Metody studia buněčné signalizace

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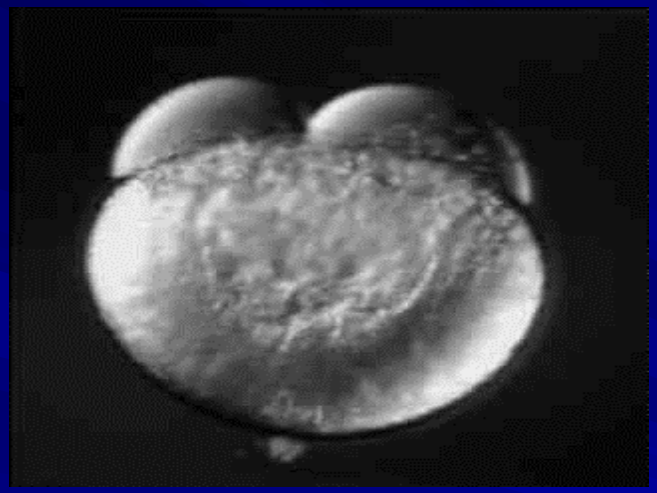
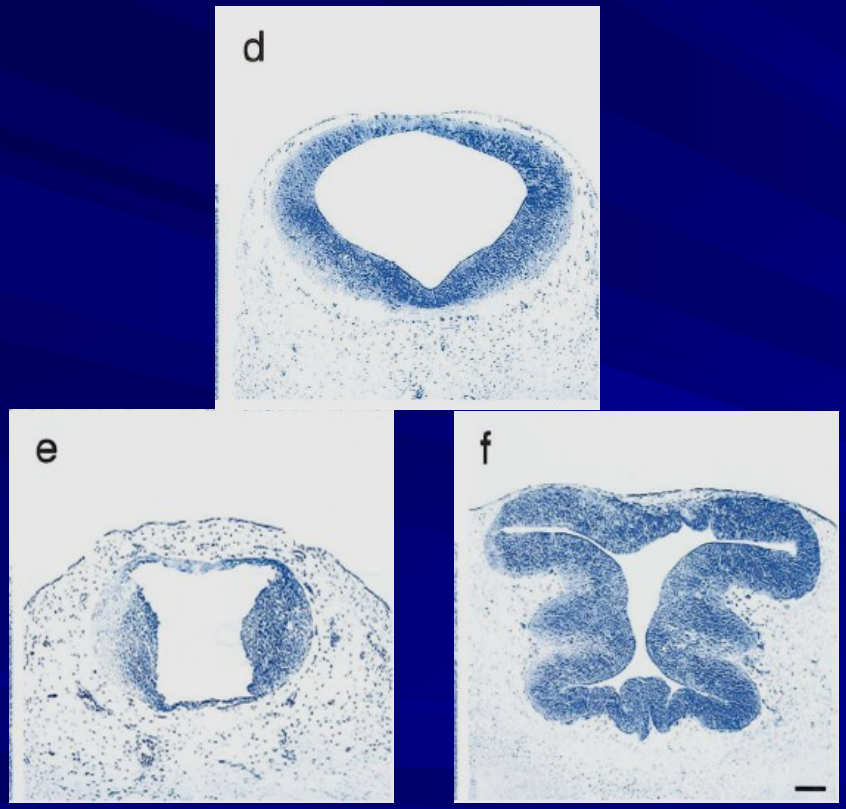
&
Oddělení cytokinetiky, Biofyzikální ústav AV ČR



Wnt signalizace

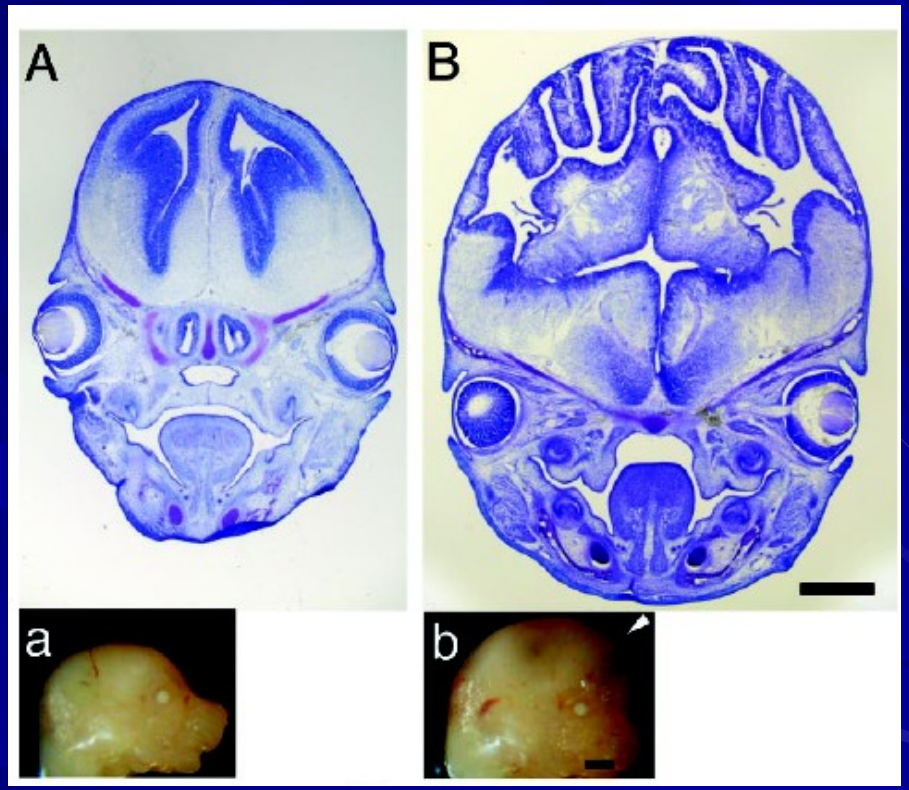
Důsledky aktivace Wnt signalizace v nervové soustavě:

midbrain (Brn4-promotor)



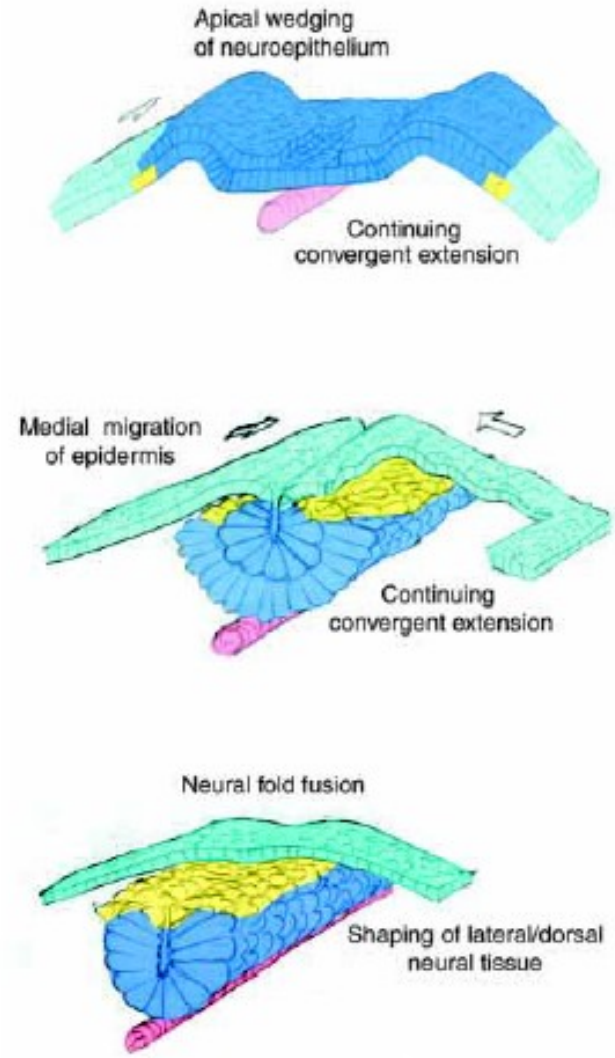
Zechner et al., 2003: Dev. Biol.;258:406-418.

cortex (nestin enhancer)

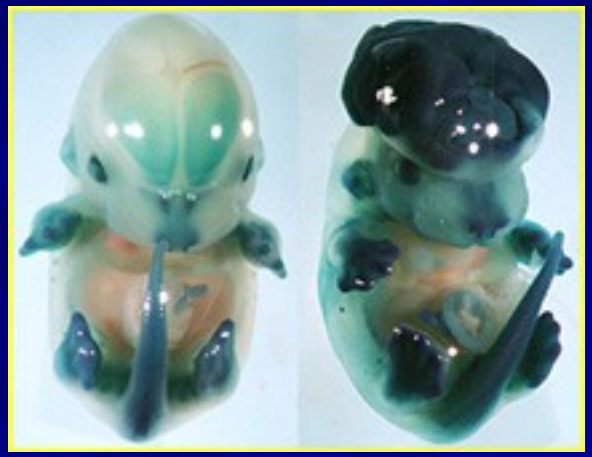


Chenn & Walsh, 2002: Science;297:365-369.

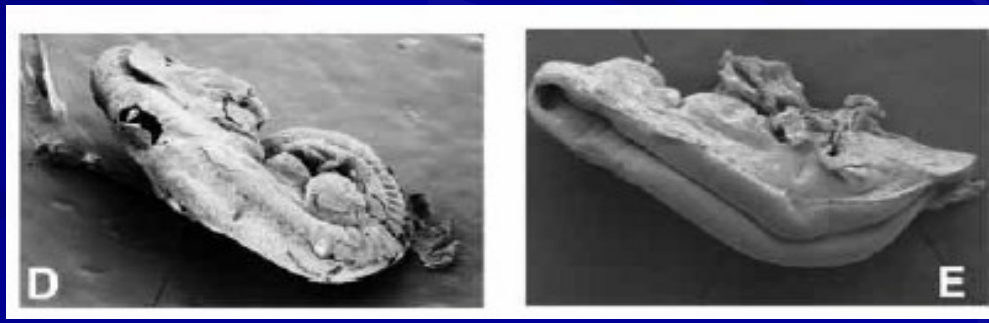
Nakanonická/PCP (Planar cell polarity) dráha způsobuje defekty v uzavírání nervové trubice



Exencephaly

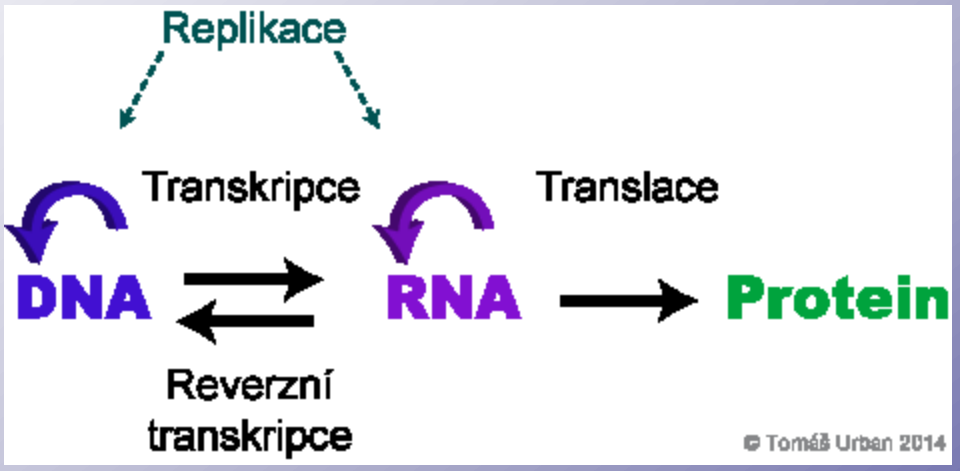


Open neural tube

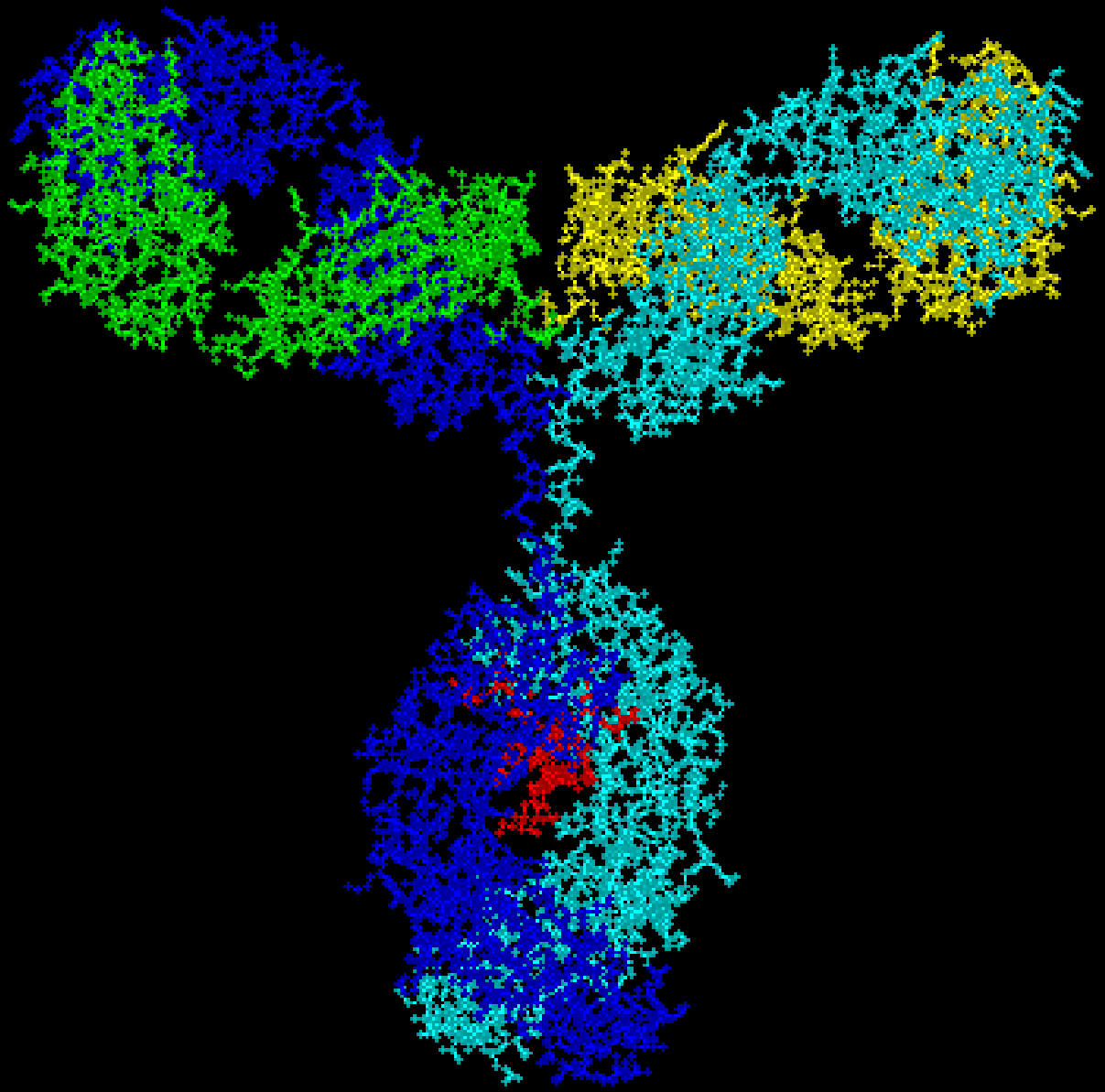


Hamblet et al., 2002, Development

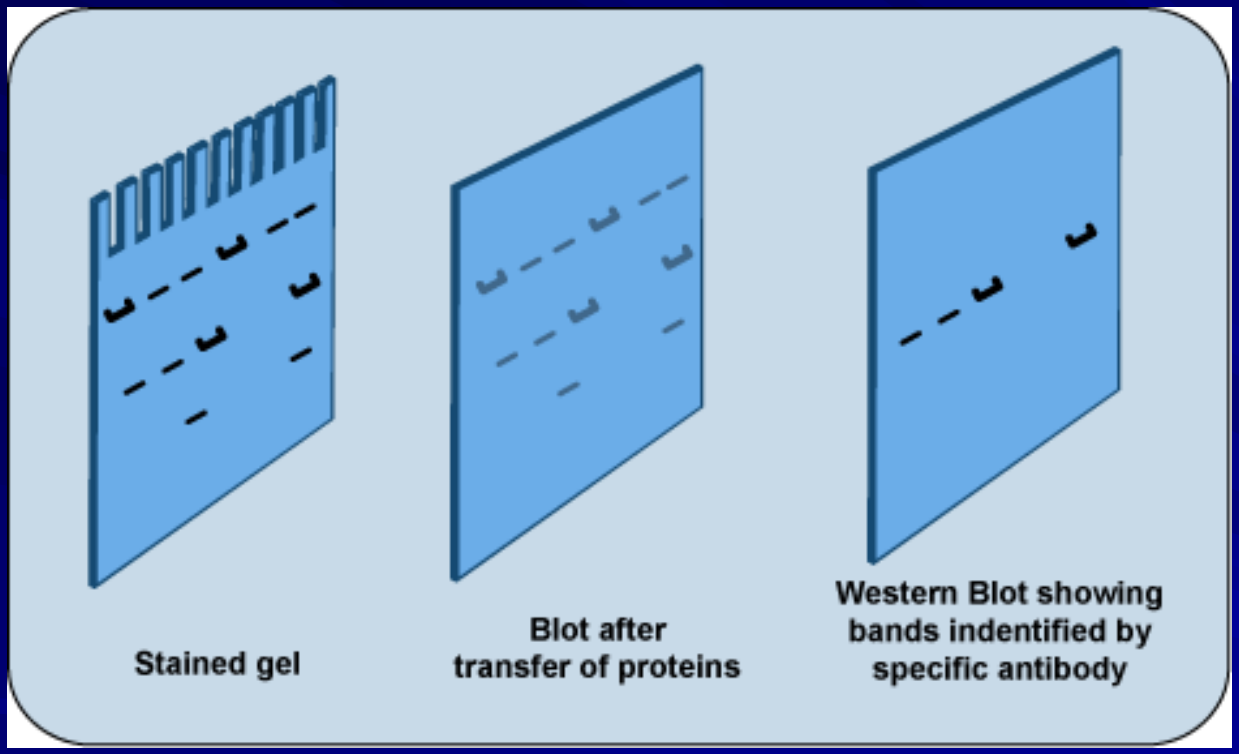
Centrální dogma molekulární biologie

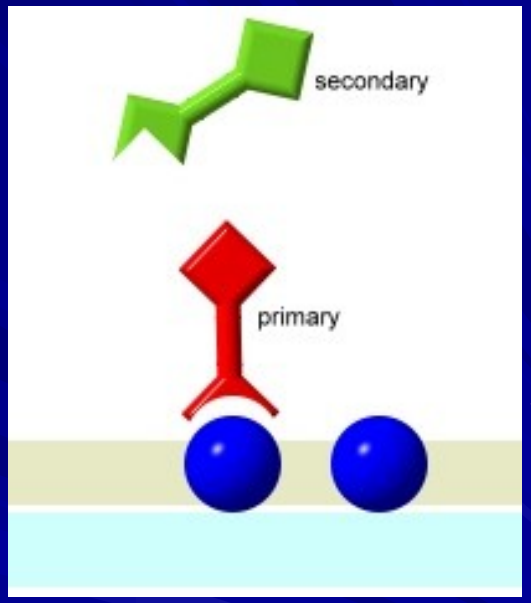


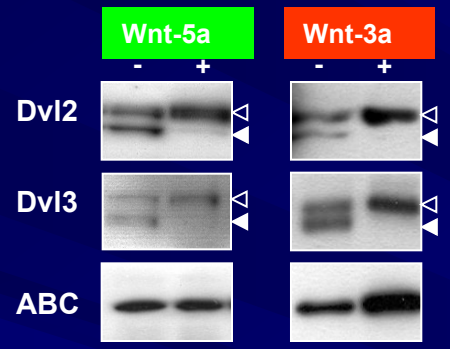
Protilátka
(imunoglobulin)



Metoda 1: Western blotting





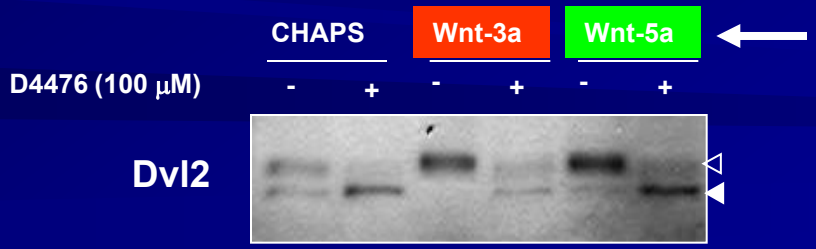


ABC – active β-catenin = β-catenin dephosphorylated on GSK3β target sites

Dvl – Dishevelled – activated by phosphorylation detected as phosphorylation dependent mobility shift

◁ PS-Dvl

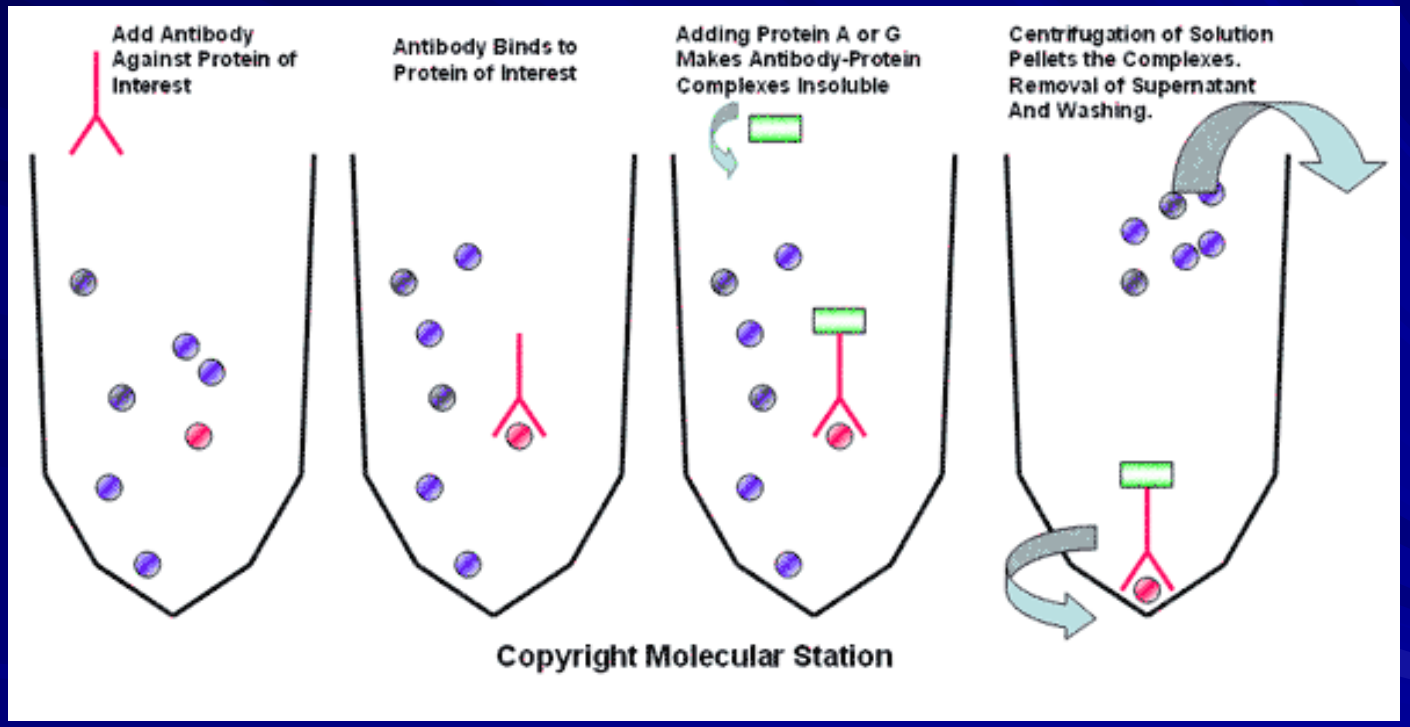
Compound	Target	Concn	Activity
PTX	Galpha i/o	100 ng/ml	No
PDBu	PKC activator	1 μM	No
Wortmannin	PI3K	50 nM	No
LY294002	PI3K	50 μM	No
PD98059	MEK1/2	10 μM	No
UO126	MEK1/2	10 μM	No
SB203580	p38	10 μM	No
JNKII inhib	JNK	6 μM	No
Genistein	PKC	50 μM	No
chelerythrine	PKC	10 μM	No
Ro-31 8220	PKC	1 μM	No
BIM I	PKC	500 nM	No
KN93	CamKII	10 μM	No
I3M	GSK-3	2 μM	No
Kenpaullone	GSK-3	6 μM	No
H89	PKA	10 μM	No
8-Br-cAMP	cAMP pathway activator	10 μM	No
8CPT-2Me-cAMP	EPAC activator	30 μM	No
SQ22536	Adenylyl cyclase	100 μM	No
MDL12330	Adenylyl cyclase	10 μM	No
PP2	Src-like	10 μM	No
AG1276	EGFR	10 μM	No
ET-18-OCH3	PLC	10 μM	No
D4476	**Casein kinase 1**	**100 μM**	**Yes**
staurosporin	Ser/Thr kinases, PKC	2 μM	No



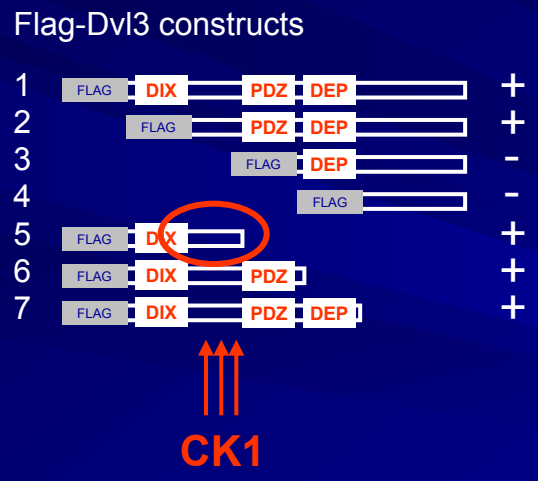
Both Wnt-3a and Wnt-5a activate Dvl2 and Dvl3 via casein kinase 1 (CK1)

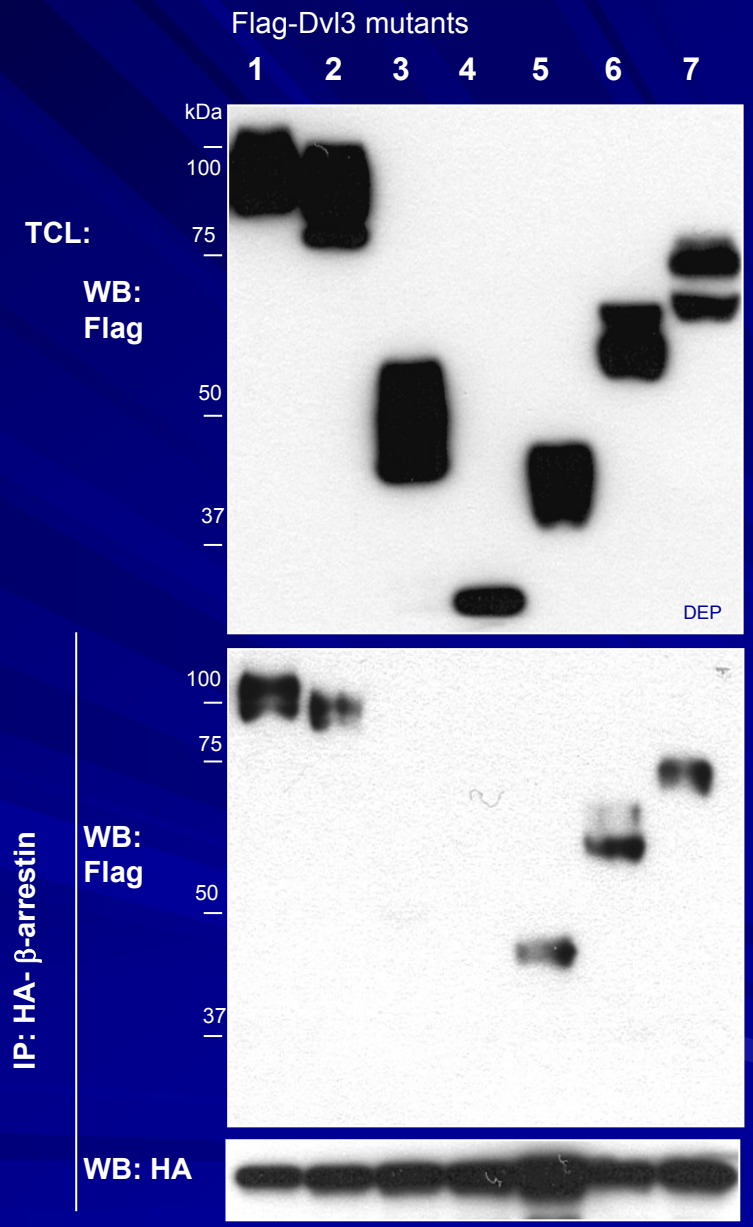
Bryja et al., J. Cell Sci., 2007

Metoda 2: Immunoprecipitace

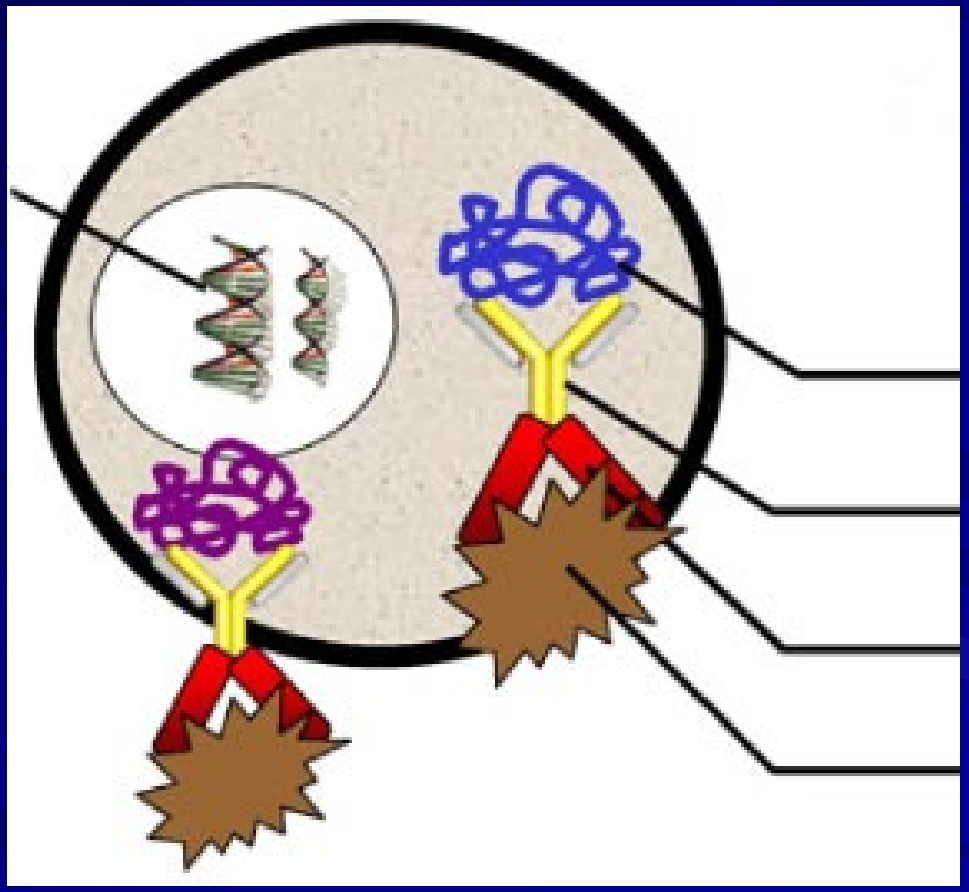


β-arrestin váže Dishevelled

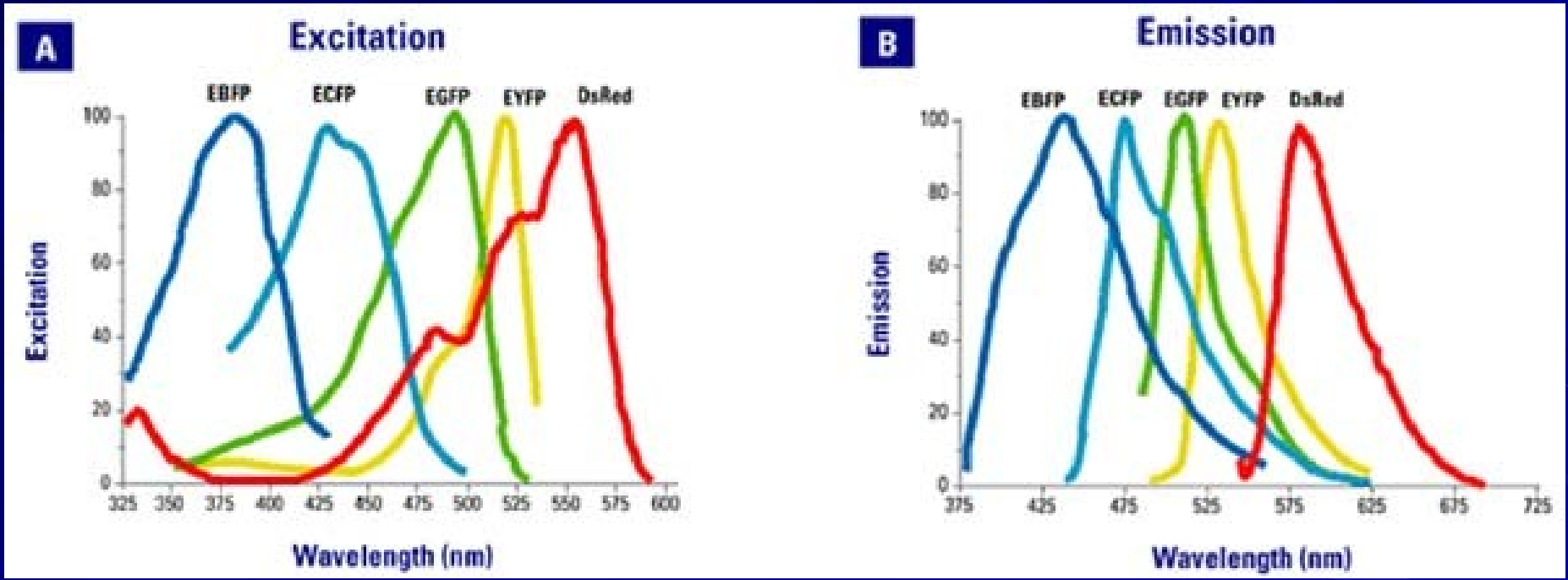




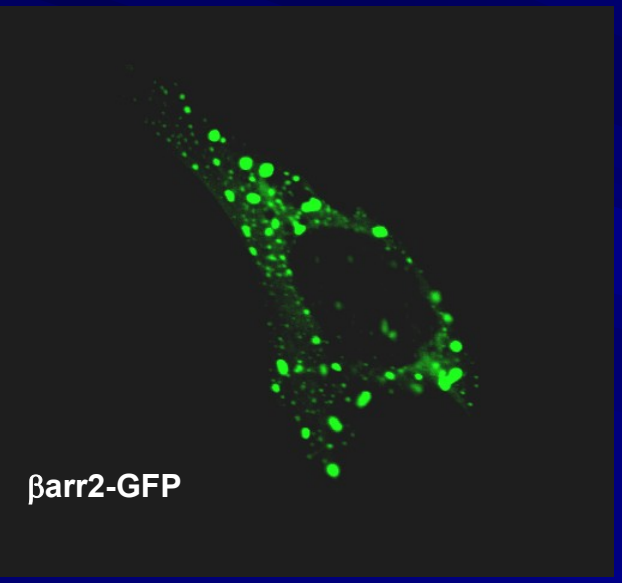
Metoda 3: Immunocytochemie

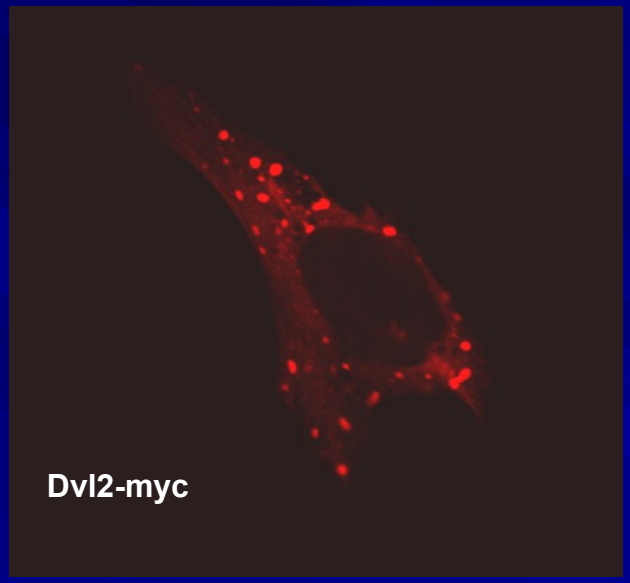


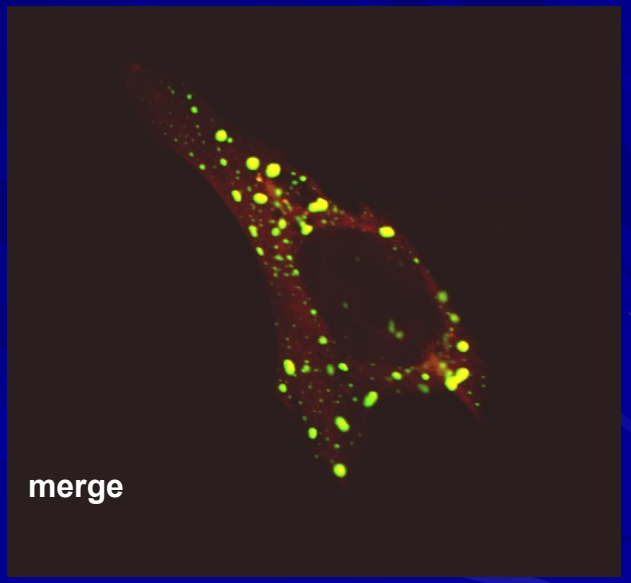
Fluorescenční proteiny



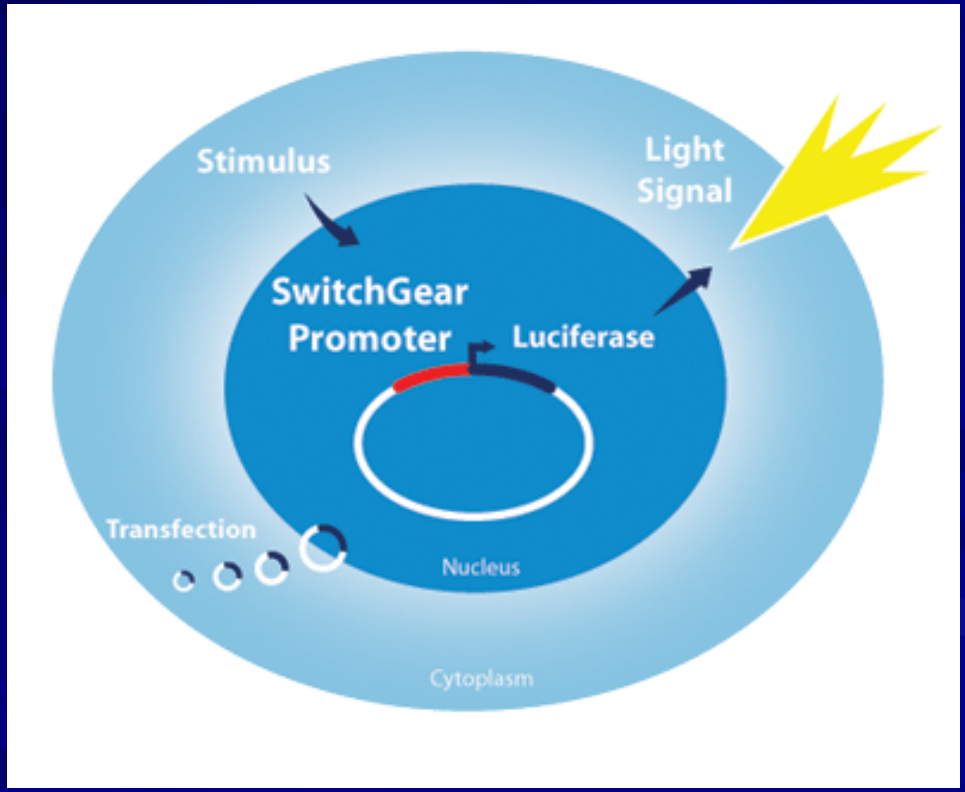
β-arrestin co-localizes with Dvl in the cytoplasm





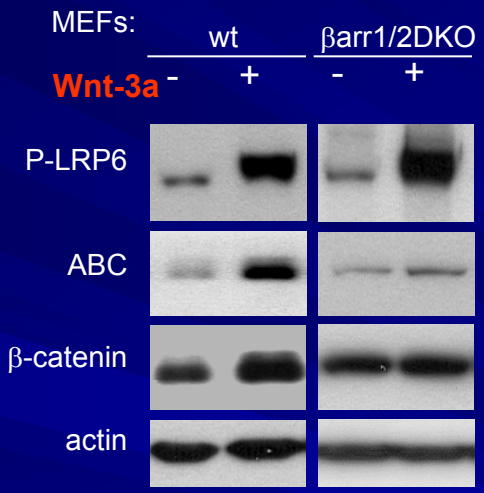


Metoda 4: Reportérové eseje – analýza aktivace promotoru

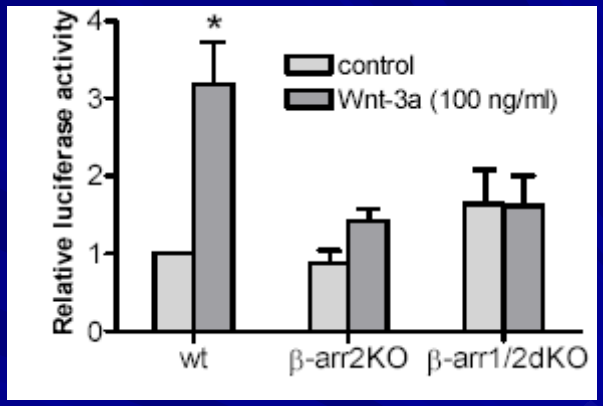


1. β-arrestin is required for β-catenin activation in vitro

β-arrestin deficient MEFs

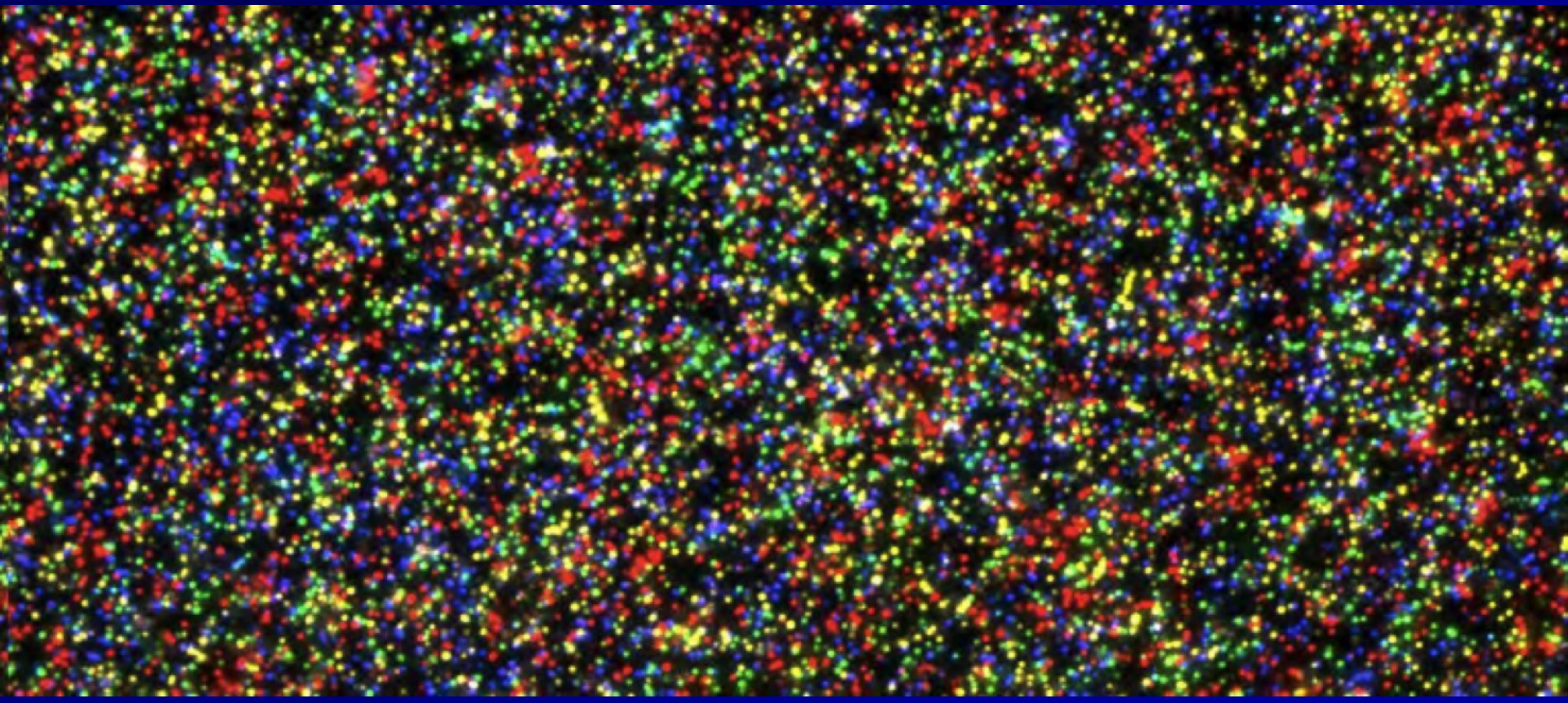


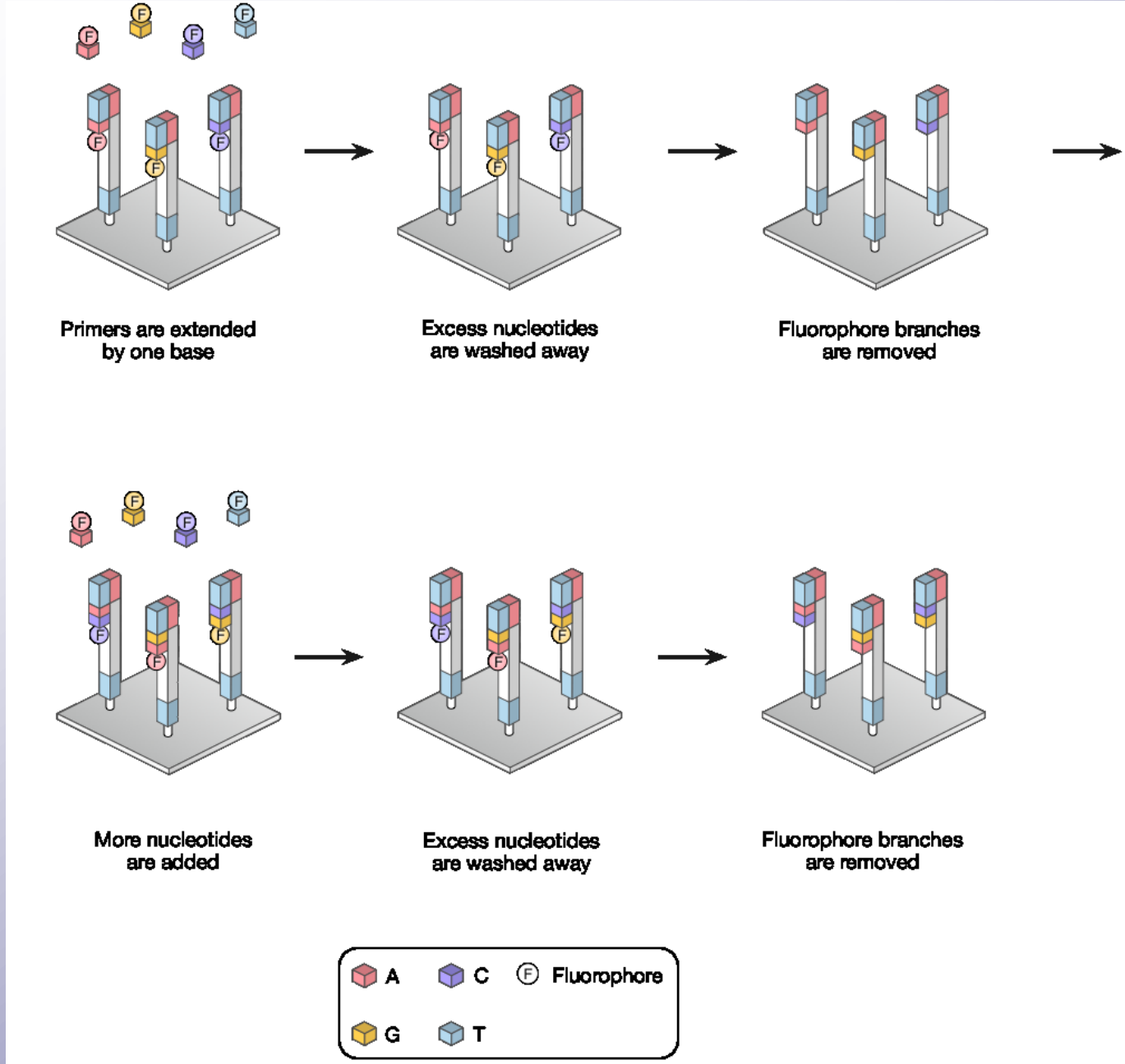
TopFlash reporter - β-catenin transcriptional activity



Is this relevant for Wnt signal transduction in vivo?

Metoda 5: RNA sekvenování (RNA Seq)

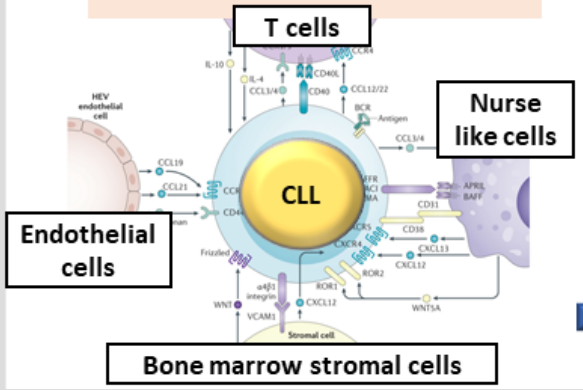



Primers are extended by one base
Excess nucleotides are washed away
Fluorophore branches are removed
More nucleotides are added
Excess nucleotides are washed away
Fluorophore branches are removed
A
C
Fluorophore
G
T

RNA sekvenování – vícedruhové kultivace mezi druhy

CK1 inhibition: In vitro direct co-culture system with primary patient CLL cells

Microenvironment studied:



Global RNA Seq analysis (SA3.1)

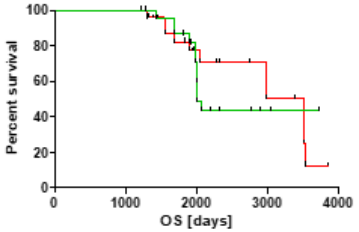
- Species-specific sequencing of human, mouse and mixed samples
- Data processing - pipeline designed to separate human and mouse reads

Focused functional analysis (SA3.2)

- Migration/invasion
- Adhesion
- Cytotoxicity
- Immunological synapse

Validation on the larger patient cohort (SA3.3)

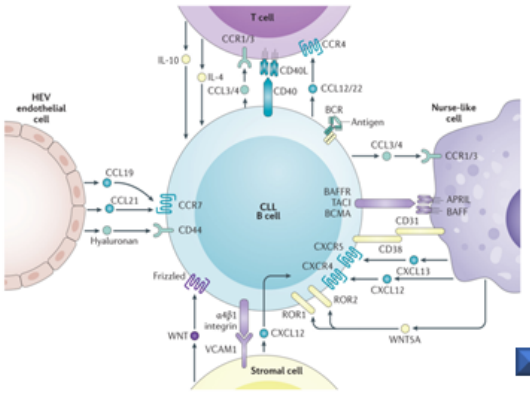
„hand picking" of genes/proteins with the clinically important function in CLL



Identification of processes controlled by CK1 inhibition in CLL cells as well as in the cells forming the microenvironment

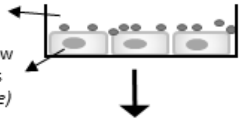
RNA sekvenování mezi druhy

Environmental interactions of CLL cells



In vitro direct co-culture system

1. CLL primary cells *(human)*
2. Bone marrow stromal cells *(mouse cel line)*



Species-specific analysis:
Gene expression

Advantages:

- Direct cell-cell contact
- Immediate lysis of cells in contact
- Mouse stromal cells (e.g. M2104 cell line) provide protective signals to CLL cells equivalent to human cell lines.
- Reproducibility

Species-specific analysis

- Library preparation
- Species-specific sequencing of human, mouse and mixed samples
- Data processing - Pipeline designed to separate human and mouse reads and remove false-detection
- Statistical analysis
- Further processing and data validation – e.g. GO terms analysis (biological processes) performed by „Panther Classification System“ online tool - Statistical overrepresentation test

Human reads

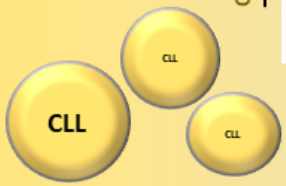
CK1 inh. sensitive	Not changed by CK1 inh.
B cell activation (GO:0042113)	cellular response to BMP stimulus (GO:0071773, GO:0071772)
lymphocyte differentiation (GO:0030098)	positive regulation of non-canonical Wnt signaling pathway (GO:2000052)
lymphocyte activation (GO:0046649)	**ERK1 and ERK2 cascade (GO:0070371, GO:0070372, GO:0070373)**
leukocyte activation (GO:0045321)	**negative regulation of extrinsic apoptotic signaling pathway (GO:2001237)**
cell activation (GO:0001775)	**cell proliferation (GO:0008283)**
leukocyte activation involved in immune response (GO:0002366)	**cell-cell adhesion (GO:0016337), cell-matrix adhesion (GO:0001952)**
cell activation involved in immune response (GO:0002263)	**regulation of Wnt signaling pathway (GO:0030111, GO:0016055, GO:0060828, GO:0198738, GO:0030178)**
regulation of cell migration (GO:0030334)	**cell motility (GO:0048870)**
regulation of cell motility (GO:2000145)	GO:0040011)
immune response (GO:0006955)	**positive regulation of MAPK cascade (GO:0043410)**
	STAT signaling (GO:0042509, GO:0046427, GO:0046426)

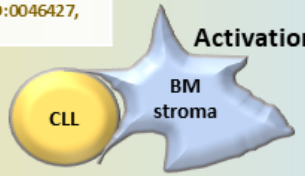
Not changed by CK1 inh.	CK1 inh. sensitive
MAPK cascade (GO:0000165)	**response to transforming growth factor beta (GO:0071559)**
cell proliferation (GO:0008283)	**regulation of cell motility (GO:2000145)**
response to fibroblast growth factor (GO:0071774)	**cell adhesion (GO:0030155), cell-cell adhesion (GO:0098609)**
semaphorin-plexin signaling pathway (GO:0071526)	**regulation of cell migration (GO:0030334)**
regulation of NIK/NF-kappaB signaling (GO:1901222)	
response to epidermal growth factor (GO:0070849)	

Mouse reads

CK1 inh. sensitive
rhythmic process (GO:0048511)
circadian rhythm (GO:0007623)
extracellular matrix organization (GO:0030198)
extracellular structure organization (GO:0043062)
regulation of cell motility (GO:2000145)
regulation of cell migration (GO:0030334)
regulation of cell adhesion (GO:0030155)
regulation of transmembrane receptor protein serine/threonine kinase signaling pathway (GO:0090092)
regulation of cellular response to transforming growth factor beta stimulus (GO:1903844)

CK1 inh.





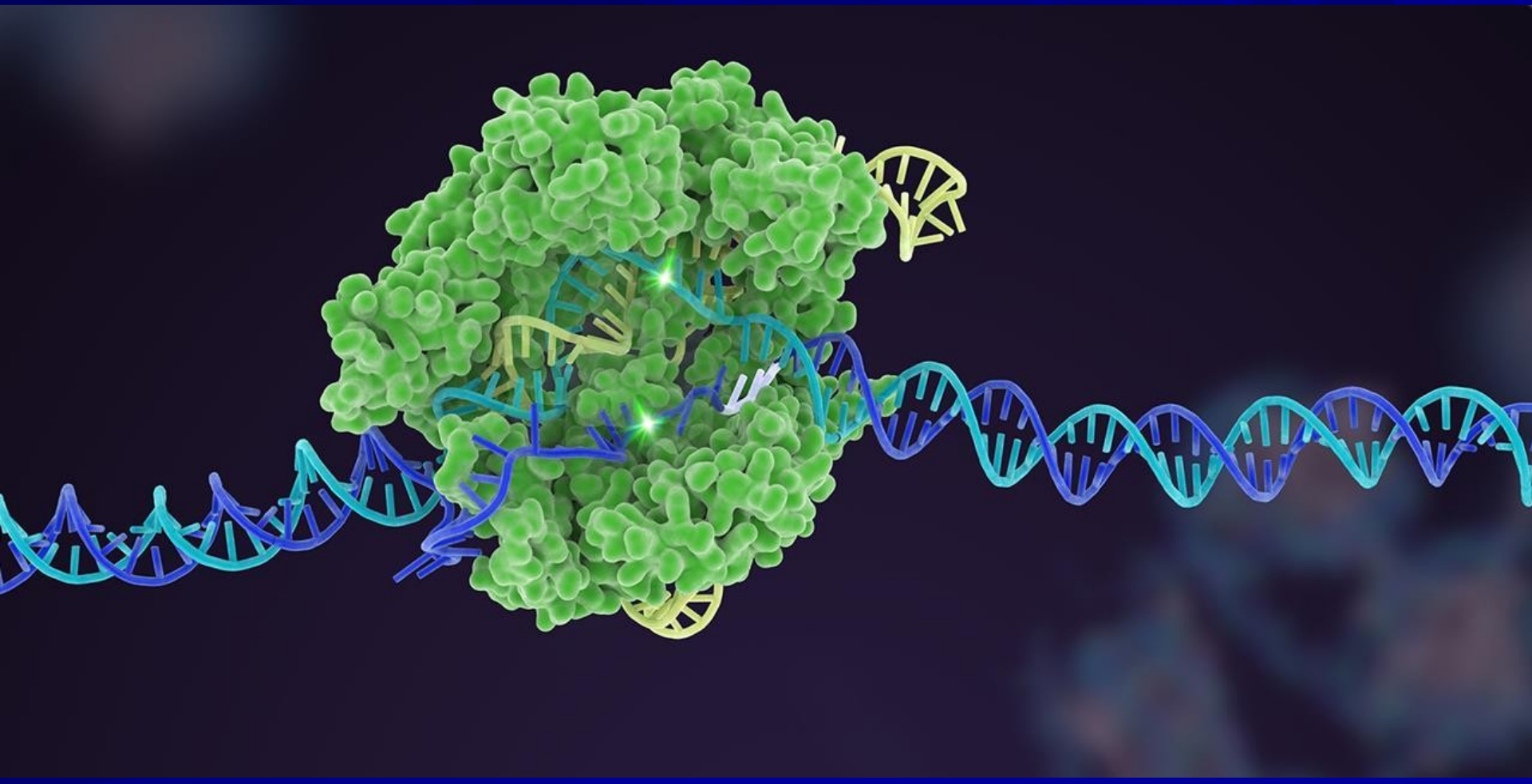
Activation of CLL cells by stroma

CK1 inh.

Metody č. 6: Genetické modifikace buněčných linií a myši

2014: Crispr/Cas9-mediated gene editing

METHOD OF THE YEAR

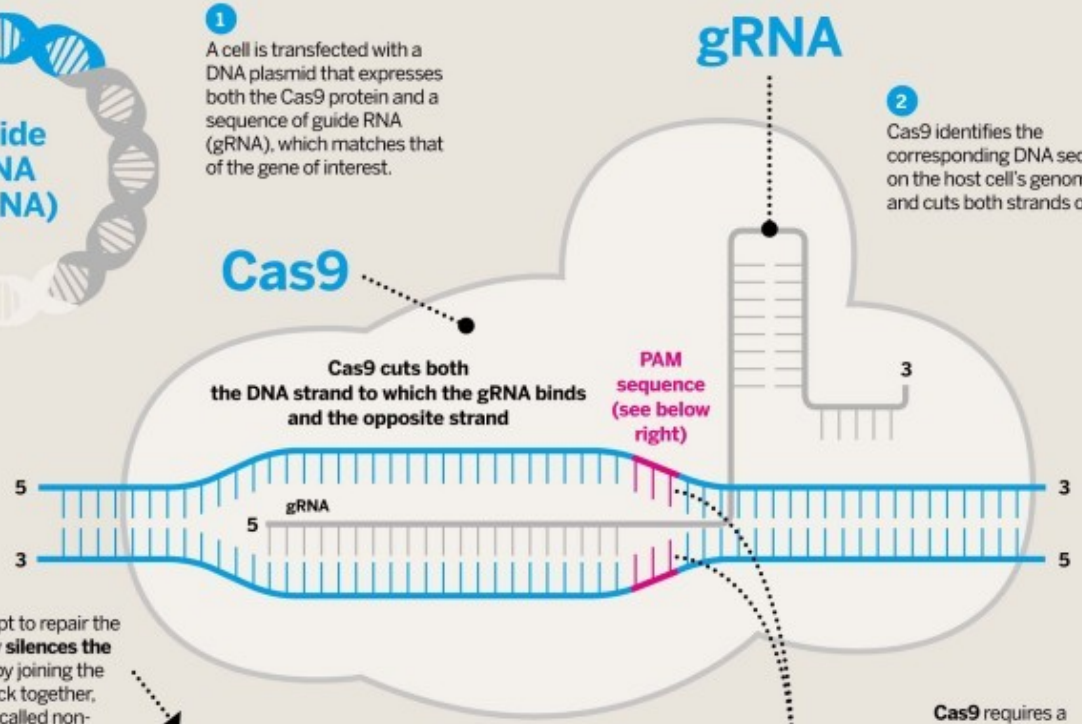


CRISPR-Cas9

How the genome editor works

1 A cell is transfected with a DNA plasmid that expresses both the Cas9 protein and a sequence of guide RNA (gRNA), which matches that of the gene of interest.

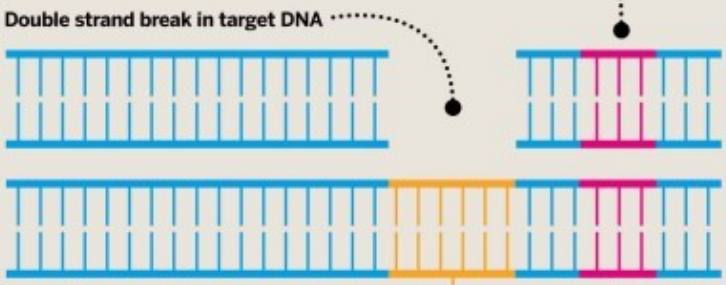
2 Cas9 identifies the corresponding DNA sequence on the host cell's genome, and cuts both strands of DNA.



3a The cell's attempt to repair the break effectively **silences the targeted gene** by joining the cleaved DNA back together, using a process called non-homologous end joining (NHEJ).

OR

3b A **faulty gene can be 'corrected'** with a replacement segment of DNA, or **a new gene altogether can be introduced**. If a modified piece of DNA whose flanking regions match the target sequence is also supplied, then there is a good chance that it will recombine with the host DNA when the cut is made, thus introducing a new or replacement gene. This pathway is known as homology directed repair (HDR).



Cas9 requires a simple and common sequence of base pairs called a **PAM sequence** to actually bind to target DNA. This feature means bacteria can prevent Cas9 from chopping up important 'memorised' sequences of foreign DNA in their own genome – by ensuring there are no PAM sequences in those regions.

What next?

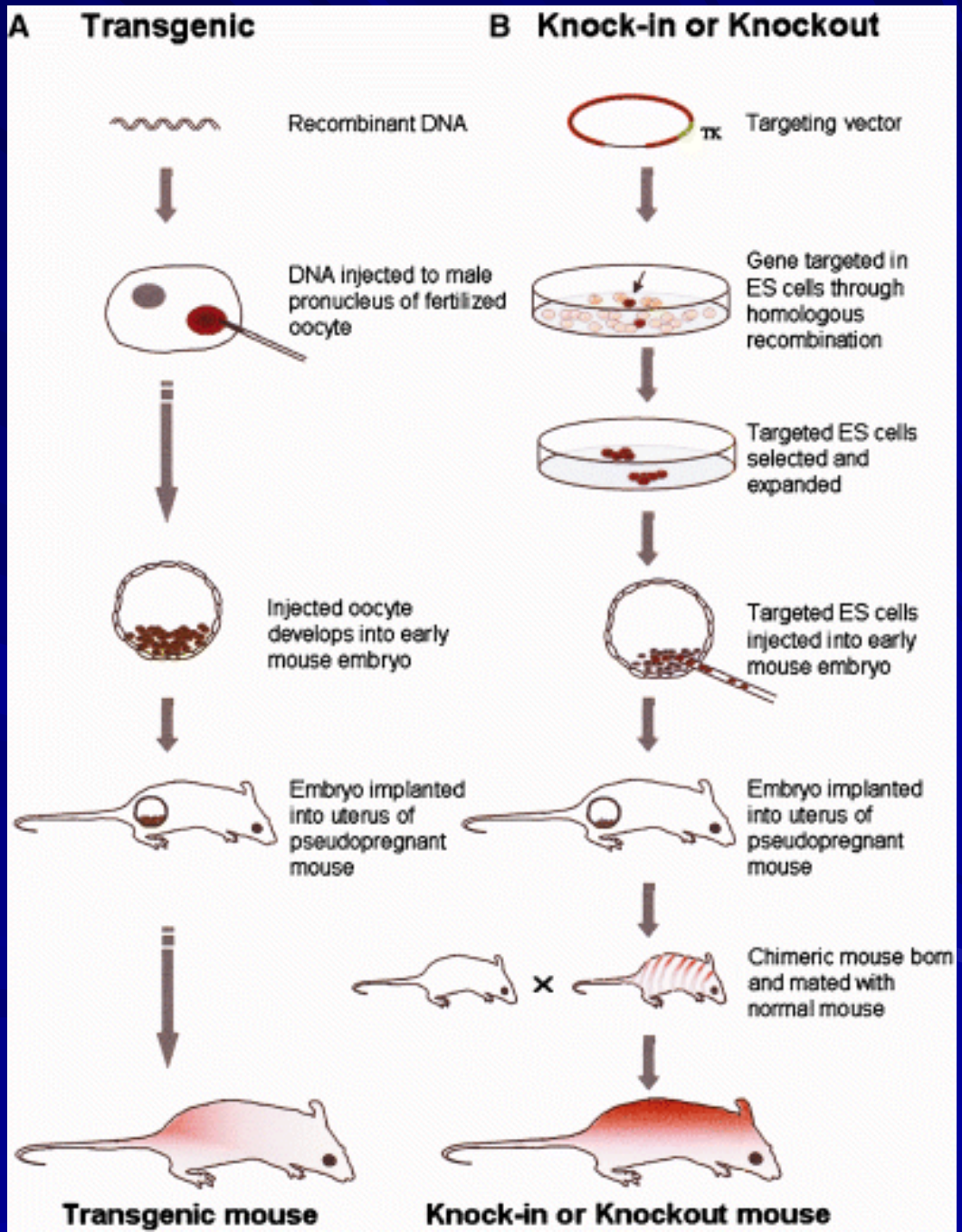
Transgenní myš

Nobelova cena 2007

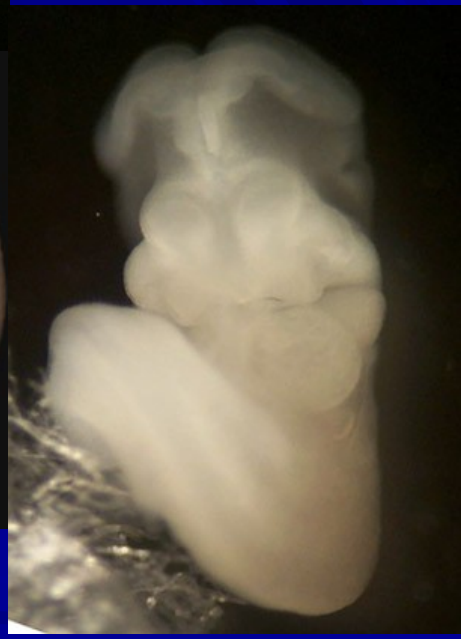
Mario R. Capecchi, **Martin J. Evans** and **Oliver Smithies**

za

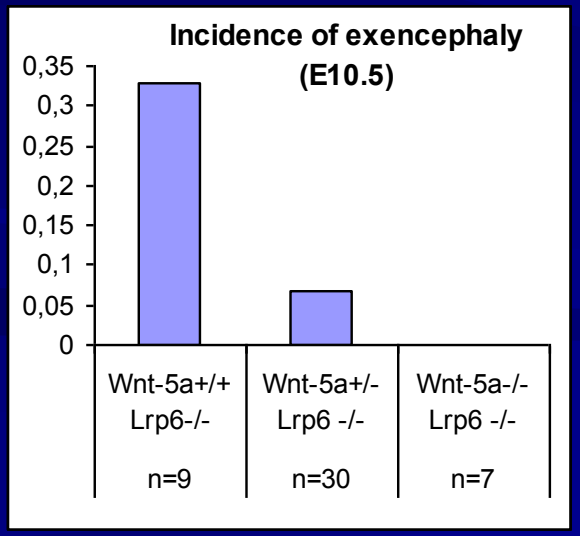
„principles for introducing specific gene modifications in mice by the use of embryonic stem cells“



Lrp6 KO embryos display exencephaly....

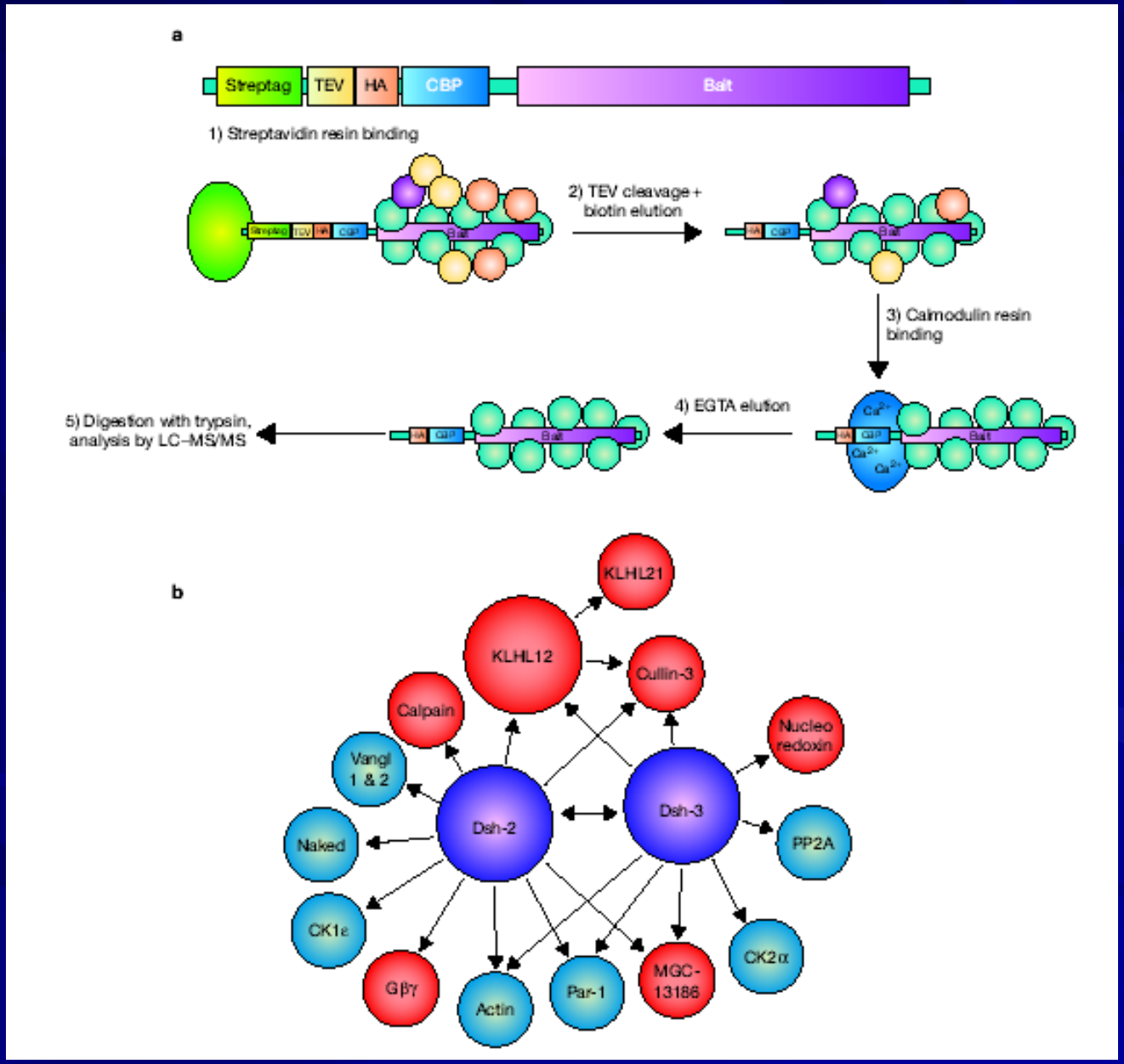


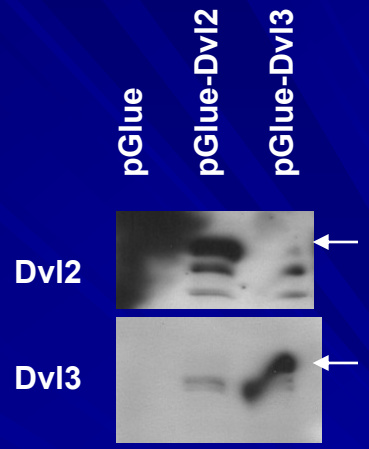
....which is rescued by loss of Wnt5a



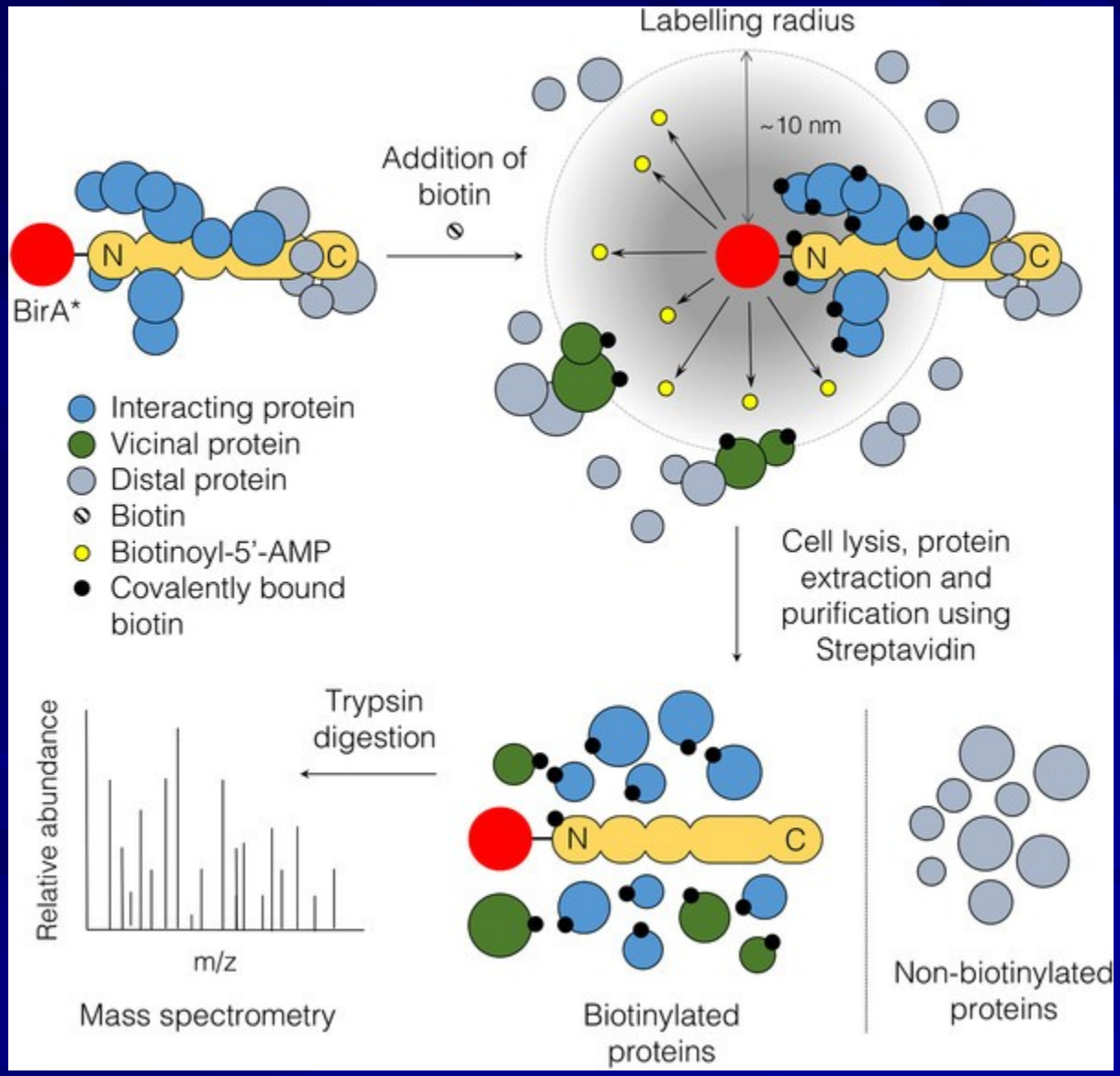
Metody č. 7: Afinitní purifikace a hmotnostní spektroskopie

Afinitní purifikace

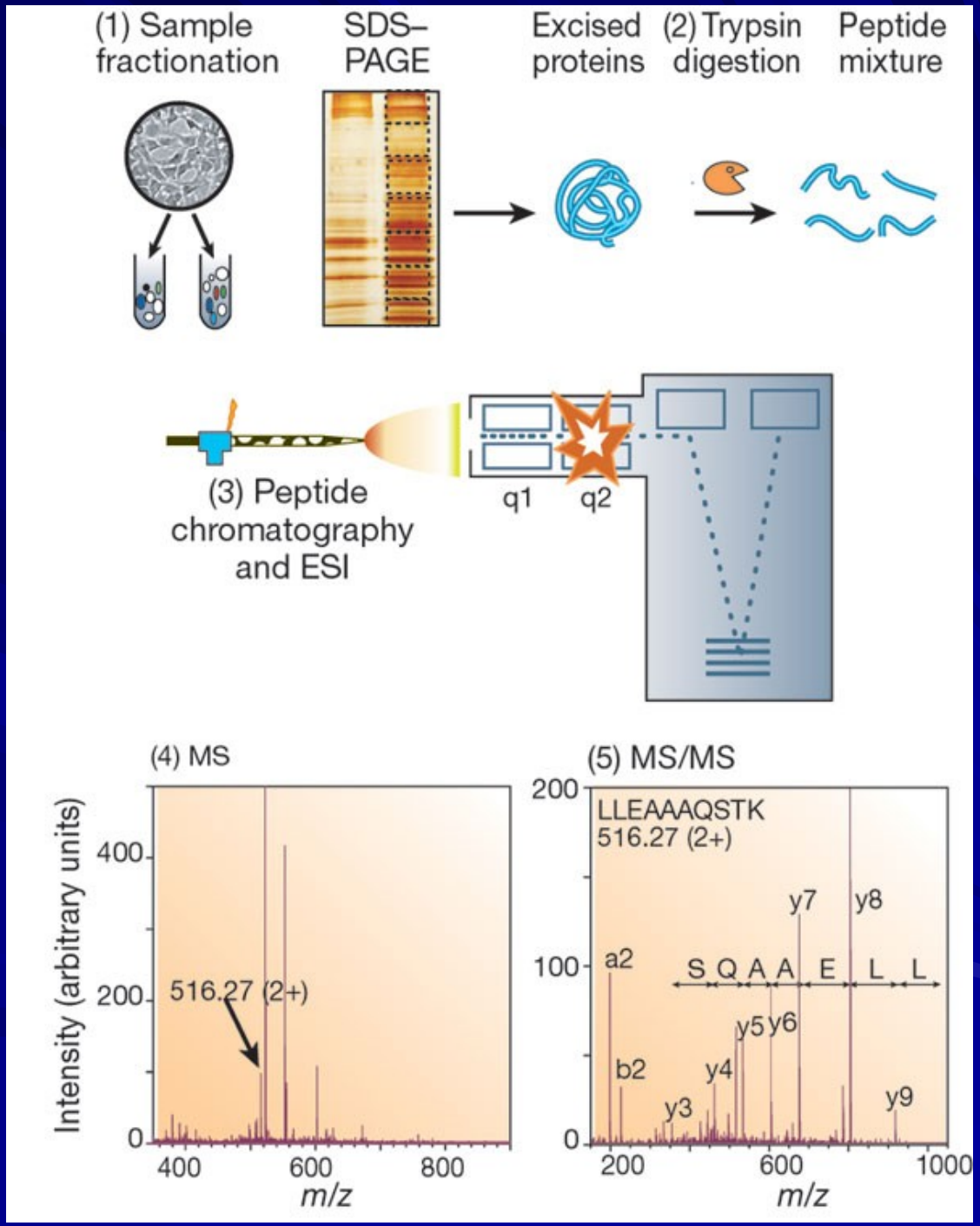




BioID – proximity labeling



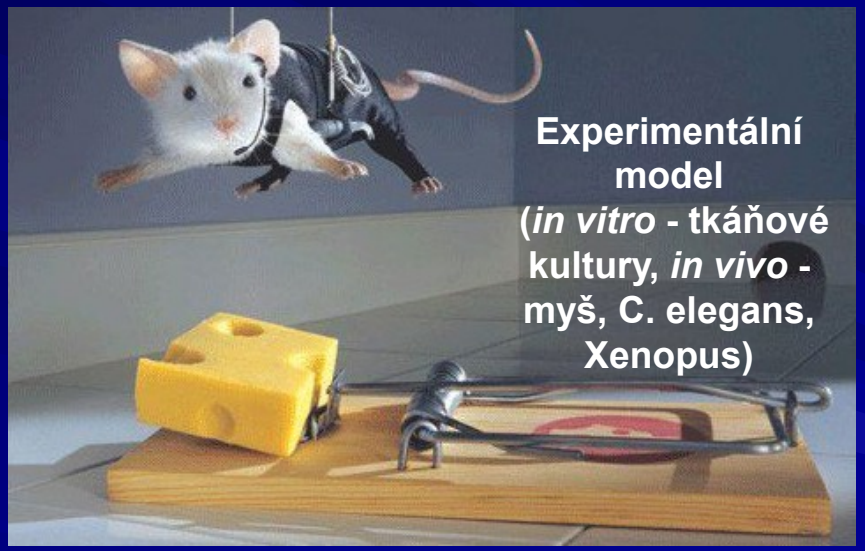
Hmotnostní spektroskopie (Mass Spec)



Děkuji za pozornost!

Celogenomové techniky

Molekulární mechanismus



Celoproteomové techniky