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Beyond the histone tale: HP1α deregulation in breast cancer epigenetics

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Heterochromatin protein 1α (HP1α) encoded from the *CBX5*-gene is an evolutionary conserved protein that binds histone H3 di- or tri-methylated at position lysine 9 (H3K9me2/3), a hallmark for heterochromatin, and has an essential role in forming higher order chromatin structures. HP1α has diverse functions in heterochromatin formation, gene regulation, and mitotic progression, and forms complex networks of gene, RNA, and protein interactions. Emerging evidence has shown that HP1α serves a unique biological role in breast cancer related processes and in particular for epigenetic control mechanisms involved in aberrant cell proliferation and metastasis. However, how HP1α deregulation plays dual mechanistic functions for cancer cell proliferation and metastasis suppression and the underlying cellular mechanisms are not yet comprehensively described. In this paper we provide an overview of the role of HP1α as a new sight of epigenetics in proliferation and metastasis of human breast cancer. This highlights the importance of addressing HP1α in breast cancer diagnostics and therapeutics.

Keywords: breast-cancer, metastasis, chromatin, epigenetics, histone-modifications, invasion, mitosis, proliferation

Abbreviations: bp, base pair; CBX, chromobox homolog; CD, chromo domain; CSC, cancer stem cells; CSD, cromo shadow domain; CTE, C-terminal extension; DNMT, DNA-methyltransferase; EMT, epithelial-to-mesenchymal transition; HDMT, histone demethylase; HMT, histone methyltransferase; HP1, heterochromatin protein 1; NTE, N-terminal extension; PEV, position effect variegation; SOMU, sumoylation; TGS, transcriptional gene silencing; TSS, transcriptional start site.

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Introduction

Modern medicine has increased breast cancer patient survival.[1] This success provides encouragement to newly diagnosed individuals. However, currently available therapeutic regimens are often poorly tolerated or involve radical surgery.[2,3] Because metastases are responsible for the vast majority of deaths attributed to breast cancer, it is essential to design novel strategies, capable of interfering with metastasis while causing fewer adverse effects. To prevent metastatic invasion by tumors into distal sites an improved understanding is needed of how individual proteins alter the metastatic processes in breast cancer. Heterochromatin Protein 1α (HP1α) has been referred to as a breast cancer metastasis suppressor, but the molecular mechanism for this suppression remains still relative elusive. To provide insights into the current knowledge we are hereby reviewing the role of HP1α in the development of primary breast tumors and the metastasis of these tumors to distal sites.

Epigenetics

In the nucleus, DNA coils around a histone octamer consisting of 8 histone molecules (2 copies each of: H2A, H2B, H3 and H4). The protein complex is wrapped with approximately 147 bp of DNA to form a nucleosome and these are connected through linker DNA and stabilized by the binding of the linker histone H1.[4,5] In this conformation DNA condenses into chromatin. There are 2 primary forms of chromatin, euchromatin and heterochromatin, and these forms are commonly characterized to be enriched with either active or silenced genes, respectively.[6] A wide array of post-translational, so called epigenetic, modifications controls the arrangement of chromatin. Such epigenetic modifications can mediate meiotically and mitotically heritable changes in gene expression and cellular phenotypes that are not controlled by the underlying DNA sequence itself.[7]

Central aspects of epigenetics include histone modifications, DNA-methylation and microRNAs.[7,8] These different mechanisms are closely interconnected and serve to regulate gene expression. The type of epigenetic modification most directly relevant to this review is histone

methylation that can generate a unique epigenetic mark specifically read by HP1 proteins. Methylation of histones can represent a mark for either gene silencing or activation and i.e. di- and tri-methylation of lysine 9 of histone H3 (H3K9me2/3) are considered hallmarks of transcriptional silent chromatin and hence preferentially found in heterochromatin.[9] In contrast, methylation of lysine 4 of the histone H3 (H3K4me) denotes transcriptional activity and this modification is predominantly localized to the promoter region of active genes in euchromatin.[9] Additions or removals of histone methyl groups are carried out by enzymes termed histone methyltransferases (HMTs) and histone demethylases (HDMTs), respectively.[10,11] Aberrant activity of HMTs can lead to epigenetic silencing of critical genes for cancer progression, such as tumor suppressor genes, and is frequently observed in breast cancer.[12,13]

The HP1 Family – Form and Function

The heterochromatin protein 1 (HP1) family was originally identified in *Drosophila melanogaster* as a component of chromatin enriched at pericentric heterochromatin and implicated in the process of chromatin packing and repression of gene expression.[14] In mammalian cells, the HP1 family is composed of 3 distinct but highly conserved non-histone protein homologs: HP1α, HP1β, and HP1γ (**Fig. 1**), encoded from the *CBX5*, *CBX1* and *CBX3* genes, respectively.[15-18] The HP1 proteins are *bone fide* transcriptional repressors, and while it still remains relative unclear how gene expression of the individual members of the HP1 family of proteins is regulated, several key observations related to the diverse biological functions of these proteins have been reported. I.e. although all 3 HP1 proteins interact specifically with di- and tri-methylated lysine 9 on the histone H3, each HP1 protein has a different chromatin distribution. Specifically, HP1α is present mainly in heterochromatic regions, HP1β is found in both hetero- and euchromatic regions and HP1γ is primarily located in euchromatic regions.[18-20] It is also described that mitotic defects occur when HP1 proteins are insufficiently expressed or improperly located within the nucleus.[21-23] Important to this review, the expression level of HP1α in breast cancer cells correlates with both clinical data and clinical outcomes in this disease.[24]

The three HP1 proteins consist of approximately 190 amino acids with a size around 22 k_D and contains an N-terminal domain termed the chromo domain (CD) and a carboxy terminal domain termed the chromo shadow domain (CSD), separated by a flexible hinge domain (**Fig. 1**).[25,26] For all 3 HP1 proteins the tethering to chromatin by the CD, CSD or heterologous DNA-binding domains mediates transcriptional repression *in cis*.[19,27] Both the CD and CSD are highly conserved among eukaryotes with a 50–70% identity of the mammalian and *Drosophila* orthologous HP1 proteins, whereas the hinge region is less conserved with 25–30% identity.[28] The CD, in part, associates HP1 to chromatin through specific interactions with di- and tri-methylated lysine 9 on the H3 histone tail (H3K9me2/3), where the affinity for CD binding increases proportionally with the degree of methylation.[19,29,30] Structural studies have shown the formation of a pocket structure of the CD fitting with H3K9 di- and tri-methylation (**Fig. 1**).[31,32] The CD also interacts with the tail of linker histone H1.4 methylated on lysine 26 that can further participate in chromatin compaction.[33] The CSD has an amino acid sequence and structure similar to that of the CD. However, the CSD functions mainly as a dimerization domain, forming homo- and heterodimers with i.e., HP1 proteins themselves (**Fig. 1**).[19,34,35] These dimers form a Y shaped interaction platform for proteins through the pentapeptide motif PxVxL (x = any amino acid) (**Fig. 1**).[34-38] Many different types of proteins contain PxVxL motifs, and several have been shown to interact with HP1 proteins through the CSD: specific examples include TIF1α and TIF1β,[17,18,39] the lamin B receptor,[40] the nuclear body component SP100,[41] the SUMO-specific protease SENP7,[42] and the chromatin assembly factor 1 subunit p150 (CAF-1p150).[43] However, there are proteins that associate with the CSD of HP1 through alternative sequence motifs, such as BRM-related gene 1 (BRG1) and pogo transposable element-derived protein with zinc finger domain (POGZ).[27,44] Interaction without requirement of the PxVxL motif is also observed for suppressor of variegation 3–9 homolog 1 (SUV39h1),[45] one of the best described interaction partners of HP1 proteins. The CSD also interacts with the first helix of the histone fold of H3, a region involved in chromatin remodeling.[37,46,47] This H3 region is abbreviated Shadock for "chromoShadow docking" and contains a variant of the PxVxL motif, PGTVAL, required for HP1 binding and besides also capable forming interaction with BRG1.[46] Efficient SWI/SNF remodeling requires this H3 contact and is inhibited in the presence of HP1 proteins. SWI/SNF ATPase activity facilitates the HP1 binding for functional detection and arrest of chromatin remodeling.[48] The H3 histone fold binding of HP1 proteins is disrupted by phosphorylation at H3Y41 by JAK2 kinase and by phosphorylation at H3T45 and H3S57 by DYRK1A kinase where the latter 2 H3 modifications also showed to have influence on the competition of HP1 proteins and BRG1 for binding to the histone fold. These complex networks of H3 histone fold modifications and interactions can thereby affect HP1 chromatin association and HP1 mediated transcriptional repression independent of H3K9 methylation.[48]

When bound to di- or tri-methylated H3K9 through the HP1 CD a subsequent recruitment of SUV39h1 causes adjacent H3K9 residues to become methylated (**Fig. 2**). This creates new binding sites for additional HP1 proteins that, in turn, will further recruit SUV39h1 proteins (**Fig. 2**, panel 4). This mechanism can explain how HP1 modulates the spread of heterochromatin into neighboring euchromatin,[29,30] a phenomenon known as position effect variegation (PEV).[49-51] PEV is shown to be suppressed when HP1 is deleted and enhanced when HP1 is duplicated.[50,51] Moreover, HP1 proteins have been shown to directly bind DNA-methyltransferases (DNMTs) via the CSD and in complex with SUV39H1.[52] These observations serve to highlight the tight interconnection between different types of

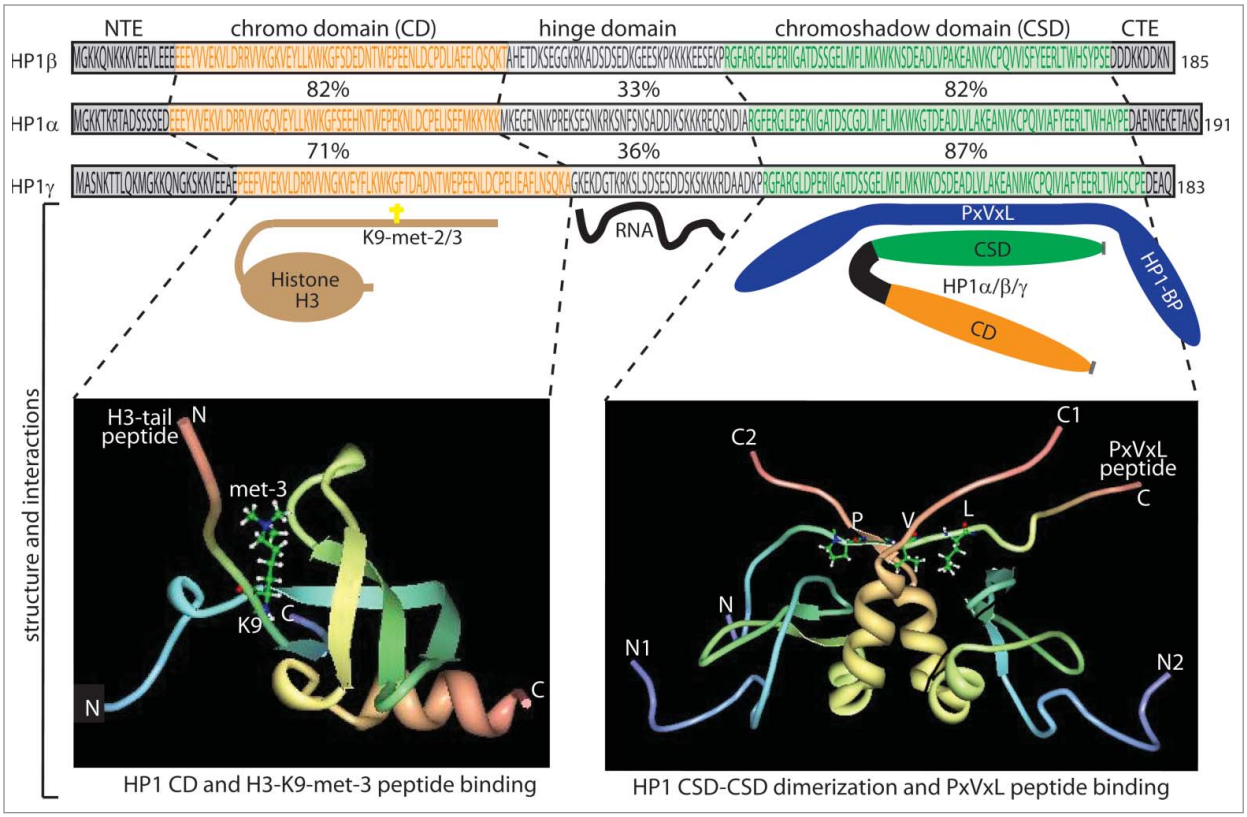

Figure 1. Schematic diagram of the HP1 domain structure. The protein domain structure is projected on the amino acid sequences of HP1α, HP1β and HP1γ with the chromo domain (CD) in orange and the chromo shadow domain (CSD) in green connected by the hinge domain. Also included are the N-terminal extension (NTE) and C-terminal extension (CTE). Percentage identity relative to HP1α is indicated between the sequences. [149] Below the diagrams are illustrated examples of key interaction partners. The CD mediates the binding of histone H3K9 di- and tri-methylated (H3K9me2/3)), the hinge mediates RNA interactions, while the CSD mediates HP1-HP1 dimerization that generates a structural platform for interaction with HP1 binding proteins (HP1-BP) including the pentapeptide motif PxVxL (x = any amino acid). Structural analyses of CD and CSD mediated protein interactions are shown in the bottom panels. The bottom panel to the left shows the 3-dimensional structure of the HP1 CD domain binding to histone H3K9 tri-methylated (Protein Data Bank code 2RSN). N- and C-terminal residues of the HP1 CD are shown together with the N- and C-terminal residues of the CD interacting H3 peptide with K9 tri-methylation. The Bottom panel to the right shows the 3-dimensional structure of the HP1 CSD domain binding to the PxVxL motif in CAF1 (Protein Data Bank code 1S4Z). N- and C-terminal residues of the 2 dimerizing HP1 CSDs are shown with numbering index 1 and 2 to distinguish the HP1 subunits. Also the N- and C-terminal residues of the CSD dimer interacting CAF1 peptide are shown with the position of the PxVxL motif indicated.

epigenetic mechanisms in fine tuning of gene regulation.

The hinge region of HP1 also contributes to the HP1 association with chromatin through interactions with histone H1 and RNA, where the RNA component is thought to be important in the maintenance and localization of HP1 proteins along the chromosome.[19,53-55] Recent studies have shown that HP1 proteins can be guided to the appropriate locations through complex formation with RNA and nuclear RNA-induced silencing complex (RISC) proteins (e.g. Argonaute).[56,57] These results suggest that HP1 together with proteins of the RNAi machinery locate in the nucleus as in transcriptional gene silencing (TGS). The mechanism of TGS is well established in *Schizosaccharomyces pombe*,[58,59] with emerging evidence of the reality of this mechanism in mammalian cells.[60,61] Interestingly, studies have shown that this mechanism is not restricted to the transcriptional activity of genes at their promoters but also at variable used exons, contributing to alternative splicing by exon inclusion or exclusion.[56,57,62] Indeed, recent studies have suggested that epigenetic mechanisms not only serve to regulate gene expression, but also influence the splicing of primary RNA transcripts.[63,64] This connection between epigenetics and alternative splicing was originally proposed 2 decades ago from the observation that the average exon is 140–150 bp long, a length strikingly similar to the 147 bp of DNA forming part of the nucleosomes.[65] Current estimates based on deep sequencing methodologies indicate that more than 90% of human genes undergo alternative splicing, which is implicated in numerous diseases including cancer.[66,67] Evidence of different mechanisms in the regulation of alternative splicing are now emerging, where histone modifications and their interaction with the non-histone proteins, such as HP1, can modulate splicing either through direct interaction with the splicing machinery,[68,69] or through regulating

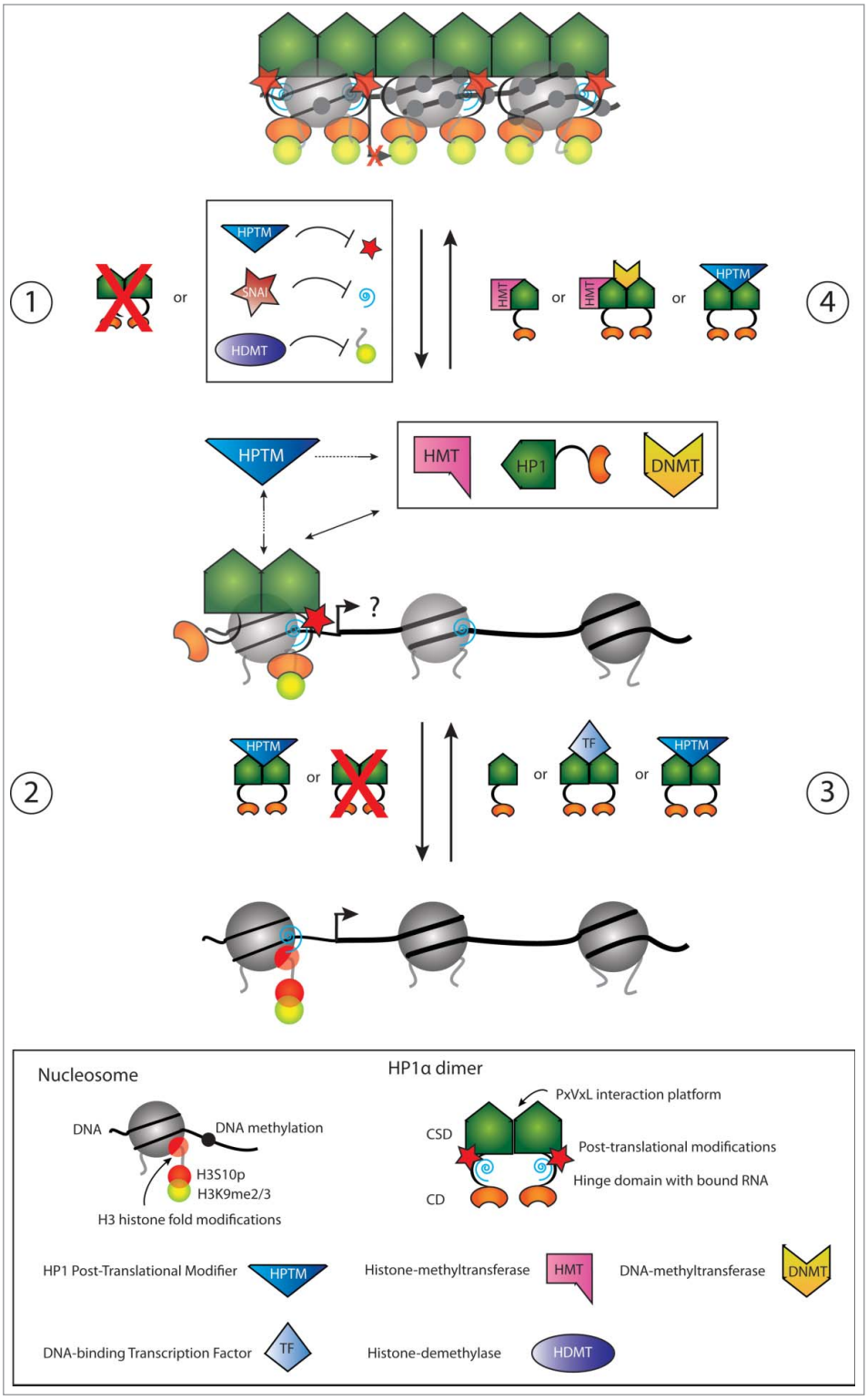

Figure 2. Schematic illustration of dynamics in HP1α chromatin release (panels 1 and 2) and recruitment (panels 3 and 4) for transcriptional regulation of pro-invasive genes in cancer progression and EMT. Panels 1 and 2. In non-metastatic cancer cells, pro-invasive genes are transcriptionally silenced through chromatin condensation mediated by HP1α. During initial stages of EMT, reduced HP1α chromatin binding can be mediated by down-regulation of HP1α expression, by Snail1 mediated repression of pericentric RNA transcripts, by alterations in the H3 modification code thereby inhibiting HP1α binding, or by alterations in the HP1α post-translational modification status (e.g., SUMO) (panel 1). The chromatin presence of HP1α can be further reduced by additional downregulation of HP1α expression, by alterations in the HP1α post-translational modification status, or by alterations in the H3 modification code inhibiting HP1α binding (panel 2). The result can be transcriptional activation of pro-invasive genes to different degrees enabeling the cell to metastasize. Panels 3 and 4. Conversely, transription of pro-invasive genes in metastasizing cells can be silenced by up-regulation in HP1α expression, by alterations in the H3 modification code allowing HP1α recruitment, by HP1α recruitment via interactions with DNA sequence specific transcription factors, by HP1α recruitment via interactions with RNA, or by alterations in the HP1α post-translational modification status (e.g. SUMO) (panel 3). Once chromatin bound, HP1α can further recruit chromatin modulating factors. Recruitment of HMTs causes H3 methylation in adjacent nucleosomes allowing the spread of HP1α, and the recruitment of DNMTs causes methylation of the underlying DNA (panel 4). Dynamics in HP1α post-translational modifications (e.g., SUMO) can participate in regulating maintenance of chromatin binding. This is altogether resulting in transcriptional silencing of pro-invasive genes and an epithelial-like phenotype.

the elongation rate by stalling of the transcribing polymerase.[56,70] These observations also suggest a role for epigenetics in other RNA processing events, such as RNA cleavage and polyadenylation in the 3'-end processing of nascent mRNA, such that histone modifications could have post transcriptional effects.[71]

Like the individual histones, the HP1 proteins have all been shown to be subjected to a variety of post-translational modifications with impact on their function and localization to chromatin.[20,55,72,73] These modifications include phosphorylation, acetylation, methylation ubiquitinylation, sumoylation and formylation, which suggest the existence of an HP1-enbodied "silencing subcode" that underlines the instructions of the histone code.[72,74,75] Although the specific effects of these different modifications are largely unknown, with notably exceptions that will be

described below, they possibly mediate a predetermination of the vast selection of binding partners available to each HP1 protein (**Fig. 2**). In relation to this, affinity purification and mass spectrometry methodologies identified in a comparative analysis the number of binding partners for HP1β and HP1γ to be 30–40 for each and around 10 for HP1α, with only partial overlap.[76] Note that Nozawa, et al. presented a larger set of HP1α interacting proteins.[44] The possibility of individual HP1 protein-protein interaction predeterminations regulated by post-translational modifications are in accordance with the observation that the individual HP1 proteins mainly associate with a single binding partner or in small protein complexes of limited entities, rather than in large complexes including all or most of the identified binding partners.[76]

How Epigenetics Control Exerted by HP1α can Influence the Onset and Pathogenesis of Breast Cancer

In the field of carcinogenesis, HP1α is of importance and has been implicated in cancers originating from a remarkable diversity of tissues including lung, colon and breast.[24,77,78] In contrast to HP1β and HP1γ, HP1α is described to be differentially expressed between cancerous and non-cancerous cells.[24] The majority of research in this regard has been conducted in relation to breast cancer. Despite these efforts, the exact roles of HP1α in breast cancer development and metastasis remain elusive and somewhat contradictory. Here, the current HP1α literature is summarized and key incongruences regarding the roles of HP1α in breast cancer are outlined together with testable hypotheses that may resolve them.

Primary tumor cells of breast carcinomas exhibit higher expression of HP1α encoding mRNA and protein compared to normal breast tissue.[24] Diverse potential cause and effect relationships between HP1α expression, putative HP1α-mediated regulation of mitosis and possible HP1α-mediated tumorigenesis are described. A role for HP1α in mitosis was postulated based on HP1α interactions with cell cycle dependent proteins[18,43,79] and the demonstration that both mRNA from the *CBX5*-gene and HP1α protein levels diminish during transient cell cycle exit.[24] Moreover, during mitosis HP1 proteins dissociate from chromosomes because of incapability to bind methylated H3K9 if the neighboring residue serine 10 is phosphorylated by Aurora B kinase (H3S10p).[44,75] POGZ is required for normal mitotic progression and for correct activation and dissociation of Aurora B kinase from chromosomes during M phase. POGZ binds HP1α uniquely of the HP1 proteins and this CSD interaction destabilizes the HP1α chromatin interaction.[44] Nielsen, et al. showed that HP1α interacts with TIF1β[18] and Wang, et al. found that the complex mediates the ubiquitination and degradation of the tumor suppressor p53.[80] Moreover, HP1α interacts with CAF-1p60 and CAF-1p150,[43,81] validated markers of cellular proliferation.[79] These CAF-1 proteins play a key role in *de novo* DNA synthesis and are required for S-phase progression in complex with HP1α.[82] In the context of these observations, however, it is difficult to reconcile how HP1α can also mediate S-phase arrest through suppression of cyclin E following direct interaction with retinoblastoma protein (Rb) or via binding of SUV39h1.[83] A direct link between acquired HP1α post-translational modifications and the HP1α mitotic functions was recently described.[84,85] Whereas HP1α is constitutively phosphorylated at the N-terminal region, the hinge domain is preferentially phosphorylated at G2/M phase of the cell cycle. This hinge domain phosphorylated form of HP1α is specifically localized to kinetochores during early mitosis.[84,85] HP1α hinge domain phosphorylation is mediated by NDR1 kinase and is required for mitotic progression and SGO1 binding to mitotic centromeres.[84,85] Cells lacking NDR kinase exhibit a loss of mitosis specific HP1α phosphorylation followed by prometaphase arrest. Altogether this points to the interconnection between HP1α post-translational modifications and accurate chromosome alignment during mitotic progression.[84,85] In this line the functional interaction between HP1 and BRCA1 also might be interesting.[86] BRCA1 is frequently mutated in inherited breast cancer. BRCA1 maintains integrity of the genome by promoting homologous recombination DNA repair. Following DNA damage, BRCA1 plays an essential role in cell cycle arrest at the G2/M boundary. HP1 proteins are required for these BRCA1 mediated functions.[86] This suggests that compromising HP1α expression could promote tumorigenesis by impairing the functions of the BRCA1 tumor suppressor.[86] BRCA1 functions are in breast cancer often inactivated by other mechanisms than mutations, called BRCAness.[87] If such BRCAness can be directly related to alterations in HP1α expression remains an important issue for future research. However, with respect to the higher observed expression of HP1α in primary tumor cells compared to normal tissue, another pair of findings failed to show a dominant role for HP1α in proliferation. Specifically, De Koning, et al. and Norwood, et al. observed no changes in cancer cell proliferation following RNAi knockdown of HP1α.[24,88] In light of their observations, De Koning, et al. proposed a novel hypothesis: increased proliferation of tumorigenic cells is accompanied by HP1α expression primarily to ensure faithful mitosis and correct chromosome segregation that could provide a selective advantage to cancer cells given their less efficient mitotic checkpoints.[89] Hopefully, future studies will reveal the roles of HP1α in cancer cells in order to explain the differential expression between primary tumor and normal tissue.

The relationships between HP1α and the invasive potential of cancer cells have been carefully addressed in recent years. HP1α was linked to a higher invasive potential of cancer cells when it was found to be down-regulated in metastatic cells of colon cancer and thyroid carcinomas relative to non-metastatic cells.[78,90] In breast cancer, HP1α has also been shown to be downregulated at the mRNA and protein level in highly invasive breast cancer cell lines (e.g., HS578T and MDA-MB-231) compared to poorly invasive breast cancer cell lines (e.g. T47D and MCF7) while HP1β and HP1γ were equally expressed.[91-93] Immunohistochemistry observations from *in vivo* samples showed that HP1α expression was reduced in cells

from metastases relative to the primary tumor in the breast corroborating these findings.[92] Alterations in the invasive potential of well characterized breast cancer cell lines were used to confirm these findings via direct functional analyses in the absence of any alterations in proliferation rate.[66,70] Poorly invasive MCF7 cells have an approximate 40% increase in their invasive potential following RNAi-mediated knockdown of HP1α expression and highly invasive MDA-MB-231 cells lost up to 50% of their invasive potential when following either transfection or transduction with a HP1α-expression vector.[88,92] Based on these data, HP1α has now been characterized as a metastasis suppressor,[88,92] which in contrast to tumor suppressors are defined as being able to suppress metastasis without affecting the growth of the tumor.[94] On the surface it seems contradictory that up-regulation of HP1α correlates with increased cell proliferation of the primary tumor and poorer clinical prognosis while down-regulation of HP1α contributes to a tumor cell's invasive potential during carcinogenesis. Closer inspection suggests that these observations constitute primary examples of the global inverse correlation that exists between cancer cell proliferation and invasion.[24,95] Within this paradigm, acquisition of an invasive phenotype is frequently incompatible with high proliferation rates. Thus, a temporal slowdown in the proliferation of tumor cells that is accompanied by a lower expression of HP1α might be the combination of conditions necessary to promote the expression of pro-invasive genes and hence metastasis (**Fig. 2**).

The observed correlation between HP1α expression and invasive potential has inspired the hypothesis that HP1α may directly be involved in the silencing of genes that potentiate cancer cell invasive potential and metastasis i.e. genes involved in the developmental program of epithelial-to-mesenchymal transition (EMT) (**Fig. 2**).[92] Furthermore, the inverse correlation between proliferation and invasive potential suggests a role for HP1α in the formation and function of cancer stem cells (CSC). CSC are a subpopulation of cancer cells that fuel tumor growth because, like normal adult stem cells i.e., haematopoietic stem cells, CSC are endowed with self-renewal and multi-lineage differentiation capacities.[96] Particularly relevant here is the knowledge that differentiated blood lymphocytes have lower expression of all 3 HP1 proteins compared to their less differentiated progenitor cells[97,98] and that HP1 proteins are important for maintaining the transcriptional integrity of haematopoietic stem cells through interactions with TIF1β.[99] Given the high degree of similarity between CSC and normal adult stem cells, a corollary to these data is that HP1α may play a critical role in maintaining the "stemness" of CSC. Originally identified in haematopoietic malignancies,[100] CSC has now been identified in a broad spectrum of solid tumors where they produce important mediators of tumor growth, promote local tumor invasion and facilitate metastasis formation.[101-103] Moreover, increasing evidence suggest that the CSC compartment itself is a heterogeneous mix of distinct CSC subpopulations (e.g., migrating vs. non-migrating CSC),[104-106] much like normal adult circulating stem cells vs. normal adult tissue-residing stem cells.[107,108] Thus, the different expression levels of HP1α between primary tumor and metastases could be reflecting, at least in part, the presence of distinct CSC subpopulations at the sites of analyses. Fully understanding the contributions of different CSC subpopulations within any given HP1α analyses is critical to accurately interpreting molecular insights regarding epigenetic contributions of HP1α in the transcriptional regulation of tumorigenesis, proliferation and invasive potential.

Recent evidences implicate HP1α in the EMT process (reviewed in ref. [109]) occurring at the initial stages of metastasis. The Snail1 transcription factor has an essential role in triggering EMT both during embryogenesis and in cancer.[110] Snail1 can repress pericentromeric transcription through the H3K4 deaminase LOXL2.[111] Millanes-Romero, et al. demonstrated that HP1α association to major satellite repeat sequences located in pericentric heterochromatin decreased during the initial steps of TGFβ-induced EMT.[111] This effect was shown to be because of a Snail1-dependent transient release of HP1α proteins from pericentric heterochromatin, rather than an effect due to transcriptional downregulation of HP1α encoding mRNA from the *CBX5*-gene. Thus, a HP1α release from chromatin is probably necessary to permit the heterochromatin reorganization occurring during EMT.[111] Since HP1α association to pericentric heterochromatin requires RNA components derived from these sites the results are consistent with an underlying Snail1 mediated down-regulation of such heterochromatic derived transcripts (**Fig. 2**, panel 1).[111] Other means such as histone modifications and HP1α post-translational modifications could participate in HP1α dynamics in heterochromatin association in EMT (**Fig. 2**). In this line it is important to note that Maison, et al. described that HP1α sumoylation in the hinge domain promoted de novo HP1α targeting to pericentric heterochromatin through interactions with pericentric heterochromatin derived RNA and accordingly could participate in seeding further HP1α localization.[55] A recent report has specifically addressed the important link between HP1α sumoylation, heterochromatin remodeling and EMT.[42] The SENP7 SUMO-specific protease is involved in breast cancer progression and interacts with the HP1α CSD through a PxVxL motif.[42,112] Sumoylated HP1α is enriched at, and silences, E2F-responsive and mesenchymal gene promoters (i.e. the EMT mesenchymal-marker vimentin) in poorly invasive epithelial cells.[42] Elevated SENP7 levels mediate an HP1α hypo-sumoylation, which abolish the silencing of these E2F-responsive and mesenchymal gene promoters, and concordantly is involved in acquisition of the EMT-like phenotype.[42] A putative not yet solved underlying complexity for the SENP7 and HP1α association to mediate gene regulated is illustrated by Maison, et al. finding that SENP7 mediated de-conjugation of HP1α sumoylation can be involved in retention of the initial targeted sumoylated HP1α to pericentric heterochromatin (**Fig. 2**).[112] However, the facts that both HP1α expression level and the distribution of HP1α through post-translational modifications are of importance in breast cancer related EMT suggests caution using absolute HP1α expression levels, mRNA and protein, as a prognostic value in future breast cancer diagnostic procedures. Instead development of

measurements of the functional HP1α amounts in relation to metastasis suppression will be more informative.

Whereas many studies have focused on HP1α pericentric heterochromatin associations, HP1α also clearly has importance for regulation of euchromatic localized genes exemplified by the involvement in regulation of E2F-responsive genes. Chromatin immunoprecipitation (ChIP) sequence data produced by LeRoy, et al.[113] revealed that only 2% of the total cellular HP1α molecules are actually associated with gene promoters, 25% are associated within the gene bodies, while the remaining HP1α is located in intergenic regions . For such reason, the conclusion that HP1α is a facilitator of a cellular pro-invasive gene transcription program may describe only a relatively small fraction of the HP1α functions in epigenetic regulation. Because a major fraction of the gene-associated HP1α is present within gene bodies and not at the promoter, it is also plausible that a primary, but not yet well described function for HP1α in gene regulation is the potential role in regulation of RNA processing i.e., alternative splicing as discussed above. Indeed both tumor progression and EMT are highly influenced by the alternative splicing of a vast number of mRNAs.[114-116] It is very intriguing that HP1α can be involved in a migratory EMT-like pathway mediated by alternative splicing besides the more conventional transcriptional repression function either in parallel or in a functional collaboration with the well-defined direct effects of Slug, Snail, Twist, ZEB1 and ZEB2 for transcriptional regulation under EMT.[117] The functional consequences of alternative splicing for EMT are well illustrated by the drastic isoform changes of CD44, which have been repeatedly linked to metastasis formation.[118,119] Brown, et al. recently showed that an ESRP1-mediated shift from CD44 expression from variant isoforms (CD44v) to the standard isoforms was necessary for cells to undergo complete EMT.[120] Interestingly, the overall level of total CD44 protein did not change significantly during this process. These observations impose another layer of complexity and emphasize that EMT is a broad concept with multiple cross-talking signaling pathways occurring in parallel. Whether HP1α plays a role in any of these classical EMT pathways or has EMT-independent mechanisms of generating an invasive phenotype of cancer cells, is urgently needed be addressed in more comprehensive studies investigating i.e. the genomic distribution of HP1α and the gene regulatory repertoire in human cells to identify cancer relevant genes regulated by HP1α.

Genetic Regulation of *CBX5* Transcription

Because of the inverse correlation between HP1α expression and the invasive potential of breast cancer cells, understanding the differential regulation of the HP1α encoding gene, *CBX5*, has been of great interest. Initial studies of the differential regulation of *CBX5* found no change in the DNA sequence or methylation status between the poorly invasive MCF7 and highly invasive mesenchymal-like MDA-MB-231 breast cancer cell lines.[121] Thus, studies concerning the differential regulation of *CBX5* have mainly focused on *cis*- and *trans*-acting elements of the promoter region, from which numerous transcription factor binding sites have been identified (e.g. YY1, E2F, and E-box elements).[93,121,122] The significance of these *cis*- and *trans*-acting elements were investigated by transient reporter assays, conducted using plasmid constructs containing different insert fragments of the promoter region with various deletions of the transcription factor binding sites in well-defined breast cancer cell lines.[121,122] Deletion of one specific MYC element resulted in an upregulation of *CBX5* mRNA expression in the highly invasive MDA-MB-231 cells.[121] In contrast, deletion of the YY1 binding sites resulted in a downregulation of *CBX5* in the poorly invasive MCF7 cells.[122] This result was further validated by RNAi mediated knockdown of YY1 in MCF7 cells that resulted in a down-regulation of *CBX5* mRNA. Furthermore, forced up-regulation of YY1 by transfection decreased the invasive potential of highly invasive HS578T cells.[122] This latter effect, however, was independent of HP1α expression, suggesting that YY1 might contribute to the regulation of *CBX5* but cannot fully account for the different levels of HP1α expression and the correlation with invasive potential. ChIP experiments have demonstrated the presence of E2F proteins at the *CBX5* promoter[123,124] but RNAi-mediated knockdown of different E2F transcription factors in both MCF7 and MDA-MB-231 cells only resulted in minor changes of *CBX5* mRNA expression.[93]

Located immediately upstream (589 bp) of the *CBX5* transcriptional start site (TSS) is the divergently transcribed *hnRNPA1*-gene. Such "head-to-head" gene arrangements are found at a surprisingly high frequency throughout the genome, with as much more than 10% of the protein-coding genes being located on opposite strands with TSSs less than 1 kb away from each other.[125-127] This bi-directional arrangement is a conserved feature among many species suggesting an ancient ancestral origin of functional importance.[128-130] Interestingly, this distinct subgroup of bi-directional promoters share several features besides the head-to-head configuration separating them from other promoters.[131] Bi-directional promoters are shown to have a higher frequency of CpG islands than other promoters, and consequently have a higher GC content.[128-130] Furthermore, the relative presence of canonical TATA box elements is significantly less for bi-directional promoters.[126,132] Finally, bi-directional promoters display an enriched occurrence of specific transcription factor binding sites, including MYC, E2F, NRF and YY1.[133] Given that the *CBX5* promoter lacks TATA box elements and contains a CpG island[121] in addition to the close proximity of *hnRNPA1* promoter and presence of the specific enriched transcription factor binding sites, it is evident that *CBX5* is a signature representative of bi-directional promoter containing genes. Whereas *CBX5* is down-regulated in highly invasive breast cancer cell lines, compared to poorly invasive breast cancer cell lines, the *hnRNPA1*-gene is evenly expressed in both types of cell lines.[92,121,122] Therefore, despite the close proximity between their transcriptional start sites, the 2 genes have been thought to be

independently regulated.[92,121,122] However, bi-directional promoters are shown to possess several features of regulatory dependence within their shared promoter region. This dependence is illustrated by the observations that promoter activity can be altered by deletion of the opposing TSS.[126] Similarly, loss of specific promoter elements of bi-directional promoters was shown to affect the transcriptional activity in both directions, suggesting that most bi-directional promoters share at least some regulatory elements.[126,134] Therefore, an element affecting the transcriptional activity of *CBX5* would also have potential to affect the activity of *hnRNPA1*. RNAi mediated knockdown of YY1 had impact on *CBX5* expression whereas *hnRNPA1* expression was unaffected.[122] But the majority of examined *cis*-elements in the promoter of *CBX5* have not been thoroughly investigated for their potential effects on the transcriptional regulation of *hnRNPA1*. A shared effect for *CBX5* and *hnRNPA1* transcription would indicate that the 2 promoters are not independently transcribed, which suggests that the reason for the differential expression of *CBX5* in breast cancer cells is not solely controlled at the promoter level, but likely involves downstream regulatory elements or a regulatory mechanism not yet identified in this context. One possibility for the cell to disconnect transcription of *CBX5* and *hnRNPA1* is the use of a downstream *CBX5* alternative promoter. Inspection of the ENCODE regulatory datasets for the *CBX5* and *hnRNPA1* bi-directional promoter in the UCSC Genome Browser, as well as published ChIP data, shows presence of poised RNA polymerase II downstream both the *CBX5* and *hnRNPA1* transcriptional start sites.[135] For *CBX5* 2 RNA polymerase II peaks are present, one 50 bp downstream the bi-directional promoter and the other 400 bp further downstream.[135] The latter peak was proposed to be a result of presence of an alternative transcriptional start site. Toward this point, Thliveris, et al. recently identified an alternative promoter located several kb downstream in *CBX5* in mice.[136] Transcription from this downstream promoter results in 2 additional HP1α encoding transcripts both containing the entire full-length coding region, but with an alternative first exon not included in the nascent transcript. However, regulation of *CBX5* was concluded to be restricted to the nascent upstream promoter, since the alternative promoter transcripts only constituted a very minor contribution of protein coding mRNA. However, it should not be ruled out that dependence on this alternative promoter during *CBX5* transcription could be dictated by anatomical location, different stages of cell cycle or developmental stages of embryogenesis. In this line, Thliveris, et al. further emphasized the potential significance of the alternative promoter, based upon the observation of a high degree of mammalian sequence conservation of the region corresponding to the alternative promoter.[136] In addition to the region corresponding to the alternative promoter, 5 other highly conserved noncoding regions of unknown function were observed in the long first intron of the mouse *CBX5* gene. This observation suggests additional regulatory elements to be involved in *CBX5* regulation. A gene that has received much attention in breast cancer research and again worth mentioning in this context is *BRCA1* with a bidirectional promoter much like *CBX5*. The *BRCA1* promoter possesses the classical features of bidirectional promoters including; located head-to-head to the divergently transcribed *NBR2* gene with a shared promoter of less than 500 bp in length,[137] contains a CpG island,[138] and bound by NRF and E2F transcription factors.[139,140] Interestingly, recent studies investigating the regulation of the *BRCA1* promoter have identified gene loop structures with intron sequences.[141] Gene loops are transient structures formed by juxtaposition of the promoter and terminator region[142–144] that can contribute to transcriptional regulation by facilitating the re-cycling of the polymerase[144–146] and enhance transcriptional directionality.[147] If the bi-directional *hnRNPA1* and *CBX5* promoter structure also uses gene loop structures for fine tuning of transcriptional regulation will be an intriguing question for future analyses by chromosome conformation methodologies.[148]

Conclusion

When considered together, the now gained data for HP1α expression and function point toward pivotal roles for HP1α in breast cancer proliferation and metastasis. The role and function of the higher level of HP1α expression observed in primary breast cancer tumors compared to normal tissue remain largely unknown and speculative. Here we call attention to the possibility of HP1α governing stem cell properties of different CSC subpopulations. We propose that HP1α serves to maintain the transcriptional integrity of non-migratory CSC. Thus, a high expression of HP1α in primary tumors of breast cancers could illustrate a dense population of non-migratory CSC. In turn, an intermediate range of HP1α expression could permit the expression or alternative splicing of pro-invasive genes and acquirement of an EMT-like phenotype while maintaining "stemness" properties, thus giving rise to migrating CSC. The reviewed data further indicates that transcription of pro-invasive genes or alternative transcript isoforms not only are dependent on the absolute HP1α expression level, but also on a complex network of post-translational modifications of HP1α proteins and epigenetic histone modifications. Understanding these modifications as well as the differential regulation of HP1α could help resolve some of the ambiguities described above. Despite much effort, little progress has been made in understanding the observed differential regulation of *CBX5* expression during breast cancer progression. History dictates genes to be perceived as linear entities confined by promoters and terminators that determine where transcription starts and ends. Hence, studies concerning the regulation of *CBX5* have been restricted to the promoter region. However, recent evidence of functional and structural relationship between the promoter, intron, exon and terminator sequences, suggests that some genes function, at least partially, as closed circuits. Because of the bi-directionally promoter composition of the, in breast cancer cells, differentially expressed *CBX5* gene and the constitutively expressed *hnRNPA1* gene, the transcriptional activity of the 2 genes is likely to be similar

affected by *trans*-factors acting within the shared promoter region. Therefore, we propose novel mechanisms of transcriptional regulation of *CBX5* in addition to the promoter region mediated regulation.

The data and hypotheses presented in this review highlight the need for future studies focused on the role of deregulated *CBX5* expression and HP1α protein functions in the development and progression of breast cancer. Not only will such studies contribute to our general mechanistic understanding of epigenetic gene regulation, but ultimately also be of benefit for future diagnostics and prognostics of breast cancer and hopefully contribute to the prevention, or even reversal, of metastases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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