Position-effect variegation revisited: HUSHing up heterochromatin in human cells

Richard T. Timms[1*], Iva A. Tchasovnikarova[1*], Paul J. Lehner[1*]

[1]Department of Medicine, Cambridge Institute for Medical Research, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 0XY, UK

*Correspondence should be addressed to rtt20@cam.ac.uk, it257@cam.ac.uk and pjl30@cam.ac.uk

Abstract

Much of what we understand about heterochromatin formation in mammals has been extrapolated from forward genetic screens for modifiers of position-effect variegation (PEV) in the fruit fly *Drosophila melanogaster*. The recent identification of the HUSH (Human Silencing Hub) complex suggests that more recent evolutionary developments contribute to the mechanisms underlying PEV in human cells. Although HUSH-mediated repression also involves heterochromatin spreading through the reading and writing of the repressive H3K9me3 histone modification, clear orthologues of HUSH subunits are not found in *Drosophila* but are conserved in vertebrates. Here we compare the insights into the mechanisms of PEV derived from genetic screens in the fly, the mouse and in human cells, review what is currently known about the HUSH complex, and discuss the implications of HUSH-mediated silencing for viral latency. Future studies will provide mechanistic insight into HUSH complex function and reveal the relationship between HUSH and other epigenetic silencing complexes.

Introduction

As a result of the many genome sequencing projects undertaken over the past two decades, we now have a detailed understanding of the precise nature of the genetic code that holds the information required to build a biological machine as complex as the human body. However merely cataloguing the exact sequence of the millions of the DNA bases A, C, G and T does not equate to being able to understand how multicellular organisms function, as this genetic information must clearly be interpreted quite differently to enable the functions of all the different cell types.

The DNA in the nucleus of eukaryotic cells is found in complex with proteins called histones, which together form a structure known as chromatin. The basic functional unit of chromatin is the nucleosome, consisting of 147 base pairs of DNA wrapped around two copies of each of the histones H2A, H2B, H3 and H4. Histones serve a dual role. First, they provide an essential role in the compaction of DNA, enabling approximately two metres of DNA to fit inside the nucleus of a human cell which has a diameter of around just ten microns across. Second, the structure of the chromatin fibre regulates the accessibility of the DNA to the plethora of factors that regulate all aspects of DNA metabolism, including gene expression, DNA replication and the repair of DNA damage. Therefore the expression of genetic information does not depend solely on the DNA sequence itself, but also upon the composition and activity of a myriad of chromatin-associated proteins.

Histone proteins can be post-translationally modified, and the exact nature and position of these modifications has dramatic consequences for chromatin function [1]. These marks either exert their effects directly, altering histone-histone or histone-DNA interactions to produce a change in nucleosomal architecture [2], or indirectly by serving as a docking site for chromatin remodelling enzymes [3]. The various histone modifications are recognised by distinct protein machineries, and hence define distinct biological entities [4]. A variety of protein domains have now been characterised that act as epigenetic ‘readers’ by specifically recognising modified histones, such as the chromodomain that binds methylated lysine residues [5], and the bromodomain that binds acetylated lysine residues [6]. Chromatin binding proteins can possess multiple such domains, leading to the attractive idea of a ‘histone code’ in which a particular combination of histone marks could be interpreted to produce a unique biological outcome [7].

In all higher eukaryotic organisms, a large fraction of the genome is packaged into an inactive form known as heterochromatin. In contrast with active euchromatin, heterochromatin is typically characterised as being highly condensed, gene-poor and less transcriptionally active. These two chromatin states were originally distinguished on the basis of differential cytological staining [8], but it is now clear that they represent two distinct biochemical entities. Euchromatin is normally associated with high levels of histone acetylation, as well as marks such as methylated lysine 4 of histone H3 which are found across active chromatin [9]. In contrast, heterochromatin is associated with low levels of histone acetylation and high levels of tri-methylated lysine-9 of histone H3 (H3K9me3). These modifications are dynamically deposited and removed by histone-modifying enzymes. In the case of H3K9me3, SET domain-containing proteins such as SUV39H1 and SETDB1 function as epigenetic writers by depositing the methyl mark [10], while the Jumonji-domain containing family of demethylases act as epigenetic erasers by removing the methyl mark [11].

Our knowledge on the mechanisms that regulate heterochromatin through the repressive H3K9me3 histone modification has come from the study of so-called "chromosomal position effects" [12], which are the main focus of this review. Position effects refer to the differences in expression observed when an identical gene is positioned at different sites in the genome. The expression level of reporter genes varies widely depending on the specific site of integration, with – broadly speaking – integration into euchromatin resulting in strong expression and integration into heterochromatin resulting in transcriptional repression [13]. The identities of the genes responsible for heterochromatin-mediated position effects have been largely studied through forward genetic screens in lower organisms. In the following sections we summarise the insights derived from these screens and consider their relevance to heterochromatin formation in man. We then focus on an analogous screen that we recently carried out in human cells which identified a novel epigenetic repressor complex, HUSH, thereby suggesting a novel route to heterochromatin formation in mammalian cells.

Forward genetics screens in *Drosophila* identify modifiers of position-effect variegation

Our understanding of the mechanisms of heterochromatin formation in higher eukaryotes is predominantly derived from classic forward genetic screens in the fruit fly *Drosophila melanogaster* for modifiers of position-effect variegation. In *Drosophila* this has

been studied extensively using the *white* gene. The *white* gene encodes a transporter which is required for the correct deposition of the pigments that give the *Drosophila* eye its characteristic red colour. When expressed normally in a euchromatic environment, the *white* gene is expressed, resulting in red pigment deposition. However, when placed into a heterochromatic environment, epigenetic silencing of the *white* gene leads to loss of pigment deposition and a mutant white eye phenotype [14]. The silencing observed is variable and only occurs in a proportion of the cells of the eye, resulting in patches of red and white colour – the so-called 'variegated' phenotype (**Figure 1**).

A particularly well-studied example is the *Inversion(1)-white-mottled-4* (w^{m4}) allele, where the white gene is subject to silencing as a result of an inversion that places it into the vicinity of heterochromatin formed at the border of the nucleolus organiser [15]. This and other such indicator strains allowed the development of forward genetic screens to identify dominant mutations in the genes that were required for heterochromatin-induced epigenetic silencing. Flies can be screened for mutations that either suppress silencing of the *white* gene, resulting in a reversion to a red eye phenotype, or for mutations that enhance silencing of the *white* gene, resulting in a more complete white eye phenotype (**Figure 1**). Overall, around 140 such suppressors of variegation (termed *Su(var)*) and 230 enhancers of variegation (*E(var)*) have been identified to date, with the molecular identities of approximately 30 genes characterised [16].

The study of two *Su(var)* genes in particular has formed the foundation for much of our present understanding of heterochromatin. *Su(var)2-5* encodes the heterochromatin-associated protein HP1a (heterochromatin protein 1a) which contains an N-terminal chromodomain and a C-terminal chromoshadow domain [17]. *Su(var)3-9* encodes the SU(VAR)3-9 protein which contains an N-terminal chromodomain and a C-terminal SET domain [18]. The latter endows the protein with histone lysine methyltransferase (HKMT) activity, which is specifically targeted towards lysine 9 of histone H3 (H3K9) [19, 20]. Both HP1a and SU(VAR)3-9 localise to pericentromeric heterochromatin and associate with each other [20, 21], suggesting that they form a core heterochromatin complex. Molecular analysis of the functions of these proteins suggests a 'read-write' mechanism for the propagation of heterochromatin and PEV. The H3K9me2/3 mark deposited by SU(VAR)3-9 forms a docking site specifically recognised by the chromodomain of HP1a [22]. The N-terminal region of SU(VAR)3-9 binds the chromoshadow domain of HP1a, thereby stabilising the

interaction between HP1a and H3K9me2/3 and allowing the further deposition of H3K9me2/3 [23].

Although this may represent a dominant mechanism at pericentromeric heterochromatin, the role of HP1a and SU(VAR)3-9 in heterochromatin-mediated silencing elsewhere in the genome is less prominent. For example, HP1α mutations have no effect on transgenes integrated in telomeric heterochromatin [24], and repression of reporters integrated along the *Drosophila* fourth chromosome are unaffected by mutations in *Su(var)3-9* but instead are de-repressed by mutations in the gene encoding dSETDB1, another H3K9 lysine methyltransferase [25–27]. Distinct sets of proteins might therefore be required for heterochromatin-mediated silencing in different heterochromatin environments. Indeed modifiers of position-effect variegation can be split into distinct groups in terms of their ability to affect silencing at different heterochromatic domains [28, 29].

An analogous forward genetic screen identifies modifiers of position-effect variegation in the mouse

Although the underlying molecular mechanisms are not as well understood in mammalian genomes as they are in *Drosophila*, similar position-effects are at work. The best evidence for this comes from an elegant recent study from the van Steensel laboratory [13]. By mapping the integration site for thousands of reporters in mouse embryonic stem (ES) cells and then correlating genomic position with expression level as measured by RNA-seq, the authors revealed that the identical gene sequence can exhibit ~1000-fold difference in expression depending on the specific site of genomic integration. An important goal, therefore, is to understand the molecular mechanisms involved and to identify the genes responsible.

To this end, the laboratory of Emma Whitelaw have carried out a large-scale genetic screen analogous to the *Drosophila* PEV screens in the mouse, with the aim of clarifying the mechanism of epigenetic gene silencing in mammals [30]. This screen was performed using a mouse strain harbouring a multicopy GFP transgene integrated on chromosome 1 [31]. The GFP reporter is under the control of an α-globin promoter and enhancer, thereby directing GFP expression to erythrocytes. This reporter shows a variegated pattern of expression, with only ~55% of erythrocytes expressing GFP as measured by flow cytometry [30]. To identify

genes that affect the epigenetic silencing at this locus, a classical forward genetic screen was performed. Male mice homozygous for the GFP transgenes were subjected to *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis and G_1 offspring analysed for changes in the pattern of GFP expression by flow cytometry, looking for mutations that either decrease or increase the degree of epigenetic silencing (analogous to the *Su(var)* and *E(var)* mutations respectively in *Drosophila)* (**Figure 2**).

Of the 4000 G_1 offspring screened, 40 strains displayed changes in GFP expression as a result of heritable, dominant-acting mutations termed *Mommes* (modifiers of murine metastable epialleles) [32]. Most of the mutations that cause the variegated phenotype in these *Momme* strains have been identified and indeed the majority map to genes encoding known epigenetic modifiers. These include the DNA methyltransferases *Dnmt1* and *Dnmt3b* [33, 34], the histone deacetylase *Hdac1* [30, 31], the chromatin-remodelling factors *Smarca5* [30, 33], *Smarcc1*, *Pbrm1*, and *Baz1b* [31, 32], the transcriptional regulator *Trim28* [35], the transcription factor *Klf1* [36], and the histone methyltransferases *Suvar39h1* and *Setdb1* [32]. Importantly the screens have also identified some novel components of the epigenetic machinery, namely *Smchd1* [37], *Rlf* [38] and *D14Abb1e* [39] (the murine orthologue of *FAM208A* or *TASOR* in humans).

Although there is significant overlap between the genes identified as modifiers of PEV in *Drosophila* and the *Momme* mutations identified in the mouse, comparing these two datasets does suggest some differences between the mechanisms of heterochromatin formation in the fruit fly as compared with mammalian cells. Several components in particular that were identified in the mouse do not have orthologues in *Drosophila*. These include the DNA methyltransferases Dnmt1 and Dnmt3b [12] and Smchd1 (structural-maintenance-of-chromosomes hinge domain containing 1), a previously uncharacterised protein shown to have a role in the maintenance of X inactivation and the hypermethylation of CpG islands associated with the inactive X [37]. These data suggest that an extra layer of transgene silencing exists in mammalian cells that involves the deposition and maintenance of DNA methylation, which is sparse in *Drosophila* [40]. An alternative possibility is that the multicopy reporter installed at a single location on chromosome 1 in the mouse screens may not be fully representative of all heterochromatic loci across the mouse genome.

The near-haploid KBM7 cell line permits forward genetic screens in cultured human cells

The genes involved in position-effect variegation in man are poorly defined, and this is largely due to the difficulty in performing analogous forward genetic screens in cultured human cells. However a major recent advance in the field of experimental genetics has been the identification and derivation of a number of haploid or near-haploid mammalian cell lines [41–44]. In a normal diploid human cell disruption of one allele of a gene is likely to have little or no phenotypic consequence, owing to compensatory gene expression from the second wild-type allele. This is a major barrier for forward genetic screens, as bi-allelic mutation of genes – at least prior to the advent of CRISPR/Cas9-technology [45] – has been difficult to achieve through chemical or insertional mutagenesis. In contrast, gene inactivation on a haploid background results in a total loss of gene function, thereby enabling classic forward genetic analysis.

Initially it was the pioneering work of the Brummelkamp laboratory that showed that the near-haploid human KBM7 cell line could be used to carry out forward genetic screens in cultured human cells [42] (**Figure 3**). The KBM7 cell line was originally derived from a patient with chronic myeloid leukaemia [41], and possesses just a single copy of each chromosome except chromosome 8 and the sex chromosomes [46]. Insertional mutagenesis with a gene-trap retroviral vector can therefore be used to create a library of knockout cells, from which mutant cells displaying the phenotype of interest can be selected. Mapping the integration sites of the gene-trap vector in the selected cells – a process which is greatly facilitated by the power of next-generation sequencing [47] – then reveals the inactivated genes which are involved in the process under investigation. Since then a fully-haploid, fibroblast-like derivative of KBM7 called HAP1 has been generated [48, 49], and two groups have reported the derivation of haploid murine ES cells [43, 44] which have also been used successfully for forward genetic screens [50, 51].

The HUSH complex mediates position-effect variegation in human cells

We recently exploited the power of non-lethal haploid genetic screens in KBM7 cells [52, 53] to directly examine the genetic basis of position-effect variegation in human cells [54]. The experimental design was directly analogous to the screens in *Drosophila* and the

mouse described above. The starting point was a green fluorescent protein (GFP) reporter gene inserted into a heterochromatic environment that was subject to epigenetic silencing, and, following insertional mutagenesis, we selected for mutants in which this epigenetic repression was relieved. A major advantage of performing the screen in cultured cells as opposed to a whole organism was that fluorescence-activating cell sorting (FACS) could be used to isolate large numbers of cells expressing defined levels of expression of the GFP reporter. In practice, near-haploid KBM7 cells were transduced with a lentiviral vector which inserted a GFP reporter construct across the genome. All the cells which had become GFP^{dim} as a result of epigenetic silencing were collected by FACS, and, following insertional mutagenesis, mutants were isolated that had reverted to a GFP^{bright} phenotype (**Figure 4**). In this way the screen was designed to examine the dominant mechanism of position-effect variegation across the genome, rather than studying heterochromatin regulation at a single reporter integration site.

The haploid screen identified four genes as being essential for reporter silencing: *SETDB1*, a histone lysine methyltransferase, and three less-well characterised genes, *FAM208A* (for which we proposed the new name *TASOR*, for transgene activation suppressor), *MPHOSPH8* (encoding M-phase phosphoprotein 8 (MPP8), a chromodomain-containing protein) and *PPHLN1* (encoding Periphilin). Subsequent proteomic and biochemical analysis revealed that TASOR, MPP8 and Periphilin exist together in a complex which we named the Human Silencing Hub (HUSH) complex. There were two initial clues as to the likely function of HUSH: (1) MPP8 had previously been shown to associate with SETDB1 [55], a histone methyltransferase responsible for depositing the repressive histone mark H3K9me3 [56], and (2) the chromodomain of MPP8 had already been shown to bind H3K9me3 [55, 57, 58]. The possibility that HUSH could both bind (through the MPP8 chromodomain) and deposit (through the recruitment of SETDB1) the repressive H3K9me3 modification suggested that the role of the HUSH complex may be in the ‘spreading’ of H3K9me3 from existing heterochromatin across the newly-integrated reporter construct to mediate epigenetic repression.

The HUSH complex mediates heterochromatin spreading through H3K9me3

A key feature of the HUSH-mediated silencing of integrated transgenes is that not all transgenes are subject to this phenomenon. Although we found HUSH to be active on both

viral and non-viral transgenes driven by multiple different promoters, we observed that only a fraction of the integrated GFP constructs in each case exhibited HUSH-mediated repression [54]. This finding suggests that transgenes are not directly targeted by HUSH in a DNA sequence-specific manner, since such a mechanism would presumably result in the silencing of all transgene integrations. Rather it seems that the position of integration into the genome is critical in determining whether HUSH-mediated silencing takes place. By mapping the genomic integration sites of the transgenes that were subject to silencing, we observed a striking correlation between high levels of H3K9me3 in the vicinity of the integration site and HUSH-mediated repression [54].

What are the implications of this result for the mechanism of HUSH-mediated silencing? Seemingly the HUSH complex is normally bound at H3K9me3-rich regions of the genome, and, upon integration of an active transgene nearby, HUSH facilitates the spreading of H3K9me3 to form new heterochromatin across the transgene and mediate transcriptional silencing. Such a mechanism would explain how transgenes landing into euchromatic regions lacking H3K9me3 are immune from the effects of HUSH. Even after repression is initially established, however, there is an ongoing requirement for HUSH to maintain the state of epigenetic repression. This suggests that there must be competing mechanisms at work that function to reverse the heterochromatic state installed through HUSH activity. This could be a passive process of progressive dilution of H3K9me3-marked histones through cell division, or an active process involving a competing H3K9me3 demethylase. In this regard it would be interesting to explore whether loss of Jumonji domain demethylases (such as KDM4D which is known to erase the H3K9me3 mark [59]) would act as enhancers of transgene silencing in human cells. Interestingly in the fission yeast *Schizosaccharomyces pombe* two recent studies have demonstrated that active H3K9 demethylation by the putative Jumonji demethylase Epe1 [60] can counteract silencing resulting from recruitment of the single yeast H3K9 methyltransferase Clr4, and that this prevents the stable maintenance of a heterochromatic domain [61, 62]. The balance between HUSH and SETDB1-mediated deposition of H3K9me3 and active demethylation could therefore also be important for preventing the aberrant spread of heterochromatin in human cells.

How does HUSH-mediated transgene silencing compare with KAP1-mediated retroviral silencing?

Integrated retroviral DNA is potently silenced in murine ES cells [63], in a process mediated by the key co-repressor KAP1 (also known as TRIM28 or TIF1β) [64]. KAP1, through its RBCC domain [65], interacts with members of the KRAB (Krüppel-associated box) domain-containing zinc finger protein (KRAB-ZFP) family of transcription factors [66]. These KRAB-ZFP transcription factors mediate the specific recognition of the viral primer binding site (PBS) of individual retroviruses. In the case of murine leukaemia virus (MLV), for example, ZFP809 bridges the viral DNA to the KAP1 co-repressor to mediate retroviral silencing [67]. Although the downstream events that lead to epigenetic repression of the integrated provirus are not fully defined, there is a central role for the H3K9 methyltransferase SETDB1 [68, 69], which is a binding partner of KAP1 [56]. SETDB1 recruitment leads to the deposition of the repressive H3K9me3 histone modification across the provirus and silencing of viral gene expression. There may also be an important role for DNA methylation [70], and the histone variant H3.3 [71].

A central feature of this model is that a different KRAB-ZFP protein would be required to specifically bind each different class of retroviral element. This is supported by the rapid expansion of the KRAB-ZFP gene family to over 350 members in humans, suggesting an evolutionary 'arms race' in which new KRAB-ZFP evolve to combat fresh retroelements as they emerge [72]. The best experimental support comes from an elegant recent study using a trans-chromosomic mouse ES cell line harbouring a copy of human chromosome 11 [73]. In the murine cellular environment, the primate-specific SINE-VNTR-Alu (SVA) and Long Interspersed Nuclear Element-1 (L1) retroelements are derepressed and become aberrantly transcribed. By screening a series of candidate human KRAB-ZFP proteins, the authors showed that expression of *ZNF91* and *ZNF93* could restore the recruitment of KAP1 to these elements and re-install transcriptional repression [73].

So how does this mechanism of KAP1-mediated viral silencing in ES cells compare to HUSH-mediated silencing of transgenes? Although there are clear similarities between the two processes, such as the shared requirement for the histone methyltransferase SETDB1 and the H3K9me3 histone mark, the key difference is that KAP1 mediates sequence-specific repression of retroelements, whereas HUSH mediates position-specific repression of any integrated transgene (**Figure 5, A and C**). This is perhaps best illustrated when considering what happens to integrated viruses following transduction of ES cells as compared with commonly used cancer cell lines. In the latter, reporter gene expression is extremely stable; the expression observed two days after infection is then maintained for weeks. The initial,

HUSH-mediated repression of viruses landing in repressed heterochromatin results in the population of 'dim' cells, but the 'bright' cells containing integrations into euchromatic environments remain stably expressed (**Figure 5B**). In ES cells, however, all of the integrations are rapidly subject to KAP1-mediated repression and become silenced over the course of a few days (**Figure 5D**). Therefore, the HUSH complex mediates the epigenetic silencing of transgenes integrating into pre-existing heterochromatin, whereas in ES cells KAP1 specifically silences viral integrations regardless of their position in the genome. In contrast to the spreading of pre-existing heterochromatin mediated by HUSH, KAP1 appears to nucleate the de novo formation of heterochromatin at specific genomic sites to which it is recruited through a KRAB-ZFP protein.

How has the HUSH complex evolved?

Why did the *Drosophila* screens for modifiers of position-effect variegation not identify HUSH? The answer is immediately apparent when examining the evolutionary conservation of the HUSH proteins: clear orthologues of HUSH complex subunits are not found in flies, but are only conserved from fish to humans. This raises another question, why has the HUSH complex evolved more recently? Although further work will be required to understand why this might be, it is clear that HUSH plays a critical role in higher organisms, as both TASOR [39] and Periphilin [74] are indispensable for murine development. Loss of HUSH subunits results in decreased H3K9me3 at hundreds of genomic loci [54], and so the HUSH complex may therefore have an essential role in heterochromatin maintenance in the early embryo, or may play a critical role in directing normal gene expression programs during early development. Furthermore, HUSH subunits are expressed in ES cells [75], and TASOR may itself be a direct target of the key pluripotency regulator Oct4 [76, 77]. Therefore, determining how loss of HUSH affects the ES cell transcriptome may be informative in further understanding its physiological role.

Although it is clear why the *Drosophila* PEV screens did not identify HUSH complex components, a more puzzling question is why our haploid genetic screen did not identify the canonical heterochromatin regulators such as HP1 and SUV39H1 that are critical for PEV in the fly. This may simply be a technical issue, as our haploid screen may not have reached saturation and hence HP1 and SUV39H1 could be false-negatives. Alternatively functional redundancy between the three human HP1 isoforms or between the highly homologous

SUV39H1 and SUV39H2 might prevent individual knockouts of these genes from generating a phenotype. It would therefore be interesting to examine the potential for cross-talk between HUSH and other heterochromatin regulators such as HP1 and SUV39H1, and to establish how the different systems divide the labour at different genomic sites or in different cell types.

The HUSH complex can mediate the epigenetic repression of integrated viruses

Although transgenesis is not a natural process, position-effect variegation is relevant in the context of retroviral infection, where viral DNA encoding active genes is integrated into the host genome. The integration preferences of human immunodeficiency virus type 1 (HIV-1) [78–81] and other retroviruses [82, 83] are well-studied and, although there is a strong bias towards integration into active transcriptional units, a significant minority of integrations occur into heterochromatin. We found evidence for HUSH-mediated silencing of both HIV-1 and murine leukaemia virus (MLV) LTR promoters through H3K9me3 [54], raising the question of whether HUSH-mediated silencing of retroviruses integrated in heterochromatin could be an important factor in the establishment or maintenance of viral latency. In the case of HIV-1, although highly active antiretroviral therapy (HAART) can reduce plasma HIV-1 below clinically detectable levels in AIDS patients, cessation of treatment leads to a rebound in plasma viraemia owing to viral reactivation from latent reservoirs in resting $CD4^+$ T cells [84]. A cure for HIV-1 infection will therefore require a pharmacological strategy to purge this latent pool. Depletion of HUSH subunits can result in reactivation of silent HIV-1 reporter viruses in cell culture models [54]. However, the dominant viral integration sites mapped in patients with persistent HIV infection are not seemingly enriched in proximity to heterochromatic regions [85, 86], although it has been questioned whether the proviruses identified in these studies are capable of lytic reactivation [87, 88]. It will therefore be important to determine the clinical relevance of HUSH-mediated silencing in HIV-1 pathogenesis, and the utility of HUSH inhibition in purging the latent pool of HIV-1 that persists in the face of antiretroviral therapy.

HUSH may also be required for the silencing and maintenance of latency of non-integrating viruses, such as the clinically important herpesviruses. Human cytomegalovirus (HCMV), for example, is a highly prevalent human pathogen that mediates life-long infection through the establishment of latency in haematopoietic stem cells [89]. Chromatinisation of

incoming viral DNA occurs minutes after initial infection [90], and, in latently infected cells, the ~250 kb HCMV genome is stably maintained as a non-replicating episome. This latent state is primarily maintained through epigenetic mechanisms, with the critical major intermediate early gene promoter coated with H3K9me3 and bound by HP1 [91]. Myeloid differentiation triggers lytic reactivation with loss of repressive histone modifications, eviction of HP1, and installation of active histone marks to facilitate lytic gene expression [92]. KAP1 was recently reporter to play a key role in HCMV latency, by directing the recruitment of SETDB1 to the viral genome to deposit H3K9me3 [93], and has also been implicated in the replication of both Epstein-Barr virus (EBV) [94] and Kaposi's sarcoma-associated herpesvirus (KSHV) [95, 96]. Whether KAP1 and HUSH co-operate in the recruitment of SETDB1 to silence these viral genomes remains to be determined.

Understanding HUSH complex function: future challenges

A key challenge for future studies will be the dissection of the molecular details of HUSH-mediated silencing (**Figure 6**). In particular, the specific roles of TASOR and Periphilin in the silencing process remain unclear. If MPP8 alone can both recognise H3K9me3 through its chromodomain [57, 58, 97], and bind SETDB1 [55] to direct further H3K9me3 deposition, then a read-write mechanism for heterochromatin spreading would seemingly need just these two components. However, the critical requirement for both TASOR and Periphilin for HUSH function [54] implicates further complexities beyond this simplistic model. TASOR is of particular interest due to its large size (1670 amino acids), yet lack of any discernible domains. The only feature currently annotated is an N-terminal 'domain of unknown function' (DUF3715), but its identity and role in HUSH-mediated repression is unknown. Periphilin is the smallest of the HUSH subunits, but also has no clearly discernible domains and indeed is predicted to be largely unstructured [98, 99].

The ability to maintain stable transgene expression is important in biotechnological applications and in gene therapy. While the development of small molecule inhibitors of HUSH or SETDB1 would be one strategy to prevent the invasion of heterochromatin into therapeutic transgenes, a more attractive option would be the identification of insulator sequences that can halt the HUSH-mediated spreading of H3K9me3. By far the best characterised insulator is HS4, identified through the study of gene regulation at the chicken β-globin locus, which can act as a 'barrier insulator' to prevent the spread of heterochromatin

[100]. The possibility of flanking therapeutic transgenes with HS4 or other such insulator sequences which might render active genes immune to HUSH-mediated heterochromatinisation might be an attractive option to mitigate unwanted silencing resulting from position effects.

Conclusion and Outlook

Recent advances in experimental human genetics now allow the re-evaluation of old questions that were previously only amenable to genetic analysis in lower eukaryotes. We exploited the near-haploid KBM7 cell line to identify genes involved in position-effect variegation in human cells, a phenomenon that has been widely studied in the fly. The haploid screen identified HUSH, an epigenetic repressor complex composed of three subunits, TASOR, MPP8 and Periphilin. The *Drosophila* genome does not possess clear orthologues of these genes, suggesting that HUSH represents a novel route to H3K9me3-mediated heterochromatin formation in mammalian cells. A central feature of HUSH function is that it appears to mediate the spreading of pre-existing heterochromatin through the reading and writing of H3K9me3, and hence is only responsible for the silencing of active transgenes when integrated into heterochromatic genomic loci.

Much further work will be required to understand in molecular detail the mechanistic basis of HUSH function, and to determine why it is critical for early mammalian development. Another key question concerns the relationship between HUSH and other silencing systems in the cell: for example, does HUSH cooperate with HP1 and SUV39H1 at certain sites or in certain cell types, or do they act independently of one another at discrete genomic loci? Finally, it is possible that modulation of HUSH function might have clinical applications. Insulating therapeutic transgenes from HUSH-mediated heterochromatin invasion could be beneficial in gene therapy, while pharmacological inhibition of HUSH function may provide a strategy for reactivating latent proviruses integrated at heterochromatic loci. A greater understanding of the molecular mechanisms of HUSH function will be essential before the feasibility of these applications can be properly assessed. More broadly, haploid genetic screens in human cells may now permit the re-evaluation of other pathways that could previously only be interrogated genetically in model eukaryotic organisms.

Acknowledgements

The authors thank Mark Dawson for helpful discussions. This work was funded by a Wellcome Trust Principal Research Fellowship to P.J.L. (084957/Z/08/Z) and a Wellcome Trust PhD studentship to I.A.T. The Cambridge Institute for Medical Research is in receipt of a Wellcome Trust Strategic Award.

Abbreviations

PEV, position-effect variegation

HUSH, Human Silencing Hub

GFP, green fluorescent protein

FACS, fluorescence-activated cell sorting

H3K9me3, tri-methylation of lysine-9 of histone H3

Su(var), suppressor of variegation

E(var), enhancer of variegation

Momme, modifier of murine metastable epiallele

HP1, heterochromatin protein 1

ES cells, embryonic stem cells

KRAB-ZFP, Krüppel-associated box domain-containing zinc finger protein

LTR, long terminal repeat

HIV-1, human immunodeficiency virus type 1

References

1. **Jenuwein T**, **Allis CD**. 2001. Translating the histone code. *Science* **293**: 1074–80.

2. **Tessarz P**, **Kouzarides T**. 2014. Histone core modifications regulating nucleosome structure and dynamics. *Nat. Rev. Mol. Cell Biol.* **15**: 703–8.

3. **Taverna SD, Li H, Ruthenburg AJ, Allis CD,** et al. 2007. How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers. *Nat. Struct. Mol. Biol.* **14**: 1025–40.

4. **Saksouk N**, **Simboeck E**, **Déjardin J**. 2015. Constitutive heterochromatin formation and transcription in mammals. *Epigenetics Chromatin* **8**: 3.

5. **Eissenberg JC**. 2012. Structural biology of the chromodomain: form and function. *Gene* **496**: 69–78.

6. **Sanchez R**, **Meslamani J**, **Zhou M-M**. 2014. The bromodomain: from epigenome reader to druggable target. *Biochim. Biophys. Acta* **1839**: 676–85.

7. **Rando OJ**. 2012. Combinatorial complexity in chromatin structure and function: revisiting the histone code. *Curr. Opin. Genet. Dev.* **22**: 148–55.

8. **Heitz E**. 1928. Das Heterochromatin der Moose. Jb. Wiss. Bot. 69: 762-818.

9. **Grewal SIS**, **Elgin SCR**. 2007. Transcription and RNA interference in the formation of heterochromatin. *Nature* **447**: 399–406.

10. **Mozzetta C**, **Boyarchuk E**, **Pontis J**, **Ait-Si-Ali S**. 2015. Sound of silence: the properties and functions of repressive Lys methyltransferases. *Nat. Rev. Mol. Cell Biol.* **16**: 499–513.

11. **Højfeldt JW**, **Agger K**, **Helin K**. 2013. Histone lysine demethylases as targets for anticancer therapy. *Nat. Rev. Drug Discov.* **12**: 917–30.

12. **Fodor BD**, **Shukeir N**, **Reuter G**, **Jenuwein T**. 2010. Mammalian Su(var) genes in chromatin control. *Annu. Rev. Cell Dev. Biol.* **26**: 471–501.

13. **Akhtar W, de Jong J, Pindyurin A V, Pagie L,** et al. 2013. Chromatin position effects assayed by thousands of reporters integrated in parallel. *Cell* **154**: 914–27.

14. **Elgin SCR**, **Reuter G**. 2013. Position-effect variegation, heterochromatin formation, and gene silencing in Drosophila. *Cold Spring Harb. Perspect. Biol.* **5**: a017780.

15. **Cooper KW**. 1959. Cytogenetic analysis of major heterochromatic elements (especially Xh and Y) in Drosophila melanogaster, and the theory of "heterochromatin". *Chromosoma* **10**: 535–88.

16. **Schotta G**, **Ebert A**, **Dorn R**, **Reuter G**. 2003. Position-effect variegation and the genetic dissection of chromatin regulation in Drosophila. *Semin. Cell Dev. Biol.* **14**: 67–75.

17. **Eissenberg JC, James TC, Foster-Hartnett DM, Hartnett T,** et al. 1990. Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of position-effect variegation in Drosophila melanogaster. *Proc. Natl. Acad. Sci. U. S. A.* **87**: 9923–7.

18. **Reuter G, Dorn R, Wustmann G, Friede B,** et al. 1986. Third chromosome suppressor of position-effect variegation loci in Drosophila melanogaster. *MGG Mol. Gen. Genet.* **202**: 481–7.

19. **Rea S, Eisenhaber F, O'Carroll D, Strahl BD,** et al. 2000. Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* **406**: 593–9.

20. **Schotta G, Ebert A, Krauss V, Fischer A,** et al. 2002. Central role of Drosophila SU(VAR)3-9 in histone H3-K9 methylation and heterochromatic gene silencing. *EMBO J.* **21**: 1121–31.

21. **James TC, Eissenberg JC, Craig C, Dietrich V,** et al. 1989. Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of Drosophila. *Eur. J. Cell Biol.* **50**: 170–80.

22. **Jacobs SA, Taverna SD, Zhang Y, Briggs SD,** et al. 2001. Specificity of the HP1 chromo domain for the methylated N-terminus of histone H3. *EMBO J.* **20**: 5232–41.

23. **Eskeland R**, **Eberharter A**, **Imhof A**. 2007. HP1 binding to chromatin methylated at H3K9 is enhanced by auxiliary factors. *Mol. Cell. Biol.* **27**: 453–65.

24. **Fanti L**, **Giovinazzo G**, **Berloco M**, **Pimpinelli S**. 1998. The Heterochromatin Protein 1 Prevents Telomere Fusions in Drosophila. *Mol. Cell* **2**: 527–38.

25. **Seum C, Reo E, Peng H, Rauscher FJ,** et al. 2007. Drosophila SETDB1 is required for chromosome 4 silencing. *PLoS Genet.* **3**: e76.

26. **Tzeng T-Y**, **Lee C-H**, **Chan L-W**, **Shen C-KJ**. 2007. Epigenetic regulation of the Drosophila chromosome 4 by the histone H3K9 methyltransferase dSETDB1. *Proc. Natl. Acad. Sci. U. S. A.* **104**: 12691–6.

27. **Brower-Toland B, Riddle NC, Jiang H, Huisinga KL,** et al. 2009. Multiple SET methyltransferases are required to maintain normal heterochromatin domains in the genome of Drosophila melanogaster. *Genetics* **181**: 1303–19.

28. **Donaldson KM**, **Lui A**, **Karpen GH**. 2002. Modifiers of terminal deficiency-associated position effect variegation in Drosophila. *Genetics* **160**: 995–1009.

29. **Phalke S, Nickel O, Walluscheck D, Hortig F,** et al. 2009. Retrotransposon silencing and telomere integrity in somatic cells of Drosophila depends on the cytosine-5 methyltransferase DNMT2. *Nat. Genet.* **41**: 696–702.

30. **Blewitt ME, Vickaryous NK, Hemley SJ, Ashe A,** et al. 2005. An N-ethyl-N-nitrosourea screen for genes involved in variegation in the mouse. *Proc. Natl. Acad. Sci. U. S. A.* **102**: 7629–34.

31. **Ashe A, Morgan DK, Whitelaw NC, Bruxner TJ,** et al. 2008. A genome-wide screen for modifiers of transgene variegation identifies genes with critical roles in development. *Genome Biol.* **9**: R182.

32. **Blewitt M**, **Whitelaw E**. 2013. The use of mouse models to study epigenetics. *Cold Spring Harb. Perspect. Biol.* **5**: a017939.

33. **Chong S, Vickaryous N, Ashe A, Zamudio N,** et al. 2007. Modifiers of epigenetic reprogramming show paternal effects in the mouse. *Nat. Genet.* **39**: 614–22.

34. **Youngson NA, Epp T, Roberts AR, Daxinger L,** et al. 2013. No evidence for cumulative effects in a Dnmt3b hypomorph across multiple generations. *Mamm. Genome* **24**: 206–17.

35. **Whitelaw NC, Chong S, Morgan DK, Nestor C,** et al. 2010. Reduced levels of two modifiers of epigenetic gene silencing, Dnmt3a and Trim28, cause increased

phenotypic noise. *Genome Biol.* **11**: R111.

36. **Sorolla A, Tallack MR, Oey H, Harten SK,** et al. 2015. Identification of novel hypomorphic and null mutations in Klf1 derived from a genetic screen for modifiers of α-globin transgene variegation. *Genomics* **105**: 116–22.

37. **Blewitt ME, Gendrel A-V, Pang Z, Sparrow DB,** et al. 2008. SmcHD1, containing a structural-maintenance-of-chromosomes hinge domain, has a critical role in X inactivation. *Nat. Genet.* **40**: 663–9.

38. **Daxinger L, Harten SK, Oey H, Epp T,** et al. 2013. An ENU mutagenesis screen identifies novel and known genes involved in epigenetic processes in the mouse. *Genome Biol.* **14**: R96.

39. **Harten SK, Bruxner TJ, Bharti V, Blewitt M,** et al. 2014. The first mouse mutants of D14Abb1e (Fam208a) show that it is critical for early development. *Mamm. Genome*

40. **Takayama S, Dhahbi J, Roberts A, Mao G,** et al. 2014. Genome methylation in D. melanogaster is found at specific short motifs and is independent of DNMT2 activity. *Genome Res.* **24**: 821–30.

41. **Andersson BS, Beran M, Pathak S, Goodacre A,** et al. 1987. Ph-positive chronic myeloid leukemia with near-haploid conversion in vivo and establishment of a continuously growing cell line with similar cytogenetic pattern. *Cancer Genet. Cytogenet.* **24**: 335–43.

42. **Carette JE, Guimaraes CP, Varadarajan M, Park AS,** et al. 2009. Haploid genetic screens in human cells identify host factors used by pathogens. *Science* **326**: 1231–5.

43. **Leeb M**, **Wutz A**. 2011. Derivation of haploid embryonic stem cells from mouse embryos. *Nature* **479**: 131–4.

44. **Elling U**, **Taubenschmid J**, **Wirnsberger G, O'Malley R,** et al. 2011. Forward and reverse genetics through derivation of haploid mouse embryonic stem cells. *Cell Stem Cell* **9**: 563–74.

45. **Hsu PD**, **Lander ES**, **Zhang F**. 2014. Development and Applications of CRISPR-Cas9 for Genome Engineering. *Cell* **157**: 1262–78.

46. **Kotecki M**, **Reddy PS**, **Cochran BH**. 1999. Isolation and characterization of a near-haploid human cell line. *Exp. Cell Res.* **252**: 273–80.

47. **Carette JE, Guimaraes CP, Wuethrich I, Blomen VA,** et al. 2011. Global gene disruption in human cells to assign genes to phenotypes by deep sequencing. *Nat. Biotechnol.* **29**: 542–6.

48. **Carette JE, Raaben M, Wong AC, Herbert AS,** et al. 2011. Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature* **477**: 340–3.

49. **Essletzbichler P, Konopka T, Santoro F, Chen D,** et al. 2014. Megabase-scale deletion using CRISPR/Cas9 to generate a fully haploid human cell line. *Genome Res.* **24**: 2059–65.

50. **Leeb M, Dietmann S, Paramor M, Niwa H,** et al. 2014. Genetic exploration of the exit from self-renewal using haploid embryonic stem cells. *Cell Stem Cell* **14**: 385–93.

51. **Monfort A, Di Minin G, Postlmayr A, Freimann R,** et al. 2015. Identification of

Spen as a Crucial Factor for Xist Function through Forward Genetic Screening in Haploid Embryonic Stem Cells. *Cell Rep.* **12**: 554–61

52. **Duncan LM, Timms RT, Zavodszky E, Cano F,** et al. 2012. Fluorescence-based phenotypic selection allows forward genetic screens in haploid human cells. *PLoS One* **7**: e39651.

53. **Timms RT, Duncan LM, Tchasovnikarova IA, Antrobus R,** et al. 2013. Haploid genetic screens identify an essential role for PLP2 in the downregulation of novel plasma membrane targets by viral E3 ubiquitin ligases. *PLoS Pathog.* **9**: e1003772.

54. **Tchasovnikarova IA, Timms RT, Matheson NJ, Wals K,** et al. 2015. Epigenetic silencing by the HUSH complex mediates position-effect variegation in human cells. *Science (80-.).* **348**: 1481–5.

55. **Kokura K**, **Sun L**, **Bedford MT**, **Fang J**. 2010. Methyl-H3K9-binding protein MPP8 mediates E-cadherin gene silencing and promotes tumour cell motility and invasion. *EMBO J.* **29**: 3673–87.

56. **Schultz DC, Ayyanathan K, Negorev D, Maul GG,** et al. 2002. SETDB1: a novel KAP-1-associated histone H3, lysine 9-specific methyltransferase that contributes to HP1-mediated silencing of euchromatic genes by KRAB zinc-finger proteins. *Genes Dev.* **16**: 919–32.

57. **Chang Y, Horton JR, Bedford MT, Zhang X,** et al. 2011. Structural insights for MPP8 chromodomain interaction with histone H3 lysine 9: potential effect of phosphorylation on methyl-lysine binding. *J. Mol. Biol.* **408**: 807–14.

58. **Li J, Li Z, Ruan J, Xu C,** et al. 2011. Structural basis for specific binding of human MPP8 chromodomain to histone H3 methylated at lysine 9. *PLoS One* **6**: e25104.

59. **Hillringhaus L, Yue WW, Rose NR, Ng SS,** et al. 2011. Structural and evolutionary basis for the dual substrate selectivity of human KDM4 histone demethylase family. *J. Biol. Chem.* **286**: 41616–25.

60. **Trewick SC, Minc E, Antonelli R, Urano T,** et al. 2007. The JmjC domain protein Epe1 prevents unregulated assembly and disassembly of heterochromatin. *EMBO J.* **26**: 4670–82.

61. **Audergon PNCB, Catania S, Kagansky A, Tong P,** et al. 2015. Restricted epigenetic inheritance of H3K9 methylation. *Science (80-.).* **348**: 132–5.

62. **Ragunathan K**, **Jih G**, **Moazed D**. 2014. Epigenetic inheritance uncoupled from sequence-specific recruitment. *Science (80-.).* **348**: 1258699.

63. **Teich NM**, **Weiss RA**, **Martin GR**, **Lowy DR**. 1977. Virus infection of murine teratocarcinoma stem cell lines. *Cell* **12**: 973–82.

64. **Iyengar S**, **Farnham PJ**. 2011. KAP1 protein: an enigmatic master regulator of the genome. *J. Biol. Chem.* **286**: 26267–76.

65. **Peng H, Begg GE, Harper SL, Friedman JR,** et al. 2000. Biochemical analysis of the Kruppel-associated box (KRAB) transcriptional repression domain. *J. Biol. Chem.* **275**: 18000–10.

66. **Lupo A, Cesaro E, Montano G, Zurlo D,** et al. 2013. KRAB-Zinc Finger Proteins: A Repressor Family Displaying Multiple Biological Functions. *Curr. Genomics* **14**: 268–

78.

67. **Wolf D**, **Goff SP**. 2009. Embryonic stem cells use ZFP809 to silence retroviral DNAs. *Nature* **458**: 1201–4.

68. **Matsui T, Leung D, Miyashita H, Maksakova IA,** et al. 2010. Proviral silencing in embryonic stem cells requires the histone methyltransferase ESET. *Nature* **464**: 927–31.

69. **Rowe HM, Jakobsson J, Mesnard D, Rougemont J,** et al. 2010. KAP1 controls endogenous retroviruses in embryonic stem cells. *Nature* **463**: 237–40.

70. **Rowe HM, Friedli M, Offner S, Verp S,** et al. 2013. De novo DNA methylation of endogenous retroviruses is shaped by KRAB-ZFPs/KAP1 and ESET. *Development* **140**: 519–29.

71. **Elsässer SJ, Noh K-M, Diaz N, Allis CD,** et al. 2015. Histone H3.3 is required for endogenous retroviral element silencing in embryonic stem cells. *Nature* **522**: 240–4.

72. **Thomas JH**, **Schneider S**. 2011. Coevolution of retroelements and tandem zinc finger genes. *Genome Res.* **21**: 1800–12.

73. **Jacobs FMJ, Greenberg D, Nguyen N, Haeussler M,** et al. 2014. An evolutionary arms race between KRAB zinc-finger genes ZNF91/93 and SVA/L1 retrotransposons. *Nature* **516**: 242–5.

74. **Soehn AS, Pham T-T, Schaeferhoff K, Floss T,** et al. 2009. Periphilin is strongly expressed in the murine nervous system and is indispensable for murine development. *Genesis* **47**: 697–707.

75. **Mortazavi A, Williams BA, McCue K, Schaeffer L,** et al. 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat. Methods* **5**: 621–8.

76. **Matoba R, Niwa H, Masui S, Ohtsuka S,** et al. 2006. Dissecting Oct3/4-regulated gene networks in embryonic stem cells by expression profiling. *PLoS One* **1**: e26.

77. **Campbell PA**, **Perez-Iratxeta C**, **Andrade-Navarro MA**, **Rudnicki MA**. 2007. Oct4 targets regulatory nodes to modulate stem cell function. *PLoS One* **2**: e553.

78. **Wang GP, Ciuffi A, Leipzig J, Berry CC,** et al. 2007. HIV integration site selection: analysis by massively parallel pyrosequencing reveals association with epigenetic modifications. *Genome Res.* **17**: 1186–94.

79. **Brady T, Agosto LM, Malani N, Berry CC,** et al. 2009. HIV integration site distributions in resting and activated CD4+ T cells infected in culture. *AIDS* **23**: 1461–71.

80. **Sherrill-Mix S, Lewinski MK, Famiglietti M, Bosque A,** et al. 2013. HIV latency and integration site placement in five cell-based models. *Retrovirology* **10**: 90.

81. **Marini B, Kertesz-Farkas A, Ali H, Lucic B,** et al. 2015. Nuclear architecture dictates HIV-1 integration site selection. *Nature* **521**: 227–31.

82. **Wu X**, **Li Y**, **Crise B**, **Burgess SM**. 2003. Transcription start regions in the human genome are favored targets for MLV integration. *Science* **300**: 1749–51.

83. **Mitchell RS, Beitzel BF, Schroder ARW, Shinn P,** et al. 2004. Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences. *PLoS Biol.* **2**:

E234.

84. **Siliciano JD**, **Siliciano RF**. 2014. Recent developments in the search for a cure for HIV-1 infection: targeting the latent reservoir for HIV-1. *J. Allergy Clin. Immunol.* **134**: 12–9.

85. **Maldarelli F, Wu X, Su L, Simonetti FR,** et al. 2014. HIV latency. Specific HIV integration sites are linked to clonal expansion and persistence of infected cells. *Science* **345**: 179–83.

86. **Wagner TA, McLaughlin S, Garg K, Cheung CYK,** et al. 2014. HIV latency. Proliferation of cells with HIV integrated into cancer genes contributes to persistent infection. *Science* **345**: 570–3.

87. **Margolis D**, **Bushman F**. 2014. HIV/AIDS. Persistence by proliferation? *Science* **345**: 143–4.

88. **Cohn LB, Silva IT, Oliveira TY, Rosales RA,** et al. 2015. HIV-1 integration landscape during latent and active infection. *Cell* **160**: 420–32.

89. **Reeves M**, **Sinclair J**. 2008. Aspects of human cytomegalovirus latency and reactivation. *Curr. Top. Microbiol. Immunol.* **325**: 297–313.

90. **Nitzsche A**, **Paulus C**, **Nevels M**. 2008. Temporal dynamics of cytomegalovirus chromatin assembly in productively infected human cells. *J. Virol.* **82**: 11167–80.

91. **Reeves MB, MacAry PA, Lehner PJ, Sissons JGP,** et al. 2005. Latency, chromatin remodeling, and reactivation of human cytomegalovirus in the dendritic cells of healthy carriers. *Proc. Natl. Acad. Sci. U. S. A.* **102**: 4140–5.

92. **Reeves MB**, **Lehner PJ**, **Sissons JGP**, **Sinclair JH**. 2005. An in vitro model for the regulation of human cytomegalovirus latency and reactivation in dendritic cells by chromatin remodelling. *J. Gen. Virol.* **86**: 2949–54.

93. **Rauwel B, Jang SM, Cassano M, Kapopoulou A,** et al. 2015. Release of human cytomegalovirus from latency by a KAP1/TRIM28 phosphorylation switch. *Elife* **4**

94. **Liao G**, **Huang J**, **Fixman ED**, **Hayward SD**. 2005. The Epstein-Barr virus replication protein BBLF2/3 provides an origin-tethering function through interaction with the zinc finger DNA binding protein ZBRK1 and the KAP-1 corepressor. *J. Virol.* **79**: 245–56.

95. **Chang P-C, Fitzgerald LD, Van Geelen A, Izumiya Y,** et al. 2009. Kruppel-associated box domain-associated protein-1 as a latency regulator for Kaposi's sarcoma-associated herpesvirus and its modulation by the viral protein kinase. *Cancer Res.* **69**: 5681–9.

96. **Cai Q, Cai S, Zhu C, Verma SC,** et al. 2013. A unique SUMO-2-interacting motif within LANA is essential for KSHV latency. *PLoS Pathog.* **9**: e1003750.

97. **Sun L, Kokura K, Izumi V, Koomen JM,** et al. 2015. MPP8 and SIRT1 crosstalk in E-cadherin gene silencing and epithelial-mesenchymal transition. *EMBO Rep.* **16**: 689–99.

98. **Yang ZR**, **Thomson R**, **McNeil P**, **Esnouf RM**. 2005. RONN: the bio-basis function neural network technique applied to the detection of natively disordered regions in proteins. *Bioinformatics* **21**: 3369–76.

99. **Kazerounian S**, **Aho S**. 2003. Characterization of periphilin, a widespread, highly insoluble nuclear protein and potential constituent of the keratinocyte cornified envelope. *J. Biol. Chem.* **278**: 36707–17.

100. **Barkess G**, **West AG**. 2012. Chromatin insulator elements: establishing barriers to set heterochromatin boundaries. *Epigenomics* **4**: 67–80.

Figure Legends

Figure 1. Forward genetic screens for modifiers of position-effect effect in the fruit fly *Drosophila melanogaster*.

Epigenetic silencing of the *white* gene occurs when it is placed into a heterochromatic environment, resulting in a variegated phenotype in the *Drosophila* eye. Following random mutagenesis, files can be screened for dominant mutations which either suppress variegation, resulting in derepression of the white gene and reversion to a red-eye phenotype, or mutations which enhance variegation, resulting in a more complete white-eye phenotype.

Figure 2. Forward genetic screens for modifiers of transgene silencing in the mouse.

The *Momme* mouse line harbours a mutilcopy GFP transgene on chromosome 1 which is subject to variable epigenetic silencing, resulting in GFP expression in approximately 55% of erythrocytes. Following random mutagenesis, mice can be screened for mutations which either increase or decrease the proportion of GFP^+ cells. Thus far, more than 40 strains which show significant changes in GFP expression have been characterised.

Figure 3. Forward genetic screens in haploid human cells.

The human KBM7 cell line, isolated from a patient with chronic myeloid leukaemia, is highly unusual in that it is haploid for all chromosomes with the exception of chromosome 8 and the sex chromosomes. Mutagenesis of this cell line using a gene-trap retrovirus therefore creates a library of total gene knockouts, which can then be screened for mutants which are defective in the process under investigation. The simplest way to do this is to apply a lethal insult (left side), whereby the rare resistant cells which grow out harbour mutations in the killing pathway, although a FACS-based method for the positive selection of cells which display altered levels of a cell surface protein or of a genetically-encoded fluorescent reporter is more widely applicable to the study of diverse cellular processes (right side). Finally, the sites of integration of the gene-trap vector are mapped in the selected cells to reveal the disrupted genes responsible for the mutant phenotype.

Figure 4. A haploid genetic screen to identify genes required for transgene silencing in human cells.

A: Near-haploid KBM7 cells were transduced with a reporter construct expressing GFP, resulting in the integration of the GFP reporter across the genome. Due to chromosomal position effects this results in a wide range of GFP levels, with a minority of integrations occurring in repressive heterochromatin leading to a GFP^{dim} phenotype.

B: To identify the genes responsible for epigenetic suppression of the GFP reporter, the GFP^{dim} cells were isolated by FACS, mutagenised with a gene-trap retrovirus, and then rare mutant GFP^{bright} cells in which derepression of the GFP reporter was lost isolated by FACS. Comparing the gene-trap integrations sites in the GFP^{bright} cells with those in the unselected mutant library identified four genes that were responsible for epigenetic repression of the transgene.

Figure 5. Contrasting the mechanisms of heterochromatin formation by KAP1 and the HUSH complex.

A and B: Spreading of pre-existing heterochromatin by the HUSH complex. The HUSH complex is localised at H3K9me3-rich regions of the genome through the chromodomain of MPP8, where, through the recruitment of SETDB1, it mediates the spreading of the repressive H3K9me3 modification to mediate the repression of transgenes integration into heterochromatin (**A**). In human cancer cell line such as HeLa, HUSH-mediated repression of integrated transgenes results in a spread of reporter gene expression, which is then stably maintained over time (**B**).

C and D: *De novo* heterochromatin formation by KAP1 mediates retroviral silencing in ES cells. Specific targeting of a retroelement by a KRAB-ZFP protein results in the recruitment of KAP1 and SETDB1 and silencing through the deposition of H3K9me3 (**C**). KAP1 is able to target the integrated proviral DNA at all genomic loci, resulting in the silencing of reporter gene expression in all cells over the course of a few days (**D**).

Figure 6. Molecular view of the HUSH complex subunits.

Schematic representations of the domain architecture of the three HUSH subunits and SETDB1 are shown. Aside from the chromodomain of MPP8 and the catalytic SET domains of SETDB1, little is currently known about the function of the other regions of the four proteins.