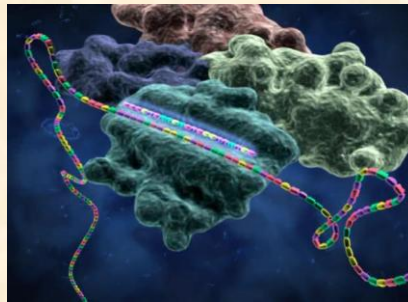


RNS interferencia a *Drosophila* genetikai analízisben



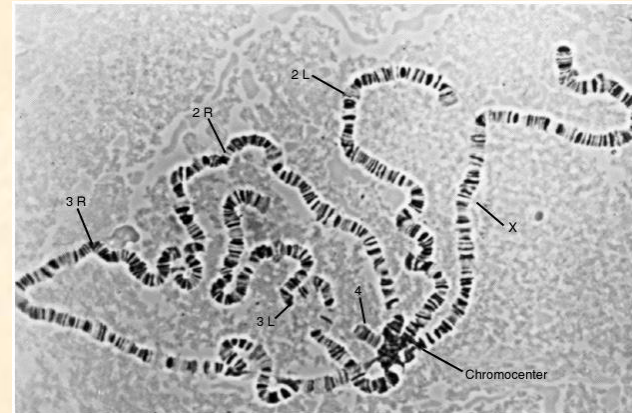
Hegedűs Krisztina

Juhász Gábor

ELTE Anatómiai, Sejt- és Fejlődésbiológiai Tanszék

Klasszikus genetikai modellállat

- 4 kromoszóma: X/Y, 2., 3., 4.
- Genom szekvencia: 2000
- Kb. 14000 gén
- a genom több mint fele konzervált
- génredundancia minimális
- <http://flybase.bio.indiana.edu/>



Thomas H. Morgan
1933 Nobel díj:
Rekombináció,
X kromoszómához
kapcsolt öröklődés



Hermann J. Muller
1946 Nobel díj:
Röntgen-sugárzás
indukálta mutagenézis

Muller's morphs:

amorph (null)

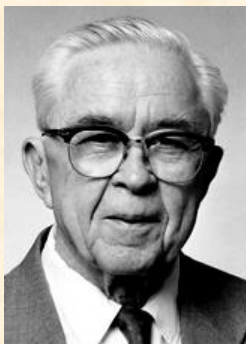
hypomorph (RNAi is általában itt)

hypermorph (túlaktiválás)

antimorph (dom-neg.)

neomorph (új funkció)

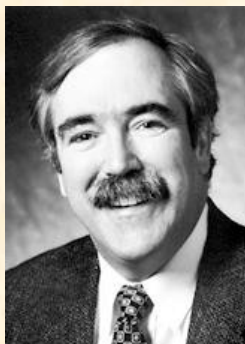
Fejlődésgenetika



Ed Lewis

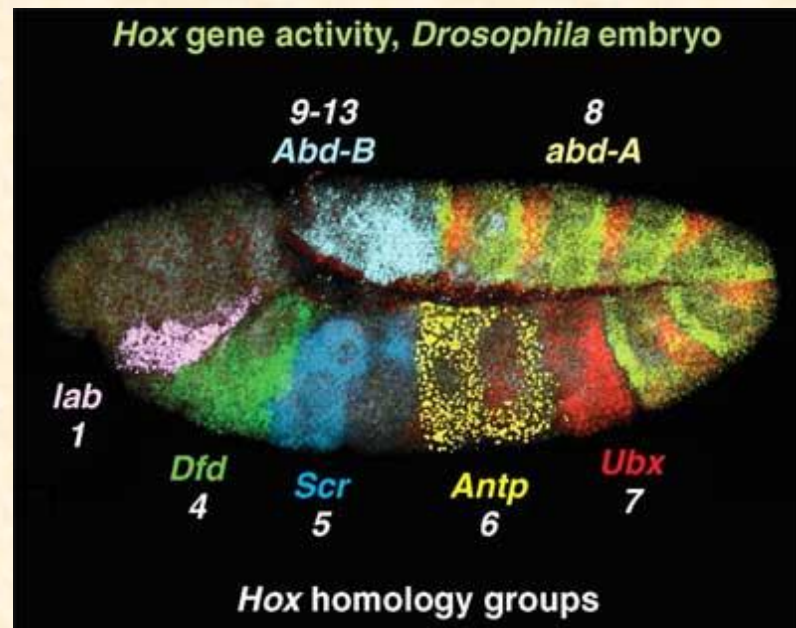


Christiane
Nüsslein-Volhard



Eric Wieschaus

1995 Nobel díj: A korai embrionális
fejlődés genetikája



A sejt- és fejlődésbiológiai folyamatokban
szerepet játszó gének felderítéséhez a
Drosophila-t régóta és igen sikeresen
használják.

Jules Hoffmann (Nobel **2011**)

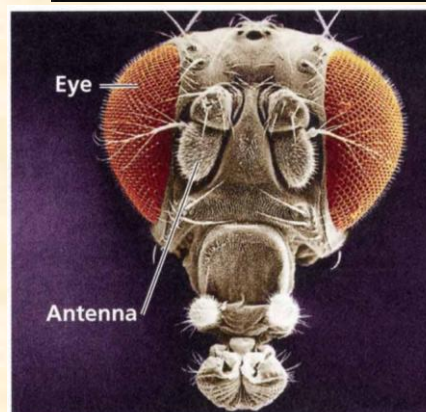


FIGURE 21.14 Homeotic mutations and abnormal pattern formation in *Drosophila*.

Drosophila in vitro rendszerek

- a Drosophila sejtek 25 fokon, normál atmoszférában tenyészthetők a muslica biológiájának köszönhetően
- ~400nt dsRNS-ek használhatók, mivel nem váltanak ki interferon választ
- off-target effektus kevésbé probléma (“kihígul”, mivel sok rövid siRNS keletkezik)
- 3 fő sejtípus érhető el:
 1. embrionális vérsejt eredetű tenyészetek: S2, Kc (transzfektálni sem kell)
 2. imágókorong eredetű tenyészet (hám): clone 8
 3. primer embrionális sejttenyészet (izom, neuron stb.)

In vitro RNSi könyvtárak

| Coverage | Description of reagents | Availability | Comments |
|--|---|--|---|
| Entire <i>D. melanogaster</i> genome | 21,396 dsRNAs, with an average length of 400 bp | PCR products for dsRNA synthesis are available from Eurogentec | Amplification was carried out using gene-specific primers designed to combine genome annotations that are available from the original BDGP/Celera data (13,672 genes) and the Sanger Center data (20,622 genes) |
| The best annotated <i>D. melanogaster</i> genes | 13,071 dsRNAs of 300–800 bp | dsRNAs are available from Ambion | Design based on Flybase v3 |
| Most <i>D. melanogaster</i> genes that are phylogenetically conserved with mammalian genes | 7,216 dsRNAs of 300–600 bp | dsRNAs are available from Open Biosystems | For each gene, the exonic sequence was amplified using gene-specific primers |
| Genes that are represented in the cDNA set 1 collection from the BDGP | 4,923 dsRNAs of variable size | dsRNAs are available from the authors of REFS 14,73 | |

www.flyrnai.org

www.flyrnai.org

BestGene Bloom DGRC DSHB EISZ ELTE Webmail ELTElefon Fluent FlyBase Gmail NIG-Fly Neptun OTKA PubMed SZTAKI VDRG Workbench

DRSC & TRiP

Drosophila RNAi Screening Center at Harvard Medical School

ABOUT ASSAYS REAGENTS PROTOCOLS APPLY PUBLICATIONS ONLINE TOOLS TRiP ENTER

Welcome to the Drosophila RNAi Screening Center (DRSC)

TALENs and CRISPRs RNAi Libraries Custom RNAi (PCR, cherry pick, etc)

Search our Database

CG, Gene Symbol or FBgn

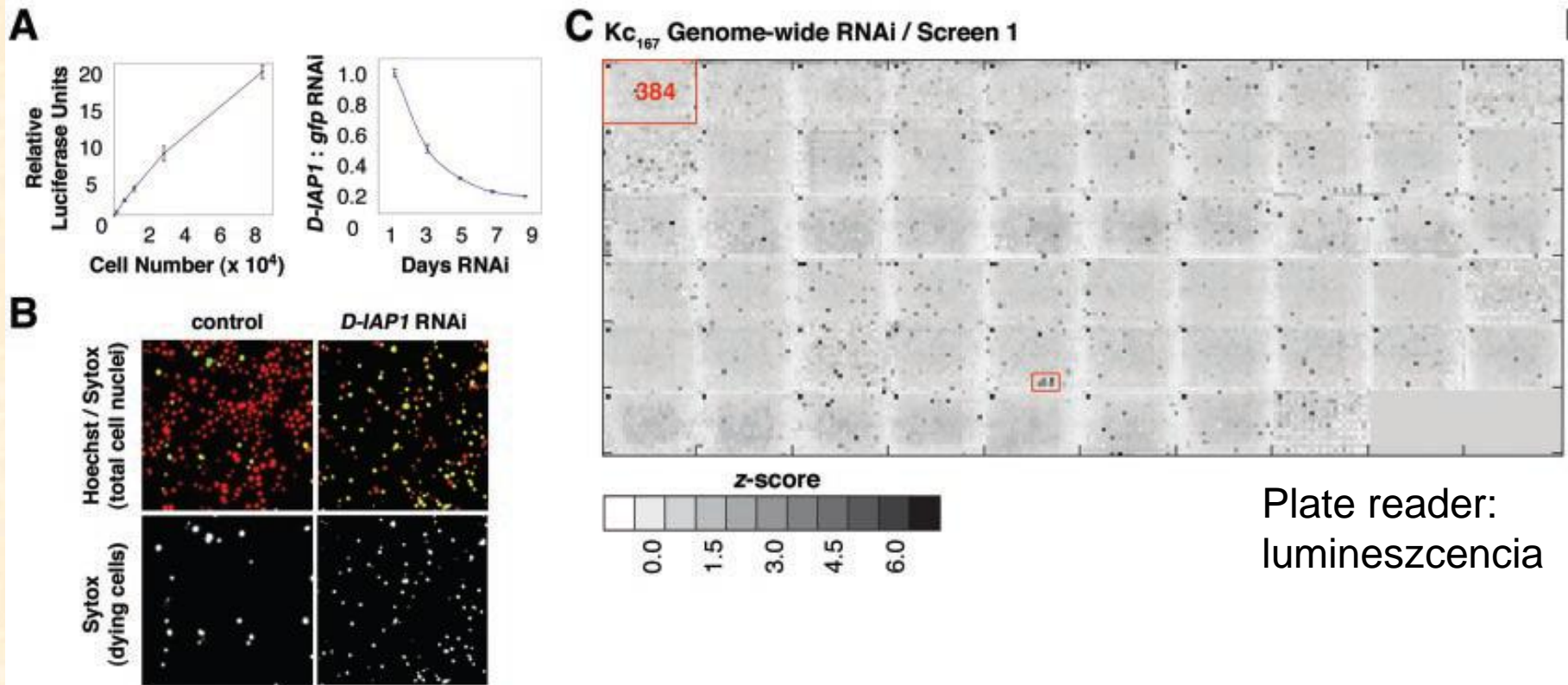
view dsRNA amplification screen

Currently Available Reagents at the DRSC

- Genome-wide RNAi
- RNAi Validation
- sub-libraries for RNAi
- UAS-x Reagents
- Rescue Fosmids (other *Drosophila* species)
- TRiP

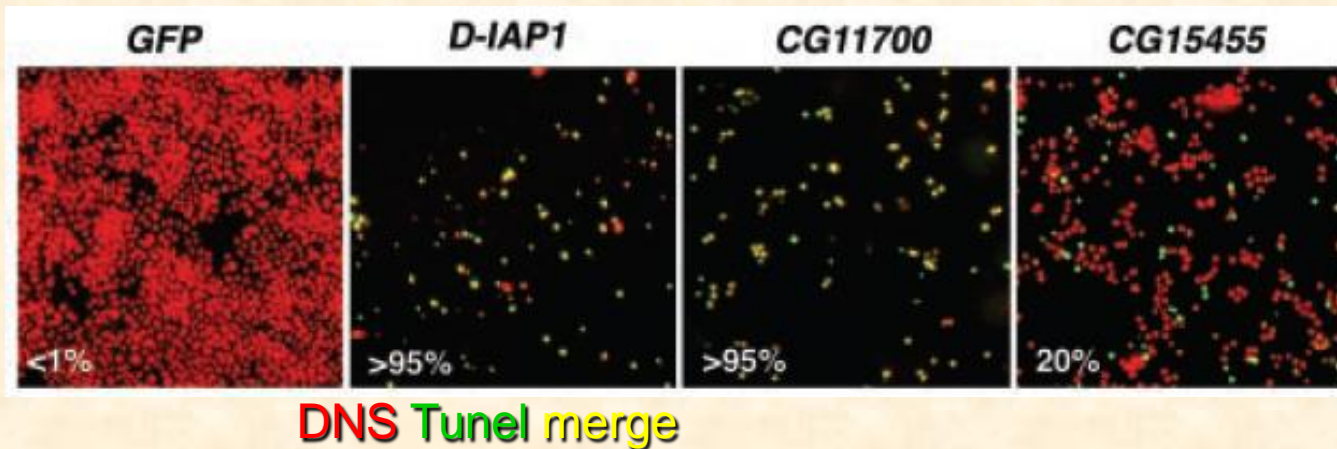


Genome-Wide RNAi Analysis of Growth and Viability in *Drosophila* Cells (2004 Science)

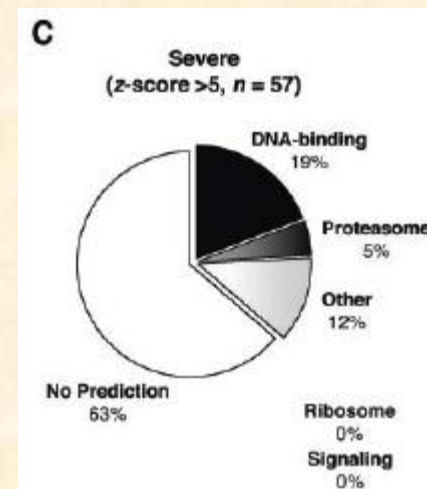
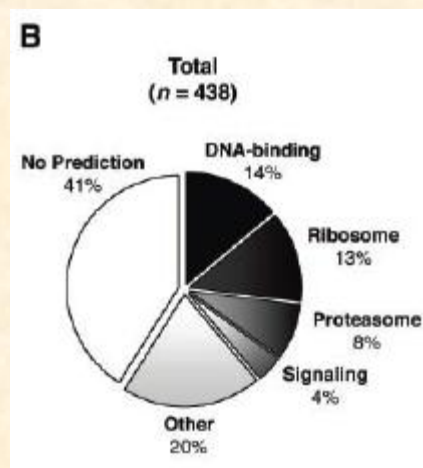


z score: szignifikancia faktor, azaz az adott gén csendesítése mennyire reprodukálható módon váltja ki a látott fenotípust

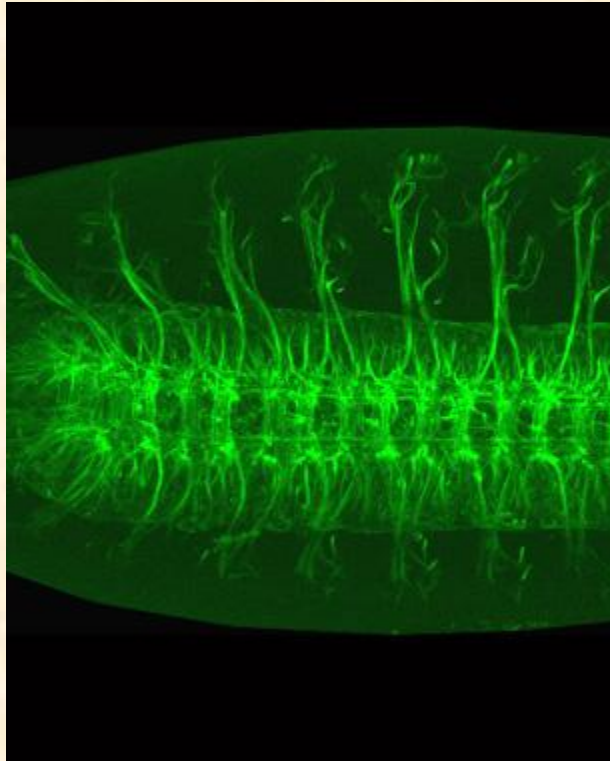
A sejtszámot befolyásoló tényezők: sejthalál, sejtnövekedés, sejtosztódás.
Sejthalálra példák:



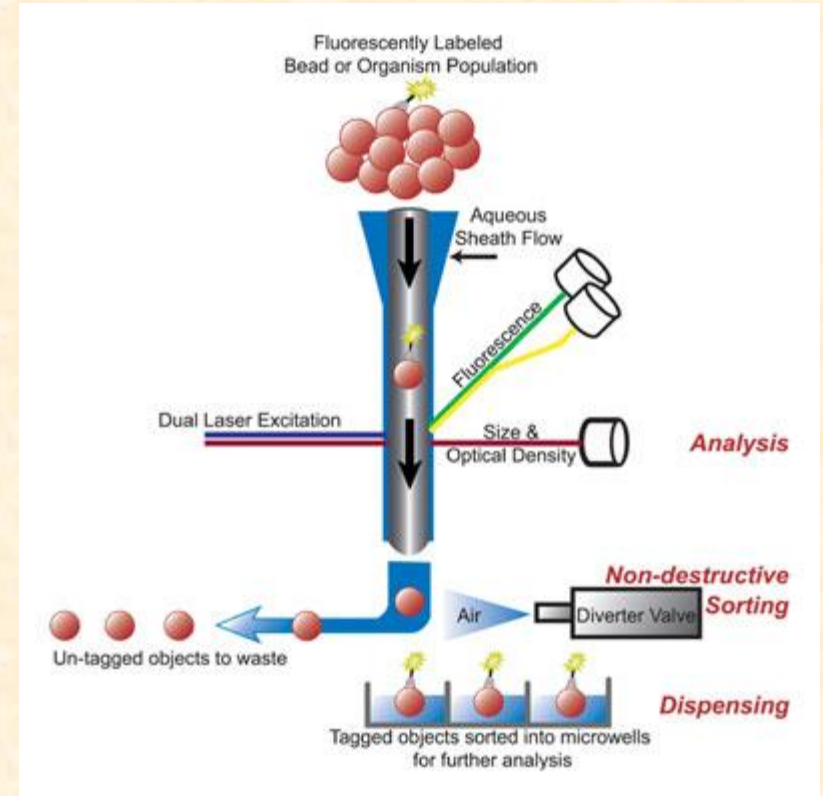
Az eredmények csoportosítása:



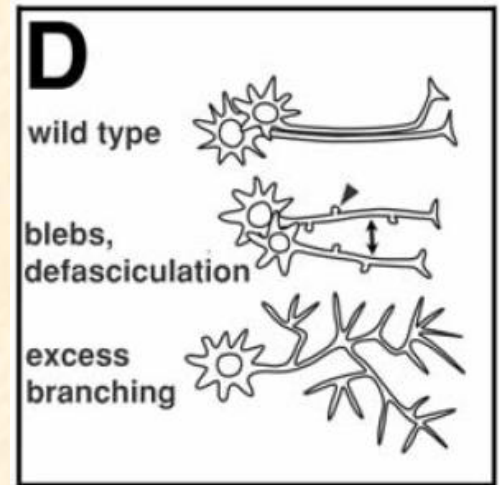
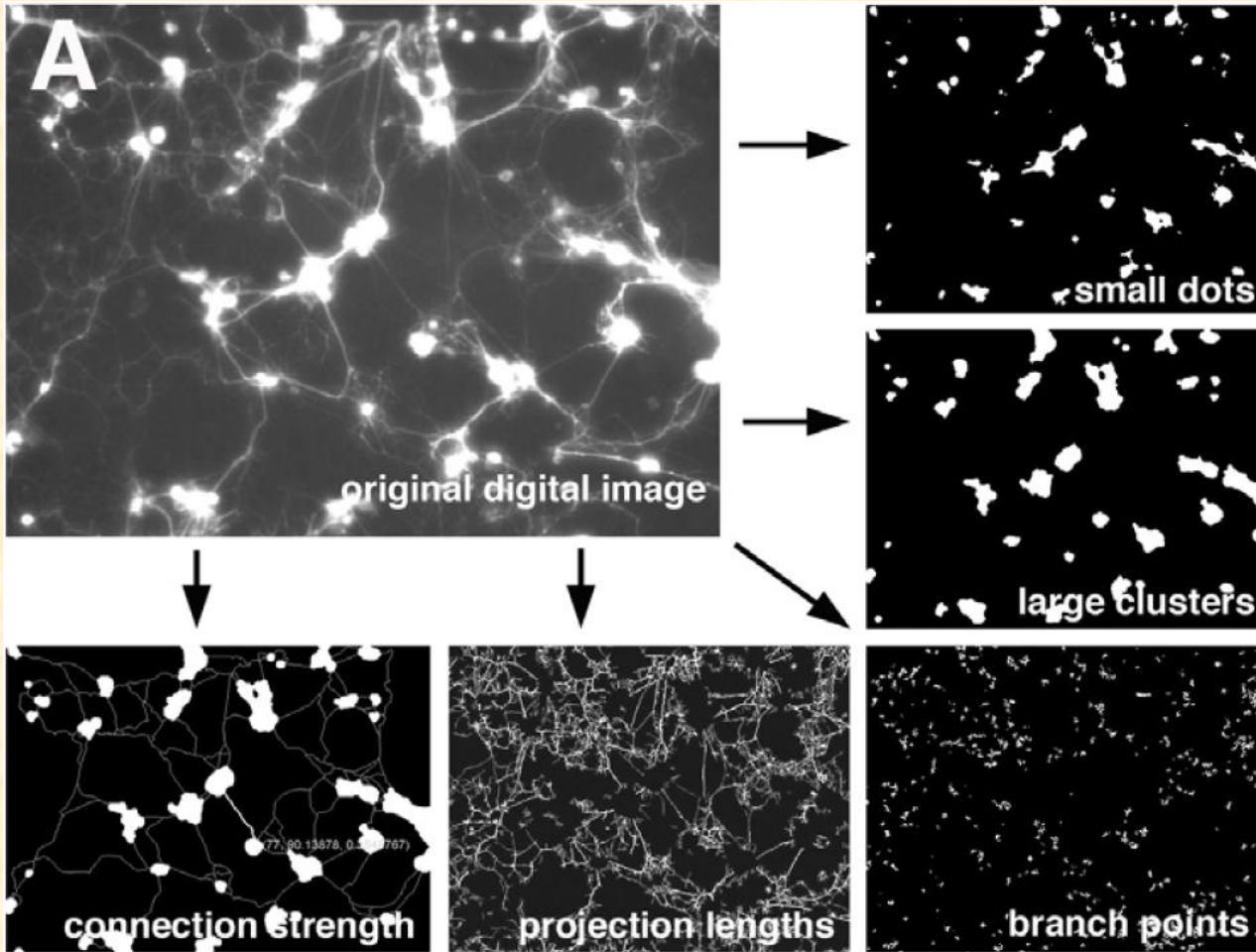
Identification of Neural Outgrowth Genes using Genome-Wide RNAi (2008 PLoS Genetics)

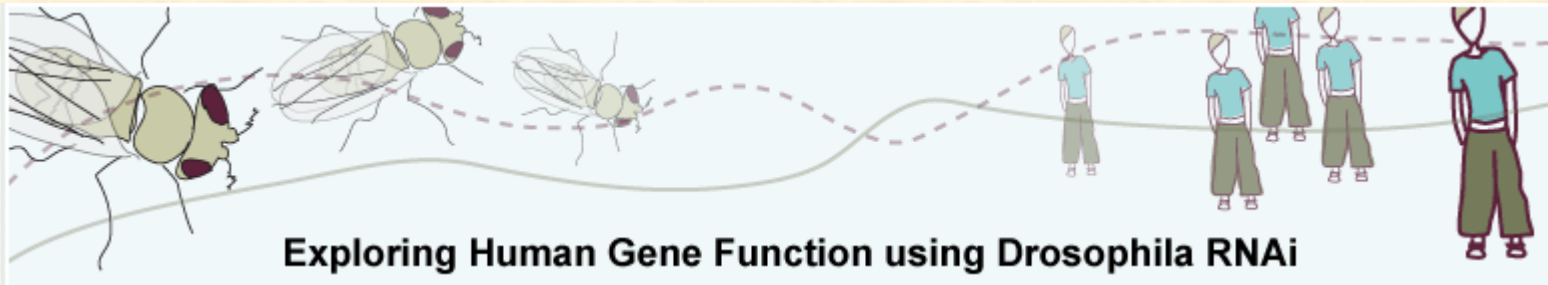


Primer sejtenyésztet: embrió disszociáció, majd GFP+ sejtek szelekciója FACS révén (Fluorescence-Activated Cell Sorting)



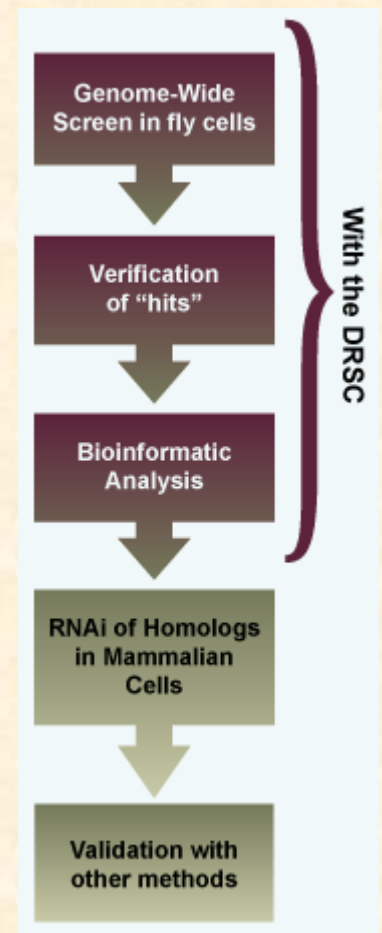
Primer neuron tenyésztet kezelem dsRNS-ekkel, majd automata mikroszkópia és szoftveres analízis.



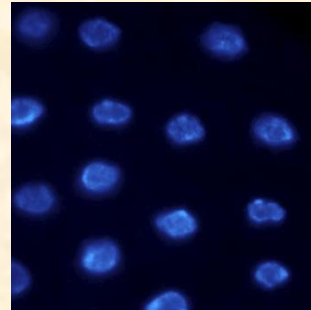
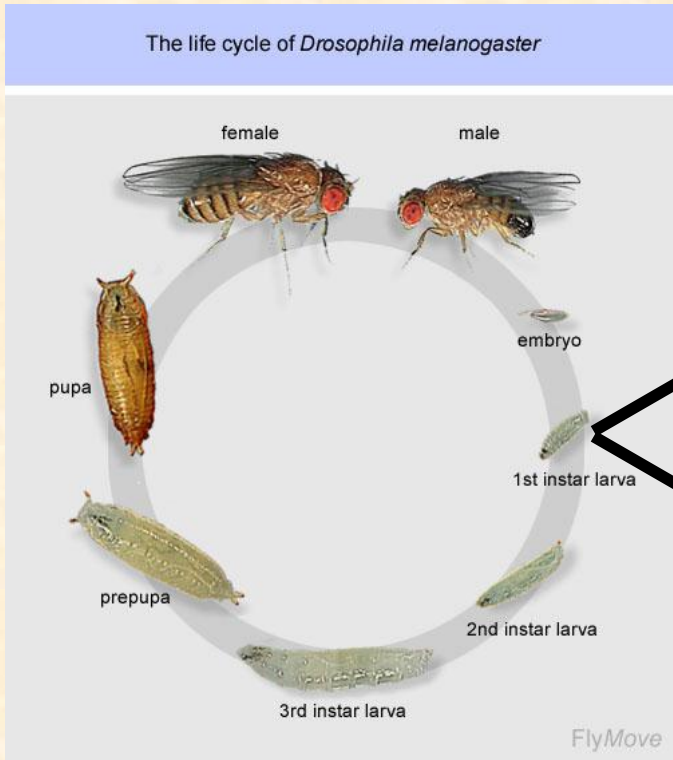


Előnyök

- Gyorsabb, olcsóbb teljes genom szűrés
- Mininális génredundancia elősegíti a funkció azonosítását
- Jól annotált genom (kódoló és nem kódoló RNS-ekre is)
- Konzervált útvonalak, homológ gének nagy száma

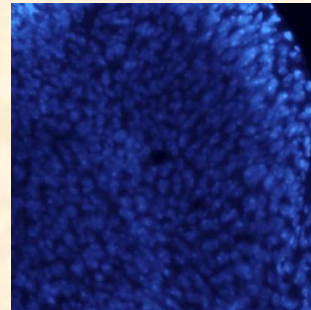


In vivo kísérletek

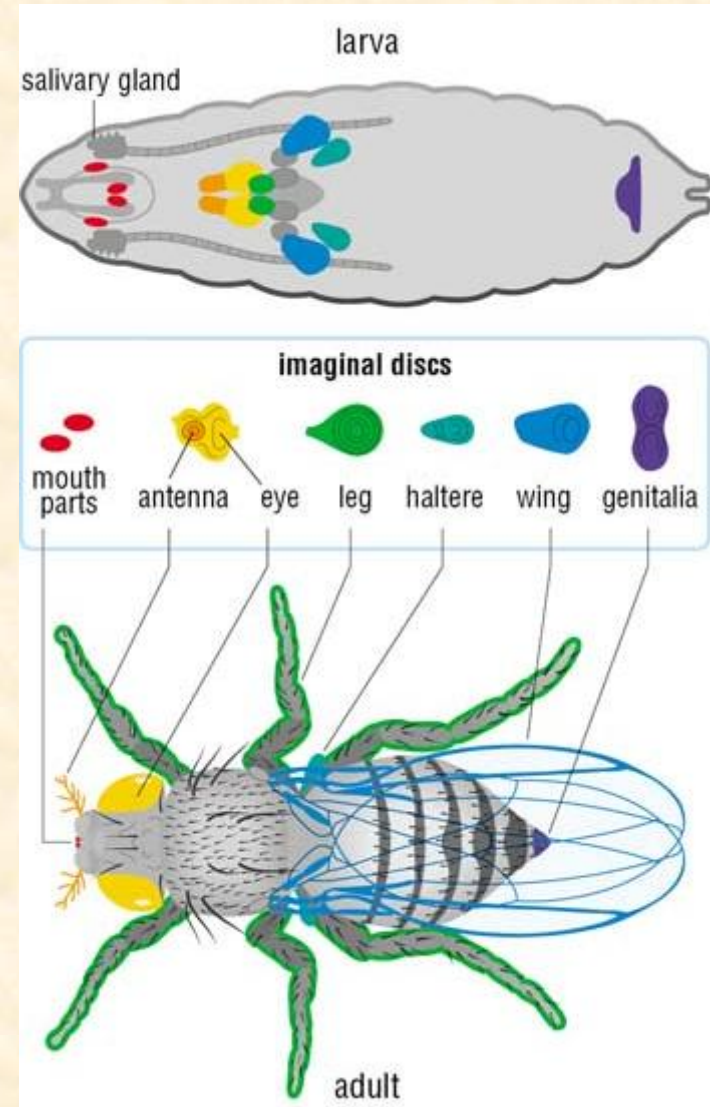


poliploid sejtek

diploid sejtek

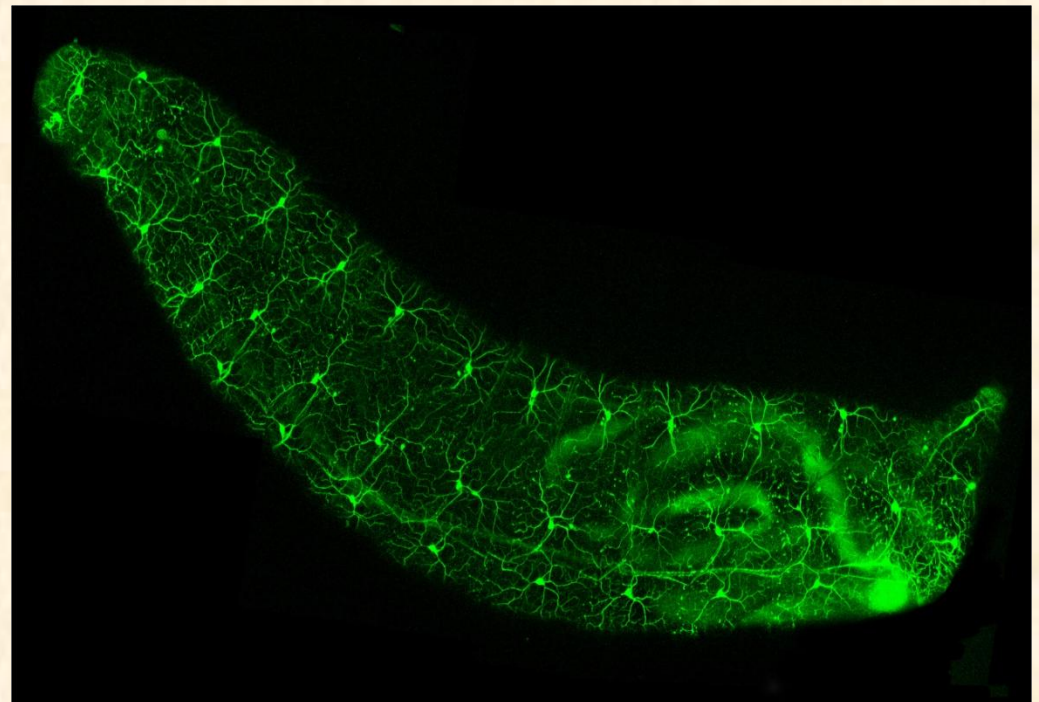
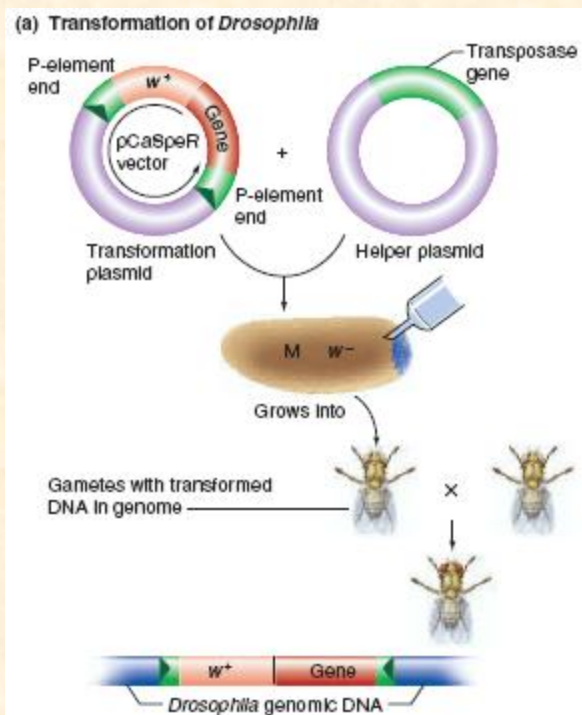


Olcsón és könnyen tartható,
az egyik legjobb molekuláris
genetikai és sejtbiológiai modell.



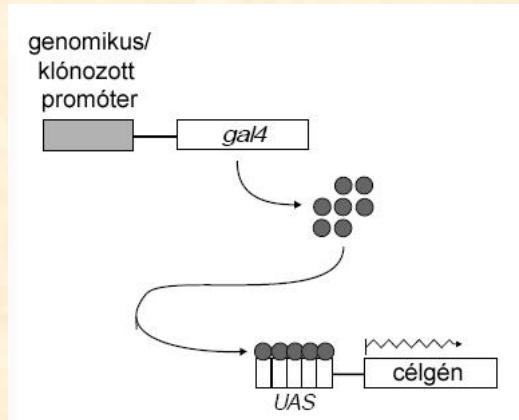
Transzformáció (transzgén)

- Az első években dsRNS-sel injektálták az embriókat, de csak gyenge és rövid ideig tartó hatást lehetett így elérni (etetés nem működik).
- A módosított P-elemet és a transzpozázt kódoló DNS-t összekeverve kell szinciciális blasztoderma stádiumú, w- embriókba injektálni. Ha a kikelő w- legyek utódai között w+ állatot találunk, az hordozza a transzgént.



Drosophila érzőneuronok a lárvában, GFP reporter gén jelölés.

Az UAS-Gal4 rendszer



Kétlépcsős transzgén rendszer:

Gal4 élesztő transzkripciós faktor, ami a megfelelő DNS-szekvenciához köt a célgén promóterében (UAS – upstream activating sequence), és elindítja a génexpressziót.

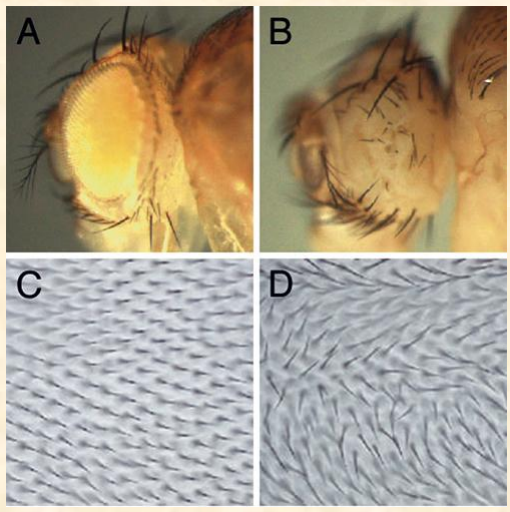
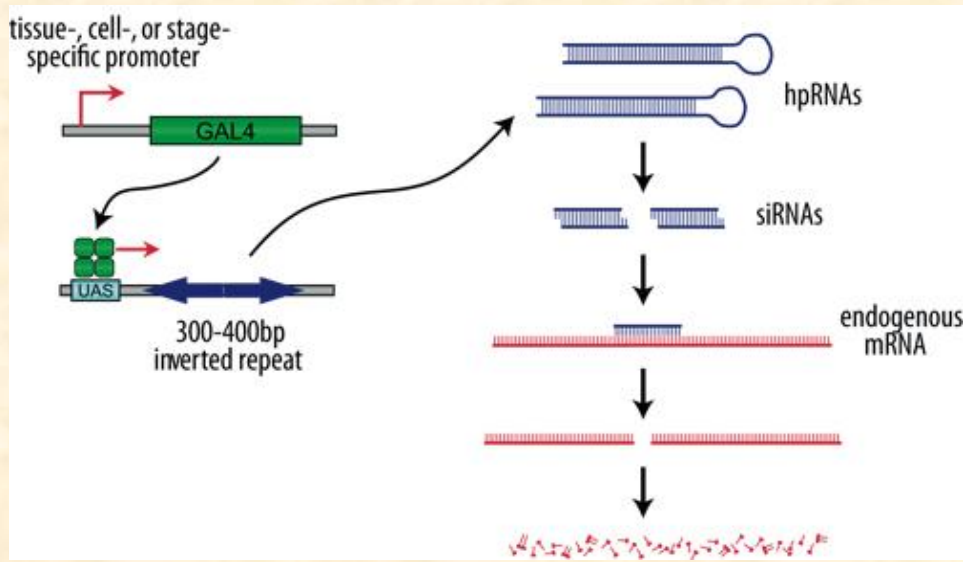


dpp-Gal4, UAS-eyeless

A Gal4 „driver”-ek bármely UAS-transzgén kapcsolgatására felhasználhatók.

1000 Gal4 vonal + 1000 UAS transzgén: 1000000 lehetséges kombináció!

In vivo UAS-RNSi könyvtárak



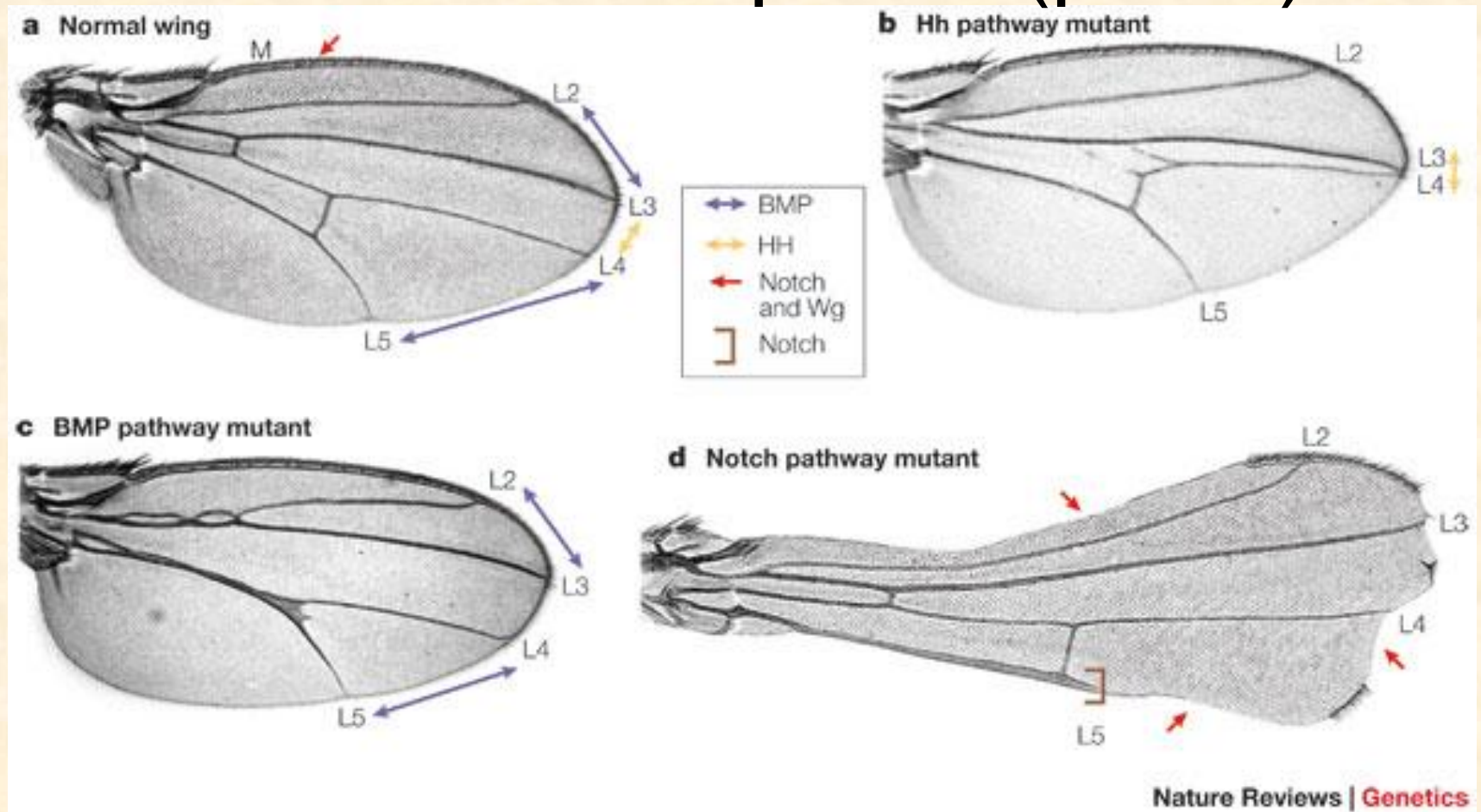
eyeless RNAi

flamingo RNAi

RNAi phenotypes. (A). Control with GAL4 driver only. (B). GAL4 driver + UAS-eyRNAi, targeting the eyeless gene. The eye is missing, as in the eyeless mutant. (C). Wing hairs in a wild-type fly all point in the same direction. (D). GAL4 driver + UAS-fmiRNAi, targeting flamingo, a gene required for planar cell polarity. The wing hairs are misorientated, as in the flamingo mutant.

- <http://www.vdrc.at/>
~13000 génre elérhető transzgén RNSi vonalak (~26000 törzs): a genom ~90%-át lefedi.
1. (GD: random) és 2. generációs (KK: irányított)
- http://flystocks.bio.indiana.edu/Browse/RNAi/RNAi_all.php
~9000 törzs
2. (irányított shRNAi) és 3. generációs (miR alapú)
- <http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp>
A Mishima-i (Japán) UAS-RNSi törzsgyűjtemény ~6200 génre (~11600 törzs).
1. generációs

Keresett fenotípusok (példa)



The *Drosophila melanogaster* wing provides an assay system for several signalling pathways. a | A wild-type wing with the wing margin (M) and the longitudinal veins L2–L5 indicated. The spacing between the L3 and L4 veins is determined during the second and third larval instars by hedgehog (HH) signalling (yellow double arrow), whereas the spacing of the L2–L3 and L4–L5 veins is determined by bone morphogenetic protein (BMP) signalling (blue double arrows). The margin, which forms at the interface between the dorsal and ventral compartments of the wing (FIG. 2b), forms in response to a reciprocal form of Notch induction that leads to production of the wingless (WG) morphogen along the margin (Notch and WG signalling indicated by red arrow). b | A wing in which reduced HH signalling has selectively narrowed the spacing between the L3 and L4 veins (yellow double arrows). c | A wing in which reduction of BMP signalling has selectively reduced the distances between the L2–L3 and L4–L5 veins leading to partial fusion of these vein pairs (blue double arrows). d | A wing with reduced Notch activity exhibiting both notches in the wing margin (red arrows) and thickened veins (bracket on L5 vein; L3 is also broader than normal). This combination of wing margin nicks and thickened veins is diagnostic for a defect in Notch signalling, whereas defects in the wing margin alone might indicate reduced WG signalling.

Második generációs könyvtárak

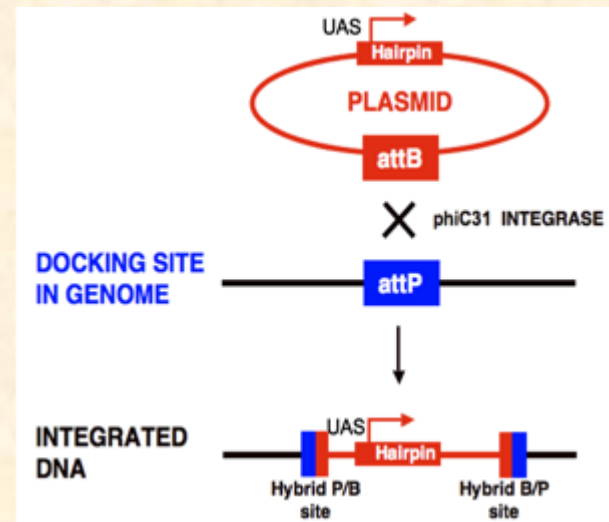
(04/2010: VDRC: ~10000, Perrimon Lab: ~2000 vonal)

Egyre jobban ismert az RNSi útvonal működése, és a *Drosophila* molekuláris genetika is fejlődik továbbra is, így optimalizálhatók a reagensek:

1. jobb hatásfokú géncsendesítés (kevesebb fals negatív)

2. kevesebb off-target
(kevesebb fals pozitív találat)

3. helyspecifikus inszerció:
phiC31 révén helyspecifikus
rekombináció meghatározott
„landing/docking platform”-ra



A helyspecifikus inszerció előnyei

- a random P-elemesnél ötször gyakrabban kapok az injektálásból transzgén állatot
- tudom, hova ült be a genomba (landing site), így nem kell kromoszómára térképezni
- minden transzgén vonal homozigóta életképes
- minden transzgén ugyanolyan jól expresszál, így kevesebb a fals negatív találat (random P elem inszerció történhet heterokromatikus régióba)
- az inszerció melletti gén aktiválása sem okoz fals pozitív találatot – a vonalak >5%-a 1. generációs könyvtárnál!
- verifikációra használhatóak a különböző 1., 2., 3. generációs könyvtárak – és mutánsok (kb. genom 60%-ra már van mutáns!)

MikroRNS alapú vonalak (3. gen.)

TRiP

Transgenic RNAi Project

at Harvard Medical School

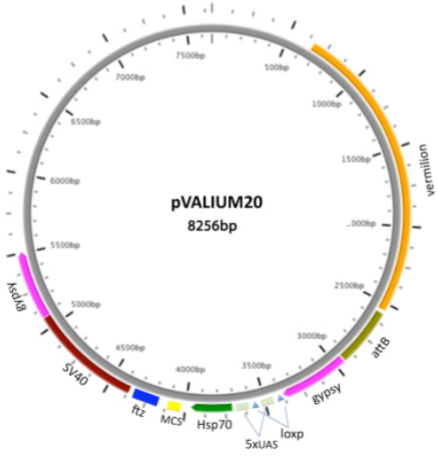
HOME ABOUT APPROACH TRIP STOCKS TRIP REAGENTS FAQ CONTACT DRSC

TRIP >> Approach - 2nd Generation

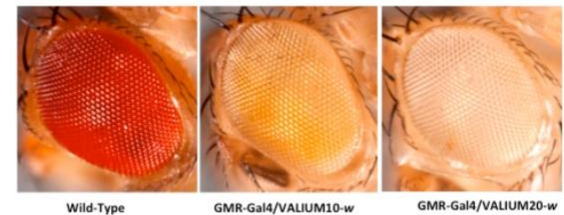
Design of the Second Generation VALIUM Vectors

The TRiP generated a series of vectors referred to as the "VALIUM series" (Vermilion-AttB-Loxp-Intron-UAS-MCS) (Ni et al., 2008; Ni et al., 2009). Here we describe VALIUM20 and VALIUM22 - the second generation of TRiP vectors. These vectors were designed to deliver siRNAs using the endogenous microRNA pathway.

Our use of the microRNA cassette is based on the shRNA design of Haley et al. (2008, 2010) with modifications (Ni et al., 2010).

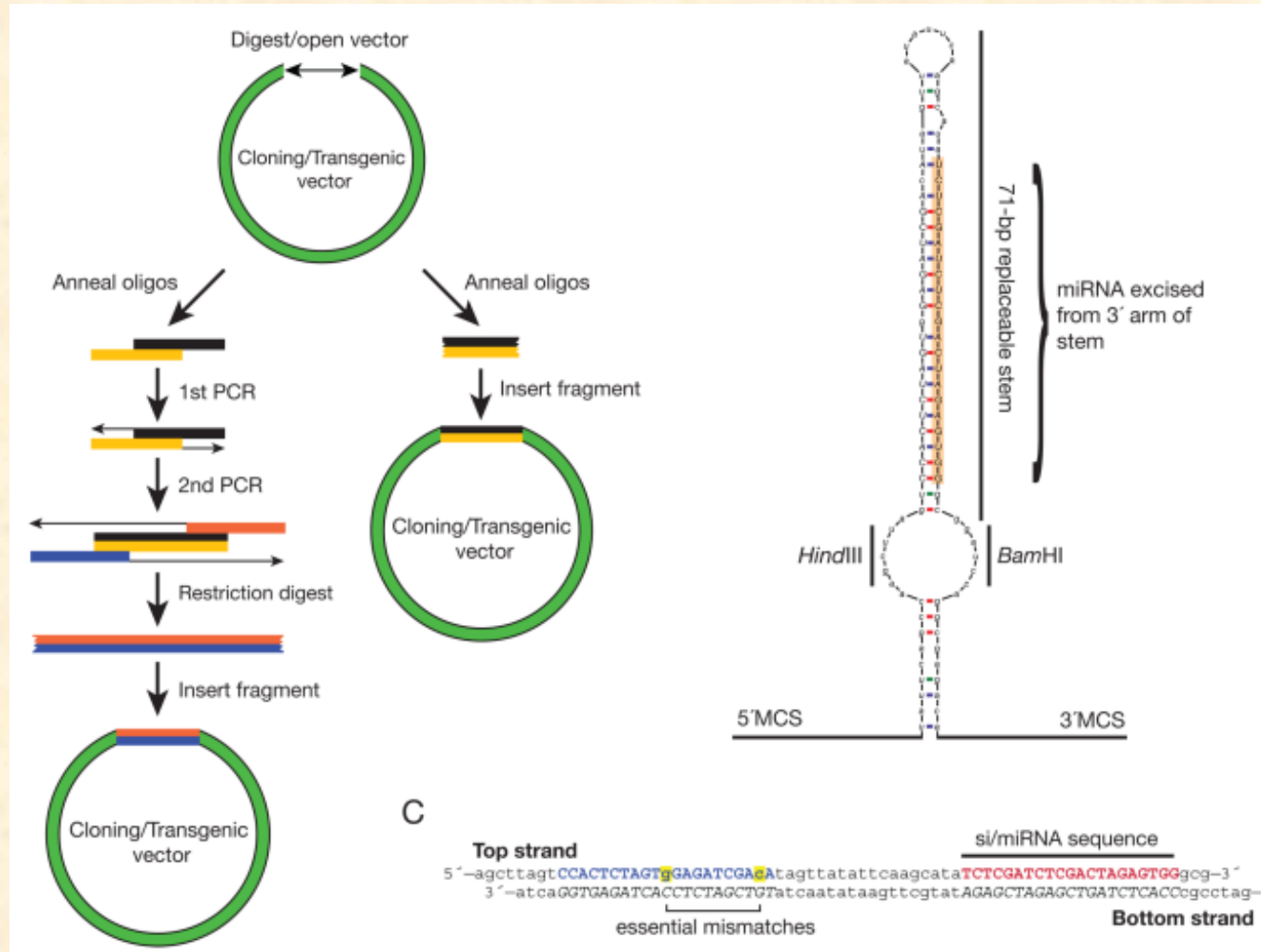


Our data shows that VALIUM20 gives a stronger knockdown than VALIUM10 in the soma, and works well in the germline (Ni et al., 2010).

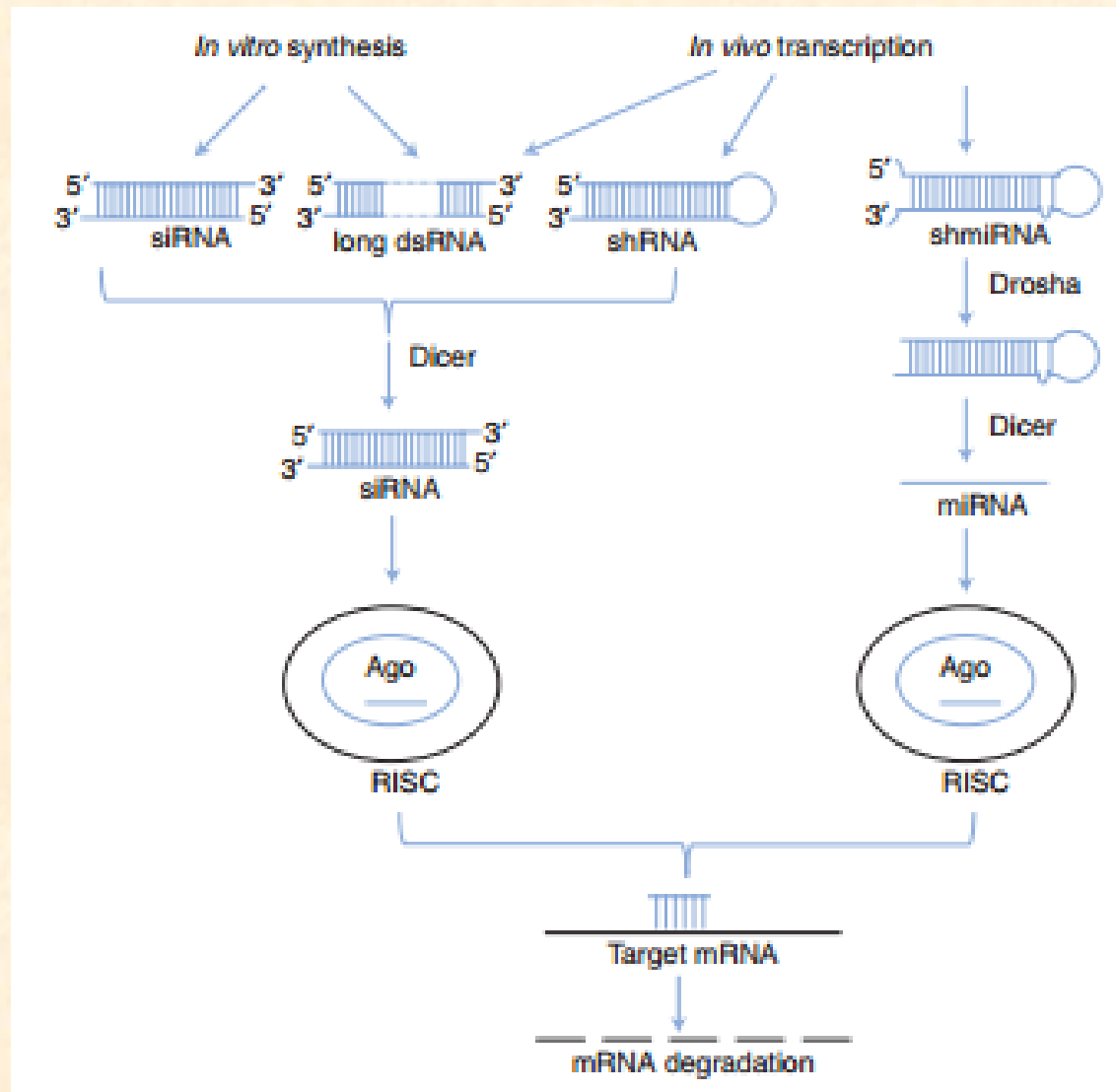


Csíravonalban is működnek, és az RNAi rescue is könnyebb ezekkel!

shmiR vektorok



siRNAs, shRNAs, shmiRNAs, and long dsRNAs pathways

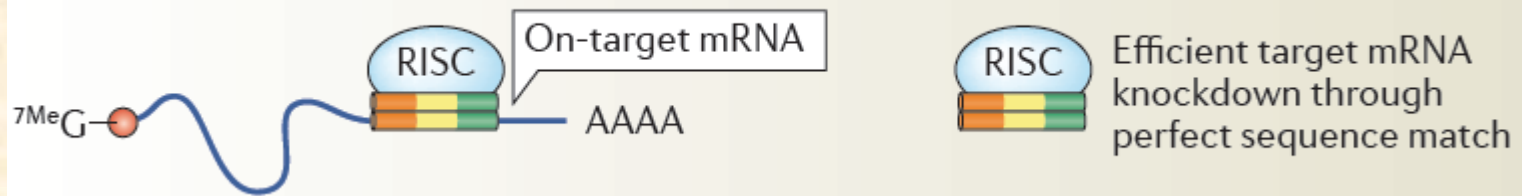


Methods for experimental verification of RNAi screen results

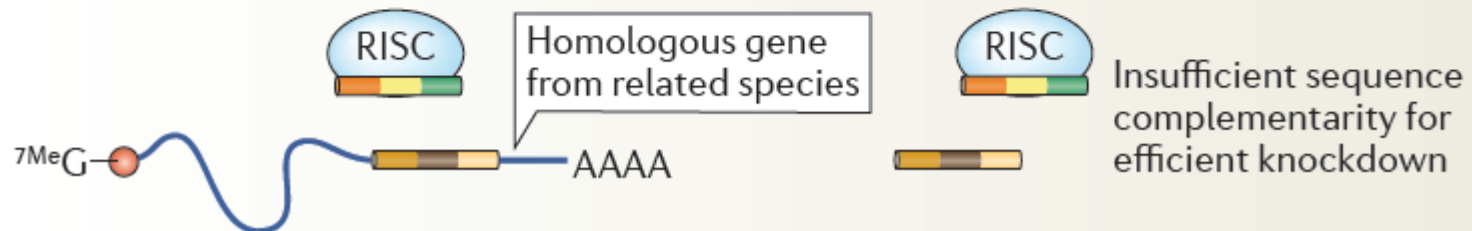
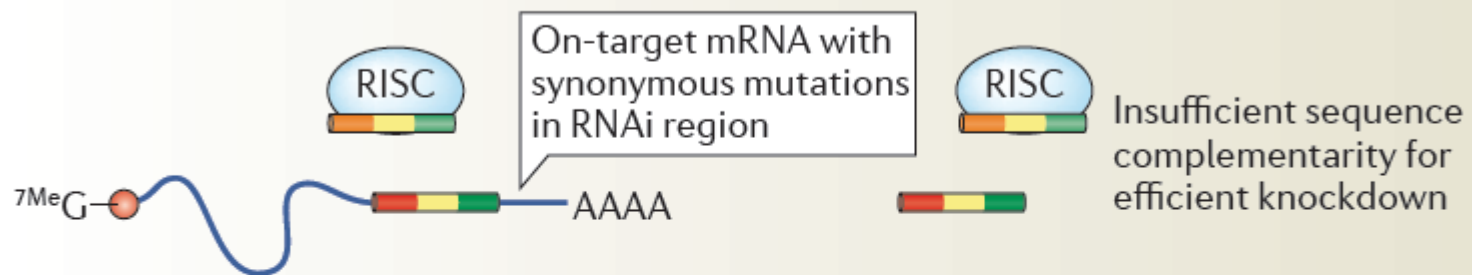
| Method | Examples | Rationale |
|--|--|---|
| Retest the reagents with the same assay | Test several replicates (including a re-synthesized or new batch of reagent); test single reagents in arrayed format after a pooled approach | Reagent-level verification |
| Retest with a related assay and/or different cell type | Switch the reporters in a dual-reporter assay; test a different cell line, marker, or antibody; test in a different cell line | Reagent-level verification |
| Retest with unique reagents | Test reagents designed to target different regions of the gene | Gene-level verification (confidence increases when more than one works) |
| Assay small molecule(s) | Test a known inhibitor of the gene product in the assay; test small molecules in parallel with RNAi and compare pathways implicated in each | Gene-level verification (correlation is suggestive of an on-target effect) |
| Determine mRNA or protein levels in the presence of the RNAi reagent | Q-PCR or immunoblotting ^a | Gene-level verification (correlation between knockdown and phenotype is suggestive of an on-target effect) |
| Rescue in the presence of the RNAi reagent ^b | Test rescue with a genomic fragment, cDNA, or open reading frame construct that evades RNAi knockdown | Gene-level verification (rescue demonstrates an on-target effect) |
| Pattern of gene expression of mRNAs corresponding to hits | Q-PCR or microarray in specific cell types, stages, and/or tissues | Gene-level verification (expression in relevant tissues or stages is suggestive of a relevant finding) |
| Pattern of expression of the proteins corresponding to hits | Immunoblotting in specific cell types, stages, and/or tissues | Gene-level verification (expression in relevant tissues or stages is suggestive of a relevant finding) |
| Subcellular distribution of proteins corresponding to hits | GFP-tagged construct or immunofluorescence | Gene-level verification (expression in relevant subcellular compartments is indicative of a relevant finding) |
| RNAi-induced phenotype in another species | Test effect of knockdown of homologs in mammalian cells as a follow-up to a nonmammalian cell screen | Gene-level verification (similar phenotype provides compelling evidence of a biologically relevant finding) |
| Correlation with a related disease or disorder | Map disease-associated regions, mutations, and amplifications | Gene- and pathway-level verification (disease association is indicative of a relevant finding) |
| Protein-protein interactions | Coimmunoprecipitation, mass spectrometry, yeast two-hybrid screen | Gene- and pathway-level verification (physical interactions among newly identified proteins or between new and established players are indicative of a relevant finding) |
| Genetic analysis in vivo | Test effects of mutations of gene hits in whole animals (same or different species than primary screen cells) | Gene and pathway-level verification [related phenotype provides compelling evidence of a relevant effect and can help refine the role(s) of the genes in specific pathways, events, or behaviors] |

Menekítés

a On-target knockdown

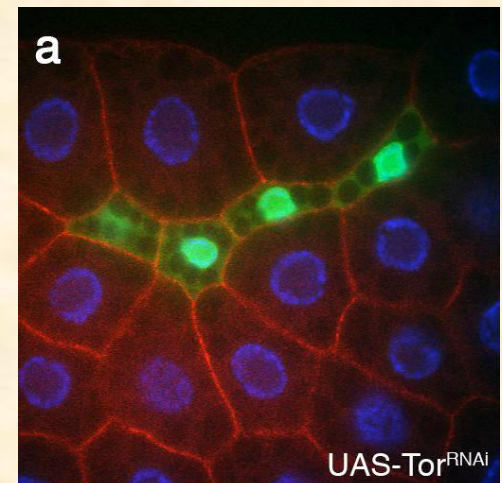
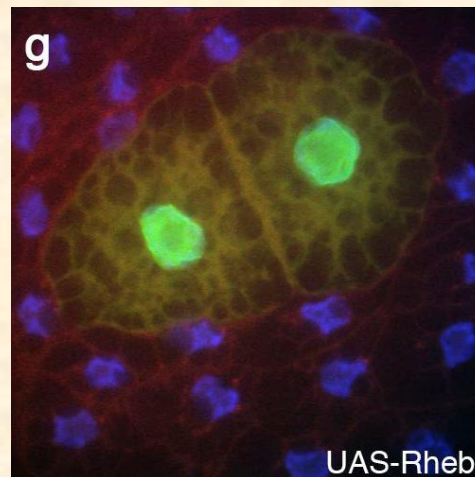
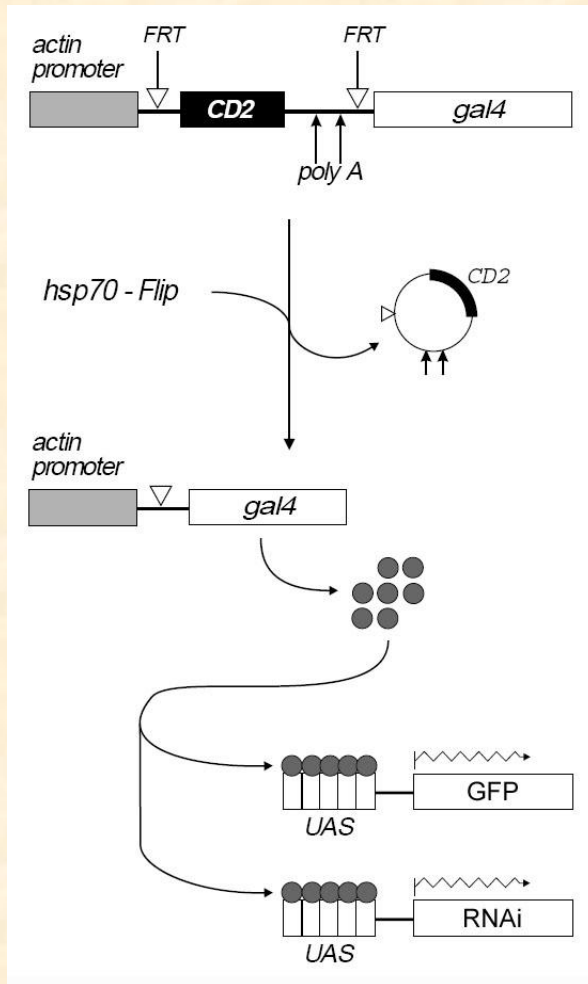


c Validation with RNAi-resistant mRNA

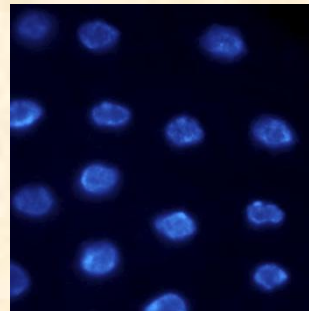
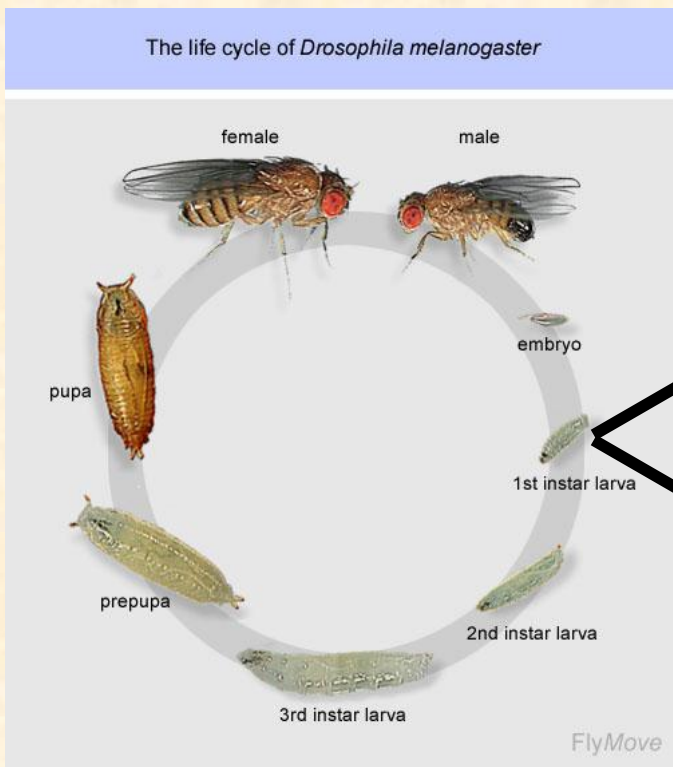


Szomatikus klónok

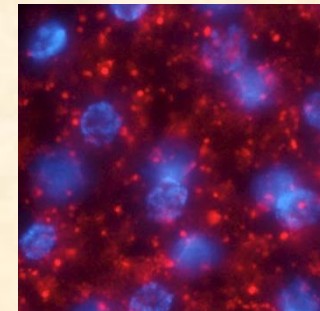
Így létre lehet hozni funkciónyeréses (túltermelő) és funkcióvesztéses (RNSi vagy domináns-negatív) klónokat UAS- vagy UAS-RNSi transzgénekkal:



Autofágia: az eukarióta sejtek lebontó és újrahasznosító folyamata



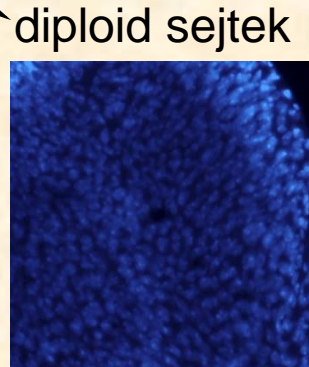
polyploid sejtek



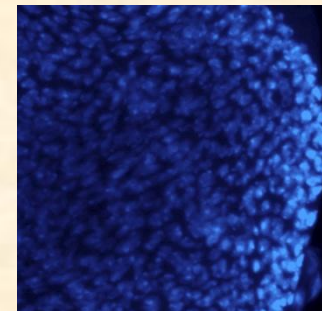
autofágia

éhezés vagy metamorfózis

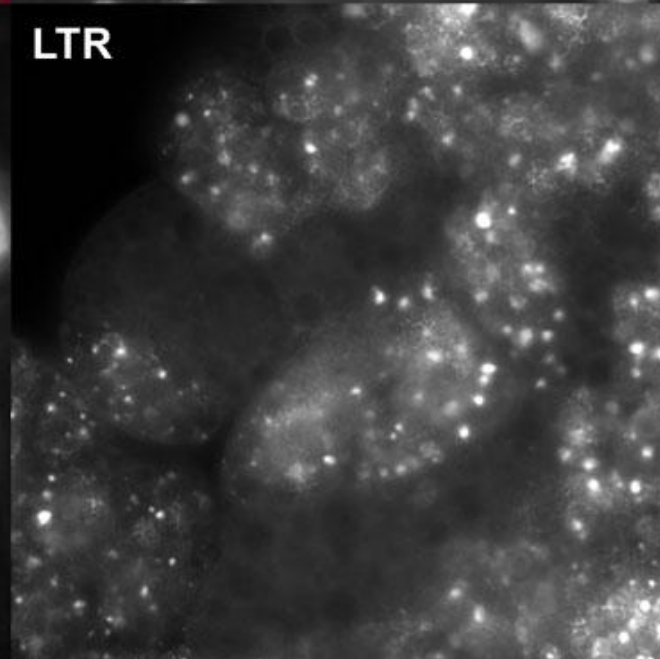
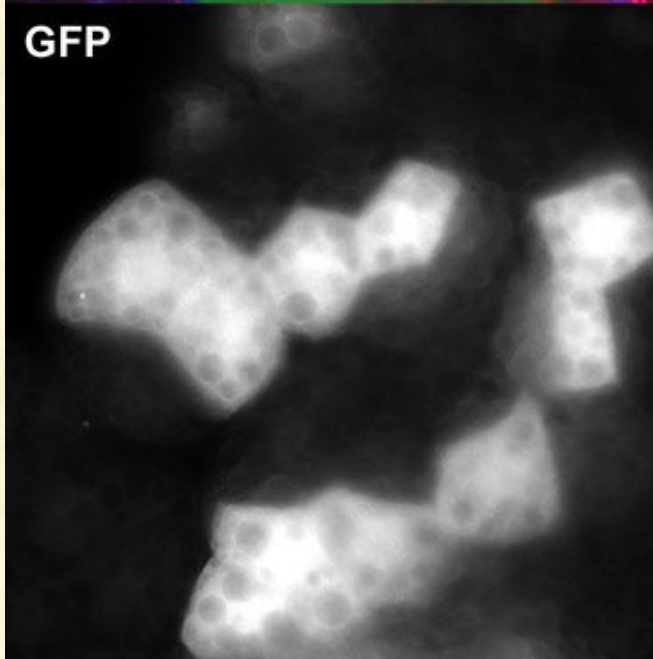
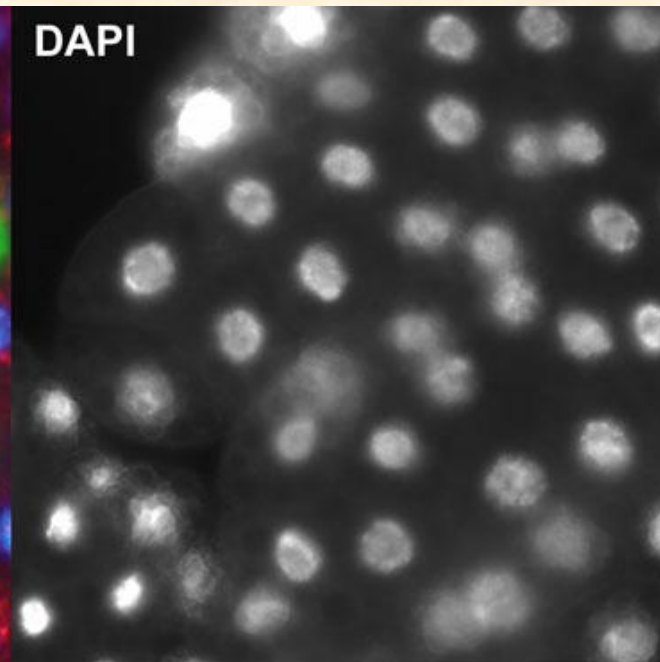
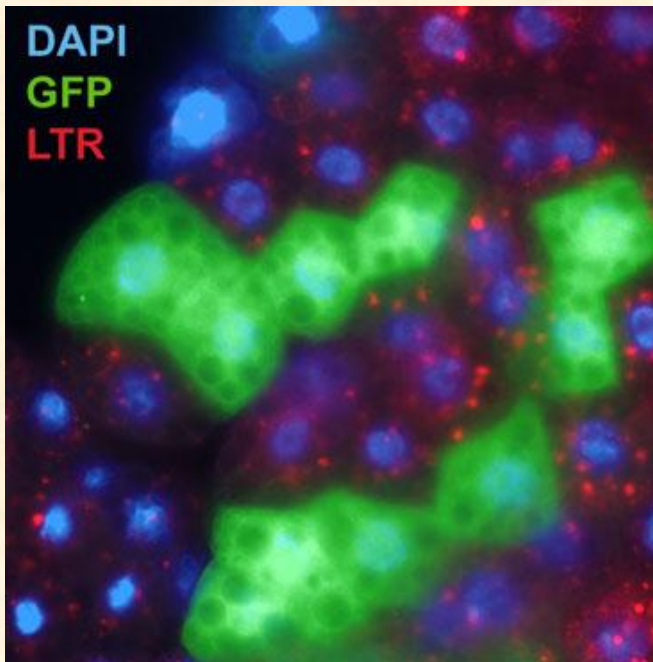
tápanyagok
energia



diploid sejtek



Atg2
RNSi



Köszönöm a figyelmet!

