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Protocol

Protocol for identification and validation of 2’3’-cGAMP-binding proteins by photoaffinity probes

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SUMMARY

Mammalian cyclic dinucleotide 2’3’-cGAMP functions as a second messenger in innate immune response. Here, we report a protocol to utilize 2’3’-cGAMP photoaffinity probes to capture 2’3’-cGAMP-binding or 2’3’-cGAMP-interacting proteins from HeLa cell lysate for in-gel visualization by fluorescent imaging or identification by SILAC–based quantitative MS. Further validation is also executed using photoaffinity probes to demonstrate the direct interaction of 2’3’-cGAMP with purified target proteins in vitro or endogenous target proteins in 293T cells.

For complete details on the use and execution of this profile, please refer to Hou et al. (2021).

BEFORE YOU BEGIN

Design and synthesis of photoaffinity probes of 2’3’-cGAMP (Probe 1–3) (Figure 1) have been reported (Hou et al., 2021). These probes bear a diazirine as the photocrosslinkable group and a terminal alkyne as the clickable group. Under irradiation at 365 nm, the probes can form a covalent connection with their binding or interacting proteins, which can be visualized in protein gel by conjugation with fluorescence tag (Rh-N3) or be identified/validated after conjugation to affinity tag (Biotin-N3) via click chemistry (Figure 2). In the competitive experiments, 2’3’-cGAMP with higher concentration can efficiently block the association between target proteins and probes, which decreases the labeling intensity or pulldown amount of target proteins by probes. From the literature (Flaxman et al., 2019), the diazirine group has a 9.0 Å labeling radius upon photoactivation and the distance constraint between the diazirine carbon and the conjugated residues is 5.0 Å, which can be considered as a general reference of distance between the probe and the target protein for crosslinking.

The protocol below describes the specific steps for endogenous EF1A1 in EF1 complex using Probe 3. However, this protocol is also suitable for Probe 1 and Probe 2 and other target proteins.

Preparation of cell lysate

© Timing: 3 days
1. Culture HeLa cells in 2 × 10-cm dishes to 90% confluency in DMEM medium with 10% FBS and 1% penicillin-streptomycin in a 5% CO₂ incubator at 37°C.

2. Remove medium from dishes, rinse cells with 5 mL PBS, and treat cells with 1 mL Trypsin-EDTA solution for 3 min in the incubator. Resuspend the cells with 2 mL DMEM medium with 10% FBS, transfer the cell suspension from 2 dishes to a 15 mL conical tube.

3. Centrifuge 5 min at 500 g, 4°C, and remove supernatant. Using the same centrifugation conditions, wash the cells 2 times with 5 mL PBS. After the last wash, resuspend the cells in 1 mL of 1% NP-40 buffer.

4. Lyse the suspension with the tube in the ice by Ultrasonic Homogenizer with 2 mm probe. Repeat two times in order to fully lyse the cells. Condition: power 20%; working time: 1 min; ultrasonic opening time: 3 s, ultrasonic turn-off time: 3 s.

5. Transfer cell lysate to 2 × 1.5 mL microcentrifuge tubes, centrifuge 15 min at 20,000 g, 4°C, and collect the supernatant in a 1.5 mL microcentrifuge tube.

6. Determine the protein concentration of the supernatant by BCA assay, and adjust the concentration to 3 mg/mL by adding 1% NP-40 buffer, and store in −80°C freezer.

Note: This preparation of cell lysate is also suitable for other cell lines.

Preparation of SILAC HeLa cell lysates

© Timing: 5 weeks

7. According to the references (Ong et al., 2002; Chen et al., 2015), culture HeLa cells in DMEM (deficient in L-arginine and L-lysine) supplemented with 10% dialyzed FBS, 1% penicillin-streptomycin, 2 mM glutamine, 4.34 mM proline, and different isotope labeled lysine and arginine (listed in the table below) in a 5% CO₂ incubator at 37°C.
8. Confirm the complete incorporation of different isotope labeled amino acids in the cells by MS analysis after cells culturing in SILAC medium for 5–7 generations.

\[ \text{Δ CRITICAL: It is critical to ensure the complete SILAC labeling of cells for the quantification and interpretation of the experimental results. The standard procedures to confirm complete SILAC labeling of cells by MS are provided in problem 5.} \]

9. Follow Step 1-6 to obtain SILAC HeLa cell lysates.

Note: The alternative method to collect cells by using cell scrapers is recommended if the interested or targeted proteins are cell membrane proteins, to prevent unnecessary digestion or damage of these proteins by collecting cells using Trypsin-EDTA.

**Preparation of purified EF1A1 in EF1 complex from 293T cells**

© Timing: 9 days

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**Figure 2.** Probe labeling of cell lysate for in-gel visualization and identification by MS
10. Culture 293T cells stably overexpressing Flag-EF1D in 50 × 10-cm dishes to 90% confluency in DMEM medium with 10% FBS and 1% penicillin-streptomycin in a 5% CO2 incubator at 37°C.

**Note:** As demonstrated in the reference (Hou et al., 2021), immunoprecipitation of Flag-EF1D using Flag beads can isolate and purify EF1A1 in EF1 complex from 293T cells, which is validated by SDS-PAGE analysis, WB using EF1A1 specific antibody and MS identification.

11. Remove medium from dishes, rinse cells with 5 mL PBS, resuspend cells in 4 mL PBS and transfer cell suspension to 5 × 50 mL conical tubes.

12. Centrifuge 5 min at 500 × g, 4°C, and remove supernatant. Resuspend the cells in 10 mL of 1% NP-40 buffer in each tube and transfer 2 × 25 mL suspension into 2 × 50 mL round-bottom centrifuge tubes.

13. Lyse the suspension by Ultrasonic Homogenizer with 6 mm probe. Repeat two times in order to fully lyse the cells. Condition: power 20%; working time: 1 min; ultrasonic opening time: 3 s, ultrasonic turn-off time: 3 s.

14. Centrifuge 15 min at 20,000 × g, 4°C, and collect the supernatant in 2 × 50 mL conical tubes.

15. Dilute the supernatant with the same amount of 0.2% NP-40 buffer, and incubate with 500 μL Flag beads (50% suspension) in each tube for 4 h at 4°C with gentle rotating.

16. Centrifuge 5 min at 4,000 × g at 4°C to remove supernatant. Resuspend the beads with 2 mL 0.2% NP-40 buffer and transfer the beads to a 15 mL conical tube.

17. Centrifuge 5 min at 4,000 × g at 4°C to collect the beads. Using the same centrifugation conditions, wash the beads 3 times with 14 mL 0.2% NP-40 buffer, followed by 3 times with 14 mL 10% glycerol in PBS.

18. Elute the bound proteins from the beads by incubation with 1 mL elution buffer (0.5 mg/mL 3× Flag peptide in PBS with 10% glycerol) for 30 min at 4°C. Collect the supernatant after centrifugation for 5 min at 4,000 × g at 4°C. Repeat elution 8 times in total.

19. Combine all elutions and concentrate to 0.5 mL by a 15 mL concentrator with 30 kD cutoff. Determine the protein concentration with Bradford assay. Store in −80°C freezer.
**Note:** As shown in Figure 3, purified EF1 complex is examined by SDS-PAGE and EF1A1 is verified by WB using EF1A1 antibody. The protein solution still contains 3× Flag peptide, which doesn’t affect probe labeling or measurement of protein concentration by Bradford assay. This preparation of the purified protein is also suitable for other overexpressed Flag-tag proteins.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| EF1A1 Mouse Monoclonal antibody | Proteintech | Cat# 67495-1-lg, RRID:AB_2882719 |
| EF1D Mouse Monoclonal antibody | Proteintech | Cat# 60085-1-lg, RRID:AB_2096996 |
| Anti-FLAG-HRP Polyclonal Conjugate avidin-HRP | Sigma-Aldrich | Cat#F7425, RRID:AB_439867 |
|                    | Jackson Immuno Research Labs | Cat# 115-035-003, RRID:AB_10015289 |

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Chemicals, peptides, and recombinant proteins** |    |            |
| Anti-Flag(DYKDDDDK) affinity gel | Bimake | Cat#B23102 |
| 3× Flag peptide | Bimake | Cat#B23112 |
| High capacity streptavidin agarose | Thermo Fisher Scientific | Cat#20359 |
| Protease inhibitor cocktail (100×) | Bimake | Cat#B14003 |
| Unlabeled L-lysine hydrochloride (Lys-0) | Silantes | Cat#211004102 |
| 4,4',5,5'-D4-L-lysine dihydrochloride (Lys-4) | Silantes | Cat#211104113 |
| 13C, 15N-L-lysine hydrochloride (Lys-8) | Silantes | Cat#211604102 |
| unlabeled L-arginine hydrochloride (Arg-0) | Silantes | Cat#210104102 |
| 13C-L-arginine hydrochloride (Arg-6) | Silantes | Cat#21204102 |
| 13C, 15N-L-arginine hydrochloride (Arg-10) | Silantes | Cat#21304102 |
| DMEM deficient in L-lysine and L-arginine | Thermo Fisher Scientific | Cat#88364 |
| Fetal Bovine Serum Dialyzed | Gibco | Cat#30067-334 |
| DMEM (high glucose, pyruvate) | Gibco | Cat#11995065 |
| Fetal Bovine Serum | Gibco | Cat#10099141 |
| PBS (Dulbecco’s phosphate-buffered saline) | BioChannel | Cat#BC-BPBS-08 |
| Trypsin-EDTA Solution with phenol red (0.25% Trypsin) | BioChannel | Cat#BC-CE-005 |
| Sequencing Grade Modified Trypsin | Promega | Cat#V5111 |
| 2′,3′-cGAMP | Zhang et al. (2013) | n/a |
| 2′,3′-cGAMP probes (Probe 1-3) | Hou et al. (2021) | n/a |
| Rhodamine-N3 | Jiang et al. (2010) | n/a |
| Biotin-N3 | Jiang et al. (2010) | n/a |
| CuBr | Bidepharm | Cat#BD122226, CAS No. 7787-70-4 |
| CuSO4 $\cdot$5H2O | China National Pharmaceutical Group Corp | Cat#10008218, CAS No. 7758-99-8 |
| TCEP | Bidepharm | Cat#BD155793, CAS No. 51805-45-9 |
| TBTA | TCI | Cat#T2993, CAS No. 510758-28-8 |
| STF-1623 | Carozza et al. (2020) | n/a |

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Critical commercial assays** |    |            |
| BCA Protein Quantification Kit | Yeasen Biotech | Cat#20201ES76 |
| Quick Start Bradford 1x Dye Reagent | Bio-Rad | Cat#500-0205 |

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Experimental models: Cell lines** |    |            |
| HeLa cells | Junying Yuan’s Lab, IRCBC | n/a |
| HEK 293T cells | Junying Yuan’s Lab, IRCBC | n/a |
| 293T cells stably overexpressing Flag-EF1D | Hou et al. (2021) | n/a |

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Software and algorithms** |    |            |
| ImageJ | National Institutes of Health | https://imagej.nih.gov/ij/ |
| "Wu Kong" platform | n/a | https://www.omicsolution.com/wkomics/main/ |
| Graphpad | GraphPad Software Inc. | https://www.graphpad.com |
**MATERIALS AND EQUIPMENT**

### 1% NP-40 buffer

| Reagent                        | Final concentration | Amount  |
|--------------------------------|--------------------|---------|
| Tris-HCl (1 M), pH 7.4         | 25 mM              | 25 mL   |
| NaCl (4 M)                     | 150 mM             | 37.5 mL |
| Glycerol                       | 10%                | 100 mL  |
| NP-40                          | 1%                 | 10 mL   |
| ddH₂O                           | n/a                | 827.5 mL|
| Total                          | n/a                | 1 L     |

Can be stored at 4°C for up to 3 months. Protease Inhibitor Cocktail (100×) will be added before use.

### 0.2% NP-40 buffer

| Reagent                        | Final concentration | Amount  |
|--------------------------------|--------------------|---------|
| Tris-HCl (1 M), pH 7.4         | 25 mM              | 25 mL   |
| NaCl (4 M)                     | 150 mM             | 37.5 mL |
| Glycerol                       | 10%                | 100 mL  |
| NP-40                          | 0.2%               | 2 mL    |
| ddH₂O                           | n/a                | 835.5 mL|
| Total                          | n/a                | 1 L     |

Can be stored at 4°C for up to 3 months. Protease Inhibitor Cocktail (100×) will be added before use.
### 1% Brij 97 buffer

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| NaCl (4 M)               | 150 mM              | 3.75 mL  |
| Triethanolamine (1 M)    | 50 mM               | 5 mL     |
| Brij 97                  | 1%                  | 1 mL     |
| ddH₂O                    | n/a                 | 90.25 mL |
| Total                    | n/a                 | 100 mL   |

Can be stored at 4°C for up to 3 months. Protease Inhibitor Cocktail (100×) will be added before use.

### 6× loading buffer

| Reagent                          | Final concentration | Amount   |
|----------------------------------|---------------------|----------|
| Tris-HCl (0.5M), pH 6.8          | 60 mM               | 24 mL    |
| SDS                              | 12%                 | 24 g     |
| Bromophenol blue                 | 0.06%               | 120 mg   |
| DTT                              | 600 mM              | 18.6 g   |
| Glycerol                         | 49%                 | 98 mL    |
| ddH₂O                            | n/a                 | 42 mL    |
| Total                            | n/a                 | 200 mL   |

Can be stored at -20°C for up to one year, and can be diluted accordingly.

### 4% SDS buffer

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| NaCl (4 M)               | 150 mM              | 3.75 mL  |
| Triethanolamine (1 M)    | 50 mM               | 5 mL     |
| SDS                      | 4%                  | 4 g      |
| ddH₂O                    | n/a                 | 91.25 mL |
| Total                    | n/a                 | 100 mL   |

Can be stored at 4°C for up to 3 months.

### Coomassie brilliant blue G-250 buffer

| Reagent                  | Final concentration | Amount   |
|--------------------------|---------------------|----------|
| Coomassie brilliant blue G-250 | 0.25%          | 2.5 g    |
| MeOH                     | 45%                 | 450 mL   |
| CH₃COOH                  | 10%                 | 100 mL   |
| ddH₂O                    | 45%                 | 450 mL   |
| Total                    | n/a                 | 1 L      |

Can be stored at 25°C for up to one year.

### Destaining buffer

| Reagent | Final concentration | Amount   |
|---------|---------------------|----------|
| MeOH    | 45%                 | 450 mL   |
| CH₃COOH | 10%                 | 100 mL   |
| ddH₂O   | 45%                 | 450 mL   |
| Total   | n/a                 | 1 L      |

Can be stored at 25°C for up to one year.

### STEP-BY-STEP METHOD DETAILS

**Labeling of cell lysates using 2’3’-cGAMP probe (Probe 3)**

© Timing: 2 days
This section describes how to visualize the potential 2’3’-cGAMP-binding or -interacting proteins present in the cell lysate based on fluorescence image of the protein gel by using Probe 3.

1. Set up the labeling assay in 1.5 mL microcentrifuge tubes as below:

| Sample No | 1 | 2 | 3 | 4 |
|-----------|---|---|---|---|
| Cell lysate (3 mg/mL) | 20 µL | 20 µL | 20 µL | 20 µL |
| 2’3’-cGAMP (1 mM) | – | – | – | + |
| Probe 3 (10 µM) | – | + | + | + |
| UV (365 nm) | + | – | + | + |

For the competitive labeling sample, add 2’3’-cGAMP (0.5 µL 40 mM stock in H₂O, final 1 mM) into 60 µg cell lysate (Sample No: 4) in the 1.5 mL microcentrifuge tubes, and incubate at 4°C for 1 h.

For non-competitive labeling samples, add the same volume of water instead of 2’3’-cGAMP into 60 µg cell lysate (Sample No: 1, 2, 3) in the 1.5 mL microcentrifuge tubes, and incubate at 4°C for 1 h.

2. Add Probe 3 (0.5 µL 0.4 mM stock in H₂O, final 10 µM) into the samples (Sample No: 2–4), respectively, and incubate at 4°C for 1 h. For the control sample, add the same volume of water instead of Probe 3 into the sample (Sample No: 1), and incubate at 4°C for 1 h.

3. Transfer the samples (Sample No: 1, 3, 4) onto the Parafilm inside of the 48-well plate lid on ice, and irradiate at 365 nm (CL-100 Ultraviolet Crosslinker, 9999 µJ/cm²) for 30 min (Figure 4). For the control sample, don’t irradiate it (Sample No: 2).

4. Collect each sample into a 1.5 mL microcentrifuge tube, and add urea (19 mg) to the final 8 M.

**Note:** The addition of urea into the sample to final 8 M increases the sample volume to 40 µL (2 times of the original volume).

△ CRITICAL: It is very important to execute protein denaturing by urea for next step of click chemistry, which eliminates the false positive labeling of proteins that don’t interact with probes directly.

5. Perform click chemistry to conjugate Rh-N₃. Add Rh-N₃ (0.9 µL 10 mM stock in DMF, final 225 µM), TBTA (0.6 µL 10 mM stock in DMF, final 150 µM), CuBr (1.5 µL 20 mM stock in DMSO, final 750 µM), CuSO₄ (1.5 µL 20 mM stock in H₂O, final 750 µM), and TCEP (1.5 µL 20 mM stock in H₂O, final 750 µM) into 34 µL each sample (final 40 µL) in order, and incubate the reaction mixture at 22°C for 2 h in the dark.

△ CRITICAL: It is very important to add the reagents in order to have maximum reaction efficiency. TCEP solution should be made freshly before use. Vortex briefly after addition of each reagent.

6. Mix the samples with 20 µL 6× loading buffer and resolve the samples (20–30 µg protein equivalent for each sample) by SDS-PAGE using a 12% acrylamide gel.

7. Fix the gel by incubation with a destaining buffer for 1 h in the dark on a mini-shaker.

**Note:** During the incubation with the destaining buffer, the background of fluorescence from unreacted Rh-N₃ will be eventually washed out from the gel. If the background fluorescence of the gel is still high, extend the period of incubation with destaining buffer to 2–4 h.

8. Scan the gel using a Typhoon 7000 imager to record the Rhodamine (Rh) fluorescence image. For representative fluorescence image figure, see Figure 2A in Hou et al. (2021).
9. Stain the gel with Coomassie blue, and record the image of protein gel with a digital camera.

**Note:** This method is also suitable for visualization of potential 2’3’-cGAMP-binding or -interacting proteins present in different subcellular fractions, tissue lysate or other protein sources. The protein bands with significantly higher fluorescence signal in Sample No. 3 than in Sample No. 1, 2 and 4 are considered as potential 2’3’-cGAMP-binding or -interacting proteins. Because there is no Probe 3 in Sample 1 and no UV irradiation in Sample 2, no obvious fluorescence signal will be seen in these two samples. Nevertheless, the level of reduced signal in Sample 4 compared with Sample 3 is dependent on the binding affinities of the potential target proteins with 2’3’-cGAMP and the probe.

### Pulldown MS experiments using Probe 3 with SILAC HeLa cell lysate

© **Timing:** 3 days

This section describes how to identify the potential 2’3’-cGAMP-binding or -interacting proteins from the SILAC cell lysates by probe pulldown and mass spectrometry.

10. For the competitive pulldown samples (Sample No: 3, 6), add 2’3’-cGAMP (25 μL of 40 mM stock in H2O, final 1 mM) into the cell lysates (1 mL of 2 mg/mL solution) in a 1.5 mL microcentrifuge tube, and incubate at 4°C for 1 h. For the non-competitive pulldown samples (Sample No: 1, 2, 4, 5), add the same volume of water instead of 2’3’-cGAMP into the cell lysates in a 1.5 mL microcentrifuge tubes, and incubate at 4°C for 1 h.

| Sample no. | 1 | 2 | 3 | 4 | 5 | 6 |
|------------|---|---|---|---|---|---|
| **Normal set of samples** | | | | | | |
| SILAC labeling | L | M | H | H | L | M |
| Cell lysate (2 mg/mL) | 1 mL | 1 mL | 1 mL | 1 mL | 1 mL | 1 mL |
| 2’3’-cGAMP (1 mM) | – | – | + | – | – | + |
| Probe 3 (10 μM) | – | + | + | – | + | + |
11. Add Probe 3 (5 μL 2 mM stock in H2O, final 10 μM) into the samples (Sample No: 2, 3, 5, 6), and incubate at 4°C for 1 h. For the control sample, add the same volume of water instead of Probe 3 into the samples (Sample No: 1, 4), and incubate at 4°C for 1 h.

12. Transfer each sample into 24-well plates (200 μL/well) on ice, and irradiate them at 365 nm (CL-100 Ultraviolet Crosslinker, 9999 μJ/cm²) for 30 min (Figure 4).

13. Collect Normal Set of Samples (Sample No: 1, 2, 3, total 3 mL 6 mg cell lysate) into a 50 mL conical tube, and add 2.8 g urea to the final 8 M. Same for Reverse Set of Samples (Sample No: 4, 5, 6, total 3 mL 6 mg cell lysate).

**Note:** The addition of urea into the sample to final 8 M increases the sample volume to 6 mL (2 times of the original volume).

△ **CRITICAL:** It is very important to execute protein denaturing by urea for next step of click chemistry, which eliminates the false positive hits.

14. Perform click chemistry to conjugate Biotin-N3. Add Biotin-N3 (135 μL 10 mM stock in DMF, final 225 μM), TBTA (90 μL 10 mM stock in DMF, final 150 μM), CuBr (225 μL 20 mM stock in DMSO, final 750 μM), CuSO4 (225 μL 20 mM stock in H2O, final 750 μM), and TCEP (225 μL 20 mM stock in H2O, final 750 μM) into 5.1 mL each sample (final 6 mL) in order, and incubate the reaction mixture at 22°C for 2 h in the dark with gentle rotating.

△ **CRITICAL:** It is very important to add the reagents in order to have maximum reaction efficiency. TCEP solution should be made freshly before use. Vortex briefly after addition of each reagent.

15. Perform protein precipitation.
   a. Add 7.98 mL methanol (ice-cold), 3 mL chloroform (ice-cold) and 6 mL water (ice-cold) into samples. Vortex and then centrifuge 40 min at 4,500 × g, 4°C.
   b. Wash the pellets with 8 mL of ice-cold methanol twice, and centrifuge 25 min at 4,500 × g, 4°C.
   c. Remove as much methanol as possible, and allow the pellets to dry for about 20 min.

16. Perform protein resolubilization.
   a. Resuspend the pellets in 600 μL 4% SDS buffer with 1 mM EDTA.
   b. Sonicate the samples by Ultrasonic Homogenizer with 2 mm probe. Repeat two times. Condition: power 4%; working time: 10 s; ultrasonic opening time: 2 s, ultrasonic turn-off time: 2 s.
   c. Adjust the SDS concentration below 0.4% by adding 6 mL 1% Brij 97 buffer.
   d. Centrifuge 15 min at 4,500 × g, 22°C. Collect the supernatants as the protein samples for the following enrichment.

17. Perform protein enrichment.
   a. Prewash High Capacity Streptavidin Agarose (40 μL 50% aqueous slurry for each sample) three times with 1 mL of 1% Brij 97 buffer in 15 mL conical tubes by centrifugation at 4,000 × g for 5 min at 22°C for each time.
   b. Incubate the protein samples with the prewashed beads in 15 mL conical tubes for 1.5 h at 22°C with gentle rotating.
   c. Centrifuge 5 min at 4,000 × g at 22°C to remove supernatant. Resuspend the beads with 1 mL 1% SDS in PBS and transfer the beads to a 1.5 mL microcentrifuge tube.
   d. Centrifuge 5 min at 4,000 × g at 22°C to collect the beads. Using the same centrifugation conditions, wash the beads 4 times with 1 mL of 1% SDS in PBS, followed by 4 times with 1 mL of 0.2% SDS in PBS and finally 4 times with 1 mL PBS. Be sure that all beads have been resuspended during the washes by inverting the tube 3–4 times.
   e. After the last wash, dry the beads with a gel loading tip.
**Note:** After protein enrichment, 2′3′-cGAMP-binding or -interacting proteins (endogenous or exogenous) have been enriched on the beads. Thus it is also feasible to detect them by western blot using their antibodies. For representative western blot figure, see Figures 1D and 2F in Hou et al. (2021).

18. Perform on-bead tryptic digestion.
   a. Resuspend the beads in 500 μL of 6 M urea in PBS and transfer to a 1.5 mL microcentrifuge tube. Add TCEP (25 μL of 200 mM) to final 10 mM, and incubate for 30 min at 22°C with gentle rotating (do not heat the samples).
   b. Add IAA (25 μL of 500 mM) to final 25 mM, and gently rotate for 30 min at 22°C in the dark.
   c. Centrifuge for 2 min at 1,400 × g to remove the supernatant. Wash beads with PBS (1 × 1 mL).
   d. Add 200 μL of 2 M Urea in PBS, 2 μL of 100 mM CaCl₂ (final 1 mM), 4 μL of 0.5 mg/mL trypsin (2 mg total) in re-suspension buffer to the beads, and incubate for 12 h at 37°C (Thermal Mixer, 1100 rpm).
   e. Centrifuge for 1 min at 4,000 × g and transfer the supernatant to a 1.5 mL microcentrifuge tube.

19. Purify digested peptides using C18 Tips.
   a. Set the pipettor to 100 μL and secure the C18 tip tightly to the end of the pipettor for optimum tip-to-pipettor seal and sample aspiration.
   b. Wet the tip by aspirating 200 μL of pure ACN and then discarding the solvent. Repeat once (keep the membrane wet).
   c. Equilibrate the tip by aspirating 200 μL of 0.1% TFA and discarding the solvent. Repeat once.
   d. Aspirate up to 200 μL of the sample (prepared in Step 18) into the C18 tip. For maximum efficiency, dispense and aspirate the sample for 10-20 cycles. Keep the sample after the last dispensation for the second round.
   e. Rinse the tip by aspirating 200 μL of 0.1% TFA and discarding the solvent. Repeat twice.
   f. Aspirate up to 200 μL of 70% ACN, and elute the solution (containing tryptic peptides) into a clean 1.5 mL microcentrifuge tube. Dry the tip.
   g. Repeat Step b-f for the second round with the kept sample at step d. Total elution is about 400 μL.

**Note:** The samples (prepared in Step 18) will be loaded to the C18 Tip twice to recover and elute more digested peptides, in case some peptides weren’t captured in the first round.

   h. Dry down the elution in a vacuum centrifuge to remove the solvent. Submit the dried peptides for nanoLC-MS/MS analysis.

20. From mass spectrometry data analysis, in the Normal Set of Samples, proteins with enrichment ratio (Medium/Light) > 1.2 and competition ratio (Medium/Heavy) > 1.2 were considered as positive hits. And in the Reverse Set of Samples, proteins with enrichment ratio (Light/Heavy) > 1.2 and competition ratio (Light/Medium) > 1.2 were considered as positive hits. Positive hits from both Normal Set of Samples and Reverse Set of Samples are seen as identified target proteins in one replicate. Totally three independent replicates for SILAC HeLa cell lysates were carried out, and the overlapped proteins of these three replicates are considered as final target proteins. For representative probe pulldown MS experiments of SILAC cell lysates, see Figures 2G and 2H in Hou et al. (2021).

**Note:** The details of Data analysis for SILAC experiments are shown here, same as in the reference (Hou et al., 2021). MS/MS raw spectra were processed using MaxQuant software (version 1.5.3.30) and thermo proteome discoverer(2.1.0.81). The human protein sequence database containing 20410 sequence entries in the Swiss-Prot database downloaded on October 12, 2018 was used for database search. Trypsin was set as the enzyme, and the maximum missed cleavage was set...
to 2. The first-search peptide mass tolerance and main-search peptide tolerance were set to 20 and 4.5 ppm. The MS/MS match tolerance was set to 0.5 Da for ITMS and 20 ppm for FTMS. A fixed carbamidomethyl modification of cysteine and variable modifications on methionine oxidation were set. “Match between runs” was applied, and the match time window was set to within 2 min. The false discovery rate (FDR) was controlled with a decoy database and set to no more than 1%. The minimum Andromeda score for modified peptides was 40, and the minimum delta score was 6 for the modified peptides. For SILAC sample, Proteome Discoverer determines the area of the extracted ion chromatogram and computes the “heavy/medium/light” ratio. Protein ratios are then calculated as the median of all the unique quantified peptides belonging to a certain protein. The ratios among proteins in their heavy and light versions were used as fold-change. We used “Wu Kong” platform (https://www.omicsolution.com/wkomics/main/) for relative Enrichment and Competition analysis of MS data. Average fold change was calculated for enrichment and competition. The p-values were resulted from the welch t-test. Alternatively, there are several options to analyze the MS data instead using the “Wu Kong” platform website. Volcano plots can be generated by (1) Graphpad; (2) EnhancedVolcano (using R script, https://www.bioconductor.org/packages/release/bioc/vignettes/EnhancedVolcano/inst/doc/EnhancedVolcano.html); (3) or a web app VolcaNoseR (https://huygens.science.uva.nl/VolcaNoseR/).

**Note:** The enrichment ratio (Medium/Light) and competition ratio (Medium/Heavy) are set according to the lowest ratio of EF1A1 in SILAC experiments. The minimum ratio in SILAC experiment is 1.2 in general. For ratio calculation, infinite ratio (with zero value as the denominator) is set as 100 for convenience.

**Note:** Identification of potential 2′3′-cGAMP-binding or -interacting proteins can also be carried out with non-SILAC cell lysates, and label-free MS analysis should be performed to obtain the target proteins.

**Note:** The probe could bind non-specifically to proteins and remain attached in the absence of crosslinking. In this protocol, we execute several steps to minimize this non-specificity. First, urea is used to denature proteins in the sample after photocrosslinking step to diminish the nonspecific binding. Second, protein precipitation and resolubilization is carried out after click chemistry to further clean up the probe from the proteins. Third, the arrangement of Normal and Reverse Set of Samples is to reduce the false positive hits generated by this non-specificity. Fourth, the experiment is repeated three times to systematically decrease the nonspecific effect. Please check the reference (Hou, et al., 2021) for the fluorescence images and MS data of probe labeling to have a better understanding.

**Labeling of endogenous EF1A1 in purified EF1 complex using Probe 3**

© Timing: 1 day

This section describes how to verify the identified protein targets as the real 2′3′-cGAMP-binding proteins by using purified target proteins and 2′3′-cGAMP Probe 3.

21. Set up the labeling assay in 1.5 mL microcentrifuge tubes as below:

| Sample No | 1      | 2     | 3     |
|-----------|--------|-------|-------|
| EF1 complex (0.2 mg/mL) | 20 µL  | 20 µL | 20 µL |
| 2′3′-cGAMP (1 mM) | –      | –     | +     |
| Probe 3 (10 µM)  | –      | +     | +     |

For the competitive labeling sample (Sample No. 3), add 2′3′-cGAMP (0.5 µL 40 mM stock in H2O, final 1 mM) into 4 µg purified EF1 complex in the 1.5 mL microcentrifuge tube, and incubate at 4°C for 1 h.
For non-competitive labeling samples (Sample No: 1, 2), add the same volume of water instead of 2’3’-cGAMP into 4 μg purified EF1 complex in the 1.5 mL microcentrifuge tube, and incubate at 4°C for 1 h.

22. Add Probe 3 (0.5 μL 0.4 mM stock in H2O, final 10 μM) into the samples (Sample No: 2, 3) respectively, and incubate at 4°C for 1 h. For the control sample, add the same volume of water instead of Probe 3 into the sample (Sample No: 1), and incubate at 4°C for 1 h.

23. Follow Step 3-9 to obtain the labeling results of endogenous EF1A1 in purified EF1 complex with Probe 3. For representative figure of probe labeling with purified proteins, see Figure 3B in Hou et al. (2021).

Note: This method is suitable for any purified proteins. The tested protein with significantly higher fluorescence signal in Sample No. 2 than in Sample No. 1 and 3 is considered as a positive hit.

**Labeling of live cells with Probe 3 to detect EF1A1 from Probe 3 bound proteins**

**Timing:** 3 days

This section describes how to validate the association between identified protein targets and 2’3’-cGAMP in cells by using probe pulldown.

24. Remove the medium from 293T cells (1 x 10-cm dish, 30% confluency), and incubate cells with fresh medium containing 0.1 mM STF-1623 (ENPP1 inhibitor) and 30 μM Probe 3 for 18 h (Sample No: 2). For the negative control, don’t incubate cells with Probe 3 (Sample No: 1).

| Sample No | 1 | 2 |
|-----------|---|---|
| 293T cells | + | + |
| Probe 3 (10 μM) | – | + |

25. Remove the medium from the dish, rinse cells with 5 mL PBS, and cover cells with 3 mL PBS containing 30 μM Probe 3 (no Probe 3 in PBS for the negative control). Put the dish on ice and irradiate it at 365 nm with CL-100 Ultraviolet Crosslinker (9999 mJ/cm²) for 30 min.

**CRITICAL:** For positive labeling of live cells, it is very important to use PBS containing the same concentration of Probe 3 as in cell culture medium to cover cells for photocrosslinking, in order to have the optimum labeling result.

26. Remove PBS buffer from the dish, add 600 μL 4% SDS buffer, lift up cells with cell scrapers and collect the suspension into a 1.5 mL microcentrifuge tube.

27. Lyse the suspension by Ultrasonic Homogenizer with 2 mm probe. Condition: power 20%, working time: 30 s, ultrasonic opening time: 2 s, ultrasonic turn-off time: 2 s.

28. Centrifuge 15 min at 20,000×g, 22°C, and collect the supernatant in a 1.5 mL microcentrifuge tube.

29. Perform click chemistry to conjugate Biotin-N3 as Step 14 (with the same final concentration of each reagent), protein precipitation as Step 15, protein resolubilization as Step 16, and protein enrichment as Step 17.

30. Mix the beads with 20 μL 2× loading buffer and heat at 95°C for 5 min. Resolve the samples by SDS-PAGE using a 12% acrylamide gel, and detect EF1A1 by western blot using EF1A1 antibody. For representative western blot figure, see Figure 5A in Hou et al. (2021).
Note: This method is also suitable for other potential 2’3’-cGAMP-binding or -interacting proteins. The tested protein with significantly higher western blot signal in Sample No. 2 than in Sample No. 1 is considered as a positive hit, which illustrates the association between the tested protein and 2’3’-cGAMP in cells.

**Labeling of live cells with Probe 3 to validate Probe 3 bound with EF1A1 in EF1 complex**

© Timing: 3 days

This section describes how to validate the association between identified protein targets and 2’3’-cGAMP in cells by using IP of target proteins. Here Flag IP of the overexpressed Flag-EF1D is applied for the protocol, which is also suitable for IP of endogenous target proteins (Hou et al., 2021).

31. Remove the medium from 293T cells and 293T cells stably overexpressing Flag-EF1D (1 × 10-cm dish, 30% confluency), and incubate cells with fresh medium containing 0.1 mM STF-1623 (ENPP1 inhibitor) and 30 μM Probe 3 for 18 h (Sample No: 1, 3). For the negative control, don’t incubate cells with Probe 3 (Sample No: 2).

| Sample No   | 1 | 2 | 3 |
|-------------|---|---|---|
| 293T cells  | + | – | – |
| 293T Flag-EF1D cells | – | + | + |
| Probe 3 (10 μM) | + | – | + |

32. Remove the medium from the dish, rinse cells with 5 mL PBS, and cover cells with 2 mL PBS containing 30 μM Probe 3 (no Probe 3 for the negative control). Put the dish on ice and irradiate it at 365 nm with CL-100 Ultraviolet Crosslinker (9999 mJ/cm²) for 30 min.

33. Remove PBS buffer from the dish, add 600 μL 1% NP-40 buffer, lift up cells with cell scrapers and collect the suspension into a 1.5 mL microcentrifuge tube.

34. Lyse the suspension by Ultrasonic Homogenizer with 2 mm probe. Condition: power 20%, working time: 30 s, ultrasonic opening time: 2 s, ultrasonic turn-off time: 2 s.

35. Centrifuge 15 min at 20,000 × g, 4°C, and collect the supernatant in a 1.5 mL microcentrifuge tube.

36. Dilute the supernatant with the same amount of 0.2% NP-40 buffer, and incubate with 20 μL Flag beads (50% suspension) in each tube for 2 h at 4°C with gentle rotating.

37. Centrifuge 5 min at 4,000 × g at 4°C to remove supernatant. Using the same centrifugation conditions, wash the beads 3 times with 0.5 mL 0.2% NP-40 buffer.

38. Elute the bound proteins from the beads by incubation with 20 μL 4% SDS buffer and heat at 95°C for 10 min. Collect the supernatant in a 1.5 mL microcentrifuge tube after centrifugation for 5 min at 4,000 × g at 22°C.

39. Perform click chemistry to conjugate Biotin-N₃ as Step 14 (with the same final concentration of each reagent).

40. Mix the samples with 10 μL 6× loading buffer, resolve the samples by SDS-PAGE using a 12% acrylamide gel, and detect biotin conjugated on EF1A1 in EF1 complex via Probe 3 by western blot using avidin-HRP. For representative western blot figure, see Figures 5B and 5C in Hou et al. (2021).

Note: This method is also suitable for other potential 2’3’-cGAMP-binding or -interacting proteins. The tested protein with significantly higher biotin signal in Sample No. 3 than in Sample No. 1 and 2 is considered as a positive hit, which illustrates the association between the tested protein and 2’3’-cGAMP in cells.
**EXPECTED OUTCOMES**

2’3’-cGAMP-based photoaffinity probes can be utilized as convenient and useful molecular tools to study biological functions of 2’3’-cGAMP. This protocol describes a general procedure to identify and validate 2’3’-cGAMP-binding or -interacting proteins. From probe pulldown MS experiments with SILAC cell lysates, the positive hits would be very likely the potential target proteins. As a proof, EF1A1 in EF1 complex was verified to associate with 2’3’-cGAMP in vitro and in cells, which reveals the new function of 2’3’-cGAMP to regulate protein translation directly. Probe labeling of purified EF1A1 in EF1 complex has the same result as 32P-2’3’-cGAMP binding assay (Hou et al., 2021), which facilitates the validation process especially in live cells.

**LIMITATIONS**

This protocol provides a feasible strategy to identify and validate 2’3’-cGAMP-binding or -interacting proteins, while some factors need to be considered in regard of results.

First, there might be no dramatic difference in probe labeling of cell lysate comparing with negative controls, either in in-gel visualization or pulldown MS experiments. This is because the endogenous abundance of potential target proteins in cell lysate might be very low (except EF1A1 which is abundant). Subcellular fractions or specifically enriched protein fractions may increase the efficacy of probe labeling and thus generate more positive hits. In our case, STING, a known 2’3’-cGAMP receptor, and SLC19A1, a reported 2’3’-cGAMP transporter, were not identified in probe pulldown MS experiments because of the low abundance of these two endogenous proteins. However, the overexpressed STING and SLC19A1 and endogenous STING were verified to associate with 2’3’-cGAMP by probe pulldown with western blot detection.

Second, probe labeling of cell lysate is carried out in the absence of endogenous cellular metabolites, which might generate some false positive hits. It is not well known whether the endogenous metabolites would affect the association between target proteins and probes. In some scenarios, probes or 2’3’-cGAMP may not interact with target proteins because of higher binding affinity of target proteins with endogenous metabolites and higher concentrations of these related metabolites. Ideally, probe labeling of live cells would identify more physiologically relevant targets. However, it will need adequate probes for the experiment since the labeling efficiency of live cells is very limited due to the low intracellular concentration of probes.

Third, there might be difficult and complicate for in vitro validation of identified target proteins. It would be better to obtain the purified target proteins in native form from mammalian cells because of protein folding and posttranslational modifications, which elevates the cost and effort to get adequate amount of purified proteins for the experiments. In our case, attempts to obtain recombinant human EF1A1 protein with good quality from E. coli, yeast or insect cells were failed. Another factor to consider is whether the target protein interacting with 2’3’-cGAMP is in free-form or protein complex, which raises the complexity of validation process. In our case, 2’3’-cGAMP associates with EF1A1 in EF1 complex in preference to free-form EF1A1.

Fourth, validation of the association between endogenous target proteins and 2’3’-cGAMP in cells might be challenging. Due to the low abundance of potential target proteins, it will require more live cells labeling with probes, which may need significant amount of probes for such experiments. It also requires good antibodies for western blot and immunoprecipitation of the endogenous target proteins. And plenty of endogenous nucleotides, such ATP and GTP, might compete with probes in association with target proteins, which sets up more obstacles for in-cell validation.

**TROUBLESHOOTING**

Problem 1

There are no positive fluorescence signals of cell lysates in the protein gel (step 8).
Potential solution
Since EF1A1 is abundant in cell lysate, labeling of EF1A1 should generate clearly positive fluorescence signal in gel comparing with the control sample. The reasons that there are no positive fluorescence signals of cell lysates might be caused from photocrosslinking and click chemistry. First, increase the irradiation time and decrease the distance between light bulbs and samples inside of the crosslinker, in order to enhance photocrosslinking efficiency. Second, the reagents for click chemistry should add into the samples in order and TCEP solution should be made freshly before use.

Problem 2
There are no positive fluorescence signals of purified target proteins in the protein gel (step 23).

Potential solution
There are several possibilities. First, there is something wrong with photocrosslinking or click chemistry. Use the labeling of cell lysate as the positive control since EF1A1 in cell lysate should give a positive fluorescence signal. And then follow the Potential solution of Problem 1 to resolve this problem. Second, the quality of purified target proteins is not good enough for the in vitro labeling with probes. Try the recombinant proteins or protein complexes expressed and purified from 293T (or 293FT) cells. Third, the purified target proteins are not the real binding or interacting proteins of 2’3’-cGAMP. The identification of these proteins in probe pulldown MS experiments might be due to that these proteins are involved in some protein complexes associating with 2’3’-cGAMP.

Problem 3
There are no positive western blot signals of target proteins in the Probe 3 pulldown of live cells (step 30).

Potential solution
First, use EF1A1 as the positive control, which should be readily detected in Probe 3 pulldown of live cells, to make sure that photocrosslinking and click chemistry are carried out successfully. Second, increase the amount of live cells for probe labeling. Third, use more sensitive and specific antibodies of target proteins for western blot. Fourth, it is helpful to try the overexpressed target proteins that have more protein abundance and sensitive tags for easier detection.

Problem 4
There are no positive signals of biotin conjugated with Probe 3 in the immunoprecipitation of target proteins from live cells (step 40).

Potential solution
First, use immunoprecipitation of EF1D as the positive control, which should produce the positive signal of biotin conjugated with Probe 3, to make sure that photocrosslinking and click chemistry are carried out successfully. Second, increase the amount of live cells for probe labeling. Third, use more potent and specific antibodies of target proteins for immunoprecipitation. Fourth, it is helpful to try the overexpressed target proteins that have more protein abundance and specific tags for more efficient immunoprecipitation.

Problem 5
Incomplete incorporation of different isotope labeled amino acids in the cells was detected by MS analysis (for step 8 in “preparation of SILAC HeLa cell lysates”).

Potential solution
Here are the standard procedures to ensure the complete SILAC labeling of cells. In SILAC experiments, cells were grown in SILAC DMEM supplemented with Lys-0, Arg-0; or Lys-4, Arg-6; or Lys-8, Arg-10. In addition, 10% dialyzed FBS and 1% penicillin-streptomycin were added in SILAC medium. After the cells have been cultured in SILAC medium for 5–7 generations, cells were collected and lysed in RIPA lysis buffer.
buffer (50 mM Tris, pH 8, 0.1% SDS, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and protease inhibitor cocktail) by sonication. The lysates were centrifuged at 18,000 g for 20 min, and the supernatants were transferred into clean tubes prior to the determination of protein concentration by BCA method. Proteins were precipitated on ice by adding 4 volumes of cold acetone and then the protein pellets were resuspended in urea buffer (100 mM Tris, pH 8.5, 8 M urea). Disulfide bonds were reduced by adding Tris(2-carboxyethyl) phosphine (TCEP) to a final concentration of 5 mM and incubating for 20 min at 22°C. Reduced cysteines were alkylated by adding iodoacetamide (IAM) to 10 mM and incubating for 15 min in the dark at 22°C. Samples were diluted with buffer (100 mM Tris, pH 8.5, 1 mM CaCl₂) to 2 M urea prior to 12-h digestion at 37°C with trypsin (Promega) using an enzyme to substrate ratio of 1:50 (w/w). Digested peptides were enriched by C18 tips for MS detection. MS/MS raw spectra were processed using PD2.1 software (version 1.5.3.30). The SwissProt human protein sequence database containing 20,410 sequence entries (download date: October 12, 2018) was used for database search. Trypsin was set as the enzyme, and the maximum missed cleavage was set to 2. The first-search peptide mass tolerance and main-search peptide tolerance were set to 20 and 4.5 ppm, respectively. Carbamidomethyl (+57.02 Da) was set as a fixed modification, and the variable modifications were oxidation of methionine (+15.99 Da) and protein N-terminal acetylation (+42.01 Da). FOR cells grown in SILAC DMEM with Lys-4 and Arg-6, lysine (+4.025 Da) and arginine (+6.020 Da) were also set as variable modification for “Medium” modification. FOR cells grown in SILAC DMEM with Lys-8 and Arg-10, lysine (+8.014 Da) and arginine (+10.008 Da) were also set as variable modification for “Heavy” modification. Once the ratio between peptides (with “Medium” or “Heavy” modification) and total peptides is greater than 99%, the SILAC labeling is considered complete.

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Hong Jiang (hongjiang@sioc.ac.cn).

**Materials availability**
Requests for reagents may be sent to Hong Jiang (hongjiang@sioc.ac.cn).

**Data and code availability**
No unique datasets or codes were generated in this study.

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**AUTHOR CONTRIBUTIONS**
H.J., Y.H., H.L., L.S., and Y.Z. established this protocol. H.J., Y.H., and H.L. wrote this manuscript.

**DECLARATION OF INTERESTS**
The authors declare no competing interests.

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