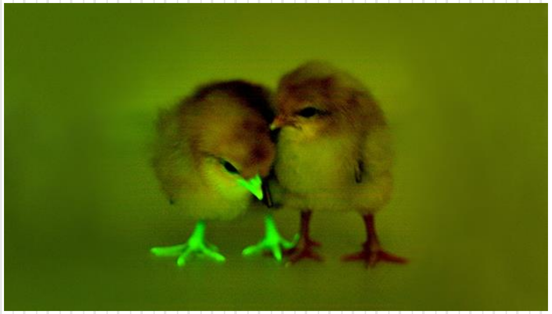
Recombinant DNA (rDNA) Technology PART II

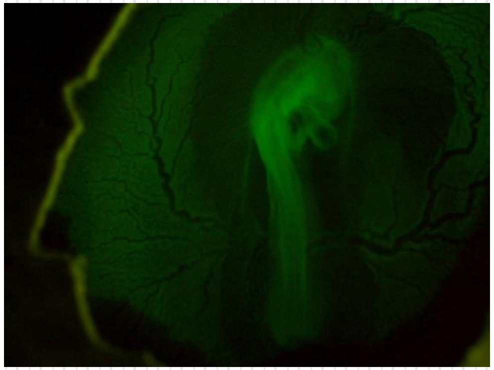
SLE254 Genetics and Genomics

Chapter 19 Concepts 12th ed

Pages 493-521

Spec topics in genetics 1 pp 687-698





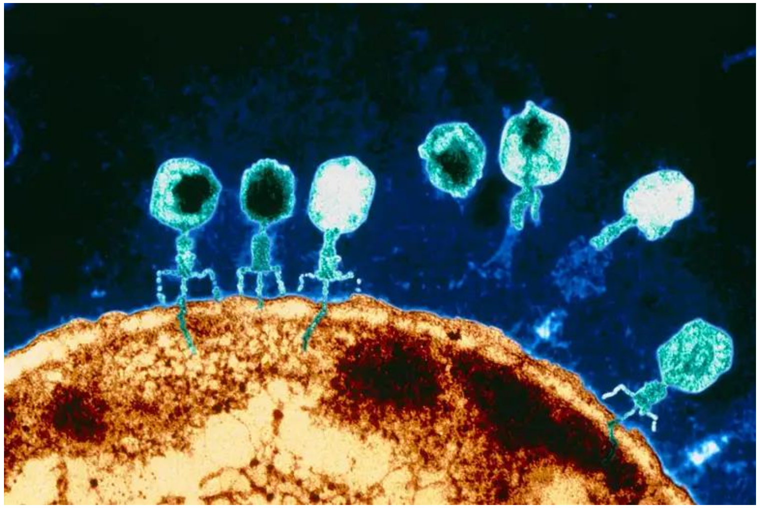
rDNA technology for studying gene function: KO and Transgenic Organisms

- Knock out (KO) of genes allows for studying gene function in vivo
  - Several approaches- CRISPR-Cas9 system a new and powerful tool
  - Can be conditional and/or tissue specific
- Transgenic animals: Knock-in animals which express or over express gene of interest
  - utilizes homologous recombination to insert a gene
  - Can be condition and/or tissue specific

CRISPR: 'Clusters of Regularly Interspaced Short Palindromic Repeats-CRISPR-associated9'

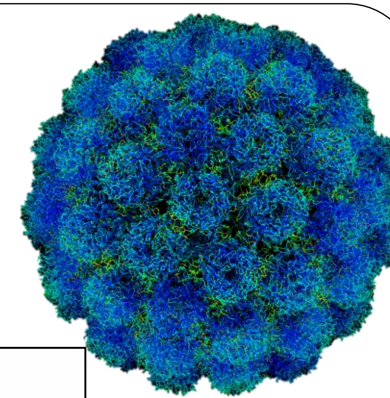
- **CRISPRs** are specialised stretches of DNA
- The protein **Cas9** is an enzyme that acts like 'molecular scissors' capable of cutting DNA.
- Where does it come from? Adapted from natural defense mechanisms in single celled prokaryotes, involved in resistance to bacteriophages.

Bacteriophage viruses attacking an *E. coli* cell



EYE OF SCIENCE/SCIENCE PHOTO LIBRARY

RE’s: How it all began…



Proc. Nat. Acad. Sci. USA
Vol. 68, No. 12, pp. 2913–2917, December 1971

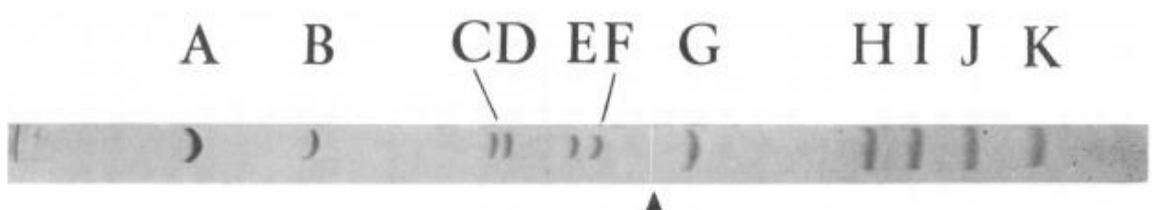
Specific Cleavage of Simian Virus 40 DNA by Restriction Endonuclease of Hemophilus Influenzae*

(gel electrophoresis/electron microscopy/DNA mapping/DNA fragments/tumor virus)

KATHLEEN DANNA AND DANIEL NATHANS

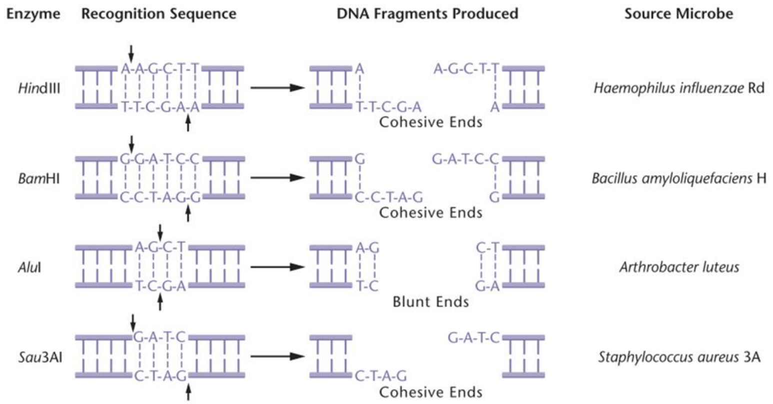
Department of Microbiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

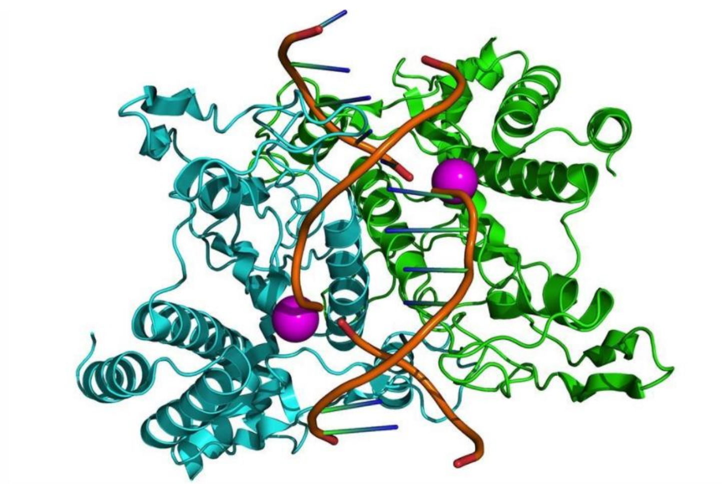
Communicated by Albert L. Lehninger, September 22, 1971



“…the restriction endonuclease should prove of general usefulness in the analysis of DNA, much as highly specific proteolytic enzymes have been used in the analysis of proteins.”

First identified and commercially used





- **Restriction enzymes** exist naturally in bacteria to attack an chop viruses up into smaller and non harmful pieces. More primitive less precise cuts. Does not confer resistance.

- **CRISPR** in it's natural state involves two RNAs and a protein. Advanced system, allows extremely precise cuts. Confers resistance to foreign genetic elements (viruses and plasmids). RNA guided nuclease

'Now, in only a few short years, CRISPR is poised to take over the DNA cutting business. If restriction enzymes are axes, CRISPR is a laser scalpel'

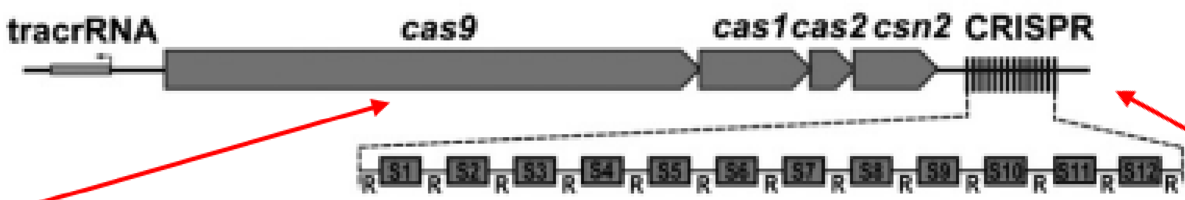
https://daily.jstor.org/revolution-will-geneticized-restriction-enzymes-crispr/

***Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero DA, Horvath P.Science. 2007 Mar 23;315(5819):1709-12. doi: 10.1126/science.1138140.**

- **2007***: Researchers demonstrated that Prokaryotes have evolved a nucleic-acid based immune system where <u>specificity</u> is dictated by CRISPR and <u>resistance</u> is provided by the Cas enzymatic machinery.
- This contrasts to Eukaryotes who utilise amino-acid based immunity.
- Not only that, but bacteria integrate new sequences derived from phage DNA as part of an 'adaptive immune response'. This provides immunity to variable phage attacks.

Definitions: example *S. thermophilus* type II *cas9* system

operon



4 Cas genes are located upstream of the 12 repeat spacer units.

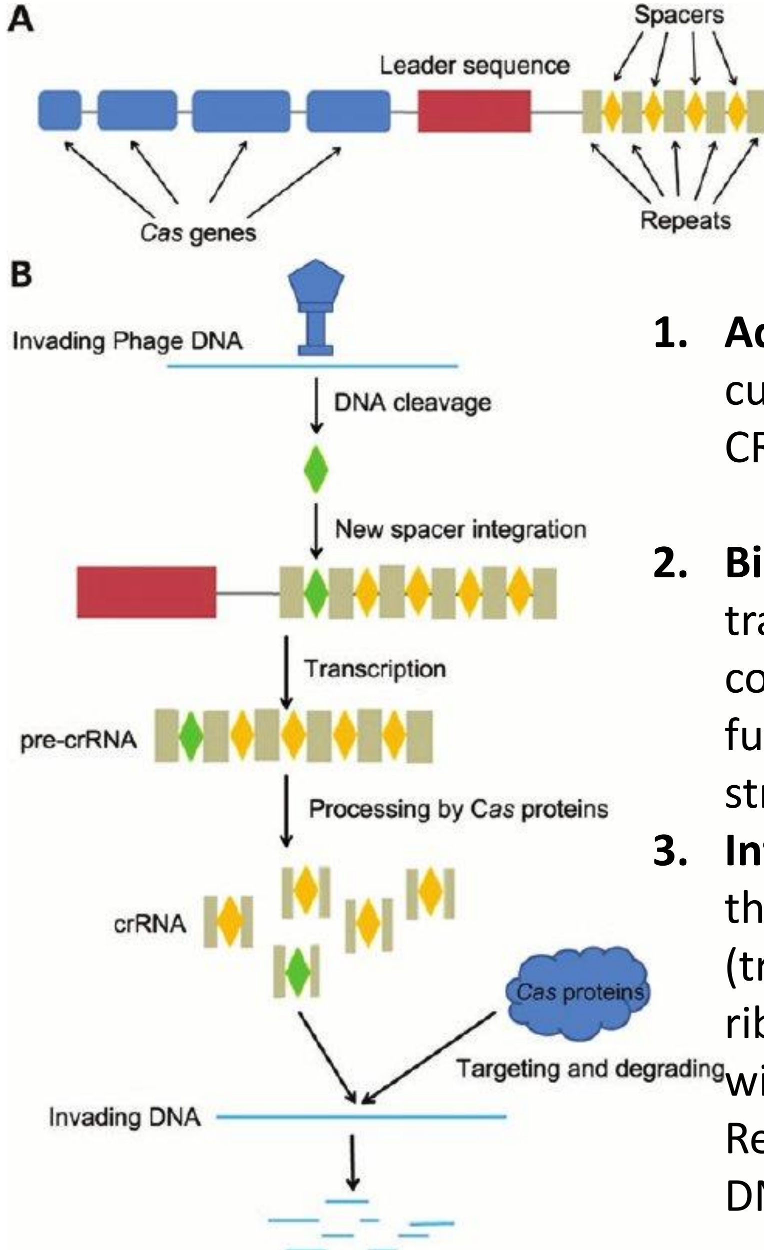
Cas9 is a large multi-domain protein

- **RuvC-**like nuclease domain (cleavage of nucleic acid, single or double stranded breaks). Cleaves the non-complementary strand
- **HNH** nuclease domain cleaves the complimentary strand

CRISPR **repeat-spacer arrays** are transcribed and processed into a set of short CRISPR RNAs **cRNAs**

Repeat spacer array: All CRISPR arrays contain a series of repeats interspersed by short sequences called **spacers** which match previously invading DNA and can incorporate new invading DNA (adaptive immunity)

https://doi.org/10.1073/pnas.1208507109 fig 1A

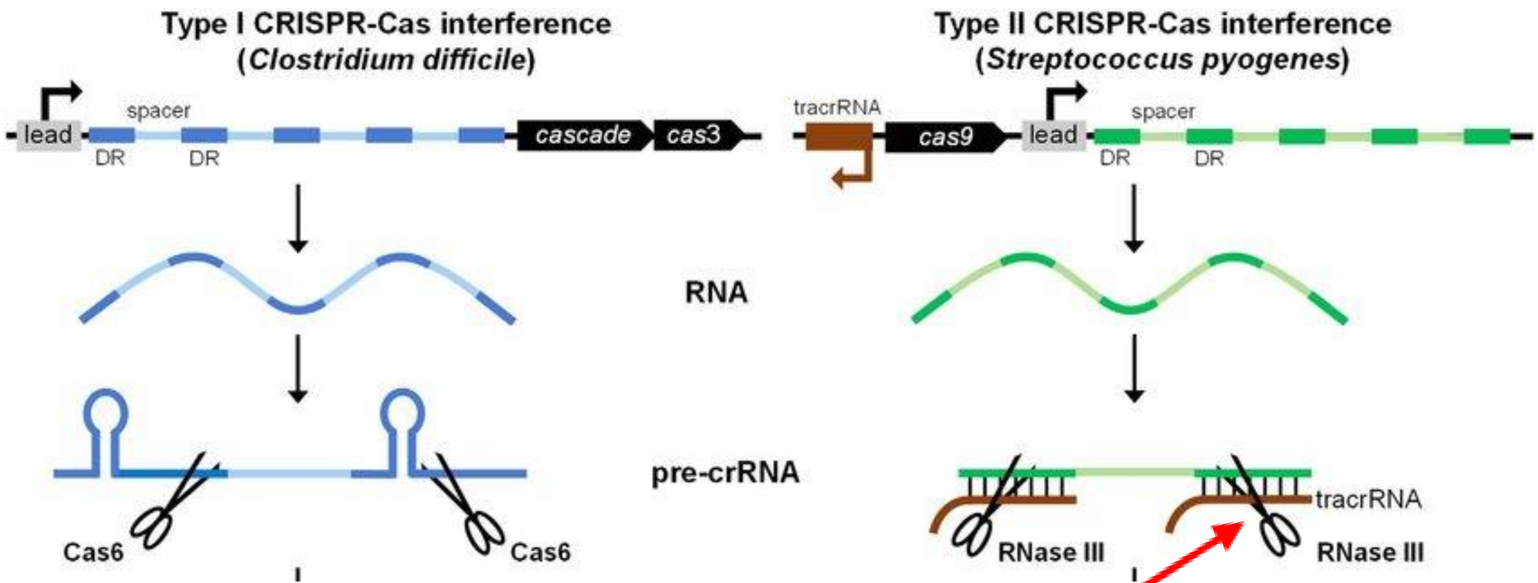


1. **Adaptation:** Invading DNA is cut and incorporated into the CRISPR locus (spacer)

2. **Biogenesis:** The locus is transcribed as single non-coding pre-crRNA that gets further processed into short stretches of mature crRNA.
3. **Interference:** Together with the a second non-coding RNA (trcrRNA) the crRNA forms a ribonucleoprotein complex with endonuclease Cas9. Recognises and cuts invading DNA (dS breaks)

https://www.researchgate.net/publication/260996402_CRISPRCas9_for_genome_editing_Progress_implications_and_challenges/figures?lo=1

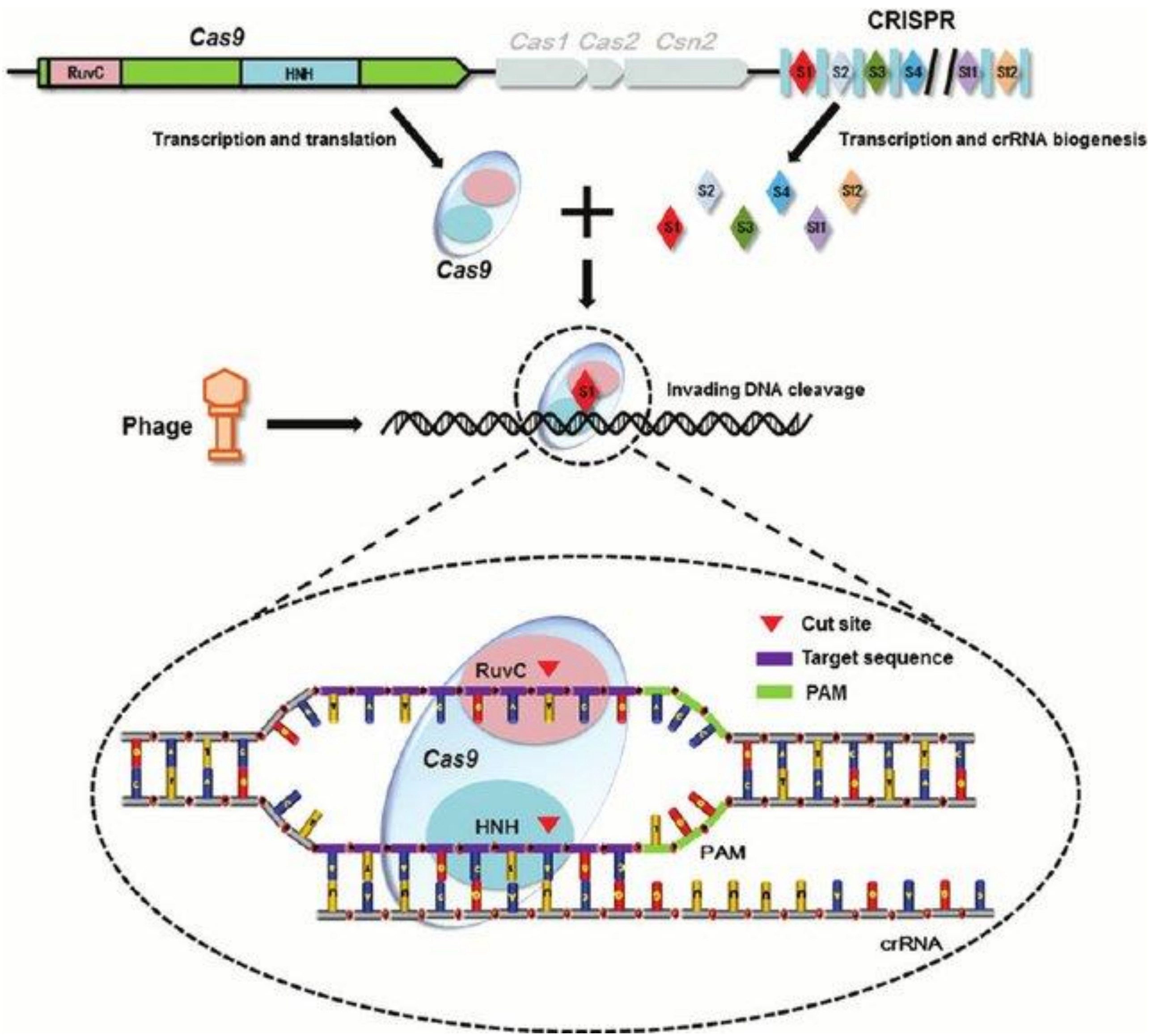
- *Translational Research* 2016 168, 15-21DOI: (10.1016/j.trsl.2015.09.008)



Leader: (lead) promotor sequence *cis* acting

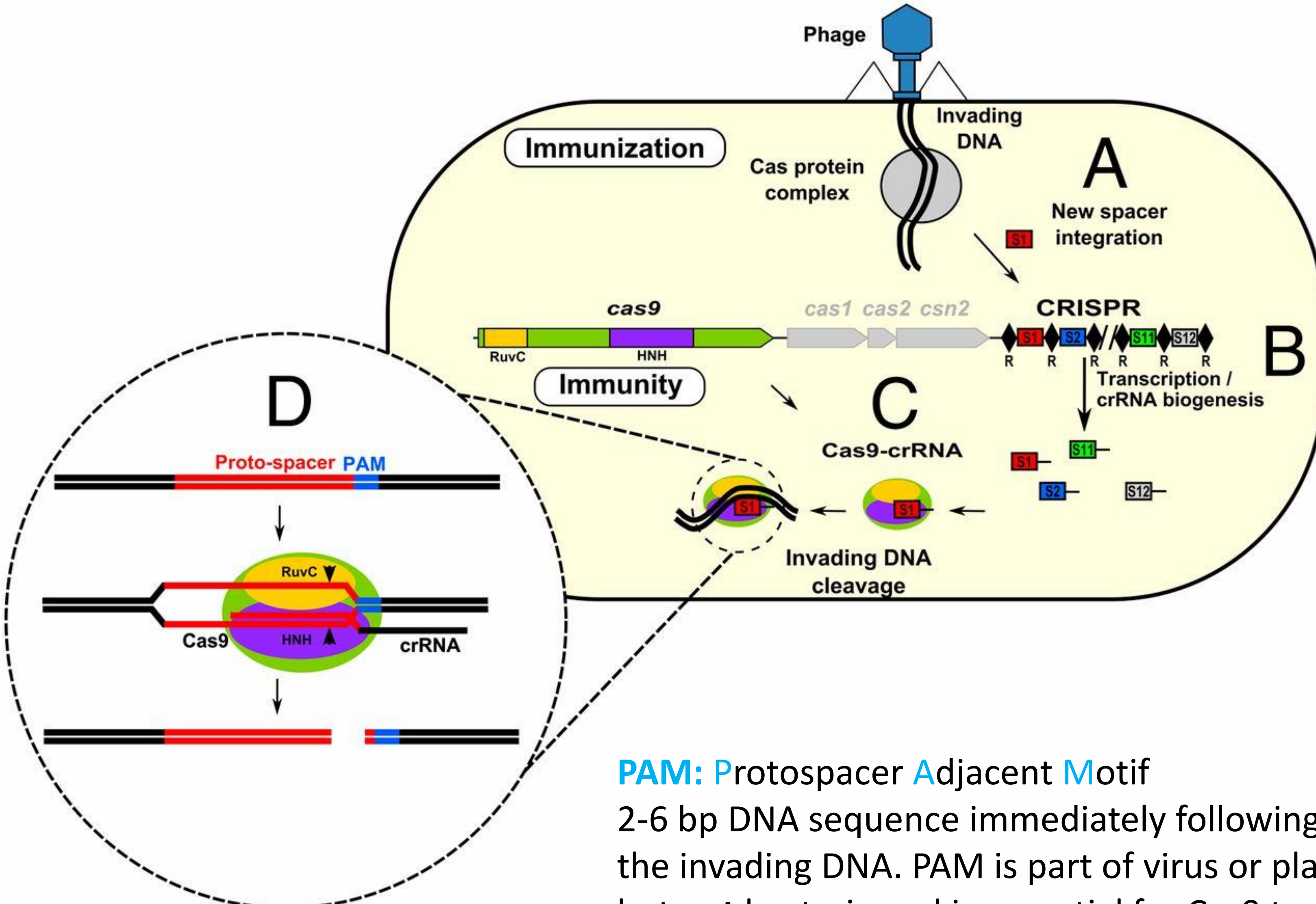
Two types of RNA Cas9:
TracrRNA: In *cas9* systems tracrRNA pairs with crRNA to form a functional **guide RNA gRNA**. *Trans* acting.

Cas9 uses the trcrRNA portion of the guide as a handle, crRNA spacer sequence guides the complex to the complementary viral sequence



https://www.researchgate.net/publication/260996402_CRISPRCas9_for_genome_editing_Progress_implications_and_challenges/figures?lo=1

Definitions



PAM: Protospacer Adjacent Motif
2-6 bp DNA sequence immediately following the invading DNA. PAM is part of virus or plasmid but **not** bacteria and is essential for Cas9 to work so the bacteria does not target it's own Cas9 complex

https://doi.org/10.1073/pnas.1208507109

How did this system become a genome editing tool?

• . 2012 Aug 17;337(6096):816-21.

doi: 10.1126/science.1225829. Epub 2012 Jun 28.

A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity

Martin Jinek[1], Krzysztof Chylinski, Ines Fonfara, Michael Hauer, Jennifer A Doudna, Emmanuelle Charpentier

domain cleaves the noncomplementary strand. The dual-tracrRNA:crRNA, when engineered as a single RNA chimera, also directs sequence-specific Cas9 dsDNA cleavage. Our study reveals a family of endonucleases that use dual-RNAs for site-specific DNA cleavage and highlights the potential to exploit the system for RNA-programmable genome editing.

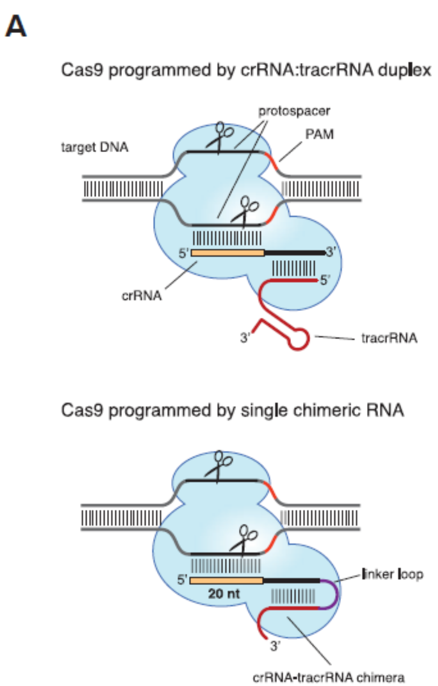
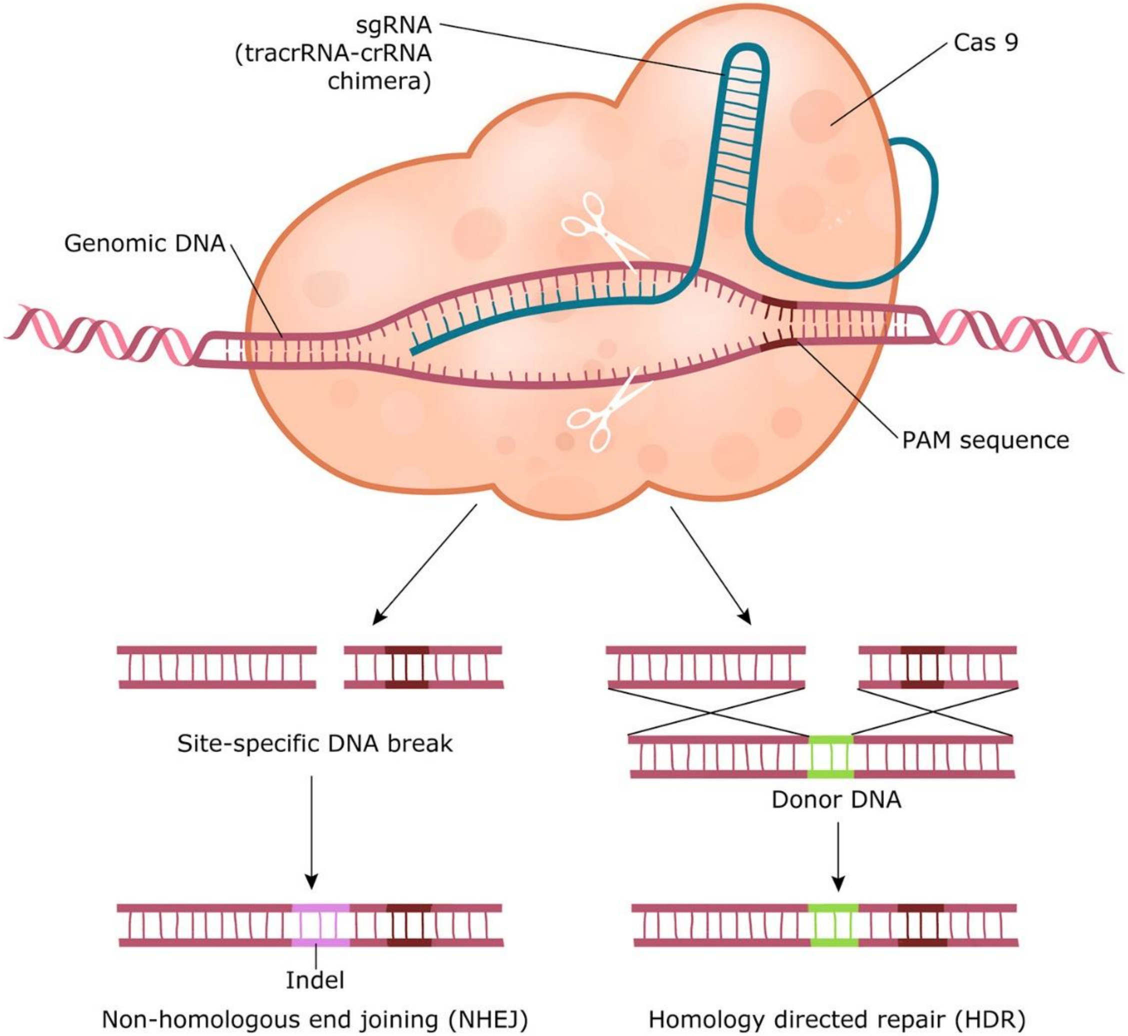


Fig. 5. Cas9 can be programmed using a single engineered RNA molecule combining tracrRNA and crRNA features. (**A**) (Top) In type II CRISPR/Cas systems, Cas9 is guided by a two-RNA structure formed by activating tracrRNA and targeting crRNA to cleave site-specifically—targeted dsDNA (see fig. S1). (Bottom) A chimeric RNA generated by fusing the 3′ end of crRNA to the 5′ end of tracrRNA. (**B**)

ing (Fig. 5A). We designed two versions of a chimeric RNA containing a target recognition sequence at the 5′ end followed by a hairpin structure retaining the base-pairing interactions that occur between the tracrRNA and the crRNA (Fig. 5B). This single transcript effectively fuses the 3′ end of crRNA to the 5′ end of tracrRNA, thereby mimicking the dual-RNA structure required to guide site-specific DNA cleavage by Cas9. In cleavage assays using plasmid DNA,

Fig 5A.

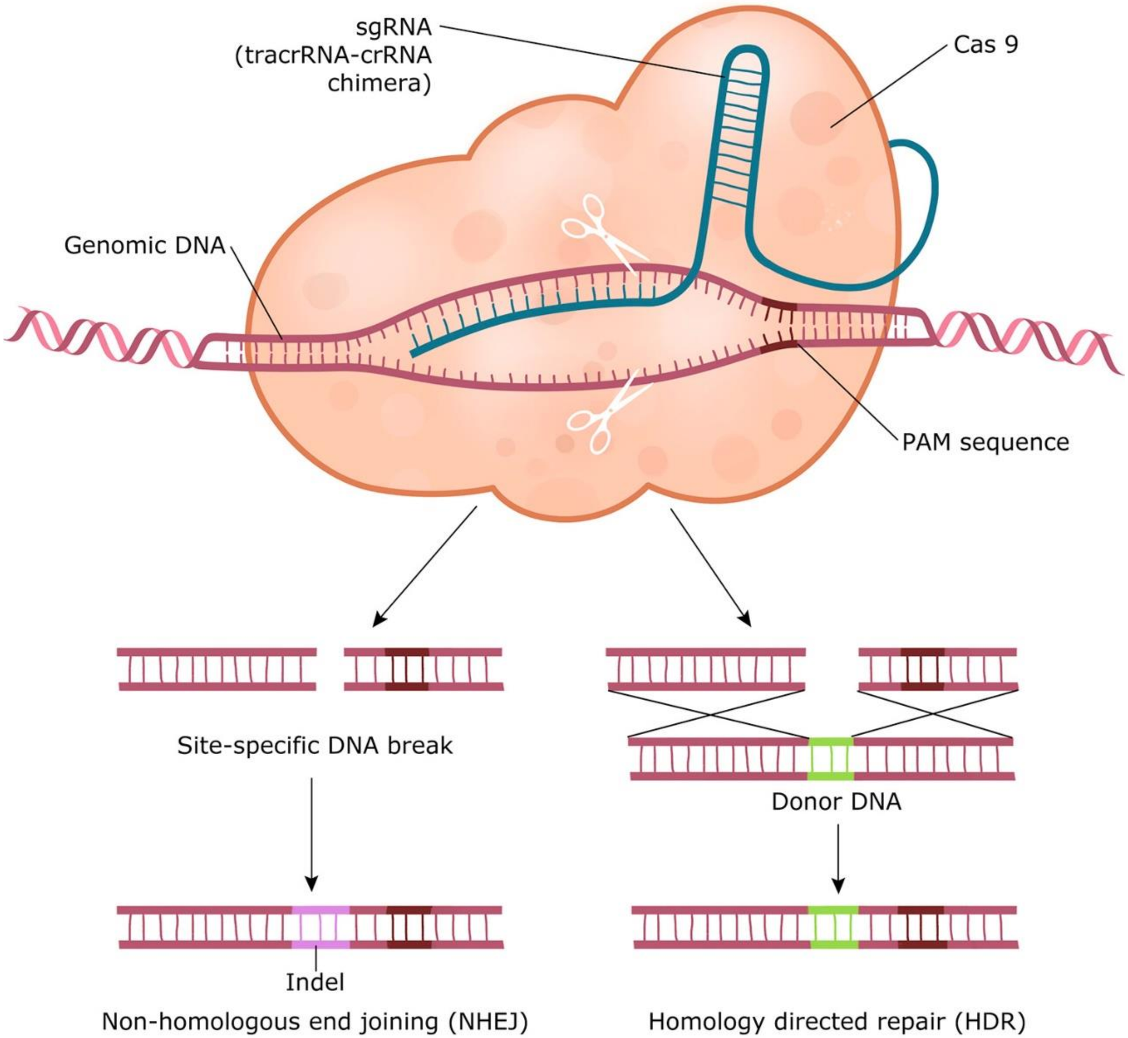
CRISPR as genome editing tool



- Cas9 recruitment to the target DNA is mediated by a chimeric single-guide RNA (sgRNA).
- It contains a protospacer recognising the target sequence followed by protopacer adjacent motif (PAM)
- The DSBs are repaired by NHEJ (indels) **or**

HDR using a synthetic donor as the template for introduced sequence changes

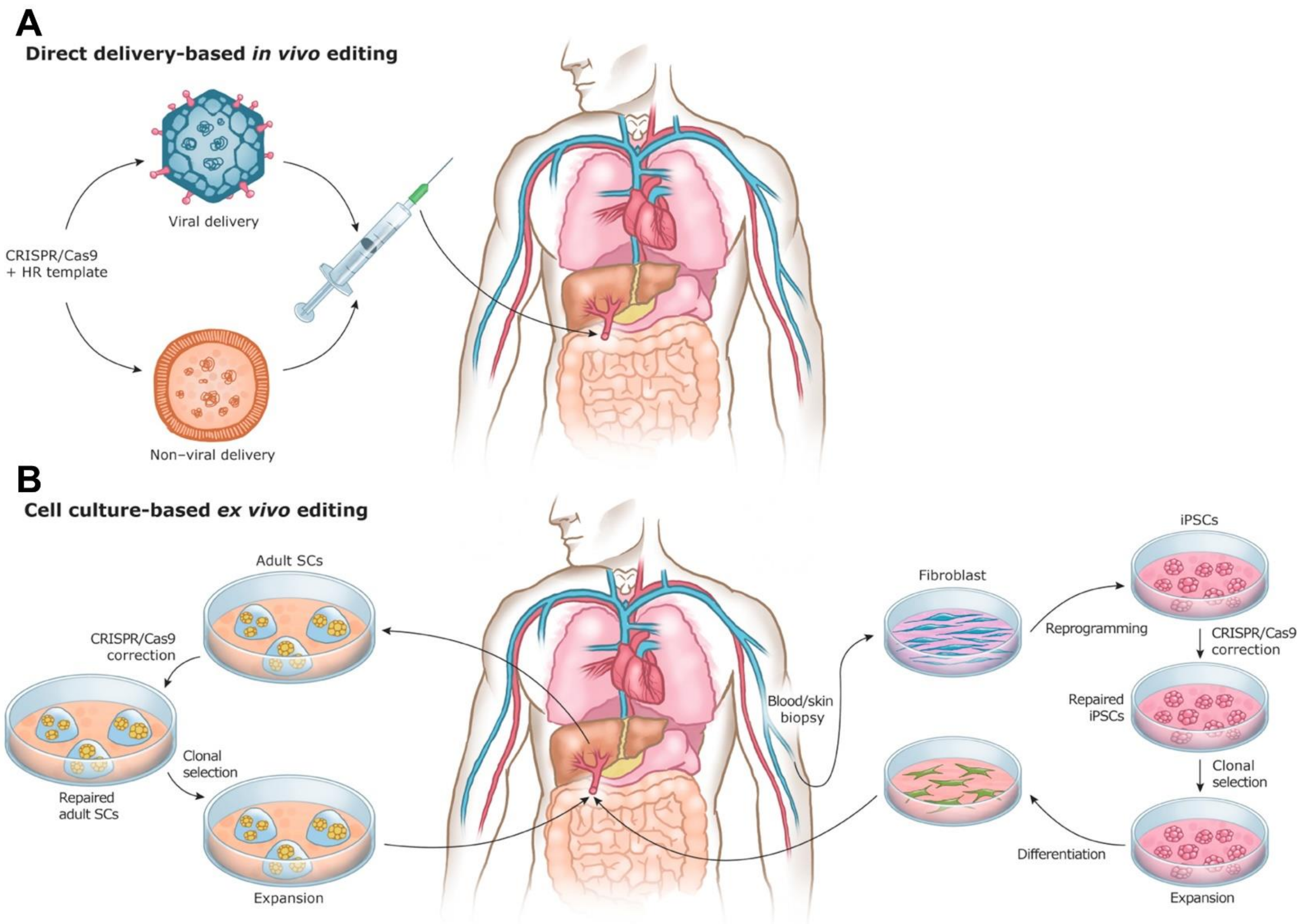
- *Translational Research* 2016 168, 15-21DOI: (10.1016/j.trsl.2015.09.008)



- **The DSB triggers genome editing via 2 different repair mechanisms in eukaryotes**

1. When you have no homologous DNA template DSBs can be repaired by **NHEJ** which leaves small insertions or deletions
2. The presence of a synthetic repair template can be repaired by **HDR** so you can introduce any sequence.
3. Aims to repair disease causing alleles by changing the DNA sequence at the exact location on the chromosome

- *Translational Research* 2016 168, 15-21DOI: (10.1016/j.trsl.2015.09.008)



Translational Research 2016 168, 15-21DOI: (10.1016/j.trsl.2015.09.008)

NHEJ is active throughout the cell cycle. First reaction robust predominant high flexibility. Error prone

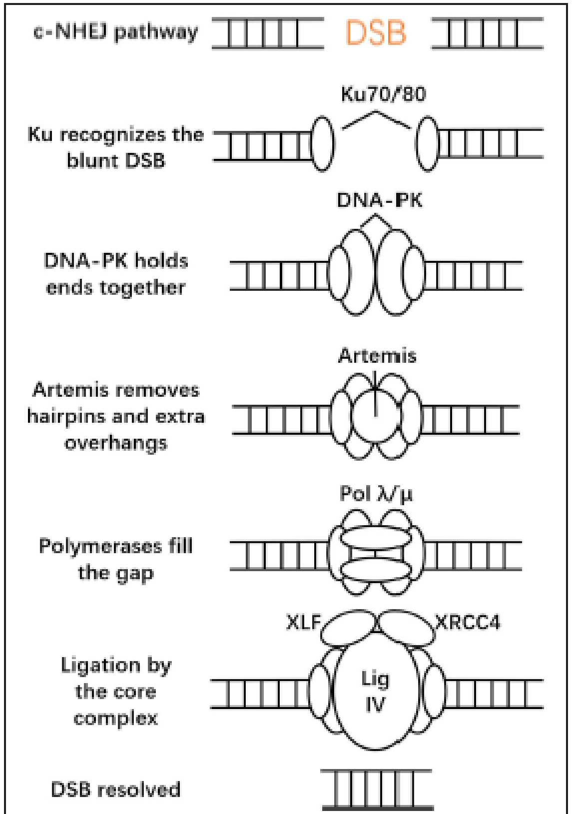


FIGURE 1 | Canonical non-homologous end joining (c-NHEJ). After CRISPR-Cas9 introduced a DSB, NHEJ is initiated by the binding of the Ku heterodimeric complex. This then forms the core complex, which is considered to recognize broken ends and keeps them together. The ends will then be ligated by various ligases.

HDR comes into action mostly in the **S** or **G2** phase of the cell cycle and relies on homologous sequences- sister chromatids

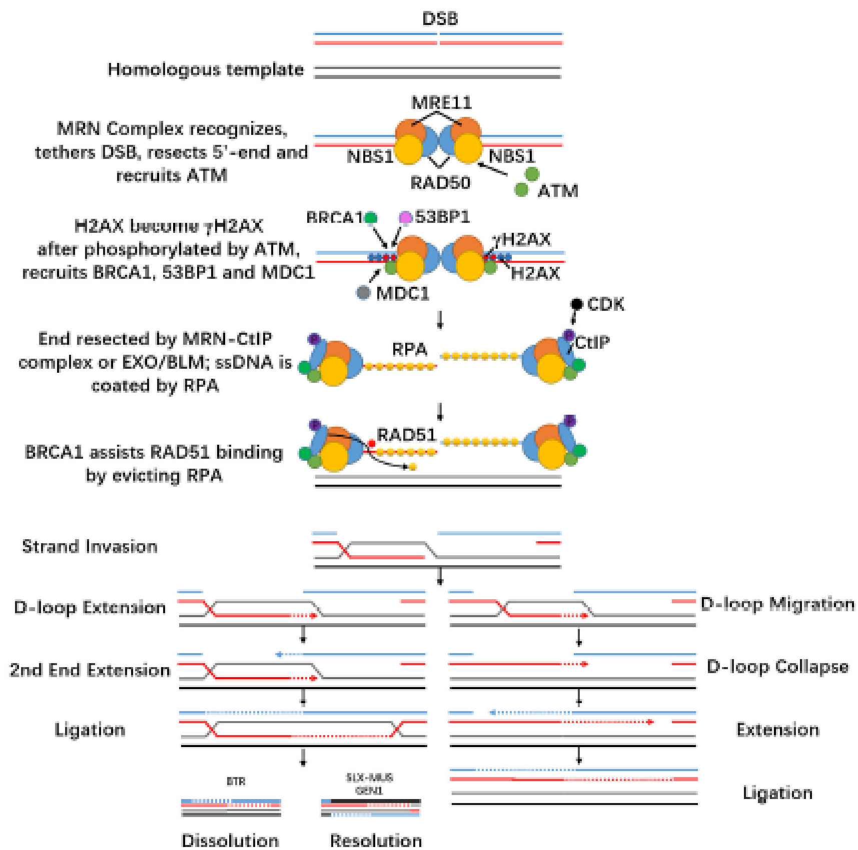
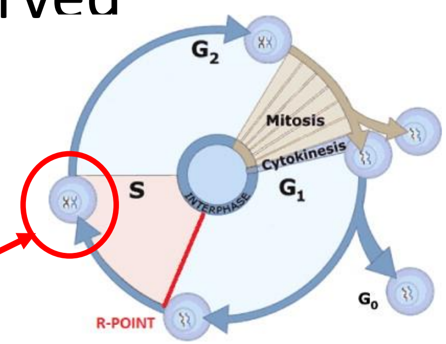


FIGURE 2 | Homology-directed repair HDR. When DSB happens in the S- or G2-phase of the cell cycle and homologous sequence exists near the DSB, DSB can be handled through the HDR pathway if the ends of the DSB are resected. Ends will be coated with various proteins and then invade homologous duplex DNA to form an exchange intermediate: the D-loop structure. Most D-loop structures will be extended by DNA synthesis (dashed arrow). The second end pairs to the D-loop and starts extension. This pathway is called the double Holliday junction pathway. Ligation generates the characteristic double Holliday junction, which may be cleaved by HJ resolvases into either crossover or non-crossover products. The synthesis-dependent strand annealing pathway is illustrated on the right. After D-loop formation, replication and branch migration take place which can lead to D-loop translocation. The translocating D-loop is unstable and collapses easily. After collapse, the extended first end may anneal to complementary ssDNA in the resected second end. Replicative extension of both ends and ligation generates non-crossover products.

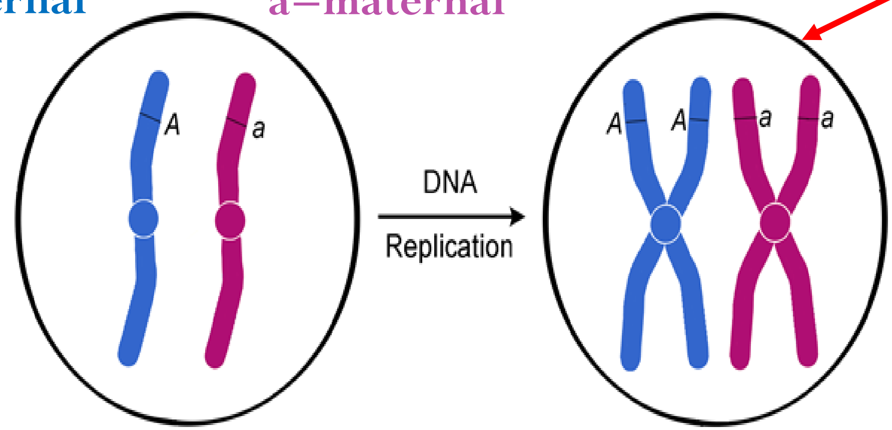
https://www.frontiersin.org/articles/10.3389/fgene.2018.00691/full

Stages of Interphase: S phase

- S phase – The period during which DNA is synthesised **(replicated)**
  - The **S** represents **synthesis**
- Create exactly two identical semi-conserved chromosomes
- Detect and fix DNA damage



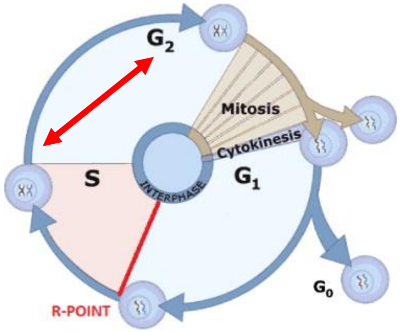
A= paternal a=maternal



Doubling of DNA but the cell is still **2n**, meaning there are still two sets of chromosomes

Stages of Interphase: G_2 phase

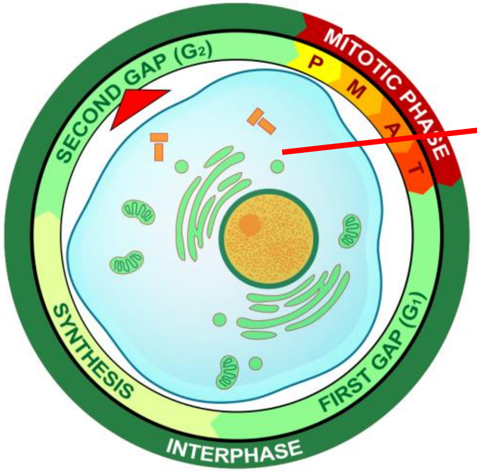
- **G_2 phase** – final subphase of Interphase in the cell cycle **directly preceding Mitosis**



Prolific growth ready for Mitosis

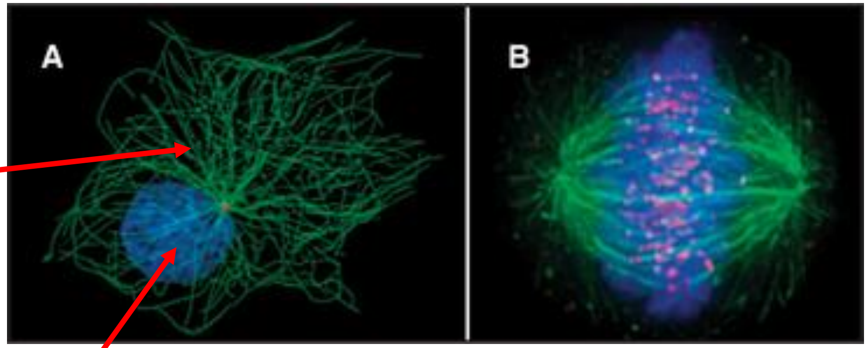
*Organelles are produced

*Increase volume of the cytoplasm



Microtubule formation

Interphase G_2 **Mitosis**



Loosely aggregated

Replicated Chromosomes indistinct and nuclear envelope intact

https://clinicalgate.com/macromolecular-assembly/

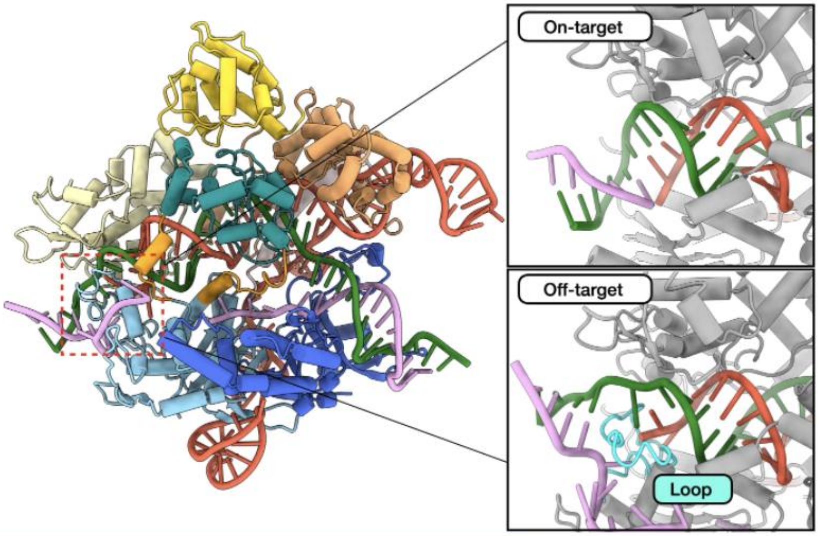
The HDR pathway is favoured – specific template guided repair

- 1. Favour HDR using chemical modification- inhibit key enzymes of NHNJ
- 2. Timed delivery of the CRISPR-Cas9 system: synchronise and capture cells as S and G2 phases or use timed delivery
- 3. Other enhancements such as modified Cas9

https://www.frontiersin.org/articles/10.3389/fgene.2018.00691/full

Why is CRISPR so error prone?

- The chimeric single-guide RNA (sgRNA) contains a 20nt guide sequence directs Cas9 to the genomic target- multiple mismatches between the guide and the target can be tolerated, but leads to off target DSBs
- If it finds one where 18/20 match it can make the edit anyway
- Cryo-electron microscopy showed a strange finger-like structure that reached out and stabilised the DNA so Cas9 could make its edit.
- The reserachers tweaked Cas9 so it pushes the finger structure away. **SuperFi-Cas9! 4k more precise**
- So far only tested *in vitro*



In this molecular model, CRISPR makes an edit to a DNA sequence (red and green). In an on-target edit (top right), the target sequence will match the guide (pink), but in an off-target edit (bottom right), it will almost match, and a finger structure (cyan) will intervene and stabilize the DNA to make the edit anyway. Jack Bravo/University of Texas at Austin

https://newatlas.com/science/crispr-gene-editing-error-correction-protein/#:~:text=Protein%20tweak%20makes%20CRISPR%20gene%20editing%204%2C000%20times%20less%20error%2Dprone,-By%20Michael%20Irving&text=In%20this%20molecular%20model%2C%20CRISPR,sequence%20(red%20and%20green).

Structural basis for mismatch surveillance by CRISPR–Cas9
Jack P. K. Bravo, Mu-Sen Liu, Grace N. Hibshman, Tyler L. Dangerfield, Kyungseok Jung, Ryan S. McCool, Kenneth A. Johnson & David W. Taylor
Nature volume 603, pages343–347 (2022)

Current Ethical debates:

- first ‘CRISPR babies’
- https://www.nature.com/articles/d41586-022-00512-w
- Disputes about who invented it

https://www.nature.com/articles/d41586-022-00629-y

Amazing/strange things done with CRISPR

https://www.cnet.com/science/how-crispr-could-save-6-billion-chickens-from-the-meat-grinder/

https://onezero.medium.com/the-7-craziest-ways-crispr-is-being-used-right-now-bcf3bd203f23

https://www.labiotech.eu/best-biotech/crispr-applications-gene-editing/

CRISPER gene editing

https://www.youtube.com/watch?v=4YKFw2KZA5o