN	0	0	0	1	P68366	Tubulin alpha-1 chain (Alpha-tubulin 1) (Testis-specific alpha-tubulin) (Tubulin H2-alpha)

6.2. Analysis of α-TRBP immunoprecipitates by LC-MSMS: complete dataset

Accession	TRBP Ab#1	TRBP Ab#2	isotype control	Uniprot	Description
UBIQ_HUMAN	9	16	0	P62988	Ubiquitin
Q9BRY2_HUMAN	9	3	0	Q9BRY2	TAR RNA binding protein 2, isoform a (TAR (HIV) RNA binding protein 2)
DICER_HUMAN	9	2	0	Q9UPY3	Endoribonuclease Dicer (EC 3.1.26.-) (Helicase with RNase motif) (Helicase-MOI)
MYH9_HUMAN	4	4	0	P35579	Myosin-9 (Myosin heavy chain, nonmuscle IIa) (Nonmuscle myosin heavy chain IIa) (NMMHC II-
ACTA_HUMAN	3	4	0	P62736	Actin, aortic smooth muscle (Alpha-actin-2)
MYH10_HUMAN	3	4	0	P35580	Myosin-10 (Myosin heavy chain, nonmuscle IIb) (Nonmuscle myosin heavy chain IIb) (NMMHC II
MYL6_HUMAN	3	1	0	P60660	Myosin light polypeptide 6 (Myosin light chain alkali 3) (Myosin light chain 3) (MLC-3) (L
TBAK_HUMAN	3	1	0	P68363	Tubulin alpha-ubiquitous chain (Alpha-tubulin ubiquitous) (Tubulin K-alpha-1)
CH60_HUMAN	3	0	0	P10809	60 kDa heat shock protein, mitochondrial precursor (Hsp60) (60 kDa chaperonin) (CPN60) (He
BIG1_HUMAN	3	0	0	Q9Y6D6	Brefeldin A-inhibited guanine nucleotide-exchange protein 1 (Brefeldin A-inhibited GEP 1)
RSSA_HUMAN	2	1	0	P08865	40S ribosomal protein SA (p40) (34/67 kDa laminin receptor) (Colon carcinoma laminin-bindi
G3P2_HUMAN	2	1	0	P04406	Glyceraldehyde-3-phosphate dehydrogenase, liver (EC 1.2.1.12) (GAPDH)
CA077_HUMAN	2	1	0	Q9Y3Y2	Protein C1orf77
CRTC_HUMAN	2	0	0	P27797	Calreticulin precursor (CRP55) (Calregulin) (HACBP) (ERp60) (grp60)
ALDOA_HUMAN	2	0	0	P04075	Fructose-bisphosphate aldolase A (EC 4.1.2.13) (Muscle-type aldolase) (Lung cancer antigen
Q5T9W7_HUMAN	2	0	0	Q5T9W7	Heat shock 90kDa protein 1, beta
TRAP1_HUMAN	2	0	0	Q12931	Heat shock protein 75 kDa, mitochondrial precursor (HSP 75) (Tumor necrosis factor type 1
Q96L09_HUMAN	2	0	0	Q96L09	LOC137392 protein (Fragment)
PGK1_HUMAN	2	0	0	P00558	Phosphoglycerate kinase 1 (EC 2.7.2.3) (Primer recognition protein 2) (PRP 2)
SET_HUMAN	2	0	0	Q01105	SET protein (Phosphatase 2A inhibitor I2PP2A) (I-2PP2A) (Template activating factor I) (TA
SNX18_HUMAN	2	0	0	Q96RF0	Sorting nexin-18 (Sorting nexin associated Golgi protein 1)
Q5TEM5_HUMAN	1	3	0	Q5TEM5	Myosin VI
DREB_HUMAN	1	2	0	Q16643	Drebrin (Developmentally regulated brain protein)

MYO6_HUMAN	1	2	0	Q9UM54	Myosin-6 (Myosin VI)
PIMT_HUMAN	1	2	0	P22061	Protein-L-isoaspartate(D-aspartate) O-methyltransferase (EC 2.1.1.77) (Protein-beta-aspart
RS19_HUMAN	1	1	0	P39019	40S ribosomal protein S19
RLA0_HUMAN	1	1	0	P05388	60S acidic ribosomal protein P0 (L10E)
ARP2_HUMAN	1	1	0	P61160	Actin-like protein 2 (Actin-related protein 2)
ARPC4_HUMAN	1	1	0	P59998	Actin-related protein 2/3 complex subunit 4 (ARP2/3 complex 20 kDa subunit) (p20-ARC)
GELS_HUMAN	1	1	0	P06396	Gelsolin precursor (Actin-depolymerizing factor) (ADF) (Brevin) (AGEL)
LDHA_HUMAN	1	1	0	P00338	L-lactate dehydrogenase A chain (EC 1.1.1.27) (LDH-A) (LDH muscle subunit) (LDH-M) (Prolif
MDHM_HUMAN	1	1	0	P40926	Malate dehydrogenase, mitochondrial precursor (EC 1.1.1.37)
MYO1D_HUMAN	1	1	0	O94832	Myosin Id
MLRM_HUMAN	1	1	0	P19105	Myosin regulatory light chain 2, nonsarcomeric (Myosin RLC)
PRDX1_HUMAN	1	1	0	Q06830	Peroxiredoxin 1 (EC 1.11.1.15) (Thioredoxin peroxidase 2) (Thioredoxin-dependent peroxide
PRDX2_HUMAN	1	1	0	P32119	Peroxiredoxin 2 (EC 1.11.1.15) (Thioredoxin peroxidase 1) (Thioredoxin-dependent peroxide
PHB2_HUMAN	1	1	0	Q99623	Prohibitin-2 (B-cell receptor-associated protein BAP37) (Repressor of estrogen receptor ac
SFPQ_HUMAN	1	1	0	P23246	Splicing factor, proline-and glutamine-rich (Polypyrimidine tract-binding protein-associat
CH10_HUMAN	1	0	0	P61604	10 kDa heat shock protein, mitochondrial (Hsp10) (10 kDa chaperonin) (CPN10) (Early-pregna
1433B_HUMAN	1	0	0	P31946	14-3-3 protein beta/alpha (Protein kinase C inhibitor protein 1) (KCIP-1) (Protein 1054)
1433E_HUMAN	1	0	0	P62258	14-3-3 protein epsilon (14-3-3E)
1433G_HUMAN	1	0	0	P61981	14-3-3 protein gamma (Protein kinase C inhibitor protein 1) (KCIP-1)
1433Z_HUMAN	1	0	0	P63104	14-3-3 protein zeta/delta (Protein kinase C inhibitor protein 1) (KCIP-1)
RS2_HUMAN	1	0	0	P15880	40S ribosomal protein S2 (S4) (LLRep3 protein)
RS25_HUMAN	1	0	0	P62851	40S ribosomal protein S25
RS3_HUMAN	1	0	0	P23396	40S ribosomal protein S3
RO52_HUMAN	1	0	0	P19474	52 kDa Ro protein (Sjogren syndrome type A antigen) (SS-A) (Ro(SS-A)) (52 kDa ribonucleopr
RL22_HUMAN	1	0	0	P35268	60S ribosomal protein L22 (Epstein-Barr virus small RNA associated protein) (EBER associat
GRP78_HUMAN	1	0	0	P11021	78 kDa glucose-regulated protein precursor (GRP 78) (Immunoglobulin heavy chain

					binding pr
AN32A_HUMAN	1	0	0	P39687	Acidic leucine-rich nuclear phosphoprotein 32 family member A (Potent heat-stable protein
ARP3_HUMAN	1	0	0	P61158	Actin-like protein 3 (Actin-related protein 3)
ACL6A_HUMAN	1	0	0	O96019	Actin-like protein 6A (53 kDa BRG1-associated factor A) (Actin-related protein Baf53a) (Ar
APT_HUMAN	1	0	0	P07741	Adenine phosphoribosyltransferase (EC 2.4.2.7) (APRT)
ARF1_HUMAN	1	0	0	P84077	ADP-ribosylation factor 1
ANXA5_HUMAN	1	0	0	P08758	Annexin A5 (Annexin V) (Lipocortin V) (Endonexin II) (Calphobindin I) (CBP-I) (Placental a
ATPA_HUMAN	1	0	0	P25705	ATP synthase alpha chain, mitochondrial precursor (EC 3.6.3.14)
ATPB_HUMAN	1	0	0	P06576	ATP synthase beta chain, mitochondrial precursor (EC 3.6.3.14)
KU70_HUMAN	1	0	0	P12956	ATP-dependent DNA helicase II, 70 kDa subunit (Lupus Ku autoantigen protein p70) (Ku70) (7
DDX39_HUMAN	1	0	0	O00148	ATP-dependent helicase DDX39 (DEAD-box protein 39) (Nuclear RNA helicase URH49)
DHX9_HUMAN	1	0	0	Q08211	ATP-dependent RNA helicase A (Nuclear DNA helicase II) (NDH II) (DEAH-box protein 9)
PUR9_HUMAN	1	0	0	P31939	Bifunctional purine biosynthesis protein PURH [Includes: Phosphoribosylaminoimidazolecarbo
CAH2_HUMAN	1	0	0	P00918	Carbonic anhydrase 2 (EC 4.2.1.1) (Carbonic anhydrase II) (Carbonate dehydratase II) (CA-I
CLH1_HUMAN	1	0	0	Q00610	Clathrin heavy chain 1 (CLH-17)
CPSF1_HUMAN	1	0	0	Q10570	Cleavage and polyadenylation specificity factor, 160 kDa subunit (CPSF 160 kDa subunit)
COF1_HUMAN	1	0	0	P23528	Cofilin-1 (Cofilin, non-muscle isoform) (18 kDa phosphoprotein) (p18)
KCRB_HUMAN	1	0	0	P12277	Creatine kinase B-type (EC 2.7.3.2) (Creatine kinase, B chain) (B-CK)
SERA_HUMAN	1	0	0	O43175	D-3-phosphoglycerate dehydrogenase (EC 1.1.1.95) (3-PGDH)
DCD_HUMAN	1	0	0	P81605	Dermcidin precursor (Preproteolysin) [Contains: Survival-promoting peptide; DCD-1]
DBPA_HUMAN	1	0	0	P16989	DNA-binding protein A (Cold shock domain protein A) (Single-strand DNA binding protein NF-
RPB8_HUMAN	1	0	0	P52434	DNA-directed RNA polymerases I, II, and III 17.1 kDa polypeptide (EC 2.7.7.6) (RPB17) (RPB

RIB2_HUMAN	1	0	0	P04844	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 63 kDa subunit precursor (E
RIB1_HUMAN	1	0	0	P04843	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 67 kDa subunit precursor (E
EEA1_HUMAN	1	0	0	Q15075	Early endosome antigen 1 (Endosome-associated protein p162) (Zinc finger FYVE domain-conta
ETFB_HUMAN	1	0	0	P38117	Electron transfer flavoprotein beta-subunit (Beta-ETF)
EF1A1_HUMAN	1	0	0	P68104	Elongation factor 1-alpha 1 (EF-1-alpha-1) (Elongation factor 1 A-1) (eEF1A-1) (Elongation
EF2_HUMAN	1	0	0	P13639	Elongation factor 2 (EF-2)
ENPL_HUMAN	1	0	0	P14625	Endoplasmin precursor (94 kDa glucose-regulated protein) (GRP94) (gp96 homolog) (Tumor rej
IF4A1_HUMAN	1	0	0	P60842	Eukaryotic initiation factor 4A-I (eIF4A-I) (eIF-4A-I)
IF4A2_HUMAN	1	0	0	Q14240	Eukaryotic initiation factor 4A-II (eIF4A-II) (eIF-4A-II)
IF32_HUMAN	1	0	0	Q13347	Eukaryotic translation initiation factor 3 subunit 2 (eIF-3 beta) (eIF3 p36) (eIF3i) (TGF-
IF5A_HUMAN	1	0	0	P63241	Eukaryotic translation initiation factor 5A (eIF-5A) (eIF-4D) (Rev-binding factor)
FOXD4_HUMAN	1	0	0	Q12950	Forkhead box protein D4 (Forkhead-related protein FKHL9) (Forkhead-related transcription f
GLU2B_HUMAN	1	0	0	P14314	Glucosidase II beta subunit precursor (Protein kinase C substrate, 60.1 kDa protein, heavy
GSTP1_HUMAN	1	0	0	P09211	Glutathione S-transferase P (EC 2.5.1.18) (GST class-pi) (GSTP1-1)
RAN_HUMAN	1	0	0	P62826	GTP-binding nuclear protein Ran (GTPase Ran) (Ras-like protein TC4) (Androgen receptor-ass
Q6GU03_HUMAN	1	0	0	Q6GU03	Heat shock 70kDa protein 9B,
HNRPF_HUMAN	1	0	0	P52597	Heterogeneous nuclear ribonucleoprotein F (hnRNP F) (Nucleolin-like protein mcs94-1)
HNRPC_HUMAN	1	0	0	P07910	Heterogeneous nuclear ribonucleoproteins C1/C2 (hnRNP C1 / hnRNP C2)
HORN_HUMAN	1	0	0	Q86YZ3	Hornerin
Q6PAU7_HUMAN	1	0	0	Q6PAU7	Hypothetical protein
Q9H797_HUMAN	1	0	0	Q9H797	Hypothetical protein FLJ21128
Q8NAT1_HUMAN	1	0	0	Q8NAT1	Hypothetical protein FLJ34828 (Glycosyltransferase)
IMB1_HUMAN	1	0	0	Q14974	Importin beta-1 subunit (Karyopherin beta-1 subunit) (Nuclear factor P97) (Importin 90)
ILF2_HUMAN	1	0	0	Q12905	Interleukin enhancer-binding factor 2 (Nuclear factor of activated T-cells 45 kDa)
Q9ULD5_HUMAN	1	0	0	Q9ULD5	KIAA1285 protein (Fragment)

LDHB_HUMAN	1	0	0	P07195	L-lactate dehydrogenase B chain (EC 1.1.1.27) (LDH-B) (LDH heart subunit) (LDH-H)
MAGM_HUMAN	1	0	0	Q9Y3D7	Mitochondria-associated granulocyte macrophage CSF signaling molecule, mitochondrial precu
DCP1A_HUMAN	1	0	0	Q9NPI6	mRNA decapping enzyme 1A (EC 3.-.-.-) (Transcription factor SMIF) (Smad4-interacting trans
NACA_HUMAN	1	0	0	Q13765	Nascent polypeptide-associated complex alpha subunit (NAC-alpha) (Alpha-NAC) (Hom s 2.02)
NUDC_HUMAN	1	0	0	Q9Y266	Nuclear migration protein nudC (Nuclear distribution protein C homolog)
HCC1_HUMAN	1	0	0	P82979	Nuclear protein Hcc-1 (Proliferation associated cytokine-inducible protein CIP29)
NPM_HUMAN	1	0	0	P06748	Nucleophosmin (NPM) (Nucleolar phosphoprotein B23) (Numatrin) (Nucleolar protein NO38)
NDKA_HUMAN	1	0	0	P15531	Nucleoside diphosphate kinase A (EC 2.7.4.6) (NDK A) (NDP kinase A) (Tumor metastatic proc
NDKB_HUMAN	1	0	0	P22392	Nucleoside diphosphate kinase B (EC 2.7.4.6) (NDK B) (NDP kinase B) (nm23-H2) (C-myc purin
PPIA_HUMAN	1	0	0	P62937	Peptidyl-prolyl cis-trans isomerase A (EC 5.2.1.8) (PPIase) (Rotamase) (Cyclophilin A) (Cy
DHB4_HUMAN	1	0	0	P51659	Peroxisomal multifunctional enzyme type 2 (MFE-2) (D-bifunctional protein) (DBP) (17-beta-
SMCA4_HUMAN	1	0	0	P51532	Possible global transcription activator SNF2L4 (SNF2-beta) (BRG-1 protein) (Mitotic growth
PROF1_HUMAN	1	0	0	P07737	Profilin-1 (Profilin I)
PHB_HUMAN	1	0	0	P35232	Prohibitin
PA2G4_HUMAN	1	0	0	Q9UQ80	Proliferation-associated protein 2G4 (Cell cycle protein p38-2G4 homolog) (hG4-1)
ANM1_HUMAN	1	0	0	Q99873	Protein arginine N-methyltransferase 1 (EC 2.1.1.-) (Interferon receptor 1-bound protein 4
SKB1_HUMAN	1	0	0	O14744	Protein arginine N-methyltransferase 5 (EC 2.1.1.-) (Shk1 kinase-binding protein 1 homolog
PDIA1_HUMAN	1	0	0	P07237	Protein disulfide-isomerase precursor (EC 5.3.4.1) (PDI) (Prolyl 4-hydroxylase beta subuni
PTD4_HUMAN	1	0	0	Q9NTK5	Putative GTP-binding protein PTD004
KPYM_HUMAN	1	0	0	P14618	Pyruvate kinase, isozymes M1/M2 (EC 2.7.1.40) (Pyruvate kinase muscle isozyme) (Cytosolic
REPS1_HUMAN	1	0	0	Q96D71	RalBP1 associated Eps domain containing protein 1 (RalBP1-interacting protein 1)
Q5VVD0_HUMAN	1	0	0	Q5VVD0	Ribosomal protein L11

Q5I0X1_HUMAN	1	0	0	Q5I0X1	Ribosomal protein L7
FUS_HUMAN	1	0	0	P35637	RNA-binding protein FUS (Oncogene FUS) (Oncogene TLS) (Translocated in liposarcoma protein
RUVB1_HUMAN	1	0	0	Q9Y265	RuvB-like 1 (EC 3.6.1.-) (49-kDa TATA box-binding protein-interacting protein) (49 kDa TBP
RUVB2_HUMAN	1	0	0	Q9Y230	RuvB-like 2 (EC 3.6.1.-) (48-kDa TATA box-binding protein-interacting protein) (48-kDa TBP
Q9HBD4_HUMAN	1	0	0	Q9HBD4	SMARCA4 isoform 2
U2AF1_HUMAN	1	0	0	Q01081	Splicing factor U2AF 35 kDa subunit (U2 auxiliary factor 35 kDa subunit) (U2 snRNP auxilia
Q6NSL3_HUMAN	1	0	0	Q6NSL3	SRRM2 protein (Fragment)
STMN1_HUMAN	1	0	0	P16949	Stathmin (Phosphoprotein p19) (pp19) (Oncoprotein 18) (Op18) (Leukemia-associated phosphop
Q9UHD2_HUMAN	1	0	0	Q9UHD2	TANK binding kinase TBK1 (NF-kB-activating kinase NAK)
TBCD1_HUMAN	1	0	0	Q86TI0	TBC1 domain family member 1
TCPA_HUMAN	1	0	0	P17987	T-complex protein 1, alpha subunit (TCP-1-alpha) (CCT-alpha)
TCPD_HUMAN	1	0	0	P50991	T-complex protein 1, delta subunit (TCP-1-delta) (CCT-delta) (Stimulator of TAR RNA bindin
TCPE_HUMAN	1	0	0	P48643	T-complex protein 1, epsilon subunit (TCP-1-epsilon) (CCT-epsilon)
TCPQ_HUMAN	1	0	0	P50990	T-complex protein 1, theta subunit (TCP-1-theta) (CCT-theta)
TCPZ_HUMAN	1	0	0	P40227	T-complex protein 1, zeta subunit (TCP-1-zeta) (CCT-zeta) (CCT-zeta-1) (Tcp20) (HTR3) (Acu
THIO_HUMAN	1	0	0	P10599	Thioredoxin (ATL-derived factor) (ADF) (Surface associated sulphydryl protein) (SASP)
PRDX3_HUMAN	1	0	0	P30048	Thioredoxin-dependent peroxide reductase, mitochondrial precursor (EC 1.11.1.15) (Peroxire
Q9UHR5_HUMAN	1	0	0	Q9UHR5	Transcriptional regulator protein HCNGP (Transcription-regulating protein)
TKT_HUMAN	1	0	0	P29401	Transketolase (EC 2.2.1.1) (TK)
TPM1_HUMAN	1	0	0	P09493	Tropomyosin 1 alpha chain (Alpha-tropomyosin)
Q7Z6L8_HUMAN	1	0	0	Q7Z6L8	Tropomyosin 1 alpha chain, isoform 6
TBBX_HUMAN	1	0	0	P68371	Tubulin beta-? chain (Tubulin beta-2 chain)
TBB2_HUMAN	1	0	0	P07437	Tubulin beta-2 chain
Q53XZ5_HUMAN	1	0	0	Q53XZ5	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, epsilon polypeptid
VATA1_HUMAN	1	0	0	P38606	Vacuolar ATP synthase catalytic subunit A, ubiquitous isoform (EC 3.6.3.14) (V-ATPase A su

VIME_HUMAN	1	0	0	P08670	Vimentin
VDAC2_HUMAN	1	0	0	P45880	Voltage-dependent anion-selective channel protein 2 (VDAC-2) (hVDAC2) (Outer mitochondrial
GRP75_HUMAN	0	3	0	P38646	Stress-70 protein, mitochondrial precursor (75 kDa glucose regulated protein) (GRP 75) (Pe
DESP_HUMAN	0	2	0	P15924	Desmoplakin (DP) (250/210 kDa paraneoplastic pemphigus antigen)
Q6PIH6_HUMAN	0	2	0	Q6PIH6	IGKV1-5 protein
MLEY_HUMAN	0	2	0	P14649	Myosin light chain 1, slow-twitch muscle A isoform (MLC1sa) (Alkali)
Q15657_HUMAN	0	2	0	Q15657	Tropomyosin isoform
RS4X_HUMAN	0	1	0	P62701	40S ribosomal protein S4, X isoform (Single copy abundant mRNA protein) (SCR10)
RL11_HUMAN	0	1	0	P62913	60S ribosomal protein L11 (CLL-associated antigen KW-12)
RL27A_HUMAN	0	1	0	P46776	60S ribosomal protein L27a
ARPC2_HUMAN	0	1	0	O15144	Actin-related protein 2/3 complex subunit 2 (ARP2/3 complex 34 kDa subunit) (p34-ARC)
ARPC3_HUMAN	0	1	0	O15145	Actin-related protein 2/3 complex subunit 3 (ARP2/3 complex 21 kDa subunit) (p21-ARC)
EPLIN_HUMAN	0	1	0	Q9UHB6	Epithelial protein lost in neoplasm
Q59FE8_HUMAN	0	1	0	Q59FE8	Epithelial protein lost in neoplasm beta variant (Fragment)
LACRT_HUMAN	0	1	0	Q9GZZ8	Extracellular glycoprotein lacritin precursor
CAZA1_HUMAN	0	1	0	P52907	F-actin capping protein alpha-1 subunit (CapZ alpha-1)
NOLA1_HUMAN	0	1	0	Q9NY12	H/ACA ribonucleoprotein complex subunit 1 (Nucleolar protein family A member 1) (snoRNP pr
HSP76_HUMAN	0	1	0	P17066	Heat shock 70 kDa protein 6 (Heat shock 70 kDa protein B
HS90B_HUMAN	0	1	0	P08238	Heat shock protein HSP 90-beta (HSP 84) (HSP 90)
HNRPD_HUMAN	0	1	0	Q14103	Heterogeneous nuclear ribonucleoprotein D0 (hnRNP D0) (AU-rich element RNA-binding protein
H2AA_HUMAN	0	1	0	P28001	Histone H2A.a (H2A/a) (H2A.2)
IGHA1_HUMAN	0	1	0	P01876	Ig alpha-1 chain C region
KV3B_HUMAN	0	1	0	P01620	Ig kappa chain V-III region SIE
PLAK_HUMAN	0	1	0	P14923	Junction plakoglobin (Desmoplakin III)
TRFL_HUMAN	0	1	0	P02788	Lactotransferrin precursor (Lactoferrin) [Contains: Kaliocin-1; Lactoferroxin A; Lactoferr
LYSC_HUMAN	0	1	0	P61626	Lysozyme C precursor (EC 3.2.1.17) (1,4-beta-N-acetylmuramidase C)
Q4LE45_HUMAN	0	1	0	Q4LE45	MYH10 variant protein (Fragment)
MYO1C_HUMAN	0	1	0	O00159	Myosin Ic (Myosin I beta) (MMI-beta) (MMIb)
Q149N3_HUMAN	0	1	0	Q149N3	Myosin, heavy polypeptide 10, non-muscle
Q149N4_HUMAN	0	1	0	Q149N4	Myosin, heavy polypeptide 10, non-muscle

MY18A_HUMAN	0	1	0	Q92614	Myosin-18A (Myosin XVIIIa) (Myosin containing PDZ domain)
Q60FE2_HUMAN	0	1	0	Q60FE2	Non-muscle myosin heavy polypeptide 9
PIGR_HUMAN	0	1	0	P01833	Polymeric-immunoglobulin receptor precursor (Poly-Ig receptor) (PIGR) [Contains: Secretory
SETX_HUMAN	0	1	0	Q7Z333	Probable helicase senataxin (EC 3.6.1.-) (SEN1 homolog)
PIP_HUMAN	0	1	0	P12273	Prolactin-inducible protein precursor (Secretory actin-binding protein) (SABP) (Gross cyst
LUC7L_HUMAN	0	1	0	Q9NQ29	Putative RNA-binding protein Luc7-like 1 (SR+89) (Putative SR protein LUC7B1)
SFXN1_HUMAN	0	1	0	Q9H9B4	Sideroflexin-1 (Tricarboxylate carrier protein) (TCC)
SSRG_HUMAN	0	1	0	Q9UNL2	Translocon-associated protein gamma subunit (TRAP-gamma) (Signal sequence receptor gamma s
TPIS_HUMAN	0	1	0	P60174	Triosephosphate isomerase (EC 5.3.1.1) (TIM) (Triose-phosphate isomerase)
TMOD3_HUMAN	0	1	0	Q9NYL9	Tropomodulin-3 (Ubiquitous tropomodulin) (U-Tmod)
TPM4_HUMAN	0	1	0	P67936	Tropomyosin alpha 4 chain (Tropomyosin 4) (TM30p1)
TPM2_HUMAN	0	1	0	P07951	Tropomyosin beta chain (Tropomyosin 2) (Beta-tropomyosin)
VEGP_HUMAN	0	1	0	P31025	Von Ebner

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8. Curriculum Vitae

Curriculum vitae

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Date of birth: 5th of August 1978; Vienna, Austria
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Education

2002 – present:	**PhD-Studies** at the University of Basel, Switzerland, **Friedrich Miescher Institute for Biomedical Research,** Basel; Doctoral Advisor: **Prof Dr. Witold Filipowicz,** (estimated graduation date: spring 2007)
1996 - 2002:	Medical Studies at the University of Vienna Degree: **Dr. med. univ.** (**MD), November 2002** MD-thesis at the **Institute of Molecular Pathology (IMP) 2001-2002, Vienna, Austria;** Doctoral Advisor: **Prof. Dr. Lukas A. Huber;** "TPA induced sequence 7 (TIS7) represses transcription in a partly histone deacetylase (HDAC) dependent manner"
1988 - 1996:	High school, Bundesgymnasium XIII, Vienna (graduated with honors)

Additional training:

LTK Modul 1: Introduction into laboratory animal science; FMI, Basel
Radiation and Biological Safety; FMI, Basel
Scientific and Technical Writing, Mediwrite, 2005
Advanced English Grammar Course, FMI 2006

Awards and Fellowships

PhD student fellowship, Novartis Research Foundation (2002 – 2007)
Wilhelm Auerswald Award 2002 (best medical thesis in Austria 2002)

Publications:

Astrid D. Haase, Lukasz Jaskiewicz, Haidi Zhang, Sébastien Lainé, Ragna Sack, Anne Gatignol, and Witold Filipowicz: **TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing** (EMBO Reports, 2005) (cited by the *Faculty of 1000*, 2005)

Ilja Vietor, Santhosh K. Vadivelu, Nikolaus Wick, Robert Hoffman, Matt Cotton, Christian Seiser, Irene Fialka, Winfried Wunderlich, Astrid Haase, Gabriala Kornikova, Gerald Bosch, and Lukas A. Huber: **TIS7 interacts with the mammalian SIN3 histone deacetylase complex in epithelial cells** (EMBO J., 2002)

International Scientific Conferences:

Keystone Symposium on small regulatory RNAs (poster), 2007
Cold Spring Harbor Laboratory RNAi meeting (**talk**), 2005
Keystone Symposium on small regulatory RNAs (poster), 2003
IMP Spring Conference 2002: "Epigenetic Reprogramming of the Genome" (poster), 2002

Local and internal meetings:

RNA meeting, Alsass 2006 (poster)
Novartis Epigenetic Meeting (poster), 2004
FMI annual meetings, 2003-2006 (posters)
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