universität wien

MASTERARBEIT

Titel der Masterarbeit

„Characterizing the biological effects of tRNA fragments on endo-siRNA targets“

verfasst von

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angestrebter akademischer Grad

Master of Science (MSc)

Wien, 2015

Studienkennzahl lt. Studienblatt:	A >066 830<
Studienrichtung lt. Studienblatt:	Molekulare Mikrobiologie, Mikrobielle Ökologie und Immunbiologie
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Zusammenfassung

Die kontrollierte, stress-induzierte Fragmentierung von tRNAs ist ein konservierter Prozess mit den verschiedensten postulierten Funktionen. Es wurde gezeigt, dass tRNA Hälften mit den Prozessen der RNAi interferieren. Das deutet darauf hin, dass tRNA Fragmente die Genexpression auf einem post-transkriptionalen Level während der Erholung von Stress beeinflussen.

Ziel dieser Arbeit war es, tRNA Fragmentierung von der zellulären Antwort auf Stress zu trennen. Dies sollte durch das Konstruieren und Testen von Expressionssystemen in Zellkultur geschehen, die es erlauben, tRNA Fragmentierung ohne jeglichen Zellstress zu induzieren und zu limitieren. Mit Hilfe von *Drosophila* S2 Zellen wurde begonnen, die Effekte von tRNA Fragmenten zu analysieren.

Zwei Expressionssysteme wurde hergestellt und ihre Fähigkeit, tRNA Fragmentierung zu induzieren wurde molekularbiologisch und biochemisch geprüft. Es wurde gezeigt, dass die Induktion von tRNA Fragmentierung durch die Expression der Endonuklease humanes Angiogenin (hANG) keine Stress-Signale hervorrief, was darauf hindeutete, dass es möglich ist, tRNA Fragmentierung von Signalwegen der Stressantwort zu trennen.

Um die stress-unabhängigen Effekte von tRNA Fragmenten auf RNAi weiter zu Untersuchen, wurden die Abundanz von mRNAs von Genen, deren Expression von *Dcr-2* und *Ago-2* Mutanten besonders nach einem Hitzeschock beeinflusst war, getestet. Die Expressionsanalyse zeigte, dass die Expression der mRNA eines bekannten Ziels einer endo-siRNA, mus-308, hochreguliert war. Weitere Untersuchungen zeigten, dass tRNA Fragmente ausreichend waren um diese hohen mRNA Mengen von mus-308 herbeizuführen.

Zusammenfassend zeigten die experimentellen Daten dieser Masterarbeit erste Versuche, den Effekt von tRNA Fragmenten ohne dein Einfluss von Stress-Signalwegen zu untersuchen. In Zukunft wird dies es möglich machen, die Hypothese zu prüfen, dass tRNA Fragmente Prozesse der RNAi beeinflussen.

Abstract

The controlled fragmentation of tRNAs is a conserved process in response to stress conditions with various proposed functions. Recently, it has been shown that tRNA halves interfere with the RNAi machinery, suggesting that tRNA fragmentation might affect gene expression on a post-transcriptional level during stress recovery.

The aim of this work was to uncouple tRNA fragmentation from the stress response by designing and testing cell culture expression systems that allow inducing but also limiting tRNA fragmentation without the application of any stress. Using *Drosophila* S2 cells this work started to analyze the downstream effects of tRNA fragments.

Two expression systems were designed and analyzed for their capability to induce and limit tRNA fragmentation by molecular as well as biochemical approaches. It was shown, that the induction of tRNA fragmentation by expressing the endonuclease human Angiogenin (hANG) did not elicit stress signaling, thus indicating that separating the fragmentation of tRNAs from other pathways of the stress response is feasible.

To extend the investigation of the effects of stress-independent tRNA fragments on the RNAi machinery, mRNA levels of genes that were affected by *Dcr-2* and *Ago-2* mutations and a Dnmt2 mutation, especially after heat shock were assessed. The expression analysis showed that the mRNA levels of a known endo-siRNA target, mus308, were upregulated. Further investigations revealed that tRNA fragments were sufficient to induce high mRNA levels of this target gene.

In summary, work presented in this thesis provides a starting point to assess the effects of tRNA fragments when uncoupled from stress responses, which should allow testing the hypothesis, which states that tRNA fragmentation affects siRNA-mediated gene silencing.

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1 Introduction

During the last few years, evidence emerged that tRNAs have many other biological functions besides their canonical role in protein synthesis. For instance, fragmentation of tRNA (Fig. 1.1) is mostly a stress-induced process that is thought to make a contribution to decreasing the overall synthesis of proteins (Thompson and Parker, 2009) but tRNA fragments can also be observed under non-stress conditions. It has been hypothesized that the fragmentation of tRNAs stalls protein synthesis because fewer intact tRNAs would decrease translation rates. However, only a fraction of all tRNAs is cleaved under stress (Yamasaki et al., 2009) and the overall abundance of mature tRNAs remains unchanged indicating that global translation is unlikely to be affected by fragmented tRNAs. tRNA fragments could stem from defective or mis-modified tRNAs. However, it was reported that yeast strains with different tRNA processing defects did not display an increased number of tRNA fragments (Thompson et al., 2008). Additionally, defective tRNAs were shown to be rather degraded by 3' or 5' exo-nucleases than being cleaved into fragments (Chernyakov et al., 2008; Copela et al., 2008). It follows that tRNA fragmentation is not a result of mis-processed or mis-modified tRNAs leading to tRNA degradation but rather a controlled yet limited process.

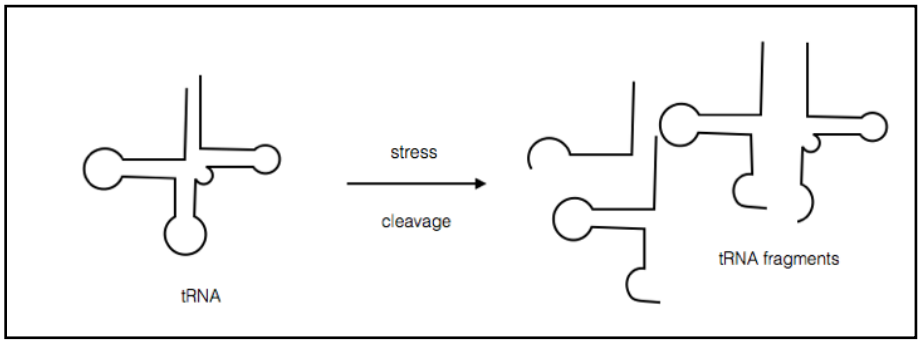

Fig. 1.1: **Fragmentation of tRNAs under stress conditions.** Fragmentation results in the formation of tRNA fragments of different lengths, corresponding to the open loop structures.

1.1 tRNA Fragments

The generation of tRNA fragments, their identities and functions vary among species, tissues and cell types. For example, tRNA fragments differ considerably in regard to their biogenesis and sequence length (13-50 nucleotides) (see Fig. 1.2) (Anderson and Ivanov, 2014). In spite of their vast heterogeneity it is possible to cluster different types of tRNA fragments as their 5'- and 3'-ends map within the open loop structures of mature tRNAs (Fig. 1.2). tRNA-derived fragments have been named tRFs (tRNA-derived RNA fragments) or tiRNAs (tRNA-derived stress-induced RNAs) (Lee et al., 2009; Yamasaki et al., 2009).

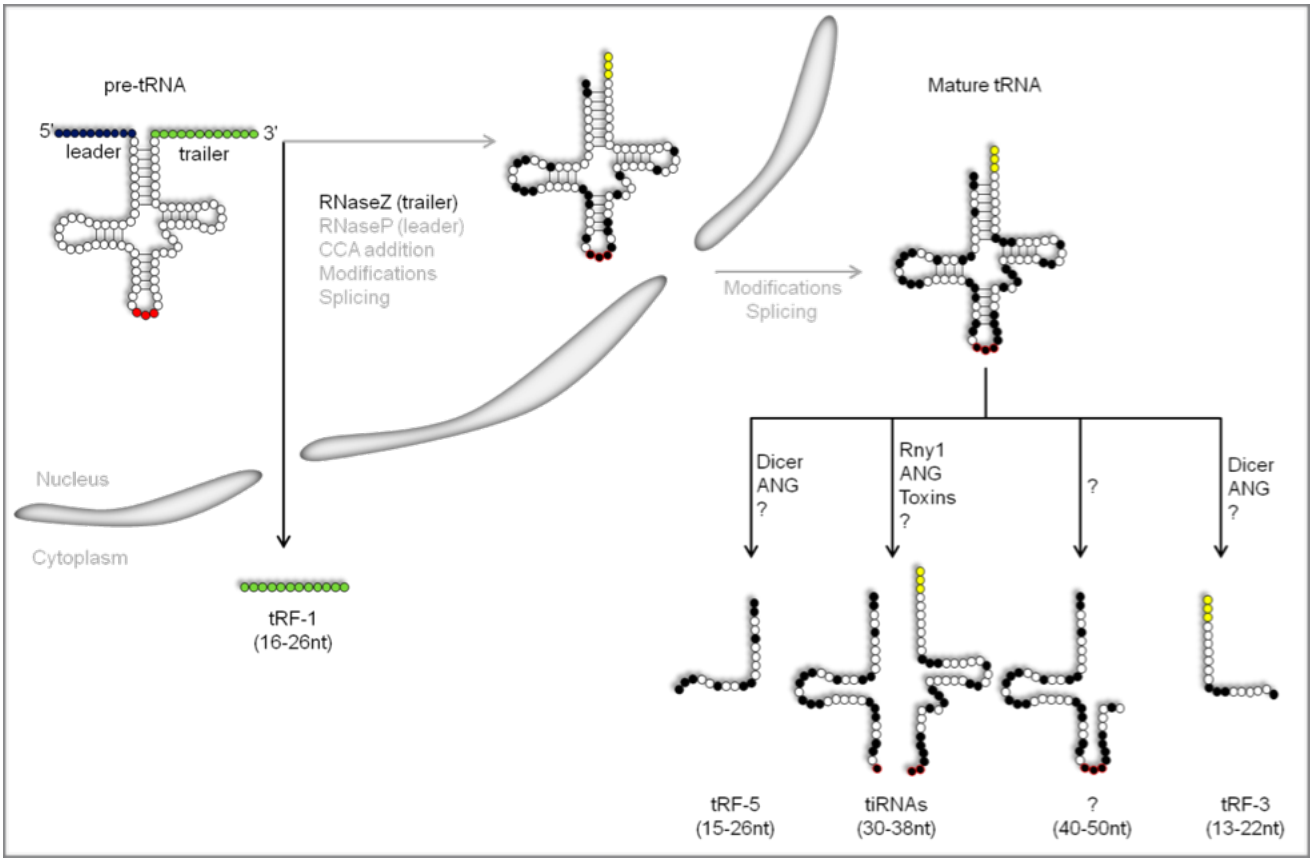

Fig. 1.2: **Different types of tRNA fragments.** tRF-1 fragments are produced by RNaseZ-mediated cleavage of pre-tRNAs. tRF-3 and tRF-5 are generated by cleavage of T- and D-loops by various endonucleases. tiRNAs are produced by cleavage in the anticodon loop. Red circles: anticodon nucleotides; black circles: nucleotides that are modified in some tRNAs; yellow circles: CCA addition at 3'-terminus; blue circles: leader sequence in pre-tRNAs; green circles: trailer sequence in pre-tRNAs. (adapted from Durdevic, 2013)

1.1.1 Generation of tRNA Fragments

The generation of stress-induced tRNA fragments was first described in *Tetrahymena thermophila* (Lee and Collins, 2005) followed by several accounts of the same phenomenon in bacteria (Haiser et al., 2008), fungi (Jöchl et al., 2008) as well as in mammals (Thompson et al., 2008). tRNA fragments are not only produced under stress but also during steady-state conditions. For instance, in human cells tRF-1 fragments are generated by RNase Z/ELAC2 and are composed of 3'-trailers of pre-tRNAs (Lee et al., 2009). In contrast, tRF-3 and tRF-5 fragments are derived from mature tRNAs and represent cleavage products of anti-codon nuclease activities (Angiogenin in mammals, Rny1 in yeast, or Dicers in mammals) (Lee et al., 2009; Thompson and Parker, 2009; Cole et al., 2009). tiRNAs are generated by Rny1 (in yeast) and Angiogenin (in mammals) by cleavage of the anticodon loop under stress conditions (Fig. 1.2). Notably, although tRNA fragments are present in many organisms, the endonucleases mediating their biogenesis have not always been identified (such as in *Drosophila*). Thus, the fragmentation of tRNAs is a process that is conserved in many organisms, involving different nucleases that produce a variety of fragments in stress as well as non-stress conditions.

Stress-induced tRNA fragments are produced by different stresses such as sodium arsenite treatment, heat shock or UV-irradiation (Yamasaki et al., 2009). However, other stresses do not elicit tRNA fragmentation at all (e.g. gamma-irradiation (Fu et al., 2009), etoposide or caffeine-treatment (Yamasaki et al. 2009)). Of note, most conditions that induce fragmentation are connected to oxidative stress (Thompson et al., 2008). Notably, it was also observed that the ratio of full-length tiRNAs to tRNAs in stressed cells is below 0.1 (Yamasaki et al., 2009). This suggested that only a low percentage of the total tRNA pool is subjected to stress-induced cleavage invoking the existence of control mechanisms that regulate which tRNAs will be fragmented. Importantly, the generation of stress-induced tRNA fragments is not mediated by all ribonucleases, as it was confirmed that, for instance, RNase Z and RNase L are not involved in the production of tiRNAs (Yamasaki et al., 2009). Fragmentation occurs mostly in mature tRNAs because few introns have been detected in cleavage products after blotting or sequencing tRNA fragments. While most tRNA fragments contain their mature 3'- and 5'-ends (Hurto, 2011) some exceptions have been reported, and it remains to be shown whether such tRNA fragmentation occurs through RNase-mediated mechanisms (Pederson, 2010). One hypothesis is that specific tRNA fragments are produced under specific stress conditions, in order to fulfill specific roles in response to a particular stressor (Hurto, 2011). In addition, it was hypothesized that the generation of tRNA fragments must be tightly controlled to prevent constant stress signaling, especially under steady-state conditions (Durdevic & Schaefer, 2013). RNases that act on tRNAs belong to the RNase A, T1 or T2 families, which are segregated from their RNA targets through compartmentalization or secretion under normal conditions (MacIntosh et al., 2001; Shapiro and Vallee, 1987). For example, Angiogenin (ANG), an RNase A family member that is expressed in mammalian cells, is bound by an RNase inhibitor, RNH1, during steady-state conditions and is released under stress conditions (Thompson and Parker, 2009). It is still not clear, whether every tRNA can undergo cleavage or how exactly the pathways work that prevent some tRNAs from entering these cleavage pathways. However, it was shown in *S.pombe* that mechanisms exist, which prevent tRNAs and rRNAs from entering into the siRNA biogenesis pathway (Bühler et al., 2008). This suggested that also other RNA sorting mechanisms might operate either constantly or only under specific conditions (i.e. stress) to prevent faulty RNase-mediated processing in higher organisms. Therefore, the generation of tRNA fragments must be a stringently regulated and even limited process that might also be highly specific in regard of the respective stress response.

That cleavage of tRNAs occurs mostly in the loops of the cloverleaf structure of tRNAs could be simply due to the fact that these regions are structurally exposed (Durdevic and Schaefer, 2013). However, that the site of tRNA cleavage is locally restricted could also be caused by the presence of RNA modifications at specific positions in tRNAs, which could influences tRNA cleavage efficiency. Post-transcriptional RNA modification is a core feature of functional tRNAs. There are several functions for tRNA modifications, which can be broadly subdivided into three classes

(Phizicky and Hopper, 2010) such as ensuring correct codon usage, guaranteeing identity and proper aminoacylation as well as providing tRNA stability. For instance, tRNA hypomethylation induces both tRNA degradation as well as tRNA fragmentation (Kadaba et al., 2004; Alexandrov et al., 2006; Schaefer et al., 2010). For example, Dnmt2/Trdmt1 is a (cytosine-5) methyltransferase, which methylates the position C38 in the anticodon loop of a few tRNAs (Goll et al., 2006, Schaefer et al., 2010). It was shown that overexpression of Dnmt2 protects *Drosophila* S2 cells from tRNA cleavage that was mediated by ectopically applied human Angiogenin (Schaefer et al., 2010). Furthermore, as most observed tRNA fragments corresponded to products resulting from cleavage in tRNA loops, it was proposed that specific modifications on their free ends prevent further degradation (Durdevic & Schaefer, 2013). Importantly it was shown in *Saccharomyces cerevisiae* that the modification pattern of tRNAs shifts significantly under stress conditions with specificity that correlated to a respective stressor (Chan et al., 2010). Taken together, these findings indicate that stress-induced tRNA fragmentation is fine-tuned by RNA modifying enzymes.

1.1.2 Functions of tRNA Fragments

Increasing experimental evidence suggests that tRNA fragments as well as tRNA nucleases and aminoacyl-synthetases are “multifunctional molecular tools“ for the regulation of various biological processes such as development, stress-response and metabolism as well as for immune responses (Hurto, 2011). Various observations indicated that tRNA fragments contribute to the multi-functionality of tRNAs beyond their essential role in protein synthesis (Fig. 1.3).

tRNA fragments were shown to inhibit protein synthesis by displacing various initiation factors from RNAs (Fig. 1.3). Importantly, only 5’-derived tiRNAs were involved in this phenomenon (Ivanov et al., 2011). When tiRNAs were transfected into cells they were able to reduce protein synthesis similarly to observations in stressed cells, in which Angiogenin had generated tRNA fragments. This indicated that not the cleavage and depletion of functional tRNAs but tRNA fragments were directly responsible of stalling translation (Emara et al., 2010). Such tRNA fragments were found in plants to have a function in intercellular signaling as it was shown that they are cleaved in extended leaves and secreted into the phloem of plants (Zhang et al., 2009). From these observations it was postulated that tRNA halves function as long-distance signals transmitting information about the metabolic state of certain tissues. In this context, further modification of tRNA fragments into plant cytokinins was discussed (Zhang et al., 2009). These findings corroborated that the observed stalling of protein synthesis was not due to depletion of tRNAs but more likely due to the interference of tiRNAs with the translation machinery.

Furthermore, it was reported that 5’-halves of tRNA-Glu targeted the tRNA 3’-end processing enzyme tRNAse Z to non-canonical cleavage sites in mRNAs (Fig. 1.3). Over 400 such mRNA targets were identified to be complementary to tRNA halves (Elbarbary et al., 2009). For example, the PP1MF mRNA, which was shown to harbor a 5’-tRNA-Glu binding site was found to be down-

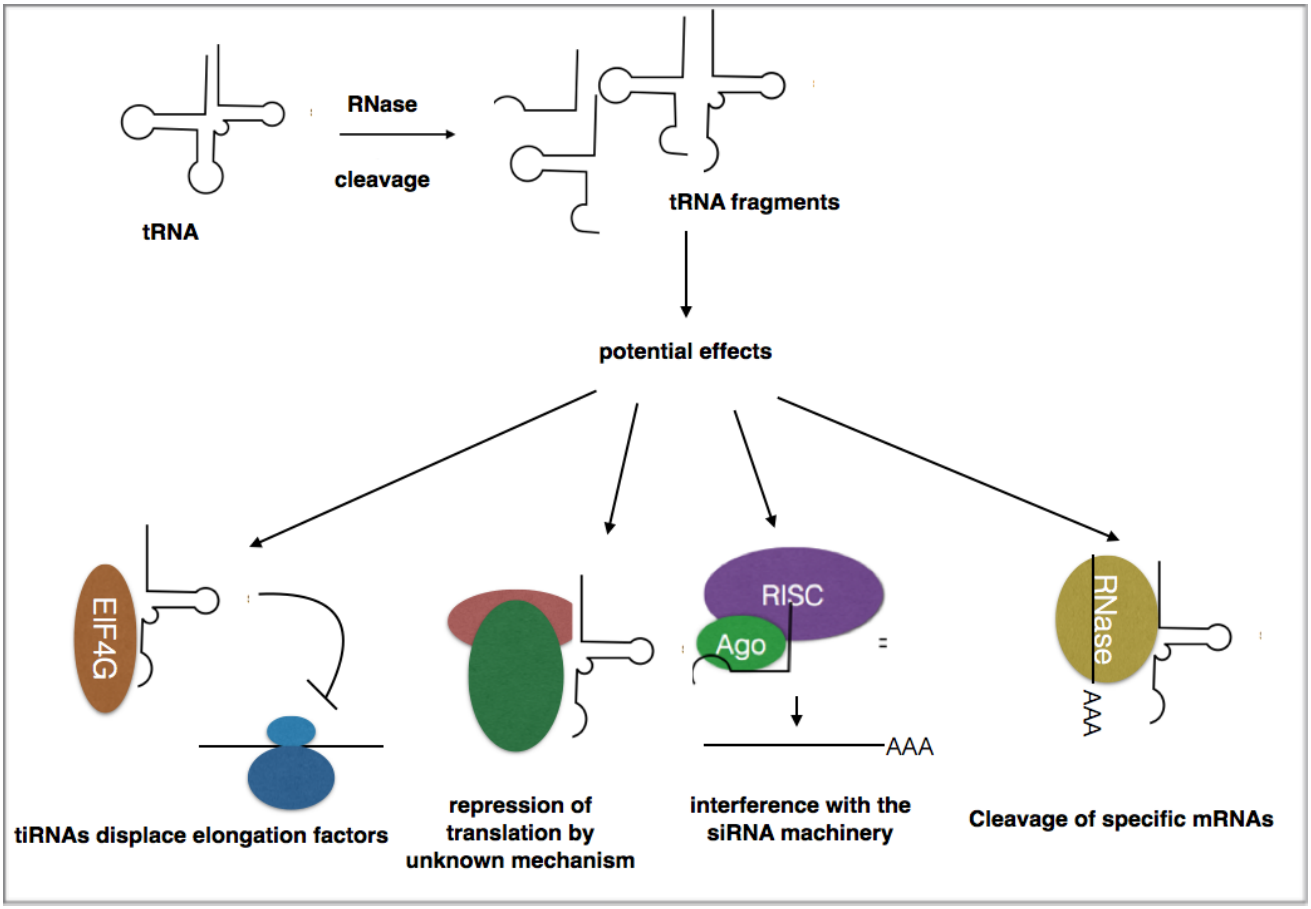

Fig. 1.3: **Examples for functions of stress-induced tRNA fragments.** (a) tiRNAs could displace initiation factors and inhibit translation. (b) tRNA fragments could interact with an unknown repression complex to stall translation. (c) tiRNAs could bind to complexes involved in post-transcriptional gene silencing such as Argonautes. (d) tRNA fragments could guide tRNase Z to cleavage sites of mRNAs (adapted from Thompson and Parker, 2009). EIF4G: eukaryotic initiation factor 4G, Ago: Argonaute-2, RISC: RNA induced silencing complex, RNase: tRNase Z.

regulated by excessive tRNA-Glu-5′-halves and tRNAse Z expression (Elbarbary et al., 2009). Thus, tRNA fragmentation does not only influence protein synthesis through interfering with the translation machinery but also through post-transcriptional gene silencing.

In addition, it has been shown that 5'-tiRNA fragments induced the formation of stress granules (SG) (Emara et al., 2010), a process that has been linked to stalled protein translation (Emara et al., 2010). However, the mechanisms by which tiRNAs trigger stress granule formation are still under investigation. As it was shown that only 5'-tiRNAs and not 3'-tiRNAs carry a 5'-monophosphate modification it was suggested that this feature was required for translational repression and SG assembly (Emara et al., 2010). Furthermore, it was reported that RNH1, the inhibitor of Angiogenin, antagonized the formation of SGs (Emara et al., 2010). As it was shown that the generation of SGs also supports cell survival (Arimoto et al., 2008), a hypothesis is that tiRNAs affect cell survival through stress granule generation (Emara et al., 2010). These findings suggested a connection between SG assembly, Angiogenin-mediated tRNA cleavage and cell survival.

In addition, tRF-fragments were reported to be involved in cell proliferation (Lee et al., 2009), to associate with Argonaute proteins during post-transcriptional gene silencing (Haussecker et al., 2010) and to function as bona fide miRNAs (Maute et al., 2013). In summary, all observations indicate that tRNA fragments are not only involved in stress responses but do also affect other cellular processes, probably also during steady-state conditions.

1.1.2.1 tRNA Fragments Are Associated with siRNA/ RNA-induced Silencing Complex (RISC) Pathways

Intriguingly, tRNA fragments have been found in physical association with Dicer and Argonaute proteins indicating interactions between tRNA processing and small-interfering RNA (siRNA) pathways (Cole et al., 2009). Small interfering RNAs (siRNAs) are crucial for the process of RNA interference (RNAi), which causes post-transcriptional silencing or inhibition of protein translation (Fire et al., 1998). There are many small RNAs that can act in RNAi. For instance, so called endo-siRNAs originate from various endogenous (genomic) sources such as transposable element sequences, from cis-natural antisense transcripts (cis-NATs) (Okamura et al., 2008) and from hairpin RNAs (hpRNAs) that form extended stem-loop structures through the presence of inverted repeats (Czech et al., 2008). Mechanistically, endo-siRNAs silence transposon expression (Sijen et al., 2003) or control endogenous mRNAs on a post-transcriptional level (Czech et al., 2008). On the other hand, exo-siRNAs are the product of exogenous dsRNAs such as viral sequences and facilitate the control of viral infections (van Rij et al., 2006). Various enzymes have been identified that process RNAs into mature siRNAs. For instance in *Drosophila,* miRNA precursors are processed into mature miRNAs by Dicer-1 (Dcr-1), whereas Dicer-2 (Dcr-2) processes all long dsRNAs into siRNAs (Lee et al., 2004).

Recently it has been shown that tRNA fragments play a role in post-transcriptional gene silencing in mammalian cells by affecting the efficiency of small RNA silencing pathways (Haussecker et al., 2010). Supporting this notion, tRNA fragments can interfere with siRNA pathway function in *Drosophila* (Durdevic et al., 2013 (2)) (See Figure 4; B(1)). Especially after heat shock, it has been shown that tRNA fragments became substrates for Dcr-2 and binding of tRNA fragments inhibited Dcr-2 activity on canonical dsRNA targets resulting in lower siRNAs production. As an example, the mRNA levels of mus-308, a target of the endogenous siRNA esi-2.1, (Czech et al., 2008) were found to be up-regulated after heat shock (Durdevic et al., 2013 (2)). This result was explained by the inhibitory effects of tRNA fragments on Dcr-2, which thereby prevented esi-2.1 maturation (Durdevic et al., 2013 (2)). However, tRNA fragments could also inhibit mature esi-2.1 from binding Ago-2, as Argonautes associated with tRNA-derived fragments (Haussecker et al., 2010; Cole et al., 2009). Notably, Ago-2 was reported to localize to stress-granules, which correlated with decreased RNAi in human cells (Detzer et al., 2010). Importantly, preliminary data using mobility testing of adult flies showed that *Dcr-2*, *Ago-2* and *Dnmt2* mutant flies responded simi-

larly to heat shock, which further strengthened the notion of a connection between the stress response, tRNA fragmentation and siRNA-pathway mediated post-transcriptional control (Durdevic et al. (2), 2013). In conclusion, there is emerging evidence that stress-dependent tiRNAs are endogenous substrates of the RNAi machinery, although it is not yet clear, whether they guide the RISC complex to specific target mRNAs or whether they function as non-specific competitors of siRNAs.

1.1.2.2 tRNA Fragments and Mobile Element Control

The RNAi machinery is essential for the control of mobile elements. As RNAi-defective mutants were reported to deregulate transposon expression in *C. elegans* (Tabara et al., 1999) it was suggested that canonical RNA interference controls mobile elements. It was proposed that the RNAi-mediated processing of mobile element transcripts could generate siRNAs, which function as guides for nucleases to degrade transposon-derived mRNAs (Sijen et al., 2003). The RNAi machinery was also reported to provide immunity against viruses in a similar way (van Rij et al., 2006). For instance, it was shown that flies with defective Ago-2 are hypersensitive to infections with Drosophila C Virus (DCV) (van Rij et al., 2006). Dcr-2 is a protein that is important for antiviral activity, which is mediated through its DexD/H-box helicase activity (Deddouche et al., 2008). Currently, it is hypothesized that those Dcr-2-dependent siRNAs guide Ago-2 to viral RNAs for degradation (van Rij and Berezikov, 2009). Importantly, flies with mutations in Dcr-2 and Ago-2 showed increased susceptibility to infections with *Drosophila* C virus (DCV), Sindbis virus, Flock House virus (FHV) and *Drosophila* X virus (Wang et al., 2006; Galiana-Arnoux et al., 2006; Zambon et al., 2006; van Rij et al., 2006). The interactions of host-derived mechanisms to eliminate RNA-based mobile elements (such as transposons and viral RNAs) by using host RNAs as effector molecules (such as siRNAs) has been discussed in the context of an evolutionary arms race between host and intruding genetic elements (Obbard et al., 2009). For example, DCV-1A, a potent suppressor of RNAi is encoded in the DCV genome (van Rij et al., 2006). Additionally, host molecules that interact with intruding elements or their respective targets evolve rapidly (Schlenke and Begun, 2003). It is important to note that RISC components can also influence mobile elements and virus-derived RNAs by regulating the transcription of genes involved in the immune response (Welker et al., 2007). Taken together, these observations indicated that RNAi components are important mediators of defenses against "parasitic" RNAs and are of major importance for organisms lacking an adaptive immune response such as invertebrates.

While tRNA fragments and mobile element-derived RNAs are both found in direct association with the RNAi machinery, these small RNAs have also been reported to be highly abundant under stress conditions (Thompson et al., 2008; Vasilyeva et al., 1999). Importantly, tRNA fragments were shown to prime TE transcription in *Drosophila* (Kikuchi et al., 1986; Arkhipova et al., 1986). Intriguingly, although mobile element control appears crucially important for genomic in-

tegrity, especially in the germ line (O'Donnel and Boeke, 2007), mobile element transcript levels and genomic re-integration rises under stress conditions, as many mobile elements have become inserted in stress-regulated genomic regions, which are accessible through chromatin remodeling during the stress response (Vasilyeva et al., 1999). This suggests that the mobilization of transposons during the stress response might be supported by an aberrant generation of tiRNAs. In summary, the physical interactions of tRNA fragments with the RNAi machinery and the pivotal role of the latter in controlling mobile elements suggest that stress-induced tRNA fragments might not only be regulators of protein translation but also affecting the expression of mobile elements during stress conditions in a process that might have been overlooked for a long time but whose significance is presently unclear.

1.2 The RNase Angiogenin and its Functions

Angiogenin is a small protein (14 kDa) belonging to the RNase A family, which is encoded in mammalian genomes but not in other species such as yeast and *Drosophila*. Angiogenin hydrolyses tRNA molecules in the anticodon loop (Saxena et al., 1992). Additionally, it was shown that the enzyme also cleaves tRNAs within their 3'-termini thereby removing the CCA-trinucleotide. CCA removal occurs much faster than tRNA cleavage in the anticodon loop suggesting quick repression of protein translation under stress conditions. CCA removal is reversible because a CCA-adding enzyme was able to repair inactivated tRNAs (Czech et al., 2013). The dinucleotide CA was shown to be the strongest recognition motif for hANG, followed by CG > UA > UG, determining a minimal sequence specificity for its various tRNA targets (Russo et al., 1996; Harper et al., 1989, Curran et al., 1993). Angiogenin is mostly found in the nucleus, but can also be detected in the cytoplasm where it is bound by its inhibitor RNH1 (Naddeo et al., 2005). tRNA fragmentation by Angiogenin was found to be a cytoplasmic process, as neomycin treatment, which prevents entry into the nucleus has no effect on Angiogenin-mediated tRNA cleavage (Yamasaki et al., 2009). Thus Angiogenin alters protein translation levels in two different ways providing an immediate and a long-term response to stress.

Angiogenin was first discovered as a protein in tumor cell-conditioned medium and it was named Angiogenin after its angiogenic activity had been described (Fett et al., 1985). Beside its nucleolytic activity on tRNAs, Angiogenin has also been shown to translocate to the nucleus and induce 18S rRNA transcription by binding to DNA, which has been discussed as reason for its angiogenic activity (Tsuji et al., 2005). Angiogenin can cause cytotoxicity as it has been shown to inhibit protein translation by 10-15 %. It was reported that the tRNA cleavage efficiency of Angiogenin is very high if compared to other RNase A family members (Saxena et al., 1992). Its ability to promote blood vessel growth has also been shown to be dependent on its ribonuclease activity (Shapiro and Vallee, 1987). It is presently unclear whether Angiogenin-mediated tRNA fragmenta-

tion or tRNA-independent functions are responsible for the diverse effects of Angiogenin activity (Anderson and Ivanov, 2014).

1.2.1 Angiogenin, tRNA Fragments and Disease

1.2.1.1 Cancer

Cancer cells show higher levels of several translation machinery components (Hanahan and Weinberg, 2000). Angiogenin was shown to be involved in rRNA transcription, which can cause accelerated proliferation rates and tumorigenesis in HeLa cells (Tsuj et al., 2005). When the entry of Angiogenin into the nucleus was blocked by neomycin treatment, its effects on proliferation were abolished (Hu, 1998). Recently, Angiogenin has become a target for anti-cancer therapeutic approaches as it was shown that Angiogenin is overexpressed in several types of cancer such as breast, gastric, liver, kidney and prostate cancers as well as in leukemia and melanoma (Montero et al., 1998; Shimoyama and Kaminishi, 2000; Hisai et al., 2003; Wechsel et al., 1999; Katona et al., 2005; Verstovsek et al., 2001; Hartmann et al.; 1999). In addition, tumor cells secrete Angiogenin, and when administered to cells Angiogenin causes transformation (Bárcena et al., 2015). Furthermore, it has been shown that Angiogenin promotes cell adhesion during metastasis in HT-29 cells (Soncin et al., 1994). It has been argued that the PI-3-K/Akt signaling, a pathway that is involved in the progression of various cancer types (Kandasamy and Srivastava, 2002; Zhong et al., 2000) is activated by Angiogenin (Kieran et al., 2008). Taken together, these observations indicate potential roles for Angiogenin in metastasis and tumor growth. In this respect, it is important to note that tRNA fragments were detected in serum and urine derived from cancer patients and it was shown that their levels correlated with the stage of certain kinds of cancers (Gehrke et al., 1979; Speer, et al., 1979). tRNA levels are generally very high in tumor cells (Pavon-Eternod, et al., 2009), and it remains to be tested whether the same holds true for tRNA fragments. However, it was also shown that Angiogenin-mediated tRNA fragmentation could directly prevent apoptosis (Mei et al., 2010) suggesting that cancer cells might exploit Angiogenin expression and tRNA fragmentation for their own survival. This hypothesis is corroborated by the observation that the nucleolytic activity of Angiogenin is important for angiogenesis (Shapiro and Vallee, 1989). Thus, further investigation is needed to ascertain the effects of stress-induced or aberrantly produced tRNA fragments on tumor growth.

1.2.1.2. Nervous System Diseases

In contrast to the effects of Angiogenin on cell proliferation, an impaired ribonucleolytic activity of Angiogenin has been associated with the manifestation of Amyotrophic Lateral Sclerosis (ALS) and Parkinson's disease (PD) (Greenway et al., 2006, van Es et al., 2013). In ALS-mouse models that carry a mutation in the SOD1 gene (one of the genes associated with the etiology of ALS) it was shown that Angiogenin treatment significantly prolonged life span (Kieran et al., 2008). The neuro-

protective activity of Angiogenin is believed to be mediated by the aforementioned PI-3-K/Akt signaling (PI-3-K), a pathway that is dysfunctional in SOD1-deficient mice but could be restored by Angiogenin treatment (Kieran et al., 2008). Notably, when PI-3-K was inhibited the neuroprotective effect of Angiogenin was abolished (Kieran et al., 2008). In addition, Angiogenin was also reported to induce cell differentiation and neurite extension as well as protecting neurons from cell death triggered by hypoxia (Subramanian et al., 2008), an observation that further supported a role for Angiogenin in cell survival. Of note, mutations in NSun2, a (cytosine-5) RNA methyltransferase that methylates various positions in tRNAs, cause increased fragmentation of tRNAs and are linked to intellectual disability phenotypes in humans, thus connecting tRNA fragments directly with neural disorders (Khan et al., 2012).

In summary, the Angiogenin-mediated production of tRNA fragments has been associated with many diseases. The available data suggest that Angiogenin levels and activity must be tightly regulated because too little or catalytically inactive Angiogenin can cause neural diseases, and too much Angiogenin might facilitate tumorigenesis. However, it is presently unclear whether Angiogenin exerts its disease-related functions directly or indirectly through tRNA fragmentation as the latter could inhibit protein translation or prevent neuronal cell death through, for instance, inhibition of apoptosis (Mei et al., 2010). Concerning the latter, tRNA fragments are good indicators of cellular stress and tissue damage, both of which are common phenotypes of the disease state (Anderson and Ivanov, 2014).

1.2.2 Control of Angiogenin Activity

To control and spatially limit the activities of Angiogenin the ribonuclease inhibitor 1 (RNH1) binds and sequesters Angiogenin. This protein regulates Angiogenin activity in different subcellular compartments (cytoplasm, nucleus, stress granules) and under different circumstances (Pizzo et al., 2013). For instance, the cellular distribution of Angiogenin and RNH1 changes in response to various growth conditions. In growth phases, Angiogenin was mainly be detected in the nucleus and in nucleoli whereas RNH1 localized to the cytoplasm and the nucleus but was excluded from nucleoli (Pizzo et al., 2013). In contrast, when cells were exposed to stress, RNH1 localized mainly to the nucleus and was also found in nucleoli whereas Angiogenin re-localized to the cytoplasm (Pizzo et al., 2013). Co-Immunoprecipitation studies indicated that under growth conditions Angiogenin is not bound by RNH1 and thus can stimulate rRNA transcription in nucleoli whereas under stress conditions, RNH1 re-localizes to nucleoli and inhibits Angiogenin (Pizzo et al., 2013). Notably, stress conditions led also to the accumulation of Angiogenin and RNH1 in stress granules. Angiogenin localization to stress granules was RNH1-mediated although both proteins did not form complexes in stress granules (Pizzo et al., 2013). RNAi-mediated knock down of RNH1 in cells that were exposed to oxidative stress showed enhanced tiRNA-mediated translational stalling. In contrast, knock down of RNH1 without additional stress resulted in an increase of tiRNAs (due to

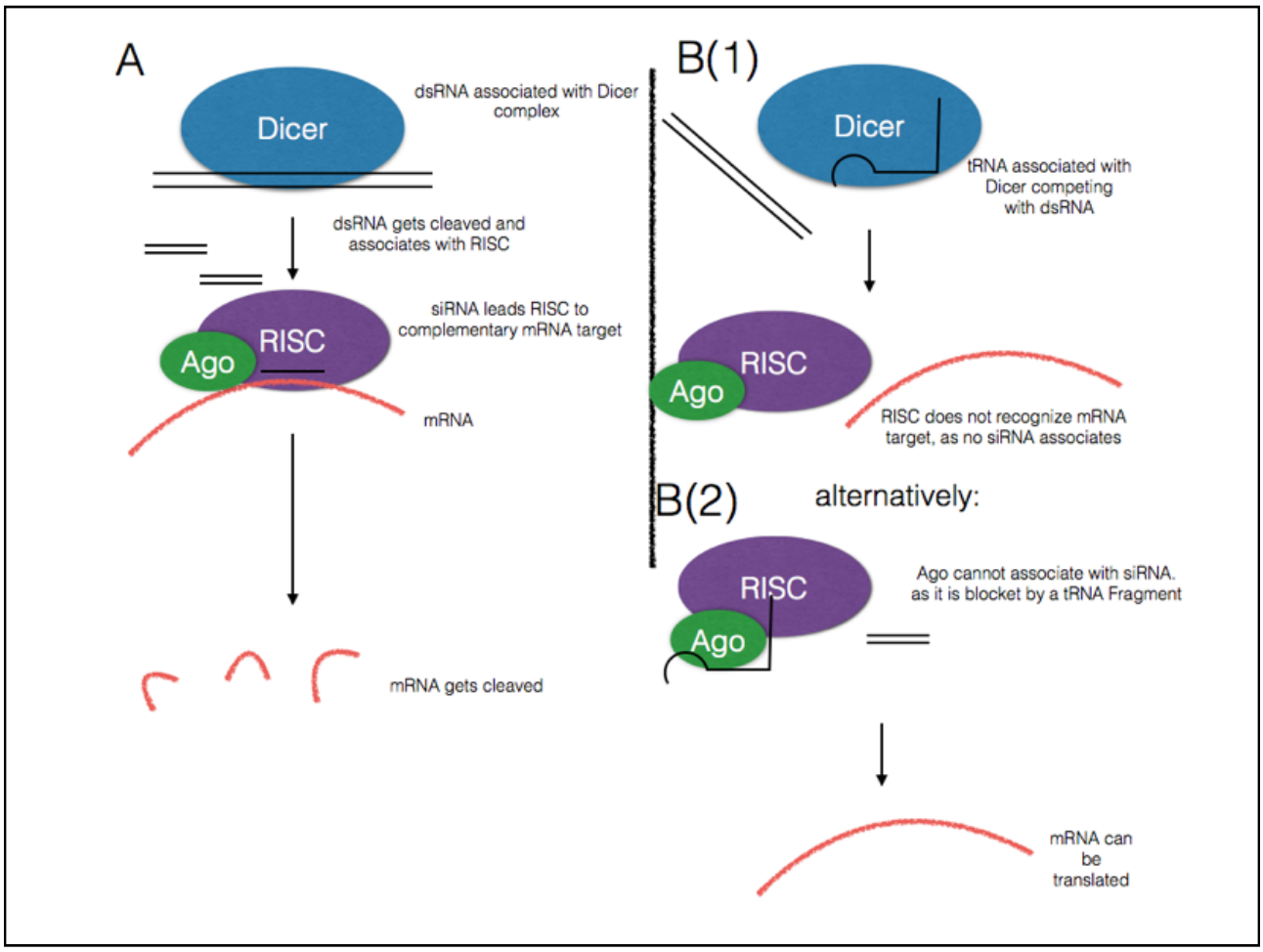

Fig. 1.4: **Proposed tiRNA mediated effects on RNAi pathways.** A: under normal conditions, dsRNAs is processed by Dicer into siRNAs, which associate with the RISC complex to induce the cleavage of siRNA-complementary mRNAs. B(1): if tRNA fragments associate with Dicer, dsRNAs cannot be cleaved at the same time any more and cannot be used by the RISC complex to target complementary mRNAs. B(2): If tRNA fragments occupy the binding position of siRNAs on Ago, the cleavage of mRNAs is also impossible. Dicer: Dicer-2. RISC: RNA induced silencing complex. Ago: Argonaute-2

un-inhibited Angiogenin activity) but caused no effects on protein translation indicating the requirement of stress- or secreted angiogenin-induced cofactors (Yamasaki et. al, 2009). In conclusion, RNH1 is a potent inhibitor of Angiogenin that controls the subcellular localization and activity of Angiogenin during growth and stress conditions.

1.3 Aim

1.3.1 Stating the Hypothesis

One unexplored consequence of stress-induced tRNA fragmentation concerns the effects of the crosstalk between tRNA fragments and small RNA-regulated pathways, which might modulate post-transcriptional gene silencing. Presently, the details and significance of these interactions remain unclear. It was indicated that high levels of tRNA fragments reduce the endonucleolytic activity of Dcr-2 on its canonical dsRNA substrates during the stress response (Durdevic et al., 2013

(2)). However, it could also be that the upregulation of certain siRNA targets (Durdevic et al., 2013 (2)) is a consequence of heat shock-induced but tRNA fragment-independent effects. To distinguish between the effect of tRNA fragments on RNAi components and the many effector mechanisms of the stress response an experimental test system is required by which tRNA fragmentation can be induced without exposure to biotic or abiotic stressors.

One such system would be exposing cells to a tRNA endonuclease (i.e. Angiogenin) and observing the effects on RISC factors such as Dicers and Argonautes as well as on gene expression. There are at least two non-exclusive scenarios that could explain the impairment of siRNA-mediated pathways through tRNA fragments:

1) tRNA fragments associate with Dcr-2 and block its activity on dsRNAs precursors thus decreasing the pool of mature and functional siRNAs. Consequently, this would decrease siRNA-mediated targeting of Ago-2 to specific mRNA targets (Fig. 1.4 B(1)).

2) tRNA fragments bind directly to Ago-2 and compete with mature siRNAs in RISC. Consequently, this would lead to reduced siRNA-mediated degradation of Ago-2-controlled mRNA targets (see Fig. 1.4 B(2)).

1.3.2 Aim of the Thesis

The aim of this Master Thesis work was to design and test experimental approaches that induce tRNA fragmentation without the application of stress conditions, thereby allowing the uncoupling of gene expression changes from transcriptional effects due to the general stress response (Fig. 1.5). To do so, tRNA fragmentation was induced in *Drosophila melanogaster* S2 cell culture using two experimental strategies:

A) tRNA fragmentation was induced using recombinant human Angiogenin. Since Angiogenin is a secreted protein it can enter cells without transfection reagents followed by cleavage of tRNAs (Anderson et al., 2009, Schaefer et al., 2010). To stop and limit Angiogenin activity, S2 cells carrying inducible constructs with an Angiogenin inhibitor (RNH1) or the (cytosine-5) methyltransferase Dnmt2 that binds and methylates some tRNAs were used.

B) S2 cells carrying an inducible human Angiogenin (hANG) construct were used and the extent of hANG activity was limited by controlling the time of hANG expression through removal of the inducing agent.

To characterize the effects of introducing Angiogenin into S2 cells gene expression changes of candidate endo-siRNA target mRNAs, which are regulated by Dcr-2 and Ago-2 function in *Drosophila (*Durdevic et al. 2013*)* were analyzed using quantitative PCR.

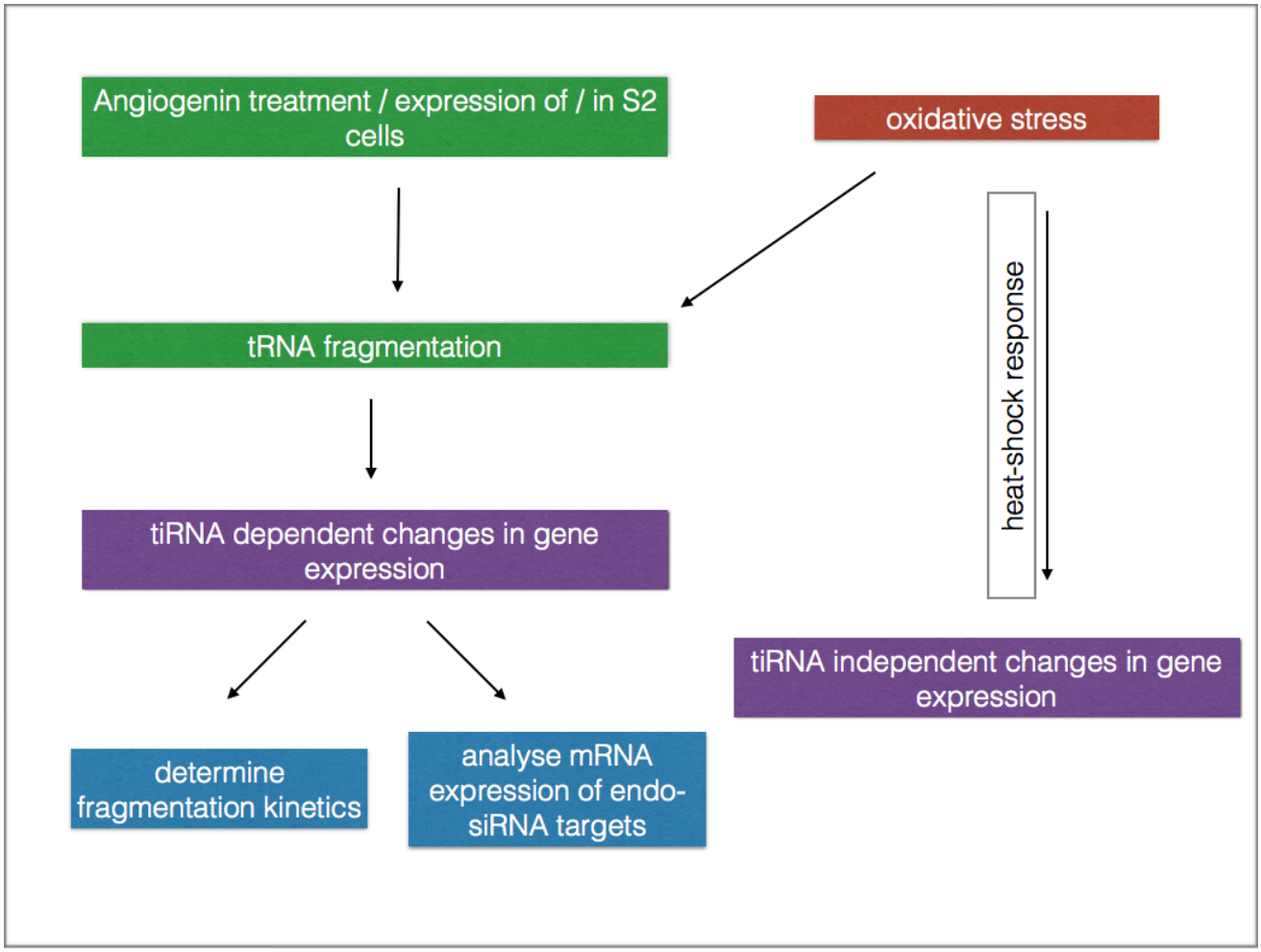

Fig. 1.5: **Experimental approaches to induce tRNA fragmentation.** Both oxidative stress and Angiogenin treatment as well as Angiogenin expression cause tRNA fragmentation. Heat shock causes additionally tiRNA independent changes in gene expression. To analyze the effects of tRNA fragmentation on endo-siRNA targets cells will be treated with Angiogenin or Angiogenin will be expressed. Subsequently, the fragmentation kinetics of the tRNAs will be assessed by northern blotting and mRNA expression will be analysed by qPCR

2 Material and Methods

2. 1 Material

2.1.1 Cell Culture

***Drosophila* S2 Cells**

S2 Ameres: virus-free S2 cell line, cultured in S2 Medium with 10 % FCS

S2 new: cultured in S2 Medium with 10 % FCS, 50 U/ml penicillin and 50 µg/ml streptomycin

S2_Cryo Medium

S2 Medium or S2 Medium for transfected cells with 10 % FCS and 10 % DMSO

S2 Medium for transfected cells

S2 Medium with 10% FCS with 25 µg/ml Blasticidin

Transfection reagents

2x HBS: 50 mM HEPES, 280 mM NaCl, 1.5 mM Na_2HPO_4, pH= 7, filter-sterilized

Sol A (36 µl 2 M $CaCl_2$, 19 µg pRmHA-3_insert, 1 µg pCoBlast, ad 300 µl)

Sol B (300 µl 2x HBS)

S2 Medium (Life Technologies)

50 ml of FCS are sterile-filtered and added to 450 ml Medium. 50 U/ml Penicillin and 50 µg/ml Streptomycin are added if required.

2.1.2 Gels

Urea Sequencing Gel (Roth)

12%: 12 ml Sequa Gel Concentrate, 10.5 ml Sequa Gel Diluent, 2.5 ml Buffer

add 200 µl 10% APS and 10 µl TEMED

SDS-PAGE Gels

5% Stacking Gel: 7.3 ml H_2O, 1 ml Buffer B, 1.7 ml Acrylamide (37.5:1), 50 µl 10% APS, 18 µl TEMED

10% Separating Gel: 6.3 ml H_2O, 6.8 ml Buffer A, 6.8 ml Acrylamide (37.5:1), 120 µl 10% APS, 20 µl TEMED

Buffer A: 34.1 g Tris-Base, 7.5 ml 10% SDS, 6 ml concentrated HCl, 200 ml H_2O, pH 8.8 ad 250 ml

Buffer B: 7.6 g Tris-Base, 5 ml 10% SDS, 5 ml concentrated HCl, 30 ml H_2O, pH 6.8 ad 50 ml

Staining Solutions for SDS-gels with Colloidal Blue Staining Kit (Invitrogen)

Fixing Solution: 40 ml H_2O, 50 ml Methanol, 10 ml Acetic Acid

Staining Solution: 55 ml H_2O, 20 ml Methanol, 20 ml stainer A (Invitrogen), 5 ml stainer B (Invitrogen)

2.1.3 Buffers

Protein Extraction Buffer

2x stock: 50 mM Tris, pH 8, 55 mM NaCl, 40 mM KCl, 20 mM EDTA , 10 % (v/v) Glycerol,

2 EDTA-free Protease Inhibitor tablets per 50 ml, 0.1% Tx-100

add freshly: 1 mM DTT, 0.25 % IGEPAL, 0.5 mM PMSF

IP Buffer

2x Stock: 10 mM Hepes pH 7.9, 10 mM KCl, 340 mM Sucrose, 1.5 mM $MgCl_2$, 10 % Glycerol, 2 EDTA-free Protease Inhibitor tablets per 50 ml

add freshly: 1 mM DTT, 0.25 % IGEPAL, 0.5 mM PMSF

Northern Blotting Buffers

Hybridization Buffer: 7.5 ml 20x SSC, 0.6 ml 1M Na_2HPO_4 pH 7.2, 21 ml 10 % SDS, 0.3 % Denhart's solution, 0.6 ml H_2O

Northern Wash Solution A: 3x SSC, 5 % SDS

Northern Wash Solution B: 1x SSC, 1 % SDS

Immunostaining Blocking Solution

2 % normal goat serum, 0.1% Triton X-100, 1x PBS, 0.05 % NaN_3

SDS-PAGE Buffers

10 x Running Buffer: 144 g Glycine, 30.3 g Tris-HCl, ad 1 l with H_2O

Blotting Buffer: 600 ml H_2O, 200 ml MeOH, 100 ml 10x Borate-buffer, 4 ml EDTA pH 8 (0.5 M), 1 ml DTT (1M), ad 1 l with H_2O

10x Borate-buffer: 15.46 g Borate acid, 900 ml H_2O, pH 8.8, ad 1 l with H_2O

qPCR Buffers

10x PCR buffer: 100 mM Tris pH 8.5, 500 mM KCl, 1.5 % Triton X-100, 20 mM $MgCl_2$

1M Trehalose: dissolve 7.56 g Trehalose in 15.5 ml H_2O, sterile-filter

2x Master Mix: 2x PCR buffer, 400 μM dNTPs, 400 μM Trehalose, 5 % Formamide, 1:10000 SYBR-1, 50 U / ml Taq Polymerase (Go-Taq, Promega)

tRNA fragmentation Buffer

0,8 ml 1 M Tris-HCl pH 7.5, 5 ml 2.5 M NaOAc, 80 μl 0.5 M EDTA, 1 ml 10% SDS, 34 ml H_2O

2.1.4 Oligonucleotides

<u>Virus detection (5'-XXX-3'):</u>

DXV fwd: GGAGTTGAAGCCACGGTTTG

DXV rev: GACGATCTTGCCAGTTGGCTCATCG

Sigma virus fwd: ATGTAACTCGGGTGTGACAG

Sigma virus rev: CCTTCGTTCATCCTCCTGAG

Nora virus fwd: AACCTCGTAGCAATCCTCTCAAG

Nora virus rev: TTCTTGTCCGGTGTATCCTGTATC

DCV fwd: TCATCGGTATGCACATTGCT

DCV rev: CGCATAACCATGCTCTTCTG

ZD257 (DCV) fwd: TAATACGACTCACTATAGGGGCGGACCCTTTGTACGACTTTTAG

ZD256 (DCV) rev: GTACAATCTCTTTTTGCTCGGAAG

RNH1 cloning primers (5'-XXX-3'):

VIE102: GCGCGGTACCATGAGCCTGGACATCCAGAGCC

VIE103: 5` GCGCAGATCTTCACTTGTAGTCGATGTCATGATCTTTATATTCACCGT
CATGGTCTTTGTAGTCGGAGATGACCCTCAGGGATGG 3`

pCR 2.1 TOPO primers, (5'-XXX-3'):

M13 fwd: GTAAAACGACGGCCAGT

M13 rev: AACAGCTATGACCATG

Northern blotting probes (5'-XXX-3'):

5' tRNA-Gly^{GCC} primer

HD 738: TCTACCACTGAACCACCGAT

5' tRNA-Asp^{GUC} primer

HD 734: CTAACCACTATACTATCGAG

5S rRNA primer

HD 242: CAACACGCGGTGTTCCCAAGCCG

qPCR primers (5'-XXX-3'):

RP 49 fwd (HD 174): CGGATCGATATGCTAAGCTGT

RP 49 rev (HD 175): GCGCTTGTTCGATCCGTA

spnE fwd (ZD 268): GCGCACGAAGGTGGTAGGTCTC

spnE rev (ZD 269): CCTTAAGTGACCGCAAGGAGACG

mus101 fwd (ZD 286): CTTCGCCGGTGTCTCTGTTTATG

mus101 rev (ZD 287): CCTTAAGCGGCTGACGCAGTG

meiotic41 fwd (ZD 288): CGAGAGCGTTAAGCAGGTCAATG

meiotic41 rev (ZD 289): CCAATGGTGGCGATTGATTCTCC

mus308 fwd (HD 507N): CTATTCTGTCTGCTACCAAC

mus308 rev (HD 508N): TCCAGAGCCAGAGCTATGTAG

cyclinE fwd (ZD 290): CGGGAGACCTTCTACTTGGCC

cyclinE rev (ZD 291): GTGGTTAAGGATGTCCCGCTCTG

hsp23 fwd (HD 647): CCATTGTTGTTGAGCCTTGC

hsp 23 rev (HD 648): GCGCCCACCTGTTTCTCCAG

hsp 70a fwd (HD 202): CCTGGAGAGCTACGTCTTCAAT

hsp 70a rev (HD 203): GTCGTTGCACTTGTCCAA

hsp 83 fwd (HD 560): CGATTAAGCGACCAGTCGAA

hsp 83 rev (HD 561): AAACGACAACTGCTCTTGAATG

SNRP fwd (HDT 031): ATCGCTTCTCGGCCTTATGGC
SNRP rev (HDT 032): CCGGCGGTACTGCAATACCGG
18S rRNA fwd (HD 200): GCCAGCTAGCAATTGGGTGTA
18S rRNA rev (HD 201): CCGGAGCCCAAAAAGCTT
gypsy fwd (HD 157): CTTCACGTTCTGCGAGCGGTCT
gypsy rev (HD 158): CGCTCGAAGGTTACCAGGTAGGTTC
Re297 fwd (ZD 86): AAAGGGCGCTCATACAAATG
Re297 rev (ZD 87): TGTGCACATAAAATGGTTCG
Re1731 fwd (ZD 90): AGCAAACGTCTGTTGGAAGG
Re1731 rev (ZD 91): CGACAGCAAAACAACACTGC

2.1.5 Antibodies

rabbit anti-Dnmt2: 1: 250
rabbit anti-HA: 1: 400
rabbit anti-FLAG: 1: 400
goat anti-hANG: 1: 250

2.1.6 Plasmids

pCR 2.1-Topo

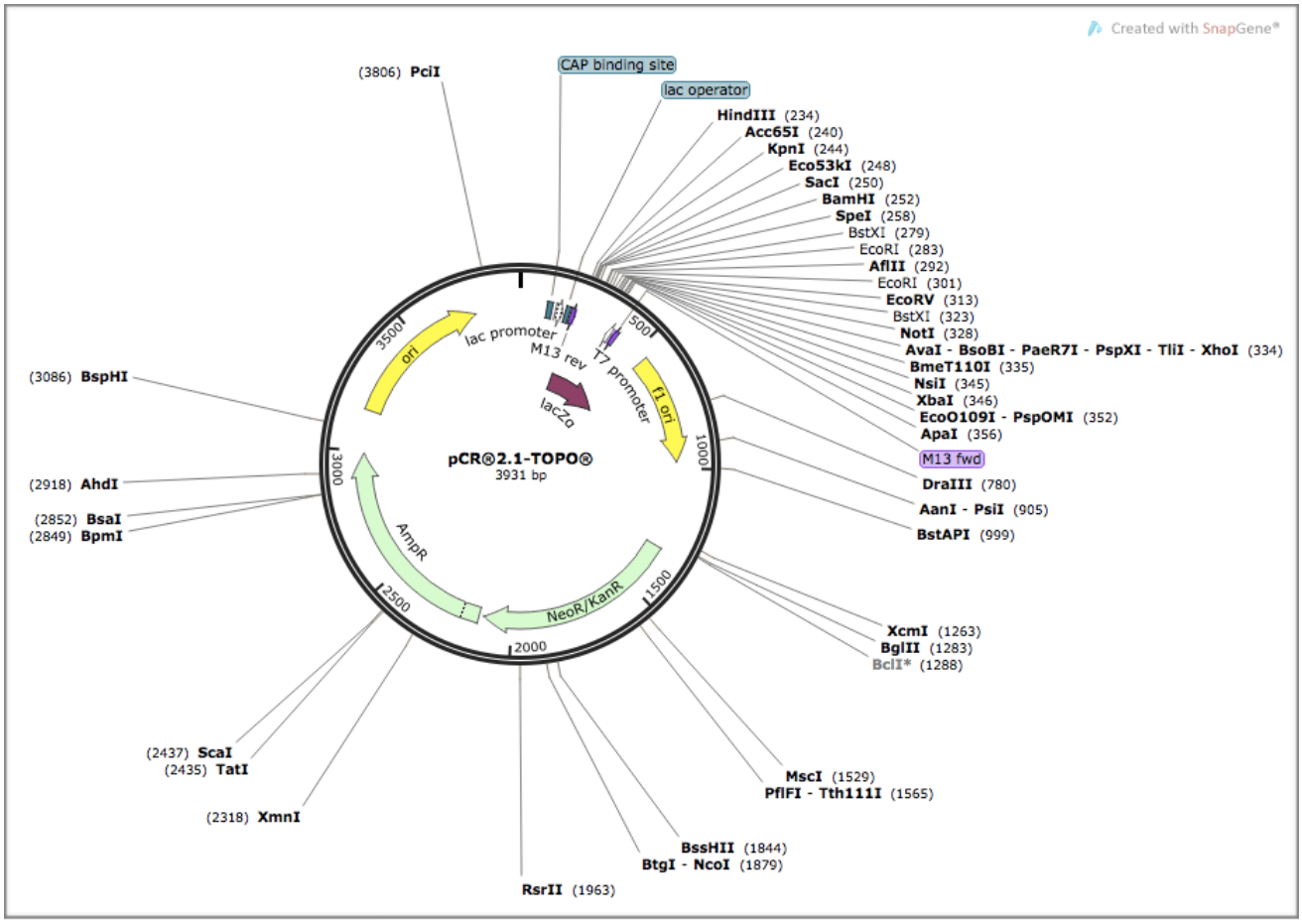

Fig. 2.1: **Vector map of PCR 2.1 Topo.**

pRmHA-3

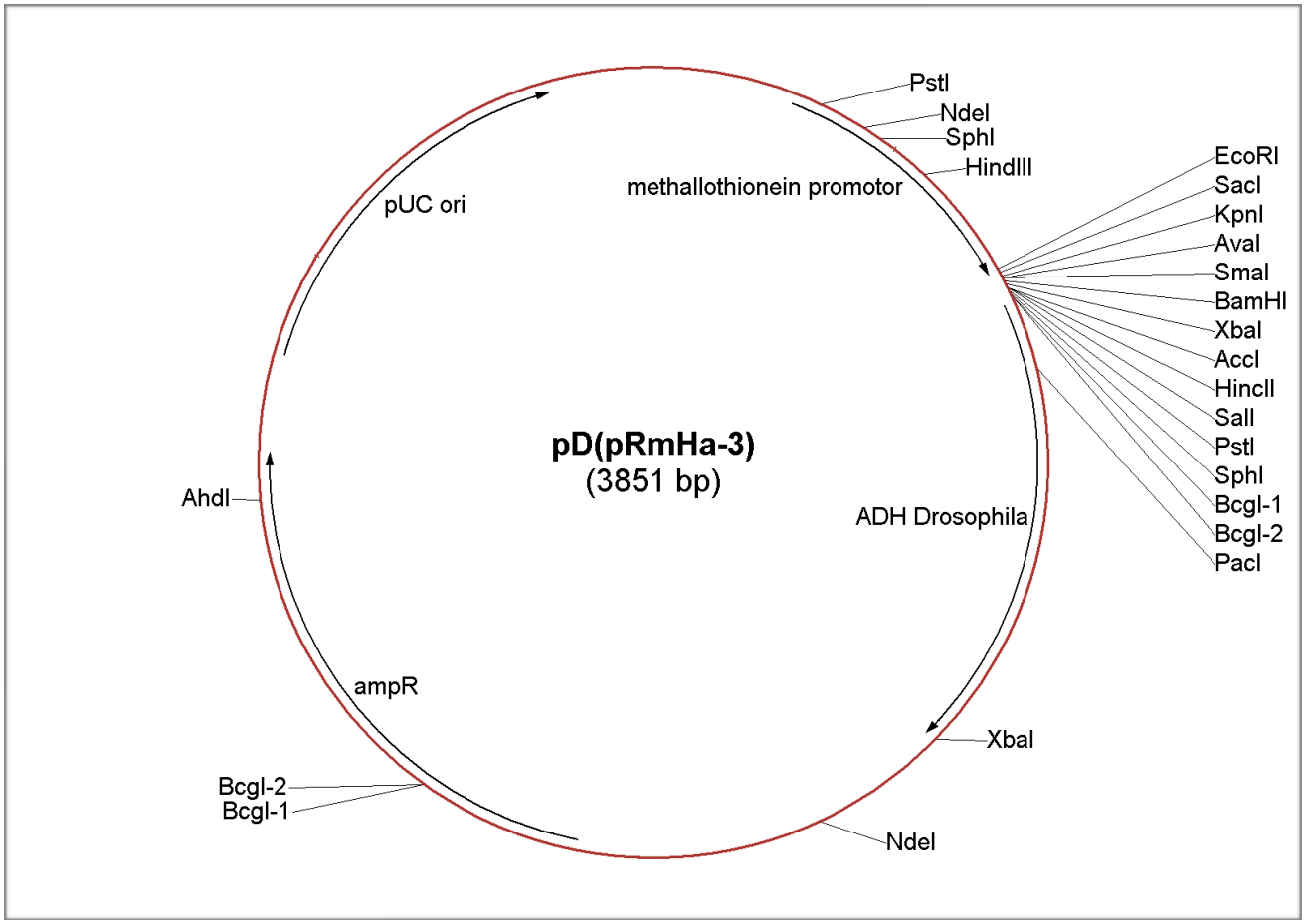

Fig. 2.2: **Vector map of pRmHa-3.**

pGEM T Easy

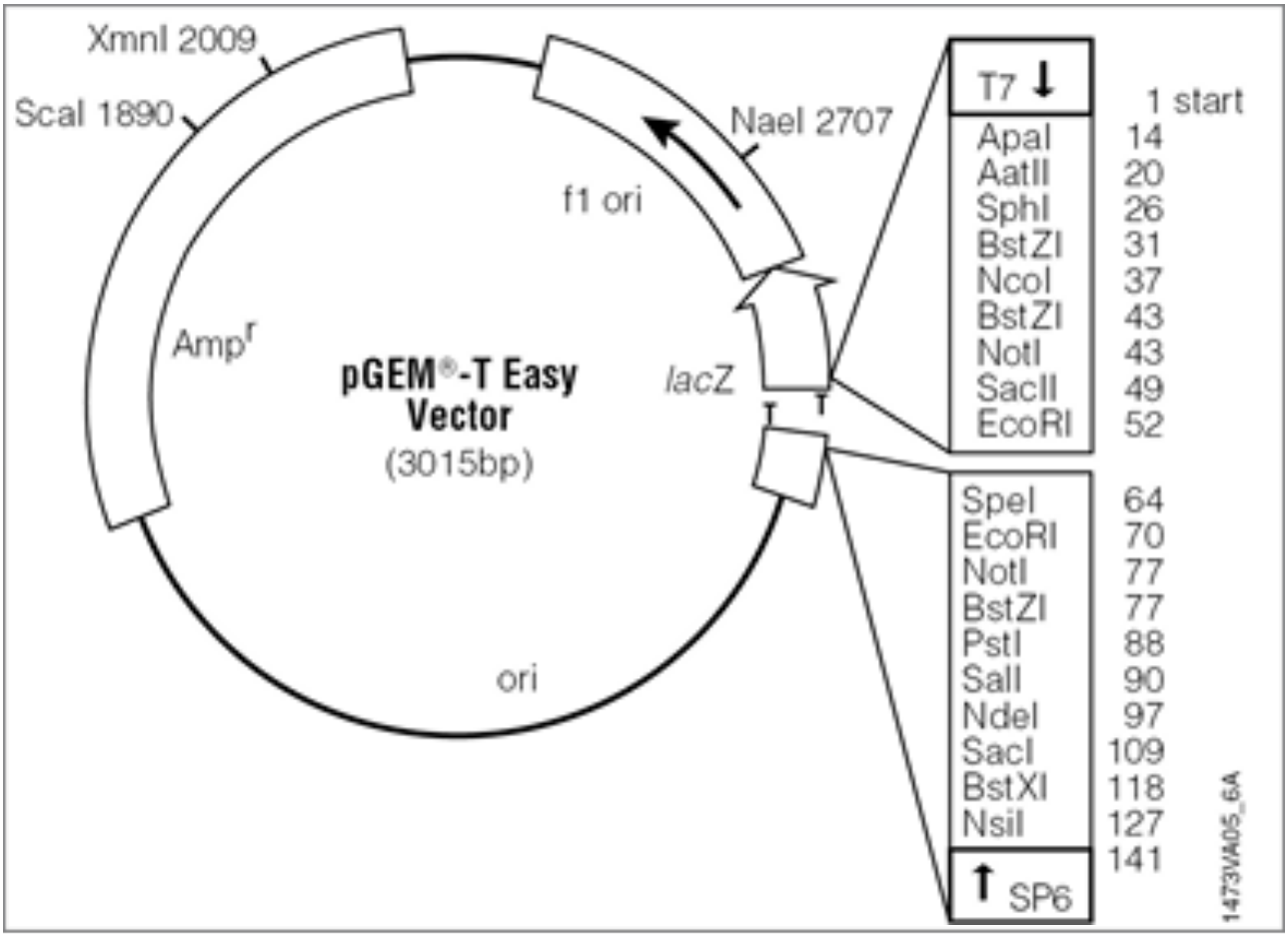

Fig. 2.3: **Vector map of pGEM T Easy.**

2.2 Methods

2.2.1 Cell Culture

Cell Culture

S2 cells were grown at room temperature in Schneider's medium with 10 % FCS and antibiotics in tissue culture flasks and passaged 1:10 when cells reached 80 % density/confluence.

Freezing S2 Cells

S2 cells were harvested for 10 minutes at 200xg and the medium was removed. Subsequently, cells were re-suspended in 1 ml cryo-medium and frozen in liquid nitrogen and stored at -80 °C. The viability of frozen cells was assessed by rapid thawing to 25°C and subsequent harvest at 200xg for 10 minutes. The cryo-medium was removed and cells were re-suspended in fresh Schneider's Medium. On the following day the medium was exchanged and cell viability was monitored by trypan-blue staining after 24, 48 and 72 hours.

Cell viability count

Cells were seeded at a density of 0.5 x 10^6 cells / ml and cultured for 48 hour to reach a density of 1.5 x 10^6 cells / ml. For each count cells were re-suspended and diluted 1:10. Subsequently, 20 μl of the cell suspension was mixed with 20 μl 0.4% Trypan-blue stain (Sigma) and viable cells were counted in a Neubauer counting chamber.

hANG treatment of S2 cells

Cells were seeded at a density of 0.5 x 10^6 cells/ml and cultured for 48 hour to reach a density of 1.5 x 10^6 cells/ml. Before hANG was applied the medium was removed and recombinant hANG was added together with fresh medium.

hANG concentration for RNH1-CoIP: 333 ng/ml

hANG concentration for tRNA fragmentation: 333 ng/ml

Treatment of S2 cells with tRNA fragments

Cells were seeded at a density of 0.5 x 10^6 cells/ml and cultured for 48 hour to reach a density of 1.5 x 10^6 cells / ml. Culture medium was replaced by serum-free medium and cells were starved for 30 minutes. tRNA fragments of two different concentrations (160 ng/ml, 300 ng/ml) were added together with fresh, serum-containing medium and cells were incubated for 24 hours before analysis.

Generating Stable Cell lines

Cells were seeded to a density of 1 x10^6 S2 cells/ml in 3 ml in a 6-well plate and were grown overnight. Solutions A and B were prepared and solution A was added drop-wise to solution B and incubated for 30 minutes at room temperature. Afterwards the solution was added drop wise to the cells and incubated for 16-24 hours. Cells were washed twice, plated in the same well and incubated for 2 days. After two days cells were harvested at 100xg for 10 minutes and re-suspended in selection Medium for transfected cells.

Protein expression in S2 Cells

S2 cells were treated with 0.7 mM $CuSO_4$ or 0.7 mM $ZnCl_2$

Immunostaining of S2 cells

Cells were harvested, diluted and centrifuged onto a Poly-Lysine-slide for 5 minutes at 200xg. Cells were fixed for 10 minutes with 4% PFA at room temperature and washed 5 minutes in PBS. Cells were blocked in blocking solution for 1 hour and incubated with the primary antibody overnight at 4°C. Cells were washed 3 times for 10 minutes with blocking solution at room temperature and incubated with the secondary antibody for 1 hour at room temperature. Cells were washed 3 times for 10 minutes with PBS and then mounted with DAPI. Slides were stored at 4°C.

2.2.2 Nucleic Acids Analysis

RNA extraction

Total RNA was isolated from S2 cells after harvesting the cells at 200xg for 10 minutes at room temperature and washed in cold PBS at 200xg for 10 minutes at 4°C. Cells were re-suspended in PBS and 1 ml Trizol and incubated for 5 minutes at room temperature. After adding 200 μl chloroform samples were centrifuged for 10 minutes at 12.000xg. The aqueous phase was transferred into a new tube, 1 volume of chloroform was added and cells were centrifuged for 10 minutes at 12.000xg. Aqueous phase was transferred into a new tube and 1 volume of isopropanol was added. After precipitation for 10 minutes at room temperature samples were centrifuged for 10 minutes at 16.000xg. The pellets were washed twice with 75% ethanol and resolve in 30-100 μl RNase-free water.

DNAse treatment & removal (Ambion)

Reagents	Amount	Incubation
RNA	3 µg	
10x DNase 1 Buffer	1 µl	
rDNase	1 µl	
H2O	ad 10 µl	20 minutes at 37°C
DNase inactivation reagent	1 µl	2 minutes at room temperature

Tubes were centrifuged at 10.000xg at room temperature for 1.5 minutes and the supernatant was transferred into a fresh tube.

cDNA Synthesis reaction

For cDNA synthesis Super Script III Transcription Kit (Invitrogen) was used.

Reagents	Amount	Incubation
RNA	1.5 µg	
Primers (random hexamers) (50 ng/µl)	1 µl	
dNTPs (10 mM)	2 µl	
H2O	to 14 µl	5 minutes at 68°C
5x Reverse Transcriptase Buffer	4 µl	
DTT (0.1 mM)	1 µl	10 minutes at 25°C
RNaseOUT (40 U/µl)	0.5 µl	60 minutes at 50°C
Reverse Transcriptase (200 U/ µl)	0.5 µl	15 minutes at 75°C
∑	20 µl	

Quantitative Polymerase Chain Reaction (qPCR)

qPCR was performed in 384-well plates using a self-made SYBR Green Mix.

qPCR reaction

Reagents	Amount
cDNA	1-3 µl
2x qPCR MMX	5 µl
Forward Primer (10 µM)	0.2 µl
Reverse Primer (10 µM)	0.2 µl
H_2O	3.6 µl
∑	10 µl

qPCR conditions

Steps	Temperature	Time	Cycles
Denaturing	95°C	15 minutes	1
Cycling	95°C	10 sec	
	60°C	30 sec	40
Melting	95°C	-	1
Cooling	40°C	10 minutes	1

Radioactive Northern Blotting

RNA was denatured for 5 minutes at 70°C and separated on a 12 % Urea-PAGE Gel. RNA was transferred to a Nylon membrane using semi dry blotting with 0.5x TBE for 30 minutes at 5 V. Blotted RNA was immobilized by UV cross-linking and subsequent incubation at 80°C for at least 1 hour or overnight. Membrane was pre-hybridized in hybridization buffer for 1 hour at 40°C and the radioactively labeled probe was added to the hybridization solution and incubated overnight at 42°C. After hybridization the membrane was washed 15 minutes in Northern wash A at 42 °C and 15 minutes in Northern wash B at room temperature. Membrane was rinsed in 1x SSC and exposed to a phosphoimager plate.

Radioactive probe labelling reaction

Reagents	Amount
Oligonucleotide (20 μM)	1 μl
10x T4 PNK Buffer	2 μl
T4 PNK (10 U/μl)	1 μl
^{32}P-γ-ATP (500 μCi)	2-5 μl
H_2O	ad 20 μl

Reaction was incubated for 1hr at 37 °C and subsequently cleaned with Micro BioSpin 6 Chromatography Columns (Bio-Rad) kit and heated at 94 °C for 5 minutes before usage.

Stripping Northern Blots

The membrane was incubated for 30 minutes in 0.1% SDS at 70 °C.

Extraction of tRNA fragments

tRNAs were analysed and excised from UREA-PAGE gel and frozen (-80 °C) for 30 minutes. 400 μl elution buffer was added and samples were incubated overnight on a spinning wheel. Tubes were centrifuged and filtered in Nanosep columns for 10 minutes at maximum speed and tRNA fragments were extracted with Phenol:Chloroform:Isoamylalkohol (25:24:1) and precipitated with ethanol.

Standard PCR settings

PCR reaction

Reagents	Amount
cDNA	2 μl
5x GoTaq Buffer (Promega)	5 μl
Forward Primer (10 mM)	1 μl
Reverse Primer (10 mM)	1 μl
dNTPs	1 μl
Taq Polymerase (5 U/μl)	0.25 μl
H_2O	ad 25 μl

PCR conditions

Temperature	Time	Cycles
94°C	3 minutes	1
94°C	30 sec	
55°C	30 sec	30
72°C	30 sec	
72°C	5 minutes	1
4°C	∞	

PCR for amplifying long fragments (RNH1)

PCR reaction

Reagents	Amount
cDNA in vector	2 µl
5x GoTaq Buffer (Promega)	10 µl
Forward Primer (10 µM)	1 µl
Reverse Primer (10 µM)	1 µl
dNTPs	1 µl
Taq Polymerase (5 U/µl)	0.5 µl
H_2O	ad 50 µl

PCR conditions

Temperature	Time	Cycles
94°C	3 minutes	1
94°C	30 sec	
55°C	30 sec	5
72°C	90 sec	
94°C	30 sec	
60°C	30 sec	25
72°C	90 sec	
72°C	10 minutes	1
4°C	∞	

Semi-quantitative PCR for determining of relative expression of mus308

PCR reaction

Reagents	Amount
cDNA	2 µl
5x GoTaq Buffer (Promega)	5 µl
Forward Primer (10 µM)	0.5 µl
Reverse Primer (10 µM)	0.5 µl
dNTPs	0.5 µl
Taq Polymerase (5 U/µl)	0.25 µl
H_2O	ad 25 µl

PCR conditions

Temperature	Time	Cycles
94°C	3 minutes	1
94°C	30 sec	
55°C	30 sec	25
72°C	30 sec	
72°C	5 minutes	1
4°C	∞	

2.2.3 Molecular Cloning Techniques

Ligation into Topo (PCR 2.1) vector (Invitrogen)

Ligation reaction

Reagents	Amount	Incubation
T4 DNA Ligase Reaction Buffer	2 µl	
PCR 2.1 vector (250 ng/µl)	2 µl	
Express Link T4 DNA Ligase (5U)	1 µl	1 hour at room temperature
PCR Product	x µl	
H2O	ad 10 µl	

Ligation into pRmHA-3 (Roche - Rapid Ligation Kit)

Ligation reaction

Reagents	1:1	3:1	ctrl	Incubation
Vector (70 ng/µl)	0.5 µl	0.5 µl	0.5 µl	
insert	1 µl	3 µl	0 µl	
H2O	4 µl	2 µl	5 µl	1 hour at room temperature
2x Ligation Buffer	10 µl	10 µl	10 µl	
5x DNA Dilution Buffer	4 µl	4 µl	4 µl	
Ligase	0.5 µl	0.5 µl	0.5 µl	

Transformation into *E. coli* XL1-blue

Competent cells were incubated with ligation reaction for 20 minutes on ice and heat-shocked for 45 sec at 42 °C. After cells were placed on ice for 2 minutes, SOC medium was added and cells were recovered at 37°C for 1 hour. Cells were plated on Amp plated (for blue white screening plates were treated with 20 µl X-Gal (20 mg/ml) and 20 µl IPTG (0.1 M)).

Plasmid Mini Prep (Promega)

Inoculated colonies were harvested at maximum speed and re-suspended in 600 µl TE Buffer. 100 µL Lysis Buffer was added and cells were inverted 5 times, then 350 µl neutralization solution was added and cells were again inverted 5 times. The lysates were centrifuged at maximum speed for 3 minutes and the supernatant was transferred into a mini column with a collection tube. The tube was centrifuged 15 seconds at maximum speed and 200 µl endotoxin removal wash was added. After tube was centrifuged for 15 seconds at maximum speed, 400 µl column wash solution was added and columns were centrifuged at maximum speed. Columns were transferred to fresh tubes and incubated for one minute in 30 µl Elution Buffer and centrifuged at maximum speed for 15 seconds.

Plasmid Midi Prep (Qiagen)

Cells were harvested for 15 minutes at 6.000xg at 4 °C and re-suspended in 4 ml Buffer P1. 4 ml Buffer P2 was added, the tube was inverted 6 times and incubated for 5 minutes at room temperature. 4 ml cooled Buffer P3 was added and tube was inverted 6 times and incubated for 15 minutes on ice. Lysate was centrifuged at 20.000xg for 30 minutes at 4°C, and the supernatant was filtered to remove of cell debris. A QIAGEN-tip 100 was equilibrated with 4 ml Buffer QBT and washed two times with 10 ml Buffer QC. Supernatant was added and DNA was eluted with 5 ml Buffer QF into a 15 ml Falcon tube. DNA was precipitated by adding 3.6 ml isopropanol followed by centrifugation for 30 minutes at 4°C at 15.000xg. DNA Pellet was washed with 70% EtOH and subsequently air dried and re-suspended in TE.

Gel & PCR Cleanup System (Promega)

Gel fragments were dissolved in Membrane Binding Solution at 65 °C and transferred into a micro-column and collection tube. Sample was incubated for one minute and subsequently centrifuged for one minute at 16.000xg. 700 µl Wash Solution was added and centrifuged for one minute at 16.000xg. Then 500 µl Wash Solution was added and centrifuged for 5 minutes at 16.000xg. Tube was again centrifuged for 5 minutes at 16.000xg after the flow-through was removed and then the column was transferred into an Eppendorf Tube. 30 µl H_2O were added and incubated for one minute at room temperature ad centrifuged at 16.000xg for one minute.

Alkaline Phosphatase Treatment (Thermo Scientific)

Phosphatase Reaction

Reagents	Amount	Incubation
Alkaline phosphatase	1 U/μg DNA	
10 x Alkaline Phosphatase Buffer	1 μl	
DNA	100 ng vector	30 minutes at 37°C
H_2O	ad 10 μl	20 minutes at 75°C

Designing expression plasmids

First a cDNA template of the respective gene was PCR amplified with primers, designed to incorporate restriction sites, as well as a FLAG-Tag (and a HA-Tag in the case of hANG). After that, the PCR product was ligated into a Topo Vector and transformed into XL1-blue cells to perform blue white screening. White colonies were picked and the correctness of the sequence was assured by sequencing. This vector was then cut at the respective restriction sites of the insert and inserted into a previously cut and de-phosphorylated vector (pRmHA-3). After the ligation was performed the vector was transformed into XL1-blue cells. The presence of the insert was confirmed by restriction digest. Finally a midi-prep was performed on the positive clones in order to transfect the plasmid DNA into S2 cells.

pRmHA-3-hANG-FLAG-HA

The human Angiogenin (hANG) coding sequence was amplified by PCR from human cDNA introducing specific restriction sites, following by digestion with restriction enzymes and ligation into pRmHA-3 (Fig. 5.1).

pRmHA-3-RNH1-FLAG

After the RNH1 construct was PCR amplified and sub-cloned it was restricted and ligated into pRmHA-3 and subsequently tested for the presence of the insert (Fig. 5.2).

2.2.4 Protein Techniques

Protein extraction

For whole protein extracts of S2 cells, cells were harvested at 200xg for 10 minutes and washed in PBS at 200xg for 10 minutes. Cells were homogenized in protein extraction buffer and incubated for 10 minutes on ice followed by centrifugation at 16.000xg for 10 minutes at 4°C. Protein concentration was measured by Bradford Assay (Roche).

Western Blotting

100 µg of protein extract was solubilized in SDS-sample buffer and denatured at 95 °C for 5 minutes and analysed by 10-15 % SDS-PAGE electrophoresis (90 V / 130 V). Proteins were transferred to cellulose membrane using semi-dry blotting in Blotting Buffer (30 minutes at 5 V=constant followed by 30 minutes 10 V=constant). The membranes were blocked in 5% non-fat dried milk/PBS for 30 minutes and incubated with primary antibody overnight at 4 °C. After incubation, membrane was washed 3 times for 5 minutes at room temperature with 5% non-fat dried milk/PBS and incubated with secondary antibody (anti-mouse: 1:10.000, anti-goat: 1:10.000, anti-rabbit: 1:10.000). Membranes were washed 3 times for 5 minutes at room temperature with 5% non-fat dried/PBS and once with 0.1 % PBS-Tween-20. Membrane was incubated in ECL solution for one minute and developed by exposure on film.

Stripping Western Blots

Cells were baked in 0.1 M glycine solution (pH 2) in a microwave (520 W, 5 minutes) and washed 3 times in PBS-T.

Staining SDS-PAGE Gels (Life Technologies)

SDS-PAGE gel was fixed in fixing solution for 10 minutes and incubated overnight in staining solution. Stained gel was de-stained in H_2O.

Immunoprecipitation

Cells were harvested at 150xg for 10 minutes at 4 °C and washed in ice-cold PBS at 150xg for 10 minutes at 4 °C. Cells were incubated for 10 minutes in 1 ml Buffer A, and subsequently homogenized with a syringe of gauge size 30.After TritonX-100 was added to a concentration of 0.1 the tubes were rotated for 15 minutes at 4 °C. Tubes were centrifuged at 480xg for 9 minutes at 4 °C, the supernatant was collected and centrifuged at 1.300xg for 5 minutes at 4 °C. The supernatant was collected and centrifuged at 16.100xg for 30 minutes at 4 °C. Protein concentration was measured using Bradford reagent, and an aliquot of total cell extract was diluted in 4x SDS Buffer to 1x SDS sample buffer concentration. For each sample, 30 µl of FLAG binding resin was centrifuged at 8.200xg for 2 minutes at room temperature, the supernatant was removed and the resin was washed 2 times for 5 minutes in 0.5 ml PBS and equilibrated in Buffer A. Resin was distributed into fresh tubes (2 for each sample) and buffer A was added to a volume of 500 µl. 3x FLAG peptide was added to a concentration of 150 ng/µl to one of the two tubes (as control experiment) and rotated for 30 minutes at room temperature. 1 mg protein extract was added to both tubes and rotated at 4 °C for 1 hr. After resins were centrifuged at 8.200xg for 30 seconds at room temperature they were washed 5 times for 5 minutes in Buffer A. 3 µl of 5 µg/µl FLAG peptide stock was added to 50 µl PBS and distributed to the samples. Samples were incubated shaking for 15 minutes at 25 °C and centrifuged at 8.200xg for 30 seconds at room temperature. Supernatant was diluted in 4x SDS loading dye to 1x SDS sample buffer and analysed with SDS-PAGE.

3 Results

3.1 Testing Potential RNA Virus Infection of S2 Cell Lines

As the components of RISC are also involved in controlling virus-derived RNAs it was important to determine whether the available S2 cell lines were infected with *Drosophila* viruses. To test the infection status the presence of the most common *Drosophila* RNA viruses was analyzed by semi-quantitative PCR. Two cell lines in use (S2-new; S2-Ameres) were analyzed along with S2 cells, which were contaminated with Flock-house virus (FHV, personal gift from Madalena Reimao Pinto, Ameres Lab). Besides FHV, cells were tested for the existence of various other *Drosophila* RNA viruses such as *Drosophila* C Virus (DCV), *Drosophila* X Virus (DXV), *Drosophila* Sigma Virus and *Drosophila* Nora Virus (DNV).

After RNA from FHV-contaminated cells was used as a positive control to test the PCR settings, S2-new and S2-Ameres cells were tested for the presence of viral RNA (Fig. 3.1).

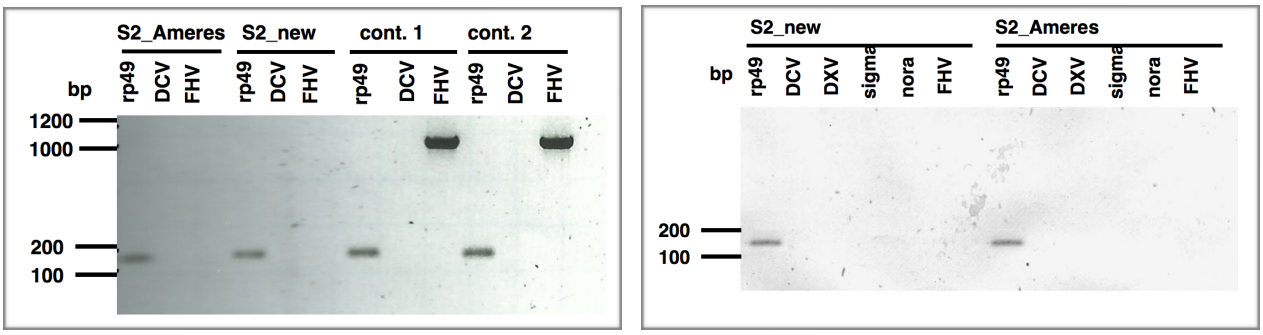

Fig. 3.1: **Assessing viral contamination in S2 cells.** (left) PCR products on 0,8 % Agarose gel of S2 cell lines and contaminated controls to test the PCR settings for assessing the infection status of S2 cells.(right) PCR products on 0,8% Agarose gel of S2 cell lines to test for the presence of various RNA viruses. rp49 = ribosomal protein 49; DCV = *Drosophila* C Virus, FHV = Flock-house Virus; DXV = *Drosophila* X Virus; sigma = *Drosophila* Sigma Virus; nora = *Drosophila* Nora Virus.

In both experiments, viral RNA was not detectable, except FHV in the positive control S2 cells, indicating that no substantial levels of viral RNAs might impact RNAi pathways in the available S2 cell lines.

3.2 Designing a System to Induce tRNA Fragmentation Without Stress

3.2.1 Inducible Expression Plasmids

Two expression plasmids, containing the coding sequences for hANG and RNH1 were designed that also contained sequences for small epitope tags (Fig. 3.2). A third expression plasmid containing the coding sequence for Dnmt2 and a FLAG-tag had already been published (Schaefer et al., 2010) and was ready for use.

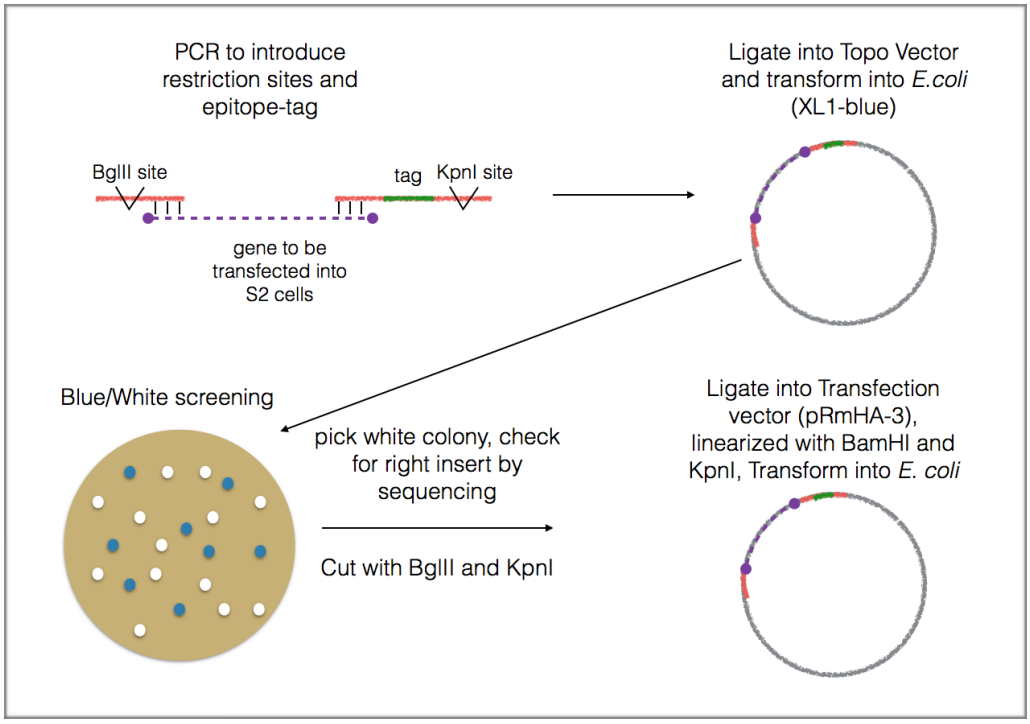

Fig. 3.2: **Workflow for constructing the expression plasmids.**

The three different expression vector systems offer different possibilities for inducing and controlling tRNA fragmentation in *Drosophila* S2 cells (see Fig. 3.3).

(A) In case of the hANG construct, hANG expression and tRNA fragmentation can be induced through the addition of copper ions (Cu++) to the cell culture medium. hANG expression can be limited by washing out Cu++ at various time points (see Fig. 3.3(A)).

(B) In case of the RNH1 construct, tRNA fragmentation can be first induced by addition of recombinant hANG to the cell culture medium, followed by the expression of RNH1 through induction with Cu++, which will limit hANG activity as RNH1 binds and inhibits hANG (see Fig. 3.3(B)).

(C) On the other hand, ectopic Dnmt2 expression should affect the extent of tRNA fragmentation in an indirect manner, as Dnmt2 binds to tRNAs and methylates the anticodon loop of three tRNAs in

Drosophila (Schaefer et al., 2010). Binding to tRNAs or methylation of these tRNAs should affect the efficiency of hANG-mediated tRNA cleavage. In this system, after addition of recombinant hANG to the cell culture medium, the induction of Dnmt2 expression through addition of Cu++ should interfere with hANG activity on three tRNAs (see Fig. 3.3(C)).

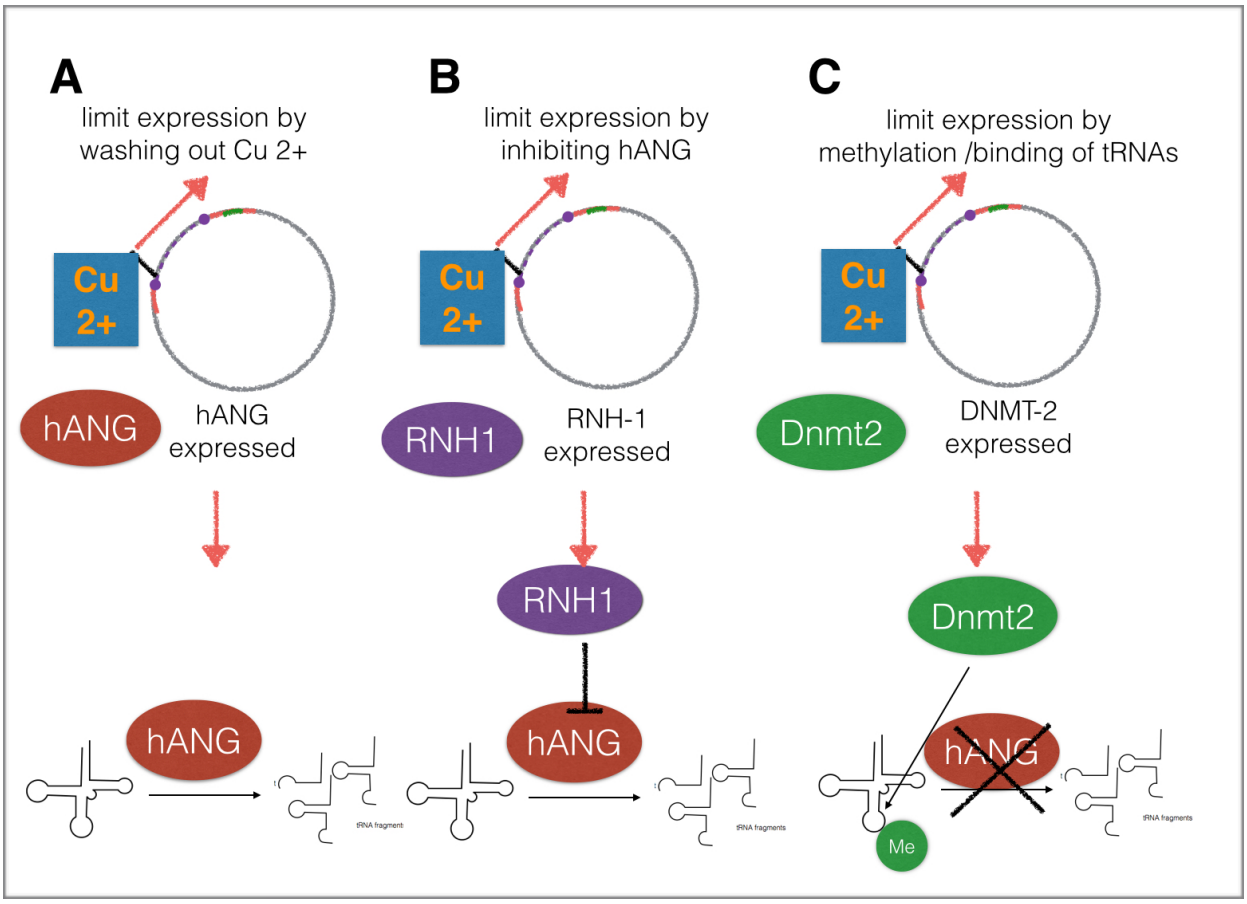

Fig. 3.3: **Expression systems for inducing and limiting tRNA fragmentation.** (A) tRNA fragmentation is induced and limited by hANG expression through application and removal of Cu++. (B) tRNA fragmentation is induced by application of ectopic hANG and limited by induction of RNH1, which binds and inhibits RNH1. (C) tRNA fragmentation is induced by application of ectopic hANG and limited by induction of Dnmt2, which methylates/binds tRNAs and thereby protects them from cleavage.

3.3 Integration of Expression Plasmids into *Drosophila* S2-Cell Lines

3.3.1 Transfection of hANG-Dnmt2- and RNH1-Containing Vectors

Individual expression plasmids were co-transfected with a vector carrying a blasticidin resistance gene into growing S2 cells (Fig. 3.4) according to the manufacturer's instructions. Two weeks after the transfection the presence of stable integrations of the respective plasmids was determined by protein expression tests.

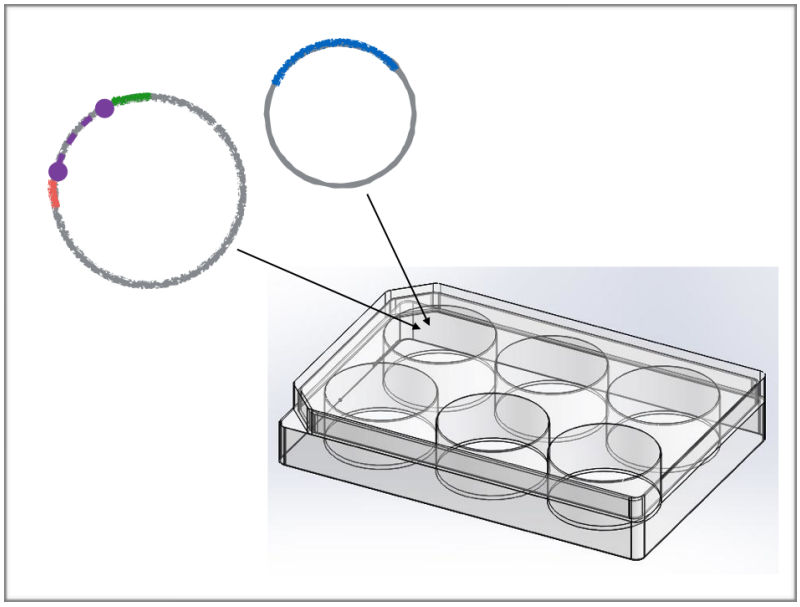

Fig. 3.4: **Transfection of expression plasmids into S2 cells.** The pRmHA-3 vector is co-transfected with a helper plasmid carrying a Blasticidin resistance gene for selection. red line: metallothionein promotor; green line: tag; dashed purple line: coding sequence, blue line: Blasticidin resistance gene

3.3.2 Expression Tests on Stably Transformed S2 cells containing the hANG Expression Vector

To test whether the transfected S2 cells express hANG-FLAG-HA cells were induced with $CuSO_4$ and harvested at several time points after induction (one, 6, 24 hours) and Western blotting using antibodies against the HA-epitope was performed on total protein extracts.

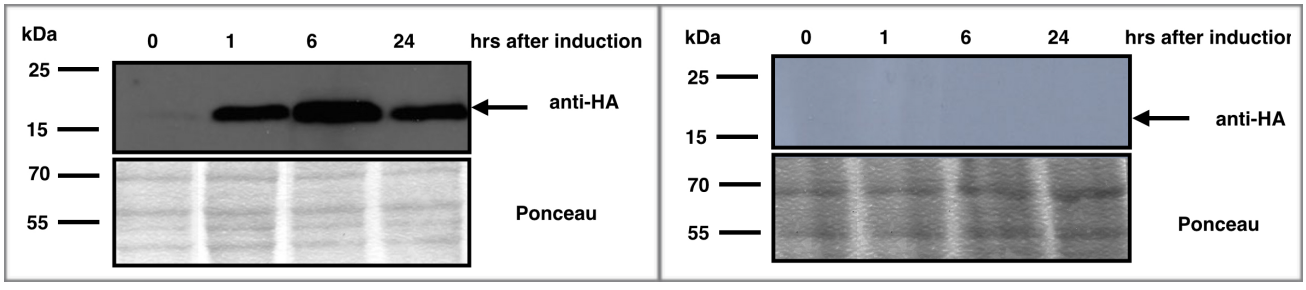

Fig. 3.5: **Cu++ induces hANG-FLAG-HA expression.** hANG-FLAG-HA expression induced by Cu++ (left) and Zn++ (right) after one, 6 and 24 hrs including an uninduced control. hANG-FLAG-HA is indicated by the arrow.

S2 cells carrying the hANG-expression vector expressed detectable amounts of hANG beginning after one hour, peaking at 6 hours and decreasing again after 24 hours (Fig. 3.5). The reduction of hANG expression after 24 hours in the presence of Cu++ could be explained by previ-

ous observations, which showed that Cu++ binds and thereby inhibits hANG (Giacomelli et al., 2015).

To circumvent an inhibition of human Angiogenin by Cu++ another divalent ion (Zn++) was used as activator of hANG expression. Zn++ was found to be an alternative inducer of metallothionein promotors (Zalewska et al., 2014). To do so, cells were incubated with $ZnCl_2$ and harvested after one, 6 and 24 hours to ensure comparability with the experiment using Cu++ as inducer. The results showed that hANG-FLAG-HA expression could be induced robustly but only temporarily using Cu++ whereas Zn++ was not able to induce Angiogenin expression in S2 cells (Fig. 3.5).

3.3.3 Expression Tests on Stably Transformed S2 cells containing the RNH1 Expression Vector

To test whether the transfected S2 cells express RNH1-FLAG cells were induced with $CuSO_4$ and harvested at several time points after induction (one, 6, 24 hours) and Western blotting using antibodies against the FLAG-epitope was performed on total protein extracts.

RNH1-FLAG expression started after 6 hours and peaked at 24 hours of induction (Fig. 3.6). The blot showed significant cross-reactivity of the anti-FLAG antibodies with various other proteins making the visualization of RNH1-FLAG problematic. Because the anti-FLAG antibodies cross-reacted with a polypeptide of approximately the same molecular weight as RNH1 an immunoprecipitation was performed to purify RNH1-FLAG from cells that were induced for 24 hours with $CuSO_4$ (Fig. 3.6). The results indicated that high levels of ectopic RNH1-FLAG could be expressed in S2 cells (Fig. 3.6).

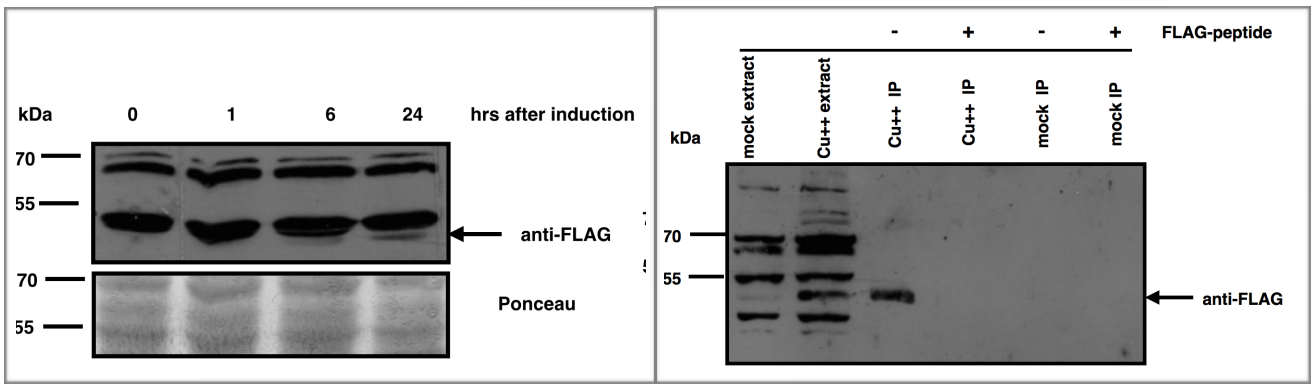

Fig. 3.6: **Cu++ induces RNH1-FLAG expression.** (left) RNH1-FLAG expression after one, 6 and 24 hrs of Cu++ induction including an uninduced control. RNH1-FLAG is indicated by the arrow.(right) Immunoprecipitation of RNH1-FLAG after 24 hrs of Cu++ induction.

3.3.4 Expression Tests on Stably Transformed S2 cells containing the Dnmt2 Expression Vector

To confirm the expression of Dnmt2-FLAG cells were induced with $CuSO_4$. Cells were harvested at several time points after induction (one, 6, 24 hours) and Western blotting was performed on total protein extracts. Low levels of Dnmt2-FLAG were detectable after one hour with increasing

amounts detectable over time (Fig. 3.7). The results indicated that high levels of ectopic Dnmt2-FLAG could be expressed in S2 cells.

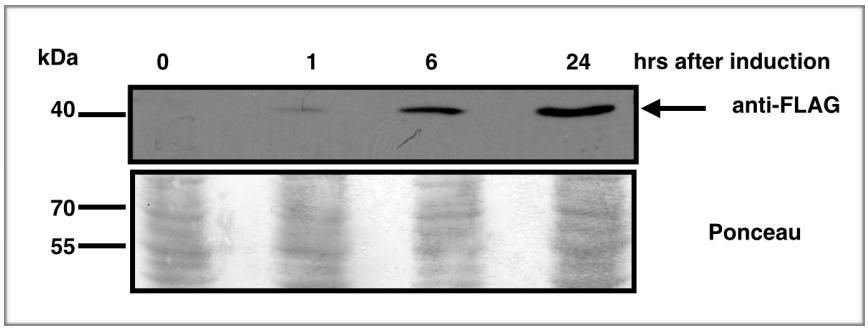

Fig. 3.7: **Cu++ induces Dnmt2-FLAG expression.** Dnmt2-FLAG expression after one, 6 and 24 hrs of Cu++ induction including an uninduced control. Dnmt2-FLAG is indicated by the arrow.

In summary, the results of these expression tests showed that all cell lines stably propagated the expression vectors with respective inserts and that expression of all proteins could be robustly induced. However, the experiments with hANG-FLAG-HA indicated that the time window for hANG expression must be kept below 24 hours because of the likely inhibitory effects of the inducer (Cu++) on the protein levels of Angiogenin.

3.4 Determining tRNA Fragmentation in S2 Cells Expressing Angiogenin

3.4.1 Kinetics of tRNA Fragmentation during Constant Induction of hANG

In order to assess the extent of tRNA fragmentation that was induced by the expression of hANG-FLAG-HA in S2 cells several experiments were performed to select the most informative time points for the subsequent investigation of tRNA fragment-mediated effects. First, hANG-FLAG-HA expression was confirmed using Western blotting. The results showed that hANG-FLAG-HA was detectable after one hour of induction but seized to be detectable after 24 and 48 hours, respectively (Fig. 3.8) confirming previous expression tests.

Next, the fragmentation of specific tRNAs (Gly-GCC, Asp-GUC) during the time course of hANG-FLAG-HA was assessed (0, two, four, 6, 8, 24, 48 hours) using Northern blotting. The results showed that tRNA fragmentation started as early as two hours after hANG-FLAG-HA induction (Fig. 3.8). The amount of detectable tRNA fragments increased until 48 hours after induction. In order to test, whether Angiogenin activity was specific for tRNAs, a probe against a ribosomal RNA (5S rRNA) was used. Notably, some 5S rRNA degradation could be observed after 48 hours of induction (Fig. 3.8), which corresponded to the time point of increased cell death (see below). Using an RNA integrity chip it was shown that hANG affected the integrity of abundant RNAs such as 18S and 28S rRNA (5.4). These results indicated that constant expression of hANG could also affect other RNAs and/or cause cytotoxicity.

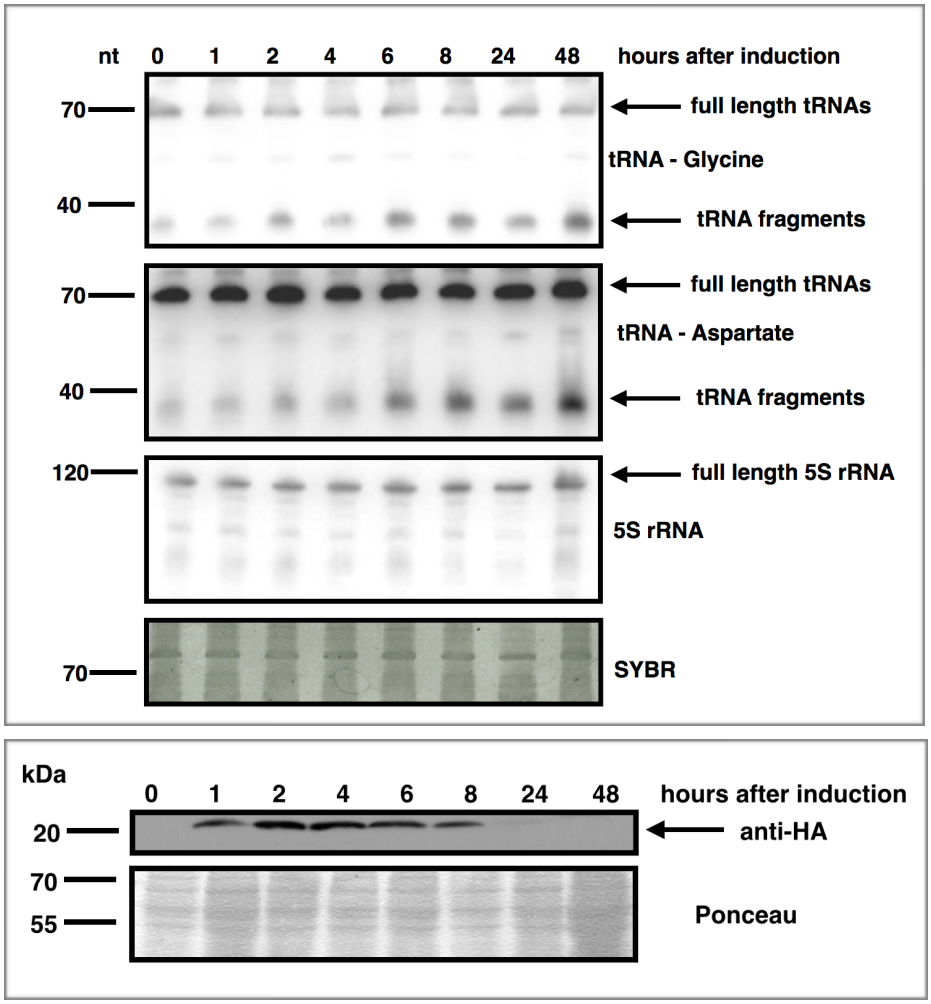

Fig. 3.8: **tRNA fragmentation and hANG-FLAG-HA expression in the course of Cu++ induction.** (top) tRNA fragmentation monitored for 0, one, two, four, 6, 8, 24 and 48 hours after Cu++ induction for the tRNAs Glycine and Aspartate and 5S rRNA. (bottom) hANG-FLAG-HA expression monitored for 0, one, two, four, 6, 8, 24 and 48 hours after Cu++ induction.

3.4.2 Kinetics of tRNA Fragmentation after Removal of hANG Induction

As 5S rRNA degradation could be observed after constant hANG-FLAG-HA expression (see Fig. 19), the extent of tRNA fragmentation and 5S rRNA degradation after removal of the inducing Cu++ was determined. Cells expressed hANG-FLAG-HA for 8 hours (pulse period) before the culture medium was exchanged and cells were cultured in fresh medium for a further 24 or 48 hours (chase period). When hANG-FLAG-HA expression was stopped after 8 hours no 5S rRNA degradation was detectable after 48 hours while tRNA fragments were still detectable at this time point (Fig. 3.9). These experiments showed that inducing hANG-FLAG-HA only temporarily followed by washing out the inducing Cu++ can reduce/limit the hANG-mediated cytotoxicity while maintaining increased levels of tRNA fragmentation.

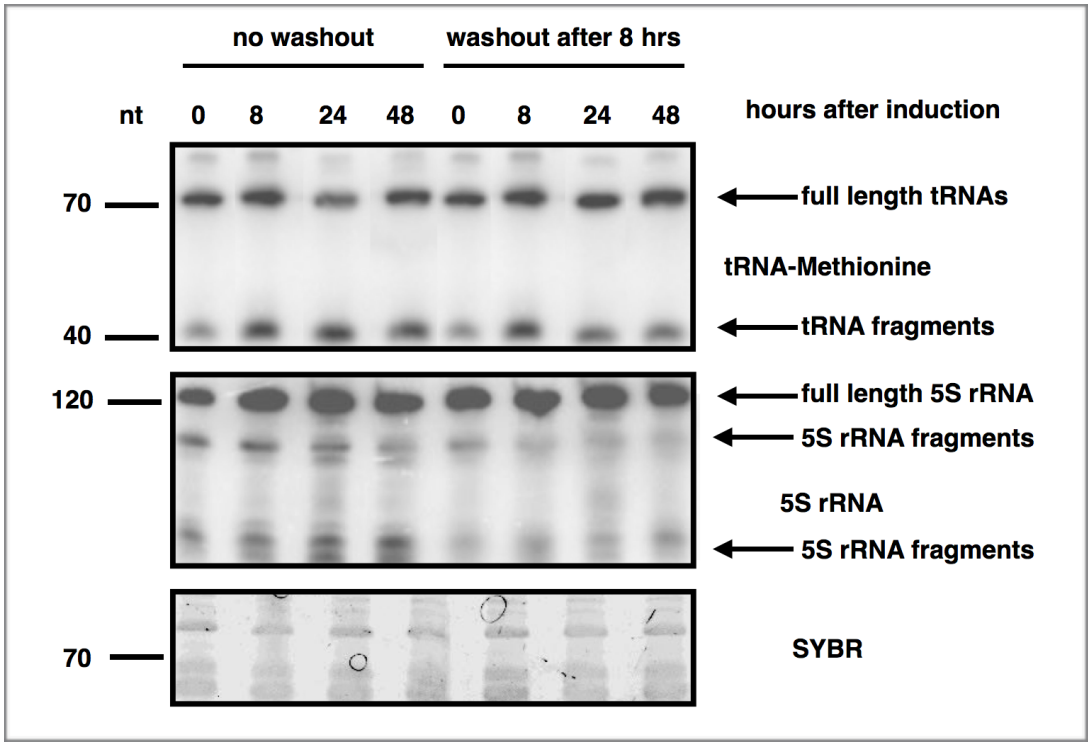

Fig. 3.9: **Constant Cu++ induction of hANG-FLAG-HA leads to 5S rRNA degradation.** tRNA fragmentation of S2 cells that were induced for 0, 8, 24 and 48 hours with Cu++ compared to cells that where induced for 0 and 8 hours with Cu++ and then cultured for 24 and 48 hours in fresh medium for the tRNA Methionine and 5S rRNA.

3.4.3 Effects on Cell Viability

Angiogenin activity affects not only tRNA fragmentation but also rRNA transcription (Tsuji et al., 2005). Constant expression of hANG-FLAG-HA in S2 cells might also cause cytotoxicity due to the degradation of other RNAs such as 5S rRNA (see Fig. 3.8). Therefore, the effect of human Angiogenin expression on S2 cell viability was determined. First, S2 cells, which expressed hANG-FLAG-HA continously were collected after 24 and 48 hours and cell viability was measured. Second, S2 cells were induced with Cu++ to express hANG-FLAG-HA for 8 hours (pulse) followed by culturing cells without Cu++ for another 24 to 48 hours (chase) in fresh medium and cell viability was measured. The results of these experiments showed that continuous expression of hANG-FLAG-HA caused a decrease in the number of living cells from 98% in controls to 85% and 74% after 24 hours and 48 hours, respectively (Fig. 3.10). Furthermore, removing the inducing Cu++ after 8 hours resulted in an increase of cell viability after further culturing for 24 and 48 hours to 94 % and 93%, respectively (Fig. 3.10). Importantly, Northern blotting for tRNA-Met^{AUG} at these time points showed that while cell viability was increased tRNA fragments were still detectable (Fig. 3.9).

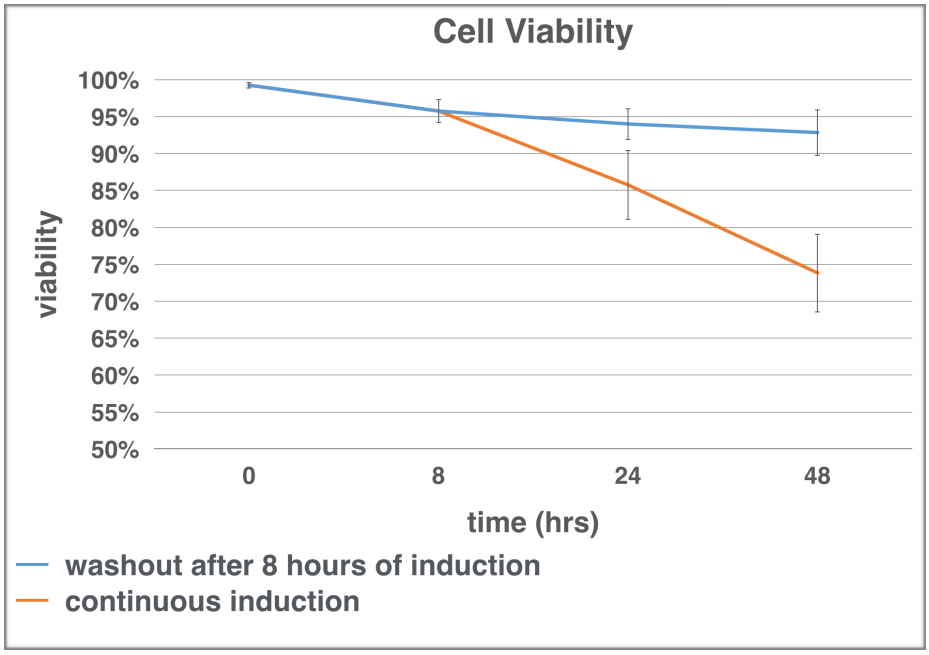

Fig. 3.10: **Limiting Cu++ induction increases cell viability.** Cell viability after induction of hANG-FLAG-HA for 48 hours compared to culturing cells in fresh medium after 8 hours of induction. Error bars indicate the standard deviation between three experiments.

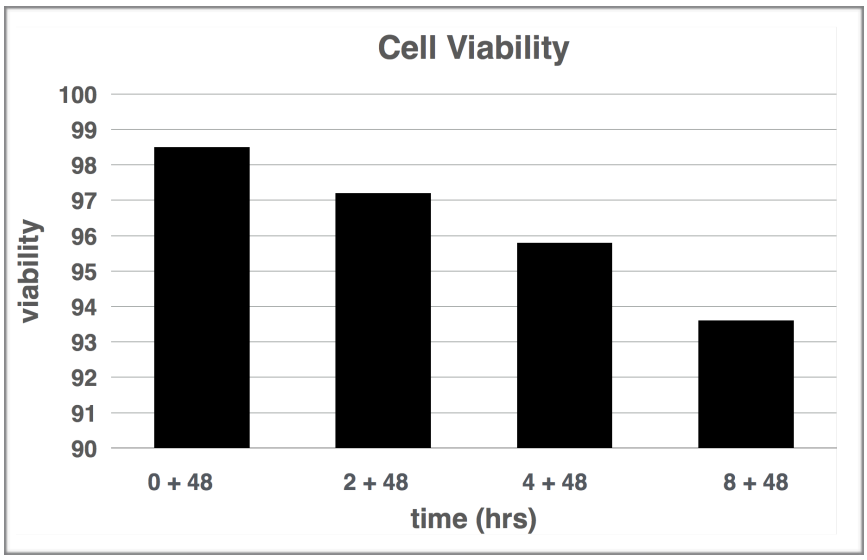

Fig. 3.11: **Limiting Cu++ induction to two hours results in viability similar to controls.** Cell viability after Cu++ induction for 0, two, four or 8 hours and subsequent culturing in fresh medium for 48 hours.

In order to assess whether limiting the time of hANG-FLAG-HA expression even further would increase cell viability the inducing Cu++ was removed after two, four or 8 hours and the cells were subsequently incubated for 48 hours in fresh medium followed by cell viability testing. The results of these experiments showed that limiting the expression of hANG-FLAG-HA to two hours and culturing the cells without Cu++ for another 48 hours (chase) resulted in a viability of 97% compared to 98% in controls (Fig. 3.11). Longer induction times decreased the cell survival, as

cells that were incubated for four or 8 hours with Cu++ and then cultured in fresh medium for 48 hours showed a viability of 96% and 94%, respectively (Fig. 3.11).

These results indicate that hANG-FLAG-HA expression for 2-8 hours followed by additional culturing of cells for 24-48 hours were best suited to test for gene expression changes as tRNA fragmentation but no cytotoxicity was observable.

3.4.4 Immunostaining for hANG-FLAG-HA Expression in S2 Cells

Because it was reported that endogenously expressed Angiogenin is both a cytoplasmic as well as a nuclear protein (Pizzo et al., 2013) the cellular distribution of ectopically expressed hANG-FLAG-HA was monitored using immunostainings on S2 cells. The localization of hANG-FLAG-HA after 8 hours of continuous expression was compared to expression for 8 hours followed by washout culturing for additional 24 or 48 hours (chase period). Using indirect immunofluorescence and antibodies against human Angiogenin showed that the sub-cellular localization of ectopic hANG-FLAG-HA changed during the chase period. 8 hours after expression of hANG-FLAG-HA (pulse period), the protein was mostly detectable in the nucleus whereas after a chase period of 24 to 48 hours hANG-FLAG-HA localized also to the cytoplasm (Fig. 3.12).

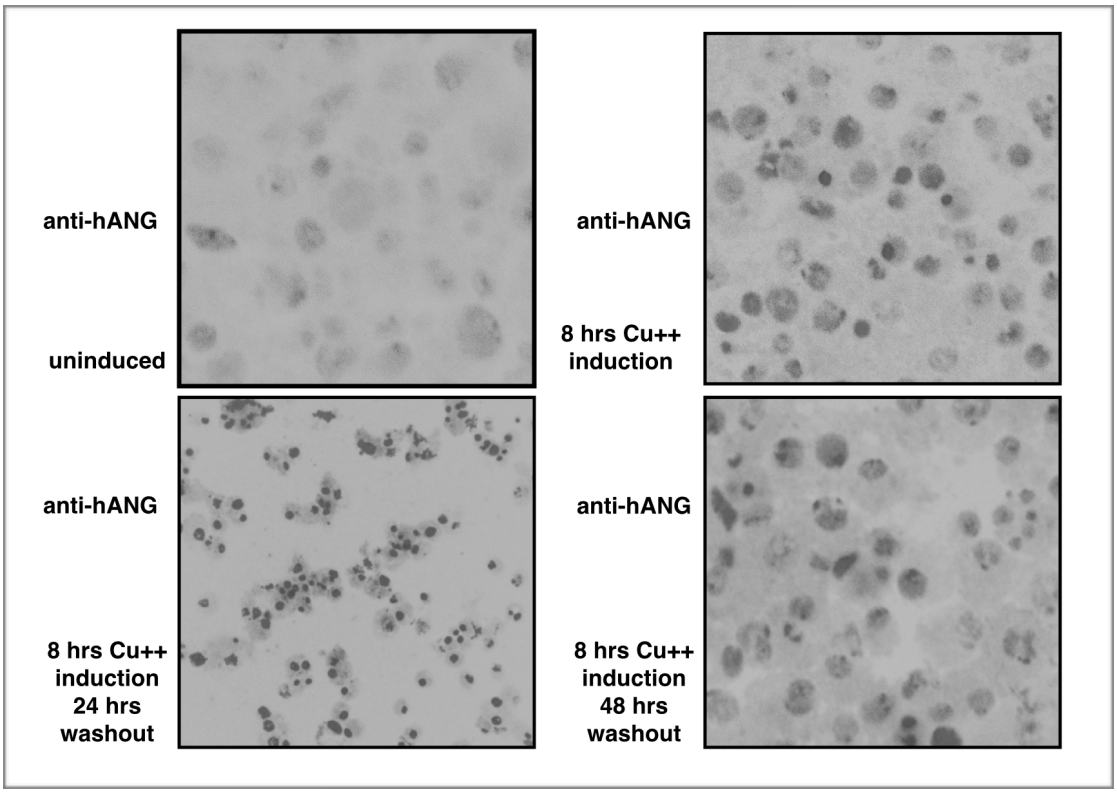

Fig. 3.12: **Localization of hANG-FLAG-HA in the course of Cu++ induction.** Immunostaining of hANG-FLAG-HA after 0 and 8 hours of induction with Cu++ compared to 8 hours of Cu ++ induction followed by culturing the cells for 24 or 48 hours in fresh medium.

These results indicated that limiting hANG-FLAG-HA expression to 8 hours and culturing cells in fresh medium for 24 or 48 hours affected its sub-cellular localization and might thereby affect its activity on tRNA fragmentation and subsequently cell survival.

3.4.5 Measurement of hANG-FLAG-HA Levels after Ectopic Expression in S2 Cells

Angiogenin expression and activity are tightly controlled in mammalian cells. To assess the extent of ectopic hANG-FLAG-HA expression in S2 cells the approximate concentration of hANG-FLAG-HA protein per cell was determined using a dilution series of recombinant human Angiogenin. First, the detection limit of commercially available anti-Angiogenin antibodies (raised against the coding sequence of human Angiogenin) was assessed after blotting and probing a serial dilution of recombinant human Angiogenin. This showed that the detection limit for this antibody preparation at the given dilution was between 1 to 50 nanograms of blotted recombinant Angiogenin (Fig. 3.13).

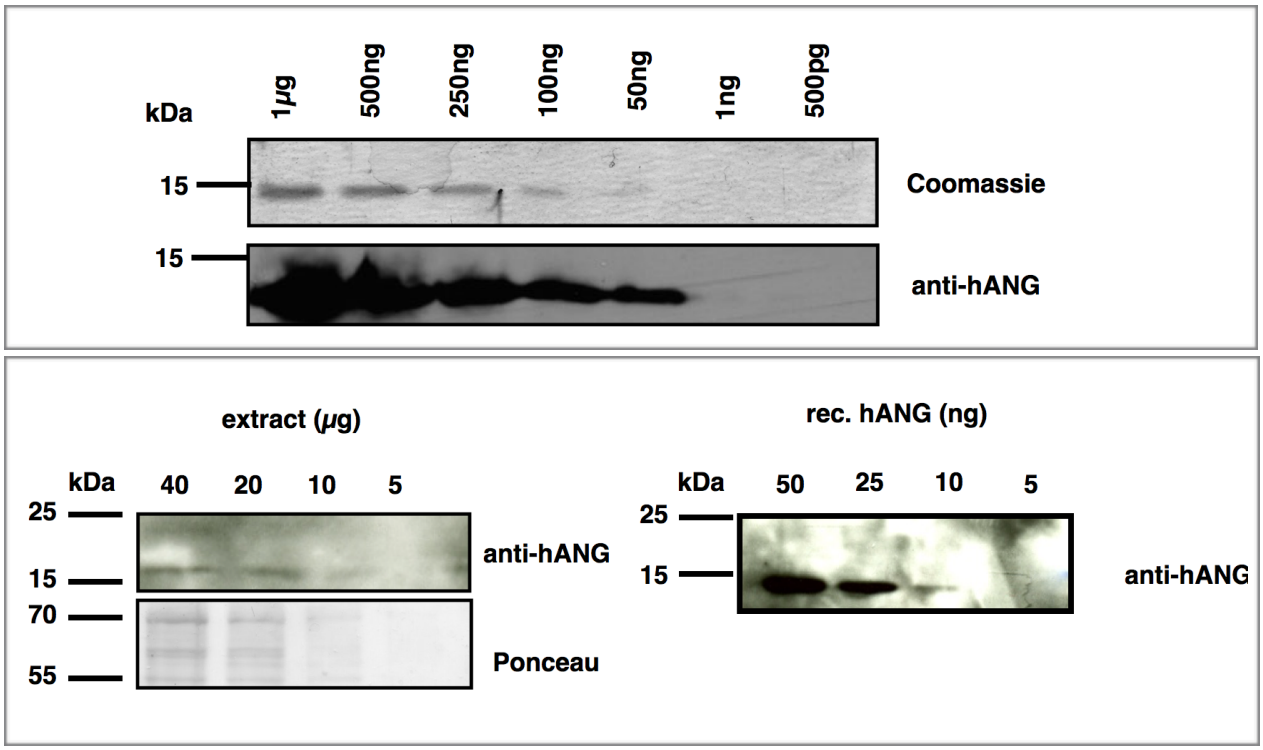

Fig. 3.13: **Assessing the concentration of hANG-FLAG-HA in induced cells.** (top) Serial dilution of recombinant hANG to assess the detection limit of the anti-hANG antibody. (bottom) dilution series of total protein extracts of cells expressing hANG-FLAG-HA (left) compared to a serial dilution of recombinant hANG close to the detection limit of the anti-ANG antibodies (right).

To draw conclusions about the concentration of ectopically expressed hANG-FLAG-HA in S2 cells, various concentrations of recombinant human Angiogenin close to the previously determined detection limit of the antibody preparation were loaded along with a dilution series of protein extracts, which were obtained from S2 cells that expressed hANG-FLAG-HA for 8 hours. Comparison of the dilution series indicated that 10 micrograms of the total S2 protein extract contained a mass of hANG-FLAG-HA that was approximately similar to 10 nanograms of recombinant human

Angiogenin (Fig. 3.13). Because 10 micrograms of total S2 protein extract were prepared from ca. 20.000 cells it can be calculated that every S2 cell contained approximately 55 femtograms of hANG-FLAG-HA after 8 hours of ectopic expression. Since human Angiogenin has a molecular weight of 14 kDa it follows that 55 femtograms of hANG-FLAG-HA correspond to about 2.3 x 10^6 hANG-FLAG-HA molecules. This result will become important when comparing the concentration of endogenously expressed human Angiogenin in mammalian cells after stress exposure with the designed hANG-FLAG-HA expression vector system in S2 cells.

3.4.6 Effect of hANG-FLAG-HA Induction on Endogenous Dnmt2 Levels

Dnmt2 has been shown to affect tRNA fragmentation of Dnmt2-substrate tRNAs (Schaefer et al., 2010). Because Dnmt2 and hANG-FLAG-HA might potentially bind to the same tRNAs it was tested whether hANG-FLAG-HA overexpression affected Dnmt2 levels in S2 cells. The results showed that the levels of endogenously expressed Dnmt2 were not changed during 48 hours of constant hANG-FLAG-HA expression (Fig. 3.14).

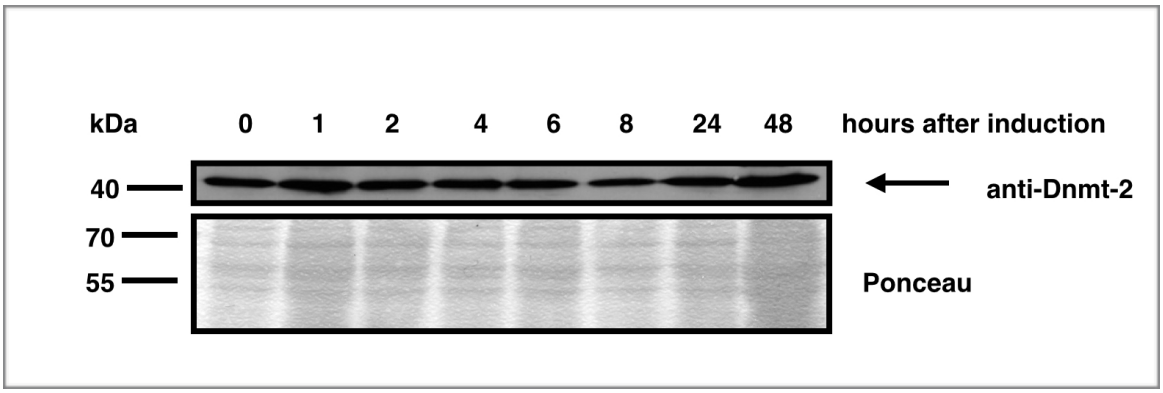

Fig. 3.14: **The effect of hANG expression on endogenous Dnmt2 levels.** Endogenous Dnmt2 expression after 0, one, two, four, 6, 8, 24 and 48 hours after hANG-FLAG-HA induction.

3.5 Determining the Inhibition of Recombinant Angiogenin by Ectopic Expression of RNH1-FLAG

RNH1 was shown to bind endogenous human Angiogenin *in vivo* (Pizzo et al., 2013). It was therefore determined if ectopically expressed RNH1-FLAG can bind and inhibit recombinant human Angiogenin in *Drosophila* S2 cells.

3.5.1 Immunoprecipitation of a Protein Complex Containing Recombinant Angiogenin and Ectopically Expressed RNH1-FLAG

To test whether RNH1-FLAG is able to bind recombinant human Angiogenin RNH1-FLAG was ectopically expressed in S2 cells for 18 hours followed by the subsequent addition of recombinant human Angiogenin to the cell culture medium (333 ng recombinant Angiogenin/ml). Cells were in-

cubated for three hours with Angiogenin in the presence of ectopically expressed RNH1-FLAG, total protein extracts were prepared and an immunoprecipitation was performed using antibodies against the FLAG-epitope. Immunoprecipitated protein complexes were analyzed by Western blotting using anti-FLAG antibodies.

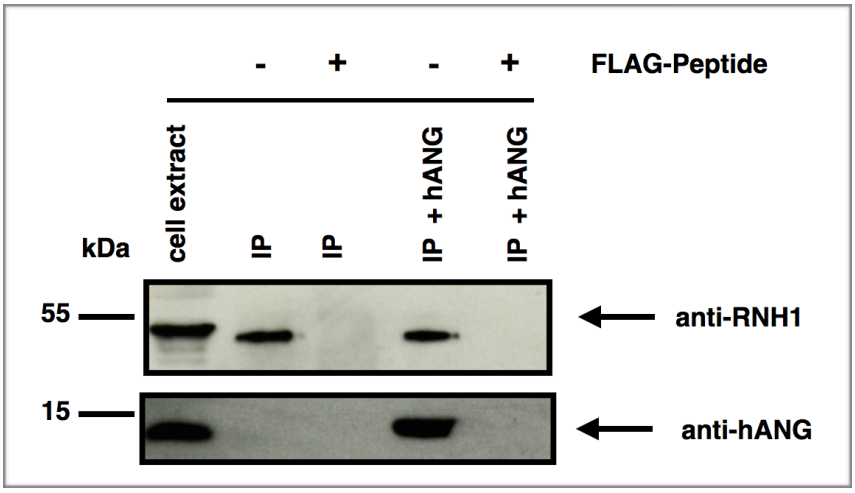

Fig. 3.15: **Co-immunoprecipitation of RNH1 and recombinant hANG.** IP of RNH1-FLAG induced for 18 hours with Cu++ and subsequently treated or not treated with recombinant hANG. The column was either (+) treated or (-) not treated with a FLAG-Peptide before incubation with the cell extract.

The results of the experiment showed that recombinant Angiogenin had entered S2 cells three hours after incubation without a transfection agent. Furthermore, recombinant Angiogenin bound to its inhibitor, RNH1, when RNH1-FLAG was ectopically expressed in S2 cells because a complex of Angiogenin and RNH1-FLAG could be efficiently purified (Fig. 3.15). These results confirmed the interaction of Angiogenin with its inhibitor suggesting that this interaction could also inhibit the activity of recombinant Angiogenin in S2 cells.

3.5.2 Angiogenin-Mediated tRNA Fragmentation in the Presence of Ectopically Expressed RNH1

Since RNH1-FLAG can bind recombinant Angiogenin in S2 cells, it was assessed whether Angiogenin-mediated tRNA fragmentation can be inhibited by this interaction.

S2 cells were first incubated with recombinant Angiogenin for 6 hours before RNH1-FLAG expression was induced for another 24 hours. The results of this experiment showed that RNH1-FLAG expression after Angiogenin treatment of cells decreased tRNA fragmentation when compared to S2 cells that were incubated with human Angiogenin without RNH1-FLAG expression (Fig. 3.16).

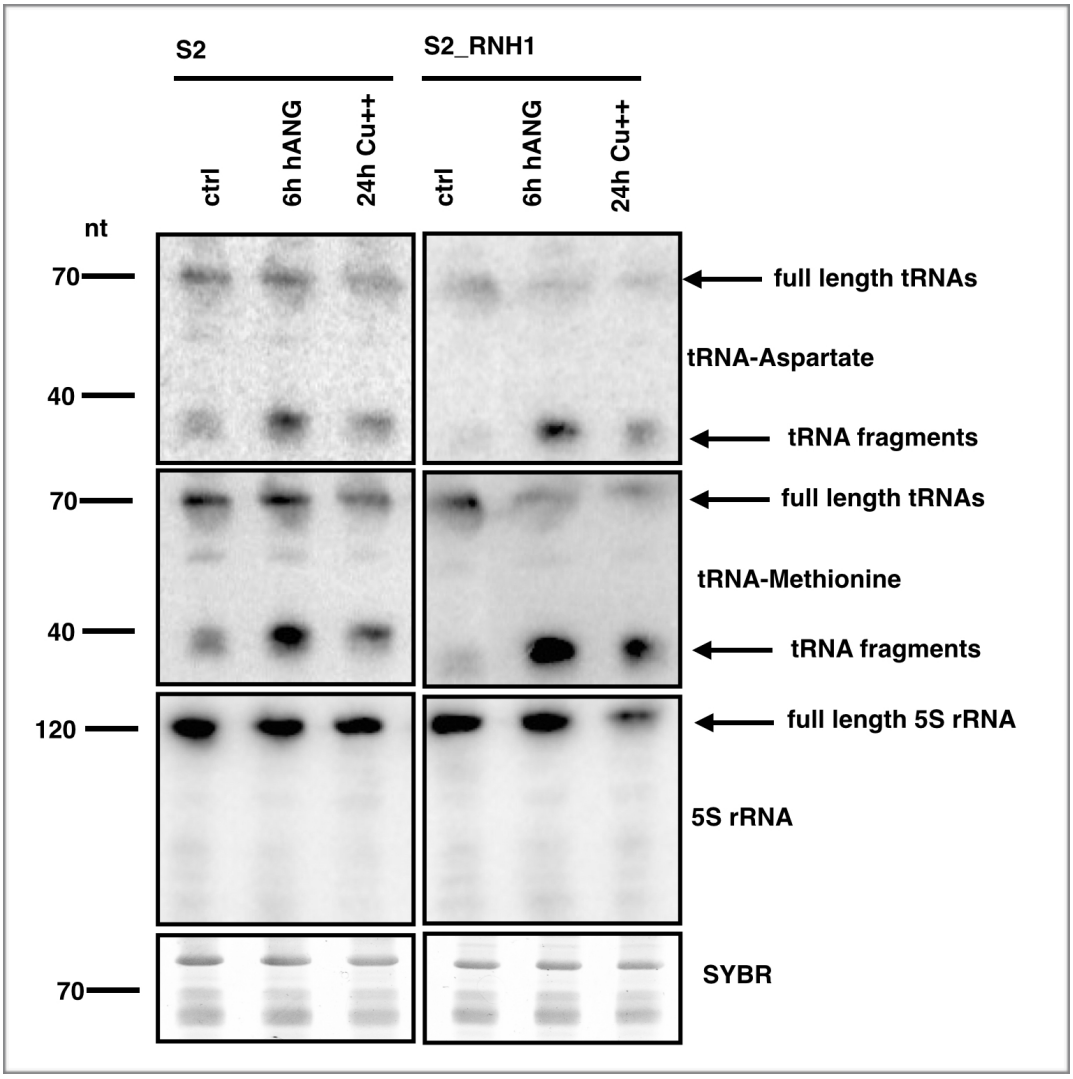

Fig. 3.16: **Capability of RNH1-FLAG to inhibit tRNA fragmentation.** tRNA fragmentation compared to S2 controls for tRNAs Aspartate and Methionine and 5S rRNA by stripping and reprobing blots: tRNA fragmentation after 6 hours of incubation with recombinant hANG followed by 24 hours induction with Cu++.

3.6 Determining of tRNA Fragmentation in S2 Cells Expressing Dnmt2

3.6.1 Kinetics of tRNA Fragmentation during Constant Induction of Dnmt2

It was shown that Dnmt2-mediated methylation of position C38 in three tRNAs prevented stress-induced fragmentation of these tRNAs (Schaefer et al., 2010). The same study showed that a heat shock and oxidative stress-induced (but still unidentified) tRNA endonuclease activity on Dnmt2-substrate tRNAs could be inhibited when Dnmt2 was ectopically expressed in S2 cells. It was tested whether ectopic Dnmt2-FLAG could stop the progression of recombinant Angiogenin-mediated tRNA fragmentation by inducing Dnmt2 expression after incubation of S2 cells with recombinant Angiogenin. The results of these experiments showed that Dnmt2-FLAG expression neither before nor after recombinant Angiogenin treatment of S2 cells was capable of limiting the extent of Angio-

genin-mediated tRNA fragmentation (Fig. 3.17) indicating that Dnmt2 expression cannot be used to interfere with the effects of recombinant Angiogenin in S2 cells.

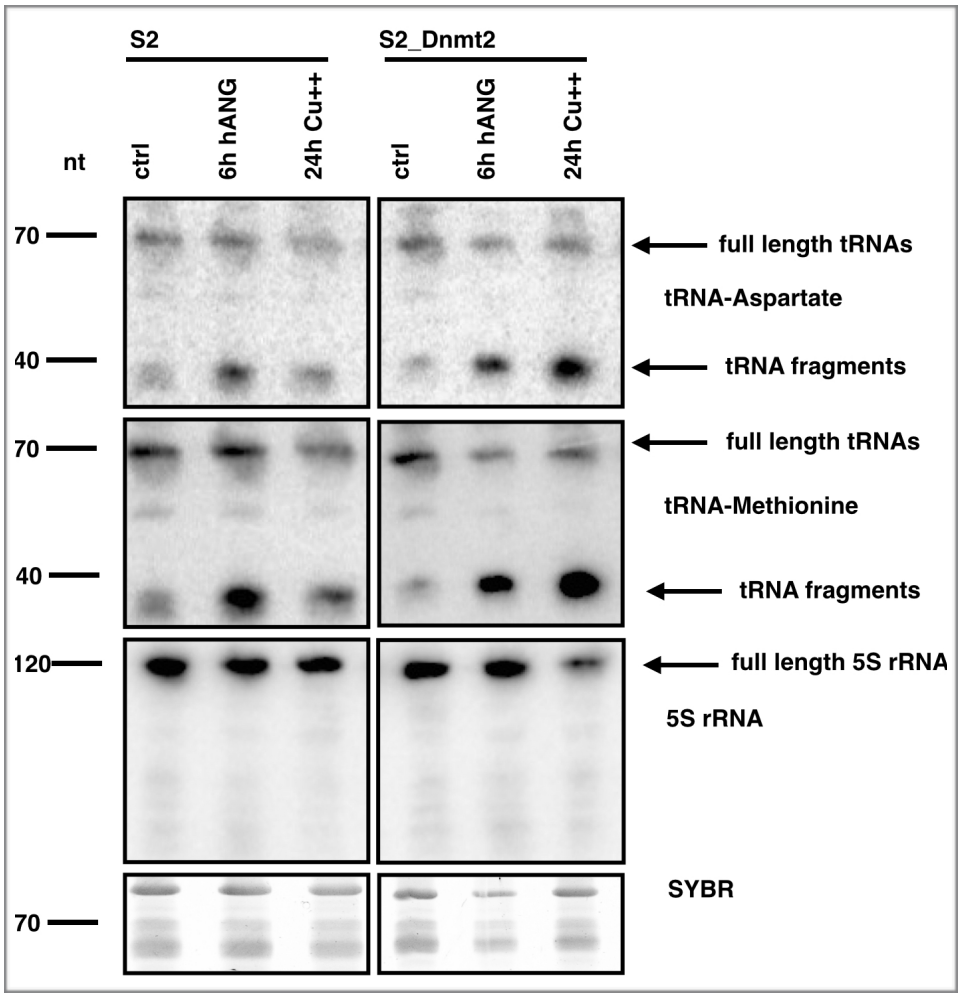

Fig. 3.17: **Effect of Dnmt2-FLAG on hANG mediated tRNA fragmentation.** tRNA fragmentation compared to S2 cell controls for tRNAs Aspartate and Methionine and 5S rRNA by stripping and reprobing blots: tRNA fragmentation after 6 hours of incubation with recombinant hANG followed by 24 hours induction with Cu++.

3.7 Physiological Effects of Ectopic Expression of hANG in S2 Cells

3.7.1 Effects on Gene Expression

To assess the influence of hANG-FLAG-HA induction and tRNA fragmentation on gene expression, mRNA levels of several candidate genes that are involved in stress responses, cell cycle regulation and DNA repair were analyzed by quantitative PCR (qPCR). Expression of these genes had been shown previously to be Dcr-2 or Ago-2-dependent in *Drosophila* and to be mis-regulated during heat shock in *Dnmt2* mutant animals (Durdevic et al., 2013 (2)). qPCR was conducted at different time points in S2 cells that expressed hANG-FLAG-HA constantly or had been induced to express hANG-FLAG-HA for 8 hours followed by a chase period of 24 to 48 hours in fresh medium.

Angiogenin affects rRNA transcription in human cells (Tsuji et al., 2005). Because ectopic hANG-FLAG-HA could be visualized not only in the cytoplasm but also in S2 cell nuclei (see above), where the protein could potentially bind to rDNA loci and affect the expression of rRNA, the expression levels of 18S rRNA during the ectopic expression of hANG-FLAG-HA were investigated. The results showed that the expression of 18S rRNA was not affected by ectopic hANG-FLAG-HA in S2 cells (Fig. 3.18) indicating that nuclear hANG-FLAG-HA does not affect rRNA transcription in a manner that was similar to the one reported in human cells.

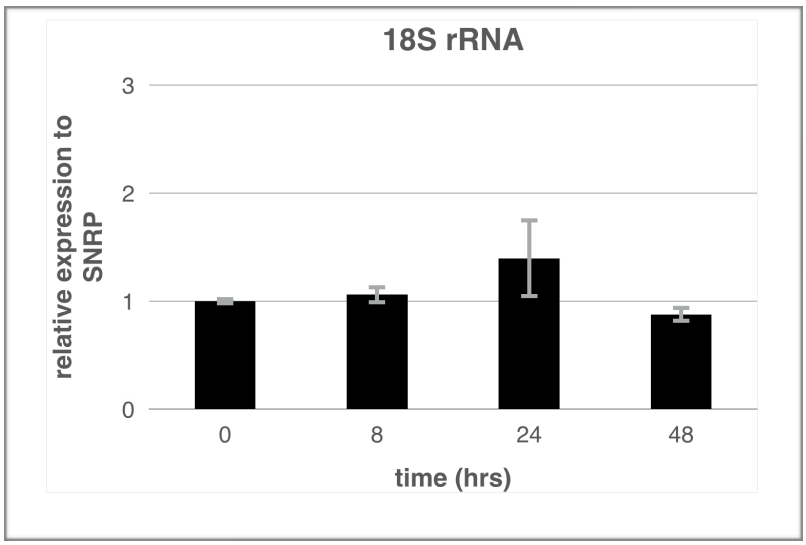

Fig. 3.18: **Expression levels of 18S rRNA. Expression after 0, 8, 24 and 48 hours of Cu++ induction.** Error bars indicate the standard deviation between three experiments.

tRNA fragmentation occurs in response to certain stress conditions. In addition, transfection of tRNA fragments into mammalian cells showed the induction of cellular stress responses including the formation of stress granules (Emara et al., 2010; Blanco et al., 2014). In order to test whether ectopic Angiogenin triggers canonical stress responses in S2 cells, which involve the activity of various chaperones, mRNA expression levels of Hsp23, Hsp70 and Hsp83 were tested. Hsp70 and Hsp23 activities are mainly involved in cellular responses to heat and cold shock as well as to hypoxia (Qin et al., 2005; Azad et al., 2009; Gong and Golic, 2006). Hsp83 acts during the heat and cold shock response but also functions during spermatogenesis (Qin et al., 2005; Neal et al., 2006; Castrillon et al., 1993). The results showed that none of the tested heat shock genes were affected in their mRNA expression by ectopic hANG-FLAG-HA or the subsequent tRNA fragmentation (Fig. 3.19).

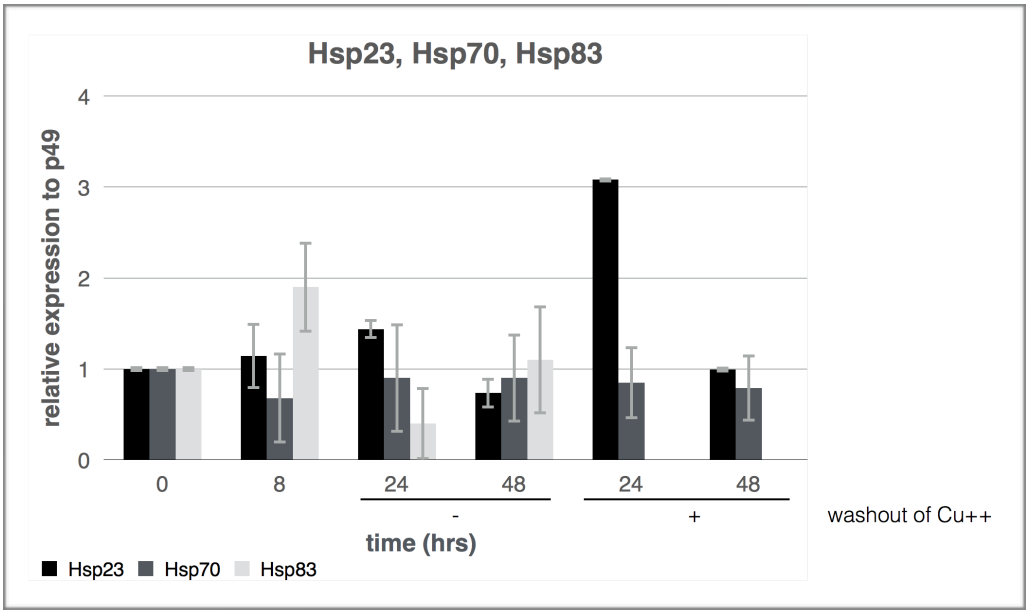

Fig. 3.19: **Expression levels of hsp23, hsp70 and hsp83 mRNA.** Expression of hsp83 after 0, 8, 24 and 48 hours and expression of hsp23 and hsp70 after 0, 8, 24 and 48 hours with or without washing out Cu++ (wo) after 8 hours. Error bars indicate the standard deviation between three experiments.

Because the aim of ectopically expressing Angiogenin in S2 cells was to induce Angiogenin-mediated rather than stress response-mediated tRNA fragmentation the results of these experiments suggested that it is possible to separate tRNA fragmentation from stress responses in S2 cells.

Recent experiments showed that the mRNA expression of various cell cycle and DNA repair genes were elevated during heat shock but were also affected by tRNA fragmentation (Durdevic et al., 2013 (2)). CyclinE, a gene that is associated with G1/S transitions (Richardson et al., 1995), and which reportedly was affected by tRNA fragmentation (Durdevic et al., 2013 (2)) did not show expression changes in the course of hANG-FLAG-HA expression (Fig. 3.20). Meiotic-41, the homolog of the human protein ATR (Hari et al., 1995) is a checkpoint serine/threonine kinase whose function as a DNA damage sensor is partly redundant with dATM, the *Drosophila* homolog of ATM (Song et al., 2004). Meiotic-41 is associated with post-replication repair and the activation of checkpoint signaling upon ionizing radiation, UV light and DNA replication stalling (Ravi et al., 2009; La Rocque et al., 2007). Meiotic-41 showed no expression changes in the course of hANG-FLAG-HA expression (Fig. 3.20). These results indicated that expression of Angiogenin does not cause the same effects on the expression of meiotic-41 and cyclinE mRNA levels as stress-induced tRNA fragmentation (Durdevic et al.,2013)

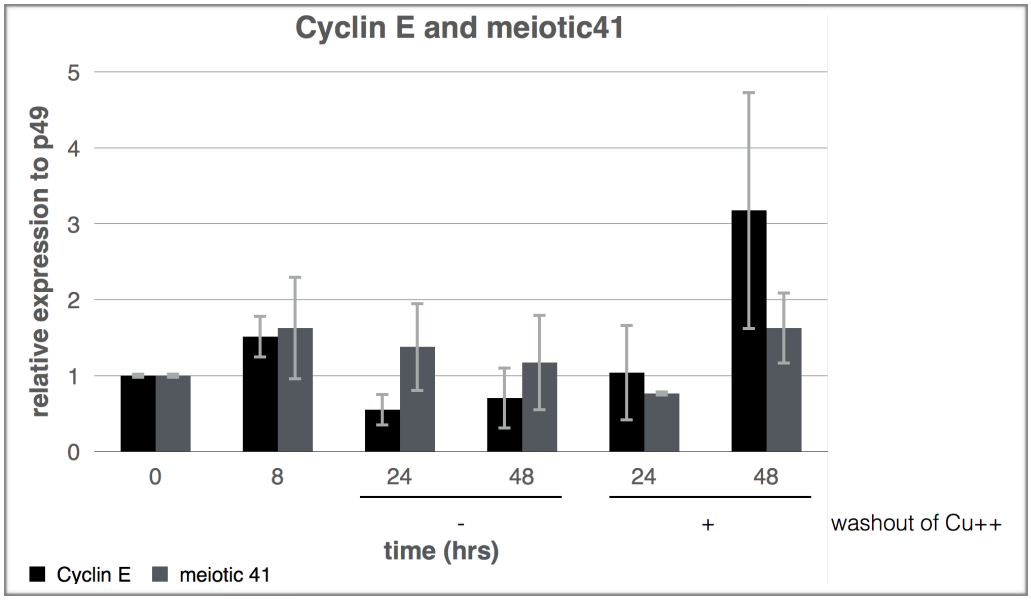

Fig. 3.20: **Expression levels of CyclinE and meiotic41 mRNA.** Expression after 0, 8, 24 and 48 hours with or without washing out Cu++ (wo) after 8 hours. Error bars indicate the standard deviation between four experiments.

Mus101 is a gene associated with DNA replication checkpoint signaling and heterochromatin condensation (Yamamoto et al., 2000). Mus308 encodes a DNA polymerase, which is involved in double-strand break repair via NHEJ and nucleotide-excision repair (Chan et al., 2010). Importantly, a 21-bp region in mus308 mRNA has been reported to match perfectly to an endo-siRNA (esi-2.1) in *Drosophila* (Czech et al., 2008). Furthermore, the biogenesis of esi-2.1 depends on Dcr-2 and Ago-2 function because mus308 mRNA expression was up-regulated in *Dcr-2* and *Ago-2* mutant animals (Czech et al., 2008).

Interestingly, the results of the experiments (Fig. 3.21) showed that both mus101 as well as mus308 mRNA expression levels were increased after expression of hANG-FLAG-HA for 8 hours followed by a chase period of 24 to 48 hours in fresh medium. Additionally, both genes showed different patterns of up-regulation, as mus101 mRNA levels increased by 91-times after 24 hours and remained elevated until the 48 hours time point (Fig. 3.21), whereas mus308 mRNA levels rose constantly and peaked at 1280-times if compared to controls after 48 hours (Fig. 3.21).

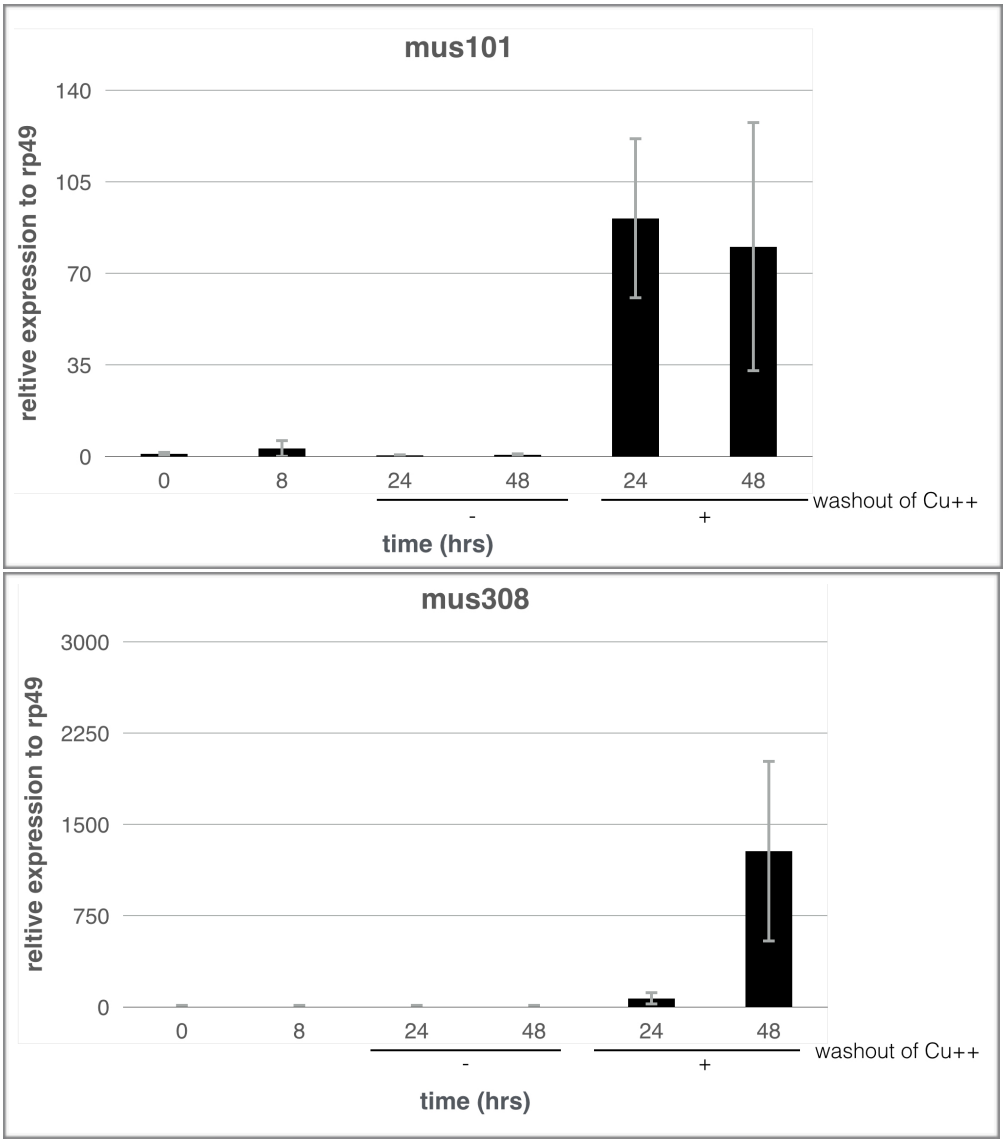

Fig. 3.21: **Expression levels of mus101 and mus308 mRNA.** Expression after 0, 8, 24 and 48 hours with or without washing out Cu++ (wo) after 8 hours. Error bars indicate the standard deviation between six experiments.

To examine, whether the up-regulation of mus101 and mus308 could also be observed after shorter pulses of hANG-FLAG-HA expression, the analysis was performed after hANG-FLAG-HA expression for two, four and 8 hours followed by a chase period in fresh medium for 48 hours. The mRNA expression data showed that the Cu++ induced expression of hANG-FLAG-HA for two and four hours was sufficient to cause the up-regulation of both mRNAs (Fig. 3.22). After a 2 hours pulse and 48 hours chase period mus101 mRNA levels were increased by 7 times and mus308 levels were increased by 30 times (Fig. 3.22).

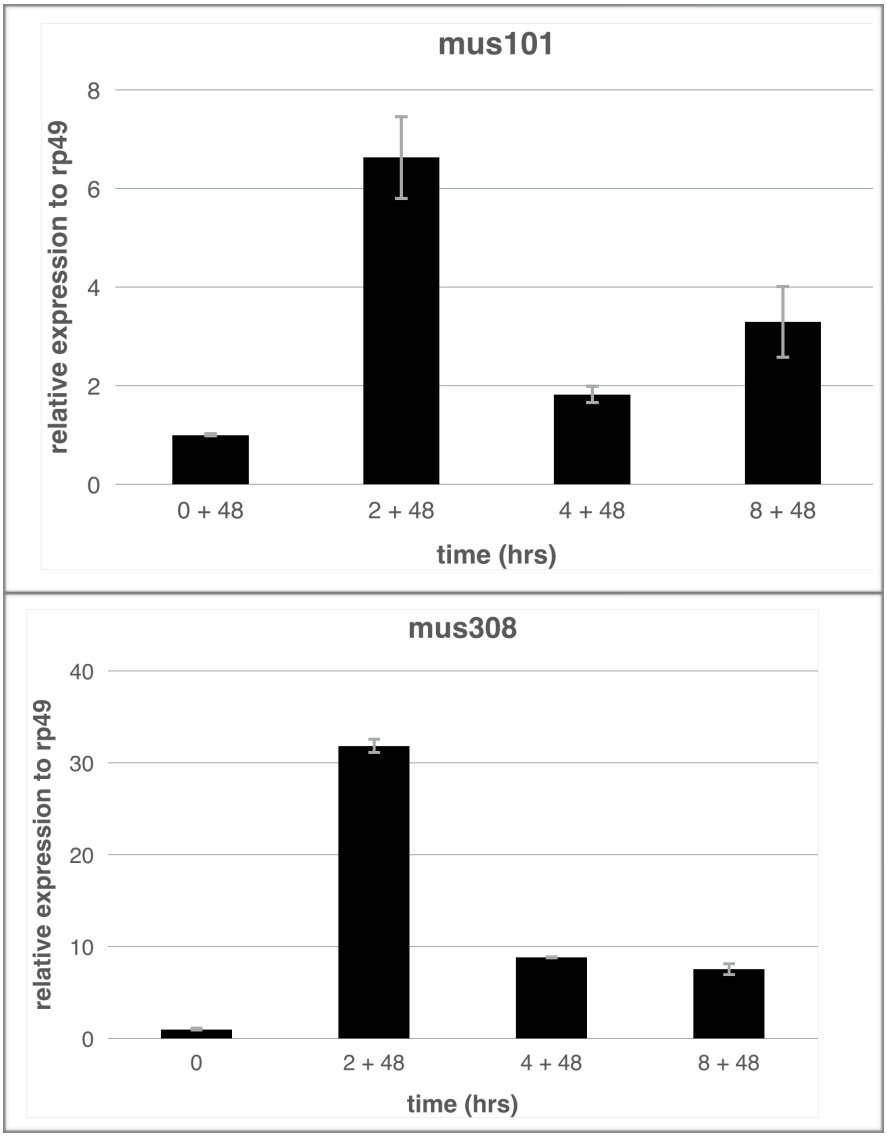

Fig. 3.22: **Expression levels of mus101 and mus308 mRNA after short Cu++ pulses.** Expresion after 0, 2, 4 or 8, hours of induction followed by 48 hours of culturing in fresh medium. Error bars indicate the standard deviation between three technical replicates.

To test whether increased expression of mus101 and mus308 mRNAs was mediated by tRNA fragments rather than by pleiotropic effects of ectopic Angiogenin expression S2 cells were incubated with tRNA fragments that were purified from S2 cell cultures (Fig. 3.23), which had been exposed to hANG-FLAG-HA expression for 8 hours. Cells were incubated for 24 hours in S2 medium containing tRNA fragments of different concentrations.

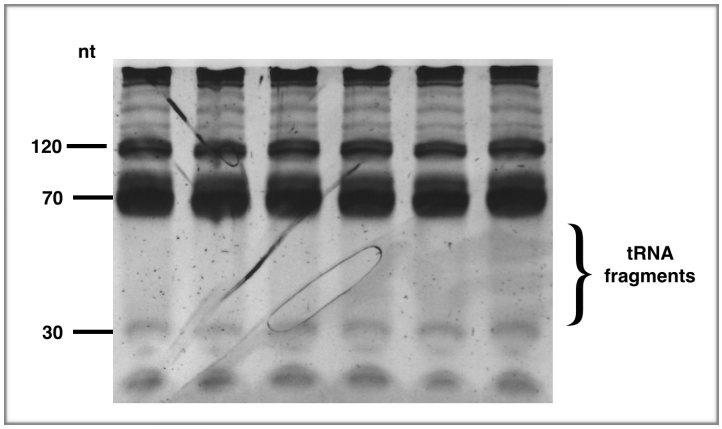

Fig. 3.23: **Regions in UREA-PAGE gel where to expect tRNA fragments.** Fragments are indicated by brace.

The results of these experiment showed that mus101 and mus308 mRNA levels were affected by treatment of S2 cells with tRNA fragments in a dose-dependent manner. A concentration of 160 nanograms tRNA fragments per milliliter culture medium (ng/ml) did not affect the expression levels of both genes (Fig. 3.24), whereas a concentration of 300 ng/ml increased mus101 expression levels 4-times and mus308 expression levels 7-times (Fig. 3.24). These data confirmed that tRNA fragments and not tRNA fragment-independent effects of hANG-FLAG-HA were the cause for the transcriptional changes that were observed for mus101 and mus308 (Fig. 3.22).

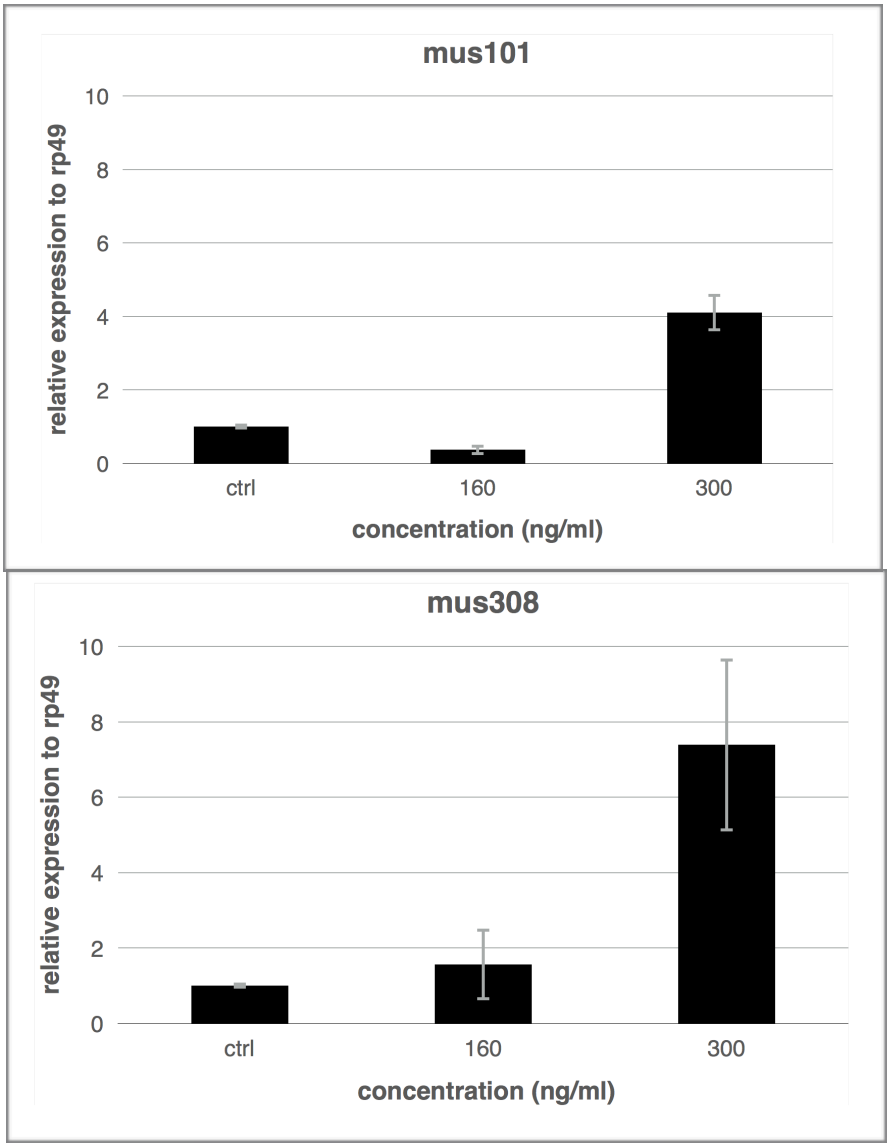

Fig. 3.24: **Expression levels of mus101 and mus308 mRNA after treatment with tRNA fragments.** Expression after treatment with tRNA fragments of different concentrations for 24 hours. Error bars indicate the standard deviation between three experiments.

To confirm the identity of the mus308 cDNA that was amplified by PCR a semi-quantitative PCR of S2 cells, which had been treated with tRNA fragments (300 ng/ml) was conducted. To this end, primers were used that encompass the region complementary to the esi-2.1 sequence, which is targeted by Ago-2-mediated mRNA cleavage (Czech et al., 2008). After increased mRNA levels at this region of the mus308 mRNA were confirmed by PCR (Fig. 3.25), the PCR fragment was sub-cloned and sequenced. The sequencing result confirmed the identity of the transcript to be originating from mus308 including the region complementary to esi-2.1 (Fig. 5.3).

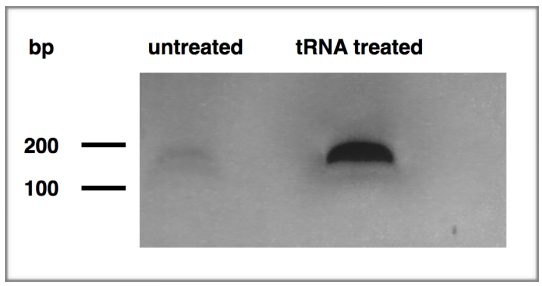

Fig. 3.25: **PCR amplification of mus308.** mus308 PCR product of semi-quantitative PCR on 0,8% Agarose Gel to visualise mus308 mRNA levels before and after treatment with 300 ng/ml tRNAs corresponding to Fig. 3.24.

3.7.2 Effects on Transposon Expression

Because tRNA fragments were shown to be able to prime the reverse transcription of retro-transposons (Kikuchi et al., 1986) it was tested whether Angiogenin-mediated tRNA fragmentation caused increased transcript levels of selected retro-transposons in the absence of stress. To this end, the relative expression of the long-terminal-repeat (LTR) retro-transposons Gypsy, Re297 and Re1371 as well as of the gene spindleE, which encodes a protein involved in retro-transposon control (Aravin et al., 2001) was assessed by qPCR on S2 cells that expressed hANG-FLAG-HA for 48 hours or only for 8 hours followed by sub-culturing in fresh medium for another 48 hours.

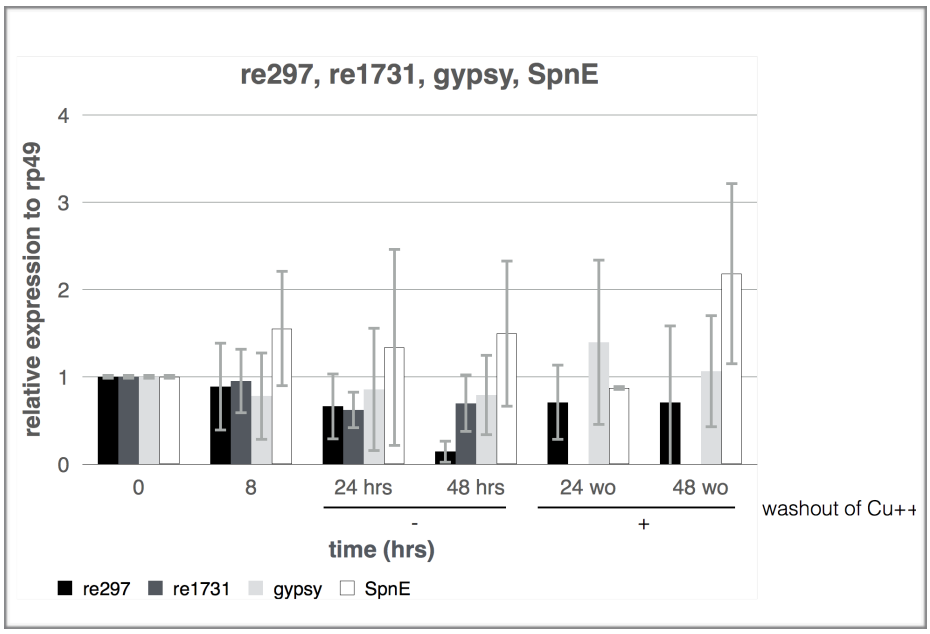

Fig. 3.26: **Expression levels of re297, re1731, gsypsy and SpnE mRNA.** Expresion of re1731 after 0, 8, 24 and 48 hours and expression of spnE, gypsy and re297 mRNA after 0, 8, 24, and 48 hours with or without washing out Cu++ (wo) after 8 hours. Error Bars indicate the standard deviation between four experiments.

The expression analysis showed (Fig. 3.26) that tRNA fragmentation had no impact on the expression levels of spindleE or the retro-transposons Gypsy, Re297 and Re1371 indicating that tRNAs that were cleaved by Angiogenin did not affect the mRNA levels of these retro-transposons.

4 Discussion

It was the aim of this Master Thesis to design and test cell culture expression systems that allow inducing but also limiting the extent of tRNA fragmentation in order to start analyzing the downstream effects of tRNA fragments. tRNA fragmentation has been observed mostly after cells were exposed to various stress conditions. However, cellular stress responses include not only tRNA fragmentation but also chromatin remodeling (Tulin and Spradling, 2003) and various signaling cascades (Wang et al., 2003) that alter gene expression, which makes it difficult to separate cause and effect of tRNA-fragment mediated processes.

In order to understand, which of the effects during the stress response and the recovery from stress are due to the biological activity of tRNA fragments it is necessary to separate general stress responses from the activity of tRNA fragments.

To do so several possibilities can be envisioned:

(1) One approach would be to express tRNA fragments from transgenes. Importantly, it is currently not known whether all or only some tRNA fragments have downstream effects. It is also not known whether tRNA fragments need to be modified and whether certain modifications are crucial for their effects. Therefore, this approach might become experimentally taunting as it is not clear whether the in vivo effects of tRNA fragments could be simulated by such expression constructs which only express specific tRNA-derived sequences.

(2) Another possibility would be to expose cells to purified exosomes, small cell-derived membranous particles that were reported to contain substantial amounts of tRNA fragments (Vojtech et al., 2014). However, exosomes do also contain significant amounts of other RNAs (non-coding and coding), which might affect recipient cells making it difficult to separate effects that are tRNA fragment-mediated from the effects elicited by other RNAs.

(3) Another approach would be to produce tRNA fragments through the expression or the cellular uptake of specific tRNA endonucleases (such as human Angiogenin, hANG) or (4) through the purification of stress-induced or tRNA endonuclease-produced tRNA fragments followed by re-introducing such tRNA-derived fragments into cells. Re-introduction could be achieved by chemical means such as transfection agents or by relying on endocytotic uptake of tRNA fragments.

In the work of this Master Thesis approaches (3) and (4) were used and validated. tRNA fragmentation was induced by the expression of human Angiogenin or by the incubation of cells with recombinant human Angiogenin. Subsequently, tRNA fragments were extracted and re-introduced into cells without the use of chemical transfection reagents.

4.1 tRNA Fragmentation Can Be Induced in S2 Cells by Expressing hANG

Ectopic expression of hANG in S2 cells as well as incubation of S2 cells with recombinant Angiogenin induced tRNA fragmentation (Figs. 3.8 and 3.16). The ectopic hANG expression experiments showed that continuous induction of hANG using relatively high concentrations of Cu++ (0.7 mM

final) decreased cell viability significantly (Fig. 3.10). Additionally, 5S rRNA degradation was also detected after continuous hANG expression, which was also confirmed for other abundant RNAs using an RNA integrity chip showing lower integrity of total RNA after expression of hANG (Figs. 3.9 and 5.4). These results could be explained by at least two hypotheses. First, Angiogenin could lose specificity when present in excess. It was reported that Angiogenin is a comparably unspecific endonuclease, which also hydrolyses ribosomal RNAs in vitro (Rybak and Vallee, 1988), while another report showed specificity for recombinant Angiogenin on tRNAs in vivo (Saxena et al., 1992). Secondly, as endogenous metallothionein promoters mediate expression of genes that are important for heavy metal homeostasis and detoxification in *Drosophila* (Egli et al., 2006) an excess of Cu++ might influence their expression levels, which could lead to increased cell death. An effect of Cu++ rather than ectopic hANG on cell viability was also suggested by experiments that showed increased cell viability after limiting the time of hANG expression by removal of the inducing agent (Cu++) and replacement with new cell culture medium (Fig. 3.10). Notably, tRNA fragmentation was still detectable after removal of Cu++. Importantly, tRNA fragments could already be detected as early as two hours after Cu++ induction and the viability of cells was significantly increased when the inducing agent (Cu++) was removed from the medium.

It follows that the ectopic hANG activity and/or the degradation of RNAs in S2 cells were probably not the cause of the observed cell death but were rather induced by the un-physiological concentrations of Cu++, which served as activator of the metallothionein promoter in the hANG-FLAG-HA expression construct. Of note, although it was reported that other metal-ions could be used to activate hANG expression from metallothionein promoters (Kurachi et al., 1988) using Zn++ in S2 cells did not cause hANG-FLAG-HA expression (Fig. 3.5). Future experiments need to be conducted to systematically analyze the effects of other metallothionein promoter-inducing ions on RNA stability and cell physiology in the *Drosophila* cell culture system.

A second approach to induce tRNA fragmentation was to introduce recombinant hANG (r-hANG) into S2 cells and limit its effects by the expression of the Angiogenin inhibitor, RNH1. Induction of RNH1 after incubation of S2 cells with r-hANG suppressed the extent of tRNA fragmentation while tRNA fragments were still detectable (Fig. 3.16). These results and the observations that the ectopic expression of hANG might cause other physiological effects such as cell death and promiscuous RNA degradation suggest that the ectopic RNH1 expression system offers a better way to induce tRNA fragmentation and to monitor its biological effects in S2 cells by using incubation of cells with r-hANG and subsequently limiting Angiogenin activities by expression of RNH1.

In conclusion, these results indicate that it is important to select appropriate time points and conditions for the exposure of S2 cells to recombinant hANG in order to achieve appreciable tRNA fragmentation, while avoiding additional RNA degradation and other effects such as increased cell death.

4.2 Cu++ Might Influence the Activity and Localization of human Angiogenin

It was recently shown that Cu++ interfered with and inhibited Angiogenin activity (Giacomelli et al., 2015). Cu++ reduced the nucleolytic activity of hANG as well as its effects on several other cellular mechanisms. Importantly, Cu++ also regulated the localization of hANG (Giacomelli et al., 2015). However, Copper does not only act as an inhibitor of hANG protein, as it was shown that Cu++ also induced the transcription of endogenous Angiogenin in human cells (Giacomelli et al., 2015). The experiments presented here showed that the RNase activity of Cu++-induced hANG expression caused tRNA fragmentation in S2 cells (Fig. 3.8). Of note, prolonged incubation of S2 cells with Cu++ did not only induce cell death but also affected the expression of hANG (Fig. 3.8) suggesting either inhibitory effects of Cu++ on hANG transcription or protein stability in S2 cells. Importantly, these observations indicate that the effects of Cu++ on Angiogenin as described in human cells might not be comparable to its effects in *Drosophila* S2 cells. Therefore, the kinetics of tRNA fragmentation and the localization of hANG in cells induced with Cu++ should be compared to cells induced with other divalent metal ions to determine Cu++-dependent effects on hANG.

Immuno-staining of ectopic hANG indicated that washing out Cu++ influenced the subcellular localization of the endonuclease as hANG was mainly detectable in the nucleus during induction (8 hours), but subsequent culturing in fresh medium led to the cytoplasmatic localization of hANG (Fig. 3.12). Notably, hANG only cleaves tRNAs in the cytoplasm (Yamasaki et al., 2009). A recent paper reported that the Angiogenin-Copper system is delicate and the equilibrium of its components is important for cellular homeostasis. Importantly, it was shown that binding of Cu++ to exogenous hANG prevents its migration into the nucleus (Giacomelli et al., 2015). Because the presented data showed that ectopic hANG does not locate to the cytoplasm of S2 cells as long as Cu++ is present, it is a testable hypothesis whether Copper generally inhibits hANG migration from the nucleus into the cytoplasm. However, it is presently unclear why ectopically expressed hANG was primarily seen in the nucleus although tRNA fragmentation (supposedly a purely cytoplasmic process) was detectable at the same time. Despite its nuclear localization, 18S rRNA transcription was not shown to be elevated (Fig. 3.18), which is in contrast to the finding that hANG promotes 18S rRNA transcription in mammalian cells (Tsuji et al., 2005).

In summary, these results suggest that interactions of Angiogenin and Cu-ions are crucial to understand both the localization and the activity of human Angiogenin in S2 cells because this interplay might affect the experimental validity of the ectopic hANG expression system. In light of these observations the incubation of cells with r-hANG and limiting tRNA fragmentation by ectopic expression of RNH1 might be a better experimental paradigm to model endonuclease-mediated tRNA fragmentation and to avoid most effects of Cu++ on human Angiogenin.

4.3 Effects of Ectopic hANG Cannot Be Controlled by Dnmt2

Ectopic expression of Dnmt2 was reported to decrease tRNA fragmentation in cells that were heat-shocked, treated with oxidative stressors or incubated with recombinant hANG (Schaefer et al., 2010). Also it was reported that Dnmt2 localized to stress granules, a cellular compartment that contains many RNAs (Anderson and Kedersha, 2009), when cells were heat-shocked (Schaefer et al., 2010). As hANG and Dnmt2 bind the same tRNAs, the overexpression of hANG could affect Dnmt2 levels. The obtained results showed that the protein levels of Dnmt2 were not subjected to changes during the course of ectopic hANG expression (Fig. 3.14), indicating that high abundance of Angiogenin and stress-independent generation of tRNA fragments did not affect Dnmt2 protein levels. Furthermore, and in contrast to previous observations, ectopic expression of Dnmt2 did not suppress the activity of r-hANG on tRNAs (Fig. 3.17). Notably, the experimental setup was different in Schaefer et al., as Dnmt2 expression was induced prior to treatment with r-hANG and incubation with r-hANG was limited to 1 hour (Schaefer et al., 2010). In contrast, in the presented experiments cells were treated with r-hANG for 6 hours before expressing ectopic Dnmt2. As the ectopic RNH1 expression system showed to be capable of limiting tRNA fragmentation under the same experimental conditions, this system is preferable to the ectopic Dnmt2 expression system, when trying to limit hANG activities.

4.4 tRNA Fragmentation Can be Disconnected from the Stress Response

The number of ectopically expressed Angiogenin molecules within each S2 cell was determined to be roughly 2.3 x 10^6 (Fig. 3.13). This number should be compared to hANG levels in cells during the stress response to conclude whether the concentration of ectopically expressed hANG is similar to endogenous hANG levels in mammalian cells. In the course of such comparison, the number of tRNA fragments that are produced by ectopic Angiogenin in S2 cells also needs to be compared to the concentration of tiRNAs that are produced in mammalian cells after stress exposure. This should facilitate further characterization of the hANG expression system in S2 cells and its suitability for studying tRNA fragment-mediated effects.

As it was the aim of the experimental paradigm to exclude mRNA expression changes due to stress-mediated signaling, it was assessed whether expression of hANG and subsequent tRNA fragmentation induced the expression of general mediators of stress responses. The analysis of mRNA levels for heat shock proteins 23, 70 and 83 did not show expression changes in the course of ectopic hANG expression (Fig. 3.19) suggesting that ectopic hANG and hANG-mediated tRNA fragmentation does not elicit stress signaling. This is in contrast to the observed tRNA fragment-mediated effect on stress pathways in mammalian cells (Blanco et al., 2014). Notably, Blanco et al. used chemical reagents to introduce 5' tRFs, which might have caused stress signaling. Also, stress was measured by a differently approach, namely by assessing the aggregation of insoluble GFP-granules (Blanco et al., 2014). To further test whether hANG-induced tiRNAs do elicit general

stress signaling, S2 cells should also be transfected with a GFP-reporter construct to measure the aggregation of insoluble GFP-granules and thereby assess the comparability of mammalian and *Drosophila* S2 cell systems. Future experiments should also include monitoring of other indicators of stress such as the formation of stress granules and the increased phosphorylation of JNK targets.

In summary, these preliminary observations indicate that ectopic tRNA fragmentation through the expression of hANG in S2 cells can be separated from the general stress response. It follows that any mRNA expression changes in S2 cells that are caused by the overexpression of human Angiogenin might not be the result of stress signaling but of tRNA fragment-mediated effects on cellular processes.

4.5 tRNA Fragmentation Affects the Expression of siRNA Targets

It was reported previously that the transcription levels of several genes involved in cell cycle regulation, DNA repair and transposable element control were elevated in *Dcr-2, Ago-2* or *Dnmt2* mutant flies (Durdevic et al., 2013 (2)). *Dnmt2* mutant flies showed higher levels of tRNA fragmentation during heat shock as the anticodon loop was not protected by (cytosine-5) methylation, suggesting that more tRNA fragments could compete with dsRNA for Dcr-2 and siRNAs for Ago-2 binding. Thus, both increased tRNA fragmentation and siRNA pathway mutations showed similar inhibition of RISC-mediated mRNA silencing.

Part of the validation of the stress-independent tRNA fragmentation paradigm was to test whether the effects on gene expression that were seen in siRNA pathway mutants are similar to the effects caused by the ectopic hANG expression system, in which high quantities of tRNA fragments should be able to interfere with the activities of Argonautes and Dicers. However, cyclin E (cell cycle), spindle E (transposon control) and meiotic 41 (DNA repair), which were reported to be up-regulated in *Dnmt2* and *Dcr-2* mutant flies after heat shock, showed no elevated expression levels after ectopically expressing hANG (Figs. 3.20 and 3.26). This showed that the effects observed in whole flies (Durdevic et al., 2013) differ from those observed in the cell culture experiments (this work). It could well be that the concentration, localisation or activity of ectopically expressed hANG is different from those of the endogenous stress-inducible (and still unidentified) tRNA nuclease in *Drosophila*. Alternatively, the influence of additional stress-related factors might affect the bioavailability of tRNA fragments (i.e. loading into stress-induced subcellular structures such as stress granules), which cannot be mimicked by overexpressing human Angiogenin in S2 cells.

Another reason for the observed discrepancy might be that previous experiments (Durdevic et al., 2013) have been performed in whole flies (containing various tissues) while S2 cell cultures contain mostly immortalized nervous and muscle system precursors. One approach to test whether flies and S2 cells respond differently to hANG-induced tRNA fragmentation would be to create flies,

which express Angiogenin in an inducible fashion in order to assess the effects of hANG on gene expression, including small RNA pathway-regulated genes.

Importantly, it has not been determined yet how the aforementioned genes are exactly affected by Dcr-2 and Ago-2. Are they genuine targets of siRNA-mediated mRNA degradation or are their mRNAs affected on the translational level? Presently, a hypothesis states that tRNA fragments could affect siRNA-mediated mRNA degradation but effects on protein translation should not be excluded. Also, these gene products could be regulated by other endo-siRNA targets, making their dependence on the siRNA machinery rather indirect. To understand this better, the association of cyclin E, spindle E and meiotic 41 mRNAs with RISC components should be assessed along with bioinformatic predictions for complementary endo-siRNAs. In addition, the finding that 18S rRNA transcription was not up-regulated when hANG was overexpressed (Fig. 3.18) confirmed that cell cycle regulation was not affected by potential proliferative signals that were induced by Angiogenin.

Small RNA pathways are known to affect the expression and stability of mobile element-derived sequences. The expression analysis in cell culture showed that hANG expression and tRNA fragmentation had no impact on the RNA levels of various retro-transposons (gypsy, RE-1731 and RE-297 (Fig. 3.26). This suggests that the presence of tRNA fragments is not sufficient to induce increased transcription or stability of retro-transposon RNA. Since tiRNA generation and transposition of mobile elements are phenomena that are observed mainly under stress conditions, it could be that chromatin reorganization, which is taking place under stress conditions might be the major cause for the reported transcription and mobility of transposon sequences. However, tRNAs and tRNA fragments have been shown to prime the reverse transcription of retro-transposons (Kikuchi et al., 1986). For instance, tRNA fragments can prime the reverse transcription of retroviruses as has been shown for mammalian cells and HIV (Saxena et al., 2002). Therefore, the effects of tRNA fragmentation on virus control after infection as well as the mobilization of mobile elements of viral origin should be monitored in future experiments

Importantly, the expression levels of mus101 and mus308 mRNAs, two genes involved in DNA repair mechanisms were shown to be highly elevated after Cu++ induction of hANG expression. Both mRNAs showed different patterns of up-regulation, as mus101 mRNA levels were constantly high, whereas mus308 levels peaked after 48 hours of hANG expression (Fig. 3.21). Both gene products were previously shown to be up-regulated in *Dnmt2* and *Dcr-2* mutant flies during recovery from heat shock (Durdevic et al., 2013 (2)). Of note, mus308 is a bona fide target of an endo-siRNA, esi-2.1 (Czech et al., 2008), whereas it is unclear whether mus101 is targeted by siRNAs. Furthermore, both mRNAs were also up-regulated after two hours of Cu++ induction followed by culturing in fresh medium (Fig. 3.22) confirming that shorter induction times did not only avoid Cu++ or hANG-induced cell death but were also sufficient to induce gene expression changes that were possibly mediated by tRNA fragments. Treatment of S2 cells with tRNA fragments

extracted from S2 cells expressing hANG showed that tRNA fragments alone affected the mRNA levels of mus308 and mus101 in a concentration-dependent fashion (Fig. 3.24), which indicated that there might exist a threshold of tRNA fragments at which the effects on gene expression become detectable. As a follow up experiment it will be interesting to assess how those concentrations correspond to the total numbers of tRNA fragments that are produced when Angiogenin is overexpressed in S2 cells or transgenic flies.

An explanation for the phenomenon that changes in the expression levels of mus101 and mus308 mRNA could only be observed after the removal of Cu++ is that hANG might cleave tRNAs in a non-processive manner. In this scenario, the overabundance of hANG in the system might not release cleaved tRNA fragments, which could inhibit their down-stream effects. In contrast, after washing out the inducing Cu++, the production of hANG will be stopped allowing the existing hANG molecules to act processively on tRNAs thereby releasing tRNA fragments while hANG molecule numbers decrease over time and during subsequent cell divisions. Alternatively, ectopic hANG might have become secreted from producing S2 cells followed by uptake into neighboring cells where hANG entered pathways that led to effective tRNA fragmentation. Additionally, other yet unexplored effects of hANG or Copper Ions might inhibit tRNA fragment-mediated effects on gene expression. To further investigate this, it would be interesting to test whether recovery times affect mus308 and mus101 mRNA up-regulation after incubating S2 cells with recombinant hANG followed by the ectopic expression of its inhibitor, RNH1.

One could argue that the expression increase of mus101 and mus308 mRNAs might be the consequence of DNA damage that was caused by nuclear Angiogenin. Importantly, Angiogenin can access DNA and induce gene transcription (Tsuji et al., 2005). Notably, meiotic 41, a gene that is also involved in DNA repair was not up-regulated after ectopic hANG expression, which argues against general or wide-spread DNA damage in S2 cells that were exposed to hANG.

In summary, these observations suggest that ectopic hANG expression might affect gene products that can be controlled by siRNA pathways. However, to extend these findings and to substantiate the proposed role of tiRNAs as *bona fide* inhibitors of RISC components whole transcriptome studies should be performed on cells that were exposed to increased levels of tRNA fragments. In addition, and to further corroborate some of the preliminary findings, changes in the association of mus308 mRNA and its complementary endo-siRNA (esi-2.1) with RISC complexes before and after the exposure of cells to tRNA fragments should be assessed. For instance, one could use various cross-linking techniques to study protein-RNA associations such as CLASH (Helwak and Tollervey 2014).

4.6 High Standard Deviation of qPCR Data Might Be due to interactions with the RISC of Target mRNAs

Of note, the standard deviation measured during the quantitative PCR within samples was very high for mus101 and mus308 cDNAs (Fig. 3.21). It is a hypothesis that this high variance is due to associations with the RISC complex: Under normal conditions, most of the transcribed mus101 and mus308 mRNAs are associated with the RISC complex through the interaction with their specific siRNA. When this is the case, tight interactions could already be a problem during RNA extraction. When hANG cleaves tRNAs into tiRNAs, they inhibit the RISC interactions with mus308 and mus101 transcripts and therefore both mRNAs are not bound to RISC and can thereby easily be extracted. This would mean that a slight change in the kinetics of RISC binding to the respective transcripts results in a large difference in the detection rates of mus308 and mus101 mRNAs.

4.7 Conclusions

Taken together, two expression systems proved to induce and subsequently limit tRNA fragmentation efficiently. Inducing tRNA fragmentation through expression of hANG in S2 cells showed that the DNA repair genes mus101 and mus308 become highly up-regulated, possibly due to the stabilization of their mRNAs. It would be the next step to assess transcriptional changes after tRNA fragmentation in a genome-wide fashion to identify other genes that are up-regulated through hANG-mediated tRNA fragmentation.

5. Appendix

5.1 Vector Maps of the constructed plasmids

5.1.1 pRmHa3_hANG

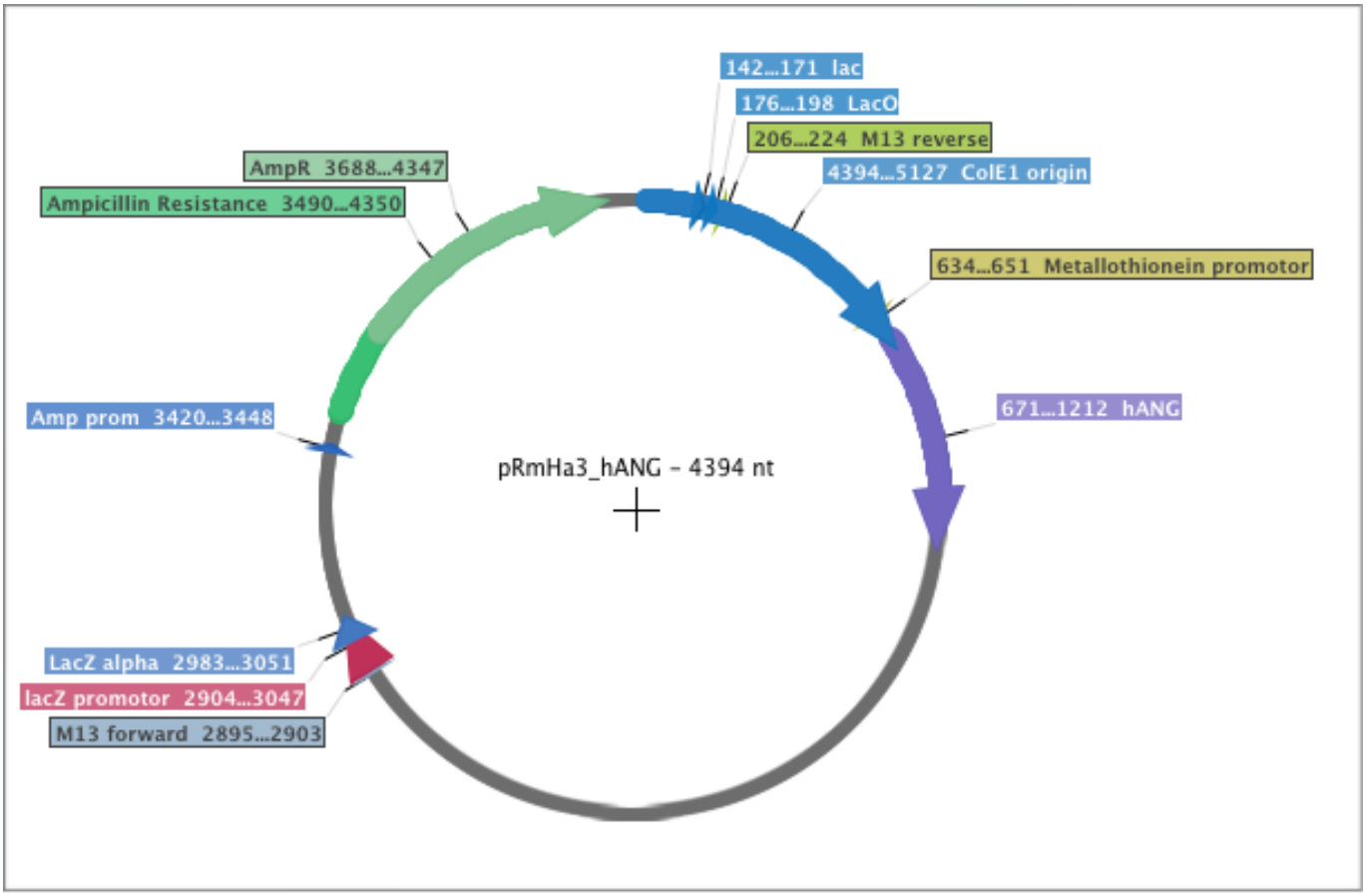

Fig. 5.1: **Vector map of pRmHa3_hANG.**

5.1.2 pRmHa3_RNH1

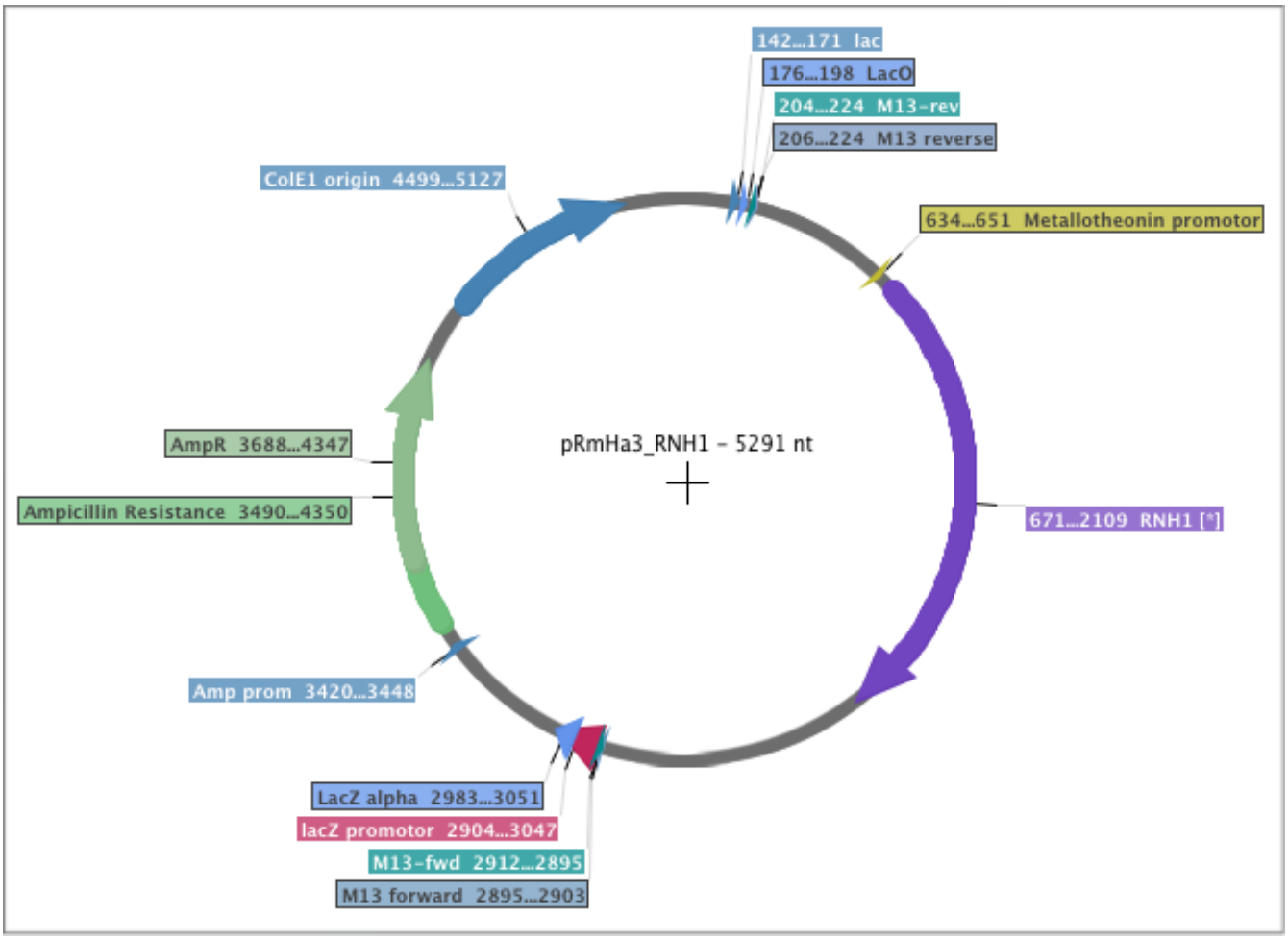

Fig. 5.2: **Vector Map of pRmHa3_RNH1.**

5.2 Sequencing of mus308

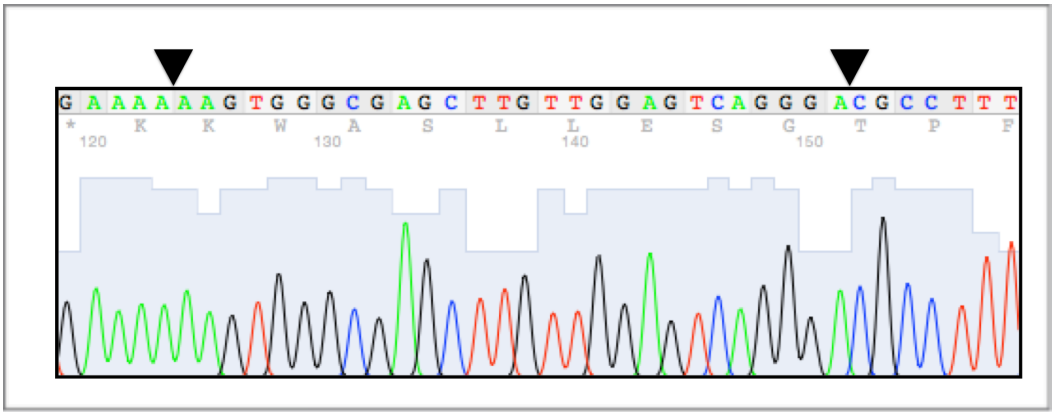

Fig. 5.3: **The mus308 PCR product contains the esi-2.1 binding site.** Sequencing data of the semi-quantitative PCR amplification of mus308 after treatment with tRNA fragments (300 ng/ml) cloned into PCR 2.1; 5' Start and 3' end of the sequence complementary to esi-2.1 is indicated by triangles.

5.3 Analysis of S2_hANG RNA integrity by Agilent Analyzer

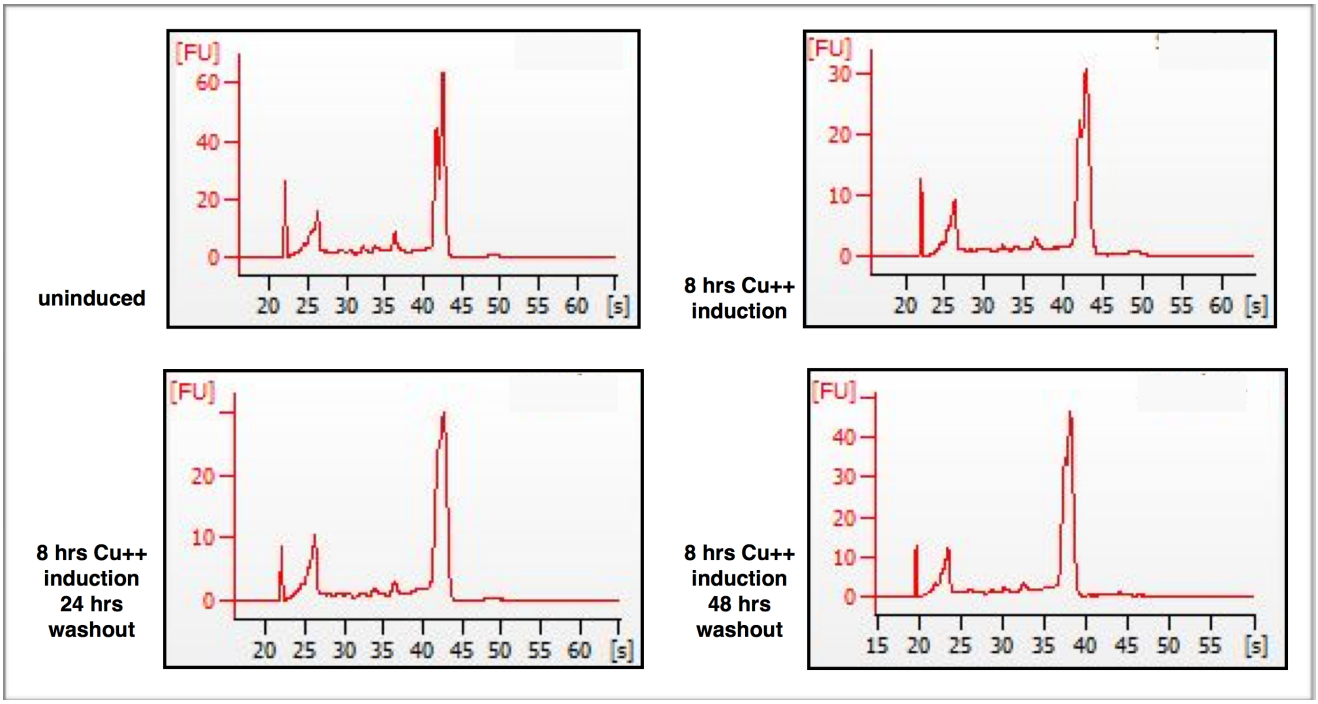

Fig. 5.4: **Agilent analysis of S2_hANG RNA integrity.** RNA integrity after 0 and 8 hours of induction with Cu++ compared to 8 hours of Cu++ induction followed by culturing the cells for 24 or 48 hours in fresh medium.

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Acknowledgements

I would like to thank Matthias Schaefer for his exceptional supervision and his constructive guidance and advice.

I also want to thank all former and current members of the Schaefer lab for the cordial and inspirational atmosphere and especially Bianca for scientific support and discussions.

Finally, I want to thank my family and my friends for their support.

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