



## Letter

## Enterovirus 71 3C proteolytically processes the histone H3 N-terminal tail during infection

Meng Miao<sup>a,b,\*</sup>, Gang Deng<sup>a</sup>, Xiaobei Xiong<sup>c</sup>, Yang Qiu<sup>c</sup>, Wenda Huang<sup>a</sup>, Meng Yuan<sup>a</sup>, Fei Yu<sup>a</sup>, Shimei Bai<sup>b</sup>, Xi Zhou<sup>c,\*</sup>, Xiaolu Zhao<sup>a,\*</sup><sup>a</sup> Hubei Key Laboratory of Cell Homeostasis, College of Life Sciences, Wuhan University, Wuhan, 430072, China<sup>b</sup> Institute of Biochemistry, College of Life Sciences and Medicine, Zhejiang Sci-Tech University, Hangzhou, 310018, China<sup>c</sup> Laboratory of RNA Virology, Wuhan Institute of Virology, Chinese Academy of Science, Wuhan, 430071, China

Dear Editor,

Enterovirus 71 (EV71) belongs to the genus *Enterovirus*, family *Picornaviridae* (Oberste et al., 1999). It was first isolated from patients with central nervous system diseases in California between 1969 and 1974 (Schmidt et al., 1974) and has spread worldwide (Solomon et al., 2010). EV71 infection usually causes mild, self-limiting hand, foot, and mouth disease in children. Acute EV71 infection may also cause severe polio-like neurological diseases and significant mortality. The spectrum of EV71-associated neurological diseases includes aseptic meningitis, brainstem and/or cerebellar encephalitis, acute flaccid paralysis (AFP), myocarditis, and rapid fatal pulmonary edema and hemorrhage (McMinn, 2002).

Like all other viruses, the replication of picornaviruses depends on hijacking the host cellular translation machinery and recruiting help from host cell proteins. Numerous host cellular machineries, including host translation and transcription machineries, have been reported to be affected by picornaviral infections (Clark et al., 1993; Etchison et al., 1982; Rose et al., 1978). Two picornaviral proteases, 2A and 3C, are responsible for the inhibitory effects. The viral protease 3C has been extensively studied and found to specifically cleave at Gln/Gly scissile pairs (Kitamura et al., 1981). The major catalytic sites of EV71 3C are His40, Glu71, and Cys147 (Matthewa et al., 1994; Shih et al., 2004). Picornaviral 3C can enter nuclei through its precursor 3CD<sup>o</sup> or 3CD, which contains a nuclear localization sequence (NLS) (Amineva et al., 2004; Sharma et al., 2004), and can cleave several host transcription factors, such as TATA-box binding proteins, p53, CstF-64, and transcription factor IIIC (Clark et al. 1991, 1993; Weidman et al., 2001; Weng et al., 2009), thus regulating viral replication within hosts.

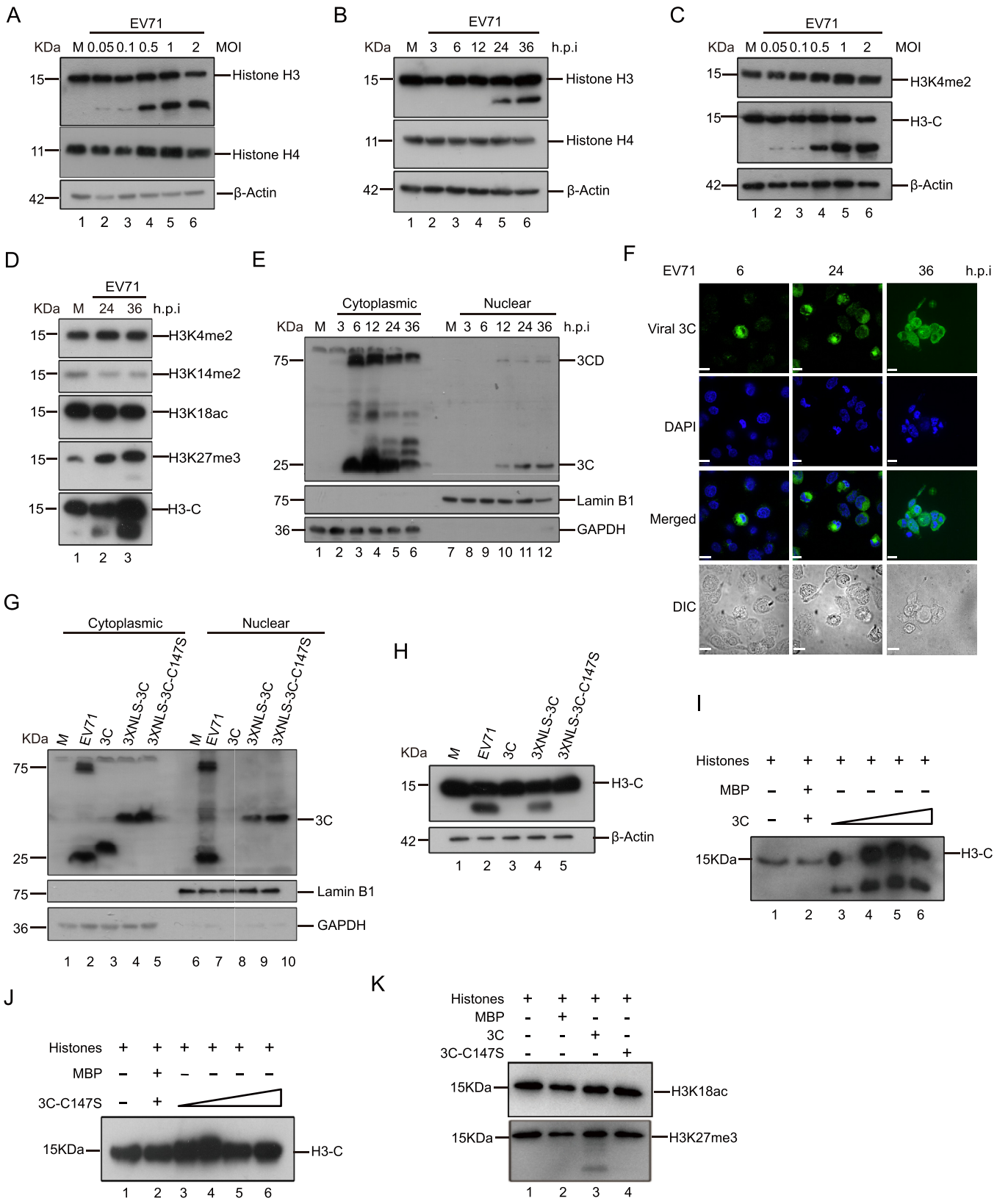
Histone proteins are essential components of chromatin in eukaryotes. Histones assemble as octamers that are wrapped by DNA every ~147 base pairs constituting the repeating unit known as the nucleosome. Post-translational modifications (PTMs) on histone tails directly

affect chromatin structure, which modulates gene expression, DNA replication, DNA repair and cell duplication (Huang H et al., 2014; Kouzarides T et al., 2007). Modifications, including acetylation, methylation, and phosphorylation, commonly occur on N-terminal histone tails and have important implications for the transcription, replication, and repair of nuclear DNA (Bhaumik SR et al., 2007). Recent studies have shown that histone covalent modification patterns change significantly upon viral infection. The genome of many DNA viruses are associate with core histone proteins to form chromatin-like structures in the nucleus, such as adenovirus, herpes simplex virus, human cytomegalovirus, Epstein-Barr virus (EBV) and human immunodeficiency virus (Horwitz et al., 2008; O'Connor et al., 2014; Placek et al., 2009; Murata T et al., 2012; Britton et al., 2014). The viral chromatin is subject to histone modifications, which has significant impact on viral gene expression and virus replication (Lieberman PM. 2006). For example, H3K9me2/3, H3K27me3, and H4K20me3 are highly enriched in the promoter of EBV transcription activator BZLF1 to silence its transcription and prevent EBV reactivation. Meanwhile, some viral pathogens can regulate host gene expression by altering host histone modifications and chromatin structure to survive and propagate in host cells (Han et al., 2012; Genin et al., 2012; Fonseca et al., 2012). For example, HIV has been reported to stimulate TLR8-dependent TNF $\alpha$  production through increasing H4 acetylation and H3K4me3 with concomitant loss of H3K27me3 at the TNF $\alpha$  promoter, which eventually activates systemic innate immune responses (Han et al., 2012).

Despite a wealth of emerging data describing the changing patterns of epigenetic signatures during infection by DNA viruses and some retroviruses, little is known about epigenetic changes that occur during RNA virus infections. Most RNA virus replication occurs in the cytoplasm of the host cell, and whether RNA virus infection can cause changes in histone modifications or chromatin structure has not been well documented. To gain an insight into the chromatin alterations that occur

\* Corresponding authors.

E-mail addresses: [miaom@whu.edu.cn](mailto:miaom@whu.edu.cn) (M. Miao), [zhouxi@whu.edu.cn](mailto:zhouxi@whu.edu.cn) (X. Zhou), [zhaoxiaolu@whu.edu.cn](mailto:zhaoxiaolu@whu.edu.cn) (X. Zhao).



(caption on next page)

during RNA virus infection, we tried to elucidate the structural and chemical changes on host cell histone proteins H3 and H4 PTMs brought by EV71 infection and the possible underlying epigenetic regulatory mechanism.

In this study, human rhabdomyosarcoma cells (RD) was infected with wild-type EV71 at a multiplicity of infection (MOI) of 0.05–2 and cells were harvested at 24 h post infection (h.p.i.). We used immunoblotting to probe whole-cell extracts with various histone antibodies for H3, H3 C terminal, acetylated lysines on histones H3K18, dimethylation at H3K4 and H3K14, trimethylation at H3K27 and H4 (Chinnadurai, 2002). When probing with a specific histone H3 antibody, we observed a faster running species of H3 (Fig. 1A). It was also observed when probing immunoblots in samples taken at time points from 3 to 36 h post virus infection, and the levels of the band markedly increased with the infection time (Fig. 1B). No evidence of changes in H4 levels was detected. Notably, this faster migrating H3 species band was observed using an H3 general antibody generated against the C terminus of histone H3, but not when using the H3K4me2 antibody specific for PTMs at the N-terminus of the H3 tail (Fig. 1C). To narrow down the cleavage site region of the H3 tail, we purified histone samples from virus-infected cells and used a combination of histone H3 PTM-specific antibodies to probe K4, K14, K18 and K27 sites at the N terminal of H3 histone (Guo et al., 2018). Because the H3K27me3 antibody detects a faster migrating band that is not detectable using the H3K4me2, H3K14me2, and H3K18ac antibodies, the cleavage site most likely lies at or within the K18–K27 region (Fig. 1D). Collectively, the results of these experiments suggest that the N-terminal tail of H3 is specifically cleaved during EV71 infection.

Further, we investigated which protease was responsible for H3 tail cleavage during EV71 infection. EV71 encodes two viral proteases, 2A and 3C, which are important for viral polypeptide processing. Picornaviral 3C can enter nuclei through its precursor 3CD, which contains a nuclear localization sequence (NLS) (Amineva et al., 2004), and can cleave some cellular transcriptional factors or regulators in the nucleus. We speculated that the N-terminal cleavage of histone H3 was likely mediated by the protease 3C encoded by EV71. First, we verified whether 3C could enter the nucleus during viral infection. Using nuclear and cytoplasmic fractions isolated from EV71-infected RD cells, we demonstrated that 3C could be detected in the nuclear fraction as early as 12 h.p.i. (Fig. 1E). Notably, the time periods and level of 3C in the nucleus were consistent with the clipped form of histone H3 (Fig. 1B). We also examined the distribution of 3C in the RD cells with an immunofluorescence assay (IFA) using confocal microscopy (Fig. 1F). The images revealed that EV71 3C was localized primarily in the cytoplasm at 6 h h.p.i.; then, 3C partially entered the nucleus at the late stage, from 24 to 36 h.p.i., which were consistent with the Western blot data (Fig. 1E).

Thereafter, we cloned the 3C protein and the catalytic activity mutant 3C-C147S (Shih et al., 2004) into mammalian cell expression vectors, and added a 3 × KKKRK NLS tag to its N-terminus to enable it to be expressed in nuclei. Primers used were shown in Supplementary Table S1. RD cells were transfected with these plasmids, and nuclear/cytoplasmic fraction extracts were prepared from transfected cells after 48 h and examined for 3C proteins using immunoblotting with an antibody against EV71 3C. As shown in Fig. 1G and 3 × NLS-3C and 3 × NLS-3C-C147S were all detected in the nuclear fraction, similar to that in the EV71 infected sample. We also examined whether histone H3 was cleaved using the C-terminal antibody of histone H3. We found that the clipped form of H3 could be detected in 3 × NLS-3C and virus infected samples, but not in 3C without 3 × NLS or 3 × NLS-3C-C147S samples (Fig. 1H). The results demonstrated that 3C protease was able to localize to the nucleus and was responsible for H3 N-tail cleavage during EV71 infection.

To further validate our findings of 3C as a histone H3 protease, we performed an *in vitro* H3 cleavage assay. First, the catalytic activities of purified recombinant EV71 wild-type 3C or mutant 3C-C147S was verified by incubation with histones extracted from RD cells at 37 °C for 1 h. The samples were then used to examine H3 cleavage using immunoblotting with the C-terminal antibody of histone H3. The clipped form of histone H3 was detected only in the samples incubated with wild-type 3C (Fig. 1I), but not in the catalytic activities of mutant 3C-C147S (Fig. 1J), which were consistent with our previous results (Fig. 1H). Upon immunoblotting using histone PTM antibodies such as H3K18ac and H3K27me3, we determined that the H3 cleavage site was between K18 and K27, similar to that in virus-infected cells (Fig. 1K).

In this study, we showed that histone H3 is proteolytically cleaved at its N-terminus during EV71 infection. Furthermore, we identified viral protease 3C as a protease responsible for the proteolytic processing of the N-terminal H3 tail. Chromatin undergoes structural and chemical changes during viral infection, which subsequently leads to differences in cellular function by altering the patterns of gene expression. The results indicate that histone proteolysis, brought about by EV71-encoded 3C protease, has a global impact on the transcription and regulation of host and viral genes.

## Footnotes

This work was supported by the National Natural Science Foundation of China grants (No. 31870827 to X Zhao, No. 31970169 to X Zhou), the National Key Research and Development Program of China grant (No. 2019YFA0802701 to X Zhao), the Zhejiang Sci-Tech University Research Startup grants (No. 19042401-Y to M Miao), the Zhejiang Sci-Tech University Youth Innovation grants (No. 20042140-Y to M Miao). The

**Fig. 1.** The Enterovirus 71 (EV71) 3C protease can enter the host cell nucleus and cleave histone H3 N tails upon viral infection. **A–D** The N-terminal tail of H3 is specifically cleaved during EV71 infection and the cleavage site lies at or within the K18–K27 region. **A** Western blotting was used to detect histone H3 after EV71 infection at different MOIs. Wild-type EV71 was rescued from infectious cDNA clone pEV-HeN09. The RD cells were infected with wild-type EV71 at MOIs from 0.05 to 2 and cells were harvested at 24 h.p.i.  $\beta$ -Actin was used as the internal control. **B** Western blot analysis of histone H3 and H4 at various h.p.i. from 3 to 36 in RD cells infected with EV71 (MOI = 0.5). Cells (**A**, **B**) were detected using histone H3 and histone H4 specific antibodies. **C** Western blotting for the detection of dimethyl-histone H3K4 (H3K4me2) and histone H3 C-terminal. RD cells were infected with EV71 at different MOI from 0.05 to 2. Cells were detected using dimethyl-histone H3K4 (H3K4me2) polyclonal antibody and histone H3 C-terminal antibody at 24 h.p.i. **D** Western blot analysis of H3K4me2, H3K14me2, H3K18ac, H3K27me3 and H3 C terminal respectively. RD cells were infected with EV71 (MOI = 0.5) or mock-infected, and cells were collected for histone purification at 24 and 36 h.p.i. Isolated histone samples were detected using combination of histone H3 PTM-specific antibodies. **E–H** The viral 3C protease can enter the cellular nucleus and be responsible for H3 N-tail cleavage. **E** Western blotting for the detection of EV71 3C protease in the cytoplasmic and nuclear fractions of EV71-infected RD cells at 3–36 h.p.i. GAPDH and Lamin B1 were used as cytoplasmic and nuclear protein controls, respectively. **F** Immunofluorescence staining of EV71 3C protease (green color) in EV71-infected RD cells at 8, 24, and 36 h.p.i. The nuclei of RD cells were stained with DAPI dye (blue color), and the merged images show the 3C and nuclei immunofluorescence signals. DIC represents bright vision. All immunofluorescence images were analyzed using confocal microscopy. Scale bar, 20  $\mu$ m. **G–H** Western blotting for distribution analysis of EV71 3C protease and its different domains in RD cells infected with EV71 (MOI = 0.5). **G** RD cells were transfected with plasmids pRK-3C, pRK-3 × NLS-3C, pRK-3 × NLS-3C-C147S, and infected with EV71 (MOI = 0.5). After 36 h, half of the cells were collected for nuclear and cytoplasmic fraction isolation. **H** The other half of the cells in **G** were used to detect histone H3 using a histone H3 C-terminal antibody. **I–K** Western blot analysis shows that recombinant EV71 3C cleaves histone H3 *in vitro*. 0.5  $\mu$ g histone samples from RD cells were treated with various quantities of recombinant wild-type 3C (**I**) and mutant 3C-C147S (**J**) proteins ranging from 1, 2, 4, and 8  $\mu$ g (lanes 3 to 6). After incubation at 37 °C for 1 h *in vitro*, samples were detected using the Histone H3 C-terminal antibody in a Western blot assay. **K** Histone samples (0.5  $\mu$ g) were incubated with 2  $\mu$ g recombinant wild-type 3C and mutant 3C-C147S. Samples were detected using acetyl-histone H3K18 (H3K18ac) polyclonal antibody, trimethyl-histone H3K27 (H3K27me3) polyclonal antibody in a Western blot assay. MBP was used as a negative control for excluding the effects of the protein tag. Experiment details were described in Supplementary materials.

authors declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.virs.2022.02.006>.

## References

- Amineva, S.P., Aminev, A.G., Palmenberg, A.C., Gern, J.E., 2004. Rhinovirus 3C protease precursors 3CD and 3CD' localize to the nuclei of infected cells. *J. Gen. Virol.* 85, 2969–2979.
- Bhaumik, S.R., Smith, E., Shilatifard, A., 2007. Covalent modifications of histones during development and disease pathogenesis. *Nat. Struct. Mol. Biol.* 14, 1008–1016.
- Britton, L.M., Sova, P., Belisle, S., Liu, S., Chan, E.Y., Katze, M.G., Garcia, B.A., 2014. A proteomic glimpse into the initial global epigenetic changes during HIV infection. *Proteomics* 14, 2226–2230.
- Clark, M.E., Hammerle, T., Wimmer, E., Dasgupta, A., 1991. Poliovirus proteinase 3C converts an active form of transcription factor IIC to an inactive form: a mechanism for inhibition of host cell polymerase III transcription by poliovirus. *EMBO J.* 10, 2941–2947.
- Clark, M.E., Lieberman, P.M., Berk, A.J., Dasgupta, A., 1993. Direct cleavage of human TATA-binding protein by poliovirus protease 3C in vivo and in vitro. *Mol. Cell Biol.* 13, 1232–1237.
- Etchison, D., Milburn, S.C., Edery, I., Sonenberg, N., Hershey, J.W., 1982. Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. *J. Biol. Chem.* 257, 14806–14810.
- Fonseca, G.J., Thillainadesan, G., Yousef, A.F., Ablack, J.N., Mossman, K.L., Torchia, J., Mymryk, J.S., 2012. Adenovirus evasion of interferon-mediated innate immunity by direct antagonism of a cellular histone posttranslational modification. *Cell Host Microbe* 11, 597–606.
- Genin, P., Lin, R., Hiscott, J., Civas, A., 2012. Recruitment of histone deacetylase 3 to the interferon- $\alpha$  gene promoters attenuates interferon expression. *PLoS One* 7, e38336.
- Guo, Q., Sidoli, S., Garcia, B.A., Zhao, X., 2018. Assessment of quantification precision of histone post-translational modifications by using an ion trap and down to 50 000 cells as starting material. *J. Proteome Res.* 17, 234–242. Jan 5.
- Han, X., Li, X., Yue, S.C., Anandaiah, A., Hashem, F., Reinach, P.S., Kozziel, H., Tachado, S.D., 2012. Epigenetic regulation of tumor necrosis factor alpha (TNF $\alpha$ ) release in human macrophages by HIV-1 single-stranded RNA (ssRNA) is dependent on TLR8 signaling. *J. Biol. Chem.* 287, 13778–13786.
- Horwitz, G.A., Zhang, K., McBrien, M.A., Grunstein, M., Kurdistani, S.K., Berk, A.J., 2008. Adenovirus small e1a alters global patterns of histone modification. *Science* 321, 1084–1085.
- Huang, H., Sabari, B.R., Garcia, B.A., Allis, C.D., Zhao, Y., 2014. SnapShot: histone modifications. *Cell* 159, 458–458.e1.
- Kitamura, N., Semler, B.L., Rothberg, P.G., Larsen, G.R., Adler, C.J., et al., 1981. Primary structure, gene organization and polypeptide expression of poliovirus RNA. *Nature* 291, 547–553.
- Kouzarides, T., 2007. Chromatin modifications and their function. *Cell* 128, 693–705.
- Lieberman, P.M., 2006. Chromatin regulation of virus infection. *Trends Microbiol.* 14, 132–140.
- Matthews, D.A., Smith, W.W., Ferre, R.A., Condon, B., Budahazi, G., et al., 1994. Structure of human rhinovirus 3C protease reveals a trypsin-like polypeptide fold, RNA-binding site, and means for cleaving precursor polyprotein. *Cell* 77, 761–771.
- McMinn, P.C., 2002. An overview of the evolution of enterovirus 71 and its clinical and public health significance. *FEMS Microbiol. Rev.* 26, 91–107.
- Murata, T., Kondo, Y., Sugimoto, A., et al., 2012. Epigenetic histone modification of Epstein-Barr virus BZLF1 promoter during latency and reactivation in Raji cells. *J. Virol.* 86, 4752–4761.
- O'Connor, C.M., DiMaggio Jr., P.A., Shenk, T., Garcia, B.A., 2014. Quantitative proteomic discovery of dynamic epigenome changes that control human cytomegalovirus (HCMV) infection. *Mol. Cell. Proteomics* 13, 2399–2410.
- Oberste, M.S., Maher, K., Kilpatrick, D.R., Pallansch, M.A., 1999. Molecular evolution of the human enteroviruses: correlation of serotype with VP1 sequence and application to picornavirus classification. *J. Virol.* 73, 1941–1948.
- Placek, B.J., Huang, J., Kent, J.R., Dorsey, J., Rice, L., Fraser, N.W., Berger, S.L., 2009. The histone variant H3.3 regulates gene expression during lytic infection with herpes simplex virus type 1. *J. Virol.* 83, 1416–1421.
- Rose, J.K., Trachsel, H., Leong, K., Baltimore, D., 1978. Inhibition of translation by poliovirus: inactivation of a specific initiation factor. *Proc. Natl. Acad. Sci. U.S.A.* 75, 2732–2736.
- Schmidt, N.J., Lennette, E.H., Ho, H.H., 1974. An apparently new enterovirus isolated from patients with disease of the central nervous system. *J. Infect. Dis.* 129, 304–309.
- Sharma, R., Raychaudhuri, S., Dasgupta, A., 2004. Nuclear entry of poliovirus protease polymerase precursor 3CD: implications for host cell transcription shutoff. *Virology* 320, 195–205.
- Shih, S.R., Chiang, C., Chen, T.C., Wu, C.N., Hsu, J.T., Lee, J.C., Hwang, M.J., Li, M.L., Chen, G.W., Ho, M.S., 2004. Mutations at KFRDI and VGK domains of enterovirus 71 3C protease affect its RNA binding and proteolytic activities. *J. Biomed. Sci.* 11, 239–248.
- Solomon, T., Lewthwaite, P., Perera, D., Cardoso, M.J., McMinn, P., Ooi, M.H., 2010. Virology, epidemiology, pathogenesis, and control of enterovirus 71. *Lancet Infect. Dis.* 10, 778–790.
- Weidman, M.K., Yalamanchili, P., Ng, B., Tsai, W., Dasgupta, A., 2001. Poliovirus 3C protease-mediated degradation of transcriptional activator p53 requires a cellular activity. *Virology* 291, 260–271.
- Weng, K.F., Li, M.L., Hung, C.T., Shih, S.R., 2009. Enterovirus 71 3C protease cleaves a novel target CstF-64 and inhibits cellular polyadenylation. *PLoS Pathog.* 5, e1000593.