Gene therapy and ‘genetic surgery’

1) Pioneering gene therapy with retroviruses: successes and failures

2) Precision genome engineering: site-specific genome cleavage and repair

Guide to readings: Gene therapy

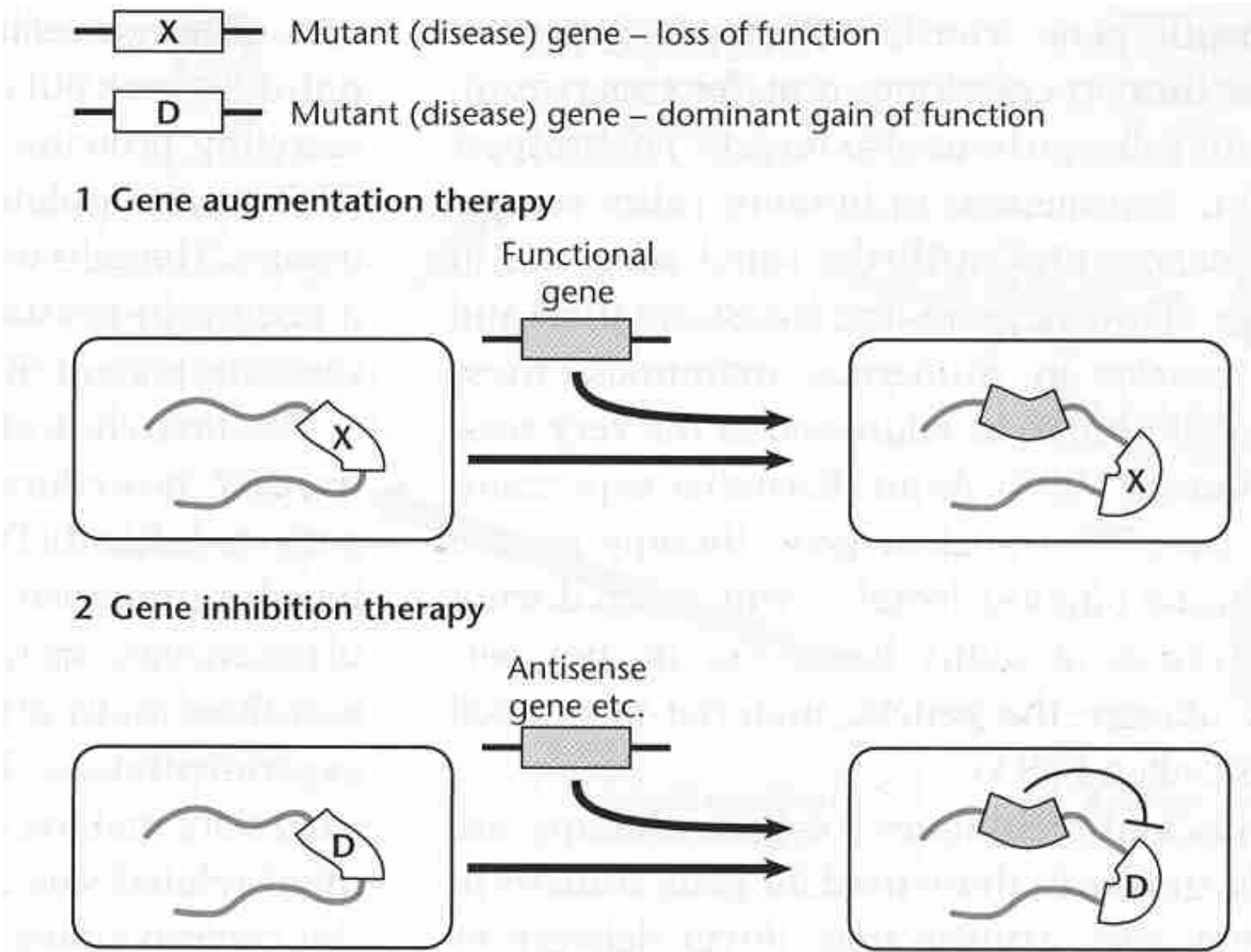
1) SCID Gene therapy. The original report of success in SCID treatment (2000)

2) Future of gene therapy. Perspectives on early gene therapy attempts (2004)

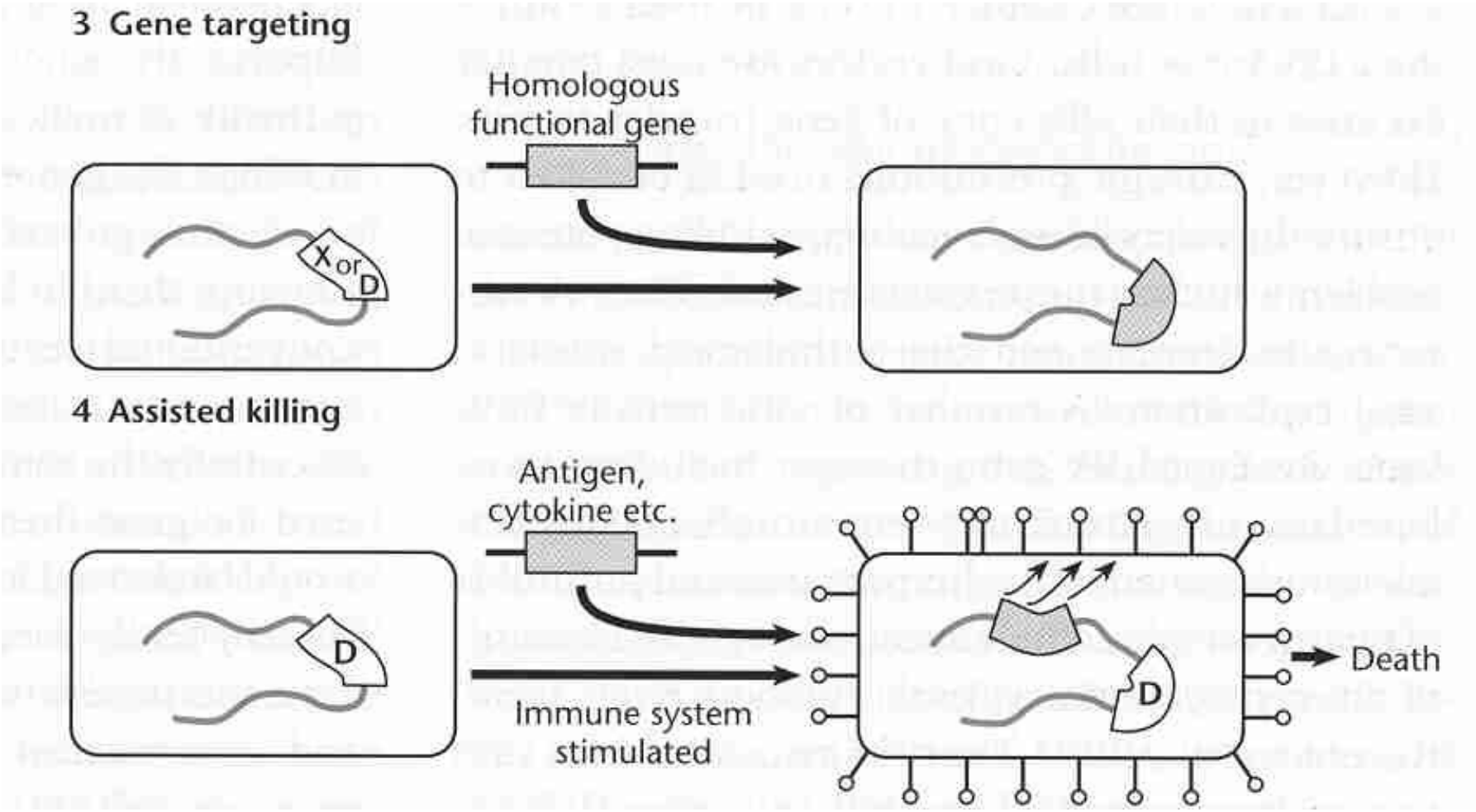
3) Gene therapy state of the art 2011. Review of the field.

4) Programmable nucleases 2014

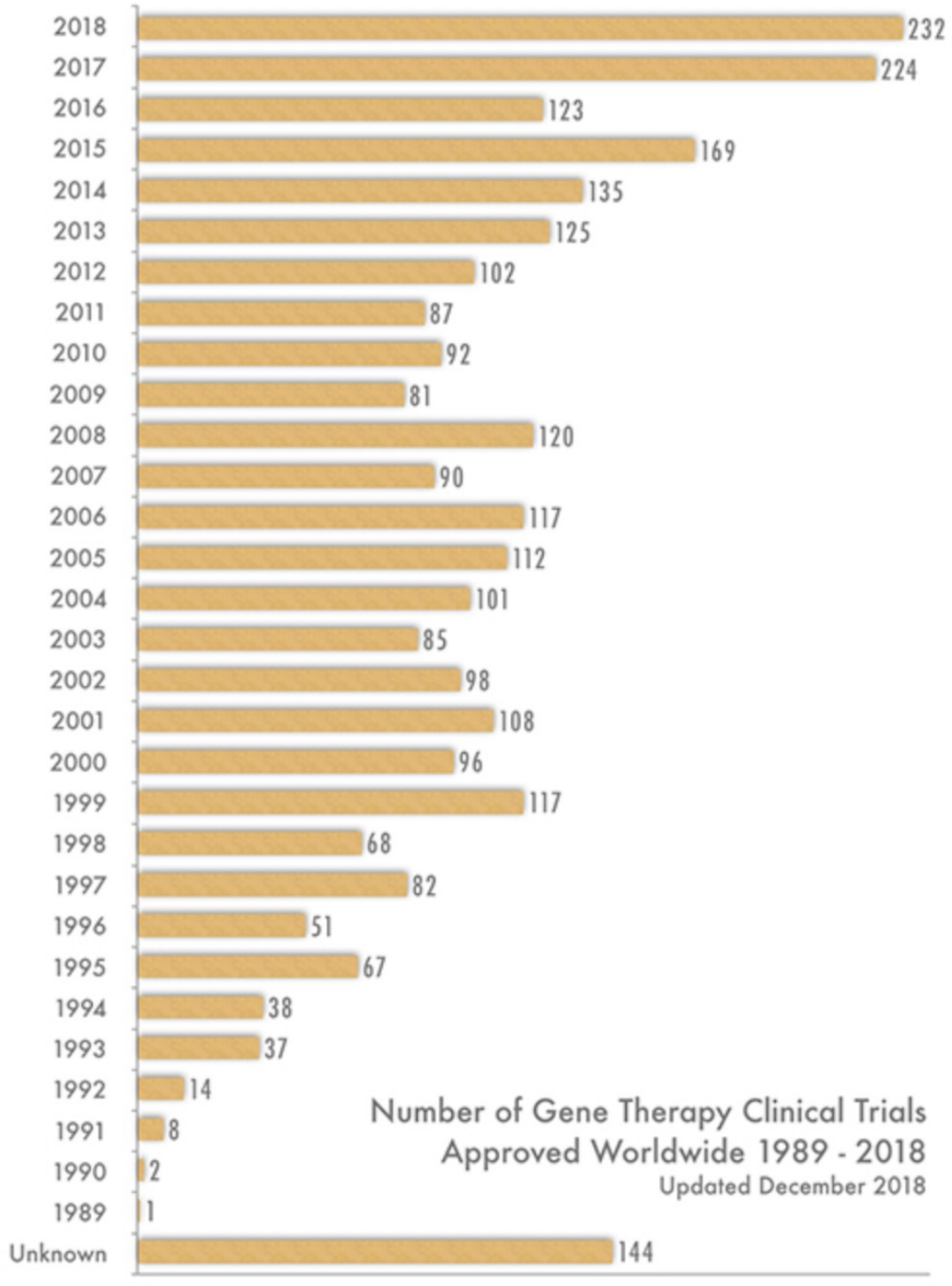
Gene therapy: treating a disease by adding a new gene



Gene therapy: treat a disease by adding a new gene



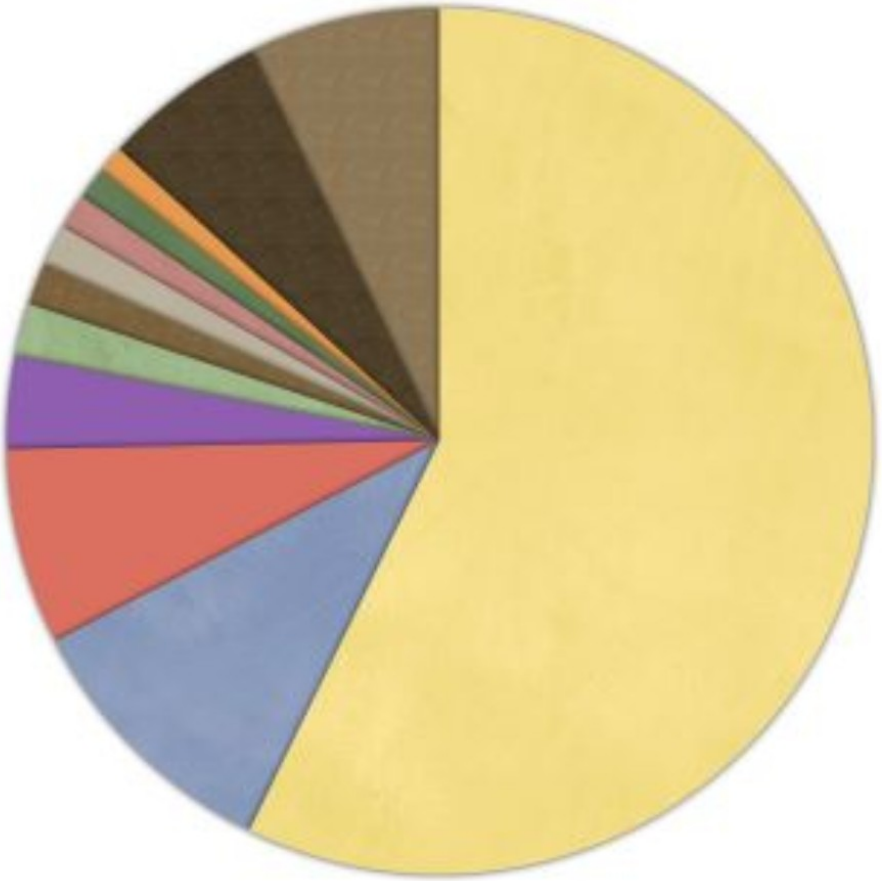
Gene therapy trials worldwide



http://www.abedia.com/wiley/

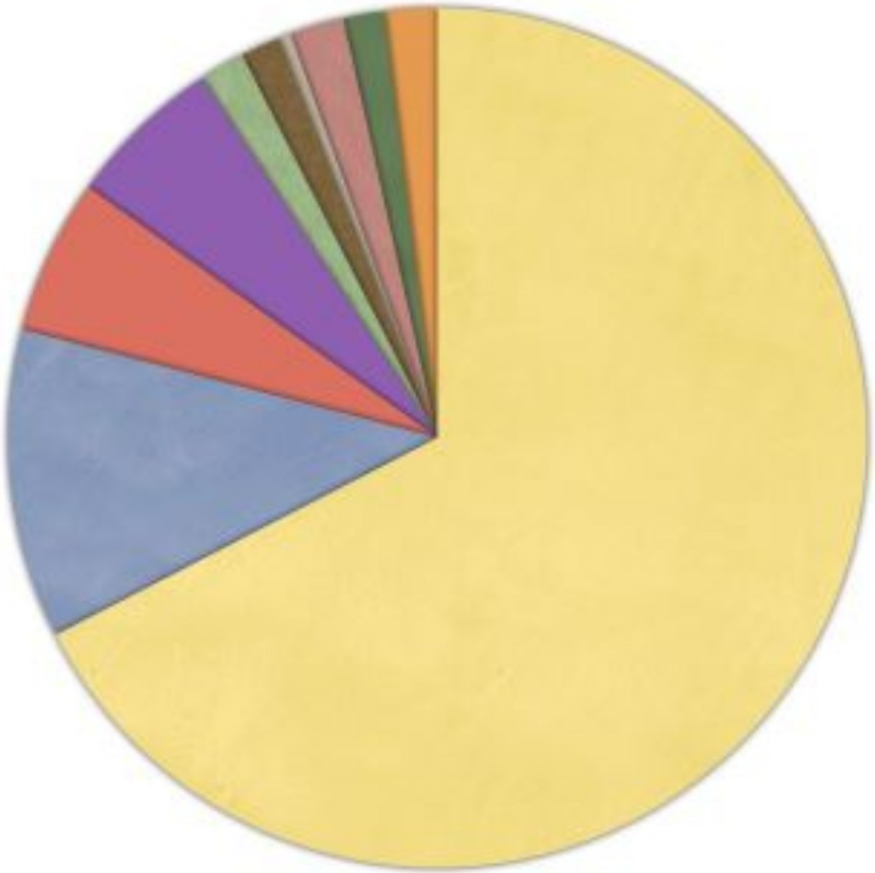
Geographical Distribution of Gene Therapy Clinical Trials

By Country



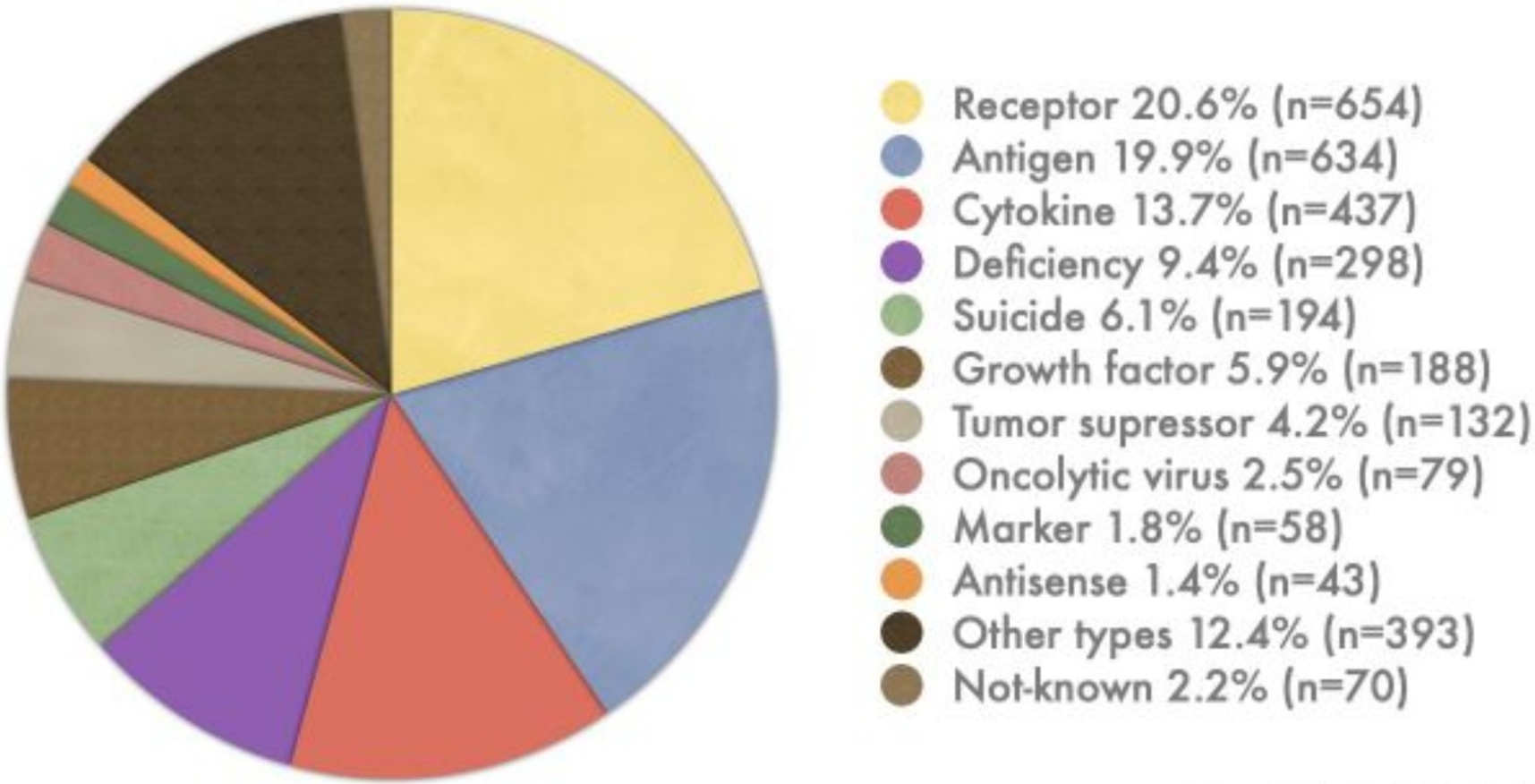
- United States 57.2% (n=1820)
- China 10.1% (n=322)
- United Kingdom 7.3% (n=233)
- Germany 3.5% (n=110)
- France 2.0% (n=63)
- Switzerland 1.6% (n=50)
- Japan 1.5% (n=48)
- Spain 1.3% (n=42)
- Netherlands 1.2% (n=38)
- Australia 1.0% (n=33)
- Other countries 6.1% (n=194)
- Multi-country 7.1% (n=227)

Indications Addressed by Gene Therapy Clinical Trials

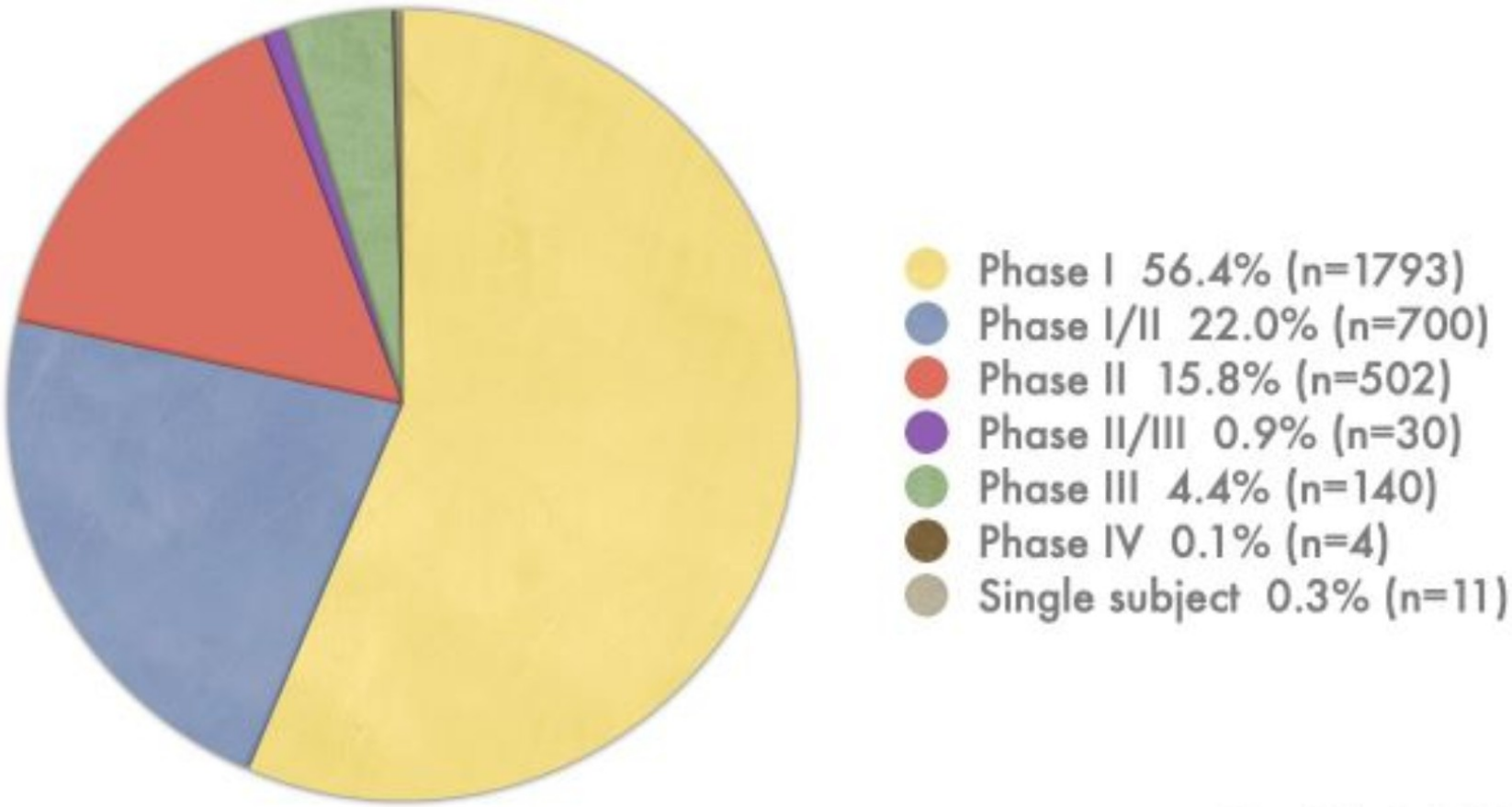


- Cancer diseases 67.4% (n=2144)
- Monogenic diseases 11.6% (n=370)
- Cardiovascular diseases 5.8% (n=186)
- Infectious diseases 5.8% (n=186)
- Neurological diseases 1.7% (n=55)
- Ocular diseases 1.5% (n=47)
- Inflammatory diseases 0.5% (n=15)
- Other diseases 2.0% (n=65)
- Gene marking 1.5% (n=49)
- Healthy volunteers 2.0% (n=63)

Gene Types Transferred in Gene Therapy Clinical Trials



Clinical Phases of Gene Therapy Clinical Trials



Phase I: Researchers test a new drug or treatment in a small group of people for the first time to evaluate its safety, determine a safe dosage range, and identify side effects.

Phase II: The drug or treatment is given to a larger group of people to see if it is effective and to further evaluate its safety.

Phase III: The drug or treatment is given to large groups of people to confirm its effectiveness, monitor side effects, compare it to commonly used treatments, and collect information that will allow the drug or treatment to be used safely.

Phase IV: Studies are done after the drug or treatment has been marketed to gather information on the drug's effect in various populations and any side effects associated with long-term use.
https://www.nlm.nih.gov/services/ctphases.html

Gene therapy vectors

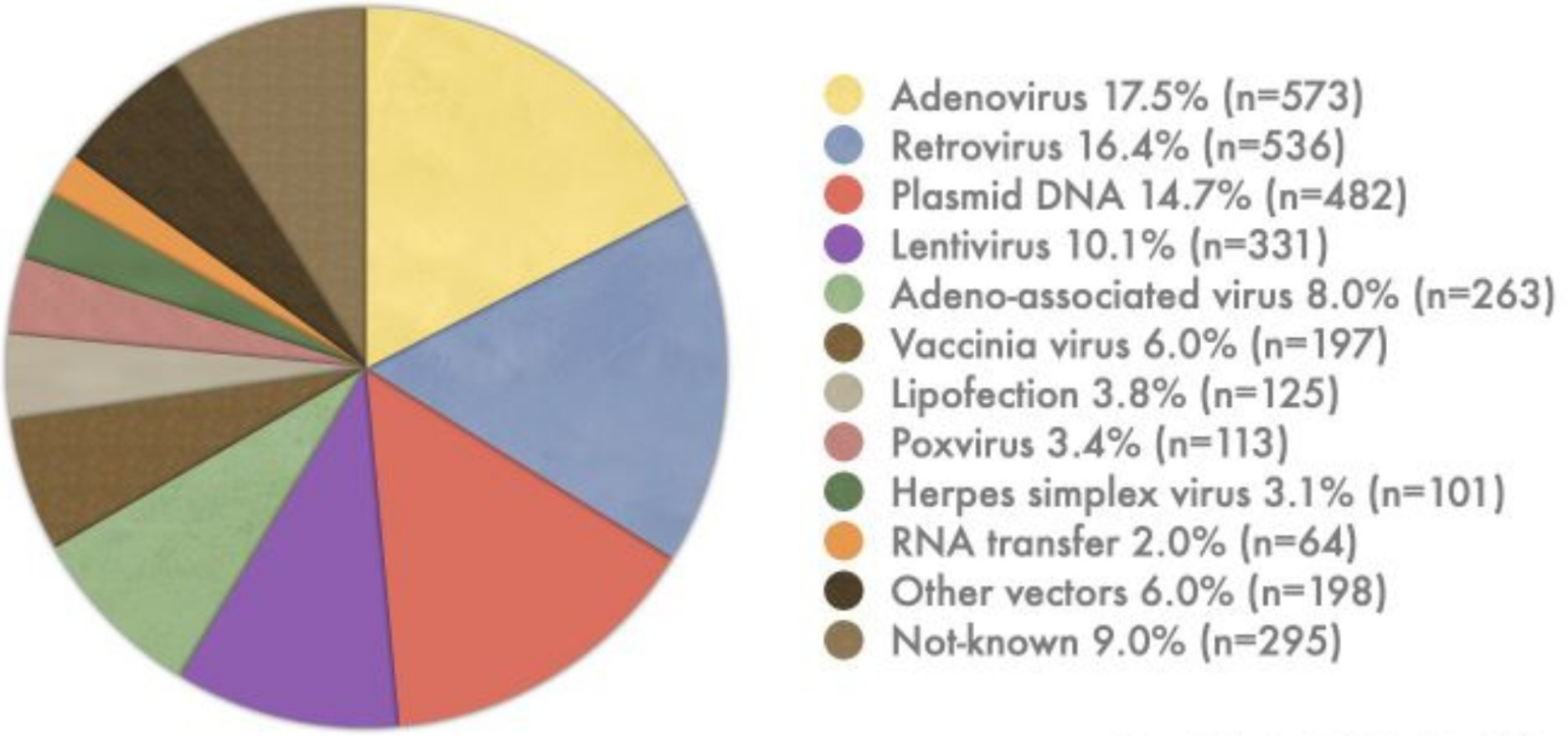
Viral: Most widely used, the viral infection and integration/replication modes bring high efficiency of transformation

- Retrovirus (actively dividing cells, semi-random integ.)
- Lentivirus (all cells, semi-random integration)
- Adenovirus (relatively toxic, replicates episomally)
- Adeno-associated virus (easy to purify, infects all cells, but DNA insert size limited to 4 kb)

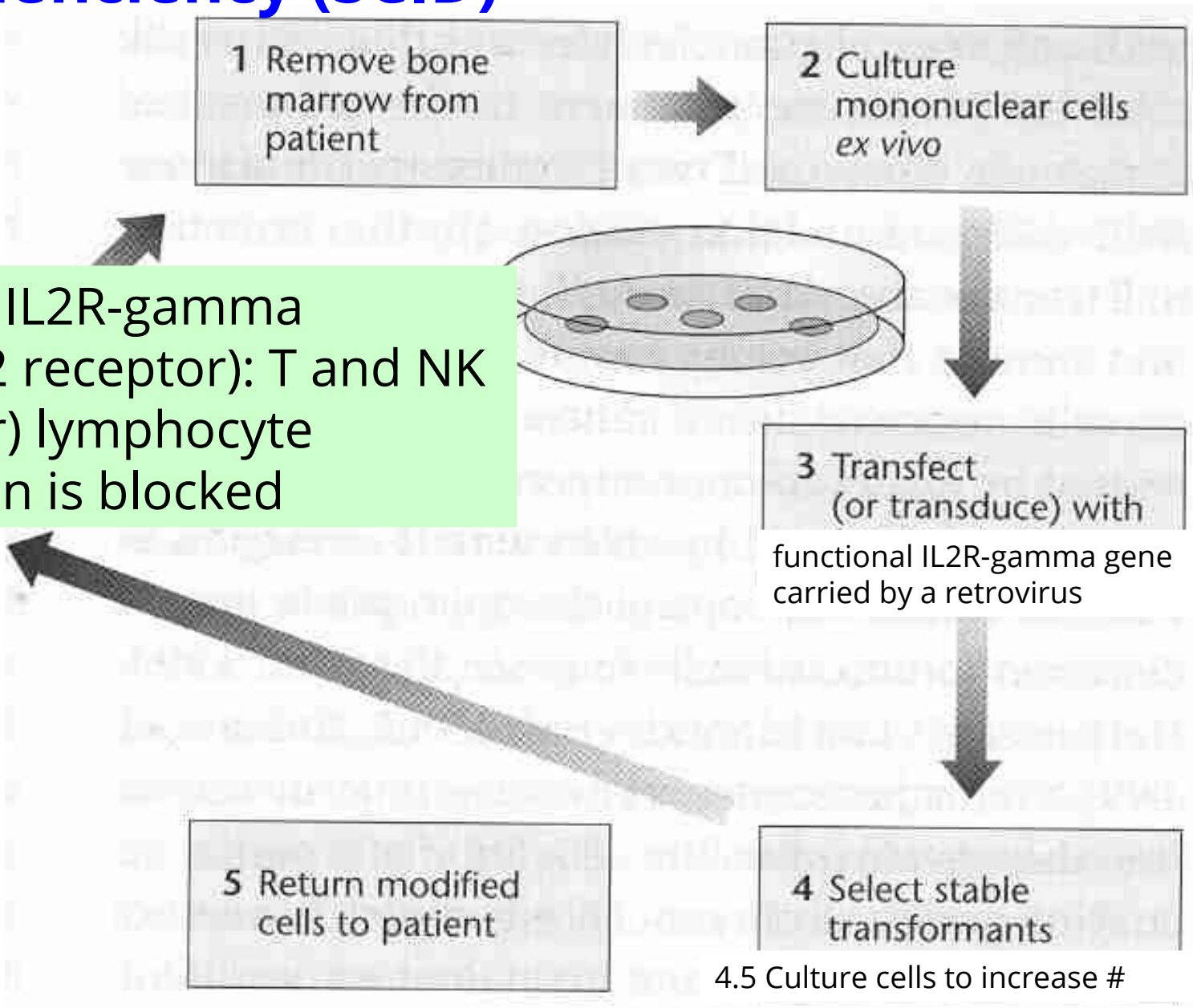
Non-viral: inefficient, because DNA must be delivered somehow (e.g. liposomes), Mainly *ex vivo* because of their low efficiency. Immune responses less of a problem, though

- Plasmids
- Minicircles (lacking unwanted plasmid DNA)

Viral transduction in ~70% of clinical trials



Ex vivo gene therapy for severe combined immunodeficiency (SCID)



Mutations in IL2R-gamma (interleukin-2 receptor): T and NK (natural killer) lymphocyte differentiation is blocked

Problems with gene therapy of SCID patients:

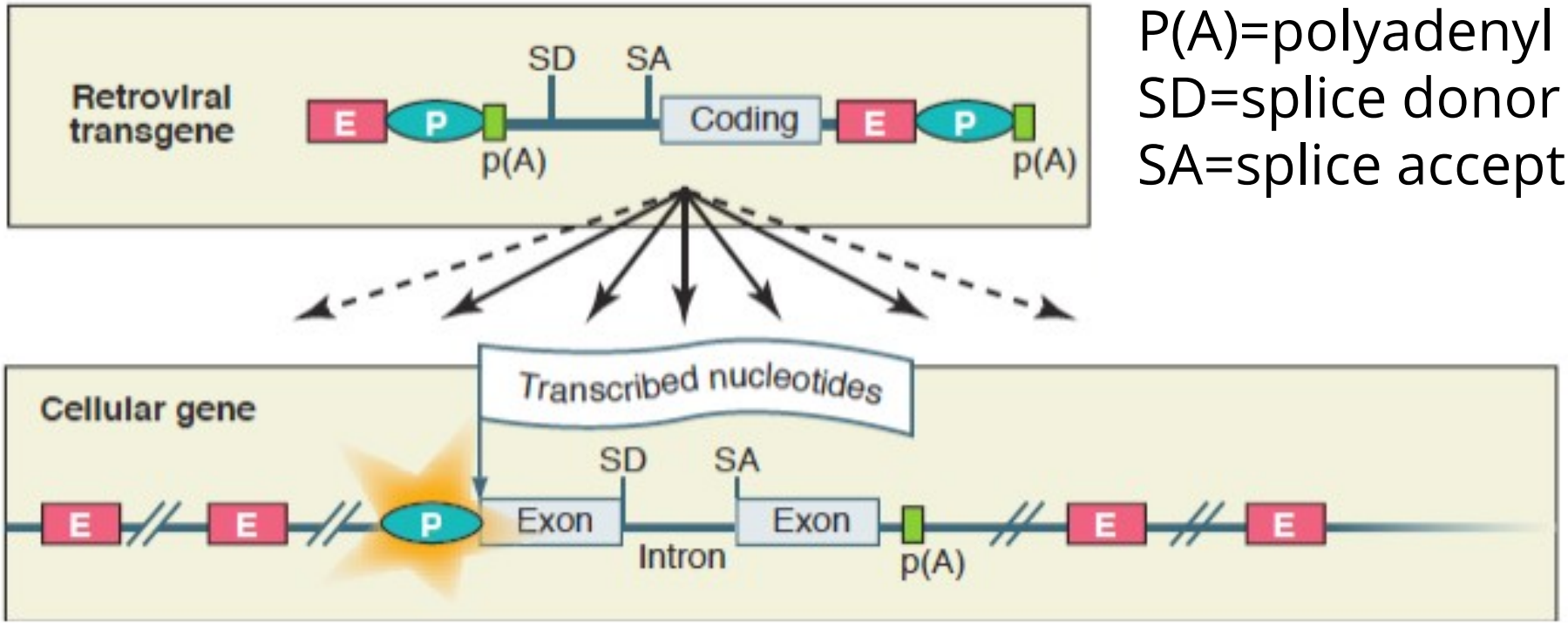
- 10 patients: bone marrow stem cells treated with retrovirus containing IL2R-gamma
- retrovirus inserted randomly into a large population of stem cells (lots of potential insertion sites)

- 9/10 patients survived an otherwise fatal disease
- BUT 2/9 developed leukemia because of insertion of virus near a proto-oncogene. The enhancers in the retroviral vector probably caused overexpression of oncogene (dominant effect)

- Oncogene dysregulation: in about 0.1 to 1% of all retroviral insertion events (or 0.01%, depends on who you ask)
- Oncogene activated cells may grow faster (selection)
- Expression of IL2R- gamma may itself be oncogenic (although this is debatable)

Retroviral integration: semi-random, but most commonly integrates near expressed genes

E=enhancer
P=promoter
P(A)=polyadenyl
SD=splice donor
SA=splice acceptor



The proto-oncogene LMO-2 was inadvertantly up-regulated in the two patients that developed leukemia

Williams & Baum (2003) *Science* **302** p. 400.

What to do about the oncogene activation problem?

- retrovirus vectors have their problems, but no better alternatives exist yet
- all medicine has side effects
- benefits still outweigh problems (recent studies: 17/18 SCID patients treated are currently alive, compared to 75% success rates of bone marrow transplants)
- clinical trials on many patients would assist in improving treatment

Cavazzana-Calvo et al. (2004)*Nature* **427**:779-81.

Following up on gene therapy using retroviruses

- Gamma retroviral insertion alters nearby growth-regulation genes by insertional mutagenesis (activation)
- 'self-inactivating vectors (mutations in U3 region of LTR) are less mutagenic in vitro and in vivo (the insertional pattern is different)
- However, self inactivating vectors do not express transgene at high enough levels
- Cancer development may also depend on other poorly understood factors: transduction protocol, target cell population characteristics
- Still more work…

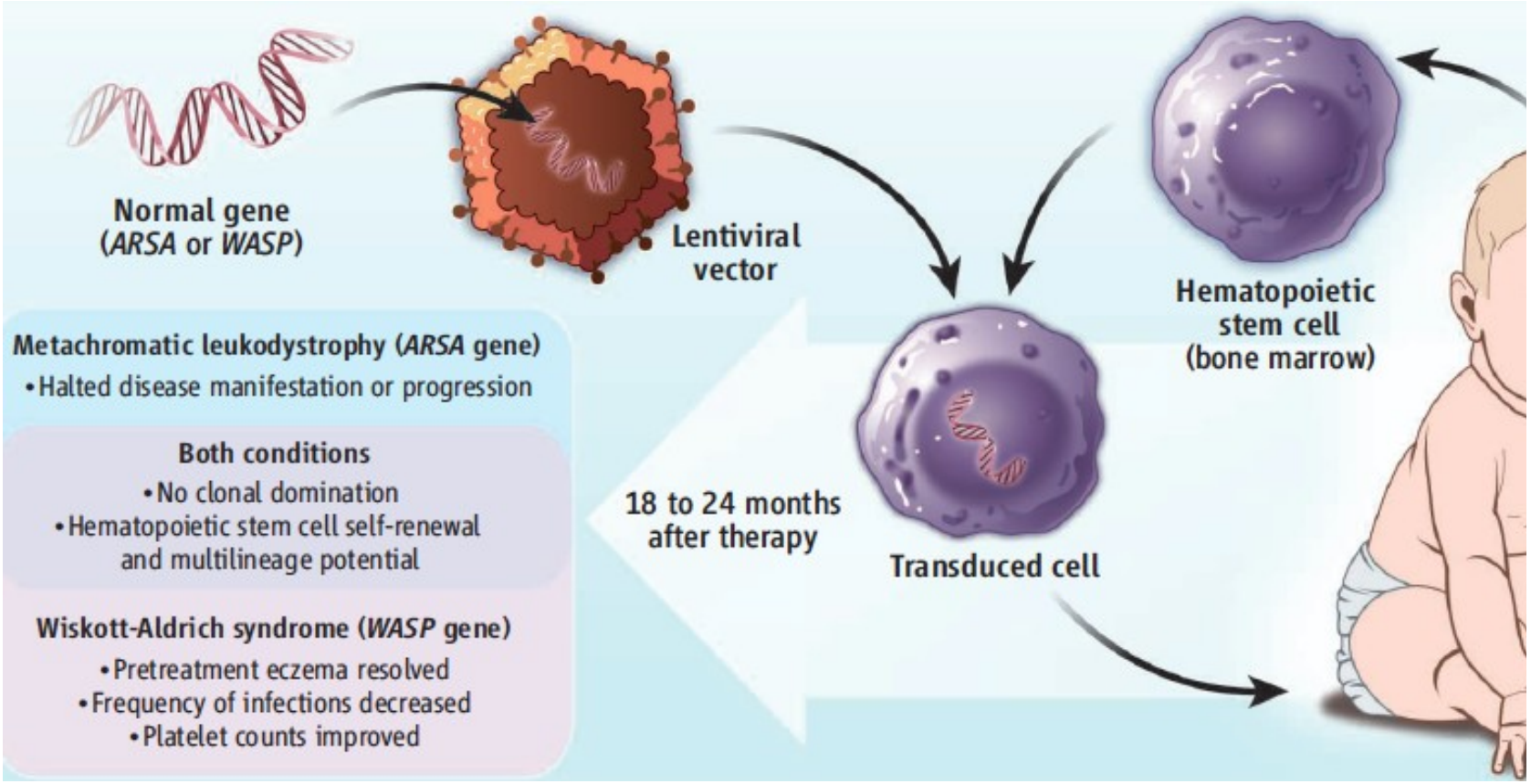
Cavazzana-Calvo (2009) *Gene Therapy* **16** p. 309.

New report on IL2R gamma gene therapy (2019)

- Lentiviral, not retroviral vector
- Engineered to have insulator sequences near end to prevent activation of nearby genes
- Patients pre-treated with busulfan (kills off immune cells) to create niche for engineered cells to grow in
- 10 infants were treated who had no sibling matches for bone marrow transplant
- All could leave isolation and go home within 3-4 months of treatment

- Long term results not yet available

More recent successes in gene therapy: single gene disorders *metachromatic leukodystrophy* and *Wiskott-Aldrich syndrome*



- Lentivirus-mediated, *ex vivo*
- No "clonal domination" was seen
- Random integration of vector (in contrast to retroviral vectors), less likely to cause abnormally proliferating cells

"Gene Therapy That Works" Science 341, 853 (2013); DOI: 10.1126/science.1242551

Important factors for gene therapy in the clinic

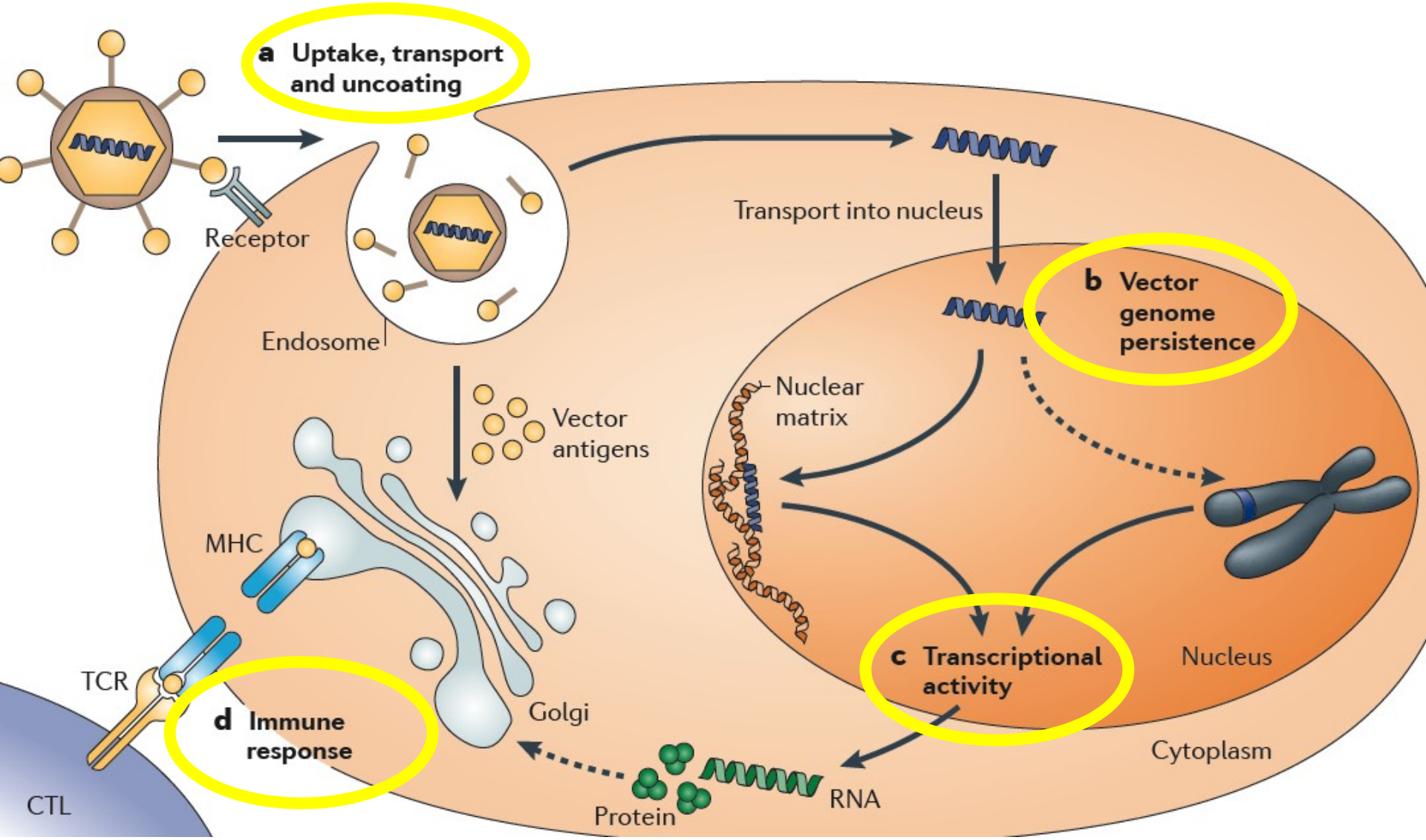
1) Production of (mostly viral) vectors is costly

2) Vector targeting/tissue specificity

   - Target cells must be treated, non-target cells must not be harmed
   - Transcription signals (promoters) must be specific for cell types that are targeted
   - It is difficult to direct vectors only to target cells
   - virus targeting is difficult to control/change

Waehler et al. (2007), Nat. Rev. Gen. ***8*** *p. 573*

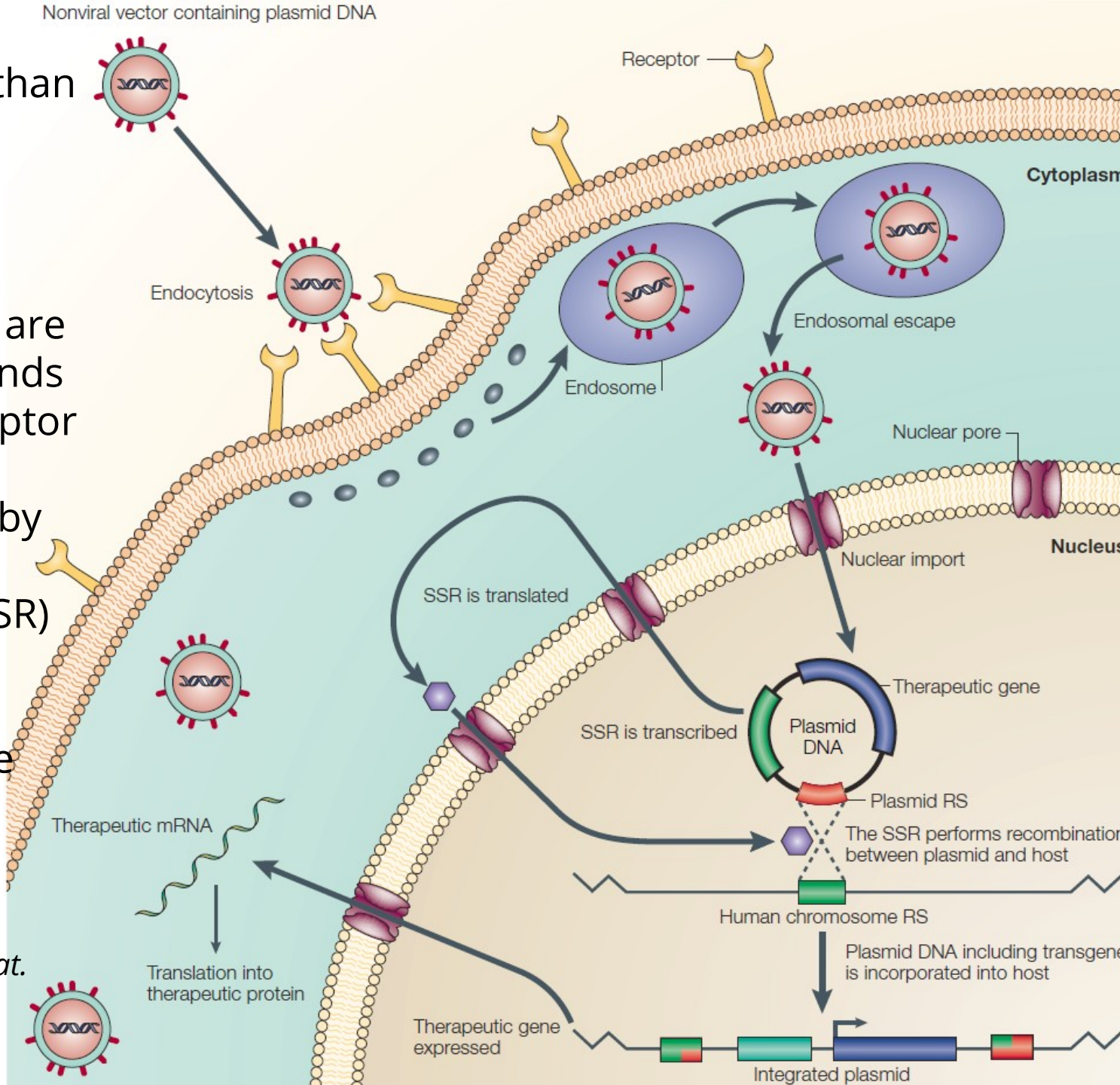
Obstacles to effective gene delivery in eukaryotic cells



Non-viral gene delivery: safer than retroviral integration?

Liposomes containing DNA are coated with ligands for specific receptor

DNA integrates by site specific recombinase (SSR) with naturally occurring pseudosites (like loxP)



Glover et al. (2005) *Nat. Rev. Genetics* **6** p. 299.

Another approach to gene insertion: targeted recombination induced by DNA breaks

DNA can be inserted by homologous recombination (gene targeting)

- Inefficient: won't happen in most cells receiving DNA
- Requires selection in culture (e.g. stem cells)

Efficiency of homologous recombination by nicking or breaking the genome at the region you want to engineer ("genome surgery")

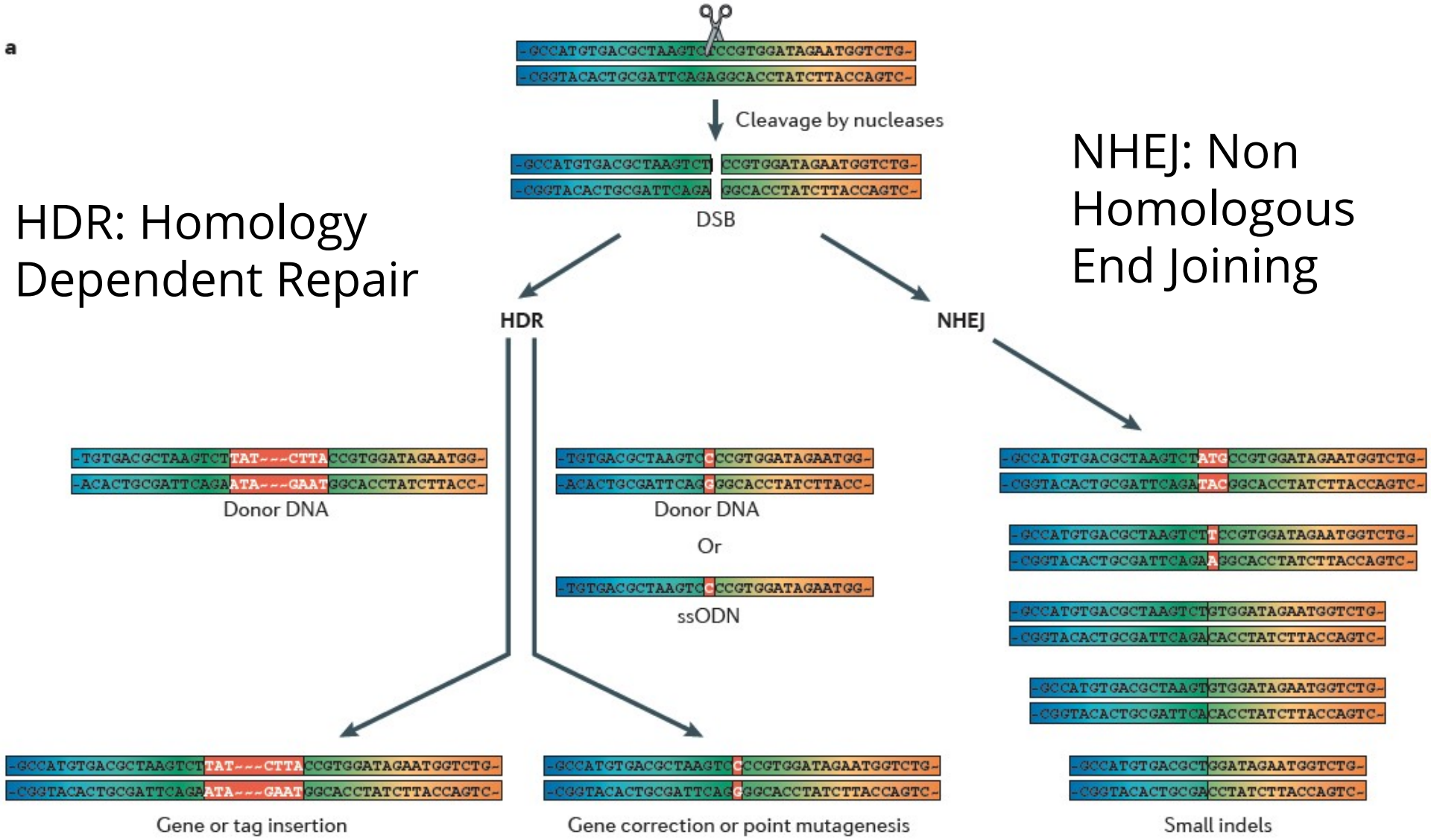
1. DNA recognition
2. DNA cleavage

- Make a machine that can do both, with selectivity for only one site in the genome

What happens after a double stranded break in DNA?

HDR: Homology Dependent Repair

NHEJ: Non Homologous End Joining



A guide to genome engineering with programmable nucleases

Hyongbum Kim[1] and Jin-Soo Kim[2,3]

NATURE REVIEWS | GENETICS
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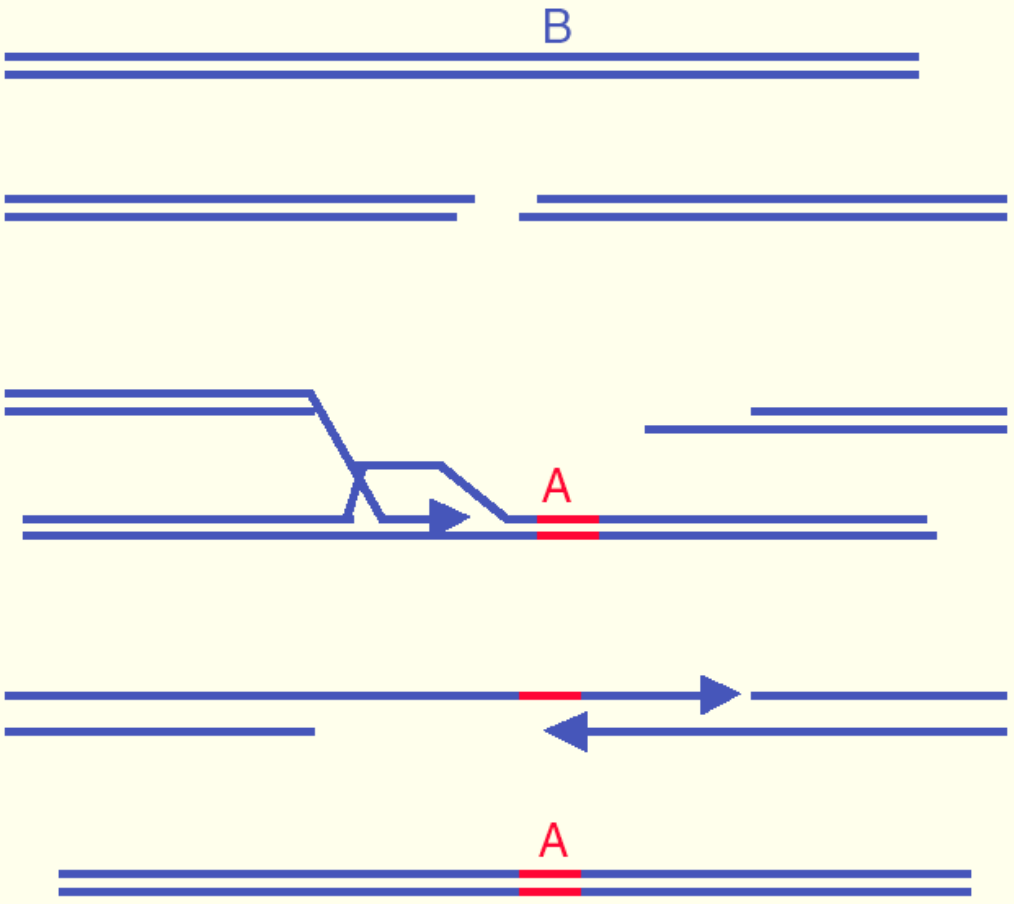
Double stranded breaks and homologous recombination



Undamaged DNA (allele B)

DSB created (spontaneous or induced, e.g., by ZFN)

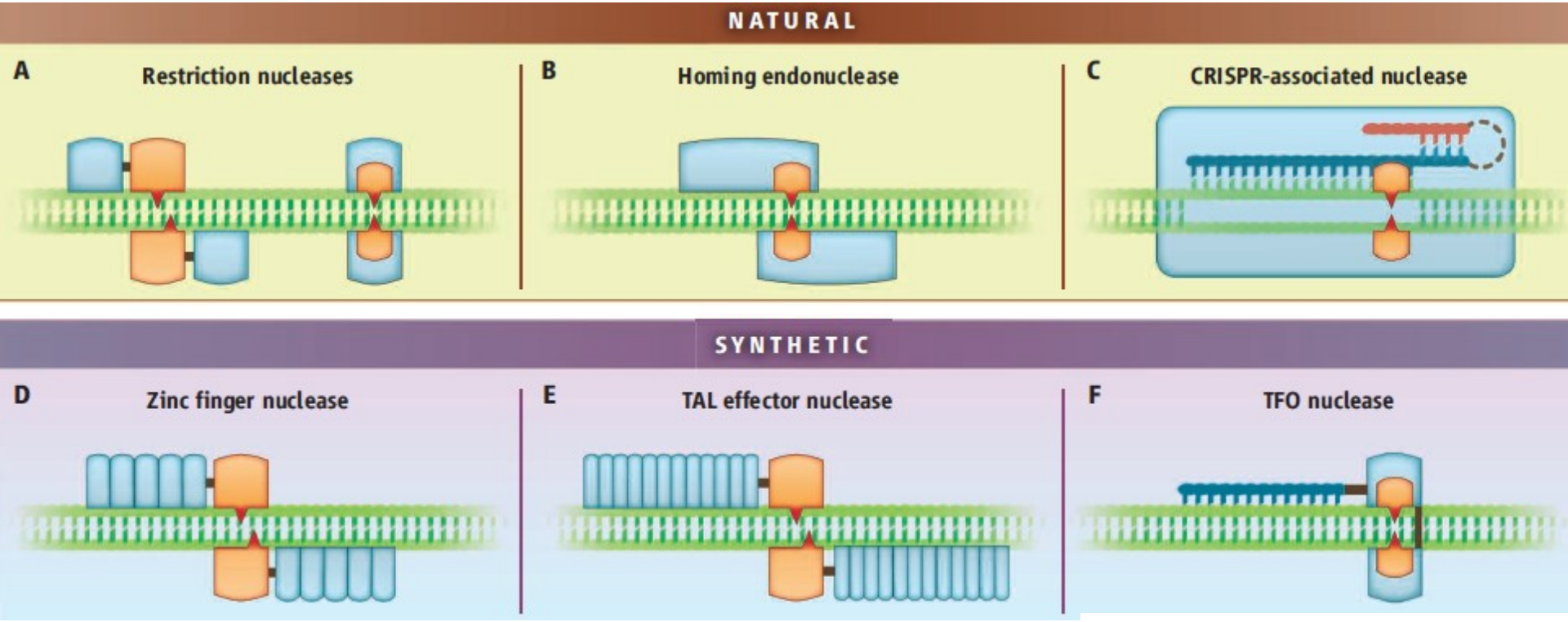
Strand invasion into undamaged homologous DNA (allele A)

In gene targeting, exogenous DNA serves as homologous DNA donor

Repairing of original strands of DNA
Gaps filled by DNA polymerase and nicks sealed by DNA ligase

Conversion of blue allele (B) into red allele (A) in region of DSB

Several strategies for defining specific DNA cut sites



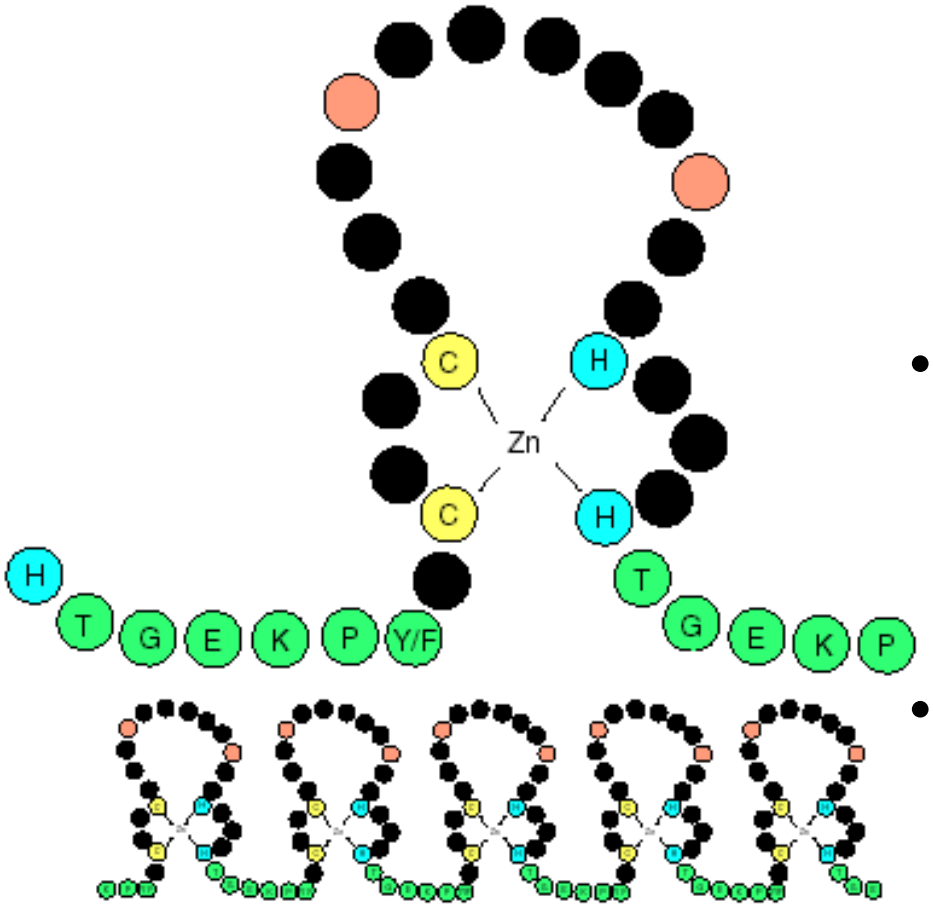
Blue: DNA recognition, Orange: DNA nuclease

New Tool for Genome Surgery
John van der Oost
Science **339**, 768 (2013);
DOI: 10.1126/science.1234726

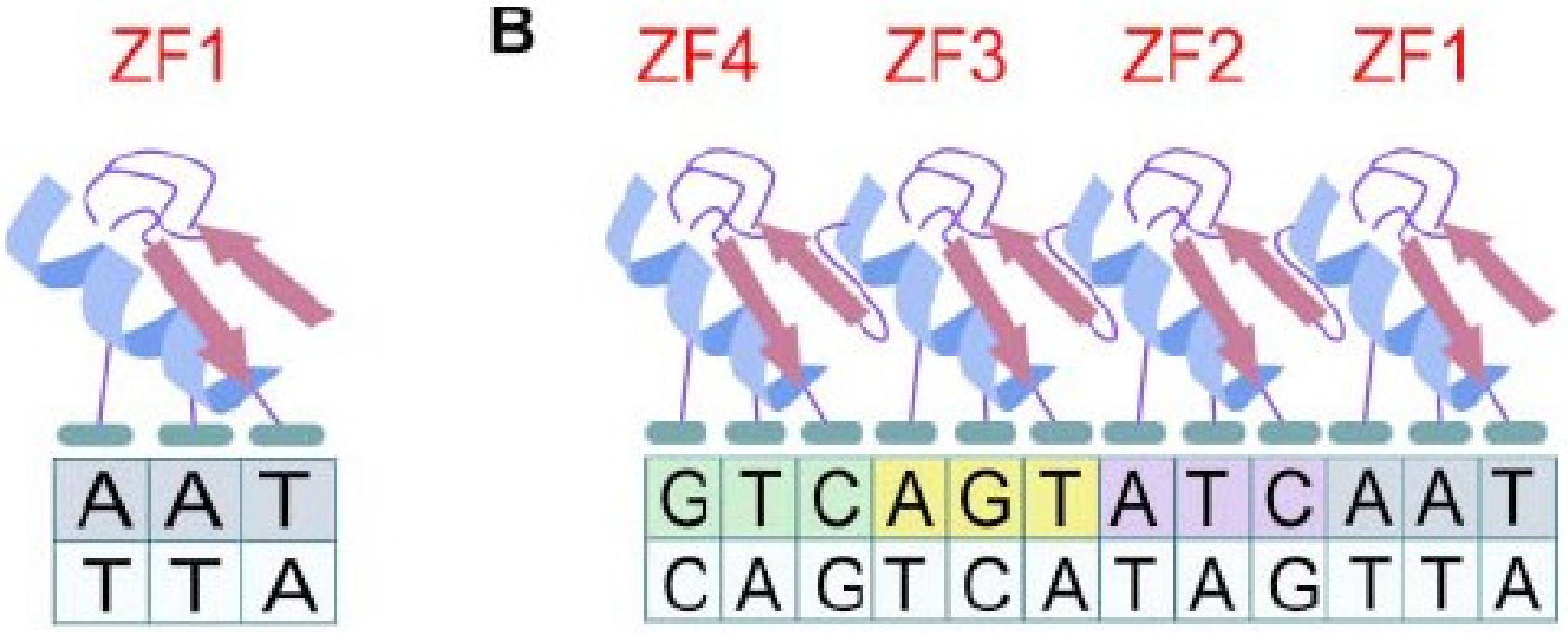
To introduce mutations, co transfect with

A) programmable nuclease
B) Targeting vector (containing desired DNA)

Zinc finger transcription factors



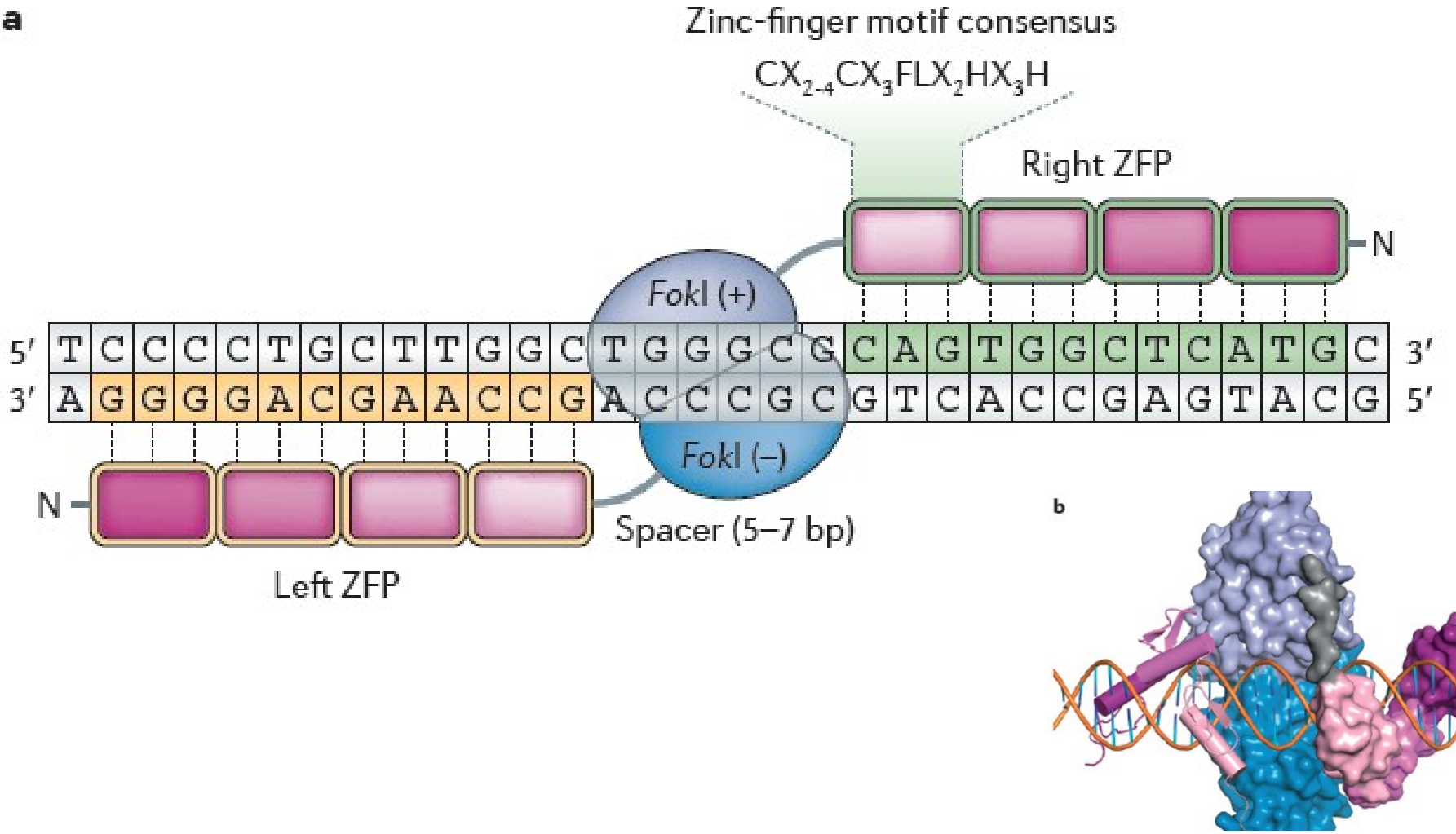
- DNA binding domains stabilized by Zn^{+2} binding
- Zn-ribbons fused together can recognize longer DNA sequences



Single Zn finger

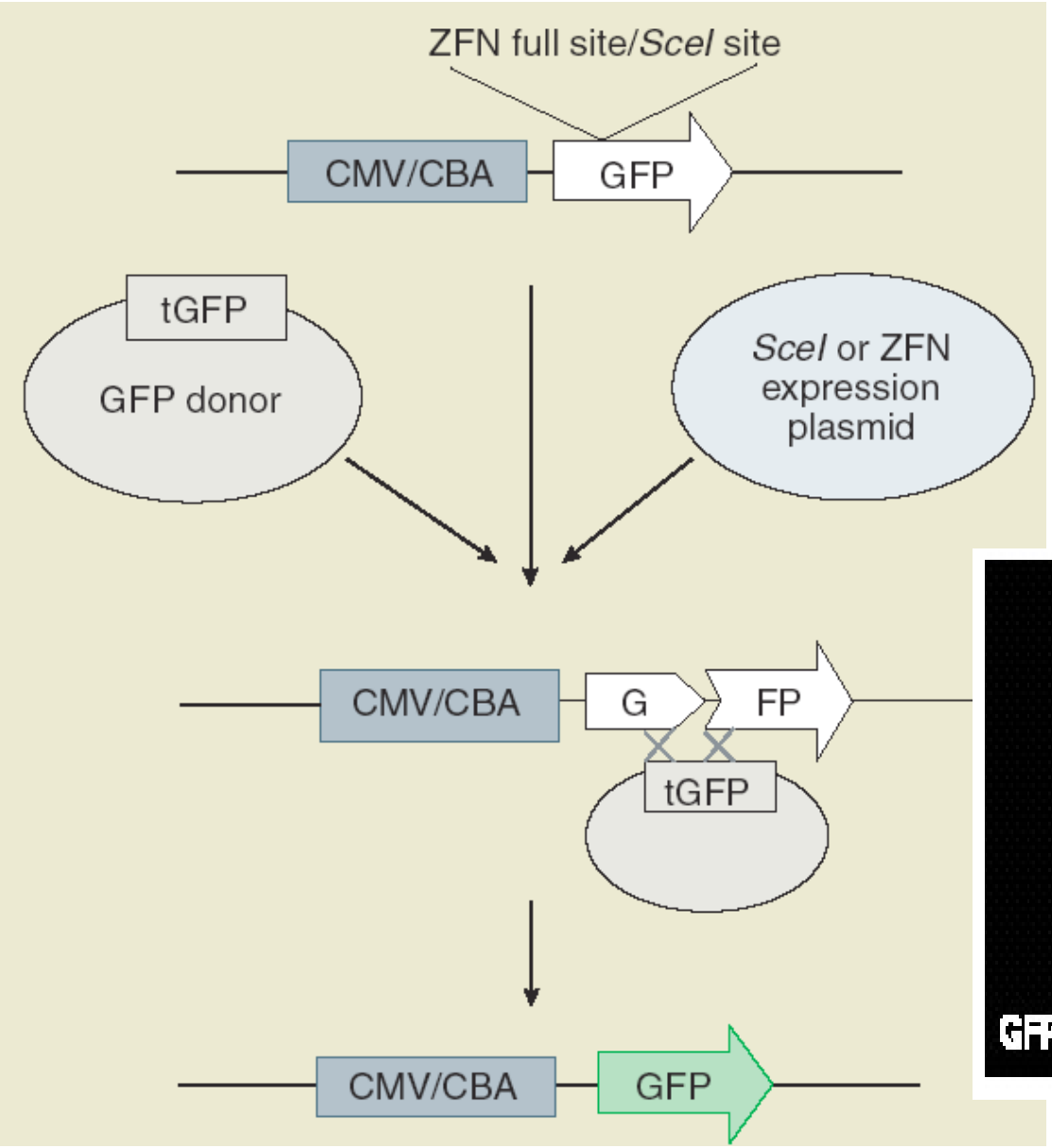
four Zn fingers in tandem

Zinc finger nuclease (ZFN) mechanism



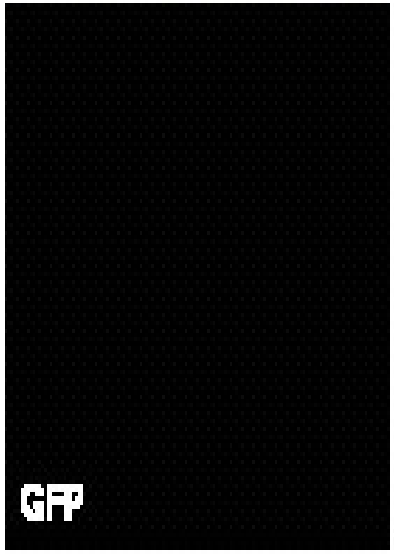
Zinc fingers can be hypothetically tailored to any DNA site; sequence must be long enough to ensure one site/genome

Test of method: make GFP functional

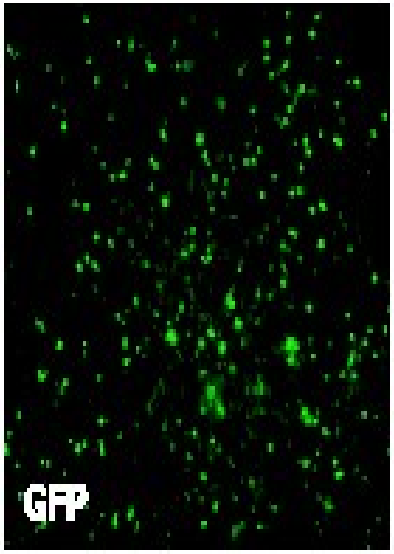


Mammalian cell culture transfection

GFP plasmid



-ZFN



+ZFN

Practical uses of ZFN-mediated recombination

- ZFN has been used to alter IL2R-gamma to wild type in cultured cells, with high efficiency

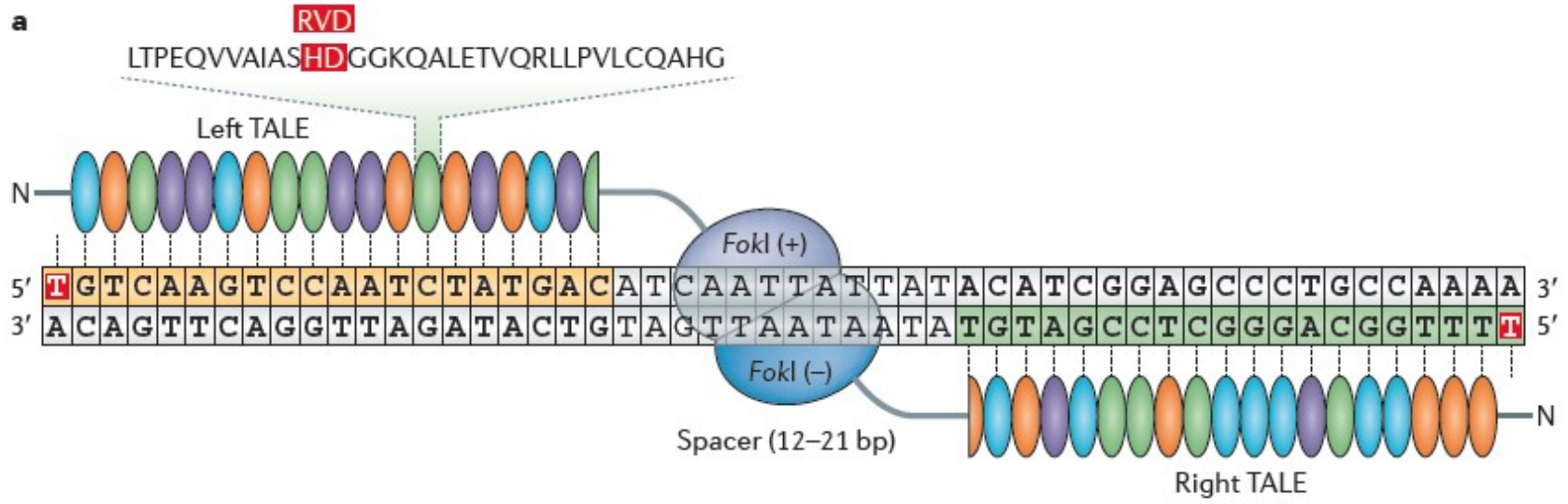
- However, ZFNs can be toxic because of “off-target” binding and genome cleavage

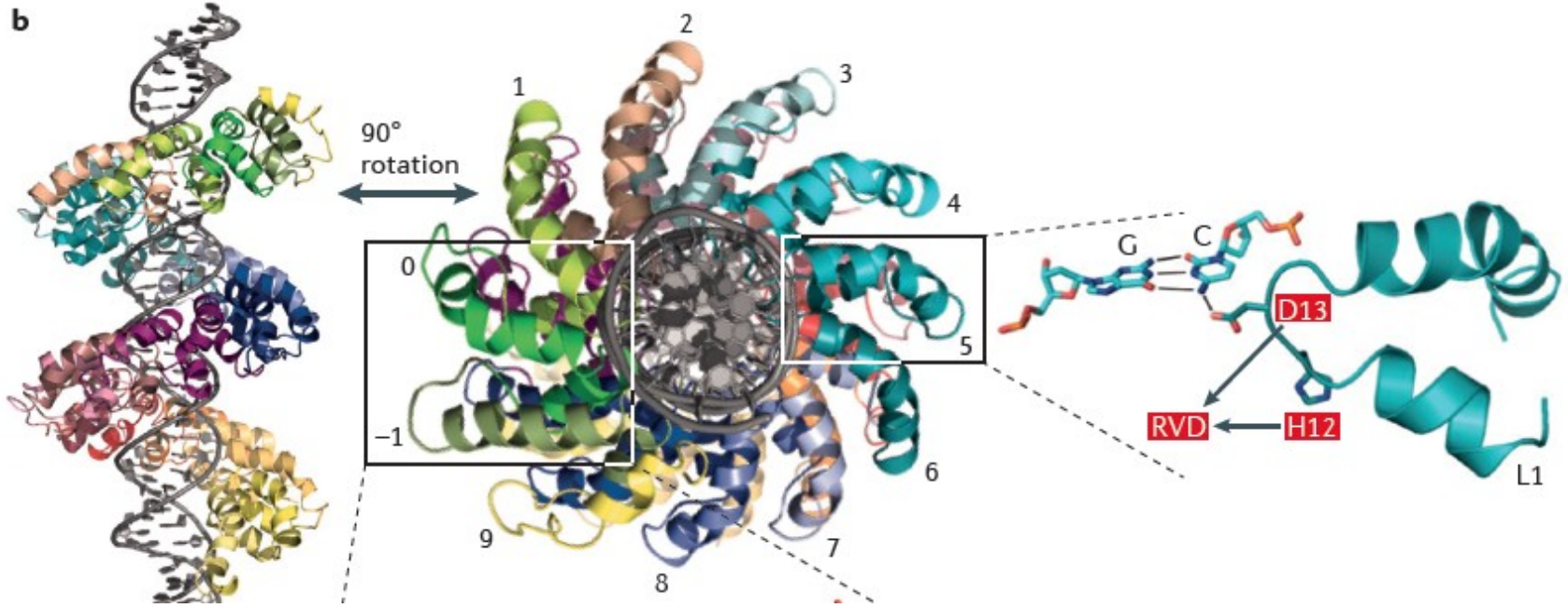
- Also, for use in gene therapy (precision genome surgery) it will be necessary to optimize systems for delivery of the ZFN and the DNA to be recombined

  - Transfection?
  - Viral delivery?
  - Direct injection?

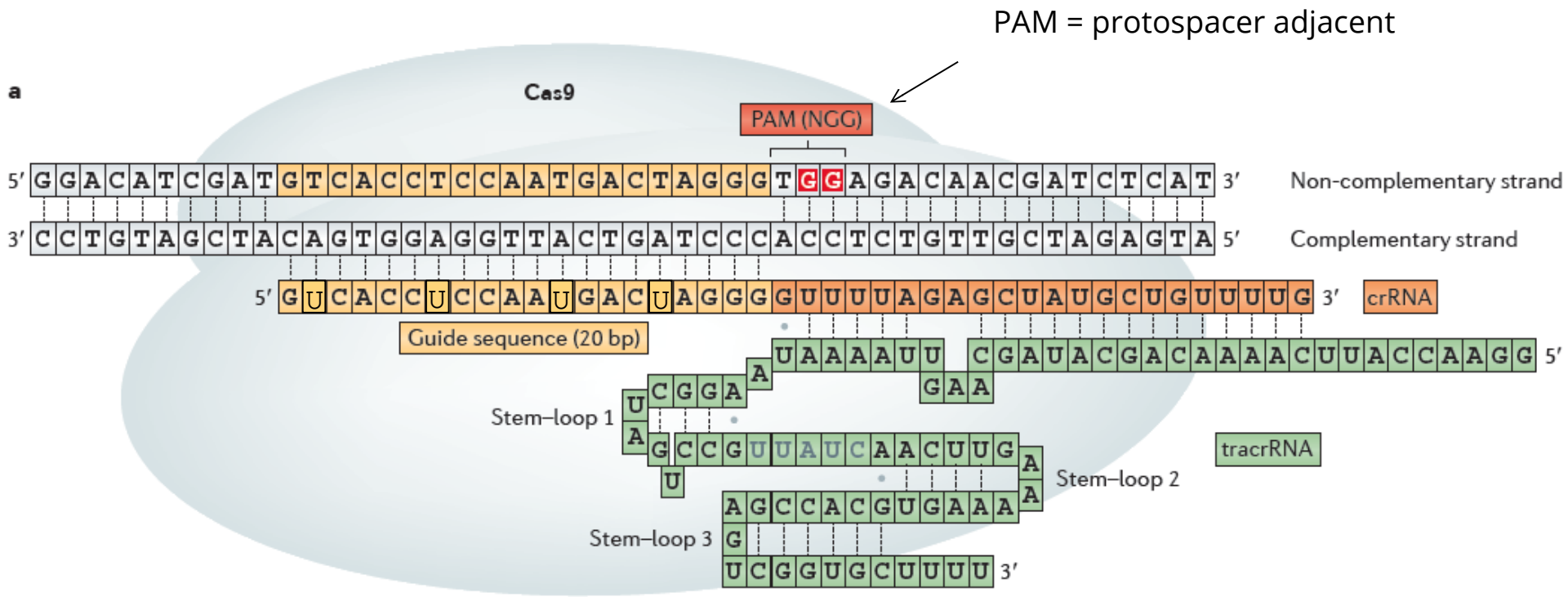
TALENs: transcription factor-like effector nucleases

Single base recognized by each repeat-variable di-residue (RVD)



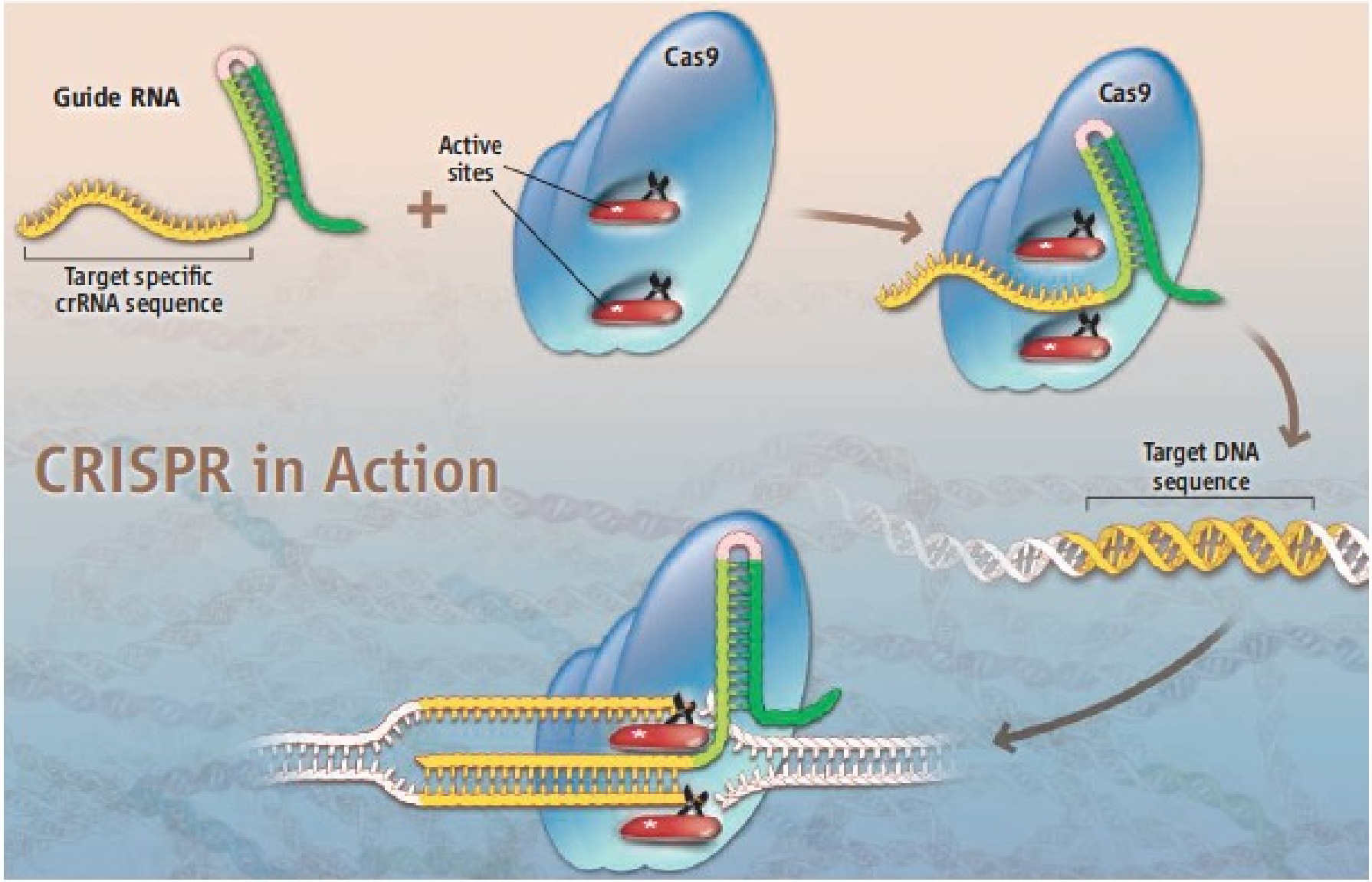


Cas9: an RNA-guided nuclease, used in microbial self-defense systems



The guide sequence-targeted interactions are very stable, and can also provide a tethering platform for proteins or RNAs, if the Cas9 nuclease activity is shut down

CRISPR: clustered regularly interspaced short palindromic repeats



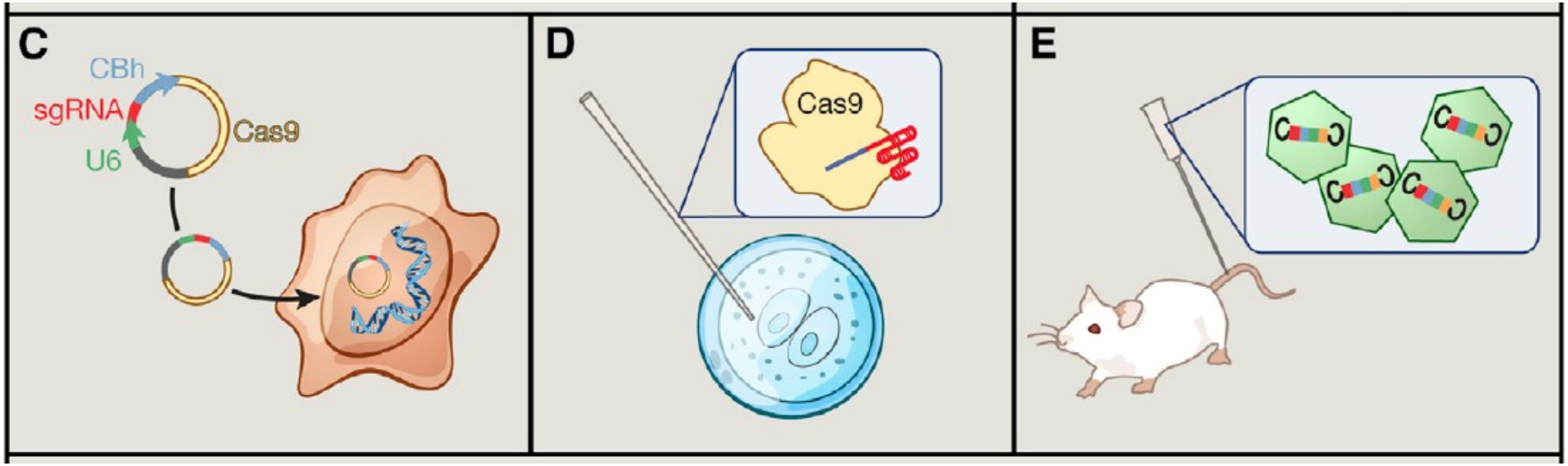
'Genetic microsurgery for the masses.' 20 DECEMBER 2013 VOL 342 SCIENCE
www.sciencemag.org

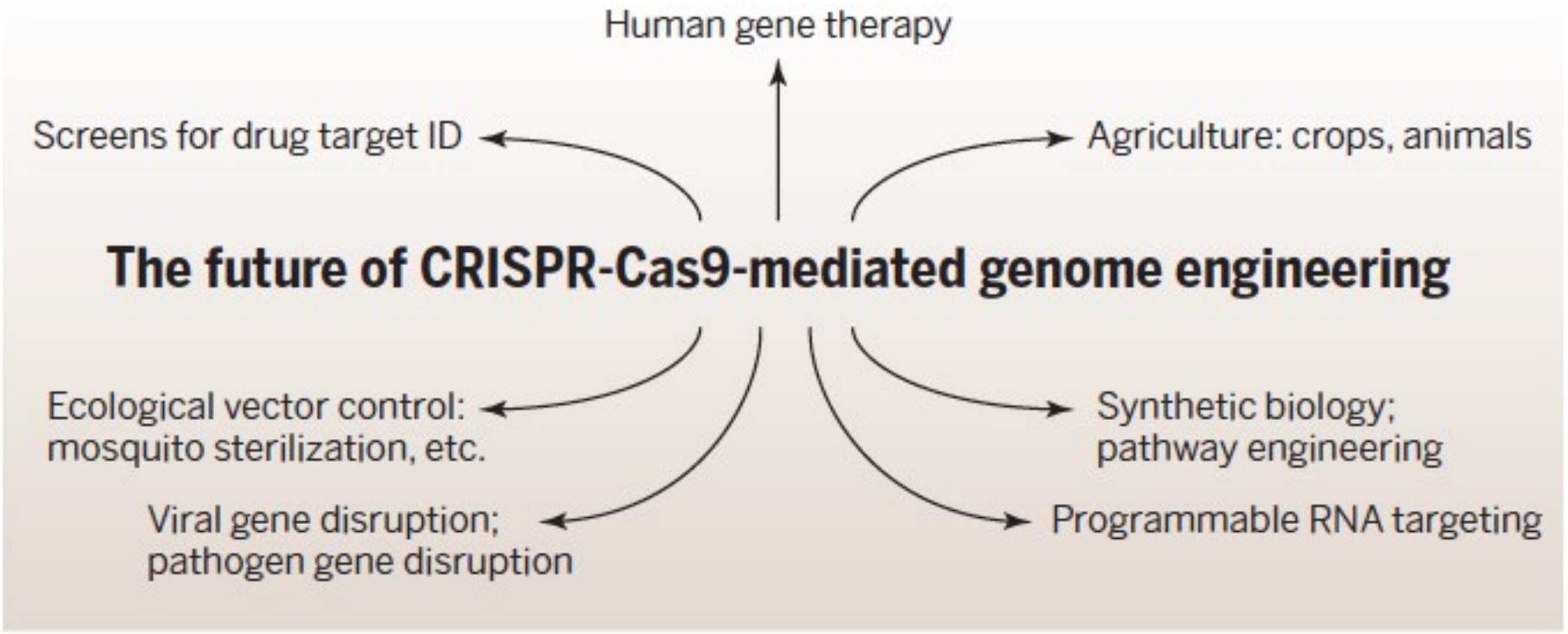
CRISPR Cas 9 is very easy to use

Transfection

Direct injection

Viral transduction





2015: Human embryos were engineered with CRISPR-Cas9

2015: Calls for ban on using CRISPR-Cas9 with human embryos, supported by the US NIH

Summary of the main nucleases for genetic surgery

	ZFNs	TALENs	RGENs
DNA targeting specificity determinant	Zinc-finger proteins	Transcription activator-like effectors	crRNA or sgRNA
Nuclease	*Fok*I	*Fok*I	Cas9
Success rate‡	Low (~24%)	High (>99%)	High (~90%)
Average mutation rate§	Low or variable (~10%)	High (~20%)	High (~20%)
Specificity-determining length of target site	18–36 bp	30–40 bp	22 bp (total length 23 bp)
Restriction in target site	G-rich	Start with T and end with A (owing to the heterodimer structure)	End with an NGG or NAG (lower activity) sequence (that is, PAM)
Design density	One per ~100 bp	At least one per base pair	One per 8 bp (NGG PAM) or 4 bp (NGG and NAG PAM)
Off-target effects	High	Low	Variable
Cytotoxicity	Variable to high	Low	Low
Size	~1 kb×2	~3 kb×2	4.2 kb (Cas9 from *Streptococcus pyogenes*) + 0.1 kb (sgRNA)

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ZFN: zinc finger nuclease
TALEN: TAL effector nuclease
RGEN: RNA guided endonuclease

Gene therapy summary

1) Gene therapy targets, types of vectors

2) Retroviral gene therapy, advantages and drawbacks

3) Precision genome engineering site-specific genome cleavage and recombinational repair