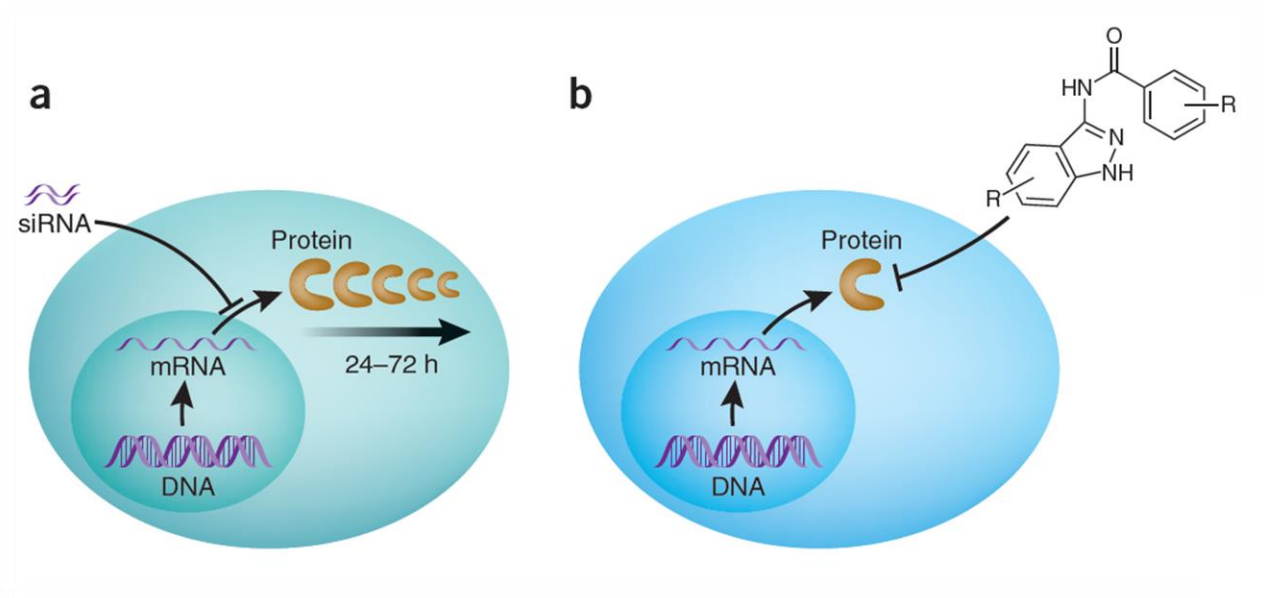
- Several methods of molecular/cell biology rely on depletion of the protein of interest
  - ➢ prediction of the effect of inhibitors

- Inhibition:
  - fast & time-flexible modulation of the activity of the target of interest
  - the protein is still present



Weiss, W. A.; Taylor, S. T.; Shokat, K. M. *Nat.Chem. Biol.* **2007**, *3*, 739.

Western Blotting is an analytical technique used in molecular biology for the qualitative detection of single proteins from a tissue sample or cells' extract.

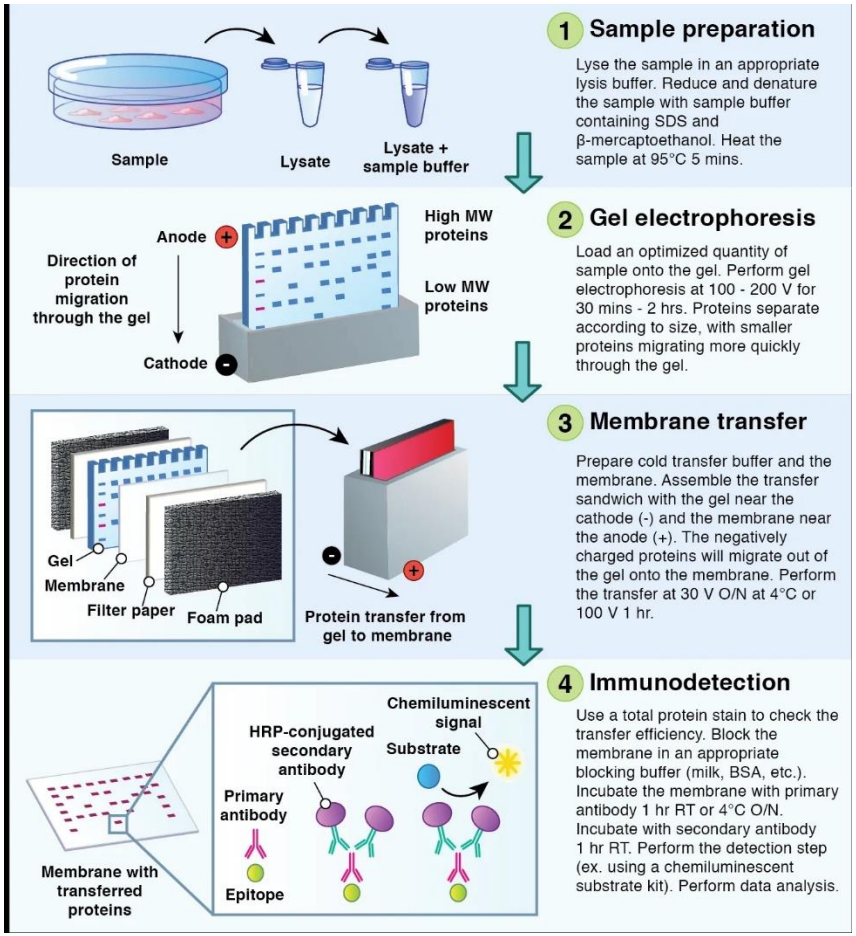
- Western blots can be used to:
  - identify a specific single protein within a complex mixture of proteins
  - evaluate the size of a protein of interest
  - measure the amount of protein expression

- Facts:
  - Name given by English biologist Edwin Southern in 1980[1]
  - Method originated in the laboratory of Harry Towbin in 1979[2] (Friedrich Miescher Institute, Basel, Switzerland)

[1] Burnette, W. N. *Analytical Biochemistry* **1981**, *112*, 195..
[2] Towbin, H.; Staehelin, T.; Gordon, J. *Proceedings of the National Academy of Sciences* **1979**, *76*, 4350.

Western blotting is achieved in 4 main steps:

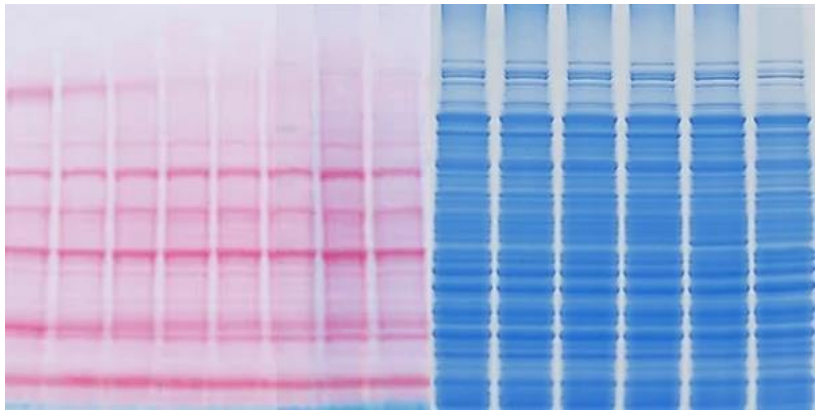
- Preparation of the sample by mixing it with a detergent called sodium dodecyl sulfate, which makes the proteins unfold into linear chains and coats them with a negative charge
- Separation of proteins by size and charge using gel electrophoresis
- Transfer of separated proteins to a solid support called a blotting membrane
- Indirect visualization of the desired protein using a specific primary antibody (binding to the desired protein) and then a secondary antibody (binding to the primary antibody). The secondary antibody is visualized through different methods (e.g., staining, immunofluorescence & radioactivity)



Picture taken from : https://www.antibodies.com/es/western-blotting

- What is the result of gel electrophoresis before membrane transfer ?

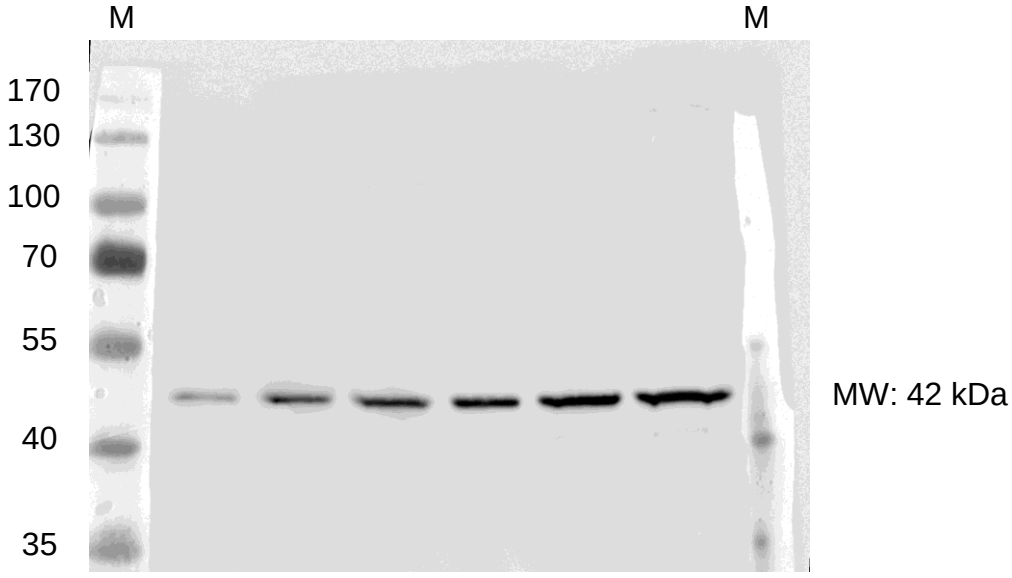
  A gel that looks like that:



Pink, Ponceau S staining; blue, Coomassie brilliant blue staining

- What is the result of membrane transfer and indirect visualization of secondary antibody ?

  A gel that looks like that:



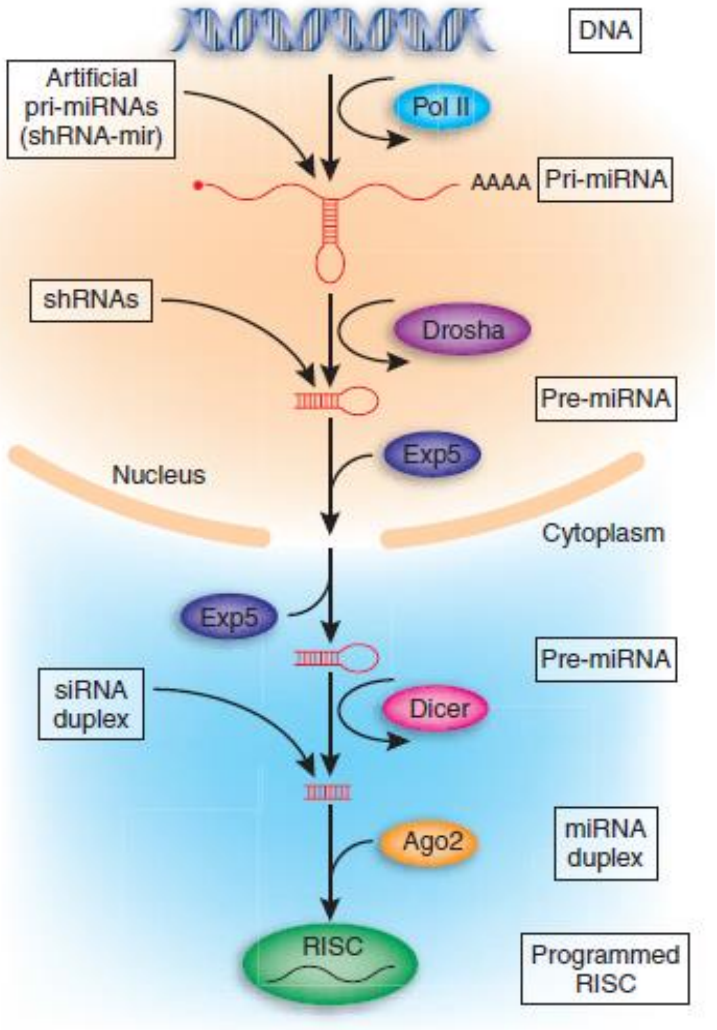
- RNAi stands for RNA interference, also known as post-transcriptional gene silencing.
- RNAi is an RNA-dependent gene silencing process = biotechnological tool capable of targeted gene inactivation.
- Fire and Mello were awarded the Nobel Prize in Medicine in 2006 for their work on the nematode worm *Caenorhabditis elegans* published in 1998[1] reporting that double-stranded RNA (dsRNA) is effective in gene silencing, driving the destruction of messenger RNA (mRNA) with sequences matching the dsRNA and thus blocking the translation of mRNA into protein.
- the first report of siRNA-mediated gene silencing in mammalian cells in 2001[2]

- Technologies used for RNAi :
  - miRNA = micro RNA
  - siRNA = small interfering RNA or silencing RNA
  - shRNA = short hairpin RNA

Educational links :

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

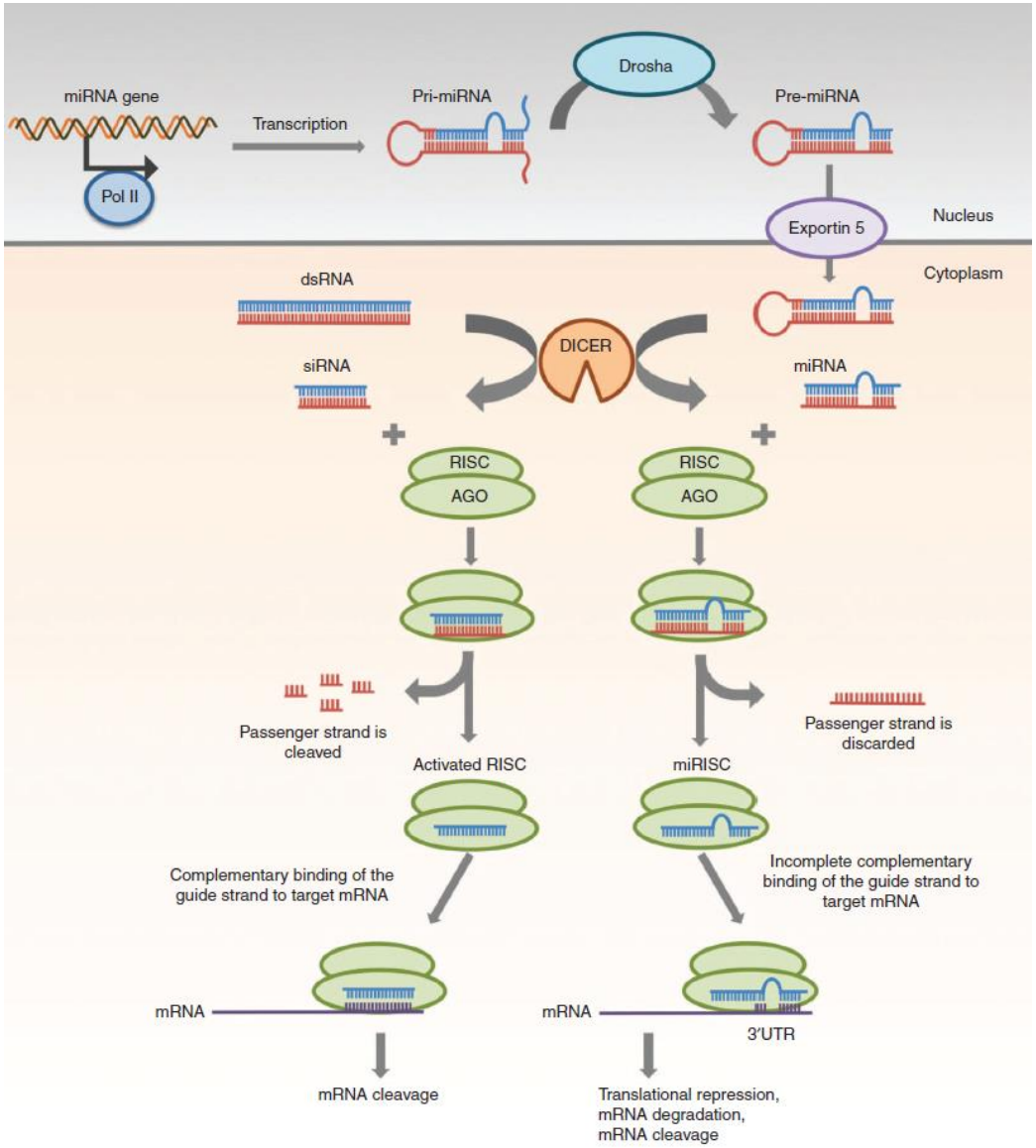
[1]Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. *Nature* **1998**, *391*, 806.
[2]Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Nature* **2001**, *411*, 494.



Artificial siRNAs can enter this pathway as synthetic siRNA duplexes, as shRNAs transcribed by Pol III or as artificial pri-miRNAs (shRNA-mir).
For simplicity, not all factors involved in miRNA biogenesis are shown.
Ago2, Argonaute-2; Exp5, Exportin-5.

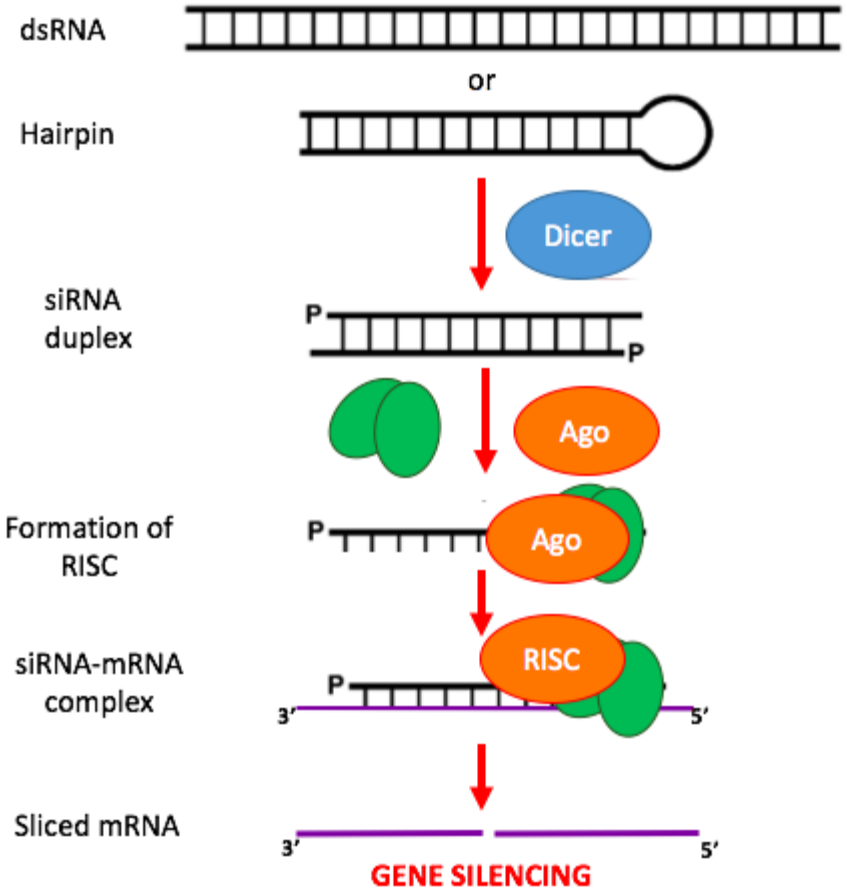
The miRNA biogenesis pathway in vertebrate cells.
Figure taken from Cullen, B. R. *Nature Genetics* **2005**, *37*, 1163.

Recommended literature :
Cullen, B. R. *Nature Genetics* **2005**, *37*, 1163.



Gene silencing mechanisms of siRNA and miRNA.
Figure taken from *Mol Ther Nucleic Acids* 2015, *4*, e252.

siRNA: dsRNA (either transcribed or artificially introduced) is processed by Dicer into siRNA which is loaded into the RISC. AGO2, which is a component of RISC, cleaves the passenger strand of siRNA. The guide strand then guides the active RISC to the target mRNA. The full complementary binding between the guide strand of siRNA and the target mRNA leads to the cleavage of mRNA.
miRNA: Transcription of miRNA gene is carried out by RNA polymerase II in the nucleus to give pri-miRNA, which is then cleaved by Drosha to form pre-miRNA. The pre-miRNA is transported by Exportin 5 to the cytoplasm where it is processed by Dicer into miRNA. The miRNA is loaded into the RISC where the passenger strand is discarded, and the miRISC is guided by the remaining guide strand to the target mRNA through partially complementary binding. The target mRNA is inhibited via translational repression, degradation or cleavage.



- Long dsRNA is cleaved by an endo-ribonuclease called Dicer. Dicer cuts the long dsRNA to form siRNA.
- Once siRNA enters the cell, it gets recruited with other proteins to form the RNA-Induced Silencing Complex (RISC).
- Once part of the RISC complex, the siRNA is unwound to form single stranded siRNA.
- The single stranded siRNA-RISC complex can scan and find a complementary mRNA.
- Once the single stranded siRNA (part of the RISC complex) binds to its target mRNA, it induces mRNA cleavage.
- The mRNA is now cut and recognized as abnormal by the cell. This causes degradation of the mRNA that is not translated into proteins.
- The gene that encodes that mRNA has been silenced.

siRNA mechanism
Figure taken from https://en.wikipedia.org/wiki/Small_interfering_RNA#Mechanism

Mechanism :

- depletion vs. inhibition

(see e.g. Nature 2016x538x477 – MCL1 knockout lethal, MCL1 inhibition OK)

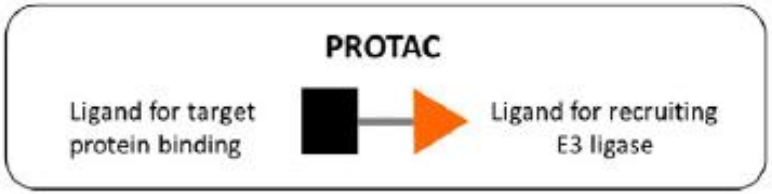
Recommended literature :

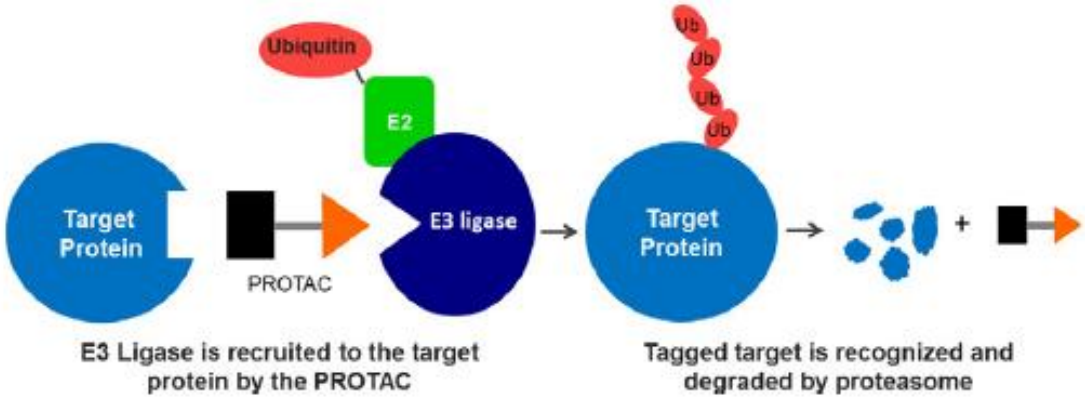
Hannon, G. J. *Nature* **2002**, *418*, 244.
Lam, J. K.; Chow, M. Y.; Zhang, Y.; Leung, S. W. *Mol Ther Nucleic Acids* **2015**, *4*, e252.

- PROTACs are currently a VERY HOT TOPIC in pharmaceutical R&D
- PROTAC = acronym for PROteolysis Targeting Chimera
- A PROTAC is a tool amongst others for Targeted Protein Degradation (TPD)
- PROTAC molecules are known as Active Degraders of targeted proteins
- First publication in 2001: Protacs: Chimeric molecules that target proteins to the Skp1–Cullin–F box complex for ubiquitination and degradation, Sakamoto, K. M.; Kim, K. B.; Kumagai, A.; Mercurio, F.; Crews, C. M.; Deshaies, R. J. *Proceedings of the National Academy of Sciences of the United States of America* **2001**, *98*, 8554.

- PROTACS are heterobifunctional small molecules (700 to 1000 Da). They consist of :
  - a binding moiety for the targeted protein to degrade
  - a binding moiety for an E3 ubiquitin ligase
  - and a linker joining the two binding moieties

- Upon binding of both moieties, a ternary complex between target protein and E3 ligase is formed, leading to polyubiquitination of the target protein (on exposed lysine residues).
- Upon dissociation of the complex, the polyubiquitinated target protein is recognized by the proteasome and subsequently degraded.
- The PROTAC molecule is then free to repeat the process (catalytic mode of action => less drug needed => less off-target toxicity).



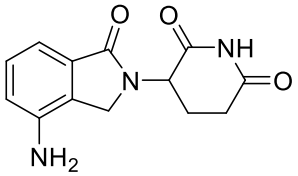


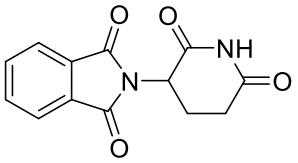
Neklesa, T. K.; Winkler, J. D.; Crews, C. M. *Pharmacology & Therapeutics* **2017**, *174*, 138.

- Why PROTACs?
- PROs
  - Catalytic mode of action.
  - The binding to the target protein can occur anywhere on the protein.
  - No requirement to target catalytic active sites of proteins or to block protein-protein interactions.
  - Target undruggable proteome.
  - Behave like traditional small molecules (exposures are dose proportional, PROTACs show good tissue distribution, optimized PROTACs show low hepatic clearance).
  - Can be very selective (ubiquitin ligase system has to transfer the ubiquitin to the exposed lysine residue on the target protein => tuning achievable even on closely related proteins depending on the positioning of the exposed lysine with respect to the ligase).
- CONs
  - Large molecules.
  - Linker design can affect the mode of action of the PROTAC (Molecular Glue[1, 2]), SAR required.
  - Acquired resistance by point mutation in targeted protein[3].

[1] Yang, J., *et al.*; *Journal of Medicinal Chemistry* **2019**, *62*, 9471.
[2] Bemis, T. A., *et al.*; *Journal of Medicinal Chemistry* **2021**, *64*, 8042.
[3] Jiang, B., *et al.*; *Nature Chemical Biology* **2021**, *17*, 675

- Design of PROTACS:
  - The Cullin 4A E3 ligase complex and the Cullin 2 E3 ligase complex are two powerful E3 ligase complexes for the design of highly potent and effective PROTAC molecules, because of the availability of potent and drug-like small-molecule ligands to recruit these degradation complexes
  - immunomodulatory imide drugs (IMiDs) = potent small-molecule ligands for cereblon, a receptor protein for the Cullin 4A complex:

lenalidomide 

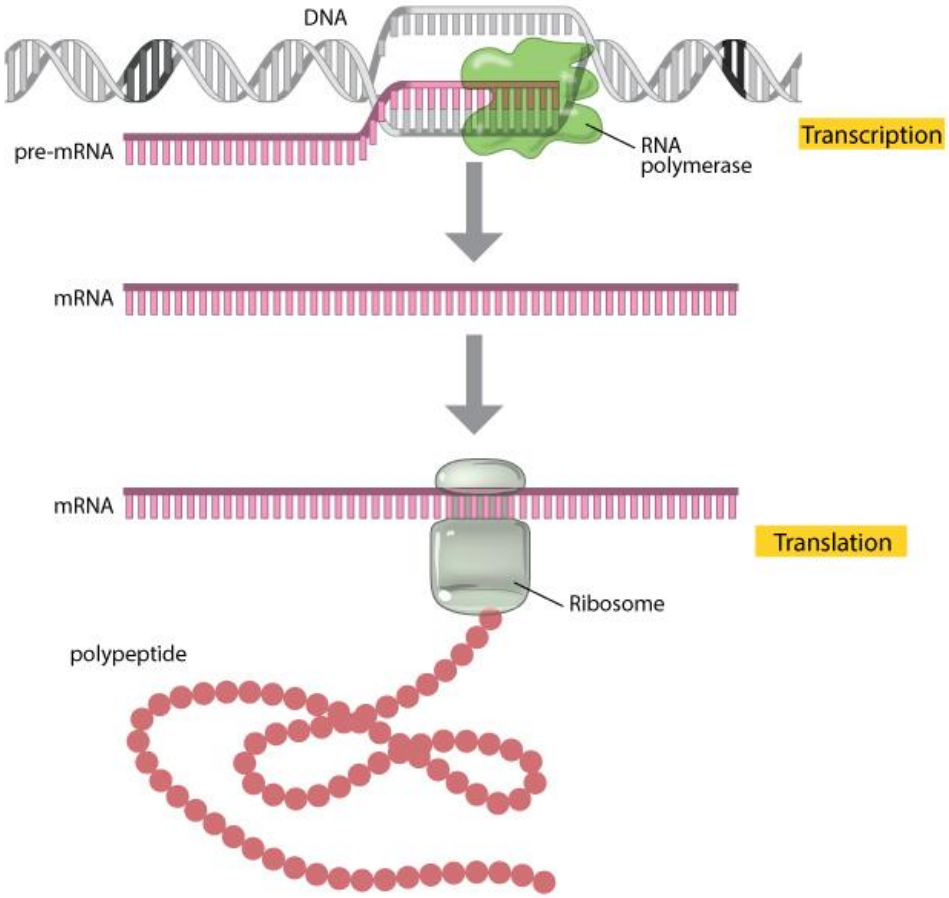
thalidomide 

From DNA to RNA to Protein

A gene is expressed through the processes of transcription and translation.

During transcription, the enzyme RNA polymerase (green) uses DNA as a template to produce a pre-mRNA transcript (primary transcript, pink).

The pre-mRNA is processed (by 5' capping, 3' polyadenylation, and alternative splicing processes) to form a mature mRNA molecule that can be translated by the ribosome to build the protein molecule (polypeptide) encoded by the original gene.



Educational links :

- https://www.nature.com/scitable/topicpage/translation-dna-to-mrna-to-protein-393/

Biotechnological tool capable of targeted gene inactivation & targeted gene editing.

Biotechnological tool revolutionizing genetic engineering.

Doudna and Charpentier were awarded the Noble Prize in Chemistry in 2020 for the development of a method for genome editing.

Origins of CRISPR:

- Discovered by basic research on how a bacteria fights a viral infection[1,2]
- CRISPR is an RNA-mediated adapted immune system in bacteria and archaea that protects them from viruses and plasmids
- Origin of CRIPSR name in 2005[3]
- First description of what is now known as CRIPSR locus by Mojica in 1993[4]

Educational links :

- https://media.hhmi.org/biointeractive/click/CRISPR/
- https://www.youtube.com/watch?v=4YKFw2KZA5o
- https://www.youtube.com/watch?v=TdBAHexVYzc
- https://www.youtube.com/watch?v=KSrSIErIxMQ

[1] Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J. A.; Charpentier, E. *Science* **2012**, *337*, 816.
[2] Mojica, F. J.; Díez-Villaseñor, C.; García-Martínez, J.; Soria, E. *J Mol Evol* **2005**, *60*, 174
[3] Jansen, R.; Embden, J. D.; Gaastra, W.; Schouls, L. M. *Mol Microbiol* **2002**, *43*, 1565
[4] Mojica, F. J.; Juez, G.; Rodríguez-Valera, F. *Mol Microbiol* **1993**, *9*, 613

- CRIPSR = acronym for Clustered Regularly Interspaced Short Palindromic Repeats

- Technology consists of a Cas9-guide RNA complex (known as Cas9 complex) that binds to PAMs:
  - Cas9 that stands for CRISPR associated protein 9. It is an endonuclease (enzyme that cuts DNA from within = molecular scissor). Cas9 activity results in DNA double-strand breaks (DSBs).

  - Guide RNA, (sgRNA, single guide RNA) synthetic sequence of RNA (about 20 nucleotides) that matches a target sequence of interest within a particular gene.

  - PAM stands for Protospacer Adjacent Motif. Commonly consists of a three-nucleotide sequence in the genome (5'-NGG-3' where N represents any nucleotide (A, C, G or T)). Cas9 recognizes and binds PAM motifs. The presence of PAM close to the targeted DNA site is a necessity.

How does it work ?

- Cas9 complex recognizes and binds to PAM motifs (that occurs about every 50 bases) until the guide RNA finds its perfectly matching sequence along the DNA strands (via Watson-Crick base pairing). Upon matching, Cas9 unwinds and pulls-apart the DNA double helix (upstream of PAM) that allows formation of a DNA-RNA helix.
- Only then the DNA-cutting activity of Cas9 is activated. Two specific cuts (on each strand of the DNA) are made, three nucleotides upstream of the PAM motif. It results in a DNA double-strand break.
- The break is repaired by naturally occurring DNA repair processes. Two main repair pathways exist to join the broken DNA strands:
  - NHEJ: Non-Homologous End Joining, an error prone repair process leading to mutation at the site of cleavage. If the targeted cleavage happens within a gene's coding region, the repair by NHEJ might inactivate the gene due to the frameshift in the coding sequence.
  - HR: Homologous Repair or Homology-directed Repair, an error free repair process leading to the original DNA strands. Cas9 (acting as a catalytic machinery) will keep on repeating its tasks until a mutation arise at the targeted site.

Recommended literature about CRISPR structures and mechanisms :

Lander, E. S. *Cell* **2016**, *164*, 18.
Jiang, F.; Doudna, J. A. *Annu Rev Biophys* **2017**, *46*, 505.

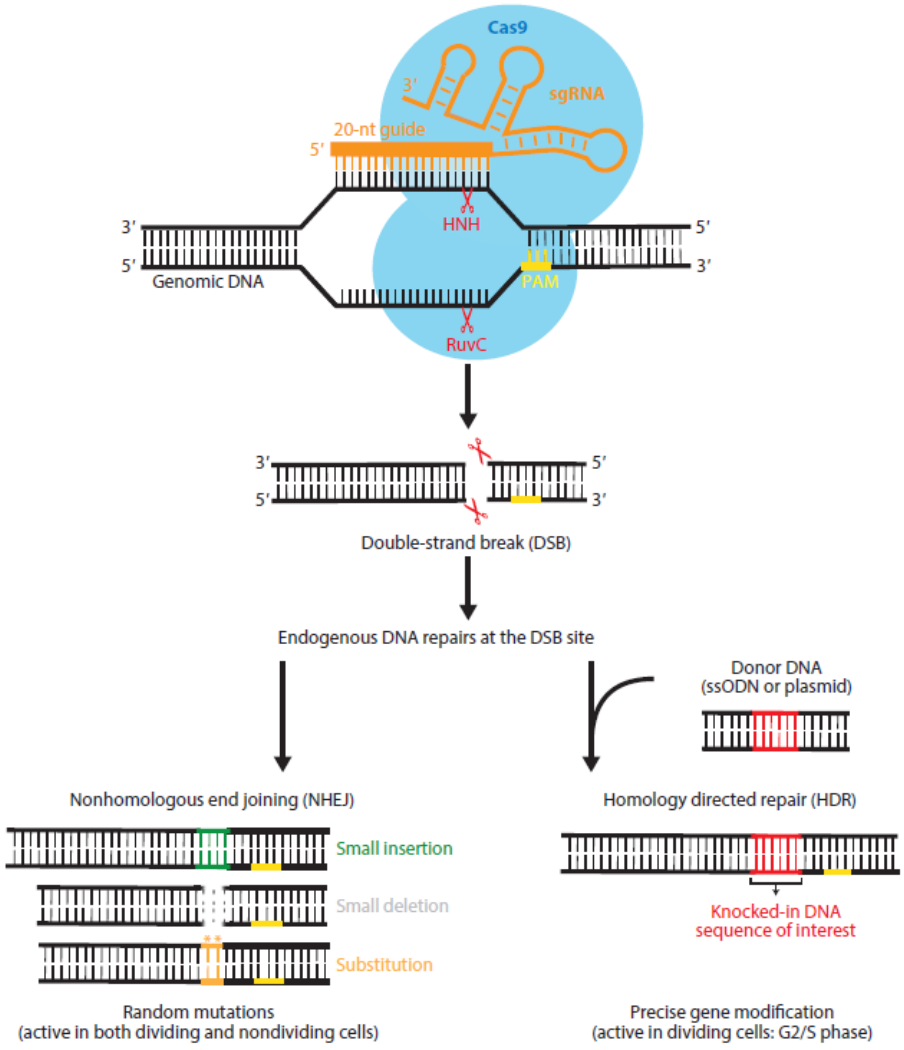


Figure taken from *Annu Rev Biophys* **2017**, *46*, 505.

Recommended literature about CRISPR structures and mechanisms :
Lander, E. S. *Cell* **2016**, *164*, 18.
Jiang, F.; Doudna, J. A. *Annu Rev Biophys* **2017**, *46*, 505.

Common targeted edits:

- conversion of DNA base pairs (e.g., point mutation = gene inactivation)
- deletion of DNA base pairs
- insertion of DNA base pairs
- a combination of the above changes (e.g., replacement of DNA base pairs = gene correction)

Recommended literature:
Anzalone, A. V.; Koblan, L. W.; Liu, D. R. *Nat Biotechnol* **2020**, *38*, 824.

CRISPR-Cas-derived genome editing agents :

- CRIPSR-Cas nucleases = nuclease variants with broaden targeting scope and specificity. 2 main nucleases Cas9 and Cas12. Engineered nuclease variants can bind to different PAM motifs.
- CRIPSR-Cas base editors = tool precisely installing point mutations without DSBs = tool to repair disease related mutations. CRISPR-Cas is engineered with a catalytically impaired Cas nuclease which is fused with a deaminase enzyme able to convert bases.
- CRIPSR-Cas transposases/recombinases = tool mediating rearrangements of large segments of DNA. Approaches with many limitations. Emerging editing technology.
- CRIPSR-Cas prime editors = tool changing an original DNA sequence by inserting a new sequence. CRISPR-Cas is engineered with a catalytically impaired Cas nuclease which is fused with a reverse transcriptase domain able to direct the synthesis of the edited DNA strand onto the 3' end of the target DNA strand. The non-edited DNA strand will then be repaired using the edited strand as a template.
- CRISPRi = CRISPR interference = CRISPR-Cas gene repression *Cell* **2013**, *152*, 1173 specifically interfere with transcriptional elongation, RNA polymerase binding, or transcription factor binding; effects are reversible
- CRISPRa = CRISPR-Cas gene activators *Nat Commun* **2018**, *9*, 2489 transcriptional activation domains fused to Cas nuclease

Recommended literature:
Anzalone, A. V.; Koblan, L. W.; Liu, D. R. *Nat Biotechnol* **2020**, *38*, 824.

CRISPR-Cas screening can be used as a tool for genetic screens :

- to validate novel drug targets.
- to study the underlying causes of diseases.
- CRISPR-Cas libraries are generated to target thousands of human genes with a large number of sgRNAs per gene.
- 3 different mechanisms : the most common is CRISPR KO (knockout) which completely inactivates a gene. The other two either activate (CRISPRa) or inhibit (CRISPRi) the expression of a gene without altering the DNA. CRISPR-Cas libraries are used to find out which genes are providing a positive (cell enrichment) or a negative (cell viability) effect.
- CRISPR-Cas screening can be done in the presence of a specific enzyme inhibitor to determine which genes promote or reduce its activity or without the presence of an inhibitor.

Pros :

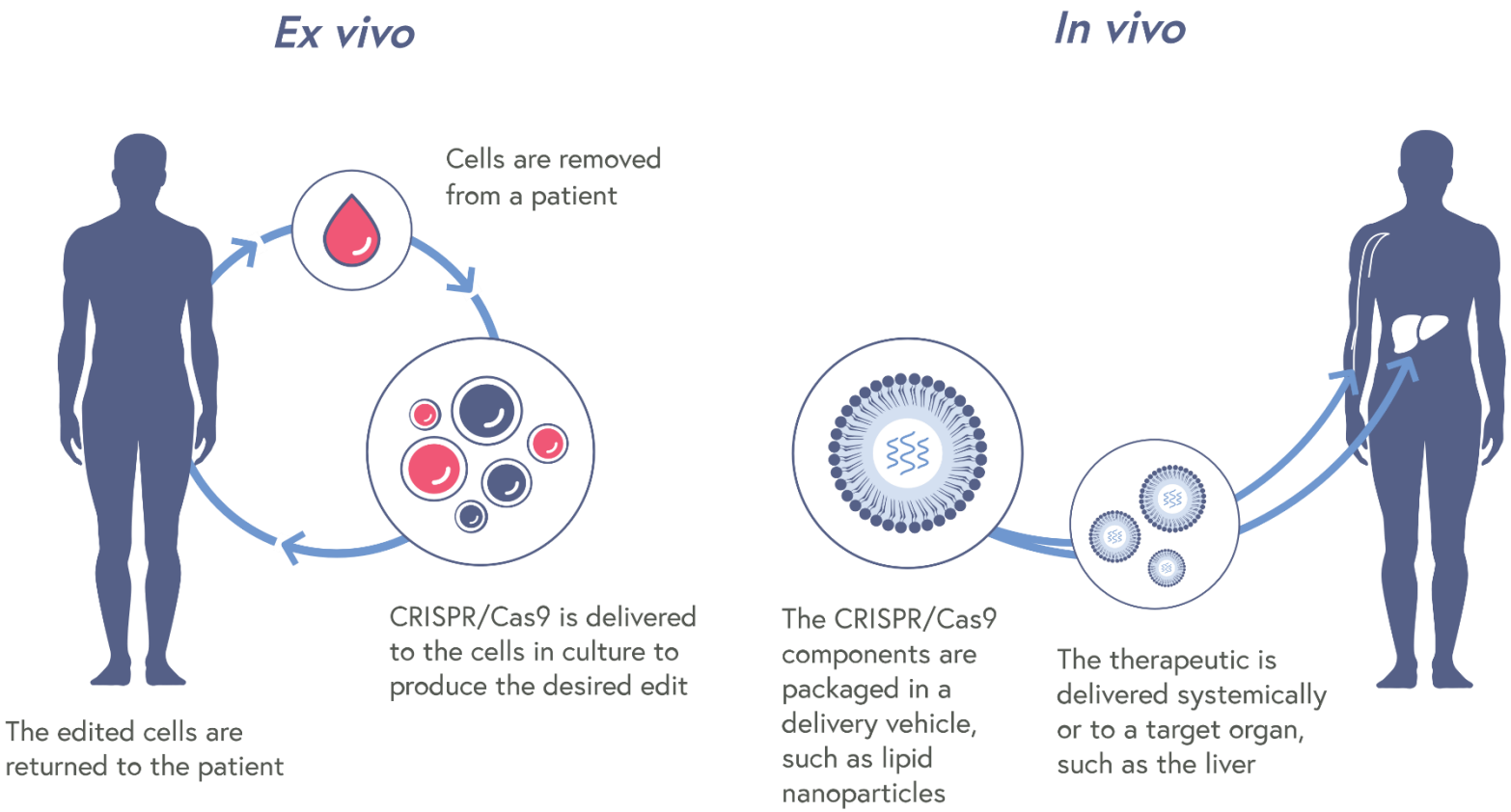
- Accelerates research in basic science
- Potential therapeutics to treat diseases such as:

cancers, Alzheimer's disease & diabetes, Duchene muscular dystrophy, cystic fibrosis, etc.

Cons :

- CRISPR-Cas specificity
- Delivery of CRISPR-Cas therapeutics *in vivo*
- Ethical issues (e.g., human genetic enhancements)

CRISPR Therapeutics aim to develop transformative gene-based medicines based on CRISP-Cas gene editing.
Cell therapy approaches in human.
Cells can either be edited *ex vivo* (outside the body) or *in vivo* (inside the body)



Picture taken from http://www.crisprtx.com/gene-editing/therapeutic-approach

Another educational link: http://www.crisprtx.com/gene-editing/crispr-cas9

- CETSA = acronym for CEllular Thermal Shift Assay
- CETSA method first described by Nordlund in 2013 published in Science.[1]
- CETSA based on the biophysical principle of ligand-induced thermal stabilization of target proteins. Ligand binding can stabilize the protein and prolong its half-life.
- This thermal stability shift phenomenon can be employed to screen for small molecule probes and to validate target engagement.
- CETSA constitutes the first broadly applicable method to assess direct drug binding in cells and tissues.
- CETSA has become a valuable tool for the validation and optimization of drug target engagement in drug discovery projects (industry & academia).

Recommended literature :
[1]Molina *et al*., *Science* **2013**, *341*, 84.

What does Thermal Shift Assay (TSA) measure ?

- TSA measures the thermal melting curves of a protein (corresponding its unfolding).
- TSA are applied only to purified proteins.
- The thermal shifts of a protein exposed to a ligand at different concentrations correlate with the IC_{50}s.

- When the protein binds to a ligand, the melting temperature T_m is typically shifted to a higher temperature (producing a thermal shift ΔT_m).

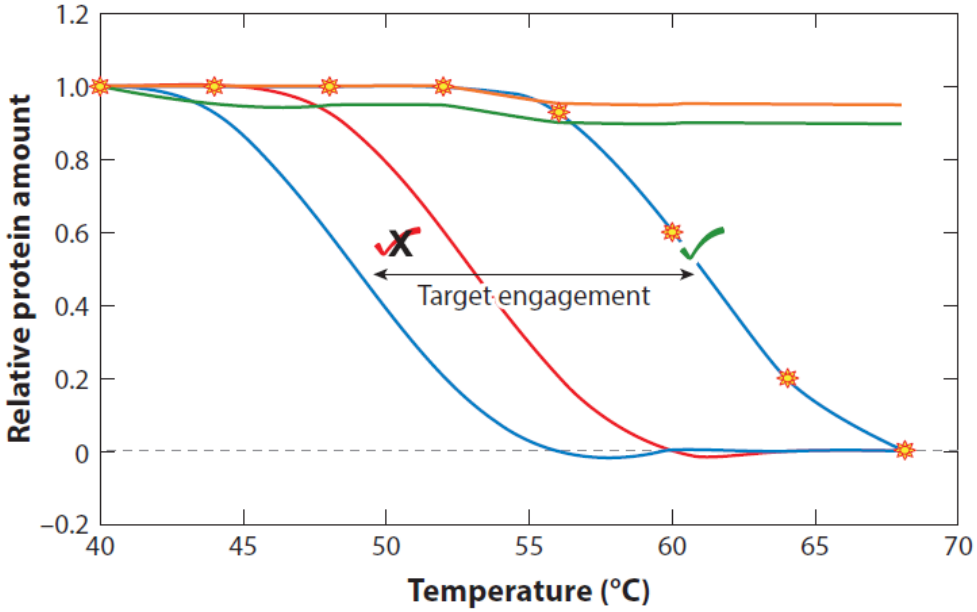


Figure taken from *Annu Rev Pharmacol Toxicol* **2016**, *56*, 141.
Schematic illustration of a thermal shift ΔT_m corresponding to target engagement.

What does CETSA measure ?

- CETSA measures the thermal melting curves of a protein in cells or tissues.

How ?

- Multiple aliquots of cell lysate are heated to different temperatures.
- After cooling, the samples are centrifuged to separate soluble fractions from precipitated proteins.
- The target protein present in the soluble fraction is quantified by Western Blotting.

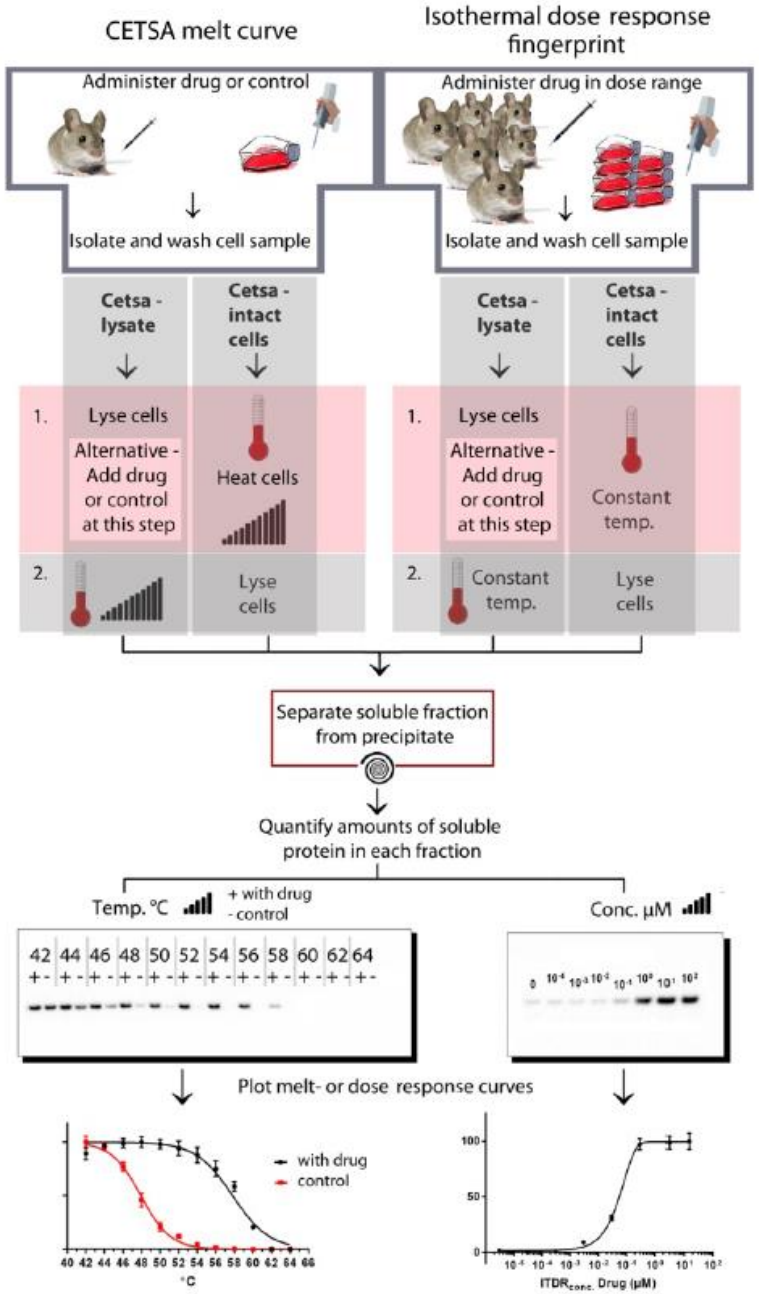


Figure taken from *Science* **2013**, *341*, 84.
Schematic illustration of CETSA melt curve and $ITDRF_{CETSA}$ procedure.
ITDRF = IsoThermal Dose Response Fingerprint

Investigation of drug concentrations effects can be done:

- on purified proteins (*in vitro*)
- on lysate aliquots or intact cells (*ex vivo*)
- on animals (*in vivo*)

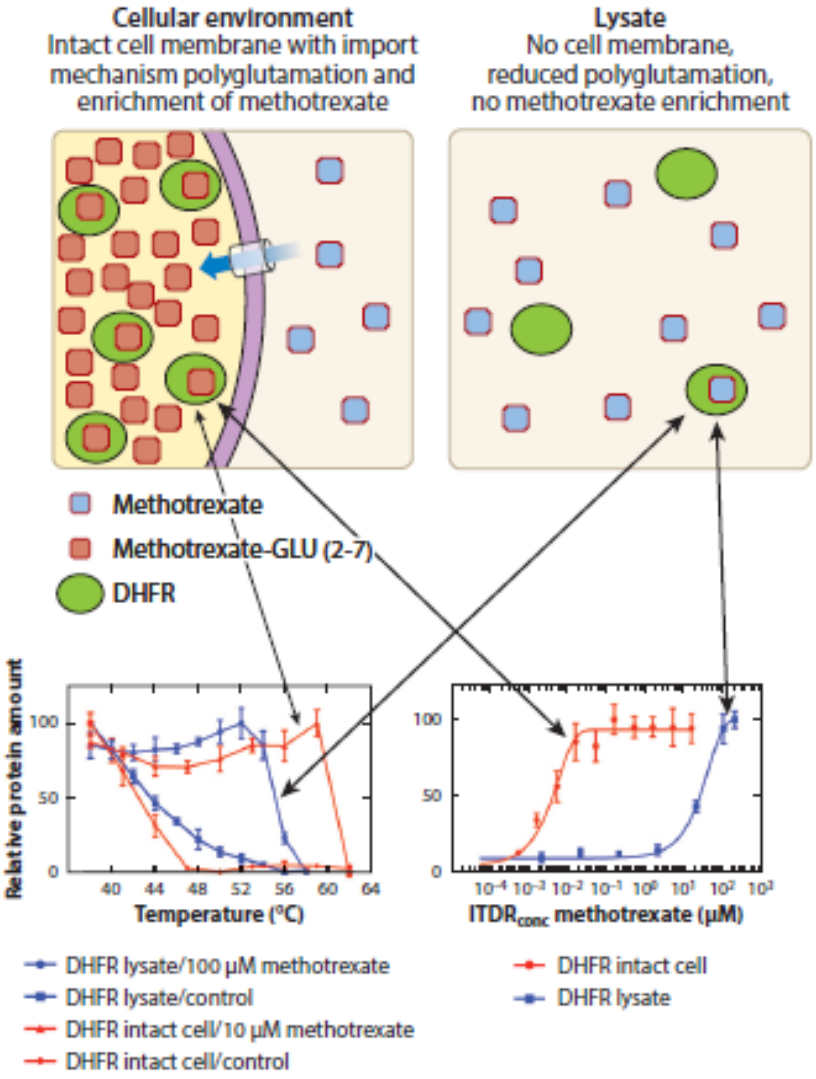


Figure taken from *Annu Rev Pharmacol Toxicol* **2016**, *56*, 141.
Illustrative examples of CETSA melting curves and ITDRFs.

When intact cells are assessed via CETSA, factors contributing to target engagement (e.g., membrane transport, accumulation, specific activation events) are detectable and distinguishable from the lysate results.

Dihydrofolate reductase (DHFR) and the drug methotrexate serve here as examples.

Methotrexate is imported by cells via specific transport mechanisms and is subsequently polyglutamated in the cell; this modification hinders it from exiting the cell, resulting in its accumulation and apparently increased concentration.

In contrast, in a cellular extract (*right*), no accumulation occurs, and methotrexate and DHFR exist in a more dilute environment and are less likely to engage with each other.

Recommended literature :
Molina *et al.*, *Annu Rev Pharmacol Toxicol* **2016**, *56*, 141

- First published proteome-wide MS-CETSA study in 2014 in Science.[1]

- MS-CETSA assay uses the tandem mass tag (TMT)-based isobaric labelling strategy.[2] Isobaric mass tags have identical overall mass but vary in terms of the distribution of heavy isotopes around their structure. The most common isobaric tag is amine-reactive (TMT which modifies lysine residues), but tags that react with cysteine residues and carbonyl groups in proteins are also available.

- MS-CETSA assay allows the relative quantification of proteins at different temperatures and different drug concentrations. Such experiment enables parallel binding studies of drugs to many proteins in the lysate, cell or tissue context.

Recommended literature :
[1]Savitski *et al*., *Science* **2014**, *346*, 1255784.
[2]Werner *et al*., *Anal Chem* **2012**, *84*, 7188