

Rare Drosha Splice Variants Are Deficient in MicroRNA Processing but Do Not Affect General MicroRNA Expression in Cancer Cells^{1,2}

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Abstract

Drosha is a key enzyme in microRNA biogenesis, generating the precursor miRNA (pre-miRNA) by excising the stem-loop embedded in the primary transcripts (pri-miRNA). The specificity for the pri-miRNAs and determination of the cleavage site are provided by its binding partner DGCR8, which is necessary for efficient processing. The crucial Drosha domains for pri-miRNA cleavage are the middle part, the two enzymatic RNase III domains (RIIID), and the dsRNA binding domain (dsRBD) in the C-terminus. Here, we identify alternatively spliced transcripts in human melanoma and NT2 cell lines, encoding C-terminally truncated Drosha proteins lacking part of the RIIIDb and the entire dsRBD. Proteins generated from these alternative splice variants fail to bind to DGCR8 but still interact with Ewing sarcoma protein (EWS). *In vitro* as well as *in vivo*, the Drosha splice variants are deficient in pri-miRNA processing. However, the aberrant transcripts in melanoma cells do not consistently reduce mature miRNA levels compared with melanoma cell lines lacking those splice variants, possibly owing to their limited abundance. Our findings show that alternative processing-deficient Drosha splice variants exist in melanoma cells. In elevated amounts, these alternatively spliced transcripts could provide one potential mechanism accounting for the deregulation of miRNAs in cancer cells. On the basis of our results, the search for alternative inactive splice variants might be fruitful in different tumor entities to unravel the molecular basis of the previously observed decreased microRNA processing efficiency in cancer.

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Introduction

MicroRNAs (miRNAs) are single-stranded noncoding RNAs of approximately 22 nucleotides (nt) involved in posttranscriptional gene regulation. Their mode of action is degradation or translational repression of target messenger RNAs (mRNAs) harboring complementary sequences [1,2]. Transcripts modulated by miRNAs are involved in a wide range of cellular functions. Consequently, dysregulation of miRNA biogenesis, and thus alteration in miRNA expression levels, contributes to multiple diseases, including various forms of cancer.

In cancer cells, aberrant expression of mature miRNAs has been frequently observed [3–9]. Deviations from the normal miRNA expression pattern could be caused, for example, by genomic deletion or mutation, epigenetic silencing, or aberrant transcription. The abundance of miRNA stabilizing factors could also potentially alter miRNA activity in cancer [10,11]. In some instances, for example, for

Abbreviations: RIIID, RNase III domain; dsRBD, dsRNA binding domain; miRNA, microRNA

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²This article refers to supplementary materials, which are designated by Table W1 and Figures W1 to W3 and are available online at www.neoplasia.com.

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mir-143 and mir-145, the precursor miRNAs (pri- and pre-miRNAs) do not differ in their quantities between normal and cancer cells, although mature miRNA levels are decreased [12]. Thus, a deficit in miRNA processing could be another alternative mechanism for a decrease in mature miRNA expression. Indeed, in some tumors, expression of key players of the miRNA biogenesis pathway is reduced or abrogated by genomic deletion and correlates with survival and mature miRNA levels [13–16]. Moreover, impairment of the miRNA biogenesis pathway by knockdown of pivotal processing factors favors tumor formation [17].

The maturation of miRNAs is a highly complex and regulated process, which is characterized by two cleavage reactions mediated by RNase III enzymes [18]. Hairpin structures of long primary transcripts (pri-miRNA) are first cleaved close to the base of the stem by the Microprocessor complex, consisting of Drosha and its binding partner DGCR8. This cropping liberates an approximately 60- to 70-nt-long hairpin structure, termed *precursor miRNA* (pre-miRNA) [19–22]. Whereas the N-terminus of Drosha is required for nuclear localization [23], the middle part of Drosha as well as the C-terminal part, harboring the two RNase III domains, RIIIDa and RIIIDb, and the dsRNA binding domain (dsRBD) are essential for pri-miRNA processing [22]. The two enzymatic domains form a processing center with two catalytic sites of RIIIDa and RIIIDb cleaving the 3'-arm and the complementary 5'-arm of the hairpin, respectively [22]. For the conversion of pri-miRNAs into pre-miRNAs, the interaction of Drosha with its cofactor DGCR8 is essential [21,22]. DGCR8 confers substrate specificity by recognizing the structural characteristics of the pri-miRNA and acts as a "molecular ruler" by determining the cleavage site within the pri-miRNA [21,24]. The basal segments, the stem as well as the terminal loop, are structural characteristics that play a role in cleavage site determination [24–26].

Although Drosha-mediated cleavage represents a critical processing step, it is not compulsory for the generation of all pre-miRNAs. Short introns that form hairpin structures resembling pre-miRNAs, termed *mirtrons*, can be spliced and debranched into pre-miRNA mimics, thereby omitting Drosha processing. The debranched hairpins are then exported, and further processed by the canonical miRNA biogenesis pathway [27–30].

After the initial cropping by the Microprocessor, the processing intermediate is exported from the nucleus by Exportin-5 in a Ran-dependent manner [25,31–33]. In the cytoplasm, Dicer removes the terminal loop in a second cleavage reaction, thereby producing the miRNA duplex of 21 to 24 nt length [34–38]. After incorporation of one selected single strand into the effector complex RISC (RNA-induced silencing complex) with an Argonaute protein [39,40], the mature miRNA causes silencing of its targets by base pairing mostly with the 3'-UTR mediating mRNA cleavage, translational repression, or mRNA degradation [1,2].

Here, we identify novel endogenous alternative splice variants of the important microRNA processing factor Drosha in melanoma cell lines and NT2 embryonal carcinoma cells. These transcripts encode proteins lacking critical functional domains, namely part of the 3'-end proximal RNase III domain and the dsRBD. These C-terminally truncated Drosha variants do not bind DGCR8 and hence also lack pri-miRNA processing activity. Although processing efficiency is impaired, the splice variants do not have a consistent major effect on the mature miRNA repertoire of the melanoma cells most likely because of the minor pool of alternative splice forms. However, the discovery of the processing-deficient Drosha variants can lead the way to a thorough

search in other tumor entities where they could be more abundant and validated as one mechanism of miRNA deregulation in cancer.

Materials and Methods

Cell Lines

Cell lines have been established from skin or lymph node metastasis and were cultured as described elsewhere [41]. Patients were experiencing histologically confirmed melanoma of the skin, mucosa, or unknown primary and were classified as stage III or IV according to American Joint Committee on Cancer.

Plasmids

Wild-type Drosha was cloned into pcDNA3.1D/V5-His-TOPO as described previously [42]. The N-terminal Flag-tag was removed and replaced by a YFP-tag using *HindIII* and *BamHI* restriction sites. The *BamHI* site and the Drosha start codon were separated by the nucleotides acc. At the end of the Drosha coding sequence, a stop codon was introduced. RIIIDb splice variants were created by deleting exon 31, exons 31 and 32, or exons 30 to 32 from pcDNA3.1D/TOPO-YFP-Drosha-V5-His. The Drosha mutant E1045Q E1222Q was generated by site-directed mutagenesis of pcDNA3.1D/TOPO-YFP-Drosha-V5-His. HA-DGCR8 was created by deleting the Flag-tag of pFLAG/HA-DGCR8, which was kindly provided by Thomas Tuschl (Rockefeller University). The expression plasmid-encoding pri-mir-145 was described previously [43]. All constructs were verified by sequencing. Primer sequences are listed in Table W1. Plasmid sequences are available on request.

Reverse Transcription and Quantitative Polymerase Chain Reaction

Total or fractionated RNA was extracted using TRIzol (Invitrogen/Life Technologies, Carlsbad, CA) and treated with DNaseI (Roche, Mannheim, Germany). RNA was reverse transcribed using RevertAid H Minus Reverse Transcriptase (Fermentas, St Leon-Rot, Germany). To test mature miRNA levels, gene-specific RT was performed using stem-loop primers shown in Table W1 according to Chen et al. [44]. For the identification of Drosha splice variants, complementary DNA (cDNA) of different cell lines was used as a template to amplify exons 29 to 35 of Drosha. Polymerase chain reaction (PCR) products were visualized on a 1.2% agarose gel using ethidium bromide. Bands that ran lower than the expected wild-type band were purified from Ma-Mel-71, Ma-Mel-75, and NT2; cloned into pCRII-TOPO (Invitrogen) by TOPO-TA cloning; and sequenced.

Quantitative PCR was performed on an ABI StepOne Plus using 2× Power SybrGreen MasterMix (Applied Biosystems/Life Technologies). Primers are shown in Table W1.

Cellular Fractionation

Nuclear and cytoplasmic fractionation was basically performed according to [45] with minor changes. In brief, Ma-Mel-71 cells were scraped off with phosphate-buffered saline and pelleted at 300g for 5 minutes at 4°C. Pellets were resuspended in 1 ml of RSB (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl₂), incubated for 3 minutes on ice and centrifuged at 1000g for 5 minutes at 4°C. The cell pellet was resuspended with four times its volume of RSBG40 (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 10% glycerol, 0.5% Nonidet P-40, 0.5 mM dithiothreitol), and 100 U/ml RNasin (Fermentas), and 10 mM ribonucleosid vanadyl complex (NEB) were used as

RNA inhibitor. After incubating for 3 minutes on ice and centrifuging at 4500g for 3 minutes at 4°C, the supernatant was saved as the cytoplasmic fraction, and the nuclear pellet was resuspended in RSBG40 containing one-tenth volume of detergent (3.3% [wt/wt] sodium deoxycholate and 6.6% [vol/vol] Tween-20) and incubated for 5 minutes on ice. Nuclei were pelleted again at 4500g for 3 minutes at 4°C, washed with RSBG40, and collected at 9300g for 5 minutes. RNA from nuclear and cytoplasmic fraction was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions.

Cycloheximide Treatment

Ma-Mel-71 human melanoma cells were grown to 80% confluence and treated either with the solvent dimethyl sulfoxide or with 50 µg/ml cycloheximide (Sigma, St Louis, MO) for 20 minutes at 37°C as described previously [46]. The cells were then washed with phosphate-buffered saline and lysed in TRIzol (Invitrogen) for total RNA isolation, DNase I treatment, reverse transcription, and quantitative PCR as described previously.

Immunoprecipitation

HEK293 cells were transfected in 10-cm dishes with a total of 24 µg of N-terminally YFP-tagged Drosha, splice variants thereof, or empty vector with or without cotransfection of HA-DGCR8 using polyethylenimine. At 48 hours after transfection, cells were lysed in 500 µl of cold lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1× complete EDTA-free protease inhibitor cocktail [Roche]) for 20 minutes at 4°C under gentle shaking followed by centrifugation at 14,000g for 15 minutes at 4°C. A ratio of 1:20 of the supernatant was saved as input sample. The remaining lysates were incubated for 2 hours with 30 µl of slurry of the GFP-nanotrap [47] with constant rotation. Beads were pelleted at 400g for 2 minutes at 4°C and washed three times with cold lysis buffer and eluted with 100 µl of sample buffer (250 mM Tris-HCl, pH 6.8, 9.2% SDS, 40% glycerol, bromophenol blue) boiling for 2 minutes. Samples were analyzed by 7.5% SDS-PAGE and Western blot using the following primary antibodies: anti-Drosha (1:500, Drosha [D28B1] XP no. 3364; Cell Signaling Technology, Danvers, MA), anti-DGCR8 (1:300, 10996-1-AP; ProteinTech Group, Inc, Chicago, IL), anti-EWS (1:1000, EWS [G-5], sc-28327; Santa Cruz Biotechnology, Inc, Santa Cruz, CA), anti-tubulin (1:1000, α/β -tubulin antibody no. 2148; Cell Signaling Technology), and anti-HA (1:500, Mono HA.11 MMS-101P; Covance, Münster, Germany).

In Vivo Processing Assay

HEK293 cells were reverse transfected with the Drosha-siRNA: 5'-GCAUGCAAGCGCGGAGUAU(dTdT) or scrambled small interfering RNA (siRNA) using Lipofectamine RNAiMAX in a six-well

plate. After 24 hours, the cells were transfected each with 1.5 µg of plasmids encoding YFP-Drosha, HA-DGCR8, and pri-mir-145 to challenge the pri-miRNA processing factors. At 48 hours after plasmid transfection, RNA was isolated using TRIzol (Invitrogen), digested with DNaseI (Roche), and reverse transcribed. The samples were analyzed for pri-miRNA and endogenous Drosha levels by quantitative PCR.

In Vitro Processing Assay

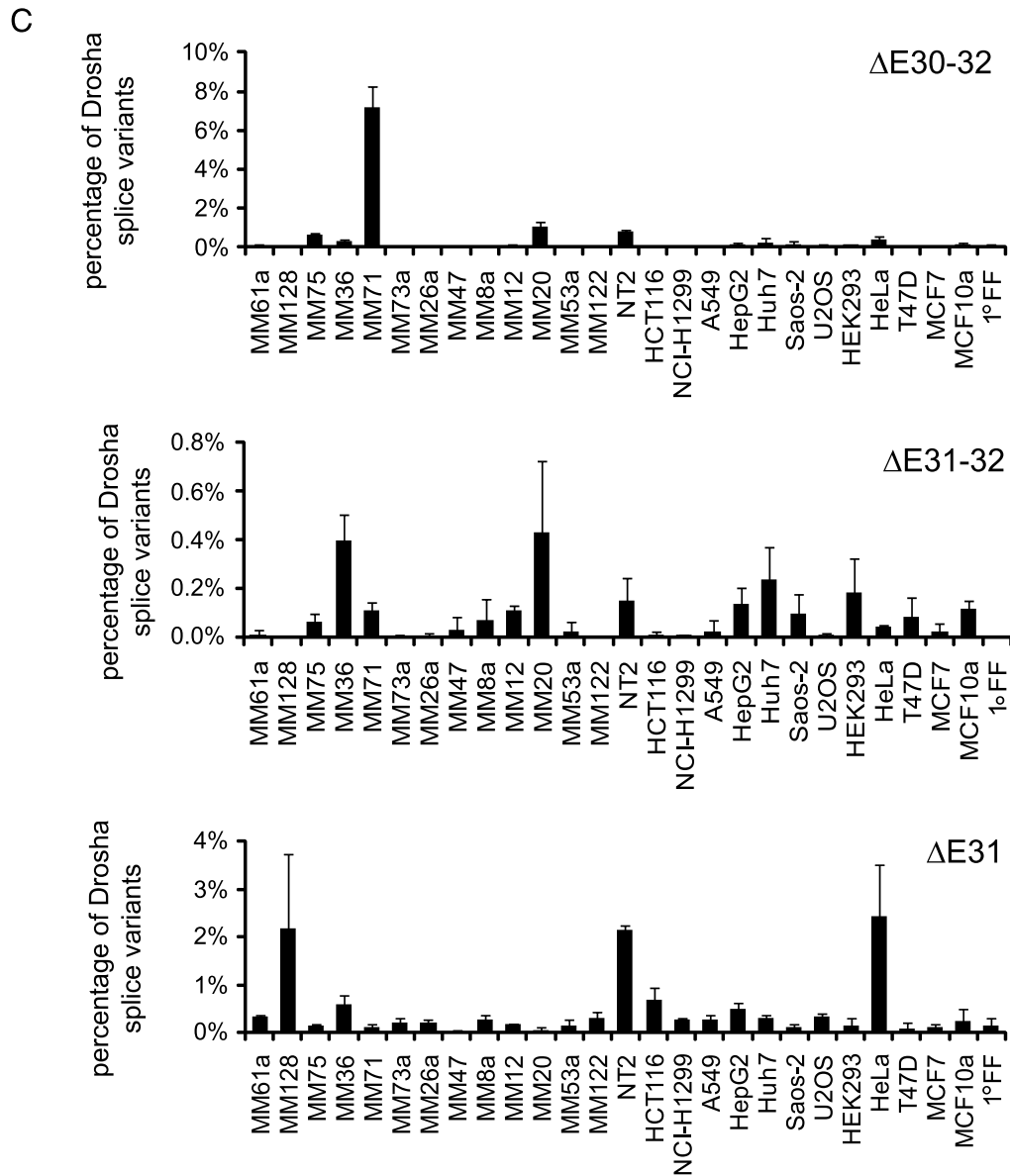
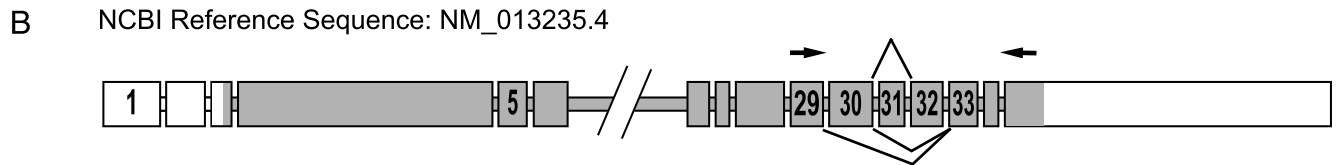
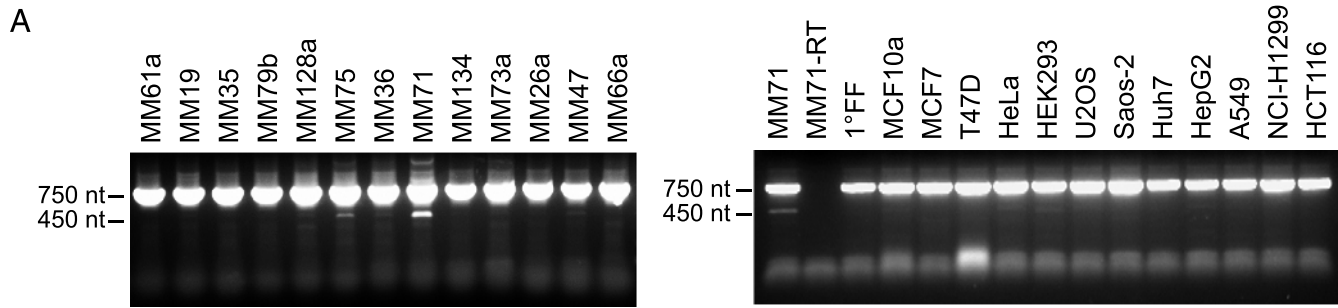
HEK293 cells were reverse transfected with the Drosha-siRNA: 5'-GCAUGCAAGCGCGGAGUAU(dTdT) or scrambled siRNA using Lipofectamine RNAiMAX in a six-well plate. After 24 hours, the cells were transfected each with 1.5 µg of plasmids encoding YFP-Drosha and HA-DGCR8. Whole-cell extract was prepared in a buffer containing 20 mM Tris-HCl (pH 8.0), 100 mM KCl by sonication, followed by centrifugation. The processing reaction (total, 35 µl) contained 40 µg of total protein, 0.5 mM ATP, 20 mM creatine phosphate, 4 mM MgCl₂, 20 U RiboLock RNase Inhibitor (Fermentas), and 10⁴ to 10⁵ counts per minute of the [³²P]-labeled transcript containing pri-15b~16-2. The assay was incubated for 30 minutes at 37°C. The T7 template for the primary transcript was amplified by PCR (Table W1). The labeled transcript was gel purified, precipitated, and allowed to fold for 10 minutes at 60°C before usage (as described in Allegra and Mertens [48]).

Array

MiRNA expression profiling for melanoma cell lines was performed on Illumina DASL (cDNA-mediated Annealing, Selection, Extension, and Ligation) platform using Human miRNA Expression Profiling V2 Panel (Illumina, San Diego, CA). Briefly, 500 ng of total RNA was polyadenylated, reverse transcribed using a biotin-labeled oligo-dT primer with a universal sequence at the 5'-end, attached to a solid phase, and annealed to miRNA-specific oligonucleotides that comprised three parts: another 5' universal PCR priming sequence, an address sequence for capturing the product on the array, and the 3' miRNA-specific sequence. The miRNA-specific oligonucleotides were extended, eluted, and subjected to PCR fluorescent universal primers. Then, single-stranded PCR products were prepared and hybridized to capture the probes immobilized on beads in the arrays. Arrays were scanned by the BeadArray Reader and intensity per bead type (miRNA) was extracted. Data were quantile normalized using BeadStudio Data Analysis Software (Illumina).

From the normalized array data, the average signal intensities for all 858 validated mature miRNAs (as annotated in the microRNA registry) and for the mirtron-derived mature miRNAs miR-877 and miR-1224-1238 were calculated. The ratio of the Drosha-dependent mature miRNAs divided by the mirtron-derived, Drosha-independent mature miRNAs was used as a normalized measure for genome-wide

Figure 1. Identification of alternative Drosha splice variants in melanoma cells. (A) Identification of alternative Drosha splice variants by reverse transcription. Drosha cDNA was amplified from exon 29 to 35 by reverse transcription in several cancer cell lines (MM = Ma-Mel). PCR products were visualized on a 1.2% agarose gel using ethidium bromide. The upper band is derived from wild-type Drosha. The lower bands are derived from splice variants lacking exons in the amplified region. (B) Schematic representation of the Drosha gene and derived alternative spliced mRNAs. Exon skipping changes the reading frame resulting in premature stop codons. Protein coding exons are shown in dark gray, noncoding exons in white. Arrowheads indicate positions of primers used to amplify exon 29 to 35. Introns are not drawn to scale. (C) Expression levels of splice variants lacking exons 31, 31 and 32, and 30 to 32 in different cancer cell lines quantified by quantitative RT-PCR in relation to total Drosha levels. Total Drosha levels were obtained by totaling the 2^{- Δ Ct} values normalized to RPLP0. The sum was set as 100%. One representative data set is shown; error bars, SEM.



Drosha-dependent processing in the cell lines. For the analysis, six cell lines with consistently detected splice variants in reverse transcription (RT)-PCR and quantitative PCR (Ma-Mel-71, Ma-Mel-128, Ma-Mel-20, Ma-Mel-36, Ma-Mel-75, and Ma-Mel-12) were compared to five cell lines with no significant signs of splice variant expression (Ma-Mel-53, Ma-Mel-73, Ma-Mel-26a, Ma-Mel-122, and Ma-Mel-8a).

Results

Drosha RIIIDb Splice Forms Exist in Several Cancer Cell Lines

To examine whether aberrant splicing of key miRNA processing factors could be a potential cause of decreased mature miRNA levels in cancer cells, we searched for functionally inactive Drosha splice variants in different sequence databases. We hypothesized that functional inactivity of Drosha would most likely require a loss of either the RNase or dsRNA binding activity, hence the loss of the RIIID or dsRBD domain. Therefore, we blasted the C-terminal region of Drosha including these three domains (NM_013235.4, nt 2985-4361) against several nucleotide databases to determine whether expressed sequence tags with aberrant splicing patterns had ever been identified.

This approach led to the identification of two Drosha transcripts lacking different parts of the functional region in melanoma (GenBank BC054003.1) and teratocarcinoma (AK308093).

To validate whether these aberrant forms exist in tumor cells, we amplified Drosha's C-terminus encoding the RIIIDb and the dsRBD from cDNA of different cancer cell lines of diverse origins, several melanoma cell lines and NT2 cells. Amplification of the wild-type isoform with primers spanning exon 29 to 35 should yield a product of 759 base pairs (bp) and the alternatively spliced isoforms of BC054003.1 and AK308093 211 and 387 bp, respectively. The wild-type product was detected in every cell line tested (Figure 1A), but amplicons, which would reflect splicing events as in BC054003.1 and AK308093, were not produced, at least not in detectable amounts. However, additional weaker PCR products of unexpected size (approximately 450 bp) were observed in NT2 cells and the melanoma cell lines Ma-Mel-71 and Ma-Mel-75 (Figure 1A). Gel purification of these products, cloning, and subsequent sequence analysis revealed that these transcripts lack exon 31 (identified from NT2), exons 31 and 32 (identified from NT2 and Ma-Mel-71), or exon 30 to 32 (identified from Ma-Mel-71, Ma-Mel-75, and Ma-Mel-20), which code for a part of the RIIIDb (Figure 1B).

To quantitate the levels of these three alternatively spliced transcripts, we performed quantitative real-time PCR using a reverse primer in exon 35 and a forward primer specific for the individual splice forms. Splice variants $\Delta E31$ and $\Delta E31-32$ contribute to less

than 3% of total Drosha levels in all cell lines tested, whereas $\Delta E30-32$ accounts for approximately 10% of all Drosha transcripts in Ma-Mel-71 cells (Figures 1C and 2E). These cells also have already a comparably low level of wild-type Drosha (Figure W1).

Drosha RIIIDb Splice Variants Yield C-terminally Truncated Proteins, Which Do Not Bind to DGCR8

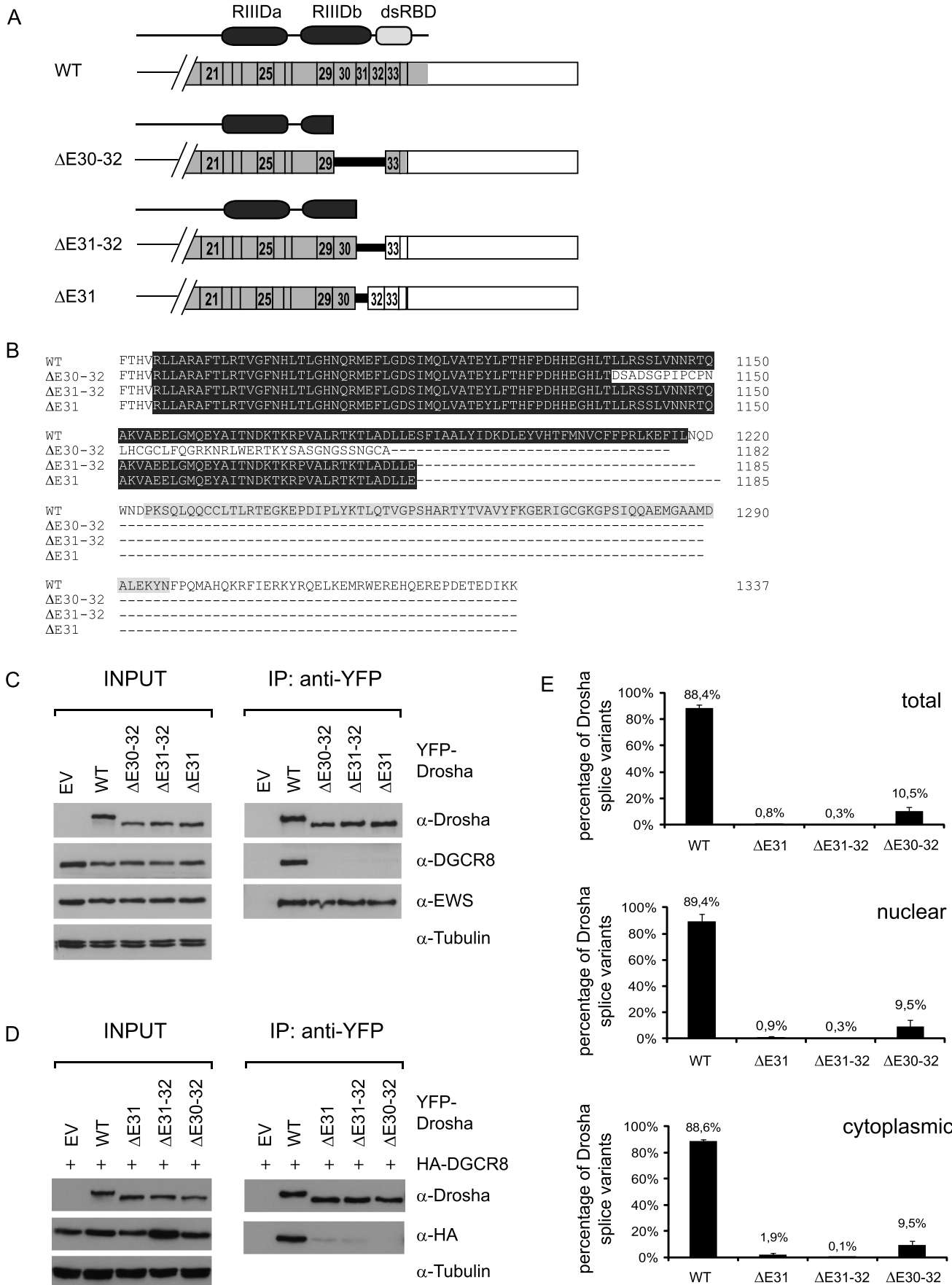
All of the three aberrant Drosha splice variants contain premature stop codons, encoding C-terminally truncated Drosha proteins. The lack of E31 or E31-32 creates a stop codon of the last two nucleotides of exon 30 and the first nucleotide of E32 or E33, respectively. Thus, $\Delta E31$ and $\Delta E31-32$ create the same protein lacking part of the RIIIDb and the dsRBD, ending with Glu1222 (E110b), the residue responsible for cutting the 5' arm of the pri-miRNA hairpin [22]. Deletion of E30-32 creates a frameshift leading to a sequence of 44 amino acids different from the wild-type before creation of a premature stop codon. The encoded protein lacks more than half of the RIIIDb and the dsRBD (Figure 2, A and B).

Processing of pri-miRNA into pre-miRNA not only requires the RNase III Drosha but also necessitates the cofactor DGCR8 [21,22], which interacts with the middle region as well as the RIIIDs of Drosha [22]. Because the alternative splice variants lack part of RIIIDb, we tested whether they are still able to bind DGCR8. To this end, we transfected HEK293 cells with plasmids encoding N-terminally YFP-tagged Drosha proteins with wild-type or alternatively spliced sequences. The expression levels of the spliced forms are similar to that of wild-type protein (Figure 2C, left panel). On YFP pull-down, endogenous (Figure 2C) or ectopic (Figure 2D) DGCR8 coprecipitated with wild-type Drosha but not with the three alternatively spliced isoforms. On the contrary, another interaction partner of Drosha, the Ewing sarcoma protein [21], was still associated with the RIIIDb splice variants to the same extent as with the wild-type (Figure 2C). Hence, lack of the dsRBD and part of the RIIIDb results in loss of DGCR8 binding but has no impact on interaction with EWS.

Drosha RIIIDb Splice Variants Exist in the Cytoplasm

Alternative splicing of exon E31, E31-32 or E30-32 generates premature termination codons, which could potentially make the variant transcripts prone to nonsense-mediated decay (NMD). To find out, whether Drosha RIIIDb alternatively spliced transcripts are degraded by NMD or whether they are stable, we examined mRNA levels of wild-type Drosha and the splice variants in nuclear and cytoplasmic fractions of Ma-Mel-71 cells. To verify the purity of the fractions, we monitored pre-45S rRNA and the tRNA^{Lys}, which showed a clear nuclear and cytoplasmic enrichment, respectively (data not shown).

Figure 2. Drosha splice variants do not bind DGCR8. (A) Schematic overview of Drosha splice variants and their protein products. The two conserved RNase III domains and the double-stranded RNA binding domain are indicated (RIIIDa, RIIIDb, dsRBD) according to the SMART program (<http://smart.embl-heidelberg.de/>). (B) Sequence alignment of Drosha wild-type and alternative splice variants. Amino acids coding for the RIIIDb are highlighted in dark gray, amino acids coding for the dsRBD in light gray. (C, D) Novel alternative splice variants do not bind DGCR8. YFP-tagged WT and alternatively spliced Drosha were overexpressed in HEK293 cells without (C) or with (D) coexpression of HA-DGCR8. Cells were lysed 48 hours after transfection, and Drosha was pulled down using the GFP-nanotrap. The eluate was analyzed by SDS-PAGE and Western blot analysis using anti-Drosha, anti-DGCR8, anti-HA, anti-EWS, and anti-tubulin antibodies. Tubulin served as loading control. EV indicates empty vector; EWS, Ewing sarcoma protein; WT, wild-type. (E) Ma-Mel-71 shows the same percentage of Drosha $\Delta E30-32$ splice variants expression in total, cytoplasmic, and nuclear fractions as determined by quantitative RT-PCR. Total Drosha levels were obtained by totaling the $2^{-\Delta C_t}$ values normalized to RPLP0. The sum was set as 100%. $n = 3$. Error bars, SEM.



The distribution of wild-type and alternatively spliced Drosha transcripts was consistent in total, nuclear, and cytoplasmic fractions (Figure 2E). Hence, the cytoplasmic stability of wild-type and alternatively spliced Drosha transcripts seems to be comparable, suggesting that the novel splice forms are not degraded by NMD. To formally prove that Drosha Δ E30-32 is not subject to NMD, we treated Ma-Mel-71 cells with cycloheximide, which inhibits NMD by blocking translation [46]. Quantification of wild-type and alternatively spliced Drosha revealed that cycloheximide treatment did not increase the expression of the alternatively spliced isoforms indicating that the isoform is not subject to NMD (Figure W2).

Drosha RIIIDb Splice Variants Are Deficient in pri-miRNA Processing Activity

Drosha processing requires DGCR8 binding and two intact RIIIDs [21,22]. To determine whether Drosha proteins generated from RIIIDb alternative transcripts are catalytically inactive, we tested their ability to process pri-miRNA *in vitro* and *in vivo*.

For the *in vitro* processing assay, lysates of cells overexpressing DGCR8 and wild-type or mutant Drosha constructs were incubated with radioactively labeled pri-miR-16-2. As negative controls, we used cells transfected with empty vector or a transdominant-negative, processing-deficient Drosha mutant (TN-Drosha) containing point mutations in both RNase III domains (E1045Q E1222Q) [49]. Only lysate containing wild-type Drosha was capable of generating pre-miR-16-2, whereas the three Drosha splice variants as well as empty vector and TN-Drosha were unable to execute pri-miRNA processing (Figure 3A).

To confirm this result *in vivo*, we determined pri-miRNA levels by quantitative PCR on endogenous Drosha knockdown and subsequent overexpression of DGCR8 and wild-type or alternatively spliced Drosha. The siRNA was designed to target the 3'-UTR of the endogenous Drosha mRNA, so that only the endogenous not the ectopically expressed Drosha was silenced. Knockdown efficiency was monitored both at the mRNA and protein levels compared with control siRNA-treated cells by quantitative PCR and Western blot. Endogenous Drosha mRNA levels dropped to less than 60% (Figure W3A), and a much more potent down-regulation was observed at protein level (Figure W3B). Ectopic Drosha constructs were not affected by the siRNA (Figure W3B). On Drosha knockdown, we observed that pri-miRNAs accumulated (data not shown).

Expression of wild-type Drosha significantly diminished pri-miRNA levels compared with cells transfected with empty vector or YFP. In accordance with the *in vitro* assay results, the Drosha splice variants did not exhibit processing activity because no change in pri-miRNA levels was observed (Figure 3B). Also, the DGCR8 mRNA, which is also a substrate for Drosha cleavage [50–52], was only processed by wild-type Drosha but not by the splice variants (Figure 3C).

Upon overexpression of TN-Drosha, pri-miRNA levels accumulated (Figure W3C) as described previously [50]. To take into consideration that Drosha splice variants could be marginally less expressed than wild-type Drosha, we transfected cells with only half of the amount of the wild-type Drosha construct (WT 12). This resulted in a comparable expression of Drosha wild-type and splice variants at the protein level (Figure W3B). Nevertheless, pri-miRNA processing efficiency closely resembles that of cells transfected with the full amount of wild-type Drosha (Figure W3C). Thus, the difference in pri-miRNA expression (Figure 3, B and C) was due to a difference in processing

activity *in vivo*, but not due to a potential difference in expression of the splice variants compared with wild-type Drosha.

Melanoma Cell Lines with Alternatively Spliced Drosha Variants Do Not Show Generally Decreased Mature MiRNA Levels

To test whether the Drosha RIIIDb splice variants affected mature miRNA levels, we determined the expression of 858 mature miRNAs using a microarray platform in eleven melanoma cell lines which consistently showed Drosha splice variants ($n = 6$) or no sign of splice variants ($n = 5$) in RT-PCR and quantitative PCR. For normalization purposes, we used average levels of mature mirtrons, which are derived from Drosha-independent substrates as they are generated by splicing [27,28]. Indeed, the average mature miRNA levels of cell lines expressing Drosha splice variants were lower than in the control cell lines, but this strong trend was not significant ($P = .096$, t test). On the contrary, the cell line with the highest level of splice variants, Ma-Mel-71, exhibited the highest mature miRNA levels within the group with splice variants (Figure 4A). Thus, these results were ambivalent with respect to the impact of Drosha splice variants on mature miRNA expression: Whereas a trend indicates decreased mature miRNA abundance coinciding with low-abundance Drosha splice variants, the cell line with the by-far highest level of processing deficient splicing variants did not follow this pattern.

Further, we investigated the levels of individual mature miRNAs, miR-17 and miR-25, relative to their respective pri-miRNA expression in cell lines expressing RIIIDb splice variants (Ma-Mel-71, Ma-Mel-75, and Ma-Mel-36) and cell lines with no detectable Drosha splice variants (Ma-Mel-8a and Ma-Mel-73a) by quantitative PCR. For both miRNAs, no significant correlation between the amount of splice variants and mature miRNA levels could be observed (Figure 4B).

Overall, these results suggest that, although alternatively spliced Drosha variants are pri-miRNA processing deficient, the amount in the tested cell lines might not be sufficient to consistently affect mature miRNA production.

Discussion

At least 30% of all human genes are predicted to be controlled by miRNAs [53]. Because many targets are involved in key cellular processes, deregulation of miRNA biogenesis and finally aberrant miRNA levels are associated with a variety of diseases, particularly cancer. Although several miRNAs are overexpressed in cancer cells, most miRNAs shows reduced expression in human tumors [3,4,8]; reviewed in references [54–56]. This phenotype could be attributed to altered levels of functional processing factors, which we hypothesized could be caused by aberrant alternative splicing. Our analyses focused on the major nuclear pri-miRNA processing enzyme Drosha, which is essential for the production of most mature miRNAs except the mirtrons. Moreover, other key factors of the miRNA biogenesis pathway might be subject to alternative splicing, as was previously shown for Dicer in neuroblastoma cell lines [57].

In this study, we identify novel alternative splice variants of the human *Drosha* gene in melanoma cell lines, which differ in their coding sequence and encode functionally inactive Drosha proteins. Skipping of exon E31, E31-32, or E30-32 changes the reading frame, leading to a premature stop codon.

Transcripts containing a stop codon that prematurely terminates translation can be eliminated by a quality mechanism, termed *nonsense-mediated decay*, to avoid the generation of truncated proteins that could

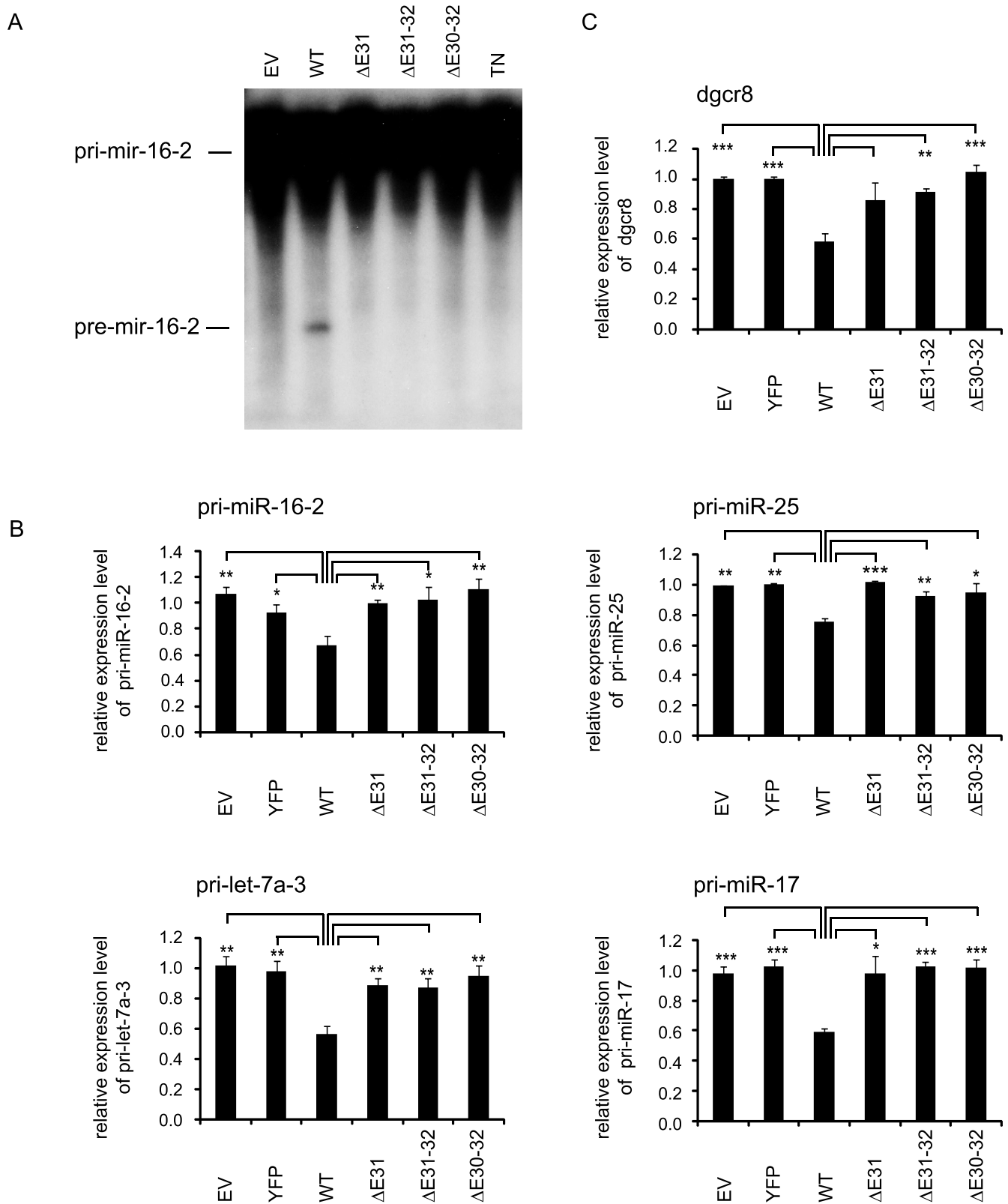


Figure 3. Drosha splice variants are defective in pri-miRNA processing activity. HEK293 cells depleted of endogenous Drosha using an siRNA against the 3'UTR of Drosha were transfected with HA-DGCR8 and siRNA-resistant Drosha constructs as indicated. EV indicates empty vector; WT, wild-type. (A) The cell lysates were incubated with labeled pri-mir-16-2 to assay pri-miRNA processing activity *in vitro*. (B, C) Quantification of several pri-miRNA levels (B) and endogenous DGCR8 mRNA levels (C) by quantitative RT-PCR normalized to cyclophilin mRNA levels. $n = 3$. Error bars, SEM. Asterisks denote statistically significant differences: * $P < .05$, ** $P < .01$, *** $P < .001$ (unpaired t test).

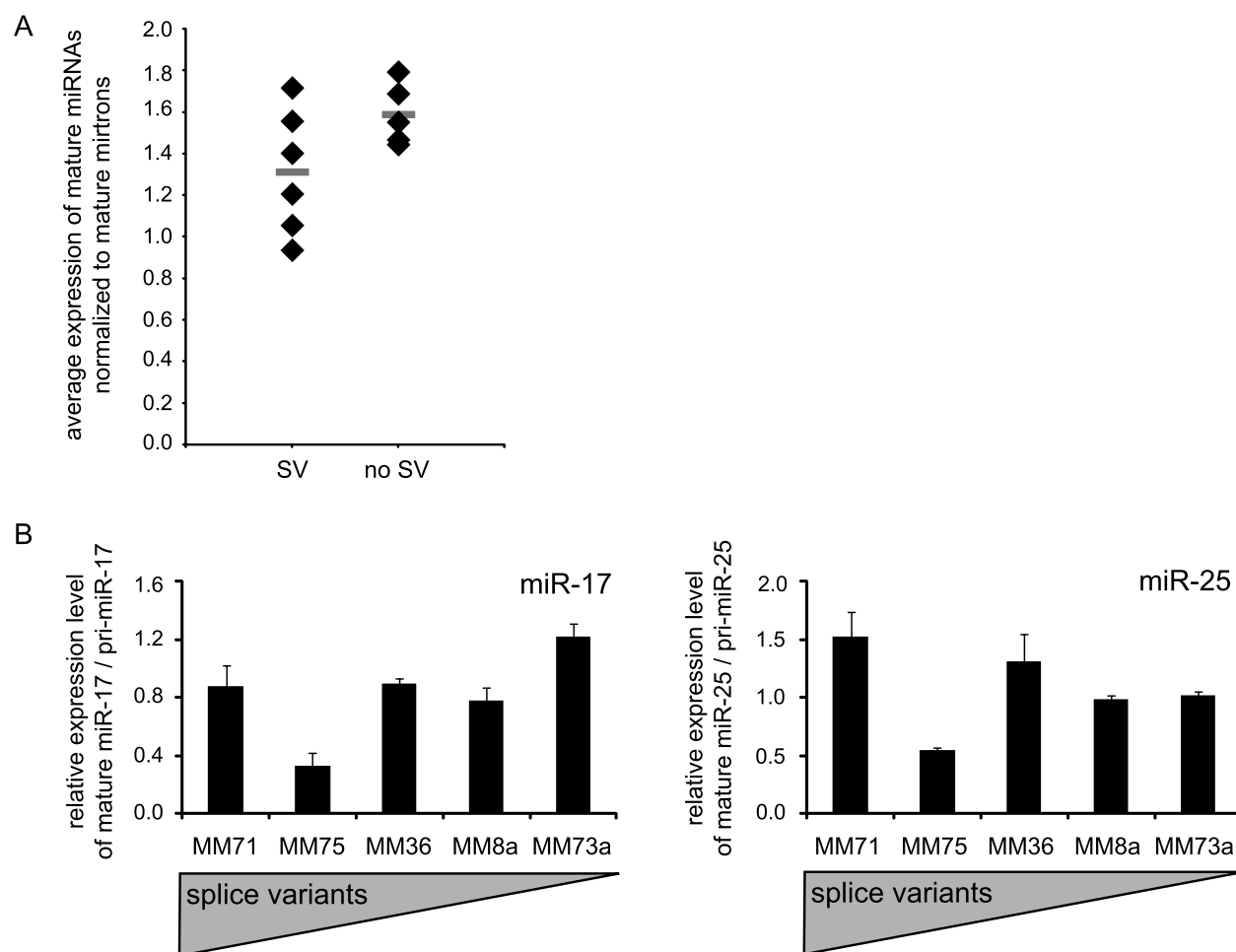


Figure 4. Cell lines with low levels of Drosha splice variants do not show consistently reduced miRNA levels. (A) Average mature miRNA expression levels from microarrays normalized to average mature mirtron levels. Cell lines with alternative splicing events at the C-terminus (SV) *versus* cell lines without detectable splicing events within exon 29 to 35 (no SV). The horizontal bars represent average values in the respective groups. (B) Expression levels of mature miRNAs, miR-25 and miR-17, normalized to the respective pri-miRNAs in different melanoma cell lines. The average miRNA level of Ma-Mel-8a and Ma-Mel-73a was set as 1. Bars indicate average expression; error bars, SEM ($n = 3$).

have dominant-negative or otherwise detrimental effects. This process itself is interestingly also regulated by microRNAs [58,59]. Stop codons are sensed as premature and elicit NMD, for example, when they are followed by an exon-exon junction located more than 50 to 55 nt downstream [60]. Drosha $\Delta E31$ and Drosha $\Delta E31-32$ splice variants fulfill this criterion, as the premature stop codon is followed by three or two exon-exon junctions, respectively, more than 55 nt downstream, and hence, they might be targeted for degradation by the NMD machinery. In contrast, removal of E30-32 creates a premature stop codon only at the last but one exon, exon 34, 5 nt upstream of the last exon-exon boundary. The most abundant transcript variant, Drosha $\Delta E30-32$, should thus escape NMD and be translated into a truncated Drosha protein. The fact that the splice variants exist in cytoplasmic fractions of Ma-Mel-71 cells to the same extent as in total lysates argues in favor of transcripts that are stable and translated rather than rapidly degraded by the cytoplasmic NMD machinery. In addition, expression of the truncated variants from plasmids leads to approximately the same level of protein as expression of wild-type Drosha. Hence, the truncation does not compromise the structure in a way that leads to degradation of the protein. Lastly, inhibiting NMD using cycloheximide in Ma-Mel-71

cells did not alter the abundance of Drosha $\Delta E30-32$ proving that it is not subject to NMD. Summing up, at least the most abundant splice variant Drosha $\Delta E30-32$ is most likely translated into a truncated Drosha protein.

The encoded truncated proteins are lacking the dsRBD and part of the RIIIDb. Previous studies have shown that the dsRBD as well as two intact RIIIDs are required for cropping of the pri-miRNA [22]. The two enzymatic RIIIDs form a single processing center with two catalytic sites by intramolecular dimerization [22]. In addition, the interaction of DGCR8 with Drosha is needed for efficient cleavage [21,22] because Drosha alone does not exhibit pri-miRNA-binding activity [61]. We demonstrated that Drosha RIIID splice variants do not bind DGCR8, showing that two complete RIIIDs are required for DGCR8 binding. Consistently, Drosha splice forms are deficient in pri-miRNA processing *in vitro* as well as *in vivo*.

Although the ectopic isoforms did not exhibit DGCR8 binding or pri-miRNA processing activity, there was no consistent impact of the endogenous alternatively spliced Drosha transcripts in melanoma cell lines on mature miRNA production. Expression profiling indicates decreased miRNA expression associated with the Drosha splice

variants, but paradoxically, Ma-Mel-71 cells, which exhibit the highest amounts of RIIID spliced transcripts among the cell lines tested, show high mature-to-pri-miRNA ratios, that is, good pri-miRNA processing. It could be possible that another factor involved in miRNA biogenesis might be altered in Ma-Mel-71 cells, which could overshadow the effect of the Drosha splice variants.

Given the importance of Drosha as a key player in miRNA biogenesis, it is most likely that sufficient amounts of alternatively spliced Drosha transcripts show a more pronounced effect on miRNA maturation. This could be envisioned in two ways: either the truncated transcripts are translated into nonfunctional proteins that execute dominant-negative functions or, in case the transcripts are degraded, wild-type RNA levels would be reduced resulting in limiting amounts of wild-type Drosha transcripts and thus less functional Drosha protein. As shown before, the artificial decrease in Drosha expression can lead to decreased miRNA activity and enhanced tumorigenesis [17]. In addition, a decrease of Drosha mRNA and protein in ovarian cancer [62] as well as neuroblastoma has been associated with a global miRNA downregulation [63] corroborating the significance of Drosha regulation in cancer. Thus, our data suggest that the search for more strongly expressed Drosha splice variants might be fruitful in other tumor entities and might provide one way to solve the many open questions about miRNA deregulation and processing defects in human cancer.

In summary, we have discovered rare splice variants of the important miRNA biogenesis factor Drosha for the first time. In melanoma, these splice variants lack parts of one RNase domain as well as the dsRBD. They thus do not bind DGCR8 and are completely deficient in miRNA processing *in vivo* and *in vitro*. However, the variants lack a consistent effect on endogenous miRNA expression in melanoma cell lines. Thus, this study shows that processing-deficient splice variants of important processing factors exist and could impact the miRNA expression patterns found in human tumors. For the future, it will be of great interest to search for tumor-enriched processing-deficient splice variants in the miRNA biogenesis pathway to understand the mechanisms underlying the frequently observed deregulation of mature miRNAs in cancer beyond the transcriptional level.

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Table W1. Primer Sequences.

Cloning		
Target	Forward	Reverse
YFP	5'-gtgtgtaagcttaccatgggtgagcaaggccgaggagc	5'-acacacggatccgagctctgtacagctcgtccatgc
Drosha ΔE31	5'-ccttggcggaccttttggatgagttcattttgaaatcag	5'-ctgattcaaaatgaactcattccaaaaggccgccaagg
Drosha ΔE31-32	5'-ccttggcggaccttttggatgactctgcagacagtg	5'-cactgtctgcagagtcattccaaaaggccgccaagg
Drosha ΔE30-32	5'-catgaaggacacttaactgactctgcagacagtg	5'-cactgtctgcagagtcattccaaaaggccgccaagg
Flag deletion to obtain HA-DGCR8	5'-ctgatcgccgcccatgracccttatgacgtgc	5'-gcagctcataagggtacatggcggcgatcag
Drosha E1045Q	5'-ggccaattgttttcaagcgrtaatagg	5'-cctattaacgcttgaacaattggcc
Drosha E1222Q	5'-ggcggacctttgcaatcattttgacg	5'-cgctgcaataatgattgcaaaaggccgccc
Specific RT for Mature miRNAs		
Target	Stem-loop Primer	
miR-17	5'-gtcgtatccagtcagggtccgaggtattcgactggatacagctacct	
miR-25	5'-gtcgtatccagtcagggtccgaggtattcgactggatacagctcagac	
PCR		
Target	Forward	Reverse
Drosha E29-35	5'-ccacaatcagagaatggaattcc	5'-gacaacagtcacagtactgagc
Quantitative PCR		
Target	Forward	Reverse
WT Drosha	5'-gcttcttccacgattgaaagag	5'-cattgtctgtcccatttcc
Drosha ΔE31	5'-ggcggaccttttggatgag	5'-cattgtctgtcccatttcc
Drosha ΔE31-32	5'-geggaccttttggatgactc	5'-cattgtctgtcccatttcc
Drosha ΔE30-32	5'-atcatgaaggacacttaactgac	5'-cattgtctgtcccatttcc
Endogenous Drosha	5'-gtgacatccagcgggaac	5'-gaagcagcctcagattttgg
Endogenous DGCR8 (5'UTR)	5'-actcgcttagtcgccagtc	5'-ggccacattgctcttttccat
pri-let-7a-3	5'-accaagaccgactgcccttt	5'-ctctgtccaccgagatatt
pri-miR-17	5'-acatcaccttgtaaaactgaagattg	5'-aaaagcactcaacatcagcag
pri-miR-25	5'-ggtcgctactcacaacacag	5'-ctcacaggacagctgaactcc
pri-miR-16-2	5'-tgttttcatcatcagatgttctg	5'-agttgtgtatccctgtcacac
miR-17	5'-gtcgtatccagtcagggtccgaggtattcgactggatacagctacct	5'-gtcagggtccgaggt
miR-25	5'-gtcgtatccagtcagggtccgaggtattcgactggatacagctcagac	5'-gtcagggtccgaggt
Cyclophilin A	5'-gtcaacccaccggtttctt	5'-ctgctgtctttggacctgtg
RPLP0	5'-ggcgacctggaagtrccaact	5'-ccatcagcaccacagccttc
Pre-45S rRNA	5'-cggctcgtgtgggtgact	5'-ctccttctccaggagcaga
tRNA ^{Lys}	5'-cccgaacaggacttgaaac	5'-gcccggatagctcagtcg
Primary MiRNA Processing		
Target	rt7_Forward	Reverse
pri-miR-15b~16-2	5'-taatacactcactataggctaggttgatgaaatccta	5'-aatacaacaattgataaaatag

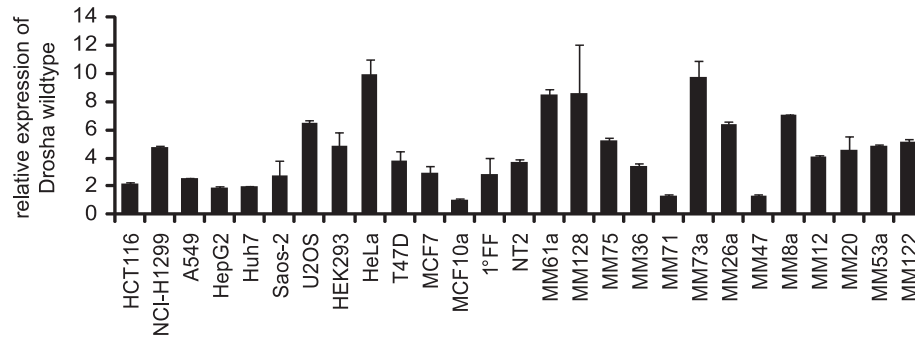


Figure W1. Drosha wild-type expression in different cell lines. Relative expression levels of wild-type Drosha mRNA were quantified by quantitative PCR using primers specific for the wild-type sequence. Values are normalized to RPLP0 levels. One representative data set is shown; error bars, SEM.

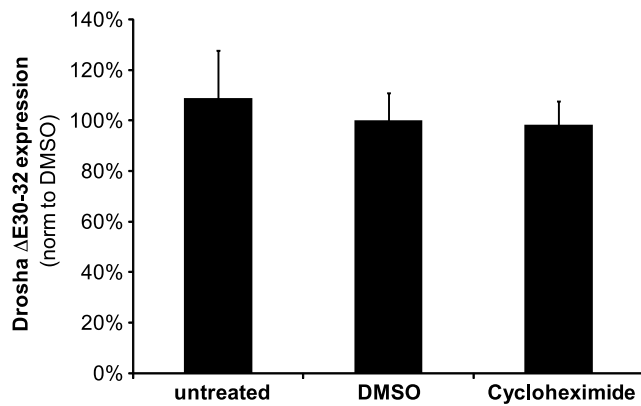


Figure W2. Drosha Δ E30-32 splice variant is not subject to NMD. Ma-Mel-71 melanoma cells were treated with cycloheximide to block the initial round of translation required for NMD. The expression of the Drosha Δ E30-32 splice variant as quantified by quantitative RT-PCR in comparison to wild-type Drosha remained unchanged when NMD was blocked, indicating that it is not subject to NMD. Depicted is the average of three experiments, with error bars representing SEM.

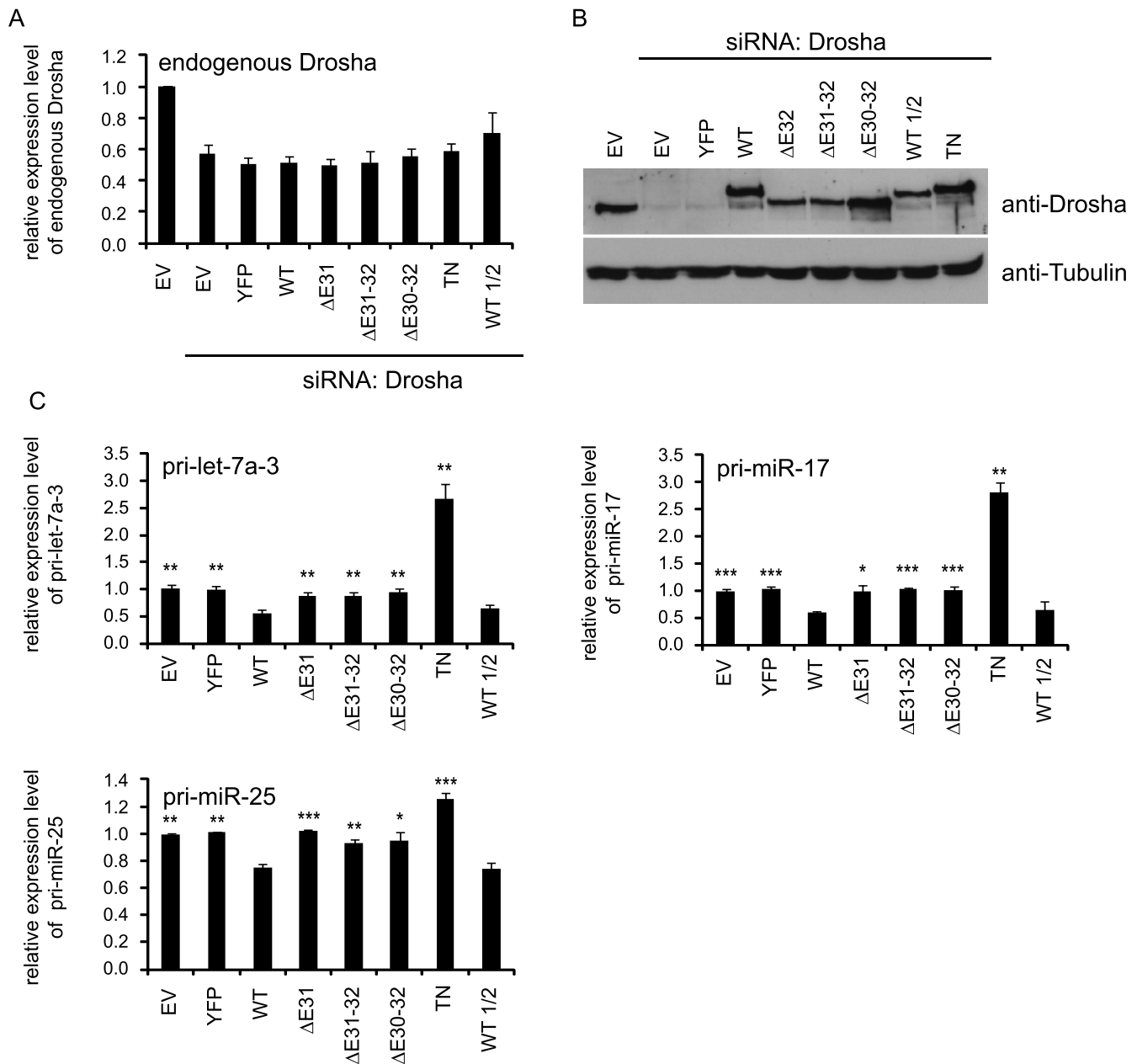


Figure W3. Drosha splice variants exhibit no pri-miRNA processing activity. HEK293 cells depleted of endogenous Drosha using a siRNA against the 3'UTR of Drosha were transfected with HA-DGCR8 and siRNA-resistant Drosha constructs as indicated. WT 1/2 indicates that half the amount of Drosha WT plasmid was used for transfection. EV indicates empty vector; WT, wild-type. (A) Endogenous Drosha levels were determined by quantitative RT-PCR to verify knockdown efficiency. (B) Protein expression levels of endogenous and ectopic Drosha were analyzed by Western blot. (C) Quantification of pri-miRNA levels by quantitative RT-PCR normalized to cyclophilin mRNA levels. $n = 3$. Error bars, SEM. Asterisks denote statistically significant differences: * $P < .05$, ** $P < .01$, *** $P < .001$ (unpaired t test).