HHS Public Access

Author manuscript

Methods Enzymol. Author manuscript; available in PMC 2016 May 04.

Published in final edited form as:

Methods Enzymol. 2016 ; 568: 113–138. doi:10.1016/bs.mie.2015.09.005.

Assays for Post-translational Modifications of Intermediate Filament Proteins

Natasha T. Snider[1] and **M. Bishr Omary**[2]

[1]Department of Cell Biology and Physiology, University of North Carolina, Chapel Hill, NC, USA

[2]Department of Molecular & Integrative Physiology, Department of Medicine, University of Michigan, Ann Arbor, MI, USA; VA Ann Arbor Healthcare System, Ann Arbor, MI, USA

Abstract

Intermediate filament (IF) proteins are known to be regulated by a number of post-translational modifications (PTMs). Phosphorylation is the best studied IF PTM, whereas ubiquitination, sumoylation, acetylation, glycosylation, ADP-ribosylation, farnesylation and transamidation are less understood in functional terms but are known to regulate specific IFs under various contexts. The number and diversity of IF PTMs is certain to grow along with rapid advances in proteomic technologies. Therefore, the need for a greater understanding of the implications of PTMs to the structure, organization, and function of the IF cytoskeleton has become more apparent with the increased availability of data from global profiling studies of normal and diseased specimens. This chapter will provide information on established methods for the isolation and monitoring of IF PTMs along with the key reagents that are necessary to carry out these experiments.

1. Introduction

1.1. Post-translational modifications of intermediate filament proteins

IF proteins are important for the maintenance of cellular function in the basal state, and are particularly important under stress and in disease states (Omary 2009, Davidson and Lammerding 2014, Gruenbaum and Aebi 2014, Homberg and Magin 2014, Toivola, Boor et al. 2015). IFs are major structural components of the cell cytoskeleton, but through their dynamic behavior and under varying cellular conditions, they have also been demonstrated to impact virtually every aspect of cellular function, including gene transcription, signaling pathways and cellular survival (Herrmann, Strelkov et al. 2009, Toivola, Strnad et al. 2010, Chung, Rotty et al. 2013). The assembly and disassembly dynamics of IF proteins, as well as their associations with other cellular components are regulated by various post-translational modifications (PTMs), summarized in **Table 1**, and a myriad of enzymes that carry out specific PTM on/off reactions (Omary, Ku et al. 2006, Hyder, Pallari et al. 2008, Snider and Omary 2014).

Correspondence to: Natasha T. Snider.

1.2 Available tools and major limitations for the study of IF protein PTMs

The extent of functional understanding regarding the role of each PTM on IF protein function is highly dependent on the availability of tools to study the particular PTM of interest. For example, phosphorylation (Roux and Thibault 2013) and ubiquitination (Sylvestersen, Young et al. 2013) can be analyzed using mass spectrometry with relative ease, whereas sumoylation (Gareau and Lima 2010), which has relatively low stoichiometry and is not easily analyzed by mass spectrometric means, is more difficult to probe. Therefore, the systems-level PTM data currently available is skewed to highlight those PTMs that can be readily tracked using proteomic platforms (Choudhary and Mann 2010, Hennrich and Gavin 2015). The combination of global proteomic data with PTM databases that catalog experimentally-determined and site-specific modifications, or that use computational approaches to predict and quantify PTMs (**Table 2**), has resulted in a wealth of information on modified residues on IF proteins. However, most of these modifications await functional assignment. For most IF protein PTMs, the use of molecular approaches (e.g. site-directed mutagenesis of modification sites), biochemical tools (pan- or site-specific PTM antibodies), chemical probes (inhibitors or activators of PTM enzymes) and transgenic mouse models, in combination with enrichment of the IF protein fraction from cells and tissues, has yielded useful insight into some of the functional roles of PTMs, although much more remains to be learned. The relative insolubility of IF proteins (particularly epidermal keratins) in nondenaturing detergent-containing buffers can be an impediment to the study of PTMs, although these limitations can be surmounted, as it was shown for the case of the type I keratin K17 (Pan, Kane et al. 2011).

1.3. Cross-talk between PTMs on IF proteins

PTMs participate in complex cross-talk mechanisms to regulate IF function. The balance of various modified forms of IF proteins is dictated by cellular conditions, such as mitosis, cell migration, stress and apoptosis. The key to resolving the information encoded by IF PTMs is to determine which PTM signatures are prevalent under a given condition and how altering the stoichiometry of IF PTMs alters IF function, distribution, interactions and, ultimately, cellular fate.

Using the database PhosphoSitePlus (Hornbeck, Zhang et al. 2015) we conducted a search for PTMs on human keratin 8 (K8) that have been reported by at least one low-throughput study, or those that appear in at least five high-throughput studies/records (**Table 3**). In this case, low-throughput refers to data generated via amino acid sequencing, site-directed mutagenesis, or the use of specific antibodies, whereas high throughput refers to studies using unbiased discovery-mode mass spectrometry. This example analysis revealed that 16% (76/483) of residues on K8 are modified by either phosphorylation, acetylation, ubiquitination, sumoylation, or methylation. The majority of the PTM sites on K8 (47/76) are modified by phosphorylation (p-Ser > p-Tyr > p-Thr). It can also be appreciated that most of the acetylation sites are also targets for ubiquitination, and one residue (Lys-285) appears to be capable of undergoing sumoylation, acetylation or ubiquitination. These observations suggest that, depending on the cellular conditions, the various PTMs may directly compete for a given site. In addition to direct competition, there is experimental

evidence for functional cross-talk between the different PTMs, such as between phosphorylation and ubiquitination, sumoylation, and acetylation (Snider and Omary 2014).

1.4. Chemical and pharmacological approaches to study context-specific IF protein PTMs

Regulation of IF proteins by PTMs occurs under specific cellular conditions, which can be modeled using chemical and pharmacological agents. An abbreviated list of representative reagents is shown in **Table 4**. Hyperphosphorylation of IF proteins can be induced by treatment with phosphatase inhibitors, such as okadaic acid or microcystin-LR. Phosphorylation, in turn, can promote IF protein sumoylation, so these two modifications are generally observed together, particularly under conditions of oxidative stress and apoptosis (Snider, Weerasinghe et al. 2011). The latter can be modeled in cells using hydrogen peroxide and anisomycin, respectively, among numerous other reagents that can induce similar responses. Increased acetylation of K8 in epithelial cells is dose-responsive to glucose levels in vitro and in vivo, and can be induced more robustly if the cells undergo transient glucose starvation prior to re-stimulation (Snider, Leonard et al. 2013). Increased *O*-glycosylation of K18 *in vitro* and *in vivo* is observed in response to PUGNAc (Ku, Toivola et al. 2010), an inhibitor of the enzyme that removes *O*-linked *N*-acetylglucosamine (*O*-GlcNAc) groups (*O*-GlcNAcase). Since many IF proteins (Rogel, Jaitovich et al. 2010) are turned over by ubiquitination and proteasomal degradation, treatment with the proteasome inhibitor MG-132 promotes the accumulation of poly-ubiquitinated IF proteins. The sections that follow describe the general methods for isolation of IF proteins from cells and tissues, followed by a summary of specific tools and approaches to study more common PTMs on IF proteins.

2. Extraction of IF proteins from tissues and cells for biochemical analysis of IF PTMs

2.1. Materials and reagents for isolation of IF proteins

Source of IF protein:

- Human or animal tissue
- Primary cells (e.g. freshly isolated hepatocytes)
- Cultured cells expressing endogenous IF proteins
- Cultured cells over-expressing IF protein(s) of interest

Antibodies and immunoprecipitation beads:

- Anti-IF protein antibodies
- Anti-PTM antibodies (pan- or site-specific)
- IgG isotype control
- Dynabeads magnetic beads (or similar)

Buffers:

- TXB: Triton-X Buffer

- ○ 1% Triton-X 100
- ○ 5mM EDTA
- ○ Bring up volume in PBS, pH 7.4

- TXB+PPI: Triton-X Buffer with Protease and Phosphatase Inhibitors

  Immediately before use, add protease and phosphatase inhibitors (follow manufacturer instructions) to the desired volume of TXB to be used in the experiment.

- HSB: High Salt Buffer:
  - ○ 10mM Tris-HCl, pH 7.6
  - ○ 140mM NaCl
  - ○ 1.5M KCl
  - ○ 5mM EDTA
  - ○ 0.5% Triton-X100
  - ○ Bring up volume in double distilled (dd) H_2O
- HSB+PPI: High Salt Buffer with Protease and Phosphatase Inhibitors

  Immediately before use, add protease and phosphatase inhibitors (follow manufacturer instructions) to the desired volume of TXB to be used in the experiment.

- PBS/EDTA:

  5mM EDTA in 1X PBS, pH 7.4. The EDTA is included to protect from calcium-activated protease-related degradation (Chou, Riopel et al. 1993)

- 2X SDS Sample Buffer
- Coomassie-based stain compatible with mass spectrometry applications (e.g. Gel Code Blue)

2.2 High Salt Extraction (HSE) of IF Proteins

1a. (*If using tissue):* Cut 10-25mg of freshly-isolated or snap-frozen tissue from liquid nitrogen storage and place directly into 1mL of ice-cold TXB+PPI on ice. Dounce (50 strokes) to a homogeneous suspension using a Potter-Elvehjem PTFE pestle and glass tube homogenizer (7mL working volume size).

1b. (*If using cells*): Collect cells by scraping (if adherent) and centrifugation at 500g for 5 min. For cells grown to 80-95% confluency on a 100mm dish (~60cm^2 growth surface area) use 2mL of 1X PBS to collect the cells. For a cell pellet with approximate volume of 50-100μL add 1-2mL of ice cold TXB+PPI and solubilize the pellet by pipetting. Leave tubes sitting on ice for 5-10 min.

2. Centrifuge tissue or cell lysate (14,000rpm) for 10 min at 4°C.

3. Collect the supernatant fraction. This is the TXB-soluble fraction, which can be used to immunoprecipitate (i.p.) the detergent-soluble pool of IF proteins (Section 2.3.).

   Note: under basal conditions ~5% of the cellular K8/K18 pool can be extracted from the human colonic cell line HT-29 in a detergent-free buffer, while a total of ~20% can be extracted using the non-ionic detergent Nonidet P-40 which is similar to the TXB buffer (Chou, Riopel et al. 1993, Omary, Ku et al. 1998). The K8/K18 soluble fraction increases during mitosis (Chou, Riopel et al. 1993) but is much smaller for epidermal keratins but likely higher for vimentin and other type III IF proteins (Lowthert, Ku et al. 1995, Omary, Ku et al. 1998). However, only 0.18-0.39% of vimentin in the rat cell line RVF-SMC was reported to be soluble (Soellner, Quinlan et al. 1985), although this may represent an underestimate based on how vimentin was isolated using single-stranded DNA-cellulose columns from [^{35}S]-methionine pulse labeled cells. In terms of keratin content in simple-type epithelial cells and tissues, K8/K18 make up 5% of total cellular protein in HT-29 cells (Chou, Riopel et al. 1993) and 0.2-0.5% of total tissue protein in mouse pancreas, liver and small intestine (Zhong, Zhou et al. 2004); while human epidermal keratins make up 25-35% of total cellular proteins in human keratinocytes (Sun and Green 1978) and 17-27% of newborn mouse keratinocytes (Feng, Zhang et al. 2013).

4. Resuspend the cell pellet in 1mL of HSB+PPI by douncing (100 strokes) and leave on the shaker in the cold room for 1hr.

   Note: the 1hr shaking step is important for obtaining clean high salt extracts. In general, the HSE purity ranks: cell line over-expressed IFs > cell line endogenous IFs > tissue IFs. Therefore, this step is the most critical when working with tissues.

5. Centrifuge the samples at 14,000 rpm for 20min at 4°C. Discard the supernatant, which contains histones and other nuclear proteins that are not solubilized in TXB (step 1).

6. Homogenize the pellet in 1mL of ice-cold PBS/EDTA buffer and centrifuge at 14,000rpm for 10 min at 4°C to obtain the IF protein-rich high salt extract (HSE).

7. Dissolve the HSE pellets in 200-400μL of 2X reducing or non-reducing SDS sample buffer that has been pre-heated to 95°C. Break up the pellet initially by pipetting/vortexing, then heat the samples for 5 min at 95°C. Vortex and pipet as needed to ensure the pellet is dissolved.

   Note: the temperature and the volume of the buffer are critical for ensuring that the entire pellet goes into solution. Dissolving the entire pellet may require 5-15 min of vortexing and pipetting per sample, depending on the pellet size.

8. Proceed with downstream analysis:

- Analyze the HSEs by SDS-PAGE followed by Coomassie stain to check the enrichment of IF proteins (an abbreviated protocol and example gel are shown in **Figure 1**).

  Note: The pellets used in the gels that are shown in Figure 1 were completely solubilized prior to loading on the gels. We have obtained HSEs of vimentin and GFAP with similar purity by following this extraction protocol (e.g., vimentin can be readily seen as noted in Figure 3B).

- Analyze the HSE samples by Western blot using PTM antibodies (pan- or site-specific)

  ○ Useful antibodies for specific PTMs are noted in each sub-section.

- If sufficient material is present on Coomassie-stained gel, excise the IF bands for proteomic analysis by mass spectrometry.

  ○ *Note*: special precautions must be taken in order to avoid contamination: gels must be handled with clean gloves and incubated in clean containers, which should only be washed using ddH2O (no soap); note that keratins are typically considered common “contaminants” of mass spectrometry experiments.

- Alternatively, proteomic analysis may be performed on the total HSE (in-solution digest)

  ○ *Note:* A consultation with a proteomics expert is necessary to discuss the various needs and options for a given experiment prior to initiating a study.

2.3. Immunoprecipitation of detergent-soluble IF proteins

1. Aliquot 50μL of Dynabeads (e.g. Dynabeads protein-G) into an Eppendorf tube, place on the magnet and aspirate storage solution.
2. Resuspend the beads in the antibody solution (1-10μg of antibody in 200μL of PBS +0.02% Tween-20) and incubate on rotator (end-over-end) at room temperature for 20 min.
3. Aspirate antibody solution and wash beads once with 200μL of PBS+0.02% Tween-20. Add 600μL of the cell or tissue lysate (save a small fraction of the original lysate to check the protein levels in the pre-i.p. input, as described in step 8).

   ○ *Note*: Lysis buffers containing different detergents (e.g. NP-40 or Empigen-BB) may be used. Empigen-containing lysis buffers solubilize a larger pool of IF protein (Lowthert, Ku et al. 1995), although they may disrupt certain interactions with IF binding partners.
4. Incubate for 3hr with rotation in a cold room.
5. Place samples on magnet and save the post-i.p. fraction for quality control in step 8.

6. Wash the beads five times with 200μL of PBS+0.02% Tween-20. For the last wash step, collect the beads in 100μL of PBS (without Tween-20) and transfer to a new tube.

7. Remove the PBS and add 60-100μL of hot non-reducing sample buffer, heat the samples to 95°C for 5min and separate the i.p. fraction from the beads on the magnet and collect it into a new tube.

   ○ *Note*: It is necessary to use non-reducing buffer so that the antibody IgG heavy chain (which, when reduced, migrates at ~50kDa) does not obscure the IF protein bands on an SDS-PAGE gel. Alternatively the antibody can be crosslinked to the beads to avoid co-elution (follow manufacturer guidelines for the specific crosslinking procedure).

8. As a quality check, analyze equivalent amounts of the pre-i.p. lysate (from step 3), post-i.p. lysate (from step 5), and the i.p. fraction (from step 7) on an SDS-PAGE gel and probe for the presence of the IF protein of interest. If significant amounts of protein remain in the post-i.p. fraction, vary the ratio of antibody to lysate in order to achieve immunodepletion.

   ○ *Note*: We generally use 3μg of anti-human keratin 18 (L2A1 or DC10 clones; see Table 1 in (Ku, Toivola et al. 2004) for list of antibodies to simple epithelial keratins) antibody per 600μL of HT-29 (human colon cancer cell line) lysate. We optimize this step based on the type and starting number of cells, rather than total protein concentration, because IF protein amounts will be highly variable depending on the starting material.

9. Proceed with downstream analysis:

   - Perform Coomassie stain to check the enrichment of IF proteins in the HSE fraction; excise the IF band of interest for proteomic analysis by mass spec.

     ○ An abbreviated protocol and example gel are shown in **Figure 2**.

   - Perform the immunoblot using antibodies to the IF protein/PTM of interest (pan- or site-specific).

   - Perform proteomic analysis of the total i.p. fraction (in-solution digest).

     ○ *Note*: antibody bead cross-linking is ideal for an in-solution digest in order to avoid signal interference from the immunoglobulins.

3. Methods for monitoring specific PTMs on IF proteins

The subsections below describe the functional significance and the available tools and methods to study several IF protein PTMs: phosphorylation, sumoylation, acetylation, glycosylation, and transamidation. For specific methods for the analysis of other PTMs, such as farnesylation (Hannoush and Sun 2010), ubiquitination (Lill and Wertz 2014), and ADP-ribosylation (Moyle and Muir 2010) we direct the readers to general protocols and previous applications of these modifications in the context of IF proteins.

3.1 Analysis of IF protein phosphorylation

Phosphorylation is the best characterized PTM of IF proteins (Omary, Ku et al. 2006, Snider and Omary 2014). Most of the known phosphorylation sites on IF proteins are Ser and Thr residues found in the globular head and tail domains, although proteomic studies have also revealed numerous phosphorylation sites in the rod domain that only recently began to undergo functional characterization (Snider, Park et al. 2013). One major function of IF phosphorylation is to facilitate the reorganization, and the assembly-disassembly dynamics of IF proteins (Izawa and Inagaki 2006, Sihag, Inagaki et al. 2007, Snider and Omary 2014). With the exception of neurofilaments, basal phosphorylation of IF proteins is minimal but becomes significantly upregulated during stress, mitosis, disease states, and in the presence of mutations that have structural effects on IF proteins. Multiple approaches can be used to study IF phosphorylation, including chemical inhibitors and activators, phospho-specific antibodies, mass spectrometry, and transgenic mice. A list of phospho site-specific antibodies targeting different human IF proteins available through major suppliers is shown in **Table 5**. Additionally, pan-pSer/Thr and pan-pTyr antibody are widely available and may be used for biochemical detection of IF protein phosphorylation sites on IF protein-enriched samples in combination with proper experimental controls. As an example, the pan-phospho-Tyr antibody pY100 (Cell Signaling) recognizes human K8 phosphorylation at Tyr-267 (Snider, Park et al. 2013).

3.1.1. Specific materials needed to study IF protein phosphorylation

- Kinase or phosphatase inhibitors
  - ○ Commercially available with varying degree of target selectivity
  - ○ *Note*: To study epithelial keratin phosphorylation we frequently use okadaic acid and microcystin-LR (Gehringer 2004) for *in vitro* and *in vivo* studies, respectively (Toivola, Zhou et al. 2002).
- Expression plasmids or siRNA against various kinases or phosphatases
  - ○ Useful for examining PTM regulation mechanisms
- Phospho-site specific or pan-phospho antibodies
  - ○ **Table 3**: most antibodies can be used for immunoblotting and some antibodies are also suitable for immunohistochemistry
- HSE fraction or IF immunoprecipitates obtained by following Protocol 2.2 and 2.3
  - ○ Samples are prepared after chemical or genetic manipulation to alter kinase or phosphatase activity or induce stress conditions
  - ○ Note: phosphatase inhibitor treatment will increase the presence of IF proteins in the detergent-soluble pool, whereas some stress conditions (e.g. oxidative stress) will lead to IF protein aggregation. Therefore, both the insoluble (HSE) and soluble (i.p.) fractions should always be examined

Figure 3 provides two different examples for monitoring keratin 8 phosphorylation. Phosphorylation of K8 Ser-74 (panel A) serves as a phosphate “sponge” during stress (Ku

and Omary 2006). Phosphorylation of K8 Tyr-267 (panel B) is important for filament organization, since the phospho-deficient Y267F mutation results in short and mostly perinuclear filaments and the phospho-mimetic Y267D mutation leads to K8/K18 aggregation (Snider, Park et al. 2013).

3.2. Analysis of IF protein sumoylation

Cytoplasmic IFs and nuclear lamins are targets for sumoylation, which entails the covalent addition of Small Ubiquitin-like Modifier (SUMO) proteins to Lysine residues on specific targets (Gareau and Lima 2010). Monosumoylation of lamin-A under basal conditions appears to be important for the proper organization of the nuclear lamina (Zhang and Sarge 2008). On the other hand, epithelial K8, K18 and K19 are modified by polymeric SUMO-2/3 chains primarily under conditions where the filament structures are altered, such as during stress or in the context of disease-associated mutations, and in these cases sumoylation likely functions (at least in part) to modulate the solubility of keratin IFs (Snider, Weerasinghe et al. 2011). Sumoylation of mammalian IF proteins can be studied in an in vitro reconstituted system or in cells and tissues. However, given that the fraction of sumoylated proteins is very small, experimental conditions that alter the stoichiometry of SUMO conjugation/deconjugation reactions are required in most cases to detect this modification on IF proteins. Sumoylation generally occurs within the classic consensus linear motif ΨKX[D/E] (Ψ and X denote a hydrophobic residue and any residue, respectively), although there are exceptions (Gareau and Lima 2010). Sequence-based predictions should be performed as a first step using the freely available programs, such as SUMOplot (Abgent), GPS-SUMO, SUMOsp, and SUMOFI to aid with the experimental analysis.

3.2.1. Specific materials needed to study IF protein sumoylation

- In vitro sumoylation components:
  - ○ Purified IF protein
  - ○ Recombinant E1 and E2 SUMO ligases
  - ○ SUMO proteins (SUMO-1, −2, or −3)
  - ○ ATP
    - ○ Note: for beginning investigations these components can be purchased as a SUMOylation kit (Enzo Life Sciences) and the reactions carried out following published procedures or manufacturer specifications. As controls, reactions lacking ATP or substrate should be performed routinely.
- SUMO antibodies
  - ○ Available from multiple commercial sources. We have used a rabbit polyclonal antibody to SUMO-2/3 (Abcam; ab3742).
  - ○ Can be used for immunoblotting, immunocytochemistry or immunoprecipitation

○ *Note*: immunoprecipitation using anti-SUMO antibodies may prove challenging in the context of hyper-sumoylation due to masking of antibody epitopes.

- HSE fraction or IF immunoprecipitates obtained by Following Protocol 2.2 and 2.3

  ○ N-ethylmalimide (20mM) should be added to the lysis buffer (e.g. TXB+PPI) to inhibit de-sumoylation during the sample processing.

  ○ Mono-sumoylation will result in a ~15kDa increase in the molecular mass of the protein, whereas hypersumoylation appears as a smear of high molecular mass complexes, some of which might be trapped at the top of the gel. Therefore, the entire membrane should be probed during immunoblotting.

Figure 4 provides examples for monitoring IF protein sumoylation biochemically and by immunofluorescence analysis. Note that the sumoylation sites on K8, K18 and K19 are located in the rod domains (Snider, Weerasinghe et al. 2011). Therefore, co-expression of K8/K18 or K8/K19 pairs results in significantly diminished sumoylation relative to expression of each protein individually, likely due to limited access of the relevant enzyme in the context of heterodimer formation that involves the rod domains of the keratin pairs.

3.3. Analysis of IF protein lysine acetylation

Acetylation of lysine residues is a common modification on numerous cellular proteins, and is best known for histones and enzymes involved in energy metabolism (Choudhary, Weinert et al. 2014). IF proteins represent a separate category of targets for this modification, with a large number of acetylation sites determined by mass spectrometry proteomic studies. However, experimental validation has only been reported on human K8, where a single residue (Lys-207) was found to be acetylated in response to glucose stimulation, inhibition or knockdown of the NAD-dependent deacetylase SIRT2, and in the presence of the NAD synthesis inhibitor FK-866 (Snider, Leonard et al. 2013). The acetylation-deficient mutant of K8 (K207R) forms filaments but lacks the ability to assemble into a dense perinuclear network that is seen with WT K8, and shows diminished phosphorylation at Ser-74 (Snider, Leonard et al. 2013). Therefore, lysine acetylation on K8 may couple the metabolic conditions to K8 phosphorylation and filament reorganization. Since there are no known consensus motifs for lysine acetylation, mass spectrometry can be used to identify acetylated sites on IFs and numerous acetylated residues on IF proteins have already been catalogued in databases like PhosphoSitePlus.

3.3.1 Specific materials needed to study IF protein lysine acetylation

- Acetyl-lysine antibodies

  ○ Note: There are a number of these available from commercial sources. However, the reactivity profiles of these antibodies vary significantly, as shown by immunoblot comparison of 3 antibodies using normal mouse liver lysates (**Figure 5A**). Therefore, multiple antibodies should be tested for their ability to recognize the IF protein of interest. We used a rabbit polyclonal anti-acetyl lysine antibody (Abcam; ab80178) to monitor human K8 acetylation.

- Inhibitor of NAD synthesis (to test involvement of NAD-dependent sirtuins)
  - ○ FK-866
- Inhibitors of Sirtuin deacetylase enzymes
  - ○ SIRT1/2 inhibitor: Salermide
  - ○ SIRT2 inhibitor: AGK-2
- pan-histone deacetylase (HDAC) inhibitor: trichostatin A

Currently the best available methods for detecting lysine acetylation on IF proteins are immunoprecipitation with a pan-acetyl lysine antibody followed by mass spectrometry analysis to identify the acetylated residues. Site directed mutagenesis to substitute candidate lysines to nonacetylatable arginines or acetylation-mimetic glutamines can narrow down the major sites, as shown for K8 (**Figure 5B**). Immunofluorescence staining analysis may also be useful in some cases. Note that the nuclear AcK staining (acetylated histones) is usually the major signal detected, although the presence/absence of cytoplasmic staining can also be examined in the context of acetylation site mutants (**Figure 5C-D**).

3.4. Analysis of IF protein *O*-linked glycosylation

Several IF proteins (K8, K13, K18, vimentin and NFs) are known to be regulated by O-linked glycosylation (Snider and Omary 2014), a modification that involves the covalent attachment of β-D-*N*-acetylglucosamine (*O*-GlcNAc) to Ser/Thr residues (Hardiville and Hart 2014). Functionally, O-linked glycosylation of K18 (Chou, Smith et al. 1992, Ku and Omary 1995) protects epithelial cells from injury by interfering with the phosphorylation and activation of stress kinases (Ku, Toivola et al. 2010). In general, *O*-linked glycosylation is important in the maintenance of metabolic homeostasis and therefore, its broader functional significance to the regulation of IF protein function may fully come to light by examining various conditions of altered metabolism or metabolic stress.

3.4.1 Specific materials needed to study IF protein *O*-linked glycosylation

- Antibodies to O-Linked N-Acetylglucosamine
  - ○ RL2, HGAC85 (Abcam, Pierce, Novus)
- Inhibitors of GlcNAc-β-N-acetylglucosaminidase
  - ○ PUGNAc, Thiamet G (Cayman Chemical, Sigma, Tocris)
- Inducers of metabolic stress
  - ○ Streptozotocin (Cayman Chemical, Sigma, Tocris)
- IF protein glycosylation-deficient mice (K18 Gly−/−)
  - ○ mice that over-express glycosylation-deficient human K18 (Ser30, Ser31 and Ser49 converted to Ala)
- Reagents for in vitro *O*-glycosylation

○ may be performed on IF immunoprecipitates or HSEs by incubation with radiolabeled UDP-(4, 5-^{3}H)-galactose and recombinant galactosyltransferase (Ku, Toivola et al. 2004) to label terminal O-GlcNAc residues.

○ non-radioactive methods for in vitro labeling of O-GlcNac modification include “click” chemistry approaches, such as the commercially available Click-iT O-GlcNAc Enzymatic Labeling System (Life Technologies).

3.5. Analysis of IF protein transamidation

Transamidation is a covalent PTM that involves formation of amide bonds between the ε-amino group of Lys and the γ- carboxyl group of Gln residues (Iismaa, Mearns et al. 2009). Epidermal and epithelial keratins are substrates of transglutaminase-2 (TG2), the calcium-dependent enzyme that catalyzes these reactions (Strnad, Harada et al. 2007). Transglutamination of type II epidermal keratins is involved in the maintenance of skin barrier function, whereas in the liver transglutamination of K8/K18 occurs in pathologic settings to promote the formation of the K8/K18-containing aggregates called Mallory-Denk bodies (Strnad Gastro 2007). Although TG2-mediated cross-linking of keratins can be monitored in vitro, methods for in vivo detection of this modification and the specific residues involved are currently lacking. Strong consensus sequences for transglutamination have not been identified, however phage display studies (Sugimura, Hosono et al. 2006, Hitomi, Kitamura et al. 2009) have revealed preferred sequences around the reactive glutamines to be: Qx(P,Y)ØD(P) and pQx(PTS)l, where: x=any; Ø=hydrophobic; p=polar; and l=aliphatic residue.

3.5.1 Specific materials needed to study IF protein transamidation

- Plasmids encoding WT IF proteins or Gln→Asn and Lys→Arg substituted mutants.
- Purified peptides containing potential transglutamination sites
- Recombinant transglutaminase enzyme
- Antibody that recognizes the isopeptide bond (antibody 81D1C2, available from abcam and Santa Cruz). However, there may be an issue with specificity of such antibodies (Johnson and LeShoure 2004).

4 Conclusions

There are several hundred known PTMs across the proteome, and we are just beginning to understand the information that they encode in order to promote adaptable functions of proteins under specific physiological and pathophysiological conditions. IF proteins are known to be regulated by a number of PTMs, but with the improvement in detection methods we will be able to uncover more details about the known IF PTMs as well as identify novel IF-targeting PTMs. Generation of new site-specific antibodies will be necessary in order to study PTM dynamics in physiologically-relevant systems. For some modifications, such as sumoylation, pharmacologic inhibitors of the conjugating and deconjugating enzymes are needed to further advance our understanding of this important modification. PTMs that target conserved IF protein domains are likely to have important

roles on multiple IF protein types, and may provide insight into novel cellular functions of IFs and how they fit into the greater function of the cell cytoskeleton. From a disease perspective, various pathologic states (in humans and in animal models) display a mix of cellular stress responses where changes in IF protein PTMs may serve as disease markers, especially given the abundance of IFs. Finally, pharmacological strategies to alter PTM regulation of IF proteins through inhibiting the activity of various enzymes may also prove to be important from a human disease perspective. The field is wide open in terms the pursuit of functional aspects of IF PTMs, and it is hoped that the tools described herein will promote this line of investigation.

Acknowledgements

The authors' work is supported by US National Institutes of Health (NIH) grants DK47918 and DK52951, and the Department of Veterans Affairs (M.B.O.); NIH grants DK093776 and DK102450 (N.T.S.); and NIH institutional grant DK34933 to the University of Michigan. M.B.O. expresses gratitude to his coworkers who have made significant contributions to our understanding of IF PTMs including Drs. Chih-Fong Chou, Nam-On Ku, Jian Liao, Pavel Strnad, Guo-Zhong Tao, Diana Toivola and Qin Zhou.

List of Abbreviations

AcK	acetyl-lysine
BHK-21	baby hamster kidney cells
Dd	double distilled
HSE	high salt extract
IF	intermediate filament
IP	immunoprecipitation
K	keratin
MS/MS	tandem mass spectrometry
O-GlcNAc	β-D-*N*-acetylglucosamine
pY	phospho-tyrosine
SUMO	small ubiquitin-like modifier
TG-2	transglutaminase-2

References

Chou CF, Riopel CL, Rott LS, Omary MB. A significant soluble keratin fraction in 'simple' epithelial cells. Lack of an apparent phosphorylation and glycosylation role in keratin solubility. J Cell Sci. 1993; 105; (Pt 2):433–444. [PubMed: 7691841]

Chou CF, Smith AJ, Omary MB. Characterization and dynamics of O-linked glycosylation of human cytokeratin 8 and 18. J Biol Chem. 1992; 267(6):3901–3906. [PubMed: 1371281]

Choudhary C, Mann M. Decoding signalling networks by mass spectrometry-based proteomics. Nat Rev Mol Cell Biol. 2010; 11(6):427–439. [PubMed: 20461098]

Choudhary C, Weinert BT, Nishida Y, Verdin E, Mann M. The growing landscape of lysine acetylation links metabolism and cell signalling. Nat Rev Mol Cell Biol. 2014; 15(8):536–550. [PubMed: 25053359]

Chung BM, Rotty JD, Coulombe PA. Networking galore: intermediate filaments and cell migration. Curr Opin Cell Biol. 2013; 25(5):600–612. [PubMed: 23886476]

Davidson PM, Lammerding J. Broken nuclei--lamins, nuclear mechanics, and disease. Trends Cell Biol. 2014; 24(4):247–256. [PubMed: 24309562]

Feng X, Zhang H, Margolick JB, Coulombe PA. Keratin intracellular concentration revisited: implications for keratin function in surface epithelia. J Invest Dermatol. 2013; 133(3):850–853. [PubMed: 23190880]

Gareau JR, Lima CD. The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. Nat Rev Mol Cell Biol. 2010; 11(12):861–871. [PubMed: 21102611]

Gehringer MM. Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response. FEBS Lett. 2004; 557(1-3):1–8. [PubMed: 14741332]

Gruenbaum Y, Aebi U. Intermediate filaments: a dynamic network that controls cell mechanics. F1000Prime Rep. 2014; 6:54. [PubMed: 25184044]

Hannoush RN, Sun J. The chemical toolbox for monitoring protein fatty acylation and prenylation. Nat Chem Biol. 2010; 6(7):498–506. [PubMed: 20559317]

Hardiville S, Hart GW. Nutrient regulation of signaling, transcription, and cell physiology by O-GlcNAcylation. Cell Metab. 2014; 20(2):208–213. [PubMed: 25100062]

Hennrich ML, Gavin AC. Quantitative mass spectrometry of posttranslational modifications: keys to confidence. Sci Signal. 2015; 8(371):re5. [PubMed: 25852188]

Herrmann H, Strelkov SV, Burkhard P, Aebi U. Intermediate filaments: primary determinants of cell architecture and plasticity. J Clin Invest. 2009; 119(7):1772–1783. [PubMed: 19587452]

Hitomi K, Kitamura M, Sugimura Y. Preferred substrate sequences for transglutaminase 2: screening using a phage-displayed peptide library. Amino Acids. 2009; 36(4):619–624. [PubMed: 18651094]

Homberg M, Magin TM. Beyond expectations: novel insights into epidermal keratin function and regulation. Int Rev Cell Mol Biol. 2014; 311:265–306. [PubMed: 24952920]

Hornbeck PV, Zhang B, Murray B, Kornhauser JM, Latham V, Skrzypek E. PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. Nucleic Acids Res. 2015; 43(Database issue):D512–520. [PubMed: 25514926]

Hyder CL, Pallari HM, Kochin V, Eriksson JE. Providing cellular signposts--post-translational modifications of intermediate filaments. FEBS Lett. 2008; 582(14):2140–2148. [PubMed: 18502206]

Iismaa SE, Mearns BM, Lorand L, Graham RM. Transglutaminases and disease: lessons from genetically engineered mouse models and inherited disorders. Physiol Rev. 2009; 89(3):991–1023. [PubMed: 19584319]

Izawa I, Inagaki M. Regulatory mechanisms and functions of intermediate filaments: a study using site- and phosphorylation state-specific antibodies. Cancer Sci. 2006; 97(3):167–174. [PubMed: 16542212]

Johnson GV, LeShoure R Jr. Immunoblot analysis reveals that isopeptide antibodies do not specifically recognize the epsilon-(gamma-glutamyl)lysine bonds formed by transglutaminase activity. J Neurosci Methods. 2004; 134(2):151–158. [PubMed: 15003381]

Ku NO, Omary MB. Identification and mutational analysis of the glycosylation sites of human keratin 18. J Biol Chem. 1995; 270(20):11820–11827. [PubMed: 7538124]

Ku NO, Omary MB. Keratins turn over by ubiquitination in a phosphorylation-modulated fashion. J Cell Biol. 2000; 149(3):547–552. [PubMed: 10791969]

Ku NO, Omary MB. A disease- and phosphorylation-related nonmechanical function for keratin 8. J Cell Biol. 2006; 174(1):115–125. [PubMed: 16818723]

Ku NO, Toivola DM, Strnad P, Omary MB. Cytoskeletal keratin glycosylation protects epithelial tissue from injury. Nat Cell Biol. 2010; 12(9):876–885. [PubMed: 20729838]

Ku NO, Toivola DM, Zhou Q, Tao GZ, Zhong B, Omary MB. Studying simple epithelial keratins in cells and tissues. Methods Cell Biol. 2004; 78:489–517. [PubMed: 15646629]

Lill JR, Wertz IE. Toward understanding ubiquitin-modifying enzymes: from pharmacological targeting to proteomics. Trends Pharmacol Sci. 2014; 35(4):187–207. [PubMed: 24717260]

Lowthert LA, Ku NO, Liao J, Coulombe PA, Omary MB. Empigen BB: a useful detergent for solubilization and biochemical analysis of keratins. Biochem Biophys Res Commun. 1995; 206(1): 370–379. [PubMed: 7529499]

Moyle PM, Muir TW. Method for the synthesis of mono-ADP-ribose conjugated peptides. J Am Chem Soc. 2010; 132(45):15878–15880. [PubMed: 20968292]

Omary MB. “IF-pathies”: a broad spectrum of intermediate filament-associated diseases. J Clin Invest. 2009; 119(7):1756–1762. [PubMed: 19587450]

Omary MB, Ku NO, Liao J, Price D. Keratin modifications and solubility properties in epithelial cells and in vitro. Subcell Biochem. 1998; 31:105–140. [PubMed: 9932491]

Omary MB, Ku NO, Tao GZ, Toivola DM, Liao J. “Heads and tails” of intermediate filament phosphorylation: multiple sites and functional insights. Trends Biochem Sci. 2006; 31(7):383–394. [PubMed: 16782342]

Pan X, Kane LA, Van Eyk JE, Coulombe PA. Type I keratin 17 protein is phosphorylated on serine 44 by p90 ribosomal protein S6 kinase 1 (RSK1) in a growth- and stress-dependent fashion. J Biol Chem. 2011; 286(49):42403–42413. [PubMed: 22006917]

Rogel MR, Jaitovich A, Ridge KM. The role of the ubiquitin proteasome pathway in keratin intermediate filament protein degradation. Proc Am Thorac Soc. 2010; 7(1):71–76. [PubMed: 20160151]

Roux PP, Thibault P. The coming of age of phosphoproteomics--from large data sets to inference of protein functions. Mol Cell Proteomics. 2013; 12(12):3453–3464. [PubMed: 24037665]

Sihag RK, Inagaki M, Yamaguchi T, Shea TB, Pant HC. Role of phosphorylation on the structural dynamics and function of types III and IV intermediate filaments. Exp Cell Res. 2007; 313(10): 2098–2109. [PubMed: 17498690]

Snider NT, Leonard JM, Kwan R, Griggs NW, Rui L, Omary MB. Glucose and SIRT2 reciprocally mediate the regulation of keratin 8 by lysine acetylation. J Cell Biol. 2013; 200(3):241–247. [PubMed: 23358244]

Snider NT, Omary MB. Post-translational modifications of intermediate filament proteins: mechanisms and functions. Nat Rev Mol Cell Biol. 2014; 15(3):163–177. [PubMed: 24556839]

Snider NT, Park H, Omary MB. A conserved rod domain phosphotyrosine that is targeted by the phosphatase PTP1B promotes keratin 8 protein insolubility and filament organization. J Biol Chem. 2013; 288(43):31329–31337. [PubMed: 24003221]

Snider NT, Weerasinghe SV, Iniguez-Lluhi JA, Herrmann H, Omary MB. Keratin hypersumoylation alters filament dynamics and is a marker for human liver disease and keratin mutation. J Biol Chem. 2011; 286(3):2273–2284. [PubMed: 21062750]

Soellner P, Quinlan RA, Franke WW. Identification of a distinct soluble subunit of an intermediate filament protein: tetrameric vimentin from living cells. Proc Natl Acad Sci U S A. 1985; 82(23): 7929–7933. [PubMed: 3865206]

Strnad P, Harada M, Siegel M, Terkeltaub RA, Graham RM, Khosla C, Omary MB. Transglutaminase 2 regulates mallory body inclusion formation and injury-associated liver enlargement. Gastroenterology. 2007; 132(4):1515–1526. [PubMed: 17408647]

Sugimura Y, Hosono M, Wada F, Yoshimura T, Maki M, Hitomi K. Screening for the preferred substrate sequence of transglutaminase using a phage-displayed peptide library: identification of peptide substrates for TGASE 2 and Factor XIIIA. J Biol Chem. 2006; 281(26):17699–17706. [PubMed: 16636049]

Sun TT, Green H. Keratin filaments of cultured human epidermal cells. Formation of intermolecular disulfide bonds during terminal differentiation. J Biol Chem. 1978; 253(6):2053–2060. [PubMed: 416022]

Sylvestersen KB, Young C, Nielsen ML. Advances in characterizing ubiquitylation sites by mass spectrometry. Curr Opin Chem Biol. 2013; 17(1):49–58. [PubMed: 23298953]

Toivola DM, Boor P, Alam C, Strnad P. Keratins in health and disease. Curr Opin Cell Biol. 2015; 32:73–81. [PubMed: 25599598]

Toivola DM, Strnad P, Habtezion A, Omary MB. Intermediate filaments take the heat as stress proteins. Trends Cell Biol. 2010; 20(2):79–91. [PubMed: 20045331]

Toivola DM, Zhou Q, English LS, Omary MB. Type II keratins are phosphorylated on a unique motif during stress and mitosis in tissues and cultured cells. Mol Biol Cell. 2002; 13(6):1857–1870. [PubMed: 12058054]

Zhang YQ, Sarge KD. Sumoylation regulates lamin A function and is lost in lamin A mutants associated with familial cardiomyopathies. J Cell Biol. 2008; 182(1):35–39. [PubMed: 18606848]

Zhong B, Zhou Q, Toivola DM, Tao GZ, Resurreccion EZ, Omary MB. Organ-specific stress induces mouse pancreatic keratin overexpression in association with NF-kappaB activation. J Cell Sci. 2004; 117(Pt 9):1709–1719. [PubMed: 15075232]

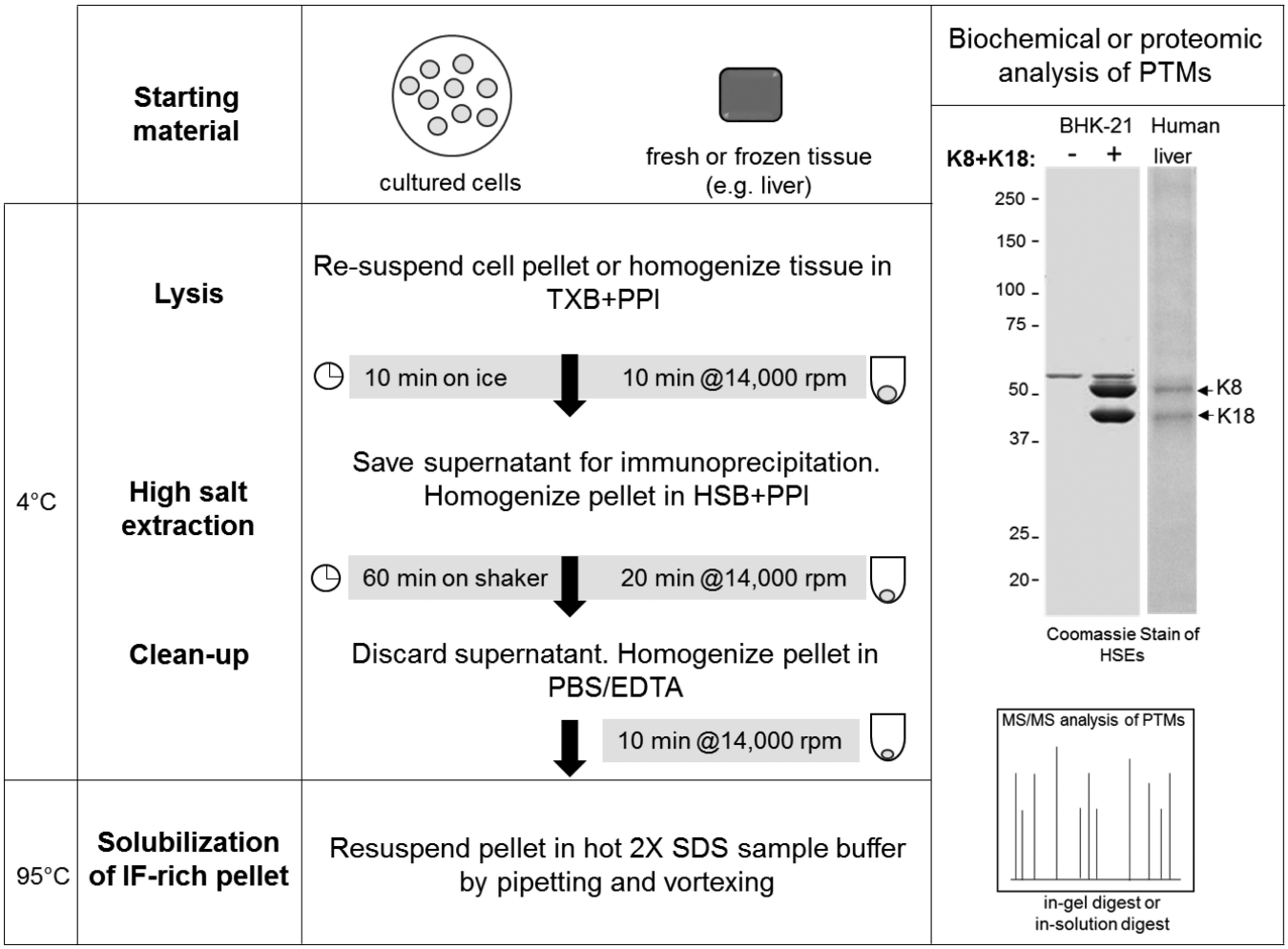

Figure 1.

Isolation of intermediate filament proteins using high salt extraction. Shown is an abbreviated version of the protocol outlined in section 2.2. In the panel on the right, BHK cells are transfected with human K8 and K18 (+) or vector alone (−) and the HSE is compared from these cells is shown in parallel with the HSE from normal human liver.

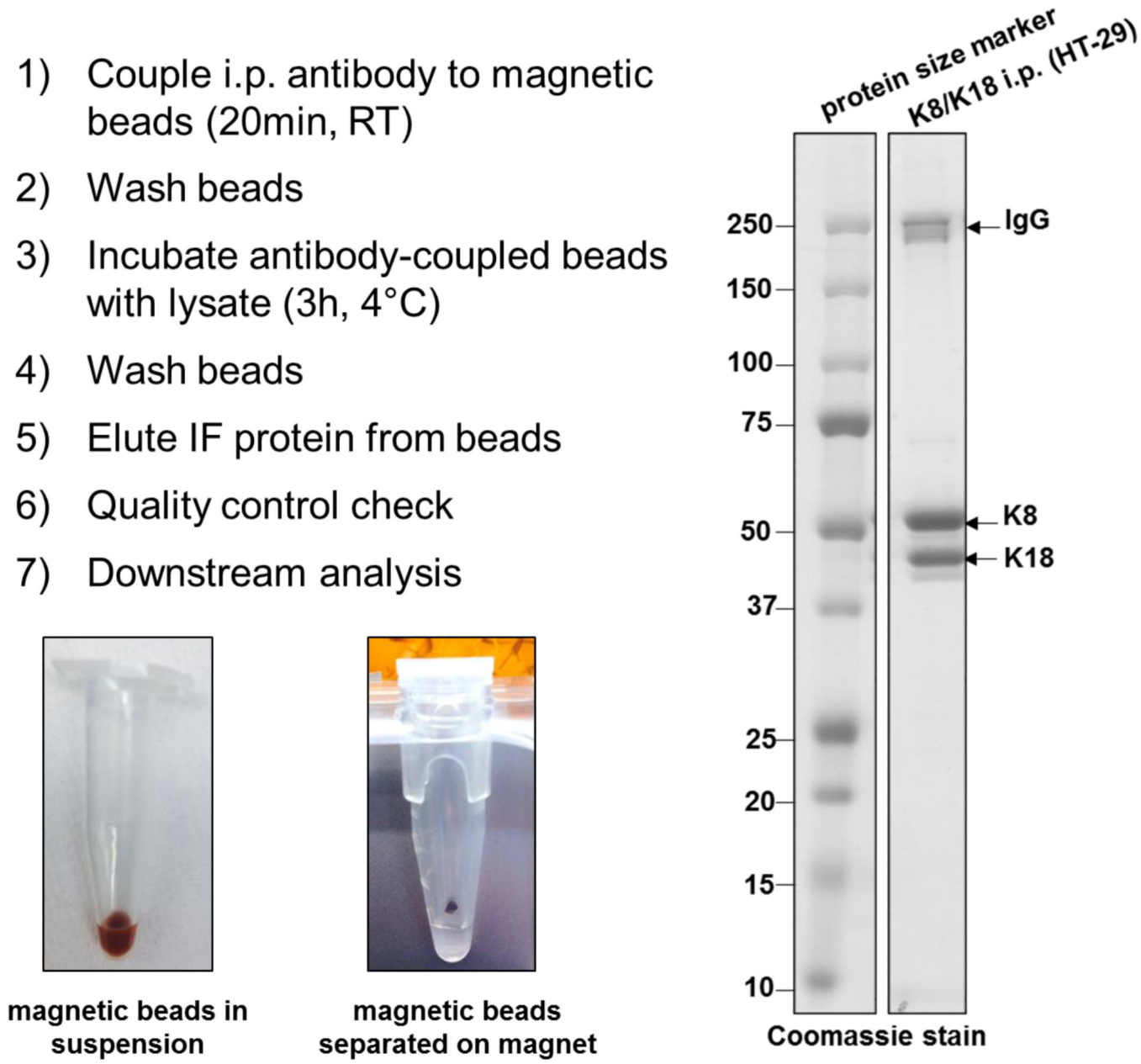

Figure 2.

Immunoprecipitation of intermediate filament proteins from detergent-soluble fractions. Shown is an abbreviated version of the protocol outlined in section 2.3. Representative Coomassie-stained gels of transfected cell- and liver tissue-extracted K8/K18 are shown on the right.

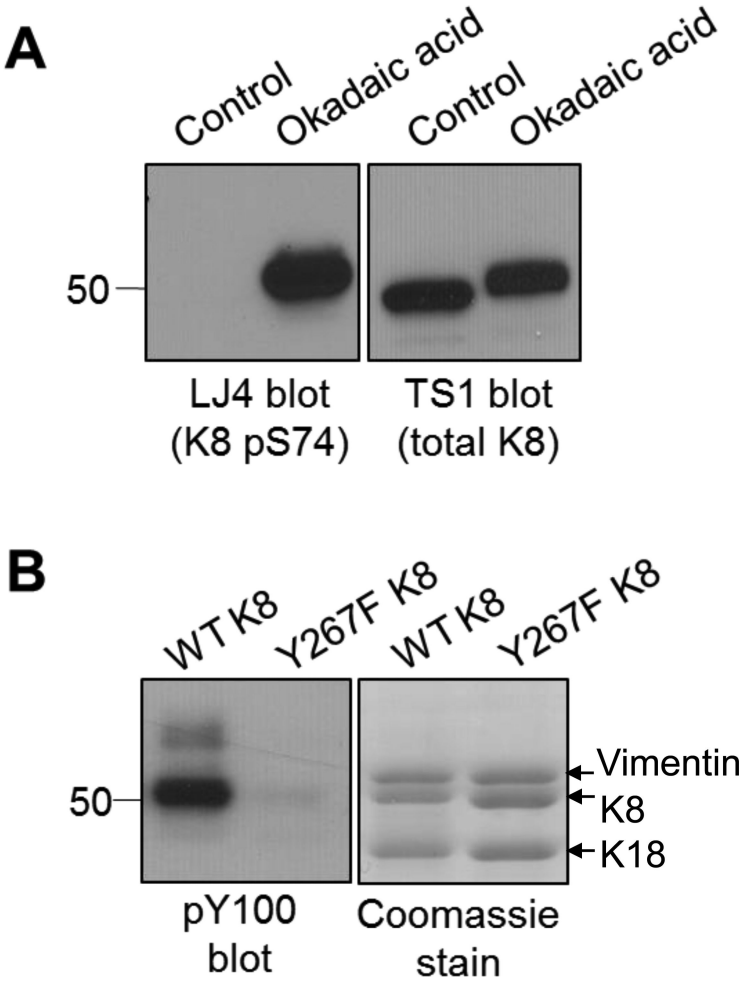

Figure 3.

Biochemical analysis of K8 serine and tyrosine phosphorylation. A, The phosphatase inhibitor okadaic acid was used to upregulate K8 Ser/Thr phosphorylation, as detected in total HepG2 cell lysates by a site-specific antibody (LJ4, which recognizes human K8 pSer-74). Total K8 was detected with the TS1 (mouse anti-human K8) antibody. Note the slight retardation in migration, which is typical of hyper-phosphorylated proteins. B, The pan-phosphotyrosine antibody pY100 antibody (Cell Signaling) was used to demonstrate that human K8 is phosphorylated at Tyr-267. In this case, the HSE fractions of BHK-21 cells expressing wild type (WT) or the phosphorylation-deficient Y267F mutant of human K8 were analyzed, and shown are the pY100 immunoblot and Coomassie stain as a loading control. WT K18 was co-transfected in each case in order to allow stabilization of the partner K18; otherwise there would be degradation of the individual keratin by the proteasome (Ku and Omary 2000). The Coomassie-stained band that migrates above K8 (arrow) corresponds to vimentin which is the major IF protein expressed in the transfected cells.

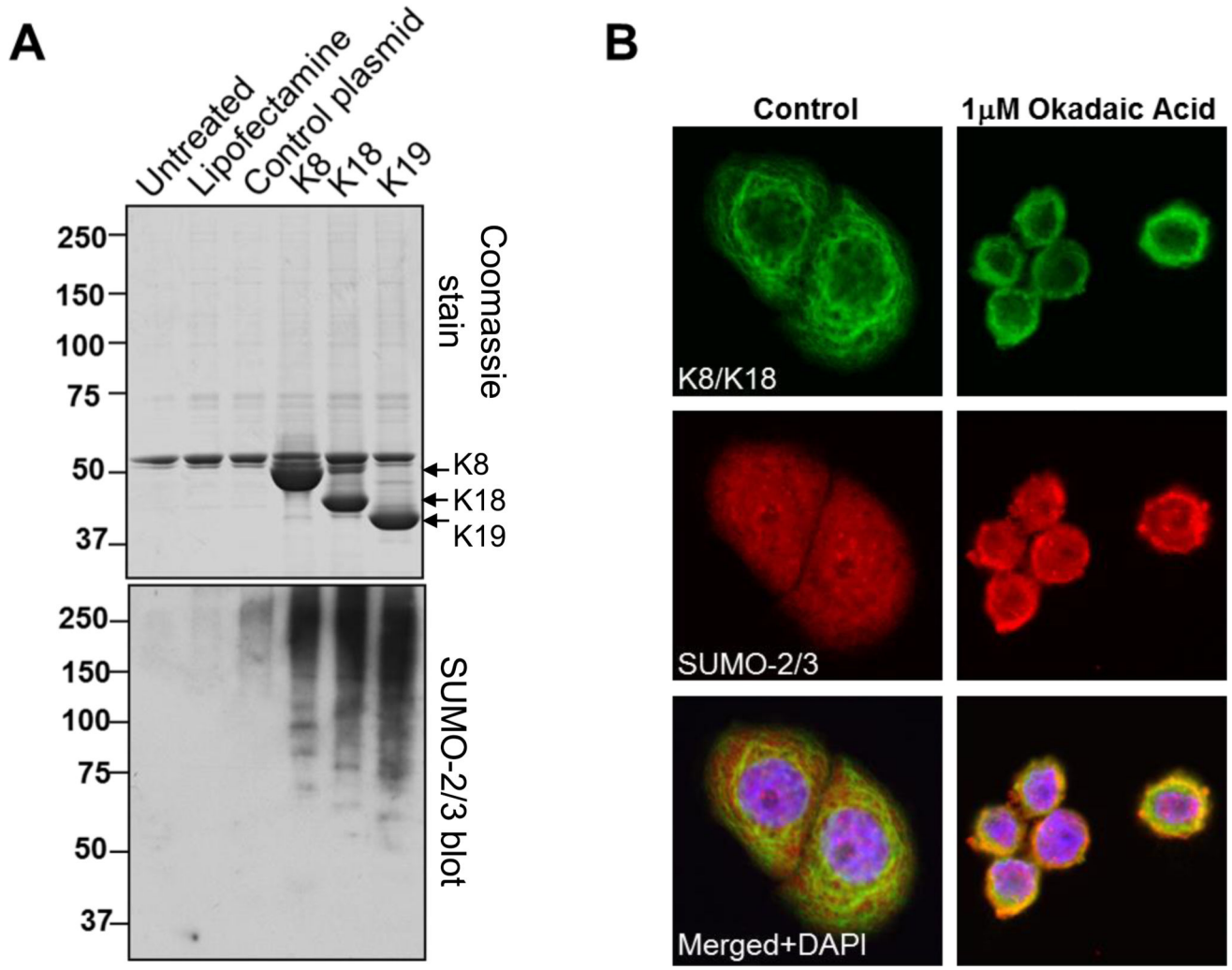

Figure 4.

Biochemical and immunofluorescence analysis of keratin sumoylation. A, extensive poly-sumoylation (observed as ubiquitin-like smears on a SUMO-2/3 immunoblot) is observed when K8, K18 and K19 are transfected individually (i.e. without their obligate type I/II partner). This is due to the fact that sumoylation sites on K8/K18/K19 are located in the rod domains and are inaccessible to the SUMO proteins and their associated enzymes under basal conditions (in the context of intact heteropolymers). Note that large amounts of protein are needed to detect a signal, which is consistent with the low stoichiometry of sumoylation (as seen by the Coomassie-stained gel in the top part of the panel). B, colocalization between an IF protein (K8, green) and SUMO-2/3 (red) is shown in the presence of the phosphatase inhibitor okadaic acid (1 μm, 45min).

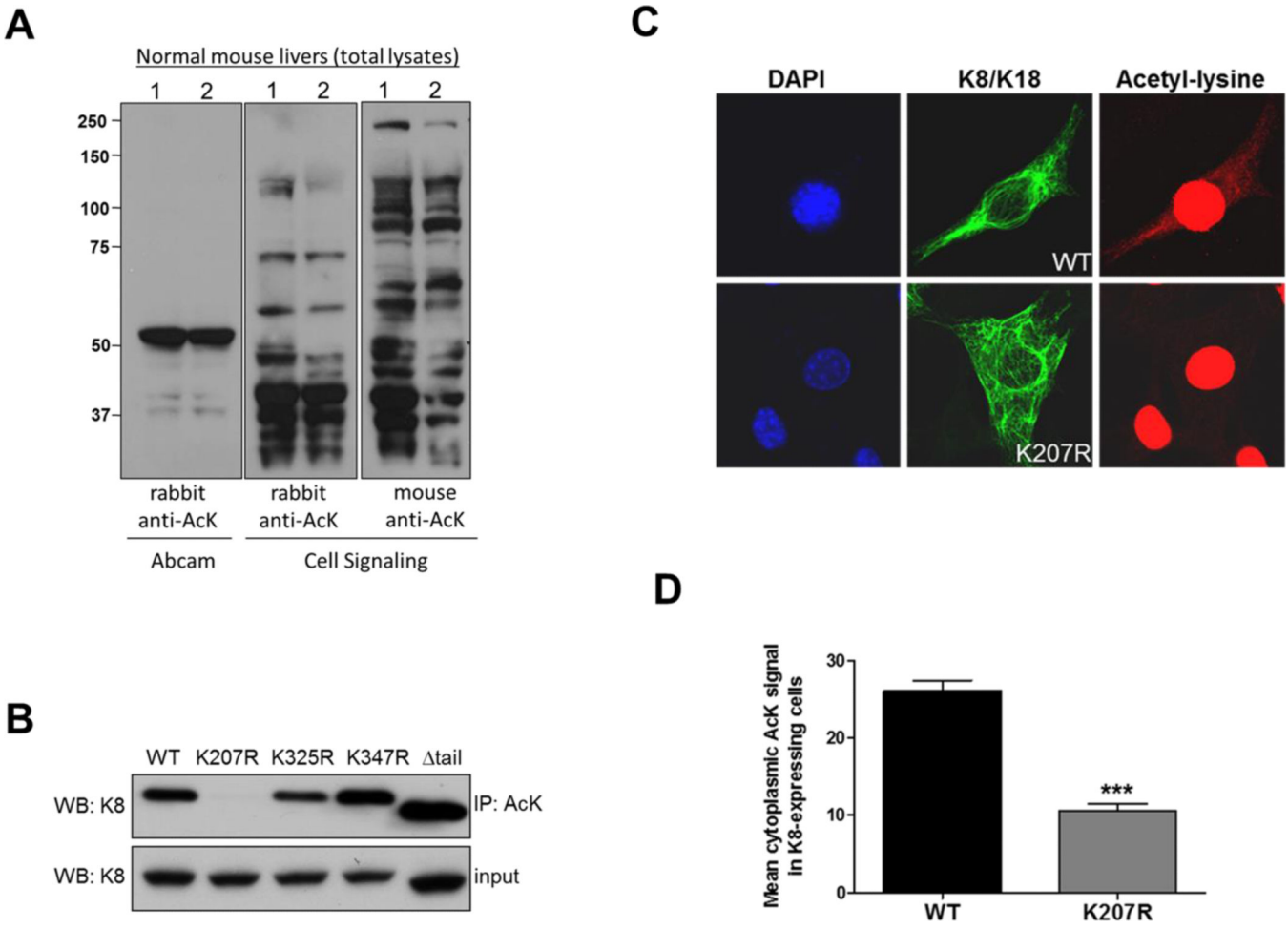

Figure 5.

Monitoring lysine acetylation on IF proteins using pan-acetyl-lysine (AcK) antibodies. **A**, Comparison of three different pan-AcK antibodies by immunoblot on total liver lysates from two control mouse livers. Note the different protein profiles detected by each antibody. **B**, Identification of Lys-207 as an acetylation site on human K8 after immunoprecipitation using an anti-AcK antibody (Abcam antibody from panel A) and immunoblotting with TS1 antibody against K8 (panel reproduced with permission from Snider et al. *J Cell Biol* 2013). **C**, Detection of K8 acetylation using immunofluorescence analysis of WT K8 and the K207R K8 mutant. Note the absence of cytoplasmic signal in the acetylation-deficient mutant. Blue=DAPI, Green=K8, Red=pan-AcK (Abcam antibody from panel A). **D**, quantification of cytoplasmic AcK signal (n=4). P<0.0001, unpaired t-test.

Table 1

Post-translational modifications of IF proteins

IF Protein	Major PTMs
Lamins	phosphorylation, farnesylation, ubiquitination, sumoylation
Keratins	phosphorylation, O-linked glycosylation, ubiquitination, sumoylation, acetylation, transamidation
Vimentin	phosphorylation, O-linked glycosylation, ubiquitination, sumoylation, ADP-ribosylation
Peripherin	phosphorylation, ubiquitination*
α-internexin	phosphorylation*
Neurofilament (-L, -M, -H)	phosphorylation, O-linked glycosylation, ubiquitination,
GFAP	phosphorylation
Nestin	phosphorylation
Desmin	phosphorylation, ubiquitination, ADP-ribosylation
Synemin	phosphorylation*
Syncoilin	phosphorylation*
Filensin (BFSP1)#	phosphorylation*
Phakinin (BFSP2)	phosphorylation*

*the modification has only been detected in high-throughput studies

#BFSP, beaded filament structural protein

Table 2

Databases* that curate experimentally determined or predicted PTMs on various proteins

Database	Web Link	Modifications	Reference
PhosphoSitePlus	www.phosphosite.org	phosphorylation, acetylation, ubiquitination, methylation, succinylation	Hornbeck PV et al (2015) PhosphoSitePlus, 2014: mutations, PTMs and recalibrations. *Nucleic Acids Research*
PTM-SD	http://www.dsimb.inserm.fr/dsimb_tools/PTM-SD/	many	Craveur P et al (2014) PTM-SD: a database of structurally resolved and annotated posttranslational modifications in proteins. *Database*
PTMCode	http://ptmcode.embl.de/	many	Minguez P et al (2012) PTMcode: a database of known and predicted functional associations between posttranslational modifications in proteins. *Nucleic Acids Research*
dbPTM	http://dbptm.mbc.nctu.edu.tw/	phosphorylation, ubiquitination, acetylation, glycosylation, succinylation, nitrosylation, glutathionylation	Lu CT et al. (2013) DbPTM 3.0: an informative resource for investigating substrate site specificity and functional association of protein posttranslational modifications. *Nucleic Acids Research*
PTMCurator	http://selene.princeton.edu/PTMCuration/	many (generates quantitative computational predictions)	Khoury GA et al (2011) Proteome-wide post-translational modification statistics: frequency analysis and curation of the swiss-prot database. *Scientific Reports*
PHOSIDA	http://www.phosida.com/	phosphorylation, acetyation, N-glycosylation	Gnad F et al. (2011) PHOSIDA 2011: the posttranslational modification database. *Nucleic Acids Research*

*Shown is a partial list of the available PTM databases. We commonly use PhosphoSitePlus.

Table 3

Example of Phosphosite database search for PTMs on human K8

# of studies/records		Residue	Type of modification	# of studies/records		Site	Type of modification
LTP*	HTP**			LTP	HTP		
2	24	9	Phosphorylation (Serine)	0	85	25	Phosphorylation (Tyrosine)
1	5	13		0	8	143	
0	44	21		1	117	204	
0	25	22		1	440	267	
5	114	24		0	106	282	
0	33	27		0	49	286	
0	19	31		0	6	419	
1	48	34		0	19	427	
0	102	35		0	49	437	
0	11	36		0	6	11	Ubiquitination (Lysine)
1	27	37		0	6	96	
0	23	39		0	9	101	
1	74	43		0	10	108	
0	21	44		0	9	117	
25	12	74		0	7	122	
0	23	104		0	10	130	
0	5	142		0	6	264	
0	19	253		0	5	285	
0	12	258		0	8	304	
0	9	274		0	22	325	
0	8	280		0	7	347	
1	6	291		0	5	352	
0	5	315		0	59	393	
0	10	330		0	11	472	
0	8	410		2	1	11	Acetylation (Lysine)
1	2	417		1	32	101	
20	27	432		0	7	108	
0	7	436		0	6	122	
0	8	442		1	4	207	
0	8	445		0	5	285	
0	9	456		1	2	393	
0	17	457		2	5	472	
0	20	475		3	38	483	
0	11	478		1	0	285	Sumoylation (Lysine)
0	24	26	Phosphorylation (Threonine)	1	0	364	

# of studies/records		Residue	Type of modification	# of studies/records		Site	Type of modification
LTP*	HTP**			LTP	HTP		
0	31	305		0	5	23	
1	1	431		0	7	32	**Methylation (Arginine)**
0	7	455		0	9	47	

PhosphoSitePlus (Hornbeck, Zhang et al. 2015) was used to conduct a search for PTMs on human keratin 8 (K8). The search was limited to modified residues that have been reported by at least one low-throughput study (LTP), or those that appear in at least five high-throughput studies/records (HTP). LTP refers to data generated via amino acid sequencing, site-directed mutagenesis, of the use of specific antibodies; HTP refers to studies using unbiased discovery-mode mass spectrometry.

*LTP, low throughput

**HTP, high throughput

Table 4

Commonly used strategies to induce IF protein PTMs

Condition	Inducer(s)	PTM(s)
Oxidative stress	hydrogen peroxide	phosphorylation, sumoylation
Apoptosis	anisomycin, Fas ligand	phosphorylation, sumoylation
Phosphatase inhibition	okadaic acid, microcystin-LR	phosphorylation, sumoylation
Nutrient stumulation	glucose	lysine acetylation
Metabolic stress	PUGNAc	*O*-linked glycosylation
Proteasome inhibition	MG-132	ubiquitination

Shown is an abbreviated list of reagents. There are many options for eliciting these types of responses for *in vitro* and *in vivo* monitoring of IF protein PTMs.

Table 5

Phospho-site specific antibodies

IF protein	phosphorylation sites	Commercial source(s)
Keratin 8	pSer-24, pSer-74, pSer-432	Abcam
Keratin 18	pSer-34, pSer-53	Abcam
Keratin 17	pSer-44	Cell Signaling
Vimentin	pSer-34, pTyr-38, pSer-39, pSer-51, pSer-55, pSer-56, pTyr-61, pSer-72, pSer-83	Abcam, Cell Signaling, Sigma, Thermo Scientific
Desmin	pSer-60	Abcam, Sigma
GFAP	pSer-8, pSer-38	Abcam, Sigma
NF-M	pSer-615/620	Abcam
NF-H	KpSP repeat (NAP4 clone)	Thermo Scientific
Lamin-A/C	pSer-22, pSer-392	Abcam, Cell Signaling, Sigma, Thermo Scientific
Lamin-B1	pSer-575	Abcam

This is a list of IF protein phospho-antibodies available from four major antibody vendors. Note that it is not an exhaustive list, as it does not include investigator-generated antibodies or antibodies from smaller suppliers. Researchers should also be aware that the nomenclature may differ depending on the antibody source. For example, K8 pSer-24 may be catalogued as K8 pSer-23 in cases where the initiator methionine is not included in the count.