

Sjögren Syndrome Antigen B (SSB)/La Promotes Global MicroRNA Expression by Binding MicroRNA Precursors through Stem-Loop Recognition*

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Chunyang Liang[‡], Ke Xiong^{‡§1}, Keith E. Szulwach[¶], Yi Zhang[‡], Zhaohui Wang[‡], Junmin Peng^{||}, Mingui Fu^{**}, Peng Jin[¶], Hiroshi I. Suzuki^{‡‡}, and Qinghua Liu^{‡2}

From the [‡]Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75390, the [§]College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, China, the [¶]Department of Human Genetics, Emory University School of Medicine, Atlanta, Georgia 30322, the ^{||}Department of Structural Biology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105, the ^{**}Department of Basic Medical Science, University of Missouri School of Medicine, Kansas, Missouri 64108, and the ^{‡‡}Department of Molecular Pathology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

Background: MicroRNA biogenesis is a multistep process regulated by RNA-binding proteins.

Results: Sjögren syndrome antigen B (SSB)/La binds stem-loop precursor microRNAs (pre-miRNA) and is required for microRNA expression.

Conclusion: La/SSB promotes global microRNA expression by stabilizing pre-miRNAs from nuclease-mediated decay.

Significance: This study reveals a novel concept of pre-miRNA holding complex that protects and escorts pre-miRNAs in microRNA biogenesis pathway.

MicroRNAs (miRNA) control numerous physiological and pathological processes. Typically, the primary miRNA (pri-miRNA) transcripts are processed by nuclear Drosha complex into ~70-nucleotide stem-loop precursor miRNAs (pre-miRNA), which are further cleaved by cytoplasmic Dicer complex into ~21-nucleotide mature miRNAs. However, it is unclear how nascent pre-miRNAs are protected from ribonucleases, such as MCPIP1, that degrade pre-miRNAs to abort miRNA production. Here, we identify Sjögren syndrome antigen B (SSB)/La as a pre-miRNA-binding protein that regulates miRNA processing *in vitro*. All three RNA-binding motifs (LAM, RRM1, and RRM2) of La/SSB are required for efficient pre-miRNA binding. Intriguingly, La/SSB recognizes the characteristic stem-loop structure of pre-miRNAs, of which the majority lack a 3' UUU terminus. Moreover, La/SSB associates with endogenous pri-/pre-miRNAs and promotes miRNA biogenesis by stabilizing pre-miRNAs from nuclease (*e.g.* MCPIP1)-mediated decay in mammalian cells. Accordingly, we observed positive correlations between the expression status of La/SSB and Dicer in human cancer transcriptome and prognosis. These studies identify an important function of La/SSB as a global regulator of miRNA expression, and implicate stem-loop recognition as a major mechanism that mediates association between La/SSB and diverse RNA molecules.

MicroRNAs (miRNAs)³ are ~21-nucleotide (nt) cellular RNAs that govern numerous biological and disease processes by directing degradation and translational repression of cognate mRNAs (1–3). It is estimated that human genome encodes up to one thousand miRNAs (1, 3, 4), which are transcribed either as independent genes, or imbedded in other genes. The majority of miRNAs are generated by sequential processing by two RNase III enzymes: Drosha and Dicer. In the nucleus, the primary miRNA (pri-miRNA) transcripts are processed by the Drosha-DGCR8/Pasha complex into ~70-nt stem-loop precursor miRNAs (pre-miRNAs) (5–8). The pre-miRNAs are exported by Exportin 5-Ran:GTP to the cytoplasm (9, 10), and are further cleaved into mature miRNAs by the Dicer-1-Loqs-PB complex in *Drosophila melanogaster* (11–13), or by the Dicer-TRBP complex in mammals (14–17). Both DGCR8/Pasha or TRBP/Loqs-PB are dsRNA-binding proteins that promote miRNA processing by stabilizing Drosha and Dicer and by facilitating their interaction with pri-miRNAs and pre-miRNAs, respectively (18–20). A number of post-transcriptional regulators of miRNA biogenesis have recently been characterized, including Lin28, KSRP, hnRNP A1, and SMAD (21–27). All of these miRNA regulators modulate the production of a small subset of miRNAs through recognition of specific sequences of pri- and/or pre-miRNAs (22, 23, 28).

In many RNA maturation processes, the precursor molecules are often bound by RNA-binding proteins or chaperones to protect them from nuclease-mediated decay and to ensure the correct processing by specific ribonucleases. In the current model, however, newly synthesized pri-/pre-miRNAs are

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² W.A. "Tex" Moncrief Jr. Scholar in Medical Research. To whom correspondence should be addressed: 5323 Harry Hines Blvd., K3.219, Dallas, TX. Tel.: 214-648-9120; Fax: 214-648-8856; E-mail: qinghua.liu@utsouthwestern.edu.

³ The abbreviations used are: miRNA, microRNA; SSB, Sjögren syndrome antigen B; MCPIP1, monocyte chemotactic protein-induced protein 1; nt, nucleotide(s); LAM, La motif; RRM, RNA recognition motif; qPCR, quantitative PCR; GSEA, gene set enrichment analysis; IP, immunoprecipitate; RNP, ribonucleoprotein.

TABLE 1
siRNA targets, primer sequences, and Northern probes

	Primer sequences
siLa1 (human) target sequence	5'-GGUCGUAGAUUUAAAGGAA-3'
siLa2 (human) target sequence	5'-GGUUAGAAGAUAAAGGUCA-3'
Cyclophilin (human) forward	5'-TGCCATCGCCAAGGAGTAG-3'
Cyclophilin (human) reverse	5'-TGCACAGACGGTCACTCAA-3'
pri-let-7a (human) forward	5'-CTCATTACACAGGAAACCGAA-3'
pri-let-7a (human) reverse	5'-CCTCATCCCACAGTGAAGAGAA-3'
pri-miR-16 (human) forward	5'-CTGACATGCTTGTTCCACTCTAGC-3'
pri-miR-16 (human) reverse	5'-CCTGTACACTAAAGCAGACAAT-3'
pri-miR-17 (human) forward	5'-GTTGTTAGAGTTTGAGGTGTT-3'
pri-miR-17 (human) reverse	5'-AGCACACAACATCAGCAGG-3'
pri-miR-21 (human) forward	5'-TACCATCGTGACATCTCCA-3'
pri-miR-21 (human) reverse	5'-CAGACAGAAGGACCAGAGTT-3'
pri-miR-23b (human) forward	5'-CAGTGTGTGCAGACAGCAC-3'
pri-miR-23b (human) reverse	5'-GTTCTCCAATCTGCAGTGA-3'
pre-let-7a (human) forward	5'-TGGGATGAGGTAGTAGGTTGT-3'
pre-let-7a (human) reverse	5'-TAGGAAAGACAGTAGATTGTATAGTT-3'
pre-miR-21 (human) forward	5'-TAGCTTATCAGACTGATGTTGA-3'
pre-miR-21 (human) reverse	5'-CGACTGCTGTTGCCATGAG-3'
pre-miR-23b (human) forward	5'-CAGGTGCTCTGGCTGCTT-3'
pre-miR-23b (human) reverse	5'-GTGGTAATCCCTGGCAATGT-3'
Anti-5.8S (human)	5'-TCCTGCAATTCACATTAATTCTCGCAG-3'
Anti-5S (human)	5'-CCGACCTGCTTAGCTTCCGAGATCA-3'
Anti-U6S (human)	5'-CGTTCCAATTTAGTATATGTGCTGCC-3'
Anti-miR-16	5'-CGCCAAUAAUUUACGUGCUGCUA-3'
Anti-miR-17	5'-CUACCUGCACUGUAAGCACUUUG-3'
Anti-miR-20	5'-CUACCUGCACUAUAGCACUUUA-3'
Anti-miR-21	5'-UCAACAUCAGUCUGAUAAAGCUA-3'
Anti-miR-23b	5'-GGUAAUCCUGGCAUUGUGAU-3'
Anti-miR-30	5'-CUUCCAGUCGAGGAGUUUACU-3'
Anti-let-7a	5'-AACUAUACAACCUACUACCUCA-3'

described as unprotected and delivered directly to Drosha or Dicer enzymes for processing. It has recently been suggested that the processing of pri-miRNAs by Drosha occurs co-transcriptionally on chromatin (29, 30). In contrast, nascent pre-miRNAs embark a long and perilous journey from the nucleus to the cytoplasm, in which they are vulnerable to degradation by many nucleases. For example, the monocyte chemotactic protein-induced protein 1 (MCP1P1)/ZC3H12A, a ribonuclease and deubiquitinase that suppresses inflammatory response (31, 32), was recently reported to antagonize Dicer by degrading pre-miRNAs to abort miRNA production (33).

Sjögren syndrome antigen B (SSB), also known as autoantigen La, is commonly associated with autoimmune disorders such as Sjögren syndrome and systemic lupus erythematosus (34). The La/SSB protein is evolutionarily conserved from yeast to human, exists abundantly in both the nucleus and cytoplasm, and plays fundamental roles in diverse processes of RNA metabolism (35). A well known function of La/SSB is its association with nascent transcripts of RNA polymerase III, including precursors of tRNAs (pre-tRNAs), through recognition of the characteristic 3' UUU termini. The binding of La/SSB protects pre-tRNAs from nonspecific exonuclease digestion and ensures correct processing of the 5' and 3' leader sequences of pre-tRNAs by specific ribonucleases, such as RNase P and RNase Z (36–38).

Both *Drosophila* and human La/SSB contain three RNA-binding motifs, a highly conserved La motif (LAM) (39), a canonical RNA recognition motif (RRM1), and an atypical RRM2, as well as a variable carboxyl (C) terminus. It has previously been shown that the amino (N)-terminal LAM-RRM1 domain of La/SSB is responsible for 3' UUU recognition (40–42). Although La/SSB is dispensable for yeast viability (43, 44), it is essential in higher eukaryotes such as *Drosophila* and mice

(45, 46). The molecular basis for this discrepancy is currently unclear. Here, we have uncovered an important function for La/SSB in promoting global miRNA expression by binding and stabilizing pre-miRNAs. Moreover, we showed that La/SSB interacts with pre-miRNAs by stem-loop recognition rather than 3' UUU recognition. These significant findings provide fresh insights into the basic process of miRNA biogenesis, the diverse functions of La/SSB in RNA metabolism, and possibly the pathogenesis of cancer and autoimmune diseases.

EXPERIMENTAL PROCEDURES

General Reagents and Antibodies—General RNA reagents were purchased from Ambion and Promega, and DNA oligos or siRNAs were synthesized by IDT. Both Dicer and human La/SSB antibodies were purchased from Santa Cruz Biotechnology, whereas actin, tubulin, and anti-FLAG antibodies were obtained from Sigma. Recombinant *Drosophila* Dicer-1·Loqs-PB and human Dicer·TRBP complexes were generated in insect cells as previously described (12, 17). Recombinant *Drosophila* and human La/SSB were produced in *Escherichia coli* and purified by nickel affinity and ion-exchange chromatography. Recombinant FLAG-tagged MCP1P1 and Drosha-DGCR8 complex was affinity purified from 293T cells following transient transfection (47).

Purification of La/SSB from S2 Extract—The cytoplasmic extract (S100) of *Drosophila* S2 cells (48) was precipitated by ammonium sulfate at 60% saturation. After a 30-min, 20,000 × g centrifugation step, the supernatant was dialyzed overnight in Buffer A (10 mM KOAc, 10 mM HEPES, pH 7.4, 2 mM Mg(OAc)₂, 2.5 mM DTT), loaded onto a Mono S column, and eluted with a 300–600 mM NaCl gradient. The fractions with peak activity were dialyzed for 4 h, loaded onto a Mono Q column, and eluted with a 0–300 mM NaCl gradient. Finally, the

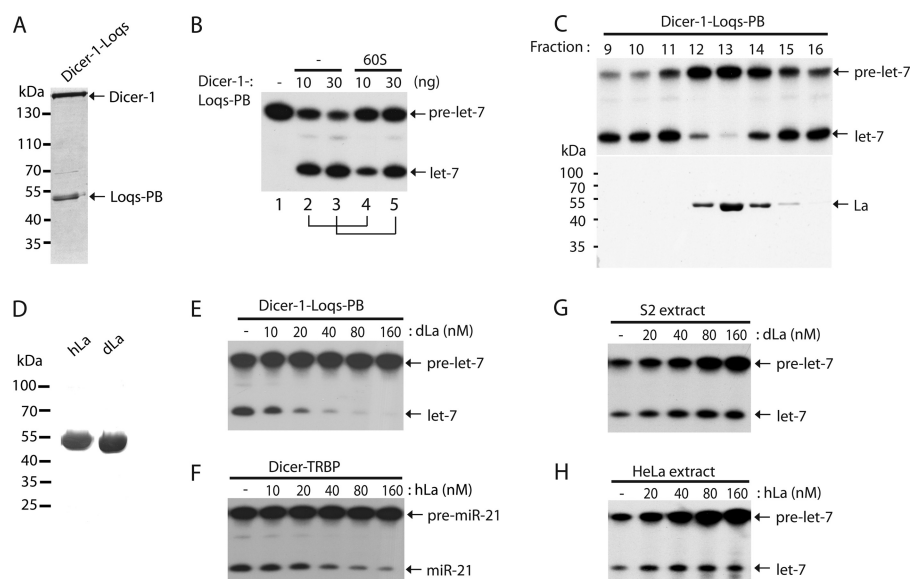


FIGURE 1. Purification and reconstitution of La/SSB as a regulator of pre-miRNA processing. *A*, Coomassie-stained SDS-PAGE showing recombinant *Drosophila* Dicer-1-Loqs-PB complex purified from insect cells. *B*, the pre-miRNA processing assay was performed in buffer (*lane 1*) or with 10 or 30 ng of recombinant Dicer-1-Loqs-PB complex in the absence (*lanes 2 and 3*) or presence (*lanes 4 and 5*) of 60% ammonium sulfate supernatant (60S) of S2 extract. *C*, purification of endogenous La/SSB from S2 extract by sequential chromatographic fractionation. At the final Mono S step, individual fractions were assayed with recombinant Dicer-1-Loqs-PB complex for pre-miRNA processing activity (*top*) or resolved by SDS-PAGE followed by silver staining (*bottom*). *D*, Coomassie-stained SDS-PAGE showing purified *Drosophila* (dLa) or human (hLa) La/SSB. *E*, the pre-miRNA processing assays were performed using ~10 nM recombinant Dicer-1-Loqs-PB complex with increasing concentrations of recombinant dLa. *F*, the pre-miRNA processing assays were conducted using ~10 nM recombinant Dicer-TRBP complex with increasing concentrations of recombinant hLa. *G*, the pre-miRNA processing assays were conducted using 10 μ g of crude S2 extract with increasing concentrations of recombinant dLa. *H*, the pre-miRNA processing assays were conducted using 10 μ g of HeLa extract with increasing concentrations of recombinant hLa.

peak fractions were directly loaded on a Smart Mono S column and eluted with a 300–600 mM NaCl gradient.

Immunoprecipitation of La/SSB Complexes—HeLa cell extract was prepared in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA) freshly supplemented with protease inhibitors. HeLa extract was incubated with anti-human La/SSB monoclonal antibody or mouse IgG for 1 h at 4 °C, and protein G-Sepharose beads were added and rotated for another 4 h. After extensive wash of the beads (4 times in RIPA buffer, 3 times in RIPA buffer with 0.5 M NaCl, and once with buffer III (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0), associated RNA was extracted from the beads by TRIzol and precipitated by ethanol.

Cross-linking and Immunoprecipitation Assay—To demonstrate *in vivo* association between La and pre-miRNAs, we transfected HeLa cells with a empty vector or FLAG-hLa/SSB expression construct. Thirty-six hours after transfection, HeLa cells were either untreated or treated with 1.0% formaldehyde before making protein extracts in RIPA buffer (49). The FLAG-La RNP complexes were immunoprecipitated from lysates using anti-FLAG (M2) antibody-conjugated affinity gel (Sigma). After extensive washing in a highly stringent condition (RIPA buffer containing 1 M NaCl and 4 M urea), the beads were resuspended in reversal buffer (50 mM Tris-Cl, pH 7.0, 5 mM EDTA, 10 mM DTT, and 1% SDS) and cooked at 70 °C for 1 h. Associated RNA was extracted by TRIzol (Invitrogen), and followed by RT-PCR to detect endogenous pre-miRNAs. The PCR primers for amplifying pri/pre-miRNAs were listed in Table 1.

RNAi Knockdown—For siRNA-mediated knockdown in HeLa cells, 1×10^6 of cells were plated in a 10-cm dish and

transfected with different siRNAs (siGFP, siLa1, or siLa2) by Lipofectamine RNAi Max (Invitrogen). After 2 days, transfected cells were replated at 1×10^6 cells/10-cm dish followed by a second siRNA transfection. Three days later, cells were harvested and total RNA was extracted by TRIzol. To generate the Tet_{on}-inducible La knockdown cells, we stably integrated a transgene that encodes four copies of a small hairpin RNA (shLa, same target sequence as siLa 2, Table 1) under control of an inducible H1 promoter, into U2OS cells expressing Tet repressor (Oligoengine). Doxycycline was added to the medium (2 μ M final concentration) to induce the expression of small hairpin RNA (shLa). The U2OS/shLa (FLAG-dLa) rescue cell line was constructed by stable integration of a FLAG-tagged *Drosophila* La cDNA transgene into the U2OS/shLa cell line. Both HeLa and U2OS cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μ g/ml of streptomycin.

Quantitative Analysis of miRNA Expression—Northern blotting for pre-miRNA and miRNA detection was performed essentially as described (50). Typically, 30 μ g of total RNA was resolved by 12% urea-PAGE and transferred to GT membrane (Bio-Rad) followed by UV cross-linking. All miRNA probes are 21-nt ssDNA or RNA oligos (IDT) that are complementary to miRNA sequences provided by miRBase. The 5S, 5.8S, and U6 probe sequences are listed in Table 1. All probes were 5' radiolabeled with [γ -³²P]ATP by T4 polynucleotide kinase (New England Biolabs). Hybridization was conducted in ultrasensitive hybridization buffer (Ambion) at 40 °C overnight. The membrane was washed 3 times at 40 °C in 2 \times SSC, 0.5% SDS, and exposed to x-ray film.

La/SSB Promotes MicroRNA Expression

Global miRNA Profiling—For RT-PCR, cDNAs were generated using the reverse transcription kit (Applied Biosystems) and amplified by PCR with *Taq* polymerase. The primer sequences are listed in Table 1. TaqMan qPCR was performed to measure the levels of specific miRNAs or pri-miRNAs according to the manufacturer's protocol (Applied Biosystems). Relative quantities were calculated using the $\Delta\Delta C_t$ method. 18S rRNA served as a loading control. Global miRNA profiling was performed using MegaPlex RT Primers and TaqMan Array microRNA Cards (Applied Biosystems, Human Pool A version 2.1 MegaPlex RT primers and Human MicroRNA A Cards version 2.0) according to the manufacturer's protocols. 750 ng of total RNA was used in Megaplex RT without pre-amplification. U6 snRNA served as the endogenous control as the average C_t varied by ≤ 0.2 between control and experimental treatments. Threshold was held constant at $0.2 \Delta R_n$ and baseline was set from cycles 3 to 16 for all miRNA. miRNA with average $C_t \geq 35$ or abnormal amplification curves in control and experimental treatments were omitted and considered as not expressed. Average relative quantities were calculated among replicates and a *t* test was used to identify miRNA differentially expressed based on a *p* value threshold of $p \leq 0.05$.

Native Gel-shift Assays—Synthetic pre-miRNAs were 5' radiolabeled with [γ - 32 P]ATP by T4 polynucleotide kinase followed by G-25 column (Ambion) purification. The pri-let-7a-1 construct, a gift from Dr. Narry Kim, was used to generate uniformly radiolabeled pri-miRNA substrate by *in vitro* transcription followed by PAGE purification. Typically, recombinant proteins and radiolabeled RNA were incubated at 37 (human proteins) or 30 °C (*Drosophila* proteins) for 30 min in a 10- μ l reaction (100 mM KOAc, 15 mM HEPES, pH 7.4, 2.5 mM EDTA, 2.5 mM DTT, pH 7.4). The reaction mixture was resolved by a 5% native PAGE and exposed to x-ray film.

Gene Set Enrichment Analysis (GSEA)—Gene expression profiling data of breast cancer patients were obtained from the NCBI Gene Expression Omnibus (GSE7390) and bioinformatics.nki.nl/data.php. The expression levels of Dicer, La/SSB, or MCP1P1 were evaluated by the corresponding probes. Genes negatively or positively associated with Dicer expression ("Dicer high/low down-regulated or up-regulated genes") were defined as 200 of the most differentially expressed genes between the top 30 cases with high and low Dicer expression. After dividing all cases into halves according to La expression, GSEA was performed with GSEA software available from the Broad Institute (51). Survival analysis was performed using the survival package of R, the survfit function, and the survdiff function. Classification of high or low expression of Dicer, La/SSB, and MCP1P1 was performed as previously described (52).

RESULTS

Purification and Reconstitution of La/SSB as a Regulator of Pre-miRNA Processing *in Vitro*—We took an unbiased biochemical approach to identify new regulators of miRNA biogenesis by supplementing recombinant Dicer-1-Loqs-PB complex (Fig. 1A) with the fractions of cytoplasmic extract (S100) of *Drosophila* S2 cells. We observed an inhibitory activity of pre-

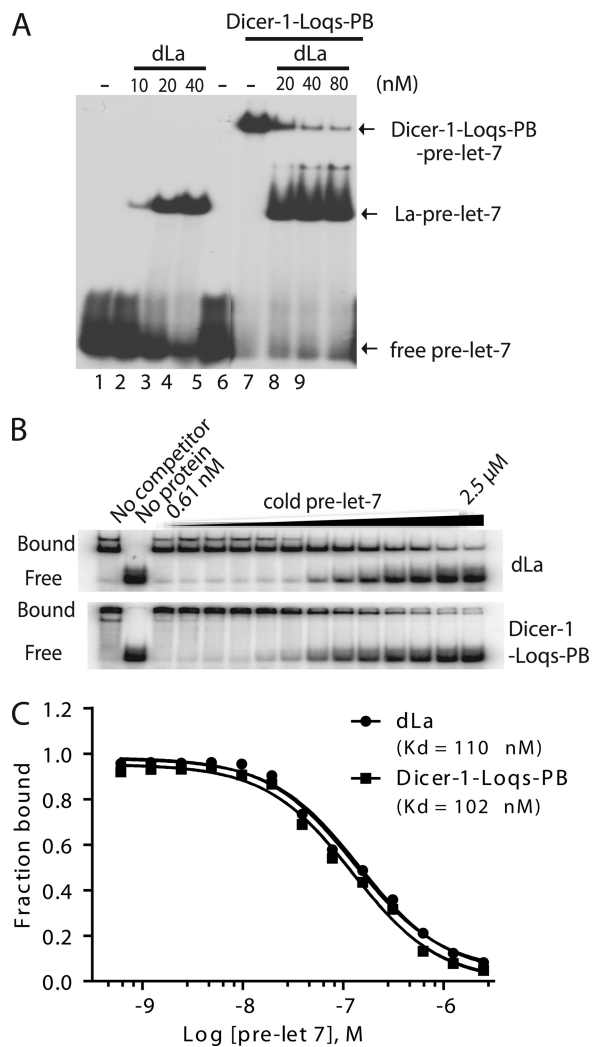


FIGURE 2. La/SSB binds pre-miRNA to regulate pre-miRNA processing *in vitro*. A, native gel-shift assays by incubating 5' radiolabeled pre-let-7 in buffer (lanes 1 and 5), recombinant dLa (lanes 2–4), Dicer-1-Loqs-PB complex alone (lane 6) or together with various concentrations of dLa (lanes 7–9). B, the competition binding assays were performed by incubating equivalent recombinant dLa or Dicer-1-Loqs-PB complex with radiolabeled pre-let-7 in the presence of increasing concentrations of cold pre-let-7. C, quantitative analysis of the data in B, and relative K_d value was measured by GraphPad Prism 5 software.

miRNA processing in the supernatant after 60% saturation of ammonium sulfate precipitation of S2/S100 (Fig. 1B). This factor was further purified to homogeneity by sequential chromatographic fractionation (Fig. 1C). At the final purification step, only a single protein of ~ 50 kDa appeared on the silver-stained polyacrylamide gel (PAGE), and correlated closely with the miRNA regulatory activity (Fig. 1C). This protein was identified as the *Drosophila* homolog of La/SSB by mass spectrometric analysis.

We generated His-tagged *Drosophila* (dLa) and human (hLa) La/SSB recombinant proteins (Fig. 1D). Addition of dLa efficiently suppressed the ability of the recombinant Dicer-1-Loqs-PB complex to process pre-miRNA *in vitro* (Fig. 1E). Likewise, hLa could inhibit pre-miRNA processing by the recombinant Dicer-TRBP complex (Fig. 1F). Intriguingly, we obtained a strikingly different result when performing pre-miRNA processing assays using crude cell extracts. Addition of

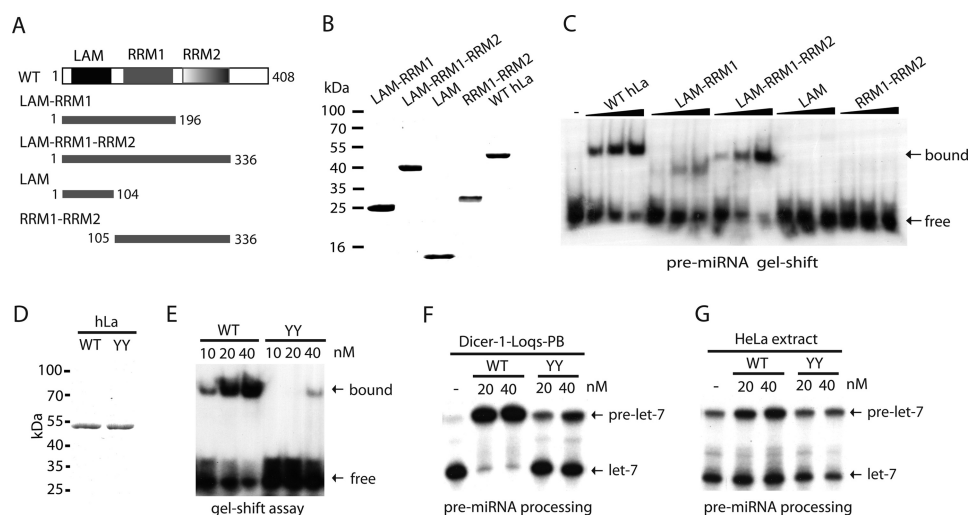


FIGURE 3. The LAM, RRM1, and RRM2 motifs of La/SSB are all required for efficient pre-miRNA binding. *A*, schematic domain structures of full-length and truncated human La/SSB. *B*, Coomassie-stained SDS-PAGE showing various purified recombinant La/SSB. *C*, native gel-shift assays comparing the binding of various recombinant La/SSB to 5' radiolabeled pre-let-7. *D*, Coomassie-stained SDS-PAGE showing wild-type (WT) and Y23A,Y24A (YY) mutant hLa recombinant proteins. *E*, native gel-shift assays comparing the ability of WT and YY mutant hLa to bind radiolabeled pre-let-7a-1. *F*, the pre-miRNA processing assays were performed with recombinant Dicer-1·Loqs-PB complex alone or together with WT or YY mutant hLa. *G*, the pre-miRNA processing assays were performed with 10 μ g of HeLa extract alone or together with recombinant WT or YY mutant hLa.

La/SSB could instead stabilize pre-miRNA and enhance miRNA production in both S2 and HeLa cell extracts (Fig. 1, *G* and *H*). The degree of miRNA increase was smaller than that of the pre-miRNA increase, suggesting a combined effect of pre-miRNA stabilization and sequestration at work.

La/SSB Is a Pre-miRNA-binding Protein—Recombinant dLa could efficiently bind pre-miRNA and compete with Dicer-1·Loqs-PB complex for pre-miRNA binding *in vitro* (Fig. 2*A*), suggesting that the binding of pre-miRNA by dLa is incompatible with that of the Dicer complex. Furthermore, recombinant dLa and Dicer-1·Loqs-PB exhibited comparable binding affinity for pre-miRNA *in vitro* (Fig. 2, *B* and *C*). A similar phenomenon was observed for recombinant hLa and Dicer·TRBP complex (data not shown). These results suggest that pre-miRNAs could be readily exchanged between La/SSB and the Dicer complex. Therefore, in a recombinant system, La/SSB could suppress miRNA production by sequestering pre-miRNA away from Dicer. However, in the crude extract or live cells, we hypothesized that La/SSB might promote miRNA expression by protecting pre-miRNAs from nuclease-mediated decay to allow for productive processing by Dicer.

The LAM, RRM1, and RRM2 Motifs of La/SSB Are All Required for Efficient Pre-miRNA Binding—To map which domains of La/SSB are important for binding pre-miRNA, we generated a series of truncated human La/SSB recombinant proteins (Fig. 3, *A* and *B*), and compared their pre-miRNA binding by gel-shift assays. Although LAM-RRM1 showed weak binding to pre-miRNA, neither LAM nor RRM1-RRM2 was able to bind pre-miRNA (Fig. 3*C*). In contrast, LAM-RRM1-RRM2 could bind pre-miRNA almost as efficiently as full-length La/SSB (Fig. 3*C*). Thus, the LAM, RRM1, and RRM2 motifs of La/SSB are all required for efficient binding of pre-miRNA. This mode of interaction is clearly distinct from the previously established mode of 3' UUU recognition by LAM-RRM1 (40–42).

La/SSB Regulates Pre-miRNA Processing by Binding Pre-miRNA—Two tyrosines (Tyr-23, Tyr-24) in the LAM of human La/SSB have previously been shown to be critical for 3' UUU binding (34). In our assays, mutations of Tyr-23 and Tyr-24 to alanine (YY) diminished the ability hLa to bind pre-miRNA (Fig. 3, *D* and *E*). Accordingly, the YY mutant also lost its ability to either inhibit pre-miRNA processing by recombinant Dicer-1·Loqs-PB complex (Fig. 3*F*), or stabilize pre-miRNA and enhance miRNA production in HeLa extract (Fig. 3*G*). These results suggest that the activity of La/SSB to regulate pre-miRNA processing is dependent on its ability to bind pre-miRNA.

La/SSB Binds Pre-miRNAs via a 3' UUU-independent Mechanism—Profiling of the miRNA database found that the majority of *Drosophila* and human pre-miRNAs lack the 3' UUU terminus (Fig. 4*A*). Among the 240 fly pre-miRNAs, only three have 3 Us out of the last four nucleotides. Among the 1,527 human pre-miRNAs, only six carry a 3' UUUU tail and 21 have 3 Us within the last four nucleotides. Accordingly, recombinant hLa could efficiently bind a number of synthetic pre-miRNAs lacking 3' UUU (data not shown). Moreover, we carefully compared the binding of hLa to mouse pre-miR-23b (lacking 3' UUU) and a modified pre-miR-23b that carried 3' UUU (Fig. 4, *B* and *C*). These experiments showed that addition of 3' UUU made little or no change to the binding of La/SSB to pre-miR-23b. Taken together, these studies suggest that La/SSB primarily binds pre-miRNAs via a 3' UUU-independent mechanism.

La/SSB Recognizes the Stem-Loop Structure of Pre-miRNAs—Because all pre-miRNAs share a stem-loop structure, we hypothesized that La/SSB could recognize the common stem-loop structure rather than specific sequences of pre-miRNAs. To test this idea, we synthesized a series of truncated forms of human pre-let-7a-1, which consists of a 21-bp imperfect double-stranded stem and a 27-nt single-stranded loop (21–27)

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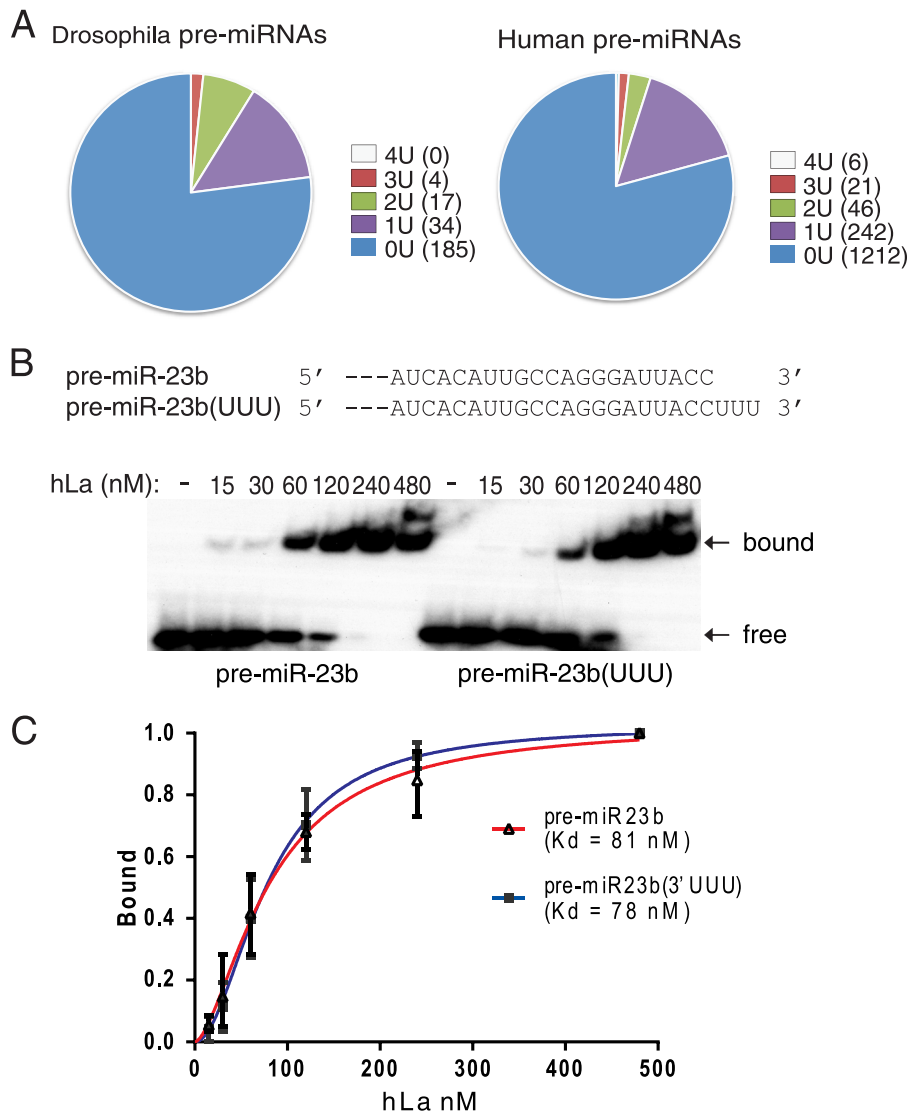


FIGURE 4. La/SSB binds pre-miRNA independent of 3' UUU terminus. *A*, bioinformatic analysis of the number of Us in the 3' terminal four nucleotides among the *Drosophila* (left) or human (right) pre-miRNAs in the miRNA database. *B*, native gel-shift assays comparing the binding of recombinant hLa/SSB to mouse pre-miR-23b and a modified pre-miR-23b(UUU). The 3' sequences of pre-miRNAs are listed above. *C*, quantitative analysis of the data in *B*, and the relative K_d value was measured by GraphPad Prism 5 software.

(Fig. 5A). Shortening the loop of pre-let-7a-1 from 27 to 4 nt reduced the binding of hLa, whereas deleting the loop abolished the binding of hLa to pre-let-7a-1, as in the case of let-7a/let* miRNA duplex (Fig. 5, *B* and *D*). Thus, the loop of pre-miRNA is critical for La/SSB binding, but in a sequence-independent manner as the binding of pre-let-7a-1 was not affected by substituting its loop with antisense sequence (data not shown). However, the La/SSB-pre-miRNA interaction cannot be explained by the loop binding alone because the binding of pre-let-7a-1 could not be competed off with an excess amount of ssRNA (data not shown).

Surprisingly, shortening the double-stranded stem of pre-let-7a-1 had little impact on the binding of hLa as shown by the similar dissociation constant (K_d) among the 21–27, 10–27, and 5–27 forms of pre-let-7a-1 (Fig. 5, *B* and *C*). Notably, removing 3' UUUC from pre-let-7a-1 did not affect hLa binding, either. However, a clear combinatorial effect was observed when both the stem and loop of pre-let-7a-1 were shortened at

the same time. For example, recombinant hLa could efficiently bind the 10–27 ($K_d = \sim 64$ nM) or 10–10 ($K_d = \sim 105$ nM) mutants, but the binding of 10–5 mutant was greatly diminished (Fig. 5, *B* and *E*). Likewise, hLa showed strong binding to the 5–27 ($K_d = \sim 88$ nM) mutant, but not to the 5–10 ($K_d = \sim 357$ nM) or 5–5 mutants (K_d was too big to be accurately measured) (Fig. 5, *B* and *F*). Taken together, these results strongly suggest that La/SSB recognizes the characteristic stem-loop structure of pre-miRNAs.

La/SSB Associates with Cellular Pre-miRNAs in Vivo—We employed a monoclonal anti-hLa/SSB antibody to immunoprecipitate (IP) the endogenous La/SSB RNA-protein (RNP) complexes from HeLa cell extract. By 5' radiolabeling of associated RNA, we detected abundant ~60–80-nt RNA species in the IPs of anti-hLa/SSB antibodies, but not in the IPs of control IgG (Fig. 6A). The La/SSB-associated RNAs likely contained pre-miRNAs because they could be efficiently cleaved by the Dicer-1-Loqs-PB complex into ~21-nt small RNA (Fig. 6B). The interac-

La/SSB Promotes MicroRNA Expression

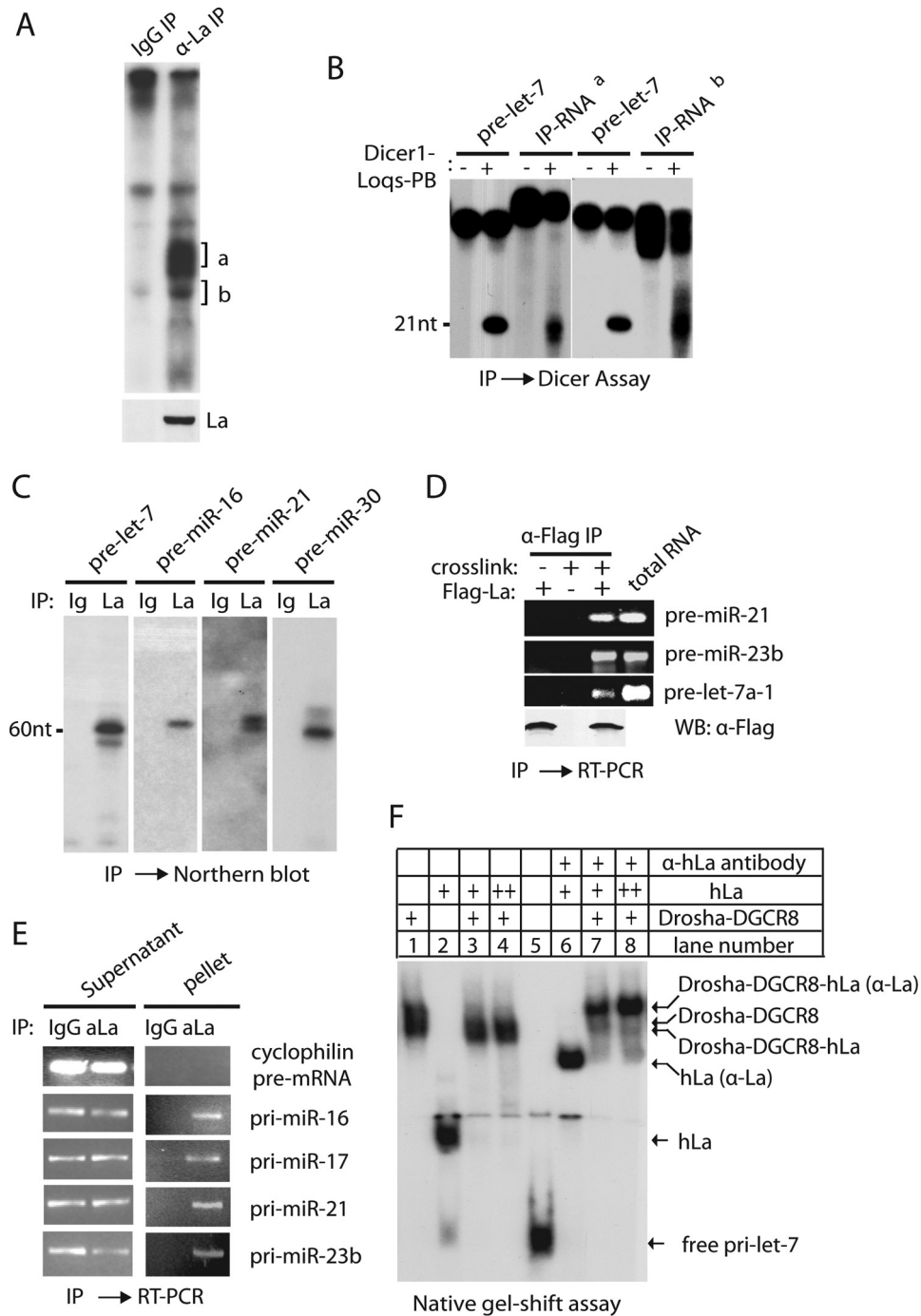


FIGURE 6. La/SSB associates with cellular pri-/pre-miRNAs in vivo. *A*, after IP of HeLa extract by mouse IgG or anti-hLa/SSB monoclonal antibodies, associated RNAs were extracted and 5' radiolabeled, resolved by 10% urea-PAGE, and visualized by autoradiography. *B*, two bands of La/SSB-associated RNA were recovered from urea-PAGE in *A* and incubated with recombinant Dicer-1·Loqs-PB complex to assay for miRNA production. Synthetic pre-let-7 was used as a positive control. *C*, Northern blotting specifically detected a number of pre-miRNAs in the IPs of anti-hLa/SSB antibodies. *D*, after transfection of a vector or FLAG-hLa construct, HeLa cells were treated with formaldehyde cross-linking before cell lysis. Immunoprecipitation of FLAG-La from lysates was carried out with stringent washing conditions followed by RT-PCR to detect the presence of endogenous pre-miRNAs in the FLAG-La RNP complexes. *E*, after immunoprecipitation of HeLa nuclear extract by mouse IgG or anti-hLa/SSB antibodies, associated RNAs were extracted from both pellets and supernatants, and followed by RT-PCR to detect the presence of pri-miRNA transcripts or cyclophilin pre-mRNA. *F*, native gel-shift assays were performed by incubating radio-labeled pri-let-7a-1 with buffer (*lane 5*), Drosha-DGCR8 complex (*lane 1*), hLa (*lane 2*), hLa + Drosha-DGCR8 (*lanes 3 and 4*), hLa + anti-La antibody (*lane 6*), hLa + Drosha-DGCR8 + anti-hLa antibody (*lanes 7 and 8*).

in repeated experiments. These findings were further validated by individual TaqMan qPCR of randomly selected miRNAs (data not shown). These results suggest that La/SSB is required for global miRNA expression *in vivo*.

La/SSB Promotes miRNA Expression by Stabilizing Pre-miRNAs—We compared the levels of pri-, pre-, and mature miRNAs between control and La/SSB-depleted HeLa cells by TaqMan qPCR and Northern blotting. Although the levels of

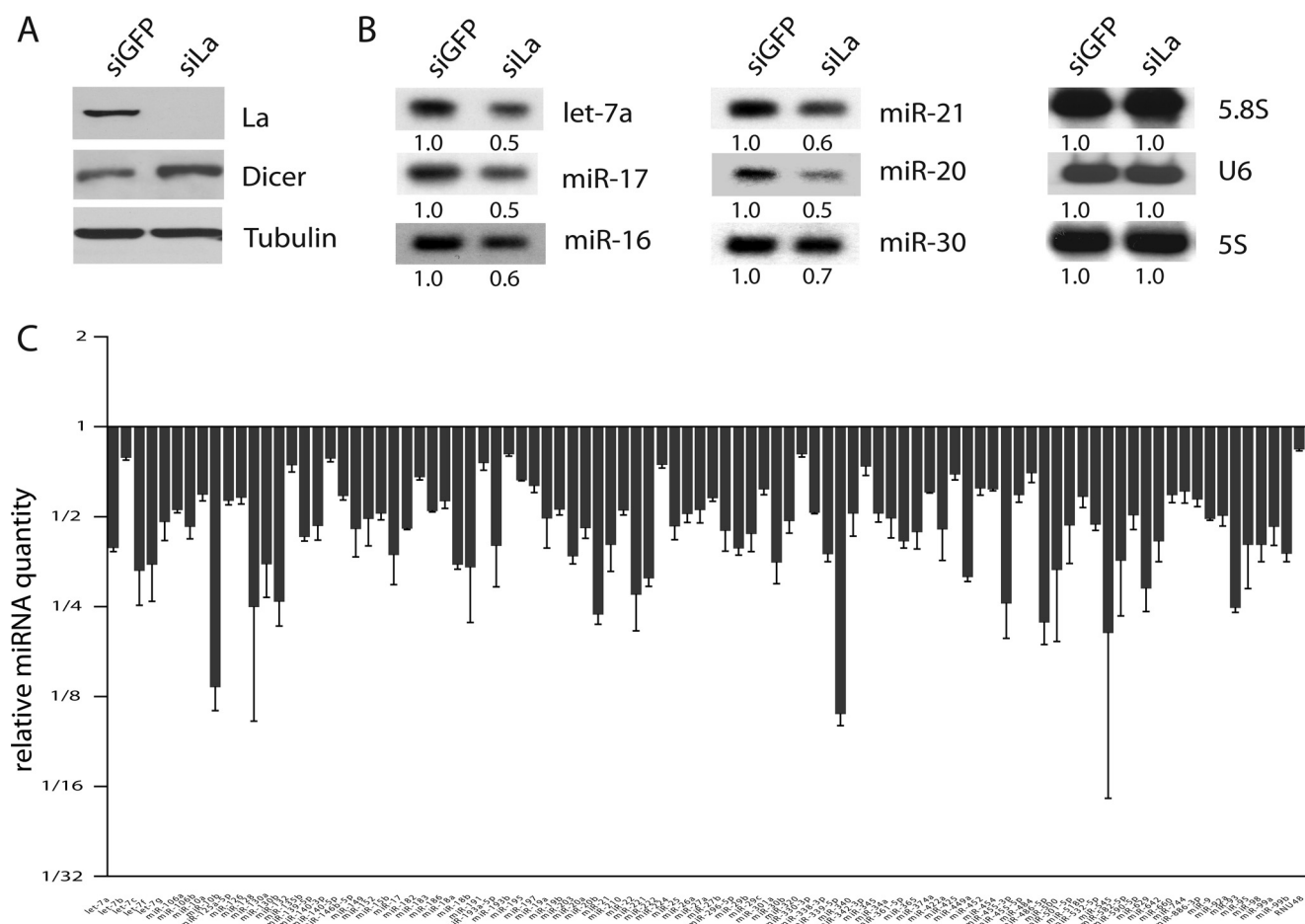


FIGURE 7. La/SSB is required for global miRNA expression *in vivo*. *A*, Western blots comparing the levels of La/SSB, Dicer, and α -Tubulin proteins between GFP-siRNA (*siGFP*) and La-siRNA (*siLa*)-treated HeLa cells. *B*, Northern blots comparing the levels of six endogenous miRNAs, U6 snRNA, and 5.8S or 5S rRNA between *siGFP*- and *siLa*-treated HeLa cells. *C*, quantitative analysis of global miRNA expression between *siGFP*- and *siLa*-treated HeLa cells. The levels of miRNAs were measured by TaqMan qPCR microRNA low density arrays and normalized to U6 snRNA (≤ 0.2 C_t difference among all samples). The relative miRNA quantity was calculated as the ratio of *siLa*/*siGFP* samples. Values represent mean \pm S.D., $n = 3$.

some pri-miRNAs remained the same, others were slightly decreased upon La/SSB depletion (Fig. 8A). In general, we detected a more substantial reduction of pre-miRNAs that was consistent with the degree of miRNA reduction in La/SSB-deficient cells (Fig. 8, *A* and *B*, and data not shown). Notably, although the level of pri-miR-23b remained unchanged, both pre-miR-23b and mature miR-23b were reduced by $\sim 30\%$ when La/SSB was depleted (Fig. 8, *A* and *B*). A similar phenomenon was observed in Tet_{on}-inducible La/SSB knockdown osteosarcoma (U2OS) cells. In these cells, induction of a short hairpin RNA (shLa) could effectively silence endogenous La/SSB expression, resulting in the concomitant reduction of pre-miRNAs and miRNAs (Fig. 8C). In another set of experiments, we also knocked down the expression of Dicer to allow for accumulation of pre-miRNAs in U2OS/shLa cells. In this case, we observed a much more dramatic reduction of pre-miRNAs when both La/SSB and Dicer were depleted (Fig. 8, *D* and *E*). Collectively, these results suggest that La/SSB promotes miRNA expression by stabilizing pre-miRNAs in mammalian cells.

La/SSB Protects Pre-miRNA from MCPIP1-mediated Degradation—In further support of this idea, we found that radiolabeled pre-miRNA was much less stable in the La/SSB-de-

pleted extract than in the control extract (Fig. 9A). Thus, the lack of La/SSB likely rendered pre-miRNAs more vulnerable to degradation by ribonucleases other than Dicer. The first example of such ribonucleases is MCPIP1, which was recently identified to inhibit miRNA production by cleaving the loop region of specific pre-miRNAs (33). Here, we showed that addition of hLa protected pre-miRNA from recombinant MCPIP1-mediated degradation *in vitro* (Fig. 9B). Furthermore, co-expression of hLa could antagonize the negative effect of MCPIP1 overexpression by stabilizing pre-miRNAs to enhance miRNA expression (Fig. 9C). Finally, the levels of both pre-miRNAs and miRNAs could be restored in La/SSB-deficient U2OS/shLa cells by expression of a FLAG-tagged *Drosophila* La/SSB transgene (Fig. 9, *D–F*). This experiment not only confirms that the miRNA phenotype is specifically due to the La/SSB deficiency, but also suggests that La/SSB plays an evolutionarily conserved role in miRNA regulation from flies to human. Therefore, our genetic and biochemical studies demonstrate that La/SSB promotes global miRNA expression by stabilizing pre-miRNAs from nuclease (e.g. MCPIP1)-mediated degradation.

Cooperative Relationships between La/SSB and Dicer in Human Cancers—It was previously reported that MCPIP1 and Dicer are in an antagonistic relationship in the transcriptome

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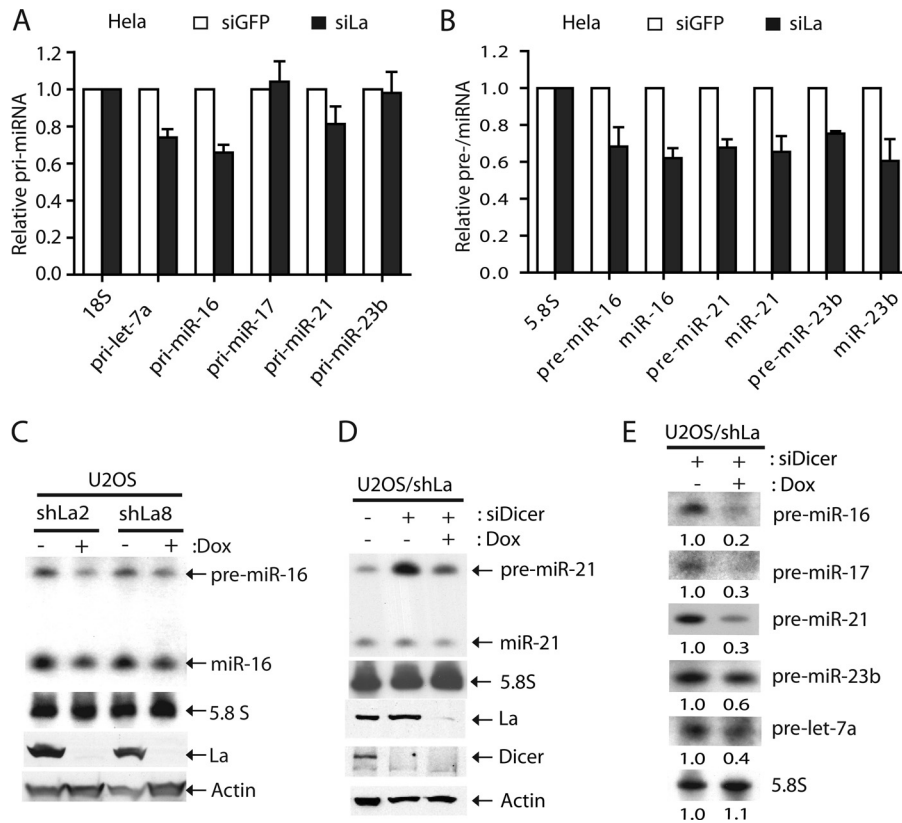


FIGURE 8. La/SSB promotes miRNA expression by stabilizing pre-miRNAs. *A*, quantitative analysis of pri-miRNA expression between siGFP- and siLa-treated HeLa cells by TaqMan PCR. 18S rRNA was used as a loading control. *B*, quantitative analysis of pre-miRNA and miRNA expression between siGFP- and siLa-treated HeLa cells by Northern blotting. 5.8S rRNA was used as loading control. *C*, Northern analysis of pre-miR-16 and miR-16 expression in two U2OS/shLa cell lines cultured in the absence or presence of 2 μ M doxycycline. Western blotting was performed to compare the levels of La/SSB or actin protein. *D*, Northern analysis of pre-miR-21 and miR-21 expression in U2OS/shLa cells, in which La/SSB, Dicer, or both were knocked down by RNAi. Western blotting was performed to compare the levels of La/SSB, Dicer, or actin protein. *E*, Northern analysis of the levels of various pre-miRNAs in U2OS/shLa cells, in which expression of La/SSB and/or Dicer was knocked down.

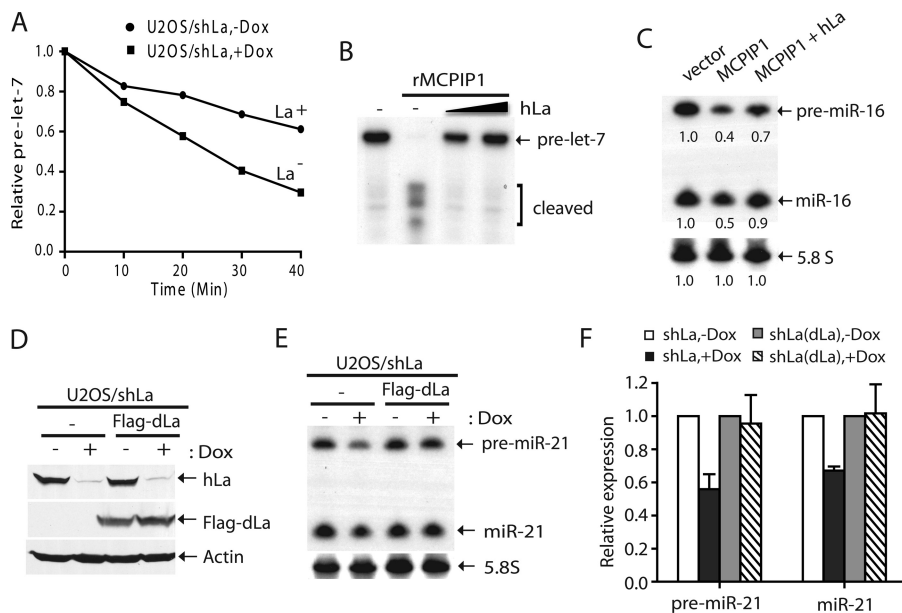


FIGURE 9. La/SSB protects pre-miRNAs from nuclease (e.g. MCPIP1)-mediated decay. *A*, a time course experiment comparing stability of a 5' radiolabeled pre-miRNA in La⁺ versus La⁻ extract prepared from U2OS/shLa cells cultured without (La⁺) or with doxycycline (La⁻). *B*, *in vitro* RNase assay showing that addition of hLa protects 5' radiolabeled pre-miRNA from recombinant MCPIP1-mediated degradation. *C*, Northern analysis of pre-miR-16 and miR-16 expression in 293T cells transiently transfected with a pri-miR-16 expression construct and vector, FLAG-MCPIP1 and/or FLAG-hLa constructs. *D*, Western blotting was performed to detect endogenous hLa, transgenic FLAG-dLa, and actin in U2OS/shLa or U2OS/shLa (+FLAG-dLa) cells. *E*, Northern analysis of pre-miR-21 and miR-21 expression in U2OS/shLa or U2OS/shLa (+FLAG-dLa) cells cultured without or with doxycycline. *F*, quantitative analysis of the data in *E* for four independent sets of experiments.

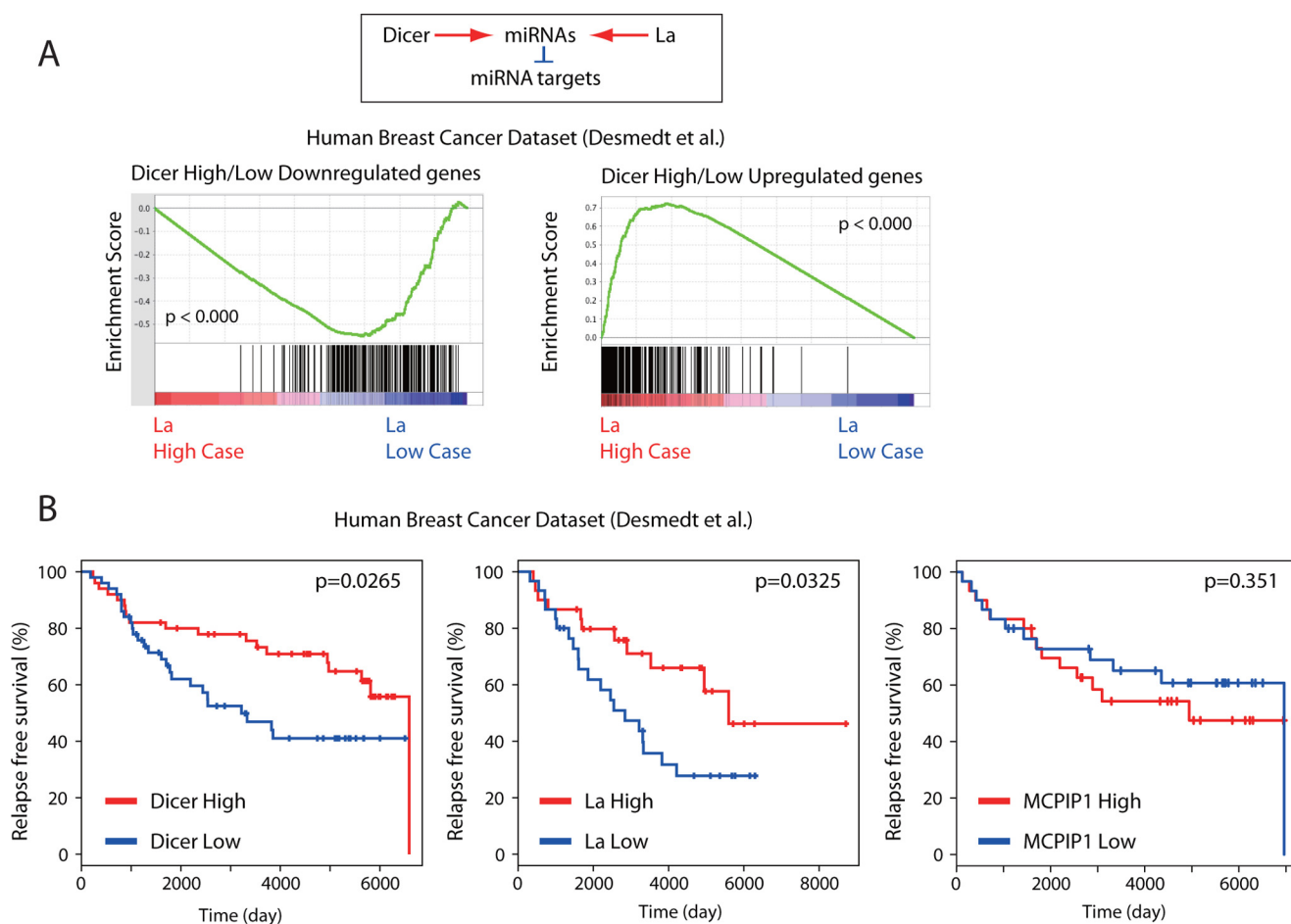


FIGURE 10. Cooperative relationships between La/SSB and Dicer in human cancers. *A*, GSEA for genes negatively or positively associated with Dicer expression (Dicer high/low down-regulated or up-regulated genes), along with patients with high and low La/SSB expression (La high case versus La low case), in the cohort of breast cancer patients. *B*, Kaplan-Meier plots representing the survival probability in this dataset, according to low or high expression levels of Dicer, La/SSB, and MCPIP1. The log-rank test p value reflects the significance of the association between low expression and Dicer and La/SSB and poor prognosis.

data of lung cancer patients (33). Thus, we analyzed the functional relationship between Dicer and La/SSB in human malignancies. To this end, we performed GSEA (51) to examine whether genes associated negatively or positively with Dicer expression status are overexpressed or underexpressed along with La/SSB expression status, using several public gene expression datasets of human malignancies. Intriguingly, we observed a potential cooperative relationship between Dicer and La/SSB in human breast cancer datasets (53). In this dataset, the Dicer high/low down-regulated genes are enriched in patients with low La/SSB expression ("La low case") (Fig. 10A). Conversely, the Dicer high/low up-regulated genes are enriched in patients with high La/SSB expression ("La high case") (Fig. 10A). This cooperative relationship between Dicer and La/SSB is opposite to the antagonistic relationship between Dicer and MCPIP1 in human cancer transcriptome (33).

Furthermore, low levels of La/SSB and Dicer were both associated with poor clinical outcome in breast cancer patients (Fig. 10B). Conversely, a high level of MCPIP1 is associated with worse prognosis. Although we failed to detect statistical significance in the latter case, the general trend is similar to what was previously reported in lung cancer patients (33). We also

observed similar correlations in another dataset of breast cancer patients (54) (data not shown). Thus, these results highlight an interesting tripartite relationship among Dicer, La/SSB, and MCPIP1 in the context of human malignancies. Taken together, these studies suggest that consistent with their critical roles in miRNA expression, the expression status of Dicer and La/SSB might have cooperative effects on the transcriptome and prognosis of certain human cancers.

DISCUSSION

In summary, we have identified a new housekeeping function of autoantigen La/SSB in promoting global miRNA expression by binding and stabilizing pre-miRNAs. We propose a revised working model for the basic process miRNA biogenesis. In the nucleus, La/SSB associates with nascent pri-miRNA transcripts and is required for the stability of some pri-miRNAs. The binding of La/SSB to pri-miRNAs is compatible with binding of the Drosha complex that processes pri-miRNAs into pre-miRNAs. Nascent pre-miRNAs are protected by La/SSB from degradation by other nucleases, such as MCPIP1. The comparable binding affinity allows pre-miRNAs to be easily transferred from La/SSB to the Dicer complex for processing into mature miRNAs. It is also plausible that this transfer process may be

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influenced by other proteins or by post-translational modifications. The work reveals a new concept of the pre-miRNA holding complex that protects and escorts nascent pre-miRNAs through the miRNA biogenesis pathway.

The La/SSB protein is well known for its versatile interactions with diverse RNA molecules, including numerous Pol III transcripts, specific cytoplasmic mRNA, and viral-encoded transcripts (35). Most of previous studies have focused on the interaction between La/SSB and 3' UUU of Pol III transcripts, such as pre-tRNAs. However, a few recent studies have reported that La/SSB associate with specific cellular and viral transcripts through a poorly understood, 3' UUU-independent mechanism (35). La/SSB has recently been shown to bind the domain IV stem-loop sequence of the internal ribosome entry site (IRES) of hepatitis C Virus mRNA (66). La/SSB recognizes the structure rather than specific sequence of domain IV RNA, and this association requires its three RNA binding motifs (i.e. LAM, RRM1 and RRM2). Herein, we have determined that La/SSB recognizes the typical stem-loop structure of pre-miRNAs. These studies implicate that stem-loop recognition may serve as a new mechanism that mediates association between La/SSB with diverse RNA lacking 3' UUU.

It is possible that additional sequence features may further modulate the binding of La/SSB to pre-miRNAs. Lin28 recruits terminal uridylyltransferase TUT4 to the polyuridylylate 3' end of a small subset of pre-miRNAs, including pre-let-7 (26, 28). In this case, however, 3' polyuridylation (addition of 5 to 19 Us) of pre-miRNA inhibits Dicer processing and leads to premature pre-miRNA degradation. It is unclear how many endogenous pre-miRNAs are uridylylated at the 3' terminus. Our studies indicate that addition of 3' UUU to a pre-miRNA have little effect on La/SSB binding. Thus, the interaction between La/SSB and pre-miRNA is primarily mediated by stem-loop recognition. This mode of interaction is similar to the mechanism of how the Dicer complex recognizes pre-miRNAs of diverse sequences.

Although La/SSB is dispensable for yeast viability (43, 44), La/SSB-deficient flies and knock-out mice die at early embryonic stage and La/SSB^{-/-} blastocysts fail to develop into embryonic stem cells *in vitro* (45, 46). These phenotypes are reminiscent of those displayed by mutant flies or mice lacking key miRNA-generating factors, such as Droscha, Dicer, Loqs, or Pasha/DGCR8 (11, 55–59). Because miRNAs do not exist in the budding or fission yeast, these observations are consistent with our theory that La/SSB plays an essential role in higher eukaryotes through regulation of global miRNA expression.

The previous and current studies suggest a dynamic process where La/SSB, MCPIP1, and Dicer compete to regulate miRNA production by modulating the levels of pre-miRNAs (33). Alterations of miRNA expression are linked to a wide range of pathological processes, including cancer (1, 3). It has been shown that the miRNA machineries can be modified by multiple mechanisms in human cancers. Such aberrant regulations include down-regulation of the miRNA processing enzymes, e.g. Dicer and Droscha (52, 60), and mutations of cofactors, e.g. TRBP, Exportin-5 (63, 64), and possibly La/SSB (this study). In several types of human malignancies, there are clear correlations between impaired miRNA processing, e.g. low expression of Droscha, Dicer, and La/SSB or high expression of MCPIP1,

and poor prognosis of cancer patients (33, 60–62). Collectively, these observations strongly suggest that a global reduction in miRNA expression, coupled with overexpression of specific oncogenic miRNAs, may be a distinct feature for certain, if not all of, human cancers.

Mounting studies indicate that miRNAs play critical roles in maintaining the homeostasis of the immune system and safeguarding autoimmunity (63). Human patients suffering Sjögren syndrome and systemic lupus erythematosus frequently produce autoantibodies against specific classes of RNA-binding proteins. These autoantigens include the commonly known La/SSB and Ro, as well as components of the miRNA pathway, such as Dicer, Argonaute, and GW182 (64, 65). Here, we have identified that autoantigen La/SSB also functions as a key regulator of global miRNA expression. Therefore, it is imperative to investigate if inactivation of La/SSB, such as by autoantibodies, down-regulation of expression, and/or phosphorylation, contributes directly to the pathogenesis of these autoimmune diseases.

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REFERENCES

1. Bartel, D. P. (2004) MicroRNAs. Genomics, biogenesis, mechanism, and function. *Cell* **116**, 281–297
2. Fabian, M. R., Sonenberg, N., and Filipowicz, W. (2010) Regulation of mRNA translation and stability by microRNAs. *Annu. Rev. Biochem.* **79**, 351–379
3. He, L., and Hannon, G. J. (2004) MicroRNAs. Small RNAs with a big role in gene regulation. *Nat. Rev. Genet.* **5**, 522–531
4. Ambros, V. (2004) The functions of animal microRNAs. *Nature* **431**, 350–355
5. Denli, A. M., Tops, B. B., Plasterk, R. H., Ketting, R. F., and Hannon, G. J. (2004) Processing of primary microRNAs by the Microprocessor complex. *Nature* **432**, 231–235
6. Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., and Shiekhattar, R. (2004) The Microprocessor complex mediates the genesis of microRNAs. *Nature* **432**, 235–240
7. Han, J., Lee, Y., Yeom, K. H., Nam, J. W., Heo, I., Rhee, J. K., Sohn, S. Y., Cho, Y., Zhang, B. T., and Kim, V. N. (2006) Molecular basis for the recognition of primary microRNAs by the Droscha-DGCR8 complex. *Cell* **125**, 887–901
8. Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Rådmark, O., Kim, S., and Kim, V. N. (2003) The nuclear RNase III Droscha initiates microRNA processing. *Nature* **425**, 415–419
9. Lund, E., Güttinger, S., Calado, A., Dahlberg, J. E., and Kutay, U. (2004) Nuclear export of microRNA precursors. *Science* **303**, 95–98
10. Yi, R., Qin, Y., Macara, I. G., and Cullen, B. R. (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev.* **17**, 3011–3016
11. Förstemann, K., Tomari, Y., Du, T., Vagin, V. V., Denli, A. M., Bratu, D. P., Klattenhoff, C., Theurkauf, W. E., and Zamore, P. D. (2005) Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein. *PLoS Biol.* **3**, e236
12. Jiang, F., Ye, X., Liu, X., Fincher, L., McKearin, D., and Liu, Q. (2005) Dicer-1 and R3D1-L catalyze miRNA maturation in *Drosophila*. *Genes Dev.* **19**, 1674–1679
13. Saito, K., Ishizuka, A., Siomi, H., and Siomi, M. C. (2005) Processing of pre-microRNAs by the Dicer-1-Loquacious complex in *Drosophila* cells. *PLoS Biol.* **3**, e235

14. Chendrimada, T. P., Gregory, R. I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K., and Shiekhattar, R. (2005) TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **436**, 740–744
15. Haase, A. D., Jaskiewicz, L., Zhang, H., Lainé, S., Sack, R., Gatignol, A., and Filipowicz, W. (2005) Mammalian Dicer finds a partner. *EMBO Rep.* **6**, 961–967
16. Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Bálint, E., Tuschl, T., and Zamore, P. D. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293**, 834–838
17. Paroo, Z., Ye, X., Chen, S., and Liu, Q. (2009) Phosphorylation of the human microRNA-generating complex mediates MAPK/Erk signaling. *Cell* **139**, 112–122
18. Carthew, R. W., and Sontheimer, E. J. (2009) Origins and Mechanisms of miRNAs and siRNAs. *Cell* **136**, 642–655
19. Kim, V. N., Han, J., and Siomi, M. C. (2009) Biogenesis of small RNAs in animals. *Nat. Rev. Mol. Cell Biol.* **10**, 126–139
20. Liu, Q., and Paroo, Z. (2010) Biochemical principles of small RNA pathways. *Annu. Rev. Biochem.* **79**, 295–319
21. Trabucchi, M., Briata, P., Filipowicz, W., Ramos, A., Gherzi, R., and Rosenfeld, M. G. (2010) KSRP promotes the maturation of a group of miRNA precursors. *Adv. Exp. Med. Biol.* **700**, 36–42
22. Trabucchi, M., Briata, P., Garcia-Mayoral, M., Haase, A. D., Filipowicz, W., Ramos, A., Gherzi, R., and Rosenfeld, M. G. (2009) The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. *Nature* **459**, 1010–1014
23. Davis, B. N., Hilyard, A. C., Lagna, G., and Hata, A. (2008) SMAD proteins control DROSHA-mediated microRNA maturation. *Nature* **454**, 56–61
24. Michlewski, G., and Cáceres, J. F. (2010) Antagonistic role of hnRNP A1 and KSRP in the regulation of let-7a biogenesis. *Nat. Struct. Mol. Biol.* **17**, 1011–1018
25. Hagan, J. P., Piskounova, E., and Gregory, R. I. (2009) Lin28 recruits the TUTase Zc3h11 to inhibit let-7 maturation in mouse embryonic stem cells. *Nat. Struct. Mol. Biol.* **16**, 1021–1025
26. Heo, I., Joo, C., Cho, J., Ha, M., Han, J., and Kim, V. N. (2008) Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. *Mol. Cell* **32**, 276–284
27. Viswanathan, S. R., Daley, G. Q., and Gregory, R. I. (2008) Selective blockade of microRNA processing by Lin28. *Science* **320**, 97–100
28. Heo, I., Joo, C., Kim, Y. K., Ha, M., Yoon, M. J., Cho, J., Yeom, K. H., Han, J., and Kim, V. N. (2009) TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. *Cell* **138**, 696–708
29. Morlando, M., Ballarino, M., Gromak, N., Pagano, F., Bozzoni, I., and Proudfoot, N. J. (2008) Primary microRNA transcripts are processed co-transcriptionally. *Nat. Struct. Mol. Biol.* **15**, 902–909
30. Pawlicki, J. M., and Steitz, J. A. (2008) Primary microRNA transcript retention at sites of transcription leads to enhanced microRNA production. *J. Cell Biol.* **182**, 61–76
31. Matsushita, K., Takeuchi, O., Standley, D. M., Kumagai, Y., Kawagoe, T., Miyake, T., Satoh, T., Kato, H., Tsujimura, T., Nakamura, H., and Akira, S. (2009) Zc3h12a is an RNase essential for controlling immune responses by regulating mRNA decay. *Nature* **458**, 1185–1190
32. Liang, J., Saad, Y., Lei, T., Wang, J., Qi, D., Yang, Q., Kolattukudy, P. E., and Fu, M. (2010) MCP-induced protein 1 deubiquitinates TRAF proteins and negatively regulates JNK and NF- κ B signaling. *J. Exp. Med.* **207**, 2959–2973
33. Suzuki, H. I., Arase, M., Matsuyama, H., Choi, Y. L., Ueno, T., Mano, H., Sugimoto, K., and Miyazono, K. (2011) MCPIP1 ribonuclease antagonizes dicer and terminates microRNA biogenesis through precursor microRNA degradation. *Mol. Cell* **44**, 424–436
34. Mattioli, M., and Reichlin, M. (1974) Heterogeneity of RNA protein antigens reactive with sera of patients with systemic lupus erythematosus. Description of a cytoplasmic nonribosomal antigen. *Arthritis Rheum.* **17**, 421–429
35. Wolin, S. L., and Cedervall, T. (2002) The La protein. *Annu. Rev. Biochem.* **71**, 375–403
36. Chakshusmathi, G., Kim, S. D., Rubinson, D. A., and Wolin, S. L. (2003) A La protein requirement for efficient pre-tRNA folding. *EMBO J.* **22**, 6562–6572
37. Fan, H., Goodier, J. L., Chamberlain, J. R., Engelke, D. R., and Maraia, R. J. (1998) 5' processing of tRNA precursors can be modulated by the human La antigen phosphoprotein. *Mol. Cell. Biol.* **18**, 3201–3211
38. Yoo, C. J., and Wolin, S. L. (1997) The La protein in *Schizosaccharomyces pombe*. A conserved yet dispensable phosphoprotein that functions in tRNA maturation. *Cell* **89**, 393–402
39. Bousquet-Antonelli, C., and Deragon, J. M. (2009) A comprehensive analysis of the La-motif protein superfamily. *RNA* **15**, 750–764
40. Alfano, C., Sanfelice, D., Babon, J., Kelly, G., Jacks, A., Curry, S., and Conte, M. R. (2004) Structural analysis of cooperative RNA binding by the La motif and central RRM domain of human La protein. *Nat. Struct. Mol. Biol.* **11**, 323–329
41. Kotik-Kogan, O., Valentine, E. R., Sanfelice, D., Conte, M. R., and Curry, S. (2008) Structural analysis reveals conformational plasticity in the recognition of RNA 3' ends by the human La protein. *Structure* **16**, 852–862
42. Teplova, M., Yuan, Y. R., Phan, A. T., Malinina, L., Ilin, S., Teplov, A., and Patel, D. J. (2006) Structural basis for recognition and sequestration of UUU(OH) 3' termini of nascent RNA polymerase III transcripts by La, a rheumatic disease autoantigen. *Mol. Cell* **21**, 75–85
43. Copela, L. A., Chakshusmathi, G., Sherrer, R. L., and Wolin, S. L. (2006) The La protein functions redundantly with tRNA modification enzymes to ensure tRNA structural stability. *RNA* **12**, 644–654
44. Yoo, C. J., and Wolin, S. L. (1994) La proteins from *Drosophila melanogaster* and *Saccharomyces cerevisiae*. A yeast homolog of the La autoantigen is dispensable for growth. *Mol. Cell. Biol.* **14**, 5412–5424
45. Park, J. M., Kohn, M. J., Bruinsma, M. W., Vech, C., Intine, R. V., Fuhrmann, S., Grinberg, A., Mukherjee, I., Love, P. E., Ko, M. S., DePamphilis, M. L., and Maraia, R. J. (2006) The multifunctional RNA-binding protein La is required for mouse development and for the establishment of embryonic stem cells. *Mol. Cell. Biol.* **26**, 1445–1451
46. Bai, C., and Tolia, P. P. (2000) Genetic analysis of a La homolog in *Drosophila melanogaster*. *Nucleic Acids Res.* **28**, 1078–1084
47. Han, J., Lee, Y., Yeom, K. H., Kim, Y. K., Jin, H., and Kim, V. N. (2004) The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev.* **18**, 3016–3027
48. Liu, Y., Ye, X., Jiang, F., Liang, C., Chen, D., Peng, J., Kinch, L. N., Grishin, N. V., and Liu, Q. (2009) C3PO, an endoribonuclease that promotes RNAi by facilitating RISC activation. *Science* **325**, 750–753
49. Niranjanakumari, S., Lasda, E., Brazas, R., and Garcia-Blanco, M. A. (2002) Reversible cross-linking combined with immunoprecipitation to study RNA-protein interactions *in vivo*. *Methods* **26**, 182–190
50. Liu, X., Park, J. K., Jiang, F., Liu, Y., McKearin, D., and Liu, Q. (2007) Dicer-1, but not Loquacious, is critical for assembly of miRNA-induced silencing complexes. *RNA* **13**, 2324–2329
51. Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., Lander, E. S., and Mesirov, J. P. (2005) Gene set enrichment analysis. A knowledge-based approach for interpreting genome-wide expression profiles. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 15545–15550
52. Martello, G., Rosato, A., Ferrari, F., Manfrin, A., Cordenonsi, M., Dupont, S., Enzo, E., Guzzardo, V., Rondina, M., Spruce, T., Parenti, A. R., Daidone, M. G., Biciato, S., and Piccolo, S. (2010) A MicroRNA targeting Dicer for metastasis control. *Cell* **141**, 1195–1207
53. Desmedt, C., Piette, F., Loi, S., Wang, Y., Lallemand, F., Haibe-Kains, B., Viale, G., Delorenzi, M., Zhang, Y., d'Assignies, M. S., Bergh, J., Lidereau, R., Ellis, P., Harris, A. L., Kljiv, J. G., Foekens, J. A., Cardoso, F., Piccart, M. J., Buyse, M., and Sotiriou, C. (2007) Strong time dependence of the 76-gene prognostic signature for node-negative breast cancer patients in the TRANSBIG multicenter independent validation series. *Clin. Cancer Res.* **13**, 3207–3214
54. van de Vijver, M. J., He, Y. D., van't Veer, L. J., Dai, H., Hart, A. A., Voskuil, D. W., Schreiber, G. J., Peterse, J. L., Roberts, C., Marton, M. J., Parrish, M., Atsma, D., Witteveen, A., Glas, A., Delahaye, L., van der Velde, T., Bartelink, H., Rodenhuis, S., Rutgers, E. T., Friend, S. H., and Bernards, R. (2002) A gene-expression signature as a predictor of survival in breast cancer.

La/SSB Promotes MicroRNA Expression

- N. Engl. J. Med.* **347**, 1999–2009
55. Hatfield, S. D., Shcherbata, H. R., Fischer, K. A., Nakahara, K., Carthew, R. W., and Ruohola-Baker, H. (2005) Stem cell division is regulated by the microRNA pathway. *Nature* **435**, 974–978
 56. Kanellopoulou, C., Muljo, S. A., Kung, A. L., Ganesan, S., Drapkin, R., Jenuwein, T., Livingston, D. M., and Rajewsky, K. (2005) Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes Dev.* **19**, 489–501
 57. Murchison, E. P., Partridge, J. F., Tam, O. H., Cheloufi, S., and Hannon, G. J. (2005) Characterization of Dicer-deficient murine embryonic stem cells. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 12135–12140
 58. Park, J. K., Liu, X., Strauss, T. J., McKearin, D. M., and Liu, Q. (2007) The miRNA pathway intrinsically controls self-renewal of *Drosophila* germline stem cells. *Curr. Biol.* **17**, 533–538
 59. Wang, Y., Medvid, R., Melton, C., Jaenisch, R., Blalock, R. (2007) DGCR8 is essential for microRNA biogenesis and silencing of embryonic stem cell self-renewal. *Nat. Genet.* **39**, 380–385
 60. Merritt, W. M., Lin, Y. G., Han, L. Y., Kamat, A. A., Spannuth, W. A., Schmandt, R., Urbauer, D., Pennacchio, L. A., Cheng, J. F., Nick, A. M., Deavers, M. T., Mourad-Zeidan, A., Wang, H., Mueller, P., Lenburg, M. E., Gray, J. W., Mok, S., Birrer, M. J., Lopez-Berestein, G., Coleman, R. L., Bar-Eli, M., and Sood, A. K. (2010) Dicer, Drosha, and outcomes in patients with ovarian cancer. *N. Engl. J. Med.* **359**, 2641–2650
 61. Melo, S. A., Roper, S., Moutinho, C., Aaltonen, L. A., Yamamoto, H., Calin, G. A., Rossi, S., Fernandez, A. F., Carneiro, F., Oliveira, C., Ferreira, B., Liu, C. G., Villanueva, A., Capella, G., Schwartz, S. Jr., Shiekhattar, R., and Esteller, M. (2009) A TARBP2 mutation in human cancer impairs microRNA processing and Dicer function. *Nat. Genet.* **41**, 365–370
 62. Melo, S. A., Moutinho, C., Roper, S., Calin, G. A., Rossi, S., Spizzo, R., Fernandez, A. F., Davalos, V., Villanueva, A., Montoya, G., Yamamoto, H., Schwartz, S., Jr., and Esteller, M. (2010) A genetic defect in exportin-5 traps precursor microRNAs in the nucleus of cancer cells. *Cancer Cell* **18**, 303–315
 63. Taganov, K. D., Boldin, M. P., and Baltimore, D. (2007) MicroRNAs and immunity. Tiny players in a big field. *Immunity* **26**, 133–137
 64. Pruijn, G. J. (2006) The RNA interference pathway. A new target for autoimmunity. *Arthritis Res. Ther.* **8**, 110
 65. Jakymiw, A., Ikeda, K., Fritzler, M. J., Reeves, W. H., Satoh, M., Chan, E. K. (2006) Autoimmune targeting of key components of RNA interference. *Arthritis Res. Ther.* **8**, R87
 66. Martino, L., Pennell, S., Kelly, G., Bui, T. T. T., Kotik-Kogan, O., Smerdon, S. J., Drake, A. F., Curry, S., and Conte, M. R. (2012) Analysis of the interaction with the hepatitis C virus mRNA reveals an alternative mode of RNA recognition by the human La protein. *Nucleic Acids Res.* **40**, 1381–1394