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Functional characterization of HDAC1 Aurora-dependent phosphorylation in mammalian cells and zebrafish development

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List of abbreviations

HDAC: Histone deacetylase

HAT: Histone acetyltransferase

Rpd3: Reduced potassium dependency 3

Hda1: Histone deacetylase 1

Sir2: Silencing Information regulator 2

HAD: HDAC association domain

NLS: Nuclear localization signal

MEF: Mouse embryonic fibroblasts

NES: Nuclear export signal

CDK1: Cyclin dependent kinase 1

HSP20: Heat shock protein 20

NuRD: Nucleosome Remodeling Deacetylase

NODE: Nanog and Oct4-associated deacetylase

MiDAC: Mitotic deacetylase

PTM: Post-translational modification

CKII: Casein kinase II

PKA: Protein kinase A

CBP: CREB binding protein

CHFR: CHeckpoint with FHA and RING domains

TNFα: Tumour necrosis factor-α

IKK2: Inhibitor of nuclear factor Kappa B kinase

Elk1: ETS domain-containing protein 1

LPS: Lipopolysaccharide

MTA1: Metastasis associated protein 1

IL1b: Interleukine 1b

MIP2: Macrophase inflammatory protein 2

HO-1: Heme oxygenase 1

Gadd45: Growth arrest and DNA damage 45

Bmp4: Bone morphogenetic protein 4

Rb1: Retinoblastoma protein 1

her6: E(spl)-related neurogenic gene

gdf11: Growth differentiation factor 11

SMC3: Structural maintenance of chromosomes 3

TSA: Trichostatin A

SAHA: Suberoylanilide Hydroxamic Acid

VPA: Valproic acid

CTCL: Cutaneous T cell lymphoma

PTCL: Peripheral T cell lymphoma

Cdk: Cyclin dependent kinase

Plk: Polo-like kinase

Nerks: NIMA related kinase

DAD/A-Box: D-Box-activating box

D-box: Destruction box

INCENP: Inner centromere protein

CPC: Passenger chromosome complex

MCAK: Mitotic Centromere-Associated Kinesin

Hec1: Highly expressed protein in cancer 1

MKLP1: Mitotic kinesin-like protein 1

Asy: Asynchronous

CIP: Calf Intestinal Phosphatase

DAPI: 4',6-diamidino-2-phenylindole

DMSO: Dimethyl sulfoxide

H3/H4: Histone 3/histone 4

HDACi: Histone deacetylase inhibitors

M: Mitosis

NEM: N-ethylmaleimide

NP-40: Nonyl Phenoxypolyethoxylethanol-40

PFA: Paraformaldehyde

PAA: Polyacrylamide

IP: Immunoprecipitation

RbAP48: Rb-associated protein

ac: Acetylated

MO: Morpholino

hpf: Hours post-fertilization

PI: Propidium iodide

FACS: Fluorescence-activated cell sorting

IHC: Immunohistochemistry

CNS: Central nervous system

ChIP: Chromatin immunoprecipitation

PCR: Polymerase chain reaction

qPCR: Quantitative PCR

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Abstract

Histone deacetylases (HDACs) are a class of modification enzymes that catalyze the removal of acetyl molecules from histone and non-histone substrates. Therefore, they play important roles in chromatin remodelling and gene expression control through regulation of histones, transcription factors, and chromatin-modifying enzymes. Class I HDACs, in particular HDAC1 and HDAC2, are ubiquitously expressed and are critical regulators of cell cycle progression, cellular proliferation and differentiation during development. Besides their subcellular localization and incorporation into multi-0subunit complexes, HDAC1 and HDAC2 can also be regulated by a plethora of post-translational modifications (PTM), which represent a complex "code" that modulates their catalytic activity, localization and complex assembly. Among the various PTMs that occur on HDAC1 and HDAC2 we identified a new mitotic-specific phosphorylation of the two enzymes driven by Aurora kinases A and B. By means of mammalian cells and zebrafish embryos, we dissected the biological role of HDAC1 Aurora-dependent phosphorylation during development, contributing to deciphering the PTM code of these deacetylases. Indeed, we demonstrated that this phosphorylation *in vitro* and *in vivo* partially and dynamically affects HDAC1 enzymatic activity**.** Moreover, we found that Aurora-driven phosphorylation of HDAC1 is critical for the maintenance of a proper proliferative and developmental plan in a complex organism and crucially regulates global histone acetylation levels in zebrafish development.

Thus, affecting its activity on histone acetylation, HDAC1 mitotic phosphorylation acts as fine-tune regulator of proper cell cycle progression, probably by modulating the expression of genes directly involved in zebrafish development.

Histone deacetylases and the regulation of the acetylome

Histone deacetylases (HDACs) are a class of modification enzymes that regulate the activity of their substrates by removing acetyl groups from lysine residues [1,2]. Histone deacetylases are so named based on their ability to deacetylate histone tails and promote chromatin compaction, which is associated with repression of gene transcription [3]. Alternatively, histone acetyltransferases (HATs), promote the acetylation of histone tails hence increasing the accessibility of DNA by relaxing the chromatin structure. Therefore they are considered transcription activators [4]. In addition to histones, a growing number of non-histone proteins has been identified as HDAC substrates [5], suggesting that these enzymes may be involved in a range of cellular processes beyond the regulation of gene expression [6].

The histone deacetylase families

HDACs are members of an ancient enzyme family found in plants, animal and fungi, as well as in Archeabacteria and Eubacteria [2]. The mammalian genome encodes 18 enzymes with deacetylase activity, which can be grouped into two families according to their mechanisms of catalysis: the classical HDAC family, whose members are zinc-dependent deacetylases, and the Sirtuin family of NAD^+-dependent enzymes [7,8]. Another criterion of classification, based on phylogenetic analysis and sequence homology to the corresponding *Saccharomyces cerevisia*e proteins, divides HDACs into four classes (Figure 1) [2,9,10]: Class I comprises HDAC1, HDAC2, HDAC3 and HDAC8, that are closely related to the yeast transcriptional regulator Rpd3 (Reduced potassium dependency 3) and are expressed in a wide range of cell types. Class II

includes HDACs homologous to the yeast Hda1 (Histone Deacetylase 1) protein, and is further divided into two subclasses: class IIa (HDAC4, HDAC5, HDAC7 and HDAC9) and class IIb (HDAC6 and HDAC10) [8]. Class II HDACs have a variety of different roles in muscle, heart and bone development and physiology and are expressed in a tissue specific manner [11]. Class III corresponds to the NAD^+-dependent Sirtuin family and is composed of seven members, Sirt1-7 [7]; they are homologous to the yeast protein Sir2 (Silencing Information regulator 2) and it has been reported to be involved in genome stability and in several metabolic pathways [12]. The only member so far belonging to Class IV is HDAC11, which is a zinc-dependent deacetylase sharing common features with both class I and class II HDACs [13]. HDAC11 was shown to regulate the balance between immune activation and immune tolerance in $CD4^+$ T-cell [14] and to play a role in oligodendrocyte differentiation [15].

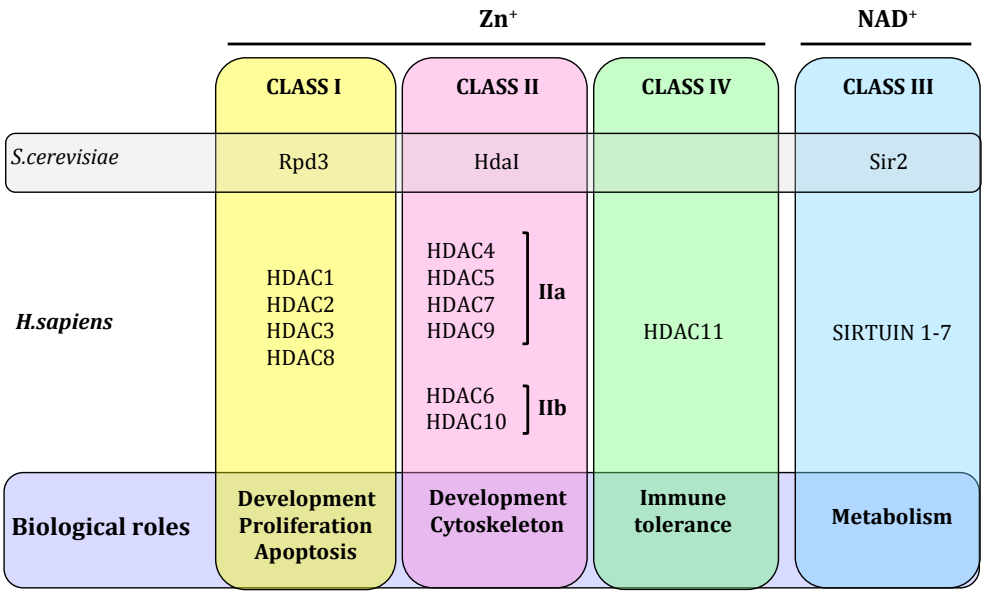


Figure 1. Classification and general biological roles of mammalian histone deacetylases

Classification of yeast and mammalian HDACs according to their mechanism of catalysis and sequence homology are schematically reported. General biological roles for each class are also indicated.

Class I HDACs: domain organization and function

Class I HDACs are ubiquitously expressed in a wide range of tissues suggesting a general role in transcription repression. Furthermore, class I HDACs interact with many lineage-specific transcription factors suggesting that these enzymes may play an important role in controlling specific transcriptional programs [16].

Class I HDACs display a modular organization (Figure 2): a well-conserved N-terminal domain that represents the two-thirds of the protein and covers the catalytic domain and a most variable C-terminal portion. The N-terminus of HDAC1 also harbours the HDAC association domain (HAD) that mediates homo/hetero-dimerization with HDAC1 or HDAC2. The two proteins differ from each other in their C-termini: a nuclear localization signal (NLS) is located in the C-terminus domain of HDAC1 [17], whereas a coiled-coil domain for protein interaction is present in HDAC2 [2]. Both HDAC1 and HDAC2 are usually localized to the nucleus. HDAC1 and HDAC2 exhibit 86% amino acid sequence identity, suggesting that their genes were probably generated from a duplication event [18,19]. HDAC1 and HDAC2 are highly related proteins, they play crucial roles in development and physiology, especially in the heart and nervous system [20], as well as in cellular proliferation, cell cycle and apoptosis. In primary mouse embryonic fibroblasts (MEFs), simultaneous deletion of HDAC1 and HDAC2 leads to strong cell cycle arrest in G1 phase followed by up-regulation of p21 and p57 [21]. Moreover, siRNA-mediated knockdown of HDAC1 and HDAC2 in different human tumour cell lines suggests that the requirement of the two deacetylases for the proper cell cycle progression may be cell-type dependent [22].

HDAC3 is evolutionarily most closely related to HDAC8, sharing an overall sequence identity of 43%. In addition to the NLS domain at the C-terminus, HDAC3 carries a nuclear export signal (NES) that enables the enzyme to shuttle from the nucleus to

the cytoplasm [23,24]. Also HDAC8 has a cytosolic localization. Increasing evidence support the role of HDAC3 in cell cycle regulation and DNA damage control, as well as in spindle assembly checkpoint and sister chromatid cohesion [25]. Conditional inactivation of HDAC3 leads to a delay in S phase during cell cycle progression and increased DNA damage and apoptosis [26,27]. Moreover, HDAC3 controls G2-M progression by regulating CDK1 (cyclin dependent kinase 1) levels in adult neural stem progenitors cells [27]. HDAC3 was shown to be associated also to hepatic lipid homeostasis [28,29] and smooth muscle differentiation [30,31].

HDAC8 plays an important role in neuroblastoma tumorigenesis. It has been described that high HDAC8 expression levels correlate with advanced stage childhood neuroblastoma and that HDAC8 ablation in neuroblastoma cells inhibits proliferation and causes cell cycle arrest [31]. HDAC8 was also found in association with HSP20 (Heat Shock Protein 20) and involved in its deacetylation affecting myometrial activity [32].

Although class I HDACs are commonly known as co-repressors, several studies in recent years have challenged this. In particular, HDAC1 and HDAC2 activity can be associated with the activation of certain genes [33,34,35,36,37] and deletion of these enzymes individually leads to deregulation of a limited set of genes, indicating a specific function of class I HDACs in transcription regulation.

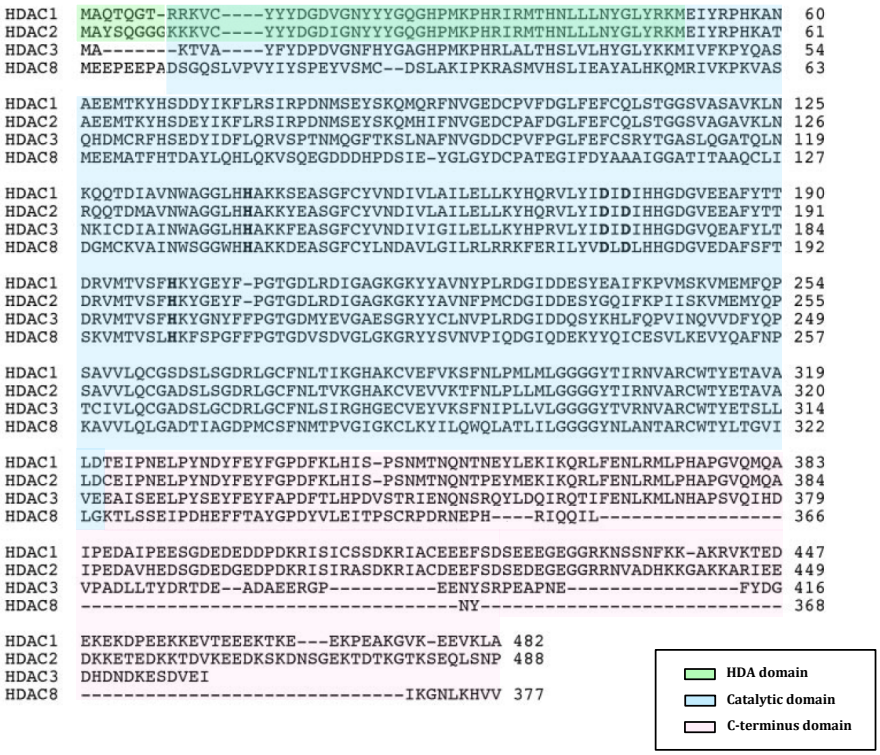


Figure 2. Alignment and functional domain organization of class I HDACs

Protein sequences of all class I HDACs were aligned using the Clustal method. The HAD domain is depicted in green, the catalytic domain in light blue and the C-terminal domain in pink. The conserved residues involved in catalysis are in boldface type. (Adapted from **[38]**)

Regulation of class I HDACs: multiprotein complexes and post-translational modification (PTMs)

Class I HDACs multiprotein complexes

Since HDAC1 and HDAC2 lack DNA binding domain and do not possess specificity for particular DNA sequences or regulatory regions, they are present in mammalian nuclei as part of multiprotein complexes [39] (Figure 3). These complexes contain proteins necessary for proper deacetylase activity and dictate the functional context of the two enzymes [40]. The best-characterized HDAC1/2-containing complexes are the Sin3A, NuRD and CoREST complexes [41,42,43,44,45]. These complexes are recruited to chromatin by transcription factors and/or histone–recognition motifs

found within complex components [46]. The Sin3A complex is essential for cellular viability: depletion of Sin3a affects the early developmental pre-implantation stage and at the cellular level leads to deregulation of genes involved in cell cycle control, DNA replication, DNA repair, apoptosis, chromatin modification and mitochondrial metabolism [47]. The NuRD (Nucleosome Remodeling Deacetylase) complex is known to be important in mouse development [48]. It has been recently associated with the DNA damage response and aging [49,50] and it also contains chromatin-remodelling ATPase activity. The REST/CoREST complex has more specific functions: it recruits HDAC1 and HDAC2 to suppress the transcription of neural genes in non-neural tissues [51,52].

Recently, two other HDAC1/HDAC2 containing complexes were characterized: the NODE (Nanog and Oct4-associated deacetylase) complex present in embryonic stem cells and the SHIP complex that have specific function during embryogenesis [53]; [54]. MiDAC (Mitotic deacetylase) is a novel mitosis-specific deacetylase complex identified by a chemoproteomics approach [55].

By contrast, HDAC3 binds exclusively to the co-repressor complex SMRT in which it is the catalytic component. Moreover, it was found that HDAC3 can associate with class II HDACs and that the interaction between HDAC3 and HDAC7 influences the enzymatic activity of HDAC7 [56]; [24].

So far HDAC8 has not been reported to associate in macromolecular complexes.

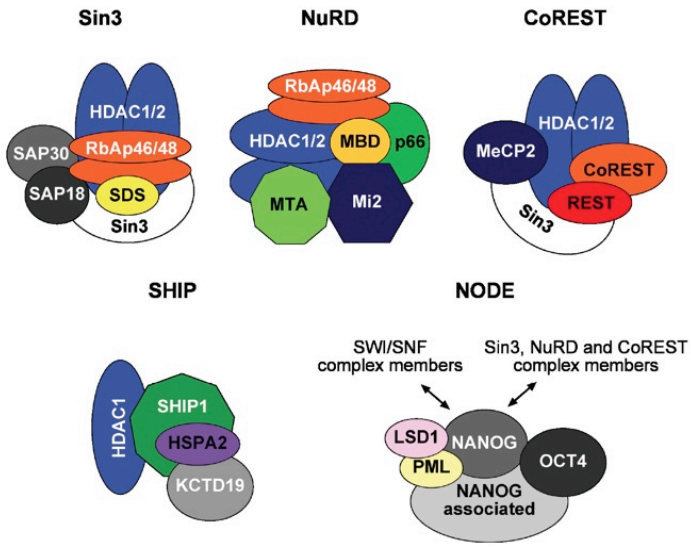


Figure 3. HDAC1/2-containing multiprotein complexes

Schematic representation of the main multiprotein complexes containing HDAC1 and HDAC2. In the NODE complex, the transcription factors Nanog and Oct4 interact with HDAC1 and HDAC2 and other members of the SIN3, NuRD, CoREST and SWI/SNF complexes. The cartoon does not report the physical interaction of the subunits. (From **[57]**)

The class I HDACs PTMs code

Besides their subcellular localization and incorporation into multi-subunit complexes, class I HDACs can also be regulated by post-translational modifications (PTMs) including phosphorylation, acetylation, ubiquitination, SUMOylation, nitrosylation and carbonylation [58,59] (Figure 4). These modifications modulate their catalytic activity, localization and complex assembly [60].

-Phosphorylation

HDAC1, HDAC2 and HDAC3 can be phosphorylated by casein kinase II (CKII). The CKII-dependent phosphorylation enhances the deacetylase activity and transcriptional repression as well as the interaction with multisubunit complex partners [38,61]. It was also reported that CKII-dependent phosphorylation on HDAC1 is constitutive throughout the cell cycle, but dispensable for its intrinsic

activity *in vitro* [62]. In addition, CKII-mediated phosphorylation of HDAC1/2 during mitosis leads to dissociation from each other but not from the multiprotein complexes components [63].

Interestingly, some viruses induce HDAC1/2 hyper-phosphorylation in the early phase of infection. Both the US3 kinase from human Herpes Simplex Virus 1 [64] and its homologue ORF66 kinase from Varicella Zoster Virus [65] induce, even if indirectly, hyper-phosphorylation of HDAC1 and HDAC2. Recently, also the non-human herpesvirus Pseudorabies Virus (PRV) US3 kinase was shown to induce phosphorylation of HDAC2, indicating a conserved effect of many herpesviruses on this deacetylase [66]. The kinase(s) directly responsible for HDAC1 and HDAC2 phosphorylation *in vivo* is unknown.

HDAC8 is phosphorylated, in its N-terminal domain, *in vitro* and *in vivo* by protein kinase A (PKA), which negatively impacts the catalytic activity [67].

- Acetylation

Class I HDACs are also subjected to reversible acetylation. HDAC1 acetylation by CREB binding protein (CBP)/p300 leads to decreased enzymatic activity [68]. Although five of the six acetylable lysines in HDAC1 are conserved in HDAC2, HDAC2 cannot be acetylated *in vitro* due to the lack of lysine 432, which seems to be the crucial residue for the HDAC1 acetylation. However, C-terminal tail swapping experiments between HDAC1 and HDAC2 showed full HDAC2 acetylation due to the substituted C-terminal region originating from HDAC1 [69]. More importantly, the crucial role of the C-terminal region in the differential regulation of the enzymatic activity of the two enzymes suggests that the C-terminal domain of HDAC1 and HDAC2, which is not conserved in the more divergent class I HDAC3 and HDAC8, has evolved to specify and finely tune the functions of two highly related proteins in the complex networks of mammalian systems [2,70].

Acetylation of HDAC3 and HDAC8 has not yet been reported.

- Ubiquitination

Poly-ubiquitination mainly targets proteins for degradation via the proteasome machinery, while mono-ubiquitination acts as a signal for different biological outputs [71]. Both HDAC1 and HDAC2 have been reported to be poly-ubiquitinated *in vitro* and *in vivo* [72,73,74]. Oh and colleagues published that the CHFR (CHeckpoint with FHA and RING domains) ubiquitin ligase downregulates HDAC1 levels by directly binding and ubiquitinating HDAC1. This functional interaction between HDAC1 and CHFR correlates with increased invasiveness and metastatic potential of prostate and breast cancer cell lines [75]. Furthermore, selective ubiquitination and depletion of HDAC1, but not of other class I HDACs, is also a critical step in the pro-inflammation response activated by tumour necrosis factor-α (TNFα) through the IKK2 (inhibitor of nuclear factor Kappa B kinase) signaling pathway [76]. This leads to a complete loss of HDAC1 occupancy at the promoter of $p21^{WAF/CIP1}$ providing evidence of a link between inflammatory signaling pathways and modulation of chromatin transcription through PTMs on histone deacetylases.

HDAC2 is specifically ubiquitinated through proteasomal degradation by the Ubc8 E2 conjugating enzyme and the RLIM E3 ligase upon treatment with the HDACs inhibitor valproic acid [73]. Finally, HDAC2 is phosphorylated and degraded by the proteasome in bronchial cells upon treatment with cigarette smoke [77].

No direct evidence of HDAC3 and HDAC8 ubiquitination has been reported so far.

- Sumoylation

Two SUMOylation sites of HDAC1 were mapped on lysines K444 and K476 [72,78]. De Pinho's laboratory reported a 60% reduction of transcriptional repression of the 2R HDAC1 mutant, even if the binding with the Sin3a protein was unaffected. Moreover, overexpression of wild type HDAC1, but not of the SUMO-deficient mutant, induced

accumulation of cells in the G2 phase of the cell cycle, suggesting that the SUMOylation mutant of HDAC1 has some impaired biological functions [72]. Furthermore, other studies have reported that SUMO modification promotes transcriptional repression of HDAC1/HDAC2 containing complexes, such as Elk1 (ETS domain-containing protein 1) or Mi-2/NuRD [79,80]. Finally, it has been demonstrated that only SUMO1 and not SUMO2 conjugation to HDAC1 promotes its ubiquitination and degradation [81]. In this way, specific SUMO paralog conjugation controls HDAC1 protein turnover in mammalian cells. It is also worth noticing that SUMOylation of the phospho-null mutant S421A, S423A of HDAC1 is increased with respect to the wild type protein [78]. This finding suggests a negative relationship between CKII phosphorylation and SUMOylation.

No data are available in literature regarding the other class I HDACs.

- Nitrosylation

Tyrosine nitration was observed in HDAC1, HDAC2 and also HDAC3 in macrophage cells after exposure to cigarette smoke with a consequent down-regulation of their protein levels [82]. Interestingly, mutation in the tyrosine Y253 of HDAC2 in human alveolar epithelial cancer cells abolished the proteasomal-dependent pathway, suggesting that tyrosine nitrosylation of HDAC2 is a stress signal to drive specifically its degradation. In neurons, cysteine (S)-nitrosylation of HDAC2 leads to chromatin remodeling, causing the release of HDAC2 from neurotrophin-dependent promoters and thereby stimulating transcription [83]. S-nitrosylation of HDAC2 also plays a role in the pathogenesis of Duchenne muscular dystrophy, where it is correlated with a decrease in HDAC2 enzymatic activity and partial rescue of the myotube differentiation abilities of cells, which are lost in the pathology [84]. Recent work showed that S-nitrosylation of HDAC2 in macrophages following lipopolysaccharide (LPS) stimulation affects the stability of the binding between HDAC2 and MTA1

(Metastasis associated protein 1), with consequent dissociation of the NuRD complex from chromatin and activation of transcription of some inflammatory genes such as IL1b (interleukine 1b), TNFα and MIP2 (macrophase inflammatory protein 2) [85].

No S-nitrosylation has been reported for HDAC1 or HDAC3 [84].

These studies suggest an emerging role of S-nytrosilation in regulating the chromatin-associated dynamics of HDAC2, in a very specific way compared to other class I HDACs, such as the highly related HDAC1.

In addition, HDAC8 can be S-nitrosylated *in vitro*, which reversibly inhibits its enzymatic activity [28]. However, this modification on HDAC8 could not be detected under physiological conditions *in vivo* [60].

- Carbonylation

HDAC1, HDAC2 and HDAC3, but not HDAC8 can be carbonylated. Carbonylation of HDAC1 does not impair its intrinsic enzymatic activity but disrupts its interactions with binding partners and histone substrates *in vivo* [86]. As a consequence, increased acetylation of lysine 9 of histone H3 was observed with concomitant transcriptional activation of some HDAC1-repressed genes, such as HO-1 (Heme oxygenase 1), Gadd45 (Growth arrest and DNA damage 45) and HSP70 (Heat shock protein 70).

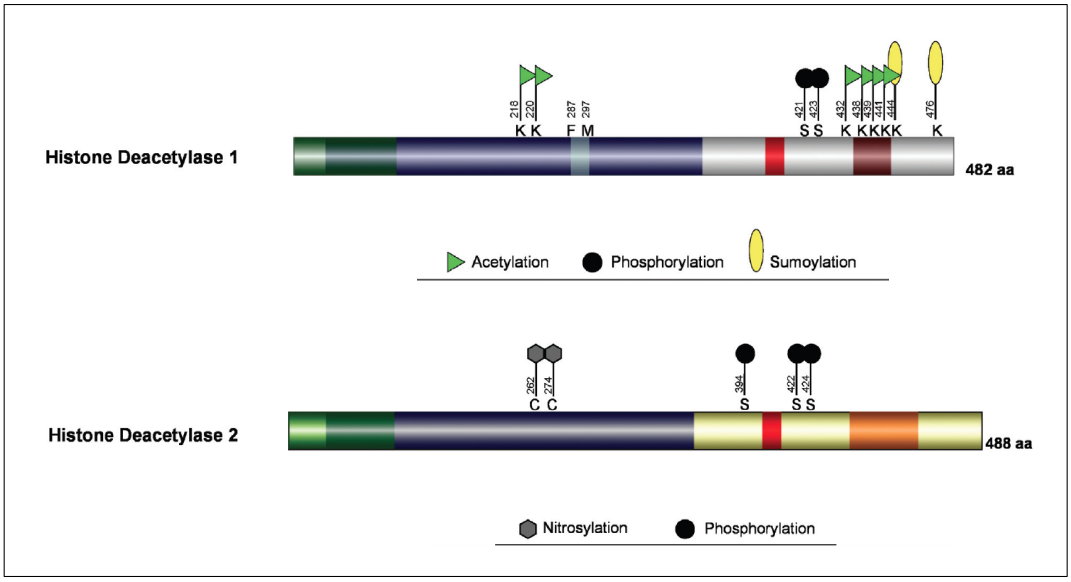


Figure 4. HDAC1 and HDAC2 post-translational modifications

Schematic overview of the posttranslational modifications occurred on HDAC1 and HDAC2 C-terminus. The letters and the numbers indicate respectively the residues and the position on which the PTM occurs. (From **[57]**)

Biology and functions of class I HDACs: the animal models

Class I HDACs: the mouse model

-Germline deletion

Every class I HDAC member has been deleted either globally or in a tissue-specific manner in mouse models. Germline deletion of *Hdac1* in mice leads to embryonic lethality before day E10.5 because of severe developmental and proliferative defects as well as growth retardation [21,87,88]. Indeed, Lagger and colleagues demonstrated that the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ is up-regulated in HDAC1 knockout mice and in HDAC1 deficient embryonic stem cells (ES). Moreover, deletion of $p21^{WAF1/CIP1}$ in HDAC1 deficient ES cells, but not in HDAC1 null mice, is able to rescue the proliferation defects [87,89]. On the contrary, the impact of *Hdac2* ablation in mice, due to cardiac defects, results in perinatal lethality [88], partial perinatal lethality [90] or partial lethality within the first months [91,92],

depending on the distinct strategies used to generate loss-of-function *Hdac2* alleles. Globally, the knockout studies of HDAC1 and 2 underline specific and distinct roles of the two highly homologous enzymes.

Knockout of HDAC3 also leads to embryonic lethality before day E9.5 caused by gastrulation defects [26,93], whereas HDAC8 ablation in mice results in perinatal lethality due to skull instability [94].

-Conditional deletion

Since germline ablation of class I HDACs results in early embryonic lethality, taking advantage of conditional cre recombinase-mediated deletion, a variety of tissue-specific HDAC knockouts have been generated.

HDAC1 and HDAC2 are usually co-expressed and often show redundancy in their function [21,95,96]. Thus, loss of either HDAC1 or HDAC2 in different cell lines such ES cells, fibroblasts, B cells, keratynocytes, cardiomyocytes and neurons exhibit only mild effects [21,60,87,95]. This is probably due to the fact that in most of these cellular systems, deletion of *Hdac1* leads to an increased HDAC2 protein level and vice versa, without corresponding changes in the mRNA levels [22]; Yamaguchi et al. 2010; Lagger et al. 2002; Wilting et al. 2010). This compensatory effect masking the loss of the other paralog, hides the knockout phenotype.

Nevertheless, knockout of HDAC1 in T cells and ES cells results respectively in increased airway inflammation and enhanced embryoid bodies differentiation, suggesting a pivotal role of HDAC1 in the modulation of the inflammatory response and in cell fate determination [97,98]. Moreover, loss of HDAC1 correlates with tumour development in skin tumour models. Evidence of HDAC1 acting as a tumour suppressor also in T and B cells were independently furnished by Dovey, Heideman and Santoro [99,100,101]. Conversely, several reports suggest a role for HDAC2 in

adult neuronal progenitor differentiation and in synaptic plasticity after neuronal maturation.

If single conditional deletion of HDAC1 or HDAC2 results in a mild phenotype, simultaneous conditional ablation of the two enzymes leads to severe defects in proliferation, differentiation and survival in many cell types and tissues. Indeed, combined loss of HDAC1 and HDAC2 causes cell cycle arrest in G1 phase and cell death [21] in fibroblasts and B cells and the failed differentiation of neuronal precursors into mature neurons (Montgomery et al. 2009). Furthermore, concomitant deletion of the two enzymes provokes de-regulation of important signaling pathways including p53/p63 pathways in epidermis [96], Bmp4 (Bone morphogenetic protein 4) and Rb1 (Retinoblastoma protein 1) in lung [102] and Wnt/ß catenin pathway in oligodendrocytes [103]. DNA damage or chromosomal abnormalities followed by apoptosis in cell types and organs upon dual loss of HDAC1 and HDAC2, is also worthy of notice [4,21,99,100,104].

Loss of HDAC3 or HDAC8 evokes a strong phenotype including developmental defects and lethality. Liver-specific deletion of HDAC3 correlates with hepatocellular carcinoma caused by DNA damage and genomic instability [105], while cardiac-specific ablation of the enzyme leads to cardiac hypertrophy and aberrant expression of cardiac metabolism genes [93]. HDAC3 also negatively regulates memory formation [106].

Finally, conditional loss of HDAC8 in neural crest cells, as for its global deletion, provokes skull instability and perinatal lethality [94].

Class I HDACs: the zebrafish model

In zebrafish, class I HDAC consists of three members: Hdac1, orthologue of the murine HDAC1 and HDAC2, Hdac3 and Hdac8.

Hdac1 is specifically required to promote neuronal specification in the developing zebrafish Central Nervous System (CNS) [107,108,109,110]. Several studies have shown that, in the hindbrain, Hdac1 represses the Notch target E(spl)-related neurogenic gene her6 thus providing expression of proneural genes in differentiating neuronal precursors [107,108,109,111,112].

Hdac1 is also required for the switch from proliferation to differentiation in the zebrafish retina and optic stalk. It promotes cell cycle exit by antagonizing Notch and Wnt signaling pathways and this correlates with repressing cyclins D1 and E2, eliciting CDK inhibitor expression and triggering neural progenitors cell cycle exit (Yamaguchi et al. 2005; Stadler et al. 2005). In contrast with the overall reduction of proliferation in mouse HDAC1 mutant embryos, due to decreased cyclin-dependent kinase activity [87], and with the reduced proliferation observed in the hindbrain of zebrafish hdac1 mutants [107], Yamaguchi and colleagues demonstrated that in zebrafish hdac1 mutants retinal cells fail to differentiate into neurons and glial cells but instead continue to proliferate.

Taken together, these evidences suggest that Hdac1 can function either as a positive or as a negative regulator of the cell cycle, depending on the tissue and the cell type in which it is active.

Hdac1 has also been found to be required for the differentiation of motoneurons from ventral neural progenitors upon hedgehog signaling [107], for normal craniofacial and fin development in zebrafish embryos [113,114] and in the organogenesis of the pancreas [115].

Moreover, it has been demonstrated that Hdac3 regulates zebrafish liver development by inhibiting growth differentiation factor 11 (gdf11) [115]. Hdac8 functions as a SMC3 (Structural maintenance of chromosomes 3) deacetylase to facilitate renewal of cohesin following its removal from chromatin in prophase or anaphase and that loss of HDAC8 activity results in decreased cohesion at localized sites to cause both cellular and clinical features of Cornelia de Lange Syndrome [116].

Class I HDACs and Cancer

Since class I HDACs, in particular HDAC1 and HDAC2, are deeply involved in cell cycle progression and proliferation [87], it is not surprising that they might be implicated in aberrant pathways occuring in tumoral cells in humans. Indeed, several HDACs were found to be deregulated (overexpressed) in various kinds of tumour or cancer cell lines, suggesting that this (de)-regulation could play a role in cancer [117,118,119,120]. Over-expression of HDAC1 is reported for breast [121], gastric [117,122], pancreatic [123,124], colorectal [125], hepatocellular [126] and prostatic [119] carcinomas. HDAC1 over-expression results in a less differentiated status of cells, enhanced proliferation and invasion, and to more advanced stage diseases with worse prognosis [127]. HDAC2 plays a major role in Adenomatous polyposis coli (APC)-colon cancer and its over-expression is present already at the early polyp stage [128]. High levels of HDAC2 are found also in cervical dysplasia and invasive carcinomas [124]. HDAC1 and HDAC2, together with HDAC3 expression are associated with poor prognosis in gastric, prostate and colorectal cancers [122,125]. In childhood neuroblastoma high HDAC8 expression is associated with advanced stage of disease and poor prognosis [31].

Histone deacetylase inhibitors (HDACi)

Given the involvement of HDACs in many types of cancer, these findings sparked a very intense interest on the possible use of HDACi for therapeutic purposes. Although the research on the therapeutic potential of HDACi was initially focused on oncology, also other areas such as neurodegeneration or autoimmunity have recently been investigated. HDACi can be grouped into four main structural classes, with a growing list of novel compounds: first, the hydroxamic acids [e.g. trichostatin A (TSA), Suberoylanilide Hydroxamic Acid (SAHA, Vorinostat, Zolinza®), and LBH589 (Panobinostat)]; second, the short chain carboxylic acids [Phenylbutyrate and Valproic acid (VPA)]; third, the benzamides [MS-275 (Entinostat)]; and fourth, the cyclic tetrapeptides [Romidepsin (FK-228 2, Istodax®)]. They all target the deacetylase enzymatic activity of HDACs by binding the active site and competing with the Zn^{2+} ion in the active pocket [129,130]. This results in increased histone acetylation following the blocking of the substrate pocket and the inactivation of the enzyme [131]. HDACi also affects biological processes other than chromatin dynamics and increases the acetylation level of non-histone proteins, such as tubulin and others [132,133,134]. A common effect of HDACi in cancer cells is the induction of the cell cycle regulator p21 [135] via a p53-independent manner. The p21 up-regulation represents the basis of the proliferation-inhibiting effects of HDACi in cancer cells [136]. Pan-HDACi cause cell cycle arrest and inhibit proliferation, leading to induction of differentiation, growth arrest, and apoptosis of transformed cells [137,138]. These effects are dose and inhibitor-dependent.

Several HDACi have been investigated in clinical trials for their potential as anticancer drugs [136,139]. The first identified HDACi is the naturally occurring antifungal antibiotic TSA [117]. TSA is one of the most potent HDACi, exerting its function in nanomolar range against HDAC1, 2, 4, 6, 7, and 9, but less efficiently

against HDAC8 [140]. Because of pharmacological side effects, TSA was not suitable for clinical use, although it appeared very promising in preclinical trials [141]. To date, three HDACi have been approved as anticancer drug in humans: Vorinostat (SAHA, Zolinza®; Merck Research Laboratories), Romidepsin (FK-228 2, Istodax®; Gloucester Pharmaceuticals) and Belinostat (Beleodaq®; Spectrum Pharmaceuticals). SAHA has been approved since 2006 as a therapy in patients with progressive, persistent, or recurrent cutaneous T cell lymphoma (CTCL) after one or more cycles of chemotherapy [142,143,144,145,146]. SAHA efficiently inhibits HDAC1, 2, 3, 4, 6, 7, and 9, similarly to TSA, but it is 5- to 30-fold less potent [140]. Conversely, Romidepsin (FK-228 2) is one of the more selective inhibitors. It strongly inhibits HDAC1 and 2, and HDAC class II enzymes at higher concentrations [147,148,149]. Since 2009 it has also been approved to treat CTCL [150]. Recently, Belinostat received its first global approval as monotherapy for relapsed or refractory peripheral T-cell lymphoma (PTCL) since it has been found to inhibit class I, II and IV HDACs [151].

The major side effects associated with HDACi are gastrointestinal symptoms, bone marrow suppression, cardiac toxicity, thrombocytopenia, neutropenia, diarrhea, nausea, vomiting and fatigue [16]. Despite these side effects, it is important to take into account that HDACi are well tolerated by the majority of patients compared to other anticancer treatments. Several HDACi have already demonstrated preclinical efficacy, either alone or in combination with other anticancer agents [141]. Moreover, an exciting finding is the observation that HDACi used at low concentrations are efficacious for treating a range of diseases that are not related to cancer. For example, HDACi exhibit anti-inflammatory properties due to a reduction of cytokine production as well as inhibition of cytokine effects [152,153]. Furthermore, HDACi have shown beneficial effects in neurodegenerative conditions and autoimmunity in

mouse models [153,154]. It is likely that a specific HDACi will have clinical benefits only in a specific tumour type and/or in a subset of patients. For future treatment and effective selection of the ‘‘right’’ HDACi for a given therapy, the availability of predictive and prognostic biomarkers will be crucial. Recently, a possible candidate cancer biomarker to evaluate HDACi sensitivity in CTCL patients has been described [155,156].

Cell cycle

The cell cycle is a complex process that directs a cell through a specific sequence of events culminating in the production of two daughter cells with an identical and intact set of chromosomes. The cell cycle is divided into two stages (Figure 5): mitosis (M), the real event of division, and interphase, the interlude between two M phases. The interphase includes G1, S and G2 phases [157]. Replication of DNA occurs in a specific part of the interphase called S phase. S phase is preceded by a gap called G1 during which the cell is preparing for DNA synthesis and is followed by a gap called G2 during which the cell prepares for mitosis. Cells in G1 can, before committing to DNA replication, enter a resting state called G0. Cells in G0 account for the major part of the non-growing, non-proliferating cells in the human body [158]. To survive many rounds of divisions through its lifetime, a cell needs to maintain its genomic stability. The proper inheritance of an intact genome is kept in check by the presence of checkpoints, which monitor the proper execution of cell cycle phases. For instance, the DNA damage and spindle assembly checkpoints ensure genomic integrity by delaying cell cycle progression in the presence of DNA or spindle damage, respectively. More recently, another checkpoint has gained attention, the antephase checkpoint that acts to prevent cells from entering mitosis in response to a range of stress agents [159].

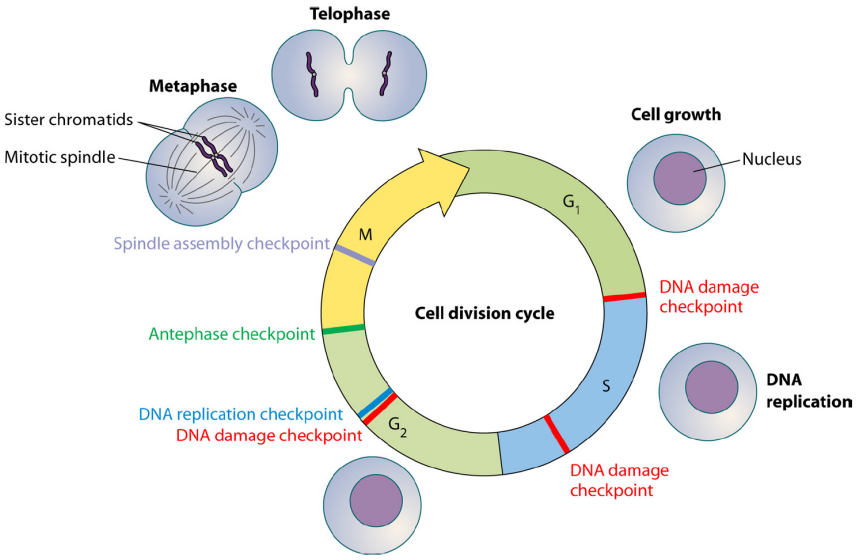


Figure 5. The cell cycle phases and checkpoints

Visual representation of the cell cycle phases. The DNA replication (blue box), DNA damage (red box) and the spindle assembly checkpoints (gray box) as well as the antephase checkpoint (green box) are reported. (From **[159]**)

Mitosis is by far the most complex phase of the whole cell cycle and it is further divided in sub-phases characterized by specific molecular events [160].

The spatiotemporal control of all the mitotic events is mainly achieved through a massive and reversible phosphorylation of key regulatory proteins, which induces conformational changes and enhances the binding for ligands in a rapid way. [161]. A high-throughput mass spectrometry screen of total phosphorylation in mitotic HeLa cells identified phosphorylation on more than 3500 proteins [162].

Critical regulators of the mitotic machinery are specific kinases and phosphatases, in turn tightly regulated in space and time.

In mammals, four main families of mitotic-specific kinases have been identified: the Cdks (Cyclin dependent kinase), the Aurora kinases, the Plks (Polo-like kinases) and the Nerks (NIMA related kinases) [160].

The Aurora kinase family

Aurora kinases are serine/threonine kinases expressed and activated specifically in mitosis. They were first identified in Drosophila and yeast [163,164]. The mammalian genome encodes three Aurora kinase proteins: Aurora A, B and C. From an evolutionary point of view, Aurora A and B originated from a common ancestor gene while Aurora C evolved from the B type [165]. Aurora C is exclusively expressed in the testis, where it plays a role during spermatogenesis [166]. Conversely, Aurora A and B are ubiquitously expressed in all tissues.

Aurora kinases possess a well-conserved catalytic domain, characterized by the presence of an auto-activation loop; for the complete activation of the enzymes, specific threonine residues in this loop (T288 for Aurora A, T232 for Aurora B and T139 for Aurora C) are required to be auto-phosphorylated. Aurora kinases A and B also have two regulatory domains involved in their degradation at the end of mitosis: a D-Box-activating box (DAD/A-Box) in the N-terminal part and a destruction box (D-box) in the C-terminal part. Aurora C seems to possess only the D-box [167] (Figure 6).

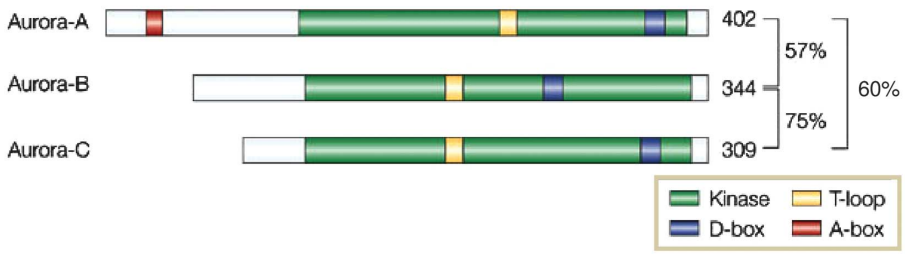


Figure 6. The Aurora kinases family

Modular domain organization of the human Aurora kinases A, B and C. The auto-activation loop (yellow) as well as the activation (red) and disruption (blue) box are reported. The percentage on the right indicates the sequence identities among the three kinases. (From **[168]**)

Despite their common origin and structural similarity, Aurora kinases A and B have distinct roles in mitosis, and are quite separated in time and space.

Aurora A is activated in late G2 by binding with specific cofactors such as the protein Ajuba and TPX (a microtubule associated protein), which then also stimulates its auto-phosphorylation in the activation loop [169,170]. Aurora A locates at centrosomes in late G2 (Figure 7). The recruitment of Aurora A on centrosomes facilitates centrosome maturation and microtubule nucleation by recruiting and thus phosphorylating centrosomal components such as γ tubulin and centrosomin [170]. Several studies have shown a role for Aurora A in promoting timely mitotic onset by controlling the centrosomal activation of the Cdk1-cyclin B complex and thus promoting the G2/M transition [169,171]. Aurora A also phosphorylates the centrosomal Cdc25B phosphatase, which is also involved in the activation of the Cdk1-cyclin B complex [172]. Aurora A is a crucial factor also for proper centrosomal migration: its depletion in fact causes the formation of monopolar spindles.

Aurora B localizes to kinethocores from prophase to metaphase, in the mid-zone during anaphase and in midbody in cytokinesis (figure 7) [167,173]. The activation of Aurora B requires the binding to INCENP (Inner centromere protein). Aurora B and INCENP, together with survivin, are part of the so-called chromosome passenger complex (CPC), which plays different roles in regulating kinetochore attachment to chromosomes, the spindle checkpoint, metaphase-anaphase transition and cytokinesis [174].

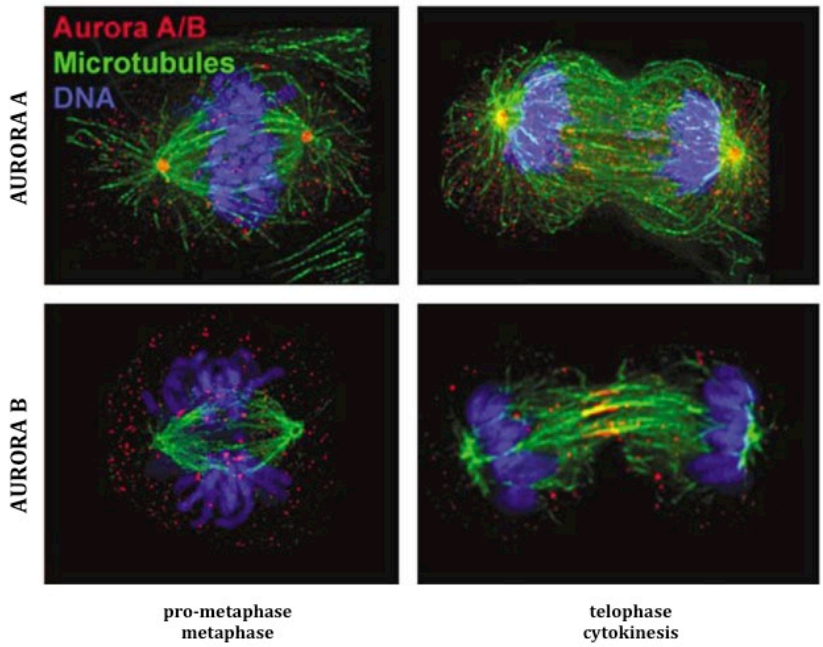


Figure 7. Aurora kinases A and B localization

Aurora A and B localized differently in time and space during mitosis. Aurora A (upper panel) locates to centrosome/spindle poles in metaphase and telophase, whereas Aurora B (lower panel) is present in the centromere from prometaphase to metaphase and then in the spindle midzone microtubles in late anaphase. (Modified from **[175]**)

In prophase, Aurora B is the main kinase involved in phosphorylation of serines 10 and 28 of histone 3 and of serine 7 of the centromeric histone variant CENPA (Centromere protein A), even though the precise biological meaning of these modifications is still unclear [176]. Aurora B is required for proper chromosome condensation and to remove sister chromatid cohesion during mitosis. Moreover, Aurora B plays a central role in the events connected with kinetochore-microtubule attachment and the spindle assembly checkpoint; for example, Aurora B phosphorylates MCAK (Mitotic Centromere-Associated Kinesin) and Hec1 (Highly expressed protein in cancer 1)/Ndc80, and this results in the inhibition of its microtubule depolymerizing activity, a process involved in the control of bipolar orientation of metaphasic chromosomes [177]. Finally, Aurora B is also required for proper cytokinesis; it localizes at the midbody and phosphorylates many proteins

involved in cytokinesis such as vimentin [178], the kinesin ZEN-4/MKLP1 (mitotic kinesin-like protein 1) [179] and the GTPase activating protein MgcRacGAP [180].

Both Aurora A and B are targets of the multisubunit ubiquitin E3 ligase APC/C, which degrades them at the exit of mitosis ensuring a low level of the kinase when cells re-enter in G1 [181,182]. Besides the APC/C also a cullin 3-based E3 ligase was shown to ubiquitinate Aurora B, leading to Aurora B degradation from mitotic chromosomes, and ensuring proper chromosome decondensation and nuclear membrane assembly in telophase [183,184].

HDAC1 and HDAC2 Aurora-dependent phosphorylation: a new PTM for the two deacetylases

(Unpublished data performed by Silvia Senese and Chiara Segrè and taken from their PhD theses)

While investigating the status of HDAC1 and HDAC2 during the different phases of cell cycle we observed a slow migrating band of both proteins appearing exclusively during mitosis (Figure 8A). These slow migrating bands disappeared upon calf intestinal phosphatase treatment, suggesting that they represent a phosphorylation specific of HDAC1 and HDAC2 (Figure 8B).

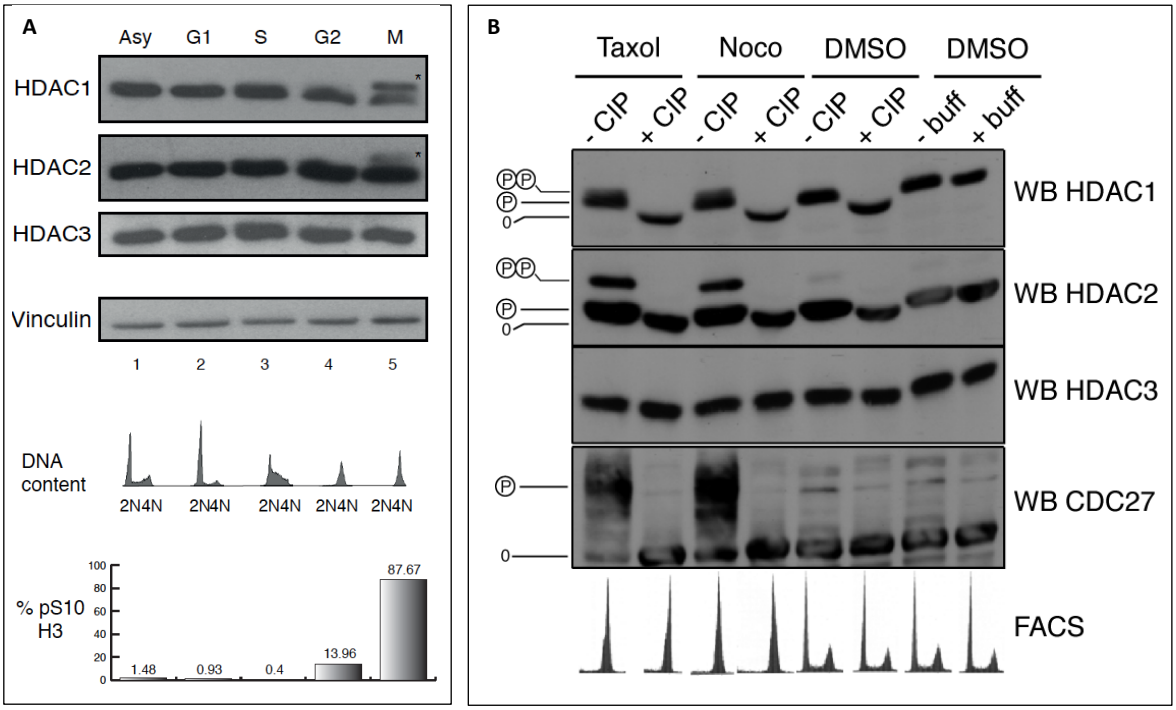


Figure 8. HDAC1 and HDAC2 are specifically phosphorylated in mitosis

A. HeLa cells were synchronized in different phases and protein lysates were analysed by western blot with the indicated antibodies. Cell synchronization was evaluated by FACS by measuring DNA content and phosphorylation levels of serine 10 of histone 3 (pS10-H3) is used as a mitotic marker. Percentages of pS10-H3-positive cells are reported. Vinculin is used as a loading control. Asterisks indicate the lower migrated bands. **B.** Mitotic and control asynchronous HeLa cell lysates were treated with calf intestinal phosphatase for 1 hour at 30°C and then samples were analysed by western blot with indicated antibodies. Cdc27c is widely phosphorylated in mitosis and here is used as positive control for the assay.

Moreover, our *in silico* analysis predicted putative consensus sites on HDAC1 and HDAC2 sequences for the mitotic Aurora kinases A and B. RNA interference experiments, with siRNA specific for the two kinases, (Figure 9) validated the hypothesis that Aurora A and B underlie HDAC1 and HDAC2 mitotic phosphorylation.

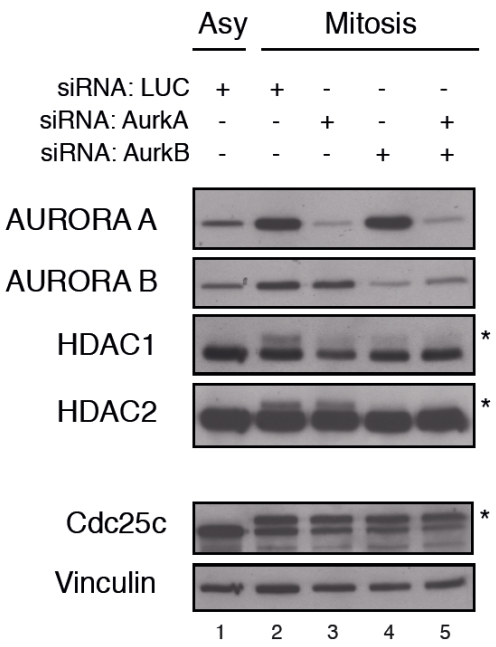
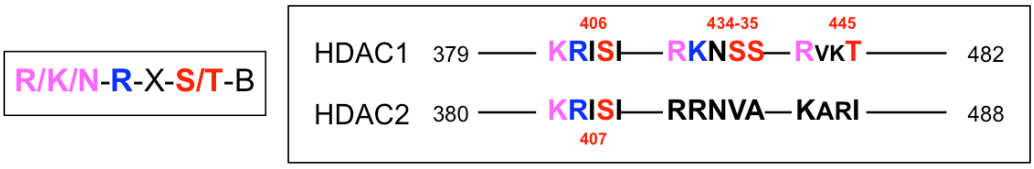


Figure 9. Aurora A and B kinases selectively phosphorylate HDAC1 and HDAC2 during mitosis

HeLa cells were subjected to two cycles of RNA interference with the indicated siRNA, synchronized at G1/S boundary and released with nocodazole. Cells were collected at 9.5 hours by mitotic shaking and analysed by western blot with the indicated antibodies. Cdc25c phosphorylation is used as mitotic marker and Vinculin as loading control. Asterisks indicate phosphorylated bands.

Aurora kinases recognize a basic sequence on their substrates and usually phosphorylate a serine residue within these motifs. Thus, once we identified on HDAC1 and HDAC2 putative consensus sequences for Aurora kinases (Figure 10A), and generated by PCR site-directed mutagenesis a series of constructs in which we mutated the phosphorylable serine with an unphosphorylable alanine, we were also able to map the Aurora phosphorylation sites on serine 406 of HDAC1 and serine 407 of HDAC2 (Figure 10B).

A



B

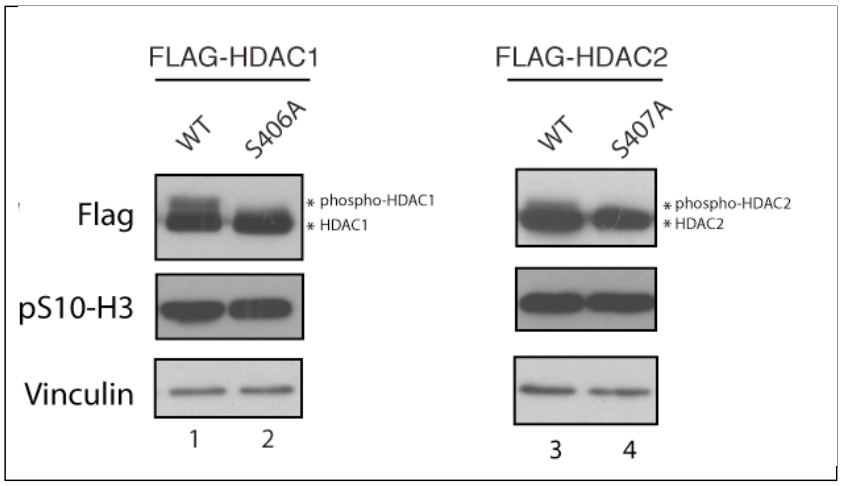


Figure 10. HDAC1 Serine 406 and HDAC2 Serine 407 are target sites for Aurora kinases *in vivo*

A. HDAC1 (aa379-482) and HDAC2 (aa 380-488) C-terminal domains. All the putative target sites for Aurora phosphorylation are reported based on the conservation of the Aurora consensus motif (left cartoon). The amino acids are indicated according to the universal letter code. X represents any aminoacids and B a hydrophobic residue except proline. **B.** HeLa cells were transfected with 10 µg of pBJ5-HDAC1-Flag or pCDNA3.1c-Flag-HDAC2. After 24h from transfection, 300nM nocodazole was added for 16 hours, cells were collected by mitotic shaking and analysed by western blot with the indicated antibody. pS10-H3 is used as mitotic marker, Vinculin as a loading control.

Furthermore, taking advantage of an antibody specific for the phosphorylated S406-HDAC1, developed in our group in collaboration with the Cogentech Biochemistry Unit at the IFOM-IEO-Campus, we observed that phosphorylation of HDAC1 on serine 406 is not constant throughout the different phases of mitosis (Figure 11). Indeed, the peak of phosphorylated HDAC1 occurs in prophase when cells have started to pack their DNA, then it decreases rapidly when cells proceed into pro-metaphase. A second wave of HDAC1 phosphorylation seemed to appear in a “crown-like” structure around

metaphase chromatin. Again, phosphorylation rapidly decreased as cells proceeded through anaphase, telophase and exit mitosis into a new interphase.

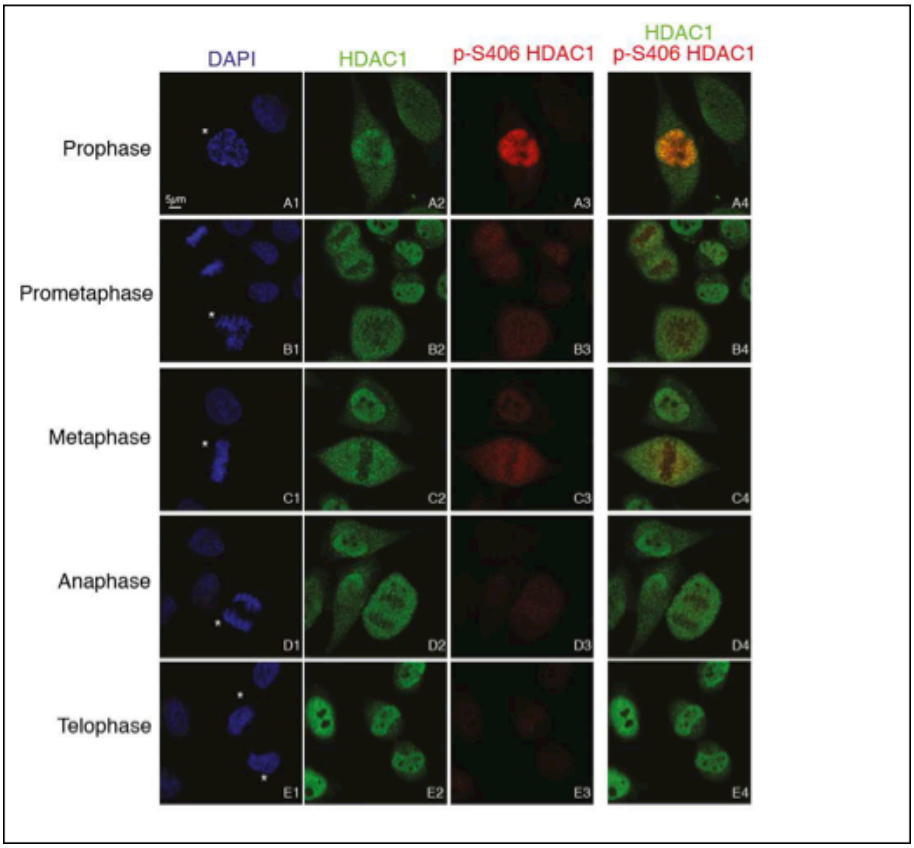


Figure 11. Phosphorylation of HDAC1 at serine 406 occurs in prophase and is removed in anaphase

HeLa cells were plated on poly-D-lysine-covered coverslips, synchronized by double thymdine block, released in fresh medium without nocodazole and fixed with paraformaldehyde 4% after 9.5 hours. Coverslips were stained using the indicated antibodies and DAPI for DNA and analyzed by confocal microscopy. Asterisks indicate the cell of interest in every field. Sub-phases of mitosis were identified by the condensation status of chromatin. A reference scale bar of 5μm is reported in panel A1.

Aim of the project

Recently, a novel mitosis-specific phosphorylation of HDAC1 and HDAC2 has been found in our group (data unpublished). We showed that HDAC1 and HDAC2 are phosphorylated, respectively, on the highly conserved residue S406 and S407 by Aurora kinases A and B and that this phosphorylation occurs only in mitosis, in a limited temporal window during pro-metaphase.

Even though the biochemical data collected suggest a function for this mitosis-specific phosphorylation during the cell cycle, the biological roles of Aurora-dependent phosphorylation of HDAC1 and HDAC2 remains still unclear.

Due to the fact that HDAC1 and HDAC2 are usually co-expressed showing redundant and overlapping functions [21,95,96] and that, in many cellular systems, a compensatory effect between the two enzymes occurs upon deletion of HDAC1 or HDAC2 [22]; Yamaguchi et al. 2010; Lagger et al. 2002; Wilting et al. 2010), it is difficult to unravel the function of the Aurora-dependent phosphorylation of HDAC1 and HDAC2 in mammalian cells. Thus, we decided to focus our attention on the HDAC1 Aurora-mediated phosphorylation, searching for a biological readout in zebrafish developing embryos. Indeed, zebrafish do not possess two distinct HDAC1 and HDAC2 genes, but have only one, which is globally more similar to human HDAC1. Moreover, like in mammals, zbHdac1 has a pivotal role in development. Thus, the aim of this project is to unravel the biology of HDAC1 phosphorylation by Aurora kinases A and B using zebrafish as an animal model.

Cell lines and culture conditions

HeLa cells were maintained in Dulbecco's modified Eagle's medium (Lonza) supplemented with 10% fetal bovine serum (Lonza), 2 mM L-glutamine (Lonza) and antibiotics (250 µg/ml penicillin and 25 µg/ml streptomycin). Cells were cultured in a humidified 37°C incubator with 5% CO2.

Cell synchronization

To synchronize cells in mitosis, 330 nM nocodazole was added for 16 hours and mitotic, rounded-up cells were harvested by shaking them off culture dishes. Cells were synchronized at the G1/S boundary by double thymidine block: cells were treated with 2 mM thymidine overnight, followed by wash and release in fresh medium. The protocol was performed for two consecutive days. The G2-enriched population is the one that remains attached to the plate upon nocodazole treatment and mitotic shaking. G1 pure population was obtained from mitotic cells released for 2 hours from nocodazole block in order to re-enter synchronously in a new G1.

If modifications were introduced, they are indicated in the corresponding experiment.

Production and purification of GST tag proteins

GST tag proteins were produced in bacteria and purified with Glutathion® beads (Sigma). Proteins were eluted using reduced glutathione and the GST tag was removed through proteolytic cleavage with PreScission protease (produced as recombinant protein at IFOM-IEO-Campus).

Quantification of purified recombinant protein was performed by SDS-PAGE and gel staining with Coomassie Brilliant Blue, by comparison with known amounts of BSA protein.

In vitro Aurora kinase assay

Aurora kinase assays were performed as previously described [185]. Briefly, 1 µg of purified substrate (HDAC1, HDAC2, HDAC3 or histone H3) were incubated with 50 ng of recombinant-purified Aurora A/TBX2 or Aurora B/INCEP kinase in the presence of 5 µCi of γ-32pATP at 30°C for 1 hour and analyzed by SDS-PAGE followed by autoradiography.

In vitro histone deacetylase assay

FLAG-tagged HDACs were transfected in cells and immune-precipitated. Purified complexes were then incubated with 100 µCi of chicken purified H^3-acetyl histones (kindly provided by Christian Seiser) in E1A buffer [(50 mM Hepes pH 7.5, 250 mM NaCl, 0.1% NP-40) PMSF, leupeptin, aprotin, sodium orthovanadate, sodium fluoride and NEM were freshly added immediately before use] for 1 hour at 30°C. H^3-acetyl groups released were extracted with a two-phase acqueous-ethyl acetate solution and radioactivity measured as counts per minute (c.p.m.) in a ß-counter scintillation machine.

Plasmids and vectors

For expression in human cells the pBJ5-HDAC1-Flag and pCDNA3.1c-Flag-HDAC2 plasmids were used. For expression of recombinant proteins in bacteria the pGEX-6P1-GST-HDAC1, pGEX-6P1-GST-HDAC2 and pGEX-6P1-GST-HDAC3 plasmids were

used. Plasmids for production of Aurora kinases have been previously described **[185,186]**. Point mutant HDAC1 and HDAC2 proteins were generated by PCR site directed mutagenesis of pBJ5-HDAC1 and pCDNA3.1c-HDAC2 wild type using primers carrying the desired mutations. For retroviral infection in HeLa cells the pBABE-PURO plasmid (empty or encoding HDAC1) was used.

For mRNA transcription and injection in zebrafish embryos, the human HDAC1 wild type, S406A and S406E constructs were subcloned into pCS2+ expression vector (Addgene) using BamHI/XhoI restriction enzymes.

Site-directed PCR mutagenesis

Plasmids encoding for wild type HDAC1 protein was used as template and was amplified through a PCR reaction with couples of primers (below reported) carrying the desired mutations. PCR products were then purified with the QIAquick® PCR Purification Kit (QIAGEN) and subjected to digestion with 20 Units of DpnI (New England Biolabs) for 1 hour at 37°C to digest bacteria-derived methylated template DNA. PCR products were then transformed by heat shock in DH5α *E.coli* strain; DNA from single colonies was extracted with the QIAGEN Plasmid Mini Kit and sequenced with a 3730XL DNA Analyzer.

Cell transfection

24 hours before transfection cells were plated in a number such as to have 50% confluence the day after. Transfections were performed according the calcium phosphate protocol. DNA containing solution was then distributed on cells and incubated for approximately 8 hours at 37°C. Cells was harvest after 72 hours for further experiments.

Infection with retroviral vectors

Packaging Phoenix Ampho cells were transfected with retroviral constructs using calcium phosphate protocol as described above to allow the production of viral particles. 24 hours prior to infection, target cells were plated in 10 cm dishes in order to reach a confluence of 50% the day of infection. The medium containing viral particles was collected, filtered with a 0.22 μM filter and added to the target cells for 6 hours in the presence of 5 μg/ml polybrene. The day after, cells were subjected to another cycle of infection. 24 hours after the second infection puromycin was added at a concentration of 1 μg/ml and cells were kept in selection for at least 4 days to select for a stable population before further experiments were performed.

Growth curves

HeLa cells were counted using the trypan blue dye and 1500 cells/well were plated in a 96-well. Every condition was plated in triplicate for all the considered time points. The day of plating is referred to as T0. Cell viability was measured every 24 hours for 4 days by CellTiter-Glo® Luminescent Cell Viability Assay according to manufacturer's instruction. The number of viable cells in culture is determined by quantitation of the ATP present, as an indicator of metabolically active cells.

Colony forming assay

HeLa cells were counted using the trypan blue dye and 3000 viable cells were plated into a 10 cm dish. Eight days after plating, cells were fixed and stained with crystal violet solution (1% crystal violet, 35% ethanol in PBS) and colonies were counted using Image J software.

Western blot analysis

For Western blot analysis cells were lysed in denaturing SDS lysis buffer [one volume of Buffer I (5% SDS, 150 mM Tris-HCl pH 6.8, 30% glycerol) + three volumes of Buffer II (25 mM Tris-HCl pH 8.3, 50 mM NaCl, 0.5% NP-40, 0.5% deoxycholate, 0.1% SDS) + aprotinin and leupeptin, sodium fluoride, sodium pyrophosphate and sodium orthovanadate].

For HDAC1 detection in zebrafish, 3 days embryos were lysed in urea buffer (8 M Urea, 0.1 M NaH2PO4, 0.01 M Tris, pH 8).

The following antibodies were used for western blot: HDAC1 mouse, clone 10E2 produced in house by Christian Seiser, HDAC1 (Abcam), HDAC2 rabbit (Abcam), HDAC3 rabbit (Abcam), vinculin mouse (Santa Cruz), phospho-serine421/423 HDAC1 (Millipore), histone H3 (Abcam), RbAp48 (Upstate), acetylated H3K9 (Abcam), phospho-serine10 histone H3 rabbit (Upstate), Cdc25c rabbit (Santa Cruz), cyclin B1 mouse (Santa Cruz), Flag mouse (Sigma), zebrafish HDAC1 rabbit (Abcam).

Immunoprecipitations

Cells were lysed in non-denaturing E1A buffer [(50 mM Hepes pH 7.5, 250 mM NaCl, 0.1% NP-40) PMSF, leupeptin, aprotin, sodium orthovanadate, sodium fluoride and NEM were freshly added immediately before use]. Immunoprecipitation (IP) was performed incubating protein extracts with the antibody of interest at ratio of 3-4 mg Ab/mg of crude extract for 16 hours at 4 °C on rotation. Then protein-A/sepharose-beads (slurry 50%) were added to the samples for 2 hours at 4 °C. Beads were then extensively washed with cold E1A buffer, loaded on SDS-PAGE and analyzed by western blot with the indicated antibodies. Total extracts (input) were loaded as a control.

Zebrafish strains and maintenance

Zebrafish strains were maintained and bred according to standard procedures. Embryos from AB wild type strain were maintained in E3 water (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4) at 28.5ºC.

Ethics statement

Fish were maintained/raised according to EU regulations on laboratory animals.

mRNAs and morpholino injections in zebrafish embryos

mRNAs were synthesized from NotI digested pCS2^{+}-hHDAC1 wt, S406A, S406E plasmids using mMessage mMachine kit (Ambion) and purified with Microcon YM-100 (Millipore) filter devices. RNA quality was assayed by means of gel electrophoresis. mRNAs was then diluted in 1X Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM HEPES, pH 7.6) at final concentration of 120 ng/λ and pressure injected into 1-2 cell stage embryos.

Morpholinos were purchased by Gene Tools, LLC. The morpholino against hdac1 (5'-TTGTTCCTTGAGAACTCAGCGCCAT-3') was targeted to the translational initiation site, as [110]. The scramble morpholino sequences was: 5'-CCTCTTACCTCAGTTACAATTTATA-3'. Morpholinos (0.15 mM) were diluted and injected as described above.

Zebrafish embryo cell dissociation

Embryos were anesthetized with tricaine and decapitated. After transient storage in PBS on ice, heads were incubated in trypsin-EDTA (Lonza) for 30 min at 37°C. Afterward, heads were transferred to fresh PBS and mechanically disgregated. This

treatment led to a single-cell suspension. Cells were harvested by centrifugation for 3 min at 3000 × g at 4°C, resuspended in PBS and used for further experiments.

FACS analysis

For FACS analysis, cell suspension obtained as above mentioned was fixed in ice-cold 70% ethanol and incubated over night on ice. DNA was stained with a solution containing 2.5 µg/ml propidium iodide (PI) (Sigma) and 250 µg/ml RNase A. Samples were acquired on a FACSCanto flow cytometer and analysis was performed using a DIVA 6.1.1 machine.

Chromatin immunoprecipitation (ChIP) and quantitative real-time PCR (qPCR)

For each batch of chromatin, 70 wild-type (AB), hHDAC1 mutants, scramble MO or hdac1 morphant embryos were enzymatically dechorionated at 72 hpf, anesthetized with tricaine and decapitated. Embryos heads were dissociated as above mentioned and fixed immediately in 1.5% formaldehyde for 10 minutes. Glycine (2.5 M) was added to quench the formaldehyde and cell suspension was washed in ice cold PBS and then lysed in RIPA buffer [(10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% Na-Deoxycholate) PMSF, leupeptin, aprotin, sodium orthovanadate, sodium fluoride were added before use]. Chromatin was sheared by sonication to give DNA fragments of approximately 300-700 bp in size. Sonicated samples were centrifuged at 12000 × g at 4°C for 15 minutes, and insoluble material was discarded. The supernatant was incubated with acetylated H3K27 antibody (Abcam) or control IgG antibody (Abcam). Dynabeads Protein G magnetic beads (Invitrogen) were added to each sample and the samples rotated at 4°C

overnight. Beads were washed six times with RIPA washing buffer (50 mM Hepes-KOH pH 7.5, 500 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% Na-Deoxycholate) at 4°C and bound complexes were eluted from the beads in Elution Buffer (1x TE, 2% SDS) at 65°C for 20 minutes with vortexing. Cross-links were reversed overnight at 65°C and DNA fragments were purified with Qiaquick purification kit (Qiagen). Quantitative real-time PCR (QPCR) of immuno-precipitated DNA was used to validate ChIP experiment. QPCR analyses were performed in triplicate and DNA abundance was normalised against Input values.

Confocal immunofluorescence microscopy

For immunostaining, 24 hpf embryos were fixed in 4% para-formaldehyde (PFA) overnight and permeabilized with acetone at -20°C for 7 minutes. Embryos were then blocked in PBS containing 0.5% Triton-X, 1% DMSO, 1% BSA and 2% sheep serum (PBST) for 2 hours at 4°C and incubated with primary antibody overnight. The next day, embryos were rinsed in PBST and incubated with secondary antibody before mounting for confocal microscopy.

The following primary antibodies were used: pS406-HDAC1 (clone BT-15 produced at IFOM-IEO Campus), pS10-histone H3 (Upstate), Flag (Abcam).

The following secondary antibodies were used: Alexa-488 conjugated mouse IgG, Alexa-433 conjugated rabbit IgG.

Immunohistochemistry (IHC)

For histological sections 72 hpf embryos were fixed in 4% PFA overnight and permeabilized in sucrose gradient. Then embryos were embedded in 2% low agarose, processed and included in paraffin before sectioned with microtome. Sections were dehydrated and treated with Na-citrate to unmask the antigen from the paraffin.

Later they were blocked for 1 hour with blocking solution (PBS, 1% BSA, 2% FBS) and incubated with primary antibody overnight. The day after, section were washed in PBS and incubated with secondary antibody. Once they were re-hydrated, antibody signal was revealed with DAB and haematoxylin staining was performed.

For immunohistochemistry, acetylated-histone antibody (clone T25, produced at IFOM-IEO Campus) was used.

Primer sequences

Mutagenesis primers for HDAC1

HDAC1 KRISI S406A	CCCTGACAAGCGCATCGCGATCTGCTCCTCTGAC
	GTCAGAGGAGCAGATCGCGATGCGCTTGTCAGGG
HDAC1 KRISI S406E	CCCTGACAAGCGCATCGAGATCTGCTCCTCTGAC
	GTCAGAGGAGCAGATCTCGATGCGCTTGTCAGGG
HDAC1 S421/423A	GTGAGGAAGAGTTCGCCGATGCGGAAGAGGAGGGAGAGG
	CCTCTCCCTCCTCTTCCGCATCGGCGAACTCTTCCTCAC

ChIP validation primers

Trim9	TGCTGATGATGCGAAGTGTG
	TAGGAAAATCAGCCGCGTTG
Phlda3	CCTCTGTGGCCAATGATCAA
	CCAACATGCCCAGATTACGG
Inpp5ka	GGCTGCGGGGAAGTTAAAAT
	GCCTTTCTGTTACTGTTCAAGC
Pck1	TCCAATCGAGTGTTTCCCCA
	ATGATTAACCCGCGGAACAC

Statistical analysis

Results are reported as means +/- standard errors. Statistical significance was calculated using Graphpad PRISM. Different algorithms used are specified for each experiment. Values of $P < 0.05$ were considered statistically significant.

Aurora kinases A and B directly phosphorylate HDAC1 *in vitro*

Since our previous findings indicated a specific role for Aurora kinases in HDAC1 mitotic phosphorylation (see Introduction), we asked whether Aurora A and B directly modified HDAC1.

Thus, we performed an *in vitro* kinase assay, in collaboration with Stefano Santaguida. Recombinant Aurora A/TPX2 or Aurora B/INCENP were incubated with 1 μg of bacterially-produced HDAC1, HDAC3 or histone 3 as substrates in the presence of [γ-^{32}P]ATP as donor of phosphate groups for 1 hour at 30°C. Histone 3 was used as a positive control for Aurora kinases activity; HDAC3 as negative control, since it is not phosphorylated in mitosis and does not possess any putative consensus site for Aurora-mediated phosphorylation. The reaction was then resolved on a SDS-PAGE and the gel exposed for autoradiography; the Coomassie staining of the gel was used as loading control (Figure 12).

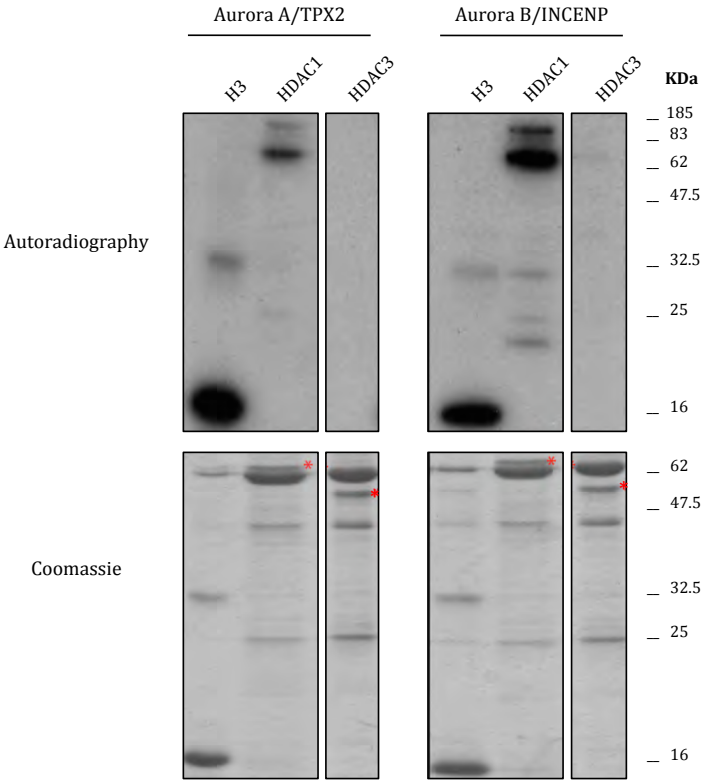


Figure 12. Aurora kinases A and B directly phosphorylate HDAC1 *in vitro*

Recombinant Aurora A/TPX2 and Aurora B/INCENP were incubated with 1 µg of HDAC1, HDAC3 or histone 3 (H3) as substrates with 5 µCi ATP (γ32P) for 1 hour at 30°C. Samples were loaded on a 14% PAA gel and incubated for autoradiography. H3 is used as a positive control for Aurora kinase activity. Coomassie-stained gels are reported as loading controls. Asterisks mark the bands corresponding to recombinant proteins. A ladder of molecular weight is reported, where KDa are kilo Daltons.

As clearly displayed in Figure 12, both Aurora A and Aurora B phosphorylate HDAC1, while HDAC3, as expected, was not modified by the two kinases.

This result shows that HDAC1 is direct substrate for Aurora kinases A and B.

Aurora kinases and Casein kinase II (CKII) phosphorylate HDAC1 independently

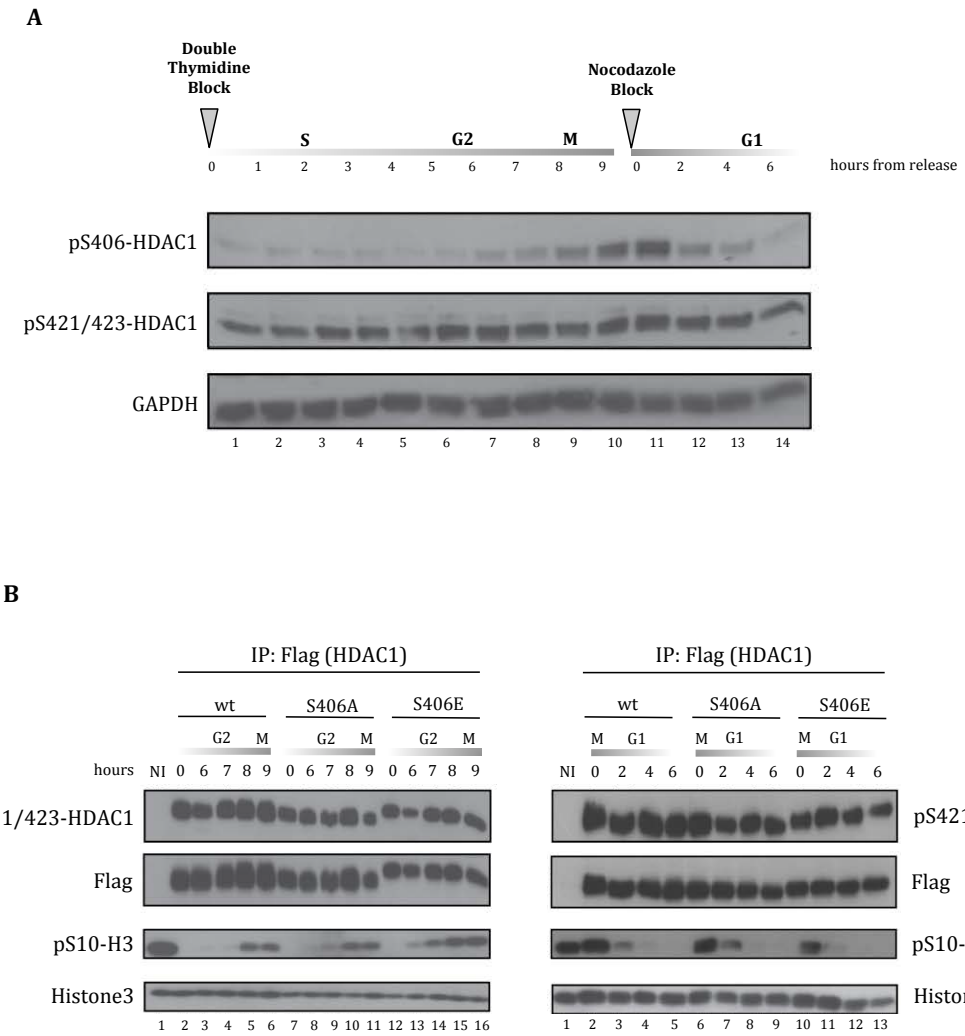
Given the well-established role of CKII in phosphorylating HDAC1 [38,62,63], we asked whether crosstalk between the basal, constitutive CKII- and the mitotic-specific Aurora phosphorylation of HDAC1 existed, thus suggesting the presence of a hierarchy in the phosphorylation code of the protein.

To investigate this hypothesis, HeLa cells expressing flag tagged HDAC1 wt, HDAC1 S406A Aurora phospho-null or HDAC1 S406E Aurora phospho-mimetic mutants were synchronized and collected at indicated time points (Figure 13A-B). Figure 13A represents the trend of the two phosphorylations during cell cycle: HDAC1 was constitutively phosphorylated by CKII at serine 421/423, whereas Aurora-dependent phosphorylation occurred only in mitosis at serine 406.

HeLa cells stably expressing flag-tagged HDAC1, HDAC1 S406A or HDAC1 S406E were synchronized at G1/S via a double thymidine block and were then released into the cell cycle in fresh medium containing 300nM nocodazole (Figure 13B, left panel). Alternatively, the cells were synchronized in M phase by treatment with 300nM nocodazole for 16 hours, purified by mitotic shaking, washed and released in fresh medium (Figure 13B, right panel). Samples were collected at the indicated time points after release and then lysed in SDS denaturing buffer. Flag-tagged HDAC1 proteins were immunoprecipitated and tested with the antibody specific for CKII phosphorylation (pS421/423-HDAC1). As shown in Figure 13B, no substantial differences in the CKII phosphorylation levels were reported upon expression of the two different HDAC1 Aurora phospho mutants neither during entry (right panel) nor exit (left panel) from mitosis. Finally, we wanted to check the opposite relationship: whether the absence of the basal CKII-phosphorylation could affect Aurora-phosphorylation of HDAC1. Flag-tagged HDAC1 wt, S406A Aurora phospho-null and

CKII phospho-null (HDAC1 S421/423A) proteins were transfected in HeLa cells. 24 hours after transfection, cells were synchronized in M phase and protein lysates were immunoprecipitated with anti flag sepharose beads and blotted with pS406-HDAC1 antibody. As reported in Figure 14C, both HDAC1 wt and HDAC1 2S mutant were equally phosphorylated on serine 406.

All the above results suggest that CKII phosphorylation of HDAC1 is not affected by the status of Aurora-phosphorylation sites and that the absence of basal CKII phosphorylation of HDAC1 does not perturb Aurora-driven phosphorylation of the protein.



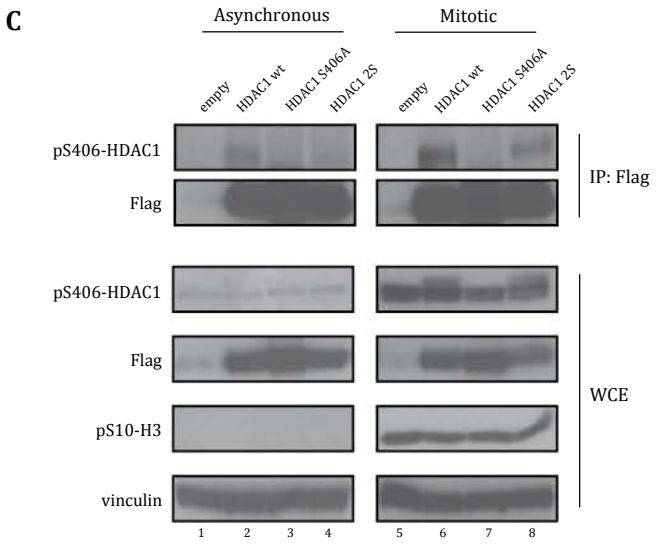


Figure 13. Aurora kinases and the CKII kinase do not crosstalk in phosphorylating HDAC1

A. HeLa cells were synchronized at the G1/S boundary and released as described in Materials and Methods (lanes 1-10). Cells were synchronized in mitosis, washed and released in fresh medium (lanes 11-14). Samples were collected every hour and analyzed by western blot with the indicated antibodies. GAPDH was used as loading control. **B.** HeLa cells stably expressing flag-tagged HDAC1 wild type, S406A or S406E mutants were synchronized at the G1/S boundary (left panel) or in M phase (right panel) as described in Materials and Methods. Samples were collected at the indicated time points after release. Cells were lysed in SDS denaturing buffer, and IP were performed against the flag tag. Samples were then analyzed by western blot with the indicated antibodies. pS10-H3 was used as marker of mitosis and total histone 3 as loading control. **C.** Flag tagged HDAC1 wt, HDAC1 S406A or HDAC1 2S mutants were transiently expressed in HeLa cells. Cells were or not synchronized in M phase. Mitotic cells were then purified and lysed in SDS denaturing buffer. Immunoprecipitation against the flag tag were performed and samples were analyzed by western blot with the indicated antibodies. pS10-H3 was used as marker of mitosis and vinculin as loading control.

Aurora kinase-mediated phosphorylation of HDAC1 reduces its deacetylase activity *in vitro* but does not affect its interaction with HDAC2 and RbAp48

Pflum and colleagues demonstrated that abrogation of the CKII-dependent phosphorylation of HDAC1 by conversion of the two serines 421-423 to unphosphorylatable alanine (HDAC1 2S mutant) entirely suppresses HDAC1 deacetylase activity and the binding with its partners [38].

Therefore we asked whether also the Aurora-dependent phosphorylation of HDAC1 could affect its enzymatic activity and complex formation.

HeLa cells were transfected with flag-tagged HDAC1 wild type or the two Aurora phospho mutants: phospho-null S406A and phospho-mimetic S406E.

24 hours after transfection, 330 nM of nocodazole was added for 16 hours, mitotic cells were collected by shake off and immunoprecipitation was performed using the flag antibody. Three quarters of the immunoprecipitated HDAC1 complexes were incubated with $^{3}CH_{3}$-acetylated histones as substrates for 1 hour at 30°C and the deacetylase activity was evaluated as radioactivity released using a scintillation counter. One fourth of the immunoprecipitation was analyzed by western blot to assess interaction with known binding partners and for normalization of the histone assay. The HDAC1 2S mutant was included as a negative control for both deacetylase and binding activity.

The results showed that, although the activity of the phospho-mimetic form of HDAC1 S406E was not completely abolished as for the HDAC1 2S mutant, it was reduced by at least 40% compared to the wild type, whereas the phospho-null HDAC1 S406A mutant showed no significant changes in its deacetylating ability (Figure 14A).

Moreover, unlike the CKII-dependent phosphorylation, the Aurora-dependent phosphorylation of HDAC1 did not affect HDAC1/HDAC2/RbAp48 catalytic core complex formation as shown in the western blot (Figure 14B).

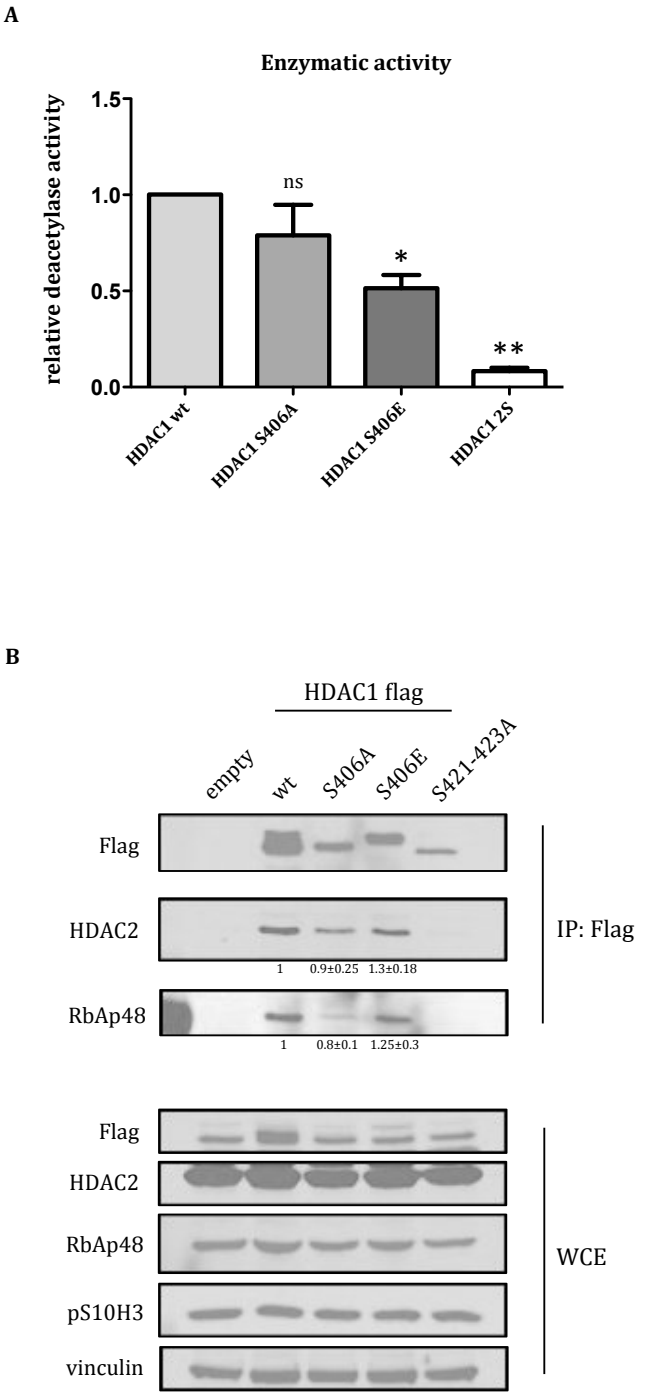


Figure 14. Aurora-dependent phosphorylation of HDAC1 is not involved in the binding with HDAC2 and RbAP48 but reduces HDAC1 enzyme activity *in vitro*

HeLa cells were transfected with 10 μg of pBJ5-HDAC-flag. After 24 hours from transfection, nocodazole was added for 16 hours and cells were collected by mitotic shake-off. Lysates were used for immunoprecipitation (IP) with the flag antibody. **A.** Three quarters of the IP were incubated with 10 μl of $^{3}CH_{3}$-acetylated histones at 30°C for 1 hour and radioactivity released was measured at the scintillation counter at count per minute (cpm); the remaining part was analyzed by Western blot **(B)**. pS10-H3 is used as mitotic marker, vinculin as loading control. For the enzymatic measurements, the scintillation counts were normalized against the amount of immunoprecipitated proteins. The average of three independent experiments was reported as fold change versus HDAC1 wt, standard error (SEM) was indicated by the error bars; the significance was calculated by one sample t test algorithm. *: P value <0.05; **: P value <0.01; ns: not significant. For the Western blot, densitometric analysis was performed using Image J and the average of three independent experiments was indicated ± SEM.

The histone assays and immunoprecipitations performed indicate that neither the abrogation nor the constitutive mimic of HDAC1 Aurora-dependent phosphorylation influence the ability of the enzyme to bind its partners RbAp48 and HDAC2. By contrast, only the phospho-mimetic form S406E, at least in the *in vitro* conditions used, has an impact on HDAC1 catalytic activity significantly decreasing its deacetylase properties.

Aurora-mediated HDAC1 phosphorylation slightly affects H3K9 deacetylase activity in mammalian cells

Since the histone assay reported above was performed *in vitro*, using histones purified from chicken cell nuclear extracts, we wanted to confirm the differences observed in the enzymatic abilities of HDAC1 wt compared to the Aurora phospho mutant in a more physiological setting. HeLa cells, expressing flag-tagged HDAC1 wt or S406A and S406E HDAC1 mutants, were synchronized in M phase and mitotic cells were collected by shake-off after 16 hours of nocodazole treatment and analyzed by SDS–PAGE and western blot (Figure 15A).

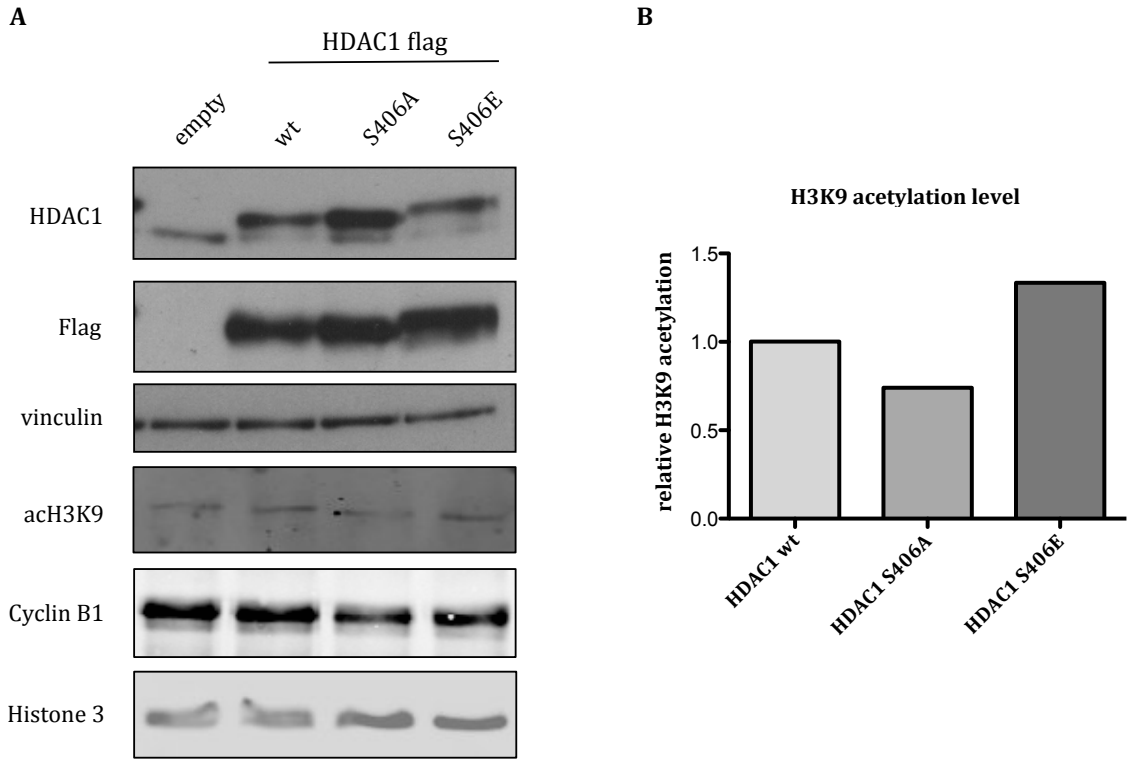


Figure 15. HDAC1 Aurora-dependent phosphorylation slightly affects H3K9 acetylation in mammalian cells

HeLa cells were stably infected with flag-tagged HDAC1 wt and Aurora phospho mutants. Nocodazole was added for 16 hours and cells were collected by mitotic shake-off. **A.** Samples were then analyzed by western blot with the indicated antibodies. Cyclin B1 was used as marker of mitosis, total Histone 3 and vinculin as loading control. Densitometric analysis **(B)** was performed using Image J. Values were reported as fold change versus HDAC1 wt. The experiment was done only once, thus no statistic analysis is included.

To test the deacetylating ability of our constructs we blotted with an antibody recognizing the acetylated lysine 9 of histone H3 (acH3K9), which has to be deacetylated in order to allow subsequent methylation of lysine 9 required for proper chromatin condensation in mitosis [187]. Overexpression of HDAC1-S406E mildly increased H3K9 acetylation levels compared to control. Conversely, lower acetylation of H3K9 was observed with the phospho-null mutant HDAC1-S406A compared to HDAC1 wt (Figure 15B).

These preliminary data indicate that the Aurora phospho-null mutant S406A of HDAC1 is equally or even more active as the wild type *in vivo* at least in deacetylating

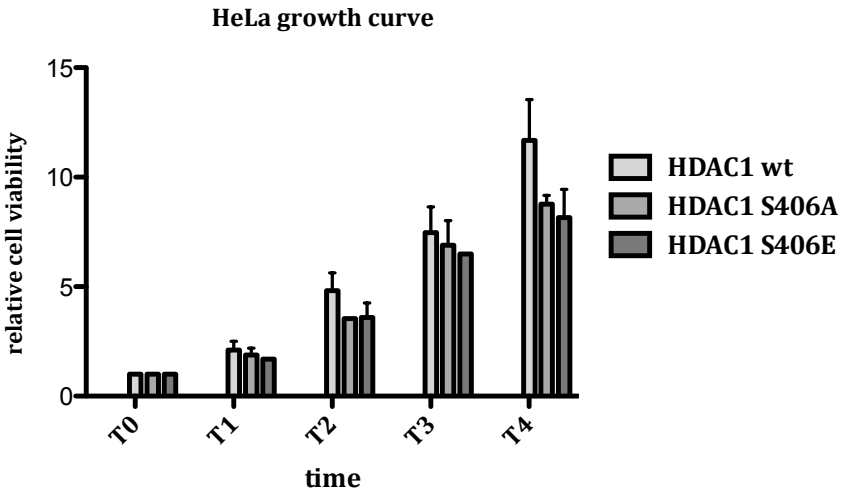
specific histone lysine substrates, such as K9, whereas the HDAC1 S406E mimetic mutant seems to fail to completely deacetylate its substrates.

Expression of HDAC1 Aurora-phospho mutants does not alter cellular proliferation and colony-forming ability compared to HDAC1 wild type cells

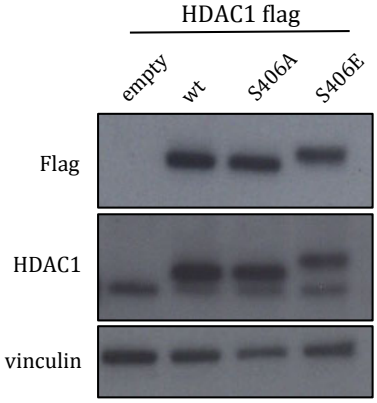
Many studies assign to HDAC1 a pivotal role in cellular proliferation, cell cycle and apoptosis [21,22,87,89].

To evaluate whether Aurora-dependent phosphorylation of HDAC1 influences the proliferation potential and clonogenicity of cells, growth curves and colony forming assays were performed. For the growth curve experiments, HeLa and MEF HDAC1 KO cells were stably infected with flag tagged HDAC1 wt, HDAC1 S406A and HDAC1 S406E constructs and plated in a 96-well plate (1500 cells/well). Cell viability was measured every 24 hours for 4 days by CellTiter-Glo® Luminescent Cell Viability Assay using quantitation of the ATP present as indicator of metabolically active cells. The graph in Figure 16A-C shows that overexpression of HDAC1 Aurora-phospho mutants did not affect cell proliferation at none of the time points considered. Western blots were performed to evaluate the efficiency of the infection (Figure 17B-D).

A


HeLa growth curve
relative cell viability
15
10
5
0
T0
T1
T2
T3
T4
time
HDAC1 wt
HDAC1 S406A
HDAC1 S406E

B


HDAC1 flag
empty
wt
S406A
S406E
Flag
HDAC1
vinculin

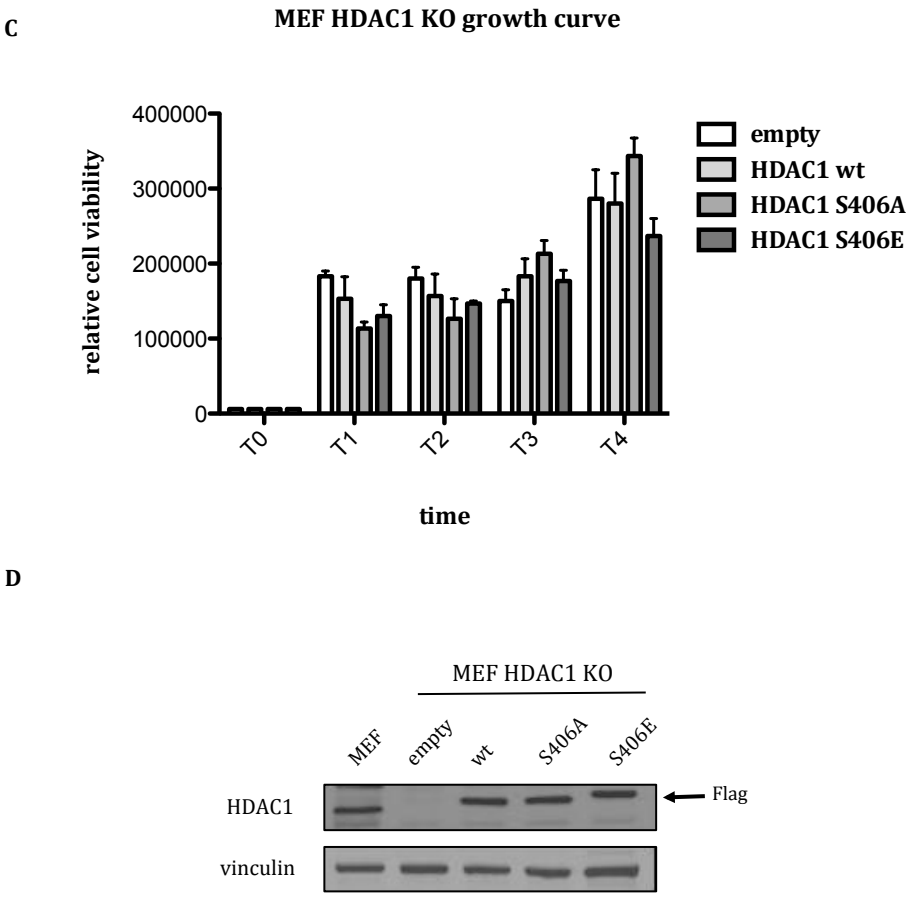


Figure 16. HDAC1 Aurora-dependent phosphorylation does not affect cellular proliferation

A-C. HeLa cells or MEF HDAC1 KO were stably infected with flag-tagged HDAC1 wt and Aurora phospho mutants. 10,000 cells were plated in 24-well plates and luminescence (as indicator of cell viability) were measured by CellTiter-Glo® every 24 hours for 4 days. The fold increases of HDAC1 construct compared to HDAC1 wt (HeLa) or empty (MEF HDAC1 KO) are reported for every time point. The average of three independent experiments was reported; the significance was calculated by two-way ANOVA algorithm, standard error (SEM) was indicated by the error bars. **B-D.** Western blot are also reported to check the over expression of the flag-tagged constructs. Vinculin is used as loading controls.

Growth curve experiments were performed in other cell lines (U2OS, MCF7, MCF10A) as well, overexpressing HDAC1 Aurora-phospho mutants alone or upon endogenous HDAC1 interference (data not shown).

For the colony forming assays, 3000 cells (a single-cell dilution) were plated in 10cm plates and colonies were stained with Crystal Violet after 8 days. The counts of colonies are reported in Figure 17; cells expressing HDAC1 S406A or HDAC1 S406E mutants seemed to have the same colony forming potential compared to cells expressing wild type HDAC1.

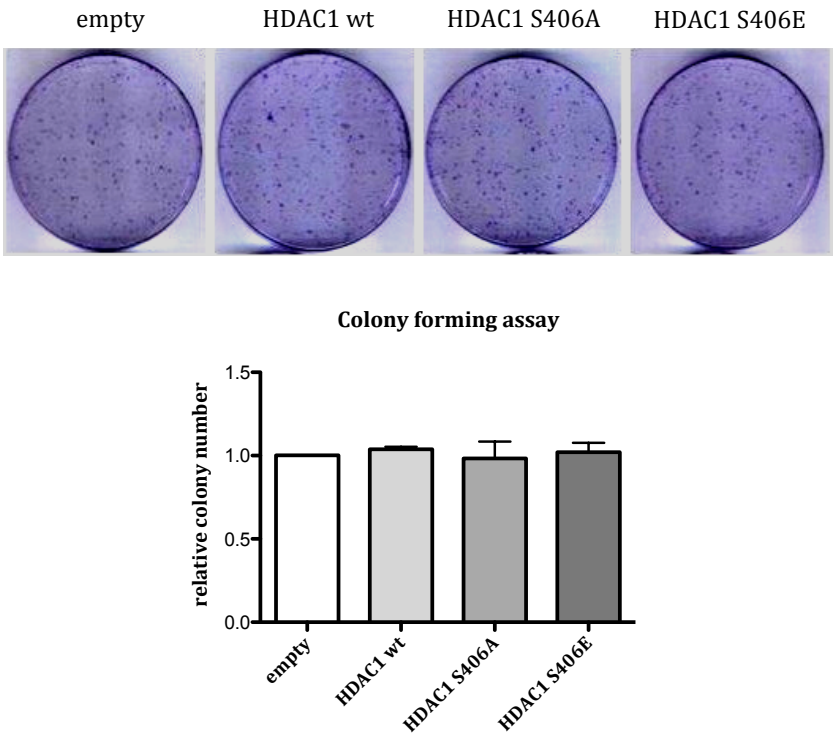


Figure 17. HDAC1 Aurora-dependent phosphorylation does not affect the clonogenic potential of the cells

HeLa cells were stably infected with flag-tagged HDAC1 wt and Aurora phospho mutants. 3000 cells were plated in 10 cm dish and colonies were stained with crystal violet after 8 days. Colonies were counting using ImageJ software. Results are reported as ratio compared to empty control. The average of three independent experiments was reported; the significance was calculated by one-way ANOVA algorithm, standard error (SEM) was indicated by the error bars.

The same trend was observed for the colony assays performed in different cell lines in presence/absence of the endogenous HDAC1 (data not shown).

All these data suggest that Aurora-dependent phosphorylation of HDAC1 is not involved in proliferation and clonogenic potential of cells.

Human HDAC1 Aurora phospho-mutants differentially rescue the phenotype of zebrafish hdac1 morphant embryos compared to wild type HDAC1

Having characterized the biochemistry of the Aurora-mediated phosphorylation of HDAC1, we searched for a biological read-out. Zebrafish is a suitable model to investigate the effects and seek for a phenotype for HDAC1 Aurora-driven phosphorylation. In zebrafish a gene encoding HDAC2 is not present, thereby overcoming the compensation mechanism that occurs in many cellular systems between HDAC1 and HDAC2 when one of the two proteins is depleted [21,22,87]. Moreover, many studies have pointed to the crucial role of HDAC1 during zebrafish development, in particular in the nervous system, the retina and the optic stalk [109].

We used zebrafish development as a model and set up the following experimental approach: zebrafish embryos were injected right after fertilization with scramble or hdac1 morpholino in the presence or absence of specific mRNAs codifying hHDAC1 wt, S406A or S406E to evaluate the rescuing potential of the different Aurora mutants compared to wild type hHDAC1 (human HDAC1). We observed that the injection of a scramble morpholino (negative control) did not affect the morphology of 72 hpf (hours post fertilization) embryos and the expression level of HDAC1 protein compared to non-injected fish (Figure 18A). On the contrary, as already known [114], MO-hdac1 injected embryos displayed multiple developmental and morphological defects due to the absence of HDAC1: reduced head size (hd), absent jaw (j), curled down tail (ct); moreover, a pericardial edema (pe) was clearly visible. Also pectoral fins (pf) were missing, otoliths (*) were smaller and close together and melanocyte migration was defective.

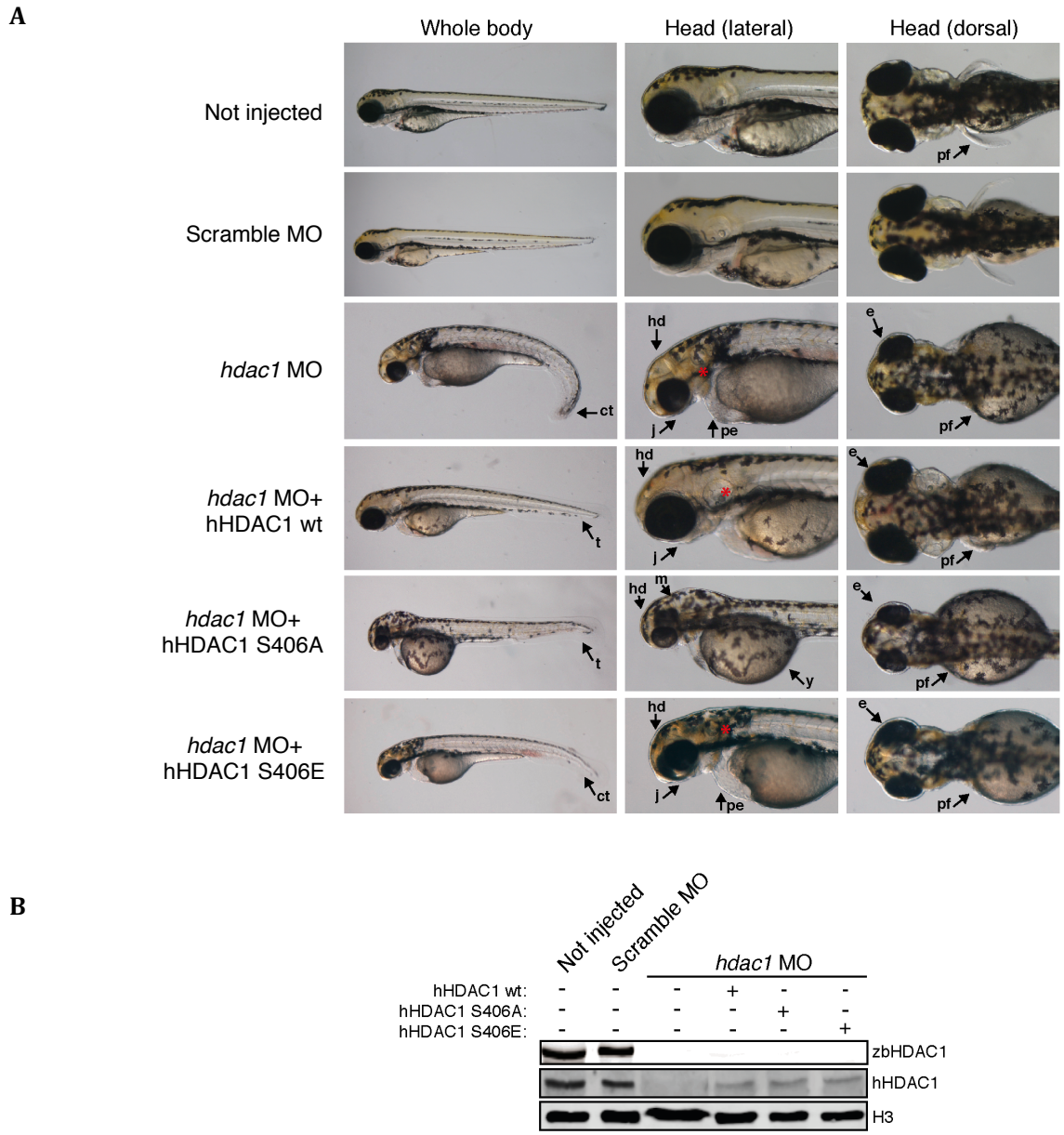


Figure 18. HDAC1 Aurora phospho-mutants differentially rescue the phenotype of zebrafish hdac1 morphant embryos compared to wild type HDAC1

A. Lateral and dorsal overviews of indicated zebrafish embryos at 72 hours post fertilization (hpf). Arrows highlight morphological differences between embryos. hd: head, m: head melanocytes, j: jaw, e: eye, pe: pericardial edema, y: yolk, pf: pectoral ns, t: tail, ct: curled down tail, *: otoliths. **B.** Western blot analysis of protein extracts from the indicated embryos collected at 72 hpf. The anti-human HDAC1 recognizes also zebrafish HDAC1. Anti-Histone3 (H3) antibody was used as a loading control.

The expression of the human HDAC1 wt by co-injection of its mRNA together with hdac1 MO significantly rescued the morphants phenotype: 90% of the embryos displayed a wild type-like phenotype with relatively normal heads, eyes and jaw structures and a straight antero-posterior body axis; reabsorption of the pericardial

edema, proper melanocyte distribution and an outline of pectoral fins are visible. Interestingly, the human Aurora phospho-null S406A mutant only partially rescued essential features of the morphant phenotype. In fact, although reduced head size, absence of pectoral fins and cell migration defects observed in morphant embryos still persisted, a proper body axis and pericardial edema were recovered. Moreover, the eye structure was less affected compared to hdac1 morpholino embryos. Notably, all the body structures showed a striking developmental delay, as indicated by the late migration of the head melanocytes and yolk reabsorption. About 56% of HDAC1 S406A injected embryos showed a wild-type-like phenotype. Conversely, the expression of the human phospho-mimetic HDAC1 S406E was not able to rescue the phenotype in a hdac1 morpholino background: strong defects in the development of the head and the eye were present, pericardial edema was not reabsorbed and jaw and pectoral fins failed to form. Furthermore, there was no recovery in the body axis: a curled down tail was present and a random distribution of melanocytes was visible, particularly in the head. About 70% of the HDAC1 S406E injected embryos had a MO hdac1-like phenotype. Figure 18B shows the corresponding western blot to verify the knock down of zebrafish endogenous hdac1 (zbHDAC1) and the expression of human HDAC1 proteins (hHDAC1): the complete depletion of the endogenous zbHDAC1 protein upon hdac1 MO injection is visible in lane 3; lanes 4, 5 and 6 display the expression of the injected human proteins in a hdac1 MO background. Taken together these results indicated that a proper balance of the Aurora-driven phosphorylation of HDAC1 is critical for the maintenance of a proper proliferative and developmental plan in a complex organism.

Aurora kinases phosphorylate human HDAC1 in zebrafish embryos

Using the BT15 antibody (produced at IFOM-IEO Campus) that specifically recognizes serine 406 phosphorylation on HDAC1 (see Figure 11, Introduction), we verified whether the orthologs of the human Aurora kinases were able to phosphorylate hHDAC1 also in zebrafish embryos. Thus, zebrafish embryos were injected with hdac1 morpholino alone or in combination with hHDAC1 Aurora phospho mutants, collected at 24 hpf and immunostained with BT15 antibody.

DAPI and p-S10H3 staining were also performed as control of the cell cycle stage (chromatin state) and the mitotic phase, respectively. Flag antibody was used to recognize cells in which flag-tagged hHDAC1 Aurora phospho mutants were distributed upon injection. Indeed, although the constructs were injected at one-cell stage right after fertilization, HDAC1 mRNAs may have randomly spread during cell division, hence producing chimerae.

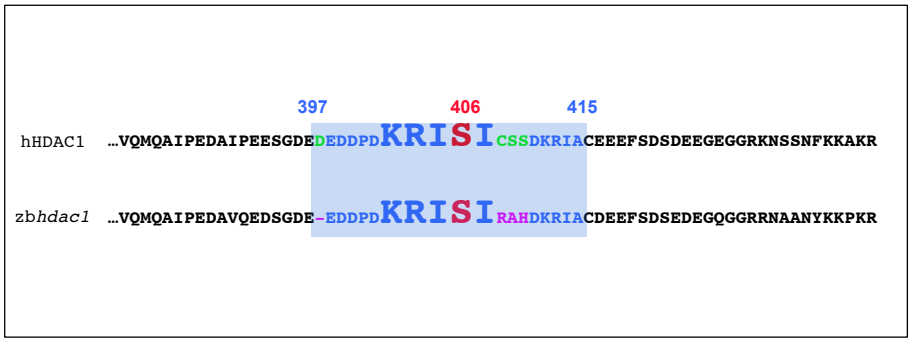


Figure 19. Human and zebrafish HDAC1 differ of 4 mismatches in the BT15 epitope

The BT15 epitope recognizing the KRISI Aurora consensus motives of HDAC1 is reported. The non-conserved residues between human and zebrafish HDAC1 are shown respectively in green and pink. The amino acids are indicated according to the universal one letter code.

As shown, in embryos injected with a scramble morpholino there were no BT15 positive cells probably because human and zebrafish HDAC1 protein differ by a 4

amino acid mismatch in the BT15 epitope (Figure 19, 20A); as expected, upon hdac1 morpholino injection, no BT15 or flag signal were detected. Embryos co-injected with hdac1 morpholino and hHDAC1 wt displayed a limited number of BT15 positive cells: indeed, among all the mitotic (p-S10H3 positive) cells, only pro-metaphase cells were recognized by the pS406 HDAC1 antibody (Figure 20B), as expected from our mammalian data that enclose HDAC1 Aurora-dependent phosphorylation in pro-metaphase (see Figure 11, Introduction). Moreover, the injection of the phospho null mutant HDAC1 S406A in a hdac1 morpholino background completely abolished the BT15 staining; on the contrary, the S406E HDAC1 mutant mimicked the Aurora driven phosphorylation of the protein, thus all the cells of the embryos injected with hdac1 MO plus S406E HDAC1 were BT15 positive, independently of the phases of the cell cycle (Figure 20A).

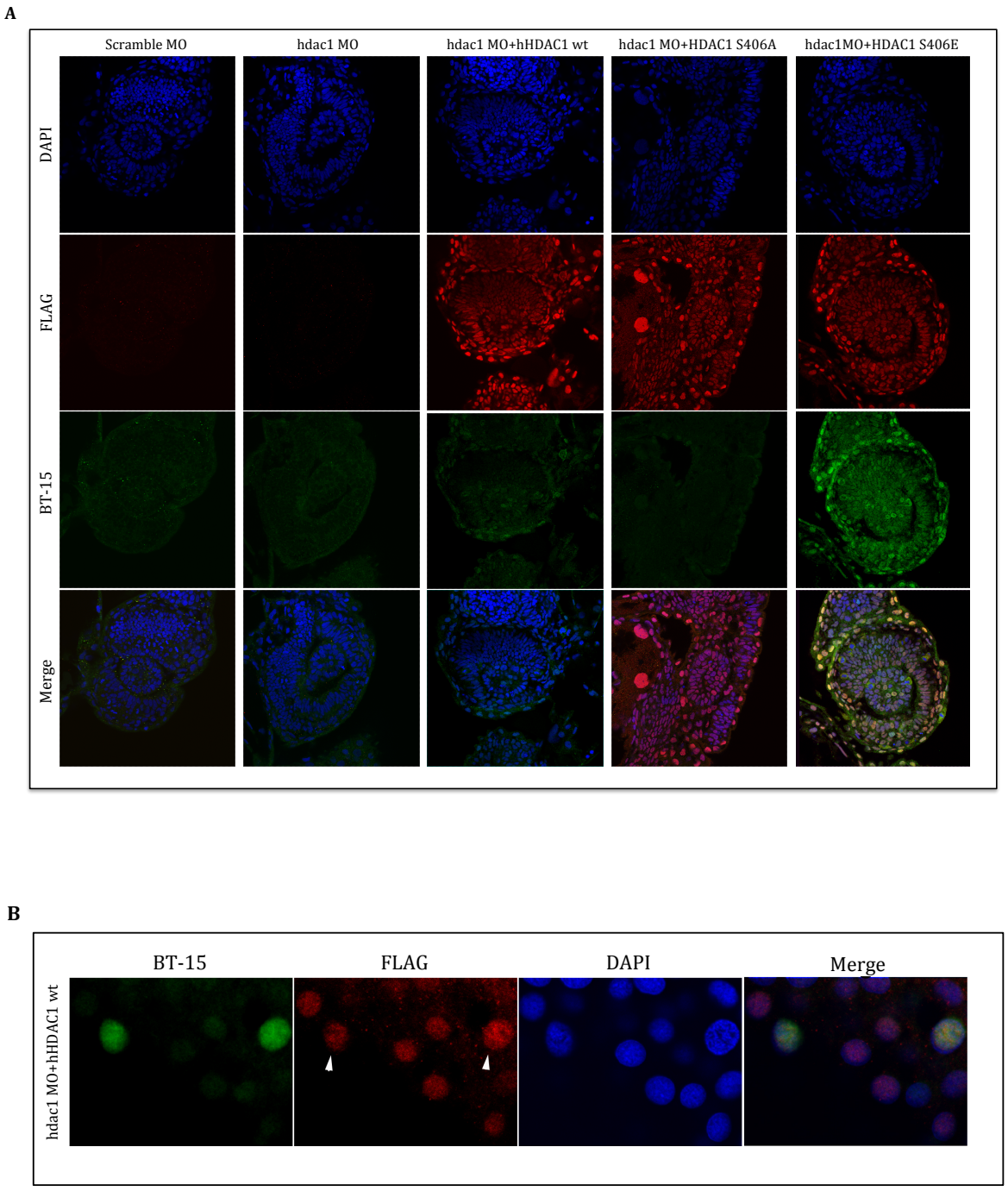


Figure 20. Aurora kinases phosphorylate hHDAC1 Aurora phospho mutants in zebrafish embryos

A. Immunofluorescence microscopy of 24 hpf zebrafish embryos. Embryos were injected at one-cell stage with scramble MO or hdac1 MO alone and in combination with hHDAC1 wt, S406A or S406E mutants, collected at 24 hpf, fixed in 4% paraformaldehyde and then immunostained with the specific antibody. **B.** Higher magnification of zebrafish gastrulae stained with the indicated antibody.

Taken together all the immunostaining data clearly suggest that Aurora kinases phosphorylate hHDAC1 also in zebrafish and that, as in mammalian cells, this phosphorylation takes place only in mitosis during pro-metaphase.

Overexpression of human HDAC1 Aurora phospho-mutants does not result in any morphological defects in zebrafish embryos

Our previous experiments demonstrated that, upon injection in zebrafish embryos together with hdac1 MO, expression of human HDAC1 Aurora phospho-mutants resulted in different phenotypes. In particular, human HDAC1 wt completely rescued the morphological defect induced by depletion of hdac1 (Figure 18), whereas HDAC1 S406A mutant caused a marked delay in the development of the embryos, and HDAC1 S406E strongly impaired the formation and growth of many embryos body structures. Therefore, we overexpressed human HDAC1 wt, S406A and S406E to evaluate their impact on expression of the endogenous zbHDAC1 and on the morphology of the embryos. Therefore, hHDAC1 wt or its Aurora phospho mutants were injected right after fertilization in zebrafish embryos and 72hpf embryos were then collected.

Figure 21A clearly displays that overexpression of human HDAC1 wt, as well as HDAC1 S406A and S406E mutants did not perturb the development and the morphology of the injected embryos, which perfectly phenocopied their un-injected counterparts. Furthermore, the related western blot (Figure 21B) showed that expression of the HDAC1 Aurora phospho-mutants did not affect the endogenous zbHDAC1 protein level.

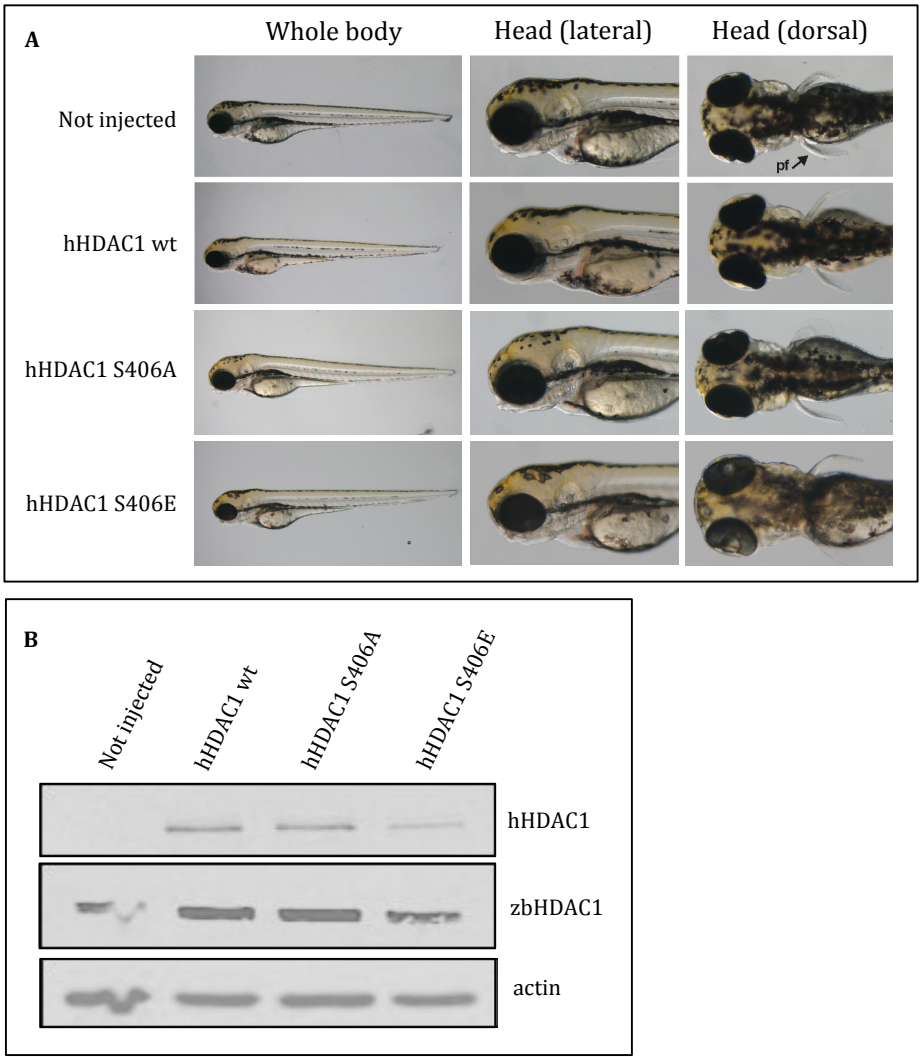


Figure 21. Overexpression of hHDAC1 Aurora phospho-mutants does not impact on developing zebrafish embryos

A. Lateral and dorsal overviews of indicated zebrafish embryos at 72 hours post fertilization (hpf). **B.** Western blot analysis of protein extracts from the indicated embryos collected at 72 hpf. The anti-human HDAC1 does not recognize zebrafish HDAC1. Anti-actin antibody was used as a loading control.

Taken together, these data do not support a possible role for the HDAC1 Aurora mutants as dominant negative and suggest that HDAC1 Aurora-dependent phosphorylation does not affect embryonic development in the presence of the endogenous hdac1.

Human HDAC1 Aurora phospho-mutants do not rescue the depletion of the G2-M population in hdac1 morphant zebrafish embryos

In light of the different effects on the zebrafish embryos development upon injection of HDAC1 Aurora-phospho mutants, we monitored cell cycle progression in embryos expressing hdac1 MO alone or in combination with human HDAC1 Aurora-phospho mutants and in control embryos. Thus, we measured the DNA content of cells from 24, 48 and 72 hpf embryos by PI staining and fluorescence-activated cell sorting (FACS) analyses. Comparing cell populations of embryos heads between scramble and hdac1 morphant embryos, we found a high and significant reduction of approximately 50% in the G2-M populations in each of the three time points considered (Figure 22).

FACS analysis

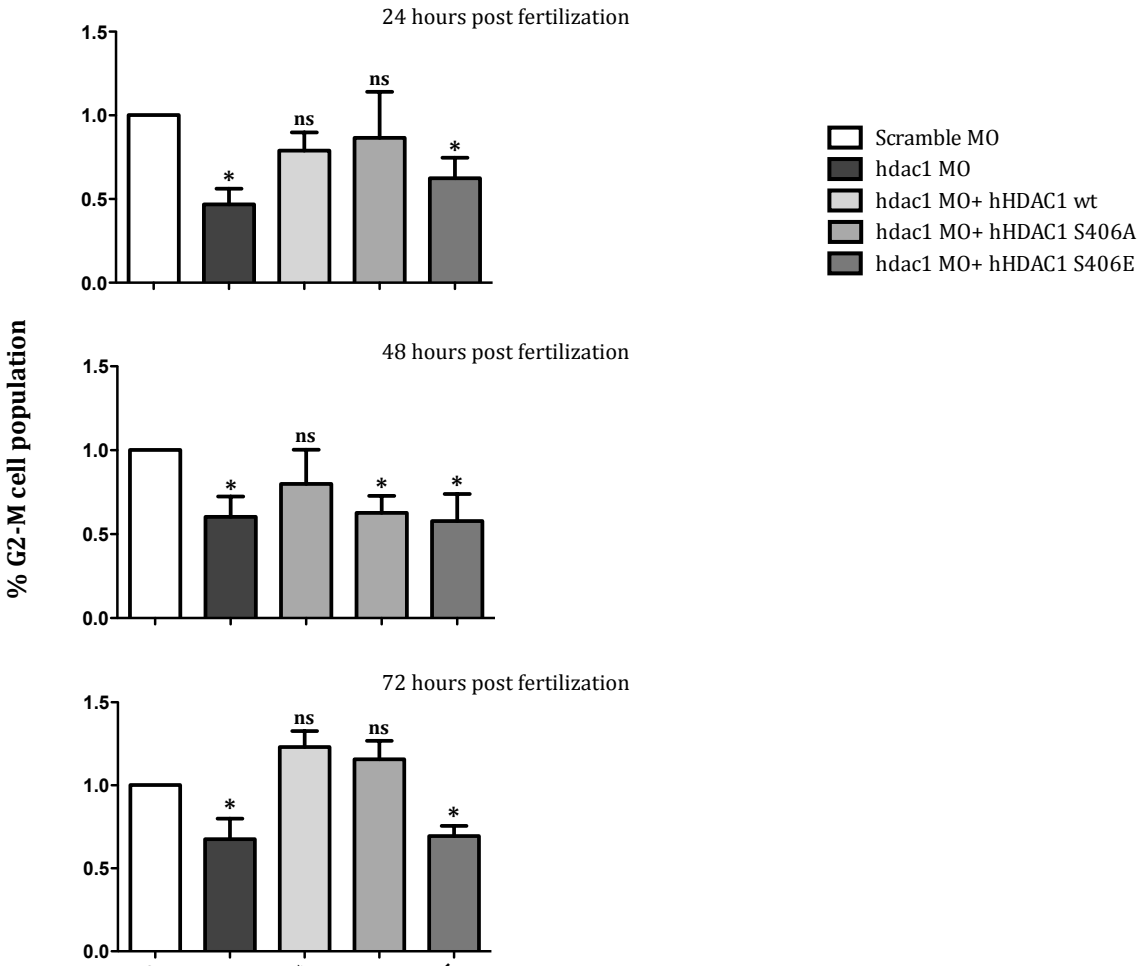


Figure 22. Human HDAC1 (hHDAC1) Aurora phospho-mutants cause the depletion of the G2-M population in hdac1 morphant zebrafish embryos

Flow cytometry analysis by PI staining of cell suspensions prepared from 24, 48, 72 hpf heads of control embryos and embryos injected at one-cell stage with hdac1 MO alone or in combination with hHDAC1 wt, hHDAC1 S406A or hHDAC1 S406E. The percentage of G2-M cells population compared with Scramble MO is reported for every sample. The average of three independent experiments was reported, standard error (SEM) was indicated by the error bars; the significance was calculated by one-way ANOVA algorithm. *: P value <0.05; ns: not significant.

Interestingly, at 24, 48 and 72 hpf there was nearly the same significant decrease in the percentage of the G2-M cells also upon co-injection of the hdac1 MO and human phospho-mimetic HDAC1 S406E, suggesting that the morphant embryos injected with HDAC1 S406E failed to revert the phenotype due to cell cycle progression impairment. On the contrary, comparing cells from heads of control embryos and embryos co-injected with hdac1 MO and human HDAC1wt, we could not detect any differences in cell cycle progression at 24 and 48 hpf, but the proportion of the cells in

G2-M phase increased by at least 20% at 72 hpf. Moreover, heads of embryos co-injected with hdac1 MO and human HDAC1 S406A displayed, compared to the control, a significant reduction of almost 40% in the G2-M population only at 48 hpf.

Taken together, these results suggest that HDAC1 Aurora phospho mutants compromise the accurate cell cycle progression in the morphant embryos, failing to restore reduction in the G2-M cell population, which most likely leads to developmental and morphological defects.

Human HDAC1 Aurora-phospho mutants regulate global histone acetylation in the developing zebrafish embryo

Our findings with the *in vitro* histone assay led to the hypothesis that the Aurora-dependent phosphorylation of HDAC1 is important in controlling the deacetylase activity of the enzyme: in particular we found that the Aurora phospho-mimetic mutant HDAC1 S406E was less efficient in deacetylating labeled histones compared to HDAC1 wt (Figure 14).

Thus, considering also the morphological data described in developing zebrafish embryos injected with hdac1 MO alone or in combination with hHDAC1 Aurora phospho mutants (Figure 18), we asked whether the differences emerging in the phenotype of hHDAC1 wt, hHDAC1 S406A and S406E embryos could be partially ascribed to a differential ability of the Aurora phospho mutants to deacetylate their substrates.

To this end, immunohistochemistry (IHC) experiments were carried out on embryo sections testing, with a pan-acetylated antibody, the deacetylating potential of hHDAC1 Aurora phospho-mutants *in vivo*.

72 hpf embryos were collected, included in agarose and then embedded in paraffin and sliced with microtome. Sectiones of hdac1 morphant embryos or embryos

injected with MO+ hHDAC1 wt, S406A or S406E mutants were immunostained with a acetylated-histone antibody and the acetylation levels in the eyes were compared to those of control embryos. Haematoxylin staining was also performed to recognize cell nuclei.

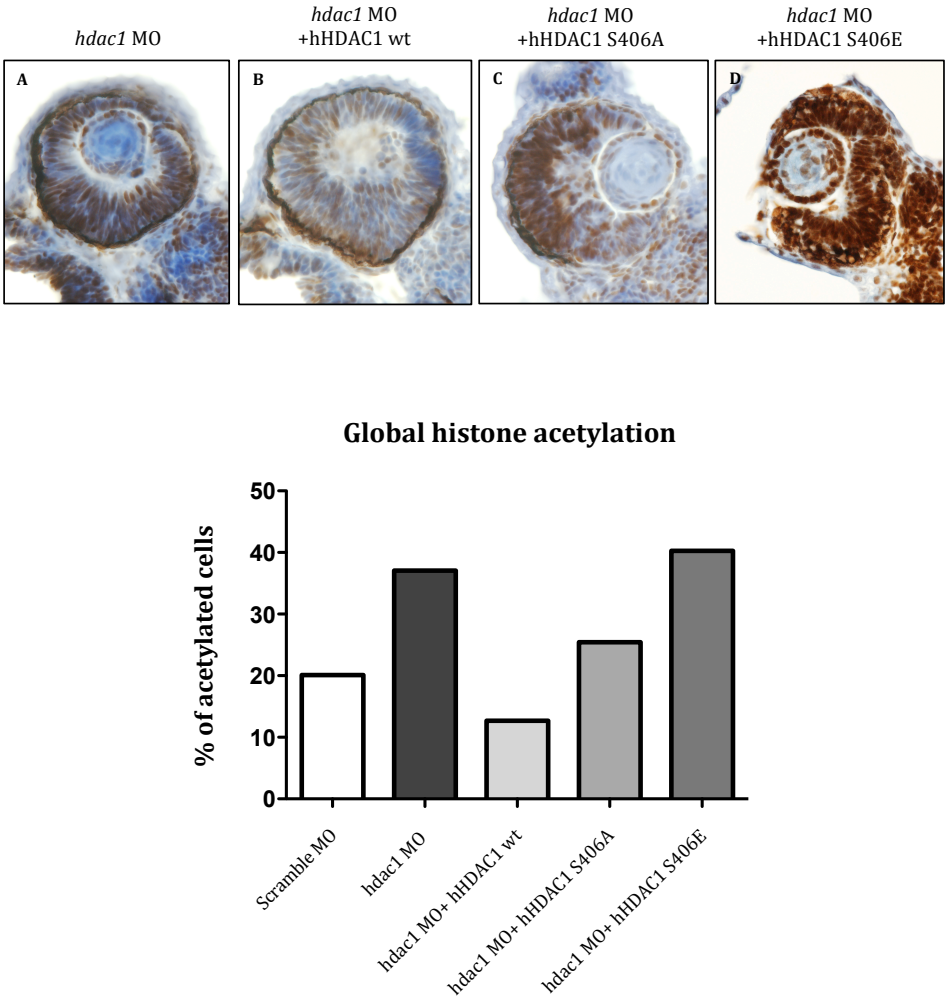


Figure 23. hHDAC1 Aurora-dependent phosphorylation affects histone acetylation *in vivo*

Immunohistochemistry staining with haematoxylin (blue) and anti-acetylated histone antibody (brown) of sections obtained by 72 hpf embryos injected at one-cell stage with scramble MO, hdac1 MO **(A)**, hdac1 MO+hHDAC1 wt **(B)**, hdac1 MO+hHDAC1 S406A **(C)**, hdac1 MO+hHDAC1 S406E **(D)**. The percentage of acetylated cells was calculated using Aperio ImageScope software and is reported for every sample. Although present in the graph, image taken from scramble MO is missing because of technical problems. The experiment was done only once, thus no statistic analysis is included.

The results are shown in Figure 23: as expected, knock down of endogenous hdac1 in morphant embryos increased the percentage of positive (acetylated) cells compared to scramble MO injected embryos (A); interestingly, we observed the same trend in the eyes of embryos injected with hdac1 MO+ hHDAC1 S406E (D), suggesting that also in a *in vivo* model the Aurora phospho-mimetic form of HDAC1 failed to deacetylate its substrates. Conversely, measuring the percentage of positive cells in control embryos and embryos co-injected with hdac1 MO and hHDAC1 wt, very slight differences were detected, indicating that hHDAC1 wt mRNA was able to restore the acetylation level in 72 hpf embryos eyes (B). Additionally, embryos expressing hdac1 MO together with hHDAC1 S406A mutant exhibited compared to the control, higher levels of eye acetylation, even though the percentage of positive cells was still much lower than those in morphant embryos (C). This result is in line with the morphological data that shows for HDAC1 Aurora phospho-null mutant an intermediate phenotype between morphant and rescued embryos.

The preliminary IHC experiment indicates that HDAC1 Aurora-dependent phosphorylation is involved in the control of the catalytic activity of the enzyme regulating global histone acetylation levels in developing zebrafish embryos.

Human HDAC1 Aurora phospho-mutants epigenetically modulate acetylation of neurogenic regulatory genes throughout the zebrafish developing CNS

Harrison and colleagues identified, via transcriptome comparative analysis of hdac1-deficient and control zebrafish embryos, a small subset of genes that specifically exhibit Hdac1-dependent expression from early neurogenesis onwards [112]. Thus, we asked whether HDAC1 Aurora mutants exert their regulation by an epigenetic mechanism. To verify this hypothesis, we performed, on heads of 72 hpf embryos

injected, at one-cell stage, with hdac1 MO alone or in combination with hHDAC1 wt, S406A or S406E, a chromatin immunoprecipitation (ChIP) experiment with anti-acetylated lysine H3K27 antibody. Since HDAC1 lacks a DNA-binding domain and as it is not directly associated with chromatin, we did not use an anti-HDAC1 antibody, but assumed that possible differences in H3K27 acetylation levels could be due to the injection of the HDAC1 Aurora phospho-mutant. The ChIP experiment was coupled to quantitative PCR to evaluate the enrichment in the acetylation of the promoter of selected genes compared to their input. A possible enrichment in the H3K27 acetylation level of the promoter can be an indirect proof of the binding of HDAC1 to the region considered. The genes used for the quantitative analysis were chosen from a list of genes involved in zebrafish developing CNS and retina, whose expression significantly changed upon hdac1 ablation published by Harrison and colleagues.

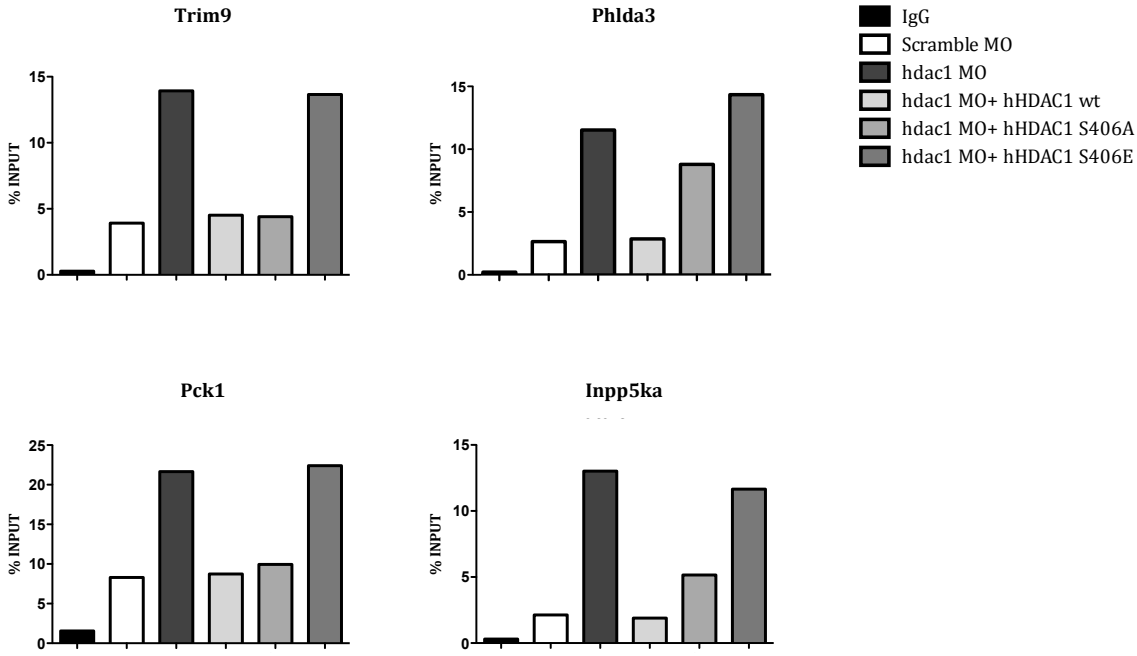


Figure 24. hHDAC1 Aurora-dependent phosphorylation plays a role in modulating acetylation of genes involved in developing zebrafish CNS

Chromatin immunoprecipitation (ChIP) analysis of H3K27 acetylated cis-regulatory regions of zbHDAC1-regulated genes. Embryos were injected with scramble MO or hdac1 MO alone or in combination with hHDAC1 aurora phospho mutants and collected at 72 hpf. Chromatin was immunoprecipitated with anti-acH3K27 antibody and control IgG and DNA content analyzed by q-PCR. Experiment done only once, thus no statistic analysis is included. Negative control was not included.

Our preliminary data, shown in Figure 24, display the trend of acetylated promoters of four genes (Trim9, Phlda3, Inpp5ka, Pck1) under hdac1 knock down and/or complementation with the hHDAC1 Aurora phospho mutants. As expected, ablation of the endogenous hdac1 led to an increased H3K27 acetylation level of each promoter of the four genes compared to the IgG control; notably, acetylated H3K27 highly increased also upon co-injection of hdac1 MO and hHDAC1 phospho-mimetic, supporting our previous findings of a reduced deacetylating activity for HDAC1 S406E mutant. On the contrary, expression of hHDAC1 wt in morphant embryos completely restored the acetylation level in the promoter of the four genes. Furthermore, comparing the H3K27 acetylation level of the selected genes in control

embryos and embryos injected with hdac1 MO+ hHDAC1 S406A, we found a similar enrichment for pck1 and trim9, but an higher acetylation level on the Phld3 and Inpp5ka promoters.

We can conclude that hHDAC1 Aurora phospho mutants modulate acetylation of genes implicated in developing CNS of zebrafish embryos.

HDAC1 and HDAC2 are believed to be the master regulators of chromatin structure and gene expression and are also deeply involved in cellular proliferation, the cell cycle, and apoptosis [21,22,87,89]. However, the molecular pathways in which HDAC1 and HDAC2 are involved during cell cycle progression remain unclear.

HDAC1 and HDAC2 are targets of a complex code of post-translational modifications (PTMs) [58]. Because PTMs influence enzymatic activity and complex formation of the two proteins, it is reasonable to assume that the key to dissect HDAC1 and HDAC2 functions lies in understanding their PTM code. Among the various PTMs that occur on HDAC1 and HDAC2, phosphorylation is the most intensively studied: HDAC1 and HDAC2 can be phosphorylated by the casein kinase II (CKII). The CKII-dependent phosphorylation enhances the deacetylase activity and the transcriptional repression as well as the interaction with multisubunit complex partners [38,61] [63].

Our lab identified a new mitotic phosphorylation of HDAC1 and HDAC2. Our previous findings demonstrated that the two enzymes are phosphorylated specifically in pro-metaphase by Aurora kinases A and B (see Figure 11, Introduction). Other enzymes critically involved in mitosis, such as the phosphatase Cdc25B [188], show the same phosphorylation profile, suggesting that HDAC1 and HDAC2 phosphorylation might be a signal to switch on/off some specific functions.

As mentioned above, HDAC1 plays a major role in controlling cell cycle progression and cell proliferation and has been found deregulated in many cancer cell lines and tissues. Moreover, Senese et al. proved that the absence of HDAC1 can arrest cells either at the G1 phase of the cell cycle or at the G2/M transition, resulting in the loss of mitotic cells, cell growth inhibition, and an increase in apoptosis, whereas HDAC2 knockdown showed no effect on cell proliferation unless HDAC1 was concurrently

knocked down [22]. Thus, we focused at understanding the biological role of the Aurora-dependent phosphorylation of HDAC1.

In accordance with the *in vivo* evidence (Figure 8-11), our *in vitro* kinase assay confirms the affinity of the Aurora A and B kinases for HDAC1 (Figure 14) also suggesting a direct interaction between the two kinases and the deacetylating enzyme (Figure 12).

Given the well-established role of CKII in phosphorylating HDAC1, we asked whether CKII was responsible for making the Aurora phosphorylation sites available. We found instead that the activity of CKII is not required for mitotic hyper-phosphorylation of HDAC1 by Aurora kinases (Figure 13). The absence of phosphorylation of HDAC1 on serine 421/423 does not impair its mitotic phosphorylation, since the HDAC1 2S mutant is phosphorylated in mitosis at levels comparable to the wild type, thus excluding a possible cross-talk between the two kinases and a possible hierarchy in the PTM code.

It is also known that the abolition of CKII-dependent phosphorylation of HDAC1 abrogates its catalytic activity and dramatically reduces the binding with HDAC2, RbAP48, Sin3a, CoREST and MTA-2 [38]. We thus asked whether the Aurora-dependent phosphorylation affects the deacetylating potential of HDAC1, acting as a fine modulator in a specific time interval of cell cycle. We found that neither the phospho-null nor the phospho-mimetic mutants of HDAC1 are deficient in their ability to bind known partners such as RbAP48 or endogenous HDAC2 (Figure 14). However, we plan to test also the interaction between HDAC1 Aurora mutants and other multiprotein complex proteins (Sin3a, CoREST and MTA-2). On the contrary, concerning the effects on the deacetylating activity, the phospho-mimetic mutant of HDAC1 displays a partial reduction on the catalytic potential compared to the wild type, even if its deacetylating ability is not completely abrogated as in the case of the

CKII phospho-null mutant (HDAC1 2S). The reduced catalytic ability of the mutant HDAC1 S406E is unlikely to be due to improper folding of the protein, since its binding with other partners is not affected. One possible explanation could be that mitotic phosphorylation of HDAC1 promotes the association with other, unknown partners that modulate the affinity of the enzyme for its substrates. Moreover, it was already reported that during mitosis HDAC1 is excluded from chromatin, even if global deacetylation activity is maintained [189]. Phosphorylation adds a negative charge to the surface of HDAC1, which could increase the electrostatic repulsion between HDAC1 and the negatively charged DNA. So we speculate that the presence of the negative charge in the HDAC1 S406E mutant could facilitate the displacement of the enzyme. Further experiments are required to corroborate this hypothesis. To this aim we generated GFP-HDAC1 fusion proteins that will be used to perform live cell imaging by time lapse microscopy to track the localization kinetics of HDAC1 wild type, phospho-null and phospho-mimetic proteins.

We also checked the activity of the different HDAC1 mutants directly *in vivo*, analyzing by western blot the acetylation status of histone H3K9 (Figure 15). Our preliminary *in vivo* data support the *in vitro* findings: indeed, we observed a slightly higher H3K9 acetylation upon overexpression of the less active S406E mutant, whereas overexpression of HDAC1 S406A mildly reduces histone acetylation compared to the control, probably because its activity cannot be modulated by Aurora-dependent phosphorylation. Thus it is always active also in mitosis.

Our preliminary data suggest that there might be an effect of S406-HDAC1 phosphorylation on its catalytic activity also *in vivo*. Nevertheless, our growth curve and colony forming assay experiments (performed both in the presence and absence of the endogenous HDAC1) do not reveal any differences among the two Aurora-

phospho mutants in terms of proliferation potential and clonogenicity of the cells compared to the wild type protein (Figure 16, 17).

The finding that Aurora-dependent phosphorylation of HDAC1 does not induce dramatic effects on cellular phenotypes is not in contrast with what our group previously reported, where depletion of HDAC1 (but not of HDAC2) caused an increase in apoptosis and proliferation defects [22]; but rather confirms our hypothesis of a fine-tuning role for HDAC1 phosphorylated S406 in proper cell cycle progression.

A modest and only partial rescuing of a mitotic phenotype was already reported for phosphorylation of another protein involved in mitosis, the phosphatase Cdc25B [188]. Cdc25B is phosphorylated by Aurora A at the G2/M transition on serine 353 [190], and this phosphorylation is inhibited when the G2/M DNA damage checkpoint is activated, probably as a consequence of Aurora A inactivation itself [191]. Overexpression of Aurora A or Cdc25B wild type caused an override of the DNA damage checkpoint of 89% and 46% respectively, while the overexpression of the Cdc25B S353A mutant induced 33% of override, and 54% when coexpressed with Aurora A. These and our data suggest that mutations in a single phosphorylation site normally do not lead to a dramatically different phenotype compared to the wild type counterparts; furthermore, it should be considered that key cellular enzymes, such as HDAC1 or Cdc25B phosphatase, possess very complex PTMs codes, and it is unlikely that a single modification event completely abrogates the protein's functions. Nevertheless, these considerations do not imply that specific, point modifications are not significant.

It has also to be taken into account that in many cellular systems, due to the well-known compensation mechanism between HDAC1 and HDAC2 [192,193], depletion of HDAC1 leads to an increased level of HDAC2 protein and vice versa [21,192,193].

Thus, it could be difficult to unravel the function of the Aurora-dependent phosphorylation of HDAC1 in the presence of HDAC2, and perhaps mammalian cells do not best suite towards the purpose of understanding the role of this phosphorylation.

Furthermore, we reasoned that proliferation and differentiation are two interconnected pathways, especially during development, where every event of cell division must be strictly regulated in space and time to successfully complete the developmental plan. It is even true that also small delays in proliferation would produce dramatic effects in the context of an organism rather than in immortalized cell cultures. As such, we searched for an *in vivo* model to investigate the biological role of this mitotic phosphorylation of HDAC1, especially considering the crucial involvement of the enzyme in development.

Zebrafish seemed to be a suitable model. Indeed, in zebrafish a perfectly coordinated and regulated cell cycle is the *conditio sine qua non* for the proper completion of the developmental program and even very slight perturbations are likely to have a dramatic impact. Unlike mammals, zebrafish do not possess two distinct HDAC1 and HDAC2 genes, but they only have one, which is more similar to human HDAC1 in terms of amino acidic sequence. Moreover, the Aurora phosphorylation consensus site is highly conserved during the evolution between zebrafish and human. As in mammals, *zb*HDAC1 (hdac1) has a pivotal role in development: hdac1 mutants show abnormalities in the heart, retina, craniofacial cartilage and pectoral fins and have reduced proliferation in hindbrain [112,114,194,195].

Our experimental approach consisted in knocking down the endogenous hdac1 in zebrafish oocytes using the morpholino strategy, and reinserting the human HDAC1 wild type and Aurora phospho mutants in order to observe possible different phenotypes during the development of the animal. While the human HDAC1 wild type

is able to rescue the phenotype of hdac1 morphants at 72 hpf (hours post fertilization), the two phospho-Aurora mutants show a different behavior (figure 18). The phenotype of the phospho-null S406A mutant injected embryos is not dramatically impaired but looks more as if it has a delay in development. It has been published that depletion of Aurora A in zebrafish causes growth retardation due to mitotic delay [196], so it may be that the phenotype observed upon injection of the HDAC1 phospho-null mutant is due to the absence of the kinase rather than a real effect of the phosphorylation on HDAC1. On the contrary, the embryos injected with the phospho-mimetic S406E are deeply affected and show defects in development and proliferation, in particular of the eye and head, phenocopying the morphology of hdac1 morphant or mutant in zebrafish embryos [114]. Perhaps the decreased enzymatic activity of the phospho-mimetic 406E mutant (Figure 14A) could be the cause of the missed morphological rescue of the embryos. This observation corroborates the hypothesis that, in a model in which HDAC2 is not present, even a difference of 40% in the deacetylating ability of the enzyme is able to substantially affect the development of the embryos. Conversely, we observed that overexpression of hHDAC1 wt or its Aurora phospho mutants does not affect endogenous hdac1 protein levels nor the development of the embryos, ruling out a possible dominant negative role for the injected constructs (Figure 21).

Moreover, we assessed that, in zebrafish, the orthologs of human Aurora kinases phosphorylate hHDAC1 wt specifically in prophase, according to the data collected in mammalian cells, whereas the injection of hdac1 MO alone or plus hHDAC1 S406A phospho-null mutant prevents the phosphorylation. On the contrary, injection of the hHDAC1 S406E mutant in hdac1 MO background mimics the phosphorylation in every cell expressing the construct (Figure 20).

Our FACS analyses on cells of heads from control and injected embryos at 24, 48 and 72 hpf, perfectly reflected what observed with the rescue experiments. Indeed, expression of the human HDAC1 wild type is able to recover the correct progression of the cell cycle in the morphants embryos, while the phospho-mimetic S406E, like hdac1 morpholino, leads to a strong reduction in the G2-M cell population in heads of developing morphants (Figure 22). This is consistent with the striking developmental defects gathered from morphological analysis. Instead, the phospho-null S406A mutant causes depletion in the number of G2-M cells as well, but only within a defined temporal window (up to 48 hpf), thus leading to a developmental delay rather than to morphological defects. It was recently shown that also Aurora kinase B phosphorylates class II HDAC 4, 5 and 9 during mitosis [197]. This finding and our results with HDAC1 demonstrate that Aurora kinases are crucial regulators of HDACs during the cell cycle and that different classes of HDACs are important in different phases of the mitosis: class I HDAC1 in prophase and early mitosis, class II HDACs in late anaphase and mitotic exit.

Many papers reported increased histone acetylation in zebrafish embryos upon treatment with the HDAC1 inhibitor TSA [198,199] or following hdac1 depletion [109,112]. Our IHC experiments with hdac1 MO reproduced these data (Figure 23) and the co-injection of hdac1 MO together with the two Aurora-phospho mutants, additionally suggest that Aurora-dependent phosphorylation of HDAC1 regulates global histone acetylation levels in developing zebrafish embryos. It is also worth noticing that our *in vivo* findings, in which expression of hHDAC1 S406E into hdac1 MO background does not rescue the enhanced histone acetylation levels compared to control embryos (Figure 23), is in line with the *in vitro* evidence on the decreased deacetylating activity of the S406E HDAC1 mutant is in zebrafish embryos. These data suggest that the Aurora phospho-mimetic mutant is not able to restore the effects

caused by endogenous hdac1 depletion, whereas hHDAC1 wt completely rescues the acetylation pathway when injected in an hdac1 MO background. On the other hand, the S406A HDAC1 mutant shows an intermediate phenotype, which contradicts our working model. Indeed, according to the activity assay we demonstrated that Aurora kinases inhibit HDAC1 deacetylating potential. Hence, the abolition of the Aurora-mediated phosphorylation should lead to a higher, or at least comparable to HDAC1 wt, deacetylating activity of the enzyme. One possible explanation could be that the persistent deacetylase activity of HDAC1 could cause a secondary effect and activate HAT (histone acetyltransferase) enzymes to compensate the presence of HDAC1 S406A mutant. Thus, the immunohistochemistry experiments must be repeated to verify this hypothesis and confirm our preliminary data.

Our ChIP experiments start to provide a further understanding on the comprehension of the HDAC1 Aurora-dependent phosphorylation biology. They show that HDAC1 epigenetically modulates histones acetylation levels of some genes involved in the zebrafish CNS development. Harrison and colleagues proved that in developing zebrafish embryos expression of genes mainly involved in the development of the CNS changes upon hdac1 ablation [112]. Here we reported that in the representative set of genes considered, the expression of hHDAC1 wt in the hdac1 MO background is able to bring their promoter acetylation levels to values comparable to those of control embryos. Conversely, the expression of hdac1 MO plus hHDAC1 S406A leads to a reduced promoter acetylation of certain genes (Trim9, Pck1), but not of others (Phlda3, Inpp5ka), suggesting that the developmental delay observed through the morphology experiment is probably due to an altered acetylation pathway exclusively in some genes, whereas upon co-injection of hdac1 MO and hHDAC1 S406E mutant a total impairment takes place and the H3K27 acetylation levels remain highly similar to those of hdac1 MO (Figure 24). These preliminary results

furnish a better explanation of the mechanism of action that leads to a differential global histone acetylation upon injection of the Aurora phospho mutants, but need to be further confirmed by the expression analysis of the genes considered.

Our working hypothesis is the following: as cells progress from G2 to metaphase, they need to condense their chromatin, monitor the process and correct any abnormalities in chromatin architecture. HDAC1 have an important role in this process since deacetylation of key residues, such as K9 of histone 3, is a prerequisite for mitotic DNA condensation, but at a certain point HDAC1 activity must be stopped and the enzyme displaced from chromatin. This could be achieved through Aurora-driven phosphorylation of HDAC1, which partially reduces its catalytic activity.

According to our results, Aurora kinases phosphorylate HDAC1 in prophase, right after cells enter mitosis. Thus, we hypothesized that this phosphorylation leads to a decrease in the deacetylating potential of the enzyme and has an effect on different levels: 1) epigenetically, modulating histones acetylation and 2) on proliferation level, controlling proper cell cycle progression.

In physiological conditions, S406 HDAC1 phosphorylation, even though very dynamically, results in increased promoter acetylation of genes expressed in the CNS or with CNS-oriented functions, thus enabling an accurate embryos' development. Thereafter, the prompt removal of phosphate group brings promoters' acetylation levels back to their basal state.

In more exacerbated conditions, i.e. mimicking HDAC1 phosphorylation, the enzyme is constitutively less active; thereby the promoters of above mentioned genes are highly acetylated. This situation lasting for quite a long time causes an overall morphological impairment and most importantly does not allow cells to go through mitosis provoking a decrease in the G2/M population. On the contrary, the absolute abrogation of the HDAC1 Aurora-dependent phosphorylation does not affect proper

cell cycle progression, but embryos display a clear developmental delay probably due to the fact that expression of genes persists at basal levels.

In conclusion, we hypothesized that while controlling the deacetylase activity of HDAC1, the Aurora-dependent phosphorylation on one hand modulates the expression of genes directly involved in zebrafish development, and, on the other hand, acts as sensor of the transcriptional process, regulating in a fine-tuned manner proper cell cycle progression.

Future work will aim at investigating in more details the mechanistic link between Aurora-dependent phosphorylation of HDAC1 and the events that take place in mitosis during development, to uncover specific genetic or protein networks in which the axis Aurora kinase/phospho-HDAC1 could be involved. Towards this aim a global and comparative analysis of the promoter acetylation (ChIPseq) or of the transcriptome (RNAseq) in embryos injected with HDAC1 Aurora mutants could be the best approach.

Therefore, our results in zebrafish strongly suggest a crucial *in vivo* role for the Aurora-driven HDAC1 phosphorylation during cell cycle progression and CNS development of zebrafish embryos.

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