

14 **rejuvenation effects induced by reprogramming in the transcriptome were**
15 **mainly independent of pluripotency gain. Decoupling of these processes al-**
16 **lowed predicting interventions mimicking reprogramming-induced rejuvena-**
17 **tion (RIR) without affecting somatic cell identity, including an anti-inflammatory**
18 **compound osthol, *ATG5* overexpression, and *C6ORF223* knockout. Overall,**
19 **we revealed specific molecular mechanisms associated with RIR at the gene**
20 **expression level and developed tools for discovering interventions that support**
21 **the rejuvenation effect of reprogramming without posing the risk of neoplasia.**

22 **Introduction**

23 Aging is associated with the buildup of molecular damage and a gradual loss of function, culmi-
24 nating in chronic age-related diseases and ultimately death (1). Searching for safe and efficient
25 interventions that can slow down or partially reverse the aging process is a major challenge in
26 the aging field (2–6). In this regard, reprogramming of somatic cells into induced pluripotent
27 stem cells (iPSCs) has been proposed as a candidate longevity intervention due to its potential
28 to rejuvenate cells in a targeted way (7, 8).

29 Pluripotency can be achieved *in vitro* by the ectopic expression of four transcription factors:
30 OCT4, SOX2, KLF4, and MYC, known as OSKM or Yamanaka factors (YFs). It was demon-
31 strated that OSKM support the generation of murine iPSCs (9) using retroviral transduction as a
32 delivery system and mouse embryonic fibroblasts (MEF) as the initial cell culture. Although this
33 original experiment was inefficient in terms of the percentage of cells that terminally achieved
34 the pluripotent state (<0.1%), more advanced *in vitro* approaches resulted in a greatly improved
35 efficiency, e.g. by down-regulation of methyl CpG-binding domain 3 (MBD3) levels (10). In
36 parallel, other approaches have been developed to induce pluripotency. In particular, the expres-

37 sion of seven other transcription factors (7F: *Jdp2-Jhdm1b-Mkk6-Glis1-Nanog-Essrb-Sall4*) re-
38 sulted in high efficiency of reprogramming (11). Therefore, it appears that the reprogramming
39 process can be attained by using different cell culture conditions, transcription factors, and small
40 molecules (12).

41 *In vivo* cell reprogramming could be accomplished by using transgenic mice with doxycycline-
42 inducible OSKM (13, 14). However, continuous expression of OSKM factors in mice leads to
43 severe forms of teratoma. Partial reprogramming protocols can overcome this problem. Some
44 of these techniques rely on the incomplete set of reprogramming factors, e.g., OSK reprogram-
45 ming (15), while others are based on a transient or temporarily controlled expression of OSKM
46 factors (16–18). The problem of oncogenesis during *in vivo* reprogramming is associated with
47 the loss of somatic cell identity in pluripotent cells. Thus, it is crucial to avoid the reset of
48 the somatic epigenetic program in order to make this technique applicable in clinical practice.
49 Recent *in vitro* experiments (19, 20) show that the decrease in epigenetic age of reprogrammed
50 cells measured by epigenetic aging clocks (21) occurs mostly prior to the stabilization phase
51 when the pluripotent state is established. However, even a short-term use of OSKM factors has
52 been shown to produce the detectable subpopulation of cells where late-stage pluripotent genes
53 are expressed (22). Therefore, independent interventions that would support Reprogramming-
54 Induced Rejuvenation (RIR) without inducing pluripotency may be of a high clinical value.

55 Since the first reprogramming experiment conducted in 2006, a massive amount of high-throughput
56 molecular data have accumulated, shedding light on the details of gene regulatory pathways
57 and their dynamics during reprogramming. These data comprise transcriptome, methylome,
58 chromatin conformation, chromatin accessibility, and other omics datasets, including single-
59 cell transcriptomes (22, 23). The resulting data on reprogramming allowed the construction of
60 various mathematical models (24–26) describing some aspects of reprogramming. However,

61 models that offer specific molecular mechanisms responsible for RIR have been lacking.

62 Here, we describe transcriptomic changes that occur in cells during reprogramming and their
63 association with mechanisms of aging and longevity. We conducted a comprehensive meta-
64 analysis of time-course gene expression datasets of mouse and human cells during multi-factor
65 reprogramming and identified robust transcriptomic signatures associated with this process. By
66 integrating them with the signatures of aging and lifespan-extending interventions, we revealed
67 genes and functional processes specifically associated with the rejuvenation and longevity ef-
68 fects of reprogramming. Using multi-tissue transcriptomic aging clocks developed for humans
69 and mice, we further observed a significant reduction of the transcriptomic age (tAge) for both
70 human and mouse cells in response to OSKM, OSK and 7F reprogramming. Remarkably, most
71 genes responsible for the rejuvenation and longevity effects of reprogramming were not in-
72 volved in the loss of somatic identity and gain of pluripotency, suggesting that these processes
73 can be separated. This allowed us to identify specific gene expression signatures of RIR and use
74 them to discover candidate chemical and genetic interventions that may induce reprogramming-
75 associated rejuvenation effects without affecting somatic cell identity.

76 **Results**

77 **Reprogramming gene expression signature captures dynamics towards** 78 **pluripotency**

79 We gathered 41 gene expression datasets of time-course cell reprogramming from 14 studies,
80 including 29 datasets for mouse cells and 12 datasets for human cells (Suppl. Table S1, Suppl.
81 Fig. S1). Each dataset represented a continuous cell reprogramming experiment conducted on a
82 specific cell line with a particular treatment, including OSKM, OSK or 7F. Most murine datasets
83 were MEF-iPSC reprogramming, whereas human studies used different types of cells. To iden-
84 tify genes, whose expression was robustly changed during the reprogramming process across

85 the datasets, we utilized mixed-effect linear models previously used to discover transcriptomic
86 signatures of lifespan-extending interventions and aging (see Methods, Fig. 1A, (27)).

87 Using this approach, we identified mouse- and human-specific gene expression signatures of
88 reprogramming, as well as common reprogramming signatures conserved across species. In
89 total, 3087, 7531, and 4807 genes changed their expression in the cells from mice, humans,
90 and both species, respectively, during reprogramming (BH-adjusted p -value < 0.05). A higher
91 number of significant genes for humans may be related to batch effects, since all corresponding
92 time-course datasets have been created by the same research group. Therefore, in this study we
93 mostly focus on the mouse signature, as it offers more reproducible and robust biomarkers of
94 cellular reprogramming.

95 To assess the quality of signatures, we checked if they recapitulate gene expression changes in
96 individual datasets used for their construction (Fig. 1B, Suppl. Fig. S2A). Both murine and
97 human signatures demonstrated a significant positive Spearman correlation with each utilized
98 dataset ($\rho > 0.66$). Clusters were generally formed by datasets from the same source (i.e.,
99 the same GSE ID) and based on the same type of treatment. Thus, classical Yamanaka factors
100 (YF), including OSKM and OSK treatments, clustered together and were separate from the 7F
101 intervention (11). In addition, human datasets clustered mainly by tissue type (Suppl. Fig.
102 S2A). Overall, the correlation analysis suggested that the constructed reprogramming signature
103 captured consistent gene expression changes observed in multiple independent experiments.

104 To investigate the expression dynamics of top genes associated with reprogramming, we vi-
105 sualized normalized expression of 5 up- and 5 downregulated genes with the lowest p -values
106 (Fig. 1C, Fig. S2B). We observed saturation of gene expression dynamics after 10 days of
107 reprogramming in mice, while in human cells top genes demonstrated sigmoid-like dynamics
108 with the saturation point at 20th day. Interestingly, several top up- and downregulated genes

109 captured by our signatures were previously shown to be associated with aging and included
110 in the GenAge database (28). Thus, *Parp1* is known as an antagonistic pleiotropic gene (29)
111 regulating genome maintenance and inflammation processes. At the same time, in cooperation
112 with *Sox2* it can function as an alternative splicing regulator during reprogramming (30). An-
113 other example is the downregulated *Zmpste24* gene, whose deficiency in mice results in nuclear
114 architecture abnormalities and signs of accelerated aging (31).

115 For additional validation of our signatures, we examined the distribution of pluripotency-associated
116 genes (see Table S2 for a full list of pluripotency genes) among those significantly perturbed
117 during reprogramming (Fig. 1D, Fig. S2C and S2D; see Table S3 for the list of top genes in
118 the signature). Notably, significantly upregulated genes (BH-adjusted p-value < 0.001) were
119 enriched by the markers of pluripotency (Fisher exact test p-value < 1^{-10}). In particular, *Zic3*,
120 *Gdf3*, *Utf1*, *Tfap2c* were reported to maintain the pluripotency state (32, 33); *Dnmt3l*, *Dnmt3b*
121 are DNA methylases, while *Tet1* is a demethylation enzyme (32); *Epcam* and *Cdh1* are mes-
122 enchymal–epithelial transition genes (34). In total, 60% (27 out of 44) pluripotency genes
123 were significantly upregulated during reprogramming according to the mouse signature. On
124 the other hand, no markers of pluripotency have been detected across significantly downregu-
125 lated genes with the exception of 3 genes (*Ccnd1*, *Ccnd2* and *Cdkn1a*) known to be activated
126 during the early stage of reprogramming and suppressed afterwards (35). Such enrichment of
127 pluripotency-associated genes within the subset of upregulated, but not downregulated, genes
128 indicates that our signature correctly characterizes the reprogramming process.

129 Finally, to compare reprogramming-associated gene expression changes across species, we ex-
130 amined the intersection of statistically significant genes (BH-adjusted p-value < 0.05) from the
131 mouse and human cell reprogramming signatures (Fig. 1E). Fisher's exact test showed signif-
132 icant co-regulation of genes during reprogramming in different species (p-value < $10e^{-10}$). In

133 particular, 4 out of the top 5 upregulated mouse genes (*Parp1*, *Rcor2*, *Jarid2*, *Epcam*) were
134 also significantly upregulated in the human signature. Similarly, 3 out of the top 5 downregu-
135 lated mouse genes (*Zmpste24*, *Msrb3*, *Tbc1d8b*) were significantly downregulated in the human
136 signatures. Therefore, although there are certain species-specific reprogramming features (36),
137 this process appears to be highly similar in human and mouse cells at the level of gene expres-
138 sion. The obtained signatures allow investigating the interplay between molecular mechanisms
139 of reprogramming and other traits, including aging and longevity.

140 **Reprogramming signatures are associated with biomarkers of longevity** 141 **and aging**

142 To explore the association between reprogramming, aging and longevity, we expanded our
143 analysis with the gene expression signatures of mammalian aging and established lifespan-
144 extending interventions identified previously (27). Aging signatures represent age-related gene
145 expression changes in individual organs (liver, brain, muscle) of mice, rats and humans; com-
146 mon changes across different tissues within a certain species (mouse, rat, human), and a global
147 signature characterizing common age-related changes across different tissues and species. Sig-
148 natures of longevity interventions include biomarkers of individual lifespan-extending interven-
149 tions (caloric restriction (CR), rapamycin, growth hormone (GH) deficiency), common biomark-
150 ers of interventions (Common) and genes, whose level of expression is correlated with mouse
151 median and maximum lifespan (Median; Maximum) (27).

152 We observed significant positive correlations between several signatures of longevity interven-
153 tions and reprogramming (mean $\rho = 0.11$, $p_{\text{adjusted}} < 0.05$) (Suppl. Fig. S3). At the same
154 time, aging-related changes demonstrated substantial negative correlation with both reprogram-
155 ming and lifespan-extending interventions (mean $\rho = -0.13$, $p_{\text{adjusted}} < 0.05$). As expected,
156 the reprogramming signatures, including human-specific, mouse-specific and the combined sig-

157 nature, clustered together, pointing to the general similarity of this process across species.
158 Aging and longevity signatures also formed separate clusters. Interestingly, reprogramming-
159 associated changes clustered together with established lifespan-extending interventions, sug-
160 gesting that in general reprogramming indeed recapitulates molecular mechanisms of longevity.
161 Thus, clustering analysis of signatures agreed with the longevity and rejuvenation effects in-
162 duced by reprogramming (Suppl. Fig. S3).

163 We next aggregated signatures within groups (Reprogramming, Aging, and Interventions) into
164 combined meta-signatures to measure statistical significance of their co-regulation. There was
165 a significant enrichment of co-regulated genes associated with reprogramming and longevity
166 interventions (Fisher's exact test, p -value = 0.00027, Fig. 2A, left panel), providing addi-
167 tional evidence of functional coherence of these two processes. Moreover, this co-regulation
168 was preserved even after removal of all pluripotency genes or epithelial-mesenchymal transi-
169 tion genes from the analysis (not shown). This suggests that the longevity-associated effect of
170 reprogramming may be uncoupled from pluripotency or the somatic identity program. Since
171 reprogramming and aging signatures demonstrated significant negative correlations in our clus-
172 tering analysis (Suppl. Fig. S3), we did not expect to find an enriched overlap of genes showing
173 the same direction of expression dynamics between aging and reprogramming. Consistently,
174 we observed a rather opposite, although not statistically significant, trend (Fisher's exact test,
175 p -value = 0.21, Fig. 2A, right panel).

176 We examined specific genes responsible for the discovered associations (Fig. 2B). For each
177 group of signatures (Reprogramming, Aging and Interventions), we selected top genes with
178 the lowest geometric mean of p -values. The first gene well-known for its association with
179 aging and longevity, *Rela*, was downregulated upon reprogramming and in response to longevity
180 interventions and upregulated during aging. *Rela* is a proto-oncogene, encoding a subunit of

181 NF- κ B, and its human ortholog is known to influence age-related inflammation (37). The role
182 of *Rela* downregulation during reprogramming is coupled with inhibition of NF- κ B pathway,
183 which was reported to block a successful reprogramming in aged and progeria cells (38).

184 *Mrpl11* encoding the 39S subunit component of mitochondrial ribosome showed the opposite
185 behavior, being positively regulated during reprogramming and by longevity interventions but
186 downregulated with age. According to the GenAge database (28), deletion of this gene in *S.*
187 *cerevisiae* decreases lifespan (39), suggesting that its level may affect longevity. However, the
188 precise mechanistic role of this gene during aging and reprogramming remains unknown.

189 The other interesting example is *Rragc*, which has positive expression dynamics in the case
190 of interventions and aging, but is downregulated during reprogramming. *Rragc* participates
191 in the relocalization of mTORC1 to the lysosomes and its subsequent activation by the GTPase
192 Rheb (40,41). *Rragc* upregulation in longevity interventions and in the aging liver signature can
193 be explained by the duality of Rag-GATOR pathway mechanism (42), depending on the source
194 of amino acids. Downregulation of *Rragc* during reprogramming may be associated with tran-
195 sient mTOR pathway suppression influencing autophagy process (43). Interestingly, although
196 expression of the gene coding for Insulin Like Growth Factor 1 (*Igf1*) was downregulated by
197 the established longevity interventions, it wasn't significantly perturbed during reprogramming.

198 Of particular interest are the genes showing the same direction of expression in all three sig-
199 nature groups: e.g. upregulated *Trappc6a* (encoding a trafficking protein particle complex that
200 tethers transport vesicles to the *cis*-Golgi membrane) (44). Surprisingly, we found one gene,
201 *Uqcrcq*, with negative dynamics in aging and reprogramming, and positive in longevity inter-
202 ventions (Fig. 2B). This gene encodes a subunit of ubiquinol-cytochrome C reductase complex
203 III, which is part of the mitochondrial respiratory chain (44).

204 **Functional enrichment analysis reveals processes associated with** 205 **reprogramming-induced rejuvenation**

206 To reveal functional processes associated with reprogramming, aging and longevity, we con-
207 ducted gene set enrichment analysis (GSEA) of identified signatures (45). Similar to the indi-
208 vidual meta-slopes (Suppl. Fig. S3), functional changes induced by reprogramming and estab-
209 lished lifespan-extending interventions generally demonstrated a significant positive correlation
210 with each other (mean $\rho = 0.23$, adjusted p-value < 0.05) and were negatively associated with
211 age-related changes (mean $\rho = -0.22$, adj. p-value < 0.05) (Fig. 2C). Remarkably, normalized
212 enrichment scores of functions were correlated even stronger than meta-slopes of individual
213 genes.

214 Certain functional terms well characterized the identified reprogramming signature (Fig. 2D).
215 For example, we observed downregulation of genes related to the Epithelial mesenchymal tran-
216 sition (EMT), the process which was shown to be suppressed during reprogramming (23, 46).
217 Among the pathways downregulated by reprogramming but upregulated with age, we observed
218 several terms related to inflammation: Inflammatory response, IL6/JAK/STAT3 signaling path-
219 way, TNF α signaling via NF κ B (adjusted geometric mean p-value < 0.007 for each term and
220 signature group). On the other hand, terms corresponding to mitochondrial function (Mitochon-
221 drial translation, ATP metabolic process) were upregulated in response to lifespan-extending
222 interventions and reprogramming but downregulated with age (adjusted p-value < 0.03 for each
223 term and signature group). This analysis pointed to the specific cellular processes associated
224 with the longevity and rejuvenation effects of reprogramming. However, reprogramming did not
225 appear to be a typical longevity intervention. In particular, it did not induce upregulation of the
226 p53 pathway (adjusted p-value = 0.004), one of the common biomarkers of lifespan-extending
227 interventions. Besides, it was also associated with downregulation of certain pathways upreg-
228 ulated by longevity interventions (27), including Heme metabolism, Hypoxia and Apoptosis

229 (adjusted p-value < 0.008 for each term in reprogramming group).

230 **Clustering analysis of gene expression dynamics during reprogramming** 231 **reveals specific trajectories of longevity-associated genes**

232 To investigate specific dynamics of expression of longevity-associated genes during reprogram-
233 ming, we performed a clustering analysis. First, we aggregated time series datasets of iPSC
234 generation in mouse cells and calculated average trajectory for each gene significantly per-
235 turbed during reprogramming (see Methods). Next, we clustered genes by their trajectory using
236 an agglomerative clustering approach. This approach resulted in 4 gene clusters selected using
237 the Elbow criterion (Fig. 4A,B). Two major clusters (2 and 3) included genes that were almost
238 monotonously up- or downregulated with time, respectively. Consistent with the data in Figure
239 1C, their expression followed a hyperbolic trajectory with a characteristic saturation at approx-
240 imately 10th day of reprogramming. The expression of genes from two other clusters (1 and
241 4) followed U-shaped curve, starting from a transient up- or downregulation, respectively, and
242 gradually returning back to the initial expression value afterwards. These genes reached their
243 peak expression value after approximately 4-6 days of reprogramming.

244 Then, each of the clusters was assessed for enrichment of longevity- and aging-associated genes
245 obtained from the previously described signatures using Fisher exact test (Fig. 4C). Upregu-
246 lated and downregulated signature genes were analyzed separately. The most significant asso-
247 ciation was observed between clusters 2-3 and signatures of longevity interventions, including
248 biomarkers of CR and GH deficiency as well as genes associated with murine median and max-
249 imum lifespan signatures (adjusted p-value < 0.05). Remarkably, genes from both of these
250 clusters were regulated by longevity interventions in the same direction. Thus, genes up- and
251 downregulated during reprogramming (clusters 2 and 3) were enriched for genes up- and down-
252 regulated in response to lifespan-extending interventions, respectively. Functional enrichment

253 analysis of the clusters revealed that genes in 2 clusters upregulated during reprogramming in-
254 cluded replication activating E2F target genes as well as genes involved in base excision repair
255 and G2-M checkpoint (adjusted p-value < 0.0032). Cluster 3, downregulated during repro-
256 gramming, was enriched with genes related to EMT, Inflammatory response and Myogenesis
257 (adjusted p-value < 0.001). Genes in clusters 1 and 4 were associated with several signatures
258 including CR (co-regulation with 1) and aging in brain and in rats (opposite regulation with 1
259 and 4). Cluster 1 following U-shape behavior was enriched for genes involved in the TNF-alpha
260 signaling pathway, Hypoxia, Protein secretion and Myogenesis (adjusted p-value < 0.003). Fi-
261 nally, cluster 4 demonstrating the opposite dynamics was functionally associated with G2-M
262 Checkpoint, E2F targets, Myc targets, and mitochondrial translation (adjusted p-value < 0.003).

263 Thus, our cluster analysis of murine cell reprogramming suggests that genes monotonously
264 changed during reprogramming show a significant co-regulated association with the biomarkers
265 of lifespan extension. Interestingly, these genes were mostly perturbed during the first 6 days of
266 reprogramming, suggesting that even transiently reprogrammed cells may acquire a longevity-
267 associated transcriptomic phenotype, consistent with the experiments in vivo (15, 16).

268 **Transcriptomic clock reveals the rejuvenation effect of reprogramming**

269 To estimate the systemic rejuvenation occurring during reprogramming, we utilized our recently
270 developed mouse and human multi-tissue gene expression aging clocks (unpublished). These
271 transcriptomic clocks (tClocks) were constructed based on more than 2,000 samples from 94
272 datasets across multiple tissues of mouse and human. We applied the clocks to predict the
273 change of transcriptomic age (tAge) during reprogramming of mouse (Fig. 3A, B) and human
274 cells (Fig. 3C). We also compared tClocks predictions with with epigenetic ages estimated using
275 Horvath clock ((21)) utilizing the dataset with both DNA methylation and gene expression
276 measured at once (47). We observed a significant positive correlation between the predictions

277 (Suppl. Fig. S5), showing consistent behavior of clocks developed using different types of
278 molecular data.

279 We found significant transcriptomic rejuvenation of murine cells during reprogramming in-
280 duced by YF (Fig. 3A). Different variants of YF treatment, including OSKM and OSK, resulted
281 in a significant decrease of tAge during reprogramming (p-value < 0.05), with the exception of
282 OK+9MS (48) treatment and OSKM accompanied by *Mbd3f* knockout (49) (Suppl. Fig. S4A).
283 Treatment of cells with the full set of 7 reprogramming factors also led to a decrease in tAge
284 (p-value = 1.43e-05) (Fig. 3C, Fig. S4A). At the same time, one-by-one removal from the cock-
285 tail of these factors displayed diverse behavior. Specifically, removals of *Esrrb*, *Nanog*, *Mkk6*,
286 *Kdm2b* (also known as *Jhdm1b*), *Jdp2* did not diminish the RIR effect, whereas removals of
287 *Glis* and especially *Sall4* blocked the rejuvenation process. Interestingly, removal of *Sall4* at
288 the same time resulted in a dramatic decrease in reprogramming efficiency (11). On the other
289 hand, removal of *Esrrb* also led to a significant decrease in reprogramming efficiency but did
290 not impair rejuvenation according to the transcriptomic clock, suggesting that the rejuvenation
291 effect can be at least partly decoupled from the pluripotency state induction. Remarkably, the
292 final tAge of reprogrammed cells subjected to YF and 7F was close to 0 for most datasets (av-
293 erage tAge = -0.0087 on day 19), which is consistent with the epigenetic data (19). It suggests
294 that features of aging are reset during reprogramming both at the gene expression and DNA
295 methylation levels.

296 To explore specific genes, whose expression change resulted in RIR, we measured the change in
297 tAge after removing each individual gene from the mouse tClock model (see Methods), further
298 referred to as a rejuvenation effect (RE) of a gene. We calculated RE for all genes with non-zero
299 coefficients in the model (337 genes in total) across all datasets where significant rejuvenation
300 was observed (adjusted p-value < 0.05). We identified 84 genes with the positive and significant

301 (adjusted p-value < 0.05) rejuvenation effect. Enrichment analysis of this set of genes indicated
302 a strong relation to Epithelial-Mesenchymal Transition (EMT) and processes involved in Ex-
303 tracellular matrix organization, including Collagen formation, Integrin cell surface interaction,
304 and others (adjusted p-value < 0.05 for all presented terms) (Fig. 3E).

305 Next, we searched for genes contributing primarily to rejuvenation according to the mouse
306 tClock model. Surprisingly, only one pluripotency-associated gene - *Ezh2* - was found among
307 the top 10 predictors of RIR (Fig. 3F). However, Polycomb-group gene *Ezh2* (50) contributed
308 19% of the total rejuvenation effect on average across datasets (adjusted p-value=0.0004). Other
309 genes in the top 10 were associated mostly with EMT (i.e., *Col3a1*, *Igfbp4*, *Postn*, *Fnl1*). We
310 further assessed the RE after removing all genes related to EMT or pluripotency from the
311 model (Fig. 3G). We observed a significant reduction of the rejuvenation effect by 37% on
312 average after removing EMT genes (p-value = 1.193e-05) and 35% on average after removing
313 pluripotency-associated genes (p-value = 1.18e-04). It's worth noting that EMT and pluripo-
314 tency gene sets have no common genes. These results provide an estimate of the impact of
315 pluripotency and EMT related genes on reprogramming-induced rejuvenation, suggesting that
316 the major part of RIR is not explained by the perturbed expression of genes associated with
317 somatic identity.

318 An analogous analysis of human cell reprogramming following OSKM treatment produced sim-
319 ilar results (Fig. 3C). Using a human multi-tissue tClock, we observed a significant rejuvenation
320 (adjusted p-value < 0.05) in almost all cell lines during reprogramming. The only exception was
321 a dataset on foreskin fibroblasts containing very few data points. Interestingly, the rejuvenation
322 effect of individual genes demonstrated high variance across human cell lines (data not shown),
323 suggesting that the rejuvenation process during reprogramming may be achieved through regu-
324 lation of various genes depending on the tissue. Consistently, human rejuvenating genes were

325 not significantly enriched in any functional terms, supporting high heterogeneity of RIR across
326 tissues.

327 To compare the rejuvenating trajectories of human and mouse cells subjected to OSKM treat-
328 ment, we aggregated normalized tAge values across the datasets for each species and applied a
329 moving average smoothing approach (Fig. 3D). We observed a rapid decrease of transcriptomic
330 age of murine cells following the shape of exponential decay. On the other hand, rejuvena-
331 tion of human cells followed a sigmoid curve. Although the transcriptomic age of cells from
332 both species was close to zero at the end of reprogramming, the RIR of human cells required
333 more time. Remarkably, this time difference was consistent with the duration of the repro-
334 gramming process, lasting, on average, for 14 and 30 days for mouse and human cell lines,
335 respectively (36).

336 **Reprogramming signature uncovers new geroprotective interventions**

337 To identify treatments that induce reprogramming-associated rejuvenation at the gene expres-
338 sion level, we selected genes displaying contrasting expression patterns according to aging and
339 reprogramming signatures (Fig. 2A, right panel). We then used these genes as a query for the
340 Connectivity MAP (CMAP) database (51) (see Methods). CMAP database contains gene ex-
341 pression profiles of human cells treated with different genetic or chemical interventions. CMAP
342 connectivity analysis provides connectivity scores as a measure of similarity between a given
343 gene set and transcription changes induced by perturbations from the database.

344 We selected top 20 perturbations showing the most significant positive or negative association
345 with the reprogramming-associated rejuvenation signature for each type of perturbation: over-
346 expression of a particular gene, treatment with a particular compound, knockdown of a gene via
347 shRNA, and knockout of a gene via CRISPR-Cas9 system. To validate the rejuvenation effect
348 of identified interventions, we applied the human and mouse aging tClocks to gene expression

349 profiles of untreated and treated samples from the CMAP database separately for each available
350 cell line. We then aggregated the obtained tAge values across cell lines using linear regression
351 models (see Methods). In the end, we obtained two estimates of rejuvenation effects for each
352 treatment, including aggregated connectivity scores from the CMAP analysis and aggregated
353 tAge values from aging clocks (Fig. 5A).

354 Among interventions that demonstrate significant rejuvenation effects based on the connectiv-
355 ity score as well as both mouse and human tClocks we observed overexpression of *HAVCR2*
356 (*TIM3*), which encodes a cell surface receptor implicated in modulating innate and adaptive im-
357 mune response (adjusted p-value = 0.028). Interestingly, overexpression of *TIM3* was shown to
358 alleviate inflammation in human patients with thyroid-associated ophthalmopathy via suppress-
359 ing the Akt/NF- κ B signaling pathway (52). At the same time, *Tim3* overexpression resulted in
360 deterioration of neuroinflammatory and neurocyte apoptosis in a rat subarachnoid hemorrhage
361 model (53). Together, these observations suggest that *TIM3* plays a significant role in regula-
362 tion of age-associated inflammatory processes, and its overexpression can be considered as a
363 treatment against inflammaging.

364 Knockdown of *ERRF1* (*MIG-6*) was also found to decrease the cellular transcriptomic age ac-
365 cording to both our clocks (adjusted p-value = 1.24e-8). Expression of *ERRF1*, which encodes
366 a negative regulator of EGFR signaling, is upregulated during the cell growth (54). Interest-
367 ingly, overexpression of *MIG-6* was shown to be sufficient to trigger premature cellular senes-
368 cence (55). In contrast, knockdown of *MIG-6* delayed the initiation of Ras-induced cellular
369 senescence (56), supporting our conclusion derived from tClock.

370 Among top interventions inducing a significant rejuvenation effect across different cell lines
371 according to the human clock, we identified knockdown of *PRKCE* (adj. p-value = 1.7e-5),
372 knockout of *C6ORF223* (adj. p-value = 1.9e-4) as well as a treatment with a chemical com-

373 pound osthoh applied at 10 μ M dose for 6 hours (adj. p-value = 0.0038). Interestingly, osthoh has
374 been shown to demonstrate anti-inflammatory effects by blocking the activation of NF- κ B and
375 MAPK/p38 pathways (57). In addition, *osthol* prevents accumulation of advanced glycation end
376 products (AGE) via the induction of *Klotho* expression (58). The rejuvenation effect of *PRKCE*
377 knockdown and *C6ORF223* knockout was also supported by experimental data. Thus, inhibi-
378 tion of *PKC* signaling was shown to maintain self-renewal and pluripotency of rat embryonic
379 stem cells (59), while *C6ORF223* accumulation was associated with age-related macular de-
380 generation (60) and was correlated in expression levels with a well-known human aging-related
381 gene *VEGF* (61, 62). Remarkably, one of the top interventions predicted by our model was over-
382 expression of *ATG5* showing a strong rejuvenation effect by human clocks (adjusted p-value =
383 1.75e-12). *ATG5* gene product is involved in autophagy, mitochondrial quality control, regula-
384 tion of the innate immune response and other cell processes. In fact, *ATG5* overexpression was
385 shown to increase lifespan of healthy mice by enhancing autophagy (63).

386 Finally, to investigate whether the treatments described above induce expression of pluripotency-
387 associated genes, we performed GSEA analysis testing if genes differentially expressed in re-
388 sponse to interventions are enriched for pluripotency genes from (64) (see Methods). After
389 obtaining NES scores for each cell line, we aggregated them using average and applied t-test
390 to assess significance of the aggregated score. As a positive control, we performed a similar
391 analysis in 2 models of overexpression of *MYC*, known to partially induce the pluripotency
392 program in cells (9, 22). The latter treatment showed a significant upregulation of pluripotency
393 genes (p-value < 8.94e-5), while it did not result in a significant reduction of cellular tAge. On
394 the other hand, *ATG5* overexpression induced a rejuvenation effect according to tClock with-
395 out activation of pluripotency genes. In fact, it even slightly suppressed pluripotency program,
396 though insignificantly (p-value = 0.083). Similar significant rejuvenation combined with neu-
397 tral effect on pluripotency (p-value = 0.128) was produced by knockout of lncRNA *C6ORF223*.

398 These examples confirm that rejuvenation and loss of somatic identity associated with repro-
399 gramming can be decoupled, and interventions separately affecting each of these processes may
400 be developed.

401 Discussion

402 Reprogramming-induced rejuvenation is a fundamental concept denoting a family of cell repro-
403 gramming approaches focused on their capacity for rejuvenation (65). These approaches gained
404 much attention in recent years as they have the potential for radical interference into aging and
405 longevity (2–7). Therefore, it is essential to understand precisely which processes during re-
406 programming lead to rejuvenation and how they can be decoupled from the loss of somatic
407 identity. In this study, we investigated these processes and provided a systemic view on reju-
408 venation during reprogramming by analyzing signatures identified from multiple time-course
409 reprogramming datasets and revealing their interplay with biomarkers of aging and lifespan
410 extension (Fig. 6A).

411 We were able to construct robust reprogramming signatures and show that: (i) mouse and human
412 signatures are well correlated with each other and share a significant number of genes regulated
413 in the same direction (Fig. 1E); and (ii) reprogramming signatures are positively correlated with
414 longevity interventions and negatively correlated with various aging signatures. In addition,
415 we discovered co-regulation of particular genes in response to reprogramming and established
416 lifespan-extending interventions including downregulation of *Rela* and upregulation of *Mrpl11*
417 previously shown to be significant biomarkers of murine longevity (28,37,38). The associations
418 between three groups of signatures - reprogramming, interventions, and aging - persist and are
419 even amplified at the level of functional enrichment (Fig. 2C, 6B). Most conspicuous functions
420 (Fig. 6C) demonstrate that reprogramming may act as a longevity intervention but not in all

421 aspects. Of note, these results are generally consistent with those obtained from single-cell
422 analysis (22). Namely, we observed that the reprogramming suppressed genes were associ-
423 ated with inflammatory response. On the other hand, upregulation of fatty acid metabolism
424 observed after transient reprogramming of mouse mesenchymal stem cells was not prominent
425 in our signatures. Multiple studies have previously demonstrated that the DNA methylation
426 age (mAge) decreases during the reprogramming process (15, 18, 19, 66). However, only a few
427 studies (20) attempted to reproduce these results at the transcriptome level using single-tissue
428 clocks (see (67) for details). To fill this gap, we utilized mouse and human clocks trained on
429 multiple tissues to predict transcriptomic age (tAge) of cells during the whole reprogramming
430 process (Fig. 6D). As expected, we observed a systematic decrease of tAge for the majority of
431 reprogramming datasets for both mouse and human cell lines (Fig. 6E, 3C,D). Notably, some
432 treatments that failed to result in successful reprogramming during the original experiment (e.g.
433 7F-Sall4, (11)), did not lead to the decrease of tAge with time. On the other hand, some of
434 the treatments that didn't lead to the gain of pluripotency significantly decreased transcriptomic
435 age of somatic cells (e.g. 7F-Esrrb, (11)). This is consistent with results of (22) reporting
436 that induction of only SK factors decreases aging score without loss of mesenchymal identity.
437 Such results support the possibility of decoupling reprogramming-induced rejuvenation from
438 the changes involved in the loss of somatic identity.

439 Next, we explored genes responsible for RIR by conducting *in silico* knockout experiments.
440 We identified several genes that contributed the most to the rejuvenation process. Among the
441 top 10 genes, there was only one pluripotency-associated gene - *Ezh2* (Fig. 3F). We also ob-
442 served several genes associated with EMT, e.g., *Col3a1*, *Igfbp4*, *Postn*, *Fn1*. In total, 37% of
443 RIR, on average, was explained by the EMT genes, and 35% of the RIR was affected by the
444 pluripotency-associated genes. Therefore, the tClock model suggests that although a part of
445 the RIR is achieved through the deregulation of genes involved in the maintenance of somatic

446 identity, a significant portion of it is orthogonal to this process. Genes responsible for that effect
447 represent perspective biomarkers allowing to search for new geroprotectors.

448 To discover such interventions, we conducted CMAP (51) connectivity analysis and revealed
449 treatments that produced rejuvenation-associated gene expression changes similar to repro-
450 gramming. We validated our hits using human transcriptomic clocks and revealed several in-
451 terventions with a potential rejuvenation effect. Consistently, some of them, including *ATG5*
452 overexpression, *C6ORF223* knockout and osthon treatment, have been previously shown to have
453 a positive effect on lifespan (57, 58, 60, 63). In addition, we tested these and other rejuvenating
454 interventions *in silico* for their ability to induce pluripotency program (5C) and observed that
455 *ATG5* overexpression and *C6ORF223* knockout did not significantly affect the expression of
456 these genes across multiple cell lines (Fig. 5H). Therefore, according to our data, these treat-
457 ments appear to produce RIR without affecting somatic cell identity. Interestingly, *MYC* over-
458 expression demonstrated the opposite effect, producing no significant rejuvenation effect but
459 inducing the pluripotency program, in agreement with its role as one of Yamanaka's factors but
460 interfering with results of (22) where induction of this factor showed little loss of mesenchymal
461 identity but also small decrease in aging score.

462 Taken together, these results indicate that the reprogramming process contains a rejuvenation
463 component that can be expressed in the gene or function dynamics. Recent *in vivo* reprogram-
464 ming demonstrated no systemic rejuvenation of all murine tissues with the exception of skin
465 and kidney tissues (18). The authors hypothesize that this is due to some tissues being more
466 susceptible to OSKM reprogramming than others. It can even be assumed that the OSKM set
467 of factors may not be suitable for *in vivo* reprogramming. Moreover, the fact that this set of
468 factors is known to be oncogenic forces researchers to develop complex treatment protocols.
469 This complexity can be avoided if the oncogenic aspect is completely excluded, which is pro-

470 posed in the RIR concept. Today, several of the possible ways to solve this problem include the
471 use of OSK reprogramming (15), reprogramming until the maturation phase achieved (20) or
472 even chemical reprogramming (12). However, understanding the mechanisms of rejuvenation
473 achieved during reprogramming may provide us with better solutions. Future rigorous studies
474 should reveal which gene networks are responsible for the regulation of the RIR process. Anal-
475 ysis of epigenetic aspects of RIR, such as methylation or histone modifications accompanying
476 expression dynamics, may be a future direction. The ultimate solution would be to construct
477 a dynamic mathematical model of RIR to predict not only a subset of transcription factors (or
478 small molecule compounds) but also other characteristics necessary for successful treatment.

479 We propose a geometric metaphor to better represent the essence of rejuvenation during cell
480 reprogramming (Fig. 6G). We represent the reprogramming process as a vector \bar{R} in the space
481 of transcriptomic signatures. We assume that this vector can be decomposed into two non-
482 orthogonal components: rejuvenation \bar{r} and pluripotency \bar{p} (here we mean the cumulative sig-
483 nature towards pluripotency). Their sum gives the original reprogramming vector $\bar{R} = \bar{r} + \bar{p}$.
484 It follows from our analysis that pluripotency may proceed without rejuvenation (Suppl. Fig.
485 3A), and rejuvenation can occur without successfully achieved pluripotency (exemplified by
486 the 7F-*Essrb* treatment Fig. 3B). It means that rejuvenation and pluripotency have co-directed
487 components (projections onto the reprogramming vector) and also have orthogonal components
488 (e.g., projection of rejuvenation vector onto the axis orthogonal to pluripotency). We argue that
489 for successful rejuvenation without the risk of pluripotency-induced tumorigenesis, we need to
490 identify the signature of "pure rejuvenation" $\bar{r} \perp \bar{p}$, i.e., a set of genes with corresponding levels
491 of expression that causes cell rejuvenation without notable shift towards pluripotency. The re-
492 sults obtained in this study using transcriptomic clock suggest that such genes include *Col3a1*,
493 *Fnl1*, *Cd24a*, while *Ezh2* is an example of a gene contributing to rejuvenation but being also
494 a marker of pluripotency. Using a multitool of transcriptomic signature analysis, we made a

495 step towards decomposing rejuvenation and pluripotency vectors that may lead to the safe and
496 efficient reprogramming-induced rejuvenation.

497 **Methods**

498 **Data collection**

499 We collected publicly available cell reprogramming datasets containing more than three time
500 points across the reprogramming process (Table S1). ESC and iPSC states were excluded since
501 they were not corresponding to any particular time point of reprogramming. We used only
502 preprocessed data (e.g., read counts) provided in the GEO database by datasets' contributors.

503 **Data preprocessing**

504 To aggregate multiple datasets into a joint signature, we utilized an approach as in our earlier
505 work (27). It consists of several steps (Fig. 1A). First, each dataset was normalized using a
506 conventional normalization technique appropriate for the given data type. RLE normalization
507 followed by log transformation was applied for RNA-seq data. Log transformation of intensi-
508 ties followed by scaling and quantile normalization was used for microarray data. Second, for
509 each gene changing its expression value with time, a linear regression model was constructed
510 using the *limma* package (68). Third, slope coefficients, their standard errors and related statis-
511 tics were extracted from models and used to represent corresponding gene regulation (positive
512 or negative). Thus, a positive or negative slope corresponds to an increasing or decreasing
513 expression of a particular gene with time in a given dataset, respectively. Finally, slope val-
514 ues from different datasets were aggregated using the mixed-effects model constructed by the
515 *metafor* package (69), with GEO ID introduced as a random term. For every gene, this model
516 produced a meta-slope, being a weighted average of slopes across all analyzed datasets. Cor-
517 responding p-values were adjusted for multiple comparisons using Benjamini-Hochberg (BH)

518 approach (70). Genes with adjusted p-value < 0.05 were considered significant and included in
519 the final reprogramming signature.

520 **Selection of datasets for the aggregated signature**

521 The critical step for constructing a correct aggregated signature is filtering out non-concordant
522 datasets. We used the Spearman correlation of dataset slopes as a concordance measure and
523 calculated pairwise correlation coefficients between all 29 mouse and 12 human datasets using
524 the union of top 350 genes in each dataset ranked by the correlation p-value of slopes. The
525 threshold of 350 genes was identified to be optimal for noise removal since it maximized the
526 number of significant pairwise correlations (Benjamini-Hochberg adjusted p-value < 0.05 and
527 absolute $\rho > 0.1$). Finally, we used the agglomerative clustering approach based on the Eu-
528 clidean distance with complete linkage to extract the largest cluster among all 29 mouse and 12
529 human datasets (Suppl. Fig. S1). As a result, 19 out of 29 mouse datasets and 11 out of 12
530 human datasets passed the selection criteria (formed a dominant cluster, see also supplementary
531 figure S1 and Methods).

532 **Signature construction**

533 Prior to signature construction, we normalized slope coefficients from different datasets based
534 on the following algorithm. First, Spearman correlation of reprogramming-related gene ex-
535 pression changes was calculated for each pair of datasets. For that, we obtained the top 350
536 statistically significant reprogramming-associated genes ranked by the correlation p-values in
537 each dataset and then formed a union of two such gene lists within a pair of datasets. Then,
538 multiple Deming regression was calculated simultaneously for each pair of datasets with sig-
539 nificant correlations using the union of top 350 genes. During this step, the cumulative squared
540 loss across all significantly correlated pairs of datasets within a certain signature was minimized

541 using the L-BFGS-B method in the R function *optim*. Normalization coefficients were allowed
542 to vary between 0.01 and 100. To establish the global minimum of the error function, multiple
543 Deming regression was calculated 10 times with random initial sets of normalization coeffi-
544 cients, and final coefficients were chosen from the run with the smallest cumulative regression
545 error. Among these 10 runs, the error minimum was the same for most runs, indicating that the
546 global minimum was achieved for each signature.

547 Then we used the *rma.mv* function in the *metafor* package (69) to construct intercept-only
548 multilevel mixed-effects model with nested random effects (71). As a response variable, we
549 used Deming-normalized slopes derived for each dataset. Since datasets originated from diverse
550 sources, we had to account for their heterogeneity across different experiments (i.e., different
551 GSE IDs) and within the same experiment (i.e., the same GSE ID), implying the multilevel
552 embedded structure of the model. Fixed effects were not considered within this model. The
553 final model can be described with the following formula:

$$\hat{s}_{ij} = \mu + \zeta_{(inGSE)ij} + \zeta_{(bwGSE)j} + \epsilon_{ij} \quad (1)$$

554 where \hat{s}_{ij} is an estimate of the true effect size s_{ij} ; μ is an actual mean of the slopes' distribution;
555 term ij denotes that some effect size i is nested in cluster j ; $\zeta_{(inGSE)ij}$ is random term cor-
556 responding to a within-GSE-ID heterogeneity; $\zeta_{(bwGSE)j}$ is a random term corresponding to a
557 between-GSE-ID heterogeneity; ϵ_{ij} is a sampling error of individual datasets, which can be es-
558 timated from a standard error of a corresponding slope. We applied this model for construction
559 of mouse, human, and combined signatures. Following the principle mentioned in the previous
560 paragraph, we selected 19 datasets for mouse signature, 11 datasets for human signature, and
561 30 datasets (19 mouse + 11 human datasets) for the combined signature.

562 **Aggregation of p-values across the signatures**

563 The aggregation of p-values within each group of signatures was conducted using the harmonic
564 mean (72). Subsequently, we adjusted aggregated p-values using BH method. We selected
565 significant genes (adjusted p-values < 0.05) to investigate the overlap of genes between repro-
566 gramming signature and signatures of aging and lifespan-extending interventions. The statisti-
567 cal significance of the overlap was assessed with Fisher's exact test.

568 **Clustering analysis**

569 To cluster genes by their expression dynamics, we first scaled all gene expression values, trans-
570 forming them into z-scores. Next, we grouped observations into 2-day periods and applied
571 one-way ANOVA considering 2-day intervals as a factor variable, testing a null hypothesis
572 that average expression is equal over all intervals. Genes with the BH adjusted p-value < 0.05
573 were considered to demonstrate significant expression change over time. We excluded genes
574 with constant expression and clustered the remaining genes using agglomerative approach with
575 correlation distance metric and complete linkage, considering time intervals as features. The
576 scikit-learn Python package (73) was used for this analysis.

577 **Prediction of transcriptomic age**

578 To investigate the dynamics of gene expression biomarkers of aging during cellular reprogram-
579 ming, we utilized multi-tissue transcriptomic mouse and human clocks based on signatures of
580 aging across different tissues identified as explained in (27). The applied clocks were based
581 on elastic net linear models that were designed to predict relative chronological age calcu-
582 lated as a real age divided by the maximum lifespan for a given species (48 months and 122
583 years for mouse and human, respectively). The missing values were omitted with the precal-
584 culated average values from the clock. Using the mouse and human clocks, we then calculated

585 the transcriptomic age (tAge) for each mouse and human sample, respectively. Change of the
586 tAge with time during reprogramming within each dataset was assessed using linear regression
587 model. The slope of the tAge change with time was considered significant if the corresponding
588 BH adjusted p-value < 0.05. For normalization of the tAge values across several OSKM-based
589 reprogramming datasets, relative tAge values were divided by the average tAge value of the first
590 time point within each dataset. After that, aggregated tAge trajectories for human and mouse
591 data were smoothed using 3-day period moving average. Standard errors were calculated for
592 smoothed tAge values in each time point.

593 **Estimation of rejuvenation effect of a gene**

594 To estimate the rejuvenation effect of a specific gene in a particular dataset, the following
595 pipeline was carried out: 1) *in silico* knockout was performed by making the expression of
596 this gene equal to 0 for all of the samples; 2) tClock was used to calculate tAge for all samples
597 in the given dataset before and after the "knockout"; 3) linear model was fitted to predict time-
598 dependent tAge trajectory before and after "knockouts"; 4) the maximum difference between
599 tAge estimates obtained from the linear model before and after "knockouts" was calculated;
600 5) the difference was normalized to the total rejuvenation effect in the dataset (the difference
601 between the tAge value at the first time point and the tAge value at the final day of reprogram-
602 ming). Thus, the result of this procedure demonstrates how the removal of certain gene affects
603 the magnitude of tAge decrease during reprogramming, corresponding to its rejuvenating effect.
604 The same approach was used to calculate the rejuvenation effect after "knocking out" the whole
605 gene set (e.g., EMT or pluripotency-associated genes).

606 **Aggregated analysis of rejuvenation-inducing interventions based on CMAP**

607 To identify treatments mimicking RIR at the gene expression level, we used CMAP query API
608 (51). As a query, genes upregulated in combined reprogramming signature but downregulated in
609 combined aging signature ('Up' subset), and genes downregulated in combined reprogramming
610 signature but upregulated in combined aging signature ('Down' subset) were used. We will
611 refer to these gene subsets as the RIR gene set.

612 The result of a CMAP query is essentially a list of perturbagens ordered by the score of as-
613 sociation between differentially-expressed gene set and the query gene set. A positive score
614 indicates a similarity between the query and effect of the given perturbagen applied to the cer-
615 tain cellular line, while a negative score indicates that these two signatures are the opposite to
616 each other (i.e., genes that are increased by treatment with the perturbagen are decreased in the
617 query, and vice versa). The magnitude of the score corresponds to the magnitude of similar-
618 ity or dissimilarity between the treatment and query. Therefore, top and bottom hits in these
619 lists represent interventions that have the strongest positive and negative associations with the
620 query, respectively. These treatments appear to be of the highest interest for the subsequent
621 investigation.

622 At the next step, we aggregated connectivity scores for each intervention with the same dosage
623 and treatment time across different cell lines using simple averaging of connectivity scores. The
624 statistical significance of the positive or negative association of the intervention across cell lines
625 was assessed using t-test with the null hypothesis that the mean of connectivity scores across
626 cell types is equal to zero. We then selected the top 20 positive and top 20 negative aggregated
627 interventions from each of four intervention types (gene overexpression, chemical compound
628 treatment, gene knockdown with shRNA, and gene knockout with CRISPR) for further analysis.

629 We downloaded gene expression data for the selected interventions and further applied tran-

630 scriptomic clocks to the gene expression profiles induced by these treatments as well as control
631 samples. Specifically, we obtained quantile normalized data from the CMAP level 3 data pre-
632 processing step. We downloaded treatment data and corresponding control data for a given
633 unique intervention-dosage-duration group indicator. After an additional data normalization
634 procedure (see "Prediction of transcriptomic age" section for details), we applied the mouse
635 and human transcriptomic clocks to the gene expression vectors. The obtained relative age
636 values were aggregated with a linear model of the following form:

$$Age \sim Cell\ Line + Treatment, \quad (2)$$

637 where *Age* is the relative tAge value, *Cell Line* is the name of corresponding cell line from
638 CMAP (factor variable), and *Treatment* is the binary variable, which indicates whether the
639 given relative tAge value is from the control or treatment subset. We fitted this model using
640 *statsmodels* python package (74). The resulting coefficient of the *Treatment* variable can be
641 interpreted as an average change in the relative tAge in response to a given intervention across
642 cell lines, while its p-value reflects the statistical significance of this change. Thus, negative
643 *Treatment* coefficient corresponds to "rejuvenation" effect while positive reflects "aging" ef-
644 fect. We paid particular attention to interventions with negative significant coefficient of the
645 treatment variable coupled with the positive aggregated connectivity scores. Such interventions
646 result in the gene expression response similar to reprogramming and opposite to aging and,
647 at the same time, contribute to rejuvenation according to the transcriptomic clock, being of a
648 particular interest.

649 Among the identified interventions, we searched for those not inducing the expression of pluripotency-
650 related genes. First, we obtained differential gene expression data from the CMAP level 5 data
651 preprocessing step for each of our top hits. Then, we performed gene set enrichment analysis
652 (GSEA) using *fGSEA* package (75) testing if the gene expression response induced by a cer-

653 tain treatment is enriched for the set of pluripotency-associated genes obtained from (64). The
 654 calculated Normalized Enrichment Scores (NES) were then aggregated using simple averaging.
 655 The statistical significance of enrichment across cell lines was assessed using t-test with the null
 656 hypothesis that the mean of NES across cell types is equal to zero.

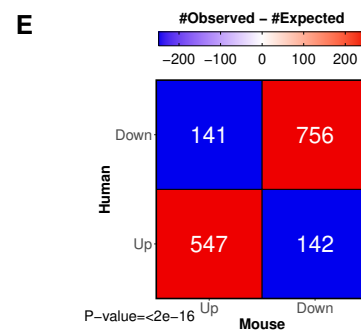
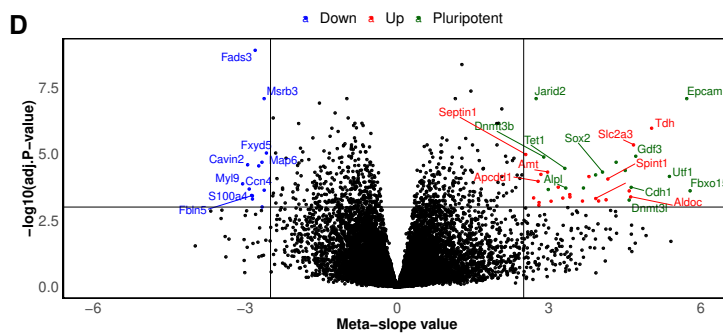
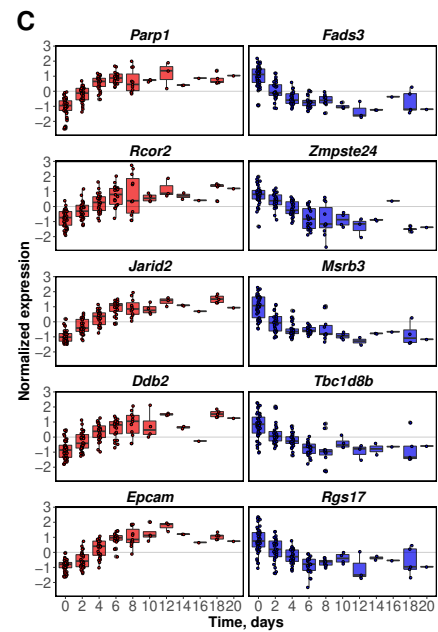
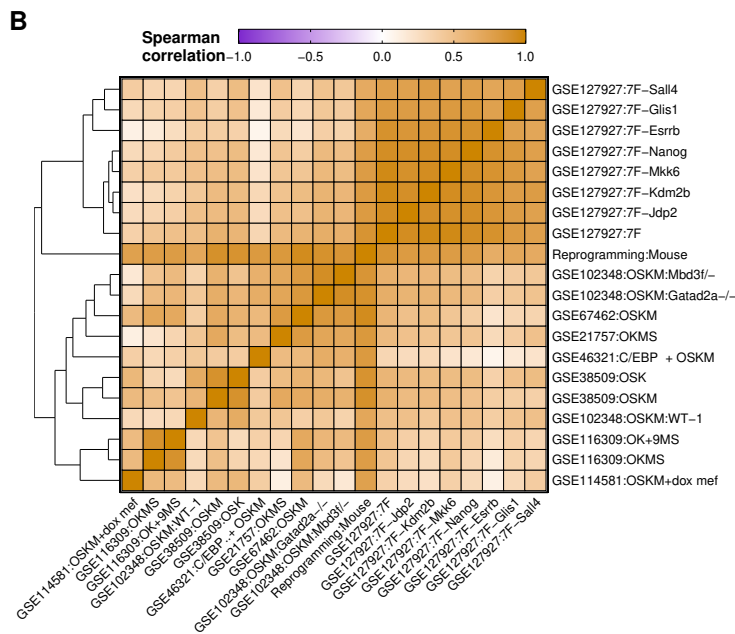
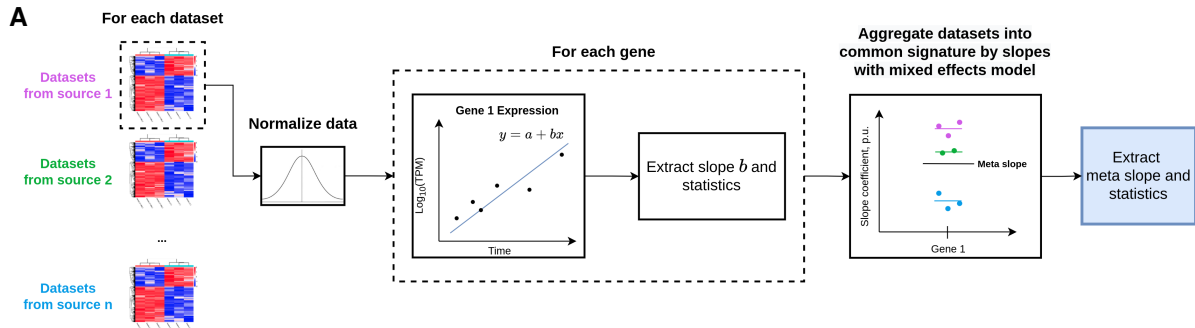


Fig.1. Construction and validation of the reprogramming signature. (A) Schematic illustration of the signature construction workflow. (B) Clustering analysis of individual mouse reprogramming datasets and the aggregated signature. GSE IDs of the datasets are accompanied by the description of reprogramming factors applied in corresponding experiments (for details, see Table S1). Cells are colored based on Spearman's correlation coefficient. (C) Expression trajectory of top five upregulated and downregulated genes with the lowest BH-adjusted p-value according to the mouse reprogramming signature. Upregulated and downregulated genes are shown in red and blue, respectively. (D) Volcano plot of meta-slope values extracted from the signature and corresponding BH-adjusted p-values. Each dot represents a single gene. Pluripotency markers are highlighted in green. Significantly upregulated and downregulated genes are shown in red and blue, respectively. The horizontal dashed line represents the significance cut-off (BH adjusted p-value < 0.001), while vertical lines represent meta-slope cut-offs ($|\log FCI| > 2.5$). (E) The overlap of significantly upregulated and downregulated genes between murine and human signatures. Only uniquely mapped orthologs according to Ensembl were considered for analysis. Numbers within cells demonstrate the observed numbers of overlapping orthologous genes, while color represents the difference between observed and expected number of genes in the corresponding cell. The p-value is calculated using Fisher's exact test.

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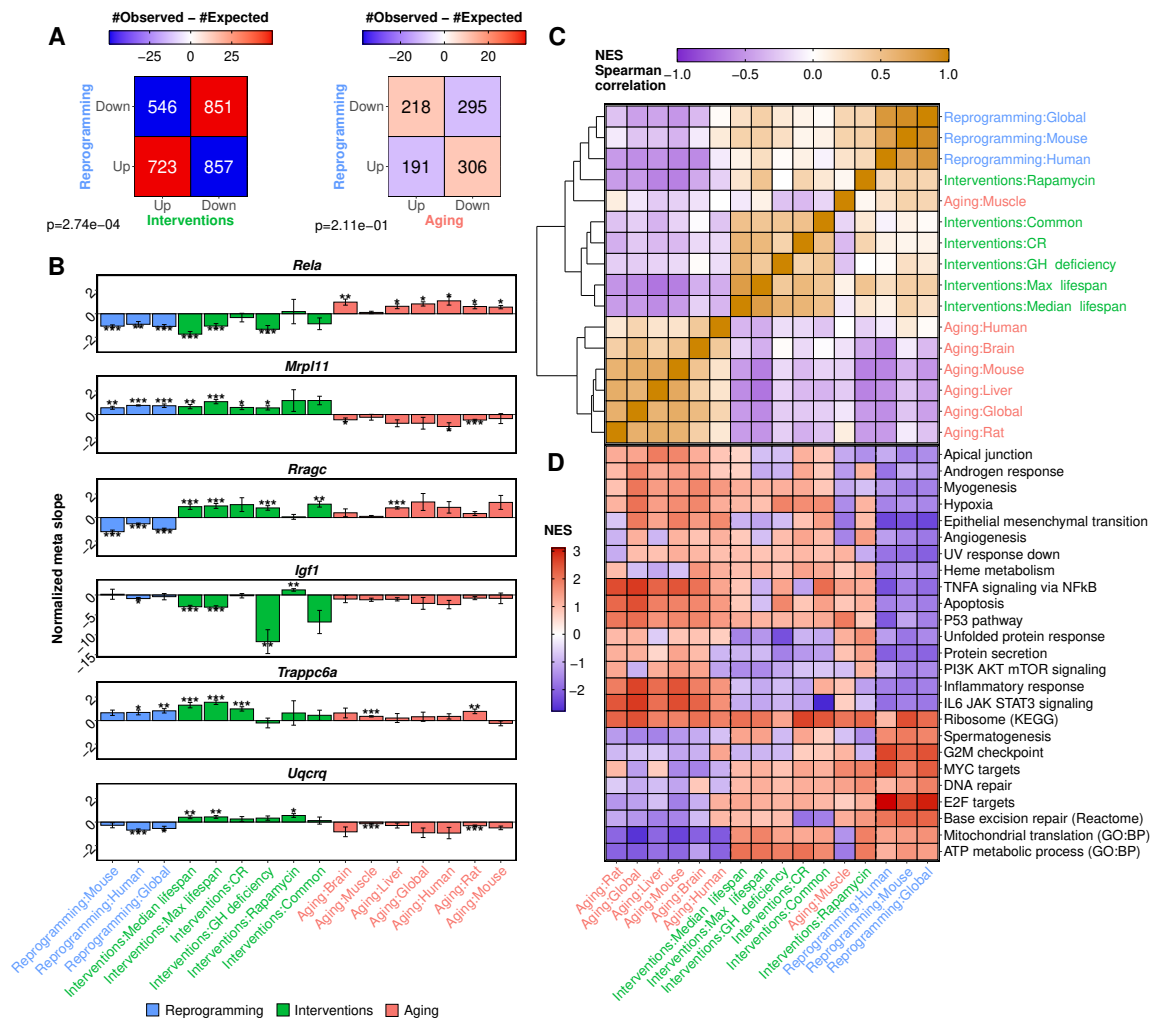


Fig. 2. The interplay between reprogramming, aging and longevity signatures. (A) The overlap of upregulated and downregulated genes between groups of signatures. Numbers within cells show the observed numbers of overlapping genes, while color reflects the difference between observed and expected values. p-values are calculated using Fisher's exact test. CR: caloric restriction, GH: growth hormone. (B) Barplots demonstrating the behavior of six particular genes across different signatures. Error bars represent standard errors of normalized meta-slopes. Annotation: * p.adjusted < 0.05; ** p.adjusted < 0.01; *** p.adjusted < 0.001. (C) Spearman correlation matrix of Normalized Enrichment Scores (NES) obtained using GSEA. (D) Functional terms across different signatures. Color represents NES values. Only the terms with at least one significant enrichment (adjusted p-value < 0.1) are shown. Dashed lines separate reprogramming and aging signature groups from the others.

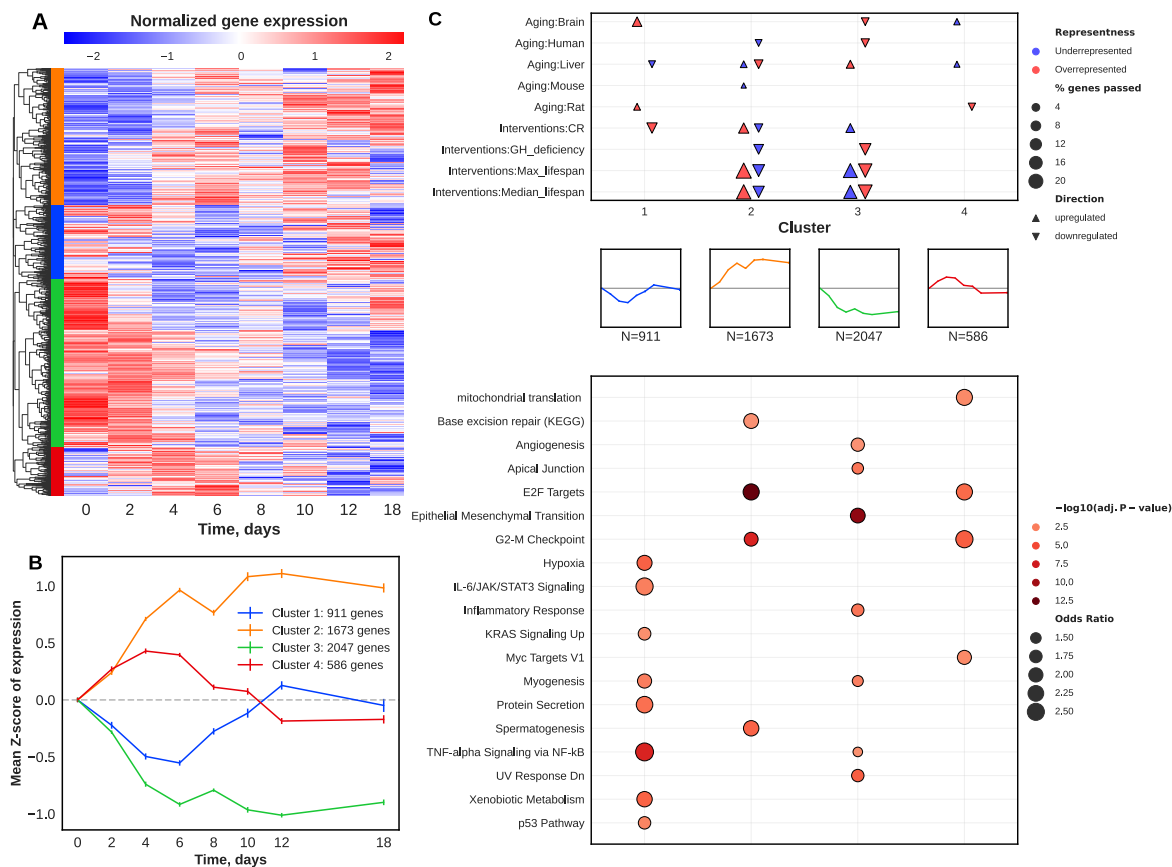


Fig. 3. Clustering analysis of murine cell reprogramming-associated gene expression changes. (A) Normalized gene expression during reprogramming. Only genes with statistically significant non-constant expression during reprogramming (ANOVA adjusted p-value < 0.05) are shown. Each row corresponds to a single gene. Colors represent four clusters chosen with the Elbow criterion. (B) Dynamics of cluster centroids during reprogramming. Error bars represent the standard error of the mean. The colors of clusters correspond to panel A. (C) Cluster enrichment analysis. Top panel: enrichment of clusters by genes associated with aging and longevity. Enrichment by up- and downregulated genes is reflected by the direction of triangles. The statistical significance of the overlap is assessed using Fisher's exact test. Overrepresentation and underrepresentation are shown in red and blue, respectively. The absence of triangle reflects non-significant results (BH-adjusted p-value \geq 0.05). Only signatures with significant enrichment in at least one cluster are shown. Triangle size represents the proportion of genes corresponding to a particular signature within a cluster. Middle panel: cluster centroids schematics (from B). Bottom panel: functional enrichment of clusters assessed with hypergeometric test. Only terms with significant enrichment in at least one cluster are shown. Color represents statistical significance of association, while size of the bubbles reflects odds ratio.

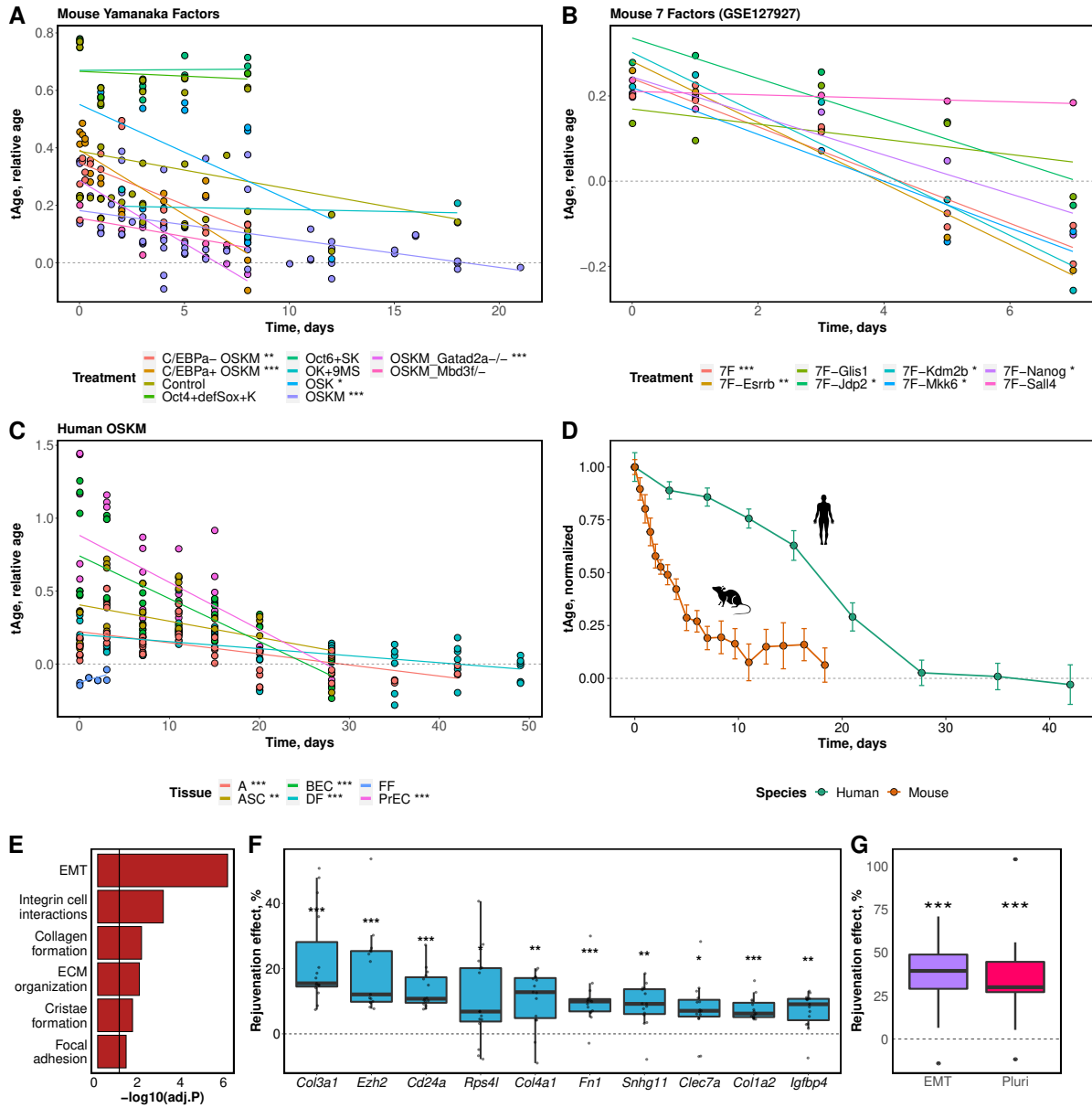


Fig. 4. The evaluation of gene expression age during reprogramming using multi-tissue transcriptomic clocks. (A) Transcriptomic age (tAge) changes during reprogramming of mouse cells induced by Yamanaka factors. Lines represent the fitted linear model for the corresponding type of treatment. Stars indicate the significance level of the corresponding linear model slope. Relative age is defined as a chronological age divided by the maximum lifespan for a given species. (B) tAge changes during reprogramming of mouse cells induced by 7 factors from (11). All notations are the same as in panel A. (C) tAge changes during reprogramming of human cell lines. All notations are the same as in panel A. Each color represents a certain cell type. (D) Aggregated trajectories of rejuvenation induced by reprogramming for mouse and human cells. The curve is smoothed using moving average and normalized by the average tAge at the first point of time. Errorbars represent standard errors of the mean. (E) Functional enrichment analysis of genes from the mouse tClock model with significant effect on rejuvenation during reprogramming. The black line shows the significance threshold (adjusted p-value=0.05). Hallmark (epithelial-mesenchymal transition), KEGG (focal adhesion), Reactome (integrin cell surface interactions, extracellular matrix organization, collagen formation), and GO:BP (cristae formation) terms are presented on the barplot. (F) Distributions of rejuvenation effects of top genes associated with murine RIR across the datasets. The top 10 genes are sorted by their average contribution to rejuvenation (see Methods) according to the mouse tClock model. (G) The portion of RIR effect caused by the regulation of EMT (green) and pluripotency-associated (orange) genes. Each boxplot reflects the distribution across individual datasets. ASC: Adipose-derived stem cell, A: human astrocytes, BEC: bronchial epithelium cells, DF: dermal fibroblasts, FF: foreskin fibroblasts, PrEC: Prostate epithelium cells, EMT: Epithelial-Mesenchymal transition, Pluri: pluripotency-associated genes. * P<0.05, ** P<0.01, *** P<0.001

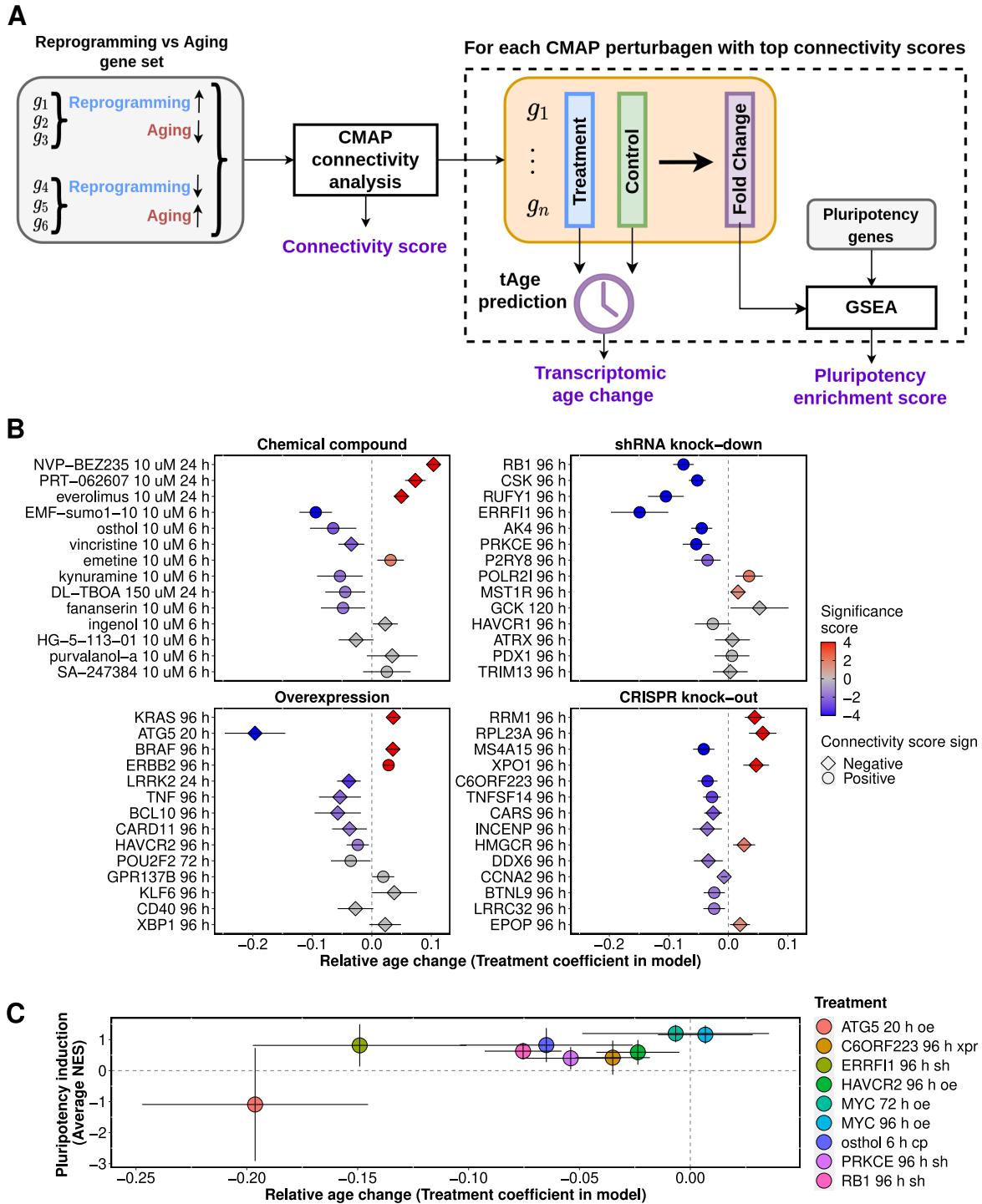


Fig. 5. Identification of rejuvenation-associated interventions using CMAP. (A) Schematic illustration of CMAP analysis workflow. (B) Relative age change for different types of interventions. Age change corresponds to the *Treatment* coefficient from the aggregation model (see Methods). Whiskers correspond to the 95% confidence interval. The significance score is computed as $-\text{Log}_{10}(\text{P-value}) * \text{Sign}(\text{Treatment coefficient})$. Grey points indicate insignificant coefficients. Circles correspond to coinciding directions of signature connectivity and tClock analysis, while other symbols correspond to opposite directions. (C) Rejuvenation- and pluripotency-inducing effects of selected interventions. Rejuvenation effect was assessed using tClock while pluripotency effect was determined with GSEA. Whiskers correspond to the 95% confidence interval. oe: overexpression, NES: Normalized Enrichment Score, h: hours, sh: gene knockdown with short hairpin RNA, xpr: gene knockout with CRISPR.

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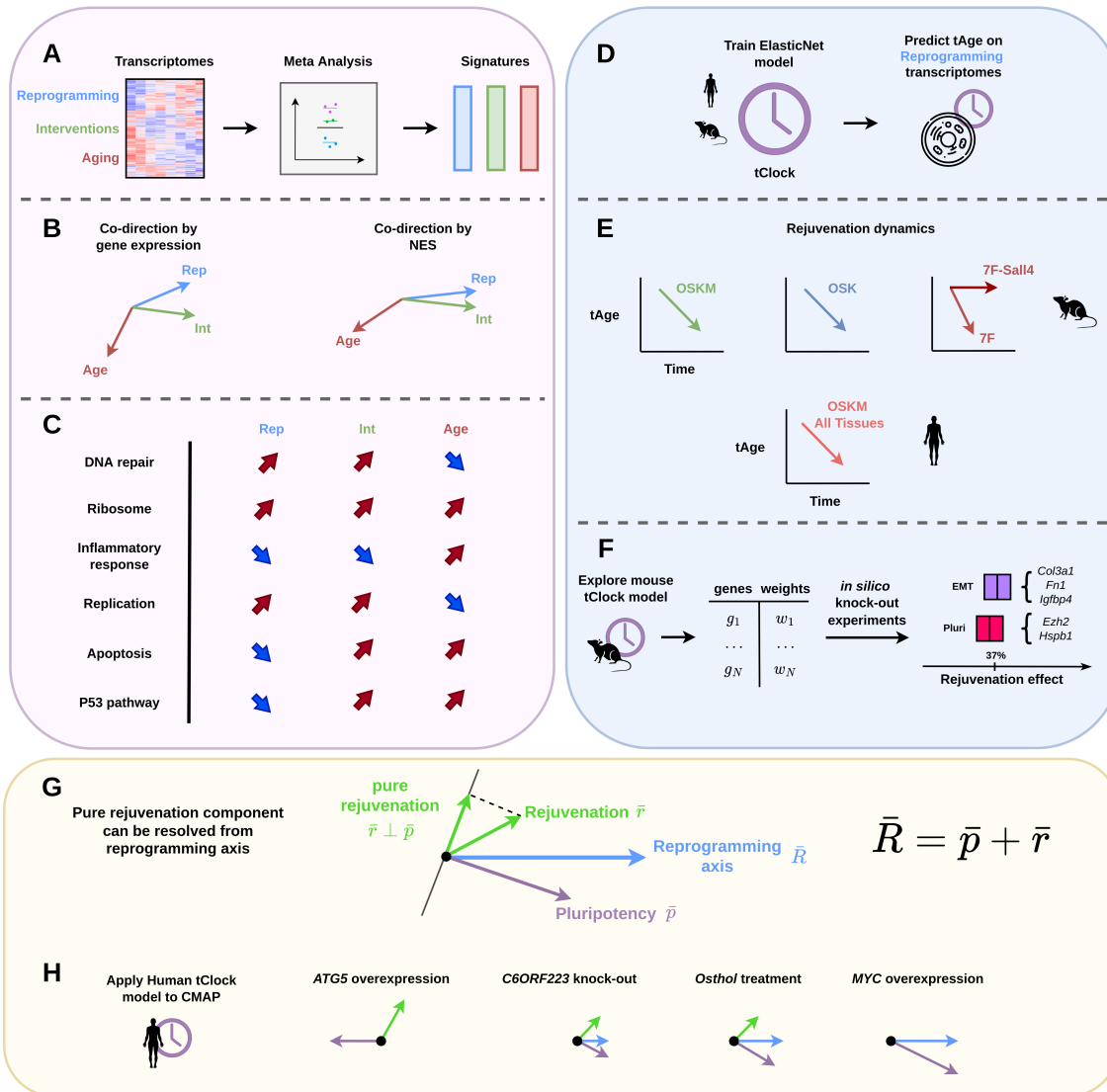


Fig. 6. Summary of the work. (A) Gene expression signatures of cellular reprogramming in mouse and human were constructed via aggregation of multiple datasets by meta-analysis technique. (B) Reprogramming signature was positively correlated with biomarkers of longevity interventions and negatively correlated with signatures of aging (meta-slopes correlation). The strength of these associations was amplified at the functional level (NES correlation). (C) Functional behavior of signatures in selected ontological terms. Red and blue arrows denote positive (upregulation) and negative (downregulation) NES scores, respectively. (D) tClock models trained on the mouse or human aging gene expression datasets were used to predict the tAge of reprogramming cells across time. (E) OSK, OSKM Yamanaka factors, and 7F factors consistently decrease tAge of murine and human cells during reprogramming. However, removal of certain factors, such as Sall4, may result in abrogation of both reprogramming and rejuvenation. (F) Genes contributing to the rejuvenation effect of reprogramming were identified. Pluripotency and EMT-associated genes are responsible for approximately 35-37% of RIR. (G) Reprogramming can be considered as a vector being a sum of two components: one moving cell toward pluripotency and the second moving cell to a rejuvenated phenotype. Investigation of gene expression signatures allows to decouple these processes. (H) New interventions affecting one of the reprogramming-induced components can be discovered using instruments provided in this work. Rep: Reprogramming; Int: Lifespan-extending interventions; Pluri: Pluripotency; EMT: Epithelial-mesenchymal transition.

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885 **Author Contributions** A.T. and S.E.D. conceived and designed this research; D.K. performed
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887 and interpretation; D.K. and A.T. wrote the manuscript with contributions from all other au-

888 thors.

889 **Competing Interests** The authors declare that they have no competing financial interests.

890 **Data and materials availability:** Additional data and materials are available online.

900 **Table S1. (separate file)** Datasets used in this study.

901 **Table S2. (separate file)** Table of pluripotency-associated genes for mouse and human.

902 **Table S3. (separate file)** Top 1000 genes from the mouse reprogramming signature sorted by
903 statistical significance.