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HDAC4 in cancer: A multitasking platform to drive not only epigenetic modifications

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Controlling access to genomic information and maintaining its stability are key aspects of cell life. Histone acetylation is a reversible epigenetic modification that allows access to DNA and the assembly of protein complexes that regulate mainly transcription but also other activities. Enzymes known as histone deacetylases (HDACs) are involved in the removal of the acetyl-group or in some cases of small hydrophobic moieties from histones but also from the non-histone substrate. The main achievement of HDACs on histones is to repress transcription and promote the formation of more compact chromatin. There are 18 different HDACs encoded in the human genome. Here we will discuss HDAC4, a member of the class IIa family, and its possible contribution to cancer development.

KEYWORDS

HDACs, enhancers, H3K27ac, MEF2, sarcomas, senescence

1 Introduction

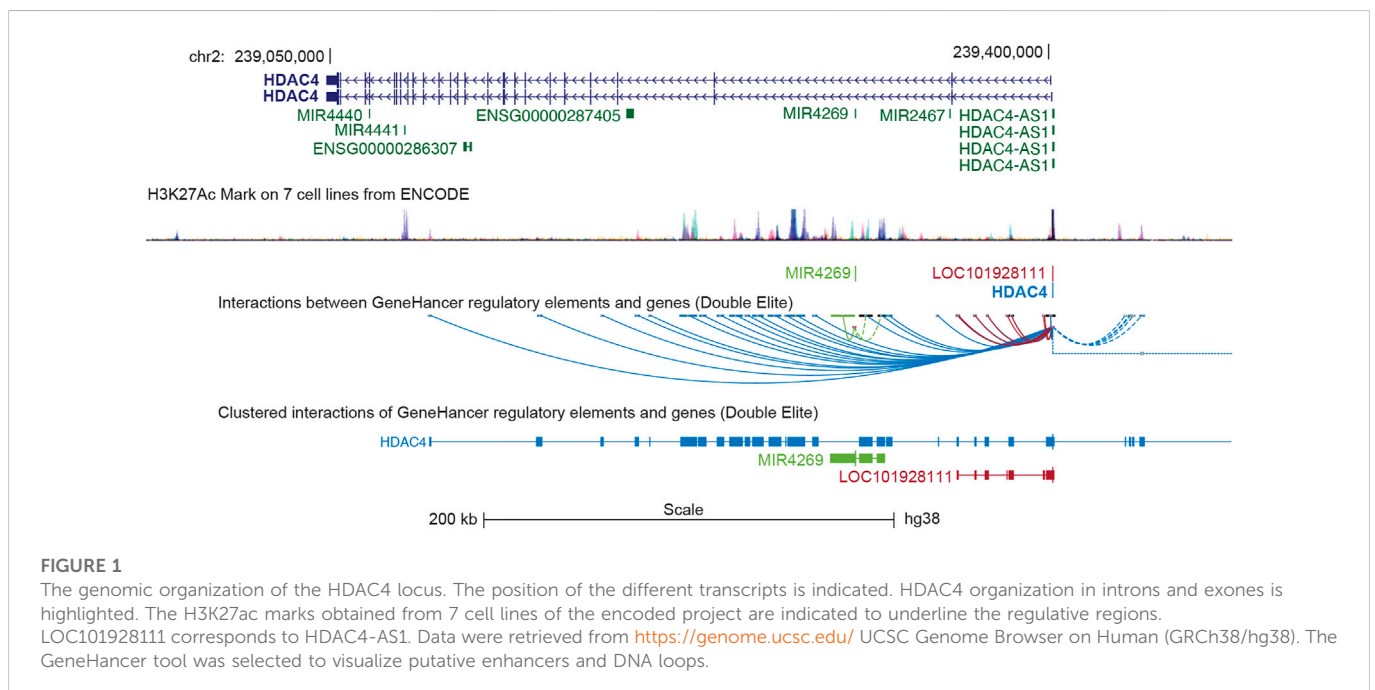
The aim of this manuscript is to provide an updated overview of the recent progresses regarding the contribution of the epigenetic regulator HDAC4 to cancer development. However, to provide readers with a critical view of the sometime controversial evidence on HDAC4 and cancer, it is essential to discuss the complex networks of regulations, interactions, and signals that influence HDAC4 activities. Therefore, the first part of the review (Chapters 2-4) is devoted to learning about the basic mechanisms of HDAC4 regulation and its ability to interact with different partners. The second part (Chapter 5) is dedicated to HDAC4 in cancer, discussing the current state of research in hematological and solid tumors and considering the principal hallmarks of cancer (Hanahan et al., 2022).

1.1 The class IIa HDACs

Histone deacetylase 4 (HDAC4) belongs to the class IIa family of deacetylases, which includes HDAC4, HDAC5, HDAC7, and HDAC9. These epigenetic regulators contribute to the regulation of the lysine acetylation/deacetylation cycle by antagonizing the action of histone acetyl transferases (HAT/KAT). In vertebrates, class IIa HDACs have negligible enzymatic activity toward acetyl-lysine. Although they possess a deacetylase domain and bind the zinc ion required for catalysis, a substitution of the critical tyrosine residue by a histidine in the catalytic pocket was selected during evolution (Lahm et al., 2007). However, by assembling into multiprotein complexes, class IIa HDACs can act as a platform and coordinate the activity of class I HDACs (Brancolini et al., 2022). The reason for this evolutionary selection is unclear. Some hypotheses have been formulated, such as the role of the deacetylase domain as a reader of

TABLE 1 The HDAC4 locus. Genes transcribed from the HDAC4 locus are indicated. Data were obtained from <https://www.ncbi.nlm.nih.gov/gene/9759> (Ensembl release 107).

Accession	Start	Stop	Length (nt)	Gene symbol	Strand	Type	Name
NC_000002.12	239048168	239401654	353486	ENSG00000068024	Minus	mRNA	HDAC4
NC_000002.12	239068817	239068914	97	ENSG00000266109	Minus	miRNA	MIR4440
NC_000002.13	239085827	239085926	99	ENSG00000264810	Minus	miRNA	MIR4441
NC_000002.14	239114858	239118842	3984	ENSG00000286307	Plus	LncRNA	
NC_000002.15	239194118	239197654	3536	ENSG00000287405	Plus	LncRNA	
NC_000002.16	239305462	239305545	83	ENSG00000265215	Plus	miRNA	MIR4269
NC_000002.17	239351724	239351804	80	ENSG00000264292	Minus	miRNA	MIR2467
NC_000002.18	239401436	239402657	1221	ENSG00000222020	Plus	LncRNA	HDAC4-AS1
NC_000002.19	239439595	239531363	91768	,ENSG00000286525	Plus	LncRNA	



acetylated histones to localize class I HDACs in competent chromatic environments. Other hypotheses include the possibility of activity against yet unknown post-translational modifications (PTMs) of lysine, which in principle, should be bulkier because of the larger catalytic pocket. Certainly, further studies are needed to clarify this still enigmatic trait of class IIa HDACs. Studies that are also critical for the development of specific inhibitors of class IIa HDACs.

Like other epigenetic regulators, the activity of class IIa HDACs is subject to tight control of various extracellular signals that allow cells to adapt their activities to the needs of the organism. Consequently, the activity of class IIa HDACs is regulated at multiple levels, including transcription, translation, and various PTMs, with phosphorylation playing an important role. Phosphorylation of class IIa controls mainly protein stability and the nuclear-cytoplasmic shuttling. Frequently, these events are responsible for the removal of the repressive influence of HDACs on gene transcription (Wang, and

Yang, 2001; Cernotta et al., 2011; Wang Z et al., 2014). Because of these multiple levels of regulation, it is not easy to identify the specific genetic alterations that may be responsible for affecting HDAC4 activities during tumorigenesis. Before discussing possible contributions of HDAC4 to cancer development and aggressiveness, we will review some basic concepts about HDAC4.

2 The HDAC4 gene

In humans, the *HDAC4* gene is located on the minus strand of chromosome 2q37.3 and is approximately 350 kbp in length (Table 1). The gene is organized into 27 exons and 26 introns. Several, mostly unverified, transcript variants of HDAC4 (>40) with different lengths have been mapped. The isoforms of HDAC4 vary in length and include between 1016 and 1113 amino acid residues. Isoform 1,

encoded by transcript variant 1 (NM_001378414.1 and NP_001365343.1), is 8,461 nucleotides (nt) long with a coding DNA sequence (CDS) of 3,270 nt and a corresponding protein length of 1,089 amino acids (aa). Various lncRNAs and miRNAs are embedded within the HDAC4 locus (Figure 1; Table 1). The HDAC4 locus is also characterized by the presence of regulatory elements, particularly enhancers, which can exert their influence both locally and through chromosomal loops at distant sites (Figure 1).

In several tissues, HDAC4 is the lowest expressed class IIa HDAC after HDAC9. Exceptions are bladder, colon, esophagus, and uterus. The ENCODE project has revealed that several transcription factors (TFs), epigenetic modifiers and architectural proteins bind to the proximal promoter of HDAC4, suggesting that HDAC4 transcription is indeed heavily regulated (Rosenbloom et al., 2009; Di Giorgio and Brancolini, 2016). Among these TFs some proto-oncogenes can be found, such as JUN, FOS, and MYC, which control the G0/G1 transition. For further information, the reader is kindly invited to consult (Di Giorgio and Brancolini 2016).

Within a large deletion on chromosome 2q37, haploinsufficiency of the HDAC4 gene has been reported to cause the 2q37 deletion syndrome, a disorder with significant intellectual impairment, brachydactyly type E (BDE), and typical facial features (Williams et al., 2010). Further studies have shown that haploinsufficiency of HDAC4 for BDE is not completely penetrant (Villavicencio-Lorini et al., 2013) and is not sufficient to cause intellectual disability (Wheeler et al., 2014). More recently, heterozygous *de novo* missense variants affecting amino acid residues involved in phosphorylation-dependent binding of 14-3-3 proteins (see below) and control of the nucleocytoplasmic shuttle, have been identified in individuals with delayed developmental milestones, intellectual disability and hypotonia, a phenotype distinct from 2q37 deletion syndrome (Wakeling et al., 2021). Although HDAC5 is the predominant family member in the central nervous system (Brancolini et al., 2021), the role of HDAC4 in controlling synaptic gene expression and the alterations in neurotransmission, learning, and memory observed in some but not all mouse models with dysregulated HDAC4 support a possible role in central nervous system (CNS) functions (Wu et al., 2016).

Genetic studies in mice have demonstrated the contribution of *Hdac4* to various differentiation and adaptation responses. *Hdac4* plays an irreplaceable role in controlling chondrocyte hypertrophy and limiting premature ossification of endochondral bone (Vega et al., 2004). A phenotype that depends in part on the upregulation of MMP13 (Nakatani et al., 2016). Tissue-specific *Hdac4* knockouts (KOs) have demonstrated a key role of the deacetylase in controlling satellite cell proliferation and mediating the skeletal muscle response to denervation (Choi et al., 2014; Marroncelli et al., 2018; Pigna et al., 2018). In the liver, deletion of *Hdac4* alters the regulation of glycogen storage (Mihaylova et al., 2011). Since *Hdac4* is a member of the class IIa HDAC family, its role in other biological responses may be underestimated due to redundancy, especially with the phylogenetically closest member, HDAC5. In addition, genetic compensatory mechanisms monitored by Jun and Mef2 may increase the expression of one family member when deficiencies occur in other members (Velasco-Aviles et al., 2022). Recruitment of HDAC4 at a specific genomic locus can trigger a fast gene silencing (Lensch et al., 2022). However, certain loci seem to be refractory to HDAC4 repressive activity even though artificially deposited through the Cas9 delivery system (Di Giorgio et al.,

2021). A result that points to the local chromatin environment as a licensing factor for HDAC4 activity.

3 The HDAC4 protein

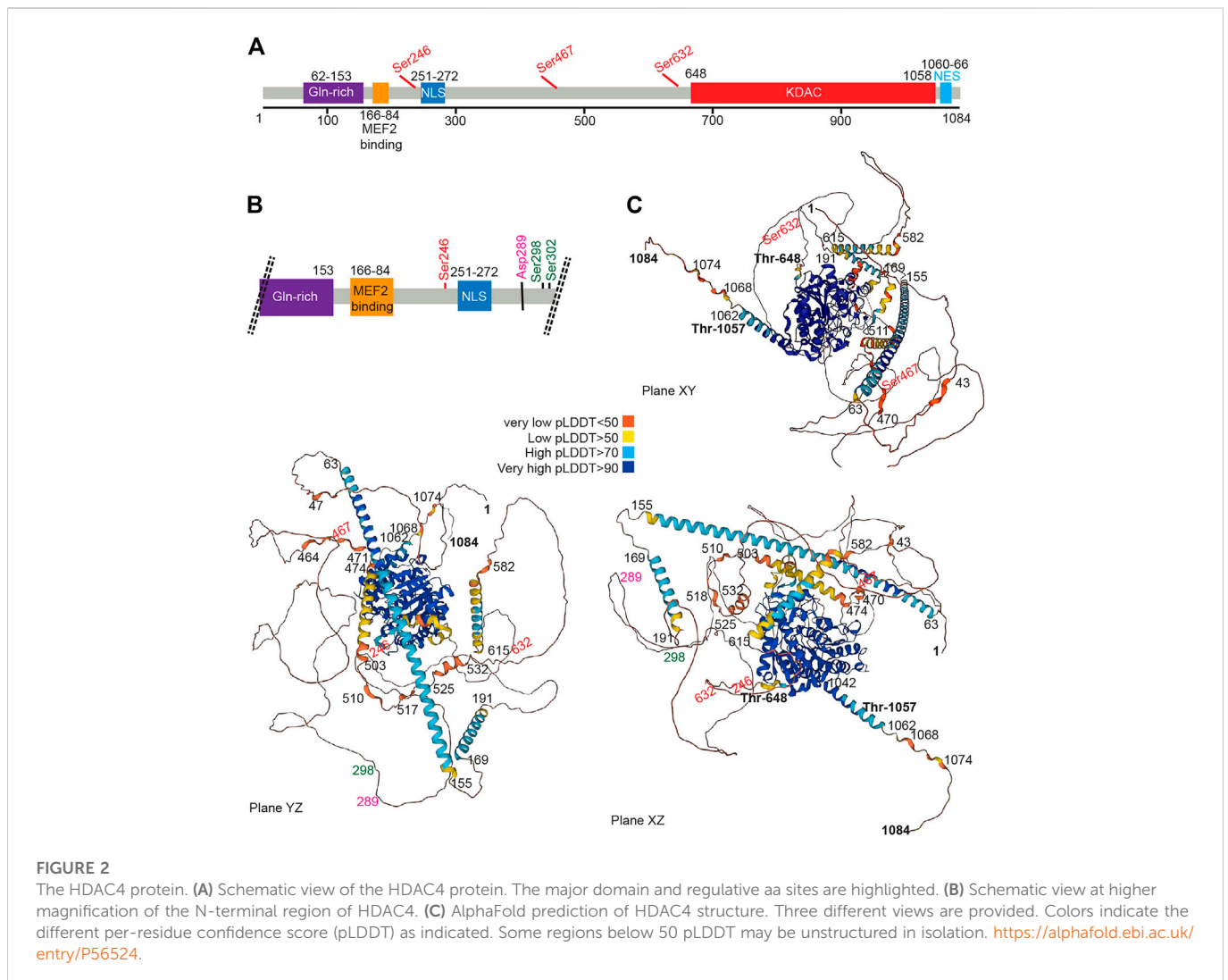
The HDAC4 protein (Figure 2) consists of a long N-terminal region responsible for protein interactions and a highly conserved C-terminal lysine deacetylase (KDAC) domain. The N-terminal region contains a lysine/arginine-rich nuclear localization sequence (NLS) spanning residues 244–279 and binding sites for transcription factors or other co-repressors (Di Giorgio et al., 2015), including members of the MEF2 family (myocyte enhancer factor-2), which was mapped to residues 166–184, (Miska et al., 1999; Wang et al., 2000). According to structural studies, this sequence can be folded into an α -helix that fits into a hydrophobic groove on the surface of a MEF2 dimer (Minisini et al., 2022). Like several other epigenetic regulators, HDAC4 cannot bind to DNA in a sequence-dependent manner (Wang et al., 2013; Torchy, et al., 2015; Hosokawa and Rothenberg, 2021). Interaction with TFs provides a strategy to recruit deacetylase to a specific region of the genome and alter chromatin. Alternative strategies can also be pursued. HDAC4, by joining multiprotein complexes containing epigenetic readers, can localize close to nucleosomes with specific histone modifications. Finally, HDAC4 and class IIa HDACs may themselves function as readers because of their low deacetylase activity, although they have not been studied in detail.

The N terminus of HDAC4 also associates with chaperone proteins, including the 14-3-3 protein, and this association allows nuclear export, thereby abolishing the repression of HDAC target genes (Grozinger and Schreiber, 2000; Wang et al., 2000; Wang B et al., 2014).

Early work has shown that overexpression of HDAC4 leads to aggregation in the nucleus and cytoplasm, suggesting the existence of a self-interaction domain (Miska et al., 1999). This hypothesis is confirmed by the presence of two domains with a high probability of forming coiled-coil structures, encompassing amino acids 67–150 and 173–184, as shown by the AlfaFold software (Jumper et al., 2021; Varadi et al., 2022). The N-terminal domain contains a glutamine-rich domain that can fold into a straight alpha helix that assembles into a tetramer (Guo et al., 2007). Accordingly, N-terminal deletion mutants show an inability to self-bind, supporting the notion that the N-terminal domain of HDAC4 contains an oligomerization domain (Kirsh et al., 2002).

The carboxy-terminal region contains the deacetylase domain with a hydrophobic pocket in which the zinc ion is coordinated. The zinc-containing domain of HDAC4 consists of residues from T648 to T1057 (Bottomley et al., 2008). This domain interacts with the NCOR1/NCOR2/HDAC3 complex, which can provide the deacetylase activity (Guenther et al., 2001; Di Giorgio et al., 2015; Hudson et al., 2015). A repeated peptide motif presents in both NCOR1 and NCOR2 is sufficient to mediate interaction with HDAC4. This peptide sequence binds near the active site of HDAC4 and requires the “closed” conformation of the zinc-binding loop on the surface of the enzyme (Hudson et al., 2015).

In addition, a hydrophobic nuclear export sequence (NES) is located between residues 1051 and 1084 at the C-terminal end of HDAC4 (Wang et al., 2000; McKinsey et al., 2001; Mathias et al., 2015). This sequence is required for CRM1-dependent nuclear export of HDAC4 and its accumulation in the cytoplasm.



3.1 Control of nucleocytoplasmic shuttling: (de)phosphorylation

HDAC4 is subject to microenvironment-dependent regulation through the action of several PTMs, which include phosphorylation, SUMOylation, and proteolytic cleavages (Mathias et al., 2015). Most of the documented PTMs control nuclear-cytoplasmic transport, the major process regulating the corepressor functions of HDAC4 (Table 2). Control of nuclear-cytoplasmic shuttling is a common strategy to influence the activities of class IIa HDACs and offers advantages in terms of response time and adaptability (Chen et al., 2020).

Phosphorylation at S246, S467, and S632 creates docking sites for 14-3-3 chaperone proteins that promote the translocation of HDAC4 from the nucleus to the cytoplasm. Because HDAC4 localization is associated with transcriptional regulation, the loss of these phosphorylation sites enhances the transcriptional activity of MEF2 (Di Giorgio et al., 2015; Mathias et al., 2015; Chen et al., 2020). Cytoplasmic sequestration appears to be caused by NLS masking because of 14-3-3 protein binding. Binding that prevents association with the importin α/β heterodimer is an obligatory step for HDAC4 nuclear import (Grozinger and Schreiber, 2000).

Many isoforms of the calcium/calmodulin-dependent kinase (CaMK) family phosphorylate HDAC4 and inhibit its accumulation in the nucleus. CaMKI preferentially phosphorylates S246. CaMKII phosphorylates residues S467 and S632 on HDAC4 by binding to a unique docking site (centred on Arg 601) that is not present in other class IIa HDACs (Backs et al., 2006; Parra and Verdin, 2010). The same serines are also phosphorylated by CaMKIV, which can also promote nuclear export by a mechanism independent of 14-3-3 protein binding (Zhao et al., 2001; Yuan et al., 2016). In cardiac cells, CaMKII δ B was described to preferentially target residue S210 of HDAC4 rather than the other HDACs of the class IIa. It was hypothesized that this phosphorylation might cause a conformational change for the recruitment of additional factors that mediate MEF2 silencing (Little et al., 2007).

Protein kinase A (PKA) phosphorylates S265 and S266 in cardiac and skeletal muscle cells, resulting in decreased HDAC4 efflux from the nucleus (Liu and Schneider, 2013). PKA can also phosphorylate serine 584. In this case, the effects are less clear, although an increase in the repressive activity of HDAC4 toward MEF2 is plausible (Doddi et al., 2019). Interestingly, parathyroid hormone (PTH) induces PKA-dependent phosphorylation of S740, leading to the export of HDAC4 to the cytoplasm and its degradation *via* a lysosomal-

TABLE 2 Main PTMs regulating HDAC4 activities.

PTM	Enzyme		Site	Effect	Reference: DOI
Phosphorylation	CamK family	CamKI	S246, S467	Nuclear export	Backs et al. (2006)
		CamKII	S467, S632	Nuclear export	Backs et al. (2006)
		CamKIV	S467, S632	Nuclear export	Zhao et al. (2001)
		CamK δ B	S210	MEF2 silencing	Little et al. (2007)
	PKD1		S246, S467	Nuclear export	Sinnott-Smith et al. (2014)
	EMK		S246	Cytoplasmic retention	Dequiedt-Smith et al. (2006)
	CTAK1		S246	Cytoplasmic retention	Dequiedt-Smith et al. (2006)
	AMPK		N/A	Nuclear export	Jiang et al. (2020)
	SIKs	SIK1, SIK2, SIK3	S246, S467, S632	Nuclear export	Berdeaux et al. (2007) , Walkinshaw et al. (2013)
	FAK		N/A	N/A	Sato et al. (2020)
	GSK3 β		S298, S302	UPS-mediated degradation	Cernotta et al. (2011)
	PKA		S265, S266, S584	Nuclear retention	Liu and Schneider (2013) , Doddi et al. (2019)
	Aurora B kinase		S265	N/A	Guise et al. (2012)
	PP2A		S246, S467, S632, S298	Nuclear import	Paroni et al. (2008)
SUMOylation	RanBP2		K559	Nuclear retention	Kirsh et al. (2002)
Proteolytic cleavage	Caspase-2 Caspase-3		D298	Apoptosis	Liu et al. (2004) , Paroni et al. (2004)
	PKA-dependent		Between Y201 and W202	Inhibition of MEF2	Backs et al. (2011)
Ubiquitylation	N/A		N/A	UPS-mediated degradation	Cernotta et al. (2011) , Potthoff et al. (2007)

dependent system ([Shimizu et al., 2014](#)). In the heart, coordinated actions of PKA and CaMKII regulate the entry and exit of HDAC4 into the nucleus ([Helmstadter et al., 2021](#)).

Importantly, PKA in macrophages could also indirectly affect the repressive activity of HDAC4 by inhibiting salt-inducible kinases (SIKs) ([Luan et al., 2014](#)).

Other kinases are involved in the phosphorylation of HDAC4, pointing to HDAC4 as an hub for different signalling pathways. An exhaustive list is provided in [Table 2](#). Protein kinase D1 (PKD1) phosphorylates residues S246 and S467, promoting nuclear export and cytoplasmic retention of HDAC4 ([Sinnott-Smith et al., 2014](#); [Pablo Tortola et al., 2021](#)).

MARK/Par-1 kinases such as EMK and C-TAK1 have been described to control HDAC4 localization by phosphorylating S246. This facilitates phosphorylation of the remaining residues required for 14-3-3 binding (S467 and S632) by other kinases, resulting in cytoplasmic retention ([Dequiedt et al., 2006](#)).

AMP-activated protein kinase (AMPK), the major sensor of energy metabolism, can phosphorylate HDAC4 and HDAC5, to promote their nuclear exclusion and the epigenomic resetting. ([Salminen et al., 2016](#); [Niu et al., 2017](#)).

In addition, salt-inducible kinase (SIK) subfamily activity is also involved in HDAC4 re-localization ([Walkinshaw et al., 2013](#)). During food intake, HDAC4 is phosphorylated and sequestered in the cytoplasm by SIK3, whose activity is upregulated in response to insulin, whereas the kinase is inactivated during fasting, leading to dephosphorylation and nuclear translocation of HDAC4. SIK2 mediates the phosphorylation and inactivation of HDAC4 in

mouse hepatocytes in response to insulin. Conversely, glucagon exposure increases HDAC4 activity through PKA-mediated inhibition of SIK2 ([Wang et al., 2011](#)). Both SIK2 and SIK3 phosphorylate HDACs at the conserved motifs for 14-3-3 binding and stimulate their nuclear export, thereby supporting MEF2-dependent transcription. However, unlike SIK2, SIK3 induces nuclear export independently of kinase activity and 14-3-3 binding ([Walkinshaw et al., 2013](#)). In muscle cells, SIK1 phosphorylates class IIa HDACs and promotes their export from the nucleus to the cytoplasm ([Berdeaux et al., 2007](#)).

Focal adhesion kinase (FAK)-mediated tyrosine phosphorylation controls the subcellular localization of HDAC4/5. Residue Y642 has been identified as the FAK-dependent tyrosine phosphorylation site for HDAC5, the closest member of the deacetylase family to HDAC4, but the precise role of Y642 phosphorylation of HDAC5 in controlling its subcellular localization remains to be determined ([Sato et al., 2020](#)).

Glycogen synthase kinase 3 β (GSK3 β) phosphorylates S298 and S302 and plays an important role in controlling the stability of HDAC4 ([Cernotta et al., 2011](#); [Wang Z et al., 2014](#); [Xiao Q et al., 2021](#)). S302 may serve as a priming phosphorylation site that promotes the subsequent phosphorylation of HDAC4 at S298. This phosphorylation provides a signal for poly-ubiquitylation; therefore, phosphorylation of HDAC4 by GSK3 β promotes UPS-mediated degradation of HDAC4 during growth arrest and senescence ([Cernotta et al., 2011](#); [Di Giorgio et al., 2021](#)).

Control of HDAC4 and other class IIa HDACs during mitosis can also be exploited by phosphorylation. In this case, it is the Aurora B kinase that phosphorylates serine 265 within the NLS. This results in

decreased association with HDAC3 and impaired repression of transcription. However, it is unclear whether other functions, unrelated to transcriptional regulation, may be affected (Guise et al., 2012). Interestingly, the role of HDAC4 in chromosome segregation has been described in TP53-defective cells (Cadot et al., 2009), which may be related to Aurora B-dependent phosphorylation.

Phosphorylation of HDAC4 is reversible, and the removal of phosphate groups is mediated by the protein phosphatase 2A (PP2A) family, which promotes the accumulation of HDAC4 in the nucleus. Specifically, the N-terminus of HDAC4 interacts with the catalytic subunit of PP2A, which dephosphorylates several serines, including 14-3-3 binding sites and S298, enabling nuclear import of HDAC4 (Paroni et al., 2008; Veloso et al., 2019; Tan et al., 2022).

Finally, a combinatorial mass spectrometry approach revealed that HDAC5 has at least 17 *in vivo* phosphorylation sites within functional domains, including NLS, NES, and KDAC domains. These novel phosphorylation sites suggest the existence of additional unexplored phosphorylation-dependent mechanisms that dynamically regulate class IIa HDACs (Greco et al., 2010). Considering that HDAC5 is the closest member of the deacetylase family to HDAC4, mass spectrometry may provide comparable results for HDAC4, suggesting novel multiple regulatory mechanisms of the deacetylase.

3.2 HDAC4 protein stability

HDAC4 protein stability is regulated by the ubiquitin-proteasome system. HDAC4 polyubiquitylation is a widely used mechanism for the radical silencing of all HDAC4 activities (both cytoplasmic and nuclear) (Renzini et al., 2022). HDAC4 polyubiquitylation has been observed under various conditions: In response to growth factor deprivation, during the onset of senescence, during hypoxia, in response to alcohol consumption in the brain or viral infection (Potthoff et al., 2007; Cernotta et al., 2011; Du et al., 2015; Griffin et al., 2017; Lu et al., 2019; Di Giorgio et al., 2021).

In osteoblasts HDAC4 serves as a brake for differentiation into osteoclasts. HDAC4 degradation is triggered by parathyroid hormone (PTH) to allow MEF2C-dependent transcription and RANKL expression. In this case, it has been proposed that the E3-ligase SMURF2 plays a role (Obri et al., 2014).

Other studies have suggested that HDAC4 levels may be under the control of lysosomal proteases. PTH leads to the export of HDAC4 to the cytoplasm through PKA-dependent phosphorylation of S740 and its degradation *via* a lysosomal-dependent system (Shimizu et al., 2014). Under excessive oxidative stress, a lysosomal serine protease released from disrupted lysosomes can generate an N-terminal fragment of HDAC4. This fragment triggers chaperone-mediated autophagy degradation of MEF2A and neuronal cell death (Zhang et al., 2014).

The involvement of lysosomes in the regulation of HDAC4 challenges the autophagic response. HDAC4 has been reported to both suppress and stimulate autophagy (Kang et al., 2014; Yue et al., 2015; Pigna et al., 2018; Yang et al., 2018). The involvement of HDAC4 in regulating autophagy may also be part of feedforward circuits that maintain malignancy (Zang et al., 2022). The relationships between HDAC4 and autophagy are complex and require further investigation. It needs to be clarified whether the influence of HDAC4 on autophagy is direct or the result of cellular stresses induced by experimental manipulation of HDAC4 levels.

Artificially manipulating HDAC4 levels may represent an alternative strategy to the use of inhibitors from a clinical perspective. Recently, the first bifunctional protein degraders of HDAC4 have been developed using proteolysis targeting chimeras (PROTACs) technology (Macabuag et al., 2022). AUTOPhagy-TARgeting Chimeras (AUTOTACs) to degrade HDAC4 *via* the macroautophagy pathway may also represent a promising strategy (Ding et al., 2022).

3.3 Additional modifications

Although phosphorylation is the main PTM of HDAC4 studied, other additional modifications may also influence HDAC4 activity and localization.

Reactive oxygen species (ROS) may control the subcellular localization and activity of HDAC4, although the mechanisms involved are not completely clear (Wang B et al., 2014; Di Giorgio and Brancolini, 2016; Schader et al., 2020).

Selective proteolysis can modulate the activity of HDAC4. Caspase 2 and caspase 3 cleave HDAC4 at D289 and generate an NLS-containing fragment with selective repressive activities (Paroni et al., 2007; Cao K et al., 2014; Zhou J et al., 2015). Phosphorylation by PKA may also render HDAC4 competent for cleavage by the serine protease under the influence of ABHD5. This N-terminal fragment represses MEF2-dependent transcription and protects mice from heart failure (Jebessa et al., 2019).

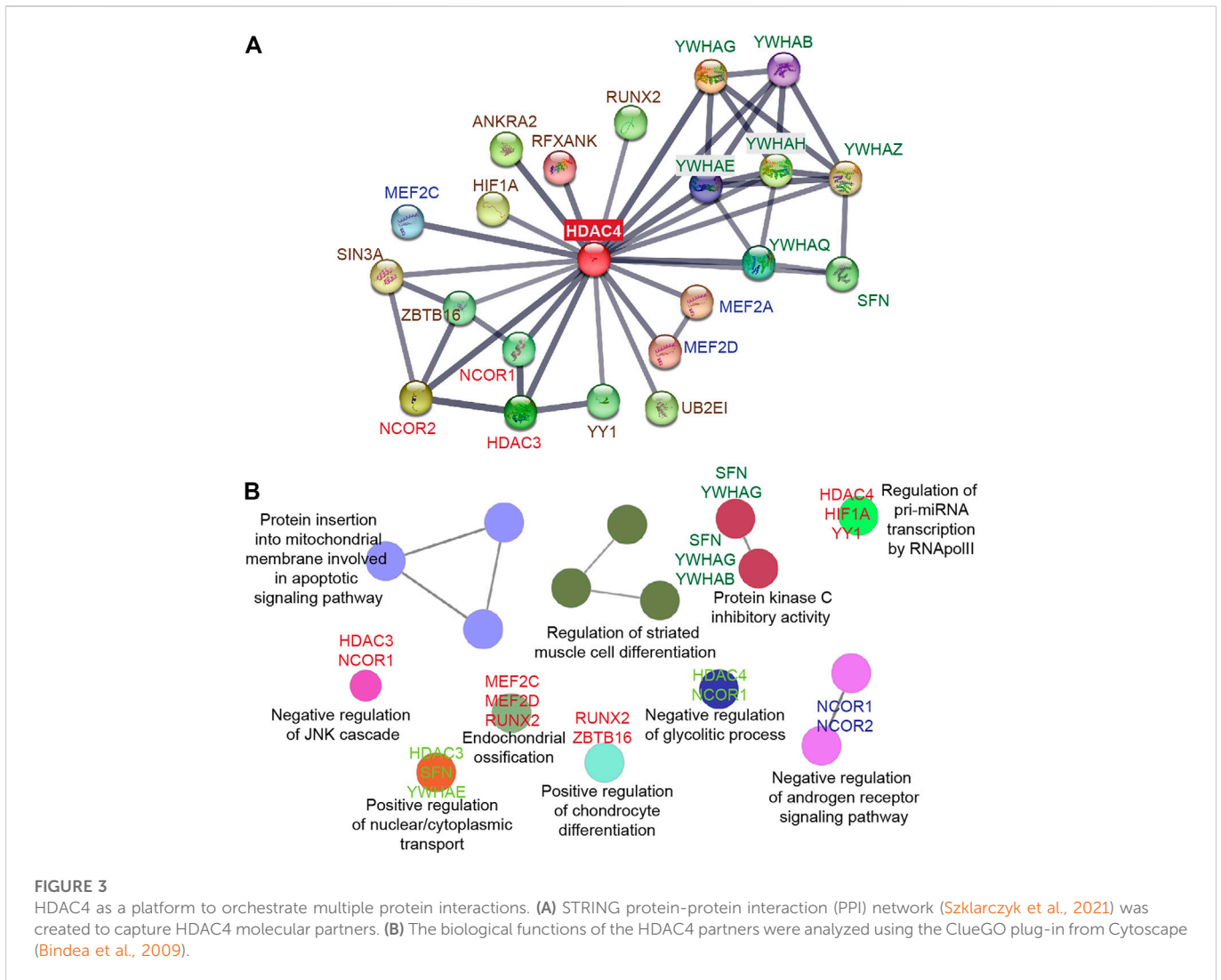
HDAC4 can be SUMOylated to lysine 559. A modification that likely occurs during translocation through the nuclear pore and enhances its repressive influence (Kirsh et al., 2002). It has been suggested that HDAC4 may regulate SUMOylation of other proteins such as IκBα, DACH1, and LXR proteins as part of a yet not clearly defined molecular complex (Lee et al., 2009; Ozcan et al., 2016; Yang et al., 2020), HDAC4 interacts with the E2 ligase Ubc9 and appears to be responsible for E3 ligase activity. An activity that may also play a role during senescence or in DNA repair (Dehennaut et al., 2013; Han et al., 2013).

4 HDAC4 as a platform to coordinate multiple tasks

The N-terminal domain of HDAC4 is a distinctive and crucial feature of class IIa deacetylases. It is responsible for homomeric interactions and is required for interactions with multiple partners, including transcription factors, co-repressors, and histone-modifying enzymes (Figure 3).

4.1 Interaction with corepressor complexes mediates histone deacetylation

In vertebrates, the HDAC4 protein is enzymatically inactive, so deacetylase activity on histone proteins depends on the formation of a protein complex containing HDAC3, a class I histone deacetylase bound to NCOR1 and NCOR2 to form the active heterotrimer. The deacetylase domain of HDAC4, particularly the edge of the hydrophobic channels leading to the catalytic site, is involved in this interaction (Kim et al., 2015). In NCOR1/NCOR2, the site of



interaction is repression domain 3 (RD3), which interacts specifically with class IIa and not class I HDACs. The RD3 domain is predicted to be intrinsically disordered and has been assigned to a conserved and repeated motif of eight amino acids, the GSI (GSI(S/T)XGXP) motif. GSI requires that the specific class IIa loop adopt the so-called “closed” configuration. Importantly, HDAC inhibitors disfavor binding to the corepressor and instead favor the open configuration of the class IIa specific loop (Hudson GM et al., 2015; Park et al., 2018).

These observations strongly suggest that HDAC4 and class IIa HDACs have evolved into pseudoenzymes that have a structural rather than a catalytic function. However, they also suggest that inhibitors of class IIa HDACs may promote acetylation, by acting as allosteric inhibitors and interfering with the binding of HDAC4 to the NCOR1/NCOR2/HDAC3 complex, even though these deacetylases no longer have catalytic activity (Hudson GM et al., 2015; Park et al., 2018).

HDAC4 activity is essential for HDAC3-mediated deacetylation of selected targets (Mihaylova et al., 2011; Lee et al., 2015), suggesting that its role as a scaffold protein is necessary for recruitment of additional functional partners.

Finally, early studies have indicated that the common splice version of HDAC9, MITR, can recruit HDAC1 via the N-terminal

region (Sparrow et al., 1999). Although molecular details are lacking, a similar interaction cannot be ruled out for other class IIa HDACs.

4.2 Interaction with transcription factors ensures DNA binding specificity

Like the other HDACs, HDAC4 does not have a DNA-binding domain, and its recruitment to specific genomic sites can be mediated by interaction with selected TFs. Of the TFs described to form complexes with HDAC4, little information are available about the bound genomic regions (Mihaylova et al., 2011; Wang Z et al., 2014; Chen and Sang 2016; Kim et al., 2016; Li et al., 2019). Thus, we do not have a complete idea of whether some TFs interacting with HDAC4, are simply substrates of the deacetylase complex, or act as Trojan horse to localize HDAC4 in specific genomic regions. In some cases, both conditions might even apply (Zhao et al., 2005).

The foremost investigated example regards the MEF2 TFs. MEF2 belongs to a family of TFs characterized by the presence of a MADS box (MCM1, Agamous, Deficiens, Serum Response Factor Box) involved in different developmental pathways. In vertebrates, the family includes four paralogous genes, MEF2A, MEF2B, MEF2C, and

MEF2D, which are involved in the control of a variety of biological functions depending on the cell type, including apoptosis, cell survival, proliferation, hypertrophy (Taylor and Hughes, 2017; Di Giorgio et al., 2018; Assali et al., 2019). HDAC4 has affinity for MEF2 *in vitro* and *in vivo* thanks to the binding of its N-terminal domain to a highly conserved region of MEF2 family members located at the junction of the MADS -box and the MEF2-specific domain (Lu et al., 2000). HDAC4 represses MEF2 transactivation through several mechanisms, including: 1) recruitment of corepressor complexes NCOR1/NCOR2/HDAC3 (Guenther et al., 2001), 2) competition for binding with transactivators such as p300 (Clocchiatti et al., 2013), 3) phosphorylation-dependent sumoylation (Grégoire et al., 2006).

The transcription factor HIF1 is the master regulator of oxygen homeostasis and consists of the subunits HIF1A and HIF1B. Under oxygen-rich conditions, HIF1A is constantly proteasomally degraded by recruiting a substrate recognition element, Von Hippel-Lindau protein (VHL), which promotes the action of an E3 ubiquitin ligase. Hypoxic conditions selectively suppress this degradation because the absence of prolyl and asparaginyl hydroxylation in HIF1A at critical residues inhibits recognition by the VHL protein (Semenza, 2012; Semenza, 2016). An interaction between HIF1A and HDAC4 has been reported. Functionally, HDAC4 regulates the posttranslational processing of HIF1A through the HSP70/HSP90 system, although, the closest family member HDAC5, seems to be more involved in this task (Chen and Sang, 2016).

4.3 Integrated models for HDAC4-dependent chromatin modulations: The platform and the positional effects

HDACs play an intermediate role in the final decision of the chromatin state at specific loci. By removing the acetyl group, they reset the epigenetic status and the pattern of protein complexes that are bound to histones and contemplates the activation of transcription. Furthermore, deacetylation can favor the intervention of other enzymes that stabilize a repressive outcome, through a wave of repressive methylations. Alternatively, Lysine acetyl transferases (KATs) can dynamically re-establish an open chromatin conformation and gene transcription.

Generally, histone deacetylation is followed by the onset of repressing modifications (e.g., H3K9me2, H3K27me3) which promote the recruitment of additional repressors to further condensate the nucleosome structure. The final goal is to impede the binding of TFs and to prevent transcription (Aloia, 2022; Franklin et al., 2022).

Overall, it seems evident that the general mechanism of HDAC4-induced transcriptional repression involves the assembly of multiprotein complexes, where HDAC4 acts as a recruitment platform and directs the activity of TFs and/or of epigenetic regulators (Hohl et al., 2013; Di Giorgio et al., 2017).

An interesting model that supports this hypothesis was proposed by Finke et al. (2022) thanks to the genetic deletion of HDAC4 in adult mouse ventricular myocytes. The loss of HDAC4 led to a whole epigenome activation, with an increase of activating histone modifications (H3K4me3, H3K9ac and H3K27ac) and a decrease of repressing modifications (H3K9me2 and H3K27me3). Also, the CHIP-seq analysis revealed an overrepresentation of MEF2 binding sites in the upregulated regions characterized by the H3K9ac and

H3K4me modifications. These findings prompt a model where HDAC4 acts as a scaffold protein that interacts with H3K9ac histones to recruit and direct enzymatically active partners—histone methyltransferase and class I HDAC to the genomic location specified by transcription factors, such as MEF2 (Finke et al., 2022).

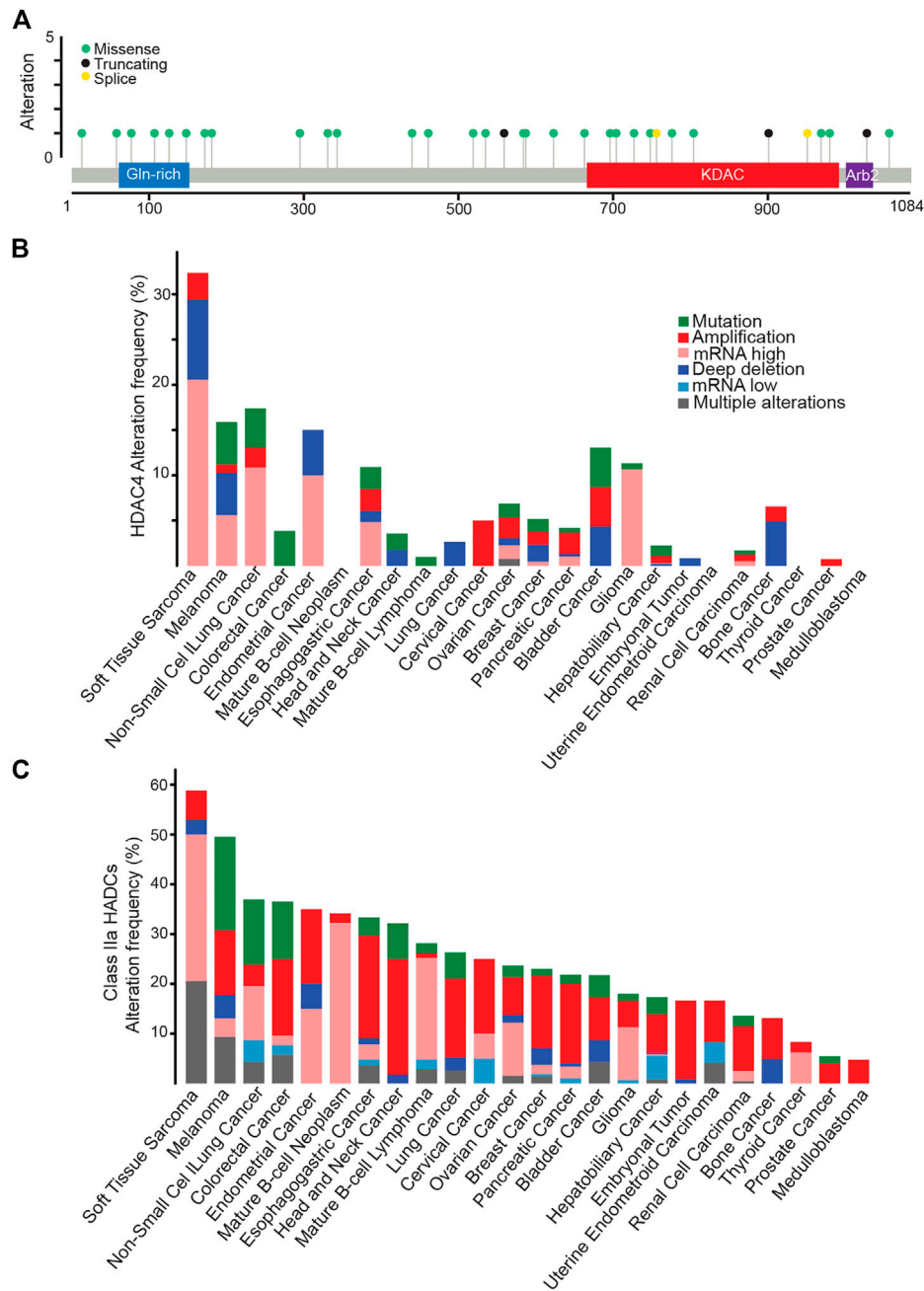
Confirmation in the human setting and integration with additional classes of epigenomic regulators are found in a comprehensive genome-wide study carried out in cellular models of leiomyosarcoma (Di Giorgio et al., 2020). The study emphasized the importance of distal region binding in the mechanism of class IIa deacetylases-mediated epigenomic regulation. First, it was demonstrated that HDAC4 controls a peculiar genetic program and possesses both shared and specific genomic binding sites compared with HDAC9, resulting respectively in the modulation of MEF2D-regulated genes and non-MEF2D-regulated genes. It was observed that the two deacetylases mainly bind intergenic regions distal from the transcription start site (TSS). Instead, MEF2D is more frequently found at promoters. Furthermore, it was noticed that, even though a region is characterized by the co-presence of MEF2D/HDAC4/HDAC9 complexes, in some regions only one member plays an active role in the epigenetic regulation. This “*dominant positional effect*” was observed in an intergenic region distal from the AHRGEF28 locus, which shows features of an enhancer and regulates the expression of ENCI thanks to chromatin looping. In fact, although both HDAC4 and HDAC9 bind this region, only HDAC9 knocked-out cells show an increase in H3K27ac levels at the enhancer and promoter sites, resulting in ENCI upregulation.

Although H3K27ac is an important epigenetic mark under the regulation of HDAC4 and class IIa HDACs, additional marks correlate with HDAC4 activity such as lysine 9 in histone 3 (H3K9ac) (Ko et al., 2013; Zhao X et al., 2022).

Other studies have not found an involvement of HDAC4 in the regulation of the global levels of H4K36ac (Liu et al., 2015; Zhu et al., 2015). However, local activities cannot be excluded as well as the compensatory action of other family members. Finally, a still poorly investigated molecular complex formed by HDAC3-HDAC4 and emerin has been proposed to regulate H4K5 acetylation at a specific locus of a novel gene with anti-aging activity (NM_026333). This regulation was observed in the heart and contributes to the regulation of the autophagic response (Osanaï et al., 2018).

5 HDAC4 dysregulations in cancer

As frequently observed for other epigenetic regulators, the contribution of HDAC4 to cancer seems to be context-dependent and therefore not easy to categorize (Bodily et al., 2011; Sandhu et al., 2012; Yim et al., 2013). Consequently, the question of whether HDACs and HDAC4 can act as tumor suppressors or oncogenes is debated (Brancolini et al., 2022). For example, high level of HDAC4 mRNA is an unfavorable prognostic marker in ovarian cancer, but favorable in pancreatic cancer (<https://www.proteinatlas.org/ENSG00000068024-HDAC4/pathology>). The molecular mechanism used by HDAC4 to exert its role in cancer is not completely understood (Wang B et al., 2014; Brancolini et al., 2022). It likely includes the interaction with the corepressor complexes NCOR1/NCOR2/HDAC3, that impacts on the transcriptional landscape of cancer cells through epigenomic resetting. Although plausible, the epigenomic mechanism does not exclude

**FIGURE 4**

HDAC4 alterations in cancer. (A) Mutational profile of HDAC4. (B) Frequency of HDAC4 alteration in solid and liquid tumors. (C) Class IIa HDACs alteration frequency in solid and liquid tumors. Data were obtained from <http://www.cbioportal.org>, WGS changes cataloged in 2,583 whole-cancer genomes and their matched normal tissues across 38 tumor types. Source data from UCSC Xena and ICGC Data Portal.

additional tasks, which independently form chromatin and DNA accessibility, are dysregulated in cancer cells in a HDAC4-dependent manner.

5.1 The landscape of genetic alterations. Is this enough?

Genetic alterations of HDAC4 in cancer mainly contemplate an increase in its expression level. Hot spot mutations are not evident and

missense mutations are scattered throughout the protein. Curiously, the few splice variants and truncating mutations usually impact on the KDAC domain, leaving the amino-terminal part of the protein potentially expressed (Figure 4A). It is important to note that in the case of HDAC9 a splicing variant lacking the KDAC domain is highly expressed in different tissues (Brancolini et al., 2021). Therefore, it is plausible that truncated versions of HDAC4 might have a biological role in cancer.

The highest percentage of HDAC4 alterations (>30%) is found in soft tissue sarcoma (STS) (Figure 4B). A condition that holds true also

when dysregulations of all members of the family are investigated (Figure 4C). Overall, almost 60% of STS patients present an alteration in a class IIa HDACs, as confirmed by previous observations (Di Giorgio et al., 2013; Di Giorgio et al., 2017; Di Giorgio et al., 2020). Point mutations are rare within this heterogeneous group of tumors. Non-small cell lung cancer and melanoma are the second tumor types for frequency of genetic alterations in HDAC4 (15–17% of cases). Not surprisingly, these tumors are characterized by high mutational burden due to high exposition to carcinogens such as UV light or cigarette smoke (Klempner et al., 2020; Kalaora et al., 2022). Similarly, mutations in the mismatch repair pathway leading to microsatellite instability can explain the selective accumulation of HDAC4 point mutations in colorectal cancer patients (Klempner et al., 2020).

The dysregulation of HDAC4 in cancer could also involve the control of its subcellular localization as the result of alterations in cell signaling. Despite the importance of nuclear accumulation of HDAC4 in tumors, little data is available. Most cancers show moderate to strong cytoplasmic HDAC4 positivity (<https://www.proteinatlas.org/ENSG0000068024-HDAC4/pathology>) and some evidence support a correlation between the nuclear accumulation of HDAC4 and tumor aggressiveness (Di Giorgio et al., 2013). In several cultured cancer cell lines, HDAC4 shows a prominent cytoplasmic localization being actively exported from the nuclei. We also must consider that HDAC4 is subject to ubiquitin-proteasome system (UPS) mediated degradation and this event is maximized in the nuclear compartment (Cernotta et al., 2011). Whether a persistent nuclear localization of HDAC4 and the possible unrestrained repressive influence is incompatible with cell fitness is a fascinating hypothesis. Too less but also too much nuclear HDAC4 might be a deleterious condition.

5.2 Mechanisms of action: Is the control of proliferation the sole mechanism?

HDAC4 is frequently amplified or overexpressed in cancer specimens and cell lines. As discussed above, *HDAC4* transcription is controlled by several oncogenes, including Jun, Fos, and Myc, arising the question of whether HDAC4 might be an oncogene itself (Di Giorgio and Brancolini, 2016). HDAC4 oncogenic activities have been proved in murine and human fibroblasts by classical *in vitro* transformation and oncogenic cooperation assays (Di Giorgio et al., 2013; Peruzzo et al., 2016; Paluvai et al., 2018). In these assays a mutant in the 14-3-3 binding sites that is predominantly localized in the nuclei shows a much stronger oncogenic activity. Supporting the putative oncogenic activity of HDAC4, mouse models with deregulated class IIa HDACs-HDAC7 or HDAC9-have been reported to develop cancer (Rad et al., 2010; Gil et al., 2016). The HDAC4 oncogenic pathway converges on the repression of MEF2 TFs. An action shared with PI3K signaling, which represses MEF2 activity through an independent route (Di Giorgio et al., 2013). The ability of HDAC4 to repress the expression of tumor suppressor genes, such as CDKN1A, further supports a positive role of the deacetylase in cancer cells growth (Liu et al., 2009; Mottet et al., 2009; Clocchiatti et al., 2015). The fact that HDAC4 might be required to regulate cell proliferation is suggested by the result of high-throughput screenings using CRISPR/Cas9 technology (Table 3). In several CRISPR-based screenings performed in different cancer cell lines, HDAC4 emerges frequently as a significant hit required for cell fitness. It is

possible that specific oncogenic transformations make cancer cells addicted to HDAC4 (Zhou et al., 2021). The dependency of some cancer cells from HDAC4 activity could be explained by its recently demonstrated role in the control of replicative senescence and oncogene-induced senescence (OIS) (see below, Di Giorgio et al., 2021).

5.3 The stability link: MiRNAs and HDAC4 in cancer

MicroRNAs (miRNAs) are a class of small non-coding RNAs of approximately 22 nucleotides that regulate gene expression at the post-transcriptional level by repressing translation and/or promoting mRNA degradation (Bartel, 2004). MiRNAs have been shown to be important regulators of a variety of biological processes and diseases, including cancer (Bracken et al., 2016). The list of miRNAs that have been proposed as regulators of HDAC4 is long (Table 4) and a separate review is necessary. Here we will discuss only a few examples.

The TF, Nuclear Factor Erythroid-2 Related Factor-2 (NRF2) is the key regulator of the antioxidant response. NRF2 signaling in cancer cells attenuates the expression of miR-1 and miR-206 by promoting the expression of HDAC4, causing a shift in glucose metabolism to the pentose phosphate pathway (PPP) (Singh et al., 2013). HDAC4 in turn represses the expression of miR-1 and miR-206, suggesting that there is a feedback loop between miR-1/miR-206 and HDAC4 that regulates glucose metabolism. In addition, HDAC4 may also function as a redox sensor. In the reduced state, it can repress miR-1/miR-206 transcription and promote PPP gene expression, whereas in the oxidized state, it is sequestered in the cytoplasm, resulting in decreased PPP gene expression.

In hepatocarcinoma cell lines, miR-200a and miR-22 downregulate HDAC4 and their expression negatively correlates with cancer proliferation and migration, suggesting their role as tumor suppressors (Zhang et al., 2010; Yuan et al., 2011). Moreover, HDAC4 mediates transcriptional repression of miR-200a by binding Sp1, resulting in an autoregulatory cycle (Yuan et al., 2011). In colon cancer cells, miR-22 reduces HDAC4 levels, which impairs cancer progression (Hu et al., 2019). The link between miR-22 and HDAC4 has also been confirmed in breast cancer. Downregulation of miR-22 increases HDAC4 levels. In a cellular model of resistance to fulvestrant, a change in miR-22 levels, both an increase and a decrease, affects cell cycle progression, reflecting its impact on multiple targets with antagonistic activities (Wang et al., 2018).

Other miRNAs involved in the control of HDAC4 mRNA stability in cancer cells are miR-145-3p and miR-145-5p. MiR-145-3p is important in limiting multiple myeloma aggressiveness and its overexpression inhibits cell proliferation and autophagy. An activity that synergizes with the action of the proteasome inhibitor bortezomib by enhancing its efficacy (Wu et al., 2020) (see below). miR-145-5p targets HDAC4 and promotes tumor suppressor p53 activation and autophagy activation. Downregulation of miR-145-5p is observed in patients with colorectal cancer (CRC) and is associated with poor prognosis. *In vitro* models of CRC, the transcription factor ATF4 is upregulated and controls the miR-145-5p/HDAC4/p53 axis by inhibiting the expression of miR-145-5p, thereby promoting tumorigenesis, autophagy, and chemoresistance to 5-FU (Zhao et al., 2022a).

In gastric tumors, low expression of miR-206 is associated with better prognosis, suggesting a possible inhibitory role of this miRNA in

TABLE 3 HDAC4 is required for the proliferation and survival of different cancer cells. Data of CRISPR screens and relative information were retrieved from the BioGRID ORCS open repository <https://orcs.thebiogrid.org>.

Cell type	Cell line	Method	Enzyme	Rank	Phenotype	PMID	Screen name
Acute myeloid leukemia	OCI-AML3	Knock-out	Cas9	1165/18663	Cell proliferation essential gene	28162770	9-PMID28162770
Acute myeloid leukemia	MOLM-13	Knock-out	Cas9	605/17995	Resistance to venetoclax	31048320	1-PMID31048320
Breast cancer	HCC1419	Knock-out	Cas9	1402/17670	Cell proliferation essential gene	29083409	72-PMID29083409
Cervical cancer	HeLa	Knock-out	Cas9	426/19113	Reduced proliferation in response to Olaparib	33257658	1-PMID33257658
Chronic myeloid leukemia	K-562	Knock-out	Cas9	89/14276	Extracellular vesicle production	30556811	2-PMID30556811
Colon cancer	HT55	Knock-out	Cas9	1679/17670	Cell proliferation essential gene	29083409	88-PMID29083409
Colon cancer	SW-620	Knock-out	Cas9	382/20111	Response to NK killer activity	34253920	3-PMID34253920
Gastric cancer	NCI-N87	Knock-out	Cas9	1120/17670	Cell proliferation essential gene	29083409	217-PMID29083409
Glioblastoma	U-138MG	Upregulation	dCas9-VP16	2/308	Resistance to temozomolide	33271924	2-PMID33271924
HIV latency	J-Lat A2	Knock-out	Cas9	389/446	Silencing latent retrovirus	31165872	1-PMID31165872
Lung cancer	A549	Knock-out	Cas9	1708/17670	Cell proliferation essential gene	29083409	6-PMID29083409
Lung cancer	NCI-H2172	Knock-out	Cas9	1577/17670	Cell proliferation essential gene	29083409	215-PMID29083409
Lung cancer	NCI-H1993	Knock-out	Cas9	1088/17958	Cell proliferation fitness gene	30971826	200-PMID30971826
Lung cancer	NCI-H23	Knock-out	Cas9	36/19875	3D cell proliferation	32238925	5-PMID32238925
Lung cancer	NCI-H23	Knock-out	Cas9	253/19970	3D cell proliferation	32238925	3-PMID32238925
Lung cancer	A549	Knock-out	Cas9	4933/14543	Response to JQ1	31406246	3-PMID31406246
Lymphoma	SU-DHL-4	Knock-out	Cas9	18581/18862	Cell proliferation	28985567	2-PMID28985567
Melanoma	K029AX	Knock-out	Cas9	1359/17670	Cell proliferation essential gene	29083409	131-PMID29083409
Regulatory t cell	CD8 ⁺ T	Knock-out	Cas9	2475/19108	Cell proliferation	30449619	2-PMID30449619
Urinary bladder cancer	MGH-U4	Knock-out	Cas9	982/16517	Resistance to cisplatin	30414698	1-PMID30414698

cancer progression. This tumor suppressive effect is thought to be mediated by inhibition of a variety of target genes, including HDAC4 (Ren et al., 2014). A similar effect is mediated by miR-125a-5p in breast cancer (Hsieh et al., 2015).

Expression of miR-155 in mouse B cells causes proliferation of pre-B cells and high-grade lymphoma or leukemia by suppressing B cell lymphoma-6 (Bcl6) via various mechanisms, including HDAC4 downregulation. Indeed, HDAC4 is a co-compressor partner of Bcl6 and a target of miR-155, also a regulatory circuit. In this context, the functions of HDAC4 are tumor suppressive. Indeed, ectopic expression of HDAC4 in diffuse large B cell lymphoma cells of the B cell type leads to reduced miR-155-induced proliferation and induction of cell death (Sandhu et al., 2012).

Various non-coding RNAs can bind competitively to miRNAs, leading to the concept of competing endogenous RNAs (ceRNAs) (Salmena et al., 2011). For example, in the case of HDAC, circ-RNA lipoprotein receptor 6 (circ-LRP6) can abrogate miR-141-3p-mediated repression of HDAC4. circ-LRP6 is highly expressed in osteosarcoma tissues and cell lines (OS), and its expression correlates with cell proliferation and lower overall survival in OS metastatic patients. In contrast, high levels of its target miR-141-3p correlate with higher overall survival (Yu et al., 2022).

Nuclear factor erythroid-2 related factor-2 (NRF2) signaling in cancer cells attenuates miR-1 and miR-206 expression by promoting the expression of HDAC4, thus inducing a shift of glucose metabolism towards the pentose phosphate pathway (PPP) (Singh et al., 2013). HDAC4 in turns increases miR-1 and miR-206 expression, suggesting the existence of a feedback loop involving miR-1/miR-206 and HDAC4 that regulates glucose metabolism. In addition, HDAC4 may act also as a redox sensor. In the reduced state it can repress miR-1/miR-206 transcription and promote PPP gene expression, whereas in the oxidized state it is sequestered in the cytoplasm resulting in decreased expression of PPP genes.

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The relation between miR-22 and HDAC4 was confirmed also in breast cancer, downregulation of miR-22 increases HDAC4 levels. In this cellular model of resistance to fulvestrant altering miR-22 levels,

TABLE 4 List of miRNAs influencing HDAC4 levels.

miRNA	Model	Biological process regulated by the miRNA/ HDAC4 axis	Reference (doi)
miR-1	Murine myoblasts	Myogenesis	Chen et al. (2006)
	Myoblasts, murine regenerating skeletal muscle	Myogenesis	Sun et al. (2010)
	Growth plate cartilage	Chondrocyte hypertrophy	Li et al. (2014)
	Hepatocellular carcinoma cells	Cancer progression	Datta et al. (2008)
	Human chordoma tissue	Cancer progression	Duan et al. (2010)
miR-9	Neural stem-cells	Neurogenesis	Davila et al. (2014)
	Waldenstrom macroglobulinemia cells	Pathogenesis	Roccaro et al. (2010)
miR-22	Huntington's and Alzheimer's disease brains	Neurodegeneration	Jovicic et al. (2013)
	Murine cardiac tissue	Cardiac hypertrophy	Huang et al. (2013)
	Hepatocellular carcinoma	Cancer progression	Zhang et al. (2010)
	Colon cancer	Cancer progression	He et al. (2019)
	Breast cancer	Cancer progression	Wang et al. (2018)
	Human primary CD4+ T cells, intestinal mucosa tissues	Differentiation of Th17 cells	Pei et al. (2018)
miR-29a	Myogenic C2C12 cell line and primary muscle cells	Myogenesis	Winbanks et al. (2011)
	Murine osteoblasts	Osteoblast differentiation	Ko et al. (2013)
miR-29b	Rat primary osteoblasts, mouse osteoblasts	Osteoblast differentiation	Li et al. (2009)
miR-125a-5p	Human breast cancer	Cancer progression	Hsieh et al. (2015)
	Breast cancer	Cancer aggressiveness	Nishida et al. (2011)
miR-128-3p	Human rheumatoid arthritis fibroblast-like synoviocytes	Rheumatoid arthritis progression	Peng et al. (2021)
miR-140	Human osteosarcoma and colon cancer cells	Chemoresensitivity	Song et al. (2009)
miR-141-3p	Human osteoblasts	Osteoblast proliferation and migration	Yu et al. (2022)
miR-145-3p	Human multiple myeloma	Autophagy, cell death, sensitivity to bortezomib	Yu et al. (2022)
miR-145-5p	Human colorectal cancer	Regulation of p53, autophagy	Zhao et al. (2022)
miR-155	Murine B-cells	B-cells proliferation	Sandhu et al. (2012)
miR-200a	Hepatocellular carcinoma cells	Cancer proliferation and migration	Yuan et al. (2011)
miR-206	Myogenic C2C12 cell line and primary muscle cells	Myogenesis	Winbanks et al. (2011)
	Primary tumors	Tumorigenesis	Singh et al. (2013)
	Neuromuscular synapse in mice	Amyotrophic lateral sclerosis	Williams et al. (2009)
	Murine spinal muscular atrophic	Muscle hypertrophy	Valsecchi et al. (2015)
	Gastric tumor	Cancer progression	Ren et al. (2014)
miR-365	Chicken chondrocytes	Chondrocyte hypertrophy	Guan et al. (2011)
	Mouse osteoblasts	Osteogenesis, glucocorticoid-induced osteoporosis	Xu et al. (2017)
miR-378a-3p	Mouse myoblasts	Myoblast differentiation, apoptosis	Wei et al. (2016)
miR-381	Mouse chondrogenic cell line	Chondrogenesis	Chen et al. (2017)
miR-483-5p	Human fetal brain	Neurogenesis	Han et al. (2013)
miR-548ah	Human hepatocarcinoma, Human hepatoblastoma	Replication and expression of Hepatitis B Virus	Xing et al. (2019)

both increasing or decreasing its levels impact on cell cycle progression, thus reflecting its influence on multiple targets with antagonizing activities ([Wang et al., 2018](#)).

Additional miRNAs implicated in the control of HDAC4 mRNA stability in cancer cells are miR-145-3p and miR-145-5p. miR-145-3p is important for limiting multiple myeloma aggressiveness and its

overexpression inhibits cell proliferation and autophagy, cooperating with the action of the proteasome inhibitor bortezomib and intensifying its efficacy (Wu et al., 2020). miR-145-5p targets HDAC4, promoting the activation of the tumor suppressor p53 and activation of autophagy. Downregulation of miR-145-5p is observed in colorectal cancer (CRC) patients and is related to poor prognosis. *In vitro* models of CRC the transcription factor ATF4 is upregulated and controls the miR-145-5p/HDAC4/p53 axis by inhibiting the expression of miR-145-5p, thus enhancing tumorigenesis, autophagy, and chemoresistance to 5-FU (Zhao L et al., 2022).

In gastric tumor, low expression of miR-206 is associated with a better prognosis, suggesting a potential inhibitory role of this miRNA in cancer progression and this tumor-suppressor action is supposedly mediated by the inhibition of a plethora of target genes, including HDAC4 (Ren et al., 2014). A similar action is mediated by miR-125a-5p in breast cancer (Hsieh et al., 2015).

miR-155 expression in mice B cells causes pre-B cell proliferation and high-grade lymphoma or leukemia by repressing B cell lymphoma-6 (Bcl6) with different mechanisms, including HDAC4 repression. In fact, HDAC4 is a corepressor partner of Bcl6 and it is a target of miR-155, again a regulative circuit. In this context HDAC4 functions are tumor suppressive. In fact, ectopic expression of HDAC4 in B cell-type diffuse large B cell lymphoma cells results in reduced miR-155-induced proliferation and induction of cell death (Sandhu et al., 2012).

Different non-coding RNAs can competitively bind to miRNAs, leading to the concept of competitive endogenous RNAs (ceRNAs) (Salmena et al., 2011). In the case of HDAC, for instance, circ-RNA-lipoprotein receptor 6 (circ-LRP6) can quench the repression mediated by miR-141-3p on HDAC4. circ-LRP6 is highly expressed in osteosarcoma (OS) tissues and cell lines and its expression correlates with cell proliferation and a lower overall survival in OS metastatic patients. On the contrary high levels of its target miR-141-3p, correlate with higher overall survival (Yu et al., 2022).

5.4 HDAC4 in hematological malignancies

Among hematological malignancies alterations in HDAC4 has been investigated in Multiple Myeloma (MM). MM is a monoclonal tumor of plasma cells (PCs) that originates from post germinal-center (GC) B cells (Kuehl and Bergsagel, 2012). MM cells are well adapted to endoplasmic reticulum (ER) stress and responsive to drugs that trigger ER and proteotoxic stress. Further increasing the level of proteotoxic stress in MM cells can have a therapeutic benefit (Shah et al., 2015). In MM, overexpression of the chaperone protein BiP and HDAC4 is associated with chemoresistance (Kikuchi S et al., 2015). In this context, the disruption of HDAC4 activity, through both inhibitors and gene silencing, enhanced cytotoxicity induced by inducer of ER-stress (Kikuchi S et al., 2015). Inhibition of HDAC4 potentiates the expression of TFs such as ATF4 and CHOP that can sustain the expression of apoptotic mediators (Brancolini and Iuliano, 2020). Of note, TMP269, a class IIa HDACs selective inhibitor, enhanced the proteotoxic stress and cell death induced by the proteasome inhibitor carfilzomib (Kikuchi S et al., 2015).

Wu et al., confirmed the overexpression of HDAC4 in MM cells and observed, as discussed above, that MIR145-3p inhibits the expression of the deacetylase. Disease progression was associated with the downregulation of miR145-3p and HDAC4 upregulation.

Mechanistically, suppression of HDAC4 triggered the upregulation of the pro-apoptotic protein BCL2L1/BIM and caused the inactivation of MTORC1 (Wu H et al., 2020). The authors proposed that alterations in this pathway might enhance autophagy ultimately leading to autophagic cell death. The key role of HDAC4 in MM survival and proliferation was also observed in other studies. Downregulation of HDAC4 triggers the upregulation of miR-29b that controls the levels of the anti-apoptotic BCL2 family member MCL1 (Amodio et al., 2016). The epigenomic surveillance of HDAC4 in MM can be exerted also directly on anti-apoptotic genes. The HDAC4–RelB–p52 complex maintains a repressive status of chromatin around proapoptotic genes such as Bim and BMF (Vallabhapurapu et al., 2015), regulating consequently MM survival and growth (Singh R et al., 2019).

It is important to note that the role of autophagy in tumor cells is complex. At a certain intensity autophagy promotes cell survival in response to metabolic stress, protects against genome damage, limits cell death and inflammation. However, an autophagic boost can lead to cell consumption and autophagic cell death (White and DiPaola, 2009). Moreover, MTORC1 is a key protein complex that regulates not only autophagy, but also cellular growth and proliferation (Saxton and Sabatini, 2017).

In conclusion, it is possible that HDAC4 plays a central role in MM through the regulation of proteostasis and autophagy. Whether this is the central mechanism, or the central mechanism is the epigenetic influence on apoptotic genes cannot be clearly defined. Certainly, the contribution of HDAC4 in several neurodegenerative diseases such as Alzheimer, Dementia and Parkinson—which are well characterized as proteinopathies—supports the proteostasis hypothesis also in tumors (Mielcarek et al., 2015).

As discussed above, epigenetic regulators could play ambiguous roles during tumorigenesis in response to specific environmental cues or genetic alterations. This ambiguity is observed also for HDAC4 in hematological malignancies and particularly in the development of acute myeloid leukemia (AML). In this context, MEF2C acts as an oncogene and sustains cancer aggressiveness and chemoresistance (Di Giorgio et al., 2018). Phosphorylation of HDAC4 through SIK3 (and in part also SIK2) determines cytoplasmic accumulation and disrupts the ability to complex with MEF2C at enhancers sites to buffer H3K27 acetylation. These enhancers regulate cell proliferation, thereby nuclear HDAC4 could inhibit the development of the disease (Tarumoto Y et al., 2018). Given these results, new therapeutic strategies have been explored to increase the nuclear pool of repressive-competent HDAC4. Inhibition of SIK3 activity using the small molecule YKL-05-099 is sufficient to reduce the proliferation of leukemia cells and prolong the survival of a mouse model of AML (MLL-AF9 translocation) (Tarumoto et al., 2020). It is important to note that SIKs have multiple targets and their inhibition might have a pro-proliferative effect where they act as tumor suppressors (Hollstein et al., 2019). A second point that needs to be considered is the possibility of off-target effects of SIK inhibitors (Sakamoto et al., 2018; Darling and Cohen, 2021).

Another tumor suppressive activity of HDAC4, investigated in leukemia cells from AML patients but also observed in various cancer cell lines, provides a link with metabolism. The target is not linked to epigenetic regulation, but it is the cytosolic enzyme 6-phosphogluconate dehydrogenase (6PGD). 6PGD is the third enzyme in the oxidative pentose phosphate pathway (PPP), which catalyzes the decarboxylating reduction of 6-phosphogluconate (6-

PG) to ribulose 5-phosphate (Ru-5-P) and produces NADPH in the presence of NADP⁺ (Shan et al., 2014). Ru-5-P and NADPH can sustain RNA synthesis and lipogenesis and Ru-5-P can also inhibit LKB1–AMPK signaling, all activities that sustain cancer cell proliferation (Lin et al., 2015). HDAC4 reduces the activity of 6PGD by deacetylating lysines 76 and 294. An undefined cytosolic HDAC4 complex seems to be involved in this activity. Unfortunately, this interesting result was not further confirmed or developed.

5.5 HDAC4 in solid tumors

Various studies have addressed possible contributions of HDAC4 in development and aggressiveness of different solid cancers. Several different biological activities have been described as target of HDAC4. The epithelial mesenchymal transition (EMT) in cancer cells is an important hallmark of increased motility and chemoresistance (Nieto et al., 2016). In lung cancer cells, low levels of AMP-activated protein kinase (AMPK) enhance glycolysis (Warburg effect) and promote EMT and metastasis, through the action of HDAC4 and HDAC5 (Feng et al., 2020). The contribution of HDAC4 to EMT was observed also in esophageal carcinoma cells (Zeng et al., 2016), but not in other cancer models, suggesting that the contribution of HDAC4 could be lineage specific (Choi et al., 2016). In nasopharyngeal carcinoma HDAC4, in complex with HDAC3–NCOR1, represses transcription from the E-cadherin promoter and augments the expression of the mesenchymal markers such as N-cadherin, Snail and Slug (Cheng et al., 2021). The role of HDAC4 in EMT and in the metastatic process required further studies since also anti-metastatic effects have been observed. ZEB1 is an important TF involved in the EMT: it favors the metastatic process by increasing the levels of integrins and the adhesion to collagen fibers. The upregulation of these activities requires the cytoplasmic accumulation of HDAC4 through the inhibition of PP2A (Paroni et al., 2007) to release the epigenetic repression on integrin gene *Itga1* (Tan et al., 2022). An anti-metastatic role of HDAC4 was also observed in ovarian cancer and is operated by the classical partner MEF2A. Mechanistically, the phosphatase PRL-3 binds HDAC4 and competes for the binding to MEF2A, which in turn is released and can promote acetylation and transcription at the SOX2 locus. This circuit allows the expansion of cancer stem cells, tumor aggressiveness and metastasis (Zhang et al., 2020). This result is in agreement with the possible involvement of MEF2 family members in EMT (Su et al., 2016; Xiao L et al., 2021).

The increased proliferative capacity of tumors is due to alterations in the normal regulation of the cell cycle, and the contribution of HDAC4 to cell cycle progression has been observed in various contexts, including normal cells and different cancer models (Wilson et al., 2008; Feng et al., 2009; Cai et al., 2018; Bindea et al., 2009; Cao et al., 2019; Ha et al., 2022). Experiments in some reports have demonstrated that the expression of CDK inhibitors (CDKN1A and CDKN2A) is regulated in an HDAC4-dependent manner. Overall, the mechanisms used by HDAC4 to influence cell cycle progression deserve further analysis. In particular, the protein complexes monitored by HDAC4 that epigenetically control transcription of these inhibitors have not been clearly elucidated. Recently, the tumor suppressor gene breast cancer type 1 BRCA1-associated protein 1 (BAP1) was shown to antagonize HDAC4 activity to reduce proliferation. BAP1 encodes a deubiquitylase that regulates the level of ubiquitylation of histone H2AK119, a repressive epigenetic

mark involved in the regulation of transcription, replication, and repair. Uveal melanoma (UM) and malignant pleural mesothelioma (MPM) are among the tumors with the highest incidence of BAP1 mutations. In BAP1-mutated UM cells, HDAC4 is upregulated, accumulates in nuclei, and is required for cell proliferation. HDAC4 controls the expression of lineage-specific genes by reducing the level of H3K27 acetylation (Kuznetsov et al., 2019). The link between BAP1 and HDAC4 may also provide a strategy for personalized therapeutic intervention, as recently proposed (Kuznetsoff et al., 2021).

Cellular senescence is a response to various cellular stresses that leads to irreversible arrest of the cell cycle and secretion of inflammatory and regenerative cytokines (Bousset and Gil, 2022). Oncogenic lesions, by enhancing cell proliferation, lead to accumulation of DNA damage and induction of senescence, also known as oncogene-induced senescence (OIS). OIS can limit the tumorigenic process and eventually promotes the elimination of tumor cells by the immune system. Senescence escape has been recognized as one of the hallmarks of cancer cells (Hanahan et al., 2022). Conversely, induction of senescence may promote tumor recurrence under certain circumstances, for example, in response to chemotherapy (Demaria et al., 2017). HDAC4 is downregulated with aging and during various forms of senescence by UPS in a GSK3 β -dependent manner (Han et al., 2016; Di Giorgio et al., 2021; Lee et al., 2022). The anti-senescence effect of HDAC4 appears to be exploited on multiple levels. Directly or indirectly, HDAC4 may reduce the transcription of CDK inhibitors, the major brakes of the cell cycle machinery that are upregulated in senescent cells (Wilson et al., 2008; Feng et al., 2009; Wang et al., 2012), or other genes of the senescence program (Lee et al., 2022). An important epigenetic strategy is employed by HDAC4 to antagonize OIS. HDAC4 can buffer the activation of typical enhancers and super-enhancers activated during senescence (Di Giorgio et al., 2021). Super-enhancers (SEs) are important regulatory domains of chromatin that span several kilobases and have a high density of TFs and other regulators of transcription to effectively control gene expression (Hnisz et al., 2017). In this activity, HDAC4 requires the action of HDAC3 to limit H3K27 acetylation. Interestingly, several of these SEs that control senescence are activated by the AP-1 TFs, in complex with the HAT p300/KAT3B (Martínez-Zamudio et al., 2020).

Similar to senescence, cell death by apoptosis is an important antiproliferative option both in terms of limiting tumor growth and eradicating it in response to therapy. As discussed above, in absence of HDAC4 MM cells are more prone to proteotoxic stress-induced cell death, possibly indicating an augmentation of the normal level of protein misfolding (Kikuchi et al., 2015). This effect could explain the increased susceptibility to TRAIL-induced cell death observed in cells deficient in HDAC4 (Lee et al., 2018). The pro-survival activities of HDAC4 could also be related to modulation of DNA damage repair. In hepatocellular carcinoma, HDAC4 contributes to the efficiency of double strand breaks (DSBs) repair, by sustaining the homologous recombination. Repair of DSBs occurs *via* two main pathways: Non-homologous end joining (NHEJ) and homologous recombination (HR). Alternative EJ repair (Alt-EJ) mechanisms may also be employed (Ceccaldi et al., 2016). In this context HDAC4 contributes to the nuclear accumulation of RAD51 (Tsai et al., 2018), a key element of the machinery for repairing DSBs (Anand et al., 2017). A possible, but not yet clearly defined, role of HDAC4 in DSBs repair has also been observed by others (Marampon et al., 2017). In pancreatic cancer cells, the serin-threonine kinase MARK2 is activated in response to paclitaxel

treatment, a microtubule disrupting agent. HDAC4 is a substrate of MARK2 and once phosphorylated promotes the activity of YAP and resistance to paclitaxel. (Zeng et al., 2022). In some cases, a direct intervention of HDAC4 in the regulation of apoptotic programs has been reported. HDAC4 can repress transcription of core elements of the apoptotic machinery, such as BMF (Vallabhapurapu et al., 2015). The pro-survival activity of HDAC4 can also be exploited by controlling the tumor microenvironment. IL24 is a cytokine that can kill cancer cells and several attempts have been made to design a therapeutic approach using this cytokine (Menezes et al., 2014). HDAC4 can repress transcription of IL24 through epigenetic control at the promoter level (Pan et al., 2010), a property it shares with other class IIa HDACs (Cutano et al., 2019). In conclusion, although there are several data confirming a role of HDAC4 in cancer cell survival, the mechanism seems to vary from case to case. We should always consider a possible redundancy with other members of the family and the engagement of compensatory mechanisms, two conditions that complicate the interpretation of the data and could explain the heterogeneity of the results obtained. Certainly, interfering with HDAC4 levels using RNAi, increases cancer cell susceptibility to different apoptotic stimuli or cellular stressors. For this reason, several attempts have been made to affect the activity of HDAC4, by isoform-specific inhibitors.

Another characteristic of cancer cells is their ability to adapt to an unfavorable metabolic environment. This hurdle can be circumvented by metabolic adaptation to oxygen deprivation or/and induction of angiogenesis, and both have been reported to be regulated by HDAC4. The transcription factor HIF1 is the master regulator of oxygen homeostasis and consists of the subunits HIF1A and HIF1B. Under oxygen-rich conditions, HIF1A is constantly degraded *via* UPS by the action of Von Hippel-Lindau protein (VHL), which promotes the action of an E3 ubiquitin ligase. Hypoxic conditions selectively suppress this degradation because the absence of prolyl and asparaginyl hydroxylation in HIF1A at critical residues inhibits recognition by the VHL protein (Semenza, 2012; Semenza, 2016). An interaction between HIF1A and HDAC4 has been reported to affect the transcriptional activity of HIF1. The mechanism appears to involve HDAC4-dependent deacetylation and stabilization of HIF1A with potentiation of its transcriptional activity (Qian et al., 2006; Geng et al., 2011; Fischer et al., 2015; Chen et al., 2016). Alternative mechanisms have also been proposed (Seo et al., 2009; Chen et al., 2016). Recently, Zhang et al. (2017) showed that Nucleus Accumbens Associated 1 (NAC1) binds to HDAC4 and prevents its cytoplasmic degradation, enhancing the adaptive response to hypoxia. On the other hand, Fan et al. (2021) have shown that HIF and VEGFA are functional downstream mediators of HDAC4 *via* the ZIP4-HDAC4-VEGFA axis in high-grade serous ovarian cancer. These two features are not necessarily distinct and can be exploited in a potential therapeutic horizon. In a “synthetic lethality” scenario (Huang et al., 2020), the combination of blocking HDAC4 and using monoclonal antibodies against VEGF, such as bevacizumab, may be promising in the overwhelming landscape of tumor resistance, and some encouraging results have already been obtained (Zhang et al., 2017).

6 Conclusion

More than two decades after its discovery, there are still many puzzling aspects and unanswered questions about HDAC4. Although

its role as a repressor of MEF2 and modulator of the epigenome is well established, experimental data suggest that alternative targeting to different genomic regions is also possible, independently of MEF2. The composition of these MEF2-independent complexes is not known. Whether the other TFs regulated by HDAC4 may also contribute to localize HDAC4-coordinated complexes to other genomic regions remains to be investigated. Another point of interest is the dynamic balance between HDAC4 acting as an epigenomic regulator and HDAC4 complexes targeting non-histone proteins. The cytoplasmic functions of HDAC4 are also poorly understood, and it is still unclear whether the composition of HDAC4 complexes formed in the cytosol differs from complexes formed in the nucleus. Although the pro-oncogenic role of HDAC4 in cancer is known, conditions under which HDAC4 activities antagonize the transformation process are conceivable. Although cancer genome projects have allowed us to explore the degree of dysregulation of several genes, including HDAC4 in tumors, we have yet to gain information about the molecular complexity of the protein and its partners.

Author contributions

EC, CG, EP, RV, and CB wrote the draft and revised it. CB designed and supervised the study. EC, CG, EP, and CB collected the data and designed the figures and tables. All the authors read the submitted version and approved it.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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