CRISPR-Cas9 Editing Chimerism in *Saccharomyces Cerevisiae*: Regulation by Cell Cycle and Its Implications for Microbial Metabolic Engineering

Zimo Hou *

Sendelta International Academy Shenzhen, Shenzhen, China

* Corresponding Author Email: Dreamy_liujia@outlook.com

Abstract. CRISPR-Cas9 editing often produces chimeric cell populations where edited and unedited cells coexist, undermining yield and reproducibility in industrial microbial processes. Modulation of the cell cycle can reduce chimerism and improve editing fidelity. The review summarizes definitions and detection methods for chimeric populations including flow cytometry and single cell sequencing and highlights representative cases from *Bacillus subtilis* cellulase production and *Escherichia coli* isobutanol synthesis that show how partial editing lowers titers and increases byproducts. Evidence is presented that chromatin accessibility and DNA repair pathway choice change across G1, S, G2 and M phases and thereby determine Cas9 cleavage efficiency and repair outcomes. S phase is associated with higher chromatin accessibility and increased homology directed repair activity that favor precise edits. G1 and M phases either favor non homologous end joining or restrict cutting, leading to higher chimerism. Practical strategies such as population synchronization, timed delivery of repair templates and integrated molecular monitoring are evaluated for their potential to reduce population heterogeneity during strain engineering and fermentation. This review aims to clarify the molecular mechanisms by which *Saccharomyces cerevisiae* cell cycle modulates CRISPR editing chimerism and to provide guidance for optimizing industrial genome engineering.

Keywords: CRISPR-Cas9, chimerism, cell cycle, chromatin accessibility.

1. Introduction

Saccharomyces cerevisiae (*S. cerevisiae*), a classic chassis microorganism in the field of biosynthesis, has been widely used in the industrial production of high-value compounds such as salidroside and artemisinin precursors [1-3]. Since the advent of the CRISPR-Cas9 technology, the accuracy and efficiency of genetic engineering modification have been significantly improved [4]. However, the widespread chimerism phenomenon during editing, defined as the coexistence of successfully edited cells with unedited or failed-to-edit cells, severely restricts the optimization of strain performance and the efficiency of product synthesis [5]. Addressing this issue is crucial for enhancing the consistency of industrial production.

The cell cycle of *S. cerevisiae* exhibits highly ordered phase-specific characteristics, with chromatin structure and accessibility changing significantly throughout the cycle. In the G1 phase, chromatin is tightly compacted, making it difficult for exogenous nucleases to access DNA [6]. In the S phase, chromatin is relaxed, and DNA double strands are exposed, facilitating nuclease binding and providing favorable conditions for the binding and cleavage of the Cas9-sgRNA complex [7]. The G2 phase retains a certain degree of chromatin relaxation, offering space for DNA repair [8]. In the M phase, chromatin is highly condensed, significantly restricting the action of exogenous molecules [9]. These dynamic changes form an important basis for influencing gene editing efficiency.

The CRISPR-Cas9 system uses single-guide RNA (sgRNA) to guide Cas9 in cleaving target DNA, generating double-strand breaks (DSBs). Subsequently, cells repair these DSBs through two main pathways: non-homologous end joining (NHEJ) and homology-directed repair (HDR). NHEJ is fast but prone to introducing mutations, making it commonly used for gene knockout; HDR relies on a homologous template to achieve precise editing, enabling site-directed mutagenesis or gene insertion [10-14]. This mechanism has been applied in the metabolic engineering of *S. cerevisiae*, such as

optimizing the synthetic pathways of artemisinin precursors and salidroside [2,3]. However, fluctuations in editing efficiency remain a bottleneck.

The presence of chimeric cells leads to unedited cells consuming substrates and competing with target cells for resources, resulting in fluctuating yields, reduced purity, and increased separation costs. Therefore, elucidating the regulatory mechanism of chimerism rate is essential for improving editing accuracy. Recent studies have shown that the cell cycle may be a key factor regulating chimerism rate: the chromatin state and repair pathway preference in different phases directly affect CRISPR cleavage efficiency and repair outcomes [15]. Studies indicate that the chimerism rate in the S phase is significantly lower than that in the G1 phase, while the M phase tends to induce a high chimerism rate [3, 5]. However, the specific regulatory mechanisms of each phase still lack systematic compilation. This review aims to analyze the interaction between the characteristics of each cell cycle phase and the key steps of CRISPR editing, clarify the regulatory mechanism of chimerism rate, and summarize the variation patterns. It is expected to provide a theoretical basis for optimizing the experimental design of *S. cerevisiae* gene editing and promoting its efficient application in the field of biosynthesis.

2. CRISPR-Cas9 Editing Chimerism

2.1. Definition and Detection of Chimeric Cells

Chimeric cells in CRISPR-Cas9 editing refer to a mixed population of cells after gene editing, where some cells have undergone the expected mutations or integrations, while others remain unedited or incompletely edited. This phenomenon arises from differences in cellular responses to CRISPR cleavage: some cells may fail to complete editing due to low Cas9 cleavage efficiency, biased repair pathway selection, or lack of repair templates, ultimately forming a genotypically heterogeneous population.

The detection of chimeric cells relies on analyzing genotypes or phenotypes at the single-cell level, with common techniques including flow cytometry and single-cell sequencing. Flow cytometry based on fluorescent reporters enables high-throughput quantification of expression changes. For example, a fluorescent protein reporter is integrated into the editing vector to distinguish edited cells from unedited cells. Single-cell sequencing can directly identify the mutation type of each cell at the genomic level (including expected site-directed substitutions and unexpected insertions/deletions), allowing the capture of low-frequency chimeric events. However, this method is relatively costly and low-throughput, making it suitable for in-depth verification of chimerism mechanisms. In addition, considering the characteristics of microorganisms, the above methods can be combined with monoclonal plate screening and digital polymerase chain reaction (digital PCR) to verify the chimerism rate from multiple dimensions.

2.2. Impacts of Chimerism on Metabolic Engineering

Chimerism is particularly prominent in multi-gene editing or complex metabolic pathway modification, directly affecting the yield and quality of target products. In a study on cellulase production by *Bacillus subtilis*, CRISPR-Cas9 was used to knock out target genes regulating competitive metabolism (e.g., lactic acid synthesis) to reduce carbon source diversion. However, approximately 30% of cells in the edited population retained the unknocked-out competitive genes, causing these cells to continuously consume glucose for lactic acid synthesis and compete with the target product (cellulase) for resources. Eventually, the fluctuation range of cellulase yield reached 25%, and the average yield was 18% lower than the theoretical value [16].

In the case of isobutanol synthesis by *Escherichia coli* (*E. coli*), a CRISPR-based strategy was adopted to integrate heterologous genes for constructing high-efficiency production strains. However, obvious chimeric mutations occurred after multi-gene editing: most cells only completed partial gene integration or no integration at all, leading to metabolic pathway disruption and accumulation of intermediate products. Ultimately, the maximum titer of isobutanol was only 1.2 g/L, far lower than

the theoretical value of homozygously edited strains (3.5 g/L), and the proportion of by-products (e.g., acetic acid) increased by 30%, significantly limiting the improvement of product titer and overall production efficiency [17].

Chimerism directly undermines synthesis efficiency by reducing metabolic homogeneity across the cell population. Unedited or failed to edit cells retain native pathways and compete with target cells for key nutrients such as carbon and nitrogen, thereby lowering the net conversion of substrate into the desired product; for example, in *Bacillus subtilis* cellulase production unknocked out cells diverted roughly 20 percent of glucose into non target metabolites, creating a shortfall of precursors for cellulase biosynthesis [16]. In addition, metabolites produced by chimeric cells decrease product purity and raise downstream separation and purification costs. Differences in growth rate and metabolic activity among genotypes within a chimeric population also drive instability in fermentation, producing larger fluctuations in product concentration that complicate industrial quality control. Taken together these effects make chimerism a central barrier to reliable CRISPR Cas9 based metabolic engineering, and reducing chimerism is therefore essential to improve yield stability and economic feasibility.

3. Regulation of Cleavage Efficiency by Cell Cycle and Chromatin Accessibility

Chromatin accessibility, defined as the ability of exogenous nucleases to bind to DNA, varies significantly across different phases of the cell cycle. This variation directly regulates the cleavage efficiency of the CRISPR-Cas9 system. The degree of chromatin relaxation is determined by factors such as nucleosome packing density and histone modification status, which exhibit periodic changes throughout the cell cycle.

In the G1 phase, a stage dedicated to cell growth and preparation for DNA replication, chromatin exists in a highly compacted state. Studies have shown that chromatin remodeling complexes (e.g., nucleosome assembly proteins) in G1 phase cells enhance the binding between nucleosomes and DNA. This structure limits the complementary pairing space between sgRNA and target DNA and hinders Cas9 protein from approaching cleavage sites, resulting in a significant reduction in CRISPR system cleavage efficiency [18]. Experiments on G1 phase-synchronized *S. cerevisiae* cells showed that the cleavage efficiency in the G1 phase was only approximately 30% of that in the S phase [19].

The S phase is a critical stage for DNA synthesis, during which chromatin is relaxed to meet the needs of DNA replication. At this stage, histone-modifying enzymes (e.g., histone acetyltransferases) are active and mediate acetylation of histones H3 and H4, weakening the interaction between nucleosomes and DNA. This exposes part of the DNA double strands and reveals target sequences [3]. This high accessibility provides sufficient binding space for the Cas9-sgRNA complex, significantly improving cleavage efficiency. Experimental data show that the CRISPR cleavage efficiency of *S. cerevisiae* cells in the S phase is more than three times that in the G1 phase, and the cleavage success rate at target sites exceeds 80% [19]. Therefore, the S phase is considered the preferred window for achieving efficient cleavage and reducing the unedited rate.

Although DNA replication is completed in the G2 phase, chromatin retains a certain degree of relaxation, with accessibility between that of the S phase and G1 phase. The cleavage efficiency of *S. cerevisiae* cells in the G2 phase is approximately 70% of that in the S phase. In the M phase, chromatin is highly condensed into visible chromosomes, and DNA is tightly wrapped around histone cores to form a superhelical structure, almost completely shielding the binding sites of exogenous nucleases. This makes it difficult for the Cas9-sgRNA complex to recognize most target sites, and the cleavage efficiency of *S. cerevisiae* in the M phase can decrease by approximately 80% compared with that in the S phase [5]. In summary, the S phase is the optimal window for CRISPR-Cas9 cleavage, while the G1 and M phases significantly inhibit cleavage activity and increase the incidence of unedited or chimeric cells.

4. Impact of Double-Strand Break Repair Pathways on Chimerism Rate

Double-strand breaks (DSBs) generated by CRISPR-Cas9 cleavage require repair through intracellular repair pathways. Different phases of the cell cycle exhibit significant differences in repair pathway preference, which directly affects the chimerism rate after editing. In general, non-homologous end joining (NHEJ) and homology-directed repair (HDR) show alternating dominance over time: when homologous templates are unavailable, the fast but error-prone NHEJ pathway dominates; in phases where sister chromatids are available as templates, HDR promotes precise repair. Thus, the cell cycle not only affects cleavage efficiency by altering chromatin accessibility but also influences the final editing results and population homogeneity by regulating the availability of repair pathways.

Cells in the G1 and M phases mainly rely on the NHEJ pathway for DSB repair. This repair method is highly random due to the lack of homologous templates, making it a core cause of high chimerism rates. In the G1 phase, DNA replication has not yet occurred, and there are no sister chromatids to serve as HDR templates, so NHEJ becomes the main repair option. NHEJ directly ligates broken ends via DNA ligase but tends to introduce base insertions, deletions, or mismatches, resulting in a large number of unexpected modifications and increasing the chimerism rate. Experiments on editing the *ura3* gene in G1 phase-synchronized *S. cerevisiae* cells showed that approximately 70% of repair events were error-prone repairs mediated by NHEJ, only 30% achieved the expected editing, and the chimerism rate was as high as 70% [19].

Although NHEJ repair is not active in the M phase, the extremely low cleavage efficiency (most cells do not generate DSBs) leads to a surge in the proportion of unedited cells, further increasing the chimerism rate. Studies have found that after CRISPR editing of M phase-synchronized *S. cerevisiae* cells, unedited cells accounted for 85% of surviving cells, and the chimerism rate exceeded 80% [5]. In addition, the DNA damage checkpoint in M phase cells is activated, and repair mechanisms are inhibited. Even if DSBs occur, they cannot be effectively repaired, leading to an increase in the proportion of failed-to-edit cells.

In contrast, the S and G2 phases have sister chromatids as homologous templates, so the HDR pathway is active, enabling precise editing and thus reducing the chimerism rate. In the S phase, sister chromatids are formed during DNA replication, providing natural templates for HDR. At the same time, the expression level of key HDR proteins (e.g., Rad51) increases significantly, promoting homologous sequence pairing and DSB repair [20]. Experiments on editing the *his3* gene in S phase-synchronized *S. cerevisiae* cells showed that 65% of repair events were precise edits mediated by HDR, only 15% resulted in editing failure, and the chimerism rate was as low as 35% [19].

Although DNA replication is completed in the G2 phase, sister chromatids have not yet separated, so HDR remains partially active. Studies have shown that the accumulation of Rad51 protein in G2 phase cells is lower than that in the S phase, but it is sufficient to support partial precise repair. In the editing of the *leu2* gene in *S. cerevisiae*, the proportion of precise editing in G2 phase cells reached 45%, and the chimerism rate was approximately 55%, significantly lower than that in the G1 and M phases [5]. However, since the G2 phase is close to mitosis, cells impose stricter time constraints on repair, so HDR efficiency is slightly lower than that in the S phase, resulting in a higher chimerism rate than in the S phase. In conclusion, the cell cycle jointly determines the chimerism rate of CRISPR-Cas9 editing by regulating chromatin accessibility and repair pathway preference. The S phase, with its high cleavage efficiency and high HDR activity, is an ideal stage for reducing the chimerism rate, while the G1 and M phases lead to high chimerism rates due to low cleavage efficiency and dominant NHEJ repair.

5. Conclusion

Chimerism in CRISPR-Cas9 editing is a key bottleneck restricting the efficiency and stability of product synthesis in industrial microorganisms. This phenomenon is closely related to the cell cycle, as different phases exhibit distinct preferences for chromatin accessibility and double-strand break repair pathways, which in turn determine cleavage efficiency and repair outcomes. In the G1 and M phases, chromatin is highly condensed and NHEJ is the dominant repair pathway; previous studies have reported that the chimerism rate in these phases can exceed 70%. In contrast, chromatin is relatively relaxed and HDR is active in the S and G2 phases, with chimerism rates of approximately 35% in the S phase and 55% in the G2 phase. Increased chimerism rates exacerbate substrate competition and reduce product purity, which is particularly evident in multi-gene modification and complex metabolic network engineering. It is recommended to perform editing during appropriate cell cycle windows, with comprehensive consideration of chromatin accessibility, repair template supply, and donor delivery timing, to reduce population heterogeneity and improve product yield and quality.

Current research still has several limitations, and the relevant rules need to be verified in a broader range of biological systems. Most studies have focused on *S. cerevisiae*, with insufficient research on the relationship between cell cycle regulation and chimerism rate in other industrial microorganisms such as *Bacillus subtilis* and *E. coli*. In addition, cell cycle synchronization methods need improvement in terms of efficiency, specificity, and scalability, making it difficult to precisely control cycle distribution in industrial-scale cultures. Future research should expand to more species, develop efficient and low-toxicity synchronization technologies, and combine single-cell sequencing with dynamic online monitoring methods to track the correlation between cycle changes and chimerism rate in real time during editing. This will provide more comprehensive theoretical and technical support for the precise regulation of industrial gene editing.

References

[1] Woolston B M, Edgar S, Stephanopoulos G. Metabolic engineering past and future. Annual Review of Chemical and Biomolecular Engineering, 2013, 4: 259-288.

[2] Ro D K, Paradise E M, Ouellet M, et al. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. Nature, 2006, 440 (7086): 940-943.

[3] Layfield J B, Vann L R, Sheppard J D. A novel method of inducing and retaining cell cycle synchronization in cultures of *Saccharomyces cerevisiae*. Journal of the American Society of Brewing Chemists, 2014, 72 (2): 102-109.

[4] DiCarlo J E, Norville J E, Mali P, et al. Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. Nucleic Acids Research, 2013, 41 (7): 4336-4343.

[5] Taubmann A. Untersuchung der DNA-Doppelstrangbruch-Reparatur über Homologe Rekombination am Übergang von der G2-Phase in die Mitose. Darmstadt, Technische Universität Darmstadt, 2015.

[6] Hartwell L H, Weinert T A. Checkpoints, controls that ensure the order of cell cycle events. Science, 1989, 246 (4930): 629-634.

[7] Diffley J F. Regulation of early events in chromosome replication. Current Biology, 2004, 14 (18): R778-R786.

[8] Weinert T A, Hartwell L H. The RAD9 gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. Science, 1988, 241 (4863): 317-322.

[9] Murray A W, Kirschner M W. Dominoes and clocks, the union of two views of the cell cycle. Science, 1989, 246 (4930): 614-621.

[10] Jinek M, Chylinski K, Fonfara I, et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science, 2012, 337 (6096): 816-821.

[11] Doench J G, Hartenian E, Graham D B, et al. Rational design of highly active single guide RNAs for CRISPR-Cas9-mediated gene inactivation. Nature Biotechnology, 2014, 32 (12): 1262-1267.

[12] Sternberg S H, Redding S, Jinek M, et al. DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. Biophysical Journal, 2014, 106 (2): 695a.

[13] Moore D P, Haber J E. Cell-cycle-regulated double-strand break repair in *Saccharomyces cerevisiae*. Genetics, 1996, 144 (3): 1399-1409.

[14] Li X, Heyer W-D. Homologous recombination in DNA repair and DNA damage tolerance. Cell Research, 2008, 18 (1): 99-113.

[15] Lawrimore J, Bloom K S, Salmon E. Point centromeres contain more than a single centromere-specific Cse4 (CENP-A) nucleosome. Journal of Cell Biology, 2011, 195 (4): 573-582.

[16] Sun T L, Huang M Z, Liu B, et al. Advances in microbial genome reduction and optimization based on synthetic biology. Science and Technology of Food Industry, 2025, 46 (1): 394-402.

[17] Bassalo M C, Garst A D, Halweg-Edwards A L, et al. Rapid and efficient one-step metabolic pathway integration in *Escherichia coli*. ACS Synthetic Biology, 2016, 5 (7): 561-568.

[18] Gavin A-C, Bösche M, Krause R, et al. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature, 2002, 415 (6868): 141-147.

[19] Juanes M A. Methods of synchronization of yeast cells for the analysis of cell cycle progression. The Mitotic Exit Network, Methods and Protocols, 2017, 1505: 19-34.

[20] San Filippo J, Sung P, Klein H. Mechanism of eukaryotic homologous recombination. Annual Review of Biochemistry, 2008, 77 (1): 229-257.