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Workflows and considerations for investigating protein interactions of viral DNA sensors

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Abstract

DNA sensors are a core component of innate immunity in mammalian cells. In response to pathogen infection, these specialized proteins sense pathogenic DNA from bacteria or viruses and initiate immune signaling cascades. These defense mechanisms rely on the rapid formation and temporal regulation of protein-protein interactions. Similarly, protein interactions underlie virus immune evasion mechanisms, as proteins from diverse viruses associate with and inhibit DNA sensors. Here, we describe experimental protocols for identifying protein interactions of DNA sensors, and discuss considerations for optimal isolation of protein complexes when targeting either endogenous or tagged proteins. Additionally, as viral infections and immune responses are known to induce prominent changes in cellular protein abundances, we provide a workflow for investigating these protein associations in the context of proteome alterations.

1. Introduction

The ability of mammalian cells to sense pathogenic DNA is a fundamental mechanism of defense against numerous microorganisms that are part of our ecosystem. Outside of RNA viruses, all other pathogens, including bacteria and DNA viruses, carry their genetic information in the form of DNA and rely on their DNA replication for the spread of infection. In response, mammalian cells have evolved a multitude of defense mechanisms that employ specialized proteins, known as pattern recognition receptors (PRRs), to detect pathogen-associated molecular patterns (PAMPs), including pathogenic double-stranded DNA (dsDNA). Diverse PRRs survey the cellular space, providing means for pathogenic DNA sensing within different cellular compartments. Upon binding to dsDNA, PRRs initiate defense programs, which include innate immune signaling, inflammatory responses, and apoptosis. For this, PRRs rely on adaptor and effector proteins to initiate immune signaling programs, amplify signals, and communicate information between subcellular compartments. Therefore, the interactions of DNA sensors with other cellular proteins are at the core of host antiviral response. In this chapter, we describe experimental protocols and considerations for identifying protein interactions of DNA sensors, and for investigating these associations in the context of proteome alterations during immune signaling.

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Fig. 1 provides examples of prominent DNA sensors and their interacting protein partners. Endosomal compartments are monitored for the presence of foreign dsDNA by the transmembrane protein Toll-like receptor 9 (TLR9). This protein binds to both bacterial (Deretic, 2012) and viral (Krug et al., 2004) unmethylated CpG DNA motifs and initiates a signaling cascade that culminates in the expression of pro-inflammatory cytokines. Like other Toll-like receptors, the cytosolic domain of TLR9 interacts with the adaptor proteins TIRAP and MyD88 to initiate the formation of the Myddosome in the cytoplasm (Bonham et al., 2014; Hemmi et al., 2000; Kawai, Adachi, Ogawa, Takeda, & Akira, 1999). The Myddosome then incorporates the serine/threonine kinases IRAK 2 and 4 (Gillen & Nita-Lazar, 2017), which act as effector proteins via TRAF6/IRF8 to activate the transcription factor NF- κ B for cytokine production (Bonham et al., 2014; Tsujimura et al., 2004).

Within the cytosol, cyclic GMP-AMP synthase (cGAS) is known as a major sensor of dsDNA. Upon binding to dsDNA in a sequence-independent manner, cGAS catalyzes the formation of 2'3'-cGAMP (Sun, Wu, Du, Chen, & Chen, 2013). This small molecule acts as a secondary messenger that binds to and activates the adaptor ER-resident protein STING, causing its conformational change and dimerization (Wu et al., 2013). STING further induces interferon expression through the TBK1-IRF3 signaling axis (Ishikawa, Ma, & Barber, 2009) (Fig. 1). Recent studies have shown that cGAS protein interactions can either promote or inhibit its immune functions. For example, the TRIM proteins TRIM14 and TRIM56 reduce cGAS degradation and enhance cGAS dimerization, respectively (Chen et al., 2016; Seo et al., 2018). Other interactions, including those with AKT1, CCP5, and CCP6, were shown to negatively regulate cGAS functions (Seo et al., 2015; Xia et al., 2016). More recently, OASL, a protein known for functioning in cytosolic RNA sensing, was found to interact with cGAS and inhibit its activity and cytokine induction (Lum et al., 2018).

Another DNA sensor functioning in the cytosol is absent in melanoma 2 (AIM2) that belongs to the PYHIN family of protein, i.e., it contains an N-terminal PYRIN domain (PYD) that mediates homotypic protein-protein interactions and a C-terminal HIN200 domain capable of binding double-stranded DNA. It is proposed that, during cellular homeostasis, AIM2 is maintained inactive via an autoinhibition mechanism that involves folding of the HIN200 domain over the PYD (Jin, Perry, Smith, Jiang, & Xiao, 2013). Upon DNA sensing, the AIM2 PYD becomes free to initiate a chain reaction of protein interactions that results in a structure known as the inflammasome. Specifically, the AIM2 PYD associates with the PYD of ASC, also known as apoptosis-associated Speck-like protein containing a caspase activation and recruitment domain (CARD), which in turn uses its CARD to recruit and activate Caspase 1 (Fernandes-Alnemri, Yu, Datta, Wu, & Alnemri, 2009; Hornung et al., 2009) (Fig. 1). The resulting inflammasome structure amplifies the danger signal from AIM2 to ultimately result in pyroptosis and inflammation caused by maturation and secretion of cytokines IL-1 β and IL-18 (Fernandes-Alnemri et al., 2009).

Although it was long thought that PRRs that sense dsDNA are exclusively partitioned outside of the nucleus in order to avoid mistaking self-DNA for that of a pathogen, investigations during the past decade have demonstrated the existence of nuclear DNA sensing mechanisms. The interferon inducible proteins IFI16 and IFIX were established as

the first proteins capable of sensing pathogen DNA within the nucleus (Diner, Li, et al., 2015; Li, Diner, Chen, & Cristea, 2012; Unterholzner et al., 2010). Like AIM2, these proteins are members of the PYHIN family, but they do not seem to predominantly use their PYRIN domains to recruit other PYD-containing proteins. Rather, they form homo-oligomers upon binding DNA. In this activated state, IFI16 and IFIX promote cytokine expression (Diner, Li, et al., 2015; Unterholzner et al., 2010) and bind to other nuclear proteins with known antiviral roles, including ND10 components PML, hDAXX, and ATRX (Diner, Li, et al., 2015; Diner, Lum, Javitt, & Cristea, 2015; Diner, Lum, Toettcher, & Cristea, 2016; Merkl & Knipe, 2019), as well as the previously discussed cGAS (Diner et al., 2016; Orzalli et al., 2015). Several studies have demonstrated that ND10 factors epigenetically repress herpes simplex virus type 1 (HSV-1) replication (Cabral, Oh, & Knipe, 2018; Lukashchuk & Everett, 2010), and a leading hypothesis suggests that association with IFI16 and IFIX can help to direct ND10 bodies to incoming viral genomes (Cabral et al., 2018; Diner et al., 2016; Everett, 2016; Merkl & Knipe, 2019). Meanwhile, nuclear cGAS was shown to stabilize IFI16 and promote innate immune signaling during HSV-1 infection (Orzalli et al., 2015).

The importance of protein-protein interactions in regulating DNA sensing is further highlighted by virus-host protein interactions through which pathogens inhibit PRRs. For example, the HSV-1 infection induces the proteasome-dependent degradation of IFI16 and, although this is still debated (Cuchet-Lourenco, Anderson, Sloan, Orr, & Everett, 2013), some studies indicate that the immediate early protein ICP0 contributes to this process (Orzalli, DeLuca, & Knipe, 2012), while human cytomegalovirus uses the major tegument protein pUL83 to bind IFI16 PYD and prevent its homo-oligomerization (Cristea et al., 2010; Li, Chen, & Cristea, 2013). The DNA sensor cGAS was also found to be inhibited by viral proteins, including by the Kaposi's sarcoma-associated herpesvirus (KSHV) proteins LANA and ORF52 (Wu et al., 2015; Zhang et al., 2016), the HSV-1 protein pUL37 (Zhang et al., 2018), and the dengue virus protein NS2B (Aguirre et al., 2017). Thus, studies of protein-protein interactions can reveal critical insights into both positive and negative regulation of DNA sensors during infection.

2. Experimental considerations when studying DNA sensor interactions

A number of experimental approaches are available for the study of protein interactions. These range from methods focused on the discovery of direct interactions, such as binary and crosslinking assays, to methods that retain both direct and indirect interactions to characterize functional protein complexes, protein interaction networks, or proximity associations within a certain subcellular location. Although many of these methods have yet to be implemented in studies of DNA sensors, these are valuable approaches that can be considered depending on the biological question that is being addressed, and we point the readers to several research papers and review manuscripts that describe some of these workflows (Chojnowski et al., 2018; Greco, Diner, & Cristea, 2014; Leitner et al., 2010; Lobingier et al., 2017; Miteva, Budayeva, & Cristea, 2013; Roux, Kim, & Burke, 2013). The method that has been so far the most widely used for studying DNA sensor protein interactions has been immunoaffinity purification (IP). IP has been either coupled with western blotting, for targeted analysis of one or several predicted interactions, or with mass

spectrometry (IP-MS), for the discovery of protein interactions and functional complexes. For example, IP-MS has been applied to investigations of cGAS (Liu et al., 2018; Lum et al., 2018; Seo et al., 2018), IFI16 (Diner et al., 2016; Diner, Li, et al., 2015; Diner, Lum, et al., 2015), as well as IFIX (Crow & Cristea, 2017; Diner, Li, et al., 2015) and AIM2 (Diner, Li, et al., 2015) protein interactions. In this chapter, we provide a robust IP-MS protocol (see Sections 4.3 and 5.3) that was proven effective for studying DNA sensors. However, prior to starting the experimental workflow, several choices and experimental considerations relevant to DNA sensors must be taken into account (Fig. 2).

IP-MS can be used to investigate a protein in different *in vitro* and *in vivo* biological contexts, with the most frequent application for DNA sensors so far being their isolation from cell culture. One important aspect that should be considered for such studies is the choice of cell type. The endogenous abundance of the protein of interest can vary among different cell types. For example, cGAS and IFI16 are present at higher levels in macrophages (as shown for differentiated THP-1 cells) than in fibroblasts (HFFs), and are not expressed in HEK293T cells (Li et al., 2012; Lum et al., 2018). This has to be taken into account if the goal is to study the interactions of an endogenous protein. However, the use of different cell types can provide the tools needed for addressing different biological questions. For example, the lack of cGAS and IFI16 expression in HEK293T cells has been instrumental in determining the minimal components needed for innate immune signaling by reconstituting the signaling axis with the co-expression of the DNA sensor and the adaptor protein STING (Orzalli et al., 2015; Sun et al., 2013; Unterholzner et al., 2010). Human or mouse fibroblasts have often been chosen to study protein interactions during infection with herpesviruses, including HSV-1 and HCMV, as it is a relevant cell type that can mimic an *in vivo* infection (Crow & Cristea, 2017; Diner et al., 2016; Lum et al., 2018; Orzalli et al., 2015). Additionally, knowledge of the high abundance of a DNA sensor in a certain cell type has provided a platform for initial studies that could be then followed up in other relevant cell types. For example, the finding that IFI16 is abundant in CEM T cells has provided the ability to perform IP-MS studies of endogenous IFI16 (Li et al., 2012). This has led to the discovery of acetylations within its nuclear localization signal, which were then confirmed via targeted MS analyses in cell types where IFI16 is present at lower levels than in CEM T cells, including macrophages and fibroblasts (Li et al., 2012).

Another important factor when choosing a cell system is that proteins can have distinct subcellular localization patterns in different cell types. For example, while IFI16 is predominantly nuclear in fibroblasts and CEM T cells, it has both nuclear and cytoplasmic localizations in THP-1 cells (Hornung et al., 2009; Unterholzner et al., 2010). Similarly, although cGAS is known to be predominantly cytoplasmic, several studies have now indicated its presence in the nuclei of different cell types (Liu et al., 2018; Orzalli et al., 2015). Therefore, an IP-MS study can provide a mixed pool of interactions from both subcellular compartments. This has to be accounted for when building protein interaction networks and, even prior to that, when optimizing the lysis buffers needed for the extraction of the protein, which may have different physical characteristics in the different subcellular compartments (e.g., localization-specific aggregation or oligomerization). The use of fractionations, such as nuclear-cytoplasmic fractionation or density gradient separations, followed by IP-MS studies in distinct fractions can help to hone in on certain localization-

dependent functions. For example, cytoplasmic fractionation was used in a recent IP-MS study of cGAS cytoplasmic interactions and regulation (Lum et al., 2018).

Another consideration is if the protein of interest needs to be tagged for IP-MS studies. Several circumstances would require or benefit from using a tagging approach. For example, one obstacle is that antibodies raised against an endogenous protein, even if successfully used in western blotting or microscopy assays, frequently lack the affinity or specificity required for IP studies. Tagging might also be preferred when one pursues the function of a specific domain or residue. In such cases, tagged domain constructs or single-point mutants can be transfected or stably expressed in cells prior to IP-MS studies. The use of a tag would afford a similar isolation strategy and the ability to compare the interactions of different mutants and domain constructs to the tagged wild type protein. For example, generation of tagged IFI16 PYRIN domain and HIN200 domain constructs provided the ability to identify functional domain-specific interactions, including the association of the IFI16 PYRIN domain with cGAS and ND10 bodies (Diner et al., 2016).

Commonly used tags include green fluorescent protein (GFP), FLAG, hemagglutinin (HA), and Protein A. The use of a fluorescent tag, such as GFP, provides the means to consecutively investigate both protein localization (by microscopy) and interactions (by IP) (Cristea, Williams, Chait, & Rout, 2005). The isolation of a tagged protein is usually more convenient due to the high specificity of the available antibodies. However, a potential drawback is that the introduction of a tag may affect protein function, or mask a certain region on the bait protein and inhibit protein interactions. Therefore, an assessment that indicates that the tagged protein retains its normal function is necessary prior to characterization of interactions. Specifically, tagged DNA sensors should retain the ability to induce innate immune signaling and elevated antiviral cytokine expression. Different locations for the tag can be considered when taking into account the position of functional domains. In a previous study that investigated the interactions of all the four members of the PYHIN family, the authors compared N- and C-terminally tagged constructs for AIM2, IFI16, IFIX and MNDA (Diner, Li, et al., 2015). Similarly, a recent cGAS interactome study started by confirming the function of N- and C-terminally tagged cGAS constructs, which led to the discovery of its association with OASL (Lum et al., 2018).

The use of tagging approaches brings another challenge relevant for DNA sensors. Overexpressed DNA sensors may induce immune signaling and proteome changes that can impact protein interactions. This can be particularly problematic when generating stably expressing cell lines. One strategy that partly ameliorates these issues and has proven useful for DNA sensors is to use an inducible expression system (e.g., as shown for doxycycline inducible-GFP-cGAS, Lum et al. (2018) and tetracycline-inducible GFP-IFI16, Diner et al. (2016)) to express the target protein shortly before the experiments and avoid consistent cytokine induction.

The selection of the IP workflow influences the selection of the appropriate control experiments that can help determine the specificity of the identified interactions (as reviewed in Miteva et al. (2013)). If a tagged protein is used, the cells in the control group should express the tag only, and be purified using the same antibody. If an endogenous protein is

purified, the control IP is frequently an IgG isolation performed in the same cell type. Alternatively, the control IP can be performed using the antibody against the endogenous protein in cells following knockdown or knockout of the bait DNA sensor.

3. Protein interactions in the context of proteome changes

The dynamic regulation of the cellular proteome underlies all biological processes, including innate immune responses. Changes in protein abundances, subcellular localizations, and posttranslational modifications (PTMs) drive the formation or disruption of protein interactions and are at the core of immune signaling events. Therefore, protein interaction studies must be investigated and understood in the broader context of proteome changes (Fig. 2). For example, the host activation of DNA sensors for recognition of viral DNA and the ability of viruses to inhibit these immune mechanisms rely on finely-tuned changes in the proteome during the progression of the virus replication cycle. Several studies have started to build a broader view of temporal proteome alterations during the progression of viral infections (Beys-da-Silva et al., 2018; Drayman et al., 2017; Forrest, Hislop, Rickinson, & Zuo, 2018; Kulej et al., 2017; Weekes et al., 2014). Relevant to immune signaling, proteome changes were also investigated upon infection with HSV-1 strains that have different abilities to induce immune responses, including elevated cytokines or apoptosis (Lum et al., 2018).

In addition to changes in protein abundance, the localization of proteins can also change during infection. For example, during active immune signaling, IRF3 dimerizes after its activation by phosphorylation and translocates into nucleus, similar to $\text{NF-}\kappa\text{B}$ (Baldwin, 1996; Hiscott et al., 1999). Therefore, protein interactions can be temporally and spatially regulated. As indicated above, PTMs are also crucial to regulating protein function during immune signaling. For example, during HSV-1 infection, the viral tegument kinase US3 hyperphosphorylates IRF3 at an atypical site, thereby inhibiting IRF3 dimerization and blocking its function (Wang, Wang, Lin, & Zheng, 2013). Another example is the acetylation on the NLS of IFI16 by the acetyltransferase p300, which modulates the localization of IFI16 and therefore its interaction with viral DNA and other defense proteins (Li et al., 2012).

Here, in addition to the protocol for immunoaffinity purification of protein complexes, we provide a protocol of proteome analysis, thereby providing a workflow to systematically study protein interactions in the context of temporal proteome alterations. The protocols are provided in the order that a user would carry these out in the laboratory, starting with the cell disruption for bait solubilization, followed by the use of the resulting cell lysate for proteome analyses and IP-MS studies.

4. Materials

4.1 Cell collection and lysis

4.1.1 Cell preparation before lysis

1. Infected/DNA-stimulated mammalian cells in tissue culture dishes or flasks.

2. 50mL conical tubes.
3. Dulbecco's phosphate-buffered saline (DPBS), pH 7.0–7.3, 1 × concentration (ice cold).
4. Plastic cell scraper.
5. Swinging bucket centrifuge set at 4 °C.
6. Protease and phosphatase inhibitor cocktail (P/PhIC) (Thermo Scientific).
7. Benzonase (Pierce).
8. Cell freezing buffer: 20mMNa-HEPES, 1.2% (w/v) polyvinylpyrrolidone, pH 7.4, 1/100 (v/v), P/PhIC added to 1 × concentration immediately before use.

4.1.2 Freezing cell pellet

1. Styrofoam container with 50-mL conical tube rack insert.
2. 50-mL conical tubes.
3. Liquid nitrogen.
4. 18-gauge needle.
5. 10 mL syringe.

4.1.3 Cryogenic cell lysis

1. Retsch MM 301 Mixer Mill.
2. Two 10 mL jars and two 20 mm (tungsten carbide or stainless steel) grinding balls (Retsch, Newtown, PA).
3. 50-mL conical tubes.
4. Liquid nitrogen.
5. Foam ice bucket.
6. Long forceps.
7. Metal spatula chilled by liquid nitrogen.
8. Windex.
9. 100% Methanol.
10. 10% bleach.
11. Ultrapure water.
12. Dry ice.

4.1.4 Detergent cell lysis

1. Foam ice bucket.
2. Microcentrifuge set to 4 °C.

3. Microcentrifuge tubes
4. Wash buffer: 20mMK-HEPES, pH 7.4, 0.11 M KOAc, 0.1% Tween-20 (v/v), 200mM NaCl, 0.6% Triton X-100.
5. Lysis Buffer: 20mMK-HEPES, pH 7.4, 0.11M KOAc, 0.1% Tween-20 (v/v), 200mM NaCl, 0.6% Triton X-100, $1 \times$ P/PhIC, 100U/mL benzonase.
6. Buffers might require optimization depending on cell type and protein of interest. See Section 5.3.3 for more information.

4.2 Proteome analysis

4.2.1 In-solution protein digestion—Store all solutions in glass containers that have been thoroughly washed with ultrapure water. Avoid using glassware that has been washed with detergents.

1. Whole cell lysate (see Section 5.1) or eluate from immunoaffinity isolation (see Section 5.3.2).
2. Refrigerated microcentrifuge capable of $14,000 \times g$ (set at 20°C).
3. LoBind pipet tips, 20–200 μL (Eppendorf).
4. Amicon Ultra-0.5 centrifugal filters, 30kDa NMWL (Millipore).
5. MS grade water (Fisher).
6. 10% sodium deoxycholate (DOC): Prepare in MS grade water and protect from light.
7. Tris-HCl buffer: 0.2M Tris-HCl, pH 8.0, in MS grade water. Store at 4°C .
8. TUD wash buffer: Prepare buffer fresh before use by mixing 1.5mL of 0.2M Tris-HCl, 0.6mL of 10% DOC, and 1.44g urea. Makes enough solution for two samples, about 1.5 mL each.
9. ABC-DOC wash buffer: 0.05M ABC, 2% DOC.
10. Trypsin, lyophilized MS-grade (Pierce). Suspended to $0.5\mu\text{g}/\mu\text{L}$, store at -80°C .
11. Digestion Buffer: Mix 1 μL of $0.5\mu\text{g}/\mu\text{L}$ trypsin stock and 99 μL of 0.05 M ABC. Prepare immediately before using.
12. 10% trifluoroacetic acid (TFA). Prepare in MS grade water, store at 4°C .
13. Ethyl acetate. Handle carefully in a chemical hood and dispose of appropriately.
14. SpeedVac concentrator.
15. TOMY Shaker.

4.2.2 Peptide fractionation with SDB-RPS StageTips

1. Microcentrifuge.
2. LoBind pipet tips, 20–200 μL (Eppendorf).

3. 14-gauge needle (Hamilton #90514).
4. Syringe plunger, 100 μ L (Hamilton #1162–02).
5. Empore SDB-RPS disks (3M #2241).
6. 50% ethyl acetate/0.5% TFA in MS grade water.
7. 0.5% TFA in MS grade water.
8. Buffer 1: 0.10 M ammonium formate, 0.5% formic acid, 40% acetonitrile (ACN) in water.
9. Buffer 2: 0.15M ammonium formate, 0.5% formic acid, 60% acetonitrile in water.
10. Buffer 3: 5% ammonium hydroxide and 80% acetonitrile in water.
11. FA solution: 1% formic acid and 1% acetonitrile in water.
12. Autosampler vials (Thermo Scientific).

4.2.3 Nanoliquid chromatography tandem mass spectrometry analysis

1. Nanoflow HPLC system such as Dionex Ultimate, Waters nano Acuity, or Agilent 1200 series.
2. Mobile phase A (MPA): 0.1% FA/99.9% water. Store in amber bottle for up to 6 months.
3. Mobile phase B (MPB): 0.1% FA/97% ACN/2.9% water. Store in amber bottle for up to 6 months.
4. Analytical column such as Acclaim PepMap RSLC 75 μ m ID $\times$ 25 cm (Dionex).
5. Mass spectrometer such as LTQ-Orbitrap Velos or Q Exactive HF (Thermo Fisher Scientific).
6. Nanospray ESI source (Thermo Fisher Scientific).
7. SilicaTip Emitter, Tubing (OD $\times$ ID) 360 μ m $\times$ 20 μ m; Tip (ID) 10 μ m (New Objective).

4.3 Immunoaffinity purification analysis

4.3.1 Conjugation of antibody to magnetic beads

4.3.1.1 Dynabeads conjugation

1. Dynabeads M-270 Epoxy (Invitrogen).
2. Round bottomed 2mL microcentrifuge tubes (Eppendorf).
3. Magnetic tube rack.
4. Tube shaker (e.g., TOMY shaker).
5. Rotating tube rack in 30 °C incubator.
6. Appropriate antibody.

7. 0.1 M Sodium Phosphate Buffer (pH 7.4): 19mM NaH₂PO₄, 81 mM Na₂HPO₄ in water.
8. 3M Ammonium sulfate: Prepared in 0.1 M Sodium Phosphate Buffer.
9. 100mM Glycine-HCl (pH 2.5): Prepare in water and adjust pH with HCl, sterilize with 0.2 µm filter.
10. Phosphate Buffered Saline (PBS).
11. 0.02% sodium azide (NaN₃) in PBS.
12. 0.5% Triton X-100 in PBS.
13. 10mM Tris-HCl (pH 8.8): Prepare in water and adjust pH with HCl.
14. 100mM Triethylamine: Prepare immediately before use in water.

4.3.1.2 Protein A/G magnetic beads conjugation

1. Pierce™ Protein A/G Magnetic Beads (ThermoFisher).
2. Round bottomed 2mL microcentrifuge tubes (Eppendorf).
3. Magnetic tube rack.
4. Rotating tube rack at 4 °C.
5. Appropriate antibody.
6. TBST buffer: Tris-buffered saline (TBS, ThermoFisher) containing 0.05% Tween-20 detergent.

4.3.2 Immunoaffinity purification

1. Polytron homogenizer (e.g., PT 10–35 Polytron from Kinematica).
2. Centrifuge and rotor rated to spin 8000 × g at 4 °C (rotor must fit a 50mL conical tube).
3. Rotating tube rack at 4 °C.
4. Heat block at 70 °C.
5. Tube shaker (e.g., TOMY shaker).
6. Vacuum evaporator (e.g., SpeedVac).
7. Magnetic tube rack for tubes under 2mL and bar magnet for larger volume tubes.
8. Round bottomed microcentrifuge 2mL tubes (for small scale IP), Protein LoBind tubes 5mL (for large scale IP) (Eppendorf).
9. Frozen cell powder or cell lysate (see Sections 5.1.2 and 5.1.3).
10. Optimized lysis buffer and wash buffer (see Section 5.3.3).
11. Antibody conjugated magnetic beads (see Section 5.3.1).

12. Elution buffer: $1 \times$ LDS Elution Buffer ($4 \times$ LDS elution buffer stock: 0.666g of Tris-HCl, 0.682 g of Tris-Base, 0.8 g of LDS, and 0.006 g of EDTA (free acid) dissolved in ultrapure dH₂O to a final volume of 10mL). Store at -20°C .
13. 500mM Tris(2-carboxyethyl)phosphine (TCEP).
14. 1M chloroacetamide (CAM): dissolve in water and store at -20°C .

5. Methods

5.1 Cell collection and lysis

Here we describe two methods of mammalian cell lysis that we have previously used for both proteome and protein-protein interaction studies: cryogenic lysis (Crow & Cristea, 2017; Diner et al., 2016; Diner, Li, et al., 2015; Diner, Lum, et al., 2015) and chemical lysis with detergent-containing buffers (Lum et al., 2018). Mechanical cell lysis may also be used, but we have found these methods to be effective at preserving the integrity of protein complexes while reducing nonspecific interactions.

5.1.1 Cell preparation before lysis

1. Aspirate media from tissue culture dish and add a small volume (~ 3 mL) of ice-cold DPBS. Quickly scrape cells and transfer to 50-mL conical tube. Repeat to collect any remaining cells.
2. Pellet cells at $250 \times g$ at 4°C .
3. Wash cell pellets with 20 mL DPBS and transfer to a pre-weighed 50-mL conical tube.
4. Pellet cells as before and aspirate media. If immediately lysing cells with a detergent-containing lysis buffer, continue directly to Section 5.1.3.
5. Weigh tube to determine wet cell pellet weight.
6. Prepare liquid nitrogen bath. Keep cells on ice.
7. Place a fresh 50-mL conical tube in the Styrofoam rack. Fill tube halfway with liquid nitrogen and leave uncovered. Fill the bottom of the rack with liquid nitrogen.
8. Add 100 μL of freezing buffer per gram of cells (see Note 1). Pipet dropwise into tube filled with liquid nitrogen.
9. Using the 18-gauge needle, punch holes in the conical tube cap before re-capping the tube with liquid nitrogen and cell material. Secure cap and gently agitate the rack in a fume hood to allow liquid nitrogen to slowly evaporate.

¹·Add 100 μL of freezing buffer for cell pellets that weigh less than 1g.

5.1.2 Cryogenic cell lysis

1. Clean the spatula, Retsch Mixer Mill jars, and grinding balls using the solutions in the following order: Windex, ultrapure water, 10% bleach, ultrapure water, and 100% methanol. Allow to air dry.
2. Use a liquid nitrogen bath in a foam ice bucket to cool both the mixing jars and the grinding balls. The jars are sufficiently cool when liquid nitrogen appears to stop bubbling. Remove materials from liquid nitrogen using long forceps.
3. Quickly transfer frozen cell pellets into mixing jar. Cell pellets can fill up to a maximum of one-third of the volume of the jar for optimal grinding—about 2.5g frozen pellets per 10mLjar. Place a single frozen grinding ball on the cell pellets, close the jar, and cool in liquid nitrogen once more.
4. Place jars in Retsch Mixer Mill. Remember to use an empty jar without a grinder ball as a balance, if only lysing one sample. Perform lysis with 10 cycles of 2min and 30s each at a frequency of 30Hz. Re-cool jars in liquid nitrogen between each cycle, and ensure jars are securely closed.
5. Open the jar and use a chilled spatula to transfer frozen cell powder to a 50 mL conical tube chilled on dry ice. Work quickly to avoid thawing sample. Store at -80°C .

5.1.3 Detergent cell lysis

1. If using frozen samples, thaw cell pellets on ice.
2. Add 500 μL of lysis buffer to each sample and vortex for 20 s.
3. Rock samples at room temperature for 10min.
4. Vortex samples for 20 s.
5. Incubate samples on ice for 10min.
6. Repeat steps 4 and 5.
7. Transfer lysates to microcentrifuge tubes and centrifuge at $8000 \times g$ for 10 min at 4°C .
8. Transfer clarified lysate to fresh microcentrifuge tubes.
9. Proceed directly to mass spectrometry preparation and analysis in the next section if performing proteome abundance quantifications. If performing protein interaction studies, please see Section 5.3 before proteomic analysis.

5.2 Mass spectrometry analysis

5.2.1 In-solution protein digestion

Day 1

1. Set microcentrifuge to 20°C .
2. Add 400 μL TUD buffer to each eluate sample.

3. Transfer each sample to separate Amicon Ultra-0.5 filters and centrifuge at $14,000 \times g$ for 10min or until liquid level reaches minimum line, about 25 μL .
4. Discard flow-through. Add 400 μL TUD buffer to filter and repeat centrifugation (see Note 2).
5. Discard flow-through. Add 300 μL TUD buffer to filter and repeat as above.
6. Repeat with another 300 μL TUD buffer.
7. Add 200 μL of ABC-DOC buffer to filter and spin at $14,000 \times g$ for 10min. Check that the retained volume is at the minimum.
8. Transfer filters to fresh collection tubes and add 100 μL of digestion buffer. Mix on TOMY shaker for 1 min.
9. Seal tube caps with parafilm and incubate overnight in 37 °C water bath.

Day 2

1. Centrifuge filters at $14,000 \times g$ for 5 min to recover digested peptides.
2. Add 25 μL MS-grade water and centrifuge at $14,000 \times g$ for 5min.
3. Repeat above step.
4. Keep flow-through with the digested peptides and discard the filter unit.
5. Add an equal volume of ethyl acetate to the sample. This should be between 100 and 150 μL , measured with LoBind pipet tip.
6. Adjust samples to 0.5% TFA with 10% TFA solution.
7. Briefly vortex samples then mix on TOMY shaker for 2 min.
8. Centrifuge at $14,000 \times g$ for 5 min.
9. Recover denser aqueous phase by pipetting from the bottom of the tube (see Note 3).
10. Proceed to “Peptide fractionation with SDB-RPS StageTips” with recovered aqueous phase.

5.2.2 Peptide fractionation with SDB-RPS StageTips

1. Cut Empore SDB-RPS disks using a 14-gauge needle. Prepare one StageTip for each sample by depositing a single Empore SDB-RPS disk into the bottom of a 200 μL pipette tip and gently tamping down with a syringe plunger (see Note 4).
2. Add half of the sample to the StageTip. Centrifuge at $2000 \times g$ until all solution passes through the Empore disk (see Note 5).

². Spray the buffer down the walls of the filter in an effort to wash all peptides down into the TUD buffer.

³. Avoid the top layer organic phase (containing ethyl acetate) and the interphase.

⁴. Be sure to not push the disk all the way to the end of the pipet tip—there should be a few mm of space left. Each disk is capable of binding about 25 μg of peptides. If needed, more disks can be layered on top.

3. Add remaining sample to the StageTip and repeat centrifugation.
4. Wash disk with 100 μ L of 50% ethyl acetate/0.5% TFA.
5. Wash disk with 100 μ L of 0.5% TFA.
6. Add 50 μ L Elution Buffer 1 to disk and collect eluate in an autosampler vial.
7. Repeat previous step with Elution Buffer 2.
8. Repeat once more with Elution Buffer 3.
9. Concentrate samples by vacuum centrifugation until the vial is almost completely dry.
10. Add FA solution to achieve final volume of 9 μ L. Quickly vortex to mix.
11. Immediately perform nLC-MS/MS analysis or store at -80°C .

5.2.3 Nanossliquid chromatography tandem mass spectrometry analysis—

For protein identification and abundance quantification, use an nLC-MS instrument with high resolution and high mass accuracy, such as those listed in Section 4.2.3. One consideration in designing a data-dependent acquisition method is if protein abundances should be measured using label-free quantification or a labeling method, such as with tandem mass tags (TMT). If the goal of the study is to compare many samples from various treatments, infection time points, or cellular fractions, TMT can be an excellent method to multiplex samples and increase instrument efficiency and accuracy of relative quantification.

5.3 Immunoaffinity purification analysis

5.3.1 Conjugation of antibody to magnetic beads—We provide protocols for two different types of magnetic beads that we previously used when studying DNA sensors, epoxy Dynabeads and Protein A/G magnetic beads. Most of our previous experiments have been performed using Epoxy Dynabeads. However, given a recent decrease in the performance of these beads, we reevaluated different types of resins. Our comparison experiments showed that Protein A/G magnetic beads had higher antibody conjugation efficiency and better purification of bait protein. As an alternative to the above mentioned beads, if the protein of interest is tagged with GFP, immunoaffinity purification of the bait via the GFP tag can also be performed using GFP-trap beads (ChromoTek), in which case the conjugation step is not needed.

The conjugation protocol for Dynabeads and Protein A/G magnetic beads can be used for conjugating antibodies directly targeting proteins of interest, antibodies against tags (e.g., GFP, FLAG), or IgG. The antibodies should be affinity purified, and, when dealing with Dynabeads, the solution in which the antibody is stored is critical. As the binding to epoxy occurs through primary amines, ensure that the antibodies used are not stored in Tris Buffer. Also, if the antibody storing solution contains other proteins, such as BSA, it would compete

⁵Loading samples into and washing the Empore disks can be done by either applying pressure with a small plastic syringe or centrifuging the StageTip in a collection tube with an adapter.

with the antibody for binding to the beads and would affect the efficiency of the immunoaffinity purification.

When determining the amount of beads needed, one must consider the amount of cellular material being used and the abundance of the protein of interest. For Dynabeads, a small scale pilot immunoaffinity purification experiment using less than 1×10^7 cells may require only 1–2mg of beads, while a larger scale IP would require 5–10mg of beads. If the protein of interest is not highly abundant, then using 10–20mg of beads could be appropriate. For Protein A/G magnetic beads, the beads are suspended as slurry in the vial. A small scale pilot experiment may require 20–25 μL (0.20–0.25 mg) bead slurry, while a larger scale experiment may need 50 μL (0.50mg) or higher amount of bead slurry.

The conjugation steps can be done at room temperature. The conjugation of Dynabeads is performed overnight, and the conjugated beads can be stored at 4 °C. The Protein A/G magnetic beads should be used immediately after the 1 h conjugation step.

5.3.1.1 Dynabeads conjugation: Day 1

1. Weigh out the appropriate amount of Dynabeads into a 2mL round-bottom tube (see Note 6).
2. Wash the beads with 1 mL of Sodium Phosphate Buffer (pH 7.4) and vortex for 30 s.
3. Mix vigorously with TOMY shaker for 15min.
4. Magnetically separate beads from solution. Once the solution becomes clear and the beads are collected on the magnet side, remove and discard the buffer using a pipette or vacuum (see Note 7).
5. Add 1 mL of fresh Sodium Phosphate Buffer and vortex for 30 s.
6. Magnetically separate beads from solution and remove the buffer as above.
7. Conjugate beads at a ratio of ~20 μL solution per mg beads. The reaction consists of three parts of solutions: antibody solution (Note 8), Sodium Phosphate Buffer, and Ammonium Sulfate, added in that order (see Note 9).
8. Seal tube caps with parafilm and rotate the slurry overnight (16h) at 30 °C. *Note:* Use a rotating rack that moves the tube end-over-end to help keep the beads suspended in the antibody solution during the conjugation period.

Day 2

⁶ 10 mg is the minimal amount to conjugate, as 200 μL is the least volume for the beads to be suspended in antibody solution during the conjugation (refer to step 7).

⁷ Removing buffer by vacuum is fast and convenient but risky because the beads may be lost. To be safe, one can always use pipette.

⁸ To calculate the necessary volume of antibody solution to use, for purified high affinity antibodies (e.g., in-house generated anti-GFP antibodies) use 3–5 μg Ab/mg beads. For commercially available antibodies that may not have as high affinity for binding, use 5–8 μg Ab/mg beads, and for conjugation of IgG use 8–10 μg IgG/mg beads.

⁹ The volume of 0.1M Sodium Phosphate Buffer depends on that of the antibody solution. For example, when conjugating 10mg of beads (200 μL total solution required), subtract the volume of antibody solution and 66.66 μL 3M Ammonium Sulfate from 200 μL . The Ammonium Sulfate solution is added last so as to reach a final concentration of 1M.

9. Place the tube on the magnetic rack and remove the supernatant as above. It is an option to keep the supernatant flow-through to determine the efficiency of bead conjugation via SDS-PAGE.
10. Wash the beads in 1 mL of Sodium Phosphate Buffer by gentle pipetting then magnetically separate beads (see Note 10). Once the solution is clear, remove and discard the buffer.
11. Wash the beads with 1 mL 100 mM Glycine-HCl, pH 2.5. This is a *fast* wash: remove the solution as soon as the beads are attached to the magnet and immediately proceed with the next wash step.
12. Wash the beads with 1 mL of 10mM Tris-HCl, pH 8.8 using the same procedure as described in step 10.
13. Wash the beads in 100mM Triethylamine. This is also a *fast* wash: proceed as is described in step 11.
14. Wash the beads four times with PBS using 1 mL for each wash. Remove the buffer following each wash.
15. Add PBS containing 0.5% Triton X-100 to the beads and mix with TOMY shaker for 15min.
16. Wash the beads twice with 1 mL PBS. Remove the buffer following each wash.
17. Suspend the beads in PBS with 0.02% NaN₃ and store at 4 °C until their use (see Note 11).

5.3.1.2 Protein A/G magnetic beads conjugation

1. Resuspend the bead slurry completely by pipetting up and down. Aliquot out the appropriate amount of beads into a 2mL round-bottom tube. DO NOT vortex the beads at any point until the protein complex elution step.
2. Wash the beads three times by adding 500 µL of TBST. Magnetically separate beads from solution and remove the buffer following each wash.
3. After the final wash, remove the tube from the magnet and begin conjugating the antibody.
4. The total volume of the conjugation reaction will be 500 µL. The solution consists of antibody and TBST. Add 10 µg of antibody per 25 µL (0.25 mg) beads slurry. The volume of TBST is 500 µL—volume of antibody (see Note 12).
5. Incubate the beads for 1h on a rotating tube rack at 4 °C, and the beads should be immediately used after the conjugation (see Note 13).

¹⁰.To avoid losing beads inside the pipette tip, after the beads attach to the magnet wall, wash the tip using the buffer inside the tube, and wait again for the beads to settle on the magnet.

¹¹.It is recommended to use the beads within 2 weeks of conjugation, as their isolation efficiency will decrease if the beads are stored for extensive periods of time (e.g., ~40% decrease after 1 month of storage). If the beads will be used immediately, NaN₃ is not necessary.

¹².25µL (0.25 mg) is the least amount of beads to operate on, as a lower amount of beads would be difficult to be collected on the magnet.

5.3.2 Immunoaffinity purification—Once the cells are collected and the beads are conjugated, the immunoaffinity purification step can proceed. A small scale IP can be done in a 1 mL volume using a 2 mL round bottomed microcentrifuge tube, while a large scale IP can be done in a 3–5 mL volume using a 5 mL Protein LoBind tube. The following protocol is for cryogenically lysed samples (see Section 5.1.2). If the cells were lysed using a detergent (see Section 5.1.3) and the samples are already in a lysis buffer solution, prepare the Wash Buffer in step 1, and proceed the protocol from step 8.

1. Prepare (i) IP Buffer for suspending the cell powder and (ii) Wash Buffer for washing the magnetic beads prior to and after the immunoaffinity purification. Chill buffers on ice. The composition of the IP Buffer is optimized as described in Section 5.3.3.
2. Bring the frozen cell powder from the -80°C storage, and keep the cells on ice.
3. Add the IP Buffer to the frozen cell powder and periodically swirl the tube until the powder is solubilized.
4. Prepare a Polytron for the homogenization of the cell lysate. Rinse the Polytron sequentially with methanol and ultrapure dH_2O , then fill a beaker with ultrapure dH_2O and allow the Polytron to run inside the beaker for 10s.
5. To homogenize the cell lysate, run the Polytron twice for 15 s at a speed of 22.5 k RPM with cooling on ice for 30s between the two steps (see Note 14).
6. If processing more than one sample, perform a rinse and a wash step in ultrapure dH_2O (see step 4) to avoid cross-contamination of samples.
7. Slowly spin the homogenized cell lysate at 4°C for 5–10 min to settle the foam.
8. To separate the insoluble fraction, centrifuge the cell lysate at $8000 \times g$ at 4°C for 10 min.
9. While waiting for the completion of the centrifugation step, equilibrate beads by wash three times with 1 mL cold Wash Buffer by gentle pipetting. To avoid drying of the beads, suspend the washed beads in 100–200 μL of Wash buffer and put them on ice.
10. Once the centrifugation (step 8) is completed, move the clarified cell supernatant to a new tube by pouring or pipetting (see Note 15). Retain the pellet and 1–5% of the supernatant for evaluating IP efficiency.
11. Add the appropriate amounts of washed beads to the cell supernatant by pipetting.

¹³Protein A/G magnetic beads can also be used in the following way: incubate antibody and cell lysate first, and then add the beads and incubate again. Our results have shown that there is no significant difference in IP efficiency, and we only provide the protocol of pre-conjugation here in this chapter.

¹⁴As foam is formed during this step, ensure that the volume of the cell lysate does not occupy more than a third of the tube to avoid over flow. Small scale IP samples can be homogenized in 5 or 15mL tubes, while larger scale IP sample can be in 50mL conical tubes.

¹⁵Carefully analyze the appearance of the cell supernatant and ensure that there are no floating or mixed insoluble particles in the supernatant. If such particles are observed, remove them by carefully pipetting before the IP. Particles may clog the beads and interfere with the affinity purification.

12. Place the tube containing the cell lysate and the beads on a slowly rotating tube rack at 4 °C and allow for 1h of incubation.
13. Once the 1 h incubation is completed, magnetically separate the beads and remove the flow through supernatant by pipetting, saving it in a fresh microcentrifuge tube to later evaluate IP efficiency.
14. Wash the beads three times with 1 mL Wash Buffer. After the third wash, move the bead slurry to a new 2mL tube.
15. Once the beads are transferred to the new tube, wash the beads two more times with 1 mL Wash Buffer, and remove the Wash solution by pipetting.
16. Add 1 mL of PBS and transfer the beads to a new 1.5 mL round bottom tube. Wash once more with 1 mL PBS to remove any residual detergent and improve the efficiency of elution.
17. Add 40 μ L Elution Buffer to the beads and incubate the tube at 70 °C for 10min (see Note 16).
18. Let the tubes cool down at room temperature for 30s and put the tubes on a TOMY shaker and mix for 10min.
19. Magnetically separate the beads from the eluate and transfer the eluate to a new microcentrifuge tube. Retain 5% of the elution for evaluating IP efficiency by SDS-PAGE. The rest of eluate can be prepared for mass spectrometry analysis.
20. Before the mass spectrometry sample preparation, the protein samples need to be reduced and alkylated. Add TCEP (1/20 of the total volume if using 500 mM stock) and CAM (1/10 of the total volume if using 500 mM stock) to reach a final concentration of 25 mM and 50 mM, respectively. Incubate at 70°C for 20min (see Note 17).
21. Proceed to preparation for mass spectrometry analysis (Section 5.2) or store samples at –20 °C.

5.3.3 Optimization of lysis buffer—The composition of the lysis buffer, i.e., detergents and salt concentration, determine the stringency of the buffer. Overall, the buffer must be stringent enough to allow the solubilization of the bait protein, but it also must be mild enough to prevent the disruption of protein interactions. It is recommended to conduct small scale IPs using lysis buffers with varying stringency prior to performing larger scale experiments. A start (mild) lysis buffer can be 1 $\times$ TBT with salt, 0.6% Triton X-100, 200 mM NaCl, 1 $\times$ PIC and PhIC, and 100U/mL Benzonase. To increase the stringency, NaCl concentration can be increase, usually up to 500 mM, and sodium deoxycholate can be added up to 0.5%. Table 1 lists examples of lysis buffers previously used to isolate DNA sensors and to study their protein interactions using various tags and cell types.

¹⁶The elution buffer is 1 $\times$ NUPAGE buffer without DTT (in-gel digestion) or 1 $\times$ LDS Elution Buffer (in-solution digestion).

¹⁷This step does not belong to the IP process, but it is convenient to conduct during the same day.

The following protocol is for cryogenically lysed samples (see Section 5.1.2). The cells can also be lysed using lysis buffers after being harvested (see Section 5.1.3) and taken straight through immunoaffinity purification.

1. Prepare cryogenically ground cell powder as described (see Section 5.1.2). Split the frozen cell powder equally into pre-chilled tubes.
2. Add different lysis buffers to each sample using 5 mL buffer per 1 g cryo-lysed cells (*see* Note 18).
3. Homogenize the cell powder in different lysis buffers by intermittent 30 s vortexing for three times with a 5 min cooling on ice in between the two vortex steps.
4. Separate the insoluble fraction by centrifugation at $8000 \times g$ for 10min at 4 °C.
5. Collect and save the supernatant as the lysate fraction.
6. Resolve the pellet with $1 \times$ NUPAGE sample buffer. Sonicate the suspension, and then boil at 95 °C for 5min. Save the solution as the pellet fraction.
7. Load equivalent amounts (5–10%) of lysate and pellet on PAGE gel and conduct western blotting, probing with antibodies against the tag or the endogenous protein.
8. Compare the relative amounts of bait protein in the lysate and pellet fractions. The lysis buffer which gives the highest amount of bait protein in the lysate fraction should be used as the lysis buffer for the formal IP experiment.

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¹⁸ Usually 0.5–1mL is good to resolve one 15 cm dish of adherent cells.

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Fig. 1.

DNA sensor immune signaling relies on protein-protein interactions. (A) Toll-like receptor 9 resides in endosome membranes to detect incoming unmethylated CpG DNA. (Step A1) The Myddosome forms after TLR9 binds to DNA, dimerizes, contacts the cytosolic protein MyD88 through interaction with the adaptor protein TIRAP, and recruits IRAK4 and IRAK1 or IRAK2. (Step A2) IRAK1 or IRAK2 activates TRAF6 to remove inhibitory proteins from NF- κ B. (Step A3) Activated NF- κ B translocates into the nucleus and promotes transcription of *IFNA* or *IFNB*. (B) Cyclic GMP-AMP synthase monitors the cytosol for double-stranded DNA. (Step B1) Upon binding to dsDNA, cGAS dimerizes and produces 2'3'-cGAMP. This activity can either be enhanced by TRIM14 or TRIM56 or repressed by AKT1 or OASL. The endoplasmic reticulum transmembrane protein STING binds to cGAMP, dimerizes, and recruits TBK-1 for activation. (Step B2) IRF3 is phosphorylated by TBK-1 and dimerizes within the cytosol. (Step B3) Dimeric phosphorylated IRF3 translocates into the nucleus and acts as a transcription factor for Type I IFN genes. (C) AIM2 recognizes dsDNA in the cytoplasm and initiates inflammasome formation. (Step C1) The HIN200 domains of AIM2 bind to dsDNA thereby allowing the AIM2 PYD to interact with the PYD of ASC. The CARD domain of ASC recognizes that of Pro-caspase 1. (Step C2) Autoactivation and cleavage of Pro-caspase 1 leads to the maturation of IL-18 and IL-1 β which are excreted from the cell to induce pyroptosis and inflammation. (D) The PYHIN proteins IFI16 and IFIX bind to herpesvirus dsDNA within the nucleus. (Step D1) IFI16 and IFIX bind to incoming viral dsDNA with their HIN200 domains while forming homo-oligomers with PYD. These oligomers then interact with ND10 bodies to facilitate repression of virus replication. (Step D2) Through unknown mechanisms, IFI16 and IFIX promote innate

immune signaling through interferon expression. (Step D3) Herpesviruses have evolved distinct mechanisms for inactivating IFI16 and IFIX: HCMV uses the tegument protein pUL83 to bind PYD and prevent oligomerization, while the HSV-1 immediate-early protein ICP0 targets PYD for degradation via the proteasome.

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Sample Preparation

Proteome Analysis during Immune Signaling

Immunoaffinity Purification of DNA sensors

Fig. 2.

Workflows for analyzing proteome changes and DNA sensor protein interactions during immune signaling. (Top) Preparation and lysis of cells prior to immunoaffinity purification and proteome analysis. Cells subjected to different experimental conditions, i.e., different time points of viral infection, are collected and lysed using either cryogenic or detergent-based techniques. Lysates are then used for either mass spectrometry proteome analysis or immunoaffinity purification of DNA sensor. (Middle) nLC-MS/MS analysis and relative quantification of protein abundances. Peptides from whole-cell lysates are produced using in-solution digestion then analyzed using either label-free or labeling quantification, such as by labeling with tandem mass tags. (Bottom) Investigation of protein-protein interactions

using immunoaffinity purification coupled to nLC-MS/MS analysis. Purification of protein complexes is performed by incubating antibody-conjugated beads with cell lysates. If targeting the endogenous protein, the control IP can be performed with IgG; if the DNA sensor bait is tagged, cells expressing the tag alone can serve as a control. Eluted protein complexes are digested either in-solution or in-gel, and peptides are analyzed by nLC-MS/MS. The specificity of protein interactions can be assessed using the Significance Analysis of INteractome (SAINT), and associations that pass the specificity filtering can be assembled into functional protein interaction networks.

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Table 1

Examples of lysis buffers for isolating DNA sensors.

DNA sensor	Tag	Cell type	Lysis buffer	Reference
TLR9	Myc-tagged	HEK293T	50mM Tris-HCl, pH 8.0, 150mM NaCl, 5mM EDTA, 1% digitonin, and 1 × PIC	Lee, Kang, and Kim (2016)
IFT16	(A,B,D) Endogenous (C,E)GFP-tagged	(A)CEM-T (B,C,D,E)HFF (C)THP-1 (C)HEK293	20mMK-Hepes, pH 7.4, 0.11M KOAc, 2mM MgCl ₂ , 0.1% Tween-20, 1 μM ZnCl ₂ , 1 μM CaCl ₂ , 0.6% Triton X-100, 200mM NaCl, (Pierce), 1 × PIC, 1 × PHIC, 100U/mL Benzonzonase.	A.Li, et al. (2012) B.Diner, Lum, et al. (2015) C.Diner, Li, et al. (2015) D. Orzalli et al. (2015) E. Diner, et al. (2016)
IFTX	GFP-tagged	HEK293	20 mM K-Hepes, pH 7.4, 0.11M KOAc, 2mM MgCl ₂ , 0.1% Tween-20, 1 μM ZnCl ₂ , 1 μM CaCl ₂ , 0.6% Triton X-100, 200mM NaCl, (Pierce), 1 × PIC, 1 × PHIC, 100U/mL Benzonzonase.	A.Diner, Li, et al. (2015) B.Crow and Cristea (2017)
AIM2	GFP-tagged	HEK293	20 mM K-Hepes, pH 7.4, 0.11M KOAc, 2mM MgCl ₂ , 0.1% Tween-20, 1 μM ZnCl ₂ , 1 μM CaCl ₂ , 0.6% Triton X-100, 200mM NaCl, (Pierce), 1 × PIC, 1 × PHIC, 100U/mL Benzonzonase.	Diner, Li, et al. (2015)
cGAS	(A,B)FLAG- tagged (C) HA- tagged (D) GFP-tagged	(A,B,C)HEK- 293 T (D)HFF	(A) 20 mM K-Hepes, pH 7.4, 0.11M KOAc, 2mM MgCl ₂ , 0.1% Tween-20, 1 μM ZnCl ₂ , 1 μM CaCl ₂ , 0.6% Triton X-100, 200mM NaCl, (Pierce), 1 × PIC, 1 × PHIC, 100U/mL Benzonzonase. (B) 1% NP-40 buffer supplemented with a complete protease inhibitor cocktail (Roche). (C) 50mM Tris, pH 7.4, 150mM NaCl, 1% Triton X-100, 1 mM EDTA (pH 8.0), 1 mM PMSF, 1 mM Na ₃ VO ₄ , 1 mM of NaF. (D) <i>Cytoplasmic fraction</i> : (20mM HEPES, pH 7.4, 10mM KCl, 2mM MgCl ₂ , 1 mM DTT, 0.5% NP-40, 1 × PIC and 1 × PhIC), 100U/mL Benzonzonase. <i>Nuclear fraction</i> : cytoplasmic lysis buffer supplemented with 200 mM NaCl, 1% Triton X-100, 0.11M KOAc, 1% Tween-20, 100U/mL Benzonzonase.	A.Diner, et al. (2016) B. Seo et al. (2018) C.Liu et al. (2018) D.Lum, et al. (2018)