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Characterization of RNAi-defective mutants -- Mut13-2 and Mut20 -- in *Chlamydomonas reinhardtii*

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CHARACTERIZATION OF RNAi DEFECTIVE MUTANTS –MUT13-2 AND MUT 20– IN *CHLAMYDOMONAS REINHARDTII*

By

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CHARACTERIZATION OF RNAi DEFECTIVE MUTANTS – MUT 13-2 & MUT 20 – IN *Chlamydomonas reinhardtii*

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RNA interference (RNAi) is a highly conserved and sequence-specific process for regulation of gene expression. At the heart of the RNAi machinery lie 21-24 nt small non-coding RNA molecules which are able to downregulate the expression of cognate sequences, by causing degradation or translational repression of mRNAs. These non-coding RNAs act via the effector RNA-induced silencing complex (RISC), which includes core proteins such as the Argonautes (AGOs). Two *Chlamydomonas reinhardtii* RNAi-defective mutants, Mut13-2 and Mut20, show deletions of various domains of the Tudor Staphylococcal Nuclease 1 (TSN1) gene. TSN1 has already been recognized as a component of the RNA-induced silencing complex in metazoans. However, the specific function of this protein in the RNAi pathway remains undefined. In this study we report that TSN1 is essential for RNAi in *Chlamydomonas reinhardtii*. Interestingly, the two examined mutants show a widely different profile of endogenous miRNAs. The small RNAs are almost completely depleted in the case of Mut20 but are only slightly reduced in level in Mut13-2. Successful complementation with an epitope-tagged TSN1 and recovery of RNAi-induced phenotypes was achieved in Mut13-2 but not in Mut20. Through immunofluorescence microscopy, the epitope-tagged TSN1 protein was found

to be predominantly cytoplasmic, although some degree of nuclear localization was also observed, which supports its association with the RISC as reported in metazoans. Yet, co-immunoprecipitation assays with the epitope-tagged TSN1 did not show association with AGO3, a core component of the RISC in *Chlamydomonas*. It is tempting to hypothesize that TSN1 may play an accessory role in the assembly of the RISC, perhaps in the loading of small RNAs onto Argonautes. This interpretation would be consistent with the reduced level of small RNAs in the mutant backgrounds and the lack of high affinity interactions with AGO proteins. However, elucidating the exact role of TSN1 will require further investigation.

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LITERATURE REVIEW

A. INTRODUCTION

Ever since its discovery in *Caenorhabditis elegans*, RNA interference (RNAi) has emerged as a major pathway for regulation of gene expression as well as a potent experimental tool (1). It is an evolutionarily conserved and a highly sequence-specific process for inhibition of gene expression, either at the level of transcription, mRNA stability or translation (2). Such potent inhibition of target mRNAs by RNAi has heralded several studies directed towards deciphering gene function via gene silencing. In case of genetic analyses, RNAi has been widely successful by providing a reverse genetics approach to functional genomics questions and in genome sequencing projects of various organisms such as *C. elegans, Drosophila melanogaster* and Humans (3-5). Genome-wide RNAi high-throughput screens (RNAi-HTS) in *Drosophila,* mammalian cells have become the primary method for high-throughput analysis gene function and have led to answering pertinent biological questions in developmental biology, signal transduction, metabolic regulation among others (6,7). Inhibition of a specific mRNA by RNAi has also proven to have valuable potential in pharmacological studies and also from a drug discovery perspective, whereby the effector molecules of RNAi – small interfering RNAs (siRNAs) seem to have an upper hand compared to small molecules and conventional drug therapies (8).

Deciphering the function of various components of the RNAi pathway has been essential towards progressive understanding of this important gene silencing mechanism. Primarily hinted in plants to begin with (9), RNAi is now found to be prevalent in almost every eukaryotic species. Besides affecting mRNA turnover and protein synthesis and stability in general, RNAi and its associated pathways are also known to regulate response to pathogens, transposons and involvement in heterochromatin formation (10,11). Post-Nobel prize winning work by Mello and Fire for characterization of double-stranded RNA (dsRNA) as the trigger for such sequence-specific gene silencing in *C. elegans* (1), cell-free systems from *Drosophila,* human cell lines and wheat germ, have greatly contributed towards our knowledge of the mechanistic details of the RNAi pathway (12-16). One such contribution from these studies is the biochemical identification of the RNA-induced silencing complex (RISC) (14). This multi-protein complex along with different other components acts as the focal point for mRNA targeting. The dsRNA trigger for RNAi is processed by an RNase III-type endoribonuclease known Dicer, into small (18-25 nt) interfering RNAs (siRNAs) (17) or certain 20-24 nt long microRNAs (miRNAs) (18-20). These siRNAs or miRNAs in association with the RISC form a siRISC or miRISC respectively and are key features in post-transcriptional regulation of gene expression in both metazoans and in plants (21). Such small RNAs have been shown to even regulate several critical junctions of life such as developmental timing, hematopoiesis, organogenesis, apoptosis and even tumorigenesis (22).

B. The RNAi mechanism

The RNAi machinery comprises of three major components – RNase III-like Dicer endoribonuclease, a small (guide) RNA and Argonaute protein (21). Hamilton and Baulcombe were first to report the presence of ~25 nt small non-coding RNAs which assist in sequence-specific defense against viral and cellular mRNAs via their sequence complementarity to the target mRNAs (17,23). While the processing of these small non-coding RNAs via Dicer (RNAse III activity) mostly occurs to a certain extent within the nucleus of the cell in most organisms, the assembly of the RISC holo-enzyme and target mRNA cleavage or repression is found to occur in the cytoplasm (20,21). The RNA-induced silencing complex (RISC) has been reported to have a wide range of size distinctions in various studies 160-550 kDa to 80S (16,21,24-28). Such size differences definitely suggest the complexity of the RISC in terms of varying in composition from one organism to the other. In part, such complexity has also hindered a more complete and universal view of the RISC assembly. But among all its various components, a ubiquitous one has been – the Argonaute (AGO) family of proteins with their PAZ and PIWI domains (29).

i. Dicer – the endoribonuclease

Compartmentalization of the canonical RNAi machinery as an upstream (nuclear) or a downstream (cytoplasmic) process has demarcated the silencing mechanism into distinct phases of dsRNA processing and RISC assembly (21,30,31). Even so, there lay differences among animals and plants in terms of the components of RNAi as well as in the different phases of the pathway. But a significant linker among such discrepancies is the requirement of Dicer as part of dsRNA processing as well as RISC function (28,32). Dicer is a class of RNAse III enzymes, which shows specificity for dsRNA (31,33). In addition to having two RNAse III domains, Dicer contains an N-terminal DEAD/DEAH box RNA helicase /ATPase domain, DUF 283 (Domain of Unknown Function), a dsRNA-binding domain (31,33). A number of Dicer homologues also possess a PAZ domain which helps in recognition of the 2 nt 3' overhang of the siRNA duplex and its incorporation into the RISC effector complex (34). PAZ stands for Piwi/Argonaute/Zwille, which are the three proteins, found to have this domain in common (35,36). The two RNAse III domains of dicer are dimerized with the help of PAZ and the dsRNA-binding domain to form a processing center which helps in the formation of products with 2 nt 3' overhangs (36). Showing minimal sequence-specificity in terms of cleavage, Dicer processes dsRNAs into staggered siRNA duplexes with 3' overhangs having 5' phosphate and 3' hydroxyl at the termini of each strand (37-39). This siRNA product is then assembled into the siRISC for downstream effects of RNAi (14,40). Dicer is also capable of processing precursor miRNAs into a similar miRISC

(41,42). In *C. elegans* and *D. melanogaster*, Dicer function requires ATP for activity (27,42-44), while mammalian Dicers do not have such requirements (45).

With regards to variability of Dicers, they are found to be present in varying numbers of one to four in different organisms (31). In *C. elegans,* Zebrafish and vertebrates such as mouse only one Dicer protein seems to be involved (43,46,47). DCR-1 is the only Dicer protein expressed in *C. elegans* (48), while there are two Dicers paralogues – DCR-1 and DCR-2 in *D. melanogaster*, each having exclusive functions (20,32). DCR-1 possesses a PAZ domain but lacks a functional helicase domain and is found to be essential for processing of miRNA precursors as well involved in miRISC-driven translational repression, while DCR-2 is responsible for processing siRNA precursors in *Drosophila* (32). Although both these Dicers have been shown to have association with the RISC, at least in *Drosophila*, they seem to have notably varying roles in this effector complex (49). In *C. elegans,* DCR-1 is found to interact with RDE-4 (a dsRNA binding protein), RDE-1 (an Argonaute protein) as well as a conserved DExH-box helicase (48). Several other studies have also revealed the presence of similar RNA helicases in post-transcriptional gene silencing. MUT 6 is a DEAH-box RNA helicase found to be involved in transposon silencing in *Chlamydomonas reinhardtii* (50), whereas SDE-3 is also a helicase involved in post-transcriptional gene silencing in *Arabidopsis thaliana* (21,48,51). Such RNA helicase activity is required downstream of siRNA formation during the repression or cleavage of the target mRNA (52). RDE-1, an Argonaute protein, exerts its RNase H activity for cleaving the passenger strand of the siRNA duplex (53), whereas RDE-4 homo-dimerizes and helps to bind preferentially to dsRNAs

(48,54). Hence, in *C. elegans*, it seems that a RDE-4/dsRNA ribonucleoprotein complex helps to recruit DCR-1/RDE-1/RNA-helicase complex through interactions between RDE-4 and DCR-1 (54). Another auxiliary dsRNA-binding protein called R2D2 is found to interact with and heterodimerize with DCR-2 in *Drosophila melanogaster* and this R2D2/DCR-2 complex is involved in sequence-specific mRNA degradation via siRNA binding (32,44). Hence it is evident that R2D2 acts as a sort of connector between the initiation and effector stages of the RNAi machinery (21,44). In terms of structure and function, R2D2 seems to be similar to the RDE-4 protein in *C. elegans* which suggests that its role is conserved among other organisms as well (21). A similar nuclear localized dsRNA binding protein – HYL1 is also known to associate with Dicer and is involved in miRNA metabolism in *A. thaliana* (55).

In *Arabidopsis thaliana,* there are four Dicer homologues (DCL1-4), and even among at least three of them, there is some variability of function (31). DCL1 is required for processing precursor miRNAs (56,57), while DCL2 processes virus-specific dsRNAs (58), and DCL3 is required for processing endo-siRNAs (58). DCL4 has been shown to be involved in trans-acting siRNA biogenesis in association with DRB4 (a dsRNA binding protein) as well as in virus-induced silencing (2,59,60). In *Chlamydomonas*, there are three Dicer-like polypeptides, of which DCL1 is shown to be involved in post-transcriptional silencing of retrotransposons such as TOC1 (61). A number of Dicers such as *Drosophila* DCR1 and *A. thaliana* DCL1 also exhibit nuclear localization signals suggesting further speciation in the roles of these critical proteins in RNAi (56,62).

ii. Small RNAs –

Small duplex RNAs have been depicted as the guide molecules for directing the RISC complex towards the target mRNA for its cleavage or translational repression. Although the term small RNAs has been widely used to depict such RNAi associated molecules, it also refers to other non-coding RNAs such as small nuclear RNAs (snRNAs) and transfer RNAs (tRNAs) (19). In fact, a group of small regulatory RNAs known as CRISPR (clustered regularly interspaced palindromic repeats) have been characterized in prokaryotes, such as bacteria, to interfere with invading viruses (63). The distinction between such different types of non-coding RNAs and eukaryotic small RNAs of the RNA silencing pathway is their size range of ~ 18-25 nt and association with the Argonaute (Ago) family of proteins (64). Ever since the first small RNA – *lin-4* was discovered in *C. elegans* (65,66), a myriad of small RNA species have been characterized in a wide array of eukaryotes especially through cloning and sequencing of size-fractionated RNAs (19,67) and more recently through deep sequencing and computational prediction methodologies (68). Although recent multitude of discoveries of non-canonical small RNAs have diversified small RNAs into various classes, they can be classified in three major classes on basis of biogenesis as well as interacting effector proteins into – microRNAs (miRNAs), small interfering RNAs (siRNAs or endo-siRNAs) and Piwi-interacting RNA (piRNAs) (19).

- ***MicroRNAs - Biogenesis & Characteristics –***

miRNAs are an almost ubiquitous class of 20-24 nt small RNAs found in both plants and animals, and in association with Ago protein effect post-transcriptional gene regulation (18). Despite their similarities, animal and plant miRNAs seem to be quite distinct with respect to their sequence, precursor structure, biogenesis and mode of action (19,22,69). Some animal miRNA genes are associated in close proximity to each other and as such a miRNA cluster is transcribed from a single polycistronic transcription unit (TU) (70), while there are other miRNAs which are produced from separate TUs (71). While animal miRNA genes are found within exons and introns, plant miRNA genes are mostly intergenic and seem to have evolved via inverted gene duplication, spontaneous evolution or through generation of miniature inverted-repeat transposable elements (MITEs) (72-76).

Transcription of most miRNA genes is carried out by RNA Polymerase II, which generates a capped and polyadenylated primary (pri-miRNA) transcript possessing a stem-loop structure (77,78). In animals, pri-miRNA is processed by Drosha (a nuclear RNase III-type protein) which cleaves at the stem of the hairpin structure to release another ~70 nt short hairpin pre-miRNA (70,79). Drosha associates with DiGeorge syndrome critical region (DGCR8) protein (in humans) also known as its homolog – Pasha (in *D. melanogaster* and *C. elegans*) to form the Microprocessor complex (80,81). This complex is able to bind the ~33 bp stem of pri-miRNA and its flanking ssRNA regions to carry out cleavage to form a pre-miRNA (82). Alternatively, pre-miRNAs can sometimes be generated, in animals, without the help of a microprocessor complex, via

splicing of pri-miRNA transcripts releasing introns (mirtrons), which resemble pre-miRNAs (83,84). The pri-miRNA sequences are stabilized with the help of a DDL (dawdle) RNA-binding protein in *A. thaliana* and SNIP1 (Smad-nuclear interacting proteins) in humans (85). In the case of plants, which lack the Drosha and Pasha proteins, the processing step from this pri- to pre-miRNA involves DCL1, a double-stranded RNA-binding protein – HYL1, a C2H2-zinc finger protein – SERRATE (SE) and a nuclear cap-binding complex – CBC (86,87). Post-nuclear processing, the pre-miRNA is exported into the cytoplasm, in case of animals, (88,89) with the help of a Ran-GTP dependent karyopherin protein – Exportin 5 (90). The pre-miRNA is then processed in the cytoplasm into a ~22 nt mature miRNA duplex with the help of the Dicer enzyme (20,42) which associates with dsRNA-binding protein – LOQS (Loquacious), TRBP (Trans-activating response RNA-binding protein) and PACT to make up a part of the RISC assembly (91-94). On the other hand, in plants, the pre-miRNA-to-mature miRNA duplex excision is carried out by DCL1 in the nucleus (18,71). In Arabidopsis, the mature miRNA duplex is stabilized by S-adenosyl methionine-dependent HEN1 methyltransferases that adds methyl groups at the 3' terminus of the duplex to prevent its uridylation and subsequent degradation by quality control mechanisms in the cytoplasm such as the RRP6 exosome subunit or SDN (Small RNA degrading nuclease) (95,96). Such mechanisms have also been described in *Chlamydomonas reinhardtii* (97). Subsequently, the plant homolog of Exportin-5, HASTY is responsible for transition of the mature miRNA duplex from the nucleus to the cytoplasm (99).

Post Dicer processing, the small RNA duplex becomes associated with an Ago protein to generate the RISC effector complex. This process requires specific strand selection where the Ago associates with the mature miRNA (guide strand) while the other miRNA* (or passenger) strand is degraded (100,101). The relative thermodynamic stability of the two ends of the miRNA duplex determines which one of them is selected as the guide strand (102,103). In case of *Drosophila*, R2D2 is able to differentiate such thermodynamic differences and along with DCR2 assists in strand selection in the RISC loading complex (RLC) (44,104), whereas in humans, TRBP and PACT may play similar roles (91,93).

Animal miRNAs tend to regulate target mRNA transcripts mostly via imperfect complementarity to multiple sites on the 3' UTR of the target mRNA and subsequent translational repression and on some occasions by mRNA decay (19). In some cases, miRNAs can also be involved in translational activation (105) and formation of heterochromatin (106). A particular seed region of the miRNA (2-7 nts) needs complementarity to the target mRNA (in animals) via Watson-Crick base pairing, even though there may be mismatches & bulges with respect to target binding (107). On the other hand, plant miRNAs have a single, highly complementary target site in the coding regions and work mainly via cleavage of the target mRNA (73). In *Chlamydomonas reinhardtii,* mRNA cleavage has also been suggested as the primary mode of regulation (108). Certain examples of translational repression have also been observed in plant miRNAs (109).

- ***Small interfering RNA (siRNA) - Biogenesis & Characteristics –***

The first species of siRNAs was discovered in plants (17) and shortly thereafter it was shown that synthetic siRNAs were able to induce an RNAi response (23). Although siRNAs employ mechanisms similar to miRNAs for biogenesis and also possess a general similarity in their regulation of the target mRNA, there are a few distinctions between them. siRNAs are ~21 nt in length and mostly associated with genomic protection against invading viral dsRNAs, transposons or transgene triggers and are dependent only on Dicer or Dicer-like proteins instead of Drosha (18,19). The precursor for the ~21 nt siRNAs has been identified as the canonical inducer of RNAi – the dsRNA which could have a multitude of sources (18,110). Besides having exogenous triggers, siRNAs have also been shown to be generated endogenously (endo-siRNAs) from transposons, sense-antisense transcripts and long stem-loop structures and hence as such differ from exo-siRNAs in having a nuclear phase in biogenesis (111-113). For generation of endo-siRNAs, plants and worms employ RNA-dependent RNA polymerases (RdRps) which is absent in mammals and flies (114). Relatively few molecules of dsRNA trigger can induce synthesis of secondary siRNAs (in presence of the target mRNA) via such RdRps (110,115). The secondary siRNAs are responsible for amplification of the RNAi response and in general lead to systemic silencing throughout the organism, leading to silencing of multiple targets (known as transitive RNAi) (18,116,117).

Nat-siRNAs (natural antisense siRNAs) are generated from convergent transcription of the same loci of the sense-antisense transcripts, while trans-acting siRNAs (ta-siRNAs) are derived from specific genomic transcripts but separate loci (118-120). In Arabidopsis, nat-siRNAs accumulation requires RNA-dependent RNA polymerases (RDR6), and they are recognized and processed by DCL2 and DCL1 into additional ~21 nt nat-siRNAs (120), while the ~21 nt ta-siRNAs are produced with the help of RDR6 and DCL4 enzyme (121,122). Other kinds of siRNAs are the heterochromatic siRNAs (hc-siRNAs) and repeat associated siRNAs (ra-siRNAs) (123). While hc-siRNAs are DCL3/RDR2-dependant and act via AGO4 for maintenance of DNA/histone methylation of transposons and chromatin remodeling (124-126), ra-siRNAs are Dicer-independent and silence retrotransposons and repetitive sequences (127).

Dicer 2-LOQS complex is involved in processing of endo-siRNAs in *Drosophila* (similar to the miRNA pathway), whereas exo-siRNAs require Dicer 1-R2D2 association (19,44,92). Human exo-siRNAs are associated with AGO1-4 of which only AGO2 has been shown to possess slicer activity, whereas Dicer-AGO2 seem to be solely involved with endo-siRNAs (128). In a canonical RNAi pathway, the siRNA guides the RISC towards the complementary target mRNA degradation. The slicer activity is carried out by the Argonaute protein (RNase H-like activity) which cleaves the phosphodiester links between target residues to give products with 5’ monophosphate and 3’ hydroxyl termini (129). Thereafter, these fragments are acted upon by cellular 5’-3’ exonucleases such as XRN1 to completely degrade the target (130). Post-cleavage the target dissociates from

the RISC which is now free to act upon additional targets thus, in some cases, showing characteristics of a multiple-turnover enzyme (131,132). In cases of partial complementarity, siRISCs can initiate silencing via translational repression or exonucleolytic degradation similar to miRNAs (18,129).

- ***Piwi-RNAs (piRNAs) – Biogenesis & Characteristics –***

piRNAs are endogenous, germ-cell specific small non-coding RNAs which have a distinct size of ~24-29 nts and are shown to associate with Aubergine (AUB) and PIWI family of proteins (133). Mostly identified in *Drosophila,* mammals and Zebrafish, piRNAs are generated from long single-stranded RNA precursors which are often produced by intergenic repetitive elements in the genome including retrotransposons and hence have a degree of similarity to ra-siRNAs (134). Similar to plant miRNAs, these non-coding RNAs are generated from piRNA clusters in *Drosophila,* although the exact mechanism of this primary processing is not clearly understood (135,136). Among the different proteins that interact with piRNAs, MILI and MIWI2 are essential for *de novo* DNA methylation of transposons in germ cells, which suggests that piRNAs are also involved in transcriptional gene silencing through DNA methylation (137,138). piRNAs have also been shown to be have an epigenetic role in transposon silencing, (139).

The PIWI clade of proteins possesses a symmetrical dimethyl arginine (sDMA), at their N termini, which is recognized by Tudor-domain containing proteins

(TDRDs) (140,141). These proteins are essential for germline development and play important roles in piRNA metabolism through sDMA binding in PIWI proteins although the exact molecular mechanism remains to be deciphered (142,143).

iii. Argonaute & the RISC complex –

At the center stage of the RNAi pathway lays the RISC complex. The RISC was originally identified by fractionation of sequence-specific nuclease activity in *Drosophila* extracts (24). Some of its known components include – Argonaute, Vasa Intronic Gene (VIG/ MUT70 – the homolog in *Chlamydomonas*), human Fragile X protein (FXR / dFXR – the homolog in *Drosophila*) and TSN1 (Tudor-Staphylococcal Nuclease) (144-146). Among these, Argonaute was the ubiquitous component in purifications from different species, while the specific biochemical roles of the others such as TSN1 are yet to be resolved. Interacting with small RNAs they are known to effect gene silencing in all currently elucidated RNAi-related pathways (147). Argonaute proteins consist of four domains – N terminal domain, PAZ domain (similar to Dicer enzymes), middle (MID) domain and the PIWI domain (148,149). Among these, the PAZ and the PIWI domains are conserved and while the PAZ domain (containing an OB fold) is shown to bind 3' end of the guide RNA (150,151), the PIWI domain has an RNase H-like fold which suggested Argonautes to have the slicer activity of the RISC (149). The MID and PIWI domains are essential towards recognition of the 5' terminal nucleotide of small RNAs (19,152). Argonaute specifically cleaves target mRNA at the phosphodiester linkages between the target nucleotides base-paired to the small RNA residues 10 and 11 from the 5' end

(21,153). The RISC loaded with the guide RNA and the specific Argonaute protein is capable of multiple rounds of target binding, cleavage in its slicing cycle (147).

Argonautes can be further classified into two major clades – AGO-like (which associates with miRNAs and siRNAs) and the PIWI-like (which interacts with piRNAs) subfamilies (147). There is also suggestion of a third clade – the group 3 Argonautes that are worm-specific and mostly contain non-slicer Argonautes (154). The numbers of Argonaute proteins vary among different organisms. The yeast, *Schizosaccharomyces pombe,* expresses only one Ago1 protein as an active slicer (155). *Arabidopsis thaliana* has ten AGO family members of which two possess slicer activity (126,156), while *Drosophila melanogaster* has five AGO proteins of which AGO1 is involved in miRNA accumulation and AGO2 for siRNA production (157,158). On the other hand, *C. elegans* has 27 Argonaute proteins while *Chlamydomonas reinhardtii* has three (61,159).

The RISC can effect gene silencing via mRNA cleavage as well as translational repression. During translational repression, the target mRNA along with AGO proteins is sequestered into cytoplasmic foci known as P-bodies or stress granules where the Argonaute proteins are able to interact with a P-body component – GW182 (160,161). In mammals and *C. elegans,* miRNAs and AGO proteins are able to co-sediment with polyribosomes, which suggest a post-initiation step of regulation of translation (19,162). Similar association with polyribosomes has also been demonstrated in *Arabidopsis,* between miRNAs and AGO1 (163). Human AGO2 has been shown to compete with eIF4E for binding to the mRNA cap structure, via its similarity to the cap binding motif

of the translation initiation factor 4E (164). miRISC mediated silencing can, as such, also take place by similarly preventing assembly of transnationally competent 80S ribosomes (165).

Purpose of this study: -

RNAi has become a method of choice for down regulating target genes and has a wide variety of applications – from medicine to crop improvement. Deciphering the function of the various components of RNAi is a crucial step towards fully understanding this important gene regulatory mechanism. The unicellular (haploid) green alga *Chlamydomonas reinhardtii* has served as a model organism for understanding the various aspects of cell biology and RNAi (123). Moreover, completion of the *Chlamydomonas* genome sequence has greatly assisted in its use as a model system and it also possesses functional RNAi machinery (108,166). Through this study, we have aimed to characterize two related RNAi-defective mutants– MUT13-2 and MUT20, generated via genetic screens in *Chlamydomonas reinhardtii*.

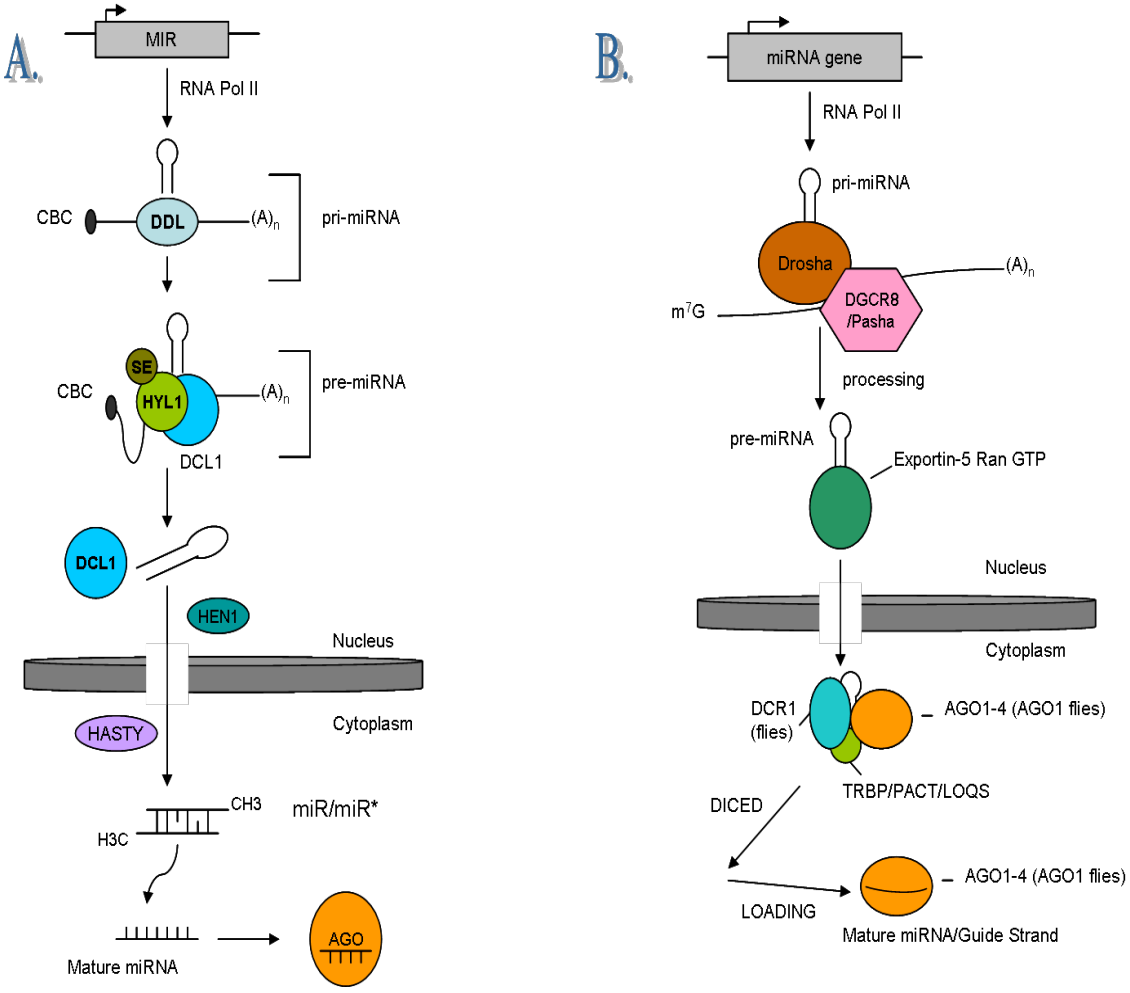


Figure 1-1. **miRNA pathway in animals and plants** –

A. Molecular pathway for miRNA biogenesis in plants. Primary (pri-) miRNAs are transcribed from MIR genes with the help of RNA Pol II and associates with stabilizing protein named Dawdle (DDL). The pri-miRNA is processed into a pre-miRNA with the help of DCL1, HYL1 and SE proteins. Post processing the mature miRNAs can also be methylated by HEN1 and are exported to the cytoplasm with the help of HASTY (an exportin-5 homolog). The guide miRNA is incorporated into the RISC complex in association with AGO family of proteins. [Reproduced and modified from - Voinnet, O. (2009) Origin, biogenesis, and activity of plant microRNAs. *Cell*, **136**, 669-687].

B. Biogenesis of canonical miRNAs in animals. The pri-miRNA is similarly generated with the help of RNA Pol II, but the pre-miRNA is formed with the help of a Drosha, DGCR8/Pasha. The pre-miRNA then associates with Exportin-5 and is exported out into the cytoplasm where it is acted upon by Dicer (DCR1 in flies) to produce the miRNA guide strand in association with AGO proteins. [Reproduced and modified from - Kim, V.N., Han, J. and Siomi, M.C. (2009) Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*, **10**, 126-139.]

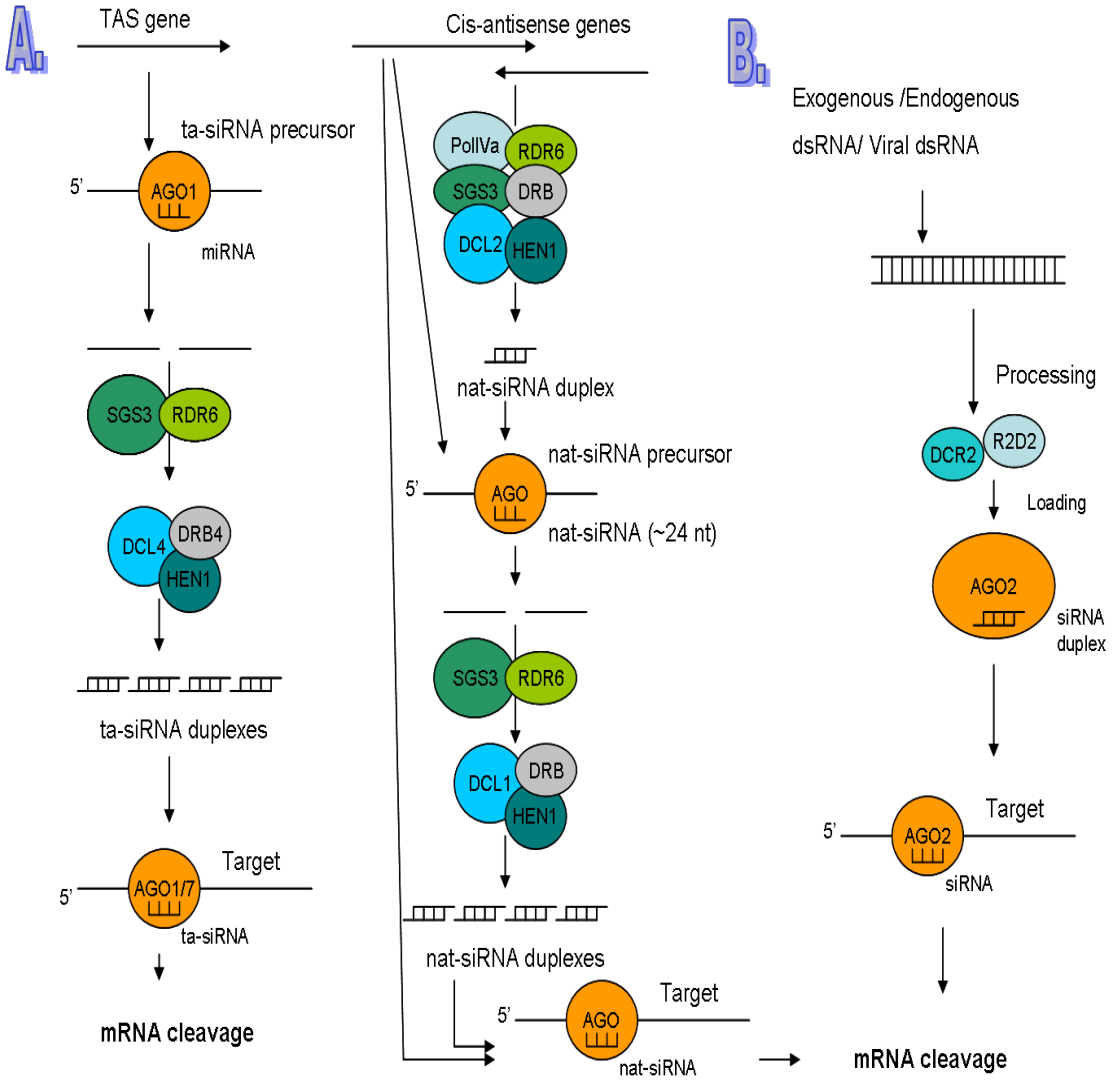


Figure 1-2. **siRNA pathways in plants and animals** –

A. ta-siRNA and nat-siRNA in plants. Besides canonical exogenous triggers for siRNA response in plants, these are the endogenous responses which result in mRNA cleavage. Generated from TAS transcripts or sense-antisense transcripts, these siRNAs are dependent on RDR6 for amplification of the silencing response and results in specific target mRNA cleavage.

B. siRNAs are also generated by exogenous triggers such viral dsRNAs or introduction of synthetic siRNAs. The endonuclease Dicer cleaves the dsRNA to generate ~20-25 nt long small RNAs of which one (guide) strand is loaded onto the RISC-loading complex along with the Argonaute protein. The Ago protein uses the guide strand (having perfect complementarity with target RNA) to direct the target mRNA cleavage. [Reproduced and modified from - Vaucheret, H. (2006) Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes Dev*, **20**, 759-771.]

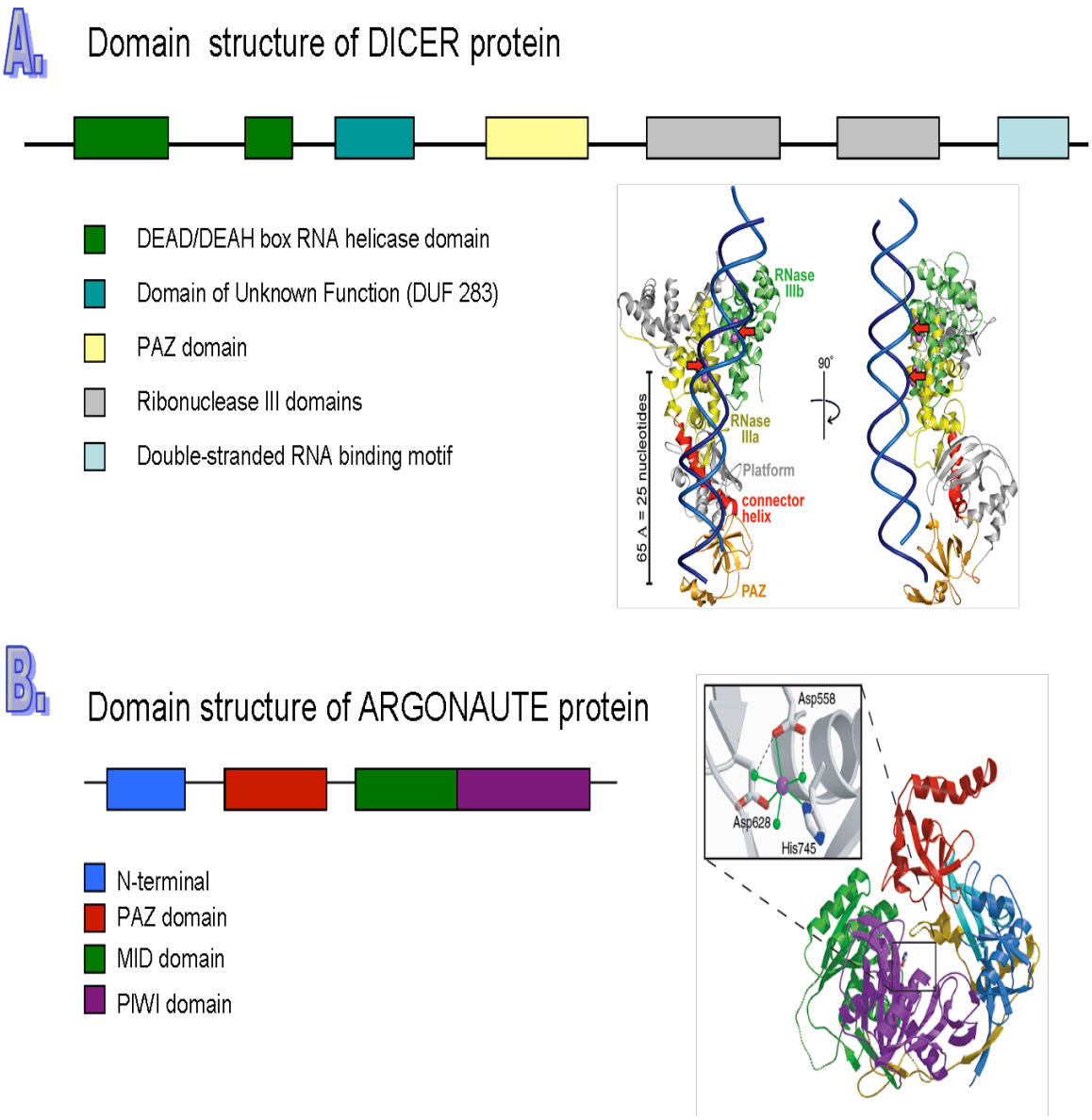


Figure 1-3. **Structure of DICER and ARGONAUTE proteins** –

A. Dicer is a member of the third class of RNase III family of enzymes and exhibits specificity for dsRNA. It contains a N-terminal DEAD/DEAH box RNA helicase domain, two Ribonuclease III (RIII) domains, a domain of unknown function (DUF 283), a dsRNA binding motif and a PAZ domain. The two RIII domains of Dicer dimerize to form a catalytic center responsible for cleaving dsRNAs. Most but not all Dicers have PAZ domains. The adjoining figure shows the front and side views of crystal structure of *Giardia* Dicer along a modeled dsRNA substrate and the red arrows show the point of cleavage. [Reproduced and modified from - Jaronczyk, K., Carmichael, J.B. and Hobman, T.C. (2005) Exploring the functions of RNA interference pathway proteins: some functions are more RISCy than others? *Biochem J*, **387**, 561-571; Macrae, I.J., Li, F., Zhou, K., Cande, W.Z. and

Doudna, J.A. (2006) Structure of Dicer and mechanistic implications for RNAi. *Cold Spring Harb Symp Quant Biol*, **71**, 73-80.]

B. Domain structure of Argonaute family of proteins. They possess four major domains – N-terminal domain, PAZ (Piwi/Argonaute/Zwille), MID (middle) domain and PIWI domain. The adjoining figure shows the crystal structure of Argonaute protein in *P. furiosus.* The domains have similar color-coding as in the key. The active site residues are listed as – Asp-Asp-His. [Reproduced and modified from - Tabara, H., Yigit, E., Siomi, H. and Mello, C.C. (2002) The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in C. elegans. *Cell*, **109**, 861-871; Tolia, N.H. and Joshua-Tor, L. (2007) Slicer and the argonautes. *Nat Chem Biol*, **3**, 36-43.]

Literature Cited

1. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, **391**, 806-811.
2. Brodersen, P. and Voinnet, O. (2006) The diversity of RNA silencing pathways in plants. *Trends Genet*, **22**, 268-280.
3. (1998) Genome sequence of the nematode C. elegans: a platform for investigating biology. *Science*, **282**, 2012-2018.
4. Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F. *et al.* (2000) The genome sequence of Drosophila melanogaster. *Science*, **287**, 2185-2195.
5. Venter, J.C., Adams, M.D., Myers, E.W., Li, P.W., Mural, R.J., Sutton, G.G., Smith, H.O., Yandell, M., Evans, C.A., Holt, R.A. *et al.* (2001) The sequence of the human genome. *Science*, **291**, 1304-1351.
6. Mohr, S., Bakal, C. and Perrimon, N. Genomic screening with RNAi: results and challenges. *Annu Rev Biochem*, **79**, 37-64.
7. Sugimoto, A. (2004) High-throughput RNAi in Caenorhabditis elegans: genome-wide screens and functional genomics. *Differentiation*, **72**, 81-91.
8. Vaishnaw, A.K., Gollob, J., Gamba-Vitalo, C., Hutabarat, R., Sah, D., Meyers, R., de Fougerolles, T. and Maraganore, J. A status report on RNAi therapeutics. *Silence*, **1**, 14.
9. Matzke, M.A. and Matzke, A.J. (2004) Planting the seeds of a new paradigm. *PLoS Biol*, **2**, E133.
10. Denli, A.M. and Hannon, G.J. (2003) RNAi: an ever-growing puzzle. *Trends Biochem Sci*, **28**, 196-201.
11. Verdel, A. and Moazed, D. (2005) RNAi-directed assembly of heterochromatin in fission yeast. *FEBS Lett*, **579**, 5872-5878.
12. Tang, G., Reinhart, B.J., Bartel, D.P. and Zamore, P.D. (2003) A biochemical framework for RNA silencing in plants. *Genes Dev*, **17**, 49-63.
13. Tijsterman, M., Ketting, R.F. and Plasterk, R.H. (2002) The genetics of RNA silencing. *Annu Rev Genet*, **36**, 489-519.
14. Hammond, S.M., Bernstein, E., Beach, D. and Hannon, G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature*, **404**, 293-296.
15. Zamore, P.D., Tuschl, T., Sharp, P.A. and Bartel, D.P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*, **101**, 25-33.
16. Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R. and Tuschl, T. (2002) Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. *Cell*, **110**, 563-574.
17. Hamilton, A.J. and Baulcombe, D.C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science*, **286**, 950-952.
18. Carthew, R.W. and Sontheimer, E.J. (2009) Origins and Mechanisms of miRNAs and siRNAs. *Cell*, **136**, 642-655.

19. Kim, V.N., Han, J. and Siomi, M.C. (2009) Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*, **10**, 126-139.
20. Bernstein, E., Caudy, A.A., Hammond, S.M. and Hannon, G.J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, **409**, 363-366.
21. Sontheimer, E.J. (2005) Assembly and function of RNA silencing complexes. *Nat Rev Mol Cell Biol*, **6**, 127-138.
22. Kim, V.N. (2005) MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol*, **6**, 376-385.
23. Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, **411**, 494-498.
24. Hammond, S.M., Boettcher, S., Caudy, A.A., Kobayashi, R. and Hannon, G.J. (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science*, **293**, 1146-1150.
25. Hutvagner, G. and Zamore, P.D. (2002) A microRNA in a multiple-turnover RNAi enzyme complex. *Science*, **297**, 2056-2060.
26. Mourelatos, Z., Dostie, J., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M. and Dreyfuss, G. (2002) miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. *Genes Dev*, **16**, 720-728.
27. Nykanen, A., Haley, B. and Zamore, P.D. (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell*, **107**, 309-321.
28. Pham, J.W., Pellino, J.L., Lee, Y.S., Carthew, R.W. and Sontheimer, E.J. (2004) A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in Drosophila. *Cell*, **117**, 83-94.
29. Carmell, M.A., Xuan, Z., Zhang, M.Q. and Hannon, G.J. (2002) The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. *Genes Dev*, **16**, 2733-2742.
30. Ketting, R.F. The many faces of RNAi. *Dev Cell*, **20**, 148-161.
31. Jaronczyk, K., Carmichael, J.B. and Hobman, T.C. (2005) Exploring the functions of RNA interference pathway proteins: some functions are more RISCy than others? *Biochem J*, **387**, 561-571.
32. Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J. and Carthew, R.W. (2004) Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. *Cell*, **117**, 69-81.
33. Lamontagne, B., Larose, S., Boulanger, J. and Elela, S.A. (2001) The RNase III family: a conserved structure and expanding functions in eukaryotic dsRNA metabolism. *Curr Issues Mol Biol*, **3**, 71-78.
34. Ma, J.B., Ye, K. and Patel, D.J. (2004) Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature*, **429**, 318-322.
35. Cerutti, L., Mian, N. and Bateman, A. (2000) Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. *Trends Biochem Sci*, **25**, 481-482.
36. Zhang, H., Kolb, F.A., Jaskiewicz, L., Westhof, E. and Filipowicz, W. (2004) Single processing center models for human Dicer and bacterial RNase III. *Cell*, **118**, 57-68.

37. Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W. and Tuschl, T. (2001) Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *EMBO J*, **20**, 6877-6888.
38. Provost, P., Silverstein, R.A., Dishart, D., Walfridsson, J., Djupedal, I., Kniola, B., Wright, A., Samuelsson, B., Radmark, O. and Ekwall, K. (2002) Dicer is required for chromosome segregation and gene silencing in fission yeast cells. *Proc Natl Acad Sci U S A*, **99**, 16648-16653.
39. Myers, J.W., Jones, J.T., Meyer, T. and Ferrell, J.E., Jr. (2003) Recombinant Dicer efficiently converts large dsRNAs into siRNAs suitable for gene silencing. *Nat Biotechnol*, **21**, 324-328.
40. Doi, N., Zenno, S., Ueda, R., Ohki-Hamazaki, H., Ui-Tei, K. and Saigo, K. (2003) Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. *Curr Biol*, **13**, 41-46.
41. Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T. and Zamore, P.D. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science*, **293**, 834-838.
42. Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J. and Plasterk, R.H. (2001) Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes Dev*, **15**, 2654-2659.
43. Bernstein, E., Kim, S.Y., Carmell, M.A., Murchison, E.P., Alcorn, H., Li, M.Z., Mills, A.A., Elledge, S.J., Anderson, K.V. and Hannon, G.J. (2003) Dicer is essential for mouse development. *Nat Genet*, **35**, 215-217.
44. Liu, Q., Rand, T.A., Kalidas, S., Du, F., Kim, H.E., Smith, D.P. and Wang, X. (2003) R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. *Science*, **301**, 1921-1925.
45. Zhang, H., Kolb, F.A., Brondani, V., Billy, E. and Filipowicz, W. (2002) Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. *EMBO J*, **21**, 5875-5885.
46. Grishok, A., Pasquinelli, A.E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D.L., Fire, A., Ruvkun, G. and Mello, C.C. (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. *Cell*, **106**, 23-34.
47. Wienholds, E., Koudijs, M.J., van Eeden, F.J., Cuppen, E. and Plasterk, R.H. (2003) The microRNA-producing enzyme Dicer1 is essential for zebrafish development. *Nat Genet*, **35**, 217-218.
48. Tabara, H., Yigit, E., Siomi, H. and Mello, C.C. (2002) The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in C. elegans. *Cell*, **109**, 861-871.
49. Paddison, P.J., Vogt, P.K., Jaskiewicz, L. and Filipowicz, W. (2008), *RNA Interference*. Springer Berlin Heidelberg, Vol. 320, pp. 77-97.
50. Wu-Scharf, D., Jeong, B., Zhang, C. and Cerutti, H. (2000) Transgene and transposon silencing in Chlamydomonas reinhardtii by a DEAH-box RNA helicase. *Science*, **290**, 1159-1162.
51. Dalmay, T., Horsefield, R., Braunstein, T.H. and Baulcombe, D.C. (2001) SDE3 encodes an RNA helicase required for post-transcriptional gene silencing in Arabidopsis. *EMBO J*, **20**, 2069-2078.

52. Tijsterman, M., Ketting, R.F., Okihara, K.L., Sijen, T. and Plasterk, R.H. (2002) RNA helicase MUT-14-dependent gene silencing triggered in C. elegans by short antisense RNAs. *Science*, **295**, 694-697.
53. Steiner, F.A., Okihara, K.L., Hoogstrate, S.W., Sijen, T. and Ketting, R.F. (2009) RDE-1 slicer activity is required only for passenger-strand cleavage during RNAi in Caenorhabditis elegans. *Nat Struct Mol Biol*, **16**, 207-211.
54. Parker, G.S., Eckert, D.M. and Bass, B.L. (2006) RDE-4 preferentially binds long dsRNA and its dimerization is necessary for cleavage of dsRNA to siRNA. *RNA*, **12**, 807-818.
55. Vazquez, F., Gasciolli, V., Crete, P. and Vaucheret, H. (2004) The nuclear dsRNA binding protein HYL1 is required for microRNA accumulation and plant development, but not posttranscriptional transgene silencing. *Curr Biol*, **14**, 346-351.
56. Schauer, S.E., Jacobsen, S.E., Meinke, D.W. and Ray, A. (2002) DICER-LIKE1: blind men and elephants in Arabidopsis development. *Trends Plant Sci*, **7**, 487-491.
57. Park, W., Li, J., Song, R., Messing, J. and Chen, X. (2002) CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. *Curr Biol*, **12**, 1484-1495.
58. Xie, Z., Johansen, L.K., Gustafson, A.M., Kasschau, K.D., Lellis, A.D., Zilberman, D., Jacobsen, S.E. and Carrington, J.C. (2004) Genetic and functional diversification of small RNA pathways in plants. *PLoS Biol*, **2**, E104.
59. Blevins, T., Rajeswaran, R., Shivaprasad, P.V., Beknazariants, D., Si-Ammour, A., Park, H.S., Vazquez, F., Robertson, D., Meins, F., Jr., Hohn, T. *et al.* (2006) Four plant Dicers mediate viral small RNA biogenesis and DNA virus induced silencing. *Nucleic Acids Res*, **34**, 6233-6246.
60. Nakazawa, Y., Hiraguri, A., Moriyama, H. and Fukuhara, T. (2007) The dsRNA-binding protein DRB4 interacts with the Dicer-like protein DCL4 in vivo and functions in the <i>trans</i>-acting siRNA pathway. *Plant Molecular Biology*, **63**, 777-785.
61. Casas-Mollano, J.A., Rohr, J., Kim, E.-J., Balassa, E., van Dijk, K. and Cerutti, H. (2008) Diversification of the Core RNA Interference Machinery in Chlamydomonas reinhardtii and the Role of DCL1 in Transposon Silencing. *Genetics*, **179**, 69-81.
62. Papp, I., Mette, M.F., Aufsatz, W., Daxinger, L., Schauer, S.E., Ray, A., van der Winden, J., Matzke, M. and Matzke, A.J. (2003) Evidence for nuclear processing of plant micro RNA and short interfering RNA precursors. *Plant Physiol*, **132**, 1382-1390.
63. Waters, L.S. and Storz, G. (2009) Regulatory RNAs in bacteria. *Cell*, **136**, 615-628.
64. Czech, B. and Hannon, G.J. Small RNA sorting: matchmaking for Argonautes. *Nat Rev Genet*, **12**, 19-31.
65. Lee, R.C., Feinbaum, R.L. and Ambros, V. (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell*, **75**, 843-854.

66. Wightman, B., Ha, I. and Ruvkun, G. (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell*, **75**, 855-862.
67. Lagos-Quintana, M., Rauhut, R., Lendeckel, W. and Tuschl, T. (2001) Identification of novel genes coding for small expressed RNAs. *Science*, **294**, 853-858.
68. Li, S.C., Pan, C.Y. and Lin, W.C. (2006) Bioinformatic discovery of microRNA precursors from human ESTs and introns. *BMC Genomics*, **7**, 164.
69. Millar, A.A. and Waterhouse, P.M. (2005) Plant and animal microRNAs: similarities and differences. *Funct Integr Genomics*, **5**, 129-135.
70. Lee, Y., Jeon, K., Lee, J.T., Kim, S. and Kim, V.N. (2002) MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J*, **21**, 4663-4670.
71. Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, **116**, 281-297.
72. Shabalina, S.A. and Koonin, E.V. (2008) Origins and evolution of eukaryotic RNA interference. *Trends in Ecology & Evolution*, **23**, 578-587.
73. Voinnet, O. (2009) Origin, biogenesis, and activity of plant microRNAs. *Cell*, **136**, 669-687.
74. Allen, E., Xie, Z., Gustafson, A.M., Sung, G.H., Spatafora, J.W. and Carrington, J.C. (2004) Evolution of microRNA genes by inverted duplication of target gene sequences in Arabidopsis thaliana. *Nat Genet*, **36**, 1282-1290.
75. Felippes, F.F., Schneeberger, K., Dezulian, T., Huson, D.H. and Weigel, D. (2008) Evolution of Arabidopsis thaliana microRNAs from random sequences. *RNA*, **14**, 2455-2459.
76. Piriyapongsa, J. and Jordan, I.K. (2008) Dual coding of siRNAs and miRNAs by plant transposable elements. *RNA*, **14**, 814-821.
77. Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H. and Kim, V.N. (2004) MicroRNA genes are transcribed by RNA polymerase II. *EMBO J*, **23**, 4051-4060.
78. Cai, X., Hagedorn, C.H. and Cullen, B.R. (2004) Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *RNA*, **10**, 1957-1966.
79. Lee, Y., Han, J., Yeom, K.H., Jin, H. and Kim, V.N. (2006) Drosha in primary microRNA processing. *Cold Spring Harb Symp Quant Biol*, **71**, 51-57.
80. Denli, A.M., Tops, B.B., Plasterk, R.H., Ketting, R.F. and Hannon, G.J. (2004) Processing of primary microRNAs by the Microprocessor complex. *Nature*, **432**, 231-235.
81. Landthaler, M., Yalcin, A. and Tuschl, T. (2004) The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. *Curr Biol*, **14**, 2162-2167.
82. Zeng, Y. and Cullen, B.R. (2005) Efficient processing of primary microRNA hairpins by Drosha requires flanking nonstructured RNA sequences. *J Biol Chem*, **280**, 27595-27603.

83. Okamura, K., Phillips, M.D., Tyler, D.M., Duan, H., Chou, Y.T. and Lai, E.C. (2008) The regulatory activity of microRNA* species has substantial influence on microRNA and 3' UTR evolution. *Nat Struct Mol Biol*, **15**, 354-363.
84. Ruby, J.G., Jan, C.H. and Bartel, D.P. (2007) Intronic microRNA precursors that bypass Drosha processing. *Nature*, **448**, 83-86.
85. Yu, B., Bi, L., Zheng, B., Ji, L., Chevalier, D., Agarwal, M., Ramachandran, V., Li, W., Lagrange, T., Walker, J.C. *et al.* (2008) The FHA domain proteins DAWDLE in Arabidopsis and SNIP1 in humans act in small RNA biogenesis. *Proc Natl Acad Sci U S A*, **105**, 10073-10078.
86. Fang, Y. and Spector, D.L. (2007) Identification of nuclear dicing bodies containing proteins for microRNA biogenesis in living Arabidopsis plants. *Curr Biol*, **17**, 818-823.
87. Kurihara, Y., Takashi, Y. and Watanabe, Y. (2006) The interaction between DCL1 and HYL1 is important for efficient and precise processing of pri-miRNA in plant microRNA biogenesis. *RNA*, **12**, 206-212.
88. Brownawell, A.M. and Macara, I.G. (2002) Exportin-5, a novel karyopherin, mediates nuclear export of double-stranded RNA binding proteins. *J Cell Biol*, **156**, 53-64.
89. Kim, V.N. (2004) MicroRNA precursors in motion: exportin-5 mediates their nuclear export. *Trends Cell Biol*, **14**, 156-159.
90. Bohnsack, M.T., Czaplinski, K. and Gorlich, D. (2004) Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA*, **10**, 185-191.
91. Chendrimada, T.P., Gregory, R.I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K. and Shiekhattar, R. (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature*, **436**, 740-744.
92. Forstemann, K., Tomari, Y., Du, T., Vagin, V.V., Denli, A.M., Bratu, D.P., Klattenhoff, C., Theurkauf, W.E. and Zamore, P.D. (2005) Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. *PLoS Biol*, **3**, e236.
93. Lee, Y., Hur, I., Park, S.Y., Kim, Y.K., Suh, M.R. and Kim, V.N. (2006) The role of PACT in the RNA silencing pathway. *EMBO J*, **25**, 522-532.
94. Saito, K., Ishizuka, A., Siomi, H. and Siomi, M.C. (2005) Processing of pre-microRNAs by the Dicer-1-Loquacious complex in Drosophila cells. *PLoS Biol*, **3**, e235.
95. Li, J., Yang, Z., Yu, B., Liu, J. and Chen, X. (2005) Methylation protects miRNAs and siRNAs from a 3'-end uridylation activity in Arabidopsis. *Curr Biol*, **15**, 1501-1507.
96. Kennedy, S., Wang, D. and Ruvkun, G. (2004) A conserved siRNA-degrading RNase negatively regulates RNA interference in C. elegans. *Nature*, **427**, 645-649.
97. Ibrahim, F., Rymarquis, L.A., Kim, E.J., Becker, J., Balassa, E., Green, P.J. and Cerutti, H. Uridylation of mature miRNAs and siRNAs by the MUT68 nucleotidyltransferase promotes their degradation in Chlamydomonas. *Proc Natl Acad Sci U S A*, **107**, 3906-3911.

98. Molnar, A., Schwach, F., Studholme, D.J., Thuenemann, E.C. and Baulcombe, D.C. (2007) miRNAs control gene expression in the single-cell alga Chlamydomonas reinhardtii. *Nature*, **447**, 1126-1129.
99. Park, M.Y., Wu, G., Gonzalez-Sulser, A., Vaucheret, H. and Poethig, R.S. (2005) Nuclear processing and export of microRNAs in Arabidopsis. *Proc Natl Acad Sci U S A*, **102**, 3691-3696.
100. Pratt, A.J. and MacRae, I.J. (2009) The RNA-induced silencing complex: a versatile gene-silencing machine. *J Biol Chem*, **284**, 17897-17901.
101. Schwarz, D.S., Tomari, Y. and Zamore, P.D. (2004) The RNA-induced silencing complex is a Mg2+-dependent endonuclease. *Curr Biol*, **14**, 787-791.
102. Schwarz, D.S., Hutvagner, G., Du, T., Xu, Z., Aronin, N. and Zamore, P.D. (2003) Asymmetry in the assembly of the RNAi enzyme complex. *Cell*, **115**, 199-208.
103. Khvorova, A., Reynolds, A. and Jayasena, S.D. (2003) Functional siRNAs and miRNAs exhibit strand bias. *Cell*, **115**, 209-216.
104. Tomari, Y., Matranga, C., Haley, B., Martinez, N. and Zamore, P.D. (2004) A protein sensor for siRNA asymmetry. *Science*, **306**, 1377-1380.
105. Filipowicz, W., Bhattacharyya, S.N. and Sonenberg, N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*, **9**, 102-114.
106. Kim, D.H., Saetrom, P., Snove, O., Jr. and Rossi, J.J. (2008) MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci U S A*, **105**, 16230-16235.
107. Winter, J., Jung, S., Keller, S., Gregory, R.I. and Diederichs, S. (2009) Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol*, **11**, 228-234.
108. Schroda, M. (2006) RNA silencing in Chlamydomonas: mechanisms and tools. *Curr Genet*, **49**, 69-84.
109. Brodersen, P., Sakvarelidze-Achard, L., Bruun-Rasmussen, M., Dunoyer, P., Yamamoto, Y.Y., Sieburth, L. and Voinnet, O. (2008) Widespread translational inhibition by plant miRNAs and siRNAs. *Science*, **320**, 1185-1190.
110. Mello, C.C. and Conte, D., Jr. (2004) Revealing the world of RNA interference. *Nature*, **431**, 338-342.
111. Ghildiyal, M., Seitz, H., Horwich, M.D., Li, C., Du, T., Lee, S., Xu, J., Kittler, E.L., Zapp, M.L., Weng, Z. *et al.* (2008) Endogenous siRNAs derived from transposons and mRNAs in Drosophila somatic cells. *Science*, **320**, 1077-1081.
112. Okamura, K., Balla, S., Martin, R., Liu, N. and Lai, E.C. (2008) Two distinct mechanisms generate endogenous siRNAs from bidirectional transcription in Drosophila melanogaster. *Nat Struct Mol Biol*, **15**, 581-590.
113. Zhou, R., Czech, B., Brennecke, J., Sachidanandam, R., Wohlschlegel, J.A., Perrimon, N. and Hannon, G.J. (2009) Processing of Drosophila endo-siRNAs depends on a specific Loquacious isoform. *RNA*, **15**, 1886-1895.
114. Chapman, E.J. and Carrington, J.C. (2007) Specialization and evolution of endogenous small RNA pathways. *Nat Rev Genet*, **8**, 884-896.
115. Meister, G. and Tuschl, T. (2004) Mechanisms of gene silencing by double-stranded RNA. *Nature*, **431**, 343-349.

116. Pak, J. and Fire, A. (2007) Distinct populations of primary and secondary effectors during RNAi in C. elegans. *Science*, **315**, 241-244.
117. Sijen, T., Steiner, F.A., Thijssen, K.L. and Plasterk, R.H. (2007) Secondary siRNAs result from unprimed RNA synthesis and form a distinct class. *Science*, **315**, 244-247.
118. Tam, O.H., Aravin, A.A., Stein, P., Girard, A., Murchison, E.P., Cheloufi, S., Hodges, E., Anger, M., Sachidanandam, R., Schultz, R.M. *et al.* (2008) Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. *Nature*, **453**, 534-538.
119. Watanabe, T., Totoki, Y., Toyoda, A., Kaneda, M., Kuramochi-Miyagawa, S., Obata, Y., Chiba, H., Kohara, Y., Kono, T., Nakano, T. *et al.* (2008) Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. *Nature*, **453**, 539-543.
120. Borsani, O., Zhu, J., Verslues, P.E., Sunkar, R. and Zhu, J.K. (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in Arabidopsis. *Cell*, **123**, 1279-1291.
121. Allen, E., Xie, Z., Gustafson, A.M. and Carrington, J.C. (2005) microRNA-directed phasing during trans-acting siRNA biogenesis in plants. *Cell*, **121**, 207-221.
122. Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gasciolli, V., Mallory, A.C., Hilbert, J.L., Bartel, D.P. and Crete, P. (2004) Endogenous trans-acting siRNAs regulate the accumulation of Arabidopsis mRNAs. *Mol Cell*, **16**, 69-79.
123. Zhao, T., Li, G., Mi, S., Li, S., Hannon, G.J., Wang, X.J. and Qi, Y. (2007) A complex system of small RNAs in the unicellular green alga Chlamydomonas reinhardtii. *Genes Dev*, **21**, 1190-1203.
124. Hamilton, A., Voinnet, O., Chappell, L. and Baulcombe, D. (2002) Two classes of short interfering RNA in RNA silencing. *EMBO J*, **21**, 4671-4679.
125. Zilberman, D., Cao, X. and Jacobsen, S.E. (2003) ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. *Science*, **299**, 716-719.
126. Qi, Y., Denli, A.M. and Hannon, G.J. (2005) Biochemical specialization within Arabidopsis RNA silencing pathways. *Mol Cell*, **19**, 421-428.
127. Vagin, V.V., Sigova, A., Li, C., Seitz, H., Gvozdev, V. and Zamore, P.D. (2006) A distinct small RNA pathway silences selfish genetic elements in the germline. *Science*, **313**, 320-324.
128. Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L. and Hannon, G.J. (2004) Argonaute2 is the catalytic engine of mammalian RNAi. *Science*, **305**, 1437-1441.
129. Tomari, Y. and Zamore, P.D. (2005) Perspective: machines for RNAi. *Genes Dev*, **19**, 517-529.
130. Orban, T.I. and Izaurralde, E. (2005) Decay of mRNAs targeted by RISC requires XRN1, the Ski complex, and the exosome. *RNA*, **11**, 459-469.
131. Forstemann, K., Horwich, M.D., Wee, L., Tomari, Y. and Zamore, P.D. (2007) Drosophila microRNAs are sorted into functionally distinct argonaute complexes after production by dicer-1. *Cell*, **130**, 287-297.

132. Rivas, F.V., Tolia, N.H., Song, J.J., Aragon, J.P., Liu, J., Hannon, G.J. and Joshua-Tor, L. (2005) Purified Argonaute2 and an siRNA form recombinant human RISC. *Nat Struct Mol Biol*, **12**, 340-349.
133. Aravin, A.A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., Gaasterland, T., Meyer, J. and Tuschl, T. (2003) The small RNA profile during Drosophila melanogaster development. *Dev Cell*, **5**, 337-350.
134. Thomson, T. and Lin, H. (2009) The biogenesis and function of PIWI proteins and piRNAs: progress and prospect. *Annu Rev Cell Dev Biol*, **25**, 355-376.
135. Ro, S., Park, C., Song, R., Nguyen, D., Jin, J., Sanders, K.M., McCarrey, J.R. and Yan, W. (2007) Cloning and expression profiling of testis-expressed piRNA-like RNAs. *RNA*, **13**, 1693-1702.
136. Lau, N.C., Seto, A.G., Kim, J., Kuramochi-Miyagawa, S., Nakano, T., Bartel, D.P. and Kingston, R.E. (2006) Characterization of the piRNA complex from rat testes. *Science*, **313**, 363-367.
137. Kuramochi-Miyagawa, S., Watanabe, T., Gotoh, K., Totoki, Y., Toyoda, A., Ikawa, M., Asada, N., Kojima, K., Yamaguchi, Y., Ijiri, T.W. *et al.* (2008) DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. *Genes Dev*, **22**, 908-917.
138. Aravin, A.A., Sachidanandam, R., Bourc'his, D., Schaefer, C., Pezic, D., Toth, K.F., Bestor, T. and Hannon, G.J. (2008) A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. *Mol Cell*, **31**, 785-799.
139. Brennecke, J., Malone, C.D., Aravin, A.A., Sachidanandam, R., Stark, A. and Hannon, G.J. (2008) An epigenetic role for maternally inherited piRNAs in transposon silencing. *Science*, **322**, 1387-1392.
140. Heo, I. and Kim, V.N. (2009) Regulating the regulators: posttranslational modifications of RNA silencing factors. *Cell*, **139**, 28-31.
141. Kim, Y.K., Heo, I. and Kim, V.N. Modifications of small RNAs and their associated proteins. *Cell*, **143**, 703-709.
142. Nishida, K.M., Okada, T.N., Kawamura, T., Mituyama, T., Kawamura, Y., Inagaki, S., Huang, H., Chen, D., Kodama, T., Siomi, H. *et al.* (2009) Functional involvement of Tudor and dPRMT5 in the piRNA processing pathway in Drosophila germlines. *EMBO J*, **28**, 3820-3831.
143. Siomi, M.C., Mannen, T. and Siomi, H. How does the royal family of Tudor rule the PIWI-interacting RNA pathway? *Genes Dev*, **24**, 636-646.
144. Caudy, A.A., Ketting, R.F., Hammond, S.M., Denli, A.M., Bathoorn, A.M., Tops, B.B., Silva, J.M., Myers, M.M., Hannon, G.J. and Plasterk, R.H. (2003) A micrococcal nuclease homologue in RNAi effector complexes. *Nature*, **425**, 411-414.
145. Caudy, A.A., Myers, M., Hannon, G.J. and Hammond, S.M. (2002) Fragile X-related protein and VIG associate with the RNA interference machinery. *Genes Dev*, **16**, 2491-2496.
146. Ishizuka, A., Siomi, M.C. and Siomi, H. (2002) A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. *Genes Dev*, **16**, 2497-2508.

147. Tolia, N.H. and Joshua-Tor, L. (2007) Slicer and the argonautes. *Nat Chem Biol*, **3**, 36-43.
148. Song, J.J. and Joshua-Tor, L. (2006) Argonaute and RNA--getting into the groove. *Curr Opin Struct Biol*, **16**, 5-11.
149. Song, J.J., Smith, S.K., Hannon, G.J. and Joshua-Tor, L. (2004) Crystal structure of Argonaute and its implications for RISC slicer activity. *Science*, **305**, 1434-1437.
150. Song, J.J., Liu, J., Tolia, N.H., Schneiderman, J., Smith, S.K., Martienssen, R.A., Hannon, G.J. and Joshua-Tor, L. (2003) The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. *Nat Struct Biol*, **10**, 1026-1032.
151. Yan, K.S., Yan, S., Farooq, A., Han, A., Zeng, L. and Zhou, M.M. (2003) Structure and conserved RNA binding of the PAZ domain. *Nature*, **426**, 468-474.
152. Ma, J.B., Yuan, Y.R., Meister, G., Pei, Y., Tuschl, T. and Patel, D.J. (2005) Structural basis for 5'-end-specific recognition of guide RNA by the A. fulgidus Piwi protein. *Nature*, **434**, 666-670.
153. Martinez, J. and Tuschl, T. (2004) RISC is a 5' phosphomonoester-producing RNA endonuclease. *Genes Dev*, **18**, 975-980.
154. Yigit, E., Batista, P.J., Bei, Y., Pang, K.M., Chen, C.C., Tolia, N.H., Joshua-Tor, L., Mitani, S., Simard, M.J. and Mello, C.C. (2006) Analysis of the C. elegans Argonaute family reveals that distinct Argonautes act sequentially during RNAi. *Cell*, **127**, 747-757.
155. Irvine, D.V., Zaratiegui, M., Tolia, N.H., Goto, D.B., Chitwood, D.H., Vaughn, M.W., Joshua-Tor, L. and Martienssen, R.A. (2006) Argonaute slicing is required for heterochromatic silencing and spreading. *Science*, **313**, 1134-1137.
156. Baumberger, N. and Baulcombe, D.C. (2005) Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs. *Proc Natl Acad Sci U S A*, **102**, 11928-11933.
157. van Rij, R.P., Saleh, M.C., Berry, B., Foo, C., Houk, A., Antoniewski, C. and Andino, R. (2006) The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. *Genes Dev*, **20**, 2985-2995.
158. Pham, J.W. and Sontheimer, E.J. (2005) Molecular requirements for RNA-induced silencing complex assembly in the Drosophila RNA interference pathway. *J Biol Chem*, **280**, 39278-39283.
159. Boisvert, M.E. and Simard, M.J. (2008) RNAi pathway in C. elegans: the argonautes and collaborators. *Curr Top Microbiol Immunol*, **320**, 21-36.
160. Liu, J., Rivas, F.V., Wohlschlegel, J., Yates, J.R., 3rd, Parker, R. and Hannon, G.J. (2005) A role for the P-body component GW182 in microRNA function. *Nat Cell Biol*, **7**, 1261-1266.
161. Sen, G.L. and Blau, H.M. (2005) Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies. *Nat Cell Biol*, **7**, 633-636.
162. Petersen, C.P., Bordeleau, M.E., Pelletier, J. and Sharp, P.A. (2006) Short RNAs repress translation after initiation in mammalian cells. *Mol Cell*, **21**, 533-542.

163. Lanet, E., Delannoy, E., Sormani, R., Floris, M., Brodersen, P., Crete, P., Voinnet, O. and Robaglia, C. (2009) Biochemical evidence for translational repression by Arabidopsis microRNAs. *Plant Cell*, **21**, 1762-1768.
164. Kinch, L.N. and Grishin, N.V. (2009) The human Ago2 MC region does not contain an eIF4E-like mRNA cap binding motif. *Biol Direct*, **4**, 2.
165. Chendrimada, T.P., Finn, K.J., Ji, X., Baillat, D., Gregory, R.I., Liebhaber, S.A., Pasquinelli, A.E. and Shiekhattar, R. (2007) MicroRNA silencing through RISC recruitment of eIF6. *Nature*, **447**, 823-828.
166. Grossman, A.R., Harris, E.E., Hauser, C., Lefebvre, P.A., Martinez, D., Rokhsar, D., Shrager, J., Silflow, C.D., Stern, D., Vallon, O. *et al.* (2003) Chlamydomonas reinhardtii at the crossroads of genomics. *Eukaryot Cell*, **2**, 1137-1150.

CHAPTER 2

CHARACTERIZATION OF RNAi DEFECTIVE MUTANTS – MUT13-2 & MUT20- IN *CHLAMYDOMONAS REINHARDTII*

INTRODUCTION

The RNA-induced silencing complex (RISC) lies at the heart of the RNAi machinery, and yet the function of a number of its components is not completely understood. In order to decipher the function of such various components, we screened for RNAi-defective mutants in *Chlamydomonas reinhardtii*. We first generated the Maa7-IR44s strain which expresses an inverted repeat (IR) transgene homologous to a ~60 bp region on the 3' UTR of the MAA7 gene (which codes for tryptophan synthase ß subunit) (1). The strain Maa7-IR44s acts via translational repression whereby the level of the tryptophan synthase ß subunit protein is reduced without changes in the corresponding transcript amount (unpublished data). We next isolated, mutants defective in down-regulation of the Maa7 protein by using random insertional mutagenesis. Two related mutants – Mut13-2 and Mut20 – were found to be deleted for a number of the essential domains of the gene Tudor Staphylococcal Nuclease 1 (TSN1) also known as P100 (transcriptional co-activator protein 100) or SND1 (staphylococcal nuclease and tudor domain 1) in mammals, *Drosophila, C. elegans,* as well as in fission yeast (2). Through this study we have sought to identify the molecular role of TSN1 in the RNAi pathway.

TSN1 is a highly conserved and ubiquitous protein in eukaryotes and is found to be involved in various cellular processes such as transcription, spliceosome assembly, RNA stability, programmed cell death as well as in RNAi. In mammals, it has been identified as a transcriptional co-activator interacting with several transcription factors such as EBNA-2 (3), c-Myb (4), STAT-6 and STAT-5 (5,6). P100 is also known to interact with

the components of the U5 small nuclear ribonucleoprotein to assist in pre-mRNA splicing and spliceosome assembly (7). TSN1 is also involved in stress tolerance in plants such as Arabidopsis (8). It is able to co-localize with G3BP (a marker for formation of stress granules) in response to various stress stimuli and affects the aggregation efficacy of these stress granules (9). In mammals, formation of such stress granules is also an indication of various disease processes such as tumor formation in hypoxic conditions (10). In this regard, Tudor-SN1 has been shown to be upregulated during human colon cancers via post-transcriptional regulation of the adenomatous polyposis coli (APC) gene (11). Knockdown of TSN1 leads to ectopic cell death and inhibits plant fertility and, intriguingly, this multifunctional protein is also known to be a natural substrate for metacaspases in the programmed cell death degradome (12). In connection with RNAi, TSN1 has been found to co-fractionate with the RISC and was the first known subunit of the RISC to possess a recognizable (staphylococcal) nuclease domain (13). Human TSN1 has been shown to be involved in degradation of inosine-containing pre-miRNAs, such as the primary transcript of human miR-142, thus providing some sort of RNA surveillance/quality control mechanism (14,15). Although the structural features of the protein suggest a preference for binding IU containing RNA, a natural substrate for TSN1 is yet to be discovered.

Such multifunctional capabilities of TSN1 can be attributed to its complex domain architecture. Tudor-SN1 is composed of four tandem repeats of staphylococcal nuclease-like domains (SN1-4) adjoining a Tudor domain fused to a C-terminal partial SN5 domain, to form an extended TSN domain (2,16). The SN domains possess dsRNA-

binding capabilities (2), and mediate interactions with STAT6 (Signal Transducer and Activator of Transcription 6) and RNA Pol II (17). The Tudor domain consists of a barrel-like fold and is a protein-protein interaction domain (16). These motifs are abundant in various proteins and have been shown to interact with symmetricaldimethylated arginines (sDMAs) such as those in the C-termini of Sm proteins partaking in snRNPs biogenesis (18,19). Interestingly, the Tudor domains of several proteins are found to interact with sDMAs containing PIWI proteins (closely related to Argonautes) and participate in the piRNA pathway (20). Our findings in *Chlamydomonas reinhardtii* suggest that the TSN1 protein is important for RNAi, as has been demonstrated in other eukaryotes, and most importantly, TSN1 deletion results in depletion of the endogenous miRNA pool. However, addressing its specific molecular role will require further investigation.

MATERIALS AND METHODS

Culture Conditions, Transformation, Selection of transgenic strains & Spot Tests

Unless otherwise noted, *C. reinhardtii* cells were grown on Tris-Acetate-Phosphate (TAP) medium at 21°C under moderate light conditions (21). For generation of transgenic strains, transformation was carried out by introduction of linearized plasmid DNA into *Chlamydomonas* cells by the glass beads procedure (22). The Maa7-IR44s strain was generated from the wild-type CC-124 background by transformation of a *Ble* transgene construct (with the *MAA7* IR sequence in its 3' UTR) as previously described (1). For generation of the RNAi-defective mutants –Mut13-2 and Mut20- via random mutagenesis, the strain Maa7-IR44s was transformed with a plasmid construct imparting resistance to paromomycin (23). The complemented strains, mut13-2-8c, mut13-2-13c, mut20-133c and mut20-167c were generated by transformation of Mut13-2 and Mut20, respectively, with a plasmid construct containing the full-length coding sequence of TSN1 (with an N-terminal FLAG-CBP-AcV5 double tag).

For phenotypic analyses of the different strains, spot tests were carried out on TAP media containing 5-fluoroindole (5-FI). The strains CC124, Maa7-IR44s, Mut13-2, Mut20, mut13-2-8c, mut13-2-13c, mut20-133c and mut20-167c were grown for two days in liquid TAP medium. For each of the strains, ~$3x10^6$ cells were collected by centrifugation at 5000 rpm for 3 minutes and then resuspended in 150 µl of TAP medium. This volume, having a concentration of $1x10^5$ cells in 5 µl was then serially

diluted to the following concentrations per 5 µl – $2.5x10^4$, $6.25x10^3$, $1.56x10^3$ and $3.9x10^2$ cells. Five µl of each of the cell dilutions were plated on solid TAP+5-FI medium as well as on TAP plates and allowed to grow under dim lights for 7-15 days.

Analysis of the extent of genomic deletions in Mut13-2 & Mut20

Whole cell genomic DNA was isolated (from Wild-type and mutant strains) using standard procedures as previously described (24). The isolated genomic DNA was used as template for PCR reactions with primers specific for the different domains of TSN1 and a neighboring Ankyrin/Histone Deacetylase (HDAC) gene, to determine the extent of the deletions in the mutants. The PCR reactions were carried out with Taq Polymerase according to the conditions mentioned in Table 1.1 & Table 1.2. The PCR products were resolved on 1.2% agarose gels and visualized by ethidium bromide staining (1,25). Actin gene DNA was used as a positive control for the all of the PCR reactions with the following conditions – 25 cycles at 94˚C for 1 min, 50˚C for 40 sec, 72˚C for 40 sec. The specific primer sequences for the different domains are indicated in Tables 1.1 and 1.2.

Table 1.1 –List of primer sequences for TSN1 domains & the PCR conditions

DOMAINS	Forward Primer	Reverse Primer	PCR conditions
SN1 domain	F1 5’ATGGCGACCGG CTGGCTGC3’	R1 5’GTCCTTGGTCC ACAGCCCCAG3’	30 cycles at – 94˚ C – 1 min 62˚ C – 30 sec 72˚ C – 2 min
SN2 domain	F-SN2 5'GCGTGCGATGT AACCGTGTG3'	R-SN2 5'CTCCCAACCCG CGAAACAAC3’	30 cycles at – 94˚ C – 1 min 55˚ C – 30 sec 72˚ C – 40 sec
SN3 domain	F-SN3 5'CTCCGCAAGCG CATCATCG3'	R-SN3 5'CGCTACACGTTC CTCCCTCCTTG3'	30 cycles at – 94˚ C – 1 min 55˚ C – 30 sec 72˚ C – 40 sec
SN4 domain	f-4 5'GCCCTTCAACC TGGCACTGG3'	r-4 5'GAGCTTGCCCTC CTTTGCTGC3'	30 cycles at – 94˚ C – 1 min 55˚ C – 40 sec 72˚ C – 40 sec
Linker Region	FF2 5’CTGCGTTGCGT GTTGCGTTG3’	RR2 5’CCACCACCTGA GAGAGAGCGAGA G3’	30 cycles at – 94˚ C – 30 sec 59˚ C – 30 sec 72˚ C – 40 sec
TSN fused domain	f'-sn5 5'GAGGCCATCAC AGCCATGCAG3'	r'-sn5 5'GGTCGCCGTAC ACGAACATGC3'	30 cycles at – 94˚ C – 1 min 55˚ C – 40 sec 72˚ C – 40 sec

Table 1.2 –List of primer sequences for HDAC/Ankyrin domains & the PCR conditions

Domains	Forward Primer	Reverse Primer	PCR Conditions
Ankyrin Repeat 1	HDAC-Fa 5’GCCTGGTCAACT TCGCGGATAG3’	HDAC-Ra 5'GGCACATCCA ACAACGCACCC 3'	30 cycles at – 94˚ C – 1 min 58˚ C – 40 sec 72˚ C – 1 min
Ankyrin Repeat 2	F-HDAC2 5'GCGTGAGAAGTT GGGCTGGATG3'	R-HDAC2 5'CTGAATGAGC TGCGCCACGA3'	30 cycles at – 94˚ C – 1 min 59˚ C – 40 sec 72˚ C – 30 sec
Ankyrin Repeat 3	HDAC-Fc 5'CACCAACCACAG AGACGGGCATC3’	HDAC-Rc 5'CCCGCTGTCGT TCTGTCGTCTC3'	30 cycles at – 94˚ C – 1 min 56˚ C – 30 sec 72˚ C – 1 min
Arginase/ Deacetylase Superfamily	F-HDAC4 5'CCAACACCGCAC CGTATGACC3'	R-HDAC4 5'GCAACAGGTT TCACACGGCG3'	30 cycles at – 94˚ C – 1 min 58˚ C – 30 sec 72˚ C – 30 sec

RNA extraction & Small RNA analyses

Standard protocols were used for nucleic acid isolation, gel electrophoresis and ^{32}P -probe labeling (24-26). Total RNA extraction was carried out with TRI reagent (Molecular Research Center) according to the manufacturer's instructions. For small RNA analyses, the isolated total RNA was resolved on 15% polyacrylamide (29:1)/ 7 M Urea gels and electroblotted onto Hybond-XL membranes (GE Healthcare) (1,27) at 0.9 Amps for 2 hours. Transferred small RNAs were UV-crosslinked to the nylon membrane (25). For detection of specific miRNAs, these blots were then hybridized with complementary DNA oligonucleotides labeled at the 5' termini with γ-^{32}P using T4 Polynucleotide Kinase (New England Biolabs). Hybridization was carried out at 40˚C for 72 hours using the High Efficiency Hybridization System – HS114 (Molecular Research Center). As a loading control, the nylon membranes were probed for U6 snRNAs using an α-^{32}P -labeled U6 probe and the hybridization was carried out at 65˚C for 24 hours (28). For analyses of the Maa7 siRNAs, total RNA was resolved on a 15% polyacrylamide (29:1)/ 7M Urea/ MOPS gel and electroblotted onto a Hybond-NX membrane (GE Healthcare) (26). The blots were then chemically crosslinked using EDC (26) and a specific α-^{32}P labeled Maa7 probe was used to detect the corresponding siRNAs. The ^{32}P radioactivity signal from the blots was detected with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) and images were quantified using the Quantity One software (BioRad).

Plasmid Construction

The TSN1 gene (512365; au5.g2631_t1) is located on chromosome 11 in the *Chlamydomonas* genome and consists of 27 exons (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). The TRI reagent-isolated RNA was treated with DNase-I (Ambion, TX, USA) for 30 min at 37˚C to remove contaminating DNA. The DNase-I treated RNA was subsequently used for first strand cDNA synthesis with SuperScript II reverse transcriptase (Invitrogen) using gene-specific primers and reaction conditions as previously described (29). Three fragments of the full length TSN1 cDNA were amplified from the synthesized cDNA template using TaKaRa LA Taq™ Polymerase (Takara Bio Inc.) with the primer sequences & PCR conditions described in Table 2.

Table 2.0 –List of primer sequences for full-length TSN1 coding sequence & the PCR conditions

Fragments	Forward Primer	Reverse Primer	PCR conditions
Fragment 1	TSN-F1’ 5’CGGTCATGAGCGACCGGCTGGCT3’	TSN-R2’ 5’CTGACCACCTCCACCACCTTGC3’	26 cycles at – 94˚ C – 30 sec 60˚ C – 30 sec 72˚ C – 2 min
Fragment 2	TSN-F3 5’GTGGAGTGGTCGCTGAACCTGATG3’	TSN-R3’ 5’TTAGCACCACGGAGCCCAGGAAG3’	28 cycles at – 94˚ C – 30 sec 58˚ C – 30 sec 72˚ C – 1 min
Fragment 3	TSN-F4’ 5’GTTTGCGGAGGAGGCGTTTGC3’	TSN-R4’ 5’CAGGATATCCTACCGCCGCACG3’	28 cycles at – 94˚ C – 30 sec 57˚ C – 30 sec 72˚ C- 2 min

The PCR products were resolved on 1.2% agarose gels and visualized by ethidium bromide staining (25). Each of these amplified fragments was cloned into the pSTBlue-1 vector (Novagen), according to standard procedures (25), and the insert sequenced using SP6 and T7 primers. The three correct fragments were then spliced together using overlapping restriction sites (Fig. 2-2A) to generate the full length TSN1 coding sequence. The full length TSN1 fragment was finally excised from the pSTBlue-1 vector and cloned into a *Chlamydomonas* expression vector containing an N-terminal FLAG-CBP-AcV5 double tag under the control of Hsp70a and psaD (photosystem I component) promoter and terminator. The insert was verified to be in-frame with the epitope tag by sequencing using psaD promoter/terminator primers.

Immunoblot Analyses

Standard protocols were used for immunoblot analyses (1). In order to detect the AcV5-tagged TSN1 protein, cells were grown to logarithmic phase and approximately $5x10^6$ cells were pelleted by centrifugation (13,000 g for 3 minutes) and resuspended in 50 µl of SDS-gel sample loading buffer (25). The samples were boiled for 5 minutes and 10 µl aliquots were resolved via 8% SDS-PAGE and electroblotted onto nitrocellulose membranes (25). For immunodetection of the AcV5-tagged TSN1 protein, the membrane was incubated overnight at 4˚C in a 1:10000 dilution of a mouse raised anti-AcV5 antibody (eBioscience 14-6995). Post-incubation, the membrane was incubated for 2 hours at room temperature in a 1:30000 dilution of a rabbit anti-mouse secondary antibody, conjugated to horseradish peroxidase (HRP). For autoradiographic detection, a chemiluminescent substrate (Immobilon™, Millipore) was used. For immunodetection of the tryptophan synthase ß (TSß) protein, an anti-TSß antibody (polyclonal raised in rabbits), kindly provided by Thomas McKnight, was used at a dilution of 1:10000. Membranes were incubated overnight at 4˚C and then treated at room temperature for 2 hours, with a 1:30000 dilution of a goat anti-rabbit secondary antibody conjugated to HRP before being used for autoradiographic detection. For immunodetection of Argonaute 3 (AGO3) protein levels in the different strains, a 8% SDS-PAGE gel was used to fractionate samples as before and the membranes with electroblotted proteins were incubated overnight at 4˚C with a 1:15000 dilution of a rabbit antibody raised against the C-terminal peptide of AGO3 conjugated to KLH (CASRSGRGAGAAEGG; GenScript). The membrane was incubated (for 2 hours at room temperature) with a

1:15000 dilution of a goat anti-rabbit secondary antibody conjugated to HRP, before being used for autoradiography. To test for equivalent loading of the lanes, Coomasie Blue staining of equivalent SDS-PAGE gels as well as immunoblotting with a polyclonal anti-histone H3 antibody (AbCam Cambridge, MA, USA ab1791) were used.

Detection of epitope-tagged TSN1 protein by Immunofluorescence Microscopy

For elucidation of the sub-cellular localization of the epitope-tagged TSN1 protein in the complemented strains, immunofluorescence microscopy was carried out using a modification of the protocol already described by Sanders and Salisbury (30). Cells were inoculated from TAP plates into 5 ml of liquid TAP media and grown for 48 hours. Cell density was determined with a hemocytometer and ~$4x10^5$ cells were sub-cultured into another 4 ml of TAP media for 48 hours. The mutant strains – Mut13-2 and Mut20 – were used as negative controls. The following steps were undertaken for preparation of slides, fixing of cells and subsequent antibody treatment before the slides were ready for imaging with a fluorescence microscope.

i. Slide Preparation:

Microscope slides were cleaned as described earlier (30) with Alconox detergent and after rinsing with ddH_2O, soaked in 5 mM ethylenediaminetetraacetic acid (EDTA) for 10 minutes, rinsed off with ddH_2O and allowed to air dry. A small area of the

slide was coated with freshly made 0.1% polyethyleneimine (PELI) solution, incubated for 1 minute and then rinsed off with ddH_2O and allowed to air-dry.

ii. Fixing cells to prepared slides:

Approximately 200 µl of cells from the culture in mid-logarithmic phase were placed onto the PELI coated area of the slide. The culture was allowed to settle for 15 minutes and then excess cells were drained off. Cells were fixed by covering the slide with 4% Paraformaldehyde in 1X Phosphate Buffered Saline (PBS) for 30 minutes. The paraformaldehyde solution was drained off immediately afterwards and the slide was washed with methanol. Fixation was continued by soaking the slide two times for 10 minutes each in methanol that was pre-chilled to –20˚C.

iii. Cell Rehydration:

Cells were rehydrated by washing/soaking the slides in 1X PBS for 15 minutes and then incubated with 1X PBS containing 0.05% Triton-X for 10 minutes. Washing was carried out twice again for 15 minutes in 1X PBS.

iv. Blocking Buffer & Antibody/ DAPI treatments:

The cells were covered with blocking buffer (see composition below), and incubated for 2 hours in a humidity chamber (Sigma H6644) and then excess buffer was drained off. Cells were covered with blocking buffer containing primary antibody (affinity purified monoclonal mouse anti-baculovirus envelope gp64 protein – Anti-AcV5, eBioscience 14-6995) at a 1:10000 dilution and incubated overnight in the humidity chamber at 4˚C. Slides were then soaked 3 times for 15 minutes each in 1X PBS. Excess primary antibody solution was drained off. Next, blocking buffer containing secondary antibody (Alexa Fluor 488 conjugated goat anti-mouse, Invitrogen A11001) at a 1:500 dilution was used

to cover the slides and incubated for 2 hours in the humidity chamber. Cells were next covered with a 5 µM 4',6-diamidino-2-phenylindole (DAPI) solution and incubated for 20 minutes at room temperature in the dark. The slides were then soaked 3 times for 15 minutes each in 1X PBS and allowed to air-dry. Coverslips were mounted over the cells using GEL-MOUNT (Electron Microscopy Sciences 17985-10).

v. Microscopy:

Cells treated in the above manner were observed and imaged using an Olympus Flouview 500 Laser Scanning Fluorescence Microscope with Olympus BX60 software version 4.3. DAPI was excited with a 405 nm diode laser and was detected between 430-480 nm. The Alexa Fluor 488 was excited with a 488 nm laser and detected between 505-525 nm. The phase contrast images were obtained using the collection of transmitted light with a 633 nm laser. Image processing and pseudocoloring were done using ImageJ (National Institutes of Health, Bethesda, Maryland, USA).

Solutions:

- 0.1% PELI – 10 µl polyethyleneimine (Sigma, P3143) dissolved in ddH2O to a final volume of 10 ml
- Blocking Buffer – 10 mM KH2PO4 pH 7.2, 10 mM K2HPO4 pH 7.2, 5% Glycerol, 5% Normal Goat Serum (BioSource PCN5000), 1% Cold Water Fish Gelatin (Sigma G7765), 1% Bovine Serum Albumin and 0.04% Sodium Azide
- 5µM DAPI solution – 5 µl of 1 mg/ml DAPI (Sigma D9542) stock diluted in 1 ml ddH_2O

Immunoprecipitation of epitope tagged-TSN1

In order to purify the FLAG-tagged TSN1 protein being expressed in the complemented strains –mut13-2-8c and mut13-2-13c- the transgenic strains were grown to midlogarithmic phase in TAP medium containing 7.5 μM 5-FI and harvested by centrifugation at 5000 rpm for 10 minutes. The Flag-Ble13 strain was used as a purification control. This transgenic strain contains a dominant selectable marker (*Ble* transgene) conferring resistance to the Bleomycin antibiotic. The Ble protein contains a FLAG-CBP-AcV5 tag and expresses as a ~19 kDa fusion protein. For each immunoprecipitation experiment, ~ $5x10^9$ cells were resuspended in lysis buffer [20 mM Tris-HCL (pH 7.5), 150 mM NaCl, 0.1 mM EDTA, 5 mM Mg-Acetate, 2.0 mM CaCl2, 10% glycerol] containing 2.0 mM Benzamidine, 0.2 mM PMSF, 5μl/ml plant protease inhibitor (Sigma). Cells were broken by two passages through a French-press at 5000 psi. An aliquot of the total lysate was stored (at -20˚C) for SDS-PAGE and immunoblot analyses. The lysate was centrifuged at 16000 g for 30 minutes at 4˚C and the supernatant (Fraction S16) was centrifuged again at 100000 g for 90 minutes at 4˚C to separate out soluble proteins (Fraction S100). The supernatant was incubated with ANTI-FLAG (M2) beads (Sigma A2220) overnight at 4˚C. After overnight incubation, the beads were collected by centrifugation (~1500 g for 5 minutes) and before proceeding to the washing steps an unbound fraction was also collected (Unbound protein fraction) from the bead supernatant. The beads were then washed with the following buffers for 15 minutes (each wash) at 4˚C:

Three washes with Washing Buffer A (20 mM Tris-HCL (pH 7.5), 300 mM NaCl, 0.1 mM EDTA, 2 mM Mg-Acetate, 2 mM $CaCl_2$, 10% glycerol) and two washes with Washing Buffer B (20 mM Tris-HCL (pH 7.5), 150 mM NaCl, 2 mM Mg-Acetate, 2 mM $CaCl_2$, 10% glycerol). Both wash buffers were supplemented with 2.0 mM Benzamidine, 0.2 mM PMSF, 5µl/ml plant protease inhibitor (Sigma) and 0.1% Triton X-100. Aliquots of the first (Wash 1) and the last wash (Wash 2) were stored to be used for SDS-PAGE and immunoblot analyses. The beads were resuspended in 2X SDS sample loading buffer (25) and the eluted proteins were resolved on a 8% SDS-PAGE gel (for FLAG-AcV5-tagged TSN1 detection) or a 15% SDS-PAGE gel (for AcV5-Flag-Ble detection as a control for non-specific association of proteins with the beads). The other fractions collected during the purification process were also separated by SDS-PAGE and subjected to immunoblot analyses with an anti-AcV5 antibody. AGO3 primary antibody was used, as described before, in immunoblotting analyses to ascertain the presence of this protein in the FLAG purified TSN1 complex.

RESULTS

Mut20 shows a larger genomic deletion compared to Mut13-2

Genomic DNA isolated from the wild-type CC-124 and the mutant strains –Mut13-2 and Mut20- was used for PCR amplification of specific domains of the TSN1 gene and the neighboring Histone Deacetylase (HDAC) gene containing Ankyrin repeats at its 5'-terminus (Figure 2-1A). The TSN1 gene (512365; au5.g2631_t1) consists of four staphylococcal domains (SN1-4), which are joined to a fused (Tudor-SN5) TSN domain via a short linker region. The SN1 domain was not detected in the PCR amplifications using as template Mut20 genomic DNA, while the region was amplified in the case of Mut13-2. The SN2-4 domains as well as the linker region and the fused TSN domain were not detected in the PCR amplifications from both Mut13-2 and Mut20 (Figure 2-1C), while all the expected PCR fragments were obtained when using as template DNA from the wild-type CC-124 (used as a positive control).

To determine the extent of the genomic deletion in both mutants, the DNA from both strains was also used for amplifying the specific domains of the neighboring Histone Deacetylase gene (512363, au5.g2630_t1). The Ankyrin repeats 1 and 2 on the 5'-terminus of the gene were detected in the PCR amplifications from both Mut13-2 and Mut20 (Figure 2-1B), but Ankyrin repeat 3 was found to be missing in Mut20 while still present in Mut13-2. Both mutants were found to lack the Arginase/Deacetylase domain, while all the motifs tested were successfully amplified in the wild-type CC-124.

Amplification of the Actin1 gene was used to assess the quality of the genomic DNA isolated from the respective strains (Figure 2-1B and C).

Taken together, the data suggests that Mut20 has a larger genomic deletion when compared to Mut13-2, including complete deletion of the TSN1 gene. In contrast, Mut13-2 may still express a truncated version of the TSN1 gene including at least the SN1 domain. The neighboring gene HDAC is also partly deleted in both mutants and it may be relevant for further characterization of the mutants and their RNAi defect. However, considering the fact, that TSN1 has been implicated in RNAi and RISC association in metazoans (2,13), we have aimed towards complementing TSN1 function in our mutants, to investigate whether this would reconstitute the RNAi phenotype.

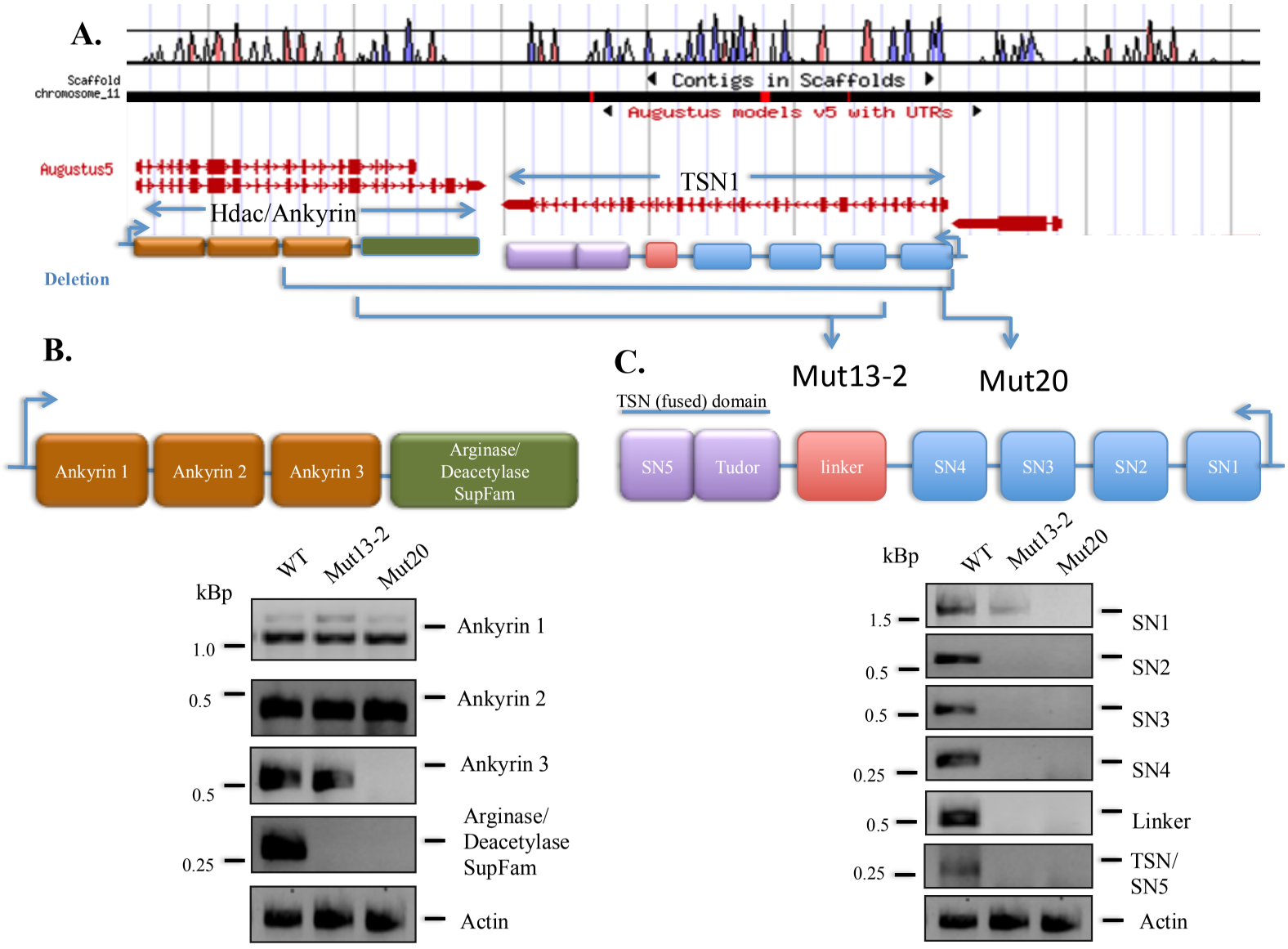

A.
Scaffold
chromosome_11
Contigs in Scaffolds
Augustus models v5 with UTRs
Augustus5
TSN1
Hdac/Ankyrin
Deletion
Mut13-2
Mut20
B.
Ankyrin 1
Ankyrin 2
Ankyrin 3
Arginase/
Deacetylase
SupFam
C.
TSN (fused) domain
SN5
Tudor
linker
SN4
SN3
SN2
SN1
kBp
WT
Mut13-2
Mut20
1.0
0.5
0.5
0.25
Ankyrin 1
Ankyrin 2
Ankyrin 3
Arginase/
Deacetylase
SupFam
Actin
kBp
WT
Mut13-2
Mut20
1.5
0.5
0.5
0.25
0.5
0.25
SN1
SN2
SN3
SN4
Linker
TSN/
SN5
Actin

Figure 2-1. Genomic Deletions in the RNAi-defective mutants –Mut13-2 & Mut20
(A) Deletions generated by random insertional mutagenesis in the two mutants are shown in a genomic context in the JGI genome browser*. **(B)** The Hdac/Ankyrin gene consisting of three ankyrin domains and one arginase/deacetylase domain. The figure illustrates that both mutants are missing the arginase/deacetylase domain while ankyrin 1 and ankyrin 2 domains are present in both. Ankyrin 3 domain is present in Mut13-2 while it is absent in Mut20. **(C)** The TSN1 gene consists of four SN domains linked to a fused TSN domain (SN5-Tudor). In case of Mut20 the full length TSN1 gene is found to be missing, while in Mut13-2 the SN1 domain still appears to be present.

The wild-type strain (CC-124) was used as a positive control. Amplification of the Actin1 gene was also carried out to test the quality of the genomic DNA template from all strains. The color-coded domains in (A) are the same as indicated in (B) and (C).
*(http://genome.jgi-psf.org/cgi-bin/browserLoad/?db=Chlre4&position=chromosome_11:1794646-1834952).

Complementation of Mut13-2 & Mut20 with epitope-tagged TSN1

In order to assemble the full-length length TSN1 coding sequence, three different fragments (I ~1.2 kb, II ~0.9 kb, III ~1.2 kb) of the TSN1 gene were amplified from the cDNA obtained by reverse-transcription of total RNA, purified from the strain Maa7-IR-44s (data not shown). The construct was cloned into a *Chlamydomonas* expression vector (NE689) under the regulatory control of Hsp70a and PsaD (Photosystem I component) dual promoter sequences to achieve constitutive expression of the insert (Figure 2-2A). Using the glass beads procedure (22), the epitope-tagged TSN1 construct was introduced into the Mut13-2 and Mut20 strains to achieve stable nuclear transformation. Transformants were initially screened by plating on 10 μM 5-FI TAP media, for recovery of the RNAi phenotype, and subsequently via immunoblot analyses to detect the expression of AcV5-tagged TSN1. The levels of tryptophan synthase ß-subunit protein (TSß) in selected strains were also ascertained by immunoblotting (Figure 2-2B). The Maa7-IR44s strain is RNAi activated and acts via the translational repression of the TSß protein (unpublished data). The mutant strains (Mut13-2 and Mut20) showed higher levels of TSß than that of the Maa7-IR44s strain indicating a defect in RNAi. Hence, as suggested from early studies in metazoans (13), it seems that TSN1 function is also essential for RNAi activity in *Chlamydomonas reinhardtii*.

In the case of the Mut13-2 transformants, mut13-2-8c and mut13-2-13c showed good levels of expression of the epitope-tagged TSN1 construct. The TSß levels for the

transformants were found to be lower than that of Mut13-2 and closer to that of Maa7-IR44s (Figure 2-2B). For phenotypic analyses, the indicated strains (Figure 2-2C) were serially diluted and cells were spotted on 10 μM 5-FI TAP medium. The growth pattern of spotted cells was inversely correlated with the TSß levels for the examined strains. The mutants as well as the wild-type CC-124 failed to grow on 5-FI while the Maa7-IR-44s strain and the Mut13-2 complemented strains showed comparable growth. This indicates that expression of the epitope-tagged TSN1 construct successfully reverted the RNAi-defective phenotype in Mut13-2.

Two Mut20 transformants –mut20-133c and mut20-167c- were chosen for further analyses after screening for transformants with appropriate levels of expression of the AcV5-tagged TSN1 protein via immunoblotting. The TSß protein levels for the Mut20 transformants -mut20-133c and mut20-167c- were a bit lower than that of Mut20 although not as low as the Maa7-IR-44s strain (Figure 2-2D). This expression analysis was also supported by spot test assays on 10 μM 5-FI TAP, where the wild-type CC-124 and the Mut20 strain died or grew poorly. In contrast, the Mut20 transformants did show some degree of survival of 5-FI, but did not grow as well as the Maa7-IR-44s strain (Figure 2-2E). This suggested that the RNAi phenotype was only partially complemented, at best, in the Mut20 complemented strains.

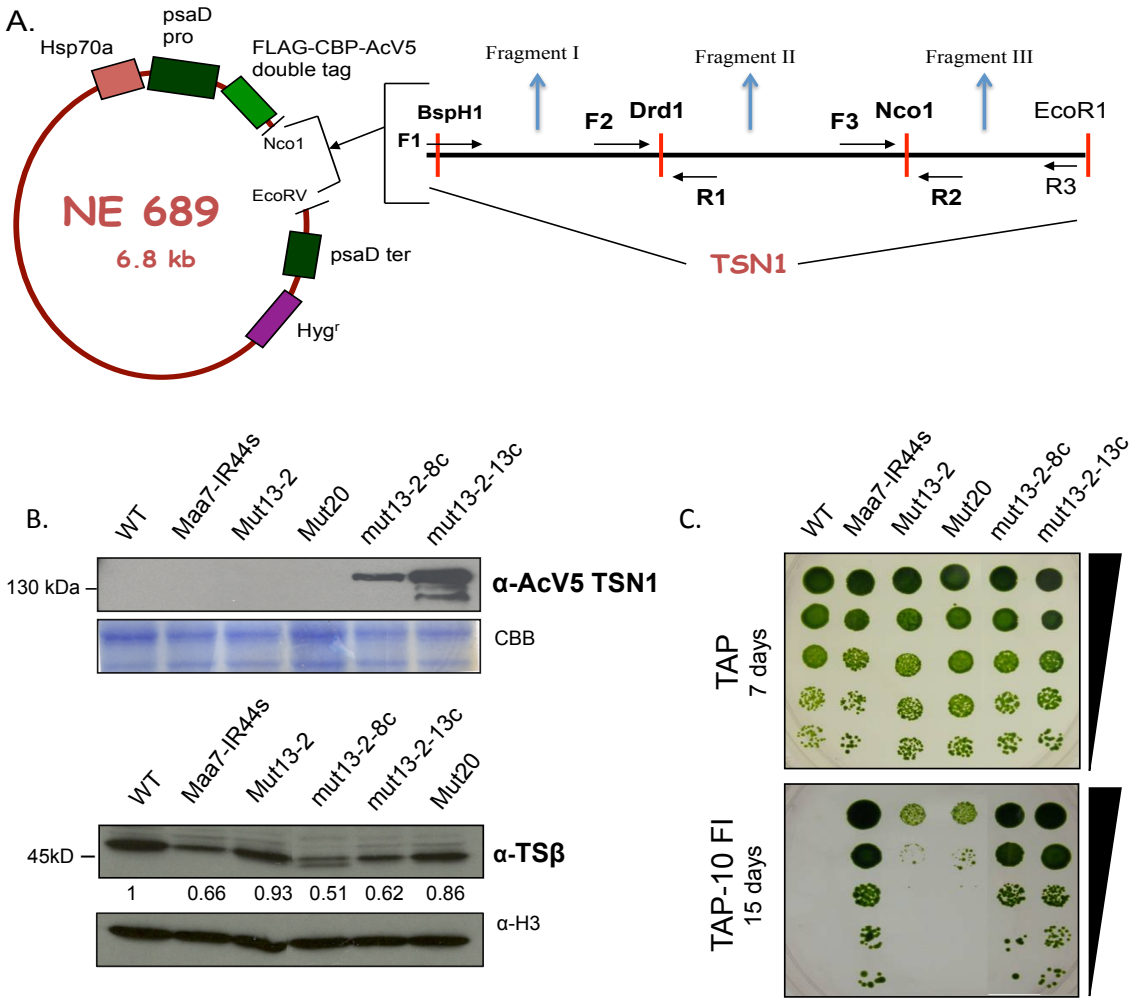


Figure 2-2. Complementation of RNAi-defective mutants with epitope-tagged TSN1
(A) The full-length TSN1 coding sequence was cloned into a *Chlamydomonas* expression vector (NE689) that contains an N-terminal FLAG-CBP-AcV5 tag. The TSN1-NE689 construct was used to transform Mut13-2 and Mut20 using the glass beads procedure (22). **(B)** Immunoblot analyses for detection of the AcV5-tagged TSN1 protein in the Mut13-2 complemented strains. A separate Coomasie-stained gel was used as a loading control. Anti-TSß antibody was used to detect the levels of the tryptophan synthase ß-subunit protein in the indicated strains. Detection of histone H3 was used as a loading control. **(C)** Phenotypic analyses – growth and survival on TAP medium and TAP containing 10 µM 5-FI for wild-type CC-124, Maa7-IR-44s, Mut13-2, Mut20 and two Mut13-2 complemented strains –mut13-2-8c and mut13-2-13c.

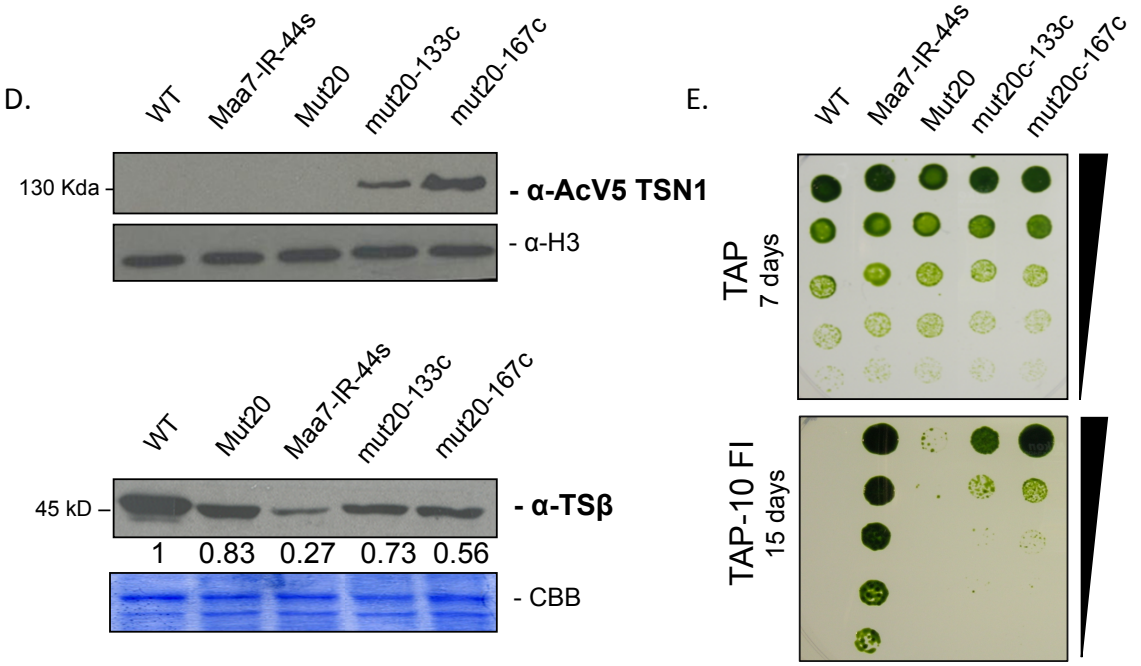


Figure 2-2 (continued). Complementation of RNAi-defective mutants with epitope-tagged TSN1 – (D) Immunoblot analyses for AcV5-tagged TSN1 shows that mut20-133c and mut20-167c express appropriate levels of tagged-TSN1 protein. Histone H3 was used as a loading control. TSß protein levels were also detected by immunoblotting in the indicated strains. The two Mut20 complemented strains showed lower levels of TSß in comparison with the mutant. An equivalent, Coomasie-stained, gel was used as loading control. **(E)** Phenotypic analyses – growth and survival on TAP and TAP medium containing 10 µM 5-FI. The wild type CC-124 and Mut20 died on 5-FI plates whereas the complemented strains –mut20-133c and mut20-167c- showed poor growth on 5-FI containing medium. Hence, the RNAi phenotype seems only partially restored in the Mut20 complemented strains.

MicroRNA & Maa7 small interfering RNA levels in Mut13-2 and Mut20

In order to investigate the levels of endogenous miRNAs in Mut13-2, Mut20 as well as in the complemented strains, total RNA isolated using TRI reagent was resolved on a denaturing polyacrylamide gel, using standard protocols (27), and subsequently probed for small RNAs using ^{32}P-labeled oligonucleotides. We probed for two species of endogenous miRNAs –miR248 and miR2726. In the case of Mut13-2, the miRNA levels did not seem to vary much as compared to the Maa7-IR44s strain, whereas Mut20 showed significant reduction in the amounts of miR248 and miR2726 (Figure 2-3A).

For the Mut13-2 complemented strain, mut13-2-8c displayed somewhat elevated levels of miRNAs whereas mut13-2-13c did not show an appreciable change in miRNA amounts (Figure 2-3A). In contrast, the Mut20 complemented strains showed only a very minor, if at all, increase in endogenous miRNAs levels (Figure 2-3B). The AGO3 protein, which is an integral component of the core RNAi machinery in *Chlamydomonas* (31), correlated in abundance with those of the endogenous miRNAs (Figure 2-3C). This indicated that in the case of the Mut20 transformants there is, at best, partial complementation of the RNAi defect. We also examined the Maa7 siRNAs, which were depleted in Mut20 in comparison with the parental strain Maa7-IR44s (Figure 2-3D). In contrast, Mut13-2 only showed a partial reduction of Maa7 siRNAs amounts. The mut13-2-8c strain showed a clear increase in Maa7 siRNA levels, thus indicating successful complementation of the Mut13-2 mutant. For the Mut20 complemented strains, however,

the Maa7siRNAs did not revert back to Maa7-IR44s levels, further confirming that the RNAi machinery is not fully recovered in these transformants.

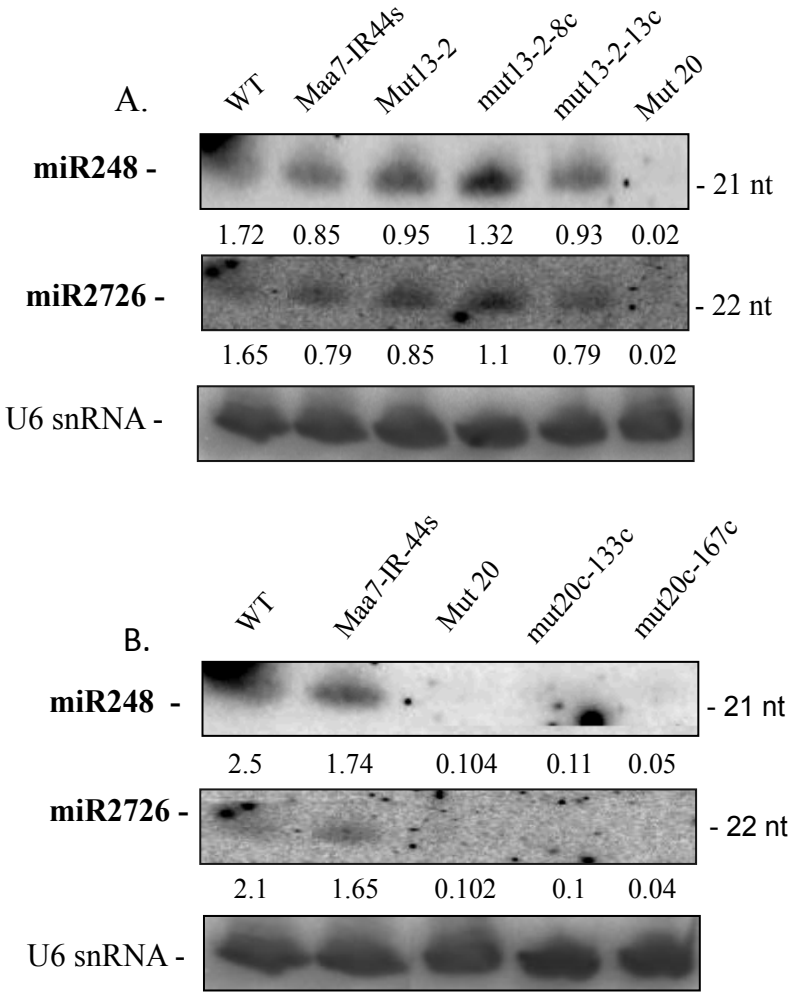


Figure 2-3. Endogenous miRNAs and Maa7 siRNAs in Mut13-2 & Mut20 - (A) Northern blotting for detection of miR248 and miR2726 in the indicated strains, using radiolabeled anti-miR248 & anti-miR2726 oligonucleotides. Wild type and Maa7-IR44s showed standard levels of the examined miRNAs. The miRNA pool was depleted in Mut20 whereas the change in Mut13-2 was not as significant. The Mut13-2 complemented strains did not show appreciable change in miRNA levels as well. Hybridization to the U6 snRNA was used as control for equivalent loading of all lanes. **(B)** Northern blotting for miR248 and miR2726 in the Mut20 complemented strains. U6 snRNA hybridization was used to assess equivalent loading of the lanes.

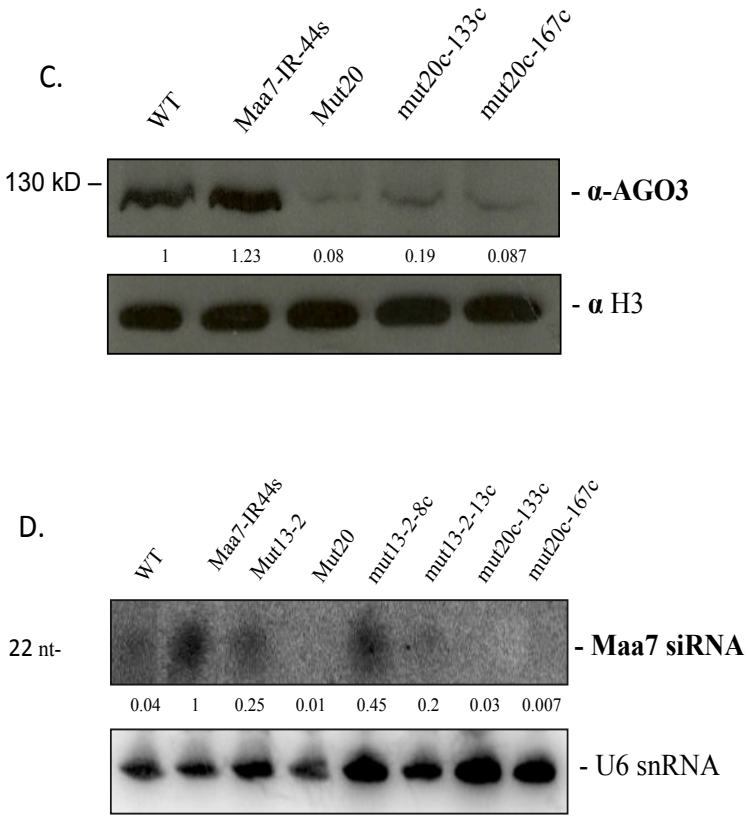


Figure 2-3. (continued)– (C) Immunoblotting analyses for detection of the AGO3 protein (an essential component of the core RNAi machinery in *Chlamydomonas*) in Wild type, Maa7-IR44s, Mut20, mut20-133c and mut20-167c strains. Detection of histone H3 was used as a loading control. **(D)** Northern blot analyses for detection of Maa7 siRNAs. The RNAi activated strain – Maa7-IR44s- showed, as expected, the highest level of Maa7siRNAs. U6 snRNA hybridization was used to detect equivalent loading of all lanes.

Subcellular localization of TSN1 in *Chlamydomonas reinhardtii*

The subcellular localization of a particular protein is to a certain extent suggestive of its function within a cell. This is especially important considering the fact that the RNAi pathway is highly compartmentalized into distinctive nuclear and cytoplasmic phases (32). To investigate the subcellular localization of the TSN1 protein in *Chlamydomonas reinhardtii*, we utilized an Alexa Fluor 488 secondary antibody to detect by fluorescence microscopy the N-terminal AcV5 tag of TSN1. The mut13-2-13c and mut20-167c transformants showed appreciable levels of expression of the tagged protein (Figure 2-3) and were selected for these experiments. The respective mutants –Mut13-2 and Mut20- were used as negative controls and revealed that there was no detectable cross-reactivity of either the primary or secondary antibodies with non-specific proteins in the fixed cells (Figure 2-4B and D).

In both transformed strains, there was a detectable Alexa Fluor 488 signal indicative of the presence of the AcV5-tagged TSN1 protein (Figure 2-4A and C). DAPI staining revealed the localization of the nuclear DNA as well as the plastid DNA nucleoids. The Alexa Fluor 488 signal for the mut13-2-13c strain showed a diffused distribution over the cells, suggesting dual localization of the TSN1 protein in the cytoplasm as well as in the nucleus. The mut20-167c strain showed greater specificity of the Alexa Fluor 488 signal concentrated mostly in the cytoplasm, although fluorescence was also visible in a perinuclear fashion (Figure 2-4C).

Approximately 110 cells from the mut20-167c images were examined individually to assess the preferential localization of the AcV5-tagged TSN1 protein (Figure 2-4E). Of the counted cells, 63% showed only cytoplasmic localization whereas 32% had both cytoplasmic and nuclear signals. These results seem to suggest a predominant cytoplasmic localization of the AcV5-tagged TSN1 protein, which supports its association with the RISC in the cytosol as revealed from other studies in metazoans (13). However, a dual nucleo-cytoplasmic localization could not be ruled out as seen with the perinuclear signals and the overlap with the DAPI stained nucleus. This provides support for the multifunctional nature of the TSN1 protein, which has also been revealed to partially localize to the nucleus in *Arabidopsis thaliana* (2,8).

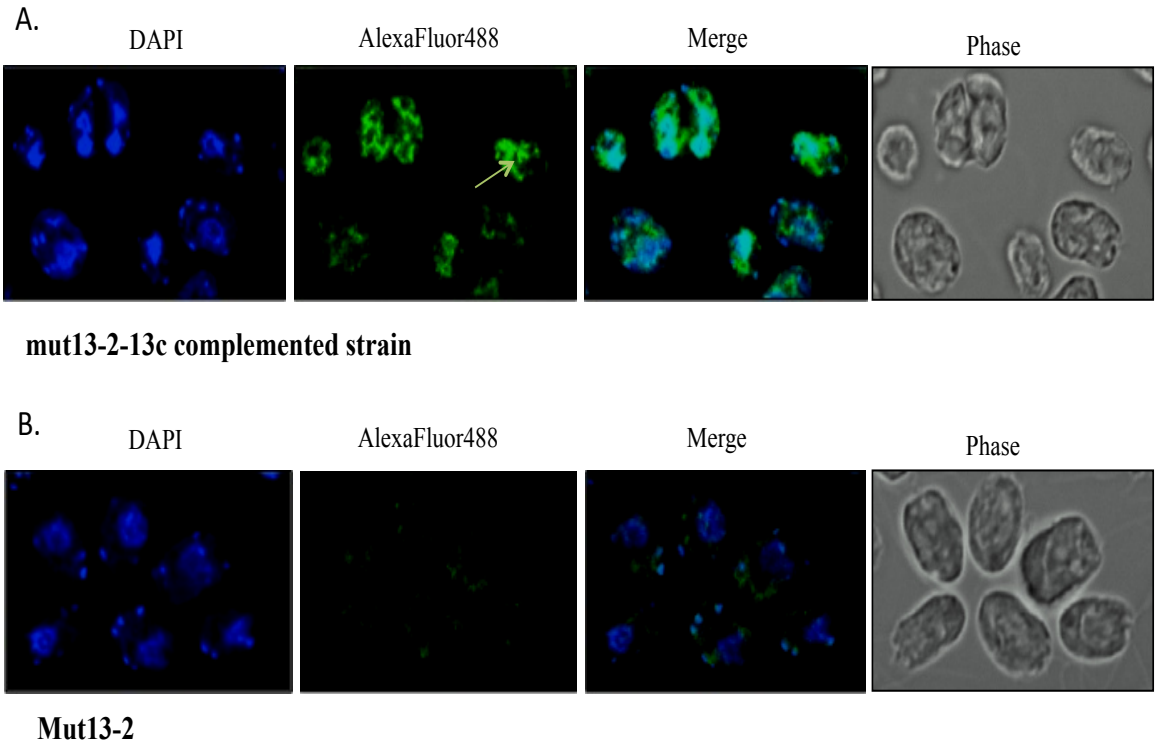
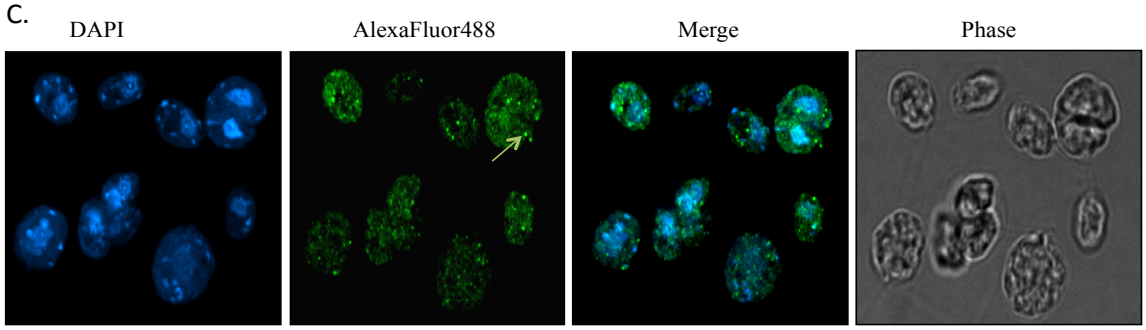


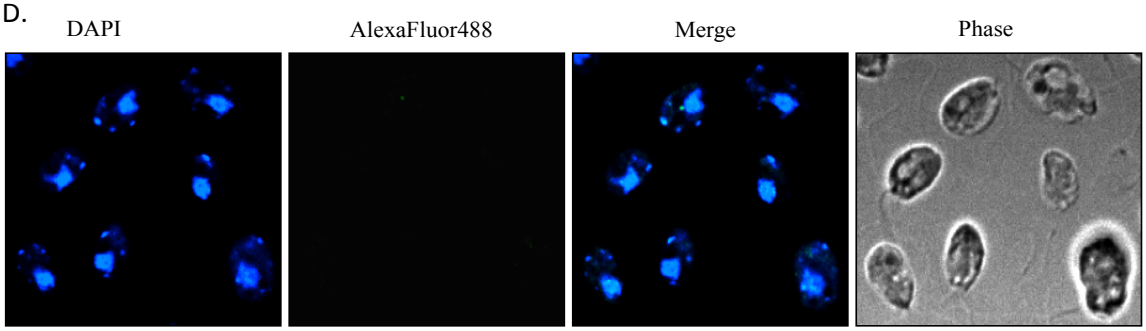
Figure 2-4. Immunofluorescence detection of epitope-tagged TSN1 – (A) The Mut13-2 complemented strain –mut13-2-13c- expressing the AcV5-tagged TSN1 protein was used to ascertain the subcellular localization of TSN1. The arrow points to the green signal indicating the TSN1 localization **(B)** Mut13-2 was used as a negative control for the experiments. No significant signal was detected for Alexa-Fluor 488. From left to right: DAPI staining (blue) of DNA, Alexa Fluor 488 bound to anti-AcV5 (green), merged image of DAPI and Alexa Fluor 488 signals and phase contrast image.

C.



mut20-167c complemented strain

D.



Mut20

E.

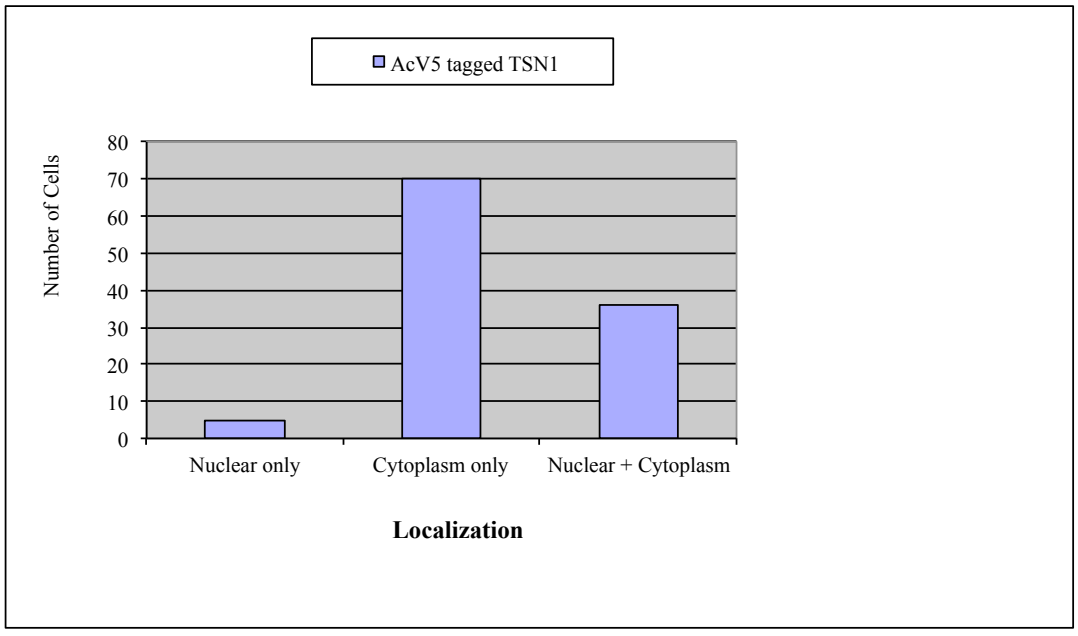


Figure 2-4 (continued). Immunofluorescence detection of AcV5-tagged TSN1 – (C) Mut20 complemented strain –mut20-167c- used to visualize the subcellular localization of tagged TSN1. The signal was mostly localized in the cytoplasm although some perinuclear localization was also detected. **(D)** Mut20 was used as a negative control. No obvious signal was detected for Alexa Fluor 488. From left to right: DAPI staining (blue) of DNA, Alexa Fluor 488 bound to anti-AcV5 (green), merged image of DAPI and Alexa Fluor 488 signals and phase contrast image. **(E)** Approximately 110 cells (mut20-167c) were individually examined for the subcellular localization of AcV5-tagged TSN1. The detected signal was mostly cytoplasmic although a number of cells showed both nuclear and cytoplasmic localization.

Co-Immunoprecipitation of AcV5-tagged TSN1

TSN1 has been shown to be a component of the RISC complex in *Drosophila, C. elegans* as well as in mammals (13,14) and this is consistent with our sub-cellular localization studies in *Chlamydomonas reinhardtii*. In *Drosophila*, a Tudor domain-containing protein –Tud- has been shown to interact with the PIWI protein AGO3 (33). Considering these facts we wanted to investigate whether TSN1, which is also a Tudor-domain containing protein, is capable of interacting with the Argonaute AGO3 in *Chlamydomonas reinhardtii*. The complemented strains –mut13-2-8c, mut13-2-13c- were used to affinity purify the epitope-tagged TSN1 protein using FLAG beads based on a previously described protocol (34). The Flag-Ble13 strain was used as a purification control for non-specific association of proteins with the FLAG beads. From each step of the purification process the following fractions (as described under Materials and Methods) were collected: Total Cell, Total Lysate, Fraction S16, Fraction S100, Unbound proteins, Wash 1 and Wash 2. These fractions along with the proteins associated with the FLAG beads were resolved by SDS-PAGE, and the AcV5-tagged TSN1 and AGO3 proteins were detected by immunoblotting using standard protocols.

The Mut13-2 complemented strain -mut13-2-13c- expressed adequate levels of the AcV5-tagged TSN1 that was retained on the FLAG beads (Figure 2-5 A). However, we could not detect association of AGO3 with FLAG beads bound TSN1 (Figure 2-5A and B). The other Mut13-2 complement –mut13-2-8c- also expresses an appropriate level of the epitope-tagged TSN1 protein (Figure 2-5C) and shows a fully restored RNAi (Figure

2-3). However, although the AGO3 protein was detected in several fractions collected during purification, it failed to associate with TSN1 retained on the FLAG beads (Figure 2-5D). These results suggest that TSN1 does not seem to interact with *Chlamydomonas* AGO3 at high affinity, although both proteins are associated with the RISC holo-enzyme in metazoans (13,14,31).

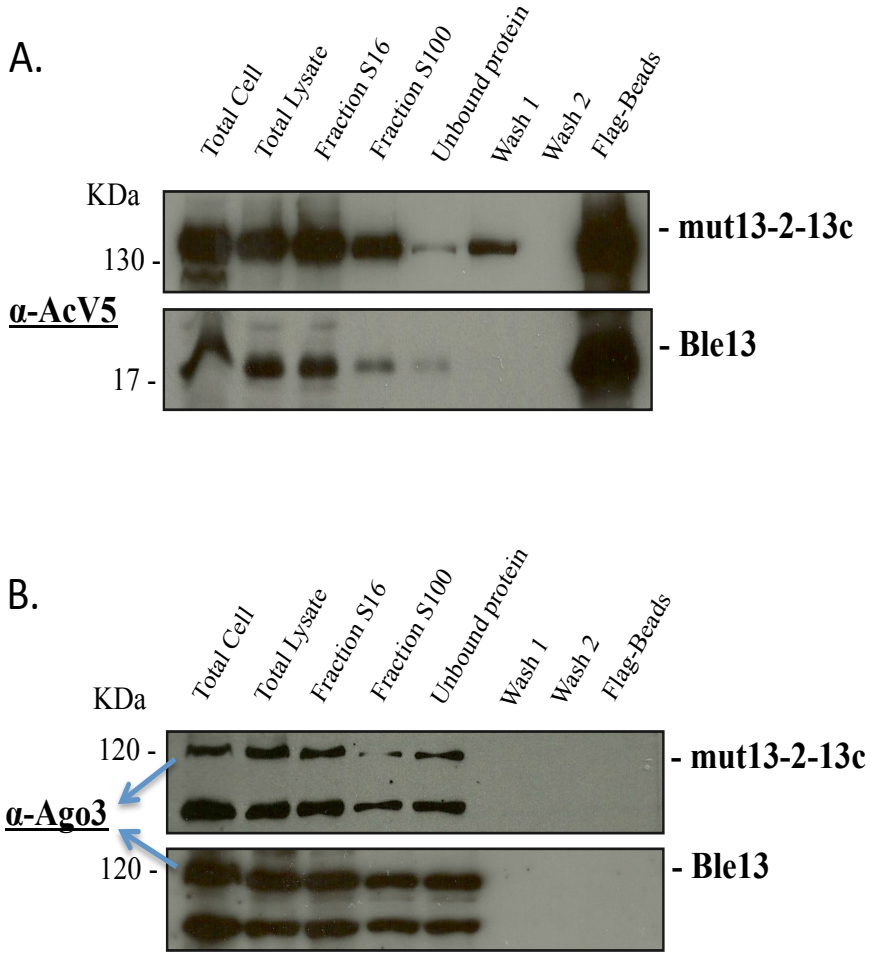


Figure 2-5. Immunoprecipitation of AcV5-tagged TSN1 – (A) Immunoblot analyses for detection of the TSN1 protein, affinity purified on FLAG beads. The Mut13-2 complemented strain –mut13-2-13c- was used for FLAG beads affinity purification. **(B)** Immunoblot analyses for the detection of the AGO3 protein in the various fractions of the indicated strain. AGO3 (~120 kDa) was detectable in mut13-2-13c but does not show association with the FLAG-beads.

The fractions collected at various steps of purification (methods section) were used for immunodetection of the tagged protein as well as for detection of AGO3. Flag-Ble13 was used as a purification control. The Ble13 strain contains a dominant selectable marker conferring resistance to the Bleomycin antibiotic. The Ble protein contains a FLAG-CBP-AcV5 tag and expresses a ~19 kDa fusion protein.

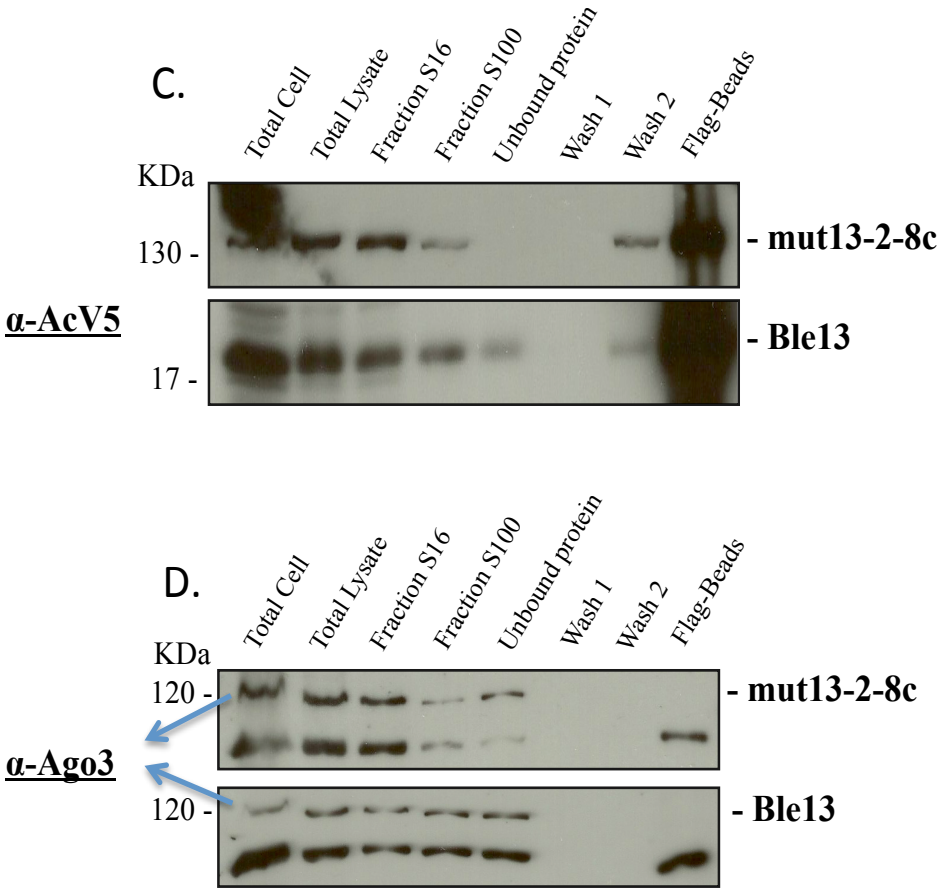


Figure 2-5 (continued). Immunoprecipitation of AcV5-tagged TSN1 – (C) Immunoblot analyses for the detection of FLAG affinity purified TSN1 protein in the mut13-2-8c complemented strain. **(D)** Immunoblot analyses for the detection of the AGO3 protein in the various fractions obtained during the purification of tagged TSN1 from mut13-2-8c. Although AGO3 (~120 kDa) was detected in the indicated fractions, it does not seem to interact with TSN1 retained on the FLAG beads.

Literature Cited

1. Rohr, J., Sarkar, N., Balenger, S., Jeong, B.R. and Cerutti, H. (2004) Tandem inverted repeat system for selection of effective transgenic RNAi strains in Chlamydomonas. *Plant J*, **40**, 611-621.
2. Li, C.L., Yang, W.Z., Chen, Y.P. and Yuan, H.S. (2008) Structural and functional insights into human Tudor-SN, a key component linking RNA interference and editing. *Nucleic Acids Res*, **36**, 3579-3589.
3. Tong, X., Drapkin, R., Yalamanchili, R., Mosialos, G. and Kieff, E. (1995) The Epstein-Barr virus nuclear protein 2 acidic domain forms a complex with a novel cellular coactivator that can interact with TFIIE. *Mol Cell Biol*, **15**, 4735-4744.
4. Leverson, J.D., Koskinen, P.J., Orrico, F.C., Rainio, E.M., Jalkanen, K.J., Dash, A.B., Eisenman, R.N. and Ness, S.A. (1998) Pim-1 kinase and p100 cooperate to enhance c-Myb activity. *Mol Cell*, **2**, 417-425.
5. Valineva, T., Yang, J. and Silvennoinen, O. (2006) Characterization of RNA helicase A as component of STAT6-dependent enhanceosome. *Nucleic Acids Res*, **34**, 3938-3946.
6. Paukku, K., Yang, J. and Silvennoinen, O. (2003) Tudor and nuclease-like domains containing protein p100 function as coactivators for signal transducer and activator of transcription 5. *Mol Endocrinol*, **17**, 1805-1814.
7. Yang, J., Valineva, T., Hong, J., Bu, T., Yao, Z., Jensen, O.N., Frilander, M.J. and Silvennoinen, O. (2007) Transcriptional co-activator protein p100 interacts with snRNP proteins and facilitates the assembly of the spliceosome. *Nucleic Acids Res*, **35**, 4485-4494.
8. dit Frey, N.F., Muller, P., Jammes, F., Kizis, D., Leung, J., Perrot-Rechenmann, C. and Bianchi, M.W. (2010) The RNA binding protein Tudor-SN is essential for stress tolerance and stabilizes levels of stress-responsive mRNAs encoding secreted proteins in Arabidopsis. *Plant Cell*, **22**, 1575-1591.
9. Gao, X., Ge, L., Shao, J., Su, C., Zhao, H., Saarikettu, J., Yao, X., Yao, Z., Silvennoinen, O. and Yang, J. (2010) Tudor-SN interacts with and co-localizes with G3BP in stress granules under stress conditions. *FEBS Lett*, **584**, 3525-3532.
10. Moeller, B.J., Cao, Y., Li, C.Y. and Dewhirst, M.W. (2004) Radiation activates HIF-1 to regulate vascular radiosensitivity in tumors: role of reoxygenation, free radicals, and stress granules. *Cancer Cell*, **5**, 429-441.
11. Tsuchiya, N., Ochiai, M., Nakashima, K., Ubagai, T., Sugimura, T. and Nakagama, H. (2007) SND1, a component of RNA-induced silencing complex, is up-regulated in human colon cancers and implicated in early stage colon carcinogenesis. *Cancer Res*, **67**, 9568-9576.
12. Sundstrom, J.F., Vaculova, A., Smertenko, A.P., Savenkov, E.I., Golovko, A., Minina, E., Tiwari, B.S., Rodriguez-Nieto, S., Zamyatnin, A.A., Jr., Valineva, T. *et al.* (2009) Tudor staphylococcal nuclease is an evolutionarily conserved component of the programmed cell death degradome. *Nat Cell Biol*, **11**, 1347-1354.

13. Caudy, A.A., Ketting, R.F., Hammond, S.M., Denli, A.M., Bathoorn, A.M., Tops, B.B., Silva, J.M., Myers, M.M., Hannon, G.J. and Plasterk, R.H. (2003) A micrococcal nuclease homologue in RNAi effector complexes. *Nature*, **425**, 411-414.
14. Scadden, A.D. (2005) The RISC subunit Tudor-SN binds to hyper-edited double-stranded RNA and promotes its cleavage. *Nat Struct Mol Biol*, **12**, 489-496.
15. Yang, W., Chendrimada, T.P., Wang, Q., Higuchi, M., Seeburg, P.H., Shiekhattar, R. and Nishikura, K. (2006) Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. *Nat Struct Mol Biol*, **13**, 13-21.
16. Friberg, A., Corsini, L., Mourao, A. and Sattler, M. (2009) Structure and ligand binding of the extended Tudor domain of D. melanogaster Tudor-SN. *J Mol Biol*, **387**, 921-934.
17. Yang, J., Aittomaki, S., Pesu, M., Carter, K., Saarinen, J., Kalkkinen, N., Kieff, E. and Silvennoinen, O. (2002) Identification of p100 as a coactivator for STAT6 that bridges STAT6 with RNA polymerase II. *Embo J*, **21**, 4950-4958.
18. Selenko, P., Sprangers, R., Stier, G., Buhler, D., Fischer, U. and Sattler, M. (2001) SMN tudor domain structure and its interaction with the Sm proteins. *Nat Struct Biol*, **8**, 27-31.
19. Cote, J. and Richard, S. (2005) Tudor domains bind symmetrical dimethylated arginines. *J Biol Chem*, **280**, 28476-28483.
20. Siomi, M.C., Mannen, T. and Siomi, H. (2010) How does the royal family of Tudor rule the PIWI-interacting RNA pathway? *Genes Dev*, **24**, 636-646.
21. E. H. Harris, *The Chlamydomonas Sourcebook – A Comprehensive Guide to Biology and Laboratory Use* (Academic Press, San Diego, CA, 1989).
22. Kindle, K.L. (1990) High-frequency nuclear transformation of Chlamydomonas reinhardtii. *Proc Natl Acad Sci U S A*, **87**, 1228-1232.
23. Sizova, I., Fuhrmann, M. and Hegemann, P. (2001) A Streptomyces rimosus aphVIII gene coding for a new type phosphotransferase provides stable antibiotic resistance to Chlamydomonas reinhardtii. *Gene*, **277**, 221-229.
24. Cerutti, H., Johnson, A.M., Gillham, N.W. and Boynton, J.E. (1997) A eubacterial gene conferring spectinomycin resistance on Chlamydomonas reinhardtii: integration into the nuclear genome and gene expression. *Genetics*, **145**, 97-110.
25. Sambrook, J. and Russell, D.W. (2006) *The condensed protocols from Molecular cloning : a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. Pall, G.S., Codony-Servat, C., Byrne, J., Ritchie, L. and Hamilton, A. (2007) Carbodiimide-mediated cross-linking of RNA to nylon membranes improves the detection of siRNA, miRNA and piRNA by northern blot. *Nucleic Acids Res*, **35**, e60.
27. Ibrahim, F., Rohr, J., Jeong, W.J., Hesson, J. and Cerutti, H. (2006) Untemplated oligoadenylation promotes degradation of RISC-cleaved transcripts. *Science*, **314**, 1893.
28. Church, G.M. and Gilbert, W. (1984) Genomic sequencing. *Proc Natl Acad Sci U S A*, **81**, 1991-1995.
29. Carninci, P., Nishiyama, Y., Westover, A., Itoh, M., Nagaoka, S., Sasaki, N., Okazaki, Y., Muramatsu, M. and Hayashizaki, Y. (1998) Thermostabilization and

thermoactivation of thermolabile enzymes by trehalose and its application for the synthesis of full length cDNA. *Proc Natl Acad Sci U S A*, **95**, 520-524.

30. Sanders, M.A. and Salisbury, J.L. (1995) Immunofluorescence microscopy of cilia and flagella. *Methods Cell Biol*, **47**, 163-169.
31. Casas-Mollano, J.A., Rohr, J., Kim, E.J., Balassa, E., van Dijk, K. and Cerutti, H. (2008) Diversification of the core RNA interference machinery in Chlamydomonas reinhardtii and the role of DCL1 in transposon silencing. *Genetics*, **179**, 69-81.
32. Ketting, R.F. (2011) The many faces of RNAi. *Dev Cell*, **20**, 148-161.
33. Nishida, K.M., Okada, T.N., Kawamura, T., Mituyama, T., Kawamura, Y., Inagaki, S., Huang, H., Chen, D., Kodama, T., Siomi, H. *et al.* (2009) Functional involvement of Tudor and dPRMT5 in the piRNA processing pathway in Drosophila germlines. *Embo J*, **28**, 3820-3831.
34. Rigaut, G., Shevchenko, A., Rutz, B., Wilm, M., Mann, M. and Seraphin, B. (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol*, **17**, 1030-1032.
35. Burroughs, A.M., Ando, Y., de Hoon, M.L., Tomaru, Y., Suzuki, H., Hayashizaki, Y. and Daub, C.O. (2011) Deep-sequencing of human Argonaute-associated small RNAs provides insight into miRNA sorting and reveals Argonaute association with RNA fragments of diverse origin. *RNA Biol*, **8**, 158-177.

CHAPTER 3

DISCUSSION & CONCLUSION

DISCUSSION & CONCLUSION

Over the last decade the RNA interference pathway has become an important subject of research. It has also been widely used as a tool for understanding gene function via the delivery of specific RNAi triggers which are able to impart long-term and heritable gene silencing in various systems such as plants and mammals (1). However, a number of issues remain unsolved regarding the RNAi process. Mechanisms such as regulation of gene expression via RNAi-mediated translational repression are now beginning to be understood properly (2,3). The myriad properties of microRNAs in various facets of biological pathways and signaling networks are continually being revealed (4,5). Interestingly, these small RNA molecules have also been found to up-regulate translational processes during certain stages of the cell cycle (6). In addition, the RISC follows a complex and ordered assembly into an effector holo-enzyme (7). However, the function of a number of the component proteins of the RISC is yet to be fully understood.

One such component of the RISC is Tudor-SN1 (TSN1), which has been reported to carry out degradation of an inosine containing miRNA precursor acting as a sort of quality control mechanism for faulty pre-miRNAs in metazoans (8,9). But a natural substrate for the protein is yet to be found. Indeed, the exact role of TSN1 in the RNAi pathway remains open to question. Considering the multi-functional nature of TSN1 and its association with the RISC it is possible that this versatile protein may have additional functions in RNAi besides just being a quality control mechanism (8,10,11).

In this study we have aimed to characterize two related mutants –Mut13-2 & Mut20– in *Chlamydomonas reinhardtii* both of which were found to be missing a number of the essential domains of the TSN1 gene. The extent of the genomic deletions was found to be larger in Mut20 when compared to Mut13-2, but both mutants showed an RNAi defect, based on their sensitivity to 5-FI and normal levels of the TSß protein. Interestingly, the Maa7 siRNAs were reduced in the case of Mut13-2 and virtually absent in Mut20. We also found that in Mut20 endogenous miRNAs were almost completely depleted whereas still detectable in Mut13-2. Hence, it can be concluded that Mut20 shows a stronger RNAi defective phenotype compared to Mut13-2. We reasoned that restoring TSN1 function might recover the RNAi-defective phenotype in the *Chlamydomonas* mutants. However, transformation of Mut20 with a construct expressing epitope-tagged TSN1 did not seem to fully restore the RNAi activity. In contrast, we successfully achieved complementation of Mut13-2 with full recovery of the RNAi phenotype and Maa7 siRNA levels. These results suggest that TSN1 is definitely important for RNAi in *Chlamydomonas reinhardtii*.

An obvious issue is the need to understand the reason(s) for the differences in RNAi defects between Mut20 and Mut13-2 as well as for the lack of complementation of Mut20. In other studies, it has been shown that the N-terminal SN domains of TSN1 are required for RNA binding and a minimum of two tandem SN domains are required for this process (9). The SN1 domain of the TSN1 gene and the neighboring Ankyrin repeat 3 domain of a neighboring Histone Deacetylase (HDAC) gene are deleted in Mut20 while are still present in Mut13-2. Hence, even if Mut13-2 expresses a truncated protein with a

single SN1 domain, it would not be able to have all the functions of a full-length TSN1 protein. If the TSN1 protein is involved in RISC assembly and interacts with different components of the RISC and the RISC-loading complex, it is possible that a truncated TSN1 protein might not allow proper loading of small RNAs onto the RISC. On the other hand, a complete absence of TSN1 could lead to a loss of the small RNAs from the RISC-loading complex. In order to fully address such hypotheses, the role of TSN1 in small RNA binding could be further investigated in RNA co-immunoprecipitation experiments involving cross-linking between TSN1 and native RNA substrates. Future experiments with affinity purification of epitope-tagged TSN1 followed by SDS-PAGE and mass spectrometry analyses might reveal TSN1 interacting partners giving us an idea of its role in the RISC complex. It is also possible that the enhanced RNAi defects, like complete depletion of the miRNAs, in Mut20 result from the deletion of the HDAC Ankyrin repeat 3 domain. However, addressing these questions will require further characterization of the mutants and their specific RNAi defects.

From our immunofluorescence microscopy studies, we found that epitope-tagged TSN1 shows a predominant cytoplasmic localization in Mut20, which supports its association with the RISC as revealed from other studies in metazoans (8,11). Nonetheless, a nuclear role for the protein could not be ruled out from the immunofluorescence detection experiments, which agrees with the reported role of TSN1 in splicing and as a transcriptional co-activator (12-14). In addition, epitope-tagged TSN1 shows a broader distribution (including a possible nuclear localization) in the Mut13-2 complemented strains. It is possible that this subcellular localization is better reflective of a fully

functional TSN1 protein whereas the epitope tagged TSN1 might be partly mislocalized and, consequently, not fully functional in the Mut20 background. A low level of expression of the epitope-tagged TSN1 protein (in the Mut20 transformants) could also be a reason for the lack of complementation of Mut20, which might require higher expression levels of the protein for its proper function.

Being a multi-functional protein, TSN1 is found to interact, in metazoans, with several other proteins such as EBNA-2 (13), c-Myb (15) and G3BP (16), but a specific RNAi component protein has not yet been characterized to associate with TSN1. In case of metazoans, TSN1 was found to be a part of the RISC along with other components such as Ago2, VIG and FXR (8). However, in our co-immunoprecipitation experiments, the epitope-tagged TSN1 did not seem to interact with the *Chlamydomonas* Argonaute polypeptide AGO3, which is an important component of the RISC and of the core RNAi machinery. This suggests that if TSN1 is a RISC component in *Chlamydomonas*, it does not appear to interact at high affinity with AGO3.

Taken together, our results suggest that TSN1 is definitely involved in RNAi in *Chlamydomonas reinhardtii* and its deletion seems to result in a depletion of the endogenous miRNAs. It can be hypothesized that TSN1 might be involved in miRNA biogenesis or even have an accessory role in RISC assembly via loading of small RNAs onto Argonautes. Yet the specific molecular role of TSN1 in RNAi will require further investigation.

Literature Cited

1. Scherr, M. and Eder, M. (2004) RNAi in functional genomics. *Curr Opin Mol Ther*, **6**, 129-135.
2. Lanet, E., Delannoy, E., Sormani, R., Floris, M., Brodersen, P., Crete, P., Voinnet, O. and Robaglia, C. (2009) Biochemical evidence for translational repression by Arabidopsis microRNAs. *Plant Cell*, **21**, 1762-1768.
3. Nottrott, S., Simard, M.J. and Richter, J.D. (2006) Human let-7a miRNA blocks protein production on actively translating polyribosomes. *Nat Struct Mol Biol*, **13**, 1108-1114.
4. Inui, M., Martello, G. and Piccolo, S. (2010) MicroRNA control of signal transduction. *Nat Rev Mol Cell Biol*, **11**, 252-263.
5. Krol, J., Loedige, I. and Filipowicz, W. (2010) The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet*, **11**, 597-610.
6. Vasudevan, S., Tong, Y. and Steitz, J.A. (2007) Switching from repression to activation: microRNAs can up-regulate translation. *Science*, **318**, 1931-1934.
7. Tomari, Y. (2009) Biochemical dissection of RISC assembly and function. *Nucleic Acids Symp Ser (Oxf)*, 15.
8. Caudy, A.A., Ketting, R.F., Hammond, S.M., Denli, A.M., Bathoorn, A.M., Tops, B.B., Silva, J.M., Myers, M.M., Hannon, G.J. and Plasterk, R.H. (2003) A micrococcal nuclease homologue in RNAi effector complexes. *Nature*, **425**, 411-414.
9. Li, C.L., Yang, W.Z., Chen, Y.P. and Yuan, H.S. (2008) Structural and functional insights into human Tudor-SN, a key component linking RNA interference and editing. *Nucleic Acids Res*, **36**, 3579-3589.
10. Sundstrom, J.F., Vaculova, A., Smertenko, A.P., Savenkov, E.I., Golovko, A., Minina, E., Tiwari, B.S., Rodriguez-Nieto, S., Zamyatnin, A.A., Jr., Valineva, T. *et al.* (2009) Tudor staphylococcal nuclease is an evolutionarily conserved component of the programmed cell death degradome. *Nat Cell Biol*, **11**, 1347-1354.
11. Scadden, A.D. (2005) The RISC subunit Tudor-SN binds to hyper-edited double-stranded RNA and promotes its cleavage. *Nat Struct Mol Biol*, **12**, 489-496.
12. Paukku, K., Yang, J. and Silvennoinen, O. (2003) Tudor and nuclease-like domains containing protein p100 function as coactivators for signal transducer and activator of transcription 5. *Mol Endocrinol*, **17**, 1805-1814.
13. Tong, X., Drapkin, R., Yalamanchili, R., Mosialos, G. and Kieff, E. (1995) The Epstein-Barr virus nuclear protein 2 acidic domain forms a complex with a novel cellular coactivator that can interact with TFIIE. *Mol Cell Biol*, **15**, 4735-4744.
14. Valineva, T., Yang, J., Palovuori, R. and Silvennoinen, O. (2005) The transcriptional co-activator protein p100 recruits histone acetyltransferase activity to STAT6 and mediates interaction between the CREB-binding protein and STAT6. *J Biol Chem*, **280**, 14989-14996.

15. Leverson, J.D., Koskinen, P.J., Orrico, F.C., Rainio, E.M., Jalkanen, K.J., Dash, A.B., Eisenman, R.N. and Ness, S.A. (1998) Pim-1 kinase and p100 cooperate to enhance c-Myb activity. *Mol Cell*, **2**, 417-425.
16. Gao, X., Ge, L., Shao, J., Su, C., Zhao, H., Saarikettu, J., Yao, X., Yao, Z., Silvennoinen, O. and Yang, J. (2010) Tudor-SN interacts with and co-localizes with G3BP in stress granules under stress conditions. *FEBS Lett*, **584**, 3525-3532.