CG920 Genomics

Lesson 7

Protein Interactions in Gene Regulations

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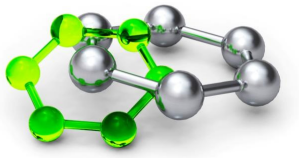

Literature

- Literature sources for Chapter 06:

- Wilt, F.H., and Hake, S. (2004). **Principles of Developmental Biology**. (New York ; London: W. W. Norton).
- Ainger, K., Avossa, D., Morgan, F., Hill, S.J., Barry, C., Barbarese, E., and Carson, J.H. (1993). Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes. J Cell Biol 123, 431-441.
- Alberts, B. (1998). The cell as a collection of protein machines: preparing the next generation of molecular biologists. Cell 92, 291-294.
- Grefen, C., Stadele, K., Ruzicka, K., Obrdlik, P., Harter, K., and Horak, J. (2008). Subcellular localization and in vivo interactions of the Arabidopsis thaliana ethylene receptor family members. Molecular Plant 1, 308-320.
- Hu, C.D., and Kerppola, T.K. (2003). Simultaneous visualization of multiple protein interactions in living cells using multicolor fluorescence complementation analysis. Nat. Biotechnol. 21, 539-545.
- Shahbabian, K., and Chartrand, P. (2012). Control of cytoplasmic mRNA localization. Cellular and molecular life sciences : CMLS 69, 535-552.
- Van Leene, J., Witters, E., Inze, D., and De Jaeger, G. (2008). Boosting tandem affinity purification of plant protein complexes. Trends Plant Sci 13, 517-520.
- Walter, M., Chaban, C., Schutze, K., Batistic, O., Weckermann, K., Nake, C., Blazevic, D., Grefen, C., Schumacher, K., Oecking, C., Harter, K., and Kudla, J. (2004). Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. Plant J 40, 428-438.

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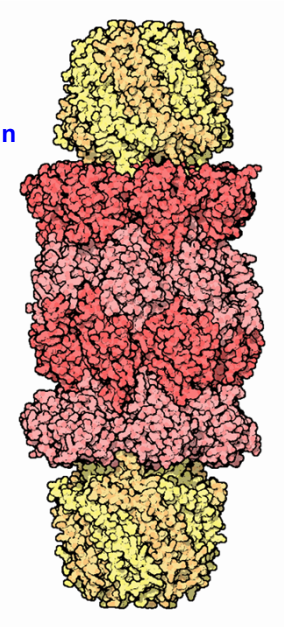
Outline

- Functional importance of the specificic interactions of proteins in the regulation of gene expression
  - Chromatin structure
  - Regulation of transcription
  - mRNA localization
  - Protein stability
  - Signal transduction
- Methods of analysis of protein interactions *in vivo*
  - Co-immunoprecipitation
  - The tandem affinity purification (TAP-tag)
  - Yeast two-hybrid assay (Y2H)
  - Bimolecular fluorescence complementation (BiFC)
  - Membrane Recruitment Assay (MeRA)
- Practical use of methods for *in vivo* studies of protein interactions

Importance of Protein Interactions

- **Functional importance of specific protein interactions**
  - Most of the proteins in the cell exist in the form of complexes which may further interact with each other
    - Proteasome
      - protein complex responsible for the degradation of obsolete proteins in the cell



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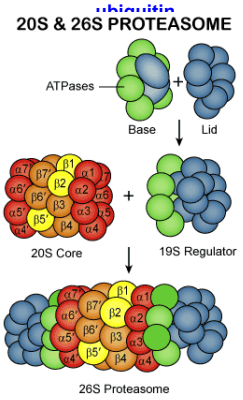
Once obsolete proteins are tagged with at least four ubiquitin molecules, they are destroyed by proteasomes. Proteasomes are voracious protein shredders, but the destructive machinery is carefully protected so that it can't attack all of the normal proteins in the cell. The proteasome, shown here from 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.

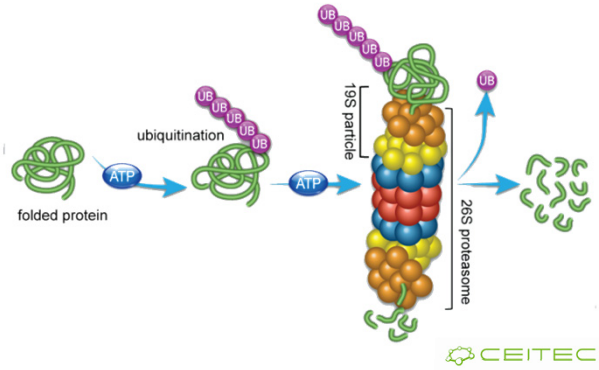
Most of the non-lysosomal proteolysis that occurs in eukaryotic cells is performed by a nonspecific and abundant barrel-shaped complex called the 20S proteasome. Substrates access the active sites, which are sequestered in an internal chamber, by traversing a narrow opening (alpha-annulus) that is blocked 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 (ref. 4), PA28 (ref. 5) and REG) are heptamers that stimulate 20S proteasome peptidase activity in vitro and may facilitate product release in vivo. Here we report the co-crystal structure of yeast 20S proteasome with the 11S regulator from Trypanosoma brucei (PA26). PA26 carboxy-terminal tails provide binding affinity by inserting into pockets on the 20S proteasome, and PA26 activation loops induce conformational changes in alpha-subunits that open the gate separating the proteasome interior from the intracellular environment. The reduction in processivity expected for an open conformation of the exit gate may explain the role of 11S regulators in the production of ligands for major histocompatibility complex class I molecules. (PDB, Whitby et al., (2000) Nature **408:** 115-120, http://www.rcsb.org/pdb/explore/explore.do?structureId=1fnt).

The importance of protein interactions

Proteasome

- Consisting of a **core**, also being designated as **20S** and **regulatory portions** (19 or 11S)
- Allows **targeted degradation** of proteins labelled by a specific marker - small polyppetide (76 aa) called





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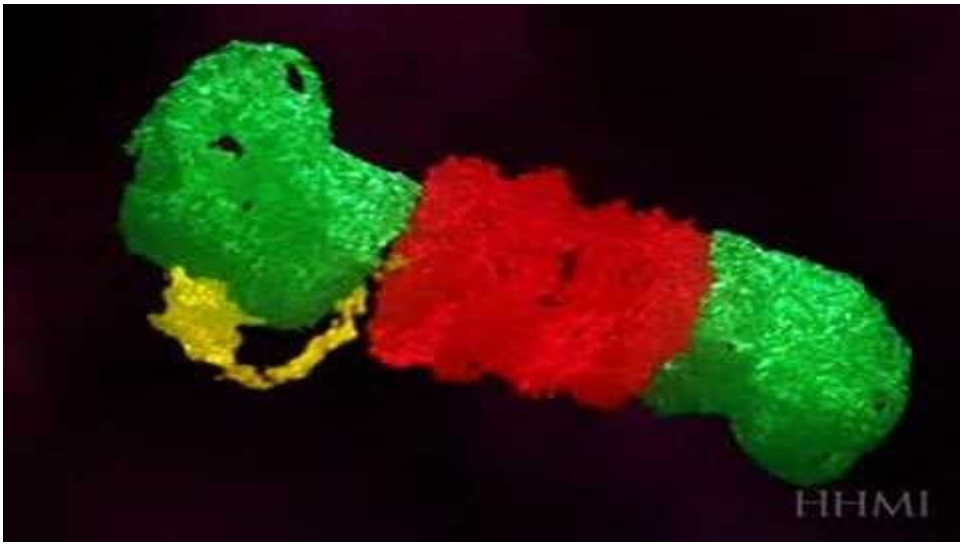
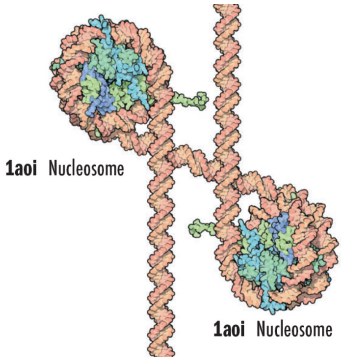
Proteasome –targeted proteolysis



Importance of Protein Interactions

- Functional importance of specific protein interactions
  - Chromatin structure



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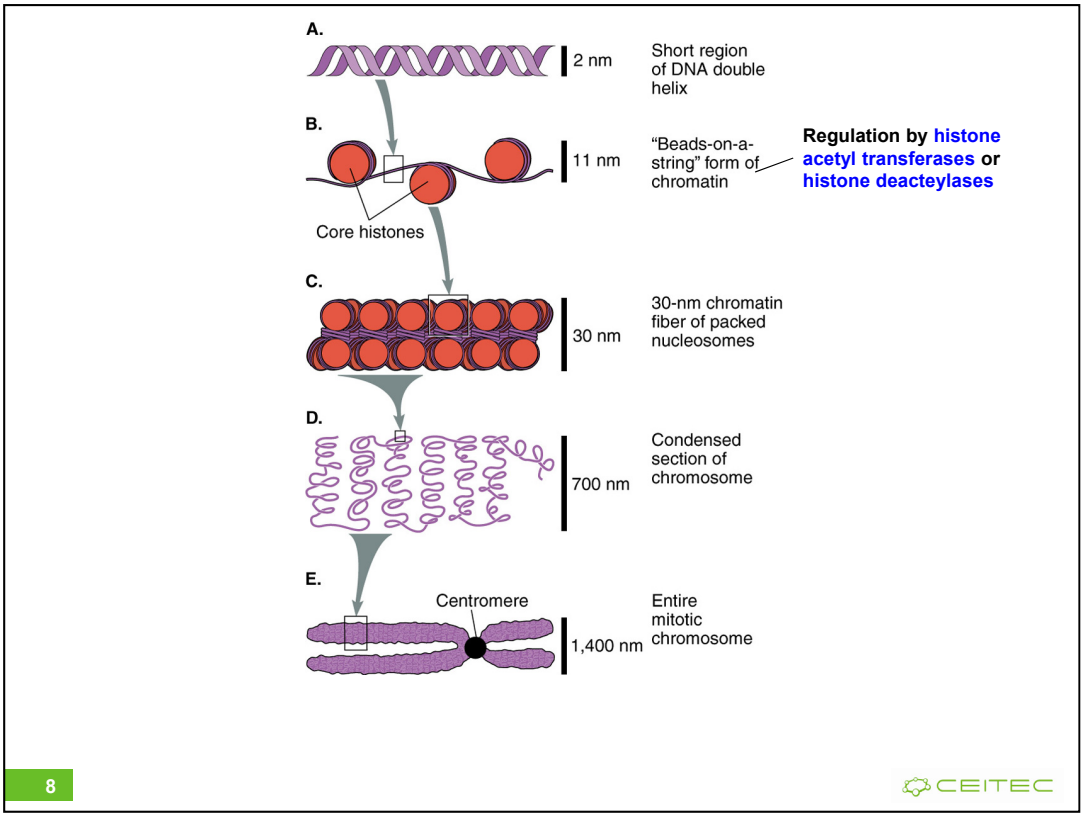
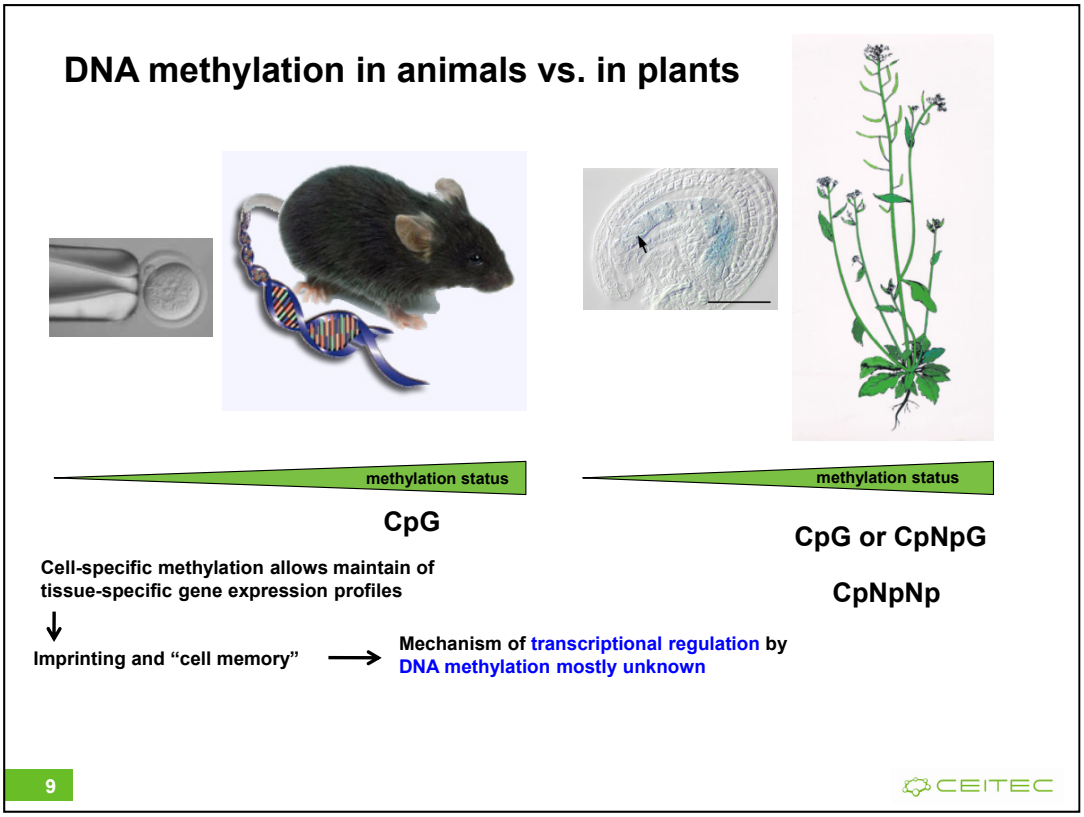


Regulation of the chromatin structure represents one of the very basal gene expression regulatory levels. Chromatin is a substrate for DNA-dependent RNA polymerases that transcript the DNA encoded information into the "words and sentences" of RNA.

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 deacteylases.



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.

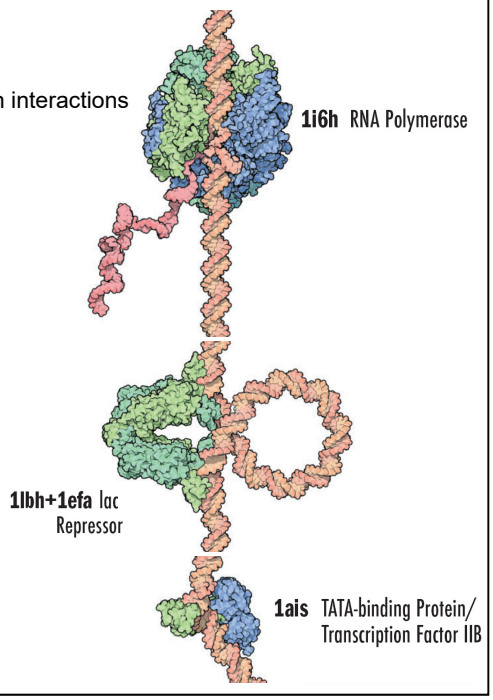
DNA methylation also stably alters the gene expression pattern in cells such that cells can "remember where they have been"; in other words, cells programmed to be pancreatic islets during embryonic development remain pancreatic islets throughout the life of the organism without continuing signals telling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

Importance of Protein Interactions

- Functional importance of specific protein interactions
  - Chromatin structure
  - Regulation of transcription



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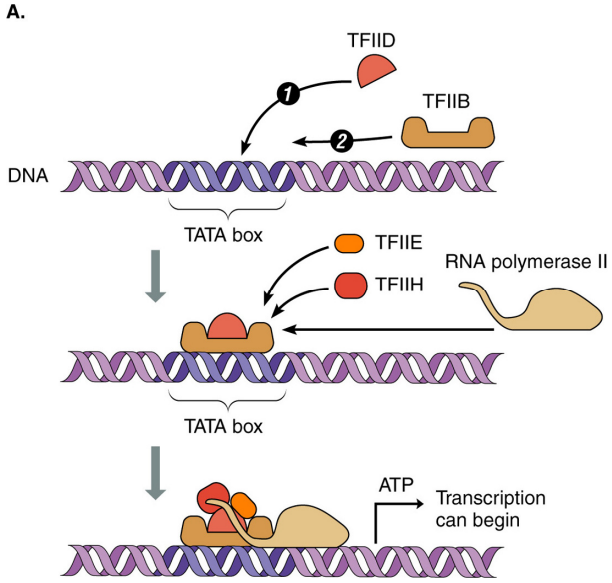
Initiation of Transcription



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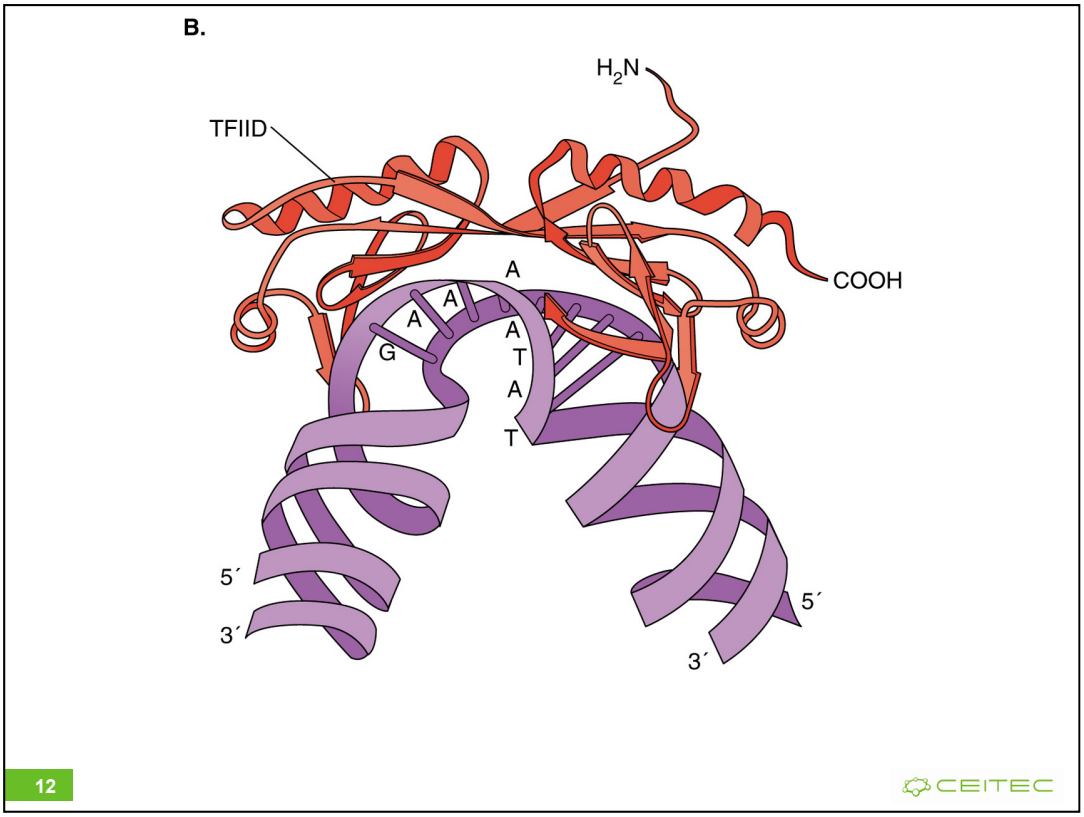
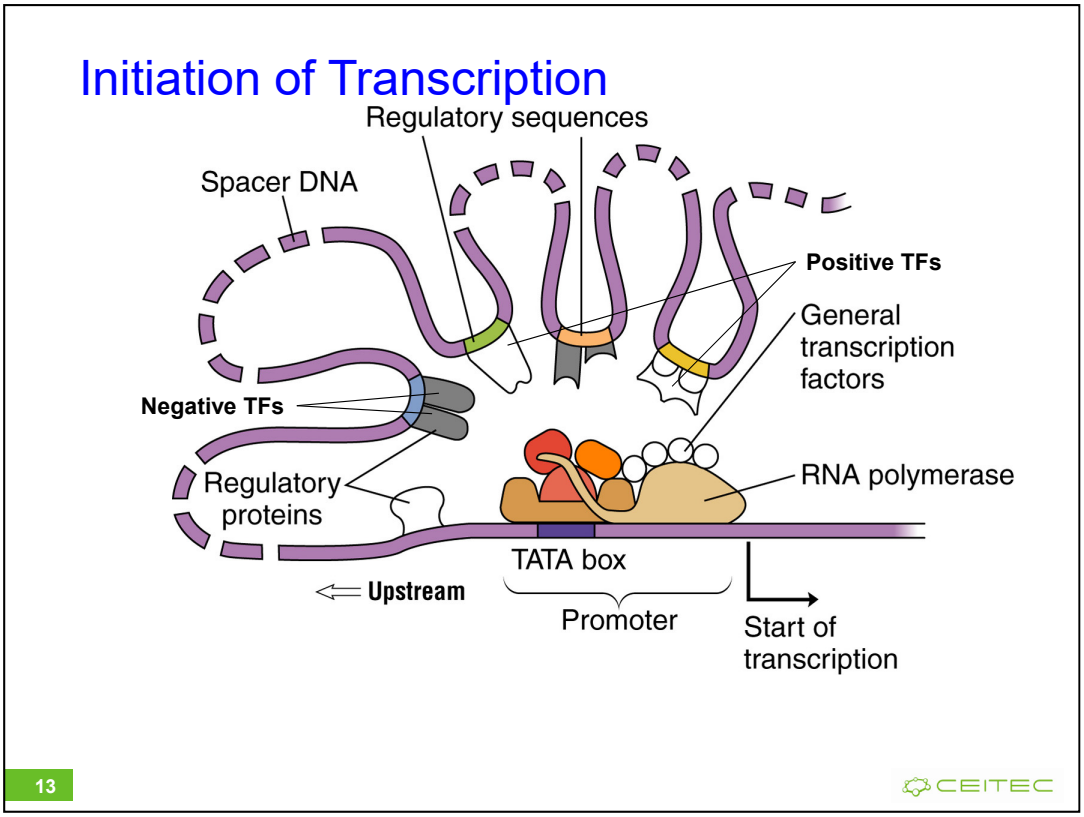
Regulation of transcription occurs via specific interaction of both general and tissue specific transcription factors (TFs) with promoter and/or enhancer sequences.

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.

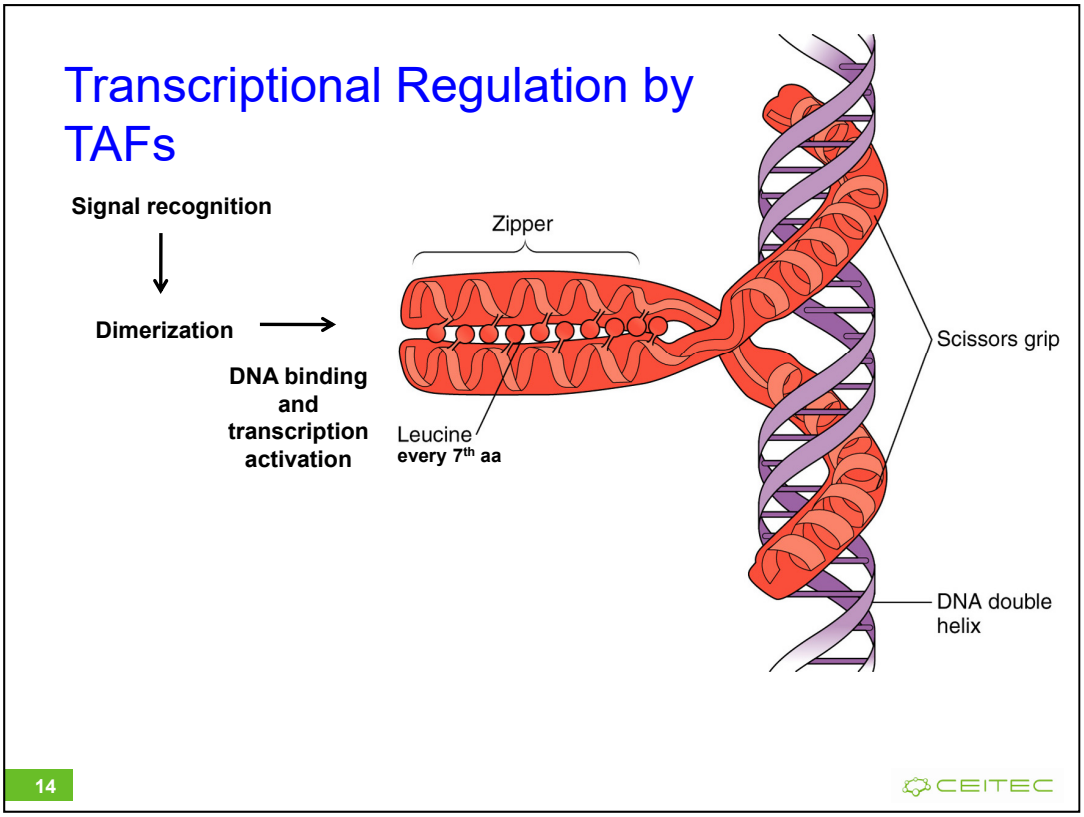
This change of confirmation provides a kind of “signature” that is recognized by other proteins and NA polymerase to recognize the proper binding site. However, there are also TATA box-less promoter, where probably other types of “signatures” occur.



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 protein 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.

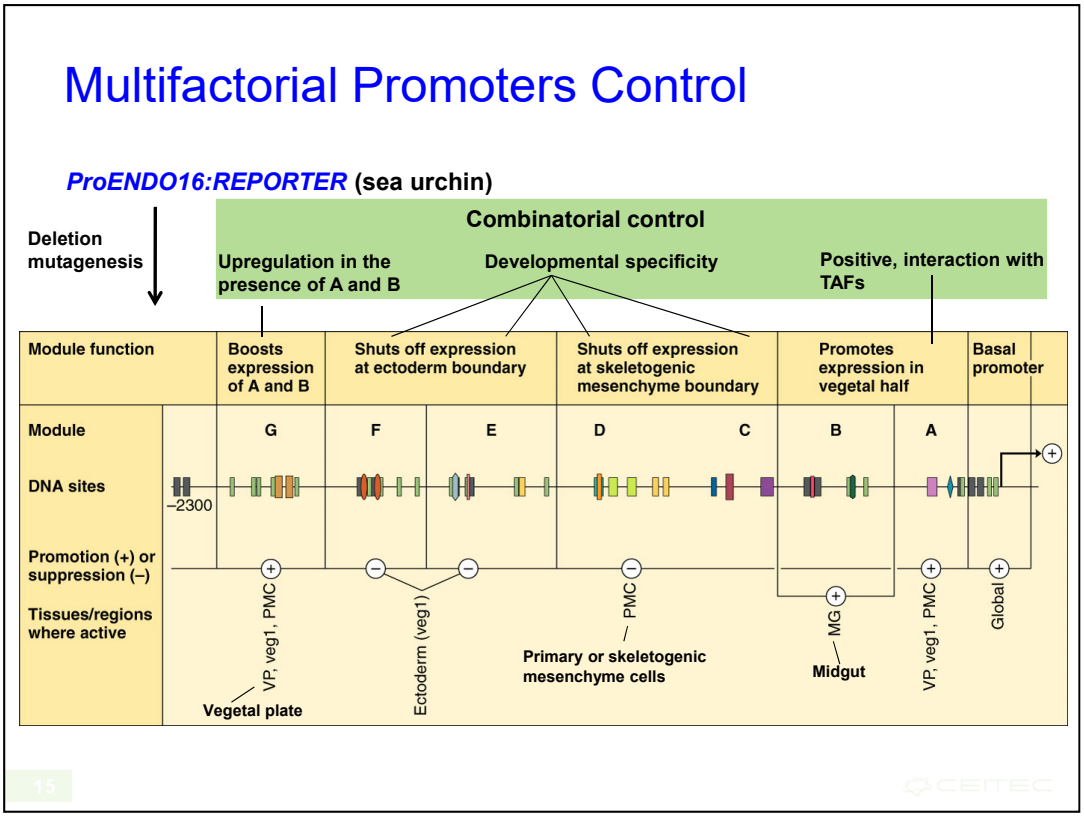
The individual positive or negative factors are complex and their activity might be regulated by their phosphorylation status or via their interaction with other proteins (i.e. momomeric or dimeric) etc..



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 7^{th} aa.

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



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.

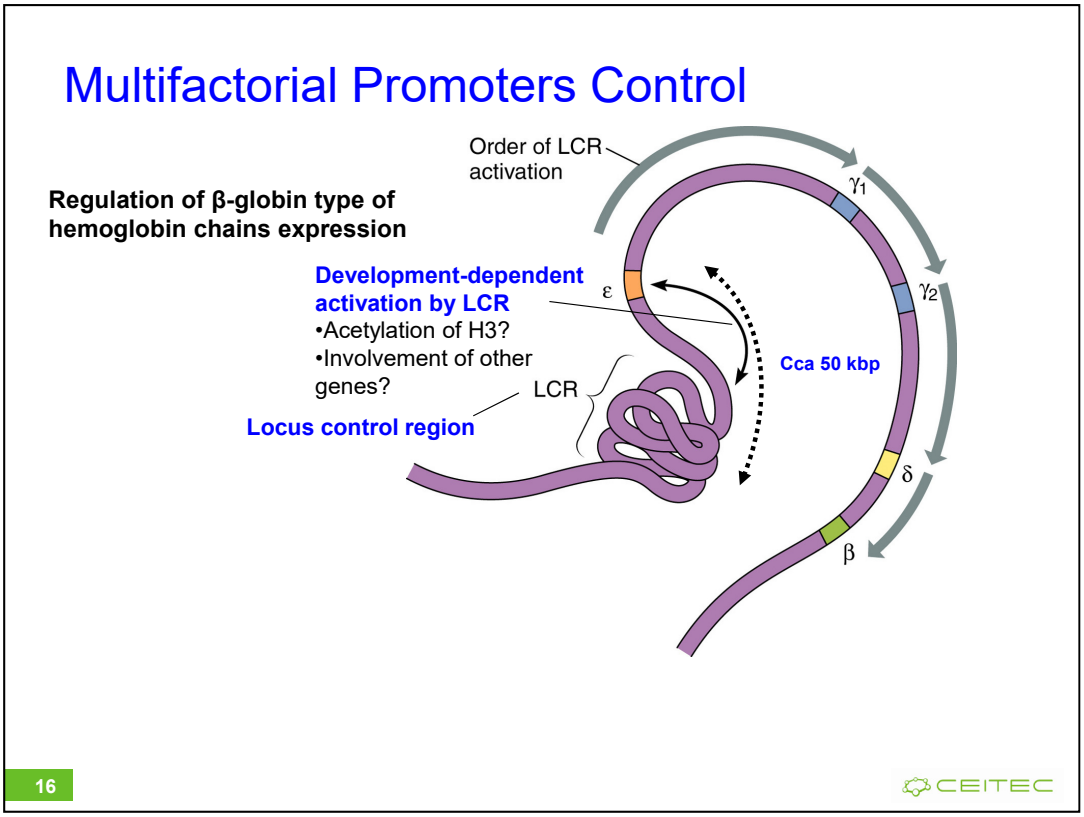
Module G enhances the expression when the A and B are active.

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.



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, γ1, γ2 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 erytrocyte 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.

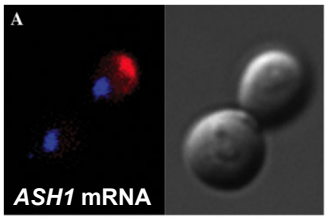
Probably, acetylation of H3 histones might play a role and possibly, other genes outside of the β-type chain family are involved in the regulation of LCR activity. That seems to be confirmed by the identification of other human genes with similar structure, suggesting common regulatory mechanisms via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

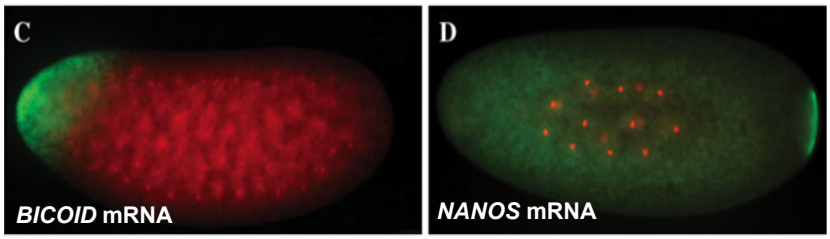
Importance of Protein Interactions

- Functional importance of specific protein interactions
  - Chromatin structure
  - Regulation of transcription
  - mRNA localization

mRNA localization

- **Importance of mRNA localization**
  - Control over spatiotemporal localization of gene product (protein)
    - Asymmetric cell division during development
    - Embryo polarization





Shahbabian and Chartrand, 2012

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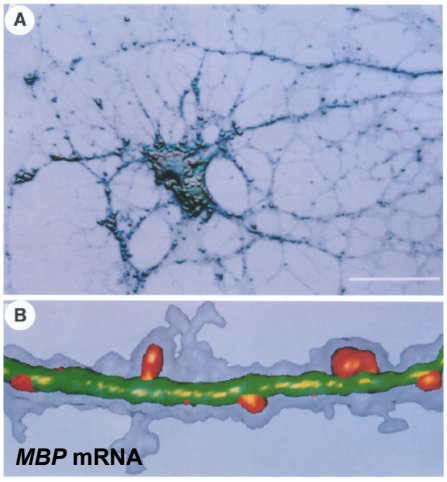
mRNA localization

- **Role of mRNA localization**
  - Attenuating the expression of potentially toxic proteins
    - Localization of expression of MYELIN BASIC PROTEIN (MBP) into myelination regions of nerve cells



Ainger et al., 1993

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

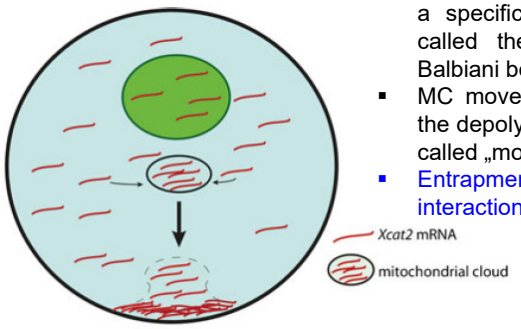
The images show localization of mRNA for MBP. Digoxigenin-labeled MBP RNA was microinjected into mouse oligodendrocytes growing in primary culture. The injected RNA appeared as small granules which were present throughout the cytoplasm and processes, and was also found dispersed in the peripheral membranes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

mRNA localization

Mechanisms

- **Diffusion and entrapment of mRNA**



Shahbabian and Chartrand, 2012

- During the early stages of *Xenopus* oogenesis, *Xcat-2* mRNA is restricted to a specific structure in the cytoplasm called the mitochondrial cloud (MC, Balbiani body)
- MC movement is partly dependent on the depolymerization of microtubuls (so-called „molecular motor“)
- Entrapment on the vegetal pole via interaction of MC and ER

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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.

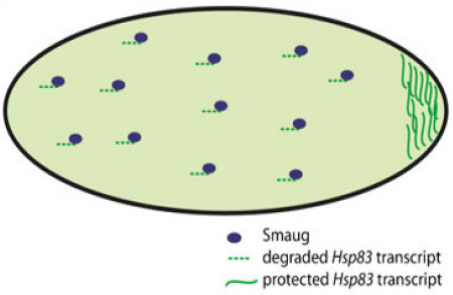
During the early stages of Xenopus oogenesis, Xcat-2 mRNA is restricted to a specific structure in the cytoplasm called the mitochondrial cloud (MC). The mitochondrial cloud, also called Balbiani body, consists mostly of mitochondria and small vesicles, and is the source of germinal granule material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

mRNA localization

Mechanisms

- **Localized mRNA degradation**
  - During embryogenesis in *Drosophila m.* *Hsp83* mRNA is localized at the posterior pole of embryo, similarly to *NANOS* mRNA
  - *Hsp83* mRNA is localized in the whole embryo, however, it is destabilized by cis elements both in 3'UTR (HDE) and in coding region (HIE).

Shahbabian and Chartrand, 2012



  - HIE elements are recognized by SMAUG protein, which mediates binding of degradation complex CCR4/POP2/NOT
  - In the posterior pole the *Hsp83* mRNA is protected from the effects of SMAUG by the so-called HPE element in 3'UTR; mechanism of this protection is still unknown

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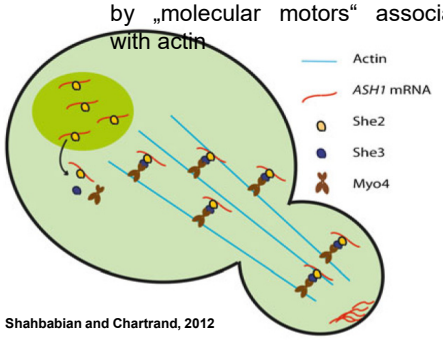
Localized stabilization of a transcript is another mechanism by which an mRNA can be subcellularly targeted. In this case, an mRNA is rapidly degraded in most parts of the cell, but it is protected from degradation at a specific location. The hsp83 mRNA, which encodes a heat shock protein in Drosophila, 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 (Shahbabian and Chartrand, 2012).

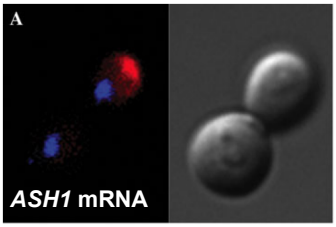
mRNA localization

Mechanisms

- **Active transport of mRNA**
  - Asymmetric Synthesis of HO1 (ASH1) is represor of the *HO* endonuclease in *S. cereviseae;* inhibition of HO results in inhibition of mating-type switching in daughter cells
  - *ASH1* mRNA is actively transported by „molecular motors“ associated with actin



Shahbabian and Chartrand, 2012



Shahbabian and Chartrand, 2012

- *ASH1* mRNA contains 4 *cis* elements (3 in the coding sequence and 1 in the 3'UTR), which are recognized by RNA-binding protein SHE2
- SHE2 interacts with SHE3, an adaptor protein, which links SHE2 to the molecular motor MYO4, which then binds to actin and allows transport of *ASH1* mRNA into the daughter cell

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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 30UTR, 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].

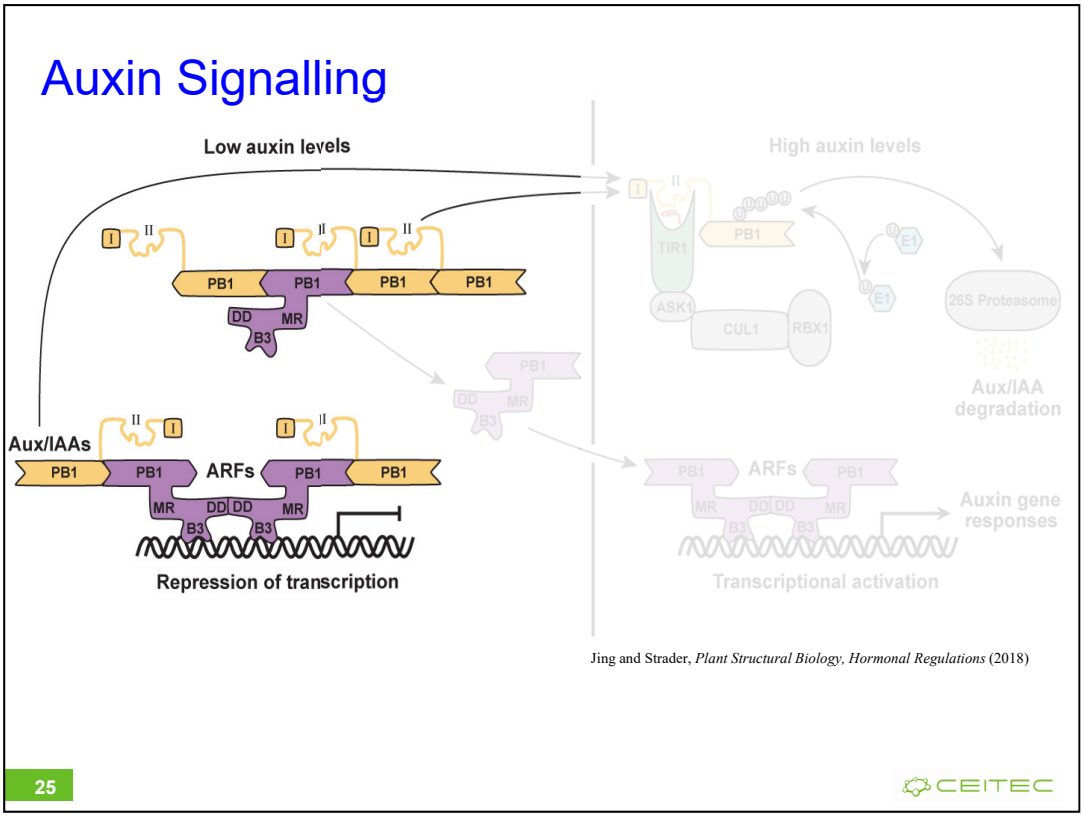
Recent studies on Myo4 helped to explain why multiple localization elements are required for proper ASH1 mRNA localization. Myo4 is a class V myosin whose main function is the transport of mRNAs to the bud tip using actin filaments [98–100]. Myo4, unlike other type V myosins, is a nonprocessive monomer 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 (Shahbabian and Chartrand, 2012).

Importance of Protein Interactions

- Functional importance of specific protein interactions
  - Chromatin structure
  - Regulation of transcription
  - mRNA localization
  - hnRNA splicing

Importance of Protein Interactions

- Functional importance of specific protein interactions
  - Chromatin structure
  - Regulation of transcription
  - mRNA localization
  - hnRNA splicing
  - Protein stability



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

Under low intracellulr 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 proteosome.

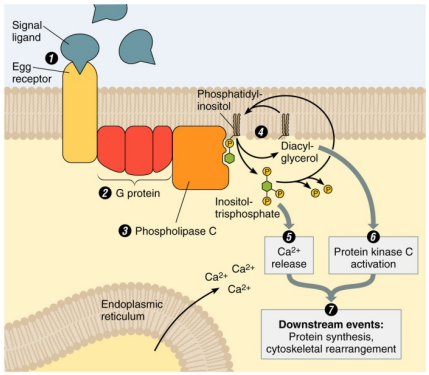
That allows ARFs to enter nucleus and activate trancription of auxin-induced genes.

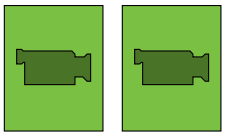
Importance of Protein Interactions

- Functional importance of specific protein interactions
  - Chromatin structure
  - Regulation of transcription
  - mRNA localization
  - hnRNA splicing
  - Protein stability
  - Signal transduction

Signal transduction

- **PI and signal transduction**
  - through G protein and phospholipase C
  - Signalling cascades using cAMP





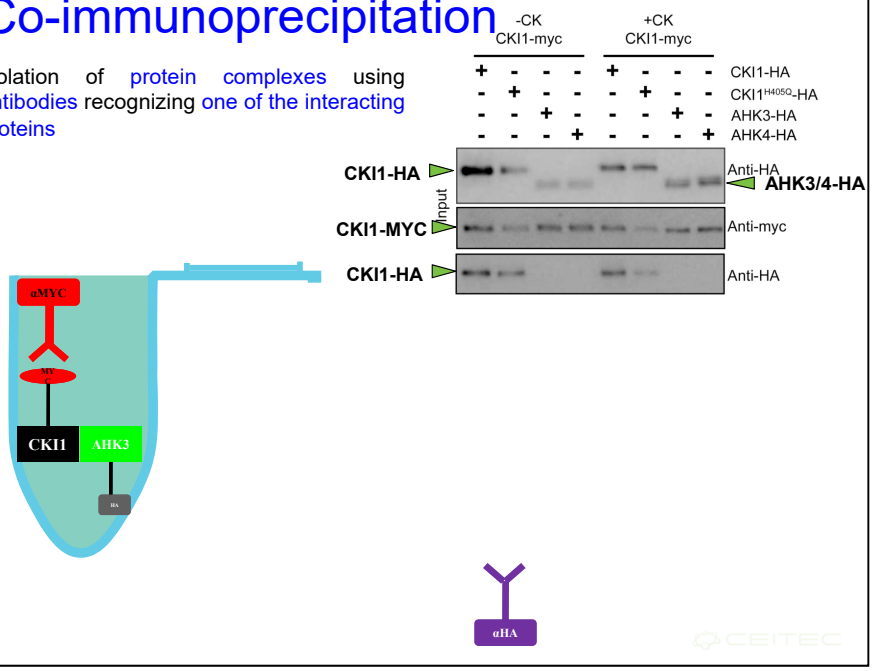
Outline

- Functional importance of the specificic interactions of proteins in the regulation of gene expression
  - Chromatin structure
  - Regulation of transcription
  - mRNA localization
  - mRNA stability
  - Protein stability
  - Signal transduction
- Methods of analysis of protein interactions *in vivo*
  - Co-immunoprecipitation

PI *in vivo*

Co-immunoprecipitation

- Isolation of protein complexes using antibodies recognizing one of the interacting proteins



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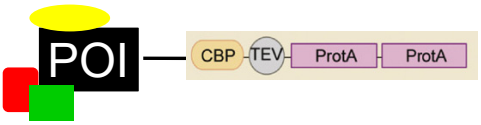
PI *in vivo*

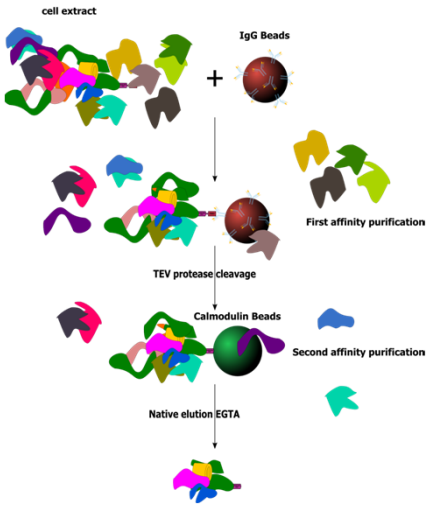
Tandem affinity purification (TAP-tag)

- Isolation of protein complexes using recombinant proteins fused with two different binding domains - tags



  - calmodulin-binding protein (CBP)
  - IgG binding domains of protein A (ProtA)
  - TEV (tobacco etch virus) protease recognition site

- Isolated protein complexes are separated using 1D ELFO and then identified by MS
- Advantage: using two independent protein domains for affinity purification -> therefore high specifity



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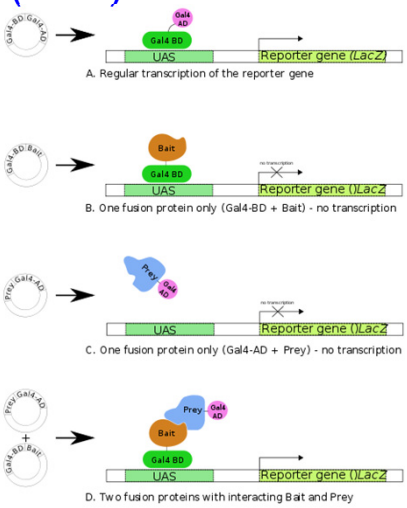
  - Yeast two-hybrid assay (Y2H)

PI *in vivo*

Yeast two-hybrid assay (Y2H)

- Isolation of protein complexes using recombinant proteins, each fused to a part of Gal4 transcription factor
  - One of the proteins (bait) fused to DNA-binding domain of Gal4 (Gal4-BD)
  - The other protein (prey) fused to activation domain of Gal4 (Gal4-AD)
- Protein interactions enable reconstitution of binding domains with activation domain and triggers the expression of a reporter gene
  - Visual detection (blue color, LacZ)
  - Auxotrophic selection (growth on medium lacking histidine, His)
- Method used for searching for interaction partners in expression libraries of individual organisms



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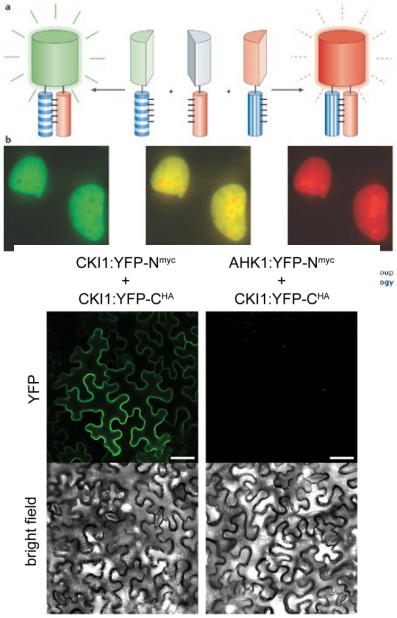
  - Bimolecular fluorescence complementation (BiFC)

PI *in vivo*

Bimolecular fluorescence complementation (BiFC)

- Protein interaction is detected by reassociation of the fluorescent protein
  - Each of the potential interaction partners is fused to one of the subunits of the fluorescent protein, e.g. YFP
  - In case of interaction, the fluorescence appears
- Apart from identification of the interaction, this method allows you to localize the interaction within the cell



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  - Membrane Recruitment Assay (MeRA)

PI *in vivo*

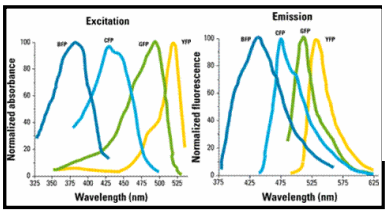
Membrane Recruitment Assay (MeRA)

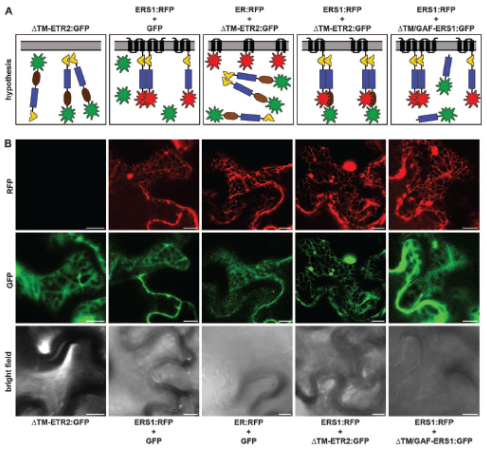
- Method for identification of interactions of cytoplasmic proteins with the membrane proteins

Membrane protein is fused with a fluorescecnt protein

Potential interaction partner is fused with another fluorescent protein with different emission spectra

- In case of interaction the localization of the cytoplasmic protein is changed – it is colocalized on the membrane with the membrane protein





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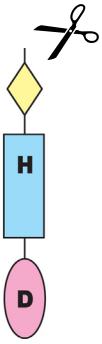
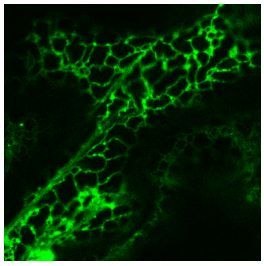
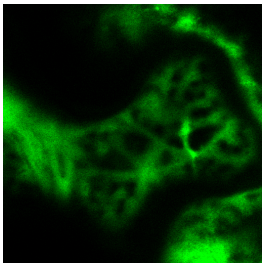
PI *in vivo*

Membrane Recruitment Assay (MeRA)







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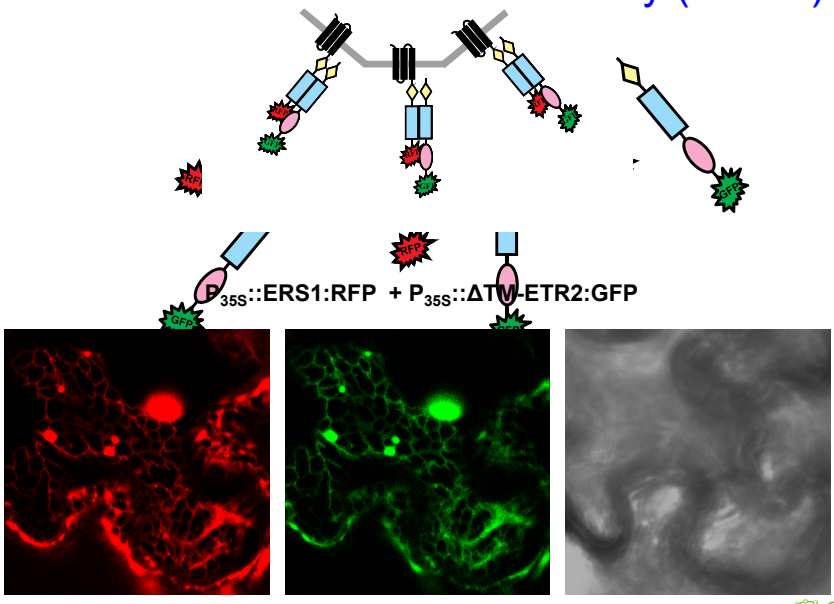
PI *in vivo*

Membrane Recruitment Assay (MeRA)



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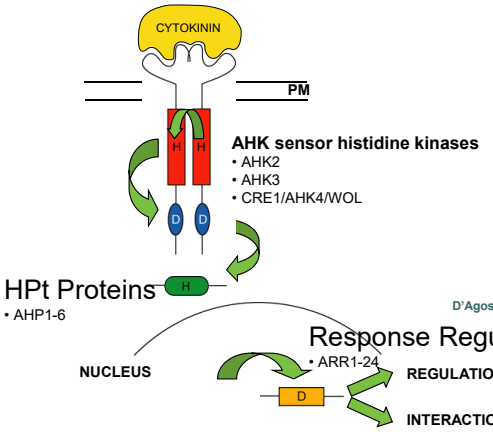
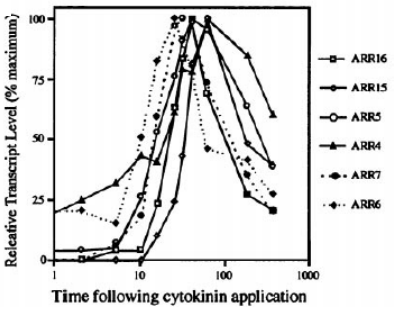
Signal Transduction via MSP

Recent Model of the CK Signaling via Multistep Phosphorelay (MSP) Pathway





D'Agostino et al., Plant Phys, 2000

CK primary response genes
- Type-A *ARRs* expression

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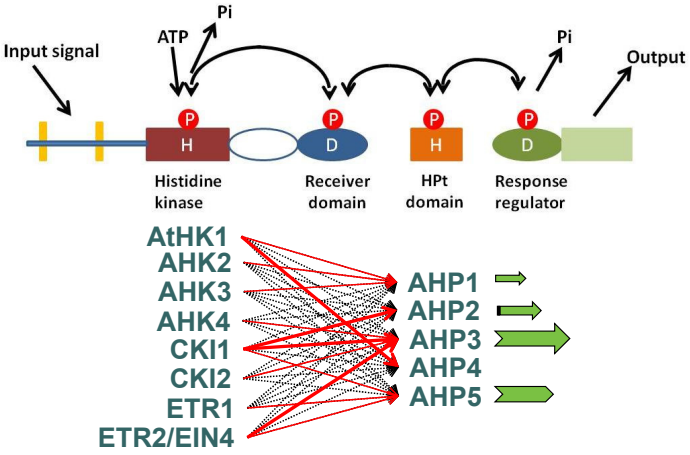
Is there any specificity in plant MSP?

- Is there ***a signalling specificity of MSP*** in plants?



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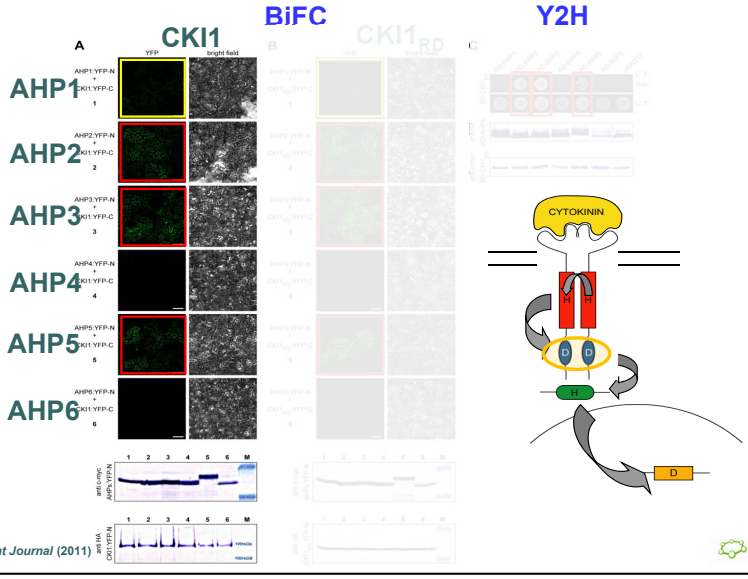
Specificity of CKI1 signalling

- ***CKI1 interacts*** *in vivo* with only ***subset of AHPs***



Pekárová et al., *Plant Journal* (2011)

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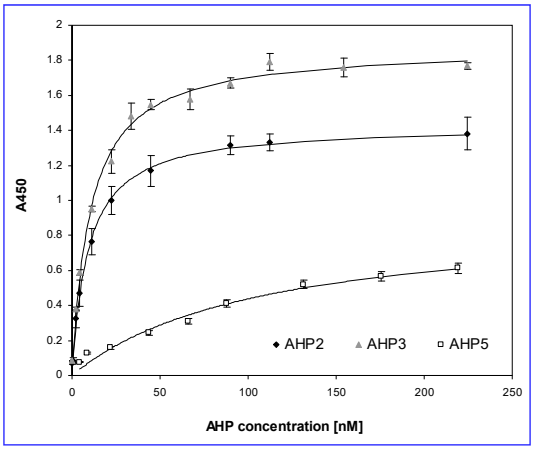
Specificity of CKI1 Signalling

- ***Specificity of CKI1 interaction*** was confirmed ***in vitro***



AHP3: K_d ~ 10,5 nM

AHP2: K_d ~ 9,17 nM

AHP5: K_d ~ 108 nM

Pekárová et al., *Plant Journal* (2011)

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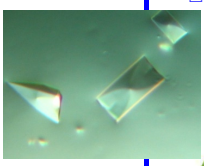
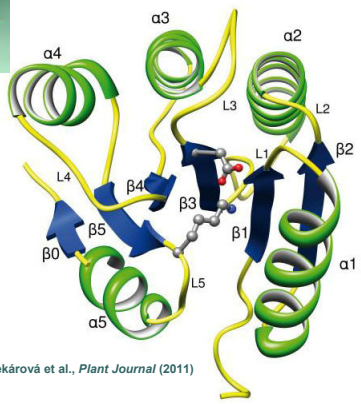
Structure of CKI1$_{RD}$

- X-ray crystallography revealed conserved ***(α/β)$_5$ structural fold*** of CKI1$_{RD}$





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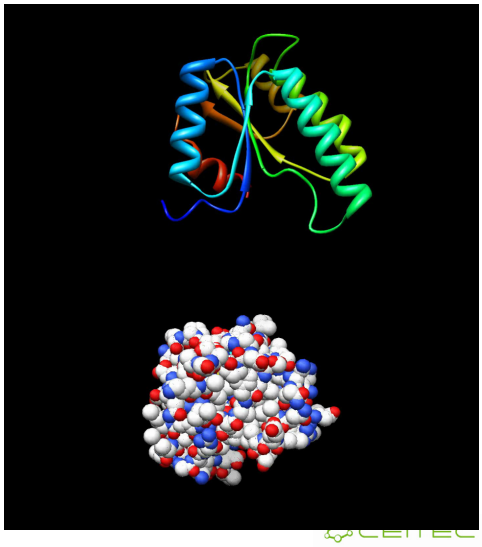
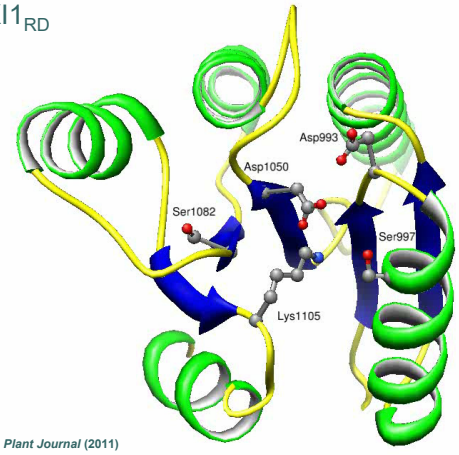


Dynamics of $CKI1_{RD}$

- ***Mg^{2+}binding*** leads to ***remodelling of active centre*** of $CKI1_{RD}$



Pekárová et al., *Plant Journal* (2011)

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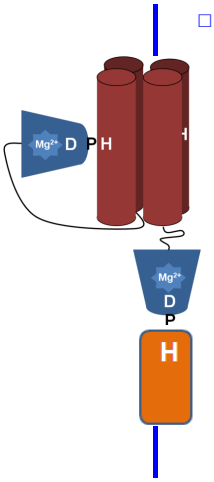
$CKI1_{RD}$ structural changes are associated with its binding specificity

- ***Mg^{2+}*- and *BeF_3^-*-induced *structural changes fine-tune binding specificity of* $CKI1_{RD}$**



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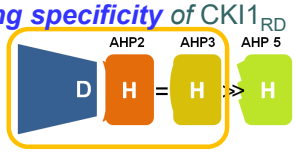

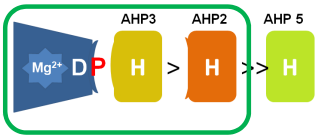

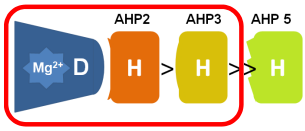

Ligand	0	Mg^{2+}	BeF_3^-
AHP2	9.17 ± 0.49	6.2 ± 0.98	11.6 ± 2.0
AHP3	10.5 ± 0.73	12.9 ± 0.72	8.0 ± 0.42
AHP5	108 ± 18	152 ± 26	119 ± 32

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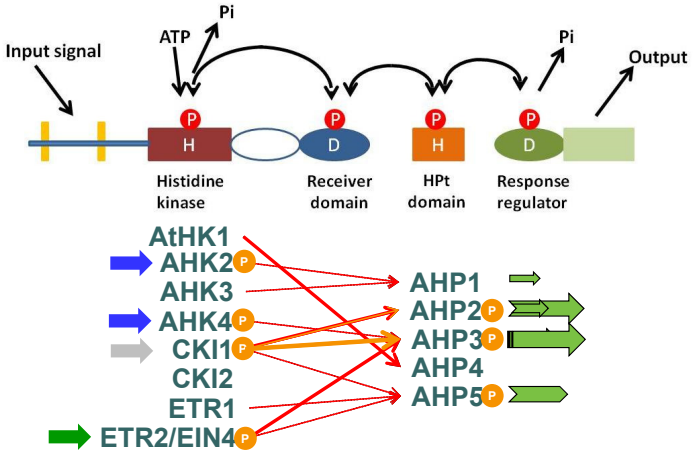
Model Suggestion

- ***YES***, there is ***signalling specificity of MSP*** in plants.



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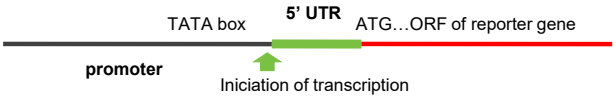
Discussion

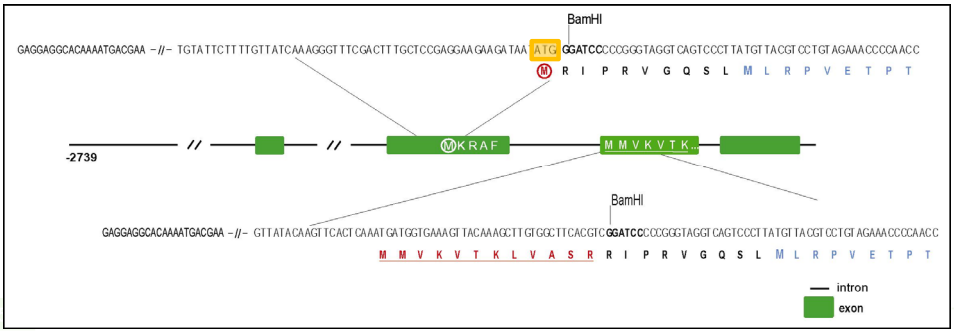
Outline

- Methods of gene expression analysis
  - Qualitative analysis of gene expression
    - Preparation of transcriptional fusion of promoter of analysed gene with a reporter gene

Transcriptional Fusion

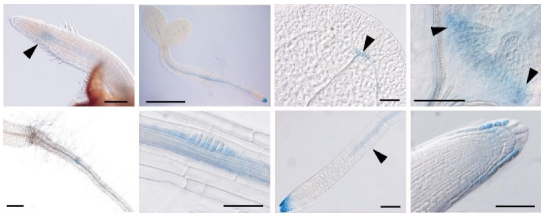
- Identification and cloning of the promoter region of the gene
- Preparation of recombinant DNA carrying the promoter and the reporter gene (uidA, GFP)

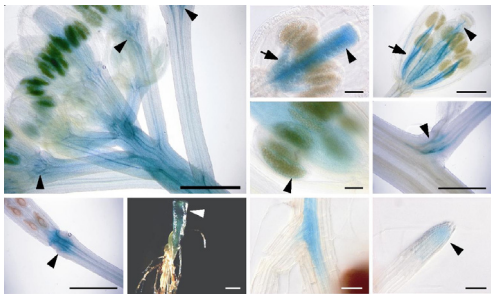




Transcriptional Fusion

- Identification and cloning of the promoter region of the gene
- Preparation of recombinant DNA carrying the promoter and the reporter gene (uidA, GFP)
- Preparation of transgenic organisms carrying this recombinant DNA and their histological analysis





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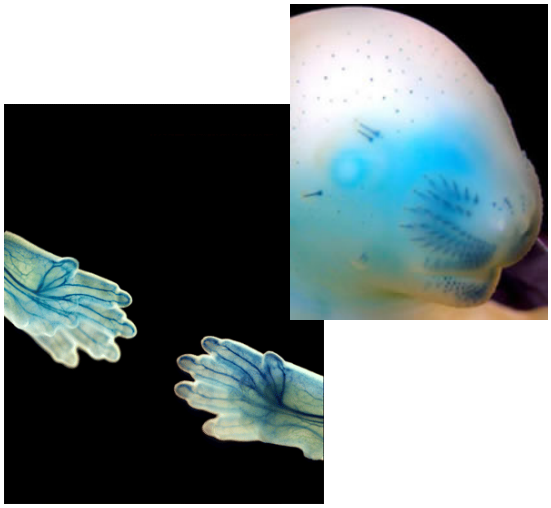
GUS Reporter in Mouse Embryos



CEITEC

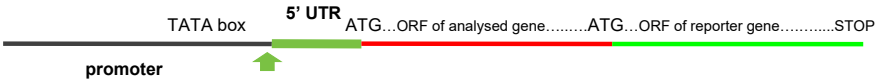
Outline

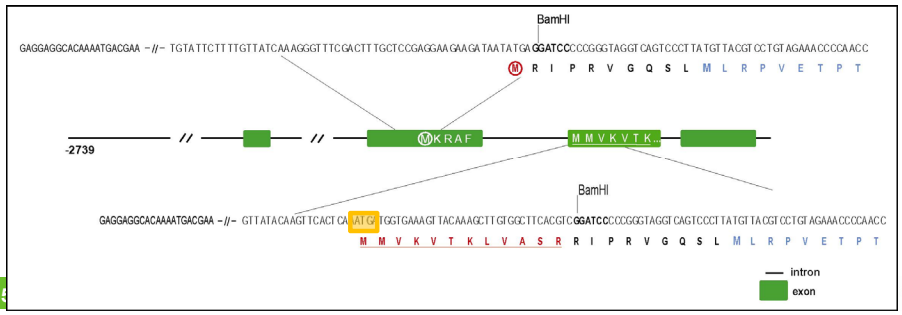
- Methods of gene expression analysis
  - Qualitative analysis of gene expression
    - Preparation of transcriptional fusion of promoter of analysed gene with a reporter gene
    - Preparation of translational fusion of the coding region of the analysed gene with reporter gene

CEITEC

Translational Fusion

- Identification and cloning of the promoter and coding region of the analyzed gene
- Preparation of a recombinant DNA carrying the promoter and the coding sequence of the studied gene in a fusion with the reporter gene (uidA, GFP)

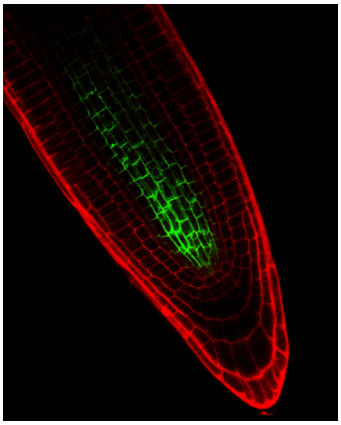




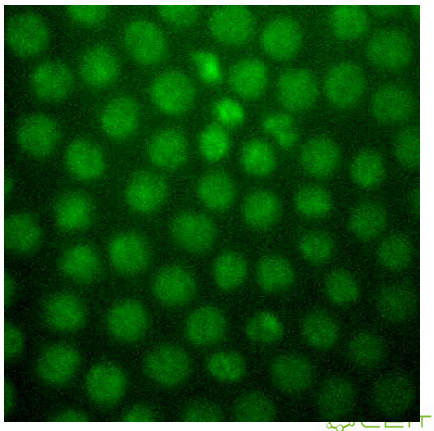
CEITEC

Translational Fusion

- Preparation of transgenic organisms carrying the recombinant DNA and their histological analysis
- Compared to transcriptional fusion, translation fusion allows analysis of intercellular localization of gene product (protein) or its dynamics

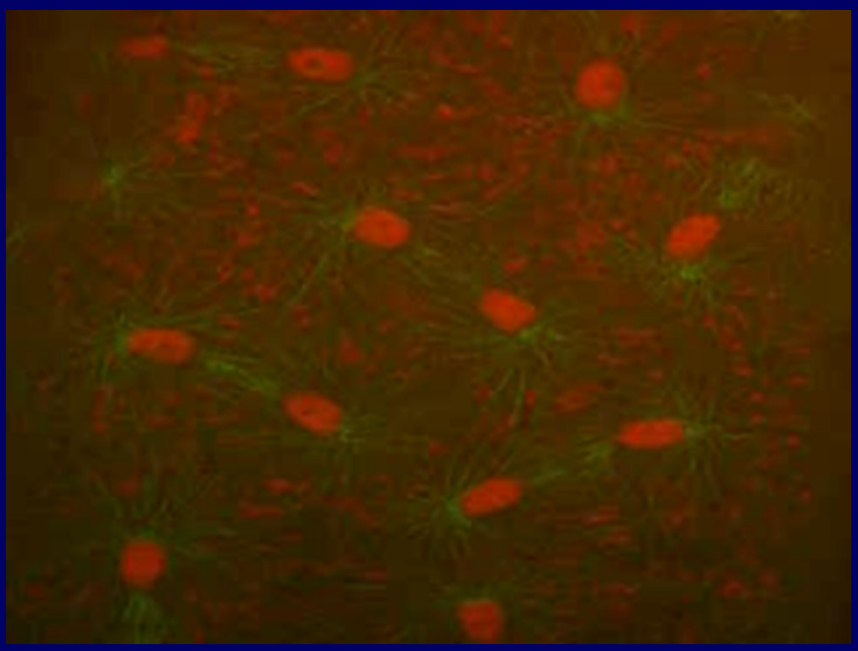


PIN1-GFP in *Arabidopsis*



Histone 2A-GFP in *Drosophila* embryo by PAM

Translational Fusion



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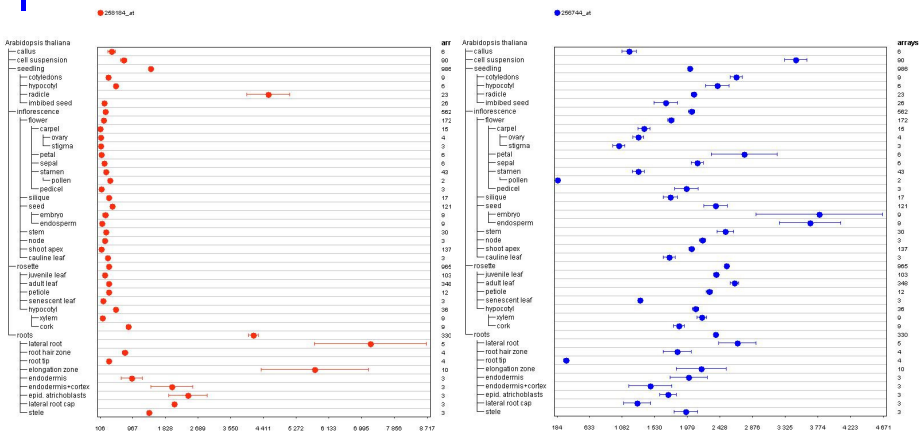
Databases

- **Analysis of expression using Genevestigator** (***AHP1*** and ***AHP2***, *Arabidopsis*, Affymetrix ATH 22K Array)



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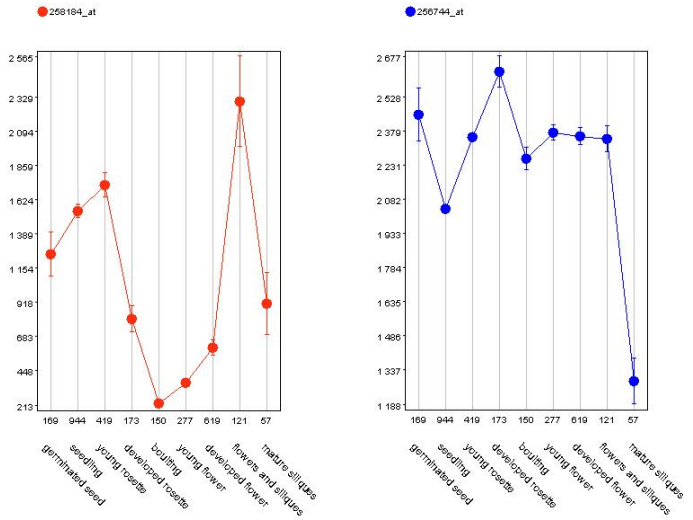
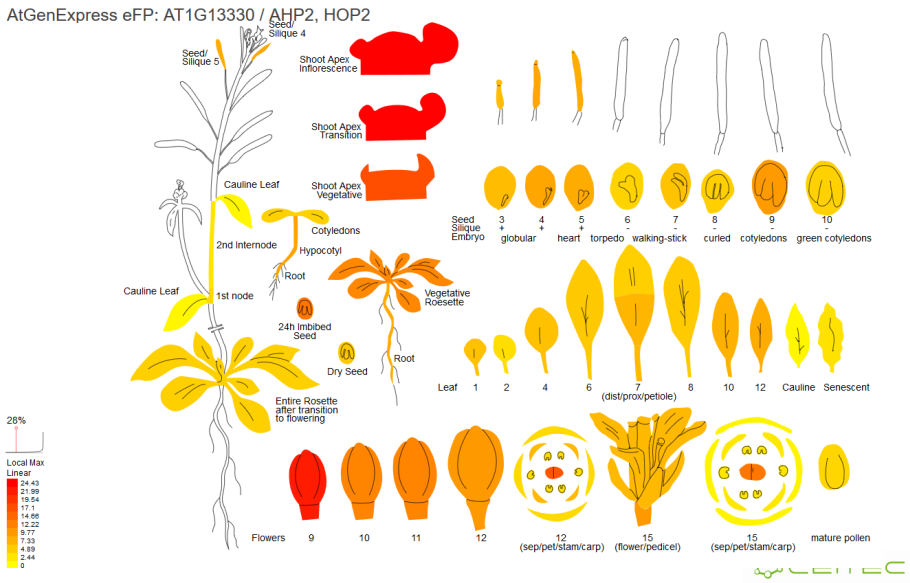


CEITEC

Databases

- **Analysis of expression using ePlant**



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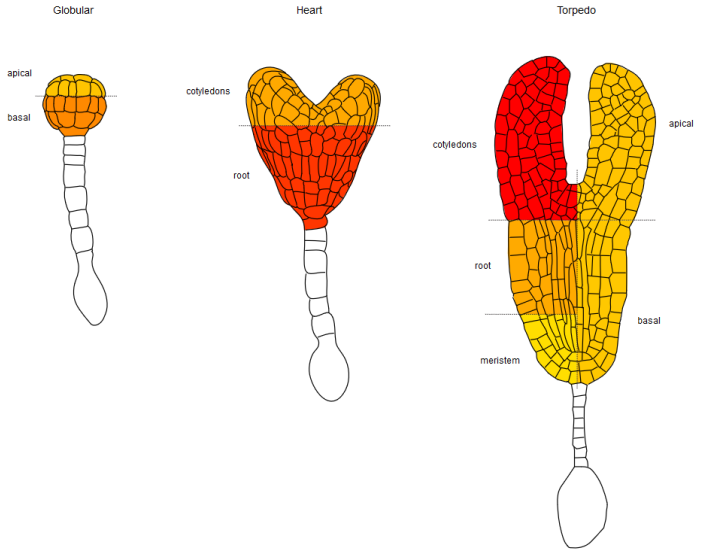
Databases

- **Analysis of expression using ePlant**



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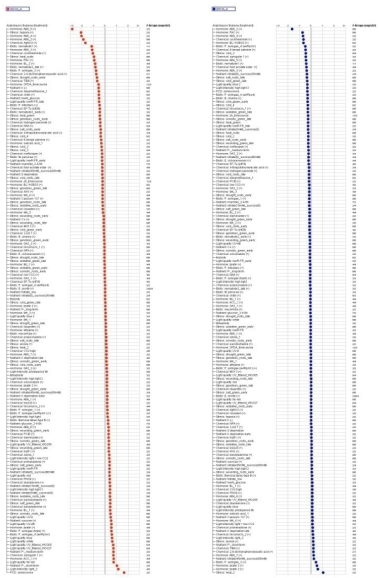

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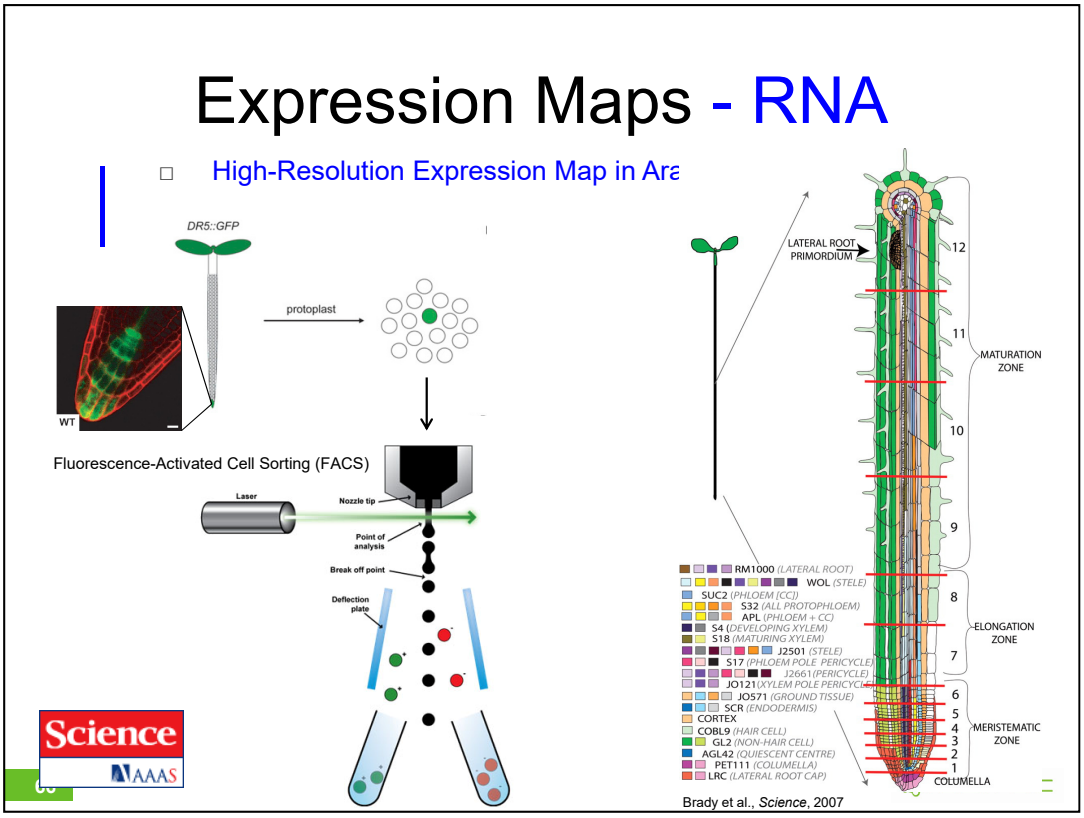
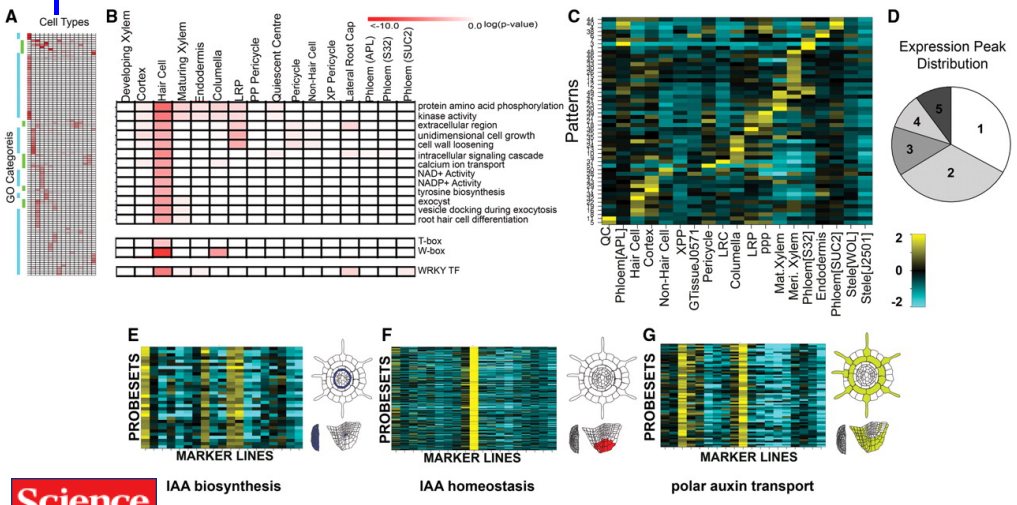


Microarray expression profiles of 19 fluorescently sorted GFP-marked lines were analyzed (*3*–*9*, *23*, *24*). The colors associated with each marker line reflect the developmental stage and cell types sampled. Thirteen transverse sections were sampled along the root's longitudinal axis (red lines) (*10*). CC, companion cells.

Expression Maps - RNA

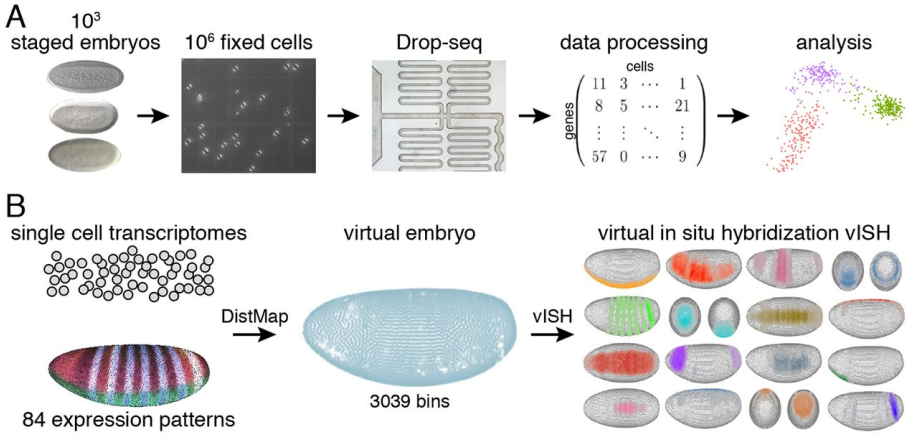
- High-Resolution Expression Map in Arabidopsis Root



(**A**) The majority of enriched GO terms (hierarchically clustered) are associated with individual cell types (blue bar). A smaller number are present across multiple cell types (green bar). (fig. S2) (**B**) GO category enrichment for hair cells confirms a previous report (*15*). Enriched cis-elements and an enriched TF family were also identified. (**C**) From the top 50% of varying probe sets, 51 dominant radial patterns were identified. Pattern expression values were mean-normalized (rows) and $\log_2$ transformed to yield relative expression indices for each marker line (columns). Marker line order is the same for all figures; see table S1 for marker line abbreviations. (**D**) Pattern expression peaks were found across one to five cell types. (**E** to **G**) Patterns where expression is enriched in single and multiple cell types support transcriptional regulation of auxin flux and synthesis. In all heat maps with probe sets, expression values were mean-normalized and $\log_2$ transformed. Expression is false-colored in representations of a root transverse section, a cut-away of a root tip, and in a lateral root primordium. (E) Auxin biosynthetic genes (*CYP79B2, CYP79B3, SUPERROOT1*, and *SUPERROOT2*) are transcriptionally enriched in the QC, lateral root primordia, pericycle, and phloem-pole pericycle (P = 1.99E^{-11}, pattern 5). All AGI identifiers and TAIR descriptions are found in table S14. (F) Auxin amido-synthases *GH3.6* and *GH3.17* that play a role in auxin homeostasis show enriched expression in the columella, just below the predicted auxin biosynthetic center of the QC (P = 8.82E^{-4}, pattern 13). (G) The expression of the auxin transporter, *PIN-FORMED2*, and auxin transport regulators (*PINOID, WAG1*) are enriched in the columella, hair cells, and cortex (P = 1.03E^{-4}, pattern 31).

Expression Maps - RNA

High-Resolution Expression Map in Drosophilla



Nikos Karaiskos et al. Science 2017;science.aan3235

Science AAAS

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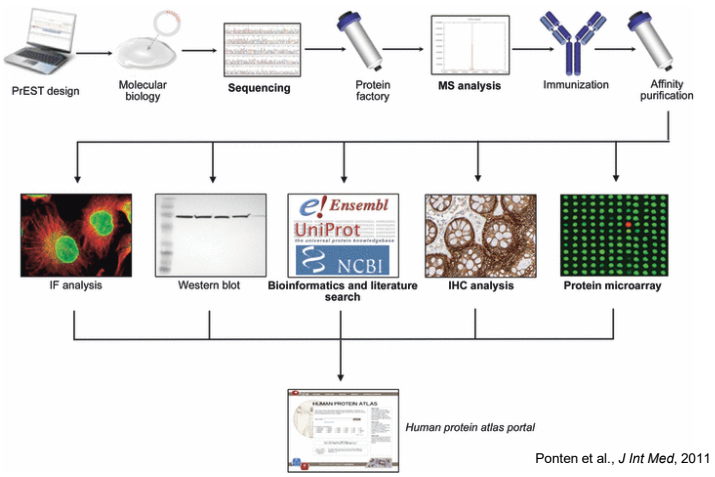
Deconstructing and reconstructing the embryo by single-cell transcriptomics combined with spatial mapping.

(A) Single-cell sequencing of the Drosophila embryo: ~1000 handpicked stage 6 fly embryos are dissociated per Drop-seq replicate, cells are fixed and counted, single cells are combined with barcoded capture beads, and libraries are prepared and sequenced. Finally, single-cell transcriptomes are deconvolved, resulting in a digital gene expression matrix for further analysis.

(B) Mapping cells back to the embryo: Single-cell transcriptomes are correlated with high-resolution gene expression patterns across 84 marker genes, cells are mapped to positions within a virtual embryo, and expression patterns are computed by combining the mapping probabilities with the expression levels (virtual in situ hybridization).

Expression Maps - Proteins

- Human Protein Atlas



Ponten et al., *J Int Med*, 2011

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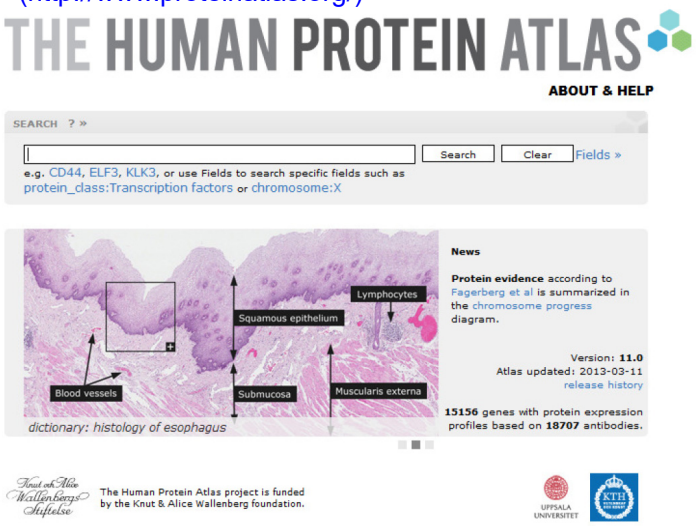
Schematic flowchart of the Human Protein Atlas. For each gene, a signature sequence (PrEST) is defined from the human genome sequence, and following RT-PCR, cloning and production of recombinant protein fragments, subsequent immunization and affinity purification of antisera results inmunospecific antibodies. The produced antibodies are tested and validated in various immunoassays. Approved antibodies are used for protein profiling in cells (immunofluorescence) and tissues (immunohistochemistry) to generate the images and protein expression data that are presented in the Human Protein Atlas (Ponten et al., *J Int Med*, 2011).

Expression Maps - Proteins

- Human Protein Atlas (http://www.proteinatlas.org/)



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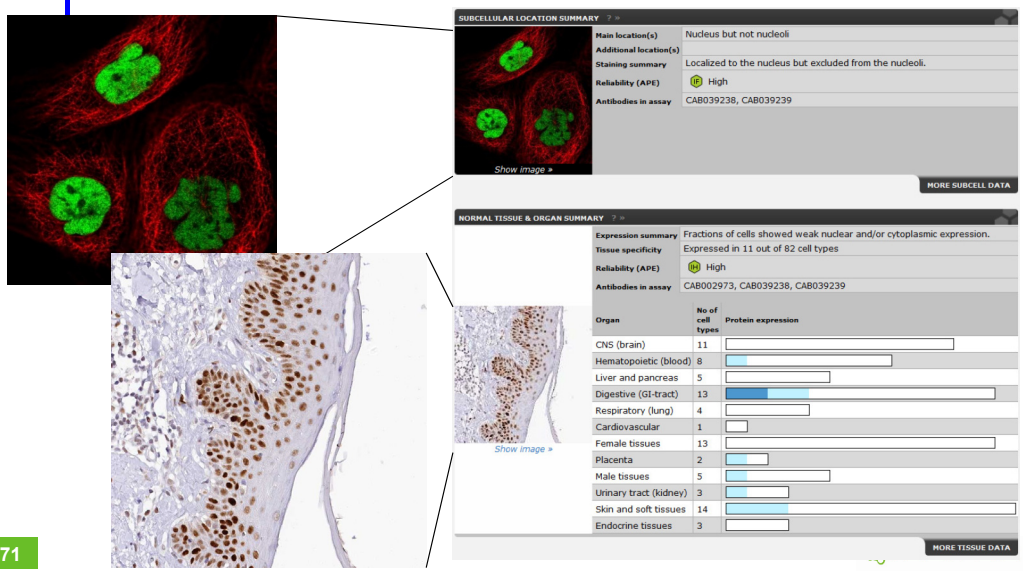

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Outline

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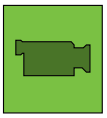
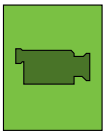
DNA Chips

- Method, which provides quick comparison of a large number of genes/proteins between the test sample and control
- Oligo DNA chips are used the most



- There are commercialy available kits for the whole genome
  - company Operon (Qiagen), 29.110 of 70-mer oligonucleotides representing 26.173 genes coding proteins, 28.964 transcripts and 87 microRNA genes of *Arabidopsis thaliana*
  - Possibility of use for the preparation of photolithography chips – facilitation of oligonucletide synthesis e.g. for the whole human genome (about 3,1 x 10^9 bp) jit is possible to prepare 25-mers in only 100 steps, by this technique



- Chips not only for the analysis of gene expression, but also for e.g. Genotyping (SNPs, sequencing with chips, …)

Affymetrix ATH1 *Arabidopsis* genome array

Critical Specifications	
Number of arrays	One
Number of sequence represented	>24,000 gene sequences
Feature size	18 µm
Oligonucleotide probe length	25-mer
Probe pairs/sequence	11
Control sequences	*E. coli* genes *bioB, bioC, bioD*. *B. subtilis* gene *lysA*. Phage P1 *cre* gene. Arabidopsis maintenance genes GAPDH, Ubiquitin, and Actin
Detection sensitivity	1:100,000*

*As measured by detection in comparative analysis between a complex target containing spiked control transcriptions and a complex target with no spikes.

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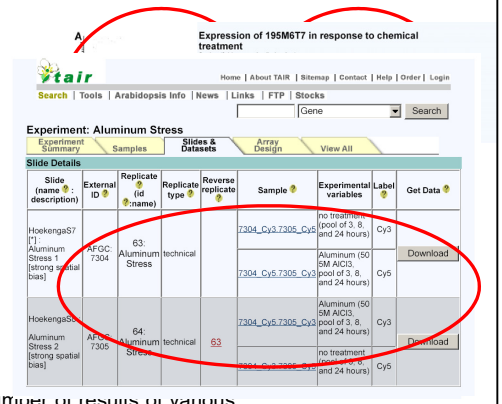
DNA Chips

- For the correct interpretation of the results, good knowledge of advanced statistical methods is required
- It is necessary to include a sufficient number of controls and repeats

- Control of accuracy of the measurement (repeated measurements on several chips with the same sample, comparing the same samples analysed on different chips with each other)
- Control of reproducibility of measurements (repeated measurements with different samples isolated under the same conditions on the same chip – comparing with each other)
- Identification of reliable measurement treshold
- Finally comparing the experiment with the control or comparing different conditions with each other -> the result



- Currently there's been a great number of results of various experiments in publicly accessible databases

Che et al., 2002

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Protein Chips

- Protein chips
  - Chips with high density containing 10^4 proteins
  - Analysis of protein-protein interactions, kinase substrates and interactions with small molecules
  - Possibility of using antibodies – more stable than proteins

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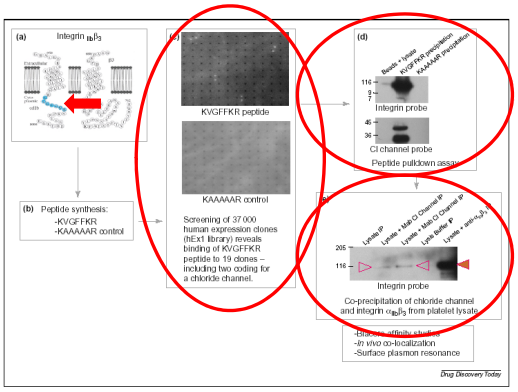
Protein Chips

- Identification of proteins interacting with integrin $\alpha_{IIb}\beta_3$ cytoplasmic domain of platelets
  - Expression of cytoplasmic part as a fusion peptide biotin-KVGFFKR
  - Analysis of binding to the protein chip containing 37.000 clones of *E. coli* expressing human recombinant proteins
  - Confirmation of interaction by pull-down analysis of peptides and by coprecipitation of whole proteins as well (e.g. chloride channel Icln)
  - Other use: e.g. in the identification of kinase substrates, when substrates are bound to the chip and exposed to kinases in the presense of radiolabeled ATP (786 purified proteins of barely, of which 21 were identified as CK2α kinase substrates; Kramer et al., 2004)



Lueking et al., 2005

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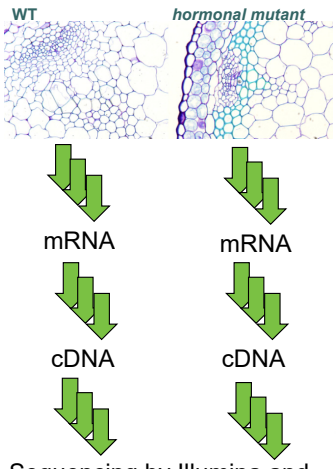
Next Gen Transcriptional Profiling

- ***Transcriptional profiling*** via ***RNA sequencing***



Sequencing by Illumina and
number of transcripts determination

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Results of –omics Studies vs Biologically Relevant Conclusions

- Transcriptional profiling yielded more then **7K differentially regulated genes**…

Ddii et al., *unpublished*

gene	locus	sample_1	sample_2	status	value_1	value_2	log2(fold_change)	test_stat	p_value	q_value	significant
AT1G07795	1:2414285-2414967	WT	MT	OK	0	1,1804	1.79769e+308	1.79769e+308	6.88885e-05	0,00039180 1	yes
HRS1	1:4556891-4558708	WT	MT	OK	0	0,696583	1.79769e+308	1.79769e+308	6.61994e-06	4.67708e-05	yes
ATMLO14	1:9227472-9232296	WT	MT	OK	0	0,514609	1.79769e+308	1.79769e+308	9.74219e-05	0,00053505 5	yes
NRT1.6	1:9400663-9403789	WT	MT	OK	0	0,877865	1.79769e+308	1.79769e+308	3.2692e-08	3.50131e-07	yes
AT1G27570	1:9575425-9582376	WT	MT	OK	0	2,0829	1.79769e+308	1.79769e+308	9.76039e-06	6.647e-05	yes
AT1G60095	1:22159735-22162419	WT	MT	OK	0	0,688588	1.79769e+308	1.79769e+308	9.95901e-08	9.84992e-07	yes
AT1G03020	1:698206-698515	WT	MT	OK	0	1,78859	1.79769e+308	1.79769e+308	0,00913915	0,0277958	yes
AT1G13609	1:4662720-4663471	WT	MT	OK	0	3,55814	1.79769e+308	1.79769e+308	0,00021683	0,0010807 9	yes
AT1G21550	1:7553100-7553876	WT	MT	OK	0	0,562868	1.79769e+308	1.79769e+308	0,00115582	0,0047149 7	yes
AT1G22120	1:7806308-7809632	WT	MT	OK	0	0,617354	1.79769e+308	1.79769e+308	2.48392e-06	1.91089e-05	yes
AT1G31370	1:11238297-11239363	WT	MT	OK	0	1,46254	1.79769e+308	1.79769e+308	4.83523e-05	0,00028514 3	yes
APUM10	1:13253397-13255570	WT	MT	OK	0	0,581031	1.79769e+308	1.79769e+308	7.87855e-06	5.46603e-05	yes
AT1G48700	1:18010728-18012871	WT	MT	OK	0	0,556525	1.79769e+308	1.79769e+308	6.53917e-05	0,00037473 6	yes
AT1G59077	1:21746209-21833195	WT	MT	OK	0	138,886	1.79769e+308	1.79769e+308	0,00122789	0,0049681 6	yes
AT1G60050	1:22121549-22123702	WT	MT	OK	0	0,370087	1.79769e+308	1.79769e+308	0,00117953	0,0048001	yes
AT4G15242	4:8705786-8706997	WT	MT	OK	0,00930712	17,9056	10,9098	-4,40523	1.05673e-05	7.13983e-05	yes
AT5G33251	5:12499071-12500433	WT	MT	OK	0,0498375	52,2837	10,0349	-9,8119	0	0	yes
AT4G12520	4:7421055-7421738	WT	MT	OK	0,0195111	15,8516	9,66612	-3,90043	9.60217e-05	0,000528904	yes
AT1G60020	1:22100651-22105276	WT	MT	OK	0,0118377	7,18823	9,24611	-7,50382	6.19504e-14	1.4988e-12	yes
AT5G15360	5:4987235-4989182	WT	MT	OK	0,0988273	56,4834	9,1587	-10,4392	0	0	yes

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Excample of an output of transcriptional profiling study using Illumina sequencing performed in our lab. Shown is just a tiny fragment of the complete list, copmprising about 7K genes revealing differential expression in the studied mutant.

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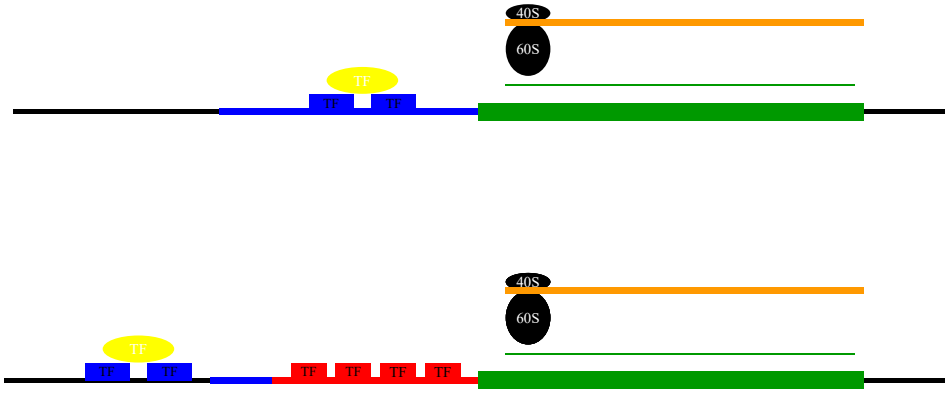
  - T-DNA activation mutagenesis

Gain-of-Function Approaches

- Methods for identification of gene function using gain-of-function approaches
  - T-DNA activation mutagenesis
    - Method enabling isolation of dominant mutants by random insertion of constitutive promoter, resulting in overexpression of the gene and therefore in corresponding phenotypic changes
    - First step: preparation of mutant library prepared by tansformation of a strong constitutive promoter or enhancer
    - Next step: search of interesting phenotypes
    - Identification of the affected gene, e.g. by plasmid-rescue

Activation Mutagenesis



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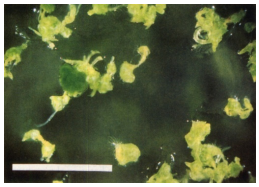
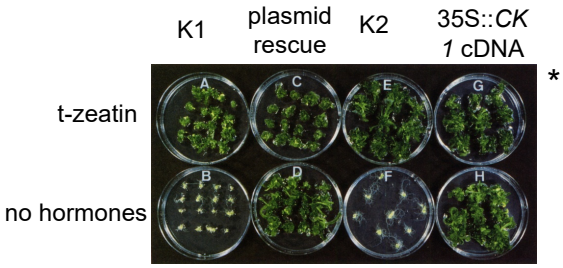
Isolation of *CKI1* Gene

- Tatsuo Kakimoto, *Science* 274 (1996), 982-985 *
- Isolation of the gene using activation mutagenesis



*

- Mutant phenotype is a phenocopy of exogenous application of cytokinins (*CKI1*, ***C***YTO***K***ININ ***I***NDEPENDENT *1*)



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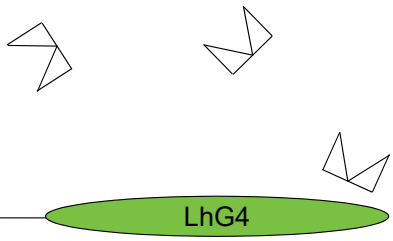
  - T-DNA activation mutagenesis
  - Ectopic expression and regulated gene expression systems

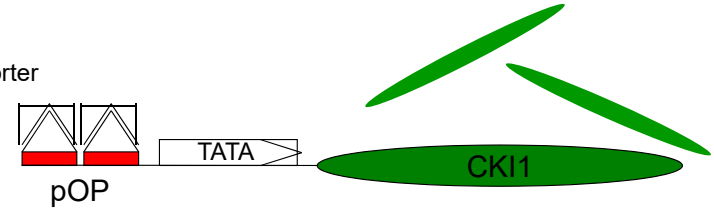
Regulated Expression Systems

activator
x

reporter

activator x reporter





pOP

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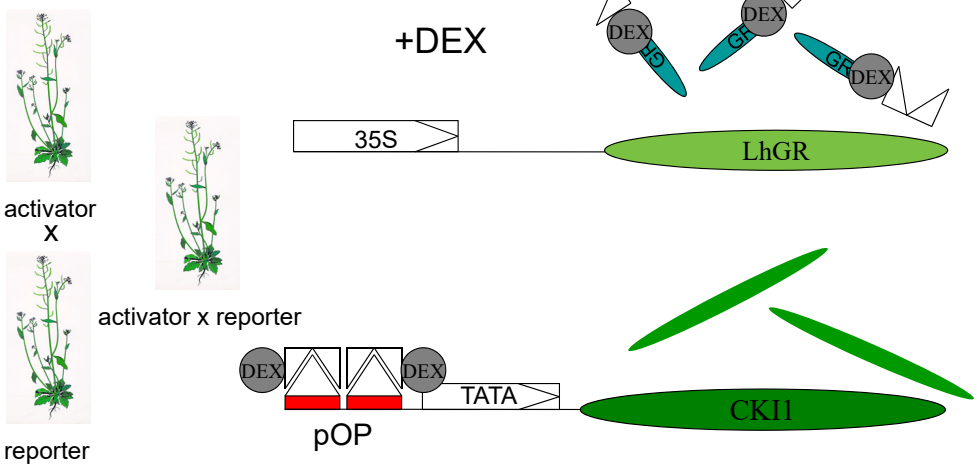
Regulated Expression Systems



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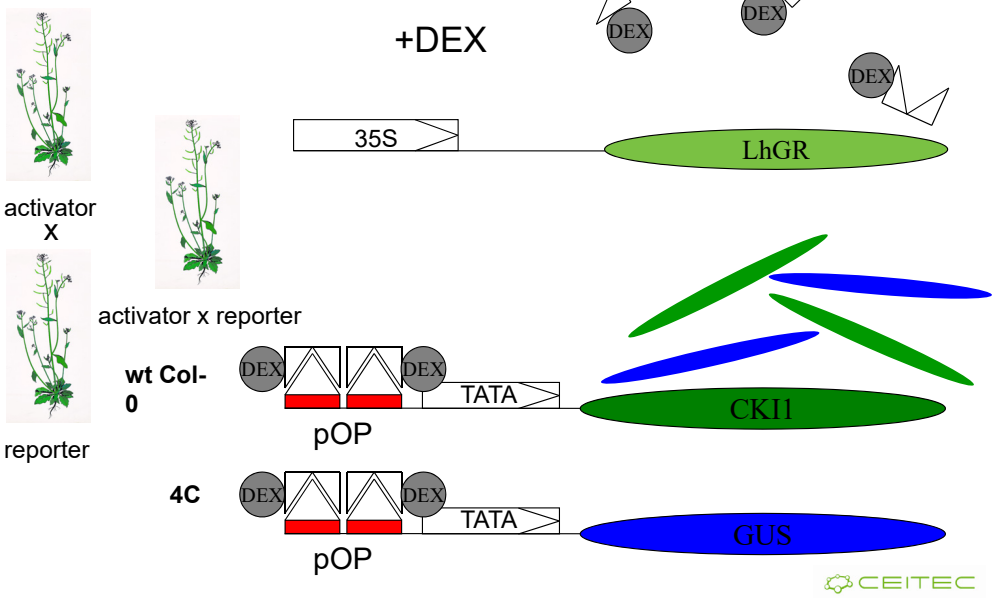
Regulated Expression Systems



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Regulated Expression Systems

- Regulatable gene expression systems
  - Time- or site-specific regulation of gene expression, leading to a change in phenotype and thereby identification of the natural function of the gene
    - pOP system
    - UAS system

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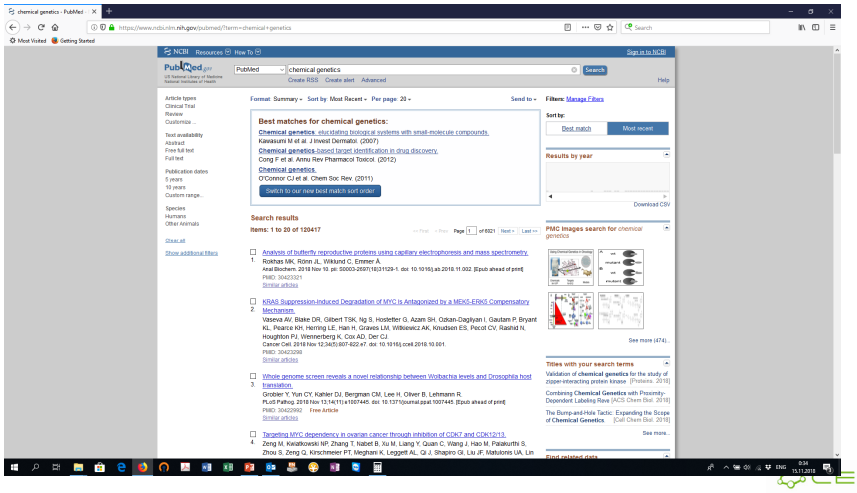
Outline

- Methods of gene expression analysis
  - Qualitative analysis of gene expression
    - Preparation of transcriptional fusion of promoter of analysed gene with a reporter gene
    - Preparation of translational fusion of the coding region of the analysed gene with reporter gene
    - Use of the data available in public databases
    - Tissue- and cell-specific gene expression analysis
  - Quantitative analysis of gene expression
    - DNA and protein chips
    - Next generation transcriptional profiling
- Regulation of gene expression in the identification of gene function by gain-of-function approaches
  - T-DNA activation mutagenesis
  - Ectopic expression and regulated gene expression systems
- Chemical Genetics

Chemical Genetics

- New trends
  - **„chemical genetics“** – more than **50.000/120.417** records in PubMed database (16.10. **2008**/15.11. **2018**, **an increase of >240 %**)



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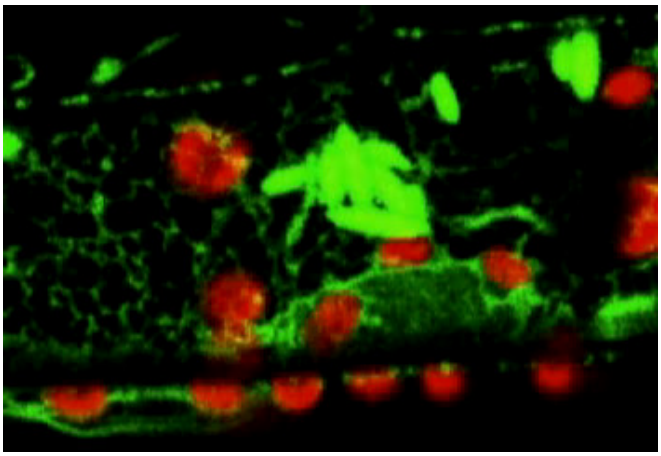
Chemical Genetics

- New trends
  - **„chemical genetics“** – more than **50.000/130.437** records in PubMed database (16.10. **2008**/24.10. **2019**, **an increase of >260 %**)
    - Like in the case of genetics, there are also „**forward**“ and „**reverse**“ genetics approaches
    - Unlike in „classical“ genetics approaches, **the subject of study** is not a gene, but a **protein**
    - Chemical genetics tries to identify either the **target protein** after a chemical treatment and after following phenotypic changes („forward“ chemical genetics) or **chemicals able to interact with protein of interest** („reverse“ chemical genetics)
    - For that purpose there are carried out **searches in the libraries** of various **chemicals** (thousands of entries, comercially available)
    - example: **analysis of endomembrane transport** in plants

Chemical Genetics

- Analysis of mechanisms of endomembrane transport by chemical genetics approaches
  - In plants cells there occurr very dynamic processes mediated mainly by endomembrane transport



GFP targeted to the ER

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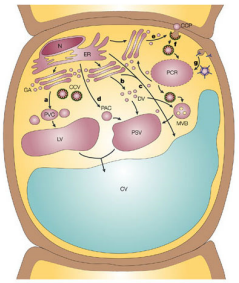
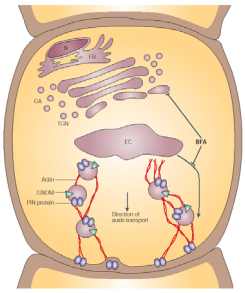
Chemical Genetics

- Analysis of mechanisms of endomembrane transport by chemical genetics approaches
  - In plants cells there occurr very dynamic processes mediated mainly by endomembrane transport (see film, GFP targeting to the ER)
  - Endomembrane transport is an important regulatory mechanism in signal transduction and regulation of cellular processes





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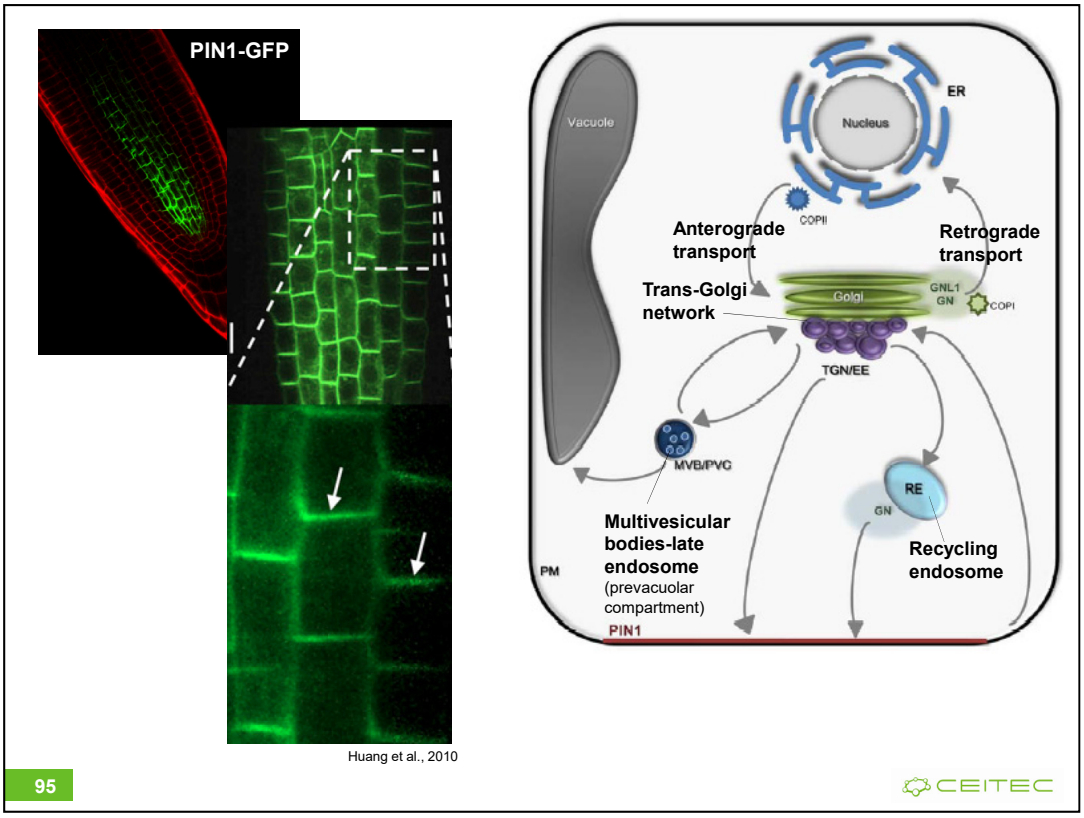


In the figure, there is simplified scheme of vesicle trafficking pathways, regulated by GNOM and its closest relative, GNOM-LIKE1 (GNL1).

Secretory and membrane proteins are synthesised at the ER (blue) and passed onto the Golgi apparatus (green) by anterograde trafficking in COPII-coated vesicles.

The retrograde route from the Golgi apparatus to the ER is regulated by the ARF-GEFs GNOM (GN) and GNL1, which regulate the recruitment of COPI coats to the Golgi membrane. On the secretory route, proteins are transported to the sorting station, the trans-Golgi network (TGN; lilac).

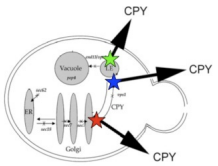
From there, proteins are either transported to the vacuole (grey) via multivesicular bodies (MVB, also called prevacuolar compartment, PVC, which corresponds to the late endosome; deep blue) or trafficked to the plasma membrane (PM).

Plasma membrane proteins like the auxin efflux carrier PIN1 (red), which accumulates at the basal PM at steady state, are continually internalised and trafficked to the TGN, which resembles the early endosome (EE) in plants.

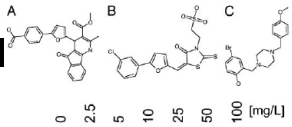
From the TGN, PIN1 is recycled to the plasma membrane via the recycling endosome (RE; light blue). This pathway is regulated by the ARF-GEF GNOM.

Chemical Genetics

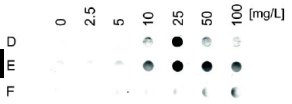
- Analysis of mechanisms of endomembrane transport by chemical genetics approaches
  - By searching in the „library“ of chemicals there were identified those, that lead to the secretion of enzyme (carboxypeptidase Y) in yeast (*S. cerevisiae)* – this enzyme is normally transported to the vacuole via the endomembrane transport
    - Analysis of changes in secretion using dot-blot and immunodetection of carboxypeptidase Y in the culture medium with monoclonal antibodies



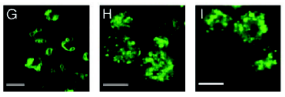
Chemical structure of sortins



Immunodetection of carboxypeptidase



Detection of vacuole phenotype (tonoplast shape) of yeast by staining with a specific color (MDY-64)



Zouhar et al., 2004

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Chemical Genetics

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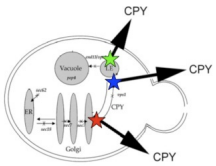
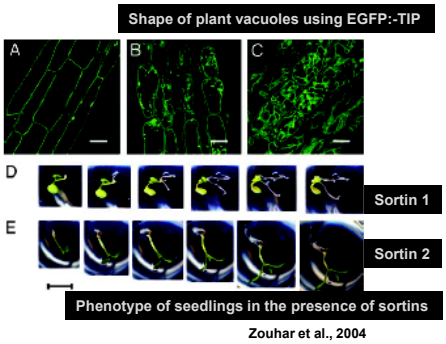
    - Analysis of changes in secretion using dot-blot and immunodetection of carboxypeptidase Y in the culture medium with monoclonal antibodies
  - Identified compounds („sortins“) were able to induce similar changes in *Arabidopsis* as well – transport mechanisms are conserved in yeast and in plants
  - For detailed identification of the molecular proces affected by one of the identified „sortins“, the analysis of its influence on a secretion of a marker protein (AtCPY) was performed – sortin 1 specifically inhibits only this secretory pathway
  - Identifcation of mutants with altered sensitivity to sortin 1 (hyper- or hypo-sensitive mutants) by EMS mutagenesis





Zouhar et al., 2004

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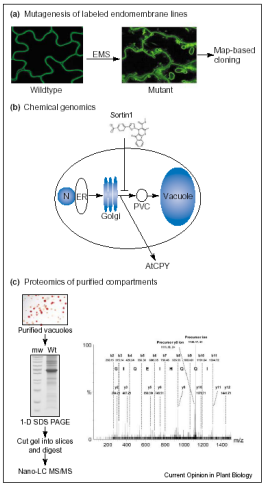
- Analysis of mechanisms of endomembrane transport by chemical genetics approaches – summary

  - GFP::d-TIP vacuole membrane (tonoplast) labelling and identification of mutations leading to altered tonoplast morphology
  - Chemical genetics in combination with classical genetics – identification of proteins participating in regulation of endomembrane transport
  - Proteomics approaches – identification and analysis of vacuole proteome



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Discussion