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1 **MicroRNAs facilitate skeletal muscle maintenance and metabolic suppression in**
 2 **hibernating brown bears**

3
 4 Short title: MyomiRs in hibernating brown bears

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34
 35 **Author contributions**

36 All authors contributed to the conception and design of the project and to the editing of the
 37 manuscript. EL, SG, JES, BC, GG, JMA, ALE, and FB conducted the brown bear experiments
 38 and provided the tissue samples. BEL and KBS conducted biochemical assays. Data analysis and
 39 assembly of the manuscript was carried out by BEL, FB, and KBS. All authors read and
 40 approved the final manuscript.

41 **Data availability statement**

42 Research data are not shared.

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55 Abstract

56 Hibernating brown bears, *Ursus arctos*, undergo extended periods of inactivity and yet
57 these large hibernators are resilient to muscle disuse atrophy. Physiological characteristics
58 associated with atrophy resistance in bear muscle have been examined (e.g. muscle mechanics,
59 neural activity) but roles for molecular signalling/regulatory mechanisms in the resistance to
60 muscle wasting in bears still require investigation. Using RT-qPCR, the present study
61 characterized the responses of 36 microRNAs linked with development, metabolism, and
62 regeneration of skeletal muscle, in the vastus lateralis of brown bears comparing winter
63 hibernating and summer active animals. Relative levels of mRNA of selected genes (*mef2a*,
64 *pax7*, *id2*, *prkaa1*, and *mstn*) implicated upstream and downstream of the microRNAs were
65 examined. Results indicated that hibernation elicited a myogenic microRNA, or “myomiR”,
66 response via MEF2A-mediated signalling. Upregulation of MEF2A-controlled miR-1 and miR-
67 206 and respective downregulation of *pax7* and *id2* mRNA are suggestive of responses that
68 promote skeletal muscle maintenance. Increased levels of metabolic microRNAs, such as miR-
69 27, miR-29, and miR-33, may facilitate metabolic suppression during hibernation via
70 mechanisms that decrease glucose uptake and fatty acid oxidation. This study identified
71 myomiR-mediated mechanisms for the promotion of muscle regeneration, suppression of
72 ubiquitin ligases, and resistance to muscle atrophy during hibernation mediated by observed
73 increases in miR-206, miR-221, miR-31, miR-23a, and miR-29b. This was further supported by
74 the downregulation of myomiRs associated with muscle injury and inflammation (miR-199a and
75 miR-223) during hibernation. The present study provides evidence of myomiR-mediated
76 signalling pathways that are activated during hibernation to maintain skeletal muscle
77 functionality in brown bears.

78 **Keywords:** atrophy; Mef2a; myomiR; noncoding RNA; ubiquitin ligase; *Ursus arctos*;

79

80 Introduction

81 Hibernators are able to endure extreme environmental conditions that cannot be tolerated
82 by most mammals. Typically, for small mammal hibernators, seasonal torpor is characterized by
83 a prolonged state of inactivity, a strong decrease in core body temperature to near-ambient
84 values, and the suppression of basal metabolic rate to levels often less than 10% of euthermic
85 values (Heldmaier, Ortmann, & Elvert, 2004; Jastroch et al., 2016; Ruf & Geiser, 2015; Storey &
86 Storey, 2010). However, the iconic hibernators, bears (*Ursidae*), stand out from these others in
87 several characteristics. Their large body mass makes it difficult to substantially decrease their
88 body temperature such that core body temperature remains at 30-35°C during hibernation and
89 they have the impressive ability to endure up to 6 months of inactivity without arousing and with
90 no food or water intake, no urination or defecation, no muscle wasting, and no accumulation of
91 nitrogen-containing waste products due to an active recycling program (Berg von Linde,
92 Arevström, & Fröbert, 2015). Previous studies of bears also showed no statistically significant
93 changes in skeletal muscle fiber size between hibernating and non-hibernating animals (Hershey,
94 Robbins, Nelson, & Lin, 2008; Tinker, Harlow, & Beck, 1998). In mammals, weight-bearing
95 activities are crucial for the maintenance of skeletal muscle mass, morphology, and protein
96 composition (Baldwin & Haddad, 2001). Yet despite drastically reduced weight-bearing activity
97 during hibernation, the skeletal muscle of bears retains most of its strength, with only a 23%
98 decrease in maximal force (elicited by electrical stimulation) (Harlow, Lohuis, Beck, & Iaizzo,
99 2001), whereas reduction in force and size as high as 60% is common in other non-hibernating
100 mammals (Baldwin & Haddad, 2001). In small hibernating mammals, up to 20-40% of muscle
101 protein content is lost at low body temperature (Steffen, Koebel, Musacchia, & Milsom, 1991;
102 Wickler, Hoyt, & van Breukelen, 2017; Yacoe, 1983). However, hibernating bears lose only 4–
103 11% of muscle protein content, and the size and number of their muscle fibres remain unchanged
104 (Tinker et al., 1998).

105 In an attempt to better understand these observations, another study investigated the
106 importance of the signalling pathways that underlie the resilience of skeletal muscle in
107 hibernating brown bears (*Ursus arctos*). The authors challenged the concept that mechanical
108 loading and neural activity of the muscles are the primary factors in maintaining skeletal muscle
109 size (Lin, Hershey, Mattoon, & Robbins, 2012). Indeed, studies on other non-hibernating
110 mammals have demonstrated that regular neural activation or electrical stimulation of muscles is
111 not enough to curtail muscle atrophy induced by mechanical unloading (Haddad, 2006; B. T.
112 Zhang et al., 2010). Given that there is decreased muscle loading during hibernation, these
113 authors studied the differences between the skeletal muscles (long distal extensor and cranial
114 tibial) of hibernating and non-hibernating *U. arctos* that had undergone transections of the
115 common peroneal nerve (Lin et al., 2012). Their results showed that active brown bears with
116 denervation exhibited significantly higher muscle mass loss as compared to hibernating bears
117 with denervation (Lin et al., 2012). The authors therefore suggested that a third factor is
118 involved, proposing that differential regulation of signalling pathways, such as those that mediate

119 the ubiquitin-mediated catabolism of contractile proteins, must be involved in atrophy resistance
120 (Lin et al., 2012).

121 The observed resistance to muscle atrophy in hibernating bears could be elicited by
122 signalling pathways that mediate changes in regulatory microRNAs. MicroRNAs are a class of
123 highly conserved small non-coding RNAs that are capable of mediating the fate of mRNA
124 transcripts in response to diverse signalling pathways in cells (Bartel, 2004; Storey & Storey,
125 2010). As a component of RNA-induced silencing complexes, microRNAs contribute to post-
126 transcriptional regulation by binding to complementary mRNAs to promote their degradation or
127 inhibit their translation into functional proteins. MicroRNAs are differentially regulated in a
128 number of small mammal hibernator models in a tissue specific manner, contributing to
129 metabolic control, antioxidant defenses, and muscle maintenance networks during hibernation
130 (Hadj-Moussa et al., 2016; Kornfeld, Biggar, & Storey, 2012; Luu, Biggar, Wu, & Storey, 2016;
131 Storey & Storey, 2010; Wu, Biggar, & Storey, 2014) but, as yet, have not been evaluated in the
132 context of bear hibernation. One direct regulator of skeletal muscle microRNA (myomiR)
133 expression is the myocyte enhancer factor-2A (MEF2A) transcription factor, and its targets
134 include miR-1, miR-133, and miR-206 (Liu et al., 2007; Rao, Kumar, Farkhondeh, Baskerville,
135 & Lodish, 2006). The MEF2A transcription factor is known to be crucial for skeletal muscle
136 development, maintenance, and regeneration (Taylor & Hughes, 2017). Previous studies have
137 demonstrated that MEF2A signalling is activated in the skeletal muscle of thirteen-lined ground
138 squirrels (*Ictidomys tridecemlineatus*) during hibernation and may contribute to muscle
139 maintenance (Tessier & Storey, 2010). Likewise, a study that profiled 10 microRNAs known to
140 affect muscle-specific factors revealed that expression of these small non-coding transcripts are
141 also differentially regulated in favour of skeletal muscle maintenance of hibernating bats (*Myotis*
142 *lucifugus*) (Kornfeld et al., 2012).

143 The present study explores whether signalling pathways that impede muscle wasting are
144 activated during hibernation in *U. arctos*. To address this, *U. arctos* mRNA and microRNA gene
145 transcript sequences were obtained from available genomic data. RNA was isolated from skeletal
146 muscle biopsies of 6 brown bears that were sampled during summer active and winter
147 hibernating conditions. Specifically, mRNA transcript levels of MEF2A were measured by RT-
148 qPCR along with a group of 36 myomiRs that are known to control skeletal muscle regeneration,
149 metabolism and/or be under the control of MEF2A (Diniz & Wang, 2016; Horak, Novak, &
150 Bienertova-Vasku, 2016; Lima et al., 2017). Furthermore, selected downstream mRNA targets
151 including myostatin (*mstn*), AMP-activated protein kinase (AMPK) catalytic subunit alpha 1
152 (*prkaa1*), paired box protein 7 (*pax7*), and inhibitor of DNA 2 (*id2*) that are known targets of the
153 subset of microRNAs assessed herein were also quantified to better understand the potential
154 roles of these microRNAs during bear hibernation.

155

156

157 **Materials and Methods**

158 Sampling of bear muscle

159 Six free-ranging brown bears (*Ursus arctos*; females; 2-3 years old), fitted with GPS collars,
160 have been followed by the Scandinavian Brown Bear Research Project (SBBRP) in the Dalarna
161 and Gävleborg counties, Sweden. Their immobilization was performed as previously reported
162 (Evans et al., 2012), to allow sampling during hibernation in late February 2015 or 2016 and
163 again during the active summer period in early June of the same years. After darting in their den
164 (winter) or from a helicopter (summer), immobilized individuals were moved onto an insulated
165 blanket prior to tissue sampling. Biopsies of the vastus lateralis muscle were collected and
166 immediately frozen on dry ice before transfer to storage at -80°C until analysis.

167
168 The mean body mass of hibernating bears (46.8 ± 8.7 kg; 25-74 kg) was not significantly
169 different ($p=0.062$; paired t-test) from that of the same bears when resampled as summer-active
170 animals (54.4 ± 7.3 kg; 37.8-83.6 kg).

171
172 The study was approved by the Swedish Ethical Committee on Animal Experiment (applications
173 #C212/9, #C47/9, #C7/12, #C268/12, and #C18/15), the Swedish Environmental Protection
174 Agency (NV-0758-14), and the Swedish Board of Agriculture (31-11102/12). All procedures
175 complied with Swedish laws and regulations.

176 Total RNA isolation

177 Total RNA was isolated from frozen tissue using TRIzol reagent (Invitrogen, Carlsbad,
178 CA), as previously described (Luu, Green, Childers, Holahan, & Storey, 2017). Briefly, samples
179 of frozen bear muscle (~50 mg) were homogenized 1:20 w/v in TRIzol reagent and then
180 combined with 300 μ L of chloroform. Samples were vigorously shaken by hand and incubated at
181 room temperature for 5 min before centrifugation at 10,000 $\times g$ for 15 min at 4°C. The aqueous
182 phase of each sample was then combined with 500 μ L isopropanol, incubated at room
183 temperature for 15 min, and then centrifuged at 12,000 $\times g$ for 15 min at 4°C to facilitate RNA
184 precipitation. RNA pellets were washed with 70% ethanol and air dried prior to resuspension in
185 autoclaved RNase-free water. To standardize concentrations across samples, RNA concentration
186 and purity (260/280 nm ratio) was assessed using a BioTek Take3 plate in a spectrophotometer
187 (BioTek, Winooski, VT). Integrity of RNA was assessed by the appearance of 28S and 18S
188 ribosomal RNA bands on a 1% agarose gel stained with SYBR Green dye.

189 RNA polyadenylation and cDNA synthesis

190 For analysis of microRNAs, samples of bear muscle RNA were polyadenylated and then
191 cDNA synthesis was conducted as previously described (Biggar, Wu, & Storey, 2014) using a
192 Poly(A) polymerase tailing kit (Epicentre, Madison, WI) with a modified adapter primer
193 (**Supplementary Table S1**) for use with short RNA molecules.

194 Synthesis of cDNA was performed separately for mRNA quantification as previously
 195 described (Holahan, Smith, Luu, & Storey, 2018). Standard oligo-dT (5'-
 196 TTTTTTTTTTTTTTTTTTTTTT-3'; where V= A, G, or C) was combined with RNA (1 µg) in
 197 a total volume of 10 µL of RNase-free water. Samples were incubated at 65°C for 5 min in an
 198 Eppendorf thermocycler, and then cooled on ice. Next, 4 µL of 5X first strand buffer, 2 µL of 0.1
 199 M DTT, 1 µL of 25 mM dNTPs, and 1 µL of MMLV reverse transcriptase enzyme (Invitrogen)
 200 were combined with the RNA/oligo-dT samples. Reactions were incubated at 42°C for 60 min in
 201 a thermocycler to facilitate cDNA synthesis.

202 Primer design

203 MicroRNAs investigated in this study were selected from the literature based on their
 204 expression in skeletal muscle and their roles in regulating muscle cell processes (Diniz & Wang,
 205 2016; Horak et al., 2016; Lima et al., 2017). In order to determine the equivalent microRNA
 206 sequences in *U. arctos*, *Mus musculus* pre-microRNA sequences were obtained from miRBase
 207 (miRBase v.22; <http://www.mirbase.org>). Each *M. musculus* pre-microRNA sequence was
 208 aligned against the *U. arctos* WGS genome (SRA Accession No. SRX156136) using Sequence
 209 Read Archive Nucleotide BLAST (NCBI SRA BLAST; <https://www.ncbi.nlm.nih.gov/sra>). The
 210 conserved *U. arctos* pre-microRNA sequences were then aligned against *M. musculus* mature
 211 microRNA sequences from miRBase using Clustal Omega (EMBL-EBI;
 212 <https://www.ebi.ac.uk/Tools/msa/clustalo/>). From these alignments, the sequences of mature *U.*
 213 *arctos* microRNA were determined. Primers for relative microRNA quantification were designed
 214 as previously described (Biggar et al., 2014) and are listed in **Supplementary Table S1**.

215 Relative quantification of mRNA levels was performed for genes that are both upstream
 216 and downstream of microRNA regulatory pathways. Transcript levels of *mef2a*, *mstn*, *pax7*,
 217 *prkaa1*, and *id2* were assessed. In order to design primers for these targets, the coding sequence
 218 of each gene was obtained from NCBI Gene (<https://www.ncbi.nlm.nih.gov/gene/>) for multiple
 219 mammals (*U. maritimus*, *H. sapiens*, *M. musculus*, *S. scrofa*, *C. lupus*, *F. catus*, *O. aries*, and *E.*
 220 *caballus*). These were aligned with Clustal Omega to determine highly conserved regions, for
 221 which primers were designed for gene expression quantification for *U. arctos* samples.
 222 Sequences deemed highly conserved and used for primer design had 100% conservation with at
 223 least 4 different mammals. Primers were further analyzed with NCBI Primer-BLAST
 224 (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to demonstrate that they would amplify the
 225 target gene in multiple mammals. All mRNA primers used in this study are listed in
 226 **Supplementary Table S1**.

227 RT-qPCR

228 Relative quantification of gene transcripts used a BioRad CFX96 Connect System
 229 (BioRad, Hercules, CA). Reagents for RT-qPCR were prepared as previously described
 230 (Pellissier, Glogowski, Heinemann, Ballivet, & Ossipow, 2006). Briefly, each amplification

231 reaction included 2 μ L of 10x buffer (100 mM Tris-HCl, pH 8.5, 500 mM KCl, 1.5% Triton X-
232 100, 20 mM MgCl₂, and 2 mM dNTPs), 6 μ L of 2 M trehalose (BioShop, Canada), 0.5 μ L of
233 100% formamide (BioShop, Canada), 0.1 μ L of 1:100 SYBR green dye (Invitrogen), 1 μ L (1U)
234 of *Taq* polymerase (BioShop, Canada), primers (synthesized by Integrated DNA Technologies,
235 250 nM final concentration), and autoclaved water to a total volume of 20 μ L. Each RT-qPCR
236 reaction was analyzed with a melt curve analysis to ensure that the reaction was not amplifying
237 more than one product and primers that amplified multiple products were not used for
238 quantification. For relative quantification of microRNAs, reactions were brought to 95°C for 3
239 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, as previously described
240 (Biggar et al., 2014). Relative quantification of mRNAs used a modified protocol, where
241 thermocycler conditions were 50 cycles of 95°C for 10 sec, 57°C for 20 sec, and 72°C for 20 sec.
242 Standard curves were performed with pooled cDNA samples and primer sets in order to assess
243 the reaction specificity and efficiency for each gene transcript, and the efficiency of the primer
244 pair was always used for quantification calculations.

245 Statistical analyses

246 Since sampling of tissues was done in a paired manner where each bear was sampled
247 (and tagged) in the winter and then sampled again the following summer, paired *t*-tests were used
248 to determine statistically significant differences ($p < 0.05$) in microRNA and mRNA levels.
249 Amplified transcripts were normalized against the expression of reference genes (*U6* small
250 nuclear RNA) for microRNA and *gapdh* for mRNA). The expression of these reference genes
251 was determined to be stable between summer and winter hibernation conditions, as instructed
252 (Schmittgen & Livak, 2008). The comparative $\Delta\Delta$ Ct method was used for relative expression
253 calculations (Schmittgen & Livak, 2008). Expression values for microRNA and mRNA were
254 presented as mean \pm SEM with $n = 6$ bears for microRNA analysis and $n = 5$ bears for mRNA
255 analysis). SigmaPlot v.12.5 was used for statistical analysis and bar graph generation (Systat
256 Software Inc., San Jose, CA).

257 **Results**

258 Relative mRNA transcript levels of selected genes involved in the MEF2A signalling
259 pathway were quantified via RT-qPCR to assess their transcriptional state in *U. arctos* vastus
260 lateralis muscle during hibernation, as compared with the summer active state of the same
261 individuals. Transcript levels of *mef2a* were 2.35 ± 0.26 fold higher during hibernation, relative to
262 summer controls (Figure 1A). Seven microRNAs under MEF2A control (miR-1a-3p, miR-1a-1-
263 5p, miR-1a-2-5p, miR-1b-5p, miR-133b-5p miR-133a-3p, and miR-206-3p) were also
264 quantified. From this group, the levels of miR-1b-5p, miR-133a-3p, and miR-206-3p increased
265 by 1.43 ± 0.19 , 1.40 ± 0.18 , and 1.65 ± 0.17 fold during hibernation, respectively (Figure 1B). The
266 other four myomiRs were not responsive to hibernation. Two downstream targets of miR-1 and
267 miR-206 were assessed with RT-qPCR to determine the potential impact of microRNA
268 upregulation during hibernation (J.-F. Chen et al., 2010; Dey, Gagan, & Dutta, 2011). Relative

269 transcript levels of *pax7* and *id2* decreased to 0.67 ± 0.07 and 0.40 ± 0.06 of summer active levels
 270 (Figure 1C).

271 Eighteen other microRNAs known for their regulatory roles in skeletal muscle (Lima et
 272 al., 2017) were also assessed in vastus lateralis muscle of active versus hibernating brown bears.
 273 Of these microRNAs, there were six that showed higher levels in hibernation. These were miR-
 274 27b-5p, miR-27a/b-3p, miR-29a-5p, miR-29a-3p, miR-29b/c-3p, and miR-33-5p that showed
 275 significantly higher levels by 1.65 ± 0.25 , 2.31 ± 0.36 , 1.30 ± 0.12 , 2.18 ± 0.18 , 2.60 ± 0.18 , and
 276 1.24 ± 0.08 fold, respectively, in hibernating versus summer active bears (Figure 2A). To
 277 investigate the potential consequences of these increases in miR-33 (Davalos et al., 2011;
 278 Rottiers et al., 2011), relative levels of *prkaa1* mRNA were quantified in bear muscle, but no
 279 changes were found between active and hibernating states (Figure 2B).

280 A subset of eleven myomiRs that are implicated in the atrophy and regeneration of
 281 skeletal muscles (Diniz & Wang, 2016) were also assessed. Specifically, miR-23a-5p, miR-
 282 23a/b-3p, miR-125b-3p, miR-199a-5p, miR-199a-3p, miR-221-3p, miR-222-5p, miR-222-3p,
 283 miR-223-5p, miR-223-3p, and miR-31-5p, were analyzed. Three microRNAs, miR-23a-5p, miR-
 284 221-3p, and miR-31-5p were elevated during hibernation by 1.32 ± 0.12 , 1.31 ± 0.11 , and
 285 1.26 ± 0.09 fold, respectively (Figure 3A). Oppositely, miR-199a-5p and miR-223-5p decreased
 286 to 0.66 ± 0.04 and 0.70 ± 0.06 , respectively, of the levels in summer active bears (Figure 3A).
 287 Separately, transcripts of the myostatin gene, *mstn*, a mRNA target implicated in skeletal muscle
 288 maintenance (Miretti, Martignani, Accornero, & Baratta, 2013) and a target of miR-27b (Figure
 289 2A) displayed no changes between summer active and hibernation conditions (Figure 3B).

290

291 Discussion

292 Studies on brown bears (*U. arctos*) have suggested that the skeletal muscle resilience of
 293 these mammalian hibernators is determined by more than just mechanical loading and neural
 294 activity (Lin et al., 2012). A third factor proposed to contribute to the resilience of hibernator
 295 skeletal muscles is the modulation of signalling pathways, such as those that may be implicated
 296 in muscle catabolism. The present study explores muscle-specific cellular signalling pathways
 297 that are highly conserved in mammals. A group of 36 myomiRs known to be crucial for skeletal
 298 muscle development, metabolism, and regeneration (Diniz & Wang, 2016; Horak et al., 2016;
 299 Lima et al., 2017) and 5 key upstream and downstream mRNA transcripts were investigated in
 300 vastus lateralis muscle from winter hibernating versus summer-active bears. The results reveal
 301 that microRNAs and mRNAs are differentially regulated in hibernating bears in a manner that
 302 would facilitate decreased metabolism, and maintenance of skeletal muscle over the winter
 303 months of torpor.

304 The observed higher levels of *mef2a* mRNA during hibernation suggest that, in a period
 305 when the bear is enduring prolonged inactivity without food or water intake (Berg von Linde et

306 al., 2015), muscle mass (and strength) is still maintained; ie. little or no atrophy occurs (Figure
307 1A). Although analyses of MEF2A transcription factor activity were not performed in this study,
308 higher *mef2a* mRNA (compared with summer) suggests that MEF2A protein abundance would
309 also be higher during hibernation, which could result in more MEF2A-mediated gene
310 transcription. Indeed, the MEF2A transcription factor is well known for its role in skeletal
311 muscle development, maintenance, and regeneration (Taylor & Hughes, 2017), and its
312 upregulation at the mRNA and protein levels have been shown to facilitate skeletal muscle
313 maintenance during hibernation in ground squirrels (Tessier & Storey, 2010, 2012). Therefore,
314 enhanced *mef2a* transcript levels may be an important underlying mechanism of skeletal muscle
315 resilience in hibernating *U. arctos*.

316 Downstream targets of the MEF2A transcription factor were also assessed in order to
317 elucidate the activated networks in skeletal muscles of bears during hibernation. MEF2A is
318 known to directly regulate skeletal muscle microRNAs, which includes miR-1, -133, and -206
319 (Liu et al., 2007; Rao et al., 2006) and thereby has important roles in the regulation of skeletal
320 muscle metabolism (Horak et al., 2016). The present study found that miR-1b-5p, miR-133a-3p,
321 and miR-206-3p all increased during hibernation (Figure 1B), which is consistent with the
322 observed increase of *mef2a* transcripts (leading to elevation of MEF2A protein). Downstream
323 consequences on myogenic signalling pathways by miR-1 and miR-206 include downregulation
324 of the *pax7* gene, that encodes a transcription factor that is responsible for upregulating *id2*
325 mRNA (J.-F. Chen et al., 2010; Dey et al., 2011). In turn, the ID2 protein is responsible for
326 downregulating the myogenic transcription factor MYOD1 that, similarly to MEF2A, has a role
327 in facilitating skeletal muscle growth and development through broad roles including the
328 expression of miR-1 and miR-206 (Horak et al., 2016). Together, this suggests that activation of
329 the MEF2A-mediated signalling pathway in hibernating bear skeletal muscle occurs through the
330 upregulation of miR-1 and miR-206 and consequent downregulation of *pax7* and *id2* (Figure
331 1C).

332 Analyses of other microRNAs in hibernating *U. arctos* included a subset of fifteen
333 microRNAs that target metabolic processes (Figure 2A) (Lima et al., 2017). Of these, six (miR-
334 27b-5p, miR-27a/b-3p, miR-29a-5p, miR-29a-3p, miR-29b/c, and miR-33-5p) were elevated
335 during hibernation, compared with the summer active condition. The increases in miR-27, miR-
336 29, and miR-33 found in hibernating bears suggest that these microRNAs may be potentially
337 facilitating decreased glucose utilization. In a previous study, miR-27 action was shown to
338 decrease glucose consumption when overexpressed in rat myoblast cell lines, and this was
339 associated with decreased GLUT4 protein levels (Zhou et al., 2016). Indeed, when antisense
340 sequences were introduced to suppress endogenous miR-27 activity in these cell lines, glucose
341 consumption and uptake were enhanced and GLUT4 protein levels increased (Zhou et al., 2016).
342 Thus, it can be postulated that elevated miR-27 in hibernating bears may be downregulating
343 GLUT4 protein in skeletal muscle. In another study, a role for miR-29a/b/c in skeletal muscle
344 glucose metabolism was indicated since these microRNAs were upregulated in a model of

345 diabetic rats (He, Zhu, Gupta, Chang, & Fang, 2007). Further experiments with cell lines showed
346 that overexpression of miR-29a/b/c inhibited insulin-stimulated glucose uptake (He et al., 2007).
347 These previous studies support the notion that increased miR-27 and miR-29 in hibernating bear
348 muscle facilitate decreased glucose metabolism in part by reducing glucose uptake, which may
349 also contribute to lowering basal metabolic rate.

350 Levels of *prkaa1* mRNA (that encodes the catalytic subunit of the nutrient sensor
351 AMPK) were also measured in hibernating *U. arctos* skeletal muscle since expression of this
352 gene known to be downregulated by miR-33 (Davalos et al., 2011; Rottiers et al., 2011). Skeletal
353 muscle AMPK has several regulatory roles in glucose and lipid metabolism, two of these being
354 to increase glucose uptake and promote fatty acid oxidation as a means of enhancing ATP
355 production under energy-stressed conditions (Collier, Bruce, Smith, Lopaschuk, & Dyck, 2006;
356 Jeon, 2016; O'Neill et al., 2014). As mentioned above, increases in miR-27, miR-29, and miR-33
357 in bear muscle may suggest that both glucose uptake and fatty acid oxidation are suppressed in
358 hibernating bears. Thus, levels of muscle *prkaa1* mRNA were assessed to determine how this
359 downstream gene of microRNA action responded in hibernation. However, mRNA levels of
360 *prkaa1* did not change in muscle of hibernating bears, compared with summer active animals,
361 suggesting that AMPK is not differentially regulated in summer vs winter, at least at the
362 transcript level (Figure 2B). AMPK activity increased in some tissues of other hibernating
363 species (e.g. in adipose of hibernating ground squirrels) during hibernation but was unchanged in
364 other tissues including skeletal muscle (Horman, Hussain, Dilworth, Storey, & Rider, 2005), an
365 observation that is consistent with the present result for bears. It is important to note that the
366 present study only assessed the mRNA levels of the catalytic subunit of AMPK in bear muscle,
367 so further investigations are warranted to obtain a more complete understanding of the role of
368 this energy/nutrient sensor in hibernating bears. It is still possible that hibernating bears are
369 conserving energy by reducing both usage of glucose and fats. Recent studies have shown that
370 enzymes involved in fatty acid oxidation are decreased in the skeletal muscle of hibernating
371 bears, which is consistent with decreased energy expenditure (Chazarin et al., 2019). Overall,
372 selected *U. arctos* myomiRs appear to be regulated in a manner that would facilitate the
373 suppression of both glucose and lipid metabolism by targeting specific signalling pathways
374 during hibernation.

375 The differentially expressed microRNAs identified in this study also have implications
376 for skeletal muscle regeneration and integrity. In particular, miR-206, miR-221, and miR-31 that
377 were elevated in hibernating bear muscle are known promoters of skeletal muscle regeneration
378 (Figures 1B and 3A) (Diniz & Wang, 2016; Greco et al., 2009; Togliatto et al., 2013). Two of the
379 microRNAs that were reduced during hibernation, miR-199a and miR-223, are associated with
380 inhibited myogenic differentiation, muscle disruption, injury, and inflammation (Figure 3A)
381 (Alexander et al., 2013; Y. Chen, Melton, Gelfond, McManus, & Shireman, 2012). Similarly to
382 miR-27b and miR-29b (discussed above), miR-23a increases in hibernating bear muscle, and
383 these three myomiRs not only increase in other species during hibernation (e.g. the little brown

384 bat, *Myotis lucifugus*), but are also associated with muscle atrophy resistance, increased MEF2A
385 activity, and decreased transcript levels of *mstn* that encodes myostatin, an inhibitor of muscle
386 growth (Figure 2A and 3A) (Kornfeld et al., 2012; Miretti et al., 2013). In the present study, *mstn*
387 mRNA levels were assessed in *U. arctos* skeletal muscle, but these did not change between
388 summer active and hibernation conditions (Figure 3B). However, a lack of microRNA
389 interaction with *mstn* mRNA cannot be ruled out since microRNAs function not only to facilitate
390 mRNA degradation, but also manage mRNA storage, during which transcript levels can remain
391 constant but their translation into protein is inhibited (Bartel, 2004). Indeed, mRNA storage
392 appears to be a common feature of metabolic rate depression in multiple species and in response
393 to a wide variety of environmental stress conditions, including hibernation (Tessier, Audas, Wu,
394 Lee, & Storey, 2014; Tessier & Storey, 2014). MiR-23a also promotes resistance to muscle
395 atrophy by disrupting protein degradation. The mechanism by which this occurs is the direct
396 suppression of two muscle-specific ubiquitin ligases, MAFbx/atrogin-1 and muscle RING-finger
397 1, that are known to be induced during muscle atrophy and atrophy-associated protein
398 degradation (Wada et al., 2011). Protein levels of ubiquitin ligases such as these are known to be
399 modulated throughout the torpor-arousal cycle of hibernating ground squirrel hearts (Y. Zhang,
400 Aguilar, & Storey, 2016), and therefore, there is potential for them to participate in the skeletal
401 muscle of bears. Together, the results from this study demonstrate how differential myomiR
402 expression in hibernating *U. arctos* may help to regulate a diverse range of mechanisms that
403 contribute to the bear's resilience to muscle atrophy.

404 An increase in miR-33 (discussed earlier for its potential role in regulating *prkaa1*
405 mRNA) and miR-29 in hibernation, as compared to active summer bears, may have a
406 cytoprotective effect in the skeletal muscle of brown bears through the downregulation of sterol
407 regulatory element-binding protein (SREBP). Overexpression of miR-33 in various cell lines
408 have shown that it directly reduces the protein levels of SREBP-1, whereas miR-33 null mice
409 had higher protein expression of SREBP-1 (Horie et al., 2013). It has also been shown that miR-
410 29 inhibits SREBP-1 expression in cancer cells (Ru et al., 2016). Although SREBP-1 has mostly
411 been studied in the liver for its roles in metabolic regulation, studies have shown that it is
412 significantly expressed in muscle cells (Ducluzeau et al., 2001; Guillet-Deniau et al., 2002), and
413 furthermore, SREBP-1 nuclear protein content induces muscle atrophy in both in vitro and in
414 vivo models (Lecomte et al., 2009). Many experiments have identified that SREBP regulates
415 many myogenic and metabolic genes in skeletal muscle (Abiola et al., 2009; Lecomte et al.,
416 2009; Rome et al., 2008). Of interest among SREBP targets are myogenic transcription factors
417 MYOD1, MYOG, and MEF2C, which have shown to decrease with SREBP overexpression
418 (Dessalle et al., 2012). Furthermore, SREBP is believed to induce skeletal muscle atrophy by
419 leveraging the ubiquitin ligase system, in part by upregulating MuRF1 mRNA (Lecomte et al.,
420 2009). The present results suggest that a similar effect might occur in bears, where
421 downregulation of SREBP by miR-33 and/or miR-29 facilitates a cytoprotective effect in skeletal
422 muscle.

423 Conclusion

424 The present study sought to assess the role of microRNAs as regulatory factors in
 425 limiting atrophy of *U. arctos* skeletal muscle during winter hibernation. Analysis of selected
 426 muscle-specific microRNAs and mRNA transcripts indicated broad modulations occurring at
 427 both transcriptional levels, which were suggestive of post-transcriptional mechanisms activating
 428 myogenic pathways, suppressing glucose and lipid metabolism, and decreasing atrophic
 429 signalling during hibernation (Figure 4). The results support a hypothesis made previously that
 430 the modulation of cellular signalling pathways is crucial for the resilience of skeletal muscles
 431 against atrophy in hibernating brown bears (Lin et al., 2012). These current results warrant
 432 continuing investigation into other regulatory controls on signalling networks (such as SREBP
 433 and/or ubiquitin ligases) in order to further our understanding the complex molecular
 434 mechanisms involved in muscle maintenance in the hibernating brown bear.

435

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652 **Figure legends**

653 **Figure 1.** MEF2A-controlled muscle microRNAs are upregulated in skeletal muscle of active
654 versus hibernating brown bears, *U. arctos*. (A) Relative transcript abundance of *mef2a* mRNA,
655 (B) Relative abundance of MEF2A-regulated microRNAs, and (C) Downstream mRNA targets
656 of miR-1 and miR-206, *pax7* and *id2* mRNA. MicroRNA and mRNA transcripts levels were
657 standardized against the expression of *U6* and *gapdh*, respectively. Data are means \pm S.E.M., $n =$
658 6 for microRNA and $n = 5$ for mRNA individuals sampled in both winter hibernating and
659 summer active states. Statistically significant differences are denoted with an asterisk (*) and
660 were analyzed with a paired *t*-test ($p < 0.05$).

661 **Figure 2.** Expression of metabolic microRNAs involved in glucose and lipid metabolism and
662 downstream targets in skeletal muscle of hibernating brown bears, *U. arctos*. (A) Abundance
663 levels of metabolic microRNAs, and (B) transcript levels of a downstream target, *prkaa1*.
664 MicroRNA and mRNA transcripts were standardized against the expression of *U6* and *gapdh*,
665 respectively. Data are means \pm S.E.M., $n = 6$ for microRNA and $n = 5$ for mRNA individuals
666 sampled in both winter hibernating and summer active states. Statistically significant differences
667 are denoted with an asterisk (*) and were determined with a paired *t*-test ($p < 0.05$).

668 **Figure 3.** Expression of microRNAs linked with skeletal muscle regeneration and maintenance,
669 and one downstream mRNA target, in skeletal muscle of the hibernating brown bear, *U. arctos*.
670 (A) Relative abundance of microRNAs implicated in muscle maintenance during hibernation,
671 and (B) transcript levels of the downstream target, *mstn*. MicroRNA and mRNA transcripts were
672 standardized against the expression of *U6* and *gapdh*, respectively. Data are means \pm S.E.M., $n =$
673 6 for microRNA and $n = 5$ individuals sampled in both winter hibernating and summer active
674 states. Statistically significant differences are denoted with an asterisk (*) and were determined
675 with a paired *t*-test ($p < 0.05$).

676 **Figure 4.** Proposed microRNA-mediated mechanism facilitating metabolic suppression and
677 skeletal muscle maintenance in *U. arctos* during hibernation. Perforated boxes denote areas of
678 the model supported by this study. Red font and asterisks denotes targets and areas warranting
679 further investigation.

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683 **Supplementary Table S1.** Primers for quantification of *U. arctos* mRNA and microRNA.