In this lesson:

CRISPR-CAS9 TECHNOLOGY APPLICATION
For one target or multiple targets

- GENOME EDITING
- RNA EDITING
- GENE REGULATION

The next generation of CRISPR–Cas technologies and applications

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Abstract | The prokaryote-derived CRISPR–Cas genome editing systems have transformed our ability to manipulate, detect, image and annotate specific DNA and RNA sequences in living cells of diverse species. The ease of use and robustness of this technology have revolutionized genome editing for research ranging from fundamental science to translational medicine. Initial successes have inspired efforts to discover new systems for targeting and manipulating nucleic acids, including those from Cas9, Cas12, Cascade and Cas13 orthologues. Genome editing by CRISPR–Cas can utilize non-homologous end joining and homology-directed repair for DNA repair, as well as single-base editing enzymes. In addition to targeting DNA, CRISPR–Cas-based RNA-targeting tools are being developed for research, medicine and diagnostics. Nuclease-inactive and RNA-targeting Cas proteins have been fused to a plethora of effector proteins to regulate gene expression, epigenetic modifications and chromatin interactions. Collectively, the new advances are considerably improving our understanding of biological processes and are propelling CRISPR–Cas-based tools towards clinical use in gene and cell therapies.

Brief history of CRISPR-Cas9 system discovery

https://youtu.be/SuAxDVBt7kQ

Molecular mechanisms

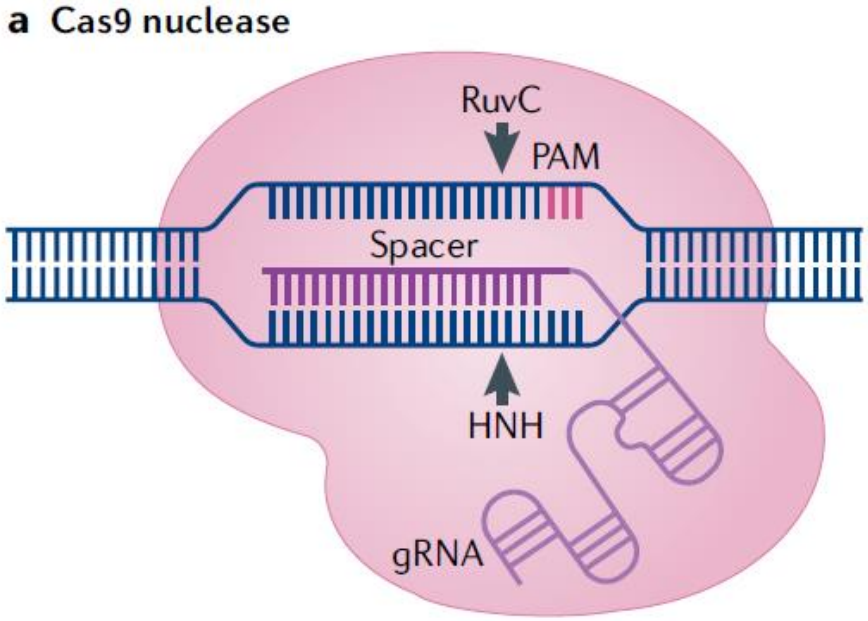
https://youtu.be/4YKFw2KZA5o

All CRISPR–Cas systems rely on CRISPR RNA (crRNA) or, in experimental CRISPR–Cas9 systems, on the guide RNA (gRNA) for guidance and targeting specificity.

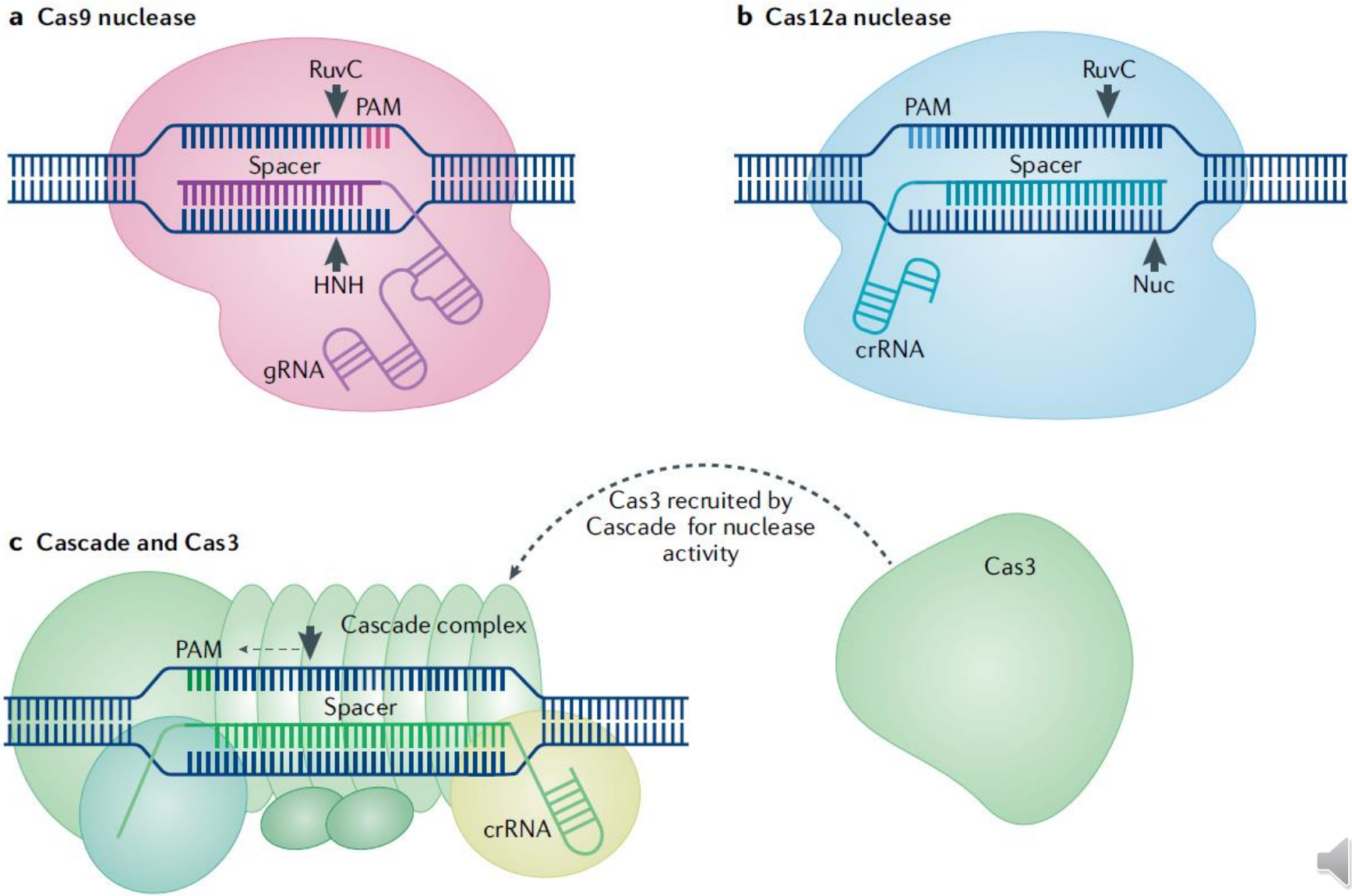
Following hybridization of the spacer part of the crRNA to a target sequence that is positioned next to a **protospacer adjacent motif (PAM)**, the Cas cleaves the target nucleic acid.

Site-specific cleavage at any locus containing a PAM can be achieved by retargeting CRISPR–Cas systems with designed crRNAs containing appropriate spacer sequences

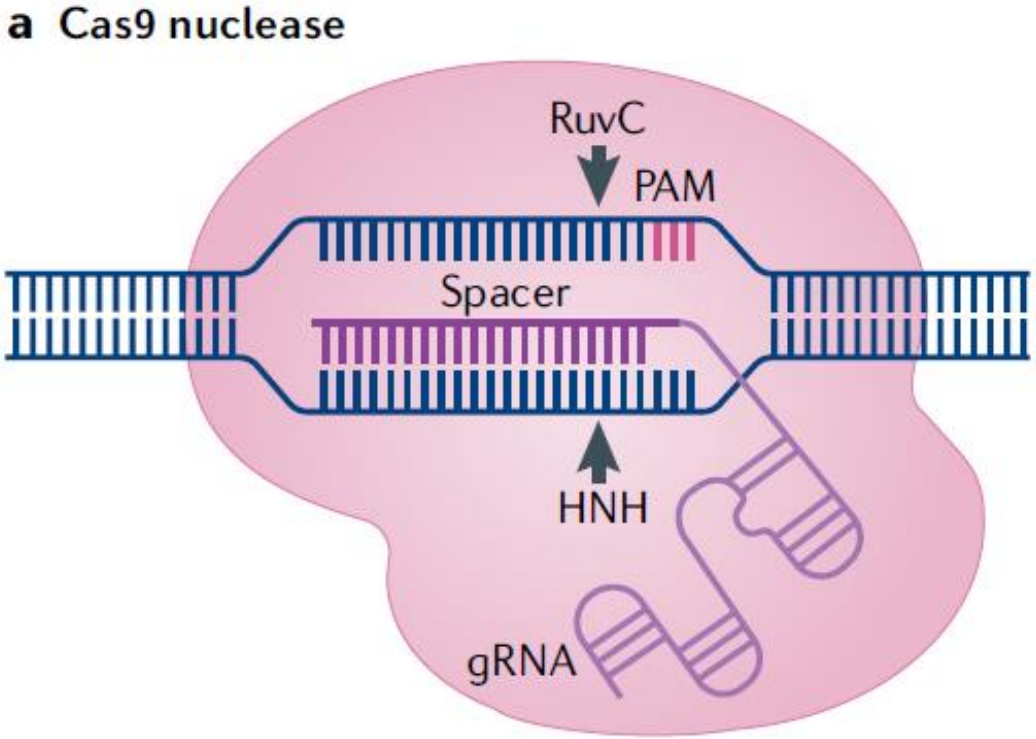
RuvC and HNH are two domains of Cas9



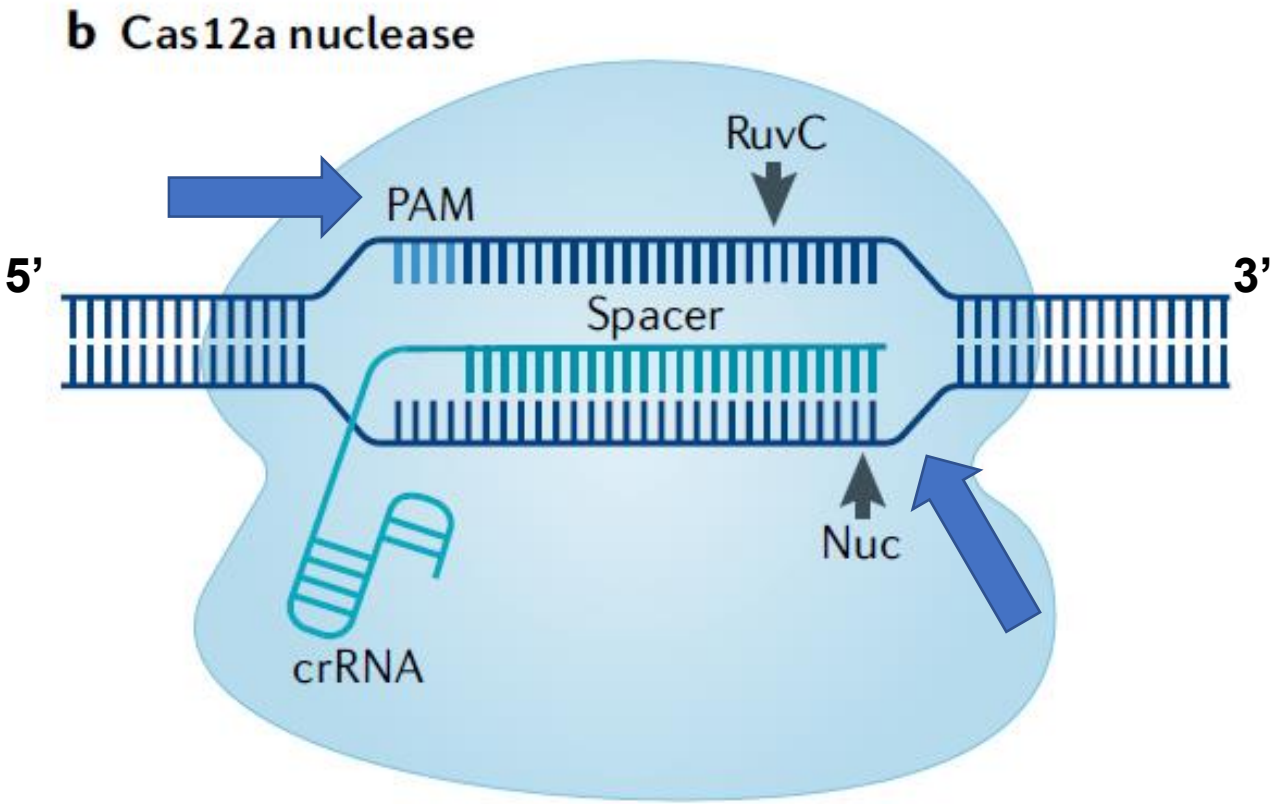
Overview of the main CRISPR–Cas gene editing tools



In engineered CRISPR–Cas9 systems, **Cas9 interacts** with the backbone of the guide RNA (gRNA). Complementary pairing of the spacer portion of the gRNA to a DNA target sequence **positioned** next to a 5‘ protospacer adjacent motif (**PAM**) results in generation of a blunt DNA double-strand break by the two Cas9 nuclease domains, RuvC and HNH



Cas12a nucleases recognize DNA target sequences with complementarity to the CRISPR RNA (crRNA) spacer positioned next to a **3' PAM**. Target recognition results in the generation of a staggered DNA double-strand break by a RuvC domain and a putative nuclease (Nuc) domain



Mechanisms and uses of gene editing

Mechanisms and uses of gene editing

Cas9 induces the DNA clevage and DNA break is **repair** by various endogenous **mechanisms**:

Non-homologous end joining versus **homology-directed repair**.

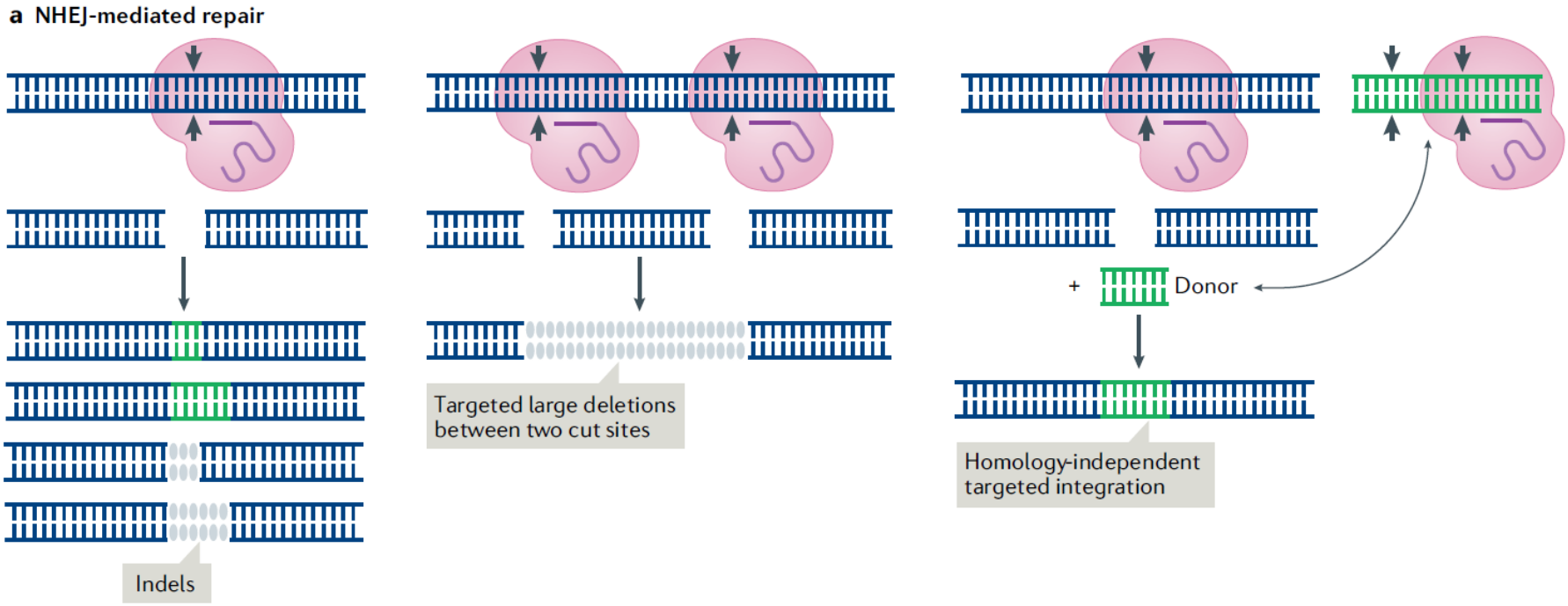
Gene Deletions

Gene Insertions

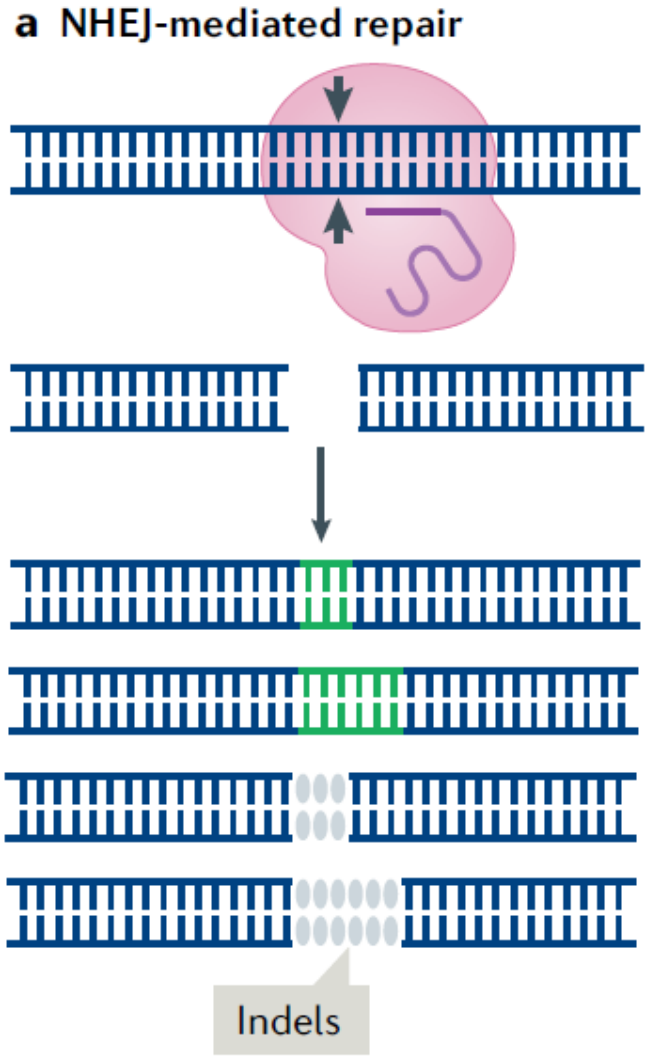
Translocations

Single-base editing

Eukaryotes predominantly repair DSBs through the error-prone **non-homologous end joining (NHEJ) pathway**, which leads to accumulation of small insertions or deletions (indels) following repeated cycles of break and repair



Non-homologous end joining (NHEJ) mediated repair



INSERTION

DELETION

Non-homologous end joining (NHEJ) mediated repair

NHEJ is referred to as "non-homologous" because the break ends are directly ligated without the need for a homologous template.

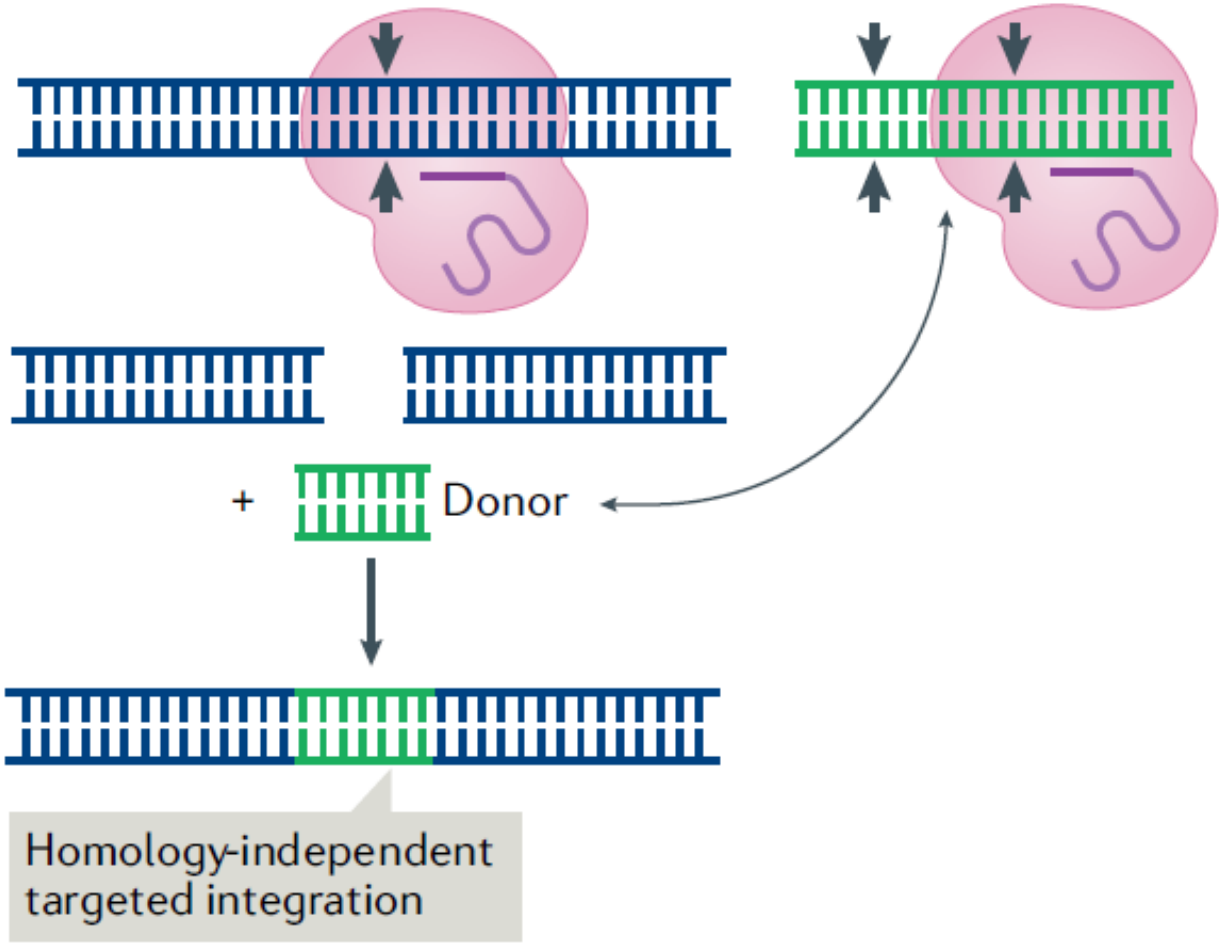
NHEJ typically utilizes **short homologous DNA sequences** called microhomologies to guide repair. These microhomologies are often present in single-stranded overhangs on the ends of double-strand breaks. When the overhangs are perfectly compatible, NHEJ usually repairs the break accurately.

Biological process and disease

Imprecise repair leading to loss of nucleotides can also occur, but is much more common when the overhangs are not compatible. Inappropriate NHEJ can lead to translocations and telomere fusion, hallmarks of tumor cells.

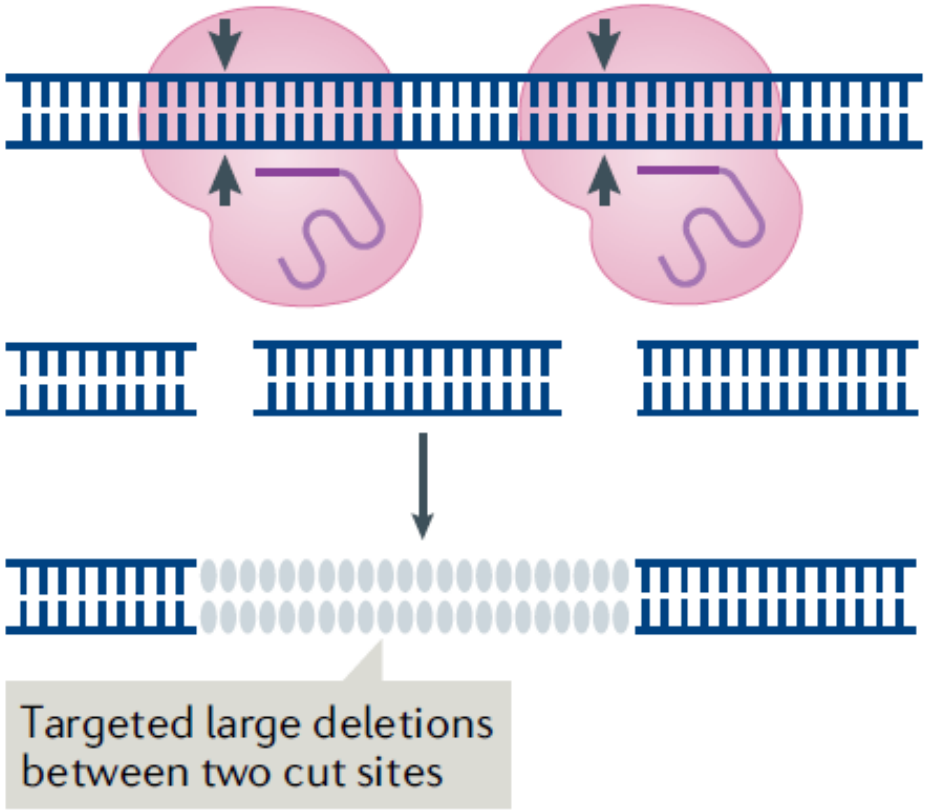
INSERTION OF GENOMIC REGION

Homology-independent targeted integrations can be directed to a single cut site by providing donor DNA that is independently targeted for cutting



DELETION OF GENOMIC REGION

Two RNAguides cut in two points and the repair between two double-strand breaks produced by simultaneous targeting of nucleases to two genomic sites.



The **homology-directed repair (HDR)** pathway can be used for genome editing and solves two problems:

- Generation of indels: unknown sequence

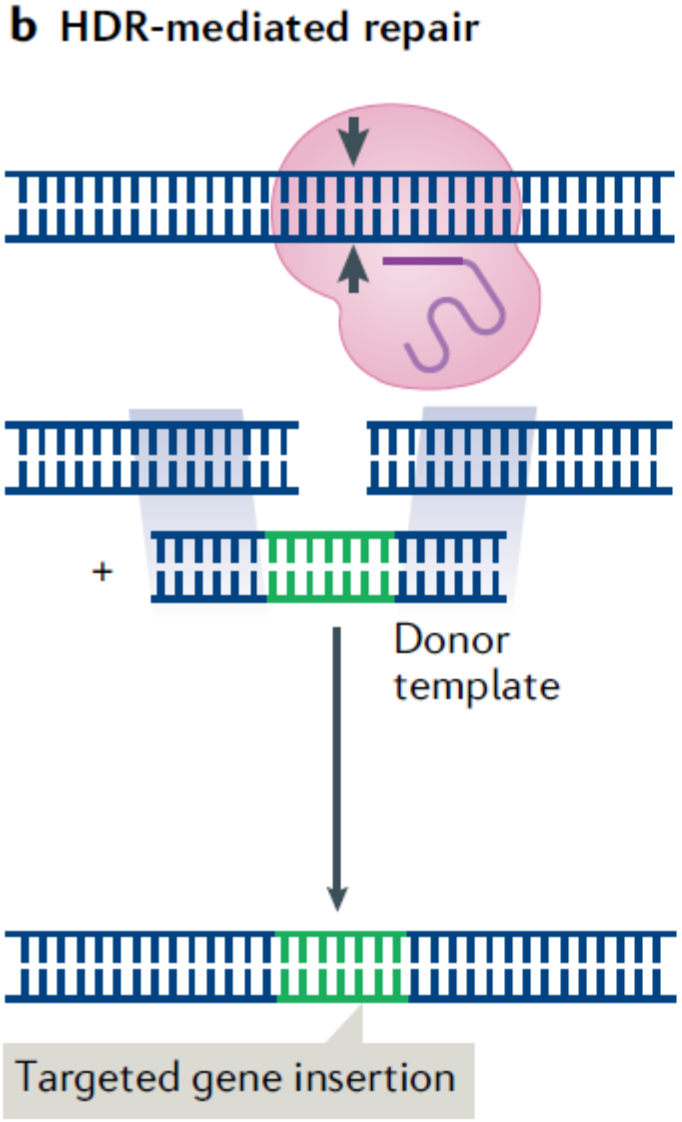
- Donor integration in random orientation

Application for:

- Mice models with missense gain-of-function mutations

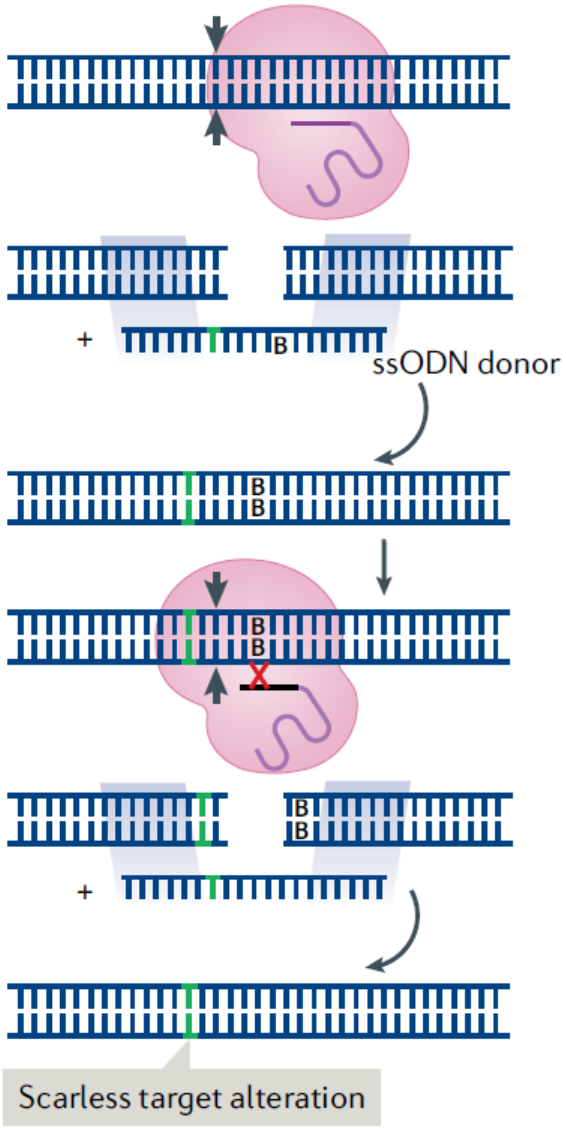
- Conditional knockout mice at high efficiency by insertion of large regulatable cassettes

The **homology-directed repair (HDR)** pathway can be used for genome editing by providing double-stranded donor templates that contain homology arms (grey rectangles) to the cut target site.



The homology-directed repair (HDR)

single-stranded oligodeoxynucleotide (ssODN) donor templates that contain homology arms (grey rectangles) to the cut target site.



Single-nucleotide alterations or insertion of larger sequences can be mediated by introducing variations into the donor template, which may also consist of plasmid DNA, viral DNA or long single-stranded DNA.

Silent mutations — also referred to as blocking mutations (B) — that prevent subsequent target site recognition by the nucleases and formation of NHEJ-mediated indels can be incorporated into the donor template along with the intended alterations

EDITING SINGLE NUCLEOTIDE VARIANTS

The most common genetic variants associated with human disease are point mutations. An ability to edit single nucleotide bases is important for the creation of genetic disease models and the development.

Targeted HDR-mediated single-base editing can be achieved by co-delivery of Cas9 and a homologous donor sequence that contains the edited nucleotide of choice.

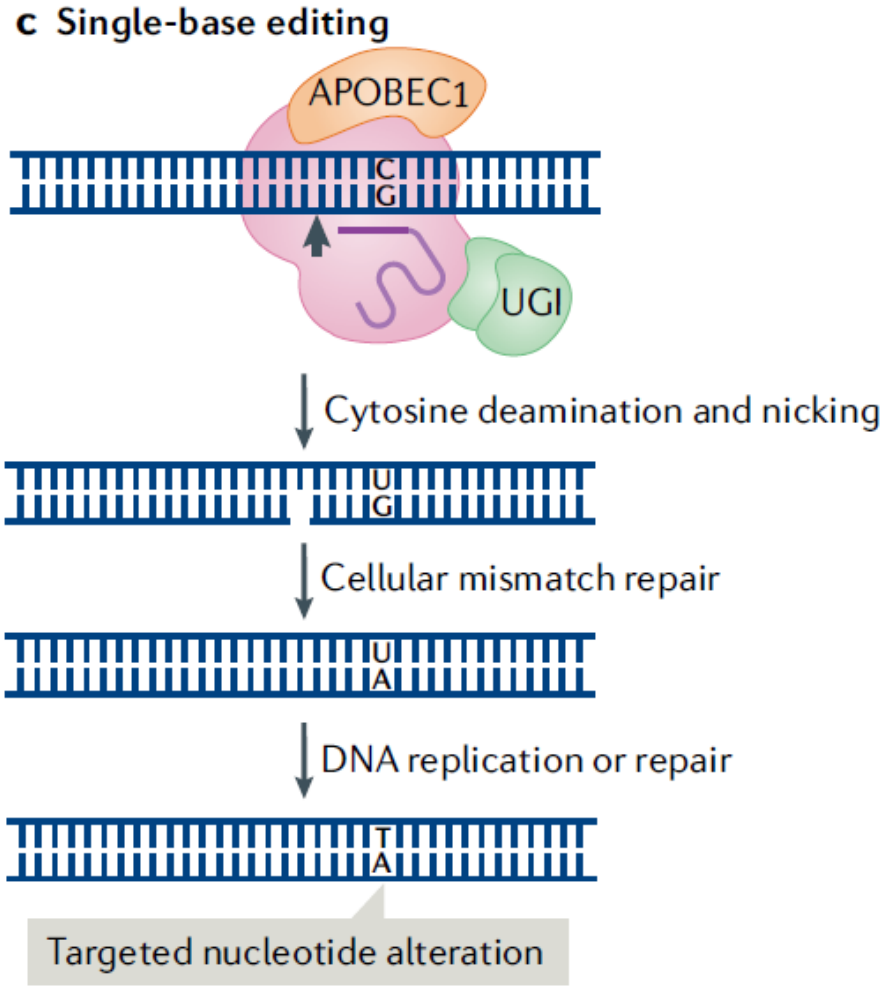
- INEFFICIENT STRATEGIES in postmitotic cells with decreased HDR activity.

- off-target mutagenesis,

- adverse effects on cell viability on-target activation of DNA repair pathways

EDITING SINGLE NUCLEOTIDE VARIANTS

For single-nucleotide C→T (or G→A) conversion, Cas9 nickase has been fused to **cytidine deaminases such as APOBEC1**. For increased base-editing efficiency , two **uracil glycosylase inhibitors (UGIs)** have been used to a base editor for prevention of cellular base excision repair.



Genome-wide pooled screens using CRISPR–Cas-based tools

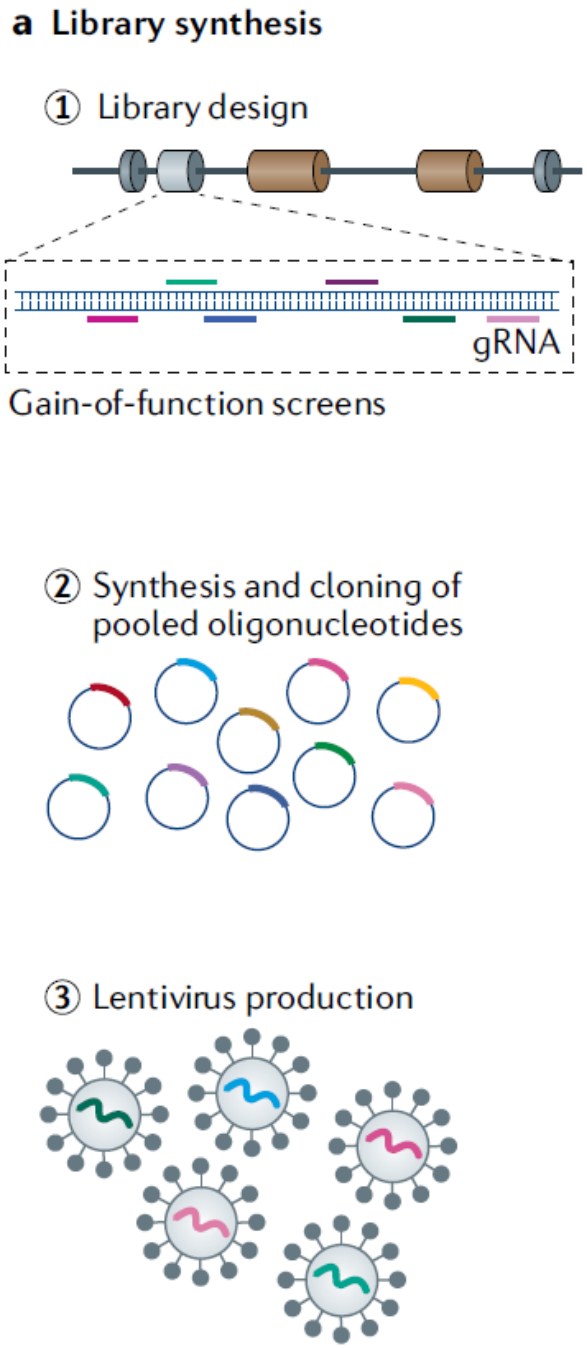
Genome-wide pooled screens using CRISPR–Cas-based tools

Genome-scale targeting of Cas9 is possible with synthesis of a guide RNA (gRNA) library.

Application.

- Screen the effect of mutation in the loss of function

- Screen the effect on gene regulation: accessible chromatin and transcription factor motifs

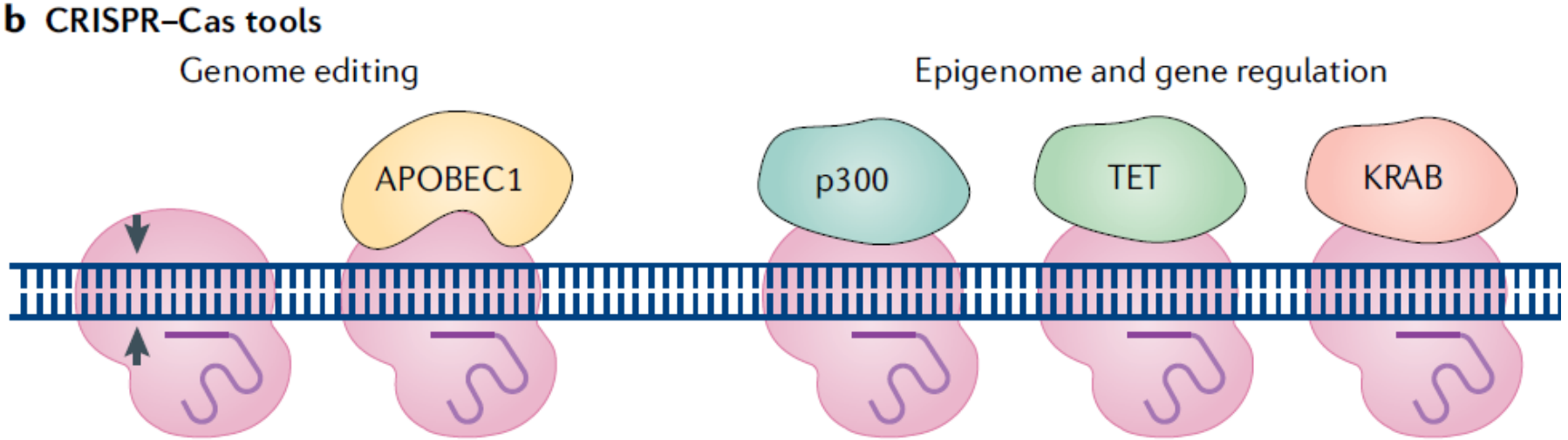


RNA GUIDE LIBRARY SYNTHESIS

The RNA guide libraries are generated by synthesizing pools of oligonucleotides, cloning them into plasmids and producing a lentivirus library that encodes the gRNAs

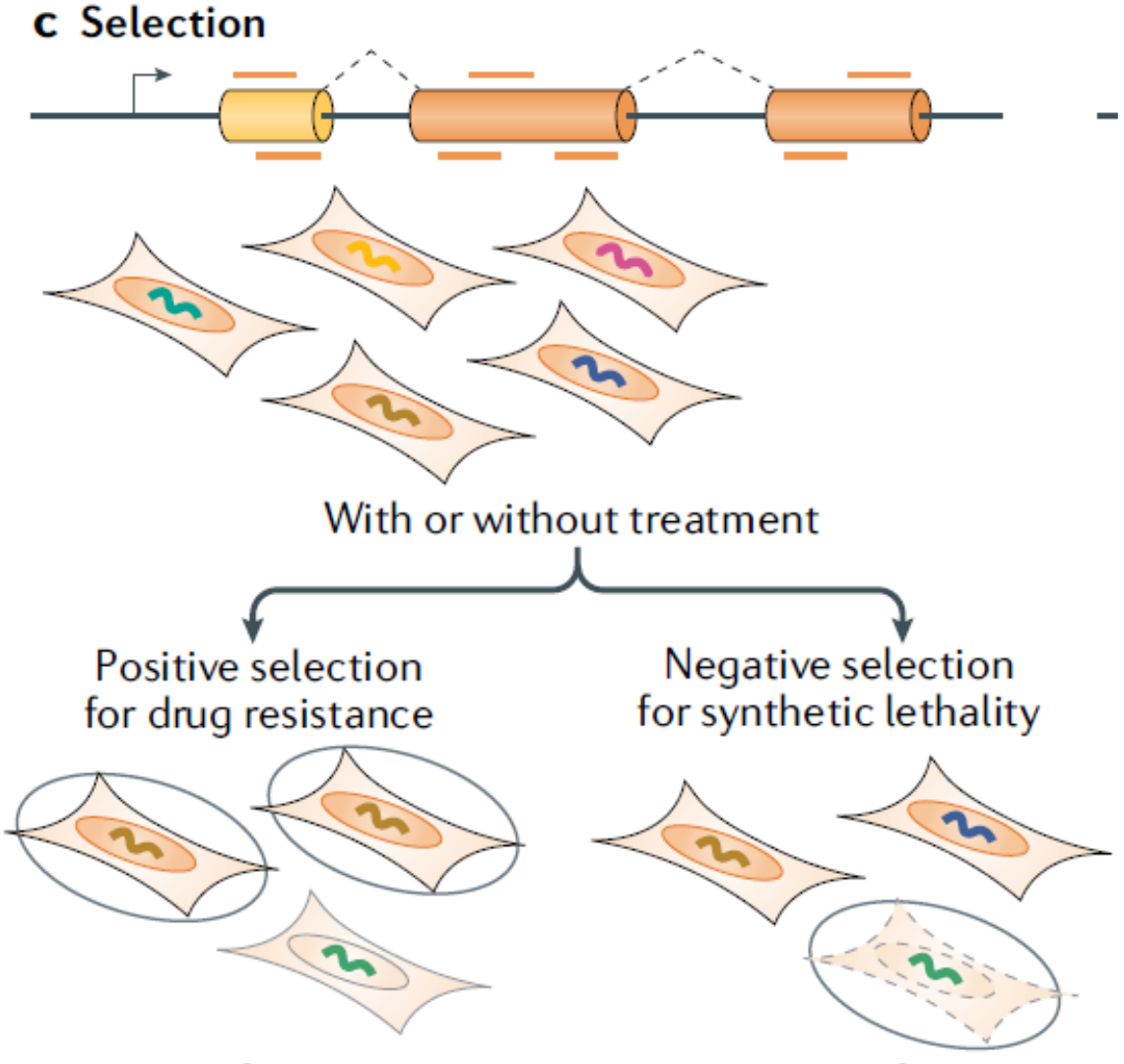
EPIGENOME AND GENE REGULATION

Fusion of Cas9 nickase or catalytically deficient Cas9 to different effector proteins can allow genome editing (for example, by the cytidine deaminase APOBEC1) or epigenome and gene regulation (for example, histone acetylation by p300, DNA demethylation by TET dioxygenases or transcription repression by Krüppel-associated box (KRAB) domains



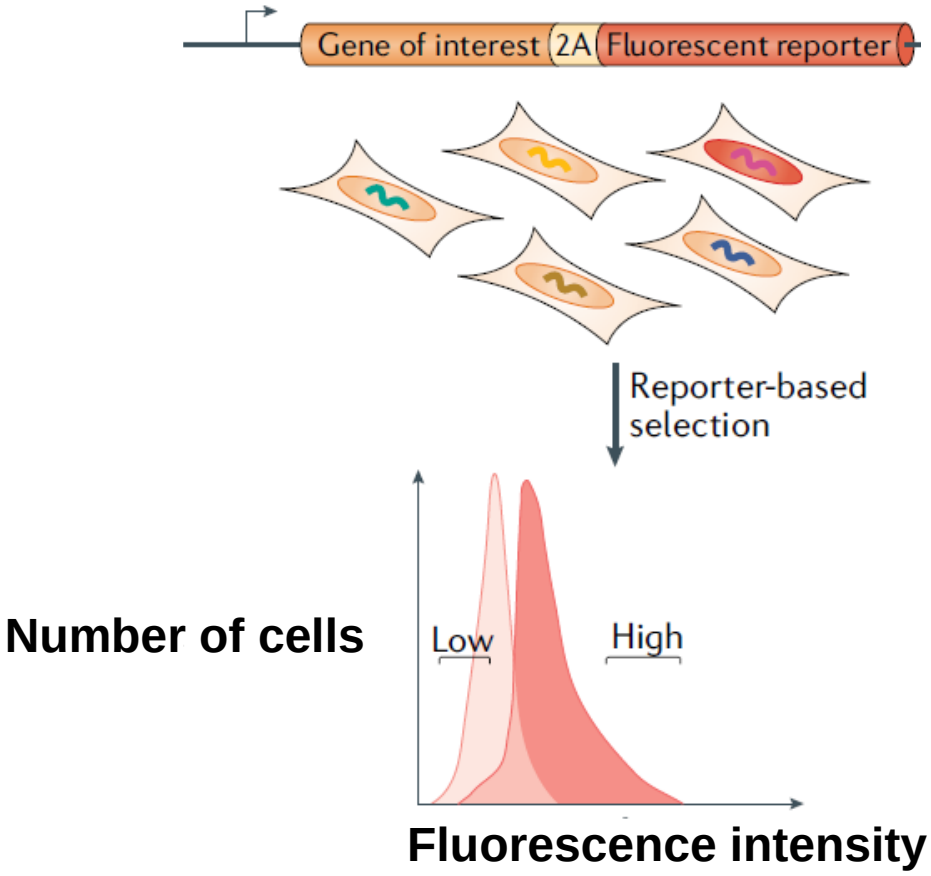
STUDY OF BIOLOGICAL FUNCTION AND RESPONSE TO DRUG

RNA GUIDE targeting a specific set of genes shows an effect as drug resistance or role in the survival.



GENE EXPRESSION AND REGULATION

Gene regulatory elements can be identified by selecting cells with altered gene expression either through direct immunofluorescence staining or through tagging an endogenous gene with a reporter. By selecting cells with low or high reporter expression, factors that affect gene expression can be identified.



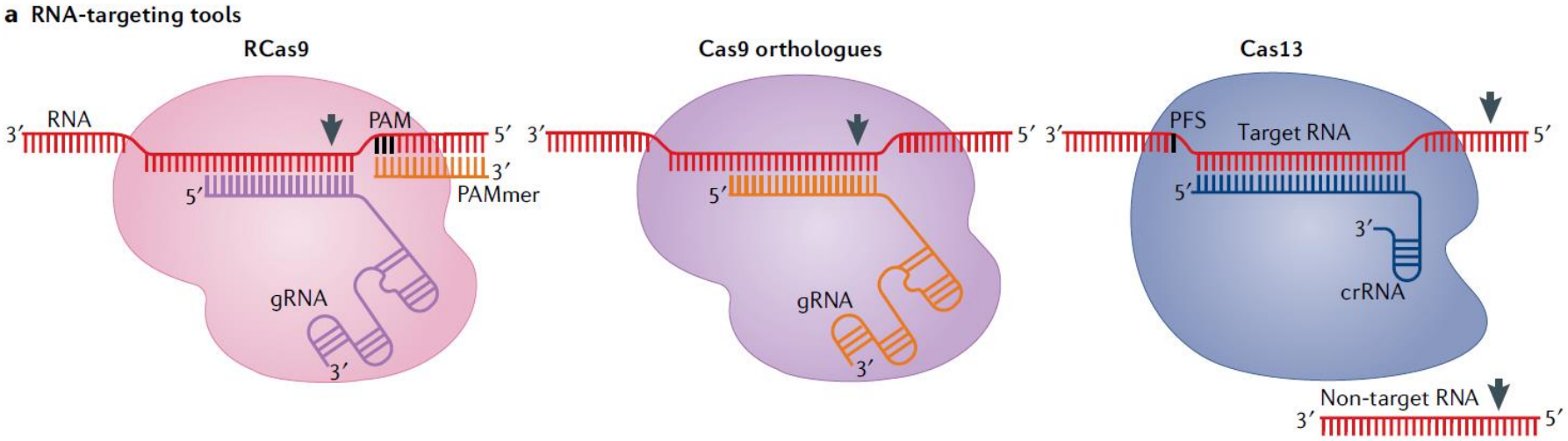
A wide range of applications is possible with CRISPR-based screens

- Next-generation sequencing and bioinformatics are used to compare the unselected gRNA library with the selected gRNA library and identify enriched and depleted gRNAs and thus specific genomic loci.
- Interrogation of gene function can identify genes involved in cell survival and proliferation or cancer genes
- drug targets can be identified on the basis of resistance or sensitivity to drugs, toxins or pathogens
- mapping the function of the non-coding genome by perturbing enhancer sequences
- modulating particular sets of genes; for example, targeted activation of all transcription factor genes to identify factors involved in stem cell differentiation
- Single Cell Analysis

CRISPR–Cas targeting RNA

the development of CRISPR–Cas technology for binding or cleaving specific RNAs has advanced RNA manipulation in living cells

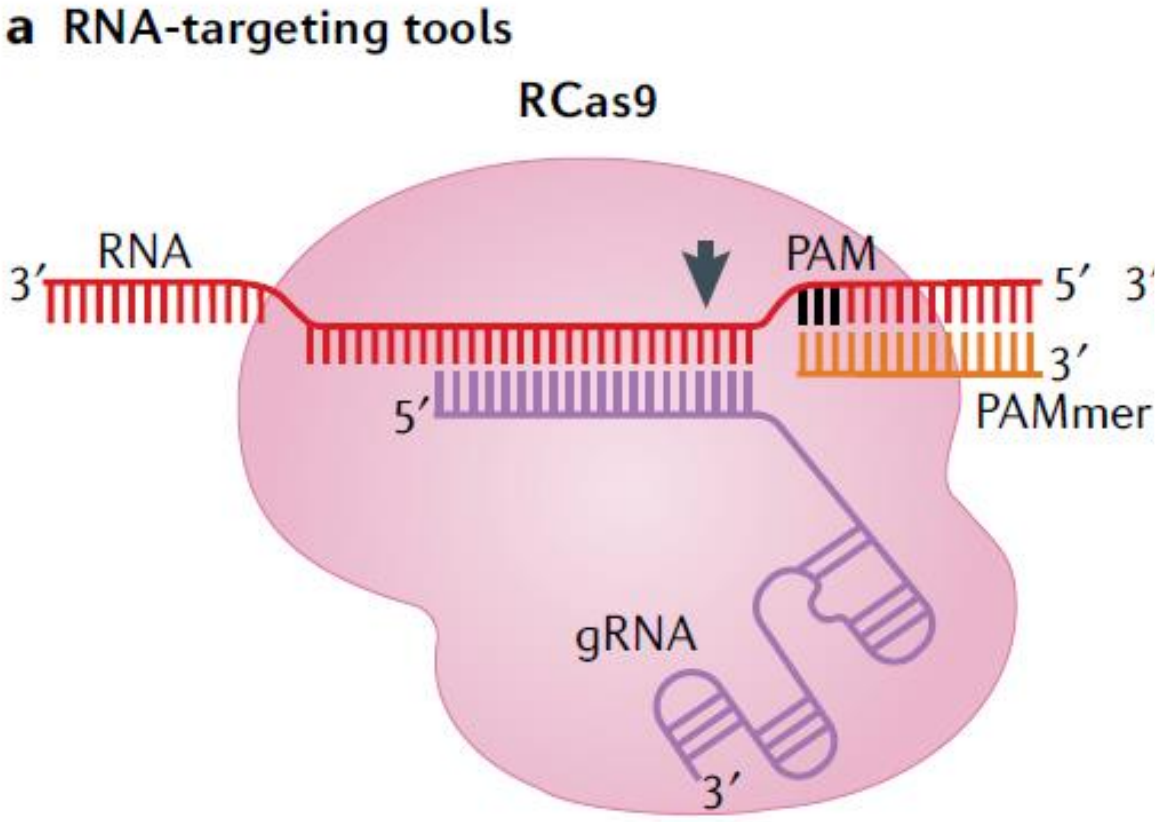
Cas9 in other species
That are specific for RNA



RNA guide specific for RNA recognition

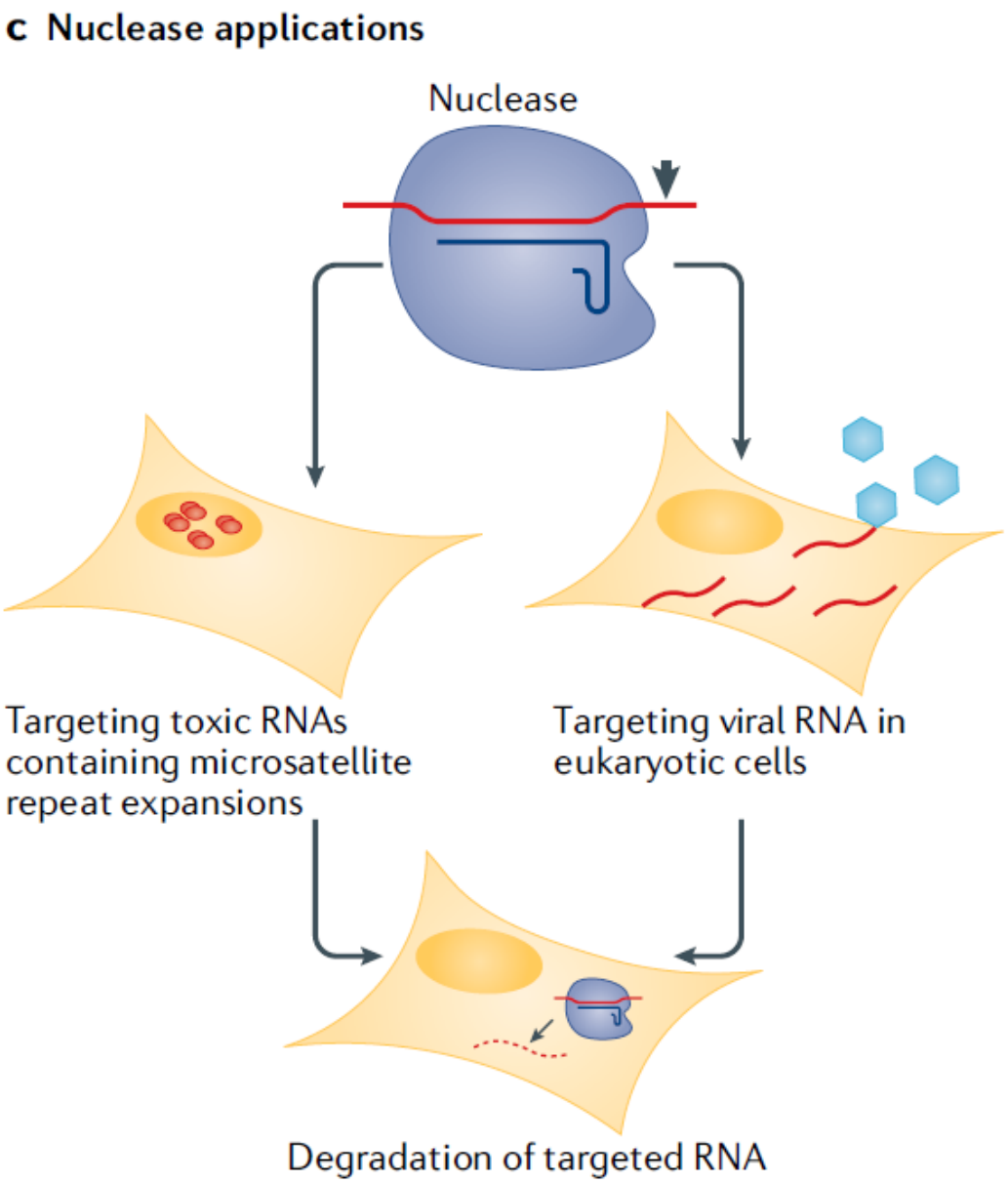
Cas13a (formerly known as C2c2) is an RNA-guided RNA-targeting nuclease

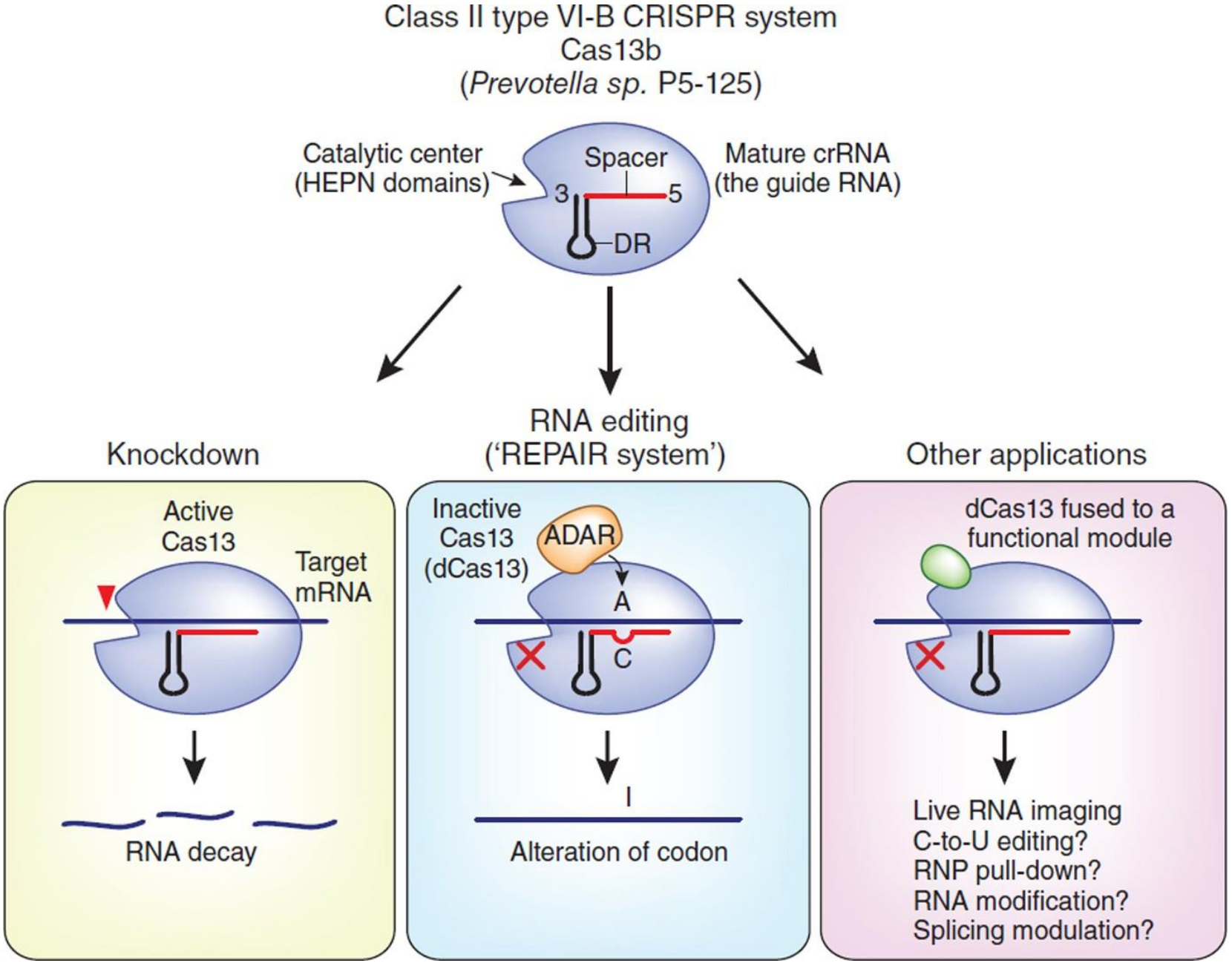
Oligonucleotide with a PAM sequence is used to direct Cas9 to single-stranded RNA target and are design for RNA sequences avoiding corresponding genomic DNA sites.



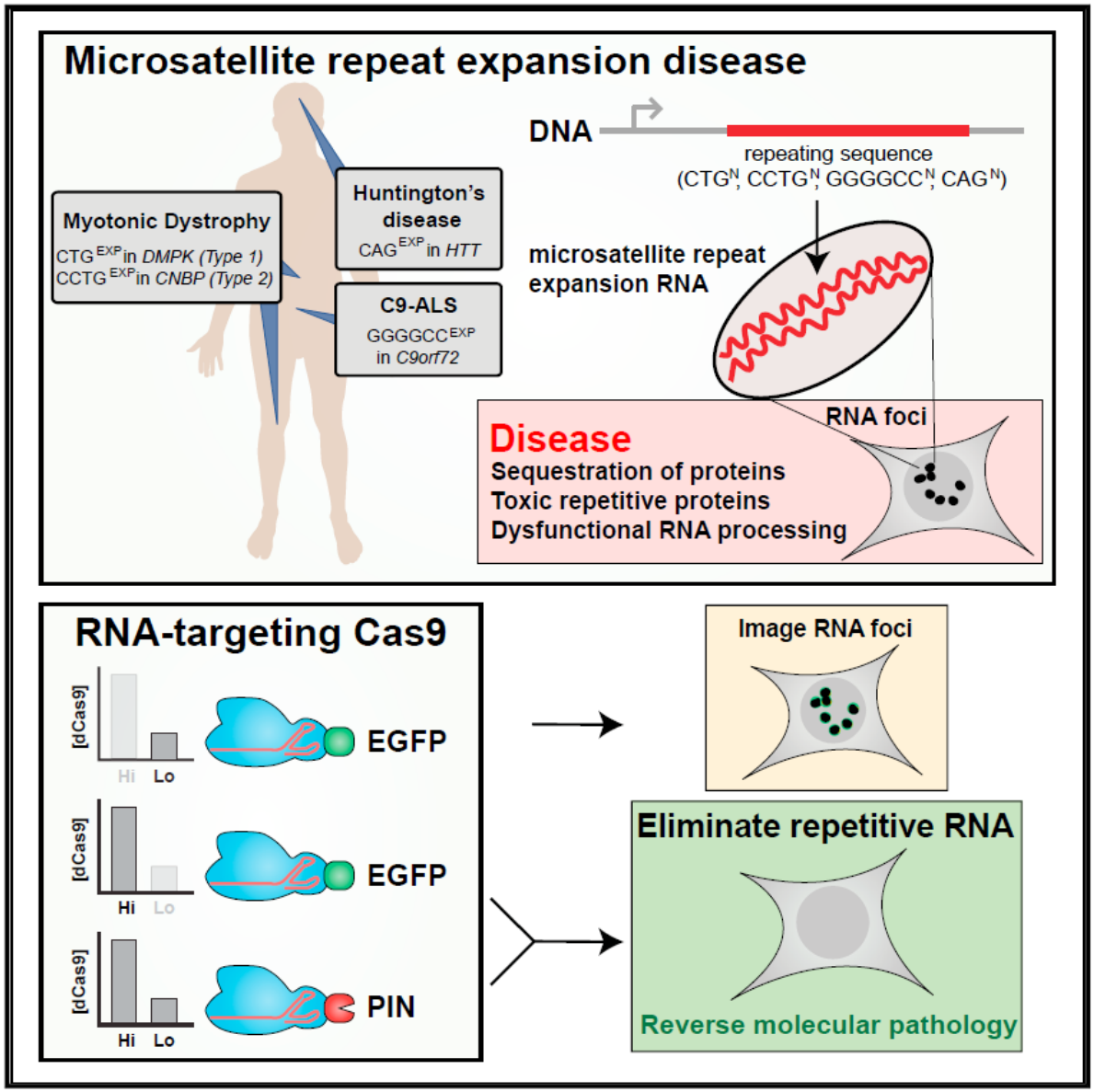
Cas13a (formerly known as C2c2)
is an RNA-guided RNA-targeting nuclease

Cas13 can be used for targeted RNA degradation in eukaryotic cells for applications such as targeting viral RNA or toxic RNAs

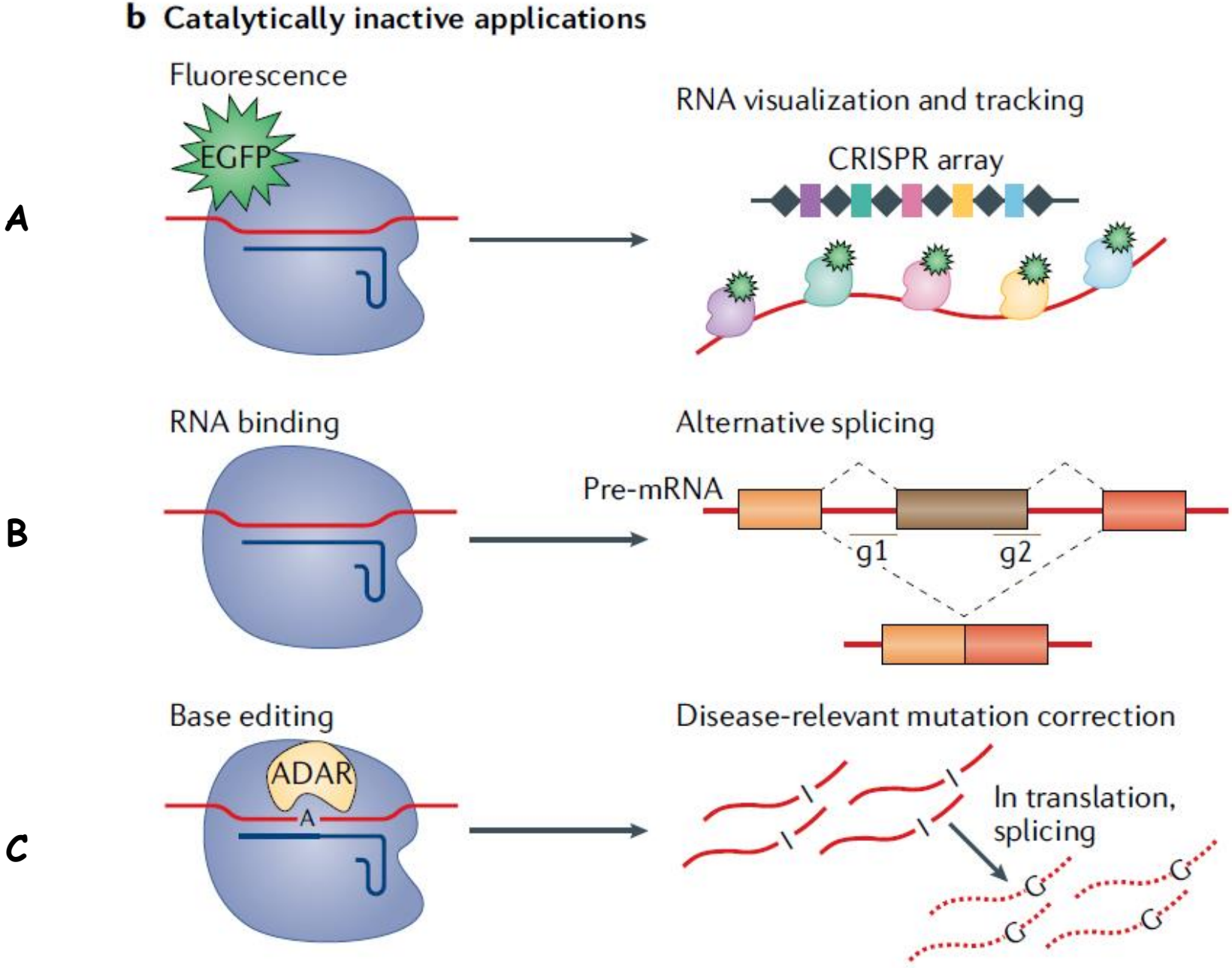



Class II type VI-B CRISPR system
Cas13b
(*Prevotella sp.* P5-125)
Catalytic center
(HEPN domains)
Spacer
3
5
DR
Mature crRNA
(the guide RNA)
Knockdown
Active
Cas13
Target
mRNA
RNA decay
RNA editing
('REPAIR system')
Inactive
Cas13
(dCas13)
ADAR
A
C
I
Alteration of codon
Other applications
dCas13 fused to a
functional module
Live RNA imaging
C-to-U editing?
RNP pull-down?
RNA modification?
Splicing modulation?

APPLICATION OF RNA-TARGETING CAS9



Batra et al., 2017


b Catalytically inactive applications
A
Fluorescence
EGFP
RNA visualization and tracking
CRISPR array
B
RNA binding
Alternative splicing
Pre-mRNA
g1
g2
C
Base editing
ADAR
A
Disease-relevant mutation correction
In translation, splicing
G
G
G
G

Catalytically deficient Cas13 maintains the capacity to bind to the targeted RNA

A For RNA visualization and tracking purposes, a fluorescent protein can be fused to the catalytically deficient Cas and colocalize with an array of CRISPR RNAs (crRNAs) or gRNAs.

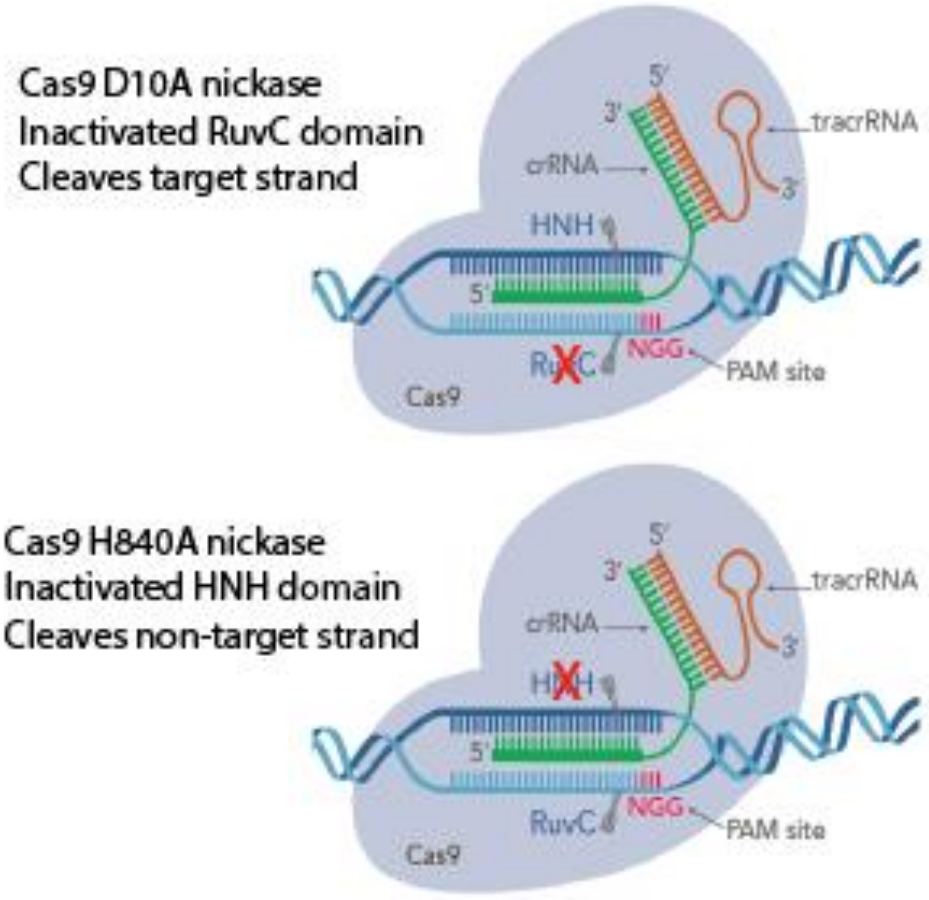
B To promote alternative splicing, catalytically deficient Cas13 can be targeted with gRNAs (g1 and g2) to bind splicing regulating *cis* elements

C Adenosine deaminase acting on RNA (ADAR) can be fused to catalytically deficient Cas for RNA A→I base editing to correct disease-relevant mutations.

Gene regulation by CRISPR-Cas

CATALYTIC DOMAINS INACTIVATION

Mutations in the RuvC (D10A) and HNH (H840A) nuclease domains destroy the catalytic activity of Cas9 while maintaining its RNA-guided DNA-targeting capacity. The CRISPR–Cas toolbox has been expanded by **fusion of this dCas9 with diverse effectors** such as transcription repressors or activators, epigenetic modifiers and fluorophores

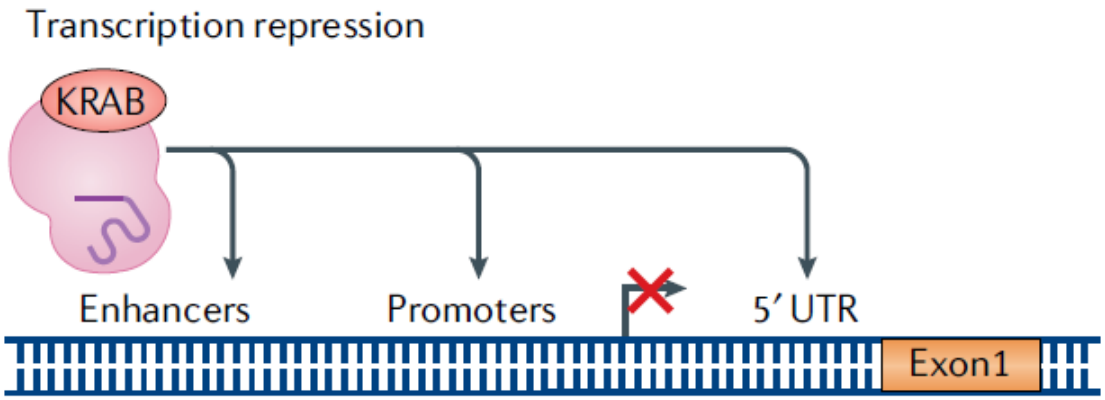


CRISPR INTERFENCE

dCas9 was tethered to transcription repressor domains such as that of **Krüppel-associated box (KRAB),** which is found in many natural zinc finger transcription factors. **KRAB is known to induce heterochromatin formation,** and changes in chromatin structure often accompany dCas9- KRAB-targeted transcription repression.

dCas9–KRAB is a robust tool in mammalian cells that can effectively silence single genes and non-coding RNAs by targeting promoter regions, 5′ untranslated regions and proximal and distal enhancer elements.

For improved repressive capabilities, dCas9 was fused to a bipartite repressor consisting of the transcription repression domains of KRAB and of methyl-CpGbinding protein 2. The versatility of dCas9–KRAB is highlighted by its capacity to repress transcription by targeting both genes and gene-regulatory regions.



CRISPR ACTIVATION

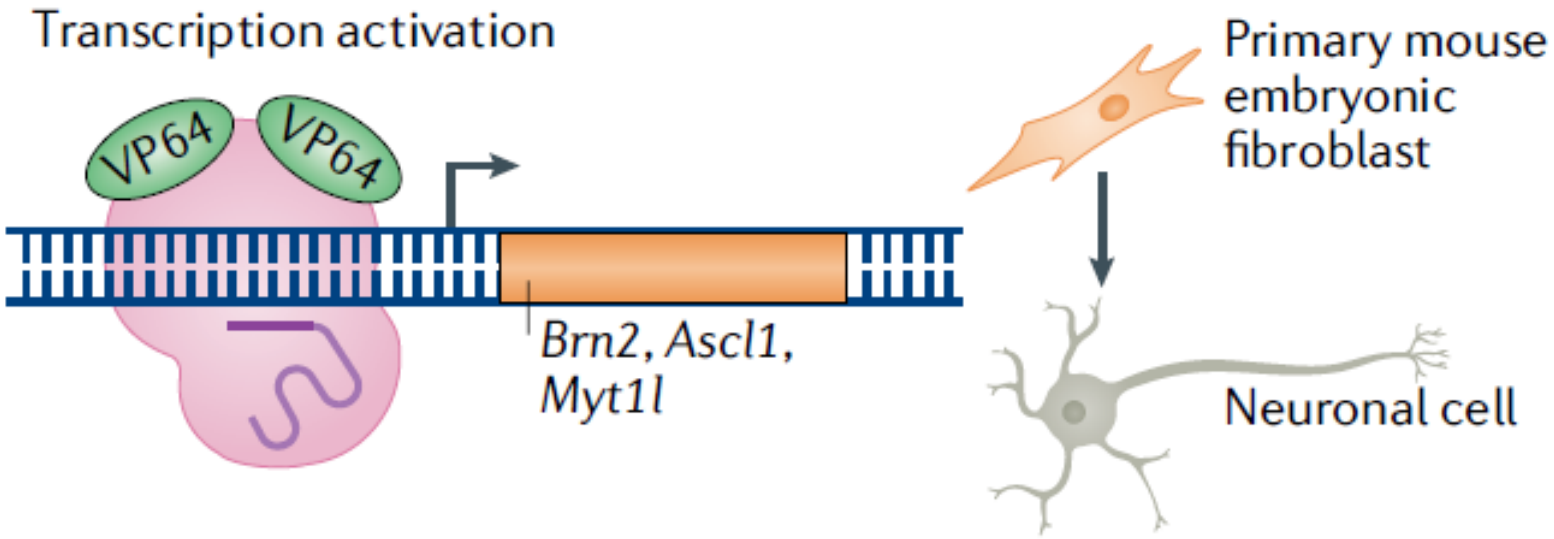
In eukaryotes, both reporter genes and endogenous genes can be activated by dCas9 **fused to the transcription activation domains** of the nuclear factor-κB transactivating subunit (p65) or to VP64 (four repeats of the herpes simplex VP16 activation domain).

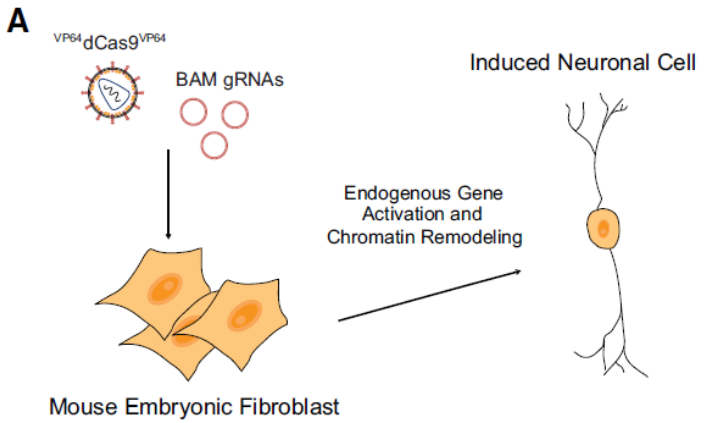
Synergistic gene activation has frequently been observed with these synthetic transcription factors by **targeting multiple gRNAs** to a promoter region.

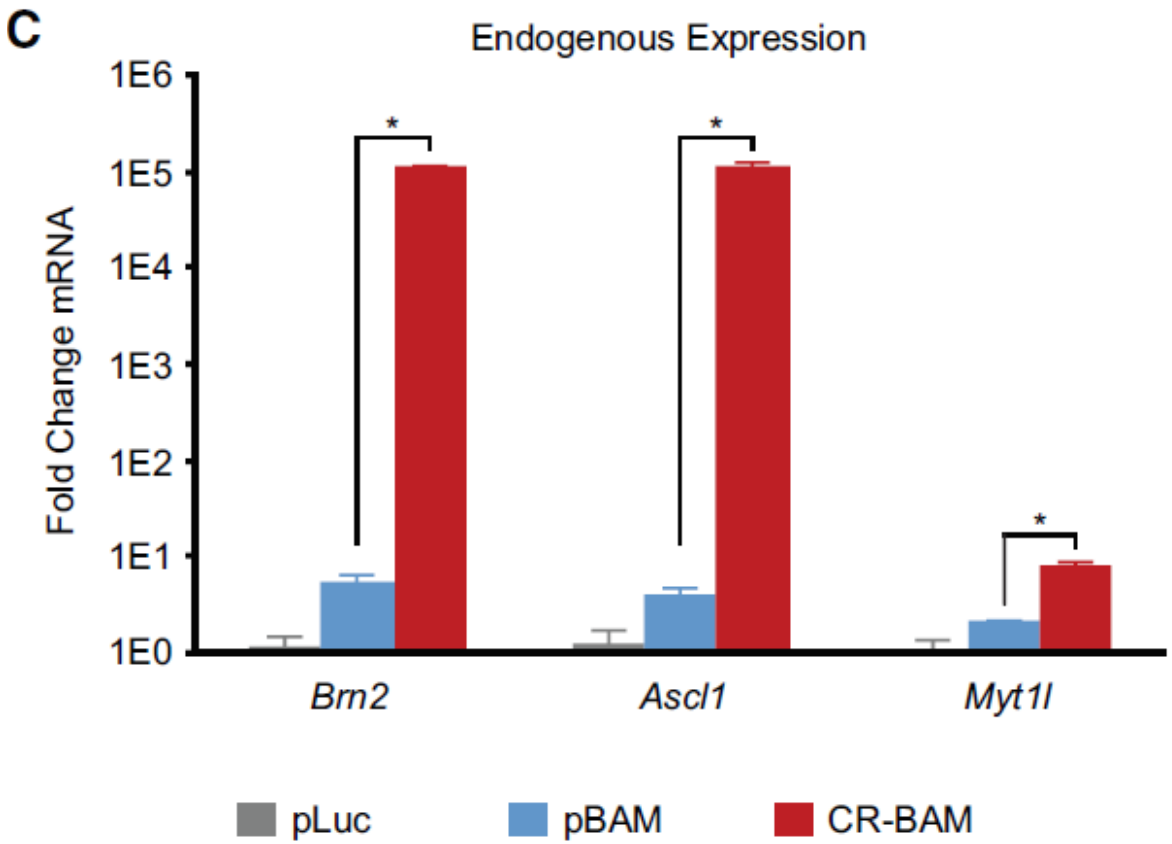
Synergy can be achieved by **combining different activator domains**.

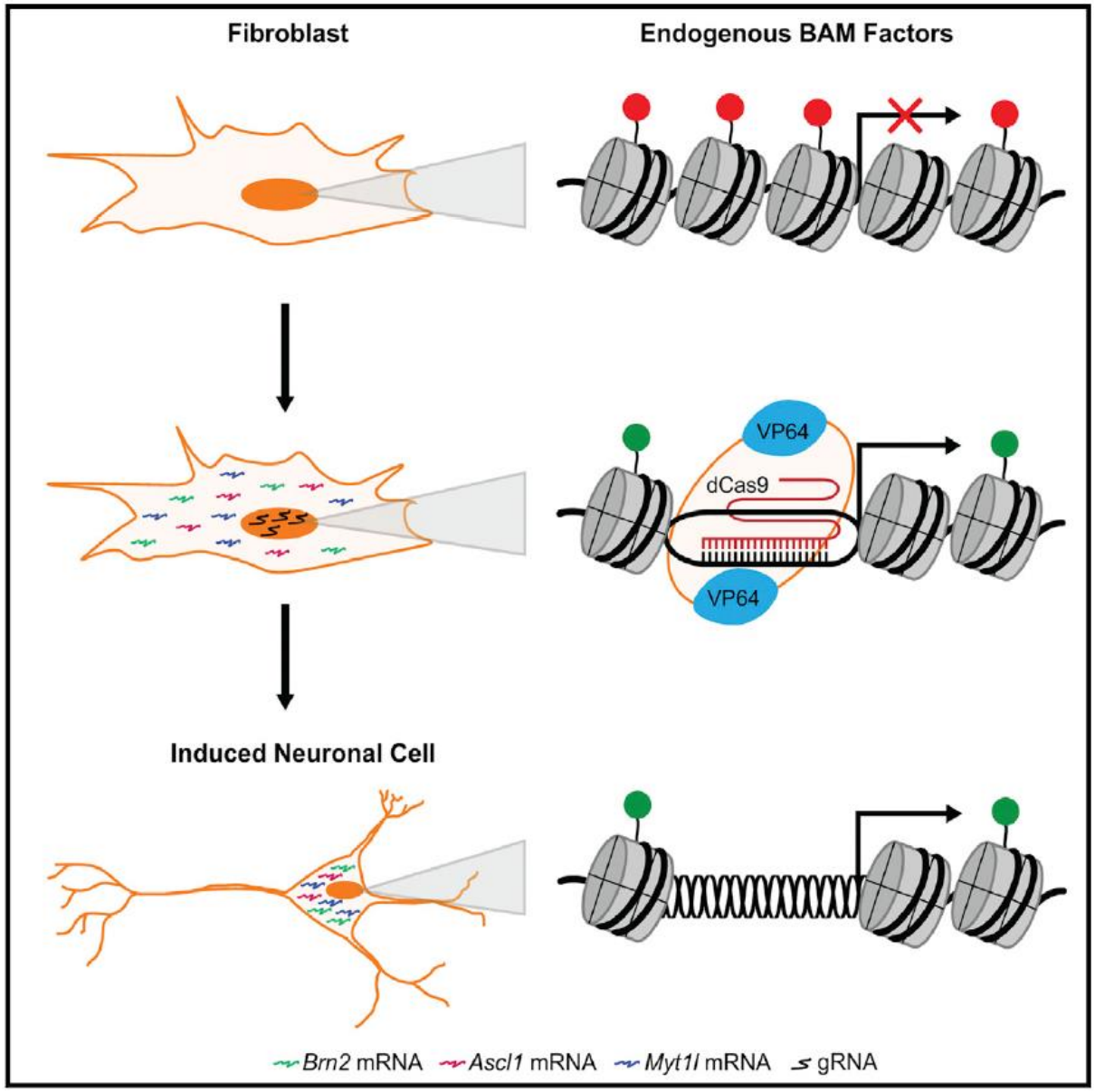
Multiplexed activation of endogenous genes can also be used for cellular reprogramming.

Transcription activation can be targeted by fusion of dCas9 to transcription activation domains such as VP64: VP64–dCas9–VP64 activated the expression of the neuronal transcription factor genes *Brn2*, *Ascl1* and *Myt1l* and thus directed the conversion of primary mouse embryonic fibroblasts into neuronal cells.

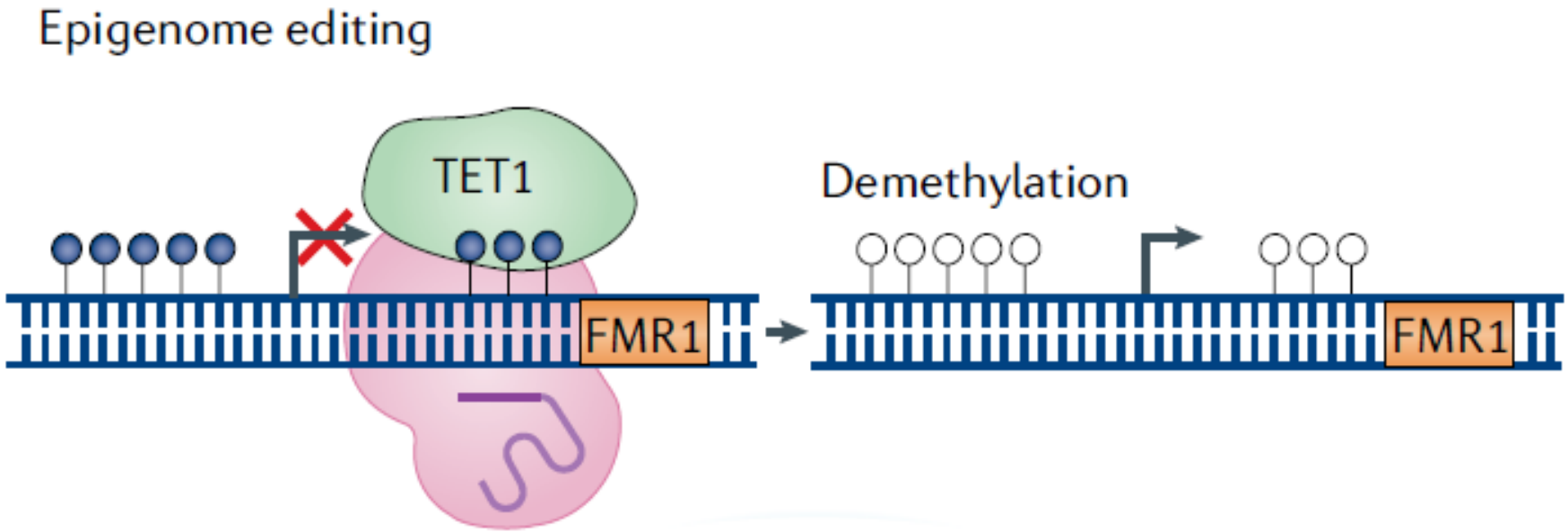



A
VP64dCas9VP64
BAM gRNAs
Induced Neuronal Cell
Endogenous Gene
Activation and
Chromatin Remodeling
Mouse Embryonic Fibroblast

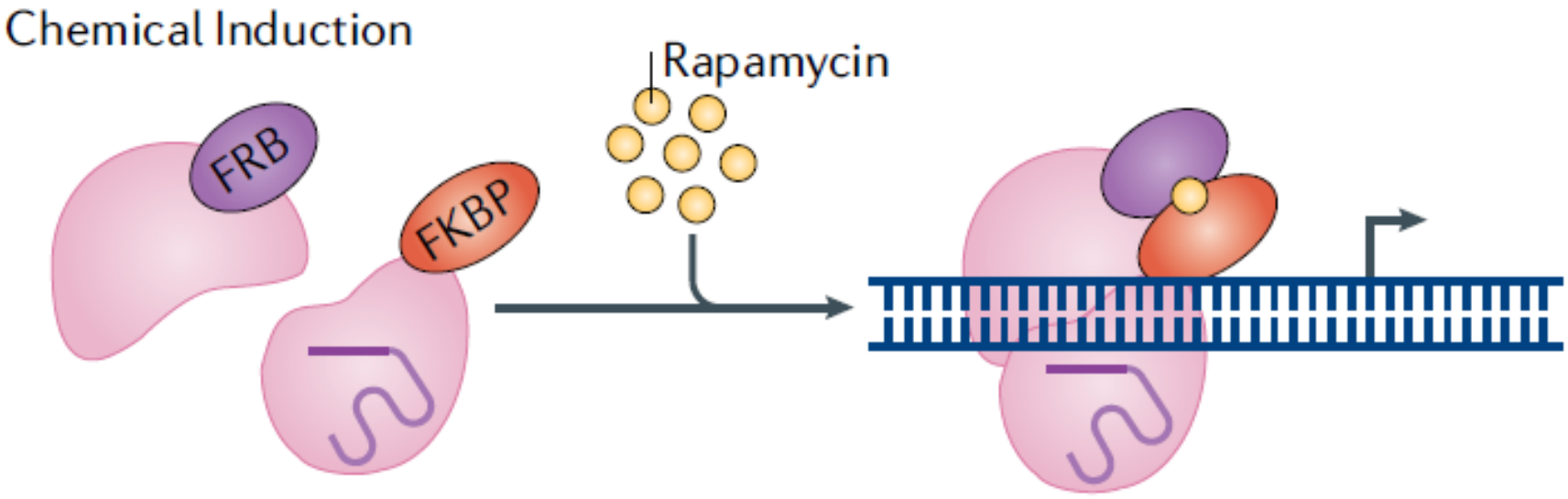

C
Endogenous Expression
Fold Change mRNA
1E6
1E5
1E4
1E3
1E2
1E1
1E0
*
*
*
Brn2
Ascl1
Myt1l
pLuc
pBAM
CR-BAM



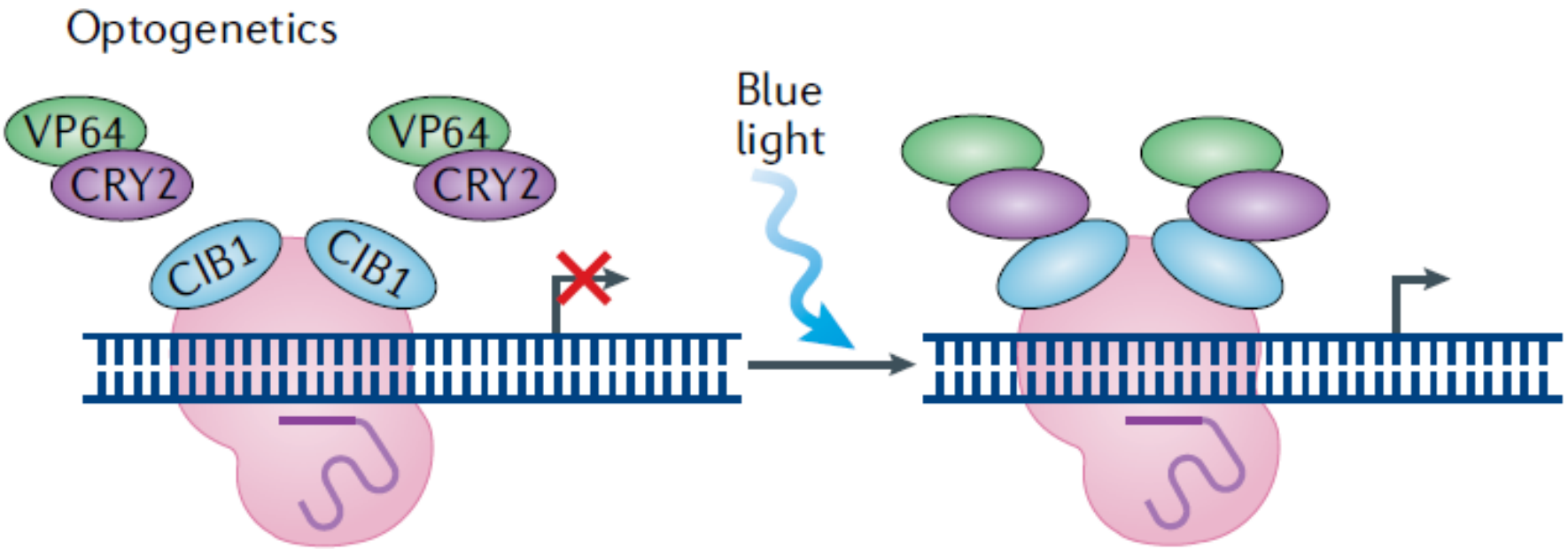
dCas9 was fused to the catalytic domain of methylcytosine dioxygenase TET1 and targeted to the *FMR1* gene to reverse the hypermethylation and silencing of the gene, which is associated with fragile X syndrome

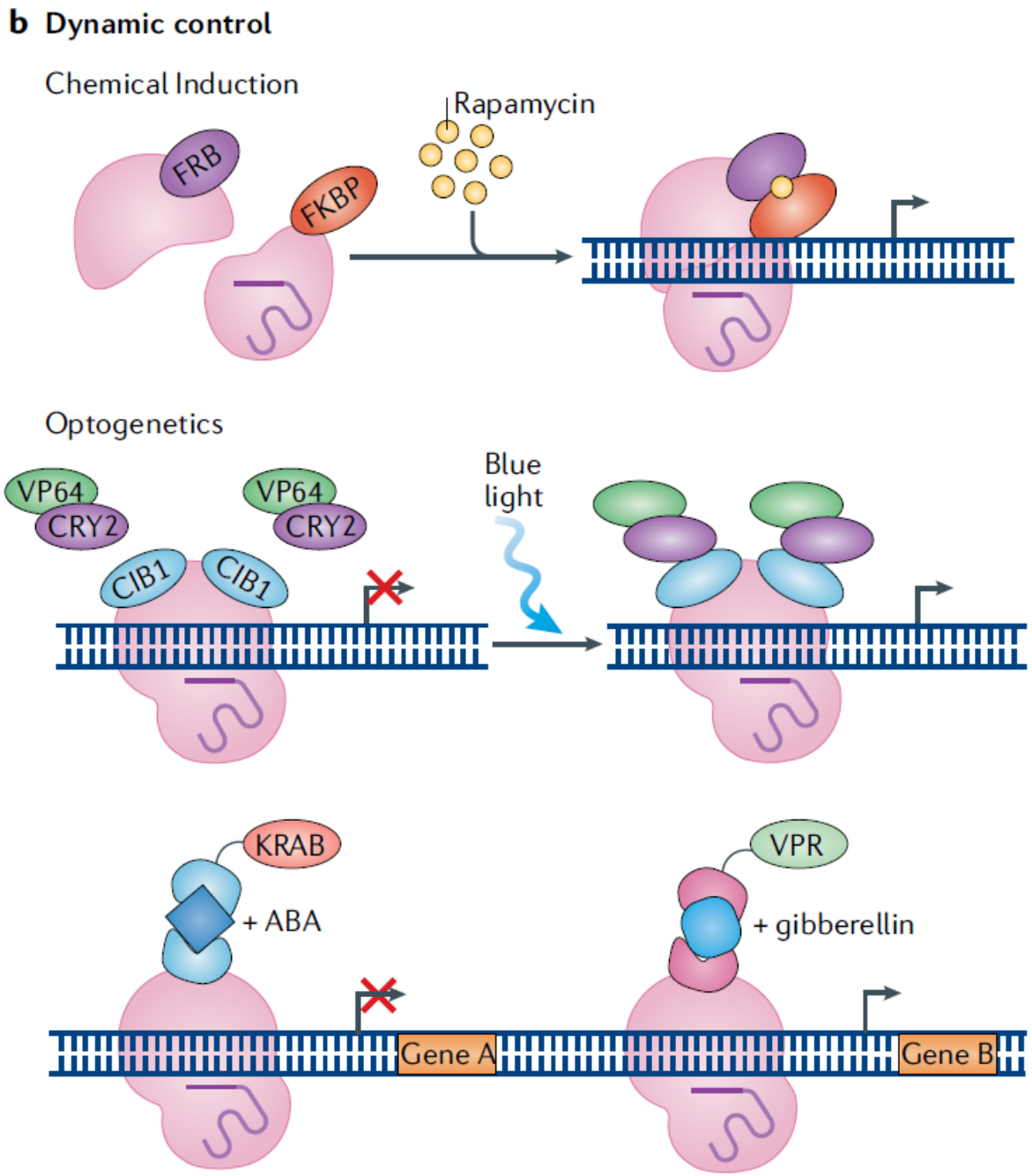


Chemical induction by rapamycin of the dimerization of split dCas9 fused to the rapamycin-binding domains FKBP and FRB activates target-gene expression.



Lightinducible dimerization of the cytochrome CRY2 with its binding partner, CIB1, can be used in photoactivatable systems.




b Dynamic control
Chemical Induction
Rapamycin
FRB
FKBP
Optogenetics
VP64
CRY2
VP64
CRY2
CIB1
CIB1
Blue
light
KRAB
+ ABA
VPR
+ gibberellin
Gene A
Gene B

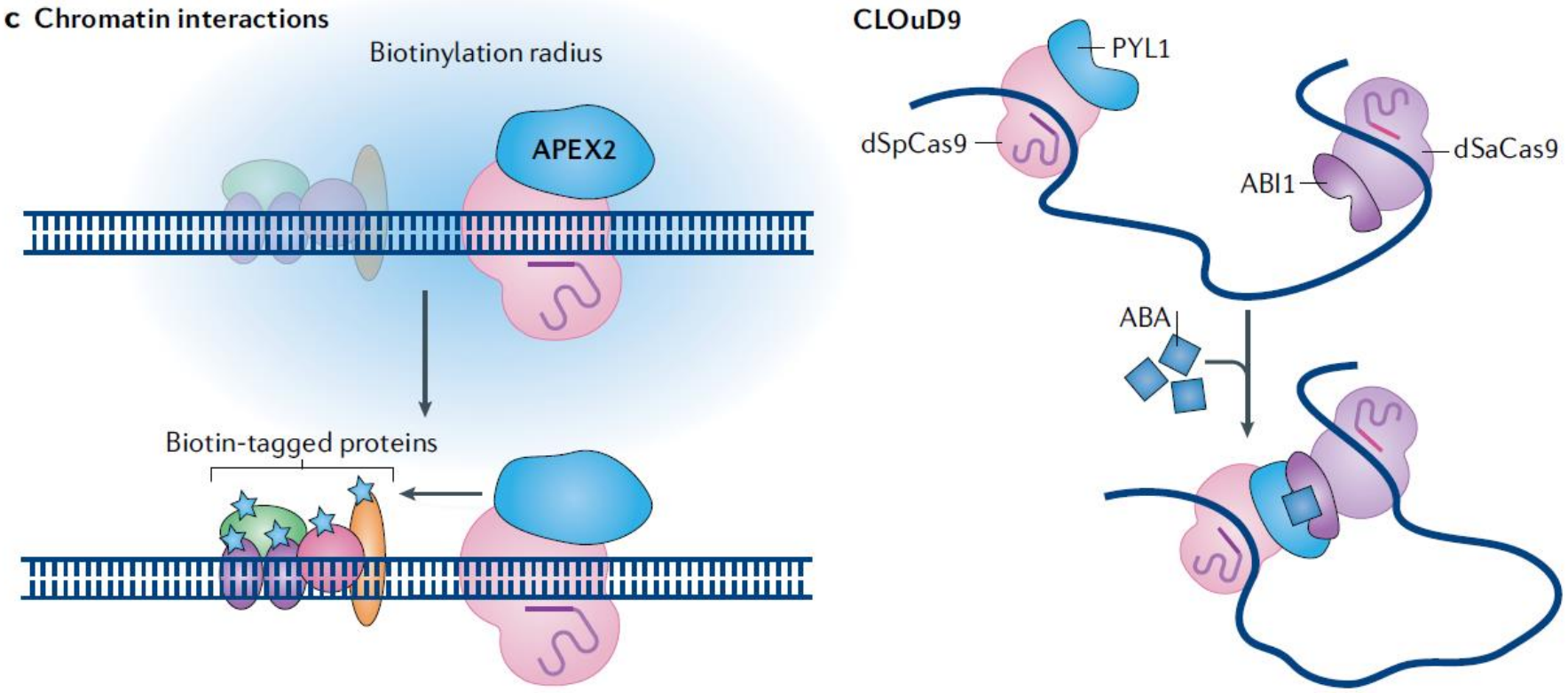
CRISPR–dCas9 tools can monitor or manipulate chromatin interactions that regulate gene expression.

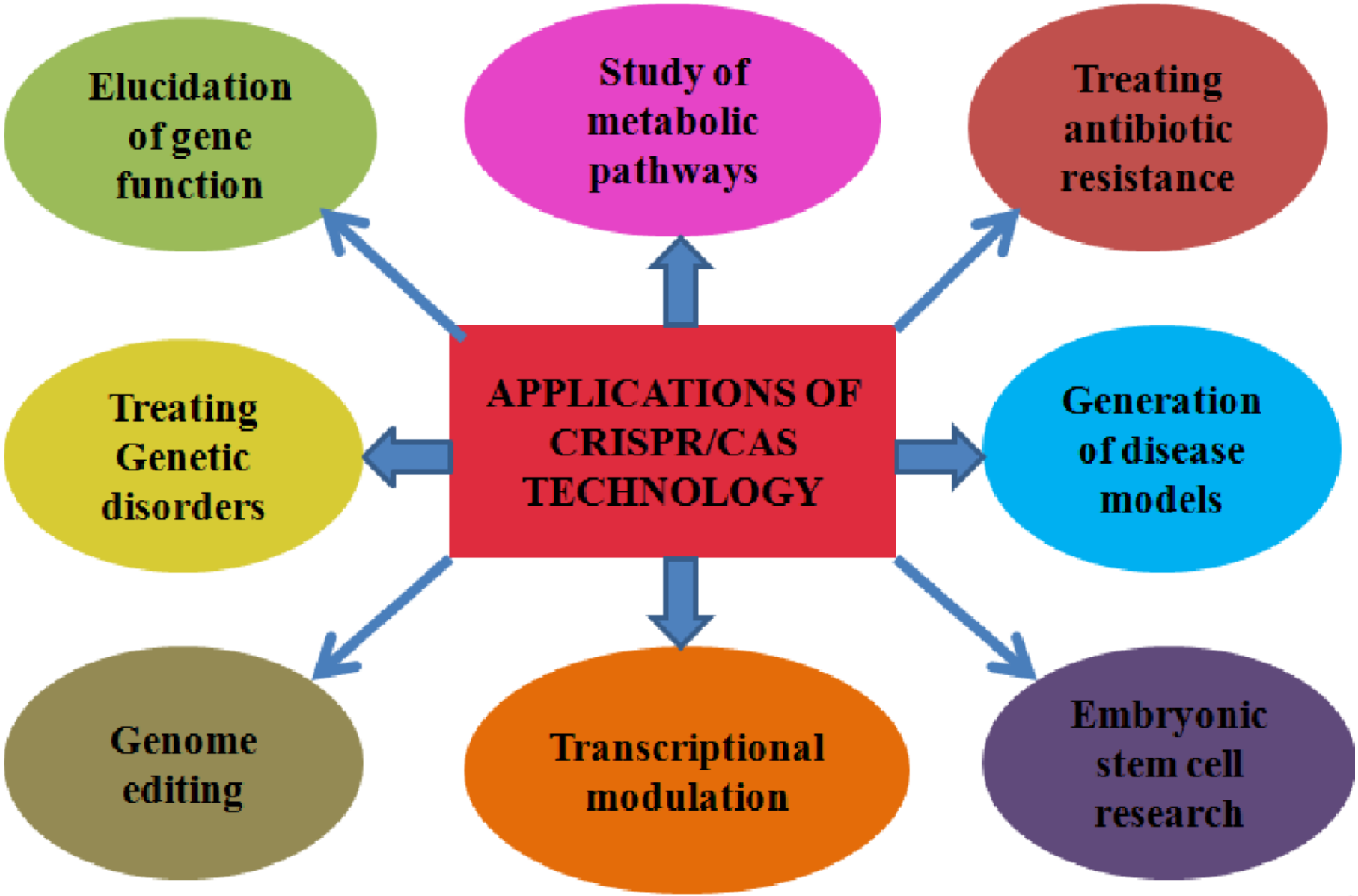
The fusion of dCas9 to the peroxidase APEX2 can be used to biotinylate proteins in the vicinity of a targeted genomic locus;
the proteins are then identified by mass spectrometry.

Distal loci can be brought into proximity using 'chromatin loop reorganization with CRISPR–dCas9' (CLOuD9).

In the CLOuD9 system, dSpCas9 and dSaCas9 targeted to distal loci are fused to the dimerizing ABA-binding proteins PYL1 and ABI1.

ABA induces targeted protein dimerization and chromatin looping, which can be reversed following its removal to restore the endogenous chromatin conformation.


c Chromatin interactions
Biotinylation radius
APEX2
Biotin-tagged proteins
CLOuD9
PYL1
dSpCas9
dSaCas9
ABI1
ABA


APPLICATIONS OF CRISPR/CAS TECHNOLOGY
Elucidation of gene function
Study of metabolic pathways
Treating antibiotic resistance
Treating Genetic disorders
Generation of disease models
Genome editing
Transcriptional modulation
Embryonic stem cell research

LONG RANGE INTERACTIONS linked to SNPs IN DISEASE

nature genetics

ARTICLES

https://doi.org/10.1038/s41588-019-0457-0

Human pancreatic islet three-dimensional chromatin architecture provides insights into the genetics of type 2 diabetes

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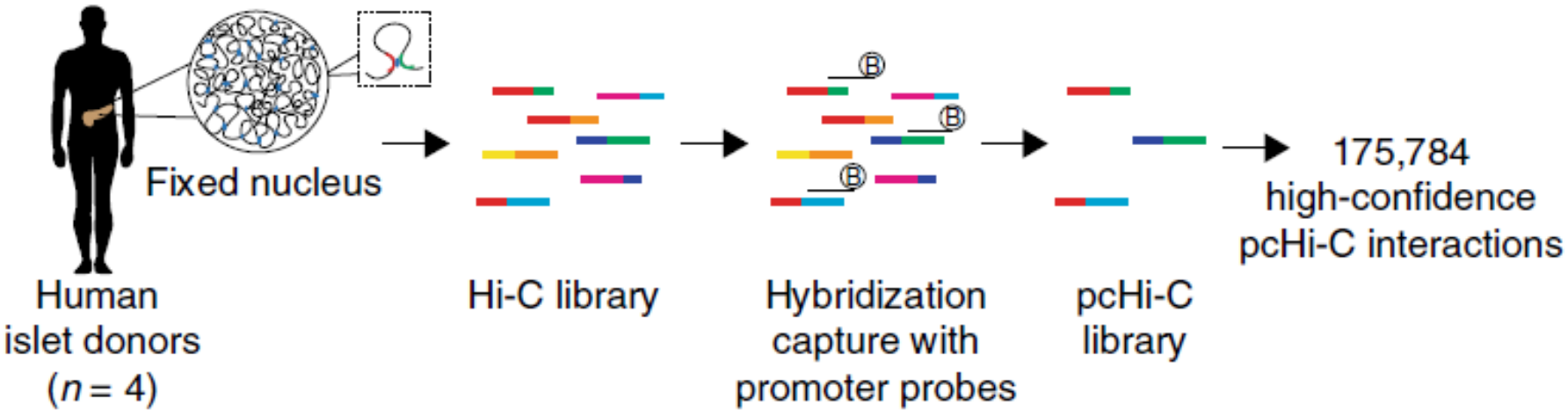
Genetic studies promise to provide insight into the molecular mechanisms underlying type 2 diabetes (T2D). Variants associated with T2D are often located in tissue-specific enhancer clusters or super-enhancers. So far, such domains have been defined through clustering of enhancers in linear genome maps rather than in three-dimensional (3D) space. Furthermore, their target genes are often unknown. We have created promoter capture Hi-C maps in human pancreatic islets. This linked diabetes-associated enhancers to their target genes, often located hundreds of kilobases away. It also revealed >1,300 groups of islet enhancers, super-enhancers and active promoters that form 3D hubs, some of which show coordinated glucose-dependent activity. We demonstrate that genetic variation in hubs impacts insulin secretion heritability, and show that hub annotations can be used for polygenic scores that predict T2D risk driven by islet regulatory variants. Human islet 3D chromatin architecture, therefore, provides a framework for interpretation of T2D genome-wide association study (GWAS) signals.

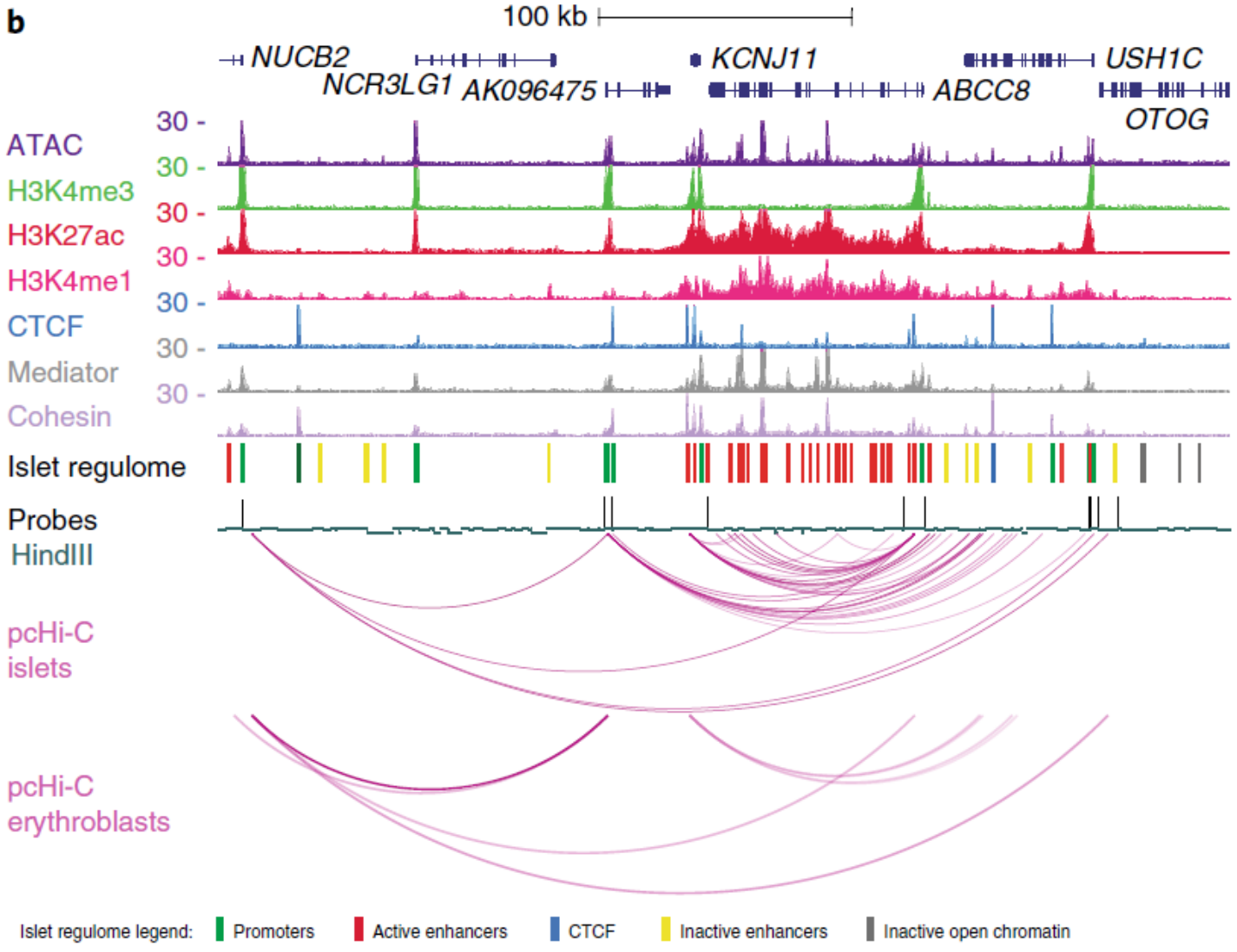
HIGHLIGHTS

- promoter capture Hi-C (pcHi-C)14 to generate a genome-scale map of interactions between gene promoters and their regulatory elements in human pancreatic islets.
- islet enhancer hubs are connected with key islet gene promoters and exhibit properties of regulatory domains.
- genome/epigenome editing to demonstrate the functional connectivity of hubs
- Validation of functional interactions between enhancers bearing 2D risk variants
- Islet can be used to partition polygenic scores to identify T2D genetic susceptibility driven by pancreatic islet regulatory variation.


Fixed nucleus
Human
islet donors
(n = 4)
Hi-C library
Hybridization
capture with
promoter probes
pcHi-C
library
175,784
high-confidence
pcHi-C interactions

Integrative map of the *KCNJ11–ABCC8* locus

human islet ATAC-seq and ChIP-seq, HindIII bait fragments and arcs representing high-confidence pcHi-C interactions in human islets and erythroblasts

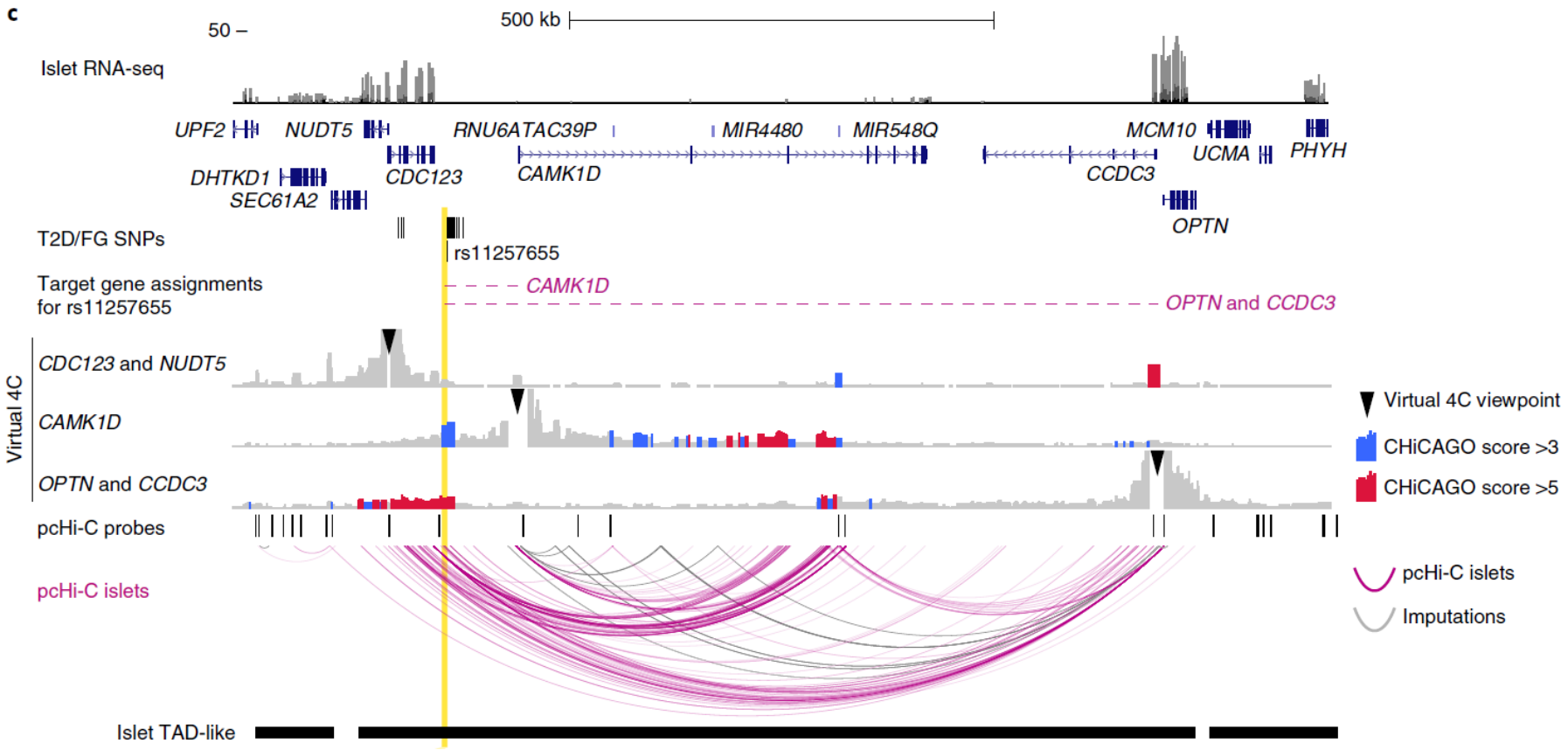


Summary of T2D-associated enhancer perturbations presented in this study

b

Enhancer SNP	Assigned genes	Genes affected by CRISPR	CRISPR/Cas9 validations CRISPRi	CRISPRa	Deletion
rs11257655	*CAMK1D*, *OPTN*	*CAMK1D*, *OPTN*	✓	✓	✓
rs4237150	*RFX3*, *RFX3-AS1*, *GLIS3*	*GLIS3*	✓	✓	✓
rs7903146	*TCF7L2*	*TCF7L2*	✓	✓	✓
rs7163757	*C2CD4A*, *VPS13C*	*C2CD4A*, *VPS13C*, *C2CD4B*	✓	✓	NT
rs1401419	*CRY2*, *PHF21A*	*CRY2*	✓	✓	NT
rs12189774	*VEGFA*	*VEGFA*	✓	✓	NT
rs7732130	*S100Z*, *ZBED3*, *snoRA47*, *ZBED3-AS1*, *PDE8B*, *WDR41*	*ZBED3*, *snoRA47*, *ZBED3-AS1*, *PDE8B*, *WDR41*	✓	✓	NT
rs58692659	*MDGA*, *ZFAND3*	*MDGA1*, *ZFAND3*	✓	✓	NT

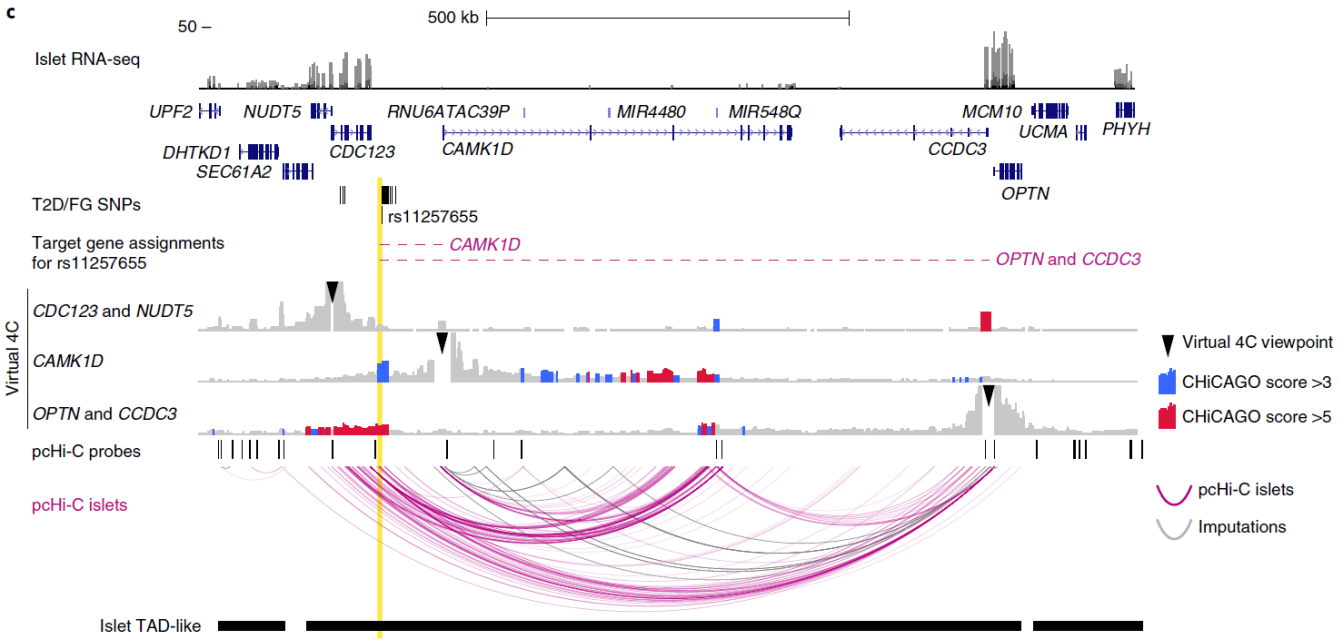
Annotation of rs11257655 to long range interaction that is involved *CAMK1D* and *OPTN*



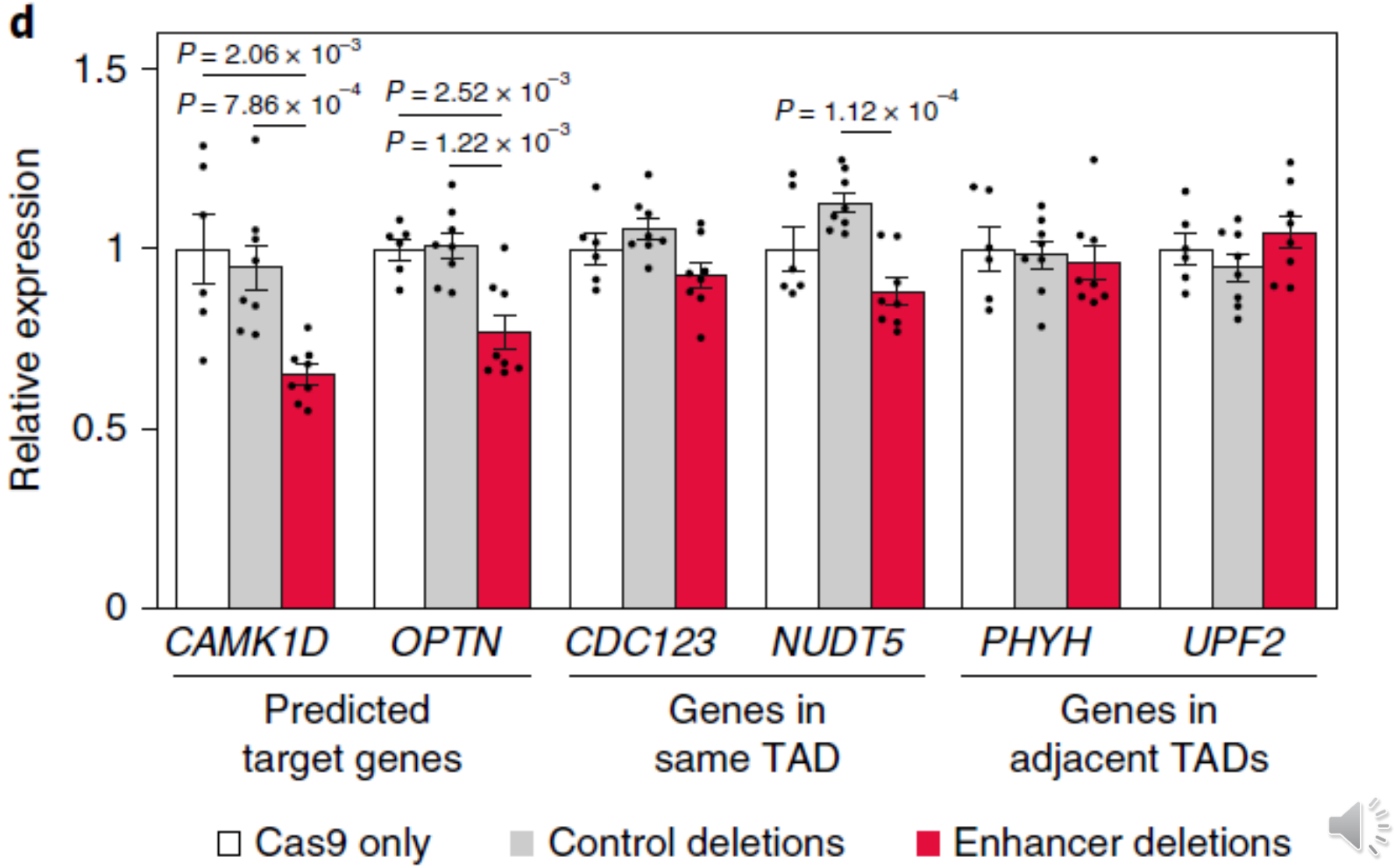
Annotation of rs11257655 to long range interaction that involved *CAMK1D* and *OPTN* .

Islet pcHi-C analysis defines gene targets of an enhancer bearing T2D-associated variants near *CDC123/CAMK1D*.

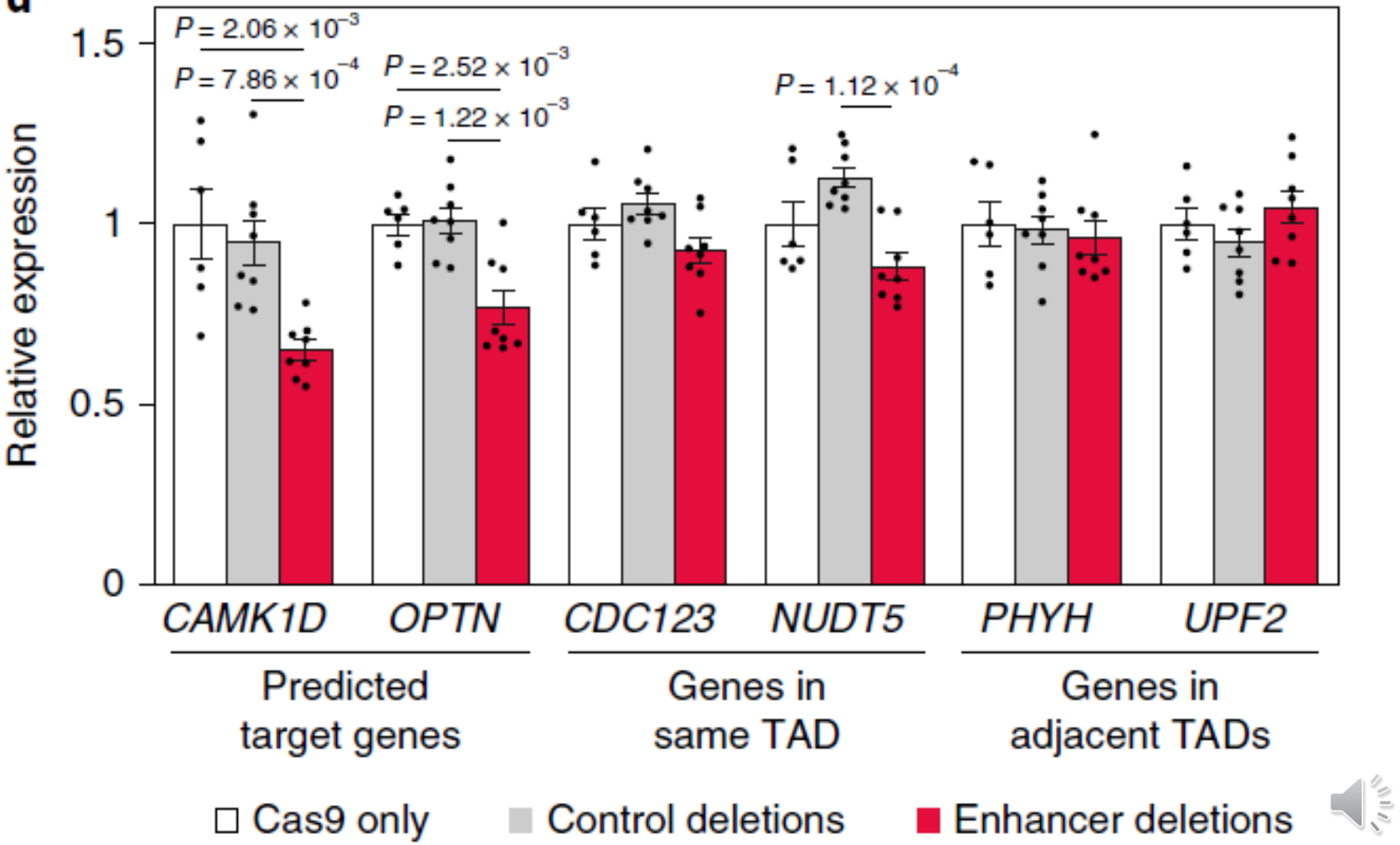
The only T2D risk credible set variant that maps to an islet enhancer in the locus (rs11257655, zoomed inset) is assigned to *CAMK1D* and *OPTN* (dashed horizontal lines). Islet pcHi-C virtual 4C representations from pooled samples show interactions stemming from both *CAMK1D* and *OPTN* promoters towards rs11257655 with *CHiCAGO* > 3, but not from *CDC123*



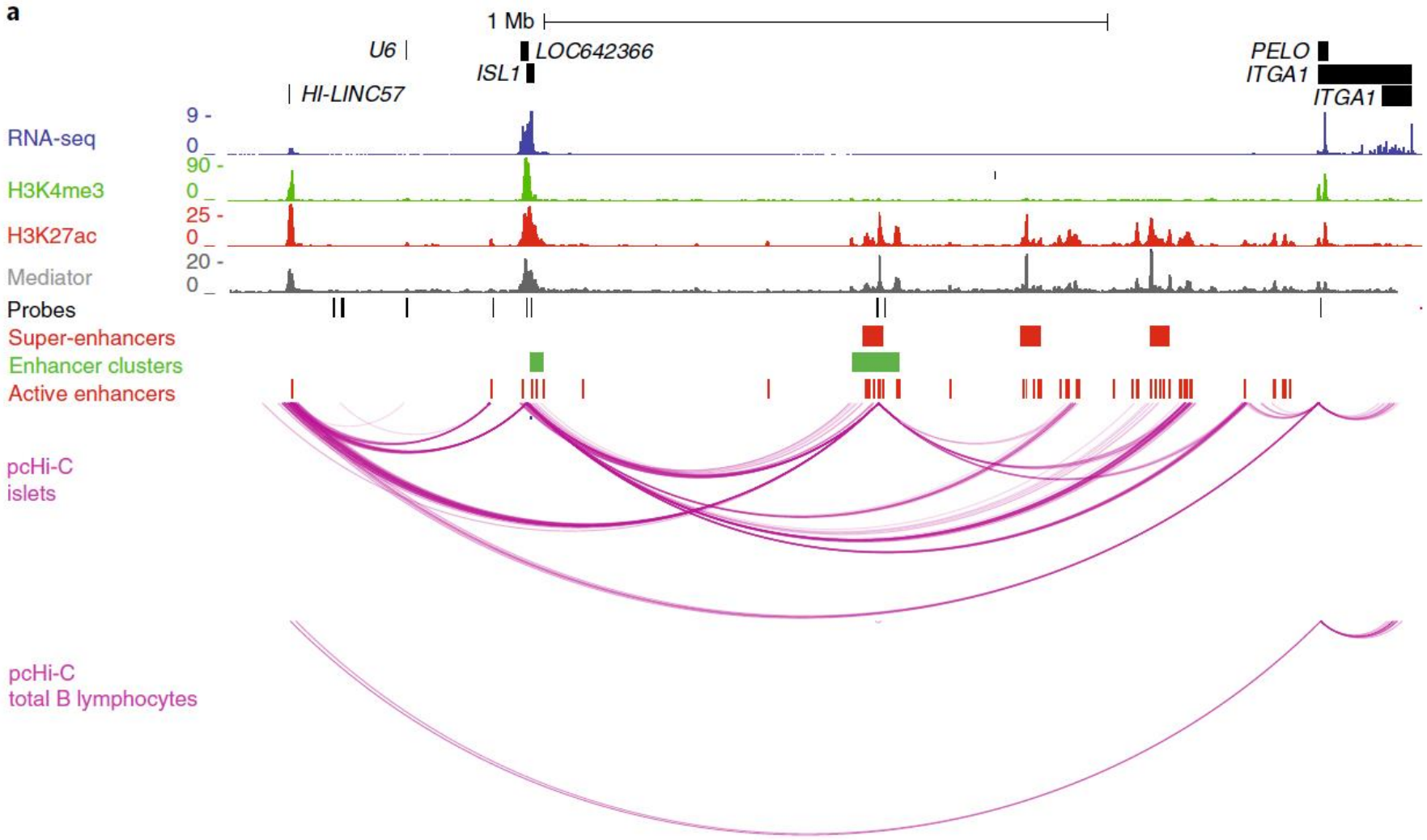
CAMK1D and *OPTN* mRNA are regulated by the rs11257655-containing enhancer



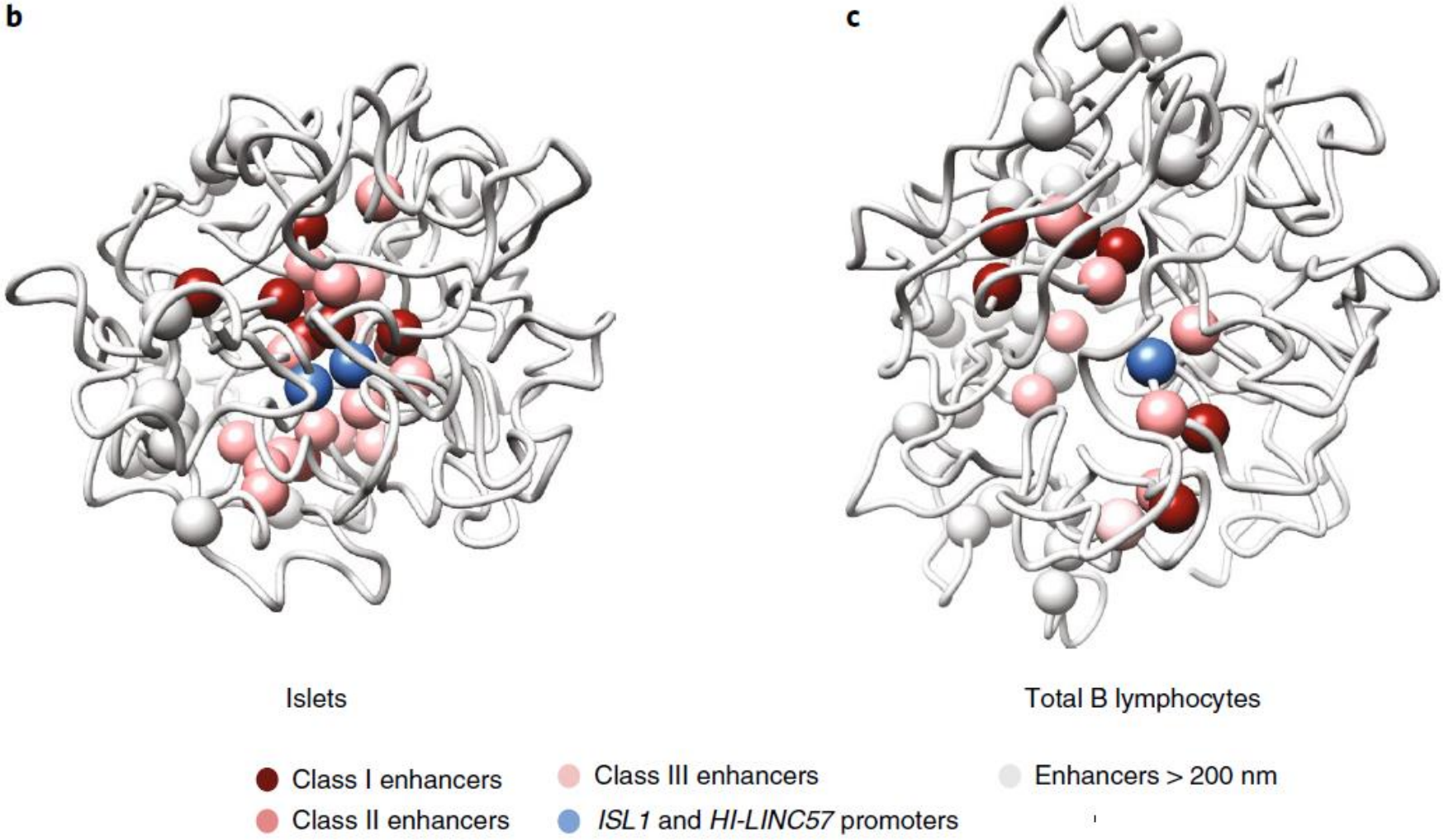
CAMK1D and *OPTN* mRNA are regulated by the rs11257655-containing enhancer. We deleted the rs11257655-containing enhancer and a nearby control region with a T2D-associated variant (rs33932777) that lacked active chromatin marks in human islets. Cas9 only: *n* = 6 (two independent experiments with triplicates). Deletions:
n = 8 (two guide RNA (gRNA) pairs in two independent experiments with biological duplicates)



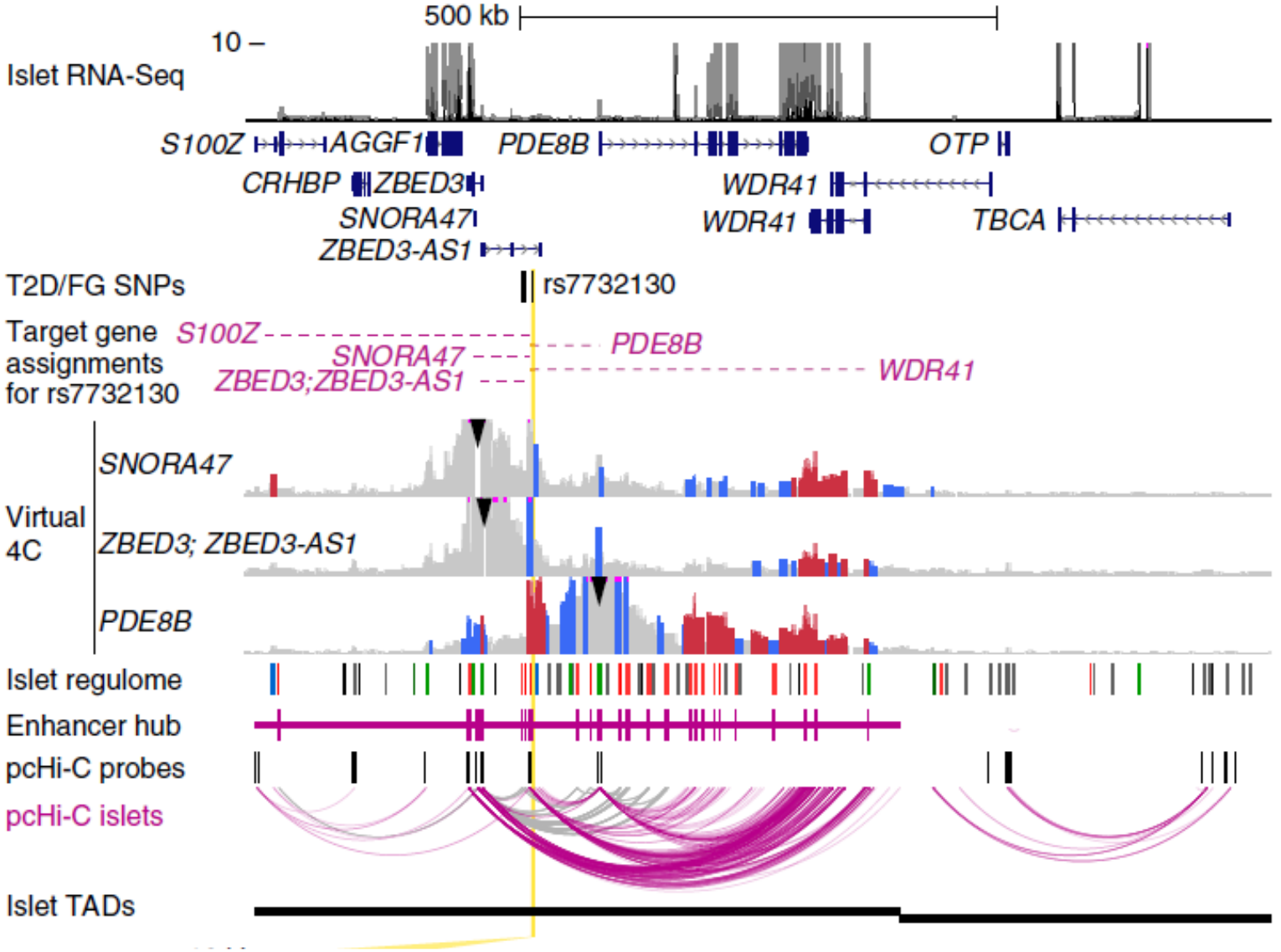
Total B lymphocytes are shown to illustrate active enhancers, super-enhancers and enhancer clusters. 3D interactions with *ISL1* and *HI-LNC57*



3D chromatin conformation models of the *ISL1* enhancer hub generated from pcHi-C libraries from human islets (b) and total B lymphocytes (c).

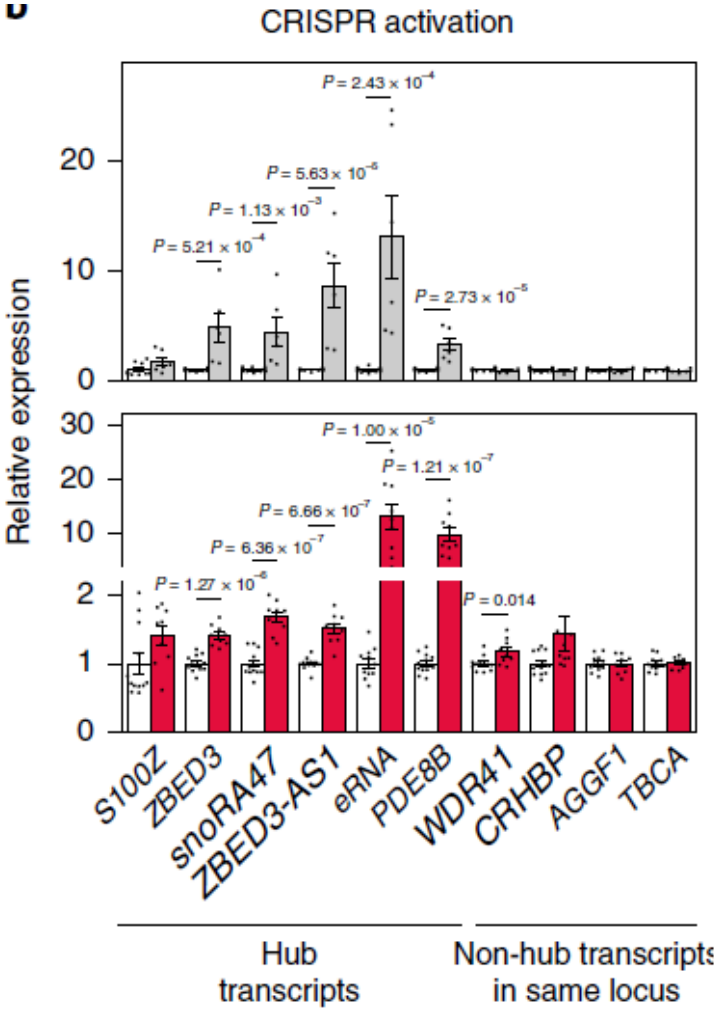


ZBED3 enhancer hub links an enhancer bearing a T2D SNP with multiple target genes



Analysis of hub and non-hub transcripts after CRISPR activation of the transcriptional start site of *ZBED3* or the rs7732130 enhancer in EndoC-βH3 cells.

□ Control gRNAs ■ ZBED3 TSS gRNAs ■ Enhancer gRNAs



CONCLUSION

- This study has systematically mapped >1,300 enhancer hubs in human islets.
- These enhancer domains are components of chromatin hubs with evolutionarily conserved noncoding sequence blocks
- enhancer hubs exhibit features of regulatory domains that control genes important for islet cell function, differentiation and diabetes. T
- enhancer hubs contain DNA variants that have a major impact on the heritability of insulin secretion.
- Hub elements define a genomic space that has direct relevance to islet function and human diabetes

APPLICATION

Polygenic scores based on islet hub variants could thus be leveraged to quantify patient-specific genetic risk acting through islet gene regulation and insulin secretion.