Applications of genome editing technologies

❑ 10. Epigenetic modifications using CRISPR

- ❑ Inactive Cas9 can be fused to epigenetic modifiers like p300, TET1, DNMT3A and LSD1 to create programmable epigenome-engineering tools.
- ❑ These tools alter gene expression without inducing double-strand breaks by modifying the methylation state of cytosines in a gene's promoter or by inducing histone modifications or methylation/demethylation.
- ❑ CRISPR epigenetic tools are specific for particular chromatin and DNA modifications, allowing researchers to isolate the effects of a single epigenetic mark.
- ❑ Another potential advantage of CRISPR epigenetic tools is their persistence and inheritance. CRISPR activators and repressors are thought to be reversible once the effector is inactivated or removed from the system.
- ❑ In contrast, epigenetic marks left by targeted epigenetic modifiers may be more frequently inherited by daughter cells. In certain cases, epigenetic modifiers may work better than activators/repressors in modulating transcription.
- ❑ However, since the effects of these tools are likely cell type- and context-dependent, it might be beneficial to try multiple CRISPR strategies when setting up your experimental system.

H3K27ac is an epigenetic modification to the DNA packaging protein Histone H3. It is a mark that indicates the acetylation at the 27th lysine residue of the histone H3 protein. H3K27ac is associated with the higher activation of transcription and therefore defined as an active enhancer mark.

Histone acetyltransferase p300 regulates transcription of genes via chromatin remodeling by allowing histone proteins to wrap DNA less tightly and functions as a transcriptional co-activator protein

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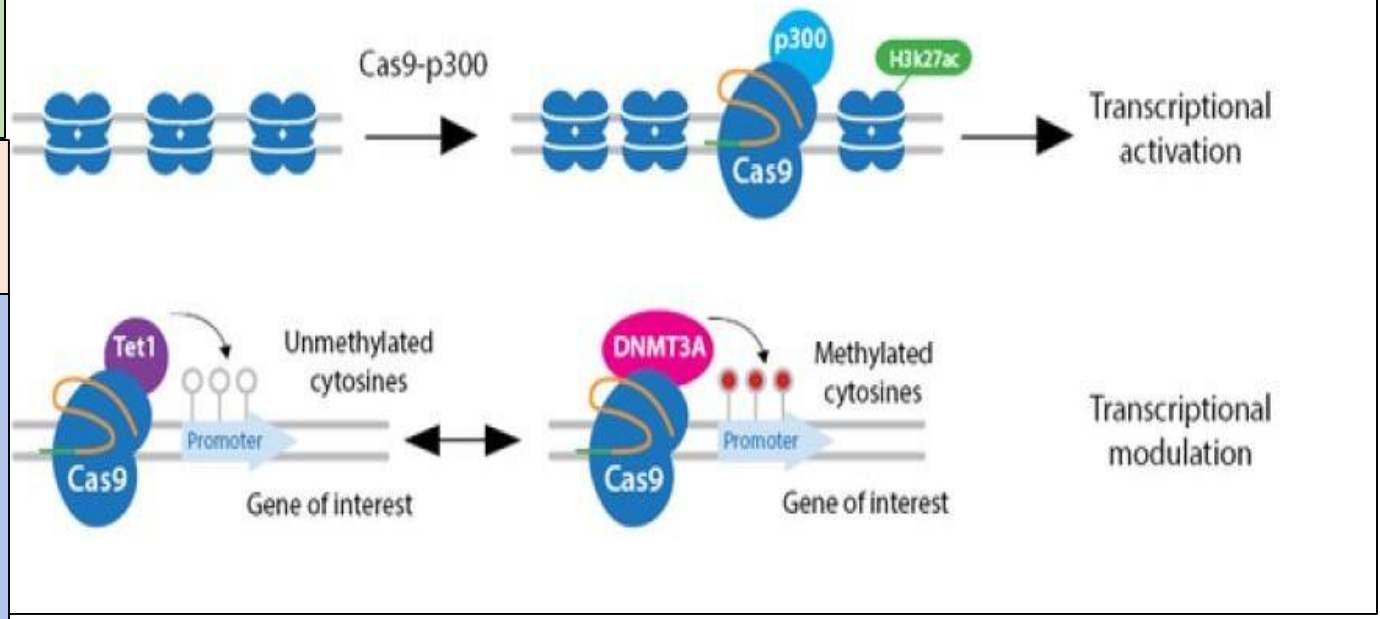
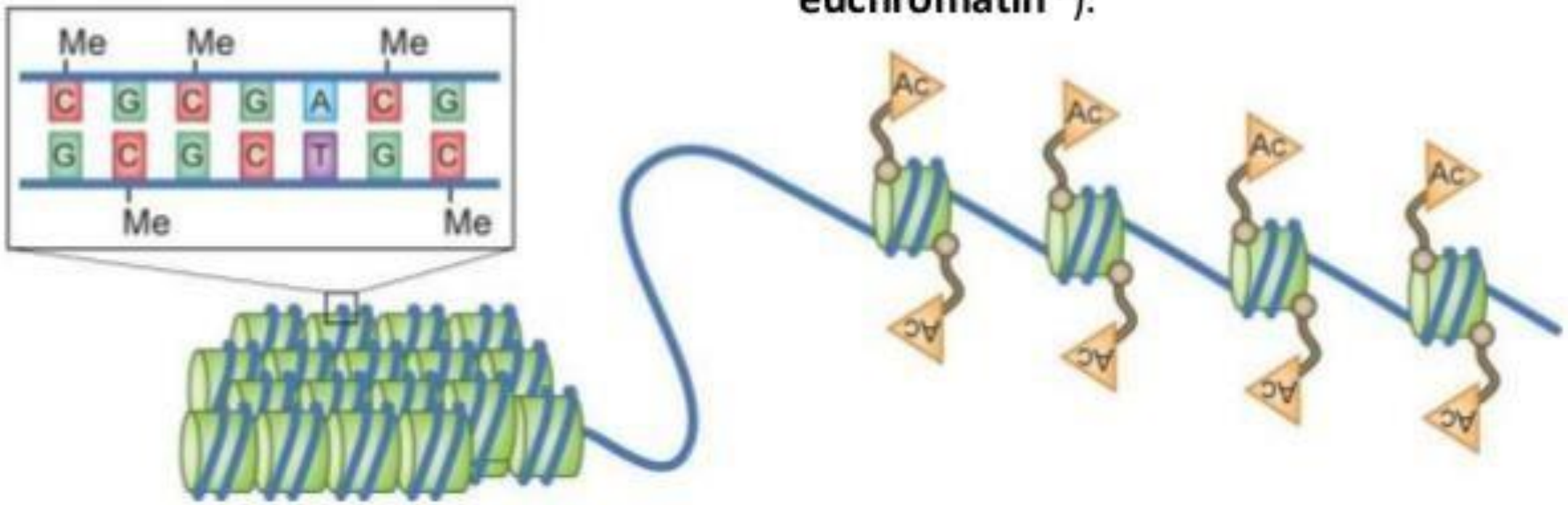


7.2.U2 Nucleosomes help to regulate transcription in eukaryotes.

Acetylation is the addition of Acetyl groups to **histones**

Acetylation promotes transcription

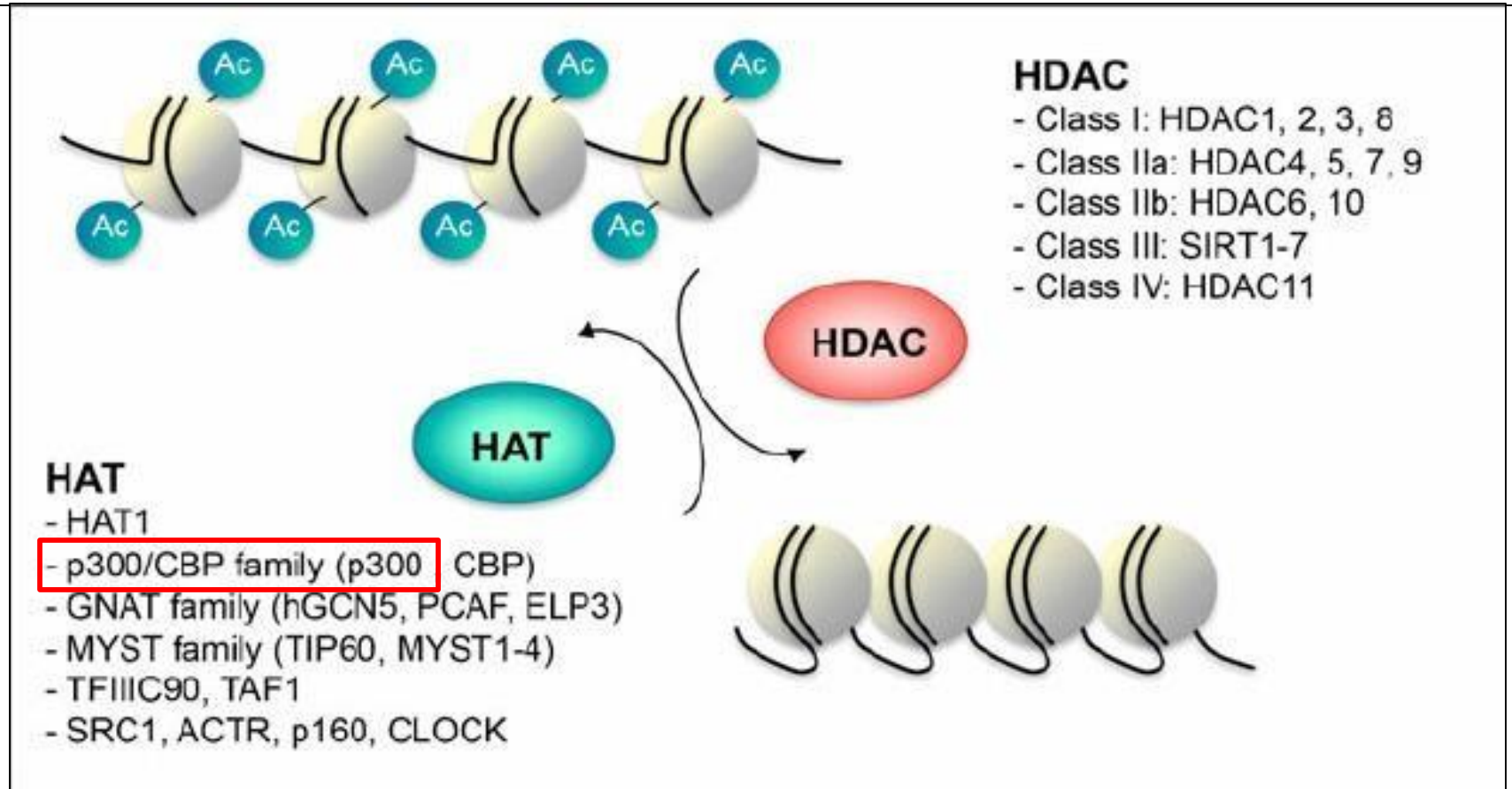
Processes that **promote transcription** **bind** the **DNA** more loosely to the **histone** making it **more accessible** to transcription factors (forming **euchromatin***).



n.b. Methylation of **histones** can also occur, this process **can both promote and inhibit** transcription.

**Chromatin is a complex of DNA, protein and RNA. Loosely packed chromatin which can be transcribed is referred to as euchromatin.*

Edited from: http://www.nature.com/neuro/journal/v13/n4/images/nn0410-405-F1.jpg


Ac
Ac
Ac
Ac
Ac
Ac
Ac
Ac
HDAC
HAT
HDAC
- Class I: HDAC1, 2, 3, 8
- Class IIa: HDAC4, 5, 7, 9
- Class IIb: HDAC6, 10
- Class III: SIRT1-7
- Class IV: HDAC11
HAT
- HAT1
- p300/CBP family (p300 CBP)
- GNAT family (hGCN5, PCAF, ELP3)
- MYST family (TIP60, MYST1-4)
- TFIIIC90, TAF1
- SRC1, ACTR, p160, CLOCK

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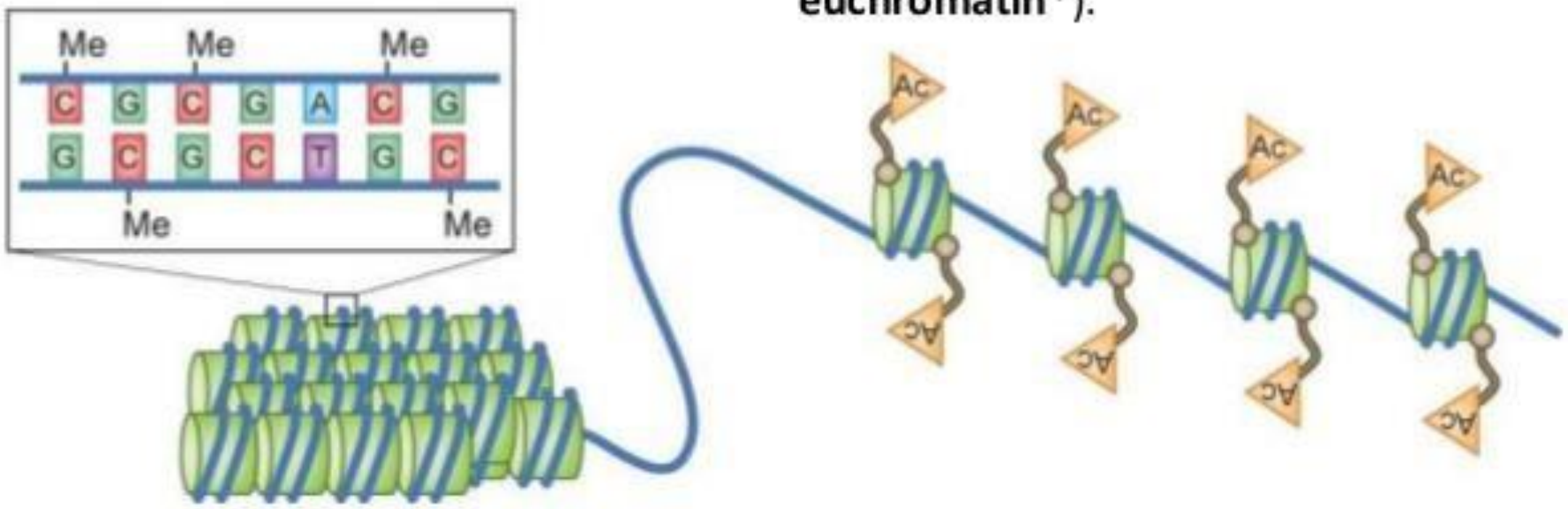

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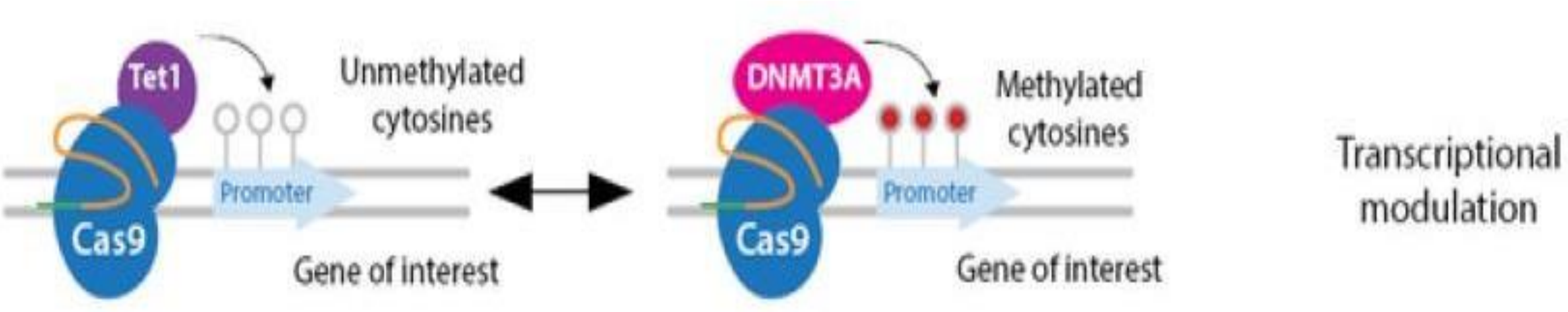
H3K4me3	Active promoters
H3K4me1	Active enhancers
H3K27ac	Active promoters and enhancers
H3K27me3	Inactive chromatin
RNA Pol II	Transcription



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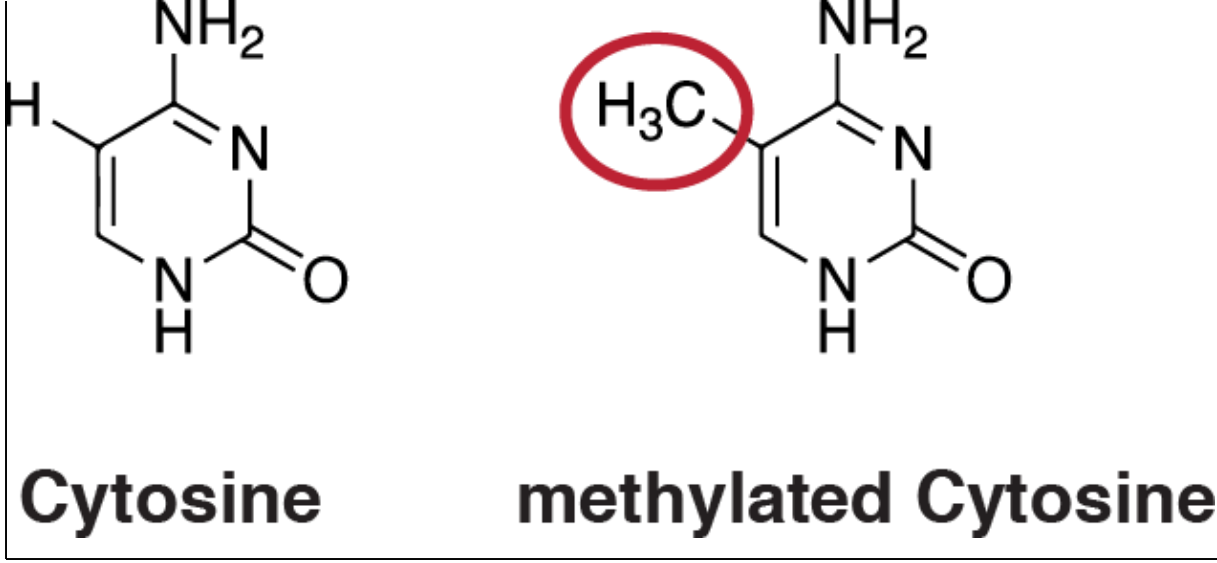
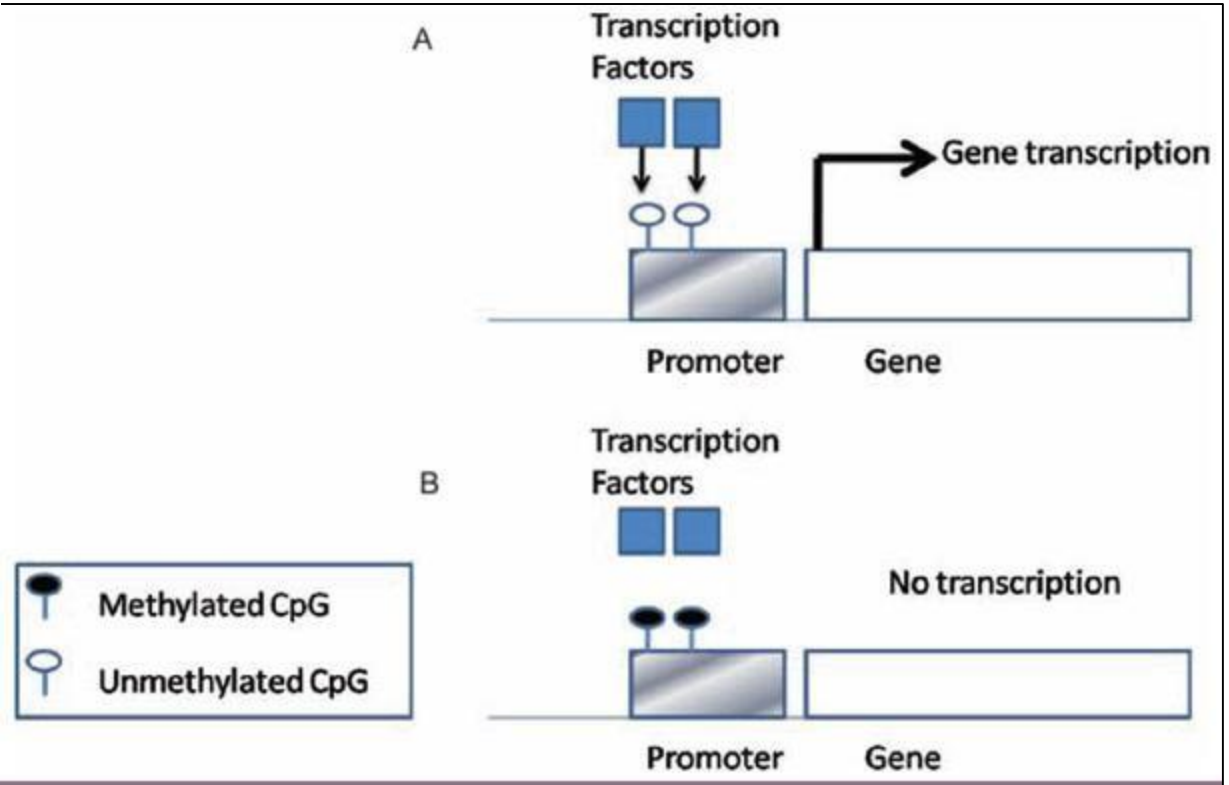
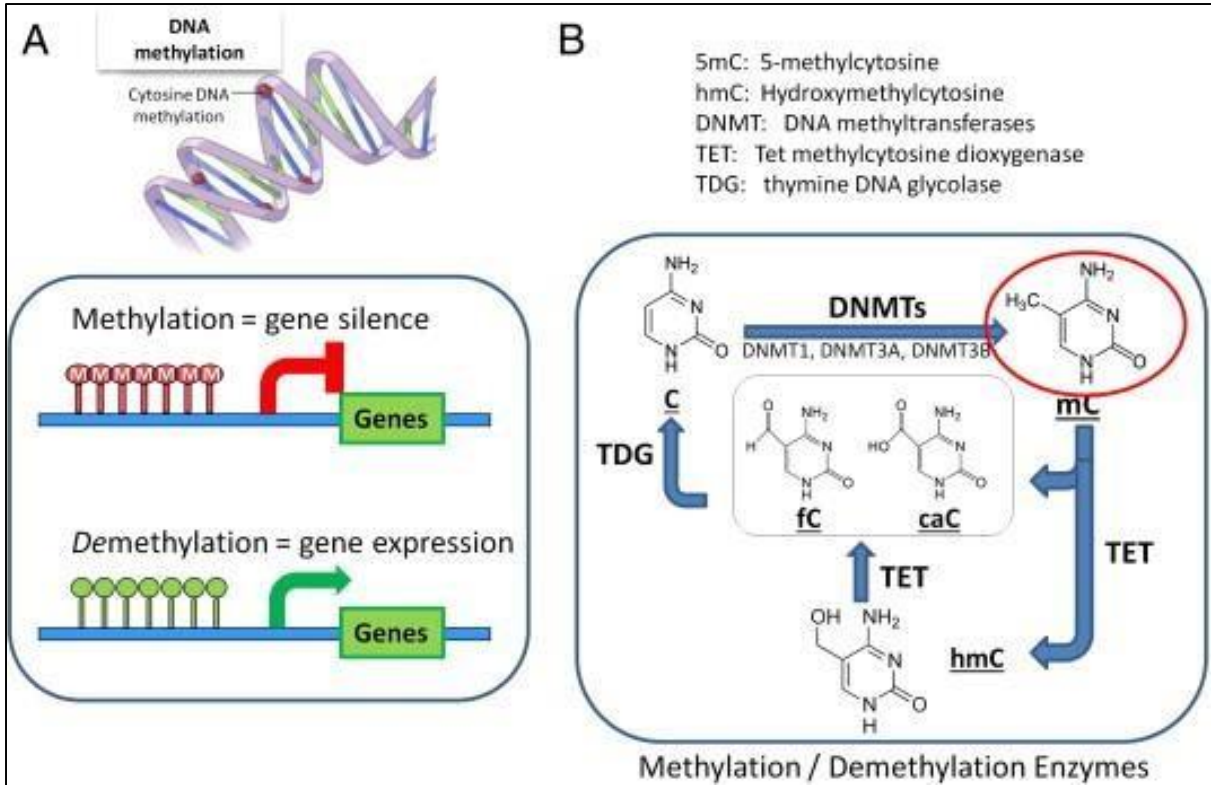
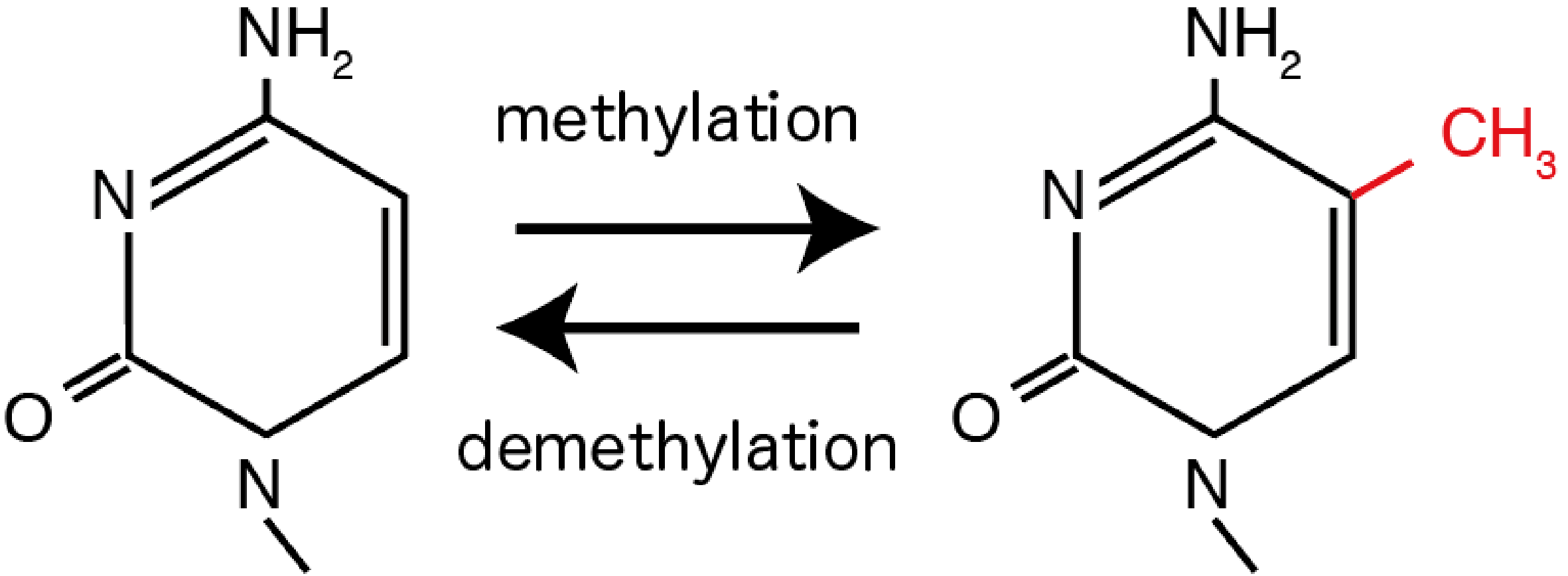
- **DNA methylation** is a biological process by which methyl groups are added to the DNA molecule.
- Methylation can change the activity of a DNA segment without changing the sequence.
- When located in a gene promoter, DNA methylation typically acts to repress gene transcription.







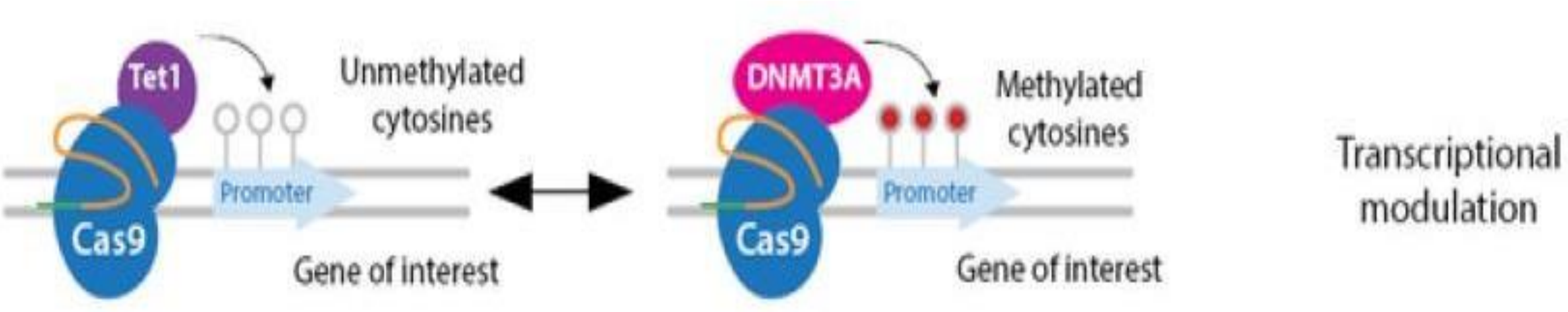


Cytosine

for switching ON the gene expression

methylated Cytosine

for switching OFF the gene expression



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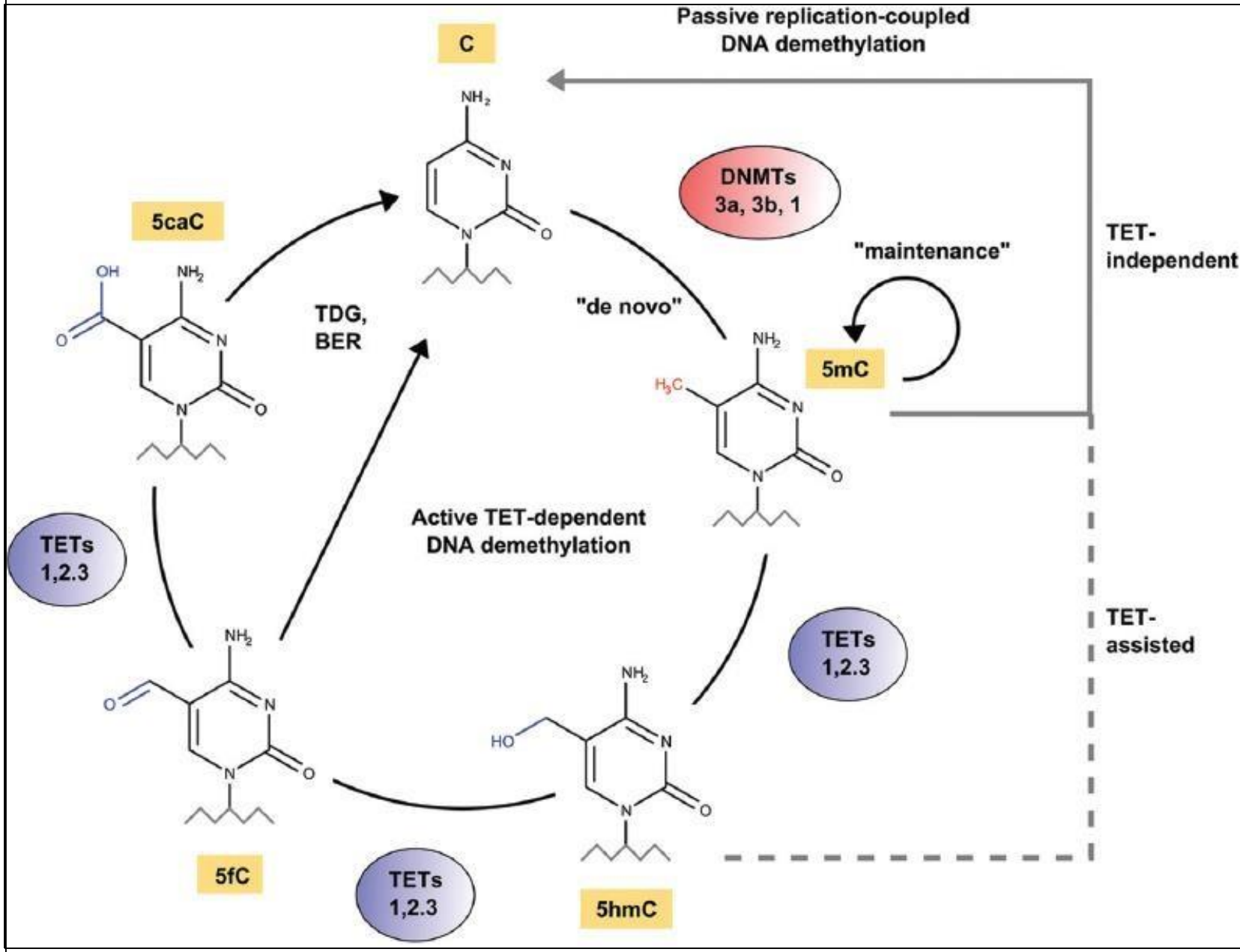
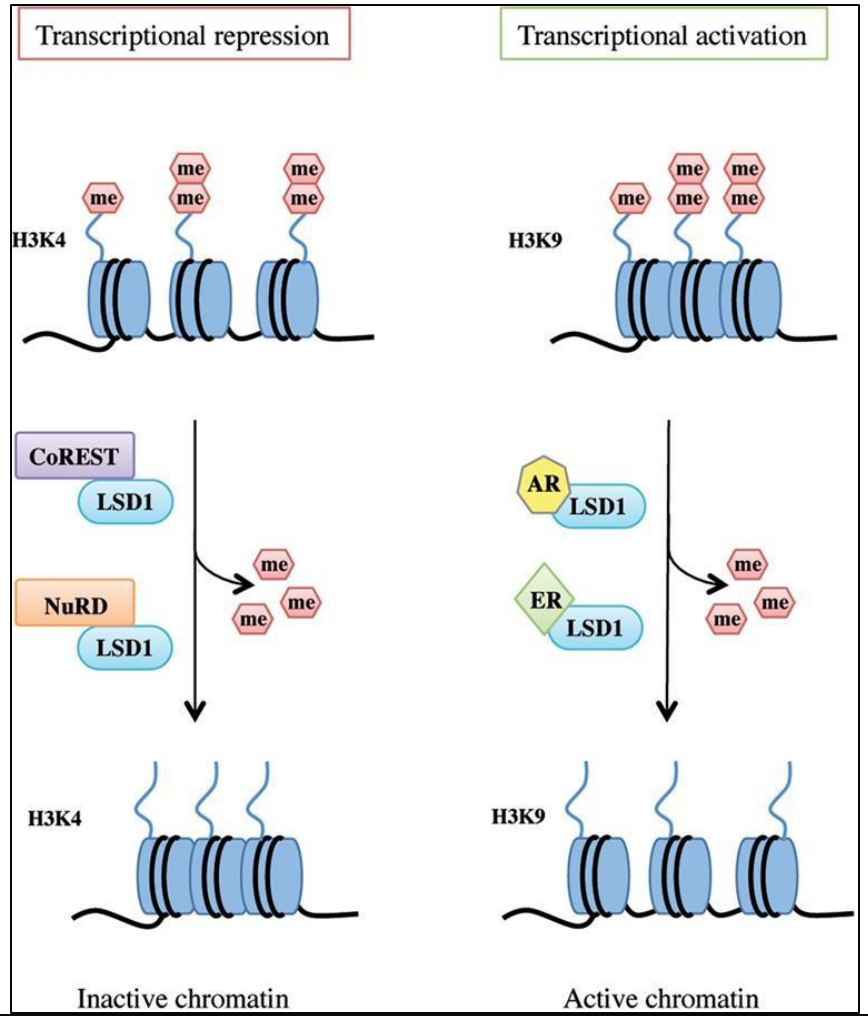
- **Pathways of active and passive DNA demethylation.**
- DNA methyltransferases (DNMTs) catalyze the methylation of cytosine by transferring a methyl group to the position C5.
- Ten-eleven translocation (TET) enzymes can catalyze the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5 hmC).
- Further TET-dependent oxidative reactions lead to the successive conversion of 5 hmC into 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC).
- 5fC and 5caC are recognized and removed by thymine-DNA glycosylase (TDG), and the created abasic site is repaired by the base excision repair (BER) pathway, generating an unmodified cytosine.
- Dilution of modified cytosines, 5mC or 5 hmC, during DNA replication can also yield unmodified cytosine, through a mechanism termed passive DNA demethylation, which is either TET-independent or TET-assisted, respectively.
- Replication-coupled dilution of 5fC and 5caC by passive DNA demethylation are not depicted.



- Ensuring epigenetic inheritance, CpG methylation is maintained during replication by the activity of DNA methyltransferase (DNMT).
- DNMT1 is the major “maintenance” methyltransferase, as it recognizes preferentially hemi-methylated DNA as a substrate and copies the pattern reliably to the daughter strand.
- On the other hand, DNMT3a and DNMT3b are classified as “de novo” methyltransferases and are indispensable for the establishment of DNA methylation patterns during early embryonic development.
- However, there is evidence that the classification of DNMTs into “maintenance” versus “de novo” is not absolute, and that enzymes share both activities to some extent in vivo.
- Particularly at repetitive sequences DNMT3A/3B fill in for DNMT1 and restore incompletely maintained DNA methylation levels.
- The model of stable persistence of DNA methylation throughout development by the cooperative action of DNMT enzymes, had to be further revised after the discovery of enzymes that actively remove DNA methylation.
- The identification of Ten-Eleven Translocation (TET) proteins, that catalyze the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5 hmC), revealed that vertebrates possess several mechanisms of active DNA demethylation.
- The active DNA demethylation pathway leads to a further oxidation of 5 hmC by TET enzymes and a final removal and replacement by unmodified cytosine through thymine DNA glycosylase (TDG)-mediated base excision repair (BER).
- The implications of the three members of the TET family (TET1, TET2 and TET3) and their oxidation products as potential epigenetic marks during pluripotency, cell differentiation, and epigenetic reprogramming are reviewed elsewhere.

Lysine-specific demethylase 1A (LSD1)

- Histone 3 lysine 4 methylation is a mark of active transcription.
- In particular, mono-methylated H3K4 denotes primed enhancer elements, di-methylated H3K4 active enhancers and promoters, and tri-methylated H3K4 transcriptionally active promoters.
- LSD1 binds to several proteins and takes part in different complexes to promote H3K4 demethylation and shape chromatin into a repressive configuration, such as the CoREST transcriptional repressor complex and the Mi-2/nucleosome remodeling and deacetylase (NuRD) complex



- Upon specific stimulation, LSD1 can also act as a transcriptional co-activator.
- For example, following androgen receptor pathway activation, LSD1 demethylates mono- and dimethyl-H3K9 repressive marks and promotes transcription of androgen target genes in prostate cancer cells.
- Moreover, upon stimulation of estrogen receptor (ER) signaling, LSD1 acts on dimethyl-H3K9 sites on enhancers and promoters of estrogen-induced genes to drive their expression.

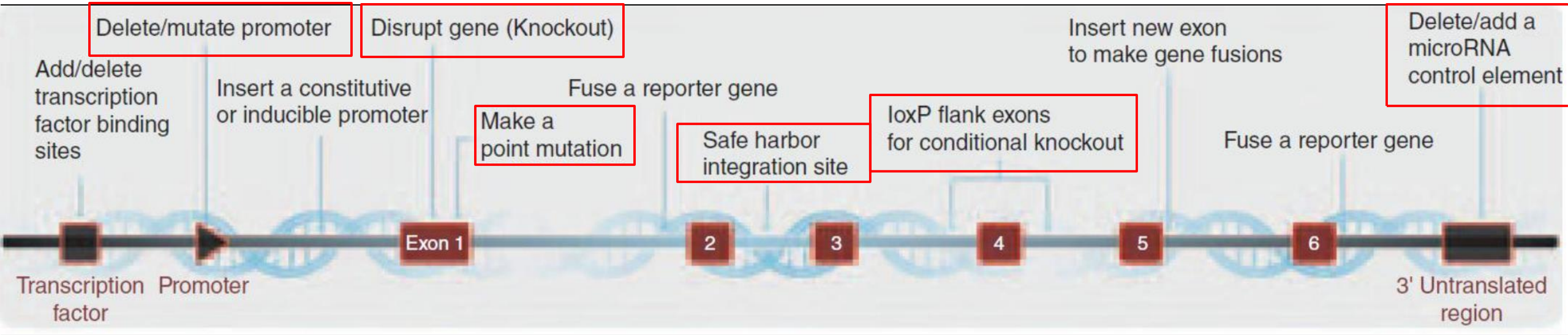
Figure. **Lysine-specific demethylase 1A (LSD1) has dual functions: both as a transcriptional repressor and activator.**
LSD1 regulates chromatin accessibility through its demethylating activity on histone H3 Lys4 and Lys9 residues.
On the left, LSD1 binds to the CoREST or nucleosome remodeling and deacetylase repressive complex thus demethylating mono- and dimethyl-group on histone H3K4 and allowing genes transcriptional repression.
On the right, following androgen receptor or estrogen receptor binding, LSD1 promotes transcriptional activation by demethylating mono- and dimethyl-group on histone H3K9.

Applications of genome editing technologies

❑ 10. Genome-Wide Screens Using CRISPR

- ❑ The ease of gRNA design and synthesis, as well as the ability to target almost any genomic locus, make CRISPR the ideal genome engineering system for large-scale forward genetic screening.
- ❑ Forward genetic screens are particularly useful for studying diseases or phenotypes for which the underlying genetic cause is not known.
- ❑ In general, the goal of a genetic screen is to generate a large population of cells with mutations in, or activation/repression of, a wide variety of genes and then use these cells to identify the genetic perturbations that result in a desired phenotype.
- ❑ Before CRISPR, genetic screens relied heavily on shRNA technology, which is prone to off-target effects and false negatives due to incomplete knockdown of target genes. In contrast, CRISPR is capable of making highly specific, permanent genetic modifications that are more likely to ablate target gene function.
- ❑ CRISPR has already been used extensively to screen for novel genes that regulate known phenotypes, including resistance to chemotherapy drugs, resistance to toxins, cell viability, and tumor metastasis.
- ❑ Currently, the most popular method for conducting genome-wide screens using CRISPR involves the use of pooled lentiviral CRISPR libraries.

Other applications



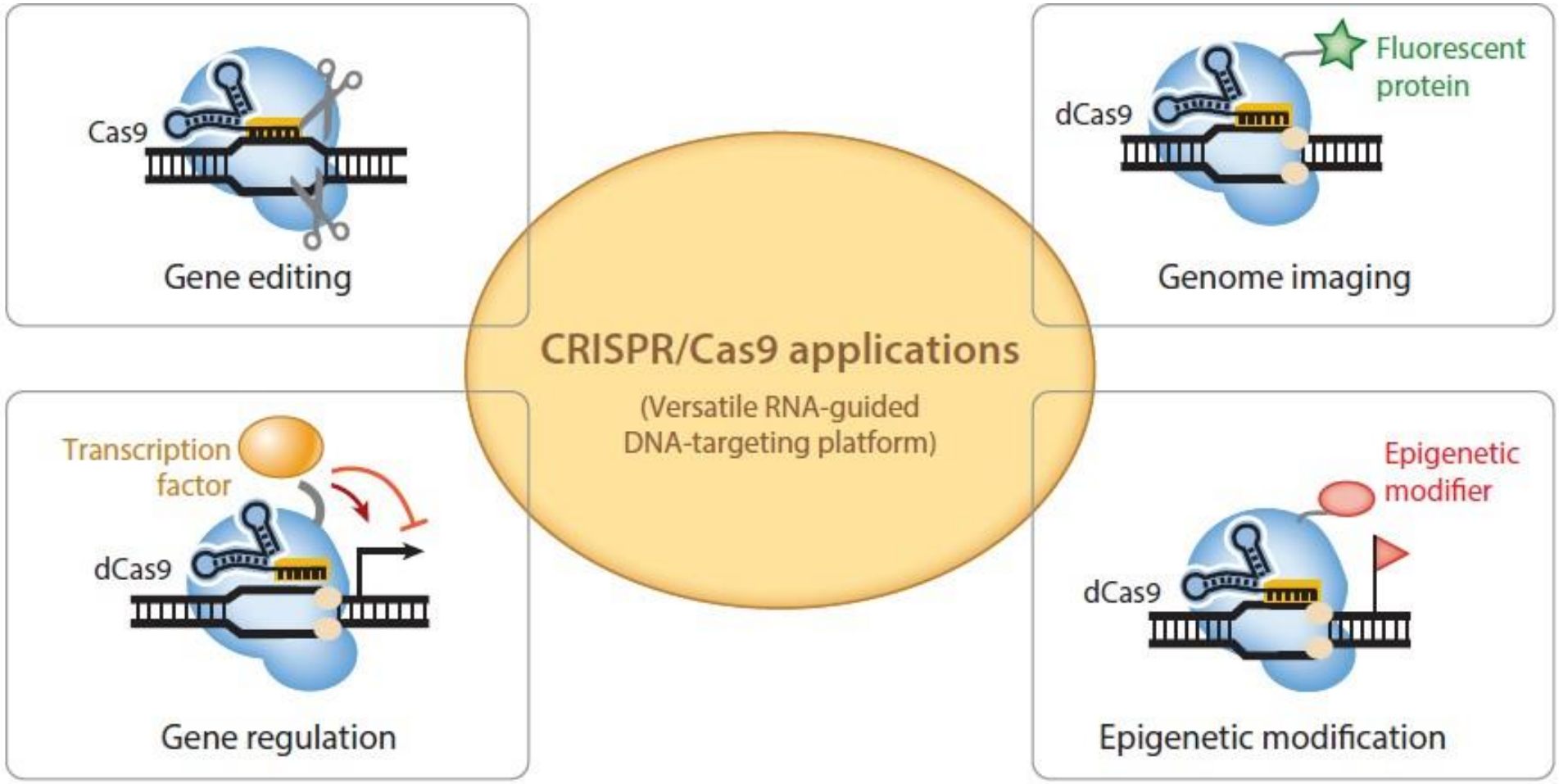


Figure 1

Overview of CRISPR/Cas9 applications. This system has been adapted and developed for gene editing, transcription regulation, chromosome imaging, and epigenetic modification. Gene editing is based on the nuclease activity of Cas9, whereas the three other applications use the catalytic, nuclease-deactivated form of Cas9 (dCas9). Fusing dCas9 to various effector domains enables the sequence-specific recruitment of transcription regulators for gene regulation, fluorescent proteins for genome imaging, and epigenetic modifiers for epigenetic modification.

Cas9 and its variants

- **Cas9 contains two nuclease domains:**
  - a HNH nuclease domain and
  - a RuvC nuclease domain.

wt Cas9

- The HNH nuclease domain is responsible for cleavage of the complementary/target DNA strands.
- The RuvC nuclease domain is responsible for cleavage of the non-complementary/non-target DNA strands.
- Both the domains are active and cleave their respective strands in DNA.

Cas9 nickase

- The **D10A** mutation inactivates the RuvC domain, so this nickase cleaves only the complementary/target strand.
- Conversely, the **H840A** mutation in the HNH domain creates a non-complementary/non-target strand-cleaving nickase.

dCas9

- Specifically, both RuvC and HNH nuclease domains can be rendered inactive by point mutations (**D10A and H840A**), resulting in a nuclease dead Cas9 (dCas9) molecule that cannot cleave target DNA.
- The dCas9 molecule retains the ability to bind to target DNA based on the gRNA targeting sequence.

Applications of genome editing technologies

❑ Cas9 nickase

- By mutating one of two Cas9 nuclease domains, researchers created the CRISPR nickase.
- Nickases create a single-strand rather than a double-strand break, and when used with two adjacent gRNAs, can lower the probability of off-target editing.
- The D10A mutation inactivates the RuvC domain, so this nickase cleaves only the target strand.
- Conversely, the H840A mutation in the HNH domain creates a non-target strand-cleaving nickase.

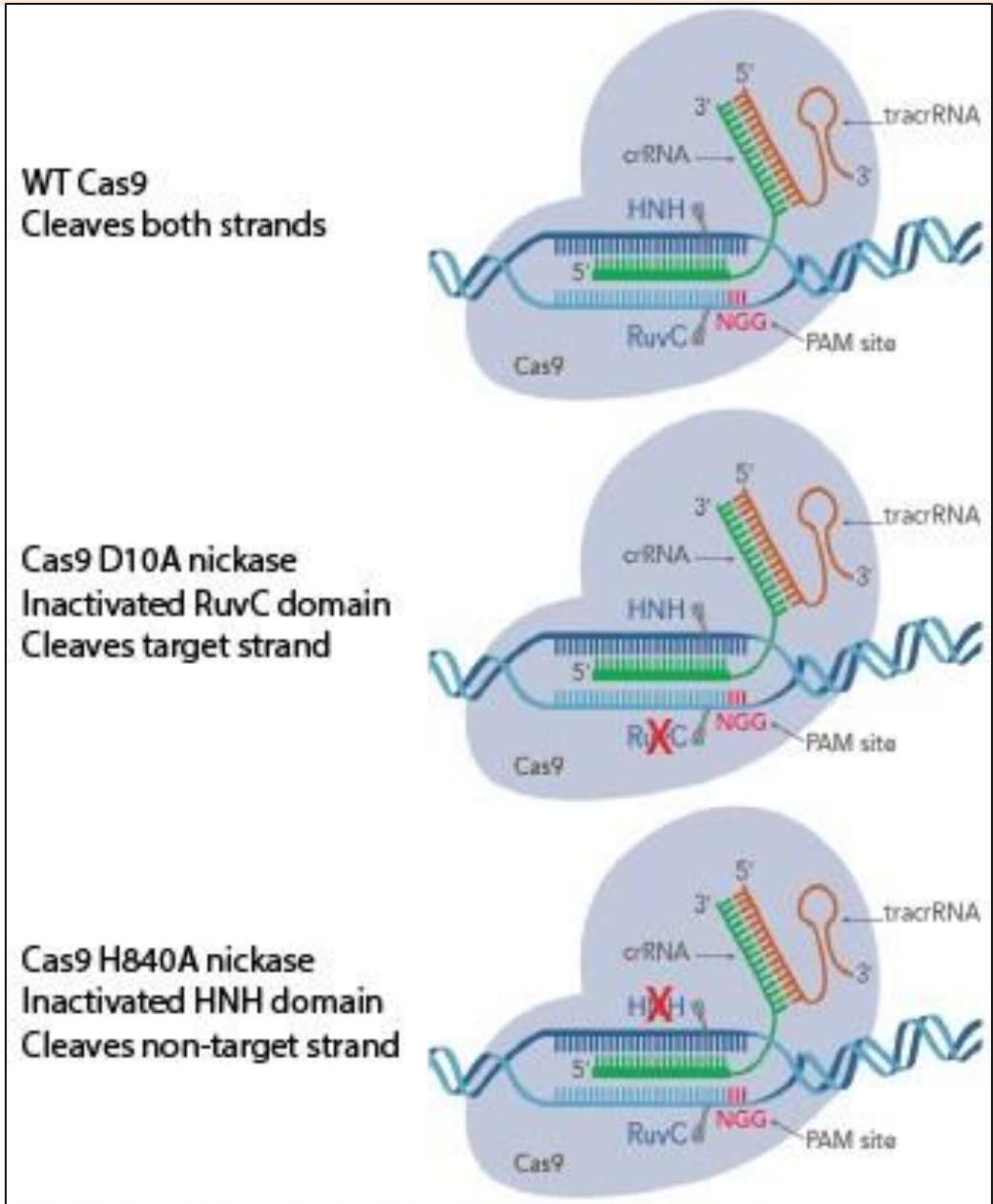
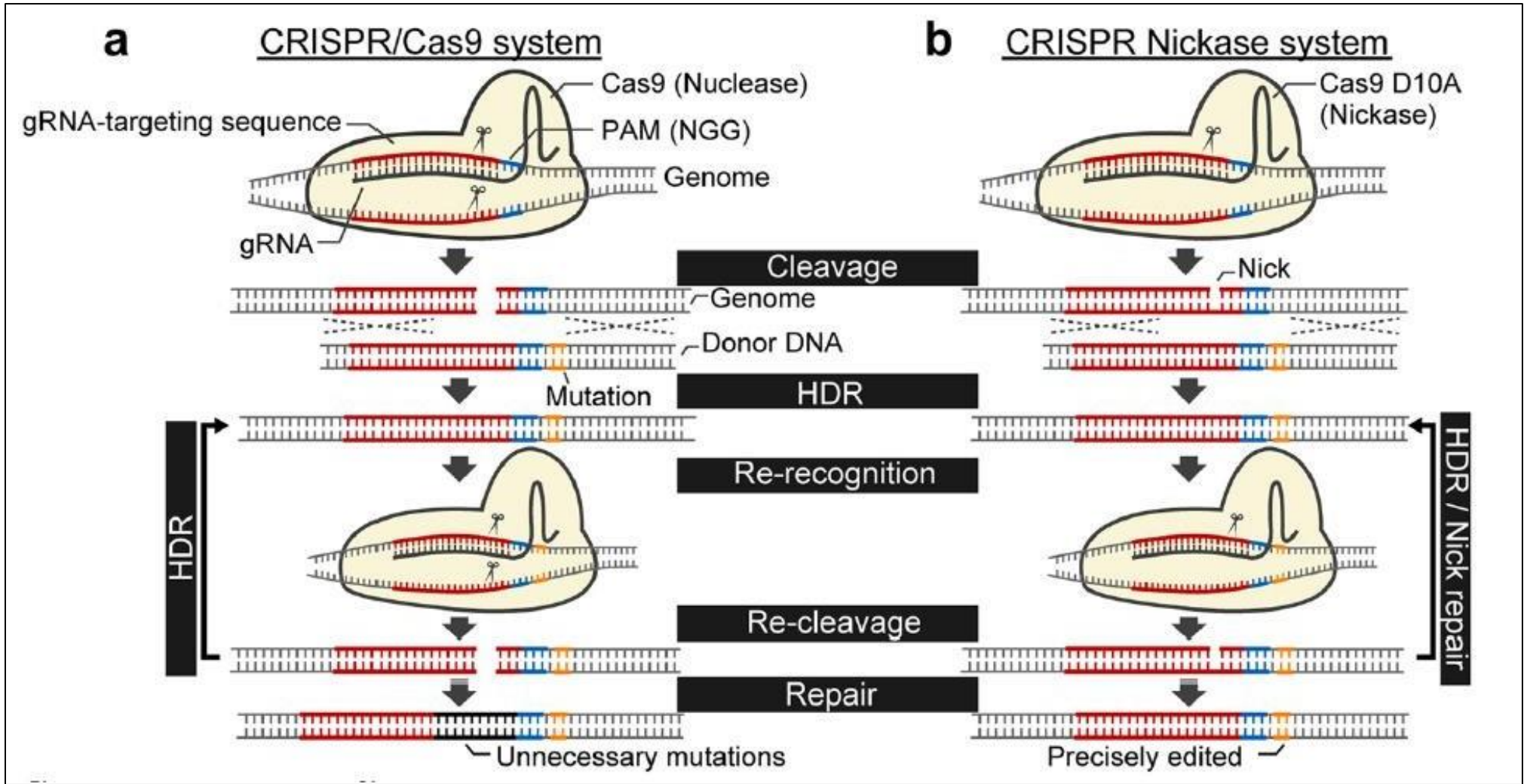


Figure 1. Cleavage capabilities of Cas9 nickases.

Precise genome-wide base editing by the CRISPR Nickase system in yeast

Atsushi Satomura[1,2], Ryosuke Nishioka[1], Hitoshi Mori[1], Kosuke Sato[1], Kouichi Kuroda[1] & Mitsuvoshi Ueda[1]

SCIENTIFIC REPORTS | 7: 2095 | DOI:10.1038/s41598-017-02013-7



Applications of genome editing technologies

❑ dCas9-FokI (fCas9)

- Genome editing by Cas9, which cleaves double-stranded DNA at a sequence programmed by a short single-guide RNA (sgRNA), can result in off-target DNA modification that may be detrimental in some applications.
- To improve DNA cleavage specificity, we generated fusions of catalytically inactive Cas9 and FokI nuclease (fCas9). DNA cleavage by fCas9 requires association of two fCas9 monomers that simultaneously bind target sites ~15 or 25 base pairs apart.
- In human cells, fCas9 modified target DNA sites with >140-fold higher specificity than wild-type Cas9 and with an efficiency similar to that of paired Cas9 'nickases', recently engineered variants that cleave only one DNA strand per monomer.
- The specificity of fCas9 was at least 4-fold higher_than that of paired nickases at loci with highly similar off-target sites.
- Target sites that conform to the substrate requirements of fCas9 occur on average every 34 bp in the human genome, suggesting the broad versatility of this approach for highly specific genome-wide editing.

Nat Biotechnol. 2014 June ; 32(6): 577–582. doi:10.1038/nbt.2909.

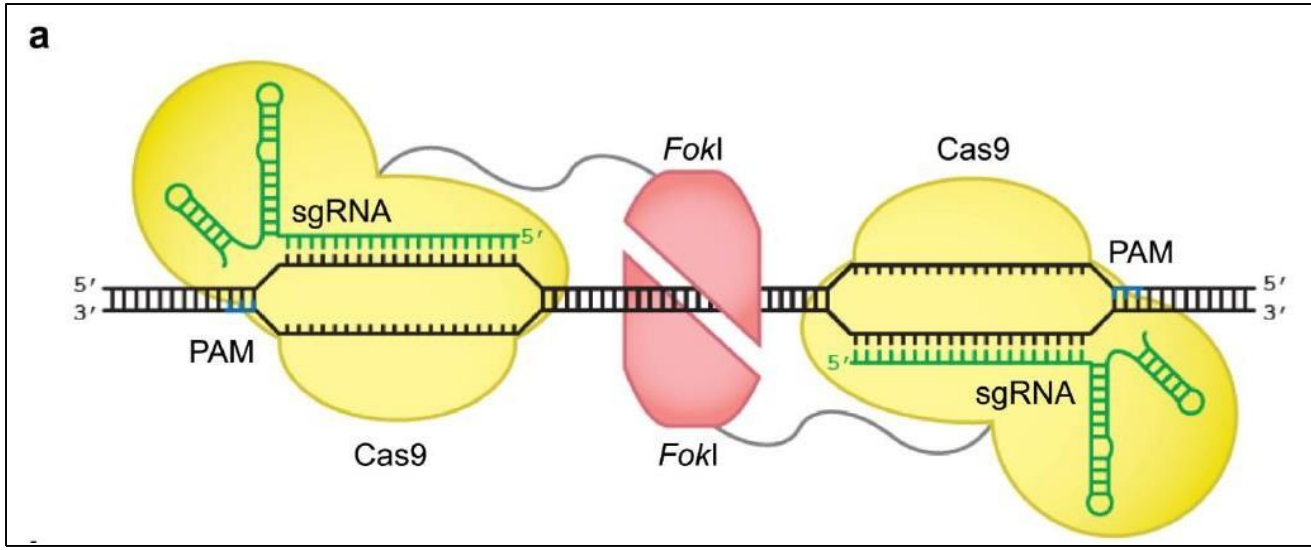
Fusion of catalytically inactive Cas9 to *FokI* nuclease improves the specificity of genome modification

John P. Guilinger[#1,2], **David B. Thompson**[#1,2], and **David R. Liu**[1,2,*]

[1]Department of Chemistry & Chemical Biology, Harvard University, Cambridge, MA, USA

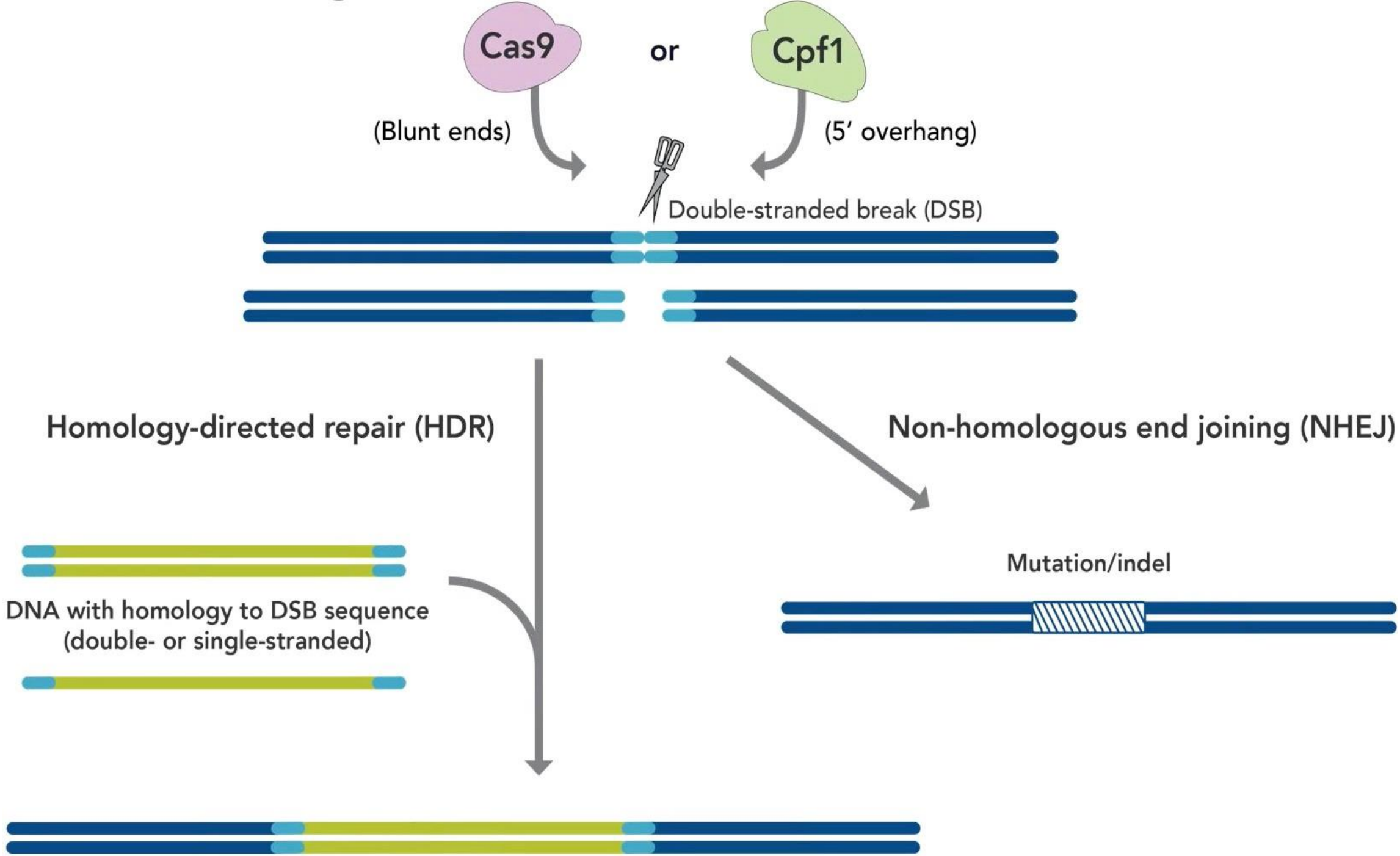
[2]Howard Hughes Medical Institute, Harvard University, Cambridge, MA, USA

[#] These authors contributed equally to this work.



Cas9 nuclease variants	Origin	PAM sites (5′ to 3′)	Specifications	Functions	Reference
Native Cas9 (SpCas9)	*Streptococcus pyrogenes*	NGG	100 nt long gRNA	Introduce double stranded breaks; create blunt ends	Mojica et al., 2005
Cas9 nickase (Cas9n)	Engineered from *S. pyrogenes*	NGG	Mutation in native Cas9 (RuvC or HNH D10, aspartate to alanine substitution)	Generate single stranded break; efficient HDR repair mechanism	Cong et al., 2013; Fauser et al., 2014
dCas9	Engineered from *S. pyrogenes*	NGG	Mutated Cas9	RNA guided transcription regulation (CRISPRi, CRISPRa); delivers GFP enabling visualization of genetic element dynamics	Hilton et al., 2015
Dimeric RNA-guided FokI nucleases (RFNs)	Engineered from *S. pyrogenes*	NGG	Fusion of dCas9 protein and FokI nuclease domain	High genome editing frequency and reduced off-target mutations	Tsai et al., 2014; Bortesi and Fischer, 2015
NmCas9	*Neisseria meningitidis*	NNNNGATT	Longer crRNA component (24 nt)	Reduced off-target effects	Hou et al., 2013
StCas9	*Streptococcus thermophilus*	NNAGAAW	On target cleavage activities	Reduced off-target effects	Horvath et al., 2008;
SaCas9	*Staphylococcus aureus*	NNGRRT or NNGRR(N)	On target cleavage activities	Reduced off-target effects	Esvelt et al., 2013
Cas9-DD (Destabilized Cas9)	*Engineered from S. pyrogenes*	NGG	Conjugation of destabilized domain to Cas9	Temporal, spatial and locus-specific control of gene expression; Increased NHEJ-mediated gene insertion efficiency	Geisinger et al., 2016; Senturk et al., 2015

- CasX
- CasY
- SpCas9-HF1
- enhanced nuclease (eSpCas9)
- hyper-accurate Cas9 (HypaCas9)
- xCas9 (Expanded PAM Recognition)
- Etc. Etc. Etc.


Cas9
or
Cpf1
(Blunt ends)
(5′ overhang)
Double-stranded break (DSB)
Homology-directed repair (HDR)
Non-homologous end joining (NHEJ)
DNA with homology to DSB sequence
(double- or single-stranded)
Mutation/indel

CRISPR/Cpf1 or CRISPR/Cas12a

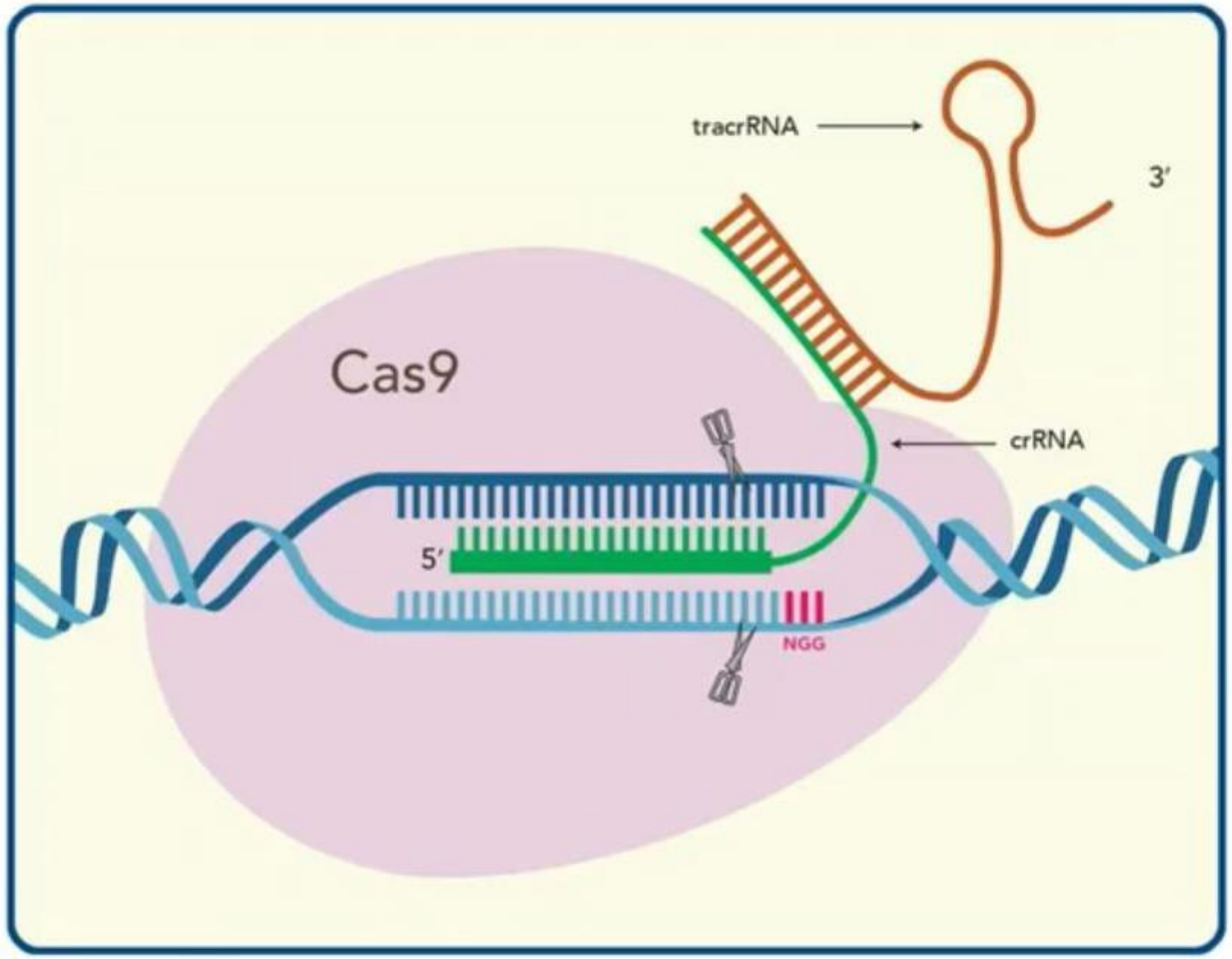
- **CRISPR/Cpf1** or also called **CRISPR/Cas12a** is a DNA-editing technology analogous to the CRISPR/Cas9 system.
- This acquired immune mechanism is found in Prevotella and Francisella bacteria.
- Like CRISPR/Cas, it is also a RNA-guided endonuclease system.
- Cas12a gene codes for an endonuclease that use a guide RNA to find and cleave viral DNA.

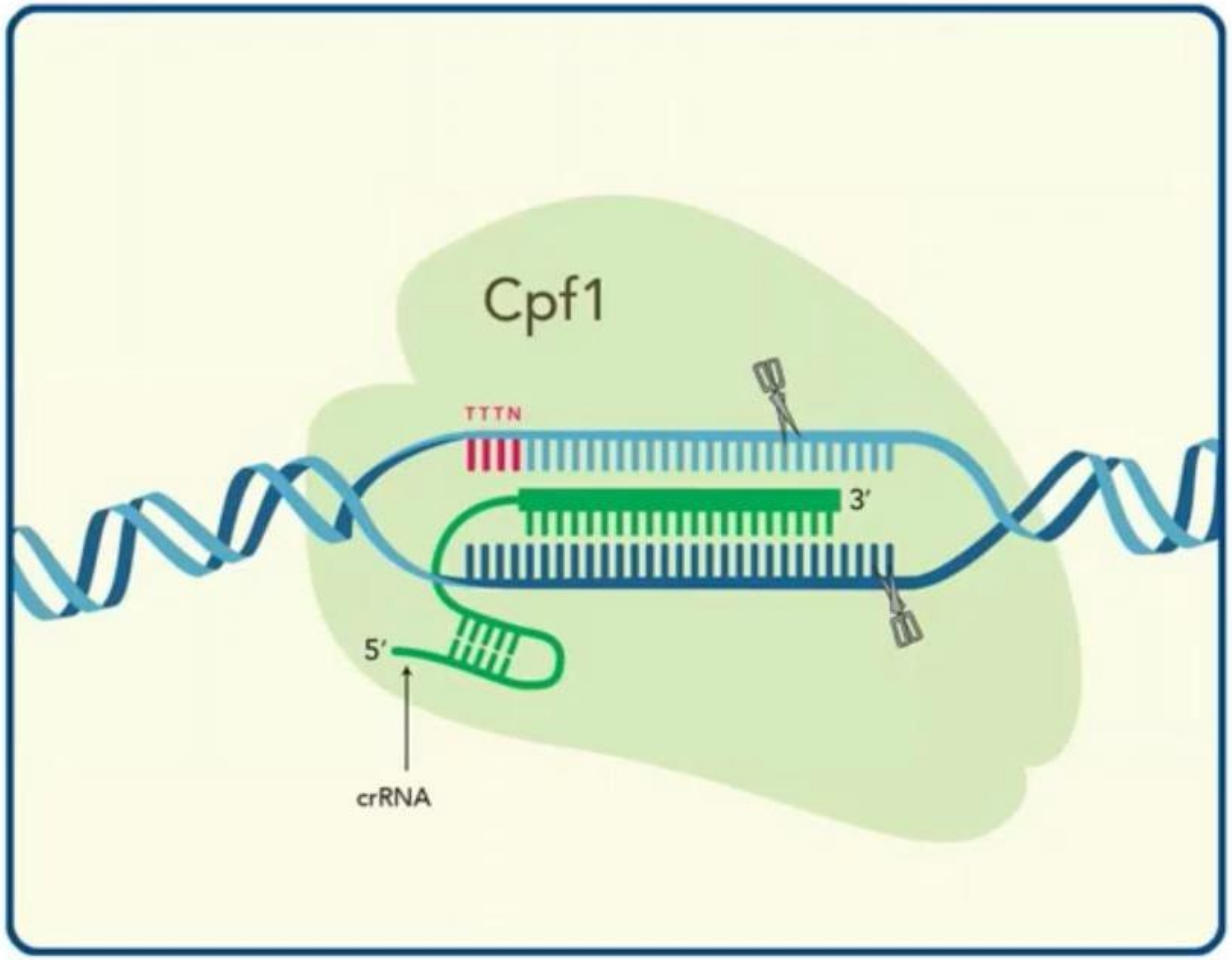
- CRISPR-Cas systems are separated into **two classes**.
- Class 1 uses several Cas proteins together with the CRISPR RNAs (crRNA) to build a functional endonuclease.
- Class 2 CRISPR systems use a single Cas protein with a guide RNA.

- Cpf1 has been recently identified as a Class 2, Type V CRISPR/Cas systems containing a 1,300 amino acid protein.

- Two candidate enzymes from Acidaminococcus and Lachnospiraceae display efficient genome-editing activity in human cells.

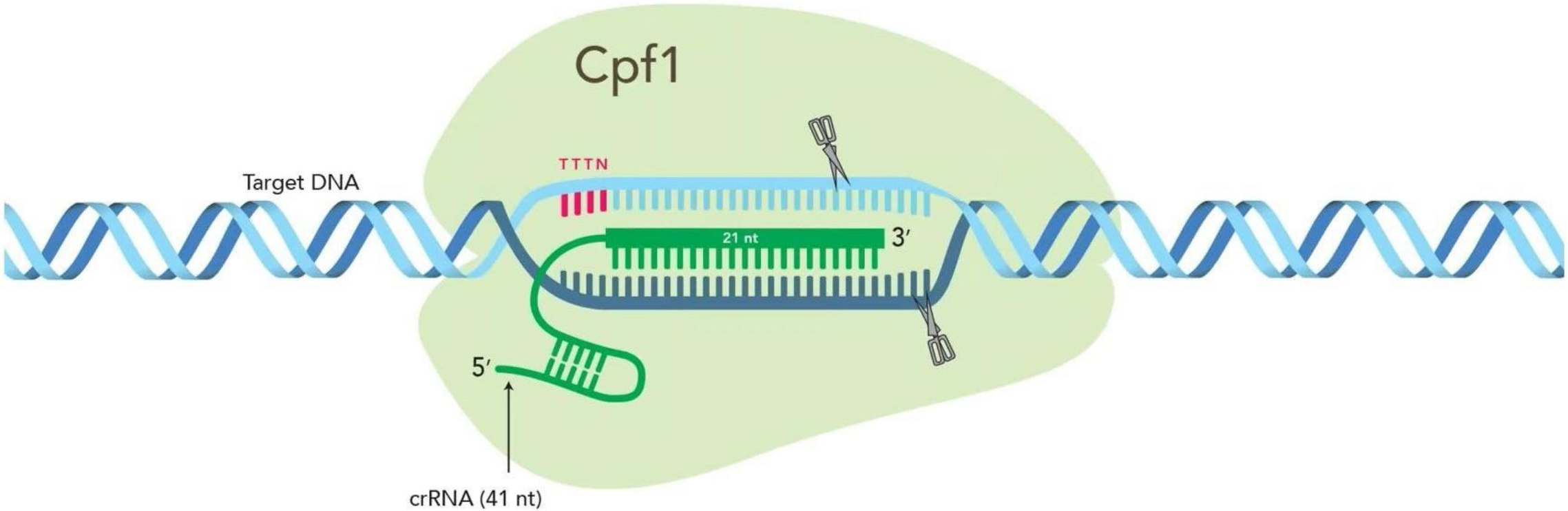
Comparison of CRISPR/Cas9 vs CRISPR/Cpf1





CRISPR/Cas9 vs CRISPR/Cpf1

CRISPR:	**CRISPR/Cas9**	**CRISPR/Cpf1**
Size	Big in size (~4.1 kb)	Small in size (~3.8 kb)
Simplicity	Complex	Simpler
Guide RNA	Cas9 requires crRNA and tracrRNA (~100 nt)	Cpf1 requires only crRNA (~42 nt)
Domains	Cas9 contains two nuclease domains: a HNH nuclease domain and a RuvC nuclease domain	Cpf1 contains only one nuclease domain: a RuvC nuclease domain
PAM	5'-NGG-3' (where "N" is any nucleobase). G-rich PAM	5'-TTTV-3' (AsCpf1; where "Y" is a pyrimidine (C/T) and "V" is A/G/C nucleobase). T-rich PAM
Blunt/Sticky	Cuts both strands in a DNA molecule at the same position, leaving behind blunt ends (blunt end cuts)	Cuts with a sticky-end DNA double-stranded break (staggered end cuts) of 4 or 5 nucleotides overhang, enhancing the efficiency of genetic insertions and specificity during DNA repair
Cutting site	Cuts target DNA closer to PAM site (proximal to recognition site). Generates indels that destroy the PAM site and prevents further rounds of cutting.	Cuts target DNA further away (18-23 nt) from PAM site (distal from recognition site). No disruption of PAM site and allows multiple rounds of cutting.



UAAUUUCUACUCUUGUAGAUNNNNNNNNNNNNNNNNNNNN

AsCpf1 constant region **21 nt protospacer region**

CRISPR-ddCpf1

- DNase-dead Cpf1 (ddCpf1)

- The E993A mutation in the RuvC domain of Acidaminococcus sp. Cpf1 (AsCpf1) generates a ddCpf1, which would lose the DNase activities against both strands of target DNA.

- Gene regulation (transcriptional activation and repression)
- Genome Imaging
- Epigenetic modifications

CRISPR/Cas14

- Dr. Doudna and her team also recently added another Cas nuclease to the CRISPR nuclease toolbox.

- The newly reported compact Cas14 nuclease from archaea bacteria shows the following special features:

  - it targets single-stranded (ss) DNA,
  - does not require PAM sequence for activation, and
  - cuts other ssDNA strands non-specifically upon binding the target sequence.

- Although Cas14a is similar to the Cas12a nuclease in the aspect that it mediates cleavage of non-target ssDNA when activated by binding to a complementary DNA sequence, there is one main difference.

- Unlike Cas12a, Cas14a requires high fidelity complementarity to the target ssDNA.

CRISPR/Cpf1 is a rising star in genome editing