Chromatin at the intersection of viral infection and DNA damage

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Abstract

During infection, viruses cause global disruption to nuclear architecture in their attempt to take over the cell. In turn, the host responds with various defenses, which include chromatin-mediated silencing of the viral genome and activation of DNA damage signaling pathways. Dynamic exchanges at chromatin, and specific post-translational modifications on histones have recently emerged as master controllers of DNA damage signaling and repair. Studying viral control of chromatin modifications is identifying histones as important players in the battle between host and virus for control of cell cycle and gene expression. These studies are revealing new complexities of the virus-host interaction, uncovering the potential of chromatin as an anti-viral defense mechanism, and also providing unique insights into the role of chromatin in DNA repair.

2. Introduction

Chromatin is a complex and dynamic structure that must be successfully navigated by viruses in order to ensure an efficient infection cycle. There are two facets to the relationship between viruses and chromatin. On one side, the incoming viral genomes may be chromatinized by the host cell, while on the other side, the virus attempts to manipulate host chromatin proteins to promote its own replication. The effects of histone deposition on incoming viral genomes is emerging as an important aspect of the virus-host interaction, and is likely to have significant implications both for lytic replication and latent maintenance of viral genomes. These topics are covered elsewhere in this focus edition. The range of host proteins targeted by viruses in their attempt to gain control of the cell has recently been expanded to include histones and other chromatin modifying proteins. We will discuss the effects of viral infection on host chromatin, with a specific focus on targeting of proteins relevant to the DNA damage response.

2.1 Histone post-translational modifications in DNA repair

Repairing chromatinized DNA is a challenge, both in terms of accessibility of the break and maintenance of the histone code and chromatin organization. Histone post-translational modifications and chromatin remodeling proteins co-ordinate to allow access of repair molecules to the DNA, and to facilitate amplification and then termination of the damage signal [1,2]. The cellular DNA damage response is a signal-transduction cascade. Lesions in DNA are detected by sensor proteins that activate kinases, which in turn lead to amplification of the signal through a series of downstream effector molecules. The major signaling kinases are ataxia telangiectasia mutated (ATM), ATM and Rad3-related (ATR) and DNA-dependent

protein kinase (DNA-PK), which are members of the phosphoinositide 3 kinase-like kinase (PIKK) family. Upon sensing a break, one or more of these kinases is activated and phosphorylates the histone H2A variant, H2AX. H2AX is constitutively expressed and distributed throughout the genome, representing approximately 10% of the total cellular H2A. H2AX contains a conserved SQ(E/D) motif and this serine residue (S139) is rapidly phosphorylated in response to DNA damage (reviewed in [3]). The phosphorylated H2AX (now referred to as γH2AX) marks the site of damage, and recruits mediator proteins to form irradiation-induced foci (IRIF), which are visible by fluorescent microscopy. γH2AX extends up to 1 megabase in mammalian cells and this spreading is thought to have a role in amplification of the DNA damage signal (reviewed in [4]). Recent work has highlighted the importance of additional post-translational modifications on H2AX and other histones in the DNA damage response. These include a novel inhibitory tyrosine phosphorylation mark on H2AX (Y142) [5], ubiquitination sites on H2A and H2AX (reviewed in [6]), sumoylation sites on the H2A variant H2AZ [7], tyrosine phosphorylation on histone H3 [8], acetylation on H2AX [9], and methylation marks on histone H4 [10,11]. The roles of these histone marks in DNA repair and the potential for their manipulation by viral infection are summarized in Figure 1 and discussed in the following sections.

2.2 The role of chromatin remodeling in DNA repair

Although the significance of post-translational modifications on H2AX is becoming increasingly well understood, several questions remain. Key among these is how ubiquitin ligases, kinases and acetyltransferases gain access to the histones at the double strand break (DSB) site. Moreover, how γH2AX spreading is controlled, what facilitates its passage through compacted chromatin and what signals the conclusion of this spreading are unknown. It has been reported that DSBs trigger local recruitment of chromatin-remodeling proteins, such as SWI/SNF, RSC, SRCAP and INO80 enzyme complexes [12,13]. These proteins co-ordinate histone eviction and decondensation of the chromatin in the region surrounding the break site and allow access of regulatory and repair enzymes to the DNA [14-17]. Recently, it was suggested that H2AX tyrosine de-phosphorylation in response to damage may adjust local chromatin structure to facilitate the access of kinases and other mediator proteins to damaged DNA [5]. In addition, the histone acetyltransferase (HAT) Tip60 acetylates H2AX and this event is required for the mobilization of γH2AX immediately after DSB break induction [9]. Further insight into the accessibility of H2AX has come from a study which demonstrated that the chromatin binding protein heterochromatin protein 1β (HP1β is phosphorylated by casein kinase II (CKII) in response to DNA damage [18]. HP1β is a chromodomain-containing adaptor molecule that binds chromatin at H3K9me3 and is implicated in gene silencing [19]. CKII-mediated phosphorylation of HP1β induces its transient dissociation from H3K9me and this is required for the phosphorylation signal to be deposited on H2AX. This model predicts that the dissociation of HP1β facilitates the formation of a break-specific open chromatin region that makes H2AX more accessible for kinases and ubiquitin ligases, and removes local repressive barriers to facilitate spreading of the γH2AX signal. This idea is attractive because targeting a histone binding protein ensures that the histone code itself remains unchanged after damage.

HP1β is also known to bind the cellular co-repressor, KAP1. In addition to HP1β, KAP1 interacts with other chromatin modifying proteins such as histone deacetylases (HDACs) [20], the H3K9 methyltransferase SETDB1 [21], and participates in gene expression and chromatin remodeling. Interestingly, KAP1 is phosphorylated by PIKKs following DNA damage, and KAP1 localizes to sites of DNA lesions [22]. The release of KAP1 from chromatin is dependent on ATM, raising the possibility that ATM may play a novel role in the phosphorylation and removal of chromatin proteins that are inhibitory to the repair process [23,24]. The role of chromatin remodeling proteins in facilitating access of repair proteins to

the break site is becoming increasingly well understood. However, how these chromatin modifications participate in the activation of ATM and concomitant induction of the signal transduction cascade has until recently remained obscure. Tip60 acetylates ATM in response to damage and this acetylation facilitates ATM autophosphorylation [25]. The recent identification of HMGN1 as a mediator of ATM activation provides another potential bridge between chromatin remodeling and signal transduction proteins. HMGN1 is required for the acetylation of H3K14 in response to IR, decreasing the retention of ATM at chromatin and increasing ATM autophosphorylation [26]. The fact that HMGN1 does not itself accumulate at DSBs suggests that it may affect the organization of ATM throughout the nucleus, raising the possibility that the activation dynamics of ATM are affected by chromatin interactions that occur before induction of a DNA break.

2.3 Viruses and the DNA damage response

As obligate intracellular parasites with small genomes, viruses have complex relationships with their hosts. Viruses have often co-evolved with the host and have developed a plethora of strategies to utilize cellular proteins to promote their own replication and facilitate maintenance of the viral genome. Many viruses are now known to interact with the DNA damage sensing and repair machinery [27]. These viruses have evolved tactics to eliminate, avoid or exploit the DNA damage machinery of the host cell. While some viruses activate DNA repair proteins, others target these cellular factors for degradation or mis-localization. The interface between viruses and the cellular DNA repair machinery is complex and it is becoming increasingly apparent that individual viruses interact with the cellular DNA repair machinery on multiple levels, inactivating certain aspects while exploiting others.

3. The effects of virally-induced DNA damage on nuclear architecture

Viral infection causes dramatic changes to the nuclear architecture as the virus attempts to take over the cell. In the initial stages of infection, host chromatin proteins can be mobilized and recruited to viral genomes. As the infection proceeds, cellular chromatin must be structurally re-organized to make room for small viral replication compartments that eventually coalesce and fill much of the nucleus. In the majority of examples of viral activation of γH2AX, no specific viral protein appears to be sufficient. In these cases, it is assumed that aberrant DNA structures on the incoming or replicating genomes may be the trigger, or that global changes to host chromatin may lead to ATM activation [26,28] and subsequent damage-independent H2AX activation. In this section, we will review some examples of viral replication leading to changes in γH2AX.

3.1 Activation and nuclear localization of γH2AX

In the case of herpes simplex virus type 1 (HSV-1), infection induces activation of DNA damage signaling [29,30], phosphorylation of H2AX and marginalization of this γH2AX to the periphery of viral replication centers [31]. In contrast, while infections with human cytomegalovirus (HCMV) also caused H2AX phosphorylation [32], the γH2AX co-localized with viral replication compartments, at least late in infection [32,33]. In this case, activation of γH2AX was shown to be dependent on viral replication as it did not occur in p53 mutant cells, in which HCMV replication is abrogated [33]. Similarly, induction of Epstein Barr virus (EBV) lytic replication [34] or polyomavirus (Py) replication [35] induced phosphorylation of a number of DNA damage markers including γH2AX.

Infection with adenovirus (Ad) deleted of early region E4 resulted in activation of a cellular DNA damage response, with accumulation of damage mediator proteins at sites of viral replication [36]. Staining for γH2AX was observed around the edges of viral centers [37]. The viral trigger of this histone phosphorylation event is unknown but the accumulation of γH2AX

was abrogated in the presence of caffeine, suggesting that the phosphorylation is mediated by cellular damage kinases. It is unclear whether the histone is modified on viral DNA or whether H2AX in cellular chromatin adjacent to viral replication centers becomes phosphorylated when damage kinases are activated in response to the virus. During infection with wild-type Ad, cellular DNA damage sensor proteins are degraded and the cellular DNA damage response is abrogated [37]. However, late during wild-type infection, H2AX is phosphorylated and the staining is distributed throughout the nucleus of infected cells, suggesting that the histone modification is on cellular DNA [38]. Control infections with a non-replicating Ad did not induce γH2AX staining, suggesting that viral replication is the trigger in this case.

Infection of cells with adeno-associated virus (AAV) also results in pan-nuclear activation of γH2AX [39,40]. Wild-type and UV-treated AAV, but not recombinant AAV vectors, were able to elicit this response suggesting that the phosphorylation was due to a component of the wild-type AAV genome and not the viral capsid. The activation of γH2AX staining by wild-type AAV was narrowed down to a region of the genome containing the p5 promoter which may function as a potential origin of replication [40]. Although AAV infection or the viral Rep protein alone could induce some diffuse nuclear γH2AX staining, the response was much more robust during replication in the presence of Ad helper functions [39]. Together these studies suggest that γH2AX is activated in response to AAV due to specific viral sequences within the p5 region and by DNA structures generated during viral replication.

3.2 Virus-induced cellular re-organization

Viral infection usually leads to the formation of viral replication compartments that fill much of the interior of the nucleus. In the case of HSV-1, these replication compartments exclude histone H1 [41], heterochromatin marks H3K9Me3 and H4K20Me3 [42], and cause the marginalization of global host chromatin [43,44], including γH2AX [31]. The exclusion of linker histones has been proposed to be due to a host cell-induced increase in H1 mobility in response to virus infection [45]. Interestingly, these histone exclusion effects were recently found to be dependent on nuclear lamin A/C. In the absence of lamin A/C, viral gene expression and replication were impaired, and this was a result of the presence of heterochromatin at viral replication compartments [42]. This led the authors to propose that lamin A/C is a molecular scaffold required both to control viral genome localization and to ensure that heterochromatin is not deposited on viral promoters during lytic infection.

In some examples, gross cellular changes induced by the virus are sufficient for phosphorylation of H2AX. For example, in the case of human immunodeficiency virus 1 (HIV-1) infection, virus-induced cell fusion was necessary and sufficient for ATM-dependent γH2AX activation [46]. This fusion is normally mediated by a gp120/CD4 interaction, and when this fusion event was prevented, H2AX phosphorylation was abolished. Surprisingly, H2AX phosphorylation could be restored by forcing cell fusion with polyethylene glycol, even in the absence of a gp120/CD4 interaction. The authors hypothesized that phosphorylation of H2AX may result from fusing of nuclei that are in different phases of the cell cycle.

4. Virus encoded proteins targeting chromatin

Viruses often inhibit or exploit specific cellular proteins to promote viral replication and facilitate maintenance of the viral genome. Recently, the repertoire of host proteins targeted directly or indirectly by viruses has been expanded to include histones. This section will consider several examples of specific viral proteins being necessary and sufficient to induce or remove post-translational modifications on histones.

4.1 Divorcing ubiquitin from histones - how a virus can remove the RING

It has been known for several years that HSV-1 and HSV-2 induce DNA damage signaling in the host cell, characterized by phosphorylation of many cellular proteins, including H2AX, and recruitment of several mediator proteins to viral replication centers [29,30,47,48]. In addition to phosphorylation, ubiquitination of H2A and H2AX has recently emerged as a central co-ordinator of the DNA damage response. Herpesviruses need to gain control of the DNA repair pathway in order to promote their efficient replication, so it is therefore not surprising that they have evolved to target this key upstream regulatory step. Recent work from our laboratory has revealed that the HSV-1 encoded E3 ubiquitin ligase, ICP0, induces the proteasome-mediated degradation of cellular ubiquitin ligases normally serving to maintain the ubiquitinated mark on H2A and H2AX (CEL, MSC and MDW, unpublished observations). ICP0 is a strong transcriptional activator important for the efficient transition from latent to lytic infection. RNF8 and RNF168 are ubiquitin ligases for H2A and H2AX that mediate the accumulation of downstream repair proteins at the sites of double-strand breaks [49-54]. We propose that by targeting RNF8 and RNF168 for proteasome-mediated degradation, ICP0 dramatically reduces the levels of ubiquitinated H2A and H2AX in the cell and prevents the retention of downstream DNA repair proteins at sites of cellular damage. We hypothesize that one reason ICP0 may prevent this immobilization is to ensure that the cellular repair factors are available to be recruited to sites of viral replication. ICP0 has previously been reported to inhibit DNA repair since its expression led to the persistence of damage-induced γH2AX foci and impaired survival in a radiosensitivity assay [55]. This was attributed to the ICP0-mediated degradation of DNA-PKcs, but it is possible that impaired DNA damage signaling due to loss of RNF8, RNF168 and ubiquitinated H2A may also contribute to this effect.

4.2 Checking in on histone phosphorylation

DNA damage signaling initiated by ATM, ATR and DNA-PKcs leads to the phosphorylation of a number of downstream effector proteins. Among these is Chk1, which is a chromatin-associated protein phosphorylated primarily by ATR. Phosphorylated Chk1 has well-documented roles in cell-cycle arrest and apoptosis (reviewed in [56]). However, unphosphorylated Chk1 is still an active kinase and it has been proposed that it is the damage-induced dissociation of Chk1 from chromatin rather than its kinase activity *per se* which promotes the checkpoint response [57]. It has recently been shown that Chk1 phosphorylates histone H3 on T11 independently of DNA damage [8]. H3T11 is then dephosphorylated when Chk1 dissociates from chromatin after damage, and this coincides with a modest reduction in H3K9 acetylation, an event normally mediated by the HAT Gcn5. As viruses attempt to gain control of the cell cycle, they commonly target checkpoint kinases such as Chk1 and Chk2. It is likely that many viruses will induce Chk1 phosphorylation as part of a wider DNA damage response [27]. However, specific examples of viral proteins that are known to affect Chk1 phosphorylation include simian virus 40 (SV40) LTag [58] and HIV-1 Vpr [59].

$\tilde{4}$.3 H2AX: intended target or innocent bystander?

Phosphorylation of H2AX is considered a hallmark of the DNA damage response, but the specific trigger is unknown in most examples of viral induction of this event. As discussed in the previous section, it has often been assumed that this phosphorylation event may result from global disorganization of the nucleus or be caused by unusual DNA structures introduced by the virus. However, several recent papers suggest that specific virally-encoded proteins can also control the phosphorylation status of H2AX directly. Transfected Tax protein from human T-cell lymphotropic virus (HTLV-1) forms nuclear foci and is sufficient to induce phosphorylation of H2AX at these sites [60]. Concomitant with the activation of γH2AX by Tax, it was noted that the viral protein also induced phosphorylation of DNA-PKcs and activation of Chk2. IR-induced phosphorylation of H2AX is typically carried out by ATM, but

in the absence of ATM, kinases such as DNA-PK can act, suggesting a possible mechanism for the effects of Tax on H2AX phosphorylation. Interestingly, when Tax-expressing cells were challenged with exogenous damage, the IR-induced increase in γH2AX was blunted and temporally delayed. These results suggest that Tax-expressing cells have an impaired capacity to respond to new damage, or that the damage recognition pathways are already "saturated" by the presence of Tax.

Previous reports indicated that infection with SV40 induced extensive DNA damage signaling with localization of many cellular repair and replication factors, including γH2AX, to large LTag-containing nuclear foci [61]. However, it was not clear whether LTag alone was sufficient to induce the response or whether viral replication structures were required. Recently, it was demonstrated that expression of LTag without a viral replication origin in normal human fibroblasts led to the induction of γH2AX foci and several other phosphorylation events indicative of a DNA damage response [58]. Interestingly, mutant LTag defective for Bub1 binding was unable to induce the formation of γH2AX foci, suggesting a role for this spindle assembly checkpoint kinase in H2AX activation.

Other viral proteins have been reported to lead to phosphorylation of γH2AX. Examples include human papillomavirus (HPV-16) E7 [62] and the EBV protein EBNA1 [63]. Interestingly, in the case of EBV, the intensity of γH2AX staining was decreased by treatment of EBNA1-expressing cells with ROS scavengers, suggesting that this viral protein may utilize a novel mechanism for viral induction of DNA damage signaling and histone modifications [64]. How these specific viral proteins lead to activation of H2AX is unclear. Possibilities include the induction of *bone fide* cellular double-strand breaks, deregulation of normal cellular DNA replication dynamics, global changes to host chromatin, or direct interaction with cellular PIKKs or other DNA repair proteins.

In the above examples, it is generally assumed that although the viral protein is the trigger, the actual phosphorylation event is performed by a cellular kinase. Recently, the first example of direct H2AX phosphorylation by a viral protein has been proposed. Tarakanova *et al.* reported that the murine gammaherpesvirus 68 (γHV68) protein orf36 and its EBV homolog, BGLF4, were necessary and sufficient to induce γH2AX phosphorylation [65]. The authors demonstrated that orf36 was necessary for the induction of γH2AX phosphorylation during γHV68 infection. They also showed that transient expression of plasmid-encoded orf36 was sufficient to cause H2AX phosphorylation and that, like other herpesviruses, the presence of ATM and H2AX are required for efficient γHV68 replication. Strikingly, immunopreciptated wild-type, but not mutant orf36, could phosphorylate recombinant H2AX *in vitro*, leading the authors to propose that the histone variant is a direct substrate of orf36. This attractive hypothesis would suggest that viral induction of the DNA damage signaling cascade can be an active event, rather than simply a "side-effect" of abnormal incoming DNA structures or intermediates generated during viral replication. Formal proof of this awaits further *in vitro* studies, since blunting of the orf36-induced phosphorylation event in ATM deficient cells raises the possibility that the viral protein may simply be stimulating ATM-mediated H2AX phosphorylation.

γHV68 has an unusual relationship with H2AX activation. In addition to encoding a potential histone kinase, γHV68 encodes another protein, M2, which increases endogenous ATM activity in an *in vitro* kinase assay. However, despite activation of this kinase, no γH2AX staining is detected in M2-expressing cells, even after treatment with DNA damaging agents [66]. This observation suggests that γHV68 M2 may specifically inhibit the ability of ATM to induce H2AX phosphorylation. γHV68 M2 is not the only viral protein known to inhibit H2AX activation. Expression of the vIRF1 protein from Kaposi's sarcoma herpes virus (KSHV) was

able to block the induction of both ATM and γH2AX by the topoisomerase inhibitor, etoposide [67].

Viral control of γH2AX induction could also be at the level of CKII-mediated phosphorylation of HP1β, the recently identified prerequisite step for H2AX phosphorylation [18]. The cellular co-repressor, KAP1, recruits HP1 family proteins to silence murine leukaemia virus (MLV) [68], and the HP1 isoform, HP1α, is known to be recruited to HIV-1 genomes [69]. Many herpesvirus genomes, including HSV-1 [70], human herpes virus 6 (HHV6) [71] and HCMV [72], are also known to co-localize with HP1 proteins in cells in which viral gene expression is repressed. This is likely an attempt to silence incoming viral genomes, and therefore may not directly affect host chromatin and γH2AX activation. However, whether these viruses have developed any counter-attack strategies against HP1 and whether these impact γH2AX activation remains to be seen. It is also interesting to note that KAP1 is phosphorylated on S824 in response to DNA damage [22] and phosphorylation of KAP1 on a different residue (S473) compromises the interaction of KAP1 with HP1β [73]. Whether viral induction of DNA damage signaling leads to phosphorylation of KAP1 on either site, and the potential implications of this modification for HP1β remain to be seen. Other viral proteins such as polyomavirus agnoprotein have been shown to bind HP1α, although the significance of this is unclear [74]. If HP1β is unable to be phosphorylated by CKII, it is constitutively bound to chromatin and prevents phosphorylation of H2AX in response to damage. Targeting of CKII might therefore be an attractive viral strategy to control H2AX phosphorylation and downstream signaling in response to damage.

4.4 A plethora of other post-translational modifications

Post-translational modifications of histones are a central theme in the recruitment of DNA repair proteins to sites of double strand breaks. Following phosphorylation of H2AX, mediator proteins are recruited and the H2AX surrounding the break site is ubiquitinated (reviewed in [6]). Poorly understood chromatin remodeling events then ensue, culminating in the exposure of methylated histone H4K20, the target of cellular SET domain methyltransferases and the binding site for the DNA damage protein 53BP1 [10,11]. We have discussed viral strategies to control the phosphorylation and ubiquitination of histones involved in DNA repair, but viral targeting of this methylation step is as yet undiscovered. A recent report hints that examples of viral control at this level may emerge. Infection with paramecium bursaria chlorella virus 1 (PBCV-1) delivers a virion-encoded SET domain histone methyltransferase (vSET) that directly modifies chromatin by methylating histone H3K27 [75]. Although H3K27 is not known to be directly involved in DNA repair, this paper demonstrates that viruses can capture histone methyltransferase functions from their hosts. It will be interesting to see whether virally-encoded histone methyltransferases are part of the weaponry used by viruses in their attempt to commandeer chromatin and control DNA repair pathways.

Acetylation of histones also provides an additional level of control in the damage signaling pathway. Tip60 is recruited to DSBs, where it acetylates histones [76]. This acetylation has a role in damage-induced histone exchange, since Tip60 is required for the mobilization of γH2AX immediately after break induction. Tip60 acetylates H2AX on K5, which promotes the UBC13-mediated polyubiquitination of γH2AX before releasing it from chromatin, and replacing it with unmodified H2AX [9,77]. Tip60 forms a stable complex with ATM and acetylates it in response to damage, facilitating ATM autophosphorylation [25]. In a similar way, DNA-PKcs also binds to Tip60, an event required for the activation of this kinase [78]. These interactions imply that viral targeting of Tip60 would result in significant effects on DNA damage signaling and repair. So far, two viruses have been shown to interact directly with Tip60 [79,80]. The most well studied example is HIV-1 Tat, for which Tip60 (Tat-interacting protein 60KDa) was named [79]. Interaction of Tat with Tip60 inhibits Tip60 HAT

activity, presumably in an attempt to limit acetylation of histones on host transcriptional units [79,81]. Adenovirus E1A binds TRRAP and Gcn5, components of the Tip60 HAT and nucleosome remodeling complex [82]. Given the recently appreciated significance of Tip60 and histone acetylation in the DNA damage response, it is likely that more examples of viral control at this level will emerge.

InadditiontoitseffectsonH2AX phosphorylation (discussed in section 4.3), γHV68 also impacts histone acetylation. The γHV68 protein M2 binds to a damaged DNA-binding protein 1 (DDB1)-containing complex that contains ATM and histone H3 and H4 [66]. Histone H3 and H4 acetylation was enhanced in M2-expressing cells, both in the presence and absence of DNA damaging agents. Since M2 expression also leads to ATM activation, the authors suggested that these histone acetylation events might induce chromatin remodeling, which in turn is known to induce autophosphorylation of ATM [28,66].

There are undoubtedly many more examples of histone acetylation controlled at multiple levels, either directly or indirectly by viruses. For example, the ICP0 protein of HSV-1 inhibits deacetylation of histones by redistributing class II HDACs [83] and displacing HDAC1/2 from the CoREST/REST/LSD1 complex [84]. ICP0 also degrades DNA-PKcs [85]. These seemingly disparate observations may be linked by the fact that DNA-PKcs serves to phosphorylate and inactivate the HAT, Gcn5 [86]. Gcn5 is normally recruited to DSB sites where it contributes to the increase of H3 and H4 histone acetylation at sites flanking the break [87]. It therefore appears that ICP0 may have evolved to target acetylation from both directions: by indirectly activating acetyltransferases and by blocking the activity of HDACs.

4.5 Prolonging the signal by targeting the γH2AX phosphatases

Although H2AX phosphorylation has long been known to be a key event in the induction of the DNA damage signaling cascade, the cellular players controlling the resolution of this signal were until recently not characterized. However, it has now been shown that protein phosphatase 2A (PP2A) dephosphorylates γH2AX. When PP2A was silenced by RNA interference, γH2AX foci persisted, DNA repair was inefficient, and cells were hypersensitive to DNA damaging agents [88]. PP2A may also play a role in the regulation of ATM autophosphorylation. ATM interacts with PP2A in undamaged cells but exposure to IR induces the phosphorylation-dependent dissociation of PP2A from ATM [89]. These data suggest that PP2A is required to resolve γH2AX foci and has an important role in both activation of ATM and the completion of repair after damage.

There are a number of examples of viruses targeting PP2A. The best characterized of these is SV40 small T antigen (STag) which interacts with PP2A and inhibits its activity. The interaction between STag and PP2A is required for SV40-mediated transformation of human cells but the exact mechanism by which this transformation is afforded is unclear. SV40 is known to induce DNA damage signaling [61,90] including LTag-mediated induction of γH2AX foci [58]. Therefore, it seems possible that large and small T antigens co-operate during a SV40 infection to prolong the phosphorylation mark on H2AX. This could be favorable for the virus, since it has been demonstrated that SV40 replication is inhibited in situations where H2AX phosphorylation might be compromised, for example in the presence of an ATM inhibitor [90]. The SV40-related viruses, polyomavirus and John Cunningham virus (JCV), also have proteins that interact with PP2A. PP2A associates with and dephosphorylates JCV agnoprotein and downregulation of PP2A by siRNA inhibited JCV replication [91].

Adenovirus also has a multifaceted relationship with PP2A and H2AX phosphorylation. Ad E4orf4 binds and relocalizes PP2A, and this interaction mediates all the known functions of E4orf4 [92] including the downregulation of Myc, possibly to counteract negative effects of Myc on Ad replication [93]. Ad is not alone in using PP2A to control myc expression; myc is

upregulated in a PP2A-dependent manner by both Py and SV40 small T antigen [94,95]. Other Ad proteins also affect DNA damage signaling and PP2A. E4orf6 interacts with E1B55K and together these form an ubiquitin ligase complex that targets several DNA repair proteins for proteasome-mediated degradation (reviewed in [96]). However, it was subsequently found that E4orf6 alone is necessary and sufficient to radiosensitize human tumor cells, suggesting that E4orf6 has additional functions in DNA repair that are independent of E1B55K [97]. Cells expressing E4orf6 displayed phosphorylation of H2AX and DNA-PKcs that extended beyond time points when DNA repair should be completed and these signaling proteins would normally be returned to their dephosphorylated state. Expression of E4orf6 did not appear to inhibit directly the re-ligation of damaged DNA ends, but the viral protein could inhibit the activation of PP2A normally observed in response to irradiation [98]. The authors suggest that the resultant prolonged signaling of DNA damage in the presence of E4orf6 falsely alerts the cell that significant levels of unrepaired damage remain and this initiates caspase-dependent and independent cell death.

While viruses such as SV40 and Ad inhibit PP2A, other viruses or viral proteins induce the activity of the cellular phosphatase. These include west nile virus (WNV) capsid protein, HIV-1 Vpr and hepatitis C virus (HCV) NS5A [99-103]. In the case of the WNV capsid protein, this PP2A activation is manifested by viral targeting of I(2)(PP2A), a PP2A inhibitor [100]. The consequences of these interactions on the γH2AX signal and viral manipulation of the DNA damage signal and cellular chromatin remain to be determined.

A second phosphatase acting on γH2AX was recently identified. Like PP2A, protein phosphatase 4 (PP4) can dephosphorylate γH2AX *in vitro* and in cells. Depletion of PP4 in cells resulted in a sustained presence of Mdc1 and Mre11 on damaged DNA, prolonged checkpoint arrest, defective repair, and hypersensitivity to DNA replication inhibitors [104, 105]. PP4 appears to have a closely related but distinct role from PP2A in mediating the dephosphorylation of H2AX. While PP2A acted on γH2AX formed by various types of exogenous damage, PP4 mainly dephosphorylated γH2AX generated during DNA replication stress [105]. This lack of redundancy led the authors to suggest that distinct phosphatases may differentially regulate γH2AX originating from different types or levels of DNA damage [105]. If this is the case, one might expect viruses would have evolved to target both phosphatases in order to control H2AX phosphorylation throughout the cell cycle. The fact that PP4 negatively regulates HDAC3 activity, combined with the observation that phosphorylation by CKII is required for HDAC3's deacetylase activity [106], suggests that PP4 may have a more widespread role in regulating DNA damage and chromatin remodeling than is currently appreciated. Future work will reveal if viruses have indeed also evolved to target PP4.

5. Cellular implications of viral control of cellular histone modifications

The widespread or dysregulated H2AX phosphorylation seen during viral infections may have severe consequences for the cell. This section will discuss how viral control of histone post-translational modifications could impact DNA repair and transformation.

5.1 Implications for DNA repair

In yeast, H2A phosphorylation-mutant strains exhibit a decreased ability to perform end-joining reactions, suggesting that this histone phosphorylation event is important in DNA repair [107]. γH2AX foci do not form after irradiation in mice in which the H2AX cannot be phosphorylated or in which a glutamic acid substitution mimics a constitutively phosphorylated H2AX. Moreover, although both of these H2AX mutants allowed initial recruitment of downstream repair factors such as Nbs1 to the damaged DNA, these proteins were not maintained at the break site [108]. Metaphase spreads and spectral karyotype analysis of

fibroblasts from H2AX-/- mice showed a marked increase in chromosomal abnormalities, including chromatid breaks, dicentric chromosomes, random translocations and complex rearrangements [109]. H2AX null cells were extremely sensitive to IR and demonstrated a defective capacity for DNA repair, as measured by DNA fragmentation assays, class switch recombination assays, gene targeting efficiencies, and homologous recombination experiments [109,110]. Thus, improperly regulated H2AX phosphorylation, as seen during the viral infections discussed above, may result in aberrant recruitment of DNA damage responders and lead to faulty DNA repair. This could result in repair proteins being recruited to undamaged cellular DNA, accumulation of cellular mutations, "repair" of viral DNA, or prevention of maintenance of repair factors at sites of cellular damage. An example of a viral protein changing histone phosphorylation status and affecting the cellular capacity for repair comes from HCV. Here the NS3/4A protein interacts with and mislocalizes ATM, causes delayed dephosphorylation of ATM and γH2AX following ionizing irradiation, and impairs the ability of the cell to repair exogenous damage as assessed by a comet assay [111].

5.2 Implications for transformation

Recent studies have suggested that aberrant, pre-cancerous cell division can trigger H2AX phosphorylation [112,113]. This is thought to be a protective mechanism since the downstream signaling events arrest cell cycle and direct the cell towards apoptosis, thereby limiting malignant progression. The authors predicted that progression from pre-cancerous lesions to mature tumors occurs in cells defective in their DNA damage response components. These cells are therefore able to evade checkpoint control, suggesting that inactivation of the DNA damage response may be a contributing factor in the switch between early lesions and cancer. In support of this prediction, there is evidence that both H2AX alleles are required for optimal protection against tumorigenesis in mice [114] and that improper levels of H2AX expression in humans may also contribute to the malignant progression (reviewed in [115]). Viral inhibition of H2AX phosphorylation or viral induction of premature γH2AX dephosphorylation might therefore be a novel mechanism by which these viruses contribute to cellular transformation.

There is also evidence of more direct links between chromatin modifications and viral transformation. Ad E1A induces a genome-wide relocalization of p300/CBP to ensure that these HATs are concentrated at genes the virus needs to be acetylated/expressed, and sequestered from genes that the virus requires to be hypoacetylated/silenced [116]. This dramatic effect of a single viral protein highlights the importance of chromatin modifications to successful infection and suggests that many more examples of virally-induced global epigenetic reprogramming will be uncovered. The overall effect of Ad E1A was global hypoacetylation of H3K18 and hypomethylation of H3K4 [117]. The acetylation changes were found to be directly attributable to the interaction of E1A with p300/CBP, since viral mutants unable to bind this HAT had no effect on the H3K18 acetylation profile. Interestingly, SV40 LTag binds and is acetylated by p300/CBP [118] and expression of SV40 LTag also leads to hypoacetylation of H3K18 [117]. This interesting parallel led the authors to propose that hypoacetylation of histones may be a general theme used by tumor virus oncoproteins in their quest to transform cells. In support of this hypothesis, the pattern of global chromatin changes seen with E1A expression (hypoacetylation of H3K18 and hypomethylation of H3K4) was strikingly similar to those seen in primary prostate cancers with poor prognosis [119].

6. Chromatin as an anti-viral defense mechanism

There are many examples of control of viral gene expression by assembly of chromatin on incoming or latent viral genomes. These have been previously discussed [120,121] and are extensively addressed elsewhere in this focus edition. In addition to traditional anti-viral defenses such as the interferon response and apoptosis, it is becoming clear that

chromatinization of viral genomes should be considered part of the host's defense to invading pathogens. For example, transcription from many viral promoters, including the HCMV IE promoter, is known to be stimulated by treatment with HDAC inhibitors [72]. More recently, chromatin control of viral latency has also emerged as an exciting area of study. Reactivation of HSV-1, EBV, and KSHV from latency is stimulated by treatment of cells with HDAC inhibitors, suggesting a role for HDACs in the silencing of viral genomes [120,122-125]. HSV-1 ICP0 is a strong transactivator of viral and cellular promoters, is required for lytic replication at low multiplicities of infection, and is necessary for the efficient reactivation of quiescent viral genomes (reviewed in [126]). We have observed that ICP0 leads to degradation of histone ubiquitin ligases RNF8 and RNF168 and a loss of ubiquitinated forms of H2A (CEL, MSC and MDW unpublished observations). Ubiquitinated H2A is strongly linked to transcriptional repression, with roles in silencing the inactive X chromosome (Xi) in mice and the promoters of polycomb-target genes such as Hox (reviewed in [127]). During lytic infection, incoming HSV-1 genomes are quickly marked by cellular histones, presumably in an attempt to silence expression of viral genes, and the latent viral genome is also nucleosome bound (reviewed in [120]). We propose that histone ubiquitination may be a novel means to silence pathogens and that removing this repressive mark on viral genomes may be one way in which ICP0 overcomes silencing during the lytic cycle and during reactivation from latency.

7. Summary and future directions

Cellular chromatin presents a challenge to viral transcription and viral manipulation of the DNA damage response. Chromatin plays an important role in the lifecycle of a diverse set of viruses, and we have reviewed some of the counter-strategies that these viruses have evolved to navigate, exploit or circumvent this potential obstacle. Viral control of histone methylation, phosphorylation and acetylation and the roles of these modifications in DNA repair are being increasingly well understood. Examples of viral interactions with cellular chromatin modifications covered in this review are summarized in Figure 1. As more post-translational modifications are uncovered, it is likely that this list will continue to expand. For example, recent work in yeast is beginning to uncover a role for histone sumoylation in DNA damage responses [7] and it has been suggested that sumoylation may serve as a block to other histone modifications [128]. It remains to be seen whether sumoylation is inversely correlated with any of the repair-associated post-translational modifications we have discussed, and whether viruses have evolved to exploit this potential blocking function of histone sumoylation. The reversible nature of histone modifications provides an extra level of control in the dynamics of chromatin remodeling.

There are already examples of virally-encoded histone kinases and viral interactions with histone phosphatases. Future work will also reveal whether viruses have evolved to encode histone phosphatases, deubiquitinases and demethylases. Investigation of the interplay between viruses, DNA repair, and the multitude of histone post-translational modifications is a productive area of research. Studying viral control of cellular epigenetics not only provides insights into the role of chromatin in DNA repair but may also lead to new opportunities for therapeutic intervention [120,129,130].

Acknowledgments

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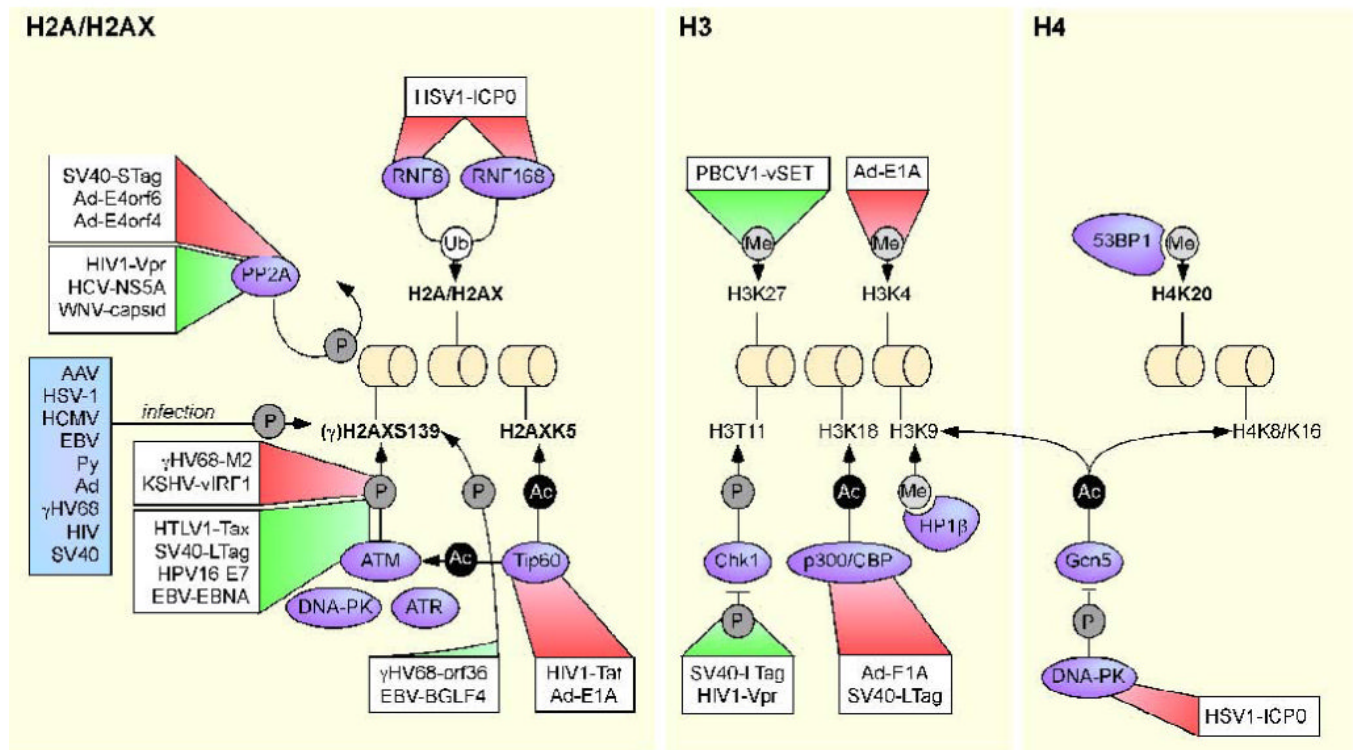
Figure 1. Viral control of cellular histone modifications
Chromatin remodeling proteins and histone post-translational modifications known to be targeted by viruses are depicted. Histone post-translational modificatons which have a known role in DNA repair are shown in bold. Effects of the viral proteins (boxed) on the chromatin-related substrates are indicated by the shaded triangles. Red triangles indicate interactions that are inhibitory to the pathways or cellular factors targeted and green triangles denote interactions that promote the activity of the pathways or cellular factors targeted. For simplicity, not all histone targets of the cellular enzymes are shown. The interactions depicted are limited to those covered in this review and many more examples of associations between viral and cellular proteins exist.

Table 1

Virus Names and Abbreviations

VIRUS NAME	ABBREVIATION
Herpes Simplex Virus	HSV
Human Cytomegalovirus	HCMV
Epstein Barr Virus	EBV
Kaposi's Sarcoma Herpes Virus	KSHV
Simian Virus 40	SV40
Adeno-associated Virus	AAV
Adenovirus	Ad
Human Immunodeficiency Virus	HIV
Human T-cell Lymphotropic Virus	HTLV
John Cunningham Virus	JCV
Murine Gamma Herpes Virus 68	γHV68
Murine Leukaemia Virus	MLV
Hepatitis C Virus	HCV
West Nile Virus	WNV
Human Herpes Virus 6	HHV6
Polyoma Virus	Py
Human Papillomavirus	HPV