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- 1 Regulation of ERK signalling pathway in the developing mouse blastocyst
- 2

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13					
14	Abstract				
15	Activation of the ERK signalling pathway is essential for the differentiation of the inner cell mass (ICM)				
16	during mouse preimplantation. We show here that ERK phosphorylation is present in ICM precursor cells,				
17	in differentiated Primitive Endoderm (PrE) cells as well as in the mature, formative state Epiblast (Epi). We				
18	further show that DUSP4 and ETV5, factors often involved in negative feedback loops of the FGF pathway				
19					
20	are differently regulated. While DUSP4 expression in PrE cells clearly depends on ERK activity, ETV5's				
	are differently regulated. While DUSP4 expression in PrE cells clearly depends on ERK activity, ETV5's localises mainly to Epi cells. Unexpectedly, ETV5 expression does not depend on ERK activation but				
21	are differently regulated. While DUSP4 expression in PrE cells clearly depends on ERK activity, ETV5's localises mainly to Epi cells. Unexpectedly, ETV5 expression does not depend on ERK activation but requires NANOG expression. Indeed ETV5, like <i>Fgf4</i> , is not expressed in <i>Nanog</i> mutant embryos. Our				

- enable the differentiation of neighbouring cells into PrE while protecting the Epi identity from autocrine
- 24 signalling.

1 Introduction

2 Signalling between cells is crucial for tissue differentiation and is required during the earliest steps of 3 embryo differentiation. During mouse preimplantation, three cell lineages are produced from two 4 successive differentiation events. The first differentiation between outer and inner cells is essentially driven by the Hippo signalling pathway leading to TE and inner cell mass (ICM) cells, respectively (Menchero et 5 6 al., 2016; Nishioka et al., 2009). During blastocyst formation around embryonic day 3.5 (E3.5), Epiblast 7 (Epi) and Primitive endoderm (PrE) cells appear within the ICM in a "salt and pepper" pattern evidenced 8 by the reciprocal and complementary expression pattern of the transcription factors NANOG and GATA6 9 (Chazaud et al., 2006; Guo et al., 2010; Kurimoto et al., 2006; Plusa et al., 2008). Numerous experiments 10 have shown that the FGF/ERK pathway balances this binary cell fate as high signals induce cells into PrE 11 whereas blocking the pathway leads ICM cells toward an Epi fate (Bessonnard et al., 2017; Chazaud et al., 12 2006; Feldman et al., 1995; Kang et al., 2013; Kang et al., 2017; Krawchuk et al., 2013; Molotkov et al., 13 2017; Nichols et al., 2009; Ohnishi et al., 2014; Saiz et al., 2016; Yamanaka et al., 2010). Interestingly, 14 Fgf4, the sole FGF ligand detected at this stage, is transcribed and secreted from Epi cells only, converting 15 neighbouring cells into PrE (Frankenberg et al., 2011; Guo et al., 2010; Kurimoto et al., 2006; Ohnishi et 16 al., 2014). Fgfr2 is specifically expressed in PrE cells (Guo et al., 2010; Kurimoto et al., 2006; Ohnishi et 17 al., 2014), however Fgfr1 which is equally expressed in Epi and PrE cells is the initial and main receptor 18 transducing the FGF activity required for PrE specification (Kang et al., 2017; Molotkov et al., 2017).

19

Despite the increasing data demonstrating the importance of the FGF/ERK pathway during blastocyst development, expression of activated phosphorylated ERK (pERK) has not been described yet. FGF target genes have been recently examined using Fgfr1/2 mutant embryos. While Dusp4 is preferentially expressed in PrE cells, as would be expected with a pERK activity required in this cell type, other genes such as Etv4/5 that are ETS-related factors and Spry2/4 are present in both cell types or preferentially expressed in Epi cells in late blastocysts at E4.5 (Kang et al., 2017). It was then proposed that pERK activity could be
also present in Epi cells where it would activate ETVs expression. Then ETVs would cut down pERK
activity in Epi cells by activating negative feedback loops involving SPRYs (Kang et al., 2017).

4

Here, we show that pERK expression begins around E3.0-E3.25 days of development concomitant with the initiation of Epi/PrE specification, and is essentially present in ICM progenitor or PrE cells. This expression is respectively increased or lost in *Klf5* and *Fgf4* mutants which is in accordance to the described phenotypes (Azami et al., 2017; Kang et al., 2013; Krawchuk et al., 2013; Ohnishi et al., 2014). We also found that DUSP4 expression tightly follows pERK activity, whereas surprisingly ETV5's is not regulated by this MAPK pathway but clearly depends on NANOG expression. Our results lead to a novel model of FGF/ERK regulation in the preimplantation embryo.

12

13 Results

14 Detection of pERK in preimplantation embryos

15 FGF/ERK signalling pathway plays pivotal roles in the segregation of Epi and PrE, yet there is currently no 16 reliable method to detect phosphorylated ERK (pERK) signal during preimplantation stages, thereby 17 hampering the understanding of how pERK regulates the fundamental process of preimplantation 18 development. We have developed two new methods to detect pERK expression that are sufficiently 19 sensitive to detect signals in preimplantation embryos. Briefly, to preserve the integrity of the phosphate 20 conjugate we add phosphatase inhibitors during the fixation step and permeabilization is carried out either 21 with Triton X100 or proteinase K (see materials and methods). Two working methods (Fig S1A) provide 22 alternative solutions to observe other labellings in co-staining experiments. Expression of pERK is evident 23 in the cytoplasm while mitotic nuclei can retain a strong labelling (Fig. S1B, 2C), as previously reported 24 (Corson et al., 2003; Shapiro et al., 1998).

1 The presence of exogenous FGF has been shown to increase the number of PrE cells (Yamanaka et al., 2 2010), and culturing embryos between E2.5 and E3.75 increases the number of pERK labelled cells as well 3 as its signal intensity, with almost all ICM cells exhibiting an elevated pERK signal compared to the 4 control (Fig. S1C). Interestingly, an increase of pERK staining can be observed after only 10 min of FGF 5 treatment (Fig S1D). To further validate these techniques, we collected E2.5 embryos and cultured them 6 until E3.75 with MEK inhibitor (PD0325901; 0.5 μ M) that blocks the pathway upstream of ERK 7 phosphorylation (Nichols et al., 2009; Yamanaka et al., 2010). The signal is totally down regulated in the 8 presence of the inhibitor (Fig. S1E), confirming the specificity of our methods. Thus, we established for the 9 first time two protocols to detect pERK during preimplantation development.

10

11 pERK expression begins during blastocyst formation

12 We analysed pERK expression during preimplantation development from 1-cell stage, thus before the 13 reported Fgf4 expression that starts in a subset of the inner cells between E3.0 and E3.25 (Guo et al., 2010; 14 Ohnishi et al., 2014). Cells expressing pERK are firstly detected around E3.0 (Fig. 1A). The number of 15 labelled cells increases with time, exhibiting graded pERK levels (Fig. 1A, 1B). A few TE cells are marked 16 (Fig S1B) and this is consistent with a required pERK activity for TE proliferation (Nichols et al., 2009). 17 Then, we examined the identity of pERK-positive ICM cells by staining NANOG and GATA6 together 18 with pERK at each developmental stage (Fig. 1B, 1D). Cell counts indicate that few cells are labelled 19 between E3.0 and E3.25 (Fig. 1C), around two to three per embryo, with a relatively weak staining that is 20 present in uncommitted cells that are doubly positive for NANOG and GATA6 (Bessonnard et al., 2014; 21 Bessonnard et al., 2017; Plusa et al., 2008; Saiz et al., 2016) (Fig. 1D). From E3.5, the number of cells with 22 a strong pERK labelling increases (Fig. 1C) and progressively correspond to GATA6 expressing cells (Fig. 23 1D). Thus ERK phosphorylation essentially occurs in PrE-specified cells as well as in a subset of 24 uncommitted cells, delineating the induction of their specification into PrE cells. However, not all PrE cells

were labelled by pERK whereas all PrE cells require FGF/ERK activity for their specification (Chazaud et al., 2006; Kang et al., 2017; Molotkov et al., 2017; Nichols et al., 2009; Yamanaka et al., 2010). This low
 frequency of labelled cells may reflect a transient nature of pERK labelling.

4

5 pERK is expressed during postimplantation development and in cultured cells

After cell specification into Epi and PrE, little is known about MAPK activity in the three cell lineages after their differentiation. During implantation (from E4.0) several FGF ligands are expressed in the Epi (*Fgf4, Fgf5, Fgf8, Fgf15, Fgf18*) and in the PrE (*Fgf3, Fgf17*), and can be transduced by *Fgfr2, Fgfr3* or *Fgfr4* (Ohnishi et al., 2014). Moreover, PDGF signalling was shown to be important for PrE cell survival (Artus et al., 2013) and could act through the MAPK pathway. Accordingly, pERK is detected in PrE cells at E4.5 (Fig 2A) and their derivatives at E6.5 (Fig 2B). Consistent with a previous report (Corson et al., 2003), strong pERK signals are detected in the extraembryonic ectoderm of E6.5 (Fig. 2B).

13 In FGF/ERK impaired embryos, NANOG expression is maintained in the E4.5 Epi while it decays in WT 14 embryos (Kang et al., 2013; Molotkov et al., 2017; Nichols et al., 2009). This suggests that FGF4 enables 15 Epi maturation by down regulating NANOG expression levels. Accordingly, we could observe pERK 16 expression in some Epi cells at E4.5 (Fig 2A), most likely through the transduction of FGFR1, the only 17 FGF receptor expressed in this tissue at this stage (Ohnishi et al., 2014). Similarly, mouse Embryonic Stem 18 (ES) cells, that are closely related to the E4.5 epiblast (Boroviak et al., 2014), express high levels of Fgf4, 19 and the FGF pathway is necessary for their exit of pluripotency and differentiation (Kunath et al., 2007; 20 Molotkov et al., 2017). A heterogeneous labelling is observed among the cells, possibly due to 21 heterogeneous levels of NANOG expression that is inducing Fgf4 secretion (Festuccia et al., 2012) and 22 also owing to a possible transient ERK phosphorylation. In the presence of the MEK inhibitor, pERK 23 staining is strongly diminished in ES cells (Fig. 2C).

24 We thus report that the ERK pathway is active in the three cell lineages derived from Epi, PrE and TE.

1 Upstream regulation of FGF/pERK through the analysis of *Fgf4*, *Klf5*, *Nanog* and *Gata6* mutant 2 embryos

Fgf4 is specifically expressed in Epi cells (Frankenberg et al., 2011; Guo et al., 2010; Kurimoto et al., 2006; Ohnishi et al., 2014) and acts as a paracrine factor to differentiate neighbouring cells (Frankenberg et al., 2011). *Fgf4* targeted inactivation leads to an absence of PrE cells and a pan expression of Epi markers in the ICM (Kang et al., 2013; Krawchuk et al., 2013). We observe an absence of pERK expression in *Fgf4* KO embryos (Fig. 3A) demonstrating that FGF4 is the sole secreted signal to activate the ERK pathway at this stage of development.

9

Our previous results showed that *Klf5* is essential for blastocyst development (Ema et al., 2008). Indeed, through a direct binding to *Fgf4* regulatory elements, KLF5 prevents precocious expression of *Fgf4* at E3.0 - E3.25 to initiate cell differentiation at the right time and to ensure a proper balance between EPI and PrE cells (Azami et al., 2017). Consistent with our previous study, *Klf5*^{-/-} embryos at E3.25 have a stronger pERK signal and in more cells compared with that of WT embryos (Fig. 3B).

15

We have previously shown (Frankenberg et al., 2011) that *Fgf4* transcription in Epi cells requires the presence of NANOG (Fig 3E). As expected, in *Nanog* mutant embryos, the lack of FGF4 leads to an absence of pERK activation (Fig 3C).

Removing one or two alleles of *Gata6* increases the number of *Fgf4* expressing cells (Fig 3E) as more cells express NANOG (Bessonnard et al., 2014). As a consequence, pERK staining can be observed throughout the ICM of *Gata6^{-/-}* embryos (Fig 3D), either due to a transcriptional activation of *Fgf4* by NANOG, or due to a lack of GATA6 repression (Fig 3E). It is interesting to note that ICM cells in *Gata6^{-/-}* embryos have an Epi identity and are nevertheless able to transduce FGF signalling through FGFR1 which is expressed in these cells (Fig 3E). This ERK activity is reminiscent of what is found in E4.5 WT Epi cells or in ES cells.

2 DUSP4 but not ETV5 is a target of pERK. 3 ETV5 and DUSP4 are considered as direct targets of the MAPK/ERK pathway (Neben et al., 2017; Patel 4 and Shvartsman, 2018) and are present in the blastocyst (Guo et al., 2010; Kang et al., 2017; Kurimoto et al., 2006). We carried out single-cell RNA analyses, and we subdivided the samples between Fgf4-positive 5 6 and Fgf4-negative populations as Fgf4 was identified as the Epi marker with the earliest binary expression 7 (Guo et al., 2010; Ohnishi et al., 2014). The transcripts of *Dusp4* are barely detected at the 16-cell stage and 8 later are preferentially expressed in *Fgf4*-negative cells identified as PrE cells (Fig 4A). On the reverse, 9 Etv5 expression in enriched in Fgf4-positive Epi cells as early as E3.25 (Fig 4A). Etv4 expression is 10 starting from E3.5, thus after the beginning of Epi/PrE specification (Fig 4A and Fig S3). At E3.75 when 11 Nanog/Gata6 mutual exclusive expression is established, Etv4 is enriched in Fgf4-negative PrE-precursor 12 cells while *Etv5* is enriched in *Fgf4*-positive EPI-precursor cells (Fig 4A).

13 At E3.25, DUSP4 expression is mainly observed in outside trophectoderm cells (Fig 4B) as reported for its 14 RNA localisation (Posfai et al., 2017), consistent with an active FGF pathway to promote TE growth 15 (Nichols et al., 2009). Later, DUSP4 expression localises to PrE, GATA6-positive, cells (Fig. 4B, C) but 16 can also be found in yet undifferentiated cells, labelled by both GATA6 and NANOG, especially at the 17 beginning of blastocyst formation (Fig 4C). Epi cells do not express DUSP4 (Fig 4C). A double labelling 18 with pERK and DUSP4 antibodies shows a high correlation of expression. However, some pERK-only 19 marked cells could be observed, probably reflecting an early activation of the pathway just preceding a 20 visible DUSP4 translation. As well, DUSP4-only labelled cells are found, illustrating a plausible transient 21 nature of ERK activation. As expected, DUSP4 expression is enhanced by FGF2 treatment and disappears 22 upon MEK inhibitor administration (Fig S2).

In *Nanog* mutants, DUSP4 expression is not activated in the ICM (Fig 4D), in accordance with the absence
of *Fgf4* in these embryos (Fig 3E, (Frankenberg et al., 2011). On the reverse DUSP4 labelling is observed

in some cells in *Gata6^{-/-}* embryos (Fig 4D). This result shows that DUSP4 does not need GATA6 presence
to be expressed but relies essentially on pERK signalling activity. DUSP4 expression is thus a faithful
reporter of ERK activity.

4

ETV5 is an ETS related transcription factor involved in the transduction of the MAPK pathway (Herriges 5 6 et al., 2015; Sharrocks, 2001) and is expressed in the blastocyst ICM (Guo et al., 2010; Kang et al., 2017). 7 By immunofluorescence we show that from E3.5 ETV5 is preferentially expressed in Epi cells as it 8 colocalises with NANOG (Fig 5A), confirming the RNA expression data (Fig 4A), however some PrE cells 9 can also express it (Fig S2B). Surprisingly, ETV5 expression is drastically down regulated upon FGF2 10 administration and is enhanced in the presence of MEK inhibitor (Fig. 5A). Thus ETV5 does not behave like a MAPK signalling reporter but as an Epi marker. Strikingly, ETV5 expression is lost in Nanog^{-/-} 11 embryos and is present throughout the ICM of Gata6^{-/-} embryos. Therefore, our data clearly indicate that 12 13 ETV5 does not depend on RTK signals but on NANOG expression during blastocyst formation.

14

15 **Discussion**

16 FGF signalling, through FGFR and ERK controls PrE differentiation in the ICM of the mouse blastocyst 17 (Brewer et al., 2016; Chazaud et al., 2006; Feldman et al., 1995; Kang et al., 2013; Kang et al., 2017; 18 Krawchuk et al., 2013; Molotkov et al., 2017; Nichols et al., 2009; Saiz et al., 2016; Yamanaka et al., 2010). 19 However the spatiotemporal expression of active, phosphorylated ERK has never been reported during 20 mouse preimplantation. We show here that pERK expression begins at the onset of Epi/PrE differentiation, 21 around E3.25, time when *Fgf4* transcripts start to be elevated in some ICM cells (Ohnishi et al., 2014). 22 Thus as soon as Fgf4 is expressed at sufficient levels pERK expression can be observed. This expression is 23 essentially present in uncommitted cells, doubly labelled by NANOG and GATA6, and in PrE cells solely 24 marked by GATA6. This is consistent with the known pERK activity which is converting uncommitted

cells into PrE (Bessonnard et al., 2017; Saiz et al., 2016) and our data show here that pERK expression
persists in PrE cells up to at least E4.5. At this stage, pERK can be observed in some Epi cells, time when
they are differentiating further through a formative phase (Smith, 2017), by down regulating NANOG and
up regulating OTX2, OCT6, FGF5 (Acampora et al., 2016; Morgani et al., 2017). Interestingly NANOG
expression is maintained in *Fgf4* and in *Fgfr1/Fgfr2* mutants (Kang et al., 2013; Molotkov et al., 2017)
indicating that Epi maturation requires FGF signalling, which is thus consistent with the observed pERK
expression.

8

9 Dual-specificity phosphatases (DUSPs) are known as transcriptional ERK targets in several models and 10 inhibit the FGF pathway in a negative feedback loop through the dephosphorylation of ERK (Amit et al., 11 2007; Brewer et al., 2016; Chu et al., 1996; Eblaghie et al., 2003; Ekerot et al., 2008; Lemmon and 12 Schlessinger, 2010; Niwa et al., 2007). We show that DUSP4 expression tightly follows pERK's, and 13 depends on it. In WT embryos DUSP4 could be considered as a PrE marker, however, its expression in 14 Gata6^{-/-} embryos, which do not contain PrE cells, indicates that this protein is linked to pERK activity 15 rather than PrE identity and is thus a reliable ERK activity reporter. Co-labelling experiments of pERK 16 with DUSP4 suggest a transient activated ERK while DUSP4 expression is more robust. Transient or 17 sustained ERK activities have been observed downstream of RTK signalling to control different cellular 18 responses. These differences in the output, despite sharing signalling intermediates, can be explained in 19 part by differences in feedback mechanisms (Lemmon and Schlessinger, 2010). The rather transient nature 20 of the pERK labelling observed during preimplantation suggests the presence of negative feedback loops 21 that can be mediated through DUSP4.

22

ETS-related molecules such as ETS-variant (ETV) 4 and 5, are transcriptionally activated by the FGF
pathway in several different developmental processes and tissues (Aulehla et al., 2008; Liu et al., 2003;

1 Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001). ETS factors are often involved in positive or 2 negative feedback loops of ERK signalling (Herriges et al., 2015; Reginensi et al., 2011), being either 3 activators or repressors of transcription and potentially regulating negative effectors of the pathway such as 4 SPROUTYs (SPRYs) (Herriges et al., 2015; Neben et al., 2017; Reginensi et al., 2011; Sharrocks, 2001; Willecke et al., 2011). Etv4, Etv5 and Spry4 have been reported to be expressed in both Epi and PrE cells 5 6 during blastocyst formation, whereas Spry2 is preferentially expressed in the Epi (Boroviak et al., 2015; 7 Kang et al., 2017; Morgani et al., 2018). Moreover, Etv4 and Spry4 expression is diminished in absence of 8 FGF/ERK signalling in both cell types (Kang et al., 2017; Morgani et al., 2018). It was then proposed that 9 these ETVS and SPRYs would suppress or dampen ERK activity through a FGF negative feedback loop, to 10 desensitise Epi cells from their own FGF4 secretion (Kang et al., 2017). However, we found that pERK is 11 almost undetectable in blastocyst Epi cells implying that either Etv4 and Sprv4 are only transcribed in 12 precursor cells with a high RNA stability or that these genes can respond to very low levels of pERK or 13 that their expression depends also on other factors. Data extraction from single-cell RNA-seq analyses 14 (Posfai et al., 2017) show that only Spry4, but not Etv4, is expressed in precursor cells at the 16-cell/E3.25 15 stages (Fig S3). Thus SPRY4, under FGF stimulation (Morgani et al., 2018) could tame the FGF/ERK 16 pathway at least in the precursor cells.

17 Spry2 is particularly interesting because its transcripts are differentially enriched between Epi and PrE cells 18 (Boroviak et al., 2015; Kang et al., 2017), and as FGF signalling must not be blocked in PrE cells. Spry2 19 expression is maintained at E3.5 and is downregulated by late blastocyst stage in Fgfr mutants (Kang et al., 20 2017), indicating that its initial expression is independent from FGF signalling. ETS-binding sites, that are 21 conserved in the mouse, have been characterized in hSpry2 promoter (Ding et al., 2003). Thus SPRY2 is a 22 good candidate downstream of ETVs to block FGF signalling in the Epi, as early as the 16-cell/E3.25 stage 23 (Fig S3). We show here that *Etv5* transcripts, like *Spry2*'s, and protein are enriched in EPI cells. 24 Surprisingly, ETV5 expression is not regulated by the FGF/ERK pathway but is induced by NANOG,

1 likely through a direct activation as NANOG binds to the Etv5 locus in ES cells (Boyer et al., 2005; Loh et 2 al., 2006; Murakami et al., 2016). Thus, this reveals a novel potential mechanism by which NANOG 3 activates the expression of FGF4 and ETV5 at the same time, on one hand to induce the paracrine PrE cell 4 differentiation, and on the other hand to protect Epi cells against an autocrine/paracrine FGF4 activity. This mechanism of direct activation of ETV5 by NANOG would seem more efficient to protect Epi cells 5 6 identity compared to an activation of ETV5 through a negative feedback loop found in other systems 7 (Herriges et al., 2015). Indeed ETV5 is already present when Epi cells are subjected to an 8 autocrine/paracrine FGF4 activity.

9

10 How ERK activity is controlled during Epi/PrE specification has remained unclear. With these novel data 11 we propose (Fig 5C) that in precursor cells of the early ICM, expressing both NANOG and GATA6, pERK 12 activity remains very low or absent due to i) KLF5 presence that limitates the number of Fgf4 transcripts 13 (Azami et al., 2017) and ii) the possible inhibition of the FGF pathway by a negative feedback mechanism 14 through SPRY4 (Kang et al., 2017; Morgani et al., 2018). Then at the beginning of Epi specification, 15 NANOG expression becomes predominant over GATA6's, and enables the transcription of both Fgf4 and 16 *Etv5.* FGF4 is secreted and binds to FGFR1, which is equally expressed among ICM cells. In Epi cells, 17 ETV5 is likely to protect the cells from the surrounding FGF4 by activating SPRY2 to attenuate or block 18 the ERK pathway. In PrE cells, NANOG is not expressed and therefore ETV5 is absent. This enables an 19 efficient FGF/ERK pathway activity, illustrated by DUSP4 expression, to differentiate the cells into PrE by 20 maintaining a high expression of GATA6 and inducing downstream PrE factors such as SOX17 and 21 GATA4.

In conclusion, our results show that NANOG is key in the binary cell differentiation by activating withinthe same cell the differentiation paracrine cues and a protective cell-autonomous mechanism.

1 Materials and methods

2 Experiments were performed in accordance with French/EU and Japanese guidelines for the care and use of

- 3 laboratory animals.
- 4

5 Embryo collection and culture.

Preimplantation embryos (CD1 (ICR) background) were collected from oviducts or uteri by flushing with
M2 medium (Sigma). Embryos were cultured in the presence of MEK inhibitor (PD0325901; 0.5 μM)
(Selleck), FGFR inhibitor (PD173074; 100 nM) (Selleck), FGF2 (Cell Guidance Systems) or FGF4 (1
μg/mL) (R&D) + heparin(1 μg/mL) (Sigma) in KSOM+AA medium (Millipore) in 4-well plates at 37°C,
5% CO₂.

11

12 Mutant Mice

Fgf4^{tm1.2Mrt} (Sun et al., 2002), obtained from MMRRC at UC Davis were crossed with Ayu1-Cre mice that 13 express Cre recombinase from zygote to generate the deletion of Fgf4 allele. $Fgf4^{+/-}$ mice were then 14 15 intercrossed to obtain Fgf4 mutant embryos. Nanog and Gata6 mutant embryos were obtained as 16 previously described (Bessonnard et al., 2014; Frankenberg et al., 2011; Mitsui et al., 2003; Sodhi et al., 17 2006) through natural mattings. *Klf5* KO embryos were generated as previously described (Azami et al., 18 2017). Reporter mice for Fgf5 (Fgf5-P2A-Venus) were generated as previously described (Khoa et al., 19 2016). All mice were maintained on the CD1 (ICR) background. Embryos genotyping was performed after 20 confocal imaging, as described previously (Azami et al., 2017; Bessonnard et al., 2014; Frankenberg et al., 21 2011; Khoa et al., 2016)

22

23 Whole mount immunostaining

24 Immunostaining of embryos were performed as described previously (Azami et al., 2017). Briefly, embryos

1 were fixed in 4% paraformaldehyde (PFA) in 1 x PBS for 15-30 min at RT. Permeabilization was 2 performed in 0.5% TritonX-100 in PBS for 15 min and embryos were incubated in blocking solution (1% 3 donkey serum, 0.1% BSA, and 0.01% Tween20 in PBS) for 1 hr at RT. Then, embryos were treated with 4 primary antibodies in blocking solution for overnight at 4°C. After washing with 0.1% Tween20 in PBS for 5 three times, embryos were treated with secondary antibodies in blocking solution for 1 hr at RT. Nuclei 6 were stained with Hoechst33342 (Thermofisher) or DAPI. To visualize DUSP4, a tyramid signal 7 amplification (TSA) reaction was carried out according to manufacturer's instructions (Thermofisher or 8 Perkin Elmer). To perform double immunostaining for pERK and DUSP4, after the TSA reaction with the 9 first rabbit antibody, the peroxydase was inactivated by a 5 min 3% H2O2 treatment. Then the second 10 rabbit primary antibody staining was carried out as usual. Primary antibodies are listed in Supplementary 11 Table S1.

12

13 Phosphorylated ERK staining

14 Visualization of phosphorylated ERK (pERK) was performed by using two protocols.

15 (1) pERK Staining with 8% PFA: embryos which were collected from uterus or oviducts were precultured 16 in KSOM+AA medium at 37°C, 5% CO₂. They were fixed in 8% PFA together with phosphatases 17 inhibitors: NaF (25 mM, Sigma), orthovanadate (2 mM, NEB) and β-glycerophosphate (50 mM, Santa 18 Cruz) for 10 min at RT. They were permeabilized 5 min in 0.5% Triton/PBS and aspecific sites were 19 blocked with 10% FBS/ 0.1% Triton/PBS for 30 min. Primary antibodies were added to the blocking 20 solution O/N at +4°C. Washes, secondary antibodies and tyramid signal amplification (TSA) reaction 21 according to manufacturer's instructions (Thermofisher or Perkin Elmer). (2) pERK staining with ProK: 22 embryos which were collected from uterus or oviducts were precultured in KSOM+AA medium at 37°C, 23 5% CO2 and fixed in 4% PFA with PhosSTOP (Roche) for 20 min on ice. Embryos were permeabilized and 24 activated with 10 ng/ml proteinase K (ProK) (Roche) in 0.1% Tween20/PBS at RT for 3-5 min, depending on developmental stages. ProK reaction was stopped in 2 mg/ml Glycine-PBS for 2 min at RT and embryos
were washed in 0.1% Tween20/PBS. Blocking reaction was performed in 0.5% donkey serum/ 0.5%
Casein/ 0.5% BSA in PBS for 30 min at RT. Then, embryos were treated with the primary antibody in
blocking solution for overnight at 4°C. After washing with 0.1% Tween20 in PBS for three times, embryos
were treated with HRP-conjugated secondary antibody in blocking solution for 1 hr at RT. Tyramide signal
amplification (Thermofisher) was used to detect pERK signal.

7

8 Confocal microscope and image analyses

9 Leica TCS SP8, SP5 or SPE confocal microscopes were used to acquire the fluorescent images of 10 immunostained embryos as described previously (Azami et al., 2017; Gasnier et al., 2013). Look up table 11 (LUT) visualization of fluorescence intensity was processed by using Fiji. The "Thermal" range of LUT 12 indicates high levels of expression in red and low levels in blue. Unless otherwise indicated, images are 13 displaying single Z sections representative of the embryo. Cell counts were performed as previously 14 described (Bessonnard et al., 2014).

15

16 Single cell RTqPCR analyses

Isolated ICM cells (after immunosurgery, (Nagy, 2003)) and morulae (16C) were incubated 10 minutes in 1X TrypLETM Express Enzyme (Gibco) at 37°C and cells were isolated by repeated mouth pipetting using pulled capillaries of serially smaller diameter openings. Each single cell was collected in 5µl of 2X Reaction Mix (Invitrogen, CellsDirect One-Step qRT-PCR Kit) and stored at -80°C or processed immediately. Genotyping of embryos was carried out using TE cells from immunosurgery with Whole Genome Amplification (WGA) using the REPLI-g kit (Qiagen) followed by PCR genotyping as above.

cDNA from desired genotypes was preamplified with 18 cycles. Quality and genotype checks were
performed by qPCR for each cell with housekeeping genes primers (RPS17 and RPL30) and primers

1	detecting the mutations. The analysed cells originate from at least 3 different embryos in each category.
2	Single-cell qPCR on a Fluidigm Biomark system (GENTYANE facility) was carried out on 48.48 or 96.96
3	Dynamic Arrays, according to the manufacturer's instructions. Cells with absent or low Ct values for
4	housekeeping gene RPS17 and RPL30 were removed from analysis (5%). Ct values were normalized with
5	the mean of housekeeping gene RPS17 and RPL30 using the 2- Δ Ct method. In boxplots, values are relative
6	to the mean of all WT 32C cells (WT-ref and WT from the transgenic background when available) to be
7	able to compare between genotypes and stages. Cells were considered in Fgf4-ve subpopulations when CT
8	values were above 35 and all other cells were placed in Fgf4+ve subpopulations. Primers used for this
9	study are listed in Supplementary table S2.
10	For the boxplots, he edges of the box represent 25th and 75th quartiles. The median is represented by the
11	central line. The whiskers extend to 1.5 times the interquartile range (25th to 75th percentile) Cells are
12	plotted individually in single-cell experiments.
13	
14	Statistical analysis
15	Statistical tests were performed with Graphpad software. Statistical significance was assessed using the
16	Wilcoxon-Mann-Whitney test (non parametric) on expression levels
17	
18	Acknowledgments
19	Images were acquired and treated at the CLIC imaging facility (Clermont-Fd). C.C. wants to thank C.
20	Pléver for genotyping the mice.
21	Author Contributions
22	C.C., C.B., T.A. and M. E. conceived and designed the experiments. C.B., T.A., L.V.E. and N.A.
23	
20	performed the experiments. P.P. designed semi-automated image analyses. C.B., C.C., T.A., N.A and M.E.
24	performed the experiments. P.P. designed semi-automated image analyses. C.B., C.C., T.A., N.A and M.E. analysed the data. C.C., T.A and M.E. wrote the manuscript.

1 Competing Interests

2 The authors declare no competing financial interests.

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7 8 Figure 1

Α E2.75 E3.0 E3.25 E3.5 E3.75 255 no ð * 0 В IANOG<mark>GAT</mark> pERK1/2 pERK1/2 Merge E3.25 E3.5 75 ЕЗ. С D 100% % of pERK expressing cells # of pERK expressing cells 90% 80% 70% 60% 50% 40% 30% 20% 10% 0% E3.25 E3.75 E3.5 E3.25 E3.75 E3.5 strong weak ■ N+ ■ G6+ ■ N+ G6 +

Figure 1: pERK expression during Epi/PrE specification. **A**. Immunolocalisation from E2.75 to E3.75. Signal intensity is colour scaled. **B**. Five-colour imaging with pERK, CDX2, NANOG and GATA6 immunostaining from E3.25 to E3.75. **C**. Number of pERK expressing cells at E3.25 (n=30 embryos), E3.5 (n=30) and E3.75 (n=30). **D**. Repartition of pERK expressing cells between NANOG+/GATA6- (N+), NANOG-/GATA6+ (G6+) and NANOG+/GATA6+ (N+G6+) populations at E3.25 (n=16 embryos), E3.5 (n=9) and E3.75 (n=11). Scale bars: 10 microns.



Figure 2: pERK immunostaining during implantation stages and in ES cells. **A**. Expression in two representative E4.5 embryos showing pERK expression in some PrE cells labelled by GATA6 and in some Epi cells (arrowheads). **B**. pERK expression at E6.5 with a strong labelling in the extraembryonic ectoderm (ExE) and some PrE cells (arrowheads). The *Fgf5-Venus* reporter labels the Epi and the embryonic part of the visceral endoderm. **C**. pERK expression in ES cells, co-stained with OCT3/4 and NANOG, cultured in absence (top panel) or presence of the MEK inhibitor (bottom panel). Scale bars: 10 (A,B) 100 (C) microns.



Figure 3: pERK expression in mutant embryos during preimplantation, at indicated stages A. pERK,
NANOG and GATA6 expression in *Fgf4* mutants (*Fgf4^{-/-}* n= 5). B. pERK, NANOG and GATA6
expression in *Klf5* mutants (*Klf5^{-/-}* n= 5). C. pERK and NANOG expression in *Nanog* mutants (*Nanog^{-/-}* n=5). D. pERK and NANOG expression in *Gata6* mutants (*Gata6^{-/-}* n=4). E. Single-cell RNA expression
of *Fgf4*, *Fgfr1* and *Fgfr2* in individual ICM cells isolated from *Nanog^{-/-}* (n= 15 cells), *Gata6^{-/-}* (n= 13 cells)
and WT (n= 24 cells) embryos at E3.25. Red asterisks indicate the significant differences of expression
between mutant and WT samples *p<0,05, **p<0,01, ***p<0,001 (Wilcoxon test). Scale bars: 10 microns.





2 Figure 4: Expression of FGF pathway related genes. A. Single-cell RNA expression of Dusp4, Etv5, Etv4, 3 Fgfr1 and Fgfr2 in single cells from the 16-cell stage embryos (n= 18 cells) and from ICM at E3.25 (n=29 4 cells), E3.5 (n=33 cells) and E3.75 (n=24 cells). At the later stages, ICM populations were subdivided into two groups defined by the presence (+) or absence (-) of Fgf4 expression in the cell. This enables 5 6 discrimination between Epi (+) and PrE (-) cells. Experiments at the 16-cell stage were not carried out for 7 Etv4 and Etv5 (n.d.: not determined). Asterisks indicate the significant differences of expression between Fgf4+ and Fgf4- cells (Wilcoxon tests: * p < 0.05, ** p < 0.01, *** p < 0.001). B. Expression of pERK, 8 9 DUSP4 and GATA6 at E3.25 and E3.75. C. Number of DUSP4 expressing cells per embryo at E3.25 10 (n=15 embryos), E3.5 (n=18) and E3.75 (n=11). The right panel indicates the percentage of cell co-expression with NANOG, GATA6 or NANOG+GATA6. D. Expression of DUSP4 and NANOG in 11 Nanog^{-/-} (Nanog^{-/-} n=5) and Gata6^{-/-} (Gata6^{-/-} n=6) embryos. Scale bars: 10 microns. 12

Figure 5



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3 Figure 5: Expression of ETV5. A. Immunostaining of ETV5, together with NANOG and SOX17, in 4 embryos cultured with either FGF2 (n=6) or MEK+FGFR inhibitors (n=17) from E2.5 to E3.75. B. ETV5 expression in Nanog^{-/-} (n=4) and Gata6^{/-} (n=3) embryos. Scale bars: 10 microns. C. Working model. In the 5 6 precursor cell (16-cell/E3.25 stage), co-expressing NANOG and GATA6, KLF5 and possibly SPRY4 7 inhibit the FGF pathway. KLF5 expression decreases during blastocyst growth. Precursor cells can differentiate into either Epi or a PrE between E3.25 and E3.75 in an asynchronous manner. In Epi cells, 8 9 NANOG induces the expression of both Fgf4 and ETV5. Upon ETV5 induction, or possibly NANOG's, 10 SPRY2 inhibits the ERK pathway, protecting the cell from autocrine as well as paracrine FGF4. In PrE 11 cells, pERK is activated by FGF4 secreted by neighbouring EPI cells, leading to the maintenance of 12 GATA6 expression and down regulation of NANOG's. Together with pERK, GATA6 induces downstream 13 PrE genes such as SOX17 and GATA4. DUSP4 is activated by pERK independently of GATA6. It 14 possibly resensitises the FGF pathway by de-phosphorylating ERK. Thick connections represent activated 15 interactions, thin arrows show tamed or extinct interactions and dotted lines are suggested activities.

1 Supplementary Information

Supplementary Figure 1



Figure S1. Validation of pERK staining in blastocysts. A. Parallel staining of pERK, GATA6 and CDX2
with the High PFA and ProK protocols. B. pERK expression at E3.75 with signal in the TE (arrow) and
ICM. The yellow arrowhead points toward a mitotic cell. C,D. pERK expression after embryo cultures for
30h (C) or 10 min (D) with FGF. E. pERK immunostaining after cultures with MEK1 inhibitor. Scale bars:
10 microns.

Supplementary Figure 2



2

Figure S2. A. DUSP4, NANOG and SOX17 immunolabelling in embryos cultured with either MEK
inhibitor or FGF2 from E2.5 to E3.75. Scale bars: 10 microns. B. Number of cells expressing ETV5 per
embryo at E3.5-E3.75 and their distribution between precursor (N+G6+), Epi (N+) or PrE cells (G6+).

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Supplementary Figure 3



9 Figure S3. Expression of indicated genes by single-cell RNA-seq extracted from (Posfai et al., 2017) at the
10 16-cell stage (33 inner and outer cells), E3.25 (32-C, 40 ICM cells) and E3.5 (64-C, 33 ICM cells).
11 Expression levels are in RPKM.

1 Table S1: List of primary antibodies used

Epitope	host	supplier	reference
pERK	rabbit	Cell Signaling	4370
GATA6	goat	R&D	AF1700
NANOG	rabbit	Abcam	ab80892
NANOG	rat	e-bioscience	14-5761
DUSP4	rabbit	Abcam	ab216576
ETV5	rabbit	Proteintech	13011-1-AP
SOX17	goat	R&D	AF1924
CDX2	mouse	Abcam	ab89949
OCT3/4	mouse	SantaCruz	Sc-5279

Table S2: list of primers used for the single-cell RTqPCR

RefSeq #	Gene	Forward	Reverse
NM_176933.4	Dusp4	AGCTCCTGGTTCATGGAAGC	ACTCAAAAGCCTCCTCCAGC
NM_023794	Etv5	CAGAACCTGGATCACAGCAA	GACTGAGGAGGGAAGGGATG
NM_010202	Fgf4	ACTACCTGCTGGGGCCTCAA	ACTCCGAAGATGCTCACCAC
NM_001079908	Fgfrl	GCTATAACCCCAGCCACAAC	AGCCAAAGTCTGCGATCTTC
NM_010207	Fgfr2	CACCAACTGCACCAATGAAC	GAATCGTCCCCTGAAGAACA
NM_001163485	Rpl30	AGTCTCTGGAGTCGATCAACT	AGCCAGTGTGCATACTCTGTAG
NM_009092	Rps17	ATGACTTCCACACCAACAAGC	GCCAACTGTAGGCTGAGTGAC