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An integrated expression atlas of miRNAs and their promoters in human and mouse

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1 **Ed sum**

2 **An atlas of microRNA expression patterns and regulators is produced by deep**
3 **sequencing of short RNAs in human and mouse cells.**

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6

7 **An integrated expression atlas of miRNAs and their promoters in human and**
8 **mouse**

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113 **MicroRNAs (miRNAs) are short non-coding RNAs with key roles in cellular**
114 **regulation. As part of the fifth edition of the Functional Annotation of**
115 **Mammalian Genome (FANTOM5) project, we created an integrated expression**
116 **atlas of miRNAs and their promoters by deep sequencing 492 short RNA (sRNA)**
117 **libraries, with matching Cap Analysis Gene Expression (CAGE) data, from 396**
118 **human and 47 mouse RNA samples. Promoters were identified for 1,357 human**
119 **and 804 mouse miRNAs and showed strong sequence conservation between**
120 **species. We also found that primary and mature miRNA expression levels were**
121 **correlated, allowing us to use the primary miRNA measurements as a proxy for**
122 **mature miRNA levels in a total of 1,829 human and 1,029 mouse CAGE**
123 **libraries. We thus provide a broad atlas of miRNA expression and promoters in**
124 **primary mammalian cells, establishing a foundation for detailed analysis of**
125 **miRNA expression patterns and transcriptional control regions.**

126 MicroRNAs¹ (miRNAs) are a class of short (21-23 nt) non-coding RNAs with key
127 roles in a wide range of biological processes including development and
128 differentiation^{2,3}, immunity⁴, reproduction⁵, and longevity⁶. Dysregulation of miRNA
129 expression has been implicated in numerous diseases⁷, including cancer^{8,9}. A detailed
130 characterization of the expression profile of miRNAs across cell types and tissues is a
131 fundamental requirement for understanding the function of miRNAs and their
132 potential role in health and disease.

133

134 MicroRNAs inhibit specific mRNAs by binding to complementary sequences, usually
135 located in the 3' UTR, leading to mRNA destabilization and a reduction in their
136 translation output¹⁰. In the canonical miRNA biogenesis pathway^{1,11}, a primary
137 miRNA transcript (pri-miRNA) is cleaved by the endoribonuclease Drosha in the
138 nucleus to excise the precursor miRNA (pre-miRNA), which is exported to the
139 cytoplasm. The pre-miRNA has a characteristic hairpin secondary structure that is
140 recognized and cleaved in the cytoplasm by the endoribonuclease Dicer, releasing the
141 mature miRNA.

142

143 Currently, the miRBase reference database of miRNAs¹² lists 1,881 pre-miRNAs in
144 human; around half (54%) are produced from intergenic non-coding pri-miRNA
145 transcripts, while the remaining 46% are excised from the introns of protein-coding
146 transcripts. A small proportion (6%) of human mature miRNAs annotated in miRBase
147 are located in multiple pre-miRNAs encoded in different genomic loci.

148

149 Several high-throughput approaches are available to measure the expression levels of
150 mature miRNAs, including high-throughput qPCR, microarray, and next-generation

151 sequencing methods¹³. Profiling pri-miRNAs, which is more challenging due to their
152 transient character, has been accomplished by RNAseq in cells expressing dominant-
153 negative Drosha¹⁴. Additionally, since most pri-miRNAs are produced by RNA
154 polymerase II and therefore have a 5' cap¹¹, they are amenable to Cap Analysis Gene
155 Expression (CAGE) profiling^{15,16}, which identifies the pri-miRNA transcription start
156 site and therefore the promoter region, while directly quantitating the pri-miRNA
157 expression level.

158

159 Here, we analyze 492 sRNA sequencing libraries to evaluate the expression patterns
160 of miRNAs in mammalian cells, with a particular emphasis on human primary cells.
161 Each sRNA library was matched to a CAGE library produced from the same RNA
162 sample, allowing us to create an integrated expression atlas of miRNAs and their
163 promoters. The expression atlas can be accessed through a web interface at
164 http://fantom.gsc.riken.jp/5/suppl/De_Rie_et_al_2017/). This work is part of the fifth
165 edition of the Functional Annotation of Mammalian Genome project
166 (FANTOM5)^{17,18}.

167 **Results**

168 **Matched miRNA and CAGE expression profiles**

169 In FANTOM5, a large collection of human and mouse primary cell types, cell lines,
170 and tissues was profiled by CAGE to identify mRNA and long non-coding RNA
171 transcription start sites and expression levels across a wide variety of biological
172 states¹⁷. Here, as a complementary data set, we produced 293 sRNA sequencing
173 libraries using FANTOM5 RNA samples from human primary cells, as well as 87
174 sRNA libraries from RNA samples of six time courses of stimulated human cells¹⁸
175 (Table 1, S1 & S2). We also incorporated previously produced CAGE and sRNA
176 sequencing libraries generated from human embryonic and induced pluripotent stem
177 cells¹⁹ (Table 1 & S1) in our analysis. In total, our sRNA sequencing data set
178 encompassed 121 distinct human cell types. In addition, we produced 6 sRNA
179 sequencing libraries from human tissues, and 42 sRNA libraries from mouse samples
180 (Table 1, S1 & S2). Most sRNA libraries were produced in biological triplicate. A
181 matching CAGE library¹⁷⁻¹⁹ generated from the same RNA sample was available for
182 492 of the 500 sRNA libraries analyzed here (Table S3).

183

184 **Establishing a robust set of miRNAs**

185 Across the sRNA libraries, expression was confirmed for 98% (1842/1877) of human
186 and 95% (1124/1186) of mouse pre-miRNAs annotated in release 21 of the miRBase
187 database¹². To assess the confidence level of annotated miRNAs, the miRBase
188 curators defined a set of five rules evaluating their secondary structure and expression
189 properties (Table 2), and used these rules to mark 295 human pre-miRNAs as high-
190 confidence annotations¹². Applying these rules to the FANTOM5 sRNA data, we
191 found that 571 human pre-miRNAs satisfied all five high-confidence rules, 224 met

192 four of them, and 1076 violated two or more rules (Figure 1a). The 795 human and
193 502 mouse (Figure S1) pre-miRNAs satisfying at least 4 out of the 5 high-confidence
194 rules were defined as the FANTOM5 robust set, and the remaining 1076 human and
195 684 mouse pre-miRNAs as the permissive set (Table S4 and Table S5). The robust set
196 encompasses 735 human and 438 mouse mature miRNAs, and covers more than 90%
197 of the high-confidence pre-miRNAs in miRBase (Figures S2 and S3), 90% of
198 miRNAs well characterized in the scientific literature (Figure S4), as well as 91%
199 (human) and 88% (mouse) of pre-miRNAs included in the manually curated
200 MirGeneDB database²⁰ (Figure S5).

201

202 **CAGE detects 3' cleavage products of Drosha**

203 In zebrafish, the Drosha cleavage site at the 3' end of pre-miRNAs was recently found
204 to be characterized by a distinctive CAGE peak²¹. We similarly observed a CAGE
205 peak immediately downstream of the 3' end of human pre-miRNA loci in the
206 ENCODE CAGE data²², and a slightly wider CAGE peak starting 1 nucleotide
207 downstream in the FANTOM5 CAGE data^{17,18} (Figure 1b, c, human; Figure S6,
208 mouse); the discrepancy between the ENCODE and FANTOM5 CAGE data is
209 expected because of differences in the sequencer technologies employed (Figure S7).
210 The ENCODE CAGE peak was found immediately downstream of the 3' end of the
211 pre-miRNA locus (Figure S8) for 19 out of 25 pre-miRNAs with a full-length
212 sequence in the FANTOM4 sRNA sequencing libraries²³, confirming that the CAGE
213 peak marks the Drosha cleavage site. FANTOM5 and ENCODE CAGE tags at the
214 peak were enriched in the nucleus (Figure S9), consistent with processing by Drosha.
215 CAGE peaks were absent at the 3' end of pre-miRNA loci encoding mirtrons (Figure

216 S10, human; Figure S11, mouse), which are excised by the spliceosomal machinery
217 instead of by Drosha²⁴.

218

219 To rule out the possibility that these CAGE tags originated from an independent
220 transcript, we analyzed the first nucleotide of the CAGE tags at the Drosha cleavage
221 site. Most CAGE tags originating from a transcription start site have an additional
222 guanine as their first nucleotide, as the 7-methylguanosine cap at the 5' end of
223 transcripts produced by RNA polymerase II can be recognized as a guanine nucleotide
224 during reverse transcription (Figure S7). No such additional guanine nucleotides were
225 found at the Drosha CAGE peak (Figure S12), confirming that the detected RNAs
226 were not due to an independent transcription initiation event. The lack of guanine
227 nucleotide enrichment also suggested that the 3' Drosha cleavage products were
228 uncapped RNAs that were nonetheless observed to some extent in the CAGE library
229 due to their cellular abundance. Alternatively, these RNAs may have a
230 hypermethylated cap, as previously found for small nucleolar RNAs (snoRNAs)
231 produced by excision from a host gene transcript²⁵: no additional guanines are found
232 as the first nucleotide of CAGE tags mapping to the 5' end of snoRNAs (Figure S12),
233 as hypermethylation of the cap prevents base-pairing during reverse transcription.

234

235 Excluding mirtrons, about half of the robust pre-miRNAs had a significant ($P < 0.05$)
236 Drosha CAGE peak (52%, human, Figure 1a; 64%, mouse, Figure S1; see Methods
237 for details). This percentage decreased from 56% for human pre-miRNAs satisfying
238 all five of the miRBase high-confidence criteria to 37% if one of the criteria was
239 violated, while only 7% of miRNAs in the permissive set had a Drosha CAGE peak
240 (Figure 1a). Similar results were obtained for mouse (Figure S1). The analysis of

241 Droscha CAGE peaks thus provided independent support for the stringency of the
242 selection criteria used to define the FANTOM5 robust and permissive set of miRNAs.

243

244 **Discovery of candidate novel miRNAs**

245 To discover potential miRNAs that had not been described previously, the miRDeep2
246 software²⁶ was applied on all unannotated sRNAs (see Methods for details). In total,
247 6,543 candidate miRNAs in human (Table S6, S7) and 1,444 in mouse (Table S8, S9)
248 were identified. Most of the candidate miRNAs were lowly expressed, with fewer
249 than 5% of them having sufficient tag counts on both arms of the pre-miRNA to
250 enable a full evaluation of the high-confidence criteria (Table 2). The 282 human and
251 34 mouse candidate miRNAs meeting at least 4 of the 5 high-confidence criteria
252 formed the robust candidate set, while the permissive candidate set consisted of the
253 remaining candidate miRNAs (Table S4 and Figure S13, human; Table S5 and Figure
254 S14, mouse). The robust candidate set comprised 279 (human) and 33 (mouse) unique
255 mature sequences, whereas the permissive candidate set provided an additional 5,826
256 (human) and 1,354 (mouse) mature sequences. Nearly 11% of robust and 5% of
257 permissive human candidate miRNAs had a significant ($P < 0.05$) Droscha CAGE
258 peak (Figure S13; Figure S14 for mouse). Validation by qPCR of a selection of robust
259 candidate miRNAs identified in monocyte and macrophage libraries confirmed their
260 expression in these cell types in multiple donors (Figure S15, Table S10).

261

262 The robust candidate set showed good concordance (127/282 or 45%) with the 3,524
263 putative miRNAs identified recently in a study of tissue- and primate-specific
264 miRNAs²⁷, whereas the permissive candidate set yielded a smaller overlap (352/6,261
265 or 6%). Few of these putative miRNAs²⁷ had a significant Droscha CAGE peak

266 (258/3,524 or 7%), which may be due to their low expression levels in the samples
267 surveyed in FANTOM5.

268

269 We conclude that the vast majority of canonical, highly expressed miRNAs had
270 already been annotated. However, our analysis also provides evidence of extensive
271 transcription of lowly expressed short RNAs from specific genomic loci.

272

273 **Expression variability of miRNAs in human primary cells**

274 The cell type dependence of expression of individual miRNAs was evaluated by
275 analyzing the distribution of miRNA abundance across the FANTOM5 primary cells
276 and tissues. First, we assessed various expression normalization strategies, and found
277 that a counts per million (cpm) normalization (i.e., dividing the tag count of each
278 miRNA by the total number of tags mapping to miRNA loci, and multiplying by
279 1,000,000) yielded the best reproducibility between different donors for the same cell
280 type, while maintaining the distinction in expression profile between different cell
281 types (Figure S16; see Methods for details). We then created a miRNA expression
282 table across the FANTOM5 samples for human (Table S11) and mouse (Table S12),
283 using cpm normalization in our further analysis. Figure 2a shows a graphical
284 overview of the human primary cells clustered by their robust miRNA expression
285 profile using Miru²⁸. An interactive heatmap of the expression data is available at
286 http://fantom.gsc.riken.jp/5/suppl/De_Rie_et_al_2017/vis_viewer/#/heatmap.

287 The expression levels of miRNAs varied greatly and were highly skewed, with on
288 average five miRNAs contributing half of the total miRNA expression in a given
289 library (Figure 2b, human; Figure S17, mouse), whereas most known and candidate
290 miRNAs were lowly expressed (Figure 2c, human; Figure S18, mouse). The extreme

291 distribution of miRNA expression across miRNAs and cell types was confirmed by
292 qPCR (Figure S19).

293

294 **Cell ontology analysis**

295 A cell type specificity index, analogous to the previously defined tissue specificity
296 index²⁹, was calculated to quantify the cell type specificity of miRNA expression
297 across the FANTOM5 collection of primary cell types (Table S13; see Methods for
298 details). Previously described highly cell type specific miRNAs included miR-122-5p,
299 miR-142-5p and miR-302a-5p, which were enriched in hepatocytes, leukocytes, and
300 pluripotent stem cells, respectively (Figure 2d). In contrast, miRNAs such as miR-
301 100-5p and miR-29a-3p were broadly expressed but specifically depleted in particular
302 cell types (leukocytes and pluripotent stem cells, respectively; Figure 2d). Candidate
303 miRNAs tended to be restricted to specific cell types, with 80% of the robust
304 candidate set and 96% of the permissive candidate set having a higher cell type
305 specificity index than the median value for robust known miRNAs (Table S13).

306

307 We then calculated the statistical significance of expression enrichment or depletion
308 of each miRNA (Table S13) with respect to cell ontology clusters (Table S14) defined
309 by the FANTOM5 cell ontology annotation^{30,31}, which organizes FANTOM5 samples
310 by cell type in a hierarchical framework (see Methods for details). Of miRNAs in the
311 robust set, 54% had enriched expression in their most significant cell ontology cluster,
312 whereas 27% were broadly expressed, with depleted expression in their most
313 significant cell ontology cluster. The remaining 19% were lowly expressed without
314 statistically significant enrichment or depletion in any cell ontology cluster;
315 understanding their functionality may need profiling in further cell types or states.

316 Pluripotent stem cells were characterized by cell type specific miRNAs, whereas cell
317 type specific depletion of broadly expressed miRNAs was predominantly found in
318 leukocytes. Examples of enriched expression not reported previously included miR-
319 488-5p in neural cells, miR-506-3p in light melanocytes, and miR-205-5p in epithelial
320 cells. MiRNAs previously not reported as broadly expressed included miR-887-3p,
321 which was present in most samples but was depleted in leukocytes.

322

323 **Identification of miRNA promoters**

324 We developed an automatic pipeline to identify miRNA promoters using Gencode
325 v19 and RefSeq transcripts as candidate pri-miRNAs and the FANTOM5 CAGE data
326 as putative transcription start sites (see Methods for details). This pipeline predicted
327 promoters for 539 robust, 623 permissive, and 3,951 candidate pre-miRNAs in human
328 (Table S15), and for 358 robust, 446 permissive, and 994 candidate pre-miRNAs in
329 mouse (Table S16). Manual curation by two independent annotators confirmed the
330 selected promoter for 512 (95%) robust pre-miRNAs; the computationally selected
331 promoter was corrected for 26 pre-miRNAs and dropped for 1 pre-miRNA. Manual
332 curation furthermore identified the promoter for an additional 196, mostly intergenic,
333 pre-miRNAs, thereby generating the—to our knowledge—largest miRNA promoter
334 collection to date (Table S17, Figure S20a). Across the human robust set, an
335 associated ENCODE RAMPAGE (RNA Annotation and Mapping of Promoters for
336 the Analysis of Gene Expression³²) 5' end was found within 300 base pairs of more
337 than 75% of the FANTOM5 curated promoters both for intergenic and intronic
338 miRNAs, outperforming the miRGen³³, Chang *et al.*¹⁴, miRStart³⁴, and TSmiR³⁵
339 collections of miRNA promoter annotations (Figure S20b). The median distance
340 between the FANTOM5 annotated miRNA promoter and the associated RAMPAGE

341 5' end was 1 nucleotide, and was thereby closer than any of the existing miRNA
342 promoter annotations (Figure S20c). RACE experiments confirmed that the transcripts
343 generated at the identified promoter extended to the mature miRNA for 6 out of 7
344 miRNAs (Figure S21, Table S18). RNA-seq data¹⁴ of cells expressing a dominant-
345 negative Drosha protein provided additional evidence for the FANTOM5 annotated
346 pri-miRNAs, with 483 out of 607 pri-miRNAs (80%) having a 5' end within 300 base
347 pairs of an RNAseq transcript assembly extending to the mature miRNA locus (Figure
348 S22).

349

350 Both in human and in mouse, promoter sequences of intronic and intergenic miRNAs,
351 like those of transcription factor genes, were highly conserved across species
352 compared to the promoter sequences of protein-coding genes and of long non-coding
353 RNAs (Figures 3a and S23, human; Figure S24, mouse). The distance between the
354 transcription start site of the pri-miRNA and the mature miRNA locus was strongly
355 conserved between human and mouse both for intergenic miRNAs and for intronic
356 miRNAs (Figures 3b and S25). While this suggests that pri-miRNA transcripts may
357 have some functional role beyond providing the substrate for pre-miRNA excision,
358 there was no evidence of substantially elevated sequence conservation across species
359 in pri-miRNAs (Figure S26).

360

361 **Correlation of mature miRNA and pri-miRNA expression levels**

362 The expression levels of mature miRNAs correlated with the CAGE expression levels
363 of the associated promoter, with comparable correlation values for intergenic and
364 intronic miRNAs (Figure 3c and S27; Table S19). The correlation was substantially
365 higher for highly differentially expressed miRNAs, and exceeded correlations found

366 for previously published^{14,33-35} miRNA promoter annotations (Figure S20d). About
367 11% of pri-miRNAs in human were polycistronic, containing multiple mature
368 miRNAs with highly correlated expression levels (Figure 3d and S28). Together this
369 suggests that miRNA expression is primarily regulated at the transcriptional level.

370

371 Using the CAGE expression level of the pri-miRNA as a proxy for the mature
372 miRNA expression level, we extended the FANTOM5 miRNA expression atlas to the
373 full breadth of the 1,829 (human) and 1,029 (mouse) libraries in the FANTOM5
374 CAGE expression compendium¹⁷⁻¹⁹. This allowed us to assess miRNA expression
375 also in samples for which only a CAGE library was available, covering an additional
376 49 primary cell types, 245 cell lines, 138 tissue types, and 13 time courses in human,
377 and an additional 48 primary cell types, 1 cell line, 234 tissue types, and 12 time
378 courses in mouse. A cell ontology analysis was performed using the CAGE
379 expression pattern of each human pri-miRNA (Tables S15 and S17) across 338 cell
380 ontology clusters (Table S20) encompassing 636 CAGE libraries. This showed
381 enriched expression of mir-202 in gonad, of mir-208a, known to be a key regulator of
382 cardiac function³⁷, in heart, as well as of multiple miRNAs in brain, including mir-
383 488, mir-556, and mir-885. Lastly, the CAGE data allowed us to measure the
384 individual contribution of each paralog to the expression of miRNAs encoded
385 multiple times in the human genome, providing evidence for differential regulation of
386 paralogs in different cell types and tissues. For example, we found that mir-128-1 was
387 expressed in most samples, while its paralog mir-128-2 was highly enriched in brain
388 (Figure 3e).

389

390 **Transcriptional regulation of miRNA expression**

391 The accuracy of the predicted miRNA promoter regions was assessed using the Motif
392 Activity Response Analysis (MARA) framework³⁸ (outlined in Figure S29 and
393 Methods). Using this framework, the expression levels of mature miRNAs were
394 predicted based on the presence of putative transcription factor binding sites in the
395 identified miRNA promoter region, and compared to the expression levels of the
396 mature miRNAs observed in the sRNA libraries. The prediction accuracy of the
397 FANTOM5 miRNA promoter atlas outperformed those of previously published
398 miRNA promoter annotations^{14,33-35} (Figure S20e).

399

400 **Discussion**

401 MicroRNAs are key factors that contribute to cellular regulation by targeting specific
402 transcripts for translational repression or for degradation. Advances in sequencing
403 technology led to an increase in sequencing depth from nearly 1,300 reads per sRNA
404 libraries in the first miRNA atlas³⁹ to nearly 4.4 million reads per library in
405 FANTOM5, allowing an accurate measurement of the expression even of lowly
406 expressed miRNAs. These lowly expressed miRNAs may be abundant in a few cells
407 in the population sampled, or in cell types, cell lines, or cellular conditions that are not
408 included in our sample collection. Alternatively, they may be a signature of the
409 ongoing evolution of the human miRNA repertoire. In particular, pervasive
410 transcription of mammalian genomes^{22,40,41} generates a large number of hairpin
411 secondary structures, which are prevalently encoded in the genome, that can act as
412 substrates for processing by Drosha in the nucleus and Dicer in the cytoplasm.
413 Whereas the majority of the sRNAs thus generated may be evolutionarily neutral and
414 remain lowly expressed, some of them may provide a selective advantage, develop
415 higher expression levels during evolution⁴², and become fixed in the genome as core

416 miRNAs. Finally, we note that in spite of the breadth and depth of the FANTOM5
417 sRNA sequencing data, most sRNAs currently annotated as miRNAs failed multiple
418 high-confidence criteria, and may belong to a different class of short non-coding
419 RNAs, such as transcription initiation RNAs²³ or DNA damage response RNAs⁴³, or
420 may be degradation products⁴⁴.

421

422 Compared to existing miRNA expression atlases^{29,39}, the FANTOM5 atlas covers the
423 widest range of normal primary cells, enabling detailed analyses of miRNA
424 expression and their contribution to establishing and maintaining cell type identity.
425 The candidate miRNAs not reported previously were in particular highly cell type
426 specific, and may therefore be missed in miRNA profiling studies in tissues rather
427 than in specific cell types.

428

429 We found extensive evidence that CAGE peaks observed at the Drosha cleavage site
430 are due to the downstream RNA fragment generated by Drosha processing of the pri-
431 miRNA. Analysis of these CAGE tags suggested that these RNA fragments do not
432 have a 7-methylguanosine cap, but may instead be uncapped or, alternatively, have a
433 non-canonical cap. For polycistronic pri-miRNAs, such a cap may play a role in
434 preventing rapid degradation of the downstream fragment, which itself may contain
435 miRNAs.

436

437 The MARA analysis allowed us to predict miRNA expression levels based on the
438 regulatory motifs found in the miRNA promoter region, indicating that transcriptional
439 regulation plays a central role in governing miRNA expression levels. Comparing the
440 promoters of miRNAs, protein-coding genes, and long non-coding RNAs showed

441 similar prevalences of transcription factor binding sites in proximal promoter regions
442 (data not shown), suggesting that the basic mechanisms of transcriptional regulation
443 are largely the same for these three classes of gene products. The identification of
444 miRNA promoter regions as described in this work therefore paves the way for a
445 detailed analysis of the transcriptional regulation of miRNA expression using the
446 same computational and experimental methods that have previously proven their
447 efficacy in the analysis of gene expression.

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463

464 **Authors' contributions**

465 P.A., G.Å., M.B., A.J.C., M.D., D.G., S.G., T.J.H., M.H., P.H., K.J.H., C.K., P.K.,
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467 Y.Y., C.A.W., K.M.S., A.R.R.F. provided RNA samples; E.A. and C.O.D. selected
468 samples from the FANTOM5 time courses; Y.I., S.N., and H.Ta. produced the sRNA
469 libraries; I.A., M.L., H.K., and T.K. managed the data; D.d.R., M.J.L.d.H., K.V.S.,
470 A.M.B., T.A., H.A., A.H., T.L., H.P., C.L., A.M., V.M., M.R. carried out the
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472 provided the cell ontology; K.M.S. created the Miru visualization; A.F., A.M.,

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474 M.R., N.B., P.S., R.D., V.M., Y.A.M. contributed to the manual miRNA promoter
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480 and M.J.L.d.H. designed the study; P.C. and Y.H. supervised the FANTOM5 project.

481

482 **Competing financial interests**

483 The authors declare no competing interests.

484

485

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590 from human protein-coding genes. *Nat. Struct. Mol. Biol.* **18**, 1075–1082
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- 592
593

594 **Figure 1: Selection of robust miRNAs and Drosha CAGE peak analysis.**

595 (a) Number of miRBase high-confidence rules (Table 2) satisfied by human pre-
596 miRNAs annotated in miRBase ($n = 1,871$). Pre-miRNAs with a statistically
597 significant ($P < 0.05$) Drosha CAGE peak are shown in orange; mirtrons are shown in
598 yellow. (b) Genomic locus of mir-223 in human with the total number of FANTOM5
599 (blue) and ENCODE (red) CAGE tags as a function of the genomic position of their
600 5' end, showing a Drosha CAGE peak at the 3' end of the pre-miRNA. FANTOM5
601 sRNA reads are shown at the bottom, colored by their read count as defined by the
602 color bar. The exact extent of the pre-miRNA was determined from FANTOM4 full-
603 length sequencing data²³. (c) Number of CAGE tags as a function of their starting
604 position relative to the 3' end of the pre-miRNA, averaged across human pre-miRNAs
605 in the robust set ($n = 795$). The 3' end of the pre-miRNA was selected as the 3' end of
606 the most prevalent sRNA on the 3' arm of the pre-miRNA in the FANTOM5 sRNA
607 data, with the position indicated as zero corresponding to the first nucleotide
608 downstream of the 3' end of the pre-miRNA.

609

610 **Figure 2: Expression profile and cell ontology analysis of mature miRNAs.**

611 (a) Miru²⁸ visualization of FANTOM5 primary cell samples based on their expression
612 profile of robust mature miRNAs. (b) Number of most abundant miRNAs
613 contributing at least 50% of the total miRNA expression in each human sRNA library
614 in FANTOM5 ($n = 420$). (c) Reverse cumulative distribution of the maximum
615 expression across the FANTOM5 samples of human miRNAs in the robust set ($n =$
616 735), permissive set ($n = 999$), and robust candidate set ($n = 279$). (d) Examples of
617 miRNAs enriched or depleted in specific primary cell samples. Expression of miR-
618 122-5p, miR-142-5p, and miR-302a-5p was enriched in hepatocytes, leukocytes, and

619 pluripotent stem cells, respectively; miR-100-5p and miR-29a-3p were broadly
620 expressed, but depleted in leukocytes and pluripotent stem cells, respectively.

621

622 **Figure 3: Analysis of the curated miRNA promoters of miRNAs in the robust set.**

623 **(a)** (left panel) Sequence conservation of the human genome, evaluated as the average
624 phastCons³⁶ score, in the promoter region of non-coding pri-miRNAs (containing
625 intergenic mature miRNAs; $n = 132$), coding pri-miRNAs (containing intronic mature
626 miRNAs; $n = 415$), transcription factor (TF)-coding transcripts ($n = 1,651$), other
627 protein-coding transcripts ($n = 15,350$), and long non-coding RNAs ($n = 1,461$). The
628 sequence conservation of randomly selected genome regions is shown in gray. The
629 shaded area corresponds to one standard deviation in the estimated mean phastCons
630 score. (right panel) The average sequence conservation at promoter regions of
631 miRNAs was higher than at the promoter regions of non-TF protein-coding genes
632 (Mann-Whitney $P = 2 \times 10^{-16}$, two-sided) and of long non-coding RNAs (Mann-
633 Whitney $P = 1 \times 10^{-35}$, two-sided). Error bars correspond to one standard deviation in
634 the estimated mean phastCons score. **(b)** Distance between the transcription start site
635 of the pri-miRNA and the 5' end of the first pre-miRNA is highly correlated between
636 human and mouse both for coding (Spearman $r = 0.90$; $n = 78$; Student $t = 18.27$; $P =$
637 2×10^{-29} two-sided) and for non-coding (Spearman $r = 0.86$; $n = 27$; Student $t = 8.33$;
638 $P = 1 \times 10^{-8}$ two-sided) pri-miRNAs, suggesting strong conservation of the genomic
639 extent of pri-miRNAs. **(c)** Expression levels of pri-miRNAs, as measured by CAGE,
640 and mature miRNAs, as measured by sRNA sequencing, were highly correlated both
641 for coding (average Spearman $r = 0.25$; $n = 362$; $P = 2 \times 10^{-53}$, Mann-Whitney U
642 test, one-sided) and non-coding (average Spearman $r = 0.27$; $n = 180$; $P = 1 \times 10^{-30}$,
643 Mann-Whitney U test, one-sided) pri-miRNAs, compared to a background

644 distribution consisting of correlations between randomly paired pri-miRNAs and
645 mature miRNAs. Correlations for polycistronic pri-miRNAs were averaged across the
646 mature miRNAs. **(d)** Expression levels between mature miRNAs originating from the
647 same pri-miRNA are highly correlated (average Spearman $r = 0.74$; $n = 1,372$; $P <$
648 10^{-100} , Mann-Whitney U test, one-sided), compared to a background distribution
649 consisting of correlations between mature miRNAs originating from different pri-
650 miRNAs. **(e)** Cell type-dependent expression of miRNA paralogs: While mir-128-1
651 was broadly expressed across most primary cell samples in FANTOM5, its paralog
652 mir-128-2 was enriched in brain samples. **(c-d)** The box extends from the lower to the
653 upper quartile, with the center line at the median; the whiskers indicate the full range
654 of the data.
655
656

657 **Table 1.**

658 Human sRNA data sets analyzed in this study.

Origin	Data collection	Number of samples	Number of cell types	
Primary cells	FANTOM5	293	118	119
	Fort <i>et al.</i> ¹⁹	6	3	
ES cells	Fort <i>et al.</i> ¹⁹	6	1	
iPS cells	Fort <i>et al.</i> ¹⁹	6	1	
Tissues	FANTOM5	6	4 tissues	
Time courses	FANTOM5	87	6 time courses	
Total number of sequenced reads: 1,519,621,910				

659

660 **Table 2.**

661 The miRBase high-confidence rules¹². As a meaningful evaluation of the second,
662 third, and fourth rule relies on accurate knowledge of the position and extent of the
663 mature miRNA on both strands of the pre-miRNA, we evaluated these three rules
664 only if the first rule was satisfied.

1.	≥ 10 tags on each arm of the pre-miRNA, or ≥ 100 tags on one arm of the pre-miRNA, with ≥ 5 tags on the other arm
2.	$\geq 50\%$ of the tags on each arm of the pre-miRNA have the same 5' end
3.	0–4 nt overhang at the mature 3' end on each arm
4.	$\geq 60\%$ of nucleotides of the mature sequence on each arm are base-paired
5.	$\Delta G < -0.2$ kcal/mole/nucleotide

665

666

667 **Methods**

668 ***Samples and library preparation***

669 Short RNA libraries were prepared following the Illumina TruSeq Small RNA Sample
670 Preparation protocol (catalog number RS-200-0012, RS-200-0024, RS-200-0036, RS-
671 200-0048) using the same RNA samples from which CAGE libraries were produced
672 previously^{17,18}, as well as one additional RNA sample without a matching CAGE
673 library. RNA samples not previously described are listed in Table S2. TruSeq Small
674 RNA Sample Prep Index Sequences were used as bar codes to allow pooling of
675 multiple samples in one library. The short RNA libraries were sequenced using the
676 Illumina HiSeq2000 sequencer in single-read, 50 base mode. The metadata of all
677 FANTOM5 RNA samples, including those used for sRNA sequencing, are available
678 in the FANTOM5 Semantic catalog of Samples, Transcription initiation And
679 Regulators⁴⁵ (SSTAR; <http://fantom.gsc.riken.jp/5/sstar>). SSTAR sample pages also
680 provide links to the FANTOM5 miRNA expression atlas web interface.

681

682 ***Data processing***

683 We extracted the short RNA sequences from the raw sequences using in-house scripts.
684 We removed linker artifact sequences using TagDust⁴⁶ version 1.13, ribosomal
685 sequences using rRNAdust¹⁷ version 1.00, and filtered against mature tRNAs,
686 ribosomal RNA, and 7SL RNA using global alignment. We mapped the remaining
687 sequences using the Burrows-Wheeler Alignment (bwa) tool⁴⁷ version 0.5.9-r16 to
688 genome assembly hg19 (human) or mm9 (mouse), including chromosome Y if the
689 donor was known to be male. Table S3 shows the number of short RNA sequences
690 mapped to the genome for each sample. Two samples had fewer than 100,000 mapped
691 tags and were discarded from the further analysis.

692

693 ***Short RNA annotation and filtering***

694 We used release 21 of the miRBase database¹², lifted over to genome assembly hg19
695 (human) or mm9 (mouse), as our reference set of known miRNAs. Four pre-miRNAs
696 in human that could not be lifted over to genome assembly hg19 and an additional six
697 human pre-miRNAs that were lifted over to unplaced chromosomes were excluded
698 from the analysis. We annotated all mapped short RNA reads mapping to genomic
699 loci for ribosomal RNA, tRNAs, the RNA component 7SL of the signal recognition
700 particle, small nuclear RNAs, small nucleolar RNAs, small Cajal body-specific RNA,
701 small cytoplasmic RNAs, and piRNAs. We corrected for cross-mapping as described
702 previously⁴⁸, discarding all mappings to unannotated loci if the short RNA sequence
703 could be mapped to an annotated locus instead.

704

705 ***Drosha CAGE peak analysis***

706 We calculated the total number of CAGE tags starting at each genomic position across
707 all 1,885 (human) and 1,202 (mouse) FANTOM5 CAGE libraries^{17,18}, as available at
708 <http://fantom.gsc.riken.jp/5/datafiles/latest/basic/>, as well as all 145 human ENCODE
709 CAGE data²², which we downloaded from
710 <http://hgdownload.cse.ucsc.edu/goldenpath/hg19/encodeDCC/wgEncodeRikenCage/>.
711 We defined the 3' end of the pre-miRNA as the 3' nucleotide of the mature miRNA
712 on the 3' arm of the pre-miRNA; the expected Drosha cleavage site is immediately
713 downstream of this nucleotide. For each pre-miRNA in the robust set, we calculated,
714 for each position with respect to the expected Drosha cleavage site, the total number
715 of CAGE tags in the CAGE libraries. We normalized by dividing by the sum over the
716 positions to obtain the CAGE profile with respect to the expected Drosha cleavage

717 site for each pre-miRNA. We then summed the CAGE profiles across the pre-
718 miRNAs to obtain the average CAGE profile with respect to the expected Drosha
719 cleavage site. Based on this profile, we selected a 9-basepair window between -2 and
720 +7 base pairs with respect to the expected Drosha cleavage site for the FANTOM5
721 CAGE data, and an 8-basepair window between -2 and +6 base pairs for the
722 ENCODE CAGE data, as the Drosha CAGE peak window for a given pre-miRNA.

723 For each pre-miRNA, we counted the number of CAGE tags with a 5' end within this
724 window, as well as the number of CAGE tags with a 5' end anywhere between the
725 pre-miRNA boundaries. Since CAGE tags tend to occur in clusters on the genome, we
726 expect the distribution of the CAGE tag counts to be heavily overdispersed compared
727 to the Poisson distribution. We therefore used the negative binomial distribution
728 instead, with the dispersion parameter r estimated by fitting the distribution to the
729 number of CAGE tags in any 8- or 9-basepair window on the human or mouse
730 genome. This resulted in a dispersion of 1.856943 and 1.616542 for the FANTOM5
731 human and mouse CAGE data, respectively (using a 9-basepair window), and
732 0.325001 for the ENCODE CAGE data (using a 8-basepair window). Using these
733 dispersion values, we calculated the statistical significance of the FANTOM5 and
734 ENCODE CAGE peaks given the number k of CAGE tags within the window, the
735 number K of CAGE tags within the pre-miRNA, the window size w , as well as the
736 genomic extent L of the pre-miRNA as $I_p(k, r)$, where I is the regularized incomplete
737 beta function and $p = \mu / (r + \mu)$, with $\mu = w K / L$ the expected number of tags at the
738 Drosha CAGE peak under the null hypothesis.

739 For human, we calculated an overall statistical significance value by combining the
740 FANTOM5 and ENCODE statistical significance into a single P -value using Fisher's
741 method.

742 The relative occurrence of CAGE tags in different subcellular fractionations (Figure
743 S9) and the bias in the first nucleotide of CAGE tags (Figure S12) were evaluated for
744 pre-miRNAs in the robust set with a statistically significant Drosha CAGE peak.

745

746 ***Identification of candidate novel miRNA***

747 Candidate novel miRNAs were identified using miRDeep2²⁶, resulting in 7,461
748 (human) and 2,034 (mouse) predicted pre-miRNAs, including 918 (human) and 590
749 (mouse) known pre-miRNAs. To avoid predicted miRNAs from failing the miRBase
750 high-confidence rules due to flaws in the predicted secondary structure, we repeated
751 the secondary structure calculation for each predicted miRNA by applying RNAfold⁴⁹
752 version 2.1.2 on the sequence of the precursor miRNA while constraining the
753 structure by allowing nucleotides in each arm of the hairpin to only base-pair to
754 nucleotides in the other arm of the hairpin. In the comparison of the candidate novel
755 miRNAs with the 3,524 tissue- and primate-specific miRNAs published recently²⁷, we
756 required the pre-miRNAs to overlap by at least 80%.

757

758 ***Validation of candidate novel miRNA expression by qPCR***

759 Fresh buffy coat was obtained from the Red Cross following approval from the human
760 research ethics committee of The University of Melbourne (ethics ID 1646608.1) and
761 material supply agreement with Red Cross (16-05VIC-21).

762 Peripheral blood mononuclear cells (PBMC) were isolated from buffy coat using
763 Ficoll hypaque (GE Healthcare, Uppsala, Sweden) as described previously⁵⁰. CD14+
764 human monocytes were isolated from PBMC using human CD14+ magnetic beads
765 (Milteny Biotec, Sydney, New South Wales, Australia). CD14+ monocytes were
766 differentiated to macrophages in complete RPMI1640 media supplemented with 10%

767 fetal calf serum and 100 ng ml⁻¹ human macrophage colony-stimulating factor (M-
768 CSF) (PeproTech, Rehovot, Israel) for 5 days. Suspended cells were removed and
769 adherent cells were washed with PBS before macrophages were collected.

770 MicroRNAs were isolated from monocytes and macrophages using mirVana miRNA
771 Isolation Kit (Life Technologies, Melbourne, Victoria, Australia) following the
772 manufacturer's protocol. Briefly, cells were lysed in lysis buffer followed by phenol
773 extraction, and miRNAs were isolated from the phenol aqueous phase using a spin
774 column followed by elution in RNase-free water. Following manufacturer's protocol,
775 cDNA synthesis was performed using miScript PCR Starter Kit (Qiagen, Hilden,
776 Germany) by ligating a poly(A) tail to the miRNA followed by reverse transcription
777 in the presence of universal tag. Samples without reverse transcriptase but with all
778 other components were included and used as negative controls.

779 Forward primers specific to the candidate novel miRNAs were designed using
780 miRprimer⁵¹ (Table S10). Real-time PCR was performed using miScript PCR Starter
781 Kit (Qiagen, Hilden, Germany) and following the manufacturer's protocol. The PCR
782 reaction was set up with the custom-made forward primers and the universal reverse
783 primer supplied with the kit. No-template controls and cDNA samples without reverse
784 transcriptase were included as negative controls. Thermal cycling was performed as
785 suggested by the manufacturer's protocol.

786 The expression levels of a wide range of miRNAs have been analyzed using our
787 miRNA PCR assay in order to evaluate the sensitivity of the assay and determine the
788 confidence of our results. Short RNAs commonly used as a reference, including
789 RNU6 and let-7a-5p (ref. 52), showed relatively high expression levels. Other
790 miRNAs that are highly conserved in metazoans or known to be expressed in myeloid
791 cells, including miR-191-5p (ref. 53), miR-15a-5p (ref. 54), miR-206 (ref. 55), miR-

792 335-5p (ref. 56) and miR-339-3p (ref. 56), were included and used as positive
793 controls, and showed moderate expression levels. Expression levels of miRNAs
794 reported to be cell markers for other cell types and assumed to be lowly expressed in
795 myeloid cells, including miR-153-3p (ref. 57) and miR-345-5p (ref. 58), were also
796 analyzed in order to determine the detection limit of the assay. Our results
797 demonstrate that the miRNA PCR assay could specifically detect the presence of the
798 target miRNAs, and measure a wide spectrum of expression levels. The expression
799 levels of the selected candidate novel miRNAs fell within the detection spectrum of
800 our miRNA PCR assay, proving the reliability of our results.

801

802 *Evaluation of miRNA expression normalization strategies*

803 We counted the number of short RNA sequences with a length between 18 and 25
804 nucleotides overlapping the mature miRNA loci in each of the primary cell samples.
805 We then applied the following normalization strategies:

- 806 • CPM (counts per million): Divide the count by the sum of counts for mature
807 miRNAs in the robust set, and multiply by 1,000,000;
- 808 • TMM (trimmed mean of M values): Apply the “calcNormFactors” function in
809 edgeR⁵⁹ with method “TMM” to the table of counts;
- 810 • RLE (relative log expression): Apply the “calcNormFactors” function in
811 edgeR⁵⁹ with method “RLE” to the table of counts;
- 812 • DESeq (effective library size): Apply the “estimateSizeFactorsForMatrix”
813 function in DESeq⁶⁰ to the table of counts;
- 814 • UQ (upper quantile normalization): Divide the count by the sum of the counts
815 of the top-25% most abundant miRNAs in each sample;

816 • UD (upper decile normalization): Divide the count by the sum of the counts of
817 the top-10% most abundant miRNAs in each sample.

818 To evaluate each normalization strategy, we divided the primary cell samples in
819 FANTOM5 into groups ($n = 96$) of independent donors of the same cell type. For
820 each cell type group, we calculated the variance for each miRNA across the donors.
821 To find the error between different cell types, we first calculated the average
822 expression for each miRNA across donors in each cell type group, and then calculated
823 the difference in the average expression between each pair of cell type groups ($n = \frac{1}{2} \times$
824 $96 \times 95 = 4,560$) for each miRNA. To evaluate the total error, we calculated the mean
825 squared error across miRNAs for each cell type group, as well as the mean squared
826 error across miRNAs for each pair of cell type groups, and took the square root of
827 each to find the root mean square (RMS) within cell type groups and between cell type
828 groups (Figure S16a). We averaged the RMS error over the $n = 96$ cell type groups,
829 and over the $n = 4,560$ pairs of cell type groups, and calculated the ratio of the
830 average RMS error within cell types to the average RMS error between cell types
831 (Figure S16b). To evaluate the standard error (Figure S16c), we calculated the mean
832 square error across cell type groups for each miRNA, as well as the mean squared
833 error across pairs of cell type groups for each miRNA, took the square root, and
834 plotted the resulting RMS value for each miRNA against its mean expression level.
835 We then used linear regression to calculate the slope of the RMS error within each
836 cell type and between different cell types as a function of the miRNA expression
837 level. Dividing these two slopes yielded the ratio in RMS error within cell types and
838 between different cell types, normalized by miRNA expression level (Figure S16d).

839

840 ***Clustering and visualisation of miRNA expression patterns***

841 MicroRNAs were clustered based on their expression patterns using the network
842 visualisation and analysis tool Miru²⁸ (<http://kajeka.com/miru/miru-about/>). The
843 Pearson correlation was calculated for each pair of miRNAs. A modified
844 Fruchterman-Rheingold algorithm was used to lay out the network graph in 3-
845 dimensional space, in which 502 nodes representing miRNAs were connected by
846 3,369 weighted, undirected edges representing correlations of at least 0.6 between
847 expression patterns. Areas of high connectivity and correlation, representing groups
848 of miRNAs with similar expression profiles, were identified using the Markov
849 clustering algorithm (MCL) with an MCL inflation value of 2.2. Clusters were
850 manually annotated based on the cell type or tissue of greatest expression. All nodes
851 in one cluster and the label describing the cluster are shown in the same color. The
852 smallest labeled cluster contains six nodes; for clarity, smaller clusters have not been
853 labeled but can be identified by groups of nodes of the same color.

854

855 **Validation of miRNA expression quantitation by qPCR**

856 Expression of selected miRNAs was measured using the TaqMan[®] MicroRNA Assay
857 (Applied Biosystems) according to its protocol. RNA samples 11544, 11624, 11705
858 (CD19+ B cells, donor1, 2, and 3), 11269, 11346, 11418 (dermal fibroblast donor1, 2,
859 and 3), 12626 (H9 embryonic stem cells), and 11523, 11603, 11684 (hepatocyte
860 donor1, 2, and 3) (Table S3) were used after confirmation of the RNA quality by
861 measuring the RIN value using a TapeStation and the 260/280 and 260/230 ratios
862 using NanoDrop. The Ct values obtained were normalized against the Ct value of
863 small nucleolar RNA SNORD48.

864

865 **Cell type specificity index**

866 Following the definition of the tissue specificity index (TSI)²⁹, we define the cell type
867 specificity index of miRNA j as

$$868 \quad \text{index}_j = \frac{1}{N-1} \sum_{i=1}^N \left(1 - \frac{x_{j,i}}{\max_{i'} x_{j,i'}} \right)$$

869 where N is the number of primary cell types in FANTOM5, and $x_{j,i}$ is the expression
870 in counts-per-million of miRNA j in cell type i , averaged over independent donors.

871

872 ***Guide strand selection***

873 For each pre-miRNA, we designated the hairpin arm with the highest expression level
874 (in counts-per-million) in any of the FANTOM5 samples as the guide strand, and
875 refer to the opposite arm as the passenger strand.

876

877 ***Cell ontology analysis***

878 We used the FANTOM5 cell ontology^{30,31} to create cell ontology clusters (Tables S14
879 and S20). We performed a likelihood-ratio test comparing the expression data
880 between the samples in each cell ontology cluster and the background, consisting of
881 all other samples listed in Tables S14 and S20, modeling the tag counts by a negative
882 binomial distribution. For each miRNA, we selected the three cell ontology terms for
883 which the expression in the cell ontology cluster compared to the background was
884 statistically most significantly higher, and the three cell ontology terms for which the
885 expression in the cell ontology cluster compared to the background was statistically
886 most significantly lower. The P -values listed in Tables S13, S15, and S17 for each
887 miRNA for specific cell ontology clusters were not corrected for multiple testing.

888

889 ***Identification of miRNA promoters***

890 Candidate pri-miRNAs consisted of transcripts annotated in Gencode⁶¹ v19 (human)
891 or vM5 (mouse) or in the NCBI Entrez Gene database⁶². For each pre-miRNA, we
892 selected all candidate pri- miRNAs with a transcription start site upstream of the pre-
893 miRNA and a 3' end downstream of the 5' end of the pre-miRNA, and defined all
894 FANTOM5 permissive CAGE peaks¹⁷ within the genomic region from 500 bp
895 upstream of the 5' end of the pri-miRNA to the 5' end of the pre-miRNA as the set of
896 candidate promoters associated with the pre-miRNA. We averaged the expression
897 level (in tags-per-million) of each candidate promoter across all FANTOM5 CAGE
898 samples, and selected the candidate promoter with the highest average expression
899 level as the (computationally predicted) promoter of the miRNA. Each human
900 miRNA in the robust set was manually curated by two annotators.

901

902 ***Validation of miRNA promoters by RAMPAGE***

903 We downloaded all 212 BAM files containing ENCODE RAMPAGE sequencing data
904 mapped to human genome assembly hg19 that were not marked as “low read depth”
905 or “low replicate concordance”. We retained the 5' end positions of RAMPAGE
906 transcripts with a 3' end within 1,000 basepairs of a pre-miRNA locus, discarding 5'
907 end positions supported by fewer than 5 RAMPAGE transcripts, and associated the
908 remaining 5' end positions with the pre-miRNA as putative transcription start sites.

909

910 ***Validation of miRNA promoters by RACE***

911 We mixed 4.0 µl 5X First-Strand Buffer, 0.5 µl DTT (100 mM; Invitrogen, catalog
912 number 70726), 1.0 µl dNTP Mix (20 mM), spun briefly in a microcentrifuge, and
913 kept at room temperature. We combined 1.0-10.0 µl with 1 µg total RNA from
914 monocytes, macrophages, and dendritic cells, 1.0 µl Random Primer Mix (N-15) (20

915 μM), and 0-9 μl sterile water to reach a total volume of 11.0 μl in separate
916 microcentrifuge tubes, mixed the contents and spun the tubes briefly. We incubated
917 these tubes at 72 °C for 3 minutes, and then cooled to 42 °C for 2 minutes. After
918 cooling, we spun the tubes for 10 seconds at 14,000 g to collect the contents at the
919 bottom. Next, we added 1.0 μl of Smarter oligo (20 μM) per reaction, and mixed well
920 by vortexing and spun the tube briefly in a microcentrifuge. We then added 0.5 μl
921 RNase Inhibitor (40 U/ μl ; Invitrogen RNaseOUT™, catalog number 10777019) and
922 2.0 μl SMARTScribe Reverse Transcriptase (100 U; Clontech, catalog number
923 639537) to the buffer mix, and mixed these reagents at room temperature. Next, we
924 added 8.0 μl of the master mix to the RNA solution, mixed the contents of the tubes
925 by gently pipetting, and spun the tubes briefly. We incubated the tubes at 42 °C for 90
926 minutes and heated the tubes at 70 °C for 10 minutes in a hot-lid thermal cycler. We
927 then added 90 μl Tricine-EDTA buffer to each tube.

928 We prepared the master mix for the first PCR by combining 2.5 μl of the cDNA
929 solution, 5.0 μl 10X Advantage 2 PCR buffer (Clontech, catalog number 639207), 1.0
930 μl dNTP Mix (10mM each) 50X Advantage 2 Polymerase Mix (Clontech), 1.0 μl of
931 the smarterRACE_forward primer at 10 pmol/ μl , 1.0 μl of the miRNA-specific outer
932 primer (Table S18) at 10 pmol/ μl , and added PCR-grade water to reach a volume of
933 50 μl . We ran a 2-step PCR program consisting of 1 minute at 95 °C, 25 cycles of 30
934 seconds at 95 °C followed by 70 seconds at 68 °C, 7 minutes at 68 °C, and finishing
935 at 8 °C. We diluted 5 μl of the primary PCR product into 245 μl of Tricine-EDTA
936 buffer.

937 We prepared the master mix for the second PCR by combining 5.0 μl of the product
938 of the first PCR after dilution with 5 μl of the 10X Advantage 2 PCR buffer, 1.0 μl
939 dNTP Mix (10 mM), 1.0 μl of 50X Advantage 2 Polymerase Mix (Clontech), 2.0 μl of

940 the Nextera_i7 primer, 2.0 µl of the miRNA-specific inner primer (Table S18), and 34
941 µl of PCR-grade water. We ran a 2-step PCR program consisting of 1 minute at 95 °C,
942 20 cycles of 30 seconds at 95 °C followed by 70 seconds at 68 °C, 7 minutes at 68 °C,
943 and finishing at 8 °C. We diluted 5 µl of the PCR product into 245 µl of Tricine-
944 EDTA buffer.

945 We prepared the master mix for the third PCR by combining 5.0 µl of the PCR
946 product of the second PCR with 5.0 µl of 10X Advantage 2 PCR buffer, 1.0 µl dNTP
947 mix (10 mM), 1.0 µl of 40X Advantage 2 Polymerase Mix (Clontech), 2.0 µl of the
948 Nextera_i7 primer, 2.0 µl of the Nextera_i5 primer, and 34 µl of PCR-grade water.
949 We purified by AMPure at a 1.8 ratio, checked 2 µl of the second PCR product on a
950 TapeStation, kept the libraries at -20 °C until sequencing, and pooled the PCR
951 products, each with a different barcode combination before paired-end sequencing on
952 a MiSeq sequencer (Illumina).

953 We mapped the sequencing data to the human genome using Blat⁶³, merged each pair
954 into a single mapped transcript, and retained transcripts that overlap an inner primer.
955 The histograms in Figure S21 show the position of the 5' end of these transcripts.

956

957 ***Promoter sequence conservation analysis***

958 We previously compiled a list of transcription factors in human and mouse¹⁷. Protein-
959 coding genes and lncRNAs consisted of all other genes annotated in the NCBI Entrez
960 Gene database⁶² as protein coding or miscRNA, respectively. For each gene in these
961 three categories, we selected the associated p1 CAGE peak as defined previously¹⁷ as
962 the gene promoter, and discarded all genes without an associated CAGE peak. We
963 then found the phastCons conservation score³⁶, obtained from the UCSC Genome
964 Browser database⁶⁴, for the alignment of 99 vertebrate organisms against the human

965 genome hg19, as a function of position relative to the transcription start site for each
966 gene and miRNA, and averaged these scores for each category at each position.

967

968 **Construction of the FANTOM5 miRNA expression atlas of miRNAs**

969 CAGE tag start site (CTSS) files³¹, excluding universal and whole body RNA
970 samples, were downloaded from <http://fantom.gsc.riken.jp/5/datafiles/latest/basic/>.

971 CAGE tag counts for technical replicates of the same RNA sample were summed for
972 each genomic position. CAGE libraries published by Fort *et al.*¹⁹ were downloaded
973 from DDBJ, accession DRA000914. The number of CAGE tags at each genome
974 position were counted to generate CTSS files, and pri-miRNA expression tables were
975 generated by summing the CAGE tags under each promoter, calculating the total
976 number of tags mapped to the genome, and using this number to normalize to tags per
977 million (tpm).

978 Mature miRNA expression tables were generated by counting the number of sRNA
979 tags to each miRNA locus, calculating the total number of tags mapping to the robust
980 miRNAs, and using this number to normalize to counts per million (cpm).

981 The CAGE and sRNA expression tables are available for download at the miRNA
982 expression viewer at

983 http://fantom.gsc.riken.jp/5/suppl/De_Rie_et_al_2017/

984 To generate the heatmap, we averaged the cpm-normalized expression values of each
985 miRNA across donors for each cell type, and converted the expression profile of each
986 miRNA to Z-scores by subtracting the mean and dividing by the standard deviation
987 across cell types. The heatmap was sorted both for cell types and for miRNAs by
988 performed centroid-linkage hierarchical clustering, using the Pearson correlation as
989 the similarity measure.

990

991 ***Motif activity response analysis (MARA)***

992 The genome-wide predictions of transcription factor binding sites were produced as
993 described previously¹⁸. Briefly, we downloaded the whole-genome alignment of the
994 human genome hg19 against 99 other vertebrate genomes, and of the mouse genome
995 mm9 against 29 other vertebrate genomes, from the UCSC Genome Browser
996 database⁶⁴, and extracted the multiple alignments of human, macaque, mouse, rat,
997 cow, horse, dog, opossum, and chicken. We divided the genome into segments and
998 realigned each segment using T-Coffee⁶⁵, and generated genome-wide transcription
999 factor binding site (TFBS) predictions using MotEvo⁶⁶ for the SwissRegulon set of
1000 position-weight matrix motifs⁶⁷ (Figure S29a). We then counted the number of
1001 predicted TFBSs for each motif in the -300 to +100 base pair base proximal promoter
1002 regions of genes in the NCBI Entrez Gene database⁶², excluding all miRNA
1003 promoters (Figure S29b). Next, we used MARA³⁸ to decompose the FANTOM5
1004 CAGE expression profiles of these promoters in terms of their associated motifs,
1005 yielding the activity profile of each motif across the FANTOM primary samples
1006 (Figure S29c). We then counted the number of TFBSs for each motif in the -300 to +
1007 100 base pair base proximal promoter region of each miRNA (Figure S29d), and
1008 predicted the miRNA expression level by calculating the weighted sum of the
1009 activities for motifs found (Figure S29e). We compared the predicted expression
1010 levels to the expression levels of the mature miRNA observed in the FANTOM5
1011 sRNA sequencing data (Figure S29f) and calculated their correlation (Figure S29g) as
1012 a measure of the accuracy of the miRNA promoter identification. Following the
1013 MARA procedure³⁸, we normalized the cpm expression values of miRNAs by adding
1014 0.5, taking the base-2 logarithm, subtracting the mean across samples, and finally

1015 subtracting the mean across miRNAs. We defined strongly differentially expressed
1016 miRNAs, included in Figure S20d and S20e, as those that had a standard deviation in
1017 expression, after normalization, across samples larger than 2.

1018

1019 ***Data availability***

1020 Raw sequencing data of the sRNA libraries are available at the DNA Data Bank of
1021 Japan (DDBJ; <http://www.ddbj.nig.ac.jp/>) under accession numbers DRA001101,
1022 DRA002711, DRA003804, and DRA003807, and for the RACE experiments at the
1023 NCBI Gene Expression Omnibus (NCBI GEO; <https://www.ncbi.nlm.nih.gov/geo/>)
1024 under accession number GSE98695.

1025

1026 **Supplemental references**

1027

1028

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