

1 **Genetic variation of human myokine signaling is dominated by biologic sex and sex**  
2 **hormones**

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4 Leandro M. Velez<sup>1,2\*</sup>, Cassandra Van<sup>1,2\*</sup>, Timothy M. Moore<sup>3</sup>, Zhenqi Zhou<sup>4</sup>, Casey Johnson<sup>1,2</sup>,  
5 Andrea L. Hevener<sup>4,5,6</sup> and Marcus M. Seldin<sup>1,2,6</sup>

6  
7 <sup>1</sup>Department of Biological Chemistry and <sup>2</sup>Center for Epigenetics and Metabolism, UC Irvine.  
8 Irvine, CA 92697, USA

9 <sup>3</sup>Department of Medicine, Division of Cardiology, David Geffen School of Medicine at UCLA,  
10 675 Charles E. Young Dr., Los Angeles, CA 90095, USA

11 <sup>4</sup>Department of Medicine, Division of Endocrinology, Diabetes, and Hypertension, and <sup>5</sup>Iris  
12 Cantor-UCLA Women's Health Research Center, David Geffen School of Medicine at UCLA,  
13 650 Charles E. Young Dr., Los Angeles, CA 90095, USA

14  
15  
16 \*Authors contributed equally

17 <sup>6</sup>Co-corresponding authors

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20  
21 To whom correspondence should be addressed:

22 Marcus Seldin

23 UC Irvine Department of Biological Chemistry

24 314 Sprague Hall

25 Irvine, CA 92697

26 Phone: 949-824-6765

27 Email: [mseldin@uci.edu](mailto:mseldin@uci.edu)

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41 **Running title:** Genetic architecture of muscle secreted proteins in humans

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44

#### 45 **Abstract/Introduction**

46 Proteins secreted from skeletal muscle, termed myokines, allow muscle to impact systemic  
47 physiology and disease. Myokines play critical roles in a variety of processes, including metabolic  
48 homeostasis, exercise improvements, inflammation, cancer and cognitive functions<sup>1-6</sup>. Despite  
49 the clear relevance of these factors in mediating a multitude of physiological outcomes, the genetic  
50 architecture, regulation and functions of myokines, as well as degree of conservation of these  
51 communication circuits remains inadequately understood. Given that biologic sex controls critical  
52 aspects of nearly every physiologic outcome, it is essential to consider when relating specific  
53 mechanisms to complex genetic and metabolic interactions. Specifically, many metabolic traits  
54 impacted by myokines show striking sex differences arising from hormonal<sup>7-10</sup>, genetic<sup>7,11</sup> or gene-  
55 by-sex interactions<sup>12,13</sup>. In this study, we performed a genetic survey of myokine gene regulation  
56 and cross-tissue signaling in humans where sex as a biological variable was emphasized. While  
57 expression levels of a majority of myokines and cell proportions within skeletal muscle showed  
58 little differences between males and females, nearly all significant cross-tissue enrichments  
59 operated in a sex-specific or hormone-dependent fashion; in particular, with estrogens. These sex-  
60 and hormone-specific effects were consistent across key metabolic tissues: liver, pancreas,  
61 hypothalamus, intestine, heart, visceral and subcutaneous adipose tissue. Skeletal muscle estrogen  
62 receptor enrichments across metabolic tissues appeared stronger than androgen receptor and,  
63 surprisingly, ~3-fold higher in males compared to females. To define the causal roles of estrogen  
64 signaling on myokine gene expression and functions, we generated male and female mice which  
65 lack estrogen receptor  $\alpha$  (*Esr1*) specifically in skeletal muscle and integrated global RNA-  
66 Sequencing with human data. These analyses highlighted mechanisms of sex-dependent myokine  
67 signaling conserved between species, such as myostatin enriched for divergent substrate utilization  
68 pathways between sexes. Several other sex-dependent mechanisms of myokine signaling were  
69 uncovered, such as muscle-derived *TNF $\alpha$*  exerting stronger inflammatory signaling in females  
70 compared to males and *GPX3* as a male-specific link between glycolytic fiber abundance and  
71 hepatic inflammation. Collectively, we provide the first genetic survey of human myokines and  
72 highlight sex and estrogen receptor signaling as critical variables when assaying myokine  
73 functions and how changes in cell composition impact other metabolic organs.

74

#### 75 **Results**

76 *Sex hormones, but not biologic sex show stronger enrichment with myokine expression:* Our goal  
77 was to perform a comprehensive survey how skeletal muscle communicates with and impacts  
78 metabolic organs. We focused these analyses on exploiting natural genetic variation to assay  
79 muscle-specific regulation of myokines and changes in cellular composition, then relate these  
80 outcomes to consequent cross-tissue signaling mechanisms (Fig 1A). Initially, we quantified  
81 differential expression of genes encoding all known secreted proteins in skeletal muscle from 210

82 male and 100 female individuals<sup>14</sup>. While several notable myokines appeared different between  
83 sexes (Fig 1B), a striking majority of all muscle secreted proteins (74%) showed no difference in  
84 expression between males and females (Fig 1C, Supplemental table 1). To understand potential  
85 sex-effects on the regulation of myokines, gene ontology enrichments were performed in muscle.  
86 Specifically, the skeletal muscle genes which showed the strongest correlation with myokines  
87 corresponding to each category (male-specific, female-specific or non-sex specific) were used for  
88 pathway enrichments. Here, the top 10 pathways which persisted in females were also observed  
89 strongly enriched within the non-sex-specific category, whereas pathways enriched for male-  
90 specific myokines were distinct (Fig 1D). Notably, the female and shared pathways suggested  
91 roles in epigenetics and RNA processing, while male-specific myokine coregulated processes were  
92 more enriched in metabolic pathways (ex. NADH metabolism) (Fig 1D). Further, a majority of  
93 myokines showed strong correlation with receptors mediating functions of androgens (androgen  
94 receptor *-AR*), estrogens (*ESRI*), or both, regardless of sex-specific expression (Fig 1E). We note  
95 that expression of hormone receptors themselves were also not significantly different between  
96 sexes (Figure 1 - Figure supplement 1). To infer causality from hormone receptor regulation, we  
97 performed RNA-sequencing on mice lacking estrogen receptor  $\alpha$  (*Esr1*) in skeletal muscle  
98 specifically and integrated these analyses with human myokine estimates. While myokines not  
99 regulated by *ESRI* showed little sex-specific modes of expression, those which were estrogen-  
100 dependent showed much stronger representation of sex-specificity, in particular in males (Fig 1F-  
101 G). Among these, the master regulator of skeletal muscle differentiation and proliferation,  
102 myostatin (*MSTN*), was strongly correlated with *ESRI* and *AR* in both sexes. Despite these  
103 hormone receptor correlations, the gene was markedly higher in males compared to females, where  
104 ablation of *ESRI* uniquely drove expression (Fig 1H). These data suggest interactions between  
105 biologic sex and *ESRI* to tightly regulate *MSTN* in males, where other factors could contribute  
106 more in females. This sex-specific regulation of myostatin also showed differences in functional  
107 annotations, as the most highly enriched pathways in males showed GO terms related to glycolytic  
108 metabolism compared to oxidative phosphorylation in females (Fig 1I). These observations are  
109 consistent with previous studies which note myostatin-dependent increases in muscle mass in  
110 males, but not females<sup>15,16</sup>, where estrogen signaling is suggested as a mechanism mediating these  
111 differences. These data demonstrate that, expression of most myokines is not different between  
112 biologic sexes; however, interactions between sex and hormone receptors likely play important  
113 roles in determining myokine regulation and local signaling.

114  
115 *Sex dominates cross-tissue pathways enriched for myokines* – Given that expression levels of most  
116 myokines appeared similar between sexes, we next assessed putative functions across organs. We  
117 applied a statistical method developed to infer cross-tissue signaling which occur as a result of  
118 genetic variation<sup>17–19</sup>. Here, we assayed the distribution of midweight bicorrelation coefficients  
119 between myokine expression levels and global gene expression measures from the same  
120 individuals in key metabolic tissues including hypothalamus, heart, intestine, pancreas, liver,  
121 subcutaneous and visceral adipose tissue. Remarkably, nearly all highly significant correlations  
122 between myokines and target organ genes showed sex-specific modes of operation (Fig 2A-H).  
123 This sex specificity also appeared more pronounced for positive correlations between myokines  
124 and target tissue genes, as compared to negative (Fig 2-H). Further, among these high significant  
125 cross-tissue circuits, myokine hormone receptor enrichment was strongly dependent on the  
126 category (ex. significant only in females) rather than target tissue (Fig 2A-H). This observation  
127 further suggests that hormone receptor levels (*ESRI* or *AR*) in muscle are a stronger determinant

128 of myokine expression compared to biologic sex; however, sex is suggested to dominate  
129 coregulated signaling processes across organs via myokines. Therefore, to gauge the relative  
130 impact of muscle steroid hormone receptors across organs, the number of significant correlations  
131 between *ESR1*, *AR* or both were quantified for each tissue. Remarkably, *ESR1* specifically as  
132 showing an order of magnitude stronger enrichment across metabolic tissues in compared to *AR*  
133 or both, where the number of significantly correlated cross-tissue male *ESR1* genes (Fig 2I) were  
134 three-fold higher than females (Fig 2J). Because both sex and *ESR1* signaling appeared critical in  
135 the regulation of myokine functions, we binned significant cross-tissue enrichments into categories  
136 taking into consideration whether myokines were driven by *ESR1* in muscle, and/or showing a  
137 sex-specific mode of inter-organ enrichment. This analysis suggested that a majority of myokines  
138 were either driven by *ESR1* and signaled robustly across sexes (Fig 2K, yellow) or signaled  
139 differently between sexes, but regulated independent of *ESR1* (Fig 2K, red). These categories  
140 appeared to a much greater result as opposed to a combination of both *ESR1*-driven myokine and  
141 sex-specific cross-tissue signaling (Fig 2K, beige) or neither (Fig 2K, seagreen). One notable  
142 example of sex-specific signaling was observed for tumor necrosis factor alpha (TNF $\alpha$ ) which  
143 showed markedly different putative target tissues (Fig 2L, left), as well as underlying functional  
144 pathways (Fig 2L, right), depending on sex. For example, overall inflammatory processes engaged  
145 by TNF $\alpha$  were substantially stronger in adipose tissue in females; however, the same pathways  
146 were higher in liver and hypothalamus in males (Fig 2L, left). Collectively, these data show that  
147 sex and related sex steroid hormones, particularly estradiol, exert dominant roles in regulating  
148 tissue and pathway engagement by myokines.

149  
150 *Muscle cell proportions are similar between sexes, but associated changes across tissues show*  
151 *sex-specificity* – To determine the impact of muscle composition on other tissues, we next surveyed  
152 muscle cellular proportions in the context of genetics and sex. Here, we integrated single-cell  
153 sequencing of human skeletal muscle<sup>20</sup> using cellular deconvolution<sup>21</sup> to estimate cellular  
154 composition (Fig 3A). Here, a proportions in admixture approach<sup>22</sup> outperformed other methods  
155 (Figure 3 - Figure supplement 1) to capture a majority of established cell populations across  
156 individuals (Supplemental Table 2). Similar to myokine expression, no differences were observed  
157 between sexes in terms of cell composition, with the exception of modest higher glycolytic fiber  
158 in males, compared to elevated oxidative fiber levels in females (Fig 3B). Additionally, no  
159 differences were observed in the correlations between compositions within males or females  
160 (Figure 3 - Figure supplement 2); however, nearly every cross-tissue enrichment corresponding to  
161 an individual muscle cell type differed between sexes (Fig 3C). Generally, differences in skeletal  
162 muscle cell abundance corresponded to changes in liver and visceral adipose tissue pathways in  
163 males, compared to pancreas in females (Fig 3C). In contrast to general myokine enrichments,  
164 cell proportions showed stronger correlations with *AR* when compared to *ESR1* across both sexes;  
165 however, the most abundant cell types were significantly enriched for both steroid hormone  
166 receptors (Fig 3D). Next, to uncover potential direct mechanisms linking changes in cell  
167 composition to peripheral tissues, we analyzed associated myokines and adopted and adjusted  
168 regression-based mediation approach. Despite few differences between sexes in terms of myokine  
169 expression and cell composition, specific myokines highly correlating with individual cell type  
170 were markedly different between males and females with the exception of one, APOD in slow-  
171 twitch fibers (Fig 3E). To determine if variation in cell compositions corresponding to sex-specific  
172 tissue signaling via myokines was predicted to be causal, we implemented adjusted regression  
173 mediation analyses<sup>23,24</sup> for glycolytic fiber composition. Because male glycolytic fiber type was

174 selectively enriched for liver pathways such as immune cell activation and regulated exocytosis  
175 (Fig 3F), the top-genes driving these enrichments were used to determine causality. The top-  
176 correlated muscle secreted protein with male glycolytic fiber type levels was secreted glutathione  
177 peroxidase 3 (*GPX3*). Here, adjusting regressions between glycolytic fiber and liver pathways on  
178 *GPX3* significantly reduced the overall significance across tissues (Fig 3G), suggesting *GPX3* as  
179 a mediator of this communication. These data point to a potential mechanism whereby muscle  
180 fiber abundance could buffer free radical generation in the liver, thereby feeding back on  
181 inflammation. This analysis appeared additionally sensitive to inferring non-dependent  
182 relationships between muscle cell types, top-ranked myokines and cross-tissue processes. For  
183 example, female glycolytic fibers were strongly enriched for pancreatic protein synthesis  
184 pathways; however, when adjusted for the top-ranked myokine *CES4A*, no changes in regression  
185 significance were observed (Fig 3F-G). These analyses show that male *GPX3* is a likely  
186 mechanism whereby fast-twitch muscle signals to liver; however, the same cell type in females  
187 drive pancreas protein synthesis independent of *CES4A*. In summary, we show that cell  
188 composition is strongly conserved between sexes, but cross-tissue signaling of altered composition  
189 differs entirely. We further suggest putative myokines and mechanisms, as well as highlight the  
190 key regulatory roles of estrogen in both sexes.

191  
192 *Conclusions and limitations* – Here we provide a population survey of skeletal muscle myokine  
193 regulation and putative functions using genetic variation and multi-tissue gene expression data.  
194 We find that in general, expression of myokines do not significantly differ between sexes;  
195 however, signaling mechanisms across tissues inferred from regressions show strong sex  
196 specificity. Steroid hormones, in particular *ESR1*, is highlighted as a key regulator of myokines  
197 and potentially interacting with biologic sex for proteins such as myostatin. Further integration  
198 with loss-of-function mouse models of *Esr1* highlighted the key roles of estradiol signaling in  
199 muscle in terms of myokine regulation and signaling across both sexes. Generation of pseudo-  
200 single-cell maps of muscle composition showed that, like myokines, muscle proportions are  
201 conserved between sexes, but inferred interorgan consequences differ substantially. When  
202 interpreting these findings, several considerations should be taken. While inter-tissue regression  
203 analyses have been informative to dissect mechanisms of endocrinology<sup>17–19,25</sup>, observations can  
204 be subjected to spurious or latent relationships in the data. While causality for inter-organ signaling  
205 can be inferred statistically using approaches such as mediation as in Fig 3H, the only methods to  
206 provide definitive validation for new mechanisms is in experimental settings. In addition, we  
207 anticipate that estimates for *ESR1* effects on myokines in this study likely represents an  
208 underestimated number of all human *ESR1*-driven myokines. The primary limitation here is that  
209 annotation of known orthologous mouse-human genes<sup>26</sup> is limited to roughly ~15% of the coding  
210 genome. Related, integration with muscle specific AR-deletion would offer intriguing information  
211 for cell composition metrics, but also limited similarly. Furthermore, cell composition estimates  
212 from single-cell sequencing data are inferred from gene expression, where histological or flow  
213 cytometry-based methods can provide more accurate direct quantifications. Clearly,  
214 morphological and structural differences between sexes have been observed in humans<sup>27</sup> which, if  
215 not apparent in gene expression, would be missed in this analysis. Future studies addressing these  
216 points will help to clarify context- and mechanism-relevant muscle-derived endocrine  
217 communication axes. In summary, this study highlights the critical nature of sex and sex steroid  
218 hormones in mediating myokine functions which should be considered when interpreting future  
219 studies of myokines.

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## 222 **Material and methods**

223 **All datasets used, R scripts implemented for analyses and detailed walkthrough guide is**  
224 **available via: <https://github.com/marcus-seldin/myokine-signaling>**

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226 *Data sources and availability* – All data used in this study can be immediately accessed via  
227 github to facilitate analysis. Human skeletal muscle and metabolic tissue data was accessed  
228 through GTEx V8 downloads portal on August 18, 2021 and previously described<sup>14</sup>. To enable  
229 sufficient integration and cross-tissue analyses, these data were filtered to retain genes which  
230 were detected across tissues where individuals were required to show counts > 0 in 1.2e6 gene-  
231 tissue combinations across all data. Given that our goal was to look across tissues at enrichments,  
232 this was done to limit spurious influence of genes only expressed in specific tissues in specific  
233 individuals. Post-filtering consists of 310 individuals and 1.8e7 gene-tissue combinations).  
234 Single-cell sequencing from skeletal muscle used for deconvolution was obtained from<sup>20</sup>. *Esr1*  
235 WT and KO mouse differential expression results are available on Github as well, where raw  
236 sequencing data has been deposited in NIH sequence read archive (SRA) under the project  
237 accession: PRJNA785746

238

239 *Selection of secreted proteins* – To determine which genes encode proteins known to be secreted  
240 as myokines, gene lists were accessed from the Universal Protein Resource which has compiled  
241 literature annotations terms for secretion<sup>28</sup>. Specifically, the query terms to access these lists  
242 were: locations:(location:"Secreted [SL-0243]" type:component) AND organism:"Homo sapiens  
243 (Human) [9606]" where 3666 total entries were found.

244

245 *Differential expression of myokines dependent on sex* – Gene expression counts matrices were  
246 isolated from the rest of the tissues, where individual genes were retained if the total number of  
247 counts exceeded 10 in 50 individuals. Next, only genes encoding secreted proteins (above) were  
248 retained, where logistic regression contrasted on sex was performed using DESeq2. Differential  
249 expression summary statistics were used for downstream binning of sex-specificity based on an  
250 empirical logistic regression pvalue <0.05. This threshold was used to reflect a least stringent  
251 cutoff where, despite potential false positive influence, genes which nominally trended toward  
252 sex-specific expression could be included in those categories. Given that the general conclusions  
253 supported very few proportions of myokines showing sex-specific patterns of expression, this  
254 conclusion would only be further exaggerated if the DE threshold were made more stringent and  
255 lessened the number of myokines in each category.

256

257 *Regression analyses across tissues* – Regression coefficients and corresponding p-values across  
258 tissues were generated using WGCNA bicorandpvalue() function<sup>22</sup>. Myokine-target gene pairs  
259 were considered significant (ex. Fig 2A-H) at a threshold of abs(bicor) > 2 standard deviations  
260 beyond the average coefficient for the given target tissue of interest. In previous studies, this  
261 threshold of 2 standard deviations reflects adaptive permutation testing pvalues <0.01<sup>17,18</sup>. For  
262 analyses estimating cumulative patterns of concordance across tissues (ex. Fig 2I-L), empirical  
263 regression pvalues (students pvalue from bicor coefficients) of 0.01 (corresponding to  
264 abs(bicor)>0.1) were used to assay global patterns. While empirical pvalues are subjected to  
265 false positives, including these enables broad visualization of both potential direct interactions

266 (ex. myokine-target gene) as well as coregulated processes across organs. It is important to note  
267 that we exclusively rely on these empirical p-values when surveying broad correlation structures,  
268 whereas much more stringent and appropriate thresholds (ex.  $p < 1e-6$  for Fig 3G) were applied  
269 when inferring direct interactions.

270  
271 *Pathway enrichment analyses* - For Fig 1I and Fig 3G, genes corresponding to p-value cutoffs were  
272 visualized using Webgestalt<sup>29</sup> to enable streamline visualization. For Fig. 1D, the top 1000 (by  
273 regression p-value) significant genes from myokines to all muscle bicorrelation analysis in  
274 females, males or non-sex specific datasets were assessed for enrichment in GO Biological Process  
275 terms using ClusterProfiler ver. 4.0.2 in R<sup>30</sup>. The resulting top ten GO terms in each dataset were  
276 integrated and plotted against the relative proportion of the p.adjusted value, and visualized in the  
277 same graph using ggplot2.

278 *Deconvolution of skeletal muscle* – Raw single-cell RNA sequencing from skeletal muscle was  
279 obtained from<sup>20</sup>. These raw counts were analyzed in Seurat where cluster analyses identified  
280 variable cell compositions. Cell type annotations were assigned based on the top 30 genes  
281 (Supplemental table 2) assigned to each UMAP cluster through manual inspection and  
282 ENRICH<sup>31</sup>. Finally, a normalized matrix of gene:cells was exported from Seurat and used to run  
283 deconvolution on skeletal muscle bulk sequencing. Using the ADAPTS pipeline<sup>21</sup>, three  
284 deconvolution methods (nnls, dcq or proportions in admixture) were compared based on ability to  
285 robustly capture cell proportions (Supplemental fig 2), where proportion in admixture showed the  
286 best performance and subsequently applied to bulk sequencing.

287 *ESR1 muscle KO generation, RNA-Seq and integration with human data* – Muscle-specific Esr1  
288 deletion was generated and characterized as previously described<sup>10</sup>. Whole quadriceps was  
289 pulverized at the temperature of liquid nitrogen. Tissue was homogenized in Trizol (Invitrogen,  
290 Carlsbad, CA, USA), RNA was isolated using the RNeasy Isolation Kit (Qiagen, Hilden,  
291 Germany), and then tested for concentration and quality with samples where RIN > 7.0 used in  
292 downstream applications. Libraries were prepared using KAPA mRNA HyperPrep Kits and  
293 KAPA Dual Index Adapters (Roche, Basel, Switzerland) per manufacturer's instructions. A total  
294 of 800-1000 ng of RNA was used for library preparation with settings 200-300 bp and 12 PCR  
295 cycles. The resultant libraries were tested for quality. Individual libraries were pooled and  
296 sequenced using a HiSeq 3000 at the UCLA Technology Center for Genomics and Bioinformatics  
297 (TCGB) following in house established protocols. Raw RNAseq reads were inspected for quality  
298 using FastQC v0.11.9 (Barbraham Institute, Barbraham, England). Reads were aligned and  
299 counted using the Rsubread v2.0.0<sup>32</sup> package in R v3.6 against the Ensembl mouse transcriptome  
300 (v97) to obtain counts. Lowly expressed genes (>80% samples with 0 counts for particular gene)  
301 were removed. Samples were analyzed for differential expression using DeSeq2 v1.28.0<sup>33</sup>.

302  
303 *Conservation of gene between mice and humans* - To find which myokines and pathways were  
304 conserved between mice and humans, all orthologous genes were accessed from MGI vertebrate  
305 homology datasets, which have been compiled from the Alliance for Genome Resources<sup>26</sup> and  
306 intersected at the gene level.

307  
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#### 314 **Author contributions**

315  
316 LV, CV, TMM, ZQ and MMS accessed raw data, performed analyses and drafted the manuscript.  
317 CJ and AH provided critical insight into data use and interpretation, as well as guided the study.  
318 All authors read and approved this manuscript.

319

#### 320 **Conflict of interest**

321

322 The authors have no conflicts of interest to declare

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#### 324 **References**

- 325 1. Severinsen, M. C. K. & Pedersen, B. K. Muscle–Organ Crosstalk: The Emerging Roles of  
326 Myokines. *Endocrine Reviews* **41**, 594–609 (2020).
- 327 2. Eckel, J. Myokines in metabolic homeostasis and diabetes. *Diabetologia* **62**, 1523–1528  
328 (2019).
- 329 3. Febbraio, M. A. & Pedersen, B. K. Who would have thought — myokines two decades on.  
330 *Nat Rev Endocrinol* **16**, 619–620 (2020).
- 331 4. Kim, S. *et al.* Roles of myokines in exercise-induced improvement of neuropsychiatric  
332 function. *Pflugers Arch - Eur J Physiol* **471**, 491–505 (2019).
- 333 5. Kim, J.-S., Galvão, D. A., Newton, R. U., Gray, E. & Taaffe, D. R. Exercise-induced  
334 myokines and their effect on prostate cancer. *Nat Rev Urol* **18**, 519–542 (2021).
- 335 6. Seldin, M. M. & Wong, G. W. Regulation of tissue crosstalk by skeletal muscle-derived  
336 myonectin and other myokines. *Adipocyte* **1**, 200–202 (2012).
- 337 7. Mauvais-Jarvis, F., Arnold, A. P. & Reue, K. A Guide for the Design of Pre-clinical Studies  
338 on Sex Differences in Metabolism. *Cell Metab* **25**, 1216–1230 (2017).



- 339 8. Mauvais-Jarvis, F. Sex differences in metabolic homeostasis, diabetes, and obesity. *Biol Sex*  
340 *Differ* **6**, 14 (2015).
- 341 9. Clegg, D. J. & Mauvais-Jarvis, F. An integrated view of sex differences in metabolic  
342 physiology and disease. *Molecular Metabolism* **15**, 1–2 (2018).
- 343 10. Ribas, V. *et al.* Skeletal muscle action of estrogen receptor  $\alpha$  is critical for the maintenance  
344 of mitochondrial function and metabolic homeostasis in females. *Sci Transl Med* **8**, 334ra54  
345 (2016).
- 346 11. Zore, T., Palafox, M. & Reue, K. Sex differences in obesity, lipid metabolism, and  
347 inflammation-A role for the sex chromosomes? *Mol Metab* **15**, 35–44 (2018).
- 348 12. Norheim, F. *et al.* Gene-by-Sex Interactions in Mitochondrial Functions and Cardio-  
349 Metabolic Traits. *Cell Metab.* **29**, 932-949.e4 (2019).
- 350 13. Chella Krishnan, K. *et al.* Sex-specific genetic regulation of adipose mitochondria and  
351 metabolic syndrome by *Ndufv2*. *Nat Metab* (2021) doi:10.1038/s42255-021-00481-w.
- 352 14. GTEx Consortium *et al.* Genetic effects on gene expression across human tissues. *Nature*  
353 **550**, 204–213 (2017).
- 354 15. Reisz-Porszasz, S. *et al.* Lower skeletal muscle mass in male transgenic mice with muscle-  
355 specific overexpression of myostatin. *Am J Physiol Endocrinol Metab* **285**, E876-888 (2003).
- 356 16. McMahon, C. D. *et al.* Sexual dimorphism is associated with decreased expression of  
357 processed myostatin in males. *Am J Physiol Endocrinol Metab* **284**, E377-381 (2003).
- 358 17. Seldin, M. M. *et al.* A Strategy for Discovery of Endocrine Interactions with Application to  
359 Whole-Body Metabolism. *Cell Metab.* **27**, 1138-1155.e6 (2018).
- 360 18. Seldin, M. M. & Lusis, A. J. Systems-based approaches for investigation of inter-tissue  
361 communication. *J. Lipid Res.* **60**, 450–455 (2019).

- 362 19. Seldin, M., Yang, X. & Lusis, A. J. Systems genetics applications in metabolism research.  
363 *Nat Metab* **1**, 1038–1050 (2019).
- 364 20. Rubenstein, A. B. *et al.* Single-cell transcriptional profiles in human skeletal muscle. *Sci Rep*  
365 **10**, 229 (2020).
- 366 21. Danziger, S. A. *et al.* ADAPTS: Automated deconvolution augmentation of profiles for  
367 tissue specific cells. *PLoS ONE* **14**, e0224693 (2019).
- 368 22. Langfelder, P. & Horvath, S. WGCNA: an R package for weighted correlation network  
369 analysis. *BMC Bioinformatics* **9**, 559 (2008).
- 370 23. Yokota, T. *et al.* Type V Collagen in Scar Tissue Regulates the Size of Scar after Heart  
371 Injury. *Cell* (2020) doi:10.1016/j.cell.2020.06.030.
- 372 24. Tingley, D., Yamamoto, T., Hirose, K., Keele, L. & Imai, K. **mediation** : R Package for  
373 Causal Mediation Analysis. *J. Stat. Soft.* **59**, (2014).
- 374 25. Li, H. & Auwerx, J. Mouse Systems Genetics as a Prelude to Precision Medicine. *Trends*  
375 *Genet* **36**, 259–272 (2020).
- 376 26. Alliance of Genome Resources Consortium. Alliance of Genome Resources Portal: unified  
377 model organism research platform. *Nucleic Acids Res* **48**, D650–D658 (2020).
- 378 27. Haizlip, K. M., Harrison, B. C. & Leinwand, L. A. Sex-based differences in skeletal muscle  
379 kinetics and fiber-type composition. *Physiology (Bethesda)* **30**, 30–39 (2015).
- 380 28. The UniProt Consortium *et al.* UniProt: the universal protein knowledgebase in 2021.  
381 *Nucleic Acids Research* **49**, D480–D489 (2021).
- 382 29. Liao, Y., Wang, J., Jaehnig, E. J., Shi, Z. & Zhang, B. WebGestalt 2019: gene set analysis  
383 toolkit with revamped UIs and APIs. *Nucleic Acids Res* **47**, W199–W205 (2019).

- 384 30. Wu, T. *et al.* clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *The*  
385 *Innovation* **2**, 100141 (2021).
- 386 31. Kuleshov, M. V. *et al.* Enrichr: a comprehensive gene set enrichment analysis web server  
387 2016 update. *Nucleic Acids Res* **44**, W90-97 (2016).
- 388 32. Liao, Y., Smyth, G. K. & Shi, W. The R package Rsubread is easier, faster, cheaper and  
389 better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Research*  
390 **47**, e47–e47 (2019).
- 391 33. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for  
392 RNA-seq data with DESeq2. *Genome Biol* **15**, 550 (2014).

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394 **Figure Legends**

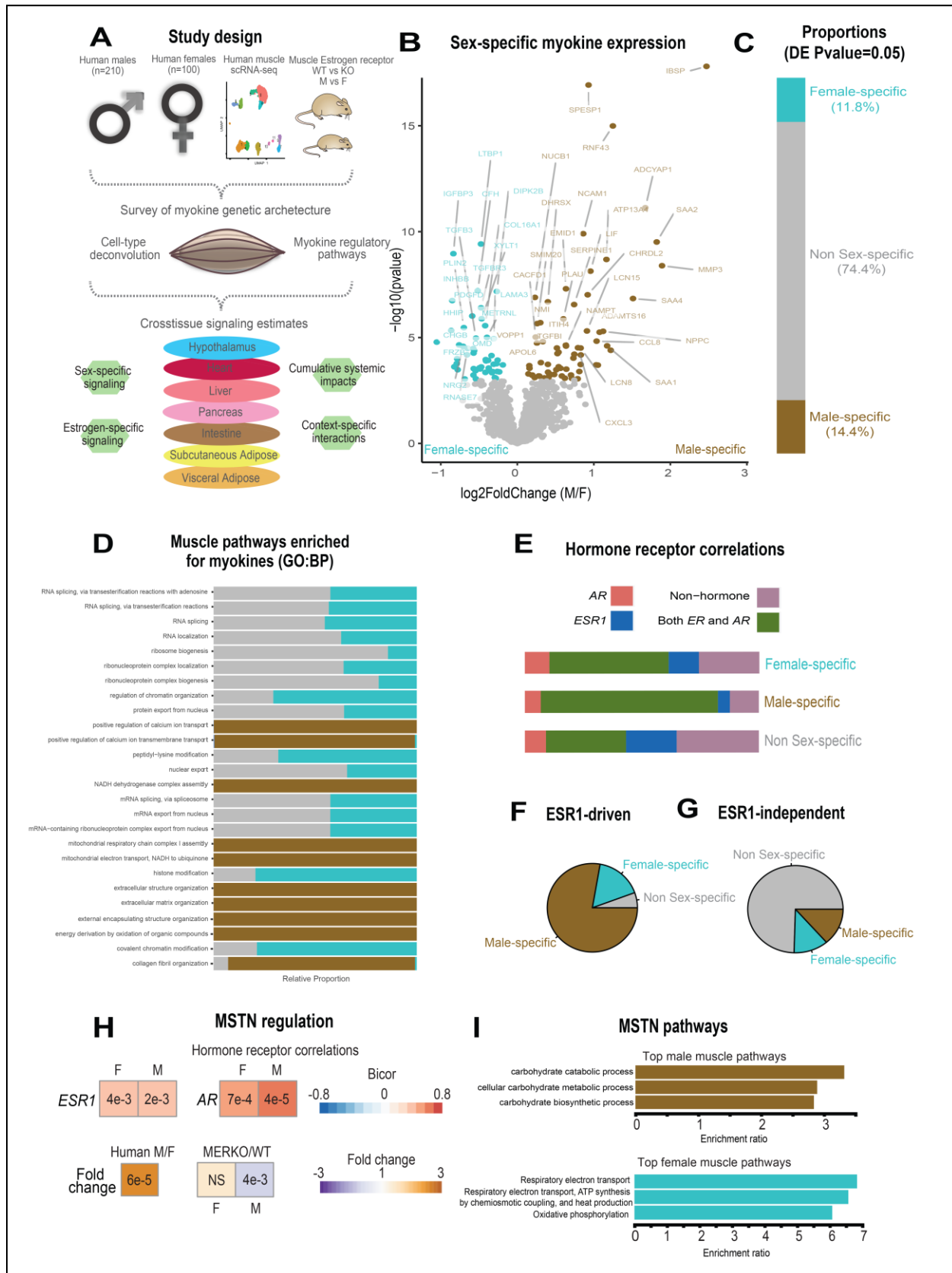
395  
396 **Figure 1. Sex and hormone effects on myokine regulation.** A, overall study design for  
397 integration of gene expression from muscle from 310 humans, single-cell RNA-seq, muscle-  
398 specific deletion of *Esr1* to infer interorgan coregulatory process across major metabolic tissues.  
399 B-C, Differential expression analysis for sex was performed on all genes corresponding to  
400 secreted proteins in skeletal muscle (Myokines). The specific genes which showed significant  
401 changes in each sex are shown as a volcano plot (B) and the relative proportions of myokines  
402 corresponding to each category at a modest logistic regression p-value less than 0.05 (C). D, for  
403 each differential expression category based on sex shown in C, myokines were correlated with  
404 all other muscle genes for pathway enrichment. Then the top 10 enriched pathways in males,  
405 females, or non-sex specific (by overall significance) were visualized together where number of  
406 genes corresponding to each category shown as a relative proportion. E, the same analysis as in  
407 D, except instead of myokines being correlated with all muscle genes, they are binned into  
408 proportions correlated with AR, ESR1, both hormone receptors, or neither. F-G, Myokines were  
409 binned into 2 categories based on significantly differentially expressed (logistic regression  
410 adjusted p-value<0.05) between muscle-specific WT and *Esr1*-KO mice (F) or those that showed  
411 no change (G), then visualized as relative proportions within each category shown in C. H,  
412 Midweight bicorrelation (bicor) coefficients (color scheme) and corresponding regression p-  
413 values (filled text) are shown for muscle MSTN and ESR1 or AR in both sexes (top). Below  
414 correlations are shown differential expression log<sub>2</sub>FC (color scheme) and corresponding logistic  
415 regression p-values (text fill) for MSTN between sexes in humans or WT vs muscle-specific  
416 ESR1 KO mice (MERKO). I, the top 3 pathways of genes which significantly (p<1e-4)  
417 correlated with muscle MSTN in males (top) or females (bottom). For human data, n=210 males  
418 and n=100 females. For mouse MERKO vs WT comparisons, n=3mice per group per sex. p-  
419 values from midweight bicorrelations were calculated using the students p-value from WGCNA  
420 and logistic regression p-values were calculated using DESeq2.

421  
422 **Figure 2. Sex and hormone effects on myokine regulation.** A-H, Key illustrating analysis for  
423 distribution of midweight bicorrelation coefficients between all myokines in skeletal muscle and  
424 global transcriptome measures are plotted between sexes (left), where proportions for 2SD >  
425 mean are subdivided into occurrence uniquely in females, males, or shared (middle). The  
426 myokines identified in each category were then binned into hormone receptor correlations for  
427 *ESR1*, *AR*, both or neither (right). This analysis was performed on all myokines across  
428 subcutaneous adipose tissue (B), visceral adipose (C), heart (D), hypothalamus (E), small  
429 intestine (F), liver (G) and pancreas (H). I-J, Significant cross-tissue correlations between muscle  
430 *ESR1*, *AR*, or both hormone receptors are colored by tissue and shown for males (I) or females  
431 (J). K, For each tissue (y-axis), the ratio of significant cross-tissue correlations per muscle  
432 myokine (x-axis) are shown and colored by categories of: either the myokine regulated by *ESR1*  
433 and/or the target tissue regression occurring specifically in one sex. L, Number of significant  
434 cross-tissue correlations with muscle TNF $\alpha$  are shown for each sex and colored by tissue as in I-  
435 L (left). The  $-\log_{10}(\text{p-value})$  of significance in an overrepresentation test (x-axis) are shown for  
436 top significant intertissue pathways for muscle TNF $\alpha$  in each sex (right).

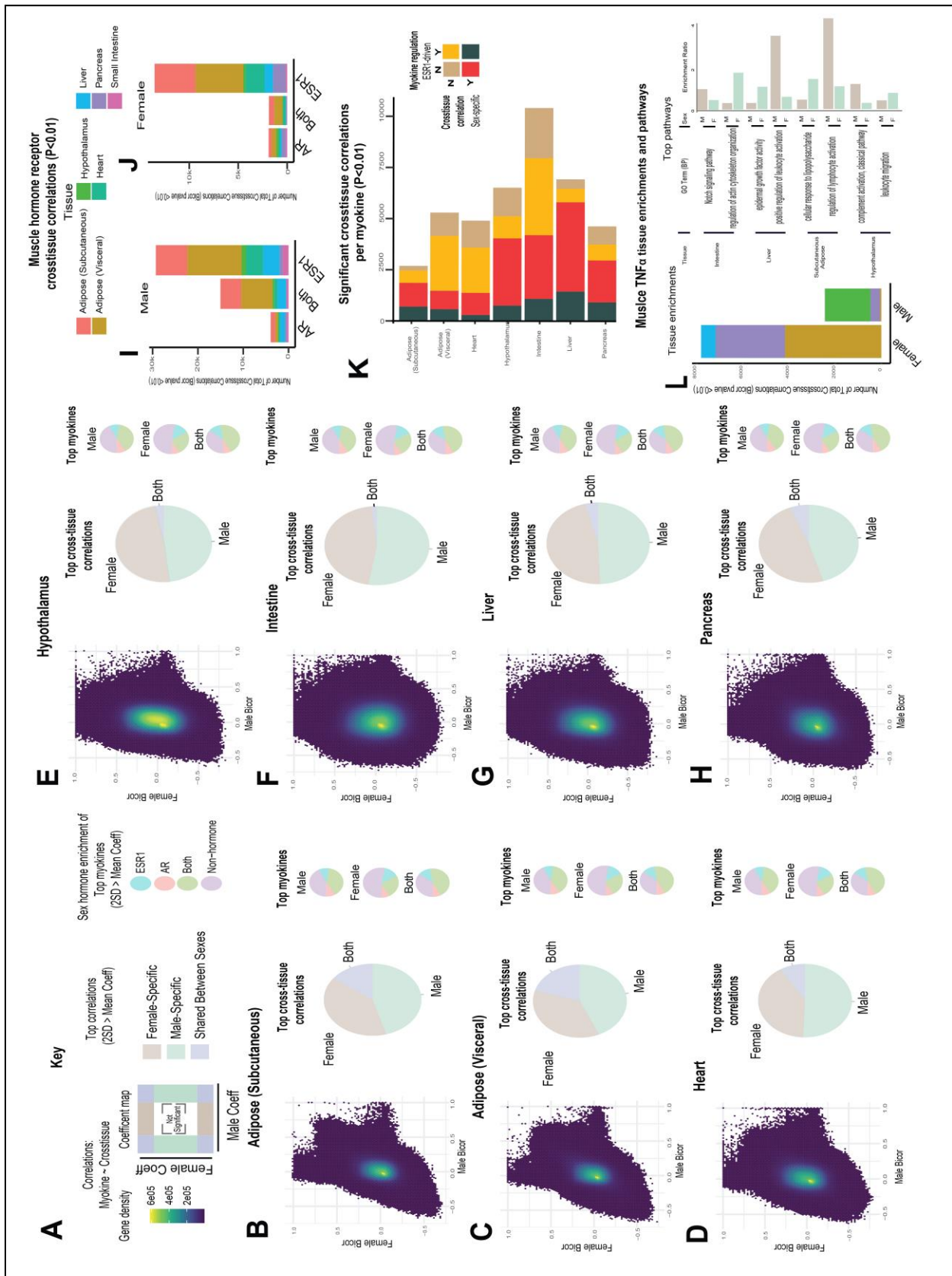
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438 **Figure 3. Genetic variation of muscle cell proportions and coregulated cross-tissue**  
439 **processes.** A, Uniform Manifold Approximation and Projection (UMAP) for skeletal muscle  
440 single-cell sequencing to deconvolute proportions. B, Mean relative proportions of pseudo-  
441 single-cell muscle cell compositions (denoted by color) between sexes. C, Number of significant  
442 cross-tissue correlations (y-axis) corresponding to each skeletal muscle type in each sex (x-axis).  
443 Target tissues are distinguished by color, where NS (male platelets) denotes that no significant  
444 cross-tissue correlations were observed. D, Heatmap showing significance of correlations  
445 between skeletal muscle hormone receptors and cell proportions, \* =  $p < 0.01$ . E, the strongest  
446 enriched myokines are plotted for each myokine (y-axis,  $-\log_{10}(\text{p-value})$  of myokine ~ cell  
447 composition) are shown for each muscle proportion for each sex (x-axis). Gene symbols for  
448 myokines are shown above each line, where red lines indicate positive correlations between  
449 myokine and cell type and blue shows inverse relationships. F, Significant cross-tissue  
450 correlated genes in liver (blue) and pancreas (purple) for muscle fast twitch glycolytic fibers  
451 ( $P < 1e-6$ ) were used for overrepresentation tests where enrichment ratio of significance (x-axis) is  
452 shown for each pathway and sex (y-axis). G, Heatmap showing the regression significance of the  
453 top 5 genes corresponding to inflammation (liver), exocytosis (liver) and protein synthesis  
454 (pancreas) for proportions of fast-twitch fiber type (un-adj). Below each correlation between  
455 fast-twitch fiber and liver or pancreas gene, the same regressions were performed while adjusting  
456 for abundance of select myokines in each sex. \*= $p < 1e-6$ .

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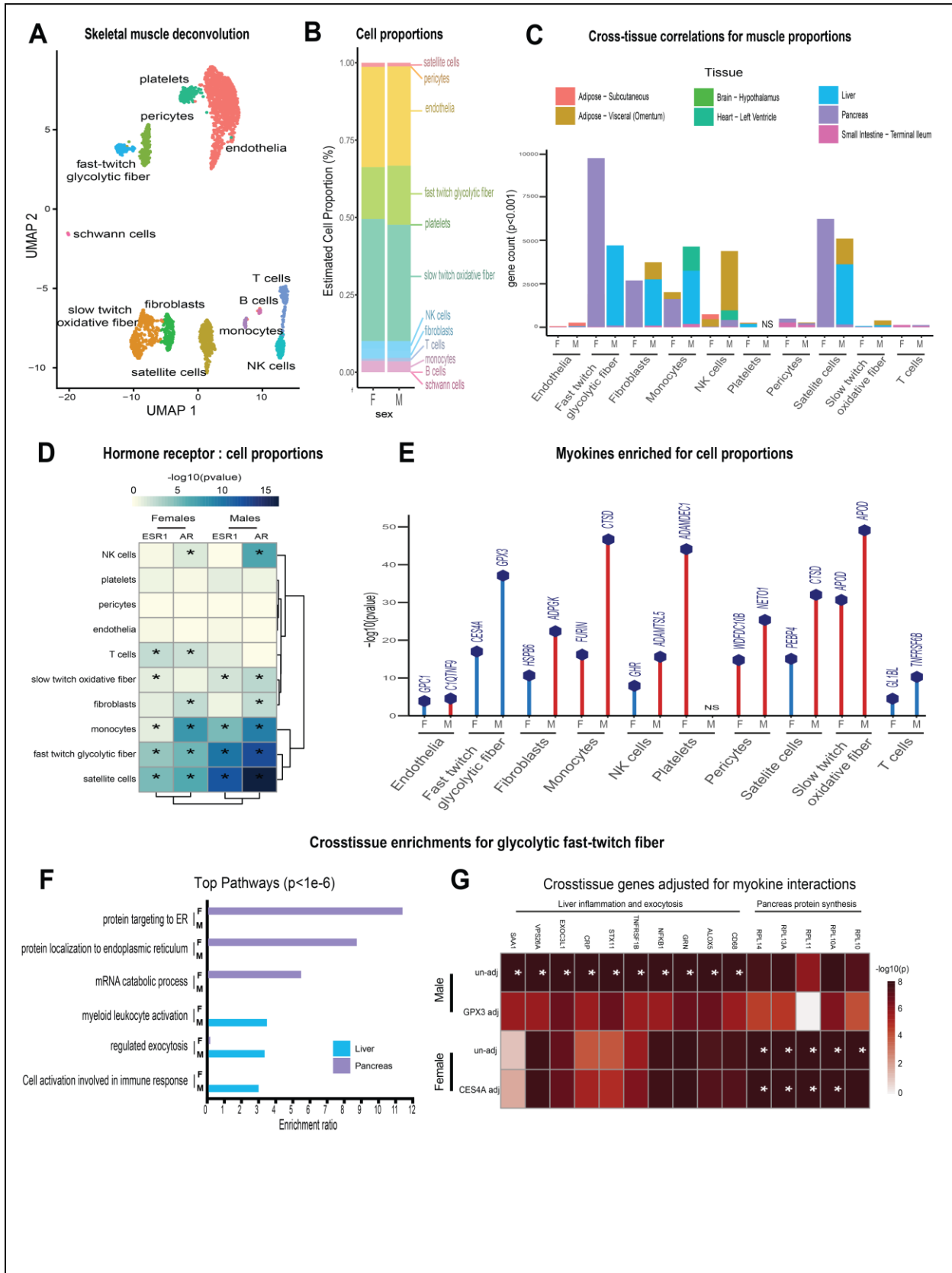
461 Figure 1



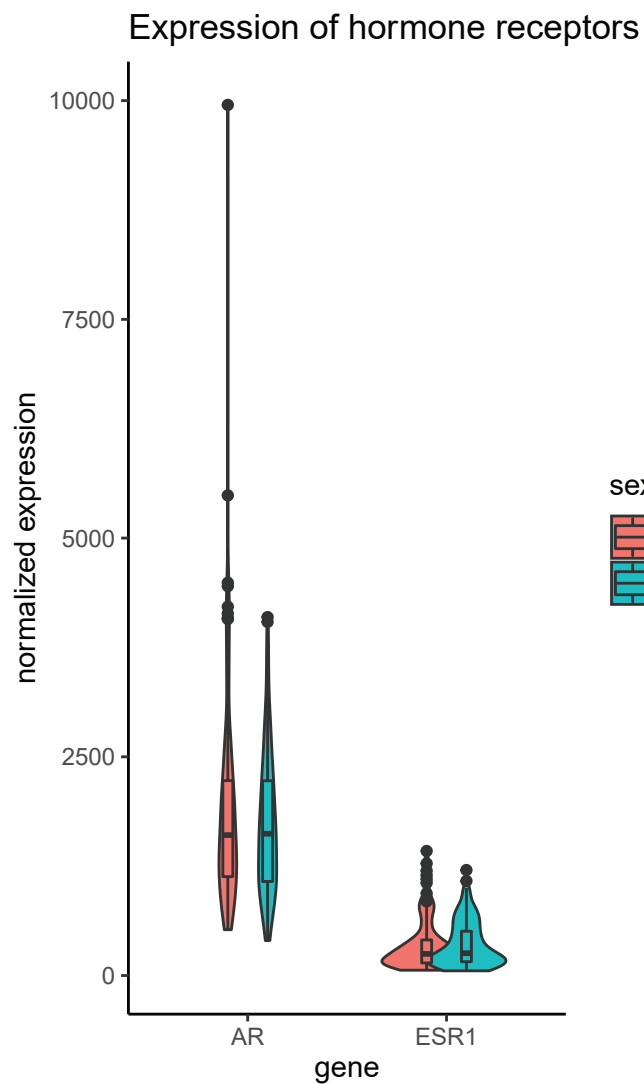
462 Figure 2



463 Figure 3

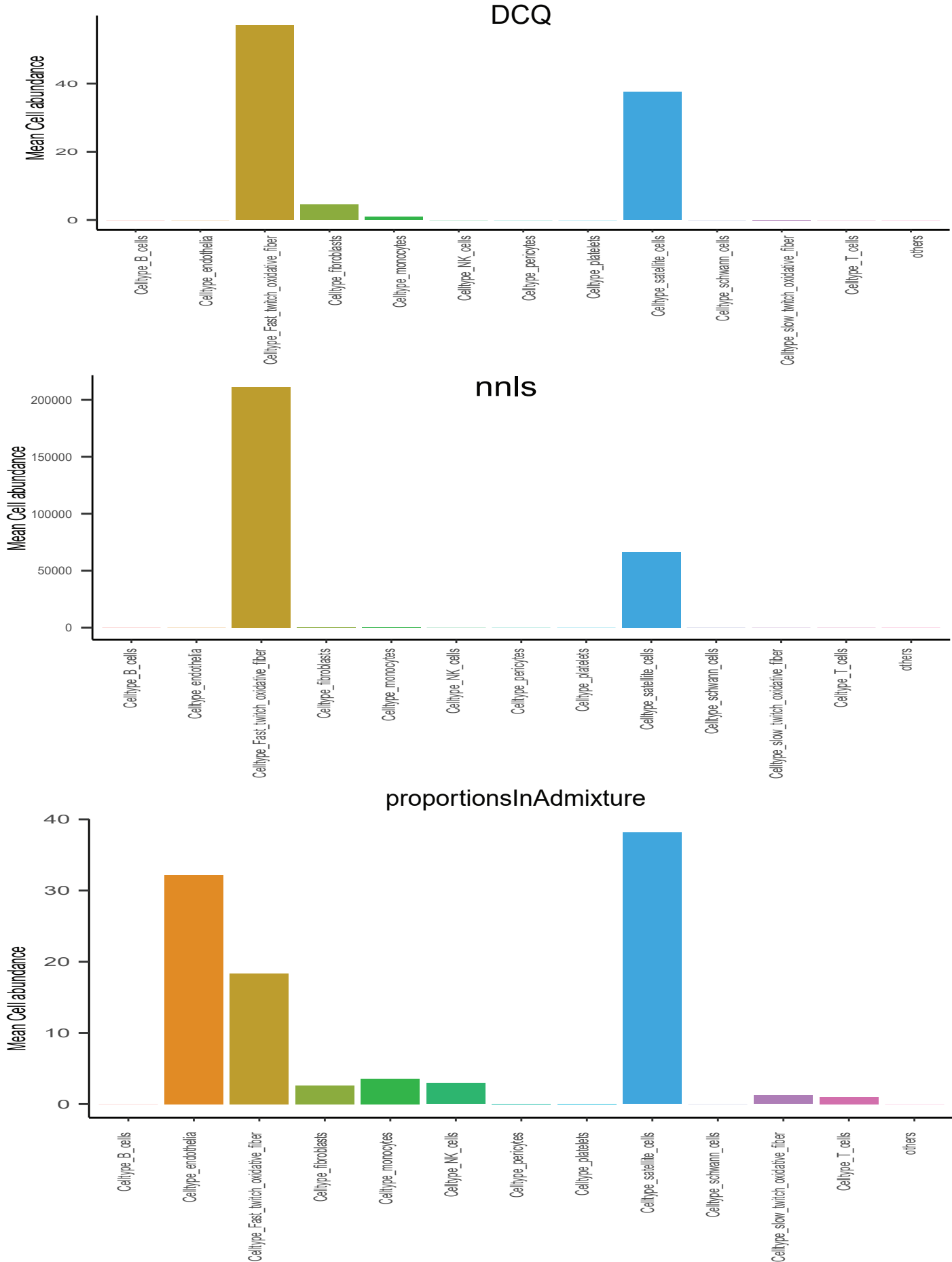


**Figure supplement 1. Skeletal muscle sex hormone receptor expression between sexes:** Normalized gene expression levels for androgen receptor (AR) or estrogen receptor (ESR1) (y-axis) in each sex (x-axis). None of the expression levels were significantly different between sexes (students t-test, two-way)





**Figure 3 | Figure Supplement 1: Comparisons of deconvolution methods.** Cell proportions were estimated from skeletal muscle sequencing available across the 310 individuals in GTEx. Here, comparisons of the three most common methods (DCQ, NNLS and proportionsInAdmixture) were plotted for each pseudo-sc-proportion, where proportionsInAdmixture method captured the largest relative number of cell types

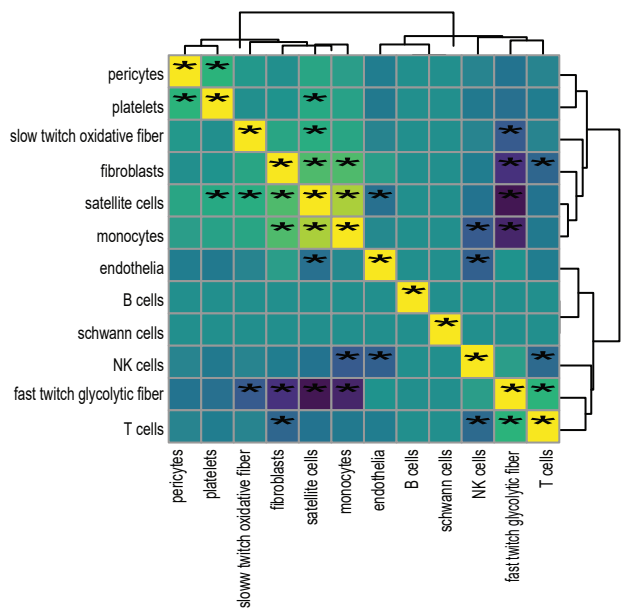


**Figure 3 Figure supplement 2. Cell composition correlations within each sex:** Heatmaps showing regressions for cell proportions in males (left) or females (right), \* = regression pvalue<0.01

### Correlations between muscle cell proportions



Males



Females

