Histone deacetylase regulation by LKB1 and PKA signaling pathways

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ABSTRACT

In humans, there are 18 known histone deacetylases (HDACs) that can be divided into four classes, with HDAC4, 5, 7 and 9 forming the class IIa subgroup. These four deacetylases are signal-responsive transcriptional co-repressors involved in a wide variety of physiological and pathological processes. In response to a spectrum of extracellular signals, class IIa HDACs become phosphorylated on three or four conserved serine residues, creating specific binding sites for 14-3-3 chaperone proteins and inducing deacetylase nuclear export and derepression of target genes. The goal of my thesis project was to identify regulatory mechanisms underlying class IIa HDAC nucleocytoplasmic trafficking. Following a literature review in Chapter I are two sets of novel findings from my thesis project. In Chapter II, I identify the salt-inducible kinases, SIK2 and SIK3 as novel class IIa HDAC kinases that induce the nuclear export of these deacetylases. I also demonstrate that SIK2/3-mediated HDAC export is stimulated by liver kinase B1 (LKB1, a major tumour suppressor for lung and other cancers) and inhibited by protein kinase A (PKA). In Chapter III, I demonstrate that PKA can also prevent nuclear export of HDAC4, 5, and 9 independent of SIK2/3 inhibition. PKA achieves this by controlling a novel phosphorylation event that is unique to these three deacetylases but absent from HDAC7. Due to the pervasive roles of LKB1, PKA, and class IIa HDACs in health and disease, these findings may lead to a better understanding of the etiology of various maladies and point to novel targets for therapeutic intervention in the future.

RÉSUMÉ

Chez l'homme, la famille d'enzymes histone déacétylase (HDAC) compte 18 membres. Ces protéines peuvent être répartis en quatre classes; dont la classe IIa est composée d'HDAC4, 5, 7 et 9. Les membres de cette classe agissent comme corépresseur de la transcription et interviennent dans une grande variété de processus physiologiques et pathologiques. En réponse à une gamme copieuse de signaux extracellulaire, les HDAC de la classe IIa sont phosphorylées sur trois ou quatre résidus sérine conservés. Cet événement crée des sites de liaison spécifiques pour les protéines 14-3-3, qui agissent comme chaperon et induisent l'exportation nucléaire des HDAC. De ce fait, la relocalisation des HDAC, anéantit la répression d'expression de gènes cibles. Le but de mon projet de thèse était d'identifier les mécanismes sous-jacents qui règlent la localisation nucléocytoplasmique de la classe IIa d'HDAC. Suite à une revue de la littérature scientifique au chapitre I, deux séries de résultats parvenant de mon projet de recherche sont présentées. Dans le chapitre II, j'identifie les protéines salt-inductible kinase, SIK2 et SIK3, comme étant kinases d'HDAC de la classe IIa qui induisent l'exportation nucléaire de ceux-ci. Je démontre ainsi que cette exportation est stimulée par liver kinase B1 (LKB1, un suppresseur de tumeur impliqué dans la pathologie de plusieurs cancers) et inhibée par la protéine kinase A (PKA). Dans le chapitre III, je démontre que la PKA peut également empêcher l'exportation nucléaire de HDAC4, 5 et 9 indépendamment de SIK2/3. PKA atteint cet objectif en dirigeant un événement de phosphorylation unique au déacétylases de la classe IIa, autres que HDAC7. En effet, puisque LKB1, PKA, et les HDAC de la classe IIa jouent un rôle omniprésent en matière de santé, ces

résultats pourraient conduire à une meilleure compréhension de l'étiologie de diverses maladies et engendrer des nouvelles cibles thérapeutiques.

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Although scientific research is by necessity often a solitary affair, the truth is that completing a PhD is dependent on the help of many people. First, I'd like to thank my supervisor, Dr. Xiang-Jiao Yang for accepting me into his lab and guiding me through my studies and training. I can still remember my interview with Dr. Yang, in which he told me to read a Cell paper on yeast histone modifications while he finished up an experiment. When he returned ten minutes later I was still trying to make sense of the abstract with a sudden worry about what I was doing there. Nevertheless, he explained the paper to me and I excitedly read every word of it during my train ride back to Toronto. This type of passion for science is never in short supply in the lab and Dr. Yang's excitement about science is contagious.

Second, I must thank all the colleagues in the lab for their camaraderie and help over the years. Special thanks go to Soroush Tahmasebi, a fellow PhD student in the lab. We never officially worked together on a project, but Soroush always lent a helping hand when I needed it. Mostly, however, I will be forever grateful for his friendship in and out of the lab. I enjoyed our constant exchange of ideas about evolution, synthetic biology, Jared Diamond books, and movies. Ryan Weist, a Master's student in the lab, also deserves special mention. Ryan worked on my main projects and translated my abstract. Our kinesiology backgrounds and shared obsessions with basketball and mixed martial arts led to an endless stream of lively discussions. Kainan Wang, a Master's student during the beginning of my PhD, was a good friend and helped me with lab techniques and making figures. Chao-Jung Wu, first a Master's student then an outstanding

lab technician, could always be counted on for technical advice and a good laugh. Finally, Serge Grégoire was extremely helpful at the beginning of my studies with his technical instruction. Of course, many other lab colleagues deserve thanks, including Lin Xiao, Cindy Degerny, Goran Gocevski, Hiroaki Taniguchi, Valérie Chénard, Minghong Xu, Songping Zhao, Yizhen Lu, Go-Woon Kim, Kezhi Yan, Mukta Ullah, Nadine Pelletier, and Jianghong Liu. This section would be incomplete without acknowledging the other great people from the MOG and the GCRC who helped make the last several years more enjoyable, including Minh, Greg, Marisa, Christine, Kelly, Ryan, Jasmine, Laurent, Dave, Laura, Jason, Ian, Chen, Charles V., Peiman, Andrea, Charles R., Edlyn, Sami, Alana, Lu, Annie, and many others who I enjoyed working and hanging out with.

Third, I would like to thank all my friends and family, especially my amazing wife Jessica. Living so close to the lab is a gift and a curse, and Jessica endured an inhumane number of my late night and weekend trips to the lab (sometimes three or four times in the same night!), even after making dinner and waiting for me to get home in the first place. In fact, and unfairly, the stress of doing a PhD often got to her more than it did to me. I am also grateful for her attending McGill, otherwise I would not have come to Montreal for my PhD and we would have missed out on many fond memories: our hikes and runs up Mount Royal, long walks to the Old Port, dining at the city's fine restaurants, and even our countless journeys, by train and car, between Montreal and Toronto.

PREFACE

The Guidelines for Thesis Preparation issued by The Faculty of Graduate Studies and Research at McGill University states that:

"As an alternative to the traditional thesis style, the research may be presented as a collection of papers of which the student is the author or co-author (i.e., the text of one or more manuscripts, submitted or to be submitted for publication, and/or published articles (not as reprints) but reformatted according to thesis requirements as described below). These papers must have a cohesive, unitary character making them a report of a single program of research."

I have chosen to follow this guideline and write a manuscript-based thesis composed of two original research manuscripts, one of which is in review for publication at Nature Communications and the other of which is in preparation to be submitted to Molecular Cell or Science Signaling. The thesis is organized into five chapters: (I) literature review; (II-III) manuscripts, each with their own preface, abstract, introduction, materials and methods, results, discussion, acknowledgements, figures and figure legends, and references; (IV) general discussion; and (V) contributions to original research.

PUBLICATIONS ARISING FROM THE WORK OF THIS THESIS

1. **Walkinshaw DR**, Tahmasebi S, Bertos NB, Yang XJ. Histone deacetylases as transducers and targets of nuclear signaling. Journal of Cellular Biochemistry, 104(5): 1541-52, 2007.

2. **Walkinshaw DR** and Yang XJ. Histone deacetylase inhibitors as novel anticancer therapeutics. Current Oncology, 15(5); 237-243, 2008.

3. Bonfils C, **Walkinshaw DR**, Besterman JM, Yang XJ, Li Z. Pharmacological inhibition of histone deacetylases for the treatment of cancer, neurodegenerative disorders, and inflammatory diseases. Expert Opinion on Drug Discovery, 3(9); 1041-1065, 2008.

4. **Walkinshaw DR**, Weist R, Lin X, Kim GW, Li, CS, Zhao SP, Yang XJ. The tumour suppressor kinase LKB1 activates SIK2 and SIK3 to stimulate nuclear export of class IIa histone deacetylases. Nature Communications, in revision, June 2011.

5. **Walkinshaw DR**, Weist R, Xiao, L, and Yang XJ. cAMP/protein kinase A signaling differentially inhibits phosphorylation and cytoplasmic localization of class IIa histone deacetylases. J. Biol. Chem., in revision, June 2011.

CONTRIBUTIONS OF AUTHORS

CHAPTER II:

The tumour suppressor kinase LKB1 activates SIK2 and SIK3 to stimulate nuclear export of class IIa histone deacetylases

I performed 95% of the assays and made most of the plasmid constructs. I also made all the figures and wrote the manuscript. Throughout the project, I optimized protocols (and developed this particular myogenesis assay in the lab), analyzed the data, and planned experiments and strategies in collaboration with Dr. Yang, who also helped edit and finalize the manuscript.

RW performed the western blots (from extracts I produced) shown in Fig. 6, and made some plasmid constructs.
LX subcloned the HA-PKA plasmid construct.
GWK analyzed MEF2 target genes.
CSL prepared the CMV-LKB1 and CMV-STRAD plasmid constructs.
SPZ made the CMV-SIK2 plasmid construct.

CHAPTER III:

cAMP/protein kinase A signaling differentially inhibits phosphorylation and cytoplasmic localization of class IIa histone deacetylases.

I performed 90% of the assays and made several plasmid constructs. I also made all the figures and wrote the manuscript. I collaborated with Dr. Yang in analyzing/interpreting data and planning experiments. Dr. Yang helped edit and finalize the manuscript.

RW made several plasmid constructs and performed some of the transfection/fluorescence microscopy experiments shown in Fig. 1D, 3C and F, and 6E. He also performed the western blots (from extracts I produced) shown in Fig. 5F and 6B.
LX prepared the HA-PKA plasmid construct.

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4.0 CHAPTER IV: GENERAL DISCUSSION

LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
AGC	kinase family comprising PKA, PKG, and PKC members
AICAR	aminoimidazole carboxamide ribonucleotide
AKAPs	A-kinase anchoring proteins
AKT	protein kinase B/AKT
Ala	alanine
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ARK5	AMPK-related kinase 5
ATM	ataxia-telangiectasia mutated kinase
ATP	adenosine triphosphate
B-RAF	v-raf murine sarcoma viral oncogene homolog B1
BRSK	brain-specific kinase/SAD
CaMK	Ca^{2+}/calmodulin-dependent protein kinase
CAMK	refers to the large kinase group that includes CaMKs
CAMKK	CaMK kinase
cAMP	cyclic AMP
CBP	CREB-binding protein
CD4/CD8	cluster of differentiation 4/8
Cdc25	cell division cycle 25
CDK	cyclin-dependent kinase
ChREBP	carbohydrate-responsive element binding protein
CK1	casein kinase 1
CLK	Cdc-like kinase
CMGC	kinase group containing CDK, MAPK, GSK3, and CLK
C-Nap1	chromosome condensation-related SMC-associated protein
CREB	cAMP-response element binding protein
CRM1	chromosome region maintenance 1
CRTC	CREB-regulated transcription coactivator
dSIK	*Drosophila* SIK
CtBP	C-terminal binding protein
EMT	epithelial-mesenchymal transition
ePK	eukaryotic protein kinase
ERK	extracellular signal-regulated kinase
ES cell	embryonic stem cell
GSK3	glycogen synthase kinase-3
HAT	histone acetyltransferase
Hda1	histone deacetylase 1 (*S. cerevisiae*)
HDAC	histone deacetylase
His	histidine
HP1	heterochromatin protein 1
IRS-1	insulin receptor substrate-1
KD	kinase-dead
KIN-29	*C. elegans* kinase-29
K-ras	Kirsten rat sarcoma viral oncogene homolog

KSR1	kinase suppressor of Ras 1
LATS1	large tumour suppressor 1
LKB1	liver kinase B1/STK11
MAPK	mitogen-activated protein kinase
MARK	microtubule affinity-regulating kinase
MEF2	myocyte enhancer factor 2
MELK	maternal embryonic leucine zipper kinase
Mirk/dyrk1B	mini brain-related kinase
MITR	MEF2-interacting transcription repressor
MLC-2	myosin light chain-2
MO25	mouse protein 25
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
MYPT1	myosin phosphatase targeting subunit 1
NAD^+	nicotinamide adenine dinucleotide
NES	nuclear export sequence
NK	Na^+, K^+-ATPase
NLS	nuclear localization signal
NUAK1	AMPK-related kinase 5 (ARK5)
NUAK2	sucrose nonfermenting AMPK-related kinase (SNARK)
Nur77	growth factor-inducible immediate early gene nur/77
Par-1	partitioning defective-1 homologue
PDE	phosphodiesterase
PGC-1α	peroxisome proliferator-activated receptor γ coactivator-1α
Pim-1	proto-oncogene serine/threonine-protein kinase pim-1
PJS	Peutz-Jeghers syndrome
PKA	protein kinase A
PKB	protein kinase B/AKT
PKC	protein kinase C
PKD	protein kinase D
PKG	protein kinase G
PKI	PKA inhibitory peptide
PME-1	phosphatase methylesterase-1
PP1β	protein phosphatase 1β
PP2A	protein phosphatase 2A
PRK	PKC-related kinase
Pro	proline
PTEN	phosphatase and tensin homologue
PTM	posttranslational modification
QIK	Qin-induced kinase
RGC	receptor guanylate cyclase
Rpd3	reduced potassium dependency 3
P90RSK	p90 ribosomal S6 kinase
SAD	synapses of amphids defective
Ser	serine
SH2	Src homology 2

SIK	salt-inducible kinase
Sir2	silent information regulator 2
SIRT	Sir2-like protein
SNF1	sucrose nonfermenting 1
SNARK	sucrose nonfermenting AMPK-related kinase
SNRK	sucrose non-fermenting-related kinase
SREBP-1c	sterol regulatory element binding protein-1c
Src	v-Src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene
STE	sterile kinases
STRAD	STE20-related adaptor protein
TAK-1	TGF-β-activated kinase-1
TBC1D1	TBC1 domain family member 1
TCS2	tuberous sclerosis complex 2
TGF-β	transforming growth factor-β
Thr	threonine
TK	tyrosine kinase
TLK	tyrosine kinase-like
TORC	transducer of regulated CREB
TSC2	tuberous sclerosis complex 2
Tyr	tyrosine
UBA	ubiquitin-associated domain
VEGF	vascular endothelial growth factor
WT	wild-type
XEEK1	xenopus early embryo kinase 1

CHAPTER I

1.0 LITERATURE REVIEW

1.1 Signal transduction and posttranslational modification

Complex signal transduction pathways have been selected for during the evolution of cellular organisms due to the speed, accuracy, and robustness with which they relay information about the extracellular environment to intracellular sites of responsive action (24, 184). One of these sites is the nucleus, where gene expression is modulated in response to extracellular cues. The biochemical events that constitute signal transduction from the plasma membrane to the nucleus include signal-dependent protein-protein interactions and posttranslational modification of proteins (74). A major form of protein posttranslational modification (PTM) is the covalent attachment of chemical moieties or polypeptides to specific amino acid residues. Hundreds of covalent PTMs have been identified, including phosphorylation, acetylation, methylation, ubiquitination, and sumoylation (320). A testament to the importance of PTMs in cellular function is the large proportion (~5%) of the eukaryotic genome that encodes for proteins that catalyze these reactions (320). Several aspects of protein function are influenced by PTMs, including catalytic activity, association with other proteins, and subcellular localization (74).

1.1.1 Protein phosphorylation

Reversible protein phosphorylation is the prototypical and most well characterized PTM. Protein kinases catalyze the transfer of the γ-phosphate from

ATP to the hydroxyl group of serine, threonine, or tyrosine residues (147). In contrast, protein phosphatases catalyze the removal of the phosphate from these residues. There are over 500 kinases in the human genome, constituting ~1.7% of all human genes (196). Although there is much sequence diversity among kinases, the majority of these contain a recognizable eukaryotic protein kinase (ePK) catalytic domain. These typical ePK domain-containing kinases are divided into 8 groups: AGC (containing PKA, PKG, and PKC families), CAMK (Ca^{2+}/calmodulin-dependent protein kinase), CK1 (casein kinase 1), CMGC (containing CDK, MAPK, GSK3, and CLK families), STE (homologues of yeast Ste7, Ste11, and Ste20 kinases), RGC (receptor guanylate cyclase), TK (tyrosine kinase), and TLK (tyrosine kinase-like) (196, 211). Moreover, five of these groups (AGC, CAMK, CK1, CMGC, and STE) are represented among the genomes of diverse eukaryotic organisms including species of alga, fungus, arthropod, chordate, fish, mammal, plant, and parasitic protozoa, suggesting that kinases found in these groups are essential for eukaryotic life (211). Kinases from other groups, including the TK group appear to be more recent products of evolution (243).

Phosphorylation alters substrate function in numerous ways. First of all, phosphorylation introduces a negative charge that alters the conformation of the substrate, leading to changes in enzymatic activity and interaction with other proteins (147, 226). For instance, the activity of many metabolic enzymes, including glycogen synthase and pyruvate kinase, is reversibly controlled by phosphorylation (169). Secondly, phosphorylated residues can also serve as docking sites for phospho-recognition domains within the substrate and in other

proteins. For example, Src homology 2 (SH2) domains bind phospho-Tyr residues, while 14-3-3 proteins bind phospho-Ser and phospho-Thr residues (269). Moreover, optimal consensus binding motifs have been recognized for these phospho-dependent binding domains. 14-3-3 proteins usually bind to the consensus sequence RSXpS/TXP (mode 1) or RXXXpS/TXP (mode 2), where X is any amino acid and pS/T represents the phosphorylated Ser or Thr residue (343). Phospho-dependent 14-3-3 binding can affect substrate enzymatic activity, subcellular localization, stability, phosphorylation status, and interaction with other proteins (76, 342).

Many signaling pathways are comprised of sequential phosphorylation cascades. A major example of this is the mitogen-activated protein kinase (MAPK) pathway (158). In general, a MAPK kinase kinase phosphorylates a MAPK kinase, which then phosphorylates a MAPK, which then phosphorylates a target protein such as a transcription factor. Moreover, many of these signaling cascades are organized through interactions with scaffold proteins, which serve as signal processing hubs by virtue of their ability to simultaneously bind multiple signaling proteins (356). For example, in the case of the yeast MAPK mating pathway, the scaffold protein Ste5 directly binds all three kinases and allosterically tunes the input-output properties of the pathway (23). Moreover, the scaffold proteins involved in cAMP/protein kinase A (PKA) signaling, termed A-kinase anchoring proteins (AKAPs) ensure that PKA activation is restricted to specific subcellular regions (282). Interestingly, another property of signal transduction is crosstalk between different signaling pathways, one example of which is the crosstalk between MAPK and PKA signaling modules (272).

1.1.2 Signaling to chromatin

A major target of signal transduction pathways is the nucleus of eukaryotic cells where the transcription of specific genes is modulated in response to extracellular cues. The eukaryotic genome is packaged into a bundle of DNA and proteins collectively termed chromatin. The fundamental repeating unit of chromatin is the histone octamer containing two copies each of histone H2A, H2B, H3, and H4, around which ~146 base pairs of double-stranded DNA is wrapped (194). These histone octamers are often compressed together into 30 nm fibers, and these fibers are often further compacted into higher-order chromatin structures (333). In its tightly packaged conformation, the DNA is inaccessible for transcription. In response to the appropriate signals, however, transcription of specific genes will occur due to a combination of chromatin remodeling, transcription factor binding, and dissociation of corepressor proteins and recruitment of coactivator proteins (18, 36, 181). All of the proteins involved in packaging DNA or in regulating accessibility to specific genomic regions are targets of signaling pathways. In particular, the flexible N-terminal tails of histone proteins are subject to an array of PTMs that in combination regulate access of the transcription machinery to DNA (143, 167).

1.1.3 Lysine acetylation

One of the major PTMs that influences chromatin-regulated processes is lysine acetylation, the transfer of an acetyl group from acetyl-coenzyme A to the ε–amino group of a lysine residue (170). Lysine acetylation was discovered nearly 50 years ago and histones were the first acetylation substrates to be

identified (5). Since then, many non-histone proteins have also been identified as acetylation substrates (58, 94, 347). In fact, proteomic analyses have identified >1000 acetylated proteins involved in many cellular processes (55). The acetylation state of a protein is governed by the opposing actions of histone acetyltransferases (HATs), which catalyze the addition of acetyl groups to lysine residues, and histone deacetylases (HDACs), which remove this modification (170). The consequence of acetylation varies from substrate to substrate (348). Some proteins are inhibited by acetylation, such as the transcriptional coactivator peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) (180, 227, 251), while others, such as the tumour suppressor transcription factor p53 are activated by acetylation (301). In the case of histones, acetylation neutralizes their positive charge thus weakening the physical association between histones and negatively charged DNA (117). Thus, histone acetylation relaxes chromatin structure and is usually associated with the activation of gene transcription, whereas HDAC-mediated deacetylation usually leads to tightening of chromatin and repression of transcription (110, 170, 239). Importantly, acetyllysine residues can also serve as docking sites for bromodomain-containing proteins, analogous to phospho-dependent binding by SH2 domains and 14-3-3 proteins (345). Many transcriptional activators possess bromodomains, and are thus recruited to chromatin in an acetylation-dependent manner (345).

1.2 Histone deacetylases (HDACs)

Given their role in reversible lysine acetylation, the function of HDACs is critical to cellular homeostasis. Moreover, HDACs are themselves subject to

PTMs like phosphorylation and thus represent important targets of many signaling pathways. HDACs are grouped into different classes according to phylogenetic analyses and sequence homology to yeast prototypes (97, 349). Class I members are homologous to yeast Rpd3 and include HDAC1, 2, 3, and 8. Class II HDACs possess catalytic domains similar to yeast Hda1 and comprise HDAC4, 5, 6, 7, 9, and 10, which are further divided into two subclasses: IIa (HDAC4, 5, 7, and 9) and IIb (HDAC6 and 10). The seven Class III members are similar to yeast Sir2 and are designated SIRT1-7. HDAC11 is the sole member of class IV and shows comparable similarity to both Rpd3 and Hda1. Members of class I/II/IV form the Rpd3/Hda1 (or "classical") family of zinc-dependent HDACs (349). Meanwhile class III HDACs comprise the sirtuin family and depend on NAD^+ for their catalytic activity (26).

1.2.1 Class IIa HDACs

Mammalian class IIa HDACs are unique among the HDAC superfamily due to their regulation by signal-dependent nucleocytoplasmic trafficking and their weak intrinsic deacetylase activity. Consistent with their signal-dependent function, the expression of HDAC4, 5, and 9 is enriched in excitable tissues such as brain, heart, and skeletal muscle, while HDAC7 is highly expressed in thymocytes, heart, and lung (317). Subsequently, it was determined that expression of HDAC4 in bone and HDAC7 in endothelial cells is of physiological importance (47, 316). Class IIa HDACs are only represented by single orthologues in *C. elegans* and *Drosophila*, and many aspects of their function and regulation are conserved in these organisms (346, 349). There is also a single

class II HDAC orthologue in the budding yeast *S. cerevisiae* and the fission yeast *S. pombe*, however, these proteins function in large multi-protein complexes involved in regulating transcription and chromatin organization, much like yeast Rpd3 and mammalian class I HDACs (171, 290, 319). This suggests that class II HDACs evolved novel functions in multicellular organisms. In support of this, metazoan class IIa HDACs, unlike their yeast counterparts, possess a long N-terminal extension containing a battery of unique sequence elements, including binding sites for transcription factors, transcriptional coactivators and repressors, and signaling proteins. These sequence elements and the interactions they permit endow these HDACs with the ability to transduce signals from the cytoplasm to the nucleus (Fig. 1).

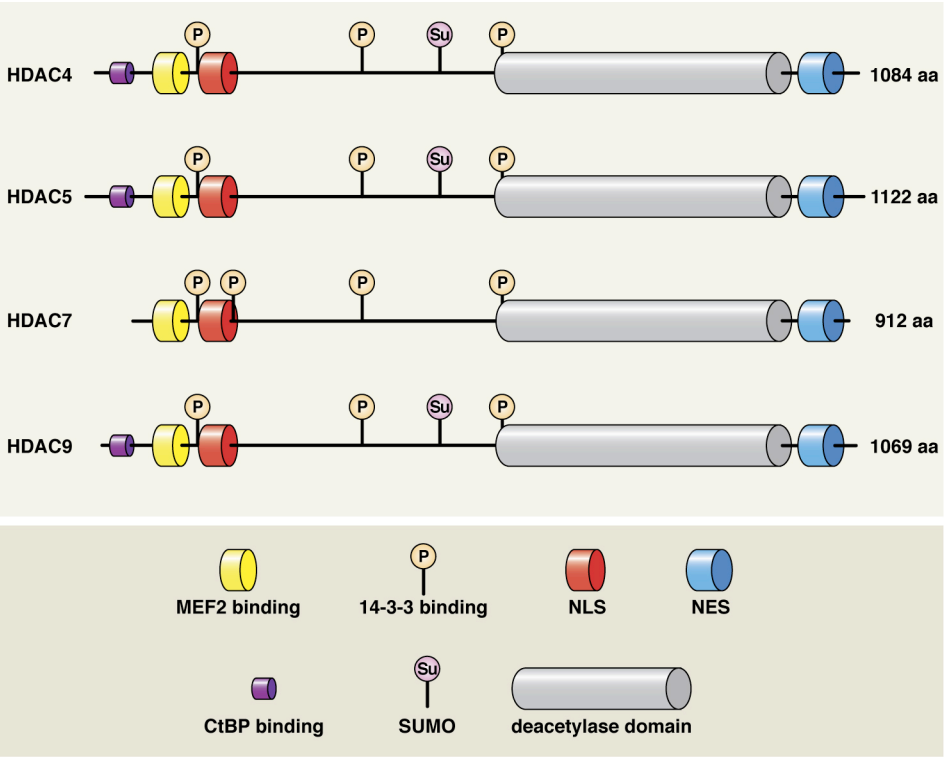

Figure 1. Domain organization of human class IIa HDACs. Class IIa HDACs possess several conserved domains. The C-terminus is composed mostly of a large deacetylase domain, while the N-terminal extension acts as an adaptor region by facilitating interactions with transcription factors such as MEF2, corepressor proteins such as CtBP, and 14-3-3 chaperone proteins. Interaction with 14-3-3 proteins is phosphorylation-dependent, and occurs on 3 or 4 serine residues. The third serine in HDAC9 may be a functional 14-3-3 binding site, but earlier reports suggested that only the first two sites were important. HDAC7 possesses a fourth 14-3-3 binding site, but its significance is not well understood. Phosphorlylation-dependent 14-3-3 binding promotes nuclear export by inhibiting the NLS and activating the NES, located at the extreme C-terminus. The N-terminal extension is also subject to other PTMs including sumoylation (SUMO).

1.2.1.1 MEF2 binding domain

One of the unique sequence elements located in the N-terminal extension of metazoan class IIa HDACs is a binding site for myocyte enhancer factor 2 (MEF2) transcription factors that is conserved in the single class IIa HDAC orthologues of *C. elegans* and *Drosophila* (346). The N-terminus also mediates

association with other transcription factors, in some cases relying on bridging cofactors to mediate the interaction (197, 206, 346). In addition to transcription factors, the N-terminus also interacts with the transcriptional repressors C-terminal binding protein (CtBP) (359) and heterochromatin protein 1 (HP1) (360).

1.2.1.2 14-3-3 binding sites, NLS, and NES

Other unique sequence elements include the 14-3-3 binding sites and nuclear localization signal (NLS) in the N-terminus as well as the C-terminal nuclear export sequence (NES). Together these elements determine the subcellular localization of class IIa HDACs. Cytoplasmic localization is dependent on three or four Ser residues that serve as 14-3-3 binding sites when they are phosphorylated (98, 151, 208, 322). These sites (S246, S467, and S632 in HDAC4) are conserved in HDAC5, 7, and 9. In some situations, the C-terminal-most 14-3-3 binding site is not functionally important in HDAC5 or HDAC9 (207, 208, 361), although one study showed that is important for HDAC5-14-3-3 binding (42). Meanwhile, HDAC7 alone possesses a fourth cryptic 14-3-3 site, but its functional significance remains unclear (72, 73, 236). Only S246 is conserved in both *C. elegans* and *Drosophila* and this site appears to be the most important mediator of subcellular localization in mammalian class IIa HDACs (349). The ability of phosphorylation-dependent 14-3-3 binding to induce cytoplasmic localization is based in part on inactivation of the NLS, which is located C-terminally adjacent to S246, and activation of the NES, located at the extreme C-terminus (209, 229, 323).

1.2.1.3 Function as a transcriptional repressor of MEF2

The MEF2 family is represented by a single orthologue in *S. cerevisiae*, *C. elegans*, and *Drosophila*, while four isoforms (MEF2A-D) are present in vertebrates (244). MEF2-dependent transcription is critical for the differentiation and/or signal-responsiveness of striated and smooth muscle, neurons, neural crest cells, chondrocytes, endothelial cells, and lymphocytes (244). MEF2 target gene transcription is either stimulated or repressed depending on whether MEF2 is bound by coactivator or corepressor proteins, respectively. Class IIa HDACs potently repress MEF2 transcriptional activity (77, 179, 192, 208, 213, 284, 321), and this is associated with histone hypoacetylation in the promoter region of MEF2 target genes (193). Interestingly, unlike class I HDACs, recombinant mammalian class IIa HDAC proteins only possess low intrinsic deacetylase activity, and it has been discovered that a single Tyr→His substitution found in mammalian class IIa HDACs, but not in the *C. elegans* or *Drosophila* orthologues, is responsible for the low activity (173). However, the class IIa HDAC deacetylase domain is active *in vivo* based on its association with class I HDACs (85, 86). Moreover, the HDAC9 splice variant MEF2-interacting transcription repressor (MITR), which lacks the deacetylase domain, as well as an HDAC7 mutant lacking the deacetylase domain, both retain the ability to repress MEF2 transcriptional activity (77, 361). In this case, the repression is likely due to the association of CtBP and HP1 (77, 359, 360). Moreover, the deacetylase activity associated with class IIa HDACs may be regulated by PTMs. HDAC4 and HDAC9 can be sumoylated on a Lys residue that is conserved in HDAC5 but

not HDAC7 (163, 242, 303), and this sumoylation may be necessary for full deacetylase and repressive activity associated with HDAC4 (163).

1.2.1.4 Signal-dependent nucleocytoplasmic shuttling

The initial observation that basal class IIa HDAC localization is cell line- and member-specific suggested that these proteins could shuttle between the nucleus and cytoplasm (98, 213, 322). Indeed, HDAC4, which is predominantly cytoplasmic in HEK293 cells, accumulates in the nucleus in response to treatment with leptomycin B, an inhibitor of the CRM1 nuclear export receptor (213, 322). Several labs demonstrated that phosphorylation-dependent 14-3-3 binding is correlated with cytoplasmic localization of class IIa HDACs (98, 151, 208, 322, 361). Moreover, phosphorylation, 14-3-3 binding, and cytoplasmic localization are associated with increased MEF2 transcriptional activity. In contrast, the opposite leads to MEF2 repression (98, 151, 192, 207, 208, 322, 361). However, this simple model (Fig. 2) misses some important nuances of class IIa HDAC localization.

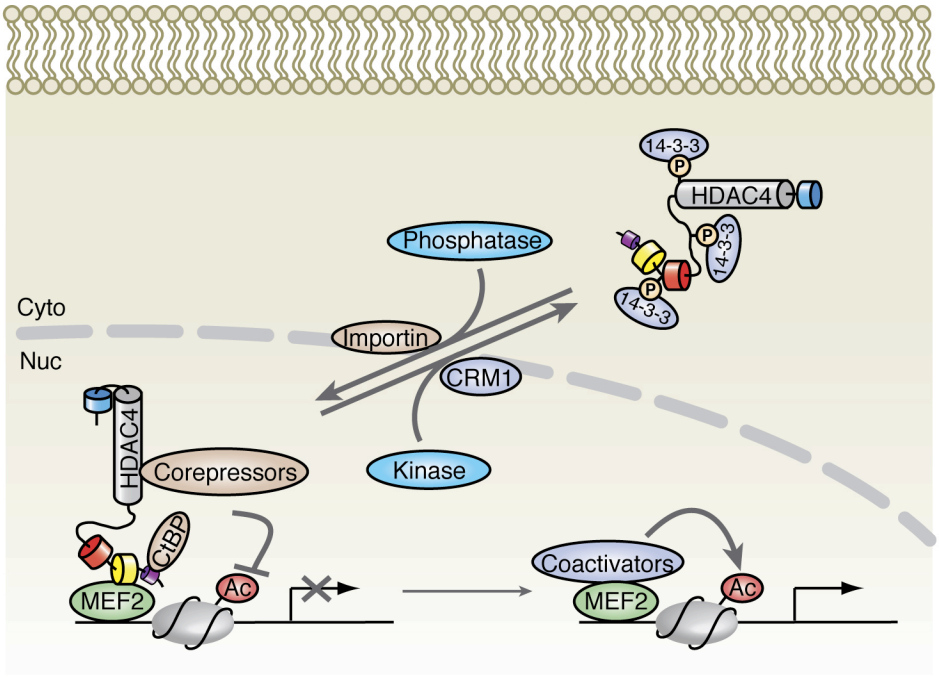

Figure 2. Regulation of class IIa HDAC nucleocytoplasmic shuttling. In the non-phosphorylated state, class IIa HDACs (represented here by HDAC4) reside in the nucleus, where they repress MEF2-dependent transcription. This repression is mediated by CtBP and other corepressor proteins, sometimes including class I HDAC complexes, in which case deacetylation of histones in the region of the MEF2 element will occur. Upon phosphorylation of conserved serine residues by various class IIa HDAC kinases, 14-3-3 proteins bind these residues, and HDAC4 is sequestered in the cytoplasm through inhibition of the NLS and activation of the NES, which likely interacts with the nuclear export receptor CRM1. MEF2 is then permitted to recruit coactivator proteins and stimulate transcription. In contrast, dephosphorylation of the 14-3-3 binding sites by class IIa HDAC phosphatases promotes nuclear import of HDAC4 by unmasking the NLS and permitting access to importin, at which point MEF2-dependent transcription is again repressed.

First, class IIa members differ in their basal localization and 14-3-3 binding. For instance, in the basal state HDAC4 is mainly cytoplasmic and constitutively bound to 14-3-3 proteins, whereas HDAC5 is nuclear and does not bind 14-3-3 or translocate to the cytoplasm unless its phosphorylation is induced (208, 322). Second, phosphorylation-dependent 14-3-3 binding is necessary but

not sufficient to cause cytoplasmic accumulation. HDAC4 and HDAC5 mutants lacking the C-terminal NES are constitutively nuclear in spite of normal phosphorylation-dependent 14-3-3 binding (208, 209, 323). The same phenomenon is seen with MITR, which naturally lacks the NES (361). However, even though these mutants do not accumulate in the cytoplasm, they still dissociate from MEF2 under phosphorylating conditions leading to derepression of MEF2 activity (208, 361). Due to the importance of phosphorylation-dependent nucleocytoplasmic shuttling in regulating class IIa HDAC repressive activity, a major focus of the HDAC field over the last decade has been the identification of kinases and phosphatases that regulate this process.

1.2.1.5 Class IIa HDAC kinases

1.2.1.5.1 CaMKs

Members of the Ca^{2+}/calmodulin-dependent protein kinase (CaMK) family were the first class IIa HDAC kinases to be identified. It was shown that CaMKIV can phosphorylate HDAC4 and HDAC5 *in vitro*, and that overexpression of active CaMKI or CaMKIV promotes nuclear export and 14-3-3 binding, leading to increased MEF2 transcriptional activity and enhanced differentiation of muscle cells (151, 193, 207, 208, 214, 361, 363). In contrast, mutation of the relevant 14-3-3 binding sites from Ser to Ala (e.g. HDAC4 S246/467/632A and HDAC5 S259/498A) prevents CaMK-induced HDAC export, 14-3-3 binding, and MEF2 activation. The use of CaMK-specific chemical inhibitors has confirmed an *in vivo* role for CaMKs as inducers of class IIa HDAC shuttling in several cell types, including neurons (27, 49, 187, 266), skeletal

muscle fibers (188), cardiomyocytes (15, 188), and immature gonadotropes (183). Moreover, diverse stimuli regulate class IIa HDAC shuttling through increased Ca^{2+} concentration and activation of CaMKs, including chronic cocaine administration (250), gonadotropin releasing hormone (183), and excitation of muscle fibers and neurons (49, 188). Interestingly, unlike CaMKI and IV, CaMKII selectively phosphorylates HDAC4 due to a unique CaMKII docking site not found in other class IIa members (15, 188). CaMKII is activated by hypertrophic stimuli in the heart, and siRNA-mediated knockdown of CaMKII or expression of a shuttling-defective HDAC4 mutant (HDAC4 S246/467/632A) prevents agonist-induced cardiomyocyte hypertrophy (15, 188).

1.2.1.5.2 PKDs

Protein kinase D (PKD) isoforms have also been shown to phosphorylate class IIa HDACs on their 14-3-3 binding sites, leading to cytoplasmic localization and MEF2 derepression. PKDs, which are downstream effectors of the protein kinase C (PKC) signaling module, are activated by several stimuli and thus control class IIa HDAC shuttling in a variety of physiological processes. Endothelin-1 and phenylalanine cause nuclear export of HDAC5 in cardiomyocytes leading to hypertrophy, and these effects are abrogated by inhibition of PKC-PKD signaling (315). In thymocytes, PKD is activated by T-cell receptor stimulation, leading to phosphorylation-dependent nuclear export of HDAC7, derepression of the MEF2 target gene Nur77 and apoptosis (73, 236). PKD1 and PKD3 were also shown to be crucial for HDAC5/7 shuttling in response to antigen receptor activation in B cells (202). In addition, vascular

endothelial growth factor (VEGF)-induced angiogenesis depends on VEGF-stimulated PKD activation, HDAC5/7 phosphorylation and nuclear export, and MEF2 derepression (103, 104, 326). Interestingly, the AKAP-Lbc scaffold protein, which assembles a complex containing PKA, PKC, and PKD, is required for maximal PKD activity toward HDAC5 during cardiomyocyte hypertrophy (42).

1.2.1.5.3 AMPK-related kinases

More recently, several members of the AMPK-related kinase family have been identified as class IIa HDACs, including salt-inducible kinase 1 (SIK1), AMP-activated protein kinase (AMPK), and microtubule affinity-regulating kinase 2 and 3 (MARK2/3) (20, 45, 72, 205). Moreover, KIN-29, the SIK orthologue in *C. elegans*, was also identified as a class IIa HDAC kinase important for regulating MEF2 activity (313). *Drosophila* SIK3 was also recently identified as an HDAC4 kinase, and this appears to be conserved in mammalian cells (325). Of note, AMPK-related kinases, CaMKs, and PKDs all belong to the CAMK supergroup of kinases (206). However, unlike CaMKs and PKDs, AMPK-related kinases are mainly activated by the upstream kinase LKB1 (see 1.3.5).

1.2.1.6 Class IIa HDAC phosphatases

Although an early class IIa HDAC study showed that inhibition of phosphatase activity increased 14-3-3 binding (98), it was not until several years later that any class IIa HDAC phosphatase was identified. The 14-3-3 binding

sites of HDAC4/5/7 were shown to be dephosphorylated by protein phosphatase 2A (PP2A) (132, 198, 235, 289) and the myosin phosphatase complex, which contains myosin phosphatase targeting subunit 1 (MYPT1) and protein phosphatase 1β (PP1β) (237). Moreover, class IIa HDAC dephosphorylation by these phosphatases is important for angiogenesis and T-cell apoptosis (198, 237). Much like class IIa HDAC kinases, it appears as though these phosphatases are regulated by diverse physiological stimuli including bile acids and nitric oxide (132, 215).

1.2.1.7 Control of localization by NLS phosphorylation

Although phosphorylation of the 14-3-3 binding sites plays a dominant role in class IIa HDAC localization, several phosphorylation events in the vicinity of the NLS are also important. The kinase Mirk/dyrk1B phosphorylates S279 of HDAC5 and the homologous site of MITR (S240), and this phosphorylation event prevents nuclear localization of MITR (69). Close to that residue, T292 of HDAC5 and the equivalent site of MITR (S253) are phosphorylated by PKC-related kinase 1/2 (PRK1/2), and this phosphorylation event also inhibits nuclear import (105). Another putative phosphorylation site in this region is S298 of HDAC4. The phospho-mimetic HDAC4 S298D mutant is defective in nuclear import, and a phosphorylation-defective HDAC4 S298A mutant partially rescues the nuclear import defect caused by knockdown of PP2A (235). Interestingly, the Mirk/dyrk1B phosphorylation site is absent from HDAC7 while the PRK1/2 site is not found in HDAC4, suggesting that differential NLS phosphorylation may underlie the differences in localization among class IIa members.

1.2.1.8 Biological roles of class IIa HDACs

All four class IIa HDACs have been knocked out in mice, and these studies have greatly aided our understanding of the physiological functions of these proteins. *Hdac4*$^{-/-}$ mice die before weaning due to impaired bone development (316). Closer inspection revealed that the skeletogenesis defect is due to precocious ossification of developing bone caused by aberrant chondrocyte hypertrophy. Mice lacking either HDAC5 or HDAC9 are viable but are hypersensitive to stress-induced cardiac hypertrophy (46, 358). Double knockout mice (*Hdac5*$^{-/-}$*Hdac9*$^{-/-}$) die during embryogenesis or shortly after birth due to cardiac abnormalities, suggesting that HDAC5 and HDAC9 play redundant roles in heart development (46). *Hdac7* deletion causes embryonic lethality due to impaired endothelial cell adhesion and rupture of blood vessels (47). Finally, deletion of any combination of 4 alleles of HDAC4, 5, or 9 (*Hdac4*$^{-/-}$*Hdac5*$^{-/-}$ or *Hdac5*$^{-/-}$*Hdac9*$^{-/-}$ or *Hdac4*$^{+/-}$*Hdac5*$^{-/-}$*Hdac9*$^{+/-}$) in skeletal muscle results in a greater percentage of slow-twitch oxidative muscle fibers (245).

Many of the phenotypes described above are the result of MEF2 hyperactivation, and this is evident from studies of *Mef2* knockout and transgenic mice. The skeletal defects exhibited by *Hdac4*$^{-/-}$ mice are rescued by the deletion of a single *Mef2c* allele (9). Conversely, homozygous deletion of *Hdac4* restores normal bone development in *Mef2c*$^{+/-}$ mice (9), suggesting that hypoactivation of MEF2 can also be deleterious. In addition, *Mef2d*$^{-/-}$ mice are resistant to cardiac hypertrophy, while Mef2d transgenic mice exhibit enhanced cardiac hypertrophy (160), similar to *Hdac5* or *Hdac9* knockout mice (46, 358). Transgenic mice expressing a hyperactive MEF2C mutant in skeletal muscle can run nearly twice

as far as WT littermates and have a higher proportion of slow-twitch oxidative muscle fibers, similar to the mice lacking 4 class IIa HDAC alleles (245). Meanwhile, the phenotype of *Hdac7*$^{-/-}$ mice was associated with upregulation of the MEF2 target gene matrix metalloproteinase 10 (47).

Mice with altered class IIa HDAC kinase activity are also informative. *CamkIIδ*$^{-/-}$ mice display attenuated cardiac hypertrophy and remodeling in response to pressure overload, and extracts from these hearts have reduced kinase activity toward HDAC4 (14). Conversely, CaMKIIδ transgenic mice have upregulated hypertrophic marker genes, increased kinase activity toward HDAC4, and increased cytoplasmic localization and 14-3-3 binding to HDAC4, along with enhanced MEF2 activity (362). Forced expression of constitutively active PKD1 in the fast-twitch glycolytic muscle fibers of transgenic mice promotes their conversion to a slow-twitch, fatigue-resistant phenotype associated with increased HDAC4/5 phosphorylation and MEF2 activity (159). In contrast, skeletal muscle-specific deletion of *Pkd1* leads to increased fatigue susceptibility of slow-twitch muscle. Transgenic mice with reduced SIK1 expression have severely damaged skeletal muscles, which exhibit reduced HDAC5 phosphorylation and cytoplasmic localization, as well as reduced MEF2 activity (20). Supporting this, overexpression of the constitutively nuclear HDAC4 S246/467/632A mutant in mouse skeletal muscle is sufficient to induce muscle damage due to inhibition of MEF2 transcriptional activity (59). Interestingly, during pathological conditions such as muscle denervation or in a mouse model of amyotrophic lateral sclerosis,

HDAC4 protein is dramatically upregulated and it relocates from the neuromuscular junction, where it colocalizes with CaMKII, to the nucleus (60).

1.3 Liver kinase B1 (LKB1)

1.3.1 Expression pattern, domains, and localization

LKB1 (Liver kinase B1/STK11) is a 433-amino acid Ser/Thr kinase possessing an N-terminal nuclear localization signal, a central catalytic (kinase) domain, and a C-terminal extension that is subject to several PTMs. Apart from the kinase domain, which is only distantly related to most other human kinase domains, LKB1 does not share significant homology to other human proteins (3). Recently, a splice variant of LKB1 was identified. In this shorter LKB1 isoform, termed $LKB1_S$ (short), the 63 amino acids at the C-terminus of the previously reported form of LKB1 ($LKB1_L$, but hereafter referred to as LKB1) are replaced by 39 unique amino acids (70, 309). LKB1 is evolutionarily conserved with single orthologues found in mouse, *Xenopus*, *C. elegans*, and *Drosophila* (113). $LKB1_S$ is found in rodents (70, 309), but it is not known whether this splice variant is present in other organisms. Although LKB1 is expressed ubiquitously, it is found at higher levels in several tissues including the cerebral cortex, ovary, testis, and skeletal muscle (259). $LKB1_S$ is also ubiquitously expressed, but is particularly abundant in haploid spermatids in the testis (70, 309). Endogenous LKB1 is predominantly localized to the cytoplasm of cells, however, when overexpressed on its own it is largely nuclear (113). This is likely due to a disruption of the stoichiometry between LKB1 and its binding partners, STRAD and MO25, since overexpression of all three proteins results in cytoplasmic

localization of LKB1 (see below), thus recapitulating its endogenous subcellular localization pattern.

1.3.2 LKB1 in Peutz-Jeghers syndrome and cancer

The human *LKB1* gene was brought to the forefront of cancer and cell signaling research as a result of the search for the genetic cause of Peutz-Jeghers syndrome (PJS), an autosomal dominant disorder characterized by cancer susceptibility, hamartomatous polyposis, and mucocutaneous pigmentation (111). Genetic analyses pointed to inactivating germline mutations in the *LKB1* gene as the causative genomic alteration in PJS (112, 142). Since the initial studies linking LKB1 to PJS, ~150 different *LKB1* germline mutations in PJS patients have been discovered, the majority of which lead to a truncated protein product (176). More diversified mutation detection methods have led to the conclusion that virtually all PJS cases are attributable to *LKB1* mutations (153). This is supported by the PJS-like phenotype observed in $LKB1^{+/-}$ mice (16, 146, 216, 253).

Somatic inactivating *LKB1* mutations have been identified in one-third of human lung adenocarcinomas (145, 260), and at lower frequencies in pancreatic cancer, melanoma, and cervical cancer (113, 331). Overall, however, the frequency of somatic *LKB1* mutations in sporadic cancers is relatively low (113). This may simply be due to difficulties associated with mutation detection (259). Alternatively, LKB1 may be inactivated in other ways. For instance, promoter hypermethylation leading to decreased LKB1 expression has been observed in breast and testicular cancers (113), and LKB1 can also be inactivated

posttranslationally in cancer (see below). *Lkb1*$^{+/-}$ mice or *Lkb1* hypomorphic mice (in which all tissues express Lkb1 at ~10% of normal levels) develop hepatocellular carcinoma (225), pancreatic cystadenomas (114), breast tumours (204), endometrial adenocarcinomas (62), and enhanced susceptibility to carcinogen-induced skin and lung tumours (99). Moreover, inactivation of *Lkb1* cooperates with mutational activation of the *K-ras* oncogene in mouse models of lung and pancreatic cancer (145, 218). Reduced expression of Lkb1 in mice also cooperates with loss of other tumour suppressors, such as *p53* (329) and *Pten* (124). LKB1 status may also be a useful prognosis indicator for cancer patients, since inactivating *LKB1* mutations and/or low LKB1 expression is associated with poor prognosis in lung (201), cervical (331), endometrial (62), breast (366), and pancreatic cancer (218).

1.3.2.1 Mechanisms of tumour suppression

Several cellular functions of LKB1 appear to underlie its tumour suppression activity. First, LKB1 is necessary for cell senescence induced by high passage number in culture (16). Second, LKB1 stimulates cell cycle arrest at the G1 stage by upregulating the cyclin-dependent kinase inhibitor p21 (278, 307, 308). Third, LKB1 can promote p53-dependent apoptosis (152). Fourth, LKB1 inhibits protein synthesis and cell growth by negatively regulating the mammalian target of rapamycin (mTOR) pathway (63, 134, 274). Fifth, through regulation of cell structure, LKB1 maintains proper polarity in several cell types (12, 113). Sixth, LKB1 inhibits angiogenesis by limiting the expression of VEGF (340, 352, 366). Finally, LKB1 suppresses epithelial-mesenchymal transition (EMT),

invasion, and metastasis of cancer cells (43, 145, 254, 366). Of note, the role of LKB1 in some of these processes is not fully defined. For instance, LKB1 has also been reported to possess anti-apoptotic (275, 340) and pro-angiogenic (191, 231) properties under some conditions.

1.3.3 Role of LKB1 in other physiological processes

1.3.3.1 LKB1 in development

Since the identification of LKB1 as a suppressor of PJS and various sporadic cancers, a plethora of other physiological processes have been shown to depend on LKB1. *Lkb1*$^{-/-}$ mice die at midgestation (146, 216, 352) as a result of vascular abnormalities and neural tube defects (352). Developmental defects are also caused by loss of the LKB1 orthologue Par-4 in *C. elegans* and *Drosophila*, and XEEK1 in *Xenopus* (199, 234, 328). These embryonic abnormalities in *Lkb1*$^{-/-}$ mice are mainly a result of cell-autonomous functions of LKB1, since tissue-specific *Lkb1* deletion or LKB1 knockdown in cultured cells recapitulates the defects observed due to global *Lkb1* deletion. For instance, endothelial cell-specific *Lkb1* knockout results in defective endothelial-to-vascular smooth muscle cell signaling and impaired angiogenesis during vascular development (191). LKB1 knockdown in cultured neurons inhibits differentiation, polarization, and migration (10, 17, 277) and disruption of LKB1 function *in vivo* impairs corticogenesis (233).

1.3.3.2 LKB1 in muscle

Skeletal and cardiac muscle-specific *Lkb1* deletion reveals numerous processes controlled by LKB1 in muscle tissue. Impaired contraction-stimulated glucose uptake (257), higher levels of intramuscular triglycerides (165), reduced capacity to increase fatty acid oxidation (304), lower mitochondrial content (35, 306), a shift in muscle fiber type (35, 305), and dramatically reduced voluntary wheel running (306) were all reported effects of *Lkb1* knockout in mouse skeletal muscle. Moreover, at 30-50 weeks of age, these mice exhibit muscle atrophy, exaggerated fatigue, and difficulty moving (305). Curiously, it was also reported that insulin sensitivity and glucose homeostasis improved in mice lacking skeletal muscle LKB1 expression (165). Consistent with the defects reported in skeletal muscle, the hearts of these mice (which also lack LKB1 expression) are dysfunctional (144, 305). Moreover, cardiac muscle-specific *Lkb1* deletion causes death and heart failure associated with pathological cardiac hypertrophy (131).

1.3.3.3 LKB1 in liver

In the liver, *Lkb1* deletion causes hyperglycemia and compromised inhibition of gluconeogenesis in response to the anti-diabetic drug metformin (276). However, the role of LKB1 in this process has been questioned recently (88). Liver-specific *Lkb1* deletion also leads to abnormal bile duct formation and reduced canalicular membrane integrity, concomitant with aberrant accumulation of bile salts, low-density lipoproteins, and nonesterified cholesterol (335). Moreover, LKB1 has been implicated in the etiology of non-alcoholic hepatic

steatosis, a condition that predisposes its victims to hepatocellular carcinoma (200).

1.3.3.4 LKB1 in pancreas

Deletion of *Lkb1* in the pancreatic endothelium results in acinar cell polarity defects, reduced islet size, and age-dependent aberrations in glucose control (114). In addition, *Lkb1* deletion in islet β-cells by means of an inducible Pdx1-*Cre*ER transgene causes hypertrophy, increased proliferation, abnormal polarity, and enhanced insulin secretion in response to glucose (90, 96). These results were largely reproduced by another study that used a different transgene (Rip2-*Cre*) to delete *Lkb1* in β-cells, however, when β-cells from these mice were tested *ex vivo*, they exhibited a decrease in glucose-stimulated insulin secretion, and these mice had a lower body weight (292). The authors suggested that the reduced body weight and enhanced insulin secretion *in vivo* (versus the diminished insulin secretion *ex vivo*) might be due to the activity of the Rip2-*Cre* transgene in neurons (292). Indeed, these mice also exhibit axon degeneration in the spinal cord and hindlimb dysfunction, suggesting that, in addition to β-cells, this transgene is also active in neurons (291).

1.3.3.5 LKB1 in haematopoietic system and immune system

Recently, the importance of LKB1 in haematopoietic stem cell function and the immune system has been uncovered. Inducible *Lkb1* deletion in the haematopoietic system of adult mice causes lethality due to the depletion of cells

from all haematopoietic lineages. This pancytopenia appeared to be the result of the exhaustion of the haematopoietic stem cell population owing to aberrant cell cycle entry, mitochondrial dysfunction, aneuploidy, and apoptosis (92, 100, 224). Meanwhile, in the immune system, thymocyte-specific *Lkb1* deletion leads to impaired survival of $CD4^+/CD8^+$ double-positive T-cells and defective production of $CD4^+$ and $CD8^+$ single-positive T-cells (41).

1.3.3.6 Physiological function of the $LKB1_S$ isoform

As mentioned above, expression of the $LKB1_S$ splice variant is enriched in testis (specifically in haploid spermatids), thus, it is not surprising that mice specifically lacking expression of the $LKB1_S$ splice variant have defects in spermatogenesis and fertility (70, 309). These mice do not display any other obvious defects.

1.3.4 Molecular Regulation of LKB1

1.3.4.1 LKB1-STRAD-MO25 complex

The kinase activity and subcellular localization of LKB1 are regulated by multiple PTMs and protein-protein interactions. *In vivo*, LKB1 exists in a heterotrimeric complex with the pseudokinase STE20-related adaptor protein (STRADα/β), and the armadillo repeat-containing mouse protein 25 (MO25α/β) (11, 28, 30, 107). Binding of these proteins to LKB1 is necessary for full cytoplasmic localization and catalytic activity of LKB1 toward substrates, including the most well characterized LKB1 phosphorylation target, AMPK (11, 28, 107, 189). Moreover, many LKB1 point mutants found in human cancers are

deficient in binding to STRAD:MO25 (29, 357), and STRAD binding is crucial for LKB1 to induce cell growth arrest (11). Crystal structure analysis of the LKB1:STRADα:MO25α complex revealed a unique mechanism for LKB1 activation. Whereas most kinases are activated when they are phosphorylated on their T-loops (a.k.a. activation loops), LKB1 activation is phosphorylation-independent, and is instead mediated by allosteric regulation via cooperative binding between STRADα and MO25α (247, 357).

1.3.4.2 Phosphorylation of LKB1

In addition to protein-protein interaction, LKB1 is also regulated via PTM, most notably phosphorylation. LKB1 autophosphorylates itself on four residues (T185, T189, T336, and T402) (3). Phosphorylation of the major autophosphorylation site, T336, appears to inhibit LKB1 function, since a phospho-mimetic mutation (T336E) prevents LKB1 from arresting cell growth (262). LKB1 is also phosphorylated on S428 by p90 ribosomal S6 kinase (RSK) (264), PKA (61, 264), and PKC-zeta (PKCζ) (341). The function of S428 phosphorylation appears to be cell context-dependent and also influenced by other phosphorylation events. Mutation of this Ser to Ala (S428A) impairs the ability of LKB1 to inhibit cell growth (264), and inhibits LKB1-dependent activation of AMPK in response to the PKCζ-activating stimuli peroxynitrite and metformin, suggesting that S428 phosphorylation activates LKB1 (339, 341). In contrast, S428 phosphorylation by p90RSK, accompanied by S325 phosphorylation by the MAPK extracellular signal-regulated kinase (ERK), inhibits LKB1 activity

toward AMPK, via disruption of LKB1-AMPK binding (82, 364). S428 phosphorylation has also been reported to have no effect on LKB1 activity (70, 87).

Moreover, the same kinase can phosphorylate LKB1 on multiple sites, as PKCζ has also been shown to phosphorylate S307 of LKB1 leading to increased cytoplasmic localization, association with STRADα, and AMPK activation (340). In this case, phosphorylation of both S307 and S428 by PKCζ-activating stimuli is required to increase LKB1 activity (340). DNA damaging agents, including ionizing radiation, induce ataxia-telangiectasia mutated (ATM) kinase-dependent LKB1 phosphorylation on T363 (4, 84, 263). LKB1 is also phosphorylated on Y261 and Y362 by the tyrosine kinase Fyn (344). Fyn-mediated phosphorylation of LKB1 prevents LKB1 from localizing to the cytoplasm and phosphorylating AMPK (344). This appears to be a dominant factor governing LKB1 subcellular localization, since constitutively active Fyn can overcome the effect of STRADα overexpression (i.e. increased LKB1 cytoplasmic localization) on LKB1 (344).

1.3.4.3 Farnesylation and acetylation of LKB1

LKB1 is also subject to other PTMs, including farnesylation and acetylation. Farnesylation (a type of prenylation) occurs on Cys 430 (61, 264). The physiological relevance of this PTM is not clear, as a farnesylation-defective LKB1 mutant displays impaired membrane localization and regulation of cell polarity in *Drosophila* (199), and impaired membrane localization in mammalian cells (61). However, another group reported no effect of farnesylation on the

kinase activity or growth suppression activity of LKB1 (264). Further, there is no crosstalk between C430 farnesylation and S428 phosphorylation (264). Acetylation of LKB1 has also emerged as an important regulator of LKB1 function. Although the acetyltransferase responsible remains unknown, SIRT1 deacetylates K48 of LKB1 leading to increased cytoplasmic localization, STRAD association, and activation in HEK293T cells (174). In fact, two stimuli that activate SIRT1, resveratrol treatment and nutrient fasting, both cause LKB1 deacetylation and activation (174, 178). In contrast, SIRT1-mediated deacetylation was shown to inhibit LKB1 function during senescence of endothelial cells (367). Thus, much like the case for S428 phosphorylation, the consequences of farnesylation and acetylation are likely context-specific.

1.3.4.4 Posttranslational modification in tumourigenesis

Posttranslational modification of LKB1 provides a means for LKB1 inactivation in sporadic tumourigenesis in the absence of *LKB1* mutations. For instance, in melanomas driven by an activating mutation in the B-RAF oncogene (B-RAF V600E), LKB1 is inactivated through S325 and S428 phosphorylation, leading to decreased AMPK activity (82, 364). In fact, LKB1 phosphorylation induced by activated B-RAF is necessary for proliferation, anchorage-independent growth, and resistance to apoptosis of melanoma cells (82, 364). Thus, although *LKB1* mutations in sporadic cancers are relatively rare (259), posttranslational inactivation of wild-type LKB1 may be responsible for some of these cancers.

1.3.4.5 Posttranslational modification of $LKB1_S$ isoform

By virtue of its possession of an alternative exon 9, some PTM sites characterized for LKB1 are not present in $LKB1_S$, including the S428 phosphorylation site and the C430 farnesylation site (70, 87, 309). Since the subcellular localization and catalytic activity of $LKB1_S$ were not different from LKB1, it was concluded that these two PTMs are not important for LKB1 regulation (70, 87). However, this issue needs to be investigated further, since 1) the function of S428 appears to be highly context-dependent (see above), and 2) other putative phosphorylation sites exist in the distinct C-terminal region of $LKB1_S$ which may act analogously to S428 of LKB1 (340).

1.3.5 LKB1 signaling through AMP-activated protein kinase (AMPK)

AMPK exists as a heterotrimeric complex, which is evolutionarily conserved in the budding yeast *S. cerevisiae*, comprised of a catalytic α subunit, and regulatory β and γ subunits (285). In mammals, there are multiple isoforms of each subunit, with α1, α2, β1, β2, γ1, γ2, and γ3, representing the pool of isoforms from which a specific AMPK trimeric complex (e.g. α1/β2/γ3) could be composed. AMPKα1/2 contain an N-terminal kinase domain, an autoinhibitory sequence, and a β-subunit interacting domain that binds the β subunit, with the latter directly associating with both the α and γ subunits (285). AMPK is activated in conditions of energy stress, manifested by an increased AMP:ATP ratio (330). AMP directly binds the γ subunit and activates AMPK by allosteric activation and also by preventing the dephosphorylation of T172 (261), a T-loop

residue that must be phosphorylated for AMPK to be active (108). Based in part on sequence similarity to *S. cerevisiae* kinases that phosphorylate the AMPKα budding yeast orthologue sucrose nonfermenting 1 (SNF1), LKB1 was identified as the long-sought AMPKα upstream kinase (107, 118, 275, 336). Although CaMKK (109, 127, 334) and TGF-β-activated kinase-1 (TAK-1) (217, 338) have since joined LKB1 as AMPK kinases, LKB1 is recognized as the major AMPK kinase in most tissues under many conditions. Basal and stress-induced AMPKα2 T172 phosphorylation and activation are impaired in LKB1-negative cell lines (107, 275, 336) and in the muscle (257, 258) and liver (276) of mice engineered to lack Lkb1 expression in those tissues.

Given its unique ability to directly sense changes in cellular energy status, it is not surprising that AMPK is a master regulator of metabolism at the cellular and whole-body level. By phosphorylating several metabolic enzymes (e.g. acetyl-CoA carboxylase) and transcriptional regulators that control metabolic gene programs (e.g. PGC-1α), AMPK modulates carbohydrate, lipid, and protein metabolism (285). For example, AMPK promotes skeletal muscle glucose uptake, fatty acid oxidation, and mitochondrial biogenesis, and inhibits hepatic gluconeogenesis and lipogenesis (285). Moreover, AMPK ensures that energy-intensive cellular processes such as division, growth, and structural changes only occur when sufficient energy is available (273). AMPK depends on LKB1-mediated phosphorylation for most of these effects.

Conversely, many effects of LKB1 are dependent on AMPK, such as several of its metabolic and tumour suppressor properties (273). For example,

LKB1 inhibits protein synthesis in response to energy deprivation through AMPK-mediated phosphorylation of tuberous sclerosis complex 2 (TSC2) and Raptor, which results in inactivation of mTOR complex 1 (mTORC1) (63, 134, 274); LKB1 induces cell cycle arrest in response to glucose deprivation through AMPK-mediated phosphorylation and activation of p53 (133, 149); and LKB1 maintains cell survival in the face of diverse stress stimuli by upregulating p53-dependent gene transcription through AMPK-mediated histone H2B phosphorylation (37). AMPK also plays an important role in LKB1-dependent control of cell polarity under energetic stress conditions (177, 212), and polarity control is increasingly being recognized as a mechanism of tumour suppression (113). Agents that activate AMPK have proven to have anti-tumourigenic effects *in vitro* and *in vivo*, and some of these, including the anti-diabetic drug metformin are in clinical trials as potential cancer therapeutics (273). Of note, many effects of LKB1 have been shown to be independent of AMPK, including some of its tumour suppression effects (113), and its effects on haematopoietic stem cell maintenance (92, 100, 224). Instead, in many cases LKB1 likely depends on one or more of the multiple AMPK-related kinases.

1.4 AMPK-related kinases

Based on sequence homology to the AMPK catalytic domain, 12 AMPK-related kinases (NUAK1, NUAK2, SAD-A, SAD-B, SIK1, SIK2, SIK3, MARK1, MARK2, MARK3, MARK4, and MELK) were identified (196). Like AMPK, all AMPK-related kinases except MELK were also shown to depend on LKB1-mediated T-loop phosphorylation for their activity (189). In addition, SNRK,

which is more distantly related to AMPK, is the only member of the 8-kinase SNRK family that is also phosphorylated and activated by LKB1 (137). Most AMPK-related kinases (all except AMPKα1/2 and NUAK1/2) possess a ubiquitin-associated (UBA) domain that is important for their phosphorylation by LKB1 and catalytic activity (138). Like AMPK, these kinases are involved in a wide range of cellular and physiological events, sometimes with overlapping roles.

1.4.1 NUAK1 and NUAK2

NUAK1/AMPK-related kinase 5 (ARK5) and NUAK2/sucrose nonfermenting AMPK-related kinase (SNARK) are required for embryonic development and have been implicated in metabolism and tumourigenesis. *Nuak1*$^{-/-}$ mice and most *Nuak2*$^{-/-}$ mice die near the end of embryogenesis due to omphalocele (a defect in abdominal wall closure leading to organs remaining outside the abdomen) and exencephaly (a condition in which the brain remains outside the skull), respectively (115, 310). In contrast, *Nuak2*$^{+/-}$ mice survive to adulthood and have an increased susceptibility to carcinogen-induced colorectal cancer, in part due to obesity and related metabolic disorders (310). Similarly, NUAK1 has been implicated as a tumour invasion- and metastasis-promoting factor (172, 296). NUAK1 can suppress cell death induced by nutrient starvation or death receptor activation (294), and it also plays an LKB1-dependent role in cell adhesion through the phosphorylation of myosin light chain-2 (MLC-2) (355), and in cell senescence and ploidy by phosphorylating large tumour suppressor 1 (LATS1) (126). NUAK2 can also phosphorylate MLC-2 and LATS1, and

NUAK2 overexpression has been shown to induce cell detachment (295). However, it appears to be dispensable for the effects on senescence and ploidy *in vivo* (126).

1.4.2 SAD-A and SAD-B

Also known as brain-specific kinases, SAD-A/BRSK2 and SAD-B/BRSK1 are evolutionarily conserved with a single orthologue in *C. elegans* and *Drosophila* (34). These kinases are highly expressed in neurons and they play key roles in regulating neuronal polarity and synaptic development in *C. elegans* and in mammals (34). Double knockout mice (*sada*$^{-/-}$/*sadb*$^{-/-}$) have difficulty moving and responding to stimuli and die shortly after birth. The mice exhibit irregularities in cortical architecture and lack distinct axons and dendrites in some neurons (164). Moreover, the microtubule-associated protein Tau was identified as a phosphorylation target of SAD-A and SAD-B in this study. Importantly, SAD double knockout mice phenocopy mice lacking LKB1 expression specifically in pyramidal neurons (277), and it was shown that the effect of LKB1 on neuronal polarity depends on signaling through SAD kinases (17, 277). SAD-B also influences entry into mitosis by phosphorylating Wee1, Cdc25C, and Cdc25B (34).

1.4.3 Microtubule affinity-regulating kinases (MARKs)

The MARK/Par-1 family has an evolutionarily conserved function in regulating cell polarity (128). Mammalian MARKs are involved in polarity control of epithelial cells and neurons through the phosphorylation of

microtubule-associated proteins (34). In fact, MARK-mediated phosphorylation of Tau has been implicated in the initiation of Alzheimer's disease. *Mark2* knockout mice exhibit several phenotypic changes including enhanced insulin sensitivity and resistance to diet-induced obesity (129), dwarfism and hypofertility (21), immune system dysfunction (130), and impaired learning and memory (270). A role for MARK3 in cell cycle control has been reported, based on both its ability to phosphorylate Cdc25C and its role as a substrate of the oncogenic kinase Pim-1 (13, 241). MARK4 might be involved in tumourigenesis, since the MARK4L isoform is upregulated in glioblastomas and in hepatocellular carcinomas (19, 154).

1.4.4 Sucrose non-fermenting-related kinase (SNRK)

Among the 8 kinases in the SNRK family, which is adjacent to the AMPK-related kinase family in the human kinome (Manning 2002), only SNRK is dependent on LKB1-mediated phosphorylation of its T-loop for its activation (137). Little is known about physiological functions of SNRK. In rats, the expression of SNRK is enriched in the testis, however analysis of a human mRNA expression database suggests a ubiquitous expression pattern (137). SNRK expression is upregulated in neurons cultured in low potassium, suggesting a potential role in cell survival (354). In zebrafish, loss of SNRK leads to defects in angioblast development that can be rescued by ectopic expression of human SNRK (57).

1.4.5 Salt-inducible kinases (SIKs)

1.4.5.1 Identification, expression, and domain organization

Salt-inducible kinase 1 (SIK1) was first cloned from the adrenal glands of rats fed a high-salt diet (327), and from neurons undergoing depolarization (83). Based on sequence similarity to SIK1, two related proteins, termed SIK2 (also called Qin-induced kinase or QIK) and SIK3 (also called QSK) were added to the SIK subfamily of AMPK-related kinases (155). This subfamily is evolutionarily conserved with a single orthologue found in *C. elegans* (KIN-29), which is most similar to SIK3, and two orthologues in *Drosophila* (dSIK2 and dSIK3) (153, 232, 300). KIN-29 is expressed in hypodermis, muscles, and neurons, and dSIK2 is highly expressed in the brain (54, 324). Mammalian SIKs are expressed in multiple tissues, with SIK1 enriched in the adrenal cortex, SIK2 in adipose tissue, and SIK3 ubiquitously expressed (155). Mammalian SIKs possess an N-terminal kinase domain and a UBA immediately C-terminal to the catalytic domain (138). SIK1 and SIK2 are of similar size (776 and 931 amino acids, respectively), while SIK3 (1263 residues) possesses a significantly extended C-terminal region (153). The major PTM sites are the T-loop LKB1 phosphorylation site (T182, T175, T163 in SIK1, 2, and 3, respectively), located near the centre of the kinase domain (189), and the PKA phosphorylation site (S577 and S587 in SIK1 and SIK2), which appears to be conserved in SIK3 at S493 (120, 156, 299) (Fig. 3). SIKs are subject to phosphorylation at other sites and these events will be discussed below.

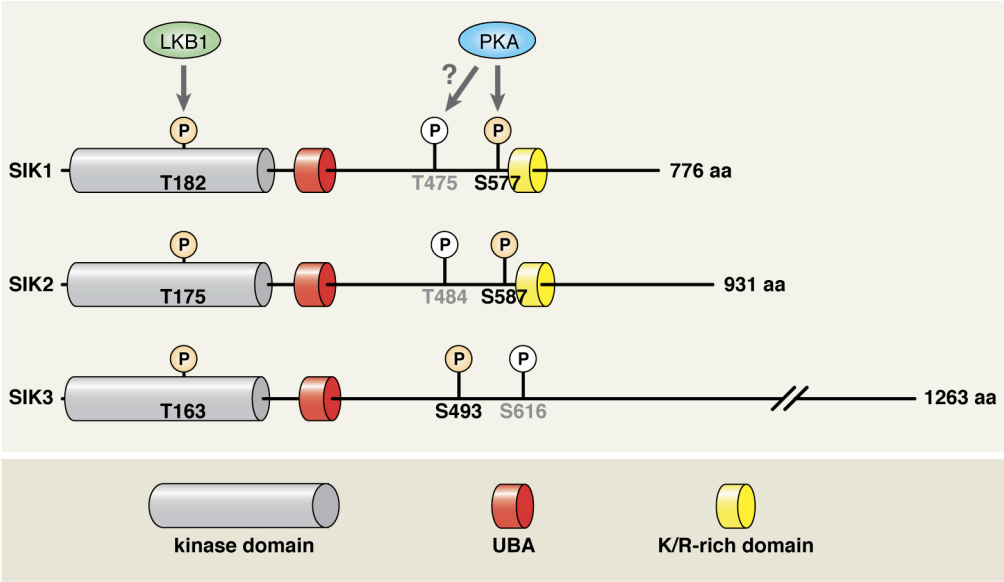

Figure 3. Domain organization of mammalian SIKs. SIKs possess an N-terminal kinase domain and an adjacent ubiquitin-associated (UBA) domain. The lysine/arginine-rich (K/R-rich) domain regulates the subcellular localization of SIK1 and SIK2. It has not been reported whether SIK3 also possesses a functional K/R-rich domain. The T-loop Thr that LKB1 phosphorylates is located in the centre of the kinase domain and the PKA phosphorylation site is located just N-terminal to the K/R-rich domain in SIK1 and SIK2, while it is more N-terminal in SIK3. Sequence inspection revealed other putative PKA phosphorylation sites in SIK1 (T475), SIK2 (T484), and SIK3 (S616). Amino acid numbers correspond to rat SIK1, mouse SIK2, and human SIK3, since these are the cDNAs that have traditionally been used for molecular analysis of these kinases.

1.4.5.2 Molecular regulation of SIK activity and localization

Several factors regulate the catalytic activity and subcellular localization of SIKs. Like most AMPK-related kinases, LKB1-mediated T-loop phosphorylation is necessary for their activation (189), in part by stimulating phospho-dependent 14-3-3 binding to this residue (2). Moreover, autophosphorylation of S186 in SIK1 acts as a priming event for T182 phosphorylation by LKB1, and sustained SIK1 activation may depend on glycogen synthase kinase-3β (GSK-3β)-mediated phosphorylation of both these

sites (106). ATP binding is also crucial for catalytic activity as mutants that cannot bind ATP (SIK1 K56M, SIK2 K49M, and SIK3 K37M) are kinase-dead (120, 156, 186). In addition, the UBA domain plays a conformational role that is required for catalytic activity (138). Other phosphorylation events that promote SIK activity include CaMK-mediated T322 phosphorylation of SIK1, and protein kinase B (PKB)/AKT-mediated S358 phosphorylation of SIK2 (71, 279).

SIK1 is localized to the nucleus and cytoplasm while SIK2 and SIK3 are fully cytoplasmic (2, 120, 186). This is partially due to an Arg- and Lys-rich region in SIK1 (residues 586-612) and the corresponding region of SIK2 (residues 596-622), since swapping these domains renders SIK1 fully cytoplasmic and SIK2 more nuclear (157). However, phosphorylation status also influences subcellular localization. PKA-mediated phosphorylation at a site adjacent to this region (SIK1 S577 and SIK2 S587) can induce SIK1 nuclear export and is necessary for the basal cytoplasmic localization of SIK2 (120, 186). PKA-mediated phosphorylation also inhibits the ability of SIKs to phosphorylate substrate proteins (20, 268), however, it seems that changes in subcellular localization induced by PKA are dispensable for this effect (157).

1.4.5.3 Regulation of CREB through CRTC phosphorylation

The most extensively studied substrates for SIKs are members of the CREB-regulated transcription coactivator family (CRTC1-3), also known as transducer of regulated CREB (TORC). SIKs phosphorylate CRTC members on a conserved Ser residue (e.g. S171 for CRTC2) (268, 299). This phosphorylation event promotes the phospho-dependent binding of 14-3-3 proteins to CRTC,

resulting in cytoplasmic sequestration of CRTC (268). This prevents CRTC from performing its nuclear role as a coactivator of the cAMP-response element binding protein (CREB) transcription factor (25, 268). Thus, SIK-mediated phosphorylation of CRTC serves to prevent CREB-dependent transcription. As mentioned above, phosphorylation of SIKs by PKA inhibits SIK-mediated phosphorylation of CRTC. Joining PKA as a negative regulator of CRTC phosphorylation is the Ca^{2+}-activated, Ser/Thr-phosphatase calcineurin, which dephosphorylates S171 of CRTC2 (25, 268). Thus, CRTC has been postulated to function as a cAMP and Ca^{2+} coincidence detector, since maximal CRTC activity (achieved when CRTC is hypophosphorylated) is only achieved when high cAMP levels fully inhibit SIKs and high Ca^{2+} levels fully activate calcineurin (268) (Fig. 4). Interestingly, *Sik1* is itself a CREB-target gene, and this provides the necessary conditions to form a negative feedback loop that resets CREB activity (20, 71). In response to cAMP stimulation, activated PKA phosphorylates CREB on S133, an event which recruits another CREB coactivator, CREB-binding protein (CBP) (8, 56) and also phosphorylates SIK1 on S577, which permits CRTC to bind CREB. After several hours of CREB stimulation, upregulation of SIK1 protein above the level at which PKA can phosphorylate every SIK1 molecule leads to an increased amount of active SIK1 thus tipping the balance toward CRTC phosphorylation and a return to basal CREB activity (20, 182). Moreover, SIK-dependent regulation of CRTC phosphorylation and CREB transcriptional activity is dependent on LKB1-mediated phosphorylation of SIKs (156).

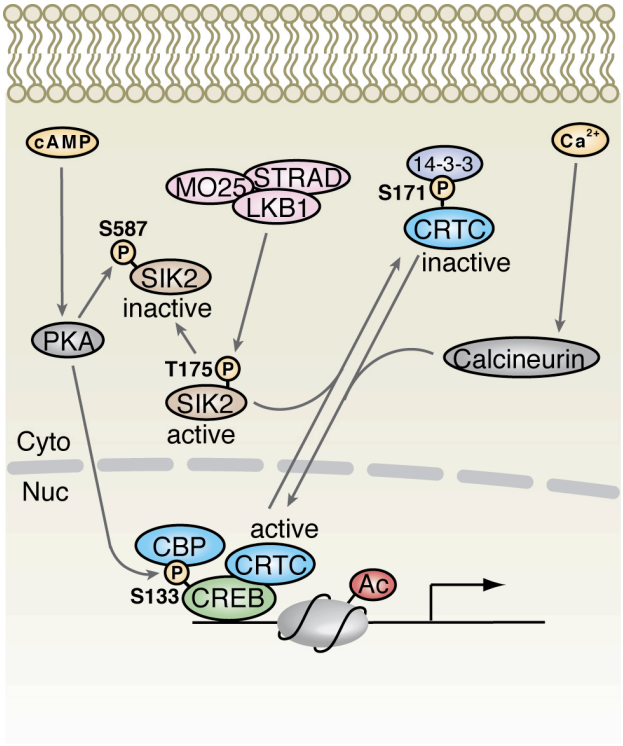

Figure 4. Regulation of CRTC-CREB signaling axis by SIK2. SIK2 activity is dependent on T-loop phosphorylation (on T175) by the upstream kinase LKB1. When active, SIK2 phosphorylates CRTC on S171, leading to 14-3-3 binding and cytoplasmic sequestration. In this condition, CREB-mediated transcription is repressed. However, dephosphorylation of CRTC permits re-entry into the nucleus where it can coactivate CREB-dependent transcription. CRTC dephosphorylation occurs due to both calcium-dependent activation of the CRTC phosphatase calcineurin, and by cAMP-mediated activation of PKA, which inactivates SIK2 by phosphorylating it on S587. PKA also stimulates CREB activity by direct phosphorylation of CREB on S133, which recruits another coactivator, CBP.

1.4.5.4 Physiological consequences of CRTC phosphorylation

In *Drosophila*, loss of dSIK2 function in neurons leads to increased CRTC activity, CREB-dependent gene transcription, higher lipid and glycogen stores, and resistance to starvation (54, 324). Rescue of these defects by neuronal dSIK2 overexpression depends on CRTC phosphorylation and CREB transcriptional activity (54). Further supporting this, loss-of-function CRTC mutant flies exhibit decreased CREB activity, lower lipid and glycogen stores, and are more sensitive to starvation and oxidative stress (324). SIK2 also controls CRTC-CREB signaling in the mammalian brain, and neurons isolated from *Sik2*$^{-/-}$ mice exhibit

enhanced survival in response to oxygen-glucose deprivation (265). Moreover, in the developing mammalian cortex, SIK1 is an integral component of the negative feedback loop that switches off CREB-dependent dendritic growth in response to persistent neuronal activity, and this also depends on SIK1-mediated phosphorylation and nuclear export of CRTC1 (182).

The SIK1-CRTC signaling axis also controls steroidogenic gene expression in the adrenal cortex. During the early stage of adrenocorticotropic hormone (ACTH) stimulation, SIK1 expression and activity is rapidly upregulated, which prevents CRTC2 from associating with CREB, resulting in repression of CREB-dependent steroidogenic gene transcription (141, 232). However, after this initial phase, SIK1 becomes inactivated by PKA-mediated phosphorylation, leading to increased CREB-dependent expression of steroidogenic genes concomitant with increased CRTC2 occupancy at the promoters of these genes (141, 232).

In the liver, the SIK-CRTC axis controls gluconeogenesis to modulate blood glucose levels in response to feeding and fasting cycles. In the fasted state, increased levels of circulating glucagon inhibit SIK1/2 activity in hepatocytes through an increase in intracellular cAMP concentration. This in turn permits the nuclear localization of hypophosphorylated CRTC2 leading to CREB-dependent transcription of genes involved in the gluconeogenic program (166). In contrast, feeding increases the level of circulating insulin which activates AKT in hepatocytes, which increases the activity of SIK2, resulting in attenuation of gluconeogenic gene transcription due to sequestration of phosphorylated CRTC2 in the cytoplasm (71). Knockdown of SIK2 in the liver of fed mice results in

increased gluconeogenesis, postprandial hyperglycemia, hyperinsulinemia, insulin resistance, and glucose intolerance (32).

In adipose tissue, endogenous SIK2 S587 phosphorylation is negatively correlated with CRTC2 S171 phosphorylation, and overexpression of the PKA-resistant SIK2 S587A mutant in the brown adipose tissue of male mice results in hyperphosphorylated CRTC2, downregulation of key CREB target genes, and hypersensitivity to high-fat diet-induced obesity (221).

The effects of cAMP signaling on melanogenesis have also recently been shown to depend on SIK2-CRTC signaling (119). CREB-dependent transcription of melanogenic genes and resulting melanin production induced by CRTC overexpression or by ultraviolet radiation is attenuated by SIK2 overexpression in melanoma cells (119). Moreover, subcutaneous injection of the cAMP agonist (and thus PKA activator) forskolin caused darker hair colour only around the injection sites, due to PKA-mediated SIK2 S587 inhibitory phosphorylation. Furthermore, crossing *Sik2*$^{-/-}$ mice (which lack an obvious phenotype) with *agouti* A^{y}/a mice suppressed the agouti hair colour phenotype resulting in brown hair instead of the normal yellow hair (119).

1.4.5.5 SIKs as class IIa HDAC kinases in neurons and myocytes

In *C. elegans*, KIN-29 phosphorylates the class IIa HDAC orthologue HDA-4, preventing it from binding MEF2 and repressing transcription of chemosensory receptor genes (313). In fact, impairment of this signaling event is responsible for the gross physiological defects seen in *kin-29* loss-of-function mutants, including small body and brood size and slow growth (175, 195, 313).

Mammalian SIKs have also been identified as class IIa HDAC kinases (20, 268). SIK1 can phosphorylate HDAC4 and HDAC5, leading to nuclear export of HDAC5 and activation of MEF2-dependent transcription (20, 298). In mice expressing a dominant-negative CREB mutant in skeletal muscle, the expression of SIK1 is decreased (20). This leads to hypophosphorylation of HDAC5, decreased MEF2 activity, and severe muscle damage. In contrast, virus-mediated re-expression of SIK1 rescues this phenotype, suggesting that SIK1-class IIa HDAC signaling in skeletal muscle is crucial for survival of myocytes and normal muscle function (20). In addition, androgen-induced SIK1 has been shown to have a protective effect against cerebral ischemia, and it was suggested that SIK1-mediated inhibition of HDAC activity may be involved (53). Although an HDAC5 peptide was uncovered in a high-throughput screen for SIK2 phosphorylation substrates (268), no reports of SIK2 as a class IIa HDAC kinase *in vivo* have been published. Moreover, during the final stages of preparing this thesis, SIK3 was identified as an HDAC4 kinase capable of inducing its nuclear export in *Drosophila* and mammalian cells (325).

1.4.5.6 SIKs in lipid and glucose metabolism

In addition to the previously described role that SIK1/2 play in hepatic gluconeogenesis through CRTC phosphorylation, they both regulate lipogenesis in the liver as well, through phosphorylation of different substrates. SIK1 influences lipogenesis by phosphorylating and inactivating the transcription factor sterol regulatory element binding protein-1c (SREBP-1c) (353). Knockdown of SIK1 in the liver increases the expression of lipogenic genes, while

overexpression of SIK1 inhibits expression of these genes, and this latter effect is abolished upon co-infection of a SREBP-1c mutant that cannot be phosphorylated by SIK1 (353).

Conversely, the effect of SIK2 on hepatic lipogenesis is mediated through the acetyltransferase p300 and the transcription factor carbohydrate-responsive element binding protein (ChREBP). The catalytic activity of p300 is inhibited by SIK2-mediated S89 phosphorylation, leading to decreased K672 acetylation of ChREBP (32). ChREBP hypoacetylation impairs its recruitment to lipogenic gene promoters and subsequent transcriptional activity. Similar to SIK1, knockdown of SIK2 (or overexpression of p300) in the liver increases lipogenic gene expression resulting in hepatic steatosis, insulin resistance, and inflammation, while these defects are rescued by SIK2/p300 co-overexpression (32). Further, hepatic steatosis and insulin resistance in obese and diabetic mice is associated with reduced hepatic SIK2 activity, increased p300 activity, and increased ChREBP K672 acetylation (32). Thus, SIK1 and SIK2 appear to be crucial regulators of glucose and fatty acid metabolism through their effects in the liver. Moreover, as described above, LKB1 has also been implicated in hepatic gluconeogenesis (276) and steatosis (200).

SIK2 also represses lipogenesis in adipocytes, although the details are not as well established as they are for the effect in liver. SIK2 is upregulated at the mRNA and protein level during adipogenesis and also in the adipose tissue of diabetic mice (78, 120). SIK2 overexpression in adipocytes reduces lipogenic gene expression and triglyceride content, while SIK2 knockdown increases lipogenic gene expression (78). Although a downstream target of SIK2-mediated

phosphorylation was not identified in this study, it was shown that SIK2 overexpression reduces the nuclear accumulation of SREBP-1. Interestingly, the authors also showed that SIK2 could be activated in adipocytes by nutrient deprivation, inhibition of ATP synthesis, and the AMP mimetic AICAR, all of which are well-characterized activators of AMPK (78). Moreover, insulin receptor substrate-1 (IRS-1), an integral component of the insulin-responsive signaling pathway in adipocytes was identified as a SIK2 phosphorylation substrate, suggesting that SIK2 may be important for regulating insulin sensitivity (120).

In addition, two recent articles have uncovered a role for the LKB1-SIK-class IIa HDAC signaling axis in the control of lipid and glucose metabolism mediated by FOXO transcription factors. Loss of *dSIK3* in *Drosophila* causes an increase in lipolysis, leading to depletion of triglyceride stores and hypersensitivity to starvation (325). This is due to hypophosphorylation of HDAC4, permitting it to remain in the nucleus where it deacetylates and activates FOXO transcription factors, leading to an increase in lipolytic gene expression (325). It was also shown that this HDAC4-FOXO pathway controls lipid and glucose metabolism in mammals (210, 325), and that in hepatocytes from *Lkb1*$^{-/-}$ mice, class IIa HDACs are hypophosphorylated and localized to the nucleus, where they activate FOXO-dependent transcription of gluconeogenic genes (210). Moreover, simultaneous shRNA-mediated knockdown of HDAC4, 5, and 7 rescues the hyperglycemia observed in diabetic mice (210). Unlike the *Drosophila* study, the specific AMPK-related kinase(s) controlling FOXO activity through HDAC phosphorylation in the mammalian liver was not identified.

However, based on the results discussed above for CRTC phosphorylation in the liver, it should be safe to postulate that several of these kinases, including SIKs, are crucial for this process.

1.4.5.7 SIKs in tumourigenesis

The earliest indication that SIKs might be involved in tumourigenesis was a report showing that SIK2 is upregulated in chicken cells transformed by one of two different oncogenes, Qin or Src. This led to the original name for SIK2, Qin-induced kinase (QIK) (337). More recently, two separate kinome-wide loss-of-function screens have implicated both SIK1 and SIK2 in tumourigenesis. SIK1 was shown to be required for p53-dependent anoikis, a type of apoptosis particular to cells that become detached from other cells (52). Loss of SIK1 promotes anchorage-independent cell growth in culture and metastatic spread and survival of cells *in vivo*. Moreover, several effects of LKB1 in this setting (promotion of p53-dependent anoikis, matrigel invasion, suppressing anchorage-independent growth) were shown to depend on SIK1. Interestingly, immune complexes isolated from detached cells containing wild-type SIK1 but not kinase-dead SIK1 (K56M) promote p53 S15 phosphorylation *in vitro* (52), and this PTM is associated with cell stress conditions (149).

Meanwhile, SIK2 was identified as a centrosome-associated kinase required for G2/M transition (1). A fraction of cellular SIK2 is constitutively associated with the centrosome, however it is held in an inactive state by PKA-mediated phosphorylation. Following centrosome doubling, PKA-mediated inhibition is relieved, and SIK2 phosphorylates C-Nap1 on S2392, which permits

separation of centrosomes at the appropriate time during mitosis. Conversely, SIK2 knockdown prevents centrosome separation and mitosis, and sensitizes ovarian cancers to the anti-tubulin drug paclitaxel (1). Loss of SIK2 also inhibits proliferation of ovarian cancer cells by arresting some cells at the G1/S stage, and this is associated with diminished AKT phosphorylation and enhanced LKB1 expression (1). In addition, genomic amplification in a diffuse large B-cell lymphoma cell line causes SIK2 overexpression (223).

More recently, SIK3 has been identified as an ovarian tumour-associated antigen (48). Interestingly, overexpression of SIK3 in ovarian cancer cells leads to increased cell proliferation and permits these cells to grow following injection into mice (48). Thus, SIKs represent attractive targets for cancer therapy and may be useful prognosis indicators. Indeed, reduced SIK1 expression is correlated with the development of metastases in breast cancer patients (52), high SIK2 expression is associated with poor survival in high-grade serous ovarian cancer patients (1), and increased expression of SIK3 is correlated with pathology in ovarian cancer patients (48).

1.4.5.8 SIKs in cell cycle control

In addition to the role of SIK2 in cell cycle regulation mentioned above, both SIK1 and SIK3 might be involved in this process. Overexpression of SIK1 in Chinese hampster ovary cells prevents cell division and leads to aneuploidy (287). *In vivo*, SIK1-mediated cell cycle control is important for its role in cardiomyogenesis. SIK1 is expressed in cardiac precursor cells during mouse embryogenesis (255), and embryoid bodies derived from *Sik1*$^{-/-}$ embryonic stem

(ES) cells give rise to fewer cardiomyocytes than embryoid bodies containing wild-type ES cells (252). It was further shown that loss of SIK1 prevented the accumulation of the cyclin-dependent kinase inhibitor $p57^{Kip2}$, which is required for withdrawal from the cell cycle. Overexpression of $p57^{Kip2}$ in this setting rescues the defect of SIK1 loss and cardiomyogenesis proceeds normally (252). In addition, a *Drosophila* genome-wide screen for kinases involved in mitotic progression identified dSIK3 as a cell cycle regulator. In fact, loss of dSIK3 resulted in altered spindle morphology, similar to the phenotype observed when LKB1 expression is lost in *Drosophila* (22). Meanwhile, overexpression of SIK3 in ovarian cancer cells promotes G1/S cell cycle progression, and this is associated with decreased expression of the cyclin-dependent kinase inhibitors p21 and p27 (48).

1.4.5.9 SIK1 in sodium sensing

Recent data also suggest that SIK1 acts as a novel component of the cellular sodium-sensing module, as follows (279). A rise in intracellular [Na^+] is translated into an increase in intracellular [Ca^{2+}] due to the reversible Na^+/Ca^{2+} exchanger. This rise in Ca^{2+} activates CaMKs, at least one of which phosphorylates SIK1 on T322 leading to increased SIK1 kinase activity toward phosphatase methylesterase-1 (PME-1). This causes the dissociation of PME-1 from the Na^+, K^+-ATPase (NK) complex, which permits the constitutively associated protein phosphatase 2A (PP2A) to become active and dephosphorylate the NK-α subunit. This in turn stimulates the catalytic activity of NK resulting in active pumping of Na^+ ions out of the cell (279). Moreover, hyperactivation of

SIK1 has been implicated in the defective Na^+ transport and hypertension caused by mutation of the α-adducin protein, and SIK1 inhibition may represent an effective treatment for this condition (286).

1.4.6 Overlapping functions of AMPK-related kinases

One striking aspect of AMPK-related kinase biology is their extensive overlapping cellular functions. For example, AMPK, NUAK1/2, and SAD-A/B are all involved in development (68, 115, 164, 310); AMPK, SAD-A/B, and MARKs are important regulators of cell polarity (17, 128, 177, 212, 277); AMPK, MARK4, NUAK1/2, and SIK1/2 have roles in tumourigenesis (1, 19, 52, 172, 296, 310); AMPK, MARK2, SIK1/2, and NUAK2 are critical for metabolism and energy homeostasis (32, 129, 285, 310, 353); and AMPK, SAD-B, MARK3, and SIK1-3 are involved in cell cycle control (1, 13, 22, 34, 68, 241, 252).

Similar substrate specificity may be the major reason for the extensive overlap in functions of AMPK-related kinases (91, 273) (Fig. 5). For instance, both Tau and Cdc25C are phosphorylated by SADs and MARKs (34, 128, 164); IRS-1 is phosphorylated by both SIK2 and AMPK (120, 136); CRTC2 is phosphorylated by SIKs, AMPK, and MARK2 (139, 156, 166, 268); and class IIa HDAC members are phosphorylated by MARK2/3, SIK1, and AMPK (20, 45, 72, 205). Interestingly, phosphorylation of several AMPK-related kinase substrates, including Cdc25C (241), CRTC (268), Raptor (102), TBC1D1 (50), MYPT1 (355), KSR1 (220), and class IIa HDACs (72), induces phosphorylation-dependent binding of 14-3-3 proteins, which in general, results in inactivation/sequestration of these proteins.

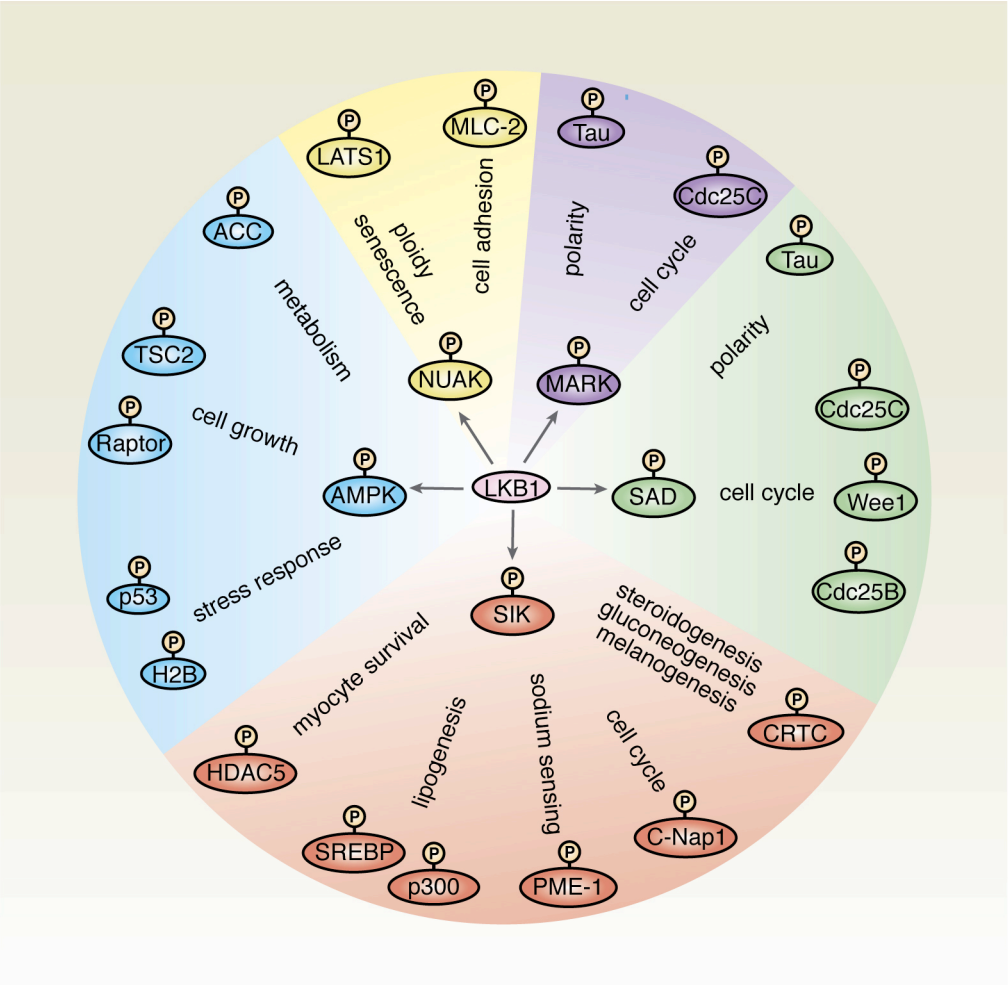

Figure 5. Cellular and physiological roles of AMPK-related kinases. Members of the five subfamilies of the AMPK-related kinase family (AMPK, SIK, MARK, NUAK, and SAD) phosphorylate numerous proteins involved in several cellular processes. All these AMPK-related kinases depend on LKB1-mediated phosphorylation of their T-loops for their activation and ability to phosphorylate these substrates.

This functional similarity has been postulated as the reason that no mutations in these kinases have been discovered in human tumours (273). Since another AMPK-related kinase could potentially compensate for one with a loss-of-function mutation, no survival advantage would be conferred on such a cell, and thus, these types of mutations would be unlikely to accumulate among a

population of cells. In contrast, LKB1 mutations are somewhat common since no other protein can compensate for LKB1 (273).

1.5 Protein kinase A (PKA)

The cAMP-dependent protein kinase, or PKA, was identified nearly 50 years ago and since that time it has served as a model for the study of Ser/Thr protein kinases. PKA is evolutionarily conserved among all eukaryotes except plants (33, 40, 67, 123) and is involved in a diverse array of physiological processes including neuronal plasticity, cardiac contractility, steroid biosynthesis, metabolism, and the immune system (302). In the basal state, PKA exists as an inactive tetramer containing two catalytic (C) subunits and two regulatory (R) subunits (89). Upon binding of two cAMP molecules to each R subunit, the cAMP-bound R subunits dissociate from the C subunits. This permits each C subunit to phosphorylate substrate proteins (89). In mammals, multiple genes encode distinct isoforms of each subunit. There are three catalytic isoforms (Cα, Cβ, and Cγ) and four regulatory isoforms (RIα, RIβ, RIIα, and RIIβ) each encoded by separate genes (281). Although there are differences in sequence and expression pattern among these isoforms and their splice variants, total C and R subunits are maintained at an equimolar ratio in cells (116).

1.5.1 Regulation of cAMP-PKA signaling

In addition to the PKA-R subunits that ensure PKA is only activated in a cAMP-dependent manner, several other proteins contribute to the tight spatio-

temporal regulation of PKA. First of all, adenylyl cyclase enzymes catalyze the production of cAMP in response to plasma membrane receptor binding by numerous hormones and neurotransmitters (293, 302). There are 10 different adenylyl cyclase isoforms in humans and they exhibit subtle differences in regulation and subcellular localization (293). PKA inhibitory peptide (PKI) is an endogenous inhibitor of PKA activity that acts by directly binding to free PKA-C subunits (66). Upon cAMP-mediated dissociation of PKA-C from PKA-R subunits, some C subunits enter the nucleus where they phosphorylate transcriptional regulators including CREB. PKI also enters the nucleus where it binds the C subunit and induces its nuclear export, thus limiting the time that PKA-C subunits are free to phosphorylate substrates (66). PKA activity and subcellular localization are also under the control of A-kinase anchoring proteins (AKAPs), which act as scaffolds to bring cAMP-PKA signaling components into close proximity (140, 282). AKAPs reside in distinct subcellular regions and they recruit PKA holoenzymes to specific locations by directly binding the R subunits (140). AKAPs also recruit phosphodiesterases (PDEs), the enzymes that break down cAMP. Moreover, PKA-mediated phosphorylation can stimulate PDE activity (271) and inhibit adenylyl cyclase activity (51, 135). Thus, negative feedback mechanisms and AKAP-mediated compartmentalization result in localized pulses of cAMP (140). An example of compartmentalized cAMP-PKA signaling was referred to above (see SIK1/2 in tumourigenesis). AKAP450 tethers PKA to the centrosome, and PKA activity in this discrete subcellular region is required to inhibit precocious SIK2-mediated phosphorylation of C-Nap1 during mitosis (1).

Exogenous factors have also been identified that modulate PKA function and these have been extensively used to dissect physiological roles for PKA in cell culture and animal models. Forskolin is a diterpene chemical derived from the plant *Coleus forskohlii* that potently activates membrane-bound adenylyl cyclase enzymes (293). Thus, treatment of cells with forskolin activates PKA by stimulating the production of endogenous cAMP. Of course cAMP, whether stimulated by endogenous ligands or by ectopically administered forskolin, is susceptible to PDE-mediated degradation. Another reagent used to stimulate PKA in cell culture is 8-Br-cAMP, a modified version of native cAMP that is more resistant to degradation by PDEs and thus permits prolonged PKA activation (332). Inhibition of PKA is popularly achieved by using H-89, an isoquinoline sulfonamide compound that potently inhibits the catalytic activity of PKA by competing with ATP for active site binding (190).

1.5.2 PKA phosphorylation targets

PKA phosphorylates hundreds of proteins, including metabolic enzymes, signaling proteins, ion channel proteins, and transcription factors (93, 272). The consensus sequence for PKA phosphorylation sites is RRXS/T, where X can be any amino acid and the S/T residue is the phospho-acceptor site (272). Mutational analysis of peptides and examination of native substrate sequences have revealed optimal surrounding sequences. For example, a Pro residue is strongly disfavoured at the P+1 position (the residue C-terminally adjacent to the phospho-acceptor site) (283, 365). It has been suggested that the presence of a Pro residue in this position has been selected against during evolution to maintain

reciprocal specificity between basophilic kinases (e.g. PKA) and Pro-directed kinases (e.g. MAPKs and cyclin-dependent kinases) (365).

By virtue of its ability to phosphorylate such a wide spectrum of proteins, PKA influences a multitude of cellular processes (272), including proliferation (288), differentiation (79), cell cycle (1), ion conductance (249), metabolism (81), microtubule dynamics (222), apoptosis (219), and autophagy (203). In some cases PKA phosphorylates multiple substrates involved in the same physiological process. For example, β-adrenergic-stimulated PKA controls excitation-contraction coupling in the heart through phosphorylation of several proteins including sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2, phospholamban, L-type Ca^{2+} channel, ryanodine receptor, troponin I, and myosin binding protein C (302). Thus, it is not surprising that a single nucleotide polymorphism in D-AKAP2 that causes dysregulation of PKA signaling leads to cardiac dysfunction (150).

1.5.3 Mouse models of PKA dysregulation

1.5.3.1 PKA catalytic subunit knockout mice

Unlike humans, mice only have two catalytic subunit genes, Cα and Cβ, which encode two and three splice variants, respectively. The Cα1 isoform is expressed in all tissues except male germ cells, where the Cα2 isoform is expressed (75). The Cβ1 isoform is ubiquitously expressed while Cβ2 and Cβ3 are restricted to the brain (101, 311, 312). The majority of mice lacking the Cα catalytic subunit ($C\alpha^{-/-}$) die perinatally and those that survive to adulthood are smaller than WT littermates (280). In contrast, although they exhibit impaired

hippocampal plasticity and strain-specific memory defects (122, 246), $C\beta^{-/-}$ mice are protected against high fat diet-induced obesity and insulin resistance (Enns 2009) and also against hypertension-induced cardiac hypertrophy (80). Complete loss of Cα and Cβ1 results in early embryonic lethality (125). Restoration of one Cα allele ($C\alpha^{+/-}C\beta^{-/-}$) permits survival past birth but these mice have severe spina bifida, and restoration of one Cβ1 allele ($C\alpha^{-/-}C\beta1^{+/-}$) results in later embryonic lethality due to neural tube defects (125).

1.5.3.2 PKA regulatory subunit knockout mice

Much like impaired PKA catalytic activity, constitutive PKA activity can also lead to dysfunction. Genetic deletion of the ubiquitously expressed RIα subunit causes embryonic lethality due to impaired mesoderm formation caused by increased basal PKA activity (7). Interestingly, mutations in the RIα gene have been identified in patients with Carney complex, an autosomal dominant neoplasia syndrome characterized by myxomas, spotty skin pigmentation, endocrine tumours, and schwannomas (44, 121, 240, 314). Global or tissue-specific $RI\alpha^{+/-}$ mice largely recapitulate the symptoms of this disease (161, 256, 318, 350, 351), and increased PKA catalytic activity has been reported in the affected tissues (148, 238, 256, 297). In addition, heart-specific $RI\alpha^{-/-}$ mice die during embryogenesis due to defective cardiac development (350). In contrast, mice lacking the RIβ subunit, which is expressed only in brain and adipose tissue (281), have normal PKA activity and do not exhibit obvious phenotypic defects except for impaired hippocampal function (31).

Similar to RIβ$^{-/-}$ mice, loss of the ubiquitously expressed RIIα subunit results in no major phenotypic changes (38), except for impaired visual cortex plasticity (248). In contrast, ablation of RIIβ, which is expressed in the central nervous system and neuroendocrine tissues (281), protects against diabetes and obesity (64, 65, 228, 267), and it has been reported that loss of RIIβ extends lifespan in male mice and enhances many aspects of health in old age (81). Interestingly, lifespan extension is also seen in budding yeast following loss of the *S. cerevisiae* PKA catalytic subunit (185). Although this seems contradictory since loss of C subunits decreases PKA activity while deletion of R subunits should increase basal PKA activity, as is the case for RIα loss, RIIβ$^{-/-}$ mice display a compensatory upregulation of RIα expression and downregulation of Cα subunit expression (6).

1.5.3.3 PKA activation and inactivation in male fertility

A delicate temporal balance between PKA activation and inactivation must be maintained for proper tissue function, and this is exemplified by the effect of PKA disruption on male fertility (162). Male mice that do not express the testis-specific Cα2 catalytic isoform are infertile (230). Sperm develop normally in these mice, but upon maturity they exhibit defective motility, suggesting that PKA activity is dispensable for spermatogenesis but required for the function of mature sperm (230). In contrast, RIα$^{+/-}$ mice and Carney complex patients with heterozygous RIα gene mutations have defective spermatogenesis leading to infertility, and this is caused by elevated PKA catalytic activity during

the early stages of spermatogenesis (39). Thus, these findings suggest that excessive PKA activity is harmful during spermatogenesis and that insufficient PKA activity is deleterious for the function of mature sperm.

1.5.4 PKA targets multiple points in signaling cascades

PKA often acts on multiple proteins involved in the same signaling pathway. For example, PKA activates CREB-dependent transcription through phosphorylation of CREB at S133, which serves as a docking site for CBP (8, 56), and it also phosphorylates SIKs to permit CRTC to enter the nucleus and bind CREB (25, 268). This multi-target action of PKA also applies to its role in repressing transcription. PKA inhibits myogenesis, in part through repression of MEF2-dependent myogenic gene transcription (79). This is likely due to multiple events, including direct phosphorylation of MEF2D on S121 and S190 (79) and promotion of class IIa HDAC nuclear localization (20, 49, 79). The latter effect has been postulated to occur based on the ability of PKA to inhibit SIK-mediated class IIa HDAC nuclear export (20, 95, 168).

1.6 Rationale for thesis project

The class IIa HDAC-MEF2 signaling axis plays an important role in regulating muscle differentiation, cardiac hypertrophy, bone and vascular development, and T-cell apoptosis. Since these HDACs repress MEF2 in a signal-dependent manner, the kinases that mediate their phosphorylation-dependent 14-3-3 binding and nuclear export are also important mediators of these physiological and pathological processes. Our lab was instrumental in establishing that class IIa HDACs shuttle between the nucleus and cytoplasm in a phosphorylation-dependent manner, and also in defining the phosphorylation sites and domains that are critical for this shuttling. It was subsequently shown that CaMKs and PKDs were the major kinases that induce nuclear export of these HDACs. After the addition of the AMPK-related kinases MARK2/3 to the list of class IIa HDAC kinases, it was tempting to believe that others would be found.

I hypothesized that other members of the AMPK-related kinase family could also phosphorylate class IIa HDACs, and thus began experiments to address this. In Chapter II, I show that SIK2 and SIK3 are potent class IIa HDAC kinases. Interestingly, I uncovered several unique attributes of SIK3 that will open new avenues of inquiry into the regulation of class IIa HDACs and MEF2 transcription factors. Moreover, since these and other AMPK-related kinases have been shown to depend on LKB1 for their activity, I hypothesized that LKB1 is an important regulator of class IIa HDAC shuttling. In Chapter II, I also demonstrate that LKB1 is not only needed for the effects of SIK2 and SIK3 but that endogenous LKB1 is necessary for phosphorylation and cytoplasmic localization of HDAC4. It was also known that PKA could inhibit the activity of SIKs toward

CRTC proteins so I hypothesized that this would also be the case for class IIa HDACs. I show that PKA does indeed prevent SIK2/3-mediated nuclear export (Chapter II).

During the course of this study I noticed that PKA could not prevent the cytoplasmic localization of HDAC7, and that PKA could still promote nuclear localization of HDAC4 or HDAC5 when a PKA-resistant SIK2 mutant (SIK2 S587A) was coexpressed. Therefore, I hypothesized that PKA-induced nuclear localization of HDAC4, 5, and 9 was independent of SIK2/3 inhibition. This led me to identify a novel phosphorylation site in these three HDACs that is regulated by cAMP/PKA signaling and is necessary for PKA-mediated nuclear import (Chapter III). Not only do these findings present several novel regulatory details and conceptual advances relating to class IIa HDACs but they also suggest new research directions for the mechanisms of LKB1 and PKA function, which are important for many physiological processes.

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CHAPTER II

2.0

The tumour suppressor kinase LKB1 activates SIK2 and SIK3 to stimulate nuclear export of class IIa histone deacetylases

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2.1 PREFACE

Phosphorylation-dependent nucleocytoplasmic shuttling is the major regulatory mechanism for modulating the repressive activity of class IIa HDACs toward genes involved in differentiation, plasticity, and death of cells. Understanding the signaling pathways that control class IIa HDAC subcellular localization is critical for our understanding of many diseases and identifying potential therapeutic targets to treat these diseases. Although several class IIa HDAC kinases had been identified, it was likely that others awaited discovery. I set out to test whether other members of the AMPK-related kinase family (in addition to MARK2/3) could phosphorylate and induce the nuclear export of class IIa HDACs. Since I observed dramatic effects of SIK2 and SIK3 on these events, they became the focus of this project. Also, due to the fact that AMPK-related kinases rely on LKB1-mediated phosphorylation for their activity, I hypothesized that LKB1 is an important regulator of class IIa HDACs. In this manuscript I confirm this hypothesis and also show that PKA negatively affects the class IIa HDAC kinase activity of SIK2 and SIK3.

2.2 ABSTRACT

HDAC4, 5, 7, and 9 comprise the class IIa subgroup of the histone deacetylase (HDAC) superfamily. These deacetylases repress the activity of transcription factors such as myocyte enhancer factor 2 (MEF2) proteins in response to extracellular cues. Phosphorylation of evolutionarily conserved serine residues induces 14-3-3 binding and nuclear export, leading to derepression of target genes. Here we identify salt-inducible kinase 2 (SIK2) and SIK3, both downstream targets of the tumor suppressor LKB1, as class IIa HDAC kinases. SIK2 and SIK3 stimulate 14-3-3 binding and cytoplasmic localization of these HDACs by directly phosphorylating these conserved motifs. As a result, SIK2 activates MEF2-dependent transcription and relieves HDAC4-mediated repression of myogenesis. In contrast, SIK3-mediated class IIa HDAC phosphorylation and nuclear export does not derepress MEF2 activity, and SIK3 is surprisingly able to induce nuclear export of constitutively nuclear HDAC4 and HDAC7 mutants. We also show that SIK2/3 are unable to induce HDAC5 export in LKB1-negative HeLa cells unless LKB1 is coexpressed, and that PKA prevents SIK2/3-induced export of HDAC5. Finally, using *Lkb1*$^{-/-}$ MEFs and cancer cell lines expressing or lacking LKB1 we show that endogenous HDAC4 localization and phosphorylation are regulated by LKB1. Thus, by regulating the nucleocytoplasmic shuttling of class IIa HDACs, the LKB1-SIK2/3 axis may represent a novel signaling module controlling MEF2-dependent cellular programs.

2.3 INTRODUCTION

Through the deacetylation of histones and other proteins, histone deacetylases (HDACs) are involved in a multitude of cellular processes. HDACs are grouped into different classes based on sequence phylogeny and homology to yeast counterparts (34, 73). Class IIa HDACs (HDAC4, 5, 7, and 9), which are homologous to yeast Hda1, repress different transcription factors, most notably members of the myocyte enhancer factor 2 (MEF2) family (25, 43, 73). These HDACs regulate several MEF2-dependent physiological and pathological processes, including the development of the vascular system, skeletal muscle, bone, and T-cells, as well as cardiac hypertrophy and neurodegeneration (25, 41). In addition, class IIa HDACs are dysregulated in a variety of cancers (47, 53, 62), with murine *Hdac7* identified as a novel candidate oncogene (56), and *HDAC4* haploinsufficiency in humans is linked to brachydactyly mental retardation syndrome (70).

Class IIa HDACs possess a unique N-terminal extension that harbors a MEF2 binding site as well as three or four conserved serine residues that serve as 14-3-3 binding sites upon their phosphorylation (43, 54, 73). Phosphorylation-dependent 14-3-3 binding promotes cytoplasmic localization through a combination of nuclear export sequence activation and nuclear localization signal inhibition (9, 22, 30, 45, 46, 51, 66, 67). This leads to derepression of MEF2 transcriptional activity, while hypophosphorylation and nuclear localization of class IIa HDACs inhibits MEF2-dependent transcription. A number of kinases have been identified that phosphorylate these conserved serine residues, including Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) (4, 30, 39, 45, 76) and

protein kinase D (PKD) isoforms (15, 65). Stimuli that activate these kinases, e.g. increased intracellular [Ca^{2+}] (71) and vascular endothelial growth factor (VEGF) treatment (24, 68), induce class IIa HDAC phosphorylation and nuclear export, leading to derepression of MEF2-dependent transcription.

Recently, additional kinases have been reported to act as class IIa HDAC kinases, including AMP-activated protein kinase (AMPK) (44), microtubule affinity-regulating kinases (MARK2 and 3) (10, 14), and salt-inducible kinase 1 (SIK1) (5, 63). All of these kinases are activated by LKB1 (40), but whether this upstream kinase is itself involved in regulating class IIa HDAC trafficking has not been addressed. Mutations in the gene encoding LKB1 cause Peutz-Jeghers syndrome (31), and LKB1 is increasingly recognized as a major tumour suppressor (59). In mammals, LKB1 phosphorylates AMPKα1 and α2 as well as 11 members of the AMPK-related kinase family (40). LKB1 relies on AMPK for many of its tumour suppressor effects as well as its effects on energy metabolism and protein translation (59). However, some LKB1 effects, including haematopoietic stem cell maintenance, are independent of AMPK (19, 23, 49), suggesting the importance of AMPK-related kinases as downstream effectors of LKB1 signaling. We set out to investigate the role of LKB1 and AMPK-related kinases, ultimately focusing on the SIK subfamily, in the regulation of class IIa HDAC trafficking.

The SIK subfamily is conserved in *C. elegans* and *Drosophila*, and there are three mammalian SIKs (31, 52). SIK1 was independently cloned from the adrenal glands of rats fed a high salt diet and also from neuronal cells following depolarization (17, 69). Two other members of the SIK family, SIK2 and SIK3

were found by genome database searches based on similarity to SIK1 (32). Mammalian SIKs are all expressed in multiple tissues, with SIK1 enriched in the adrenal cortex, SIK2 in adipose tissue, and SIK3 ubiquitously expressed (32). SIK1 is important for myocyte and neuron survival (5, 38), cardiac myogenesis (57), steroidogenesis (52), sodium sensing (61), p53-dependent anoikis (11), and hepatic lipogenesis and gluconeogenesis (35, 74). Meanwhile, SIK2 is also involved in hepatic lipogenesis and gluconeogenesis (6, 13), as well as melanogenesis (26), adipocyte metabolism (27, 48), and mitotic spindle formation and tumourigenesis (1). SIK1/2 mediate several of these effects by phosphorylating members of the CREB-regulated transcription coactivator (CRTC) family, which causes 14-3-3 binding and cytoplasmic sequestration of CRTC, leading to impaired cAMP-response element binding protein (CREB) transcriptional activity. This is reversed by PKA-mediated phosphorylation of these kinases (e.g. on S587 of SIK2) (58). Although such a regulatory mechanism for SIK3 has yet to be exhaustively investigated, SIK3 has been shown to induce cytoplasmic sequestration of CRTC2 (33). SIKs share an N-terminal kinase domain, but SIK3 possesses a unique C-terminal extension (52). However, compared to SIK1 and SIK2, little is known about SIK3.

Here we show that SIK2 and SIK3 phosphorylate class IIa HDACs leading to their cytoplasmic localization. Under the same experimental conditions, SIK1 is unable to do so. In contrast to SIK2, SIK3 exhibits several unique properties, such as an inability to activate MEF2, and the ability to induce class IIa HDAC nuclear export independent of its kinase activity and to promote cytoplasmic accumulation of putative constitutively nuclear HDAC4 and HDAC7 mutants. In

addition, using a variety of experimental techniques we show that SIK2/3 depend on LKB1 for their ability to act as class IIa HDAC kinases, and that endogenous LKB1 is required for proper phosphorylation and subcellular localization of HDAC4. Moreover, we show that PKA inhibits nuclear export of these deacetylases by counteracting LKB1 and SIK2/3. These results thus add SIK2 and SIK3 to the growing list of class IIa HDAC kinases and further present LKB1 as an important regulator of these deacetylases.

2.4 MATERIALS AND METHODS

Cell culture. HEK293, HeLa, H1299, and C2C12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), while A549 cells were maintained in F12/DMEM (1:1 ratio). All media were supplemented with 10% fetal bovine serum (FBS, Sigma) and 1% penicillin/streptomycin (Invitrogen), except for C2C12 cells during differentiation, which entailed switching the cells to DMEM containing 5% horse serum (Sigma). HEK293FT cells were maintained in DMEM containing 10% heat-inactivated FBS, plus 400 µg/ml G418, non-essential amino acids, pyruvate, and glutamate (FT medium). *Lkb1*$^{-/-}$ MEF cells stably expressing empty Flag vector or Flag-LKB1 as well as IRES-driven GFP were a kind gift from Russell Jones (McGill University) and were described previously (8). These cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

Plasmid constructs and antibodies. GFP- and Flag-tagged wild-type (WT), triple mutant (TM; S246A/467A/632A), V1066A, and L1062A HDAC4 constructs, as well a plasmid encoding HA-14-3-3ß have been previously described (66, 67). Both V1066A and L1062A were prepared on the HDAC4 mutant 1-1069, which lacks the C-terminal acidic stretch (67). An expression plasmid for mouse HDAC5 was generously provided by S. Khochbin (INSERM) (37) and used to create GFP- and Flag-tagged HDAC5 using derivatives of pEGFP-C2 (BD Biosciences) and pCDNA3.1 (Invitrogen), respectively. GFP-HDAC7 was derived from a pEGFP-C2 derivative and an expression plasmid for mouse HDAC7 (GenBank accession number AF207749). GFP-HDAC7-1-591 was the unintentional byproduct of a subcloning experiment where a 0.84 kb

HindIII/ApaI fragment from the coding sequence was inserted after the HindIII site encoding K590 and L591, so this mutant contains residues 1-591 of murine HDAC7 and unrelated residues until an in-frame stop codon. GFP-HDAC9 was constructed from a pEGFP-C2 derivative by linking an oligoduplex consisting of DAC103 (5'-AA TTC ATG CAC AGT AT-3') and DAC104 (5'-GA TCA TAC TGT GCA TG-3') to the coding sequence of human HDAC9. cDNAs for SIK1, SIK2, SIK3, LKB1, CRTC2, and PKA-Cα1 (hereafter referred to as PKA) were purchased from Open Biosystems. SIK1, SIK2, and SIK3 were subcloned into pcDNA3.1 derivatives with and without an HA tag, PKA was subcloned into an HA tag-containing pcDNA3.1 derivative, while LKB1 was subcloned into an untagged pcDNA3.1 derivative. SIK1 and a kinase-dead mutant were also kindly provided by Hiroshi Takemori (Osaka University) (27). mCh-CRTC2 was constructed using a pEGFP-C2 derivative in which the GFP coding sequence was replaced by an mCherry (mCh) coding sequence. SIK2 and SIK3 mutants were generated by PCR-mediated site-directed mutagenesis using the Pfu polymerase system (Fermentas). All mutants were verified by automatic sequencing. The 3xMEF2-luc construct has been previously described (21). MCK-luc was a kind gift from Josephine Nalbantoglu (Montreal Neurological Institute) (36). MCK-GFP was generously provided by John McDermott (York University) (16). pLove-GFP lentivirus plasmid was purchased from Addgene, and lentivirus plasmids bearing shRNAs targeting LKB1 were purchased from Open Biosystems: shLKB1-2 (5′ –CCGG-GCCAACGTGAAGAAGGAAATT-CTCGAG-AATTTCCTTCTTCACGTTGGC-TTTTT-3′) and shLKB1-5 (5′-CCGG-CATCTACACTCAGGACTTCAC-CTCGAG-

GTGAAGTCCTGAGTGTAGATG-TTTTT-3′). The LKB1 coding sequence was subcloned into pENTR11 (Invitrogen) and recombined with the pLenti6/V5-DEST lentivirus vector (Invitrogen) following the manufacturer's instructions to yield pLenti6-LKB1 lentivirus construct.

Anti-phospho-S246 (HDAC4) polyclonal rabbit antibody was prepared by immunization of rabbits with a peptide representing residues 241-251 of HDAC4 (LRKTApSEPNLKC, where pS is phospho-Ser and the Cys was added for conjugation). Antibodies were affinity-purified from rabbit serum using SulfoLink gel (Pierce) linked to the phosphopeptide through its free Cys. The specificity of the antibody toward phospho-S246 versus non-phosphorylated peptide was confirmed by dot blotting (Fig. S4). The anti-HDAC4 polyclonal antibody was affinity-purified from rabbit antisera that were previously described (66). This purified antibody is highly selective for HDAC4 and does not cross-react with any other class IIa HDACs (Fig. S6).

Cell transfection. For western blot experiments (including IPs), 0.2 X 10^6 HEK293 or HeLa cells were plated in 6 cm plates and transfections were performed with 10 µl Superfect reagent (Qiagen) and 5 µg total DNA. For fluorescence microscopy and reporter gene assays, 0.04 X 10^6 HEK293, HeLa, or C2C12 cells were plated per well in 12 well plates. For HEK293 and HeLa cells, transfections were performed with 3 µl Superfect (Qiagen) and 1.5 µg total DNA. For C2C12 cells, transfections were performed with 3 µl Lipofectamine 2000 (Invitrogen) and 1.5 µg total DNA. For HeLa cells, Superfect was used for fluorescence microscopy but Lipofectamine 2000 was used for reporter gene

assays because we could not detect luciferase signals in HeLa cells using Superfect. Experiments were performed 24-48 h post-transfection.

Co-immunoprecipitation. To analyze 14-3-3 binding to HDAC4/5, expression plasmids for Flag-tagged HDACs were transfected into HEK293 cells along with constructs expressing HA-SIK2 or HA-SIK3, and in some experiments an HA-14-3-3ß construct was also transfected. Transfections were performed in 6 cm plates using 10 µl Superfect reagent and 5 µg of total DNA. About 48 h post-transfection, cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in 0.5 mL buffer K (20 mM sodium phosphate pH 7.0, 150 mM KCl, 30 mM sodium pyrophosphate, 0.1% NP-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na_3VO_4, 25 mM ß-glycerophosphate, and protease inhibitors). For affinity purification of Flag-tagged proteins, 200 µl of extract was added to 10 µl of M2 agarose beads (Sigma) and rotated at 4°C for 2 hours. Following 4 washes with buffer K, bound proteins were eluted with 2 µl Flag peptide (Sigma) in 25 µl buffer K.

Immunoblotting. After addition of 3 X SDS sample buffer, whole cell extracts and IP extracts were boiled for 5 min and then separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked in PBS-Tween 20 (PBS-T) with 20% horse serum for 1 hour at room temperature and then incubated overnight at 4°C with anti-HA (Covance), anti-Flag (Sigma), anti-14-3-3ß (H-8; Santa Cruz), anti-phospho-S246 (HDAC4), anti-LKB1 (Ley 37D/G6; Santa Cruz), and anti-α-tubulin (Sigma) antibodies. For detection of endogenous HDAC4, phospho-HDAC4 (S246), LKB1, and α-tubulin, 5% milk in PBS-T was used as the blocking solution. Membranes were then washed in PBS-

T (6 X 8 min), and then incubated in the appropriate secondary antibody conjugated to HRP for 1 hour at room temperature. Following another set of 6 X 8 min washes in PBS-T, membranes were incubated in PBS (2 X 5 min), and then visualized on film after 5 min incubation in Supersignal enhanced chemiluminescent solution (Pierce).

***In vitro* kinase assay.** HEK293T cells were transfected with Flag-tagged HDAC5, SIK2 WT, SIK2 K49M, SIK3 WT, or SIK3 K37M. Flag-tagged proteins were purified as above, except both the final washing step and the elution were performed with kinase buffer (50 mM Tris pH 7.5, 0.1 mM EGTA, 1 mM DTT, 10 mM NaF, 0.1 mM Na_3VO_4 and protease inhibitors) instead of buffer K. *In vitro* kinase (IVK) assays were performed in a total volume of 40 µl comprising 20 µl IVK buffer (50 mM Tris pH 7.5, 5 mM MgOAc, 5 mM $MnCl_2$, 0.1 mM EGTA, 1 mM DTT, 10 mM NaF, 0.1 mM Na_3VO_4 and protease inhibitors), 150 ng Flag-HDAC5, 10 ng Flag-kinase (SIK2 WT, SIK2 K49M, SIK3 WT, or SIK3 K37M), and 100 pmol ATP. Reactions were carried out at 30°C for 1 h and were stopped upon addition of 20 µl ice-cold 3 X SDS sample buffer. Samples were then boiled for 5 min, separated by SDS-PAGE, and transferred to nitrocellulose membranes, which were blocked in PBS-Tween 20 (PBS-T) with 20% horse serum (HS) for 1 hour at room temperature. For the kinase input blot, 100 ng Flag-kinase was resolved by SDS-PAGE. Membranes were incubated with anti-Flag (Sigma; 1:1000) and anti-phospho-S246 (HDAC4; 1:20,000) primary antibodies overnight at 4°C.

Reporter gene assays. MEF2-dependent luciferase assays were performed using a 3xMEF2-luc construct and were previously described (21).

Myogenesis assay. Plasmids encoding MCK-GFP and mCherry vector were cotransfected into C2C12 cells alone or in combination with Flag-HDAC4 in the presence or absence of constitutively active SIK2 (HA-SIK2 S587A) or kinase-dead SIK2 (HA-SIK2 K49M). After 48h recovery in growth medium (GM), cells were incubated in DMEM containing 5% horse serum (differentiation medium; DM) for 48h. After 48h in DM, myotubes were visualized by green fluorescence microscopy. Transfection efficiency was monitored by visualizing mCherry protein expression via red fluorescence microscopy. To quantify the extent of myogenesis, transfections were also performed with MCK-luciferase (MCK-luc) plasmid instead of MCK-GFP, plus a ß-galactosidase expression plasmid to monitor transfection efficiency. Forty-eight hours after switching from GM to DM, cells were lysed in luciferase lysis buffer (0.2 M potassium phosphate [0.017 M KH_2PO_4 + 0.183 M K_2HPO_4], 1% Triton X-100, 2 mM DTT, 10% glycerol) and MCK-luc activity was assessed using a luminometer. Luciferase values were divided by ß-galactosidase values and a value of 1.0 was arbitrarily assigned to the reporter-only condition to facilitate normalization across three independent experiments.

Lentivirus production and infection. Lentivirus plasmids were transfected into HEK293FT cells using the reverse transfection method. Briefly, 10 µg of lentivirus plasmid, along with the helper vectors psPAX2 (6.5 µg) and pMD2.G (3.5 µg) were incubated with 45 µl Lipofectamine 2000 according to the manufacturer's instructions and then added to 10 cm plates. HEK293FT cells (8.0 x 10^6) were then seeded onto the DNA/Lipofectamine mixture. Throughout the transfection and virus production phases, HEK293FT cells were grown in FT

medium in the absence of antibiotics. Virus-containing medium was collected from the plates on the 2nd and 3rd day following transfection, filtered, pooled, and then frozen in aliquots at -80°C. For infection, H1299 or A549 cells were seeded in 6-well plates (1.0×10^6) in FT cell medium without antibiotics and containing 6 µg/µl polybrene. Cells were split and re-plated in 10 cm dishes 48h after infection, and after a further 48h, cells were harvested for immunoblotting.

Immunofluorscence microscopy. *Lkb1*$^{-/-}$ MEFs stably infected with either empty vector or Flag-LKB1 were seeded on glass coverslips. Once cells had reached ~60% confluency, coverslips were washed twice with PBS, fixed with 2% paraformaldehyde for 20 min, then washed three times with PBS. Cells were then permeabilized with 0.2% Triton X-100 for 10 min, followed by washes with 100 mM glycine (3 x 5 min each). After blocking with IF buffer (PBS with 0.2% Triton X-100 and 0.05% Tween-20) containing 2% BSA for 45 min, cells were incubated overnight at 4°C with anti-HDAC4 antibody (1:500). After primary antibody incubation, cells were rinsed with IF buffer (3 x 5 min), then incubated in AlexaFluor 568 goat anti-rabbit IgG (H +L) secondary antibody (Invitrogen, Molecular Probes) (1:1000) at room temperature for 45 min, followed by washes with IF buffer (3 x 5 min). Cells were then counterstained with DAPI for 5 min at room temperature, then washed with ddH_2O (2 x 5 min) and mounted on slides for microscopy.

Statistical analysis. Data are presented as means ± SEM. For experiments with more than two conditions, one-way ANOVAs were performed with a Bonferroni post-hoc test. For experiments with two conditions, unpaired

two-tailed Student's *t*-tests were performed. $p<0.05$ was considered statistically significant.

2.5 RESULTS

SIK2 and SIK3 induce nuclear export of class IIa HDACs. Since four reported class IIa HDAC kinases belong to the AMPK-related kinase family (5, 10, 14, 44), we set out to investigate whether other AMPK-related kinases play a role in class IIa HDAC regulation and also whether LKB1 is important for class IIa HDAC trafficking. To address these issues we tested the ability of AMPK-related kinases to induce cytoplasmic localization of class IIa HDACs. Specifically, we transfected HEK293 cells with expression plasmids for GFP-tagged HDAC4, 5, 7, or 9 together with empty vector or expression plasmids for several AMPK-related kinases. Western blotting was performed with an anti-GFP primary antibody to confirm that GFP-HDACs were correctly expressed (Supplemental Figures; Fig. S1A). Overexpression of AMPK, MARK1, MARK2, MARK3, NUAK1, NUAK2, SNRK, or NIM1 (all of which are related to AMPK and, except for NIM1, are activated by LKB1) (2, 29, 40) in HEK293 cells had no major effect on class IIa HDAC localization (data not shown). We also failed to detect any major effect of SIK1 expression on class IIa HDAC subcellular localization in HEK293 cells (Fig. 1A; *v*, *vi*, *vii*, *viii*) except for a relatively small change in the proportion of cells with GFP-HDAC9 showing a pancellular localization pattern versus a nuclear pattern (Fig. S1B). However, expression of SIK1 did cause a major increase in the cytoplasmic accumulation of CRTC2 (Fig. S2A), suggesting that the kinase was properly expressed. Thus, although some of these results are not consistent with previous reports (5, 10, 14, 44, 63), they demonstrate that these kinases do not alter class IIa HDAC localization in this particular experimental system.

In contrast, expression of either SIK2 or SIK3 caused a dramatic relocalization of GFP-HDAC5 (Fig. 1A; *x* vs *ii* and *xiv* vs *ii*) and GFP-HDAC9 (Fig. 1A; *xii* vs *iv* and *xvi* vs *iv*) from the nucleus to the cytoplasm (see Fig. S1B for quantification). In the case of HDAC4 (Fig. 1A; *i*) and HDAC7 (Fig. 1A; *iii*), which displayed a cytoplasmic and/or pancellular localization pattern in HEK293 cells the effect is necessarily minor compared to that seen for HDAC5 and HDAC9. Nevertheless, expression of SIK2 and SIK3 resulted in a more completely cytoplasmic localization pattern compared to empty vector control (Fig. 1A and Fig. S1B). Since their effects on class IIa HDAC localization were so dramatic, we chose to narrow our focus toward SIK2 and SIK3 for subsequent experiments.

To test whether the cytoplasmic accumulation of class IIa HDACs in response to overexpression of SIK2/3 was due to stimulation of nuclear export or inhibition of nuclear import, we treated cells with leptomycin B (LMB), an inhibitor of the nuclear export receptor CRM1 (18). Since LMB inhibits CRM1-dependent nuclear export of class IIa HDACs without having an effect on nuclear import (66), it represents a useful tool to separate these two opposing processes. We found that SIK2/3-mediated cytoplasmic accumulation of GFP-HDAC5 was completely abolished by treatment with LMB (Fig. S3), indicating that these kinases stimulate nuclear export of IIa HDACs.

To begin to address the mechanism through which SIK2 and SIK3 act on class IIa HDACs to stimulate their nuclear export, we created kinase-dead (KD) mutants of both kinases by substituting a lysine residue in their kinase domains with a methionine residue (K49M for SIK2 and K37M for SIK3). These

mutations have been shown to render SIK2 and SIK3 catalytically inactive (27, 33). SIK2 K49M was unable to induce nuclear export of HDAC5 or HDAC9, indicating that SIK2 kinase activity is necessary for this effect (Fig. 1B). Surprisingly, SIK3 K37M retained the ability to cause nuclear export of these HDACs, suggesting that the kinase activity of SIK3 is dispensible for its effect on class IIa HDAC nuclear export (Fig. 1B).

SIK2/3 increase class IIa HDAC phosphorylation and 14-3-3 binding. Our observation that SIK2 kinase activity is crucial for its ability to induce nuclear export of HDAC5 and HDAC9 prompted us to examine whether SIK2 (and SIK3) could phosphorylate class IIa HDACs. In addition, nuclear export is controlled by phosphorylation of conserved serine residues and binding to 14-3-3 proteins, which use these phospho-serine residues as docking sites (22, 30, 45, 66). Thus, we performed immunoprecipitation experiments to see whether overexpression of these kinases increased class IIa HDAC phosphorylation and 14-3-3 binding. For this we coexpressed HA-tagged SIK2 or SIK3 along with Flag-HDAC4 or Flag-HDAC5 and performed western blotting with an antibody that recognizes phospho-S246 of HDAC4 and the corresponding phosphorylated residue (S259) of HDAC5 (Fig. S4). Phosphorylation of this serine increased when SIK2 or SIK3 was coexpressed along with HDAC4 or HDAC5 (Fig. 2A, lane 4, Fig. 2A, lane 2 and 3). This phosphorylation event corresponded to an increase in 14-3-3 binding, as seen when HA-14-3-3 was cotransfected (Fig. 2C, lane 3) or when endogenous 14-3-3 was measured (Fig. 2A, lane 4). SIK3 overexpression also caused an increase in 14-3-3 binding (data not shown). Moreover, the increase in both S246 phosphorylation and 14-3-3 binding was

abolished when SIK2 K49M was overexpressed instead of SIK2 WT (Fig. 2A, lane 5). To test whether SIK2 and SIK3 could directly phosphorylate class IIa HDACs, we performed *in vitro* kinase (IVK) assays with purified Flag-tagged WT and KD SIK2 and SIK3. When WT SIK2 or SIK3 was incubated with purified Flag-HDAC5 in the presence of ATP, we observed an increase in S259 phosphorylation of HDAC5 (Fig. 2D). In contrast, no *in vitro* phosphorylation was observed when KD mutants (SIK2 K49M and SIK3 K37M) were incubated with Flag-HDAC5 (Fig. 2D). Together, these results demonstrate that SIK2 and SIK3 are class IIa HDAC kinases capable of inducing phosphorylation *in vitro* (Fig. 2D) and *in vivo* (Fig. 2A-C).

SIK3 promotes nuclear export of class IIa HDACs through a unique mechanism. Previous studies have shown that HDAC4 triple mutant (TM; S246/467/632A), or equivalent mutant versions of HDAC5/7, are resistant to kinase-induced nuclear export (4, 30, 45, 65), likely due to a failure to bind 14-3-3 proteins (45). As a positive control, we found that CaMKIV, a well-characterized class IIa HDAC kinase, had no effect on HDAC4 TM localization (Fig. 3A). We next tested whether this was also the case for SIK2 and SIK3. Consistent with the lack of 14-3-3 binding to HDAC4 TM (Fig. 2A), SIK2 was unable to induce nuclear export of HDAC4 TM (Fig. 3A). Surprisingly, unlike SIK2 and CaMKIV, SIK3 was still able to induce nuclear export of HDAC4 TM (Fig. 3A). To further investigate this issue we decided to test the ability of these 3 kinases to cause nuclear export of several HDAC4 mutants. In addition to the serine residues (246, 467, and 632) that serve as 14-3-3 binding sites when phosphorylated, HDAC4 also possesses a nuclear export sequence (NES) at its C-

terminus (46, 67). An HDAC4 mutant lacking the NES (HDAC4 1-1040) and two other mutants, each with an inactivating mutation in the NES (HDAC4 V1066A and HDAC4 L1062A), were similar to the HDAC4 TM in that all mutants displayed a basal nuclear localization pattern (Fig. 3A). However, unlike the TM, HDAC4 1-1040, V1066A, and L1062A were all responsive to CaMKIV-mediated nuclear export (Fig. 3A). SIK3 also induced nuclear export of these mutants, however, SIK2 was unable to alter their subcellular localization (Fig. 3A). Another HDAC4 mutant, HDAC4 1-669, which lacks the deacetylase (DAC) domain as well as the NES, behaved even more strikingly. Only SIK3 was able to cause its nuclear export, with both SIK2 and CaMKIV lacking this ability (Fig. 3A).

While addressing this unique property of SIK3, we noticed a fourth potential 14-3-3 binding site at S1036 of HDAC4 (Fig. 3B), so we constructed a GFP-HDAC4 quadruple mutant (QM; S246/467/632/1036A) to see whether this potential phosphorylation site was involved in the ability of SIK3 to induce nuclear export of HDAC4 mutants that are insensitive to SIK2. However, SIK3 induced nuclear export of this mutant as well (Fig. 3C). Finally, we tested whether this property of SIK3 was unique to HDAC4 or whether other class IIa HDACs could be similarly regulated. An HDAC7 mutant, 1-591, displayed the same shuttling dynamics as the HDAC4 NES mutants, as both SIK3 and CaMKIV, but not SIK2, could induce nuclear export of this mutant (Fig. 3D). Taken together, these results indicate that SIK3 has properties distinct from SIK2 and from another class IIa HDAC kinase, CaMKIV.

Differential effects of SIK2 and SIK3 on MEF2 activity. Many of the biological effects of class IIa HDACs can be attributed to their inhibition of MEF2 transcription factors (25, 55). Thus, we used MEF2 activity as a read-out of class IIa HDAC activity under the influence of SIK2 and SIK3. For this we performed reporter gene assays with a construct that expresses luciferase under the control of three MEF2 binding sites (3xMEF2-luc). When HDAC4 (Fig. 4A), HDAC5 (Fig. 4B), HDAC7 (Fig. 4C), or HDAC9 (Fig. 4D) was coexpressed with MEF2D, there was a major suppression of MEF2 transcriptional activity. However, upon the addition of SIK2, this suppression was abrogated (Fig. 4A-D). This result was expected since SIK2 caused these HDACs to localize to the cytoplasm, where they are unable to repress MEF2 transcriptional activity. Moreover, both HDAC4 TM and HDAC7 TM were resistant to SIK2-mediated derepression of MEF2 activity (Fig. 4A, 4C), further supporting the role of 14-3-3 binding and nuclear export in SIK2-mediated regulation of class IIa HDACs.

However, in the case of SIK3, which also induced phosphorylation and nuclear export of IIa HDACs, there was no derepression of MEF2 activity (Fig. 4A-D). The lack of effect with SIK3 was not due to nuclear exclusion of MEF2, as we saw no change in MEF2D localization following overexpression of SIK2 or SIK3 (Fig. 4E). Nor was it due to MEF2-mediated trapping of class IIa HDACs in the nucleus. Overexpression of MEF2D did increase the nuclear localization of HDAC4, as reported previously for MEF2C (67), but both SIK2 and SIK3 overcame this change in basal localization to induce nuclear export of HDAC4 (Fig. 4F) and HDAC5 (Fig. S5). Thus, as expected for a class IIa HDAC kinase,

SIK2 stimulated MEF2 transcriptional activity in the presence of these HDACs, while SIK3 unexpectedly lacked this ability.

SIK2 rescues HDAC4-mediated repression of myogenesis. Due to the well-known role of MEF2 in skeletal muscle (50) we tested whether SIK2 could also cause class IIa HDAC export and MEF2 derepression in C2C12 cells, a mouse myoblast cell line (72). In contrast to its effect in HEK293 cells, WT SIK2 did not cause HDAC5 nuclear export or derepression of MEF2-dependent transcription in C2C12 cells (Fig. 5A-B). We postulated that this could be due to strong cAMP/PKA signaling in these cells (60), so we used SIK2 S587A, which is resistant to PKA-mediated phosphorylation (58). Indeed, SIK2 S587A was competent in inducing HDAC5 cytoplasmic localization in addition to derepressing MEF2 activity in C2C12 cells (Fig. 5A-B).

To see whether the class IIa HDAC kinase activity of SIK2 was relevant in a more physiological setting, we performed myogenesis assays in C2C12 cells, which readily form myotubes when switched from medium containing 10% FBS (growth medium; GM) to medium containing 5% horse serum (differentiation medium; DM). To visualize myogenesis, we transfected C2C12 cells with a GFP construct driven by the muscle creatine kinase (MCK) promoter (MCK-GFP). The MCK promoter contains a MEF2 binding site and is highly dependent on MEF2 for its activation in myogenic cells (3). This reporter gene provides a readout of MEF2 activity in the context of a full-length native promoter, and it also allows the visualization of myotubes since GFP is expressed pancellularly. Consistent with its dependence on MEF2 activity, myotube formation was strongly repressed by cotransfection of HDAC4, whereas in the absence of

HDAC4, MCK-GFP was highly expressed after 48 h in DM (Fig. 5D). This repression was rescued by co-expression of constitutively active SIK2 (S587A), but not by KD SIK2 (K49M) (Fig. 5D). To quantify this result, we repeated the same assay using a different reporter gene construct. In this case, we used the MCK-luc plasmid, in which luciferase expression is driven by the MCK promoter. Consistent with the results for the MCK-GFP experiment, the MCK-luc quantification showed that SIK2 S587A but not SIK2 K49M overexpression rescued HDAC4-mediated suppression of MCK promoter activity during C2C12 cell differentiation (Fig. 5C). Thus, SIK2 can rescue HDAC4-mediated repression of myogenesis and this effect is associated with SIK2-induced phosphorylation-dependent nuclear export.

LKB1 is necessary for SIK2/3-mediated class IIa HDAC nuclear export. SIK2 and SIK3 are members of the AMPK-related kinase family, whose members are all targets of the upstream kinase LKB1 (40). LKB1 phosphorylates AMPK-related kinases on a threonine residue in the T-loop, an event that is crucial for their kinase activity (40). Thus, we performed experiments to test whether LKB1 is necessary for SIK2/3 to act as class IIa HDAC kinases. To address this, we employed HeLa cells, which do not express LKB1 (64). Using transient transfection in a manner similar to that applied to HEK293 cells (Fig. 1), we found that neither SIK2 nor SIK3 could cause nuclear export of GFP-HDAC5 in HeLa cells (Fig. 6A). This result was in contrast to what we showed in HEK293 cells, ie. robust cytoplasmic accumulation of HDAC5 when cotransfected with SIK2 or SIK3 (Fig. 1A, Fig. S1B). Because LKB1 expression status is not the only genetic difference between HeLa cells and HEK293 cells,

we performed add-back experiments to demonstrate the specificity of this effect. While SIK2/3 did not cause nuclear export of HDAC5 in HeLa cells, this ability was restored upon addition of LKB1 (Fig. 6A). Similar to our observations with HEK293 cells, we found that SIK1 was unable to induce HDAC5 nuclear export even when LKB1 was cotransfected (Fig. 6A), whereas cotransfection of SIK1 and LKB1 did cause cytoplasmic localization of CRTC2 (Fig. S2B). Consistent with these subcellular localization experiments, we also found that cotransfection of LKB1 along with SIK2 was necessary for SIK2 to derepress MEF2 activity in HeLa cells (Fig. 6B). In addition, coexpression of LKB1 was needed for SIK2 to induce S246 phosphorylation of endogenous HDAC4 in these cells (Fig. 6C). To a lesser extent the same result was seen for phosphorylation of the corresponding residue (S155) of endogenous HDAC7 (Fig. 6C). These results point to the importance of the upstream kinase LKB1 for the class IIa HDAC kinase activity of SIK2 and SIK3.

PKA reverses SIK2/3-dependent nuclear export of HDAC5. Although previous reports with CRTC2 (33, 58) and our experiments in C2C12 cells suggest that SIK-induced HDAC5 cytoplasmic accumulation would be sensitive to PKA signaling, we next sought to test this hypothesis directly. Like its effect on subcellular localization of CRTC2 (Fig. S2B), overexpression of PKA prevented cytoplasmic localization of HDAC5 induced by SIK2/LKB1 and SIK3/LKB1 (Fig. 6A). We also observed this effect in HEK293 cells without cotransfecting LKB1 (data not shown). This result demonstrates that PKA exerts a dominant effect on SIK2/3. Moreover, it suggests that SIK2/3 activity is

influenced by multiple upstream signals, which ultimately affect the downstream targets of SIK2/3, such as class IIa HDACs.

LKB1 promotes phosphorylation and cytoplasmic localization of endogenous class IIa HDACs. In view of the importance of LKB1 in SIK2/3-induced nuclear export of class IIa HDACs, we next tested whether endogenous LKB1 expression was required for proper localization of class IIa HDACs. We employed two approaches to address this issue: lentivirus-mediated knockdown or add-back of LKB1 and genetic deletion. First we examined whether LKB1 expression regulates HDAC4 S246 phosphorylation status. For this, we used two human lung cancer cell lines, one of which lacks expression of LKB1 (A549 cells) and one of which expresses LKB1 (H1299 cells). We expressed LKB1 in A549 cells and observed a higher level of HDAC4 S246 phosphorylation (and S155 phosphorylation of HDAC7) compared to overexpressing GFP as a control (Fig. 7A). Western blotting confirmed that LKB1 expression was only detectable in cells infected with an LKB1-expressing lentivirus (Fig. 7A). We observed the opposite effect when LKB1 was knocked down in cells that normally express it. Specifically, we knocked down LKB1 in H1299 cells using lentiviruses bearing shRNAs targeting two different sequences of human LKB1 (Fig. 7B). LKB1 expression was virtually undetectable after infection with either shLKB1-2 or shLKB1-5, and this was associated with a decrease in HDAC4 S246 phosphorylation (and to a lesser extent, HDAC7 S155 phosphorylation) compared to a scrambled shRNA that does not reduce LKB1 protein levels (Fig. 7B).

We also performed studies using *Lkb1*$^{-/-}$ MEFs to complement the lentivirus experiments. HDAC4 S246 phosphorylation (and to a lesser extent HDAC7 S155 phosphorylation) was lower in *Lkb1*$^{-/-}$ MEFs expressing empty vector compared to *Lkb1*$^{-/-}$ MEFs with re-introduced LKB1 (Fig. 7C). This was consistent with the dependence of HDAC4 S246 phosphorylation on LKB1 expression in the lentivirus experiments (Fig. 7A-B). Finally, we examined the localization of endogenous HDAC4 using immunofluorescence microscopy in *Lkb1*$^{-/-}$ MEFs. The anti-HDAC4 primary antibody we used is highly specific to HDAC4 and does not react with any other class IIa HDAC (Fig. S6). HDAC4 localization in *Lkb1*$^{-/-}$ MEFs was predominantly pancellular whereas it was more cytoplasmic in *Lkb1*$^{-/-}$ MEFs with restored LKB1 expression (Fig. 7D). We quantified this effect by observing the number of cells displaying nuclear exclusion (a marked absence of staining in the nucleus) of HDAC4. A graph summarizing the data from three independent experiments shows an ~2-fold increase in HDAC4 nuclear exclusion in *Lkb1*$^{-/-}$ cells re-expressing LKB1 versus cells lacking LKB1 expression (Fig. 7E; 55% vs 27%, p=.0017). Taken together, these results demonstrate that LKB1 plays an important role in endogenous HDAC4 phosphorylation and subcellular localization.

2.6 DISCUSSION

Identification of SIK2/3 as class IIa HDAC kinases. Phosphorylation-dependent 14-3-3 binding and nuclear export of class IIa HDACs has emerged as a novel signaling module that controls numerous physiological processes (43, 54, 73). Here we identify SIK2 and SIK3 as class IIa HDAC kinases, based on their ability to phosphorylate HDAC4 and HDAC5 *in vivo* and *in vitro* (Fig. 2). Moreover, SIK2 and SIK3 induce nuclear export of class IIa HDACs (Fig. 1), increase 14-3-3 binding to these HDACs (Fig. 2 and data not shown), and SIK2 stimulates MEF2 transcriptional activity (Fig. 4), all hallmarks of class IIa HDAC kinases. We also observed these effects of SIK2 in a more physiological setting, as SIK2 S587A reversed HDAC4-mediated inhibition of myogenesis, a cellular process in which MEF2 plays a critical role (55).

Differential effects of SIK2 and SIK3 on class IIa HDACs. We have uncovered several differences possessed by SIK2 and SIK3 relating to their effects on class IIa HDACs. First, as would be expected for a class IIa HDAC kinase, KD SIK2 (K49M) is unable to induce nuclear export of HDAC5/9, but surprisingly, SIK3 K37M retains this ability (Fig. 1B). Second, an HDAC4 triple mutant (TM; S246/467/632A) lacking all three phosphorylatable serines that serve as 14-3-3 docking sites is refractory to SIK2- and CAMKIV-induced cytoplasmic accumulation, yet it unexpectedly relocates to the cytoplasm when SIK3 is overexpressed (Fig. 3A). This result points to a novel mechanism regulating class IIa HDAC subcellular localization. Moreover, the above results suggest that the effect of SIK3 on class IIa HDAC subcellular localization is independent of phosphorylation. Further understanding of the underlying molecular mechanism

may open exciting new avenues of research into the regulation of class IIa HDACs.

A third difference between SIK2 and SIK3 is their differential effect on MEF2-dependent transcription. Our results demonstrate that while SIK2 strongly activates MEF2, SIK3 has no effect on MEF2 transcriptional activity (Fig. 4). Although we did not uncover the cause of this effect, we did rule out two possibilities. First, SIK3 did not alter the subcellular localization of MEF2 (ie. MEF2 remained in the nucleus) (Fig. 4E). Second, overexpression of MEF2 is known to sequester HDAC4 in the nucleus (67), but SIK3 was still able to cause nuclear export of HDAC4 and HDAC5 in the presence of overexpressed MEF2 (Fig. 4F and Fig. S5). Therefore, it is clear that SIK2 and SIK3 display unique characteristics that are presumably related to differences in their domain structure. While SIK2 and SIK3 are similar in their N-terminal catalytic domains, SIK3 has a long C-terminal extension not present in SIK2 (32). Work is ongoing in our lab to address whether this C-terminal extension is responsible for the distinct properties of SIK3.

Role of LKB1 in class IIa HDAC nucleocytoplasmic shuttling. Since SIK2 and SIK3 rely on LKB1 for their kinase activity (40) and their ability to cause nuclear export of CRTC2 (33), we tested whether LKB1 was necessary for SIK2/3 to induce HDAC5 nuclear export. Using HeLa cells, which do not express LKB1, we found that SIK2/3 were unable to alter HDAC5 localization unless LKB1 was cotransfected (Fig. 6A). These experiments with HeLa cells suggested that LKB1 could be an important endogenous regulator of class IIa HDACs. Indeed, we showed that endogenous S246 phosphorylation of HDAC4,

and phosphorylation of the equivalent site of HDAC7 (S155) was increased after expressing LKB1 in LKB1-negative A549 cells (Fig. 7A), while S246 (and S155) phosphorylation was decreased after knocking down LKB1 in LKB1-positive H1299 cells. Moreover, HDAC4 S246 (and HDAC7 S155) phosphorylation was higher in *Lkb1*$^{-/-}$ MEFs with re-introduced LKB1 compared to *Lkb1*$^{-/-}$ MEFs expressing empty vector (Fig. 7C). Consistent with this, the pancellular distribution of endogenous HDAC4 seen in *Lkb1*$^{-/-}$ MEFs was markedly more cytoplasmic upon re-expression of LKB1 (Fig. 7D-E). Thus, LKB1 plays an important role in phosphorylation-dependent nuclear export of class IIa HDACs.

The importance of LKB1 in class IIa HDAC localization is also suggested by the high percentage of LKB1-dependent kinases that have been reported as class IIa HDAC kinases, including AMPK (44), MARK2/3 (10, 14), and SIK1 (5, 63). Although we did not observe any major effect of these kinases on class IIa HDAC localization in our experimental system, these kinases may function in other cellular contexts. This is suggested by the context-dependent regulation of SIK2 activity in different cells lines, such as HeLa and C2C12, used in our studies. It will be interesting to investigate which AMPK-related kinases are responsible for basal localization or changes in class IIa HDAC localization induced by different stimuli. In addition, as LKB1 is known to play roles in many physiological processes, it will be important to test whether class IIa HDAC shuttling is important in these LKB1-dependent processes. Related to this, we have shown that class IIa HDAC phosphorylation is influenced by LKB1 expression status in two lung cancer cell lines (Fig. 7A-B), and LKB1 has a previously characterized role in lung cancer pathogenesis (42).

PKA as a regulator of class IIa HDACs. Consistent with its role as a negative regulator of SIKs, we found PKA prevented nuclear export of HDAC5 induced by expression of LKB1/SIK2/3 in HeLa cells (Fig. 6A). This result is consistent with reports showing that PKA inhibits SIK1-mediated phosphorylation and nuclear export of HDAC4 (20) and HDAC5 (5). However, there are other ways in which PKA might ultimately lead to inhibition of MEF2 activity (Fig. 8A). Indeed, PKA phosphorylates LKB1 (12) and MEF2D (16), and it also controls a novel phosphorylation site in class IIa HDACs to regulate their subcellular trafficking (see Chapter III).

Conclusion. The results of this study suggest that the SIK2/3-class IIa HDAC signaling module represents a novel branch downstream from LKB1 (Fig. 8B). This signaling axis lies parallel to the SIK-CRTC2 and SIK-p300 pathways that control hepatic gluconeogenesis and lipogenesis, respectively (Fig. 8B) (6, 13, 33). Of note, several LKB1-dependent kinases, including SIK2 (1), are involved in tumourigenesis, and mouse *Hdac7* has been recently identified as a novel candidate oncogene (56), while the expression of each class IIa HDAC has been shown to be dysregulated in certain cancers (47, 53, 62). Thus, it will be interesting to test whether alterations in class IIa HDAC phosphorylation and localization are involved in cancers with dysregulated LKB1-dependent signaling.

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2.9 FIGURE LEGENDS AND FIGURES

Figure 1. Effect of SIKs on class IIa HDAC subcellular localization. (A) HEK293 cells were transiently transfected with expression plasmids for GFP-HDAC4, 5, 7, and 9 in combination with pCDNA empty vector, or an expression plasmid encoding SIK1, SIK2, or SIK3, as indicated. The next day, fluorophore-tagged proteins were visualized in live cells using green fluorescence microscopy. (B) HEK293 cells were transiently transfected with expression plasmids for GFP-HDAC5 or GFP-HDAC9 together with either SIK2 K49M or SIK3 K37M (both kinase-dead mutants), as indicated. The next day, GFP-tagged proteins were visualized by green fluorescence microscopy as in (A).

Figure 1

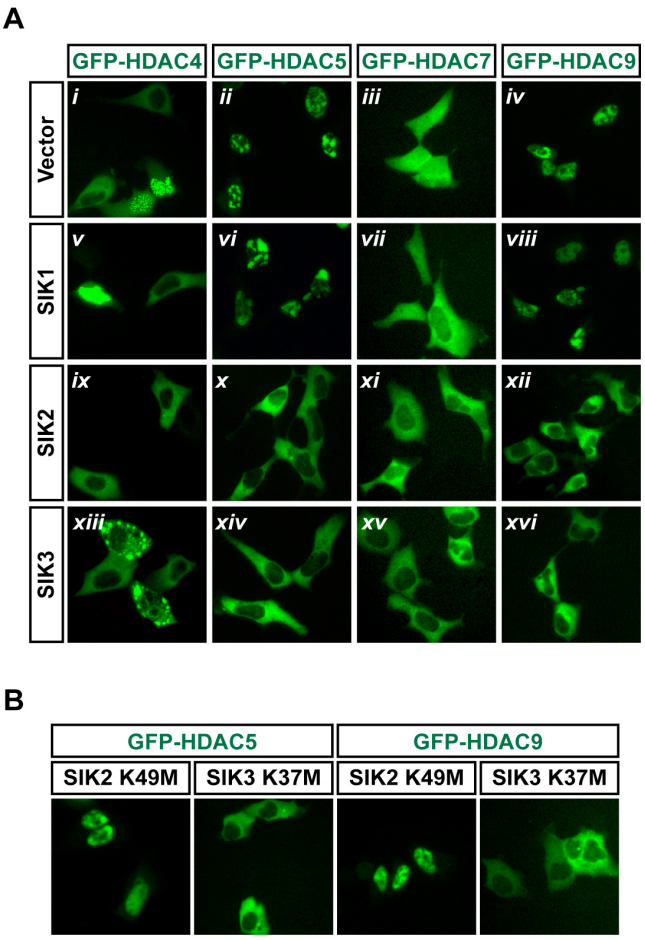

A
GFP-HDAC4
GFP-HDAC5
GFP-HDAC7
GFP-HDAC9
Vector
SIK1
SIK2
SIK3
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii
xiii
xiv
xv
xvi
B
GFP-HDAC5
GFP-HDAC9
SIK2 K49M
SIK3 K37M
SIK2 K49M
SIK3 K37M

Figure 2. SIK2/3 phosphorylate HDAC4/5 leading to increased 14-3-3 binding. (A) HEK293 cells were transiently transfected with expression plasmids encoding Flag-tagged wild-type (WT) HDAC4 (lanes 3-5) or triple mutant (TM; S246/467/632A) HDAC4 (lanes 6-7). In some cases, an expression plasmid for HA-tagged SIK2 WT (lanes 2, 4, and 7) or HA-SIK2 K49M (lane 5) was cotransfected. Forty-eight hours later, cells were harvested in buffer K and Flag-tagged proteins were immunoprecipitated with M2 agarose beads and eluted with Flag peptide. Whole cell extracts (Input) and immunoprecipitates (IP) were separated by SDS-PAGE and immunoblotting was performed with antibodies directed against the indicated proteins. (B) As in (A), except that Flag-HDAC5 was transfected alone (lane 1) or together with either HA-SIK2 (lane 2) or HA-SIK3 (lane 3). (C) As in (A), except that an expression plasmid encoding HA-14-3-3ß was transfected alone (lane 1) or together with Flag-HDAC5 (lane 2) or Flag-HDAC5 and HA-SIK2 (lane 3). (D) *In vitro kinase* (IVK) assays were performed with Flag-tagged proteins purified from HEK293T cells. Reactions were performed with the indicated combinations of purified proteins (150 ng Flag-HDAC5 and 10 ng Flag-kinase) and 100 pmol ATP in IVK buffer for 1 h at 30°C. Reactions were stopped by the addition of SDS sample buffer and then boiled for 5 min before gel electrophoresis. Western blots were performed using antibodies directed against the indicated proteins. For the input blot, 100 ng of purified Flag-SIK2 or Flag-SIK3 WT or KD was subject to SDS-PAGE and western blotting using anti-Flag antibody.

Figure 2

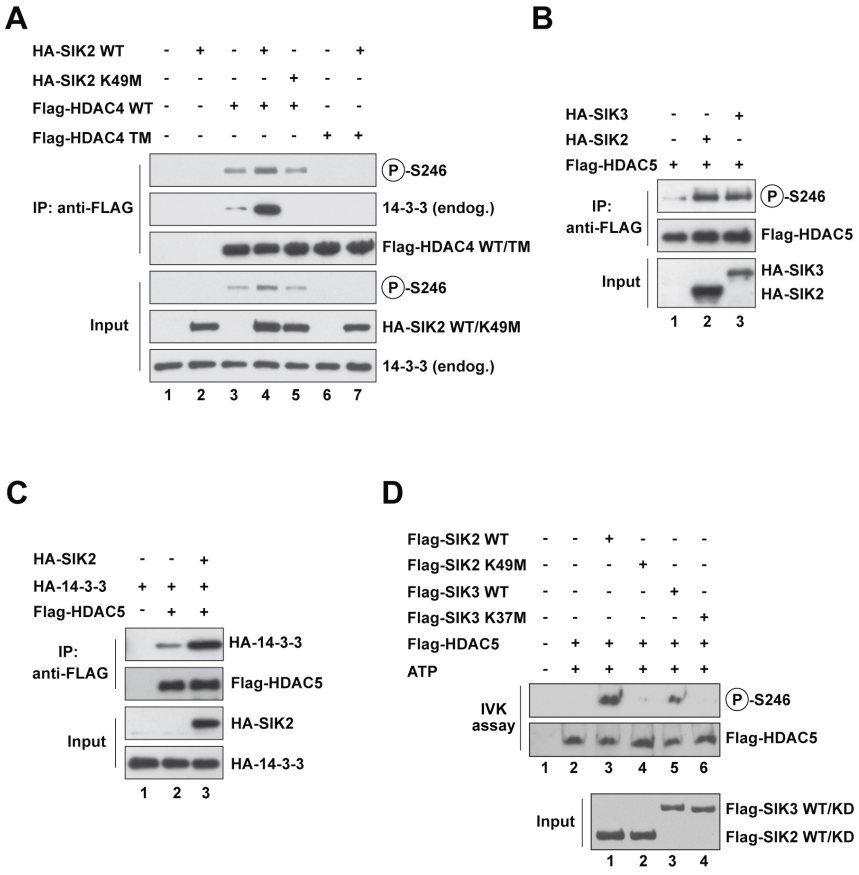

A
HA-SIK2 WT - + - + - - +
HA-SIK2 K49M - - - - + - -
Flag-HDAC4 WT - - + + + - -
Flag-HDAC4 TM - - - - - + +
IP: anti-FLAG
Input
P-S246
14-3-3 (endog.)
Flag-HDAC4 WT/TM
P-S246
HA-SIK2 WT/K49M
14-3-3 (endog.)
1 2 3 4 5 6 7
B
HA-SIK3 - - +
HA-SIK2 - + -
Flag-HDAC5 + + +
IP: anti-FLAG
Input
P-S246
Flag-HDAC5
HA-SIK3
HA-SIK2
1 2 3
C
HA-SIK2 - - +
HA-14-3-3 + + +
Flag-HDAC5 - + +
IP: anti-FLAG
Input
HA-14-3-3
Flag-HDAC5
HA-SIK2
HA-14-3-3
1 2 3
D
Flag-SIK2 WT - - + - - -
Flag-SIK2 K49M - - - + - -
Flag-SIK3 WT - - - - + -
Flag-SIK3 K37M - - - - - +
Flag-HDAC5 - + + + + +
ATP - + + + + +
IVK assay
Input
P-S246
Flag-HDAC5
1 2 3 4 5 6
Flag-SIK3 WT/KD
Flag-SIK2 WT/KD
1 2 3 4

Figure 3. Unique properties of SIK3-mediated nuclear export of HDAC4 and HDAC7. (A) HEK293 cells were cotransfected with expression plasmids for the indicated GFP-HDAC4 mutants and either pCDNA vector, SIK2, SIK3, or CaMKIV. Proteins were visualized using green fluorescence microscopy (*right*). Schematic diagram for each HDAC4 mutant compared to WT HDAC4 (*left*). (B) (*top*) Schematic diagram of HDAC4 protein with phosphorylatable serines (246, 467, 632) and hypothetical phosphorylatable serine (1036) highlighted. NLS, nuclear localization signal; NES, nuclear export sequence; DAC, deacetylase domain. *(bottom)* Sequence alignment of three known phosphorylation sites (S246, S467, S632) and fourth hypothetical phosphorylation site (S1036), highlighted in red box, plus surrounding sequence. Identical amino acids are highlighted in grey boxes. (C) HEK293 cells were transfected with GFP-HDAC4 S246/467/632/1036A (quadruple mutant; QM) together with either pCDNA vector or a SIK3 expression plasmid. Fluorescence microscopy was performed as in (A). (D) as in (A) except that a GFP-HDAC7-1-591 mutant was cotransfected with the indicated plasmids.

Figure 3

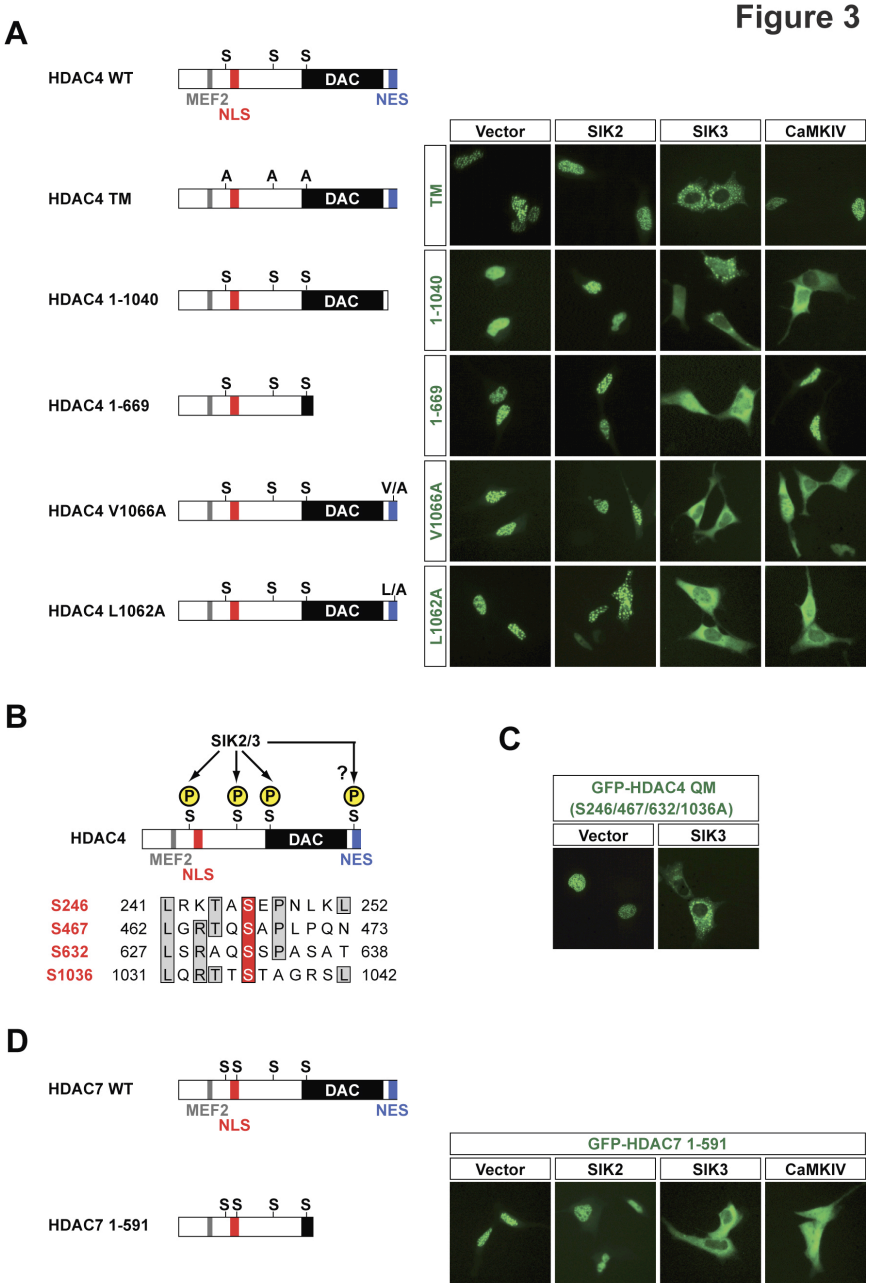

A
HDAC4 WT
S S S
DAC
MEF2
NLS
NES
HDAC4 TM
A A A
HDAC4 1-1040
HDAC4 1-669
HDAC4 V1066A
V/A
HDAC4 L1062A
L/A
Vector
SIK2
SIK3
CaMKIV
TM
1-1040
1-669
V1066A
L1062A
B
SIK2/3
?
P
HDAC4
S246 241 L R K T A S E P N L K L 252
S467 462 L G R T Q S A P L P Q N 473
S632 627 L S R A Q S S P A S A T 638
S1036 1031 L Q R T T S T A G R S L 1042
C
GFP-HDAC4 QM
(S246/467/632/1036A)
Vector
SIK3
D
HDAC7 WT
S S S S
HDAC7 1-591
GFP-HDAC7 1-591
Vector
SIK2
SIK3
CaMKIV

Figure 4. SIK2 but not SIK3 derepresses MEF2 transcriptional activity. (A-D) HEK293 cells were transfected with a luciferase construct driven by three MEF2 binding sites (3xMEF2-luc) along with HA-SIK2 or HA-SIK3 plasmids where indicated by 2 or 3, respectively. Where indicated, a MEF2D expression plasmid was cotransfected along with expression plasmids for wild-type (WT) or triple-mutant (TM) HDAC4 (A), WT HDAC5 (B) WT HDAC7 or TM HDAC7 (C), or WT HDAC9 (D). Luciferase activity was measured with a luminometer and divided by ß-galactosidase activity to control for transfection efficiency. The first column in all panels (reporter genes with no other plasmid cotransfected) was arbitrarily assigned a value of 1.0. Data are presented as means ± SEM (error bars) from three independent experiments. Western blot showing equal expression of HA-SIK2 and HA-SIK3 (A, *inset*). (E) HEK293 cells were cotransfected with GFP-MEF2D and either pCDNA vector, or expression plasmids encoding SIK2 or SIK3. Subcellular localization of GFP-MEF2D was monitored by green fluorescence microscopy. (F) As in (E), except that GFP-HDAC5 was cotransfected with MEF2D and either pCDNA vector, or expression plasmids encoding SIK2 or SIK3.

Figure 4

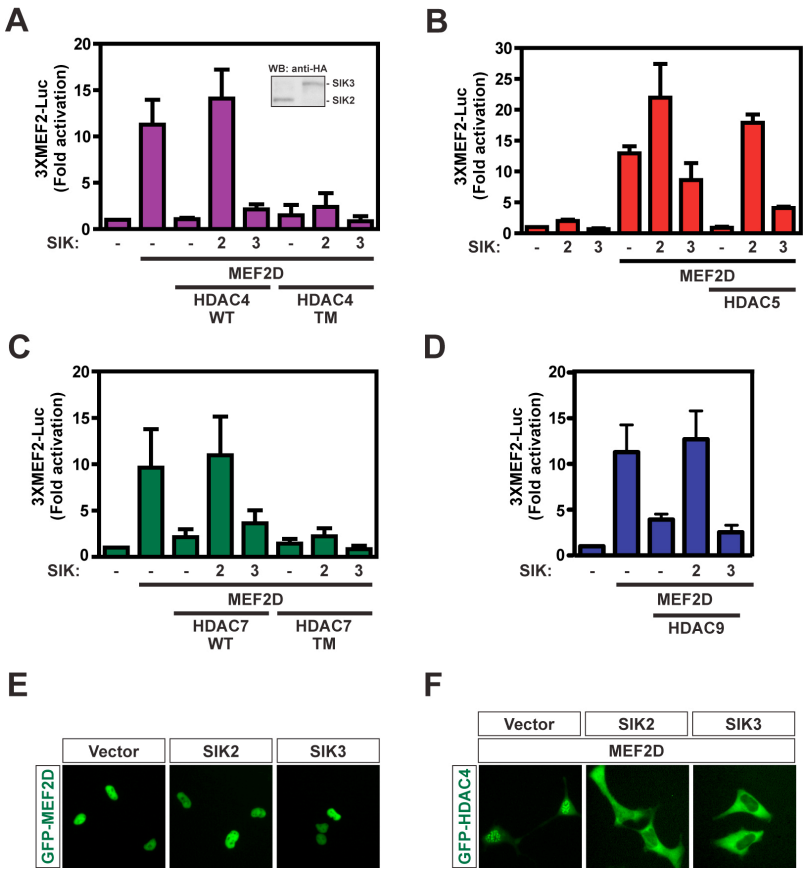

A
B
C
D
E
F
3XMEF2-Luc (Fold activation)
WB: anti-HA
SIK3
SIK2
SIK:
MEF2D
HDAC4 WT
HDAC4 TM
HDAC5
HDAC7 WT
HDAC7 TM
HDAC9
Vector
SIK2
SIK3
GFP-MEF2D
GFP-HDAC4

Figure 5. SIK2 reverses HDAC4-mediated inhibition of myogenesis. (A) C2C12 cells were transiently transfected with a GFP-HDAC5 expression plasmid together with either pCDNA vector or expression plasmids encoding SIK2 WT or SIK2 S587A. Green fluorescence microscopy was performed to monitor subcellular localization of GFP-HDAC5. (B) C2C12 cells were cotransfected with a 3xMEF2-luc plasmid together with the indicated plasmids as well as a β-galactosidase expression vector. Luciferase assays were performed as in Fig. 4. (C) C2C12 cells were cotransfected with a plasmid bearing the luciferase gene driven by the muscle creatine kinase promoter (MCK-luc) along with the indicated plasmids and a β-galactosidase expression vector. Two days posttransfection, cells were shifted from growth medium (GM; 10% FBS) to differentiation medium (DM; 5% HS), and luciferase assays were performed after 2 d in DM. *** indicates $p<0.001$ versus first bar. (D) As in (C), except that the MCK promoter construct contained the GFP gene instead of the luciferase gene and an mCherry expression vector was cotransfected instead of a β-galactosidase expression vector to control for transfection efficiency. Green fluorescence microscopy was performed to monitor MCK promoter activity and myotube formation following 2 d in DM.

Figure 5

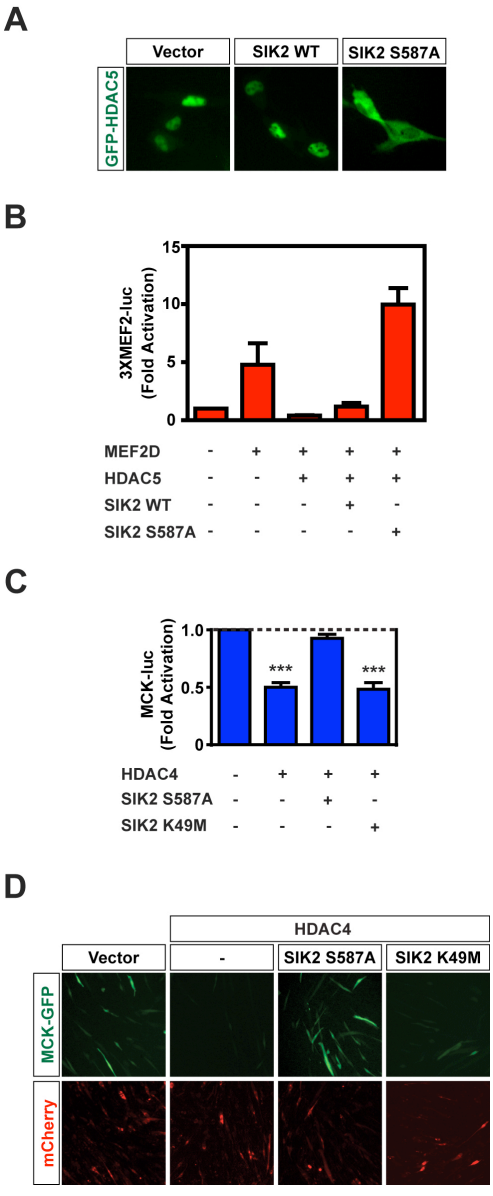

A
Vector
SIK2 WT
SIK2 S587A
GFP-HDAC5
B
3XMEF2-luc
(Fold Activation)
15
10
5
0
MEF2D - + + + +
HDAC5 - - + + +
SIK2 WT - - - + -
SIK2 S587A - - - - +
C
MCK-luc
(Fold Activation)
1.0
0.5
0
***
***
HDAC4 - + + +
SIK2 S587A - - + -
SIK2 K49M - - - +
D
HDAC4
Vector
-
SIK2 S587A
SIK2 K49M
MCK-GFP
mCherry

Figure 6. LKB1 is required for SIK2/3-mediated class IIa HDAC nuclear export. (A) HeLa cells were transiently transfected with an expression plasmid encoding GFP-HDAC5 together with the indicated expression plasmids. Subcellular localization was monitored by green fluorescence microscopy. (B) HeLa cells were transfected with a 3xMEF2-luciferase construct together with the indicated expression plasmids. Luciferase activities were measured using a luminometer and divided by ß-galactosidase activity. Corrected luciferase values were normalized to the reporter-only condition (first bar), which was given the arbitrary value of 1.0. * indicates $p<0.05$ versus first bar. (C) HeLa cells were transfected with HA-SIK2 (lanes 2 and 3) together with (lane 3) or without (lane 2) an LKB1 expression plasmid. Cells were harvested in buffer K and subjected to SDS-PAGE followed by western blotting with antibodies against the proteins indicated on the right side of the blots. The position of the band representing either HDAC4 or HDAC7 is indicated to the right of the phospho-S246 blot (*top blot*). The size of the indicated proteins in kilodaltons (kDa) is shown to the left of the blots.

Figure 6

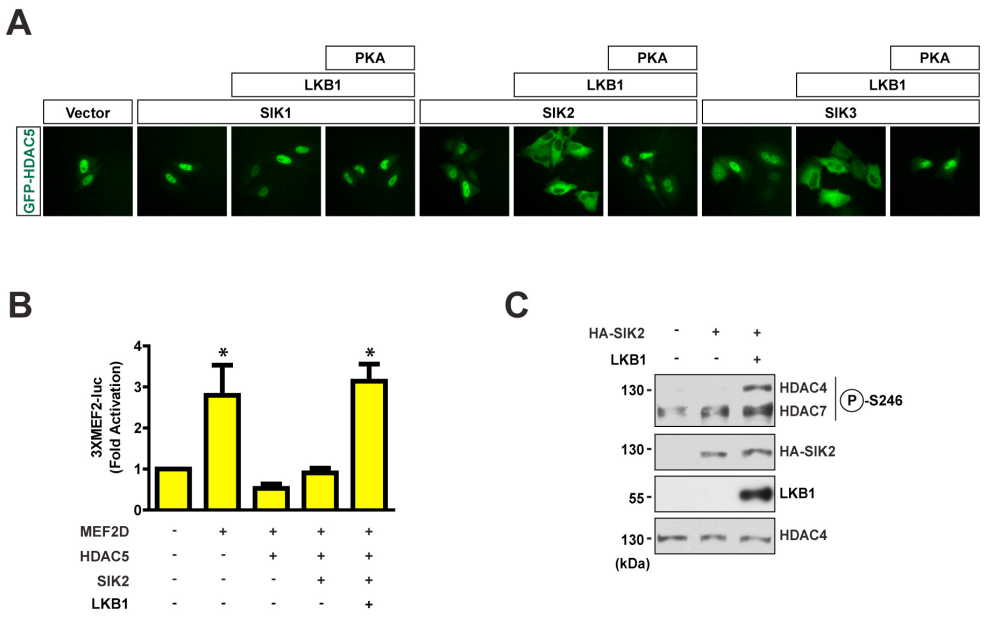
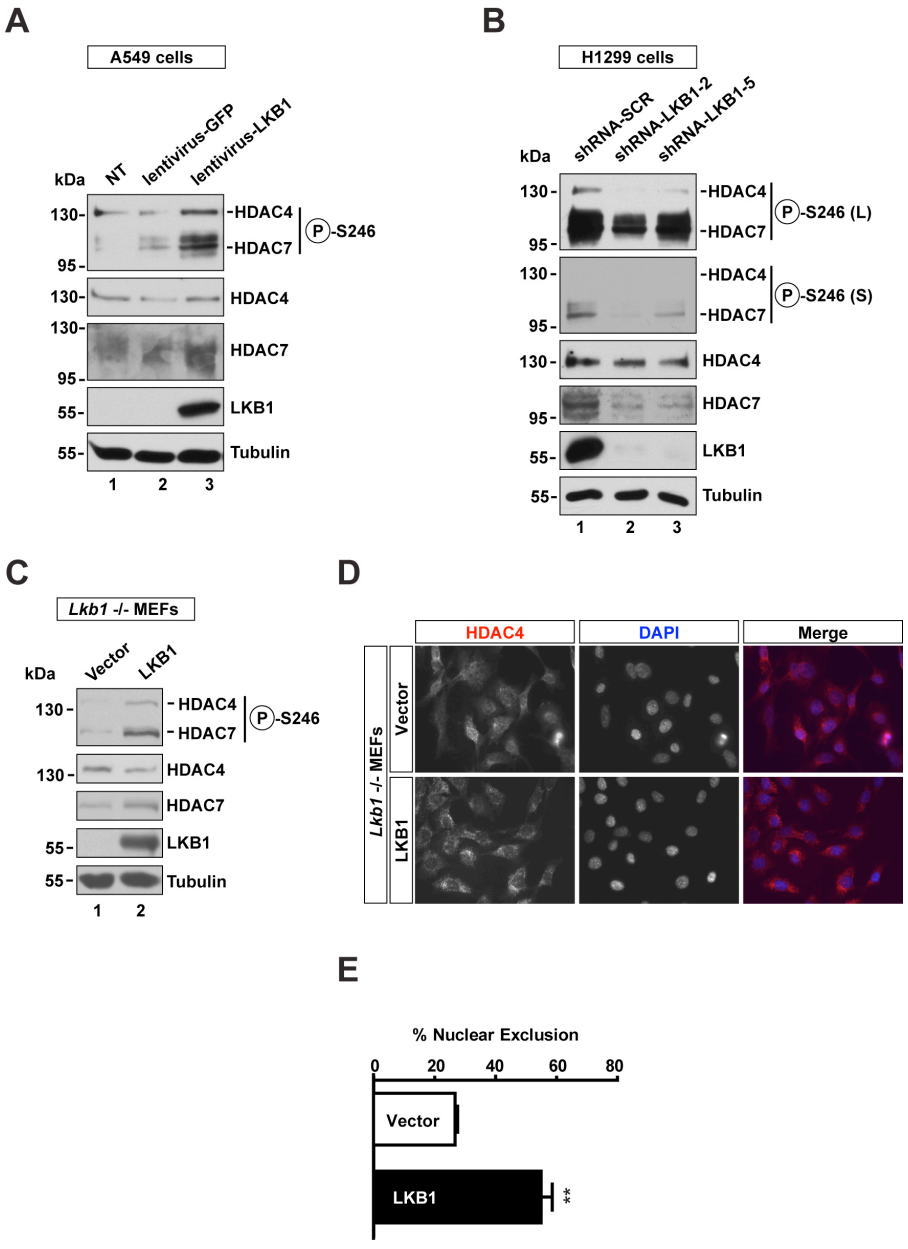

A
PKA
LKB1
Vector
SIK1
SIK2
SIK3
GFP-HDAC5
B
3XMEF2-luc
(Fold Activation)
MEF2D
HDAC5
SIK2
LKB1
C
HA-SIK2
LKB1
130
HDAC4
HDAC7
P-S246
HA-SIK2
55
LKB1
HDAC4
(kDa)

Figure 7. LKB1 is important for endogenous HDAC4 localization and phosphorylation. (A) A549 cells (LKB1 negative) received no treatment (NT, lane 1) or were infected with lentiviruses containing the coding sequence for GFP (lane 2) or LKB1 (lane 3). Whole cell extracts were subject to SDS-PAGE followed by immunoblotting with antibodies directed against the indicated proteins. The position of the band representing either HDAC4 or HDAC7 is indicated to the right of the phospho-S246 blot (*top blot*). The size of the indicated proteins in kilodaltons (kDa) is shown to the left of the blots. (B) H1299 cells (LKB1 positive) were infected with lentiviruses containing either a scrambled shRNA (SCR, lane 1) or shRNAs targeting LKB1 (LKB1-2, lane 2; LKB1-5, lane 3). Western blot analysis was performed as in (A). (C) Whole cell extracts were prepared as in (A) from *Lkb1*$^{-/-}$ MEF cells expressing empty Flag vector (Vector) or Flag-tagged LKB1 (LKB1) and western blot analysis was performed as in (A). (D) Immunofluorescence microscopy of *Lkb1*$^{-/-}$ MEF cells expressing empty Flag vector (Vector) or Flag-tagged LKB1 (LKB1). Cells were co-stained with anti-HDAC4 primary antibody (left panels) and DAPI (middle panels) to visualize nuclei. (E) Quantification of HDAC4 subcellular localization. Percentage of cells with nuclear exclusion of HDAC4 was determined for 3 independent experiments. Data are presented as means ± SEMs (error bars). ** indicates $p<0.01$ vs Vector.

Figure 7


A
A549 cells
kDa
NT
lentivirus-GFP
lentivirus-LKB1
130-
95-
130-
130-
95-
55-
55-
HDAC4
HDAC7
P-S246
HDAC4
HDAC7
LKB1
Tubulin
1 2 3
B
H1299 cells
kDa
shRNA-SCR
shRNA-LKB1-2
shRNA-LKB1-5
130-
95-
130-
95-
130-
95-
55-
55-
HDAC4
HDAC7
P-S246 (L)
HDAC4
HDAC7
P-S246 (S)
HDAC4
HDAC7
LKB1
Tubulin
1 2 3
C
Lkb1 -/- MEFs
kDa
Vector
LKB1
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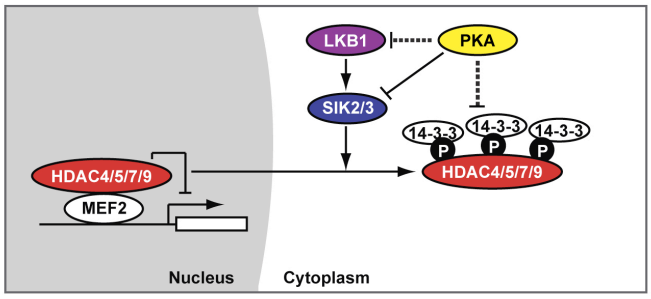
130-
55-
55-
HDAC4
HDAC7
P-S246
HDAC4
HDAC7
LKB1
Tubulin
1 2
D
HDAC4
DAPI
Merge
Lkb1 -/- MEFs
Vector
LKB1
E
% Nuclear Exclusion
0 20 40 60 80
Vector
LKB1
**

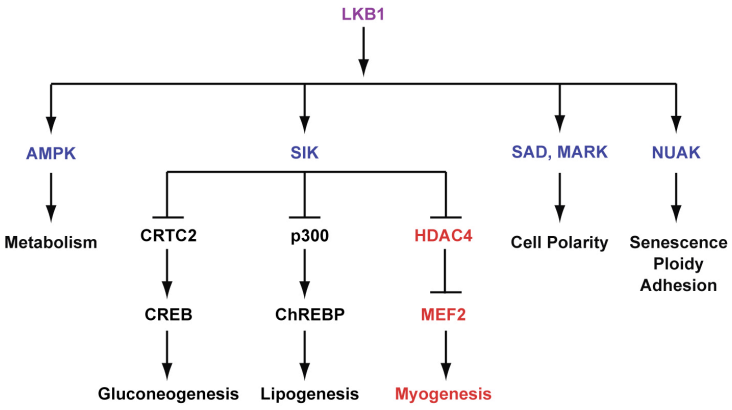
Figure 8. LKB1-SIK2/3-HDAC4 pathway as a novel signaling module. (A) Cartoon showing effect of the PKA-sensitive LKB1-SIK2/3 pathway on nucleocytoplasmic shuttling of class IIa HDACs. The dotted lines denote events that await formal demonstration or require further investigation. (B) Schematic showing the major downstream targets of LKB1 and the predominant physiological process that they each regulate. LKB1-dependent kinases belong to several subfamilies within the AMPK-related kinase family. LKB1 controls cell metabolism by signaling through the AMPK subfamily (59); cell polarity through the SAD and MARK subfamilies (7); and cell senescence and ploidy as well as cell adhesion through the NUAK subfamily (28, 75). Among the three signaling branches downstream from the SIK subfamily are events controlled by the transcriptional coactivators CRTC2 and p300 (6, 13, 33), as well as the SIK2/3-class IIa HDAC pathway that we have characterized in this paper.

Figure 8

A


LKB1
PKA
SIK2/3
14-3-3
14-3-3
14-3-3
P
P
P
HDAC4/5/7/9
HDAC4/5/7/9
MEF2
Nucleus
Cytoplasm

B


LKB1
AMPK
SIK
SAD, MARK
NUAK
Metabolism
CRTC2
p300
HDAC4
Cell Polarity
Senescence
Ploidy
Adhesion
CREB
ChREBP
MEF2
Gluconeogenesis
Lipogenesis
Myogenesis

Supplemental Figure S1

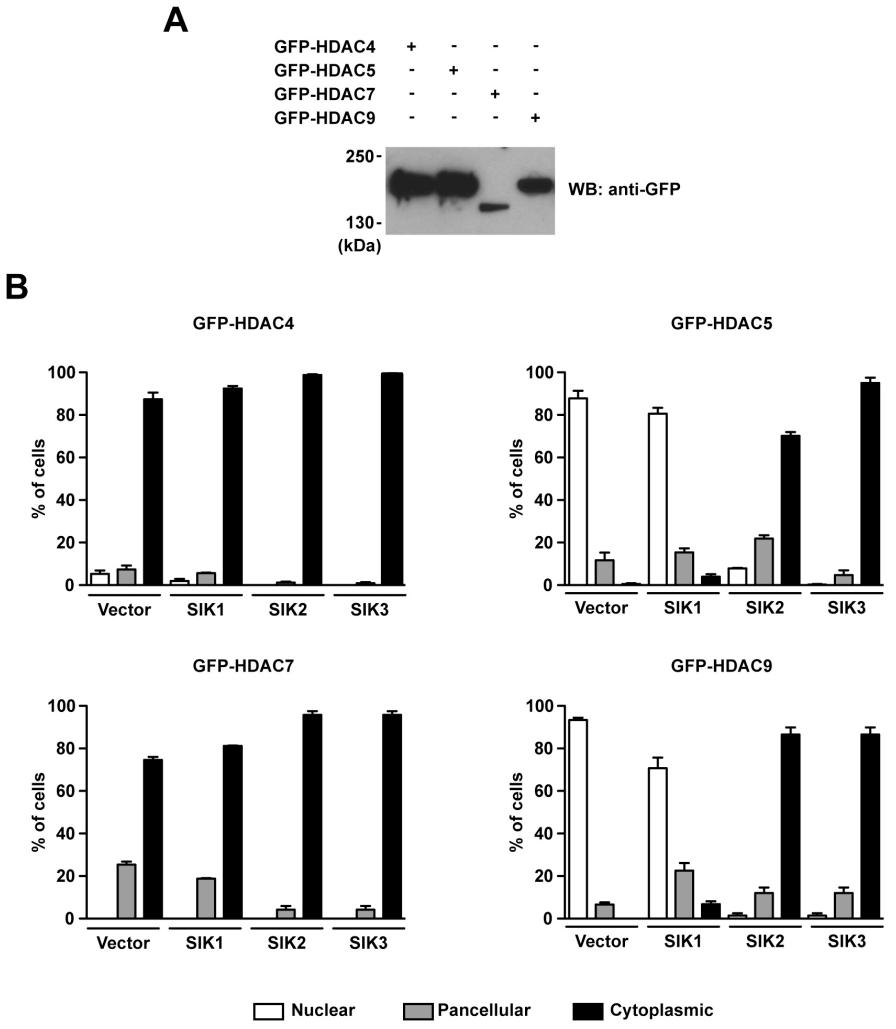


Fig. S1. Quantification of subcellular localization of class IIa HDACs.

(A) Western blot of GFP-tagged class IIa HDACs transiently expressed in HEK293 cells. Whole cell extracts were subject to SDS-PAGE followed by western blotting using anti-GFP primary antibody.

(B) Quantification of class IIa HDAC subcellular localization as depicted in Fig. 1. At least 100 cells were counted for all conditions over 3 independent experiments. Data are presented as mean +/- SEM (error bars).

Supplemental Figure S2

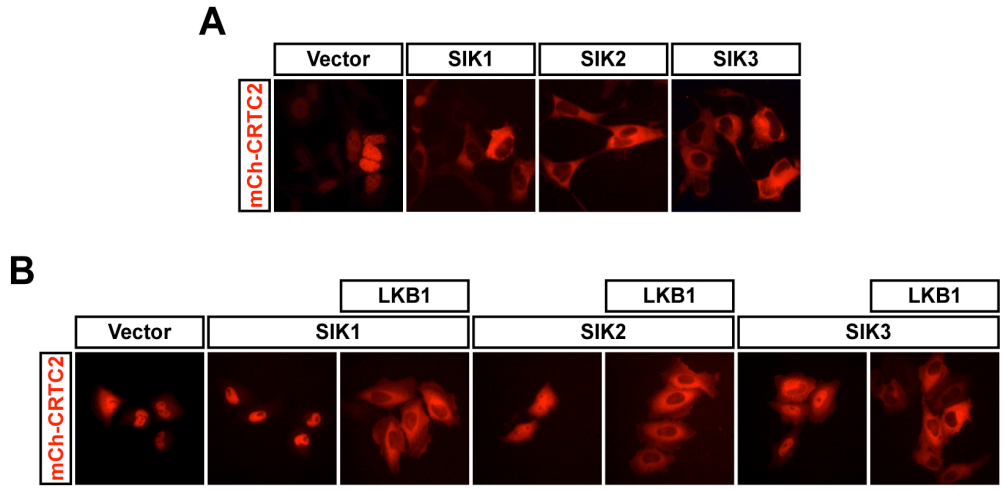


Fig. S2. Cytoplasmic accumulation of CRTC2 in response to SIK1,2, and 3.

(A) Red fluorescence microscopy images of mCh-CRTC2 localization upon cotransfection with empty vector, or expression plasmids for SIK1, SIK2, or SIK3 in HEK293 cells.
(B) As in (A) except that HeLa cells were used. Where indicated, an expression plasmid for LKB1 was also cotransfected.

Supplemental Figure S3

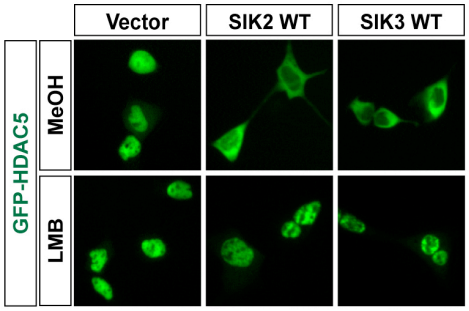


Fig. S3. SIK2/3-mediated cytoplasmic accumulation of HDAC5 is due to stimulation of nuclear export and not inhibition of nuclear import.

Green fluorescence microscopy images of GFP-HDAC5 cotransfected with either empty vector or an expression plasmid encoding either SIK2 or SIK3, followed by treatment with either MeOH or leptomycin B (LMB) for 60 minutes.

Supplemental Figure S4

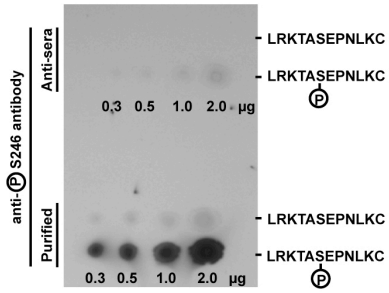


Fig. S4. Anti-phospho-Ser246 (HDAC4) antibody validation.

Peptide dot blotting to analyze the anti-phospho-Ser246 antibody of HDAC4. Regular and phosphopeptides were spotted on membranes at indicated amounts for blotting with anti-sera from rabbits immunized with the phosphopeptide conjugated to McKLH (top) or with the antibody affinity-purified from the sera using SulfoLink gel (Pierce) linked to the phosphopeptide through its free Cys (LRKTApSEPNLKC, where pS is phospho-Ser; corresponding to residues 241-251 of HDAC4, with the Cys added for conjugation).

Supplemental Figure S5

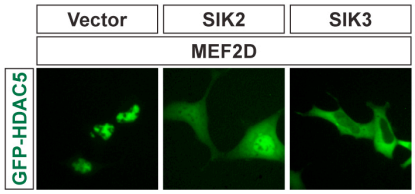


Fig. S5. SIK3 causes export of HDAC5 in the presence of MEF2D.

Green fluorescence microscopy images of GFP-HDAC5 cotransfected with MEF2D along with empty vector, or expression plasmids encoding SIK2 or SIK3.

Supplemental Figure S6

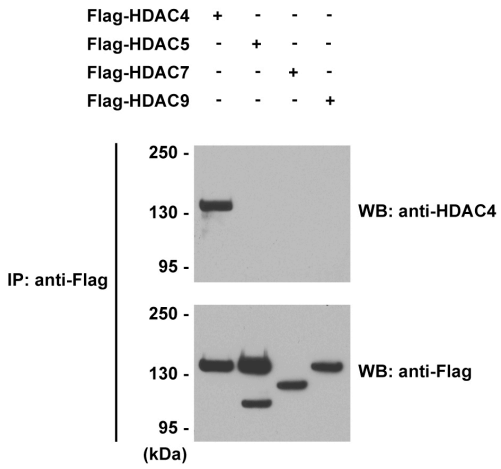


Fig. S6. Anti-HDAC4 antibody validation.

The indicated Flag-tagged class IIa HDACs were transiently transfected in HEK293 cells, which were harvested 48 h later in buffer K. Whole cell extracts were subject to immunoprecipitation with M2-agarose beads and Flag-tagged proteins were eluted with Flag peptide. Immunoprecipitates were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane and western blotting was performed using either anti-HDAC4 or anti-Flag primary antibodies.

CHAPTER III

3.0

cAMP/protein kinase A signaling differentially inhibits phosphorylation and cytoplasmic localization of class IIa histone deacetylases

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3.1 PREFACE

In Chapter II, I tested the ability of PKA to inhibit SIK-mediated nuclear export of class IIa HDACs. This was based on previously published work showing that PKA-mediated phosphorylation of SIKs could inhibit their ability to induce cytoplasmic localization of the CREB coactivator proteins, CRTC1-3. I confirmed the hypothesis that this is also the case for class IIa HDACs by showing that PKA prevented SIK2-mediated phosphorylation and nuclear export of these HDACs, as well as derepression of MEF2 transcriptional activity. However, during these experiments I observed effects that lead me to hypothesize that PKA promotes nuclear localization of class IIa HDACs through another mechanism not involving inhibition of SIKs. This exciting prospect led me to identify a novel phosphorylation site in HDAC4, 5, and 9 that controls their nuclear localization in response to cAMP/PKA signaling. Since HDAC7 does not possess this site and is insensitive to cAMP/PKA, these results identify a novel mechanism for the differential regulation of class IIa HDACs. In addition, these findings strengthen the concept of class IIa HDACs as novel effectors of cAMP/PKA signaling.

3.2 ABSTRACT

Regulated shuttling of proteins between the nucleus and the cytoplasm represents an important tool that cells use to respond to extracellular signals. Class IIa HDACs (HDAC4, 5, 7, and 9) are exported from the nucleus to the cytoplasm in response to phosphorylation on specific serine residues (including S246). It has also recently been shown that cAMP/PKA promotes nuclear localization of these HDACs, although the mechanism behind this remains unknown. Here we show that mutation of a single serine residue located in the nuclear localization signal (S266 in HDAC4 and S279 in HDAC5) to alanine prevents cAMP/PKA-mediated nuclear localization of HDAC4 and 5. Moreover, engineering HDAC7 (which lacks this residue) to match the HDAC4/5 sequence increases its sensitivity to cAMP. Using a novel antibody that recognizes phosphorylated S266, we demonstrate that cAMP signaling decreases S266 phosphorylation. In addition, we show that phosphorylation of S246 is crucial for basal S266 phosphorylation, suggesting crosstalk between known and novel phosphorylation sites. Finally, adrenocorticotropic hormone, which uses cAMP as a second messenger, also causes a decrease in endogenous HDAC4 S266 phosphorylation.

3.3 INTRODUCTION

Cells respond to changes in their extracellular environment through the activation of signal transduction pathways that relay information from the plasma membrane to the nucleus, where gene expression can be altered to adapt to environmental changes. The histone deacetylase (HDAC) superfamily is a major target of such signaling cascades (66). HDACs remove acetyl groups that have been attached to lysine residues of target proteins by histone acetyltransferase (HAT) enzymes. Traditionally, deacetylation of histones and transcription factors by HDACs has been associated with transcriptional repression, however, deacetylation is associated with transcriptional activation in certain cases (56), and is now known to control DNA damage repair (5, 47), transcription elongation (9, 28, 30), heterochromatin assembly (14), retrotransposon silencing (7) and antisense RNA-mediated gene silencing (8). The opposing actions of HDACs and HATs ensure dynamic turnover of acetyllysine residues, and thus represent important targets of cell signaling cascades. Indeed, HDAC enzymatic activity and subcellular localization are regulated in a signal-dependent manner through changes in protein-protein interaction and posttranslational modification (41, 52, 60).

Class IIa HDACs (HDAC4, 5, 7, and 9) shuttle between the nucleus and the cytoplasm in response to changes in phosphorylation status. Phosphorylation of conserved serine residues (S246, 467, and 632 in HDAC4) promotes nuclear export by a combination of increased 14-3-3 binding, inhibition of the nuclear localization signal (NLS) and activation of the nuclear export sequence (NES) (21, 45, 46, 61, 62). The primary consequence of nuclear export is derepression

of target gene transcription mediated by transcription factors such as myocyte enhancer factor 2 (MEF2) proteins (41, 52). The list of class IIa HDAC kinases has steadily grown since the founding members of this group, Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) were identified (44). The most recent additions to this list are salt-inducible kinase 2 (SIK2) and SIK3, which we recently identified as class IIa HDAC kinases (see Chapter II). In contrast, dephosphorylation of these serine residues by phosphatases such as protein phosphatase 2A (PP2A) and myosin phosphatase (PP1ß/MYPT1), promotes nuclear localization of class IIa HDACs and repression of MEF2-dependent transcription (27, 42, 50, 51). In addition to the canonical phosphorylation-dependent nucleocytoplasmic shuttling of class IIa HDACs, other mechanisms might also control their subcellular localization. Additional phosphorylation sites have been identified in the NLS that regulate nuclear import, including S298 of HDAC4 (50), and both S240 and S253 of MEF2-interacting transcription repressor (MITR), the HDAC9 splice variant (11, 26). Thus, subcellular localization of class IIa HDACs is finely tuned by multiple phosphorylation events that are regulated by diverse signaling cascades.

Many hormones act on cells through G protein-coupled receptor (GPCR)-induced activation of adenylyl cyclase, which generates the second messenger cyclic 3', 5'-adenosine monophosphate (cAMP). cAMP exerts many biological effects, in part through the activation of cAMP-dependent protein kinase (also called protein kinase A; PKA). This occurs through binding of cAMP to the regulatory subunits of the PKA holoenzyme, leading to the release of active catalytic subunits that are now free to phosphorylate substrates (16). cAMP

signaling is tightly controlled temporally and spatially by phosphodiesterases (PDEs) that breakdown cAMP, and A-kinase anchoring proteins (AKAPs) that serve as scaffolds to localize cAMP/PKA signaling nodes to discrete subcellular compartments (2, 64). PKA phosphorylates substrate proteins on serine or threonine residues found predominantly as part of the consensus sequence RRxS/T (55). PKA phosphorylates multiple proteins involved in a wide range of cellular processes, including apoptosis and cell survival, metabolism, transcription, ion channel transport, and muscle contraction (55). A major PKA substrate is cAMP-response element binding protein (CREB), a transcription factor that becomes activated by PKA-mediated phosphorylation (10, 48, 57). However, cAMP/PKA signaling can also repress transcription factors. For example, MEF2-dependent transcription is inhibited by cAMP/PKA, in part by PKA-mediated phosphorylation of MEF2D (13). However, cAMP signaling is also associated with increased nuclear localization of HDAC4 and HDAC5 (3, 13). Thus, the inhibitory effect of cAMP/PKA signaling toward MEF2 may involve regulation of class IIa HDAC nucleocytoplasmic shuttling. Related to this, negative regulation of SIKs by PKA may be responsible for its effect on class IIa HDACs (4, 19) (also see Chapter II). Alternatively, PKA-mediated activation of PP2A has also been implicated in increased nuclear localization of HDAC4 (32).

Here we propose a novel mechanism underlying cAMP/PKA-mediated nuclear localization of class IIa HDACs. cAMP/PKA signaling appears to induce nuclear localization through the regulation of a novel phosphorylation site in the NLS of HDAC4, 5 and 9. Disruption of this site (S266 in HDAC4) by

mutagenesis prevents cAMP/PKA-mediated nuclear localization, and transferring this site to HDAC7 (which does not possess it) renders its subcellular localization more sensitive to cAMP/PKA signaling. Through the use of a novel phospho-S266 antibody we show that this phosphorylation event is negatively regulated by cAMP/PKA in different cellular contexts including ACTH stimulation in Y1 adrenocorticotropic tumour cells. Moreover, we observed crosstalk between S246 phosphorylation (which controls 14-3-3 binding and nuclear export) and S266 phosphorylation. These results thus add another layer of complexity to the mechanisms controlling the signal-dependent regulation of class IIa HDACs.

3.4 MATERIALS AND METHODS

Cell culture and treatments. HEK293, HeLa, and C2C12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Sigma), and 1% penicillin/streptomycin (Invitrogen), except for C2C12 cells during differentiation, which entailed switching the cells to DMEM containing 2% horse serum (Sigma). 8-Br-cAMP (1 mM), forskolin (10 μM), H-89 (10 μM), okadaic acid (100 nM), staurosporine (1 μM), KN-62 (10 μM), Bis I (10 μM), PD 98059 (10 μM), and kenpaullone (5 μM) were purchased from Calbiochem and used at the indicated concentrations. Leptomycin B (Sigma) was used at a concentration of 10 ng/ml, while ACTH (Bachem) was used at 1 μM.

Plasmid constructs and antibodies. GFP- and Flag-tagged WT, S246A, and triple mutant (TM; S246A/467A/632A) HDAC4 constructs have been previously described (61). An expression plasmid for mouse HDAC5 was generously provided by S. Khochbin (INSERM) (34) and used to create GFP-tagged HDAC5 using a pEGFP-C2 (BD Biosciences) derivative. GFP-HDAC7 was derived from a pEGFP-C2 derivative and an expression plasmid for mouse HDAC7 (GenBank accession number AF207749). GFP-HDAC9 was constructed from a pEGFP-C2 derivative by linking an oligoduplex consisting of DAC103 (5'-AA TTC ATG CAC AGT AT-3') and DAC104 (5'-GA TCA TAC TGT GCA TG-3') to the coding sequence of human HDAC9. cDNAs for SIK2, SIK3, and PKA-Cα1 (hereafter referred to as PKA) were purchased from Open Biosystems, and subcloned into pCDNA 3.1 derivatives. HDAC4 (S266A, S265A, S266D, P267A), HDAC5 (S279A, S279D), HDAC7 (N197S, K196S/N197S), and SIK2

(S587A, T484A, T484A/S587A) mutants were generated by PCR-mediated site-directed mutagenesis using the Pfu polymerase system according to the manufacturer's instructions (Fermentas). All mutants were verified by automatic sequencing.

Anti-phospho-S246 (HDAC4) polyclonal rabbit antibody was prepared by immunization of rabbits with a peptide representing residues 241-251 of HDAC4 (LRKTApSEPNLKC, where pS is phospho-Ser and the Cys was added for conjugation). Antibodies were affinity-purified from rabbit serum using SulfoLink gel (Pierce) linked to the phosphopeptide through its free Cys. The specificity of the antibody toward phospho-S246 versus non-phosphorylated peptide was confirmed by dot blotting (Chapter II, Fig. S4). Anti-phospho-S266 (HDAC4) polyclonal rabbit antibody was prepared as above using a peptide representing residues 262-273 of HDAC4 (ERRSpSPLLRRKDC, where pS is phospho-Ser and the Cys was added for conjugation). The specificity of the antibody toward phospho-S266 versus non-phosphorylated peptide was confirmed by dot blotting (Fig. 5). The anti-HDAC4 polyclonal antibody was affinity-purified from rabbit antisera that were previously described (61). This purified antibody is highly selective for HDAC4 and does not cross-react with any other class IIa HDACs (Chapter II, Fig. S6).

Immunofluorescence microscopy. C2C12 and HeLa cells were seeded on glass coverslips. Once cells had reached ~60% confluence, they were treated with 1 mM 8-Br-cAMP for 30 min. Coverslips were then washed twice with phosphate-buffered saline (PBS), fixed with 2% paraformaldehyde for 20 min then washed three times with PBS. Cells were then permeabilized with 0.2%

Triton X-100 for 10 min, followed by washes with 100 mM glycine (3 x 5 min each). After blocking with IF buffer (PBS with 0.2% Triton X-100 and 0.05% Tween-20) containing 2% BSA for 45 min, cells were incubated overnight at 4°C with anti-HDAC4 antibody (1:500). After primary antibody incubation, cells were rinsed with IF buffer (3 x 5 min), then incubated in AlexaFluor 488 goat anti-rabbit IgG (H +L) secondary antibody (Invitrogen, Molecular Probes) (1:1000) at room temperature for 45 min, followed by washes with IF buffer (3 x 5 min). Cells were then counterstained with DAPI for 5 min at room temperature, then washed with ddH_2O (2 x 5 min) and mounted on slides for microscopy.

Cell transfection. For western blot experiments (including IPs), 0.2 X 10^6 C2C12 cells were plated in 6 cm plates and transfections were performed with 15 µl Lipofectamine 2000 (Invitrogen) and 5 µg total DNA according to the manufacturer's instructions. For fluorescence microscopy, 0.04 X 10^6 HEK293 or 0.06 X 10^6 C2C12 cells were plated in 12 well plates. For HEK293 cells, transfections were performed with 3 µl Superfect (Qiagen) and 1.5 µg total DNA. For C2C12 cells, transfections were performed with 3 µl Lipofectamine 2000 (Invitrogen) and 1.5 µg total DNA. Experiments were performed 24-48 h post-transfection.

Immunoprecipitation. The day after transfection, C2C12 cells were washed twice with ice-cold PBS and lysed in 0.25 mL buffer K (20 mM sodium phosphate pH 7.0, 150 mM KCl, 30 mM sodium pyrophosphate, 0.1% NP-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na_3VO_4, 25 mM ß-glycerophosphate and protease inhibitors). For affinity purification of Flag-tagged proteins, 200 µl of extracts was added to 10 µl of M2 agarose beads (Sigma) and rotated at 4°C for 2

h. Following 4 washes with buffer K, bound proteins were eluted with 2 µl Flag peptide (Sigma) in 25 µl buffer K.

Immunoblotting. After addition of 3 X SDS sample buffer, whole cell extracts and IP extracts were boiled for 5 min and then separated by SDS-PAGE and transferred to nitrocellulose membranes. For IP experiments, membranes were blocked in PBS-Tween 20 (PBS-T) with 20% horse serum for 1 h at room temperature and then incubated overnight at 4°C with anti-Flag (Sigma), anti-phospho-S266 (HDAC4), and anti-phospho-S246 (HDAC4) antibodies. For detection of endogenous proteins, membranes were blocked in PBS-T with 5% milk for 1 h at room temperature and then incubated overnight at 4°C with anti-HDAC4, anti-phospho-S266, anti-phospho-S246, and anti-α-tubulin (Sigma) antibodies. Membranes were then washed in PBS-T (6 X 8 min), and then incubated in the appropriate secondary antibody conjugated to HRP for 1 hour at room temperature. Following another set of 6 X 8 min washes in PBS-T, membranes were incubated in PBS (2 X 5 min), and then visualized on film after 5 min incubation in Supersignal enhanced chemiluminescent solution (Pierce).

Statistical analysis. Data are presented as means ± SEM. One-way ANOVAs were performed with a Bonferroni post-hoc test. $p<0.05$ was considered statistically significant.

3.5 RESULTS

cAMP/PKA signaling prevents nuclear export of HDAC4, 5, and 9. To test the effect of PKA signaling on class IIa HDAC localization, we treated C2C12 myoblasts with 8-Br-cAMP and performed immunofluorescence microscopy using an anti-HDAC4 antibody. Following 30 min of treatment with 8-Br-cAMP, HDAC4 localization shifted from a pancellular distribution to a predominantly nuclear pattern (Fig. 1A). We also observed a similar effect after cotransfection of GFP-HDAC4 with a PKA expression plasmid in both C2C12 and HEK293 cells (Fig. 1B). In contrast to HDAC4, HDAC5 exhibited a basal nuclear localization pattern (Fig. 1C). Therefore, in order to see whether cAMP/PKA signaling had a similar effect on HDAC5, we first had to induce a cytoplasmic localization pattern for HDAC5. This was achieved by cotransfecting GFP-HDAC5 with SIK2, SIK3, or CaMKIV in HEK293 cells. As was the case for HDAC4, PKA prevented the cytoplasmic localization of HDAC5, whether induced by SIK2/3 (Fig. 1C), or by CaMKIV (Supplemental Figures; Fig. S1). Similarly, SIK2 induced nuclear export of HDAC9, and PKA abolished this effect (Fig. 1E). Thus, PKA prevents nuclear export of HDAC4, 5, and 9. However, as opposed to its counterparts, HDAC7 is less sensitive to PKA. Its cytoplasmic localization pattern was not significantly altered by cotransfection of PKA in these cells (Fig. 1D). The effects on HDAC5 and HDAC7 were also seen when C2C12 cells were treated with 8-Br-cAMP (Fig. 3).

Effect of PKA on class IIa HDAC localization is independent of SIK inhibition. One possible mechanism behind the effect of PKA on nuclear export is through inhibition of class IIa HDAC kinases, which induce nuclear export of

these HDACs through phosphorylation-dependent 14-3-3 binding (41, 52, 60). Indeed, PKA is known to inhibit SIKs and we showed that PKA inhibited SIK2/3-mediated nuclear export of HDAC5 and 9 (Fig. 1C and 1E). PKA has been shown to phosphorylate SIK2 on S587, and mutation of this residue to Ala (S587A) renders SIK2 resistant to PKA-mediated inhibition (54). Thus, we tested whether PKA could still have its effect on HDAC5 localization when SIK2 S587A was used to export HDAC5 to the cytoplasm. Surprisingly, PKA was still able to prevent nuclear export of HDAC5 caused by SIK2 S587A (Fig. 1F). We found the same thing using another SIK2 mutant (T484A) in which a second putative PKA consensus phosphorylation motif was disrupted, and also when a double mutant bearing both these mutations (T484A/S587A) was used to relocate HDAC5 to the cytoplasm (Fig. 1F). Furthermore, to compliment the transient transfection experiments, we monitored the localization of endogenous HDAC4 in response to 8-Br-cAMP in cells with defective SIK signaling. For this we used HeLa cells, in which SIKs are inactive (29) owing to the lack of expression of their upstream activator, the tumor suppressor kinase LKB1 (59). In these cells, HDAC4 was more nuclear following 8-Br-cAMP treatment (Fig. 1H). Although PKA does inhibit SIKs, these results suggest that this is not the underlying cause of the effect of cAMP/PKA on class IIa HDAC localization. Moreover, since HDAC7 is less sensitive to PKA, our focus shifted to examine differences in the sequences of class IIa HDACs that may ultimately be responsible for the differential sensitivity to cAMP/PKA signaling.

A novel regulatory motif controls HDAC4 localization in response to cAMP/PKA signaling. To determine whether sequence differences between

HDAC7 and the other class IIa HDACs were behind their differential sensitivity to cAMP/PKA signaling, we performed a sequence alignment comparison. There are two PKA consensus phosphorylation (RRxS/T) sites in HDAC4, S266 and T707. Strikingly, S266 is conserved in HDAC5 (S279) and HDAC9 (S240), but is absent from HDAC7 (Fig. 2A). In addition, this site is located in the NLS and is adjacent to the N-terminal 14-3-3 binding site (S246). We created a GFP-HDAC4 S266A expression plasmid, in which Ser 266 is mutated to Ala. This mutation had no effect on basal localization of HDAC4, but it prevented nuclear localization induced by PKA overexpression in HEK293 cells (Fig. 2B). HDAC4 S266A was also resistant to 8-Br-cAMP treatment in C2C12 cells (Fig. 2C-D). These results suggest that S266 may represent a novel PKA phosphorylation site, so we also created a S266D mutant, in which the negatively charged aspartic acid residue mimics the negative charge of a phosphate group. In that case, we expected the S266D mutant to be constitutively nuclear, but we saw no change in basal HDAC4 localization (Fig. 2E). In fact, the S266D mutant behaved exactly as the S266A mutant did, as it was also resistant to cAMP-mediated nuclear localization (Fig. 2E). We also created mutants to investigate the importance of the sequence surrounding S266. Mutation of S265 to Ala (S265A) had a very minor effect on localization as this mutant largely accumulated in the nucleus following 8-Br-cAMP treatment (Fig. 2E). In contrast, HDAC4 P267A was totally unresponsive to 8-Br-cAMP treatment and remained in the cytoplasm like HDAC4 S266A (Fig. 2E). Thus, the Pro residue adjacent to S266 is also crucial for cAMP-mediated nuclear localization of HDAC4.

We next tested whether HDAC4 S266A and P267A are defective in general nuclear import or whether the effect is specific to cAMP/PKA-mediated nuclear localization. For this we applied two stimuli known to induce nuclear localization of class IIa HDACs. First, we treated C2C12 cells with leptomycin B (LMB), an inhibitor of the nuclear export receptor CRM1 (15, 17, 33, 49). Since class IIa HDACs constantly shuttle between the nucleus and cytoplasm, inhibition of nuclear export by LMB results in nuclear accumulation of these HDACs (45, 61). As expected, LMB treatment induced nuclear localization of WT HDAC4 (Figure 2C and 2D). The nuclear localization of HDAC4 S266A was slightly reduced compared to WT HDAC4, but it was still predominantly nuclear in response to LMB (Fig. 2C-D). HDAC4 S266D and P267A mutants also accumulated in the nucleus after LMB treatment (Fig. 2F). Another stimulus known to cause nuclear accumulation of HDAC4 is overexpression of MEF2C (6, 62). We showed that this is also the case for MEF2D (Fig. 2G). Like the effect seen for LMB, HDAC4 S266A and S266D mutants also moved to the nucleus upon overexpression of MEF2D (Fig. 2G). Taken together, these results demonstrate that HDAC4 S266 and P267 are important for cAMP/PKA-mediated nuclear accumulation, but are not crucial for general nuclear import of HDAC4.

HDAC5 S279 is important for cAMP-mediated nuclear accumulation. Since the HDAC4 cAMP/PKA-responsive motif is conserved in HDAC5 (Fig. 2A), we next sought to determine whether HDAC5 shuttling in response to cAMP/PKA is regulated in a similar fashion. Like in HEK293 cells (Fig. 1C), HDAC5 is predominantly nuclear in C2C12 cells (Fig. 3A). Thus, we used SIK2 S587A to move HDAC5 into the cytoplasm in order to see the effect of cAMP on

nuclear import of HDAC5. Also similar to HEK293 cells, PKA prevented the cytoplasmic localization of HDAC5 induced by SIK2 S587A (Fig. 3A-B). Next, we did the same experiment with GFP-HDAC5 S279A, in which the residue equivalent to S266 of HDAC4 is mutated to Ala. Strikingly, HDAC5 S279A was significantly more cytoplasmic than WT HDAC5 (Fig. 3A-B). This is in contrast to what we observed with HDAC4 (Fig. 2B-D). However, this can be explained by the fact that HDAC4 is already fully cytoplasmic in the basal state, and thus cytoplasmic localization cannot be increased. In addition, HDAC5 S279A was also more cytoplasmic than WT HDAC5 in response to SIK2 S587A cotransfection (Fig. 3A-B). Conversely, whereas WT HDAC5 returned to the nucleus in response to 8-Br-cAMP treatment (even in the presence of coexpressed SIK2 S587A), HDAC5 S279A remained fully cytoplasmic following 8-Br-cAMP treatment (Fig. 3A-B). Thus, like S266 in HDAC4, the equivalent residue in HDAC5 (S279) is necessary for cAMP-mediated nuclear accumulation. Similar results were obtained in HEK293 cells using PKA overexpression instead of 8-Br-cAMP treatment. Specifically, PKA was ineffective in preventing SIK2 T484A/S587A-induced HDAC5 S279A export (Fig. 3D). This was also true for HDAC5 S279D (Fig. 3D). These results contrast sharply with those seen in Fig. 1E, where PKA prevented SIK2 T484A/S587A-mediated nuclear export of WT HDAC5. Moreover, the basal localization of HDAC5 S279D, much like HDAC5 S279A was less nuclear than WT HDAC5 (Fig. S2). As seen for HDAC4, neither HDAC5 S279A nor S279D had a significant effect on general nuclear import induced by LMB treatment (Fig. 3C and 3E).

cAMP-responsive sequence of HDAC4/5 is functional when transferred to HDAC7. As discussed earlier, HDAC7 is the only class IIa HDAC that lacks the PKA consensus phosphorylation site corresponding to S266 of HDAC4 (Fig. 2A). To further test the importance of this motif in conferring cAMP/PKA sensitivity to class IIa HDACs, we engineered HDAC7 mutants to possess the same regulatory sequence as HDAC4 and 5. We created an HDAC7 N197S mutant that gives HDAC7 a Ser residue equivalent to HDAC4 S266 and HDAC5 S279, and also an HDAC7 K196S/N197S mutant that recreates the full motif found in the other class IIa HDACs. Whereas WT HDAC7 remained cytoplasmic in response to 8-Br-cAMP treatment, both HDAC7 N197S and HDAC7 K196S/N197S displayed a partial localization shift to the nucleus following 8-Br-cAMP treatment (Fig. 3F). Thus, this novel cAMP-responsive sequence is a major determinant of class IIa HDAC localization in response to cAMP/PKA signaling.

Effect of cAMP on HDAC4 localization is mediated by PKA. PKA is the major protein activated by increased cAMP concentration, and both cAMP and PKA stimulate nuclear localization of HDAC4 (Fig. 1A-B). However, it remained a possibility that the effect of cAMP signaling on HDAC4 subcellular localization was mediated through non-PKA targets. To test this we employed the PKA inhibitor H-89 to see if the effect of 8-Br-cAMP on HDAC4 was altered. Pre-treatment of C2C12 cells with H-89 abolished the change in HDAC4 localization upon 8-Br-cAMP treatment (Fig. 4). Thus, PKA is necessary for cAMP-induced nuclear localization of HDAC4. Next, we checked for the involvement of protein phosphatase 2A (PP2A), a target of PKA, in this process.

Unlike the results with H-89, pre-treating cells with okadaic acid (a PP2A inhibitor) had no effect on the change in HDAC4 localization induced by 8-Br-cAMP (Fig. 4), suggesting that PP2A is not involved in this regulation. Moreover, HDAC4 S246A (which is slightly less cytoplasmic than WT HDAC4) still became more nuclear in response to 8-Br-cAMP treatment (Fig. 4). These last two results indicate that the effect of cAMP on HDAC4 localization is not due to dephosphorylation of the 14-3-3 binding sites that induce nuclear export. Instead, as suggested by the results in Fig. 2 and 3, cAMP/PKA signals specifically to the novel regulatory sequence containing S266.

HDAC4 S266 phosphorylation decreases in response to cAMP signaling. Due to the dramatic effects of S266A (and S279A) mutation on HDAC4 (and HDAC5) responsiveness to cAMP signaling, we hypothesized that this residue represents a novel phosphorylation site. To investigate this possibility, we created an antibody to detect HDAC4 only when phosphorylated on S266 (Fig. 5A). This antibody recognized a single band of the correct size in whole cell extracts from cells that had been transfected with Flag-HDAC4 WT, but not Flag-HDAC4 S266A (Fig. 5B), demonstrating its specificity *in vivo*. However, activation of PKA by treatment with 8-Br-cAMP or forskolin unexpectedly caused a decrease in S266 phosphorylation (Fig. 5C). This effect was stable whether C2C12 cells were pre-incubated in growth medium (10% FBS; GM) or differentiation medium (2% horse serum; DM) (Fig. 5D). 8-Br-cAMP or forskolin treatment also decreased endogenous S266 phosphorylation (Fig. 5E). We also examined the phosphorylation status of the nearby S246 residue. Endogenous HDAC4 S246 phosphorylation was also decreased after 30

min of 8-Br-cAMP or forskolin treatment (Fig. 5E). However, phosphorylation of the equivalent residue of HDAC7 (S155) was unaltered by these treatments (Fig. 5E). To better gauge the timing of phosphorylation changes, we performed time course experiments in which C2C12 cells were exposed to 8-Br-cAMP for different amounts of time (from 5 to 180 min). Endogenous HDAC4 S266 phosphorylation was significantly lower by 10 min of treatment (Fig. 5F). This effect was somewhat transient as the S266 phosphorylation level returned slightly by 90 min of treatment, but was still lower than the basal state. In contrast, S246 phosphorylation exhibited a slightly slower rate of decline with phosphorylation still depressed at 180 min (Fig. 5F). Interestingly, HDAC7 S155 phosphorylation (as measured with the anti-phospho-S246 antibody), did eventually decline but at a significantly slower rate than HDAC4 S246 phosphorylation (Fig. 5F). Related to the subcellular localization results in Fig. 4, H-89 treatment partially abrogated the cAMP-induced decline in S266 phosphorylation, while okadiac acid treatment had no effect (Fig. S3).

Influence of subcellular localization and S246 phosphorylation on HDAC4 S266 phosphorylation. To help characterize the mechanisms controlling S266 phosphorylation, we treated cells with LMB to see whether S266 phosphorylation status was affected by altered HDAC4 localization. LMB treatment in C2C12 cells resulted in nuclear accumulation of HDAC4 (Fig. 2C) and this was associated with a dramatic decrease in S266 phosphorylation, while S246 phosphorylation was unaltered (Fig. 6A). Time course experiments showed that S266 phosphorylation continued to decrease in response to LMB for the duration of the 180 min experiment, while S246 phosphorylation never changed

during the 3 h time course (Fig. 6B). We next tested whether the effect of cAMP on S266 phosphorylation was altered by mutation of S246. Strikingly, we noticed a major decrease in basal S266 phosphorylation using an HDAC4 construct with a S246A mutation (Fig. 6C). Although the basal S266 phosphorylation level was much lower than for WT HDAC4, there was still a further decrease in S266 phosphorylation after 8-Br-cAMP treatment (Fig. 6C). This result suggests that S246 phosphorylation influences basal S266 phosphorylation (Fig. 6F) but not its response to cAMP. We observed the same effect when we used HDAC4 TM (which contains S467A and S632A mutations in addition to S246A). Thus, these changes were not simply due to subcellular localization changes, since HDAC4 S246A is still largely cytoplasmic in C2C12 cells, while HDAC4 TM is predominantly nuclear (Fig. 6E). Conversely, there was no change in basal S246 phosphorylation when S266 was mutated (Fig. 6C-D), however there was a slightly muted response to cAMP treatment (Fig. 6C).

Hormone-induced cAMP regulates endogenous S266 phosphorylation status. Next we investigated whether S266 phosphorylation status was affected by treatment with a hormone that induces an increase in cAMP concentration. Adrenocorticotropic hormone (ACTH) acts on adrenal cells by increasing the intracellular cAMP concentration (18). We treated Y1 adrenocortical tumour cells with different amounts of ACTH to see if endogenous HDAC4 S266 phosphorylation was influenced by a natural cAMP-producing stimulus. Only the higher dose that we used (1 μM ACTH) induced a drop in S266 phosphorylation (Fig. 7A). We next used this dose of ACTH to perform time course experiments. ACTH treatment resulted in a decrease in S266 phosphorylation at 10 and 30 min,

and the phosphorylation returned to the basal level by 60 min (Fig. 7B). The effects seen with ACTH were less potent than those observed using 8-Br-cAMP (Fig. 7B), and this likely reflects the enhanced resistance toward PDE-mediated degradation possessed by 8-Br-cAMP compared to native cAMP (65). Nevertheless, these findings point to S266 phosphorylation as a physiologically relevant target of diverse cAMP/PKA signaling modules.

3.6 DISCUSSION

Signal-dependent regulation of class IIa HDAC subcellular localization is an important signaling module involved in diverse physiological processes, including skeletal and cardiac muscle differentiation (40), skeletal muscle plasticity (31, 37, 38), cardiac hypertrophy (43), angiogenesis (22, 24, 42, 63), T-cell selection (52), and neurodegeneration (39). Underlying these physiological effects is a complex interplay between canonical phosphorylation-dependent 14-3-3 binding (41, 52) and other modifications that are related to subcellular localization, such as NLS phosphorylation. Mutation of S298 to Ala prevents nuclear import of HDAC4 (50), while phosphorylation of S240 and S253 of the HDAC9 splice variant MITR affects nuclear localization as well (11, 26). Thus, multiple upstream signals converge at the level of class IIa HDAC posttranslational modification to produce an output involving alterations in subcellular localization.

cAMP/PKA regulation of class IIa HDACs is independent of SIK inhibition or PP2A activation. Previous studies showed that cAMP signaling increased the nuclear localization of HDAC4/5 in hippocampal neurons (3), skeletal muscle cells (13), smooth muscle cells (19), and chondrocytes (32). Inhibition of SIK-mediated nuclear export (19) and stimulation of PP2A-mediated nuclear import (32) were presented as underlying mechanisms. Since the subcellular distribution of class IIa HDACs is dependent on the balance between nuclear export-promoting events and nuclear import-promoting events, various stimuli will differ in their importance depending on the cellular context. The above mechanisms may be important in cellular contexts where cytoplasmic

localization is heavily dependent on SIK activity, as opposed to other class IIa HDAC kinases, and where nuclear localization is preferentially dependent on PP2A-mediated dephosphorylation. Nevertheless, our early observation that HDAC7 is much less sensitive to cAMP signaling (Fig. 1D and 3F) suggested that other mechanisms were involved since HDAC7 is regulated by SIKs and PP2A in a manner no different from its counterparts (42) (also see Chapter II). This was confirmed by our finding that PKA prevented nuclear export of HDAC5 induced by PKA-resistant SIK2 mutants (Fig. 1E) and that cAMP treatment increased the nuclear localization of HDAC4 in LKB1-negative HeLa cells, which are defective in SIK activity (Fig. 1F). In addition, pretreating cells with the PP2A inhibitor okadaic acid had no effect on cAMP-induced nuclear localization of HDAC4 in C2C12 cells (Fig. 4). These results suggested that there was another mechanism responsible for the cAMP-responsiveness of class IIa HDACs.

A novel phosphorylation site in the NLS of HDAC4, 5, and 9 regulates cAMP/PKA-responsiveness. Instead of exerting its effect through the known 14-3-3-binding sites, we suggest that cAMP/PKA acts on class IIa HDACs through a novel phosphorylation site located in the NLS. Mutation of this site to a non-phosphorylatable residue (S266A in HDAC4) rendered HDAC4 totally unresponsive to cAMP treatment or PKA overexpression (Fig. 2). In addition, the equivalent mutation in HDAC5 (S279A) inhibited cAMP/PKA from preventing nuclear export induced by PKA-resistant SIK2 S587A (Fig. 3). The ability of this phosphorylation site to inrease the cAMP sensitivity of HDAC7 in an "add-back" HDAC7 K196S/N197S mutant confirmed the specificity of this residue in conferring cAMP/PKA-responsiveness to class IIa HDACs. Interestingly, nuclear

import of the HDAC4 S266A (or HDAC5 S279A) is not generally impaired as these proteins still accumulated in the nucleus in response to inhibition of nuclear export by LMB (Fig. 2 and 3). This is consistent with another report, which showed no effect of HDAC4 S266A mutation on nuclear accumulation in response to treatment of ratjadone, another nuclear export inhibitor (50). Thus, S266 phosphorylation is involved specifically in cAMP/PKA-induced nuclear accumulation of class IIa HDACs, but is not crucial for protein import in general. Interestingly, substituting an Asp residue for the Ser (S266D in HDAC4 and S279D in HDAC5) had the exact same effect as the S266A (or S279A) mutation did (Fig. 2 and 3). Although this was somewhat unexpected, there is precedent for the inability of an Asp residue to mimic phosphorylation, for example in the case of CREB S133D, which has no effect on CREB transcriptional activity (48). Moreover, our finding that HDAC5 S279D is more cytoplasmic than WT HDAC5 (Fig. S2) is supported by a similar finding involving the HDAC9 splice variant MITR (11).

We selected this potential phosphorylation site for analysis because it was part of a PKA consensus phosphorylation motif (Fig. 2A). Since we initially thought that PKA would phosphorylate this site, we were surprised to find that S266 phosphorylation decreased upon cAMP/PKA treatment (Fig. 5). In contrast, during the latter stages of our project, another group reported that PKA phosphorylates S279 of HDAC5 (the site equivalent to S266 of HDAC4) (23). Assuming that this phosphorylation site is regulated similarly in HDAC4 and HDAC5 (the surrounding sequence is identical), this discrepancy might be due to the different techniques employed to measure this phosphorylation event, namely

our use of a phospho-specific antibody specific to this sequence, compared to their use of a general PKA consensus site phospho-specific antibody and P^{32} incorporation in an *in vitro* kinase assay. Moreover, it is possible that our results with the phospho-specific antibody could be due to antibody interference by phosphorylation of S265. In fact, another study published during the latter stages of our project identified both S278 and S279 (equivalent to S265 and S266 of HDAC4) as *in vivo* phosphorylation sites in HDAC5 (20), and we found that our anti-phospho-S266 antibody is incapable of recognizing a peptide phosphorylated on both S265 and S266 (Fig. S6). However, a number of findings lend support to the results of our current study. First, we showed that an HDAC4 P267A mutant is also cAMP-resistant (to the same extent as HDAC4 S266A), indicating that the Pro is important for S266 phosphorylation. Many kinases phosphorylate Ser residues immediately preceding a Pro residue (e.g. proline-directed kinases), however, a Pro residue at the P+1 position (the residue immediately C-terminal to the phospho-Ser) is strongly disfavoured among native PKA substrates prevents PKA-mediated phosphorylation when artificially placed in this position of PKA substrate peptides (58, 67). Second, we found that mutating the other potential phosphorylation site (S265 in HDAC4) to Ala had a minor impact on subcellular localization, indicating that even though S265 may be phosphorylated *in vivo* under some circumstances (20) it does not appear to be important for HDAC4 nuclear accumulation in response to cAMP/PKA. Third, Ha et al. (23) also reported that the HDAC5 S279D mutant was more nuclear than WT HDAC5, which is the opposite of what we have shown for HDAC5 (Fig. 3) and what others have shown for MITR (11).

Thus, the identity of the kinase responsible for S266 phosphorylation *in vivo* remains an outstanding issue. S266 also conforms to the consensus phosphorylation sequence for several other kinases, including CaMK, PKC, MEK/ERK, and GSK-3β. We did not observe any decrease in endogenous S266 phosphorylation after treatment with inhibitors directed against these kinases (Fig. S4). Interestingly, Myrk/dyrk1B, a kinase that regulates myoblast cell survival, can phosphorylate S240 of MITR (equivalent to S266 of HDAC4) (11). Thus, it will be important to test whether endogenous S266 phosphorylation depends on Mirk/dyrk1B or another kinase. Moreover, okadaic acid, an inhibitor of PP2A did not have an effect on S266 phosphorylation in response to cAMP treatment (Fig. S3). Thus, whether cAMP/PKA decreases S266 phosphorylation through the inhibition of a S266 kinase or the activation of a S266 phosphatase remains an important unresolved issue.

Crosstalk between S246 and S266 phosphorylation. Due to its role in subcellular localization of class IIa HDACs, we tested whether S246 phosphorylation was involved in S266 phosphorylation status. We found a striking relationship between the established and novel phosphorylation sites in HDAC4. HDAC4 S246A had dramatically reduced basal S266 phosphorylation (Fig. 6C). This effect was not enhanced by simultaneous mutation of the other two 14-3-3 binding sites in HDAC4, as HDAC4 TM (S246/467/632A) had the exact same effect on S266 phosphorylation as HDAC4 S246A did (Fig. 6D). Interestingly, HDAC4 S246A was largely cytoplasmic in C2C12 cells, while HDAC4 TM was totally nuclear (Fig. 6E). Thus, S246 phosphorylation itself (and not merely a change in subcellular localization affected by S246 mutation) is

an important determinant of basal S266 phosphorylation status (Fig. 6F). However, it does not appear that S246 phosphorylation plays a major role in cAMP-mediated reduction of S266 phosphorylation. Although cAMP treatment caused both S266 and S246 phosphorylation to decrease, the effect was seen at earlier time points for S266 phosphorylation (Fig. 5F), indicating that this was independent of the S246 phosphorylation status. Moreover, although basal S266 phosphorylation was reduced by mutation of S246, it still decreased further following cAMP treatment (Fig. 6C). Interestingly, although S246 is highly conserved from *Drosophila* and *C. elegans* to humans (66), the phosphorylation site identified in this study, S266, is not present in *Drosophila*, and the surrounding sequence is more divergent in *C. elegans*, *A. mellifera*, *S. purpuratus*, and *C. intestinalis* than the sequence surrounding S246 is (Fig. S5). Thus, S266 phosphorylation may represent an additional layer of control that has evolved more recently than the canonical regulatory mechanisms. Thus, these results demonstrate interplay between known and novel phosphorylation sites, and suggest that multisite phosphorylation of class IIa HDACs may occur in a hierarchical manner, as has been suggested by others (12).

Specificity versus redundancy among class IIa HDACs. It is interesting that the cAMP/PKA-responsive motif is absent from HDAC7 (Fig. 2A). Although much redundancy exists between class IIa members, including their highly conserved deacetylase domains, MEF2 binding activity, and 14-3-3 binding sites (66), several unique features are displayed across the IIa subclass. First, the basal subcellular localization differs in some cell types, with HDAC4 and 7 being predominantly cytoplasmic and HDAC5 and 9 mainly restricted to

the nucleus (Fig. 1). Second, HDAC4 and HDAC7 each possess a unique caspase cleavage site not found in any other class IIa HDAC (36, 53). Third, CaMKII only phosphorylates HDAC4, owing to its possession of a unique CaMKII docking site (1, 35). In addition to differences in tissue-specific expression, these unique attributes likely account for some of the distinct biological roles of these proteins, which are exemplified by the different phenotypes exhibited by mice with deletion of specific class IIa HDACs (25). A number of possible explanations exist for the impaired cAMP/PKA-responsiveness of HDAC7. Perhaps having one class IIa HDAC remain in the cytoplasm upon cAMP stimulation in certain cells provided a survival advantage. Alternatively, nuclear localization of a class IIa HDAC upon cAMP stimulation may be detrimental to a particular tissue in which HDAC7 is preferentially expressed. In this case, loss of the cAMP/PKA-responsive motif in HDAC7 (but not in other class IIa HDACs) might have been selected for.

Conclusion. We have shown that cAMP/PKA signaling promotes nuclear localization of HDAC4, 5, and 9, but not HDAC7. The reason for this differential effect is a novel phosphorylation site that is present in HDAC4, 5, and 9, but is absent from HDAC7. Mutation of this site (S266A in HDAC4) or of the Pro immediately downstream from it (P267A in HDAC4) prevents cAMP/PKA-mediated nuclear localization of HDAC4. Mutation of this region in HDAC7 to match the sequence in HDAC4/5 (HDAC7 K196S/N197S) enhances the ability of HDAC7 to respond to cAMP/PKA signaling. Using a novel phospo-S266 antibody, we show that cAMP treatment reduces S266 phosphorylation. This effect was also seen with ACTH, a hormone that acts through cAMP. Moreover,

phosphorylation of the 14-3-3 binding site S246 is crucial for basal S266 phosphorylation status, suggesting hierarchical multisite phosphorylation of class IIa HDACs. Given the importance of cAMP/PKA signaling in a diverse range of physiological processes, further investigation of this novel regulatory mechanism may help to uncover previously unrecognized biological roles of class IIa HDACs.

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3.9 FIGURE LEGENDS AND FIGURES

Figure 1. Effect of PKA on class IIa HDAC localization is independent of SIK inhibition. (A) Immunofluorescence microscopy of endogenous HDAC4 subcellular localization in C2C12 cells using anti-HDAC4 antibody following 30 min treatment with 1 mM 8-Br-cAMP. (B) C2C12 cells (*left*) or HEK293 cells (*right*) were transiently transfected with a GFP-HDAC4 expression plasmid, together with either pCDNA empty vector or an expression plasmid encoding PKA. GFP-HDAC4 localization was visualized using green fluorescence microscopy. (C) As in (B), except HEK293 cells were transiently transfected with a GFP-HDAC5 expression plasmid together with pCDNA empty vector, or an expression plasmid encoding SIK2 (*top*) or SIK3 (*bottom*), with or without an expression plasmid for PKA. (D) As in (B) except HEK293 cells were transiently transfected with a GFP-HDAC7 expression plasmid together with pCDNA empty vector or a PKA expression plasmid. (E) As in (C, *top*) except a GFP-HDAC9 expression plasmid was transfected. (F) HEK293 cells were co-transfected with GFP-HDAC5 and the indicated SIK2 mutant expression plasmid (SIK2 T484A, SIK2 S587A, or SIK2 T484A/S587A) with (*bottom*) or without (*top*) a PKA expression plasmid. (G) As in (A) except that LKB1-negative HeLa cells were used for immunofluorescence microscopy.

Figure 1

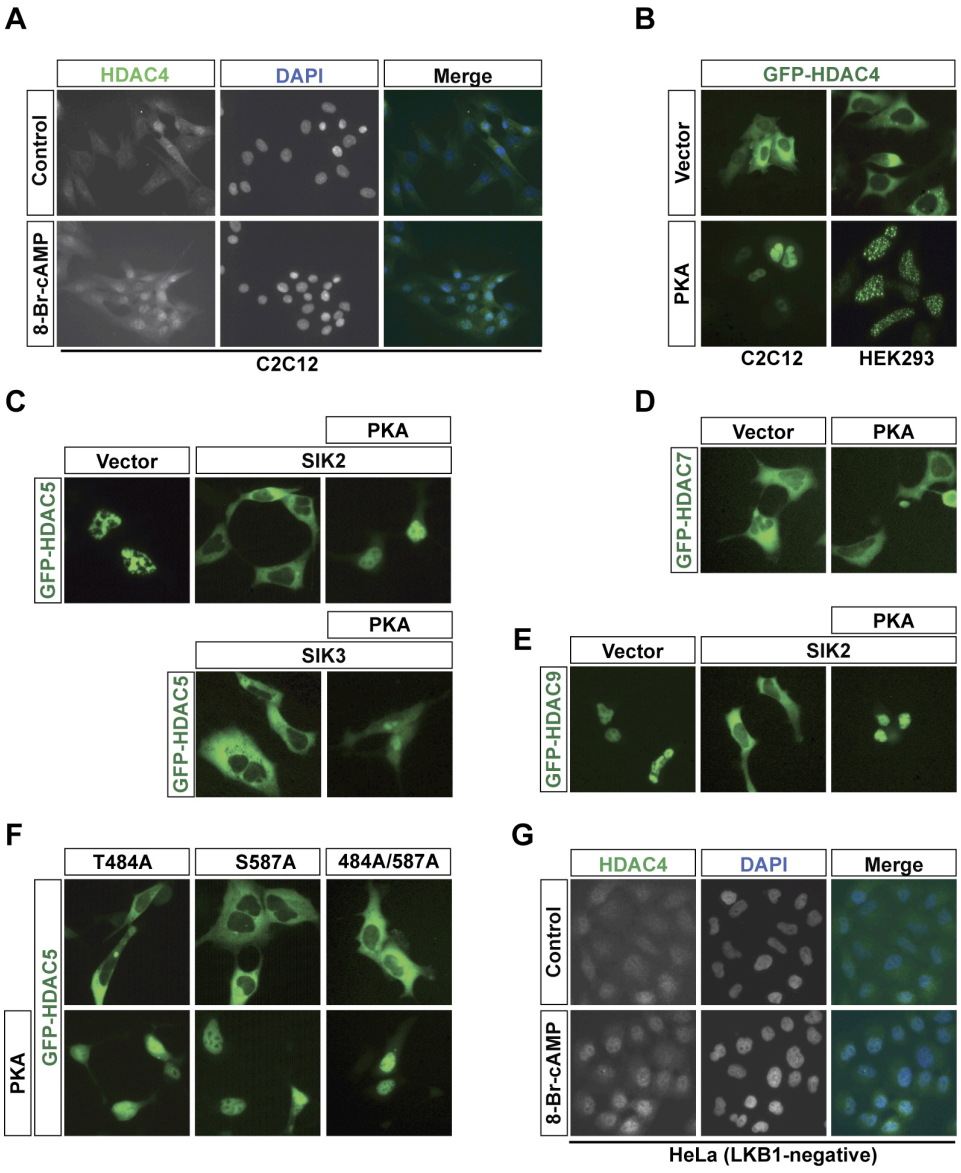

A
HDAC4
DAPI
Merge
Control
8-Br-cAMP
C2C12
B
GFP-HDAC4
Vector
PKA
C2C12
HEK293
C
PKA
Vector
SIK2
GFP-HDAC5
PKA
SIK3
GFP-HDAC5
D
Vector
PKA
GFP-HDAC7
E
PKA
Vector
SIK2
GFP-HDAC9
F
T484A
S587A
484A/587A
PKA
GFP-HDAC5
G
HDAC4
DAPI
Merge
Control
8-Br-cAMP
HeLa (LKB1-negative)

Figure 2. cAMP/PKA effect on HDAC4 localization depends on novel regulatory motif. (A) Sequence alignment of 14-3-3 binding site and nuclear localization signal (NLS) of human class IIa HDACs. Identical residues are highlighted in dark grey boxes, while similar residues are highlighted in light grey boxes. S246 of the 14-3-3 binding site and S266 (of the PKA consensus phosphorylation site, RRSS) are presented in white text. (B) HEK293 cells were transiently transfected with an expression plasmid encoding GFP-HDAC4 S266A together with pCDNA empty vector or a PKA expression plasmid. (C) C2C12 cells were transfected with GFP-HDAC4 WT or S266A expression plasmids and then treated with 1 mM 8-Br-cAMP for 30 min or 10 ng/ml leptomycin B (LMB) or vehicle (70% MeOH) for 1 h before HDAC4 localization was observed by green fluorescence microscopy. (D) Quantification of (C). At least 100 cells were counted for each condition for 3 independent experiments. *** indicates $p<.001$ vs. S266A for cAMP, ** indicates $p<.01$ vs. S266A for LMB, $$$ indicates $p<.001$ vs. S266A for vehicle and S266A for cAMP. (E) As in (C) except expression plasmids for GFP-HDAC4 S266D, S265A, and P267A were transfected prior to 8-Br-cAMP treatment. (F) As in (C) except expression plasmids for GFP-HDAC4 S266D and P267A were transfected prior to LMB treatment. (G) As in (C) except GFP-HDAC4 WT, S266A, or S266D was cotransfected with empty vector (*top*) or a MEF2D expression plasmid (*bottom*).

Figure 2

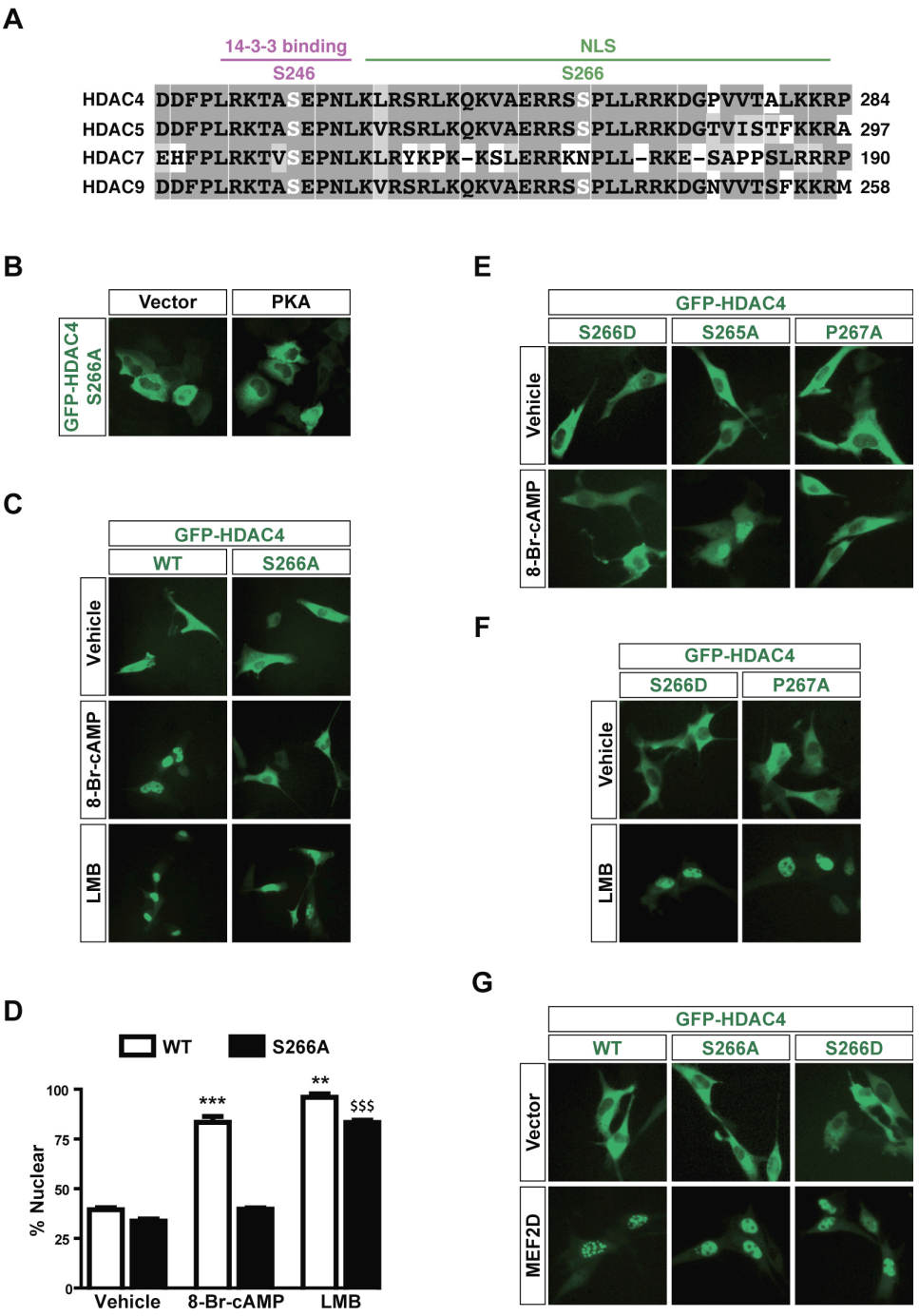

A
14-3-3 binding
S246
NLS
S266
HDAC4 DDFPLRKTASEPNLKLRSRLKQKVAERRSSPLLRRKDGPVVTALKKRP 284
HDAC5 DDFPLRKTASEPNLKVRSRLKQKVAERRSSPLLRRKDGTVISTFKKRA 297
HDAC7 EHFPLRKTVSEPNLKLRYKPK-KSLERRKNPLL-RKE-SAPPSLRRRP 190
HDAC9 DDFPLRKTASEPNLKVRSRLKQKVAERRSSPLLRRKDGNVVTSFKKRM 258
B
Vector
PKA
GFP-HDAC4 S266A
C
GFP-HDAC4
WT
S266A
Vehicle
8-Br-cAMP
LMB
D
WT
S266A
% Nuclear
100
75
50
25
0
***
**
$$$
Vehicle
8-Br-cAMP
LMB
E
GFP-HDAC4
S266D
S265A
P267A
Vehicle
8-Br-cAMP
F
GFP-HDAC4
S266D
P267A
Vehicle
LMB
G
GFP-HDAC4
WT
S266A
S266D
Vector
MEF2D

Figure 3. cAMP/PKA-mediated inhibition of HDAC5/7 nuclear export depends on the same mechanism as HDAC4. (A) C2C12 cells were cotransfected with expression plasmids for GFP-HDAC5 WT or S279A along with empty vector or a SIK2 S587A expression plasmid. Cells were then treated with vehicle (H_2O) or 1 mM 8-Br-cAMP for 30 min, followed by green fluorescence microscopy. (B) Quantification of (A). At least 100 cells were counted for each condition for 3 independent experiments. *** indicates $p<.001$ vs. WT under same condition, ### indicates $p<.001$ vs. S279A under vehicle condition with empty vector transfected, $$$ indicates $p<.001$ vs. WT in two other conditions. (C) As in (A) except GFP-HDAC5 S279A was cotransfected with empty vector (*left*) or SIK2 S587A (*right*), prior to treatment with vehicle (70% MeOH, *top*) or 10 ng/ml LMB (*bottom*) for 1 h. (D) HEK293 cells were transfected with expression plasmids for GFP-HDAC5 S279A or S279D together with empty vector or SIK2 T484A/S587A with or without PKA. (E) As in (C), except a GFP-HDAC5 S279D expression plasmid was used. (F) C2C12 cells were transfected with expression plasmids for GFP-HDAC7 WT, N197S, or K196S/N197S, prior to treatment with 8-Br-cAMP.

Figure 3

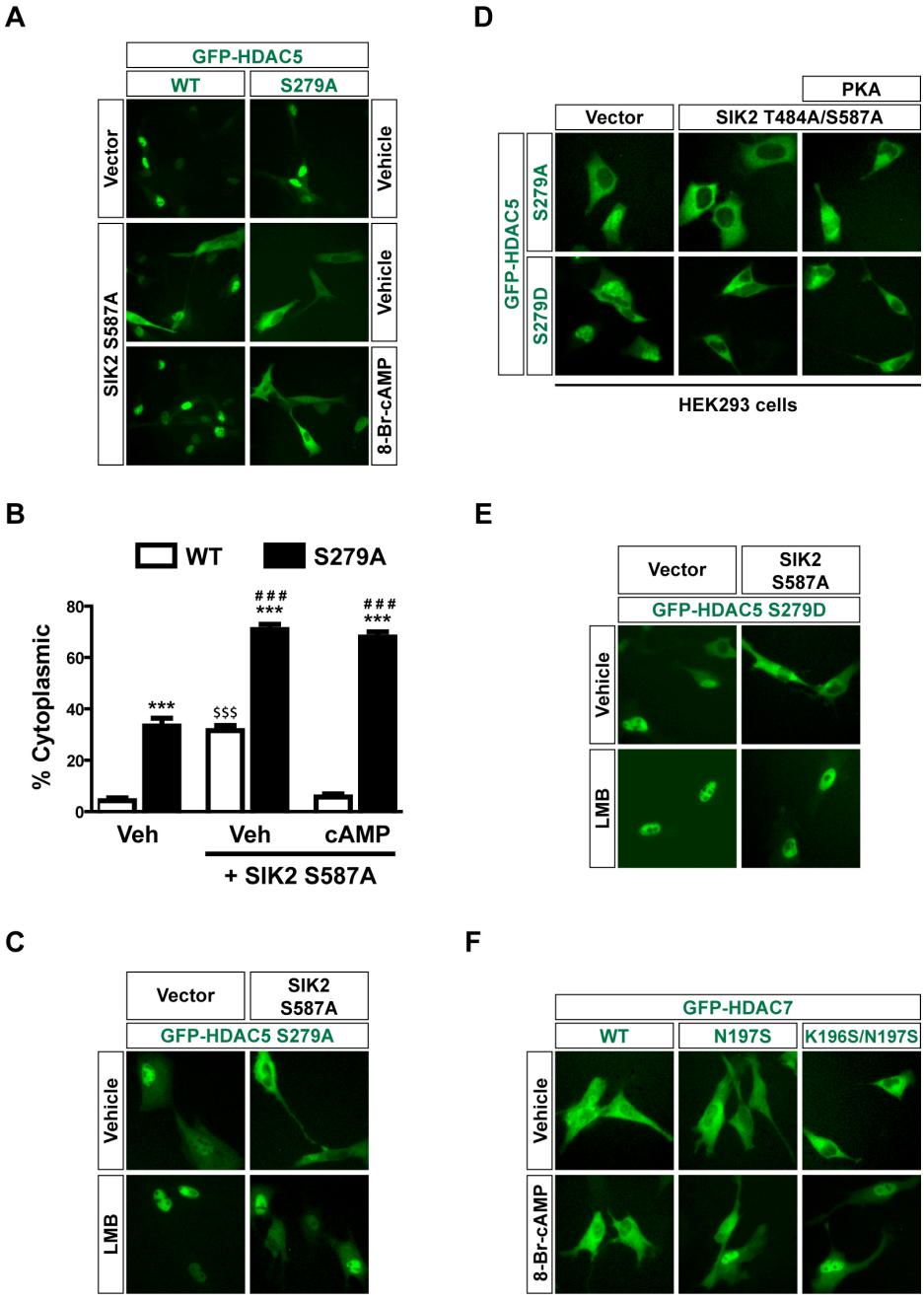

A
GFP-HDAC5
WT
S279A
Vector
SIK2 S587A
Vehicle
Vehicle
8-Br-cAMP
D
PKA
Vector
SIK2 T484A/S587A
GFP-HDAC5
S279A
S279D
HEK293 cells
B
WT
S279A
% Cytoplasmic
80
60
40
20
0
***
$$$
###
***
###
***
Veh
Veh
cAMP
+ SIK2 S587A
E
Vector
SIK2 S587A
GFP-HDAC5 S279D
Vehicle
LMB
C
Vector
SIK2 S587A
GFP-HDAC5 S279A
Vehicle
LMB
F
GFP-HDAC7
WT
N197S
K196S/N197S
Vehicle
8-Br-cAMP

Figure 4. H-89 treatment inhibits cAMP-mediated nuclear localization of HDAC4. C2C12 cells were transiently transfected with expression plasmids for GFP-HDAC4 WT, S266A, or S246A. Cells were pre-treated with vehicle (H_2O), the PKA inhibitor H-89 (10 μM), or the PP2A inhibitor okadaic acid (OA; 100 nM) for 1 h, followed by treatment with vehicle (H_2O) or 1 mM 8-Br-cAMP for 30 min. Subcellular localization of the GFP-HDAC4 constructs was monitored by green fluorescence microscopy.

Figure 4

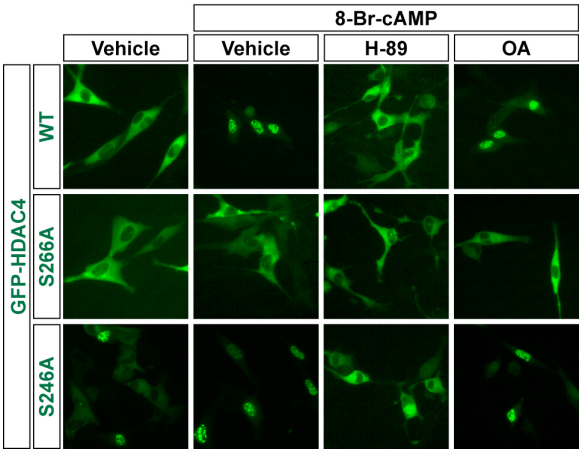


Figure 5. S266 is a novel HDAC4 phosphorylation site regulated by cAMP signaling. (A) Different amounts of regular (*top*) or phospho-peptide (*bottom*), in which S266 is phosphorylated, were spotted on a nitrocellulose membrane, blocked for 2 h in PBS-T containing 20% horse serum and then incubated overnight at 4°C with anti-phospho-S266 (HDAC4) primary antibody (1:5000 dilution). (B) HEK293 cells were transfected with an expression plasmid for Flag-HDAC4 WT or S266A. Forty-eight hours later, cells were harvested in buffer K and Flag-tagged proteins were immunoprecipitated with M2 agarose beads and eluted with Flag peptide. Immunoprecipitates (IP) were separated by SDS-PAGE and immunoblotting was performed with anti-phospho-S266 or anti-Flag antibodies. (C) C2C12 cells were transfected with a Flag-HDAC4 expression plasmid, and treated with DMSO (lane 1), 1 mM 8-Br-cAMP (lane 2), or 10 μM Forskolin (lane 3) for 30 min. Cells were harvested and IPs and immunoblots were performed as in (B). (D) As in (C) except cells were cultured in growth medium (GM), containing 10% FBS, or in differentiation medium (DM), containing 2% horse serum, for 3 h prior to 8-Br-cAMP treatment. (E) C2C12 cells were treated with DMSO (lane 1), 1 mM 8-Br-cAMP (lane 2), or 10 μM Forskolin (lane 3) for 30 min. Whole cell extracts were subject to SDS-PAGE and immunoblotting was performed using anti-phospho-S266, anti-phospho-S246, or anti-HDAC4 primary antibodies. Position of bands representing HDAC4 or HDAC7 detected by the anti-phospho-S246 antibody is indicated to the immediate right of the blot. Protein size in kilodaltons (kDa) is indicated to the left side of the blots. (F) C2C12 cells were treated with vehicle

(H_2O) for 30 min or 1 mM 8-Br-cAMP for the indicated times (in min). Immunoblotting of endogenous proteins was performed as in (E).

Figure 5

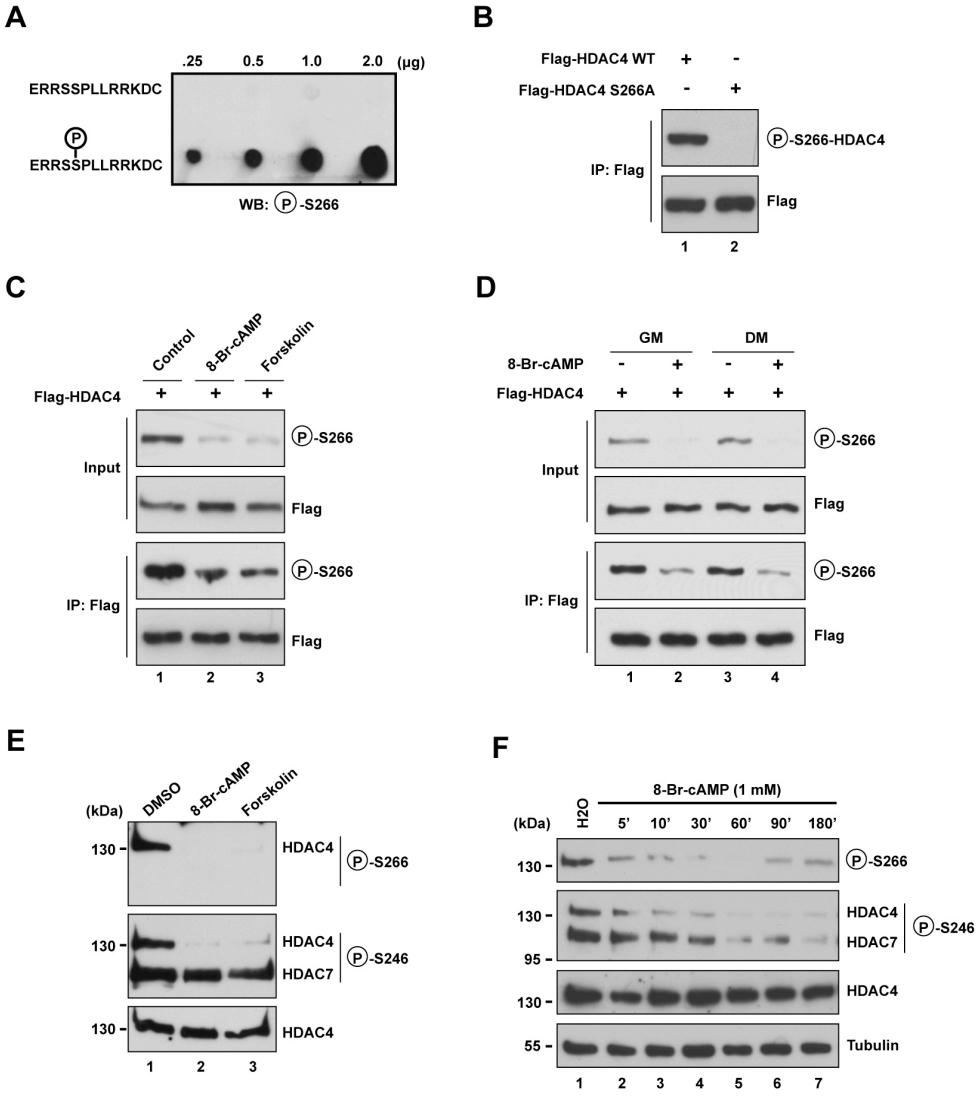


Figure 6. Crosstalk between S246 and S266 phosphorylation. (A) C2C12 cells were treated with vehicle (70% MeOH) or 10 ng/ml leptomycin B for 1 h. Whole cell extracts were separated by SDS-PAGE and immunoblotting was performed using anti-phospho-S266, anti-phospho-S246, or anti-HDAC4 antibodies. Position of bands representing HDAC4 or HDAC7 detected by the anti-phospho-S246 antibody is indicated to the immediate right of the blot. Protein size in kilodaltons (kDa) is indicated to the left side of the blots. (B) Time course experiment as in (Figure 5F) except that the vehicle (70% MeOH) treatment was for 1 h and 10 ng/ml leptomycin B was used instead of 8-Br-cAMP. (C) C2C12 cells were transfected with expression plasmids for Flag-tagged HDAC4 WT, S266A, or S246A, and treated with 1 mM 8-Br-cAMP (lane 2, 4, 6) or vehicle (H_2O; lane 3, 5, 7). Cells were harvested in buffer K and Flag-tagged proteins were immunoprecipitated with M2 agarose beads and eluted with Flag peptide. Immunoprecipitates (IP) were separated by SDS-PAGE and immunoblotting was performed with anti-phospho-S266, anti-phospho-S246, or anti-Flag antibodies. (Low), low exposure time; (High), high exposure time. (D) As in (C) except Flag-HDAC4 WT, S266A, and S246/467/632A (triple mutant; TM) expression plasmids were transfected. (E) C2C12 cells were transfected with expression plasmids for GFP-HDAC4 S246A or TM. Subcellular localization was monitored by green fluorescence microscopy. (F) Schematic of crosstalk between S246 and S266 phosphorylation. S246 (as well as S467 and S632) phosphorylation is mediated by kinases such as CaMKs, PKDs, and SIKs. Phosphorylation of S246 stimulates phosphorylation of S266.

Figure 6

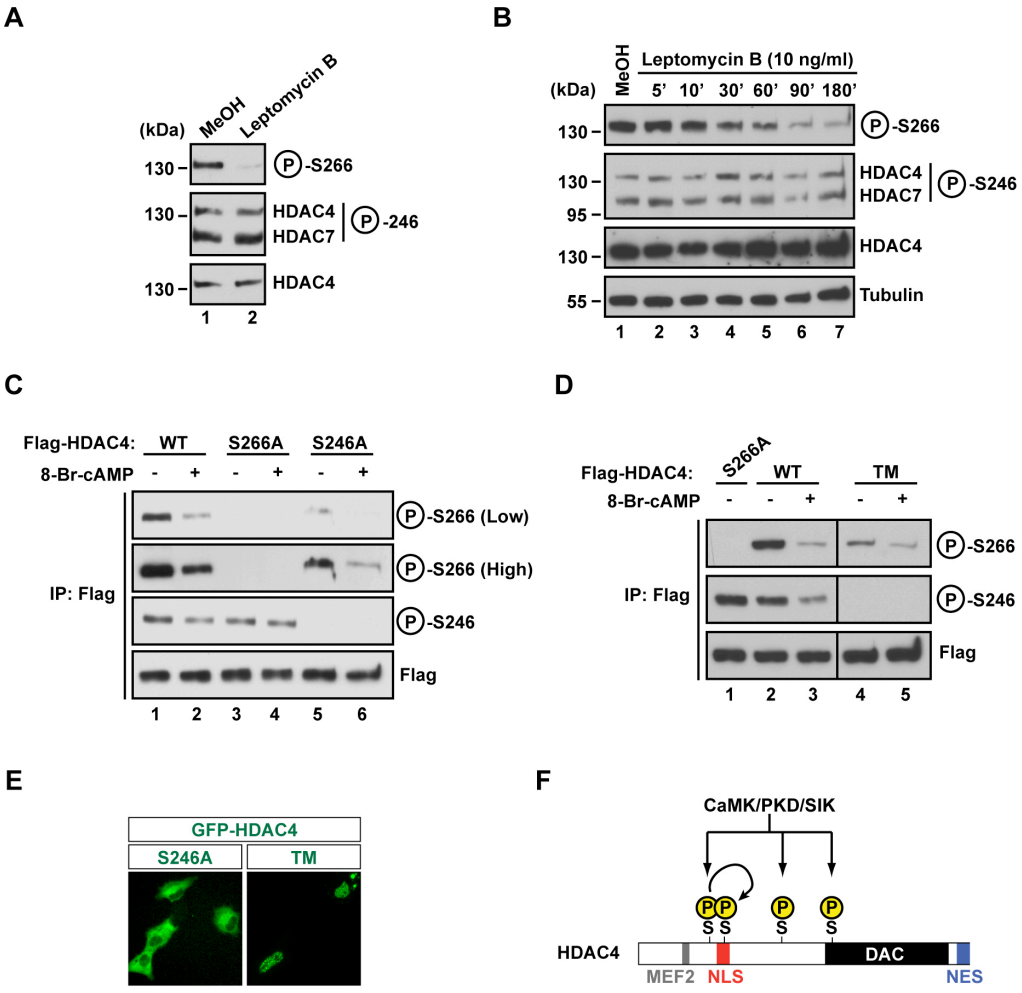
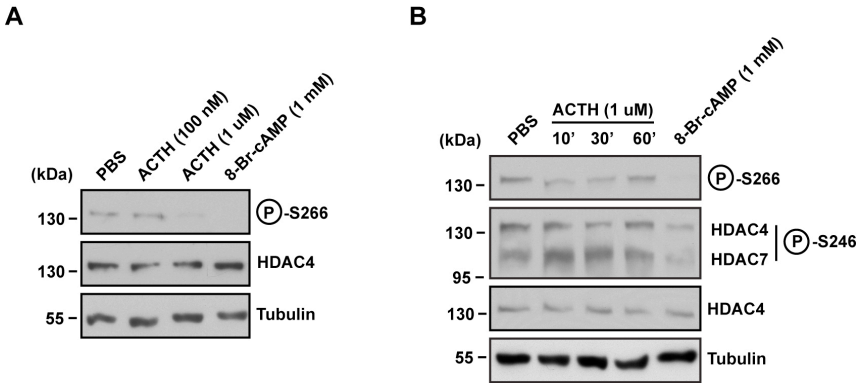

A
(kDa)
MeOH
Leptomycin B
130
P-S266
130
HDAC4
HDAC7
P-246
130
HDAC4
1 2
B
MeOH
Leptomycin B (10 ng/ml)
(kDa)
5' 10' 30' 60' 90' 180'
130
P-S266
130
95
HDAC4
HDAC7
P-S246
130
HDAC4
55
Tubulin
1 2 3 4 5 6 7
C
Flag-HDAC4: WT S266A S246A
8-Br-cAMP - + - + - +
IP: Flag
P-S266 (Low)
P-S266 (High)
P-S246
Flag
1 2 3 4 5 6
D
Flag-HDAC4: S266A WT TM
8-Br-cAMP - - + - +
IP: Flag
P-S266
P-S246
Flag
1 2 3 4 5
E
GFP-HDAC4
S246A
TM
F
CaMK/PKD/SIK
P P P P
S S S S
HDAC4
DAC
MEF2
NLS
NES

Figure 7. ACTH affects endogenous S266 phosphorylation level in Y1 cells. (A) Y1 cells were treated with vehicle (PBS) or different concentrations (100 nM or 1 μM) of adrenocorticotropic hormone (ACTH), or 1 mM 8-Br-cAMP for 30 min. Whole cell extracts were subject to SDS-PAGE and immunoblotting was performed using anti-phospho-S266, anti-HDAC4, or anti-α-tubulin primary antibodies. Protein size in kilodaltons (kDa) is indicated to the left side of the blots. (B) Y1 cells were treated with vehicle (PBS) for 60 min, with 1 μM ACTH for different times (10, 30, or 60 min), or with 1 mM 8-Br-cAMP for 30 min. Immunoblotting was performed as in (A) except that anti-phospho-S246 antibody was also used. Position of bands representing HDAC4 or HDAC7 detected by the anti-phospho-S246 antibody is indicated to the immediate right of the blot.

Figure 7



Supplemental Figure S1

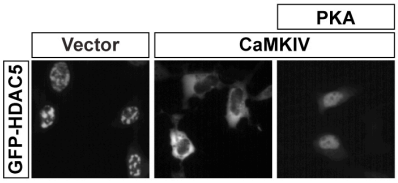


Fig. S1. PKA reverses CaMKIV-mediated nuclear export of HDAC5.

HEK293 cells were transiently transfected with GFP-HDAC5 together with empty vector, or expression plasmids for CaMKIV, or CaMKIV and PKA.

Supplemental Figure S2

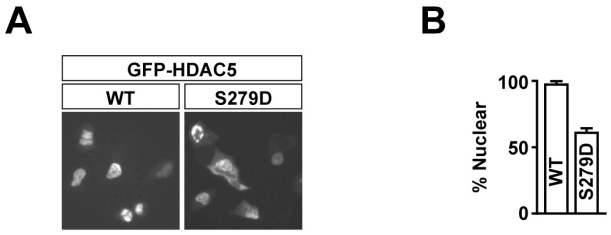


Fig. S2. HDAC5 S279D is less nuclear than wild-type HDAC5.

(A) Representative fluorescence microscopy images of GFP-HDAC5 WT or GFP-HDAC5 S279D following transient transfection in HEK293 cells.
(B) Quantification of GFP-HDAC5 WT and GFP-S279D subcellular localization following transient transfection in C2C12 cells. At least 100 cells were counted for each condition over 2 independent experiments. Data are presented as mean +/- SD (error bars).

Supplemental Figure S3

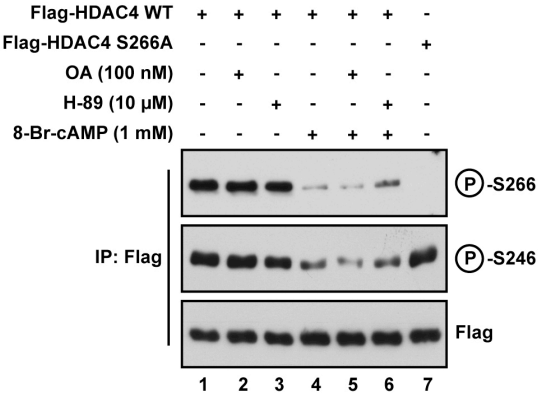


Fig. S3. Effect of OA and H-89 treatment on S266 phosphorylation.

C2C12 cells were transiently transfected with Flag-HDAC4 WT or Flag-HDAC4 S266A. The following day, cells were pre-treated with 100 nM okadaic acid (OA) or 10 μM H-89 for 1 h, followed by treatment with vehicle (H2O) or 1 mM 8-Br-cAMP for 30 min, as indicated. Cells were harvested and Flag-tagged proteins were immunoprecipitated with M2 agarose beads. Immunoblotting was performed using antibodies directed agains the indicated proteins.

Supplemental Figure S4

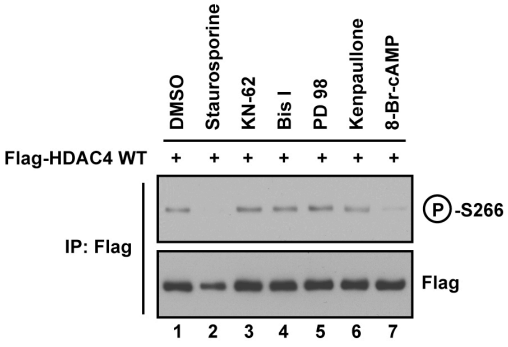


Fig. S4. Effect of various kinase inhibitors on S266 phosphorylation.

C2C12 cells were transiently transfected with Flag-HDAC4 WT. The following day, cells were treated with either DMSO, 8-Br-cAMP, or inhibitors of the following kinases: general S/T kinases (Staurosporine, 1 μM), CaMK (KN-62, 10 μM), PKC (Bis I, 10 μM), MEK/ERK (PD 98059 10 μM), or GSK-3β (Kenpaullone 5 μM). Cells were harvested and Flag-tagged proteins were immunoprecipitated with M2 agarose beads. Immunoblotting was performed using antibodies directed against the indicated proteins

Supplemental Figure S5

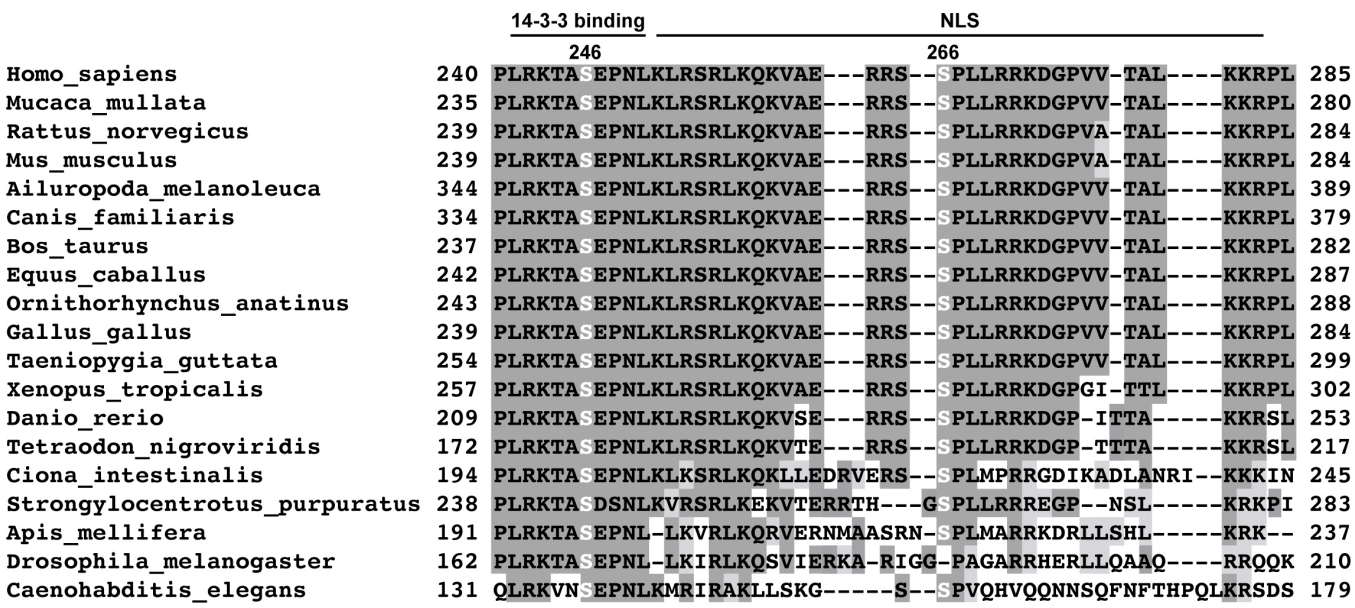


Figure S5. Comparison of the HDAC4 NLS region from different organisms.
After an initial BLAST search for HDAC4 homologues from different species, amino acid sequences encompassing the first 14-3-3 binding site and the adjacent NLS region were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The aligned file was imported to Adobe Illustrator CS4 for further editing. Identical residues are highlighted by dark grey boxes while similar residues are shaded by light grey boxes. The S246 14-3-3 binding site and the PKA/cAMP-sensitive phosphorylation site S266 are each depicted as a white"S". Genbank accession numbers used for the alignment are: H. sapiens (NP_006028); M. mulatta (XP_001099472); R. norvegicus (XP_001067733); M. musculus (NP_997108); A. melanoleuca (XP_002922520); C. familiaris (XP_849514); B. taurus (XP_002686643); E. caballus (XP_001497201); O. anatinus (XP_001510939); G. gallus (NP_989644); T. guttata (XP_002190608); X. tropicalis (XP_002932071); D. rerio (NP_001034447); T. nigroviridis (CAF98995); C. intestinalis (XP_002128142); S. purpuratus (XP_797761); A. mellifera (XP_391882); D. melanogaster (NP_727682); C. elegans (AAZ82858).

Supplemental Figure S6

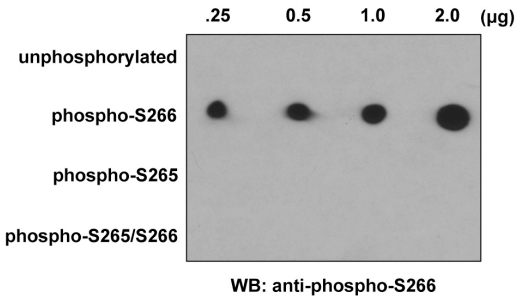


Fig. S6. HDAC4 S265 phosphorylation interferes with anti-phospho-S266 antibody recognition.

The indicated peptides were spotted on membranes at the indicated amounts for blotting with the purified anti-phospho-S266 (HDAC4) primary antibody. The peptides correspond to the peptide used for producing the antibody (see Materials and Methods), however, their phosphorylation status is altered as indicated. The anti-phospho-S266 antibody only recognizes the peptide phosphorylated solely on S266, while it does not recognize the unphosphorylated peptide or peptides with either S265 alone phosphorylated or both S265 and S266 phosphorylated.

CHAPTER IV

4.0 GENERAL DISCUSSION

Of the 18 human HDACs, the class IIa members HDAC4, 5, 7, and 9 are unique in their function as novel signal transducers. These HDACs shuttle between the nucleus and the cytoplasm in response to phosphorylation of 14-3-3 binding sites by various kinases. Thus, class IIa HDAC-mediated gene repression is signal-dependent and finely tuned by an array of extracellular signals. In Chapters II and III, I characterized novel mechanisms - involving the tumour suppressor kinase LKB1 (Chapter II) and the archetypal signaling kinase PKA (Chapter III) - that control class IIa HDAC nucleocytoplasmic shuttling in muscle and cancer cells (Fig. 1). These findings point to class IIa HDACs as potential mediators of some of the physiological and pathological processes controlled by LKB1 and PKA, and also provide novel insight into the molecular events that control class IIa HDACs. In this section I will discuss the implications of my findings and raise questions that may guide future research into these topics.

4.1 Identification of SIK2 and SIK3 as class IIa HDAC kinases

In Chapter II, I identified SIK2 and SIK3 as novel class IIa HDAC kinases. Overexpression of SIK2 or SIK3 induced nuclear export of all class IIa HDACs, with the most dramatic effects seen for the basally nuclear HDAC5 and HDAC9 (Ch. II Fig. 1A). I confirmed that this effect was due to

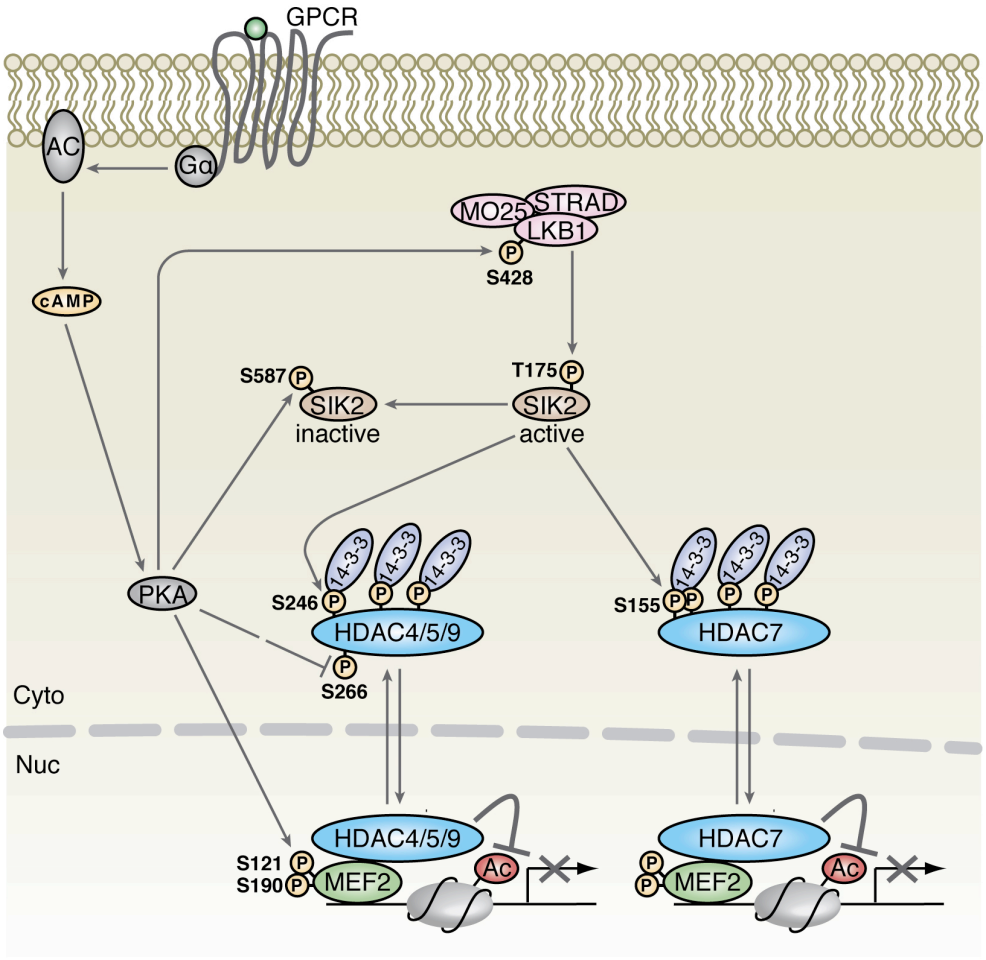


Figure 1. Summary of major thesis findings. I found that upon phosphorylation by LKB1, SIK2 (and SIK3) become activated and phosphorylate class IIa HDACs and induce 14-3-3 binding and nuclear export. This prevents these HDACs from repressing MEF2-dependent transcription. I also found that PKA-mediated phosphorylation of SIK2 on S587 prevents the ability of SIK2 to induce nuclear export of these HDACs. I further showed that PKA, which is activated by cAMP produced by adenylyl cyclase (AC) in response to ligand binding to G protein-coupled receptors (GPCRs), prevents the nuclear export of HDAC4, 5, and 9, but not HDAC7. This is due to the ability of PKA to decrease HDAC4 S266 phosphorylation. PKA has also been reported to phosphorylate LKB1 on S428, although the exact consequences of this phosphorylation event remain unclear, and PKA can inhibit MEF2 activity through direct phosphorylation of MEF2D on S121 and S190. See text for details.

phosphorylation-dependent 14-3-3 binding by demonstrating that SIK2 and SIK3 phosphorylatedd HDAC4 and 5 *in vitro* and *in vivo* on the N-terminal 14-3-3 binding site (S246 in HDAC4 and S259 in HDAC5) and promoted 14-3-3 binding to HDAC4 and HDAC5 (Ch. II Fig. 2). Since MEF2 transcription factors are the major targets of class IIa HDAC repressive activity, stimulation of MEF2 activity is another expected property of class IIa HDAC kinases. SIK2 derepressed MEF2 activity in the presence of HDAC4, 5, 7, and 9 (Ch. II Fig. 4), and also rescued HDAC4-mediated repression of myogenesis (Ch. II Fig. 5). Moreover, kinase-dead SIK2 could not induce nuclear export (Ch. II Fig. 1B) or 14-3-3 binding (Ch. II Fig. 2A), while the 14-3-3-binding-defective HDAC4 S246/467/632A mutant was refractory to SIK2-mediated cytoplasmic localization and derepression of MEF2 activity (Ch. II Fig. 3A and 4A).

Unexpectedly, I observed several unusual properties of SIK3 in the context of class IIa HDAC regulation. First, kinase-dead SIK3 still induced nuclear export of HDAC5 and HDAC9 (Ch. II Fig. 1B), suggesting that its kinase activity is dispensible for its effect on HDAC shuttling. Second, SIK3 induced nuclear export of the purportedly constitutively nuclear HDAC4 S246/467/632A mutant (Ch. II Fig. 3). To my knowledge, no other kinase has been reported to possess this ability. Furthermore, this result suggests that class IIa HDAC nuclear export can be regulated independently of phosphorylation-dependent 14-3-3 binding. Third, despite the potent ability of SIK3 to induce class IIa HDAC nuclear export and phosphorylation, SIK3 was unable to derepress MEF2 activity in the presence of these HDACs. This result is just as surprising as the first two, and may point to a novel mechanism for MEF2 regulation. I tested a few

hypotheses to explain these unexpected findings but the mechanism involved remains to be uncovered. Future work on this issue will involve testing SIK3 mutants to see if the long C-terminal extension (that is not possessed by SIK1 or SIK2) is responsible. Since these effects are opposite to those expected for canonical class IIa HDAC trafficking and MEF2 derepression, answers to these questions may broaden our understanding of this important signaling axis.

4.2 AMPK-related kinases control class IIa HDAC shuttling

Before I started working on this project, MARK2 and MARK3 had been identified as class IIa HDAC kinases (7, 9), and during my studies, SIK1 and AMPK were added to this list (3, 21). Although overexpression of these kinases did not induce HDAC5 nuclear export in my hands (Ch. II Fig. 1 and data not shown), it is clear that several AMPK-related kinases can influence class IIa HDAC phosphorylation and/or localization under certain circumstances. Thus, with my work identifying SIK2 and SIK3, nearly half of the AMPK-related kinases (SIK1, SIK2, SIK3, AMPK, MARK2, and MARK3) have been reported as class IIa HDAC kinases. Of note, during the final preparation of this thesis, SIK3 was reported as a class IIa HDAC kinase in *Drosophila* and mammalian cells by another group (33). Similar substrate specificity is a common theme of AMPK-related kinase function (see section 1.4.6 of Chapter I) that may permit distinct members to perform this function in different tissues and/or in response to different stimuli. Thus, it will be important to test which of these kinases is involved in regulating class IIa HDAC shuttling during specific physiological and pathological processes in different tissues.

4.3 Role of LKB1 in class IIa HDAC subcellular trafficking

Importantly, all the AMPK-related kinases identified as class IIa HDAC kinases rely on LKB1-mediated T-loop phosphorylation for their activity (19). Indeed, during the final preparation of this thesis, a report was published showing that phosphorylation of class IIa HDACs is dependent on LKB1 expression in the liver (22), thus confirming my results obtained in lung cancer cells and *Lkb1*$^{-/-}$ MEFs. As shown in Chapter II, I found that HDAC4 phosphorylation and subcellular localization was dependent on expression of the tumour suppressor LKB1. Specifically, HDAC4 was more nuclear in *Lkb1*$^{-/-}$ MEFs expressing empty vector compared to *Lkb1*$^{-/-}$ MEFs re-expressing LKB1 (Ch. II Fig. 7). In addition, HDAC4 S246 phosphorylation status was sensitive to LKB1 expression in lung cancer cell lines. Ectopic expression of LKB1 in the LKB1-negative A549 lung cancer cell line increased endogenous HDAC4 S246 phosphorylation. Conversely, shRNA-mediated knockdown of LKB1 from the LKB1-positive H1299 lung cancer cell line reduced endogenous HDAC4 S246 phosphorylation (Ch. II Fig. 7). These results suggest that class IIa HDAC shuttling may be an important downstream event in cancers with loss of LKB1 expression or function. Related to this, several studies have indicated an emerging role for class IIa HDACs in tumourigenesis.

4.4 Class IIa HDACs in cancer

Recent studies have implicated dysregulation of chromatin-modifying proteins such as HDACs in tumourigenesis. For instance, overexpression of specific HDACs occurs in several cancer types (5), and histone hypoacetylation is

characteristic of most cancers (4). Accordingly, several HDAC inhibitors have entered the clinical trial stage for a variety of cancer indications (12). Although many effects of HDAC inhibitors are attributable to the inhibition of class I HDACs, recent findings suggest that class IIa HDACs are also important for tumourigenesis.

First, MEF2 transcription factors, the major targets of class IIa HDAC function, may be involved in tumourigenesis. For example, *Mef2C* and *Mef2D* have been identified as novel candidate cancer genes in mouse genome-wide transposon mutagenesis studies (20, 28, 32), while the human *MEF2D* gene is rearranged in acute lymphoblastic leukemia (ALL), leading to enhanced transcriptional activity and increased ability to promote cell growth (27, 35). Second, *Hdac7* was also identified as a candidate cancer gene in a mouse transposon mutagenesis screen (28), and altered expression of class IIa HDACs has been observed in several cancer types. Expression of HDAC5 and HDAC9 is associated with poor survival in medulloblastoma patients (23), while higher expression of HDAC7 and HDAC9 is associated with poor prognosis in childhood ALL (24). HDAC7 is upregulated in 81% of pancreatic adenocarcinomas, while it is reduced in other pancreatic cancer subtypes (26). In contrast, HDAC4 is downregulated in chondrosarcoma cells, leading to increased expression of VEGF, a critical factor for tumour growth and metastasis (31).

Third, experimental manipulation of class IIa HDAC expression has dramatic effects on cancer cell function. For instance, siRNA-mediated knockdown of HDAC4 induces growth arrest and apoptosis of cancer cells *in vitro* (6, 25, 34), and impairs the growth of xenograft tumours derived from

human colon and glioblastoma cells (25, 34). Similarly, knockdown of HDAC5 and HDAC9 decreases the growth and viability of medulloblastoma cells (23), while HDAC7 knockdown in various cancer cell lines results in G1/S cell cycle arrest and senescence (37).

Although signal-dependent nucleocytoplasmic shuttling is the dominant mode of class IIa HDAC regulation, few studies have specifically examined this trafficking in cancer. However, it has been reported that an oncogenic Ras mutant stimulates nuclear localization of HDAC4 (36), and DNA damaging agents induce nuclear import of HDAC4, which may be important for DNA repair and G2/M cell cycle arrest (2, 11). In addition, HDAC4 shuttles to the nucleus and the cytoplasm in response to androgens and anti-androgens, respectively, in hormone-sensitive prostate cancer cells. However, in hormone-refractory prostate cancer HDAC4 is constitutively nuclear (16). HDAC7 also shuttles from the cytoplasm to the nucleus in response to hormone occupancy of the androgen receptor (17). Thus, class IIa HDAC shuttling may be involved in some cancers, and based on my results with *Lkb1*$^{-/-}$ MEFs and lung cancer cells, it will be interesting to investigate the phosphorylation and subcellular localization of class IIa members in tumour samples from patients with *LKB1* mutations.

4.5 Regulation of class IIa HDACs by cAMP/PKA signaling

In Chapter II, I demonstrated that PKA prevents SIK2 and SIK3 from inducing nuclear export of class IIa HDACs (Ch. II Fig. 6). This was expected based on the fact that PKA-mediated phosphorylation of SIK2 on S587 prevents SIK2 from phosphorylating CRTC and inducing its nuclear export (29). During

the course of this study other papers reported that PKA had this effect on SIK1-mediated HDAC phosphorylation and export (3, 13, 18). Together, these results show that class IIa HDAC localization is not only the result of a balance between kinase and phosphatase activity, but that different kinases can have opposite effects on nuclear export. In the case of the LKB1-SIK-HDAC axis, this is particularly interesting since PKA can also phosphorylate LKB1 on S428 (see section 1.3.4.2 of Chapter I). Although the consequence of this phosphorylation event remains unclear, it suggests that PKA may target this signaling pathway at more than one point. In fact, PKA can also phosphorylate MEF2D directly, and this is at least partially responsible for the repression of MEF2 activity and myogenesis associated with cAMP/PKA signaling (10).

However, while working on this project I observed that PKA could only promote the nuclear localization of HDAC4, 5, and 9, while HDAC7 appeared to be insensitive to PKA (Chapter III Fig. 1). Since HDAC7 responds to SIK2 and SIK3 just like the other class IIa members do, this result suggested that the effect of PKA on HDAC localization was independent of SIK inhibition. Based on this observation, I decided to test the effect of PKA on HDAC5 nuclear export induced by the PKA-resistant SIK2 S587A mutant. In this setting PKA still induced full nuclear localization of HDAC5 (Chapter III Fig. 1).

4.6 Identification of a novel PKA-sensitive phosphorylation event

Next we noticed that there are two consensus PKA phosphorylation sites in class IIa HDACs. One of these, T707 of HDAC4 is also found in the other class IIa members, but the other one, S266 of HDAC4 is only present in HDAC5

and 9. Thus, the S266 site satisfies the criterion that whatever the underlying mechanism, it should affect HDAC4, 5, and 9, but not HDAC7. In fact, S266 turned out to be the key to the differential regulation of class IIa HDACs by PKA. In Chapter III, I showed that an HDAC4 S266A mutant is fully cytoplasmic even in the presence of overexpressed PKA and in response to 8-Br-cAMP treatment (Ch. III Fig. 2). The equivalent mutation in HDAC5 (S279A) also prevented PKA- or 8-Br-cAMP-induced nuclear localization (Ch. III Fig. 3). Interestingly, these mutants still accumulated in the nucleus in response to leptomycin B treatment, indicating that this residue does not control general nuclear import (Ch. III Fig. 2 and 3). Instead, S266 appears to be specific for cAMP/PKA-mediated nuclear import.

We prepared an antibody to recognize phospho-S266 and I found that overexpression of PKA does not increase S266 phosphorylation (data not shown). Moreover, cAMP treatment decreases endogenous S266 phosphorylation (Ch. III Fig. 5). Thus, I concluded that PKA does not phosphorylate S266, but instead leads to the dephosphorylation of S266, presumably through the activation of a S266 phosphatase or the inactivation of a S266 kinase. Related to this, it has been reported that PKA can activate PP2A by direct phosphorylation (1), however, I showed that treatment with okadaic acid, which inhibits PP2A, had no effect on cAMP-mediated nuclear import of HDAC4 (Ch. III Fig. 4). Since HDAC4 was phosphorylated on S266 in the basal state, I treated cells with inhibitors for several potential S266 kinases. This site matched the consensus sequence for CaMK, PKC, ERK1/2, and GSK-3β. However, treatment of cells with inhibitors for these kinases (KN-62, Bis I, PD 98059, and kenpaullone, respectively) did not

reduce basal S266 phosphorylation (Ch. III Fig. S4). Another candidate for the S266 kinase is Mirk/Dyrk1B, which was shown to phosphorylate the equivalent site in the HDAC9 splice variant MITR (8). Future work testing whether Mirk/Dyrk1B also targets this site in HDAC4 and HDAC5, and whether such an effect is sensitive to cAMP/PKA signaling is warranted. However, to my knowledge no link between cAMP/PKA signaling and Mirk/Dyrk1B function has ever been reported.

During the final stages of work on this project, two papers were published that support a role for HDAC5 S279 (the site equivalent to HDAC4 S266) in nuclear import (14, 15). Like my findings, Ha et al. (15) showed that cAMP treatment causes nuclear import of WT HDAC5 but not of the HDAC5 S279A mutant. However, in contrast to my results, they reported that the HDAC5 S279D mutant is more nuclear than WT HDAC5, whereas I showed that the HDAC5 S279D mutant is more cytoplasmic than WT HDAC5 (Ch. III Fig. 3). Also in contrast to my results, they showed that PKA directly phosphorylates this site (15). Although this issue needs to be clarified experimentally, the sequence surrounding S279 (or S266 in HDAC4) does not support direct phosphorylation by PKA, due to the presence of a Pro residue immediately C-terminal to S279 (and S266). Phospho-peptide screening and analysis of native PKA substrates demonstrated that Pro is strongly disfavoured at this position for PKA phosphorylation sites (30, 38). In fact, placement of a Pro residue at this position in different peptides "vetoes" substrate recognition by PKA (38). Clearly, additional studies are required to definitively identify the S266 (and S279) kinase.

Greco et al. (14) did not study cAMP/PKA signaling, but they did identify S279 of HDAC5 as an *in vivo* phosphorylation site in a proteomic screen. Of note, they also showed that the N-terminally adjacent S278 is also phosphorylated *in vivo*. Moreover, they found that each site could be phosphorylated alone or simultaneously with the other site *in vivo* (14). I tested our phospho-S266 antibody to see whether phosphorylation of the adjacent Ser would block antibody-antigen recognition. Indeed, the antibody does not recognize a peptide phosphorylated on both S265 and S266 (data not shown). This suggests that cAMP-induced loss of S266 phosphorylation may be due to the inability of the antibody to bind when S265 is also phosphorylated. Future work aimed at resolving this issue should help in the identification of the kinase(s) and phosphatase(s) that control this phosphorylation event in the basal state and in response to cAMP/PKA signaling.

4.7 Summary

Through the work presented in Chapters II and III, I have characterized several novel aspects of class IIa HDAC subcellular trafficking (Fig 1). I identified two novel class IIa HDAC kinases and also showed that the kinase upstream from them, the tumour suppressor LKB1, is important for endogenous shuttling of these HDACs. I also identified a novel cAMP/PKA-sensitive phosphorylation site in the NLS that controls trafficking in response to cAMP. These findings will not only be useful for an understanding of class IIa HDAC function and regulation, but they also establish these HDACs as potential mediators of effects downstream from the LKB1 and PKA signaling pathways.

Since these kinases are involved in such a wide range of physiological processes and disease states, the mechanisms I have identified and characterized may provide novel targets for therapeutic intervention.

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CHAPTER V

5.0 CONTRIBUTION TO ORIGINAL RESEARCH

1. Identified SIK2 and SIK3 as class IIa HDAC kinases that can phosphorylate these HDACs *in vitro* and *in vivo* and induce their nuclear export and 14-3-3 binding.

2. Demonstrated that SIK2 can activate MEF2-dependent transcription and rescue HDAC4-mediated repression of myogenesis.

3. Showed that kinase-dead SIK3 can still induce HDAC5 and HDAC9 export; that WT SIK3 can stimulate nuclear export of the constitutively nuclear HDAC4 S246/467/632A mutant; and that SIK3-mediated class IIa HDAC export does not derepress MEF2 transcriptional activity.

4. Identified the tumour suppressor kinase LKB1 as necessary for SIK-mediated class IIa HDAC export, and showed that LKB1 is required for endogenous HDAC4 cytoplasmic localization and S246 phosphorylation.

5. Demonstrated that PKA prevents SIK-mediated class IIa HDAC nuclear export.

6. Showed that PKA is unable to prevent HDAC7 cytoplasmic localization, and that PKA still inhibits the PKA-resistant SIK2 S587A mutant from inducing class IIa HDAC nuclear export.

7. Identified S266 of HDAC4 (and the equivalent S279 of HDAC5) as a novel cAMP/PKA-sensitive phosphorylation site. Mutation of this residue to Ala prevents cAMP/PKA-mediated nuclear import of HDAC4 and HDAC5. Addition of this site to HDAC7 (HDAC7 K196S/N197S) partially restores its PKA sensitivity.

8. Demonstrated that S246 phosphorylation is required for basal S266 phosphorylation of HDAC4.

9. Characterized novel phospho-specific antibody that recognizes phospho-S266 of HDAC4 and showed that cAMP/PKA signaling decreases S266 phosphorylation.