SUPPLEMENTAL MATERIALS AND METHODS:

Linker ligation

In order to determine the precise nature of the small RNAs and verify that the miRNAs are generated by an endonucleolytic processing event rather than non-specific degradation, total RNA, or small RNA isolated using the miRvana kit (Ambion) was ligated on the 3’ end to Linker1 (IDT) using T4 RNA ligase 2 truncated (NEB) as per the manufacturer’s instructions. The ligated RNA was analyzed by RT-PCR using a combination of adapter (3’ linker) and miRNA-specific primers (miR-1225 Forward, miR-1228 forward and miR-877 forward). Products were subsequently cloned and sequenced. This strategy only allows detection of molecules that have a 3’ hydroxyl, like known miRNAs. Using this methodology we verified the predicted miR-877 mature miRNA (Supplemental Figure 2C), both mature miR-1228 and mature miR-1228* (Supplemental Figure 2B), and several species of mature miR-1225-5p with two to four guanines on the 3’ end (Supplemental Figure 2A). Although the versions of miR-1225-5p previous sequenced from humans had six to seven guanines on the 3’ end, the shorter miR-1225 sequence was previously detected in macaque (40). This data indicates 3’ heterogeneity of miR-1225.

Sequencing

For mRNA transcripts, RT-PCR amplicons were sequenced directly using gene specific primers (See Supplemental Table 1). For miRNAs, stemloop RT-PCR products were inserted into the pGem-T Easy Vector (Promega) and sequenced using a T7 promoter primer. All sequencing was preformed at the Northwestern University Genomics Core Facility.

In vitro Transcription

Wild-type and Δss plasmids were used as templates in PCR reactions. The forward primers were T7ABCF1, T7LRP1, T7PKD1, T7DHX30 that are specific to the 5’ end of the host gene and contain a T7 promoter sequence. The reverse primers were targeted to the exon following the mirtron/simtron (Gene specific reverse) or to the 3’ end of the predominate miRNA species (miRNA specific reverse) in order to generate substrates that should be recognized by gene specific amplification or stemloop RT-PCR, respectively. The PCR products were used as templates for in vitro transcription with a T7 RNA polymerase (Promega) to make RNA transcripts. Transcription reactions were carried out in the presence of $^{32}$P-UTP and 7Me-GpppG cap analog (NEB) to make pre-mRNA. RNA was purified by gel electrophoresis (80). The resulting synthetic RNA was reverse transcribed as described above and subjected to radiolabelled RT-PCR or stemloop RT-PCR.

qRT-PCR

Total RNA collected from HEK-293T cells transiently transfected with wild-type, Δss, or empty vector control (-) was reverse transcribed using the Taqman microRNA reverse transcription kit (Applied Biosystems) and Taqman microRNA assay kit (Applied
Biosystems PN4427975: miR-1225 TM2764, miR-1228 TM002919 and snoRNA48 TM001006). qPCR was carried out with Taqman universal master mix II, no UNG (Applied Biosystems) on an Applied Biosystems (ABI) 7500 Real-Time PCR System using the ABI 7500 detection software. For each experiment, samples were analyzed in triplicate. Values greater than +/- one standard deviation within the triplicate measurements were not considered.

Construction of plamids

For construction of the miRNAs expressed in an intergenic context, pcDNA-1228, the miRNA containing introns were PCR amplified with Phire polymerase (NEB) using the previously constructed minigenes as templates and primers with restriction sites incorporated on the termini. PCR products were digested with BamHI and EcoRI and inserted into the similarly digested pcDNA3.1+ plasmid (Invitrogen) using T4 DNA ligase.

For pmiRGLO-luciferase reporter plasmid construction, oligonucleotides miR-1228 Target 3' UTR and miR-1228 Target 3' UTR R were annealed and inserted into an XbaI- and XhoI-digested pmiRGLO plasmid (Promega) as per manufacturer’s instructions.

Immunoprecipitation

Immunoprecipitation was performed as described in the methods of the main text

Luciferase assays

Assays were performed as described in the methods of the main text. Where HEK-293T cells were transfected with 0.4 µg, 1 µg or 1.5 µg of minigene plasmid and 10 ng of pmiRGLO (Promega) using OptiFect (Invitrogen). miR-877 was transfected with the luciferase reporter plasmid for miR-1228 as a mismatch control.
SUPPLEMENTAL REFERENCES:

40. Berezikov, E., Chung, W.J., Willis, J., Cuppen, E. and Lai, E.C. (2007) Mammalian mirtron genes. *Mol Cell*, 28, 328-336.

80. Jodelka, F.M., Ebert, A.D., Duelli, D.M. and Hastings, M.L. (2010) A feedback loop regulates splicing of the spinal muscular atrophy-modifying gene, SMN2. *Hum Mol Genet*, 19, 4906-4917.

81. Cikos, S., Bukovska, A. and Koppel, J. (2007) Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. *BMC Mol Biol*, 8, 113.
SUPPLEMENTAL FIGURE LEGENDS:

Supplemental Table 1. Primer and siRNA sequences.

Supplemental Figure 1. Validation of the specificity of the stem-loop RT-PCR method for detection of miRNAs. (A) Diagram of the DNA templates used for in vitro transcription to generate synthetic mRNAs and miRNAs. The primer locations that were used for in vitro transcription are indicated. (B) Stemloop RT-PCR only amplifies RNA with 3’ ends that match the stem-loop primer. Internal sequences that match the stemloop primer are not amplified. In vitro transcribed RNA was generated using the T7 Forward and Gene Specific Reverse primers for both wildtype (WT) and splicing-deficient (Δss) transcripts. The in vitro transcribed RNA was reverse transcribed in the presence (+) or absence (-) of reverse transcriptase using stemloop primers (to detect miRNA specifically) or gene specific primers (to detect and demonstrate the quality of the synthetic RNA). The cDNA was amplified via radiolabelled RT-PCR. (C) Synthetic miRNAs are specifically detected using the stem-loop RT-PCR method. RNA with the 3’ end corresponding to the stemloop primer only amplifies in the presence of reverse transcriptase. In vitro transcribed RNA was generated using the T7 Forward and miRNA specific primers for both wildtype (WT) and splicing-deficient (Δss) transcripts. The in vitro transcribed RNA was reverse transcribed in the presence (+) or absence (-) of reverse transcriptase using stemloop primers. The cDNA was amplified via radiolabelled RT-PCR. M indicates a synthetic size marker for the mature miRNA species. • Indicates a non-specific primer dimer.

Supplemental Figure 2. Sequencing of miRNA. DNA sequencing traces of cloned (A) pre-miR-1225 and mature miR-1225-5p, (B) mature miR-1228 and (C) mature miR-877. The location of forward and linker-specific or stemloop primers used to clone and sequence the RNAs are shown. All samples were PCR amplified with indicated primers, ligated into pGEM-T Easy vector (Promega) and sequenced.

Supplemental Figure 3. qPCR analysis of simtrons. RNAs from wild-type (WT), splicing deficient (Δss), and empty vector (-) transfections for miR-1225 and miR-1228 were amplified via Taqman qPCR (Applied Biosystems). snoRNA48 (sno48) was used as a control. miRNA abundance was analyzed via the Liu and Saint method (81) and normalized using the equation: miRNA/sno48, n=5. Bars represent the average expression +/-SEM.

Supplemental Figure 4. Simtron 1228 processing is context independent and associates with Argonaute proteins. (A) Control, Dicer (Dicer-/-) or DGCR8 (DGCR8-/-) knockout mouse embryonic stem cells were transiently transfected with the intergenic wild-type minigene (WT), or intergenic splicing-deficient minigene (Δss). Minigene-derived miRNAs and endogenous miR-16 were analyzed by stemloop RT-PCR. sno65 was analyzed as a loading control. (B) Graph shows quantitation of miR-1228 abundance using the equation: (miRNA_Dicer-/- or DGCR8-/-/sno65)/(miRNA_control/sno65). Bars represent the average values +/- SEM, n=4 for Dicer/- and n=3 for DGCR8-/. The horizontal dotted line indicates normalized control cell levels. (C) Luciferase expression in HEK-293T cells transiently co-transfected with pmiRGLO containing matching miRNA target sequences or mis-match miRNA target sequences and two different concentrations (0.4,
1 or 1.5 µg) of the wild-type (WT) or splicing-deficient (Δss) minigene. Horizontal dotted line represents the normalized mismatch control value. The following equation was used to analyze the data: 

\[
\frac{((\text{Luciferase Target} / \text{Renilla})_{\text{miR\_match}})_{\text{avg}}}{((\text{Luciferase Target} / \text{Renilla})_{\text{miR\_mismatch}})_{\text{avg}}}
\]

Each sample was analyzed in triplicate. Bars represent the average +/- SEM, n=3 independent experiments. (D) miR-1228 derived from WT and Δss minigenes were co-transfected with pFLAG-GFP, pFLAG-Ago1, pFLAG-Ago2, pFLAG-Ago3, pFLAG-Ago4 or mock (-) into HEK-293T cells. FLAG-tagged proteins were immunoprecipitated from cell lysates and associated miRNAs were analyzed by radiolabelled stemloop RT-PCR. Un indicates the unbound fraction, and IP is the immunoprecipitated miRNA. For wild-type minigenes, unbound is 1/20 of IP. For Δss minigenes, unbound is 1/5 of IP. Sno65 is a control non-coding RNA. miR-16 is a canonical miRNA control. (E) The graphs represent the percent of the pre-miRNA in the IP fraction as determined using the equation: \(\frac{\text{IP}}{\text{IP}+(\text{Un}*20 \text{ or } 5)}*100\). Left panel: WT miR-1228, Right panel: Δss miR-1228.
| Primer Name | Sequence |
|-------------|----------|
| miR-16      | GTCGTATCCAGTGCAGGGTGAGGTATTCGCACTGGATACGACCGCCAA |
| miR-1225    | GTCGTATCCAGTGCAGGGTGAGGTATTCGCACTGGATACGACCCCCCCC |
| miR-1228    | GTCGTATCCAGTGCAGGGTGAGGTATTCGCACTGGATACGACGGGGGG |
| miR-1226    | GTCGTATCCAGTGCAGGGTGAGGTATTCGCACTGGATACGACCTAGGG |
| miR-877     | GTCGTATCCAGTGCAGGGTGAGGTATTCGCACTGGATACGACCCCTG |
| miR-877 + 1 NT 3' | GTCGTATCCAGTGCAGGGTGAGGTATTCGCACTGGATACGACTGTCCC |
| miR-1228 + 2 NT 3' MT | GTCGTATCCAGTGCAGGGTGAGGTATTCGCACTGGATACGACCTGGGG |
| miR-1228 +1 NT 3' MT | GTCGTATCCAGTGCAGGGTGAGGTATTCGCACTGGATACGACTGGGGG |
| RNA linker 1 | nAppCTGTAGGCACCACTCAAT/3ddC/ |
| miRNA Specific | | |
| miR-16 Forward | TAGCACGACGTAAA |
| miR-1225-Forward | GTGGGTACGGCCCA |
| miR-1228 Forward | TCACACCTGCTTC |
| miR-877 Forward | GTAGGAGAGATGG |
| snoRNA65 Forward | TQACTCCTGCTCCGAAGCAT |
| miRNA Specific Reverse | | |
| mir-1225 Reverse | CCCCCCACAGGGCGGTACCC |
| miR-1228 Reverse | GGGGGGCGAGGACATGTTG |
| miR-1226 Reverse | AGGGAAACACAGGCTTGTGA |
| miR-877 Reverse | CCCTGCCACCATCTCCT |
| Gene Specific | | |
| PKD1 Forward | CGAAGCTTGCTCCTGCTCTTCTG |
| DHX30 Forward | CGAAGCTTACTCATCAAACAGTTC |
| LRP1 Forward | CGAAGCTTACAGGCCCTGTG |
| DBR1 EXON 5 Forward | TTTGCCGCTTGTAGCAG |
| DBR1 EXON 8 Reverse | CTGTGGACTATAATCC |
| Drosha Forward | CATGCACAGATTCTCTGTA |
| Drosha Reverse | GTCTCCTGCATAACTCCA |
| DGC88 Reverse | TATCAGATCCCTCAACAGAG |
| DGC88 Reverse | TCTTGAGGGCTGTAGGAT |
| GAPDH Forward | GAAAGGCTAGAGCTTGAGATC |
| GAPDH Reverse | GAAGATGTGATTGGGTTC |
| XPO5 Forward | CCTCCCGAGACCAACAAG |
| XPO5 Reverse | CTTCAGAGAAGACCCT |
| 3' linker | ATGATGGTCCTAC |
| Vector specific | | |
| pTT3 Reverse | GGTCGAGGTCCGGGATCC |
| pCI Forward | GACTCACTATAGGCTCC |
| pCI Reverse | GTAAGTATAGGCTCC |
| siRNA | | |
| siDGCR8 | Sense: CAUCGGGACAAAGAGUGUAUTT Antisense: AUCACACUUCUGUGCCAUAGTT |
| siXPO5 | Sense: AGAUGUUGCGACACUAATT Antisense: UUUAGUGUUGAAGAAACAUUGG |
| Cloning | | |
| HindIII PKD1 ex 43 For | CGAAGCTTGCTCCTGCTCTTCTG |
| Enzyme | PKD1 ex 46 Rev | Sequence |
|--------|---------------|----------|
| BamHI  | CGGGATCCCTGAGAGGGTGAGGG |
| XhoI   | GGCGGCGCCCTGATGACAGAGGC |
| NcoI   | GGACGCTGACCCGATGACAGAGGC |
| BamHI  | LRP1 Ex 48 For | Sequence |
| HindIII| CGGAATTCCAGGAGGGTGAGGG |
| BamHI  | DHX30 ex Rev | Sequence |
| miR-1225-5p Target 3' UTR | CTCGAGGAGAGGGTGAGGG |
| miR-1225-5p Target 3' UTR R | CTAGAGATCAAGGAGGGTGAGGG |
| miR-877 Target 3' UTR | TCGGGATCCCTGAGAGGGTGAGGG |
| miR-877 Target 3' UTR R | CTAGAGATCAAGGAGGGTGAGGG |
| miR-1228 Target 3' UTR | TCGGGATCCCTGAGAGGGTGAGGG |
| miR-1228 Target 3' UTR R | CTAGAGATCAAGGAGGGTGAGGG |
| Mirtron intron 1225 BamHI | CGGAGATCCCTGAGAGGGTGAGGG |
| Mirtron intron 1225 EcoRI | CGGAGATCCCTGAGAGGGTGAGGG |
| MT Mirtron intron 1225 BamHI | CGGAGATCCCTGAGAGGGTGAGGG |
| MT Mirtron intron 1225 EcoRI | CGGAGATCCCTGAGAGGGTGAGGG |
| Mirtron intron 1228 BamHI | CGGAGATCCCTGAGAGGGTGAGGG |
| Mirtron intron 1228 EcoRI | CGGAGATCCCTGAGAGGGTGAGGG |
| MT Mirtron intron 1228 BamHI | CGGAGATCCCTGAGAGGGTGAGGG |
| Mirtron intron 877 BamHI | CGGAGATCCCTGAGAGGGTGAGGG |
| Mirtron intron 877 EcoRI | CGGAGATCCCTGAGAGGGTGAGGG |
| MT Mirtron intron 877 BamHI | CGGAGATCCCTGAGAGGGTGAGGG |
| MT Mirtron intron 877 EcoRI | CGGAGATCCCTGAGAGGGTGAGGG |
| Mirtron intron 1228 BamHI | CGGAGATCCCTGAGAGGGTGAGGG |
| Mirtron intron 1228 EcoRI | CGGAGATCCCTGAGAGGGTGAGGG |
| MT Mirtron intron 1228 BamHI | CGGAGATCCCTGAGAGGGTGAGGG |
| Mirtron intron 1228 EcoRI | CGGAGATCCCTGAGAGGGTGAGGG |
| MT Mirtron intron 1228 BamHI | CGGAGATCCCTGAGAGGGTGAGGG |
| MT Mirtron intron 1228 EcoRI | CGGAGATCCCTGAGAGGGTGAGGG |
| Quik change PKD1 5' SS For | TCAAAGGAGAGGGTGAGGG |
| PKD1 5' SS Rev | GCCCGTACCCTGCTCCTGAG |
| PKD1 3' SS For | TGCCGCCCCTGCTCCTGAG |
| PKD1 3' SS Rev | GTGCCGAAAGGGGGGGG |
| ABCF1 5' SS For | TCGAGAAGCTAGAGGAGAG |
| ABCF1 3' SS Rev | CATCTCCACTGAGGGAGAG |
| ABCF1 3' SS For | CTCTCCCACCCTGAGGGAG |
| ABCF1 3' SS Rev | CTCTCCCACCCTGAGGGAG |
| DDX30 5' SS For | CCATTAACAGTTGAGGGCATG |
| DDX30 5' SS Rev | GCATGCCCTCAAAGTGTTATG |
| DDX30 3' SS For | GTTGTCCTCATAGGAGGCCCAG |
| DDX30 3' SS Rev | CGCGTGCCTCCTCATAGGAGG |
| LR1 5' SS For | GACTGCCGACCTGAGGGGG |
| LR1 5' SS Rev | CCCGGCCGACCTGAGGGGG |
| LR1 3' SS For | CGCGGCGACCTGAGGGGG |
| LR1 3' SS Rev | CGCTGCCAGGAGGAGG |
| Drosha glu1222 Forward | GGGCCGACCTTGGATGACAGAGG |
| Drosha glu1222 Reverse | CGCGCTCAATAGTTGAGG |
| Drosha glu 1045 Forward | GCCACATGCTTACACGAG |
| Drosha glu 1045 Reverse | CCTTACAGCTTACACTAGT |

*In vitro Transcript.*
| T7 PKD1 Forward | TAATACGACACTATAGGTGTGTACGGTTCAGGTGAACAGC |
|----------------|-----------------------------------------|
| PKD1 ex46 Reverse | AAGACTCACCTGGAGGAGG |
| T7 pri-miR-16-1 Reverse | TAATACGACACTATAGGCAGGCCATATTGTGCTGCCTC |
| pri-miR-16-1 Reverse | CGGGATCCAGAATCATACTAAAAATAAC |
| T7 ABCF1 Forward | TAATACGACACTATAGGAGGTAGTAGCAGATGAG |
| T7 ABCF1 ex14 Reverse | ATGGCCACCTGGCCAGGG |
Havens et al., Supplemental Fig. 2

A  miR-1225

B  miR-1228

C  miR-877
Supplemental Fig. 3

qPCR of miR-1225

Relative miRNA Expression

WT  Δss  -

qPCR of miR-1228

Relative miRNA Expression

WT  Δss  -
