CRISPR/Cas9 mediated genome editing of epigenetic factors for cancer therapy

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Abstract:

Recent advances in engineered recombinant nuclease have provided facile and reliable methods for genome editing. Especially with the development of CRISPR/cas9 system, the discovery of various versions of Cas9 proteins and delivery carriers, it is now practicable to introduce desired mutations into genome, to correct disease related mutations, and to activate or suppress genes of interest. Epigenetic regulators are often disturbed in cancer cells and are essential for the transformation of normal cells to the cancerous. Tumor-related epigenetic alterations or epigenetic factor mutations play a major part during the multistep of carcinogenesis and affect a variety of cancer-related genes and a wide range of cancerous phenotypes. Therefore, epigenetic regulatory enzymes might be candidate targets for cancer therapy. In this review, we will discuss prospects of CRISPR/Cas9 based genome editing in targeting epigenetics for cancer gene therapy.

Introduction

Classical epigenetics is defined as a heritable molecular mechanism that changes gene expression patterns without alterations in DNA sequence. This mechanism involves multiple DNA and histone covalent modifications, which constitute an essential platform for the actions of chromatin remodeling and gene expression regulators [1,2]. In the last decade, epigenetics has become an important topic of cancer research and accumulating evidence showed that malignant transformation of normal cells and tumor maintenance require extensive epigenomic reconfigurations [2-4]. DNA methyl-transferases (DNMTs) and various histone modification enzymes, such as NSD2, EZH2 and LSD1, were dysregulated in cancerous cells causing gene expression reprogramming in a genome wide scale [5-11]. Recently, multiple epigenetic factors, including histones themselves, were found to be recurrently muted in multiple types of human cancer, including glioblastoma, hematological malignancies, chondrosarcoma and osteosarcoma, which directly linked dysregulated epigenetic control with carcinogenesis [12-15]. Further, emerging evidence indicates that these epigenetic abnormalities are also essential for tumor maintenance[16,17]. Based on those evidence, targeting epigenetic regulatory enzymes might be important strategies for cancer therapy[18,19].

Recent rapid advances in engineered nuclease-mediated genome editing provide efficient and convenient tools for gene therapy. To date, three major nucleases, zinc finger nucleases (ZFNs)[20], transcription activator-like effector nucleases (TALENs) [21] and the clustered regularly interspaced short palindromic repeat (CRISPR) Cas9 nuleases[22], have been developed to induce site-specific genome modifications. Compared with ZFNs and TALENs, CRISPR-Cas9 is much easier to be reprogrammed to new targets and has significant higher amount of targetable sites [22,23]. Another advantage of CRISPR/Cas9 is the ability to manipulate multiple genes simultaneously. These advantages of CRISPR/Cas9 system have substantially improved our ability to make desired changes in the genome, which provides a promising tool for targeted gene therapy[24]. Since the pioneering works showed that the bacteria type II CRISPR system is reprogrammable[25,26], numerous efforts have been focusing

on exploring the modes of action of CRISPR/Cas9 system and developing new versions of Cas9 proteins and carriers. Based on these efforts, a list of CRISPR/Cas9 based toolkits are already available to introduce desired mutations into genome, to correct disease related mutations, and to activate or suppress the expression of certain genes[27-32]. These exciting advances in this field are beginning to show remarkable promise in clinical applications[24,33]. Here, we will discuss some prospects of CRISPR/Cas9 based technology to target cancer epigenome as potential cancer therapy.

Epigenetic regulation

Epigenetic mechanisms, including DNA methylation and various histone modifications, constitute an essential environment for gene expression regulation, thus playing important roles in normal cell differentiation, proliferation and function[34]. DNA methylation usually takes place at the 5' carbon of the cytosine ring within CpG dinucleotides in the mammalian genomes, resulting in gene silencing[2]. In mammalian cells, there are three main DNA methyltransferases (DNMTs): DNMT1, DNMT3A and DNMT3B[34]. Of those, DNMT1 is responsible for the maintenance of pre-existing methylation patterns after DNA replication during cell divisions [35], whereas DNMT3A and DNMT3B target previously unmethylated CpGs yielding novel methylation patterns [36,37]. Another member of the DNMT3 family, DNMT3-like (DNMT3L) has no catalytic activity and functions as a regulator of DNMT3A and DNMT3B[38,39]. DNA methylation is reversible both at a specific genomic locus and in a global scale. The mechanisms of DNA demethylation can be either passive or active[40]. The passive process takes place on newly synthesized DNA strands during DNA replication when DNMT1 function is absent. Active DNA demethylation occurs via removal of a methyl group that has been modified in a DNA replication-independent manner. For example, it was recently shown that the ten-eleven translocation (TET) protein family could sequentially modify DNA methylation, which initiates an important active mechanism of DNA demethylation[41]. Tet family comprises at least three members, TET1-3, all of which can oxidize methylated cytosine into 5-hydroxyl-methyl cytosine, and subsequently into carboxyl- and

formyl-methyl-cytosine[42,43]. These modified methyl groups may be actively removed via DNA repair pathways resulting in active demthylation. Also, the oxidized methyl groups are not recognized by DNMT1, leading to passive demethylation during replication[42]. Dynamic and reversible DNA methylations through DNMTs and TETs are critical for gene regulation and various cellular functions.

Histone modification is another major type of epigenetic mechanism that occurs on specific amino acid residues of the histone proteins. The post-translational histone modifications play fundamental roles in gene regulation and cell differentiation by forming synergistic signaling platforms within specific genomic loci, which are recognized by specific readers with diverse functions ranging from transcriptional regulation of specific genes to large scale chromatin remodeling[44]. Currently, a large body of histone modifications have been discovered, including lysine acetylation and methylation, arginine methylation, serine and threonine phosphorylation and glycosylation, ADP ribosylation, lysine ubiquitylation as well as SUMOylation. Among them, acetylation and methylation are the best known histone modifications. Depending on which amino acids in the histone are modified and how many methyl groups are added, histone methylations can either increase or decrease gene transcription[45,46]. Methylation events such as H3K4me2/3 and H3K79me3 weaken chemical attractions between histone tails and DNA helix, thus facilitating DNA to uncoil from nucleosomes and to be available for transcription factor proteins and RNA polymerase. On the contrary, methylations such as H3K9me2/3, H3K27me2/3, and H4K20me3 are associated with repression. Histone methylation is relatively stable because its half-life is approximately equal to the half-life of histones themselves[47]. However, like DNA methylation, histone methylation is also reversible. The methyl groups on given histone amino acid residues can be removed by specific demethylases. LSD1, also known as KDM1A, was the first demethylase to be discovered, which performs the demethylation of di- and monomethyl lysine 4 and lysine9 in histone H3[48]. After that, more histone demethylases were identified that utilize similar or different reaction mechanisms in demethylation [49]. Histone acetylation is

another major type of histone modifications that usually occurs at lysine residues within the N-terminal histone tails, and such acetylations are catalyzed by histone actylases (HATs) and removed by histone deacetylases (HDACs) [50]. Some acetylation and methylation can take place on the same amino acid residues of histone protein, and counteract each other. A good example is the acetylation and methylation of the histone 3 tail, in which H3K9 trimethylation (H3K9me3) is a crucial epigenetic mark of heterochromatin and has been associated with transcriptional repression[51], whereas H3K9 acetylation is associated with transcriptional activation and marks euchromatin[52]. These histone and DNA modifications are tightly regulated by numerous epigenetic regulators, which bear mutations in many human diseases associated with abnormal epigenetic patterns.

Epigenetic dysregulation in cancer

It has been increasingly recognized that epigenetic abnormalities play essential roles in both the genesis and progression of cancer cells. Among them alterations in the methylation pattern of genomic DNA are common. A global loss of DNA methylation coupled with hypermethylation at specific loci characterizes a significant portion of human cancer. Global DNA hypomethylation could activate genes that are commonly silenced in normal cells such as viral and parasitic transposons, and affect genome stability. On the other hand, gene specific DNA hypermethylation is often found in the promoter regions and therefore causes abnormal silencing of tumor suppressor genes such as PTEN, TP53, BRCA1, ATM etc [53] . The maintenance of those abnormal hypermethylation requires the continuous activity of DNMTs . Therefore, cancer cells may be more sensitive to the changes in DNMT activity than normal cells, which represents the key rational for DNMT inhibition in cancer therapies [54]. In support to this notion, evidence from mouse model showed that haploinsufficiency of Dnmt1 was sufficient to delay progression of leukemogenesis and to impair LSC self-renewal, but did not disturb normal hematopoiesis[55].

Histone modifications are also frequently dysregulated in cancer cells. As mentioned above,

some histone modifications counteract each other. The imbalanced histone modifications lead to dysregulated gene expression and cell growth, and are often found in cancerous cells. In some cases, such as prostate and ovarian tumors, a decreased level of H3K9 acetylation was observed and correlated with severe histological grading and the clinical outcomes [56-58]. However, in other cases, such as hepatocellular carcinoma and gastric adenocarcinoma, increase in H3K9ac levels and decrease in H3K9me3 are associated with poor prognosis [59]. In consistent with these observations, numerous histone modification enzymes are mutated in cancerous cells and contribute to malignant phenotypes. For example, HDACs, LSD1 and EZH2, a histone methyltransferase responsible for H3K27 methylation, are often overexpressed in many human cancers, including prostate, breast, ovarian and colon cancer, and lead to epigenetic alterations that promote tumor growth, invasion, and metastasis [7,9,11,60]. The rapidly growing list of mutant epigenetic factors found in cancer includes, but is not limited to, the DNA methyltransferase DNMT3B, DNA demethylation enzyme TET2, histone methylation enzyme MLL3, EZH2 and NSD2, and the isocitrate dehydrogenase IDH1 and IDH2 whose mutant proteins generate 2-HG that inhibits several epigenetic enzymes including TET2 and JMJD3 [61-66]. Interestingly, mutations in histone proteins themselves can impact on their epigenetic modifications. For example, mutations in histone 3 (H3) has been found to be recurrently mutated in several cancers [12]. The H3 mutations are usually heterozygous and take place on its K27, K36, and G34 amino acid residues, which were demonstrated to induce abnormal loss or insufficiency of methylations [14,15]. The epigenetic consequences of such genetic changes were further supported by in vitro functional test and in vivo animal models [67-69]. These evidence strongly supports the notion that epigenetic mechanisms may act as driving forces during carcinogenesis and support cancer cell survival and proliferation. Based on this notion, epigenetic regulatory enzymes are considered as important targets for cancer therapy [3]. Several drugs targeting DNA methylation and histone acetylation have been approved for cancer treatment in clinic [3]. DNMT inhibitors, such as the cytidine analogues 5-azacytidine and 5-aza-2'deoxycytidine, can be incorporated into the daughter strands of DNA during DNA replication, which leads to DNA demethylation because

they cannot be methylated by the DNMTs [70,71]. HDAC inhibitors directly block the action of HDAC and result in hyperacetylation of histones. Both of them have been shown to inhibit the growth of a variety of cancers or to restore the sensitivity of cancer cells to chemical drugs and radiation-induced cell cycle arrest and apoptosis [54,60]. Currently, there are a number of additional small molecules with epigenetic modulation activity are undergoing preclinical evaluation and clinical trials, such as G9a, Ezh2 and IDH inhibitors [72-76]. However, most of these molecules showed limited specificity to their target enzymes and target cells, thus causing unpredictable side effects. Therefore, the traditional strategies of epigenetic therapy need to be further improved to target epigenetic programs in a tumor specific manner.

Targeting cancer epigenetic factors by Cas9 nuclease

In recent years, advances in the development of genome editing tools have made it more accessible to induce targeted DNA break in a sequence-specific and programmable manner. ZFNs, TALENs and the recently developed CRISPR/Cas9 system can all be programmed to generate targeted DSBs in genomic DNA of interest [20,22,24,77]. In most cases, DSBs are repaired by NHEJ mechanism that can result in random insertions and/or deletions. In the presence of a repair template, DSBs may also be repaired by HDR mechanism that generates designed modifications albeit at a much lower frequency [78]. ZFNs and TALENs are chimera nucleases that combine programmable and sequence-specific DNA-binding modules with the non-sequence specific DNA cleavage domain from the Fok1 endonuclease [20,77]. Upon binding to their targets, ZFNs or TALENs form heterodimers and cause DSBs in regions between their binding sites. In contrast, CRISPR/Cas9 targets and cleaves its target DNA through a programmable short guide RNA binding to its complementary DNA strand and a Cas9 protein, respectively. In addition, Cas9 protein recognizes the protospacer recognition motif (PAM) of target DNA that is usually 2-5 bp unique sequence adjacent to the RNA binding site[22,25]. Through the binding of guide RNA and Cas9 protein to the complementary strand and PAM motif, Cas9 cleaves the DNA double strands at several base pairs upstream of the PAM motif to generate a DSB. For the most widely used SpCas9 system, the cleavage

site is about 3-4 base pairs upstream of the NGG PAM [25]. Reprogramming CRISPR/Cas9 system for new targets is unprecedentedly easy, requiring only the presence of PAM in the target DNA and changing the first ~20 bases in guide RNA. When a donor template is given, this system can achieve precise genome correction [28]. And this effect may be further enhanced by simultaneously inhibiting the NEJM pathway. In two recent independent reports, when the NEJM pathway was inhibited by the ligase IV inhibitor SCR7, the efficiency of template guided HDR could be enhanced up to 20 fold [79,80]. These advancements have made CRISPR/Cas9 system promising in a wide range of biomedical research.

As discussed above, targeting epigenetic regulators with small molecules is effective in cancer therapy with limitation in specificity. Therefore, it is attractive to design CRISPR/Cas9-based strategies to target these cancerous epigenetic regulators in a more specific manner. For example, DNMT1 and several other factors carrying cancer related mutations or causing epigenetic-silencing are good candidates for testing such strategies. As mentioned above, because cancerous cells are more sensitive to the level of DNMT1 activity than normal cells, targeting this enzyme should cause limited side-effects. Cancer specific mutations, such as H3K27M and IDH1 R132H, are demonstrated to be driving forces for the proliferation of malignant cells, and therefore reprogramming Cas9 nuclease to target these specific mutant sequences may achieve mutant allele specific disruption.

Manipulating the epigenetic patterns of target loci by dCas-effectors

Besides genetic mutations of epigenetic regulators, direct manipulating the epigenetic patterns of target genes is also available through nuclease inactivated Cas9 (dead Cas9, dCas9) based methods[29,31-33]. Like zinc finger effectors and transcription activator–like (TAL) effectors[81,82], dCas9 can also be linked with effectors and target them to specific loci to activate or repress gene expression. When fused to transcriptional activation domains like VP64 domain and VP64-p65-Rta fusion domain, dCas9 effectors activate gene expression at different levels depending on the strength of activator used and the relative dCas9 binding position to transcription start site[83,84]. Interestingly, dCas9 can specifically interfere with transcriptional elongation and result in 10- to 300-fold repression of mRNA production when it

was directed to the non-template strand of gene bodies, which is thought due to a "roadblocking" effect on the way of RNA polymerase during transcription elongation[85]. Likewise, dCas9 can also be redirected to the regions near which were initially occupied by RNA polymerase and inhibit transcription initiation[85]. To achieve transcriptional inactivation, dCas9 had also been fused with transcriptional repressor domains, such as Krüppel-associated box (KRAB) repressor domain that induce long-range transcriptional repression through the spread of heterochromatin[86,87].Triggered by the positive results from the fusion of transcriptional regulators with those reprogrammable DNA binding proteins, a list of epigenetic regulators had also been tested if they can be redirected to specific genomic region and induce desired local epigenetic modifications [88-91]. Histone methyltransferase domains from G9A and Suv39H1 had been fused to zinc finger proteins, which led to local methylation of histone H3K9 and the consequent repression of target gene expression[51]. Histone demethylase domain from LSD1 fused with TALE efficiently removed H3K4me2 and H3K27ac from the enhancers of target loci and causes down-regulation of proximal genes. Recently, dCas9 had been fused with histone acetyltransferase domain from p300 and demethylase domain from LSD1 to specifically alter local histone modification, leading to robust transcriptional activation or repression of target genes[88,92]. DNA modification enzymes had also been fused with programmable nucleases to alter the methylation state of target loci[89,93]. Methyltransferase domain from DNMT3a fused with zinc finger protein caused site-specific DNA methylation and repression of the target gene[93], while TET1 hydroxylase catalytic domain fused with TALE led to DNA demethylation and transcriptional activation of the target genes[89]. Unlike reprogrammable transcriptional effectors whose function require the presence of themselves at the target site, reprogrammable epigenetic factors that were redirected to local genomic regions altered the levels of nearby histone or DNA modifications and might have long-term effect on gene regulation. Especially in the case of DNA methylation, induced site-specific DNA methylation caused stably and heritably repression of target genes.

The feature that dCas9 effectors can achieve either repression or activation of cancer

related genes make it a very useful tool in cancer gene therapy by activating tumor suppressor genes or inhibiting tumor driving genes. In many cancers, some epigenetic factors, such as TET families that function as tumor suppressors, were usually inactivated by epigenetic mechanisms, while other epigenetic factors, such as LSD1, EZH2 and NSD2 that function as tumor drivers, were overexpressed by either epigenenetic or genetic mechanisms. Therefore, it may be effective to design CRISPR/Cas9 based transcriptional regulators to restore or suppress the expression of these enzymes in such cancers. Further, dCas9-effectors don't result in genetic changes and therefore can be reversible. In theory, such methods can be applied to target the driver genes of cancer and the genes essential for cancer maintenance or drug resistance. Moreover, these methods can also target the epigenetically silenced tumor suppressors, which otherwise are "undruggable". Thus, dCas9-fused epigenetic regulators can be a powerful tool to reversibly manipulate the epigenetic patterns and thus regulate the expressions without genetic changes of both oncogenes and tumor suppressors for cancer treatment.

Delivery of CRISPR-Cas9 system to cancer cells

Theoretically, Cas9 can be delivered as mRNAs, proteins, non-viral DNA and viruses (Figure 1). mRNAs are not sufficiently stable for long term gene therapy purposes. However, for the action of Cas9 nuclease, even transient expression and function can leave the genetic alteration permanent, which is why Cas9 mRNA is widely used in both cell culture and model organisms such as Drosophila, zebrafish, Xenopus and mouse[94] [95,96] [97]. Notably, ZFN mRNAs have been approved in clinical trial for the treatment of HIV infection by disrupting the CCR5 gene in isolated human CD4+ T lymphocytes (NCT02388594, https://clinicaltrials.gov). It is therefore interesting to test the feasibility of delivering Cas9 mRNA for therapeutic usage. Compared with mRNAs, proteins are much more stable and easier to be produced in large scale. Although the cell-penetrating peptides (CPP) and Cas9 fusion protein is difficult to be expressed and purified by genetic method, in vitro CPP–conjugated recombinant Cas9 protein and CPP-complexed guide RNAs have been shown to cause efficient gene

disruptions [27]. Given that CPP containing protein drugs are in clinical trials, CPP-conjugated Cas9 system is worthy of further evaluation as well[98].

Non-viral plasmid DNAs are among the most stable formulations for gene transfer and are widely used for ex vivo delivery of Cas9 and gRNA that are either combined in a single plasmid or separated into two plasmids. For in vivo delivery, only hydrodynamic gene transfer method was reported, in which a relatively large volume of plasmid DNA solution was quickly injected into mouse circulation through tail vein to create hydrodynamic pressure to enable the plasmid DNA to escape the vasculature between the endothelial cells lining the hepatic sinusoids and allow plasmids to enter the cells, mainly hepatocytes [99]. However, the efficiency was rather low. Besides, it is not clear whether this delivery method has specificity to cancerous cells that include primary HCC or tumor cells metastasized from other organs to liver. Therefore, the development of gene transfer methods targeting cancer, which allow for efficient and specific expression of the CRISPR-Cas9 system in tumor tissues, would provide a major advancement in cancer treatment.

Basically, cancer specific expression of transgenes can be achieved either by targeting delivery of therapeutic vector to tumor tissue or by controlling transgene expression with a tumor specific promoter. Cancer-specific promoters, such as the promoter of the telomerase gene, are active specifically in cancerous cells and minimize transgene expression in normal cells [100,101]. Ligand-directed targeting is a more popular choice to delivery transgene specifically to cancerous cells that express corresponding receptors on their surface. By utilizing cancer specific ligands/receptors, this method can achieve high delivery efficiency and specificity, thus having great potential in cancer gene therapy [102]. The past several years have witnessed a rapid development of biodegradable nano-carriers based on lipid and various polymers for cancer specific gene delivery [103-106]. Folate coated liposome, for example, showed significantly higher capability of delivering DNA and small molecule drugs to cells expressing folate receptor than uncoated liposome [106]. Folate receptor is highly expressed in a wide range of epithelial malignant cells, such as ovarian, cervical and lung cancer cells, and absent or weakly expressed in normal cells[104]. This property makes

folate-targeted approaches promising in cancer specific gene transfer. Recently, we tested a folate-coated liposome vector carrying Cas9 plasmid as an approach to treating ovarian cancer. In a mouse ovarian cancer model, intraperitoneal injection of folate-conjugated liposome carrying cas9 plasmids targeting DNMT1 resulted in 80-90% reduction in tumor volume without any detectable acute toxicity (unpublished data). As the size of plasmid is pivotal for efficient gene delivery through endosomal uptake and cytoplasmic transportation towards the nucleus, there is a great need to reduce the length of Cas9 coding region. Fortunately, a smaller Cas9 orthologue from Staphylococcus aureus (SaCas9) was recently isolated and shown to edit genome with efficiencies similar to those of SpCas9 [107]. Based on the fact that truncated SpCas9 protein with Rec2 domain deletion still preserved about 50% cleavage activity, there may still be room to reduce SaCas9 size while keeping its activity at an acceptable level [108]. In addition, minicircle plasmid has shown great benefit of reducing plasmid size and minimizing immuno-reactions by removing bacteria derived DNA sequences [109], which can generate a SaCas9 plasmid less than 4 kbs long. Therefore, combining these techniques, we shall see a big improvement of non-viral delivery of Cas9 system.

Adenoviral, retroviral, lentiviral, and adeno-associated viral (AAV) vectors are the major viral vectors used in gene therapy. Compared with non-viral vector, viral vectors generally offer higher delivery efficiency and longer term of transgene expression. Adenovirus and lentivirus have been used to deliver cas9 ex vivo and achieved highly efficient gene disruption. However, a concern of using virus, especially adenovirus, is its immunogenicity, which may hamper their use in repeated treatments. AAV is relative small and causes a very mild immune response, making it a very attractive delivery vector for gene therapy. As mentioned above, the discovery of SaCas9 of a smaller size makes it possible to use AAVs as Cas9 carriers. In in vivo experiments, AAV-mediated systemic delivery of Cas9 caused above 40% gene disruption in mouse liver [107]. Given that AAV infection is usually tissue specific, AAV based Cas9 delivery may be useful in treating malignant tumors from certain tissues.

Perspectives:

The rapid progression of genome editing technology has brought great benefits for both basic science and clinical research. Hopefully in the near future, with further development of CRISPR/Cas9 system, this system can be designed to be smaller and to recognize any possible PAM thus to target any DNA sequences of interest, and to function in a more specific manner. Although applications of CRISPR/Cas9 in clinic are just taking place, rapid advances in this field and other additional technologies, such as targeted delivery and nanotechnology, will undoubtedly further expand its therapeutic use in cancer treatment.

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Reference：

1 House, S. H. Epigenetics in adaptive evolution and development: The interplay between evolving species and epigenetic mechanisms: Extract from Trygve Tollefsbol (ed.) (2011) Handbook of Epigenetics - The New Molecular and Medical Genetics. Chapter 26. Amsterdam, USA: Elsevier, pp. 423-446. *Nutrition and health* **22**, 105-131, doi:10.1177/0260106014537160 (2013).

2 Vogel, T. & Lassmann, S. Epigenetics: development, dynamics and disease. *Cell and tissue research* **356**, 451-455, doi:10.1007/s00441-014-1916-7 (2014).

3 Kanwal, R., Gupta, K. & Gupta, S. Cancer epigenetics: an introduction. *Methods in molecular biology* **1238**, 3-25, doi:10.1007/978-1-4939-1804-1_1 (2015).

4 Ushijima, T. Cancer epigenetics: now harvesting fruit and seeding for common diseases. *Biochemical and biophysical research communications* **455**, 1-2, doi:10.1016/j.bbrc.2014.11.008 (2014).

5 Morishita, M. & di Luccio, E. Cancers and the NSD family of histone lysine methyltransferases. *Biochimica et biophysica acta* **1816**, 158-163, doi:10.1016/j.bbcan.2011.05.004 (2011).

6 Yang, P. *et al.* Histone methyltransferase NSD2/MMSET mediates constitutive NF-kappaB signaling for cancer cell proliferation, survival, and tumor growth via a feed-forward loop. *Molecular and cellular biology* **32**, 3121-3131, doi:10.1128/MCB.00204-12 (2012).

7 Kleer, C. G. *et al.* EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 11606-11611, doi:10.1073/pnas.1933744100 (2003).

8 Sellers, W. R. & Loda, M. The EZH2 polycomb transcriptional repressor--a marker or mover of metastatic prostate cancer? *Cancer cell* **2**, 349-350 (2002).

9 Varambally, S. *et al.* The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* **419**, 624-629, doi:10.1038/nature01075 (2002).

10 Shao, G. B. *et al.* [Histone demethylase LSD1 and its biological functions]. *Yi chuan = Hereditas / Zhongguo yi chuan xue hui bian ji* **32**, 331-338 (2010).

11 Hayami, S. *et al.* Overexpression of LSD1 contributes to human carcinogenesis through chromatin regulation in various cancers. *International journal of cancer. Journal international du cancer* **128**, 574-586, doi:10.1002/ijc.25349 (2011).

12 Behjati, S. *et al.* Distinct H3F3A and H3F3B driver mutations define chondroblastoma and giant cell tumor of bone. *Nature genetics* **45**, 1479-1482, doi:10.1038/ng.2814 (2013).

13 Lewis, P. W. *et al.* Inhibition of PRC2 activity by a gain-of-function H3 mutation found in pediatric glioblastoma. *Science* **340**, 857-861, doi:10.1126/science.1232245 (2013).

14 Schwartzentruber, J. *et al.* Driver mutations in histone H3.3 and chromatin remodelling genes in paediatric glioblastoma. *Nature* **482**, 226-231, doi:10.1038/nature10833 (2012).

15 Wu, G. *et al.* Somatic histone H3 alterations in pediatric diffuse intrinsic pontine gliomas and non-brainstem glioblastomas. *Nature genetics* **44**, 251-253, doi:10.1038/ng.1102 (2012).

16 Chen, C. *et al.* MLL3 is a haploinsufficient 7q tumor suppressor in acute myeloid leukemia. *Cancer cell* **25**, 652-665, doi:10.1016/j.ccr.2014.03.016 (2014).

17 Zuber, J. *et al.* RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. *Nature* **478**, 524-528, doi:10.1038/nature10334 (2011).

18 Miozzo, M., Vaira, V. & Sirchia, S. M. Epigenetic alterations in cancer and personalized cancer treatment. *Future oncology* **11**, 333-348, doi:10.2217/fon.14.237 (2015).

19 Chessum, N., Jones, K., Pasqua, E. & Tucker, M. Recent advances in cancer therapeutics. *Progress in medicinal chemistry* **54**, 1-63, doi:10.1016/bs.pmch.2014.11.002 (2015).

20 Urnov, F. D., Rebar, E. J., Holmes, M. C., Zhang, H. S. & Gregory, P. D. Genome editing with engineered zinc finger nucleases. *Nature reviews. Genetics* **11**, 636-646, doi:10.1038/nrg2842 (2010).

21 Bogdanove, A. J. & Voytas, D. F. TAL effectors: customizable proteins for DNA targeting. *Science* **333**, 1843-1846, doi:10.1126/science.1204094 (2011).

22 Hsu, P. D., Lander, E. S. & Zhang, F. Development and applications of CRISPR-Cas9 for genome engineering. *Cell* **157**, 1262-1278, doi:10.1016/j.cell.2014.05.010 (2014).

23 Doudna, J. A. & Charpentier, E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* **346**, 1258096, doi:10.1126/science.1258096 (2014).

24 Cox, D. B., Platt, R. J. & Zhang, F. Therapeutic genome editing: prospects and challenges. *Nature medicine* **21**, 121-131, doi:10.1038/nm.3793 (2015).

25 Jinek, M. *et al.* A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821, doi:10.1126/science.1225829 (2012).

26 Cong, L. *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-823, doi:10.1126/science.1231143 (2013).

27 Ramakrishna, S. *et al.* Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. *Genome research* **24**, 1020-1027, doi:10.1101/gr.171264.113 (2014).

28 Hisano, Y. *et al.* Precise in-frame integration of exogenous DNA mediated by CRISPR/Cas9 system in zebrafish. *Scientific reports* **5**, 8841, doi:10.1038/srep08841 (2015).

29 Cheng, A. W. *et al.* Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. *Cell research* **23**, 1163-1171, doi:10.1038/cr.2013.122 (2013).

30 Kearns, N. A. *et al.* Cas9 effector-mediated regulation of transcription and differentiation in human pluripotent stem cells. *Development* **141**, 219-223, doi:10.1242/dev.103341 (2014).

31 Larson, M. H. *et al.* CRISPR interference (CRISPRi) for sequence-specific control of gene expression. *Nature protocols* **8**, 2180-2196, doi:10.1038/nprot.2013.132 (2013).

32 Xu, T., Li, Y., Van Nostrand, J. D., He, Z. & Zhou, J. Cas9-based tools for targeted genome editing and transcriptional control. *Applied and environmental microbiology* **80**, 1544-1552, doi:10.1128/AEM.03786-13 (2014).

33 Nelles, D. A., Fang, M. Y., Aigner, S. & Yeo, G. W. Applications of Cas9 as an RNA-programmed RNA-binding protein. *BioEssays : news and reviews in molecular, cellular and developmental biology*, doi:10.1002/bies.201500001 (2015).

34 Handel, A. E., Ebers, G. C. & Ramagopalan, S. V. Epigenetics: molecular mechanisms and implications for disease. *Trends in molecular medicine* **16**, 7-16, doi:10.1016/j.molmed.2009.11.003 (2010).

35 Hirasawa, R. *et al.* Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. *Genes & development* **22**, 1607-1616, doi:10.1101/gad.1667008 (2008).

36 Yokochi, T. & Robertson, K. D. Preferential methylation of unmethylated DNA by Mammalian de novo DNA methyltransferase Dnmt3a. *The Journal of biological chemistry* **277**, 11735-11745, doi:10.1074/jbc.M106590200 (2002).

37 Wang, Z. G. & Wu, J. X. [DNA methyltransferases: classification, functions and research progress]. *Yi chuan = Hereditas / Zhongguo yi chuan xue hui bian ji* **31**, 903-912 (2009).

38 Chedin, F., Lieber, M. R. & Hsieh, C. L. The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 16916-16921, doi:10.1073/pnas.262443999 (2002).

39 Suetake, I., Shinozaki, F., Miyagawa, J., Takeshima, H. & Tajima, S. DNMT3L stimulates the DNA

methylation activity of Dnmt3a and Dnmt3b through a direct interaction. *The Journal of biological chemistry* **279**, 27816-27823, doi:10.1074/jbc.M400181200 (2004).

40 Schubeler, D. Function and information content of DNA methylation. *Nature* **517**, 321-326, doi:10.1038/nature14192 (2015).

41 Kroeze, L. I., van der Reijden, B. A. & Jansen, J. H. 5-Hydroxymethylcytosine: An epigenetic mark frequently deregulated in cancer. *Biochimica et biophysica acta* **1855**, 144-154, doi:10.1016/j.bbcan.2015.01.001 (2015).

42 Shen, L., Song, C. X., He, C. & Zhang, Y. Mechanism and function of oxidative reversal of DNA and RNA methylation. *Annual review of biochemistry* **83**, 585-614, doi:10.1146/annurev-biochem-060713-035513 (2014).

43 Ito, S. *et al.* Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* **466**, 1129-1133, doi:10.1038/nature09303 (2010).

44 Butler, J. S., Koutelou, E., Schibler, A. C. & Dent, S. Y. Histone-modifying enzymes: regulators of developmental decisions and drivers of human disease. *Epigenomics* **4**, 163-177, doi:10.2217/epi.12.3 (2012).

45 du Preez, L. L. & Patterton, H. G. Secondary structures of the core histone N-terminal tails: their role in regulating chromatin structure. *Sub-cellular biochemistry* **61**, 37-55, doi:10.1007/978-94-007-4525-4_2 (2013).

46 Tessarz, P. & Kouzarides, T. Histone core modifications regulating nucleosome structure and dynamics. *Nature reviews. Molecular cell biology* **15**, 703-708, doi:10.1038/nrm3890 (2014).

47 Mosammaparast, N. & Shi, Y. Reversal of histone methylation: biochemical and molecular mechanisms of histone demethylases. *Annual review of biochemistry* **79**, 155-179, doi:10.1146/annurev.biochem.78.070907.103946 (2010).

48 Shi, Y. *et al.* Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119**, 941-953, doi:10.1016/j.cell.2004.12.012 (2004).

49 Kooistra, S. M. & Helin, K. Molecular mechanisms and potential functions of histone demethylases. *Nature reviews. Molecular cell biology* **13**, 297-311, doi:10.1038/nrm3327 (2012).

50 Wang, C., Fu, M. & Pestell, R. G. Histone acetylation/deacetylation as a regulator of cell cycle gene expression. *Methods in molecular biology* **241**, 207-216 (2004).

51 Snowden, A. W., Gregory, P. D., Case, C. C. & Pabo, C. O. Gene-specific targeting of H3K9 methylation is sufficient for initiating repression in vivo. *Current biology : CB* **12**, 2159-2166 (2002).

52 Karmodiya, K., Krebs, A. R., Oulad-Abdelghani, M., Kimura, H. & Tora, L. H3K9 and H3K14 acetylation co-occur at many gene regulatory elements, while H3K14ac marks a subset of inactive inducible promoters in mouse embryonic stem cells. *BMC genomics* **13**, 424, doi:10.1186/1471-2164-13-424 (2012).

53 Baylin, S. B. & Herman, J. G. DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends in genetics : TIG* **16**, 168-174 (2000).

54 Gnyszka, A., Jastrzebski, Z. & Flis, S. DNA methyltransferase inhibitors and their emerging role in epigenetic therapy of cancer. *Anticancer research* **33**, 2989-2996 (2013).

55 Trowbridge, J. J. *et al.* Haploinsufficiency of Dnmt1 impairs leukemia stem cell function through derepression of bivalent chromatin domains. *Genes & development* **26**, 344-349, doi:10.1101/gad.184341.111 (2012).

56 Zhen, L., Gui-lan, L., Ping, Y., Jin, H. & Ya-li, W. The expression of H3K9Ac, H3K14Ac, and H4K20TriMe in epithelial ovarian tumors and the clinical significance. *International journal of gynecological cancer :*

official journal of the International Gynecological Cancer Society **20**, 82-86, doi:10.1111/IGC.0b013e3181ae3efa (2010).

57 Mohamed, M. A. *et al.* Epigenetic events, remodelling enzymes and their relationship to chromatin organization in prostatic intraepithelial neoplasia and prostatic adenocarcinoma. *BJU international* **99**, 908-915, doi:10.1111/j.1464-410X.2006.06704.x (2007).

58 Seligson, D. B. *et al.* Global histone modification patterns predict risk of prostate cancer recurrence. *Nature* **435**, 1262-1266, doi:10.1038/nature03672 (2005).

59 Park, Y. S. *et al.* The global histone modification pattern correlates with cancer recurrence and overall survival in gastric adenocarcinoma. *Annals of surgical oncology* **15**, 1968-1976, doi:10.1245/s10434-008-9927-9 (2008).

60 De Souza, C. & Chatterji, B. P. HDAC Inhibitors as Novel Anti-Cancer Therapeutics. *Recent patents on anti-cancer drug discovery* (2015).

61 Jin, B. *et al.* DNA methyltransferase 3B (DNMT3B) mutations in ICF syndrome lead to altered epigenetic modifications and aberrant expression of genes regulating development, neurogenesis and immune function. *Human molecular genetics* **17**, 690-709, doi:10.1093/hmg/ddm341 (2008).

62 Tefferi, A., Lim, K. H. & Levine, R. Mutation in TET2 in myeloid cancers. *The New England journal of medicine* **361**, 1117; author reply 1117-1118, doi:10.1056/NEJMc091348 (2009).

63 Bodor, C. *et al.* EZH2 mutations are frequent and represent an early event in follicular lymphoma. *Blood* **122**, 3165-3168, doi:10.1182/blood-2013-04-496893 (2013).

64 Oyer, J. A. *et al.* Point mutation E1099K in MMSET/NSD2 enhances its methyltranferase activity and leads to altered global chromatin methylation in lymphoid malignancies. *Leukemia* **28**, 198-201, doi:10.1038/leu.2013.204 (2014).

65 Wang, X. X. *et al.* Somatic mutations of the mixed-lineage leukemia 3 (MLL3) gene in primary breast cancers. *Pathology oncology research : POR* **17**, 429-433, doi:10.1007/s12253-010-9316-0 (2011).

66 Yen, K. E., Bittinger, M. A., Su, S. M. & Fantin, V. R. Cancer-associated IDH mutations: biomarker and therapeutic opportunities. *Oncogene* **29**, 6409-6417, doi:10.1038/onc.2010.444 (2010).

67 Moran-Crusio, K. *et al.* Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. *Cancer cell* **20**, 11-24, doi:10.1016/j.ccr.2011.06.001 (2011).

68 Funato, K., Major, T., Lewis, P. W., Allis, C. D. & Tabar, V. Use of human embryonic stem cells to model pediatric gliomas with H3.3K27M histone mutation. *Science* **346**, 1529-1533, doi:10.1126/science.1253799 (2014).

69 Hashizume, R. *et al.* Pharmacologic inhibition of histone demethylation as a therapy for pediatric brainstem glioma. *Nature medicine* **20**, 1394-1396, doi:10.1038/nm.3716 (2014).

70 Wongtrakoongate, P. Epigenetic therapy of cancer stem and progenitor cells by targeting DNA methylation machineries. *World journal of stem cells* **7**, 137-148, doi:10.4252/wjsc.v7.i1.137 (2015).

71 Yang, X., Lay, F., Han, H. & Jones, P. A. Targeting DNA methylation for epigenetic therapy. *Trends in pharmacological sciences* **31**, 536-546, doi:10.1016/j.tips.2010.08.001 (2010).

72 Liu, F. *et al.* Discovery of a 2,4-diamino-7-aminoalkoxyquinazoline as a potent and selective inhibitor of histone lysine methyltransferase G9a. *Journal of medicinal chemistry* **52**, 7950-7953, doi:10.1021/jm901543m (2009).

73 Knutson, S. K. *et al.* A selective inhibitor of EZH2 blocks H3K27 methylation and kills mutant lymphoma cells. *Nature chemical biology* **8**, 890-896, doi:10.1038/nchembio.1084 (2012).

74 Qi, W. *et al.* Selective inhibition of Ezh2 by a small molecule inhibitor blocks tumor cells proliferation. *Proceedings of the National Academy of Sciences of the United States of America* **109**, 21360-21365,

doi:10.1073/pnas.1210371110 (2012).

75 Rohle, D. *et al.* An inhibitor of mutant IDH1 delays growth and promotes differentiation of glioma cells. *Science* **340**, 626-630, doi:10.1126/science.1236062 (2013).

76 Wang, F. *et al.* Targeted inhibition of mutant IDH2 in leukemia cells induces cellular differentiation. *Science* **340**, 622-626, doi:10.1126/science.1234769 (2013).

77 Li, T. & Yang, B. TAL effector nuclease (TALEN) engineering. *Methods in molecular biology* **978**, 63-72, doi:10.1007/978-1-62703-293-3_5 (2013).

78 McVey, M. & Lee, S. E. MMEJ repair of double-strand breaks (director's cut): deleted sequences and alternative endings. *Trends in genetics : TIG* **24**, 529-538, doi:10.1016/j.tig.2008.08.007 (2008).

79 Maruyama, T. *et al.* Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nature biotechnology*, doi:10.1038/nbt.3190 (2015).

80 Chu, V. T. *et al.* Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nature biotechnology*, doi:10.1038/nbt.3198 (2015).

81 Wilson, K. A., Chateau, M. L. & Porteus, M. H. Design and Development of Artificial Zinc Finger Transcription Factors and Zinc Finger Nucleases to the hTERT Locus. *Molecular therapy. Nucleic acids* **2**, e87, doi:10.1038/mtna.2013.12 (2013).

82 Wright, D. A., Li, T., Yang, B. & Spalding, M. H. TALEN-mediated genome editing: prospects and perspectives. *The Biochemical journal* **462**, 15-24, doi:10.1042/BJ20140295 (2014).

83 Maeder, M. L. *et al.* CRISPR RNA-guided activation of endogenous human genes. *Nature methods* **10**, 977-979, doi:10.1038/nmeth.2598 (2013).

84 Chavez, A. *et al.* Highly efficient Cas9-mediated transcriptional programming. *Nature methods* **12**, 326-328, doi:10.1038/nmeth.3312 (2015).

85 Qi, L. S. *et al.* Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* **152**, 1173-1183, doi:10.1016/j.cell.2013.02.022 (2013).

86 Gilbert, L. A. *et al.* CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* **154**, 442-451, doi:10.1016/j.cell.2013.06.044 (2013).

87 Groner, A. C. *et al.* KRAB-zinc finger proteins and KAP1 can mediate long-range transcriptional repression through heterochromatin spreading. *PLoS genetics* **6**, e1000869, doi:10.1371/journal.pgen.1000869 (2010).

88 Hilton, I. B. *et al.* Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nature biotechnology* **33**, 510-517, doi:10.1038/nbt.3199 (2015).

89 Maeder, M. L. *et al.* Targeted DNA demethylation and activation of endogenous genes using programmable TALE-TET1 fusion proteins. *Nature biotechnology* **31**, 1137-1142, doi:10.1038/nbt.2726 (2013).

90 Mendenhall, E. M. *et al.* Locus-specific editing of histone modifications at endogenous enhancers. *Nature biotechnology* **31**, 1133-1136, doi:10.1038/nbt.2701 (2013).

91 Keung, A. J., Bashor, C. J., Kiriakov, S., Collins, J. J. & Khalil, A. S. Using targeted chromatin regulators to engineer combinatorial and spatial transcriptional regulation. *Cell* **158**, 110-120, doi:10.1016/j.cell.2014.04.047 (2014).

92 Kearns, N. A. *et al.* Functional annotation of native enhancers with a Cas9-histone demethylase fusion. *Nature methods* **12**, 401-403, doi:10.1038/nmeth.3325 (2015).

93 Rivenbark, A. G. *et al.* Epigenetic reprogramming of cancer cells via targeted DNA methylation. *Epigenetics : official journal of the DNA Methylation Society* **7**, 350-360, doi:10.4161/epi.19507 (2012).

94 Gratz, S. J. *et al.* Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease.

Genetics **194**, 1029-1035, doi:10.1534/genetics.113.152710 (2013).

95 Hwang, W. Y. *et al.* Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature biotechnology* **31**, 227-229, doi:10.1038/nbt.2501 (2013).

96 Nakayama, T. *et al.* Simple and efficient CRISPR/Cas9-mediated targeted mutagenesis in Xenopus tropicalis. *Genesis* **51**, 835-843, doi:10.1002/dvg.22720 (2013).

97 Shen, B. *et al.* Efficient genome modification by CRISPR-Cas9 nickase with minimal off-target effects. *Nature methods* **11**, 399-402, doi:10.1038/nmeth.2857 (2014).

98 Vasconcelos, L., Parn, K. & Langel, U. Therapeutic potential of cell-penetrating peptides. *Therapeutic delivery* **4**, 573-591, doi:10.4155/tde.13.22 (2013).

99 Xue, W. *et al.* CRISPR-mediated direct mutation of cancer genes in the mouse liver. *Nature* **514**, 380-384, doi:10.1038/nature13589 (2014).

100 Kanegae, Y. *et al.* High-level expression by tissue/cancer-specific promoter with strict specificity using a single-adenoviral vector. *Nucleic acids research* **39**, e7, doi:10.1093/nar/gkq966 (2011).

101 Kim, Y. C., Kim, B. G. & Lee, J. H. Thymosin beta10 expression driven by the human TERT promoter induces ovarian cancer-specific apoptosis through ROS production. *PloS one* **7**, e35399, doi:10.1371/journal.pone.0035399 (2012).

102 Xu, X., Ho, W., Zhang, X., Bertrand, N. & Farokhzad, O. Cancer nanomedicine: from targeted delivery to combination therapy. *Trends in molecular medicine* **21**, 223-232, doi:10.1016/j.molmed.2015.01.001 (2015).

103 Won, Y. W., Lim, K. S. & Kim, Y. H. Intracellular organelle-targeted non-viral gene delivery systems. *Journal of controlled release : official journal of the Controlled Release Society* **152**, 99-109, doi:10.1016/j.jconrel.2011.01.013 (2011).

104 Hattori, Y. & Maitani, Y. Folate-linked lipid-based nanoparticle for targeted gene delivery. *Current drug delivery* **2**, 243-252 (2005).

105 Jin, L., Zeng, X., Liu, M., Deng, Y. & He, N. Current progress in gene delivery technology based on chemical methods and nano-carriers. *Theranostics* **4**, 240-255, doi:10.7150/thno.6914 (2014).

106 He, Z. Y. *et al.* Folate-linked lipoplexes for short hairpin RNA targeting claudin-3 delivery in ovarian cancer xenografts. *Journal of controlled release : official journal of the Controlled Release Society* **172**, 679-689, doi:10.1016/j.jconrel.2013.10.015 (2013).

107 Ran, F. A. *et al.* In vivo genome editing using Staphylococcus aureus Cas9. *Nature* **520**, 186-191, doi:10.1038/nature14299 (2015).

108 Nishimasu, H. *et al.* Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* **156**, 935-949, doi:10.1016/j.cell.2014.02.001 (2014).

109 Chen, Z. Y., He, C. Y. & Kay, M. A. Improved production and purification of minicircle DNA vector free of plasmid bacterial sequences and capable of persistent transgene expression in vivo. *Human gene therapy* **16**, 126-131, doi:10.1089/hum.2005.16.126 (2005).

Figure Legend:

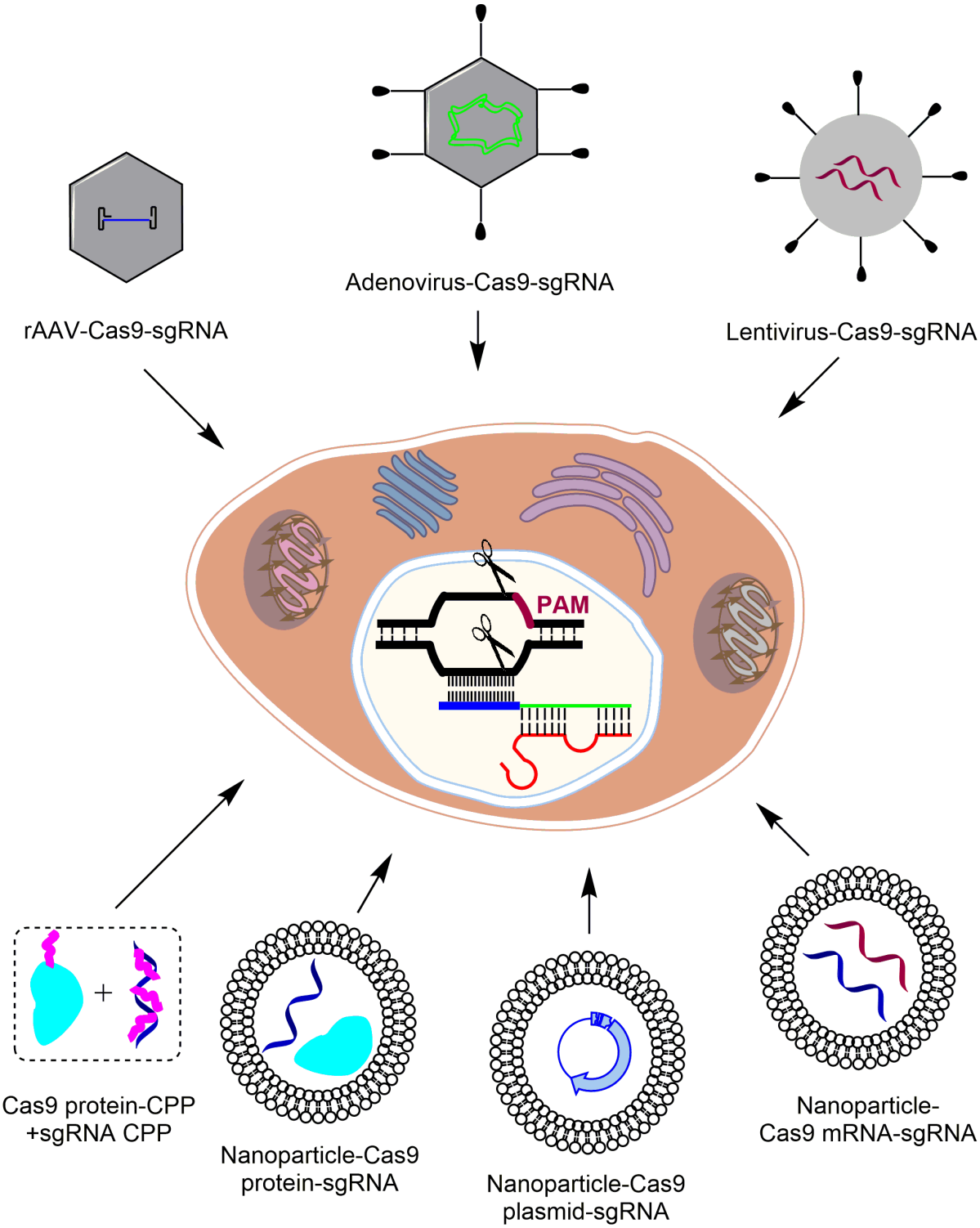

Figure 1. Overview of delivery systems for CRISPR–Cas9. Schematics summarizing the delivery of CRISPR–Cas9 through proteins, mRNAs, non-viral plasmid DNA and various viruses. For virus and plasmid vector delivery, Cas9 and gRNA have been either combined into one single vector or separated into two vectors. For mRNA delivery, Cas9 mRNA and sgRNA are in vitro synthesized and encapsulated in nanoparticles. For protein delivery, Cas9 proteins and sgRNAs are either conjugated with cell penetrating peptides (CPP) or encapsulated in nanoparticles.