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Antagonistic role of hnRNP A1 and KSRP in the regulation of Let-7a biogenesis

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Abstract

The pluripotency promoting proteins Lin28a and Lin28b act as posttranscriptional repressors of let-7 miRNA biogenesis in undifferentiated embryonic stem cells. The levels of mature let-7a differ substantially in cells lacking Lin28 expression, indicating the existence of additional mechanism(s) of posttranscriptional regulation. Here, we present evidence supporting a role for hnRNP A1 as a negative regulator of let-7a. HnRNP A1 binds the conserved terminal loop of pri-let-7a-1 and inhibits its processing by Drosha. Levels of mature let-7a negatively correlate with hnRNP A1 levels in somatic cell lines. Furthermore, hnRNP A1 depletion increased pri-let-7a-1 processing by cell extracts, whereas its ectopic expression decreased let-7a production *in vivo*. Finally, hnRNP A1 binding to let-7a interferes with the binding of the KH-type splicing regulatory protein KSRP, known to promote let-7a biogenesis. We propose that hnRNP A1 and KSRP play antagonistic roles in the posttranscriptional regulation of let-7a expression.

Keywords

hnRNP A1; microRNAs; Drosha; Let-7a; oncogenesis

MicroRNAs (miRNAs) are endogenous single-stranded RNAs (ssRNAs) that base-pair with target mRNAs and negatively regulate their expression (reviewed by[1]). They have important regulatory roles in many different biological processes and miRNA dysregulation can contribute to various diseases, including cancer[2]. Their biogenesis involves a nuclear phase catalyzed by the RNase III Drosha enzyme, which results in the production of stem loop precursors, termed pre-miRNAs[3,4]. Following pre-miRNA export to the cytoplasm, there is an additional processing by the type III ribonuclease Dicer, resulting in the production of mature miRNAs (reviewed by[5]).

Drosha is part of a large multiprotein complex, termed the microprocessor, which also includes its partner, DGCR8, a double-stranded RNA-binding protein that is deleted in the DiGeorge syndrome[6,7], as well as several associated proteins such as the DEAD-box helicases p68/p72 and a number of hnRNP proteins[8,9]. A smaller microprocessor complex comprising just Drosha and DGCR8 is necessary and sufficient to process miRNAs. The associated proteins present in the larger microprocessor complex may act to provide regulation either directly or by recruiting regulatory factors. The Drosha cleavage of pri-miRNA precursors occurs co-transcriptionally on both independently transcribed as well as

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in intron-encoded miRNAs[10,11]. Furthermore, pri-miRNA processing has been shown to be enhanced by its coupling to transcription[12].

Interestingly, miRNA production is a highly regulated process that could be modulated at different steps throughout the biogenesis pathway (for a review see[13]). It has been reported that several pri-miRNA precursors fail to be processed by Drosha during early mouse development or during cancer progression[14]. The RNA helicases p68 and p72, which are part of the larger microprocessor complex, have been shown to be required for the processing of a subset of miRNAs[15]. Only recently, it was shown that estrogen-bound ERα blocks the Drosha processing of a subset of miRNAs that are dependent on p68/p72[16]. The posttranscriptional regulation of miRNA processing is not always inhibitory. For instance, transforming growth factor beta (TGF-β) and bone morphogenetic protein (BMP) signaling pathways positively regulate the Drosha-mediated processing of miR-21[17]. Cytoplasmic processing by Dicer can also be regulated in such a way that the expression of the mature miRNA is restricted to only a fraction of the tissues where the pre-miRNA is initially expressed[18].

The let-7 family of miRNAs is present in multiple copies in different genomes, with 10 mature let-7 family sequences produced from 13 precursors in humans (reviewed by[19]). The pluripotency promoting proteins Lin28a and Lin28b, which are exclusively expressed in undifferentiated cells and turned off following differentiation, bind to the terminal loop of let-7 precursors and block their processing in undifferentiated ES cells, acting at the level of Drosha[20-22] and/or Dicer[23,24]. The strong induction of let-7 during neural differentiation of embryonic stem (ES) cells also involves a feedback loop mechanism, whereby Lin-28 is downregulated by miR-125 and let-7, allowing processing of pre-let-7 to proceed[24].

HnRNP A1 is a nucleo-cytoplasmic shuttling protein with roles in many aspects of mRNA metabolism. It is involved in alternative splicing regulation antagonizing the activity of the SR family of proteins and also influences constitutive splicing by modulating the conformation of mammalian pre-mRNAs (for reviews see[25,26]). The shuttling ability of hnRNP A1 has been linked to its post-splicing roles such as internal ribosome entry site (IRES)-mediated translation and mRNA stability. Our lab has recently shown that hnRNP A1 specifically binds to a miRNA cluster containing miR-18a, and facilitates the Drosha-mediated processing of miR-18a but not of other members of the cluster[27]. We showed that hnRNP A1 binds to the conserved terminal loop of pri-miR-18a and induces a relaxation at the stem, creating a more favorable cleavage site for Drosha[28]. Furthermore, we found that 14% of all human pri-miRNAs have terminal loops that are well-conserved throughout evolution and suggested that they could act as landing pads for trans-acting factors that regulate biogenesis of miRNAs. An RNA chromatography approach combined with Mass Spectrometry analysis revealed that hnRNP A1 also binds to the terminal loop of pri-let-7a miRNA, which harbors a perfect hnRNP A1 consensus-binding site (UAGGGA/U)[28,29]. The splicing factor KSRP (KH-type splicing regulatory protein) contains four K homology RNA-binding domains and promotes exon inclusion of the *c-src* alternative exon through an intronic splicing enhancer[30]. It has been recently shown to be a component of both Drosha and Dicer complexes and positively regulates the biogenesis of a subset of miRNAs, including miR-155[31] and let-7[32]. Altogether, there is ample evidence to suggest the existence of trans-acting factors that bind to conserved terminal loops and can influence positively or negatively the processing of specific miRNAs.

Here, we report that hnRNP A1 binds specifically to the conserved terminal loop of the let-7a precursor and blocks its Drosha-mediated processing in somatic cells, where Lin28 is not expressed. We propose a model whereby hnRNP A1 antagonizes the function of the positively acting factor KSRP in the biogenesis of let-7a miRNA.

RESULTS

hnRNP A1 binds to the terminal loop of pri-let-7a-1

The hnRNP A1 protein has a modular structure with two copies of the RNA recognition motif (RRM) at its N-terminus and a Gly-rich auxiliary domain at the C-terminal protein (reviewed by[33]). The crystal structure of a proteolytic fragment consisting of both N-terminal RRMs (termed unwinding protein 1, or UP1) revealed that the two RRMs are antiparallel and held in close contact forming a single entity with an extended RNA-binding surface[34],[35]. By contrast, the Gly-rich domain seems to be responsible for cooperative binding, which starts with binding to a high-affinity site followed by cooperative spreading in a 3′-to-5′ direction[36].

We previously reported the binding of hnRNP A1 to the conserved terminal loop of pri-let-7a-1[28]. Several members of the let-7 family of miRNAs have perfect (let-7a-1, let-7f-2) or close to perfect (let-7f-1, let-7a-2, let-7c, let-7d) hnRNP A1 consensus binding site, (UAGGGA/U) as defined by SELEX[29] (Supplementary Fig. 1). In order to analyze the interaction between the terminal loop of pri-let-7a and hnRNP A1, we performed electrophoretic gel mobility shift analysis (EMSA) using UP1, to avoid cooperative spreading during RNA binding[36]. We observed that UP1, but not the individual RRMs (RRM1 or RRM2), was able to bind efficiently to the terminal loop of wild-type pri-let-7a-1 (Supplementary Fig. 2a-c); this binding was competed by an excess of unlabeled let-7a-1 terminal loop but not of miR-18a or miR-379 loops (Fig. 1b, and Supplementary Fig. 2d, respectively). Consistently, UP1 did not bind to an oligo in which the wild-type terminal loop sequence was replaced with a GCAA motif (pri-let-7a-1_loop_mt1) (Fig. 1). Importantly, a let-7a-1 terminal loop sequence with a mutation in the hnRNP A1 consensus binding site (pri-let-7a-1 GGG/CCC mutant) did not bind UP1 efficiently (Supplementary Fig. 2e), neither could it compete UP1 binding to a let-7a-1 wild-type sequence (Supplementary Fig. 2f). Although miR-18a, miR-379 and let-7a-1 GGG/CCC stem loops were not effective competitors in this assay, their addition resulted in faster migrating UP1/let-7a-1 complexes (Supplementary Fig. 2d,f). One possible explanation is that the let-7a-1 terminal loop sequence might have two binding sites for UP1, so the other miRNAs used as competitors could compete for UP1 binding to the lower affinity site in-let-7a-1, resulting in a faster migrating complex.

Inverse correlation between hnRNP A1 and let-7a levels in human cells

Lin28 is expressed specifically in undifferentiated cells and is strongly downregulated upon differentiation, whereas KSRP has been shown to promote let-7 biogenesis[32]. Thus, a dominant role for Lin28 over KSRP would explain the posttranscriptional repression of let-7 biogenesis in embryonic cells. This creates the paradox of certain cells and tissues where the levels of let-7 miRNAs are low despite the absence of Lin28 expression, strongly suggesting the existence of yet an unidentified repressor of let-7a processing. We hypothesized that the binding of hnRNP A1 to the terminal loop of let-7a could have an inhibitory role. First, we analyzed the levels of hnRNP A1 protein in different cell lines and correlated those with the levels of known let-7a regulatory factors in various cell types. As expected, the levels of the negative factor Lin28a, which is predominantly expressed in undifferentiated cells, were undetectable in 293T, HeLa or Astrocytoma cell lines, but showed high abundance in undifferentiated P19 mouse cells (Supplementary Fig. 3a). By contrast, the levels of the positive let-7a factor KSRP were similar in all cell types (Fig. 2a). Western blot analysis revealed that hnRNP A1 is highly abundant in P19 undifferentiated mouse cells and in human 293T cells, with an intermediate level in HeLa cells and low levels in Astrocytoma cells (Fig. 2a and Supplementary Fig. 3a). In these cell lines the levels of mature, endogenous let-7a were significantly different. We could establish a strong correlation

between high levels of hnRNP A1 protein and low levels of let-7a, as shown in 293T cells, suggesting that hnRNP A1 could be acting as a repressor of let-7a biogenesis (Fig. 2). Importantly, these changes could not be attributed to different rates of let-7a transcription, as the levels of the primary transcripts were comparable across all cell lines analyzed (Supplementary Fig. 3b,c). In agreement with our previous results[27], we observed the highest levels of miR-18a in 293T cells (Supplementary Fig. 4a). Comparable levels of control miR-16 ensured that the general miRNA processing machinery was equally functional in 293T, HeLa and Astrocytoma cells (Supplementary Fig. 4b).

To further confirm these findings we analyzed the levels of endogenous let-7a in cells with ectopic expression of hnRNP A1. Transient transfection of an epitope-tagged hnRNP A1 expression vector into HeLa or Astrocytoma cells resulted in reduced levels of mature let-7a miRNA in both cell types (Fig. 3a-d). As expected, overexpression of Lin28a in HeLa cells, where this protein is normally not expressed, also resulted in significantly reduced levels of let-7a (Fig. 3b). Intriguingly, we could not detect any fluctuations in the levels of pre-let-7a-1 upon hnRNP A1 overexpression. This might be a consequence of a tight control of pre-miRNA processing by Dicer and/or pre-miRNA turnover by a yet unidentified nuclease. A similar phenomenon has been observed during the Lin28-mediated regulation of let-7 biogenesis in ES cells[23]. Unexpectedly, increased expression of Lin28 elicited only a small reduction in the levels of mature let-7a in Astrocytoma cells (Fig. 3d). This could be due to several reasons. It may well be that co-factors required for the repressive effect of Lin28, such as the 3′ terminal uridylyl transferase (TUTase) that mediates pre-let-7 uridylation and subsequent blockade of let-7 processing are absent or expressed at lower levels in Astrocytoma cells[37-39]. Alternatively, it remains possible that an additional activator, other than KSRP, is present in Astrocytoma cells.

In order to elucidate which step of let-7a biogenesis was affected by hnRNP A1 overexpression, we used qRT-PCR with specific primers designed to cover either the upstream region or to span the pri-let-7a-1 stem loop structure (Fig. 3e). The microprocessor cleaves pri-miRNAs in a co-transcriptional manner only marginally affecting the overall stability and integrity of the host transcript[10, 11], thus, the upstream primers can detect both un-cleaved and cleaved pri-let-7a-1 transcripts, reflecting changes in transcription and/or RNA stability. By contrast, the set of primers encompassing the pri-let-7a-1 stem-loop only detect the pool of pri-let-7a-1 transcripts that has not been cleaved by Drosha. Increased expression of hnRNP A1 in HeLa cells resulted in a 4.5 fold accumulation of unprocessed pri-let-7a-1 molecules with no apparent change in the rate of pri-let-7a-1 transcription and/or stability (Fig. 3f). Interestingly, overexpression of Lin28a greatly reduced the levels of mature let-7a (Fig. 3b), while only resulting in 1.5 fold accumulation of unprocessed pri-let-7a-1 transcripts. This is in agreement with previous reports showing a dual role of Lin28 at the Drosha and Dicer levels of processing (reviewed by[13]). The levels of overexpressed hnRNP A1 and Lin28a are shown on Supplementary Fig. 5a.

hnRNP A1 blocks the Drosha-mediated processing of let-7a

To test a direct involvement of hnRNP A1 in the biogenesis of let-7a we performed *in vitro* processing assays using control, hnRNP A1-depleted or hnRNP L-depleted HeLa cell extracts (see Supplementary Fig. 5b for levels of hnRNP A1 depletion). As shown in Fig. 4a, the let-7a-1primary transcript (pri-let7-a1) was readily processed *in vitro* in the presence of HeLa extracts (lane 2), rendering a product of ~72 nts that corresponds to pre-let-7a. We observed that the efficiency of Drosha cleavage of pri-let-7a-1 transcript was significantly enhanced in the absence of hnRNP A1 (Fig. 4a, lane 3). Depletion of hnRNP L that was previously shown to bind to the terminal loop of let-7a[28], didn't affect the efficiency of pri-let-7a-1 cleavage (Fig. 4a, lane 4), indicating that binding per se is not necessarily indicative of function. In agreement, increasing amounts of recombinant hnRNP A1 protein strongly

inhibited the microprocessor-induced cleavage of pri-let-7a-1 *in vitro*, in a dose-dependent manner (Fig. 4b). By contrast, addition of the positive modulator KSRP had a slight stimulatory effect on the pri-let-7a-1 processing, perhaps suggesting that KSRP is not a limiting factor in these extracts (data not shown). In vitro processing of pri-let-7a-1 harboring a GGG/CCC mutation in its terminal loop, which has been shown to abrogate binding of the positive factor KSRP, was significantly reduced when comparing with the processing of wild-type pri-let-7a-1 (Supplementary Fig. 6). This confirms the positive role of KSRP in the biogenesis of let-7a-1[32]. It should be noted that the GGG/CCC mutation also abrogates hnRNP A1 binding, but lack of binding of the positive KSRP factor is dominant. Altogether, these results strongly suggest a negative role for hnRNP A1 in the processing of pri-let-7a-1 and are suggestive of antagonistic roles for hnRNP A1 and KSRP in the processing of let-7a-1. We have previously shown that hnRNP A1 also binds to the terminal loop of pri-miR-101; however, no change in its rate of processing was observed upon depletion of hnRNP A1 (data not shown) indicating that the sensitivity towards hnRNP A1 levels could be a specific feature of selected pri-miRNAs.

HnRNP A1 binds and remodels the terminal loop of pri-let-7a-1

In order to map the precise binding site/s of hnRNP A1 in pri-let-7a-1 transcript and to determine whether this binding influences the RNA architecture we analyzed its RNA secondary structure in the presence or absence of recombinant hnRNP A1 protein. Footprint analysis, was carried out using Pb(II)-lead ions that cleave single-stranded and relaxed nucleotides and also Ribonuclease T1 that preferentially cleaves RNA after G nucleotides present in single-stranded and loose conformations. It should be noted that G residues present in highly stable RNA duplexes are protected from ribonuclease T1 cleavage; however, those G residues placed in short stretches of base pairs that are situated in flexible RNA regions, such as those present in the pri-let-7a-1 terminal loop, are efficiently recognized by ribonuclease T1[40,41]. This analysis revealed that hnRNP A1 was largely confined to the 5′ and 3′ ends of the terminal loop region (Fig. 5). Additionally, binding of hnRNP A1 to pri-let-7a-1 resulted in an opening of the terminal loop secondary structure, visible through appearance of new lead ions cleavages in the center of the loop. We could also detect minor Pb(II)-lead ions cleavages upon hnRNP A1 addition in the stem (Fig. 5a). This is most likely due to the unwinding/annealing properties of hnRNP A1[42-44]. Structure probing of the pri-let-7a-1 GGG/CCC mutant revealed an open conformation of the terminal loop, with three C-G base pairs, stacked immediately adjacent to the loop (Supplementary Fig. 7a,b). Such conformation prevented recognition by RNase T1. In agreement with our EMSA assays, we could not detect any hnRNP A1 footprints to a pri-let-7a-1 transcript harboring an artificial terminal loop (pri-let-7a-1_loop_mt1), composed of a GCAA motif (Supplementary Fig. 8a,b) or to a transcript harboring a mutated hnRNP A1 binding site (pri-let7a-1 GGG/CCC mutant) (Supplementary Fig. 8c,d).

Antagonism between KSRP and hnRNP A1 determines the processing of let-7a-1

We hypothesized that hnRNP A1 could be acting as a pri-let-7a-1 inhibitor by direct competition with the positive factor, KSRP. To gain more insight into this, we performed footprint analysis of the pri-let-7a-1 transcript in the presence or absence of recombinant KSRP protein (Fig. 6). The terminal loop of pri-let-7a-1 harbors two GGG triplets that have been shown to be optimal binding sites for the KH3 domain of KSRP[32]. Footprint analysis, using Pb(II)-lead ions or Ribonuclease T1 revealed that addition of KSRP recombinant protein generated a very similar pattern, as the one observed with addition of hnRNP A1, with KSRP binding being confined to the 5′ and 3′ ends of the terminal loop region (Fig. 6). This would suggest that both proteins could be recognizing similar sequences. Indeed, the hnRNP A1 SELEX sequence within the terminal loop of let-7a-1 comprises a GGG triplet that was found to be the binding site for KSRP[32].

In order to analyze the interaction between the terminal loop of pri-let-7a and KSRP, we performed EMSA analysis. We confirmed a previous report and showed that recombinant KSRP protein was able to bind efficiently to the terminal loop of wild-type pri-let-7a-1 (Fig. 7a)[32]. Moreover, addition of excess of unlabeled let-7a-1 terminal loop sequence abolished KSRP binding to far greater extent than addition of miR-18a or miR-379 terminal loop sequences (Fig. 7a). Importantly, different EMSA migration rates of pri-let-7a-1/KSRP and pri-let-7a-1/UP1 complexes allowed us to examine whether hnRNP A1 and KSRP could bind pri-let-7a-1 terminal loop cooperatively or in a mutually exclusive manner (Fig. 7b). We maintained the levels of KSRP protein constant and increased the levels of UP1. We observed a significant decrease in the abundance of the pri-let-7a-1/KSRP complex and a concurrent increase in the presence of the pri-let-7a-1/UP1 complex, suggesting that hnRNP A1 is competing with KSRP for binding to the pri-let-7a-1 terminal loop (Fig. 7b). We also observed a minor, slowly-migrating band when both proteins where present in the reaction. This might be a consequence of simultaneous occupancy of the RNA by both UP1 and KSRP or due to direct protein-protein interaction. Accordingly, it was previously reported that hnRNP A1 and KSRP proteins might be able to interact with each other[45]. In a reciprocal experiment maintaining a constant level of UP1 and adding increasing concentrations of KSRP also resulted in a shift towards KSRP occupancy (Supplementary Fig. 9).

DISCUSSION

The large extent of post-transcriptional regulation of microRNA processing has been recently uncovered. This regulation has been shown to act at both nuclear and cytoplasmic processing steps and can be either stimulatory or inhibitory (for a review see[13]). The DEAD-box RNA helicases p68 and p72, which are associated to the large microprocessor complex, are required for the Drosha-mediated processing of a subset of miRNAs[15] and many factors that have positive or negative influence in Drosha-mediated processing seem to be recruited by these proteins. Thus, p68/p72 may be acting as a scaffold to promote recruitment of Drosha itself or of auxiliary factors, such as estrogen-bound ERα that has an inhibitory role in Drosha processing of a subset of miRNAs[16]. Interestingly, they also act to recruit TGF-beta- and BMP-specific SMAD signal transducers to the precursor of miR-21 and promote its Drosha-mediated processing[17]. In a similar fashion, the tumor suppressor gene, p53, positively regulates the processing of several miRNAs with tumor suppressive functions, via association with Drosha and p68[46].

We have recently reported that 14% of human pri-miRNAs have terminal loops that display a high degree of conservation throughout evolution, and we proposed that they could serve as landing pads for many different RNA-binding proteins that could promote or block their processing[28]. The developmentally regulated RNA-binding protein Lin28 binds to the conserved terminal loop region of the Let-7d precursor[21] and has been shown to block let-7 production acting at the level of Drosha and/or Dicer processing. A dominant negative role for Lin28 in the processing of let-7 in undifferentiated cells has been clearly established (reviewed by[47]). Upon differentiation, the expression of Lin28 proteins is turned off and this results in the activation of the biogenesis of let-7 family members. Despite this developmental regulation, the levels of mature let-7a differ substantially in differentiated human cells, despite a similar level of pri-let-7 sequences, indicating the presence of a yet unidentified posttranscriptional repressor in differentiated cells[48]. The RNA-binding protein nuclear factor 90 (NF90) and its heteromeric partner, NF45, have many roles in gene expression, including transcription and translational regulation[49]. These proteins that are associated with the microprocessor complex act as negative regulators of Drosha processing. This inhibition seems to be non-specific, since all miRNAs tested, including pri-let7-a, pri-miR-15a/16-1, pri-miR-21 and pri-miR-30a, have been shown to be inhibited[50]. Altogether,

this evidence suggests a general, non-specific role for the association of the NF90-NF45 complex with pri-miRNAs via the dsRBD present in NF90[50].

We had previously shown that hnRNP A1 binds to the conserved terminal loop of let-7a-1[28]. Here, we have presented evidence showing that hnRNP A1 blocks the microprocessor-mediated processing of let-7a-1. We show a perfect correlation between high levels of hnRNP A1 and low levels of let-7a mature miRNA. Furthermore, we show that the binding site of hnRNP A1 to the terminal loop of pri-let-7a sequences overlaps with the binding site for the positive regulatory factor, KSRP. Thus, a competition for the binding to the conserved terminal loop of let-7a between a repressor and an activator may explain the posttranscriptional regulation of let-7a production in human cells (Fig. 8). We cannot rule a situation where simultaneous occupancy of pri-let-7a-1 terminal loop by both KSRP and hnRNP A1 could occur (Fig. 8b); however, we clearly established that high levels of hnRNP A1 result in the abrogation of let-7a-1 processing. Importantly, the levels of the miRNA repressor NF90 protein are similar in 293T and HeLa cells[51], which display extremely low or high levels of endogenous mature let-7a miRNA, respectively (Fig. 2c). Thus, whereas a correlation between the levels of hnRNP A1 and endogenous let-7a can be established, this is not the case for NF90/45. Furthermore, miR-16, which was shown to be inhibited by the NF90-NF45 complex remains constant in the cell lines studied (Supplementary Fig. 4a).

We had previously shown that hnRNP A1 can facilitate microprocessor-mediated processing of miR-18a in a context-dependent manner. Binding of hnRNP A1 to the stem in miR-18a results in the relaxation of some nucleotides involved in strong Watson-Crick pairing in the unbound pri-miR-18a molecule, creating a more favorable cleavage site for Drosha[28]. Here, we provide evidence that hnRNP A1 negatively regulates the processing of pri-let-7a-1. This apparent discrepancy can be attributed to the substantially different sequence-structure characteristics of let-7a-1 and miR-18a stem loops. Thermodynamically the miR-18a stem loop is much less stable than the stem loop of let-7a-1 (ΔG values of −22.00 kcal/mol and −35.60 kcal/mol, respectively). Such relaxed conformation of the miR-18a stem loop may allow for efficient binding of hnRNP A1 and remodeling of critical residues that are cleaved by Drosha. Importantly, the terminal loop of let-7a-1 has the ability to bind the positive factor KSRP, unlike the terminal loop of miR-18a, which thus far was only shown to bind hnRNP A1. This suggests that the combination of structural and sequence characteristics of pri-miRNAs will determine the outcome of the regulation exerted by RNA-binding proteins i.e. the same RNA-binding protein, can bind to conserved terminal loop sequences in two different pri-miRNAs and because of their sequence and structural variations influence their processing in opposite ways.

Multiple evidence points to a role for the let-7 family of miRNAs in tumor suppression. First, several members of this family of miRNAs are repressed in human cancers. This results in a general de-repression of let-7 mRNA targets, such as K-ras, c-myc and HMGA2, resulting in cellular transformation. Conversely, ectopic expression of let-7g in K-Ras(G12D)-expressing murine lung cancer cells suppress tumor development[52]. In agreement, the RNA-binding proteins Lin28a and Lin28b that are negative regulators of let-7 processing in embryonic cells, are overexpressed in some primary human tumors and human cancer cell lines leading to derepression of let-7 targets[53]. Thus, the inhibitory role of hnRNP A1 in the production of mature let-7a miRNA is suggestive of a link between high levels of hnRNP A1 and cancer. Interestingly, several members of the hnRNP family of proteins, including hnRNP A1 and the related proteins hnRNP A2/B1, show high expression in cancer cells and this elevated expression has been used as a biomarker to estimate cancer progression[54],[55]. HnRNP A1 and related hnRNP proteins are very pleiotropic in function with an involvement in many aspects of RNA processing including alternative splicing regulation, IRES-mediated translation, mRNA stability and also miRNA biogenesis[56]. Thus,

it may well be that the association of increased levels of hnRNP A1 and related proteins with cellular transformation could reflect not only their role in miRNA biogenesis (miR-18a and let-7a-1) but also in other RNA processing events.

In summary, hnRNP A1, a general RNA binding protein that has been implicated in the regulation of alternative splicing and in facilitating the production of miR-18a, has been shown here to have an inhibitory role in the Drosha-mediated processing of let-7a in human cells that lack Lin28 expression. This model would predict that variations in the relative levels of hnRNP A1 and KSRP will determine the steady-state levels of let-7a in differentiated and transformed human cells (Fig. 8).

METHODS

EMSA analysis

5′^{32}P-labeled let-7a transcripts (the same as used for RNA structure probing) were incubated with recombinant hnRNP A1 - UP1, hnRNP A1 - RRM1, hnRNPA1- RRM2 proteins expressed in *E.coli* (a gift from Michael Sattler, Munich, Germany) or recombinant GFP-KSRP (in the range from 50 ng to 800 ng) for one hour at 4°C in buffer A: (20 mM Hepes-KOH (pH 8.0), 150 mM KCl, 1.5mM $MgCl_2$, 0.2 mM EDTA, 0.1% (w/v) Triton X-100. Where indicated, an excess of cold RNA competitor was added prior addition of the protein. Competitor sequences are as follow: let-7a-1 loop (5′-GUUGUAUAGUUUUAGGGUCACACCCACCACUGGGAGAUAACUAUACAAUC), mir-18a loop (5′AGUGCAGAUAGUGAAGUAGAUUAGCAUCUACUGCCCUAAG and miR-379 loop (5′-AACGUAGGCGUUAUGAUUUCUGACCUAUGUA). Where indicated (Supplementary Fig. 1e,f), pri-miRNA transcripts were used, instead. The samples with added loading buffer (10mM Tris-HCl, 3% sucrose and dyes) were loaded on 8% (w/v) non-denaturing gel in the case of UP1 (Fig. 1 and Supplementary Fig. 1) and run at constant power of 8W. For experiments using GFP-KSRP samples were loaded on 5% (w/v) non denaturing gel (Fig. 7).

Cell culture

293T, HeLa, and human Brain Astrocytoma 1321N1 cells were grown in DMEM (Invitrogen) supplemented with 10% foetal calf serum (FCS). Mouse teratocarinoma P19 cells were grown in DMEM supplemented with 7.5% Calf Serum (Sigma) and 2.5% Foetal Bovine Serum. Knockdown of hnRNP A1 was achieved by transfection of different set of siRNAs (hnRNP A1-targeted smart pool of siRNAs from Dharmacon), as previously described[28].

Western blots

293T, HeLa or Astrocytoma cells were scraped and sonicated in lysis buffer (20 mM Hepes-KOH (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 5% (w/v) glycerol). Total protein extracts (50-100μg) were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes (Whatman) in 25 mM Tris-base, 40 mM glycine, and 20% methanol in a Genie Blotter unit (Idea Scientific Company) at 12V for 1 hr. Nonspecific binding sites were blocked by incubation of the membrane with 1:10 western blocking solution (Roche) in TBST (20 mM Tris (pH 7.5), 137 mM NaCl, and 0.1% Tween 20). Proteins were detected using the following primary antibodies diluted in 1:20 western blocking solution in TBST: mouse monoclonal anti-hnRNP A1 (clone 4B10; 1:1000, Santa Cruz Biotechnology), rabbit polyclonal anti-Lin28a (1:1000, Cell Signalling Technology), mouse monoclonal anti-T7 (1:10,000, Novagen), mouse monoclonal anti-hnRNP L (1:1000, Sigma), mouse-monoclonal anti-KSRP (clone 4C10, 1:500, Sigma), mouse-monoclonal anti-GAPDH (1:10000, Sigma), mouse-monoclonal anti-beta-tubulin

(1:10,000, Sigma). Following washing in TBST, blots were incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (Pierce) and detected with SuperSignal West Pico detection reagent (Thermo Scientific). The membranes were stripped using ReBlot Plus Strong Antibody Stripping Solution (Chemicon) equilibrated in water, blocked in 1:10 western blocking solution in TBST, and reprobed as described above.

Northern blots

Total RNA (10-20μg) from different cell lines, prepared using Trizol reagent (Sigma), was resolved on a 10% (w/v) denaturing polyacrylamide gel (7.5 M urea) and transferred to Hybond N membranes (Amersham Pharmacia Biotech). The membranes were UV-crosslinked and prehybridized for 4 h at 40 °C in 1 SSC, 1% (w/v) SDS and 200 μg/ml of sperm-salmon DNA (Sigma). Probes corresponding to let-7a, miR-18a and miR-16 were synthesized using the mirVana™ miRNA Probe Construction Kit (Ambion) and left to hybridize overnight in 1 SSC, 1% (w/v) SDS and 200 μg/ml of single-stranded DNA. Following hybridization, the membranes were washed four times at 50 °C in 0.2 SSC and 0.2% (w/v) SDS for 30 min each. The blots were then analysed using a PhosphorImager (Molecular Dynamics). For reblotting, membranes were incubated in wash solution for one hour at 70°C and washed again for another hour at 40 °C.

Pri-miRNA substrates and *in vitro* processing assays

Pri-miRNA substrates were prepared by standard *in vitro* transcription with T7 RNA polymerase in the presence of [α-^{32}P]GTP. The templates used to generate pri-let-7a-1 and pri-let-7a-1_loop_mt1 were described previously[28]. The template for pri-let-7a-1 GGG/CCC transcript was derived from the corresponding wild-type plasmid by PCR amplification with oligonucleotides let-7a_1_GGG/CCCfor (GGTTGTATAGTTTTACCCTCACACCCACCACTG) and let-7a_1_GGG/CCCrev CAGTGGTGGGTGTGAGGGTAAAACTATACAACC) using Pfu polymerase (Promega), followed by T4 ligation (Roche) and transformation of DH5alpha competent cells (Invitrogen). Total HeLa extracts were prepared from ~ 3×10^6 control or hnRNP A1-depleted cells and resuspended in 1ml of buffer D (20mM Hepes-KOH pH7.9, 100mM KCl, 0.2mM EDTA, 0.5mM DTT, 0.2mM PMSF, 5% (w/v) glycerol), as previously described[27]. The suspension was sonicated and centrifuged for 5min at 10,000g, and the supernatant used for in vitro assays. Template DNAs were previously linearized with SpeI. Assays were performed in 30μl reaction mixtures containing 50% (v/v) total or hnRNP A1-depleted HeLa extract, 0.5 mM ATP, 20 mM creatine phosphate, 3.2 mM $MgCl_2$, and 20,000 cpm (~10 fmol) of each pri-miRNA. When indicated T7-hnRNP A1 or GFP-KSRP protein were added at specified concentrations 5 min prior to the reaction. Reactions were incubated at 30°C for 90 min followed by phenol-chloroform extraction, precipitation, and 8% (w/v) denaturing gel electrophoresis.

Purification of recombinant proteins

Recombinant hnRNP A1 protein was obtained as previously described[57]. For overexpression of hnRNP A1, we used the mammalian expression plasmid pCGT-A1, which carries a T7-tagged version of hnRNP A1[58]. In brief, T7 tagged hnRNP A1 protein was expressed in the 293T cells were transiently transfected with pCGT7-hnRNP A1 vector. Forty-eight hours after transfection, cells were scrapped and sonicated in lysis buffer. Recombinant hnRNP A1 protein was purified using anti-T7 affinity chromatography (Novagen). Recombinant KSRP was obtained using a His-tagged GFP-KSRP mammalian expression vector (a gift from Douglas Black, UCLA). Following transient transfection in 293T cells cells were scrapped and sonicated in lysis buffer. Recombinant GFP-KSRP protein was purified using HisPur Purification Kit (Thermo Scientific). The epitope-tagged Lin28a expression plasmid was constructed by amplifying a Lin-28a cDNA with specific

primers and the resulting PCR product was subcloned as a *Nhe*I-*Bam*HI fragment into the *Xba*I-*Bam*H I sites of the pCG-T7 expression vector.

A detailed methods section covering RNA probing and footprint analysis methods used in this paper is presented in the Supplemental material section.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009; 136:215–233. [PubMed: 19167326]
2. Kumar MS, Lu J, Mercer KL, Golub TR, Jacks T. Impaired microRNA processing enhances cellular transformation and tumorigenesis. Nat. Genet. 2007; 39:673–677. [PubMed: 17401365]
3. Han J, et al. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev. 2004; 18:3016–3027. [PubMed: 15574589]
4. Zeng Y, Yi R, Cullen BR. Recognition and cleavage of primary microRNA precursors by the nuclear processing enzyme Drosha. EMBO J. 2005; 24:138–148. [PubMed: 15565168]
5. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. Nat. Rev. Mol. Cell Biol. 2009; 10:126–139. [PubMed: 19165215]
6. Shiohama A, Sasaki T, Noda S, Minoshima S, Shimizu N. Molecular cloning and expression analysis of a novel gene DGCR8 located in the DiGeorge syndrome chromosomal region. Biochem. Biophys. Res. Commun. 2003; 304:184–190. [PubMed: 12705904]
7. Landthaler M, Yalcin A, Tuschl T. The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. Curr. Biol. 2004; 14:2162–2167. [PubMed: 15589161]
8. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. Processing of primary microRNAs by the Microprocessor complex. Nature. 2004; 432:231–235. [PubMed: 15531879]
9. Gregory RI, et al. The Microprocessor complex mediates the genesis of microRNAs. Nature. 2004; 432:235–240. [PubMed: 15531877]
10. Kim YK, Kim VN. Processing of intronic microRNAs. EMBO J. 2007; 26:775–783. [PubMed: 17255951]
11. Morlando M, et al. Primary microRNA transcripts are processed co-transcriptionally. Nat. Struct. Mol. Biol. 2008
12. Pawlicki JM, Steitz JA. Primary microRNA transcript retention at sites of transcription leads to enhanced microRNA production. J. Cell Biol. 2008; 182:61–76. [PubMed: 18625843]
13. Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat. Cell Biol. 2009; 11:228–234. [PubMed: 19255566]
14. Thomson JM, et al. Extensive post-transcriptional regulation of microRNAs and its implications for cancer. Genes Dev. 2006; 20:2202–2207. [PubMed: 16882971]
15. Fukuda T, et al. DEAD-box RNA helicase subunits of the Drosha complex are required for processing of rRNA and a subset of microRNAs. Nat. Cell Biol. 2007; 9:604–611. [PubMed: 17435748]

16. Yamagata K, et al. Maturation of MicroRNA Is Hormonally Regulated by a Nuclear Receptor. Mol. Cell. 2009; 36:340–347. [PubMed: 19854141]
17. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. Nature. 2008; 454:56–61. [PubMed: 18548003]
18. Obernosterer G, Leuschner PJ, Alenius M, Martinez J. Posttranscriptional regulation of microRNA expression. RNA. 2006; 12:1161–1167. [PubMed: 16738409]
19. Roush S, Slack FJ. The let-7 family of microRNAs. Trends Cell Biol. 2008; 18:505–516. [PubMed: 18774294] 19 Viswanathan SR, Daley GQ, Gregory RI. Selective blockade of microRNA processing by Lin28. Science. 2008; 320:97–100. [PubMed: 18292307]
20. Viswanathan SR, Daley GQ, Gregory RI. Selective blockade of microRNA processing by Lin28. Science. 2008; 320:97–100. [PubMed: 18292307]
21. Newman MA, Thomson JM, Hammond SM. Lin-28 interaction with the Let-7 precursor loop mediates regulated microRNA processing. RNA. 2008; 14:1539–1549. [PubMed: 18566191]
22. Piskounova E, et al. Determinants of microRNA processing inhibition by the developmentally regulated RNA-binding protein Lin28. J. Biol. Chem. 2008; 283:21310–21314. [PubMed: 18550544]
23. Heo I, et al. Lin28 mediates the terminal uridylation of let-7 precursor MicroRNA. Mol. Cell. 2008; 32:276–284. [PubMed: 18951094]
24. Rybak A, et al. A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. Nat. Cell Biol. 2008; 10:987–993. [PubMed: 18604195]
25. Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat. Rev. Genet. 2002; 3:285–298. [PubMed: 11967553]
26. Chen M, Manley JL. Mechanisms of alternative splicing regulation: insights from molecular and genomics approaches. Nat. Rev. Mol. Cell Biol. 2009; 10:741–754. [PubMed: 19773805]
27. Guil S, Caceres JF. The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a. Nat. Struct. Mol. Biol. 2007; 14:591–596. [PubMed: 17558416]
28. Michlewski G, Guil S, Semple CA, Caceres JF. Posttranscriptional regulation of miRNAs harboring conserved terminal loops. Mol. Cell. 2008; 32:383–393. [PubMed: 18995836]
29. Burd CG, Dreyfuss G. RNA binding specificity of hnRNP A1: significance of hnRNP A1 high-affinity binding sites in pre-mRNA splicing. EMBO J. 1994; 13:1197–1204. [PubMed: 7510636]
30. Min H, Turck CW, Nikolic JM, Black DL. A new regulatory protein, KSRP, mediates exon inclusion through an intronic splicing enhancer. Genes Dev. 1997; 11:1023–1036. [PubMed: 9136930]
31. Ruggiero T, et al. LPS induces KH-type splicing regulatory protein-dependent processing of microRNA-155 precursors in macrophages. FASEB J. 2009
32. Trabucchi M, et al. The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. Nature. 2009; 459:1010–1014. [PubMed: 19458619]
33. Dreyfuss G, Matunis MJ, Pinol-Roma S, Burd CG. hnRNP proteins and the biogenesis of mRNA. Annu. Rev. Biochem. 1993; 62:289–321. [PubMed: 8352591]
34. Shamoo Y, Krueger U, Rice LM, Williams KR, Steitz TA. Crystal structure of the two RNA binding domains of human hnRNP A1 at 1.75 A resolution. Nat. Struct. Biol. 1997; 4:215–222. [PubMed: 9164463]
35. Xu RM, Jokhan L, Cheng X, Mayeda A, Krainer AR. Crystal structure of human UP1, the domain of hnRNP A1 that contains two RNA-recognition motifs. Structure. 1997; 5:559–570. [PubMed: 9115444]
36. Okunola HL, Krainer AR. Cooperative-binding and splicing-repressive properties of hnRNP A1. Mol. Cell Biol. 2009; 29:5620–5631. [PubMed: 19667073]
37. Hagan JP, Piskounova E, Gregory RI. Lin28 recruits the TUTase Zcchc11 to inhibit let-7 maturation in mouse embryonic stem cells. Nat. Struct. Mol. Biol. 2009; 16:1021–1025. [PubMed: 19713958]
38. Heo I, et al. TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. Cell. 2009; 138:696–708. [PubMed: 19703396]

39. Lehrbach NJ, et al. LIN-28 and the poly(U) polymerase PUP-2 regulate let-7 microRNA processing in Caenorhabditis elegans. Nat. Struct. Mol. Biol. 2009; 16:1016–1020. [PubMed: 19713957]
40. Sobczak K, de Mecer M, Michlewski G, Krol J, Krzyzosiak WJ. RNA structure of trinucleotide repeats associated with human neurological diseases. Nucleic Acids Res. 2003; 31:5469–5482. [PubMed: 14500809]
41. Michlewski G, Krzyzosiak WJ. Molecular architecture of CAG repeats in human disease related transcripts. J. Mol. Biol. 2004; 340:665–679. [PubMed: 15223312]
42. Kumar A, Wilson SH. Studies of the strand-annealing activity of mammalian hnRNP complex protein A1. Biochemistry. 1990; 29:10717–10722. [PubMed: 1703006]
43. Pontius BW, Berg P. Renaturation of complementary DNA strands mediated by purified mammalian heterogeneous nuclear ribonucleoprotein A1 protein: implications for a mechanism for rapid molecular assembly. Proc. Natl. Acad. Sci. U. S. A. 1990; 87:8403–8407. [PubMed: 2236048]
44. Munroe SH, Dong XF. Heterogeneous nuclear ribonucleoprotein A1 catalyzes RNA.RNA annealing. Proc. Natl. Acad. Sci. U. S. A. 1992; 89:895–899. [PubMed: 1371011]
45. Ruggiero T, et al. Identification of a set of KSRP target transcripts upregulated by PI3K-AKT signaling. BMC. Mol. Biol. 2007; 8:28. [PubMed: 17437629]
46. Suzuki HI, et al. Modulation of microRNA processing by p53. Nature. 2009; 460:529–533. [PubMed: 19626115]
47. Ding XC, Weiler J, Grosshans H. Regulating the regulators: mechanisms controlling the maturation of microRNAs. Trends Biotechnol. 2009; 27:27–36. [PubMed: 19012978]
48. Landgraf P, et al. A mammalian microRNA expression atlas based on small RNA library sequencing. Cell. 2007; 129:1401–1414. [PubMed: 17604727]
49. Reichman TW, et al. Selective regulation of gene expression by nuclear factor 110, a member of the NF90 family of double-stranded RNA-binding proteins. J. Mol. Biol. 2003; 332:85–98. [PubMed: 12946349]
50. Sakamoto S, et al. The NF90-NF45 complex functions as a negative regulator in the microRNA processing pathway. Mol. Cell Biol. 2009; 29:3754–3769. [PubMed: 19398578]
51. Berglund L, et al. A genecentric Human Protein Atlas for expression profiles based on antibodies. Mol. Cell Proteomics. 2008; 7:2019–2027. [PubMed: 18669619]
52. Kumar MS, et al. Suppression of non-small cell lung tumor development by the let-7 microRNA family. Proc. Natl. Acad. Sci. U. S. A. 2008; 105:3903–3908. [PubMed: 18308936]
53. Viswanathan SR, et al. Lin28 promotes transformation and is associated with advanced human malignancies. Nat. Genet. 2009; 41:843–848. [PubMed: 19483683]
54. Tockman MS, et al. Prospective detection of preclinical lung cancer: results from two studies of heterogeneous nuclear ribonucleoprotein A2/B1 overexpression. Clin. Cancer Res. 1997; 3:2237–2246. [PubMed: 9815620]
55. Ghigna C, Moroni M, Porta C, Riva S, Biamonti G. Altered expression of heterogenous nuclear ribonucleoproteins and SR factors in human colon adenocarcinomas. Cancer Res. 1998; 58:5818–5824. [PubMed: 9865741]
56. Martinez-Contreras R, et al. hnRNP proteins and splicing control. Adv. Exp. Med. Biol. 2007; 623:123–147. [PubMed: 18380344]
57. Cazalla D, Sanford JR, Caceres JF. A rapid and efficient protocol to purify biologically active recombinant proteins from mammalian cells. Protein Expr. Purif. 2005; 42:54–58. [PubMed: 15878828]
58. Caceres JF, Misteli T, Screaton GR, Spector DL, Krainer AR. Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. J. Cell Biol. 1997; 138:225–238. [PubMed: 9230067]

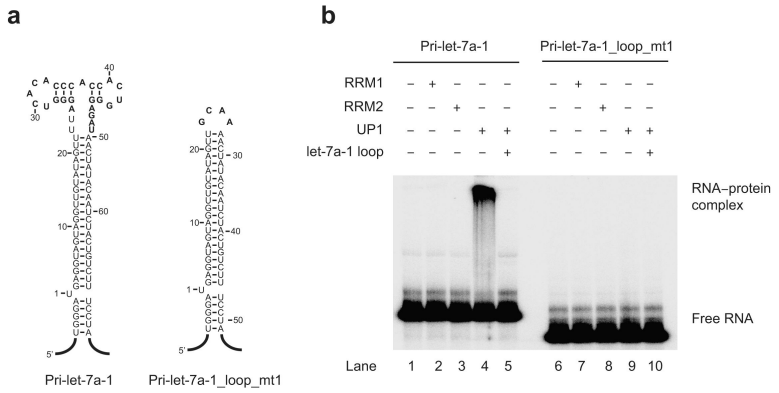

Figure 1.

HnRNP A1 specifically binds to the terminal loop of pri-let-7a-1 (**a**) Validated secondary structure of let-7a-1 wild-type and a terminal loop mutant, in which the wild-type terminal loop sequence has been replaced with a GCAA motif (pri-let-7a-1_loop_mt1) (**b**) EMSA analysis of wild-type and a loop mutant sequence (pri-let-7a-1 and pri-let-7a-1_loop_mt1, respectively), with the UP1 protein. Native gel electrophoresis with 5′32^{P}-labeled transcripts (100×10^{3}/~2.5 pmol) incubated in the presence of recombinant RRM1, RRM2 or UP1 fragments of hnRNP A1 (200 ng). Let-7a-1 loop indicates unlabeled RNA competitor (100 pmol)

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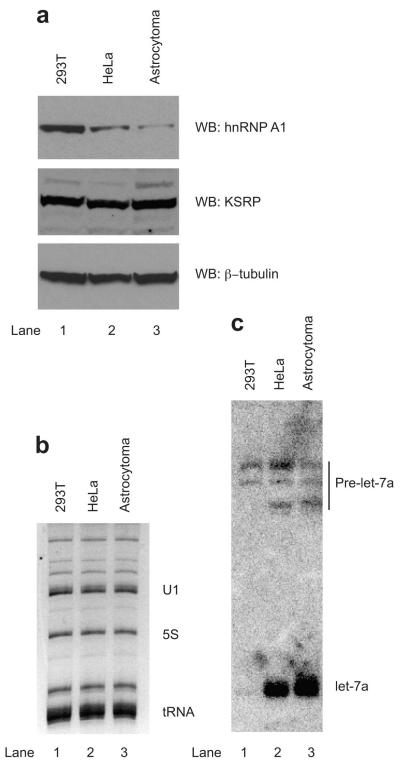

Figure 2.

Levels of mature let-7a correlate negatively with the levels of hnRNP A1 in human cells. (**a**) Western blot analysis of whole cells extracts from 293T, HeLa and Astrocytoma 1321N1 cells shows different levels of hnRNP A1 expression and similar levels of KSRP, respectively. (**b**,**c**) Northern blots of total RNA from 293T, HeLa and Astrocytoma 1321N1 cells reveal different levels of mature let-7a. As a loading control, ethidium bromide stain of the RNA is shown on panel b. The black bar on panel c denotes let-7a precursors.

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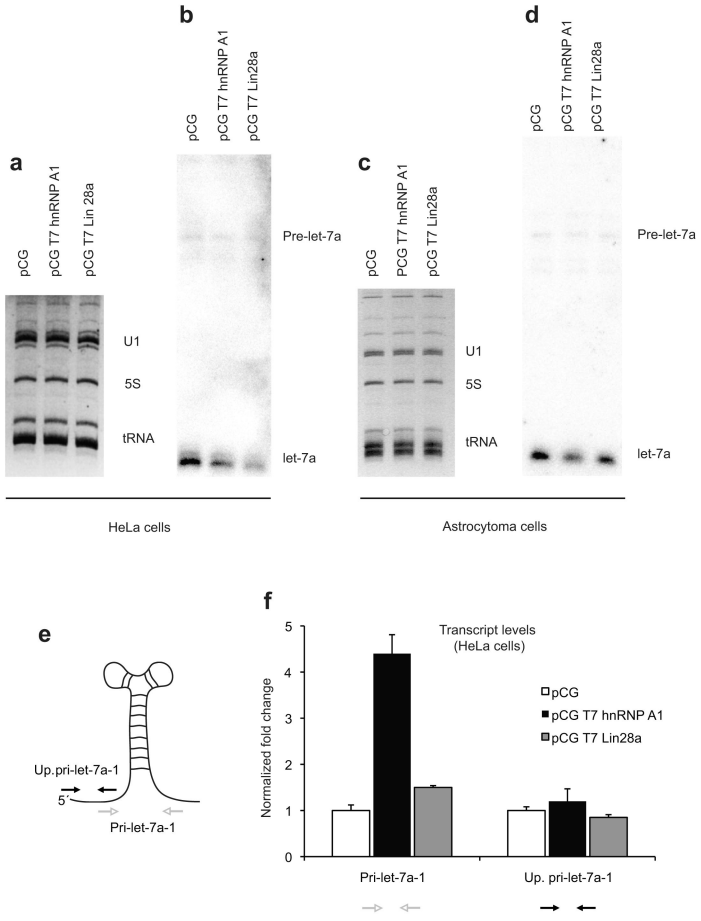

Figure 3.

Ectopic expression of hnRNP A1 in HeLa and Astrocytoma 1321N1 cells reduces the levels of endogenous mature let-7a. (**b,d**) Northern blots of let-7a using total RNA from HeLa and Astrocytoma cells that have been transiently transfected with a control plasmid (pCG), pCG T7 hnRNP A1, of pCG T7 Lin28a (**a,c**) As loading controls, ethidium bromide stains of total RNA are shown. (**e**) Schematic of the secondary structure of pri-let-7a-1 RNA. Light grey arrows indicate qRT-PCR pair of primers used to detect the levels of pri-let-7a-1 (subjected to Drosha cleavage), whereas black arrows indicate qRT-PCR pair of primers used to detect the levels of upstream region (up.) of pri-let-7a-1 (representing total levels of pri-let-7a-1 transcript) (**f**) Real Time qRT-PCR on RNAs from HeLa cells overexpressing hnRNP A1 reveals a substantial increase in the levels of uncleaved pri-let-7a-1 transcripts (light grey arrows) and at the same time no change in the total levels of pri-let-7a-1 transcript (black arrows). The values were normalized against GAPDH mRNA. The fold change of corresponding RNA fragments abundance mediated by overexpression of either pCG T7 hnRNP A1 of pCG T7 Lin28a was plotted relative to values from a control transfection set to one. Mean values and standard deviations of two independent experiments are shown.

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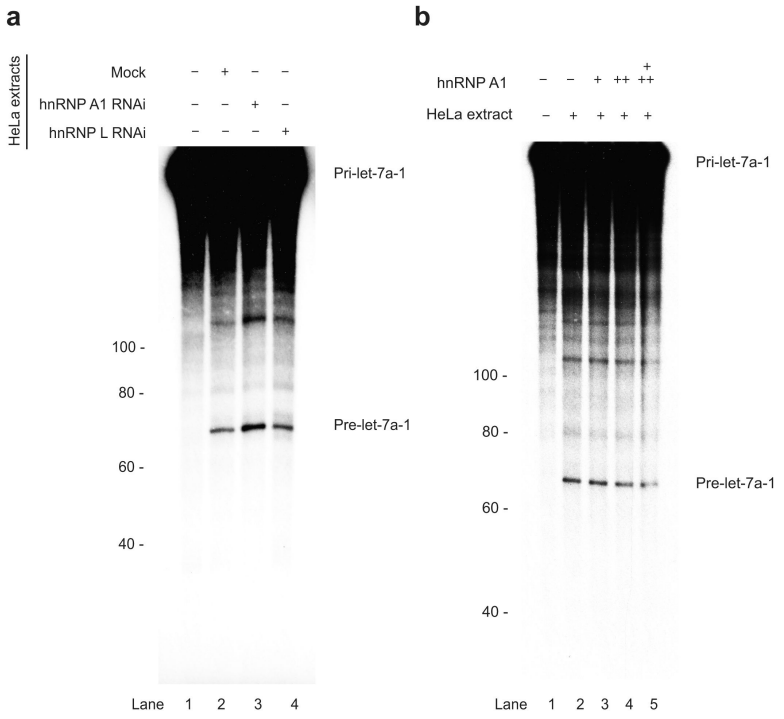

Figure 4.

HnRNP A1 negatively regulates the Drosha-mediated processing of let-7a-1 (**a**) In vitro processing of pri-let-7a-1 is enhanced in the hnRNP A1-depleted extracts. Internally radiolabeled pri-let-7a-1 transcripts (100×10^3 c.p.m.) were incubated in the presence of either control HeLa extracts or hnRNP A1-depleted extracts. Lanes (−) shows negative controls with no extract added. Products were analyzed on an 8% polycrylamide gel. Numbers on the left hand side represent RNA size marker. (**b**) In vitro processing of pri-miRNAs pri-let-7a-1 performed in HeLa cell extracts in the presence of increasing amounts of recombinant hnRNP A1 (+, 200 ng, ++, 400ng, +++, 800ng).

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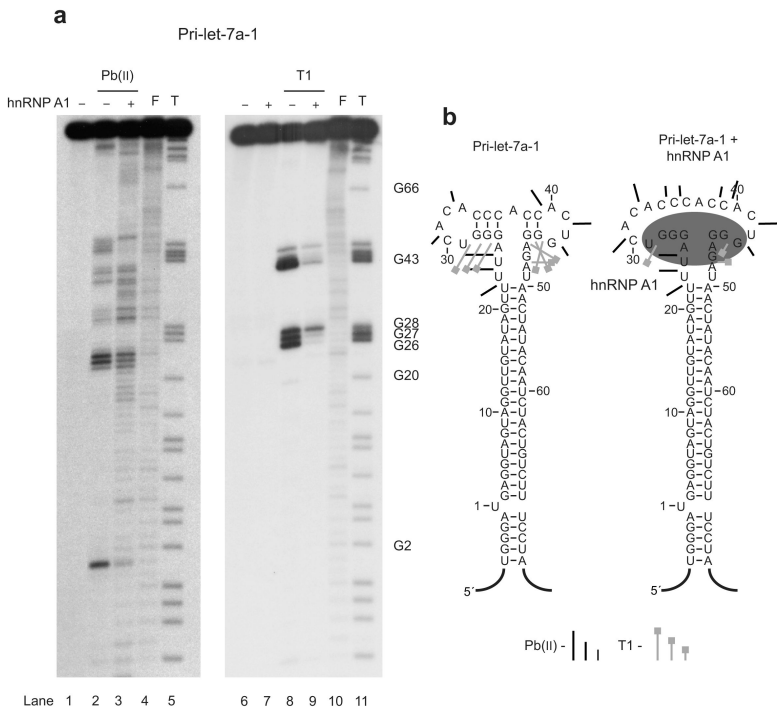

Figure 5.

HnRNP A1 binds and remodels the terminal loop of pri-let-7a-1. (**a**) Footprint analysis of the pri-let-7a-1/hnRNP A1 complex. Cleavage patterns were obtained for 5′ 32^{P}-labeled transcripts (100 × 10^{3}/2.5 pmol) incubated in the absence or presence of recombinant hnRNP A1 protein (+, 200 ng) treated with Pb (II)-lead ions (0.5 mM), left panel); or ribonuclease T1 (1.5 units/μl), right panel. F and T identify nucleotide residues subjected to partial digest with formamide (every nucleotide) or ribonuclease T1 (G-specific cleavage), respectively. Electrophoresis was performed in a 10% polyacrylamide gel under denaturing conditions. Positions of selected G residues are indicated. (**b**) Proposed structure of free and hnRNP A1-bound pri-let-7a-1. The sites and intensities of cleavages generated by structure probes (presented below), located at the places of hnRNP A1 binding are shown. Nucleotides are numbered from the 5′ site of Drosha cleavage.

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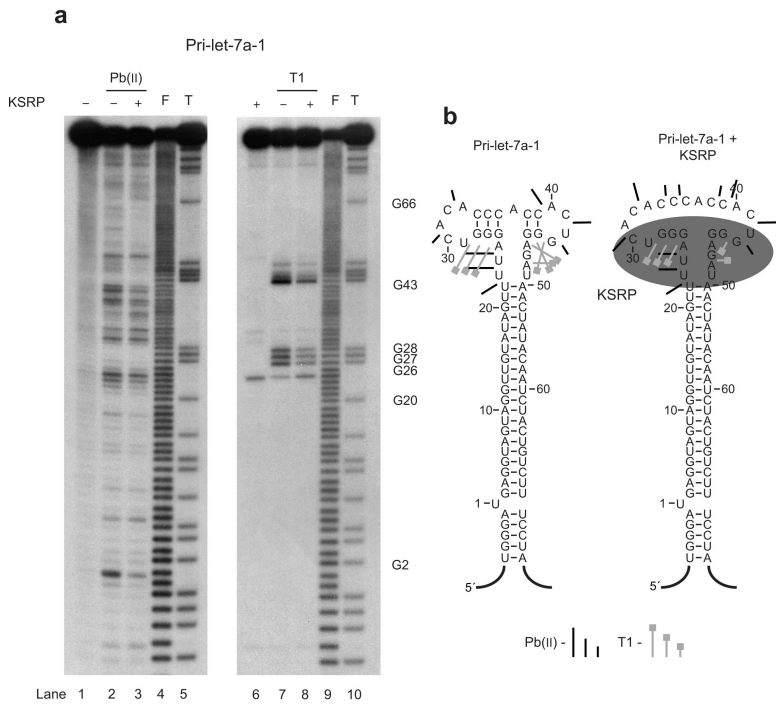

Figure 6.

KSRP binds to the terminal loop of pri-let-7a-1. (**a**) Footprint analysis of the pri-let-7a-1/GFP-KSRP complex. Cleavage patterns were obtained for 5′^{32}P-labeled transcripts (100 × 10^3/2.5 pmol) incubated in the absence or presence of recombinant GFP-KSRP protein (+, 200 ng) treated with Pb (II)-lead ions (0.5 mM); left panel or ribonuclease T1 (1.5 units/μl), right panel. F and T identify nucleotide residues subjected to partial digest with formamide (every nucleotide) or ribonuclease T1 (G-specific cleavage), respectively. Electrophoresis was performed in a 10% polyacrylamide gel under denaturing conditions. Positions of selected G residues are indicated. (**b**) A proposed structure of free and GFP-KSRP-bound pri-let-7a-1. The sites and intensities of cleavages generated by structure probes (presented below), located at the places of GFP-KSRP binding are shown. Nucleotides are numbered from the 5′ site of Drosha cleavage.

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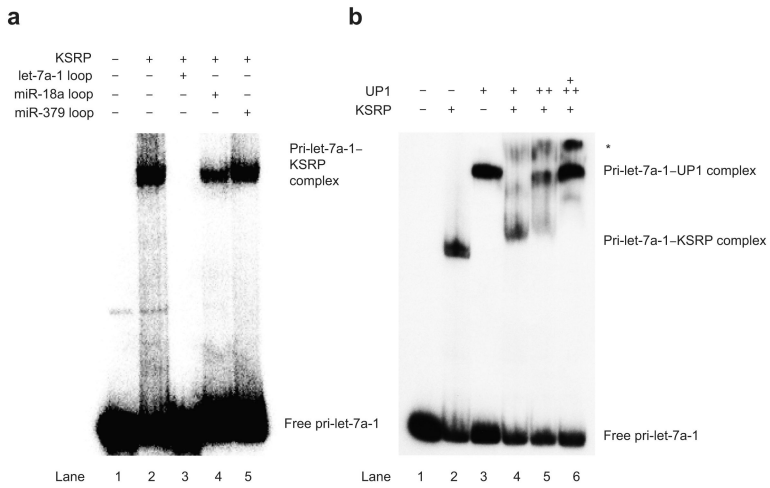

Figure 7.

KSRP and hnRNP A1 compete for binding to the pri-let-7a-1 terminal loop. (**a**) EMSA analysis of wild-type 5′^{32}P-labeled pri-let-7a-1 transcripts (100 × 10^3/~2.5 pmol) with GFP-KSRP protein (+, 200 ng) in the absence of presence of various unlabeled RNA competitors (100 pmol). (**b)** EMSA analysis of wild-type 5′^{32}P-labeled pri-let-7a-1 transcripts (5 × 10^3/~0.125 pmol) with GFP-KSRP protein (+, 200 ng, lane 2), UP1 (+, 200 ng, lane 3)), or a constant amount of GFP-KSRP (+, 200 ng) incubated together with increasing amounts of UP1 (+, 200 ng, ++, 400 ng, +++, 800 ng, lanes 4-6). * indicates a slower migrating complex that only appears in the presence of both UP1 and KSRP and could reflect simultaneous occupancy

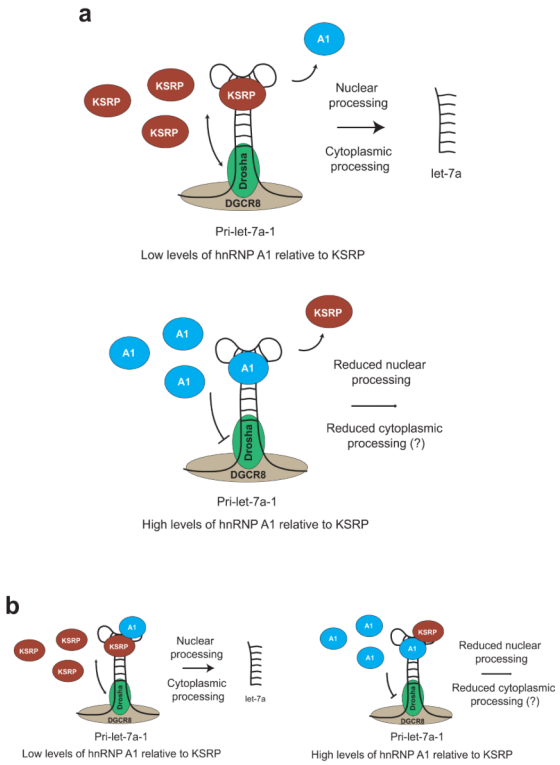

Figure 8.

Cartoon displaying the antagonism of KSRP and hnRNP A1 in the post-transcriptional regulation of let-7a processing. (**a**) This model illustrates how the relative levels of KSRP and hnRNP A1 may influence a direct competition between a repressor and an activator binding to the same sequence and determine the processing of let-7a-1 (**b**) Simultaneous occupancy of pri-let-7a-1 terminal loop by both KSRP and hnRNP A1 cannot be ruled out; however, high levels of hnRNP A1 always result in the abrogation of let-7a-1 processing.

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