

1 **Transcriptomics reveal stretched human pluripotent stem cell-derived**  
2 **cardiomyocytes as an advantageous hypertrophy model**

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## 13 **Summary statement**

14 Distinct hypertrophic gene expression changes in mechanically stretched human induced pluripotent  
15 stem cell-derived cardiomyocytes reveal the utility of these cells as an advantageous *in vitro* model for  
16 mechanical overload-induced hypertrophy.

## 17 **Abstract**

18 Left ventricular hypertrophy, characterized by hypertrophy of individual cardiomyocytes, is an  
19 adaptive response to an increased cardiac workload that eventually leads to heart failure. Previous  
20 studies using neonatal rat ventricular myocytes (NRVMs) and animal models have revealed several  
21 hypertrophy- and mechanical load-associated genes and signaling pathways. However, these models  
22 are not directly applicable to humans. Here, we studied the effect of cyclic mechanical stretch on gene  
23 expression of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) using RNA  
24 sequencing. HiPSC-CMs showed distinct hypertrophic changes in gene expression at the level of  
25 individual genes and in biological processes. We also identified several differentially expressed genes  
26 that have not been previously associated with cardiomyocyte hypertrophy and thus serve as attractive  
27 targets for future studies. When compared to previously published data attained from stretched  
28 NRVMs and human embryonic stem cell-derived cardiomyocytes, hiPSC-CMs displayed a smaller  
29 number of changes in gene expression, but the differentially expressed genes revealed more  
30 pronounced enrichment of hypertrophy-related biological processes and pathways. Overall, these  
31 results establish hiPSC-CMs as a valuable *in vitro* model for studying human cardiomyocyte  
32 hypertrophy.

## 33 **Introduction**

34 The prevalence of cardiovascular diseases, including coronary artery disease and hypertension, is  
35 increasing rapidly, from approximately 271 million in 1990 to 523 million in 2019 (University of  
36 Washington, Institute of Health Metrics and Evaluation, 2021). However, treatment strategies have not  
37 evolved correspondingly; hence, cardiovascular disease is the leading cause of death (Roth et al.,  
38 2017). Hypertension and myocardial infarction increase cardiac workload, causing structural and  
39 functional changes in the myocardium (Frey et al., 2004). These changes include left ventricular  
40 hypertrophy, which is characterized by cardiomyocyte enlargement. Although it is initially an adaptive  
41 response to physiological and pathological stimuli, such as mechanical stretch or neurohumoral  
42 activation, prolonged hypertrophy leads to contractile dysfunction and heart failure.

43 In response to hypertrophic stimuli, cardiomyocytes not only increase in size but also increase their  
44 protein synthesis, sarcomeres become disorganized, and specific changes in gene expression occur

45 (Lorell and Carabello, 2000). The early genetic response to stretch is activation of immediate early  
46 response genes, such as proto-oncogenes *FOS* and *JUN*, components of the transcription factor AP-1.  
47 This is followed by upregulation of natriuretic peptide B (BNP) coding gene (*NPPB*) and reactivation  
48 of fetal genes such as natriuretic peptide A (*NPPA*), myosin-7 (*MYH7*), and skeletal muscle  $\alpha$ -actin  
49 (*ACTA1*). A variety of effectors and signaling pathways mediate the hypertrophic response (Heineke  
50 and Molkentin, 2006). For example, studies in animal models and in neonatal rat ventricular myocytes  
51 (NRVMs) have identified mitogen-activated protein kinase (MAPK) (Rose et al., 2010), protein kinase  
52 C (PKC) (Palaniyandi et al., 2009) and bromodomain and extraterminal domain (BET) proteins  
53 (Borck et al., 2020) as potential mediators transducing the hypertrophic response in cardiomyocytes.  
54 However, controversial results of the exact role of these signal transducers have also been published,  
55 which can partially be explained by the use of different experimental models.

56 Cardiomyocyte hypertrophy has commonly been studied in animal models *in vivo* or in isolated  
57 NRVMs *in vitro* (Heineke and Molkentin, 2006). However, studies conducted with animals or animal  
58 cells are not always directly translatable to humans. Human induced pluripotent stem cell-derived  
59 cardiomyocytes (hiPSC-CMs) offer a unique possibility to investigate human cardiomyocytes and  
60 abolish the effects of species differences. However, although hiPSC-CMs beat spontaneously, they are  
61 relatively immature by structural, metabolic and electrophysiological properties (Robertson et al.,  
62 2013; Földes et al., 2014). We and others have previously shown that hiPSC-CMs respond to  
63 endothelin-1 (ET-1) by increasing the expression of pro-B-type natriuretic peptide (proBNP) and the  
64 corresponding gene (*NPPB*) along with other hypertrophy-related genes, although no morphological  
65 change was observed (Pohjolainen et al., 2020; Carlson et al., 2013). On the other hand, Földes et al.  
66 (Földes et al., 2014) showed that hiPSC-CMs lack the hypertrophic response to  $\alpha$ -adrenergic stimuli.  
67 Hence, it seems that not all hypertrophic signaling pathways are functional in hiPSC-CMs.

68 The aim of this study was to characterize the transcriptomic response of hiPSC-CMs to mechanical  
69 stretch. In addition, we compared stretch-induced gene expression changes to previously published  
70 data from stretched NRVMs and human embryonic stem cell-derived cardiomyocytes (hESC-CMs)  
71 (Rysä et al., 2018; Ovchinnikova et al., 2018). We also used our model to test the involvement of  
72 different signaling pathways in mechanical load-induced hypertrophy of hiPSC-CMs by  
73 pharmacological inhibition of several signaling molecules.

## 74 **Results**

### 75 **Mechanical stretch induces natriuretic peptide gene expression in hiPSC-CMs**

76 The mechanical stretch model of hiPSC-CMs was first validated by measuring the mRNA expression  
77 of *NPPA* and *NPPB*, hallmark genes of cardiomyocyte hypertrophy (Ogawa et al., 1995). After 24 h of

78 cyclic mechanical stretch, hiPSC-CMs showed increased expression of both *NPPA* and *NPPB* (Fig.  
79 1A,B). At 48 h and 72 h, the upregulation of the *NPPA* and *NPPB* mRNA levels was not statistically  
80 significant, although increased gene expression was observed in each independent experiment.

### 81 **Mechanical stretch-induced genome-wide gene expression program in hiPSC-CMs**

82 To identify genome-wide gene expression changes regulated by mechanical stretch, we performed  
83 RNA sequencing (RNAseq) at 24 h, 48 h and 72 h of mechanically stretched hiPSC-CMs and their  
84 unstretched controls. Principal component analysis showed strong separation of stretched and control  
85 samples at 24 h and 48 h defined by two principal components (Fig. S1). However, after 72 h of  
86 stretching, no clear difference between stretched and unstretched groups was detected, while  
87 separation of individual experiments was seen instead. These findings suggest strong conserved early  
88 responses to stretch and increased biological variation over time.

89 Of the 30,861 genes identified in our samples, 134 genes showed differential expression ( $FC > 1.5$ ,  
90 false discovery rate (FDR)-adjusted  $p < 0.05$ ) after stretching. Our analysis identified 75, 28 and 2  
91 upregulated genes in response to 24 h, 48 h and 72 h of stretch, respectively (Fig. 1C). In addition, 12,  
92 30 and 0 genes were downregulated in response to 24 h, 48 h and 72 h of stretch, respectively (Fig.  
93 1D). Venn diagrams demonstrated minor overlaps between time points, indicating time-dependent  
94 regulation of gene expression. The top 12 differentially expressed genes at each time point are  
95 presented in Fig. 1E,F. The full data are available in Dataset S1.

96 Multiple hypertrophy-associated genes were upregulated. These include fetal genes coding for  
97 natriuretic peptides (*NPPA* and *NPPB*), skeletal alpha actin 1 (*ACTA1*), and transgelin (*TAGLN*), and  
98 several genes encoding contractile proteins, such as cardiac alpha actin (*ACTC1*), myosin light chain 3  
99 (*MYL3*), troponins (*TNNI3*, *TNNC1*), and tropomyosin 2 (*TPM2*). In addition to contractile proteins,  
100 other cytoskeletal proteins, such as alpha- and beta-tubulins (*TUBA4A*, *TUBA1A*, *TUBB2A*, *TUBB6*),  
101 alpha-actinin 1 (*ACTN1*), nestin (*NES*) and keratins (*KRT8*, *KRT18*), were upregulated. These changes  
102 confirm that mechanical stretching of hiPSC-CMs induces alterations in gene expression, which are  
103 characteristic for cardiomyocyte hypertrophy. The upregulated genes mainly encode enzymes (26),  
104 exosomal proteins (20) and cytoskeletal proteins (15), while the downregulated genes encode enzymes  
105 (6) and transcription factors (6) (Table S2).

106 We chose eleven genes for validation by qRT-PCR. The selection was first based on differential  
107 expression of both up- and downregulated genes. Second, both protein-coding and noncoding  
108 (*LINC00648*, *PTPRG-AS1*) genes were chosen. In addition, different protein-coding genes were  
109 selected, including hypertrophy-associated secreted peptides (*NPPA*, *NPPB*), cytoskeletal proteins  
110 (*ACTA1*, *ACTC1*, *ACTN1*, *TNNI3*), a transcription factor (*CSRP3*) and a transporter protein

111 (*SLC16A9*). Overall, similar results were obtained with both qRT-PCR and RNAseq (Fig. 2), except  
112 for *ACTN1*, which showed downregulation in qRT-PCR and slight upregulation in RNAseq after 72 h  
113 of stretch (Fig. 2A).

#### 114 **Comparison of differentially expressed genes in hiPSC-CMs, NRVMs and hESC-CMs**

115 We compared our data with the NRVM data published by Rysä et al. (Rysä et al., 2018) to elucidate  
116 similarities and differences between these two *in vitro* cardiomyocyte models from different species.  
117 Both we and Rysä et al. (Rysä et al., 2018) used time points of 24 h and 48 h; hence, these were  
118 chosen for comparison, although the equivalency of these time points between species has not been  
119 proven. Overall, the number of differentially expressed genes was drastically different; for example,  
120 after a 48-h stretch, over 600 genes were upregulated in NRVMs, while only 28 genes were  
121 upregulated in hiPSC-CMs (Fig. 3). Interestingly, 21 differentially expressed genes showed similar  
122 changes in both cell models. In fact, 3 genes were upregulated in both cardiomyocyte types at both  
123 time points: *CASQ1*, *TIMP1* and *TUBB2B*. We did not identify genes that were consistently  
124 downregulated in both CM types at 24h and 48 h. Comparison of cell types after 24 h of stretch  
125 showed that 14 genes were upregulated in both cell types, while no commonly downregulated genes  
126 were identified (Fig. 3C). After 48 h of stretch, 8 genes were upregulated, and 2 genes were  
127 downregulated in both cell types (Fig. 3D). Cross comparison of different time points revealed 5  
128 upregulated genes and 2 downregulated genes in hiPSC-CMs after 48 h of stretch and in NRVMs after  
129 24 h of stretch (Table S3). In addition, 20 upregulated genes and one downregulated gene in hiPSC-  
130 CMs after a 24-h stretch were similarly differentially expressed in NRVMs after a 48-h stretch. Hence,  
131 the differentially expressed genes showed the most similarity between upregulated genes in hiPSC-  
132 CM at 24 h and in NRVMs at 48 h. Only three genes showed opposing expression in hiPSC-CMs and  
133 NRVMs: *MASP1*, *ENO3*, and *CES1* were upregulated in hiPSC-CMs but downregulated in NRVMs.

134 We also compared our differential gene expression data at 48 h with that from 48 h stretched hESC-  
135 CMs reported by Ovchinnikova et al. (Ovchinnikova et al., 2018). Of 936 differentially expressed  
136 genes in stretched hESC-CMs, only 13 genes were similarly expressed in hiPSC-CMs: four genes  
137 were upregulated, and nine genes were downregulated. The upregulated genes included *TUBB2B*,  
138 which was upregulated in all cell types (hiPSC-CMs, NRVMs and hESC-CMs) after 24 h (not studied  
139 in hESC-CMs) and 48 h of stretching. Other upregulated genes were *DUSP13*, *ACAT2* and *ENO3*. In  
140 addition, one of the genes downregulated in both hiPSC-CMs and NRVMs, *ZNF519*, was also  
141 downregulated in hESC-CMs. In hiPSC-CMs and hESC-CMs, no changes in opposite directions were  
142 observed in any differentially expressed gene. All common differentially expressed genes and their  
143 fold changes are presented in Table S3.

## 144 **Functional analysis of differentially expressed genes in stretched hiPSC-CMs**

145 To identify the biological functions regulated by the differentially expressed genes, we performed  
146 Gene Ontology (GO) enrichment analysis. The GOrilla analysis recognized 18,992 genes out of  
147 30,861 gene terms entered. Only 15,554 of these genes were associated with a GO term, and these  
148 were used for enrichment calculation. Enriched GO terms were found only for upregulated genes. The  
149 enriched biological processes of upregulated genes at all time points are presented in Figs. 4A and S2.  
150 All enriched processes were highly related to cardiomyocyte hypertrophy. Actin filament-based  
151 movement, more specifically actin-myosin filament sliding and muscle filament sliding, was the most  
152 enriched biological process. Other enriched processes can be grouped under four categories: muscle  
153 contraction, secretion, regulation of cell death, and steroid biosynthesis. This result indicates that very  
154 specific biological processes are activated in hiPSC-CMs in response to stretching.

155 We also searched for enriched GO terms for molecular function and cellular components. Again,  
156 enriched GO terms were only found for upregulated genes. Three molecular functions were  
157 significantly overrepresented: structural molecule activity, structural constituent of the cytoskeleton,  
158 and calcium-dependent protein binding (Fig. S3A). Among the cellular components, 14 GO terms,  
159 especially terms related to extracellular vesicles, supramolecular complexes and cytoskeleton, were  
160 enriched (Fig. S3B). These analyses confirm that structural and cytoskeletal protein-coding genes are  
161 among the most upregulated genes in stretched hiPSC-CMs. The complete data of the GO analyses are  
162 available in Dataset S2.

## 163 **Comparison of the functional analysis of hiPSC-CMs and NRVMs**

164 To compare stretch-induced enriched biological processes of hiPSC-CMs and NRVMs, we performed  
165 a similar GO analysis for upregulated genes of NRVMs studied by Rysä et al. (Rysä et al., 2018). In  
166 line with a higher number of upregulated genes in NRVMs compared to hiPSC-CMs, more enriched  
167 biological processes were found (71 GO terms). Hence, while a very limited number of specific  
168 processes were enriched in stretched hiPSC-CMs, a broad range of biological processes were detected  
169 in NRVMs. The most evidently enriched biological processes associated with upregulated genes in  
170 NRVMs were RNA metabolic processes, response to stimulus, biosynthetic processes, cellular  
171 component biogenesis, developmental processes, and regulation of cell death (Fig. 4B). Upregulated  
172 genes from both hiPSC-CMs and NRVMs thus share GO terms associated with the regulation of  
173 apoptosis and steroid biosynthesis.

174 To further discover the functionality of the differentially expressed genes, KEGG and Reactome  
175 pathway analyses were performed. KEGG pathway analysis revealed 11 and 10 enriched pathways in  
176 upregulated genes in hiPSC-CMs and NRVMs, respectively (Fig. 5A,B). However, none of the

177 pathways was common to both cell types. In hiPSC-CM, the pathways included cardiac- and  
178 cardiomyocyte-associated pathways, while the enriched terms in NRVMs were heterogeneous, and  
179 half of them were cancer-associated. In turn, Reactome pathway analysis resulted in 31 and 17  
180 enriched pathways for hiPSC-CMs and NRVMs, respectively (Fig. 5C,D). Three pathways were  
181 enriched in both cell types: striated muscle contraction, HSP90 chaperone cycle for steroid hormone  
182 receptors (SHRs), and the role of GTSE1 in G2/M progression after the G2 checkpoint.

### 183 **Enriched transcription factor targets of stretch-induced genes**

184 Several transcription factors (TFs) are associated with cardiomyocyte hypertrophy (Heineke and  
185 Molkentin, 2006; Kohli et al., 2011). Hence, we analyzed which TF target sites were enriched in  
186 response to stretch. In our analysis, 19 and 18 TF binding sites were enriched in upregulated genes of  
187 stretched hiPSC-CMs and NRVMs, respectively (Fig. 6A,B). Most of the enriched binding sites were  
188 for serum response factor (SRF), which had 5 enriched binding sites in both cell types. SRF controls  
189 the expression of genes regulating the cytoskeleton during development and cardiac hypertrophy  
190 (Coletti et al., 2016; Nelson et al., 2005). In addition, two binding sites for the transcription factor c-  
191 Jun, which is part of the AP-1 complex and is involved in cardiomyocyte hypertrophy and increased  
192 steroidogenic gene expression (Windak et al., 2013; Lan et al., 2007), were enriched in both hiPSC-  
193 CMs and NRVMs. Both cell types also had an enriched binding site for transcription factor E2-alpha  
194 (TCF3) and for nuclear factor erythroid-derived 2 (NFE2). In hiPSC-CMs, two binding sites for  
195 myocyte enhancer Factor 2A (MEF2A) were enriched. MEF2A is known to regulate multiple cardiac  
196 structural genes and to be activated by several hypertrophic signaling pathways (Czubryt and Olson,  
197 2004; Xu et al., 2006; Han and Molkentin, 2000). In NRVMs, two binding sites for both MYC proto-  
198 oncogene protein and heat shock transcription factor 1 (HSF1) were enriched.

### 199 **Differentially expressed lncRNAs**

200 Of the 7,818 long noncoding RNAs (lncRNAs) expressed in our samples, only one lncRNA was  
201 differentially expressed after 24 h of stretch: *LINC00648* was downregulated by 50% ( $p=0.011$ )  
202 relative to the unstretched control. *LINC00648* also showed downregulation by 30% in hESC-CMs  
203 after a 48-h stretch (Ovchinnikova et al., 2018). In hiPSC-CMs, after 48 h of stretch, one lncRNA  
204 (*LINC00702*) was upregulated, while five lncRNAs (*AUXG01000058.1*, *AZINI-AS1*, *LAMTOR5-AS1*,  
205 *LINC01341*, *PTPRG-AS1*) were downregulated. There is no previous report of these lncRNAs being  
206 involved in cardiomyocyte hypertrophy. Predicted putative interaction partners of the differentially  
207 expressed lncRNAs are shown in Table S4. None of these interaction partners was differentially  
208 expressed ( $>1.5$ -fold) in response to stretch. However, an interaction partner of *AZINI-AS1*, a gene  
209 encoding Egl-9 family hypoxia inducible factor 3 (*EGLN3*), was upregulated 1.48-fold ( $p=0.0454$ ).

210 EGLN3 is a prolyl hydroxylase that is activated by hypoxia and may regulate cardiomyocyte apoptosis  
211 (Liu et al., 2010).

## 212 **The effects of p38 MAPK, MEK1/2, PKC and BET inhibition on the stretch response**

213 To gain further insight into the signaling routes mediating the stretch response in hiPSC-CMs, the  
214 involvement of p38 MAPK, MEK1/2 and PKC signaling pathways as well as BET in the stretch  
215 response of hiPSC-CMs was examined by pharmacological inhibitors and analysis of *NPPA* and  
216 *NPPB* mRNA expression after mechanical stretch for 24 h. In this experiment, *NPPA* expression was  
217 not significantly affected by stretch, while a 5.6-fold increase ( $p=0.008$ ) in *NPPB* expression was  
218 detected in 0.1% DMSO-treated stretched cells compared to unstretched cells (Fig. 7). The MEK1/2  
219 inhibitor U0126 at a concentration of 10  $\mu\text{M}$  decreased both *NPPA* and *NPPB* gene expression (40%,  
220  $p=0.008$  and 80%,  $p=0.008$ , respectively), while the cPKC inhibitor Gö6976 at 1  $\mu\text{M}$  increased *NPPB*  
221 expression 1.8-fold ( $p=0.016$ ). In addition, the p38 MAPK inhibitor SB203580 at 10  $\mu\text{M}$  and the BET  
222 inhibitor JQ1 at 300 nM showed a tendency toward increased *NPPA* and *NPPB* expression. An  
223 inhibitor of all PKC isoforms, Gö6983 at 1  $\mu\text{M}$ , did not affect basal *NPPA* or *NPPB* gene expression.

224 The effect of compounds on the stretch-induced hypertrophic response was evaluated by *NPPB*  
225 expression (Fig. 7B). None of the treatments could fully block the stretch response, although U0126  
226 significantly decreased stretch-induced *NPPB* expression compared to DMSO-treated stretched cells  
227 (70%,  $p=0.016$ ). SB203580 and Gö6976 slightly increased the *NPPB* stretch response relative to  
228 DMSO, while Gö6983 tended to decrease it. The BET inhibitor JQ1 did not affect the stretch-induced  
229 increase in *NPPB* expression.

## 230 **Discussion**

231 Prolonged mechanical load leads to maladaptive changes in the heart, including cardiomyocyte  
232 hypertrophy and left ventricular hypertrophy, which are major causes of heart failure (Heineke and  
233 Molkenin, 2006). Understanding the molecular mechanisms that underlie the development of left  
234 ventricular hypertrophy is essential for finding new treatments for heart failure. Identification of genes  
235 and pathways involved mechanical stretch response of cardiomyocytes is therefore of great interest.

236 The optimal *in vitro* model of cardiomyocyte hypertrophy would use adult human cardiomyocytes.  
237 However, they are difficult to obtain and culture long-term, so the most commonly used *in vitro*  
238 hypertrophy models employ NRVMs. To study this phenomenon in human cells and reduce the use of  
239 experimental animals, we used hiPSC-CMs. *In vitro* models of hiPSC-CMs are increasingly used in  
240 disease modeling and drug development (Ovics et al., 2020; Protze et al., 2019; Karakikes et al.,  
241 2015). To our knowledge, the transcriptional responses of hiPSC-CMs to cyclic mechanical stretch  
242 have not been characterized before. We validated the model by measuring the expression of well-



243 established mechanical stress-responsive genes, *NPPA* and *NPPB* (Ogawa et al., 1995), and showed  
244 that cyclic mechanical stretch leads to *NPPA* and *NPPB* gene expression responses comparable to  
245 those of NRVMs (Pikkarainen et al., 2003; Rysä et al., 2018).

246 We then performed RNAseq to identify additional gene expression changes involved in the  
247 hypertrophic response. Surprisingly, the number of differentially expressed genes in the stretched  
248 hiPSC-CMs was drastically lower than in the stretched NRVMs or hESC-CMs (Ovchinnikova et al.,  
249 2018; Rysä et al., 2018). In the NRVM study (Rysä et al., 2018), the species difference and the  
250 different methods (microarray in the NRVM study and RNAseq in the present hiPSC-CM analysis)  
251 may partly explain the difference. Furthermore, the hiPSC-CM cultures used in the current analysis  
252 were  $\geq 95\%$  pure cardiomyocytes, while NRVMs isolated from the heart usually contain other cardiac  
253 cell types, such as fibroblasts and endothelial cells, despite enrichment with preplating. In agreement  
254 with this, a broad range of gene expression changes was observed in NRVMs, including several  
255 changes associated with cardiac fibroblast activation such as upregulation of  $\alpha$ -smooth muscle actin  
256 (*ACTA2*), a hallmark of fibroblast activation, and overrepresentation of GO terms of extracellular  
257 matrix and collagen-containing extracellular matrix. Similar changes in gene expression were not  
258 detected in stretched hiPSC-CM, suggesting that pure cardiomyocyte cultures are more suitable to  
259 study the changes occurring specifically in cardiomyocytes. In the hESC-CM study (Ovchinnikova et  
260 al., 2018),  $>98\%$  pure cardiomyocytes were used, but the magnitude and frequency of the stretch  
261 applied were different. Moreover, the age and maturation level of the cardiomyocytes were not  
262 described in the hESC-CM study, and these differences may influence the comparison of the two  
263 human cardiomyocyte models. Although transcriptionally hESC-CMs and hiPSC-CMs are very  
264 similar (Gupta et al., 2010), their responses to stretch were different. As NRVMs, hESC-CMs also  
265 seem to respond to stretching by inducing a broad range of gene expression changes, while in hiPSC-  
266 CMs, differentially expressed genes are more defined. Only one gene, *TUBB2B*, coding for tubulin  
267 beta-2A chain, a constituent of microtubules, was upregulated in all cardiomyocyte types. In hiPSC-  
268 CMs, other forms of alpha- and beta-tubulins were also upregulated. The increase in the expression of  
269 microtubules is strongly associated with cardiac hypertrophy; thus, this change was expected  
270 (Caporizzo et al., 2019). In contrast, one gene, *ZNF519*, coding for zinc finger protein 519, was  
271 downregulated in all cell models. *ZNF519* has not been characterized in cardiomyocytes, and its  
272 potential role in the development of cardiomyocyte hypertrophy remains to be established.

273 In response to stretch, two central changes occur in cardiomyocytes: (1) several genes normally  
274 expressed only in embryonic or fetal hearts are reactivated, and (2) the expression of sarcomeric and  
275 other constitutive proteins is increased (Kuwahara et al., 2012; Hoshijima and Chien, 2002). Here, we  
276 showed that these changes occur also in hiPSC-CMs in response to stretching. The upregulation of

277 contractile proteins was also reflected in the GO enrichment analysis, where most enriched processes  
278 were associated with actin-myosin filament sliding and muscle contraction.

279 Although the differentially expressed genes and their numbers differed among the studies using  
280 different cardiomyocyte models, some similarities in the enriched processes and pathways were  
281 discovered. Regulation of cell death and sterol biosynthesis were enriched in the upregulated genes in  
282 all cell types. Apoptosis has previously been linked to hypertrophy in multiple studies both in rodents  
283 and in humans (Okada et al., 2004; Mohamed et al., 2016; Fujita and Ishikawa, 2011; Condorelli et al.,  
284 1999). Although the upregulation of genes associated with steroid biosynthesis has been reported in  
285 previous studies (Rysä et al., 2018; Ovchinnikova et al., 2018), its role in cardiomyocyte hypertrophy  
286 has not been characterized. Increased steroid synthesis might be needed for the growth of  
287 cardiomyocytes or may be associated with changes in energy metabolism. Steroid biosynthesis is  
288 downregulated in the neonatal mouse heart within the first nine days of postnatal life, during which the  
289 heart loses its regenerative capacity (Talman et al., 2018). Hence, it can be speculated that increased  
290 steroid synthesis is a part of the fetal program that is reactivated in response to stress.

291 Several genes associated with both apoptosis and cardiomyocyte hypertrophy, such as *CRYAB*, *ENO1*  
292 and *GSTO1*, were among the upregulated genes in hiPSC-CMs (Kumarapeli et al., 2008; Chis et al.,  
293 2012; Captur et al., 2020; Dulhunty et al., 2001; Piaggi et al., 2010; Manupati et al., 2019; Wang, K. et  
294 al., 2021; Wang, L. et al., 2019). *ENO1*, which codes for the glycolytic enzyme  $\alpha$ -enolase and is  
295 normally highly expressed in embryonic and fetal heart but only weakly in adult heart, has shown to  
296 increase during hypertrophy in animal models (Gao et al., 2018; Keller et al., 1995; Zhu et al., 2009).  
297 This is in line with previous evidence of a metabolic switch from fatty acid to glycolysis during  
298 pathological hypertrophy (Lehman and Kelly, 2002). Furthermore, one study has shown compensatory  
299 increase in  $\alpha$ -enolase expression to protect cardiomyocytes from hypertrophy (Gao et al., 2018).  
300 Interestingly, after a 48-h stretch, the most upregulated genes were neuropeptide galanin and GMAP  
301 prepropeptide coding gene *GAL*. Galanin is expressed mainly in the nervous system and in some  
302 peripheral organs, but no expression in cardiomyocytes has been reported (Palkeeva et al., 2019).  
303 However, its receptors are expressed in various cell types, including cardiomyocytes, and it has been  
304 suggested to be cardioprotective (Fang et al., 2013; Studneva et al., 2020; Serebryakova et al., 2019;  
305 Palkeeva et al., 2019; Martinelli et al., 2021).

306 All cardiomyocyte types included in the present comparisons, hiPSC-CMs, hESC-CMs and NRVMs,  
307 are considered relatively immature and do not fully correspond to adult cardiomyocytes in terms of  
308 their sarcomere structure, metabolism, or electrophysiological properties (Robertson et al., 2013).  
309 However, based on our comparison, stretched hiPSC-CMs were the only cell model in which  
310 biological processes of muscle contraction and actin-myosin filament sliding were enriched among the  
311 upregulated genes. In view of *in vivo* cardiac overload, these are the most important processes to

312 enhance in order to preserve cardiac pump function. However, these changes could also imply  
313 maturation of hiPSC-CMs, but this is unlikely because they were accompanied by upregulation of the  
314 fetal gene program and apoptosis-associated genes. Moreover, hiPSC-CMs were the only cells in  
315 which upregulated genes had enrichment of pathways for hypertrophic cardiomyopathy. On the  
316 contrary, these pathways were enriched among downregulated genes of stretched hESC-CMs  
317 (Ovchinnikova et al., 2018). Taken together, hiPSC-CMs show distinct hypertrophic changes in gene  
318 expression at the levels of individual genes and biological processes, indicating that cyclic stretching  
319 of hiPSC-CMs is an advantageous *in vitro* model for studying mechanically induced cardiomyocyte  
320 hypertrophy.

321 Finally, we applied our model to study signaling pathways associated with cardiomyocyte  
322 hypertrophy. Although p38 MAPK, MEK1/2-ERK1/2, PKC and BET are widely studied in animal  
323 models, their role in human cardiomyocytes is poorly known. In this study, the tested inhibitors of  
324 these transducers could not block the *NPPB* stretch response completely, indicating that these  
325 signaling pathways are not necessary for mechanical stretch-induced hypertrophy of hiPSC-CMs.  
326 Previous studies using MEK1/2 and BET inhibitors have demonstrated that MEK1/2 and BET mediate  
327 ET-1-induced proBNP and *NPPB* expression in hiPSC-CMs (Pohjolainen et al., 2020; Duan et al.,  
328 2017). Thus, in hiPSC-CMs, ET-1 and mechanical stimulation seem to induce BNP expression  
329 through different signaling pathways.

330 In conclusion, in the present study, we showed that mechanical stretching of hiPSC-CMs is a relevant  
331 *in vitro* model for studying human cardiomyocyte hypertrophy. We elucidated stretch-induced  
332 transcriptional changes and identified biological processes and pathways associated with these gene  
333 expression changes. The changes, including activation of the fetal gene program and upregulation of  
334 constitutive protein coding genes, were characteristic of cardiomyocyte hypertrophy. Comparison to  
335 previous data of stretched NRVMs and hESC-CMs demonstrated that hiPSC-CMs revealed more  
336 defined changes in gene expression and that differentially expressed genes were restricted to cardiac  
337 and hypertrophy-related genes. In addition, we identified several differentially expressed genes with  
338 no or weak previous association with cardiomyocyte hypertrophy. These results can be used to further  
339 elucidate hypertrophic signaling pathways and to discover potential pharmacological targets and  
340 biomarkers of cardiomyocyte hypertrophy.

## 341 **Materials and Methods**

### 342 **Compounds and reagents**

343 The MEK1/2 inhibitor U0126 and the p38 MAPK inhibitor SB203580 were purchased from Tocris  
344 Bioscience (Bristol, UK), the pan-PKC inhibitor Gö6983 was purchased from STEMCELL

345 Technologies (Vancouver, Canada), and the classical PKC inhibitor Gö6976 and the BET inhibitor  
346 JQ1 were purchased from Merck (Darmstadt, Germany). Growth factor-reduced Matrigel was  
347 purchased from Corning (Bedford, MA, USA), and the small-molecule inhibitors Y-27632,  
348 CHIR99021 and Wnt-C59 were purchased from Tocris Bioscience (Bristol, UK). All other cell culture  
349 reagents were purchased from Gibco (Paisley, UK).

### 350 **Human induced pluripotent stem cell-derived cardiomyocytes**

351 The hiPS(IMR90)-4 line was purchased from WiCell (Madison, WI, USA). Cells were maintained in  
352 Essential 8 medium on Matrigel-coated 6-well plates at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.  
353 Cells were passaged 1:15 approximately every four days using Versene and regularly tested negative  
354 for mycoplasma contamination. Cardiomyocytes were differentiated as described previously  
355 (Pohjolainen et al., 2020; Burridge et al., 2014; Karhu et al., 2018). When the cultures were 80-95%  
356 confluent, differentiation was initiated by the addition of 6 µM CHIR99021 (Day 0) to RPMI 1640  
357 medium supplemented with B-27 without insulin (RB-). On Day 1 or Day 2, the medium was changed  
358 to fresh RB-. On Day 3, fresh RB- containing 2.5 µM Wnt-C59 was added. On Days 5, 7 and 9, RB-  
359 was changed to fresh RB-. On Day 11, metabolic selection of cardiomyocytes was started by changing  
360 the RB- to RPMI 1640 without glucose supplemented with B-27 (with insulin). On Day 13, the cells  
361 were fed fresh metabolic selection medium. From Day 15 onwards, the cardiomyocytes were cultured  
362 in RPMI 1640 supplemented with B-27 (RB+). On Days 15 or 17, the differentiated hiPSC-CMs were  
363 dissociated and seeded at a density of 700,000-800,000 cells/well in RB+ supplemented with 10%  
364 fetal bovine serum on flexible collagen I-coated 6-well BioFlex® culture plates (Flexcell International  
365 Corporation, Hillsborough, NC, USA) with additional Matrigel coating. The hiPSC-CMs were  
366 allowed to attach for 48 h, after which the medium was changed to serum-free RB+. Cardiomyocytes  
367 were maintained by changing fresh RB+ approximately every four days until the start of experiments  
368 on Days 29-43.

### 369 **Cyclic mechanical stretch**

370 The hiPSC-CMs were exposed to cyclic mechanical stretch by applying vacuum suction to the  
371 BioFlex® plates with an FX-5000 Tension System (Flexcell International Corporation). For  
372 pharmacological assays, compounds were added one hour before starting the stretch. Equibiaxial  
373 stretch was applied for 24 h, 48 h or 72 h in two-second cycles (0.5 Hz) to induce 10 to 21% stretch.  
374 Unstretched control cells were from the same differentiation and were seeded on BioFlex® plates at  
375 the same time, but no stretch was applied.

## 376 **RNA isolation**

377 Cells were lysed in 350  $\mu$ l of RA1 lysis buffer (Macherey-Nagel, Düren, Germany) supplemented with  
378 1%  $\beta$ -mercaptoethanol and stored at -80 °C (maximum one month) before RNA isolation. RNA was  
379 isolated using a NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's instructions.  
380 Analysis of the RNA concentration and quality was performed with a NanoDrop 1000  
381 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) for qRT-PCR and with 4200  
382 TapeStation (Agilent, Santa Clara, CA, USA) for RNAseq. One of the sample pairs at 24 h time point  
383 was omitted from the RNAseq due to poor quality (RNA integrity number < 9).

## 384 **Quantitative Reverse Transcription PCR (qRT-PCR)**

385 cDNA was synthesized from 100–500 ng of total RNA in 10  $\mu$ l reactions with a Transcriptor First  
386 Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) according to the manufacturer's protocol  
387 using random hexamer primers and an MJ Mini Personal thermal cycler (Bio-Rad, Hercules, CA,  
388 USA). The cDNA was diluted 1:10 in PCR grade H<sub>2</sub>O and stored at -20 °C. Commercial TaqMan®  
389 Gene Expression Assays (Thermo Fisher Scientific) listed in Table S1 were used with LightCycler®  
390 480 Probes Master reagent (Roche) according to the manufacturer's instructions. A LightCycler® 480  
391 Real-Time PCR System (Roche) was used to analyze 4.5  $\mu$ l of the cDNA dilution in 10  $\mu$ l reactions on  
392 a white LightCycler® 480 Multiwell Plate 384 (Roche). To confirm the absence of PCR  
393 contamination, no-template controls were used. Each reaction was run at least in triplicate, and the  
394 average of the technical replicates was used in the analysis as n=1. Grubbs' test was used to identify  
395 outliers within technical replicates and identified outliers were excluded from the analysis. The  $2^{-\Delta\Delta Ct}$   
396 method was used to analyze the relative gene expression using *ACTB* and *18S rRNA* as reference  
397 genes.

## 398 **RNA sequencing**

399 RNAseq was performed as single-end sequencing for a read length of 75 bp with an Illumina NextSeq  
400 500 sequencer (Illumina, San Diego, CA, USA) in high output runs using a NEBNext Ultra  
401 Directional RNA Library Prep kit (New England Biolabs, Ipswich, MA, USA) including rRNA  
402 depletion. Data quality was analyzed by FastQC, and quality trimming was applied to the data with  
403 Trimmomatic software (Bolger et al., 2014). The sample reads were aligned against the Genome  
404 Reference Consortium Human Build 38 patch release 13 (GRCh38.p13, GCA\_000001405.28)  
405 reference with Spliced Transcripts Alignment to a Reference (STAR) (Dobin et al., 2013). The  
406 mapping quality was assessed with Qualimap (Okonechnikov et al., 2016). Read quantification was  
407 created with featureCounts (Liao, Yang et al., 2014), and differential expression with quality  
408 assessment was performed with DESeq2 (Love et al., 2014).

## 409 **Functional enrichment analysis of gene sets**

410 GO enrichment of differentially expressed genes was analyzed with GOrilla (version updated 27<sup>th</sup> of  
411 February 2021, available at <http://cbl-gorilla.cs.technion.ac.il/>) (Eden et al., 2009). The tool searched  
412 for GO terms that were enriched in the target set compared to the background set. A list of upregulated  
413 or downregulated genes was entered as target sets. As the background set, we used all expressed genes  
414 in our dataset (30,861 genes), defined as genes with a detected signal in at least two samples in one  
415 treatment group. A relatively low p value threshold of  $p < 0.0001$  was used for running the analysis, as  
416 no correction for multiple testing was applied. FDR-adjusted p values were calculated after the  
417 analysis, and an FDR-adjusted p value  $< 0.05$  was considered significant.

418 WebGestalt (version 2019, available at <http://www.webgestalt.org/>) was used for KEGG and  
419 Reactome pathway analyses and for transcription factor target analysis (Liao, Yuxing et al., 2019).  
420 The same target and reference gene sets as in the GO enrichment analysis were used. The Benjamini-  
421 Hochberg method was used for multiple testing, and a significance level of  $< 0.05$  was used. KEGG  
422 Mapper (available at <https://www.genome.jp/kegg/mapper.html>) was used to determine the cellular  
423 functions of proteins representing the differentially expressed genes [24]. Encyclopedia of RNA  
424 Interactomes (ENCORI; available at <http://starbase.sysu.edu.cn/>) was used to predict the putative  
425 interaction partners of differentially expressed lncRNAs (Li et al., 2014).

## 426 **Dataset comparison**

427 Our dataset of stretched hiPSC-CMs was compared to datasets of the two other studies. We used data  
428 from differentially expressed genes of stretched NRVMs by Rysä et al. (Rysä et al., 2018), who  
429 isolated NRVMs from 2- to 4-day-old Sprague–Dawley rats and stretched cells with the FlexCell  
430 vacuum system, similar to the present study (0.5 Hz, 10-25% elongation). We also compared our data  
431 to the data of stretched hESC-CMs obtained by Ovchinnikova et al. (Ovchinnikova et al., 2018), who  
432 had used a slightly different stretching protocol: cyclic stretch with elongation from 0% to 15% was  
433 applied at a frequency of 1 Hz with the FlexCell system.

## 434 **Statistical analysis**

435 Each treatment group comprised 3–5 independent experiments of cells from individual  
436 differentiations. Sample size was determined by initial analysis of *NPPA* and *NPPB* responses  
437 measured by qRT-PCR and previous study on NRVMs (Rysä et al., 2018). Each qRT-PCR included  
438 technical replicates, and the average was calculated for statistical analysis to represent  $n=1$ . Statistical  
439 analysis of the qRT-PCR results was performed in IBM SPSS Statistics 25 software. Student's t-test  
440 for independent samples was performed to compare the stretched and unstretched samples. A  
441 nonparametric Mann–Whitney U test was applied for the normalized qRT-PCR data of the

442 pharmacological inhibitor experiments. A value of  $p < 0.05$  was considered statistically significant.  
443 Statistical analysis of the RNAseq results was performed as a pairwise comparison of stretched and  
444 unstretched samples using the Wald test in DESeq2 software. Genes with a fold change (FC)  $> 1.5$  and  
445 Benjamini-Hochberg adjusted  $p < 0.05$  were defined as differentially expressed.

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## 451 **Competing interests**

452 No competing interests declared.

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## 457 **Data Availability Statement**

458 The RNAseq data are freely available at the NCBI Gene Expression Omnibus  
459 (<http://www.ncbi.nlm.nih.gov/geo>, accession number GSE186208). All other data are available from  
460 the corresponding author upon reasonable request.

## 461 **Author contributions statement**

462 Lotta Pohjolainen: Conceptualization, Methodology, Validation, Formal analysis, Investigation,  
463 Writing – original draft preparation

464 Heikki Ruskoaho: Conceptualization, Methodology, Writing – review and editing, Supervision,  
465 Project administration, Funding acquisition

466 Virpi Talman: Conceptualization, Methodology, Writing – review and editing, Supervision, Project  
467 administration, Funding acquisition

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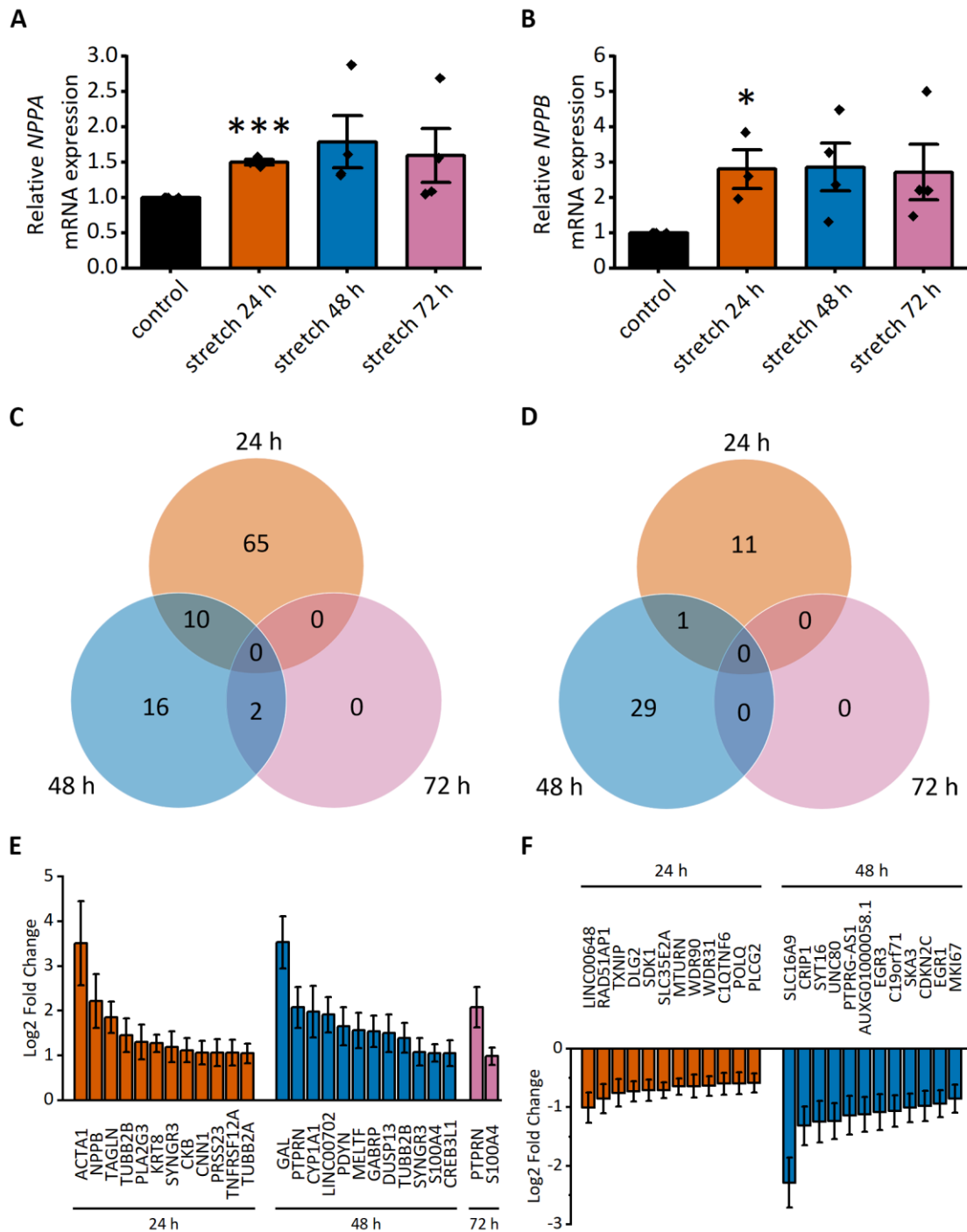
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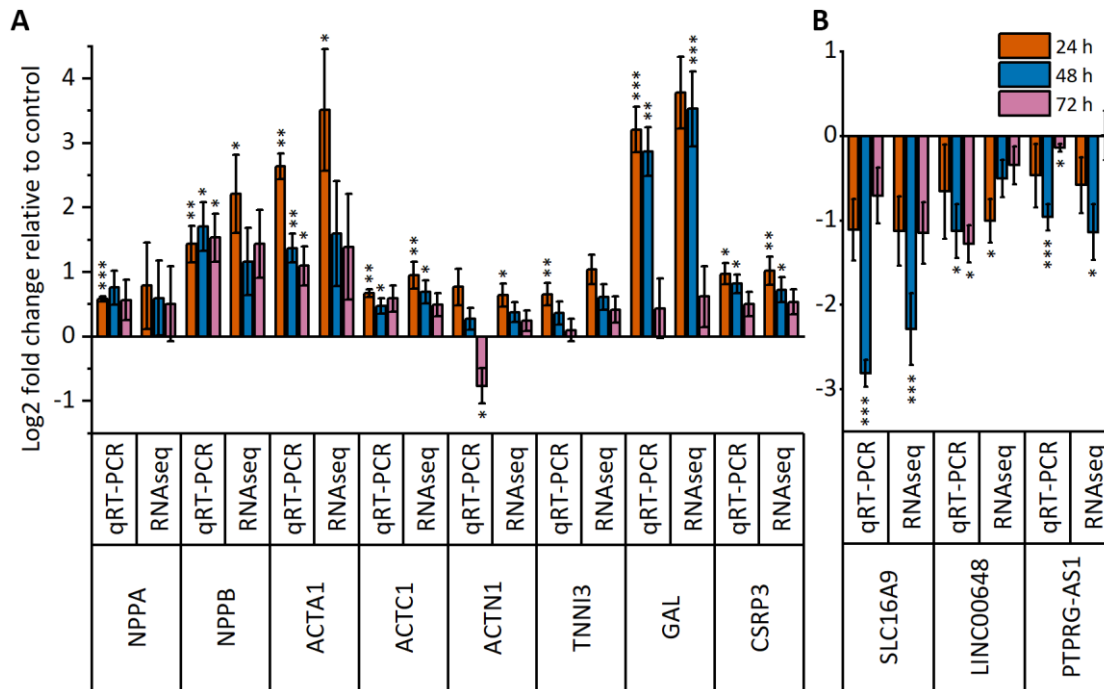
643 **Figures**



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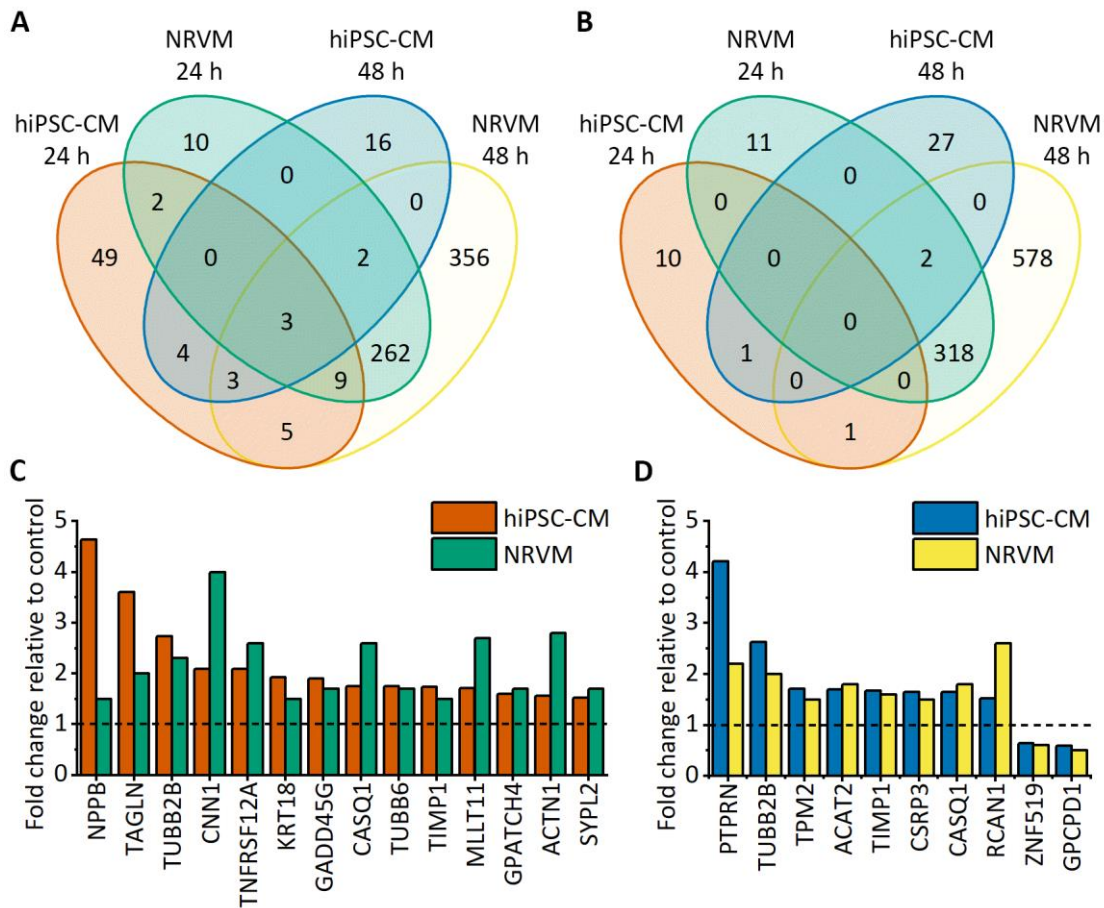
645 **Figure 1. Mechanical stretch-induced differential gene expression of human induced pluripotent**  
 646 **stem cell-derived cardiomyocytes (hiPSC-CMs).** A-B, HiPSC-CMs respond to stretch by increased  
 647 expression of hypertrophy-associated genes NPPA (natriuretic peptide A; A) and NPPB (natriuretic  
 648 peptide B; B). mRNA expression of 24 h, 48 h and 72 h stretched hiPSC-CMs measured with qRT-  
 649 PCR was normalized to the unstretched control. Bars present the mean, dots present individual values,  
 650 and error bars present the standard error. \* $p < 0.05$ , \*\*\* $p < 0.001$  vs. unstretched control, Student's t-test

651 for independent samples. C-D, Venn diagrams show the number of upregulated (C) and  
 652 downregulated (D) genes after 24 h, 48 h and 72 h of cyclic stretch measured with RNA sequencing.  
 653 Differential expression was defined as a >1.5-fold change compared to the unstretched control. E-F,  
 654 The expression of the top 12 up- (E) and downregulated (F) genes is presented as log<sub>2</sub>-fold change  
 655 relative to the unstretched control ± standard error (n=3 for 24 h, n=4 for 48 h and 72 h, n represents  
 656 biological replicates of cells from individual differentiations). Only statistically significant  
 657 (Benjamini-Hochberg adjusted p<0.05) results are presented.



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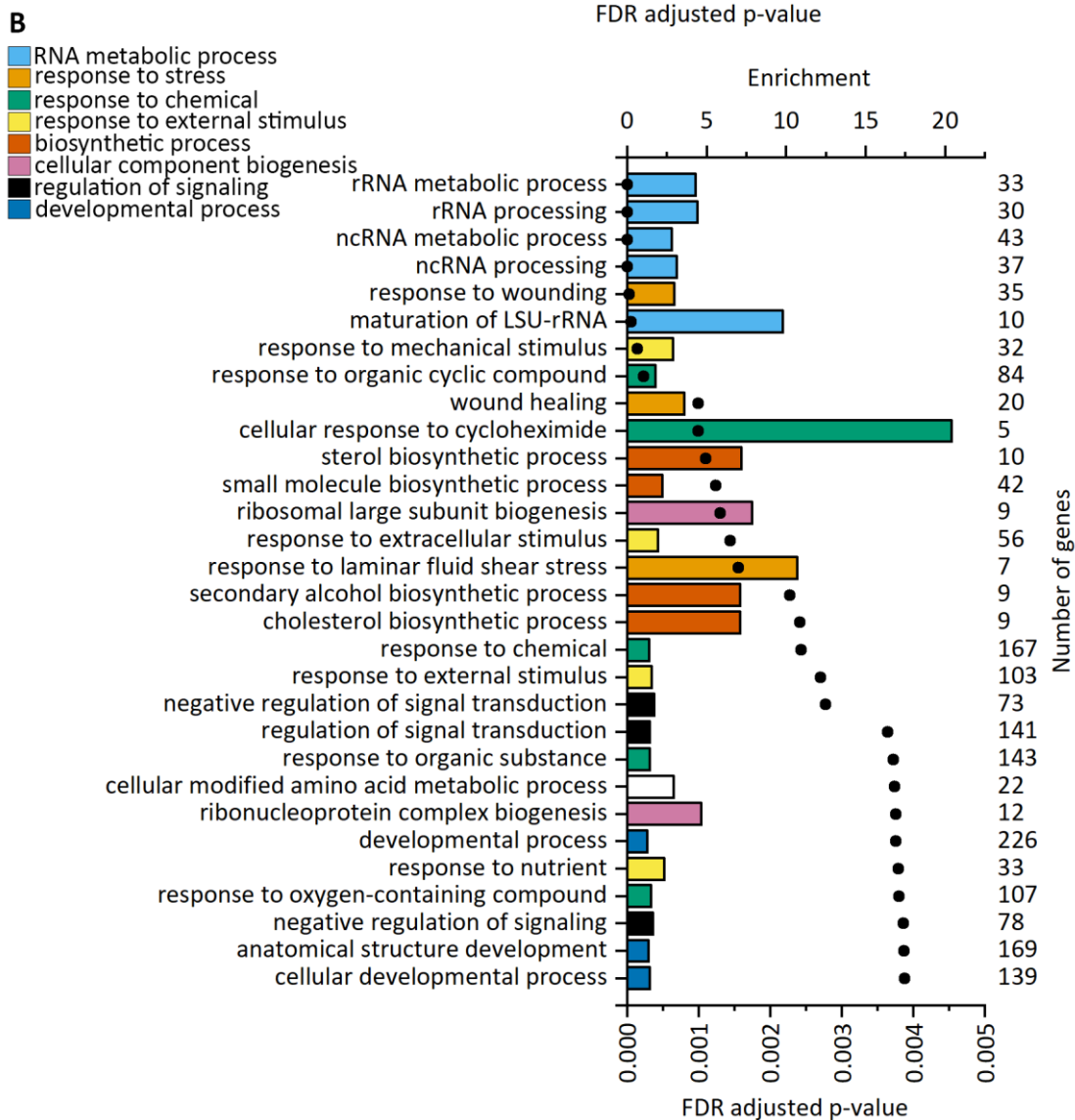
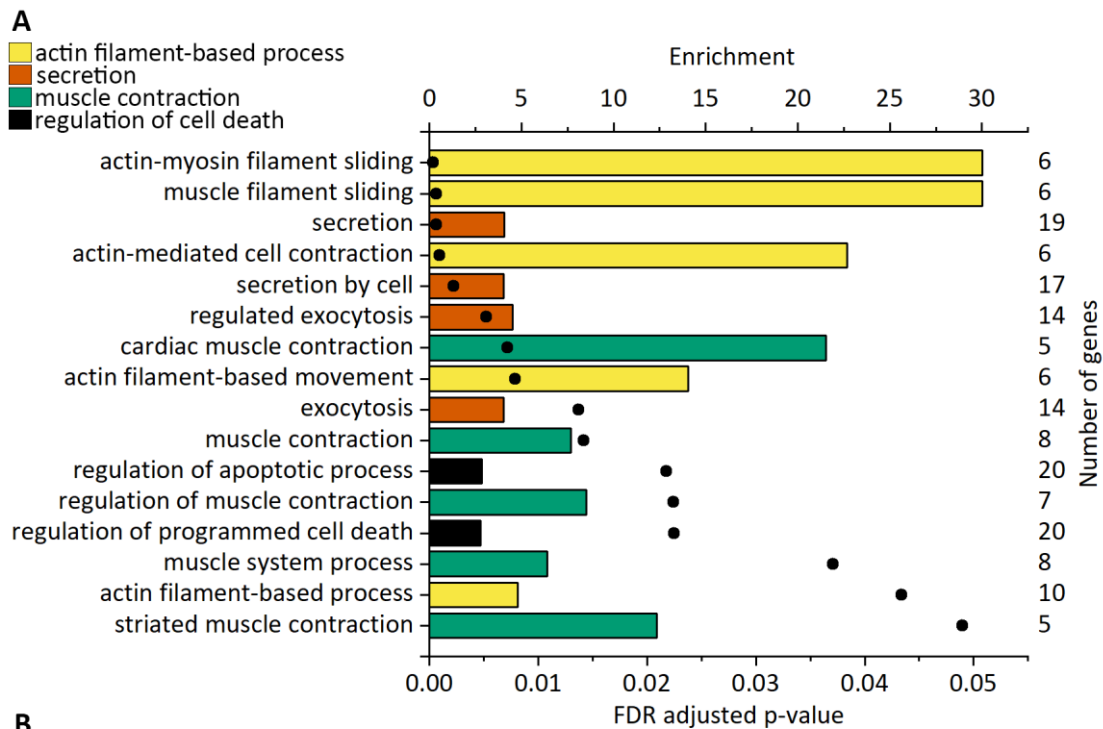
659 **Figure 2. Time-dependent changes in gene expression of selected genes.** Differential expression of  
 660 11 selected genes after 24 h, 48 h and 72 h of stretch was validated by qRT-PCR and RNA  
 661 sequencing. The results are presented as log<sub>2</sub>-fold change relative to the unstretched control ±  
 662 standard error (n=3 for 24 h, n=4 for 48 h and 72 h, n represents biological replicates of cells from  
 663 individual differentiations) for upregulated (A) and downregulated (B) genes. \*p<0.05, \*\*p<0.01,  
 664 \*\*\*p<0.001 vs. unstretched control, Student's t-test for independent samples (qRT-PCR), Wald test  
 665 (RNAseq).



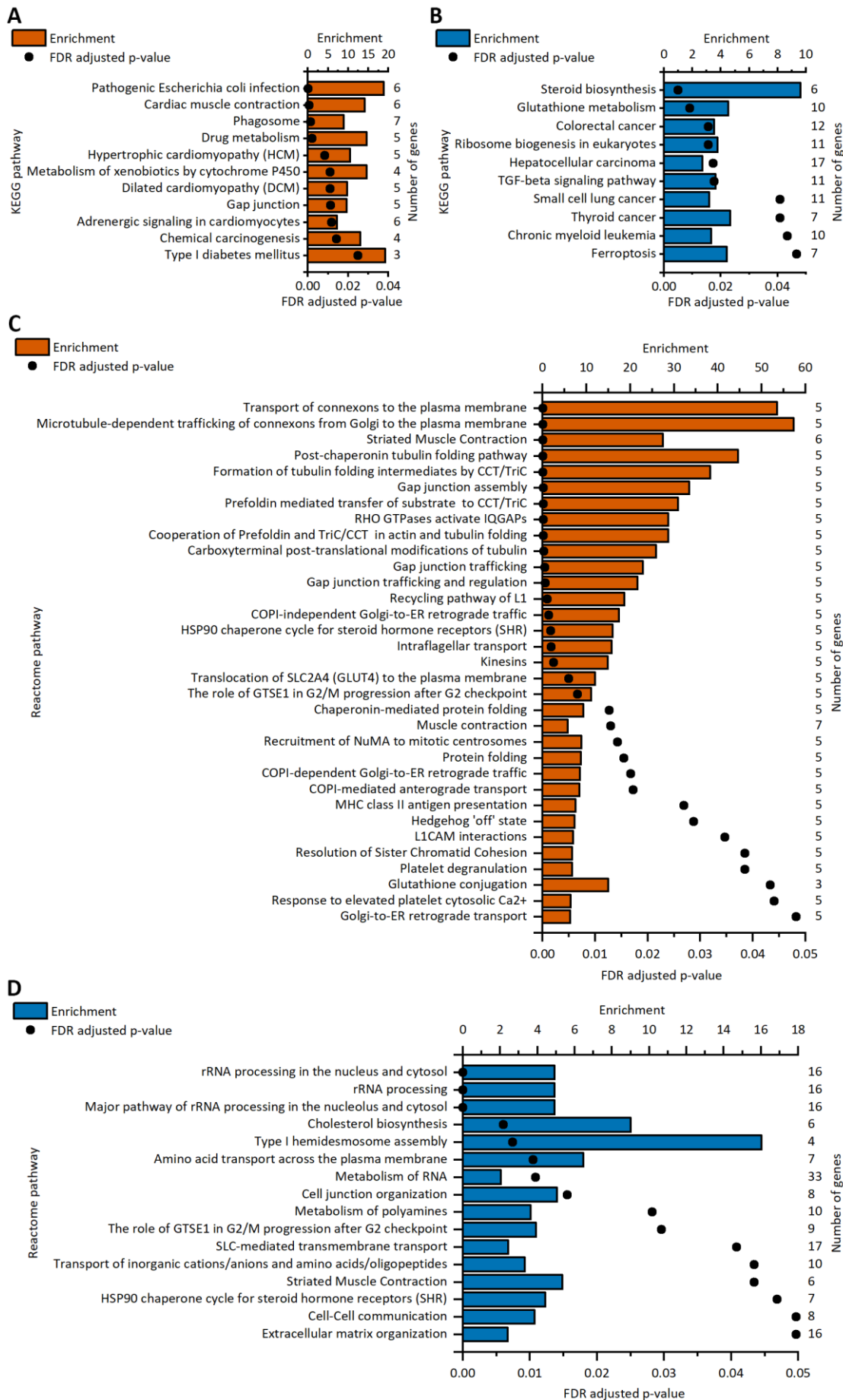
666

667 **Figure 3. Comparison of differentially expressed genes in human induced pluripotent stem cell-**  
 668 **derived cardiomyocytes (hiPSC-CMs) and neonatal rat ventricular myocytes (NRVMs) after 24**  
 669 **h and 48 h of stretch.** NRVM gene expression data are from Rysä et al. (Rysä et al., 2018). A-B,  
 670 Venn diagrams show the number of upregulated (A) and downregulated (B) genes after 24 h and 48 h  
 671 of stretch in hiPSC-CMs and NRVMs. C-D, Expression of genes differentially regulated in both cell  
 672 types after 24 h (C) and 48 h (D) of stretching normalized to the unstretched control (n=3 for 24 h  
 673 hiPSC-CMs, n=4 for 48 h and 72 h hiPSC-CMs, and n=5 for NRVMs).

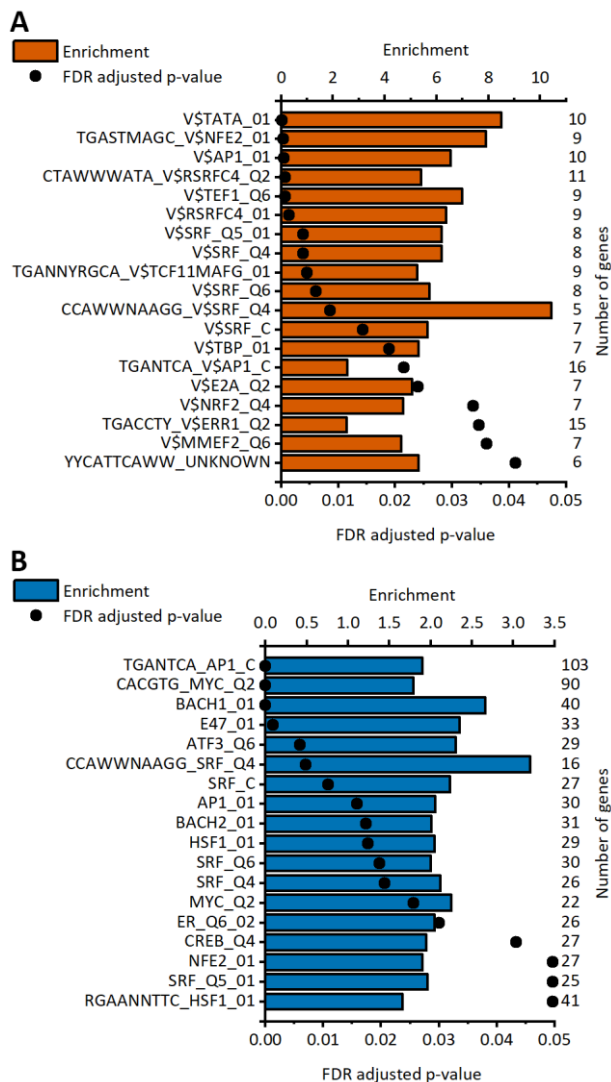




675 **Figure 4. Enriched biological processes in upregulated genes after cyclic stretch in human**  
676 **induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs; A) and neonatal rat**  
677 **ventricular myocytes (NRVMs; B).** Gene Ontology (GO) enrichment analysis was performed with  
678 GOrilla. For each significantly enriched GO term, enrichment values are presented as bars, and FDR-  
679 adjusted p values are presented as dots. The number of upregulated genes associated with each GO  
680 term is shown on the right. The upregulated genes in the NRVMs used for the analysis are from Rysä  
681 et al. (Rysä et al., 2018). The top 30 terms for NRVMs are shown.

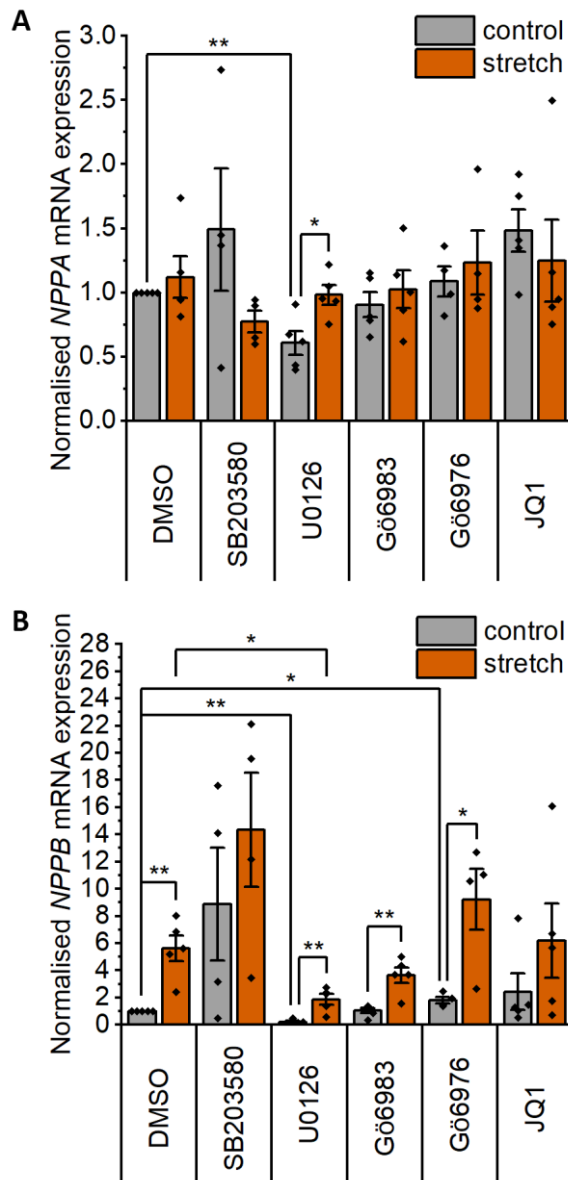


683 **Figure 5. Enriched pathways in upregulated genes after cyclic stretch in human induced**  
 684 **pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) and neonatal rat ventricular**  
 685 **myocytes (NRVMs).** A-B, KEGG pathway analyses of upregulated genes of stretched hiPSC-CMs  
 686 (A) and NRVMs (B). C-D, Reactome pathway analyses of upregulated genes of stretched hiPSC-CMs  
 687 (C) and NRVMs (D). For each significantly enriched pathway term, enrichment values are presented  
 688 as bars, and FDR-adjusted p values are presented as dots. The number of upregulated genes associated  
 689 with each term is shown on the right. The upregulated genes in the NRVMs used for the analysis are  
 690 from Rysä et al. (Rysä et al., 2018).



691

692 **Figure 6. Enriched transcription factor target sites in the upregulated genes of the stretched**  
 693 **human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs; A) and neonatal rat**  
 694 **ventricular myocytes (NRVMs; B).** Enrichment values for each significantly enriched target site are  
 695 presented as bars, and FDR-adjusted p values are presented as dots. The number of upregulated genes  
 696 associated with each target site is shown on the right. The upregulated genes in NRVMs used for the  
 697 analysis are from Rysä et al. (Rysä et al., 2018).



698

699 **Figure 7. Effects of p38 mitogen-activated protein kinase (p38 MAPK), mitogen-activated**  
700 **protein kinase kinase 1/2 (MEK1/2), protein kinase C (PKC) and bromodomain and**  
701 **extraterminal domain (BET) inhibitors on stretch-induced hypertrophic gene expression.** The  
702 following inhibitors were used: SB203580 at 10  $\mu$ M to inhibit p38 MAPK, U0126 at 10  $\mu$ M to inhibit  
703 MEK1/2, Gö6983 at 1  $\mu$ M to inhibit all PKC isoforms, Gö6976 at 1  $\mu$ M to inhibit classical PKC  
704 isoforms, and JQ1 at 300 nM to inhibit BET. Natriuretic peptide A (NPPA; A) and natriuretic peptide  
705 B (NPPB; B) mRNA expression was measured after cyclic mechanical stretch for 24 h with qRT-  
706 PCR, and the results are presented as the fold change relative to the unstretched control. Bars present  
707 the mean, dots present individual values, and error bars present the standard error of mean (n=5,  
708 except for SB203580 and Gö6976 n=4, n represents biological replicates of cells from individual  
709 differentiations). \*p<0.05, \*\*p<0.01, Mann-Whitney U test.