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High-sensitivity measurements of multiple kinase activities in live single cells

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Summary

Increasing evidence has shown that population dynamics are qualitatively different from single cell behaviors. Reporters to probe dynamic, single cell behaviors are desirable, yet relatively scarce. Here we describe an easy-to-implement and generalizable technology to generate reporters of kinase activity for individual cells. Our technology converts phosphorylation into a nucleocytoplasmic shuttling event that can be measured by epifluorescence microscopy. Our reporters reproduce kinase activity for multiple types of kinases, and allow for calculation of active kinase concentrations via a mathematical model. Using this technology, we made several experimental observations that had previously been technically unfeasible, including stimulus-dependent patterns of c-Jun N-Terminal Kinase (JNK) and Nuclear Factor kappa B (NF- κ B) activation. We also measured JNK, p38 and ERK activities simultaneously, finding that p38 regulates the peak number, but not the intensity, of ERK fluctuations. Our approach opens the possibility of analyzing a wide range of kinase-mediated processes in individual cells.

Introduction

Ongoing efforts have shown that multicellular systems are best understood as a combination of heterogeneous single cell behaviors (Lahav et al., 2004; Shankaran et al., 2009; Tay et al., 2010). Intrinsic noise generates cell-to-cell variation that can be critical for cellular survival, development and differentiation (Balazsi et al., 2011). In response to changing environments, cells also generate complex signaling dynamics that encode relevant information for gene expression, proliferation or stress responses. Indeed, bulk population dynamics are often qualitatively different from single cell behaviors (Albeck et al., 2013; Cai et al., 2008; Mettetal et al., 2008; Purvis et al., 2012; Santos et al., 2007).

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Author Contributions

S.R. conceived the initial idea for KTR technology. S.R., B.T.B., S.C. and M.W.C. did the experiments. J.J.H. implemented the model and performed the mathematical analysis presented in Figure 4. S.R., J.J.H. and M.W.C. wrote the paper. M.W.C. supervised the project.

Dynamic single cell reporters are essential to study single cell biology, yet the number and type of molecular events that can be dynamically monitored in an individual cell is small. Such reporters have led to the successful measurement of metabolic state (Berg et al., 2009), transcription factor localization (Cai et al., 2008), second messenger concentration (Zhao et al., 2011) and even protein activities (Ting et al., 2001; Zhang et al., 2001) in live single cells. In the latter category, kinase activities are of particular interest. It has been estimated that 30% of cellular proteins are phosphorylated on at least one residue (Cohen, 2000; Ptacek et al., 2005). Kinases are known to regulate multiple and diverse biological functions, including the cell cycle, the innate immune response, development and cell differentiation (Ubersax and Ferrell, 2007).

To date, FRET sensors have been the most commonly used method to measure kinase activity dynamically in single cells (Fosbrink et al., 2010; Fritz et al., 2013; Ting et al., 2001; Zhang et al., 2001). Such sensors, based on Förster resonance energy transfer, have provided exciting new insights into how kinases are activated in single cells. However, FRET sensors have encountered challenges to their widespread adoption. They often have a low signal-to-noise ratio and do not accurately reflect the downregulation of the kinase, most likely because the closed conformation of the FRET sensor is highly stable and its phosphorylated sites can not be accessed by the phosphatases (Komatsu et al., 2011). Perhaps most significantly, they require two fluorescent proteins, and thus limit the number of outputs that can be observed simultaneously in any given cell. As signaling networks are known to be highly integrated, it would be highly desirable to measure a particular kinase activity together with multiple other activities or states in the same living cell.

Here we describe a novel strategy to generate genetically encoded biosensors for kinase activity named Kinase Translocation Reporters (KTRs). Our approach is based on the concept of converting phosphorylation into a nucleocytoplasmic shuttling event. Although phosphorylation-regulated nucleocytoplasmic translocation in certain specific and naturally occurring proteins has been reported (Gu et al., 2004; Komeili and O'Shea, 1999; Nardozzi et al., 2010), and in some cases, used as single-cell reporters (Hahn et al., 2009; Hao et al., 2013; Spencer et al., 2013), this concept has never before been exploited to make a general class of synthetic reporters.

We have extensively explored the sequence space and generated a set of rules that allowed us to generate reporters for multiple types of kinases, including MAP and AGC kinases. Our method uses a single fluorescent protein for each kinase and reliably recapitulates kinase activity dynamics – both the up- and down-regulation – in live single cells. Using KTR technology, we measured c-Jun N terminal Kinase (JNK) activity dynamics in single cells within the context of the innate immune signaling network, showing that different inputs are encoded with different dynamics. Finally, as a proof of principle of the multiplexing capabilities of KTR technology, we have dynamically measured JNK, p38 and ERK activities simultaneously in single living cells and gained new insight on how p38 regulates ERK signaling.

Results and discussion

A synthetic reporter of kinase activity based on nuclear translocation

Our initial goal was to construct a single color reporter for JNK activity translating phosphorylation into a localization change. JNK is a central Stress Activated Protein Kinase (SAPK) that coordinates multiple physiological processes like the stress response, innate immune signaling or development (Bagowski and Ferrell, 2001). We found that the transcription factor c-Jun had a potential NES nearby one of the two major JNK phosphorylation sites (specifically, the S73 site) (Figure 1A). We hypothesized that the phosphorylation state of this site could impact the nuclear export of c-Jun, leading to the conversion of a phosphorylation event into nucleocytoplasmic shuttling event.

Accordingly, we fused different c-Jun fragments to the fluorescent protein Clover (Lam et al., 2012), and analyzed whether JNK activity (stimulated by the protein synthesis inhibitor anisomycin) altered the localization of any of these constructs. We found that fragments of c-Jun without its C-terminal NLS have a weak but reproducible localization change from the nucleus to the cytoplasm (Figure 1B). Further fragment analysis revealed that a minimal peptide of 55 amino acids (Jun⁽²⁹⁻⁸⁴⁾), containing the JNK docking site, two phosphorylation sites (S63 and S73) and the identified NES, is sufficient to alter the localization of the fluorescent protein in the presence of anisomycin (Figures 1B, S1A and S1B). This translocation can be blocked with both a specific JNK inhibitor as well as a non-phosphorylatable mutant construct (S73A). We concluded that the shuttling event depends on JNK phosphorylating S73 (Figures 1B, S1C and S1D). Quantitative Western blot analysis revealed that expressing Jun⁽²⁹⁻⁸⁴⁾-Clover does not alter endogenous c-Jun phosphorylation by JNK and that both are phosphorylated and dephosphorylated with similar dynamics (Figures S2A and S2B). Moreover, c-Jun dependent gene expression remained unaltered in Jun⁽²⁹⁻⁸⁴⁾-Clover expressing cells (Figure S2C). These results suggested that phosphorylation near the c-Jun NES can enhance nuclear export, and that such phosphorylation is being converted into a localization change without changing the endogenous dynamics or consequences of JNK and c-Jun activities.

In order to improve the dynamic range of the observed localization change, we rationally designed a set of mutant variants to explore the sequence space of Jun⁽²⁹⁻⁸⁴⁾ (Figure 1C) (see Supplementary Information for a complete description and analysis). We reasoned that incorporating a negatively regulated NLS (Hahn et al., 2009; Komeili and O'Shea, 1999; Nardozi et al., 2010) together with the positively regulated NES, in the same engineered construct, could greatly increase the dynamic range. We began by exploring NES and NLS variants independently. We found that a suboptimal bipartite NLS (bNLS) can be negatively regulated by phosphorylation at either end of the linker region (see Supplementary Information for complete details). In contrast, negatively charged amino acids within the NES sequence increase its basal export activity, which is sufficient to explain why phosphorylation conditionally increases the export rate of the NES.

By combining a negatively phospho-regulated NLS with a positively phospho-regulated NES into a single construct, we were able to increase the dynamic range of nucleocytoplasmic transport by threefold (Figures 1D, S2D-S2F and Supplemental Video).

We designated this new construct the JNK Kinase Translocation Reporter (JNK KTR). Importantly, the localization change observed with JNK KTR was readily visible (Figure 1E) and quantifiable (Figure 1F). This localization change could be specifically inhibited by targeting JNK, but not p38 (Figures 1E, 1G, S3A and S3B). We also observed that a p38 inhibitor caused hyper-activation of JNK KTR (see below for further details). Furthermore, a non-phosphorylatable mutant (JNK KTR^{AA}) localized strictly to the nucleus, whereas a phosphomimetic mutant (JNK KTR^{EE}) was restricted to the cytoplasm, indicating that the negative charge introduced by the phosphorylation is responsible for the change in import and export activities (Figures 1E and S3A).

JNK KTR dynamics reflect JNK activity

To validate the dynamics of JNK KTR, we analyzed JNK activity in response to the cytokine IL-1 β as it induces a weaker and more physiological response than anisomycin. IL-1 β is a proinflammatory cytokine involved in the innate immune signaling response that activates multiple signaling pathways including the JNK, p38 and NF- κ B pathways (Weber et al., 2010). We compared JNK KTR dynamics with the most common techniques used to detect JNK activity: Western blot (WB) (Figure 2A), immunofluorescence (IF) (Figures 2B and 2C) and a FRET-based JNK reporter (JNKAR) (Figures 2D-2F), all in response to the same dose IL-1 β . Results showed that JNK and c-Jun phosphorylation measured by WB or IF strongly correlates with JNK KTR data at both population and single-cell levels (Figures 2A-2C and S3C-S3G). Importantly, the level of JNK KTR expression did not impact the measured cytoplasmic:nuclear (C/N) ratios of the JNK KTR (Figures S3H and S3I). Moreover, in contrast to the JNK KTR data, the JNKAR FRET reporter (Komatsu et al., 2011) measurements failed to capture the down-regulation of the kinase (Figure 2F).

To test the functionality of KTR technology in other cell types, we also expressed JNK KTR in HeLa, HEK-293 and RAW 264.7 cells. In all cell types, anisomycin induced an expected change in localization (Figure S3J). Taken together, these results suggest that JNK KTR translocation reliably represents JNK activity dynamics and can be successfully implemented in a diverse panel of cell lines.

KTR technology can be applied to multiple types of kinases

We hypothesized that the design principles we discovered for JNK KTR could be used to generate synthetic single cell reporters for other kinases. To test this hypothesis we followed two different strategies, depending on the mechanism of specificity for each kinase. For kinases that operate through distant docking sites (e.g., MAP Kinases), we exchanged the JNK docking site with other docking sites found in substrates for other kinases (Chang et al., 2002) (i.e. Mef2C for p38 and Elk1 for ERK). Using this approach, we constructed functional KTRs for ERK and p38 (Figures 3A-3C and S4A-4C). The KTRs are much more sensitive than the localization of the respective kinase, as we verified with experiments tracking MAP Kinase localization (Compare Figure 3 with Figures S4D-4G).

In contrast to MAP kinases, AGC kinases target substrates based on the context of the phosphorylation site. We therefore decided to adapt our KTR construction, mutating a naturally occurring substrate to introduce a bNLS and a NES without interfering with the

critical residues for phosphorylation. Using this approach, we converted a fragment of the PKA substrate HDAC8 (which has no localization signals naturally) into a dynamic single cell reporter (Figure 3D). In each case, the KTR was both responsive and specific to the expected stimulus and inhibitor (Figures 3 and S4C).

These results indicate that KTR technology can be applied to other kinases simply and with minimal optimization. Moreover, recently reported mammalian CDK2 and yeast PKA kinase reporters (Hao et al., 2013; Spencer et al., 2013) have the same naturally occurring arrangement of bNLS, NES and phosphorylation sites in their sequences. As a result, the KTR strategy has been successful for three classes of kinases (MAPKs, CDKs and AGC kinases), suggesting that the technology is generalizable.

Calculating the single-cell concentration of active kinase via a mathematical model

The KTR technology offered an exciting new possibility to estimate, quantitatively, the concentrations of active kinase in individual cells. To do this, we used the chemical reactions of the KTR (phosphorylation, dephosphorylation, import, and export) to construct a mathematical model of the system (see Supplementary Equations for details). The model describes the quantitative relationship between the dynamics of active kinase and the dynamics of the KTR. We then calibrated the model specifically for the JNK KTR (Figure 4A). To estimate the import and export rate constants of the unphosphorylated and phosphorylated states of the KTR, we treated cells expressing the non-phosphorylatable JNK KTR^{AA} or the phospho-mimetic JNK KTR^{EE} construct with the nuclear export inhibitor leptomycin B (Figures S5A and S5B).

Importantly, a similar approach to estimate concentrations of active kinase in single cells could not be used with FRET sensors. This is because no method is currently known for producing a constitutively phospho-mimetic FRET sensor, and without such a control it is impossible to know the fraction of active wild-type sensor. As a result, critical parameters of the system remain unspecified. In contrast, the phospho-mimetic construct required for KTR models is straightforward to implement, and leads to novel calculations of active kinase concentration.

In addition to providing the import and export rate constants, the JNK KTR^{AA} and the JNK KTR^{EE} constructs also give us the expected C/N ratio of the wild-type JNK KTR, if 0% or 100% of the wild-type JNK KTR molecules in a cell were phosphorylated. For a KTR to be most useful and for accurate estimation of active kinase concentrations, the KTR should be responsive to the range of active kinase concentrations under baseline and stimulated conditions. Based on our parameterization with the JNK KTR^{AA} and JNK KTR^{EE} constructs, we calculated the relationship between active JNK concentration and steady state C/N ratio (Figure 4B). Interestingly, JNK KTR reaches only about 80% saturation in response to IL-1 β , TNF- α , and LPS. We conclude that the responsive range of the JNK KTR is almost ideally matched to the range of active JNK induced by physiological stimuli.

We noticed that all four of the non-phosphorylatable JNK KTR mutants (JNK KTR^{AA}, JNK KTR^{AE}, JNK KTR^{EA}, and JNK KTR^{EE}) exhibited a similar degree of cell-to-cell variability in the C/N ratio (Figure S5C). In addition, the C/N ratios of two different non-

phosphorylatable mutants in the same cell were highly correlated (Figures S5D and S5E). This suggests that variability in the C/N ratio of the wild-type JNK KTR and all other KTRs is caused not only by variability in levels of active kinase, but also by noise in the import and export rates. To distinguish between these two sources of variability in individual cells, we generated a cell line expressing both JNK KTR-Clover and an in-cell control construct, JNK KTR^{AE}-mRuby2 (Lam et al., 2012) (Figures 4C, S5D and S5E). Although this construct cannot be phosphorylated, the bNLS and NES signals are still functional. As a result, the C/N ratio variability is caused strictly by differences in general import and export rates.

Surprisingly, although the C/N ratios of two non-phosphorylatable mutants (JNK KTR^{AE} and JNK KTR^{EA}) correlated well in single cells, the C/N ratios of JNK KTR^{AE} and wild-type JNK KTR correlated very weakly (Figures S5D and S5E). This observation suggests that the variability of the wild-type JNK KTR is primarily caused not by noise in import and export rates, but by variability in basal JNK activity. A similar finding has been made in yeast, where basal MAP kinase activity has been shown to accelerate the response time of the High Osmolarity Glycerol (HOG1) pathway (Macia et al., 2009). However, little is known about this phenomenon in mammalian MAPK pathways.

We also observed the dynamics of the wild-type JNK KTR and in-cell control in response to IL-1 β . Our subsequent analysis showed that nuclear import and export noise is negligible compared to the localization change induced by JNK activation (Figure 4C). We then used the model to estimate the concentration of active kinase over time in single cells (Figures 4D, 4E and S5F-S5H, see Supplementary Equations for details). Our analysis suggests that the baseline concentration of active JNK is between 0 and 15 nM, and that upon treatment with IL-1 β , active JNK concentration peaks between 20 and 80 nM (Figure S5G). Consistent with the data from Western blots (Figures 2A, S2A, S2B S3C and S3D), the C/N ratio of the JNK KTR closely tracks the estimated concentration of active JNK (Figures 4D and 4G).

Taken together, these results indicate that for most applications, the C/N ratio of the wild-type KTR, even without an in-cell control, is a sensitive and accurate proxy for kinase activity. However, the in-cell control remains useful for generating more accurate calculations of kinase activity in single cells. In addition, the in-cell control can also be used to compare ratios between cell lines and to calibrate the system, when a stimulus affects general import or export rates. Finally, the simplicity of the KTR model structure, as well as the straightforward, standardizable method for model parameterization, all suggest that the method could be readily applied to other KTRs to obtain active kinase concentrations in single cells.

KTR technology applied to novel biological measurements

Finally, we were anxious to use KTR technology to perform novel measurements – in particular, multifactorial analysis of live single cells. We first investigated how JNK and the transcription factor NF- κ B act in a coordinated fashion to generate specificity in innate immune signaling. Accordingly, we introduced JNK KTR-mCerulean3 (Markwardt et al., 2011) into previously described RelA^{-/-} 3T3 p65-DsRed H2B-EGFP cells (Tay et al., 2010) and isolated a clonal line. We then stimulated cells with three different innate immune inputs

(TNF- α , IL-1 β and LPS), spanning three orders of magnitude in concentration, and measured p65 and JNK KTR nucleocytoplasmic translocation dynamics in each individual cell (Figures 5A, 5B and S6). Translocation of JNK KTR (indicating activation of JNK) was transient in most cells, with a time to peak that correlated well with the time to peak of nuclear p65 (Figures 5B and 5C). In particular, for both JNK KTR and p65, the timing of the response was slower for LPS than for either TNF- α or IL-1 β . In addition, in response to TNF- α and IL-1 β , JNK KTR translocation tended to precede p65 translocation by 6–12 minutes – a finding made possible by the fine time resolution offered by KTR technology.

At the single cell level, we found that the JNK response varied between cells, most notably in the basal state (i.e., unstimulated cells growing in 1% fetal calf serum), as well as during down-regulation of activity (Figures 5D and S6). We also observed that JNK KTR oscillated in some, but not all, cells upon stimulation with TNF- α or IL-1 β (Figures 5D, S7A and S7B). These findings would have been very difficult or impossible to obtain using population assays, which blend together asynchronous cellular responses, or using the JNK FRET sensor, which is too slow in reporting down-regulation of JNK (Figure 2F).

Detailed 2-dimensional analysis of the single cell response – taking JNK and p65 simultaneously into account – showed that the amplitude and the time to the first peak of JNK and p65 are differently used to encode dose at the single cell level. For example, stimulation with either TNF- α or IL-1 β yields JNK and p65 peak intensities that are positively correlated with one another across ligand concentration (Figures 5E and 5F). In contrast, cells respond to LPS with timing that is independent of concentration and without correlation between peak JNK and p65 amplitudes (Figure 5G).

The correlation between the timing of the response for JNK and p65 was different. In this case, the time to the first peak of activity for both reporters seemed to be correlated and dose dependent under both LPS and IL-1 β stimulation conditions, but not for TNF- α (Figures 5H-5J). These data strongly suggest that multiple innate immune inputs can be differently interpreted by the same signaling network by generating different dynamic patterns of key effectors.

Finally, as MAP kinases are known to regulate each other, we explored how the three MAP kinases JNK, ERK and p38 dynamically interact in single cells. Accordingly, we generated a cell line expressing 4 different fluorescent proteins (4C cell line); ERK KTR-Clover, p38 KTR-mCerulean3, JNK-KTR-mRuby2 and H2B-tdiRFP (Filonov et al., 2011) (Figure 6A). This cell line allowed us to simultaneously measure the activity dynamics of all 3 MAP kinases in single cells over entire cell cycles (Figure 6B). Our data confirmed that ERK activity in NIH 3T3 cells fluctuates under basal conditions with dynamics similar to those previously described in MCF-10 cells (Albeck et al., 2013) (Figure 6B). Interestingly, previous studies (Shankaran et al., 2009), showed periodic oscillations of ERK localization upon EGF stimulation. However, in NIH 3T3 cells, the basal fluctuations are visible only with ERK KTR, as ERK localization itself does not oscillate (Figures S4F and S4G). This observation highlights the utility of using KTR technology, as a more direct assessment of kinase activity.

Moreover, our data suggests that the basal activity of both ERK and p38 correlates with the cell cycle. In particular, we found that ERK fluctuations are more frequent in the beginning of the cell cycle, whereas basal activation of p38 tends to be higher towards the end of the cell cycle (Figure 6C).

Next, we considered the interactions between the kinases. Using experiments at the population level, others have previously described the inhibition of ERK by p38 (Jensen et al., 2013; Westermarck et al., 2001). This inhibition is important because p38 has been shown to regulate cell cycle progression (Joaquin et al., 2012). Therefore, a better understanding of how p38 regulates ERK can provide some insight into how SAP kinases regulate the cell cycle. To study this interaction at the single cell level, we measured the dynamics of ERK, p38 and JNK activation in response to anisomycin. Upon anisomycin treatment, activation of JNK was more transient than activation of p38 (Figure 6D).

Moreover, after treatment with anisomycin, inhibition of JNK only affected JNK, whereas inhibition of p38 caused an increase in both JNK and ERK activities. These results suggest that p38 activity represses both the ERK and JNK pathways (Figure 6E).

Furthermore, the single cell analysis revealed that p38 inhibits ERK fluctuations, by reducing the number of peaks rather than their intensity (Figures 6F, S7C and S7D). Given that the time between ERK fluctuations has been shown to encode proliferation signals (Albeck et al., 2013), ERK inhibition by p38 provides a plausible mechanism for cell cycle regulation upon stress. These observations demonstrate that KTR technology has an unprecedented potential to enable the generation of multidimensional datasets.

In summary, KTR technology is an easy-to-implement, generalizable strategy for developing reporters of kinase activity in live cells. We have shown how KTRs enable measurement of single cell activation dynamics of multiple signaling pathways simultaneously and with high time resolution. Furthermore, construction and parameterization of a mathematical model to represent KTR activity is both mathematically and experimentally straightforward, and enables the estimation of active kinase concentrations. While we emphasize that FRET sensors remain powerful tools in this area, particularly in their capacity to elucidate the subcellular distribution of kinase activity, the multiplexing capabilities of KTR technology open the possibility of measuring single cell dynamics of signaling networks rather than signaling pathways by relatively simple and cost-effective means. We anticipate that this strategy will contribute significantly to our understanding of the mechanisms underlying dynamic signal transduction in individual living cells.

Experimental Procedures

Cell line generation

Engineered sequences were generated by Gibson assembly (Gibson et al., 2009) into pENTR vectors, then sequenced and transferred to pLenti DEST vectors using Gateway® Cloning Technology. Lentiviral vectors were transfected into 293FT cell line together with 3rd generation packaging plasmids. 48 hours later, packaged lentivirus were used to infect

3T3s. 24 hours post infection, drug selection was added (Puromycin 1 μ g/ml, Blasticidin 1 μ g/ml or Hygromycin, 50 μ g/ml (InvivoGene)). 3–5 days later cells were imaged to confirm fluorescent protein expression.

Cells were cultured in DMEM (Invitrogen) supplemented with 2 mM L-glutamine (Gibco), 100 U/ml penicillin, 100 μ g/ml streptomycin (Gibco), and 10% fetal bovine serum (Omega Scientific) or fetal calf serum (Colorado). Derivates of RelA^{-/-} 3T3 were cultured in 10% FBS while derivates of NIH3T3 were cultured in 10% FCS.

Imaging

Cells were seeded at 7,000 cells/well onto glass coverslip 96-well plates (Nunc) coated with 10 μ g/ml fibronectin (Millipore). Next day, media was changed to imaging media (DMEM without phenol red, with 1% FBS or FCS, see above) at least one hour prior to imaging. Cell lines without a genetically encoded nuclear marker were incubated with 50 ng/ml Hoechst (Sigma) to assist nuclear segmentation. Cells were imaged with a Nikon Eclipse Ti fluorescence microscope controlled by Micromanager (Edelstein et al., 2010). Temperature (37°C), CO₂ (5%), and humidity were held constant during the experiments. 5 blank positions (media only) were imaged for every experiment and used to flat field the rest of the images.

Image quantification

Flatfielding and image registration were performed using custom Matlab software. Segmentation and object quantification was performed with CellProfiler (Kamentsky et al., 2011). Nucleus and a 5-pixel wide cytoplasm ring (cytoring) were segmented for each cell, using Hoechst or H2B fused fluorescent proteins, and quantified in each channel. Tracking and manual curation were performed using custom Matlab and Python software. Median intensity values for each object were extracted and used to calculate ratios. KTRs cytoring over nuclear intensities were calculated to intuitively reflect activity. Nuclear p65 intensity was normalized using the cytoplasmic intensity under basal conditions. For Figure 1D and S2D-F, basal and 30 min post anisomycin cytoplasmic over nuclear ratio was quantified. Dynamic range was calculated for each cell as (anisomycin-basal)/basal C/N ratio and normalized by the mean wild type dynamic range. For heat maps shown in Figures 5B, 6C and 6E each trace was normalized between 0 and 1. When more than one KTR was compared (Figures 6D and 6E), each trace was normalized using as minimum and maximum the inhibitor and stimulated mean ratios obtained in Figure 3.

FRET analysis

For FRET JNKAR experiments, H2B-mRuby2 3T3 cells were transfected with JNKAR1EV plasmid (Komatsu et al., 2011) (kindly donated by Dr. Kazuhiro Aoki) 24 hours prior to imaging. The mRuby2 image was used to segment the nuclei. YFP, CFP and FRET images were taken and corrected for signal bleed through as previously described (Feige et al., 2005). FRET image was divided by CFP image and the resulting image was used to quantify FRET signal in the cytoplasm.

Immunofluorescence

Cells were seeded at 12,000 cells/well onto glass coverslip 8-well imaging slides (Lab-teck) coated with 10 µg/ml fibronectin (Millipore). Next day, media was changed to imaging media (DMEM without phenol red, with 1% FBS or FCS) at least one hour prior to stimulation. Cells were stimulated for indicated times, fixed with 4% paraformaldehyde and permeabilized with PBS 0.5% TritonX100. Blocking and antibody incubations were done in PBG buffer (PBS, 0.2% cold water fish gelatin (Sigma), 0.5% BSA (Sigma)). Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb (4668, Cell Signaling) and Phospho-c-Jun (Ser63) II Antibody (9261, Cell Signaling) were used as primary antibodies. Goat anti-Rabbit (Cy5 ®) (ab97077, abcam) secondary antibody was used to detect primary antibodies. 10ng/ml DAPI was used to stain the nuclei for segmentation purposes. 10 multichannel images (DAPI, mClover and Cy5) were taken for each IF condition. Images were segmented as described above and median intensities for mClover and Cy5 channels were obtained. JNK phosphorylation was calculated as the sum of nuclear and cytoplasmic intensities whereas JUN phosphorylation corresponded to nuclear Cy5 intensity.

Quantitative Western Blotting

Cells were grown to 80% confluence onto 6-well plates, media was changed to 1% FBS or FCS media for one hour prior to stimulation. After stimulation, cells were harvested and proteins extracted in 150 µl of RIPA buffer containing protease and phosphatase inhibitors. Proteins were resolved in 4–12% gradient SDS PAGE gels (BioRad) and transferred into low autofluorescence PVDF membranes (Millipore). Blocking and antibody incubations were done with LICOR Blocking buffer. Primary antibodies used were: Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit (4668, Cell Signaling), Phospho-c-Jun (Ser63) Rabbit (9261, Cell Signaling), Phospho-c-Jun (Ser73) Antibody (9164, Cell Signaling), JNK1 (2C6) Mouse (3708, Cell Signaling), c-Jun (5B1) Mouse (ab119944, abcam) and GFP Rabbit (ab290, abcam). Membranes were scanned using a LICOR scanner and quantified using ImageStudioLite software.

Quantitative gene expression analysis

Cells were grown to 80% confluence onto 6-well plates, media was changed to 1% FBS or FCS media for one hour prior to stimulation. After stimulation, cells were harvested and RNA was extracted using RNeasy Mini Kit (74104 Quiagen). cDNA was produced using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (4374966, Applied Biosystems). qPCR was performed using Taqman gene expression assay (Life Technologies).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

Kinase substrates were engineered to convert phosphorylation to localization changes.

Genetically encoded biosensors are named Kinase Translocation Reporters (KTR)

A mathematical model calculates active kinase concentrations from KTR data.

KTRs enable the generation of unprecedented multidimensional data in single cells.

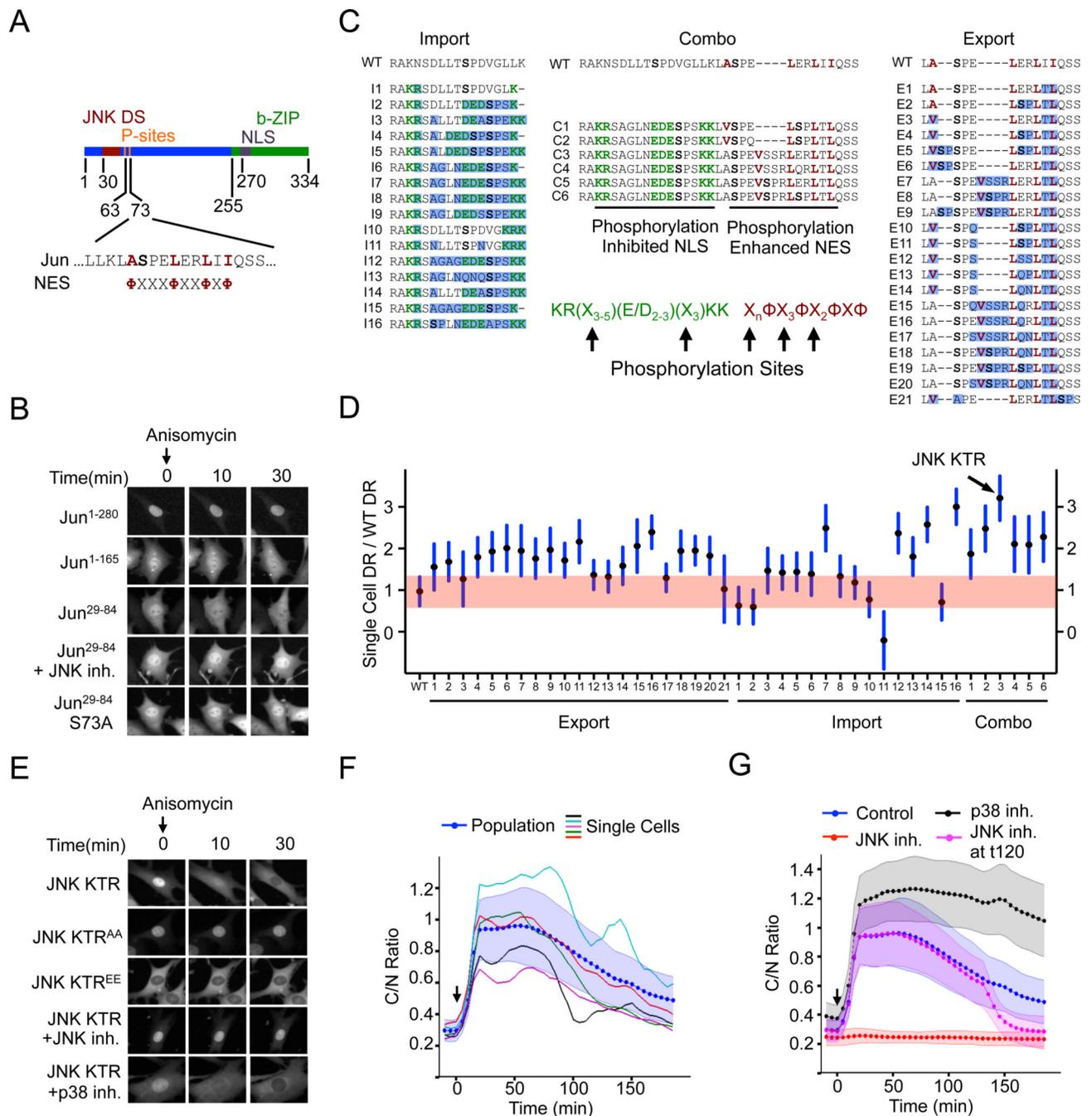


Figure 1. Development of Kinase Translocation Reporter (KTR) technology

(A) Schematic representation of mouse c-Jun protein showing JNK docking site (JNK DS), phosphorylation sites (P-sites), basic leucine zipper DNA binding domain (b-ZIP) and nuclear localization signal (NLS). Consensus nuclear export signal (NES, Φ indicates hydrophobic amino acid) and serine 73 sequence context are shown. (B) 3T3 cells expressing indicated c-Jun fragments fused to Clover were stimulated with anisomycin (50 ng/ml) and imaged at indicated time points. Where indicated (+ JNK inh.), cells were preincubated for 45 minutes with 10μM JNK inhibitor VIII. Representative cells are shown

for each construct or condition. (C) Engineered protein sequences aligned to the wild type c-Jun sequence. Residues involved in nuclear import, nuclear export or phosphorylation are shown in green, purple or bold respectively. Changes from wild type sequence are highlighted in blue. (D) 3T3 cells expressing the Jun²⁹⁻⁸⁴-Clover variants shown in panel c were treated with anisomycin (50 ng/ml) and imaged over time. Dynamic range was calculated for each cell as (anisomycin-basal)/basal C/N ratio and normalized by the mean wild type dynamic range. Pink band corresponds to the observed wild type dynamic range variation. Data is represented as the mean \pm standard deviation (SD) from more than 50 cells for each variant. (E) 3T3 cells expressing JNK KTR (WT or with phosphoresidues mutated to alanine, AA, or glutamate, EE) were stimulated with anisomycin (50 ng/ml) and imaged at indicated time points. Where indicated (+ JNK inh. or + p38 inh.), cells were preincubated for 45 minutes with 10 μ M JNK inhibitor VIII or 10 μ M SB203580. Representative cells are shown for each construct or condition. (F) 3T3 JNK KTR cells were stimulated with anisomycin (50ng/ml), imaged and quantified as described in Methods. C/N refers to cytoplasmic over nuclear intensities. Data is represented as the mean \pm SD from 158 cells. (G) 3T3 JNK KTR cells were pretreated with 10 μ M JNK inhibitor VIII or 10 μ M SB203580 (JNK inh. and p38 inh. respectively) and stimulated with anisomycin (50 ng/ml). When indicated (JNK inh. at t120) cells were treated with 10 μ M JNK inhibitor VIII. Images were quantified as described in Methods. Data represents mean \pm SD from more than 100 cells. See also Figures S1 and S2

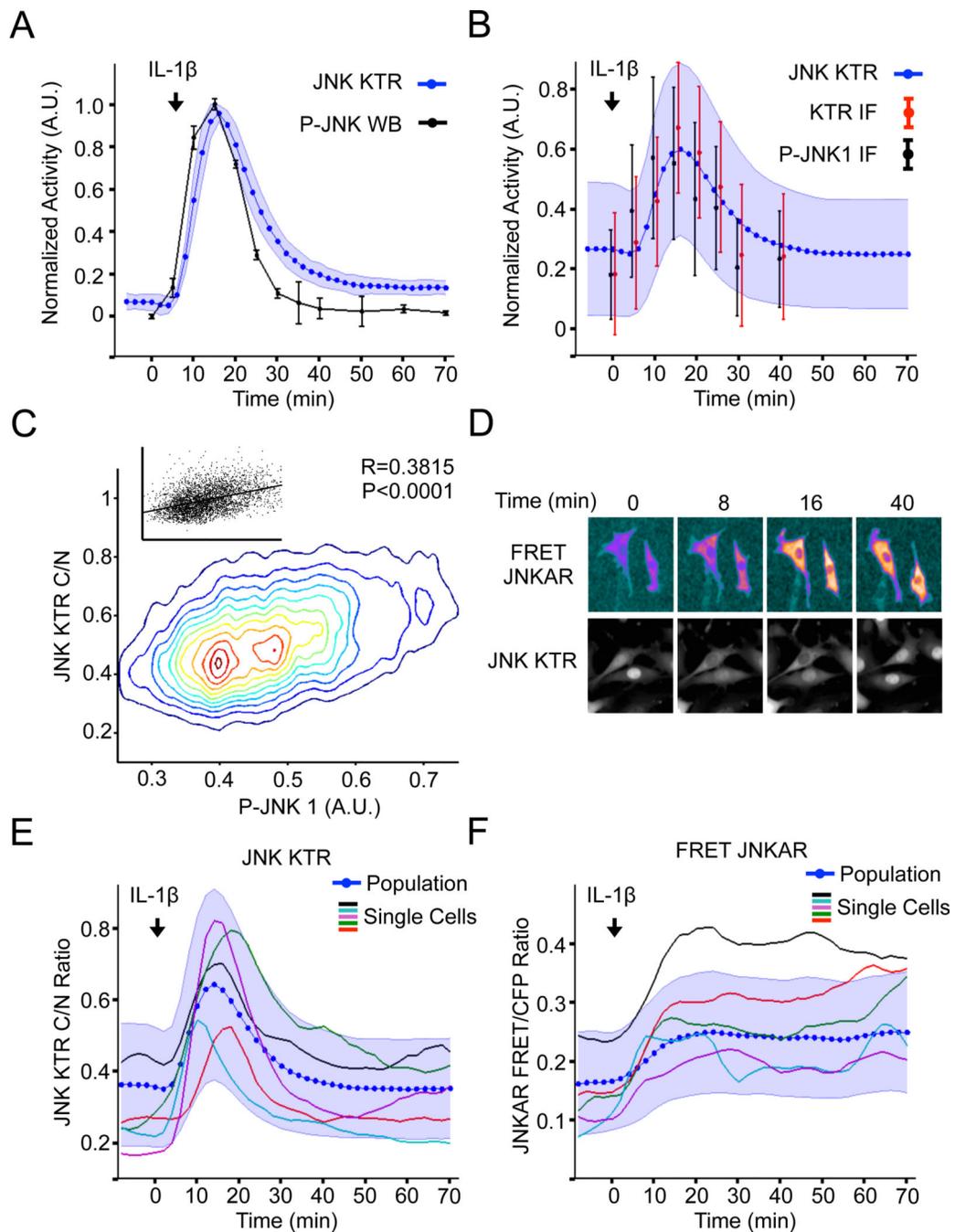


Figure 2. JNK KTR dynamics validation

(A) JNK KTR cells were stimulated with IL-1 β (1 ng/ml), imaged and quantified as described in Methods. Three independent experiments were performed resulting in 980 single cells measured. KTR data represents the mean \pm SD from the 3 experiment means (averaged to mimic *in silico* WBs). P-JNK WB data is calculated as the fraction of phosphorylated over total and represents the mean \pm SD from 3 independent experiments. All datasets were normalized between 0 and 1 for comparison. (B) 3T3 JNK KTR cells were stimulated with IL-1 β (1 ng/ml) for indicated times and fixed with 4% PFA for quantitative

IF analysis. 10 images were taken for each time point and quantified as described in Methods. For each cell C/N KTR ratio (red) and phospho-JNK intensity (black) were determined. All datasets were normalized between 0 and 1 for comparison. Data represents the mean \pm SD from more than 500 cells for each time point obtained from 2 independent experiments. IF data is overlaid on the dynamic JNK KTR dataset (blue) used in Figure S6. Note that in this case, JNK KTR dynamic data represents the mean \pm SD from all individual cells (n=980), obtained in 3 independent experiments. (C) IF data obtained in Panel b represented as contour scatter plot. Single cell JNK KTR ratio and phospho-JNK intensity from all time points are shown. Contour color represent areas of increasing data point density. Raw scatter plots fitted to a linear regression are shown together with Pearson correlation value R and P values. (D) 3T3 JNK KTR cells and 3T3 expressing FRET JNK Activity Reporter (JNKAR) were stimulated with IL-1 β (1 ng/ml) and imaged at indicated time points. FRET image was calculated as described in Methods. Representative cells are shown for each technique over time. (E) 3T3 JNK KTR single cell dynamic data used in Figure S7. 5 randomly selected single cell traces are shown. (F) FRET JNKAR cells were stimulated with IL-1 β (1 ng/ml) imaged and quantified as described in Methods. Data represents the mean \pm SD from all individual cells (n=67) obtained from 2 independent experiments. 5 randomly selected single cell traces are shown. See also Figure S3.

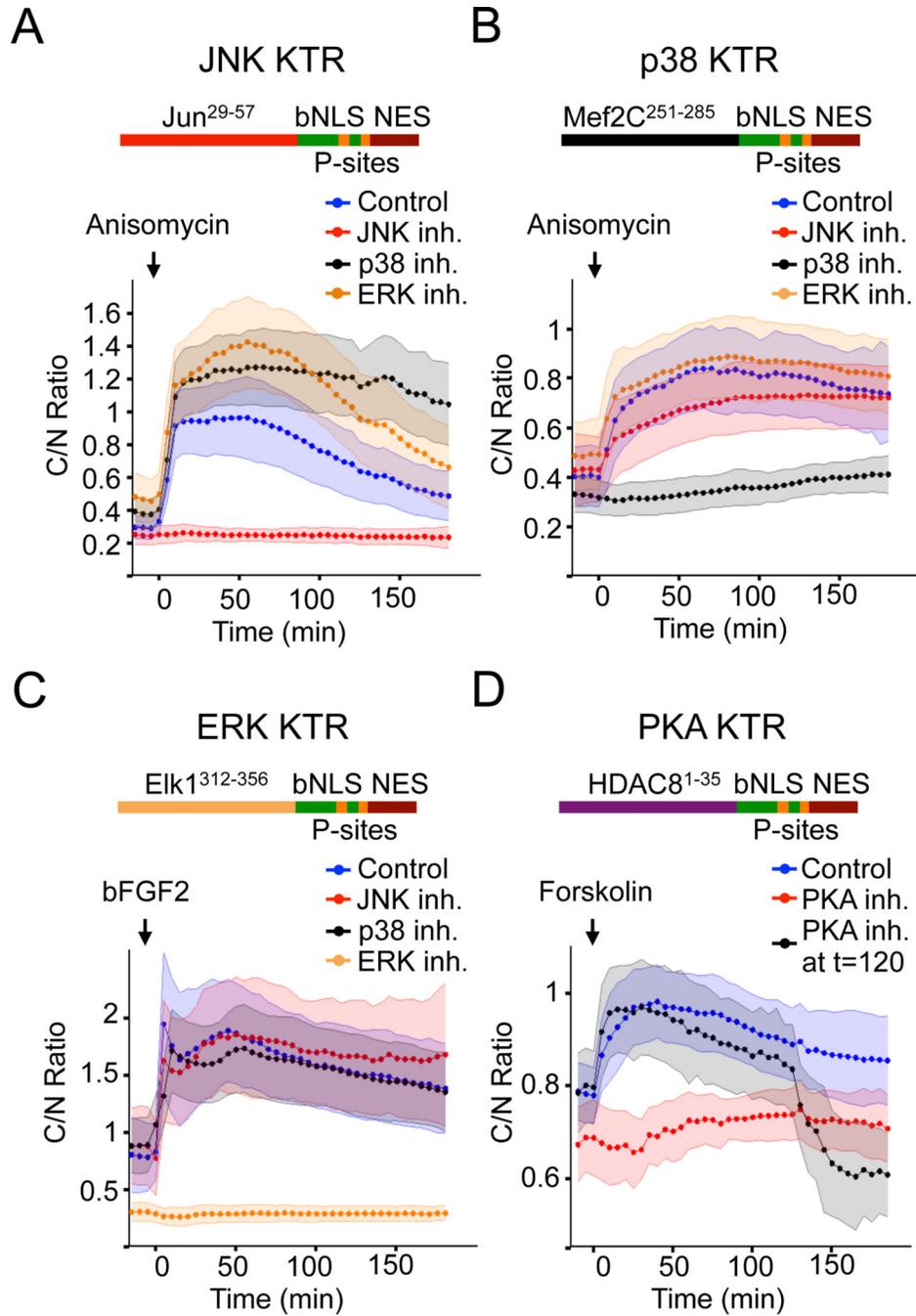


Figure 3. KTR technology is generalizable to other kinases

(A) 3T3 cells expressing JNK KTR were stimulated with anisomycin (50 ng/ml), imaged and quantified as described in Methods. Schematic representation of the engineered reporter is shown for all panels. Data represents the mean \pm SD of more than 100 cells. Cells were preincubated with media (Control), 10 μ M JNK inhibitor VIII (JNK inh.), 10 μ M SB203580 (p38 inh.) or 100 nM PD032591 (ERK inh.). (B) 3T3 cells expressing p38 KTR were stimulated with anisomycin (50 ng/ml), imaged and quantified as described in Methods. Cells were pretreated or not with kinase inhibitors as in panel a. (C) 3T3 cells expressing

ERK KTR were stimulated with basic fibroblast growth factor-2 (bFGF2, 100 ng/ml), imaged and quantified as described in Methods. Cells were pretreated or not with kinase inhibitors as in panel a. (D) 3T3 cells expressing PKA KTR were stimulated with the PKA activator forskolin (10 μ M) imaged and quantified as described in Methods. Cells were pretreated with the specific PKA inhibitor H89 (30 μ M) (+ PKA inh.) or not (Control). Where indicated H89 (30 μ M) was added at time 120 minutes (+ PKA inh. at t=120). See also Figure S4.

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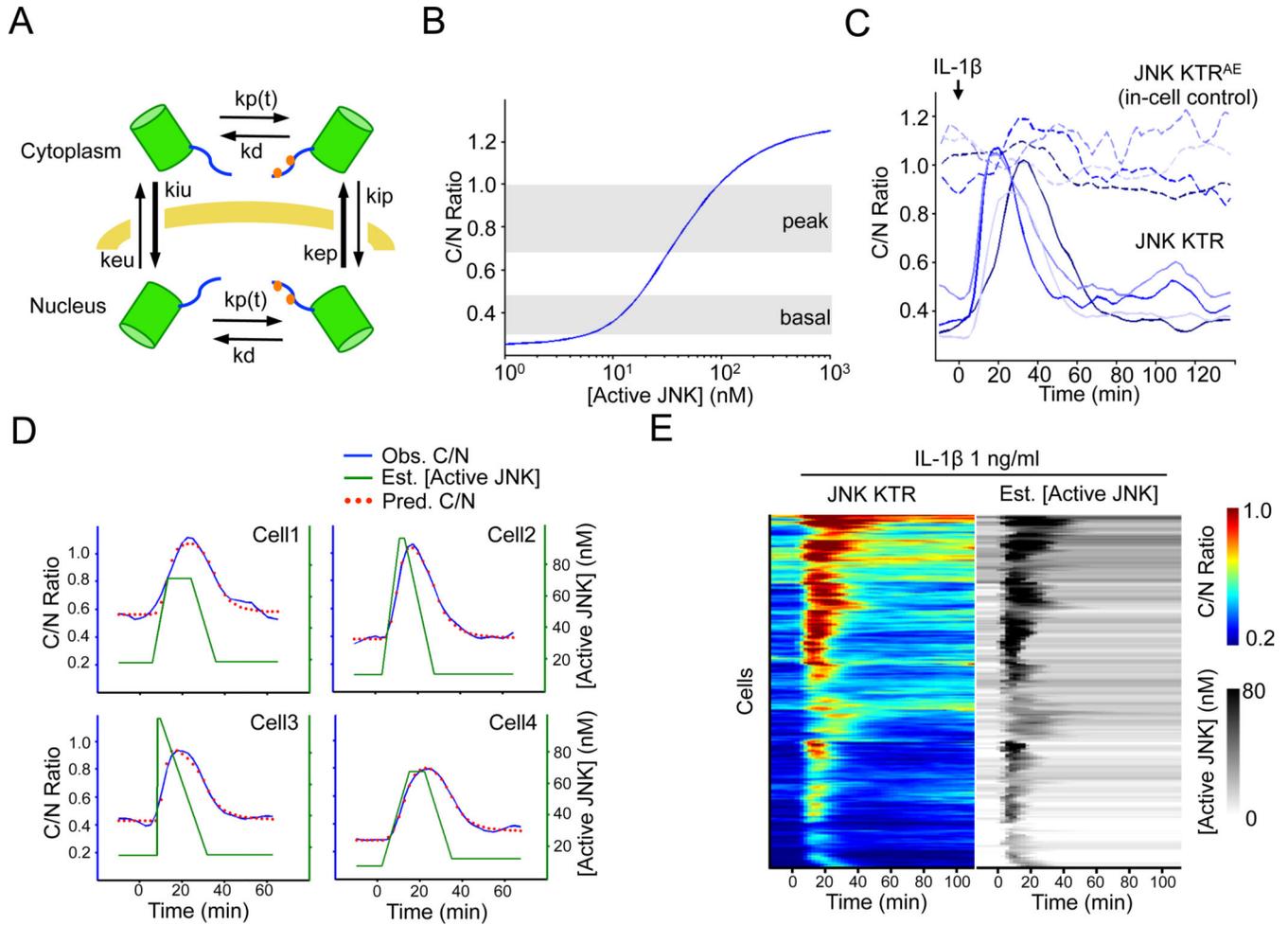


Figure 4. KTR modeling allows estimating active JNK kinase concentration in single cells
 (A) Schematic representation of the mathematical model, see Supplementary Equations for a complete description of the parameters depicted in this figure. (B) Model based relationship between steady state C/N JNK KTR ratio and concentration of active JNK. Grey areas indicate the range of C/N ratios observed (at basal or peak times) when cells are stimulated with 1 ng/ml IL-1 β (data represents the distribution of C/N ratio between 25th and 75th percentiles). (C) 3T3 cells expressing JNK KTR Clover (solid line) and JNK KTR^{AE}mRuby2 (super index indicates A and E mutations on the phosphorylation sites) (dashed line) were stimulated with IL-1 β (1 ng/ml), imaged and quantified as described in Methods. The activation dynamics for each protein in four randomly chosen individual cells are shown. (D) Cells were treated as in Panel c. Corrected C/N ratio (blue line), estimated active kinase concentration obtained as described in Methods (green line) and fitted C/N ratio using the estimated concentration of active kinase for that particular cell (red dots) are shown for four randomly chosen individual cells. (E) Cells were treated as in Panel b. Each row of the heat map corresponds to an individual cell. Corrected JNK KTR C/N ratio and estimated concentration of active JNK are displayed in tandem for each cell. No normalization was used for the corrected JNK KTR C/N ratio. Heat maps represent 195 cells and two independent experiments. See also Figure S5.

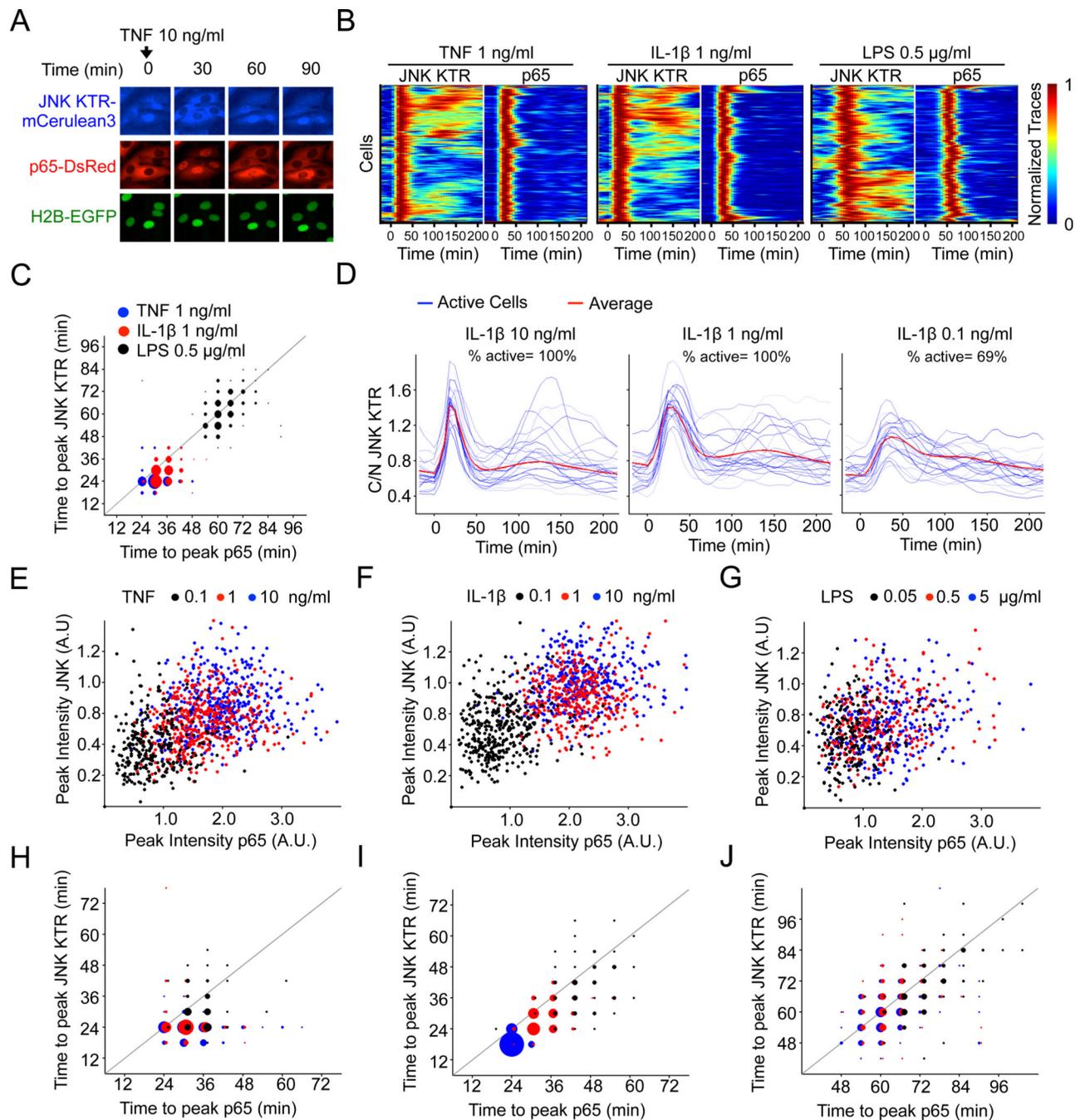


Figure 5. KTR technology provides a new dimension for analyzing the innate immune signaling network

(A) RelA^{-/-} 3T3 cells expressing H2B-EGFP and p65-DsRed were infected with JNK KTR-mCerulean3 and a clonal cell line was isolated (cell line 3B8). 3B8 cells were stimulated with TNF- α (10 ng/ml) and imaged at indicated times. Representative cells are shown for each channel over time. (B) Clonal line 3B8 was stimulated with the indicated concentration of TNF- α , IL-1 β or LPS, imaged and quantified as described in Methods. Each row of the heat map corresponds to a single cell; JNK KTR and nuclear p65 (p65) dynamics are displayed in tandem for each cell (>300 cells total, three independent experiments), with

each trace normalized between 0 and 1 for each reporter. (C) The amount of time elapsed at the first peak of activity for JNK KTR and p65-dsRed. Data comes from Panel b, and dot size represents the number of cells. Gray line indicates $x=y$. (D) Clonal line 3B8 was stimulated with indicated concentrations of IL-1 β , imaged and quantified as described in methods. 25 randomly chosen cells (blue) and the average of all cells (>300) (red) are shown. (E-J) 3B8 cells were stimulated with indicated concentrations of TNF- α (E and H), IL-1 β (F and I) or LPS (G and J). Correlations between JNK KTR and p65 peak amplitude (E, F and G) or time to first peak (H, I and J) are shown. Dot size represents the number of cells. Gray line indicates $x=y$. See also Figures S6 and S7.

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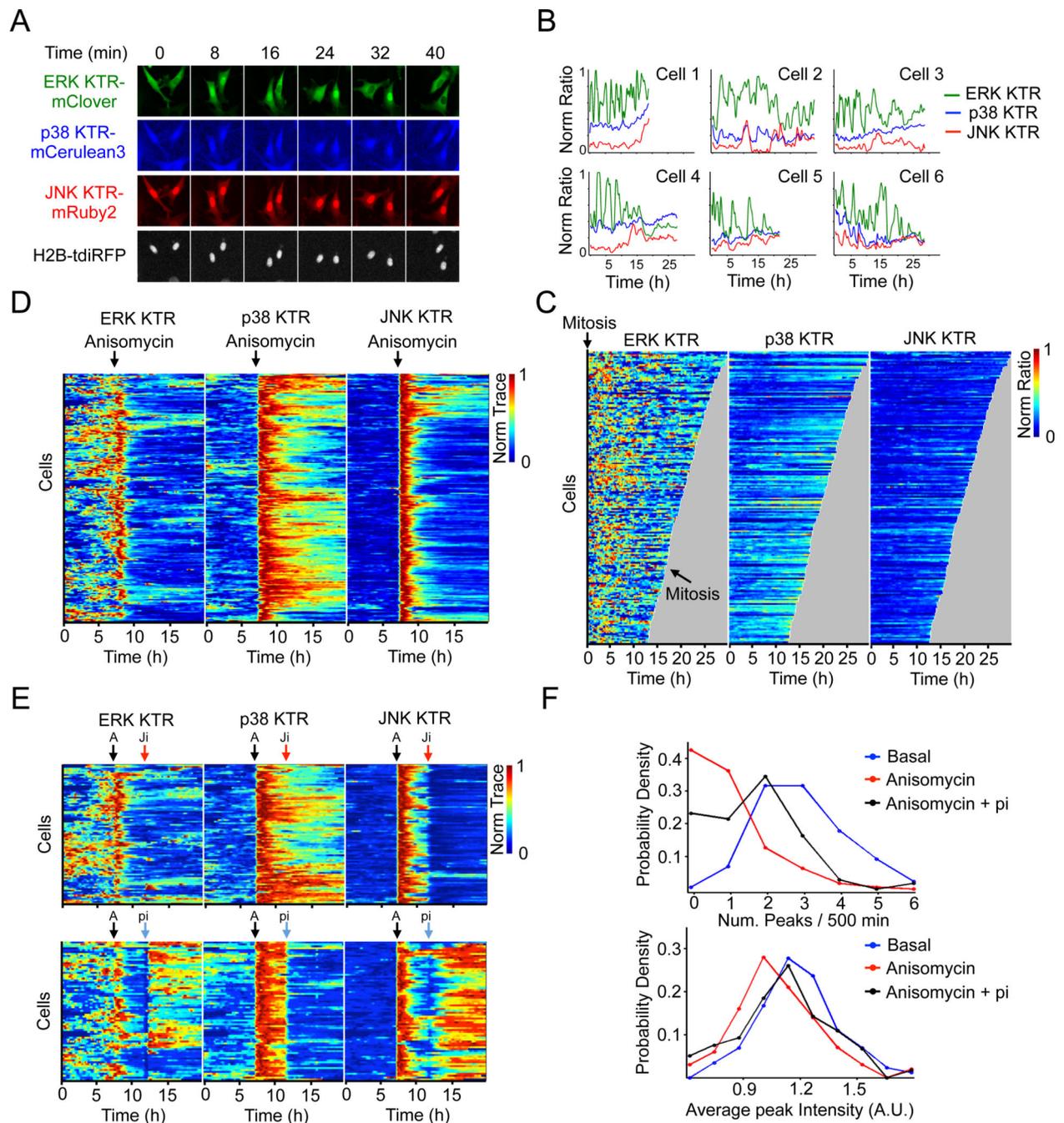


Figure 6. KTR technology allows output multiplexing

(A) 3T3 cells expressing indicated fusion proteins (4C cell line) were imaged under basal conditions over time. Representative cells are shown for each time point. (B and C) 4C cell line was imaged every 8 minutes without stimulation and quantified as described in Methods without stimulation. Six representative cells (b) and heat maps for 196 cells (c) are shown. For each cell, ERK, p38 and JNK KTRs were quantified from mitotic exit to mitotic entry. (D and E) 4C cell line was stimulated with Anisomycin (A) (50 ng/ml) where indicated (black arrow) and treated with 10 μ M JNK inhibitor VIII (Ji) or 10 μ M SB203580 (pi) (red

or blue arrows respectively). Images were taken every 8 minutes and quantified as described in Methods. Heat maps for more than 100 cells are shown. (F) Peaks of ERK activity from data presented in Panels c and e were identified using custom software. Distributions of number of peaks per 500 minutes (top) and average peak intensity (bottom) are shown for 3 conditions; No stimuli (blue), Anisomycin 50 ng/ml (red) or Anisomycin 50 ng/ml and 10 μ M SB203580 (pi) (black). See also Figure S7.