Posttranscriptional Crossregulation between Drosha and DGCR8

Jinju Han, Jakob S. Pedersen, S. Chul Kwon, Cassandra Belair, Young-Kook Kim, Kyu-Hyeon Yeom, Woo-Young Yang, David Haussler, Robert Blelloch, and V. Narry Kim

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mouse ES cell, A3-1 culture and siRNA transfection

A3-1 mouse embryonic stem cell line was cultured on gelatin-coated dishes with DMEM (WelGENE) containing 20% FBS (Hyclone), 100 units/ml penicillin, 100 μg/ml streptomycin, 1x non-essential amino acid (Gibco), 1x nucleoside mix (Sigma), 100μM 2-mercaptoethanol (Amresco), and 0.15% LIF. LIF was from the conditioned media from a CHO cell line secreting human LIF.

A3-1 cells were reverse-transfected with siRNAs for 48 hrs with Lipofectamine 2000 (Invitrogen). Briefly, Lipofectamine was diluted in OptiMEM I Reduced Serum Medium (Invitrogen), mixed gently, and incubated for 15 min. Subsequently, siRNAs and OptiMEM I was added and incubated for 15 min. After the incubation, the total mixture was added into a 10-cm dish with complete growth medium containing 2×10^6 cells.

Luciferase reporter constructs.

Reporter constructs were generated by inserting the 5' region of human DGCR8 gene into pCMV-firefly luciferase vector. CMV promoter was amplified using forward (5'-GCTAGCGATGTACGGGCCAGATATAC-3') and reverse (5'-GCTAGCGAATTCAATTTCGATAAGCCAGTAAGCAG-3') primers and inserted into pGL3-basic vector (Promega). To amplify *DGCR8* first exon-first intron fore part, we used forward (5'-gaattcGGCGGTCGGTCGGTGAGG-3') and reverse (5'-ctcgagGCTGGGTGACATCACAA-3') primers. To make pGL3-DG Hairpin, we amplified *DGCR8* first intron rear part-second exon part which contains the hairpins, using forward (5'-ctcgagAGTCGCTTCACCTTCAGTG-3') and reverse (5'-aagctttgtAAGCTCCGTAGAAGTTGAAG-3') primers. To make pGL3-DG ΔHairpin, we used the same forward primer for pGL3-DG Hairpin and a reverse primer (5'-aagcttGAGTCTGTCTTCATGCCGGA-3'). Pri-miR-16-1 was amplified by using forward (5'- TGATAGCAATGTCAGCAGTG-3') and reverse (5'-GTAGAGTATGGTCAACCTTA-3') and inserted in the 3' UTR of pCMV-firefly luciferase vector.

Luciferase assay

HeLa cells were seeded on 24- or 6-wells at one day before transfection. In 24-well scale, 80ng of pCMV-firefly luciferase constructs and 40ng of pCMV-renilla vectors were co-transfected. Forty four nM of siRNAs were co-transfected at the same time using Lipofectamine2000 (Invitrogen). Forty eight hours after transfection, luciferase activity was measured using Dual-Luciferase Reporter System (Promega) according to the manufacturer's instruction.

Microarray analysis

To analyze the changes of mRNA expression in Drosha- and DGCR8-depleted cells, we transfected siGFP, siDrosha, or siDGCR8 into HeLa cells and incubated for 24 or 48 hrs. Experiments were duplicated for each siRNA treatment and at each time point. The RNA was extracted using Trizol reagent (Invitrogen) and treated with DNase I (Takara). Affymetrix microarray platform (U133 ver 2.0 plus) was used to analyze the mRNA expression. For the analysis of mRNA expression in Dicer- and Ago2-depleted HeLa cells, similar method was used except that a different Affymetrix array platform (HuGene ver 1.0) was used. We

averaged the expression level of each gene from duplicated samples from 24 and 48 hrs, and computed the expression ratio between the signals of each siRNA-transfected sample with those of siGFP-control sample. Because we used two different microarray platforms, only the gene that probed in both platforms were used for data analysis. The ratio value was normalized to make the distributions of signals from different platforms become similar. Total number of selected genes was 16,309. Full data sets have been submitted to the Gene Expression Omnibus (GEO) repository (GSE13640).

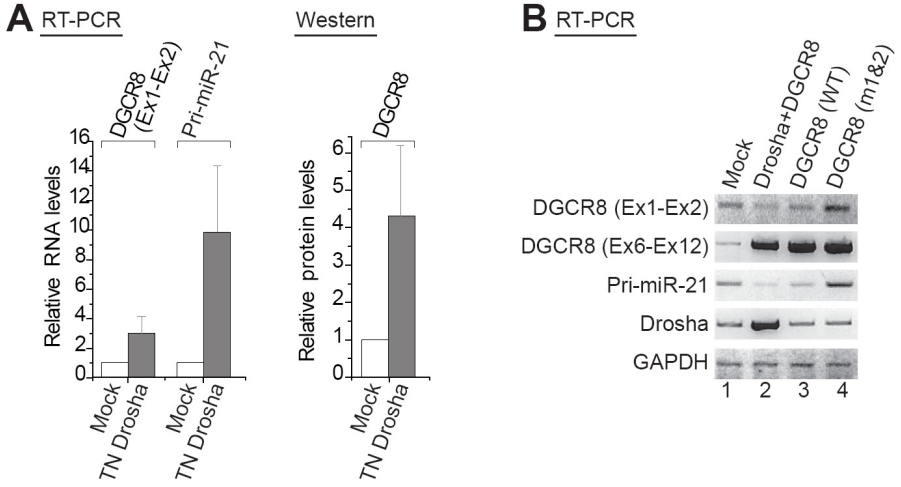


Figure S1. The DGCR8 levels increase following transfection of transdominant Drosha or DGCR8

(A) Quantitation of Figure 1C. The transcript levels were determined by semi-quantitative RT-PCR and by measuring the band intensity using densitometer (Multi Gauge program, Fuji). The RNA levels were normalized against GAPDH. Three (for RT-PCR) and five (for Western) biologically independent experiments were performed for quantification (mean ± s.d.).

(B) RT-PCR analysis after over-expression of the transdominant negative DGCR8 mutant. DGCR8 (m1&2) is a mutant lacking the RNA binding activity. Wild type (WT) Drosha and DGCR8 were transfected as controls. To detect endogenous DGCR8 mRNA, a primer set Ex1-Ex2 was used for PCR. To verify the transfection of DGCR8 plasmid, the primers Ex6_F and Ex12_R were used.

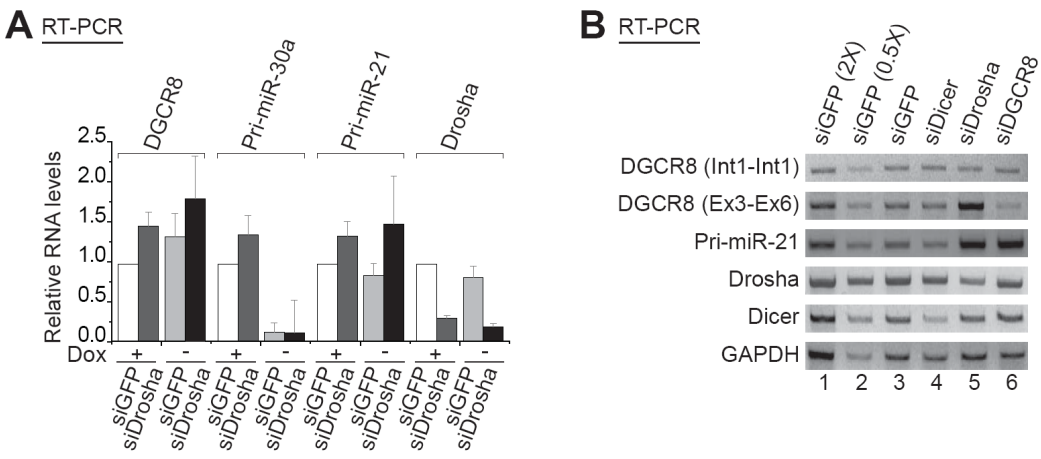


Figure S2. DGCR8 is down-regulated after the completion of transcription and splicing
(A) RT-PCR was performed to measure the steady state levels of mRNAs in miR-30a-inducible cells transfected with siRNAs. Total RNA of miR-30a-inducible HeLa cells were prepared after siDrosha transfection and doxycycline treatment as in Figure 1D. The RNA levels were normalized against GAPDH mRNA levels. Two biologically independent experiments were performed for quantification (mean ± s.d.).
(B) RT-PCR analysis of DGCR8 intronic region after Dicer, Drosha, or DGCR8 knockdown in HeLa cells. Twice (2x) or a half (0.5x) the amount of cDNA from siGFP treated cells were used for semi-quantitative RT-PCR (lanes 1 and 2).

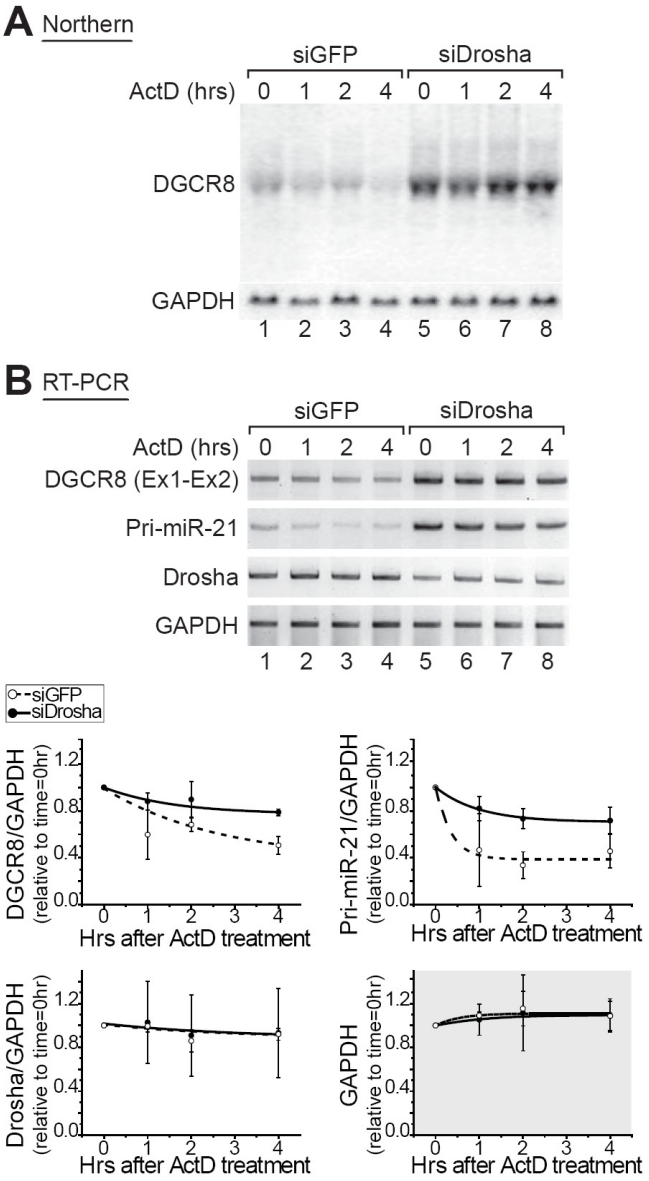


Figure S3. The DGCR8 mRNA is stabilized when Drosha is depleted
(A) Representative northern blot. Five μg/ml of ActD was treated to HEK293T cells 40 hrs after siDrosha transfection. Total RNAs were prepared at the indicated time point after ActD treatment. The quantitated results are shown in Figure 1E.
(B) RT-PCR analysis of the DGCR8 mRNA. RNA samples were prepared as in (A). The band intensity was measured by Multi Gauge program (Fuji) and normalized against GAPDH mRNA. Fractions of DGCR8 mRNA relative to time 0 hr were plotted. Three independent experiments were performed for quantification (mean ± s.d.).

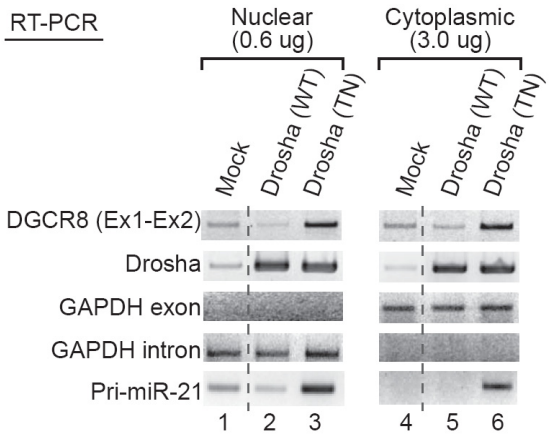


Figure S4. Nuclear degradation of DGCR8 mRNA
RT-PCR analysis following subcellular fractionation of HEK293T cells where transdominant negative Drosha mutant, Drosha (TN) was over-expressed. Nuclear and cytoplasmic RNA fractions were obtained after 72hr post-transfection. For first strand cDNA synthesis, 0.6μg of the nuclear RNA and 3.0μg of the cytoplasmic RNA was used. Though the juxtaposed lanes are not contiguous, all of them are from a single gel.

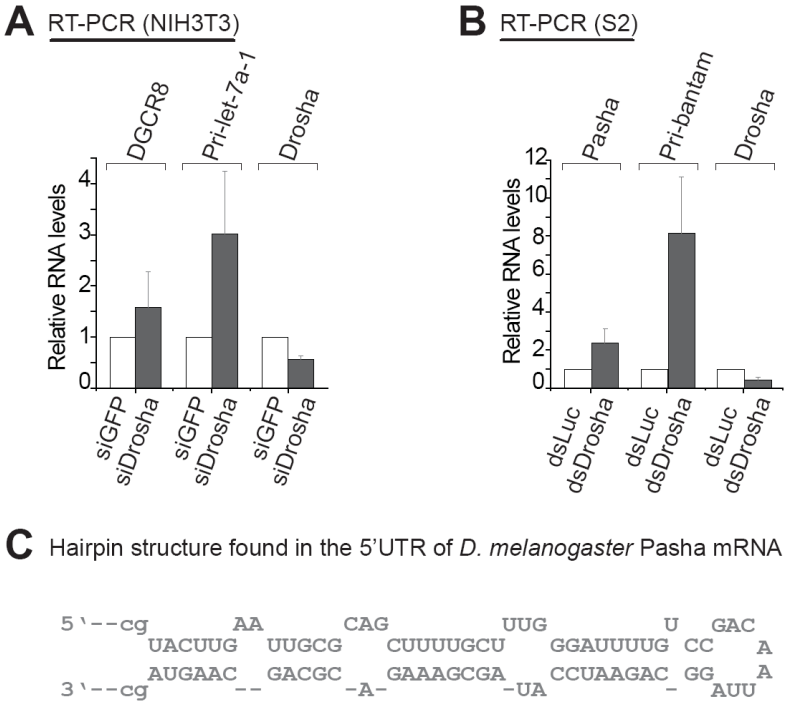


Figure S5. Conservation of regulation in mouse and fly cells.
(A and B) Quantification of the semi-quantitative RT-PCR results shown in Figures 3A and 3B. Three independent experiments were performed for quantification (mean ± s.d.).
(C) The hairpin structure in the 5' UTR of the *Drosophila* Pasha mRNA.

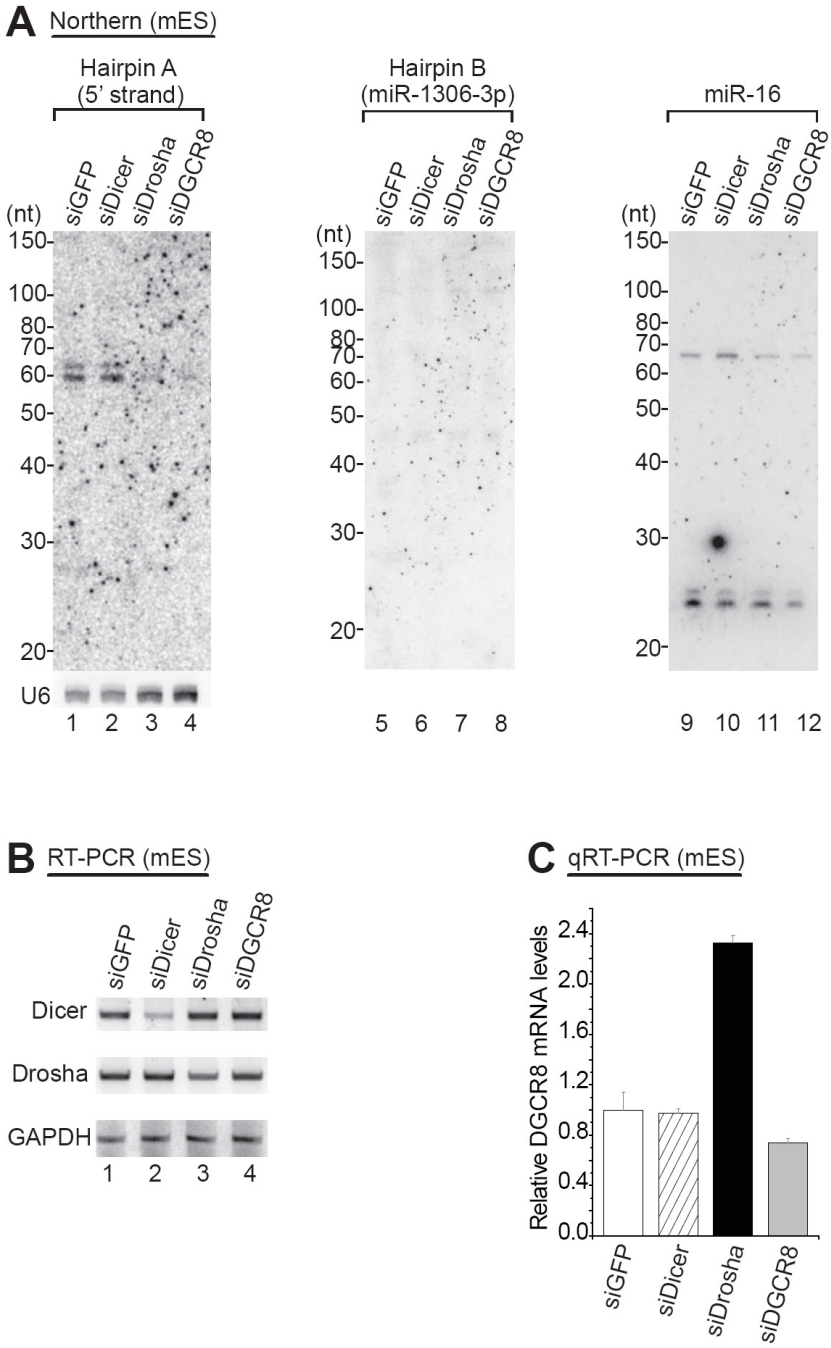


Figure S6. Cleavage of DGCR8 hairpins in mouse ES cells, A3-1
(A) Northern blot assay to detect the cleavage fragments from the hairpin A (left panel, lanes 1-4), hairpin B (miR-1306; middle panel, lanes 5-8), or miR-16 (right panel, lanes 9-12). Total RNAs are prepared from mouse ES cells transfected with the indicated siRNAs. Probe is complementary to the 5' stem region of hairpin A (left), miR-1306 (middle), or miR-16 (right). Small RNAs under 200nt are enriched after siRNA transfection. As a loading control, U6 snRNA was probed. All the panels are from the same gel.
(B) RT-PCR analysis to confirm RNAi of Dicer or Drosha. The same RNA was used as in (A).
(C) Quantitative real time PCR (qRT-PCR) to measure the DGCR8 levels after RNAi. The same RNA was used as in (A).

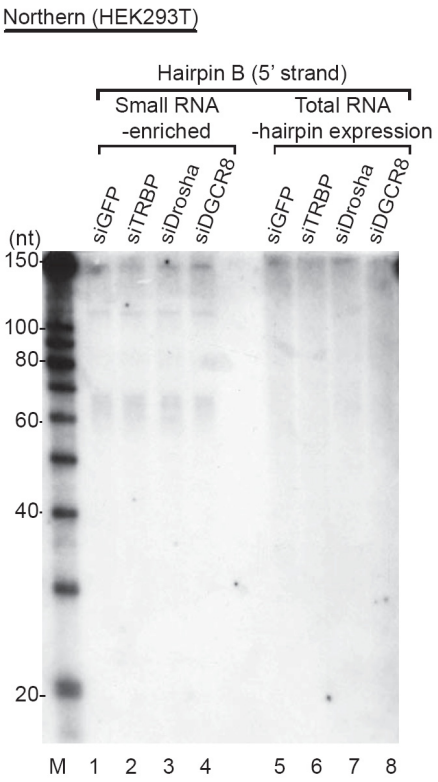


Figure S7. The hairpin B does not release a detectable level of small RNAs in HEK293T *in vivo*

Northern blot assay following RNAi against TRBP, Drosha, or DGCR8. RNA fraction enriched with small RNA (lanes 1 to 4) was prepared from HEK293T cell. HEK293T total RNAs were prepared after over-expression of the hairpins of the DGCR8 (lanes 5 to 8). The probe is complementary to the 5' strand of the hairpin B, marked as a gray underline in Figure 2D.

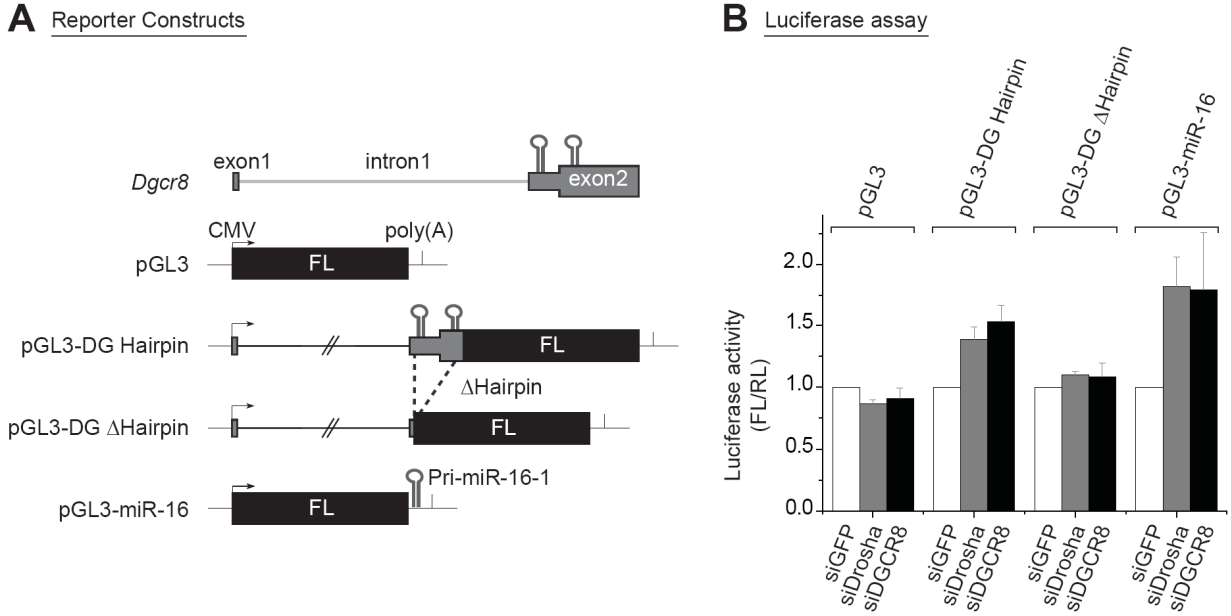


Figure S8. DGCR8 hairpins are required for Microprocessor-mediated down-regulation
(A) Schematic presentation of the luciferase reporters used for the analyses. pGL3 plasmid contains a firefly luciferase gene (FL) downstream of the CMV promoter. To generate pGL3-DG Hairpin, the 5' sequences of the DGCR8 gene was inserted upstream of FL in frame. The pGL3-DG ΔHairpin construct was made by deleting the two hairpins from pGL3-DG Hairpin. pGL3-miR-16 that contains pri-miR-16-1 hairpin in the downstream of FL was used as a control.
(B) Luciferase assay. HeLa cells were transfected with siRNA and reporter plasmids. Renilla luciferase (RL) expression plasmid was co-transfected for normalization. Relative luciferase activity (FL/RL) was determined from three biologically independent experiments (mean ± s.d.).

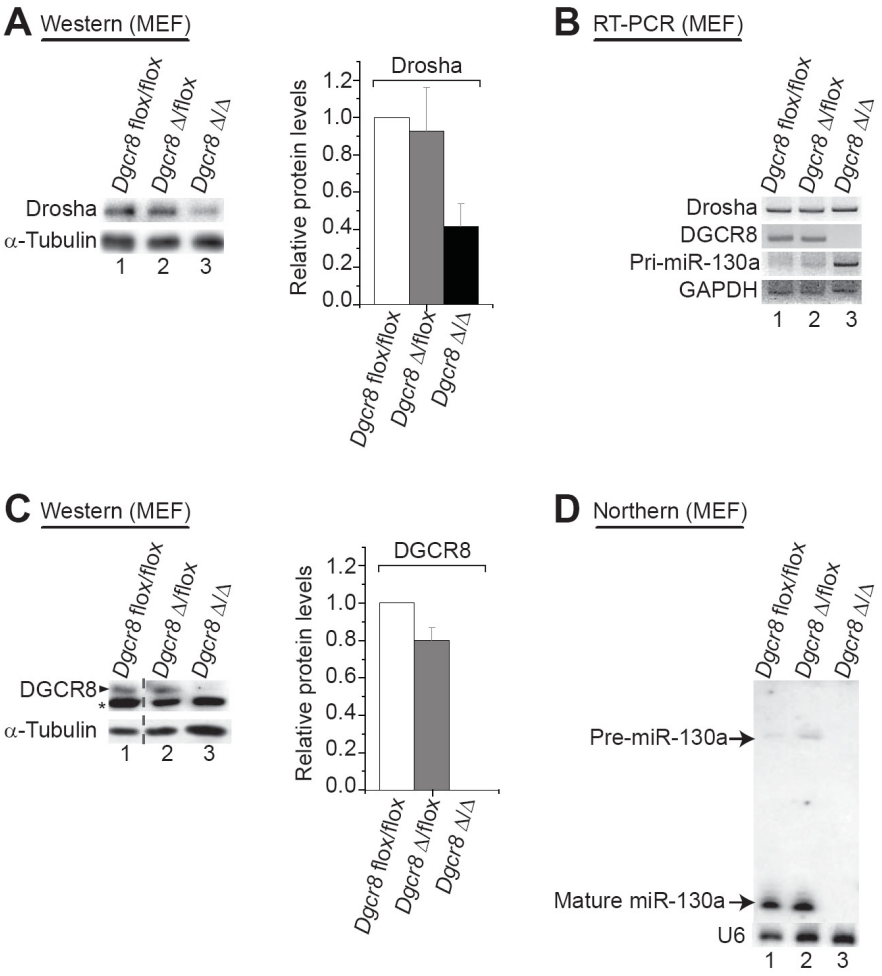


Figure S9. Expression levels of DGCR8 mRNA or protein in *Dgcr8* KO MEF cells
(A) Western blot analysis of Drosha protein in mouse embryonic fibroblast (MEF). flox/flox, Δ/flox, and Δ/Δ represent wild type, heterozygote, and KO homozygote, respectively. The protein levels were determined by measuring the band intensity using densitometer. DGCR8 levels were normalized against α-Tubulin level. Data from three independent experiments were used for quantitation (mean ± s.d.).
(B) RT-PCR analysis in MEF.
(C) Western blot analysis of DGCR8 protein in MEF. An asterisk represents a non-specific band which serves as a loading control. The protein levels were quantitated as in (A). Three independent experiments were performed for quantitation (mean ± s.d.).
(D) Northern blot analysis of miR-130a in MEF.

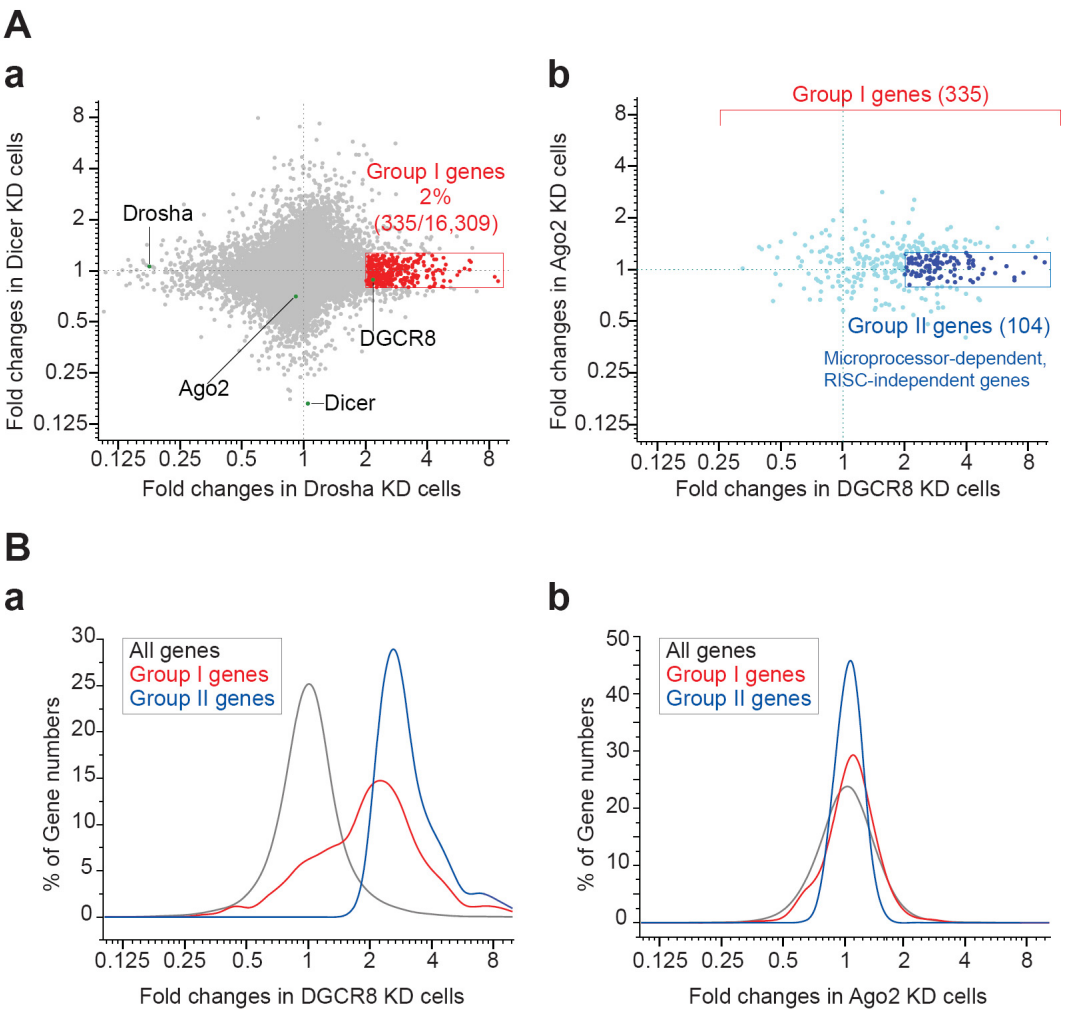


Figure S10. Regulation of mRNAs by Microprocessor

Microarray analysis following knockdown (KD). HeLa cells were transfected with siRNA against Drosha, Dicer, DGCR8, or Ago2. Total RNA was analyzed by Affymetrix chip. Four biological replicates were used for each gene KD. Four biological replicates from cells treated with siRNA against GFP were taken to calculate fold changes.

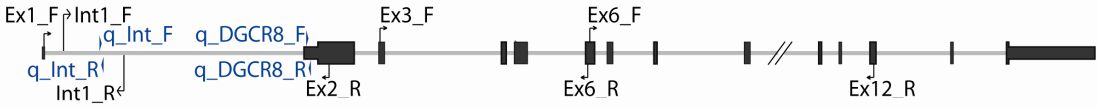
(Aa) Genes that are up-regulated more than 2 folds in Drosha KD and changed less than 0.25 fold in Dicer KD are shown in red (referred to as Group I). Group I contains 335 out of 16,309 total genes detected.

(Ab) Group I genes are then plotted based on their fold changes in DGCR8 KD (x-axis) and Ago2 KD (y-axis). Presented as dark blue dots (Group II), 104 genes are up-regulated over 2 fold in DGCR8 KD and changed less than 0.25 fold in Ago2 KD.

(Ba) Fold changes of Group I (red) and Group II (blue) genes in comparison to all genes in DGCR8 KD cells. This plot shows that the majority of the genes suppressed by Drosha are also down-regulated by DGCR8.

(Bb) Fold changes of Group I (red) and Group II (blue) genes in comparison with all genes in Ago2 KD cells. There is no significance differences in the expression distribution of group I and II genes compared to all genes when Ago2 is depleted, indicating that Group I and II genes are not regulated by Ago2.

A DGCR8 mRNA (NM_022720)



B Human DGCR8 primers for RT-PCR and quantitative real-time PCR

Primer name	sequence (5'->3')
Ex1_F	GGCGGTCGGTCGGTGAGGCTTTC
Ex3_F	GAATTCACGATGGAGAGACAAGTGTGCAG
Ex6_F	GAATTCCAGCCAATCAGAAGCTCATTAC
Ex2_R	CTTAGAGAAGGATCCTTTGG
Ex6_R	GCGGCCGCTCACAAGATGGGCCTCTCGGACTC
Ex12_R	GCGGCCGCTCACCCCCAGTTCTTGACATGTGG
Int1_F	CTGCCGGCTTTAACACAGTTTG
Int1_R	GCACATTTCCTACCCCAAGGTC
q_DGCR8_F	GGCGGTCGGTCGGTGAGGCTTTC
q_DGCR8_R	ATGGAGACAGATGAGAGCCCC
q_Int_F	GGCGGTCGGTCGGTGAGGCTTTC
q_Int_R	ATGGAGACAGATGAGAGCCCC

Figure S11. Human DGCR8 primers for RT-PCR
A schematic presentation of DGCR8 gene (RefSeq NM_022720.5) and the PCR primer binding sites. Boxes represent exons; thin boxes for untranslated region (UTR) and thick boxes for coding sequences, respectively. Lines stand for introns.

Primers for RT-PCR

Target gene for amplification	Direction of primer	Sequence (5'->3')
Human Drosha	Forward	CCTAGTCTGGAAGAAGGAGGATTAGC
	Reverse	CAAAGTTAAGTGTCCTTCATGATGATCTGGG
Human Pri- miR-21	Forward	GTTCGATCTTAACAGGCCAGAAATGCCTGG
	Reverse	ACCAGACAGAAGGACCAGAGTTTCTGATTA
Human GAPDH (exon)	Forward	TGTCATCAATGGAAATCCCATCACC
	Reverse	CATGAGTCCTTCCACGATACCAAAG
Human GAPDH intron	Forward	AGGCAACTAGGATGGTGTGGCTC
	Reverse	TGAGAAAGGTGGGAGCCTCAGTC
Mouse DGCR8	Forward	ATGGAGACATATGAGAGTCCC
	Reverse	TCAGCTGCGTTCTACTCCTGTGTACAG
Mouse Drosha	Forward	CACCCACTCCAACTACAAGAGC
	Reverse	CGTTGGTGATGGCATACTCC
Mouse Dicer	Forward	CTGAGCTTAGGAGATCCGAGG
	Reverse	CTTCCACGGTGACTCTGACC
Mouse Pri-let-7a-1	Forward	GGATCCAGAGGCTTATAGCCCAGGTG
	Reverse	CTCGAGCTATGAGACCCCATGAATGC
Mouse GAPDH	Forward	TGACATCAAGAAGGTGGTGAAGCAG
	Reverse	GGTCCACCACCCTGTTGCTGTAG
Fly Drosha	Forward	CAAGAGCGAAAACGAAGTCAGAG
	Reverse	GCAAAGAGGTCCGTCGTTCA
Fly Pasha	Forward	TCCCAATACTAGCGAATGCTGG
	Reverse	TGCAGAATTCGGTGACTCGG
Fly pri-Bantam	Forward	ACGAACGAAAAGCGTTTGTAACTC
	Reverse	ATATCGCTATATCTGTGTAGGCACACC
Fly GAPDH	Forward	GAACTGAAACTGAACGAGAG
	Reverse	CTTGCGGATTATGCAACAGT

Figure S12. Primers for RT-PCR

Primers for quantitative real-time PCR

Target gene for amplification	Direction of primer	Sequence (5'->3')
Human Pri-miR-16-1	Forward_q	GAAAAGGTGCAGGCCATATTGT
	Reverse_q	CGCCAATATTTACGTGCTGCTA
Human Pri-let-7a-1	Forward_q	GATTCCTTTTCACCATTCACCCTGGATGTT
	Reverse_q	TTTCTATCAGACCGCCTGGATGCAGACTTT
Human Drosha	Forward_q	TAGGCTGTGGGAAAGGACCAAG
	Reverse_q	GTTCGATGAACCGCTTCTGATG
Human Actin_b	Forward_q	TTCAACACCCCAGCCATGTA
	Reverse_q	TCACCGGAGTCCATCACGAT
Mouse DGCR8	Forward_q	AGGTCTCTGTGCTCCCAAGAAG
	Reverse_q	TGGTCATCATTGGCTGTACACTT
Mouse GAPDH	Forward_q	CCGTGTTCCTACCCCCAAT
	Reverse_q	CTTCACCACCTTCTTGATGTCATC

Figure S13. Primers for quantitative real time PCR