The missing link*er*: emerging trends for H1 variant-specific functions

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Major advances in the chromatin and epigenetics fields have uncovered the importance of core histones, histone variants and their post-translational modifications (PTMs) in modulating chromatin structure. However, an acutely understudied related feature of chromatin structure is the role of linker histone H1. Previous assumptions of the functional redundancy of the 11 nonallelic H1 variants are contrasted by their strong evolutionary conservation, variability in their potential PTMs, and increased reports of their disparate functions, sub-nuclear localizations and unique expression patterns in different cell types. The commonly accepted notion that histone H1 functions solely in chromatin compaction and transcription repression is now being challenged by work from multiple groups. These studies highlight histone H1 variants as underappreciated facets of chromatin dynamics that function independently in various chromatin-based processes. In this review, we present notable findings involving the individual somatic H1 variants of which there are seven, underscoring their particular contributions to distinctly significant chromatin-related processes.

The rate of discovery in the field of chromatin biology has been remarkable over the last few decades. This progress is considerable given that less than a century ago, in 1928, Emil Heitz first coined the terms euchromatin and heterochromatin, describing the simple concepts of light versus dark staining patterns of chromosomes, respectively (Heitz 1928). No one understood how chromosomes adopted these different structures or what molecules were involved. It was not until 1974 that Olins and Olins (1974) first observed the nucleosomes as "beads on a string" via electron microscopy. This structure, DNA packaged tightly around a core of histone proteins, was proposed by Roger Kornberg (1974). A few months later, Pierre Chambon (Oudet et al. 1975) termed this repeating unit of chromatin the "nucleosome" and provided both visual and biochemical evidence supporting the model proposed by Kornberg (1974).

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In the 46 yr that have passed since this discovery, we as a field have fixated on the nucleosome structure and rightly so. We have dissected most of its intricacies and now have a fuller understanding of core histones and how their variants and post-translational modifications (PTMs) affect chromatin structure and gene expression. We have evolved into many subfields, such as those focusing on the chromatin remodeling enzymes that can alter the structure of the nucleosome, the DNA contacts, the spacing between nucleosomes, histone-modifying complexes that change histone biochemistry, and the histone chaperones that assemble and disassemble the nucleosome. However, one major chromatin component considered as being essential for the formation of heterochromatin has received little attention: the family of linker histones (H1).

Our intent in this review is to emphasize the likelihood that the H1 family of variants may also be key to understanding several fundamental processes in chromatin biology and to highlight key aspects of this frequently overlooked cadre of participants in chromatin biology. Here, we discuss the proposed functions of human and mouse somatic H1 variants.

The somatic H1 variants: what is known and what is unclear?

Eleven H1 variants have been described in humans and mice. This group includes four germline-specific variants: three testis-specific (H1t, H1T2, and HILS1) and one oocyte-specific (H1oo). The scope of this review will focus on the seven somatic (non-germline-specific) variants: H1.1-H1.5, H1.0, and H1x. Here we use the human nomenclature to avoid confusion (Table 1). Like histones composing the nucleosome, the variants can be further subdivided into DNA replication-dependent variants (H1.1–H1.5), which are mainly expressed in S-phase and replication-independent variants (H1x and H1.0).

Evolutionary conservation of H1 variants

Comparison of the mouse and human H1 gene sequences reveals that each variant is more similar to its ortholog than other intra-species variants (Fig. 1A–C; Drabent et al. 1995), suggesting that these variants have been evolutionarily conserved. The human replication-dependent H1 variants do exhibit considerable sequence similarity to each other (Fig. 1B). H1.3 and H1.4 are the most similar to the replication-dependent variants with an 86% overlap, whereas, H1.1 and H1.5 are the most divergent with only a 66.4% overlap (Drabent et al. 1995; Pan and Fan 2016). In contrast, replication-independent variants show very little sequence similarity to the other somatic variants, and even less sequence similarity to each other (Fig. 1B,C; Drabent et al. 1995; Pan and Fan 2016).

H1 structure and general function

The somatic H1 variants in mammals all have a similar structure, consisting of a highly conserved central globular domain and less conserved N- and C-terminal tails. A generally recognized function of linker histones is their binding to and facilitating the folding of chromatin into a more compact form. H1 accomplishes compaction via its globular domain that binds to DNA entry/exit points of the nucleosome at the dyad axis (center of the nucleosomal DNA), forming a structure called the chromatosome (Fig. 2). Unfortunately, as much of the N- and C-terminal tails of H1 are intrinsically disordered, resolving the complete structure of H1 bound to the nucleosome by X-ray crystallography is a challenge. Very recently, using cutting edge cryo-EM techniques, the structure of avian H1 (H5) bound to a dedocanucleosome has been determined at 3.6 Å resolution, revealing the atomic structure of an almost full-length linker histone H5 (G Li and P Zhu, pers. comm.). It has also come to our attention that cryo-EM structures of chromatosomes prepared with full-length H1x, H1.0, and H1.4 variants have been completed and will be available very soon (Zhou et al. 2020).

After the discovery of this effect of H1 on chromatin structure and the transcriptionally repressed chromatin states inherently incurred upon H1 incorporation, H1 was proposed as being a general repressor of transcription (Croston et al. 1991; Laybourn and Kadonaga 1991). However, more recent studies show that this is not always the case. In fact, some H1 variants are associated with active gene expression, suggesting that the presence of H1 does not always result in chromatin structures that are impermissible to transcription (Table 3, below; Fan et al. 2005; Sancho et al. 2008; Zheng et al. 2010; Kamieniarz et al. 2012; Izzo et al. 2013; Mayor et al. 2015).

Table 1. *H1 variant nomenclature for humans and mice*

	Human gene symbol	Human protein	Mouse protein
Cell cycle-independent	*H1FX*	H1x (H1.10)	H1X
	H1F0	H1.0 (H1°)	H1(0)
Cell cycle-dependent	*HIST1H1A*	H1.1	H1a
	HIST1H1B	H1.5	H1b
	HIST1H1C	H1.2	H1c
	HIST1H1D	H1.3	H1d
	HIST1H1E	H1.4	H1e

Protein names are the most frequently used nomenclature for each variant; alternative commonly used names are in parentheses.

Variability and discrepancies in H1 variant interaction with chromatin

There are still many gaps in our understanding of H1-dependent chromatin compaction. The following evidence highlights how important it is for future studies to clearly distinguish between H1 variants when studying chromatin dynamics. First, the H1 variants display a broad range of both affinity for and ability to compact chromatin (Table 2; Clausell et al. 2009; Öberg et al. 2012; Perišić et al. 2019). The H1 C-terminal tail, which is also important for chromatin compaction and organization (Bednar et al. 2017; Turner et al. 2018), varies in sequence and length between the variants, likely explaining their differences. Accordingly, two weak compactors of chromatin, H1.1 and H1.2, both have a shorter C-terminal tail compared with those of the other H1 variants (Clausell et al. 2009). It has also been reported that the PTMs associated with the H1 variants can alter their affinity for chromatin and their compacting ability (Perišić et al. 2019); e.g., when acetylated at lysine 34, H1.4 has a decreased affinity for chromatin (Table 4, below; Kamieniarz et al. 2012).

Second, whether H1 binding to the nucleosome is equidistant to each DNA entry/exit point (on-dyad) or closer to one DNA entry/exit point (off-dyad) (Fig. 2) has been debated in the field; models for each have been proposed (Song et al. 2014; Zhou et al. 2015; Bednar et al. 2017). Structural data for H1.5 and H1.0 binding supports the on-dyad model (Bednar et al. 2017). However, many in the field have accepted the off-dyad model based on cryo-EM data using H1.4 (Song et al. 2014). The mechanism by which H1 binding influences the compaction and structure of chromatin is still unclear (recently reviewed [Öztürk et al. 2020]). The Bai laboratory (Zhou et al. 2015) proposed that H1 binding on-dyad would lead to more compact chromatin than binding off-dyad using ultracentrifugation sedimentation coefficients; however, the Schlick laboratory (Perišić et al. 2019) came to the opposite conclusion using mesoscale modeling. The possibility that H1 can bind to the nucleosome both on- and off-dyad has also been proposed (Perišić et al. 2019), stressing the limitation of these structural approaches in capturing a highly dynamic H1/nucleosome interaction that is likely occurring in vivo.

Very recent cryo-EM structures of the H5-chromatin fiber with high resolution supports the off-dyad binding mode (G Li, pers. comm.), indicating that these discrepancies in H1 binding may not result from the different linker histone variants used in the respective study. Instead, given that the on-dyad binding of H1/H5 are mainly observed in the mononucleosome particle and the off-dyad binding

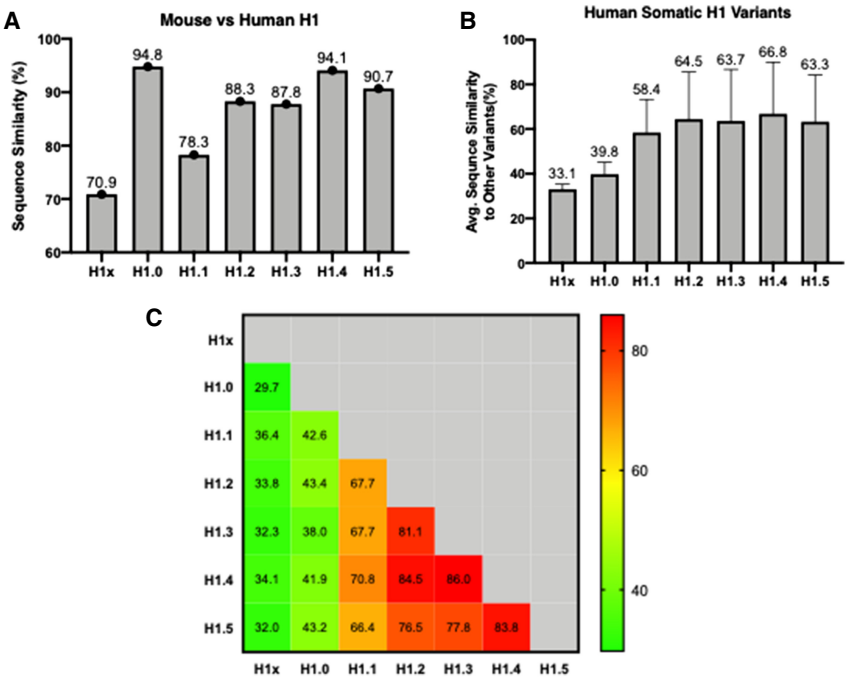

Figure 1. Sequence similarity of human H1 variants to other somatic variants and mouse ortholog. Graphs were produced using data published in Pan and Fan (2016). (*A*) Percent sequence similarity between human and mouse H1 orthologs. (*B*) Average sequence similarity of human H1 variants to the other somatic variants; error bars represent standard deviation. (*C*) Heat map representing the sequence similarity between individual somatic H1 variants.

are found in the compacted 30-nm fiber, it is likely that the globular domains of linker histone H1/H5 may switch from an on-dyad to an off-dyad location due to the asymmetric geometry and orientation of linker DNAs during the folding of higher-order 30-nm fiber from the open nucleosome arrays (G Li, pers. comm.).

However, the intriguing possibility that the human somatic variants have different effects on chromatin structure and perhaps distinct roles in local chromatin structure has not been ruled out and is under active investigation. This hypothesis is supported by evidence of the distinct chromatin structures and the dynamics of chromatosomes containing different human H1 variants (Zhou et al. 2020). Additionally, an important possibility to keep in mind is that chromatin structures in vivo are likely not as uniform as those established in vitro (e.g., the 30-nm fiber) and thus, a nuclear pool of somatic variants with varying abilities to compact and bind to chromatin may facilitate the stability of different chromatin structures. This possibility is strongly supported by the functional diversity of factors with which each of the variants interact (Table 2). Thus, it is very important to take note of the specific H1 variant being used in structural modeling and chromatin assays.

Perspectives and challenges of H1 variant research

Several challenges make it difficult to address the outstanding research questions regarding H1. For starters, the seven somatic H1 variants are differentially expressed in stem versus differentiated cells and some have been shown to be up- or down-regulated in cancer (Fig. 3). Additionally, some of the H1 variants are differentially expressed in the cells of different adult mouse tissues (for a previous review, see Pan and Fan 2016), making it difficult to compare studies using different model systems. A formidable challenge is the lack of reliable variant-specific antibodies, which limits the scope of many investigations into variant-specific differences. While a few variants have been reported to have specific antibodies, e.g., H1x, H1.0, and H1.2, their reliability and specificity are still unclear. Discrepancies have been found in ChIP-seq data acquired using an antibody specific to endogenous H1.2 versus tagged H1.2 in the same cells (Millán-Ariño et al. 2014). In accordance, concerns can be raised regarding the interference from an ectopic tag added to H1 with its overall function and/or chromatin binding dynamics. Moreover, many of the somatic H1 variants are cell cycle regulated, thus raising additional concerns that artificial

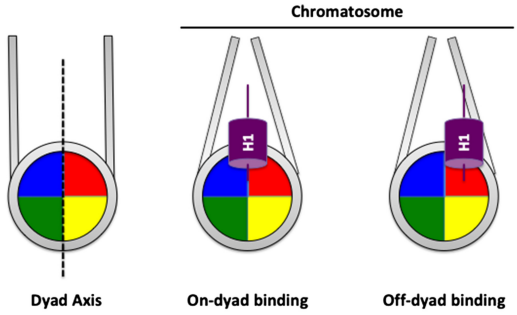

Figure 2. The proposed binding models of H1 of the chromatosome. The dyad axis is the central point of the nucleosome as demonstrated in the *left* panel. H1 has been shown to bind on-dyad (shown in the *middle* panel) or off-dyad (shown in the *right* panel). The implications of such binding modes are discussed in the text.

Table 2. *Summary of chromatin dynamics, protein interactions, and probable function for each somatic H1 variant*

Variants	Affinity for chromatin[a]	Ability to compact chromatin[b]	Protein interactions	Hypothesized functions
H1x	Lowest	High	RNF3; UBC13	Unclear, but possible role for H1x in DNA damage signaling and in the interphase nucleoli.
H1.0	Intermediate	High	Nap1; SET/Taf1β; many components of the nucleolus	In differentiated cells H1.0 creates a chromatin environment impermissible to DNA replication, represses transcription, and possibly exhibits tumor-suppressing activity.
H1.1	Low	Weakest	BAF	Unclear, but H1.1 is associated with a more relaxed chromatin and less differentiated cell state.
H1.2	Intermediate	Weak	Cul4A; PAF1; RNAPII; pRb; p53; YB1; PURα; DNA-PK; Bak; ATM; HUWE1; RNF8-UBC13; RNF168; PARP1; PARP3	H1.2 is an inhibitor of p53 and ATM via direct binding, and depending on which pathway is activated, dissociation of H1.2 from these proteins can activate apoptotic or prosurvival DNA damage repair.
H1.3	Intermediate	Intermediate	DMNT1; DMNT3B; HDAC3; SMRT; NCOR1	Overall it appears that H1.3 plays a role in transcriptional repression of hormone signaling-dependent genes. The literature also suggests a role of H1.3 in the regulation of microtubule dynamics and potentially a tumor-promoting role.
H1.4	High	High	EZH2; G9a; HP1; L3MBSTL1; JMJD2/KDM4; Aurora B kinase; p300; SirT1; CDK9; PKA; GCN5; TAF1	H1.4 has been shown to regulate transcription at specific regions, either repressing or activating as a function of its PTMs, and to regulate chromatin compaction during mitosis via modulation of HP1 bindings to chromatin.
H1.5	High, but may be more dependent on Nap-1 than other variants	High	Msx1; FoxP3	H1.5 appears to maintain condensed chromatin and gene repression at specific gene family clusters in differentiated cells and at prodifferentiation genes in undifferentiated cells.

Please note that references are listed throughout text.
[a]Determined by measuring the amount of each human H1 variant required to generate a nucleosomal repeat length of 200 bp on minichromosomes, and a band shift assay of mononucleosomes incubated with the H1 variants (Clausell et al. 2009).
[b]Measured using atomic force microscopy of purified minichromosomes assembled with each human H1 variant (Clausell et al. 2009).

manipulation of the total H1 variant pool by overexpression may interfere with overall function and/or chromatin binding dynamics.

In 2003, the Skoultchi laboratory (Fan et al. 2003) reported that a single knockout of some H1 variants (H1.2, H1.3, or H1.4) does not have a significant effect on the phenotype of mouse embryonic stem cells (mESCs). However, the simultaneous knockout of these three variants (H1.2, H1.3, and H1.4) that results in ~50% reduction in total H1, inhibits differentiation and is embryonic lethal, suggesting that H1 is essential. However, these variants may be functionally redundant. Following this study, the field moved toward a general acceptance of H1 variants being functionally redundant, once again removing H1 from the spotlight.

Fortunately, recent studies in human fibroblasts and cancer cells have countered the assumption of H1 functional redundancy and discovered variant-specific functions (Table 2). For example, H1.2 appears to be unique in that it has a low ability to compact chromatin and instead functions in DNA damage response (DDR) pathways, and H1.4 is important for mediating HP1 chromatin binding and chromatid separation during mitosis (Table 2).

The discrepancy between these studies and previous knockout experiments in mESCs highlights how important it is to ascertain the general applicability of the findings obtained from one model system. Of note, ESCs contain the lowest H1 to nucleosome ratio, with the greatest ratio being found in fully differentiated cells (Woodcock et al. 2006). These contrasting features suggest that the function of H1 may be less important in ESCs, consistent with chromatin being more open/accessible in ESCs (Efroni et al. 2008; Gaspar-Maia et al. 2011). Moreover, the

Table 3. *H1 variant effect on transcription*

Variant	Activator/ repressor	Cell type	Evidence
H1x	Activator	Breast cancer	ChIP-seq using an antibody specific to H1x shows its enrichment at gene-rich chromosomes, RNA polymerase II-enriched regions, and hypomethylated CpG islands (Millán-Ariño et al. 2014). H1x-enriched regions are highly expressed, while H1x-depleted regions are repressed (Mayor et al. 2015).
H1.0	Repressor	Mouse fibroblasts; hESCs; cancer cells	Overexpression of H1.0 in mouse fibroblasts results in a decrease in transcription of cell cycle regulated and housekeeping genes (Brown et al. 1996). During human ESC (hESC) differentiation, H1.0 is recruited to pluripotency genes (Terme et al. 2011). In human cancer cells, knockdown of H1.0 causes an up-regulation of genes involved in stem cell maintenance including those related to PRC2 (Torres et al. 2016; Mayor et al. 2015). ChIP-seq using tagged H1.0 and antibodies to endogenous H1.0 shows preferential accumulation of H1.0 at a number of known transcriptionally silent genomic regions including satellite regions, retrotransposable elements, nucleolus-associated DNA repeats, rDNA spacers, telomeric regions, and those comprising H3K27me3 (Torres et al. 2016; Cao et al. 2013; Mayor et al. 2015).
H1.1	No clear evidence		
H1.2	Both	**Activator** cancer cells; mouse fibroblasts	**Activator** Analysis of gene expression upon overexpression or knockdown of H1.2 reveals an increase or decrease in the expression of some genes, respectively (Brown et al. 1996; Sancho et al. 2008). Phosphorylation of H1.2 at S172 results in localization of H1.2 to active transcription sites (Talasz et al. 2009). H1.2 is the only variant able to bind to and recruit Cul4A and the PAF1 complex to RNAPII transcription sites via a direct interaction with the S2-phospho form of RNAPII. This H1.2-mediated recruitment is necessary for gene transcription (Kim et al. 2013). Phosphorylation of H1.2 at serine 173 is enriched in interphase nucleoli and associated with transcribing rDNA, perhaps acting as a facilitator of RNAPI transcription (Zheng et al. 2010).
		Repressor mESCs; cancer cells	**Repressor** Genome-wide analysis of H1.2 binding to chromatin using an ectopic tag or an H1.2 variant-specific antibody shows that H1.2 negatively correlates with transcriptionally active regions, being enriched at intergenic regions, gene-poor chromosomes, lamina-associated domains (LADs), and primarily associated with inactive promoters (Mayor et al. 2015; Clausell et al. 2009; Cao et al. 2013; Kim et al. 2015). Direct interaction of H1.2 with pRb at E2F target genes is essential for silencing of E2F gene transcription (Munro et al. 2017). H1.2 represses p53-mediated transcription via a direct interaction with p53 and corepressors YB1 and PURα, blocking the ability of p300-mediated chromatin acetylation and activation of p53 target genes (Kim et al. 2008).
H1.3	Repressor	mESCs; mouse fibroblasts; cancer cells	ChIP-seq of tagged H1.3 in mESCs reveals the lack of H1.3 at gene-rich regions and active promoters, enriched instead at sites with H3K9me3, major satellite elements, and LINES (Cao et al. 2013). H1.3 alone is able to restore DNA methylation at the imprinting control regions (ICRs) of *H19* and *GTL2*, which are lost in mESCs having a triple knockout of H1.2, H1.3, and H1.4. H1.3 restores DNA methylation at these ICRs via its direct interaction with DMNT1 and DMNT3B, thereby recruiting the complex to ICRs. This occurrence inhibits the methyltransferase SET7/9 from

Continued

Table 3. *Continued*

Variant	Activator/ repressor	Cell type	Evidence
			binding to chromatin and catalyzing methylation of H3 at lysine 4, associated with active transcription (Yang et al. 2013). H1.3 was identified as the specific H1 variant responsible for modulating the chromatin structure and transcriptional activation of the *MMTV* promoter. Dephosphorylation of H1.3 is associated with inhibition of gene expression at this promoter (Banks et al. 2001). HDAC3/H1.3 complex copurifies with the SMRT and NCOR1 nuclear receptor corepressor complexes (Patil et al. 2016).
H1.4	Both	**Repressor** cancer cells; mouse fibroblasts; mESC	**Repressor** EZH2- and G9a-mediated methylation of H1.4 at K26 recruits HP1 to chromatin and results in compaction and repression of transcription (Patil et al. 2016; Kuzmichev et al. 2004; Daujat et al. 2005; Trojer et al. 2009).
		Activator cancer cells; human pluripotent carcinoma cells (NT2) mESCs; iPS cells	**Activator** ChIP-seq using a phosphorylation-specific antibody demonstrates that H1.4 phosphorylated at serine 187 is positively associated with the transcription of rDNA, and is enriched at the response elements of the transcriptionally active MMTV-glucocorticoid receptor or the estrogen receptor after treatment of cells with dexamethasone or estradiol, respectively (Zheng et al. 2010). A separate study finds this phosphorylated H1.4 to be enriched at the TSS of genes encoding pluripotency factors in stem cells (Liao and Mizzen 2017). H1.4 acetylated at lysine 34 (H1.4K34ac) is enriched at the TSS of active genes, correlating with the presence of H3K4me3 and RNAPII. H1.4K24Ac positively affects transcription by recruiting the TAF1 subunit of TFIID via a direct interaction with the TAF1 bromodomain (Kamieniarz et al. 2012).
H1.5	Repressor	Myoblast cells; human T cells; human lung fibroblasts	H1.5 is recruited to the core enhancer region of the *MYOD* gene by Msx1 resulting in repressive chromatin at the *MYOD* locus, thus inhibiting MyoD expression (Lee et al. 2004). FoxP3 and H1.5 cooperate to repress IL-2 expression in human T cells (Mackey-Cushman et al. 2011). In differentiated cells, H1.5 preferentially binds to and represses gene regions that encode membrane and membrane-related proteins. H1.5 binding to these regions is required for SIRT1 binding, H3K9me2 enrichment, and chromatin compaction (Li et al. 2012).

somatic H1 variants are post-translationally modified differently in stem versus differentiated cells (Table 4, below; Kamieniarz et al. 2012). Here, emphasis is placed on these criteria when interpreting and comparing studies of H1 variants between model systems and when discussing their proposed functions.

DNA replication-independent variants (H1x and H1.0)

H1x

H1x is the least conserved and the most divergent somatic variant (Fig. 1A–C; Drabent et al. 1995; Happel et al. 2005; Pan and Fan 2016). H1x is ubiquitously expressed in mammalian cells (Yamamoto and Horikoshi 1996), but its expression has been shown to be higher in differentiated cells (Fig. 3; Terme et al. 2011). Although H1x is expressed in a DNA replication-independent manner (Happel et al. 2005), its nuclear distribution has been shown to be cell cycle-dependent: During G1 phase, H1x is concentrated in the nucleoli, but upon entry into S-phase H1x is evenly dispersed in the nucleus until the next G1 phase (Stoldt et al. 2007; Chen et al. 2018). The purpose of this nucleolar localization in G1 remains unclear. H1x is not localized to sites of active RNA polymerase I transcription (Stoldt et al. 2007). It has been suggested that this cell cycle-dependent shuttling of H1x is a mechanism to regulate its activity (Stoldt et al. 2007).

Interestingly, H1x has the lowest affinity for chromatin, but is among the variants with the greatest ability to compact chromatin (Table 2; Clausell et al. 2009). Contrary to its ability to compact chromatin, a few studies using ChIP-seq of endogenous H1x show it to be associated with more accessible, transcriptionally active chromatin:

Table 4. *H1 variant-specific PTMs with proposed function*

Variant	PTM	Enzymes	Cell model	Function
H1.2	S172-P[a]	?	Tumorigenic cells (HeLa; HEK 293)	Results in localization of H1.2 to sites of active DNA replication and transcription (Talasz et al. 2009).
	S173-P	?	Tumorigenic cells (HeLa S3)	Enriched in interphase nucleoli and associated with actively transcribing rDNA, perhaps acting as a facilitator of RNAPI transcription (Zheng et al. 2010).
	T146-P	Writer: DNA-PK	Tumorigenic cells (H1299; U2OS)	Impedes H1.2 binding to p53, thus relieving p53 of H1.2-mediated repression, allowing p53 to activate transcription of its target genes (Kim et al. 2012).
H1.3	S174-P	?	Tumorigenic cells (HeLa)	Unclear, but unlike S174 site analogs in other variants, this H1.3 phosphorylation does not exhibit cell cycle dependency, suggesting a unique function (Chen et al. 2016).
H1.4	K26ac	Writer: p300-HAT Eraser: SirT1	Tumorigenic cells (HEK 293)	Deacetylation of H1.4K26 by SirT1 is associated with transcriptional repression at promoter regions in facultative heterochromatin (Vaquero et al. 2004).
	K26me	Writer: EZH2/G9a Eraser: JMJD2/KDM4	Tumorigenic cells (HeLa; HEK 293; HL60); mouse fibroblasts (NIH 3T3 cells); mESCs	Recruits HP1 to chromatin resulting in chromatin compaction and repression of transcription (Patil et al. 2016; Kuzmichev et al. 2004; Daujat et al. 2005; Trojer et al. 2009).
	K34ac	Writer: GCN5 Eraser: class I and II HDACs	Tumorigenic cells (HeLa; MCF7); iPS cells	Enriched at the TSS of active genes, and positively affects transcription by recruiting the TAF1 subunit of TFIID via a direct interaction with the TAF1 bromodomain. Additional sites of enrichment are sites hypersensitive to DNase I as well as CTCF binding sites, suggesting it marks regulatory regions. Of interest, this PTM is up-regulated in iPS cells (Kamieniarz et al. 2012).
	S187-P	Writer: CDK9	Tumorigenic cells (HeLa S3; HeLa); human pluripotent carcinoma cells (NT2); mESCs	Enriched at transcriptionally active rDNA genes in the interphase nucleoli and at the transcription start sites (TSSs) of genes encoding pluripotency factors in stem cells, but not differentiated cells (Zheng et al. 2010; Liao and Mizzen 2017).
	S27-P	Writer: Aurora B kinase	Mouse fibroblasts (NIH 3T3 cells); tumorigenic cells (HeLa; HEK 293; HL60; MCF7)	Prevents HP1 from binding to H1.4K26me. Is most enriched on metaphase chromatin, suggesting it modulates HP1 binding to chromatin during mitosis (Daujat et al. 2005; Hergeth et al. 2011).
	S35-P	Writer: PKA	Tumorigenic cells (HeLa; HEK 293)	Causes H1.4 dissociation from mitotic chromatin. Mutation at this site results in mitotic defects (Chu et al. 2011).
	T18-P	Writer: PLK1(?)	Immortalized mouse embryonic fibroblasts; immortalized human epithelial cells (hTERT-RPE1)	Results in SET-mediated eviction of H1.4 from chromatin during the prophase-metaphase transition. Mutation causes incomplete chromatid arm resolution (Krishnan et al. 2017).
H1.5	S172-P[a]	?	Cancer cells (HeLa; HEK293)	Results in localization of H1.5 to sites of active DNA replication and transcription (Talasz et al. 2009).

(K) Lysine, (S) serine, (T) threonine, (ac) acetylation, (me) methylation, (P) phosphorylation.
[a]S172 phosphorylation is specific to both H1.2 and H1.5.

H1x is enriched at gene-rich chromosomes, RNA polymerase II-enriched regions, and hypomethylated CpG islands (Mayor et al. 2015; Millán-Ariño et al. 2016). In fact, H1x-enriched regions are highly expressed, while H1x-depleted regions are repressed (Table 3; Millán-Ariño et al. 2014; Mayor et al. 2015).

It is possible that H1x functions differently depending on its cellular localization. For example, its high concentration in the nucleolus could support its increased binding to nucleolar chromatin and facilitate nucleolar chromatin condensation of transcriptionally inactive ribosomal genes. The RNA polymerase I transcription factor UBF is able to out-compete H1 histones for binding chromatin (Kermekchiev et al. 1997), thus preventing the high nucleolar concentration of H1x from inhibiting essential ribosomal gene transcription. Moreover, H1x relocalization into the nucleoplasm during S phase may foster the maintenance of genome stability by inhibiting transcription at active genes, thus preventing collisions between the replication and transcription machineries. This latter possibility is supported by two pieces of evidence: H1x is enriched at sites of DNase-hypersensitivity

	Stem Cells	Differentiated Cells	Cancer Cells
H1x	Lower	Higher	Unknown
H1.0	Not Expressed	Very High	Decreased
H1.1	Higher	Very Low	Unknown
H1.2	Lower	Higher	Increased
H1.3	Higher	Lower	Increased
H1.4	Lower	Higher	Decreased
H1.5	Higher	Lower	Unknown

Figure 3. H1 variant expression levels in stem cells, differentiated cells, and cancer cells. Stem cells are known to have a more open chromatin compared with the more compact chromatin landscape of differentiated cells. Upon tumorigenesis, the chromatin landscape can be dysregulated, resulting in a more open chromatin. The expression of specific H1 variants is altered depending on cell state. References for each of the variant expression levels are cited and further discussed throughout the text.

(Millán-Ariño et al. 2016), and H1x is one of the few variants that is modified by K63-linked ubiquitin chains upon the occurrence of double-strand DNA breaks, serving as an important mark of recognition by factors involved in DNA repair signaling (Thorslund et al. 2015). Alternatively, it is also possible that H1x is sequestered in the nucleoli during G1 for reasons that are not yet clear.

H1.0

H1.0 is the most conserved variant between mouse and human (Drabent et al. 1995; Pan and Fan 2016) and the most abundant variant in adult somatic cells (Fig. 1A; Terme et al. 2011). It has been nicknamed the "replacement linker histone" due to its lack of expression in stem cells and its accumulation in terminally differentiating cells (Fig. 3; Sirotkin et al. 1995; Terme et al. 2011). H1.0 expression has also been shown to decrease in cancer cells (Fig. 3; Gabrilovich et al. 2002; Medrzycki et al. 2012). In accordance, H1.0 forms highly compacted chromatin (Table 2; Gunjan et al. 1999; Clausell et al. 2009). H1.0 has also been shown to accumulate in nondividing cells and its overexpression in cycling cells inhibits DNA replication (Sirotkin et al. 1995; Brown et al. 1996; Torres et al. 2016).

Unsurprisingly, H1.0 is also an inhibitor of transcription (Table 3; Brown et al. 1996; Terme et al. 2011; Kalashnikova et al. 2013; Mayor et al. 2015; Zhang et al. 2015). Its overexpression in mouse fibroblasts results in a decrease in transcription, including that of cell cycle regulated and housekeeping genes (Brown et al. 1996). During human ESC (hESC) differentiation, H1.0 is recruited to pluripotency genes and genes important for differentiation (Terme et al. 2011). In human cancer cells, H1.0 knockdown gives rise to an up-regulation of genes involved in stem cell maintenance including those related to Polycomb Repressive Complex 2 (PRC2) (Torres et al. 2016), which catalyzes methylation of lysine 27 of histone H3, a histone modification associated with chromatin repression (Kuzmichev et al. 2002). Additionally, H1.0 preferentially accumulates at a number of known transcriptionally silent genomic regions, including satellite regions, retrotransposable elements, nucleolus-associated DNA repeats, rDNA spacers, telomeric regions, and those comprising H3K27me3 (Cao et al. 2013; Mayor et al. 2015; Torres et al. 2016). The histone chaperones Nap1 and SET/Taf1β help to overcome repression by binding to and evicting H1.0 (Zhang et al. 2015).

H1.0 is the most enriched H1 variant in the nucleolus, showing the greatest enrichment at nucleolus-associated chromatin domains (NADs) (Mayor et al. 2015). A proteomics study in multiple human cell lines using a HaloTag-H1.0 fusion protein shows that ~88% of H1.0 binding partners have been identified as components of the nucleolus, including proteins involved in core splicing and rRNA biogenesis (Kalashnikova et al. 2013; Szerlong et al. 2015). Only a third of these interactions are mediated by the C-terminal domain (CTD) of H1.0, and thus it is possible that its disordered N-terminal domain (NTD) mediates some, if not many, of the H1.0 protein interactions. Whether or not these interactions are functionally relevant is yet to be elucidated.

DNA replication-dependent variants (H1.1–H1.5)

H1.1

Of the somatic cell cycle-dependent linker histone variants, H1.1 shows the lowest mouse to human conservation and the most intraspecies divergence (Fig. 1A–C; Drabent et al. 1995; Ponte et al. 1998; Pan and Fan 2016). H1.1 exhibits a very different DNA chromatin binding profile from that of H1.2-H1.5, suggesting that H1.1 may also have a unique function (Izzo et al. 2013). H1.1 is overrepresented at promoter regions and CpG islands, and shows enrichment at intergenic regions and chromatin domains rich in the Polycomb group of proteins (PcG), including PRC2 (Yu et al. 2019).

H1.1 expression is associated with a less differentiated cell state (Terme et al. 2011). Its expression in mice is restricted to the thymus, spleen, and testis, tissues known to have higher populations of stem-like cells (Fig. 3; Franke et al. 1998; Wiśniewski et al. 2007). H1.1 has a low affinity for chromatin and has one of the weakest chromatin compaction capabilities of all H1 variants (Table 2; Clausell et al. 2009), thus resulting in a more relaxed chromatin structure in undifferentiated cells. H1.1 exchange occurs rapidly and between physically separated regions of chromatin (Lever et al. 2000). These dynamic interactions with chromatin are probably a result of its

short C-terminal tail compared with most of the other H1 variants (Clausell et al. 2009).

Sparse research has been conducted into the specific functions of H1.1. H1.1 has been shown to directly interact with barrier to autointegration factor (BAF) (de Oca et al. 2005). The implications of this interaction are not well-understood, but deletion of BAF reduces the expression of ESC markers (Sox2, Oct4, and Nanog). This scenario suggests that BAF helps maintain the pluripotent state (Cox et al. 2011), perhaps in collaboration with H1.1. Mass spectrometry analysis has uncovered that H1.1 is heavily post-translationally modified, having acetyl, methyl, and phospho groups in human cells (Starkova et al. 2017). Further investigations are needed to identify other H1.1 protein interactors and the consequences of its depletion on gene expression.

H1.2

H1.2 expression increases upon cellular differentiation (Fig. 3; Lennox and Cohen 1983; Doynova et al. 2017). Notably, H1.2 is overexpressed in many cancer cells (Fig. 3; Nagel et al. 2013; Kim et al. 2015) and its depletion from cancer cells has been shown to decrease proliferation (Sancho et al. 2008; Li et al. 2018). H1.2 has an intermediate affinity for chromatin, but a weak chromatin compaction ability (Table 2; Clausell et al. 2009). H1.2 is the most well-studied somatic variant, but our understanding of its function is not straightforward. First, it is debatable as to whether H1.2 is an activator or repressor of transcription as both such activities have been reported (Brown et al. 1996; Kim et al. 2008, 2013, 2015; Sancho et al. 2008; Zheng et al. 2010; Cao et al. 2013; Izzo et al. 2013; Millán-Ariño et al. 2014; Mayor et al. 2015; Chen et al. 2018). Second, multiple unique biological functions of H1.2 have been proposed. We begin by introducing what is known about H1.2 in regards to transcriptional regulation.

Analysis of gene expression upon overexpression or knockdown of H1.2 reveals that its increased levels are associated with activation of some genes, while its depletion is associated with the repression of others, many being cell cycle genes (Table 3; Brown et al. 1996; Sancho et al. 2008). These findings suggest that H1.2 is involved in the activation of gene expression, but a direct transcriptional role has not been established. In accordance, phosphorylation of H1.2 at serine 172 (H1.2S172-P) results in its localization to sites of active DNA replication and transcription (Talasz et al. 2009). This notion of H1.2 association with transcriptionally-active genes is further supported by a study using ectopically-tagged versions of the somatic variants (H1x was not tested), in which H1.2 is the only variant that binds Cul4A (E3 ubiquitin ligase) and the PAF1 complex (transcription elongation factor) and recruits them to RNAPII transcription sites via a direct interaction with the serine 2-phospho form of RNAPII (Fig. 4A; Kim et al. 2013). H1.2 knockdown severely impairs the ability of the Cul4A and PAF1 complexes to generate active histone PTMs as well as to facilitate transcriptional elongation. This study directly links H1.2 to transcription and suggests that H1.2-mediated recruitment is necessary for gene transcription (Kim et al. 2013). Additionally, H1.2 is also phosphorylated at serine 173 (H1.2S173-P) (Table 4), again appearing to be associated with active transcription as it is enriched in interphase nucleoli and associated with transcribing rDNA, perhaps acting as a facilitator of RNAPI transcription (Zheng et al. 2010).

Contrary to these findings, genome-wide analysis of H1.2 chromatin binding using an ectopic tag or H1.2 variant-specific antibody shows that H1.2 binding negatively correlates with transcriptionally active regions. In this case, H1.2 is enriched at intergenic regions, gene poor chromosomes, lamina-associated domains (LADs), and primarily associated with inactive promoters (Table 3; Cao et al. 2013; Millán-Ariño et al. 2014; Kim et al. 2015; Mayor et al. 2015). Nonetheless, there are some discrepancies in these results as well. For example, one group using antibodies specific to endogenous H1.2 in breast cancer cells finds that H1.2 is enriched at and required for expansion of H3K27me3 (Millán-Ariño et al. 2014), a histone PTM associated with facultative heterochromatin. Another group using endogenously-tagged H1.2 in mESCs finds that H1.2 is depleted from chromatin at H3K27me3 and instead enriched at H3K9me3 marks, another histone PTM primarily associated with constitutive heterochromatin (Cao et al. 2013). These discrepancies could be explained by a differential function of H1.2 in pluripotent versus more differentiated cells; data showing a change in the relative abundance of H1.2-specific PTMs upon differentiation supports this hypothesis (Liao and Mizzen 2017). Given that discrepancies have been reported in the ChIP-seq patterns when pull-down experiments are performed using a H1.2 variant-specific antibody versus ectopically-tagged H1.2 in the same cells (Millán-Ariño et al. 2014), further caution in the interpretation of these results is warranted. It is unclear if this discrepancy is due to a lack of specificity of the H1.2 antibody or possible interference of the H1.2 tag with protein function and chromatin binding.

Additional evidence for the role of H1.2 in transcriptional repression is in its direct interaction with pRb at E2F target genes, which is essential for pRb recruitment to and silencing of E2F gene transcription (Fig. 4B; Munro et al. 2017). Also, H1.2 has been shown to repress p53-mediated transcription via a direct interaction with p53 and corepressors YB1 and PURα. This latter complex blocks the ability of p300-mediated chromatin acetylation and activation of p53 target genes (Fig. 5B; Kim et al. 2008). Both of these discoveries were made using ectopically-tagged H1.2.

Cumulatively, this data suggests that H1.2 is neither a general repressor nor an activator of transcription in isolation, but may function as either in a context-dependent manner. This dynamic may depend upon interactions with other proteins and/or its PTMs (Table 4). Indeed, H1.2-mediated repression of p53 target genes can be negated by DNA-dependent protein kinase (DNA-PK)-mediated phosphorylation of H1.2 at its threonine 146 residue

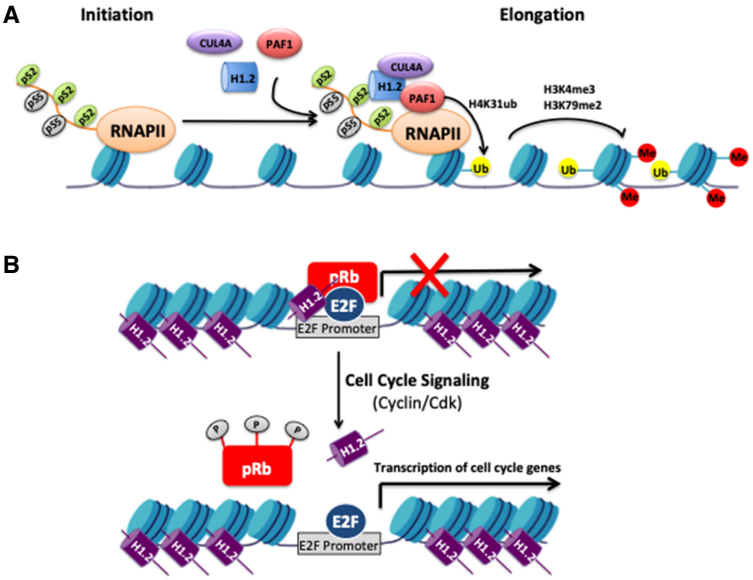

Figure 4. H1.2 can both activate and repress transcription. (*A*) Model of H1.2 facilitation of transcription elongation by recruitment of Cul4A and PAF1 to RNAPII. After transcription initiation and phosphorylation of the serine 2 (pS2) repeats within the RNAPII C-terminal tail, H1.2 selectively binds to pS2 and recruits both CUL4A and PAF1 to target genes. This recruitment leads to H4K31 ubiquitination (Ub), and trimethylation or dimethylation of H3K4 (H3K4me3), and H3K79 (H3K79me2), respectively, which facilitate productive transcription elongation and maintenance of active gene transcription (Kim et al. 2013). (*B*) Model showing H1.2 mediates pRb recruitment to and silencing of E2F gene transcription. In the absence of cell cycle stimuli, H1.2 recruits pRb to E2F-regulated promoters, thereby silencing gene expression. Upon cell cycle signaling, pRb is phosphorylated by Cyclin-CDK, resulting in the dissociation of the pRb-H1.2 complex from chromatin and activation of cell cycle-associated genes by E2F, resulting in cell cycle progression (Munro et al. 2017).

(T146), which impedes H1.2 binding to p53 (Kim et al. 2012). More research on the PTMs and binding partners of H1.2 is needed to establish a clear understanding of its role in gene transcription.

A major function of H1.2 as reported by many groups is its function in DDR (Fig. 5A–D; Konishi et al. 2003; Giné et al. 2008; Kim et al. 2008, 2012; Okamura et al. 2008; Rulten et al. 2011; Thorslund et al. 2015; Mandemaker et al. 2017; Li et al. 2018; Nánási et al. 2020). However, this H1.2 function is not quite straightforward. First, H1.2 has been shown to be associated with both the induction of apoptosis (Fig. 5A; Konishi et al. 2003; Giné et al. 2008; Okamura et al. 2008; Nánási et al. 2020) and in the promotion of a prosurvival DDR (Fig. 5B–D; Kim et al. 2012; Thorslund et al. 2015; Mandemaker et al. 2017). Second, H1.2 has been shown to associate with and mediate the function of both p53 (Kim et al. 2008, 2012) and ATM (Fig. 5B,C; Li et al. 2018).

One of the clearest roles of H1.2 in the literature is its transmission of the apoptotic signal from the nucleus to

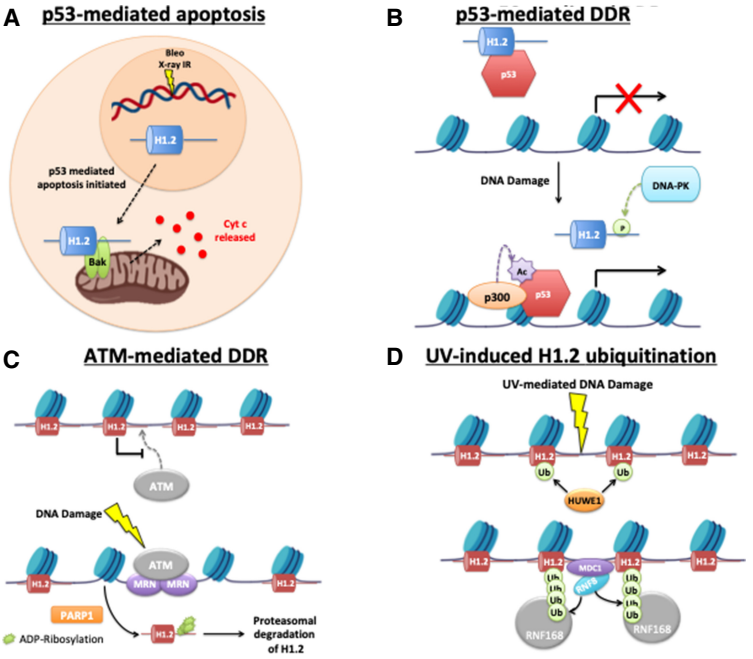

Figure 5. H1.2 is involved in numerous DNA damage response pathways. Schematic models of H1.2 proposed functions in response to DNA damage. (*A–D*) H1.2 mechanisms in response to bleomycin, etoposide, or X-ray (*A–C*) and in response to UV irradiation (*D*). (*A*) Upon induction of apoptosis, induced by Bleo or X-ray, but not UV irradiation, H1.2 is translocated from the nucleus to the cytoplasm where it interacts with Bak in the mitochondria and induces apoptosis in a p53-dependent manner (Konishi et al. 2003; Okamura et al. 2008). (*B*) In normal cells, H1.2 binds to and inhibits p53 from activating target genes. When cells are treated with DNA damage inducing agents (etoposide or bleomycin), p53 is acetylated by p300 and H1.2 is phosphorylated by DNA-PK. These modifications block H1.2 from interacting with and inhibiting p53, thus allowing the recruitment of chromatin remodeling and transcription factors to p53 target promoters (Kim et al. 2012). (*C*) H1.2 binds to chromatin and inhibits the recruitment and activation of ATM. Upon DNA damage with etoposide or ionizing radiation, but not UV irradiation, PARP1 PARylates H1.2. This results in H1.2 displacement from chromatin and proteosomal degradation. Loss of H1.2 from chromatin allows ATM to be recruited and activated by MRN at DNA breaks (Li et al. 2018). (*D*) HUWE1 ubiquitinates H1 in response to UV-mediated DNA damage. This H1.2-ub serves as a substrate for RNF8-mediated K63-linked poly- ubiquitylation. These K63-chains are recognized and bound by RNF168, which activates DDR (Mandemaker et al. 2017).

the cytoplasm in response to DNA damage (Fig. 5A; Konishi et al. 2003; Giné et al. 2008; Okamura et al. 2008; Nánási et al. 2020). These studies demonstrate that in response to DNA damaging agents such as X-ray, etoposide, bleomycin, and doxorubicin, H1.2 is translocated from the nucleus to the cytoplasm in a p53-dependent manner. Of note, UV irradiation was unable to cause H1.2 transport to the cytoplasm (Konishi et al. 2003; Okamura et al. 2008). Once in the cytoplasm, H1.2 colocalizes with Bak (Bcl-2 homologous antagonist/killer) in the mitochondria, where it releases Cytochrome C to initiate apoptosis in a Bak-dependent manner (Konishi et al. 2003; Okamura et al. 2008). This translocation of H1.2 apparently occurs independently of ATM signaling (Giné et al. 2008). In fact, H1.2 depletion renders cells resistant to apoptosis, stressing the importance of H1.2 translocation to the cytoplasm in transmitting an apoptotic signal (Konishi et al. 2003). It is unclear how H1.2 translocation to the cytoplasm is stimulated, but phosphorylation of its T146 residue (H1.2T146-P) together with p300-mediated acetylation of p53 has been shown to block p53-H1.2 binding, allowing p53 to activate transcription of its target genes such as *BAX* and thereby promote cell death (Kim et al. 2012). Thus, it is possible that H1.2T146-P primes H1.2 for such translocation to the cytoplasm, but this possibility has not yet been explored.

Interestingly, two studies using UV irradiation (which is unable to induce H1.2 transport to the cytoplasm) show an important role for H1.2 in activating the DNA damage repair pathway (Fig. 5D; Thorslund et al. 2015; Mandemaker et al. 2017). These studies demonstrate that H1.2 is ubiquitinated at many sites by the E2 ligase HUWE1 upon UV damage, priming H1.2 for further modification via RNF8-UBC13 and K63-linked ubiquitin chains. The H1.2 K63 ubiquitin chain is bound by RNF168 with high affinity, thereby recruiting it to double-strand DNA breaks and inducing DNA damage repair signaling.

Additionally, ectopically-tagged H1.2 has been shown to interact with ATM via its C-terminal tail and prevent aberrant ATM loading onto chromatin and activation of DNA damage signaling (Fig. 5C; Li et al. 2018). Interestingly, upon DNA damage induced by irradiation or etoposide (but not UV), H1.2, but not other H1 variants, is PARylated on its C terminus by PARP1. This event results in the rapid displacement of H1.2 from chromatin and its subsequent degradation resulting in ATM activation and enhanced cell survival. H1.2 knockout in HeLa cells results in an increased formation of γ-H2AX foci upon DNA damage (Li et al. 2018). PARP3 has also been shown to PARylate H1.2, but the mechanistic details have not been further investigated (Rulten et al. 2011).

Together, these results point to H1.2 playing a role in determining cell fate upon DNA damage. Upon UV-mediated DNA damage, H1.2 undergoes multiple rounds of ubiquitination allowing it to recruit DNA damage response proteins to the damage site. However, the H1.2 response to DNA damage elicited by X-ray or etoposide treatment depends upon whether the event is mediated through p53 or ATM.

The role of H1.2 in inhibiting p53 and ATM may explain why it is expressed at lower levels in mESCs than in differentiated cells and is then further elevated in cancer cells. As stem cells are known for their robust DNA damage response in order to maintain genome stability, it is appropriate that an inhibitor of two major DNA damage response pathways such as H1.2 would be expressed at lower levels in these cells. Also, *P53* and *ATM* are both known to be frequently silenced and dysregulated in cancer cells, suggesting that elevated H1.2 expression is advantageous for tumor growth.

H1.3

In several mouse tissues, H1.3 levels decline soon after birth (Lennox and Cohen 1983; Terme et al. 2011), and human ESCs have greater expression levels of H1.3 compared with differentiated cells (Fig. 3; Lennox and Cohen 1983; Terme et al. 2011). Interestingly, pluripotent transcription factors (OCT4, SOX2, and NANOG) are detected at the *H1.3* promoter, suggesting that H1.3 could play a role in maintaining pluripotency and self-renewal (Terme et al. 2011). In accordance, the *H1.3* gene promoter is trimethylated at H3K27 and silenced in AMLs that exhibit a better prognosis and greater chance of survival (Garciaz et al. 2019). On the other hand, H1.3 knockout mice develop normally (Fan et al. 2001) and knockdown of H1.3 in hESCs does not affect self-renewal (Terme et al. 2011), thus questioning its role in pluripotency.

H1.3 is intermediate in both its affinity for chromatin and its chromatin compaction ability (Table 2; Clausell et al. 2009). H1.3 is depleted from gene-rich regions and active promoters and is enriched instead at sites with H3K9me3 (but not H3K27me3), major satellite elements, LINES, and the inactive X-chromosome (Parseghian and Hamkalo 2001; Cao et al. 2013). These data suggest a role for H1.3 in localized, transcriptionally repressed regions of the genome (Table 4).

Interestingly, H1.3 expression alone is able to restore DNA methylation at the imprinting control regions (ICRs) of *H19* and *GTL2*, which are lost in mESCs with a triple knockout of H1.2-H1.4 (Yang et al. 2013). H1.3-mediated restoration of DNA methylation at these ICRs entails a direct interaction between its C terminus and DMNT1 and DMNT3B, thereby recruiting the complex to ICRs and inhibiting the methyltransferase SET7/9 from binding to chromatin and methylating lysine 4 of histone H3 (H3K4me), a modification associated with active transcription (Fig. 6A; Yang et al. 2013). Using H3 methyltransferase assays performed with chromatin in vitro and ChIP-qPCR of ICRs, the authors reported a decrease in dimethylated H3K4 (H3K4me2) as evidence of H1.3-mediated inhibition of SET7/9 methyltransferase activity. However, SET7/9 catalyzes only monomethylation, not dimethylation of H3K4 (Nishioka et al. 2002; Xiao et al. 2003), and it has been proposed that SET7/9 cannot catalyze the transfer of a methyl group to monomethylated H3K4 (Wilson et al. 2002). Can it be that other methyltransferases are also inhibited by H1.3 in the

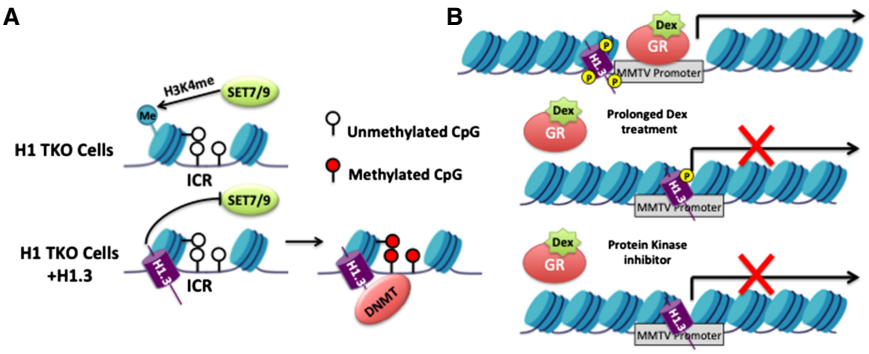

Figure 6. H1.3 regulates chromatin status at imprint control regions (ICRs) and the *MMTV* promoter. (*A*) Model demonstrating how H1.3 promotes DNA methylation and gene silencing at the *H19* and *GTL2* ICRs. mESC with a TKO of H1.2-H1.4 lose CpG methylation at the *H19* and *GTL2* ICR. Expression of only H1.3 is able to restore this defect. First, H1.3 binds and inhibits SET7/9-mediated methylation of H3K4 (H3K4me) at this region. Next, H1.3 recruits DNMT1 and DNMT3B to chromatin through binding by its CTD thereby promoting CpG methylation of the *H19* and *GTL2* ICRs (Yang et al. 2013). (*B*) Model demonstrating that prolonged treatment with dexamethasone results in dephosphorylation of H1.3 and silencing of the *MMTV* promoter. Upon prolonged treatment with dexamethasone (Dex), total phosphorylation of H1.3 (H1.3P) is decreased and the *MMTV* promoter becomes refractory. Additionally, treatment with staurosporine (a protein kinase inhibitor) results in complete dephosphorylation of H1.3 and silencing of the *MMTV* promoter. Dephosphorylated H1.3 is believed to bind to the *MMTV* promoter, restrict chromatin remodeling and transcription factor access, steps necessary for transcriptional activation of *MMTV* (Banks et al. 2001).

context of this mechanism and/or does the specificity of the H3K4me2 antibody require validation?

Another example of a specific role for H1.3 in site-specific transcriptional repression is evident in mouse cells treated for a prolonged period of time with dexamethasone (glucocorticoid agonist). This treatment causes dephosphorylation of H1.3 (and also of H1.4 and H1.5) and renders the *MMTV* promoter refractory to hormonal stimulation (Fig. 6B; Banks et al. 2001); the phosphorylation status of specific H1 variants was detected by mass spectrometry. In the same study, treatment with staurosporine (a protein kinase inhibitor) dephosphorylated only H1.3 and led to transcriptional repression of the *MMTV* promoter (Fig. 6B; Banks et al. 2001). This study implicates H1.3 as the specific H1 variant responsible for modulating the chromatin structure of the *MMTV* promoter, and this function is dependent on its phosphorylation status. The specific phosphorylation sites involved in this function have yet to be resolved.

Of the cell cycle-dependent H1 variants, H1.3 shows the strongest increase in expression levels during S phase (14-fold increase in mRNA expression) (Meergans et al. 1997). Additionally, H1.3 is phosphorylated at its serine residue 174 (S174), but unlike S174 site analogs in other variants, this phosphorylation does not exhibit cell cycle dependency, suggesting a distinct function for H1.3 in the cell cycle (Table 4; Chen et al. 2016). In support of this notion, H1.3, but not other variants, forms a stable complex with HDAC3 and its cofactors (Patil et al. 2016). This HDAC3/H1.3 complex increases in abundance during the G2/M phases of the cell cycle, presumably to deacetylate H3K9 during mitosis. The HDAC3/H1.3 complex localizes to polar microtubules and spindle poles in mitotic cells, also suggestive of a role for H1.3 in the regulation of microtubule dynamics.

A cumulative review of H1.3-specific functions and interactions reveals a trend of H1.3-mediated transcription repression of specific genomic regions that are responsive to nuclear hormone receptors. First, as discussed above, the *MMTV* promoter, which is induced by the glucocorticoid hormone, is silenced by dephosphorylation of H1.3 (Banks et al. 2001). Second, HDAC3, which complexes with H1.3, has a unique role in modulating transcription of nuclear receptors (You et al. 2013). Indeed, the HDAC3/H1.3 complex copurifies with the SMRT and NCOR1 nuclear receptor corepressor complexes, which are known to interact with RAR/RXR in a retinoic acid (RA) hormone regulated manner. In the presence of RA, the complex is displaced from RAR/RXR-regulated genes and is now free to interact with p300/CBP (Mottis et al. 2013). Third, steroid hormones have been shown to increase the expression of genes encoding *H19* and *GTL2*, which are transcriptionally silenced upon H1.3 expression (Yang et al. 2013). Other interesting correlations in this regard are that both hormone signaling and H1.3 expression are decreased in aging (Happel et al. 2008) and that H1.3 is up-regulated in many aggressive cancers (Medrzycki et al. 2012; Bauden et al. 2017; Garciaz et al. 2019), specifically in high-risk ovarian cancer in which many cases present with silencing of the nuclear estrogen (ER) and progesterone (PR) receptors (Harding et al. 1990; Medrzycki et al. 2012).

H1.4

H1.4 expression increases soon after birth in many mouse tissues (Lennox and Cohen 1983). Its expression is significantly reduced in malignant adenocarcinomas compared with benign adenomas (Fig. 3; Medrzycki et al. 2012). Together, these data suggest that elevated H1.4 expression is associated with a differentiated cell state. In accordance, H1.4 has a high affinity for chromatin resulting in its compaction (Table 2; Clausell et al. 2009; Öberg et al. 2012). Interestingly, H1.4 has been shown to be both an inhibitor and activator of transcription (Table 3). The PTMs of H1.4 appears to alter its ability to either repress or activate transcription (Table 4).

The most extensively studied site of the PTMs in H1.4 is lysine 26 (H1.4K26) (Table 4). H1.4K26 is methylated (H1.4K26me) by two methyltransferases: PcG protein-associated EZH2 within the PRC2 complex and G9a (Fig. 7A; Kuzmichev et al. 2004; Daujat et al. 2005; Trojer et al. 2009; Weiss et al. 2010; Ruan et al. 2012). This EZH2/G9a methylation site is specific to H1.4 (Weiss et al. 2010). EZH2- and G9a-mediated methylation of H1.4K26 recruits HP1α, H1β, and/or HP1ɣ by binding to the HP1 chromodomain, resulting in chromatin compaction and repression of transcription (Fig. 7C; Kuzmichev et al. 2004; Daujat et al. 2005; Trojer et al. 2009; Weiss et al. 2010). Despite K26 being conserved in many of the H1 variants, only H1.4K26me can recruit HP1 (Weiss et al. 2010). The affinity of HP1 binding to H1.4K26me was determined using an isothermal titration calorimetry (ITC) assay, and shown to be comparable with that of its binding affinity to H3K9me3. Thus, H1.4K26me could have a role in tethering HP1 to chromatin, possibly explaining how HP1 is targeted to regions of chromatin lacking methylated H3K9 (Ruan et al. 2012).

H1.4K26me appears to be regulated via multiple mechanisms. First, JMJD2/KDM4 demethylates this H1.4 site, and not any other known methylation sites on other variants (Fig. 7B; Trojer et al. 2009; Weiss et al. 2010). Second, phosphorylation of a neighboring serine 27 residue (H1.4S27-P) by the Aurora B kinase prevents HP1 from binding to H1.4K26me (Fig. 7D; Daujat et al. 2005). H1.4 is the only variant that is targeted for phosphorylation by Aurora B kinase. H1.4S27-P is cell cycle regulated and is most enriched on metaphase chromatin, suggesting that the status of H1.4 PTM can modulate HP1 binding to chromatin during mitosis (Hergeth et al. 2011). Additionally, H1.4K26 has also been shown to be acetylated (H1.4K26ac) by the p300 histone acetyltransferase (HAT) and deacetylated by SirT1 (Vaquero et al. 2004). SirT1 interacts with and deacetylates H1.4K26ac at promoter regions in facultative heterochromatin; deacetylation of H1.4K26ac by SirT1 is associated with transcriptional repression.

Another reported functional PTM of H1.4 is the phosphorylated version of serine 187 (H1.4S187-P). ChIP-seq using a phosphorylation-specific antibody demonstrates that this modification is enriched in the nucleoli during interphase, and is positively associated with the transcription of rDNA, suggesting that it may facilitate RNAPI activity (Zheng et al. 2010). This study also reports a similar function for H1.2S173-P, but only H1.4S187-P is enriched at the response elements of the transcriptionally active MMTV-glucocorticoid receptor or the estrogen receptor after treatment of cells with dexamethasone or estradiol, respectively (Zheng et al. 2010). An additional study finds that cyclin-dependent kinase 9 (CDK9) is the kinase that phosphorylates H1.4S187 and that H1.4S187-P is enriched at the transcription start sites (TSS) of genes encoding pluripotency factors in stem cells (Liao and Mizzen 2017). Interestingly, this PTM is depleted from such sites upon differentiation, in a manner that correlates with the decrease in their expression (Liao and Mizzen 2017). Thus, these data cumulatively suggest that phosphorylation at H1.4S187 converts H1.4 into an activator of transcription by both RNAPI and RNAPII.

Three additional H1.4 PTMs have been functionally analyzed. H1.4 serine 35 (H1.4S35) is phosphorylated by protein kinase A (PKA) during mitosis (Chu et al. 2011). H1.4S35-P accumulates immediately after H3S10 phosphorylation, and causes H1.4 dissociation from mitotic chromatin. Mutation at this site, resulting in a loss of phosphorylation, causes mitotic defects. Second, phosphorylation of H1.4T18 (H1.4T18-P) results in SET-mediated eviction of H1.4 from chromatin during the prophase-metaphase transition (Krishnan et al. 2017). Accordingly, SET knockout or overexpression of H1.4 with a T18A mutation causes incomplete arm resolution, most likely due to the lack of H1.4 eviction from chromatin.

The last H1.4 PTM that has been studied is acetylation of H1.4 at lysine 34 (H1.4K34ac) by GCN5 (Kamieniarz et al. 2012). H1.4K34ac is deacetylated by class I and II HDACs, but not sirtuins. H1.4K34ac is enriched at the TSS of active genes, and correlates with the presence of H3K4me3 and RNAPII. Sites of H1.4K34ac enrichment also colocalize with sites hypersensitive to DNase I as well as CTCF binding sites, suggesting it marks regulatory regions. H1.4K34ac positively affects transcription by

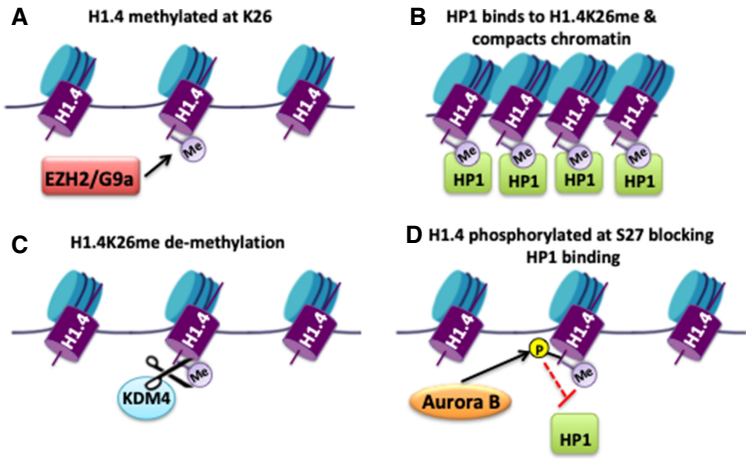

Figure 7. H1.4K26me recruits HP1 resulting in chromatin compaction. (*A*) H1.4 is specifically methylated at lysine 26 (H1.4K26me) by EZH2 or G9a (Kuzmichev et al. 2004; Daujat et al. 2005; Trojer et al. 2009; Weiss et al. 2010; Ruan et al. 2012). (*B*) H1.4K26me is demethylated specifically by JMJD2/KDM4 (Trojer et al. 2009; Weiss et al. 2010). (*C*) H1.4K26me recruits HP1α, H1β, and/or HP1ɣ by binding to the HP1 chromodomain, resulting in chromatin compaction and repression of transcription (Kuzmichev et al. 2004; Trojer et al. 2009; Weiss et al. 2010). (*D*) Aurora B kinase-mediated phosphorylation (P) of H1.4 at the neighboring serine 27 residue prevents HP1 from binding to H1.4K26me (Hergeth et al. 2011).

recruiting the TAF1 subunit of TFIID via a direct interaction with the TAF1 bromodomain. H1.4K34ac increases the mobility of H1.4 on chromatin, which is most likely due to its increased affinity to chromatin. In line with this, H1.4K24ac has been shown to be elevated in iPS cells.

Cumulatively, H1.4 has been shown to be a highly versatile H1 variant, in part due to its multiple PTMs. Its most likely functions are in transcription regulation either contributing to transcription repression or activation as a function of the presence of its specific PTMs, and in regulation of chromatin compaction during mitosis. Additional reports of H1.4 functions demonstrate that its depletion in human lung fibroblasts disrupts the localization of laminas at the nuclear periphery. Depletion in breast cancer cells results in a slower growth rate and a decreased population of cells in S phase, not due to G1 arrest, but instead to cell death in a manner unrelated to the process of apoptosis (Sancho et al. 2008; Izzo et al. 2013). Despite the abundance of literature regarding H1.4, there still remains many gaps in our understanding of its functions and how its PTMs regulate these functions. However, it is clear that histone H1 variants do not function exclusively to repress and compact chromatin.

H1.5

H1.5 expression is most elevated in pluripotent cells (Fig. 3; Lennox and Cohen 1983; Lee et al. 2004; Terme et al. 2011). Additionally, H1.5 has a very high affinity for chromatin and an ability to compact chromatin (Table 2). Similar to H1.3, H1.5 expression in mouse tissues declines after birth (Lennox and Cohen 1983) and in hESCs, the *H1.5* gene is occupied by the OCT4, SOX2, and Nanog pluripotency factors (Terme et al. 2011). Despite these similarities, H1.5, but not the other variants, has been shown to preferentially associate with two transcription factors involved in the process of differentiation: Msx1 and FoxP3 (Lee et al. 2004; Mackey-Cushman et al. 2011).

Msx1 recruits H1.5 to the core enhancer region of the *MYOD* gene, which establishes repressive chromatin at the *MYOD* locus, thus inhibiting MyoD expression and myogenic differentiation of myoblast cells (Lee et al. 2004). RNAi of H1.5 abrogates the ability of Msx1 to inhibit MyoD and differentiation, suggesting a role for H1.5 in maintaining pluripotency and self-renewal.

FoxP3 also specifically interacts with H1.5 in a manner that is not dependent on DNA-binding (Lee et al. 2004; Mackey-Cushman et al. 2011). Functionally, FoxP3 and H1.5 cooperate to repress IL-2 expression in human T cells. That H1.5 is essential for FoxP3 programming of regulatory T (Treg) cells rests on the following findings: Silencing of H1.5 expression in T cells inhibits the ability of FoxP3 to suppress IL-2 expression, and silencing of H1.5 expression in human Treg cells impairs the Treg function to suppress target T cells. Moreover, an independent study finds that H1.5 expression is higher in activated, replicating T-cells compared with nonreplicative T-cells (Gréen et al. 2011).

Interestingly, H1.5 has been shown to exhibit blocks of enrichment at genic and intergenic regions in differentiated human cells, but not in ESCs (Li et al. 2012). In differentiated cells, H1.5 preferentially binds to and represses gene regions that encode membrane and membrane-related proteins. H1.5 binding to these regions is required for SIRT1 binding, H3K9me2 enrichment, and chromatin compaction.

Cumulatively, these results suggest that H1.5 maintains condensed chromatin and gene repression at specific gene family clusters in differentiated cells and at prodifferentiation genes in undifferentiated cells. The abundance of phosphorylation at specific H1.5 sites declines upon differentiation of pluripotent cells (Liao and Mizzen 2017) and the amount of methylated H1.5 is higher in differentiated cells than in ESCs (Starkova et al. 2019), possibly explaining the changes observed in the genomic distribution and functionality of H1.5. Similar to H1.2, phosphorylation of H1.5 Serine 172 (H1.5S172-P) results in its localization to sites of active DNA replication and transcription in cancer cells (Talasz et al. 2009). While the function of H1.5S172-P remains unclear, it likely decreases the chromatin compaction ability of H1.5.

Conclusions and future perspectives

The limited knowledge regarding H1 variant functions is a major gap in chromatin biology that has been overlooked up until recently. H1 variant expression levels change during differentiation and are dysregulated in cancer, further stressing the importance of the roles these variants play in the maintenance or the generation of specific cellular states. Many key mechanisms that regulate the chromatin landscape and gene expression before, during, and after differentiation have remained elusive. Moreover, how cancer cells are able to escape the constraints of compacted chromatin in differentiated tissues still remains unclear.

In many cases, research focused on linker histones investigated the family of somatic H1 variants as one, either using antibodies incapable of discriminating between variants or using a pool of H1 variants in reconstitution experiments. In fact, some publications do not even specify which variant(s) are used, thus making it difficult to interpret results. This review treated members of the family of linker histones as separate proteins and organized peer-reviewed literature that differentiates between the H1 variants or specifies the variant studied; even those intending to study the general function of H1, but reported using a specific variant in the Methods section. This approach led to the uncovering of some very interesting trends in H1 variant-specific expression patterns and functions.

We have proposed functions for each of the somatic H1 variants based on a cumulative review of the literature (Table 2). The functions of H1x and H1.1 remain the most elusive, although H1x is shown to be the only variant associated with activation and not repression of

transcription, (Table 3). H1.0 has a well-documented role in maintaining a differentiated cell state with more compact chromatin. H1.2 is the most unique variant, and may play a larger role in mediating DDR than chromatin compaction. In fact, many of the proposed functions of H1.2 do not involve direct modulation of chromatin structure, suggesting that we have been oversimplifying and underappreciating the functional potential of this family of linker histones. H1.3 is shown in a few studies to function in the transcriptional repression of hormone signaling-dependent gene regulation and regulation of microtubule dynamics during mitosis. Like H1.3, H1.4 is also functional in mitosis. H1.4 regulates chromatin compaction during mitosis via modulation of HP1 binding to chromatin. Interestingly, H1.4 also exhibits the ability to regulate transcription at specific genomic regions; either facilitating the repression or activation of transcription, depending on its acquired PTMs. H1.2 is the only other variant that has both transcriptional activating and repressive functions. H1.5 seems to function in maintaining condensed chromatin and gene repression at specific gene family clusters in differentiated cells and at prodifferentiation genes in undifferentiated cells. Much more research is needed to gain a fuller picture of H1 variant-specific functions.

One clear trend that emerges is the differences between variant expression in differentiated cells versus stem cells: H1x, H1.0, H1.2, and H1.4 are all expressed at higher levels in differentiated cells. Knowing that the chromatin landscape of differentiated cells is more compact, it is not surprising to find that all of these variants have a greater ability to compact chromatin. The only exception is H1.2, which has only weak chromatin compaction ability, supporting that its primary function is in regulating DDR via inhibiting p53 and ATM, rather than maintaining a compact chromatin state. DNA repair signaling is incredibly important in maintaining genomic stability in pluripotent cells, perhaps explaining why an inhibitor of p53 and ATM would be expressed at lower levels in stem cells.

An inverse trend is seen in variants expressed at higher levels in stem cells: H1.1, H1.3, H1.5. H1.1 and H1.3 levels show a lower affinity for chromatin and a weaker ability to compact chromatin, in line with the open chromatin landscape of stem cells. H1.5 is an exception, showing high affinity for chromatin and an ability to compact chromatin. This may be due to its role in binding to and silencing prodifferentiation genes. However, it is also important to consider that H1.5 has a weak affinity for chromatin in the absence of NAP1, suggesting that NAP1-mediated chaperoning to specific regions of chromatin may be necessary. Additionally, the amount of H1.5 phosphorylation is higher in pluripotent cells, and one specific phosphorylation site, H1.5S172-P, is enriched at sites of active replication and transcription, suggesting that the gain or loss of specific PTMs is an additional mechanism of regulating H1 variants during differentiation (Talasz et al. 2009).

Another clear trend is the discrepancies in the chromatin compaction ability of variants that are up-regulated versus those that are down-regulated in cancer. H1x, H1.0, and H1.4 all have high chromatin compaction ability and are reported as being down-regulated in cancer cells. This decrease in highly compacting H1 variants most likely supports the opening of chromatin. This scenario could in turn facilitate an increase in DNA replication and transcription, as well as promote a more plastic chromatin state that can foster mutations and an altered 3D genomic organization; all of which facilitates the adaptation and survival of tumorigenic cells. Additionally, H1.2 and H1.3, both weaker compactors of chromatin, are up-regulated in some cancers, also likely supporting a more open chromatin structure. Moreover, there are other implications as to why increased H1.2 and H1.3 expression would be beneficial to cancer cells.

First, H1.2 has been shown to be essential for proliferation in breast cancer cells, but not normal breast cells or mESCs. It is clear that H1.2 plays a role in inhibiting the DDR proteins, p53 and ATM, therefore it is plausible that cancer cells up-regulate H1.2 as a mechanism to avoid cell cycle arrest or apoptosis in response to DNA damage. In accordance, H1.2 depletion in two breast cancer cell lines, T47D (p53-null) and MCF7 (wild-type p53), results in G1 arrest and cell death, respectively (Sancho et al. 2008). Second, H1.3 has been shown to be a transcriptional repressor of hormone responsive genes. Hormone receptor silencing is an important biomarker in many cancers such as breast and ovarian cancer. Thus, it is very plausible that in addition to maintaining a more relaxed chromatin structure, H1.3 also facilitates the silencing of hormone receptors, which can facilitate tumorigenesis.

Additionally, this review highlights the marked importance of PTMs in modulating variant function (Tables 3,4), stressing the need for the further characterization of H1 variant-specific PTMs. H1.2, H1.3, and H1.4 change in their ability to activate or repress transcription based on their respective PTMs. Mass spectrometry analysis of linker histone PTMs has detected an abundance of H1 PTMs, many of which are variant-specific, but their functional importance has yet to be investigated. Additionally, significant differences in the location and nature of PTMs are found in H1.3–H1.5 when comparing ESCs with differentiated cells (Starkova et al. 2017, 2019).

The field is now in a prime position to tap into this reservoir of information regarding the functional importance of the linker histone variants. We suspect that this challenge will be aptly met through the design of innovative approaches to distinguish among the linker histone variants, ultimately filling these gaps and advancing the field of chromatin biology.

Competing interest statement

D.R. is a cofounder of Constellation Biotechnology and Fulcrum Biotechnology.

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