농학석사학위논문

MicroRNA 생합성에 관여하는 DROSHA N-말단의 Contribution 에 대한 연구

Contribution of DROSHA N-terminus to MicroRNA Biogenesis

2021년 2월

서울대학교 대학원

농생명공학부 응용생명화학전공

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A Dissertation for the Degree of Master of Science

Contribution of DROSHA N-terminus to MicroRNA Biogenesis

February 2021

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이 논문을 농학석사학위논문으로 제출함

2020년 12월

서울대학교 대학원

농생명공학부 응용생명화학전공

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채광웅의 석사학위논문을 인준함

2021년 1월

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ABSTRACT

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MicroRNAs(miRNAs) are small non-coding RNAs of ~22nucleotides (nt) in length , which regulate post-transcription by silencing target gene. MicroRNA maturation is initiated by RNase III enzyme DROSHA that cleaves primary microRNA. DROSHA contains N-terminal proline-rich (P-rich) and arginine/serine-rich (R/S-rich) domains, a central domain (CED), two RNase III domains (RIIIDa and RIIIDb), and a dsRNA-binding domain (dsRBD). Although the role and structure of DROSHA are well documented, the role of DROSHA N-terminus (residues 1-390) is not yet known. In this study I did research on direct and indirect contribution of DROSHA N-terminus to miRNA processing. To check direct contribution, electrophoretic mobility shift assay (EMSA) was conducted between zebrafish DROSHA N-terminus and pri-miRNA. DROSHA N-terminus has a non-specific weak binding affinity to pri-miRNAs. To confirm indirect contribution, I experimented qRT-PCR to measure pri-miRNA upon knockdown of several RNA-binding protein (RBP) that

bind to DROSHA N-terminus. As a result, a subset of DROSHA N-terminus-binding-RBPs had marginal effects on diverse pri-miRNA processing. My studies broaden understanding of DROSHA N-terminus and further suggest the possibility that RBPs binding with DROSHA N-terminus engage in pri-miRNA processing.

Keywords: DROSHA, DROSHA N-terminus (residues 1-390), electrophoretic mobility shift assay, microRNA, pri-miRNA, qRT-PCR, RNA-binding protein,

Student Number: 2019-24410

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LIST OF ABBREVIATIONS

AGO	Argonaute
bp	Base pair
cDNA	Complementary DNA
DMEM	Dulbecco's modified eagle medium
DNA	Deoxynucleic acid
EMSA	Electrophoretic mobility shift assay
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GST-FH	Glutathione S-transferase, FLAG peptide and six histidine
miRNA	MicroRNA
nt	Nucleotide
NLS	Nuclear Localization Signal
PCR	Polymerase chain reaction
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
qRT-PCR	Quantitative real-time PCR
RIPA	Radioimmunoprecipitation assay
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
mRNA	Messenger RNA
rRNA	Ribosomal RNA
siRNA	Small interfering RNA

TBE	Tris-borate-EDTA
TGF-β	Transforming growth factor-β

INTRODUCTION

MicroRNAs(miRNAs) are small non-coding RNAs of ~22nucleotides (nt) in length , which regulate post-transcription by silencing to target gene (Bartel, 2018; Ha & Kim, 2014). Lin-4, the first miRNA, was discovered in the nematode, *C.elegans*, in 1993 by screening genome wide (R. C. Lee, Feinbaum, & Ambros, 1993; Wightman, Ha, & Ruvkun, 1993). Several years later, The second miRNA, let-7, was found in , *C.elegans* (Reinhart et al., 2000). Ever since it became known that the let-7 miRNA is conserved among a wide range of different species (Lagos-Quintana, Rauhut, Lendeckel, & Tuschl, 2001; Lau, Lim, Weinstein, & Bartel, 2001; R. C. Lee & Ambros, 2001; Pasquinelli et al., 2000), a number of miRNAs have been identified and thousands of confidence miRNA sequences have been uploaded in the miRNA database (miRBase) (Kozomara & Griffiths-Jones, 2011). In the canonical pathway of miRNA biogenesis, two RNase III enzymes, DROSHA and DICER, are concerned. RNA Polymerase II transcribes miRNA genes to longer precursor transcripts called as primary miRNA (pri-miRNA). RNase III enzyme DROSHA initially cleaves a pri-miRNA to release a ~60-80 nt short hairpin miRNA (pre-miRNA). After that, DICER cleaves a pre-miRNA near the hairpin loop to produce a ~22nt miRNA duplex (Denli, Tops, Plasterk, Ketting, & Hannon, 2004; Gregory et al., 2004; Grishok et al., 2001; Han et al., 2004; Hutvagner et al., 2001; Ketting et al., 2001; Kwon et al., 2016; Y. Lee et al., 2003; Nguyen et al., 2015). The duplex is loaded into Argonaute protein (AGO), and one strand is unwound. As a result, The miRNA-induced silencing complex (miRISC) is formed (Hammond, Boettcher, Caudy, Kobayashi, & Hannon, 2001; Tang et al., 2013). Positions 2-7 (relative to the 5' end) of miRNA in the miRISC recognize complementary sites of its targets to effect post-transcriptional repression (Bartel, 2009; Jonas & Izaurralde, 2015).

Human DROSHA contains two catalytic RNase III domains (RIIIDs) and one double stranded RNA-binding domain (dsRBD) at its carboxyl terminus, which perform critical role in pri-miRNA processing. Two RIIIDs form intramolecular dimer to develop a catalytic domain to process pri-miRNA. The first RIIID (RIIIDa) and the second RIIID (RIIIDb) cleaves 3' strand and 5' strand of substrate pri-miRNA stem, respectively (Han et al., 2004). As a result of pri-miRNA cleavage, A pre-miRNA with a two-nucleotide-long 3' overhang is produced, which is necessary for interaction with XPO5 transporting pre-miRNA to cytoplasm (Okada et al., 2009). dsRBD in human DROSHA interacts with pri-miRNA substrate, but this interaction is insufficient. For this reason, DROSHA replenishes its dsRNA-binding ability by interacting with its co-partner DGCR8 which perform critical roles with its dsRBDs (Han et al., 2004).

Although much is known about the catalytic domains such as central domain (CED), RIIIDs, and dsRBD which plays core role in microRNA processing, little is known about regulatory domains, P-rich and RS-rich domain at the amino terminal region of DROSHA. Furthermore, catalytic domain of DROSHA are highly conserved among metazoan orthologs while regulatory domains, N-terminus of human DROSHA, are not conserved (Kwon et al., 2016). Despite of poor conservation of its N-terminus, its several functions have been reported. Human DROSHAs provide a platform for its stability control by several post-translational modifications. For example, N-terminus of DROSHA is a target for ubiquitination which leads proteins to be degraded, and acetylation at N-terminus inhibit its degradation to control stability (Tang et al., 2013). Under stress condition, N-terminus of DROSHA is phosphorylated by p38 MAPK to be localized to cytoplasm, followed by degradation (Yang et al., 2015). In addition of these functions, N-terminus of DROSHA interacts with RNA-binding protein (RBP) CBP80 and RNA polymerase II, which regulates gene expression in RNA cleavage-independent manner (Gromak et al., 2013). Of note, phosphorylation at serine300 and serine302

by glycogen synthase kinase 3 beta (GSK3β) is responsible for nuclear translocation of human DROSHA (Tang, Li, Tucker, & Ramratnam, 2011; Tang, Zhang, Tucker, & Ramratnam, 2010). Similarly, DROSHA splice isoforms different from its N-terminal sequence showed distinctive distribution, which showed nuclear localization function of DROSHA N-terminus (Dai et al., 2016; Link, Grund, & Diederichs, 2016).

Although several roles of human DROSHA N-terminus were reported and previous research reported that N-terminus of DROSHA is dispensable for microRNA processing, the effects of DROSHA N-terminus on pri-miRNA processing is still elusive (Han et al., 2004). Here, I observed differentially processed subset of certain pri-miRNA in absence of N-terminus of DROSHA. I became interested in certain function of N-terminus of human DROSHA in miRNA processing aside from other reported functions such as nuclear localization, stability control, and protein-protein interaction. Dr. Doo Young Lee. performed small RNA sequencing using DROSHA knockout cell line with variant forms of ectopic DROSHA with or without N-terminus of DROSHA. So subsets of pri-miRNAs which are dependent on DROSHA N-terminus to be fully processed into mature microRNAs are classified. Furthermore I did an experiment to know the direct and indirect contribution of DROSHA N-terminus to pri-miRNA processing.

MATERIALS AND METHODS

Plasmids

pCK-DROSHA-Flag and pCK-Flag-DGCR8 were kindly provided by Dr. V. Narry Kim (Seoul National University). To generate truncated DROSHA in its N-terminus (pCK-DROSHA$^{\Delta N220}$-FLAG, pCK-DROSHA$^{\Delta N390}$-Flag), Dr. Doo Young Lee. subcloned truncated DROSHA-FLAG into HindIII and NotI site of pCK vector. Triple NLS of SV40 were ligated to pCK-DROSHA$^{\Delta N390}$-Flag to prepare pCK-DROSHA $^{\Delta N390}$-3XNLS. For luciferase reporter assay, pri-miRNAs containing its genomic pre-miRNA sequences and its flanking sequences(~120bp) from genomic DNA of HeLa cells or plasmid constructs used elsewhere(D. Lee, Nam, & Shin, 2017; D. Lee, Park, Park, Kim, & Shin, 2019) were subcloned into XhoI and NotI site of psicheck-2 vector (Promega), kindly provided by Daehyun Baek (Seoul National University). N-terminal sequence of *G. gallus* DROSHA (residues 1~354) is amplified from cDNA, a gift from Dr. Jae Yong Han (Seoul National University) and the DROSHA coding sequences of *X. tropicalis* (residues 1~346*)* and *D. rerio* (residues 1~307) were synthesized through gBLOCK gene synthesis (IDT co.), followed by subcloning into pCK-DROSHA$^{\Delta N390}$-Flag. All Cloning procedures were performed by using the overlap cloner DNA cloning kit (Elpis biotech).

Cell culture and transfection

HEK293T cells and its DROSHA KO derivatives were cultured in DMEM (Welgene) supplemented with 9% FBS (Welgene). HCT116 cells and its DROSHA KO derivatives were cultured in McCoy's 5A (Welgene) supplemented with 9% FBS

(Welgene). Sf9 cells were cultured in Sf-900™ II SFM (Gibco) supplemented with 5% FBS (Welgene). All procedures for transfection of plasmid and oligonucleotides are performed with lipofectamine 2000 (ThermoFisher scientific), according to the manufacturer's instructions.

CRISPR/Cas9-mediated gene knockout

To generate DROSHA KO HEK293T cells, single-guide RNAs targeting the 20th coding exon of DROSHA gene were annealed and ligated into pSpCas9(BB)-2A-Puro (Addgene; ID 48139) vector. HCT116 cells transfected with CRISPR/Cas9-mediated knockout constructs were screened by selective medium containing 3 μg/ml of puromycin (Gibco). Single cell-derived colonies were isolated and expanded. The absence of DROSHA protein of isolated monoclonal cells was confirmed by western blotting and genotyping ensured frame-shifting mutations in the DROSHA gene. DROSHA KO HCT116 cells, a gift from Dr. V. Narry Kim (Seoul National University), were generated as previously described. (Kim, Kim, & Kim, 2016)

Western blotting analysis

Cultured cells were washed with ice cold 1X PBS and scraped to collect cell pellet. Cell pellets were incubated with RIPA buffer (50mM Tris-HCl at pH 8.0, 150mM NaCl, 1 % NP-40, 0.1 % SDS, 0.1% sodium deoxycholate, 1mM DTT and 1X complete Protease Inhibitor Cocktail [Roche Life Science]) on ice for 30 min with intermittent vortexing to obtain crude extract. The supernatant containing total protein was collected by centrifugation at 15,000 rpm for 10 min. 30 to 50 μg of resulting cell lysate were separated on a 10 % SDS-polyacrylamide gel and transferred to Immobilon-P PVDF membrane (EMD Millipore). The antibody used for western blotting were rabbit anti-FLAG (Sigma, F7425), rabbit anti-α-tublin

(Abcam, ab52866), mouse anti-hnRNP A1 (4B10; EMD Millipore, 05-1521), rabbit anti-DROSHA (Abcam, ab12286), rabbit anti-DGCR8 (a gift from Dr. V. Narry Kim), rabbit anti-DICER1 (Cell Signaling Technology, 5362T), rabbit anti-DDX5 (Abcam, ab21696), rabbit anti-DDX17 (Abcam, ab24601), rabbit anti-TDP43 (Proteintech, 12892-1-AP), mouse anti-hnRNP C1/C2 (a gift from Dr. V. Narry Kim) and rabbit anti-RNA PolII (Santa cruz, sc-9001) IgG antibodies. The secondary antibodies for chemiluminescent detection were horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG antibodies (Jackson ImmunoResearch, West Grove, PA, USA).

Expression and purification of recombinant proteins

To obtain DROSHA N-terminus GST-FLAG-tagged protein, oligonucleotides corresponding to the GST-FLAG peptide were inserted into the pFastBac-His plasmid (Invitrogen). Recombinant human and zebrafish DROSHA N-terminus proteins were expressed in insect cells using the Bac-to-Bac Baculovirus Expression System (Invitrogen) according to the manufacturer's instructions. Briefly, recombinant baculovirus DNA was transfected using Cellfectin II Reagent (Invitrogen) into Sf9 cells grown at 26°C in Sf-900 III medium (Invitrogen). Sf9 cells were infected with the recombinant virus for 48 h, harvested and washed with PBS. To obtain eGFP GST-FLAG-HIS-tagged protein, the bacterial expression system was used. The bacterial expression plasmids were introduced into Rosetta2(DE3) *E. coli* cells (EMD Millipore), which were grown at 37°C to OD_{600} of ~0.6 and treated with 0.5 mM IPTG at 16°C overnight to induce protein expression. Briefly, 500ml of bacterial culture was harvested. SF9 Cells and Rosetta2 were lysed in lysis buffer (20 mM Tris-Cl, pH 7.4, 100 mM KCl, 0.1% Triton X-100, 10% glycerol, 0.2mM EDTA, and 1× EDTA-free Protease Inhibitor cocktail [Roche]) and sonicated five 6-s bursts at

35% amplitude. After centrifugation at 18,500 rpm at 4°C for 25 min, the supernatant was binding to anti-FLAG M2 Affinity Gel (Sigma), washed with wash buffer (20 mM Tris-Cl (pH7.4), 1M KCl, 0.2 mM EDTA, 10% Glycerol) and eluted with a 3× FLAG peptide (Sigma) and stored in aliquots containing 10% (v/v) glycerol at –80°C.

Native gel mobility-shift assay

Native gel mobility-shift assay was carried out with the following condition: 0.2nM of radiolabeled pri-miR-21, 22, 221, 222, 100nM-1μM of recombinant zebrafish DROSHA N-terminus, 1mM DTT, 1μg of BSA (Takara) in binding buffer (20mM HEPES at pH 7.4, 50mM KCl, 0.5mM EDTA, 10% (v/v) Glycerol). The reaction was incubated at 37°C for 10 min. RNA–protein complexes were analyzed by 6% native polyacrylamide gel electrophoresis at 120V in a 4°C cold room.

Quantitative real-time PCR

To measure pri-miRNA levels, siRNA of RBP candidates binding with DROSHA N-terminus was transfected to knock-down endogenous RBPs expression in HEK293T parental cells. After 48 hours post-transfection of siRNA, the total RNA extraction was performed using RiboEx solution (GeneAll Biotechnology Co.), according to the manufacturer's instuctions. Total RNA was also treated with DNaseI (Takara) to prevent from contamination with genomic DNA. Purified total RNA was reverse transcribed with PrimeScript reverse transcriptase (Takara) using either oligo dT or random hexamer to synthesize cDNA. Quantitative RT-PCR was performed based on SYBR Green using *AccuPower® 2X GreenStar™ qPCR Master Mix (Bioneer) and analyzed with* LightCycler 480 Instrument II (Roche Life Science). Pri-miRNA levels were normalized to GAPDH.

Small RNA sequencing and analysis

Small RNA sequencing libraries were prepared according to the protocol described elsewhere(Fu, Wu, Beane, Zamore, & Weng, 2018) with minor modifications. In brief, 15 μg of Total RNA in DROSHA KO HCT116 cells was isolated with RiboEX solution and separated with formamide loading buffer on 15 % urea-polyacrylamide gel at 15 W for 45 min. RNAs between 18 – 30 nt RNA markers were purified and 3' adaptor was ligated with T4 RNA Ligase 2, truncated (New England Biolabs). The resulting RNAs were resolved on 15 % urea-acrylamide gel. 35 – 52 nt RNA was purified and 5' adaptor was ligated with T4 RNA Ligase (Ambion). The ligated RNA was reverse transcribed with PrimeScript (Takara), amplified with Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific). Sequencing procedure was conducted with HiSeq 2500 platform (Illumina).

Small RNA northern blot analysis

Total RNA was prepared using RiboEX and 10 μg of total RNAs were separated on 12.5 % urea-polyacrylamide gel at 15 W, transferred to Hybond N+ membrane (GE Healthcare) at 5 V for 1 hr, fixed by UV crosslinking (0.12 J), and baked at 80 °C. Radioisotope was labeled to 5' end of DNA oligonucleotides complementary to miR-21-5p, miR-221-3p, miR-222-3p, let-7i-5p, let-7g-5p, miR-320a-3p, U6 snRNA and 5S rRNA by T4 Polynucleotide Kinase (New England Biolabs). After overnight hybridization, phosphorimaging was performed using a BAS-2500 image analyzer (Fujifilm).

RESULTS

N-terminus of DROSHA affects the subset of mature microRNAs abundance

To explore the contribution of DROSHA N-terminus to the miRNA processing, Dr. Doo Young Lee. generated DROSHA knockout (KO) HEK293T cells using CRISPR/Cas9 system. A guide RNA was designed to target the 20th coding exon of the DROSHA gene. Puromycin-resistant clones were isolated and western blot analysis and genotyping confirmed that endogenous DROSHA KO cell lines were successfully established. DROSHA KO HCT116 cells were generated by RNA-guided endonuclease as described elsewhere (Kim et al., 2016). Considering that primary microRNA is processed by DROSHA to form precursor microRNA to be further processed into mature microRNA duplex, accumulation of pri-miRNAs caused by low processing efficiency of DROSHA results in corresponding mature miRNAs depletion. Thus, Dr. Doo Young Lee. performed small RNA deep sequencing on ~18-30 nt RNA from DROSHA KO HCT116 cells upon expression of either Wild-type or Δ390-3xNLS (Figure 1A). Based on the data from small RNA sequencing data. Dr. Doo Young Lee. analyzed the abundance difference of mature microRNA processed by either Wild-type or Δ390-3xNLS DROSHA. As a result, 764 of mature microRNAs level were decreased while 329 of which were increased upon processing by Δ390-3xNLS as compared with processing by Wild-type DROSHA (Figure 1B).

DROSHA N-terminus is conserved not in its sequences but in function on primary microRNA processing among vertebrates

The observation that N-terminus of human DROSHA is necessary to process a subset of primary microRNA prompted to study whether the N-terminus of DROSHA is conserved. Multiple alignment of vertebrate DROSHA among *G.gallus , X.tropicalis* and *D.rerio* revealed that N-terminus of vertebrate DROSHA (1-390) has low homology while RIIIDa (916-1056) and dsRBD (1234-1374) is highly conserved in its sequences (Figure 2). So There was a need to find out if the N-terminus of DROSHA is conserved not in its amino acid sequences but in function on primary microRNA processing among vertebrates. To confirm that conservation of DROSHA N-terminal function, Dr. Doo Young Lee. done remote homology detection using HHPred. Remote homology detection using HHPred showed that human (*H.sapiens*) DROSHA N-terminus and zebrafish (*D.rerio*) DROSHA N-terminus have mutual proteins which contain high sequence homology such as HPRP28, U1-70K, PRP8 whereas DROSHA N-terminus of fly as invertebrate was revealed only its short region has low homology with PRP8 (Figure 3). As confirmed in small RNA sequencing data, the levels of miR-21-5p, miR-221-3p and let-7i-5p were decreased by Δ220, Δ390 and Δ390-3xNLS, Interestingly, *Dre*N'-Δ390 could nearly rescue miRNAs level to which level processed by Wild-type DROSHA. On the other hand, miR-222-3p and let-7g-5p which was not influenced by presences of N-terminus of human DROSHA is also processed to the similar level by all constructs transfected in the DROKO HCT116 cells (Figure 4). These data suggested that N-terminus of DROSHA is conserved among vertebrates in microRNA processing functions caused not by sequences but common motif.

Purification of DROSHA N-terminus expressed in insect cell

To set up an electrophoretic mobility shift assay (EMSA), I initially tried to purify the human and zebrafish DROSHA N-terminus protein from *E. coli* systems but failed to do so due to poor expression. I next employed baculovirus expression

system based on a sf9 cell line, derived from the parental Spodoptera frugiperda cell line IPLB-Sf-21-AE. The DROSHA N-terminus proteins contained Glutathione S-transferase, FLAG peptide and six histidine (GST-FH) tag. For protein expression, I first extracted five plasmids with cloned human DROSHA N-terminus (*Hsa*N') genes and four plasmids with cloned zebrafish DROSHA N-terminus (*Dre*N') genes from *E. coli* DH10Bac strain. Each of the four bacmids was transfectioned into the sf9 cell, and the protein expression was checked (Figure 5A). After each of the four P1 virus stocks infected with the sf9 cell, the protein expression was checked (Figure 5B). P2 virus stocks of *Hsa*N' #2 and *Dre*N' #3 with high protein expression in P1 virus infection infected with the sf9 cell, and the protein expression was checked (Figure 5C). *Hsa*N' #2 protein expression was too low in the P2 virus infection, so I selected *Dre*N' #3 in the P3 virus stock and it infected with a large scale of sf9 cell to express the protein. After Harvesting the large scale of sf9 cell infected P3 virus, I used anti-flag Affinity gel to purify the protein. I checked *Dre*N'-GST-FH in the band of expected size by silver staining (Figure 6A). Also The eGFP-GST-FH to be used as the negative control of EMSA was purified from *E. coli* (Figure 6B).

DROSHA N-terminus has a non-specific weak binding affinity to pri-miRNAs

To confirm the direct contribution of DROSHA N-terminus to pri-miRNA processing, I experimented EMSA with pri-miRNA-21, 221 dependent on N-terminus (Figure 7A) and pri-miRNA-22, 222 independent on N-terminus (Figure 7B) in small RNA seq. I used purified eGFP-GST-FH that binds to RNA as a negative control. And as positive control, I used V5 fused DGCR8 which had already been purified by Dr. Doo Young Lee. The RNA substrates were internally labeled by [γ - ^{32}P] UTP. I analyzed DROSHA N-terminus binding affinity with pri-miRNA-21, 22. In buffers and eGFP-GST-FH, the band's shift was not visible. No band shift was observed at low concentration *Dre*N'-GST-FH. However as the concentration of

*Dre*N'-GST-FH increased, the band's shift occurred. But in the similar concentration of DGCR8, which is known to bind to pri-miRNA, the band's shift became more severe. And there was no difference in band shift in pri-miRNA-21, 22. The result of EMSA with pri-miRNA-221, 222 also showed similar result to EMSA with pri-miRNA-21, 22. These results suggest that DROSHA N-terminus has a non-specific weak binding affinity.

There are RBPs to bind with DROSHA N terminus

The RNA-binding protein Lin-28 negatively regulates let-7 maturation by direct interactions with terminal loop of let-7 precursor. From the evidence that N-terminus of DROSHA interacts with RBPs in cleavage-independent manner, Dr. Doo Young Lee. hypothesized DROSHA N-terminus interacts heterogenous complex of RNA-binding protein to differentially process a subset of pri-miRNA. Dr. Doo Young Lee. immunoprecipitated variant forms of DROSHA (Wild-type, Δ390-3xNLS, DreN'-Δ390) and analyzed which RBPs interacts with N-terminus of DROSHA. Axillary cofactors, DDX5, DDX17 (p82/p72) and TDP-43, appeared to interact with DROSHA N-terminus and especially TDP-43 interacts with DROSHA in RNA-dependent manner. Interestingly, hnRNA-associated RBP, hnRNP A1 and hnRNP C1/C2 were also co-immunoprecipitated with DROSHA (Figure 8). These results suggested that N-terminus of DROSHA interacts with a variety of heterogenous RBP complex not yet been fully identified and can have influences on pri-miRNA processing.

Some RBP affect pri-miRNA processing.

To confirm the indirect contribution of DROSHA N-terminus to pri-miRNA processing, I experimented qRT-PCR of pri-miRNA for the knockdown of the RBP

candidates that bind to DROSHA N-terminus (Figures 8 and 9). HEK293T cells were transfected with siRNA against the RBP candidates and siGFP as negative control. After that, I checked quantification of pri-miRNA levels for DROSHA N-terminus dependent and independent pri-miRNA. The expression levels of specific pri-miRNAs were higher than 1.5-fold at the knockdown of RBP candidates compared to control. The results suggested that specific DROSHA N-terminus binding RBPs affect specific or diverse pri-miRNA processing. But unlike the results I expected, N-terminus independent pri-miRNAs were seen affected by the RBP candidates. In conclusion, I think that the RBP candidate's influence is greater than that of the RBP candidates itself rather than the effect of its association with DROSHA N-terminus in pri-miRNA processing.

DISCUSSION

In this study, I observed only a subset of pri-miRNA is not processed by DROSHA truncated in its N-terminus. To uncover why this dependency on DROSHA N-terminus happens, I attempted several approaches. I hypothesized that N-terminus of DROSHA may directly or indirectly interacts preferentially with a subset of pri-miRNA in processing the pri-miRNA by microprocessor. To figure out whether N-terminus of DROSHA itself has binding affinity to pri-miRNA and contribute to differential expression in a miRNA, I performed EMSA. Due to the difficulties in expressing and purifying N-terminus of human DROSHA, N-terminus of zebra fish DROSHA was used in EMSA instead of one of human DROSHA. Interestingly, result from EMSA demonstrated that N-terminus of zebrafish interacts with pri-miR-21, 22, 221 and 222 in a concentration dependent manner. This result suggested that N-terminus of DROSHA itself interacts with pri-miRNAs. However, there were no significant differences in binding affinity between pri-miRNAs dependent on N-terminus and not dependent on N-terminus. From this understanding, I focused on the indirect contribution of N-terminus of DROSHA.

Several studies implied possibilities that RNA-binding proteins interacting with N-terminus of DROSHA to regulate specific microRNAs maturation. For example, the Smad protein, the signal transducer of transforming growth factor ß (TGF-ß)/ bone morphogenetic protein, binds DROSHA to enhance pri-miR-21 processing by DROSHA.(Davis, Hilyard, Lagna, & Hata, 2008) Furthermore, not only pri-miR-21 but a subset of pri-miRNA containing conserved motifs in its stem regions recruits Smad proteins to be cleaved by direct binding to DROSHA in a

Smad-dependent manner.(Davis, Hilyard, Nguyen, Lagna, & Hata, 2010) Interestingly, two rare mutations of DROSHA in N-terminus (P100L and R279L) were unable to interacts with Smad proteins and are associated with abnormal vascular phenotype in hereditary hemorrhagic telangiectasia patients, suggesting that DROSHA directly interacts with Smad proteins through its N-terminus and influences abundance of a subset of microRNAs.(Jiang et al., 2018) Several RBP candidates were chosen by co-immunoprecipitated with DROSHA. I experimented qRT-PCR of pri-miRNA for the knockdown of the RBP candidates(Figure 8) that bind to DROSHA N-terminus. Unfortunately, it seemed that the RBP candidate's influence was greater than that of the RBP candidates itself rather than the effect of its association with DROSHA N-terminus in pri-miRNA processing.

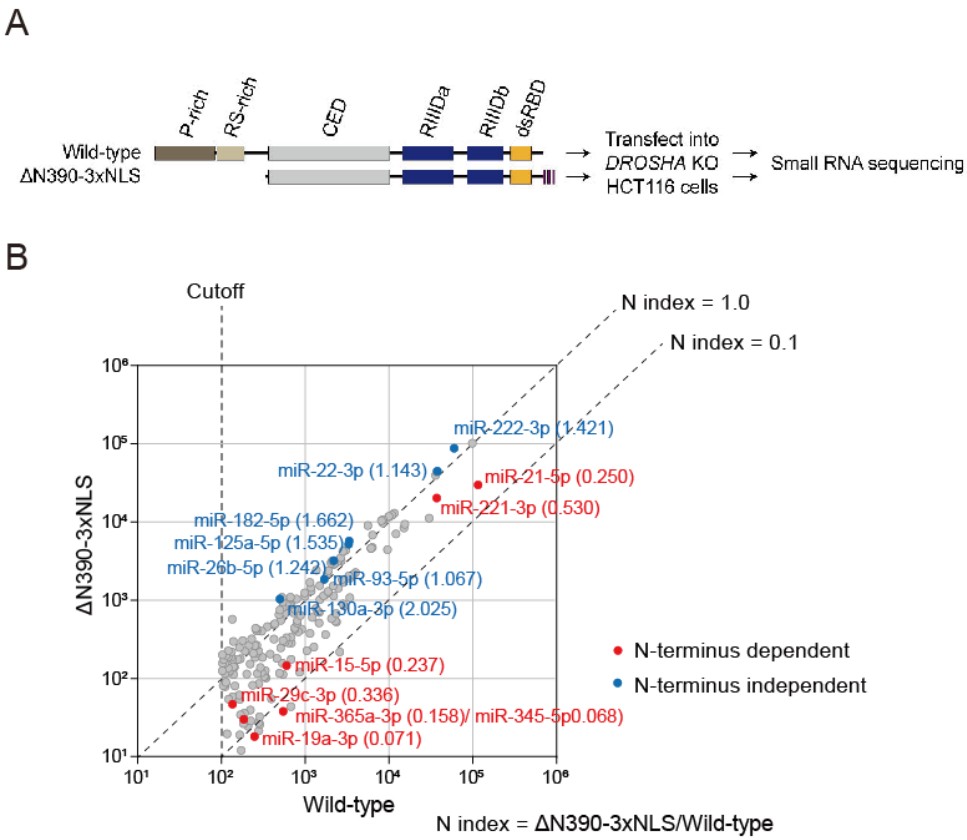
In conclusion, expecting that N-terminus of DROSHA interacts with various and heterogeneous complex of RNA-binding proteins, it seems possible that a subset of microRNA could be affected by interaction between RNA-binding protein and DROSHA N-terminus similarly to Smad Proteins. So, mass spectrometry analysis should be performed to figure out more RBPs that combine with DROSHA N-terminus. Furthermore, knockdown experiment based on results from mass spectrometry analysis will deepen specific function of DROSHA N-terminus in pri-miRNA processing.

FIGURES AND TABLES

Figure 1. The impact of DROSHA N-terminus on the HCT 166 miRNAome.

(A) Wild type DROSHA and 1-390 residues deletion DROSHA fused 3XNLS (nuclear localization signal) was transfected into *DROSHA* KO HCT 166 cells to make a difference of N-terminus.

(B) Small RNA sequencing data. N index = miRNA levels of DROSHA $^{\Delta N390}$-3XNLS / miRNA levels of wild type DROSHA. Blue dots are N-terminus independent miRNAs and Red dots are N-terminus dependent miRNAs.



This experiment was conducted by Dr. Doo Young Lee.

Figure 2. Analysis of conserved DROSHA between vertebrates.

Multiple alignment of vertebrate DROSHA among *H.sapiens*, *G.gallus , X.tropicalis* and *D.rerio.* N-terminus of vertebrate DROSHA (residues 1-390) has low homology while RIIIDa (residues 916-1056) and dsRBD (residues 1234-1374) is highly conserved in its sequences.

Multiple alignment of vertebrate DROSHA(*H. sapiens, G. gallu, X. tropicalis and D. rerio*)

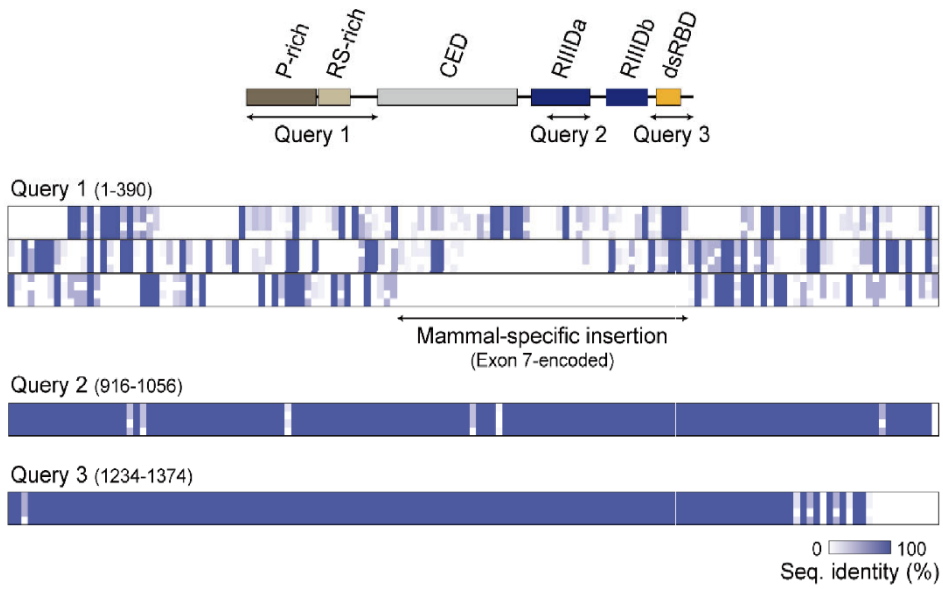


Figure 3. Remote homology detection of DROSHA N-terminus using HHPred.

Human (*H.sapiens*) DROSHA N-terminus and zebrafish (*D.rerio*) DROSHA N-terminus have mutual proteins which contain high sequence homology such as HPRP28, U1-70K, PRP8 whereas DROSHA N-terminus of fly as invertebrate was revealed only its short region has low homology with PRP8.

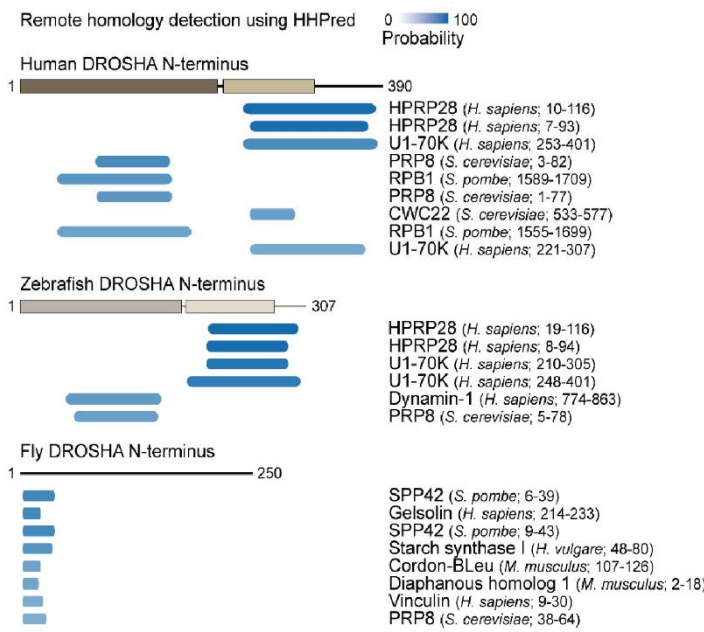
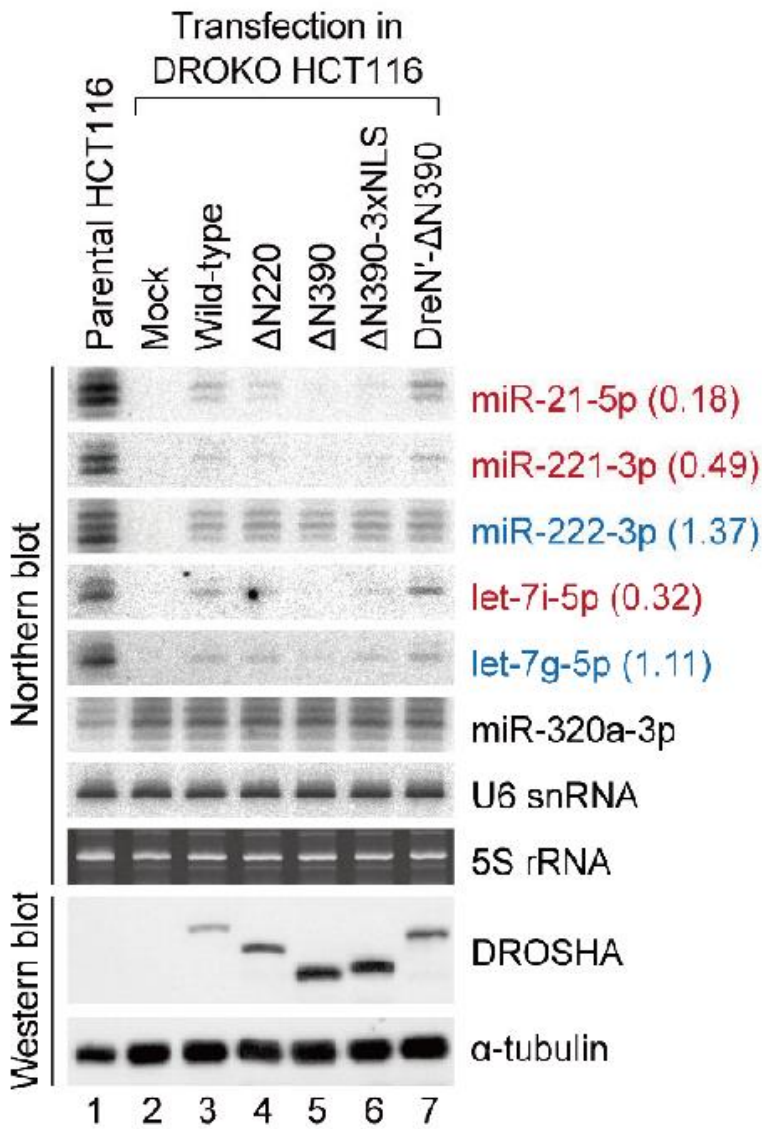

Remote homology detection using HHPred
0
100
Probability
Human DROSHA N-terminus
1
390
HPRP28 (H. sapiens; 10-116)
HPRP28 (H. sapiens; 7-93)
U1-70K (H. sapiens; 253-401)
PRP8 (S. cerevisiae; 3-82)
RPB1 (S. pombe; 1589-1709)
PRP8 (S. cerevisiae; 1-77)
CWC22 (S. cerevisiae; 533-577)
RPB1 (S. pombe; 1555-1699)
U1-70K (H. sapiens; 221-307)
Zebrafish DROSHA N-terminus
1
307
HPRP28 (H. sapiens; 19-116)
HPRP28 (H. sapiens; 8-94)
U1-70K (H. sapiens; 210-305)
U1-70K (H. sapiens; 248-401)
Dynamin-1 (H. sapiens; 774-863)
PRP8 (S. cerevisiae; 5-78)
Fly DROSHA N-terminus
1
250
SPP42 (S. pombe; 6-39)
Gelsolin (H. sapiens; 214-233)
SPP42 (S. pombe; 9-43)
Starch synthase I (H. vulgare; 48-80)
Cordon-Bleu (M. musculus; 107-126)
Diaphanous homolog 1 (M. musculus; 2-18)
Vinculin (H. sapiens; 9-30)
PRP8 (S. cerevisiae; 38-64)

Figure 4. Functionally conservation between human and zebrafish DROSHA N-terminus.

miR-21-5p, miR-221-3p and let-7i-5p are N-terminus dependent miRNAs. On the other hand, miR-222-3p and let-7g-5p are N-terminus independent miRNAs. U6 snRNA, 5S rRNA, and miR-320a-3p served as loading controls.



This experiment was conducted by Dr. Doo Young Lee.

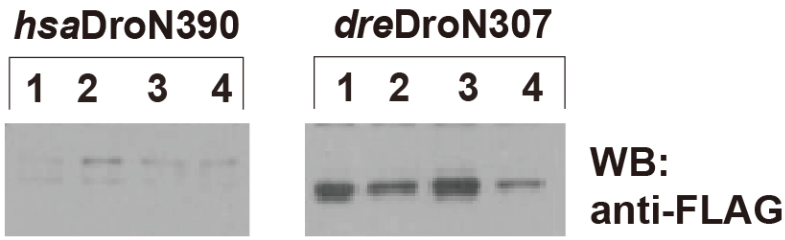
Figure 5. Recombinant protein expression test in SF9 cells.

(A) Expression test of human and zebrafish DROSHA N-terminus GST-FH after recombinant baculovirus DNA was transfected into Sf9 cells.

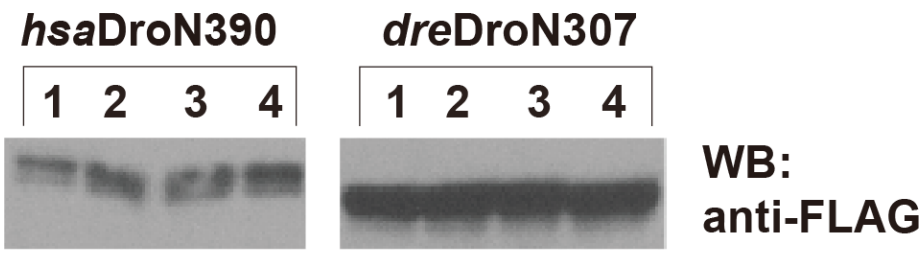
(B) Expression test of human and zebrafish DROSHA N-terminus GST-FH after Sf9 cells were infected by P1 virus stock.

(C) Expression test of human and zebrafish DROSHA N-terminus GST-FH after Sf9 cells were infected by P2 virus stock.

A Western blotting



B Western blotting



C Western blotting

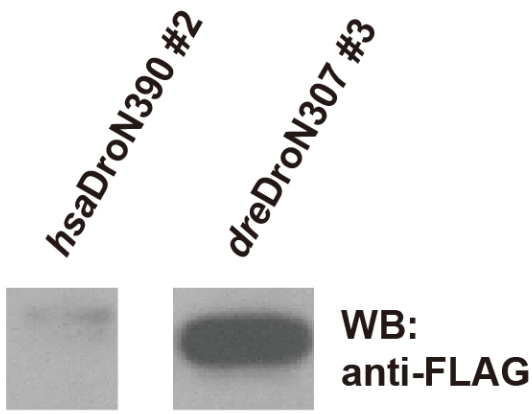


Figure 6. Purification of recombinant proteins for EMSA.

(A) Silver staining of recombinant zebrafish DROSHA N-terminus tagged GST-FLAG-HIS produced in and purified from Sf9 cells.

(B) Coomassie brilliant blue staining of recombinant eGFP tagged GST-FLAG-HIS produced in and purified from *E. coli*.

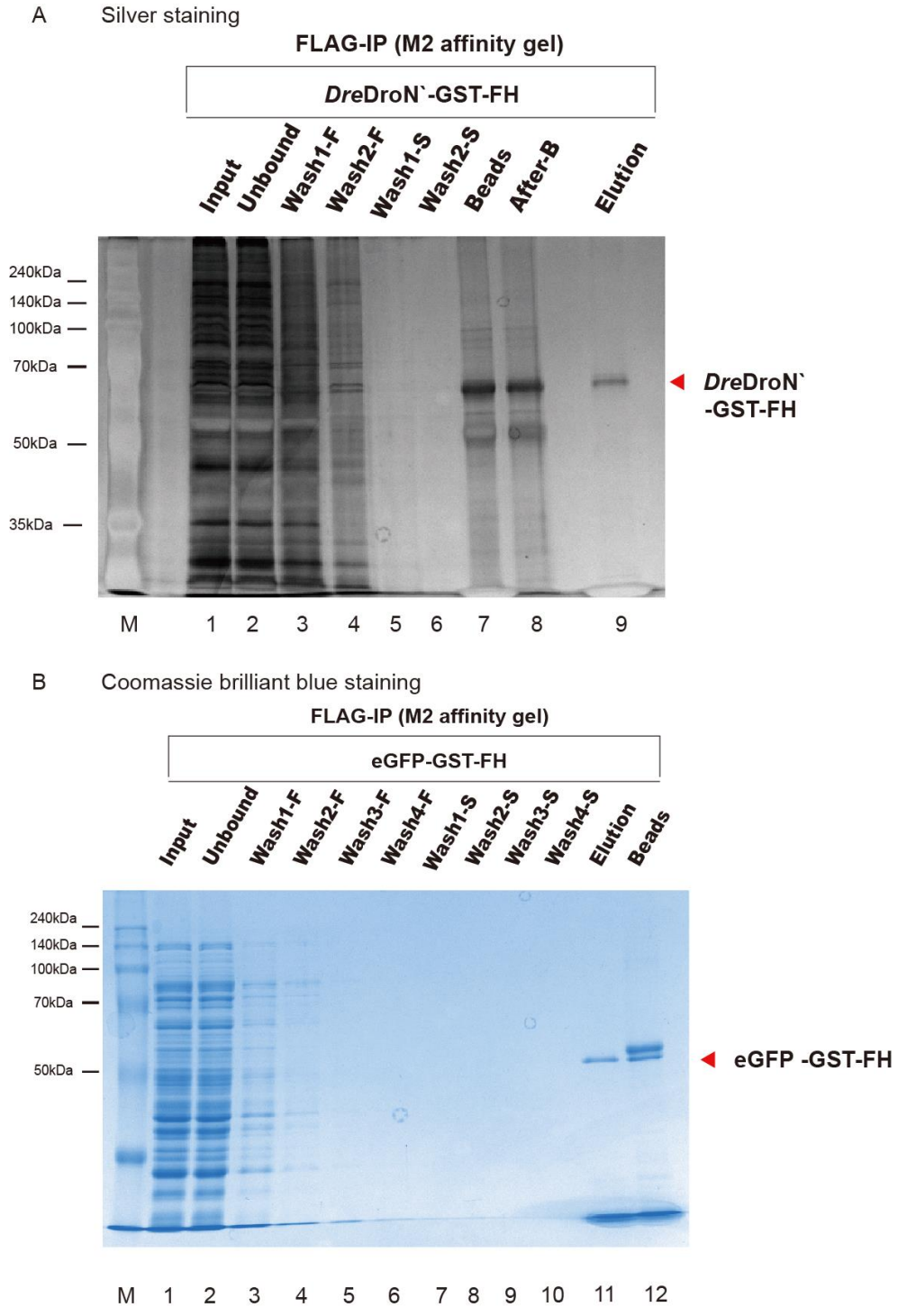
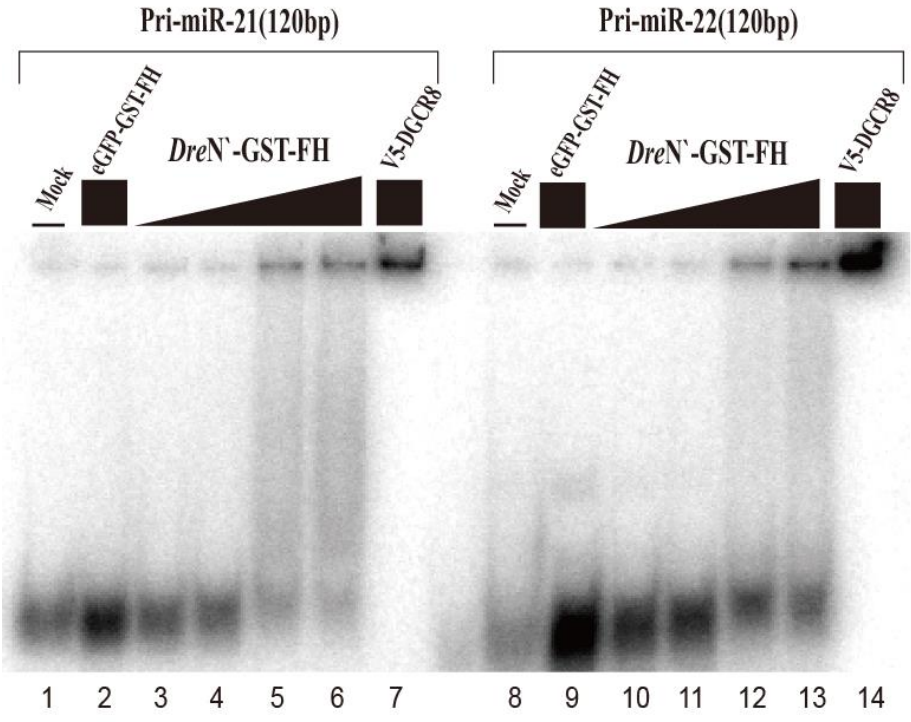

A
Silver staining
FLAG-IP (M2 affinity gel)
DreDroN`-GST-FH
Input
Unbound
Wash1-F
Wash2-F
Wash1-S
Wash2-S
Beads
After-B
Elution
240kDa
140kDa
100kDa
70kDa
50kDa
35kDa
DreDroN`
-GST-FH
M 1 2 3 4 5 6 7 8 9
B
Coomassie brilliant blue staining
FLAG-IP (M2 affinity gel)
eGFP-GST-FH
Input
Unbound
Wash1-F
Wash2-F
Wash3-F
Wash4-F
Wash1-S
Wash2-S
Wash3-S
Wash4-S
Elution
Beads
240kDa
140kDa
100kDa
70kDa
50kDa
eGFP -GST-FH
M 1 2 3 4 5 6 7 8 9 10 11 12

Figure 7. A non-specific weak binding affinity to pri-miRNAs of DROSHA N-terminus.

Native gel mobility-shift assay of zebrafish DROSHA N-terminus with pri-miR-21, 22, 221 and 222. Pri-miR-21 and pri-miR-221 are N-terminus dependent pri-miRNAs. Pri-miR-22 and pri-miR-222 are N-terminus independent pri-miRNAs. EGFP-GST-FH served as negative control. V5-DGCR8 served as positive control.

A EMSA_pri-miR-21 ,22 (120bp) with *Dre* N`



B EMSA_pri-miR-221 ,222 (120bp) with *Dre* N`

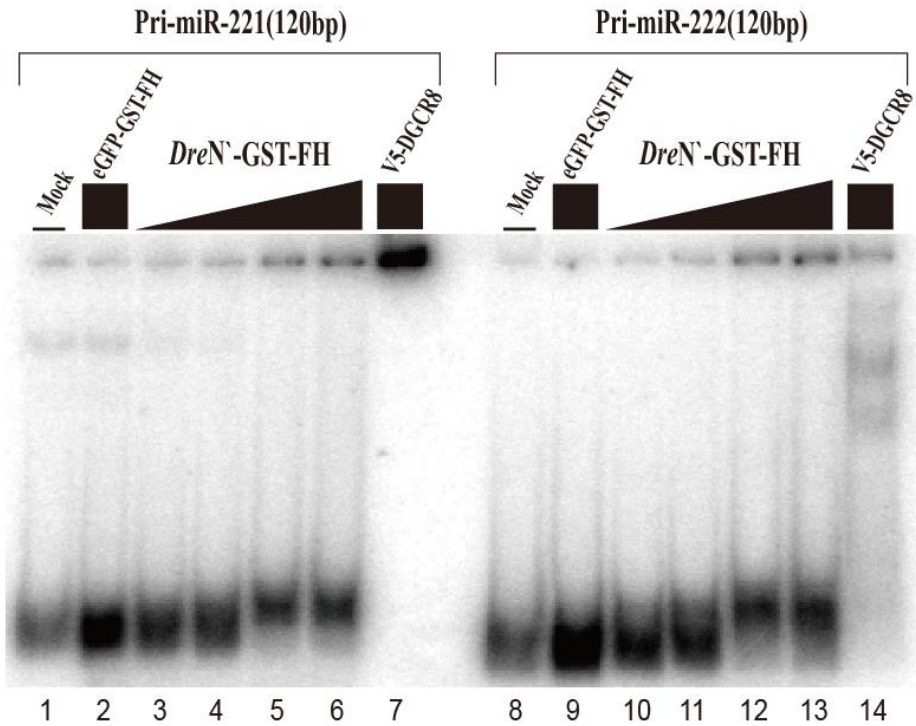
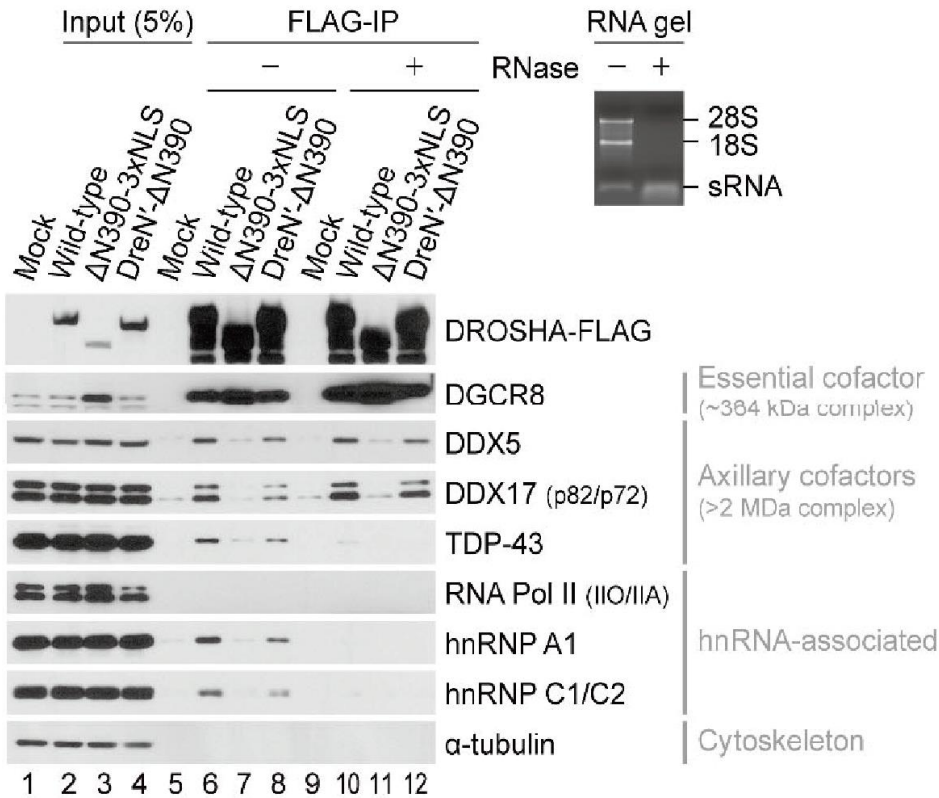


Figure 8. The RBP candidates binding with DROSHA N-terminus.

Western blot analysis of co-immunoprecipitated variant forms of DROSHA (Wild-type, Δ390-3xNLS, *Dre*N'-Δ390). Alpha tubulin served as loading and immunoprecipitation control. sRNA served as RNase control.

Western blotting



This experiment was conducted by Dr. Doo Young Lee.

Figure 9. The impact of the RBP candidates to pri-miRNA processing.

Relative expression levels of pri-miRNA were examined in the RBP candidates knock-down HEK293T cells by qRT-PCR. Red square is N-terminus dependent pri-miRNAs and Blue square is N-terminus independent pri-miRNAs. SiGFP served as negative control. Pri-miRNA levels were normalized to GAPDH. Relative expression levels were normalized to that of siGFP (n=3, mean ± SD).

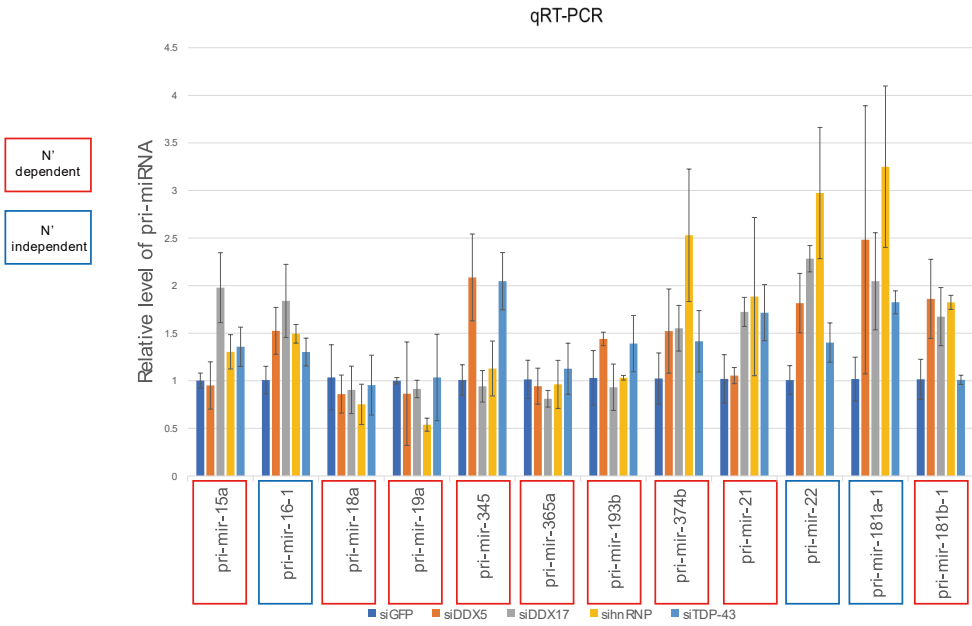

qRT-PCR
N' dependent
N' independent
Relative level of pri-miRNA
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
pri-mir-15a
pri-mir-16-1
pri-mir-18a
pri-mir-19a
pri-mir-345
pri-mir-365a
pri-mir-193b
pri-mir-374b
pri-mir-21
pri-mir-22
pri-mir-181a-1
pri-mir-181b-1
siGFP
siDDX5
siDDX17
sihnRNP
siTDP-43

Table 1. List of DNA oligonucleotide sequences used in this study.

DNA oligonucleotide	Sequence (5'→3')	Purpose
pFB-Hsa_outer_F	TCTCGGTCCGAAACCATGATGCAGGGAAACACATG	Cloning of recombinant protein pFastBac plasmids
pFB-Hsa_inner_R	AGGTTTTCCGAGCTCGAATTTTTTTCCTTGATTGAGGTATAGTTCT	
pFB-Z_outer_F	TCTCGGTCCGAAACCATGTCTTTCCATGCTGGCCG	
pFB-Z_inner_R	AGGTTTTCCGAGCTCGAATTTCTGCTCCTGCTGGAGTTCAC	
T7-pri-miR-21 (120) FP	GAATTAATACGACTCACTATAGGGACATCTCCATGGCTGTACCACCT	Invitro transcription for EMSA
Pri-miR-21 (120) RP	ATGGTCAGATGAAAGATACCAAAATGTCA	
T7-pri-miR-22 (120) FP	GAATTAATACGACTCACTATAGGGTCTCACGCCCTCACCTGGCTGAG	
Pri-miR-22 (120) RP	CCTCCTCCTCGAAGCCAGGGGC	
T7-pri-miR-221 (120) FP	GAATTAATACGACTCACTATAGGGGCAAGCTGAACATCCAGGTCTGGG	
Pri-miR-221 (120) RP	GCCAATGGAGAACATGTTTCCAGGTAGC	
T7-pri-miR-222 (120) FP	GAATTAATACGACTCACTATAGGGGCTGCTGGAAGGTGTAGGTACCC	
Pri-miR-222 (120) RP	TGAAGCAGAAGCTAGAAGATGCCATCAGAG	
qPCR pri-mir-15a F	ATTCTTTAGGCGCGAATGTG	Quantitative RT-PCR
qPCR pri-mir-15a R	CAATATGGCCTGCACCTTTT	
qPCR Pri-miR-16-1 F	GCTCTTATGATAGCAATGTCAGCA	
qPCR Pri-miR-16-1 R	CAACCTTACTTCAGCAGCACA	
qPCR Pri-miR-181a-1 F	GTGAACATTCAACGCTGTCG	
qPCR Pri-miR-181a-1 R	TCCACCTTTGGTTTCCTGTC	

qPCR Pri-miR-181b-1 F	GACAGGAAACCAAAGGTGGA	Quantitative RT-PCR
qPCR Pri-miR-181b-1 R	ACAGTTCAACCCACCGACAG	
qPCR Pri-miR-345 F	ACCCAAACCCTAGGTCTGCT	
qPCR Pri-miR-345 R	AACCAAGTGGGTCAGAGAGG	
qPCR Pri-miR-18a F	CCTGCTGATGTTGAGTGCTT	
qPCR Pri-miR-18a R	TTGCTTGGCTTGAATTATTGG	
qPCR Pri-miR-19a F	CCAATAATTCAAGCCAAGCAA	
qPCR Pri-miR-19a R	AAATAGCAGGCCACCATCAG	
qPCR Pri-miR-365a F	GTTACCGCAGGGAAAATGAG	
qPCR Pri-miR-365a R	CCGAGGAATACTGCAAGAGC	
qPCR Pri-miR-193b F	GGGACTCACTTCTTGGGAAA	
qPCR Pri-miR-193b R	CTTTGAGGGCCAGTTGGATA	
qPCR Pri-miR-374b F	GGAGTGGTGCTCCTCTGAAG	
qPCR Pri-miR-374b R	GCCATAGACACGGACAATGA	
qPCR Pri-miR-21 F	GGCAACACCAGTCGATGGGC	
qPCR Pri-miR-21 R	TGCCACCAGACAGAAGGACCA	
qPCR Pri-miR-22 F	GAGCCTGTTCCTCTCACGCC	
qPCR Pri-miR-22 R	CTCCTCCTCGAAGCCAGGGG	
qPCR GAPDH F	GAGTCAACGGATTTGGTCGT	
qPCR GAPDH F	GACAAGCTTCCCGTTCTCAG	

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ABSTACT IN KOREAN

마이크로RNA는 약 22개의 뉴클레오타이드로 이루어진 짧은 비암호화 RNA의 일종으로 타겟 유전자의 발현을 침묵하는 방식으로 후속 전사를 조절한다. 마이크로RNA의 성숙은 RNase Ⅲ 효소인 DROSHA에 의해 마이크로RNA 1차 전구체가 잘려 시작된다. 현재 DROSHA의 역할과 구조는 잘 알려져 있다. DROSHA는 N 말단에 프롤린 리치 그리고 아르기닌, 세린 리치 도메인, 중앙도메인, 두개의 RNase Ⅲ 도메인 그리고 dsRNA 결합 도메인으로 이루어져 있다. 하지만 DROSHA의 N 말단 (1-390 residues)의 역할은 아직 잘 알려져 있지않다. 이번 연구에서 DROSHA N말단이 마이크로RNA 생합성에 직접적으로 그리고 간접적으로 어떤 영향을 주는지에 대해 연구하였다. 직접적인 영향을 알기 위해 제브라다니오 DROSHA의 N 말단과 마이크로RNA 1차 전구체의 전기영동 이동성 변화분석을 하였다. 그 결과로 DROSHA의 N 말단은 마이크로RNA 1차 전구체에 비특이적 약한 결합 친화력을 가진다는 것을 확인할 수 있었다. 간접적인 영향을 확인하기 위해 DROSHA N 말단과 결합하는 RNA결합 단백질 후보군들을 knockdown하여 마이크로RNA 1차 전구체의 qRT-PCR을 시행하였다. DROSHA의 N 말단과 결합하는 특정 RNA 단백질들이 특정 또는 다양한 마이크로RNA 생합성에 영향을 준다는 것을 확인했다. 결론적으로 본 연구는 DROSHA의 N 말단에 대한 이해를 확장시켜 주었고 더 나아가 DROSHA의 N말단과 결합하는 RNA 결합 단백질들이 마이크로RNA 생합성에 관여할 수 잇는 가능성을 제시해준다.

주요어: 마이크로RNA, 마이크로RNA 1차 전구체, 전기영동 이동성 변화분석, DROSHA, DROSHA N 말단 (1-390 residues), qRT-PCR, RNA 결합 단백질

학번: 2019-24410