Supplementary Discussion

accompanying Ponsioen et al.

Quantifying single-cell ERK dynamics in colorectal cancer organoids reveals EGFR as an amplifier of oncogenic MAPK pathway signaling

Genetically encoded ERK biosensors present an excellent tool for live monitoring of ERK dynamics at the single-cell level in PDOs. At this moment, PDOs constitute the most representative model to capture intercellular heterogeneities *in vitro*[1]. ERK can be monitored using either FRET-based sensors, which change conformational state when phosphorylated by endogenous ERK[2,3] or Kinase Translocation Reporters (KTRs), whose phosphorylation by ERK induces translocation from the nucleus to the cytosol[4]. Of note, since both sensor types are also subject to local phosphatases, they actually report the kinase-phosphatase equilibrium[2]. It is exactly this equilibrium (not the kinase activity *per se*) that orchestrates downstream target activation and, thus, cellular responses. The FRET sensor, which behaves as endogenous MAPK targets, thus accurately reports local heterogeneities in this equilibrium, resulting either from kinase or phosphatase variabilities.

While KTR-reporters are appreciated for intuitive and straightforward single-channel readout, their quantitative analysis presents technical hurdles for application in 3D organoids. First, the ratiometric quantitation of nuclear-versus-cytosolic fluorescence requires high degrees of optical sectioning to avoid mixing of signal from these adjacent compartments. ROI assignment for these compartments is generally feasible in flattened 2D cultured cells, but troublesome in densely packed 3D organoids, where cytosolic space is very limited and cytosolic fluorescence from neighboring cells cannot be objectively separated. Altogether, the limited cytosolic signal will introduce noise in the nucleus-to-cytosol output ratio. Furthermore, the fact that single-channel KTR images frequently lack the contrast needed to assign the nuclear compartment, necessitates the additional expression of a nuclear marker[5], complicating mutagenesis strategies, necessitating double laser excitation and undermining the advantage of being a single-channel sensor. Finally, our attempts to standardize double calibration using super-inhibition and super-activation were frustrated by inconsistent behavior of the ERK-KTR biosensor at extreme levels (mainly questionable PMA-induced plateaus). Thus, KTRs are excellent for qualitative assessments, but not ideal for quantitative analyses, especially in 3D tissue.

In contrast, ratiometric YFP/CFP readout of the NLS-tagged FRET biosensors in 3D organoids yielded reliable, reproducible ERK dynamics of single-cells over long time intervals. YFP/CFP ratios remain unaffected when absolute fluorescence intensities are influenced by subtle changes in e.g. cell shape, orientation, cross-sectioning or expression level. Our custom-made analysis script (see Methods) enabled 'plane-picking', i.e. the selection of z-planes with optimal trans-sectioning of the analyzed nucleus. Careful plane-picking is essential for rigorous analysis, since it eliminates the risk of artefactual ratio changes due to low-intensity out-of-focus pixels. Furthermore, laser scanning with ultra-short dwell times (8000 Hz resonant scanner) prevents photobleaching or detectable cell damage over long intervals (>24 hr)[6], while signal-to-noise (S/N) ratios allowed appreciation of small and dynamic ratio changes in individual cells. The importance of the latter is exemplified by the subtle FRET decreases induced

upon super-inhibition, revealing minor residual ERK activities under pharmacological pathway inhibition (selumetinib in Fig. 3, afatinib in Fig. 4). Importantly, FRET sensors performed consistently under our double-calibration procedure. The consecutive full inactivation followed by full activation of sensor states generates quantitative data that are amenable to comparative analyses, either cell-to-cell or PDO-to-PDO.

The CDC25C-derived substrate sequence of the EKAREV biosensor (as well as the ERK sensor EKAR2G[7]) suffers from an undesired sensitivity to mitotic CDK1/cyclinB signaling (Extended Data Fig. 1). The same accounts for those of hELK1 and hRSK (Extended Data Fig. 2b) as well as the ELK1 domain in ERK-KTR (Fig. 1g and Extended Data Fig. 1g), suggesting that physiological ERK and CDK1 may share downstream signaling targets. Our studies indicate that rationally designed point mutations in the CDC25 substrate sequence of EKAREV(Tq) (K424P+K426W; PW-mutagenesis) presents the optimal solution to significantly overcome sensor activation by CDK1 (Fig. 1d,e). Of note, the mild residual FRET changes during M-phase are not consequential of ERK (i.e. insensitive to sel+SCH). However, in contrast to the parental EKAREV(Tq), the PW-mutated EKAREN4 and EKAREN5 are completely devoid of cell-cycle-related effects during G_2 phase (Fig. 1). Thus, unless cells are in M-phase, PW-mutagenesis eliminates all false positive signal. This is particularly relevant, since during G_2 phase, in contrast to M-phase, cells cannot be readily identified based on morphology and are thus not straightforwardly excluded from analyses.

Accidentally, PW-mutagenesis was also accompanied by advantageous ENhancement of the FRET range (Fig. 1f). Since YFP/CFP ratios were specifically elevated in phosphorylated (i.e. closed-state) sensors (Extended Data Fig. 2e,f), we hypothesize that both mutated residues, located nearby the phosphorylated threonine, increase the affinity between the phosphorylated CDC25C sensor domain and the PIN1 ligand domain, thereby favoring the closed state of the reporter. Anticipating on long-term imaging in 3D organoids, we inserted Turquiose2, which outcompetes the original eCFP with respect to brightness and photostability[8]. Whereas we here registered FRET through ratiometric imaging, Turquoise2 opens venues for fluorescence lifetime imaging (FLIM) thanks to its single-exponential decay[9]. Lifetime measurement is independent of signal intensity and FLIM may therefore surface as a successful acquisitional strategy in 3D samples. For reasons that are still under debate, however, lifetime detection regularly displays poor lifetime ranges as compared to ratiometric readout.

Furthermore, we introduced 169 silent mutations into Turquoise2 (Extended Data Fig. 4a) to ENhance genomic stability[10]. Indeed, loss of either fluorophore has been extremely rare in our PDOs despite months of culturing. This 'recombination-immune' Turquoise2 cDNA can be transferred to other FRET sensors to overcome similar obstacles (note, however, that this variant is not monomeric due to the intentional affinity-promoting residues K206A and V224L).

EKAREV was originally designed with a greatly lengthened intramolecular linker in order to minimize FRET in non-phosphorylated sensor molecules, thus enlarging FRET increase upon ERK-mediated phosphorylation[3]. However, inspired by reported mathematical modeling (Komatsu et al., 2011), we shortened the linker (with 34%, from 118 to 78 amino acids) to sacrifice some of this FRET range (Extended Data Fig. 3a,b) in favor of detection threshold (Fig. 2b). Using co-expressed ERK-KTR-mCherry as an unbiased reference sensor[11,12], we extensively compared performances of EKAREV(Tq), EKAREN4 and EKAREN5 in detecting the subtle spontaneous ERK activity oscillations of HeLa cells. EKAREV(Tq) and EKAREN5 perform comparably well in the low ERK activity range

(Extended Data Fig. 2c-g and Supplementary Movie 2), likely due to different virtues. EKAREV(Tq), on the one hand, displays steeper relationship between ERK activity and FRET (Fig. 2d), which is favorable in terms of signal-to-noise (S/N) ratio. On the other hand, EKAREN5 displays the lowest detection level (Fig. 2b), while FRET increase as a function of ERK activity is less steep (summarized in Fig. 2h). The latter, in turn, explains that EKAREN5 saturates at higher ERK levels than EKAREV(Tq). Thus, EKAREN5 shows optimized detection range, performing well in the low and high range of physiological ERK levels (Fig. 2h,i).

To our knowledge, the effects of expression levels on absolute FRET ratio (Extended Data Fig. 2e,f) had not been properly described to date. We hypothesize that the effect is due to the intentional inter-fluorophore affinity between Turquoise2 and YPet. While this affinity contributes to the successful design of EKAREV (prolonging the closed conformation, thereby increasing phosphorylation-induced FRET change[3]), it expectedly also causes intermolecular clustering of non-phosphorylated sensor molecules, leading to (undesired) ERK-independent intermolecular FRET. In line with this notion, baseline YFP/CFP ratios increase with expression level (Extended Data Fig. 2e), also in the non-phosphorylatable, ERK-insensitive Thr(48)-Ala mutant (Extended Data Fig. 2f). In contrast, YFP/CFP of saturated sensors is less influenced by expression level increases (Extended Data Fig. 2e'), possibly because intramolecular interactions disable intermolecular complexing. Although it is important to keep these aspects in mind when employing EKAREV or EKAREN sensors, it must be noted that in order to determine these relationships, we intentionally generated a tremendous range of expression levels (Extended Data Fig. 2e). Opposed to this, we perform biologically relevant FRET experiments at standardized expression levels, preferably in the 'medium' range (Extended Data Fig. 2g), which combine satisfactory signal-to-noise ratio with minimized sensor concentrations so as to match those of the endogenous kinases and phosphatases.

Finally, we addressed the issue of whether sensor dimerization could introduce a bias in the relative rate constants of phosphorylation versus dephosphorylation. We verified that a >30-fold difference in expression levels had no effects on kinetics (Extended Data Fig. 3d). Furthermore, our analyses on fluctuating HeLa cells (Fig. 2) and oscillations in PDOs (Fig. 3) showed no signs of temporal misrepresentations. However, we cannot exclude that FP-dimerization causes minute discrepancies between physiological response and sensor signal in a concentration-dependent manner.

Sensor expression level is among a series of potential ERK-independent determinants for absolute YFP/CFP ratios in 3D organoids. As another example, the ERK-insensitive Thr(48)-Ala mutant sensors revealed that depth within the fluorescent organoid is a variable influencing the FRET ratio. This results from the fact that blue and yellow emission are differentially affected by scattering in the 3D tissue (Extended Data Fig. 5). These considerations underscore the essence of calibration for the interpretation of each individual FRET analysis. We strongly advertise our experimental protocol encompassing total inactivation and total activation of the sensor. We enforced pathway 'super-inhibition' using MEK and ERK inhibitors at high concentrations (both 5μM). Our interpretations build on the notion, that total pathway shutdown is followed by sensor dephosphorylation by endogenous phosphatases. Although we cannot formally prove that this is always the case, it is worth noting that the effects of afatinib and super-inhibition on ERK are perfectly mirrored in pERK levels in Western blot analyses on the same EKAREN5-expressing *BRAF*^{V600E} mutant PDO lines (Fig. 4e). To enforce pathway 'super-activation', we added PMA, which

hyperactivates ERK through PKC and RAF[13], combined with subsequent addition of phosphatase inhibitor okadaic acid (OA), since in PDOs PMA alone appeared insufficiently reproducible in enforcing sensor saturation. Eventually, the toxic OA ended experiments, but max-FRET ratios could consistently be derived from individual cells before death induction. While both manipulations are important for quantification, the effect of super-inhibition was especially crucial to interpret drug efficacies in ERK pathway inhibition, since it had the potential to uncover minimal residual ERK activities by subtle FRET reductions. Conversely, we interpreted a lack of effect upon super-inhibition as full pathway inhibition by prior treatment (circles in Fig. 4c,d).

Both in 2D cultured cells and in 3D organoids, nuclear localization of EKAREN5 is key to single-cell analysis. It must be noted that ERK can fulfill separate roles in the cytosol[14]. Thus, in the current studies, FRET signals must be interpreted as ERK activity penetrating the nuclear compartment, where it is known to encounter a plethora of targets for the execution of its genetic programs. For the potential study of differential ERK functions in cytosol and nucleus, we generated EKAREN4 and EKAREN5 variants available with a nuclear exclusion signal (NES) or without any targeting motif (Extended Data Fig 4d).

We present the next-generation, ENhanced EKAREN biosensors for ERK (Fig. 2h,i). By virtue of its extended detection range, EKAREN4 is the sensor of choice for the study of strong ERK signals, for instance those driving induction of senescence[15]. EKAREN5, by virtue of its low detection threshold, is the optimal ERK biosensor to study subtle spontaneous fluctuations or inhibitor efficacies.

References Supplementary Discussion

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