Exercises week 5, important background information:

General:

- **Understand the applied methods some of which are illustrated below in this handout.**

- **Be able to discuss Figure 1-5.**

Figure 1 :
For SELEX method (Figure 1B, see page 3 of this handout)
How could you confirm that the Bound (B) complex indeed contains the indicated polypeptides and is not an agglomerate of DNA only?

Figure 2:
Explain

Figure 3:
Explain and understand methods
If you had doubts about the specificity of the anti-53BP1 antibody, how would you test if the detected foci indeed correspond to 53PB1?
(For Cre-Lox system see page 5; For FISH see page 4, for IF see page 7)

Figure 4:
Explain; (for Pot1a and Pot1b paralogs in the mouse, see page 2)

Figure 5:
Explain figure, understand 5'end mapping by STELA, Southern blot (see page 6)

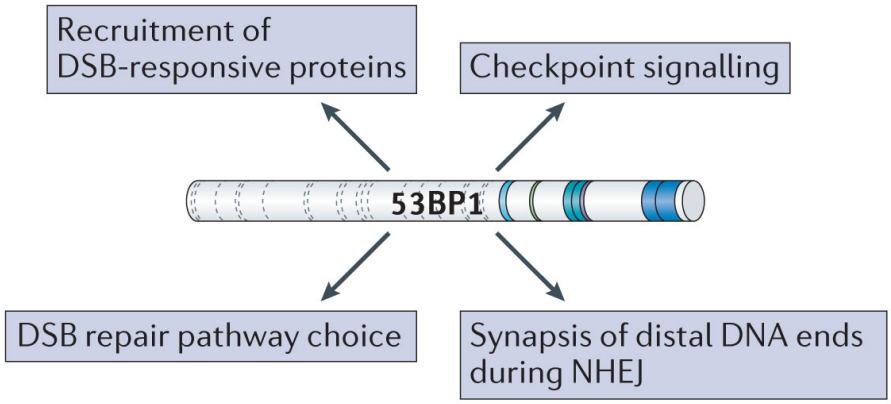
Human POT1, TPP1; and Pot1a and Pot1 b alleles in mice:

Human telomeres are protected by shelterin, a complex that includes the POT1 single-stranded DNA binding protein. TPP1 elevates the ssDNA binding affinity of POT1. In addition, TPP1 via its interaction with TIN2, which in turn binds TRF1 and TRF2, helps recruiting POT1 to telomeres. POT1 represses ATR signaling and homology directed repair (HDR).

In contrast to humans, mouse telomeres contain two POT1 paralogs, POT1a and POT1b (Genes separated by speciation are called orthologs. Genes separated by gene duplication events are called paralogs).

Double-knockout cells promote DNA damage signaling at chromosome ends and senescence. POT1a/b are largely dispensable for repression of telomere fusions. POT1a, but not POT1b, is required to repress a DNA damage signal at telomeres.

53BP1:



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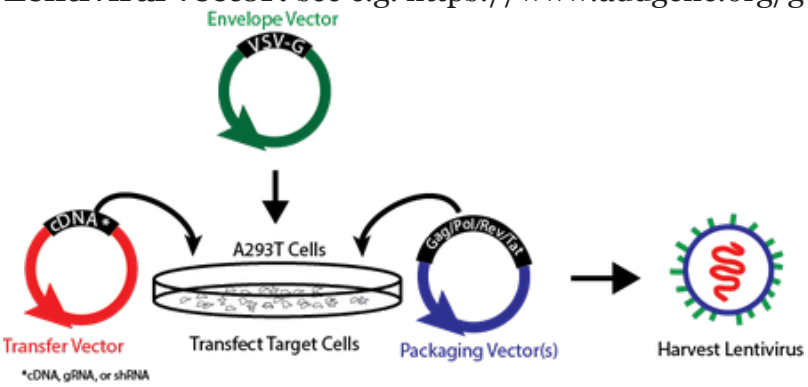
53BP1 is recruited to damaged chromatin. 53BP1 and cofactors protect the broken DNA end, inhibit long-range end resection and thus promote NHEJ.

SELEX (*Systematic Evolution of Ligands by EXponential Enrichment*)

Adapted from doi: 10.1016/j.biochi.2015.04.015 :

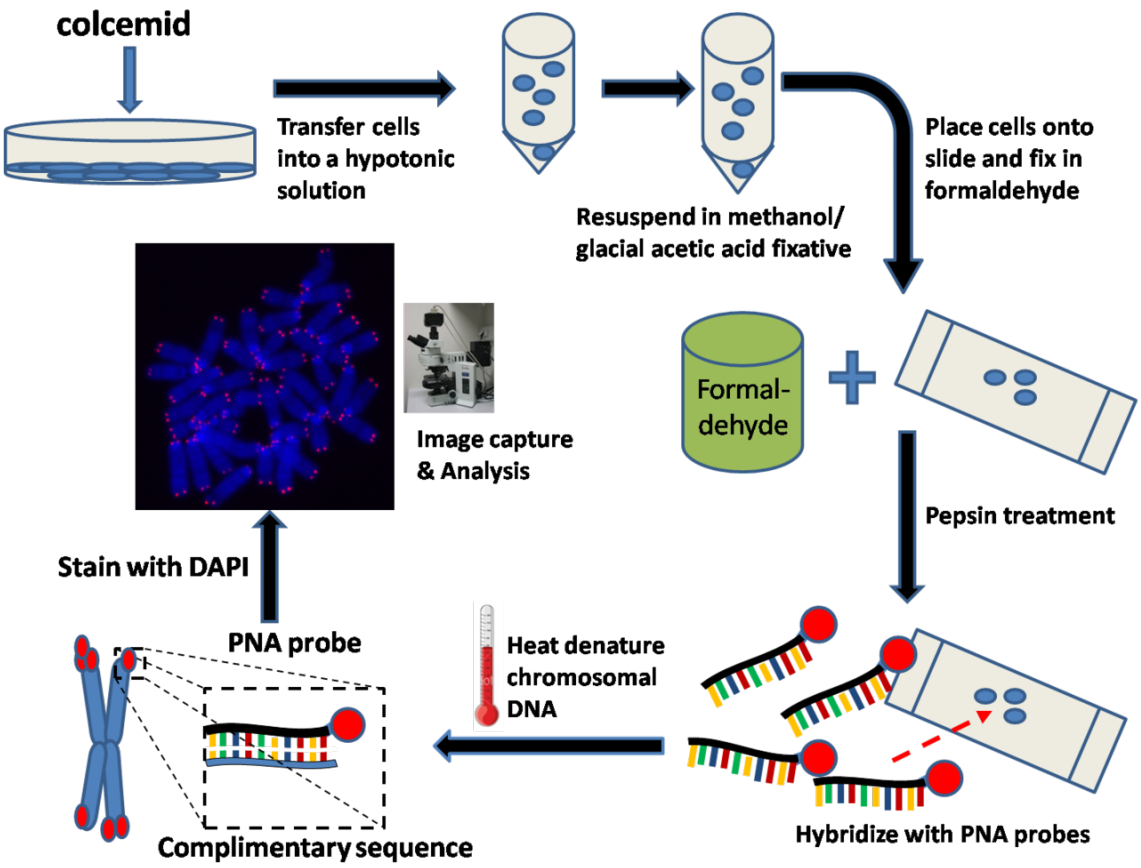
SELEX was used to reexamine the DNA-binding specificity of human POT1.
An oligonucleotide library containing a random core of 35 nucleotides flanked by PCR priming sequences was synthesized
(5'- CAGTAGCACACGACATCAAG $(N)_{35}$TGCATGTCTCGTGTCAGTTG-3').
A Flag-tagged human POT1 protein was produced. The oligonucleotide library was incubated with the Flag-POT1 to allow for the formation of protein/DNA complexes. With beads coated by the anti-Flag M2 antibody, protein/DNA complexes containing Flag-POT1 were captured along with their associated DNA. The recovered ssDNA molecules were then PCR amplified, converted back to ssDNA, and subjected to additional rounds of selection. After six consecutive rounds of SELEX, the selected and reamplified DNA was cloned and sequenced

Lentiviral vector: see e.g. https://www.addgene.org/guides/lentivirus/



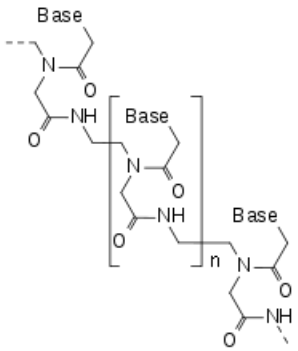
- Lentiviral vectors can carry relatively large transgenes
- Can be used for gene editing (e.g. deliver CRISPR elements to the cell)
- Ability to transduce both dividing and non-dividing cells
- Integration into the host genome and stable expression of the transgene

Telomere FISH protocol: https://en.wikipedia.org/wiki/Q-FISH

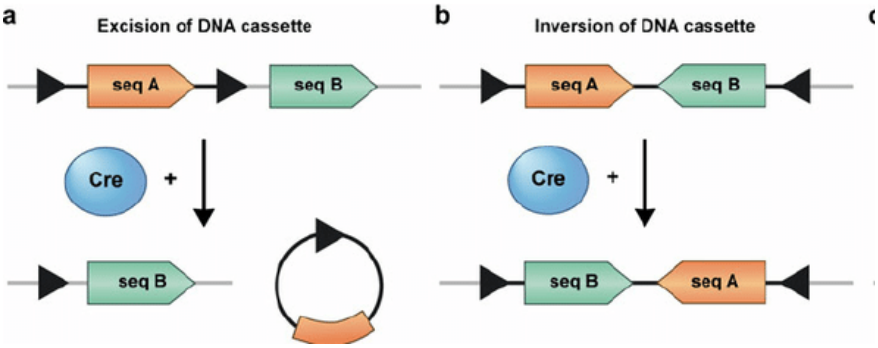


Colcemid: Colcemid acts to arrest cells in the metaphase state by disrupting microtubules in mitotic cells.

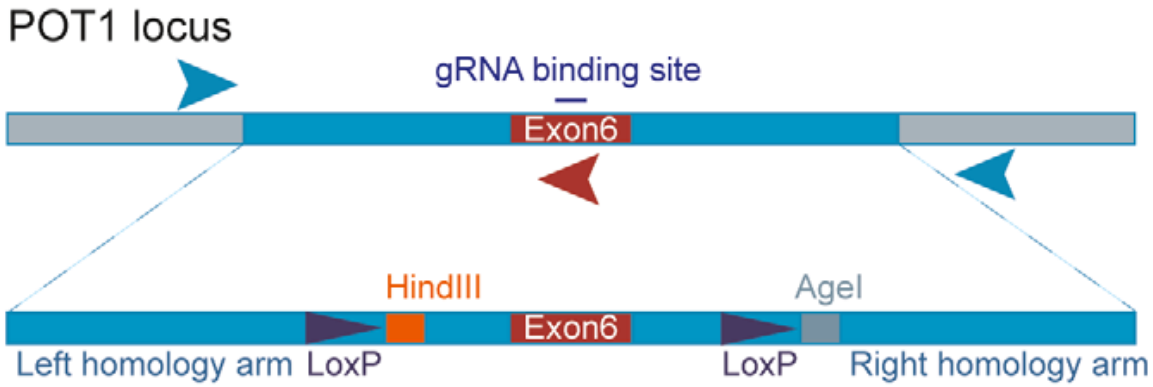
PNA: non-charged backbone: → higher melting temperature



Cre/loxP system :

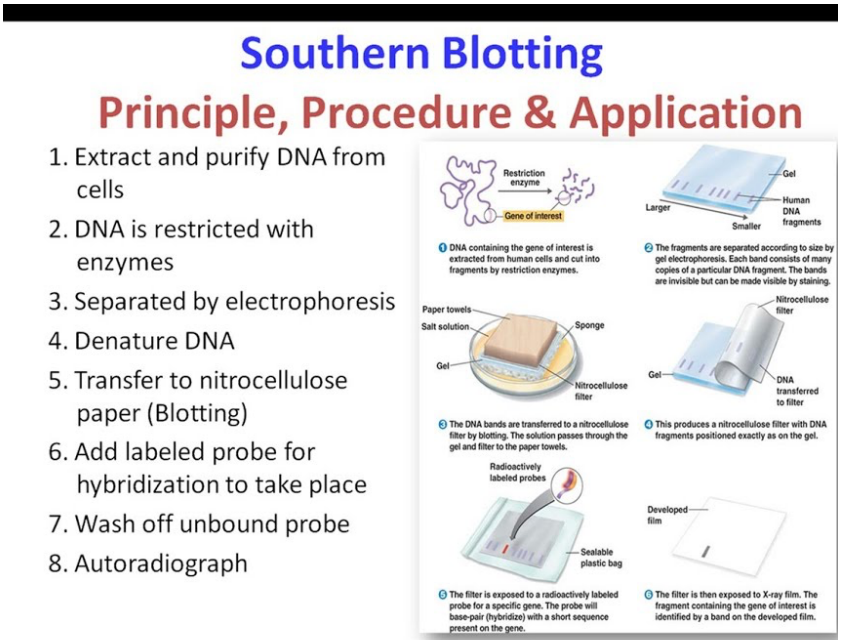


Cre recombinase is derived from bacteriophage P1. It catalyzes site-specific recombination between lox P sites (34 bp sequence). DNA sequences found between two **loxP** sites are said to be "floxed".
Cre recombinase-regulated gene expression necessitates transgenic constructs harbouring a DNA sequence of interest (seq) flanked by loxP sites (black triangle) on either side. a In cells expressing Cre recombinase, such floxed DNA elements are excised if loxP sites are oriented in parallel (head-to-tail). b If loxP sites are anti-parallel-oriented (head-to-head), Cre-mediated recombination results in the inversion of the floxed DNA element.
CreERT2 encodes a Cre recombinase (Cre) fused to a mutant estrogen ligand-binding domain (ERT2) that requires the presence of **tamoxifen** (**abbreviated as 4-OHT in the paper**) for activity.



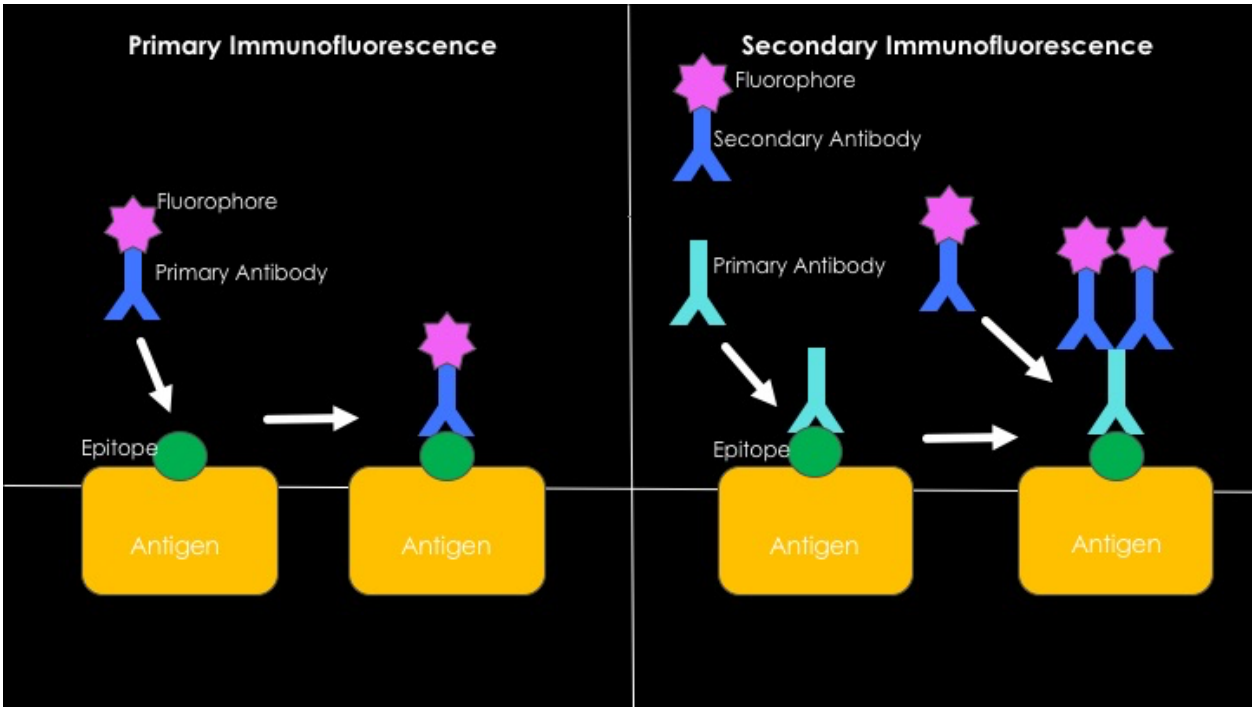
Schematic drawing of the repair template used for CRISPR/Cas9-mediated gene editing in HEK293E cells. Positions of primers for genotyping PCR are marked with arrows.

Southern blot and hybridization principle:



For obtaining telomeric restriction fragments, the genomic DNA is digested with frequent cutting (4 bp recognition sequence) restriction enzymes. Thus, the genomic DNA is cleaved into small fragments but the telomeric TTAGGG-repeats remain intact. For detecting single stranded TTAGGG-repeats present as 3’ overhangs at telomeres, the restricted DNA is NOT denatured prior to hybridization (Supplementary Figure 4b, left). For detecting total TTAGGG-repeats, the restricted chromosomal DNA is denatured prior to hybridization.

Immunofluorescence:



These figures demonstrates the basic mechanism of immunofluorescence. Primary immunofluorescence is depicted on the left, which shows an antibody with a fluorophore group bound to it directly binding to the epitope of the antigen for which it is specific. Once the antibody binds to the epitope, the sample can be viewed under fluorescent microscope to confirm the presence of the antigen in the sample. Conversely, secondary immunofluorescence is depicted to the right, which shows that first an untagged primary antibody binds to the epitope of the antigen in a mechanism similar to the one described above. However, after the primary antibodies have bound to their target, a secondary antibody (tagged with a fluorophore) comes along. This secondary antibody's binding sites are specific for the primary antibody that's already bound to the antigen, and therefore the secondary antibody binds to the primary antibody. This method allows for more fluorophore-tagged antibodies to attach to their target, thus increasing the fluorescent signal during microscopy.

Epitope tagging is a technique in which a known epitope is fused to a recombinant protein by means of genetic engineering. By choosing an epitope for which an antibody is available, the technique makes it possible to detect proteins for which no antibody is available.

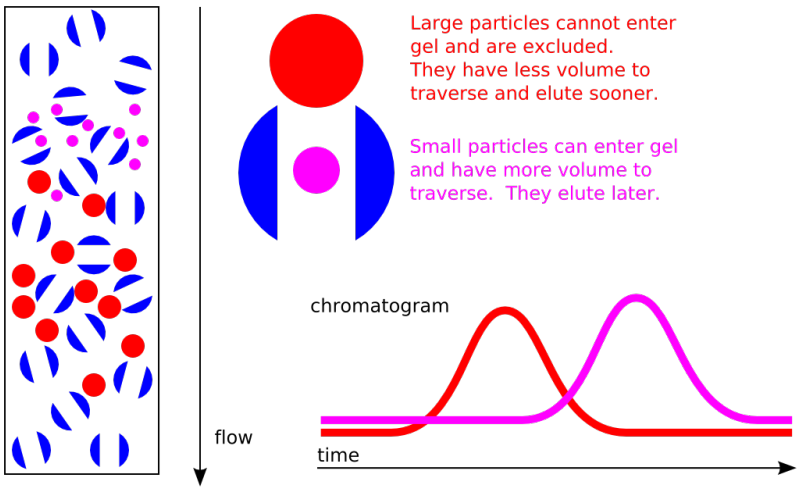
Table 1. Common Epitope Tags

Name	Sequence	Detection	Purification	Reference
FLAG	DYKDDDDK	M1,M2, M5	Immunoaffinity	1
6 × His	HHHHHH	Anti-His	Metal affinity	2
HA	YPYDVPDYA	12CA5	Immunoaffinity	4
c-myc	EQKLISEEDL	9E10	Immunoaffinity	5
GST	220 aa GST	Anti-GST	Glutathione	3
Protein A	IgG-binding domain	IgG	IgG	10
CD	18 aa exon	12CA5	Immunoaffinity	19
Strep-tag	WSAPQFEK	Strep-Tactin	Strep-Tactin	11
MBP	Maltose-binding protein	Anti-MBP	Maltose	13
CBD	Chitin-binding domain	Anti-CBD	Chitin	14
S-tag	S-peptide	Anti-S peptide	S-peptide	16
Avitag	GLNDIFEAQKIEWHE	Avidin	Avidin	12
CBP	CBP peptide	Anti-CBP	Calmodulin	15
TAP	Calmodulin- and IgG-binding domains	Anti-CBP	Calmodulin and IgG	15
SF-TAP	Strep Tag II and FLAG	Anti-FLAG	Strep-Tactin	28

GST, glutathione-S-transferase; CBP, calmodulin-binding peptide.

GFP and derivatives: https://en.wikipedia.org/wiki/Green_fluorescent_protein

Size exclusion chromatography/gel filtration:



…Fractionation according to Stokes' radius