Palladium-triggered deprotection chemistry for protein activation in living cells

Jie Li1, Juntao Yu2, Jingyi Zhao1, Jie Wang2, Siqi Zheng1, Shixian Lin1, Long Chen1, Maiyun Yang1, Shang Jia1, Xiaoyu Zhang3 and Peng R. Chen1,2*

Employing small molecules or chemical reagents to modulate the function of an intracellular protein, particularly in a gain-of-function fashion, remains a central task because of their remarkable advantages in facilitating a gain-of-function study rather than the widely adopted, inhibitor-based loss-of-function study of target proteins1. For example, a panel of small-molecule activators, the majority of which are allosteric activators2, has been developed for switching on the intrinsic activity of enzymes and provides a way to ascertain the sufficiency of an enzyme in triggering a specific signalling pathway or cellular response. Another intriguing example is the chemical rescue strategy in which an external small molecule is utilized to restore the wild-type activity of a protein that contains active-site mutations, which enables the dissection of its specific roles in modulating intracellular signal transduction3,4. However, typically these small-molecule protein activators are identified by high-throughput screening or discovered by serendipity, and are thus not directly transferrable to other proteins1. Alternatively, mechanism-based photocaged versions of proteins or small effector molecules have been developed, and allow spatial and temporal activation of a protein under living conditions5,6. Such methodologies rely on light-mediated removal of the caging group as a way to modulate the functionality or localization of the target proteins in vitro and in vivo. Among these approaches, direct caging of proteins via a genetically encoded unnatural amino acid (UAA) bearing a photocleavable group allows control of protein activity in a site-specific fashion7–9. However, ultraviolet irradiation on live cells may trigger surface-receptor internalization10, alter the intracellular signalling networks and cause additional cytotoxic effects (Supplementary Fig. 1). In addition, the poor penetration capability renders these photocaged UAA incapable of being further developed for utilization in deep tissues or intact animals. Inspired by the aforementioned chemical rescue approach, we envisioned that direct blockage of an essential residue on a protein of interest by a cleavable group may generate a ‘chemically caged’ protein with the wild-type activity temporally switched off. The subsequent addition of a membrane-permeable and biocompatible cleavage reagent may drive the elimination of this caging group and thus restore the native functionality to a protein within its intracellular context. Such a ‘chemical decaging’ strategy may offer an attractive technique based on small molecules and generally applicable for manipulating the activity of proteins within living systems (Fig. 1a).

Lysine is a key amino acid in protein posttranslational modifications (PTMs) and transduction of intracellular signals11, and it also plays essential roles in various enzymes such as protein kinases12. We are interested in developing a biocompatible chemical control strategy to cage and release the ε-amine from a lysine, which may allow modulation of the function, structure and/or localization of a protein that relies on a key lysine residue. In our efforts towards searching for a desired biocompatible cleavage group to modulate protein activity chemically via the liberation of free lysine, we turned our attention to allyl and propargyl, two commonly used protecting groups for alcohol/amine and carbonate/carbamate, that can be deprotected by organometallic catalysts under mild or living conditions13–20 (Fig. 1b). Herein we report the development of a palladium-mediated chemical decaging strategy to control lysine-dependent activation of intracellular proteins. By using biocompatible palladium catalysts identified from this study, we successfully liberated the ε-amine from a proparglyoxycarbonyl (Proc)-‘caged’ lysine analogue (3, Fig. 1c) that was genetically and site-specifically incorporated into an intracellular protein, allowing facile restoration of the wild-type activity to the chemically caged protein with its biological roles investigated within living cells.

Results and discussion

Identifying palladium catalysts for the biocompatible and efficient deprotection of caged lysine. We started by synthesizing two fluorogenic rhodamine derivatives, Proc-protected rhodamine

1 Synthetic and Functional Biomolecules Center, Beijing National Laboratory for Molecular Sciences, Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China, 2 Peking-Tsinghua Center for Life Sciences, Beijing 100871, China, 3 College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou 730000, China.
*e-mail: pengchen@pku.edu.cn
Figure 1 | A chemical decaging strategy for protein activation in living cells. a, A key amino acid (for example, a lysine residue in this study) on a protein of interest may be site-specifically replaced by its genetically encoded analogue that bears a caging group 'X', rendering the protein inactive. Intracellular addition of an external small molecule or biocompatible catalyst would trigger a deprotection reaction to remove this caging group, and so release the decaged amino-acid side chain with restored protein activity inside living cells. b, Deprotection chemistry as the potential chemical decaging method. In contrast to the deprotection of the propargyl group, which can be carried out with palladium catalysts in all the typical oxidation states (0, +2 and +4), the allyl group can only be deprotected by Pd(0) species. c, Structures of lysine (Lys, 1), pyrrolysine (Pyl, 2), N-terminal-propargoxy carbonyl-L-lysine (Proc-Lys, 3) and N-allyl-propargoxy carbonyl-L-lysine (Aloc-Lys, 4). The liberation of lysine’s ε-amino from 3 and 4 can be controlled by palladium-mediated propargyl carbamate cleavage and allyl carbamate cleavage reactions, respectively.

110 (Proc-R110, 5) and allyloxy carbonyl (Aloc)-‘caged’ rhodamine 110 (Aloc-R110, 6) as the reporters to assess the efficiency of palladium-mediated depropargylation and deallylation reactions, respectively (Fig. 2a). These two probes are virtually nonfluorescent until the conversion into a highly fluorescent compound (Φ = 0.91, excitation (E_x) = 485 nm, emission (E_y) = 520 nm) via the cleavage of protection groups (Fig. 2a). These fluorogenic substrates can link the deprotection yield directly with a fluorescent readout, which may offer a facile approach for an assessment of the reaction efficiency catalysed by different palladium species in vitro and in vivo. As bulky and negatively charged ligands may have membrane-permeability issues, simple and single-component palladium sources are preferred to facilitate the cellular entry, and may also be less toxic than many palladium complexes that contain phosphine ligands. Therefore, as shown in Fig. 2b, a total of six simple and air-stable palladium species were surveyed. Among these, C4 is an air-stable palladium(0) precursor and C5 is a simple and stable Pd(II) compound, but is readily converted into Pd(0) species on nucleophilic (for example, water) attack through reductive elimination. In addition, three commonly used and water-soluble ligands (L1, L2 and L3) were used in the complex with simple palladium sources for comparison. The elimination efficiency catalysed by each of the palladium species was evaluated systematically by analysing the enhanced fluorescence (Supplementary Method 2). Interestingly, all six simple palladium catalysts were found to catalyse the depropargylation reaction on Proc-R110 more efficiently than the deallylation reaction on Aloc-R110 (Fig. 2c), which is in line with previous reports that strong reducing reagents and/or phosphine ligands are necessary to promote the simple deallylation reaction mediated by a palladium source. We found that two simple palladium compounds, allyl-Pd2Cl2 (C5, allylpalladium(II) chloride dimer) and Pd(dbath), (C4) (dbath = dibenzylidene acetone), were the most-efficient catalysts for both types of elimination reactions (Fig. 2c, Supplementary Fig. 2). In contrast, the addition of water-soluble ligand L1 to C2 increased the deallylation efficiency to a certain extent, but not the depropargylation efficiency. In addition, although ligands L2 and L3 have been used previously to facilitate the palladium-catalysed Suzuki–Miyaura and copper-free Sonogashira cross-coupling reactions on proteins, respectively, both of these ligands were found to decrease significantly the Pd(OAc)2(C1)-mediated depropargylation on Proc-R110.

To confirm further that these identified palladium species can depropargylate the Proc-protected lysine amino acid effectively, and that this reaction is catalytic, we synthesized N-α-propargoxy carbonyl-lysine (Proc-Lys) (Fig. 1c, 1), as well as 9-fluorenylmethoxy carbonyl (Fmoc)-protected Proc-Lys (Proc-Fmoc-Lys; structure shown in Supplementary Fig. 3). The reaction yields of both C4- and C5-mediated depropargylation on Proc-Lys and Proc-Fmoc-Lys were analysed by liquid chromatography–mass spectrometry (LC-MS), which showed that a catalytic loading of either of these two palladium species in the absence of any additional reagents can release free lysine with high efficiency (Fig. 2d and Supplementary Table 1). In particular, a 10% loading of C4 or C5 in PBS buffer (pH 7.4) at 37 °C drove the depropargylation reaction to > 80% completion (Fig. 2d). In contrast, neither of these palladium catalysts can efficiently liberate the Aloc-protected lysine analogue, N-α-allyloxy carbonyl-lysine (Aloc-Lys, 4, Fig. 1c) or the Fmoc-protected Aloc-Lys (Aloc-Fmoc-Lys; structure shown in Supplementary Fig. 3) under the same conditions without additional reducing agents (Fig. 2d and Supplementary Table 1). Furthermore, our additional experiments indicated that the two most-efficient palladium compounds, allyl-Pd2Cl2 and Pd(dbath), may catalyse the depropargylation reaction via Pd(0) species with free amine and hydroxycetone as the final products (Supplementary Fig. 4). Taken together, our results indicate that the Proc group can be deprotected more effectively than the Aloc group by simple palladium catalysts. As the bioorthogonality of the alkyne moiety is well-established, this Proc group and our identified palladium reagents may serve as a biocompatible ‘protection group/catalyst pair’ for lysine activation under living conditions.

Protein-based verification of palladium reagents for deprotection of lysine. Next, we aimed to evaluate systematically the aforementioned palladium reagents for propargyl carbamate cleavage in the context of a carrier protein. Proc-Lys is an analogue of pyrrolysine (Pyl, 2, Fig. 1c), the 22nd naturally occurring amino acid encoded by a Pyl-tRNA synthetase (PylRS)/tRNA_CUA* pair in Archaea species. This Pyl-based system has been adapted for incorporation of various lysine-derived UAAs in response to an in-frame amber codon on target proteins in various living
Proc-Lys residue (Supplementary Fig. 7), which showed a deprotection efficiency followed by LC-MS-MS analysis on digested peptides that contained the specific reaction site on GFP-N149-ProcLys were validated by Supplementary Fig. 6). Furthermore, the reaction yield and the deprotection yields reaching 90 and 78%, respectively (Fig. 3a, d). LC-MS analysis was employed to compare the molecular weight (MW) of intact GFP-N149-ProcLys protein before and after deprotection (Fig. 3a). To examine the compatibility of our identified palladium catalysts, allyl2Pd2Cl2 and Pd(dba)2, are highly efficient propargyl carbamate cleavage reagents compatible with intact proteins.

Palladium-mediated propargyl carbamate cleavage in living cells.

To examine the compatibility of our identified palladium compounds within living cells, we first characterized their cytotoxicity by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. Allyl2Pd2Cl2 and Pd(dba)2 exhibited negligible toxicity to HeLa cells (three hours incubation, Supplementary Fig. 11) at a concentration of 10 μM. The nucleus and membrane integrity of the cells after palladium treatment were also confirmed by Hoescht 33342 nuclear staining and propidium iodide staining, respectively (Supplementary Fig. 8). Next, to exclude the possibility that the potential cell-damaging effects stimulated by these palladium compounds were not detectable until after a longer incubation time, we extended the toxicity analysis of these two palladium compounds to a longer timescale. First, HeLa cells and another five different cell lines (HEK293T, CHO, Caco-2, A549 and NIH3T3) were treated with 10 μM allyl2Pd2Cl2 or Pd(dba)2 for 24 h. No significant cytotoxic effect was observed in any cell line, consistent with our previous observations that the water-soluble phosphine ligand L1 was investigated, which decreased the efficiency for both the C4- and C5-mediated eliminations (Fig. 3a). Also, unlike Cu(II) ions utilized in the CuAAC reaction, working concentrations of these palladium species did not generate detectable reactive oxidative species (ROS) in the presence of reducing agents (Supplementary Fig. 8), and they also exhibited negligible damaging effects on GFP as well as on firefly luciferase, a more weakly folded protein (Supplementary Figs 9 and 10). Taken together, we demonstrated that our identified palladium catalysts, allyl2Pd2Cl2 and Pd(dba)2, are highly efficient propargyl carbamate cleavage reagents compatible with intact proteins.

**Figure 2 | Catalyst screening for palladium-mediated deprotection reactions.**

**a.** Structures of Proc-caged and Aloc-caged fluorogenic rhodamine 110 dyes (Proc-R110, 5; Aloc-R110, 6) that can be converted readily into the highly fluorescent R110 (7) (λex = 485 nm, λem = 520 nm) on deprotection triggered by palladium catalysts. The fluorescence turn-on property of these two fluorophores can be used to screen catalysts in depropargylation and deallylation reactions, respectively. 

**b.** The chemical formula and structure of palladium species and the water-soluble ligands used in this study. The water-insoluble palladium species were prepared as DMSO stock solutions and diluted into water before usage. 

**c.** Confirmation of the catalytic property of the identified highly efficient palladium catalysts in the deprotection of Proc-Lys. Deprotection of Aloc-Lys by the same palladium catalysts was used for comparison.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Substrate†</th>
<th>Product</th>
<th>Catalyst (10 mol%)</th>
<th>Yield (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Proc-Lys</td>
<td>Lys</td>
<td>C4</td>
<td>84</td>
</tr>
<tr>
<td>2</td>
<td>Aloc-Lys</td>
<td>Lys</td>
<td>C4</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>Proc-Lys</td>
<td>Lys</td>
<td>C5</td>
<td>82</td>
</tr>
<tr>
<td>4</td>
<td>Aloc-Lys</td>
<td>Lys</td>
<td>C5</td>
<td>22</td>
</tr>
</tbody>
</table>

*Yields were determined by LC analysis (Supplementary Methods 3).†PBS buffer (pH = 7.4), 37 °C, 8 h.
Pd(dba)$_2$ for three hours and then cultured for another 24 hours in fresh media before being analysed by MTS assay, and all showed high viability (>90%) (Fig. 4a). Additionally, the incubation times of palladium compounds were extended to 24 hours and 48 hours before being subjected to MTS assay to confirm the biocompatibility on a long timescale (Supplementary Fig. 13). Before being subjected to MTS assay to confirm the biocompatibility on a long timescale (Supplementary Fig. 13). Furthermore, the intracellular uptake and distribution of allyl$_2$Pd$_2$Cl$_2$ was analysed by inductively coupled plasma mass spectrometry (ICP-MS) after treating cells with 10$^{-5}$ M Pd loading was sufficient for in vivo depropargylation, live HeLa cells after depropargylation reactions were subjected to flow cytometric analysis or in-cell fluorescence assay (Supplementary Method 10). Both results indicate that 10$^{-5}$ M Pd loading was sufficient for in vivo depropargylation with high activity and low toxicity (Supplementary Fig. 16). In addition, to demonstrate the generality of this depropargylation chemistry in different cell lines, palladium-mediated deprotection of 5 was monitored in the aforementioned six cell lines by in-cell fluorescence assay and showed that 5 was readily converted into fluorogenic compound 7 in all cases (Fig. 4c). Fluorescence imaging was also conducted to verify the intracellular generation of 7 as well as the integrity of the nucleus and membrane in all these cell types (Supplementary Fig. 17). Taken together, the results for our two identified depropargylation palladium reagents establish satisfying biocompatibility and elimination efficiency in living cells.

**Palladium-mediated lysine liberation on intracellular proteins.** To apply our strategy of palladium-mediated lysine liberation to proteins within living cells, we first extended the Proc-Lys.
allyl\textsubscript{2}Pd\textsubscript{2}Cl\textsubscript{2}-mediated depropargylation reaction on GFP-Y40-deprotection efficiency. Following this protocol, we quantified the labelled by an azide-containing fluorophore through CuAAC. The after cells that harboured GFP-Y40-ProcLys were treated with 'clickable' handle (details in Methods). As shown in Fig. 4d, by taking advantage of the terminal alkyne moiety on Proc-Lys as reaction. To measure the depropargylation efficiency on proteins within living cells. POI, protein of interest. incorporation method to proteins expressed in mammalian cells. Following a protocol shown in Method

Figure 4 | Palladium-mediated propargyl carbamate cleavage within living cells. a, Cytotoxicity analysis of the two identified palladium reagents in six different cell lines (HeLa, CHO, HEK293T, NIH3T3, Caco-2 and A549). Cells were all treated with palladium compounds for three hours and then cultured for another 24 hours in fresh media before being subjected to MTS assay. Error bars represent ±1 s.d. from three independent experiments. b, Cellular uptake and distribution of allyl\textsubscript{2}Pd\textsubscript{2}Cl\textsubscript{2} analysed by ICP-MS. The palladium contents in all three cell fractions significantly increased after palladium treatment. Error bars represent ±1 s.d. from three independent experiments. c, In-cell fluorescence assay of palladium-induced decaging of S within different cell lines. Error bars represent ±1 s.d. from three independent experiments. d, Scheme illustrating the utilization of the alkyne moeity from the Proc group to quantify the depropargylation efficiency on proteins within living cells. POI, protein of interest. e, In-gel fluorescence analysis for determining the depropargylation yield on the GFP model protein within HeLa cells. Cells expressing GFP-Y40-ProcLys were treated with palladium compounds followed by cell lysis and labelling with an azide-Cy3 probe via a CuAAC reaction. The relative abundance of GFP-Y40-ProcLys in the cell lysate can be quantified by in-gel fluorescence analysis. Shown are the in-gel fluorescence and Coomassie staining of SDS-PAGE with the immunoblotting verification for GFP expression (anti-His) and equal loading (anti-\textalpha-tubulin). f, Quantifying the reaction yield of depropargylation on GFP-Y40-ProcLys inside HeLa cells through (1) the protocol shown in d and (2) the LC-MS-MS analysis of the target peptides after trypsin digestion. Although our current lysine-decaging efficiency on intracellular proteins is still low, this strategy may serve as a useful gain-of-function tool in rescuing the native activity of a target protein that can generate an amplifiable signal, such as an enzyme, a transcription
Palladium-triggered activation of a bacterial phosphothreonine lyase within host cells. To show that our palladium-triggered lysine depoprygalgal strategy can effectively modulate the function of an enzyme that contains a catalytic lysine, we focused on OspF, a phosphothreonine lyase known to be secreted into host cells through a *Shigella* Type III secretion system. On entering the host cells, OspF acts as an epigenetic modulator by irreversibly dephosphorylating mitogen-activated protein kinases (MAPKs), such as phosphorlyated Erk (p-Erk), which results in altered host inflammatory transcriptional responses. An unusual α-elimination mechanism was employed by OspF to remove irreversibly the phosphate group from phosphothreonine in the conserved Thr-Glu-Tyr (T-E-Y) motif on p-Erk, required for MAPK activity, to generate dehydrobutyrylamine bearing an unsaturated double bond. In a previous study showed that both Lys102 and Lys134 are essential residues for the phosphothreonine lyase activity of OspF towards MAPKs. In particular, it was proposed that Lys134 is the key catalytic residue that can donate a pair of electrons to remove the α-hydrogen of phosphorylated Thr219 on Erk. We found that the wild-type OspF protein (WT-OspF) can effectively dephosphorylate the p-Erk protein in vitro (Fig. 5b, lane 4). Interestingly, when Proc-Lys was site-specifically incorporated into OspF at position Lys134, the generated OspF variant (OspF-K134-Proc-Lys) exhibited no dephosphorylating activity on p-Erk (Fig. 5b, lane 3). Therefore, we confirmed that our Proc group can serve as a caging moiety to mask the activity of a specific lysine residue, and thus the associated enzymatic activity, from its embedded lysine-dependent protein. As expected, the addition of 10 μM allyl2Pd2Cl2 led to a dramatic decrease in luciferase activity (Fig. 6a). OspF-mediated irreversible dephosphorylation by OspF (Fig. 6a). Erk was known to undergo phosphorylation and dephosphorylation cycles, which determine its localization in the cytoplasm versus the nucleus of eukaryotic cells. Moreover, after a nucleus translocation of p-Erk from cytoplasm, the accumulation of Erk, even after its dephosphorylation, has been observed within the nucleus and this event, termed 'nucleus sequestration', resulted in retention of Erk in the nucleus to various degrees, depending on the source and intensity of extracellular stimulation. However, the intracellular localization of Erk damaged by OspF, via permanent dephosphorylation by OspF (Fig. 6a). Erk was known to undergo phosphorylation and dephosphorylation cycles, which determine its localization in the cytoplasm versus the nucleus of eukaryotic cells. Moreover, after a nucleus translocation of p-Erk from cytoplasm, the accumulation of Erk, even after its dephosphorylation, has been observed within the nucleus and this event, termed 'nucleus sequestration', resulted in retention of Erk in the nucleus to various degrees, depending on the source and intensity of extracellular stimulation. However, the intracellular localization of Erk damaged by OspF, via permanent dephosphorylation remains elusive. First, we found that GFP-tagged OspF-K134-Proc-Lys was retained within the nucleus, which indicates that the nucleus localization of OspF was independent of its catalytic activity and that the OspF-mediated dephosphorylation reaction occurs within the nucleus (Fig. 6b). Next, the localization of GFP-fused Erk (GFP-Erk) was monitored before and after the palladium-induced decaging of OspF-K134-Proc-Lys in HeLa cells that coexpress GFP-Erk and OspF-K134-Proc-Lys. In untreated cells, the overexpressed GFP-Erk accumulated in the nucleus after stimulating cells with phorbol 12-myristate 13-acetate (PMA), consistent with previous report. Rescued OspF, via the palladium-mediated decaging of OspF-K134-Proc-Lys, caused a gradual loss of nucleus retention of GFP-Erk. After 180 minutes, 12% of the total GFP-Erk pool was
exported to cytoplasm (Fig. 6c,d, Supplementary Fig. 21). By contrast, GFP-Erk remained within the nucleus throughout the experiment for palladium-treated cells without the expression of WT-OspF or OspF-K134-ProcLys, or for cells without palladium treatment (Supplementary Fig. 22). Together, these observations reveal that the OspF-dephosphorylated Erk was irreversibly exported from the nucleus to the cytosol, which led to an accumulation of damaged Erk in the cytoplasm (Fig. 6a). The impairment of Erk’s subcellular balance between the nucleus and the cytoplasm may largely contribute to OspF’s virulence effects within host cells. Therefore, our palladium-triggered decaging strategy was able to turn on sufficient OspF activity to address its pathological roles in modulating Erk’s phosphorylation level and thus the MAPK signalling cascade.

Figure 5 | Modulating the activity of a bacterial phosphothreonine lyase OspF via palladium-mediated decaging of lysine in vitro and in vivo.

a. Mechanism of the OspF-catalysed irreversible dephosphorylation of its substrate p-Erk. The key catalytic lysine residue 134 on OspF is highlighted in red. Our palladium-triggered Proc-Lys depropargylation strategy on OspF may modulate its irreversible dephosphorylation activity on p-Erk (shown in the yellow-shaded box).
b. The dephosphorylation assay on p-Erk performed in vitro. Purified WT-OspF can dephosphorylate p-Erk effectively (lane 4), and the OspF-K134-ProcLys variant protein exhibited no dephosphorylation activity on p-Erk (lane 3). The addition of palladium compounds can convert the Proc-Lys residue into free Lys and thus restore OspF’s dephosphorylation activity (lanes 1 and 2).
c. The p-Erk dephosphorylation assay performed in vivo. In contrast to WT-OspF, the expressed OspF-K134-ProcLys protein inside the cells exhibited no dephosphorylation activity on p-Erk (lanes 4 and 5). The addition of palladium compounds in live cells can restore OspF’s dephosphorylation activity (lanes 1 and 2).
d. Time-dependent OspF-K134-ProcLys activity restoration in vivo.
e. Dual luciferase assay measures the restoration of relative WT-OspF activity from OspF-K134-Proclys on palladium treatment in living cells. The relative luminescence intensity is proportional to the level of intracellular p-Erk. Columns 2 and 4 verify that the addition of allyl2Pd2Cl2 in cells bearing OspF-K134-Proclys caused a significant decrease of p-Erk level within HeLa cells. Error bars represent ±1 s.d. from three independent experiments. The relative intensities (Rel. int.) of WT-OspF and OspF-K134-Proclys in living HeLa cells are shown for comparison.
OspF is the prototype of a newly identified OspF family of phosphothreonine lyases that, as yet, has no homologues in eukaryotic cells. The extremely high specificity of OspF towards MAPKs (Erk and p38) made it an attractive protein-based inhibitor for such kinases that have no potent small-molecule inhibitors currently available. Recent work used OspF as a tool to rewire kinase pathways in yeast and immune cells, which may provide a new strategy to engineer cells for therapeutic or biotechnological applications.

However, OspF irreversibility dephosphorylates its substrates and thus permanently impairs MAPK signalling pathways, and so the controlled activation of this enzyme with spatial and temporal precision is desired. Our chemical decaging strategy may create a 'proenzyme' form for OspF that would rely on the spatial–temporal activation of this enzyme with spatial and temporal precision. This was achieved by rational design and catalyst screening for a biocompatible protection group/catalyst pair on fluorogenic small-molecule reporters as well as on intact fluorescent proteins, followed by validating the efficiency and compatibility with live cells. The Proc moiety served as an effective caging group to mask the activity of a lysine residue from an intact protein, whereas our identified palladium catalysts provided a facile approach to convert this genetically encoded chemically caged lysine analogue into free lysine, which led to rescued protein activity under living conditions. This study represents the first example, to our knowledge, of a transition metal utilized to activate a target protein specifically within its native cellular context. Our strategy may be generally applicable to control chemically the liberation of...
key lysine residue(s) from a given protein, and so facilitate intracellular manipulation of its lysine-dependent activity. In addition, our work extends the rapidly expanding toolkit of palladium-mediated intracellular cleavage reactions from small molecules to proteins, which may ignite more interest in exploiting transition metals for the manipulation of proteins under living conditions. Importantly, whereas our palladium catalysts alone did not cause the change in the Erk phosphorylation level in vitro and in vivo, our results indicate that ultraviolet irradiation triggered significant p-Erk dephosphorylation when applied to living HeLa cells. Therefore, our chemical decaging strategy developed here offers a complimentary approach based on small molecules to the commonly used photocaging methods that rely on photosensitive UAAs for site-specific control of protein activity, particularly when ultraviolet-induced photodamage becomes a concern. Furthermore, in comparison with small-molecule allosteric activators or the chemical rescue strategies, our approach possesses unique features:

(1) As a result of its mechanism-based design, this method is generally applicable to virtually any given protein that contains the lysine of interest.

(2) The mutant proteins utilized in the chemical rescue strategy usually exhibit low, but detectable, wild-type activity, which may interfere with the subsequent activation process to a certain extent, particularly when the protein has an amplifiable signal (for example, enzymes). In contrast, in our method the catalytic lysine residue is caged directly, which completely ablates the protein activity and thus creates a clean background until the cleavage reagent-mediated decaging.

(3) Sometimes an in vitro activation compound turns out to be an inhibitor for the same enzyme within cells, probably because of the interference with protein’s intracellular functions, such as substrate recruitment. In contrast, our strategy only creates a point mutation in the active pocket, but does not affect other areas of the protein. This, in conjunction with the single-component palladium reagent we used, could largely avoid such problems.

(4) Instead of non-covalent small-molecule binders that reversibly avoid such problems.

(5) Instead of non-covalent small-molecule binders that reversibly avoid such problems.

(6) Instead of non-covalent small-molecule binders that reversibly avoid such problems.

(7) Instead of non-covalent small-molecule binders that reversibly avoid such problems.

(8) In contrast, our strategy only creates a point mutation in the active pocket, but does not affect other areas of the protein. This, in conjunction with the single-component palladium reagent we used, could largely avoid such problems.

A fast-growing list of UAAs, including various caging analogues of the canonical 20 amino acids, have been genetically encoded in diverse living cells as well as in multicellular organisms. This, in conjunction with the rapidly emerging biofriendly usage of transition metals in various intracellular chemical conversions, may enable a reservoir of protecction chemistry to control the release of amino-acid residues other than lysine. Such a biocompatible chemical decaging strategy would permit small-molecule catalysts to mediate selectively the function of an intracellular protein of interest, particularly in a gain-of-function and site-specific fashion.

Methods
The chemical synthesis procedures and detailed protocols are all included in the Supplementary Information.

Propargyl carbamate cleavage reaction on purified proteins. Palladium reagents (Sigma) were prepared either as a 10 mM stock in DMSO solution (1:1,000 dilution with water solution when used) or directly dissolved in water for Pd(NO3)2 and Na2PdCl4. Phosphine ligands (P(Ph3)2N4)2 (IK Chemicals) were prepared as 20 mM stock solutions in pure water. All these materials were prepared just before use.

For Proc-Lys incorporation, a plasmid that encoded the target protein carrying an in-frame amber mutation was co-transfected with a plasmid expressing the PyrRS/tRNA-Pro (Sigma) pair into cells via X-tremeGENE HP (Roche) in DMEM (10% FBS) supplemented with 1 mM Proc-Lys. Cells were allowed to grow for an additional 24 hours to express the desired protein bearing a site-specifically incorporated Proc-Lys residue. For depropargylation on proteins, cells that harbored the Proc-Lys-incorporated proteins were further cultured in DMEM without Proc-Lys and FBS for 180 minutes, followed by treatment with palladium reagents (10 μM) in fresh DMEM for another 180 minutes to allow the propargyl carbamate cleavage reaction to proceed inside mammalian cells.

Quantifying propargyl carbamate cleavage efficiency on intracellular proteins. The Proc-Lys-bearing protein within cells before and after the palladium-mediated depropargylation were labelled by an azide-Cy3 probe (200 μM final concentration) in the Cu(2+)/BTTAA (BTTAA = bis(t-butyl)tetramethyl-1-azoniumyl)aminomethyl)-1H,1,2,3-triazol-4-acetic acid) (100 μM final concentration, Cu/BTTAA = 1:2) mediated CuAAC reaction in cell lysate (2 mg ml−1 final concentration, quantified by bicinchoninic acid assay). The labelling reaction was allowed to proceed for one hour at 30 °C before being quenched by BCS and 5 × SDS sample buffer. The labelled lysates were then subjected to SDS–PAGE and in-gel fluorescence analysis (Typhoon-FLA9500, GE) to quantify the propargyl carbamate cleavage efficiency on proteins. In a separate approach, the target protein was purified or immunoprecipitated from cells and subjected to in-gel trypsin digestion. The deprotection efficiency was quantified by comparing the Proc-Lys protected and deprotected peptides from LC-MS/MS analysis.

Palladium-mediated depropargylation of OspF in mammalian cells. HeLa cells were seeded into a 24-well corning plate to confluence in DMEM that contained 10% FBS and penicillin/streptomycin. Palladium-mediated depropargylation were labelled by an azide-Cy3 probe (200 μM final concentration) in the Cu(2+)/BTTAA (BTTAA = bis(t-butyl)tetramethyl-1-azoniumyl)aminomethyl)-1H,1,2,3-triazol-4-acetic acid) (100 μM final concentration, Cu/BTTAA = 1:2) mediated CuAAC reaction in cell lysate (2 mg ml−1 final concentration, quantified by bicinchoninic acid assay). The labelling reaction was allowed to proceed for one hour at 30 °C before being quenched by BCS and 5 × SDS sample buffer. The labelled lysates were then subjected to SDS–PAGE and in-gel fluorescence analysis (Typhoon-FLA9500, GE) to quantify the propargyl carbamate cleavage efficiency on proteins. In a separate approach, the target protein was purified or immunoprecipitated from cells and subjected to in-gel trypsin digestion. The deprotection efficiency was quantified by comparing the Proc-Lys protected and deprotected peptides from LC-MS/MS analysis.

Dual luciferase assay to monitor the phosphorylation level of Erk. The in vivo dual luciferase assay on p-Erk was performed following the manufacturer’s instructions (Promega) and a previous report. Plasmids that encode Gal4-Elk, Gal4-luc and pRL-TK were transfected in combination with plasmids used in the above western blotting analysis. Cells after transfection and palladium treatment were incubated in fresh medium for another 12 hours before the relative p-Erk levels were assayed to determine the separation of soluble fractions from cell debris and SDS–PAGE analysis. Western blotting analysis was carried out to detect the full-length OspF protein as well as the dephosphorylation level of p-Erk by using antibodies against OspF, p-Erk1/2 or Erk1/2.

Live-cell fluorescence imaging. HeLa cells were seeded into a 24-well corning plate and allowed to grow to approximately 90% confluence in DMEM (10% FBS) that contained penicillin/streptomycin. Transfection was carried out using the same protocol as in the western blotting analysis. After transfection, cells were grown for 18 hours and transferred onto an eight-well Lab-Tek chambered coverslip for another eight hours in DMEM and in the absence of Proc-Lys. The p-Erk level in cells were first elevated by PMA (0.01%) followed by treatment with palladium reagents (10 μM) for three hours and fluorescence imaging by a Zeiss LSM 700 laser confocal microscope with a 40× oil-immersion objective in a scan zoom (averaging 16). The mean fluorescence intensities of nucleus (F0) and cytoplasm (F0) were quantified using ZEN software to enable the F0/F0 ratio to be determined according to the formula F0/F0 = (F0 − Fc)(F0 − Fc), where F0 is the mean background fluorescence intensity.
Received 14 August 2013; accepted 5 February 2014; published online 16 March 2014

References


Acknowledgements

This work was supported by research grants from National Natural Science Foundation of China (21225206 and 91313301) and the National Key Basic Research Foundation of China (2010CB912302). We thank F. Shao and O. Schneewind for the donation of plasmids.

Author contributions

P.R.C. conceived and designed the experiments. J.L., J.Y., J.Z., W.Z., S.Z., S.L., L.C., M.Y., S.J. and X.Z. performed the experiments. P.R.C., J.L., J.Y. and J.W. analysed the data. P.R.C., J.L. and J.Y. prepared the figures and co-wrote the paper, with input from all the authors.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to P.R.C.

Competing financial interests

The authors declare no competing financial interests.