A core viral protein binds host nucleosomes to sequester immune danger signals

Daphne C. Avgousti1,2, Christin Herrmann3,4, Katarzyna Kulej1,2, Neha J. Pancholi2,3, Nikolina Sekulic4,5, Joana Petrescu2,6, Rosalynn C. Molden3, Daniel Blumenthal7, Andrew J. Paris8, Emigdio D. Reyes9,12, Philomena Ostapchuk9, Patrick Hearing9, Steven H. Seeholzer10, G. Scott Worthen11, Ben E. Black4,5, Benjamin A. García4,5 & Matthew D. Weitzman1,2

Viral proteins mimic host protein structure and function to redirect cellular processes and subvert innate defenses1. Small basic proteins compact and regulate both viral and cellular DNA genomes. Nucleosomes are the repeating units of cellular chromatin and play an important part in innate immune responses2. Viral-encoded core basic proteins compact viral genomes, but their impact on host chromatin structure and function remains unexplored. Adenoviruses encode a highly basic protein called protein VII that resembles cellular histones. Although protein VII binds viral DNA and is incorporated with viral genomes into virus particles3–5, it is unknown whether protein VII affects cellular chromatin. Here we show that protein VII alters cellular chromatin, leading us to hypothesize that this has an impact on antiviral responses during adenovirus infection in human cells. We find that protein VII forms complexes with nucleosomes and limits DNA accessibility. We identified post-translational modifications on protein VII that are responsible for chromatin localization. Furthermore, proteomic analysis demonstrated that protein VII is sufficient to alter the protein composition of host chromatin. We found that protein VII is necessary and sufficient for retention in the chromatin of members of the high-mobility-group protein B family (HMGB1, HMGB2 and HMGB3). HMGB1 is actively released in response to inflammatory stimuli and functions as a danger signal to activate immune responses6,7. We showed that protein VII can directly bind HMGB1 in vitro and further demonstrated that protein VII expression in mouse lungs is sufficient to decrease inflammation-induced HMGB1 content and neutrophil recruitment in the bronchoalveolar lavage fluid. Together, our in vitro and in vivo results show that protein VII sequesters HMGB1 and can prevent its release. This study uncovers a viral strategy in which nucleosome binding is exploited to control extracellular immune signalling.

As viruses commandeer cellular functions to promote viral production, they induce numerous cellular changes. Manipulation of host chromatin is important for viral takeover of cellular functions8–11. Although there are known examples of viral control by manipulating gene expression2,9,12, an alternative strategy for immune evasion could exploit cellular chromatin to affect extracellular signalling. Genomes of DNA viruses are compacted and packaged into virus particles with small basic proteins encoded by the host or virus. Adenoviruses encode protein VII, a small basic protein packaged with viral genomes3–5. We hypothesize that protein VII contributes to host chromatin manipulation. We investigated protein VII localization during infection, and found it present in both viral replication centres stained for viral DNA-binding protein (DBP; Fig. 1a and Extended Data Fig. 1a), and in cellular chromatin stained for histone H1 and 4′,6-diamidino-2-phenylindole (DAPI; Fig. 1b). These observations suggest that protein VII functions on both viral and host genomes. To determine the impact of protein VII on cellular chromatin, we generated cell lines with inducible expression. In multiple cell types we observed that protein VII accumulation altered nuclear DNA into a punctate appearance (Fig. 1c and Extended Data Fig. 1b, c). We tested whether other basic proteins produce similar effects on chromatin. Viral core protein V and the precursor of protein VII (preVII) localized to nucleoli and did not affect chromatin appearance (Extended Data Fig. 1d). Taken together, our data demonstrate that protein VII is sufficient to alter cellular chromatin and is distinct from other small basic proteins.

To affect cellular chromatin at the nucleosome level during infection, we reasoned that protein VII must be abundant and associated with histones. Acid extraction of histones4,13 from infected cells revealed viral proteins VII and V isolated with cellular histones (Fig. 1d), as verified by western blot (Extended Data Fig. 2a) and mass spectrometry (MS). Protein VII abundance was comparable to cellular histone levels (Fig. 1d). We further analysed association of protein VII with cellular chromatin by salt fractionation of nucleic acids14. We found protein VII with cellular histones and DNA in high-salt fractions (Fig. 1e and Extended Data Fig. 2b–d). Ectopically expressed protein VII is also found in high-salt fractions, in contrast to other viral proteins that elute at low salt (Fig. 1e and Extended Data Fig. 2b). These data suggest that protein VII is highly abundant and tightly associated with cellular chromatin.

We hypothesized that protein VII interacts with chromatin by forming complexes with DNA, histones or nucleosomes, and examined protein VII interactions in vitro. Purified recombinant protein VII binds to DNA9 (Extended Data Fig. 2e,f). We reconstituted nucleosomes in vitro with recombinant histone proteins on 195 base pairs (bp) of DNA17. Protein VII changed nucleosome mobility upon native gel electrophoresis (SDS–PAGE), and confirmed that complexes contained core histones with protein VII (Fig. 1f, bottom). Unlike protamines13, protein VII forms complexes with nucleosomes but does not appear to replace histones. Next, we examined whether protein VII association

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1Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania 19104, USA. 2Division of Cancer Pathobiology, Department of Pathology and Laboratory Medicine, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. 3Cell and Molecular Biology Graduate Group, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania 19104, USA. 4Department of Biochemistry and Biophysics, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania 19104, USA. 5Department of Pulmonary, Allergy, and Critical Care Medicine, Hospital of the University of Pennsylvania, and the Department of Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, Pennsylvania 19104, USA. 6Division of Neopatology, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. 7Division of Neonatology, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. 8Division of Neonatology, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. 9Division of Molecular Genetics and Microbiology, School of Medicine, Stony Brook University, Stony Brook, New York 11794, USA. 10Protein and Proteomics Core, Children’s Hospital of Philadelphia, Philadelphia, Pennsylvania 19104, USA. Present address: Biotechnology Centre of Oslo and Department of Chemistry, University of Oslo, Oslo 0316, Norway.
with nucleosomes affects DNA wrapping using micrococcal nuclease (Mnase) digestion followed by DNA fragment analysis. We found that protein VII pauses nucleosomal DNA digestion at approximately 165 bp, the point at which DNA strands cross over the nucleosome dyad (Fig. 1g and Extended Data Fig. 3a). By contrast, nucleosome digestion alone paused with core particles at approximately 150 bp, suggesting that protein VII encumbers DNA access. Unlike linker histone binding that is dependent on DNA length, protein VII protects against Mnase digestion on the nucleosome core particle of 147 bp (Extended Data Fig. 3b). Protein VII alone protects DNA from Mnase digestion, as would be expected given its role in the viral core. Together, these data demonstrate that protein VII binds directly to nucleosomes and limits DNA accessibility at the DNA entry/exit site.

Post-translational modifications (PTMs) on histones are central to regulating chromatin structure. Owing to the histone-like nature of protein VII (ref. 3), we hypothesized that it is subject to post-translational modification similar to histones. PreVII was previously proposed to be acetylated by N-terminal addition during protein synthesis. We noted that protein VII contains conserved lysine residues within an AKKRS motif, similar to the commonly modified canonical histone motif ARSK. We therefore purified protein VII from histone extracts over an adenovirus infection time course by reverse-phase high-performance liquid chromatography (RP-HPLC; Fig. 2a and Extended Data Fig. 4). Consistent with observations from histone extracts (Fig. 1d), protein VII levels were comparable to endogenous histones. We digested purified protein VII and preVII with chymotrypsin to distinguish the two proteins, and analysed peptides by tandem mass spectrometry (MS/MS). We identified several PTMs, with modifications (Fig. 2b and Extended Data Figs 5, 6b). Interestingly, we identified acetylation sites on ectopically expressed protein VII but not on protein VII in virus particles (Extended Data Fig. 6a). We speculated that this provides a possible mechanism for distinguishing protein VII bound to cellular chromatin from protein destined for packaged virus. To investigate the relevance of the identified PTMs, we mutated identified sites in infected cells. Underlined residues represent moieties that may also be modified in identified peptides (see Extended Data Fig. 5). ac, acetylated; P, phosphorylated.

Figure 1 | Protein VII is sufficient to alter chromatin and directly binds nucleosomes. a, b, Adenovirus serotype 5 (Ad5)-infected small airway epithelial cells (SAECs) stained for protein VII (red) with DBP (a) or histone H1 (b) (green), and DAPI (grey, blue in merge). hpi, hours post-infection. c, Protein–VII–haemagglutinin (HA)-induced cells over 4 days showing HA (green) and DAPI (grey, blue in merge). d, doxycycline. a-c, Scale bars, 10 μm. d, SDS–PAGE of histone extract from Ad5-infected cells showing protein V and protein VII. e, Western blot of chromatin fractionation from nuclei of Ad5-infected cells, induced for protein–VII–HA, or unind. f, Protein VII binds to nucleosomes (Nucs). Protein bands from native gel stained with Coomassie (top) were subjected to two-dimensional analysis by SDS–PAGE (bottom). g, Protein VII protects nucleosome complexes from Mnase digestion. Bioanalyzer curves represent nucleosome alone (black) or protein–VII–nucleosome complexes (orange).

Figure 2 | Post-translational modifications on protein VII contribute to chromatin localization. a, RP-HPLC analysis of histone extracts. Viral proteins V, VII and preVII are indicated at 24 hpi. b, Primary sequence of protein VII with modified residues identified in infected cells. Underlined residues represent sites that may also be modified in identified peptides (see Extended Data Fig. 5). c, Immunofluorescence showing DAPI (grey, blue in merge) and protein VII (red) as wild type or with alanine substitutions at PTM sites (ΔPTM), K3A or K3Q. Scale bar, 10 μm.

Post-translational modifications on protein VII contribute to chromatin localization. a, RP-HPLC analysis of histone extracts. Viral proteins V, VII and preVII are indicated at 24 hpi. b, Primary sequence of protein VII with modified residues identified in infected cells. Underlined residues represent sites that may also be modified in identified peptides (see Extended Data Fig. 5). c, Immunofluorescence showing DAPI (grey, blue in merge) and protein VII (red) as wild type or with alanine substitutions at PTM sites (ΔPTM), K3A or K3Q. Scale bar, 10 μm.
related to immune responses (Extended Data Fig. 7c). The top four proteins enriched in chromatin fractions by protein VII were SET (also known as TAF-1), a protein previously shown to interact with protein VII22,23, and HMGB1, HMGB2 and HMGB3 (Fig. 3a). The HMGB proteins are alarmins with multiple functions as activators of immunity and inflammation24. HMGB1 is a nuclear protein normally only transiently associated with chromatin24,25. Cells also release HMGB1 as an extracellular danger signal that promotes immune responses after injury or infection26. We confirmed increased chromatin association of HMGB1 and HMGB2 by analysis of fractionated nuclei, upon protein VII expression and during adenovirus infection (Fig. 3b). We verified that these changes are not due to altered HMGB1 expression levels (Extended Data Fig. 8a, b). We demonstrated direct binding of recombinant protein VII to HMGB1 in vitro and confirmed HMGB1 co-immunoprecipitation with protein VII (Fig. 3c). We visually observed reorganization of HMGB1 and HMGB2 distribution upon protein VII expression, and at late stages of infection (Fig. 3d–f and Extended Data Fig. 8g). We also showed reorganization of HMGB1 distribution by vector transduction to express protein-VII–monomeric GFP (mGFP). Recovery of FRAP signal in time-course images (left) with quantification and diffusion coefficients (right). Scale bar, 5 μm. i, FRAP experiment with HMGB1–monomeric GFP (mGFP). Recovery of FRAP signal in time-course images (left) with quantification and diffusion coefficients (right). Scale bar, 5 μm. D, diffusion coefficient; t(1/2), halftime of recovery. j, Schematic showing loxp strategy for deleting protein VII. j, Western blots comparing 293 and 293-Cre cells infected with Ad5−flox−VII virus. k, Salt fractionation in nuclei from j.

Figure 3 | Protein VII directly binds HMGB1 and is necessary for retention of the alarmin in cellular chromatin. a, Volcano plot for proteomics analysis of one representative biological replicate of the high-salt fraction. The y axis represents −log₂ statistical P value and the x axis represents log₂ protein fold-change between uninduced or protein-VII-expressing cells (homoscedastic two-tailed t-test, P < 0.05 red dots; n = 3 technical replicates). b, Nuclear fractionation shows that HMGB1 and HMGB2 normally elute from nuclei at low salt concentrations but are retained in high-salt fractions by protein-VII–HA. d, day; dox, doxycycline. c, Protein VII interacts with HMGB1 in pull-down of recombinant HMGB1–glutathione S-transferase (GST) (left, Coomassie-stained SDS–PAGE) and immunoprecipitation of HMGB1 (right, western blots). d, e, Protein VII expression alters localization of HMGB1 (d) and HMGB2 (e). Immunofluorescence shows protein-VII–HA (green) colocalized with HMGB1 (d) and HMGB2 (e) (red) in cellular chromatin (DAPI, grey, blue in merge). f, Same as d at 18 hpi with Ad5 DBP (green). g, Protein-VII–GFP relocalizes HMGB1 (red) to chromatin with DAPI (grey, blue in merge). rAd, recombinant adenosivirus. d–g, Scale bars, 10 μm.

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We then tested whether protein VII prevents HMGB1 release in cell donors (Fig. 4a). Consistent with cell culture experiments, we demonstrated adenovirus infection in precision-cut lung slices from human immune responses. We therefore visualized endogenous HMGB1 during the early phase of infection in human immune responses (Extended Data Fig. 10d). Our study reveals that chromatin localization, and that protein VII affects the chromatin association of host proteins. Finally, we show that protein VII in cellular chromatin leads to sequestration of HMGB1 in vivo (Fig. 4d). Together, these data suggest that protein VII functions in cellular chromatin to retain HMGB1 as a mechanism to blunt immune responses.

In addition to known roles on packaged viral DNA, we show that protein VII interacts with cellular chromatin and binds nucleosomes. We suggest that protein VII PMTs contribute to chromatin localization, and that protein VII affects the chromatin association of host proteins. Finally, we show that protein VII in cellular chromatin leads to sequestration of HMGB1 family members, contributing to abrogated immune responses (Extended Data Fig. 10d). Our study reveals that chromatin retention of signalling molecules by a viral protein may represent a previously unrecognized immune evasion strategy.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Figure 4 | Protein VII prevents HMGB1 release. a, Precision-cut lung slices infected with Ad5 or transduced to express protein VII–GFP. Endogenous HMGB1 (red) is redistributed in cells with virus (DBP-top) and protein–VII–GFP (bottom). b, Protein–VII–GFP is sufficient to inhibit HMGB1 and HMGB2 release in THP-1 cells. Numbers indicate relative intensities of bands quantified with ImageJ. c, Enzyme-linked immunosorbent assay (ELISA)-based quantification of HMGB1 in supernatants. d, Schematic for investigating protein VII in a mouse lung injury model. e, Expression of protein VII–VII–GFP decreases HMGB1 in mouse BAL fluid quantified by ELISA. f, Neutrophils in bronchoalveolar lavage (BAL) fluid are significantly fewer in mice expressing protein–VII–GFP. Data are mean ± s.d., biological replicates: *P = 0.01, **P = 0.001, ***P = 0.003, ****P = 0.0003, t-test. We then tested whether protein VII prevents HMGB1 release in cell culture and in vitro models. We expressed GFP or protein–VII–GFP in macrophage-like THP-1 cells, and confirmed that protein–VII–GFP was sufficient to alter chromatin and HMGB1 localization (Extended Data Fig. 9d). Cells were treated to stimulate inflammasomes, and HMGB1 content was analysed in supernatants. Protein VII expression resulted in reduced levels of HMGB1 and HMGB2 in supernatants (Fig. 4b, c). Subsequently, we employed a murine model of lipopolysaccharide (LPS)-induced lung injury to investigate the impact of protein VII on HMGB1 release and neutrophil recruitment in vivo (Fig. 4d). We confirmed that protein VII was expressed in transduced mouse lungs (Extended Data Fig. 10a–c) and retained mouse HMGB1 (Extended Data Fig. 9e, f). We exposed mice to inhaled LPS to induce HMGB1 release and neutrophil recruitment to alveoli. Bronchoalveolar lavage fluid obtained 24 h after LPS exposure showed that mice transduced to express protein VII had significantly less HMGB1 and fewer neutrophils than mice expressing GFP (Fig. 4d–f). Together, these data suggest that protein VII functions in cellular chromatin to retain HMGB1 as a mechanism to blunt immune responses.

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1. Eldes, N. C. & Malik, H. S. The evolutionary conundrum of pathogen mimicry. Nature Rev. Microbiol. 7, 787–797 (2009).
2. Smale, S. T., Tarakhovsky, A. & Natoli, G. Chromatin contributions to the regulation of innate immunity. Annu. Rev. Immunol. 32, 489–511 (2014).
3. Litchwa, M. A. & Sung, M. T. A histone-like protein from adenovirus chromatin. Nature 267, 552–554 (1977).
4. Chatterjee, P. K., Vaya, M. E. & Flint, S. J. Adenoviral protein VII packages intracellular viral DNA throughout the early phase of infection. EMBO J. 5, 1635–1644 (1986).
5. Vaya, M. E., Rogers, A. E. & Flint, S. J. The structure of nucleic acid proteins released from adenovirons. Nucleic Acids Res. 11, 441–460 (1983).
6. Kang, R. et al. HMGB1 in health and disease. Mol. Aspects Med. 40, 1–116 (2014).
7. Lotze, M. T. & Tracey, K. J. High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. Nature Rev. Immunol. 5, 331–342 (2005).
8. Paschos, K. & Allday, M. J. Epigenetic reprogramming of host genes and virus. Viral Micro. 18, 439–447 (2010).
9. Marazzi, I. et al. Suppression of the antiviral response by an influenza histone mimic. Nature 483, 428–433 (2012).
10. Ferrari, R., Berk, A. J. & Kurdistani, S. K. Viral manipulation of the host epigenome for oncogenic transformation. Nature Rev. Genet. 10, 290–294 (2009).
11. Knipe, D. M. et al. Snapshots: chromatin control of viral infection. Virology 435, 141–156 (2013).
12. Ferrari, R. et al. Adenovirus small IEA1 plays the lysine acetylases p300/CBP and tumor suppressor Rb to repress select host genes and promote productive virus infection. Cell Host Microbe 16, 663–674 (2014).
13. Wykes, S. M. & Krawetz, S. A. The structural organization of sperm chromatin. J. Biol. Chem. 278, 29471–29477 (2003).
14. Lin, S. & Garcia, B. A. Examining histone posttranslational modification patterns by high-resolution mass spectrometry. Methods Enzymol. 512, 3–28 (2012).
15. Shechter, D., Dorrman, H. L., Allis, C. D. & Hake, S. B. Extraction, purification and analysis of histones. Nature Protocols 2, 1445–1457 (2007).
16. Teves, S. S. & Henikoff, S. Salt fractionation of nucleosomes for genome-wide profiling. Methods Mol. Biol. 833, 421–432 (2012).
17. Falk, J. S. et al. Chromosomes. CENP-C reshapes and stabilizes CENP-A nucleosomes at the centromere. Science 348, 699–703 (2015).
18. White, A. E., Hieb, A. R. & Luger, K. A quantitative investigation of linker histone interactions with nucleosomes and chromatin. Sci. Rep. 6, 19122 (2016).
19. Kouzarides, T. Chromatin modifications and their function. Cell 128, 693–705 (2007).
20. Feder, M. J. & Daniell, E. A. Acetylation of histone-like proteins of adenovirus type 5 J. Virol. 35, 637–643 (1980).

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21. Robinson, C. M. et al. Molecular evolution of human adenoviruses. Sci. Rep. 3, 1812 (2013).
22. Gyurcsik, B., Haruki, H., Takahashi, T., Mihara, H. & Nagata, K. Binding modes of the precursor of adenovirus major core protein VII to DNA and template activating factor I: implication for the mechanism of remodeling of the adenovirus chromatin. Biochemistry 45, 303–313 (2006).
23. Haruki, H., Okuwaki, M., Miyagishi, M., Taira, K. & Nagata, K. Involvement of template-activating factor I/SET in transcription of adenovirus early genes as a positive-acting factor. J. Virol. 80, 794–801 (2006).
24. Scaffidi, P., Misteli, T. & Bianchi, M. E. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature 418, 191–195 (2002).
25. Sapojnikova, N. et al. Biochemical observation of the rapid mobility of nuclear HMGB1. Biochim. Biophys. Acta 1729, 57–63 (2005).
26. Lu, B. et al. Novel role of PKR in inflammasome activation and HMGB1 release. Nature 488, 670–674 (2012).
27. Koziol-White, C. J., Damera, G. & Panettieri, R. A. Jr. Targeting airway smooth muscle in airways diseases: an old concept with new twists. Expert Rev. Respir. Med. 5, 767–777 (2011).
28. Ueno, H. et al. Contributions of high mobility group box protein in experimental and clinical acute lung injury. Am. J. Respir. Crit. Care Med. 170, 1310–1316 (2004).
29. Johnson, J. S. et al. Adenovirus protein VII condenses DNA, represses transcription, and associates with transcriptional activator E1A. J. Virol. 78, 6459–6468 (2004).
30. Kohli, R. et al. Adenovirus core protein VII protects the viral genome from a DNA damage response at early times after infection. J. Virol. 85, 4135–4142 (2011).

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Author Information All proteomic raw files have been deposited in the Chorus database under project number 1047 (https://chorusproject.org/). Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.D.W. (weitzmanm@email.chop.edu).

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Author Contributions D.C.A. and M.D.W. conceived the project and designed experiments; D.C.A., C.H., N.S., J.P., N.J.P. and E.D.R. performed the experiments; D.C.A., C.H. and J.P. generated constructs and cell lines; K.K., R.C.M., S.H.S. and B.A.G. performed MS analysis; P.O. and P.H. generated Ad5-flox-VII virus and provided 293-Cre cell line; D.C.A. and D.B. performed the FRAP experiments; A.J.P. and G.S.W. conducted all mouse experiments; B.E.B. and B.A.G. designed experiments and interpreted the data; D.C.A. and M.D.W. interpreted the data and wrote the manuscript and all authors were involved in editing the manuscript.

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METHODS

Cells. Primary SAECs, U2OS, HeLa, 293, THP-1 and A549 cells were obtained from the American Type Culture Collection (ATCC) and grown according to the provider’s instructions. Cell lines were not authenticated or tested for mycoplasma. Acceptor cells for generation of inducible cell lines were provided by P. Hearing. Animal Welfare Assurance Number A3442-01. C57BL/6J male mice aged 8–10 weeks were housed in specific-pathogen-free (SPF) conditions in an animal facility at the Children’s Hospital of Philadelphia. All studies in mice were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. U2OS or HeLa acceptor cells along with plasmid expressing the Cre recombinase. Recombined clones were selected by puromycin resistance (1 μg·ml⁻¹) and induced with doxycycline (0.2 μg·ml⁻¹) to express the desired protein. Protein expression was verified by immunofluorescence and western blot. All figures shown are after 4 days of induction unless otherwise stated. Protein VII and preVII were also verified by HPLC purification and MS analysis. Point mutations were generated by gene synthesis from Genewiz. 293-Cre cells were provided by P. Hearing.

Viruses and infections. Wild-type Ad5, Ad9, Ad12 and recombinant adenovirus vectors expressing only GFP were propagated in 293 cells as previously described38. Recombinant adenovirus vector with protein VII–GFP replaced in the E1 region was a gift from D. Curiel33. Infections were carried out as described previously14 using a multiplicity of infection of 10 for primary cells and cell lines for Ad5 infections. Ad9 and Ad12 infections were carried out with a multiplicity of infection of 50 and 20, respectively. Ad5–flox VII was generated by P. Hearing and also prepared using standard methods in 293 cells. lacO sites were added flanking protein VII in the Ad5 genome resulting in protein VII deletion during infection of 293 cells expressing Cre recombinase.

Antibodies. Primary antibodies were purchased from Covance (HA MMS-101R), Abcam (H1 ab24950 and ab1791), HMGB1 ab18256, HMGR2 ab67282), Millipore (H2A 07-146, proserfactin-C AB3786), and Santa Cruz (Ku86 sc5280, tubulin sc69969). The antibodies to DBP, adenoviral late proteins, terminal protein and Ku86 (H2A 07-146, prosurfactin-C AB3786), and Santa Cruz (Ku86 sc5280, tubulin sc69969). The antibodies to DBP, adenoviral late proteins, terminal protein and Ku86 (H2A 07-146, prosurfactin-C AB3786), and Santa Cruz (Ku86 sc5280, tubulin sc69969).

Immunofluorescence. Cells were grown on glass coverslips in 24-well plates and either infected or induced with doxycycline (0.2 μg·ml⁻¹). Cells were harvested at the indicated time points, washed in PBS, fixed in 4% paraformaldehyde for 15 min and post-fixed with 100% ice-cold methanol for 5 min. Coverslips were then blocked and stained as previously described16 and mounted using ProLong Gold Antifade Reagent (Life Technologies). Immunofluorescence was visualized using a Zeiss LSM 710 Confocal microscope (Cell and Developmental Microscopy Core at UPenn) and ZEN 2011 software. Images were processed using ImageJ and assembled with Adobe CS6.

Immunoblotting. Western blot analysis was carried out using standard methods. Briefly, equal amounts of total protein lysates were separated by SDS–PAGE and transferred to a nitrocellulose membrane (Millipore) for at least 30 min at 30 V. Membranes were stained with ponceau to confirm protein loading and blocked in 5% milk in TBST containing 0.1% azide. Membranes were incubated with primary antibodies overnight, washed for 30 min in TBST and incubated with secondary antibodies conjugated to horseshad peroxidase (Jackson Laboratories) for 1 h. Membranes were washed again and proteins were visualized with Pierce ECL Western Blotting Substrate (Thermo Scientific) and detected using a Synergy G-Box.

Mice. All mice were housed in specific-pathogen-free (SPF) conditions in an animal facility at the Children’s Hospital of Philadelphia. All studies in mice were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The National Institutes of Health and the Institutional Animal Care and Use Committee, Children’s Hospital of Philadelphia Animal Welfare Assurance Number A3442-01. C57BL/6J male mice aged 8–10 weeks were used for experiments. Mice were sedated with ketamine and xylazine. Once sedated, mice underwent orotrachial intubation, as previously described37, as stated in the Methods section. Once sedated, mice underwent orotrachial intubation, as previously described37, as stated in the Methods section.

Preparation of salt fractions for MS analysis. All chemicals used for preparation of MS samples were of at least sequencing grade and purchased from Sigma-Aldrich, unless otherwise stated. The 600 mM salt fraction was used for LC-MS/MS analysis. The 0.1% Triton X-100 detergent was removed from samples before MS analysis by precipitation using chloroform (CHCl₃)-methanol (MeOH) precipitation44. The protein pellet from CHCl₃-MeOH precipitation was resuspended in 6 M urea and 2 M thiourea in 50 mM ammonium bicarbonate. Samples were reduced with 10 mM DTT for 1 h at room temperature and then carbamidomethylated with 20 mM iodoacetamide for 30 min at room temperature in the dark. Afterwards, alkylated proteins were digested first with endopeptidase Lys-C (Wako, MS grade) for 3 h, after which the solution was diluted 10 times with 20 ml ammonium bicarbonate. Subsequently, samples were digested with trypsin (Promega) at an enzyme-to-substrate ratio of approximately 1:50 for 12 h at room temperature. The samples were acidified with 5% formic acid (FA) to a pH < 3 and desalted using Poros Oligo R3 RP columns (PerSeptive Biosystems) packed in a P200 stage tip with C₈ 3 M plug (3M Bioanalytical Technologies). Purified peptide samples were dried by lyophilization and stored at −20 °C until further analysis. This procedure was carried out for three biological replicates.

Nano-LC-MS/MS and analysis of salt fractions. Samples were loaded onto a 16 cm C₁₈–AQ column (inner diameter 75 μm, 3 μm beads, Dr, Maisch GmbH, Germany) using an Easy nano-flow HPLC system (Thermo Fisher Scientific). The nano-LC was coupled to an Orbitrap Fusion Tribrid Mass Spectrometer (Thermo Fisher Scientific) via a nanoelectrospray ion source (Thermo Fisher Scientific). The samples were acquired with a resolution of 150,000 (Δm/z 1.7) together with a 120 min linear gradient from 2–30% buffer B (95% acetonitrile, 0.1% formic acid) to a (pH < 3 and desalted using Poros Oligo R3 RP columns (PerSeptive Biosystems) packed in a P200 stage tip with C₈ 3 M plug (3M Bioanalytical Technologies). Purified peptide samples were dried by lyophilization and stored at −20 °C until further analysis. This procedure was carried out for three biological replicates.
N-terminal acetylation, and fixed modification of carbamidomethyl cysteine. Each iBAQ value was log-transformed and subsequently normalized by the average protein abundance within each run. Biological process association analysis and process network enrichment were performed using the GeneGo MetaCore pathways analysis package with FDR < 5%; each Gene Ontology term was ranked using P-value enrichment.

**Purification of recombinant protein-VII–His.** Protein VII was cloned from genomic DNA isolated from adenovirus-infected HeLa cells into a PET21a backbone to generate a C-terminal hexahistidine tag. Positive colonies were selected in DH5α cells, sequenced, and transformed into BL21 (DE3) cells (NEB C2527). The purification of insoluble protein-VII–His was adapted from existing protocols to purify histone proteins from *Escherichia coli*.

Histones were prepared for MS analysis as described previously. Histones were reconstituted with reducing agent (Invitrogen) and boiled. Supernatants and lysates were precipitated overnight at 4 °C with a final concentration of 20% trichloroacetic acid (TCA) and washed and 200 μl of serum-free RPMI was added. To stimulate the inflammasome, samples were alkylated in 20 mM iodoacetamide in 50 mM ammonium bicarbonate for 30 min in the dark. Samples were digested with chymotrypsin or Arg-C, at an enzyme-to-substrate ratio of approximately 1:20 for 8 h at 37 °C. The samples were acidified to pH 2.5 with 100 μl of 1 M formic acid and desalted using a P200 stage tip columns packed with C18, 3 μm plug (3M Bioanalytical Technologies). Purified peptide samples were dried by lyophilization and stored at −20 °C until further analysis.

**Nano-LC-MS analysis of histone PTMs.** The nano-LC-MS/MS analysis was performed as previously described.

**Nano-LC-MS/MS analysis of protein VII peptides.** The nano-LC-MS/MS analysis was performed in triplicate for each sample. Samples were loaded onto a 16 cm C18–AQ column (inner diameter 75 μm, 3 μm beads, Dr. Maisch GmbH) using an Easy nano-flow HPLC system (Thermo Fisher Scientific). The nano-LC was coupled to an Orbitrap Velos Pro Mass Spectrometer (Thermo Fisher Scientific) via a nanoelectrospray ion source (Thermo Fisher Scientific). Peptides were loaded into B (0.1% formic acid) and eluted with a 45 min linear gradient from 2 to 30% buffer B (95% acetonitrile, 0.1% formic acid). After the gradient, the column was washed with 90% buffer B. Mass spectra were acquired using a data-dependent acquisition method with the top 15 most intense ions. Signals were acquired with 1,000 count charges were selected for HCD fragmentation using normalized collision energy of 36, a maximum injection time of 100 ms and an AGC target of 50,000. Fragments were analysed in the orbitrap.

**Nano-LC-MS/MS analysis of protein VII peptides.** The nano-LC-MS/MS analysis was performed as previously described. The nano-LC-MS/MS analysis was performed in triplicate for each sample. Samples were loaded onto a 16 cm C18–AQ column (inner diameter 75 μm, 3 μm beads, Dr. Maisch GmbH) using an Easy nano-flow HPLC system (Thermo Fisher Scientific). The nano-LC was coupled to an Orbitrap Velos Pro Mass Spectrometer (Thermo Fisher Scientific) via a nanoelectrospray ion source (Thermo Fisher Scientific). Peptides were loaded into B (0.1% formic acid) and eluted with a 45 min linear gradient from 2 to 30% buffer B (95% acetonitrile, 0.1% formic acid). After the gradient, the column was washed with 90% buffer B. Mass spectra were acquired using a data-dependent acquisition method with the top 15 most intense ions. Signals were acquired with 1,000 count charges were selected for HCD fragmentation using normalized collision energy of 36, a maximum injection time of 100 ms and an AGC target of 50,000. Fragments were analysed in the orbitrap.

**Data processing of protein VII spectra.** Raw mass spectrometer files were analysed using Proteome Discoverer (v.1.4, Thermo Scientific). MS/MS spectra were converted to .mgf files and searched against the UniProt adenovirus C serotype 5 database using Mascot (v.2.5, Matrix Science). Database searching was performed with the following parameters: precursor mass tolerance 10 p.p.m.; MS/MS mass tolerance 0.05 Da; enzyme chymotrypsin (Promega) or Arg-C (Roche), with two missed cleavages allowed; fixed modification was cysteine carbamidomethylation; variable modifications were methionine oxidation, serine/threonine/tyrosine phosphorylation, lysine acetylation and methylation, asparagine and glutamine deamidation. Specifically, phosphorylation, acetylation, and methylation were searched separately, not as co-existing modifications. Peptides were filtered for <1% FDR, Mascot ion score >20 and peptide rank 1.

**Co-immunoprecipitation of protein-VII–HA.** AS49 cells were cultured to express protein VII with doxycycline for 4 days as described earlier. Approximately 4 × 10^6 cells were harvested and pelleted for each immunoprecipitation reaction. Cell pellets were resuspended in 500 μl of IC wash buffer with protease inhibitors (20 mM HEPES pH 7.9, 110 mM KCl, 2 mM MgCl2, 150 mM NaCl, 0.1% Tween-20, 0.1% Triton X-100) and incubated on ice for 2 h. Samples were then incubated on ice for 1 h with 5 μl of benzobenzene (Millipore) added to each sample to digest DNA to ~150 bp, which was confirmed by DNA isolation and agarose gel analysis. Samples were then sonicated in a Diagenode Bioruptre for 30 s on and 30 s off for five rounds at 4 °C and centrifuged at 14,000 g for 15 min at 4 °C. Supernatants were then incubated rotating for 1 h at 4 °C with 30 μl of HA-conjugated magnetic beads (Thermo Scientific) and washed three times for 5 min in IC buffer. Isolated proteins were eluted with 100 μl of 2 mg ml^−1 HA peptide (Thermo Scientific) for 20 min rotating at 37 °C and separated on a SDS–PAGE gel. For protein separation by SDS–PAGE the NuPAGE 1 DE System was used (NuPAGE Novex Bis-Tris Protein Gels, Invitrogen). Uninduced cells were used as a negative control. The immunoprecipitation was carried out in biological triplicate and pull-down of protein–VII–HA and HMGB1 was confirmed by western blot analysis.

**Quantitative PCR.** Genomic DNA was isolated using the PureLink Genomic DNA kit (Thermo Scientific). Quantitative PCR was performed using primers specific for viral DBP (5′-GCCATTGGGCGCCAGAAGAA and 5′-CTGGTCCAGGATTCTCTCGGTGAT), protein VII (5′-GGCGGGT ATGGTCACTTGTGC and 5′-CACCAATAACAGTCTTGGGC), and cellular β-actin (5′-CCACGGGGAACCAAGGCAC and 5′-GGATGATGTCAC). Values for DBP and protein VII were normalized internally to tubulin and to the 4 h time point to control for any variation in virus input. RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed using the High Capacity RNA to cDNA Kit (Applied Biosystems). Quantitative PCR was performed using primers specific for HMGB1 and GAPDH.
(5'-TAACCTAAACATGGGCAAGGAG and 5'-TAGCAGACATGGTCTTCCAC) and β-actin (5'-GGGACTACCGCCCTTCTGAG and 5'-GGTCTCAACATGATCTGGGTC). Quantitative PCR was performed using the standard protocol for Sybr Green (Thermo Scientific) and analysed using the Viia 7 Real-Time PCR System (Thermo Scientific).

**Precision-cut lung slice immunofluorescence.** Precision-cut lung slices were obtained and prepared as previously described. De-identified human lung tissue from donors was obtained from the National Disease Research Interchange. Analysis of human samples was approved by the University of Pennsylvania Internal Review Board. Samples were infected with 10⁵ plaque-forming units (p.f.u.) of Ad5 per slice or 10⁹ GC of rAd protein-VII–GFP for 24 h. Samples were fixed in 4% PFA at room temperature and washed three times in PBS. Samples were permeabilized with 0.5% Triton X-100 and washed twice more in PBS. Samples were then incubated with 3% BSA and 0.03% Triton X-100 in PBS for 1 h to block. Primary antibodies (DBP or HMGB1) were incubated in the same buffer for 1 h and then samples were washed three times in PBS with 3% BSA, incubated with secondary antibodies and DAPI for 1 h, and washed three more times. Whole slices were mounted on slides with mounting solution and imaged by confocal microscopy.

**FRAP.** Full-length HMGB1 was cloned from pcDNA3.1 Flag-hHMGB1 (Addgene 31609) into pEGFP-N1 containing a L221K mutation to prevent dimerization of GFP molecules. A549 cells were induced to express protein VII for 4 days with doxycycline in glass-bottom dishes. Cells were then transfected with the construct that constitutively expresses HMGB1 with a monomeric GFP C-terminal tag. FRAP was carried out using standard methods on a Zeiss LSM 710 confocal microscope. Diffusion coefficients were calculated using the 'simFRAP' algorithm (http://imagej.nih.gov/ij/plugins/sim­frap/index.html), a simulation based approach to FRAP analysis.

**Statistical analyses.** Statistical details are reported in each figure legend. Statistical analyses were performed on at least three different biological replicates, unless otherwise stated in the figure legend. The sample size was chosen to provide enough statistical power to apply parametric tests (one- or two-tailed homoscedastic t-test). The t-test was considered a valuable statistical test since binary comparisons were performed and the number of replicates was limited. Furthermore, we applied the homoscedastic t-test assuming that the variance between the two data sets would remain homogeneous due to the use of the same cell lines in culture with and without protein VII expression. No samples were excluded as outliers (this applies to all proteomics analyses described in this manuscript). Proteins with a P value smaller than 0.05 were considered to be significantly altered between the two tested conditions for two-tailed and one-tailed t-test. Data distribution was assumed to be normal but this was not formally tested. The nano-LC-MS/MS analysis was performed in triplicate for each sample to determine technical variation.

31. Khandelia, P., Yap, K. & Makeyev, E. V. Streamlined platform for short hairpin RNA interference and transgenesis in cultured mammalian cells. Proc. Natl Acad. Sci. USA 108, 12793–12804 (2011).

32. Kozarsky, K. F., Jooss, K., Donahee, M., Strauss, J. F., III & Wilson, J. M. Effective treatment of familial hypercholesterolaemia in the mouse model using adenovirus-mediated transfer of the LDLR receptor gene. Nature Genet. 13, 152–156 (1996).

33. Orazio, N. I., Naeger, C. M., Karlseder, J. & Weitzman, M. D. The adenovirus E1b55K/E4orf6 complex induces degradation of the Bloom helicase during infection. J. Virol. 85, 1887–1892 (2011).

34. Le, L. P. et al. Core labeling of adenovirus with EGFP. Virology 351, 291–302 (2006).

35. Reich, N. C., Sarnow, P., Duprey, E. & Levine, A. J. Monoclonal antibodies which recognize native and denatured forms of the adenovirus DNA-binding protein. Virology 128, 480–484 (1983).

36. Lilley, C. E., Chaurushiya, M. S., Boutell, C., Everett, R. D. & Weitzman, M. D. The intrinsic antiviral defense to incoming HSV-1 genomes includes specific DNA repair proteins and is counteracted by the viral protein ICP0. PLoS Pathog. 7, e1002084 (2011).

37. Das, S., MacDonald, K., Chang, H.-Y. S. & Mitzner, W. A simple method of mouse lung intubation. J. Vis. Exp. 73, e50318 (2013).

38. Jeyaseelan, S., Chu, H. W., Young, S. K. & Worthen, G. S. Transcriptional profiling of lipopolysaccharide-induced acute lung injury. Infect. Immun. 72, 7247–7256 (2004).

39. Nick, J. A. et al. Role of p38 mitogen-activated protein kinase in a murine model of pulmonary inflammation. J. Immunol. 164, 2151–2159 (2000).

40. Zaret, K. Micrococcal nuclease analysis of chromatin structure. Curr. Protoc. Mol. Biol. Chapter 21, Unit 21.1 (2005).

41. Wessel, D. & Flügge, U. I. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. 138, 141–143 (1984).

42. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nature Biotechnol. 26, 1367–1372 (2008).

43. Schwannhaußser, B. et al. Global quantification of mammalian gene expression control. Nature 473, 337–342 (2011).

44. Tanaka, Y. et al. Expression and purification of recombinant human histones. Methods 33, 3–11 (2004).

45. Luger, K., Rechtsteiner, T. J., Flaus, A. J., Waye, M. M. & Richmond, T. J. The octamer is the major form of CENP-A nucleosomes at human centromeres. Nature Struct. Mol. Biol. 20, 687–695 (2013).

46. Hasson, D. et al. The octamer is the major form of CENP-A nucleosomes at human centromeres. Nature 467, 347–351 (2010).

47. Kulej, K., Avgousti, D. C., Weitzman, M. D. & Garcia, B. A. Characterization of histone post-translational modifications during virus infection using mass spectrometry-based proteomics. Methods 90, 3–20 (2015).

48. Cooper, P. R. & Panettieri, R. A. Jr. Steroids completely reverse albuterol-induced β₂-sympathomimetic action in human small airways. J. Allergy Clin. Immunol. 122, 734–740 (2008).

49. Zacharias, N. I., Naeger, C. M., Karlseder, J. & Weitzman, M. D. The intrinsic antiviral defense to incoming HSV-1 genomes includes specific DNA repair proteins and is counteracted by the viral protein ICP0. PLoS Pathog. 7, e1002084 (2011).

50. Das, S., MacDonald, K., Chang, H.-Y. S. & Mitzner, W. A simple method of mouse lung intubation. J. Vis. Exp. 73, e50318 (2013).

51. Blumenthal, D., Goldstein, L., Edlin, M. & Gheber, L. A. Universal approach to FRAP analysis of arbitrary bleaching patterns. Sci. Rep. 5, 11655 (2015).
Extended Data Figure 1 | Adenovirus protein VII distorts chromatin.

a, Protein VII localizes to cellular chromatin and viral replication centres in U2OS cells similarly to SAECs in Fig. 1a. b, Protein VII messenger RNA levels measured by quantitative PCR showing that after 4 days of induction in the A549 cell line, the level of protein VII transcripts is approximately 10% of that measured during infection at 16 hpi. Despite the low relative level, this amount of protein VII is sufficient to cause dramatic changes in the nucleus (graph shows mean ± s.d., n = 3 biological replicates).

c, Inducible cell lines of U2OS and HeLa expressing protein-VII–HA show chromatin localization and distortion, similar to A549 cells in Fig. 1c. d, Inducible A549 cell lines expressing viral protein V, the precursor for protein VII (preVII) or cellular protamine PRM1 with C-terminal HA tags. Although all three proteins possess a large number of charged residues, none are sufficient to distort cellular chromatin or increase nuclear size as observed with mature protein VII. Scale bars, 10 μm.
Extended Data Figure 2 | Protein VII associates tightly with chromatin and binds DNA and nucleosomes in vitro. **a**, Western blot analysis showing protein VII in histone extracts from infected HeLa cells at 24 hpi. Chromatin fractionation of lysates from A549 cells that were uninfected (mock) or infected for 24 h with Ad5. Viral and cellular proteins were detected by western blotting with various antibodies as indicated. **c**, Agarose gel analysis of DNA extracted from nuclear fractionation experiments, indicating that the size of DNA is between 100 and 200 bp and elutes predominantly in the higher-salt fractions.

**b**, Chromatin fractionation of cells induced to express protein VII, indicating that protein VII is present in the highest-salt fraction from the first day of induction. **e, f**, Recombinant protein-VII–His binds DNA. Incubating increasing molar amounts of protein VII with 195 bp DNA results in shifts by native gel electrophoresis, indicating protein-VII–DNA complex formation. Staining with either ethidium bromide (e) or Coomassie (f) are shown to verify the presence of DNA and protein, respectively. **g**, Ethidium bromide staining shows DNA content of nucleosome shifts from gel in Fig. 1f.
Bioanalyzer Analysis of MNase digested nucleosomes and protein VII-nucleosome complexes

Extended Data Figure 3 | Bioanalyzer examination of MNase-digested nucleosomes and protein-VII–nucleosome complexes. 

a, 195 bp nucleosomes or protein-VII–nucleosome complexes were incubated with MNase for the indicated times, the reaction was stopped, DNA was extracted and analysed. As in Fig. 1g, nucleosomes are shown in black and protein-VII–nucleosome complexes in orange. The presence of protein VII pauses digestion at 165 bp, suggesting that protein VII is blocking access to the DNA. 

b, 147 bp nucleosomes or protein-VII–nucleosome complexes were incubated with MNase for the indicated times, the reaction was stopped, DNA was extracted and analysed. Graphs show nucleosomes in grey and protein-VII–nucleosome complexes in orange. The presence of protein VII completely blocks digestion even after nucleosomes alone have been digested well beyond the core particle. In contrast to what would be expected for linker histones, protein VII protects the core nucleosome particle from digestion. These data indicate that protein VII may be masking the substrate for MNase through complex formation. This represents a unique mechanism of nucleosome binding and suggests a model for blocking DNA access in cellular chromatin during infection.

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Extended Data Figure 4 | Purification of protein VII from infected cells.

a, Coomassie-stained SDS–PAGE analysis of fractions from RP-HPLC in Fig. 2a. The bands in fraction 38–41 min correspond to histone H1. Protein VII and V, as indicated, were verified by MS analysis (data not shown). The slight upward shift of the protein VII bands in the later peak corresponds to the higher abundance of protein preVII, as seen by HPLC in Fig. 2a. b, Western blot analysis of protein VII in HPLC fractions from a. c, Time course of infection followed by histone extraction and HPLC analysis. MS analysis verified peaks in each sample as indicated.
Extended Data Figure 5 | Representative mass spectra. a–f. Annotated MS/MS spectra of identified peptides of protein VII containing PTMs (a–c, acetylated peptides; d–f, phosphorylated peptides). The images represent the observed fragment ions collected using MS/MS collision-induced dissociation (CID). Coloured lines represent matches between observed and expected fragment ions of the given peptides. Specifically, green lines represent not fragmented precursor mass, blue lines represent matches with y-type fragments, red lines with b-type fragments, and yellow boxed masses represent fragments containing PTM neutral losses (for example, ions that lost the phosphorylation during fragmentation).
Extended Data Figure 6 | See next page for caption.
Extended Data Figure 6 | Acetylated protein VII spectra from virus particles and analysis of total histone PTM changes upon protein VII expression. 

**a.** Liquid chromatography-mass spectrometry (LC-MS) analysis of unmodified and modified chymotryptic peptide AKKRSĐQHPYVRGRHY. On the left, nano-LC-MS-extracted ion chromatograms of protein VII peptides identified in the histone extracts of adenovirus infected cells (Inf) or viral particles (VP). The top left represents the modified form, while the bottom left represents the unmodified form. Non-modified forms were detected in both conditions for Inf and VP, while the acetylated form was unique for the infected sample only (Inf). On the right, full MS spectrum of the modified (top) and unmodified (bottom) peptide. Circled mass represents the monoisotopic signal of the peptide. **b.** Summary of post-translational modifications detected on protein VII. Peptides shown were identified during infection at various time points with the mature protein VII in the top row and preVII in the bottom row. The numbers in brackets for preVII indicate the location of the same moiety in mature protein VII. Acetylation sites were detected in approximately 3% of peptides for mature protein VII and 2% of peptides in preVII. Phosphorylation was detected in approximately 1% of peptides for mature protein VII and preVII.

c, d, Quantification of histone H3 (c) and H4 (d) PTMs in protein-VII–HA-induced (+dox) and -uninduced (−dox) A549 cells from the analysis of crude histone mixtures (n = 3 biological replicates). Positions of PTMs are listed along the x axis. Modification type is indicated by colour as shown. y Axis represents the cumulative extent of PTMs relative to the total histone H3 or H4, respectively. **e.** Breakdown of the histone marks (H3K14ac, H3K27me1, H3K36me3, H4K20me1, H4K20me2 and H4K20me3) found to be significantly different (n = 3 biological replicates) in terms of relative abundance between the protein-VII–HA-induced and -uninduced states (<5% homoscedastic two-tailed t-test). Mean ± s.d.
Extended Data Figure 7 | Bioinformatic analysis of proteins enriched in the high-salt fraction upon protein VII expression. a, Venn diagram showing overlap between three biological replicates of high-salt fraction proteins significantly enriched compared with uninduced cells. b, Proteins found significantly enriched in the protein-VII–HA–induced state compared with uninduced (<5% homoscedastic t-test) in all three biological replicates (‘VII–HA induced’ indicates proteins identified only in protein-VII–HA–induced condition). c, d, Classification of proteins significantly enriched in minimum two out of three biological replicates (protein-VII–HA–induced versus uninduced) according to process network enrichment and Gene Ontology biological process (GeneGo MetaCore pathways analysis package; false discovery rate (FDR) < 5%); each Gene Ontology term was ranked using −Log10(p-value) enrichment.

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Extended Data Figure 8 | Protein VII retains HMGB1 and HMGB2 in chromatin. a, Western blot of adenovirus-infected or doxycycline-treated A549 cells showing the relative levels of protein VII expression. HMGB1 levels do not change upon infection or protein VII expression. Tubulin is shown as a loading control. b, Quantitative PCR analysis of mRNA transcripts of HMGB1 in various cell types as indicated (for A549, n = 3 biological replicates; for THP-1, n = 2 biological replicates; mean ± s.d.). The levels of HMGB1 do not significantly change. c, Immunofluorescence analysis of a time course of protein-VII–HA (red) induction shown with HMGB1 (green) and DAPI (grey, blue in merge) in A549 cells. Expression of protein-VII–HA results in a change to the HMGB1 distribution upon expression. d, HMGB1 (green) localization changes between 12 and 24 hpi of wild-type adenovirus in A549 cells, and adopts a pattern similar to protein VII as in Fig. 1a. DBP (red) is shown as a marker of infection, DNA is stained with DAPI (blue in merge). e, Same as d showing that HMGB2 adopts the same pattern as HMGB1 during Ad5 infection at 24 hpi.
f, Multiple cells showing the same pattern of HMGB1 relocalization upon expressing protein-VII–GFP as in Fig. 3g.
g, HMGB1 retention in the high-salt fraction is conserved across adenovirus serotypes. Western blot analysis of HMGB1 from salt-fractionated A549 cells infected with Ad5, Ad9 or Ad12 as shown. Scale bars, 10 μm.
Extended Data Figure 9 | Protein VII is necessary and sufficient for chromatin retention of HMGB1 in human and mouse cells.

**a**, **b**, Replication of Ad5-flox-VII virus on 293 or 293-Cre cells. Quantitative PCR analysis of viral genomic DNA over a time course of infection (a) shows the DBP gene is increasing exponentially in 293 and 293-Cre cells when infected with Ad5-flox-VII virus. In contrast, PCR for the protein VII gene (b) demonstrates deletion in 293-Cre cells (n = 2 biological replicates, mean ± s.d.).

**c**, Salt fractionation of 293-Cre cells infected with wild-type Ad5, indicating that the Cre recombinase does not interfere with the ability of protein VII to retain HMGB1 in the high-salt chromatin fraction. Protein VII is also necessary for the chromatin retention of HMGB2. **d**, THP-1 cells transduced to express protein-VII–GFP results in chromatin distortion and HMGB1 retention in chromatin. Immunofluorescence of transduced PMA-treated THP-1 cells showing protein-VII–GFP (green), HMGB1 (red) and DNA (grey; blue in merge). **e**, Transduction to express protein-VII–GFP is sufficient to relocalize mouse HMGB1 in mouse embryonic fibroblast (MEF) cells. **f**, Salt fractionation of mouse embryonic fibroblast cells transduced to express protein-VII–GFP. Human Ad5 protein VII is sufficient to retain mouse HMGB1 in the high-salt fraction in MEF cells. The control vector expressing GFP alone does not have this effect.

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Extended Data Figure 10 | Transduction of mouse lungs demonstrating expression of GFP or protein-VII–GFP. a, Sections of mouse lungs transduced to express protein-VII–GFP or GFP co-stained for HMGB1. GFP signal shows multiple cell types transduced in both cases. Protein-VII–GFP has a more distinct nuclear signal than GFP, which also appears cytoplasmic. Two sections for each condition are shown to indicate transduction efficiency. b, Same as a but co-stained for prosurfactant-C to mark type II pneumocytes. Some cells are positive for both, confirming that multiple cell types were transduced. c, Zoomed images of individual epithelial cells from mouse lungs showing the characteristic protein-VII–GFP pattern colocalizing with DAPI in the nucleus. GFP only is mostly cytoplasmic. d, Schematic summarizing function of protein VII during infection. Newly synthesized protein VII late during infection can be post-translationally modified and binds to HMGB1, sequestering it on the cellular chromatin and preventing its release. Unmodified protein VII is packaged in viral progeny.