Aplikovaná chemie a biochemie

Přednáška č. 3

Proteinové techniky (2)

Proteiny (2):

- funkce jednotlivých domén;
- posttranslační modifikace (fosforylace, acetylace, glykosylace);
- regulace odborávání proteinu (ubikvitinace; proteazomální degradace);
- manipulace s proteinem (overexprese; dominant negative constructs, antisense oligonuclotides, siRNA).

Proteiny se skládají z domén, definovaných na základě struktury a funkce:

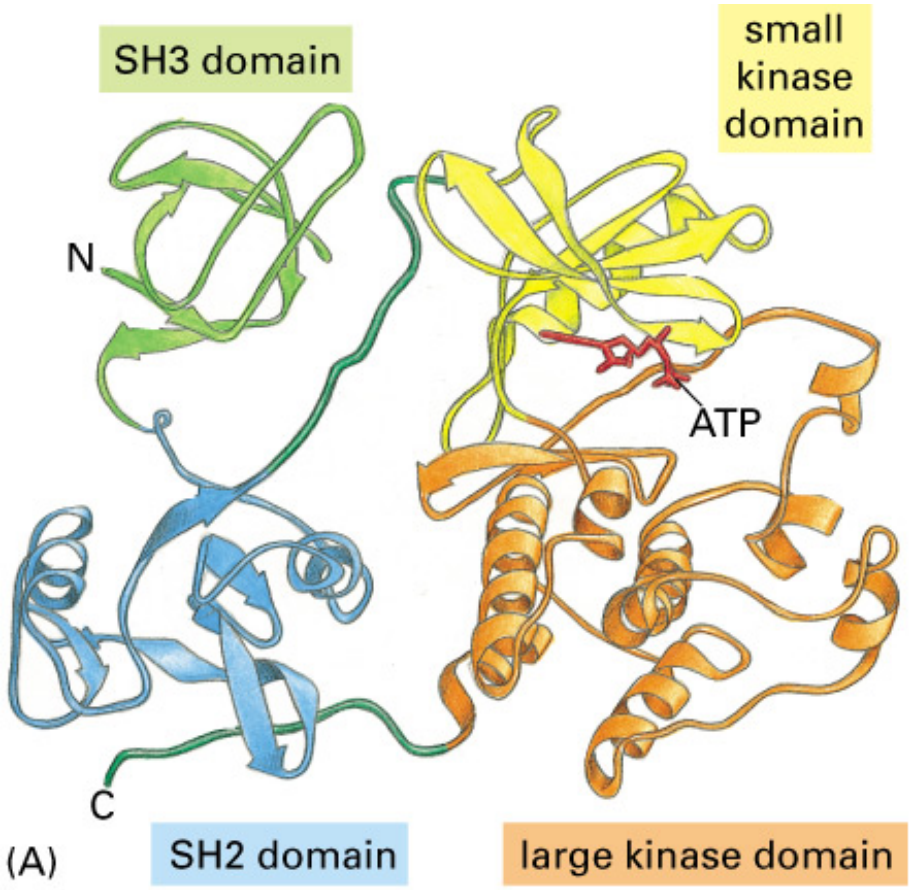
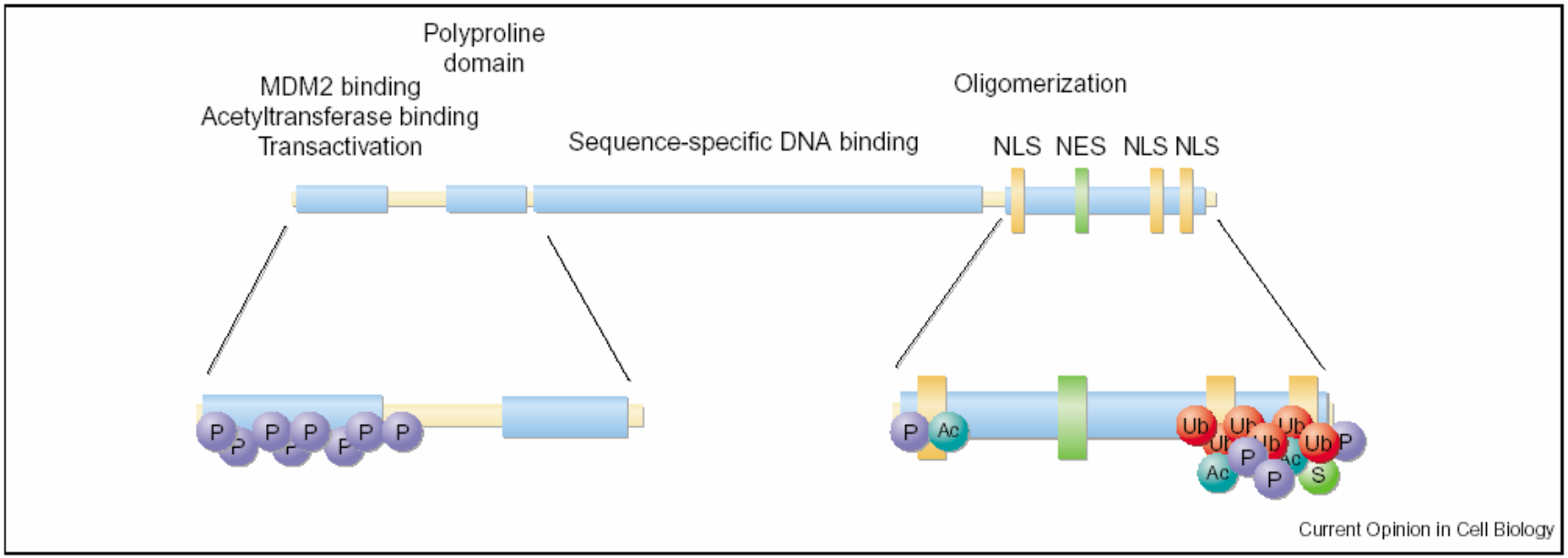


Figure 3–12 part 1 of 2. Molecular Biology of the Cell, 4th Edition.

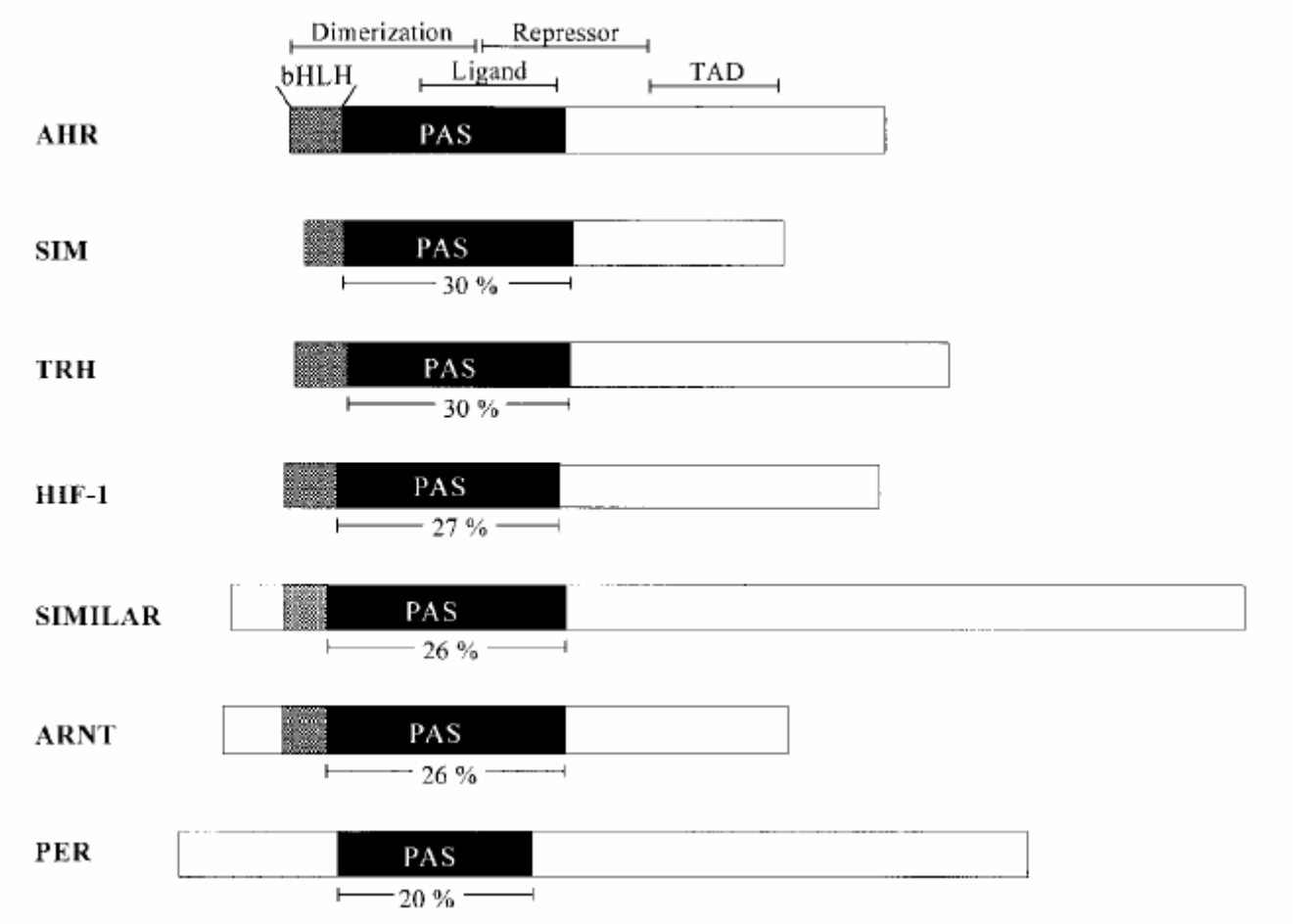
Primární a sekundární struktura proteinu může naznačit jeho funkci:



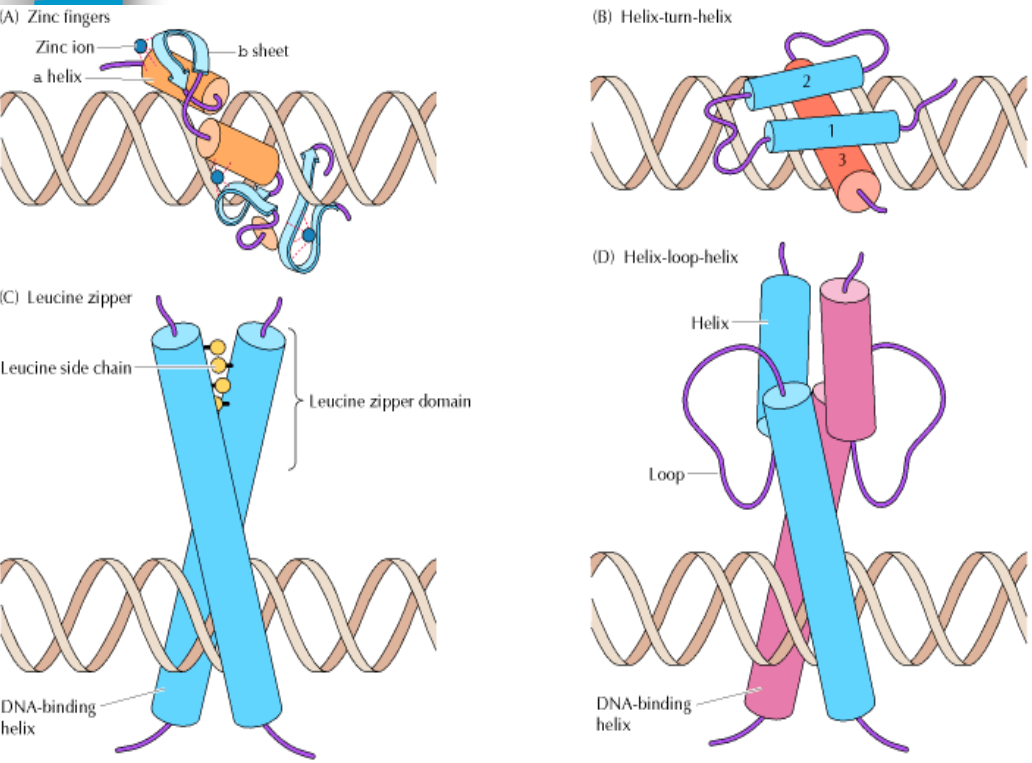
Cartoon of p53, showing the major functional domains and the positions of modifications within the amino and carboxyl termini. Ac, acetylation; NES, nuclear export signal; NLS, nuclear localization signal; P, phosphorylation; S, sumoylation; Ub, ubiquitination.

P53 domain structure

Proteinové domény často charakterizují celou rodinu proteinů:

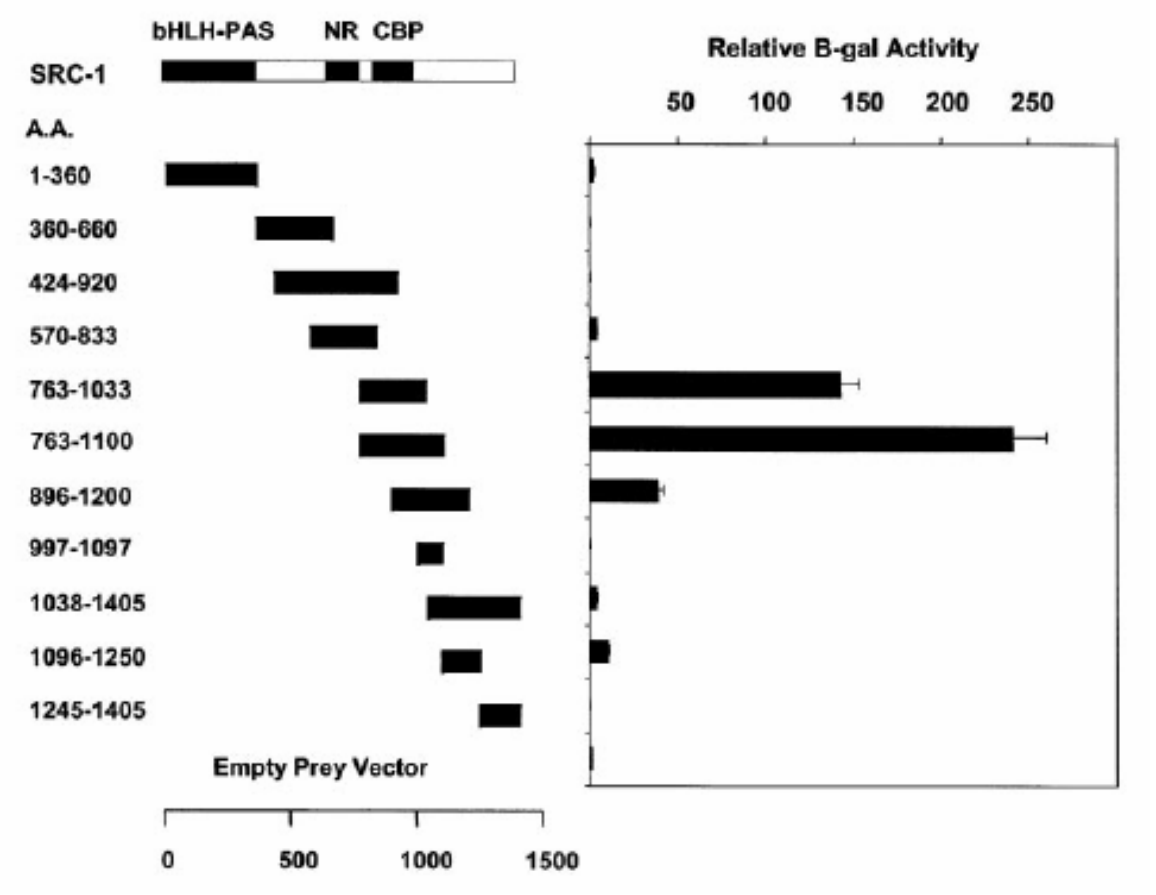


Různé strukturní domény mohou plnit stejnou funkci:

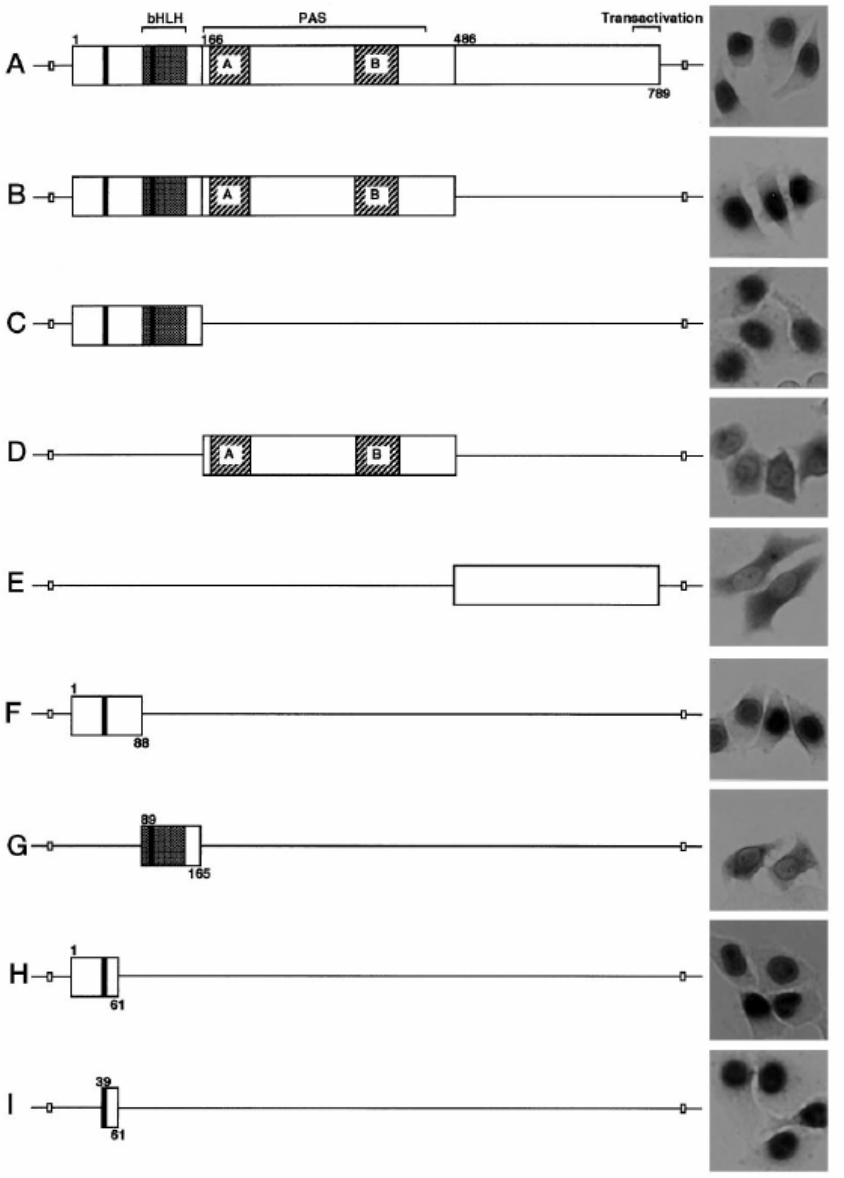


Families of DNA-binding domains (A) Zinc finger domains consist of loops in which an α helix and a β sheet coordinately bind a zinc ion. (B) Helix-turn-helix domains consis of three (or in some cases four) helical regions. One helix (helix 3) makes most of the contacts with DNA, while helices 1 and 2 lie on top and stabilize the interaction. (C) The DNA-binding domains of leucine zipper proteins are formed from two distinct polypeptide chains. Interactions between the hydrophobic side chains of leucine residues exposed on one side of a helical region (the leucine zipper) are responsible for dimerization. Immediately following the leucine zipper is a DNA-binding helix, which is rich in basic amino acids. (D) Helix-loop-helix domains are similar to leucine zippers, except that the dimerization domains of these proteins each consist of two helical regions separated by a loop

Příklad – funkce domén v interakci proteinů:

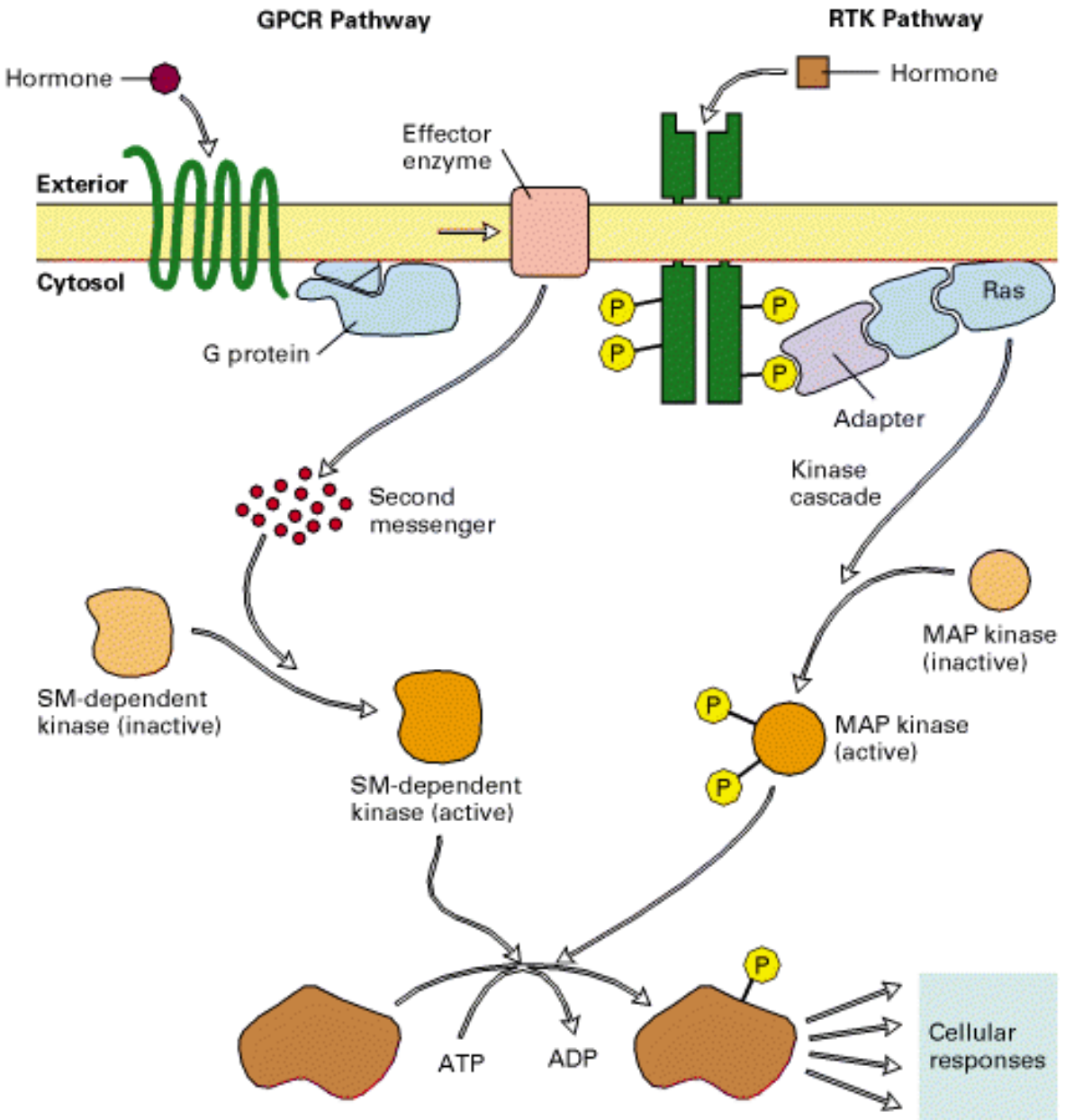


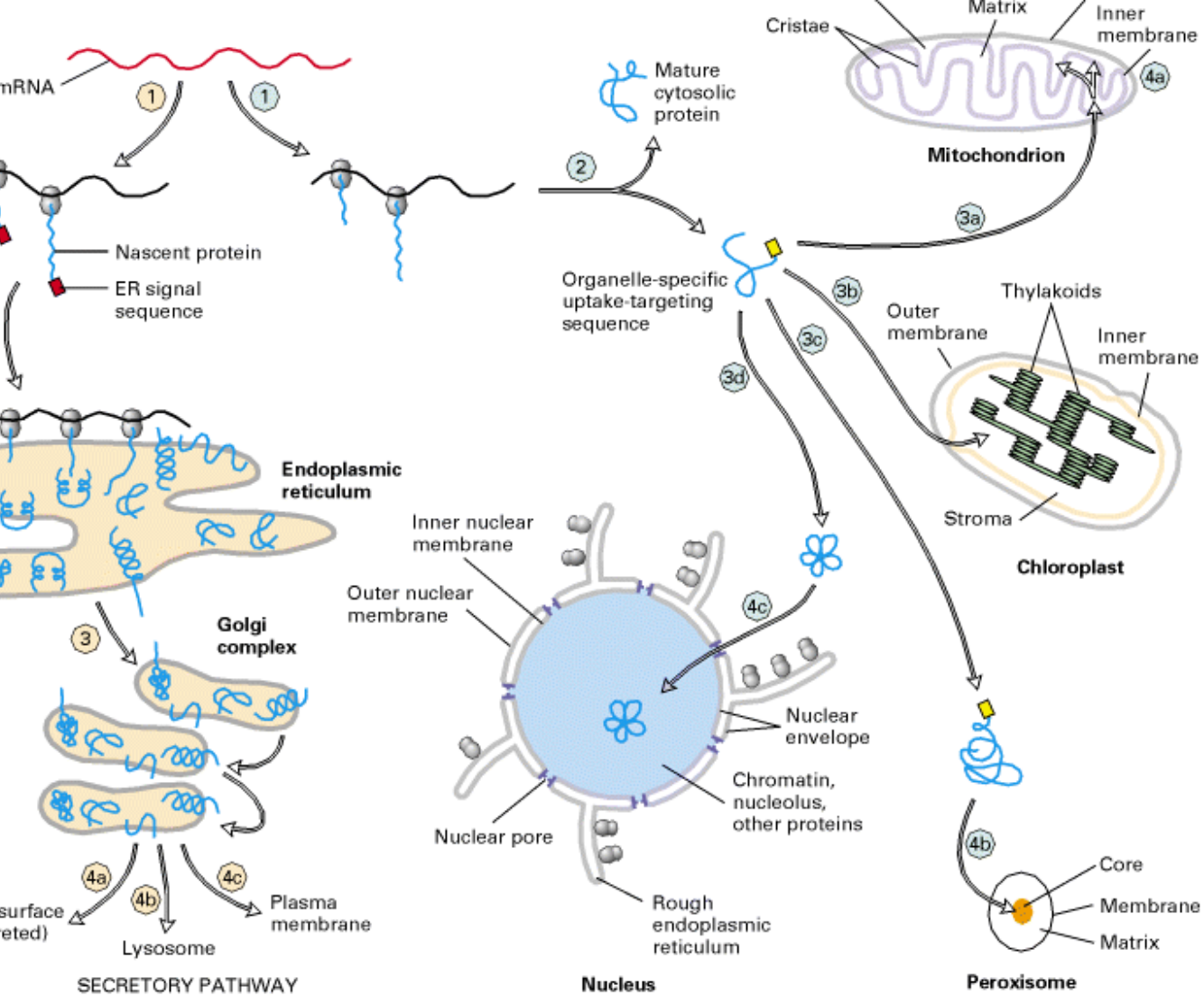
Identification of the region responsible for the nuclear localization of ARNT. Various portions of ARNT were synthesized using PCR, and the resulting fragments were fused to the modified β-Gal control vector. An expression vector of β-Gal/ARNT-(1–789) fusion gene was delivered into the indicated cells by means of electroporation. After a 48-h incubation at 37 °C, the cells were fixed and stained with 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside solution. The subcellular localization of the fusion proteins were examined by microscopy.



Regulace hladiny, aktivity a lokalizace proteinu – dynamický proces:

- posttranslační modifikace;
- vazba ligandu;
- interakce protein-protein;
- štěpení inaktivní formy proteinu;
- degradace proteinů (lysozóm, protеazóm)


GPCR Pathway
RTK Pathway
Hormone
Hormone
Effector enzyme
Exterior
Cytosol
G protein
P
P
P
P
Ras
Adapter
Kinase cascade
Second messenger
MAP kinase (inactive)
SM-dependent kinase (inactive)
MAP kinase (active)
SM-dependent kinase (active)
ATP
ADP
Cellular responses



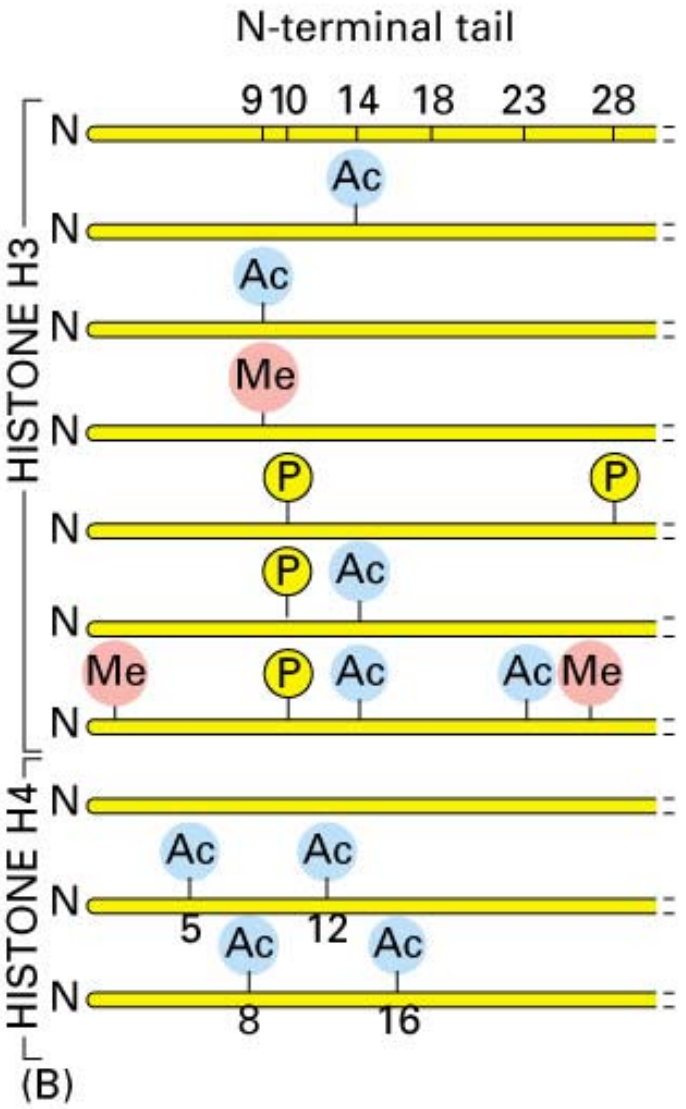
Overview of sorting of nuclear-encoded proteins in eukaryotic cells.

All nuclear-encoded mRNAs are translated on cytosolic ribosomes. Ribosomes are directed to the roug endoplasmic reticulum (ER) by an ER signal sequence And these proteins move to the Golgi complex, from whence they are further sorted to several destinations

fter synthesis of proteins lacking an ER signal sequence is completed on free bosomes, the proteins are released into the cytosol and those with an organelle-pecific uptake-targeting sequence are imported into the mitochondrion, eroxisome, or nucleus.

Modification	Donor molecule	Example of modified protein	Protein function
Phosphorylation	ATP	Glycogen phosphorylase	Glucose homeostasis; energy transduction
Acetylation	Acetyl CoA	Histones	DNA packing; transcription
Myristoylation	Myristoyl CoA	Src	Signal transductio
ADP-ribosylation	NAD	RNA polymerase	Transcription
Farnesylation	Farnesyl pyrophosphate	Ras	Signal transductio
γ-Carboxylation	HCO_3^-	Thrombin	Blood clotting
Sulfation	3′-Phosphoadenosine-5′-phosphosulfate	Fibrinogen	Blood-clot formation
Ubiquitination	Ubiquitin	Cyclin	Control of cell cycle

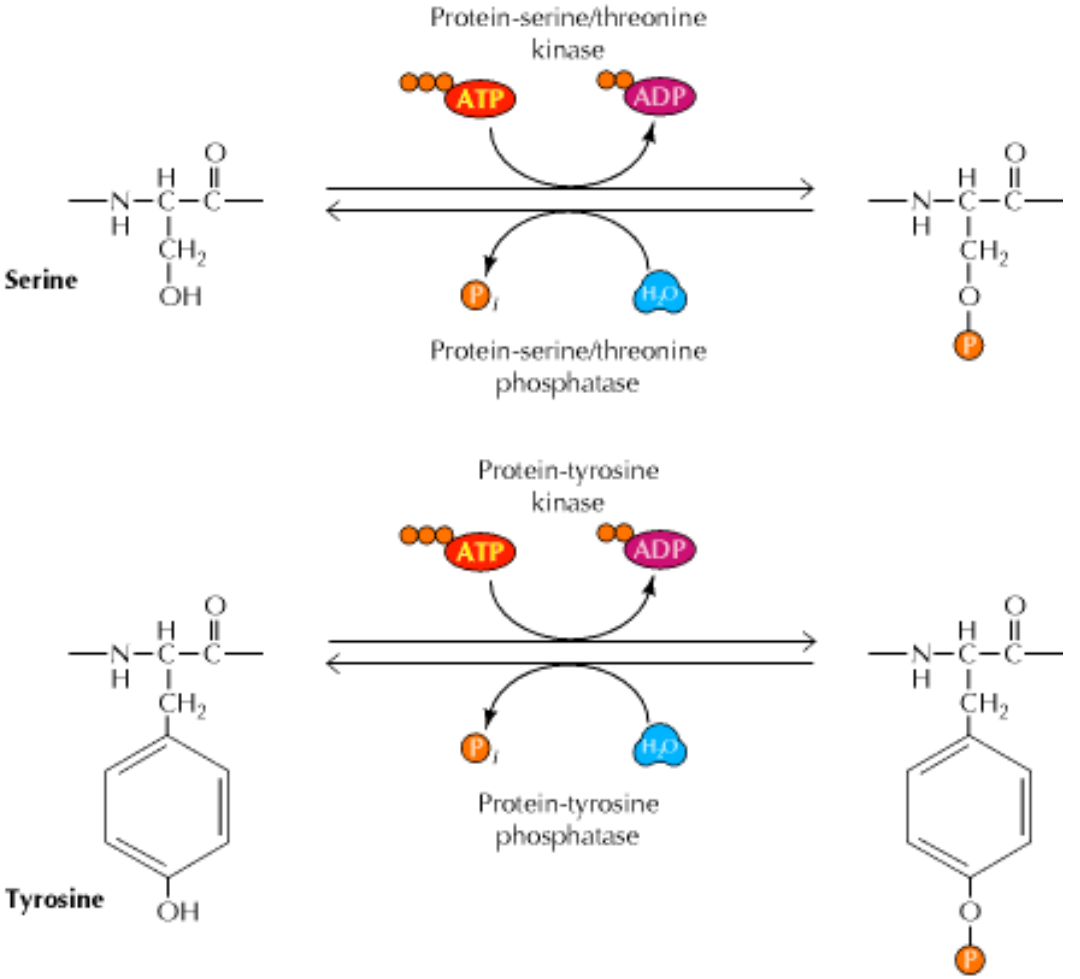
Posttranslační modifikace:



modification state	"meaning"
unmodified	gene silencing?
acetylated	gene expression
acetylated	histone deposition
methylated	gene silencing/ heterochromatin
phosphorylated	mitosis/meiosis
phosphorylated/ acetylated	gene expression
higher-order combinations	?
unmodified	gene silencing?
acetylated	histone deposition
acetylated	gene expression

Figure 4–35 part 2 of 2. Molecular Biology of the Cell, 4th Edition.

Protein kinases and phosphatases. Protein kinases catalyze the transfer of a phosphate group from ATP to the side chains of serine and threonine (protein-serine/threonine kinases) or tyrosine (protein-tyrosine kinases) residues. Protein phosphatases catalyze the removal of phosphate groups from the same amino acids by hydrolysis.



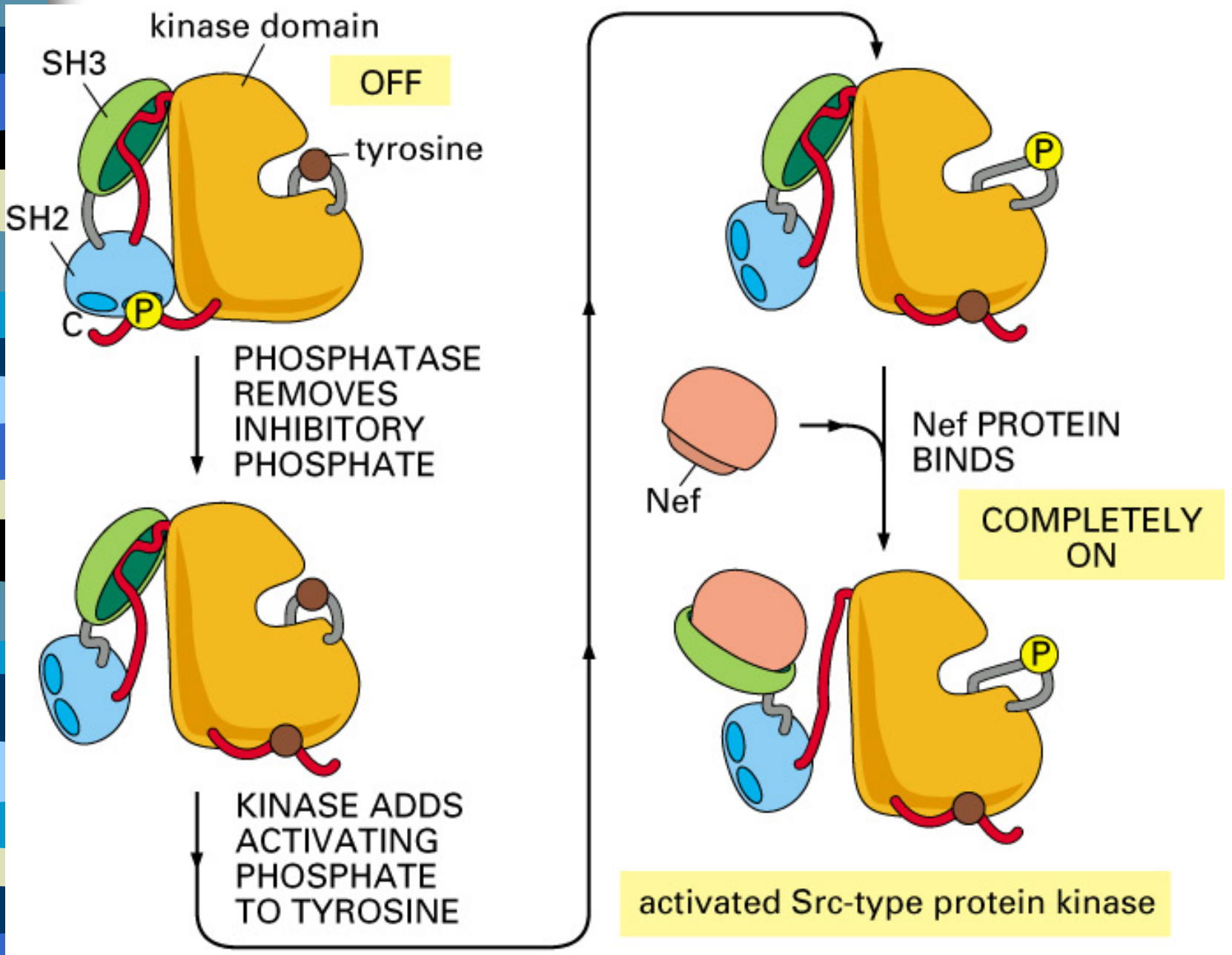
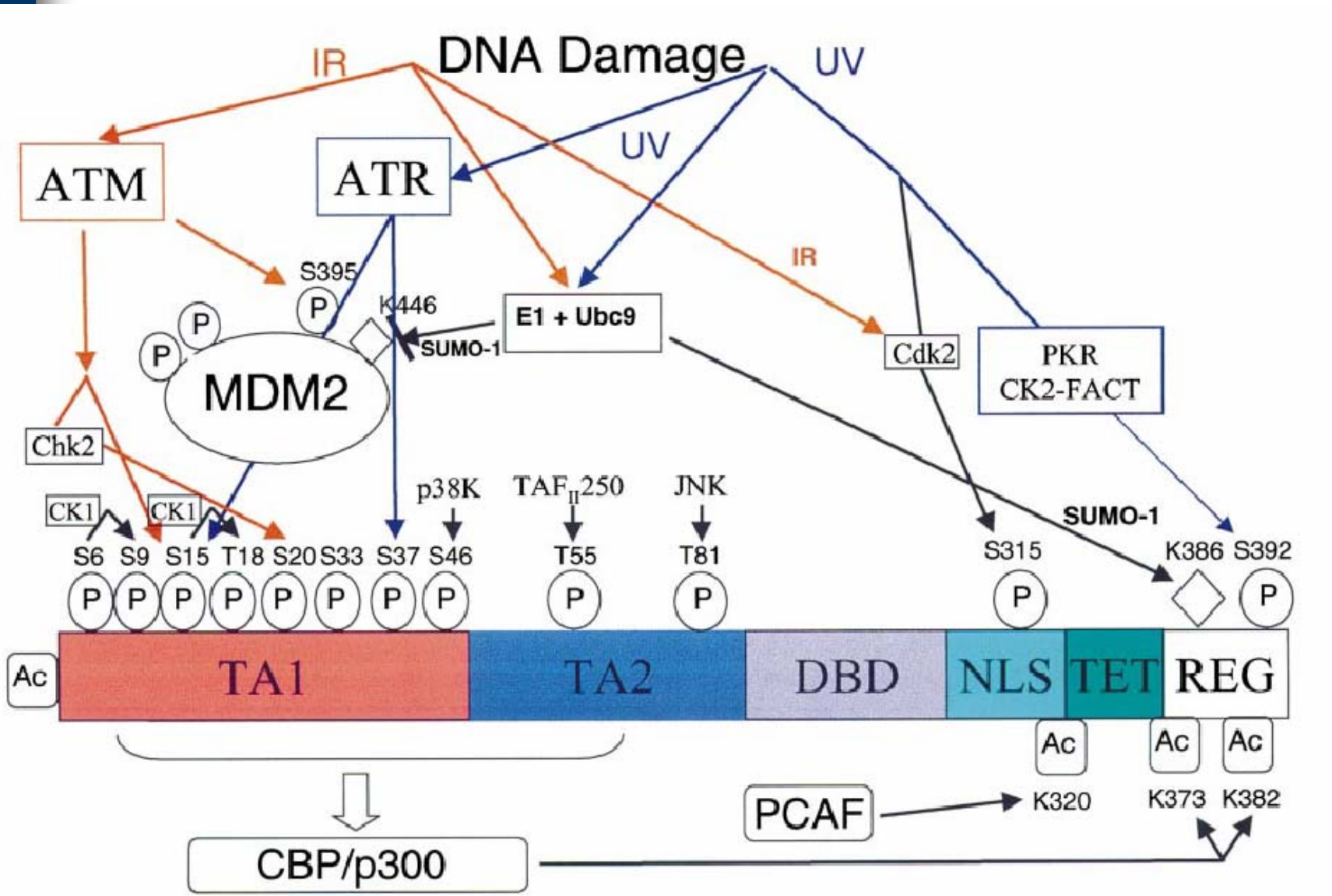
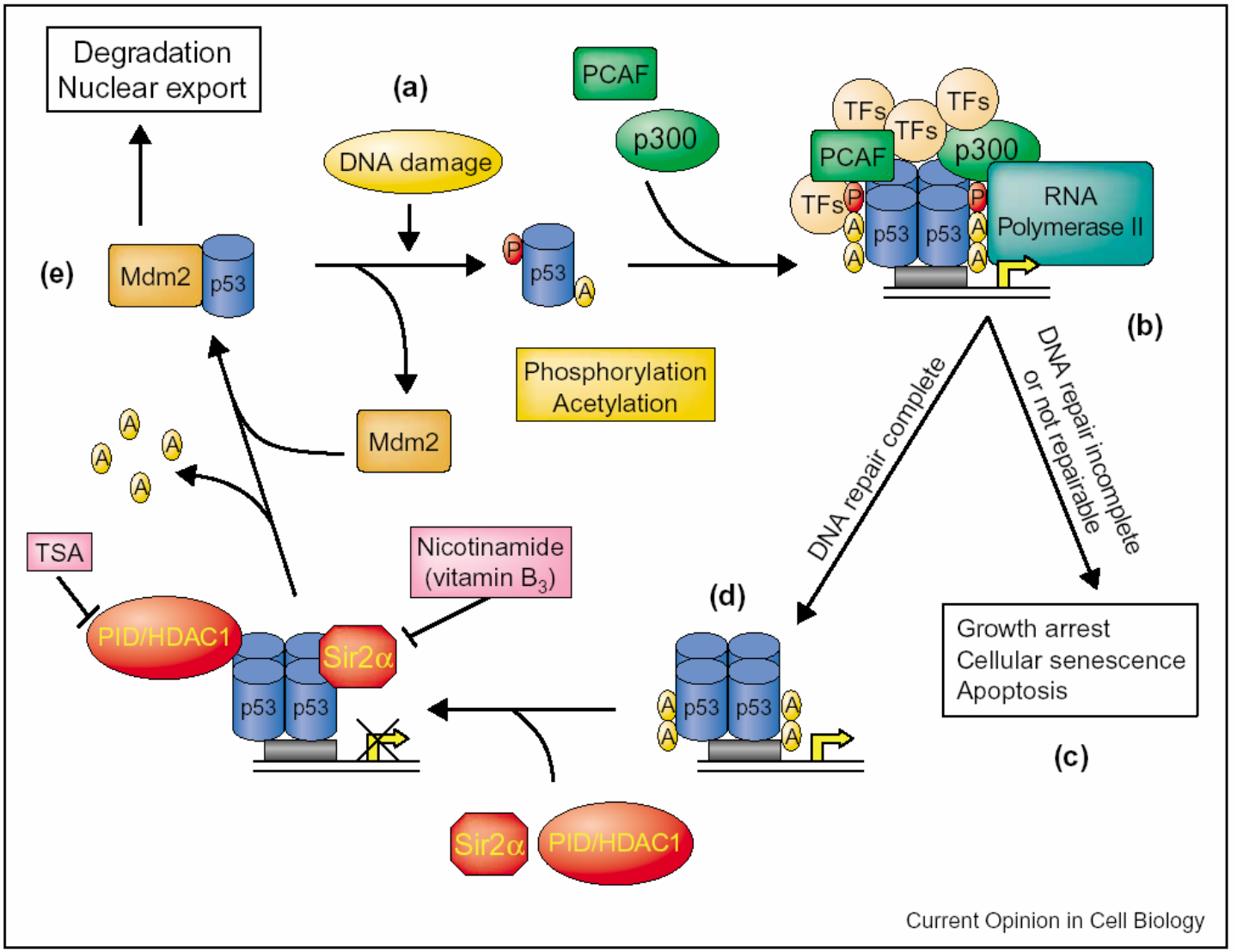
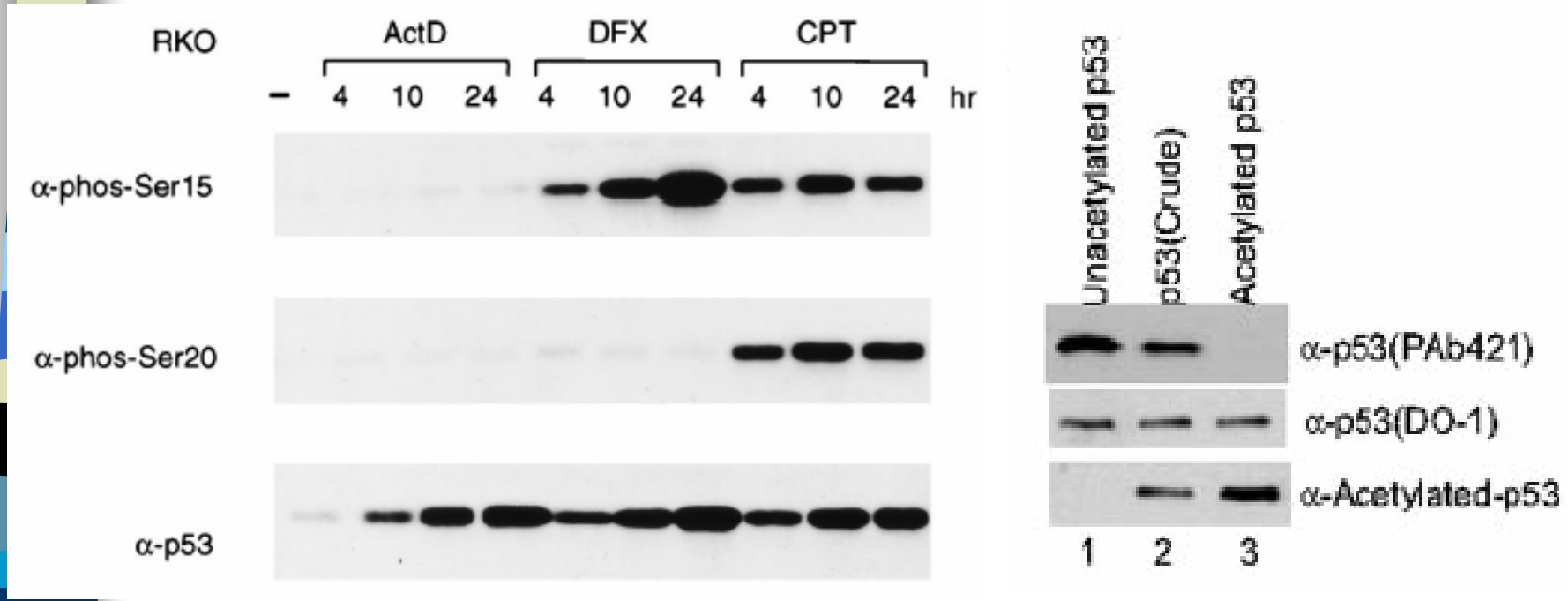


Figure 3–68. Molecular Biology of the Cell, 4th Edition.

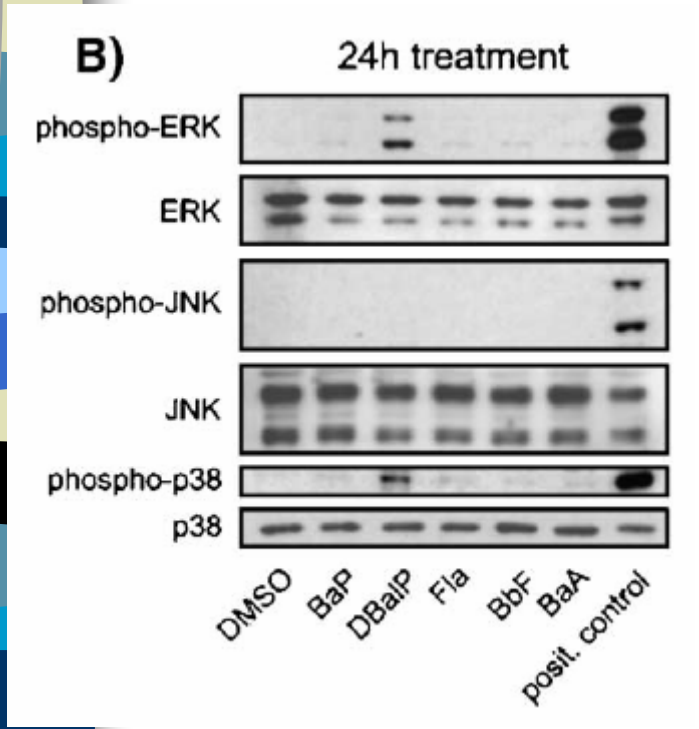

DNA Damage
IR
UV
UV
IR
ATM
ATR
S395
P
K446
SUMO-1
E1 + Ubc9
P
P
MDM2
Cdk2
PKR
CK2-FACT
Chk2
CK1
CK1
p38K
TAF$_{II}$250
JNK
SUMO-1
S6 S9 S15 T18 S20 S33 S37 S46
T55
T81
S315
K386 S392
P
Ac
TA1
TA2
DBD
NLS
TET
REG
Ac
Ac
Ac
PCAF
K320
K373 K382
CBP/p300


Degradation
Nuclear export
(a)
DNA damage
PCAF
p300
TFs
TFs
TFs
PCAF
p300
TFs
RNA
Polymerase II
p53
p53
(e)
Mdm2
p53
Phosphorylation
Acetylation
(b)
Mdm2
DNA repair complete
DNA repair incomplete
or not repairable
TSA
Nicotinamide
(vitamin B3)
PID/HDAC1
Sir2α
p53
p53
(d)
Growth arrest
Cellular senescence
Apoptosis
(c)
Sir2α
PID/HDAC1
Current Opinion in Cell Biology

Příklady PT modifikací – detekce specifickými protilátkami



Příklady PT modifikací – detekce pomocí specifické protilátky



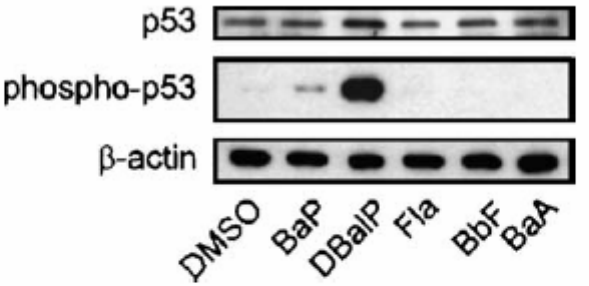
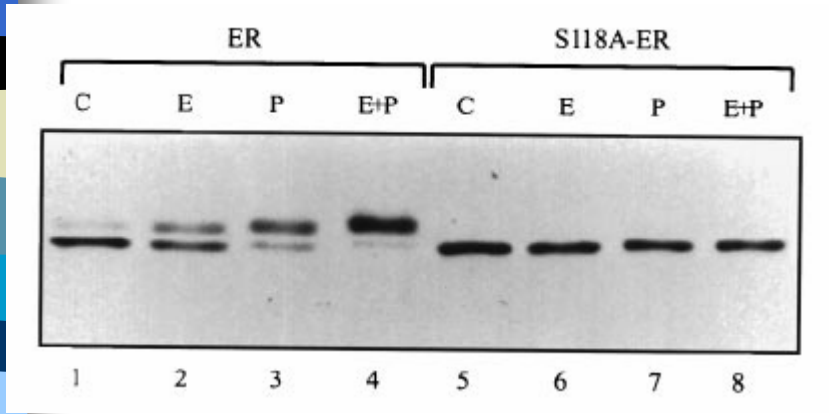


Fig. 3. DBalP induces p53 phosphorylation at serine 15 following the 24-h incubation. Confluent WB-F344 cells were treated with Fla (10 μM), BaA (10 μM), BbF (10 μM), BaP (10 μM), DBalP (100 nM) or DMSO as vehicle (0.1%). Cell lysates were prepared and Western blotting performed as described in Materials and methods. The results shown here are representative of three independent experiments.

Příklady PT modifikací – detekce podle MW a pI



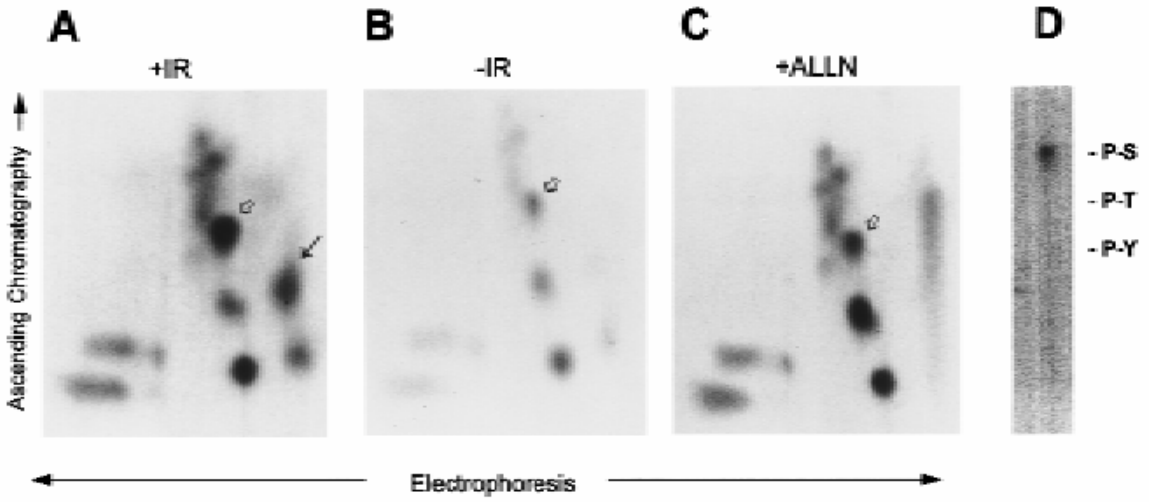
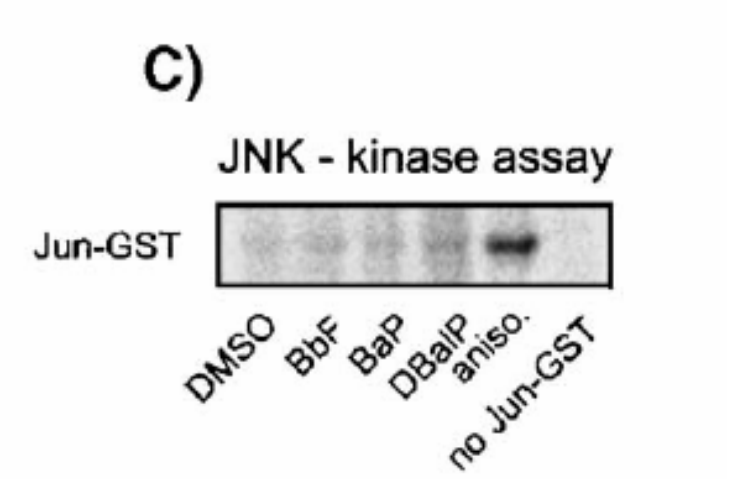
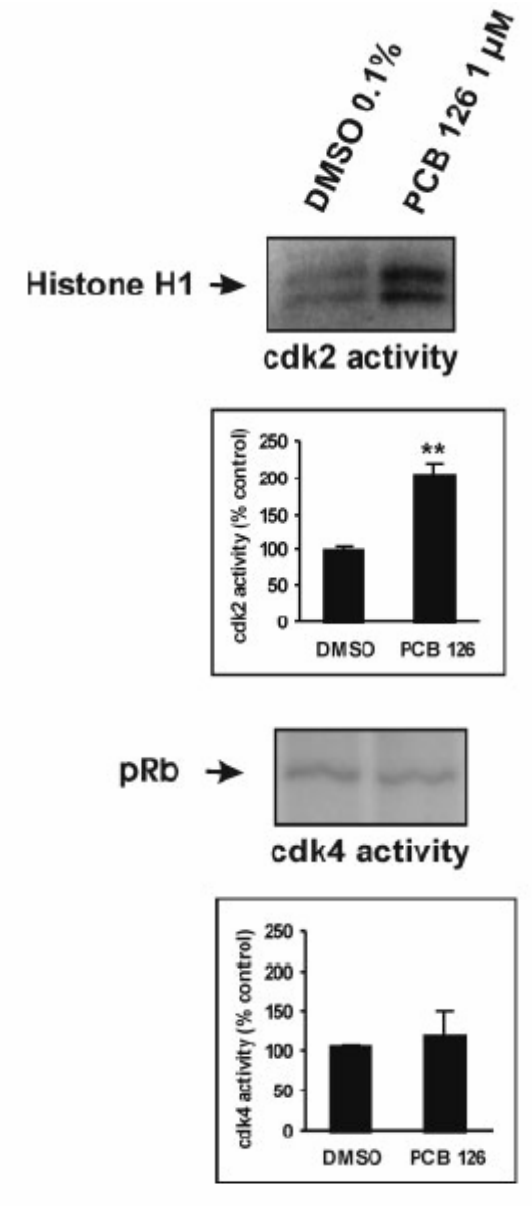


Figure 2. IR induces phosphorylation of two serines within the first 24 amino acids of p53 in vivo. p53 was immunoprecipitated from ^{32}P-labeled ML-1 cells that were either given 2 Gy irradiation (*A*), untreated (*B*), or treated with 20 μm of ALLN (*C*). Proteins were resolved by 10% SDS-PAGE and electrophoretically transferred to PVDF membrane. Radiolabeled p53 was cut from the membrane and digested with TPCK–trypsin. Radiolabeled peptides were resolved by electrophoresis at pH 3.5 in the first dimension followed by ascending chromatography in the second dimension. The unique, irradiation-induced p53 phosphopeptide (*A*, black arrow) was eluted from the cellulose and subjected to phosphoamino acid analysis. The position of the unlabeled phosphoamino acid markers are indicated (*D*). A singly phosphorylated p53 synthetic peptide corresponding to amino acids 1–24 (Ac 1–24; serine-15 P) comigrated with an in vivo peptide in all three maps (*A–C*, open arrow). The unique, IR-induced phosphopeptide comigrated with a synthetic, triply phosphorylated p53 peptide comprising amino acids 1–24 (Ac 1–24; serine-9 P, serine-15 P, serine-20 P) (*A*, black arrow).

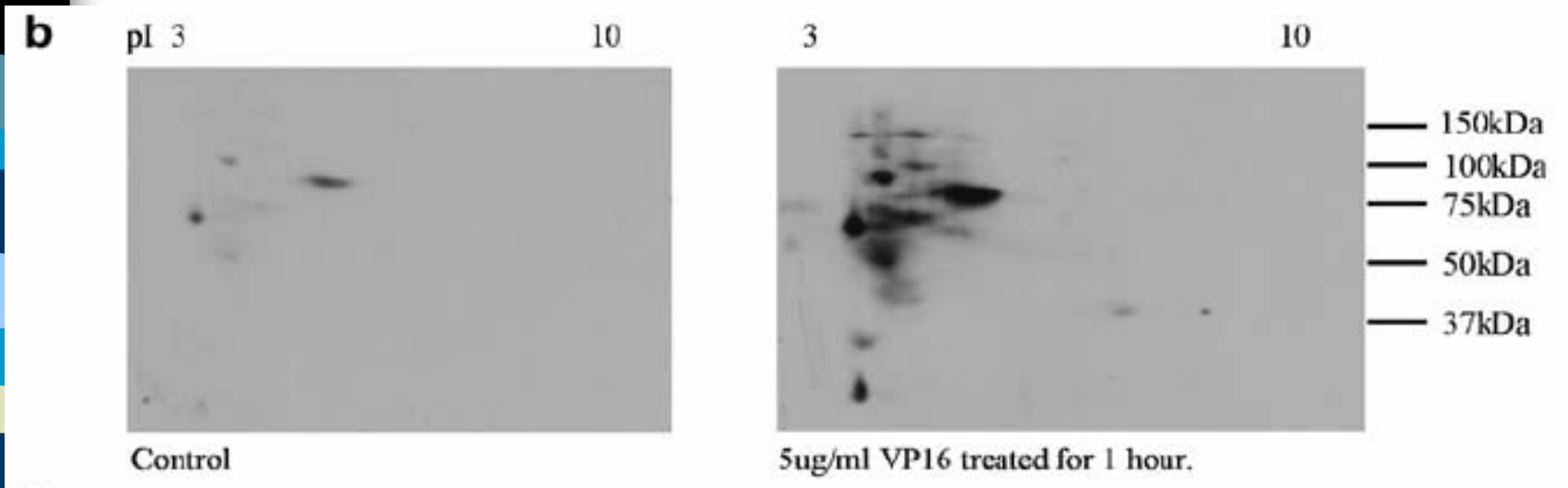
Příklady PT modifikací – detekce aktivity modifikujících enzymů





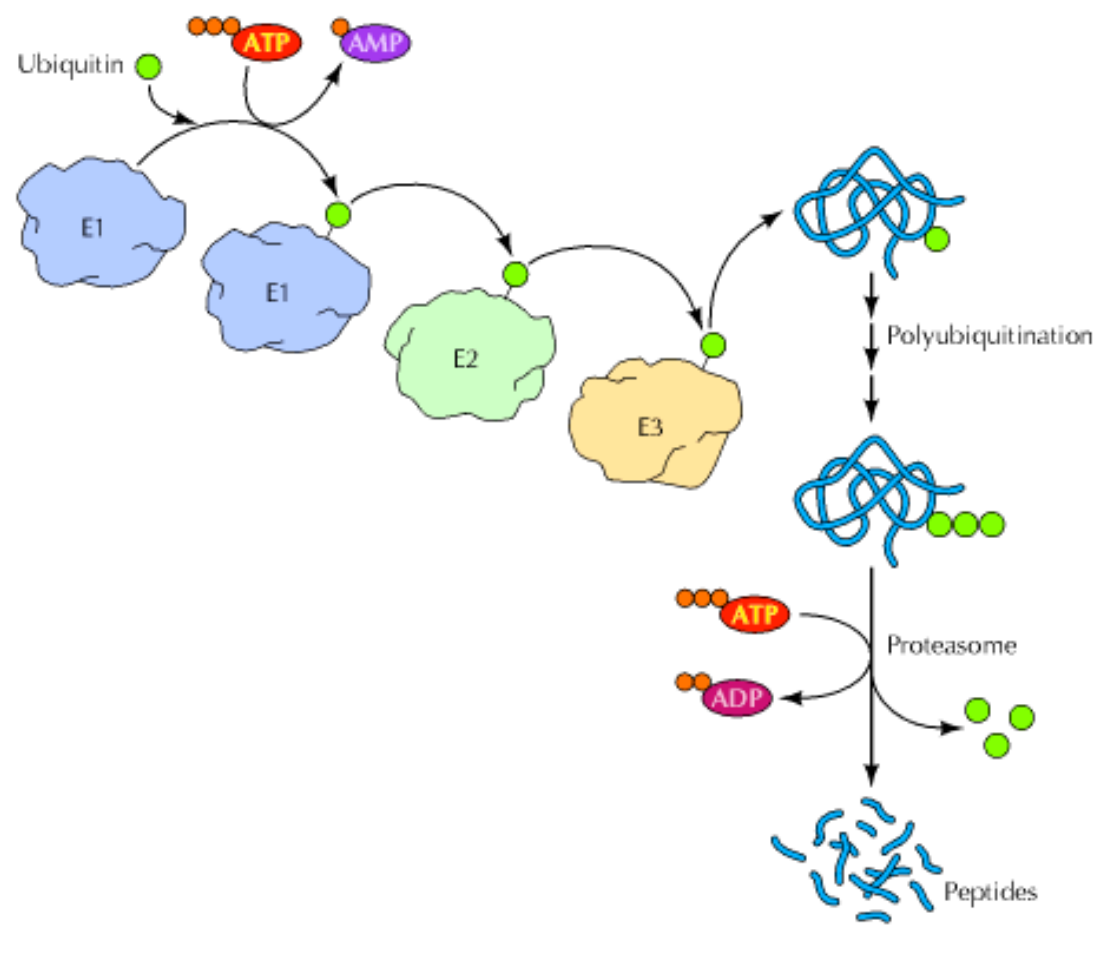
Příklad proteomové analýzy – karbonylace proteinů v průběhu apoptózy

Spot Number	Protein (accession number)	Function	No. matched peptides
1	BiP (glucose regulated protein grp78) (p11021)	Chaperone	10
2	α enolase (P06733)	Metabolism	8
3	Fructose bis-phosphate aldolase (P09972)	Metabolism	5
4	c-AMP-dependent PDE (Q9NP56)	Signal transduction	5
5	Phosphoglycerate mutase (P18669)	Metabolism	8
6	Triose phosphate isomerase (P00938)	Metabolism	11
7	Actin (P02570)	Cytoskeleton	10
8	Plexin B2 (20270190)	Transmembrane receptor	7



Proteinová degradace:

The levels of proteins within cells are determined not only by rates of synthesis, but also by rates of degradation. The half-lives of proteins within cells vary widely, from minutes to several days, and differential rates of protein degradation are an important aspect of cell regulation. Many rapidly degraded proteins function as regulatory molecules, such as transcription factors. The rapid turnover of these proteins is necessary to allow their levels to change quickly in response to external stimuli. Other proteins are rapidly degraded in response to specific signals, providing another mechanism for the regulation of intracellular enzyme activity. In addition, faulty or damaged proteins are recognized and rapidly degraded within cells, thereby eliminating the consequences of mistakes made during protein synthesis. In eukaryotic cells, two major pathways—the ubiquitin-proteasome pathway and lysosomal proteolysis—mediate protein degradation.



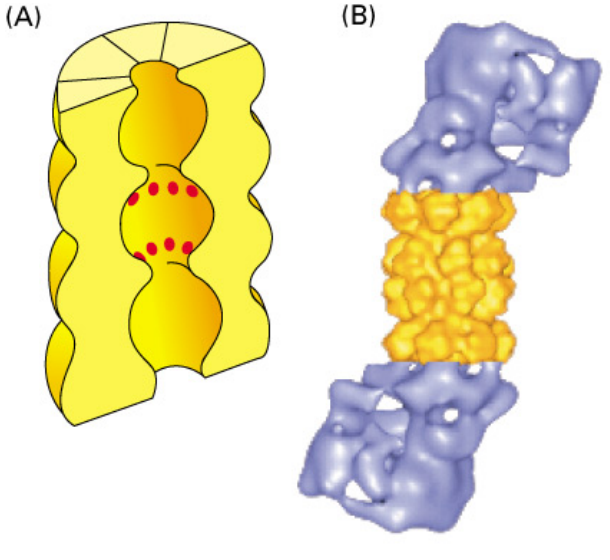
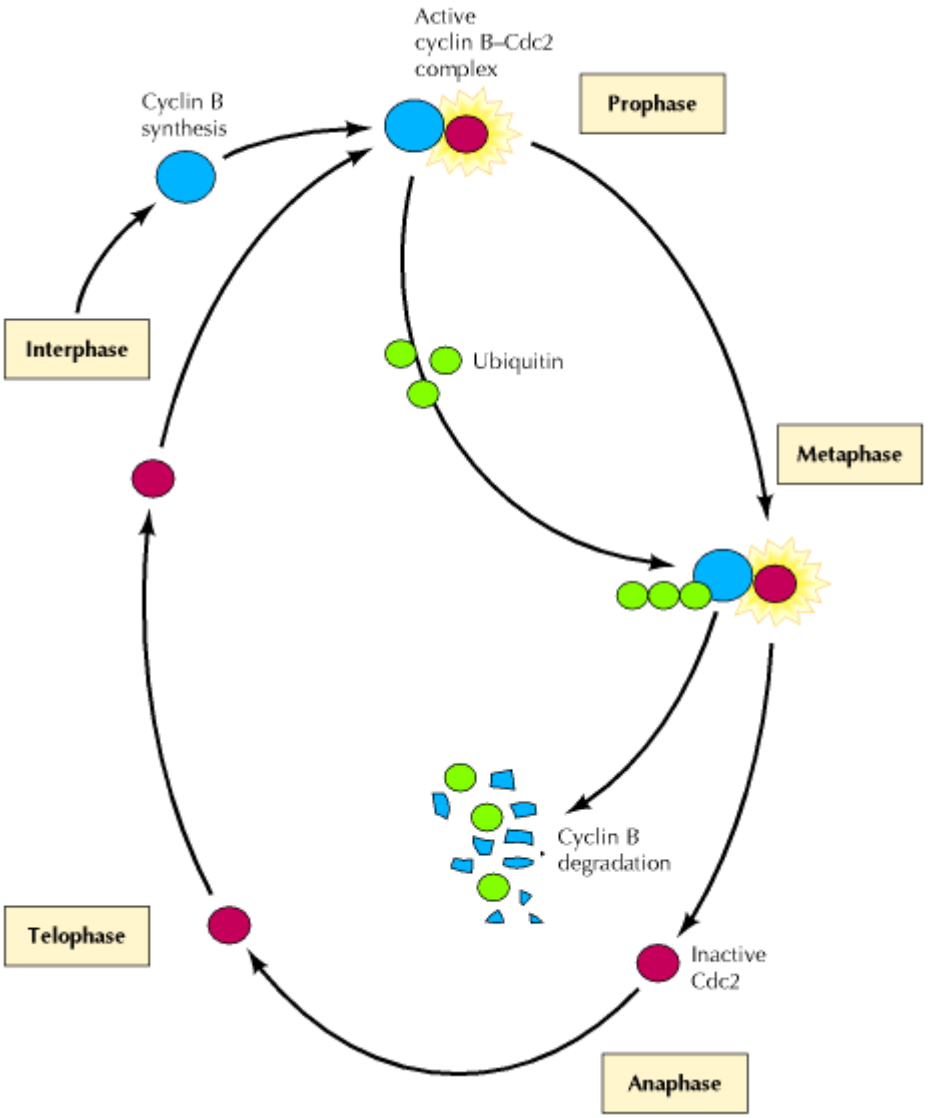


Figure 6–86. Molecular Biology of the Cell, 4th Edition.

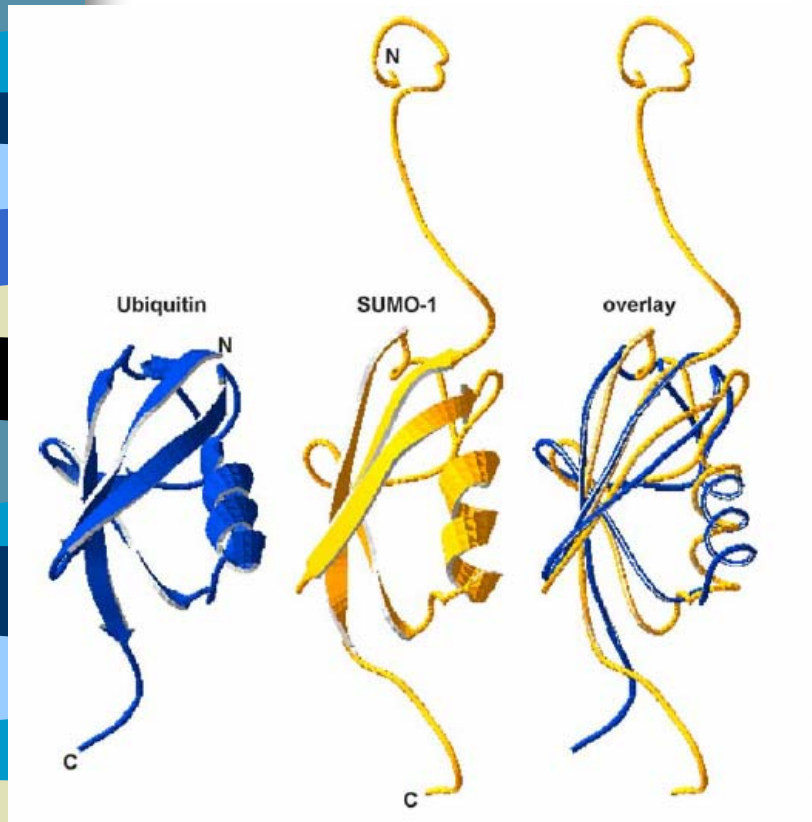
The ubiquitin-proteasome pathway Proteins are marked for rapid degradation by the covalent attachment of several molecules of ubiquitin. Ubiquitin is first activated by the enzyme E1. Activated ubiquitin is then transferred to one of several different ubiquitin-conjugating enzymes (E2). In most cases, the ubiquitin is then transferred to a ubiquitin ligase (E3) and then to a specific target protein. Multiple ubiquitins are then added, and the polyubiquinated proteins are degraded by a protease complex (the proteasome).

Cyclin degradation during the cell cycle.

The progression of eukaryotic cells through the division cycle is controlled in part by the synthesis and degradation of cyclin B, which is a regulatory subunit of the Cdc2 protein kinase. Synthesis of cyclin B during interphase leads to the formation of an active cyclin B–Cdc2 complex, which induces entry into mitosis. Rapid degradation of cyclin B then leads to inactivation of the Cdc2 kinase, allowing the cell to exit mitosis and return to interphase of the next cell cycle.



Další typy modifikací – SUMO, sentrin, NEDD



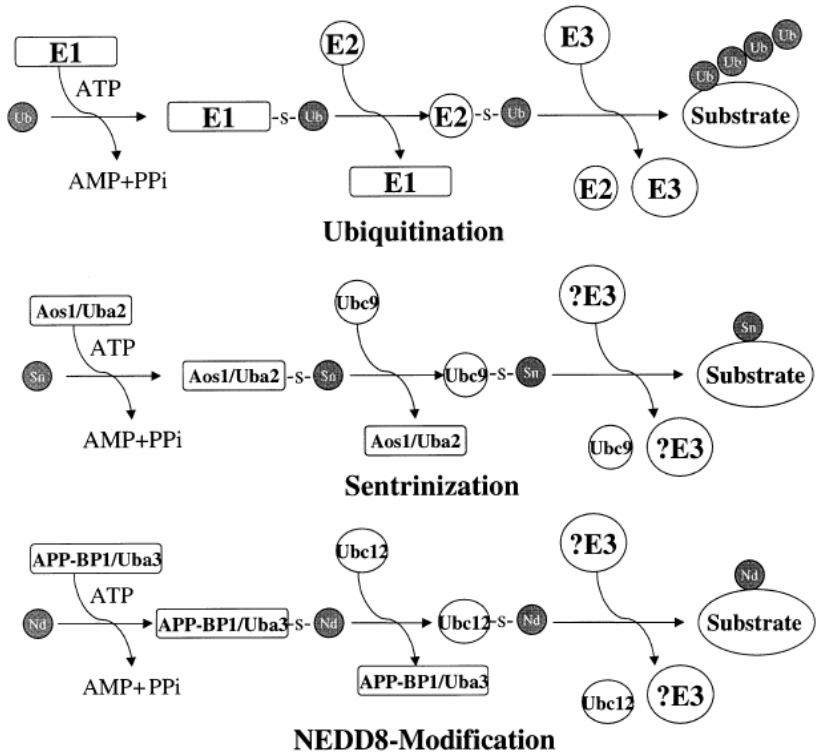
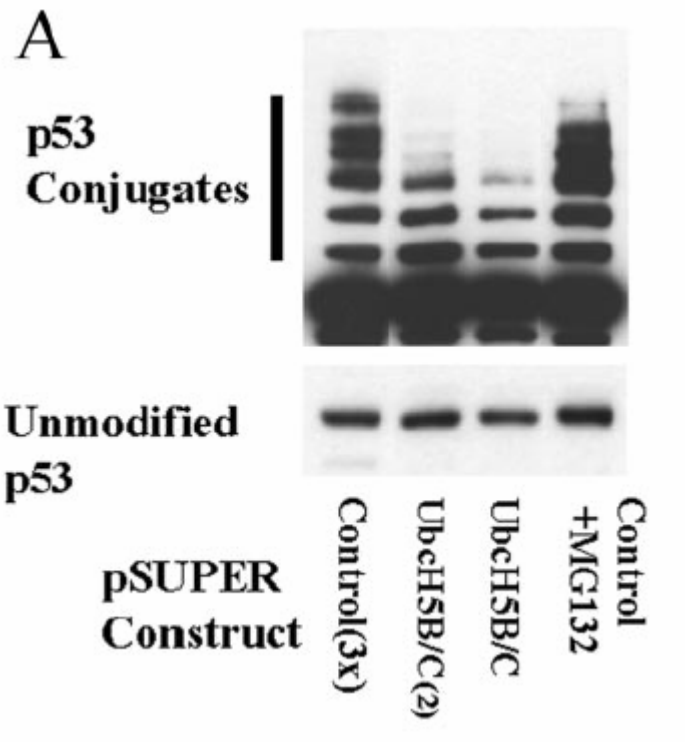
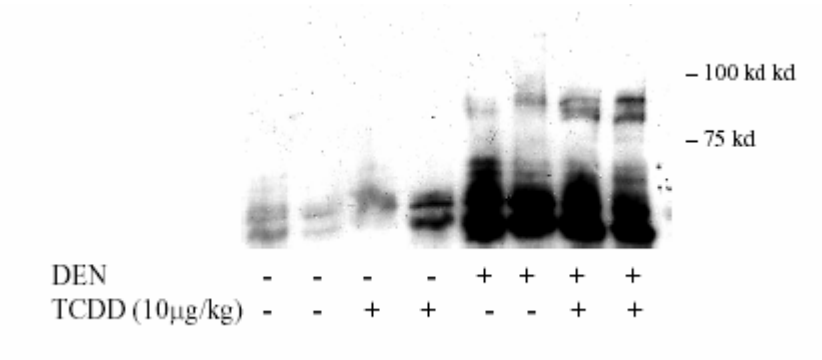


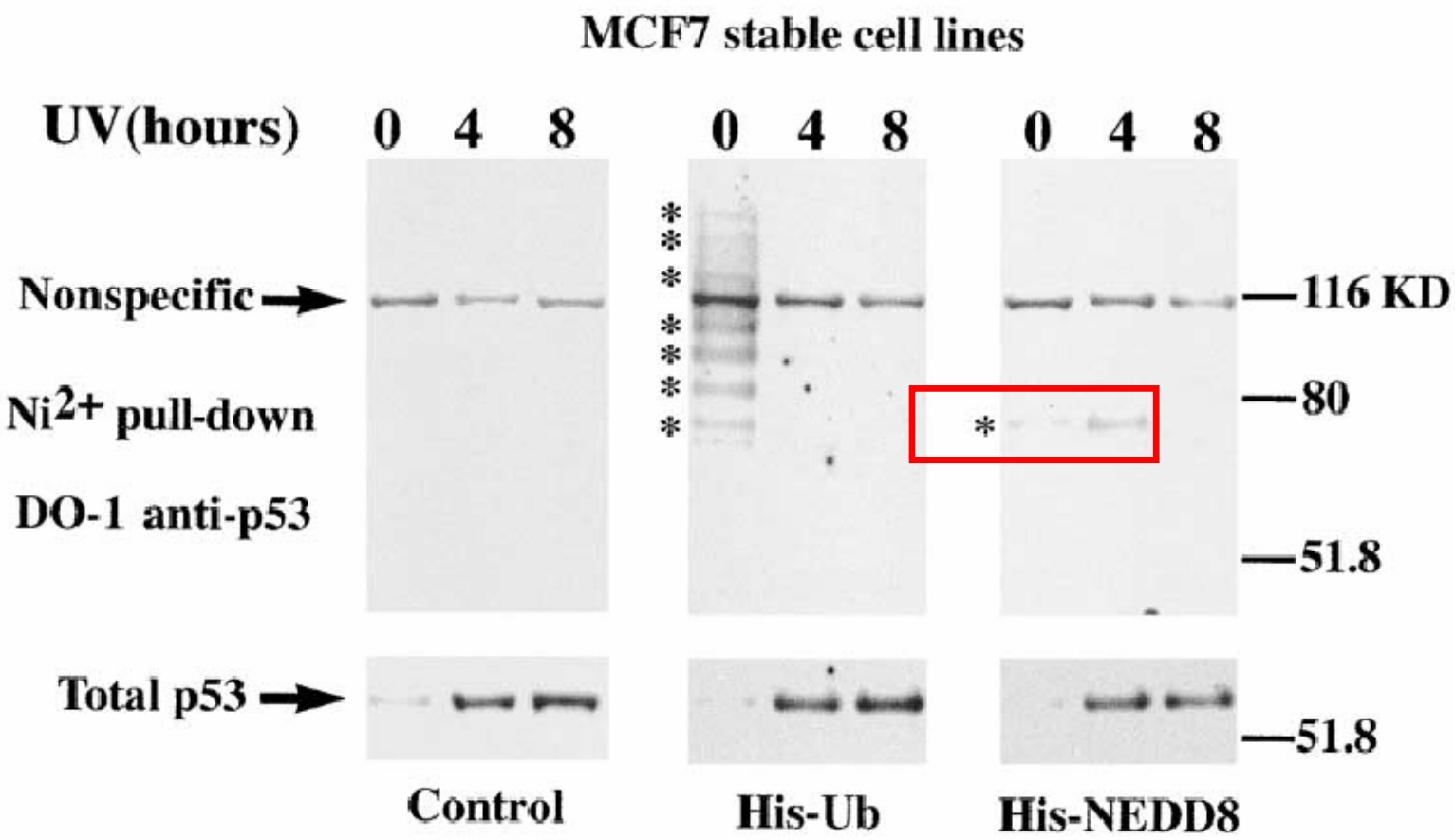
Fig. 1. Comparison of ubiquitination, sentrinization, and NEDD8 modification pathways.

Detekce ubikvitinace – shift assay:

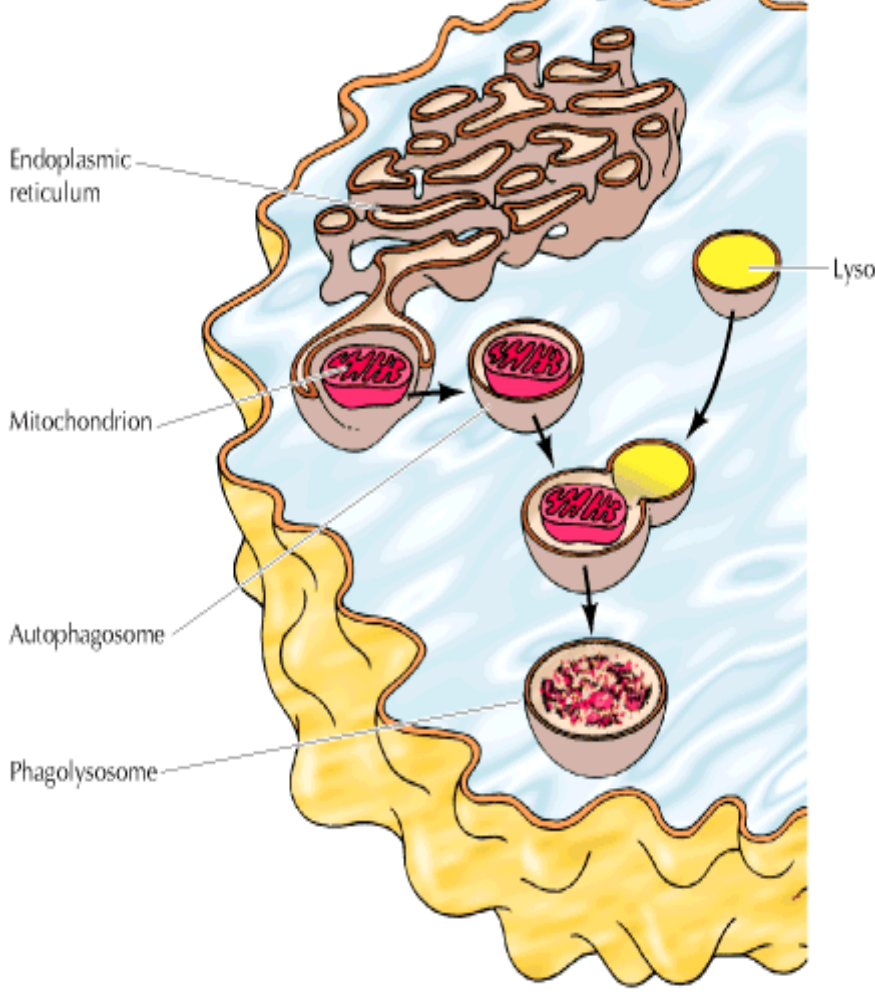




Detekce neddylace – rekombinantní protein:

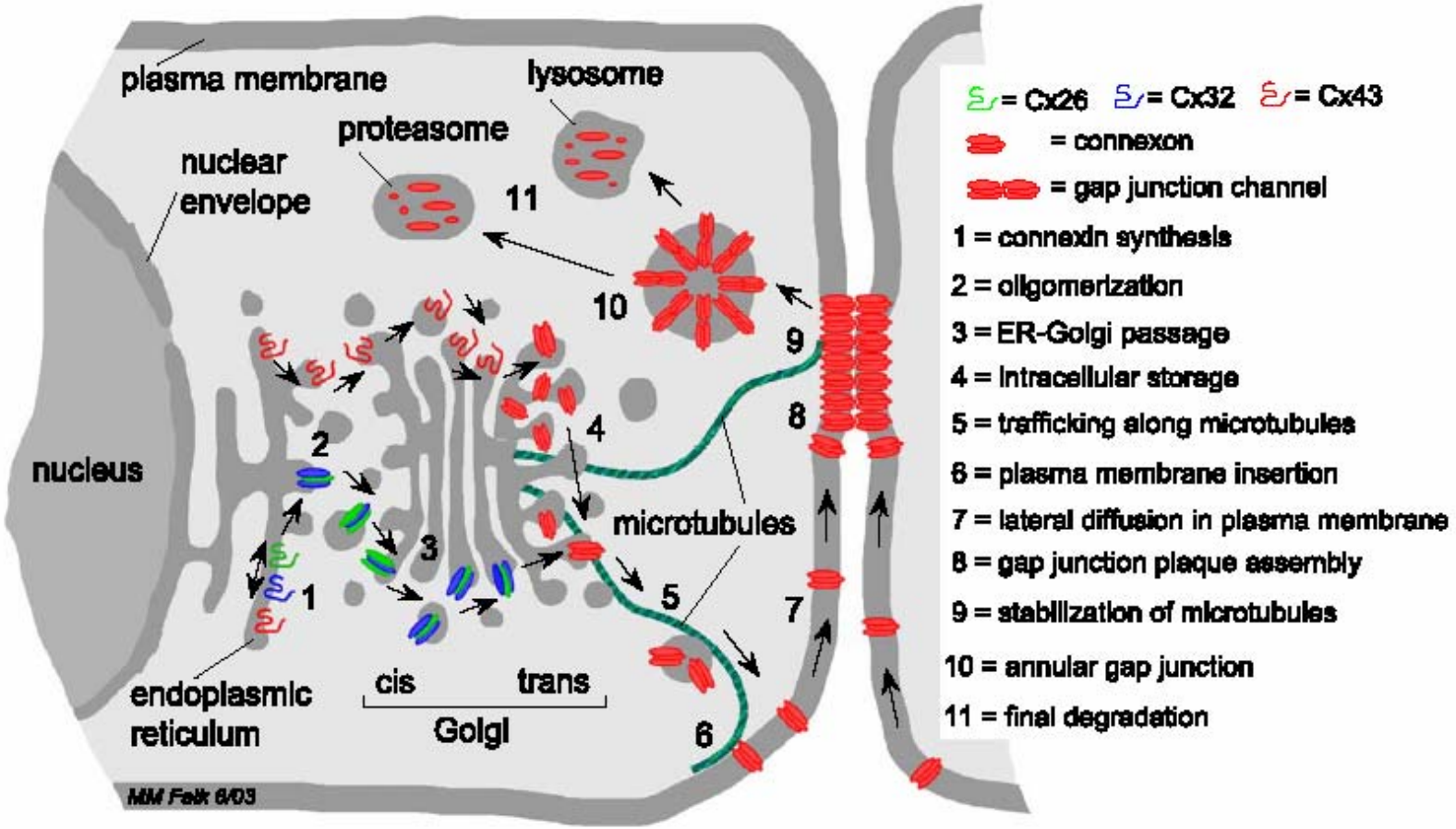


he other major pathway of protein egradation in eukaryotic cells involves the ptake of proteins by **lysosomes**. ysosomes are membrane-enclosed rganelles that contain an array of igestive enzymes, including several roteases. They have several roles in cell etabolism, including the digestion of xtracellular proteins taken up by ndocytosis as well as the gradual turnover f cytoplasmic organelles and cytosolic roteins. The containment of proteases nd other digestive enzymes within sosomes prevents uncontrolled egradation of the contents of the cell. herefore, in order to be degraded by sosomal proteolysis, cellular proteins ust first be taken up by lysosomes. One athway for this uptake of cellular roteins, **autophagy**, involves the formation f vesicles (**autophagosomes**) in which small reas of cytoplasm or cytoplasmic rganelles are enclosed in membranes erived from the endoplasmic reticulum.

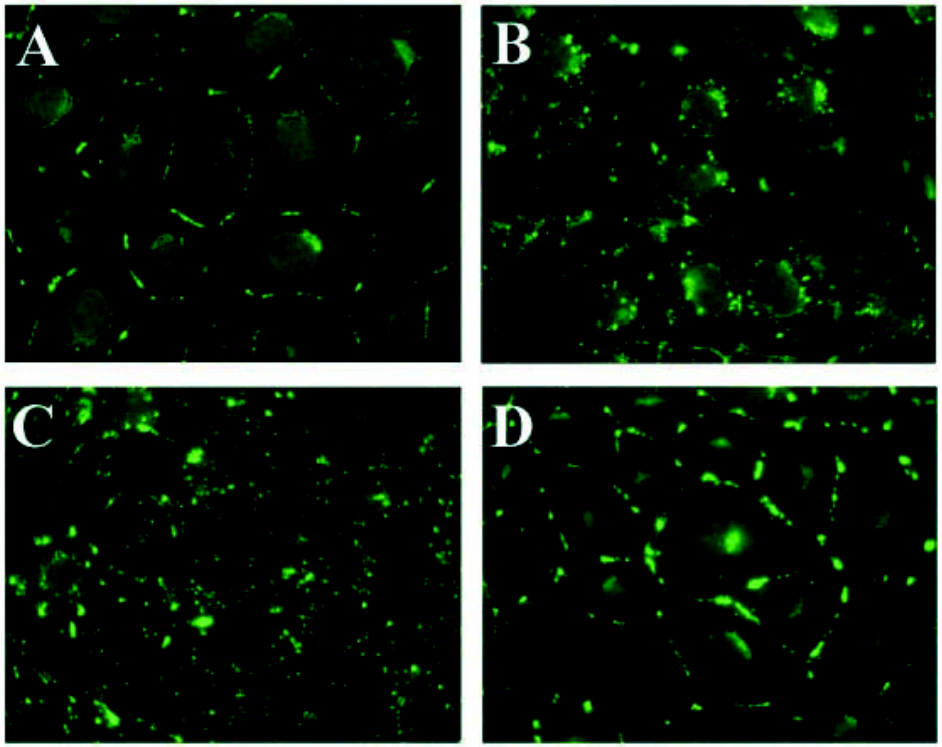


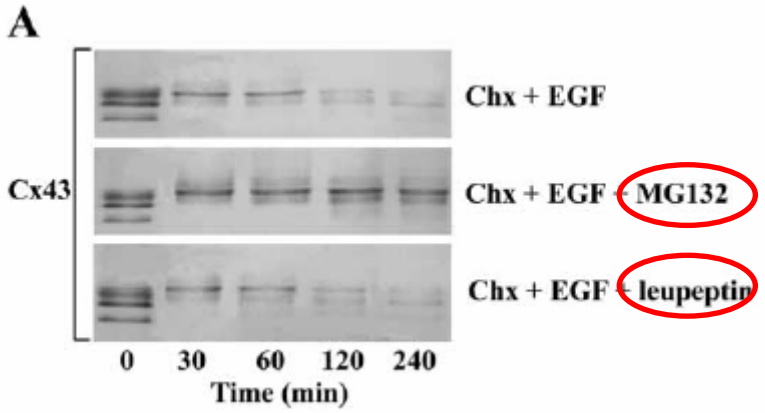
Lysosomes are able to degrade cytosolic proteins in a selective manner. The proteins degraded by lysosomal proteases under these conditions contain amino acid sequences simila to the broad consensus sequence Lys-Phe-Glu Arg-Gln.

Životní cyklus konexinů:



Dráhy regulující „protein trafficking“ lze studovat pomocí specifických inhibitorů:





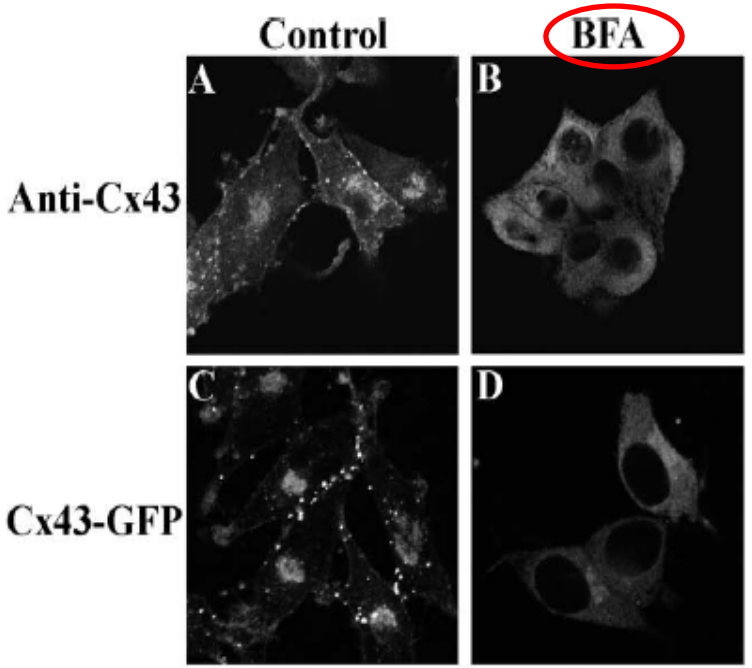


Fig. 3. Effects of cycloheximide and MG132 on EGF-induced relocalization of Cx43. IAR20 cells were either left untreated (A) or treated with 50 ng ml^{-1} EGF for 30 minutes (B). (C) Cells were preincubated with 10 μg ml^{-1} cycloheximide for 2 hours, then exposed to EGF at a final concentration of 50 ng ml^{-1} and incubated for 30 minutes in the sustained presence of cycloheximide. (D) Cells were preincubated with 10 μM MG132 for 30 minutes, then exposed to EGF at a final concentration of 50 ng ml^{-1} and incubated for 30 minutes in the sustained presence of MG132. Cells were fixed, immunostained with anti-Cx43 antibodies and visualized using fluorescence microscopy.

Práce s DNA a RNA:

- manipulace s proteinem (overexprese; dominant negative constructs, antisense oligonuclotides, siRNA);
- detekce exprese mRNA
- příprava a izolace plazmidů, izolace genomové DNA, transfekce živočišných buněk