# The influence of eukaryotic chromatin state on CRISPR-Cas9 editing efficiencies

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**Abstract**

CRISPR/Cas technologies have rapidly become routine in many laboratories. Despite this, the efficiency of CRISPR/Cas9 functioning cannot entirely be predicted, and it is not fully understood which factors contribute to this variability. Recent studies indicate that heterochromatin can negatively affect Cas9 binding and functioning. Investigating chromatin factors indicates that 5-cytosine methylation does not directly block Cas9 binding. Nucleosomes, however, can completely block Cas9 access to DNA in cell-free assays and present a substantial hurdle in vivo. In addition to being associated with an open chromatin state, active transcription can directly stimulate DNA cleavage by influencing Cas9 release rates in a strand-specific manner. With these insights and a better understanding of genome-wide chromatin and transcription states, CRISPR/Cas9 effectiveness and reliability can be improved.



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# The influence of eukaryotic chromatin state on CRISPR–Cas9 editing efficiencies

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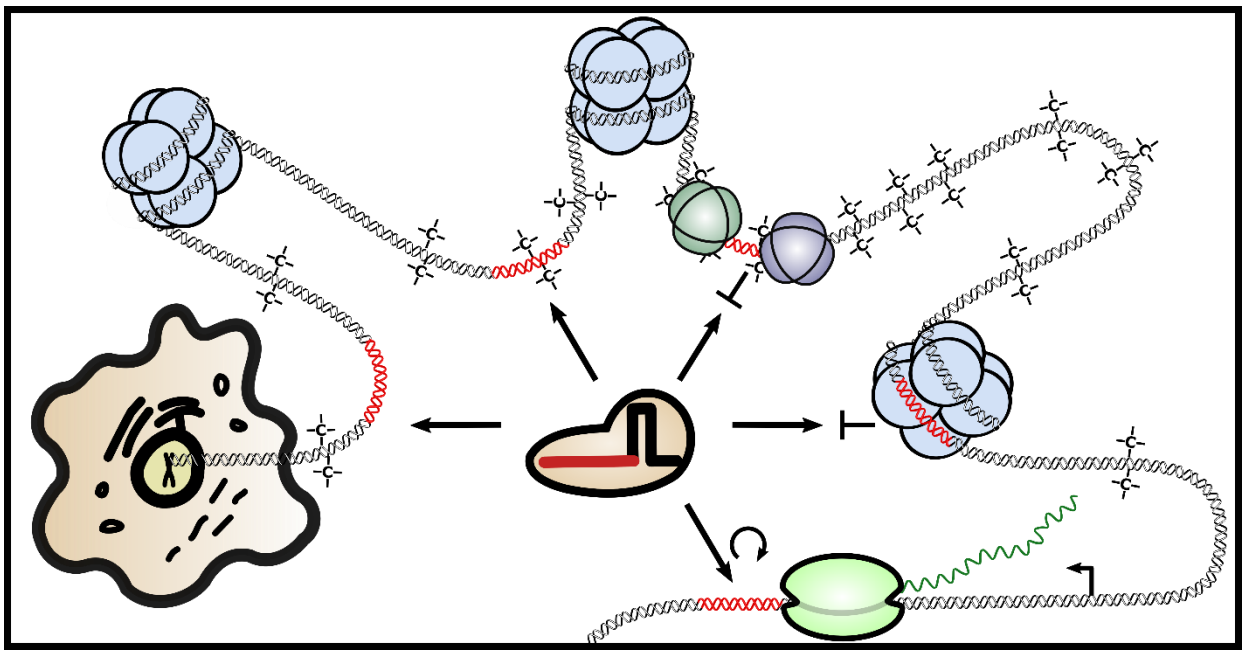
## Influence of chromatin on CRISPR-Cas9 functioning

## Graphical abstract



## Highlights

- CRISPR/Cas9 is the method of choice for site-directed genetic or transcriptional manipulation
- Genomic loci differ in chromatin configuration which affects Cas9 functioning
- 5-cytosine DNA methylation does not directly impair Cas9 binding/cleavage
- Nucleosomes can completely block Cas9 access in cell-free assays and present a substantial hurdle *in vivo*
- Active gene transcription can increase Cas9 cleavage efficiency independent of chromatin parameters

## Abstract

CRISPR/Cas technologies have rapidly become routine in many laboratories. Despite this, the efficiency of CRISPR/Cas9 functioning cannot entirely be predicted, and it is not fully understood which

factors contribute to this variability. Recent studies indicate that heterochromatin can negatively affect Cas9 binding and functioning. Investigating chromatin factors indicates that 5-cytosine methylation does not directly block Cas9 binding. Nucleosomes, however, can completely block Cas9 access to DNA in cell-free assays and present a substantial hurdle *in vivo*. In addition to being associated with an open chromatin state, active transcription can directly stimulate DNA cleavage by influencing Cas9 release rates in a strand-specific manner. With these insights and a better understanding of genome-wide chromatin and transcription states, CRISPR/Cas9 effectiveness and reliability can be improved.

## Introduction

Sequence-specific DNA targeting proteins form the cornerstone of increasingly common techniques to modify specific genomic loci at will. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas9) system, originally found in *Streptococcus pyogenes*, has become the most widely adopted DNA targeting platform [1,2]. However, despite many algorithms and sgRNA design tools, there is much-unexplained variability in efficiencies, even between the same target sequences in different cell types. Furthermore, it has been observed that sgRNAs performing well *in vitro* can underperform *in vivo* [3] and that partially matching off-target DNA sequences can exhibit higher Cas9 cleavage rates than their fully matching on-target site [4–6]. These results suggest that factors beyond the sgRNA/target sequence influence Cas9 functioning.

Recent work indicates that the way DNA is modified and packaged in the nucleus – collectively referred to as chromatin state – can have significant effects on Cas9 binding and may help explain variation in functional efficiency. ChIP-seq screens found dCas9 off-target binding to be enriched in open chromatin [7–10], and chromatin accessibility has been found to positively correlate with CRISPR/Cas9 efficiency [11]. Importantly, not only the binding of (d)Cas9 may be affected by the local chromatin state, but also the intended outcome.

Understanding the role of chromatin in genome targeting applications is especially important when chromatin states differ between experimental models and intended applications (e.g. cell cultures may not resemble target cells in patients). Here, we review the influence of chromatin states on Cas9 functioning. We contrast cell-free (generally chromatin-free) assays and *in vivo* experimental evidence and discuss strategies to increase Cas9 efficiency.

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| **(d)Cas9 and sgRNA** | Cas9 is a protein with intrinsic nuclease activity that cuts a specific DNA sequence *via* base complementarity using a short RNA molecule called the single guide RNA (sgRNA). The nuclease complex can be retargeted to another genomic locus simply by changing the sgRNA sequence. Since its introduction as a genome-editing tool in 2012 [12,13], Cas9 has been adapted for far more than just site-directed DNA cleavage. Most commonly this is achieved through the direct fusion of effectors to a nuclease deactivated Cas9 (dCas9) to allow for site-directed effects such as transcriptional modification. |
| **Euchromatin and heterochromatin** | Chromatin refers to a cell's DNA and its associated macromolecules and modifications. Euchromatin describes segments of chromatin that are relatively unpacked and accessible to regulatory factors. In contrast, |

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| | heterochromatin consists of densely packed nucleosomes, crowded by non-histone proteins hampering accessibility. |
| **DNase hypersensitivity (DHS)** | DNase hypersensitivity reflects open chromatin states by measuring the accessibility of DNA to the DNase I enzyme. Euchromatin is more prone to cleavage than regions of heterochromatin. |
| **ChIP-seq** | Chromatin Immunoprecipitation sequencing (ChIP-seq) is a method to identify the sequences at which a protein or protein complex interacts with DNA. In the context of this review, (d)Cas9 can be crosslinked to and isolated with DNA it has (transiently) bound, and the location can be identified by mapping the sequences back to the genome. |

## Heterochromatin states can impede Cas9 access

The best controlled experimental evidence of chromatin influence on Cas9 editing outcomes and binding comes from studying otherwise identical sgRNA target sequences with allele-specific chromatin states. For example, three sgRNAs were targeted to the CpG Island (CGI) containing *p16INK4a* locus, of which the two alleles have different chromatin states in HCT116 cells: one repressed (hypermethylated), the other expressed (hypomethylated) [14]••. Sequencing of individual clones showed that for two of the three sgRNAs there was no significant difference in Cas9 mutagenesis rate between the two alleles. The third sgRNA did show a significant difference: of the 18 screened clones, 2/18 had a mutation in the repressed allele, and 17/18 had a mutation at the transcriptionally active one. This preference was also reflected in a lower Cas9 binding on the heterochromatin allele over the euchromatin allele.

Similar to the above study, Kallimasioti-Pazi and colleagues targeted CGIs on differentially imprinted alleles [15]••: the repressed heterochromatin maternal allele accumulated Cas9-mediated mutations slower than the paternal allele. The most substantial difference in mutagenesis was observed when Cas9 exposure was brief and when intracellular Cas9 expression was low. More subtle effects were observed for higher Cas9 expression levels.

Heterochromatin-euchromatin differences comprise a heterogeneous mix of many factors. Separating the contribution of individual factors is challenging and depends on modulating single chromatin components with *in vitro* assays. To date DNA methylation and nucleosome occupancy have been the best studied and below we evaluate their effect on functional Cas9 binding.

## 5-Methylcytosine does not directly hinder Cas9 binding

Cleavage of DNA by Cas9 can be susceptible to modifications at the site of gRNA:DNA hybridisation. For example, the presence of large glucosylated hydroxymethylcytosine in the DNA target site of Cas9 allows T4 phages to escape Cas9-mediated bacterial immunity [16,17], although this can depend on how the gRNA is designed [17,18]. Methylation of T4 DNA target sites was not found to protect the phages [18]. Likewise, a cell-free Cas9 cleavage assay confirmed complete cleavage of both methylated and unmethylated DNA and three tested sgRNAs induced mutations at a methylated locus in cells [19]••. Similarly, using dCas9 fused to transcriptional activators, hypermethylated loci could

successfully be activated [20,21]. The absence of direct effects of DNA methylation was further demonstrated by studying the differentially methylated *p16INK4a* alleles in HCT116 cells [14]: by using purified genomic DNA (maintaining DNA methylation while removing other chromatin factors), the binding preference observed *in vivo* for the hypomethylated *p16INK4a* allele was lost.

For suboptimal, relatively weakly-binding off-targets, CpG methylation might play a role: a bioinformatic analysis identified DNase hypersensitivity as the strongest predictor of off-target binding for four different sgRNAs [8]•. However, considering sites containing CpG dinucleotides, CpG methylation negatively correlated with binding and became the strongest predictor of dCas9 binding. These data suggest that CpG methylation reflects chromatin accessibility beyond DNase hypersensitivity and that CpG methylation (indirectly) affects binding at off-target sites [22].

The effect of methylation is further nuanced by its microchromatin context as we described for dCas9 and zinc-finger fusions to VP64 [23]. Like earlier studies, dCas9-VP64 -even when targeted to methylated sites- effectively upregulated genes. However, methylated target sites located in CGIs seemed recalcitrant to dCas9-VP64 upregulation. This difference was reflected in reduced binding as measured by ChIP-seq. Interestingly, targeting VP64 with smaller zinc finger proteins to these same sites within the methylated CGIs did result in upregulation of transcription. This finding supports the idea that not CpG methylation of the target sequence itself, but the recruited methylation-associated factors affect Cas9 binding

In summary, methylation does not seem to have a direct effect on Cas9 binding or effectivity. If it does, it is likely weak and may only be detected under sub-optimal conditions. However, *in vivo* CpG methylation (especially in CGIs) may be indicative of other chromatin factors that influence Cas9 binding.

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| **DNA methylation** | The most common modification to DNA in mammals is the methylation of cytosine, forming 5-methylcytosine (5-mC). Importantly, differential DNA methylation has been recognised as a disease mechanism, for example cancer or immune disorders [24,25]. Therefore, these regions have been forwarded as potential therapeutic targets for engineered DNA-targeting platforms highlighting the importance of understanding the influence of DNA methylation on Cas9 functioning [1]. |
| **CpG islands (CGIs)** | Short stretches of CpG (cytosine nucleotide followed by guanine) rich DNA are termed are CpG islands. For CpG islands in promoter regions, hypermethylation associates with gene inactivation. |

## Nucleosomes can completely block functional Cas9 binding to DNA in cell-free assays but are more tolerant *in vivo*

To find a mechanistic basis for the effects of chromatin on functional Cas9 binding, research has focussed on the smallest unit of chromatin organisation, the nucleosome. In cell-free assays, the packaging of DNA in nucleosomes impedes DNA cleavage by Cas9, presumably by hindering access to the DNA and preventing efficient binding [9,26,27]. DNA at the entry or exit sites of nucleosomes can

be less shielded than sites at the centre of the nucleosome to which access is almost abolished [26,27]. However, another study found complete obstruction along the full length of the nucleosomal DNA [9].

Although these *in vitro* assays mostly make use of DNA substrates with artificially strong nucleosome positioning sequences resulting in strong, stable nucleosomes, most cellular nucleosomes are dynamic. Nucleosome "breathing" is assumed to occur frequently and refers to the temporary unwrapping of stretches of nucleosomal DNA, and may thereby shortly expose these DNA sequences to binding by (d)Cas9. Indeed, when a natural nucleosome positioning sequence was investigated the DNA was found to be far more permissible to Cas9 cleavage near the nucleosome edge [26]. However, the DNA at the centre of the nucleosome remained almost completely resistant to cleavage. Upon chromatin remodelling, the nucleosomal DNA, otherwise inaccessible to Cas9 cleavage, became a viable substrate in these cell-free systems [9,26].

Engineered cells in which local heterochromatin formation can be induced or reversed [28,29] demonstrated that targeting the same site under a more relaxed chromatin structure increased Cas9-mediated mutagenesis. Bioinformatics analysis indicated that while nucleosomes allow binding and cutting *in vivo*, sgRNAs classified as highly effective were predominantly found in low nucleosome occupancy regions [9].

Together, these results point to a model in which functional Cas9 binding is strongly occluded by nucleosomes, but access can be gained during natural nucleosome remodelling and breathing [11,26]. This may be influenced by endogenous chromatin remodelling enzymes, transcription, and DNA replication.

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| **Nucleosomes** | Nucleosomes are the primary constituent of chromatin, being formed of 147 pbs of DNA wrapped around a complex of eight histone proteins. |

## Genetic and epigenetic editing outcomes can be affected by target chromatin states

Under certain conditions not only the binding of the Cas9 complex may be affected by the local chromatin state, but also the functional outcome of the treatment. We have observed this when studying the effect of epigenetic editors fused to dCas9. Targeting methylated or unmethylated sites can result in a difference in the length of time transcriptional upregulation of a gene is sustained. This is likely not due to the efficiency of editing but instead reflects the permissiveness of the local chromatin state for maintaining the newly introduced epigenetic mark [23]. Likewise, the observed differences in Cas9 gene editing efficiency at heterochromatin sites may in part be due to changes in repair outcomes. Differential repair fidelity or dynamics could result in different perceived rates of mutagenesis without reflecting Cas9 inaccessibility.

Although numerous studies have evaluated Cas9 mediated mutagenesis, few explicitly report the chromatin state of the target and even fewer perform well-controlled comparisons between the same site under differing chromatin states. Those studies that do, show no apparent differences in effects. For example, no significant difference was found in the ratio of homology-directed repair over error-

prone non-homologous end joining repair for the same locus on the two alleles with different chromatin states and mutation patterns were similar [15]. The same was found using an inducible heterochromatin assay: while the rate of mutagenesis was severely affected by the closed chromatin, the resulting mutations were similar [29]. These results are surprising, as chromatin states have been found to affect endogenous DNA repair in complex ways [30].

Kallimasioti-Pazi *et al.* speculated that the chromatin remodelling events associated with Cas9 binding might obscure differences in repair outcomes [15]. This is supported by studies finding that dCas9 binding results in locally increased chromatin accessibility, which even increases editing efficiencies of other co-delivered Cas constructs [31–33]. The authors noted that during early points in their time course experiments (<24 h) a higher proportion of the mutations on the heterochromatin allele were single nucleotide deletions compared to the open chromatin allele. This time-dependent phenomenon has previously been observed by Overbeek *et al.* and is likely due to re-cutting of smaller mutations despite sgRNA mismatches [34].

Finally, it has been noted that the transcriptional state of a gene can correlate with editing efficiency [3]. However, it is challenging to disentangle transcription from chromatin factors that vary with transcriptional state. Interestingly, a recent study found that only particular sgRNAs showed higher mutation rates targeting a transcriptionally activated gene compared to its uninduced state [35]••. Cas9:sgRNA complexes that directly base-paired to the DNA strand serving as a template for RNA polymerase, not those binding the complementary strand, showed an increase in mutagenesis rate. Excluding chromatin effects with *in vitro* transcription assays showed that RNA polymerases could displace specifically oriented Cas9 proteins from DNA, freeing them to cut other DNA molecules in the solution. The authors suggest that the dislodging of Cas9 by the RNA polymerase exposes the cut ends which increases the opportunity for error-prone repair or additional cleavage events on transcribed genes. However, this phenomenon cannot account for all chromatin-related effects on Cas9. In the study by Fujita *et al.* the only sgRNA that showed a difference between the open and closed allele should not have been affected by sense transcription of the targeted gene [14]. Moreover, a sgRNA targeted nearby in the same orientation showed no significant difference in targeting efficiency between the two alleles. Notwithstanding, Cas9 displacement by RNA polymerases is an exciting new finding and should be taken into account in future studies investigating the influence of chromatin state on Cas9.

## Conclusions

Current evidence points towards chromatin states playing a significant role in Cas9 binding and functioning. CpG methylation does not seem to affect Cas9 binding and cleavage directly. However, CpG methylation may correlate with other factors that can obstruct Cas9, with limited evidence indicating this may be especially important in CGI contexts. Conceptually, the methylation in a CpG island context may result in the recruitment of methyl-binding proteins and chromatin remodelers that are not attracted to the same extent by more sporadic methylation. Nucleosomes show a clear and robust ability to obstruct Cas9 cleavage in cell-free assays, while *in vivo* nucleosomes seem more dynamic allowing nucleosomal DNA to be more permissive for Cas9 cleavage. These findings are summarised in Figure 1.

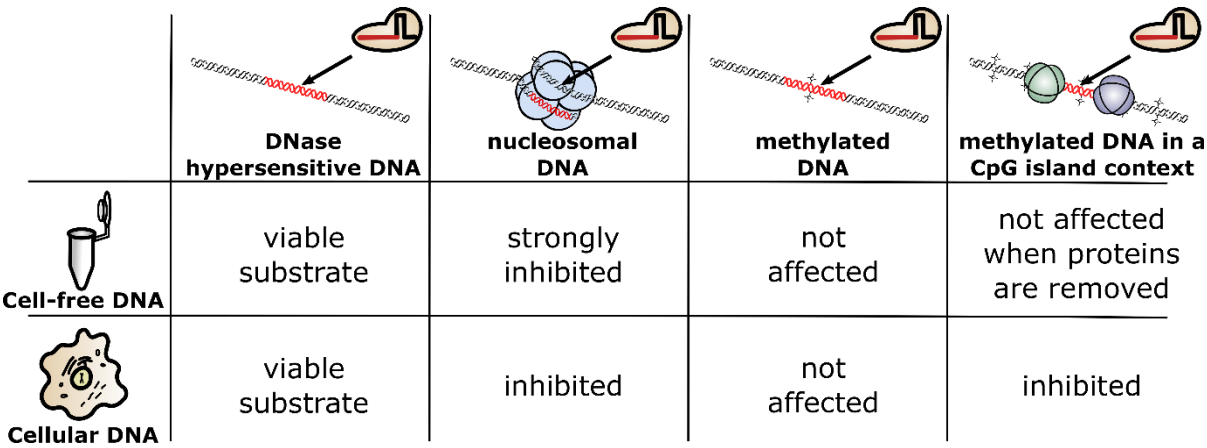


*Figure 1: The influence of chromatin factors on functional Cas9 binding. This takes into account both direct evidence of binding and indirect evidence from functional outcomes such as mutagenesis by Cas9 or transcriptional modulation by dCas9 fusions to effector domains. The various methyl-binding proteins, massively recruited to CpG islands when methylated, are only indicated as sporadic complexes. Note that this is schematic: relative sizes of DNA, methyl groups (+), histones, and Cas9 are not accurate.*

More broadly, heterochromatin can substantially obstruct Cas9 mediated editing, especially under suboptimal conditions such as low Cas9 expression, inefficient sgRNAs, or partial mismatches. The repair results of Cas9 based editing in euchromatin and heterochromatin are more similar than expected, warranting further investigation. Finally, transcription can have its own positive effect on Cas9 editing by displacing Cas9, increasing the rate at which cut ends are exposed but possibly having an inhibitory effect on some dCas9-based applications where extended binding is beneficial.

The practical consequences of the experimental results discussed in this review are that the chromatin and transcriptional state of a target site should be taken into account when translating *in vitro* CRISPR experiments to *in vivo* situations. *In vitro* assays can assist in identifying highly active sgRNAs but cannot predict general applicability given the impact of chromatin factors on Cas9 functioning. Although gene editing of an actively transcribed locus seems rather straightforward and gene modulation studies use combinations of sgRNAs, studies relying on effects of individual or few sgRNAs have to be carefully interpreted. Future sgRNA design tools should exploit available databases on chromatin context and gene transcription such as www.encodeproject.org or ihecepigenomes.org to increase Cas9 reliability. Importantly, chromatin effects should be better taken into account during experimental design: inclusion of appropriate controls will provide insights and minimise overestimations of general applicability of any given sgRNA.

## References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

## Acknowledgements

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**Using in vivo and cell-free assays, this work shows that RNA polymerase can dislodge specifically orientated Cas9 from DNA it has already cleaved. The authors suggest that this exposes the ends to DNA repair pathways thereby increasing Cas9 turnover and overall mutagenesis rates. This has important implications for evaluating the influence of chromatin state on Cas9 as changes in gene transcription and chromatin state are highly interdependent.**