HDAC6 activity is not required for basal autophagic flux in metastatic prostate cancer cells

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Abstract

Histone deacetylase 6 is a multifunctional lysine deacetylase that is recently emerging as a central facilitator of response to stress and may play an important role in cancer cell proliferation. The histone deacetylase 6-inhibitor tubacin has been shown to slow the growth of metastatic prostate cancer cells and sensitize cancer cells to chemotherapeutic agents. However, the proteins histone deacetylase 6 interacts with, and thus its role in cancer cells, remains poorly characterized. Histone deacetylase 6 deacetylase activity has recently been shown to be required for efficient basal autophagic flux. Autophagy is often dysregulated in cancer cells and may confer stress resistance and allow for cell maintenance and a high proliferation rate. Tubacin may therefore slow cancer cell proliferation by decreasing autophagic flux. We characterized the histone deacetylase 6-interacting proteins in LNCaP metastatic prostate cancer cells and found that histone deacetylase 6 interacts with proteins involved in several cellular processes, including autophagy. Based on our interaction screen, we assessed the impact of the histone deacetylase 6-inhibitor tubacin on autophagic flux in two metastatic prostate cancer cell lines and found that tubacin does not influence autophagic flux. Histone deacetylase 6 therefore influences cell proliferation through an autophagy-independent mechanism.

Keywords: Autophagy, metastatic prostate cancer, HDAC6, tubacin

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Introduction

Histone deacetylase 6 (HDAC6) is a cytoplasmic lysine deacetylase that has a role in maintaining homeostasis in response to cellular stressors.¹ HDAC6 facilitates aggregation and turnover of misfolded proteins and is protective during misfolded-protein stress;^{2,3} its ubiquitin-binding domain is required for heat shock factor 1 (HSF1) activation and downstream induction of cytoprotective chaperone proteins;⁴ and its deacetylase activity is required for efficient fusion of autophagosomes and lysosomes for autophagic flux.⁵ Despite this critical role in homeostasis, how HDAC6 is regulated, the proteins it interacts with, its substrates and cell-type specific functions are only recently coming to light.^{6–8}

The cytoprotective functions of autophagy suggest this process would be important in maintaining cell viability under conditions of heightened stress, such as during rapid proliferation or nutrient limitation. This is particularly relevant to cancer because the tumor microenvironment is an inherently stressful niche characterized by low nutrient concentrations, hypoxia, and damaging reactive oxygen species (ROS). Furthermore, metastatic cancers are likely evolved to require autophagy in response to selective pressures exerted throughout the process of dissemination.^{9–11} Consequently, autophagy is under investigation as a therapeutic target in cancer cells with the goal of inhibiting its cytoprotective function to slow growth or sensitize cancer cells to established therapeutic agents.^{12,13}

Consistent with its role in cell maintenance and stress response, HDAC6 inhibition (HDAC6i) or depletion has been shown to slow cancer cell growth and migration and sensitize cancer cells to chemotherapeutic agents.^{14–16} A cytoprotective role in cancer cells is also supported by associative clinical data showing a tendency toward increased expression of HDAC6 in patient tumors¹⁷ (The Cancer Genome Atlas datasets^{18,19}). The HDAC6 inhibitor tubacin²⁰ has been shown to slow growth and sensitize LNCaP metastatic prostate cancer cells to chemotherapeutic agents.¹⁴

We hypothesized that growth retardation and a synergistic decrease in cell viability in combination with other therapeutic agents may be a result of decreased autophagic flux in response to HDAC6i. To test this hypothesis, we first characterized the proteins that interact with HDAC6 in LNCaP cells to gain insight into the protein's role in metastatic prostate cancer cells. We found HDAC6 to interact with proteins associated with autophagy and subsequently tested whether HDAC6i influences autophagic flux²¹ in two metastatic prostate cancer cell lines.

Materials and methods

Chemicals and reagents

Tubacin (N₁-[4-[(2*R*,4*R*,6*S*)-4-[[[4,5-diphenyl-2-oxazolyl]thio]methyl]-6-[4-(hydroxymethyl)phenyl]-1,3-dioxan-2-yl]phenyl]-N₈-hydroxyoctanediamide) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA) and resuspended in dimethyl sulfoxide (DMSO) (EMD Millipore, Darmstadt, Germany). Ammonium chloride (NH₄Cl) (Sigma-Aldrich) was prepared in sterile water. Mitochondrial dye MitoTracker Deep Red FM was purchased from Life Technologies (Carlsbad, CA, USA) and resuspended in DMSO. Primary antibodies for p62 (Santa Cruz Biotechnology), LC3A/B (Cell Signaling Technology, Danvers, MA, USA), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology), cytochrome c (CYCS) (Santa Cruz Biotechnology), ubiquitin (Ub) (Santa Cruz Biotechnology), acetylated- α -tubulin (Sigma, St. Louis, MO, USA), α -tubulin (Sigma) and β -actin (Sigma) were used in accordance with the manufacturer's protocol. HRP-conjugated secondary antibodies (Santa Cruz Biotechnology) for Western Blotting detection and AlexaFluor-conjugated secondary antibodies for immunofluorescence/confocal imaging (Life Technologies) were used in accordance with the manufacturer's protocol.

Cell lines and culture conditions

PC3 and LNCaP prostate cancer cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in RPMI-1640 media with L-glutamine supplemented with fetal bovine serum (FBS, 50 ml FBS/500 ml media) at 37°C 5% CO₂. Cell lines were validated by Idexx Radil (Columbia, MO, USA) on December 24, 2012. Cells were treated under the indicated conditions prior to harvest. Treatment reagents were used at the following concentrations: 8 μ M tubacin, 30 mM NH₄Cl.

DMSO or other appropriate carrier was used as needed for control treatments.

Protein preparation and Western blot analysis

Protein lysates were prepared in radioimmunoprecipitation assay (RIPA) protein lysis buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM TRIS pH 8.0) supplemented with protease inhibitor cocktail (Thermo Scientific, Waltham, MA, USA). Lysates were cleared of insoluble material by centrifugation (10 min, 13,000r/min) at 4°C and quantitated by the DCA Protein Assay (BioRad, Hercules, CA, USA). Equal amounts of protein were separated by SDS-PAGE and blotted to a PVDF membrane (BioRad) using NuPAGE reagents and equipment in accordance with the manufacturer's protocol (Life Technologies). Membranes were blocked and probed for the indicated proteins following standard protocols. Membranes were stripped and reprobed as needed. Membranes were incubated in SuperSignal West Femto Reagent (Thermo Scientific) and developed on the AlphaInnotech FluorChem 8900 system for visualization (San Jose, CA, USA). Densitometric analyses were performed on the native membrane image using AlphaInnotech FluorChem 8900 software. For each membrane, the relative densitometric value of each replicate for a given probe was normalized to the corresponding relative level of the normalizing protein (β -actin). DMSO (control) was set to 1.

Immunofluorescent staining

Cells were grown and treated on glass coverslips. Mitochondria were stained using MitoTracker Deep Red FM probe following the manufacturer's protocol. The MitoTracker probe was added at 200 nM during the final 30 min of cell treatment and then cells were prepared for immunostaining. Briefly, cells were rinsed in PBS and fixed/permeabilized in ice-cold 100% methanol for 10 min at –20°C. Cells were placed in blocking buffer (1X PBS, 5% BSA, 0.3% Triton X-100). Cells were probed with primary antibodies in antibody dilution buffer (1X PBS, 1% BSA, 0.3% Triton X-100) overnight at 4°C. After incubation with secondary antibodies cells were rinsed three times in PBS, with the final rinse containing DAPI nuclear stain. Coverslips were mounted on glass slides using ProLong Gold Antifade Reagent (Life Technologies) and allowed to set. Slides were visualized on a Zeiss LSM Meta Confocal Microscope (Oberkochen, Germany) at the Center for Genome Research and Biocomputing (CGRB) at Oregon State University. Colocalization quantification was done using Coloc 2 (Fiji distribution for ImageJ2).²²

Quantitative real-time PCR

Total RNA was harvested by TRIzol reagent in accordance with the manufacturer's protocol (Life Technologies). cDNA was prepared from 1 μ g RNA using the SuperScript III kit from Life Technologies. Approximately 50 ng cDNA was amplified by Fast SYBR Green Reagent in accordance with the manufacturer's protocol (Life Technologies) on a

7900HT Real Time PCR Machine (Applied Biosciences, Life Technologies).

Primers: GAPDH (sense 5'-CGAGATCCCTCCAAAATCAA-3', antisense 5'-TTCACACCCATGACGAACAT-3'); p62 (sense 5'-CATCGGAGGATCCGAGTGTG-3', antisense 5'-TTCTTTTCCCTCCGTGCTCC-3'); HDAC6 (sense 5'-CAGCACAGTCTTATGGATGG-3', antisense 5'-CGGTGGATGGAGAAATAGAG-3'). Relative expression was determined by the Comparative Ct Method ([delta] [delta] Ct = [delta]Ct_{sample} - [delta]Ct_{reference}). Graphing was done using GraphPad Prism Software (La Jolla, CA, USA). Graphs depicts mean + SEM for two independent experiments.

HDAC6 immunoprecipitation and liquid chromatography tandem mass spectrometry

LNCaP cell lysate (3 mg) was immunoprecipitated with HDAC6 antibody in 1% Triton X-100, 137 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl pH 8.0 overnight at 4°C. The following day, Protein G agarose (Life Technologies) was added and incubated for an additional 2 h. Immunocomplexes were isolated by low-speed centrifugation and washed twice in 50 mM ammonium bicarbonate. The last wash was removed and immunocomplexes were resuspended in 50 µl 0.5 M urea, 5 mM DTT, 50 mM Tris pH 8.0. Samples were then sonicated in a water bath (2X 1 min), vortexed and spun down. Each sample was digested with 500 ng Trypsin Gold (Promega, Madison, WI, USA) at 37°C.

Liquid chromatography tandem mass spectrometry (LC-MS/MS) was carried out as described previously.²³ High-confidence protein identifications were made under the following conditions: Protein Threshold 99.0%, Minimum # of Peptides = 2, Peptide Threshold 5% FDR.

Results

Identification of HDAC6-interacting proteins in LNCaP cells

HDAC6 has been shown to have a significant role in many cellular processes in various cell types.^{17,24,25} However, the proteins that interact with HDAC6 in metastatic prostate cancer cells have not been fully characterized. To address this issue we characterized the proteins that HDAC6 interacts with in an unbiased manner. We identified 52 high-confidence proteins as the major HDAC6-interacting proteins in LNCaP cells (Table 1). As expected, we identified many well-characterized HDAC6 interacting proteins, including components of the cytoskeletal network^{26,27} and heat shock proteins.^{27,28} We also identified more recently reported HDAC6-interacting proteins such as myosin-9⁷ (Table 1).

Thirty of the HDAC6-associated proteins we identified were found to be involved in autophagy. We identified proteins that are characterized macroautophagy substrates and mediators, including mitochondria-associated proteins, ribosomes, actin and tubulin (Table 1).^{3,29-32} HDAC6 interaction with tubulin was confirmed by co-immunoprecipitation and mitochondrial association was assessed by confocal microscopy (Figure S1).

We also identified proteins associated with chaperone-mediated autophagy. Chaperone-mediated autophagy (CMA) delivers a subset of cytosolic proteins to the lysosome via heat shock cognate 71 kDa protein (hsc70) and the lysosomal receptor LAMP2A.³³ Hsc70 has recently been reported as an HDAC6 substrate.⁷ Our LC-MS/MS screen for HDAC6 interacting proteins also identified hsc70 as a prominent HDAC6-interactor in LNCaP cells (Table 1). In addition to hsc70, we also identified multiple recently characterized CMA substrates involved in regulation of cellular energy production, including α -enolase (ENO1), pyruvate kinase (PKM) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)^{34,35} (Table 1).

Tubacin does not inhibit autophagy in metastatic prostate cancer cells

The interaction of HDAC6 with proteins and substrates involved in autophagy in LNCaP cells suggests that tubacin may slow LNCaP growth by interfering with autophagic flux. To test this hypothesis we treated LNCaP and PC3 metastatic prostate cancer cells with tubacin and monitored autophagic flux (Figure 1). Ammonium chloride (AC) was used to assess delivery of substrates to the lysosome. The selective autophagy adaptor protein p62 (sequestosome 1, SQSTM1) was monitored in addition to LC3-II turnover to assess macroautophagy.^{21,36}

Tubacin did not influence overall HDAC6 protein level in either cell-type and AC treatment indicated that HDAC6 is not turned over through the lysosomal pathway (Figure 1). Tubacin did lead to an increase in p62 protein (Figure 1), suggesting impaired p62-selective flux. We did not observe an increase in p62 comparing AC treatment to the tubacin/AC combination treatment (Figure 1), indicating that the increase in p62 observed during tubacin treatment is not the result of an increase in gene expression. However, the p62 response in PC3 cells was not consistent (Figure S2). We observed on several occasions that tubacin enhanced p62 degradation (decrease in p62 protein with no associated decrease in gene expression). When assessing macroautophagy based on LC3-II levels, tubacin did not significantly impair or enhance flux under any treatment conditions (Figure 1 Lane 2 and Figure S2).

Tubacin did not influence CMA in either cell type as determined by GAPDH levels (Figure 1). However, we did not observe accumulation of GAPDH in AC-treated cells, suggesting no significant turnover of GAPDH under these experimental conditions.

The observed influence of tubacin on p62 levels suggests that although macroautophagy is occurring unimpeded, p62 substrate degradation may be specifically altered. To test this hypothesis we assayed turnover of global poly-ubiquitinated protein levels and mitochondria (Figure 2). Tubacin did not alter global poly-ubiquitinated proteins or mitochondria levels (as assessed by cytochrome c (CYCS)) despite increased p62 levels (Figure 2). No change in mitochondrial levels was also observed in circumstances where tubacin increased p62 degradation (Figure S2).

Table 1 HDAC6-interacting proteins identified by mass spectrometry

HDAC6-interacting proteins in LNCaP cells	Accession	EUSC	Assignment
40S ribosomal protein S15	4506687	2	AS (31)
40S ribosomal protein S15a	14165469	2	AS (31)
40S ribosomal protein S17-like	312284072	3	AS (31)
40S ribosomal protein S18	11968182	2	AS (31)
40S ribosomal protein S3 isoform 1	15718687	2	AS (31)
40S ribosomal protein S5	13904870	2	AS (31)
40S ribosomal protein S7	4506741	5	AS (31)
40S ribosomal protein SA	59859885	2	AS (31)
60 kDa heat shock protein, mitochondrial	31542947	9	MP (32)
60S acidic ribosomal protein P2	4506671	3	AS (31)
60S ribosomal protein L21	18104948	2	AS (31)
60S ribosomal protein L3 isoform a	4506649	2	AS (31)
Actin, cytoplasmic 2	316659409	7	AM (5)
alpha-enolase isoform 1	4503571	6	AS (35)
ATP synthase subunit alpha, mitochondrial isoform c	382546190	3	MP (32)
ATP synthase subunit beta, mitochondrial precursor	32189394	6	MP (32)
ATP-dependent RNA helicase DDX3X isoform 2	301171467	4	
D-3-phosphoglycerate dehydrogenase	23308577	4	
E3 ubiquitin-protein ligase TRIM21	15208660	4	
Elongation factor 1-alpha 1	4503471	2	
Elongation factor 2	4503483	3	
Eukaryotic initiation factor 4A-I isoform 1	4503529	3	
Fatty acid synthase	41872631	10	AS (35)
FK506-binding protein 15	150010552	8	
Glyceraldehyde-3-phosphate dehydrogenase isoform 2	378404908	3	AS (35)
Heat shock 70 kDa protein 1A/1B	167466173	2	MP (32)
Heat shock cognate 71 kDa protein isoform 2	24234686	4	H6S (7),AM (33)
Heat shock protein HSP 90-beta isoform a	20149594	5	H6S (28)
Heterogeneous nuclear ribonucleoprotein K isoform b	14165435	3	
Heterogeneous nuclear ribonucleoprotein R isoform 1	156151394	16	
Heterogeneous nuclear ribonucleoprotein U isoform b	14141161	4	
Histone deacetylase 6	13128864	6	AM (5)
Histone H2B type 1-B	10800140	2	
Keratin, type I cytoskeletal 18	40354195	5	
Lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex, mitochondrial precursor	392494079	2	MP (32)
myc box-dependent-interacting protein 1 isoform 1	21536400	27	
Myosin light polypeptide 6 isoform 1	17986258	2	
Myosin-10 isoform 1	365192532	2	AM (50)
Myosin-9	12667788	5	H6S (7)
Non-POU domain-containing octamer-binding protein isoform 1	224028244	2	
Nucleolin	55956788	2	
Nucleophosmin isoform 1	10835063	6	
PRKC apoptosis WT1 regulator protein	55769533	2	
protein-L-isoaspartate(D-aspartate) O-methyltransferase isoform 1	226530908	7	
Pyruvate kinase isozymes M1/M2 isoform e	332164779	2	AS (34,35)
RNA-binding protein FUS isoform 3	283135173	3	
Stress-70 protein, mitochondrial precursor	24234688	4	MP (32)
T-complex protein 1 subunit epsilon	24307939	5	
transformation/transcription domain-associated protein isoform 1	347360922	2	
Tubulin alpha-1B chain	57013276	10	H6S (26), AM (3)
Tubulin beta chain	29788785	20	H6S (26), AM (3)
Tubulin beta-4B chain	5174735	5	H6S (26), AM (3)

EUSC: exclusive unique spectrum count; H6S: HDAC6 substrate; AS: autophagy substrate; MP: mitochondrial protein; AM: autophagy mediator.

Figure 1 Tubacin does not influence autophagy in metastatic prostate cancer cells. LNCaP and PC3 cells were treated with control (DMSO, Lane 1), tubacin (tub, Lane 2), ammonium chloride (AC, Lane 3), or tubacin and AC (tub/AC, Lane 4) for 24 h prior to harvesting for protein analysis. To assess autophagic flux, membranes were probed for the selective autophagy adaptor and substrate p62, the chaperone mediated autophagy substrate GAPDH, and autophagosome marker and autophagy substrate LC3. One representative experiment from at least three independent experiments is pictured. p62 levels (relative to DMSO control) are indicated. p62 was normalized to the loading control β -actin

Tubacin leads to the appearance of HDAC6 puncta in PC3 cells

Accumulation or degradation of p62 in response to tubacin suggests direct regulation of p62 stability independent of autophagy. p62 is also a known HDAC6-interacting protein.³⁷ We observed colocalization of p62 and HDAC6 under basal conditions in PC3 cells (Figure 3). Tubacin treatment led to an increase in p62 and HDAC6 puncta (Figure 3). Tubacin also led to an increase in association between p62 and HDAC6 foci: HDAC6-only foci were not observed in tubacin-treated cells, and colocalization analysis noted an increase in association as assessed by Pearson's R and Spearman's rank correlation (Figure S3). Analysis of LC3 by immunofluorescence showed an increase in LC3 puncta, indicating an increase in autophagosome formation in response to tubacin (Figure 4). LC3 puncta were also found to colocalize with p62 foci in tubacin-treated cells.

Discussion

A number of recent studies have provided evidence supporting the further development of HDAC6 as a cancer therapeutic target. Selective inhibition of HDAC6 deacetylase activity slows cancer cell proliferation and sensitizes cancer cells to chemotherapeutic agents in a preclinical model of leukemia¹⁶ and similar results have been found in prostate cancer cells *in vitro*.¹⁴ The observation that HDAC6 knockout mice are viable and are largely normal³⁸ also suggests that systemic HDAC6 inhibition

will have limited off target adverse effects, a desirable characteristic of any clinical target. Despite these promising results, how HDAC6i slows prostate cancer cell proliferation is poorly understood.

In this investigation, we characterized the HDAC6-interacting proteins in LNCaP metastatic prostate cancer cells to gain insight into the role of HDAC6 in prostate cancer cells and how HDAC6i slows cancer cell proliferation. We found HDAC6 to interact with many proteins associated with autophagy (Table 1) and subsequently tested the hypothesis that HDAC6i slows proliferation by impairing basal autophagic flux. Our results show that tubacin does not impair macroautophagy in metastatic prostate cancer cells, suggesting flux-inhibition is not responsible for slowed cancer growth associated with tubacin treatment.

Autophagic flux independent of HDAC6 tubulin deacetylase activity was not expected given its known role in mediating efficient autophagosome-lysosome fusion.⁵ HDAC6 was found to associate with proteins and substrates known to be involved in, or degraded by, autophagy (Table 1, Figure S1 and Figure 3) and the presence of HDAC6 at the site of autophagosomes can also be inferred from complete overlap of both LC3 and HDAC6 puncta with p62 foci in tubacin-treated cells (Figures 3 and 4). Despite this association, HDAC6 activity is dispensable for autophagic flux in metastatic prostate cancer cells. This may suggest a unique role for HDAC6 in prostate cell or prostate cancer cell biology relative to other cell types.

Figure 2 Tubacin does not influence global ubiquitinated protein levels or mitochondrial turnover. Known p62 and HDAC6 substrates were assessed to determine whether altered p62 levels influenced substrate degradation. PC3 and LNCaP cells were treated as in Figure 1. Membranes were probed for poly-ubiquitin (Ub) and mitochondrial marker cytochrome c (CYCS). One representative blot from at least three independent experiments is shown (same samples depicted in Figure 1)

Figure 3 Tubacin leads to an increase in HDAC6 foci in PC3 cells. PC3 cells were treated with control (DMSO) or tubacin for 24 h prior to processing for HDAC6 (red) and p62 (green) visualization by confocal microscopy. HDAC6 formed cytoplasmic foci in response to tubacin. HDAC6 foci strongly colocalized with p62 foci. Scale bar is 10 μ m

Cancer cells undergo vast changes in metabolism and intracellular signaling pathways during transformation to enable rapid proliferation and enhanced survival.³⁹ Metastatic prostate cancer cells are particularly prone to constitutive activation of the PI3K/AKT/mTOR signaling axis,^{40,41} a key regulator of autophagy,⁴² and both LNCaP and PC3 cell lines that were used in our experiments contain mutations in PTEN, a negative regulator of the PI3K signaling axis.^{43,44} This may suggest that these cell lines, and perhaps metastatic prostate cancers in general, evolve to allow autophagic flux independent of well-established regulators.

In addition to monitoring LC3-II levels, the autophagy adaptor and substrate p62 was assessed to evaluate flux. We unexpectedly found that tubacin influences p62 stability independent of autophagic flux (Figure 1 and Figure S2). HDAC6 and p62 physically interact in mouse embryonic fibroblasts (MEFs) and p62 has been proposed to influence HDAC6 activity.³⁷ We also found a close association between these two proteins in metastatic prostate cancer cells (Figure 3); however, we found p62 levels to be dependent on HDAC6 activity (Figure 1 and Figure S2), suggesting a co-regulatory relationship. The observation that the outcome of HDAC6i on p62 protein level is variable also suggests the involvement of

Figure 4 Tubacin leads to the appearance of LC3 puncta in PC3 cells. PC3 cells were treated with control (DMSO) or tubacin for 24 h prior to processing for p62 (green) and LC3 (red) visualization by confocal microscopy. Tubacin led to an increase in LC3 puncta in PC3 cells. LC3 puncta colocalized with p62 foci. Scale bar is 10 μ m

other factors -HDAC6 and p62 are both multifunctional proteins known to interact with an array of other proteins.^{7,24,45–47} Variable p62 levels may also be a result of overlapping function with other autophagy adaptors and shared substrates (e.g. neighbor of BRCA1 gene 1 (NBR1) or optineurin (OPTN)^{48,49}). Further work will be needed to understand how HDAC6 may regulate p62 stability.

Localization analyses suggested that tubacin stimulates autophagy as assessed by an increase in LC3 puncta that colocalized with p62 (Figure 4). However, we observed no accumulation of LC3-II protein in the flux assay (Figure 1, Lanes 3 and 4), confirming no increase in autophagic activity. Autophagy-independent p62 activity may explain why p62 degradation was observed to be independent of LC3-II level (Figure 1 and Figure S2) and why we did not observe global changes in two p62 substrates (Figure 2). An alternative hypothesis also taking into account the appearance of HDAC6 foci may suggest that tubacin leads to more efficient trafficking across the cytoskeletal network as a result of increased microtubule acetylation.^{8,50–52} Enhanced trafficking may lead to the accumulation of HDAC6 and p62 at degradation sites, leading to the appearance of discrete foci without significantly affecting overall autophagic vesicle fusion. This would support a model where tubacin does not actively cause HDAC6 re-localization, but instead passively leads to deposition of HDAC6 at sites of protein degradation. This suggests that p62/HDAC6 foci represent either protein bodies that are de-ubiquitinated and degraded,⁵³ or are sites of ubiquitin-independent basal protein turnover.⁵⁴ It is unlikely that HDAC6 foci represent stress granules or P bodies as HDAC6 activity is required for their formation⁶ and we did not observe obvious signs of cell stress. Further work will be needed to evaluate these various interpretations.

Our unbiased LC-MS/MS screen for HDAC6-interacting proteins suggested a role for HDAC6 in CMA (Table 1). A test of whether tubacin influenced CMA in metastatic prostate cancer cells using GAPDH as a reporter indicated no major role (Figure 1). However, inhibition of lysosomal degradation by AC also revealed no increase in GAPDH, indicating no significant turnover under our experimental conditions (lysosomal turnover of GAPDH has been detected within 2 h of inhibition of degradation in liver³⁵). This may reflect cell-type specific metabolism and suggests HDAC6 may influence cellular energetics not through altering degradation of metabolic enzymes, but instead through direct regulation. This will be an interesting area of future research in understanding how HDAC6i may slow cancer growth.

In this investigation, we showed that tubacin does not impede basal autophagic flux; however, this does not necessarily mean HDAC6 deacetylase activity is dispensable for flux in other circumstances. HDAC6 is known to have a context-dependent role in autophagic flux,⁵ suggesting HDAC6 may be required in a stimulus-dependent manner.⁵⁵ This will be particularly relevant in future work exploring whether HDAC6 has a role in flux following exposure to chemotherapeutic agents.¹⁴

Author contributions: GWW, SW and YF generated the data. GWW, SW, YF, CSM, DEW, RHD, VIP and EH contributed to overall study design, evaluation of data and manuscript preparation.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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