1    # Common modes of ERK induction resolve into context

2    # specific signalling via a combination of signal duration and

3    # cell type specific transcriptional repression

4    Marta Perera[1] and Joshua M Brickman[1*]

5    [1]reNEW UCPH – The Novo Nordisk Foundation Center for Stem Cell Medicine.

6    Faculty of Health and Medical Sciences, University of Copenhagen. Blegdamsvej

7    3B, 2200, Copenhagen N, Denmark.

8    *Corresponding author: joshua.brickman@sund.ku.dk

## 9 Abstract

10 Fibroblast Growth Factor (FGF) signalling via ERK exerts diverse roles in

11 development and disease. In mammalian preimplantation embryos and naïve

12 pluripotent stem cells ERK promotes differentiation, whereas in primed pluripotent

13 states closer to somatic differentiation ERK sustains self-renewal. How can the same

14 pathway produce different outcomes in two related cell types? To explore context-

15 dependent ERK signalling in embryonic development and differentiation we

16 generated cell and mouse lines that allow for tissue- and time-specific cell intrinsic

17 ERK activation. Using these tools, we find variations in response to ERK are mostly

18 mediated by repression of transcriptional targets that occur in tandem with

19 reductions in chromatin accessibility at regulatory regions. Furthermore, immediate

20 early ERK responses are largely shared by different cell types, but become refined

21 producing cell-specific programs as increasing durations of signalling interface with

22 cell specific gene regulatory networks. Induction in naïve pluripotency is

23 accompanied by chromatin changes, whereas in later stages it is not, suggesting

24 that chromatin context doesn't shape signalling response. Altogether, our data

25 suggest that cell-type specific responses to ERK signalling exploit the same

26 immediate early response, but then sculpt it to specific lineages via repression of

27 distinct cellular programs and downstream indirect stimulation of available enhancer

28 networks.

## Introduction

Cell fate decisions during embryonic development are driven by a limited number of extracellular signals that operate at the precise time and place to activate distinct lineage-specific programs. Given there is a finite number of signals but thousands of different cell types, it becomes necessary to repurpose these signals to produce cell type-specific outcomes. Fibroblast Growth Factor (FGF) signalling *via* Extracellular signal-Regulated Kinase (ERK) is a prime example of this problem, exploiting the same set of kinases to produce diverse responses in multiple tissues during embryonic development (Corson et al., 2003; Dorey and Amaya, 2010). FGF/ERK plays a fundamental role in coordinating cell fate choice and is one of the most frequent pathways mutated in cancer (Brewer et al., 2016; Dorey and Amaya, 2010; Ornitz and Itoh, 2015).

Early development in mammals is concerned with the segregation of the embryonic Epiblast from the extra-embryonic lineages required for post implantation development, the Trophectoderm (TE) and the Primitive Endoderm (PrE); and FGF/ERK is required to make both. First, it is important for the segregation of outside cells into the TE from the inner cells into the Inner Cell Mass (ICM) (Lu et al., 2008; Nichols et al., 1998; Nichols et al., 2009). Then, just before implantation at embryonic day (E)3.5, FGF/ERK is necessary to drive segregation of PrE from the Epiblast (Chazaud et al., 2006; Krawchuk et al., 2013; Yamanaka et al., 2010). This segregation is initiated by Epiblast cells secreting FGF4, which activates ERK in a neighbouring cell, inducing PrE identity (Azami et al., 2019; Chazaud et al., 2006; Frankenberg et al., 2011; Kang et al., 2017; Molotkov et al., 2017). As a result, culture of pre-segregation mouse embryos with either FGF4 or an FGF/ERK signalling inhibitor produces an ICM composed entirely of PrE or Epiblast cells, respectively (Nichols et al., 2009; Schrode et al., 2014; Yamanaka et al., 2010).

Based on the capacity of ERK inhibition to block PrE differentiation, mouse Embryonic Stem Cells (mESCs) can be efficiently derived by the *ex vivo* expansion

2

57     of peri-implantation Epiblast in the presence of an FGF/ERK signalling inhibitor

58     (Ying et al., 2008). This supports their expansion as cells with the competence to

59     differentiate into all lineages, a property termed pluripotency. As the gene

60     expression state in these cells resembles the earliest stage of embryonic

61     development, they are called naïve (Nichols and Smith, 2009). Stimulation of ERK

62     induces naïve cells to exit naïve pluripotency, supressing the expression of factors

63     known to support pluripotency at the same time as activating a differentiation

64     program by acting directly on enhancer activity (Hamilton et al., 2019). However,

65     the activity of ERK rapidly changes at implantation. Post-implantation Epiblast cells

66     can also be expanded as pluripotent stem cell cultures, but in these cases FGF/ERK

67     is required for their expansion and to block differentiation into somatic lineages

68     (Brons et al., 2007; Tesar et al., 2007). Their gene expression profiles approximate

69     later stages of embryonic development than naïve cells, and therefore are termed

70     primed (Endoh and Niwa, 2022; Morgani et al., 2017; Nichols and Smith, 2009).

71     FGF/ERK is not the only pathway to promote opposing outcomes in pre- vs post-

72     implantation Epiblast: at the naïve stage WNT/ß-catenin activation (or GSK3

73     inhibition) blocks differentiation to promote pluripotency, while in primed Epiblast

74     induces differentiation (Sumi et al., 2013; Ying et al., 2008). *In vivo*, the Epiblast

75     progresses out of the naïve state through two morphogenetic changes: first,

76     Epiblast cells become polarized to produce rossette-like structures at E5.0, then a

77     central lumen arises and the cells form a cup-shaped Epiblast at E5.5 (Bedzhov and

78     Zernicka-Goetz, 2014; Shahbazi et al., 2017). The formation of these morphological

79     features has been exploited to identify the temporal hierarchy of WNT and FGF

80     signalling. Naïve mESCs and *ex vivo* cultured embryos can be captured in a rosette-

81     like state in the presence of FGF and WNT inhibition, and then advanced further

82     into Epiblast development upon FGF/ERK release (Neagu et al., 2020). This led to

83     the identification of Rosette Stem Cells (RSCs), analogous to the E5.0 Epiblast

84     (Endoh and Niwa, 2022; Neagu et al., 2020). RSCs can be expanded in the presence

3

85    of both WNT and FGF inhibitors, and then progressed towards primed pluripotency

86    *via* FGF/ERK activation to generate Epiblast-Like Cells (EpiLCs) (Hayashi et al.,

87    2011). The existence of these defined culture models that mimic the progression

88    through naïve into primed Epiblast development provides an ideal platform to

89    investigate the molecular cues that regulate FGF/ERK signalling context.

90    In this paper, we have developed mouse and cell culture models that allow time-

91    and cell type-specific ERK activation by exploiting a combination of our intrinsic c-

92    RAF induction model (Hamilton and Brickman, 2014; Hamilton et al., 2019) and Cre-

93    mediated recombination. We have used these models to map responses to ERK

94    signalling as cell states progress from naïve to primed pluripotency. When we

95    examine early signalling responses, we find a striking conservation of inductive

96    events across cell types. However, later signalling responses are more variable

97    between cell types and are mediated by repression of cell type-specific

98    transcription. Finally, we have investigated the mechanism of ERK-mediated context

99    responses by mapping FGF-dependent regulatory regions in each cellular context.

100    In naïve pluripotency, ERK triggers functional chromatin remodelling, while in

101    primed pluripotency, it exploits the existing accessible regions.

## Results

103    To investigate the cellular responses to ERK in mouse pre- and post-implantation

104    Epiblast, we designed a strategy that allowed us to induce ERK *in vivo* and *in vitro*.

105    We took advantage of a previously published genetic model (Hamilton and

106    Brickman, 2014; Hamilton et al., 2019) which employs a constitutively active c-RAF

107    (*Raf1*) to activate the kinase cascade upstream of ERK (Fig. 1A). The engineered c-

108    RAF kinase is fused to a tamoxifen (4OHT)-specific ligand binding domain of the

109    oestrogen receptor (ER$^{T2}$), allowing for precise temporal activation upon 4OHT

110    supplementation (Fig. 1A). To enable *in vivo* lineage-specific Cre-mediated ERK

111    activation, we introduced a lox-stop-lox cassette upstream of the inducible c-RAF.

112    We furthermore tagged the inducible c-RAF with HA and followed it by a T2A self-

113    cleaving peptide and a H2B-mCherry reporter, allowing us to visualise transgene

114    expression based on either H2B-mCherry fluorescence or HA immunodetection (Fig.

115    1B). We targeted mESCs and verified ERK induction and mCherry expression (Figs

116    S1A-E) before injecting into morula-stage embryos to produce chimeric mice, which

117    were then crossed to obtain a stable homozygous line. The line was called CRAFR26

118    (Conditional RAF in Rosa26 locus).

119    To validate the system, we crossed homozygous CRAFR26 females with

120    heterozygous Sox2Cre males (Hayashi et al., 2002), which expresses Cre in the

121    Epiblast lineage. We observed mCherry signal in the Epiblast of E6.5 embryos (Fig.

122    1C) and induced the c-RAF kinase with 4OHT for 2 or 4 hours. 4OHT-treated

123    embryos produced higher pERK expression in the Epiblast compared to non-treated

124    embryos (Vehicle) (Figs 1D, S1F-H), or non-floxed embryos (Sox2Cre$^{+/+}$) (Figs S1F-H).

125    To investigate the context-dependent roles of ERK *in vitro*, CRAFR26 mESCs were

126    transiently transfected with Cre, generating a cell line that constitutively expressed

127    *cRAF-HA-T2A-H2B-mCherry* from the *Rosa26* locus (Fig. 1E). The cells show

128    homogeneous induction of pERK after 2 or 4 hours of 4OHT treatment, rapidly

129    reversed by a MEK inhibitor (MEKi, see Methods) (Fig. 1F). Cells were cultured in

5

130    the presence of FGF receptor inhibitor (FGFRi) for 48 hours before and during the

131    induction, to ensure that the source of ERK signalling comes from the exogenous

132    construct. We detected SOX2 during ERK induction to confirm that cells maintained

133    their ESC identity (Fig. 1F).

**134    ERK activates an early shared transcriptional response and later cell type-specific**

**135    programs**

136      To precisely map the transcriptional response to ERK in *in vitro* pluripotent cell

137    types around implantation, we differentiated targeted ESCs into RSCs, which are

138    analogous to the E5.0 Epiblast (Neagu et al., 2020), and EpiLCs, related to the E5.5

139    Epiblast (Hayashi et al., 2011) (Fig. 2A). We analysed the transcriptome of these cells

140    by bulk RNA-sequencing (RNA-seq) before and after ERK stimulation and reversion

141    with MEKi (Fig. 2A). Principal Component Analysis (PCA) projection shows that the

142    biggest change is driven by developmental time: there is a stepwise progression

143    ESCs-RSC-EpiLCs as cells transition from naïve to primed pluripotency (Fig. 2B).

144    Naïve ESC populations express naïve pluripotency markers, while EpiLC populations

145    express primed pluripotency markers (Fig. 2C). RSCs, as an intermediate population,

146    show a transcriptome that clusters between ESCs and EpiLCs (Fig. 2B) and express

147    both naïve and primed markers (Fig. 2C). As expected ERK repressed pluripotency

148    genes in naïve ESC (Hamilton et al., 2019), but in EpiLCs it induced them (Fig. 2D),

149    consistent with the role of FGF in supporting primed pluripotency (Greber et al.,

150    2010; Tesar et al., 2007). This included genes normally associated with naïve

151    pluripotency, such as *Klf2*, *Klf4*, *Esrrb* and *Prdm14* (Fig. 2D). We confirmed that ERK-

152    regulated genes defined in Serum/LIF ESCs (Hamilton et al., 2019) were also being

153    up- and down-regulated in our ESC samples (Fig. S2A). Finally, we investigated

154    whether we could detect differences between early (upregulated at 2h) vs late (at

155    4h) genes. Overall, the ERK transcriptional response could be separated between

156    early ERK-responsive or MAPK canonical genes, that are upregulated in all cells

157 (Figs 2E-F), and late responsive genes which were more specific to each cell type

158 (Fig. 2F).

**ERK-mediated gene repression is cell type-specific while activation is more**

**ubiquitous**

161     Since we obtained a time resolved dataset, we defined genes regulated by ERK in

162 all conditions by clustering the differentially expressed genes according to the

163 dynamics of activation and repression in response to induction and inhibition of the

164 pathway across time (Figs S3A-C, Table S1). For activated genes, we selected

165 clusters with highest expression at 4h (cluster 2 ESC, cluster 3 RSC, cluster 3 EpiLC

166 in Figs S3A-C). For repressed genes, we selected clusters where their lowest

167 expression is at 4h (clusters 1 & 3 ESC, clusters 1, 2 & 4 RSC, cluster 4 EpiLC in Figs

168 S3A-C). These clusters were compared between cell types to determine the extent

169 to which ERK regulates common sets of genes in different contexts (Figs 3A-B,

170 Table S2). While the strict overlap between cell type-specific activated genes was

171 surprisingly low (Fig. 3A), the overall tendency trend of change is similar in all three

172 cell types, with 70% of genes activated by ERK in ESCs exhibiting a positive fold

173 change in EpiLCs, and the mean is always above zero (Fig. 3C). This contrasts with

174 ERK- repressed genes, where almost no overlap is observed (Fig. 3B),only 50% of

175 genes repressed in ESCs show negative fold changes in EpiLCs and the mean of the

176 fold change is zero (Fig. 3D). Thus, while ERK-activated genes showed modest

177 overlap between cell types, ERK-repressed genes were even more context-

178 dependent (Figs 3A-B). For both repression and activation, the response in RSCs

179 and naïve ESCs is far more similar than that observed in EpiLCs.

180     To understand the functional role of ERK-activated genes in each context, we

181 extracted genes lists annotated as a specific GO Term and compared their fold

182 changes upon ERK activation in each cell type. We found that genes annotated as

183 endoderm related (GO:000792) were induced by ERK in both naïve and primed

184 (Fig. 3E), consistent with the ability of these cell types to make primitive and

7

185  definitive endoderm (Anderson et al. 2017). However, genes related to the neural

186  lineage (GO:0061351) and stem cell maintenance (GO:0019827) were better

187  induced by ERK in primed cells (Fig. 3E).

188  Given that ERK-repressed genes were more context-dependent than ERK-

189  activated genes (Figs 3A-B), this suggests that repression of a set of genes might

190  play a more important role in shaping specific responses than activation. We found

191  genes related to placenta development repressed in RSCs and angiogenesis in

192  EpiLCs (Fig. S3D, Table S3). These data support the idea that ERK activates genes

193  related to lineage progression but in specific cell types it also has an important role

194  in suppressing non lineage-specific gene expression.

**_In vivo_ ERK responses recapitulate the _in vitro_ transcriptome**

196  To complement the analysis of _in vitro_ responses to ERK in naïve vs primed stages,

197  we sequenced the transcriptome from _in vivo_ pre- and post-implantation Epiblast

198  before and after ERK activation (Fig. 4A). PCA of the uninduced samples suggests

199  that the major alterations in gene expression were due to _in vitro_ adaptation, but it

200  also illustrated a very clear separation of the naïve (ESCs and the pre-implantation

201  Epiblast) from the primed (EpiLCs and the post-implantation Epiblast) states (Fig.

202  4B). To investigate the differences between _in vivo_ and _in vitro_, we extracted the

203  most significant genes driving variation across PC1, but we found them to be mostly

204  related to biosynthesis and metabolism, as one might expect from _in vitro_

205  adaptation, and not related to developmental processes (Fig. S4A). Moreover, E3.5

206  Epiblast cells expressed naïve pluripotency markers (Fig. 4C), similar to ESCs (Fig.

207  2C) while E5.5 Epiblast cells expressed primed pluripotency markers (Fig. 4C),

208  similar to EpiLCs (Fig. 2C).

209  We then computed correlation coefficients between _in vivo_ and _in vitro_ ERK-

210  induced samples and found that ERK-induced E3.5 Epiblast correlated more closely

211  to the ERK-induced ESC samples, while E5.5 Epiblast correlated better with EpiLCs

212  (Fig. 4D). We also observed that the E3.5 Epiblast upregulated 58% of endoderm-

8

213    related genes upon ERK induction (Fig. 4E), and E5.5 Epiblast showed 54% of stem

214    cell-annotated genes being upregulated upon ERK induction (Fig. 4E), which

215    recapitulated the effect observed *in vitro*.

216     While we see ERK inducing a global endoderm signature in E3.5 Epiblast (Fig. 4E),

217    we saw more robust *Sox17* induction compared to *Gata4* or *Gata6* after 4h of ERK

218    (Figs. 5A, S5A-C, Table S4). We therefore sought to validate the capacity of

219    intracellular ERK induction to functionally promote GATA6-positive PrE in the

220    blastocyst. We isolated E3.5 CRAFR26 embryos and treated them with either 4OHT

221    or FGF4 for 4h. Then, the embryos were left to develop until late blastocyst stage

222    (E4.5) in the presence of an FGFRi to prevent paracrine signalling (Fig. 5B). In ERK-

223    induced blastocysts, we observed that 96% of the ICM cells expressed the PrE

224    marker GATA6 at E4.5 (Figs 5B-C), and this induction was more efficient than the

225    same length of treatment (4h) with FGF4 (Figs 5B-C). These surprising observations

226    suggest that *Sox17* is induced independently by ERK, rather than downstream of

227    GATA4 and GATA6, and that it may be one of the earliest immediate responses to

228    ERK. Although GATA6 is believed to be an early marker of mouse PrE at a protein

229    level (Artus et al., 2011), the induction of *Sox17* mRNA may be an earlier response

230    to ERK than *Gata6* mRNA.

231    **ERK triggers the remodelling of the chromatin landscape**

232     We hypothesised that cell type-specific ERK responses could be mediated either

233    by regulatory regions already differentially available when ERK signalling is

234    activated, or by ERK triggering the reorganisation of the accessible regions to direct

235    transcriptional changes. To distinguish between these two possible scenarios, we

236    performed Assay for Transposase-Accessible Chromatin using sequencing (ATAC-

237    seq) (Buenrostro et al., 2013; Grandi et al., 2022) (Fig. 6A), and assessed which

238    regions were differentially accessible between cellular states (Table S5), and upon

239    ERK induction (Table S6).

240 We validated the changes observed by ATAC-seq in ESCs by exploiting our

241 existing data on enhancer activity in response to ERK (Hamilton et al., 2019). We

242 found that opening regions gained cofactor binding, including EP300 and Mediator

243 (Med24) occupancy, as well as an increase in the active enhancer histone

244 modification H3K27 acetylation (H3K27ac) (Fig. S6A). We also observed that regions

245 that were becoming less accessible in response to ERK were losing EP300, Med24

246 or H3K27ac (Fig. S6A). Super enhancers, large cooperative DNA elements known to

247 regulate transcription in pluripotent cells (Hnisz et al. 2013), were not remodelled

248 upon ERK activation (Fig. S6B), consistent with our previous observations (Hamilton

249 et al., 2019).

250 To validate cell type-specific changes in our dataset, we analysed the ATAC-seq

251 signal at ESCs- and EpiLCs-specific enhancers (Buecker et al. 2014). We found that

252 ESC-specific enhancers were more accessible in ESCs and closed down

253 progressively in RSCs and EpiLCs, while EpiLC enhancers were more accessible in

254 EpiLCs than in ESCs. (Fig. S6C). Interestingly, both sets of enhancers were at an

255 intermediate accessible state in RSCs (Fig. S6C), although RSC expressed mostly

256 naïve genes (Fig. 2C). Finally, we assessed changes at the set of ERK-activated and

257 repressed enhancers we previously defined based on EP300 and H3K27ac changes

258 (Hamilton et al. 2019.). We found that, although these enhancers were defined in

259 naïve ESCs, ERK-activated enhancers appeared to become more accessible with

260 signalling in all three cell types (Fig. 6B). In contrast, while ERK-repressed enhancers

261 became less accessible in naïve ESCs, this phenomenon was negligible in RSCs and

262 EpiLCs (Fig. 6B), providing further support for the notion that cell type-specific

263 responses depend on signalling-mediated repression.

264 **ERK-activated regulatory regions are shared between cellular contexts while**

265 **repressed regions are more cell type-specific**

266 While we observed that the positively regulated naïve ESC enhancers were

267 responding to signalling at some level in all three cell types, we wished to take a

268    non-biased approach to identify cell type-specific ERK-regulated regions. Thus, we

269    compared the sets of peaks that were opening and closing with ERK induction in

270    each cell type. Consistent with the RNA-seq and the ERK-induced sequences (Fig.

271    6B), there is considerable overlap in regions that opening in response to ERK (Fig.

272    6C). The overlap of opening regulatory regions was considerable, with 307 shared

273    regulatory regions between all three cell types (Fig. 6C), showing higher overlap

274    than the induced gene sets (Fig 3A). ERK closed regions appear again almost

275    mutually exclusive (Fig 6D), suggesting that the transcriptional mechanisms

276    governing repression are more specific to each cell type, whereas activation is

277    shared between cellular contexts.

278        To assess the commonalities in ERK-responsive regions, we extracted the enriched

279    motifs in each set of opening regions and found that most motifs were shared

280    between the 3 cell states and were related to ERK-canonical factors (ETS–AP-1

281    motifs) (Fig. 6E, Table S7). However, motifs in the closing regions were more

282    enriched in specific contexts (Fig. 6F, Table S7). We found motifs related to naïve

283    pluripotency being closed in ESCs and RSCs (TCF, OCT motifs), and motifs related

284    to later development stages being decommissioned in EpiLCs (FOX, SOX motifs)

285    (Fig. 6F, Table S7). To further confirm that repression appears cell type-specific

286    while activation seems relatively ubiquitous, we determined the genes nearest to

287    opening and closing regulatory regions. We found a large number of opening

288    regions mapped to the same genes in different cell types, but a high degree of

289    specificity in negative regulation (Fig. S6D).

290        We then wanted to discern whether the regulatory regions were merely being

291    remodelled or were occupied by different Transcription Factors (TFs). We examined

292    changes in TF occupancy based on the predicted capacity of a bound TF to block

293    the activity of Tn5 transposase and create a footprint in the ATAC-seq signal. We

294    exploited the computational tool TOBIAS (Bentsen et al., 2020) to extract TF

295    footprints from ATAC-seq data and predict changes in TF occupancy upon ERK

296  induction, obtaining a differential binding score. We found that the ERK-canonical

297  TFs (ETS–AP-1) showed similar increased TF-binding scores upon ERK in all cells

298  (Fig. 6G, Table S8). Consistent with this, we observed 53% of footprints to be

299  shared in all cell types in the 307 commonly opening regions, with a core set of

300  ETS–AP-1 TFs in 38% of these in all cell types (Fig. S6E, Table S8).

301    When we look for global TF binding release in response to ERK, we see little

302  change in all three cell types (Fig. S6F). Notably, the small number of TFs that are

303  significantly unbound show some cell type-specificity, with nuclear hormone

304  receptors being unbound in ESCs and FOX/SOX TFs losing binding in EpiLCs (Fig.

305  6H). However, consistent with our previous observations, the great majority of TFs

306  remain associated with binding sites in cis-regulatory elements following ERK-

307  mediated repression, including pluripotency factors like SOX2 in naïve ESCs (Fig.

308  S6F, Table S8). In summary, while ERK mediated activation appears to be triggered

309  by canonical ETS–AP-1 TFs, cell type-specific repression appears to be mediated by

310  decommissioning in the absence of robust reduction to lineage-specific TF binding.

311  **ERK mediates distinct cis-regulatory strategies in specific contexts**

312    In response to ERK stimulation we observed alterations in accessibility of

313  regulatory regions, both previously defined as enhancers and new regions reported

314  here. We next sought to investigate whether this ERK-mediated remodelling had an

315  impact on ERK-regulated genes. By combining the RNA-seq with the ATAC-seq, we

316  could distinguish between two strategies: either ERK is regulating genes in the

317  proximity of already accessible regions, or factors downstream of ERK are directly

318  influencing accessibility to produce changes in gene expression.

319    A significant number of regions were specifically accessible in each cell type

320  before ERK induction (at 0h) (Fig. S7A, Table S5), and these regions were enriched

321  for different TF motifs (Fig. S7B, Table S7). When we analysed the $Log_2FoldChange$

322  (LFC) of the genes closest to these peaks, we found that in EpiLCs the genes found

323  next to already accessible regions were more strongly upregulated than in ESCs and

324 RSCs (Fig. 7A, Table S9). This suggested that signalling acts on accessible

325 regulatory sequences in EpiLCs, as exemplified in the *Spred3* enhancer,

326 progressively accessible in RSCs and EpiLCs, while its transcription is activated by

327 ERK solely in EpiLCs (Fig. 7B).

328  The observation that available regions do not seem to be exploited by ERK in

329 naïve ESCs suggested a requirement for remodelling in the ERK response in naïve

330 ESC. We therefore investigated whether the regions remodelled by ERK were

331 driving significant changes in gene expression, and found a strong correlation

332 between changes in accessibility and transcription at the nearest gene in naïve

333 ESCs, but not in the other two cell types (Fig. 7C, Table S9). This was measured as a

334 significant different in transcriptional change from nearest genes to opening vs

335 closing regions (Fig. 7C). This was manifested when we examined the *Zic3*

336 enhancer. Its transcript was upregulated at a comparable fold change in all three

337 cell types, while its enhancer was opening upon ERK induction in naïve cells but did

338 not correlate with transcript levels in primed cells (Fig. 7D). Taken together our

339 observations suggest that the chromatin landscape already present before the

340 signal arrives can produce a significant impact in the signalling output, given that

341 ERK exploits a preconfigured set of targets in EpiLCs, whereas in naïve ESCs it

342 drives the rearrangement of its target loci.

13

## Discussion

343    How the same signal can produce different effects depending on the cellular or

345    tissue context is a long-standing question in developmental biology. While

346    FGF/ERK is a fundamental mediator of diverse processes, including proliferation,

347    survival and differentiation (Dorey and Amaya, 2010; Turner and Grose, 2010), in

348    numerous tissues across development (Corson et al., 2003), it is difficult to fathom

349    how it achieves this diversity of regulation through a set of conserved immediate

350    early TFs. To investigate the context-dependent cellular responses to ERK, we have

351    developed a tool that works both *in vivo* and *in vitro* and allows for precise,

352    homogeneous and controlled activation of ERK in the tissue and time of choice and

353    found that context-dependent transcription appears to depend largely on the

354    capacity of this pathway to supress cell type-specific transcription.

355    One of the advantages of our model is that it overcomes heterogeneity and

356    feedback inhibition. Historically this has complicated the assessment of responses to

357    signalling, as the immediate early response involves feedback inhibition. Our

358    previous findings suggest that the range of enhancer regulation and

359    phosphorylation induced intrinsically in naïve ESCs is the same as that induced by

360    FGF4 in FGF4 mutant cells, but that ERK phosphorylation occurs synchronously

361    throughout the culture. This is likely the reason that four hours of ERK induction *via*

362    4OHT converts the ICM to entirely GATA6-positive PrE over the course of a

363    subsequent 24-hour period, similar to that achieved only when FGF4 is present for

364    the full 24 hours. What does this suggest about the network involved in Epiblast-

365    endoderm segregation, that has always been modelled based on GATA6-FGF-

366    NANOG? Perhaps that the induction of GATA6 may not be a direct transcriptional

367    response to FGF/ERK, but rather its levels are stabilised someplace downstream of

368    the immediate early response that includes induction of *Sox17* mRNA.

369    Given the effective homogenous response achieved with the c-RAF model, we

370    have been able to produce quantitative data on changes in enhancer and promoter

14

371   accessibility that suggest cell type-independent regulatory regions that acquire

372   similar signalling-induced accessibility in all cell types. However, regions that

373   become decommissioned in response to signalling were found to be cell type

374   specific.

375     In naïve ESCs, the regions losing accessibility upon ERK induction are

376   characterized by the presence of TCF motifs (Fig. 6F). ESCs and RSCs are cultured

377   in nearly identical conditions, with the only difference being that canonical WNT

378   signalling is activated in ESC *via* GSK3 inhibition and inhibited in RSC with IWP2

379   (see Methods). In naïve ESCs, canonical ß-catenin signalling exploits TCF3 to

380   support pluripotency, by associating with these regulatory regions and

381   counteracting TCF3-mediated repression(Athanasouli et al., 2023; Watanabe and

382   Dai, 2011). Yet canonical WNT signalling also induces PrE differentiation in ESC and

383   can do so in virtually identical ESC media with one key difference, the removal of

384   the FGF block (Anderson et al., 2017). How does the same pathway support both

385   activities? In response to ERK activation in naïve ESCs, we found decommissioned

386   regulatory regions enriched in TCF binding, suggesting an FGF-dependent

387   alteration in WNT activity preventing it from activating the TCF dependent

388   pluripotency network. As a result, WNT activation now triggers endoderm

389   differentiation based on an ERK dependent set of accessible TCF targets.

390     In the Epiblast lineage, where WNT is inhibited prior to ERK signalling in RSCs,

391   repression by ERK once again targets naïve ESC enhancers, but this time in regions

392   enriched in POU/OCT motifs such that signalling promotes the transition into

393   primed pluripotency (Fig. 6F). These regions could reflect the acquisition of

394   bivalency at pluripotency enhancers in RSCs, enabling them to both move forward

395   developmentally, while retaining the capacity to revert to naïve ESCs (Neagu et al.,

396   2020). Finally in EpiLCs, where FGF/ERK supports self-renewal, we identify the FOX

397   factors prominent in gastrulation stage differentiation.

398    Historically it has been difficult to reconcile the role of FGF/ERK in neural

399    differentiation with so called default neural induction. We previously observed that

400    anterior neural identity can be achieved in the presence of ERK inhibition (Hamilton

401    and Brickman, 2014), and similar results were obtained when BMP receptor mutants

402    were cultured in a block to the FGF receptor (Di-Gregorio et al., 2007). However,

403    this contradicts experiments in the chick embryo (Linker and Stern, 2004; Lunn et al.,

404    2007) and ESC differentiation (Kunath et al., 2007; Stavridis et al., 2007), where it

405    was argued that naïve to primed transition requires ERK. Our findings provide a

406    means to reconcile these observations, by suggesting that context-dependent

407    repression by ERK drives endoderm differentiation in the presence of WNT

408    signalling, but Epiblast differentiation in its absence. The presence of POU motifs in

409    ERK repressed RSC enhancers could also suggest that FGF/ERK represses the

410    overlapping pluripotent/neural programs regulated by POU and SOX proteins, such

411    that signalling supports the maintenance of Epiblast identity over its differentiation

412    towards anterior neural (Jaeger et al., 2011).

413    At a regulatory level, the notion that cell type-specific response is largely about

414    repression has a certain logic. Immediate early genes activated downstream of ERK

415    are widely studied and are likely to be context independent. This contrasts with

416    repression, that depends on the regulatory program transcribed in the target cell

417    type. While activation is more ubiquitous, relying on a conserved set of factors, it

418    would not be entirely - as time and duration would eventually alter the

419    transcriptional program in the responding cell type. Thus common immediate early

420    TFs would be influenced by time and the emergent network downstream of the

421    signal would interact with cell type specific factors to produce a unique response.

422    For example, we observe pluripotency genes are induced in EpiLCs only after four

423    hours, and are unlikely to be regulated directly by ETS–AP-1 factors.

424    While this work focuses on FGF/ERK, the question of context is universal to all

425    signalling pathways. Here we show that these ubiquitous factors can act identically

16

426   in different cell types, but their capacity to induce transcription is not always

427   correlated with alterations in chromatin structure. We found that the cis-regulatory

428   strategy by which a signalling generates a transcriptional output requires

429   remodelling in naïve ESCs, but not in EpiLC, suggesting that the same signal can

430   employ different strategies depending on the context. This signal adaptability to

431   the cellular context and chromatin has also been observed in other settings during

432   development (Delás et al., 2023), but the means by which these pathways can effect

433   changes in remodelling in one cell type, but not require it in another remains

434   unknown. However, it does suggest that the canonical view of signalling specificity

435   being governed by chromatin context and accessibility is not correct, as in this

436   instance we observe the same immediate early response, but in one instance this

437   involves remodelling and other not, suggesting that the first wave of ERK induction

438   can overcome chromatin accessibility.

439   Overall, our data provides a framework for signal-dependent context specificity

440   where both transcriptional and chromatin changes in response to ERK are more

441   universal in activation than repression. As we found that context-dependent

442   repression is mediated mainly by deactivation of enhancers, leaving cell specific TFs

443   bound to their targets, this response is, by definition, context-dependent. Activation

444   of ERK-responsive genes relies largely on commonly activated genes triggered as

445   immediate early response to ETS–AP-1 canonical factors, but this can be

446   propagated in time through existing cell-specific states. Whether activation exploits

447   chromatin remodelling or not would depend on the existence of prebound core

448   TFs, which could render cells plastic to respond to signalling and differentiate in

449   specific contexts (Hamilton et al., 2019; Knudsen et al., 2023; Redó-Riveiro et al.,

450   2024).

17

# Materials and methods

## *Generation of the CRAFR26 construct*

The CRAFR26 construct was engineered from two previous versions of the BXB plasmid (Hamilton and Brickman, 2014; Hamilton et al., 2019) that were digested and ligated to obtain a BXB-T2A-mCherry plasmid. Then, this fragment was introduced downstream of a lox-stop-lox cassette using the BigT plasmid (Srinivas et al., 2001), and then between the ROSA26 locus homology arms using the R26 plasmid (Srinivas et al., 2001).

## *Generation of CRAFR26 cells and mouse line*

The CRAFR26 construct was transfected into mESCs using a published gRNA directed to the Rosa26 locus (Gu et al., 2018), and the Cas9 plasmid PX458 (Addgene, #48138). Clonal colonies were initially screened by PCR, and then further validated by Southern blot (see probe sequence in Table S10), Sanger sequencing and in house karyotyping. For *in vitro* ERK induction experiments, the cells were lipofected with a pCAG-CRE plasmid to remove the STOP codon. The cells were then validated by flow cytometry, western blot and immunostaining.

To generate the CRAFR26 mouse line, correctly targeted CRAFR26 mESCs (Agouti background) were injected into C57BL/6 mouse embryos and chimera contribution was assessed by fur colour. Male chimeras were crossed with C57BL/6 females to obtain heterozygous F1s. Pups were genotyped from ear snips by PCR.

Primers and genotyping PCR as follows:

| | 5' to 3' sequence | PCR Parameters | Expected product |
|---|---|---|---|
| External Forward | CTTGCTCTCCCAAAGTCGCT | 95°C, 5 min;<br>10 cycles of: 95°C, 30 s; 59°C, 30s, ramp -0.5°C/cycle; 72°C, 30 s<br>24 cycles of: 95°C, 30 s; 54°C, 30s;<br>72°C, 30 s;<br>72°C, 10 min | 300 bp (tg band) |
| Internal Reverse | CTGGAAAGACCGCGAAGAGT | | |
| External Forward | CTTGCTCTCCCAAAGTCGCT | 95°C, 5 min;<br>35 cycles of: 95°C, 30 s; 54°C, 30s;<br>72°C, 30 s;<br>72°C, 10 min | 600 bp (wt band) |
| External Reverse | AGAAGGAGCGGGAGAAATGG | | |

18

### *Mouse husbandry*

Mouse lines (*Mus musculus*) were maintained under 12h light cycles in designated facilities at the University of Copenhagen. Natural mating was set up in the evening, and females were checked for plugs the following morning, which was established as embryonic day 0.5 (E0.5). Mouse heterozygous Sox2Cre (Hayashi et al., 2002) females were mated with homozygous CRAFR26 males to produce constitutive CRAFR26 expression in pre-implantation embryos, and Sox2Cre males were mated with homozygous CRAFR26 females to produce expression of CRAFR26 in the Epiblast lineage in post-implantation embryos (Hayashi et al., 2003). Sox2Cre females were superovulated to obtain larger numbers of embryos per animal. Superovulation was performed by intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (PMSG, Sigma) followed by 5 IU human chorionic gonadotrophin (hCG, Chorulon, Intervet) 42 to 50 hours later. The females were mated immediately following hCG injection.

Animal work was authorized by the Danish National Animal Experiments Inspectorate under project license 2018-15-0201-01520 and performed in accordance with Danish national guidelines and European legislation.

### *mESCs culture*

CRAFR26 mESCs were routinely cultured on 0.1% gelatine-coated flasks in 2i/LIF media (Ying et al., 2008) consisting of N2B27 media supplemented with 3 µM of the GSK3 inhibitor Chir (Axon, CHIR99021), 1 µM of the MEKi PD03 (PD0325901, Sigma PZ0162) and 1000 U/ml of LIF (prepared in house). N2B27 media was prepared as previously described (Knudsen et al., 2023). For passaging, confluent flasks were washed with PBS, incubated for 3 minutes in accutase (Sigma, A6964), and tapped to release the cells from the flask. Accutase was inactivated by adding medium and the cells were centrifuged, resuspended and replated onto new gelatine-coated flasks. All mESCs lines used in this study were routinely karyotyped and tested for mycoplasma.

For differentiation into RSCs, mESCs were plated into N2B27 media supplemented with 2 µM IWP2 (Millipore, 681671), 1 µM of the MEKi PD03 (PD0325901, Sigma, PZ0162) and 1000 U/ml of LIF (prepared in house). After 3 days cells were passaged as RSCs (Neagu et al., 2020).

501    For EpiLC differentiation, RSCs were plated in clumps at high confluency (50,000 cells/cm$^2$),

502    on Fibronectin-coated plates (Millipore, FC010), into N2B27 media supplemented with 12

503    ng/ml bFGF (Peprotech, 450-33-100µG), 20 ng/ml Activin A (Peprotech, 120-14E-200µG)

504    and 1 µM XAV939 (Sigma, X3004). Media was changed after 24h and EpiLCs were collected

505    after 48h.

506    For ERK induction *in vitro*, mESCs and RSCs were cultured for 48h before the experiment in

507    250 nM of the FGFRi PD17 (PD173074, Sigma, P2499) instead of PD03. For EpiLCs, PD17

508    incubation was reduced to 2h prior to induction. Then, the c-RAF kinase construct was

509    induced by adding 250 nM 4OHT (4-Hydroxytamoxifen, Sigma, H7904) to the media.

510    *Immunofluorescence staining of cells*

511    Cells were cultured for immunostaining in 8-well slides (Ibidi, 80826), and then stained and

512    imaged on the plate. Cells were fixed for 10 minutes with 4% Formaldehyde (Fisher

513    Scientific, PI-28906) at room temperature, then washed 3 times with PBS, then treated with

514    cold methanol for 10 minutes at -20°C and washed again 3 times with PBS. Cells were

515    blocked for 2 hours at room temperature in 10% Donkey Serum (Sigma, D9663), 0.3%

516    Triton X-100 (Sigma, T8787) in PBS. Primary antibodies (see Table S11) were incubated

517    overnight at 4°C in 1% BSA (Sigma, A2153), then washed 3 times. Secondaries were

518    incubated for 2 hours at room temperature also in 1% BSA, and further washed 3 times.

519    DAPI (Molecular Probes, D1306, 1 µg/ml) was then added for 5 minutes and washed twice

520    with PBS. Cells were imaged in a Zeiss 780 confocal microscope.

521    *Isolation of mouse embryos*

522    Timed pregnant females were euthanised by cervical dislocation. E2.5 embryos were

523    flushed from the oviducts using M2 medium (Sigma, M7167), and E5.5-E6.5 embryos were

524    dissected from the uterus. For Sox2Cre; CRAFR26 embryos, they were briefly imaged in a

525    Deltavision Widefield Screening microscope to confirm their genotype by mCherry

526    expression.

527    For longer culture of E2.5 embryos the zona pellucida was removed by brief incubation in

528    acidic Tyrode's solution (Sigma, T1788) and then the embryos were incubated in KSOM

529    drops (Millipore, MR-101-D) covered in mineral oil (NidOil, Nidacon, NO-400K) at 37°C. To

530    induce ERK, 4OHT (4-Hydroxytamoxifen, Sigma, H7904) was added at 250 nM for the times

531    indicated. FGFRi (PD173074, Sigma, P2499) was added at 250 nM. FGF4 supplemented

532    with heparin was added at 500 ng/ml. IVC2 media was used for culture of E5.5-E6.5

533    embryos (Bedzhov et al., 2014).

534    *Immunofluorescence staining of E4.5 mouse embryos*

535    E4.5 embryos were fixed in 4% Formaldehyde (Fisher Scientific, PI-28906) at room

536    temperature for 15 minutes, then washed twice in PBS. Embryos were permeabilized for 15

537    minutes in 0.25% Triton X-100 (Sigma, T8787) in PBS/PVP (3 mg/ml PVP

538    (Polyvinylpyrrolidone, Sigma, PVP40) in PBS). Blocking was performed for 1 hour in 2%

539    Donkey Serum (Sigma, D9663), 0.1% BSA (Sigma, A2153), 0.01% Tween20 (Sigma, P1379)

540    in PBS/PVP. Primary antibodies (see Table S11) were added to blocking buffer and

541    incubated overnight at 4°C. Embryos were washed 3 times for 10 minutes in blocking

542    buffer, and then secondary antibodies were added to blocking buffer and incubated for 2

543    hours. Secondary antibodies were washed 3 times for 10 minutes in blocking buffer. DAPI

544    (Molecular Probes, D1306, 1 µg/ml) was added to blocking buffer and incubated for 15

545    minutes at room temperature before washing once more in blocking buffer and mounted

546    for imaging. Imaging was performed in a Leica Stellaris confocal microscope.

547    *Immunofluorescence staining of E5.5-E6.5 mouse embryos*

548    E6.5 embryos were fixed in 4% Formaldehyde (Fisher Scientific, PI-28906) at room

549    temperature for 30 minutes, and then washed 3 times for 10 minutes in PBS. Embryos were

550    permeabilized for 30 minutes in 0.5% Triton X-100 (Sigma, T8787) in PBS. Blocking was

551    done overnight in 2% Donkey Serum (Sigma, D9663), 0.1% BSA (Sigma, A2153), 0.1%

552    Tween20 (Sigma, P1379) in PBS. Primary antibodies (see Table S11) were added to blocking

553    buffer and incubated for 48h at 4°C. Embryos were washed 3 times for 15 minutes in

554    blocking buffer, and then washed overnight. Secondary antibodies were added to blocking

555    buffer and incubated overnight. Secondary antibodies were washed 3 times for 15 minutes

556    and washed overnight in blocking buffer. DAPI (Molecular Probes, D1306, 1 µg/ml) was

557    added to blocking buffer and incubated for 2 hours at room temperature before washing 3

558    times for 15 minutes in blocking buffer and mounted for imaging. All overnight incubations

21

559 were done at 4°C with gentle shaking in the dark. Imaging was performed in a Leica

560 Stellaris confocal microscope.

*Bulk RNA-sequencing (RNA-seq) of cells*

562 RNA was extracted using the RNEasy Mini Kit (Qiagen, 74106) following manufacturer's

563 instructions. 4 biological replicates were collected per sample (ESCs vs RSCs vs EpiLCs) and

564 per condition (0h vs 2h vs 4h vs rev), defined as 2 different passages of 2 independent

565 clones. RNA was quantified by Nanodrop and 1 µg of RNA per sample was treated for

566 ribosomal RNA depletion using the NEBNext rRNA Depletion Kit (NEB, E6350) and then

567 immediately followed by Library prep using NEBNext Ultra II kit (NEB, E7770). Libraries

568 were pooled and sequenced using a H75 kit from Illumina in a NextSeq500 sequencer

569 following manufacturer's instructions.

*Bulk RNA-sequencing (RNA-seq) of E3.5 mouse Epiblast*

571 To obtain an ICM with only Epiblast cells, E2.5 Sox2Cre; CRAFR26 embryos were cultured

572 in KSOM supplemented with 250 nM PD17 (PD173074, Sigma, P2499) for 24h. Then, to

573 induce ERK, the media was supplemented with either 250 nM 4OHT (4-Hydroxytamoxifen,

574 Sigma, H7904) for ERK induction, or ethanol at the same dilution for control conditions.

575 To collect the ICM, the trophectoderm layer was removed by blastocyst immunosurgery as

576 previously described (Linneberg-Agerholm et al., 2023; Solter and Knowles, 1975). 4

577 biological replicates were collected per condition (treatment vs control), each containing a

578 group of 10 ICMs. The samples were placed into a 1.5ml tube with lysis buffer from the

579 Arcturus™ PicoPure™ RNA Isolation Kit (Applied Biosystems, KIT0204), and followed with

580 RNA extraction according to manufacturer's instructions. RNA was quantified using a

581 Qubit™ RNA High Sensitivity (HS) kit (Invitrogen, Q32852). Libraries were prepared from 2

582 ng of RNA per sample using the NEBNext® Single Cell/Low Input RNA Library Prep Kit for

583 Illumina (NEB, E6420). Libraries were pooled and sequenced using a P3-100 kit from

584 Illumina in a NextSeq2000 sequencer following manufacturer's instructions.

*Bulk RNA-sequencing (RNA-seq) of E5.5 mouse Epiblast*

586 To induce ERK in E5.5 Sox2Cre; CRAFR26 embryos, they were cultured in IVC2 media

587 (Bedzhov et al., 2014) supplemented with either 250 nM 4OHT (4-Hydroxytamoxifen, Sigma

22

588    H7904) for ERK induction, or ethanol at the same dilution for control conditions. The

589    Trophectoderm and Parietal Endoderm were removed by dissection using forceps. Then,

590    the Epiblast was separated from the Visceral Endoderm by 8 minutes incubation on ice in a

591    solution of 2.5% pancreatin (Sigma, P3292) and 0.5% trypsin (Sigma, T4799). 4 biological

592    replicates were collected per condition (treatment vs control), each containing a group of

593    10 isolated Epiblasts. The samples were immediately placed into a 1.5 ml tube with 350 μl

594    of lysis buffer, as per manufacturer's instructions of the Micro RNEasy Kit (Qiagen, 74004),

595    used for RNA extraction. After extraction, RNA was quantified using a Qubit™ RNA High

596    Sensitivity (HS) kit (Invitrogen, Q32852). Libraries were prepared from 7 ng of RNA per

597    sample using the NEBNext® Single Cell/Low Input RNA Library Prep Kit for Illumina (NEB,

598    E6420). Libraries were pooled and sequenced using a P3-100 kit from Illumina in a

599    NextSeq2000 sequencer following manufacturer's instructions.

600    *Bulk RNA sequencing data analysis*

601    Raw reads were processed with bcl2fastq (v 2.19.1) and STAR (v 2.5.3a) was used to map

602    sequencing reads and to generate the count table (Dobin et al., 2013). Genes with less than

603    10 counts were discarded from downstream analysis. 1 sample of 0h EpiLCs and 1 sample

604    of 0h E3.5 were discarded at Quality Control. Principal component analysis and differential

605    expression analysis were performed using the DESeq2 (Love et al., 2014) and factoextra

606    (Kassambara and Mundt, 2020) packages in R, significance was defined as abs(log2FC) >

607    1.5 and adjusted p < 0.05 for *in vitro* samples, and abs(log2FC) > 0.75 and adjusted p <

608    0.05 for *in vivo* samples. Principal component analysis was computed using the prcomp

609    function. Hierarchical clustering was performed using the hclust function. Enrichment of GO

610    terms was determined using the clusterProfiler package (Wu et al., 2021; Yu et al., 2012).

611    Heatmaps were created using ComplexHeatmap (Gu et al., 2016).

612    *ATAC-seq*

613    ATAC-seq or Omni-ATAC was performed as described in Grandi et al., 2022. Briefly, 50,000

614    cells were collected in a 1.5 ml DNA LoBind tube (Eppendorf, 0030108051), and lysed for 3

615    minutes on ice in 0.1% IGEPAL, 0.1% Tween-20, 0.01% Digitonin (Promega, G9441). Nuclei

616    were washed and pelleted for 10 minutes at 500g, and then treated for 30 minutes at 37°C

617    with Tn5 transposase (Illumina, 20034197). The DNA was extracted using DNA Clean and

23

618 Concentrator 5-kit (Zymo Research, D4014), and libraries were prepared according to

619 (Buenrostro et al., 2013). Libraries were quantified using the NEBNext Library Quant Kit

620 (NEB, E7630S), then pooled at same molarity and paired-end sequenced with a P2-100 kit

621 from Illumina in a NextSeq2000 sequencer following manufacturer's instructions.

622 *ATAC-seq data analysis*

623 Raw reads were processed with bcl2fastq, adapters were trimmed with cutadapt, reads

624 were mapped to mm10 using bowtie2. ChrM, duplicate reads and reads in blacklisted

625 regions were removed. Peaks were called using macs2 (v 2.2.7.1) (--broad -f BAMPE --

626 qvalue 0.001) and consensus peaks per condition were defined from two replicates per

627 condition using the DiffBind package (Stark and Brown, 2011). We removed promoter

628 regions using the publicly available Eukaryotic Promoter Database (Dreos et al., 2015;

629 Périer et al., 1998). Reads within defined peaks were counted using subread featureCounts

630 -p v2.0.3 (Liao et al., 2014). Counts were used to generate a count table for downstream

631 differential enrichment analysis using DESeq2 (Love et al., 2014) , to obtain the Log2 Fold

632 Change (log2FC) enrichment per peak before and after ERK induction. Significance was

633 defined as abs(log2FC) > 1.5 and padj < 0.01. Overlap of peaks between conditions was

634 determined using bedtools intersect (v2.31.0) (Quinlan and Hall, 2010). Motif analysis and

635 peak annotation was performed with HOMER (v4.11, findMotifsGenome.pl -size 200; and

636 annotatePeaks.pl) (Heinz et al., 2010). Heatmaps and Profile Plots were created with

637 deeptools (v3.5.1) (Ramírez et al., 2016). TF footprinting and differential binding score was

638 determined using TOBIAS (Bentsen et al., 2020). Correction and scoring were done in

639 merged bam files with both replicates, against all consensus peaks. BINDetect was

640 performed in the peak set of interest. TF binding motifs for footprinting analysis were

641 downloaded from the latest version of JASPAR (Rauluseviciute et al., 2023; Sandelin et al.,

642 2004).

24

## 643 Competing interest statement

644 The authors declare no competing interests.

## 645 Acknowledgements

## 653 Author contributions

654 MP and JMB conceived the study, designed and interpreted experiments. MP performed all
655 experiments and analysed the data. MP and JMB wrote the manuscript.

## 656 Funding

## 664 Data and materials availability

665 Previously published Hamilton et al. 2019 data that were used here are available in the
666 Gene Expression Omnibus (GEO) repository under accession number GSE132444.
667 Previously published Buecker et al. 2014 data that were used here are available in the Gene
668 Expression Omnibus (GEO) repository under accession number GSE56138. The RNA-seq
669 and ATAC-seq datasets generated in this study have been deposited in the Gene
670 Expression Omnibus (GEO) repository and are available under these accession numbers:
671 GSE259232 for RNA-seq in ESCs, GSE259233 for RNA-seq in RSCs, GSE259234 for RNA-
672 seq in EpiLCs, GSE259235 for RNA-seq in E3.5, GSE259236 for RNA-seq in E5.5,
673 GSE259237 for the ATAC-seq. Reagents generated in this study are available upon
674 reasonable request to the corresponding author.

# References

Anderson, K. G. V., Hamilton, W. B., Roske, F. V., Azad, A., Knudsen, T. E., Canham, M. A., Forrester, L. M. and Brickman, J. M. (2017). Insulin fine-tunes self-renewal pathways governing naive pluripotency and extra-embryonic endoderm. *Nature Cell Biology* 19, 1164–1177.

Artus, J., Piliszek, A. and Hadjantonakis, A.-K. (2011). The primitive endoderm lineage of the mouse blastocyst: Sequential transcription factor activation and regulation of differentiation by Sox17. *Developmental Biology* 350, 393–404.

Athanasouli, P., Balli, M., De Jaime-Soguero, A., Boel, A., Papanikolaou, S., van der Veer, B. K., Janiszewski, A., Vanhessche, T., Francis, A., El Laithy, Y., et al. (2023). The Wnt/TCF7L1 transcriptional repressor axis drives primitive endoderm formation by antagonizing naive and formative pluripotency. *Nat Commun* 14, 1210.

Azami, T., Bassalert, C., Allègre, N., Valverde Estrella, L., Pouchin, P., Ema, M. and Chazaud, C. (2019). Regulation of the ERK signalling pathway in the developing mouse blastocyst. *Development* 146, dev177139.

Bedzhov, I. and Zernicka-Goetz, M. (2014). Self-Organizing Properties of Mouse Pluripotent Cells Initiate Morphogenesis upon Implantation. *Cell* 156, 1032–1044.

Bedzhov, I., Leung, C. Y., Bialecka, M. and Zernicka-Goetz, M. (2014). In vitro culture of mouse blastocysts beyond the implantation stages. *Nat Protoc* 9, 2732–2739.

Bentsen, M., Goymann, P., Schultheis, H., Klee, K., Petrova, A., Wiegandt, R., Fust, A., Preussner, J., Kuenne, C., Braun, T., et al. (2020). ATAC-seq footprinting unravels kinetics of transcription factor binding during zygotic genome activation. *Nat Commun* 11, 4267.

Brewer, J. R., Mazot, P. and Soriano, P. (2016). Genetic insights into the mechanisms of Fgf signaling. *Genes Dev.* 30, 751–771.

Brons, I. G. M., Smithers, L. E., Trotter, M. W. B., Rugg-Gunn, P., Sun, B., Chuva De Sousa Lopes, S. M., Howlett, S. K., Clarkson, A., Ahrlund-Richter, L., Pedersen, R. A., et al. (2007). Derivation of pluripotent epiblast stem cells from mammalian embryos. *Nature* 448, 191–195.

Buecker, C., Srinivasan, R., Wu, Z., Calo, E., Acampora, D., Faial, T., Simeone, A., Tan, M., Swigut, T. and Wysocka, J. (2014). Reorganization of Enhancer Patterns in Transition from Naive to Primed Pluripotency. *Cell Stem Cell* 14, 838–853.

Buenrostro, J. D., Giresi, P. G., Zaba, L. C., Chang, H. Y. and Greenleaf, W. J. (2013). Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nat Methods* 10, 1213–1218.

26

711 Chazaud, C., Yamanaka, Y., Pawson, T. and Rossant, J. (2006). Early Lineage Segregation
712     between Epiblast and Primitive Endoderm in Mouse Blastocysts through the Grb2-
713     MAPK Pathway. *Developmental Cell* 10, 615–624.

714 Corson, L. B., Yamanaka, Y., Lai, K.-M. V. and Rossant, J. (2003). Spatial and temporal
715     patterns of ERK signaling during mouse embryogenesis. *Development* 130, 4527–
716     4537.

717 Delás, M. J., Kalaitzis, C. M., Fawzi, T., Demuth, M., Zhang, I., Stuart, H. T., Costantini, E.,
718     Ivanovitch, K., Tanaka, E. M. and Briscoe, J. (2023). Developmental cell fate choice
719     in neural tube progenitors employs two distinct cis-regulatory strategies.
720     *Developmental Cell* 58, 3-17.e8.

721 Di-Gregorio, A., Sancho, M., Stuckey, D. W., Crompton, L. A., Godwin, J., Mishina, Y. and
722     Rodriguez, T. A. (2007). BMP signalling inhibits premature neural differentiation in
723     the mouse embryo. *Development* 134, 3359–3369.

724 Dobin, A., Davis, C. A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson,
725     M. and Gingeras, T. R. (2013). STAR: ultrafast universal RNA-seq aligner.
726     *Bioinformatics* 29, 15–21.

727 Dorey, K. and Amaya, E. (2010). FGF signalling: diverse roles during early vertebrate
728     embryogenesis. *Development* 137, 3731–3742.

729 Dreos, R., Ambrosini, G., Périer, R. C. and Bucher, P. (2015). The Eukaryotic Promoter
730     Database: expansion of EPDnew and new promoter analysis tools. *Nucleic Acids*
731     *Research* 43, D92–D96.

732 Endoh, M. and Niwa, H. (2022). Stepwise pluripotency transitions in mouse stem cells.
733     *EMBO reports* 23, e55010.

734 Frankenberg, S., Gerbe, F., Bessonnard, S., Belville, C., Pouchin, P., Bardot, O. and
735     Chazaud, C. (2011). Primitive Endoderm Differentiates via a Three-Step Mechanism
736     Involving Nanog and RTK Signaling. *Developmental Cell* 21, 1005–1013.

737 Grandi, F. C., Modi, H., Kampman, L. and Corces, M. R. (2022). Chromatin accessibility
738     profiling by ATAC-seq. *Nat Protoc* 17, 1518–1552.

739 Greber, B., Wu, G., Bernemann, C., Joo, J. Y., Han, D. W., Ko, K., Tapia, N., Sabour, D.,
740     Sterneckert, J., Tesar, P., et al. (2010). Conserved and Divergent Roles of FGF
741     Signaling in Mouse Epiblast Stem Cells and Human Embryonic Stem Cells. *Cell Stem*
742     *Cell* 6, 215–226.

743 Gu, Z., Eils, R. and Schlesner, M. (2016). Complex heatmaps reveal patterns and
744     correlations in multidimensional genomic data. *Bioinformatics* 32, 2847–2849.

745 Gu, B., Posfai, E. and Rossant, J. (2018). Efficient generation of targeted large insertions by
746     microinjection into two-cell-stage mouse embryos. *Nature Biotechnology* 36, 632–
747     637.

748 Hamilton, W. B. and Brickman, J. M. (2014). Erk Signaling Suppresses Embryonic Stem Cell
749         Self-Renewal to Specify Endoderm. *Cell Reports* 9, 2056–2070.

750 Hamilton, W. B., Mosesson, Y., Monteiro, R. S., Emdal, K. B., Knudsen, T. E., Francavilla, C.,
751         Baarkai, N., Olsen, J. V. and Brickman, J. M. (2019). Dynamic lineage priming is
752         driven via direct enhancer regulation by ERK. *Nature* 575, 355–360.

753 Hayashi, S., Lewis, P., Pevny, L. and McMahon, A. P. (2002). Efficient gene modulation in
754         mouse epiblast using a Sox2Cre transgenic mouse strain. *Mechanisms of
755         Development* 119, S97–S101.

756 Hayashi, S., Tenzen, T. and McMahon, A. P. (2003). Maternal inheritance of Cre activity in a
757         Sox2Cre deleter strain. *genesis* 37, 51–53.

758 Hayashi, K., Ohta, H., Kurimoto, K., Aramaki, S. and Saitou, M. (2011). Reconstitution of the
759         Mouse Germ Cell Specification Pathway in Culture by Pluripotent Stem Cells. *Cell*
760         146, 519–532.

761 Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y. C., Laslo, P., Cheng, J. X., Murre, C.,
762         Singh, H. and Glass, C. K. (2010). Simple Combinations of Lineage-Determining
763         Transcription Factors Prime cis-Regulatory Elements Required for Macrophage and
764         B Cell Identities. *Molecular Cell* 38, 576–589.

765 Hnisz, D., Abraham, B. J., Lee, T. I., Lau, A., Saint-André, V., Sigova, A. A., Hoke, H. A. and
766         Young, R. A. (2013). Super-Enhancers in the Control of Cell Identity and Disease.
767         *Cell* 155, 934–947.

768 Jaeger, I., Arber, C., Risner-Janiczek, J. R., Kuechler, J., Pritzsche, D., Chen, I.-C.,
769         Naveenan, T., Ungless, M. A. and Li, M. (2011). Temporally controlled modulation of
770         FGF/ERK signaling directs midbrain dopaminergic neural progenitor fate in mouse
771         and human pluripotent stem cells. *Development* 138, 4363–4374.

772 Kang, M., Garg, V. and Hadjantonakis, A.-K. (2017). Lineage Establishment and Progression
773         within the Inner Cell Mass of the Mouse Blastocyst Requires FGFR1 and FGFR2.
774         *Developmental Cell* 41, 496-510.e5.

775 Kassambara, A. and Mundt, F. (2020). factoextra: Extract and Visualize the Results of
776         Multivariate Data Analyses.

777 Knudsen, T. E., Hamilton, W. B., Proks, M., Lykkegaard, M., Linneberg-Agerholm, M.,
778         Nielsen, A. V., Perera, M., Malzard, L. L., Trusina, A. and Brickman, J. M. (2023). A
779         bipartite function of ESRRB can integrate signaling over time to balance self-renewal
780         and differentiation. *Cell Systems* 14, 788-805.e8.

781 Krawchuk, D., Honma-Yamanaka, N., Anani, S. and Yamanaka, Y. (2013). FGF4 is a limiting
782         factor controlling the proportions of primitive endoderm and epiblast in the ICM of
783         the mouse blastocyst. *Developmental Biology* 384, 65–71.

784      Kunath, T., Saba-El-Leil, M. K., Almousailleakh, M., Wray, J., Meloche, S. and Smith, A.
785          (2007). FGF stimulation of the Erk1/2 signalling cascade triggers transition of
786          pluripotent embryonic stem cells from self-renewal to lineage commitment.
787          *Development* 134, 2895–2902.

788      Liao, Y., Smyth, G. K. and Shi, W. (2014). featureCounts: an efficient general purpose
789          program for assigning sequence reads to genomic features. *Bioinformatics* 30, 923–
790          930.

791      Linker, C. and Stern, C. D. (2004). Neural induction requires BMP inhibition only as a late
792          step, and involves signals other than FGF and Wnt antagonists. *Development* 131,
793          5671–5681.

794      Linneberg-Agerholm, M., Sell, A. C., Redo-Riveiro, A., Proks, M., Knudsen, T. E., Perera, M.
795          and Brickman, J. M. (2023). Enhancer status in the primitive endoderm supports
796          unrestricted lineage plasticity in regulative development. 2023.05.20.540779.

797      Love, M. I., Huber, W. and Anders, S. (2014). Moderated estimation of fold change and
798          dispersion for RNA-seq data with DESeq2. *Genome Biology* 15, 550.

799      Lu, C. W., Yabuuchi, A., Chen, L., Viswanathan, S., Kim, K. and Daley, G. Q. (2008). Ras-
800          MAPK signaling promotes trophectoderm formation from embryonic stem cells and
801          mouse embryos. *Nature Genetics* 40, 921–926.

802      Lunn, J. S., Fishwick, K. J., Halley, P. A. and Storey, K. G. (2007). A spatial and temporal
803          map of FGF/Erk1/2 activity and response repertoires in the early chick embryo.
804          *Developmental Biology* 302, 536–552.

805      Molotkov, A., Mazot, P., Brewer, J. R., Cinalli, R. M. and Soriano, P. (2017). Distinct
806          Requirements for FGFR1 and FGFR2 in Primitive Endoderm Development and Exit
807          from Pluripotency. *Developmental Cell* 41, 511-526.e4.

808      Morgani, S. M., Nichols, J. and Hadjantonakis, A.-K. (2017). The many faces of Pluripotency:
809          in vitro adaptations of a continuum of in vivo states. *BMC Developmental Biology*
810          17, 7.

811      Neagu, A., van Genderen, E., Escudero, I., Verwegen, L., Kurek, D., Lehmann, J., Stel, J.,
812          Dirks, R. A. M., van Mierlo, G., Maas, A., et al. (2020). In vitro capture and
813          characterization of embryonic rosette-stage pluripotency between naive and primed
814          states. *Nature Cell Biology* 22, 534–545.

815      Nichols, J. and Smith, A. (2009). Naive and Primed Pluripotent States. *Cell Stem Cell* 4,
816          487–492.

817      Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I.,
818          Schöler, H. and Smith, A. (1998). Formation of Pluripotent Stem Cells in the
819          Mammalian Embryo Depends on the POU Transcription Factor Oct4. *Cell* 95, 379–
820          391.

821   Nichols, J., Silva, J., Roode, M. and Smith, A. (2009). Suppression of Erk signalling
822         promotes ground state pluripotency in the mouse embryo. *Development* 136,
823         3215–3222.

824   Ornitz, D. and Itoh, N. (2015). The fibroblast growth factor signaling pathway. *WIREs Dev*
825         *Bio* 4, 215:266-215:266.

826   Périer, R. C., Junier, T. and Bucher, P. (1998). The Eukaryotic Promoter Database EPD.
827         *Nucleic Acids Research* 26, 353–357.

828   Quinlan, A. R. and Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing
829         genomic features. *Bioinformatics* 26, 841–842.

830   Ramírez, F., Ryan, D. P., Grüning, B., Bhardwaj, V., Kilpert, F., Richter, A. S., Heyne, S.,
831         Dündar, F. and Manke, T. (2016). deepTools2: a next generation web server for
832         deep-sequencing data analysis. *Nucleic Acids Research* 44, W160–W165.

833   Rauluseviciute, I., Riudavets-Puig, R., Blanc-Mathieu, R., Castro-Mondragon, J. A., Ferenc,
834         K., Kumar, V., Lemma, R. B., Lucas, J., Chèneby, J., Baranasic, D., et al. (2023).
835         JASPAR 2024: 20th anniversary of the open-access database of transcription factor
836         binding profiles. *Nucleic Acids Research* gkad1059.

837   Redó-Riveiro, A., Al-Mousawi, J., Linneberg-Agerholm, M., Proks, M., Perera, M., Salehin,
838         N. and Brickman, J. M. (2024). Transcription factor co-expression mediates lineage
839         priming for embryonic and extra-embryonic differentiation. *Stem Cell Reports* 19,
840         174–186.

841   Sandelin, A., Alkema, W., Engström, P., Wasserman, W. W. and Lenhard, B. (2004). JASPAR:
842         an open-access database for eukaryotic transcription factor binding profiles. *Nucleic*
843         *Acids Research* 32, D91–D94.

844   Schrode, N., Saiz, N., Di Talia, S. and Hadjantonakis, A. K. (2014). GATA6 levels modulate
845         primitive endoderm cell fate choice and timing in the mouse blastocyst.
846         *Developmental Cell* 29, 454–467.

847   Shahbazi, M. N., Scialdone, A., Skorupska, N., Weberling, A., Recher, G., Zhu, M., Jedrusik,
848         A., Devito, L. G., Noli, L., Macaulay, I. C., et al. (2017). Pluripotent state transitions
849         coordinate morphogenesis in mouse and human embryos. *Nature* 552, 239–243.

850   Solter, D. and Knowles, B. B. (1975). Immunosurgery of mouse blastocyst. *Proc Natl Acad*
851         *Sci U S A* 72, 5099–5102.

852   Srinivas, S., Watanabe, T., Lin, C., William, C. M., Tanabe, Y., Jessell, T. M. and Costantini,
853         F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into
854         the ROSA26 locus. *BMC Developmental Biology* 1,.

855   Stark, R. and Brown, G. (2011). DiffBind: differential binding analysis of ChIP-Seq peak data.

856    Stavridis, M. P., Lunn, J. S., Collins, B. J. and Storey, K. G. (2007). A discrete period of FGF-
857         induced Erk1/2 signalling is required for vertebrate neural specification.
858         *Development* 134, 2889–2894.

859    Sumi, T., Oki, S., Kitajima, K. and Meno, C. (2013). Epiblast Ground State Is Controlled by
860         Canonical Wnt/**β**-Catenin Signaling in the Postimplantation Mouse Embryo and
861         Epiblast Stem Cells. *PLOS ONE* 8, e63378.

862    Tesar, P. J., Chenoweth, J. G., Brook, F. A., Davies, T. J., Evans, E. P., Mack, D. L., Gardner,
863         R. L. and McKay, R. D. G. (2007). New cell lines from mouse epiblast share defining
864         features with human embryonic stem cells. *Nature* 448, 196–199.

865    Turner, N. and Grose, R. (2010). Fibroblast growth factor signalling: From development to
866         cancer. *Nature Reviews Cancer* 10, 116–129.

867    Watanabe, K. and Dai, X. (2011). A WNTer Revisit: New Faces of **β**-Catenin and TCFs in
868         Pluripotency. *Science Signaling* 4, pe41–pe41.

869    Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., Feng, T., Zhou, L., Tang, W., Zhan, L., et
870         al. (2021). clusterProfiler 4.0: A universal enrichment tool for interpreting omics data.
871         *The Innovation* 2, 100141.

872    Yamanaka, Y., Lanner, F. and Rossant, J. (2010). FGF signal-dependent segregation of
873         primitive endoderm and epiblast in the mouse blastocyst. *Development* 137, 715–
874         724.

875    Ying, Q. L., Wray, J., Nichols, J., Batlle-Morera, L., Doble, B., Woodgett, J., Cohen, P. and
876         Smith, A. (2008). The ground state of embryonic stem cell self-renewal. *Nature* 453,
877         519–523.

878    Yu, G., Wang, L.-G., Han, Y. and He, Q.-Y. (2012). clusterProfiler: an R Package for
879         Comparing Biological Themes Among Gene Clusters. *OMICS: A Journal of
880         Integrative Biology* 16, 284–287.

881

## Figure legends

**Figure 1.** A new mouse and mESC tool to activate ERK in a time- and tissue-specific manner.

A. Diagram of the strategy to control ERK activation and inhibition. Culture with FGF Receptor inhibitor (FGFRi) inhibits the endogenous FGF/ERK pathway. The exogenous c-RAF-ER$^{T2}$ is activated with tamoxifen (4OHT). The activation can be rapidly reverted by adding MEK inhibitor (MEKi), upstream of ERK.

B. Outline of the CRAFR26 mouse line construct. A lox-stop-lox cassette prevents transcription unless Cre recombinase is expressed. A constitutively active form of the kinase c-RAF produces phosphatase-independent ERK induction. The c-RAF is fused with an ER$^{T2}$, rendering it inactive unless 4OHT is present. An HA-tag allows detection of the construct by Western blot or immunofluorescence. A T2A self-cleaving peptide separates the c-RAF from an H2B-mCherry fluorescent protein that labels all cells that express the construct. To activate the pathway during embryonic development, Sox2Cre heterozygous males were crossed with CRAFR26 homozygous females to obtain Sox2Cre$^{tg/+}$;Rosa26$^{CRAF/+}$ E6.5 embryos. Embryos were then treated with either 250 nM of 4OHT (to activate ERK) or ethanol (Vehicle control) for the indicated times.

C. Live imaging of E6.5 Sox2Cre$^{tg/+}$;Rosa26$^{CRAF/+}$ embryos shows expression of the mCherry fluorescent protein in the Epiblast, confirming expression of the CRAFR26 construct restricted to the Epiblast, driven by the Sox2Cre.

D. Sox2Cre$^{tg/+}$;Rosa26$^{CRAF/+}$ E6.5 embryos were fixed and stained after the indicated treatment (Vehicle or 4OHT). HA-tag detection shows expression of the construct restricted to the Epiblast driven by the Sox2Cre. pERK staining shows induction of the ERK pathway after 4OHT treatment, and no induction when treated with vehicle. Dashed lines indicate the location of the Epiblast. See quantification and n numbers in Fig. S1G-H.

E. The CRAFR26 cell line was transfected with a pCAG-Cre plasmid to remove the lox-stop-lox cassette and allow for constitutive expression of the construct in vitro. The c-RAF kinase is only active upon treatment with 4OHT. CRAFR26 mESCs lines were routinely cultured in 2i/LIF, and 48 hours before the experiment they were plated in FGFRi (see Methods). Induction of the c-RAF kinase was achieved by supplementation of 250 nM of 4OHT to the culture media for the indicated times. Reversion (rev) of the ERK activation was performed by addition of MEKi. Cells were collected at 0h (FGFRi), 2h or 4h of ERK induction, and reversion (4h of induction plus 2h of MEKi).

F. CRAFR26 mESCs were fixed at the indicated times of ERK induction and stained for pERK to show ERK induction, and SOX2 to confirm maintenance of mESC pluripotency during the induction. mCherry fluorescence indicates homogeneous expression of the CRAFR26 construct.

32

916    **Figure 2.** Pluripotent *in vitro* RNA-seq timecourse captures an early universal and a late

917    specific ERK transcriptional response.

918    A. Diagram depicting the *in vitro* cell types collected to investigate the context-dependent

919    responses to ERK in naïve (Embryonic Stem Cells, ESCs), primed (Epiblast-Like Cells, EpiLCs) and

920    intermediate (Rosette Stem Cells, RSCs) pluripotent populations. All samples were cultured in FGFRi

921    for 48 hours before beginning the experiment except for EpiLCs, which were in FGFRi for 2 hours.

922    Then ERK is activated for 2h or 4h by addition of 4OHT. To explore reversion (rev) of ERK target

923    genes, MEKi was added for an additional 2h after the induction. 4 biological replicates were

924    collected for each condition (0h, 2h, 4h and rev). 1 sample of EpiLC 0h was discarded during Quality

925    Control.

926    B. Principal Component Analysis (PCA) of the in vitro samples sequenced. PC1 contains most of the

927    variance (72%) and separates naïve ESCs from primed EpiLCs, with RSCs at the intermediate stage.

928    Symbol represents the treatment course described in Fig 2A.

929    C. Heatmap showing the expression of pluripotency markers (orange for naïve markers, blue for

930    primed and black for shared or core pluripotency) of the in vitro cell types before ERK induction (0h

931    as described in Fig 2A). ESCs and RSCs express mostly naïve markers while EpiLCs show higher

932    expression of primed markers. Each column represents a represents an individual biological

933    replicate. ESCs n=4, RSCs n=4, EpiLCs n=3. The colour scale indicates normalised counts in log2

934    scale, scaled by row.

935    D. Heatmap illustrating the conversion of the role of ERK in naïve vs primed pluripotent cell types.

936    The colour scale represents the log2 fold change (LFC) for each indicated sample between 4h of ERK

937    activation and 0h. While in ESCs and RSCs ERK downregulates most pluripotent genes, in EpiLCs

938    pluripotent genes are being upregulated upon ERK.

939    E. The genes annotated as the GO term MAPK cascade (GO:0000165) were plotted in average

940    expression changes (scaled from -1 to 1) between the conditions described in Fig 2A. Each line

941    represents a gene. The thickest line represents the average of all genes.

942    F. Barplots indicating the log2 fold change (LFC) of either 2h or 4h of ERK induction compared to the

943    baseline at 0h, as indicated. Early activated ERK genes are upregulated at 2h in all cell types, while

944    lineage specific late genes become up- or downregulated at 4h, showing cell type-specific

945    behaviour.

33

946    **Figure 3.** Repressed ERK targets are more specific to each cellular context than activated

947    responses.

948    A. Venn diagram showing the proportion of ERK-activated genes that are shared between cell types.

949    The ERK-activated genes in each stage were defined from k-means clustering (See Fig. S3A-C and

950    Methods) of all differentially expressed genes between all conditions (differentially expressed were

951    defined as padj < 0.05 and abs (LFC) > 1.5).

952    B. Venn diagram showing the proportion of genes repressed by ERK that are shared between cell

953    types. The ERK-repressed genes in each stage were defined from k-means clustering (See Fig. S3A-C

954    and Methods) of all differentially expressed genes between all conditions (differentially expressed

955    were defined as padj < 0.05 and abs (LFC) > 1.5).

956    C. Violin Plot of the log2 fold change (LFC) for each indicated cell type between 4h of ERK activation

957    and 0h, for the activated set of genes found specifically in ESC, RSC and EpiLC, respectively.

958    D. Violin Plot of the log2 fold change (LFC) for each indicated cell type between 4h of ERK activation

959    and 0h, for the repressed set of genes found specifically in ESC, RSC and EpiLC, respectively.

960    E. Violin Plot of the log2 fold change (LFC) for each indicated cell type between 4h of ERK activation

961    and 0h, for the genes annotated as the indicated GO Term.

34

962    **Figure 4.** Overlap between *in vivo* and *in vitro* responses to ERK.

963    A. Diagram of the *in vivo* populations collected for RNA-seq. Both pre-implantation (E3.5) and post-
964    implantation (E5.5) Epiblast were isolated after 4h of treatment with either 4OHT (for ERK activation),
965    or ethanol (Vehicle control). 4 replicates for each condition were collected, and 1 sample of E3.5 0h
966    was discarded during Quality Control.

967    B. Principal Component Analysis (PCA) of the 0h samples collected for RNA-seq shows that, while *in*
968    *vitro* and *in vivo* populations cluster separately in PC1, PC2 separates naïve populations (ESCs and
969    E3.5 Epiblast) from the primed populations (EpiLCs and E5.5 Epiblast), with the intermediate RSCs
970    population in the middle. Each dot represents an individual biological replicate. ESCs n=4, RSCs
971    n=4, EpiLCs n=3, E3.5 n=3, E5.5 n=4.

972    C. Heatmap showing the expression of pluripotency markers (orange for naïve markers, blue for
973    primed and black for shared or core pluripotency) of the *in vivo* populations before ERK induction.
974    E3.5 Epiblast expresses higher expression of naïve markers than post-implantation E5.5 Epiblast,
975    which expresses primed markers. Each column represents an individual biological replicate. E3.5
976    n=3, E5.5 n=4. The colour scale indicates normalised counts in log2 scale.

977    D. Correlation matrix for *in vivo* vs *in vitro* 4h induced samples, showing higher correlation for E3.5
978    with ESC, and E5.5 with EpiLCs. Count matrices from RNA-seq were filtered for genes differentially
979    expressed in E3.5 or E5.5, respectively, and covariances between all samples were calculated.
980    Differentially expressed genes were defined as padj < 0.05 and abs (LFC) > 0.75). The colour scale
981    indicates correlation coefficient.

982    E. Violin Plot of the log2 fold change (LFC) for each indicated sample between 4h of ERK activation
983    and 0h, for the genes annotated as the indicated GO Term.

984    **Figure 5.** ERK induction in pre-implantation embryos is more efficient than FGF4 in

985    inducing PrE.

986    A. Barplot indicating the log2 fold change (LFC) of either 2h or 4h of ERK induction compared to 0h,

987    as indicated. *Sox17* shows stronger transcriptional induction upon ERK than *Gata4* and *Gata6*, both

988    *in vivo* (E3.5) and *in vitro* (ESC).

989    B. E3.5 embryos were isolated and treated as indicated for 24h before fixing. Staining for SOX2

990    (Epiblast) and GATA6 (PrE) shows that ERK induction for 4h induces PrE at the same efficiency as

991    FGF4 treatment for 24h. FGFRi (PD17) was added at 250 nM. FGF4 supplemented with heparin was

992    added at 500 ng/ml. 4OHT was added at 250 nM.

993    C. Quantification of immunofluorescence images from Fig. 5B. Only ICM cells were scored. PrE cells

994    are scored at GATA6+ cells. Epiblast (Epi) cells are scored at SOX2+ cells. Double positive

995    GATA6/SOX2 were also observed. None double negative cells were observed in the ICM.

36

**Figure 6.** Chromatin accessibility cell-specific changes upon ERK induction *in vitro.*

A. Diagram depicting the *in vitro* cell types collected for ATAC-seq, before and after 4h of ERK activation by addition of 4OHT. 2 biological replicates were collected for each condition. All cells were pre-treated with FGFRi for 48h before ERK induction, except for EpiLCs which were pre-treated for 4h.

B. Scaled signal intensity from ATAC-seq of ESC, RSC and EpiLC comparing 0h and 4h of ERK induction, on the ERK-regulated enhancers defined in Hamilton et al., 2019.

C. Venn diagram showing the overlap between regions that gained accessibility upon ERK induction (Opening regions, padj < 0.01 and LFC > 1.5), comparing between the different cell types described in Fig. 6A.

D. Venn diagram showing the overlap between regions that lost accessibility upon ERK (Closing regions, padj < 0.01 and LFC < 1.5), comparing between the different cell types described in Fig. 6A.

E. Ternary plot depicting the motifs enriched in the opening regions between 0h and 4h of ERK, comparing p-value scores across ESC, RSC and EpiLC. The axes represent -log10 of p-value.

F. Ternary plot depicting the motifs enriched in the closing regions between 0h and 4h of ERK, comparing p-value scores across ESC, RSC and EpiLC. The axes represent -log10 of p-value.

G. Footprinting predicted binding score were calculated for all consensus peaks at 0h and 4h. Binding scores are normalised to number of binding sites and accessible regions. Then a differential score is computed to illustrate the fold change in binding between 4h and 0h of ERK induction. TFs of the indicated families were grouped together, showing enriched binding of TFs from the canonical ERK factors (AP-1/ETS) upon ERK induction.

H. Footprinting predicted binding score were calculated for all consensus peaks at 0h and 4h. Binding scores are normalised to number of binding sites and accessible regions. Then a differential score is computed to illustrate the fold change in binding between 4h and 0h of ERK induction. TFs of the indicated families were grouped together, showing distinct families of TFs being unbound upon ERK in specific cellular contexts.

37

1023     **Figure 7.** ERK mediates context-specific strategies in chromatin accessibility to drive

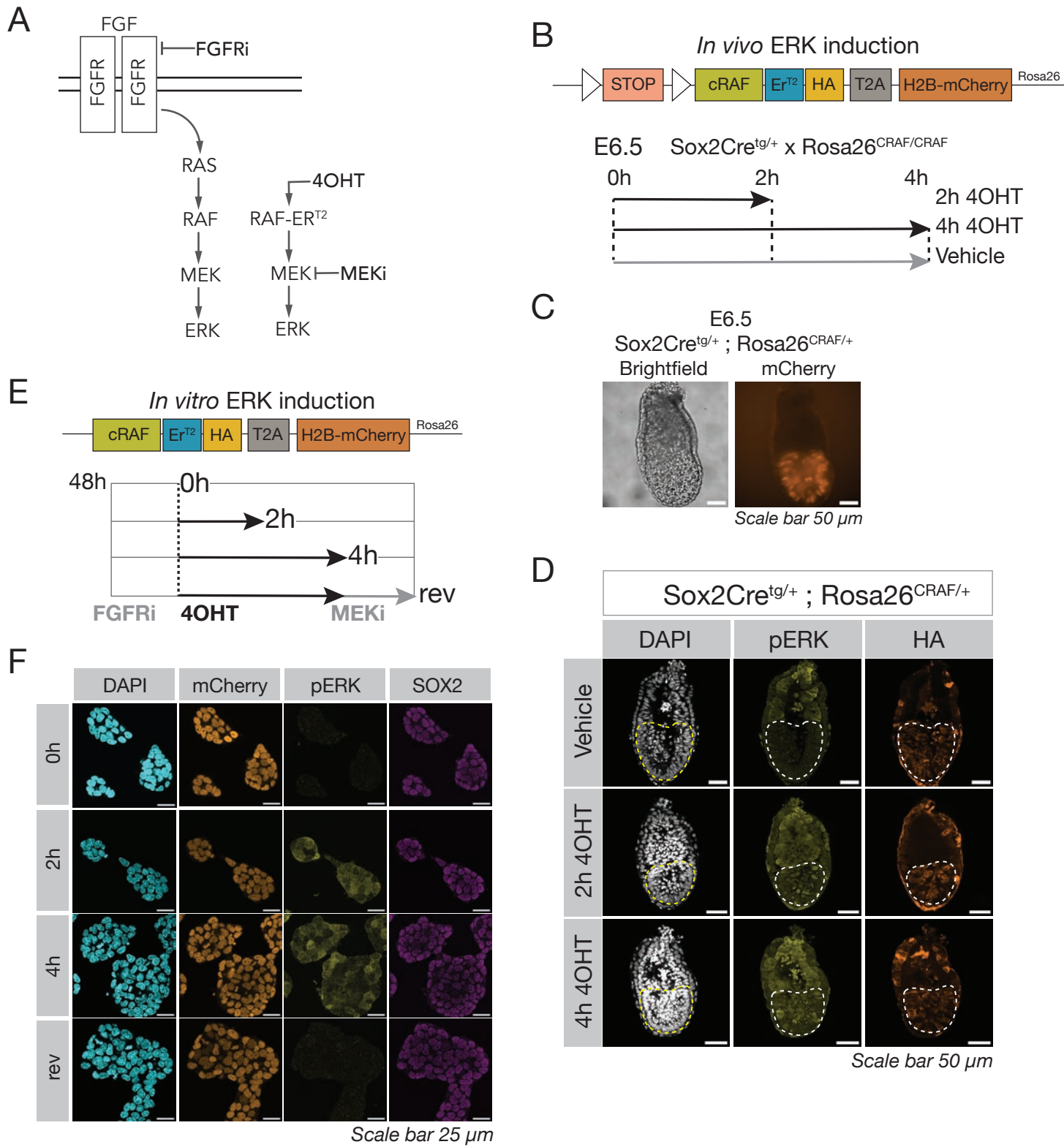1024     transcriptional responses.

1025     A. The ATAC-seq peaks were classified as specifically accessible in each cell type at 0h as shown in

1026     Fig. S7A. Then, the log2 Fold Changes (LFC) of the transcription at the closest genes are shown for

1027     each category of peaks. RNA-seq LFC values were filtered for padj < 0.05. **** represents p-value <

1028     0.0001 from Kruskal-Wallis test. n.s. represents a non-significant p-value > 0.01.

1029     B. Genome browser tracks (IGV v.2.16.0) showing ATAC-seq coverage for the *Spred3* enhancer and

1030     the corresponding normalized count for RNA-seq for the *Spred3* transcript. The regulatory region

1031     downstream of the TSS is available in RSCs and increasingly in EpiLCs before ERK induction (left) but

1032     transcription is only activated in EpiLCs upon ERK induction (right).

1033     C. The ATAC-seq peaks were classified as more (Opening) or less (Closing) accessible with ERK.

1034     Then, the log2 Fold Changes (LFC) of the transcription at the closest genes are shown for each

1035     category of regions. RNA-seq LFC values were filtered for padj < 0.05. **** represents p-value <

1036     0.0001 from Kruskal-Wallis test. n.s. represents a non-significant p-value > 0.01.

1037     D. Genome browser tracks (IGV v.2.16.0) showing ATAC-seq coverage for the *Zic3* enhancer and the

1038     corresponding normalized count for RNA-seq for the *Zic3* transcript. We found similar induction of

1039     transcription in all cell types (right). However, the regulatory region downstream was being made

1040     accessible with ERK induction in ESCs and RSCs but not in EpiLCs (left).
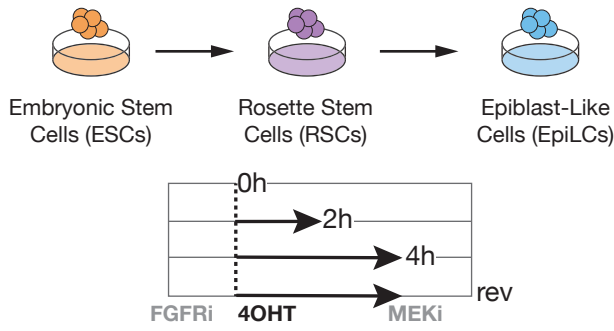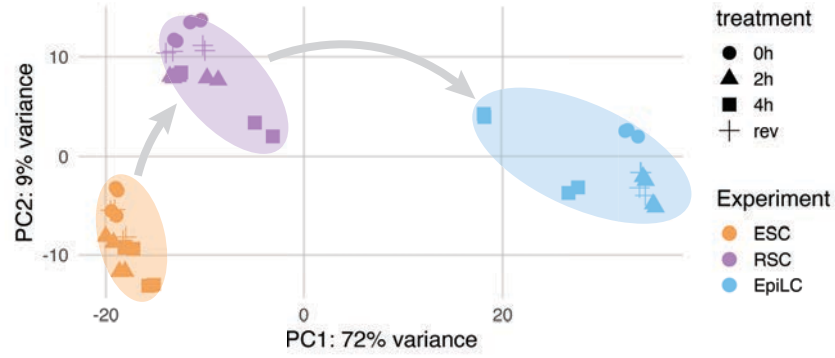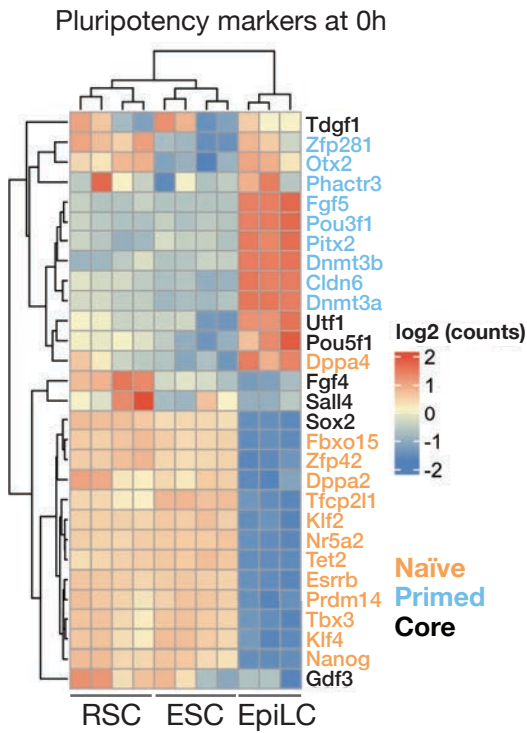
**Figure 1**

A

B — *In vivo* ERK induction

C — E6.5 Sox2Cre$^{tg/+}$ ; Rosa26$^{CRAF/+}$

*Scale bar 50 µm*

D — Sox2Cre$^{tg/+}$ ; Rosa26$^{CRAF/+}$

*Scale bar 50 µm*

E — *In vitro* ERK induction

F

*Scale bar 25 µm*

# Figure 2

A — *In vitro*

Embryonic Stem Cells (ESCs) → Rosette Stem Cells (RSCs) → Epiblast-Like Cells (EpiLCs)

Timeline: FGFRi, 4OHT (0h), 2h, 4h, MEKi, rev

B — PC1: 72% variance / PC2: 9% variance
treatment: 0h (circle), 2h (triangle), 4h (square), rev (plus)
Experiment: ESC, RSC, EpiLC

C — Pluripotency markers at 0h
log2 (counts); Naïve / Primed / Core
RSC, ESC, EpiLC

D — Pluripotency markers upon ERK activation
LFC 4h vs 0h; Naïve / Primed / Core
EpiLC, ESC, RSC

E — GO:0000165 MAPK cascade genes
ESC, RSC, EpiLC; Expression changes; 0h 2h 4h rev
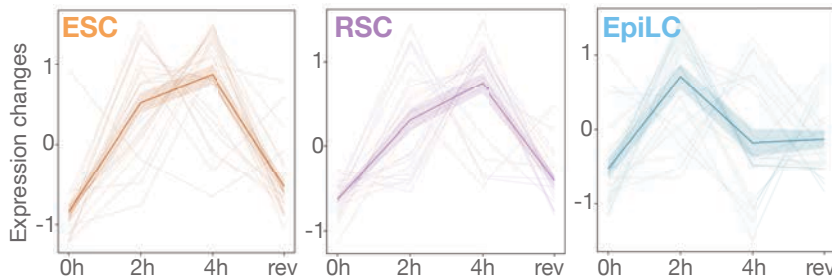
F — Early genes - Common MAPK; LFC vs 0h
Dusp5, Dusp6, Egr2, Egr3, Fos, Fosb, Fosl2, Jun, Junb, Spry2, Spry4
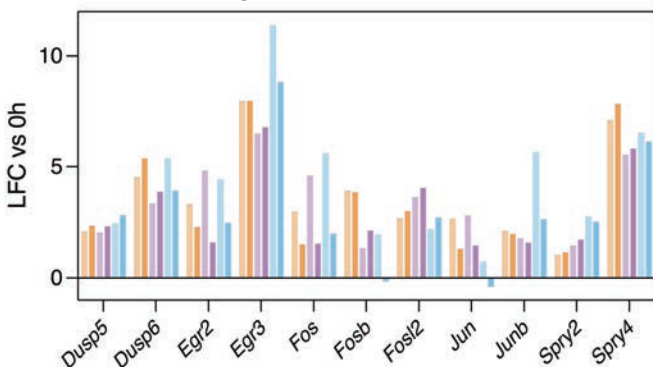
Late genes - Cell type specific; LFC vs 0h
Sox17, Wnt6, Nanog, Sox2
ESC_2h, ESC_4h, RSC_2h, RSC_4h, EpiLC_2h, EpiLC_4h

# Figure 3

# Figure 4

**Figure 5**

Scale bar 50μm

# Figure 6

# Figure 7

**A** Accessible regions

Transcription activated at accessible regions

Transcription at nearest genes from regions accessible at 0h

ESC-specific regions
RSC-specific regions
EpiLC-specific regions

**C** Active chromatin remodelling

Correlation between transcription and accessibility

Transcription at nearest genes from differentially accessible regions

ESC-specific regions
RSC-specific regions
EpiLC-specific regions

**B**
ATAC-seq
RNA-seq Normalized counts
*Spred3* (10kb downstream of TSS)

**D**
ATAC-seq
RNA-seq Normalized counts
*Zic3* (10kb upstream of TSS)