Supramolecular Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713649759

Genetically encoded fluorescent indicators to visualise protein phosphorylation by c-Jun NH_2-terminal kinase in living cells

Hideyuki Suzuki[a]; Moritoshi Sato[a]
[a] Graduate School of Arts and Sciences, The University of Tokyo, Meguro-ku, Tokyo, Japan

Online publication date: 23 June 2010

To cite this Article Suzuki, Hideyuki and Sato, Moritoshi(2010) 'Genetically encoded fluorescent indicators to visualise protein phosphorylation by c-Jun NH_2-terminal kinase in living cells', Supramolecular Chemistry, 22: 7, 434 — 439
To link to this Article: DOI: 10.1080/10610278.2010.484864
URL: http://dx.doi.org/10.1080/10610278.2010.484864

Supramolecular Chemistry
Vol. 22, Nos. 7–8, July–August 2010, 434–439

Taylor & Francis
Taylor & Francis Group

Genetically encoded fluorescent indicators to visualise protein phosphorylation by c-Jun NH_2-terminal kinase in living cells

Hideyuki Suzuki and Moritoshi Sato*

Graduate School of Arts and Sciences, The University of Tokyo, Komaba, Meguro-ku, Tokyo 153-8902, Japan

(*Received 25 January 2010; final version received 7 April 2010*)

We have developed genetically encoded fluorescent indicators for c-Jun NH_2-terminal kinase (JNK), a serine/threonine protein kinase that regulates a wide variety of cellular functions. The indicator was designed based on fluorescence resonance energy transfer (FRET) between two fluorescent proteins. Protein phosphorylation of the indicator by JNK changed its FRET efficiency due to its phosphorylation-dependent conformational alternation, allowing us to detect protein phosphorylation by JNK under a fluorescence microscope using the present indicator. We also have shown that the indicator is genetically targetable to subcellular compartments, such as the cytosol and the nucleus, to monitor subcellular JNK activities there. The present fluorescent indicators provide a powerful tool to reveal spatio-temporal dynamics of protein phosphorylation by JNK in living cells.

Keywords: fluorescence; indicator; FRET; kinase

Introduction

Protein phosphorylation plays a central role in cellular signalling processes (*1*). More than 500 protein kinases are encoded by the human genome. c-Jun NH_2-terminal kinase (JNK) is one of the most intensely studied protein kinases (*2–4*). JNK is a serine/threonine protein kinase that belongs to the mitogen-activated protein kinase (MAPK) family (*5*). JNK is activated by a wide variety of extracellular stimuli, such as cytokines, reactive oxygen species and natural products. The activated JNK phosphorylates various substrate proteins, and is involved in the regulation of cellular function, such as apoptosis, neurodegeneration and inflammatory responses.

We have previously reported a general approach for visualising protein phosphorylation in living cells (*6*). This approach, which is based on the development of genetically encoded fluorescent indicators, has been utilised for several serine/threonine kinases and tyrosine kinases, such as extracellular signal-regulated kinase (ERK) (*7*), Akt (*8*) and Src (*9*, *10*). These fluorescent indicators have provided spatio-temporal information about protein kinase activation in living cells. Here, we describe a genetically encoded fluorescent indicator, nicknamed 'JuCKY', to visualise protein phosphorylation by JNK. JuCKY was devised based on our general approach for visualising protein phosphorylation. We demonstrated that phosphorylation of JuCKY by JNK changes the efficiency of fluorescence resonance energy transfer (FRET) within the fluorescent indicator. We have visualised the spatio-temporal dynamics of protein phosphorylation by JNK in living cells using the present indicators.

Results and discussion

Design of JuCKY

JuCKY is a tandem fusion protein containing cyan fluorescent protein (CFP), phosphorylation recognition domain, JNK-binding domain (JBD), substrate domain and yellow fluorescent protein (YFP; Figure 1). We used a forkhead-associated 2 (FHA2) domain (*11*), derived from yeast Rad53p for the phosphorylation recognition domain of JuCKY. We used an amino acid sequence of D^{44}PVG-SLKPHLRAKNSDLLT**A**^{63}PDVGLLKLA**T**^{73}PELERL^{79} for the substrate domain of JuCKY. This amino acid sequence is derived from a cellular JNK substrate protein c-Jun (*12*), except for alanine residue 63 and threonine residue 73. The threonine residue 73 was substituted from an original serine because the FHA2 domain binds to phosphothreonine but not to phosphoserine. The threonine residue 73 of the substrate domain is preferentially phosphorylated by JNK. The alanine residue 63 was substituted from an original serine to restrict the phospho-acceptor residue to the threonine residue 73 in JuCKY. We used an amino acid sequence of E^{22}SGPYGYSN-PKILKQSMTLNLA^{43}, derived from c-Jun (*12*), for JBD. In addition to the substrate domain, JBD also provides the present fluorescent indicator with a basis of its highly sensitive and selective response to JNK.

*Corresponding author. Email: cmsato@mail.ecc.u-tokyo.ac.jp

ISSN 1061-0278 print/ISSN 1029-0478 online

DOI: 10.1080/10610278.2010.484864
http://www.informaworld.com

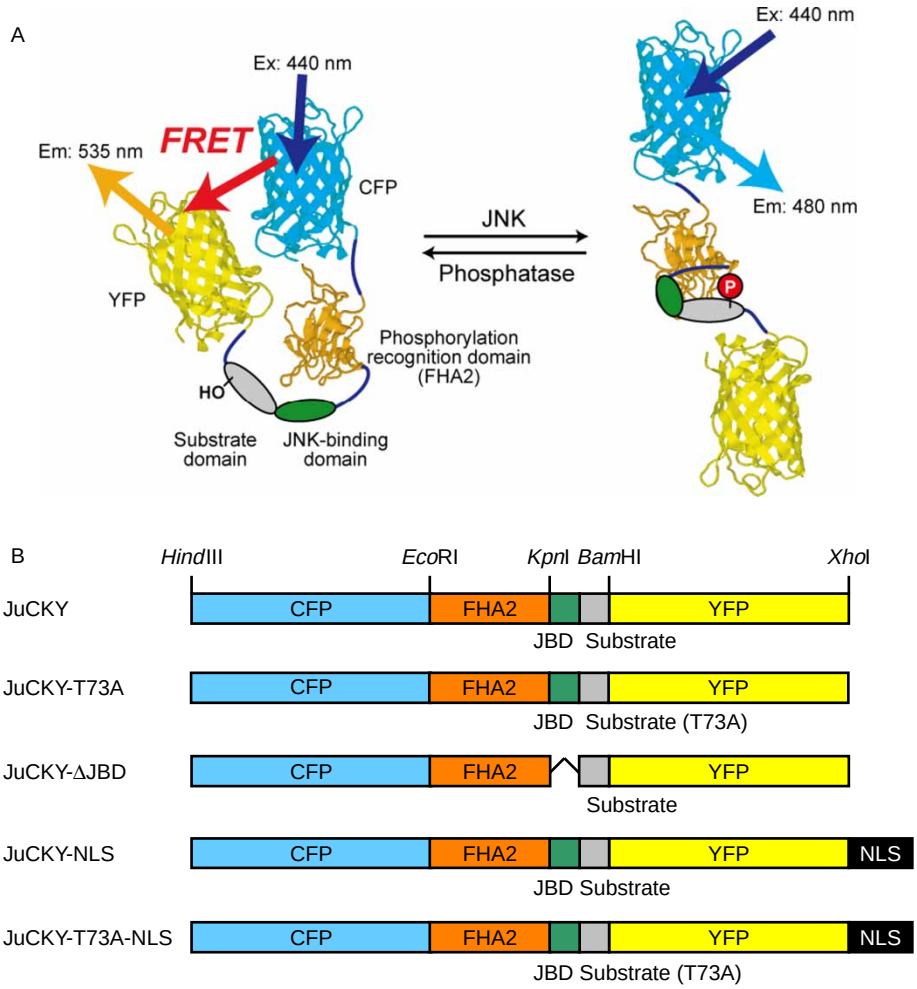

Figure 1. Design of the present fluorescent indicator, named JuCKY, to visualise protein phosphorylation by JNK. (A) Principle of JuCKY. JuCKY consists of CFP, FHA2 domain, JBD, substrate domain and YFP. CFP and YFP are differently coloured mutants of *A. victoria* fluorescent proteins. Upon phosphorylation, the substrate domain binds with FHA2 domain, resulting in the decrease in the FRET efficiency from CFP to YFP. (B) Schematic representations of domain structures of JuCKY and its variants. Restriction sites are shown above the bars. JBD, JBD derived from c-Jun; NLS, nuclear localisation sequence.

CFP and YFP located at both ends of JuCKY are differently coloured mutants of green fluorescent protein (GFP) derived from a jellyfish *Aequorea victoria* (*13*). Phosphorylation of the substrate domain by JNK and subsequent binding of the phosphorylated substrate domain with the adjacent FHA2 domain changes the efficiency of FRET from the donor fluorophore CFP to the acceptor fluorophore YFP (Figure 1(A)). We thus measure changes in fluorescence emission ratios of the donor and acceptor fluorophores to visualise protein phosphorylation by JNK under a fluorescence microscope.

Phosphorylation of JuCKY and its FRET change

We constructed cDNA that encodes JuCKY (Figure 1(B)). HeLa cells were then transfected with the cDNA and subjected to immunoblot analysis. We confirmed the expression of JuCKY using anti-GFP antibody (Figure 2(A)). The HeLa cells expressing JuCKY were stimulated with anisomycin (*14*, *15*), an antibiotic that inhibits proteins synthesis and then activates endogenous JNK in the cell. The cells were subject to immunoblot analysis using anti-phospho-c-Jun antibody (Figure 2(A)). The result shows that JuCKY is indeed phosphorylated by endogenous JNK in the cell. We also showed that phosphorylation of JuCKY is induced with the same time course as endogenous c-Jun, in response to the cellular stimulation.

To examine subcellular localisation of JuCKY, we expressed the indicator in HeLa cells. YFP fluorescence of JuCKY was observed in the cytosol but not in the nucleus (Figure 2(B)). This shows that JuCKY, which has a molecular weight of 75 kDa, is localised in the cytosolic compartment of a HeLa cell when expressed in the cell.

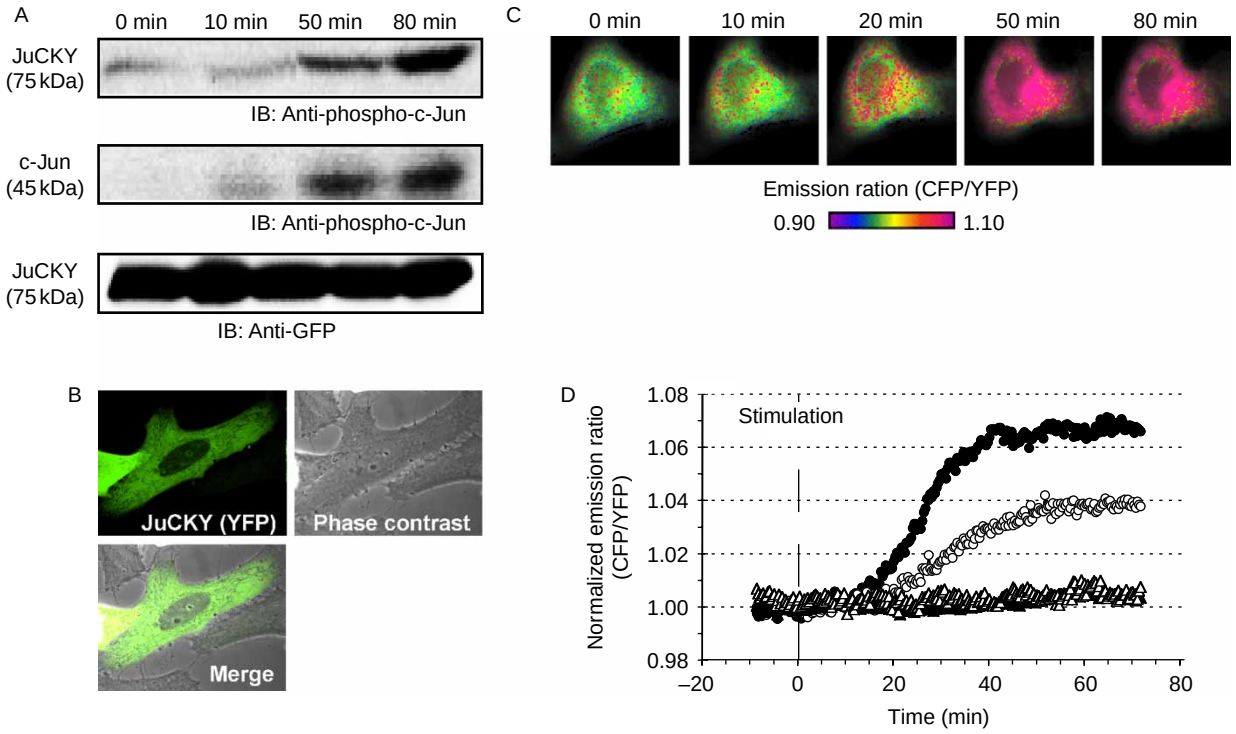

Figure 2. Characterisation of JuCKY. (A) Phosphorylation of both JuCKY and c-Jun by JNK. HeLa cells expressed with JuCKY were stimulated with 10 μg/ml of anisomycin for indicated time at room temperature. The whole-cell lysates were assayed by immunoblotting with anti-phospho-c-Jun antibody or anti-GFP antibody. (B) Subcellular localisation of JuCKY in the cytosol of a HeLa cell. The images were taken using a confocal laser scanning microscope with a 100 × objective. (C) Pseudocolour images of the CFP/YFP emission ratios before and after the addition of 10 μg/ml of anisomycin, obtained from the HeLa cells expressing JuCKY. (D) Time course of JuCKY (filled circle and open circle) or JuCKY-T73A (filled triangle and open triangle) in HeLa cells upon stimulation with 10 μg/ml anisomycin (filled circle and filled triangle) or 10 μg/ml of IL-1α (open circle and open triangle). The results (A) to (D) represent typical experiments from three independent trials.

We next stimulated JuCKY-expressing HeLa cells with anisomycin and monitored both CFP and YFP fluorescence of the indicator under a dual-fluorescence microscope. Approximately 15 min after the anisomycin stimulation, a substantial increase was observed in the emission ratio of CFP to YFP (Figure 2(C) and (D)), indicating that phosphorylation of JuCKY provokes a decrease in FRET from its CFP to YFP. The CFP/YFP emission ratio of JuCKY reached a plateau in 40 min after the anisomycin stimulation (Figure 2(C) and (D)). Anisomycin inhibits peptidyl transferases, thereby interfering protein synthesis in living cells (*16*). The anisomycin-induced cellular stress leads to the activation of JNK (*14*). JuCKY visualised the induction of JNK activation upon cellular stress caused by anisomycin stimulation (Figure 2(D)). JuCKY emitted FRET signals in response to various cellular stimulations, such as interleukin 1α (IL-1α) (*17*) that activates JNK in living cells (Figure 2(D) and see Figure 1 of the Supplementary Information, available online). Anisomycin is one of the most potent activators of JNK. The result obtained with JuCKY is consistent with the potency of the stimulator substances that activate JNK. In contrast to JuCKY, a negative control indicator JuCKY-T73A, in which the phospho-acceptor threonine within the substrate domain was replaced with an alanine (Figure 1(B)), showed no significant change in the emission ratio upon anisomycin stimulation (Figure 2(D)). This confirms that the observed FRET signals of JuCKY are phosphorylation dependent.

Protein kinase selectivity of JuCKY

We examined the selectivity of JuCKY using pharmacological techniques. When HeLa cells expressing JuCKY were pretreated with SP600125 (*18*), a specific inhibitor of JNK, the anisomycin-induced phosphorylation of JuCKY was completely abolished (Figure 3(A)). However, in the case of other protein kinases relevant to JNK, that is, ERK and p38 MAPK, their specific inhibitors U0126 (*19*) (inhibitor of ERK) and SB203580 (*20*) (inhibitor of p38 MAPK), had no effect on the anisomycin-induced phosphorylation of JuCKY (Figure 3(B) and (C)). This result confirms that JuCKY is phosphorylated by JNK but not by other protein kinases, including ERK and p38 MAPK. The specific response of JuCKY for JNK

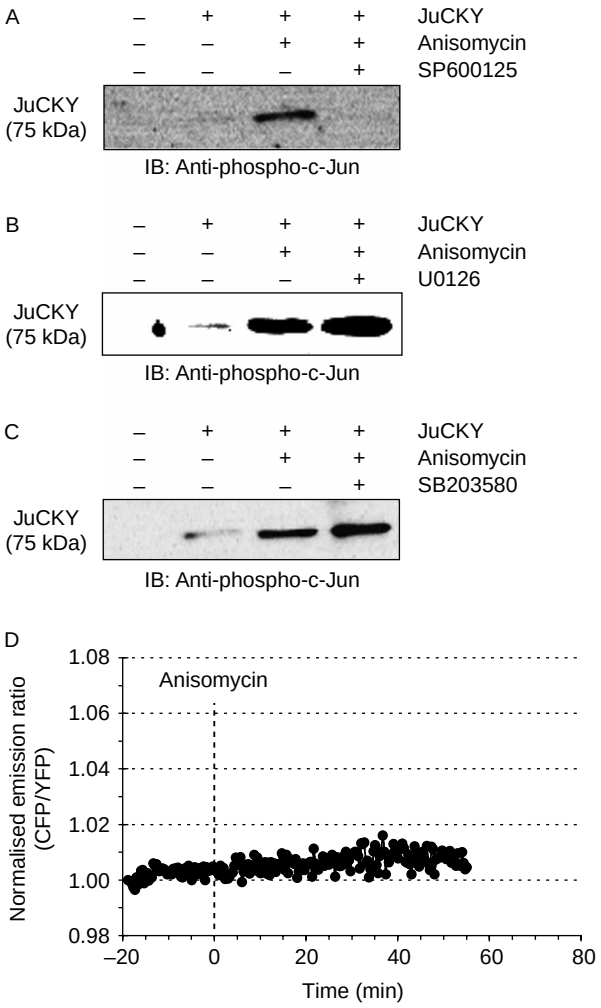

Figure 3. Protein kinase selectivity of JuCKY. (A–C) Immunoblot analysis of phosphorylation of JuCKY in the presence of the protein kinase inhibitor SP600125 (A), U0126 (B) or SB203580 (C). HeLa cells expressed with JuCKY were stimulated with 10 μg/ml of anisomycin. The whole-cell lysates were assayed by immunoblotting with anti-phospho-c-Jun antibody. (D) Time course of JuCKY-ΔJBD in HeLa cells upon stimulation with 10 μg/ml of anisomycin. The results (A) to (D) represent typical experiments from three independent trials.

guarantees the accurate analysis of JNK activation using JuCKY in living cells.

JuCKY is constructed to have a JBD (Figure 1(A) and (B)). To examine the effect of JBD on the response of JuCKY, we constructed JuCKY-ΔJBD, in which JBD is truncated (Figure 1(B)). The anisomycin-induced FRET response of JuCKY-ΔJBD was decreased nearly to the basal level (Figure 3(D) and see Figure 1 of the Supplementary Information, available online). This result demonstrates that JBD within JuCKY underlies the high sensitivity of JuCKY through its efficient and selective interaction with JNK.

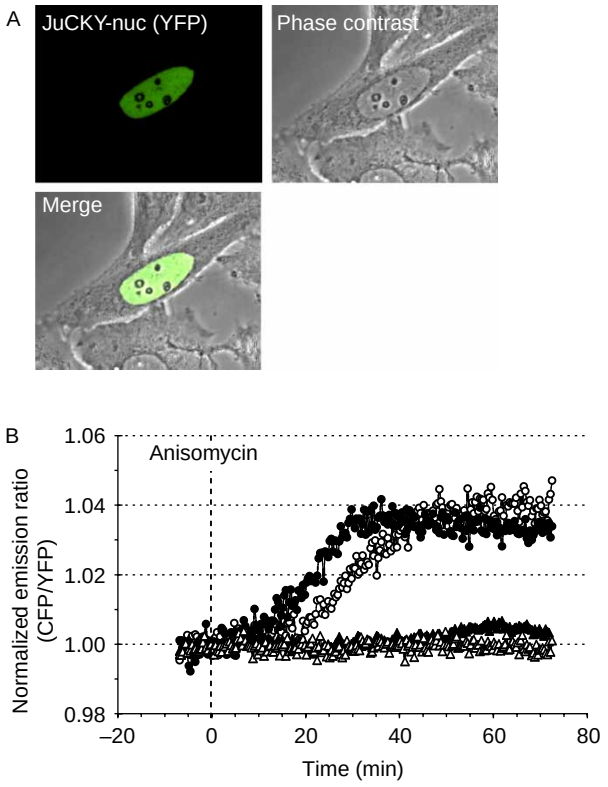

Figure 4. Imaging protein phosphorylation by JNK in the nucleus. (A) Subcellular localisation of JuCKY-NLS in the nucleus of a HeLa cell. The images were taken using a confocal laser scanning microscope with a 100× objective. (B) Time course of JuCKY-NLS (open circle), JuCKY-T73A-NLS (open triangle), JuCKY (filled circle) or JuCKY-T73A (filled triangle) in HeLa cells upon stimulation with 10 μg/ml of anisomycin. The results (A) and (B) represent typical experiments from three independent trials.

Protein phosphorylation by JNK in the nucleus

JNK phosphorylates a variety of substrate proteins when activated in the cell (*21*). The JNK signalling has extensively been studied in the nucleus, in particular, because JNK phosphorylates more than 10 nuclear transcriptional factors, such as c-Jun, c-Myc and p53, that regulate the expression of key genes for cellular functions (*21*). This is why subcellular dynamics of JNK in the nucleus is remarkable. To develop a nuclear fluorescent indicator for protein phosphorylation by JNK, we connected a nuclear localisation sequence (NLS) of PKKKRKVEDA with JuCKY (Figure 1(B)). We named this indicator 'JuCKY-NLS'. When expressed in HeLa cells, YFP fluorescence of JuCKY-NLS was observed in the nucleus (Figure 4(A)). The images indicate that JuCKY-NLS is precisely localised in the nucleus, and thereby allows visualisation of nuclear JNK activities using JuCKY-NLS. This is in contrast to JuCKY that visualises the cytosolic JNK activities (Figure 2(B)).

We compared subcellular JNK activities in the cytosol with those in the nucleus upon cellular stimulations. HeLa cells were separately expressed with JuCKY and JuCKY-NLS and then stimulated with anisomycin. JuCKY-NLS exhibited substantial FRET response in the nucleus like JuCKY in the cytosol upon anisomycin stimulation (Figure 4(B)). Its negative control, JuCKY-T73A-NLS, exhibited no significant response as well as JuCKY-T73A (Figure 4(B)). The result reveals that JNK is also activated in the nucleus, in addition to the cytosolic compartment. The comparison between the two indicators, JuCKY and JuCKY-NLS, also showed that the nuclear activation of JNK is slightly delayed compared with its cytosolic activation upon anisomycin stimulation.

In the present study, we have developed a fluorescent indicator, named JuCKY. JuCKY exhibited substantial changes in the FRET efficiency upon its selective phosphorylation by JNK. This allowed us to visualise protein phosphorylation by JNK, an index of the JNK activity, using JuCKY in living cells. JuCKY is customised for JNK, based on our general approach to develop genetically encoded fluorescent indicators for protein phosphorylation (*6*). In addition to the JNK-selective substrate domain, we also introduced a JBD in JuCKY, which selectively and efficiently binds with JNK. We showed that this domain plays a key role in the development of highly sensitive and selective indicators. In addition to c-Jun, of which JBD we used, a variety of JNK substrates have their own JBDs (*21*). Further improvement of JuCKY may be possible using more efficient JBD than that of c-Jun. The present approach is not limited to the JNK indicators but appears to be generally applicable to the development of fluorescent indicators for a wide spectrum of protein kinases.

We have also shown that JuCKY is genetically targetable to subcellular compartments by connecting subcellular localisation sequences, as represented by the NLS for the nuclear localisation. No powerful methods have previously been available for monitoring subcellular activation of JNK with a high spatial and temporal resolution, despite the protein kinase playing a key role in the regulation of many transcription factors in the nucleus. JuCKY and JuCKY-NLS have allowed to clearly visualise different dynamics of JNK activation between the cytosol and the nucleus, respectively. Our interest is not limited to the nucleus. This is because many substrate proteins of JNK, localising in various subcellular compartments, have been identified, such as mitochondrial substrate proteins and substrate proteins in the plasma membrane (*21*). We believe that the present approach provides a powerful tool to reveal subcellular dynamics of protein phosphorylation by JNK in living cells.

Materials and methods

Materials

All cloning enzymes and *Trans* IT-LT1 were purchased from Takara Bio, Inc. (Shiga, Japan). Fetal bovine serum, Hank's balanced salt solution and pcDNA3.1 were purchased from Invitrogen Co. (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium, anisomycin, SP600125, U0126, SB203580 and IL-1α were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Anti-phospho-c-Jun antibody was purchased from Upstate, Inc. (Charlottesville, VA, USA). Anti-GFP antibody was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

Plasmid construction

For constructing cDNAs of the present indicators, fragment cDNAs of CFP, FHA2 with JBD and the substrate domain to its C terminus, YFP and YFP with NLS were generated by standard polymerase chain reaction (PCR). Each cDNA fragment was subcloned into pBluescript SK(+) vector. All PCR fragments were sequenced with an ABI310 genetic analyser (Applied Biosystems, Foster City, CA, USA). Each cDNA encoding JuCKY was subcloned into a mammalian expression vector, pcDNA3.1(+), at the sites shown in Figure 1(B). All the amino acid sequences of the indicators presented are listed in the Supplementary Information, available online.

Cell culture and transfection

HeLa cells were cultured in Eagle's minimum essential medium, supplemented with 10% fetal bovine serum and 2 mM L-glutamine, at 37°C in 5% CO_2. The cells were transfected with the *Trans* IT-LT1 reagent in glass-bottomed dishes and plastic culture dishes for fluorescence imaging of living cells and immunoblot analysis, respectively.

Imaging of cells

Twenty-four hours after the transfection, the culture medium was replaced with Hank's balanced salt solution. The cells were imaged at room temperature on a Carl Zeiss Axiovert 200 microscope with a cooled charge-coupled device camera, Cool-SNAP HQ (Roper Scientific, Tucson, AZ, USA), controlled by MetaFluor (Universal Imaging Co., West Chester, PA, USA). The exposure time at 440 ± 10 nm excitation was 100 ms. Fluorescence images were obtained through 480 ± 15 and 535 ± 12.5 nm filters with a 40× oil immersion objective (Carl Zeiss, Jena, Germany).

Immunoblot analysis

Twenty-four hours after the transfection, the cells were stimulated with 10 μg/ml of anisomycin at room

temperature in the presence and absence of protein kinase inhibitors. The cells were lysed with an ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10 mM NaF, 2 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The whole-cell lysates were separated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. The membrane was probed with anti-phospho-c-Jun antibody to detect phosphorylated JuCKY, and anti-GFP to detect both phosphorylated and unphosphorylated JuCKY. The obtained signal was quantified using an image analyser, LAS-1000plus (Fuji Photo Film Co., Tokyo, Japan). The antibody is raised against a phosphoserine-containing peptide, K^{70}LApSPELERLIIQC^{82}, corresponding to amino acids 70–82 of human phospho-c-Jun. Our results of Figure 2(B) show that the antibody is cross-reactive with the phosphorylated substrate sequence of JuCKY, in which the phosphoacceptor is not a serine but a threonine.

Acknowledgements

This work was supported by grants from the Japan Science and Technology Agency (JST), Japan Society for the Promotion of Science (JSPS) and the Takeda Science Foundation (to M.S).

References

(1) Hunter, T. *Cell* **2000**, *100*, 113–127.
(2) Johnson, G.L.; Nakamura, K. *Biochim. Biophys. Acta* **2007**, *1773*, 1341–1348.
(3) Dhanasekaran, D.N.; Reddy, E.P. *Oncogene* **2008**, *27*, 6245–6251.
(4) Wagner, E.F.; Nebreda, A.R. *Nat. Rev. Cancer* **2009**, *9*, 537–549.
(5) Johnson, G.L.; Lapadat, R. *Science* **2002**, *298*, 1911–1912.
(6) Sato, M.; Ozawa, T.; Inukai, K.; Asano, T.; Umezawa, Y. *Nat. Biotechnol.* **2002**, *20*, 287–294.
(7) Sato, M.; Kawai, Y.; Umezawa, Y. *Anal. Chem.* **2007**, *79*, 2570–2575.
(8) Sasaki, K.; Sato, M.; Umezawa, Y. *J. Biol. Chem.* **2003**, *278*, 30945–30951.
(9) Hitosugi, T.; Sasaki, K.; Sato, M.; Suzuki, Y.; Umezawa, Y. *J. Biol. Chem.* **2007**, *282*, 10694–10706.
(10) Hitosugi, T.; Sato, M.; Sasaki, K.; Umezawa, Y. *Cancer Res.* **2007**, *67*, 8139–8148.
(11) Durocher, D.; Taylor, I.A.; Sarbassova, D.; Haire, L.F.; Westcott, S.L.; Jackson, S.P.; Smerdon, S.J.; Yaffe, M.B. *Mol. Cell* **2000**, *6*, 1169–1182.
(12) Hibi, M.; Lin, A.; Smeal, T.; Minden, A.; Karin, M. *Genes Dev.* **1993**, *7*, 2135–2148.
(13) Miyawaki, A.; Tsien, R.Y. *Methods Enzymol.* **2000**, *327*, 472–500.
(14) Bogoyevitch, M.A.; Ketterman, A.J.; Sugden, P.H. *J. Biol. Chem.* **1995**, *270*, 29710–29717.
(15) Hazzalin, C.A.; Le Panse, R.; Cano, E.; Mahadevan, L.C. *Mol. Cell Biol.* **1998**, *18*, 1844–1854.
(16) Hansen, J.L.; Moore, P.B.; Steitz, T.A. *J. Mol. Biol.* **2003**, *330*, 1061–1075.
(17) Weber, A.; Wasiliew, P.; Kracht, M. *Sci. Signal.* **2010**, *3*, 1–6.
(18) Bennett, B.L.; Sasaki, D.T.; Murray, B.W.; O'Leary, E.C.; Sakata, S.T.; Xu, W.; Leisten, J.C.; Motiwala, A.; Pierce, S.; Satoh, Y.; Bhagwat, S.S.; Manning, A.M. *Proc. Natl Acad. Sci. USA* **2001**, *98*, 13681–13686.
(19) Favata, M.F.; Horiuchi, K.Y.; Manos, E.J.; Daulerio, A.J.; Stradley, D.A.; Feeser, W.S.; Van Dyk, D.E.; Pitts, W.J.; Earl, R.A.; Hobbs, F.; Copeland, R.A.; Magolda, R.L.; Scherle, P.A.; Trzaskos, J.M. *J. Biol. Chem.* **1998**, *273*, 18623–18632.
(20) Cuenda, A.; Rouse, J.; Doza, Y.N.; Meier, R.; Cohen, P.; Gallagher, T.F.; Young, P.R.; Lee, J.C. *FEBS Lett.* **1995**, *364*, 229–233.
(21) Bogoyevitch, M.A.; Kobe, B. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 1061–1095.