

CG020 Genomika

Přednáška 7

Proteinové interakce v genových regulacích

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Genomika 07

▪ Zdrojová literatura

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Osnova

- Funkční význam specifických interakcí proteinů v regulaci genové exprese
 - Struktura chromatinu
 - Regulace transkripce
 - Lokalizace mRNA
 - Stabilita proteinů
 - Přenos signálu
- Metody analýzy proteinových interakcí *in vivo*
 - Koimunoprecipitace
 - Tandemová afinitní purifikace (TAP-tag)
 - Kvasinkový dvouhybridní test (Y2H)
 - Bimolekulární fluorescenční komplementace (BiFC)
 - Analýza zprostředkované membránové vazby (MeRA)

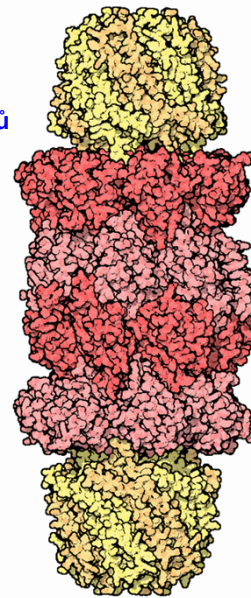
Význam interakcí proteinů

- **Funkční význam specifických interakcí proteinů**

- Většina proteinů v buňce existuje ve formě komplexů, které mohou dále navzájem interagovat

- **Proteazom**

- proteinový komplex zodpovědný za degradaci nepotřebných proteinů v buňce



1fnt

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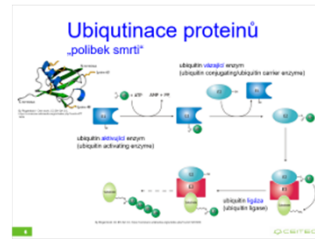
Once obsolete proteins are tagged with at least four ubiquitin molecules, they are destroyed by proteasomes. Proteasomes are voracious protein shredders, but the destructive machinery is carefully protected so that it can't attack all of the normal proteins in the cell. The proteasome, shown here from PDB entry 1fnt, is shaped like a cylinder, with its active sites sheltered inside the tube. The caps on the ends regulate entry into the destructive chamber, where the protein is chopped into pieces 3 to 23 amino acids long.

Most of the non-lysosomal proteolysis that occurs in eukaryotic cells is performed by a nonspecific and abundant barrel-shaped complex called the 20S proteasome. Substrates access the active sites, which are sequestered in an internal chamber, by traversing a narrow opening (alpha-annulus) that is blocked in the unliganded 20S proteasome by amino-terminal sequences of alpha-subunits. Peptide products probably exit the 20S proteasome through the same opening. 11S regulators (also called PA26, PA28 and REG) are heptamers that stimulate 20S proteasome peptidase activity in vitro and may facilitate product release in vivo. (PDB, Whitby et al., (2000) Nature **408**: 115-120, <http://www.rcsb.org/pdb/explore/explore.do?structureId=1fnt>).

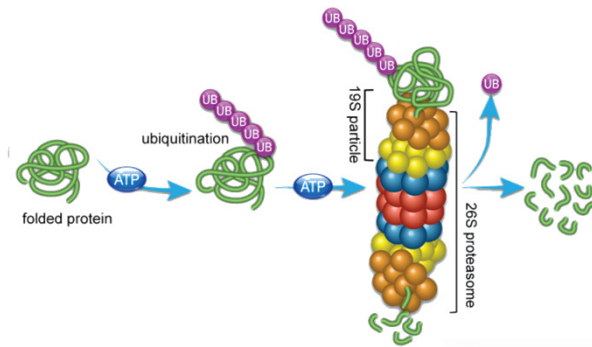
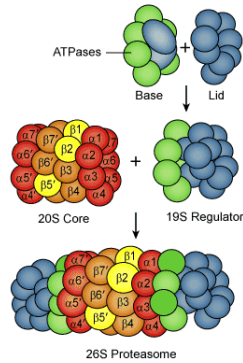
Význam interakcí proteinů

Proteazom

- Skládá se z centrálního komplexu označovaného jako 20S a regulačních částí (19S, někdy také 11S)
- Umožňuje cílenou degradaci proteinů označených specifickou značkou, malým proteinem (76 aa) - ubiquitinem

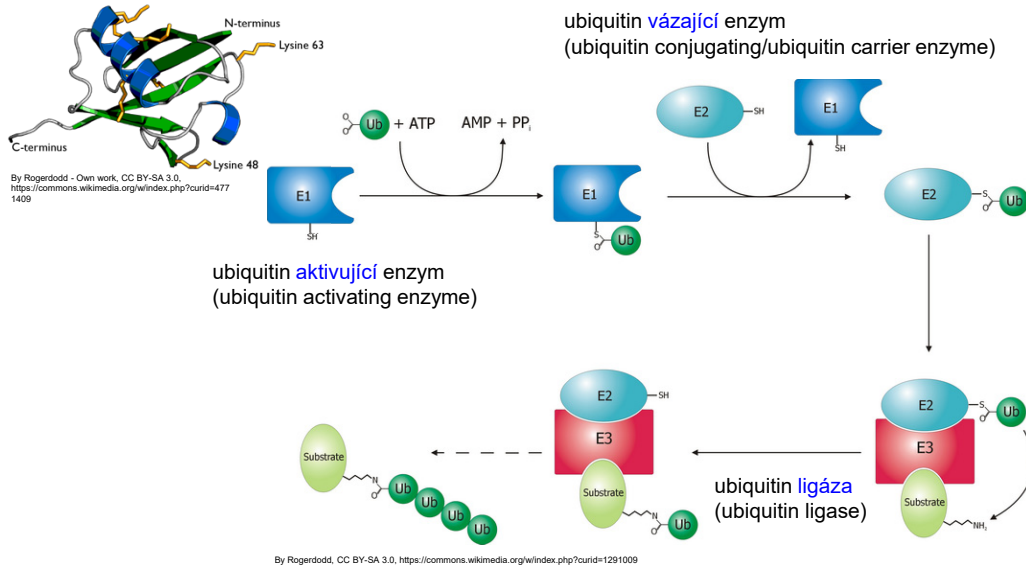


20S & 26S PROTEASOME

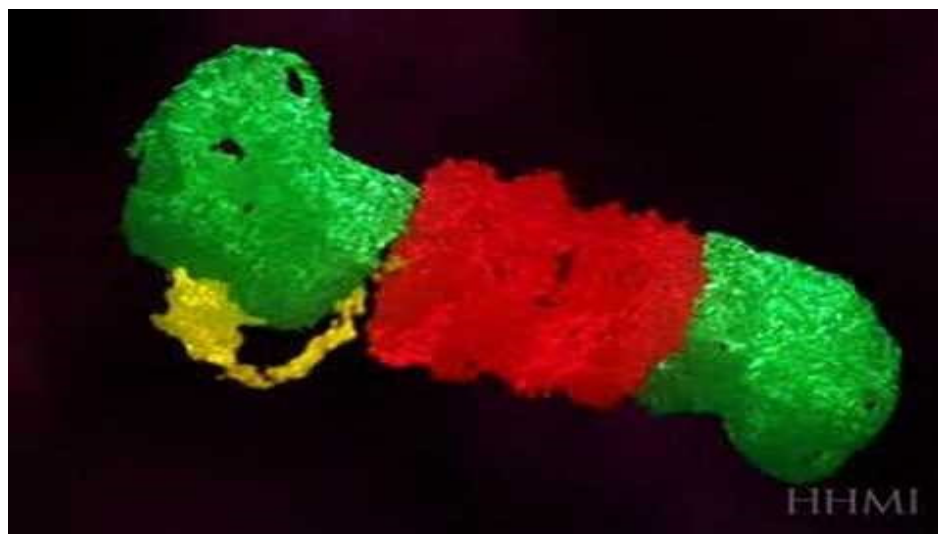


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Ubiquitinace proteinů „polibek smrti“

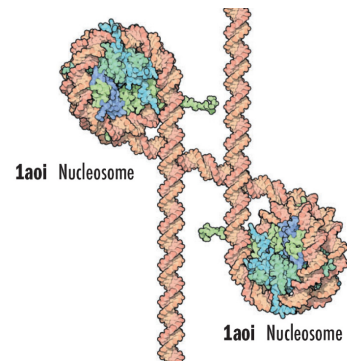


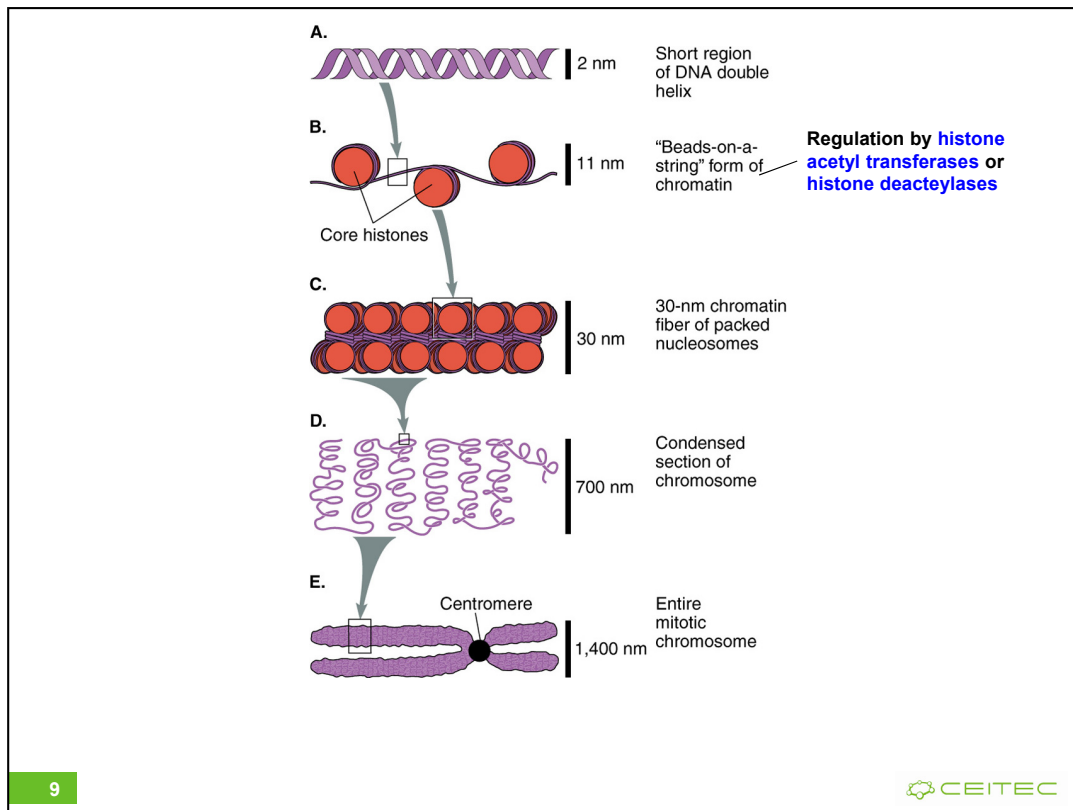
Proteasome –řízená proteolýza



Význam PI

- Funkční význam specifických interakcí proteinů
- Struktura chromatinu

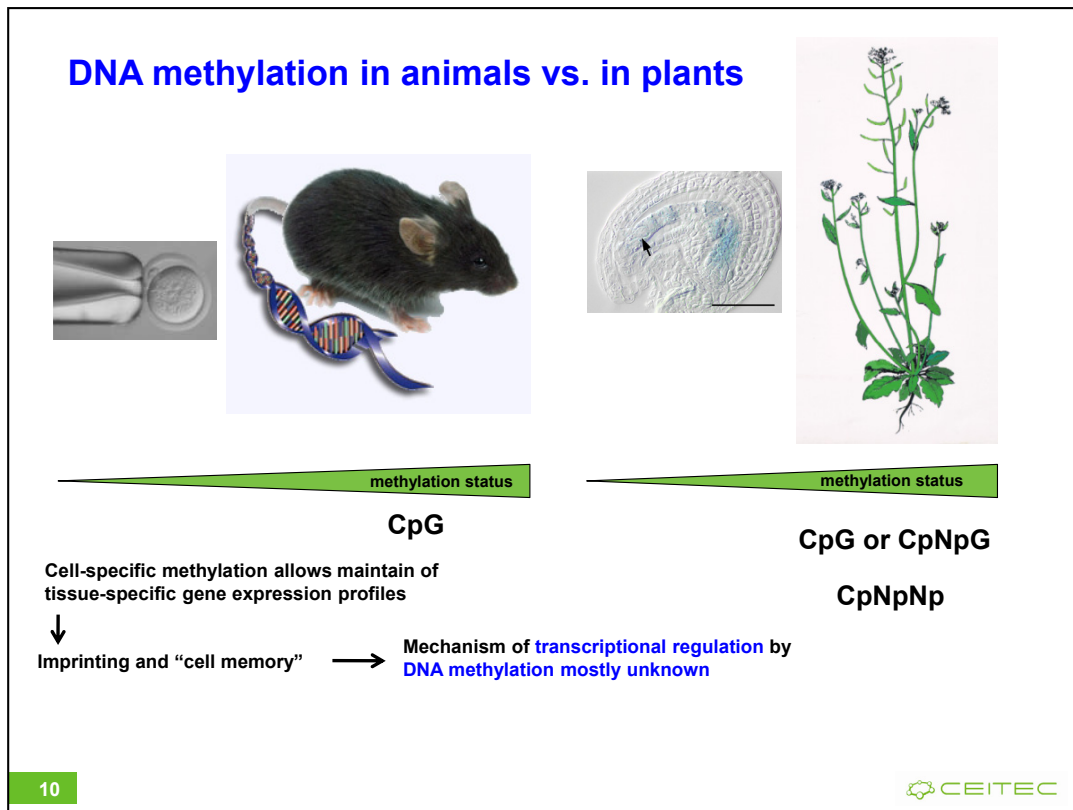




Regulation of the chromatin structure represents one of the very basal gene expression regulatory levels. Chromatin is a substrate for DNA-dependent RNA polymerases that transcribe the DNA encoded information into the “words and sentences” of RNA.

Regulation of chromatin structure and its accessibility to DNA-dependent RNA polymerases depends on many factors, one of the most important is the regulation of chromatin binding to nucleosomes and chromatin methylation.

Regulation of chromatin interaction with histones, the positively charged proteins forming the core of nucleosomes, is performed via modification of acetylation status of the N-terminal portion of histones, especially histones H3 and H4. This occurs via action of histone acetyl transferases or histone deacetylases.



Modification of the chromatin methylation is performed via DNA methyltransferases.

Interestingly, there is difference in the methylation in animals and in plants.

In animals, the methylation takes place mostly on the cytosine that occurs next to guanosine (the sequence is denoted as CpG). In mammals, 60-90% of all CpGs are methylated.

In plants, cytosines are methylated both symmetrically (CpG or CpNpG) and asymmetrically (CpNpNp), where N is any nucleotide.

Methylation status is usually “reset” in the zygote and is reconstituted during development again. E.g. the methylation is very low in the mouse embryo at the blastula stage, however, DNA derived from later stages when organogenesis is initiated is substantially more modified by methylation.

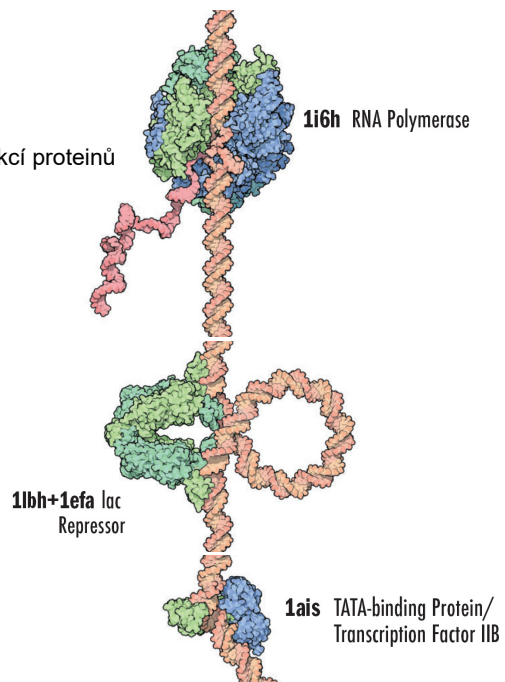
DNA methylation also stably alters the gene expression pattern in cells such that cells can “remember where they have been”; in other words, cells programmed to be pancreatic islets during embryonic development remain pancreatic islets throughout the life of the organism without continuing signals telling them that they need to remain islets.

DNA methylation is involved in the genomic imprinting, i.e. the genes originating from both parents are often diversely methylated, which results into differential expression of parental genomes (for the importance of the imprinting in the parental conflict and epigenetics, see the lecture “Bi0580 Developmental genetics” by prof. Vyskot).

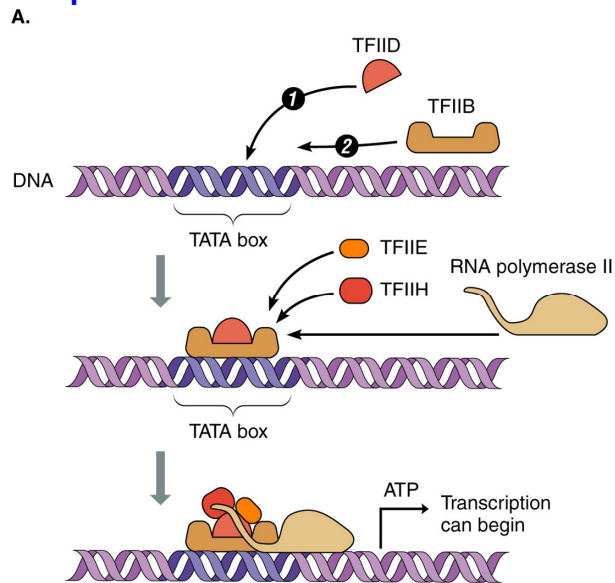
Up to know it is not clear how methylation regulates transcription. Possibly, methylation status affects chromatin configuration or binding general repressor factors.

Význam PI

- Funkční význam specifických interakcí proteinů
 - Struktura chromatinu
 - Regulace transkripce



Iniciace Transkripce

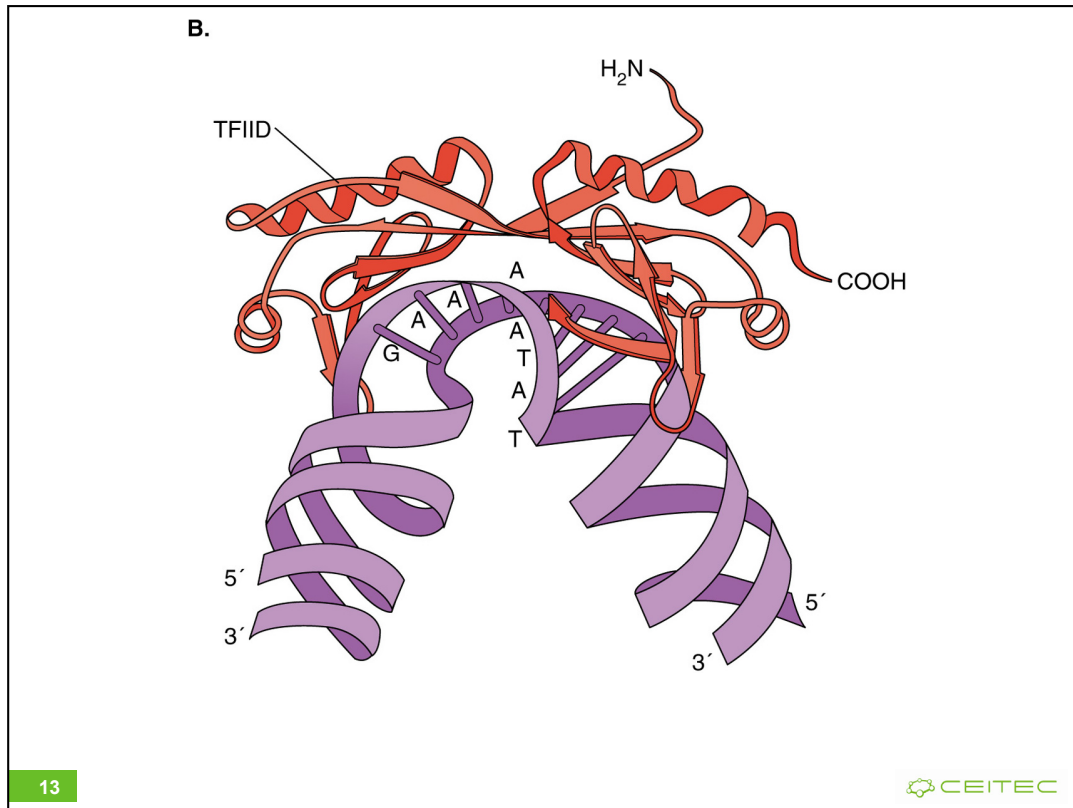


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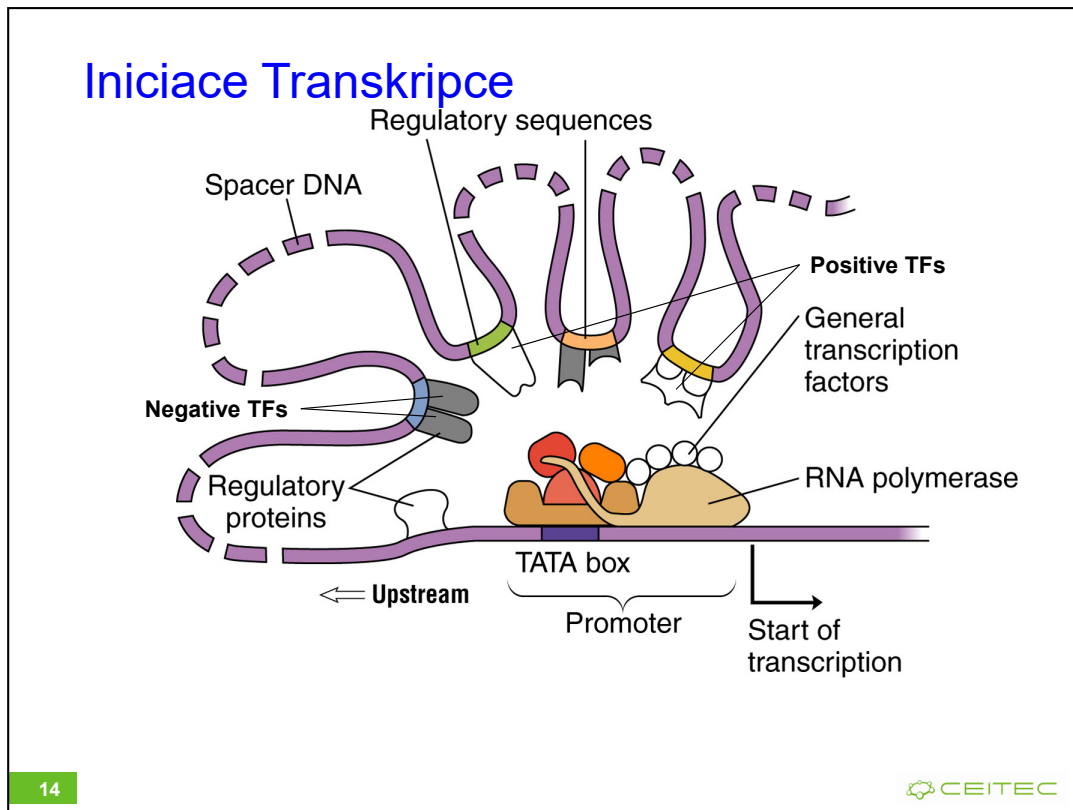
Regulation of transcription occurs via specific interaction of both general and tissue specific transcription factors (TFs) with promoter and/or enhancer sequences.

The scheme above shows simplified subsequent formation of the complex of TFs involved in the regulation of transcription. Interaction of general TFIID with the TATA box induces distortion of the DNA structure (see the next slide).



Induction of structural changes upon interaction of TFIID with DNA. This may be important for the assembly of other TFs involved in the formation of transcription initiation complex.

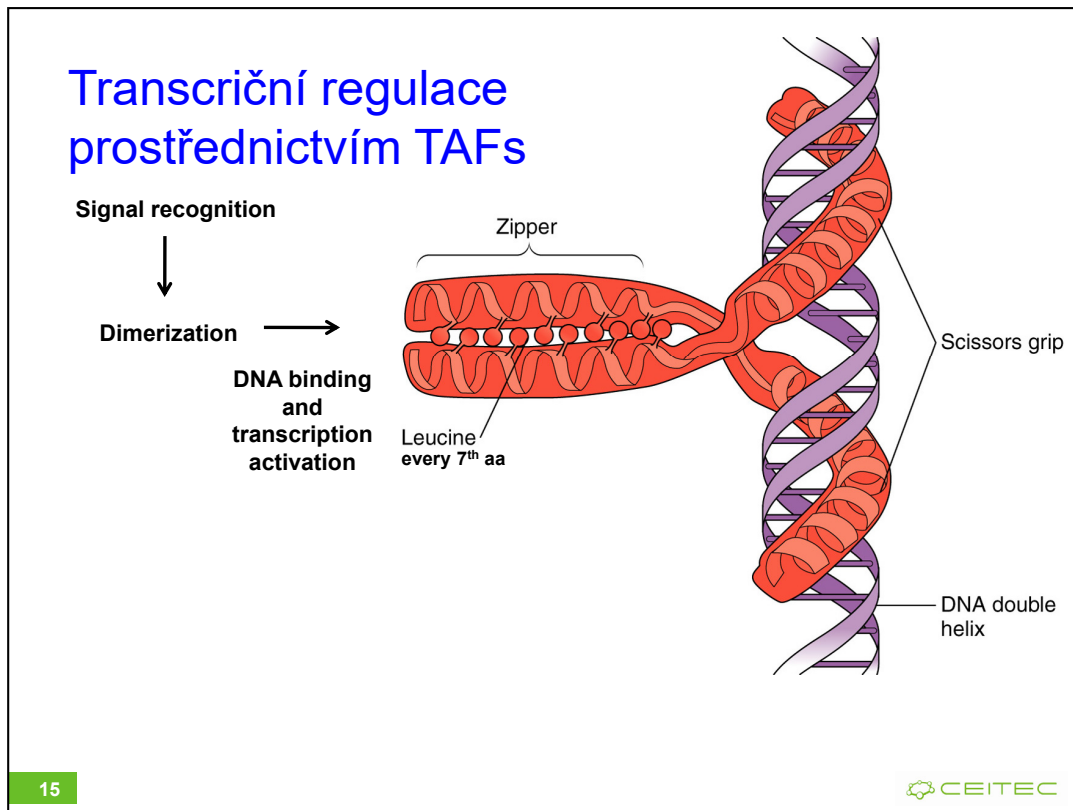
This change of conformation provides a kind of “signature” that is recognized by other proteins and NA polymerase to recognize the proper binding site. However, there are also TATA box-less promoter, where probably other types of “signatures” occur.



The scheme showing the formation of the transcription initiation complex and the interaction of both positive (open symbols) and negative (solid symbols) factors.

These proteins bind to the regulatory sequences that might be hundreds or even thousands of base pairs away from the promoter. These proteins interact with each other and with the RNA polymerase, integrating thus many signals into a “yes” or “no” response of the basal promoter, i.e. the region adjacent to the TATA box and recognized by the RNA polymerase.

The individual positive or negative factors are complex and their activity might be regulated by their phosphorylation status or via their interaction with other proteins (i.e. monomeric or dimeric) etc..



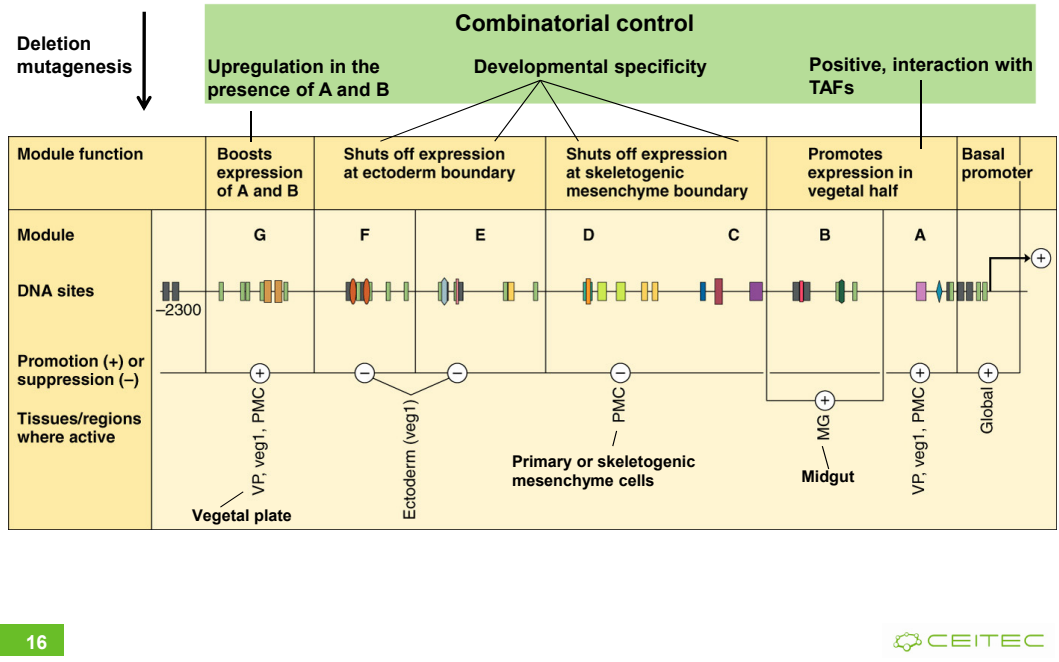
There is a whole family of transcription activating factors (TAFs) that interact with signalling molecules, e.g. steroid hormones, thyroid hormones or retinoic acid and in a response to the signal transfer to the nucleus where they regulate transcription.

One of the type of TAF are leucine zipper or bZIP type TAFs. These TAFs are dimeric, with leucine-rich hydrophobic face formed by the Leu that occurs every 7th aa.

That allows the factor to take the proper configuration, which provides the dimer with the ability to bind DNA via charged aa.

Multifaktoriální kontrola promotorů

*Pro*ENDO16:REPORTER (sea urchin)



An example of the “microprocessor”-like acting promoter is a promoter of the *endo16* gene from the sea urchin.

There have been identified several gene regulatory modules in the *endo16* gene that have positive or negative regulatory role. These modules were identified via formation of deletion mutants of the transcriptional fusions with reporter gene.

The analysis has revealed that the module A has a positive function and must interact with its cognate TAFs for transcription to occur.

Module G enhances the expression when the A and B are active.

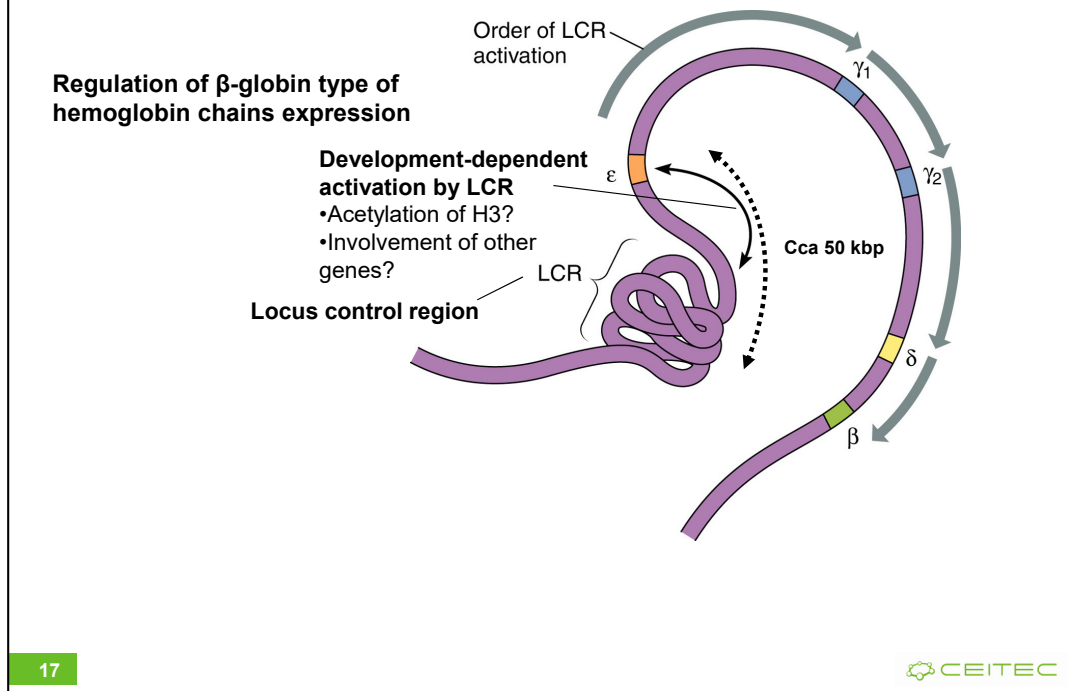
C, D, E and F are responsible for the specificity of the expression of *endo16* during sea urchin development.

Each of the modules has several protein interaction sites, some of them general, other unique. Site for the protein SpGCF1 is present in many modules and is probably responsible for looping of chromatin, allowing thus bringing of distal regulatory modules close to the basal promoter.

This type of regulation, i.e. based on the different activities of diverse regulatory sequences is sometimes called *combinatorial* and is common for development of many living creatures.

In the combinatorial type of regulations, some modules may act synergistically, some of them antagonistically, some may have both positive and negative roles (e.g. the module B, see the figure). This variability allows very precise and responsive regulations towards changing environmental conditions.

Multifaktoriální kontrola promotorů



An example of the combinatorial gene regulation is the regulation of β -globin type of hemoglobin chains of humans.

As discussed in the Lesson 5 (course Bi8940 Developmental biology), the type of hemoglobin produced by the fetus changes during development. The hemoglobin present in the erythrocytes is composed of two α - and two β -type chains. The β -type hemoglobin chains are of several developmental types, produced by ϵ , γ_1 , γ_2 and β (in this order). In addition, there is minor adult type of β -type hemoglobin, called δ globin.

The genes for the β -type chains are aligned on the chromosome in the order, in which they are expressed during development (see the figure).

For the expression of individual cell types is distinctive an upstream regulatory sequence called locus control region (LCR). LCR is located about 50 kbp away from the most proximal ϵ gene.

The LCR structure is different in erythrocyte precursor cells in comparison to other cells that could be demonstrated by the changes in the sensitivity to low concentrations of DNase, suggesting low amount of nucleosomes bound.

For the expression of the particular genes, the interaction of their regulatory sequences with LCRs is necessary. Because of LCR can interact only with one regulatory sequence at a time, only one type of genes for the particular β -type chain is activated (the first interaction of LCR with ϵ gene, which is later in development replaced by the other one, is shown by the double-headed arrow).

The underlying molecular mechanisms of the specific pattern of the LCR movement from the most proximal towards the most distal gene cannot be satisfactory explained.

Probably, acetylation of H3 histones might play a role and possibly, other genes outside of the β -type chain family are involved in the regulation of LCR activity. That seems to be confirmed by the identification of other human genes with similar structure, suggesting common regulatory mechanisms via LCRs.

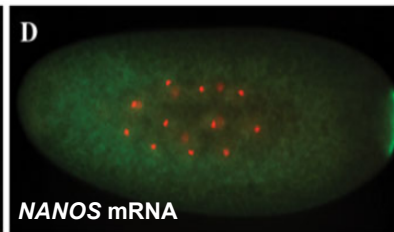
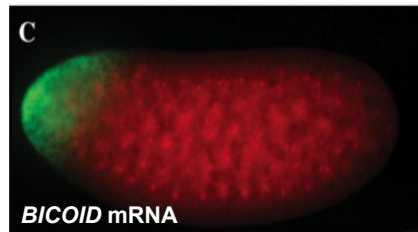
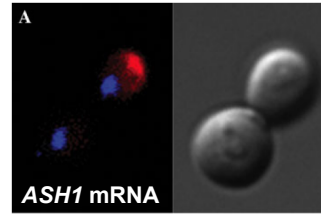
For

Význam PI

- Funkční význam specifických interakcí proteinů
 - Struktura chromatinu
 - Regulace transkripce
 - Lokalizace mRNA

Lokalizace mRNA

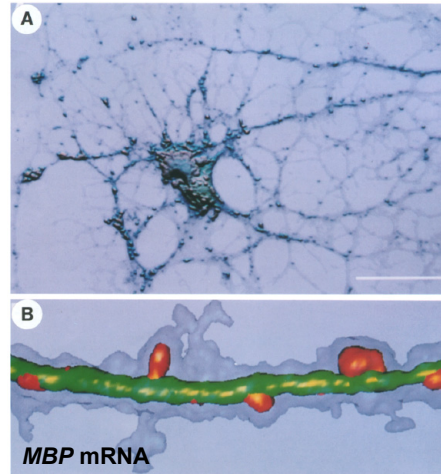
- Význam lokalizace mRNA
 - Lokalizace proteinového produktu genu v čase a místě
 - asymetrické dělení během vývoje
 - polarizace embrya



Shahbadian and Chartrand, 2012

Lokalizace mRNA

- **Role lokalizace mRNA**
 - Omezení exprese potenciálně toxických proteinů
 - lokalizace exprese mRNA pro MYELIN BASIC PROTEIN (MBP) do oblasti myelinizace nervových buněk



Ainger et al., 1993

Myelin basic protein (MBP) is a protein believed to be important in the process of myelination of nerves in the nervous system.

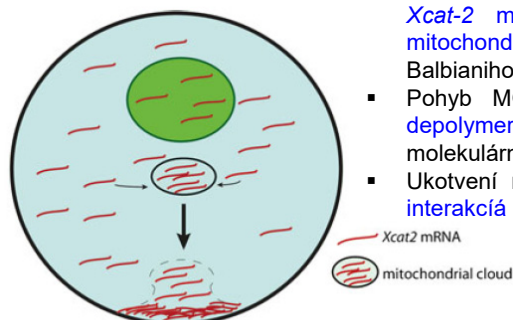
The images show localization of mRNA for MBP. Digoxigenin-labeled MBP RNA was microinjected into mouse oligodendrocytes growing in primary culture. The injected RNA appeared as small granules which were present throughout the cytoplasm and processes, and was also found dispersed in the peripheral membranes of the cell.

To analyze the three dimensional distribution of microinjected labeled MBP mRNA throughout the cell, consecutive optical sections through a single oligodendrocyte were collected, reconstructed, and visualized using volume rendering (Fig. A) or isosurface rendering (Fig. B) techniques. An oligodendrocyte microinjected with MBP mRNA, visualized by volume rendering is shown in Fig. A. RNA granules were observed throughout the perikaryon and in some, but not all, processes. The granules in the perikaryon and in the processes appeared to be equivalent in size. In some regions the granules in the processes were aligned in tracks. Although not apparent from this image, the nucleus was devoid of granules.

Lokalizace mRNA

Mechanizmy

▪ Difúze a ukotvení mRNA



Shahbadian and Chartrand, 2012

- Během ranné oogeneze u drápatky je **Xcat-2 mRNA** lokalizována do tzv. **mitochondriálního oblaku** (MO, Balbianiho tělísko)
- Pohyb MO je částečně závislý na **depolymerizaci mikrotubulů** (tzv. molekulární motor)
- Ukotvení na vegetálním pólu je dáno **interakcí MO s ER**

Another well studied example of the diffusion-entrapment mechanism is the *Xenopus* Xcat-2 mRNA, which encodes a Nos related zinc-finger RNA-binding protein.

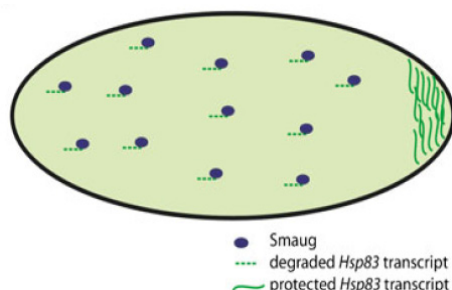
During the early stages of *Xenopus* oogenesis, Xcat-2 mRNA is restricted to a specific structure in the cytoplasm called the mitochondrial cloud (MC). The mitochondrial cloud, also called Balbiani body, consists mostly of mitochondria and small vesicles, and is the source of germinal granule material [32]. The movement of the MC in the cytoplasm results in the localization of the Xcat-2 mRNA at the vegetal cortex (Shahbadian and Chartrand, 2012).

Lokalizace mRNA

Mechanismy

▪ Lokalizovaná degradace mRNA

- V embryogenezi u *Drosophila m.* dochází k **polární lokalizaci Hsp83 mRNA**, podobně jako *NANOS* mRNA
- *Hsp83* mRNA je lokalizována v celém embryu, zde je však **destabilizována prostřednictvím cis elementů** jak v 3'UTR (HDE), tak v kódující oblasti (HIE)
- **HIE elementy** jsou rozpoznávány proteinem **SMAUG**, který zprostředkovává vazbu **degradačního komplexu CCR4/POP2/NOT**
- V oblasti **posteriorního pólu** je *Hsp83* mRNA chráněna před účinkem SMAUG tzv. **HPE elementem v 3'UTR**; mechanismus této ochrany je dosud neznámý



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Localized stabilization of a transcript is another mechanism by which an mRNA can be subcellularly targeted. In this case, an mRNA is rapidly degraded in most parts of the cell, but it is protected from degradation at a specific location. The *hsp83* mRNA, which encodes a heat shock protein in *Drosophila*, is a well-characterized example of this kind of localization (Fig. 2b). This transcript is localized at the posterior pole of the early *Drosophila* embryo by the selective stabilization of the mRNA at the posterior pole and degradation of the transcript elsewhere in the cytoplasm.

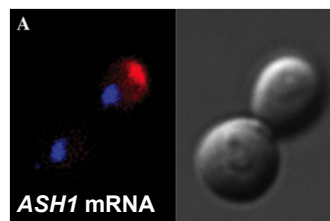
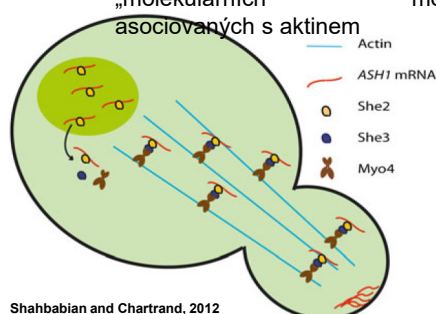
The level of *hsp83* mRNA, which is a maternally encoded transcript, decreases more rapidly in embryos than in unfertilized eggs, which suggests that two separate mechanisms control the stability of this transcript [38]. These two independent pathways, which are called “maternal” and “zygotic” pathways, use maternally and embryonic encoded proteins, respectively, to degrade the *hsp83* transcript [38]. By analyzing the 3'UTR of *hsp83* mRNA, a region from nucleotides 253–349 was identified as the *Hsp83* degradation element (HDE), which directs the destabilization of this mRNA in unfertilized eggs. However, this region has no effect in the zygotic degradation pathway, and transcripts without the HDE domain are subject to degradation by the embryonic degradation machinery [38]. The *hsp83* ORF has also been shown to affect the stability of the transcript. A region at the 3' end of the ORF, which comprises 615 nucleotides, has been found to be responsible for this destabilization, and was consequently called *Hsp38* instability element (HIE) [39]. This region, which has the major effect in the destabilization of the transcript, functions together with the HDE for complete degradation. The HIE domain contains six stem-loop structures that are recognized by the maternally encoded RNA-binding protein Smaug [39, 40]. It was shown that in Smaug mutants, degradation and thus localization of *hsp83* mRNA are impaired. Smaug recruits the CCR4/POP2/NOT deadenylase complex, triggering deadenylation and thus degradation of the *hsp83* transcript [40]. Although Smaug is present throughout the pole plasm, the *hsp83* mRNA is protected from Smaug action at the posterior pole. This protection is related to a 57 nt region in the 3'UTR (nucleotides 351–407) downstream of HDE, which is called HPE (*Hsp83* protection element). HPE is sufficient to confer stability to an unstable transcript at the pole plasm [40]. The mechanism by which this domain functions is not clear, and may include interaction of trans-acting factors that block the availability of the transcript to Smaug (Shahbadian and Chartrand, 2012).

Lokalizace mRNA

Mechanismy

▪ Aktivní transport mRNA

- Asymmetric Synthesis of HO1 (ASH1) je represor *HO* u *S. cerevisiae*; inhibice *HO* endonukleázy v dceřinných buňkách zabraňuje změně párovacího typu
- *ASH1* mRNA je aktivně transportována prostřednictvím „molekulárních motorů“



Shahbadian and Chartrand, 2012

- *ASH1* mRNA obsahuje 4 cis elementy (3 v CDS a 1 ve 3'UTR), které jsou rozpoznávány RNA vazebným proteinem SHE2
- SHE2 umožňuje prostřednictvím SHE3 vazbu na „molekulární motor“, MYO4, který se váže na aktin a umožňuje transport *ASH1* mRNA do dceřinné buňky

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Shahbadian and Chartrand, 2012

Localization of *ASH1* mRNA is essential for the asymmetric distribution of *Ash1*, which acts as a transcriptional repressor of the *HO* endonuclease and results in inhibition of mating-type switching in daughter cells [88, 89]. The *ASH1* mRNA contains four localization elements, three in the coding sequence (E1, E2A, and E2B) and one overlapping the end of the coding sequence and the 3'UTR (E3) [25, 90]. While the presence of these four elements leads to an optimal localization, deletion analysis revealed that each element is sufficient for localization of a reporter mRNA to the bud.

When each of these elements was inserted in multiple copies in the 3'UTR, the new constructs showed nearly normal localization. However, for these mRNAs, the asymmetric distribution of *Ash1* was impaired, suggesting that the position of these elements is important for *Ash1* sorting but not for *ASH1* mRNA localization [91]. Although the primary sequences of the four *ASH1* localization elements are different, they all fold into a stem-loop structure that contains a few conserved nucleotides [92, 93]. All four elements interact with the same RNA binding protein called *She2*, which is involved in the localization of bud-localized mRNAs in *S. cerevisiae*.

She2 forms a tetramer under physiological conditions, and mutations that disrupt this tetrameric state abolish its RNA-binding capacity and impair *She2*-dependent localization to the bud tip [94]. *She2* interacts directly with the C-terminal domain of *She3*, an adaptor protein that links the *She2*-mRNA complex to the molecular motor *Myo4* (Fig. 2c) [55, 95, 96]. Recent evidence also suggests that *She3*, besides its role in connecting the *She2*-RNA complex to *Myo4*, is itself able to bind RNA and acts synergistically with *She2* to increase the affinity and specificity of RNA binding [97].

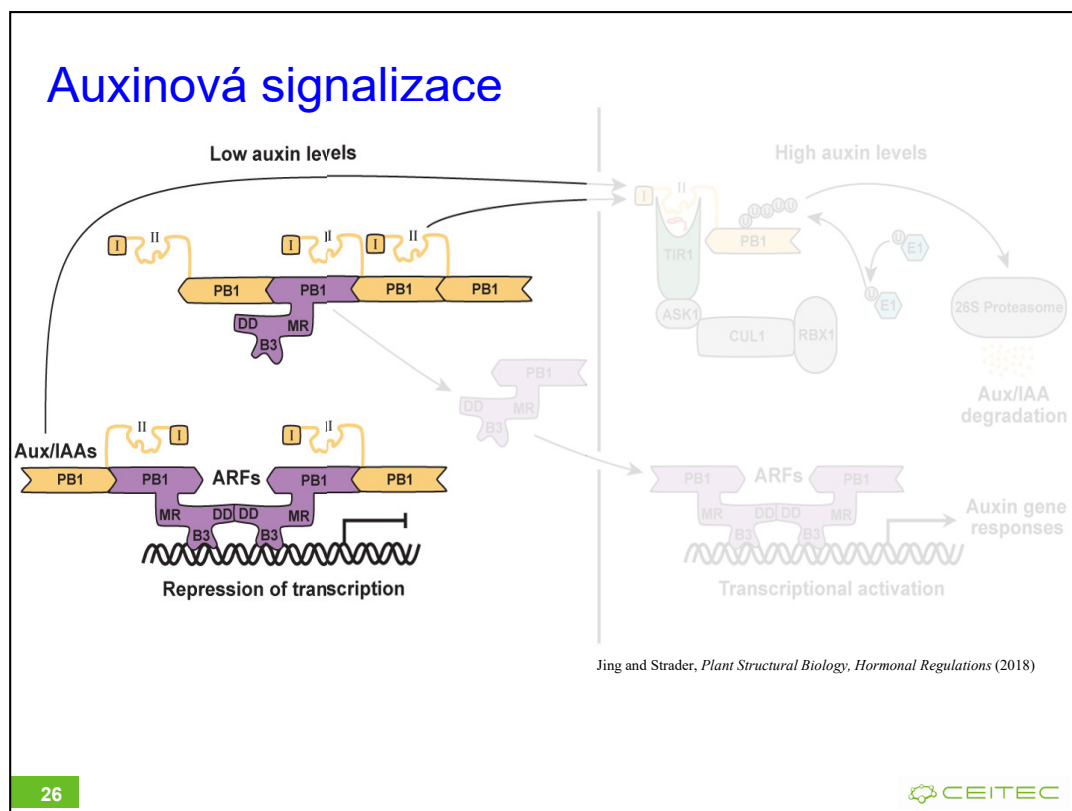
Recent studies on *Myo4* helped to explain why multiple localization elements are required for proper *ASH1* mRNA localization. *Myo4* is a class V myosin whose main function is the transport of mRNAs to the bud tip using actin filaments [98–100]. *Myo4*, unlike other type V myosins, is a nonprocessive monomer *in vivo*, but it becomes processive when present in the form of oligomers [101, 102]. Purification of the localization complex associated with a single localization element revealed that multiple copies of *Myo4* are associated with this RNA [103]. Moreover, increasing the number of *Myo4* attached to the *ASH1* mRNA increased the efficiency of localization of this transcript. These results suggest that each localization element interacts with higher order protein complexes in which a *She2* tetramer may recruit multiple copies of *Myo4*, thus ensuring a continuous and processive movement of the mRNP complex into the bud. Moreover, it is possible that a *She2* tetramer binds simultaneously to the localization elements of a single transcript or, alternatively, to those of different mRNAs. This would bring multiple mRNAs together within a single complex in which several *Myo4* molecules modulate their transport to the bud tip (Shahbadian and Chartrand, 2012).

Význam PI

- Funkční význam specifických interakcí proteinů
 - Struktura chromatinu
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 - Lokalizace mRNA
 - Sestřih hnRNA

Význam PI

- Funkční význam specifických interakcí proteinů
 - Struktura chromatinu
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 - Lokalizace mRNA
 - Sestřih hnRNA
 - **Stabilita proteinů**



Scheme of the auxin signaling pathway as an example of the role of protein stabilization leading to regulation of gene expression.

Under low intracellular auxin concentrations, the transcription activators of auxin-regulated genes, which are called auxin responsive factors (ARFs), are in a complex with negative regulators of transcription, so called AUX/IAA proteins. In the complex, ARFs can not activate transcription.

After auxin is imported into the cell, it binds to the TIR1 protein, that allows interaction with AUX/IAA-ARF complex and targets AUX/IAA protein for the degradation via proteasome.

That allows ARFs to enter nucleus and activate transcription of auxin-induced genes.

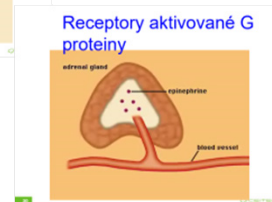
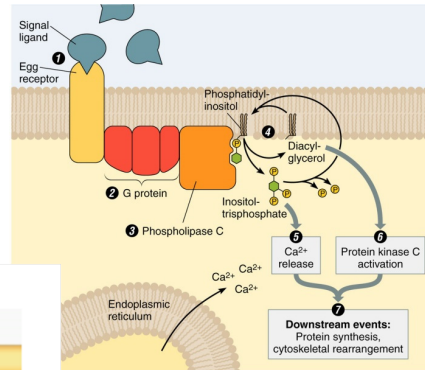
Význam PI

- Funkční význam specifických interakcí proteinů
 - Struktura chromatinu
 - Regulace transkripce
 - Lokalizace mRNA
 - Sestřih hnRNA
 - Stabilita proteinů
 - Přenos signálu

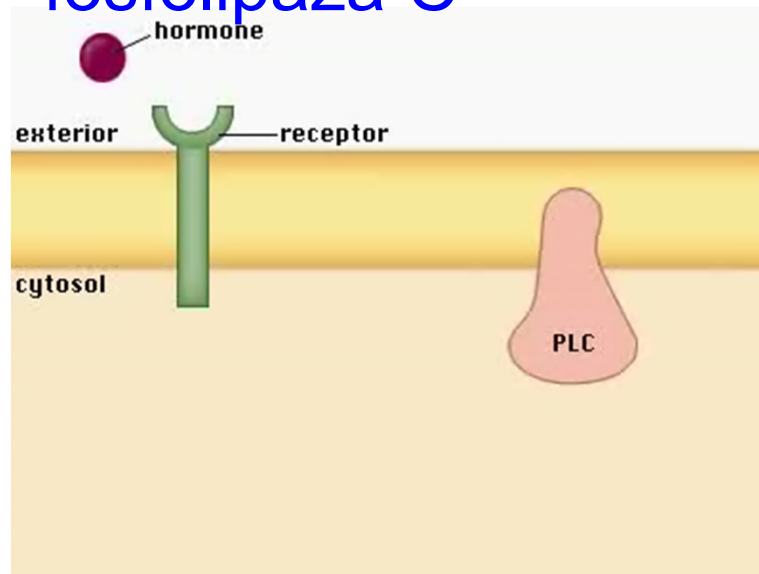
PI a přenos signálu

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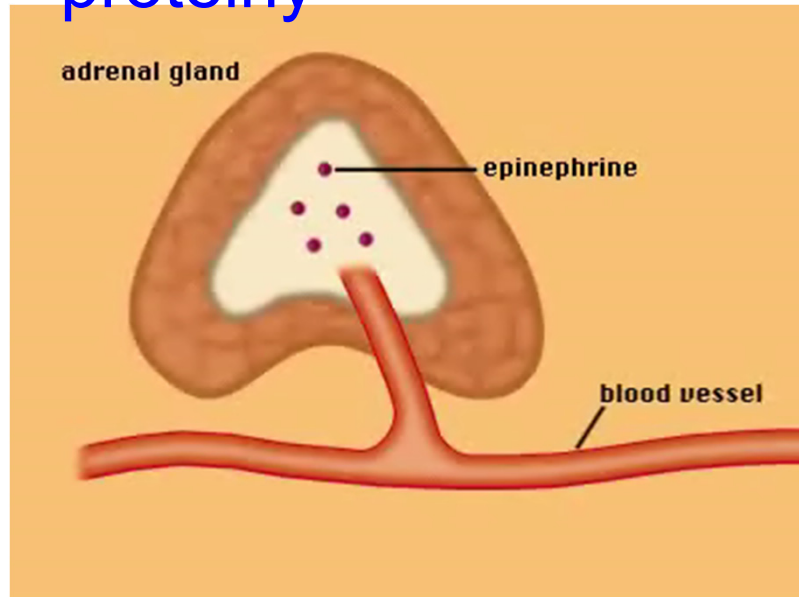
- prostřednictvím G proteinu a fosfolipasy C
- Signální kaskády využívající cAMP



Přenos signálu a fosfolipáza C



Receptory aktivované G proteiny



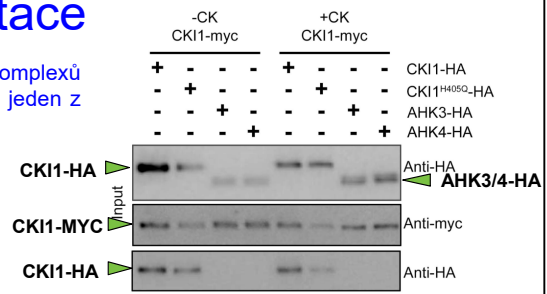
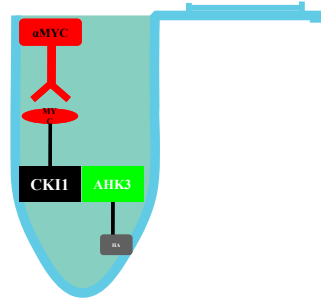
Osnova

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 - Struktura chromatinu
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 - Koimunoprecipitace

PI *in vivo*

Koimunoprecipitace

- založena na izolaci **proteinových komplexů** pomocí **protilátek** rozpoznávajících **jeden z interagujících proteinů**



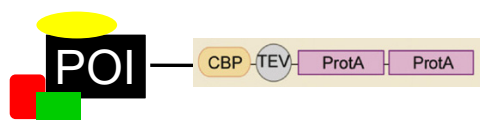
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 - Tandemová afinitní purifikace (TAP-tag)

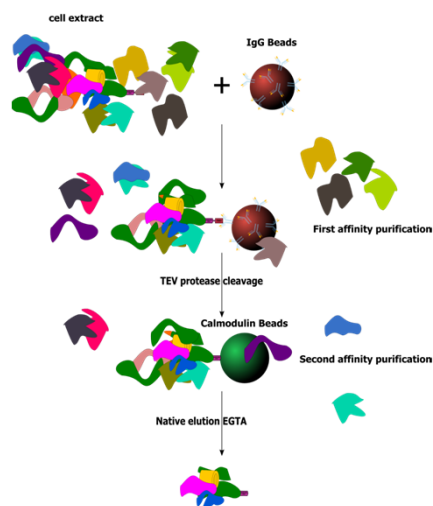
PI *in vivo*

Tandemová afinitní purifikace (TAP-tag)

- izolace proteinových komplexů pomocí rekombinantních proteinů, fúzovaných s dvěma různými vazebnými doménami



- calmodulin-binding protein (CBP)
- IgG vazající domény proteinu A (ProtA)
- místo rozpoznávané specifickou proteázou z TEV viru (tobacco etch virus)
- proteiny izolovaných komplexů jsou po rozdělení na 1D ELFO identifikovány pomocí MS
- výhodou je použití dvou nezávislých proteinových domén pro afinitní purifikaci a tedy velká specifita



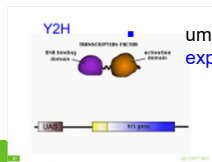
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 - Kvasinkový dvouhybridní test (Y2H)

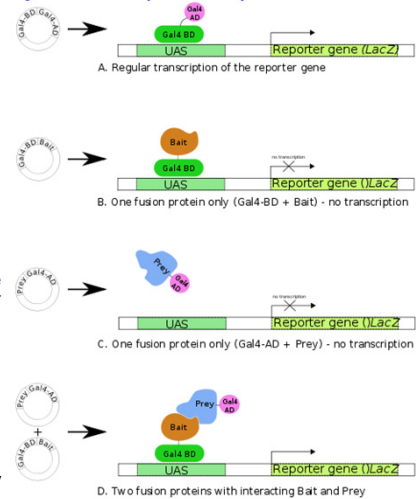
PI *in vivo*

Dvouhybridní kvasinkový test (Y2H)

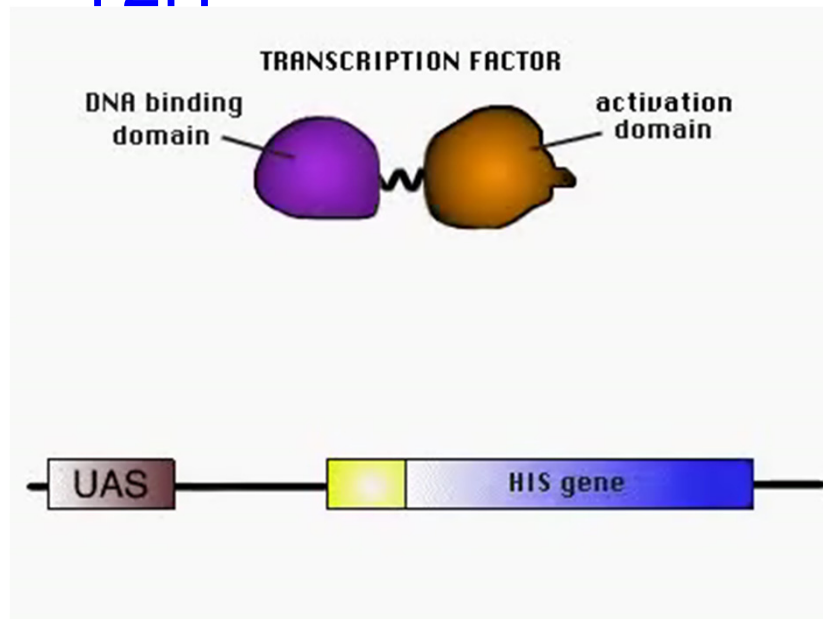
- izolace proteinových komplexů pomocí rekombinantních proteinů, každý z nich fúzovaný s částí transkripčního faktoru Gal4
- jeden z proteinů (návnada, bait) fúzovaný s DNA vazebnou doménou Gal4 (Gal4-BD)
- druhý z proteinů (kořist, prey) fúzovaný s aktivační doménou Gal4 (Gal4-AD)
- Interakce proteinů umožní rekonstituci vazebné domény s aktivační doménou a spuštění reportérového genu
 - vizuální detekce (modré zbarvení, LacZ)
 - auxotrofní selekce (růst na médiu bez histidinu, His)



umožňuje vyhledávání interakčních partnerů v expresních knihovnách jednotlivých organismů



Y2H



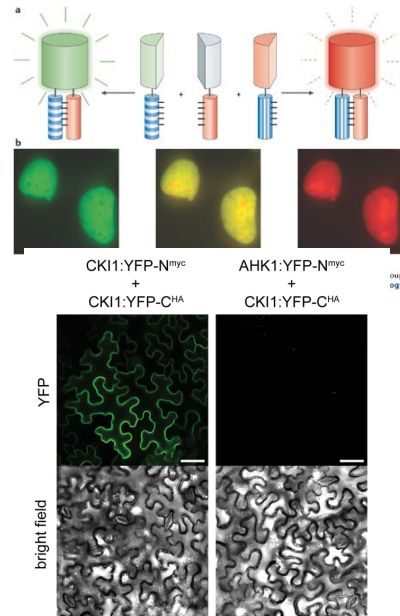
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 - Bimolekulární fluorescenční komplementace (BiFC)

PI *in vivo*

bimolekulární fluorescenční komplementace (BiFC)

- Proteinová interakce je detekována na základě reasociace fluoreskujícího proteinu
- každý z potenciálních interakčních partnerů je fúzován s jednou z podjednotek fluoreskujícího proteinu, např. YFP
- při interakci dojde ke znovuobnovení fluorescence
- Kromě identifikace vlastní interakce umožňuje i lokalizovat interakci v buňce



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 - Analýza zprostředkované membránové vazby (MeRA)

PI *in vivo*

Analýza zprostředkované membránové vazby (MeRA)

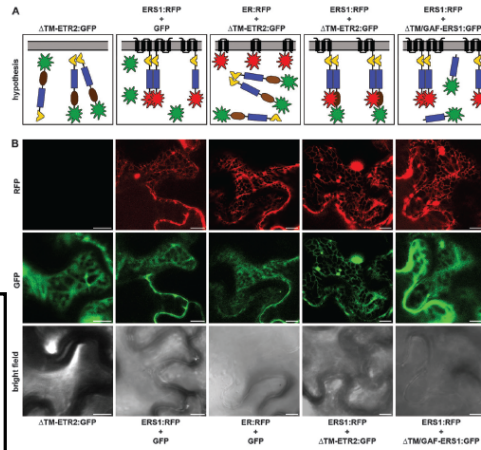
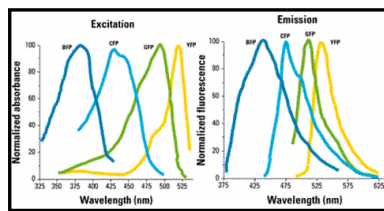
- Umožňuje identifikaci interakcí cytoplazmatických proteinů s membránovými proteiny



membránový protein je fúzován s fluoreskujícím proteinem

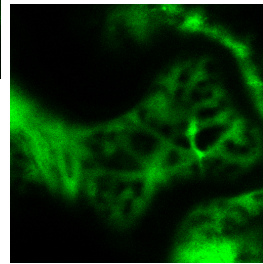
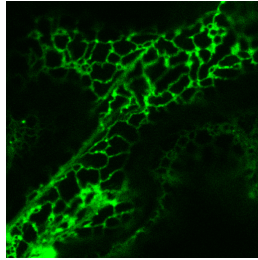
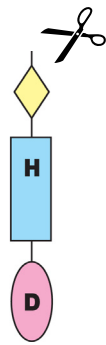
potenciální ineterakční partner je fúzován s jiným fluoreskujícím proteinem, lišícím se svým emisním spektrem

- v případě interakce dojde ke změně lokalizace cytoplazmatického proteinu na membránu (kolokalizaci s membránovým proteinem)



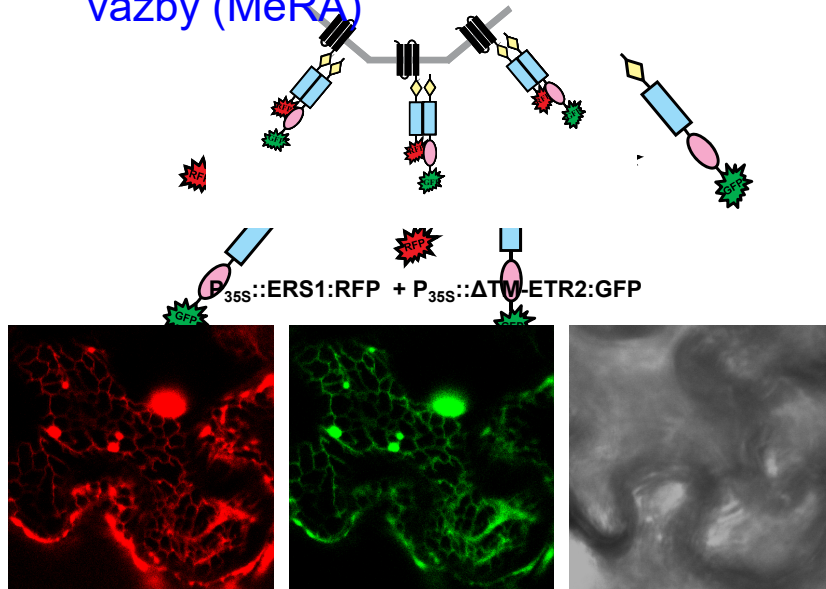
PI *in vivo*

Analýza zprostředkované membránové vazby (MeRA)



PI *in vivo*

Analýza zprostředkované membránové vazby (MeRA)



Klíčové koncepty

- Proteiny a jejich interakce jsou zásadním mechanismem regulace genové exprese
- Podílejí se na regulaci
 - Struktury chromatinu
 - Iniciaci transkripce
 - Lokalizace mRNA
 - Zprostředkovávají regulaci genové exprese v *odpovědi na různé typy signálů*
- Proteinové interakce lze detekovat *in vivo* např. pomocí
 - Koimunoprecipitace
 - Tandemové afinitní purifikace (TAP-tag)
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 - Bimolekulární fluorescenční komplementace (BiFC)
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Diskuse