



universität
wien

DISSERTATION

Titel der Dissertation

Processing, Stability and RNA Editing of microRNAs in
Xenopus oocytes and eggs

Verfasser

Mag. Dominik Muggenhumer

angestrebter akademischer Grad

Doktor der Naturwissenschaften (Dr. rer. nat.)

Wien, im Dezember 2010

Studienkennzahl lt. Studienblatt: A 091 441

Dissertationsgebiet lt.
Studienblatt: Genetik-Mikrobiologie

Betreuer: Ao. Univ.-Prof. Dr. Michael Jantsch

Table of contents

Abstract	1
Zusammenfassung	2
Introduction	3
RNA interference (RNAi)	3
Post-transcriptional gene regulation by microRNAs (miRNAs).....	4
Biogenesis of miRNAs and their assembly into ribonucleoproteins	4
Targets of miRNAs and modes of action	7
Regulation of miRNA gene transcription	8
Post-transcriptional regulation of miRNA maturation	9
A role for miRNAs in animal differentiation and development.....	12
<i>Xenopus</i> life cycle	13
<i>Xenopus laevis</i> oocytes and eggs.....	14
Early development	14
Maturation	15
Cytoplasmatic polyadenylation regulates mRNA translation during maturation.....	17
miRNAs in <i>Xenopus</i> development	18
RNA editing	19
RNA A-to-I editing by ADAR Deaminases	19
A-to-I RNA editing of miRNA precursors.....	22
Material and Methods	24
Buffers.....	24
Cloning of microRNA variants containing a T7 promoter.....	25
T7 <i>in vitro</i> transcription	28
Northern Blotting	28
Recombinant N-terminal myc-tagged Drosha	29
Immunoprecipitation (IP) of recombinant Drosha	29
Drosha <i>in vitro</i> processing assay.....	30
Cloning and sequencing of pre-microRNAs	30
Specific aims of the thesis	32
1) Regulation of miRNA biogenesis during <i>Xenopus</i> oogenesis.....	32
2) Impact of RNA editing on pri-miRNA processing <i>in vivo</i>	32

Results.....	34
Processing and stability of microinjected miRNAs in <i>Xenopus laevis</i> oocytes and eggs	34
Pre-miRNAs are exported and processed to mature miRNAs in oocytes	34
Processing of pre-miRNAs in the cytoplasm is enhanced in eggs	36
Degradation of pri-miRNAs in oocyte germinal vesicle	37
Processing of pri-miRNAs is activated in eggs	39
Comparison of pri-miRNA processing in oocyte and egg cytoplasm	40
Confirmation of successful processing by miRNA cloning and sequencing.....	41
Endogenous <i>Xenopus</i> microRNA processing is enhanced in eggs.....	42
Dissecting the processing pattern of <i>Xenopus</i> microRNA-148a	44
Processing pattern of xtr-pri-miRNA-148a in eggs.....	44
Processing pattern of xtr-pri-miRNA-148a in oocytes	46
Processing pattern of xtr-pre-miRNA-148a in oocytes and eggs	47
Regulation of Drosha activity.....	50
Detection of processing in transcriptionally silent cells	50
Analysis of recombinant Drosha activity in <i>Xenopus</i> oocytes and eggs	52
Impact of RNA editing on pri-miRNA processing <i>in vivo</i>	56
Discussion	60
Developmental activation of pri-miRNA processing	60
Developmental regulation of Drosha activity	62
Impact of RNA editing on pri-miRNA processing <i>in vivo</i>	64
References	66
Curriculum Vitae	86
Danksagung	87

Table of figures

Figure 1. Biogenesis of miRNAs and their assembly into ribonucleoproteins	6
Figure 2. Life cycle of the African clawed frog <i>Xenopus laevis</i>	13
Figure 3. <i>Xenopus</i> oocyte maturation	15
Figure 4. Deamination of adenosine to inosine by ADARs	20
Figure 5. RNA editing of pri-miRNA-142	22
Figure 6. RNA editing of pri-miRNA-151	23
Figure 7. N7myc-Drosha-poly(A) cloned in pBluescript KS vector	29
Figure 8. Hairpin structure of pre-miRNA-21	34
Figure 9. Nuclear injection of pre-miRNAs are exported to the cytoplasm and processed to mature microRNAs	35
Figure 10. Cytoplasmic injections of pre-miRNAs are processed to mature miRNA.	36
Figure 11. Hairpin structure and flanking region of pri-miRNA-21	37
Figure 12. Nuclear injection of pri-miRNAs leads to degradation	38
Figure 13. Injection of pri-miRNAs in eggs leads to processing to pre-miRNAs	39
Figure 14. Comparison of microinjected pri-miRNAs in eggs and oocytes	40
Figure 15. Confirmation of appropriate Drosha cleavage	41
Figure 16. Northern Blot of endogenous miRNA-101 and miRNA-148a	42
Figure 17. Quantification of relative endogenous miRNA expression in oocytes and eggs	43
Figure 18. Processing of pri-xtr-miRNA-148a is activated in eggs	44
Figure 19. Quantification of pri- to pre-miRNA-148a processing in oocytes and eggs	45
Figure 20. Nuclear injected pri-xtr-miRNA-148a is degraded in the nucleus	46
Figure 21. Nuclear injection of pre-miRNA-148a shows export and processing	47
Figure 22. Processing of pre-xtr-miRNA-148a is enhanced in eggs	48
Figure 23. Quantification of pre- to mature miRNA processing in oocytes and eggs	48
Figure 24. Processing of pri-miRNAs can still be detected in transcriptionally silent cells	51
Figure 25. Drosha cleavage assay using immunoprecipitated myc-tagged Drosha is active in oocytes and eggs	53
Figure 26. Quantification of pri- to pre-miRNA-29b-1 processing in oocytes and expressing recombinant Drosha	54
Figure 27. Expression of recombinant myc-tagged Drosha in oocytes and eggs reveals processing activity in oocytes	55
Figure 28. Variants of pri-miRNA-142 used in processing analysis	57
Figure 29. Processing of pri-miRNA-142 variants in <i>Xenopus</i> eggs	58
Figure 30. Processing of pre-edited pri-miRNA-142 is reduced <i>in vivo</i>	59

Abstract

MicroRNAs (miRNAs) comprise a large family of small ~21nt nucleotide long endogenous, single-stranded noncoding RNAs that regulate gene expression. The biogenesis of miRNAs is mediated by the two RNase III-like enzymes Drosha and Dicer. Fully grown *Xenopus* oocytes are transcriptionally inactive and arrested at meiotic prophase I. Maturation to eggs by progesterone allows progression of the oocytes into meiosis II leading to germinal vesicle breakdown (GVBD) accompanied by a complex network of regulation of stored maternal RNAs.

In order to gain insight in the regulation of miRNA biogenesis during *Xenopus* oogenesis we performed miRNA precursor microinjection experiments in oocytes and eggs. We were able to show the activation of pri-miRNA processing and the enhancement of endogenous miRNA expression upon maturation to eggs.

Consequently, tests on the developmental regulation of Drosha enzyme were performed, suggesting that Drosha is neither transcriptionally activated nor post-translationally inactivated upon maturation.

Further, the effects of RNA editing, a post-transcriptional single nucleotide modification, on miRNA processing were studied in *Xenopus* eggs. Microinjection experiments indicate an important role for RNA editing in blocking pri-miRNA processing *in vivo*.

Zusammenfassung

Die Familie der mikroRNAs (miRNAs) spielt eine wichtige Rolle bei der post-transkriptionellen Genregulation. Sie besteht aus kleinen ~21 Nukleotid-langen endogenen, einzelsträngigen, nicht-kodierenden RNA Molekülen. Die Biogenese von miRNAs wird von den beiden RNase III-Enzymen Drosha und Dicer reguliert.

Ausgewachsene *Xenopus* Oozyten sind transkriptionell inaktiv und arretieren in der meiotischen Prophase I. Maturation mittels des Hormons Progesteron führt die Oozyten weiter in die Meiose II, was zum Zusammenbruch der zellulären Kernstruktur und der Aktivierung eines komplexen Regulationsnetzwerks von maternal gespeicherten RNA Molekülen führt.

Um die Regulierung der Reifung von miRNA-Molekülen während der Entwicklung von Oozyten zu Eiern zu untersuchen wurde ein Mikroinjektionssystem von miRNA-Vorläuferstufen etabliert. Wir konnten eine Aktivierung der Prozessierung von primären miRNA-Transkripten (pri-miRNA) sowie eine Erhöhung der Expression endogener miRNAs nachweisen. Weitere Experimente zur Untersuchung der Regulation des Enzyms Drosha während der Maturation zu Eiern konnten zeigen, dass dieses weder transkriptionell noch post-translational reguliert wird.

Weitere Studien zum Effekt von RNA-Editierung, einer post-transkriptionellen Einzelnukleotid-Modifikation, auf die Prozessierung von miRNAs wurden in *Xenopus* Eiern durchgeführt. Mikroinjektionsexperimente konnten eine wichtige Rolle der RNA-Editierung bei der Hemmung der Prozessierung von pri-miRNAs *in vivo* belegen.

Introduction

RNA interference (RNAi)

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism that regulates gene expression in animals and plants by the formation of double-stranded RNA (dsRNA). The first evidence that RNAi is mediated by dsRNA came from injection experiments in *Caenorhabditis elegans* (Fire et al., 1998). Initially, the phenomenon of post-transcriptional gene silencing (PTGS) was described in plants and quelling fungi (Jorgensen, 1990, Romano and Macino, 1992). Further, the sequence specific effector molecules of RNAi were identified as short RNA species (21-25nt) in plants undergoing PTGS (Hamilton and Baulcombe, 1999).

During RNAi, dsRNA (produced by transgenes, viruses, repeat elements, supplied by microinjection, transfection, etc.) is processed to ~21nt small interfering RNAs (siRNAs) containing 2nt 3' overhangs. These siRNAs are incorporated into an RNA-induced silencing complex (RISC), which mediates the degradation of messenger RNAs (mRNAs) with sequence complementarity to the siRNA.

A second, very similar process is silencing mediated by microRNAs (miRNAs). These are also short (~ 21nt) RNAs that are excised from genome-encoded precursors folding into dsRNA-like hairpins. Different to siRNAs, miRNAs carry out their silencing function by base pairing to the 3'-untranslated region (3'-UTR) of target mRNAs and consequently inhibit protein accumulation. One further difference is that siRNAs exhibit perfect complementarity in the central part of the siRNA-mRNA duplex, whereas miRNA-mRNA duplexes are characterized by bulges or other irregularities. Although their mode of action seems to be different, miRNA and siRNA pathways are very similar and involve similar and in part identical machinery components (e.g. Dicer, Argonautes).

Post-transcriptional gene regulation by microRNAs (miRNAs)

MicroRNAs (miRNAs) are defined as 21-23nt long, non-protein-coding RNAs that are generated from endogenous hairpin-shaped transcripts (Bartel, 2004). Mature miRNAs and Argonaute (AGO) form the RNA-induced silencing complex (RISC) mediating post-transcriptional gene silencing by imperfect complementary base-pairing with target mRNAs, usually in the 3' untranslated region (3'-UTR).

Target mRNA binding inhibits protein synthesis either by repressing translation and/or by deadenylation and subsequent degradation of mRNA targets (reviewed in Filipowicz et al., 2008).

Biogenesis of miRNAs and their assembly into ribonucleoproteins

Transcription of primary transcripts, termed pri-miRNAs, from miRNA genes mainly by RNA polymerase II are usually several kilobases long and contain local stem-loop structures.

The first step in maturation is nuclear cleavage of the pri-miRNA at the stem of its hairpin structure. This reaction is mediated by the microprocessor complex consisting of the 160kD sized RNase III-like enzyme Drosha and its cofactor DiGeorge syndrome critical region 8 (DGCR8). DGCR8, harboring two double-stranded RNA binding domains (dsRBDs), recognizes the double stranded stem and the single-stranded flanking region of the pri-miRNA. Its catalytical partner Drosha cleaves the substrate ~11bp away from the ssRNA-dsRNA junction (Han et al., 2006). Drosha and DGCR8 regulate each other post-transcriptionally and furthermore DGCR8 stabilizes the Drosha protein via protein-protein interaction (Han et al., 2009).

Cleavage by the microprocessor complex releases a short ~60-70nt hairpin, termed pre-miRNA, having a 2nt 3' overhang (Lee et al., 2002). Pre-miRNAs are bound by exportin 5 (EXP5), a Ran-dependent importin-beta-related transport receptor, at the 14bp dsRNA stem along with a short 1-8nt 3' overhang (Kutay et al., 1998, Zeng and Cullen, 2004).

Nuclear export occurs through nuclear pore complexes, which are large proteinaceous channels embedded in the nuclear membrane (Nakielnny and Dreyfuss, 1999). EXP5 cooperatively binds to pre-miRNAs and the GTP-bound form of the cofactor Ran in the nucleus. Hydrolysis of GTP in the cytoplasm releases the pre-miRNA cargo of EXP5. (Yi et al., 2003, Bohnsack et al., 2004, Lund et al., 2004)

Following export from the nucleus, pre-miRNAs are cleaved by the ~200kD RNase III-like enzyme Dicer (complexed with TAR RNA binding protein [TRBP]) in the cytoplasm (Chendrimada et al., 2005). Dicer measures from the terminus of the pre-miRNA releasing ~22nt miRNA duplexes with protruding 2nt 3'ends similar to small interfering RNA (siRNAs) (Ma et al., 2004, Macrae et al., 2006).

The resulting miRNA duplexes are assembled into ribonucleoprotein (RNP) complexes called micro-RNPs (miRNPs) or miRNA-induced silencing complexes (miRISCs). Loading of miRNA duplexes in miRNP complexes is a dynamic, but so far not well understood process, that is usually coupled to pre-miRNA processing by Dicer (reviewed in Rana, 2007).

The catalytical component of miRNPs is the Argonaute (AGO) protein family (Hock and Meister, 2008). Generally, the strand with the 5' terminus located at the thermodynamically less-stable end of the duplex is selected to function as a mature miRNA. The strand that gets selected for incorporation into RISC is called guide strand. The other strand, termed passenger strand (or miRNA*), is normally degraded by exoribonucleases (Ramachandran and Chen, 2008, Chatterjee and Grosshans, 2009). Depending on the availability of mRNA targets and on the activity of different accessory proteins the ratio of miRNA to miRNA* can vary in certain tissues or developmental stages (Chiang et al., 2010, Krol et al., 2004, Ro et al., 2007). Moreover, certain miRNA duplexes can produce two functional miRNAs targeting different populations of mRNA targets (Okamura et al., 2008).

In human all four Argonaute proteins known (AGO1-4) are functioning in miRNA mediated translational repression. Additionally AGO2 has a RNase H-like P-element induced wimpy testis (PIWI) domain and can therefore cleave mRNA at the centre of the siRNA-mRNA duplex in RNAi (Tolia and Joshua-Tor, 2007).

A schematic summary of the miRNA biogenesis pathway can be found in Figure 1.

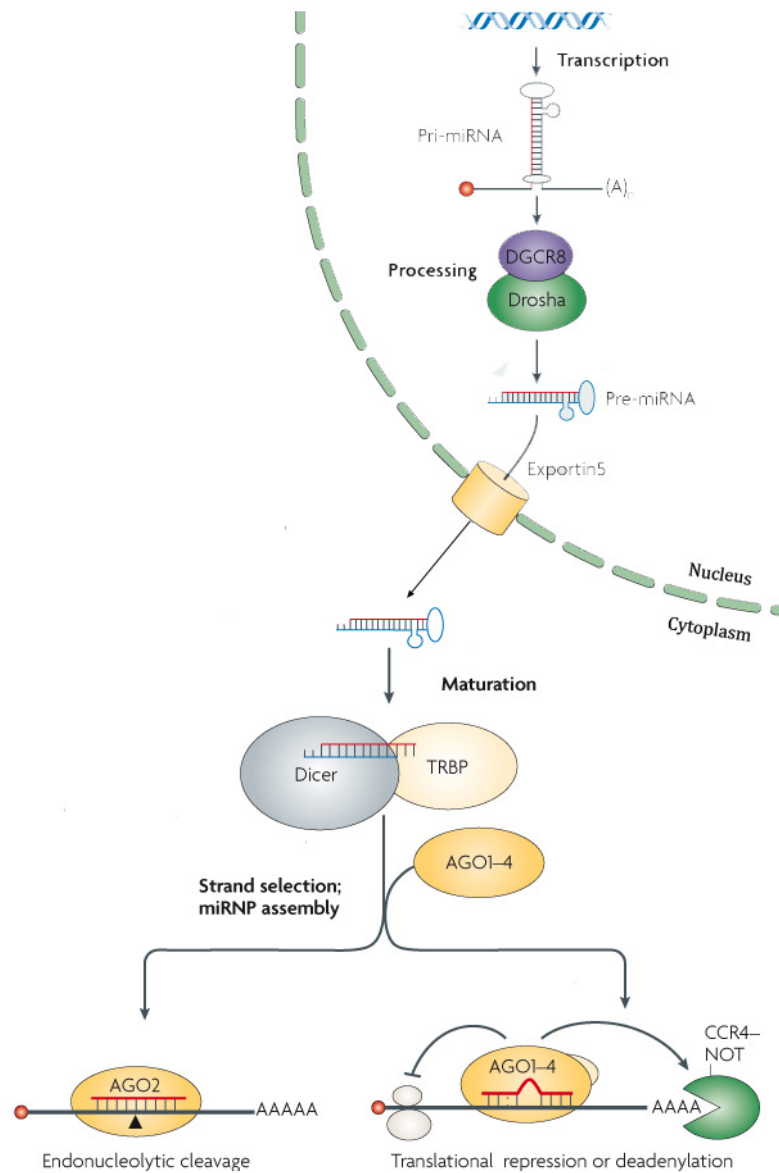


Figure 1. Biogenesis of miRNAs and their assembly into ribonucleoproteins

Canonical miRNA genes are transcribed to generate pri-miRNAs. The initiation step is mediated by the microprocessor complex (Drosha-DGCR8) generating 60-70nt pre-miRNAs. Nuclear export by EXP5-RanGTP is followed by cleavage to miRNA duplexes by Dicer in the cytoplasm (modified from Filipowicz et al., 2008)

Targets of miRNAs and modes of action

In most cases metazoan miRNAs pair imperfectly in the 3'-UTR of their mRNA targets according to a set of rules defined by experimental and bioinformatic studies (Grimson et al., 2007).

The most stringent rule is the perfect and contiguous base pairing of miRNA nucleotides 2 to 8, which represents the “seed” region (Brennecke et al., 2005, Lewis et al., 2005). Another rule is the preclusion of AGO-mediated endonucleolytic cleavage of mRNA through bulges and mismatches in the central region (position 10-12) of the miRNA-mRNA duplex (reviewed in Bartel, 2009). To stabilize the interaction a reasonable complementarity to the 3' half of the miRNA must be given, as well as an AU-rich neighborhood improves site efficacy. In general, accessibility of binding sites has an important effect on miRNA-mediated repression (Kertesz et al., 2007, reviewed in Filipowicz et al., 2008). Importantly, effective repression requires multiple sites for the same or different miRNAs (Doench and Sharp, 2004, Grimson et al., 2007, Nielsen et al., 2007).

Possible mechanisms of the miRNA mediated post-transcriptional gene repression are deadenylation followed by decapping and degradation, repression of translational initiation and blocking of translational elongation (Behm-Ansmant et al., 2006, Wu et al., 2006, Humphreys et al., 2005, Pillai et al., 2005, Maroney et al., 2006, Nottrott et al., 2006, Petersen et al., 2006, reviewed in Chekulaeva and Filipowicz, 2009).

The 182 kDa glycine-tryptophan protein (GW182), in concert with the other core components of miRISC, mediates the deadenylation of the target mRNAs. The GW182 amino-terminal part contains GW repeats that interact with AGO protein, while the carboxyterminal part interacts with poly(A) binding protein (PABP) and recruits the CCR4 and CAF1 deadenylases (Eulalio et al., 2009).

Translationally inactive eukaryotic mRNA assembled in repressive miRNPs usually accumulate in discrete cytoplasmic foci known as P-bodies (also known as GW-bodies) (reviewed in Eulalio et al., 2007). Under stress conditions stress granules (SG) can be formed, that also contain repressed mRNAs (reviewed in Anderson and Kedersha, 2009).

Regulation of miRNA gene transcription

Expression of miRNAs can be regulated by transcription factors in a tissue-specific or developmental-specific manner. Autonomously expressed miRNA gene promoters contain CpG islands, TATA box sequences, initiation elements and certain histone modifications (Ozsolak et al., 2008, Corcoran et al., 2009). Their similarity to protein-coding genes indicate that the promoters of miRNA genes are controlled by transcription factors (TFs), enhancers, silencing elements and chromatin modifications (reviewed in Krol et al., 2010).

The transcription factor PITX3 stimulates transcription of miRNA 133b, which then suppresses PITX3 transcription. This negative autoregulatory feedback loop controls dopaminergic neuron differentiation (Kim et al., 2007). The c-Myc oncogenic transcription factor (Myc) and V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (N-Myc) both stimulate expression of the miR-17-92 oncogenic cluster in lymphoma cells (O'Donnell et al., 2005) and miR-9 in neuroblastoma cells, but also inhibit the expression of several tumour suppressor miRNAs (e.g., miRNA 15a) by direct binding of myc to miRNA promoters (O'Donnell et al., 2005, Ma et al., 2008, Chang et al., 2008). Expression of miRNA-34 and miRNA-107 families, which enhance cell cycle arrest and apoptosis, can be stimulated by p53 (He et al., 2007). The transcriptional repressor, RE1 silencing transcription factor (REST) prevents transcription of the miRNA-124 promoter in neuronal progenitors and non-neuronal cells by recruiting histone deacetylases and the methyl CpG binding protein MeCP2 (Conaco et al., 2006).

Promoter methylation by the DNMT1 and DNMT3b DNA methyltransferases regulates transcription of miRNA-148a, miRNA-34b/c, miRNA-9 and let-7 (Han et al., 2007).

Post-transcriptional regulation of miRNA maturation

There are several positive and negative posttranscriptional regulators of microRNA biogenesis:

Drosha can target hairpins in DGCR8 mRNAs and facilitate its degradation, whereas DGCR8 can interact with the Drosha middle domain thereby stabilizing Drosha (Han et al., 2009, Triboulet et al., 2009). Heme can also bind to DGCR8 and facilitate pri-miRNA processing by promoting dimerisation (Faller et al., 2007).

The microprocessor complex normally only consists of Drosha and DGCR8 protein, but can form a larger complex that contains RNA helicases, double stranded RNA binding proteins (dsRBDs), heterogeneous nuclear ribonucleoproteins and Ewing's sarcoma proteins (Gregory et al., 2004). The DEAD-box RNA helicases p68 (DDX5) and p72 (DDX17) were identified as part of the microprocessor complex. Depletion of p68 (DDX5) and p72 (DDX17) reduces the expression of several miRNAs (and pre-miRNAs) without concurrent changes in pri-miRNAs (Fukuda et al., 2007). In cancer cells, the tumor suppressor p53 can associate with p68 (DDX5) and facilitate the maturation of a restricted population of pri-miRNAs in response to DNA damage (Suzuki et al., 2009).

The heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) can specifically bind to pri-miRNA-18a (a member of the miRNA-17-92 cluster) via its RNA Recognition Motifs (RRM) to facilitate its processing (Guil and Caceres, 2007). Drosha cleavage is increased via binding to the hairpin-loop to change the conformation, thereby creating a more favorable cleavage site for Drosha (Michlewski et al., 2008). Also the single-strand RNA binding protein KH-type splicing regulatory protein (KSRP) can bind to the terminal loop of many miRNA precursors (Trabucchi et al., 2009). KSRP interacts with single-strand AU-rich-element-containing mRNAs and is a key mediator of mRNA decay (Gherzi et al., 2004). Interaction of KSRP with both Dicer and Drosha promotes target miRNA maturation (Trabucchi et al., 2009).

Transforming factor- β (TGF- β) and bone morphogenetic factors (BMPs) can recruit ligand specific signal transducers (SMAD proteins) to pri-miRNA-21 transcripts in complex with the RNA helicase p68 (DDX5). This promotes the processing of pri-miRNA-21 to pre-miRNA-21 thereby increasing the expression of miRNA-21 (Davis et al., 2008). SMAD proteins bind a consensus sequences (R-SBE) within the stem

region of the primary transcripts which can be found in 20 miRNAs (T/B-miRs) regulated by TGF- β and BMPs (Davis et al., 2010).

The best studied negative regulator of miRNA biogenesis is Lin28. This developmentally regulated RNA binding protein uses multiple mechanisms to repress the maturation of the let-7 miRNA family, which is derived from 12 precursor transcripts that contain nine mature miRNA sequences. Lin28 is necessary and sufficient for blocking microprocessor-mediated cleavage of pri-let-7 miRNAs (Viswanathan et al., 2008). Lin28 binds to the terminal loop of pri-let-7 and acts as a competitor for Drosha (Newman et al., 2008, Piskounova et al., 2008). Lin28 is abundant in the cytoplasm suggesting that this may be the primary interaction with let-7 precursors (Balzer and Moss, 2007). Binding of Lin28 to pre-let-7 competes with Dicer binding to inhibit Dicer processing (Rybak et al., 2008). Additionally, Lin28 recognizes a tetra-nucleotide sequence motif (GGAG) in the terminal loop of let-7. This recruits TUTase4 (TUT4) to promote 3'-uridylation of pre-let-7 thereby inhibiting Dicer cleavage and leading to degradation of pre-let-7 by an unidentified nuclease activity (Heo et al., 2008, Heo et al., 2009).

A negative feedback loop is formed when Dicer mRNA is targeted by let-7 in the coding region, broadly influencing miRNA biogenesis (Forman et al., 2008). In contrast to Lin28, which is restricted to the let-7 family, the NF90/NF45 heterodimer can downregulate the maturation of a broader spectrum of miRNA precursors (Sakamoto et al., 2009). The NF90 subunit contains two dsRBDs that recognize the stem of pri-miRNAs in a sequence independent way, explaining interaction with a broad range of miRNA precursors (Chang and Ramos, 2005). Interaction with the stem of pri-miRNAs blocks binding of DGCR8 and subsequent Drosha cleavage (Sakamoto et al., 2009).

Dicer is not only involved in the cleavage of pre-miRNAs but also has a crucial role in miRISC loading (Carthew and Sontheimer, 2009). Addition of TRBP and removal of Dicer's amino terminal helicase domain stimulates catalytical activity of human Dicer (Ma et al., 2008). Dicer activity can also be modulated by proteolysis or by changing its specificity towards DNA (Lugli et al., 2005, Zhang et al., 2002, Nakagawa et al., 2010).

Phosphorylation of TRBP in the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK) pathway stabilizes the Dicer-TRBP complex to enhance miRNA production (Paroo et al., 2009).

A role for miRNAs in animal differentiation and development

In 1993, the first small RNA, *lin-4*, was discovered by forward genetics in *C. elegans* acting in the heterochronic pathway to control developmental timing (Lee et al., 1993, Wightman et al., 1993). Since then the number of known small RNAs has increased substantially revealing functions in almost every biological process, including developmental timing, cell differentiation, cell proliferation, apoptosis, metabolic control, transposon silencing and antiviral defense (reviewed in Bushati and Cohen, 2007). Mouse and human ES express a unique set of miRNAs that are downregulated upon differentiation (Houbaviy et al., 2003, Suh et al., 2004).

Dicer-mutant mice, arrest in development and die during gastrulation, before axis formation, while Dicer-deficient ES cells are viable, but do not form mature miRNAs and fail to differentiate *in vitro* and *in vivo* (Bernstein et al., 2003, Kanellopoulou et al., 2005). Development of mouse oocytes requires Dicer-dependent miRNA biogenesis for normal mouse embryo development (Murchison et al., 2007, Tang et al., 2007). Differentiation and patterning in other Dicer-depleted tissue is normal, but morphogenesis of hair follicles, lung epithelium, and limbs is perturbed (Andl et al., 2006, Harfe et al., 2005, Harris et al., 2006, Yi et al., 2006). In early zebrafish development Dicer-depleted embryos develop normally but arrest after 8 days. They die within 2-3 weeks when the maternal Dicer is running out and they are depleted of mature miRNAs (Wienholds et al., 2003).

Taken together, these studies suggest that miRNAs play important roles in cell differentiation and are required in a broad range of biological processes during animal development. To answer the question of what aspect of their biology is being regulated the identification of further miRNAs and their targets will be needed for a deeper understanding of their roles in development.

***Xenopus* life cycle**

Xenopus laevis (African clawed frog) is a species of South African aquatic frog that can grow to a length of 12cm (5 inch). The average life span ranges from 5 to 15 years with some animals recorded to have lived for 20 years.

Unlike other model organisms *Xenopus* does not have a short generation time and genetically simplicity (it is tetraploid). To reach sexual maturity the frog has to grow for 1 to 2 years.

However, *Xenopus* is an important model organism in developmental biology because of its large and easy manipulable embryo. *Xenopus* oocytes provide an excellent microinjection and expression system to study early vertebrate development.

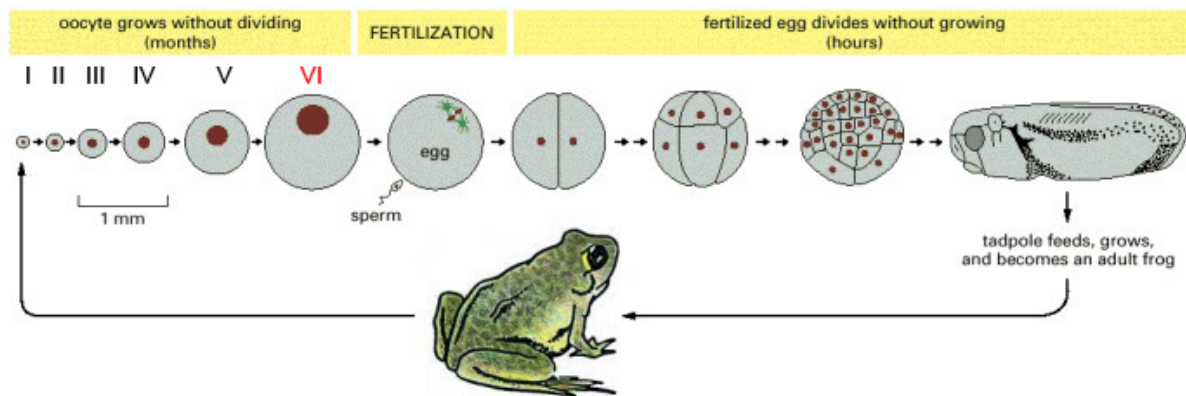


Figure 2. Life cycle of the African clawed frog *Xenopus laevis*.

Oocytes develop from Stage I to Stage VI and mature to eggs by progesterone (Dumont, 1972). The fertilized egg undergoes gastrulation and forms a multicellular tadpole within a day or two. Amphibian metamorphosis then grows the tadpole to an adult frog (modified from Alberts, 2002).

***Xenopus laevis* oocytes and eggs**

Early development

Xenopus oocyte maturation starts with a cell that is only slightly larger than a typical somatic cell. The meiotic cycle starts with one round of replication and enters meiotic prophase, during which chromosomes condense, the homologs pair and meiotic recombination occurs.

The oocyte develops for several months in a G₂-like growth phase, with active transcription, an intact germinal vesicle and partially decondensed chromatin (Ferrell, 1999). Once the oocyte has grown from stage I to stage VI it can remain for years in meiotic diplotene, which resembles a G₂ phase arrest state (see Figure 2) (Dumont, 1972). Stage VI oocytes are very large cells of about 1.3mm in diameter and an intracellular volume of 1μl which is 10⁶ times the size of a typical somatic cell (Hausen and Riebesell, 1991). The oocyte also has a huge nucleus, termed germinal vesicle, that is 300-400nm in diameter residing in the darkly pigmented animal hemisphere (Ferrell, 1999).

The accumulated material in the oocyte cytoplasm includes energy sources and energy-producing organelles (yolk and mitochondria), the enzymes and precursors for DNA, RNA and protein synthesis, stored messenger RNAs, structural proteins and morphogenetic regulatory factors that control early embryogenesis (Gilbert, 2000).

During the diplotene stage of meiotic prophase I so called lampbrush chromosomes can be observed even under light microscope (Old et al., 1977). These are sites of active RNA synthesis and stretch out in large loops of DNA.

Maturation

In response to gonadotropic hormones secreted by the pituitary gland, follicle cells synthesize and release the steroid hormone progesterone that initiates maturation of oocytes (Fortune et al., 1975).

The maturing oocyte reenters meiosis I, undergoes germinal vesicle breakdown (GVBD), and completes the first meiotic division by emitting the first polar body (Ferrell, 1999). GVBD can be scored by an appearing white spot at the animal pole, resulting from rearrangement of cortical pigment granules.

Entry into the mitotic (M) phase of the cell cycle (in both meiosis and mitosis) is regulated by mitosis-promoting factor, or MPF (originally called “maturation-promoting factor” after its meiotic function) (see Figure 3). MPF contains two subunits, cyclin B and the p34^{cdc2} protein (a cyclin-dependent-kinase).

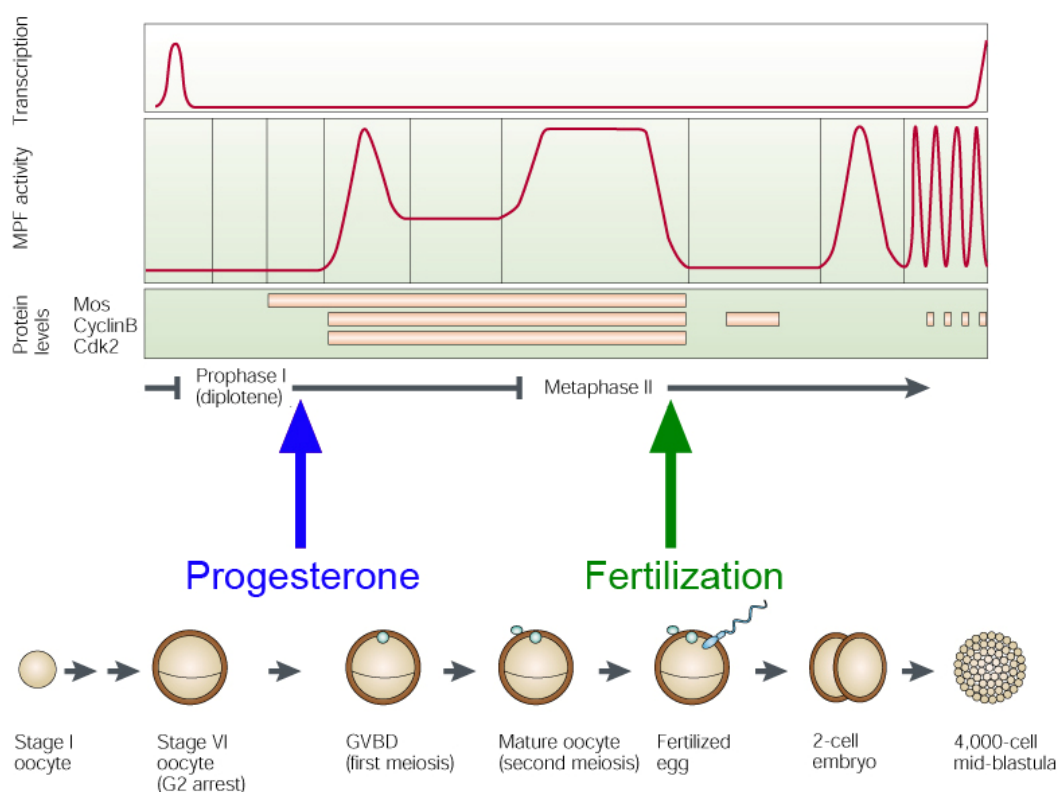


Figure 3. *Xenopus* oocyte maturation

Progesterone stimulation activates the M-Phase promoting factor (MPF) and Mos-Kinase. This guides oocytes from meiotic prophase I to meiosis II (modified from Mendez and Richter, 2001).

Progesterone stimulation turns the components present in the oocyte, into an active MPF by mediating polyadenylation of the maternal *c-mos* mRNA that has been stored in the cytoplasm (Sagata et al., 1988, Sagata et al., 1989, Sheets et al., 1995). This leads to translation of the 39-kDa phosphoprotein c-mos, which has an important role in releasing the egg from its dormancy. The protein can only be detected during oocyte maturation and is destroyed quickly upon fertilization. Inhibition of c-mos translation (by injecting c-mos antisense oligos into the oocyte) blocks GVBD and the resumption of oocyte maturation (Sagata et al., 1988). The c-mos protein activates a phosphorylation cascade that phosphorylates and activates the p34 subunit of MPF (Ferrell and Machleder, 1998, Ferrell, 1999). The active MPF allows GVBD and the chromosomes go through the first meiotic division and the prophase of the second meiotic division.

In metaphase of the second meiotic division the egg is again arrested by combined activity of c-mos and cyclin-dependent kinase 2 (cdk2) (Gabrielli et al., 1993). These two proteins are subunits of a cytostatic factor (CSF) which is the product of the c-mos proto-oncogene. CSF blocks cell cycles in metaphase by preventing the degradation of cyclin (Masui, 1974, Castro et al., 2001).

Fertilization releases the block in metaphase of the second meiotic division by calcium ion flux thereby activating the calcium binding protein calmodulin (Zhang and Yuan, 1998). Calmodulin activates calmodulin-dependent protein kinase II, which inactivates p34, and calpain II, a calcium-dependent protease that degrades c-mos (Watanabe et al., 1989, Lorca et al., 1993) Therefore CSF is inactivated and subsequent cyclin degradation completes the meiotic division.

Cytoplasmatic polyadenylation regulates mRNA translation during maturation

Mos, cyclin B and other dormant mRNAs present in the *Xenopus* oocyte contain short poly(A) tails (~20-40nt long). Translation of these mRNAs can only take place when the length is elongated to 150nt by poly(A) polymerases (reviewed in Mendez and Richter, 2001).

Two elements in the 3'-UTR of those dormant mRNAs are required for the cytoplasmatic polyadenylation and subsequent translational activation: The hexanucleotide AAUAAA and the cytoplasmic polyadenylation element (CPE) with the general consensus sequence UUUUUUAU (Fox et al., 1989, McGrew et al., 1989, McGrew and Richter, 1990, Barkoff et al., 2000). The timing of polyadenylation may be regulated by the precise sequence of the CPE, the number of copies of the CPE, the distance between the CPE and the hexanucleotide, or sequences adjacent to the CPE (reviewed in Mendez and Richter, 2001).

Initiation of maturation by progesterone activates the serine/threonine protein kinase Eg2, phosphorylating CPEB at serine residue 174 (Andresson and Ruderman, 1998, Mendez et al., 2000a). CPEB is a highly conserved, sequence-specific RNA binding protein harboring a Zinc finger domain and two RNA recognition motifs (RRM) bound to CPEs (Hake and Richter, 1994, Stebbins-Boaz et al., 1996). Phosphorylation of CPEB increases the affinity of CPEB for CPSF, a cleavage and polyadenylation specificity factor. Therefore, CPEB stabilizes the interaction of CPSF to the AAUAAA sequence by recruiting poly(A) polymerase to the end of the mRNA (Fox et al., 1992, Bilger et al., 1994, Dickson et al., 1999, Mendez et al., 2000b).

The newly elongated poly(A) tail can then recruit translation initiation factors through its association with Poly(A) binding protein (PABP). This helps forming a complex of the transcriptional initiation factors eIF4G and eIF4E resulting in the initiation of translation (reviewed in Radford et al., 2008).

miRNAs in *Xenopus* development

In *Xenopus*, miRNA genes are predominantly positioned within introns of protein-coding and non-protein-coding RNA Polymerase II-transcribed genes. The genes of miRNAs were also located in pre-mRNA exons and positioned intergenically between known protein-coding genes and clustered throughout the genome, indicating the potential for the cotranscription and coordinate expression of miRNAs located in a given cluster. Comparison of *Xenopus tropicalis* and *Xenopus laevis* blots revealed comparable expression profiles, although several miRNAs exhibited species-specific expression. Differences in the tissue specific expression profile of pri-miRNAs compared to mature miRNA expression profile argue for post-transcriptional regulation of miRNA expression in *Xenopus* (Tang and Maxwell, 2008). Nuclear microinjection experiments in *Xenopus laevis* oocytes revealed efficient export of pre-miRNAs to the cytoplasm mediated by EXP5 (Bohnsack et al., 2004, Lund et al., 2004). Cytoplasmic Dicer processing can be detected in *Xenopus* oocytes and is further increased in eggs (Lund and Dahlberg, 2006). During *Xenopus laevis* development from oocyte stage to tadpole stage a variety of miRNAs start to be expressed. Most of these miRNAs emerged at a specific stage and were continuously expressed until the tadpole stage (Watanabe et al., 2005).

In *Xenopus laevis* embryogenesis miRNA-427 mediates the rapid deadenylation of maternal mRNAs after the midblastula transition (MBT). By MBT, the stage when the embryonic cell cycle is remodeled and zygotic transcription of mRNAs is initiated, each embryo has accumulated $\sim 10^9$ molecules of miRNA-427 processed from multimeric pri-miRNA 427 transcripts synthesized after fertilization (Lund et al., 2009). Analysis of the spatiotemporal expression of miRNAs in *Xenopus tropicalis* embryogenesis revealed that many miRNAs, that gave specific expression patterns, are present in neuronal tissues (Walker and Harland, 2008).

RNA editing

RNA editing is a post-transcriptional mechanism that alters the nucleotide sequence of its RNA substrate from the DNA sequence encoded by the corresponding gene in the genome.

The phenomenon of RNA editing was first discovered in kinetoplast protozoa as the deletion or insertion of uridine nucleotides (Benne et al., 1986). Since then, many other RNA editing mechanisms were discovered, grouped in either insertion/deletion of nucleotides or nucleotide modification (reviewed in Gott and Emeson, 2000). The best characterized mechanism of RNA editing by base modification are C-to-U deamination and A-to-I deamination (reviewed in Keegan et al., 2001, Alfonzo et al., 1997).

Editing in the coding region of an mRNA can change the protein coding sequence by replacing single nucleotides or by insertion or removal of stop codons. Additionally, splice sites can be changed and RNA stability can be influenced, affecting splice efficiency and alternative splicing decisions. Consequently, RNA editing can increase the number of different functional proteins derived from a given gene (reviewed in Bass, 2002).

The most widespread type of RNA editing in higher eukaryotes is the conversion of A-to-I, which is interpreted as guanosine by the translation or splicing machinery.

RNA A-to-I editing by ADAR Deaminases

The hydrolytic deamination of adenosine to inosine (6-deaminated inosine, see Figure 4) is mediated by ADAR (adenosine deaminase acting on RNA) enzymes. ADARs are targeting dsRNA via their double-stranded RNA binding domains (dsRBDs), which are present on all members of the protein family. ADARs were first discovered in *Xenopus laevis* eggs and embryos for their dsRNA unwinding activity and later discovered to be dsRNA specific adenosine deaminases (Bass and Weintraub, 1987, Bass and Weintraub, 1988).

ADAR enzymes probably evolved from the functionally and structurally related adenosine deaminases that act on tRNAs (ADATs) (Gerber et al., 1998).

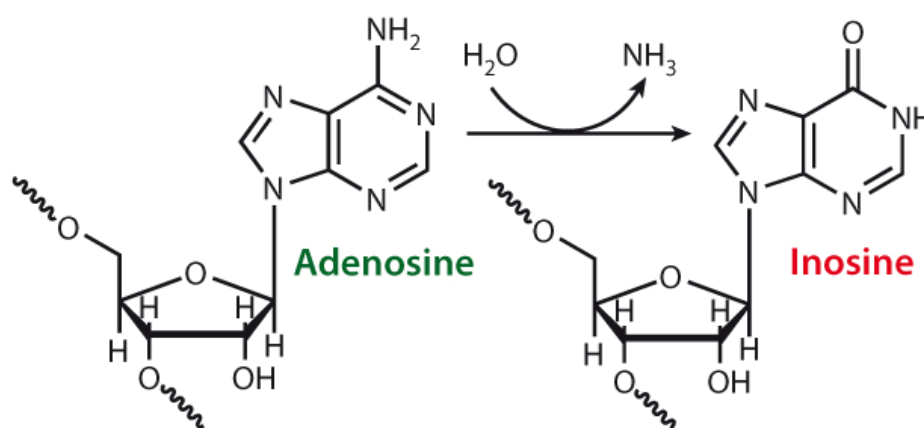


Figure 4. Deamination of adenosine to inosine by ADARs

During A-to-I RNA editing a hydrolytic deamination reaction converts adenosine to inosine (from Nishikura, 2010).

In vertebrates, three ADAR family members are known (ADAR1-3), where ADAR1 has two translational products: an interferon inducible 150kD shuttling protein (ADAR1p150) and a constitutively expressed 110kD shuttling protein (ADAR1p110) (Patterson and Samuel, 1995). ADAR1 and ADAR2 have enzymatic activity, whereas for ADAR3 (also having a conserved deaminase domain) so far no activity has been described (Gerber et al., 1997).

ADAR enzymes harbor a conserved catalytic deaminase domain at the carboxyterminal end, and a variable number of dsRBDs at the aminoterminal end (reviewed in Lunde et al., 2007). The dsRNA binding motif of dsRBDs was first discovered in *Drosophila* staufen and in *Xenopus laevis* RNA binding protein A (Xlrpba) and regulates numerous biological processes such as RNA localization, RNA interference, RNA processing and RNA editing (St Johnston et al., 1992, reviewed in Doyle and Jantsch, 2002).

ADAR targets both intermolecular and intramolecular dsRNAs of >20 base pairs (bp) (two turns of the dsRNA helix) as substrates (Nishikura et al., 1991). In long, completely base paired dsRNA (>100bp), including many adenosine residues editing, occurs non-selectively. In contrast, long but partially dsRNAs containing mismatched bases, bulges and loops (imperfect dsRNAs) as well as short dsRNAs (~20-30bp) are edited selectively (Lehmann and Bass, 1999).

Base modification by hydrolytic deamination in A-to-I editing in the coding region of mRNA can create, delete or alter the meaning of a codon resulting in a diversification of their protein functions (reviewed in Bass, 2002). Protein coding RNA targets of ADARs are mammalian glutamate receptor (GluR), mammalian serotonin receptor (5-HT_{2C}R), human potassium channel Kv1.1, squid Kv1.1A, *Drosophila* Na⁺ channel and hepatitis delta virus antigen (Higuchi et al., 1993, Burns et al., 1997, Hoopengardner et al., 2003, Rosenthal and Bezanilla, 2002, Reenan et al., 2000, Polson et al., 1996). In most cases, RNA editing of these protein coding genes results in the generation of protein isoforms leading to the modification of ion channel/neurotransmitter receptor functions (Seeburg and Hartner, 2003). Editing has been detected within viral transcripts and in tRNAs but the vast majority of A-to-I editing sites are noncoding sequences (Cattaneo et al., 1988, Polson et al., 1996, Laforest et al., 1997). Frequent targets are 5' and 3' UTRs and intragenic retrotransposon elements, such as Alu and long interspersed elements (LINEs) (Levanon et al., 2004, Athanasiadis et al., 2004, Morse et al., 2002, reviewed in Nishikura, 2006). A-to-I editing can also alter RNA stability by inosine to uridine binding, which can only form one hydrogen bond and thereby weakens RNA strand pairing.

Further, accumulating evidence suggests an interaction of A-to-I RNA editing on the RNAi pathway thereby modulating the efficacy of RNAi (reviewed in Nishikura, 2006, Nishikura, 2010). Precursors of certain miRNAs are A-to-I edited having important implications on their processing, expression and function of mature miRNAs (Luciano et al., 2004, Blow et al., 2006, Yang et al., 2006, Kawahara et al., 2007a, Kawahara et al., 2007b).

A-to-I RNA editing of miRNA precursors

A-to-I RNA editing of miRNA precursors by ADAR1 and ADAR2 was first described for miRNA-22 followed by miRNA-151, miRNA-197, miRNA-223, miRNA-376a, miRNA-379, miRNA-99a and miRNA-1-1, miRNA-142, miRNA-143 (Luciano et al., 2004, Blow et al., 2006, Calin and Croce, 2006).

Editing of the specific sites +4 and +5 of pri-miRNA-142 (see Figure 5) is sufficient and necessary to block cleavage by the microprocessor complex *in vitro* (Yang et al., 2006). Consistently, in *Adar1*^{-/-} or *Adar2*^{-/-} mice levels of miRNA-142 expression is substantially increased (Yang et al., 2006). Further, highly edited pri-miRNA-142 is degraded *in vitro* by Tudor staphylococcal nuclease (Tudor-SN), a nuclease that cleaves hyper-edited dsRNA substrates containing multiple I-U base pairs (Yang et al., 2006, Scadden, 2005).

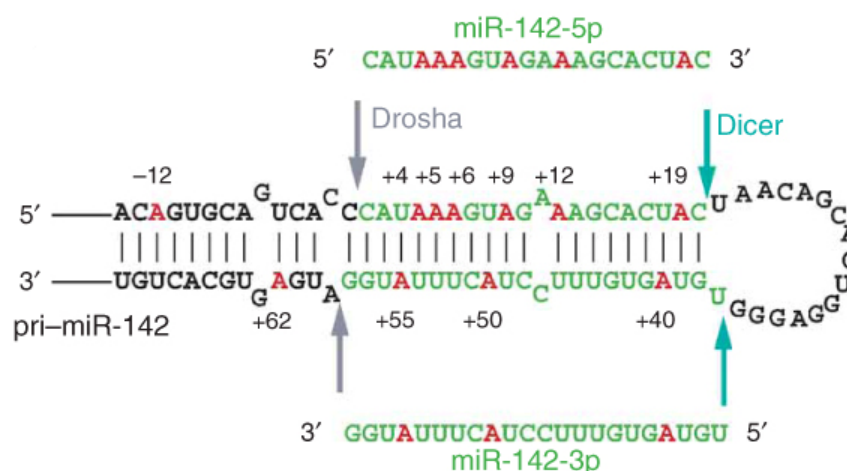


Figure 5. RNA editing of pri-miRNA-142

Editing of site +4 and +5 blocks processing of the microprocessor complex *in vitro* (taken from Yang et al., 2006)

Downstream processing steps are also affected by RNA editing: pri-miRNA-151 has a major (+3) and a minor ADAR1 editing site (-1) on its antisense strand near the Dicer cleavage site (see Figure 6). Editing of these sites had no effect on Drosha cleavage but inhibited Dicer cleavage, leading to accumulation of edited pre-miRNA-151 in the cytoplasm (Kawahara et al., 2007a).

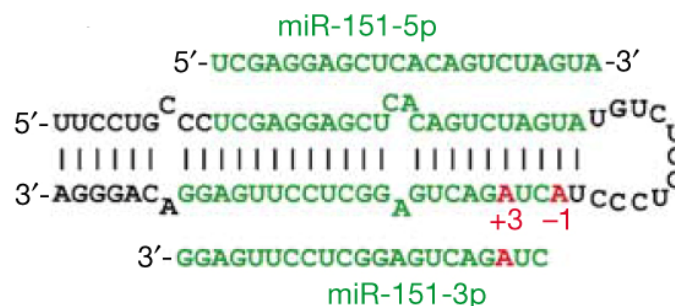


Figure 6. RNA editing of pri-miRNA-151

Editing of site +3 and -1 blocks cleavage by Dicer (taken from Kawahara et al., 2007a)

Another miRNA that is edited is encoded by the miRNA-376 cluster. This cluster is maternally imprinted and consists in human of six, in mouse of three miRNA-376 genes. High editing levels in the mouse brain were detected at site +4 (by ADAR2) and site +44 (by ADAR1). These editing sites are located in the middle of the 5'-proximal half "seed" region of miRNA 376-5p and 3p strands that are critical for miRNA hybridization to their targets.

Endogenous expression of a predicted target of edited miRNA-376a, PRPS1, a mouse protein involved in purine metabolism and uric acid synthesis, was dependent upon ADAR2 and edited miRNA-376a. It was specifically shown, that in the cortex of ADAR2^{-/-} mice, PRPS1 protein and uric acid levels were ~2-fold higher compared to wild type mice (Kawahara et al., 2007b). These findings suggest the redirection of silencing target genes by edited miRNAs.

Material and Methods

Xenopus laevis oocytes and eggs were stored in OR2-buffer at 16°C. Oocytes were matured to eggs by adding 20µg/ml progesterone and incubation at 16°C o/n.

Xenopus laevis eggs were selected for visible germinal vesicle breakdown (GVBD). Nuclear and cytoplasmic injections were performed using WPI Nanoliter 2000™ microinjector. Extraction of total RNA was carried out using Invitrogen™ TRIzol reagent according to manufacturer's protocol. RNA was separated on 10% 29:1-Acrylamide:bisAcrylamide Gels containing 8M UREA in 1xTBE.

DNA and RNA molecules were precipitated in 3x Vol. 96% EtOH and $\frac{1}{10}$ x Vol. 3M NaOAc pH=5.2.

Buffers

OR2 buffer: 82,5mM NaCl, 2,5mM KCl, 1mM CaCl₂, 1mM MgCl₂, 1mM Na₂HPO₄, 5mM HEPES pH=8.3

5:1 Isolation buffer pH=7.0: 83mM NaCl, 17mM KCl, 6,5mM Na₂HPO₄, 3.5mM KH₂PO₄, 3mM MgCl₂ was added directly before isolation

NET-2: 150mM NaCl; 50mM Tris pH7.4; 0.05% NP40

20x SSC, pH=7.0: 3M NaCl, 300 mM tri-Sodium citrate dihydrate

10xTBE pH=8.3: 1liter: 108g Tris base; 55g boric acid; 40ml 0.5M EDTA

50xTAE: 1 liter: 242g Tris base; 57ml glacial acetic acid, 18.6g EDTA

2xTY: 1 liter: 10g yeast extract, 16g tryptone, 5g NaCl – for plates add 15g bacto agar

1xTE: 10mM Tris pH7.5; 1mM EDTA

Church & Gilbert buffer (Church and Gilbert, 1984): for 1 liter: 7% SDS; 1% BSA; 1mM EDTA; 500 ml 2xNaHPO₄ pH7.2 (1Liter 2xNaHPO₄: 34g Na₂HPO₄·7H₂O; 4 ml 85% H₃PO₄)

Urea loading buffer: 8M urea; 5mM Tris pH7.5; 0.5% (w/v) bromophenol blue; 0.5% (w/v) xylene cyanol FF; 20mM EDTA, pH8.0.

All RNase sensitive assays were carried out with 1:2000 DEPC treated reagents.

Cloning of microRNA variants containing a T7 promoter

To produce DNA constructs that can be *in vitro* transcribed by T7 RNA Polymerase miRNA sequences were PCR amplified from 1µg of genomic DNA.

PCR reactions were performed using Fermentas™ Taq Polymerase and supplied reagents according to manufacturer's protocol.

PCR cycles: 5min 95°C, 40x (30sec T_m=55°C, 30sec 72°C, 30sec 95°C), 5min 72°C

PCR products were cloned in Promega™ pGEM-Teasy vector and verified by sequencing.

PCR primers (T7 RNA Polymerase promoter sequences underlined):

T7 pre-miRNA-21

Forward+T7 5'-TAATACGACTCACTATAGGGCTTATCAGACTGATGTT-3' (MJ2267)

Reverse 5'-AGAGCCCATCGACTGGT-3' (MJ2268)

T7 pri-miRNA-21

Forward+T7 5'-TAATACGACTCACTATAGGACATCTCCATGGCTGTACC-3' (MJ2265)

Reverse 5'-ATGGTCAGATGAAAGATA-3' (MJ2266)

T7 pri-miRNA-29b-1 Forward+T7

5'-TAATACGACTCACTATAGGGTCCTGGGTTTATTGTAAG-3' (MJ2354)

Reverse 5'-TTCTTTTGCTGTTGGTAGTGCAG-3' (MJ2355)

T7 pre-xtr-miRNA-148a (based on T7 prei xtr-miRNA-148a)

Forward+T7 5'-TAATACGACTCACTATAGGAGTTCTGTGACACTTAGACTCT-3' (MJ2847)

Reverse 5'-ACAAAGTTCTGTAGTGCACTGA-3' (MJ2848)

T7 prei xtr-miRNA-148a (cloning intermediate to create pri-xtr-miRNA-148a)

Forward+T7

5'-TCTTGGGTTTATTGTAAGAGAGCATTATGAATAGTCTTTTAAATCAAAGTTCTGTG-3' (MJ2845)

Reverse 5'-CAGACTCCCAAACAAAGTTC-3' (MJ2846)

T7 pri-xtr-miRNA-148a (based on T7 prei xtr-miRNA-148a)

Forward+T7 5'-TAATACGACTCACTATAGGGTCTTGGGTTTATTGTAAGAGAGC-3'
(MJ2844)

Reverse

5'-TTCTTTTGCTGTTGGTAGTGCAGCGCAGCCAGACTCCCAAACAAAGTTC-3'
(MJ2851)

T7 prei-miRNA-142 (cloning intermediate to create pri-miRNA-142 variants)

Forward+T7 5'-TAATACGACTCACTATAGGACGGACAGACAGACAGTGC-3'
(MJ2122)

Reverse 5'-ATCTCCGAAGCCCACAGTAC-3' (MJ2121)

T7 pri-miRNA-142 (based on T7 prei-miRNA-142)

Forward+T7

5'-TAATACGACTCACTATAGGGTCTTGGGTTTATTGTAAGAGAGCATTATGAACG
GACAGACAGACAGTGCA-3' (MJ2587)

Reverse

5'-TTCTTTTGCTGTTGGTAGTGCAGCGCAGCTATCTCCGAAGCCCACAGTAC-3'
(MJ2612)

T7 pri-miRNA-142 pre-edited: A-to-G at position +4, +5 (based on T7 prei-miRNA-142)

(1) Forward 5'-GACAGACAGACAGTGCAGTCACCCATGGAGTAG-3' (MJ2364)

(1) Reverse 5'-ATCTCCGAAGCCCACAGTAC-3' (MJ2121)

(2) Forward+T7

5'-TAATACGACTCACTATAGGGTCTTGGGTTTATTGTAAGAGAGCATTATGAACG
GACAGACAGACAGTGCA-3' (MJ2587)

(2) Reverse

5'-TTCTTTTGCTGTTGGTAGTGCAGCGCAGCTATCTCCGAAGCCCACAGTAC-3'
(MJ2612)

T7 pri-miRNA-142 degenerated CC: A-to-C at position +4, +5, +6 (based on T7 prei-miRNA-142)

(1) Forward 5'-GACAGACAGACAGTGCAGTCACCCATCCCGTAG-3' (MJ2365)

(1) Reverse 5'-GCCCACAGTACACTCATCCATCCCGTAGGAAAC-3' (MJ2366)

(2) Forward+T7

5'-TAATACGACTCACTATAGGGTCTTGGGTTTATTGTAAGAGAGCATTATGAACG
GACAGACAGACAGTGCA-3' (MJ2587)

(2) Reverse

5'-TTCTTTTGCTGTTGGTAGTGCAGCGCAGCTATCTCCGAAGCCCACAGTAC-3'
(MJ2612)

**T7 pri-miRNA-142 degenerated GG: A-to-G at position +4, +5, +6 (based on T7
prei-miRNA-142)**

(1) Forward 5'-GACAGACAGACAGTGCAAGTCACCCATGGGGTAG-3' (MJ2670)

(1) Reverse 5'-GCCCACAGTACACTCATCCATGGGGTAGGAAAC-3' (MJ2671)

(2) Forward+T7

5'-TAATACGACTCACTATAGGGTCTTGGGTTTATTGTAAGAGAGCATTATGAACG
GACAGACAGACAGTGCA-3' (MJ2587)

(2) Reverse

5'-TTCTTTTGCTGTTGGTAGTGCAGCGCAGCTATCTCCGAAGCCCACAGTAC-3'
(MJ2612)

T7 *in vitro* transcription

T7-microRNA constructs cloned in Promega™ pGEM-T easy vector were linearized by digestion using 1µl (10u) Pml (XmnI) restriction enzyme (Fermentas, 2h – 37°C). PCR was performed on the linearized vector to create an upstream of T7 extended PCR template for *in vitro* transcription.

PCR cycles: 5min 95°C, 40x (30sec Tm=55°C, 30sec 72°C, 30sec 95°C), 5min 72°C

PCR products were checked on a 1.5%-TAE Agarose Gel and eluted for *in vitro* transcription using PeqLab™ elution kit.

T7 *in vitro* transcription using 500ng of template DNA was performed in 10mM DTT, 1xStratagene™ transcription buffer, NTPs (0.5mM GTP, 0.5mM CTP, 0.5mM UTP, 0.1mM ATP), 30µCi [α-32P]ATP, 1µl (40u) Ribolock™ RNase Inhibitor and T7 Polymerase. The reaction was incubated for 2h at 37°C, purified using Sephadex G-25 columns and precipitated.

Radiolabeled RNA *in vitro* transcripts were separated on 10% 29:1 AA:bisAA gels (8M UREA in 1xTBE), exposed on Kodak™ X-ray films, appropriate bands were cut out and eluted in 400µl elution buffer (500mM NH₄OAc, 0.2%SDS, 100mM EDTA) o/n. Eluted samples were purified by Sephadex G-25 columns and precipitated.

Northern Blotting

Northern Blots were performed as described earlier (Pall and Hamilton, 2008) using semi-dry blotting technique. Northern Blot probes xtr-miRNA-101 5'-CTTCAGTTATCACAGTACTGTA-3' (MJ2521) and xtr-miRNA-148a 5'-ACAAAGTTCTGTAATGCACTGA-3' (MJ2523) were 5'-end labeled with 20µCi [γ-32P] ATP by Fermentas™ PNK according to manufacturer's protocol.

Recombinant N-terminal myc-tagged Drosha

Recombinant N7myc-Drosha was cloned based C7myc-Drosha obtained from my supervisor Michael Jantsch. A 3' Poly-(A)-tail was added downstream the Drosha coding sequence (Figure 7).

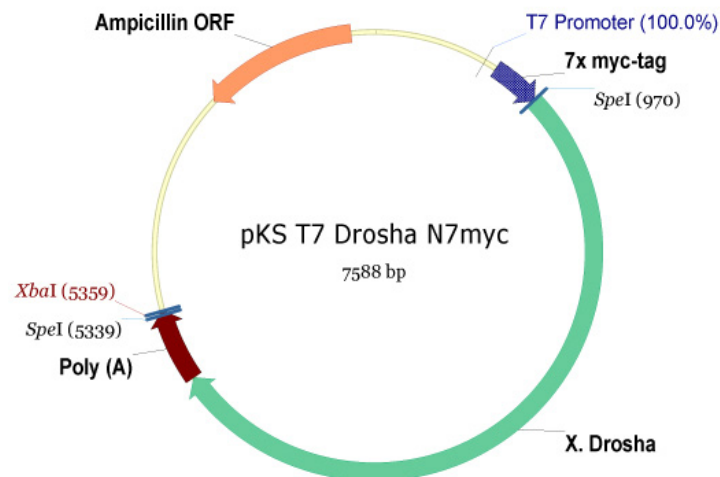


Figure 7. N7myc-Drosha-poly(A) cloned in pBluescript KS vector

The *Xenopus* Drosha containing a 3' Poly-(A)-sequence was cloned into pBluescript II KS vector.

The Plasmid was linearized using 10u Fermentas™ XbaI restriction enzyme and *in vitro* transcribed using T7 RNA polymerase (as described before).

N7myc-Drosha-Poly-(A) was injected in oocytes and incubated o/n. Next, half of the oocytes were matured to eggs in OR2-buffer containing 20µg/ml progesterone and incubated o/n.

Immunoprecipitation (IP) of recombinant Drosha

Oocytes and eggs (10 cells) injected with N7myc-Drosha-Poly(A) RNA were grinded and 100µl NET-2 buffer was added. The mixture was spun down for 5min at 14000rpm and the supernatant was transferred in a fresh tube. An aliquot of one cell (10µl) was transferred in a fresh tube for Western Blotting.

α-myc antibody 9E10 was coupled to Sepharose A beads (4mg beads in 5ml 9E10) o/n at 4°C.

Coupled antibody was washed 4x in NET-2 buffer and filled up to 500µl with NET-2 buffer. Cell extracts were added and rotated for 1h at 4°C. The IP was washed four times in NET-2 buffer and filled up to 500µl NET-2 buffer. An aliquot of one cell (50µl) was transferred in a fresh tube for Western blotting.

The rest of the IP was washed in 500µl 6.4mM MgCl₂ and used for Drosha *in vitro* processing assay.

Drosha *in vitro* processing assay

This experiment was performed using immunoprecipitated N7myc-Drosha according to a protocol by the Narry Kim lab:

http://www.narrykim.org/in_vitro_Drosha_processing.pdf

Cloning and sequencing of pre-microRNAs

Radiolabeled pri-microRNAs were microinjected in eggs and total RNA was extracted and separated on a 10% - 29:1 AA:bisAA Gel as described earlier. Putative pre-microRNA bands were cut out, eluted and precipitated as described above.

Eluted putative pre-microRNAs were labeled with 20µCi [γ-32P] by Fermentas™ PNK ATP according to manufacturer's protocol.

1st Ligation (3'Adapter): Precipitated putative pre-miRNA were resuspended in 16µl dH₂O and 2µl of 200µM RNA-3'Adapter 5'-UCGUAUGCCGUCUUCUGCUUGU-3' (MJ2440), 4µl 10x NEB™ T4 RNA Ligase Buffer, 1µl (40u) Fermentas™ Ribolock, 16µl 50% PEG6000 and 1µl (20u) NEB™ T4 RNA Ligase were added.

The reaction was incubated for 2h at 37°C. Another 1µl of NEB™ T4 RNA Ligase was added and the reaction was incubated for another 2h. The reaction was then precipitated and separated a 10% - 29:1 AA:bisAA Gel (8M UREA in 1xTBE).

Putative pre-microRNA+3'Adapter bands were cut out, eluted and precipitated.

2nd Ligation (5'Adapter): Precipitated putative pre-microRNAs were resuspended in 16µl dH₂O and 2µl of 200µM RNA-5'Adapter 5'-GUUCAGAGUUCUACAGUCCGACGAUC-3' (MJ2439), 4µl 10x NEB™ T4 RNA Ligase Buffer, 1µl (40u) Fermentas™ Ribolock, 16µl 50% PEG6000 and 1µl NEB™ T4 RNA Ligase were added.

The reaction was incubated for 2h at 37°C. Another 1µl (20u) NEB™ T4 RNA Ligase was added and the reaction was incubated for another 2h. The reaction was then precipitated and separated on a 10% - 29:1 AA:bisAA Gel (8M UREA in 1xTBE). Putative pre-microRNA+3'Adapter+5'Adapter bands were cut out, eluted and precipitated.

Reverse transcription (RT): The precipitated putative pre-microRNAs+3'Adapters+5'Adapters were resuspended in 11µl dH₂O and 100ng of RT-primer 5'-CAAGCAGAAGACGGCATACGA-3' (MJ2438) was added, heated up to 70°C for 5min and immediately put on ice. 4µl Fermentas™ 5x RT-buffer, 2µl dNTPs (10mM each), 1µl (40u) Fermentas™ Ribolock and 1µl (200u) Fermentas RevertAid™ H Minus Reverse Transcriptase were added. The reaction was incubated for 5min at room temperature, 5min at 37°C, 60min on 42°C, stopped for 10min at 70°C and precipitated. The synthesized cDNA was resuspended in 20µl dH₂O.

RT-PCR: 5µl (25%) of the resuspended cDNA were transferred in a fresh eppendorf tube and 1µl (100ng) of forward primer 5'-GTTCTACAGTCCGACGATCGCT-3' (MJ2436) and 1µl (100ng) of reverse primer 5'-AAGCAGAAGACGGCATACGAAAC-3' (MJ2437), 5µl 10x Fermentas™ PCR buffer, 4µl MgCl₂ (25mM), 1µl dNTPs (10mM each), 1µl Fermentas Taq-Polymerase™ and 32µl dH₂O were added.

Control reactions without cDNA and without Fermentas™ Revertaid respectively were also performed.

PCR-cycles: 5min 95°C, 25x (30sec 55°C, 30sec 72°C, 30sec 95°C), 5min 72°C

RT-PCR-products were separated on 1.4% TAE Agarose Gel, cut out under UV-light and eluted in 30µl elution buffer using PeqLab™ elution kit.

5µl of eluted RT-PCR products were ligated in 0.5µl (25ng) Promega™ pGEM-T easy vector by adding 2µl 10x Fermentas™ Ligation buffer, 1µl (5u) Fermentas™ T4 DNA Ligase, 11.5µl dH₂O and incubating the reaction at 16°C o/n.

The successful ligation was transformed into XL1-blue competent E. coli cells, plated out on 2xTy+Amp plates (containing 5mM IPTG and 1mg X-Gal) and incubated at 37°C o/n. White colonies were selected, grown in 3ml 2xTy+Amp medium o/n and plasmid DNA was extracted by Mini-Prep.

Plasmid DNA was sent to the Institute of Botany, University of Vienna for sequence analysis. Sequencing primer: 5'-CGCCAGGGTTTTCCCAGTCACGAC-3' (MJ004)

Specific aims of the thesis

1) Regulation of miRNA biogenesis during *Xenopus* oogenesis

Gene regulation by miRNAs plays a pivotal role in animal development and differentiation (reviewed in Bushati and Cohen, 2007). In early *Xenopus* development miRNA expression is regulated in a stage and tissue specific manner (Tang and Maxwell, 2008, Watanabe et al., 2005).

The objective of this study was to reveal the regulation of pri- and pre-miRNAs processing in different stages of *Xenopus* oogenesis and upon editing.

By using oocytes as a model, we were thus able to follow the path of a particular miRNA through various processing steps by injecting the precursor miRNAs.

We therefore cloned miRNA precursor constructs of the ubiquitous miRNA-21 containing an upstream T7 promoter and *in vitro* transcribed them using T7 RNA Polymerase. The precursor miRNA molecules were investigated by establishing a RNA microinjection system in *Xenopus* stage VI oocytes and eggs. Nuclear injections of miRNA precursors were performed in combination with U3 snoRNA/U6 snRNA as positive nuclear retention controls. RNA samples were generated by radiolabeled T7 Polymerase *in vitro* transcription using [α -³²P]ATP. After injection and incubation RNA was extracted and analyzed PAA-Gels.

2) Impact of RNA editing on pri-miRNA processing *in vivo*

A-to-I RNA editing is mediated by ADAR enzymes, which were originally discovered in *Xenopus laevis* eggs and embryos as an unwinding activity and soon after as a dsRNA adenosine deaminase (Bass and Weintraub, 1987, Rebagliati and Melton, 1987, Bass and Weintraub, 1988, Wagner et al., 1989). Tudor-SN, a specific ribonuclease targeting hyper-edited dsRNA, was shown to be active in *Xenopus laevis* oocyte extracts (Scadden and Smith, 2001b, Scadden, 2005).

Studies on edited miRNAs have shown that RNA editing can strongly affect the processing and stability of pri and pre-miRNAs (reviewed in Ohman, 2007).

A-to-I RNA editing by ADARs can block pri-miRNA-142 processing in cell extracts and transfected ADAR suppresses mature miRNA-142 expression. Further, expression of mature miRNA-142 was increased in ADAR *null* mice. Cleavage of

edited pri-miRNA-142 by Tudor-SN *in vitro* might explain that no cellular accumulation of edited pri-miRNA-142 could be detected (Yang et al., 2006).

Our goal was to study the impact of RNA editing on miRNA processing and stability by directly following the path of pri-miRNA-142 maturation *in vivo*. Therefore, several editing variants of pri-miRNA-142 containing an upstream T7 promoter sequence were cloned and *in vitro* transcribed using T7 RNA Polymerase in the presence of [α -32P]ATP. To evaluate processing levels and stability of the pri-miRNA-142 editing variants *in vivo* we performed RNA microinjection experiments in *Xenopus* eggs and analyzed them on PAA-Gels.

Results

Processing and stability of microinjected miRNAs in *Xenopus laevis* oocytes and eggs

Pre-miRNAs are exported and processed to mature miRNAs in oocytes

Previous work in *Xenopus* oocytes revealed that nuclear injected pre-miRNAs are exported to the cytoplasm (Lund et al., 2004). Moreover cytoplasmatically injected pre-miRNAs are processed to mature microRNAs by Dicer (Lund and Dahlberg, 2006).

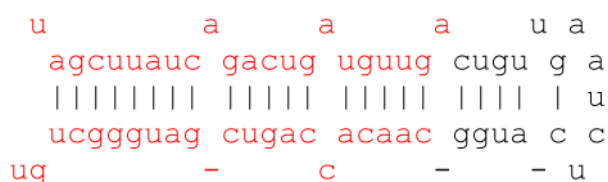


Figure 8. Hairpin structure of pre-miRNA-21

The sequence of pre-miRNA-21 is from miRBASE <http://www.mirbase.org>

Analysis of nuclear microinjection experiments of ubiquitous pre-miRNA-21 (Figure 8) in oocytes confirmed an efficient export to the cytoplasm and subsequent processing to mature miRNA (see Figure 9). This is in agreement with other work published earlier (Lund et al., 2004).

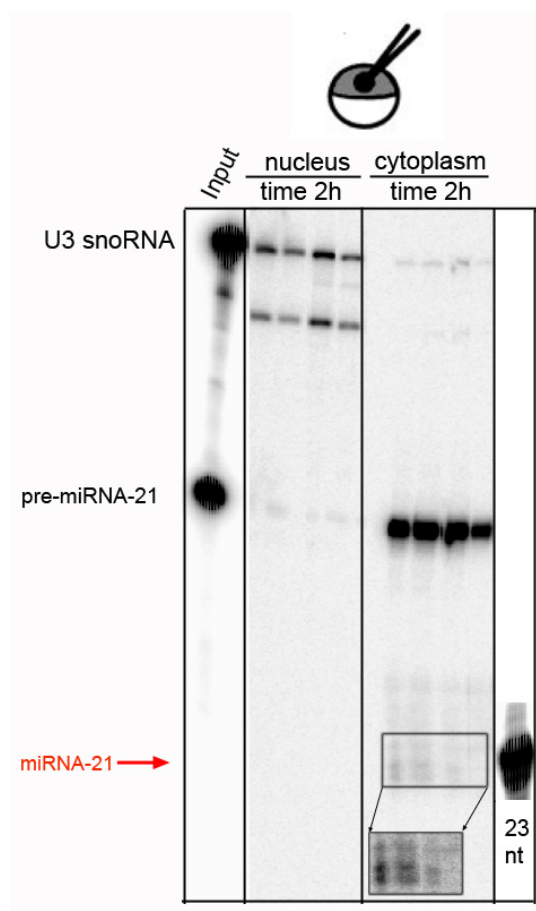


Figure 9. Nuclear injection of pre-miRNAs are exported to the cytoplasm and processed to mature microRNAs

Stage VI oocytes were coinjected with pre-miRNA-21 and U3 snoRNA and incubated for 2 hours. RNA extraction of nuclei (left) and cytoplasm's (right) reveals pre-miRNA export and processing to the cytoplasm while U3 snoRNA remains stable.

Processing of pre-miRNAs in the cytoplasm is enhanced in eggs

In 2006, Lund & Dahlberg documented enhanced processing of pre-miRNAs in eggs compared to oocytes. In order to recapitulate this experiment we used our established microinjection system. We were able to confirm an increase in Dicer processing upon maturation in a comparative assay (shown in Figure 10).

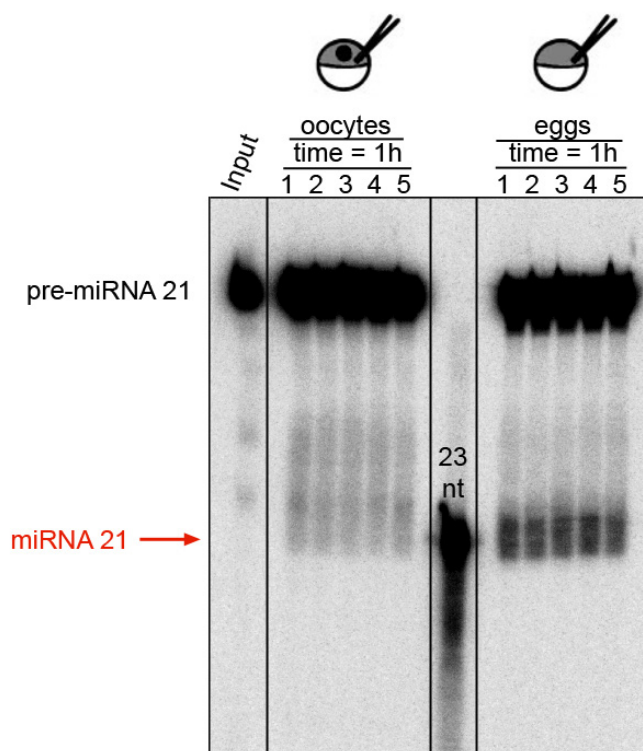


Figure 10. Cytoplasmic injections of pre-miRNAs are processed to mature miRNA

Stage VI oocytes (left) and eggs (right) were injected with pre-miRNA-21 and incubated for 1h. Gel electrophoresis of extracted RNA shows enhanced processing to mature miRNA.

In summary, nuclear injections of pre-miRNAs in oocytes showed export to the cytoplasm and subsequent cleavage by Dicer. When directly injected in the cytoplasm of oocytes and eggs a boost of Dicer activity was observed upon maturation. These results are consistent with work published earlier (Lund et al., 2004, Lund and Dahlberg, 2006).

Degradation of pri-miRNAs in oocyte germinal vesicle

To study the efficacy of Drosha mediated cleavage and to study possible differences arising by RNA editing, we examined the fate of pri-miRNAs in the oocyte germinal vesicle. In somatic cells, pri-miRNAs are cleaved by Drosha in the nucleus, exported to the cytoplasm and processed by Dicer.

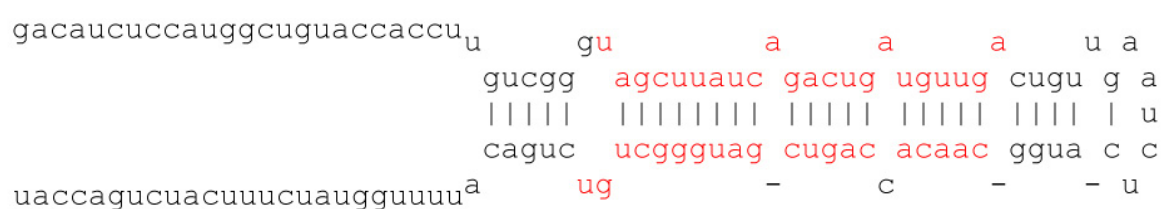


Figure 11. Hairpin structure and flanking region of pri-miRNA-21

The sequence of pri-miRNA-21 is from miRBASE <http://www.mirbase.org>

To test for Drosha activity we performed nuclear microinjection experiments using ubiquitous pri-miRNA-21 (Figure 11) in the oocyte germinal vesicle. Interestingly, no Drosha cleavage but degradation by an unknown factor was observed in the *Xenopus* germinal vesicle (see Figure 12).

In addition, misinjections of pri-miRNAs into the cytoplasm (not shown) appeared to leave the pri-miRNAs stable in this cellular compartment.

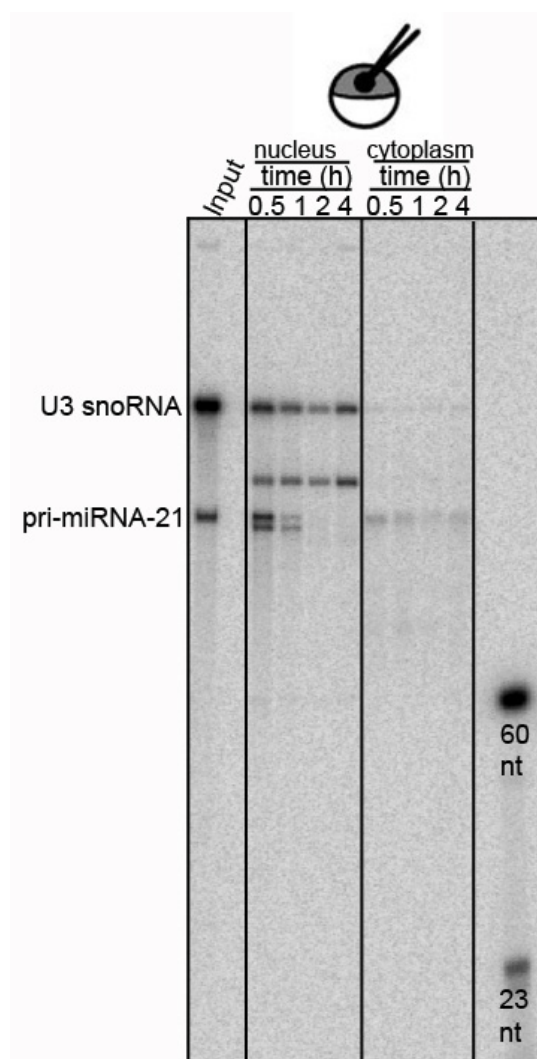


Figure 12. Nuclear injection of pri-miRNAs leads to degradation

Pri-miRNA-21 and nuclear retained U3 snoRNA were coinjected in stage VI oocytes and incubated for up to 4 hours. RNA extraction of nuclei (left) and cytoplasm (right) indicates degradation while U3 snoRNA remains in the nucleus.

Processing of pri-miRNAs is activated in eggs

With the finding, that pri-miRNAs remain stable in the oocyte cytoplasm and are degraded in the germinal vesicle (Figure 10) we turned our focus on studying the efficiency of Drosha mediated cleavage in eggs. Since it was shown that Dicer activity is stimulated by oocyte maturation we wondered whether the same would be true for Drosha activity. Therefore, to test this possibility we matured stage VI oocytes to eggs by the addition of progesterone. Maturation to eggs leads to germinal vesicle breakdown (GVBD) and therefore only cytoplasmic injections could be performed.

As depicted in Figure 13, microinjection experiments of ubiquitous pri-miRNA-21 (Figure 11) clearly showed activation of Drosha mediated processing to pre-miRNA-21 in eggs.

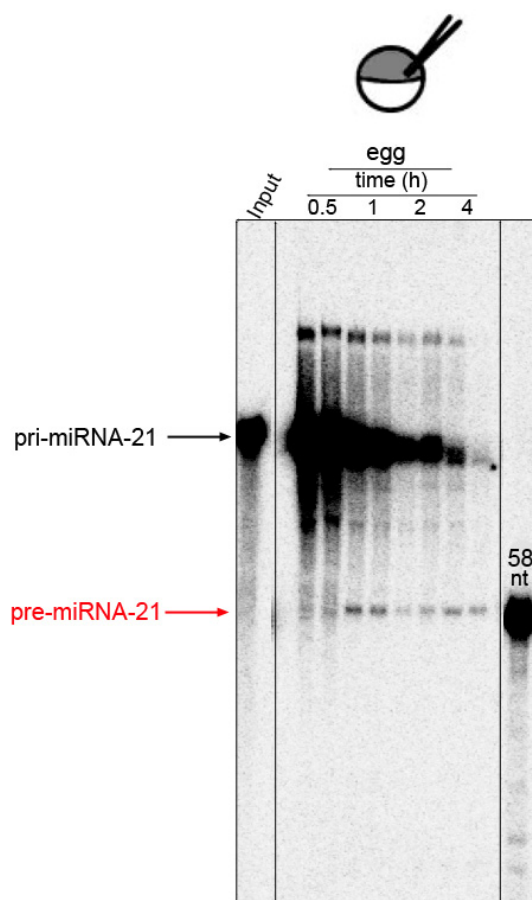


Figure 13. Injection of pri-miRNAs in eggs leads to processing to pre-miRNAs

Upon maturation to eggs pri-miRNA-21 was microinjected and incubated for up to 4 hours. RNA was extracted and analyzed on a PAA-Gel showing an activation of pri-miRNA-21 processing to pre-miRNA-21 in eggs.

Comparison of pri-miRNA processing in oocyte and egg cytoplasm

To comparatively investigate the different processing pattern of pri-miRNAs in oocytes and eggs, injection experiments were carried out simultaneously in both cell types.

Therefore, both pri-miRNA-21 and the stem cell specific pri-miRNA-29b-1 were injected in the cytoplasm of stage VI oocytes and eggs and incubated for up to 4 hours.

As shown in Figure 14, this comparative assay confirms that pri-miRNAs are stable in the oocyte cytoplasm but get processed in eggs.

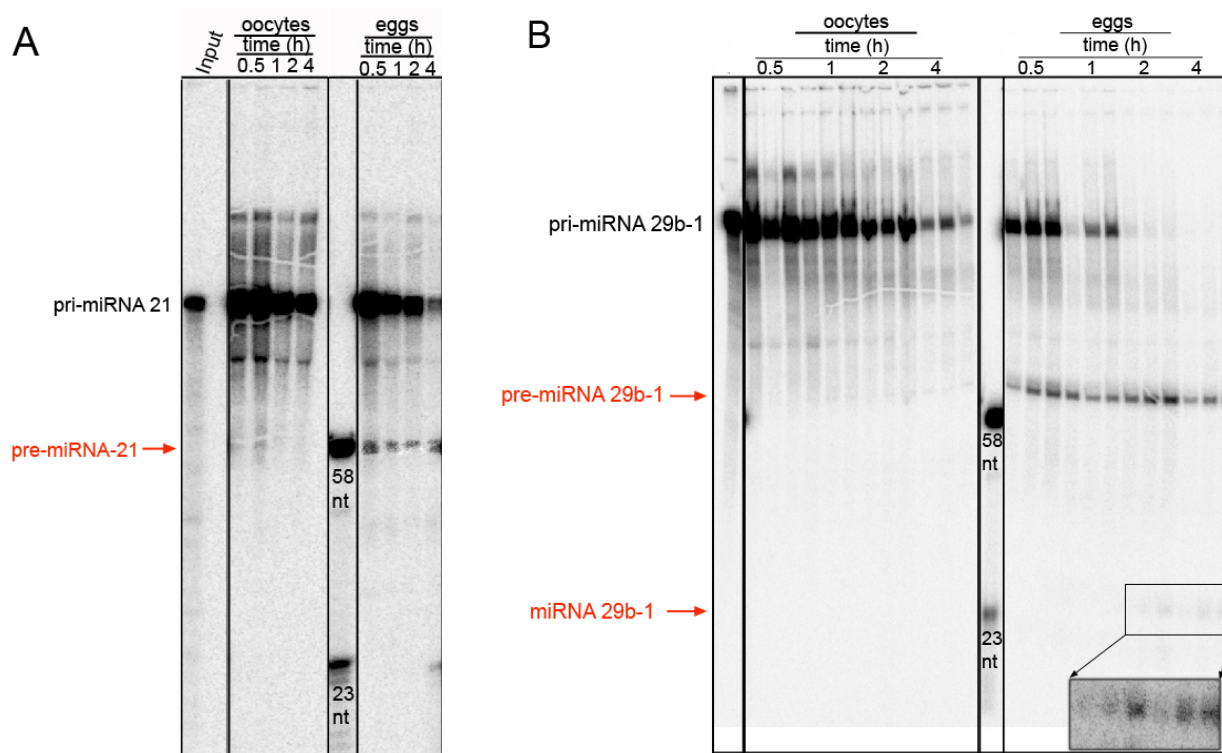


Figure 14. Comparison of microinjected pri-miRNAs in eggs and oocytes

Microinjected pri-miRNA-21 (A) and pri-miRNA-29b-1 (B) in oocytes (left) and eggs (right) were incubated for up to 4 hours. RNA extraction and analysis of this comparative assay clearly demonstrates the activation of pri-miRNA processing in eggs.

These results confirm the nuclear export and subsequent Dicer processing of pre-miRNAs in *Xenopus* oocytes published earlier (Lund et al., 2004, Lund and Dahlberg, 2006). Additionally, activation of pri-miRNA processing was detected in *Xenopus* eggs, whereas pri-miRNAs are degraded in the nucleus and stable in the cytoplasmic compartment of *Xenopus* oocytes.

Confirmation of successful processing by miRNA cloning and sequencing

To confirm successful Drosha processing of pri-miRNAs to pre-miRNAs in *Xenopus* eggs, the obtained putative pre-miRNA band was cut out and eluted. After ligation of a 3'- and 5'-Adapter to the putative pre-miRNA molecule RT-PCR was performed. The resulting cDNA was cloned into a pGEM-T easy™ Vector and sequenced.

To avoid contamination with endogenous pre-miRNA molecules human pri-miRNA-29b-1 was used for microinjection. The resulting pre-miRNA has no homologue in *Xenopus*. Sequence analysis, shown in Figure 15, confirmed appropriate processing of microinjected pri-miRNA-29b-1 to pre-miRNA-29b-1 in *Xenopus* eggs.

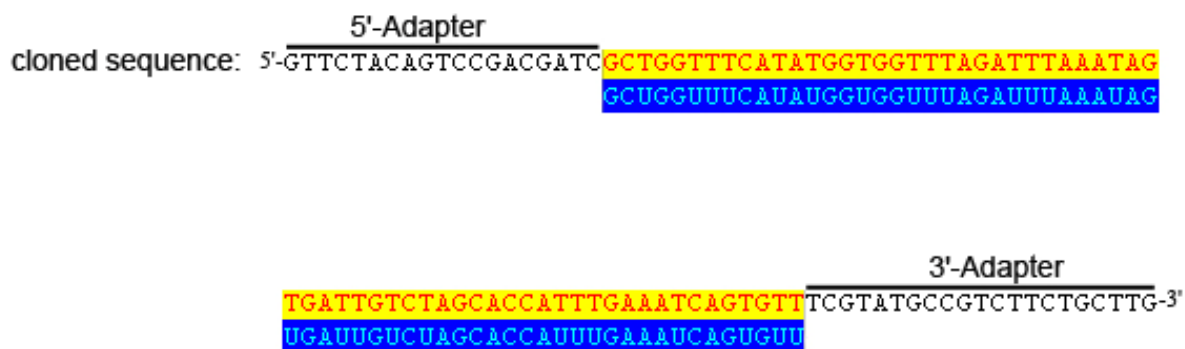


Figure 15. Confirmation of appropriate Drosha cleavage

Sequence analysis was performed using the RT-PCR primer MJ2437 and subsequent PCR primer pair MJ2436 and MJ2437. Sequence alignment of cloned putative pre-miRNA-29b-1 (yellow) with miRBASE annotated pre-miRNA-29b-1 (blue) revealed proper Drosha processing of microinjected pri-miRNA-29b-1 in *Xenopus* eggs.

Endogenous *Xenopus* microRNA processing is enhanced in eggs

Since oocyte maturation to eggs boosts the processing of injected pre-miRNAs and activated processing of pri-miRNAs, we were raising the question if endogenous miRNA levels are also affected by the process of maturation. Thus, Northern Blot experiments to compare endogenous levels of miRNAs known to be expressed in oocytes were carried out. We selected two miRNAs that were shown to be expressed in *Xenopus* germline cells: xtr-miRNA-101 and xtr-miRNA-148a (Armisen et al., 2009)

Probes of xtr-miRNA-101 and xtr-miRNA-148a on Northern blots reveal that endogenous miRNA processing is enhanced in eggs (see Figure 16 and Figure 17)

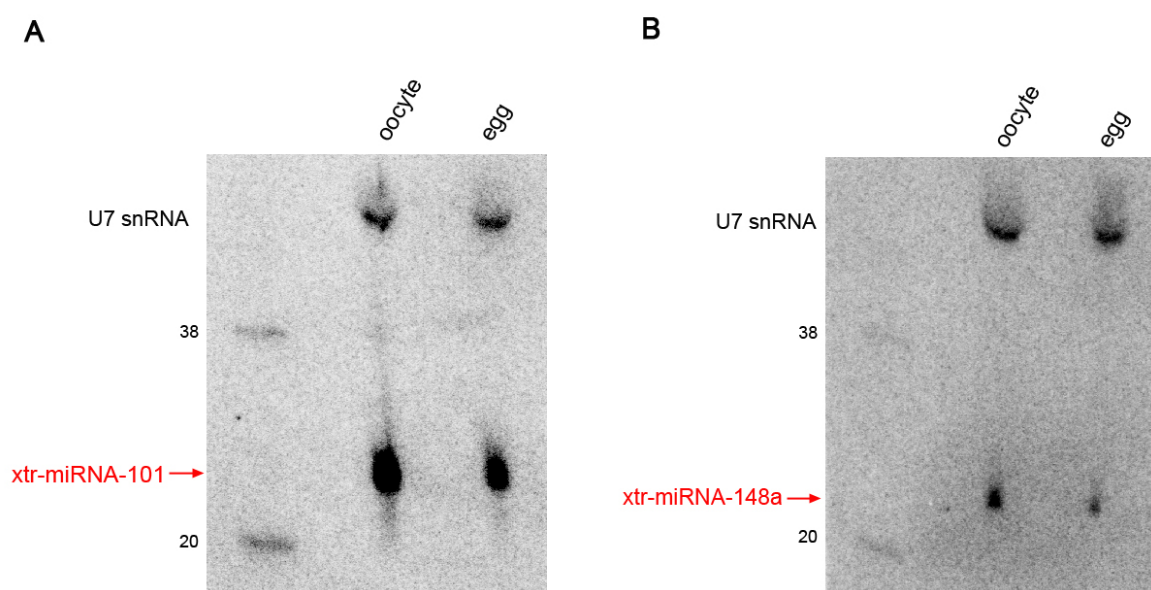


Figure 16. Northern Blot of endogenous miRNA-101 and miRNA-148a

An equal amount of oocyte and egg total RNA (35 cells, equal RNA amount confirmed by RiboGreen™ Fluorometric quantification) was separated on a PAA-Gel, semi-dry blotted and probed with labeled antisense DNA oligo probes.

(A) Expression of xtr-miRNA-101 and

(B) Expression of xtr-miRNA-148a in oocytes and eggs.

Relative pri- to pre-miRNA processing rates of the endogenous xtr-miRNA-101 and xtr-miRNA-148a based on absolute intensities and relative to U7snRNA expression were calculated. As demonstrated in Figure 17, these quantifications show that endogenous miRNA expression levels are approximately doubled upon maturation.

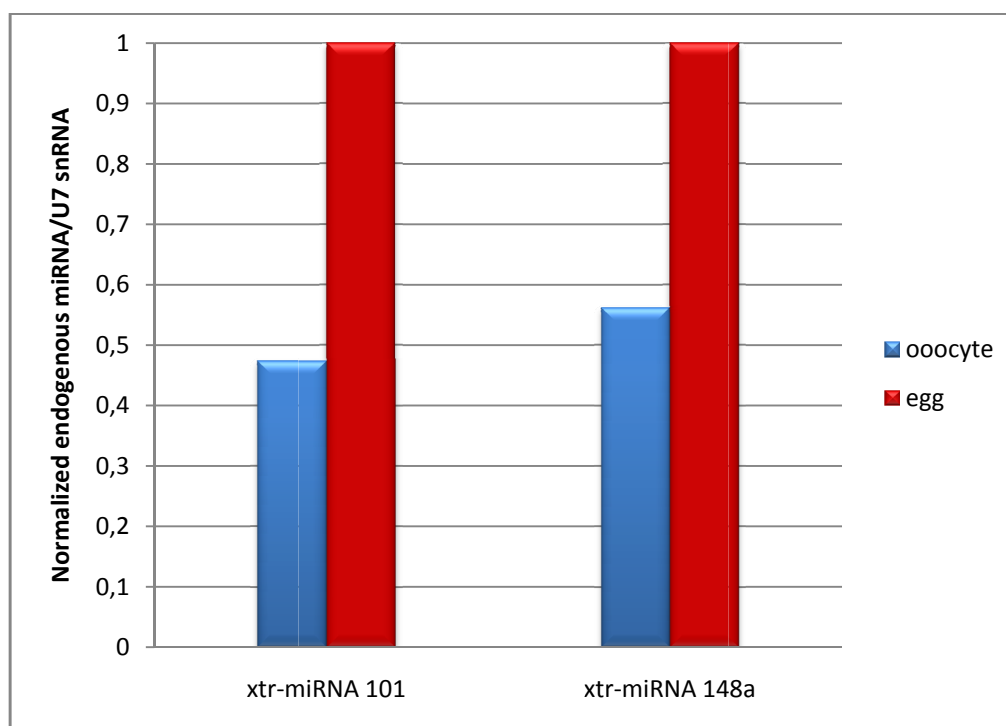


Figure 17. Quantification of relative endogenous miRNA expression in oocytes and eggs

Normalized expression of xtr-miRNA-101 (left) and xtr-miRNA-148a (right) relative to U7 snRNA was calculated using Bio-Rad™ QuantityOne software. Endogenous miRNA expression is strongly enhanced in eggs.

To sum up, quantification of endogenous miRNA levels showed a striking increase in mature miRNA levels upon maturation to eggs. This result fits with the previous miRNA precursor microinjection observations, that pri-miRNA processing is activated and pre-miRNA processing is enhanced in eggs.

Dissecting the processing pattern of *Xenopus* microRNA-148a

Processing pattern of xtr-pri-miRNA-148a in eggs

To answer the question to which extent enhanced processing of the endogenous xtr-miRNA 148a in eggs is regulated by Drosha and Dicer, we cloned and *in vitro* transcribed [α - ^{32} P]ATP body labeled *Xenopus* xtr-pre- and xtr-pri-miRNA-148a. This approach allows us to track all steps of miRNA-148a processing during maturation.

Comparable microinjections of xtr-pri-miRNA-148a in oocytes and eggs clearly demonstrate the activation of Drosha processing in eggs (Figure 18). Further, the resulting xtr-pre-miRNA-148a is cleaved by Dicer.

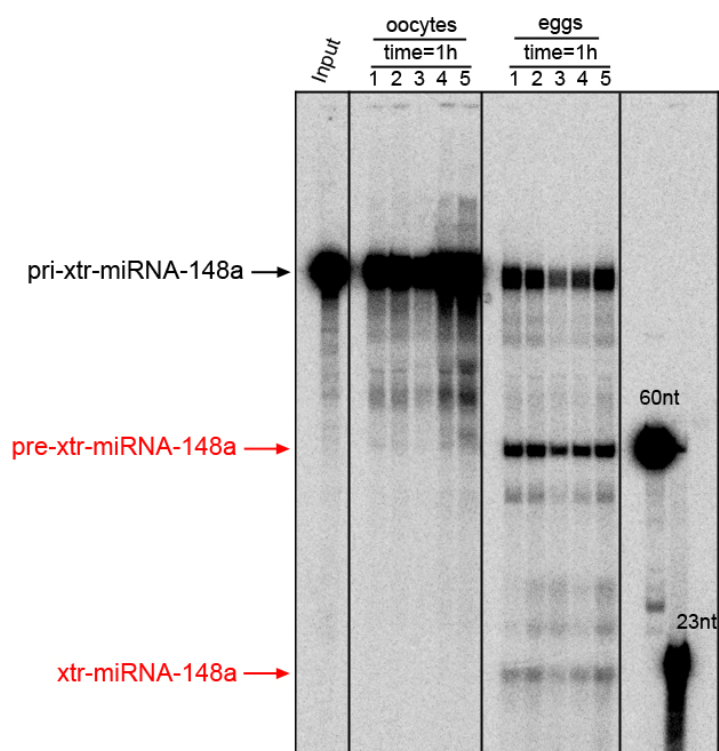


Figure 18. Processing of pri-xtr-miRNA-148a is activated in eggs

Comparable microinjections of pri-xtr-miRNA-148a in oocytes and eggs were performed. After incubation for 1h efficient Drosha and Dicer cleavage in eggs (right) could be detected. In oocytes (left) pri-miRNA-148a remains stable in the cytoplasm.

As depicted in Figure 19, relative pri- to pre-miRNA-148a processing rates based on absolute intensities were calculated and clearly demonstrate a complete activation of pri-miRNA-148a processing in eggs.

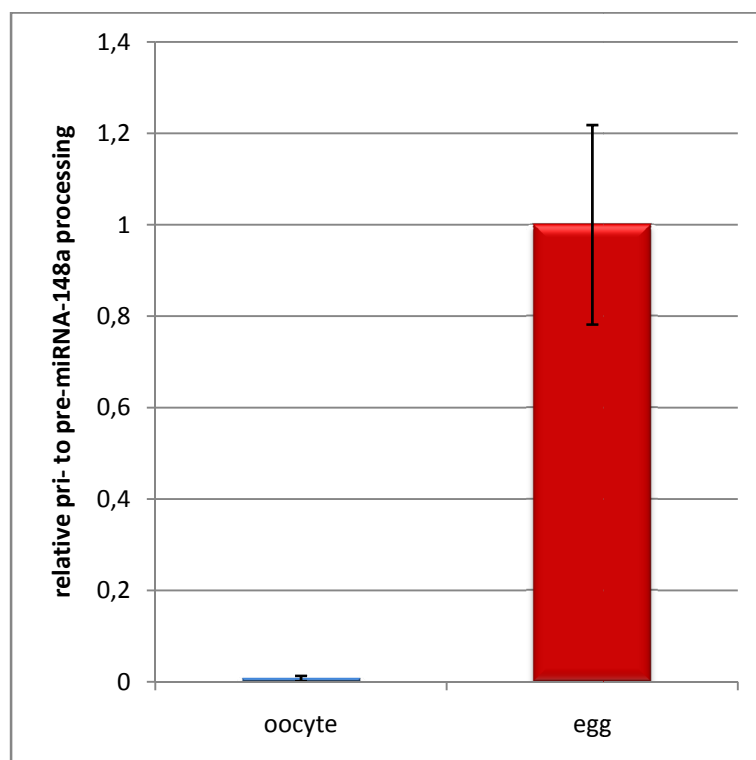


Figure 19. Quantification of pri- to pre-miRNA-148a processing in oocytes and eggs

Processing of microinjected pri-xtr-miRNA-148a to pre-xtr-miRNA-148a based on absolute intensities was calculated using Bio-Rad™ QuantityOne software. Drosha processing is activated upon maturation to eggs.

Processing pattern of xtr-pri-miRNA-148a in oocytes

We further analyzed the processing pattern of xtr-miRNA-148a precursors in oocytes performing nuclear injections. Degradation by an unknown factor is detected for pri-miRNA-148a (as detected for pri-miRNA-21). No processing or export to the cytoplasm was detectable while the nuclear retention and stability control U6 snRNA remains stable in the nucleus (Figure 20).

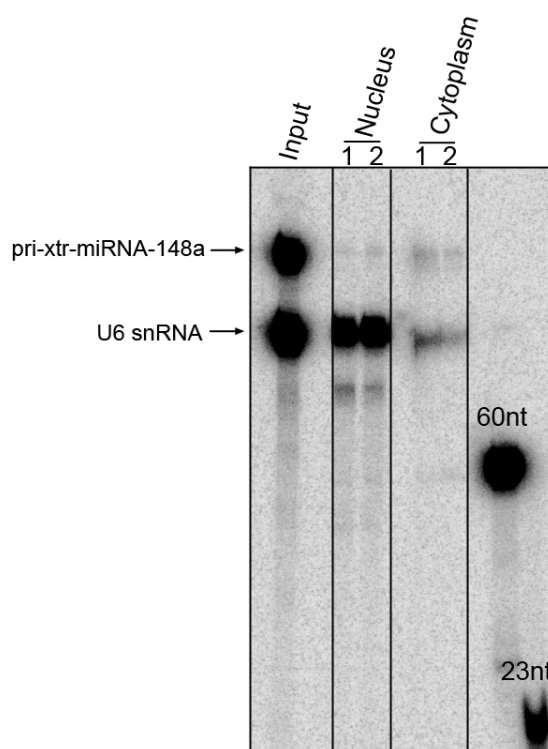


Figure 20. Nuclear injected pri-xtr-miRNA-148a is degraded in the nucleus

Oocyte nuclear (left) microinjection of pri-xtr-miRNA-148a together with U6 snRNA indicates degradation of the pri-miRNA by an unknown factor, whereas U6 snRNA remains stable in the nucleus.

Processing pattern of xtr-pre-miRNA-148a in oocytes and eggs

After having shown that pri-miRNAs are unstable in nuclei of *Xenopus* oocytes, we wanted to analyze the fate of nuclear injected pre-miRNAs. To turn our focus on differences in Dicer mediated processing we performed microinjection experiments using xtr-pre-miRNA-148a.

Nuclear microinjection showed export and subsequent processing by Dicer in the cytoplasm (see Figure 21).

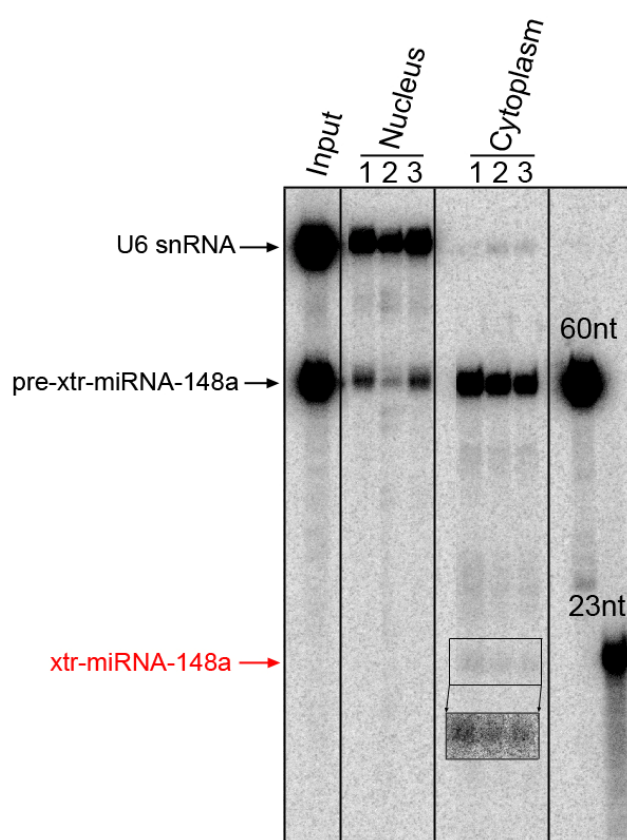


Figure 21. Nuclear injection of pre-miRNA-148a shows export and processing

Nuclear microinjected pre-xtr-miRNA-148a and the nuclear retention control U6 snRNA were incubated for 1h and extracted using Trizol reagent. Analysis on a PAA-Gel revealed successful export to the cytoplasm and processing by Dicer.

Previous studies had shown a marked increase of Dicer activity upon oocyte maturation (Lund and Dahlberg, 2006). We were therefore investigating comparative Dicer processing activity in oocytes and eggs by injecting pre-miRNA 148a in both cell types (Figure 22).

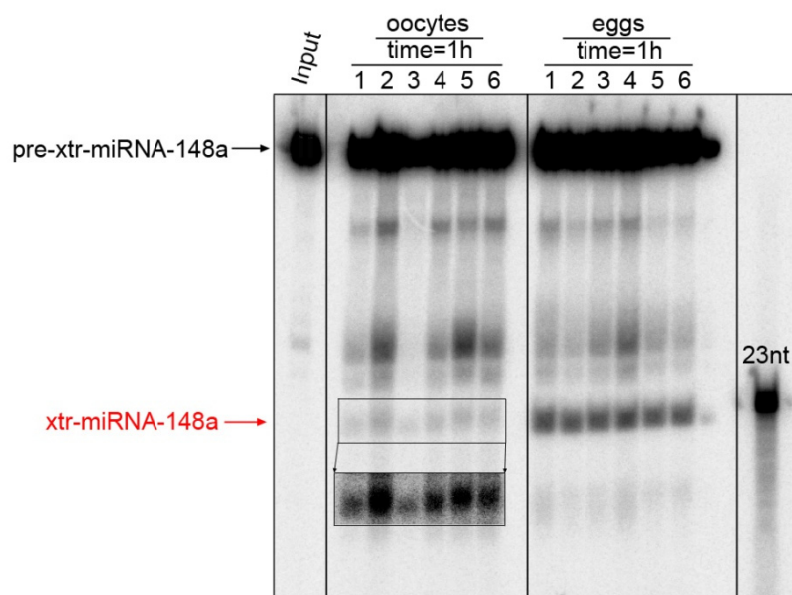


Figure 22. Processing of pre-xtr-miRNA-148a is enhanced in eggs

Comparative cytoplasmic microinjections of pre-xtr-miRNA-148a in oocytes and eggs were incubated for 1h, extracted and analyzed on a PAA-Gel. Processing of pre-xtr-miRNA-148a by Dicer is enhanced in eggs.

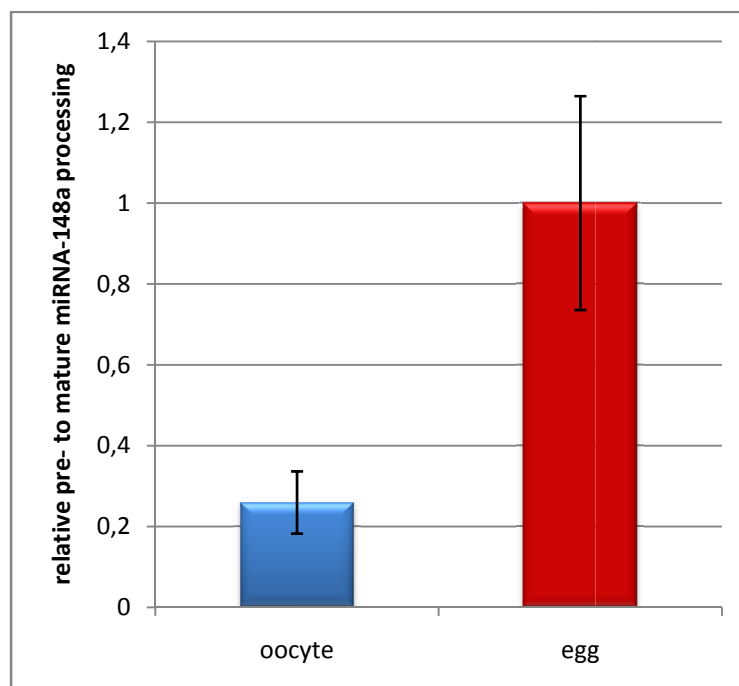


Figure 23. Quantification of pre- to mature miRNA-148a processing in oocytes and eggs

Processing of microinjected pre-xtr-miRNA-148a to xtr-miRNA-148a was calculated using Bio-Rad™ QuantityOne software. A strong increase in Dicer activity is observed upon maturation to eggs.

Figure 23 shows a comparative analysis of relative pri- to pre-miRNA-148a processing based on absolute intensities in oocytes and eggs. An almost four-fold boost of Dicer activity in eggs can be detected upon maturation.

In summary, these experiments show that the increase in endogenous miRNA-148a levels in eggs depends on the activation of Drosha as the rate limiting step and additionally on an increased activity of Dicer.

Regulation of Drosha activity

Oocyte maturation leads to activation of pri-miRNA processing in eggs. This result raises the questions how Drosha processing is activated in this developmentally regulated manner. Therefore, we turned our focus on the regulation of the key enzyme Drosha.

Detection of processing in transcriptionally silent cells

To check for transcriptional regulation of Drosha activity, eggs were pre-treated with the transcriptional inhibitor Actinomycin D (AMD). Consequently, the processing pattern of microinjected pri-miRNA-29b-1 (stem cell specific) was examined in the AMD treated cells and untreated cells as a control.

As shown in Figure 24, comparable analysis of AMD treated (A) and untreated cells (B) do not show a block of Drosha processing in transcriptionally silent cells.

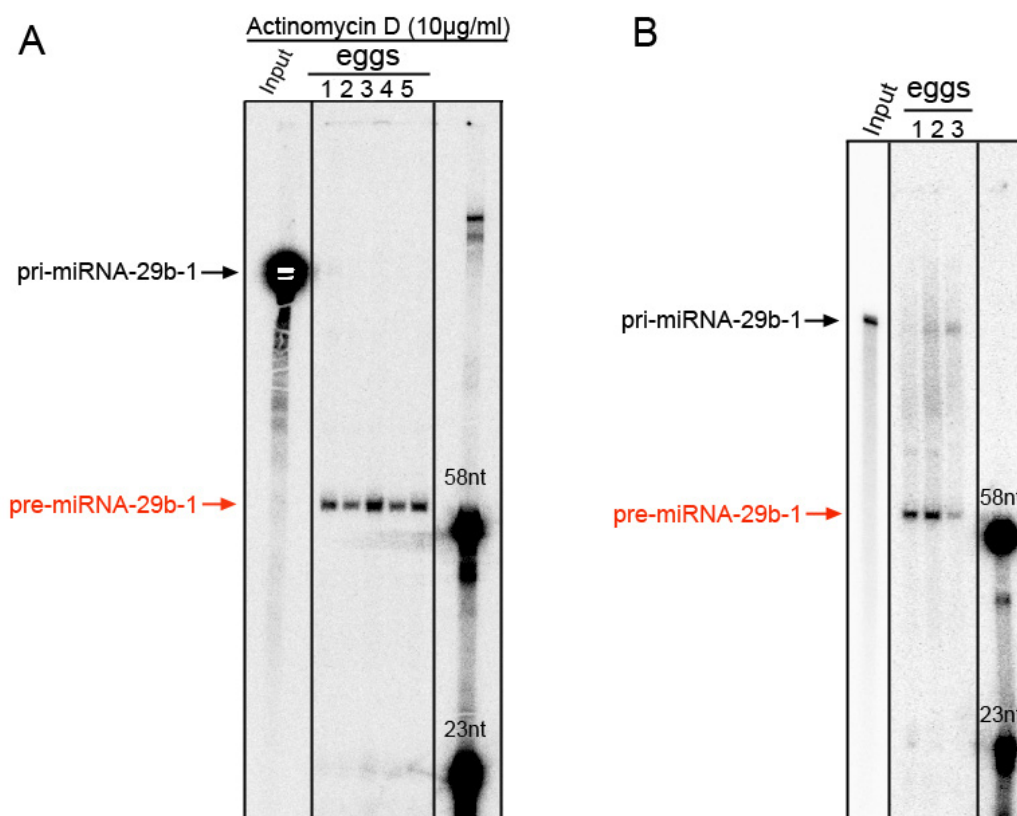


Figure 24. Processing of pri-miRNAs can still be detected in transcriptionally silent cells

Oocytes were pre-incubated with 10µg/ml Actinomycin D and then matured to eggs by adding 20µg/ml progesterone. Eggs were injected with pri-miRNA-29b-1, incubated for 2h and processing was analyzed on a PAA-Gel. Processing activity could be detected in the transcriptionally silent cells (A) comparable to AMD untreated control cells (B).

Additionally, tests using the translational inhibitor Cycloheximide (250µg/ml) were performed while initiating maturation. We observed a block of maturation confirming previous work that protein synthesis is strictly required for oocyte maturation (Wasserman and Masui, 1975, Schuetz and Samson, 1979).

Analysis of recombinant Drosha activity in *Xenopus* oocytes and eggs

In order to study the activity of Drosha during oocyte maturation at the protein level, an amino-terminally 7xmyc-tagged recombinant *Xenopus* Drosha enzyme (N7myc-Drosha) was cloned. N7myc-Drosha RNA contains a start-codon and the 3' untranslated region of the *Xenopus* NO38 cDNA harboring a poly(A)+ tail to stabilize injected RNAs (Peculis and Gall, 1992). Translation of stored maternal mRNAs is controlled by the length of the poly(A) tail. Elongation of poly(A) mediated by CPEB facilitates translation of dormant mRNAs during oocyte development (reviewed in Mendez and Richter, 2001).

To examine the regulation of Drosha activity in oocytes and eggs N7myc-Drosha was microinjected in oocytes and half of the cells were then matured to eggs. Cleavage was examined in immunoprecipitation (IP) and subsequent pri-miRNA *in vitro* processing assays using cell extracts of oocytes and eggs.

As displayed in Figure 25, cells expressing recombinant Drosha establish a distinct processing pattern while uninjected control cells do not show processing activity *in vitro*.

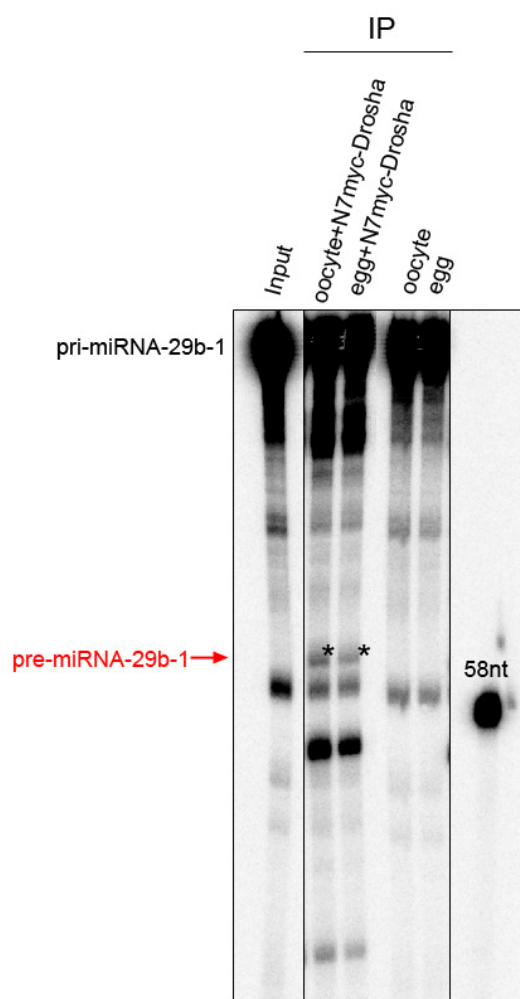


Figure 25. Drosha cleavage assay using immunoprecipitated myc-tagged Drosha is active in oocytes and eggs

N7myc-Drosha RNA was injected in oocytes and half of the cells were matured to eggs. Immunoprecipitation was performed using α -myc antibody. The pri-miRNA-29b-1 *in vitro* processing assay reveals activity of recombinant Drosha enzyme in oocytes and eggs. The asterisk indicates the band for pre-miRNA-29b-1.

To further check for differences in pri- to pre-miRNA-29b-1 *in vitro* processing levels, absolute intensities were quantified and relative processing rates were calculated. No differences in Drosha activity could be detected in oocytes and eggs expressing recombinant Drosha compared to eggs not expressing recombinant Drosha (see Figure 26).

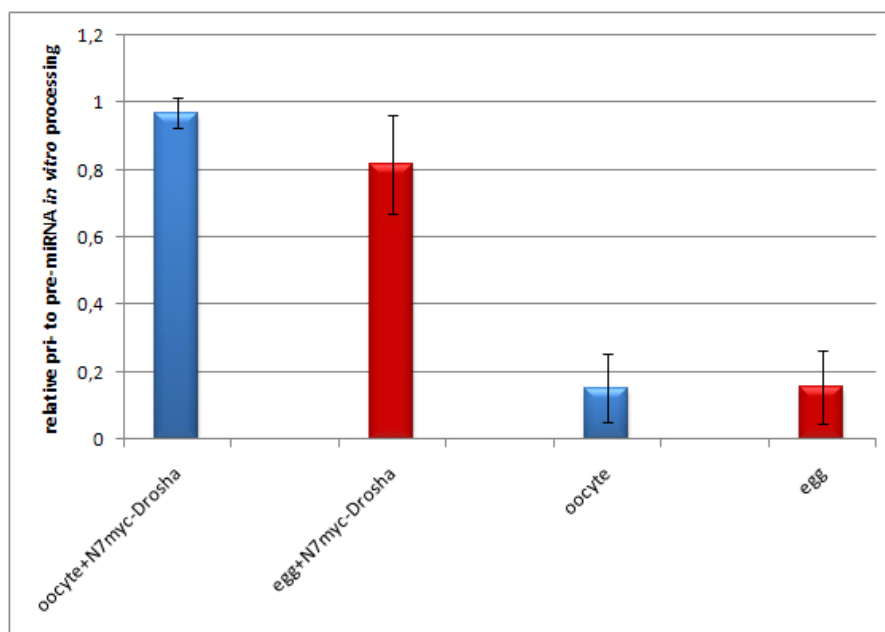


Figure 26. Quantification of pri- to pre-miRNA-29b-1 processing in oocytes and expressing recombinant Drosha

Immunoprecipitated recombinant Drosha *in vitro* pri- to pre-miRNA-29b-1 processing rates were calculated based on absolute intensities using Bio-Rad™ QuantityOne software. Oocytes (column 1) and eggs (column 2) expressing recombinant Drosha do not differ in processing levels. Control oocytes (column 3) and eggs (column 4) expressing no recombinant Drosha do not exhibit a distinct processing pattern.

Immunoprecipitation and subsequent *in vitro* processing assays of recombinant Drosha expression reveal comparable levels of pri- to pre-miRNA-29b-1 processing and no block or inhibition of Drosha activity in oocytes.

To test for possible inhibitory cofactors that regulate Drosha activity *in vivo*, recombinant Drosha enzyme was expressed in oocytes and half of the cells were then matured to eggs. Microinjected pri- to pre-miRNA-29b-1 processing pattern was analyzed on a PAA-Gel in Figure 27. It is obvious that recombinant Drosha is active in oocytes *in vivo* (Figure 27B). This in turn suggests that no inhibitor of Drosha activity is present in oocytes.

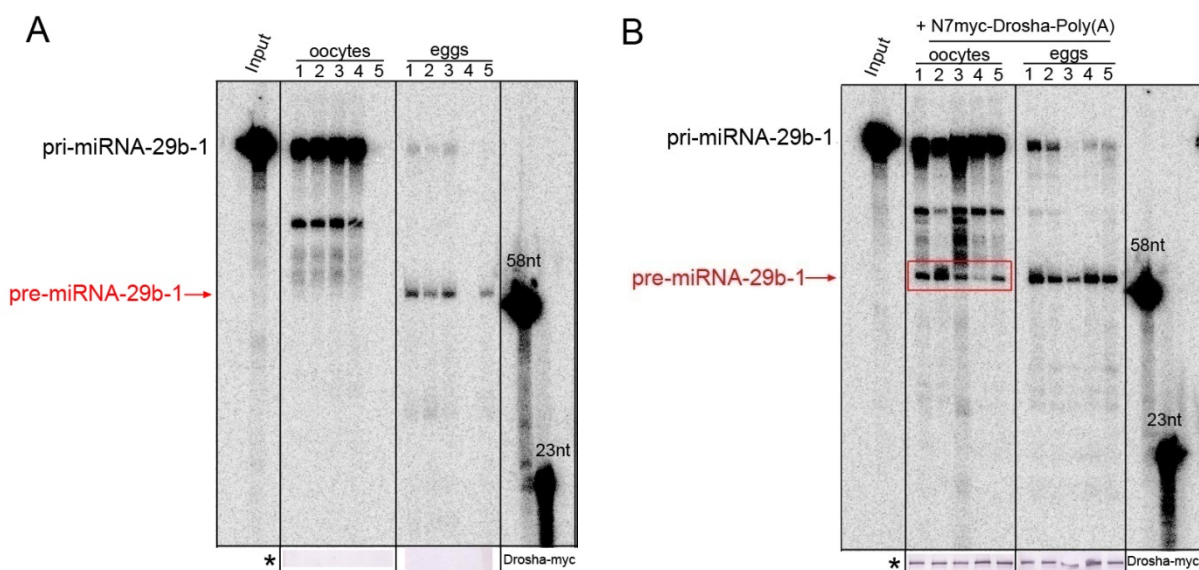


Figure 27. Expression of recombinant myc-tagged Drosha in oocytes and eggs reveals processing activity in oocytes

Oocytes were either uninjected (A) or microinjected with N7myc-Drosha RNA (B) and then partly matured to eggs. Consequently, oocytes and eggs were microinjected with pri-miRNA-29b-1, incubated for 1h and analyzed on a 10%-29:1 AA:bisAA Gel. Recombinant Drosha protein is active in *Xenopus* oocytes. The asterisks mark the expression of recombinant Drosha examined by Western blotting.

Taken together, these experiments confirm recombinant Drosha *in vitro* and *in vivo* activity in stage VI oocyte extracts and stage VI oocytes. We were not able to detect a general block or inhibition of enzymatic Drosha activity at this stage of development. Thus, expression of recombinant *Xenopus* Drosha in stage VI oocytes is sufficient to regain pri- to pre-miRNA processing activity.

Impact of RNA editing on pri-miRNA processing *in vivo*

ADAR enzymes, the mediators of A-to-I RNA editing, were originally discovered in *Xenopus laevis* eggs and embryos as an unwinding activity and soon after as a dsRNA adenosine deaminase (Bass and Weintraub, 1987, Rebagliati and Melton, 1987, Bass and Weintraub, 1988, Wagner et al., 1989). Further, the ribonuclease Tudor-SN, specifically targeting hyper-edited dsRNA, was shown to be active in *Xenopus laevis* oocyte extracts (Scadden and Smith, 2001b, Scadden, 2005).

RNA editing of pri- and pre-miRNAs can significantly alter miRNA processing patterns or silencing targets. As previously shown, RNA editing at position +4 and +5 is both, necessary and sufficient, to block pri-miRNA-142 processing *in vitro* (Yang et al., 2006, Kawahara et al., 2007a, Kawahara et al., 2007b).

Activation of pri-miRNA processing in *Xenopus* eggs provides an excellent tool to investigate the effects of RNA editing on Drosha processing *in vivo*. Several editing variants of pri-miRNA-142 containing an upstream T7 promoter sequence were cloned and *in vitro* transcribed using T7 RNA Polymerase. To evaluate processing levels of the pri-miRNA-142 editing variants, RNA microinjection experiments in eggs were performed and analyzed on PAA-Gels.

We analyzed the effect of RNA editing on Drosha mediated processing *in vivo*, using wild-type pri-miRNA-142 (Figure 28A) and further designed a pre-edited clone mimicking A-to-I editing by the introduction of G at position +4 and +5 (Figure 28B). Introduction of structural alterations, by using guanosine instead of inosine in the pre-edited variant, is assumed to be equivalent to those of the A-to-I edited pri-miRNA 142. We anticipated for the pre-edited variant that replacing A-U Watson-Crick pairs at position +4 and +5 with G•U wobble pairs (instead of two isosteric I•U wobble pairs) is equivalent to those of the A-to-I edited pri-miRNA-142 (Yang et al., 2006, Masquida and Westhof, 2000). A mutation, that precludes RNA editing, was introduced as an editing control by replacing A with C at position +4, +5 and +6 and U with C as their counterparts (Figure 28C). Further, a compensatory mutation was established, having A exchanged with G at position +4, +5 and +6 and U with G as their counterparts in the hairpin (Figure 28D).

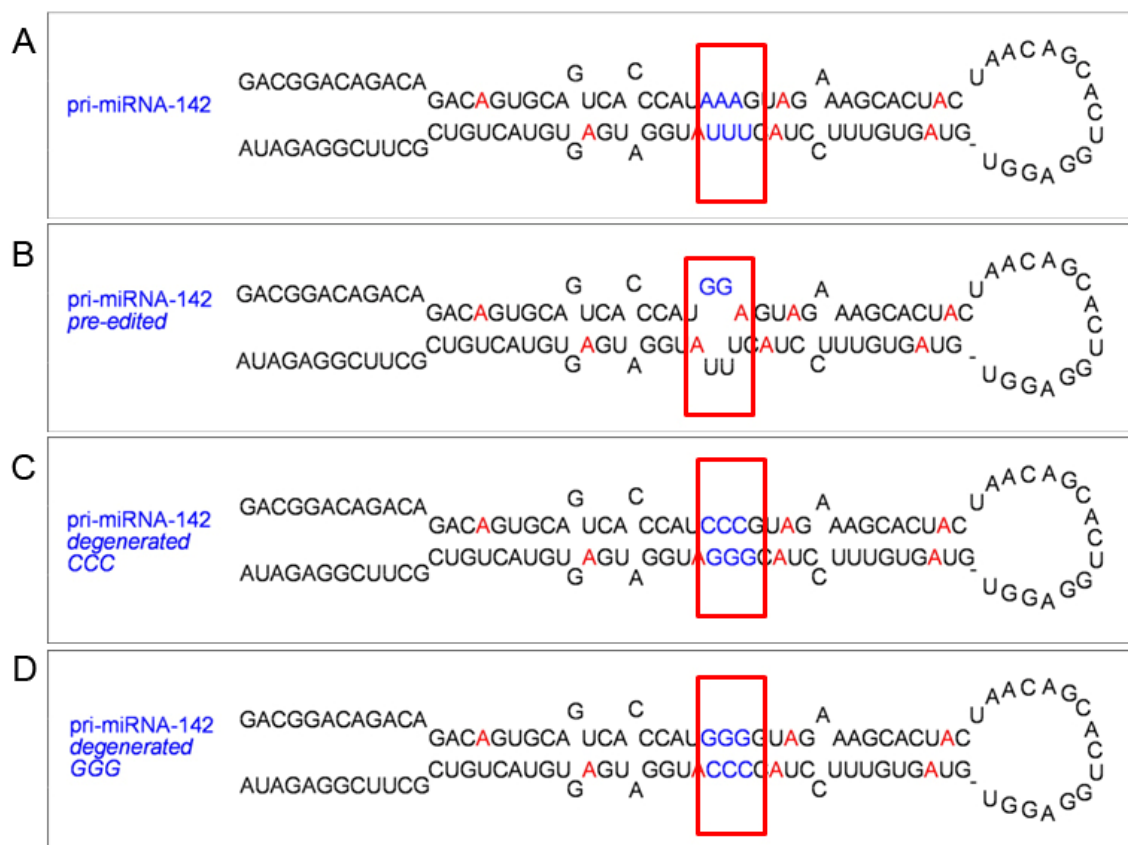


Figure 28. Variants of pri-miRNA-142 used in processing analysis

- (A) pri-miRNA-142 : sequence annotated on www.mIRbase.org
- (B) pri-miRNA-142 : pre-edited: A-to-G at position +4,+5
- (C) pri-miRNA-142 : degenerated CCC: A-to-C at position +4,+5,+6 (mutated editing site)
- (D) pri-miRNA-142 : degenerated GGG: A-to-G at position +4,+5,+6 (compensatory mutated editing site)

T7 RNA Polymerase mediated *in vitro* transcription of pri-miRNA-142 variants was performed in the presence of [α - ^{32} P]ATP. Equal amounts of the body-labeled pri-miRNA-142 variants were microinjected and the processing rates were analyzed on PAA-Gels (see Figure 29).

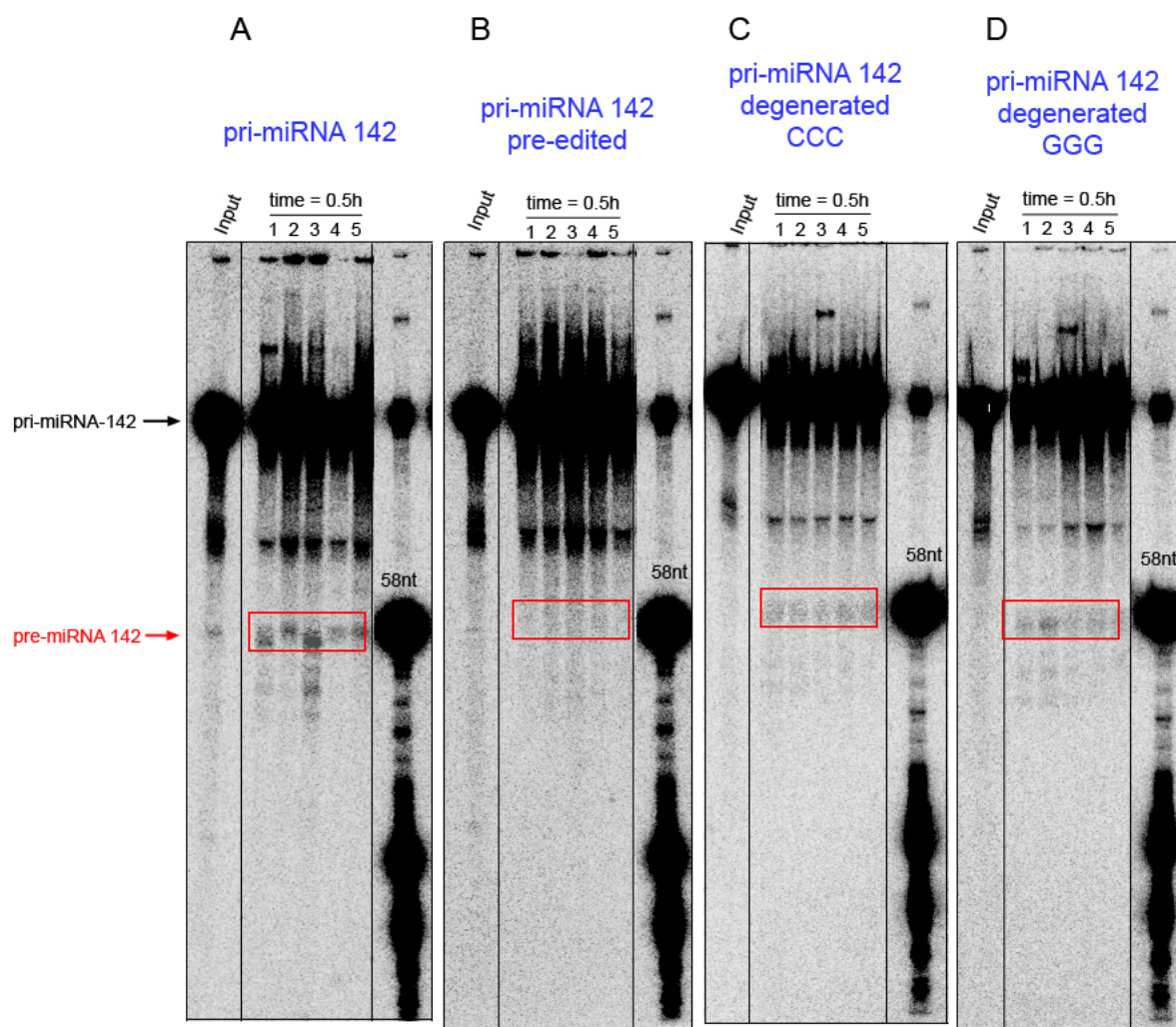


Figure 29. Processing of pri-miRNA-142 variants in *Xenopus* eggs

Microinjection of (A) pri-miRNA-142, (B) pri-miRNA-142 pre-edited, (C) pri-miRNA-142 degenerated CCC and (D) pri-miRNA-142 degenerated GGG in eggs. After an incubation of 0.5h the extracted RNA was analyzed on a 10% PAA-Gel.

Further, pri- to pre-miRNA-142 processing rates depending on absolute intensities were calculated. As displayed in Figure 30, in contrast to the wild type and the control variants the pre-edited pri-miRNA-142 processing level is strongly reduced *in vivo*.

Cloning and sequence analysis of microinjected pri-miRNA-142 variants did not reveal *de novo* A-to-I RNA editing in eggs. Hence, pri-miRNA-142 variants remain stable and are not targeted by Tudor-SN which degrades hyperedited RNA molecules (Scadden, 2005).

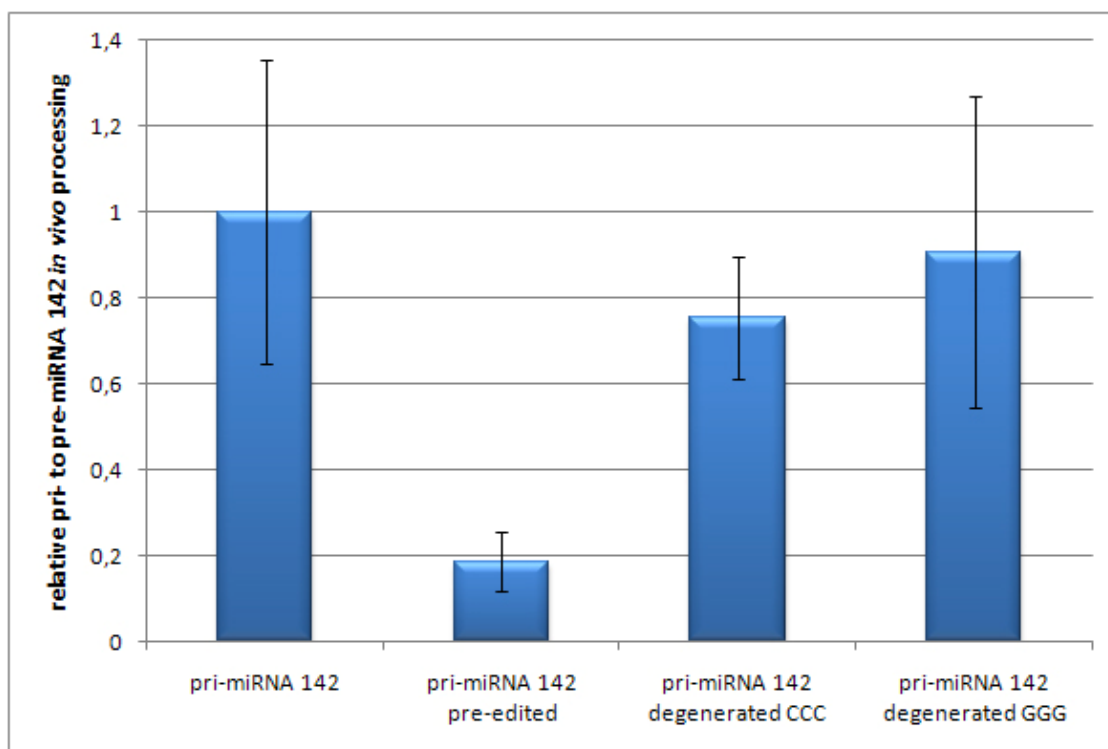


Figure 30. Processing of pre-edited pri-miRNA-142 is reduced *in vivo*

Processing levels of pri-miRNA-142 variants were calculated from absolute intensities using Bio-Rad™ QuantityOne software. A dramatic drop in processing for the pre-edited variant of pri-miRNA-142 can be detected.

In summary, this comparative assay reveals a striking reduction of pre-miRNA-142 levels *in vivo* only for pre-edited pri-miRNA 142. The wild-type and control pri-miRNA-142 variants exhibit comparable processing rates. Moreover, no *de novo* A-to-I RNA editing of pri-miRNA-142 could be detected leaving the pri-miRNA-142 variants stable in the *Xenopus* eggs.

Discussion

Gene regulation by miRNAs plays an important role in the development and differentiation of animals (reviewed in Bushati and Cohen, 2007). Biogenesis of miRNAs can be controlled by the regulation of miRNA gene transcription, by post-transcriptional modification of miRNA maturation and post-translational regulation of the key enzymes Drosha and Dicer influencing the processing of miRNA precursors (reviewed in Krol et al., 2010).

The maturation of *Xenopus* oocytes to eggs is accompanied by a cessation of transcription and a complex network of translational activation and repression of stored maternal mRNAs (reviewed in Mendez and Richter, 2001). Recently it became clear that oocyte maturation can boost Dicer activity and that miRNAs are expressed in a stage specific manner in *Xenopus* development (Lund and Dahlberg, 2006, Watanabe et al., 2005, Lund et al., 2009, Armisen et al., 2009).

In order to gain insight in the regulation of miRNA maturation in *Xenopus* oogenesis we created a miRNA precursor microinjection system in *Xenopus* stage VI oocytes and eggs. Evaluating the processing pattern of the pre- and pri-miRNAs allowed us to track all steps in miRNA biogenesis during the maturation from oocytes to eggs.

Developmental activation of pri-miRNA processing

Data from microinjection experiments in oocytes confirmed previous work that pre-miRNAs are exported from the nucleus and processed by Dicer in the cytoplasm (Lund et al., 2004). Consequently, comparative analysis of Dicer cleavage also revealed enhanced processing in eggs as described earlier (Lund and Dahlberg, 2006).

We were able to provide new insight that pri-miRNA processing is activated upon maturation to eggs. Further, pri-miRNAs are degraded by an unknown factor in the germinal vesicle of stage VI oocytes. G2 arrested stage VI oocytes are transcriptionally inactive, which does not rule out that miRNA gene transcription and processing occurs earlier in development. However, in contrast to somatic cells, oocytes at this stage of development obviously do not have active nuclear Drosha.

Further, microinjection experiments into the cytoplasm of oocytes could show that pri-miRNAs remain stable in this cellular compartment. Additionally, comparative experimental evidence in both cell types could clearly demonstrate the activation of pri-miRNA processing in eggs. In Northern Blot experiments the biological importance of the Drosha activation could be confirmed by showing a strong increase of endogenous xtr-miRNA-101 and xtr-miRNA-148a upon maturation. However, we cannot distinguish if pri-miRNA transcription levels are also elevated or if pri-miRNAs already present in the oocyte are processed upon maturation. Enhanced endogenous miRNA levels could also be explained by processing of pre-miRNAs stored in the cytoplasm. This is rather unlikely, because Dicer is also active in stage VI oocytes, a stage lasting for several months.

Taken together, these facts might argue for a possible storage of (maternal) pri-miRNA molecules in the cytoplasm as observed for maternal mRNAs. Maternally inherited mRNAs are stored during the long period of oogenesis and remain dormant in oocytes. One important startpoint for mobilizing the stored mRNA is maturation, when several cell-cycle-control proteins are starting to be translated (reviewed in Radford et al., 2008). However, so far no experimental evidence has documented storage of miRNA precursor molecules in oocytes.

To further assess the distinct processing pattern of *Xenopus* miRNA precursors we performed microinjection experiments using xtr-pri- and xtr-pre-miRNA-148a. Comparison of pri-miRNA-148a Drosha processing rates in oocytes and eggs revealed a complete activation of processing upon maturation to eggs. This result goes in line with the activation of pri-miRNA processing demonstrated in earlier experiments. In contrast to Drosha activation, xtr-pre-miRNA-148a Dicer processing can already be detected in stage VI oocytes. Maturation then intensifies xtr-pre-miRNA-148a Dicer processing up to four times.

On the whole these results demonstrate a developmentally regulated onset of Drosha and increase of Dicer activity leading to enhanced of miRNA biogenesis. Accordingly, Drosha processing is the rate limiting step in enhancing mature xtr-miRNA-148a levels during maturation. This potentially has important implications on miRNA mediated gene regulation during oogenesis and fertilization. Previous work showed that miRNAs play an important role in the turnover of maternal mRNAs during the switch from maternal to zygotic gene expression by mediating

deadenylation of target mRNAs (Giraldez et al., 2006, Bushati et al., 2008). Thus, the function of the miRNA upregulation and which targets are affected in eggs have to be elucidated.

Developmental regulation of Drosha activity

Stage VI oocytes are assumed to be transcriptionally inactive (reviewed in Mendez and Richter, 2001). However, to investigate a possible activation of Drosha RNA transcription upon maturation we applied the transcriptional inhibitor Actinomycin D (AMD). Microinjection of pri-miRNAs in eggs matured in the presence of AMD showed no differences in pri-miRNA Drosha processing. We therefore conclude that Drosha activity is not influenced by transcription during maturation.

We examined post-translational regulation as another possible regulator of Drosha activity in eggs. Therefore evaluation of Drosha regulation at the protein level by *in vitro* processing experiments using immunoprecipitated recombinant *Xenopus* Drosha revealed no differences of activity when recombinantly expressed in oocytes and matured to eggs. This approach does not rule out the possibility that Drosha might be regulated by potential interaction partners or inhibitors *in vivo*.

Consequently, tests for inhibitory cofactors that regulate Drosha activity *in vivo* using recombinant protein were carried out. Microinjection experiments using pri-miRNA in oocytes and eggs that express recombinant Drosha showed Drosha activity in both cell types. Thus, Drosha protein is not blocked or inhibited in stage VI oocytes.

Additional tests on Drosha regulation at the protein level were performed using the translational inhibitor Cycloheximide while initiating maturation. We observed a block of maturation confirming previous work showing that protein synthesis is strictly required for oocyte maturation (Wasserman and Masui, 1975, Schuetz and Samson, 1979).

In summary, these experiments demonstrate that no post-translational blockage or inhibitory cofactors of Drosha could be identified in stage VI oocytes. Hence, expression of recombinant Drosha is sufficient to regain pri-miRNA processing activity in stage VI oocytes. However, we cannot exclude that a post-translational inhibition or modification, leading to inactivation of Drosha enzyme, occurs earlier in development.

Another approach evaluating developmental regulation of Drosha is investigating an important factor modulating translation of mRNAs in the developing oocyte. CPEB, a highly conserved, sequence-specific RNA binding protein targeting the cytoplasmic polyadenylation element, regulates mRNA translation by cytoplasmic polyadenylation of mRNAs (reviewed in Mendez and Richter, 2001). Induction of translation in early development by polyadenylation seems to be a characteristic of all metazoans (Fox et al., 1989, McGrew et al., 1989, Tay et al., 2000, Minshall et al., 1999, Gebauer et al., 1994, Salles et al., 1994).

In order to investigate cytoplasmic polyadenylation regulating the translation of Drosha, we are performing experiments comparing the length of Drosha mRNAs in the process of maturation. Further, tests studying translational regulation of Drosha activity are performed using RNase H and morpholino assays to perturb Drosha RNA levels. These experiments are still pending and will be presented elsewhere.

Impact of RNA editing on pri-miRNA processing *in vivo*

ADAR proteins and proteins of the RNAi machinery, such as the microprocessor complex (Drosha-DGCR8), Dicer and TRBP contain dsRBDs. In general, there is no sequence specificity for dsRBDs which makes these proteins potential competitors for RNA binding (Saunders and Barber, 2003, reviewed in Nishikura, 2006).

Moreover, extensively A-to-I edited dsRNA *in vitro* by ADAR generates less siRNA and reduced RNAi (Scadden and Smith, 2001a). The double-stranded hairpin of miRNA precursors are common targets of ADAR1 and ADAR2 (Luciano et al., 2004, Blow et al., 2006, Calin and Croce, 2006). Recent studies detected RNA editing of several adenosine residues of pri-miRNA 142. *In vitro* experiments using Drosha/DGCR8 complexes revealed that editing of position +4 and +5 located in the fold-back dsRNA stem near the Drosha cleavage site is both, necessary and sufficient, to inhibit Drosha cleavage (Yang et al., 2006).

In order to gain insight into the effects of RNA editing on pri-miRNA-142 processing *in vivo*, we investigated different editing variants of pri-miRNA-142 using our established *Xenopus* eggs microinjection system.

We analyzed four different variants of pri-miRNA 142: a wild-type variant, a pre-edited pri-miRNA (A-to-G at position +4, +5), a mutated variant that precludes editing as well as a compensatory mutated variant. In the pre-edited variant two A-U Watson-Crick pairs at position +4 and +5 were replaced with G•U wobble pairs instead of two isosteric I•U wobble pairs (Yang et al., 2006, Masquida and Westhof, 2000).

When compared to wild-type and control variants processing of the pre-edited pri-miRNA-142 is strikingly reduced *in vivo*. Reduction in processing levels might be explained by the introduced structural change in the hairpin of the pre-edited pri-miRNA-142 variant. This could lead to a reduced DGCR8 binding and/or subsequent Drosha cleavage. Further, it cannot be excluded that the pre-edited pri-miRNA variant is a prevalent dsRNA target for ADAR enzyme. The dsRBDs harboring proteins DGCR8 and ADAR are putative competitors for binding the double-stranded hairpin. Enhanced ADAR binding would have an antagonistic effect on processing by the microprocessor complex. Additional experiments elucidating the role of

competitive dsRNA binding will provide further insight the reduction of edited pri-miRNA-142 processing *in vivo*.

Another important finding is high stability of pri-miRNA-142 upon microinjection in the egg. Sequencing of microinjected pri-miRNA-142 could not show *de novo* RNA editing *in vivo* leaving unprocessed pri-miRNA-142 stable in the egg. This result is in contrast to previous work where unprocessed pri-miRNA-142 was A-to-I edited and therefore a potential target for degradation by Tudor-SN (Yang et al., 2006, Scadden, 2005).

However, reduced processing of pre-edited pri-miRNA-142 *in vivo* goes in hand with results published for inhibition of pri-miRNA-142 processing *in vitro* and pre-miRNA-151 *in vivo* processing (Yang et al., 2006, Kawahara et al., 2007a). This further proves the importance of RNA editing as an antagonist of miRNA biogenesis and consequently miRNA mediated gene silencing.

References

- ALBERTS, B. 2002. *Molecular biology of the cell*, New York, Garland Science.
- ALFONZO, J. D., THIEMANN, O. & SIMPSON, L. 1997. The mechanism of U insertion/deletion RNA editing in kinetoplastid mitochondria. *Nucleic Acids Res*, 25, 3751-9.
- ANDERSON, P. & KEDERSHA, N. 2009. RNA granules: post-transcriptional and epigenetic modulators of gene expression. *Nat Rev Mol Cell Biol*, 10, 430-6.
- ANDL, T., MURCHISON, E. P., LIU, F., ZHANG, Y., YUNTA-GONZALEZ, M., TOBIAS, J. W., ANDL, C. D., SEYKORA, J. T., HANNON, G. J. & MILLAR, S. E. 2006. The miRNA-processing enzyme dicer is essential for the morphogenesis and maintenance of hair follicles. *Curr Biol*, 16, 1041-9.
- ANDRESSON, T. & RUDERMAN, J. V. 1998. The kinase Eg2 is a component of the *Xenopus* oocyte progesterone-activated signaling pathway. *EMBO J*, 17, 5627-37.
- ARMISEN, J., GILCHRIST, M. J., WILCZYNSKA, A., STANDART, N. & MISKA, E. A. 2009. Abundant and dynamically expressed miRNAs, piRNAs, and other small RNAs in the vertebrate *Xenopus tropicalis*. *Genome Res*, 19, 1766-75.
- ATHANASIADIS, A., RICH, A. & MAAS, S. 2004. Widespread A-to-I RNA editing of Alu-containing mRNAs in the human transcriptome. *PLoS Biol*, 2, e391.
- BALZER, E. & MOSS, E. G. 2007. Localization of the developmental timing regulator Lin28 to mRNP complexes, P-bodies and stress granules. *RNA Biol*, 4, 16-25.
- BARKOFF, A. F., DICKSON, K. S., GRAY, N. K. & WICKENS, M. 2000. Translational control of cyclin B1 mRNA during meiotic maturation: coordinated repression and cytoplasmic polyadenylation. *Dev Biol*, 220, 97-109.
- BARTEL, D. P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 116, 281-97.

- BARTEL, D. P. 2009. MicroRNAs: target recognition and regulatory functions. *Cell*, 136, 215-33.
- BASS, B. L. 2002. RNA editing by adenosine deaminases that act on RNA. *Annu Rev Biochem*, 71, 817-46.
- BASS, B. L. & WEINTRAUB, H. 1987. A developmentally regulated activity that unwinds RNA duplexes. *Cell*, 48, 607-13.
- BASS, B. L. & WEINTRAUB, H. 1988. An unwinding activity that covalently modifies its double-stranded RNA substrate. *Cell*, 55, 1089-98.
- BEHM-ANSMANT, I., REHWINKEL, J., DOERKS, T., STARK, A., BORK, P. & IZAURRALDE, E. 2006. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev*, 20, 1885-98.
- BENNE, R., VAN DEN BURG, J., BRAKENHOFF, J. P., SLOOF, P., VAN BOOM, J. H. & TROMP, M. C. 1986. Major transcript of the frameshifted coxII gene from trypanosome mitochondria contains four nucleotides that are not encoded in the DNA. *Cell*, 46, 819-26.
- BERNSTEIN, E., KIM, S. Y., CARMELL, M. A., MURCHISON, E. P., ALCORN, H., LI, M. Z., MILLS, A. A., ELLEDGE, S. J., ANDERSON, K. V. & HANNON, G. J. 2003. Dicer is essential for mouse development. *Nat Genet*, 35, 215-7.
- BILGER, A., FOX, C. A., WAHLE, E. & WICKENS, M. 1994. Nuclear polyadenylation factors recognize cytoplasmic polyadenylation elements. *Genes Dev*, 8, 1106-16.
- BLOW, M. J., GROCOCK, R. J., VAN DONGEN, S., ENRIGHT, A. J., DICKS, E., FUTREAL, P. A., WOOSTER, R. & STRATTON, M. R. 2006. RNA editing of human microRNAs. *Genome Biol*, 7, R27.

- BOHNSACK, M. T., CZAPLINSKI, K. & GORLICH, D. 2004. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA*, 10, 185-91.
- BRENNECKE, J., STARK, A., RUSSELL, R. B. & COHEN, S. M. 2005. Principles of microRNA-target recognition. *PLoS Biol*, 3, e85.
- BURNS, C. M., CHU, H., RUETER, S. M., HUTCHINSON, L. K., CANTON, H., SANDERS-BUSH, E. & EMESON, R. B. 1997. Regulation of serotonin-2C receptor G-protein coupling by RNA editing. *Nature*, 387, 303-8.
- BUSHATI, N. & COHEN, S. M. 2007. microRNA functions. *Annu Rev Cell Dev Biol*, 23, 175-205.
- BUSHATI, N., STARK, A., BRENNECKE, J. & COHEN, S. M. 2008. Temporal reciprocity of miRNAs and their targets during the maternal-to-zygotic transition in *Drosophila*. *Curr Biol*, 18, 501-6.
- CALIN, G. A. & CROCE, C. M. 2006. MicroRNA signatures in human cancers. *Nat Rev Cancer*, 6, 857-66.
- CARTHEW, R. W. & SONTHEIMER, E. J. 2009. Origins and Mechanisms of miRNAs and siRNAs. *Cell*, 136, 642-55.
- CASTRO, A., PETER, M., LORCA, T. & MANDART, E. 2001. c-Mos and cyclin B/cdc2 connections during *Xenopus* oocyte maturation. *Biol Cell*, 93, 15-25.
- CATTANEO, R., SCHMID, A., ESCHLE, D., BACZKO, K., TER MEULEN, V. & BILLETER, M. A. 1988. Biased hypermutation and other genetic changes in defective measles viruses in human brain infections. *Cell*, 55, 255-65.
- CHANG, K. Y. & RAMOS, A. 2005. The double-stranded RNA-binding motif, a versatile macromolecular docking platform. *FEBS J*, 272, 2109-17.
- CHANG, T. C., YU, D., LEE, Y. S., WENTZEL, E. A., ARKING, D. E., WEST, K. M., DANG, C. V., THOMAS-TIKHONENKO, A. & MENDELL, J. T. 2008.

- Widespread microRNA repression by Myc contributes to tumorigenesis. *Nat Genet*, 40, 43-50.
- CHATTERJEE, S. & GROSSHANS, H. 2009. Active turnover modulates mature microRNA activity in *Caenorhabditis elegans*. *Nature*, 461, 546-9.
- CHEKULAEVA, M. & FILIPOWICZ, W. 2009. Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr Opin Cell Biol*, 21, 452-60.
- CHENDRIMADA, T. P., GREGORY, R. I., KUMARASWAMY, E., NORMAN, J., COOCH, N., NISHIKURA, K. & SHIEKHATTAR, R. 2005. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature*, 436, 740-4.
- CHIANG, H. R., SCHOENFELD, L. W., RUBY, J. G., AUYEUNG, V. C., SPIES, N., BAEK, D., JOHNSTON, W. K., RUSS, C., LUO, S., BABIARZ, J. E., BLELLOCH, R., SCHROTH, G. P., NUSBAUM, C. & BARTEL, D. P. 2010. Mammalian microRNAs: experimental evaluation of novel and previously annotated genes. *Genes Dev*, 24, 992-1009.
- CHURCH, G. M. & GILBERT, W. 1984. Genomic sequencing. *Proc Natl Acad Sci U S A*, 81, 1991-5.
- CONACO, C., OTTO, S., HAN, J. J. & MANDEL, G. 2006. Reciprocal actions of REST and a microRNA promote neuronal identity. *Proc Natl Acad Sci U S A*, 103, 2422-7.
- CORCORAN, D. L., PANDIT, K. V., GORDON, B., BHATTACHARJEE, A., KAMINSKI, N. & BENOS, P. V. 2009. Features of mammalian microRNA promoters emerge from polymerase II chromatin immunoprecipitation data. *PLoS One*, 4, e5279.
- DAVIS, B. N., HILYARD, A. C., LAGNA, G. & HATA, A. 2008. SMAD proteins control DROSHA-mediated microRNA maturation. *Nature*, 454, 56-61.

- DAVIS, B. N., HILYARD, A. C., NGUYEN, P. H., LAGNA, G. & HATA, A. 2010. Smad Proteins Bind a Conserved RNA Sequence to Promote MicroRNA Maturation by Drosha. *Mol Cell*, 39, 373-384.
- DICKSON, K. S., BILGER, A., BALLANTYNE, S. & WICKENS, M. P. 1999. The cleavage and polyadenylation specificity factor in *Xenopus laevis* oocytes is a cytoplasmic factor involved in regulated polyadenylation. *Mol Cell Biol*, 19, 5707-17.
- DOENCH, J. G. & SHARP, P. A. 2004. Specificity of microRNA target selection in translational repression. *Genes Dev*, 18, 504-11.
- DOYLE, M. & JANTSCH, M. F. 2002. New and old roles of the double-stranded RNA-binding domain. *J Struct Biol*, 140, 147-53.
- DUMONT, J. N. 1972. Oogenesis in *Xenopus laevis* (Daudin). I. Stages of oocyte development in laboratory maintained animals. *J Morphol*, 136, 153-79.
- EULALIO, A., BEHM-ANSMANT, I. & IZAURRALDE, E. 2007. P bodies: at the crossroads of post-transcriptional pathways. *Nat Rev Mol Cell Biol*, 8, 9-22.
- EULALIO, A., TRITSCHLER, F. & IZAURRALDE, E. 2009. The GW182 protein family in animal cells: new insights into domains required for miRNA-mediated gene silencing. *RNA*, 15, 1433-42.
- FALLER, M., MATSUNAGA, M., YIN, S., LOO, J. A. & GUO, F. 2007. Heme is involved in microRNA processing. *Nat Struct Mol Biol*, 14, 23-9.
- FERRELL, J. E., JR. 1999. *Xenopus* oocyte maturation: new lessons from a good egg. *Bioessays*, 21, 833-42.
- FERRELL, J. E., JR. & MACHLEDER, E. M. 1998. The biochemical basis of an all-or-none cell fate switch in *Xenopus* oocytes. *Science*, 280, 895-8.

- FILIPOWICZ, W., BHATTACHARYYA, S. N. & SONENBERG, N. 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*, 9, 102-14.
- FIRE, A., XU, S., MONTGOMERY, M. K., KOSTAS, S. A., DRIVER, S. E. & MELLO, C. C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, 391, 806-11.
- FORMAN, J. J., LEGESSE-MILLER, A. & COLLIER, H. A. 2008. A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence. *Proc Natl Acad Sci U S A*, 105, 14879-84.
- FORTUNE, J. E., CONCANNON, P. W. & HANSEL, W. 1975. Ovarian progesterone levels during in vitro oocyte maturation and ovulation in *Xenopus laevis*. *Biol Reprod*, 13, 561-7.
- FOX, C. A., SHEETS, M. D., WAHLE, E. & WICKENS, M. 1992. Polyadenylation of maternal mRNA during oocyte maturation: poly(A) addition in vitro requires a regulated RNA binding activity and a poly(A) polymerase. *EMBO J*, 11, 5021-32.
- FOX, C. A., SHEETS, M. D. & WICKENS, M. P. 1989. Poly(A) addition during maturation of frog oocytes: distinct nuclear and cytoplasmic activities and regulation by the sequence UUUUUAU. *Genes Dev*, 3, 2151-62.
- FUKUDA, T., YAMAGATA, K., FUJIYAMA, S., MATSUMOTO, T., KOSHIDA, I., YOSHIMURA, K., MIHARA, M., NAITOU, M., ENDOH, H., NAKAMURA, T., AKIMOTO, C., YAMAMOTO, Y., KATAGIRI, T., FOULDS, C., TAKEZAWA, S., KITAGAWA, H., TAKEYAMA, K., O'MALLEY, B. W. & KATO, S. 2007. DEAD-box RNA helicase subunits of the Drosha complex are required for processing of rRNA and a subset of microRNAs. *Nat Cell Biol*, 9, 604-11.
- GABRIELLI, B. G., ROY, L. M. & MALLER, J. L. 1993. Requirement for Cdk2 in cytostatic factor-mediated metaphase II arrest. *Science*, 259, 1766-9.

- GEBAUER, F., XU, W., COOPER, G. M. & RICHTER, J. D. 1994. Translational control by cytoplasmic polyadenylation of c-mos mRNA is necessary for oocyte maturation in the mouse. *EMBO J*, 13, 5712-20.
- GERBER, A., GROSJEAN, H., MELCHER, T. & KELLER, W. 1998. Tad1p, a yeast tRNA-specific adenosine deaminase, is related to the mammalian pre-mRNA editing enzymes ADAR1 and ADAR2. *EMBO J*, 17, 4780-9.
- GERBER, A., O'CONNELL, M. A. & KELLER, W. 1997. Two forms of human double-stranded RNA-specific editase 1 (hRED1) generated by the insertion of an Alu cassette. *RNA*, 3, 453-63.
- GHERZI, R., LEE, K. Y., BRIATA, P., WEGMULLER, D., MORONI, C., KARIN, M. & CHEN, C. Y. 2004. A KH domain RNA binding protein, KSRP, promotes ARE-directed mRNA turnover by recruiting the degradation machinery. *Mol Cell*, 14, 571-83.
- GILBERT, S. F. 2000. *Developmental Biology*, 6th edition, Sinauer Associates Inc.
- GIRALDEZ, A. J., MISHIMA, Y., RHEL, J., GROCOCK, R. J., VAN DONGEN, S., INOUE, K., ENRIGHT, A. J. & SCHIER, A. F. 2006. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science*, 312, 75-9.
- GOTT, J. M. & EMESON, R. B. 2000. Functions and mechanisms of RNA editing. *Annu Rev Genet*, 34, 499-531.
- GREGORY, R. I., YAN, K. P., AMUTHAN, G., CHENDRIMADA, T., DORATOTAJ, B., COOCH, N. & SHIEKHATTAR, R. 2004. The Microprocessor complex mediates the genesis of microRNAs. *Nature*, 432, 235-40.
- GRIMSON, A., FARH, K. K., JOHNSTON, W. K., GARRETT-ENGELE, P., LIM, L. P. & BARTEL, D. P. 2007. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell*, 27, 91-105.

- GUIL, S. & CACERES, J. F. 2007. The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a. *Nat Struct Mol Biol*, 14, 591-6.
- HAKE, L. E. & RICHTER, J. D. 1994. CPEB is a specificity factor that mediates cytoplasmic polyadenylation during *Xenopus* oocyte maturation. *Cell*, 79, 617-27.
- HAMILTON, A. J. & BAULCOMBE, D. C. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science*, 286, 950-2.
- HAN, J., LEE, Y., YEOM, K. H., NAM, J. W., HEO, I., RHEE, J. K., SOHN, S. Y., CHO, Y., ZHANG, B. T. & KIM, V. N. 2006. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell*, 125, 887-901.
- HAN, J., PEDERSEN, J. S., KWON, S. C., BELAIR, C. D., KIM, Y. K., YEOM, K. H., YANG, W. Y., HAUSSLER, D., BLELLOCH, R. & KIM, V. N. 2009. Posttranscriptional crossregulation between Drosha and DGCR8. *Cell*, 136, 75-84.
- HAN, L., WITMER, P. D., CASEY, E., VALLE, D. & SUKUMAR, S. 2007. DNA methylation regulates MicroRNA expression. *Cancer Biol Ther*, 6, 1284-8.
- HARFE, B. D., MCMANUS, M. T., MANSFIELD, J. H., HORNSTEIN, E. & TABIN, C. J. 2005. The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. *Proc Natl Acad Sci U S A*, 102, 10898-903.
- HARRIS, K. S., ZHANG, Z., MCMANUS, M. T., HARFE, B. D. & SUN, X. 2006. Dicer function is essential for lung epithelium morphogenesis. *Proc Natl Acad Sci U S A*, 103, 2208-13.
- HAUSEN, P. & RIEBESELL, M. 1991. *The Early Development of Xenopus Laevis: An Atlas of the Histology*, Berlin, Springer Verlag.
- HE, L., HE, X., LIM, L. P., DE STANCHINA, E., XUAN, Z., LIANG, Y., XUE, W., ZENDER, L., MAGNUS, J., RIDZON, D., JACKSON, A. L., LINSLEY, P. S., CHEN, C., LOWE, S. W., CLEARY, M. A. & HANNON, G. J. 2007. A

- microRNA component of the p53 tumour suppressor network. *Nature*, 447, 1130-4.
- HEO, I., JOO, C., CHO, J., HA, M., HAN, J. & KIM, V. N. 2008. Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Mol Cell*, 32, 276-84.
- HEO, I., JOO, C., KIM, Y. K., HA, M., YOON, M. J., CHO, J., YEOM, K. H., HAN, J. & KIM, V. N. 2009. TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. *Cell*, 138, 696-708.
- HIGUCHI, M., SINGLE, F. N., KOHLER, M., SOMMER, B., SPRENGEL, R. & SEEBURG, P. H. 1993. RNA editing of AMPA receptor subunit GluR-B: a base-paired intron-exon structure determines position and efficiency. *Cell*, 75, 1361-70.
- HOCK, J. & MEISTER, G. 2008. The Argonaute protein family. *Genome Biol*, 9, 210.
- HOOPENGARDNER, B., BHALLA, T., STABER, C. & REENAN, R. 2003. Nervous system targets of RNA editing identified by comparative genomics. *Science*, 301, 832-6.
- HOUBAVIY, H. B., MURRAY, M. F. & SHARP, P. A. 2003. Embryonic stem cell-specific MicroRNAs. *Dev Cell*, 5, 351-8.
- HUMPHREYS, D. T., WESTMAN, B. J., MARTIN, D. I. & PREISS, T. 2005. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. *Proc Natl Acad Sci U S A*, 102, 16961-6.
- JORGENSEN, R. 1990. Altered gene expression in plants due to trans interactions between homologous genes. *Trends Biotechnol*, 8, 340-4.
- KANELLOPOULOU, C., MULJO, S. A., KUNG, A. L., GANESAN, S., DRAPKIN, R., JENUWEIN, T., LIVINGSTON, D. M. & RAJEWSKY, K. 2005. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev*, 19, 489-501.

- KAWAHARA, Y., ZINSHTEYN, B., CHENDRIMADA, T. P., SHIEKHATTAR, R. & NISHIKURA, K. 2007a. RNA editing of the microRNA-151 precursor blocks cleavage by the Dicer-TRBP complex. *EMBO Rep*, 8, 763-9.
- KAWAHARA, Y., ZINSHTEYN, B., SETHUPATHY, P., IIZASA, H., HATZIGEORGIOU, A. G. & NISHIKURA, K. 2007b. Redirection of silencing targets by adenosine-to-inosine editing of miRNAs. *Science*, 315, 1137-40.
- KEEGAN, L. P., GALLO, A. & O'CONNELL, M. A. 2001. The many roles of an RNA editor. *Nat Rev Genet*, 2, 869-78.
- KERTESZ, M., IOVINO, N., UNNERSTALL, U., GAUL, U. & SEGAL, E. 2007. The role of site accessibility in microRNA target recognition. *Nat Genet*, 39, 1278-84.
- KIM, J., INOUE, K., ISHII, J., VANTI, W. B., VORONOV, S. V., MURCHISON, E., HANNON, G. & ABELIOVICH, A. 2007. A MicroRNA feedback circuit in midbrain dopamine neurons. *Science*, 317, 1220-4.
- KROL, J., LOEDIGE, I. & FILIPOWICZ, W. 2010. The widespread regulation of microRNA biogenesis, function and decay. *Nat Rev Genet*, 11, 597-610.
- KROL, J., SOBCZAK, K., WILCZYNSKA, U., DRATH, M., JASINSKA, A., KACZYNSKA, D. & KRZYZOSIAK, W. J. 2004. Structural features of microRNA (miRNA) precursors and their relevance to miRNA biogenesis and small interfering RNA/short hairpin RNA design. *J Biol Chem*, 279, 42230-9.
- KUTAY, U., LIPOWSKY, G., IZAURRALDE, E., BISCHOFF, F. R., SCHWARZMAIER, P., HARTMANN, E. & GORLICH, D. 1998. Identification of a tRNA-specific nuclear export receptor. *Mol Cell*, 1, 359-69.
- LAFOREST, M. J., ROEWER, I. & LANG, B. F. 1997. Mitochondrial tRNAs in the lower fungus *Spizellomyces punctatus*: tRNA editing and UAG 'stop' codons recognized as leucine. *Nucleic Acids Res*, 25, 626-32.

- LEE, R. C., FEINBAUM, R. L. & AMBROS, V. 1993. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*, 75, 843-54.
- LEE, Y., JEON, K., LEE, J. T., KIM, S. & KIM, V. N. 2002. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J*, 21, 4663-70.
- LEHMANN, K. A. & BASS, B. L. 1999. The importance of internal loops within RNA substrates of ADAR1. *J Mol Biol*, 291, 1-13.
- LEVANON, E. Y., EISENBERG, E., YELIN, R., NEMZER, S., HALLEGGER, M., SHEMESH, R., FLIGELMAN, Z. Y., SHOSHAN, A., POLLOCK, S. R., SZTYBEL, D., OLSHANSKY, M., RECHAVI, G. & JANTSCH, M. F. 2004. Systematic identification of abundant A-to-I editing sites in the human transcriptome. *Nat Biotechnol*, 22, 1001-5.
- LEWIS, B. P., BURGE, C. B. & BARTEL, D. P. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell*, 120, 15-20.
- LORCA, T., CRUZALEGUI, F. H., FESQUET, D., CAVADORE, J. C., MERY, J., MEANS, A. & DOREE, M. 1993. Calmodulin-dependent protein kinase II mediates inactivation of MPF and CSF upon fertilization of *Xenopus* eggs. *Nature*, 366, 270-3.
- LUCIANO, D. J., MIRSKY, H., VENDETTI, N. J. & MAAS, S. 2004. RNA editing of a miRNA precursor. *RNA*, 10, 1174-7.
- LUGLI, G., LARSON, J., MARTONE, M. E., JONES, Y. & SMALHEISER, N. R. 2005. Dicer and eIF2c are enriched at postsynaptic densities in adult mouse brain and are modified by neuronal activity in a calpain-dependent manner. *J Neurochem*, 94, 896-905.
- LUND, E. & DAHLBERG, J. E. 2006. Substrate selectivity of exportin 5 and Dicer in the biogenesis of microRNAs. *Cold Spring Harb Symp Quant Biol*, 71, 59-66.

- LUND, E., GUTTINGER, S., CALADO, A., DAHLBERG, J. E. & KUTAY, U. 2004. Nuclear export of microRNA precursors. *Science*, 303, 95-8.
- LUND, E., LIU, M., HARTLEY, R. S., SHEETS, M. D. & DAHLBERG, J. E. 2009. Deadenylation of maternal mRNAs mediated by miR-427 in *Xenopus laevis* embryos. *RNA*, 15, 2351-63.
- LUNDE, B. M., MOORE, C. & VARANI, G. 2007. RNA-binding proteins: modular design for efficient function. *Nat Rev Mol Cell Biol*, 8, 479-90.
- MA, E., MACRAE, I. J., KIRSCH, J. F. & DOUDNA, J. A. 2008. Autoinhibition of human dicer by its internal helicase domain. *J Mol Biol*, 380, 237-43.
- MA, J. B., YE, K. & PATEL, D. J. 2004. Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature*, 429, 318-22.
- MACRAE, I. J., ZHOU, K., LI, F., REPIC, A., BROOKS, A. N., CANDE, W. Z., ADAMS, P. D. & DOUDNA, J. A. 2006. Structural basis for double-stranded RNA processing by Dicer. *Science*, 311, 195-8.
- MARONEY, P. A., YU, Y., FISHER, J. & NILSEN, T. W. 2006. Evidence that microRNAs are associated with translating messenger RNAs in human cells. *Nat Struct Mol Biol*, 13, 1102-7.
- MASQUIDA, B. & WESTHOF, E. 2000. On the wobble GoU and related pairs. *RNA*, 6, 9-15.
- MASUI, Y. 1974. A cytostatic factor in amphibian oocytes: its extraction and partial characterization. *J Exp Zool*, 187, 141-7.
- MCGREW, L. L., DWORKIN-RASTL, E., DWORKIN, M. B. & RICHTER, J. D. 1989. Poly(A) elongation during *Xenopus* oocyte maturation is required for translational recruitment and is mediated by a short sequence element. *Genes Dev*, 3, 803-15.

- MCGREW, L. L. & RICHTER, J. D. 1990. Translational control by cytoplasmic polyadenylation during *Xenopus* oocyte maturation: characterization of cis and trans elements and regulation by cyclin/MPF. *EMBO J*, 9, 3743-51.
- MENDEZ, R., HAKE, L. E., ANDRESSON, T., LITTLEPAGE, L. E., RUDERMAN, J. V. & RICHTER, J. D. 2000a. Phosphorylation of CPE binding factor by Eg2 regulates translation of c-mos mRNA. *Nature*, 404, 302-7.
- MENDEZ, R., MURTHY, K. G., RYAN, K., MANLEY, J. L. & RICHTER, J. D. 2000b. Phosphorylation of CPEB by Eg2 mediates the recruitment of CPSF into an active cytoplasmic polyadenylation complex. *Mol Cell*, 6, 1253-9.
- MENDEZ, R. & RICHTER, J. D. 2001. Translational control by CPEB: a means to the end. *Nat Rev Mol Cell Biol*, 2, 521-9.
- MICHLEWSKI, G., GUIL, S., SEMPLE, C. A. & CACERES, J. F. 2008. Posttranscriptional regulation of miRNAs harboring conserved terminal loops. *Mol Cell*, 32, 383-93.
- MINSHALL, N., WALKER, J., DALE, M. & STANDART, N. 1999. Dual roles of p82, the clam CPEB homolog, in cytoplasmic polyadenylation and translational masking. *RNA*, 5, 27-38.
- MORSE, D. P., ARUSCAVAGE, P. J. & BASS, B. L. 2002. RNA hairpins in noncoding regions of human brain and *Caenorhabditis elegans* mRNA are edited by adenosine deaminases that act on RNA. *Proc Natl Acad Sci U S A*, 99, 7906-11.
- MURCHISON, E. P., STEIN, P., XUAN, Z., PAN, H., ZHANG, M. Q., SCHULTZ, R. M. & HANNON, G. J. 2007. Critical roles for Dicer in the female germline. *Genes Dev*, 21, 682-93.
- NAKAGAWA, A., SHI, Y., KAGE-NAKADAI, E., MITANI, S. & XUE, D. 2010. Caspase-dependent conversion of Dicer ribonuclease into a death-promoting deoxyribonuclease. *Science*, 328, 327-34.

- NAKIELNY, S. & DREYFUSS, G. 1999. Transport of proteins and RNAs in and out of the nucleus. *Cell*, 99, 677-90.
- NEWMAN, M. A., THOMSON, J. M. & HAMMOND, S. M. 2008. Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. *RNA*, 14, 1539-49.
- NIELSEN, C. B., SHOMRON, N., SANDBERG, R., HORNSTEIN, E., KITZMAN, J. & BURGE, C. B. 2007. Determinants of targeting by endogenous and exogenous microRNAs and siRNAs. *RNA*, 13, 1894-910.
- NISHIKURA, K. 2006. Editor meets silencer: crosstalk between RNA editing and RNA interference. *Nat Rev Mol Cell Biol*, 7, 919-31.
- NISHIKURA, K. 2010. Functions and regulation of RNA editing by ADAR deaminases. *Annu Rev Biochem*, 79, 321-49.
- NISHIKURA, K., YOO, C., KIM, U., MURRAY, J. M., ESTES, P. A., CASH, F. E. & LIEBHABER, S. A. 1991. Substrate specificity of the dsRNA unwinding/modifying activity. *EMBO J*, 10, 3523-32.
- NOTTROTT, S., SIMARD, M. J. & RICHTER, J. D. 2006. Human let-7a miRNA blocks protein production on actively translating polyribosomes. *Nat Struct Mol Biol*, 13, 1108-14.
- O'DONNELL, K. A., WENTZEL, E. A., ZELLER, K. I., DANG, C. V. & MENDELL, J. T. 2005. c-Myc-regulated microRNAs modulate E2F1 expression. *Nature*, 435, 839-43.
- OHMAN, M. 2007. A-to-I editing challenger or ally to the microRNA process. *Biochimie*, 89, 1171-6.
- OKAMURA, K., PHILLIPS, M. D., TYLER, D. M., DUAN, H., CHOU, Y. T. & LAI, E. C. 2008. The regulatory activity of microRNA* species has substantial influence on microRNA and 3' UTR evolution. *Nat Struct Mol Biol*, 15, 354-63.

- OLD, R. W., CALLAN, G. H. & GROSS, K. W. 1977. Localization of histone gene transcripts in newt lampbrush chromosomes by in situ hybridization. *J Cell Sci*, 27, 57-79.
- OZSOLAK, F., POLING, L. L., WANG, Z., LIU, H., LIU, X. S., ROEDER, R. G., ZHANG, X., SONG, J. S. & FISHER, D. E. 2008. Chromatin structure analyses identify miRNA promoters. *Genes Dev*, 22, 3172-83.
- PALL, G. S. & HAMILTON, A. J. 2008. Improved northern blot method for enhanced detection of small RNA. *Nat Protoc*, 3, 1077-84.
- PAROO, Z., YE, X., CHEN, S. & LIU, Q. 2009. Phosphorylation of the human microRNA-generating complex mediates MAPK/Erk signaling. *Cell*, 139, 112-22.
- PATTERSON, J. B. & SAMUEL, C. E. 1995. Expression and regulation by interferon of a double-stranded-RNA-specific adenosine deaminase from human cells: evidence for two forms of the deaminase. *Mol Cell Biol*, 15, 5376-88.
- PECULIS, B. A. & GALL, J. G. 1992. Localization of the nucleolar protein NO38 in amphibian oocytes. *J Cell Biol*, 116, 1-14.
- PETERSEN, C. P., BORDELEAU, M. E., PELLETIER, J. & SHARP, P. A. 2006. Short RNAs repress translation after initiation in mammalian cells. *Mol Cell*, 21, 533-42.
- PILLAI, R. S., BHATTACHARYYA, S. N., ARTUS, C. G., ZOLLER, T., COUGOT, N., BASYUK, E., BERTRAND, E. & FILIPOWICZ, W. 2005. Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science*, 309, 1573-6.
- PISKOUNOVA, E., VISWANATHAN, S. R., JANAS, M., LAPIERRE, R. J., DALEY, G. Q., SLIZ, P. & GREGORY, R. I. 2008. Determinants of microRNA processing inhibition by the developmentally regulated RNA-binding protein Lin28. *J Biol Chem*, 283, 21310-4.

- POLSON, A. G., BASS, B. L. & CASEY, J. L. 1996. RNA editing of hepatitis delta virus antigenome by dsRNA-adenosine deaminase. *Nature*, 380, 454-6.
- RADFORD, H. E., MEIJER, H. A. & DE MOOR, C. H. 2008. Translational control by cytoplasmic polyadenylation in *Xenopus* oocytes. *Biochim Biophys Acta*, 1779, 217-29.
- RAMACHANDRAN, V. & CHEN, X. 2008. Degradation of microRNAs by a family of exoribonucleases in *Arabidopsis*. *Science*, 321, 1490-2.
- RANA, T. M. 2007. Illuminating the silence: understanding the structure and function of small RNAs. *Nat Rev Mol Cell Biol*, 8, 23-36.
- REBAGLIATI, M. R. & MELTON, D. A. 1987. Antisense RNA injections in fertilized frog eggs reveal an RNA duplex unwinding activity. *Cell*, 48, 599-605.
- REENAN, R. A., HANRAHAN, C. J. & GANETZKY, B. 2000. The mle(napts) RNA helicase mutation in *Drosophila* results in a splicing catastrophe of the para Na⁺ channel transcript in a region of RNA editing. *Neuron*, 25, 139-49.
- RO, S., PARK, C., YOUNG, D., SANDERS, K. M. & YAN, W. 2007. Tissue-dependent paired expression of miRNAs. *Nucleic Acids Res*, 35, 5944-53.
- ROMANO, N. & MACINO, G. 1992. Quelling: transient inactivation of gene expression in *Neurospora crassa* by transformation with homologous sequences. *Mol Microbiol*, 6, 3343-53.
- ROSENTHAL, J. J. & BEZANILLA, F. 2002. Extensive editing of mRNAs for the squid delayed rectifier K⁺ channel regulates subunit tetramerization. *Neuron*, 34, 743-57.
- RYBAK, A., FUCHS, H., SMIRNOVA, L., BRANDT, C., POHL, E. E., NITSCH, R. & WULCZYN, F. G. 2008. A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. *Nat Cell Biol*, 10, 987-93.

- SAGATA, N., OSKARSSON, M., COPELAND, T., BRUMBAUGH, J. & VANDE WOUDE, G. F. 1988. Function of c-mos proto-oncogene product in meiotic maturation in *Xenopus* oocytes. *Nature*, 335, 519-25.
- SAGATA, N., WATANABE, N., VANDE WOUDE, G. F. & IKAWA, Y. 1989. The c-mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs. *Nature*, 342, 512-8.
- SAKAMOTO, S., AOKI, K., HIGUCHI, T., TODAKA, H., MORISAWA, K., TAMAKI, N., HATANO, E., FUKUSHIMA, A., TANIGUCHI, T. & AGATA, Y. 2009. The NF90-NF45 complex functions as a negative regulator in the microRNA processing pathway. *Mol Cell Biol*, 29, 3754-69.
- SALLES, F. J., LIEBERFARB, M. E., WREDEN, C., GERGEN, J. P. & STRICKLAND, S. 1994. Coordinate initiation of *Drosophila* development by regulated polyadenylation of maternal messenger RNAs. *Science*, 266, 1996-9.
- SAUNDERS, L. R. & BARBER, G. N. 2003. The dsRNA binding protein family: critical roles, diverse cellular functions. *FASEB J*, 17, 961-83.
- SCADDEN, A. D. 2005. The RISC subunit Tudor-SN binds to hyper-edited double-stranded RNA and promotes its cleavage. *Nat Struct Mol Biol*, 12, 489-96.
- SCADDEN, A. D. & SMITH, C. W. 2001a. RNAi is antagonized by A→I hyper-editing. *EMBO Rep*, 2, 1107-11.
- SCADDEN, A. D. & SMITH, C. W. 2001b. Specific cleavage of hyper-edited dsRNAs. *EMBO J*, 20, 4243-52.
- SCHUETZ, A. W. & SAMSON, D. 1979. Nuclear requirement of post-maturational cortical differentiation of amphibian oocytes: effects of cycloheximide. *J Exp Zool*, 210, 307-19.
- SEEBURG, P. H. & HARTNER, J. 2003. Regulation of ion channel/neurotransmitter receptor function by RNA editing. *Curr Opin Neurobiol*, 13, 279-83.

- SHEETS, M. D., WU, M. & WICKENS, M. 1995. Polyadenylation of c-mos mRNA as a control point in *Xenopus* meiotic maturation. *Nature*, 374, 511-6.
- ST JOHNSTON, D., BROWN, N. H., GALL, J. G. & JANTSCH, M. 1992. A conserved double-stranded RNA-binding domain. *Proc Natl Acad Sci U S A*, 89, 10979-83.
- STEBBINS-BOAZ, B., HAKE, L. E. & RICHTER, J. D. 1996. CPEB controls the cytoplasmic polyadenylation of cyclin, Cdk2 and c-mos mRNAs and is necessary for oocyte maturation in *Xenopus*. *EMBO J*, 15, 2582-92.
- SUH, M. R., LEE, Y., KIM, J. Y., KIM, S. K., MOON, S. H., LEE, J. Y., CHA, K. Y., CHUNG, H. M., YOON, H. S., MOON, S. Y., KIM, V. N. & KIM, K. S. 2004. Human embryonic stem cells express a unique set of microRNAs. *Dev Biol*, 270, 488-98.
- SUZUKI, H. I., YAMAGATA, K., SUGIMOTO, K., IWAMOTO, T., KATO, S. & MIYAZONO, K. 2009. Modulation of microRNA processing by p53. *Nature*, 460, 529-33.
- TANG, F., KANEDA, M., O'CARROLL, D., HAJKOVA, P., BARTON, S. C., SUN, Y. A., LEE, C., TARAKHOVSKY, A., LAO, K. & SURANI, M. A. 2007. Maternal microRNAs are essential for mouse zygotic development. *Genes Dev*, 21, 644-8.
- TANG, G. Q. & MAXWELL, E. S. 2008. *Xenopus* microRNA genes are predominantly located within introns and are differentially expressed in adult frog tissues via post-transcriptional regulation. *Genome Res*, 18, 104-12.
- TAY, J., HODGMAN, R. & RICHTER, J. D. 2000. The control of cyclin B1 mRNA translation during mouse oocyte maturation. *Dev Biol*, 221, 1-9.
- TOLIA, N. H. & JOSHUA-TOR, L. 2007. Slicer and the argonautes. *Nat Chem Biol*, 3, 36-43.

- TRABUCCHI, M., BRIATA, P., GARCIA-MAYORAL, M., HAASE, A. D., FILIPOWICZ, W., RAMOS, A., GHERZI, R. & ROSENFELD, M. G. 2009. The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. *Nature*, 459, 1010-4.
- TRIBOULET, R., CHANG, H. M., LAPIERRE, R. J. & GREGORY, R. I. 2009. Post-transcriptional control of DGCR8 expression by the Microprocessor. *RNA*, 15, 1005-11.
- VISWANATHAN, S. R., DALEY, G. Q. & GREGORY, R. I. 2008. Selective blockade of microRNA processing by Lin28. *Science*, 320, 97-100.
- WAGNER, R. W., SMITH, J. E., COOPERMAN, B. S. & NISHIKURA, K. 1989. A double-stranded RNA unwinding activity introduces structural alterations by means of adenosine to inosine conversions in mammalian cells and *Xenopus* eggs. *Proc Natl Acad Sci U S A*, 86, 2647-51.
- WALKER, J. C. & HARLAND, R. M. 2008. Expression of microRNAs during embryonic development of *Xenopus tropicalis*. *Gene Expr Patterns*, 8, 452-6.
- WASSERMAN, W. J. & MASUI, Y. 1975. Effects of cyclohexamide on a cytoplasmic factor initiating meiotic maturation in *Xenopus* oocytes. *Exp Cell Res*, 91, 381-8.
- WATANABE, N., VANDE WOUDE, G. F., IKAWA, Y. & SAGATA, N. 1989. Specific proteolysis of the c-mos proto-oncogene product by calpain on fertilization of *Xenopus* eggs. *Nature*, 342, 505-11.
- WATANABE, T., TAKEDA, A., MISE, K., OKUNO, T., SUZUKI, T., MINAMI, N. & IMAI, H. 2005. Stage-specific expression of microRNAs during *Xenopus* development. *FEBS Lett*, 579, 318-24.
- WIENHOLDS, E., KOUDIJS, M. J., VAN EEDEN, F. J., CUPPEN, E. & PLASTERK, R. H. 2003. The microRNA-producing enzyme Dicer1 is essential for zebrafish development. *Nat Genet*, 35, 217-8.

- WIGHTMAN, B., HA, I. & RUVKUN, G. 1993. Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell*, 75, 855-62.
- WU, L., FAN, J. & BELASCO, J. G. 2006. MicroRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci U S A*, 103, 4034-9.
- YANG, W., CHENDRIMADA, T. P., WANG, Q., HIGUCHI, M., SEEBURG, P. H., SHIEKHATTAR, R. & NISHIKURA, K. 2006. Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. *Nat Struct Mol Biol*, 13, 13-21.
- YI, R., O'CARROLL, D., PASOLLI, H. A., ZHANG, Z., DIETRICH, F. S., TARAKHOVSKY, A. & FUCHS, E. 2006. Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs. *Nat Genet*, 38, 356-62.
- YI, R., QIN, Y., MACARA, I. G. & CULLEN, B. R. 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev*, 17, 3011-6.
- ZENG, Y. & CULLEN, B. R. 2004. Structural requirements for pre-microRNA binding and nuclear export by Exportin 5. *Nucleic Acids Res*, 32, 4776-85.
- ZHANG, H., KOLB, F. A., BRONDANI, V., BILLY, E. & FILIPOWICZ, W. 2002. Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. *EMBO J*, 21, 5875-85.
- ZHANG, M. & YUAN, T. 1998. Molecular mechanisms of calmodulin's functional versatility. *Biochem Cell Biol*, 76, 313-23.

Curriculum Vitae

Dominik Muggenhumer

Personal Data

Date and place of birth: 29 December 1978, Ried i. I., Austria
Nationality: Austrian

Education

2006-present	PhD candidate in Genetics-Microbiology, University of Vienna PhD Thesis: „Processing, Stability and RNA editing of microRNAs in <i>Xenopus</i> oocytes and eggs“, Max F Perutz Laboratories (MFPL), Department of Chromosome Biology Supervisor: Dr Michael Jantsch
1999-2006	MSc in Genetics-Microbiology, University of Vienna Diploma Thesis: „Transgenic RNAi screen: R1-R6 Photoreceptor targeting in the <i>Drosophila</i> Visual System“, Institute of Molecular Biotechnology (IMBA) Supervisor: Dr Barry Dickson
1994-1998	BORG Grieskirchen, grammar school (additional tuition in Chemistry, Physics and Biology), School leaving certificate: Matura

Scientific Presentations

Poster presentation at the RNA 2008 - 13th Annual Meeting of the RNA Society, July 28-August 3 2008, Berlin, Germany

Poster presentation at the Keystone Symposia: The Biology of RNA silencing, April 25-30, 2009, Victoria BC, Canada

Danksagung

Ich möchte mich bei Dr. Michael Jantsch für die Möglichkeit, unter seiner Betreuung und auf Basis der ihm zuerkannten wissenschaftlichen Fördermittel des FWF und MFPL, meine Doktorarbeit durchzuführen sehr herzlich bedanken. Weiters danke ich meinen Kolleginnen und Kollegen aus dem Jantsch-Labor sowie allen Mitarbeitern des Departments für Chromosomenbiologie für die vielen interessanten Stunden in- und abseits des Labors.

Ein spezieller Dank geht natürlich an meine Eltern für die Ermöglichung meines Studiums und die liebevolle Unterstützung über all die Jahre.

Darüber hinaus bedanke ich mich bei meiner ganzen Familie, ganz besonders bei Edith und Andi, die immer offene Türen und ein offenes Ohr in Wien für mich hatten.

Danke auch an alle Freundinnen und Freunde, die mich auf dem Weg zu meiner Promotion begleitet und unterstützt haben.