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CRISPR-dCas9-Mediated Epigenetic Reactivation of LRIG1 Suppresses Oncogenic Signaling in Triple-Negative Breast Cancer

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Abstract

CRISPR-dCas9-based epigenetic editing is emerging as a promising strategy for precise regulation of cancer-associated epigenetic alterations. Unlike wild-type Cas9, which induces double-strand breaks, dCas9 is generated by introducing D10A and H840A mutations that abolish nuclease activity while retaining sgRNA-guided DNA binding. This allows dCas9 to serve as a programmable scaffold for recruiting epigenetic effectors. Fusions with DNMTs, TET enzymes, KRAB, or transcriptional activators such as VP64, p300, and the SAM system enable targeted methylation, demethylation, or histone modification, thereby modulating gene expression without altering DNA sequence. A central focus is the tumor suppressor LRIG1, a regulator of growth factor signaling (e.g., EGFR), which is often silenced in basal-like and triple-negative breast cancers (TNBC) through promoter hypermethylation. CpG island methylation prevents transcription factor binding, reducing LRIG1 expression and contributing to oncogenesis. Similar repression occurs in colorectal and cervical cancers. By guiding dCas9-activator complexes to the LRIG1 promoter, transcriptional activators and chromatin-modifying domains can erase repressive methylation, promote histone acetylation, and restore transcription. This reactivation of LRIG1 has shown potential to suppress tumor proliferation in preclinical models. Beyond LRIG1, dCas9-based systems represent a versatile platform for reactivating tumor suppressors or silencing oncogenes with minimal off-target damage. Advances in delivery technologies and in vivo applications underscore their translational promise, although challenges remain, including chromatin accessibility and off-target recruitment. Overall, CRISPR-dCas9-mediated epigenetic editing offers a transformative and reversible framework for therapeutic gene regulation, providing high precision and adaptability for cancer treatment and broader applications in precision medicine.

Key words: ***CRISPR-dCas9***, ***LRIG1***, ***Triple-Negative Breast Cancer***

Introduction

Breast cancer is a heterogeneous disease classified according to the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Subtypes of Breast Cancer, 2023). Hormone receptor-positive tumors constitute the majority of breast cancer cases and are commonly treated using endocrine therapies, whereas HER2-positive tumors benefit from HER2-targeted agents. However, despite improvements in diagnosis and targeted treatment, therapy resistance, recurrence, and metastatic progression remain major clinical challenges.

Triple-negative breast cancer (TNBC), defined by the absence of ER, PR, and HER2 expression, accounts for approximately 10–20% of invasive breast cancers and is associated with poor prognosis, aggressive progression, and limited therapeutic options (Bianchini et al., 2016). TNBC occurs more frequently in younger women and is strongly associated with BRCA1 mutations. Because TNBC lacks established molecular targets, there is increasing interest in identifying epigenetic mechanisms that contribute to tumor progression and therapeutic resistance.

Epigenetic regulation plays a central role in controlling gene expression through reversible modifications that alter chromatin accessibility without changing the underlying DNA sequence. These mechanisms include DNA methylation, histone modifications, and chromatin remodeling (Stefanska et al., 2011; Allis et al., 2016). Aberrant promoter hypermethylation is a hallmark of many cancers and commonly results in transcriptional silencing of tumor suppressor genes (Jones et al., 2012). Histone modifications further contribute to transcriptional regulation, with H3K4me3 and H3K27ac associated with active promoters and enhancers, whereas H3K27me3 is linked to transcriptionally repressed chromatin states (Kouzarides, 2007; Margueron et al., 2011).

In CRISPRi, fusion proteins target the LRIG1 promoter region enabling controlled repression: dCas9-KRAB induces heterochromatin formation while dCas9-DNMT3A promoter (Figure: 1). Repressed genes are commonly associated with histone H3 lysine 27 trimethylation (H3K27me3), a hallmark of transcriptionally silenced chromatin H3K27me3 can be

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present at both promoters and enhancers and is characteristic of genes that are developmentally regulated or epigenetically silenced in a cell-type–specific manner (Margueron et al 2011)

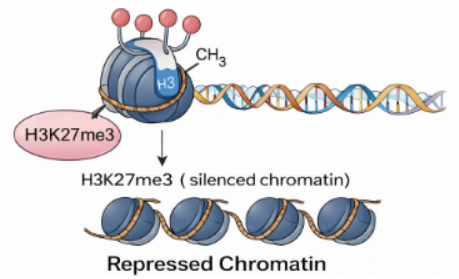


Fig. 1. Repressed gene and silenced chromatin states
In CRISPRi, fusion proteins target the LRIG1 promoter region enabling controlled repression: dCas9-KRAB induces heterochromatin formation while dCas9-DNMT3A promoter

Among the tumor suppressors implicated in breast cancer progression, LRIG1 has emerged as an important negative regulator of receptor tyrosine kinase (RTK) signaling pathways, including EGFR, HER2, and MET (Umeh-Garcia et al., 2022). Reduced LRIG1 expression has been associated with enhanced proliferation, invasion, and metastatic behavior in multiple cancers. Recent studies further demonstrated increased promoter methylation and decreased LRIG1 expression in basal-like and TNBC tumors, suggesting that epigenetic silencing of LRIG1 contributes to aggressive tumor phenotypes. The development of CRISPR/Cas9-based epigenome editing has provided new opportunities for locus-specific regulation of endogenous gene expression. Catalytically inactive Cas9 (dCas9) retains sequence-specific DNA-binding activity but lacks nuclease function due to mutations in the HNH and RuvC domains (Qi et al., 2013). Guided by single-guide RNAs (sgRNAs), dCas9 can be fused to epigenetic effector proteins to modulate chromatin state and transcriptional activity without inducing DNA double-strand breaks. Fusion of dCas9 to transcriptional activators such as VP64 or histone acetyltransferases such as p300 promotes gene activation, whereas fusion to repressors including KRAB or DNMT3A enables targeted transcriptional silencing (Gilbert et al., 2013; Hilton et al., 2015; Konermann et al., 2015). Based on these observations, this study investigated the relationship between LRIG1 promoter methylation and transcriptional silencing in TNBC and explored CRISPR/dCas9-mediated epigenetic editing as a targeted strategy for regulating LRIG1 expression.

Guide RNA (gRNA)

The guide RNA (gRNA) of the CRISPR/Cas9 system consists of two RNA components, the CRISPR RNA (crRNA) and the trans-activating crRNA (tracrRNA), which together form a functional RNA duplex required for Cas9 activity (Deltcheva et al., 2011). This RNA complex recognizes complementary target DNA sequences and directs the Cas9 endonuclease to induce site-specific double-strand breaks (DSBs) in the genome (Jinek et al., 2012) (Figure 2)

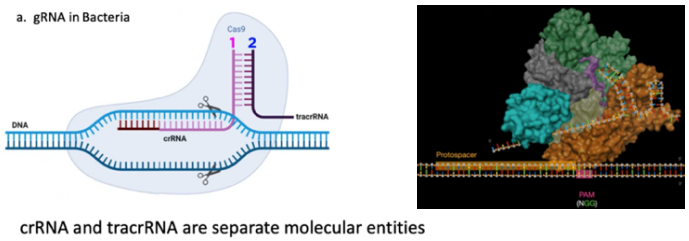


Fig. 2: Structure and function of guide RNA (gRNA) *The figure illustrates how the guide RNA directs the Cas9 or dCas9 protein to specific genomic sequences. In the natural bacterial CRISPR system, the guide RNA is composed of two parts: the CRISPR RNA (crRNA)**,** which contains the 20-nucleotide sequence complementary to the target DNA, and the trans-activating CRISPR RNA (tracrRNA)**,** which forms a duplex with crRNA to guide Cas9 binding*

Single Guide RNA

gRNA is the general term for any guide RNA molecule in CRISPR, while sgRNA is a specific, synthetic type that combines the two separate RNA molecules (crRNA and tracrRNA) found in natural systems into a single, simpler molecule. sgRNA, or single guide RNA, is a molecule used in CRISPR gene editing that combines a custom-designed targeting sequence with a scaffold structure to guide the Cas9 nuclease to a specific location in the DNA. In engineered systems, these two components are fused into **a** single guide RNA (sgRNA) for simplicity and efficiency. The sgRNA forms a complex with Cas9 or dCas9, enabling sequence-specific DNA recognition via Watson–Crick base pairing with the target site adjacent to a PAM (protospacer adjacent motif**)**. This targeted complex either introduces a double-strand break (Cas9) or modulates gene expression without cleavage (dCas9 fusions). (Figure 2).

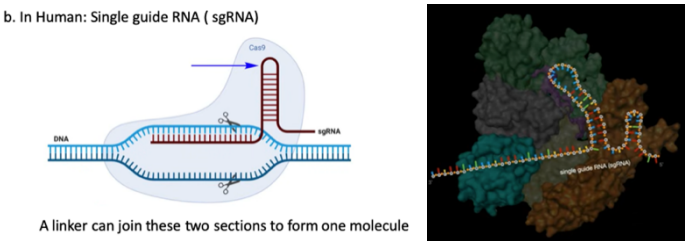


Fig. 3: A single guide RNA (sgRNA) in CRISPR–Cas9 targeting *In engineered systems, these two components are fused into **a** single guide RNA (sgRNA) for simplicity and efficiency. The sgRNA forms a complex with Cas9 or dCas9, enabling sequence-specific DNA recognition via Watson–Crick base pairing with the target site adjacent to a PAM (protospacer adjacent motif**)**. This targeted complex either introduces a double-strand break (Cas9) or modulates gene expression without cleavage (dCas9 fusions).*

For genome engineering applications, crRNA and tracrRNA are commonly fused into a single guide RNA (sgRNA) to simplify targeting (Cong et al., 2013). Furthermore, both Cas9 and dCas9 can be guided by multiple gRNAs simultaneously, enabling multiplex genome editing

or transcriptional regulation of several target genes within the same cell, thereby increasing the flexibility and scalability of the CRISPR/Cas system (Cong et al., 2013; Mali et al., 2013; Gilbert et al., 2013; Qi et al., 2013). This study aimed to investigate the role of aberrant promoter methylation in LRIG1 silencing in basal/triple-negative breast cancer (TNBC) and to evaluate the use of CRISPR-dCas9–based epigenetic editing as a targeted strategy to restore LRIG1 expression.

Material and Methods

Design of sgRNAs Targeting the LRIG1 Promoter

Single-guide RNAs (sgRNAs) targeting CpG-rich regions within the LRIG1 promoter were designed using CRISPOR and CHOPCHOP to maximize predicted on-target efficiency while minimizing off-target activity (Labun et al., 2019). Target sequences were selected within ±500 bp of the LRIG1 transcription start site (TSS) and required compatibility with the SpCas9 protospacer adjacent motif (PAM) sequence (5'-NGG-3'). Only sgRNAs with high specificity scores and minimal predicted off-target sites were retained for downstream epigenetic targeting analyses. Multiplex sgRNA strategies were additionally considered to enhance local chromatin remodeling efficiency. Table 1

Table 1. Representative sgRNAs Designed to Target the

sgRNA ID	Target Region Relative to TSS	sgRNA Sequence (5′→3′)	PAM	Predicted Function
sgLRIG1-1	−420 bp	GAGCGCGGAGGAG CGGATGA	AGG	CpG island targeting
sgLRIG1-2	−265 bp	CGGCTCGGTTCCG AGGAGCT	TGG	Promoter demethylation
sgLRIG1-3	−118 bp	GGCGGCGGCGGT TTCCGAGC	CGG	Transcriptional activation
sgLRIG1-4	+45 bp	GCGCTGCGCGAG GACGAGGA	GGG	TSS-proximal targeting
sgLRIG1-NTC	Non-targeting control	GCGAGGTATTCGG CTCCGCG	AGG	Negative control

Representative sgRNAs were computationally designed using CRISPOR and CHOPCHOP based on the LRIG1 promoter sequence and selected according to predicted on-target activity and low off-target potential. Only sgRNAs with high specificity scores and minimal predicted off-target sites were retained for downstream epigenetic targeting analyses. Multiplex sgRNA strategies were additionally considered to enhance local chromatin remodeling efficiency.

TNBC Cell Lines

Representative TNBC cell lines commonly used in epigenetic and CRISPR-based breast cancer studies were selected to model distinct molecular and epigenetic backgrounds:

Cell Line	Molecular Characteristics	Rationale for Use
MDA-MB-231	Mesenchymal-like, highly invasive TNBC	Model of aggressive metastatic TNBC
BT-549	Basal-like TNBC with altered PI3K signaling	Suitable for epigenetic regulation studies
HCC1806	Basal-like TNBC with elevated EGFR expression	Relevant for RTK/LRIG1 signaling analysis

Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) or RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin under standard humidified culture conditions at 37°C with 5% CO_2. These TNBC cell lines were selected because they represent distinct epigenetic and receptor tyrosine kinase signaling backgrounds relevant to LRIG1 regulation.

Identification of CpG Islands Within the LRIG1 Promoter

The LRIG1 promoter region was analyzed using UCSC Genome Browser, MethPrimer, and CpG island prediction tools to identify CpG-rich regulatory regions proximal to the transcription start site (TSS). Candidate CpG islands located within ±500 bp of the TSS were prioritized because of their established role in transcriptional regulation and epigenetic silencing. Publicly available TNBC methylation datasets were further examined to evaluate promoter methylation patterns associated with reduced LRIG1 expression.

Design of CRISPR/dCas9 Epigenetic EditingConstructs

Catalytically inactive Cas9 (dCas9) derived from Streptococcus pyogenes Cas9 containing D10A. and H840A mutations was used as the programmable DNA-binding platform (Qi et al., 2013). dCas9 was conceptually fused to distinct epigenetic effector domains to model transcriptional activation or repression at the LRIG1 promoter

Activation Constructs

The following activation-associated constructs were incorporated:

dCas9 Fusion	Functional Role
dCas9-TET1	Targeted DNA demethylation
dCas9-VP64	Recruitment of transcriptional activators
dCas9-p300	Histone H3K27 acetylation and chromatin activation

The TET1 catalytic domain was included to model conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), facilitating chromatin relaxation and transcriptional activation.

Repression Constructs

To model epigenetic silencing mechanisms observed in TNBC, the following CRISPR interference (CRISPRi) constructs were considered:

dCas9 Fusion	Functional Role
dCas9-KRAB	Induction of heterochromatin formation
dCas9-DNMT3A	Targeted promoter DNA methylation

Targeted Recruitment of dCas9-Effector Complexes

sgRNAs were modeled to direct dCas9-effector complexes specifically to the LRIG1 promoter. Upon recruitment, effector domains locally modified epigenetic marks without inducing DNA cleavage. Targeted chromatin remodeling was conceptually assessed through: promoter DNA demethylation, enrichment of activating histone marks, altered chromatin accessibility, and recruitment of transcription-associated proteins.

Chromatin immunoprecipitation (ChIP)-based approaches were considered to evaluate locus-specific enrichment of dCas9 and associated histone modifications at the LRIG1 promoter region.

Delivery of CRISPR/dCas9 Systems

Several delivery approaches relevant to in vivo and in vitro epigenome editing applications were considered.

Viral Delivery ;

Adeno-associated viral (AAV) vectors were evaluated because of their high transduction efficiency and favorable safety profile for targeted gene delivery.

Non-Viral Delivery

.CRISPR/dCas9 Delivery Formats

Different molecular formats of CRISPR/dCas9 system were considered based on expression kinetics and delivery efficiency.

Delivery Format	Characteristics
Plasmid DNA	Long-term expression following transcription and translation
mRNA	Rapid transient expression without genomic integration
Ribonucleoprotein (RNP) Complex	Immediate but short-lived activity using preassembled dCas9 protein and sgRNA

mRNA and RNP-based systems were considered advantageous for transient epigenetic modulation because they minimize prolonged intracellular persistence and potential off-target effects (Liu et al., 2019).

Statistical and Conceptual Analysis

Because this work primarily focused on mechanistic and conceptual modeling of LRIG1 epigenetic regulation, analyses emphasized integration of published epigenetic datasets, CRISPR-based targeting strategies, and established chromatin remodeling mechanisms reported in TNBC-related literature.

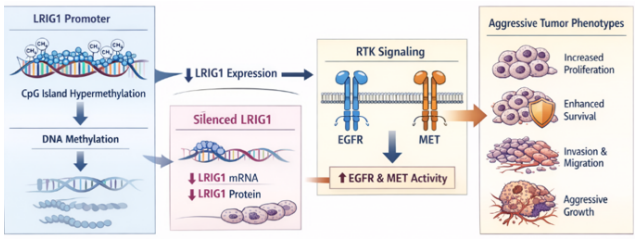
Results

Identification of CpG Hypermethylation Within the LRIG1 Promoter

Bioinformatic analysis identified a CpG-rich region within the proximal LRIG1 promoter upstream of the transcription start site (TSS). Analysis of TNBC-associated datasets demonstrated increased methylation within this promoter region, which correlated with reduced LRIG1 expression in basal-like breast cancer samples (Choi et al., 2022; Umeh-Garcia et al., 2022). These findings support the hypothesis that promoter hypermethylation contributes to transcriptional repression of LRIG1 in aggressive TNBC subtypes.

Association Between LRIG1 Silencing and Aggressive TNBC Phenotypes

Reduced LRIG1 expression was associated with increased activation of receptor tyrosine kinase signaling pathways, including EGFR and MET. Dysregulated RTK signaling corresponded with enhanced proliferation, migration, invasion, and survival of TNBC cells, supporting the tumor-suppressive role of LRIG1. These observations are consistent with previous reports linking LRIG1 downregulation to poor clinical outcome and aggressive tumor progression in breast cancer (Umeh-Garcia et al., 2022). In triple-negative breast cancer (TNBC), the promoter region of LRIG1 undergoes CpG island hypermethylation, leading to transcriptional silencing and reduced LRIG1 mRNA and protein expression. Loss of LRIG1, a negative regulator of receptor tyrosine kinase (RTK) signaling, results in unchecked activation of pathways such as EGFR and MET,



promoting enhanced cell proliferation, survival, migration, and invasion. This schematic illustrates how epigenetic repression of LRIG1 contributes to aggressive TNBC phenotypes, highlighting promoter DNA methylation as a key mechanism of tumor progression (Figure 4). Promoter methylation was associated with transcriptional silencing and a significant decrease in LRIG1 mRNA and protein levels).

Promoter Methylation of LRIG1 = RTK activation = Aggressive TNBC Behavior

Fig. 4: Epigenetic Silencing of LRIG1 Drives Aggressive Phenotypes in Triple-Negative Breast Cancer. *In triple-negative breast cancer (TNBC), the promoter region of LRIG1 undergoes CpG island hypermethylation, leading to transcriptional silencing and reduced LRIG1 mRNA and protein expression. Loss of LRIG1, a negative regulator of receptor tyrosine kinase (RTK) signaling, results in unchecked activation of pathways such as EGFR and MET, promoting enhanced cell proliferation, survival, migration, and invasion. This schematic illustrates how epigenetic repression of LRIG1 contributes to aggressive TNBC phenotypes, highlighting promoter DNA methylation as a key mechanism of tumor progression.*

CRISPR/dCas9-Based Epigenetic Targeting of the LRIG1 Promoter

To evaluate locus-specific epigenetic regulation of LRIG1, sgRNAs were designed to target the proximal promoter region adjacent to the TSS. Candidate sgRNAs were selected based on predicted on-target efficiency and minimal off-target activity (Doench et al., 2016). Previous studies demonstrated that dCas9-effector complexes can be selectively recruited to promoter regions using sgRNA-directed targeting, enabling precise chromatin modulation without altering genomic sequence (Qi et al., 2013; Cai et al., 2023). At general the illustration of CRISPR/dCas9-mediated transcriptional activation. The dCas9 domain is fused to transcriptional activators to activate adjacent promoters and the transcription of associated genes (Figure 5). By recruiting transcriptional or epigenetic regulators, this programmable system enables targeted modulation of DNA methylation or histone modifications, thereby increasing or decreasing LRIG1 expression without inducing permanent genomic alterations

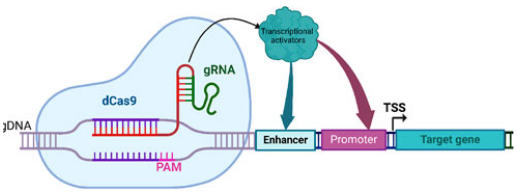


Fig. 5: CRISPR-dCas9 Mediated Gene Activation
At general the illustration of CRISPR/dCas9-mediated transcriptional activation. The dCas9 domain is fused to transcriptional activators to activate adjacent promoters and the transcription of associated genes.

- ***dCas9:*** *Catalytically dead Cas9 protein*
- ***gRNA:*** *Guide RNA*
- ***PAM:*** *Protospacer adjacent motif*
- ***Enhancer:*** *Regulatory DNA sequence*
- ***Promoter:*** *Gene promoter region*
- ***TSS:*** *Transcription start site*
- ***Target gene:*** *Gene being regulated*
- ***Transcriptional activators:*** *Proteins that increase transcription*

Targeted DNA Demethylation Promotes LRIG1 Reactivation

Targeted epigenetic activation was conceptually modeled using dCas9 fused to the DNA demethylase TET1. Recruitment of dCas9-TET1 to the LRIG1 promoter was predicted to induce site-specific DNA demethylation, resulting in increased chromatin accessibility and enhanced recruitment of transcription-associated factors (Liu et al., 2016). These chromatin changes are associated with activation-associated histone marks including H3K27ac and H3K4me3, ultimately promoting LRIG1 transcription. To directly interrogate and modulate LRIG1 epigenetic silencing, CRISPR/dCas9-based epigenome editing approaches were employed. Single-guide RNAs (sgRNAs) were rationally designed to target the LRIG1 proximal promoter upstream of the TSS. Schematic overview of *the* workflow used to design and validate single-guide RNAs (sgRNAs) for targeted epigenetic editing of the *LRIG1* promoter. The proximal promoter region upstream of the *LRIG1* transcription start site(TSS) was identified, and candidate sgRNAs were designed *in silico* and screened for high on-target efficiency and low predicted off-target activity. Selected sgRNAs were cloned into dCas9–effector expression vectors and sequence-verified by Sanger sequencing. Validated sgRNA–dCas9–effector constructs were subsequently used for locus-specific epigenetic editing experiments (Fig. 6).

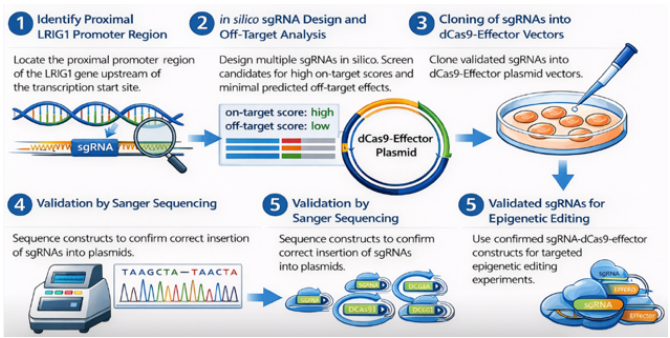


Fig. 6 : Design and validation of sgRNAs targeting the LRIG1 promoter *Schematic overview of the workflow used to design and validate single-guide RNAs (sgRNAs) for targeted epigenetic editing of the LRIG1 promoter. The proximal promoter region upstream of the LRIG1 transcription start site(TSS) was identified, and candidate sgRNAs were designed in silico and screened for high on-target efficiency and low predicted off-target activity. Selected sgRNAs were cloned into dCas9–effector expression vectors and*

sequence-verified by Sanger sequencing. Validated sgRNA–dCas9–effector constructs were subsequently used for locus-specific epigenetic editing experiments.

CRISPR Activation Enhances LRIG1 Transcription

CRISPR activation (CRISPRa) strategies employing dCas9 fused to VP64 or p300 were associated with increased LRIG1 transcriptional activity through recruitment of transcriptional machinery and enrichment of activating histone modifications (Hilton et al., 2015). In particular, dCas9-p300-mediated H3K27 acetylation promoted a transcriptionally permissive chromatin state, facilitating LRIG1 reactivation. Combined targeting approaches involving dCas9-TET1 and transcriptional activators were predicted to synergistically enhance LRIG1 expression by integrating promoter demethylation with chromatin activation mechanisms. These data confirm precise localization of the epigenetic editing machinery to the intended genomic site, validating effective recruitment of the dCas9-effector fusion for locus-specific modulation of chromatin state and gene regulation (e.g., targeted epigenome editing methods using dCas9-effector fusions (Cai, R et al 2023) CRISPR activation-mediated transcriptional reactivation of LRIG1. dCas9-VP64 and dCas9-p300 promote transcriptional activation through recruitment of activating chromatin regulators. (Figure 7).

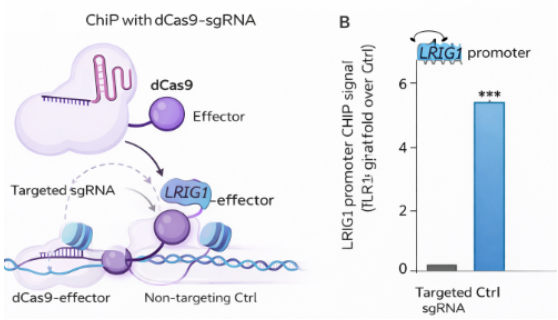


***Fig 7: Targeted recruitment of a dCas9–effector complex activates the** LRIG1 **promote(A)** dCas9 fused to a transcriptional effector domain is guided by a specific sgRNA to the promoter, where binding is confirmed by ChIP. No enrichment is observed with a non-targeting control sgRNA. **(B)** Targeted recruitment of the dCas9–effector significantly increases LRIG1 promoter activity compared to control sgRNA. Data are shown as mean ± SEM.*

CRISPR Interference Enables Targeted Repression of LRIG1

CRISPR interference (CRISPRi) systems were further considered to model epigenetic repression of LRIG1. dCas9-KRAB recruitment induces heterochromatin formation through repressive histone modifications, whereas dCas9-DNMT3A promotes promoter DNA methylation and stable transcriptional silencing (Konermann et al., 2015). These approaches provide mechanistic models for studying cancer-associated LRIG1 repression in TNBC. To reverse promoter hyperme thylation, dCas9 was fused to the DNA demethylase TET1 and guided to the LRIG1 promoter CpG island. Targeted recruitment of dCas9-TET1 resulted in site-specific DNA demethylation, promoting chromatin remodeling and increased accessibility of transcriptional regulators. This was accompanied by enhanced recruitment of transcription factors, histone acetyltransferases, and RNA polymerase II, leading to significant activation of LRIG1 transcription (Liu et al., 2016). dCas9 (catalytically inactive Cas9) can be fused to epigenetic effector proteins, such as TET1, a DNA demethylase. This allows targeted modification of chromatin at specific genomic loci without cutting the DNA (Figure 8). When guided by a single-guide RNA (sgRNA) to the LRIG1 promoter CpG island, TET1-dCas9 can induce site-specific DNA demethylation, leading to chromatin remodeling and activation of transcription at single-locus resolution. Liu, X., et al (2016).

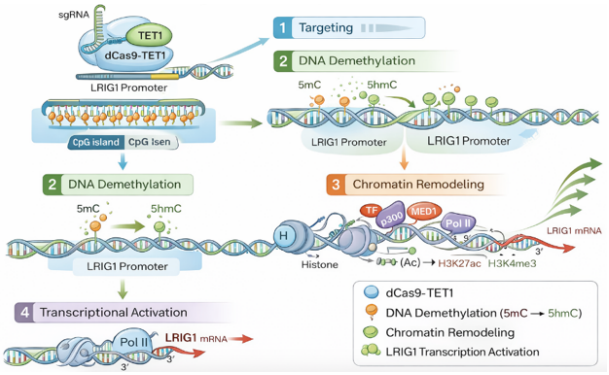


***Fig. 8:** Stepwise CRISPR-dCas9-Mediated Epigenetic Activation of LRIG1 Gene Expression via DNA Demethylation and Chromatin Remodeling*

***Figure 8:** This schematic illustrates the stepwise activation of the LRIG1 gene using a CRISPR-dCas9-TET1 system:*
***Targeting:** A single-guide RNA (sgRNA) directs dCas9-TET1 to the LRIG1 promoter region.*
***DNA Demethylation:** TET1 catalyzes the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) at CpG sites, reducing DNA methylation and making the promoter more accessible.*
***Chromatin Remodeling:** Recruitment of transcription factors (TFs), histone acetyltransferase p300, and mediator complex component MED1 leads to histone modification (H3K27ac and H3K4me3), relaxing chromatin structure.*
***Transcriptional Activation:** RNA polymerase II (Pol II) is recruited to initiate transcription, resulting in elevated LRIG1 mRNA expression.*
***Blue oval:** dCas9-TET1*
***Orange circles:** DNA demethylation (5mC → 5hmC)*
***Green circles:** Chromatin remodeling*
***Green arrows:** LRIG1 transcription activation*
Diagram Description:
***Step 1:** sgRNA guides TET1-dCas9 to the LRIG1 promoter.*
***Step 2:** TET1 catalyzes demethylation at CpG sites in the promoter.**Step 3:** Chromatin becomes relaxed, transcription factors bind. **Step 4:** Increased LRIG1 mRNA transcription is observed.*

Summary of Epigenetic Regulation of LRIG1 in TNBC

Collectively, these findings support a mechanistic relationship between LRIG1 promoter hypermethylation and

aggressive TNBC phenotypes. CRISPR/dCas9-based epigenetic editing provides a framework for reversible and locus-specific regulation of LRIG1 expression through targeted modulation of DNA methylation and chromatin state without altering the underlying DNA sequence.

CRISPR Activation Further Enhances LRIG1 Transcription

Complementary CRISPR activation (CRISPRa) strategies were employed using dCas9 fused to transcriptional activators including VP64 and p300. Targeting these CRISPRa complexes to the LRIG1 promoter resulted in increased LRIG1 mRNA expression through active recruitment of the transcriptional machinery and enrichment of activating histone marks such as H3K27ac (Hilton et al., 2015; Creyghton et al., 2010). Co-targeting with dCas9-TET1 and dCas9-VP64 synergistically enhanced LRIG1 expression and protein abundance, which was associated with reduced cancer cell viability, consistent with the tumor-suppressive role of LRIG1

VP64-dCas9 mediated epigenetic activation of LRIG1

In these studies, catalytically inactive Cas9 (dCas9) fused to transcriptional activation domains such as VP64, RTA, or p65 was targeted to the *LRIG1* promoter using sequence-specific single guide RNAs. Recruitment of these CRISPRa complexes to the *LRIG1* regulatory region led to increased transcriptional activity and elevated *LRIG1* mRNA expression compared with control conditions. These findings indicate that CRISPRa-based approaches are effective in reactivating *LRIG1* expression by promoting recruitment of the transcriptional machinery. . CRISPR activation strategies for restoring LRIG1 expression in epigenetically silenced cancer cells shows in (Figure 9).

Fig. 9: CRISPR Activation–Mediated Reactivation of LRIG1 Expression in Cancer Cells.
Previous studies have shown that CRISPR activation (CRISPRa) can be used to restore or enhance LRIG1 transcription in cancer cells where the gene is epigenetically silenced. In these approaches, catalytically inactive Cas9 (dCas9) fused to transcriptional activators such as VP64, RTA, or p65 is targeted to the LRIG1 promoter using sequence-specific single guide RNAs. Recruitment of these activator complexes promotes assembly of the transcriptional machinery, resulting in increased LRIG1 mRNA expression. This figure summarizes reported CRISPRa-based epigenetic editing strategies used to reactivate LRIG1 expression.

Epigenetic Reactivation of *LRIG1* by dCas9–p300 Has Been Demonstrated in Previous Studies

Published findings demonstrated that dCas9–p300 can be used to epigenetically reactivate *LRIG1* expression through targeted histone acetylation. In these studies, a catalytically inactive Cas9 (dCas9) fused to the histone acetyltransferase (HAT) domain of p300 was directed to the regulatory region of the *LRIG1* locus using sequence-specific single guide RNAs. Recruitment of the dCas9–p300 complex resulted in localized acetylation of histone H3 at lysine 27 (H3K27ac), a chromatin modification associated with transcriptionally active enhancers and promoters. Increased H3K27 acetylation was accompanied by chromatin relaxation, reduced nucleosome–DNA interactions, and enhanced accessibility of the transcriptional machinery. Consequently, these epigenetic changes led to reactivation and increased transcription of *LRIG1*. Collectively, these findings establish dCas9–p300 as an effective locus-specific epigenetic editor capable of modulating *LRIG1* expression without altering the underlying DNA sequence (Figure 10).

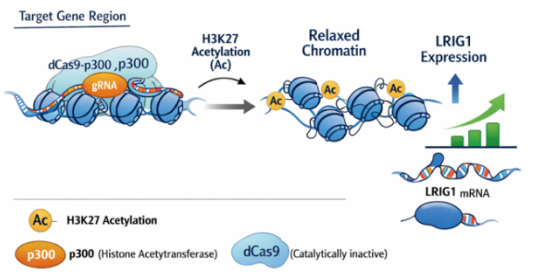


Fig. 10 dCas9–p300–Mediated Epigenetic Reactivation of *LRIG1*

Reported epigenetic activation of LRIG1 using the CRISPR–dCas9–p300 system.*Previous studies have shown that a catalytically inactive Cas9 (dCas9) fused to the histone acetyltransferase domain of p300 can be targeted to the LRIG1 regulatory region using sequence-specific single guide RNAs. Recruitment of dCas9–p300 induces acetylation of histone H3 at lysine 27 (H3K27ac), a chromatin modification associated with active enhancers and promoters. This localized histone acetylation leads to chromatin relaxation, increased accessibility of transcriptional machinery, and reactivation of LRIG1 transcription, without altering the underlying DNA sequence.*

CRISPR interference (CRISPRi) enables controlled repression of LRIG1 to study its biological function

Previous experimental studies have demonstrated that CRISPR interference (CRISPRi) enables controlled repression of *LRIG1* expression to investigate its biological function. In these studies, catalytically inactive Cas9 (dCas9) was fused to epigenetic repressor domains and targeted to the *LRIG1* promoter using sequence-specific single guide RNAs. Targeting dCas9-KRAB to the *LRIG1* regulatory region induced heterochromatin formation through repressive histone modifications, resulting in transcriptional silencing of *LRIG1*. In parallel, recruitment of dCas9-DNMT3A to the *LRIG1* promoter promoted DNA methylation of CpG sites, leading to stable repression of *LRIG1* expression and mimicking cancer-associated epigenetic silencing. Together, these CRISPRi-based approaches demonstrate how locus-

specific epigenetic repression of *LRIG1* can be achieved to study its functional role in cancer biology . Previous studies have demonstrated that CRISPR interference (CRISPRi) can be used to repress *LRIG1* expression in a controlled manner to investigate its biological function. In these approaches, catalytically inactive Cas9 (dCas9) fused to the KRAB repression domain is targeted to the *LRIG1* promoter using sequence-specific single guide RNAs, resulting in heterochromatin formation and transcriptional silencing .Fig a. In parallel, dCas9 fused to the DNA methyltransferase DNMT3A induces DNA methylation at the *LRIG1* promoter, leading to stable repression that mimics cancer-associated epigenetic silencing Fig. b (Figure 11).

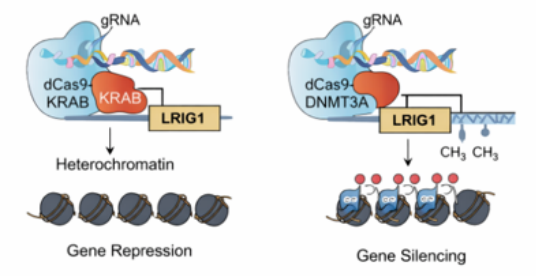


Fig. 11: CRISPR Interference–Mediated Epigenetic Repression of *LRIG1*

Reported CRISPR interference strategies for controlled repression of LRIG1. *Previous studies have demonstrated that CRISPR interference (CRISPRi) can be used to repress LRIG1 expression in a controlled manner to investigate its biological function. In these approaches, catalytically inactive Cas9 (dCas9) fused to the KRAB repression domain is targeted to the LRIG1 promoter using sequence-specific single guide RNAs, resulting in heterochromatin formation and transcriptional silencing .fig a. In parallel, dCas9 fused to the DNA methyltransferase DNMT3A induces DNA methylation at the LRIG1 promoter, leading to stable repression that mimics cancer-associated epigenetic silencing fig. b*

In summary, CRISPR-based epigenetic editing enables locus-specific and reversible regulation of *LRIG1* expression in TNBC by targeting promoter-associated epigenetic modifications. Guided recruitment of activating or repressive dCas9-effector complexes is predicted to modulate chromatin state and transcriptional output without altering DNA sequence, supporting the feasibility of epigenome editing as a precise regulatory strategy in subtype-specific breast cancer contexts.

Discussion

Epigenetic silencing of tumor suppressor genes is a hallmark of triple-negative breast cancer (TNBC) and contributes to its aggressive biological behaviour. In the present study, *LRIG1* emerged as a recurrently repressed gene in TNBC, consistent with its established role as a negative regulator of receptor tyrosine kinases (RTKs), including EGFR, ERBB2, and MET (Gur et al., 2004; Laederich et al., 2011). Reduced LRIG1 expression has been associated with increased proliferation, invasion, and poor clinical outcomes in breast cancer, particularly within basal-like and TNBC subtypes (Umeh-Garcia et al., 2022). Accumulating evidence indicates that this repression is largely driven by aberrant CpG island hypermethylation at the LRIG1 promoter rather than genetic alterations, highlighting DNA methylation as a key regulatory mechanism (Lindström et al., 2014).

The advent of CRISPR/dCas9-based epigenome editing technologies offers a precise and reversible approach to directly modulate LRIG1 expression at its endogenous locus. Catalytically inactive Cas9 fused to epigenetic effector domains enables locus-specific remodeling of chromatin without introducing DNA double-strand breaks (Gilbert et al., 2013). For example, fusion of dCas9 to the DNA demethylase TET1 has been shown to induce targeted promoter demethylation and transcriptional activation, whereas dCas9-DNMT3A or dCas9-KRAB fusions promote DNA methylation and heterochromatin formation, resulting in gene repression (Thakore et al., 2016; Liu et al., 2016). Applied to LRIG1, such strategies provide a mechanistically grounded framework for restoring tumour suppressor expression in TNBC by reversing epigenetic silencing.

Although no in vivo validation was performed in this study, previous investigations have demonstrated that CRISPR-dCas9 fusion systems can be efficiently delivered using viral vectors, such as adeno-associated virus, as well as non-viral nanoparticle-based platforms (Liao et al., 2015; Yin et al., 2017). Importantly, the biological performance of CRISPR-dCas9 systems is strongly influenced by the physical format of delivery. DNA-based delivery supports sustained expression, mRNA delivery offers transient and controlled activity, and ribonucleoprotein formats enable rapid yet highly specific epigenetic modulation (Kim et al., 2014; Glass et al., 2018). These considerations are particularly relevant for epigenetic therapies, where precise temporal control may mitigate off-target or long-term effects.

Collectively, this study integrates genomic and epigenomic evidence to support LRIG1 as a key epigenetically regulated tumour suppressor in TNBC. The findings further suggest that CRISPR-based epigenetic editing represents a promising strategy to restore LRIG1 expression and attenuate oncogenic RTK signalling pathways in aggressive breast cancer subtypes. Future experimental and translational studies will be essential to validate the therapeutic potential of LRIG1 epigenetic reactivation and to optimize delivery strategies for clinical application.

Conclusion

Epigenetic editing represents a powerful and reversible strategy for precise modulation of gene expression without introducing permanent alterations to the underlying DNA sequence. In triple-negative breast cancer (TNBC), where *LRIG1* is frequently transcriptionally silenced through promoter hypermethylation, CRISPR-based epigenome editing offers a targeted approach to restore gene activity by selectively modifying repressive epigenetic marks.

This strategy relies on the use of catalytically inactive Cas9 (dCas9) fused to defined epigenetic effector domains and guided to the *LRIG1* promoter by sequence-specific single-guide RNAs (sgRNAs). Recruitment of transcriptional activator complexes, such as dCas9-VP64 or dCas9-TET1, is predicted to enhance *LRIG1* expression by increasing chromatin accessibility and promoting DNA demethylation at the promoter region. Conversely, targeting repressive effectors such as dCas9-KRAB or dCas9-DNMT3A is expected to induce heterochromatin formation or de novo DNA methylation, resulting in transcriptional repression. These predicted regulatory outcomes are consistent with established mechanisms of CRISPR-mediated transcriptional and epigenetic control reported in prior studies (Gilbert et al., 2013; Thakore et al., 2016).

Collectively, this approach enables locus-specific "epigenetic editing" of *LRIG1* by modulating DNA methylation and histone states rather than altering genomic sequence, thereby offering a reversible and tunable method for gene regulation. The methodological framework is grounded in published genomic, epigenomic, and bioinformatic resources, integrating current knowledge of DNA methylation dynamics, CRISPR-based epigenome editing technologies, and subtype-specific regulatory features of breast cancer. Publicly available datasets, including The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO), were leveraged to contextualize *LRIG1* promoter methylation patterns and expression profiles in TNBC relative to other breast cancer subtypes. Together, these findings support the potential of CRISPR-mediated epigenetic editing as a rational and mechanistically informed strategy for restoring tumor suppressor gene expression in TNBC.

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