Current Biology, Vol. 14, 2162–2167, December 14, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.cub.2004.11.001

The Human DiGeorge Syndrome Critical Region Gene 8 and Its *D. melanogaster* Homolog Are Required for miRNA Biogenesis

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Summary

MicroRNAs (miRNAs) represent a family of small noncoding RNAs that are found in plants and animals (for recent reviews, see [1–5]). miRNAs are expressed in a developmentally and tissue-specific manner and regulate the translational efficiency and stability of partial or fully sequence-complementary mRNAs. miRNAs are excised in a stepwise process from double-stranded RNA precursors that are embedded in long RNA polymerase II primary transcripts (pri-miRNA) [6–10]. Drosha RNase III catalyzes the first excision event, the release in the nucleus [11–13] of a hairpin RNA (pre-miRNA), which is followed by export of the pre-miRNA to the cytoplasm [14–16] and further processing by Dicer to mature miRNAs [17–22]. Here, we characterize the human DGCR8, the DiGeorge syndrome critical region gene 8, and its *Drosophila melanogaster* homolog. We provide biochemical and cell-based readouts to demonstrate the requirement of DGCR8 for the maturation of miRNA primary transcripts. RNAi knockdown experiments of fly and human DGCR8 resulted in accumulation of pri-miRNAs and reduction of pre-miRNAs and mature miRNAs. Our results suggest that DGCR8 and Drosha interact in human cells and reside in a functional pri-miRNA processing complex.

Results and Discussion

Depletion of *D. melanogaster* dmDGCR8 by RNAi Results in Pri-miRNA Accumulation and Reduction of Pre- and Mature miRNAs

A genome-wide, two-hybrid interaction screen for *D. melanogaster* proteins recently revealed an interaction between Drosha and the WW domain-containing protein dmDGCR8 (CG1800-PA, Accession: NP_651879) [23], which is homologous to the human DiGeorge syndrome critical region gene 8 protein, DGCR8 [24]. We therefore refer to the *D. melanogaster* homolog as dmDGCR8 throughout the manuscript. The DGCR8 orthologs contain two dsRNA binding motifs (Figure 1). This observation prompted us to investigate if these proteins are involved in maturation of primary miRNA transcripts. RNAi-based reverse-genetic methods have been widely applied to study gene function in *D. melanogaster* S2 suspension cell cultures [25]. We adapted this method for inactivation of dmDGCR8 as well as several other genes known to be involved in nuclear and cytoplasmic miRNA processing. DsRNA of about 500 base pair (bp) in length, directed against the coding region of the respective targets, was added without transfection reagent to the S2 cells growing in suspension culture medium. To examine miRNA processing, we selected to analyze mir-2 gene clusters, which were previously shown to be expressed in S2 cells [26] (Figure 2A). After 5 days, total RNA was isolated and probed for the presence of the closely sequence-related mature miR-2a and -2b (collectively referred to as miR-2) and their precursors. Depletion of Drosha and dmDGCR8 resulted in a 4.5- and 12-fold reduction of mature miR-2, respectively, and a 10- to 12-fold reduction of the pre-miR-2 hairpin when compared to a control experiment using GFP dsRNA (Figure 2C). In contrast, depletion of the cytoplasmic pre-miRNA processing factor Dicer-1 [21] resulted in a 6.5-fold increase of pre-miRNA and no measurable reduction of mature miRNA. Depletion of Dicer-2, which is the enzyme predominantly responsible for generating siRNAs from long dsRNA in *D. melanogaster* [21], had no significant effect on miRNA processing.

In order to characterize the molecular defect in miRNA processing induced by dmDGCR8 depletion, we looked for accumulation of unprocessed primary miRNA transcripts (pri-miRNAs) by quantitative RT/PCR (qRT/RCR) analysis. *D. melanogaster* expresses members of the miR-2/-13 family from four different loci [27] (Figure 2A). The first and third cluster are embedded in sense orientation within the introns of protein-coding genes, whereas the second and fourth genes appear to have their own promoter [27]. We were able to detect expression for the first, third and fourth miR-2/-13 loci by qRT/PCR analysis using total RNA from untreated S2 cells (Figure S1 in the Supplemental Data available with this article online). Knockdown of dmDGCR8 or Drosha resulted in a 5- to 23-fold accumulation of pri-miRNAs for intronic miR-2 loci 1 and 4 (Figure 2D).

Knockdown of Human DGCR8 Results in Reduction in Pre- and Mature miRNAs

A reciprocal BLAST search of the protein database using the *D. melanogaster* dmDGCR8 coding sequence identified a gene in the DiGeorge syndrome critical region, DGCR8, as the likely human homolog. The DGCR8 protein is 40% identical to dmDGCR8 and shares a highly similar domain structure (Figure 1). In order to determine whether DGCR8 functions in miRNA maturation, we depleted the protein in cultured human cells by RNAi and examined the effect on miRNA biogenesis for a selection of human miRNA genes (Figure 3A). Total RNA was isolated from cells 6 days after transfection, with siRNA

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Figure 1. Domain Structure of the *D. melanogaster* and *H. sapiens* Homologs of Drosha and DGCR8 and the DGCR8 Functionally Related *A. thaliana* HYL1

The WW domain (WW) is a protein module with two highly conserved tryptophans that bind proline-rich peptide motifs (Pro-rich). The dsRNA binding motif (dsRBD) and the RNase III catalytic domain (RNase III) are also indicated.

duplexes targeting either GFP, DGCR8, hDrosha, or Dicer and probed for four different miRNA genes by Northern analysis (Figure 3B). Specific knockdown of mRNAs was confirmed by qRT-PCR (Figure S2). Similar to results observed for miR-2 in S2 cells, depletion of hDrosha or DGCR8 in HeLa S3 and HEK 293 cells resulted in a reduction of pre-miR16 and mature miR-16 compared to RNA isolated from untreated cells or cells transfected with GFP control siRNA (Figure 3B). Knockdown of Dicer in both human cell types led to the expected increase of the pre-miRNA hairpin. To examine whether the decrease in pre- and mature miRNA levels by knockdowns of DGCR8 or hDrosha is restricted to miR-16, we probed RNA extracted from HeLa cell knockdowns with probes specific for miR-18, miR-21, or miR-27a, which derive from distinct miRNA primary transcripts (pri-miRNAs) (Figure 3B). As for miR-16, probing for miR-21 revealed a decrease in pre- and mature miRNA levels in hDrosha- and DGCR8-depleted cells. A reduction of mature miR-18 and miR-27a, for which only the mature miRNA was detectable in the control experiments, was observed in DGCR8, hDrosha, and Dicer knockdowns, as compared to controls.

Because the reduction of pre-miRNAs and mature miRNAs could be a consequence of decreased pri-miRNA transcription in DGCR8 and hDrosha knockdown

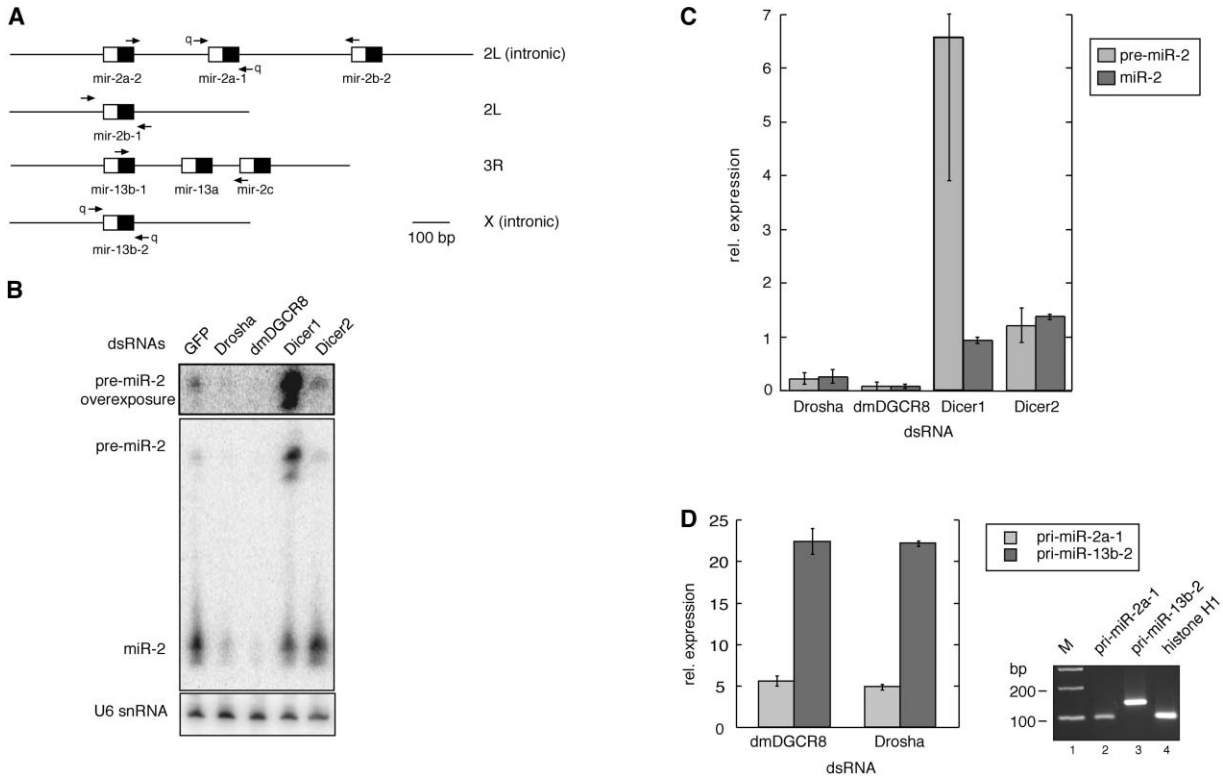

Figure 2. Knockdown of dmDGCR8 and Drosha Reduces Mature and Pre-miRNA Accumulation

(A) Genomic organization of mir-2 and mir-13 genes. The precursor structure is indicated as a box, and the location of the miRNA within the precursor is shown in black; the chromosomal location is indicated to the right. PCR primers for the amplification of the primary transcripts are shown as arrows.

(B) Total RNA was isolated 5 days after initial exposure of *D. melanogaster* S2 cells to the indicated target genes. The RNA was separated on 12% denaturing polyacrylamide gels, transferred to a Nylon membrane, and probed with 5′ radiolabeled mir-2a antisense oligodeoxynucleotide (MIR2as) (top panel) and reprobed for U6 snRNA (U6as) (bottom panel). It is presumed that the probe for miR-2a will also cross-hybridize to miR-2b.

(C) After normalization for loading, the relative ratios for mature and precursor RNAs were calculated and normalized to GFP dsRNA experiments.

(D) Relative primary transcript accumulation, as determined by qRT/PCR with primer pairs shown above. The gene knockdown source of RNAi is indicated on the x axis. Relative pri-miRNA expression with respect to endogenous histone H1 mRNA level was normalized to the control GFP-dsRNA-treated S2 cells. The error bars are derived from duplicate qRT/PCR using the same source of cDNA.

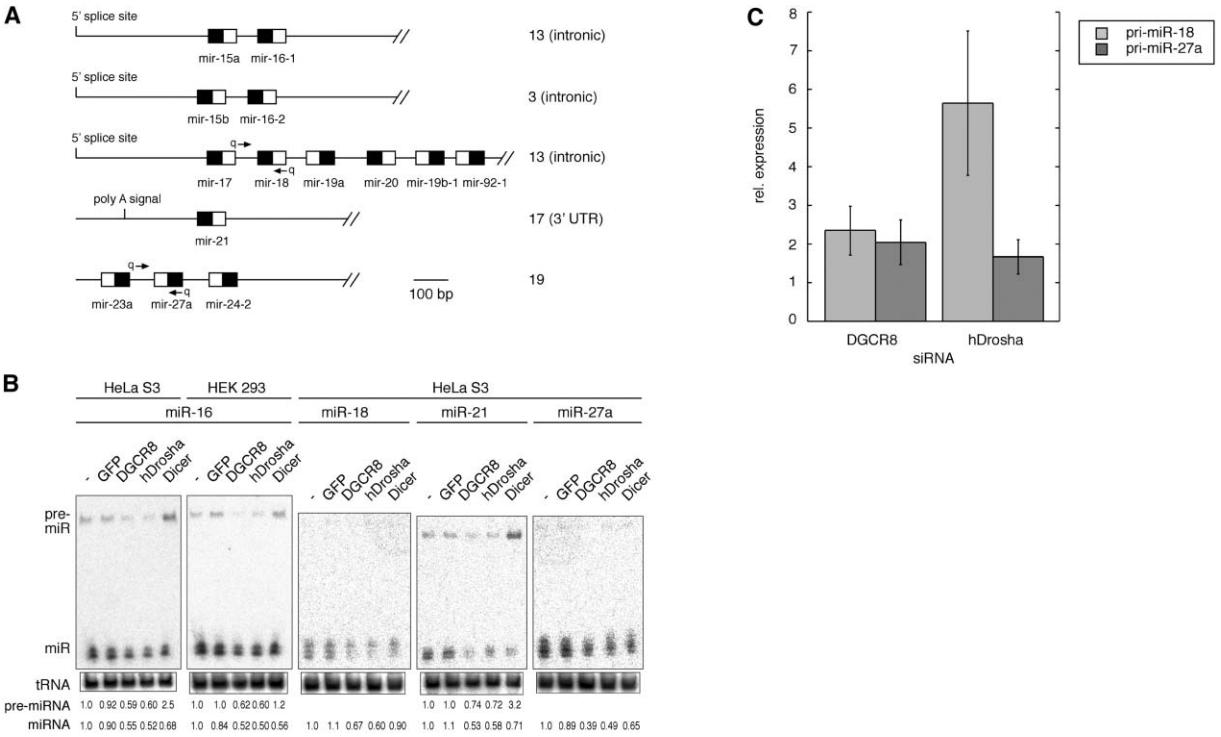

Figure 3. Knockdown of DGCR8 and hDrosha Reduces Mature and Pre-miRNA Accumulation

(A) Genomic organization of selected human miRNA genes. For explanation of symbols and labeling, see the legend for Figure 2A.

(B) miRNA Northern analysis. RNA extracted from HeLa and HEK 293 cells 6 days after transfection with siRNAs was blotted and probed with radiolabeled DNA oligonucleotides. Equal loading of the gels is shown by reprobing of each blot for valine tRNA. After normalization for loading, the relative ratios for mature and precursor RNAs were calculated.

(C) Relative primary transcript accumulation as determined by qRT/PCR with primer pairs shown above. The gene knockdown source of RNAi is indicated on the x axis. Relative pri-miRNA expression with respect to endogenous U6 snRNA level was normalized to the control GFP siRNA-treated cells.

cells, we used a qRT/PCR assay to monitor the relative expression level of two pri-miRNAs [28]. miRNA-specific reverse primers were used to convert pri-miR-18 and pri-miR-27a into cDNA. For qPCR analysis, PCR primers were positioned such that the resulting amplification product would span the hDrosha-processing site (Figure 3A). Knockdown of DGCR8 or hDrosha resulted in a 2- to 5.5-fold increase in the relative amounts of pri-miR-18 and pri-miR-27a compared to the GFP siRNA control (Figure 3C). The observed decrease of pre- and mature miRNAs levels in DGCR8 knockdown cells, with a simultaneous increase in pri-miRNAs, indicates that DGCR8 is necessary for miRNA maturation.

Knockdown of DGCR8 Interferes with miRNA-Guided Cleavage Activity in Cell Culture

To assess the role of miRNA-processing factors directly in living cells, we applied a HeLa cell-based positive-EGFP readout assay that senses endogenous miR-21-guided target RNA cleavage [29, 30]. The EGFP mRNA, which carries a fully complementary miR-21 sequence in its 3′ UTR, is constantly cleaved by the endogenously expressed miR-21 (Figure 4) but derepressed by transfection of an anti-miR-21 2′-O-methyl oligoribonucleotide that blocks endogenous miR-21 ribonucleoprotein complexes. A control anti-miR-7 2′-O-methyl oligoribonucleotide was unable to derepress EGFP. Similar to the depletion of Ago2, the putative RISC endonuclease [30–32], knockdowns of DGCR8 and hDrosha with validated siRNAs resulted in an upregulation of EGFP compared to cells transfected with control siRNA targeting lamin A/C, further supporting that DGCR8, like hDrosha, is required for miR-21 maturation in vivo.

hDrosha and DGCR8 Interact and Are Present in a Functional Complex

To determine whether hDrosha also interacts with DGCR8, as might be expected from the *D. melanogaster* 2-hybrid analysis, we performed coimmunoprecipitations (co-IPs) of transiently expressed, epitope-tagged hDrosha and DGCR8. HEK 293 cells were either transfected with pmyc-hDrosha alone or transfected simultaneously with pmyc-hDrosha and pFLAG/HA (as empty vector control) or pFLAG/HA-DGCR8. Total lysates from the transfected cells were immunoprecipitated with anti-myc or anti-FLAG antibodies, and the precipitated protein complex was analyzed by SDS-PAGE followed by Western blotting (Figure 5A). myc-hDrosha and FLAG/HA-DGCR8 coimmunoprecipitated when either antibody was used for IPs. The treatment of the anti-FLAG IPs with a cocktail of RNase A and RNase T1 had no obvious effect on the coprecipitation, suggesting the interaction of myc-hDrosha and FLAG/HA-DGCR8 was RNA independent (Figure 5A). No interaction could be detected in cells

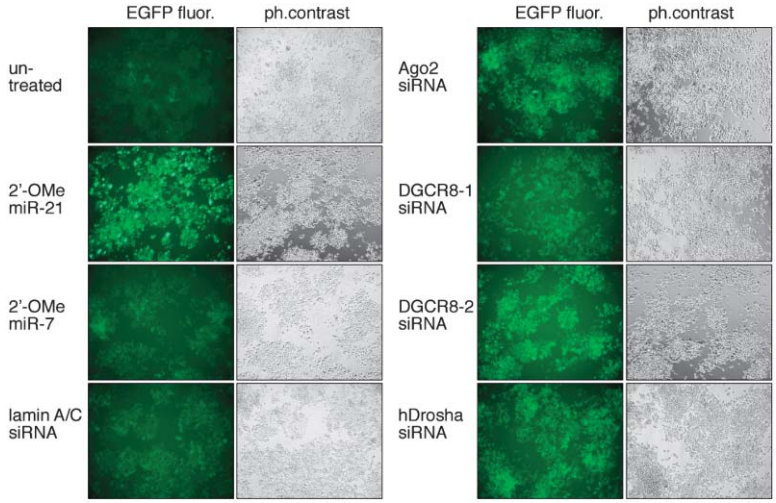

Figure 4. miR-21-Guided Cleavage Activity Is Disrupted in HeLa Cell Culture upon Knockdown of General miRNA-Processing Factors

A stable HeLa cell line that expresses EGFP carrying a sequence with perfect complementarity to miR-21 in its 3′ UTR was transfected with 2′-O-methyl oligoribonucleotides complementary to miR-21 or the control miR-7. siRNAs cognate to lamin A/C as control, Ago2, DGCR8 (two independent targeting sites), and hDrosha. Fluorescence and phase-contrast images were recorded 6 days after transfection with a Zeiss Axiovert 200 inverted fluorescence microscope.

singly transfected with pmyc-hDrosha or cotransfected with the empty expression vector pFLAG/HA.

The hDrosha and DGCR8 protein-protein interaction is likely mediated through two conserved domains. The N terminus of Drosha contains a proline-rich region [33]. This domain generally provides a ligand for binding to a WW motif [34]. Such a motif has previously been identified in the DGCR8 protein sequence [24].

The interaction of hDrosha and DGCR8 suggests that both proteins reside in a functional protein complex. hDrosha executes pri-miRNA processing in the nucleus, and immunopurified hDrosha was able to cleave a pri-miRNA, releasing a pre-miRNA hairpin [12]. We expressed myc-hDrosha by transient transfection in HEK 293 cells and isolated the protein by immunoprecipitation (IP) from total cell lysate by using anti-myc antibody. The immunoprecipitated complex or protein G sepharose beads were incubated with radiolabeled pri-miR-27a. Only the immunoprecipitated complex cleaved the long pri-miR-27a into a smaller RNA of the expected length of about 64 nt (Figure 5B, lane 4), whereas recombinant mouse Dicer generated small RNAs of about 22 nt (Figure 5B, lane 7).

Interestingly, anti-FLAG-immunoprecipitated complexes from lysates prepared from cells cotransfected with pmyc-hDrosha and pFLAG/HA-DGCR8 also cut the pri-miR-27a and released a major cleavage product of about 64 nt (Figure 5B, lane 6). This cleavage product was not detected in complexes derived from pFLAG/HA-GFP-cotransfected control cells (Figure 5B, lane 5). In summary, these data suggest that FLAG/HA-DGCR8 and myc-hDrosha interact in vivo and are present in a functional pri-miRNA-processing protein complex.

In plants, miRNA accumulation depends on the activity of the predominantly nuclear proteins DCL1, HEN1, and HYL1 [20, 35–41]. *dcl1* null alleles are embryonic lethal, whereas partial loss-of-function *dcl1* mutants are viable [42], but show reduced miRNA accumulation and developmental defects [20, 35]. *hen1* and *hyl1* null alleles exhibit reduced miRNA levels and developmental defects that overlap with those of partial loss-of-function *dcl1* mutants [20, 36, 38, 40], suggesting that DCL1, HEN1 and HYL1 act together in the nucleus. The HYL1 protein, which also contains a tandem dsRBD (Figure 1), may play a molecular role in miRNA primary transcript recognition in plants, similar to DGCR8 in animals.

Several possible models can be envisioned for explaining the function of DGCR8. DGCR8, containing a tandem dsRNA binding domain (dsRBD), is involved in the recognition of miRNA precursors prior or concomitant to cleavage, and it positions Drosha, containing a single dsRBD, at the miRNA hairpin stem base. Alternatively, DGCR8 functions in stabilizing and handing over the Drosha-cleaved pre-miRNA to the nuclear export complex. For both of these models, one might predict either mutually exclusive/sequential or simultaneous/cooperative miRNA recognition. Structural and biochemical analyses have shown that two dsRNA binding motifs bind to regions of 11 to 16 bp of dsRNA [43–45], suggesting the possibility for simultaneous binding of DGCR8 and Drosha to a miRNA stem loop structure. Independent of these proposed models, our study demonstrates that the Drosha-interactor DGCR8 is involved in miRNA biogenesis in *D. melanogaster* and humans.

Supplemental Data

Supplemental Data including Experimental Procedures and two additional figures are available at http://www.current-biology.com/cgi/content/full/14/23/2162/DC1/.

Acknowledgments

We thank Y. Dorsett, P. Landgraf, G. Meister, and R. Müller for critical reading of the manuscript; M. Lagos-Quintana, J. Chen, R. Müller, M. Hannus, and C. Echeverri (Cenix Bioscience) for reagents and technical assistence; J.-B. Ma for discussion; and R. Lührmann for support. This work was also financially supported by National Institutes of Health grant R01 GM068476-01.

Received: October 14, 2004
Revised: November 1, 2004
Accepted: November 1, 2004
Published online: November 4, 2004

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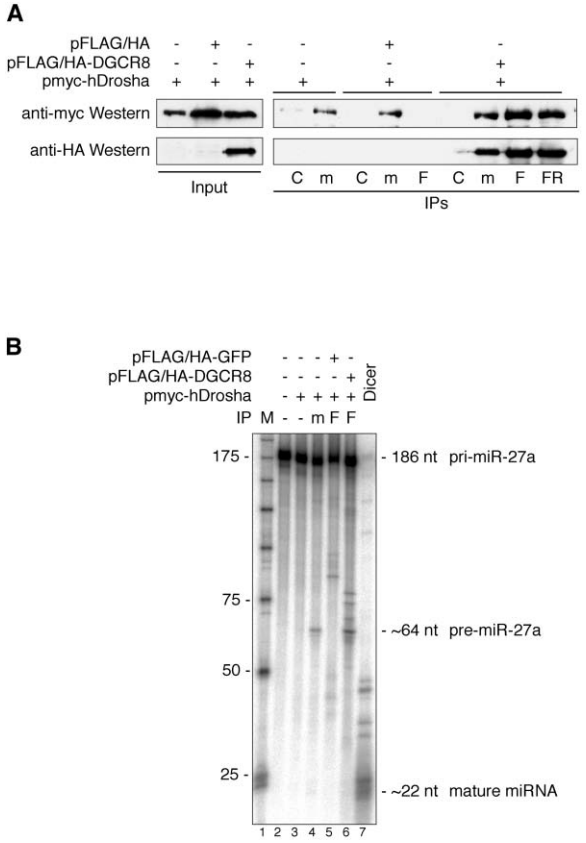

Figure 5. hDrosha and DGCR8 Interact and Reside in a Functional pri-miRNA-Processing Complex

(A) Co-IP of hDrosha and DGCR8 from transfected HEK 293 cells. 293 cells were transiently transfected with plasmids pmyc-hDrosha and pFLAG/HA-DGCR8 expressing full-length myc-tagged hDrosha and FLAG/HA-tagged DGCR8, respectively. The empty vector was used as a control. As a control (abbreviated as C), the cell extract was bound to protein G-Sepharose (Amersham) with no antibody or preabsorbed with monoclonal anti-myc antibody (m) or with anti-FLAG antibody agarose in the absence (F) or presence of RNase A/RNase T1 mix (FR). Stoichiometric equivalents of the input and the immune complexes were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with anti-HA or anti-myc antibody. IP, immunoprecipitation.

(B) myc-hDrosha was expressed in HEK 293 alone and with FLAG/HA-tagged GFP or DGCR8. Total cell extracts were used for IPs as described in (A). ^{32}P-labeled pre- miR-27a was incubated with beads containing IP complexes, extracted with phenol/chloroform, and separated on a 6% polyacrylamide gel. The left lane shows a radiolabeled 25 bp DNA ladder with sizes indicated (M). The right lane shows RNA cleavage reaction products after incubation with recombinant mouse Dicer.

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