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Approaches for Studying the Subcellular Localization, Interactions, and Regulation of Histone Deacetylase 5 (HDAC5)

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Abstract

As a member of the class IIa family of histone deacetylases, the histone deacetylase 5 (HDAC5) is known to undergo nuclear–cytoplasmic shuttling and to be a critical transcriptional regulator. Its misregulation has been linked to prominent human diseases, including cardiac diseases and tumorigenesis. In this chapter, we describe several experimental methods that have proven effective for studying the functions and regulatory features of HDAC5. We present methods for assessing the subcellular localization, protein interactions, posttranslational modifications (PTMs), and activity of HDAC5 from the standpoint of investigating either the endogenous protein or tagged protein forms in human cells. Specifically, given that at the heart of HDAC5 regulation lie its dynamic localization, interactions, and PTMs, we present methods for assessing HDAC5 localization in fixed and live cells, for isolating HDAC5-containing protein complexes to identify its interactions and modifications, and for determining how these PTMs map to predicted HDAC5 structural motifs. Lastly, we provide examples of approaches for studying HDAC5 functions with a focus on its regulation during cell-cycle progression. These methods can readily be adapted for the study of other HDACs or non-HDAC-proteins of interest. Individually, these techniques capture temporal and spatial snapshots of HDAC5 functions; yet together, these approaches provide powerful tools for investigating both the regulation and regulatory roles of HDAC5 in different cell contexts relevant to health and disease.

Keywords

HDAC5; Protein complexes; Immunoaffinity purification; Phosphorylation; Protein interactions; Immunofluorescence microscopy; Cell cycle; Kinase inhibition; siRNA knockdown; In-gel digestion

1 Introduction

Histone deacetylase 5 (HDAC5) is a member of the class IIa family of HDACs. Being implicated in cardiac development, the misregulation of HDAC5 is known to be associated with cardiac hypertrophy and myopathies [1, 2]. Misregulation of HDAC5 is further linked

to other human diseases, including tumorigenesis [3], viral infection-induced diseases [4], and drug abuse-induced epigenetic alterations [5-7] (Fig. 1a).

Class IIa HDACs (HDAC4, HDAC5, HDAC7, and HDAC9) are known to have limited intrinsic enzymatic activity, owing to an amino acid substitution within their catalytic domains when compared to the prominent class I deacetylases (e.g., HDAC1 and HDAC2) [8]. Thus, rather than being established as regulators of substrate acetylation levels, the main functions of HDAC5 and the other class IIa enzymes are connected to their critical ability to act as transcriptional repressors through participation in multi-protein complexes [9, 10] (Fig. 1b). In the nucleus, HDAC5 associates with co-repressor complexes, including the nuclear co-repressor complex (NCOR) and the class I enzyme HDAC3 to repress transcriptional targets [9]. HDAC5 is also capable of repressing transcriptional targets independently of these co-repressor complexes through association with transcription factors, such as MEF2 [11]. The HDAC5-MEF2 association is mediated through the N-terminal region of class IIa HDACs that contains transcription factor binding sites, as well as a nuclear localization signal (NLS) (Fig. 2). The C-terminus of HDAC5 contains the deacetylation domain (DAC) and nuclear export sequence (NES) [12]. The domain features of HDAC5 were established through the generation of mutated HDAC5 constructs and subsequent observation of the impact on localization patterns and enzymatic activity, as well as through comparison with other class IIa HDACs [13-18]. Moreover, putative localization signals were fused to GFP to assess their impact on localization, allowing the definition of NLS and NES motifs [19].

Since its discovery, HDAC5 has been the focus of intensive study. Numerous signaling pathways have been shown to converge on phosphorylation sites within HDAC5, including CaMK family members, protein kinases A, C, and D, and Aurora B [2, 20-30]. In particular, HDAC5 has been the target of investigation for its roles in heart development and disease [26, 31-33], especially with respect to its change in localization during hypertrophic conditions. An important feature of class IIa HDACs is their ability to shuttle in and out of the nucleus. In conjunction with the NLS and NES, HDAC5 subcellular localization is regulated by site-specific phosphorylation. Phosphorylation of two sites (Ser259 and Ser498) flanking the NLS promotes binding of 14-3-3 chaperone proteins and nuclear export [11, 19, 34], while phosphorylation of Ser279 within the NLS is important for nuclear localization of HDAC5 [16, 29] (Fig. 2). The kinases responsible for individual phosphorylations have been identified by examining consensus kinase target motifs, and confirmed by activation/inhibition studies and site-directed mutagenesis of target residues [7, 19, 29, 30]. While the nuclear roles of HDAC5 in regulating transcription are the best understood, HDAC5 roles in the cytoplasm and outside of transcriptional control remain poorly defined. A recent investigation of HDAC5 has revealed a cell cycle-dependent regulation of class IIa HDACs, including HDAC5, HDAC4, and HDAC9, by Aurora B-mediated phosphorylation [30]. Phosphorylation of HDAC5 within the NLS is accompanied by midzone and midbody localization of HDAC5 within a phosphorylation gradient during mitosis [30]. Using immunoaffinity purification of EGFP-tagged HDAC5, numerous phosphorylation sites, including those detailed above, were identified [16] (Fig. 2). Site-specific mutations of HDAC5 has provided further insight into the functional roles of individual phosphorylations, for example, identifying a new point of regulation of its nuclear

localization [16, 30, 35]; however, numerous sites await further characterization. The impact of phosphorylation on protein function can be assessed through the effect of phosphorylation site mutation on subcellular localization, protein interactions, and, possibly, transcriptional repression functions or enzymatic activity.

This chapter describes several methods that have proven valuable for studying the subcellular localization, protein interactions, posttranslational modifications, and activity of HDAC5 (Fig. 1b). We start by discussing the value and the challenges associated with studying either endogenous or tagged versions of HDAC5, and by describing a method for generating stable cell lines expressing tagged HDAC5. We next describe a frequently used microscopy approach for assessing the sub-cellular localization of HDAC5 in fixed and live cells. Given that two of the most critical regulators of HDAC5 functions are its protein interactions and posttranslational modifications, we dedicate the next two sections to methods for isolating HDAC5 protein complexes and identifying its interactions and modifications, and for predicting associated HDAC5 structural motifs. Lastly, we provide examples of methods for studying HDAC5 functions and activity, by focusing on its regulation during the cell-cycle progression and its assessment of enzymatic activity.

2 Materials and Equipment

2.1 Studying Endogenous and Tagged HDAC5 in Cell Culture

2.1.1 Generating Cell Lines Expressing GFP-Tagged HDAC5

1. HDAC5-EGFP construct in retroviral vector (e.g., pLXSN).
2. Phoenix cells (production cell line)
3. 6-well tissue culture plates or T75 flasks.
4. Tissue culture dishes (10 or 15 cm) or flasks (T75 or T175).
5. Incubator set at 37 °C, 5 % CO₂.
6. DMEM supplemented with 10 % FBS (DMEM+) or RPMI supplemented with 10 % FBS (RPMI+).
7. DPBS.
8. Wild-type HEK293 cells (or other recipient cell type).
9. FuGene transfection reagent.
10. Centrifuge and rotor capable of spinning at 1000 × *g* for 5 min.
11. Serological pipettes.
12. Sterile 15 mL conical tubes.
13. Centrifuge adaptors for 15 mL conical tubes.
14. 0.45 µm low-binding membrane filter (e.g., cellulose acetate or polysulfonic membrane filters).
15. Polybrene.

16. G418.
17. Materials for FACS analysis (*see* Subheading 2.5.2).
18. Materials for immunofluorescence microscopy (*see* Subheading 2.2).

2.1.2 Cell Culture for Studies of Endogenous or Tagged HDAC5

1. Sterile DPBS (Dulbecco's phosphate-buffered saline (1×), liquid) (DPBS).
2. DMEM++ (500 mL DMEM (Dulbecco's modified Eagle media (1×), liquid) + 50 mL fetal bovine serum + 5 mL penicillin–streptomycin).
3. 1× trypsin (5 mL trypsin Stock + 45 mL sterile DPBS).
4. Cell stock (typically frozen stock in an 85 % FBS/10 % DMSO solution, stored at −144 °C).
5. Water bath set at 37 °C.
6. Incubator set at 37 °C, 5 % CO₂.
7. Sterile tissue culture hood with UV sterilization capability.
8. Centrifuge with swinging bucket rotor capable of spins at 254×*g* at room temperature.
9. Conical tube adaptors for centrifuge rotor.
10. 15 mL conical tubes.
11. 50 mL conical tubes.
12. Serological pipettes and pipettor.
13. Tissue culture dishes (100 mm or 150 mm) or flasks (T25 or T75).
14. Light microscope for visualizing cells (optional).
15. Hemocytometer (optional).

2.2 Assessing Subcellular Localization of HDAC5

2.2.1 Fixed Cell Imaging

1. Starting cell culture (ca. 1×10^6 cells).
2. Poly-D-lysine.
3. Glass coverslips.
4. Glass microscopy slides or glass-bottom dishes.
5. Phosphate buffered saline (DPBS).
6. 2 % paraformaldehyde in DPBS.
7. 0.1 M glycine in DPBS (0.375 g glycine in 50 mL DPBS).
8. 0.1 % Triton X-100 in DPBS (500 µL 10 % Triton X-100 in 49.5 mL DPBS).

9. 0.2 % Tween in DPBS (100 μ l 100 % Tween in 49.9 mL DPBS).
10. BSAT solution: 2 % BSA, 0.2 % Tween in DPBS (1 g BSA and 100 μ L 100 % Tween in 49.9 mL DPBS).
11. Primary antibodies.
12. Fluorescent secondary antibodies.
13. DAPI or To-Pro3 dye (if staining DNA).
14. Anti-fade solution/mounting media (e.g., Aqua PolyMount).
15. Nail Polish.
16. Aluminum Foil.

2.2.2 Live Cell Imaging

1. Starting cell culture (ca. 1×10^6 cells) expressing fluorescently tagged proteins.
2. Glass-bottom dishes (35 mm glass-bottom dishes).
3. DMEM++ (*see* Subheading 2.1.2)
4. Hank's balanced saline solution (HBSS) or phosphate buffered saline (DPBS).
 - a. HBSS is available commercially or can be made to the following specifications: 0.137 M NaCl, 5.4 mM KCl, 0.25 mM Na_2HPO_4 , 0.1 g glucose, 0.44 mM KH_2PO_4 , 1.3 mM CaCl_2 , 1.0 mM MgSO_4 , 4.2 mM NaHCO_3 .
5. Live-cell compatible fluorescent dyes (e.g., ER-Tracker, MitoTracker, LysoTracker, Hoechst) dissolved in DMSO according to manufacturer's instructions.
6. Opti-MEM or other dye-free media for imaging.
7. Aluminum Foil.
8. Fluorescence microscope capable of live cell imaging and laser excitation at wavelengths corresponding to fluorescent tags.

2.3 Defining HDAC5 Protein Interactions

2.3.1 Harvesting Cells

1. Sterile DPBS (*see* Subheading 2.1.2).
2. $1\times$ trypsin (*see* Subheading 2.1.2).
3. DMEM++ or standard culture media (*see* Subheading 2.1.2).
4. Protease inhibitor cocktail.
5. Freezing buffer (20 mM Na-HEPES/1.2 % PVP (w/v), pH=7.4).

For 50 mL, use 0.2383 g HEPES (MW = 238.3 g/mol) and 0.6 g polyvinylpyrrolidone. Adjust the pH to 7.4 with NaOH. Adjust volume to 50 mL with dH₂O. Sterile filter solution and store at room temperature.

2.3.2 Cryogenic Cell Lysis

1. Ball mill-type homogenizer (mixer mill) capable of mechanically disrupting frozen tissue and cells within removable grinding chambers, such as a Retsch Mixer Mill.
2. Grinding chamber (Stainless steel or tungsten carbide).
3. Grinding ball (Stainless steel or tungsten carbide).
4. Hot H₂O (tap water is sufficient).
5. Windex.
6. 10 % bleach solution.
7. Ultrapure H₂O.
8. Methanol.
9. Metal spatula.
10. Liquid nitrogen bath.
11. 50 mL conical tubes.
12. Long-handled tongs.

2.3.3 Conjugation of Magnetic Beads to Antibodies

1. Superparamagnetic beads with surface epoxy groups (e.g., this protocol was specifically optimized for M-270 Epoxy Dynabeads) (Store at 4 °C).
2. Affinity purified antibody against tag or protein of interest (e.g., GFP or FLAG or HDAC5).
3. 0.1 M Na-phosphate buffer, pH 7.4 (Na-phosphate buffer). Prepare solution as 19 mM NaH₂PO₄, 81 mM Na₂HPO₄ and adjust pH to 7.4. Filter-sterilize and store at 4 °C.
4. 3 M ammonium sulfate (AmSO₄). Prepare solution in 0.1 M sodium phosphate buffer, pH 7.4. Filter-sterilize and store at 4 °C.
5. 100 mM glycine-HCl, pH 2.5. Prepare in H₂O and adjust to pH 2.5 with HCl. Filter-sterilize and store at 4 °C.
6. 10 mM Tris, pH 8.8. Prepare in water and adjust to pH 8.8 with HCl. Filter-sterilize and store at 4 °C.
7. 100 mM triethylamine. Prepare a fresh solution in water on the day of bead washing steps. **CAUTION:** Triethylamine must be handled in a chemical hood

and be disposed of as hazardous chemical waste. Triethylamine is toxic and flammable.

8. DPBS, pH 7.4 (*see* Subheading 2.1.2).
9. DPBS containing 0.5 % Triton X-100.
10. DPBS containing 0.02 % sodium azide (NaN_3). **CAUTION:** Sodium azide must be handled in a chemical hood and disposed appropriately. Sodium azide is a toxic solid compound.
11. Tube rotator kept at 30 °C.
12. Magnetic tube rack separator.
13. TOMY micro tube mixer.
14. 2 mL round bottom tubes.
15. Ultrapure water (e.g., from a Milli-Q Integral Water Purification System).

2.3.4 Immunoaffinity Purifications of Endogenous or Tagged HDAC5

1. Frozen cell powder stored at $-80\text{ }^{\circ}\text{C}$ prior to use.
2. 3 M NaCl, filter-sterilized.
3. 10 % Triton X-100 in Milli-Q H_2O , filter-sterilized.
4. 10× TBT with salts, filter-sterilized. Store at 4 °C.

To prepare 100 mL of 10× TBT:

- a. 4.766 g HEPES (MW 238.31).
 - b. 10.797 g potassium acetate (MW 98.15).
 - c. 0.407 g magnesium chloride hexahydrate (MW 203.30).
 - d. 2.0 μL 0.5 M ZnCl_2 .
 - e. 2.0 μL 0.5 M CaCl_2 .
 - f. Adjust pH to 7.4 with 5 M KOH.
 - g. Add 1.0 mL Tween 20.
 - h. Add Milli-Q H_2O up to 100 mL.
 - i. Sterile filter and store at 4 °C.
5. Mammalian protease inhibitor cocktail containing inhibitors with broad specificity against serine proteases, cysteine proteases, acid proteases, and aminopeptidases.
 6. Phosphatase inhibitor cocktail containing sodium orthovanadate, sodium molybdate, sodium tartrate, and imidazole.

7. Phosphatase inhibitor cocktail containing cantharidin, (-)-*p*-Bromolevamisole, and calyculin A.
8. DNase I (1 mg/mL stock).
9. Milli-Q H₂O or other Ultrapure H₂O.
10. Optimized lysis buffer (prepared immediately before isolations). Store at 4 °C.
11. Magnetic beads conjugated with antibodies. Store at 4 °C.
12. 50 mL conical tubes.
13. Immersion tissue homogenizer/emulsifier (e.g., Polytron from Kinematica) or vortex for smaller-scale isolations.
14. Centrifuge and rotor capable of spinning at 8000 × *g*, set at 4 °C with 50 mL conical tube adaptors.
15. Tube rotator kept at 4 °C.
16. 2 mL round-bottom tubes.
17. 1.5 mL tubes.
18. Bar magnets (for conical tubes) and magnetic separation rack (for micro tubes).
19. 4× LDS elution buffer. For 10 mL solution: dissolve 0.666 g of Tris–HCl, 0.682 g of Tris base, 0.8 g of LDS, and 0.006 g of EDTA (free acid) in ultrapure H₂O. Store at –20 °C.
20. 10× reducing agent (e.g., 1 M DTT).
21. 10× alkylating agent (e.g., 1 M iodoacetamide, light-sensitive, store at –20 °C).
22. Heating block or bath set at 70 °C.

2.3.5 In-Gel Digestion of Isolated Protein Complexes

1. Milli-Q water.
2. SimplyBlue SafeStain.
3. HPLC grade water.
4. 100 % acetonitrile (ACN).
5. 0.1 M ammonium bicarbonate (ABC).
6. 0.1% formic acid.
7. 0.1 % formic acid–50 % ACN.
8. 0.5 µg/µL trypsin stock.
9. 10% TFA stock.
10. Nu-PAGE gel containing separated immunoaffinity isolated samples.
11. Low-protein-binding or low-retention pipet tips.

12. Gel slicer capable of generating 1 mm slices.
13. Razor blade for gel lane excision.
14. Metal spatula or pick for separating gel slices.
15. Ceramic surface for separating gel slices.
16. Optional: multichannel pipettor and reagent reservoirs.

2.3.6 Peptide Extraction and Sample Desalting (StageTips)

1. 1.5 mL tubes.
2. Low-protein-binding or low-retention pipet tips.
3. HPLC grade water.
4. 100% acetonitrile (ACN).
5. 0.1 M ammonium bicarbonate (ABC).
6. 0.1 % formic acid.
7. 0.1 % formic acid–50 % ACN.
8. 10% TFA stock.
9. 0.2 % TFA (wash buffer).
10. 0.10 M ammonium formate–0.5 % formic acid–40 % ACN (hereafter referred to as Elution #1).
11. 0.15 M ammonium formate–0.5 % formic acid–60 % ACN (hereafter referred to as Elution #2).
12. 5% ammonium hydroxide–80 % ACN (hereafter referred to as Elution #3).
13. 1 % FA/4 % ACN (Dilution Solution).
14. 16 G Needle and plunger.
15. Hydrophilic sulfonated poly(styrene-divinylbenzene) copolymer disks (e.g., SDB-RPS Empore disks).
16. SpeedVac or vacuum concentrator.

2.4 Investigating Regulation of HDAC5 by Posttranslational Modification

2.4.1 Identification of Posttranslational Modifications—All reagents from Subheadings 2.3.4, 2.3.5, and 2.3.6.

2.4.2 Characterizing PTM Functions

1. FASTA sequence of interest.
2. Computer with internet access.
3. E-mail address to retrieve results (Optional).

2.4.3 Structure Predictions

1. FASTA sequence for protein of interest.
2. List of PDB accession numbers for structures to be included/excluded.
3. Computer with internet access.
4. E-mail address to retrieve results (Optional).

2.5 Cell Cycle Dependent Functions of HDAC5

2.5.1 G1/S, G2/M, and Cytokinesis Arrests

1. Cell stocks or cells growing in culture, plus standard culturing materials (*see* Subheading 2.1.2).
2. DMSO (negative control).
3. Hydroxyurea suspended in DMSO.
4. Nocodazole suspended in DMSO.
5. Hesperadin suspended in DMSO.
6. DPBS (*see* Subheading 2.1.2).
7. Trypsin (*see* Subheading 2.1.2).
8. DMEM++ (*see* Subheading 2.1.2).

2.5.2 Flow Cytometry

1. Cell stocks or cells growing in culture, plus standard culturing materials (*see* Subheading 2.1.2).
2. DPBS (*see* Subheading 2.1.2).
3. 1× Trypsin (*see* Subheading 2.1.2).
4. DPBS supplemented with 2–5 % FBS.
5. 15 mL conical tubes.
6. 95% Ethanol.
7. Propidium iodide (1 mg/mL).
8. RNase A (10 mg/mL).
9. Barrier pipet tips.
10. Flow cytometry-compatible culture tube.
11. Ice bucket.
12. Aluminum foil.
13. Microcentrifuge capable of rotation at $500 \times g$, set at room temperature.
14. Vortex.

15. Flow cytometer (e.g., BD LSRII FACS) and software (e.g., BD FACS Diva).

2.5.3 Kinase Inhibition Studies

Small-Molecule Kinase Inhibitors

1. Cell stocks or cells growing in culture, plus standard culturing materials (*see* Subheading 2.1.1).
2. DMSO (negative control).
3. Hydroxyurea suspended in DMSO.
4. Nocodazole suspended in DMSO.
5. Hesperadin suspended in DMSO.

siRNA-Mediated Knockdown of Kinases

1. Cell stocks or cells growing in culture, plus standard culturing materials (*see* Subheading 2.1.1).
2. Lipofectamine RNAiMAX Reagent or similar transfection reagent.
3. Opti-MEM medium (Life Technologies).
4. siRNA complex targeting kinase.
5. RNase-free barrier pipet tips.
6. RNase-free 1.7 mL tubes.
7. Incubator set at 37 °C, 5 % CO₂.
8. Sterile tissue culture hood with UV sterilization capability.

2.6 HDAC5 Enzymatic Activity

1. All reagents from the Subheading 2.3 *Immunoaffinity isolations*.
2. Fluorometric HDAC activity assay kit. The following protocol has been optimized for the Fluor-de-Lys® HDAC fluorometric activity assay kit, but may be adapted for other fluorometric kits.
3. 96-well plate with optical bottom.
4. 1.5 mL tubes.
5. Microplate-reading fluorimeter capable of excitation at 350–380 nm and detection at 450–480 nm.

3 Methods

3.1 Studying Endogenous and Tagged HDAC5 in Cell Culture

Depending on the biological questions of interest, experiments can be performed by studying either endogenous or tagged HDAC5. The studies on endogenous HDAC5 most frequently rely on the use of specific anti-HDAC5 antibodies. For example, the protein

expression levels of endogenous HDAC5 can be studied across cell types and under different stimuli, treatments, or disease conditions using anti-HDAC5 antibodies and western blotting. The protein levels can be compared to HDAC5 mRNA levels, which can be assessed by qRT-PCR. Anti-HDAC5 antibodies also allow visualization of the subcellular localization of HDAC5 (e.g., [19]) (see Subheading 3.2) and have the potential to allow the identification of HDAC5 protein interactions via immunoaffinity purification assays (see Subheading 3.3). Anti-HDAC5 antibodies can also be designed to recognize a certain modified state of HDAC5 (Fig. 2), for example via the generation of antibodies against site-specific phosphorylations (i.e., phosphoSer259 [36, 37], phosphoSer278 [30], phosphoSer498 [38]). Custom antibody generation can be achieved through the synthesis of a peptide corresponding to the site of modification and a sufficiently long flanking region to impart specificity. Noteworthy, although this has not been yet used extensively in HDAC5 studies and, therefore, it is not covered in this chapter, the endogenous HDAC5, unmodified or modified, can also be detected by targeted mass spectrometry analyses (as in Refs. [35, 39, 40]). Efficient digestion of HDAC5 can be achieved with trypsin, and the use of additional enzymes can further expand the protein sequence coverage and aid in the identification of PTMs (see the in-gel digestion method in Subheading 3.3.5).

Making use of tagged HDAC5 constructs can be beneficial in numerous studies. One valuable application is in live cell studies, in which the dynamic localization of the enzyme can be monitored in real time using fluorescently tagged-HDAC5 constructs. Such studies have been used effectively to visualize the nuclear–cytoplasmic shuttling of HDAC5 (e.g., [16]). The tagging of HDAC5 has also proved valuable when studying its protein interactions. Antibodies against a tag, such as green fluorescent protein (GFP) [41], tend to provide effective isolations of the protein of interest, usually by having higher affinity and specificity than the antibodies against the endogenous protein. Importantly, tagged constructs provide a mean to study HDAC5 mutants that assess the functions of posttranslational modifications. HDAC5 mutants that either mimic or abolish a modification state can be used to study the functions of individual modification sites (e.g., [16, 30, 35]).

When working with a tagged protein construct, it is critical that the functionality of the tagged protein is assessed and validated. It may be practically impossible to rule out all potential effects of tagging, as these studies are limited by the current knowledge regarding the protein functions and the assays available. However, as an example, the functionality of HDAC5-EGFP was assessed by visualization of its subcellular localization by immunofluorescence microscopy, by confirming that the tagged HDAC5 retained its interaction with HDAC3 by immunoaffinity purification, and thereby its *in vitro* enzymatic activity via a fluorometric assay. Several important parameters have to be considered when generating tagged constructs, including the selection of the promoter driving the expression of the tagged proteins. Greater overexpression of HDAC5 can be achieved using the pCMV promoter in certain cell types [42, 43], while a moderate stable expression can be achieved using retrovirus or lentivirus transduction, such as with pLXSN [44]. Avoiding a substantial overexpression of a protein can be critical when constructing cell lines, as protein accumulation can be toxic to the cell and can contribute to the formation of protein aggregates. In this chapter, we provide a protocol for generating stable cell lines that express tagged-HDAC5. This protocol has been successfully used in HDAC5 studies, as well as in studies of

the other ten HDACs and of the seven sirtuins [10, 16, 39, 45-47]. As our laboratory has used this protocol for studying HDAC5 in both plated (HEK293) and suspension (CEM T) cells, differences in the respective protocols are indicated.

3.1.1 HDAC5 Cell Line Generation

Generation of Stable HDAC5-Flag-EGFP Cell Lines via Retroviral Transduction

1. DAY 1: plate Phoenix cells in a 6-well plate (3×10^5 cells in 2 mL media per well). Plating is best performed in the afternoon to allow cells to grow overnight (ca. 24 h). Incubate at 37 °C, 5 % CO₂ overnight.
2. DAY 2: transfect Phoenix cells in afternoon with 1 µg pLXSN-HDAC5-Flag_EGFP plasmid + 3uL FuGene transfection reagent. Incubate at 37 °C, 5 % CO₂ overnight.
3. In the morning of Day 3, wash cells with fresh DMEM+ media.
4. DAY 3: plate HEK293 cells in a 6-well plate (3×10^5 cells in 2 mL media per well). Culture cells overnight (ca. 24 h). For suspension cells (e.g., CEM T) wait to split cells until Day 5. Incubate all cell cultures at 37 °C, 5 % CO₂.
5. DAY 4: collect the supernatant from the cell culture containing released viral particles in a 15 mL conical tube.
6. Centrifuge collected supernatant from **step 5** at $1000 \times g$ for 5 min and reserve resulting supernatant.
7. Filter the supernatant from **step 6** using a 0.45 µm low-binding membrane filter (e.g., cellulose acetate or polysulfonic). The filtered fraction is the Viral Media.
8. Aspirate the media from HEK293 cells (from **step 4**) and add filtered Viral Media such that the volume is ca. 2 mL. Add polybrene (1:1000). Incubate at 37 °C, 5 % CO₂ overnight. Alternatively, dilute a confluent T25 suspension culture 1:10 and add 1 mL of culture to each well. Add 1 mL of Viral Media and 2 µL of polybrene and incubate at 37 °C, 5 % CO₂ overnight.
9. DAY 5: change the media, or if the cells are sufficiently confluent transfer adherent cells to a 10 cm dish (or T25 flask for suspension cells). Incubate at 37 °C, 5 % CO₂ overnight.
10. DAY 6: add G418 (300 µg/mL) to the media. Incubate at 37 °C, 5 % CO₂.
11. For subsequent expansions on days 7–12, split cells following standard procedures, but continue to add G418 to the media (at 300 µg/mL).
12. For cells intended to stably express a fluorescently tagged protein, examine fluorescent signal by immunofluorescence microscopy to assess incorporation.
13. DAY 13: or after ca. 1 week following the initial G418 selection, collect ca. 1×10^5 cells for fluorescence activated cell sorting (FACS) to isolate and retain the fluorescent population.

3.1.2 Cell Culture—The following protocol is described for use of adherent cells. For suspension cells, such as CEM T cells, the trypsin steps should be omitted, and RPMI 1640 media should be substituted for DMEM. Both adherent and suspension cells can be cultured in filter-cap flasks or cell culture dishes. All cell culture work is to be done under the hood. Spray anything that will go into the hood with 70 % ethanol. Latex or nitrile gloves and a lab coat should be worn for all tissue culture and cell culture work. Spills under the hood can be cleaned using bleach.

1. Prepare sterile solutions for cell culture.
 - a. DPBS.
 - b. DMEM++ (500 mL DMEM Media + 50 mL fetal bovine serum+5 mL penicillin–streptomycin).
 - c. 1× trypsin (5 mL trypsin stock + 45 mL sterile DPBS).
2. One hour prior to working with cells, warm media, DPBS, and trypsin solution in a 37 °C water bath.
3. Thaw frozen aliquot of cell stock. Cell stocks are typically frozen in an 85 % FBS, 10 % DMSO solution and are stored long-term at –144 °C.
4. Pipet 5 mL of DMEM++ into a sterile 15 mL tube using a serological pipet.
5. Pipet cell stock solution into the same tube as in **step 4**, mixing the stock solution with the 5 mL of media. Pipet up and down gently to suspend cells in media.
6. Centrifuge 15 mL tubes at ca. $254 \times g$ for 5 min at room temperature in a swinging bucket rotor.
7. Add 8 mL fresh DMEM++ to every plate to which cells will be added.
8. When the spin in **step 6** is finished, aspirate the supernatant and discard. Aspiration steps can be performed using an aspirating pipet and tubing connected to a vacuum collection flask. **CAUTION:** Be careful not to disturb the cell pellet at the bottom of the tube or aspirate cell material when removing the supernatant.
9. To divide the cells among plates, add 2 mL of fresh DMEM++ for each plate (e.g., for three plates, pipet 6 mL of fresh DMEM++ into the tube) and mix up and down by pipetting to suspend the cell pellet.
10. Add 2 mL of cell suspension to each plate. Rock the plate back and forth a few times to distribute the cells across the surface of the plate.
11. Examine plates under the light microscope to ensure cell transfer after plating.
12. Place plates in the 37 °C incubator and allow to adhere and grow.
13. Check plates daily to assess confluence and media color. Cells should be ready to split when confluent in 3–4 days. Once >70% confluent, split cells 1:3 or 1:4.
14. Aspirate old media.

15. Wash cells on plate with 2 mL DPBS. Aspirate DPBS.
16. Add 2 mL 1× Trypsin to plate and tip plate to spread solution over the surface. Allow to sit for 1–2 min. Tap the edges of the plate firmly to dislodge cells from surface of the plate. While incubating the plates with trypsin, prepare fresh plates (3–4 new plates per original plate) and add 8 mL of DMEM++ to each new plate.
17. Once cells are no longer adhering to the surface of the original plate, add an appropriate amount of fresh DMEM++ to the plate and pipet up and down to mix. For example, add 4 mL of DM++ for a total volume of 6 mL (including Trypsin solution) for distribution to three new plates.
18. Add 2 mL of this cell suspension to each fresh plate and rock plate to distribute cells.
19. Return cells to incubator.

3.2 Assessing the Subcellular Localization of HDAC5

HDAC5 localization can be examined either in fixed cells to capture snapshots of HDAC5 localization [10, 16, 29, 30, 35], which is the most commonly used technique and is described in this section, or by live cell imaging to assess dynamic localizations of HDAC5 in different contexts (e.g., altered phosphorylation status, varying cell cycle stages) [16, 38] (*see* Subheading 3.2.1).

Fixation of cells allows imaging the endogenous HDAC5 using commercial antibodies and assessing its co-localization with other proteins of interest. Antibodies raised against specific phosphorylated sites can further provide spatial and temporal information about the posttranslational status of HDAC5. Localization studies can be further aided by the use of cells expressing fluorescently tagged HDAC5. Such methods allow for visualizing the protein via direct fluorescence, which can prevent potential cross-specificity of antibodies used for immunostaining. Alternatively, antibodies against the fluorescent tag can also be used for visualization. The protocol for examining the localization of tagged HDAC5 in fixed cells is identical to that of endogenous HDAC5, with the exception of the antibodies used. The visualization of fluorescently tagged HDAC5 (either by direct fluorescence or antibodies against the tag) can be performed in tandem with the visualization of other proteins using antibody staining procedures to assess their co-localization. The protocols detailed in the next two sections describe the preparation of cells for studies of HDAC5 localization in fixed or live cells and includes instructions on how to prepare cells expressing fluorescently tagged HDAC5 (e.g., HDAC5-EGFP) for live cells imaging, and how to incorporate in these studies staining of cellular organelles, such as trackers for ER (indicated below) or mitochondria.

3.2.1 Preparing Cells for Visualization of Fixed Cells

1. DAY 0: Coat coverslips with poly-D-lysine solution (diluted a 1 mg/mL stock 1:100) and incubate overnight (or at least 8 h) at room temperature.

2. DAY 1: Plate 5×10^5 cells on cover slips in a 6-well plate. Incubate at 37 °C, 5 % CO₂ overnight. Cells may be plated on glass-bottom dishes or EZ-Slides as an alternative to coverslips.
3. DAY 2: Check to ensure that cells are ca. 80–90 % confluent.
4. Wash coverslips 1× with 1 mL DPBS. Dispense DPBS with a pipet onto the side of the well rather than directly over top of the coverslip to avoid disturbing the cell monolayer.
5. Fix cells with 1 mL 2 % Paraformaldehyde for 15 min at 40 °C and incubate on a plate shaker with gentle rocking.
6. Wash 3 × 5 min with 1 mL 0.1 M glycine in DPBS by gentle rocking on a shaker.
7. Permeabilize cells with 1 mL 0.1 % Triton X-100 in DPBS for 15 min, while gently rocking on plate shaker.
8. Wash 3 × 5 min with 1 mL 0.2 % Tween in DPBS. Incubate on plate shaker (gentle).
9. Block cells with 1 mL 2 % BSA, 0.2 % Tween in DPBS, 60 min, on plate shaker (gentle). Alternatively, cells can be blocked at 4 °C overnight.
10. Aspirate blocking solution and add 1 mL of the primary antibody solution. For example, for visualizing HDAC5-EGFP by indirect fluorescence, Roche anti-GFP antibodies can be diluted at 1:5000 in 2 % BSA, 0.2 % Tween in DPBS to make the primary antibody solution. EGFP-tagged HDAC5 can also be visualized using direct fluorescence, in which case the antibody incubation steps can be omitted. Aurora B visualization can be achieved by preparation of a 1:1000 anti-Aurora B antibody solution in 2 % BSA, 0.2 % Tween in DPBS. Wrap the plate in aluminum foil to protect from light. Incubate for 30–60 min at room temperature on a plate shaker (gentle).
11. Wash cells 3 × 5 min with 1 mL 0.2 % Tween in DPBS on a plate shaker (gentle).
12. Add 1 mL secondary antibody solution. Wrap the plate in foil to protect from light and incubate for 30–60 min at room temperature on a plate shaker (gentle).
13. Wash cells 3 × 5 min with 1 mL 0.2 % Tween in DPBS, Plate shaker (gentle).
14. Stain cells in 1 mL 1 μM DAPI or To-Pro3. Wrap the plate in foil to protect from light and incubate for 30–60 min at room temperature on a plate shaker (gentle).
15. Wash cells 3 × 5 min with 1 mL 0.2 % Tween in DPBS on a plate shaker (gentle). Keep cells in the last wash.
16. To mount coverslips, drop ca. 15 μL of anti-fade solution onto the slide.
17. Place cover slip on top of the slide such that the cell monolayer and anti-fade solution are sandwiched between the slide and the coverslip.

18. Wipe off the excess anti-fade solution and seal the edges of slide/coverslip with nail polish.
19. Store the slide at 4 °C in the dark prior to imaging. Slides can be stored for 1–2 weeks at 4 °C in the dark before signal deterioration.

3.2.2 Preparing Cells for Live Cell Imaging by Immunofluorescence Microscopy

Immunofluorescence Microscopy by Live Cell Imaging

1. DAY 0: plate cells on glass-bottom dishes (e.g., 35 mm glass-bottom dishes) in DMEM++
2. DAY 1: Dilute ER-Tracker solution to 500 nM in HBSS Solution and warm to 37 °C.
3. Aspirate DMEM ++ and wash cells with 1 mL HBSS.
4. Aspirate HBSS wash and add 1 mL of 500 nM ER-Tracker/HBSS staining solution to dish.
5. Wrap dish in foil and incubate cells in staining solution at 37 °C, 5 % CO₂ for 30 min.
6. Aspirate staining solution and add 1 mL of Opti-MEM media to dish.
7. Wrap dish in foil and proceed to fluorescence imaging.

3.3 Defining HDAC5 Protein Interactions

Given the nuclear–cytoplasmic shuttling of HDAC5, many of its functions are modulated through the formation of temporal and spatial protein interactions (Fig. 1b). Therefore, similar to the studies of other histone deacetylases, there has been a great interest in characterizing these HDAC5 interactions and their regulation by posttranslational modifications. Immunoaffinity purification in conjunction with mass spectrometry has become a robust and routine approach for identifying HDAC5 protein interactions in different biological context in an effective and unbiased manner. Although important insights have been gained from such studies, it is evident that further analyses will be needed to expand this knowledge and characterize tissue-, cell type-, and subcellular localization-specific HDAC5 protein interactions in the context of health or disease states. The protocol presented in this section has been successfully implemented in HDAC5 protein interactions studies [10, 16, 30, 35], and contains as main steps: (1) cell harvesting, (2) cryogenic cell lysis, (3) conjugation of magnetic beads with antibody, (4) immunoaffinity purification of HDAC5, and (5) analysis of interacting proteins using in-gel digestion with trypsin and mass spectrometry. This workflow can be used to study the protein interactions of either endogenous or tagged HDAC5. The isolation of endogenous HDAC5 would require the use of an anti-HDAC5 antibody with validated affinity and specificity. The isolation of tagged HDAC5 requires the use of antibodies against the tag. For example, we have effectively isolated HDAC5-EGFP using magnetic beads conjugated with anti-EGFP antibodies [10, 16, 30, 35, 41]. It is important to note that the antibodies used for these workflows, regardless if

these are against a protein or a tag, should be purified. Otherwise, all the other proteins present in the antibody solution would also bind to the magnetic beads, leading to the presence of numerous non-specific associations. The protocol described below is suitable for both adherent and suspension cells (*see* Note 1). Additional protocols for immunoaffinity purification of proteins from other tissue types or from studies incorporating metabolic labeling have been described in detail elsewhere [48].

3.3.1 Harvesting and Freezing Cells

1. Aspirate media from cell culture.
2. Wash with 8 mL DPBS to remove dead cells and wash away serum.
3. Aspirate DPBS wash.
4. Add 2 mL 1× trypsin–EDTA in DPBS and place the dish on a rocker. Tap gently to loosen cells.
5. Quench trypsin digest with 6 mL DMEM/10 % FBS/1 % (Pen/Strep) and transfer to conical tube (or larger autoclaved cell harvesting centrifuge tubes if the volumes are large).
6. Spin the samples at $\sim 900 \times g$ for 5 min at 4 °C to pellet cells.
7. Aspirate media.
8. Wash cell pellet in 50 mL DPBS.
9. Re-pellet cells ($900 \times g$ for 5 min at 4 °C).
10. Wash the cell pellet again in 50 mL DPBS.
11. Re-pellet cells ($900 \times g$ for 5 min at 4 °C).
12. Record the weight of the final cell pellet (*see* Note 2).
13. Suspend pelleted cells in freezing buffer (20 mM Na-HEPES/1.2 % PVP (w/v), pH = 7.4), using $\sim 100 \mu\text{L}$ for 0.5 g of cell powder.
14. Poke 5–10 small holes in the cap of a fresh 50 mL falcon tube with a syringe needle. Place the tube in a liquid nitrogen bath and allow to cool, and fill half of the tube with liquid nitrogen (*see* Note 3).
15. Using a pipet, allow the suspended cell solution to fall drop-wise into the falcon tube from **step 14**. The cell droplets will flash freeze into small spheres upon contact with the liquid nitrogen. Continue this process until the whole cell solution is frozen into small spheres.

¹The volumes provided for reference are for adherent cells in culture plates with 100 mm diameter; adjust volumes accordingly for larger dishes. For cells in suspension, trypsinization steps should be omitted.

²The mass of the wet cell pellet can be most easily calculated by recording the mass of the empty tube and the final mass of the tube including cell pellet after harvesting. To avoid overestimation of the mass of the pellet, try to remove as much of the DPBS as possible from the pellet without aspirating cell material.

³For freezing cell material in the liquid nitrogen bath, it is helpful to secure a Styrofoam tube rack in the bottom of another Styrofoam box to hold the falcon tube upright. This can be easily achieved by using pipet tips to pin the rack down in the nitrogen bath. Alternatively, a metal tube rack can be carefully placed in the liquid nitrogen bath to hold the conical tubes.

16. Re-cap the falcon tube with the cap with holes and agitate the tube to allow the residual liquid nitrogen to escape. Replace the lid with a fresh cap that has no holes.
17. Store frozen cell spheres at -80°C until ready to proceed with cryo-disruption.

3.3.2 Cryogenic Cell Lysis

1. Wash mill chambers, grinding ball, plastic washer, and metal spatula in the following order with:
 - a. Hot H_2O .
 - b. Windex.
 - c. Bleach.
 - d. Windex.
 - e. Methanol.
2. Allow mill chamber and ball to dry in the hood for ca. 15 min.
3. Once dried, prechill the mill chamber and the ball together in liquid nitrogen until the boiling appearance stops (ca. 3–5 min).
4. Using long-handled tongs, remove the mill chamber and ball from the liquid nitrogen.
5. Add cell or tissue material to be ground to the deeper half of chamber.
6. Place the ball on top of sample.
7. Tightly screw on the other half of the mill chamber and then submerge the entire chamber containing cell material and grinding ball in the liquid nitrogen bath for 5 min.
8. Using the tongs, remove the mill chamber from the nitrogen back and lock the chamber into the pulverizer and press start (setting: 30 Hz, 2.5 min/cycle). Ensure that the machine is balanced.
9. Re-freeze chamber.
10. Repeat **steps** 8–9 seven times for mammalian cells or 11 times for bacterial cells or 20 times for yeast cells.
11. Label a fresh 50 mL conical tube and place upright in a stand in the liquid nitrogen bath to keep tube cool.
12. Unscrew the mill chamber taking care not to spill any cell/tissue powder. Scrape sample into 50 mL conical using a prechilled metal spatula. Transfer the ball between sides of the mill chamber and scrape to maximize yield.
13. Clean mill chamber, ball, washer, and spatula as in **step 1** and allow to dry in hood.

3.3.3 Conjugation of Magnetic Beads—The protocol described below has been optimized for the conjugation of high-affinity antibodies to magnetic M-270 Epoxy Dynabeads; however, this protocol can be adapted for the use of different bead diameters or conjugation chemistries (e.g., carboxylic acid, tosylated, streptavidin, amine) for isolation of other targets (e.g., glycoproteins or biotinylated proteins). This protocol is compatible with in-house purified antibodies, as well as many commercially available antibodies against both affinity tags and endogenous proteins. Therefore, the protocol can be used for studying endogenous and tagged HDAC5. For example, for tagged HDAC5, the magnetic beads can be conjugated with IgG for Protein A-tagged HDAC5, with anti-GFP antibodies for HDAC5-EGFP, and with anti-FLAG for isolating HDAC5-FLAG.

1. DAY 0, afternoon: Weigh the necessary amount (start with ca. 4–5 mg beads/isolation) of magnetic beads (M-270 Epoxy Dynabeads. in a round-bottom 2 mL tube using clean metal spatula and a sensitive balance (*see* Note 4).
2. Wash beads with 1 mL sodium phosphate buffer; vortex for 30 s; seal cap with Parafilm.
3. Mix for 15 min on TOMY shaker (speed #7, room temperature).
4. Place tube against magnetic rack and remove the ferrous buffer once the beads have separated from the buffer solution.
5. Wash beads with 1 mL Na-phosphate buffer; vortex 30 s.
6. Place tube against magnetic rack and remove buffer.
7. Prepare antibody solution to resuspend beads. Use 5 µg Ab/1 mg beads for purified, high-affinity, antibodies. To calculate the amount of antibody, AmSO_4 , and Na-phosphate buffer required for antibody solution, assume a total volume of 20 µL per mg of beads. The final concentration of AmSO_4 should be 1 M (i.e., one-third of the total volume if starting with a 3 M stock). The remaining volume can be made up with Na-phosphate buffer (i.e., $\text{Volume}_T - \text{Volume}_{\text{Ab}} - \text{Volume}_{\text{AmSO}_4} = \text{Volume}_{\text{Na-phosphate}}$). Add the 3 M AmSO_4 last to limit protein precipitation.
8. Carry out the conjugation overnight on a rotor at 30 °C. Use Parafilm to seal the top of the tube and rotate on y-axis.
9. DAY 1, morning: place the tube on a magnet. Remove supernatant and wash beads with:
 - a. 1 mL NaPhosphate buffer.
 - b. 1 mL 100 mM glycine–HCl (fast wash) (*see* Note 5).
 - c. 1 mL 10 mM Tris.

⁴Bead conjugation is easier to perform with bead amounts of >10 mg. Therefore, it is advisable to conjugate the beads for the bait and control and replicates at the same time.

⁵In the “fast wash” steps, the beads should not be left to sit in the wash solution to minimize prolonged exposure of the antibody-conjugated bead solution to the highly acidic (pH = 2.5) glycine solution or to highly basic triethylamine solution.

- d. 1 mL freshly prepared 100 mM triethylamine solution (fast wash) (140 μ L + 10 mL Milli-Q H₂O in hood) (*see* Note 5).
 - e. 1 mL DPBS (wash 4 $\times$).
 - f. 1 mL DPBS + 0.5 % Triton X-100 for 15 min.
 - g. 1 mL DPBS
10. Resuspend beads for storage at 4 °C in DPBS–0.02 % NaN₃ and use within 2–3 weeks (*see* Note 6). Store at C_f ca. 15 μ L/ mg beads.

3.3.4 Immunoaffinity Purification of HDAC5 With Interacting Partners

1. Resuspend frozen cell powder in an appropriate volume of lysis buffer (LB), e.g., 10 mL LB/g cells. It is advisable to optimize the lysis buffer composition for each isolation of HDAC5, as the efficiency of isolation can be cell-, tissue-, and stimulus-type dependent. However, as a starting point, the buffer detailed below provides an example of a previously optimized lysis condition that was successfully used to isolate HDAC5 from HEK293, U2OS, and CEM T cells [10, 16, 30, 35]. The buffer composition is: 20 mM HEPES–KOH, pH 7.4, 0.1 M potassium acetate, 2 mM MgCl₂, 0.1 % Tween 20, 1 μ M ZnCl₂, 1 μ M CaCl₂, 0.5 % Triton X-100, 250 mM NaCl, 4 μ g/mL DNase I, 1/100 (v/v) protease, and phosphatase inhibitor cocktails. Therefore, 10 mL of this lysis buffer can be prepared by mixing the following reagents:
 - a. 833 μ L 3M NaCl.
 - b. 500 μ L 10 % Triton X-100.
 - c. 1 mL 10 $\times$ TBT with salts.
 - d. 100 μ L protease inhibitor cocktail (for mammalian cells).
 - e. 100 μ L phosphatase inhibitor cocktail (*see* Subheading 2.3.4, **item 6**).
 - f. 100 μ L phosphatase inhibitor cocktail (*see* Subheading 2.3.4, **item 7**).
 - g. 40 μ L DNase I (1 mg/mL stock).
 - h. 7.33 mL Milli-Q H₂O.
2. Mix with Polytron for 20 s; or vortex for 30 s for small volumes or viral samples.
3. Incubate for 5 min with agitation at room temp for DNaseI activity (*see* Note 7).
4. Centrifuge at 4 °C for 10 min at 8000 $\times g$.
5. Place conjugated Dynabeads on magnet and remove buffer.
6. Wash 3 $\times$ with 1 mL of WB and resuspend in 100 μ L of WB.

⁶The isolation efficiency after 1 month of storage at 4 °C decreases to ca. 60 % of the original performance. Therefore, use the magnetic beads within 2 weeks after conjugation.

⁷Addition of DNase is important for the analysis of proteins that are known to bind to DNA in order to determine the dependency of co-isolating proteins on the presence of DNA in immunoaffinity purifications. In lieu of DNase I, benzonase can also be used for efficient degradation of DNA in samples [69].

7. Transfer lysate S/N to clean container (conical or culture tube)—make sure no particles are present, as these can clog the magnetic beads and lead to non-specific associations. Save pellet for Western blot analysis.
8. Add washed beads to cell lysate S/N.
9. Incubate at 4 °C with gentle rotation (5 min to 3 h (*see* Note 8)).
10. Place tube on magnet.
11. Transfer flow-through to another tube and store at 4 °C for Western blot analysis.
12. Wash beads 4× with 1 mL WB (during first and fourth washes, transfer beads to fresh tubes) and 2× with 1 mL DPBS.
13. Elute proteins with 30 µL 1× LDS sample buffer (EB) or 1× (LDS SB/Reducing Agent).
14. Agitate on TOMY shaker (setting #7) for 10 min and place sample at 70 °C for 10 min. At this point, samples can be kept at 4 °C (if running gel within next 24 h) or at −20 °C for longer. Keep 10 % for Western.
15. Carry on either with **steps 16–22** for determining the efficiency of HDAC5 isolation or with Subheading 3.3.5 for preparing the samples for mass spectrometry analysis.
16. Perform Western blotting to check IP efficiency using the following samples:
 - a. Whole cell lysate (WCL): Precipitate protein with four volumes of 100 % acetone (ice cold) and place at −20 °C for at least 30 min (can leave this overnight). Spin precipitated protein solution at 3000 × *g* for 10 min at 4 °C. Wash pellet 2× with 75 % acetone. Use 1 % of precipitated FT protein (instead of 10 % because there should be a lot of protein present) to prepare samples for Western blotting.
 - b. Pellet: suspend pellet in water; boil and vortex if necessary; dissolve pellet as well as possible. Use 10 % of pellet to prepare samples for Western blotting.
 - c. Flow-through (FT): Take 10 % of flow-through volume. Precipitate protein with four volumes of 100 % acetone (ice cold) and place at −20 °C for at least 30 min (can leave this overnight). Spin precipitated protein solution at 3000 × *g* for 10 min at 4 °C. Wash pellet 2× with 75 % acetone. Allow to dry for 15 min (*see* Note 9). Add LDS SB and prepare samples for Western blotting.

⁸Incubation time should be optimized for efficient recovery of the bait protein while minimizing non-specific background proteins. It is best to use as short an incubation time as possible, as extended times lead to the accumulation of nonspecific associations and the loss of weak interactions [41]. For HDAC5 isolations, incubations of 30–60 min are sufficient to capture the protein and its interacting partners.

⁹Allowing the precipitated protein to dry for too long will make the pellet hard to resuspend. Insufficient drying retains traces of acetone, which will interfere with the running of the gel. The dried pellet should be dull white and opaque in appearance. A translucent pellet indicates that the pellet is not dry. A bright white pellet indicates that the pellet may be overly dry.

- d. Magnetic Beads: Resuspend beads in LDS SB and use 10 % of beads to prepare samples for Western blotting. Eluate: Take 10 % of IP eluate and prepare samples for Western blotting.
17. Prepare samples in LDS sample buffer (4×) such that final sample concentration is 1× LDS sample buffer.
18. Add NuPAGE reducing agent or DTT (10× stocks) to a final concentration of 1×.
19. Heat at 70 °C for 10 min.
20. Add iodoacetamide (final = 10 %) to sample and incubate in dark for 20–30 min at room temperature.
21. Separate samples on gel, transfer to a PVDF or nitrocellulose membrane, incubate with antibody against the bait protein or tag to determine efficiency of isolation.
22. Upon confirmation of efficient isolation of bait protein by Western blotting, separate remaining eluate by 1D SDS-PAGE.
 - a. Add reducing agent (e.g., DTT) to final concentration of 100 mM.
 - b. Heat sample at 70 °C for 10 min.
 - c. Add iodoacetamide at 10 % (from 1 M stock, final [IAA] = 100 mM); incubate in dark for 20 min on ice.

3.3.5 In-gel Digestion of HDAC5 Co-isolated Proteins (See Note 10)—Proteins co-isolated with HDAC5 can be analyzed by mass spectrometry or by western blotting. This section discusses in-gel digestion techniques for proteomic-based mass spectrometry analysis; however, in-solution digestion can provide a less time-consuming alternative to in-gel methods. The advantages of using in-gel digestion lie in the ability of gel resolution to remove detergents from IP samples, as well as to resolve complex protein samples [49]. Use of in-solution digestion [48] can minimize sample loss and aid in reproducibility for higher-throughput analyses [10, 50, 51]. The protocol described in this section includes enzymatic digestion using trypsin; however, additional and/or alternative enzymes may be appropriate for the generation of specific target peptides or for the analysis of other proteins of interest. When selecting an enzyme for protein digestion, *in silico* digestions of the protein sequences can be performed to determine the expected cleavage products and assess enzyme suitability, which can be achieved using online platforms such as the MS-Digest utility (<http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msdigest>) of Protein Prospector (Baker, P.R. and Clauser, K.R. <http://prospector.ucsf.edu>).

1. To prepare remaining eluates for mass spectrometry analysis, separate samples on a pre-cast NuPAGE Gradient 4–12 % using a MOPS SDS or MES SDS running buffer. Use the following settings to separate proteins: 100 V for 5 min;

¹⁰Preparation for in-gel digestion can also be performed on same day as digestion to shorten protocol. For very complex samples or if loading > 50 µg of protein per lane, full gel resolution can be performed to distribute sample complexity across the gel length. However, for the majority of protein complexes, ideal gel resolution is achieved when the dye-front is between a third and half way down the full gel length.

200 V for ca. 20 min. Allow the dye front to progress ca. one-third of the way down the gel.

2. After removing the gel from the pre-cast cassette, wash 3×5 min in Milli-Q dH₂O.
3. After third wash, stain gel using Simply Blue SafeStain for 30–60 min.
4. Destain gel in Milli-Q dH₂O and store gel in Milli-Q dH₂O at 4 °C until ready to process for digestion.
5. In-gel digestion can be performed in either individual 1.7 mL tubes or in wells of a 96-well plate with sealing mat (useful when more than four samples are processed at a time). Rinse the 96-well plate with Milli-Q water and shake/tap out excess liquid. Each well/tube is considered a single sample (*see* Note 11).
6. Prepare destaining solution on the day of digestion by mixing 2.0 mL of 100 % ACN and 2.0 mL of 0.1 M ABC in a solution basin (*see* Note 12).
7. Excise a single gel lane from the gel and cut the gel lane into 1 mm slices. When using a 96-well format, do not put more than four 1 mm slices into each well.
8. Add 90 μ L of destaining solution to each well and agitate at 4 °C for 10 min. Aspirate and discard (*see* Note 13).
9. Add 90 μ L of fresh destaining solution to each well and agitate at 4 °C for 10 min.
10. While destaining aliquot the following amounts of the following solutions into separate basins to allow use of the multi-channel pipette for dehydration and rehydration steps:
 - a. 5 mL of 100 % ACN for dehydration steps.
 - b. 5 mL of 50 mM ABC for rehydration steps.
11. Aspirate the destaining solution from wells and add 90 μ L ACN to each well. Invert the plate several times and incubate until gel pieces are white (ca. 1 min).
12. Remove ACN and add 90 μ L of 50 mM ABC to each well. Invert the plate several times and shake at 4 °C until the gel pieces swell and become translucent (ca. 5 min).
13. Aspirate ABC and add 90 μ L of 100 % ACN to dehydrate, and incubate as above.
14. Remove ACN and add 50 mM ABC to samples and let rehydrate as above.
15. Aspirate ABC and perform a final (third) dehydration with 90 μ L of 100 % ACN.

¹¹Working solution volumes in this protocol have been calculated for 16 samples, which corresponds to the average number of wells needed to process two gel lanes in which samples have been resolved one-third of the way down the gel (ca. 8 fractions per lane), assuming that 90 μ L of solution are used per well, unless noted otherwise.

¹²If using 96-well plates, all solutions can be added/removed from wells by a multichannel pipettor, keeping same set of tips for each sample set for all steps performed on this day.

¹³For all aspirations, ensure that gel pieces remain in the wells and do not stick to the pipet tips. To minimize loss of gel pieces, avoid pushing the pipet tip down to the bottom of well and limit the number of gel pieces in each well.

16. Aspirate ACN. Allow the residual ACN in wells to evaporate during preparation of the trypsin solution (*see step 13*).
17. Mix 331.5 μL of 50 mM ABC and 8.5 μL of 0.5 $\mu\text{g}/\mu\text{L}$ trypsin in an 1.7 mL tube.
18. Add 20 μL of trypsin–ABC solution to the dehydrated gel pieces. Allow gel pieces to rehydrate and swell (ca. 5 min).
19. Add 30–50 μL of 50 mM ABC to gel pieces, using a sufficient volume to fully cover the gel pieces. Seal the plate and incubate for 10 min at 37 °C. Check gel pieces after 10 min of incubation to ensure they are still fully covered by ABC, then incubate overnight at 37 °C.

3.3.6 Peptide Extraction and Sample Desalting (StageTips)—Following peptide extraction, samples can be desalted using either StageTips (as described in this section) [50, 52] or just prior to liquid chromatographic separation on the analytical column through the use of online trap columns.

1. Add an equal volume (relative to amount of ABC added on Day 2, **step 15**) of 1 % formic acid to each sample and incubate for 4 h at room temperature.
2. Transfer the extracted peptides to a clean 1.7 mL tube. Avoid transferring large gel pieces. Use a different tip for each well; however, the tip can be reused for the same well in **step 4** below. Adjacent samples can be pooled at this step, if desired, for samples corresponding to gel regions with lighter staining/ lower estimated protein content.
3. Add an equal volume (*see step 1*) of 0.5 % formic acid–50 % ACN solution to the gel pieces and incubate for an additional 2 h at room temperature.
4. After 2 h, combine the second extraction (**step 3**) with the first peptide extraction (**step 2**).
5. SpeedVac samples to ~20 μL to remove ACN. During SpeedVac concentration, assemble StageTips. Assemble one StageTip for each sample.
 - a. Cut Empore SDB disk out using the 16 gauge needle and use the plunger to pack the disk into a P200 tip.
 - b. Tap the disk down into place until it is approximately $\frac{3}{4}$ of the way down from the lowest graduated ring of the P200 tip.
 - c. Each disk binds ca. 15 μg of peptides. Additional disks can be layered or a larger disk diameter can be selected if needed (e.g., for 25–30 μg , use 14 gauge needle).
6. Dilute each sample to approximately 40 μL with 0.5 % TFA.
7. Adjust samples to 1 % TFA (*see Note 14*).

¹⁴The final volume of the sample loaded over the StageTip should be between 50 and 150 μL . The final concentration of ACN should be <10 %.

8. Pipet samples over the top of the StageTip. Hold StageTip and flick wrist to remove excess air between the sample and the top of the SBD disk.
9. Place each StageTip into a collection tube and centrifuge tips at $1000\text{--}2000 \times g$ until the entire sample has passed through the disk (*see* Note 15). Flow-through can be saved if desired for analysis of the efficiency of isolation.
10. Pipet 100 μL wash buffer over the top of the StageTip and centrifuge as above until all wash solution has passed through the disk (*see* Note 16).
11. If fractionating sample, continue to **step 13**. If performing a single elution, apply 50 μL of the Elution #3 solution to the top of each StageTip. Proceed to **step 14**.
12. Elute peptides from the disk into autosampler vials by applying air pressure with the syringe. Each elution should take approximately 15 s.
13. For fractionation, perform stepwise elutions into separate autosampler vials using Elution #1, #2, and #3 solutions. Proceed to **step 14**.
14. Concentrate the samples to approximately 1 μL in the SpeedVac (*see* Note 17).
15. Add Dilution Solution (1 % FA/4 % ACN) to achieve the desired peptide concentrations (e.g., 9 μL for routine LC-MS/MS analysis).

3.4 Investigating Regulation of HDAC5 by Posttranslational Modification

3.4.1 Identification of Posttranslational Modifications (PTMs)—Immunoaffinity approaches coupled with mass spectrometry analysis can also be used for the identification of posttranslational modifications, including phosphorylation, acetylation, sumoylation, and ubiquitination, among others. Enrichment for the isolation of specific modifications can be achieved through the use of antibody-based enrichments (e.g., using pan-anti-acetyl or anti-phospho antibodies) [53-55] or through the use of affinity chromatography methods that make use of metal-based resins with affinity for phosphorylated peptides such as IMAC (Ni-based) or TiO_2 enrichments [56-59].

3.4.2 Characterizing PTM Functions

Amino Acid Substitutions: The impact of posttranslational modifications on HDAC5 localization, activity, and function can be assessed by generation of site-specific null or mimetic mutants at identified sites of modification (Fig. 2). For example, a phosphorylated serine residue can be mutated to alanine to simulate an unmodified state, while mutation of the residue to aspartic acid can mimic constitutive phosphorylation. Specifically, mutation of Ser259 and Ser298 to alanine results in a loss of association with 14-3-3 proteins and increased nuclear localization of HDAC5 [15, 60]. Alternatively, mutation of Ser279 to alanine results in cytoplasmic localization of HDAC5 [16, 29]. Similarly, phosphorylated threonine or tyrosine residues can be mutated to glutamic acid to generate phosphomimetics.

¹⁵Choose a centrifuge speed that gives a flow rate over the disk of approximately 50 μL sample/min.

¹⁶Empty collection tubes if necessary.

¹⁷For fractionated samples, the three elution solutions will concentrate at different rates. The concentration speed of Elution #3 is the fastest, then Elution #2, then Elution #1. For reference, concentration of Elution #3 takes 8–12 min in our SpeedVac (on the no-heat setting).

Acetylation of lysine can be mimicked with replacement of lysine with glutamine, while mutation of lysine to arginine would eliminate potential acetylation of the site while retaining the charge state of the amino acid. These mutations can be achieved by site-directed mutagenesis using a variety of available kits and cloning techniques.

3.4.3 Motif Searching—Determining potential enzymes that can act on PTM sites can also provide insight into the regulation and function of individual modifications. Algorithms such as those found at gps.biocuckoo.org or expasy.org that search amino acid sequences for motifs matching established consensus sequences can be employed for prediction of multiple modifications.

1. Obtain FASTA sequence of interest.
2. Navigate to <http://www.cbs.dtu.dk/services/NetPhosK/>
3. Paste FASTA sequence into “Submission” window or upload FASTA submission using the “Choose file” radio button.
4. Select filtering type (for rapid prediction, select “Prediction without filtering”).
5. Click submit to begin the search.

3.4.4 Structure Predictions—While a crystal structure for HDAC5 has not yet been published, the complete structure of another deacetylase, HDAC8, and of the deacetylase domain of HDAC4 have been investigated by X-ray crystallography and modeling [61-63]. Due to the high degree of sequence homology among HDACs, especially within the enzymatic domain, these known structures provide a useful platform for further predicting HDAC5 structural elements. These structural predictions can be informative when investigating how certain posttranslational modifications may impact HDAC5 functions.

Predictions of HDAC5 structural conformation can be generated based on the primary amino acid sequence of HDAC5 and its similarity in sequence and motifs to other previously crystallized structures. Predictions for HDAC5 can thus be developed based on PDB structures currently available in the RCSB PDB databank. Online structural prediction platforms, such as I-TASSER (<http://zhanglab.ccmb.med.umich.edu/I-TASSER/>), allow for searching of protein FASTA sequences and also allow addition of customizable restraints to the search space through the addition of inclusion and exclusion lists for similar proteins and protein motifs with solved crystal structures [64-66]. Such user-defined restraints and curated structure libraries can further refine the algorithm for individual proteins.

1. Obtain FASTA sequence of HDAC5.
2. Navigate to <http://zhanglab.ccmb.med.umich.edu/I-TASSER/> in the web browser.
3. Copy and paste FASTA sequence into the box under the heading “Copy and paste your sequence here.”
4. Optional: enter an e-mail address in the corresponding box to retrieve I-TASSER prediction results.

5. If desired, select additional constraints for structural modeling by selecting the appropriate filter by clicking on the black triangle next to each “Option” category. (i.e., Option I: Assign additional restraints & templates to guide I-TASSER modeling, Option II: Exclude some templates from I-TASSER template library, Option III: Specify secondary structure for specific residues)
6. Click the “Run I-TASSER” button to execute the modeling algorithm.

3.5 Cell Cycle-Dependent Functions of HDAC5

Accumulating evidence has demonstrated that histone deacetylases are regulated during the progression of the cell cycle. For example, class IIa HDACs (HDAC4, HDAC5, and HDAC9) were shown to be substrates of the mitotic kinase Aurora B, and protein interactions and phosphorylations of HDAC5 were reported to change during the cell cycle. These reports demonstrate the value and the need for future studies that characterize the cell cycle-dependent functions of HDACs. Cell cycle arrests in conjunction with protein immunoaffinity purifications, imaging techniques, and examination of posttranslational modifications can provide important insights into HDAC5 functions (Fig. 1b). The protocols presented in this chapter can be used to assess diverse biological functions of HDAC5 during cell cycle progression, and have been selected given their successful use in previous HDAC5 studies [30]. The protocols describe the use of HEK293 cells, but similar methods can be used for studies in other cell types.

3.5.1 Arresting Cells in G1/S, G2/M, and Cytokinesis

1. Seed HEK293 cells expressing HDAC5-EGFP and culture to 30% confluence.
2. To arrest cells in G1/S: add hydroxyurea to standard media DMEM++ to a final concentration of 4.0 mM and incubate for 40 h.
3. To arrest cells in G2/M: add nocodazole to standard media DMEM++ to a final concentration of 100 ng/mL and incubate for 20 h.
4. To block the completion of cytokinesis: add hesperadin to DMEM++ to a final concentration of 20–60 nM and incubate for 24 h.
5. Harvest cells by trypsinization and centrifugation.
6. Lyse cells and analyze lysates by SDS-PAGE and Western blotting.

3.5.2 Analysis of Cell Cycle Stage by Flow Cytometry

1. Wash cells with 5 mL DPBS.
2. Perform trypsin digestion.
3. Transfer into 5 mL DPBS + 2–5 % FBS serum in a 15 mL conical (to quench trypsin reaction).
4. Spin at $250 \times g$ for 5 min.
5. Aspirate supernatant.
6. Vortex pellet and add 1.5 mL of cold DPBS.

7. Slowly add 3 mL of cold 95 % EtOH in drop wise fashion.
8. Store overnight at 4 °C (can be stored up to 2 weeks).
9. Spin cells at $1800 \times g$ for 5 min.
10. Pour off supernatant and resuspend in 5 mL DPBS.
11. Spin at $1800 \times g$ for 5 min.
12. Pour off supernatant and resuspend in 5 mL DPBS.
13. Spin at $1800 \times g$ for 5 min.
14. Pour off supernatant and use a barrier pipette tip to resuspend in 500 μ L propidium iodide (PI)–RNase mixture (400 μ L PI (1 mg/ mL)+200 μ L RnaseA (10 mg/mL)) (final [PI]=40 μ g/mL).
15. Transfer cell solution to a flow cytometry compatible tube.
16. Cover tube in foil and incubate at room temperature for 1 h in the dark.
17. Vortex tube immediately prior to flow cytometry analysis to limit cell clumping.
18. Acquire and record PI signal using a BD LSRII FACS instrument and the BD FACS Diva program.

3.5.3 Kinase Inhibition Studies—As HDAC5 is known to be heavily modified by phosphorylation at multiple sites, kinases play critical roles in regulating its functions. Many studies have aimed to define the kinases that modulate HDAC5 phosphorylations and the downstream impact of these modifications [2, 6, 16, 24, 29, 30, 67] and reviewed in Ref. [68]. While not all the used protocols can be covered here, one commonly used approach is to inhibit kinases and study the impact of that inhibition on HDAC5 localization, interactions, and transcriptional regulatory functions. Inhibition of specific kinases can be achieved at a global level using Staurosporine or at a more specific level using selective kinase inhibitors (e.g., hesperadin to inhibit Aurora B). Small molecule inhibitors should be selected based on high selectivity toward targets and low cytotoxicity. Selective kinase inhibition can also be achieved through siRNA mediated knockdown of target kinases.

3.5.4 Small-Molecule Kinase Inhibitors

1. Seed HEK293 cells expressing HDAC5-EGFP and culture to 30% confluence.
2. Add hesperadin (or other selective kinase inhibitors) to DMEM++ to a final concentration of 20–60 nM and incubate for 24 h.
3. Harvest cells by trypsinization and centrifugation.
4. Lyse cells and analyze lysates by Western blotting, room temperature-PCR, or immunofluorescence microscopy.

3.5.5 siRNA-Mediated Knockdown of Kinases

1. Seed HEK293 cells and culture to 60–80 % confluence.

2. Dilute Lipofectamine RNAiMAX reagent in Opti-MEM medium. For a 6-well dish, dilute 9 μ L Lipofectamine RNAiMAX in 150 μ L Opti-MEM.
3. Dilute siRNA in Opti-MEM Medium. For a 6-well dish, dilute 30 pmol of siRNA complex in 150 μ L Opti-MEM.
4. Mix diluted Lipofectamine solution (**step 3**) and siRNA solution (**step 4**) 1:1.
5. Incubate the combined solution for 5 min at room temperature.
6. Add combined solution (siRNA–lipid complex) to plated cells.
7. Incubate cells at 37 °C for 24–72 h.
8. Analyze transfected cells for efficient knockdown by Western blotting, reverse transcription-qPCR, or immunofluorescence microscopy.

3.6 Enzymatic Activity of HDAC5

The innate deacetylase activity of HDAC5 against acetylated lysine is limited in vivo due to the absence of a catalytic tyrosine residue present in the enzymatic domains of other HDACs. In HDAC5 and the other class IIa HDACs (4, 7, and 9), this tyrosine residue is replaced with histidine [8]. However, HDAC5 seems to retain activity toward a trifluoroacetylated lysine substrate [8], which can be used to assess enzymatic activity immunoaffinity purified class IIa HDACs. It is important to consider that HDAC5 closely associates with the class I enzyme HDAC3 in vivo, resulting in ambiguity in analysis of immunoaffinity purified HDAC5. Therefore, to determine HDAC5 activity, the co-isolation of HDAC3 must also be assessed. Alternatively, cell lines expressing shHDAC3 may be suitable for determining the individual contributions of HDAC5 to enzymatic activity. Assessment of deacetylation activity most commonly involves the use of a fluorometric assay. Following immunoaffinity isolation of HDAC5 (and HDAC3) using magnetic beads, the fluorometric enzymatic assay can be performed while the proteins themselves are bound to the beads. Following the fluorometric assay, protein samples can be eluted from the beads for additional analysis of protein expression levels and co-isolating proteins by Western blotting.

3.6.1 Fluorometric Deacetylase Assay

1. Start with 0.2 g cells/enzymatic assay. Use 2 mg beads per 0.2 g cells and 1 mL of lysis buffer per 0.2 g cells.
2. Follow the protocol above for immunoaffinity isolation of protein complexes (*see Subheading 3.3.4 Immunoaffinity purification of HDAC5 with interacting partners steps 1–12(b)*).
3. Wash 3 $\times$ with buffer used for enzymatic activity assay (e.g., phosphatase buffer or assay buffer).
4. After the final wash, resuspend the sample in a small volume buffer (300 μ L) and split it to 5 round bottom tubes (60 μ L in each). The remaining tubes are for individual assay conditions as indicated below.

- a. Tube 1 is reserved for Western blotting. Proceed to elution in 20 μ l 1 $\times$ TEL buffer.
 - b. Tube 2: Time point 1 (e.g., time = 0 min), No inhibitor (-TSA).
 - c. Tube 3: Time point 1 (e.g., time = 0 min), +Inhibitor (+TSA) (*see* Note 18).
 - d. Tube 4: Time point 2 (e.g., time = 90 min), No inhibitor (-TSA).
 - e. Tube 5: Time point 2 (e.g., time = 90 min), +Inhibitor (+TSA).
5. Re-split the sample from each tube (**step 4b-e (Tubes 2-5)**) into three round bottom tubes for three replicates.
6. Place all tubes on magnetic rack, discard supernatant. Set up the enzymatic reactions on the magnetic beads, taking into account the following pre-steps:
 - a. Prepare master mixes with 400 μ M substrate $\pm$ 2 μ M TSA in assay buffer and pre-warm to 37 $^{\circ}$ C.
 - b. Add 20 μ L of the appropriate master mix solutions to Tubes 4 and 5 and mix well.
 - c. Allow Tubes 2 and 3 to stand at 37 $^{\circ}$ C. Do not add master mixes to these tubes at this time.
7. Allow the reactions in Tubes 4 and 5 to proceed for 90 min at 37 $^{\circ}$ C, flick the tubes to mix intermittently during the incubation.
8. Quench the deacetylation reaction by adding 40 μ l of 1 $\times$ Developer in assay buffer. Also add the appropriate master mix solutions to Tubes 2 and 3, followed by an immediate addition of the developer.
9. After the development step, briefly spin, place tubes on magnetic rack, and transfer all supernatants to individual wells on 96-well plates for fluorescence measurements. Save the beads for **step 12**.
10. Read samples in a microplate-reading fluorimeter capable of excitation at a wavelength in the range 350–380 nm and detection of emitted light in the range 450–480 nm (e.g., Synergy Mx fluorometer). Completion of signal development can be assessed by taking fluorescence readings at 5 min intervals. The Developer reaction is complete when the fluorescence readings reach a maximum and plateau. Fluorescence signal remains stable for at least 60 min. after reaching the maximum plateau.
11. Elute the protein from the magnetic beads with 20 μ l 1 $\times$ TEL buffer to assess isolation by Western blotting.

¹⁸TSA (trichostatin A) treatment of samples is included to inhibit HDAC activity and serve as a control to demonstrate that measured deacetylation activity in parallel experiments is dependent on the presence of HDAC activity.

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a HDAC5 in development and disease

b Cellular functions of HDAC5

Fig. 1.

Exploring the broad impact of HDAC5 on human health and disease and its essential intracellular functions. **(a)** HDAC5 is implicated in muscle development, cardiac disease, and neuron survival (adapted from Ref. [68]) **(b)** Within the cell, HDAC5 shuttles between the nucleus and the cytoplasm and has critical roles in transcriptional control, cell cycle regulation, and localization-dependent protein interactions

Fig. 2.

HDAC5 is heavily modified by posttranslational modification (reproduced from Ref. [35]).

(a) Phosphorylation and acetylation of HDAC5 spans the protein sequence. HDAC5 domains are indicated: *MEF* mef2 binding domain (*blue*); *NLS* nuclear localization signal (*green*); *AD* acidic domain (*red*); *DAC* deacetylation domain (*purple*); *NES* nuclear export sequence (*yellow*). (b) Numerous posttranslational modifications of HDAC5 map to the external surface of the predicted protein structure