

1 Cell type-dependent differential activation of ERK by oncogenic KRAS in
2 colon cancer and intestinal epithelium

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1 Abstract

2 Mutations activating the KRAS GTPase or the BRAF kinase are frequent in colorectal cancer and are
3 thought to constitutively activate the terminal mitogen-activated protein kinase, ERK. Using mass
4 cytometry, we found graded phosphorylation of ERK anti-correlated with cell differentiation in patient-
5 derived colorectal cancer organoids, independent of KRAS mutational status. Reporter, single cell
6 transcriptome and mass cytometry analyses showed that transgenic KRAS^{G12V} activated ERK in a cell
7 type-specific pattern in mouse intestinal organoids. In contrast, transgenic BRAF^{V600E} triggered high ERK
8 activity and downstream gene expression in all intestinal cell types, followed by epithelial
9 disorganisation. Quantitative network modelling from perturbation data revealed that activation of
10 ERK is shaped by cell type-specific MEK to ERK feed forward and negative feedback signalling. We
11 identified dual-specificity phosphatases as candidate modulators of ERK activity between intestinal cell
12 types. Furthermore, we found that oncogenic KRAS, together with β -Catenin, favoured expansion of
13 crypt cells with high ERK activity. Our experiments highlight key differences between ERK activity
14 elicited by the BRAF or KRAS oncogenes in colorectal cancer and find unexpected heterogeneity in a
15 signalling pathway with fundamental relevance for cancer therapy.

1 Introduction

2 Multiple signalling pathways, including the mitogen-activated protein kinase (MAPK) and the Wnt/ β -
3 Catenin cascades, form a network controlling cellular turnover in the intestinal epithelium ¹.
4 Collectively, activities within the signalling network control stem cell maintenance, cell proliferation,
5 differentiation into absorptive enterocyte and secretory cells, and apoptosis. Wnt/ β -Catenin and
6 MAPK activities are regionalized within the folded single-layered intestinal epithelium. Both are high
7 in crypts harbouring stem cells and low in differentiated cells that have migrated away from the crypt
8 base. Oncogenic mutations activating β -Catenin and MAPK perturb intestinal homeostasis and thereby
9 drive colorectal cancer (CRC) initiation and progression.

10 Multiple MAPK modules transduce signals downstream of receptor tyrosine kinases, such as EGFR, and
11 RAS family GTPases. The consecutively activated RAF, MEK and ERK kinases represent a MAPK module
12 frequently activated in cancer. Upon activation, ERK can phosphorylate and activate a series of
13 transcription factors orchestrating a complex cellular response that often is pro-proliferative ². In the
14 normal intestine, EGFR to ERK signalling is initiated by ligands from the crypt microenvironment, which
15 are secreted by e.g. epithelial Paneth cells of the small intestine, Reg4+ secretory niche cells of the
16 large intestine, or adjacent fibroblasts ^{3,4}. In CRC, ERK activity is supposedly more cell-autonomous due
17 to oncogenic mutations activating KRAS, NRAS or BRAF (found in 45%, 5% and 10% of CRCs,
18 respectively) ^{5,6}, or by *de novo* expression of EGFR ligands such as amphiregulin ⁷. Signal transduction
19 to ERK is a main determinant of cancer development and therapy response ^{5,6,8}.

20 Recent studies suggest that the relationship between ERK-activating mutations, ERK activity and
21 phenotypic outcome in CRC is complex. Firstly, mutations in KRAS and BRAF are associated with distinct
22 CRC development routes: KRAS, but not BRAF, mutations frequently occur as secondary events after
23 mutations activating Wnt/ β -Catenin in the conventional CRC progression sequence ^{9,10}. Conversely,
24 BRAF, and less frequently KRAS, mutations precede activation of Wnt/ β -Catenin in the alternative
25 serrated progression route ^{11,12}. The observed disequilibrium between KRAS and BRAF mutations in
26 the conventional versus serrated pathways of CRC evolution suggest the existence of functional
27 differences, resulting in distinctive effects on clinical course and treatment efficacy ¹³. Secondly, ERK
28 activity appears to be heterogeneous in genetically identical CRC cells. Cells at the invasive front
29 frequently exhibited higher ERK phosphorylation levels compared to cells in central areas of the same
30 cancer, and CRCs with activating KRAS mutations also showed heterogeneous ERK activity ¹⁴. Previous
31 studies already showed heterogeneous Wnt/ β -Catenin activity in cancer specimens, suggesting a more
32 general paradigm of graded pathway activities in CRC ¹⁵. Furthermore, CRC cells have been shown to
33 exhibit functional differences within a cancer, as only few CRC cells, so-called cancer stem cells, could
34 initiate new tumours in xenografts ¹⁶⁻¹⁹. Gradients of surface markers such as EphB2 were found to

1 distinguish CRC stem cells with high malignant potential ²⁰. CRC subtypes can share similarities with
2 cell types of the normal crypt, such as stem cells, enterocytes or secretory cells in bulk cell analysis ²¹.
3 Finally, because of variable signal transduction and differentiation states, genetically identical CRC
4 clones exhibit variable proliferative potential and therapeutic response ²².

5 Experimental techniques with cellular resolution, ranging from fluorescent reporters ²³ to single-cell
6 transcriptome analyses ^{24,25} and mass cytometry ²⁶ hold the promise to disentangle the relationship
7 between oncogenes, cell differentiation states and cell signal transduction while taking into account
8 cellular heterogeneity. Here, we ask whether oncogenic forms of KRAS or BRAF show cell-to-cell
9 heterogeneity in their proclivity to activate ERK. For this, we use patient-derived and mouse transgenic
10 organoid cultures that maintain the cell hierarchy of tissue *in vitro* ²⁷. We assess signalling network
11 states with cellular resolution by mass cytometry and use BRAF^{V600E} and KRAS^{G12V} transgenes to assess
12 immediate impact of the oncogenes on cell signal transduction, gene expression programs and
13 phenotypic outcome. We discover strong functional differences between the BRAF and KRAS
14 oncogenes and find that signal transduction from KRAS to ERK is shaped by different strengths of feed
15 forward and negative feedback in a cell type-specific manner.

1 Results

2 ERK activity is graded in KRAS-mutant CRC

3 To investigate whether oncogenic KRAS enforces constitutive activity of MEK and ERK kinases, we
4 examined patient-derived three-dimensional CRC organoid cultures by immunohistochemistry. We
5 found heterogeneous phosphorylation of both, MEK and ERK, in organoids with no mutations in the
6 EGFR-RAS-ERK cascade (line OT326), as well as in KRAS-mutant organoids (line OT227, carrying a
7 KRAS^{G13D} mutation) (Fig. 1a).

8 We next used mass cytometry to analyse cell differentiation markers and MEK and ERK
9 phosphorylation side-by-side in the patient-derived organoids (Fig. 1b). We selected the two organoid
10 lines used above, as well as line OT302, harbouring a KRAS^{G12D} mutation. We found that cells of all
11 three organoid lines formed gradients with respect to levels of EphB2, a known marker of CRC
12 hierarchies linked to metastasis and therapy response²⁰. In two of the three lines (OT326 and OT302),
13 a substantial proportion of EphB2-low cells was marked by cleaved Caspase 3, suggesting apoptotic
14 removal of cells at the end of their life span. Intriguingly, all three lines displayed gradients of
15 phosphorylated MEK and ERK that were largely congruent with EphB2. These results indicate that
16 patient-derived CRC organoids contain phosphorylation gradients of MEK and ERK kinases along an
17 axis defined by cell differentiation. The observed gradient formed regardless of oncogenic activation
18 of the upstream KRAS GTPase, and in the absence of tumour stroma that is not present in the
19 organoids.

20 BRAF^{V600E}, but not KRAS^{G12V}, induces high levels of ERK activity and epithelial 21 disorganisation in intestinal organoids

22 As MEK and ERK activities were graded along a differentiation axis in patient-derived CRC organoids
23 irrespective of mutational status of KRAS, we asked whether oncoproteins activating the RAS-ERK
24 signalling axis exert their activities in a cell type-specific manner. To study this question, we employed
25 intestinal organoids of transgenic mic carrying doxycycline-inducible single copy constructs encoding
26 tdTomato linked to KRAS^{G12V}, BRAF^{V600E}, or firefly luciferase (FLUC) as a control in the Gt(ROSA26)Sor
27 locus (Fig. 2a)^{28,29}.

28 We initiated organoid cultures by embedding intestinal crypts from FLUC-, KRAS^{G12V}-, and BRAF^{V600E}-
29 inducible mice into extracellular matrix, as described before²⁷. When we induced oncoprotein
30 production by adding doxycycline to the culture media, BRAF^{V600E} led to irreversible disintegration of
31 organoids within 1-2 days, whereas transgenic KRAS^{G12V} or the FLUC control protein were well
32 tolerated, even after several passages (Fig. 2b). To examine whether the BRAF oncogene has
33 detrimental effects on the epithelium beyond the previously reported loss of stem cells^{29,30}, we

1 examined histology at ultrastructural level of the induced organoids using transmission electron
2 microscopy (Fig. 2c). We found that control and KRAS^{G12V}-induced organoids show the expected tissue
3 structure, that is, a single-layered polarized epithelium with continuous apical and basal surfaces as
4 well as a brush border at the apical side. Desmosomes, providing lateral cell adhesion, were clearly
5 visible. In contrast, BRAF^{V600E}-induced organoids displayed a continuous basal surface, whereas the
6 apical side was grossly distorted, although it contained a brush border as evidence of polarisation.
7 Nuclei were pleomorphic and no longer lined up basally but scattered at different positions. Cells were
8 still attached to each other by desmosome bridges, indicating that the ongoing epithelial
9 disorganisation was taking place in the presence of lateral cell adhesion.

10 To ascertain whether the epithelial disorganisation provoked by BRAF^{V600E} was correlated with MAPK
11 activity, we measured phosphorylation of ERK. We found that induction of BRAF^{V600E}, but not KRAS^{G12V},
12 resulted in high phospho-ERK levels in intestinal organoids, as determined by capillary protein analysis
13 (Fig. 2d). BRAF^{V600E}-induced organoid disintegration could be counteracted by inhibition of the BRAF-
14 downstream MEK and ERK kinases using AZD6244/Selumetinib ³¹ and BVD-523/Ulixertinib ³²,
15 respectively, but not by inhibition of the upstream EGFR tyrosine kinase receptor family using
16 AZD8931/Sapitinib ³³ (Fig. 2e), showing that the induced phenotype is due to excessive MEK-ERK
17 activity. Indeed, only 24 h after BRAF^{V600E} induction almost all direct ERK target genes ³⁴ were activated,
18 whereas conditional expression of KRAS^{G12V} had no obvious effect on bulk organoid transcription
19 (Supplementary Fig. 1).

20 [Single cell RNA sequencing reveals disruption of intestinal differentiation trajectories by](#) 21 [BRAF^{V600E}](#)

22 To uncover potential cellular heterogeneity in response to the oncogenes, we performed single cell
23 transcriptome analyses. We induced FLUC control, BRAF^{V600E}- and KRAS^{G12V}-transgenic organoids for 24
24 h, prepared single cell suspensions, and stained them with a fluorescent antibody against the crypt cell
25 marker CD44 ³⁵, and with a fluorescent dye to eliminate dead cells. Using single cell sorting on the
26 transgene-expressing organoids we next acquired samples of CD44-high crypt and CD44-low villus cells
27 (see Supplementary Fig. 2 for FACS gating strategy), which were subjected to single cell RNA
28 sequencing. In total, we obtained transcriptomes of 167 cells with >1000 detected genes each that
29 were used for further analysis. Single cell transcriptomes could be assigned to six interconnected
30 clusters with help of k-means clustering and were visualised in a t-SNE-based representation (Fig. 3a).
31 Mapping of signature genes for intestinal stem cells (ISCs), proliferative TA cells, differentiated
32 enterocytes ²⁰ and secretory Paneth cells ⁴, and the CD44 status as inferred from flow cytometry (Fig.
33 3b-c) confirmed the calculated differentiation trajectories (grey overlay in Fig. 3a): undifferentiated
34 CD44-high ISC and TA cell signature genes were enriched in clusters 1 and 2 while Paneth cell marker

1 genes were highest in cluster 2, indicating the differentiation route for secretory crypt cells; expression
2 of enterocyte signature genes increased gradually in clusters 3-5, marking the CD44-low absorptive
3 lineage.

4 We next considered the distribution of cells expressing specific transgenes (Fig. 3b): FLUC control and
5 KRAS^{G12V}-expressing cells intermingled throughout the clusters 1-5 of the normal cell differentiation
6 trajectories. BRAF^{V600E}-expressing cells were in contrast depleted from the central clusters 2-4, and
7 instead formed outsider cluster 6, composed entirely of BRAF-induced cells. Notably, cells in cluster 6
8 uniformly expressed high levels of ERK target genes, regardless of whether they were sorted as CD44-
9 high or CD44-low. Furthermore, cluster 6 cells also highly expressed *Anxa10*, which has been identified
10 as a marker for BRAF-positive serrated adenoma³⁶ (Supplementary Fig. 3). The single cell analysis thus
11 showed that BRAF^{V600E} imposed a specific gene expression program onto intestinal cells, independent
12 of their prior differentiation state. Transcriptomes of KRAS^{G12V}-induced cells, in contrast, were
13 undistinguishable from FLUC control cells; however, we observed that KRAS^{G12V}-induced cells showed
14 a shift towards CD44-high undifferentiated cell types compared to FLUC controls (see Supplementary
15 Fig. 2b).

16 ERK-dependent reporter activity and single cell analyses identify KRAS^{G12V}-responsive 17 intestinal cells

18 To visualize ERK activity with single cell resolution in organoids, we employed the Fra-1-based
19 integrative reporter of ERK (FIRE) that translates ERK kinase activity into stability of a nuclear yellow-
20 green venus fluorescent protein (Fig. 4a)²³. FIRE fluorescence in organoids cultured in normal growth
21 medium containing EGF was strong in crypts, whereas differentiated villus tissue was largely FIRE-
22 negative (Fig. 4b). In EGF-free medium, ERK activity in the crypt base persisted, likely due to autocrine
23 and paracrine signals from EGF-producing Paneth cells⁴.

24 We next conditionally expressed FLUC control, KRAS^{G12V}-, or BRAF^{V600E}-encoding transgenes in FIRE-
25 transfected organoids (Fig. 4c). Transgene induction was often variable, as inferred by tdTomato
26 fluorescence, allowing to compare individual tdTomato-positive cells with transgene-negative
27 neighbouring tissue. tdTomato-FLUC control transgene expression had no influence on FIRE activity. In
28 contrast, expression of KRAS^{G12V} resulted in increased FIRE signals in crypt cells, which consistently
29 displayed stronger reporter activity compared to adjacent KRAS^{G12V}-negative cells. Surprisingly, a large
30 majority of villus cells remained FIRE negative, despite strong tdTomato-KRAS^{G12V} positivity. We
31 confirmed the differential signal transduction from KRAS^{G12V} to ERK using phospho-ERK
32 immunohistochemistry (Fig. 4d). In line with our FIRE reporter data, p-ERK-positive cells were largely
33 absent in central differentiated (Ki67-negative) villus areas of organoids, despite strong tdTomato-

1 KRAS^{G12V} staining. Taken together, our results show that ERK activity in differentiated villus epithelial
2 cells can neither be increased by EGF in the medium nor by induction of oncogenic KRAS^{G12V}. However,
3 when we induced BRAF^{V600E}, we found widespread and strong FIRE signals across the complete
4 organoid (Fig. 4c). This suggests a strict and cell-type specific control of signal transduction by
5 oncogenic KRAS, but not BRAF, in intestinal epithelial cells.

6 Since FIRE fluorescence could distinguish cells responsive to KRAS^{G12V}, we next used the reporter to
7 assist selection of cells for single cell RNA sequencing. Our aim was to define cell types with high ERK
8 activity, either in response to KRAS^{G12V} or as part of the normal cell hierarchy. For this, we induced
9 organoids with the integrated ERK reporter for KRAS^{G12V} or FLUC, prepared single cell suspensions and
10 sorted cells by FACS into 96-well plates for transcriptome analysis (see Supplementary Fig. 4 for FACS
11 gating strategy). We focussed on single cells with high transgene (tdTomato) signal and either positive
12 or negative for FIRE (venus) fluorescence (Fig. 5a). In total, we obtained 197 single cell transcriptomes.
13 K-means clustering into 8 groups and t-SNE-based visualisation revealed the cell type distribution (Fig.
14 5b, c). Cluster 1 was enriched for undifferentiated crypt (ISC and TA) marker genes, whereas clusters
15 2-4 were defined by Paneth cell signature genes (Fig 5d; Supplementary Fig. 5). Cluster 2 was enriched
16 for Paneth cell markers such as *Lyz1*, encoding Lysozyme³⁷, and several genes encoding Defensins,
17 while other cluster-defining genes such as *Mptx1* and *Agr2* in cluster 4 hint at a high degree of Paneth
18 cell heterogeneity. Clusters 5-8 formed a differentiation trajectory for absorptive cells, with *Ifabp1* as
19 the top defining genes for clusters 5-7 (Supplementary Fig. 5)

20 Using this information, we assessed the distribution of transcriptomes derived from KRAS^{G12V}-induced
21 FIRE-high cells (Fig. 5c, d). These were confined to distinct aggregates encompassing the
22 undifferentiated cell zone of cluster 1, as well as transcriptomes inhabiting the outer right rim of the
23 tSNE representation that we above assigned to be derived from late-stage enterocytes and Paneth
24 cells. Immunofluorescence microscopy using the Paneth cell marker Lysozyme confirmed high FIRE
25 activity in this cell type after KRAS^{G12V} induction (Supplementary Fig. 6). In contrast, a central area of
26 the tSNE plot encompassing the largest clusters 5 and 6 of bulk enterocytes was almost devoid of
27 KRAS^{G12V}-producing FIRE-high cells but harboured many KRAS^{G12V}/FIRE-low cells, confirming that
28 enterocytes generally cannot activate ERK, even when expressing oncogenic KRAS^{G12V}; however, a
29 specific subset of presumably late-stage enterocytes displayed high ERK activity.

30 KRAS^{G12V} interacts with GSK3 β inhibition in modulating intestinal cell type markers

31 In order to understand how β -Catenin-, and MAPK- networks interact in controlling cell differentiation
32 and ERK phosphorylation in intestinal epithelium, we performed a network perturbation study using
33 kinase inhibitors, followed by mass cytometry in KRAS^{G12V}-inducible and FLUC control organoids. For
34 this, we induced the transgenes in 3 days old organoids, subsequently treated them with an GSK3 β

1 inhibitor (CHIR99021) for 24 h to stabilize β -Catenin³⁸, and used MEK and p38 inhibitors (AZD6244 and
2 LY2228820/Ralimetinib³⁹, respectively) for 3 h to inhibit key kinases as part of the intestinal cell
3 signalling network (Fig. 6a). We measured a total of 160 000 transgene-positive cells, representing 12
4 multiplexed samples.

5 To discern the immediate effects of KRAS^{G12V} and stabilized β -Catenin on intestinal cell hierarchies, we
6 assessed the distribution of cell type markers (Fig. 6b). As a positive control for the effect of GSK3 β
7 inhibition on β -Catenin activity, treatment with CHIR99021 increased levels of the β -Catenin target
8 protein Axin2⁴⁰. We observed that both, induction of KRAS^{G12V} and treatment with the GSK3 β inhibitor,
9 increased median levels of crypt cell markers EphB2, CD44 and CD24, and for all three proteins,
10 KRAS^{G12V}-induced cells that were additionally treated with the GSK3 β inhibitor had the highest levels.
11 These results are in line with prior evidence that oncogenic KRAS and β -Catenin activities can inhibit
12 or reverse intestinal cell differentiation and provide clonal benefits linked to crypt cell fate^{41,42}.

13 We used k-means clustering to allocate KRAS^{G12V}-induced cells to six clusters defined by levels of cell
14 type- and surface markers CD24, CD44, EphB2, Krt20 and apoptosis marker cleaved Caspase 3 (Fig. 6c).
15 p-ERK-positive cells were enriched in clusters 5 and 6, while cleaved Caspase 3-positive cells were
16 found in Cluster 5 (Fig. 6d-e). Based on gradual loss of the crypt cell markers EphB2, CD44 and CD24,
17 we concluded that Clusters 1-4 represent a crypt-to-villus gradient, interconnecting with Cluster 5 at
18 the end of the differentiation trajectory (Fig. 6f). Clusters 3 and 4 had the lowest phospho-MEK and
19 phospho-ERK levels, and also contained lowest levels of the Wnt/ β -Catenin target Axin2, in agreement
20 with differentiated villus cell status. High levels of CD24 marked p-ERK-positive cells in Cluster 6 as
21 presumptive Paneth cells⁴. Interestingly, we observed that fractions of cells allocated to the clusters
22 were modified by both KRAS^{G12V} induction and GSK3 β inhibition (Fig. 6g): both treatments increased
23 the percentage of cells in cluster 1, representing the presumptive undifferentiated crypt cells, as
24 inferred from high levels of markers such as EphB2 and CD44^{1,20}. The combination of KRAS^{G12V} and
25 GSK3 β inhibition had the greatest effect and furthermore strongly decreased the fraction of cells in
26 the apoptotic cell cluster 5. These data suggest that oncogenic KRAS and β -Catenin stabilisation can
27 both favour crypt cell fate over differentiation, at least on the level of cell type marker expression.

28 [Quantitative modelling reveals cell type-specific differences in ERK feedback regulation](#)

29 To quantitatively dissect differences in signalling in the cell types, we assigned the cells measured
30 under different perturbed conditions to the 6 clusters defined previously according to their shortest
31 Euclidian distance (Fig. 6c). For each condition and each cluster, we calculated average
32 phosphorylation levels of MEK, ERK, 4EBP1, p38, ribosomal protein S6 and total protein levels of I κ B α
33 and Axin2, forming a Wnt-, MAPK-, NF κ B- and mTOR network (Fig. 7a,b). Cellular signalling states
34 varied strongly between the cell clusters 1 to 6. However, comparison between FLUC control and

1 KRAS^{G12V}-expressing organoid cells showed that signalling within each cell type was very comparable,
2 except for a slight increase in phosphorylation levels of MEK and ERK in clusters 5 and 6 in cells
3 expressing KRAS^{G12V}.

4 Using this data, we next employed network modelling using Modular Response Analysis (MRA)^{43,44}.
5 This approach allows to quantify the signal transmission from perturbation-response data and, by
6 using likelihood ratio tests, to pinpoint which signalling routes are different between the clusters. The
7 method requires fold-changes in network node activity after perturbation (calculated from data shown
8 in Fig. 7a) and a literature-derived network (as shown in Fig 7b) as input and calculates so-called
9 response coefficients that reflect the strength of signalling interactions. When we applied this
10 modelling framework to the signalling perturbation data of the 6 clusters for the KRAS mutant organoid
11 data, we observed that only 4 out of 11 signalling routes significantly differed between clusters with
12 two of them constituting the feed-forward and feedback signalling paths between MEK and ERK (Fig.
13 7b, c). In clusters 5 and 6, which were the clusters that showed elevated phosphorylation levels of MEK
14 and ERK levels after KRAS^{G12V} induction, the model unveiled that these cells enable RAS-ERK signalling
15 by two mechanisms: signal transmission from MEK to ERK was enhanced, while ERK-dependent
16 negative feedback inhibition of RAF and upstream components was attenuated when compared to the
17 other clusters. In contrast, clusters 3 and 4, which exhibited the lowest phospho-ERK levels that were
18 also not increased by KRAS^{G12V}, MEK-to-ERK feed forward signal transduction was low, and this
19 coincided with strong ERK-dependent feedback inhibition. Cluster 1, which based on surface marker
20 expression represent undifferentiated crypt cells, had strong feedback inhibition according to the
21 model. This cluster had intermediate phospho-ERK levels as measured by CyTOF (Fig. 6f) and was FIRE
22 positive (Fig. 4c). However, phospho-ERK levels were unresponsive to KRAS^{G12V} in this cluster and our
23 model predicts that this could be due to strong ERK feedback.

24 To model differences between KRAS^{G12V}-induced and control cells within each cluster, we employed
25 comparative MRA modelling that resulted in cluster-specific signalling models that consider the
26 influence of KRAS^{G12V} on the signalling network per cluster (Fig. 7d). We could discern only few
27 differences. Most importantly, KRAS^{G12V} enhanced signalling from ERK to S6 and MEK to ERK in clusters
28 5 and 6, respectively. Furthermore, we observed that KRAS^{G12V} modulated the effect of Wnt/Axin2
29 signalling on mTOR and p38 in cluster 2.

30 As our functional studies and the modelling showed that RAS-ERK signal transduction can be differently
31 wired between cell types, we tested whether this was also true for CRC cell lines. Indeed, when we
32 compared ERK phosphorylation in response to transfected KRAS^{G12V} in SW48 and Caco2 CRC cells (that
33 have no mutations in KRAS, NRAS or BRAF), we found that Caco2 cells were KRAS^{G12V}-responsive, while
34 SW48 cells were KRAS^{G12V}-insensitive (Supplementary Fig. 7)

1 Our cluster-specific signalling data (Fig. 7a) showed correlated activities of RAS-ERK, Wnt/ β -Catenin
2 and other signalling pathways, as they were generally higher in presumptive crypt cell clusters 1 and
3 6, but lower in the presumptive villus enterocyte clusters 3 and 4. Contrarily, the MRA approach
4 suggested that Axin2 as read-out of Wnt/ β -Catenin signalling was a negative regulator of RAS-ERK. We
5 reasoned that, as we stimulated Wnt/ β -Catenin signalling by inhibiting GSK3 β , attenuation of RAS-ERK
6 could be caused by other targets of GSK3 β . To more directly assess the effect of β -Catenin on RAS-ERK,
7 we therefore performed further experiments in transgenic organoids in which we induced transgenic
8 stabilised β -Catenin or withdrew Wnt ligands. The data show that supplementation and abrogation of
9 β -Catenin activity both result in lower ERK phosphorylation in organoids (Supplementary Fig 8).

10 As the results of the modelling pinpointed differences in signal transduction from KRAS^{G12V} to ERK to
11 feed-forward and feedback signalling between MEK and ERK, we investigated which molecular
12 mechanism might attenuate ERK activation in intestinal cells. For this we sorted FLUC control or
13 KRAS^{G12V}-induced organoids with respect to their FIRE reporter levels, as above (Fig. 5A) and performed
14 bulk low-input RNA sequencing. When inspecting 267 genes whose products are implicated in the
15 MAPK signalling pathway, we noticed three dual-specificity phosphatases among a total of 20
16 differentially expressed genes. As these phosphatases dephosphorylate ERK, we consider the DUSP1,
17 DUSP5 and DUSP6 gene products as candidate mediators of attenuated MEK-ERK signal transmission
18 that we observe in the differentiated cells of clusters 3-4.

1 Discussion

2 The mass cytometry, reporter assay and single cell RNA sequencing data that we present here support
3 a model of cell type-specific regulation of the terminal MAP kinase ERK (Fig. 8). Cell-intrinsic differences
4 in signal transduction to ERK also result in cell type-specific response to oncogenic KRAS. Our
5 quantitative network model suggests that ERK activity can be attenuated by increased feedback
6 inhibition and reduced feed-forward signalling from MEK to ERK. In agreement, transcriptome analysis
7 showed that dual-specificity phosphatases (DUSPs) are selectively expressed in cells with low ERK
8 activity and may therefore contribute to cell type-specific suppression of ERK. In addition, we found
9 that crypt cells expanded in organoids upon induction of KRAS^{G12V}, and this effect was increased when
10 KRAS^{G12V} was combined with high β -Catenin activity. These cells had elevated ERK activity, likely due
11 to low expression of DUSPs, but were variable in their feedback regulation and thus, KRAS^{G12V}-to-ERK
12 signal transduction.

13 The unexpected disparities in the levels of ERK phosphorylation in different cell types of the intestinal
14 epithelium extend our understanding of how KRAS, the most prevalent oncogene in CRC, exerts its
15 effects. Local differences of ERK activity have recently been found in clinical specimens of CRC,
16 including KRAS-mutant CRC [15]. In this previous study, ERK levels were generally higher in cancer cells
17 adjacent to stromal cells at the invasive front, and lower in more central areas of the cancer specimen,
18 in line with modulation of ERK activity by cues from the microenvironment. Our results agree with a
19 model of dynamic ERK activity in cancer tissues, and we find in addition that ERK is not only regulated
20 via external cues from the microenvironment, but also by the cell-intrinsic differentiation state.

21 Quantitative modelling of ERK activity from perturbation data revealed that the markedly distinct
22 abilities of KRAS^{G12} to activate ERK are due to different strengths of two network connections between
23 cell types, namely MEK to ERK feed forward and ERK to MEK negative feedback signalling. Negative
24 feedback within the pathway is well known, and has been linked to induction of cellular senescence ⁴⁶
25 and to therapy resistance in CRC ^{44,47}. Our study has identified dual-specific phosphatases as candidate
26 genes modulating MEK-to-ERK signalling. Indeed, the *Dusp1* and *Dusp5* and *Dusp6* genes we have
27 identified to be differentially regulated between RAS-to-ERK-responsive and -insensitive intestinal
28 cells, encode ERK-specific phosphatases ⁴⁸. Dual-specificity phosphatases targeting ERK have
29 previously been implicated in resistance of non-small lung cancer to anti-EGFR therapy ⁴⁹. It remains
30 to be seen whether differences in RAS-ERK signalling beyond the mutational status of BRAF, NRAS and
31 KRAS have prognostic value in CRC. Today, CRC patients with wildtype status of the predictive markers
32 KRAS, NRAS and BRAF are eligible for anti-EGFR therapy ^{50,51}. However, the treatment also generates
33 mixed outcomes among eligible patients, showing the need to identify novel markers and mechanisms
34 contributing to differences in EGFR-RAS-ERK signal transduction and therapy response.

1 Paneth cells and enterocytes represent the main differentiated cell types of the intestinal epithelium,
2 and we found that they have marked differences in their abilities to activate ERK. It is of note that the
3 former reside in the Wnt/ β -Catenin-high crypt compartment, while the latter inhabit the villus with
4 low β -Catenin activity. Indeed, a functional role for Wnt/ β -Catenin in the activation of ERK in intestinal
5 epithelium has been proposed before ⁵². In our experiments using organoids, p-ERK levels were
6 lowered when we increased or decreased β -Catenin activity (Supplementary Fig. 8A,B). We
7 hypothesize that this is due to a loss of all crypt cells or of phospho-ERK-high Paneth cells in the models
8 with low and high β -Catenin activity, respectively. Our data agree with a recent publication proposing
9 negative interaction between Wnt/ β -Catenin and ERK signals in intestinal stem cells ⁵³. Indeed,
10 activation of β -Catenin by treatment with a GSK3 β inhibitor increased the fraction of cells positive for
11 stem cell marker CD44, but not levels of MEK or ERK phosphorylation per cell in the CyTOF
12 experiments.

13 ERK activity is regulated on multiple levels, including its subcellular localisation ⁵⁴. We used different
14 approaches to quantify ERK activity. While mass cytometry and capillary protein analysis measure total
15 levels of ERK phosphorylation, the FIRE reporter assess nuclear ERK activity only²³. Comparing these
16 activity measures for the crypt cells show divergence of total and nuclear activity. While all crypt cells
17 appeared FIRE positive, the presumptive undifferentiated crypt cell cluster 1 showed only intermediate
18 levels of ERK phosphorylation in the CyTOF analysis. Further analysis will be required to understand
19 how subcellular ERK activity is controlled in a cell type-specific manner.

20 In our experiments, signal transduction from BRAF^{V600E} to ERK was independent of cellular context.
21 Extending previous studies ^{29,30}, we found that high levels of ERK activity induced by oncogenic
22 BRAF^{V600E} are not tolerated in the intestine. This is in contrast to CRC and cell lines, where BRAF^{V600E}
23 amplifications exist and are selected for by MEK inhibition ^{55,56}. It thus appears that the corridor for
24 acceptable ERK activity is tuneable during CRC progression and under selective pressure exerted, for
25 instance, by targeted therapy. Therefore, our findings are reminiscent of the “just right” signalling
26 model that has been proposed to explain step-wise increases of β -Catenin activity in CRC progression
27 ⁵⁷.

1 Materials and Methods

2 Generation of transgenic mice

3 Transgene cassettes were constructed by linking tdTomato to human BRAF(V600E), KRAS(G12V)
4 and/or murine stabilized mutant Ctnnb1 (S33A, S37A, T41A, S45A) or firefly luciferase via 2A peptides,
5 and subsequent cloning of these gene combinations into a doxycycline-inducible expression cassette
6 flanked by heterologous loxP sites, and integrated into a previously modified Gt(ROSA)26Sor locus of
7 F1 hybrid B6/129S6 embryonic stem cells by Cre recombinase-mediated cassette exchange, as
8 described previously²⁸. Transgenic animal experiments were approved by Berlin authorities LAGeSo
9 (G0185/09, G0143/14).

10 Organoid and cell lines culture

11 Mouse organoid cultures were initiated and propagated as described before²⁷, using 48-well plates
12 with 15 μ l droplets of Matrigel (Corning) per well overlaid with 300 μ l crypt culture medium containing
13 EGF (50 ng/ml), Noggin (100 ng/ml) and R-Spondin1 (functionally tested from R-Spondin-conditioned
14 medium; CCM-REN). Transgenes were induced by addition of 2 μ g/ml doxycycline to the medium.

15 To obtain adenomatous organoids (spheroids) after induction of stabilized β -Catenin, R-Spondin was
16 removed after induction of the transgene encoding β -Catenin^{stab} alone or in combination with
17 KRAS^{G12V}. Spheroids were dissociated with TrypLE (Gibco) for 3 min and Rho kinase inhibitor Y27632
18 (10 μ M) was added to the culture medium after passaging to prevent anoikis.

19 For viral transfection, a protocol from reference⁵⁹ was employed, with modifications: organoids were
20 cultured in the presence of Y27632 and the GSK3 β inhibitor CHIR99021 for two days. Next, organoids
21 were disaggregated into single cells using TrypLE (Gibco) for 5 min at 37°C. Cell suspensions were spin-
22 oculated in an ultra-low adhesion round bottom 96-well plate with the virus at 300 g for 45 min.
23 Subsequently, cells were resuspended in Matrigel, and cultured for 2 days in CCM-REN supplemented
24 with Y27632 and CHIR99021. Medium was then replaced by CCM-REN containing 2 μ g/ml Puromycin
25 to select for transfected cells. As viral transfection initially resulted in organoid pools that were
26 heterogeneous for FIRE reporter activity, single FIRE positive organoids were manually selected and
27 propagated before experimental analysis.

28 Organoid survival was scored as follows: cultures were passaged, inhibitors and doxycycline were
29 applied with the culture medium directly after passaging. Individual wells were imaged using the z-
30 stack function of BioZero observation and analyser software (Keyence) on day 1 and 4 (organoids) or
31 day 1 and 6 (spheroids), and full focus reconstructed images were used for quantification.

1 Patient derived organoids (PD3Ds) were obtained from the biobank of the Charité - Universitätsmedizin
2 Berlin and experiments were approved by the ethics commission of Charité - Universitätsmedizin Berlin
3 (EA1/011/18). Cells were cultivated in 24 well plates (Corning) in medium containing bFGF (20 ng/ml,
4 Sigma Aldrich) and human-EGF (50 ng/ml, Sigma Aldrich), as published ⁶⁰. Medium was exchanged
5 every three days.

6 SW48 and Caco2 CRC cells were cultured in L-15 and DMEM, respectively, supplemented with 10%
7 fetal bovine serum. Cells were transfected using Lipofectamin 3000 Transfection Reagent (Thermo
8 Fischer) with vectors encoding BRAF^{V600E}, KRAS^{G12V} or FLUC linked to tdTomato and the pTet-on
9 Advanced Vector (Clontech). Cells were starved in medium containing 0.1% fetal bovine serum and
10 induced with 2 µg/ml doxycycline 48h after transfection. 24 h later, cells were harvested using TrypLE,
11 washed, rested for 30 min at 37°C in starvation medium and fixed in 4% PFA for 15 min at 37°C. Fixed
12 cells were washed in PBS/1% BSA, permeabilised in MeOH at -20°C over night, and immunostained
13 with Alexa Fluor 488 mouse anti-ERK1/2 (pT202/pY204) antibody (1:10; 612592, BD Bioscience) for 30
14 min.

15 The following inhibitors (SelleckChem) were employed: AZD6244 (10 µM), BVD-523 (3 µM), AZD8931
16 (50 nM), CHIR99021 (6µM), Gefitinib (1 µM), LY2228820 (200nM).

17 **Mass Cytometry (CyTOF)**

18 For CyTOF analysis, we used the following pre-conjugated antibodies (Fluidigm) as per manufacturers
19 recommendation: CD24 (for mouse: 150-Nd, 3150009B, for human: 169-Tm, 3169004B), CD44 (for
20 mouse: 162-Dy, 3162030B, for human: 166-Er, 3166001B), cleaved Casp3 (142-Nd, 3142004A), p-H2AX
21 [S139] (147-Sm, 3147016A), p-Akt [S473] (152-Sm, 3152005A), p-p38 [T180/Y182] (156-Gd,
22 3156002A), Ki67 (162-Dy, 3168007B), IκBα (164-Dy, 3164004A), p-ERK1/2 [T202/Y204] (171-Yb,
23 3171010A), p-S6 [S235/236] (175-Lu, 3175009A).

24 For antibodies not available as metal-conjugates, we used the Maxpar Antibody Labeling Kit (Fluidigm)
25 according to manufacturer's instructions for addition of the respective metal tags: Axin2 (145-Nd,
26 Abcam, ab32197, 2 µg/ml), p-MEK1/2 [S217/221] (151-Eu, CST, 41G9, 2 µg/ml), EphB2 (158-Gd, BD,
27 2H9, 2 µg/ml), p-4e-BP1 [T37/46] (170-Er, CST, 236B4, 2 µg/ml), Krt20 (176-Yb, CST, D9Z1Z, 2 µg/ml).
28 The yield after conjugation was determined using a NanoDrop spectrometer measuring the absorbance
29 at 280 nm wavelength.

30 For measurements, organoids were harvested in PBS and digested to a single cell solution in 1:1
31 Accutase (Biolegend) and TrypLE (Gibco) with addition of 100 U/ml Universal Nuclease (Thermo
32 Scientific) at 37°C. Cells were counted and a maximum of 500.000 cells were stained with 5 µM Cell-ID
33 Cisplatin (Fluidigm) in PBS for 5 min at 37°C. After washing, cells were resuspended in their respective
34 growth medium and allowed to rest for 30 min at 37°C. Subsequently, cells were resuspended in

1 BSA/PBS solution, mixed 1:1.4 with Proteomics Stabilizer (Smart Tube Inc.) and incubated for 10 min at
2 room temperature. Afterwards the cells were frozen at -80°C for storage.
3 One day prior to analysis, cells were thawed in a 37°C water bath and mixed with Maxpar Cell Staining
4 Buffer (CSB, Fluidigm). We used the Cell-ID 20-Plex Pd Barcoding Kit (Fluidigm) to label different
5 samples and performed a downscaled version of the manufacturer's recommended protocol. Cells
6 were washed again in CSB, then in Barcode Perm Buffer (Fluidigm). After resuspension in 200 µl
7 Barcode Perm Buffer, 25 µl of the diluted Barcoding Reagents were added to the respective samples
8 and incubated for 30 min at room temperature. Afterwards cells were washed twice in CSB, pooled
9 into one tube and counted. 3×10^6 cells were then stained with a surface antibody cocktail for 30 min
10 at room temperature. After washing in CSB, cells were refixed in 2% methanol-free formaldehyde
11 solution (Pierce; diluted in Maxpar PBS, Fluidigm) for 10 min at room temperature. Cells were washed
12 in CSB and put on ice for 10 min. Next, cells were permeabilized by adding 4°C methanol for 15 min.
13 Cells were washed twice in CSB and incubated with a phospho-protein antibody cocktail for 30 min at
14 room temperature. Cells were washed twice in CSB and incubated with 62.5 nM Cell-ID Intercalator-Ir
15 in Maxpar PBS for 20 min at room temperature. Cells were washed in Maxpar PBS and fixed in 2%
16 methanol-free formaldehyde overnight at 4°C. The day after, cells were washed with CSB and then
17 twice with Milli-Q water. Cell number was adjusted to $2.5 - 5 \times 10^5$ /ml with Milli-Q water, cells were
18 filtered through a 20 µm cell strainer (CellTrics, Sysmex) and supplemented 1:10 with EQ Four Element
19 Calibration Beads (Fluidigm). Data was acquired on a Helios CyTOF system. Mass cytometry data was
20 normalized using the Helios software and bead-related events were removed. Doublets were excluded
21 by filtering for DNA content (^{191}Ir and ^{193}Ir) vs. event length, and apoptotic debris removed by a filter in
22 the platin channel (^{195}Pt). De-convolution of the barcoded sample was performed using the CATALYST
23 R package version 1.5.3⁶¹.

24 **Capillary protein quantification**

25 Protein sample preparation and quantification of p-ERK was performed as previously described⁵⁸ using
26 a WES capillary system (12–230 kD Master kit α-Rabbit–HRP; PS-MK01; Protein Simple) and the
27 antibody p-ERK/2(T202/Y204) (1:50; #9101, Cell Signal). Raw p-ERK values were normalized to vinculin
28 (1:30; #4650; Cell Signal).

29 **Immunohistochemistry**

30 Immunohistochemistry was done on paraformaldehyde (PFA)-fixed and paraffin-embedded tissues.
31 Organoids were fixed in 4% PFA for 30 minutes, while intestines were fixed over night at room
32 temperature. Subsequently, tissues were dehydrated in a graded ethanol series, followed by xylene.
33 Tissues were paraffine-embedded, sectioned at 4µm and mounted on Superfrost Plus slides (Thermo
34 Fisher Scientific). Sections were deparaffinised, rehydrated, bleached for 10 min in 3% H₂O₂. Antigens

1 were retrieved using 10mM Na-citrate, pH 6 for 20 minutes at boiling temperature. The following
2 antibodies were used: P-ERK (T202/Y204; #4370 CellSignal); P-MEK (S217/221; #9121 CellSignal), anti-
3 RFP (1:200; #600-401-379 Rockland). ImmPRESS secondary antibody and NovaRED substrate kits
4 (Vector Labs, Burlingame, CA, USA) were used for signal detection, according to manufacturer's
5 protocols.

6 **Immunofluorescence and microscopy**

7 For immunofluorescence imaging, organoids were washed with PBS and fixed in-well with 4% PFA for
8 30 min at 37°C. Fixation was stopped with PBS containing 100nm Glycine. Cells were blocked and
9 permeabilised with blocking buffer (PBS containing 1% BSA, 0.2 % Triton X100, 0.05 % Tween-20) for
10 at least 2.5 h at room temperature. Samples were incubated for 36 h at 4°C with primary antibody
11 against lysozyme (1:250; ab108508, Abcam) diluted in blocking buffer. After washing with IF-buffer
12 (PBS containing 0.1% BSA, 0.2 % Triton X100, 0.05 % Tween-20), samples were incubated for 24 h at
13 4°C with secondary antibody Alexa Fluor 647 anti-rabbit (1:500, 4414, Molecular Probes) diluted in IF-
14 buffer. Samples were counterstained for 5 min at room temperature using 0.5 µg/ml DAPI. After
15 washing with IF-buffer, stained cultures were released from the Matrigel and collected in PBS. Samples
16 were washed, resuspended in remaining PBS and mounted on slides using Vectashield Antifade
17 Mounting Medium (H-1000, Vector).

18 Immunofluorescence and FIRE reporter images were taken with a Leica TSC SPE confocal microscope
19 using an ACS 20x oil-immersion objective, solid-state lasers (405, 488, 532 and 635 nm) as sources of
20 excitation and LAS X operating software (Leica). Light microscopy images of cultures were taken with
21 a Biozero microscope using a Plan Apo 4x NA 0.20 objective and Biozero observation and analyser
22 software (Keyence).

23 For transmission electron microscopy, organoids were induced for 24 h, removed from Matrigel and
24 fixed in a buffer containing 2% PFA and 2.5% glutaraldehyde at 4°C. Regions of approximately 100-150
25 µm² showing representative sections through organoids were imaged on a 120 kV Tecnai Spirit
26 transmission electron microscope (FEI) equipped with a F416 CMOS camera (TVIPS). Micrographs were
27 recorded automatically at a final magnification of 4400x (2.49 nm pixel size at object scale) and -10 µm
28 defocus using Legion⁶² and then stitched using TrakEM2⁶³.

29 **Flow cytometry and fluorescence-activated cell sorting (FACS)**

30 Flow cytometry of anti-p-ERK-stained CRC cells resuspended in PBS/1%BSA cells was done using an
31 Accuri cytometer (BD). Cells were gated for populations displaying different tdTomato fluorescence
32 values (negative, low, medium and high), which correlates with transgene expression. For each

1 population, the mean anti-p-ERK fluorescence values were determined and normalised to the
2 tdTomato negative fraction of the corresponding FLUC control experiment.

3 For fluorescence-activated cell sorting of organoid cells, single cell suspensions from induced organoids
4 were prepared by digestion with TrypLE (Thermo Fisher Scientific) in the presence of 2 mM EDTA and
5 200 u/ml DNase I. Digestion was monitored by visual inspection and stopped by crypt culture medium
6 containing 0.2% bovine serum albumin. Cell suspensions were filtered through 30µm Celltrix filters and
7 stained with an anti-CD44-antibody conjugated to Allophycocyanin (APC; clone IM7, BioLegend) and
8 the Green or Near-IR Live/Dead Fixable Dead Cell Stain Kits (Life Tech) for subsequent exclusion of
9 dead cells. Single cells were sorted into the 96-well plates of the Precise WTA Kit with predispensed
10 library chemistry using a BD FACSAriaII SORP (BD) and a gating strategy as displayed in Supplementary
11 Figure 3. Cells were sorted into quadrants of plates to minimize batch effects between plates. For later
12 analysis of CD44 positivity of the subsets, sorts were performed as index sorts.

13 **RNA sequencing and bioinformatic analyses**

14 For single cell RNA sequencing, the Precise WTA Kit (BD) was used, according to the manufacturers'
15 instructions. Sequences were produced using NextSeq and/or HiSeq chemistry (Illumina). Cluster
16 generation on NextSeq 500 followed the instructions of the manufacturer, at a final loading
17 concentration of 2 pM on a High-Output-Flowcell. 1% PhiX was added as quality control, at least 40
18 million paired reads per pool were gained during a Paired-End-75 run. Library-pools running on the
19 HiSeq4000-system were prepared according to Illumina recommendations, loaded with 200 pM
20 concentration and sequenced during a Paired-End-75 run. Again, 1% PhiX was added as quality control,
21 and at least 40 million read-pairs per pool were targeted.

22 Single cell RNA-sequencing data was pre-processed using the BD Precise Whole Transcriptome Assay
23 Analysis Pipeline v2.0⁶⁴. Quality control was performed using scater⁶⁵. Read counts were normalised
24 using the trimmed mean of median values (TMM) approach provided with edgeR⁶⁶. Normalised read
25 counts were used for k-means clustering and t-SNE visualisation. Differentiation trajectories in t-SNE
26 plots were determined using slingshot⁶⁷, with intestinal stem cell cluster 1 as predefined origin.
27 Differentially expressed genes were called on log-transformed raw counts using a hurdle model
28 provided with R package MAST⁶⁸. Top-10 signature genes per cluster were identified by comparing
29 average gene expressions within cluster to average gene expressions across all other clusters. For bulk
30 cell RNA sequencing, organoids were induced for 24 h with 2 µg/ml doxycycline in CCM-REN medium
31 and subsequently dissociated, as described before⁵⁸. RNA-seq reads were aligned to the mouse
32 genome GRCm38 using STAR aligner with GENCODE annotation vM11. Differentially expressed genes

1 were called using DESeq2. scRNA-seq and bulk RNA-seq data are available in the GEO repository under
2 accession numbers GSE115242 and GSE115234, respectively.

3 **Mathematical Modelling**

4 We quantified and locally adjusted the signaling networks from the KRAS mutant perturbation data
5 using an adjusted version of Modular Response Analysis as implemented in the R package STASNet,
6 version 1.0.1⁶⁹ as follows: As input data, we derived representative mean and standard error of the
7 mean values from the single cell CyTOF data (trimming the lower and upper 5% of cells) and a literature-
8 informed prior network. As the apparent response pattern across clusters was similar, it was decided
9 to use a joint modeling approach, i.e. we first quantified the response coefficients for all 6 clusters by
10 a single set of coefficients using a combination of latin hypercube sampling with subsequent Levenberg-
11 Marquardt fitting ($n=4 \times 10^4$) and then iteratively derived and quantified the significantly different
12 signaling coefficients between clusters using a likelihood ratio test. Afterwards we searched for
13 biologically justifiable link extensions lacking in the network to better describe the data. The whole
14 procedure was repeated until no further justifiable link additions could be found, followed by a removal
15 round of statistically insignificant links. (see Supplementary HTML file).

16 **Statistical information**

17 Error bars in figures denote standard deviations. p-values are calculated from two-tailed unpaired t-
18 tests. *, ** and *** denote p-values <0.05 , <0.01 and <0.001 , respectively.

19

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7

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12

13 Author contributions

14 RB, TS, ML, PR, CG, SiS, DK, NM, BF, IAES conducted, analysed, and interpreted experiments; FU, TS,
15 BK performed bioinformatic analyses; MM, NB, CS, PR, TM, BGH conceived, designed, interpreted
16 experiments and/or supervised parts of the study; MM, NB, BK wrote the manuscript.

17

18 Conflict of interest

19 The authors declare that they have no conflict of interest.

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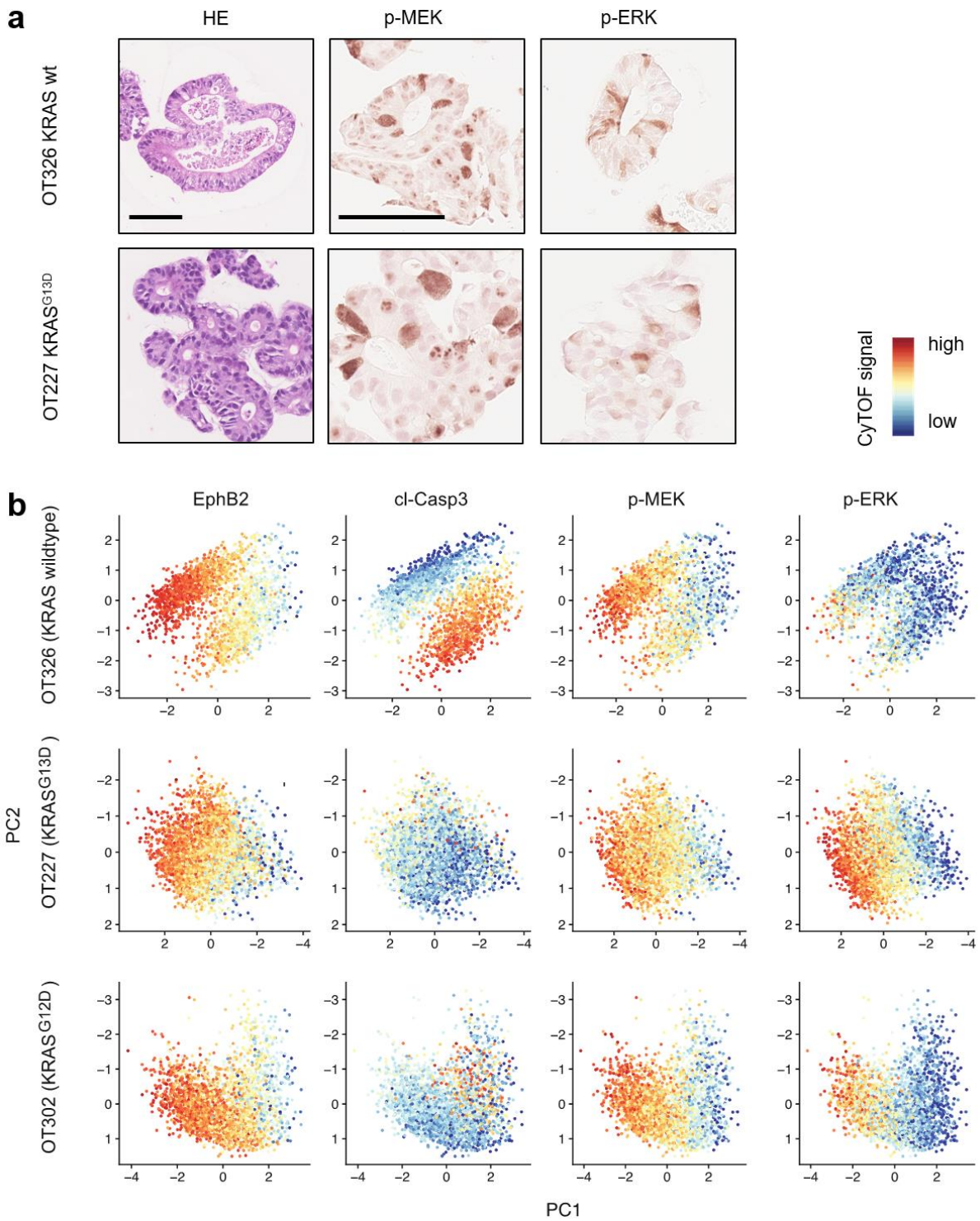
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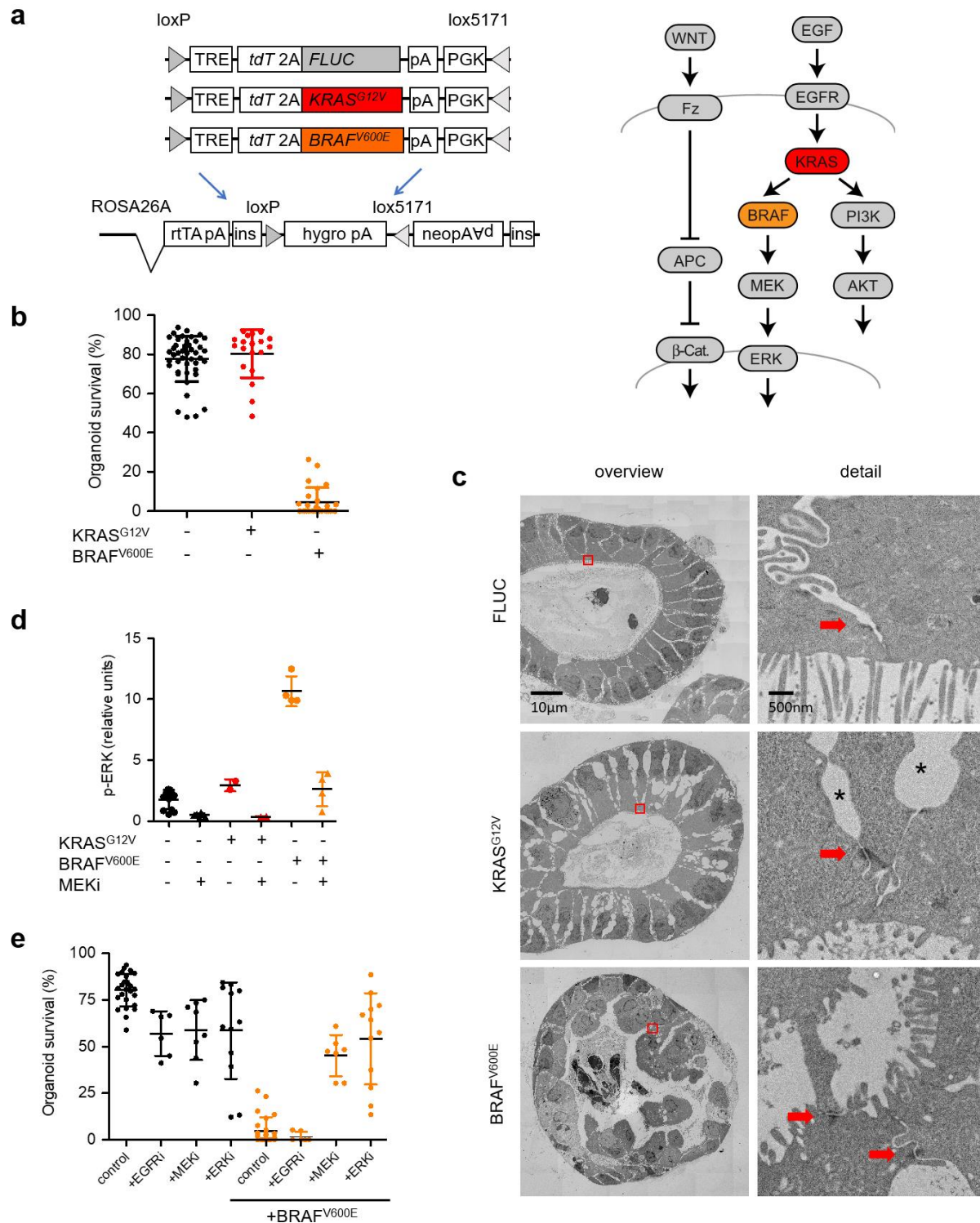
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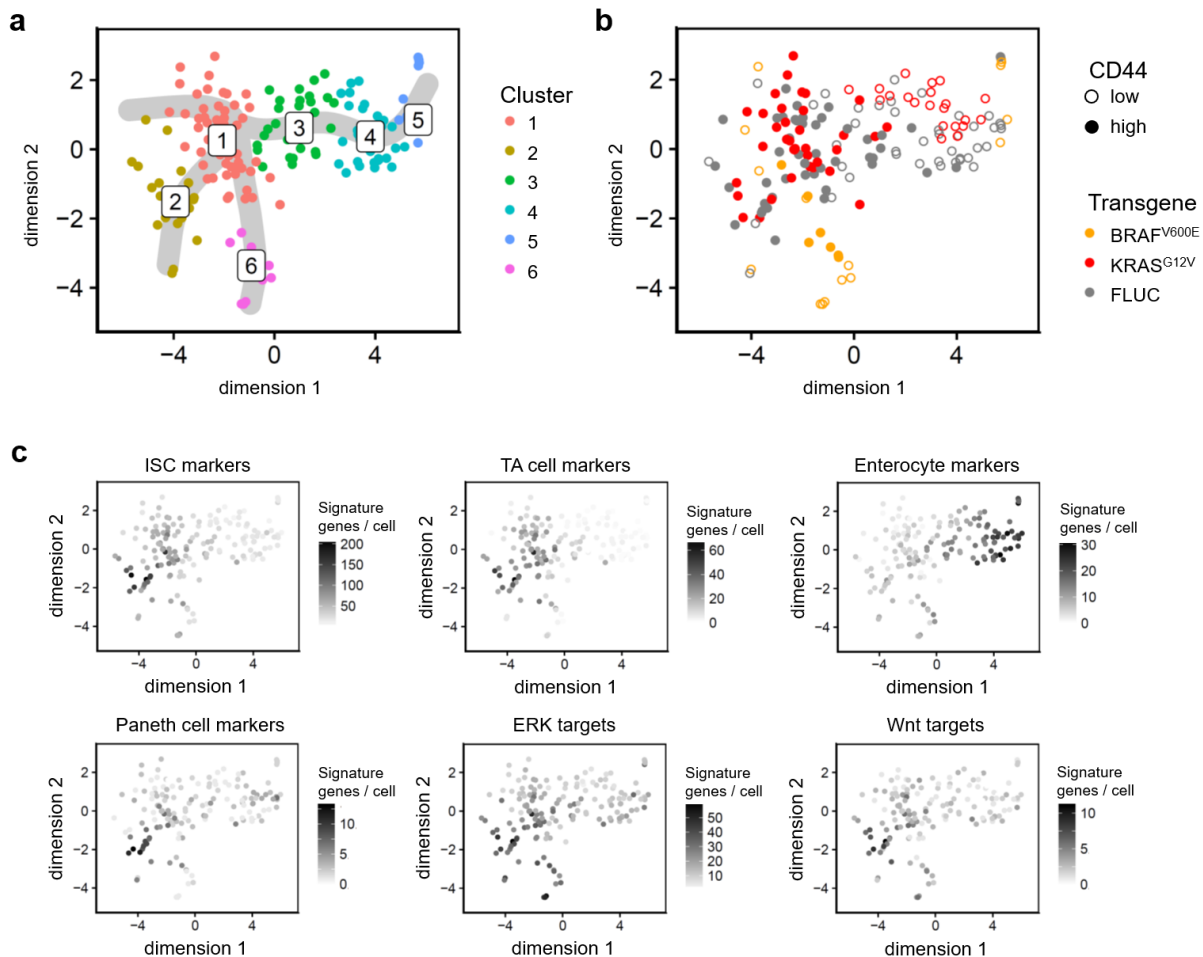
2 **Figure 1: Patient-derived colorectal organoids display graded MEK and ERK phosphorylation,**
3 **regardless of KRAS mutational status.** a) Hematoxylin-eosin (HE) staining and phospho-MEK and
4 phospho-ERK immunohistochemistry of two PD3D lines OT326 and OT227 that are KRAS-wildtype and
5 KRAS-mutant, respectively. Scale bars denote 100 μ m for HE and immunohistochemistry. b) CyTOF
6 analysis of PD3Ds. Principal component analyses, colour coded for EphB2, cleaved Caspase, phospho-
7 MEK and phospho-ERK are shown. Red, yellow and blue colours of the scale represent high,
8 intermediate and low signals, respectively.



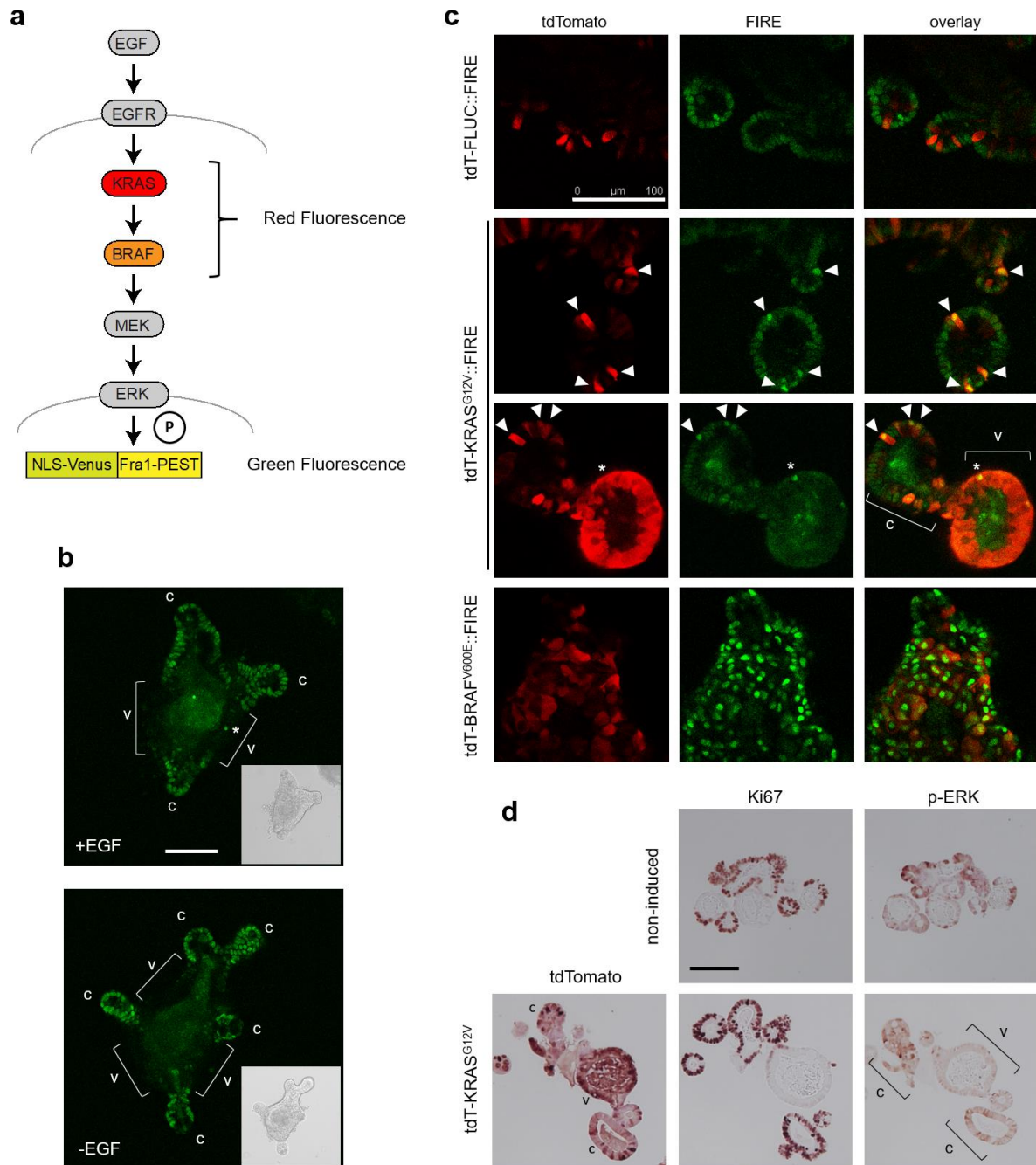
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2 **Figure 2: Transgenic induction of BRAF^{V600E}, but not KRAS^{G12V} disrupts intestinal organoids due to**
3 **high ERK activity.** a) Simplified representations of transgenes (to the left) and the RAS-ERK and Wnt/ β -
4 Catenin pathways (to the right), indicating the relative positions of the KRAS and BRAF proto-
5 oncogenes. b) Organoid survival 4 days after induction of oncogenic KRAS^{G12V} or BRAF^{V600E}. Organoids
6 are counted 1 day after passaging, and fractions of surviving organoids were calculated at day 4.
7 Organoid survival was judged by presence of crypt domains and a continuous basal surface. Control

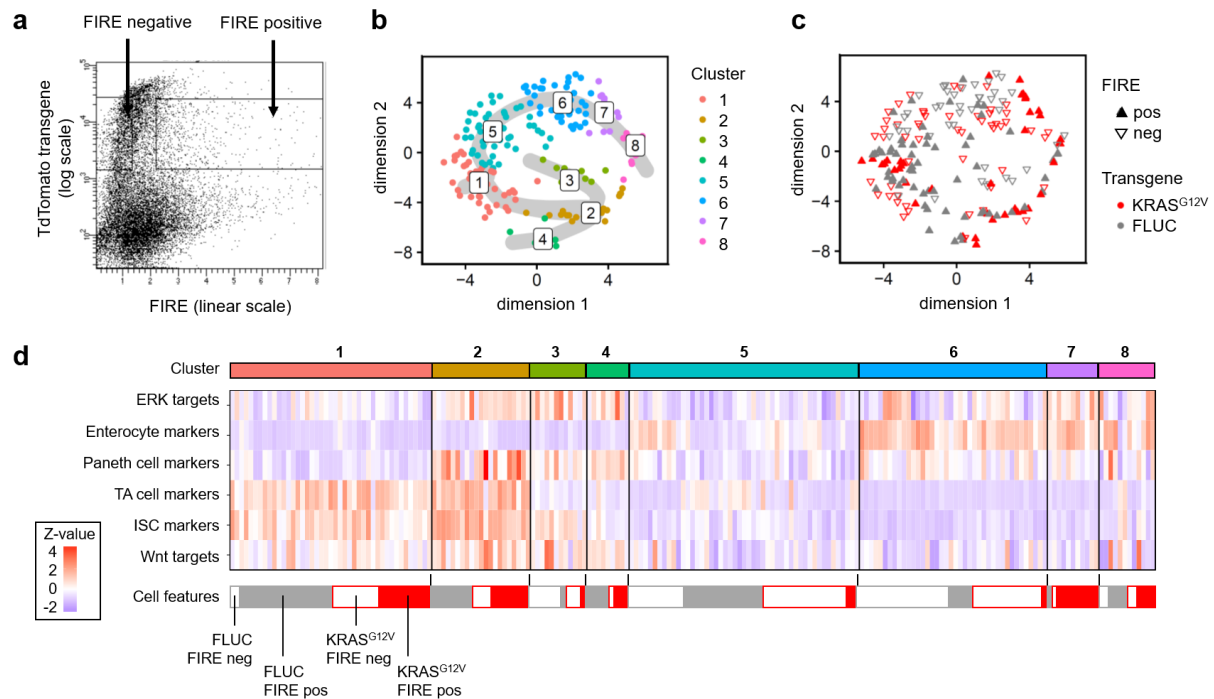
1 organoids comprise of mixed non-induced cultures of KRAS^{G12V} and BRAF^{V600E} lines. c) Electron
2 microscopy reveals loss of epithelial integrity after BRAF^{V600E} induction. Images of the intestinal
3 organoid epithelium, 24 h after induction of control FLUC, KRAS^{G12V} or BRAF^{V600E} transgenes. Detailed
4 views (right) represent a zoom into areas marked by red boxes in the overviews (left). Detailed views
5 show apical surfaces of adjacent enterocytes with brush border. Red arrows mark desmosomes.
6 Intercellular vacuoles, most visible in the KRAS^{G12V} model (marked by *) are likely fixation-induced
7 artefacts, see ref. ²⁷. Scale bars are 10 μ m in the overview panels and 500 nm in the detailed view
8 panels. d) Quantification of ERK phosphorylation in organoids, 24 h after induction of control, BRAF or
9 KRAS transgenes, using a capillary protein analysis. e) Quantification of organoid survival, 4 days after
10 inhibition of EGFR, MEK, ERK and/or induction of BRAF^{V600E}, as in panel B). Error bars in panels B, D, E
11 denote standard deviations.



1
2 **Figure 3: Single cell RNA sequencing of transgenic organoids reveals differential effects of BRAF^{V600E}**
3 **or KRAS^{G12V} on gene expression and intestinal cell hierarchies.** All panels: t-SNE visualisations and
4 clustering of organoid single cell transcriptomes clustered with k-means, 24 h after induction of FLUC
5 control, BRAF^{V600E} or KRAS^{G12V} transgenes. a) Colour code for six k-means clusters, and inferred
6 differentiation trajectories starting at cluster 1 shown as grey overlay. b) Colour code for transgene
7 and CD44 positivity, as inferred from flow cytometry. CD44 positivity was used to direct cell selection,
8 and thus is not representative for the cell population. For CD44 status of the cell populations, see
9 Supplementary Fig. 2. c) Mapping of cell- and pathway-specific differentiation signatures. Numbers of
10 signature genes detected are given per single cell transcriptome.

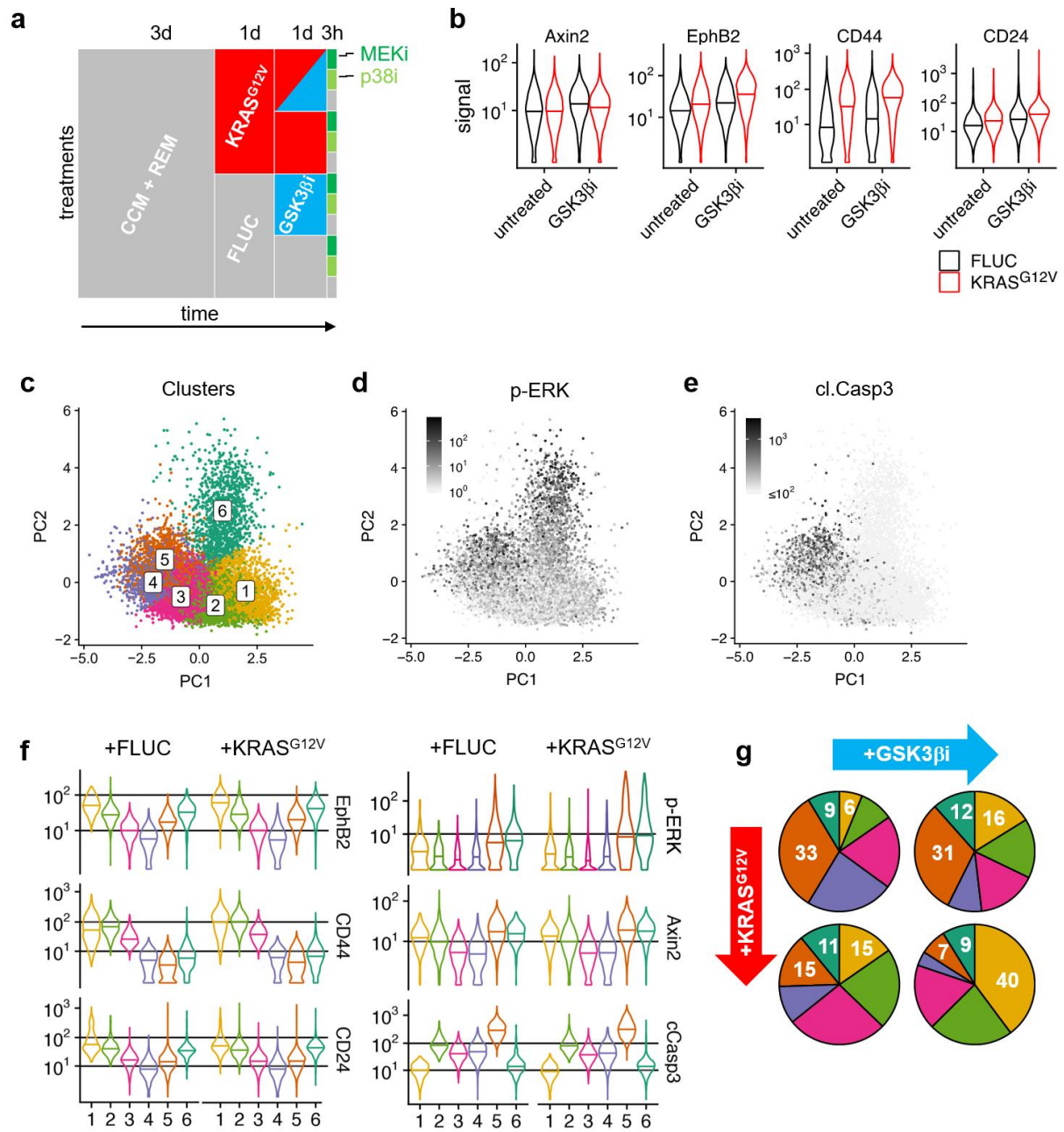


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2 **Figure 4: Visualisation of ERK activity by FIRE reveals KRAS^{G12V}-responsive cells.** a) Schematic
3 representation of the relevant signalling pathway and reporter b) FIRE activity in wildtype intestinal
4 organoids, in the presence and absence of EGF in the culture medium, as indicated. Asterisk marks
5 isolated FIRE-high villus cell. c) Fluorescence microscopy images showing transgene expression (red),
6 FIRE activity (green), and overlays in intestinal organoids, taken 2 days (FLUC, KRAS) or 1 day (BRAF)
7 after transgene induction. Arrow heads mark KRAS^{G12V}/FIRE high crypt cells, asterisk marks FIRE high
8 villus cell, c and v demarcate crypt and villus areas, respectively. d) Immunohistochemistry of
9 tdTomato, Ki67 and p-ERK in intestinal organoids, as indicated. c and v demarcate crypt and villus
10 areas, respectively. Scale bars are 100 μm in all panels.



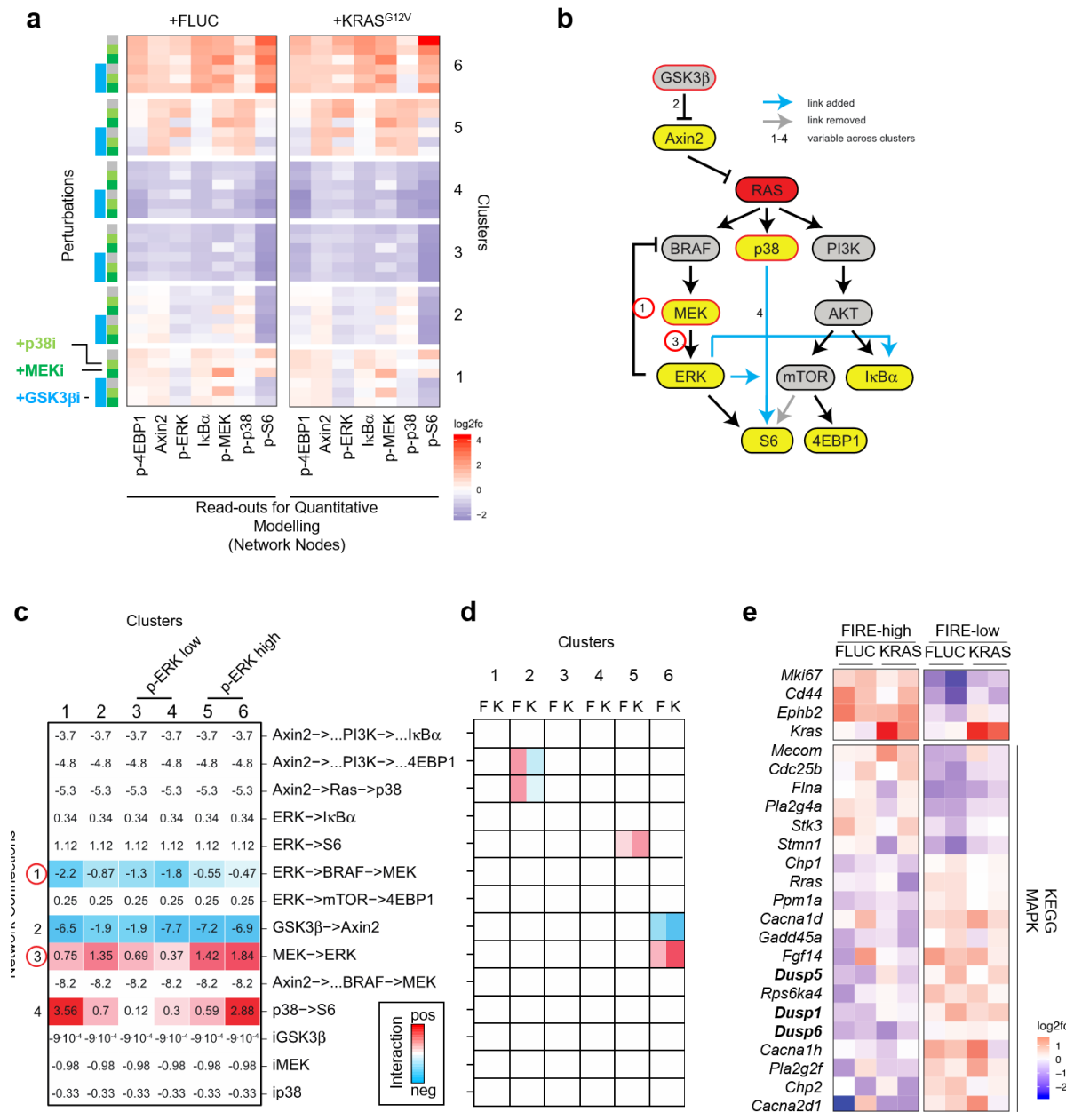
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 2 **Figure 5: Single cell RNA sequencing of FIRE transgenic organoids reveals populations of KRAS^{G12V}-**
 3 **responsive and -unresponsive cells** a) Diagram of fluorescence-activated cell sort gates for FIRE-
 4 negative and -positive cells. b) t-SNE visualisation colour-coded for eight clusters identified with k-
 5 means clustering. Inferred differentiation trajectories starting at cluster 1 are shown as grey overlay.
 6 c) tSNE visualisation displaying colour codes for transgene and FIRE positivity. Filled upward-pointing
 7 triangles: FIRE-high; outlined downward-pointing triangles: FIRE-low. Red: KRAS^{G12V}; grey: FLUC. d)
 8 Heat map of z-transformed signature scores per cell for cluster cell type identification. Signature scores
 9 correspond to number of expressed signature genes per cell normalised to gene detection rate and
 10 signature length. Blue: low target gene signature abundance; Red: high target gene signature
 11 abundance. Cluster colour codes are given above, and transgene and FIRE positivity codes are given
 12 below the heatmap.

13



1
 2 **Figure 6: CyTOF analysis reveals KRAS^{G12V}- and GSK3β inhibitor-responsive p-ERK high cell clusters.**
 3 a) Schematics for generation of network perturbation data by CyTOF. In short, organoids were
 4 established from KRAS^{G12V}- and FLUC transgenic mice, induced for transgene expression after 3 days,
 5 and treated with GSK3β inhibitor for 1 day and with MEK and p38 inhibitors for 3 hours before
 6 harvesting. Finally, 12 samples were subjected to multiplexed CyTOF analysis. b) Distributions of cell
 7 type markers in organoid cells induced for FLUC or KRAS^{G12V} transgenes plus/minus GSK3β inhibitor
 8 treatment. Central lines of violin plots denote median values. c) PCA showing colour code of k-means
 9 clustering in KRAS^{G12V}-induced cells by EphB2, CD44, CD24, Krt20, cleaved Caspase 3 signal strength. d-
 10 e) Mapping of signal strength for p-ERK and cleaved Caspase3 on PCA, as in c). f) Distribution of EphB2,
 11 CD44, CD24, Axin2, p-ERK and cleaved Caspase 3 signals in clusters 1-6, as above. Central lines of violin

- 1 plots denote median values g) Fractions of cells in clusters 1-6, in organoid cells induced for FLUC or
- 2 KRAS^{G12V} transgenes plus/minus GSK3 β inhibitor treatment. Numbers denote percentages of cells in
- 3 clusters 1, 5, 6.
- 4



1
2 **Figure 7: Network quantification identifies cell type-specific differences in KRAS to ERK signal**
3 **transduction.** a) Protein phosphorylation or abundance CyTOF data, demultiplexed by treatment and
4 cell clusters, as in Fig. 6C. Log₂ fold changes to average untreated FLUC-induced control line are given.
5 b) Signalling network structure used for modelling. The network was re-parametrized from a starting
6 network, using the experimental data to remove and add connections, denoted by grey and blue
7 arrows, respectively. c) Signalling quantification of identifiable network links using Modular Response
8 Analysis. After an initial round of optimally parametrizing all clusters with a joint parameter set, a
9 greedy hill climbing procedure was conducted to identify and quantify the significantly different
10 signalling routes that are coloured to show the similar strengths. Numbers 1-4 in b) and c) show
11 network connections with significant differences between clusters. Red circles mark MEK-ERK and ERK-
12 MEK connections identified as having different strengths in clusters with high versus low ERK

1 phosphorylation after KRAS^{G12V} induction. The model further noted control of S6 protein via p38 to be
2 preferentially in presumptive crypt cell clusters 1 and 6, and a decreased response of Axin2 to GSK3 β
3 inhibition in clusters 2 and 3. d) MRA modelling of differences between KRAS^{G12V}-induced and FLUC
4 control cells within each cluster. K and F mark KRAS^{G12V} and FLUC control cluster pairs, respectively.
5 Cluster pairs exhibiting KRAS^{G12V}-specific differences are shown in red and blue, indicating regulation
6 strengths (see also Supplementary HTML file). e) Colour-coded gene expression data from cells sorted
7 by high and low FIRE activity, as indicated. Upper panel shows marker genes (Mki67, encoding Ki67,
8 for proliferative cells, Cd44 and Ephb2 for crypt cells, and Kras), lower panel shows 20 significantly
9 regulated genes between the conditions. 269 genes encoding MAPK network components in KEGG
10 were tested. Bold letters indicate dual-specificity phosphatases.

11

