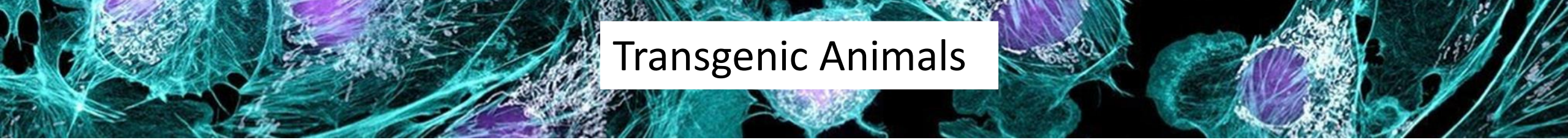
Advanced Cell Biology and Biotechnology

ACBB 2021/22

…the lecture of November 12th is about to begin…

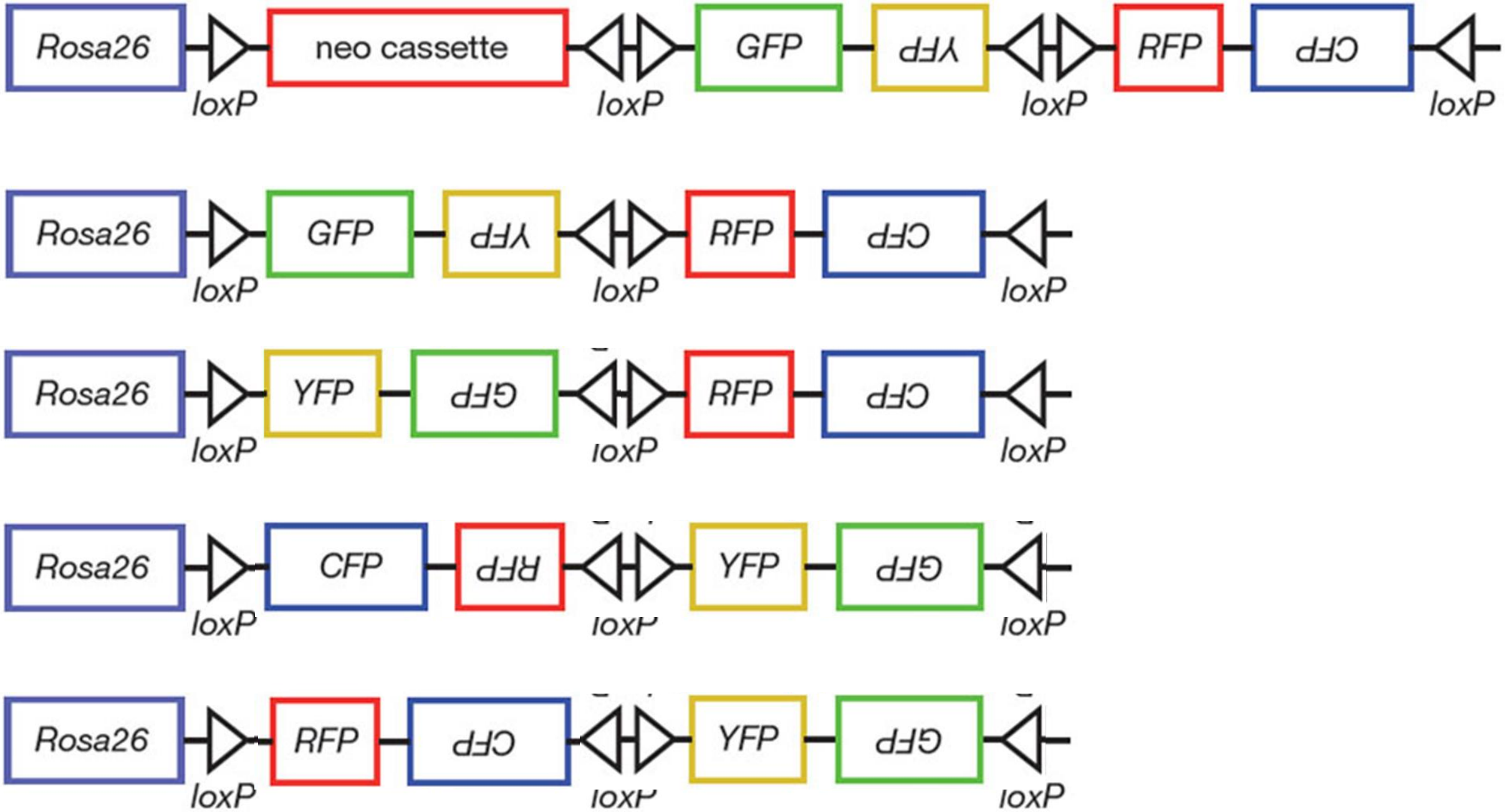


Transgenic Animals

1- DNA microinjection
- random insertion

2- embryonic stem cell-mediated gene transfer
- random insertion
- homologous recombination (double selection)
- Rosa26 locus
- genomic analysis to identify genetically modified animals

- knock-out animals
- knock-in animals
- conditional knock-out (cre-lox technique, inducible systems)
- siRNA
- CRISPR-CAS9


Rosa26
loxP
neo cassette
loxP
GFP
YFP
loxP
RFP
CFP
loxP
Rosa26
loxP
GFP
YFP
loxP
RFP
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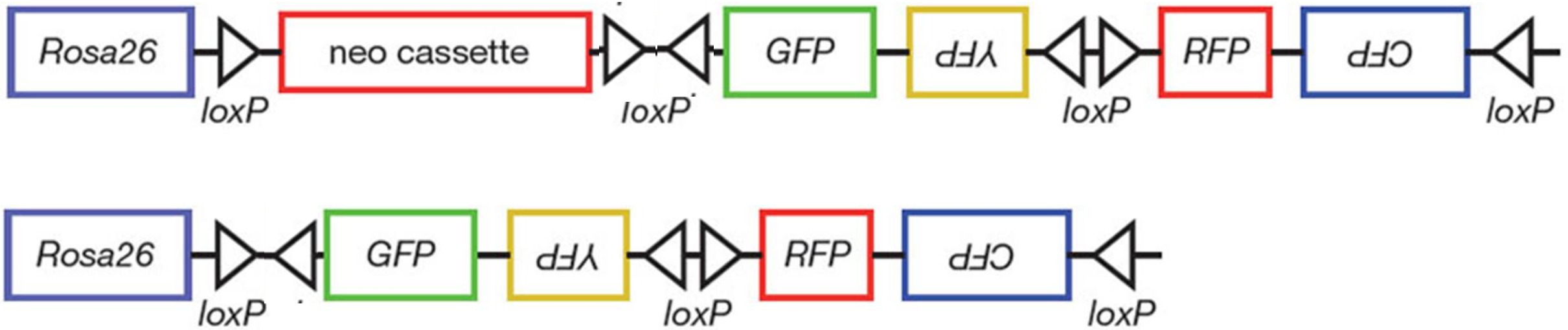
YFP
GFP
loxP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CFP
loxP

INDUCIBLE SYSTEMS TO REGULATE CRE RECOMBINASE ACTIVITY

- Tamoxifen

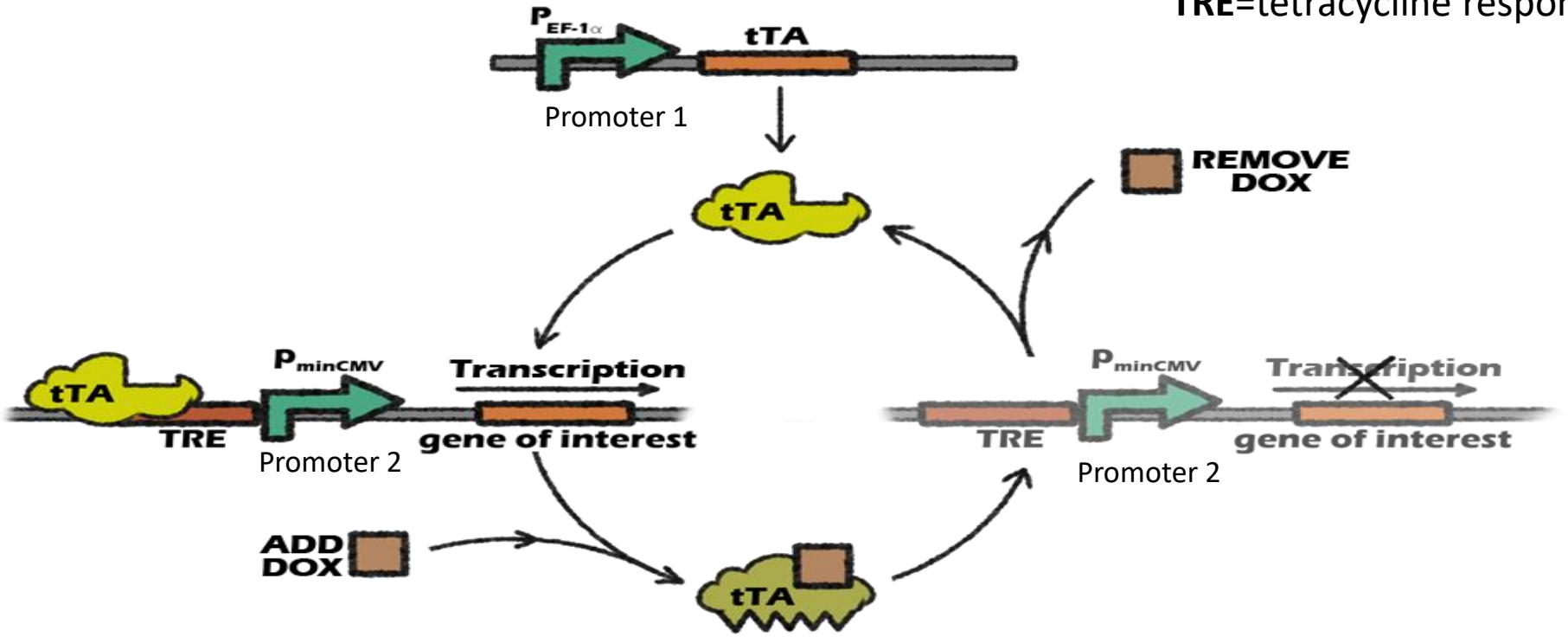
Inducible systems to regulate CRE recombinase expression
or expression of a gene of interest

- Tet-OFF
- Tet-ON

- Virus mediated expression

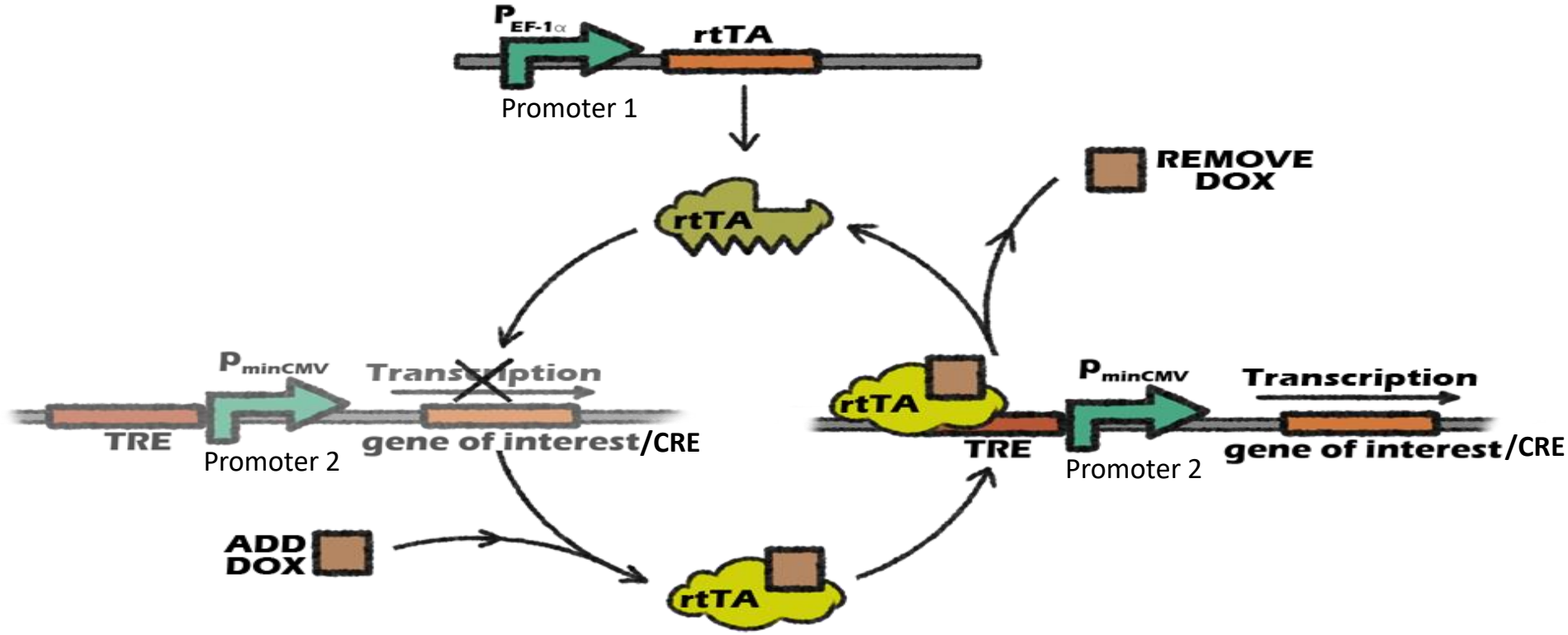
Tet-OFF (tetracycline switch-off transcription)

TRE=tetracycline responsive element



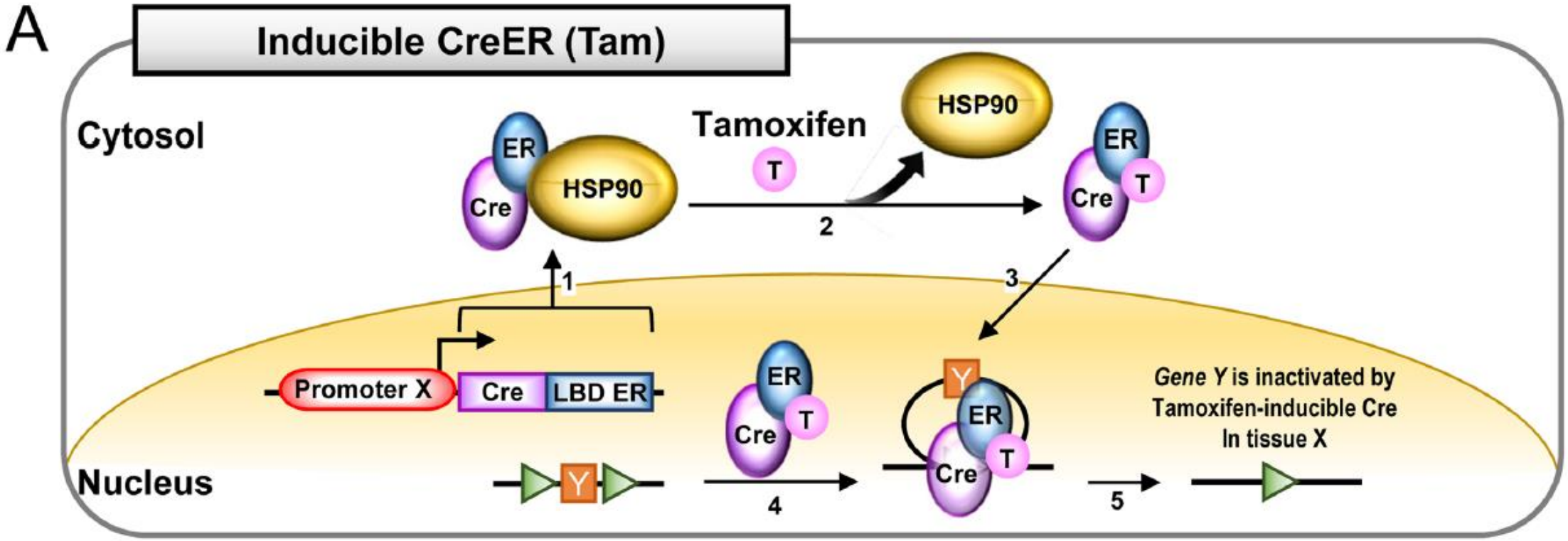
tetracycline Transactivator factor (**tTA**) is a hybrid transcription factor resulting from the fusion of the prokaryotic Tet Repressor, **TetR**, with an eukaryotic transcriptional transactivation domain.
TetR confers **sequence specific DNA binding** and **sensitivity to tetracyclines.**
Binding of the antibiotic (**tetracycline** or **doxycycline**) dramatically lowers the affinity of **tTA** to its binding sites, TRE. (**Doxycycline** has excellent tissue penetration and low toxicity in eukaryotes).
The **Tet-OFF** system can be used to obtain **inducible repression** of a specific gene.
A construct for **tTA expression** must be prepared and inserted into the mouse.

Tet-ON (tetracycline switch-on transcription)



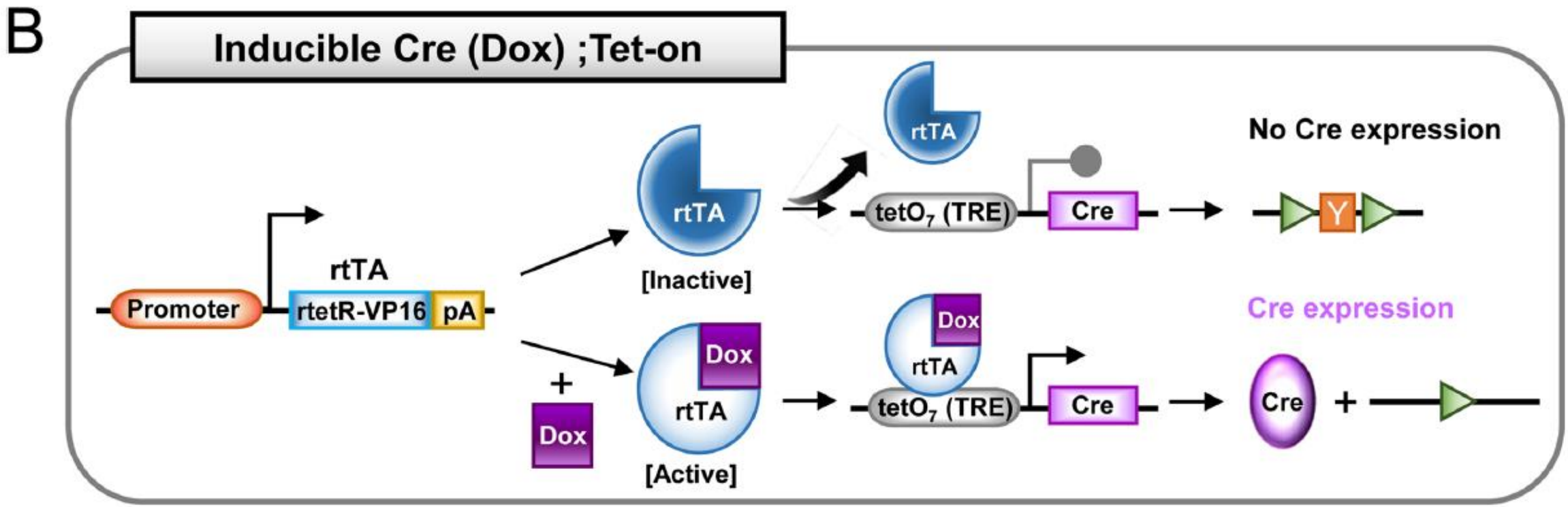
rtTA (**reversal tetracycline Transactivator factor**) differs from tTA by a few point mutations within TetR which result in a complete reversal of tetracycline responsiveness of this transcription factor.
Binding of the antibiotic (**tetracycline** or **doxycycline**) dramatically **increases** the affinity of **rtTA** to its binding sites, **TRE**.
The **Tet-ON** system can be used to obtain **inducible expression of CRE** or inducible expression of a specific gene.
A construct for **rtTA expression** must be prepared and inserted into the mouse.

Tamoxifen (Tam)-inducible System of estrogen receptor fused to Cre (CreER).



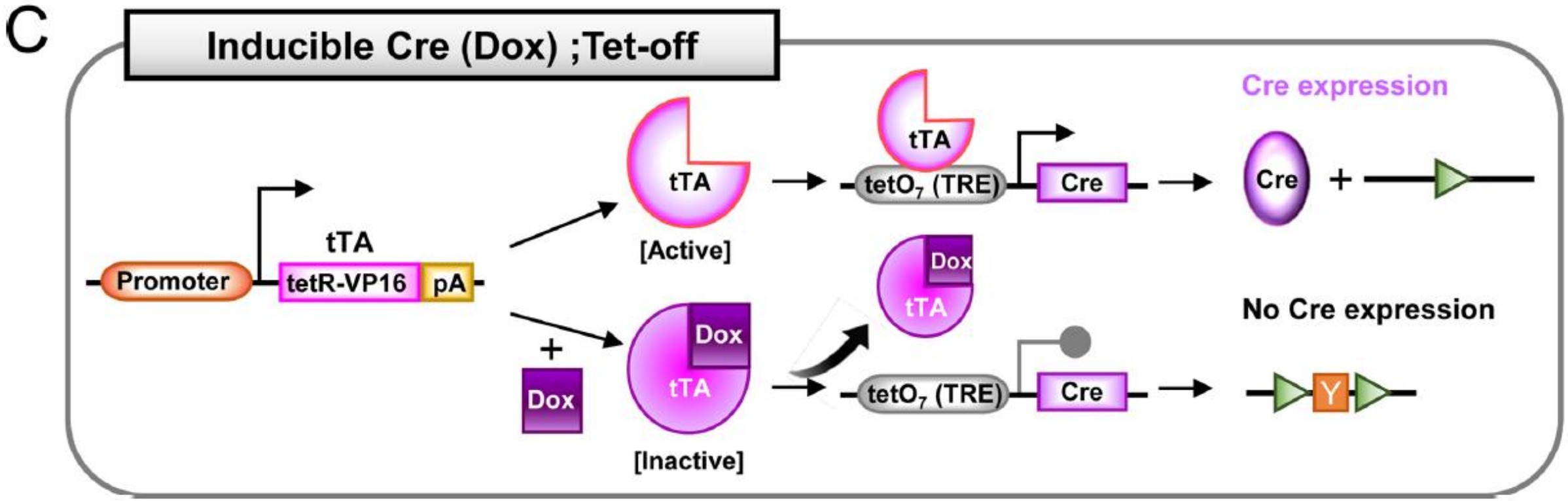
(A) In the absence of tamoxifen, expressed fusion protein, CreER, interacts with heat shock protein 90 (HSP90) and exists in cytoplasm (1).
Administration of Tam disrupts the interaction of HSP90 with CreER (2). Interaction of ER with Tam induces nuclear translocation of Cre (3). In the nucleus, the CreER recognizes the loxP sites (4) and inactivates the gene Y in tissue X (5).

Doxycycline (Dox)-induced Tetracyclin (Tet)-on system.



(B) In Tet-on system, ubiquitous or tissue-specific promoter driven rtTA is expressed. In the absence of Dox, inactivated rtTA is unable to bind $tetO_7$ (7 repeats of a 19 nucleotide tetO minimal promoter, also referred to as TRE) sequence of cre gene. Cre is not expressed. Following Dox administration, Dox interacts with the rtTA and allows to activate. Activated rtTA binds to $tetO_7$ promoter of cre and induces the Cre expression.

Doxycycline (Dox)-induced Tetracyclin (Tet)-off system.



(C) In Tet-off system, in the absence of Dox, activated tTA is able to bind $tetO_7$ (TRE) sequence of Cre and induces the Cre expression.
Upon Dox administration, tTA interacted with Dox is inactivated. Inactivated rTA is not able to bind to $tetO_7$ promoter and therefore Cre expression is inhibited.

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- Tet-OFF
- Tet-ON

- Virus mediated expression

 Nucleic Acids Research, 2001, Vol. 29, No. 16 e80

Adenovirus-mediated Cre deletion of floxed sequences in primary mouse cells is an efficient alternative for studies of gene deletion

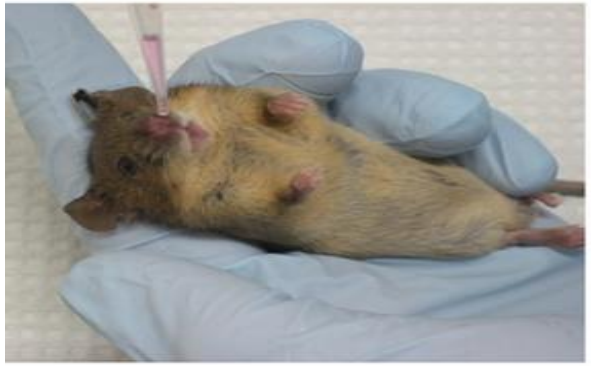
Sandrine Prost*, Sharon Sheahan, Dominic Rannie and David J. Harrison

Nature Protocols **4**, 1064 - 1072 (2009)
Published online: 25 June 2009 | doi:10.1038/nprot.2009.95

Subject Category: Model organisms

Conditional mouse lung cancer models using adenoviral or lentiviral delivery of Cre recombinase

Michel DuPage[1,3], Alison L Dooley[1,3] & Tyler Jacks[1,2]



The development of animal models of lung cancer is critical to our understanding and treatment of the human disease. Conditional mouse models provide new opportunities for testing novel chemo preventatives, therapeutics and screening methods that are not possible with cultured cell lines or xenograft models. This protocol describes how to initiate tumors in two conditional genetic models of human non-small cell lung cancer (NSCLC) using the **activation of oncogenic K-ras** alone or in combination with the loss of function of p53. **We discuss methods for sporadic expression of Cre in the lungs through engineered adenovirus or lentivirus, and provide a detailed protocol for the administration of the virus by intranasal inhalation or intratracheal intubation.** The protocol requires 1–5 min per mouse with an additional 30–45 min to set-up and allow for the recovery of mice from anesthesia. Mice may be analyzed for tumor formation and progression starting 2–3 weeks after infection.

Tissue specific expression and inducible systems
can also be used to obtain **inducible tissue specific death**

Tissue specific expression and inducible systems can also be used to obtain **inducible tissue specific death**

Example:

frontiers in NEUROSCIENCE

REVIEW ARTICLE
published: 02 May 2011
doi: 10.3389/fnins.2011.00064

Genetic methods to identify and manipulate newly born neurons in the adult brain

Itaru Imayoshi[1,2,3], Masayuki Sakamoto[1,4,5] and Ryoichiro Kageyama[1,4]**

Nestin is an intermediate filament protein specifically expressed by neural stem/progenitor cells in the developing nervous system and the adult brain.

How can you obtain nestin expression specific inducible cell death?

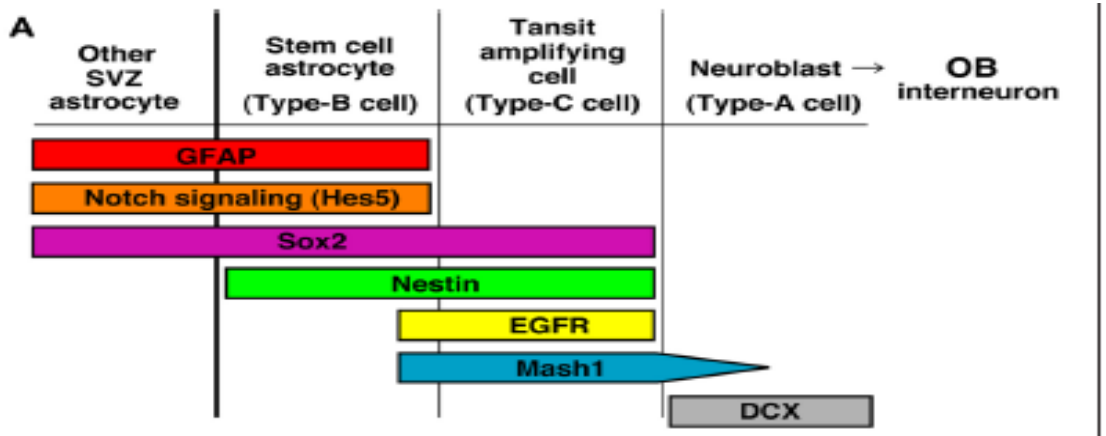
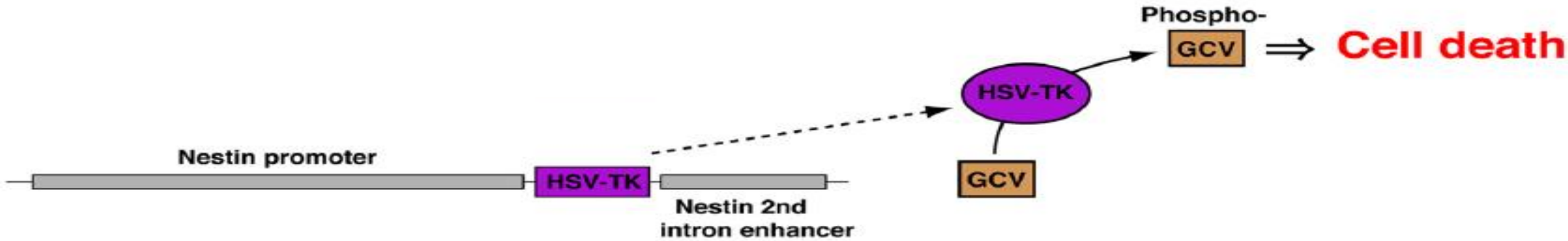
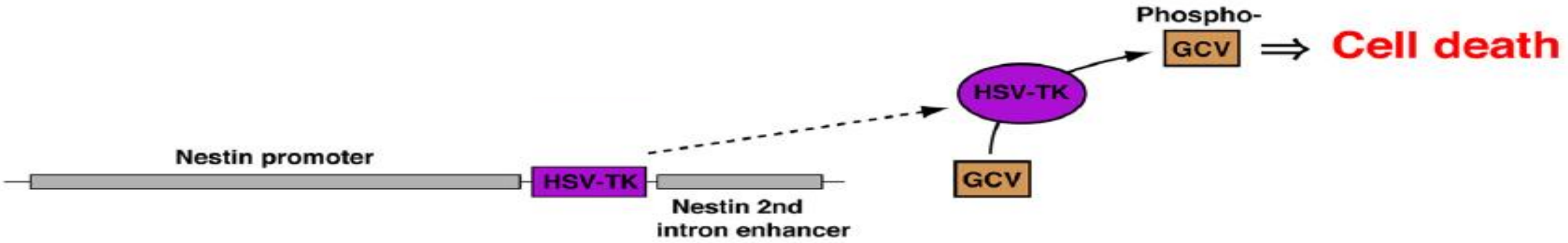


FIGURE 1 | Current view of the sequence of neurogenesis from NSCs in the adult brain. (A) Generation of new interneurons in the olfactory bulb (OB) from NSCs in the subventricular zone of the lateral ventricle (SVZ/LV). **(B)** Generation of new granular neurons in the dentate gyrus (DG) of the hippocampus from NSCs in the subgranular zone (SGZ).

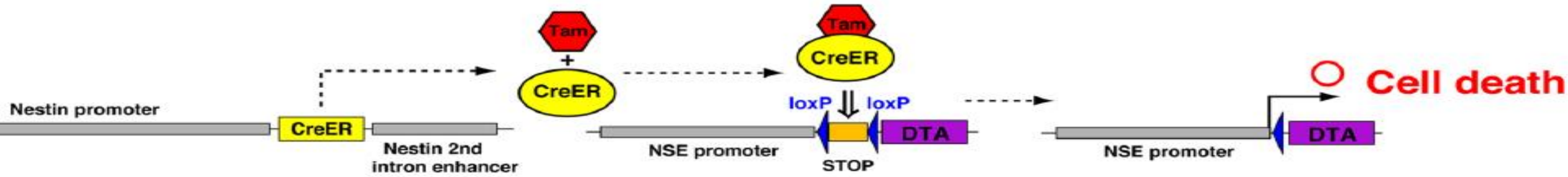

Phospho-
GCV
⇒
Cell death
HSV-TK
GCV
Nestin promoter
HSV-TK
Nestin 2nd
intron enhancer

THYMIDINE KINASE

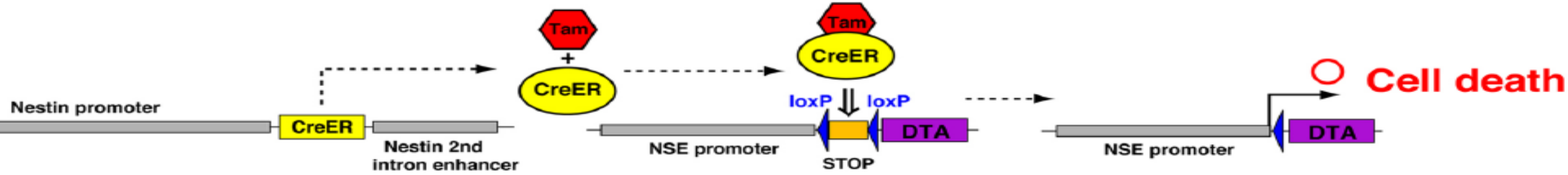


Administration of **Ganciclovir** (GCV) to mice carrying the transgene Nestin-TK results in death of dividing cells expressing herpes simplex virus **thymidine kinase** (HSV-TK).

HSV-TK produces toxic metabolites that disrupt DNA synthesis and results in the death of dividing cells.

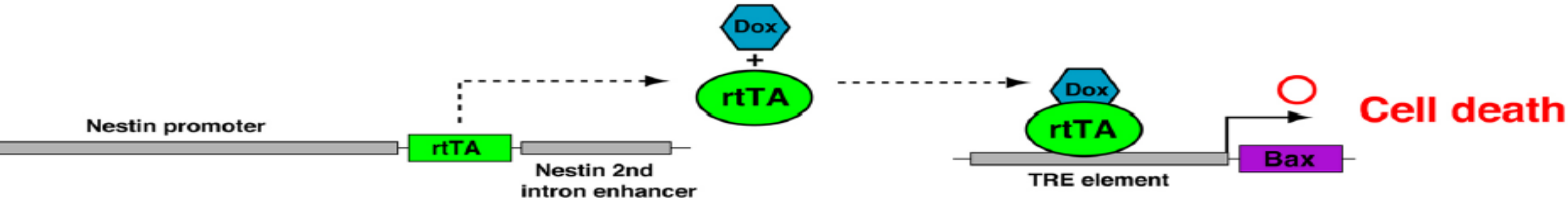

Tam
+
CreER
Nestin promoter
CreER
Nestin 2nd
intron enhancer
Tam
CreER
loxP
loxP
NSE promoter
STOP
DTA
Cell death
NSE promoter
DTA

TAMOXIFEN –CRE/ER RECOMBINASE DRIVEN EXPRESSION OF DTA

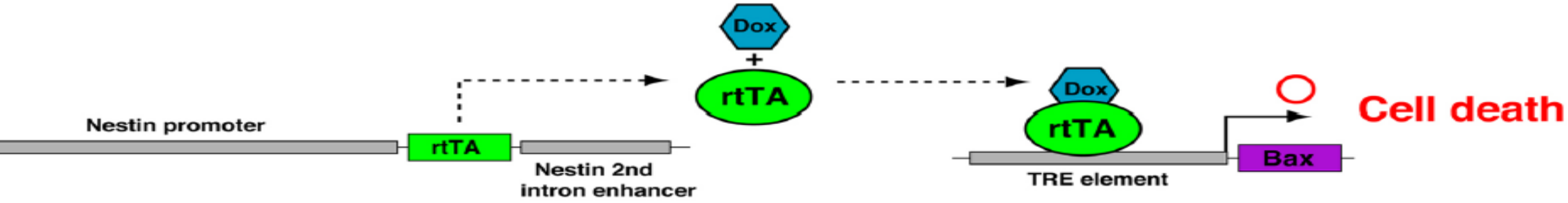


In the Nestin-CreER/ NSE-DTA mouse

- **Nestin-CreER** drives the expression of a tamoxifen (Tam)-inducible form of Cre in NSCs
- **Cre-inducible diphtheria toxin fragment A** (DTA) is engineered into the locus of the neuron-specific enolase (NSE) gene.
- activated CreER leads to the recombination of loxP sites and removal of the STOP cassette upstream of the DTA gene, thus allowing the expression of DTA from the NSE promoter.


Dox
+
rtTA
Nestin promoter
rtTA
Nestin 2nd
intron enhancer
Dox
rtTA
TRE element
Bax
Cell death

DOXYCICLINE INDUCED EXPRESSION OF BAX



In the **Nestin-rtTA/TRE-Bax mice**, doxycycline (Dox) activates the rtTA protein, which binds to seven TetO sequences (TRE) to drive the expression of the pro-apoptotic protein Bax, which activates the apoptosis pathway in NSCs. Alternatively, also DTA could be used.

TRANSGENIC ANIMALS

- Two methods to produce transgenic animals:

    1- DNA microinjection
        - random insertion
    2- embryonic stem cell-mediated gene transfer
        - random insertion
        - homologous recombination (double selection)
        - Rosa26 locus
        - genomic analysis to identify genetically modified animals
- knock-out animals
- knock-in animals
- conditional knock-out (cre-lox technique, inducible systems)

- siRNA
- CRISPR-CAS9

The Nobel Prize in Physiology or Medicine 2006

Photo: L. Cicero

Andrew Z. Fire

Prize share: 1/2

Photo: J. Mottern

Craig C. Mello

Prize share: 1/2

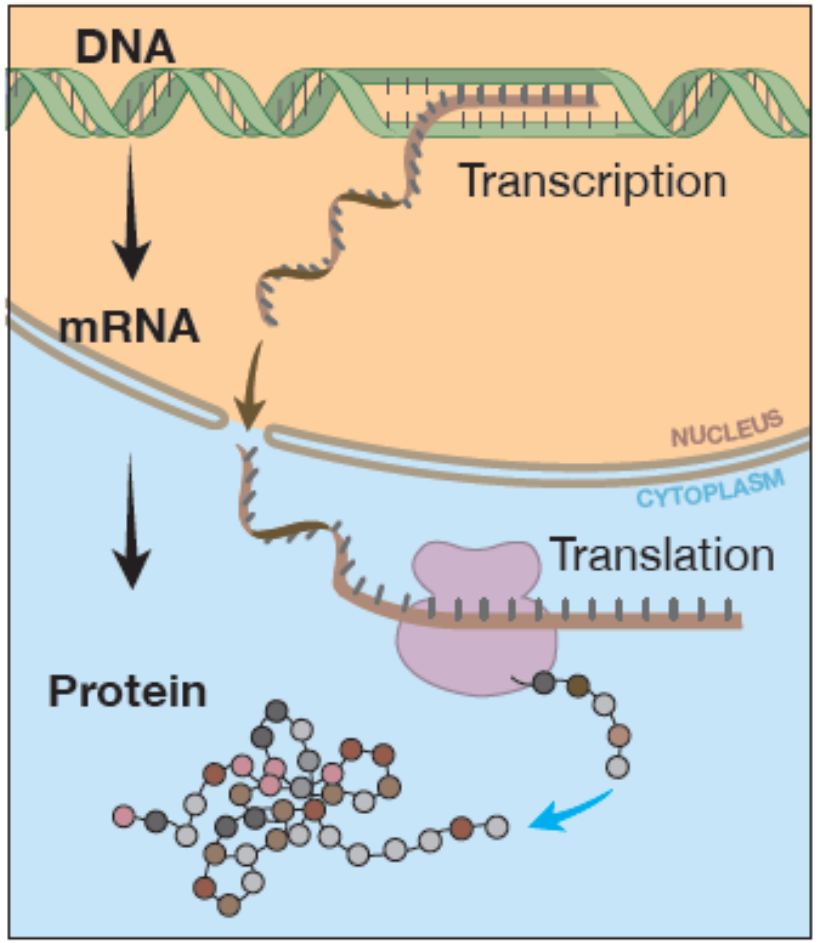
The Nobel Prize in Physiology or Medicine 2006 was awarded jointly to Andrew Z. Fire and Craig C. Mello *"for their discovery of RNA interference - gene silencing by double-stranded RNA"*

https://www.nobelprize.org/nobel_prizes/medicine/laureates/2006/press.html

RNA interference

— gene silencing by double-stranded RNA

1. The central dogma

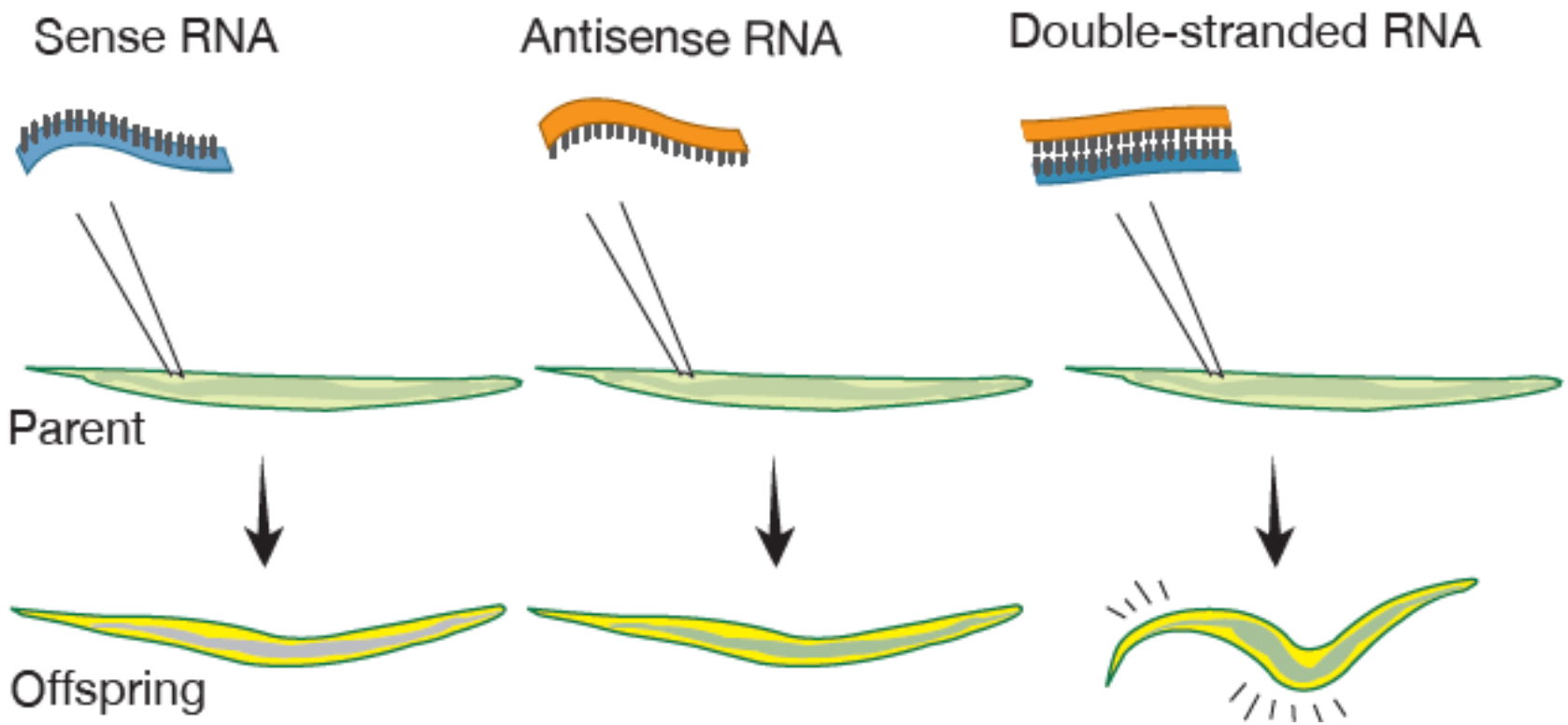


Our genome operates by sending information from double-stranded DNA in the nucleus, via single-stranded mRNA, to guide the synthesis of proteins in the cytoplasm.

https://www.youtube.com/watch?v=cK-OGB1_ELE

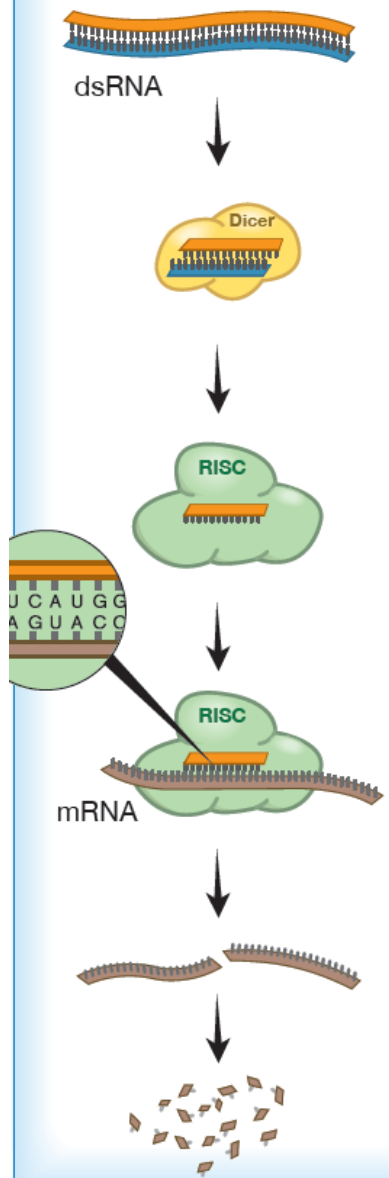
2. The experiment

RNA carrying the code for a muscle protein is injected into the worm *C. elegans*. Single-stranded RNA has no effect. But when double-stranded RNA is injected, the worm starts twitching in a similar way to worms carrying a defective gene for the muscle protein.



3. The RNAi mechanism

RNA interference (RNAi) is an important biological mechanism in the regulation of gene expression.



Double-stranded RNA (dsRNA) binds to the protein Dicer …

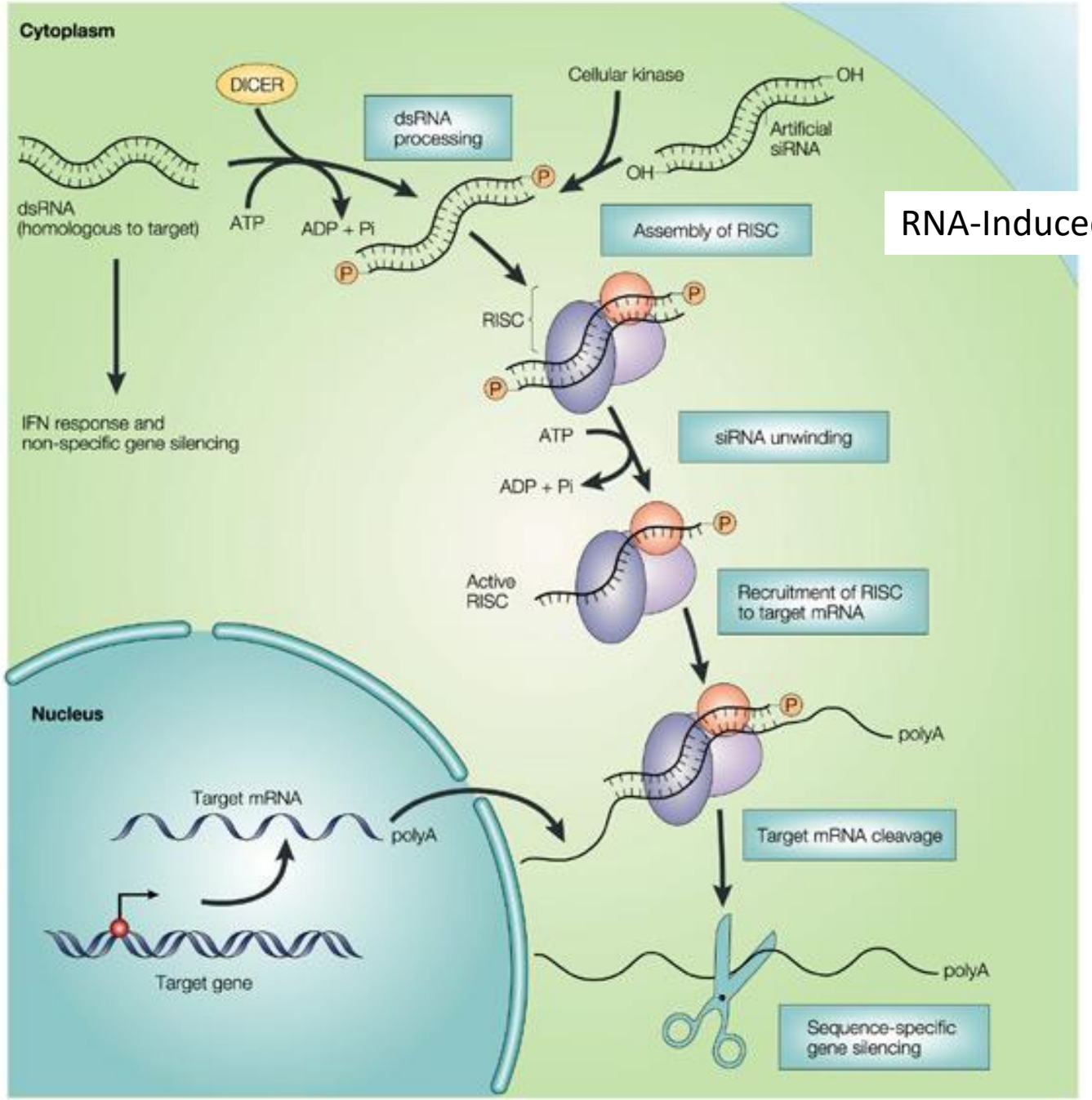
… which cleaves dsRNA into smaller fragments.

One of the RNA strands is loaded into a RISC complex…

…and links the complex to the mRNA strand by basepairing.

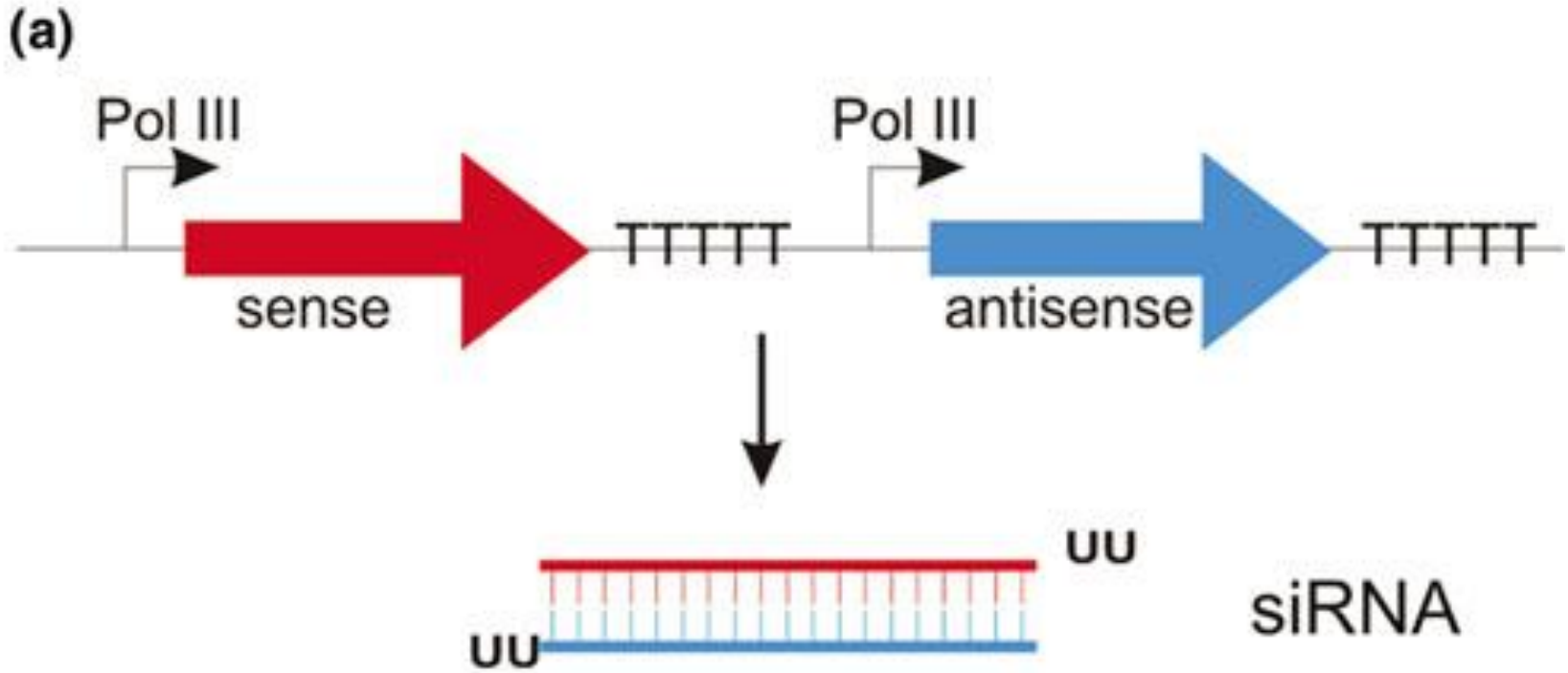
mRNA is cleaved and destroyed. No protein can be synthesized.

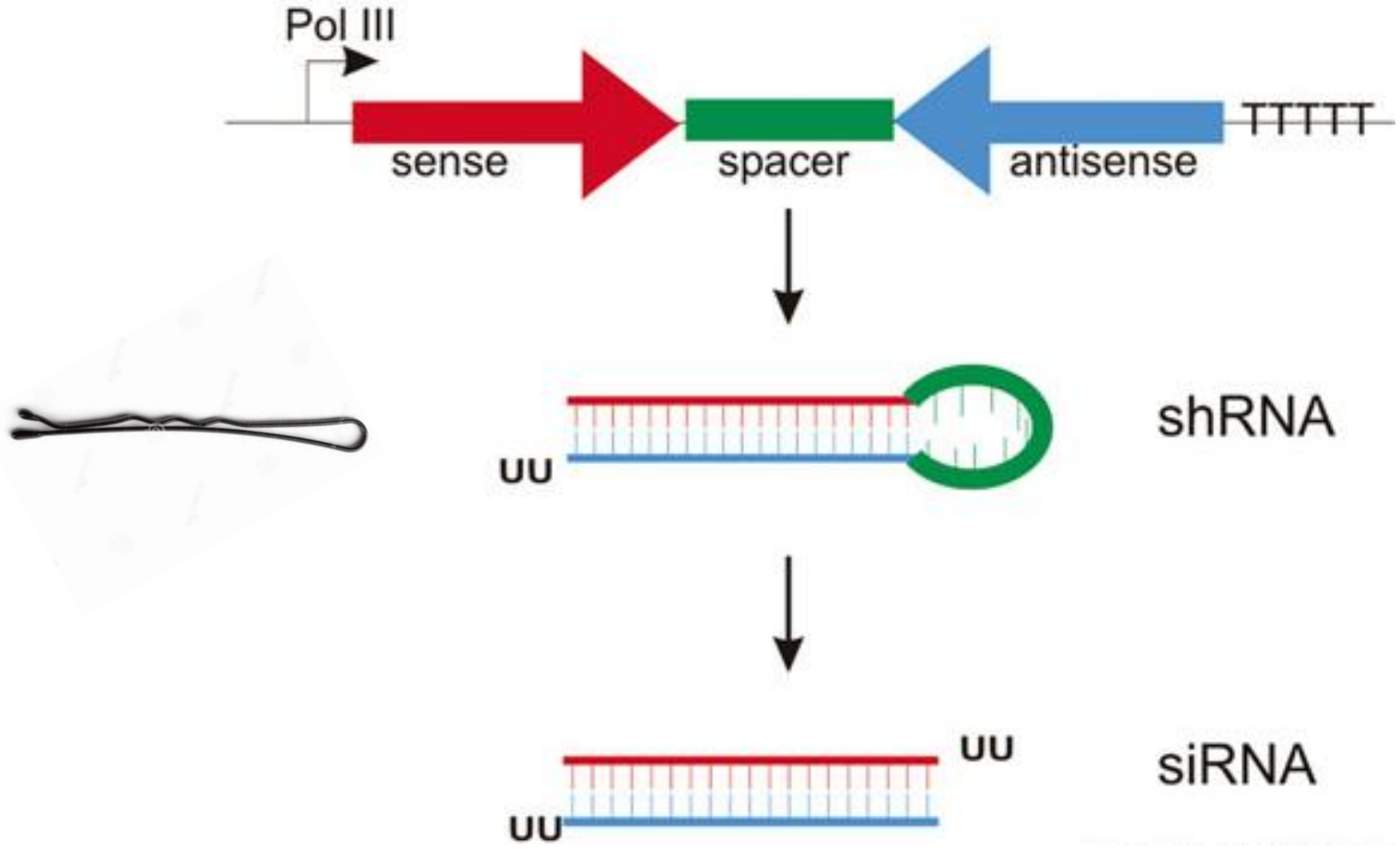
RNA interference



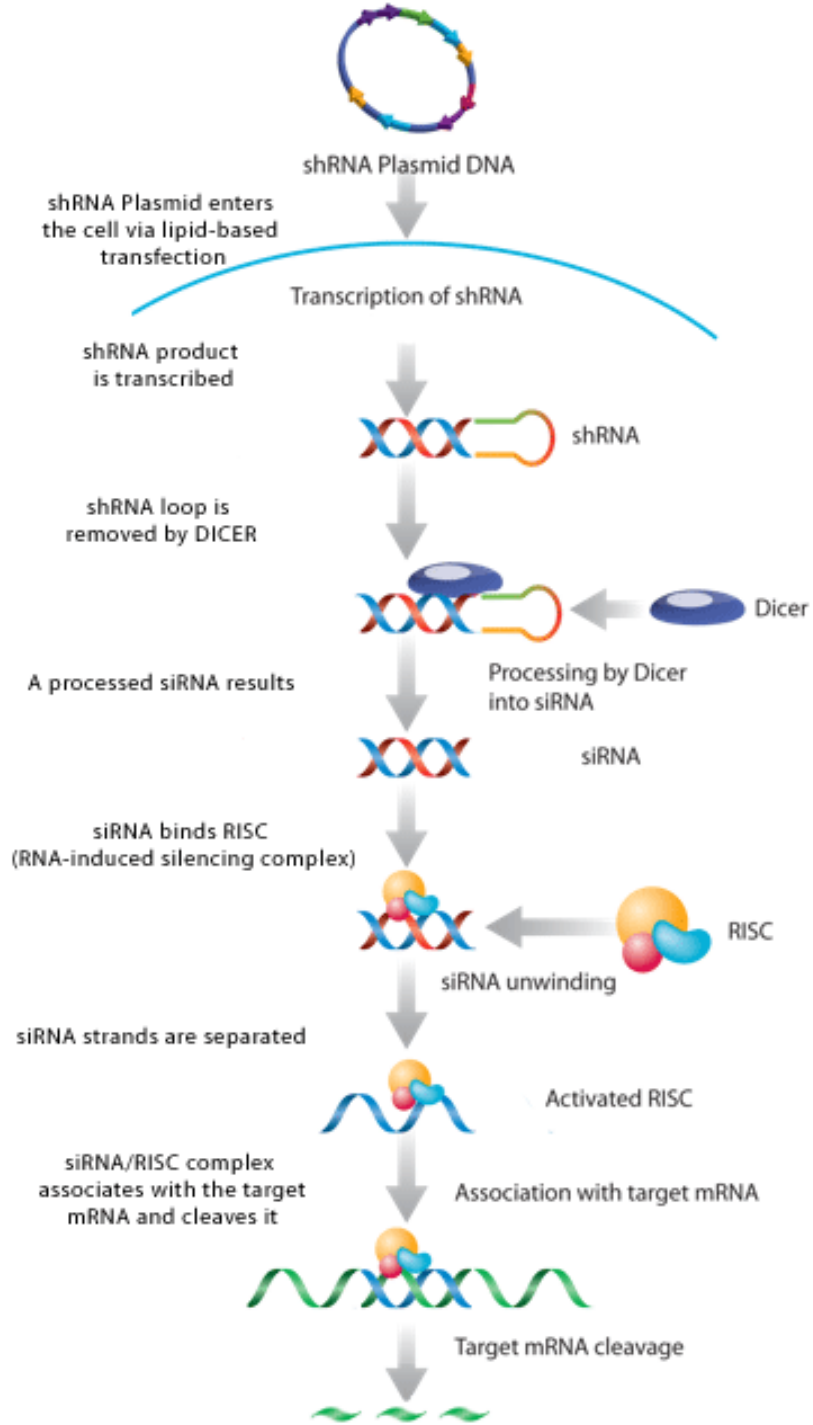
RNA-Induced Silencing Complex (RISC)

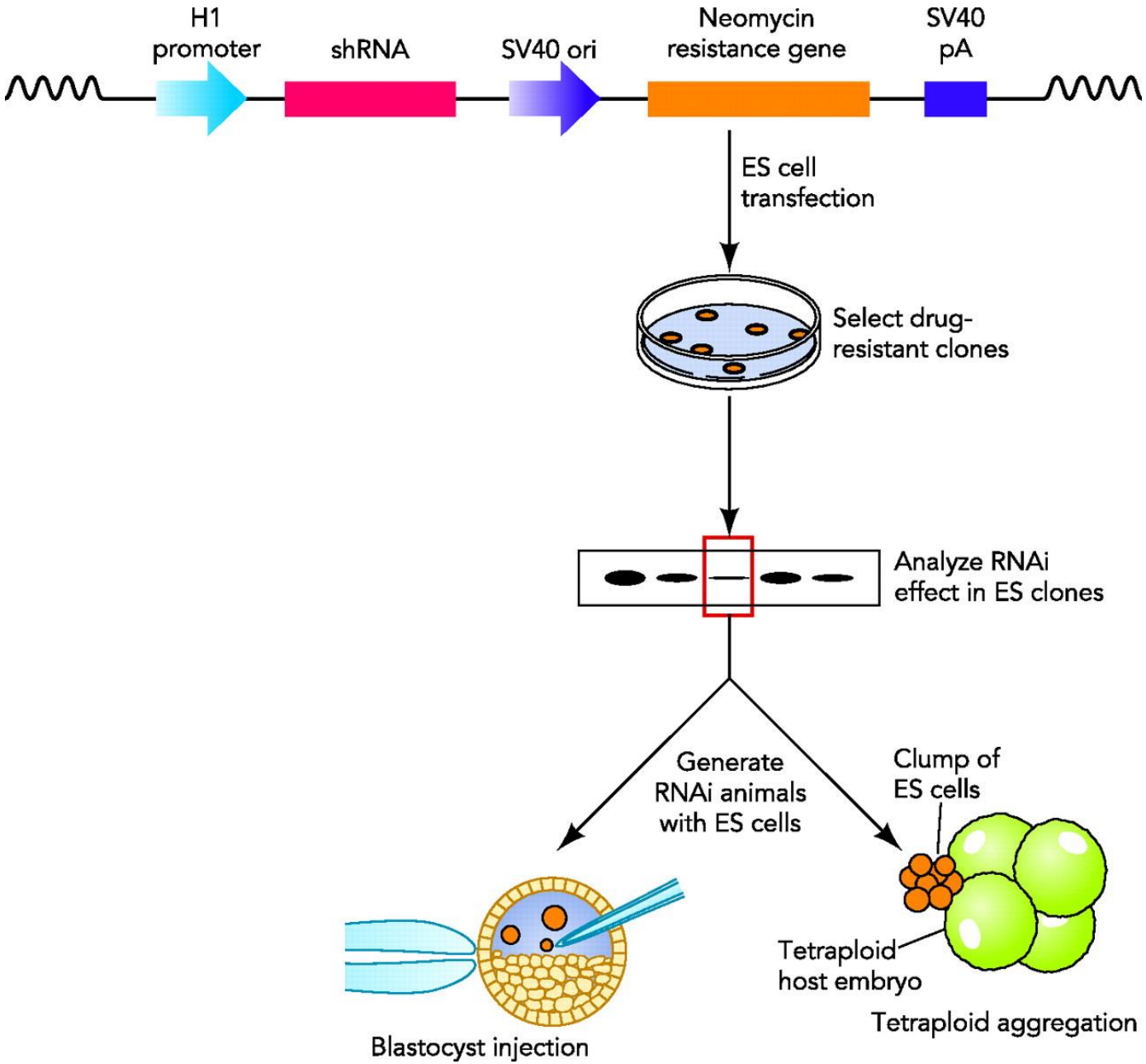
Expression of siRNAs inside the cells, which strategies can be used?

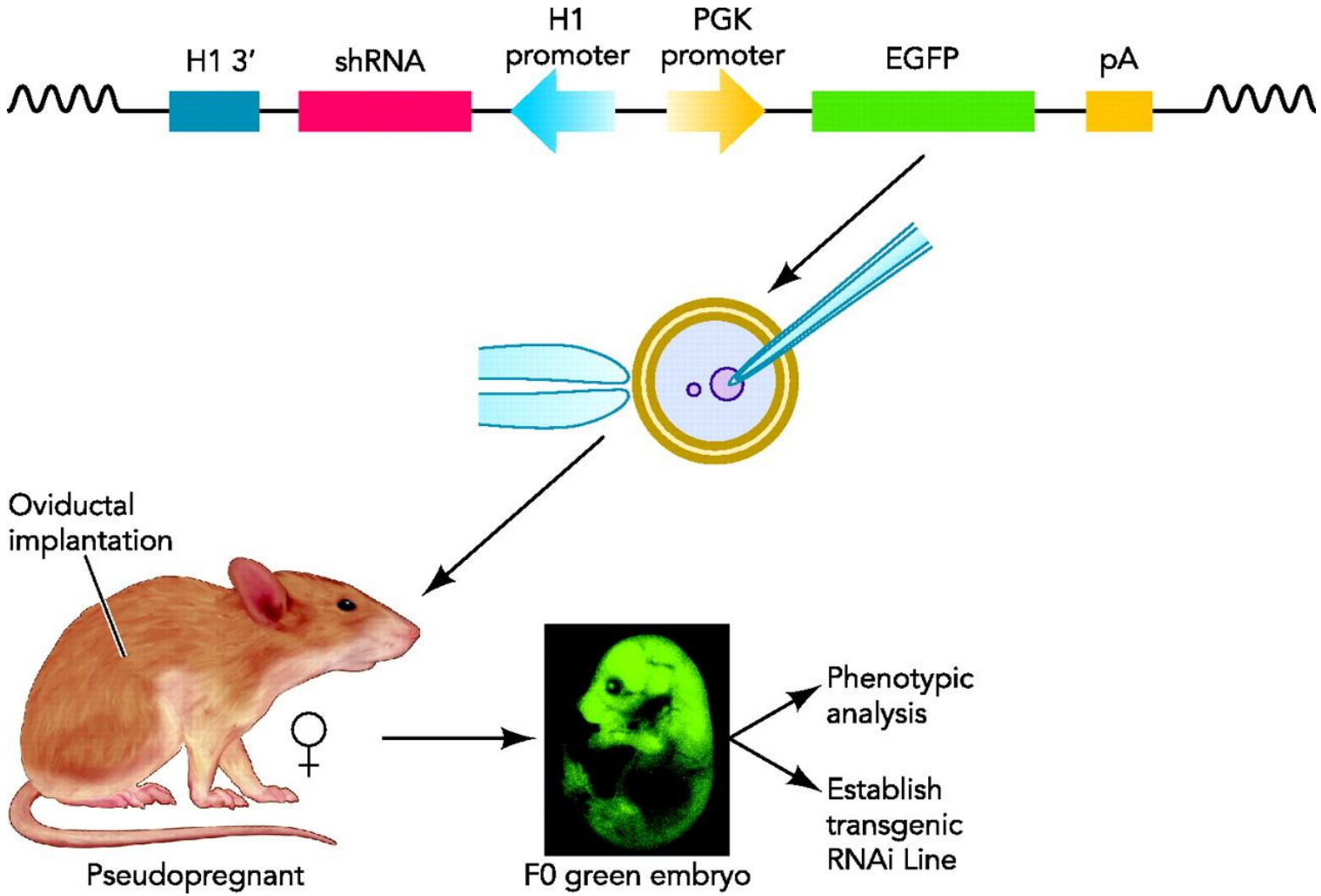

(a)
Pol III
Pol III
TTTTT
TTTTT
sense
antisense
uu
uu
siRNA



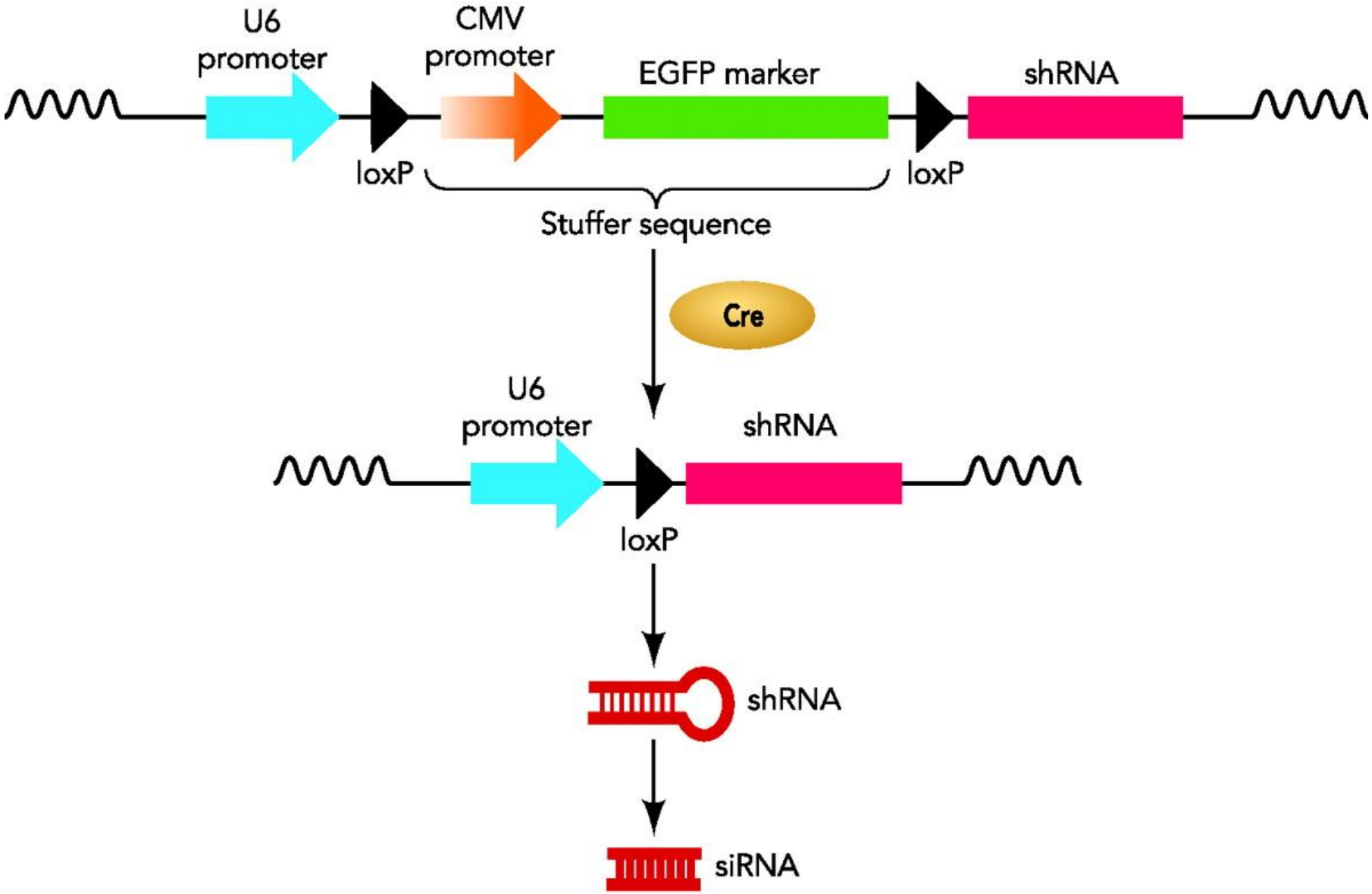
A **short hairpin RNA** or **small hairpin RNA** (**shRNA**) is an artificial RNA molecule with a hairpin turn that can be used to silence target gene expression via **RNA interference** (**RNAi**). Expression of shRNA in cells is typically obtained by delivery of plasmids or through viral or bacterial vectors. shRNA is a good mediator of RNAi in that it has a relatively low rate of degradation and turnover.

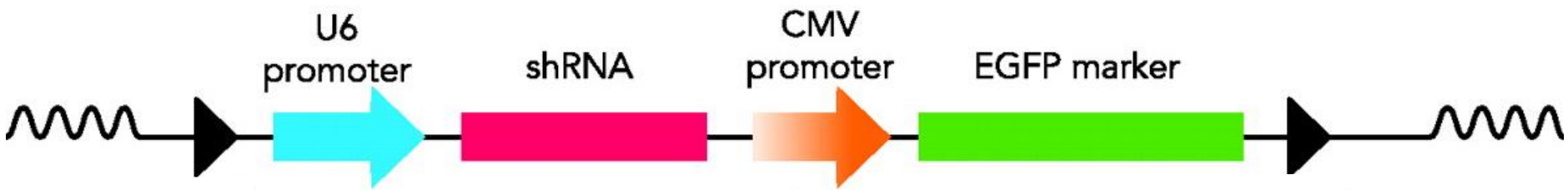

shRNA Plasmid DNA
shRNA Plasmid enters the cell via lipid-based transfection
Transcription of shRNA
shRNA product is transcribed
shRNA
shRNA loop is removed by DICER
Dicer
Processing by Dicer into siRNA
A processed siRNA results
siRNA
siRNA binds RISC (RNA-induced silencing complex)
RISC
siRNA unwinding
siRNA strands are separated
Activated RISC
siRNA/RISC complex associates with the target mRNA and cleaves it
Association with target mRNA
Target mRNA cleavage

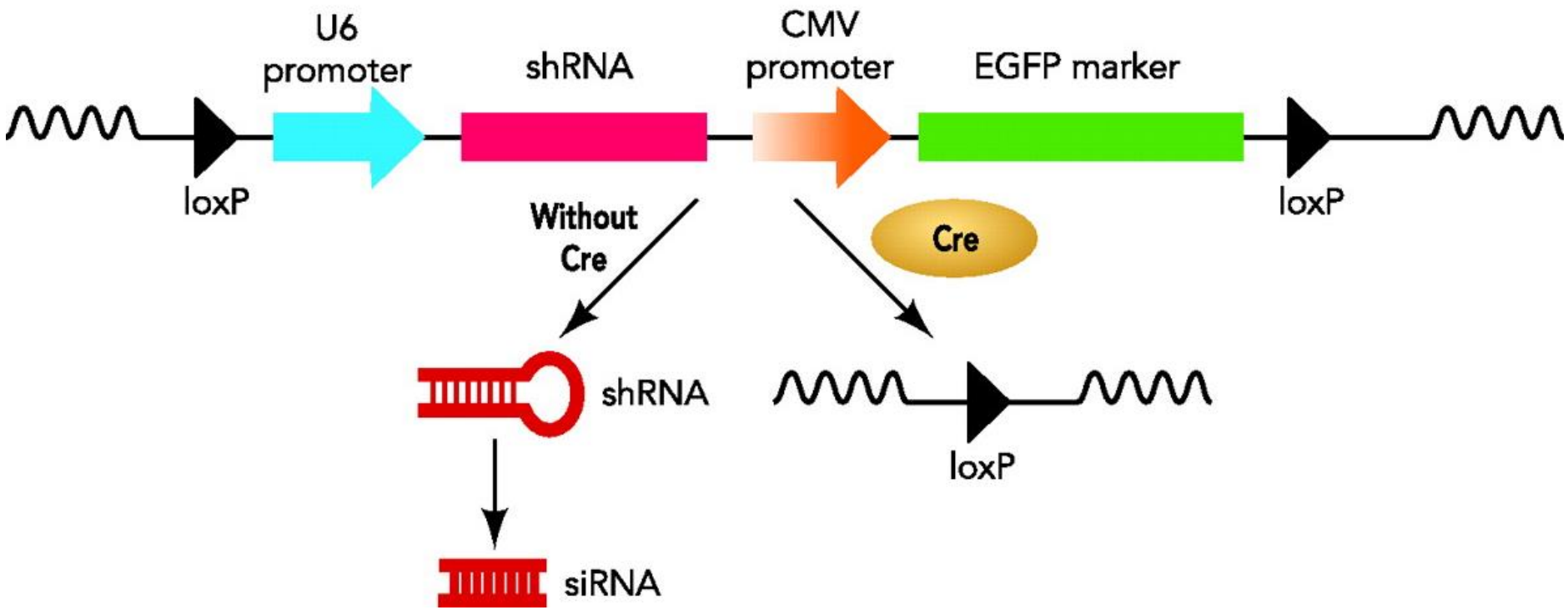

H1
promoter
shRNA
SV40 ori
Neomycin
resistance gene
SV40
pA
ES cell
transfection
Select drug-
resistant clones
Analyze RNAi
effect in ES clones
Generate
RNAi animals
with ES cells
Clump of
ES cells
Tetraploid
host embryo
Blastocyst injection
Tetraploid aggregation


H1 3′
shRNA
H1 promoter
PGK promoter
EGFP
pA
Oviductal implantation
Pseudopregnant
F0 green embryo
Phenotypic analysis
Establish transgenic RNAi Line


U6
promoter
CMV
promoter
EGFP marker
shRNA


U6
promoter
CMV
promoter
EGFP marker
shRNA
loxP
loxP
Stuffer sequence
Cre
U6
promoter
shRNA
loxP
shRNA
siRNA


U6
promoter
shRNA
CMV
promoter
EGFP marker

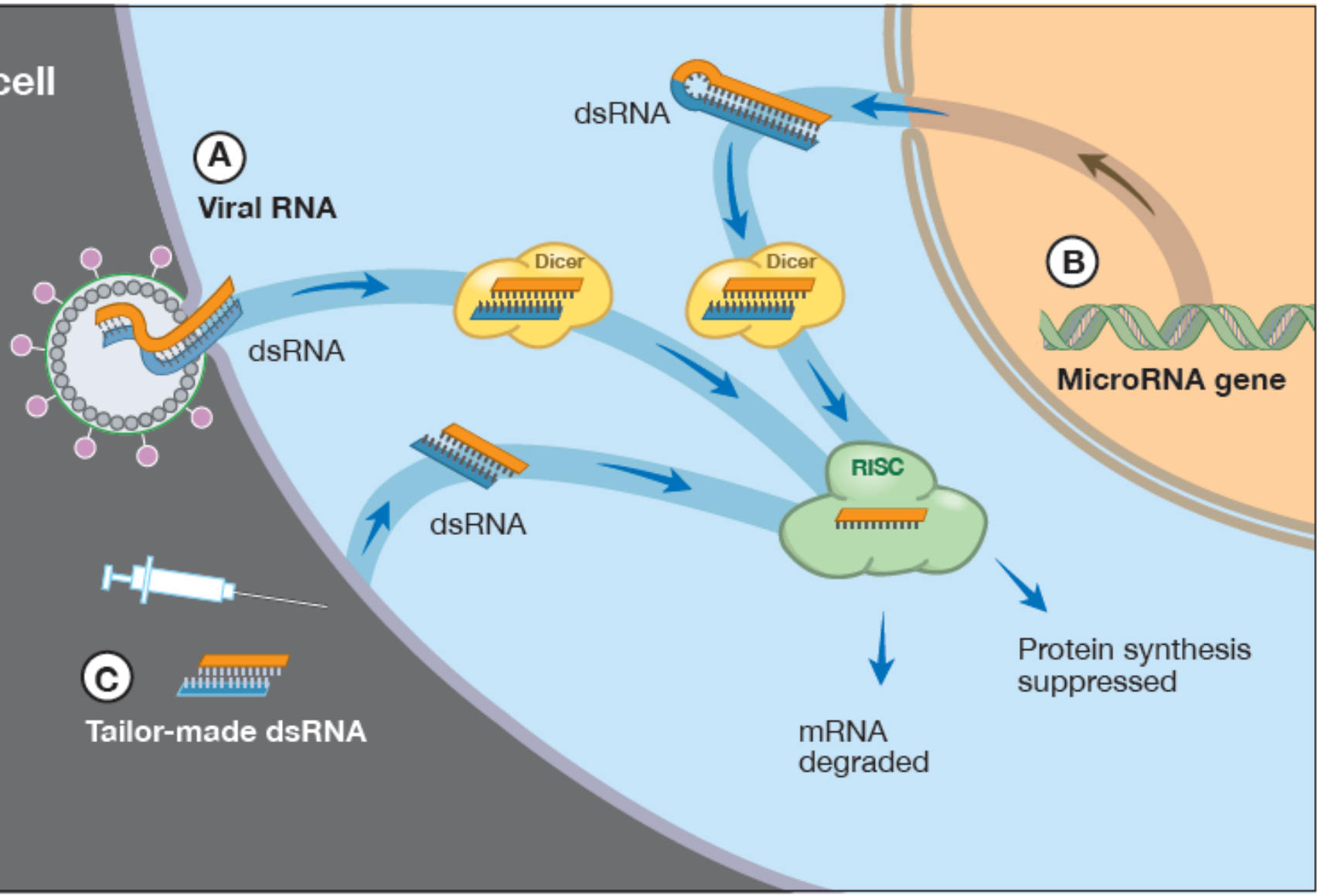

U6
promoter
shRNA
CMV
promoter
EGFP marker
loxP
loxP
Without
Cre
Cre
shRNA
loxP
siRNA

4. Several processes in the cell use RNAi

A. When an RNA virus infects the cell, it injects its genome consisting of double-stranded RNA. RNA interference destroys the viral RNA, preventing the formation of new viruses.

B. Synthesis of many proteins is controlled by genes encoding microRNA. After processing, microRNA prevents the translation of mRNA to protein.

C. In the research laboratory, dsRNA molecules are tailor-made to activate the RISC complex to degrade mRNA for a specific gene.



microRNA (miRNA)

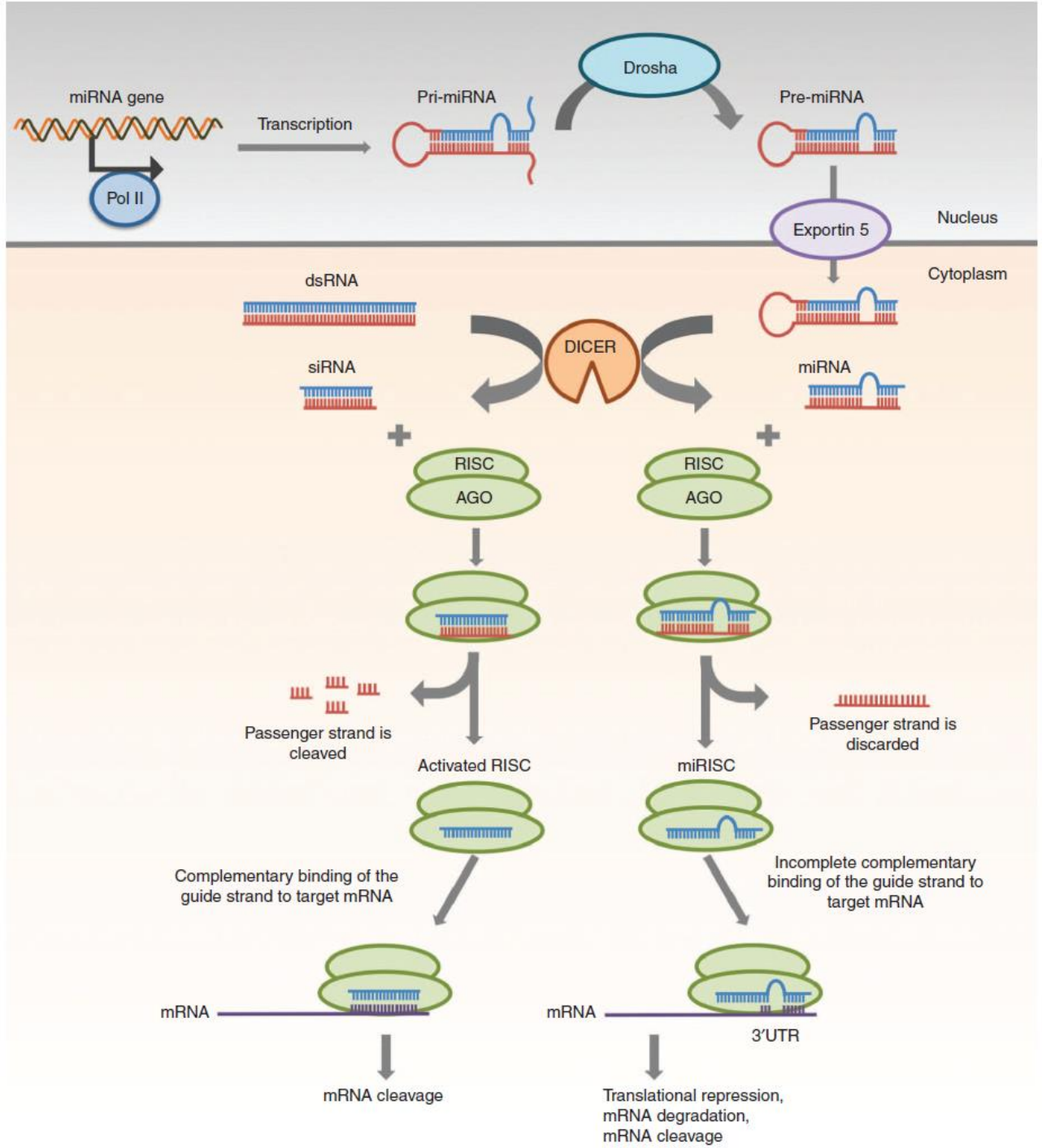
- small non-coding RNA molecule
- found in plants, animals and some viruses
- RNA silencing and post-transcriptional regulation of gene expression
- majority of miRNAs are located within the cell, some miRNAs are circulating in extracellular environment, including various biological fluids and cell culture media
- miRNAs function via base-pairing with complementary sequences within mRNA molecules
- mRNA molecules are silenced, by one or more of the following processes:
    - cleavage of the mRNA strand into two pieces,
    - destabilization of the mRNA through shortening of its poly(A) tail
    - less efficient translation of the mRNA into proteins by ribosomes.

- the human genome may encode over 1000 miRNAs which are abundant in many mammalian cell types and appear to target about 60% of the genes of humans and other mammals.

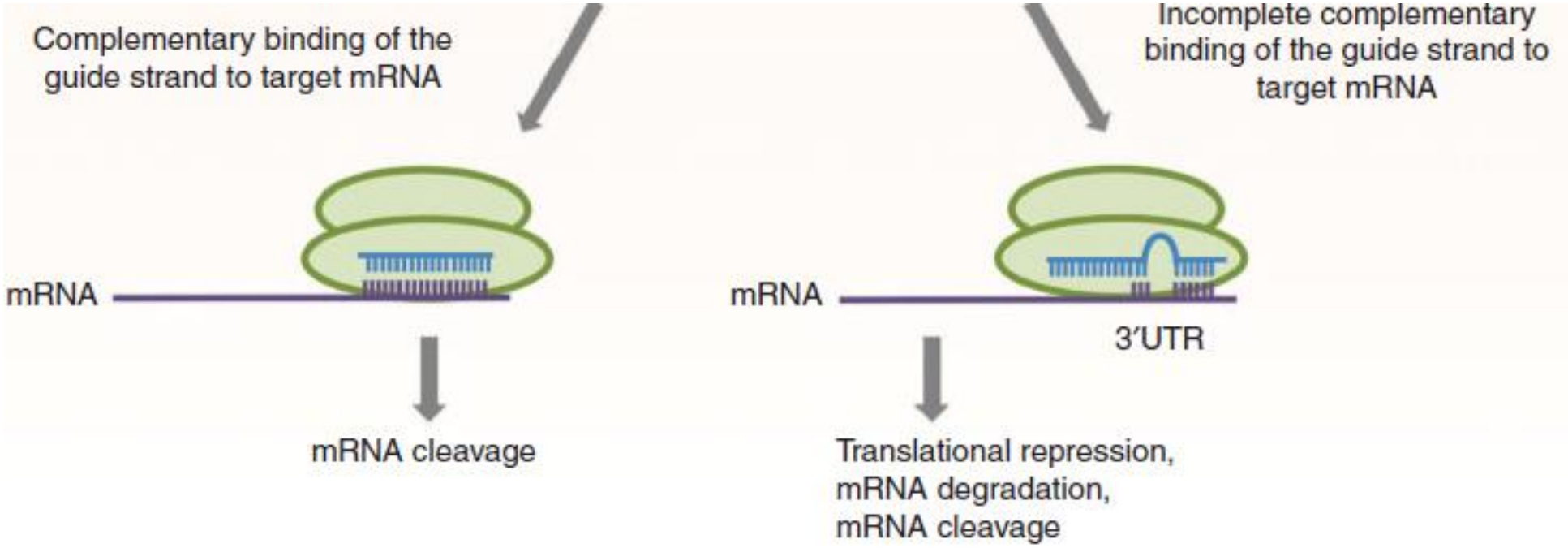
- **one miRNA targets many genes, each gene is target of many miRNA**

Table 1 Comparison of general properties between siRNA and miRNA

	siRNA	miRNA
Prior to Dicer processing	Double-stranded RNA that contains 30 to over 100 nucleotides	Precursor miRNA (pre-miRNA) that contains 70–100 nucleotides with interspersed mismatches and hairpin structure
Structure	21–23 nucleotide RNA duplex with 2 nucleotides 3'overhang	19–25 nucleotide RNA duplex with 2 nucleotides 3'overhang
Complementary	Fully complementary to mRNA	Partially complementary to mRNA, typically targeting the 3' untranslated region of mRNA
mRNA target	One	Multiple (could be over 100 at the same time)
Mechanism of gene regulation	Endonucleolytic cleavage of mRNA	Translational repression
		Degradation of mRNA
		Endonucleolytic cleavage of mRNA (rare, only when there is a high level of complementary between miRNA and mRNA)
Clinical applications	Therapeutic agent	Drug target
		Therapeutic agent
		Diagnostic and biomarker tool


miRNA gene
Pol II
Transcription
Pri-miRNA
Drosha
Pre-miRNA
Exportin 5
Nucleus
Cytoplasm
dsRNA
siRNA
DICER
miRNA
RISC
AGO
RISC
AGO
Passenger strand is cleaved
Activated RISC
miRISC
Passenger strand is discarded
Complementary binding of the guide strand to target mRNA
Incomplete complementary binding of the guide strand to target mRNA
mRNA
mRNA
3′UTR
mRNA cleavage
Translational repression, mRNA degradation, mRNA cleavage

siRNA



- siRNA could switch off the target gene, but also affect expression of unspecific targets
- which are the possible controls in RNA interference experiments to verify that the phenotype observed depends on the absence of the gene which is the siRNA target?

siRNA

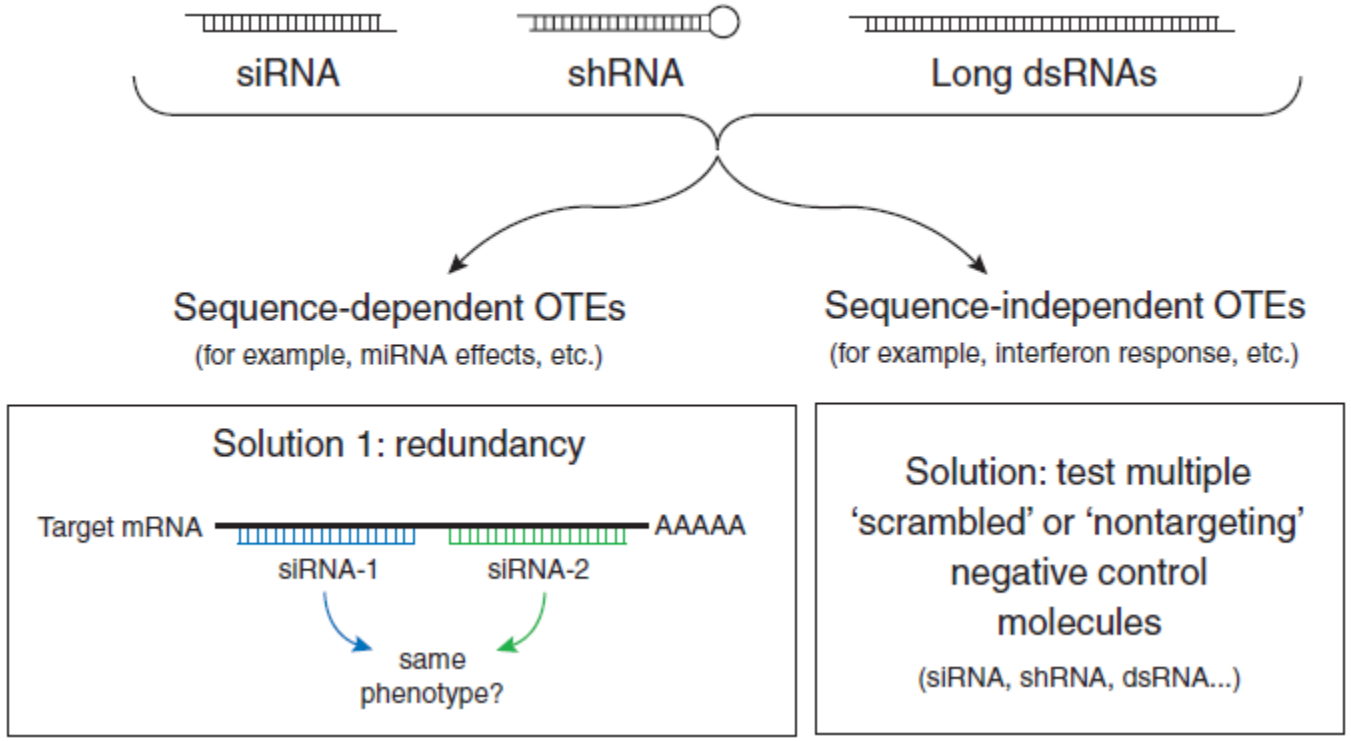


Figure 1 | Appropriate experimental controls to minimize risks of misinterpretation of RNAi data due to off-target effects (OTEs).

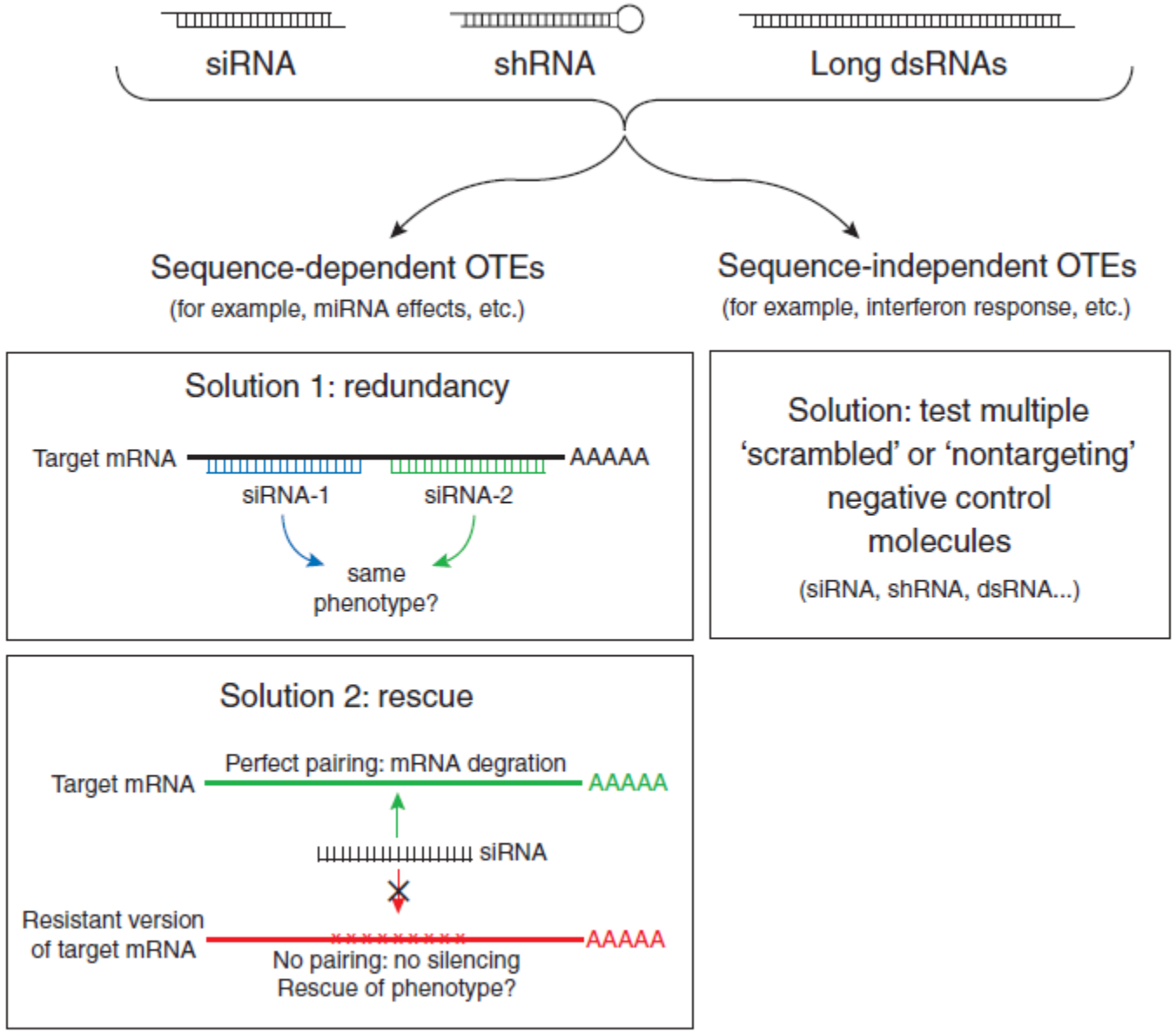


Figure 1 | Appropriate experimental controls to minimize risks of misinterpretation of RNAi data due to off-target effects (OTEs). siRNA-like molecules, vector-based shRNAs and long dsRNAs trigger detectable off-target effects in all major systems studied to date, from mammalian cells to *D. melanogaster* and *C. elegans*. Simple solutions are available to minimize the risk that an observed phenotype may arise from an off-target effect rather than the targeted gene's loss of function.

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Minimizing the risk of reporting false positives in large-scale RNAi screens

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Large-scale RNA interference (RNAi)-based analyses, very much as other 'omic' approaches, have inherent rates of false positives and negatives. The variability in the standards of care applied to validate results from these studies, if left unchecked, could eventually begin to undermine the credibility of RNAi as a powerful functional approach. This Commentary is an invitation to an open discussion started among various users of RNAi to set forth accepted standards that would insure the quality and accuracy of information in the large datasets coming out of genome-scale screens.

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siRNA

Transfection of small RNAs globally perturbs gene regulation by endogenous microRNAs

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Abstract

Transfection of small RNAs (si/miRNAs) into cells typically lowers expression of many genes. Unexpectedly, increased expression of genes also occurs. We investigated whether this upregulation results from a saturation effect, i.e. competition for intracellular small RNA processing machinery between the transfected si/miRNAs and the endogenous pool of microRNAs (miRNAs). To test this hypothesis, we analyzed genome-wide transcript responses from more than 150 published transfection experiments in 7 different cell types. We show that endogenous miRNA targets have significantly higher expression levels following transfection, consistent with an impaired effectiveness of endogenous miRNA repression. Further confirmation comes from concentration and temporal dependence. Strikingly, the profile of endogenous miRNAs can largely be inferred by correlating miRNA sites with gene expression changes after transfections. The saturation and competition effects present practical implications for miRNA target prediction, the design of si/shRNA genomic screens and siRNA therapeutics.