regulation of gene transcription, it is important to develop sensitive and accurate methods for detecting them, ideally within intact, living mammalian cells. Existing methods, such as chromatin immunoprecipitation and immunofluorescence staining, require cell lysis or membrane permeabilization and therefore sacrifice spatial and/or temporal information. Here we report the development of a fluorescent reporter that can detect phosphorylation of histone H3 at serine 28 in intact, single cells in real time.

The reporter design is shown in Figure 1a. A peptide substrate corresponding to the 30 N-terminal amino acids of histone H3 is joined to a phosphoserine/threonine-binding
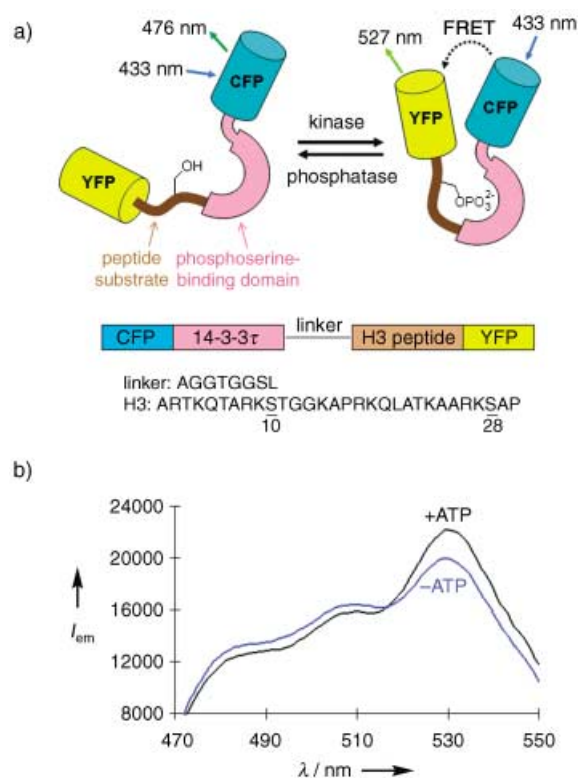
Phosphorylation Reporters

A Genetically Encoded Fluorescent Reporter of Histone Phosphorylation in Living Cells**

Chi-Wang Lin and Alice Y. Ting

Histones, the scaffolding proteins around which DNA is wrapped in chromatin, participate in transcriptional regulation by controlling steric access to DNA and providing docking sites for nuclear proteins. One of the major mechanisms for this regulation is post-translational modification; phosphorylation, acetylation, and methylation of the N-terminal tails of histone proteins, especially histones H3 and H4, alter the shape, charge, and hydrophobicity of the nucleosome core particle, affecting its interactions with transcription factors, polymerases, and other chromatin-associated proteins.[1] Since post-translational modifications of histones represent one of the earliest control points for the

Figure 1. a) Reporter design and domain structure. The two serine-phosphorylation sites in the H3 peptide are underlined. b) Normalized reporter emission spectra before (blue) and after (black) phosphorylation by Msk-1 kinase (excitation at 433 nm).

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module by a flexible linker, and this fusion protein is sandwiched between a pair of green-fluorescent protein (GFP) mutants capable of fluorescence resonance energy transfer (FRET): cyan-fluorescent protein (CFP) and yellow-fluorescent protein (YFP).[2–4] On enzymatic phosphorylation of the histone H3 peptide, the phosphoserine/threonine-binding domain should form an intramolecular complex with the phosphopeptide, altering the spatial relationship between the flanking CFP and YFP units and changing the FRET level. Dephosphorylation of the substrate peptide by a phosphatase should cause the intramolecular complex to dissociate, restoring the original FRET level. We previously used this general design to develop phosphorylation reporters for consensus substrates of the epidermal growth factor

receptor (EGFR), Src kinase, and Abl kinase,[3] while others have used this approach to develop reporters for protein kinase A,[4] protein kinase C,[5] and the insulin receptor.[6] The phosphoserine/threonine-binding module in the reporter described herein, which represents the first for histones, is the signaling protein 14-3-3τ,[7] which consists of 232 amino acids and naturally binds to the phosphorylated form of residues 615–644 of Cbl,[8] a sequence similar to residues 1–30 of H3.

We constructed the reporter gene by standard cloning methods (GenBank accession no. AY422821), expressed the His$_6$-tagged recombinant protein in *E. coli*, and purified it by nickel affinity chromatography. In in vitro assays with purified Msk-1 kinase, which phosphorylates both serine 10 and 28 of H3,[9] the reporter gave a 25 % increase in the YFP/CFP emission ratio (Figures 1 b and 2 a). Leaving out either ATP or
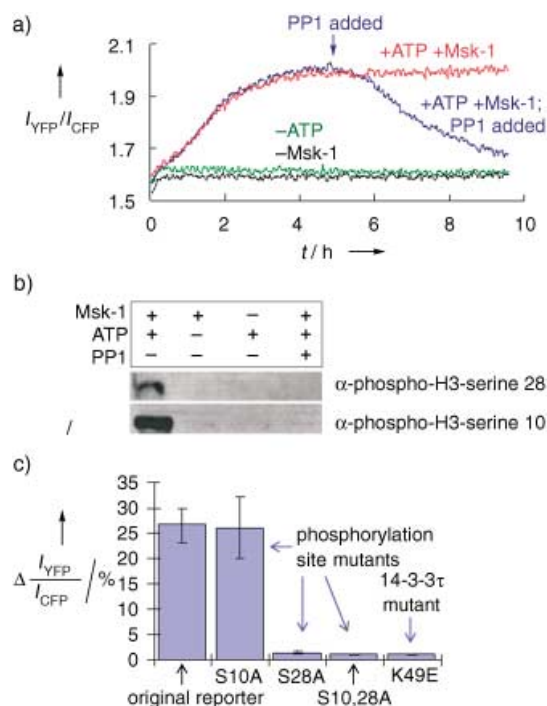

Figure 2. a) Change in the reporter YFP/CFP emission ratio over time under various reaction conditions. b) Immunoblot analysis of the four reactions in (a) after 10 h with anti-phospho-H3-serine 10 and -serine 28 antibodies. c) Maximal emission ratio changes observed in phosphorylation reactions with four reporter mutants. Each measurement was performed in triplicate.

the enzyme abolished the FRET change (Figure 2 a). To examine reporter reversibility, we treated the phosphorylated reporter protein with PP1, a broad-specificity serine/threonine phosphatase.[10] Over about 5 h, the FRET returned to its original level (Figure 2 a). Reporter protein from each reaction in Figure 2 a was also analyzed by an anti-phospho-H3-serine 10 and -serine 28 immunoblot. Figure 2 b shows a good correspondence between the FRET level and this traditional method for determining the phosphorylation state of a protein. Lastly, the phosphorylation rate of the in vitro reporter is very similar to that of full-length H3 under

identical conditions (data not shown), which indicates that the former is a good mimic of the latter.

Because the N-terminus of H3 contains two potential phosphorylation sites (serine 10 and serine 28), we performed site-directed mutagenesis on the reporter to determine which site(s) were responsible for the FRET increase. Figure 2 c shows that mutation of serine 10 to a phosphorylation-incompetent alanine had no effect on the reporter response, whereas mutation of serine 28 to alanine abolished the FRET change. These results indicate that the reporter specifically responds to serine 28 phosphorylation. Because we wondered whether this specificity was a consequence of the reporter geometry or reflected an inherent selectivity of the 14-3-3τ domain for phosphoserine 28, we constructed another reporter identical to the original but with the 14-3-3τ and histone peptide domains transposed. Interestingly, this reporter was responsive to phosphorylation at both the serine 10 and serine 28 positions (see Supporting Information), which suggests that geometrical constraints determine the specificity of the original reporter.

A charge-reversal mutation in the binding pocket of the 14-3-3τ domain (K49E), expected to abolish the binding affinity of the 14-3-3τ domain for phosphoserine,[7] also eliminated the FRET response (Figure 2 c). This is consistent with the designed mechanism for sensing of phosphorylation through FRET. We wondered, however, whether the known dimerization potential of 14-3-3τ,[7] also demonstrated in a previous 14-3-3τ-based reporter,[4] would lead to dimerization of our reporter. Analytical ultracentrifugation confirmed that our reporter exists as a dimer in solution (data not shown). Therefore, the fluorescence response of our reporter might be due either to true intramolecular binding or to intermolecular binding across the dimer interface.

To test the histone phosphorylation reporter in living cells, we appended a nuclear localization sequence (PKKKRK) to the C-terminus of the reporter gene and introduced the plasmid DNA into HeLa cells by lipofection. The reporter was efficiently expressed after about 12 h. Because previous studies have shown that serine 28 phosphorylation levels transiently increase during the mitotic (M) phase of the cell cycle,[11] we monitored the FRET level of our reporter in dividing HeLa cells over 26 h. As shown in Figure 3 and in the movie provided as Supporting Information, the reporter displayed a rapid increase in YFP/CFP emission ratio (15–25 %) that, in eight separate cells, closely preceded breakdown of the nuclear envelope (as measured by reporter distribution in the cell) by 5–15 min. The high FRET was sustained for several hours and then dropped slowly after cytokinesis, or separation of the cytoplasm into daughter cells. A control experiment with the S28A mutant of the reporter did not give a FRET change during cell division, demonstrating that the response of the reporter is not an artifact of changes in reporter concentration or cellular pH over the course of cell division (see Figure 3 b).

Elevated serine 10 and 28 phosphorylation levels have both been linked to mitosis,[12, 13] but the precise molecular roles of these modifications have yet to be elucidated. Our study provides a high-resolution picture of serine 28 phosphorylation onset and disappearance in single living HeLa
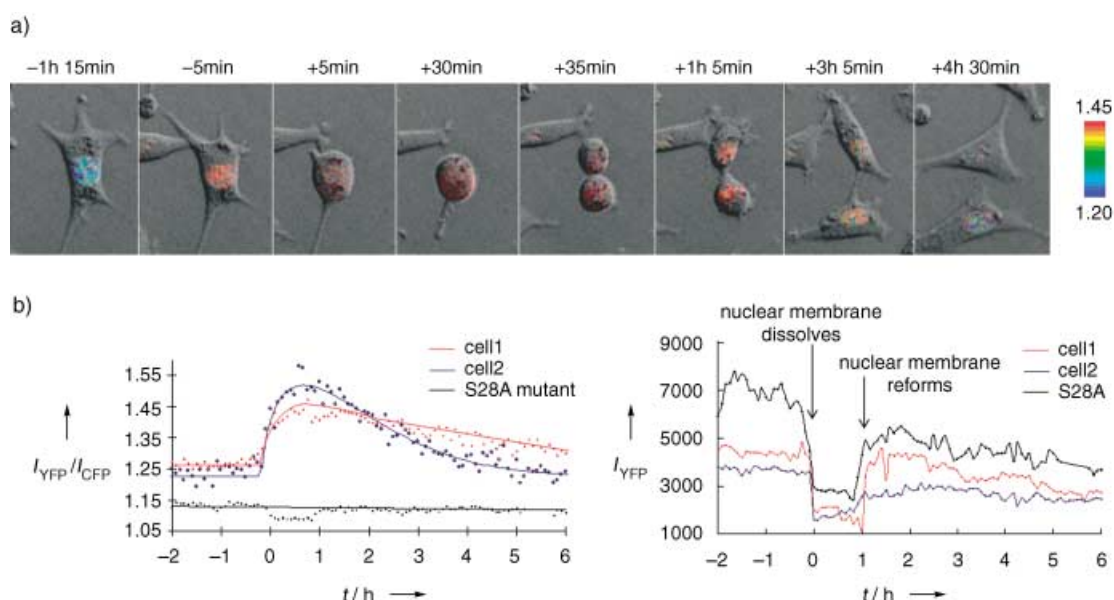
Figure 3. a) Reporter response during HeLa cell division. The bar on the right shows the correlation between YFP/CFP emission ratio and cell pseudocolor. The indicated times refer to hours/minutes after nuclear-membrane breakdown. b) Emission ratio and YFP emission intensity as a function of time after nuclear-membrane breakdown for two cells expressing the reporter and one cell expressing the S28A reporter mutant (t = time after nuclear-membrane breakdown).

cells during cell division. We find a previously unobserved correlation between serine 28 phosphorylation onset and nuclear-envelope breakdown, which may suggest a mechanism for coordinating changes in chromatin and cell morphology during the M phase. Future investigation will follow up on this intriguing possibility. In another avenue for future study, if the phosphorylation reporter were physically anchored to chromatin (for example, through genetic fusion to one of the four histone proteins), spatial resolution might be further increased.

The work described herein also demonstrates the capabilities and limitations of current methodology for the design of fluorescent reporters. Without a recognition element known a priori to bind the phosphorylated histone sequence, we developed a reporter for H3 serine 28. In fact, the 14-3-3τ domain consensus recognition sequence is quite different from the peptide sequence flanking serine 28.[14] Either 14-3-3τ binding is significantly more promiscuous and context-dependent than previously thought, or our reporter design can accommodate recognition domains of only modest intermolecular affinity for the substrate peptide. On the other hand, the impressive, geometry-sensitive specificity of our reporter for H3 serine 28 over other phosphorylation-competent sites in the same substrate peptide indicates a keen dependence on reporter design elements such as spacing and orientation of the binding domain and the substrate peptide.

In conclusion, this reporter, and similar ones such as histone methylation reporters[15] and acetylation reporters under development in our laboratory, should allow the study of transcriptional regulation in cells with greatly improved spatiotemporal resolution.

Experimental Section

The reporter gene was ligated into the pRSETB vector (Invitrogen, Carlsbad, CA) between the *Bam*HI and *Eco*RI sites, which installs a His$_6$ tag at the N-terminus. For protein expression, the plasmid was introduced into BL21(DE3) bacteria (Stratagene, La Jolla, CA) and the cells were grown in Luria Broth supplemented with ampicillin (100 µg mL^{-1}) at 37°C until OD$_{600}$ 0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.4 mM to induce reporter expression. The cells were grown for 3 h at 30°C and then harvested by centrifugation. Cells were lysed by sonication at 4°C (six 30-second pulses at half-maximal power with 1 min between each pulse) in lysis buffer (50 mM Tris pH 7.8, 300 mM NaCl, 4 mM PMSF, and $^1/_4$ EDTA-free protease inhibitor cocktail tablet (Roche, Indianapolis, IN) per 10 mL of lysis buffer). The His$_6$-tagged reporter was purified from the lysate using a Ni-NTA agarose column (Qiagen, Valencia, CA) following the manufacturer's protocol. Fractions containing the reporter were pooled and transferred into TBS (140 mM NaCl, 3 mM KCl, 25 mM Tris pH 7.4) by two rounds of dialysis for storage in aliquots at −80°C. Typical yields were 100–500 µg of protein per 0.5 L culture. Msk-1 kinase was prepared in an identical manner using the Msk-1-expression plasmid (His$_6$-tagged human Msk-1 in pET-11 vector (Novagen, Madison, WI)) that we received from Prof. Dr. Jiahuai Han.

Reaction conditions for the in vitro phosphorylation assays were as follows: 1–5 µM reporter, 0.6 mM ATP, 20 mM Hepes pH 7.7, 10 mM MgCl$_2$, 0.1 mM EGTA, 0.5 mM DTT, and 4 µM of Msk-1. Reactions were incubated at 30°C for the indicated times. The phosphorylation state of the reporter from the in vitro assays was analyzed by Western blot using 12% SDS-PAGE gel, anti-phospho-H3-serine 10 antibody (Cell Signaling Technology, Beverly, MA) or anti-phospho-H3-serine 28 antibody (Upstate Biotechnology, Charlottesville, VA) (both at 1/1000 dilution for 3 h at 25°C), and goat anti-rabbit-HRP conjugate or goat anti-mouse-HRP conjugate (1/1000 dilution for 2 h at 25°C) (Bio-Rad, Hercules, CA). The HRP was visualized by chemiluminescence (Supersignal West Pico, Pierce, Rockford, IL).

pcDNA3 (Invitrogen, Carlsbad, CA) plasmid containing the reporter gene between the *Bam*HI and *Eco*RI sites was introduced

into HeLa cells by transfection with Fugene 6 (Roche, Indianapolis, IN). Images were collected 8–24 h after transfection on a Zeiss Axiovert 200M inverted epifluorescence microscope with differential interference contrast (DIC). Cells were maintained in 10% fetal bovine serum in phenol red-free DMEM (Invitrogen, Carlsbad, CA) at 37°C under 5% CO_2 during imaging with an environmental control system (Solent Scientific, Portsmouth, UK) that housed the microscope stage. At each time point, four images were collected in rapid succession (automated, using OpenLab software (Improvision, Lexington, MA)): a CFP image (420DF20 excitation, 450DRLP dichroic, 475DF40 emission), a FRET image (420DF20 excitation, 450DRLP dichroic, 530DF30 emission), a YFP image (495DF10 excitation, 515DRLP dichroic, 530DF30 emission), and a DIC image (775DF50 emission). Fluorescence images were background corrected. Acquisition times ranged from 100 to 800 ms. The emission ratio image was generated by dividing the FRET image by the CFP image. The data shown in Figure 3a are a merge between the emission ratio image and the DIC image. The YFP intensity graph shown in Figure 3b was generated from the YFP images.

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