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Quantitative NMR analysis of Erk activity and inhibition by U0126 in a panel of patient-derived colorectal cancer cell lines☆

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ABSTRACT

We comparatively analyzed the basal activity of extra-cellular signal-regulated kinase (Erk1/2) in lysates of 10 human colorectal cancer cell lines by semi-quantitative Western blotting and time-resolved NMR spectroscopy. Both methods revealed heterogeneous levels of endogenous Erk1/2 activities in a highly consistent manner. Upon treatment with U0126, an inhibitor of mitogen-activated protein kinase kinase (MEK) acting upstream of Erk1/2, Western-blotting and NMR congruently reported specific modulations of cellular phospho-Erk levels that translated into reduced kinase activities. Results obtained in this study highlight the complementary nature of antibody- and NMR-based phospho-detection techniques. They further exemplify the usefulness of time-resolved NMR measurements in providing fast and quantitative readouts of kinase activities and kinase inhibitor efficacies in native cellular environments. This article is part of a Special Issue entitled: Inhibitors of Protein Kinases (2012).

1. Introduction

Quantitative methods to assess physiological and pathophysiological kinase activities allow us to better understand how altered cellular signaling behaviors steer human diseases such as cancers [1]. Quantitative activity profiling methods are equally important in kinase inhibitor studies and thereby constitute important tools in drug discovery approaches [2,3]. In reflection of this, several new methods to observe multiple kinase activities in complex biological samples have recently been introduced including fluorescence- [4–6] and mass spectrometry-based approaches [7,8]. Despite continuous efforts to develop new tools for direct kinase activity profiling in complex cellular environments, antibody-based phospho-detection techniques are still most commonly used [9,10]. Central to these assays are immuno-reagents that either recognize phosphorylated amino acids in downstream kinase substrates, whose phosphorylation states are used to indirectly infer the activities of upstream-acting enzymes, or, as in the case of mitogen-activated protein kinases (MAPKs), antibodies that bind to phosphorylated activation loop residues of MAPKs to determine cellular concentrations, and thereby activities, of catalytically active pools of kinases [11–13]. Evidently, these antibody-based methods do not actually reveal the 'activities' of cellular kinases, but rather whether downstream substrate molecules are modified, or whether activation loop residues are phosphorylated. In many instances, however, these types of measurements serve as good proxies for actual enzymatic activities, as has been shown in many studies. In this study, we directly compare the application of semi-quantitative Western blotting to assess the activity of a cellular MAPK with quantitative analyses by time-resolved, high-resolution nuclear magnetic resonance (NMR) spectroscopy [14].

NMR monitoring of reversible phosphorylation reactions offers several advantages over other analytical methods [15]. First, it provides atomic-resolution readouts of the actual phosphorylation states of individual protein or peptide residues without having to rely on indirect detection schemes (Fig. 1a). Second, NMR spectroscopy is a non-invasive and non-disruptive biophysical technique that can be employed to follow post-translational modification reactions such as protein phosphorylation in a continuous, time-resolved fashion (Fig. 1b). Third, NMR is inherently quantitative. Resonance signals of modified and unmodified substrate species are detected side-by-side in the same NMR experiment and can thus be integrated at different time points of phosphorylation reactions to yield quantitative modification levels (Fig. 1c). This enables deductions of kinetic reaction parameters such as protein phosphorylation rates. Fourth, NMR readouts can be obtained in complex mixtures such as cell extracts, or whole live cells, which enables quantitative *in situ* measurements of cellular phosphorylation reactions in

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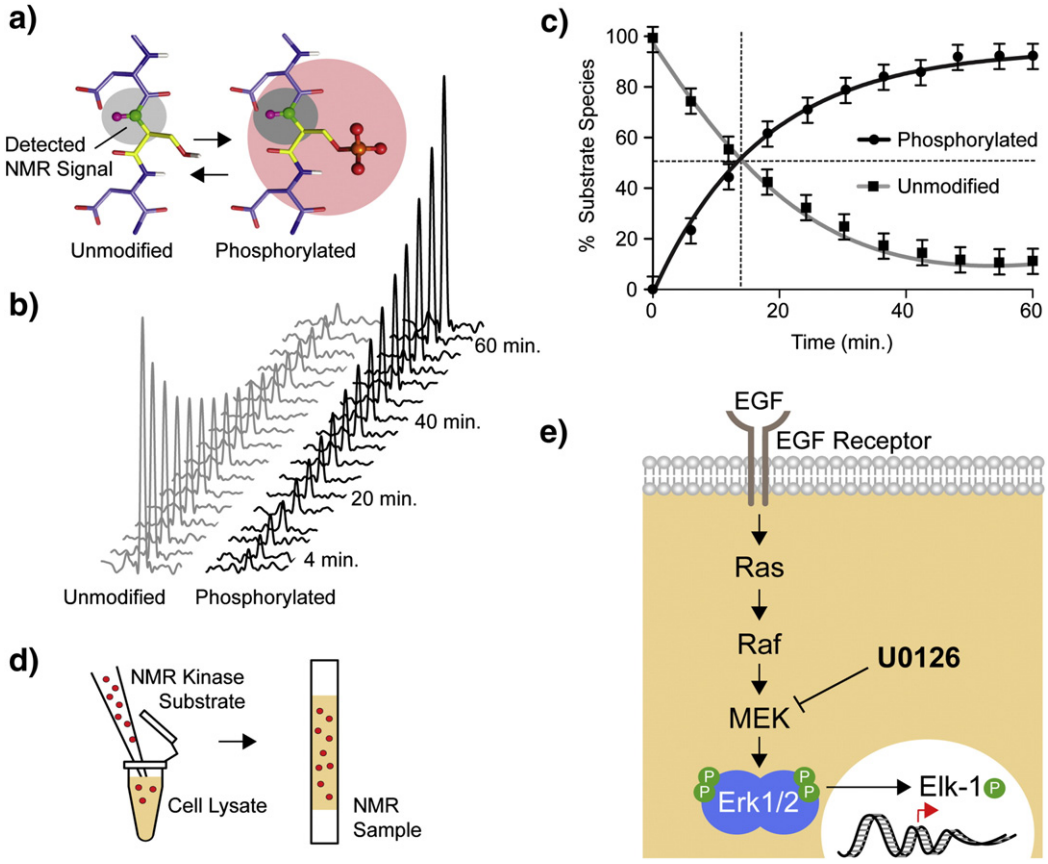

Fig. 1. NMR characteristics of protein phosphorylation. a.) Covalent addition of a phosphate group to an isotope-labeled serine residue for example, changes the chemical environment of neighboring atomic nuclei (indicated by a red sphere). In turn, this leads to changes in NMR resonance frequencies and the appearance of new NMR signals that correspond to the phosphorylated form of the substrate molecule. Different sets of NMR signals of unmodified and phosphorylated protein/peptide residues are detected by ^{1}H-^{15}N backbone-amide correlation NMR experiments. b.) Measurements of continuous phosphorylation reactions by consecutive NMR experiments produce time-series of NMR spectra with progressively disappearing resonance signals of unmodified substrate residues and gradually increasing NMR signals of phosphorylated substrate resonances. c.) By quantifying these signal intensities at individual measurement time-points, time-resolved modification trajectories can be obtained. d.) Addition of isotope-labeled, NMR-visible proteins, or peptides to non isotope-labeled, native cellular environments enables NMR monitoring of endogenous phosphorylation reactions in real time. e.) Schematic representation of one part of the MAPK signaling pathway.

these environments without the need for sample processing, or purification (Fig. 1d). Fifth, protein de-phosphorylation behaviors are detected along the same experimental rationale. Therefore, quantitative assessments of cellular kinase and phosphatase activities are obtained in parallel, with no required changes in experimental setups (Fig. 1a). For these reasons, NMR studies of protein phosphorylation and de-phosphorylation events in native, cellular environments are becoming increasingly popular [14,16–18]. To investigate whether NMR spectroscopy can be used to directly report cellular kinase activities in a disease-relevant setting, we characterized the endogenous levels of p44/p42 MAPK activity (also referred to as extracellular signal-regulated kinase 1/2 (Erk1/2)), in a panel of patient-derived colorectal cancer (CRC) cells [19].

In response to cell surface receptor tyrosine kinase (RTK) signaling, for example *via* the epidermal growth factor (EGF) receptor and its downstream effectors, the small GTPase p21/Ras and the Ser/Thr-kinase c-Raf 1 [20–22], mitogen-activated protein kinase kinases (MEK1/2) become activated and phosphorylate activation loop residues Thr202, Tyr204 of Erk1, and Thr185, Tyr187 of Erk2, and thereby activate both kinase isoforms [23,24] (Fig. 1e). In turn, active Erk1/2 phosphorylates a great number of target proteins that include many of the transcription factors that eventually mediate oncogenic transformations, such as Elk-1 [25–27]. Therefore, antibodies against phospho-Erk are widely used to infer oncogenic Erk1/2 activities and cellular levels of phospho-Erk often serve as indicators for cancer progression and tumorigenicity [28,29]. A recent analysis of 64 CRC cell lines revealed a broad range of Erk1/2 activity among different cancer cells derived from the same cell type of origin. In 5 of these cell lines, each with similar levels of phospho-Erk, treatment with U0126 resulted in varying degrees of Erk1/2 inactivation [19]. U0126 is a drug that acts on Erk1/2 *via* inhibition of the MEK1/2 kinases upstream of Erk [30,31] (Fig. 1e). Using semi-quantitative Western blotting and time-resolved NMR spectroscopy, we comparatively analyzed Erk1/2 activities in cell lysates prepared from these CRC cell lines and measured differences in Erk1/2 inhibition by U0126. Our data reveal a high degree of consistency between these two methods and provide a strong argument for NMR spectroscopy as a complementary tool for quantitative studies of cellular phosphorylation reactions.

2. Materials and methods

2.1. Preparation of CRC cell lysates

The following CRC cell lines were grown as previously described [19]: Colo 678, Colo 741, HCT 116, HDC 8, LS 123, CCK 81, HCA 46, HDC 57, HRA 19, and LS 174T. For each NMR experiment ~5–10 × 10^6 cells were harvested 24 h after final feeding at ~70–80% confluency. For experiments with the MEK inhibitor, cells were incubated with either 10 μM of U0126 in DMSO (0.01% (v/v)) (Cat.# 662009, Calbiochem) or in DMSO (0.01% (v/v)) alone, for 30 min as previously described [31]. Cells were harvested by scraping them on ice into lysis buffer: 20 mM HEPES pH 7.4, 1.5 mM $MgCl_2$, 10 mM KCl, 1% (v/v) NP-40, 2× protease

inhibitor cocktail (Cat.# 11697498001, Roche), 1.4 μg/mL pepstatin A (Cat.# P4265, Sigma Aldrich), 4× final concentration of both phosphatase inhibitor cocktails 2 and 3 (Cat.# P5726, P0044, Sigma Aldrich) and 0.4 mM PMSF. In this buffer, whole cell lysates were then prepared as previously described [19]. Total protein concentration was determined by Bradford assay. Aliquots were snap-frozen in liquid nitrogen and stored at −80 °C.

2.2. Erk1/2 activity/phosphorylation levels by semi-quantitative Western blotting

35 μg total protein CRC cell lysates were loaded onto a gradient (4–20%) SDS-PAGE (BioRad). After gel electrophoresis, proteins were transferred onto PVDF membranes using the Trans-Blot® Turbo Transfer system (BioRad). PVDF membranes were blocked in 5% BSA in TBS-T for 1 h, before probing with 1:1000 anti-phospho-Thr202/Tyr204 Erk1/2 (Cat.# 9101, Cell Signaling Technology) or 1:1000 anti-total Erk1/2 (Cat.# 9102, Cell Signaling Technology) antibodies followed by secondary anti-rabbit HRP antibody at 1:10,000 dilution (Cat.# 97051, Biosciences). Sample loading was confirmed by probing with 1:5000 anti-actin (Cat.# CP01, Calbiochem), followed by a secondary anti-mouse HRP antibody at 1:10,000 dilution (Cat.# A9917, Sigma Aldrich). Membranes were developed using SuperSignal West Pico Chemiluminescent Substrate (Cat.# 34087, Thermo Scientific). Detection of the chemiluminescence signal was performed using a BioRad Molecular Imager and ImageLab software (BioRad) was used to quantify the respective signal intensities [32]. Ratios for phospho-Erk over total Erk1/2 ($Erk_{phospho}/Erk_{total}$) were determined for each cell line.

2.3. Erk1/2 NMR substrate

An optimized Erk1/2 substrate peptide containing the so-called 'DEF' docking site for Erk1/2 *i.e.* FXFP, based on amino acids 386–399 of human Elk-1 [33], was chosen, with a Lys to Ala mutation at position +3 with respect to the phosphorylatable Ser residue (*i.e.* GYAPRSPA**A**LA*KFQFP*A phosphoacceptor Ser underlined, Lys to Ala mutation in bold, 'DEF' sequence in italics). The Lys to Ala mutation effectively abolished cross-reactivity with the members of the cyclin-dependent family of kinases (data not shown). ^{15}N isotope-labeled Erk1/2 substrate peptide was produced according to a previously published solid-phase peptide synthesis (SPPS) protocol, with site-selective ^{15}N isotope incorporation [34]. *In vitro* kinase reactions with commercial Erk confirmed phosphorylation of the canonical kinase substrate site.

2.4. NMR spectroscopy

Cell lysates were thawed on ice and diluted in lysis buffer to a final lysate protein concentration of 3 mg/mL in 350 μL of sample volume. End concentrations of 333 μM ATP, 3.33 mM $MgCl_2$ and 10% D_2O were added for measurements in 5 mm diameter Shigemi NMR tubes. The lysates were spiked with the ^{15}N isotope-labeled Erk1/2 kinase substrate to a final concentration of 50 μM and the resulting samples were immediately loaded into the NMR spectrometer. Consecutive sets of identical 1D ^{1}H-^{15}N SOFAST-HMQC experiments [35] were recorded at 298 K using 2048 transients, a sweep width of 16.6 (^{1}H) ppm and an inter-scan delay of 0.1 s (experimental time ~6 min). At the end of each reaction, sample temperatures were reduced to 281 K, and single 1D (as above)- and 2D ^{1}H-^{15}N SOFAST-HMQC NMR spectra with 1024 transients, sweep widths of 16.6 (^{1}H) ppm and 19 (^{15}N) ppm, 32 complex points and inter-scan delay of 0.1 s (experimental time ~11 min) were recorded. pH measurements before and after the lysate reactions confirmed that NMR signal intensity variations were not affected by changes in solution conditions. All NMR experiments were acquired on a 600 MHz Bruker Avance spectrometer equipped with a cryogenically cooled triple-resonance $^{1}H(^{13}C/^{15}N)$ probe (TCI). Additional Erk1/2 kinase substrate samples were measured in reactions with 500 enzymatic units (U) of commercial, recombinant Erk (Cat.# P6080L, New England Biolabs) as *in vitro* Erk1/2 activity references. All data were processed and analyzed in Topspin 2 (Bruker) and iNMR 3.6.3.

2.5. Quantitative analyses from NMR data

The NMR signal intensities of 50 μM unmodified and fully phosphorylated Erk1/2 kinase substrate reference samples were used to accurately quantify the individual phosphorylation levels in samples at intermediate time points. Integrated NMR signal intensities were then converted into picomoles (pmol) of modified Erk1/2 substrate based on the known starting and end concentrations of the substrate (*i.e.* 50 μM). At all time points of the reaction this quantification could be cross checked, whereby the sum of the NMR signals of the unmodified and modified substrate species added to the total of 50 μM. In cell lysates with low kinase activities, NMR signals of the phosphorylated substrate species were below the detection limit (~1.5 nanomole of p-Erk substrate) for the first few measurement time points so that complete modification trajectories were not determined. Instead, for these lysates we established the final concentrations of phospho-species at the respective reaction end points by NMR experiments that we recorded at lower temperatures, and with more transients, for optimal signal to noise. In cell lysates with high endogenous kinase activities, individual data points were fitted to exponential buildup/decay curves with GraphPad Prism 5.0. The fit of these curves allowed for a comparison of the initial rates (pmol/min) of kinase activities in each of the cell lysates. In turn, by comparing these rates to reference *in vitro* experiments with 500 units (U) of commercial Erk, we delineated specific kinase activities in terms of unit equivalents, or U_{equ}.

3. Results

To measure Erk activity in colorectal cancer cell lysates, we initially probed for the total amount of cellular Erk1/2 in protein concentration-adjusted lysates from 10 CRC cell lines (Fig. 2a). Western blot detection using an Erk1/2 specific antibody revealed the characteristic double-band of p42 MAPK (Erk2) and p44 MAPK (Erk1) [36]. In line with previous results, the detected amounts of cellular Erk1 and Erk2 varied only slightly between replicate experiments of lysates made from the same cell types, or in lysates from the different cell lines. Quantitative integration of Western blot intensities of the Erk1/2 signals revealed comparable levels of both kinase isoforms. A different picture emerged when we probed the same panel of CRC cell lysates with a phospho-Erk antibody (Fig. 2a). We detected substantial variations in the relative amounts of cellular phospho-Erk, which was in good agreement with previously published data [19]. Cell lines with high endogenous levels of phospho-Erk, such as Colo 678, Colo 741, HCT 116 and HDC 8, also exhibited the highest phospho-Erk levels in the previous study by Kress et al. [19]. We further determined that LS 123, HCA 46 and HRA 19 lysates contained intermediate levels of phospho-Erk. We found very low levels of phospho-Erk in the remaining CCK 81, HDC 57 and LS 174T lysates. By determining the individual ratios of phospho-Erk *versus* total Erk1/2, we established that Colo 678, Colo 741, HTC 116 and HDC 8 constituted 'high Erk1/2 activity' cells with ratios above 1, whereas LS 123, HCA 46, and HRA 19 scored as 'intermediate Erk1/2 activity' cells with ratios of ~0.1–1. CCK 81, HDC 57 and LS 174T cells were classified as 'low Erk1/2 activity' with ratios below 0.1 (Fig. 2b).

Next, we asked whether the different amounts of cellular phospho-Erk that we measured by Western blotting translated into observable differences in the individual modification rates of the Erk1/2 substrate peptide that we were to monitor directly in the respective cell lysates by time-resolved NMR spectroscopy. NMR modification trajectories of the individual phosphorylation reactions were recorded as independent triplicates in all 10 CRC lysates. We found that 'high Erk1/2 activity' cell lines Colo 678, Colo 741, HTC 116 and HDC 8, but also 'intermediate' LS 123 cells yielded quantitative phosphorylation curves, although LS 123 lysates were close to the detection limit (Fig. 2c). We did not detect

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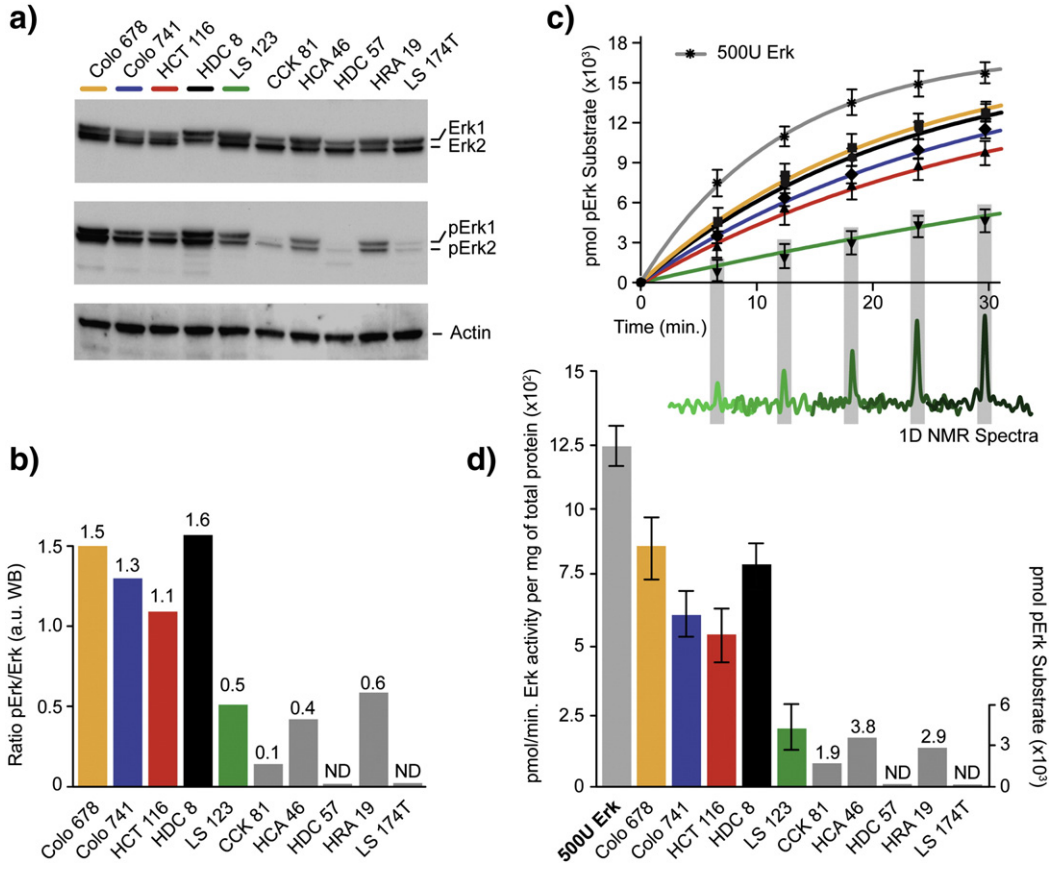

Fig. 2. Erk1/2 activities in lysates of 10 colorectal cancer cell lines. a.) Western blot analysis of Erk1/2 (top panel) and phospho-Erk1/2 (middle panel) levels in lysates of 10 CRC cell lines. Actin levels are shown as loading controls in the bottom panel. b.) Semi-quantitative analysis of cellular Erk1/2 activities determined as Western blot signal intensity ratios (arbitrary units, a.u.) of phospho-Erk over total Erk. Cell line-specific ratios are indicated above the bar graphs. N.D. not detectable. c.) Phosphorylation build-up curves as determined by time-resolved 1D ^{1}H-^{15}N correlation NMR experiments in lysates of Colo 678 (yellow), Colo 741 (blue), HCT 116 (red), HDC 8 (black) and LS 123 (green) cells. Signal intensity measurements of phospho-NMR resonances of the modified Erk1/2 substrate (pErk Substrate) are translated into picomol equivalents of modified substrate species. A reference build-up curve of reactions with 500 U of commercial Erk is shown in grey. Experimental NMR spectra of individual time-points of the LS 123 lysate reaction are shown below. Data were measured in triplicates and are shown as means ± s.d. d.) Initial rates (pmol/min/mg of lysate protein) of kinase activities for cell lysates with higher Erk1/2 activities. Absolute levels of phosphorylated Erk1/2 NMR substrates (pErk Substrate) of lysates with low Erk1/2 activities are shown as grey bars on the right.

quantifiable levels of Erk1/2 phosphorylation throughout the first halves of the reactions in lysates of HCA 46, HRA 19 and 'low' Erk1/2 activity CCK 81, HDC 57 and LS 174T cells, and thus we did not obtain fully time-resolved modification curves. In those cases, we determined the absolute concentrations of phosphorylated Erk1/2 NMR substrates after 35 min of lysate incubation and at NMR spectrometer settings that afforded higher overall 'intermediate' (*i.e.* at a lower temperature and with an increased number of scans, see Materials and methods for details). In addition, we ran *in vitro* reference reactions with 500 enzymatic units (U) of commercial Erk to quantitatively deduce the unit equivalents of active Erk1/2 in these lysates (see below). Based on these data, we calculated the respective rates of phosphorylation in picomoles (pmol) per minute (min) per milligram (mg) of total lysate protein (Fig. 2d). Our analysis revealed that the qualitative differences in phospho-Erk over total Erk1/2 ratios that we had determined as arbitrary units of Western blot signal intensities (Fig. 2b) were in good agreement with the quantitative rates of Erk1/2 phosphorylation that we had measured by time-resolved NMR spectroscopy (Fig. 2d). Moreover, the determined endpoint concentrations of phosphorylated Erk1/2 peptides (pErk substrate) in CCK 81, HCA 46 and HRA 19 lysates consistently recapitulated the cellular levels of phospho-Erk that we had detected by Western blotting. In HDC 57 and LS 174T lysates, neither NMR spectroscopy, nor Western blotting revealed any Erk1/2 activity (Fig. 2b and d). Based on the *in vitro* phosphorylation rate that we had determined for 500 U of Erk, we calculated the following mean unit equivalents (U_{equ}) of cellular Erk1/2 activities for the different cell lysates: ~360 U_{equ} in Colo 678 cells, ~250 U_{equ} in Colo 741 cells, ~220 U_{equ} in HCT 116 cells, ~320 U_{equ} in HDC 8 cells and ~90 U_{equ} in LS 123 cells.

Having determined the basal Erk1/2 activities in a panel of CRC cell lines, we set out to address the suitability of time-resolved NMR spectroscopy to quantitatively delineate the inhibitory potential of a well-characterized, commercial MEK antagonist, U0126 [30]. We incubated the four 'high Erk1/2 activity' cell lines and the 'intermediate' LS 123 line with 10 μM of U0126 for 30 min, prior to preparing lysates, according to the protocol by Rice et al. [31]. As expected, semi-quantitative Western blotting showed that the relative levels of phospho-Erk *versus* total Erk were drastically altered in U0126 treated cells (Fig. 3a and b). 'High Erk1/2 activity' Colo 678, Colo 741 and HCT 116 cells, and also LS 123, displayed marked reductions in their cellular phospho-Erk levels, whereas HDC 8 cells did not exhibit similarly diminished amounts of active kinase. A semi-quantitative analysis of the Western blots revealed that phospho-Erk levels were 100% reduced in Colo 741, HCT 116 and LS 123 cells, and only partially reduced in Colo 678 and HDC 8 cells (Fig. 3b). We similarly performed time-resolved NMR measurements in lysates of U0126-treated Colo 741, HCT 116 and LS 123 cells and found no detectable levels of Erk1/2 phosphorylation, even after prolonged peptide incubation (data not shown). Modification trajectories in U0126-treated Colo 678 and HDC 8 lysates revealed a reduction in Erk1/2 activities that was evidently reflected in the respective modification curves (Fig. 3c). We calculated that the apparent changes in phosphorylation rates corresponded to ~85% and ~55% reductions in kinase activities, respectively (Fig. 3d). These numbers were in good agreement

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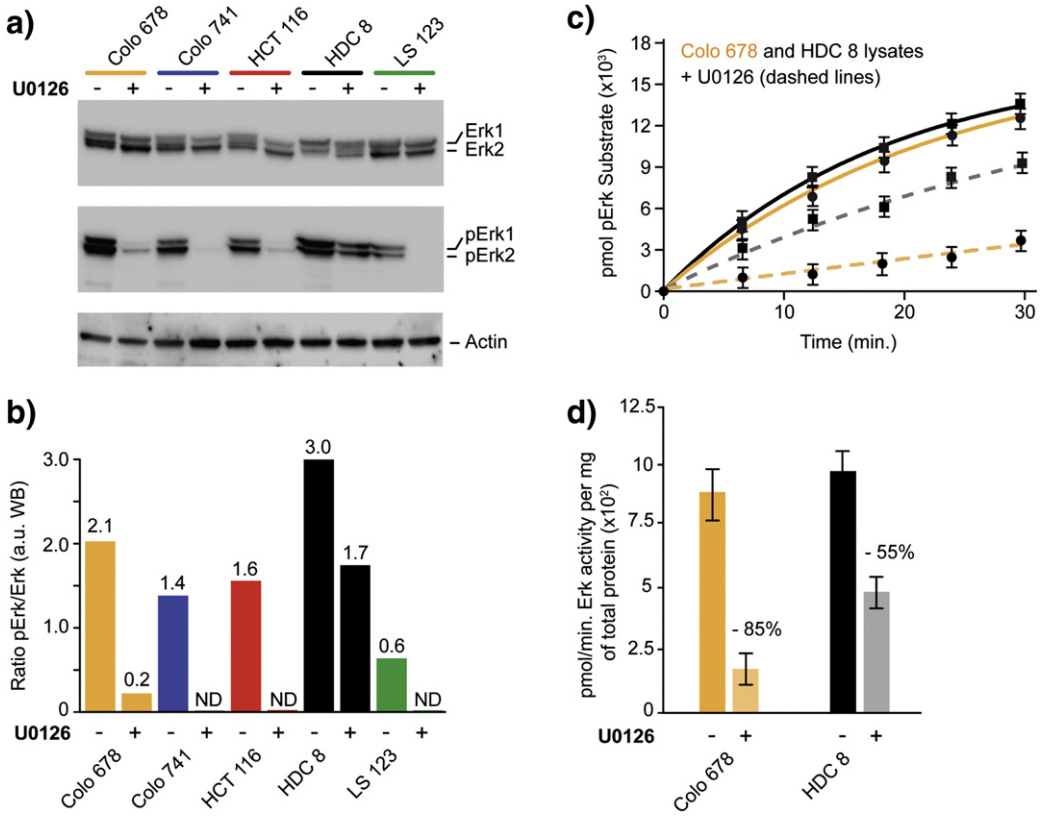

Fig. 3. Effect of U0126 treatment on Erk1/2 activities. a.) Western blot analysis of Erk1/2 inhibition by U0126. Five CRC cell lines with higher Erk1/2 activities were treated with (+) and without (−) 10 μM of the MEK inhibitor. Total Erk (top panel), phospho-Erk (middle panel) and actin loading control are shown. b.) Relative changes in phospho-Erk ratios between treated (+) and vehicle-treated (−) cells were determined by semi-quantitative Western blot analysis as in Fig. 2b. Cell line-specific ratios are indicated above each bar. c.) Comparison of time-resolved phosphorylation trajectories of Colo 678 (yellow) and HDC 8 (black) cell lysates after treatment with 10 μM U0126 (dashed lines) or DMSO 0.01% vehicle (solid lines). Data were measured in triplicates and are shown as means ± s.d. d.) Initial rates (pmol/min/mg lysate protein) of kinase activities for Colo 678 (yellow) and HDC 8 (black) cell lysates after treatment with 10 μM U0126, or DMSO 0.01% vehicle. Data show mean inhibitions of ~85% in Colo 678 and of ~55% in HDC 8 cells after U0126 treatment (n = 3, shown as means ± s.d.).

with the changes in phospho-Erk levels that we had determined by Western blotting (Fig. 3b).

4. Discussion and conclusions

In this study, we comparatively analyzed the ability of semi-quantitative Western blotting and time-resolved, high-resolution NMR spectroscopy to derive quantitative information about cellular Erk1/2 kinase activities in a panel of 10 different, patient-derived colorectal cancer cell lines. Our data suggest that both methods produce highly congruent results. Western blotting is well suited to quickly obtain qualitative and semi-quantitative information about cellular levels of active kinases, whereas NMR spectroscopy additionally provides quantitative information about individual phosphorylation rates, which can be determined accurately as units of picomol substrate turnover, per minute of lysate incubation, per milligram of total lysate protein. When *in vitro* phosphorylation reactions at defined enzyme activities are used as a reference, unit equivalents (U_{equ}) of endogenous kinase activities can be obtained directly. This kind of quantitative information is of particular importance when kinase inhibitor efficacies are to be determined.

Within the tested panel of cell lines, we confirmed high basal Erk1/2 activities in Colo 678, Colo 741, HCT 116 and HDC 8 lysates and intermediate/low levels of kinase activities in LS 123, CCK 81, HCA 46 and HRA 19 lysates. No detectable levels of Erk1/2 phosphorylation were found in HDC 57 and LS 174T lysates, neither by Western blotting, nor by NMR spectroscopy. Upon U0126 treatment, Erk1/2 activities were greatly diminished in Colo 741, HCT 116 and LS 123 lysates, whereas residual activities were measured in Colo 678 and HDC 8 lysates. In this regard, it is interesting to note that both Colo 678 and HDC 8 cells harbor the same activating mutation in p21/Ras, also called oncogenic KRAS *i.e.* Gly12Asp, which has previously been suggested to contribute to differences in response behaviors upon U0126 treatment in a number of cancer cells [19,28,37–39]. In the context of colorectal cancer, this may specifically affect the plasticity of other MAPK signaling pathways, whereby parallel c-Jun N-terminal (JNK), or p38 mitogen-activated protein kinase (p38 MAPK) cascades are activated to maintain cell proliferation under conditions of MEK inhibition [40]. Indeed Erk1/2, JNK and p38 MAPK share similar canonical substrate recognition motifs [33] and JNK and p38 MAPK activities are likely to be preserved under conditions of U0126 treatment, as there are few reported off-target effects for this drug [41]. By using a single NMR peptide reporter to measure cellular Erk1/2 activities, off-target effects of the inhibitor, or phosphorylation of the reporter by other cellular kinases such as JNK, or p38 MAPK are not monitored and cannot be excluded. In such cases, further NMR experiments with additional kinase reporters may be employed to provide a more detailed description of possible cross-inhibition effects and off-target phosphorylation behaviors. However, it has been argued previously that the presence of the 'DEF' specificity motif ameliorates possible off-target effects of this particular Erk1/2 sequence in functioning as a JNK, or p38γ/δ substrate [33]. Probably the most striking argument for the specificity of the measured residual Erk1/2 activity may come from the fact that congruent levels of active phospho-Erk were similarly detected by Western blotting. Together, these observations suggest a reduced inhibitory potential of U0126 in Colo 678 and HDC 8 cells for reasons that remain to be determined.

In summary, we analyzed Erk1/2 activities of a panel of 10 CRC cell lines by NMR and Western blotting and demonstrated the usefulness

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of high-resolution NMR measurements to quickly and accurately assess cellular kinase activities and their selective inhibitions by time-resolved readout schemes. The possibility to extend NMR-based phospho-detection approaches to other kinases in complex environments makes it an ideal tool for future *in situ* kinase activity studies.

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