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OP Education for Competitiveness

INVESTMENTS IN EDUCATION DEVELOPMENT

IMG Institute of Molecular Genetics of the ASCR, v. v. i.

Cell tracking and manipulation in genetically modified mice

An insight from digestive trackt: intestine and liver

Bohumil Fafílek

Institute of Molecular Genetics of the ASCR, v.v.i.

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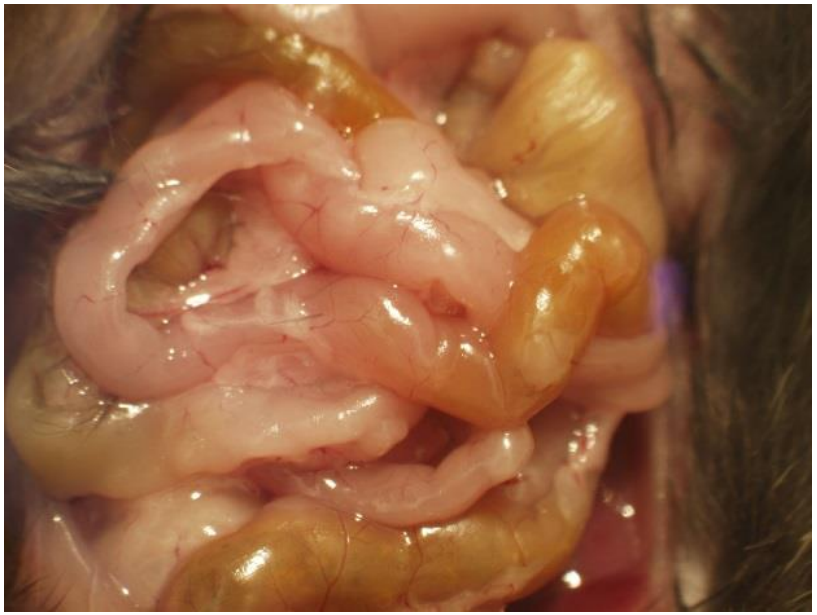

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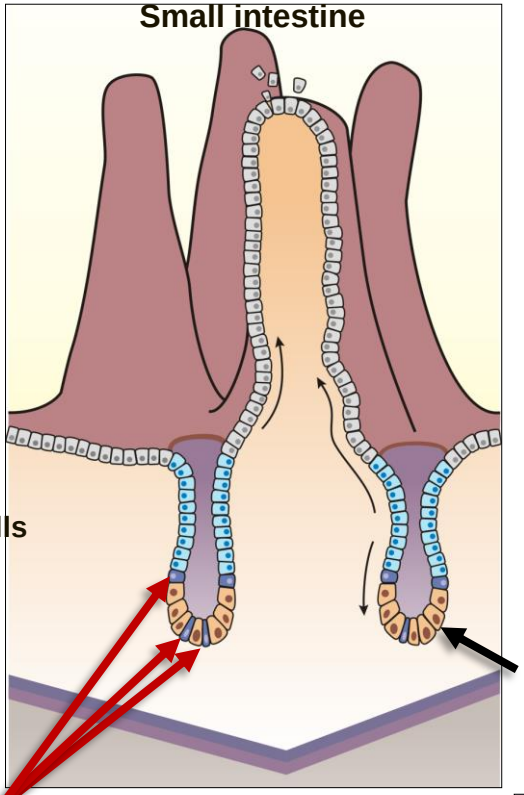


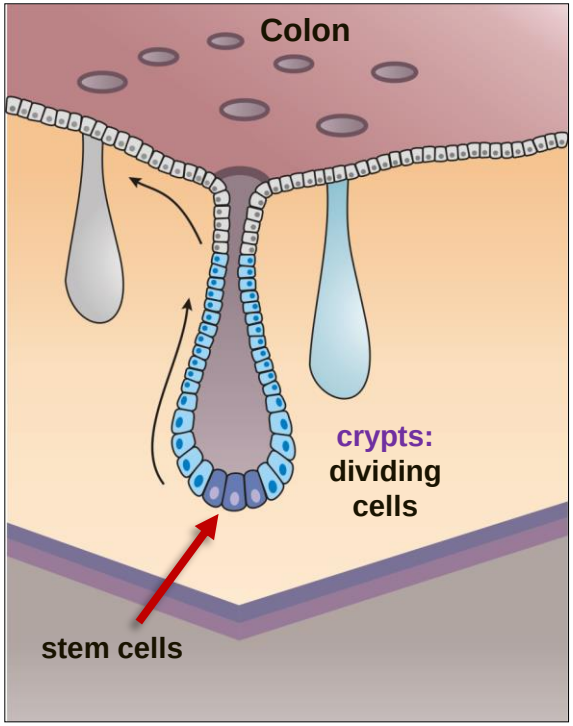
crypts: dividing cells

stem cells (dark blue)

Epithelial lining represents one of the most intensively self-replenishing organ in mammals

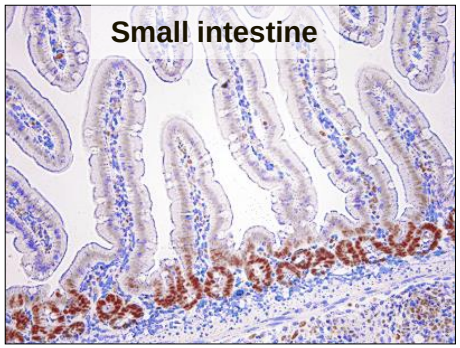
Intestinal villi/colon lumen: non-dividing cells
life span – 5 days

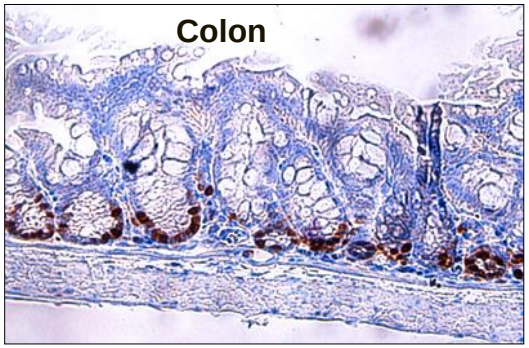
Paneth cells (yelow) are postmitotic



anti-Ki67 staining marks proliferating cells

Ki67 - brown nuclei
counterstained with hematoxylin – blue nuclei





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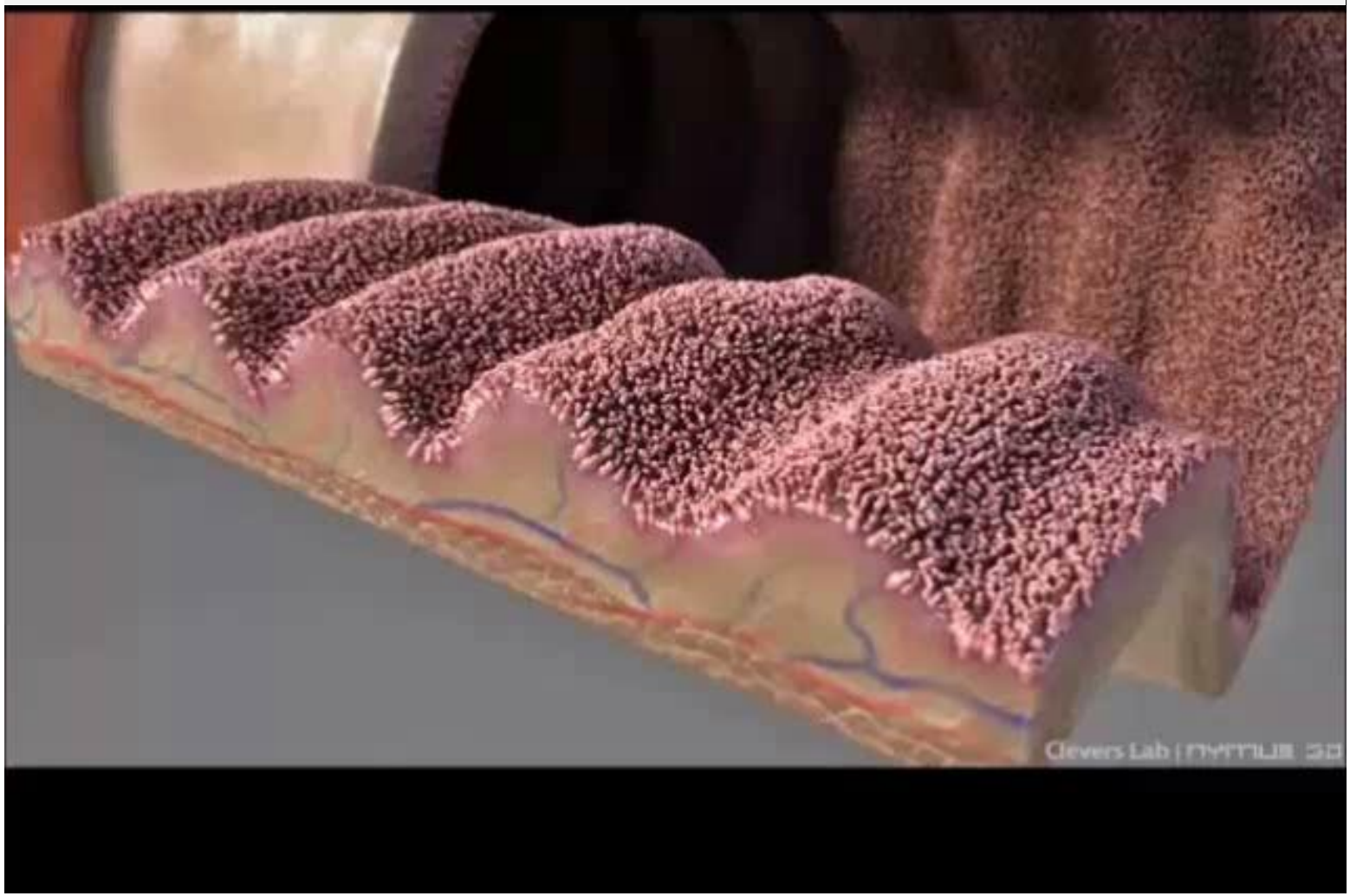
Dynamic and organized cell turnover in the intestine represents an attractive paradigm for tissue maintenance studies



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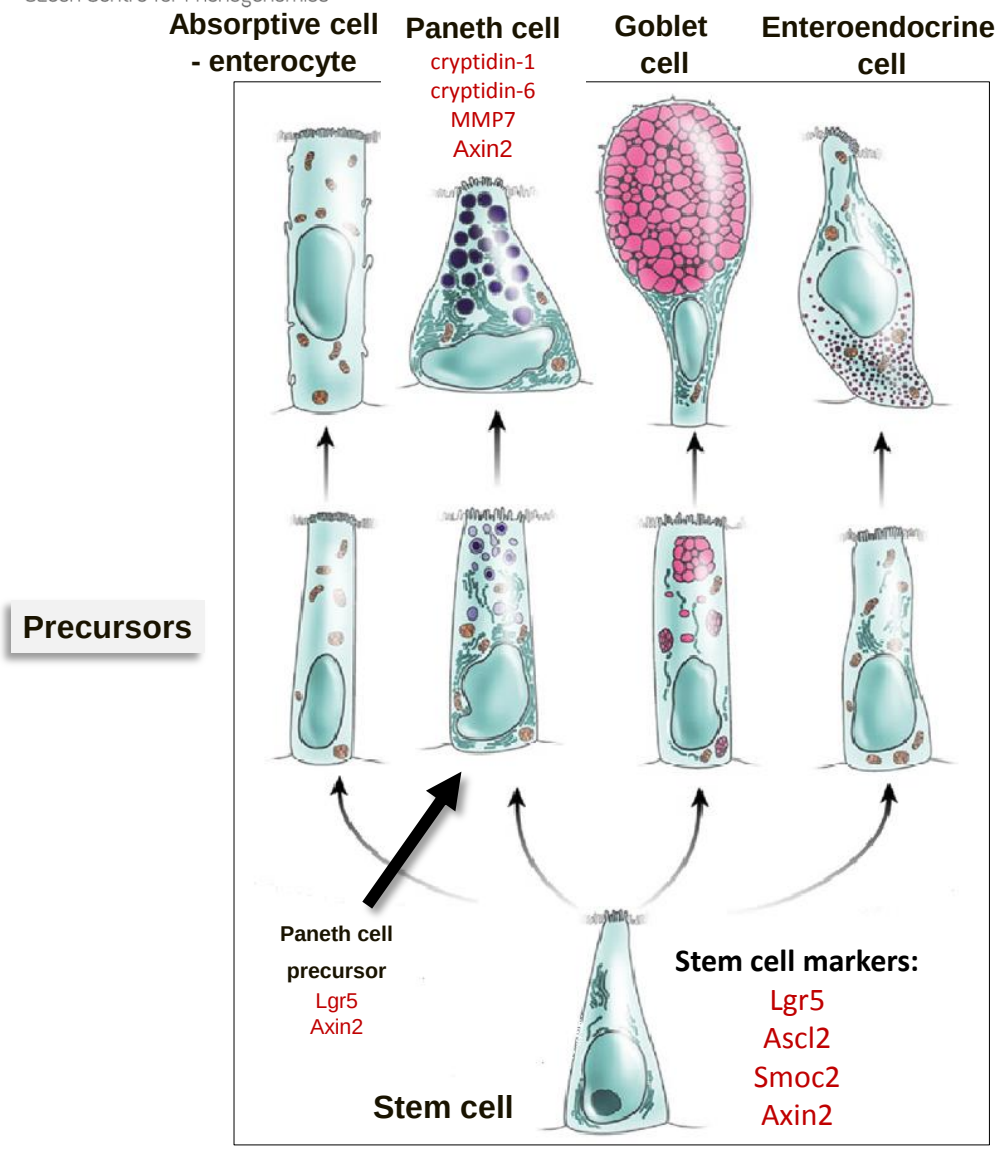
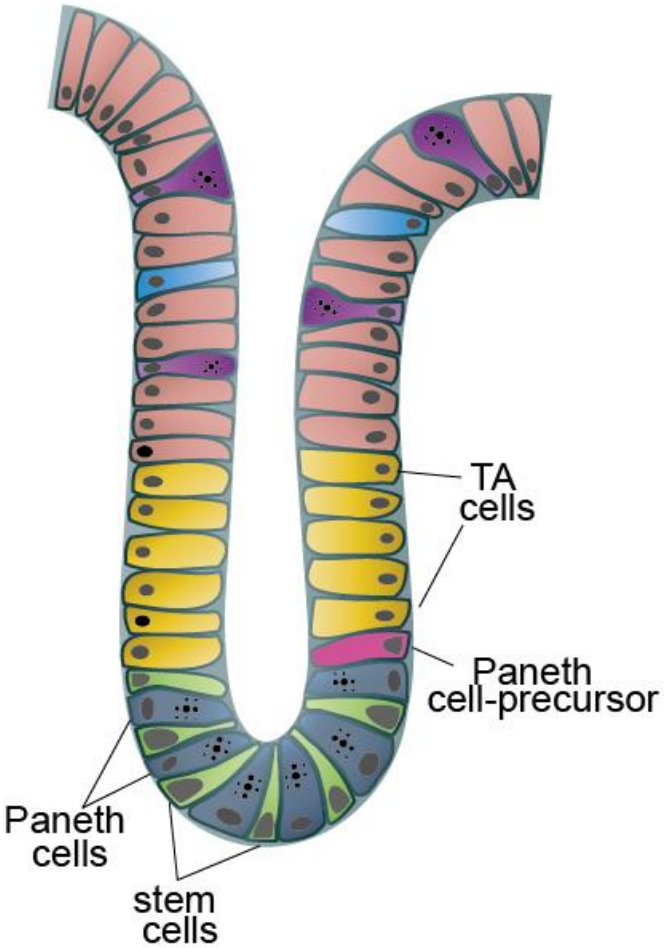


© Barker and Clevers, 2009

Cellular architecture in the crypt of the small intestine



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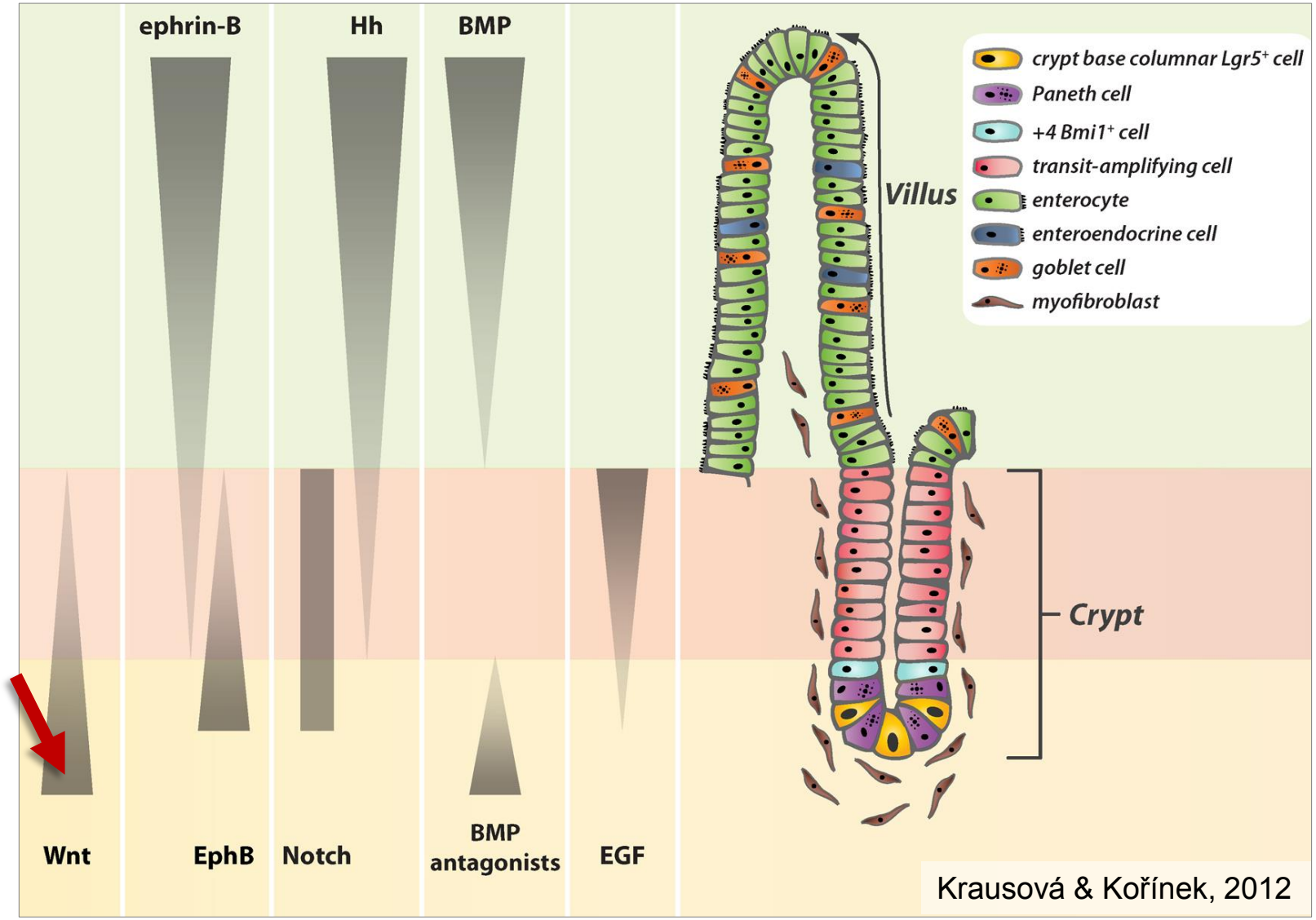

Krausová & Kořínek, 2012

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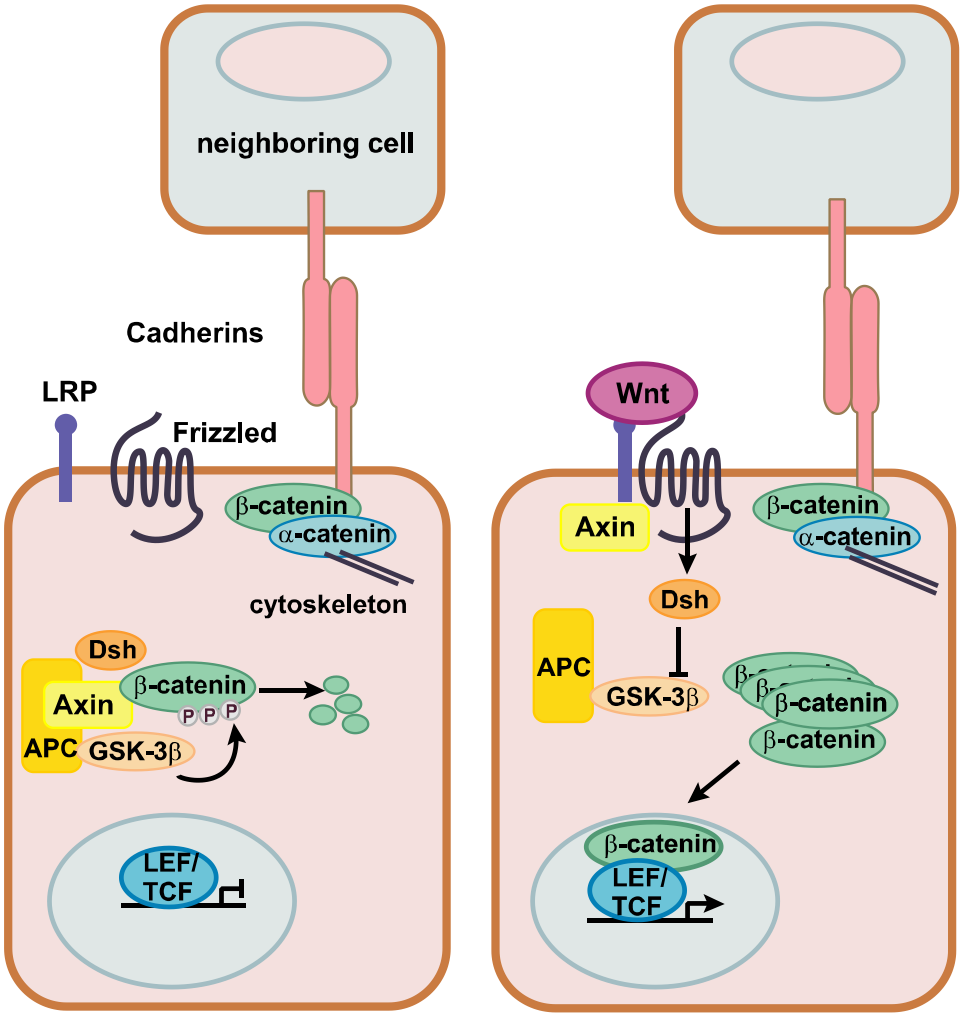
The Wnt signaling pathway



adopted from Reya and Clevers, 2006

- **The Wnt pathway is active in many adult tissues**
- **Biological effects:** stem cells maintenance, cell proliferation, cell differentiation
- **Target genes in the intestine**
  c-myc, Cyclin D1, Lef-1, Lgr5, Axin2
- **Target genes in the liver**
  glutamine synthetase
  glutamate transporter 1
  ornithine aminotransferase
  Axin2
- **Some inhibitors of Wnt signaling are targets of the pathway**

  – negative feedback loop

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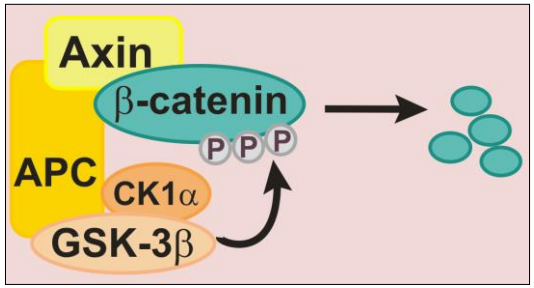
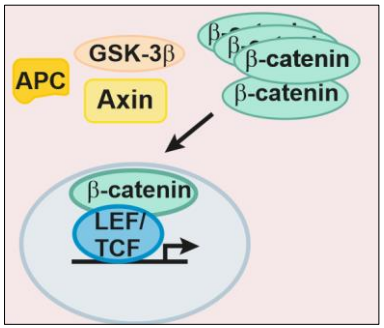
Wnt signaling in intestinal cancer

- **75-85% cases of cancer of the colon and rectum: inactivated APC gene**
- **5-10 %** of sporadic tumors contain mutations in genes encoding other components of the Wnt pathway (e.g. β-catenin, Axin1/2)
- **>90 % of all tumors display aberrant Wnt signaling** essential for tumor initiation

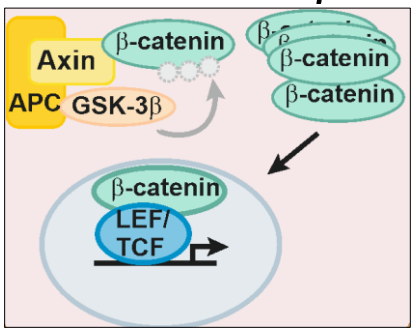
Cells with intact APC



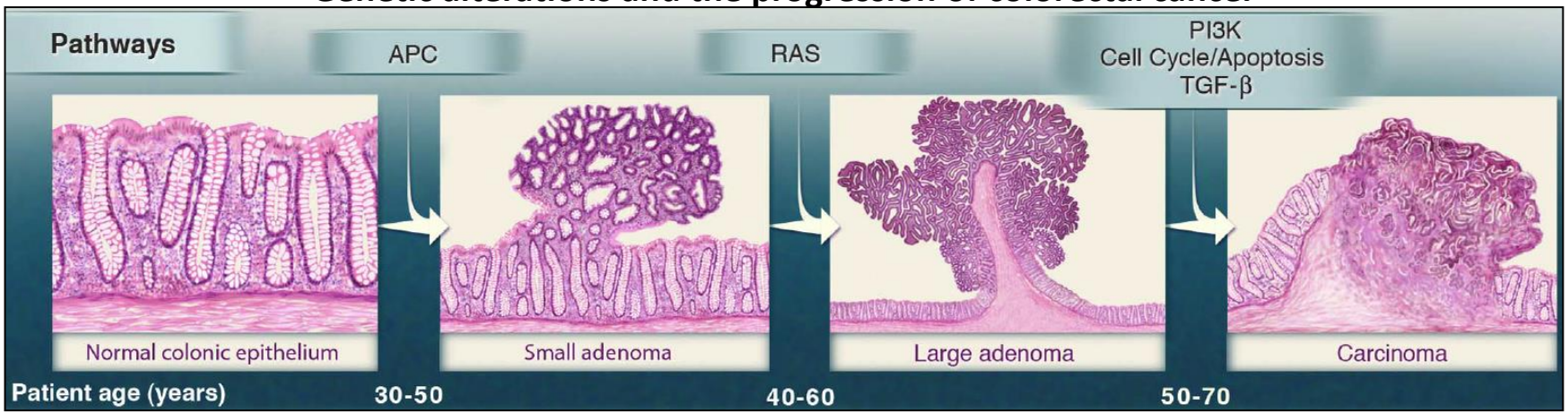
Cells with mutated APC



Cells with mutated β-catenin



Genetic alterations and the progression of colorectal cancer



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- colorectal cancer cell lines with aberrantly activated Wnt signaling pathway

Colo320 DLD-1 LS174T SW480

Chromatin Immunoprecipitation (Tcf4)

DNA Microarray

TROY, NKD1

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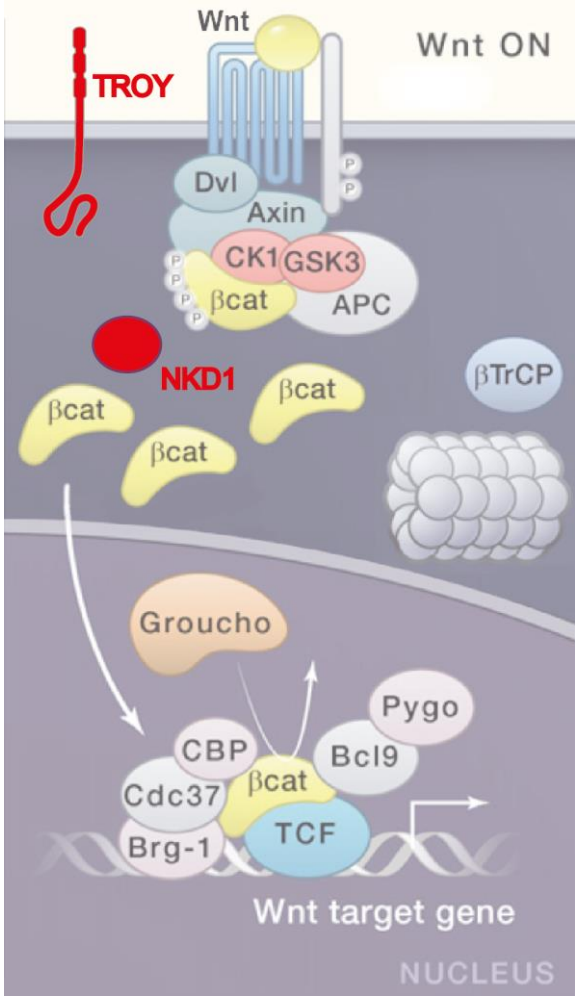
NKD1 and TROY are the Wnt signaling target genes

- **Nkd1** (drosophila naked cuticle homologue 1) is negative regulator of Wnt signaling (van Raay *et al.*, 2007,2011)
- drosophila *nkd* is required for its embryonic development (Zeng *et al.*, 2000)
- Nkd1 interacts with Dvl (Yan *et al.*, 2001) and β-catenin (van Raay *et al.*, 2011)
- Nkd1 mutant mice display in impaired spermatogenesis (Li *et al.*, 2005)
- Nkd1/Nkd2 double mutant mice are viable and fertile (Zhang *et* al., 2007)

- ***Troy*** (Tnf-receptor family member [19] expressed in the whole embryo) encodes a transmembrane protein
- Troy was described to participate in NF-κB (Hashimoto *et al.*, 2005), JNK (Eby *et al.*, 2000), RhoA (Shao *et al.*, 2005) and Rac1 signaling (Paulino *et al.*, 2010)
- Troy defficient mice has no major phenotype



adapted from Li *et al.*, 2012

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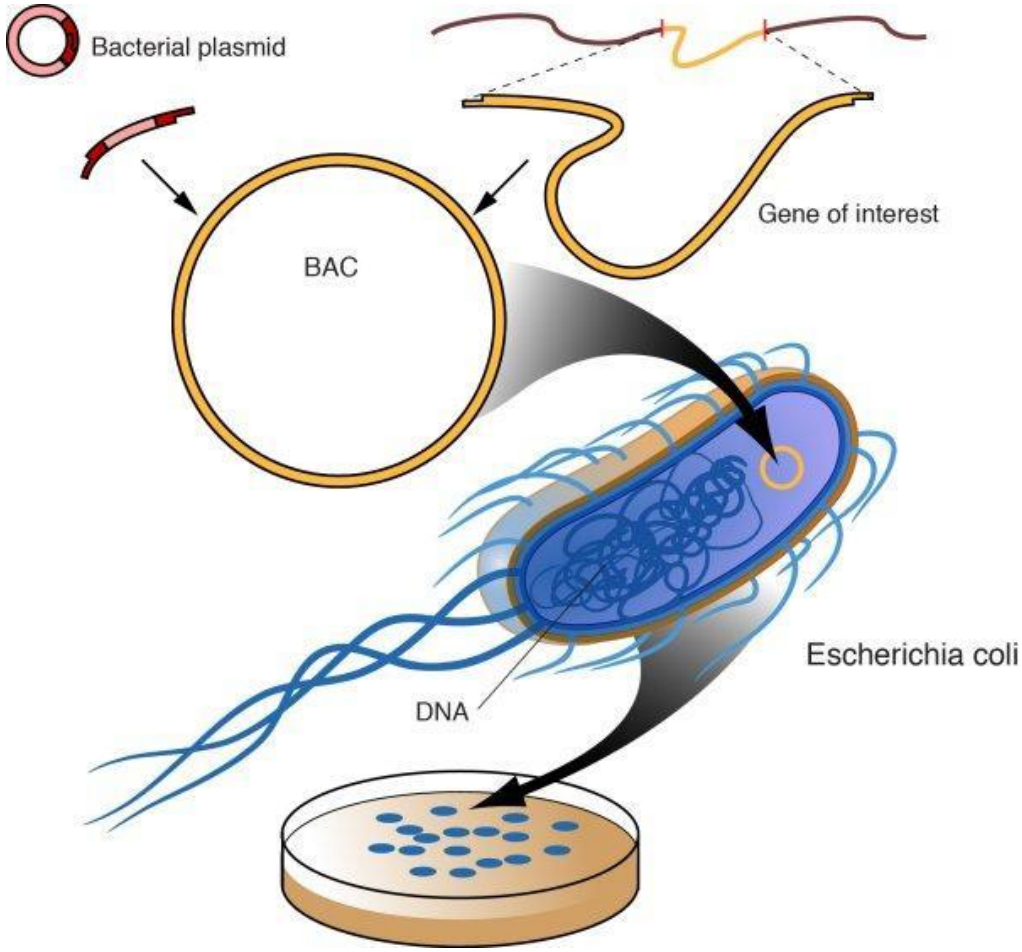
Bacterial Arteficial Chromosome recombineering:

large pieces of DNA can be manipulated in bacteria



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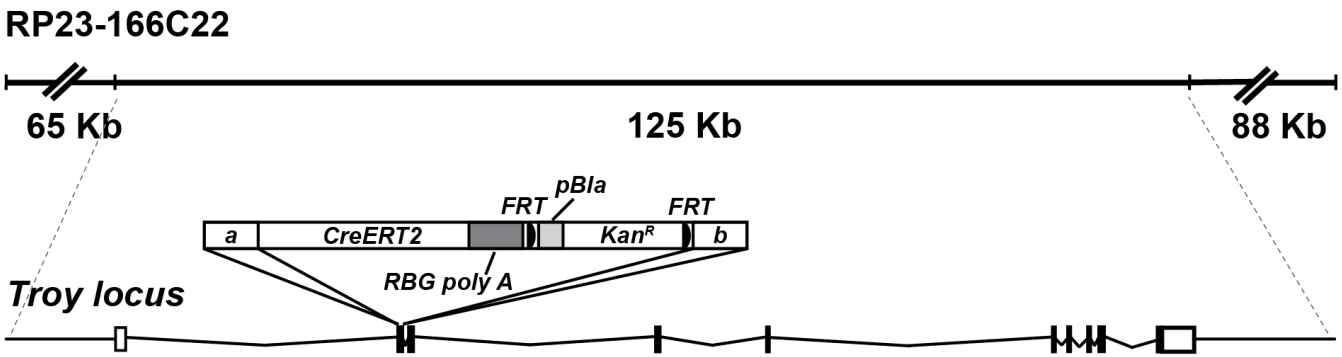
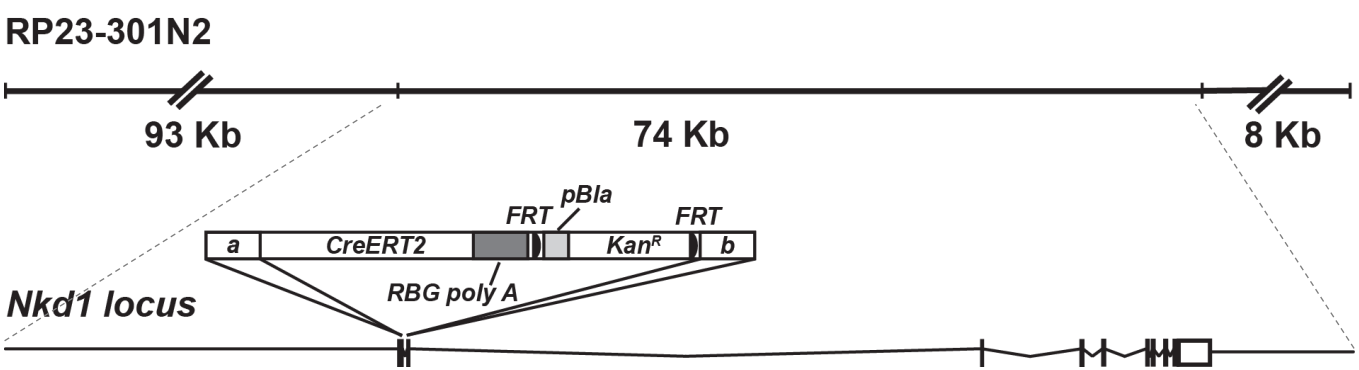
Bacterial Arteficial Chromosome recombineering:

regulated Cre expression from Troy and Nkd1 locus





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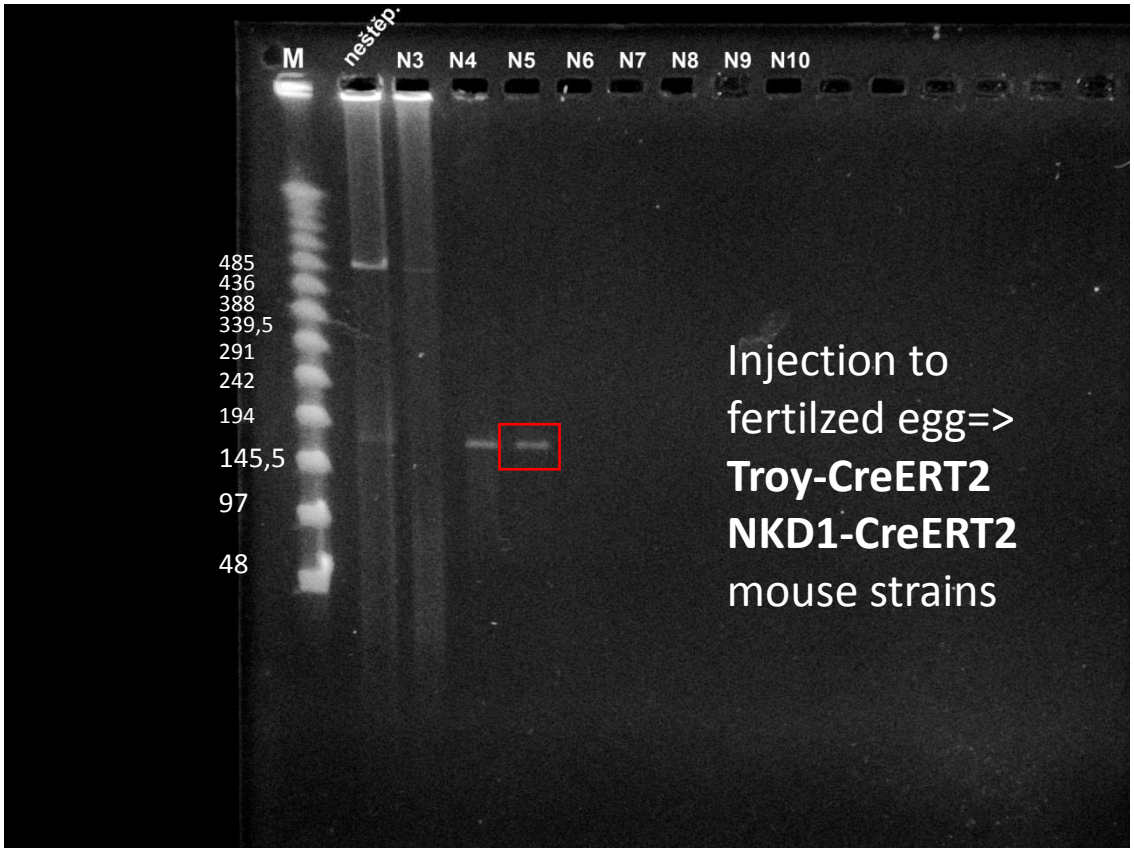
BAC purification on sepharose column, Pulse Field gel electrophoresis



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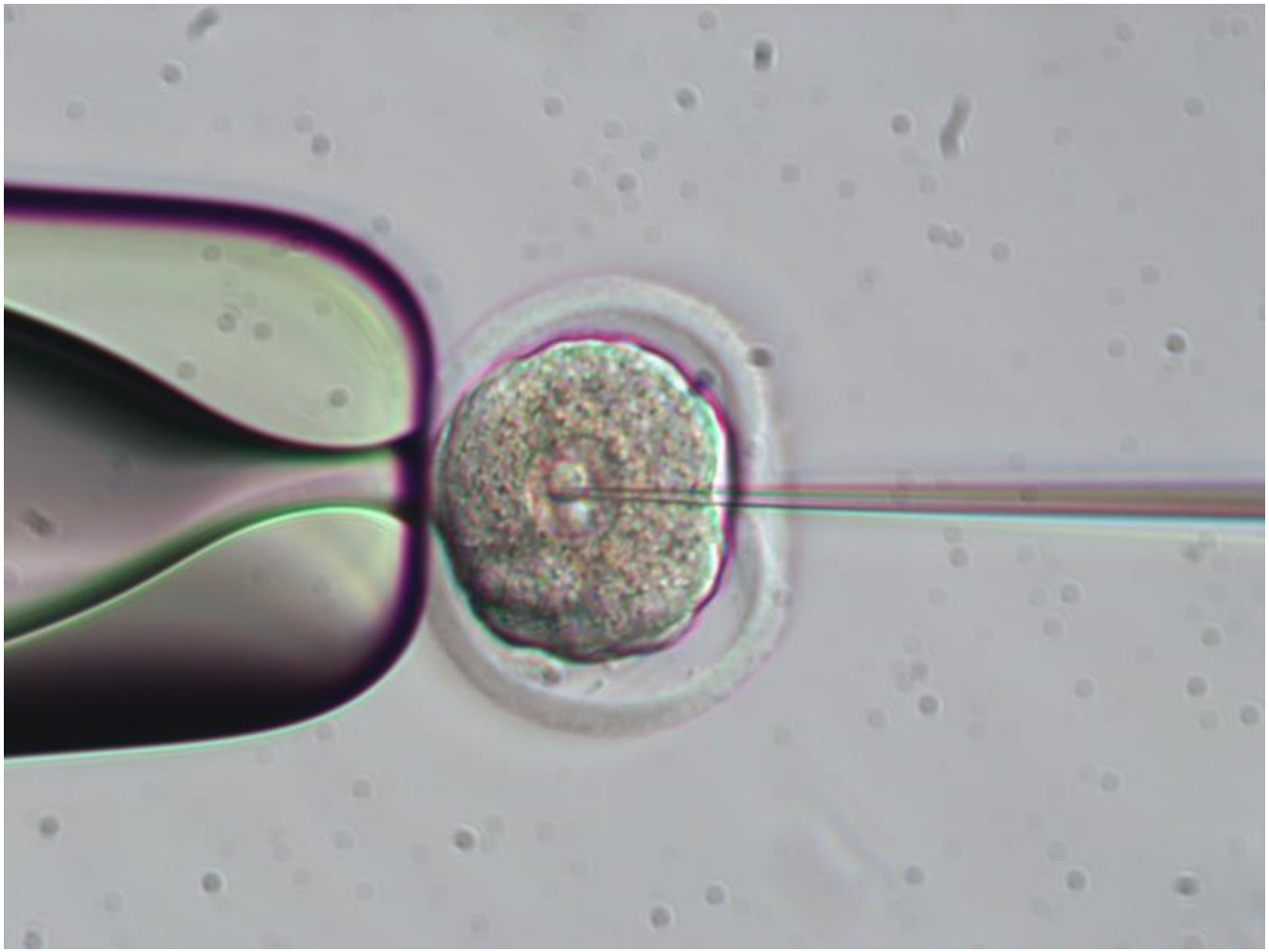
BAC injection into mouse oocyte



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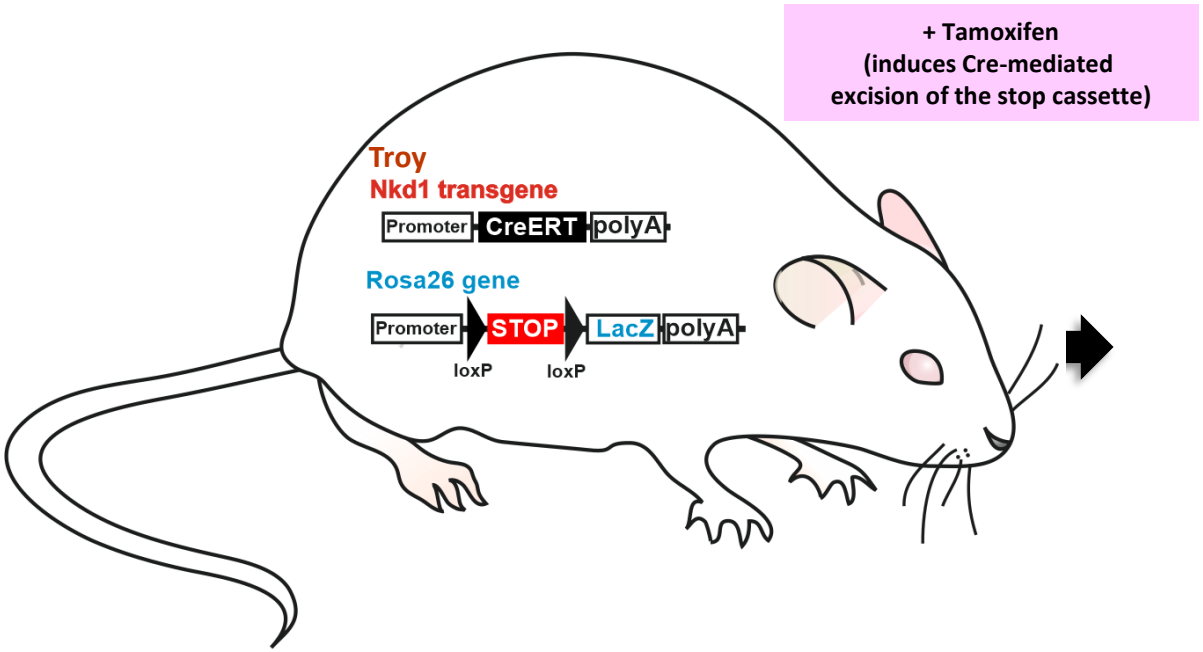
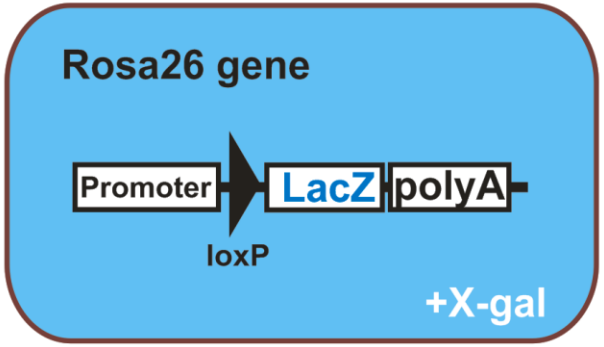
Reporter induction in Nkd1/Troy-positive cells in mice

Nkd1/Troy BAC transgenic mouse crossed to Rosa26 reporter mouse



Situation in genomic DNA of Nkd1/Troy$^+$ cells



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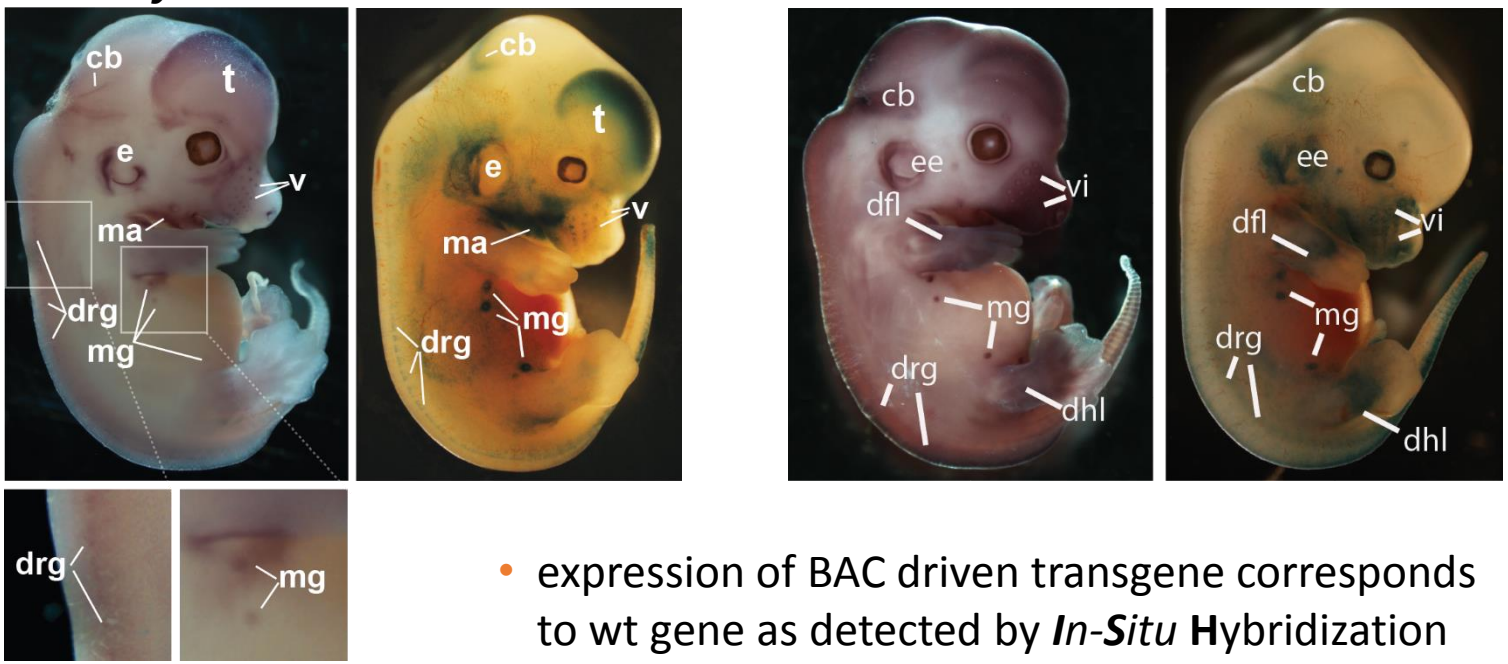
Crossbreeding **Troy/Nkd1-CreERT2** with **ROSA-LacZ** (reporter strain)

***Troy* ISH**

ROSA-LacZ/ **TROY**-CreERT2

***Nkd1* ISH**

ROSA-LacZ/ **NKD1**-CreERT2



- expression of BAC driven transgene corresponds to wt gene as detected by ***In-Situ* Hybridization**

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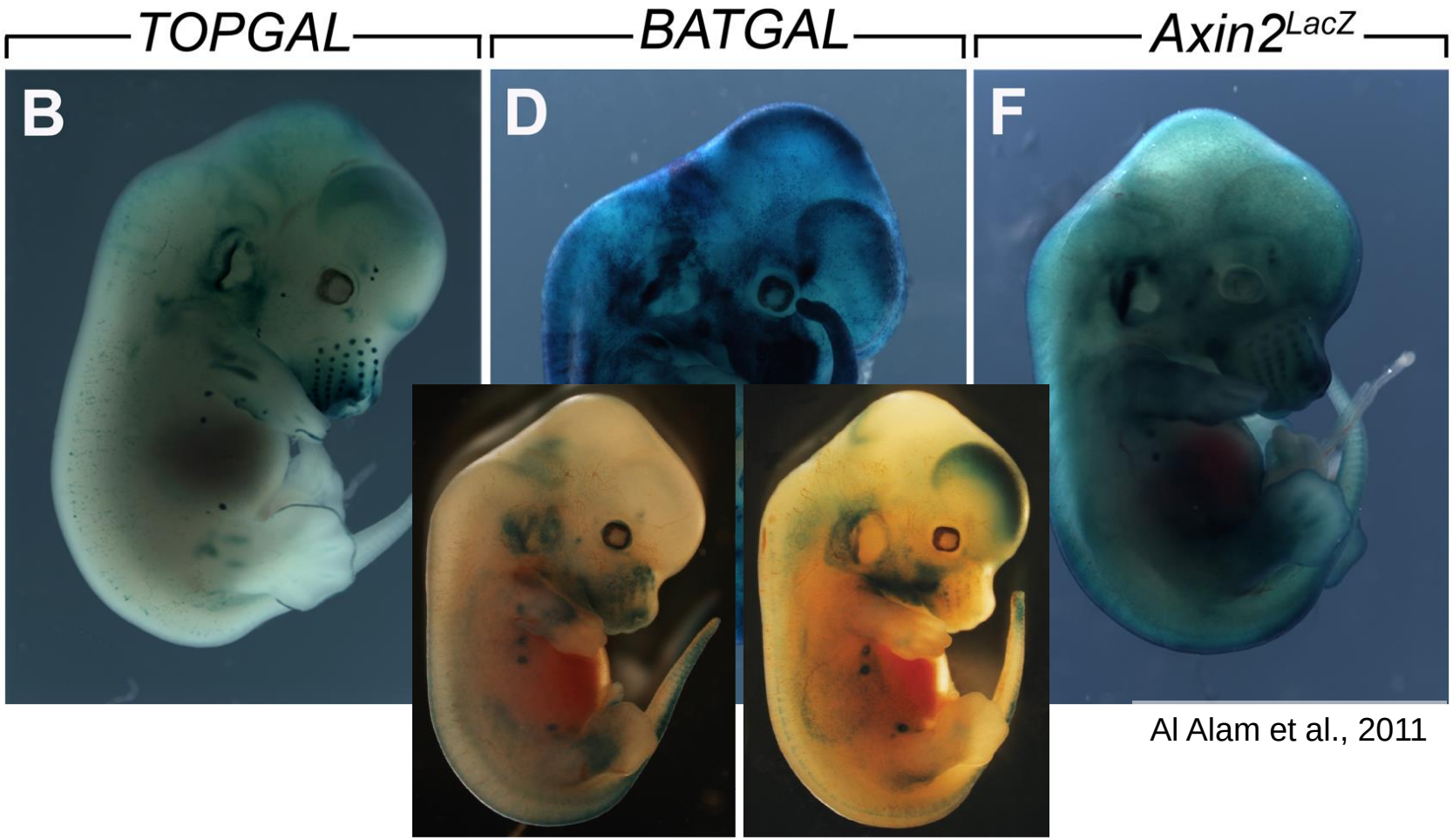
- sites of expression of BAC driven transgene corresponds to sites of activation of established Wnt reporter strains



Al Alam et al., 2011

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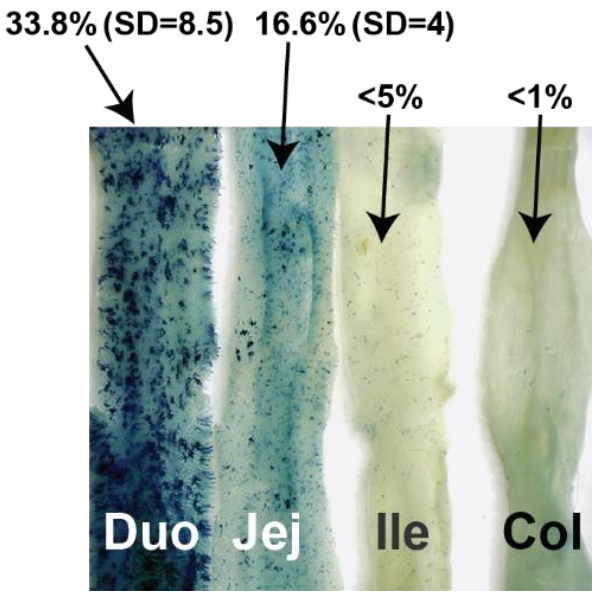
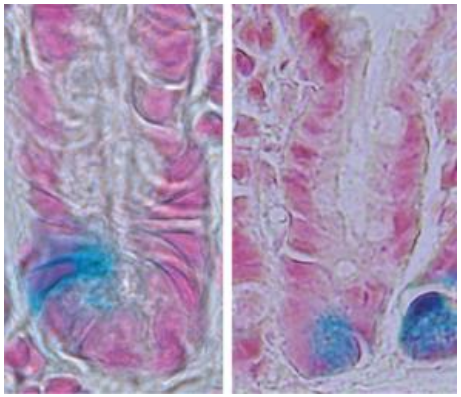
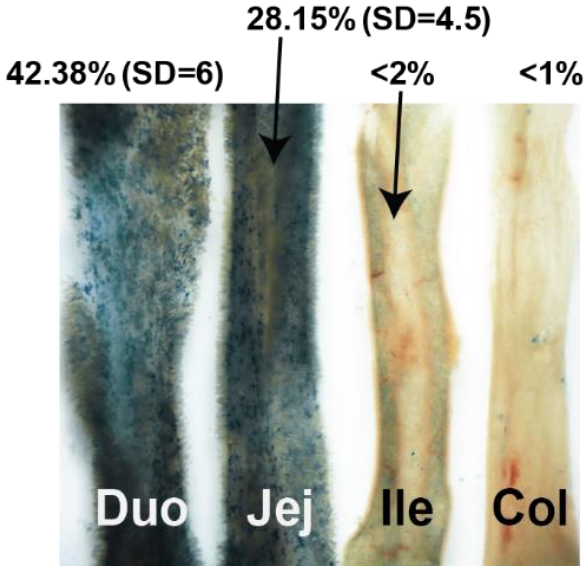
Expresion of Troy-CreERT2 and Nkd1-CreERT2 differs in the intestine

ROSA-LacZ/**TROY**-CreERT2





ROSA-LacZ/**NKD1**-CreERT2



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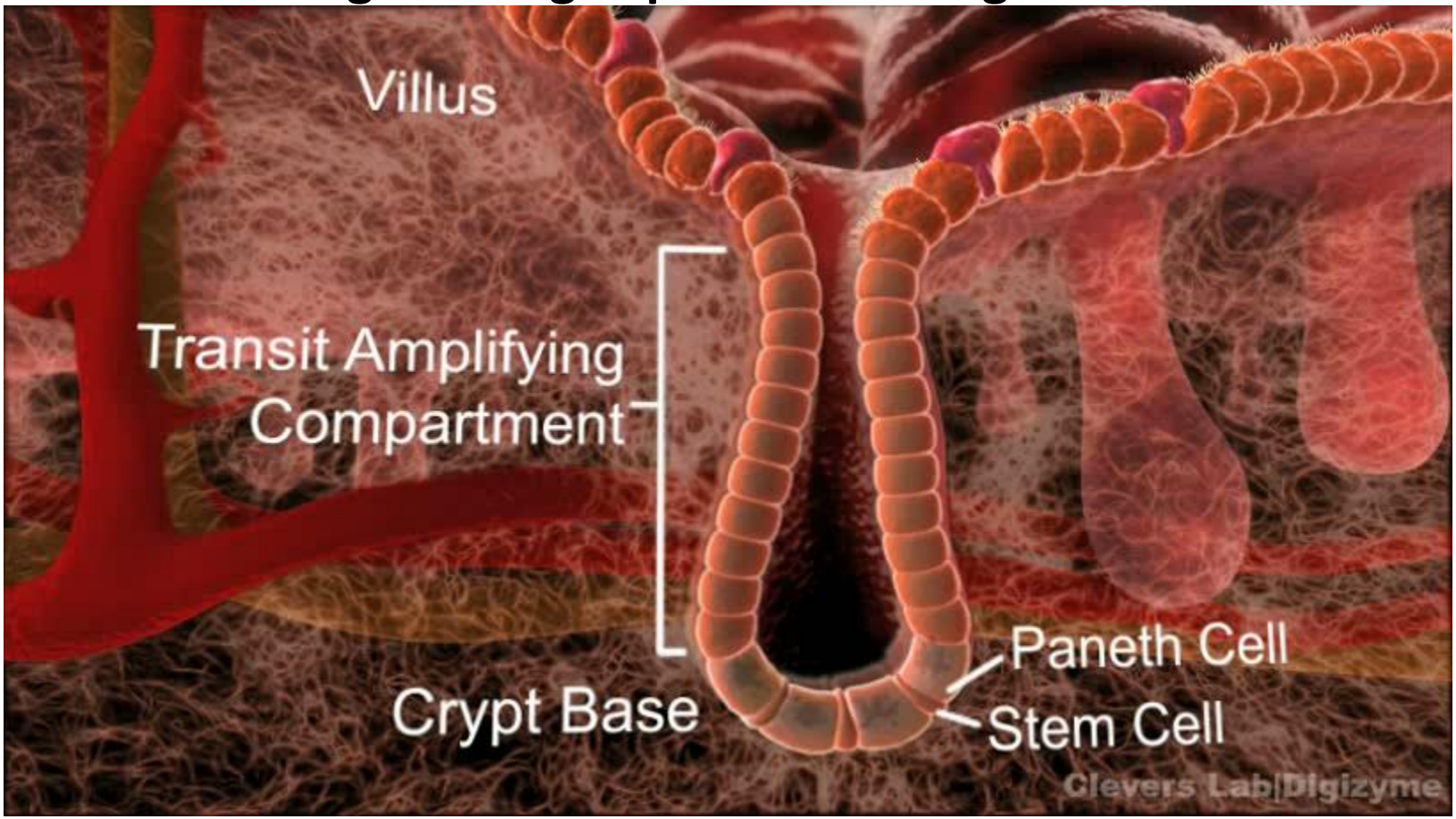
Lineage tracing: a prove of hitting stem cell



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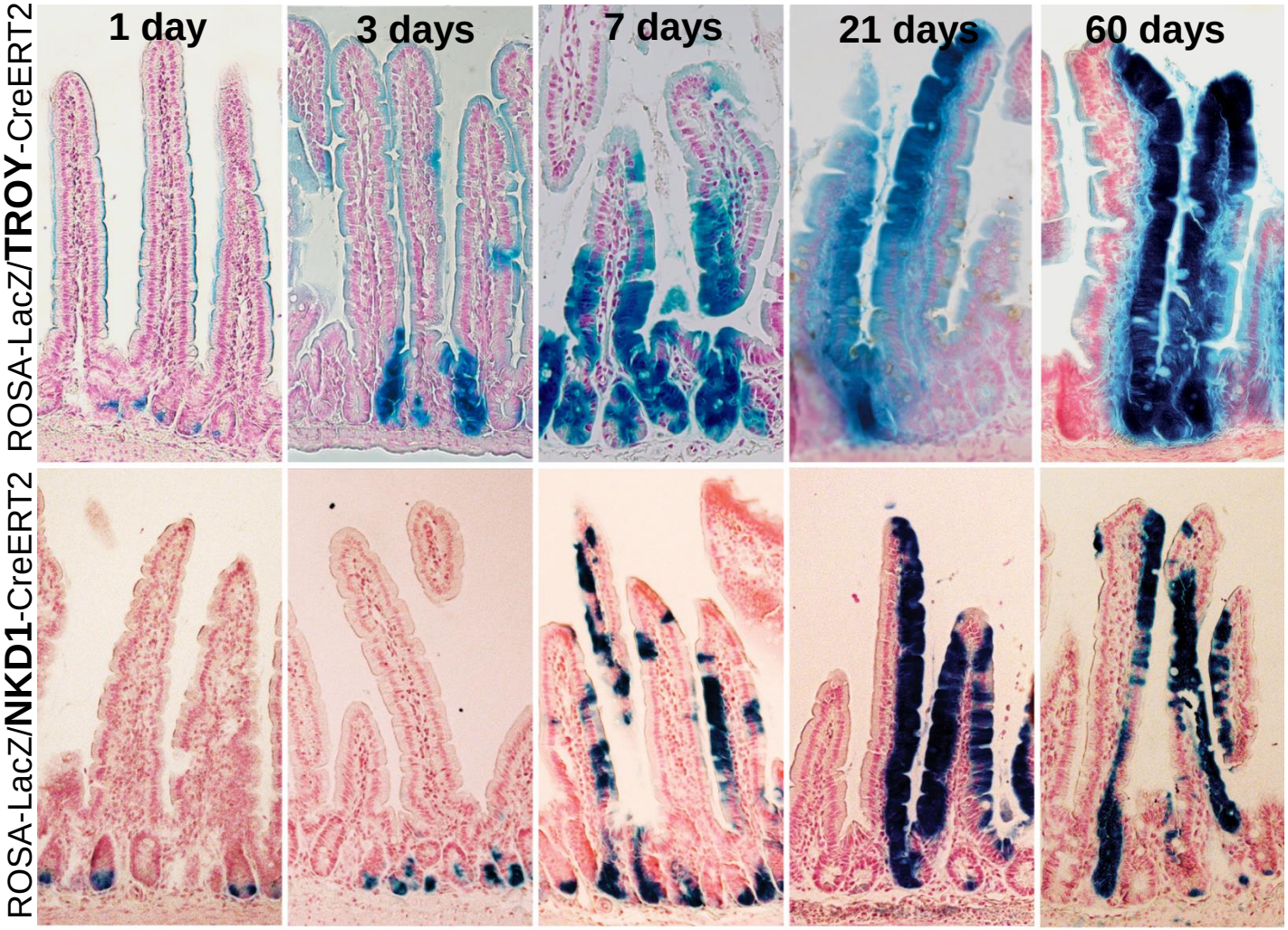
Troy and *Nkd1* expressing cells are different



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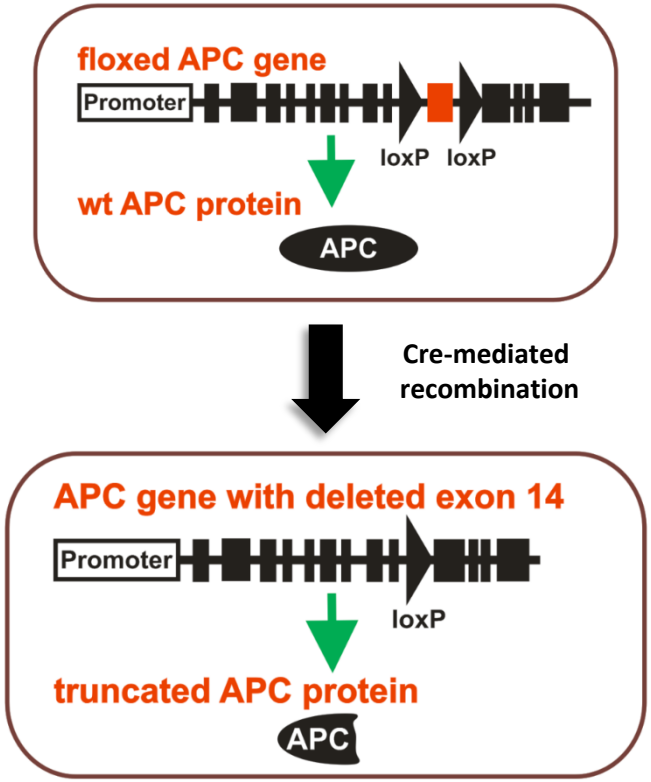
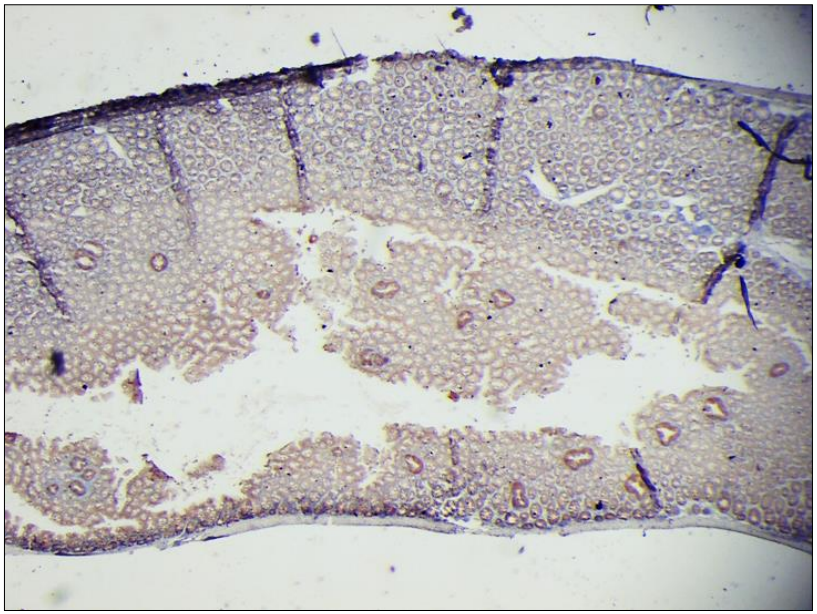
Cre-mediated loss of APC function: model to cancer initiation



APC conditional knock-out (APC CKO)

Microadenomas in the small intestine



Immunohistochemical staining with an anti-β-catenin antibody 2 weeks after recombination

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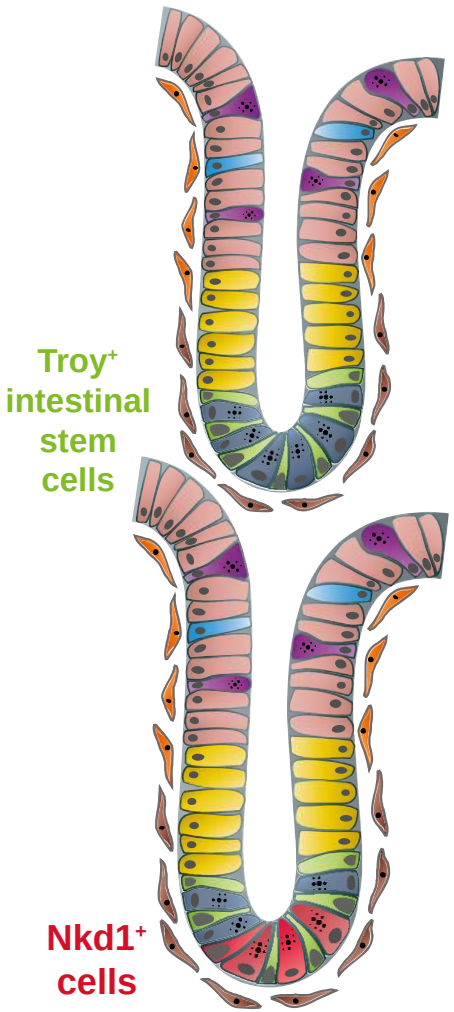
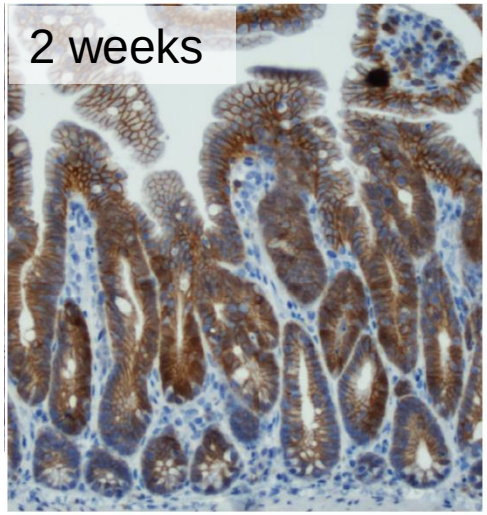
Different Cre lines give different output

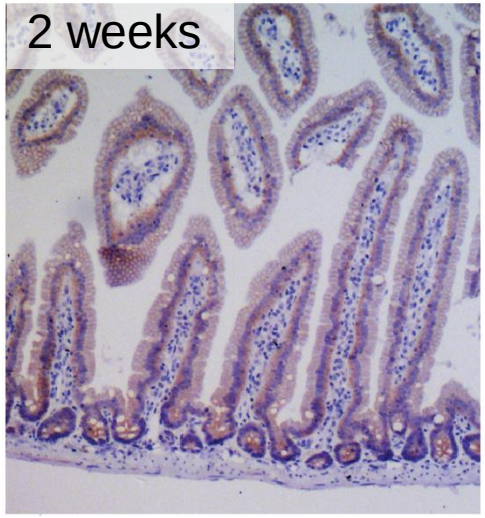


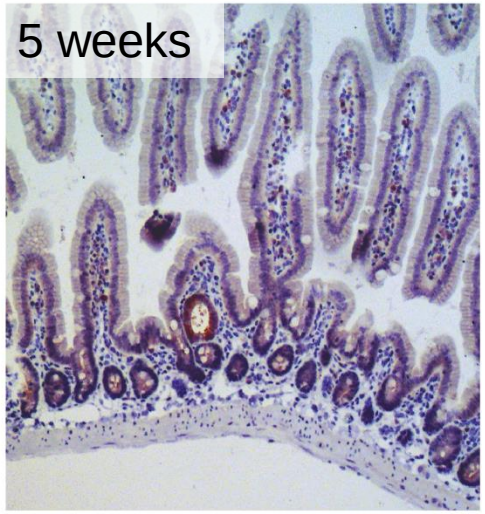
APC CKO/**TROY**-CreERT2



Will not survive

APC CKO/**NKD1**-CreERT2





β-catenin

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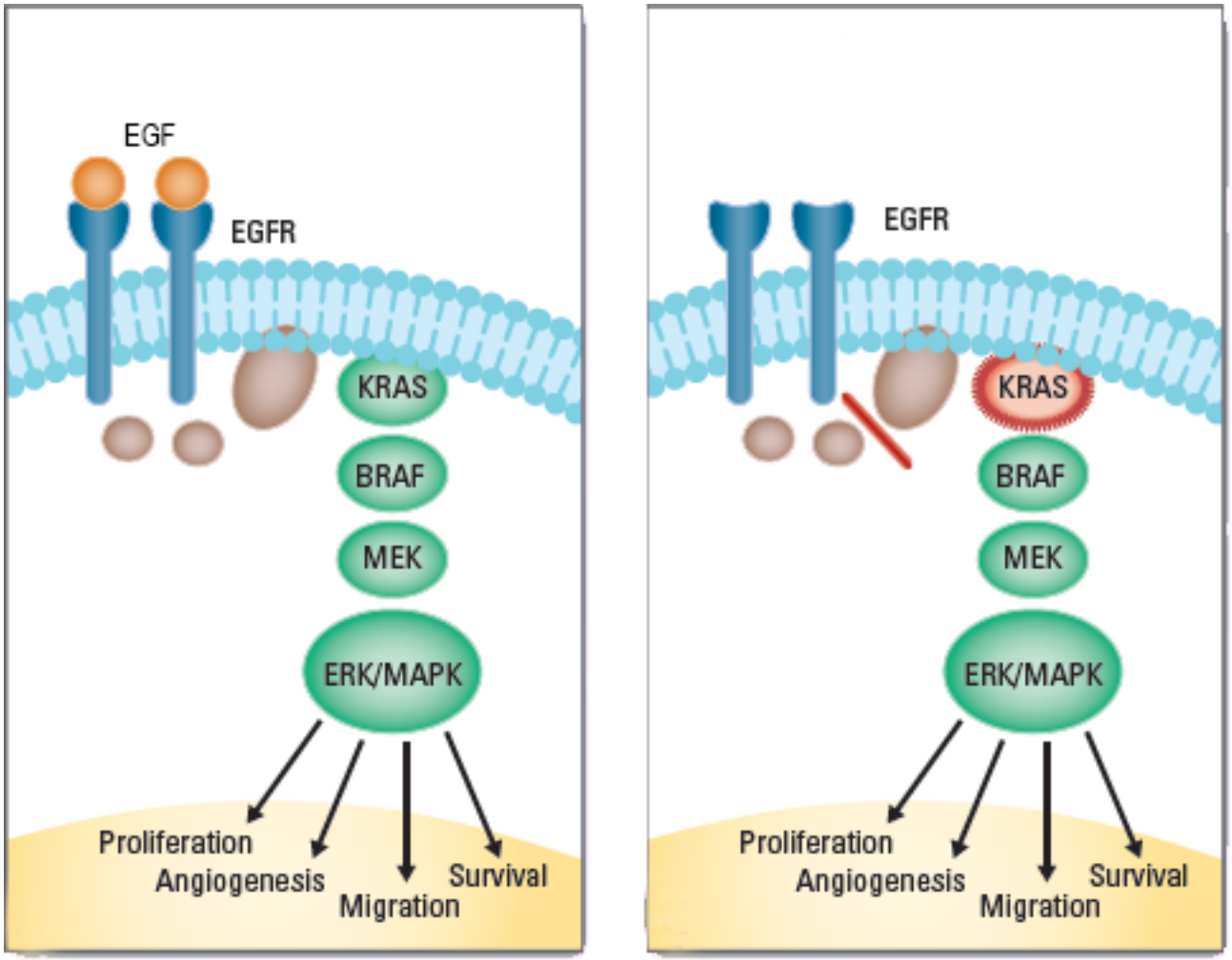
K-Ras signalling



- K-Ras is mutated in 30-60% cases of colorectal cancer
- The most common *KRAS* mutations in codons 12 and 13 are activation mutations, causing the GTPase domain of Ras being insensitive to inactivation by GAP

Adapted from GE Healthcare

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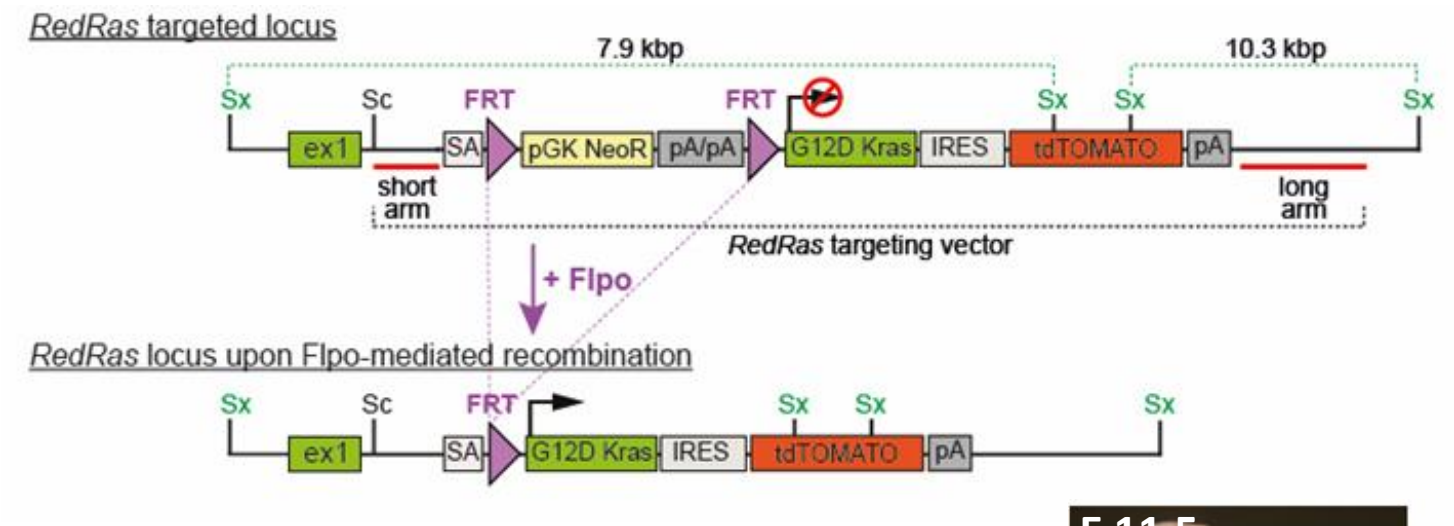
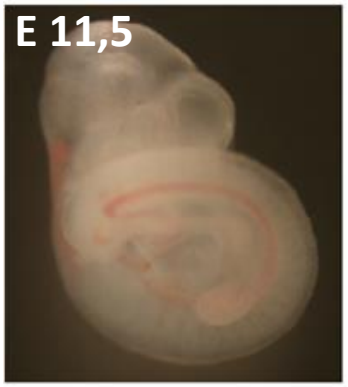
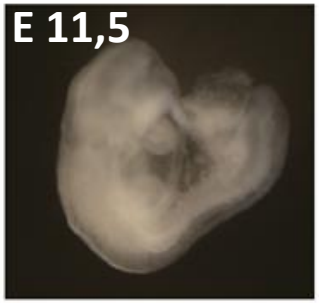
Flpo mediated activation of oncogenic K-Ras



- oncogenic K-Ras activation is embryonically lethal
- tdTomato expression allows tracking of G12D Kras positive cells (too weak fluorescence in the embryo)



RedRas⁺, ACTB-Flpe⁻



RedRas⁺, ACTB-Flpe⁺

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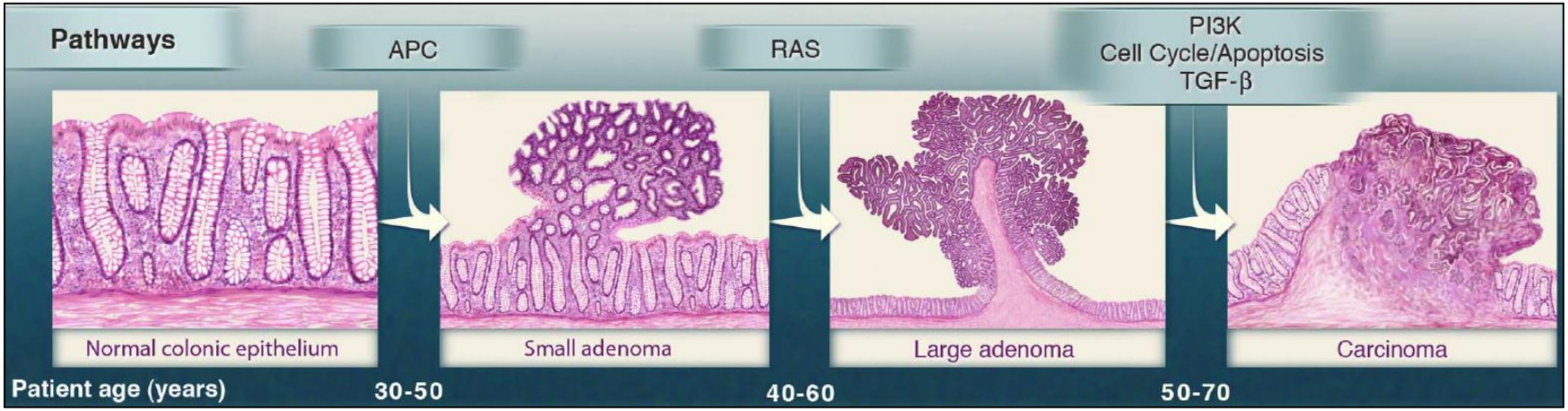
Mouse models for mimicking colorectal cancer development in mice

- APC-CKO/Troy(Nkd1)-CreERT2 – model to aberrant Wnt signaling
- Adeno-Flpo/RedRas – model to oncogenic K-Ras signaling
- Crossing to p53 mutant strain to further promote progression into carcinoma
- Models for testing cancer therapeutics



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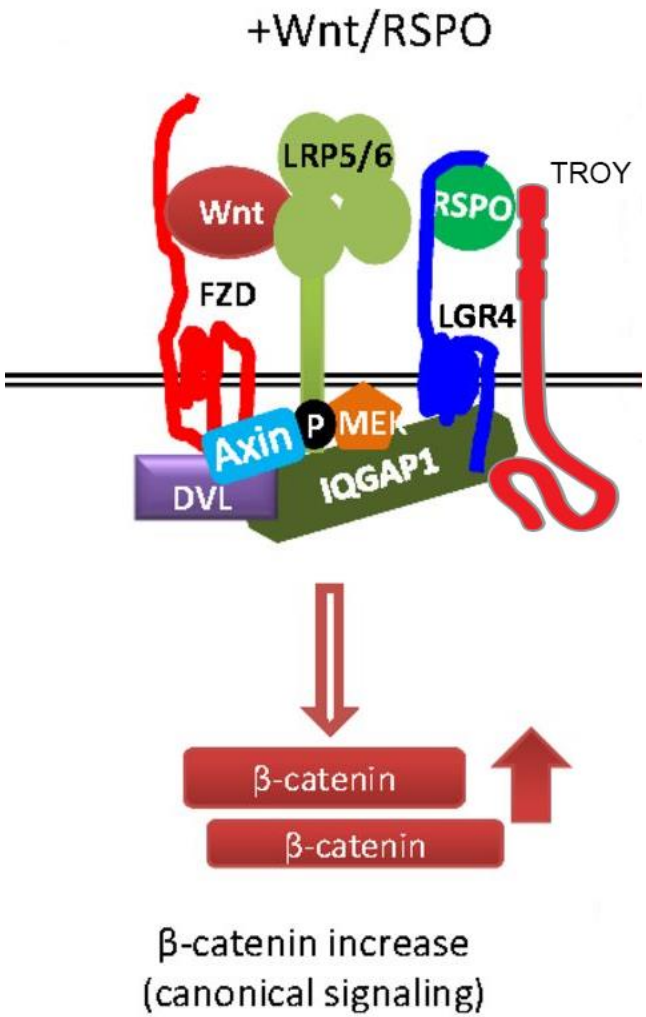
Complex regulation of Wnt signalling

- Lgr4 is the main receptor for Wnt signaling agonist Rspo
- Lgr4 helps, via scaffolding protein IQGAP1, to hold the β-catenin destruction complex at the membrane



- Troy is Wnt signalling target gene
- Troy inhibits Wnt signalling by so far unknown mechanism

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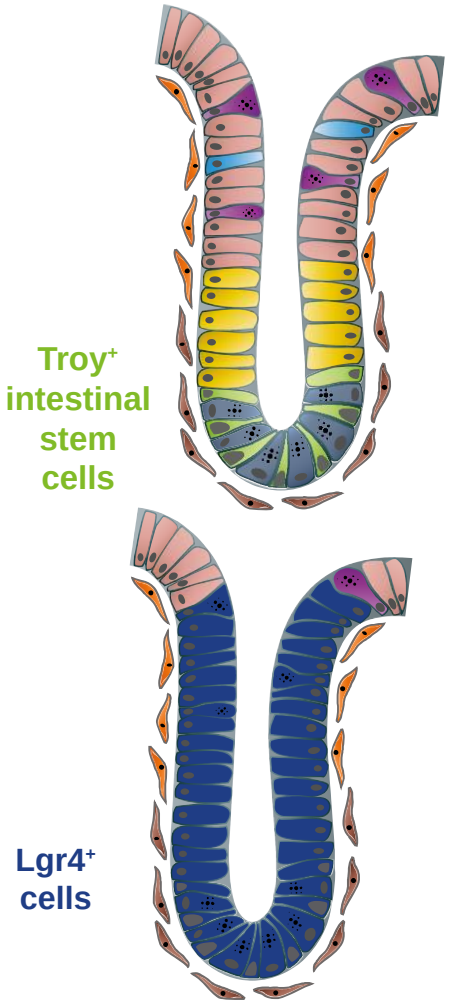
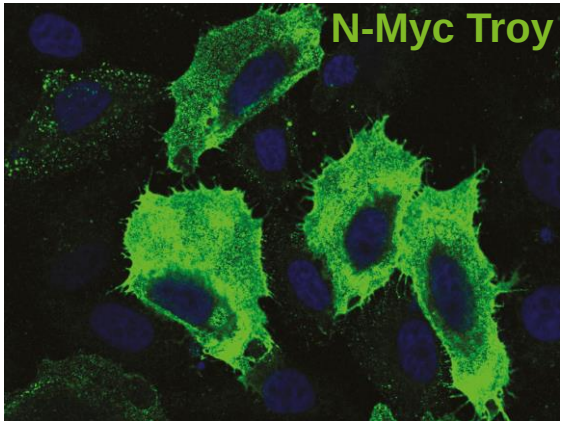
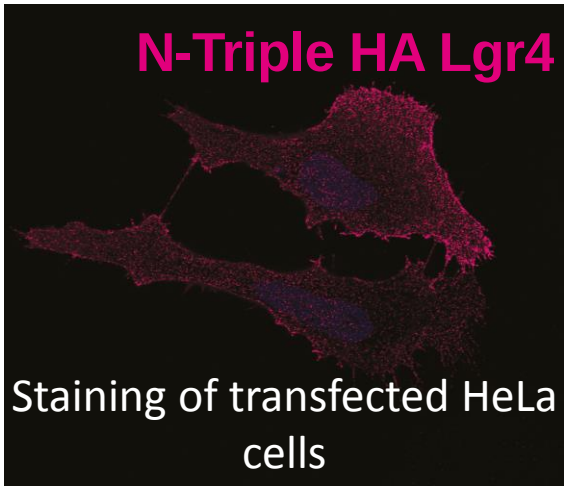
Lgr4 and Troy transgenic mice generated by TALen technology







- Troy is produced specifically in the intestinal stem cell
- N-Myc tag will allow stem cell labeling and sorting
- Lgr4 is produced on all the cells of the intestinal crypt
- HA tag protein will label Wnt/Rspo receiving cells

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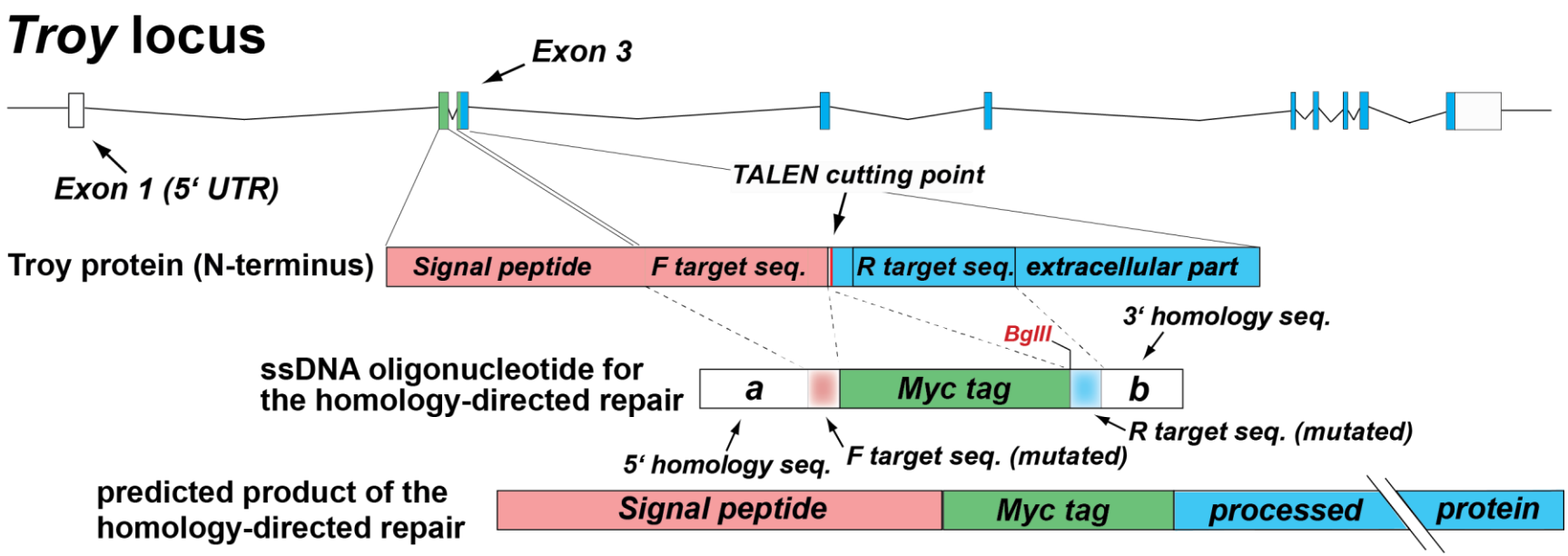
TALEN-generated N-Myc Troy mouse



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TALENs generates mosaic (multiallelic) mice

Expected DNA sequence

Left TALen binding site **Myc Tag insert** **Right TALen binding site**

```
TGGTTTTTCTTTTATAGGCATGTAAAGTGAGTTGCGAATCTGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGAGATCTGGAGATTGtAGaCAGCAaGAATTCAAGGATCGATCTGGAAA
```

mouse 1

```
TGGTTTTTCTTTTATAGGCATGTAAAGTGAGTTGCGAA.....................................................GCAGGCAGCAGGAATTCAAGGATCGATCTGGAAA
TGGTTTTTCTTTTATAGGCATGTAAAGTGAGTTGCGAATCTGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGAGATCTGGAGATTGCAGGCAGCAGGAATTCAAGGATCGATCTGGAAA
TGGTTTTTCTTTTATAGGCATGTAAAGTGAGTTGCGAA..............GCTGATCTCAGAGGAGGACCTGAGATCTGGAGATTGtAGaCAGCAGGAATTCAAGGATCGATCTGGAAA
```

mouse 2

```
TGGTTTTTCTTTTATAGGCATGTAAAGTGAGTTGCGAA.......................................ACCGGAGATTGCAGGCAGCAGGAATTCAAGGATCGATCTGGAAA
TGGTTTTTCTTTTATAGGCATGTAAAGTGAGTTGCGAA.......................................ACCGGAGATTGCAGGCAGCAGGAATTCAAGGATCGATCTGGAAA
TGGTTTTTCTTTTATAGGCATGTAAAGTGAGTTGCGAA.....................................................GGCAGCAGGAATTCAAGGATCGATCTGGAAA
TGGTTTTTCTTTTATAGGCATGTAAAGTGAGTTGCGAA.....................................................GGCAGCAGGAATTCAAGGATCGATCTGGAAA
```

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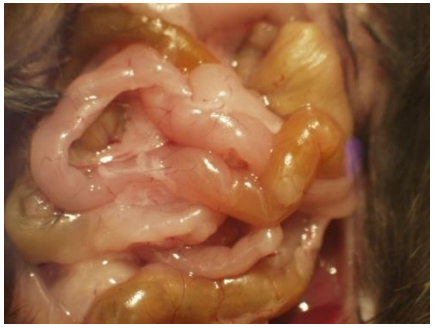
Intestines: Conclusions I



- two strains of tamoxifen inducible Cre-ERT2 recombinase can promote loss of APC with different intensity
- Oncogenic K-Ras signalling together with hyperactive Wnt signalling will be used to further promote oncogenic process in mouse model
- N-terminal labeling of Wnt signaling antagonist and stem cell specific marker Troy will allow tracking of stem cell
- N-terminal labeling of Lgr4 will allow tracking of Wnt signaling receiving cells
- TALENs with ssDNA-mediated dsDNA-break-repair generates more than two alleles
- Transgenic mouse models of colorectal cancer will be used for tracking, isolation and characterization of cancer stem cell

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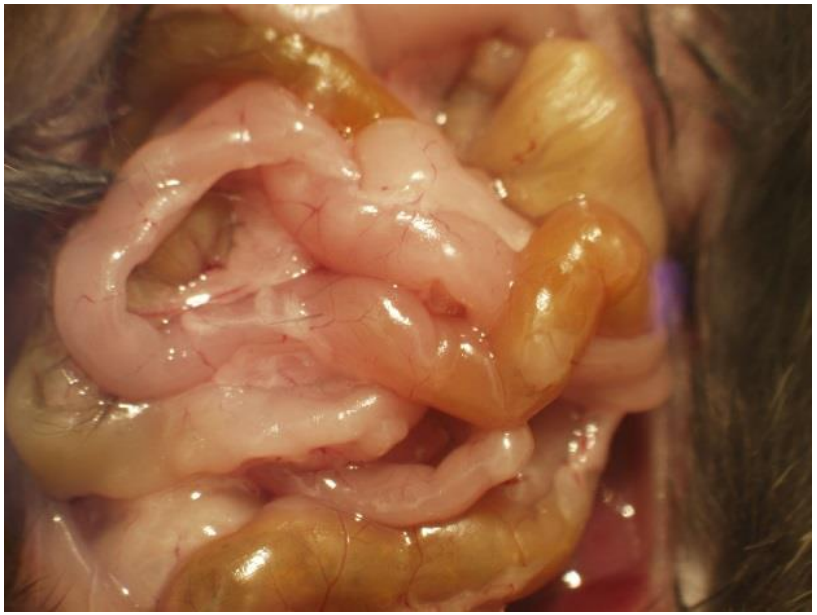

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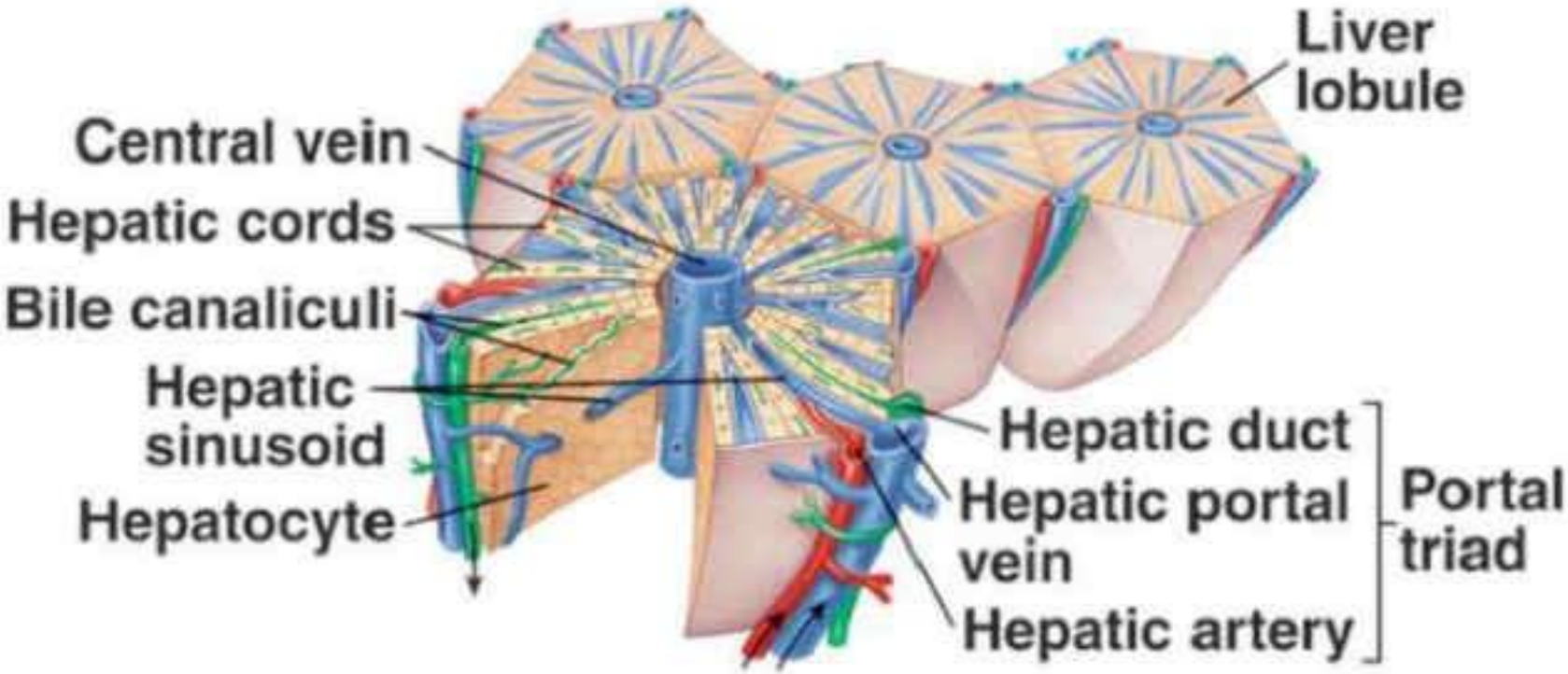
Liver lobule: basic functional unit of liver



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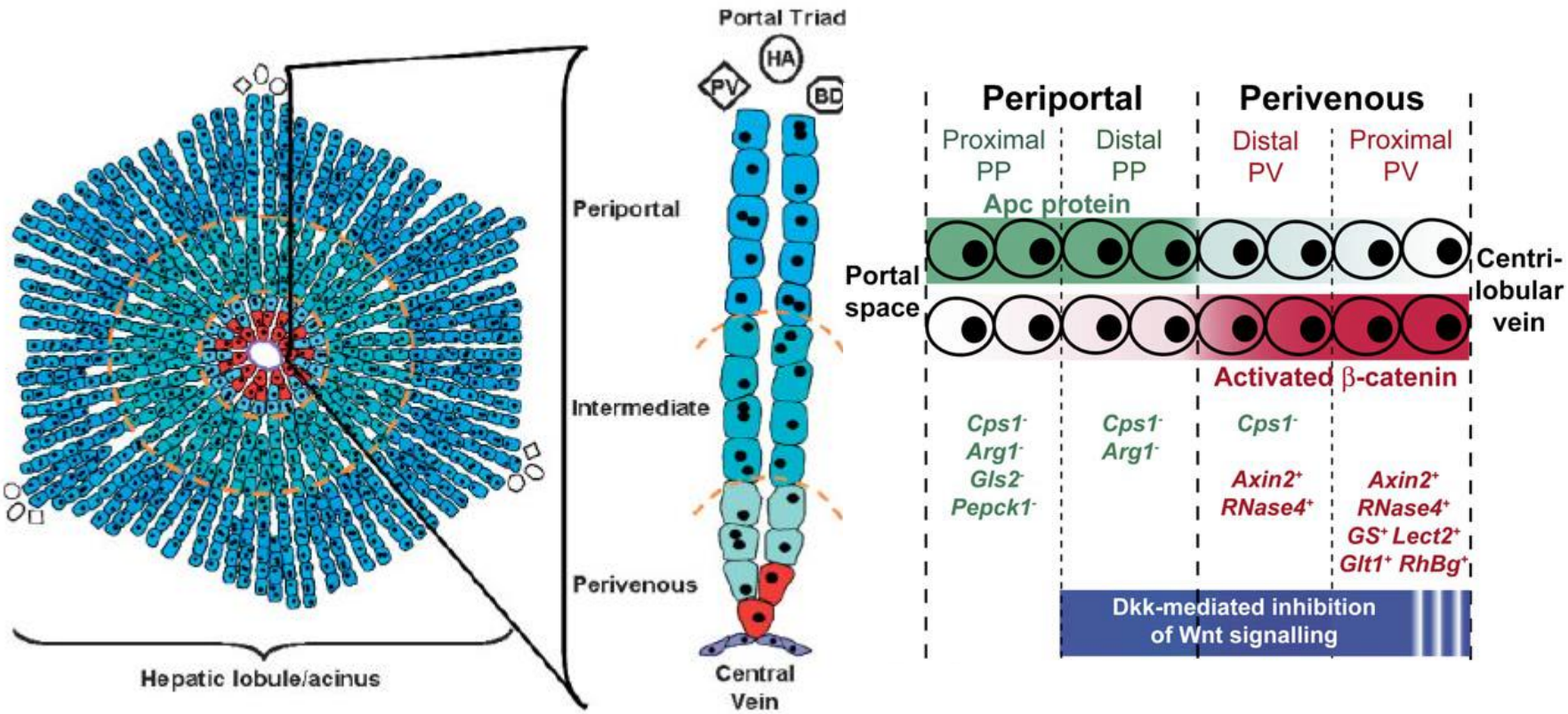
Liver: zonated organ where zones are defined by APC/Wnt proteins



Burke *et al.*, 2006

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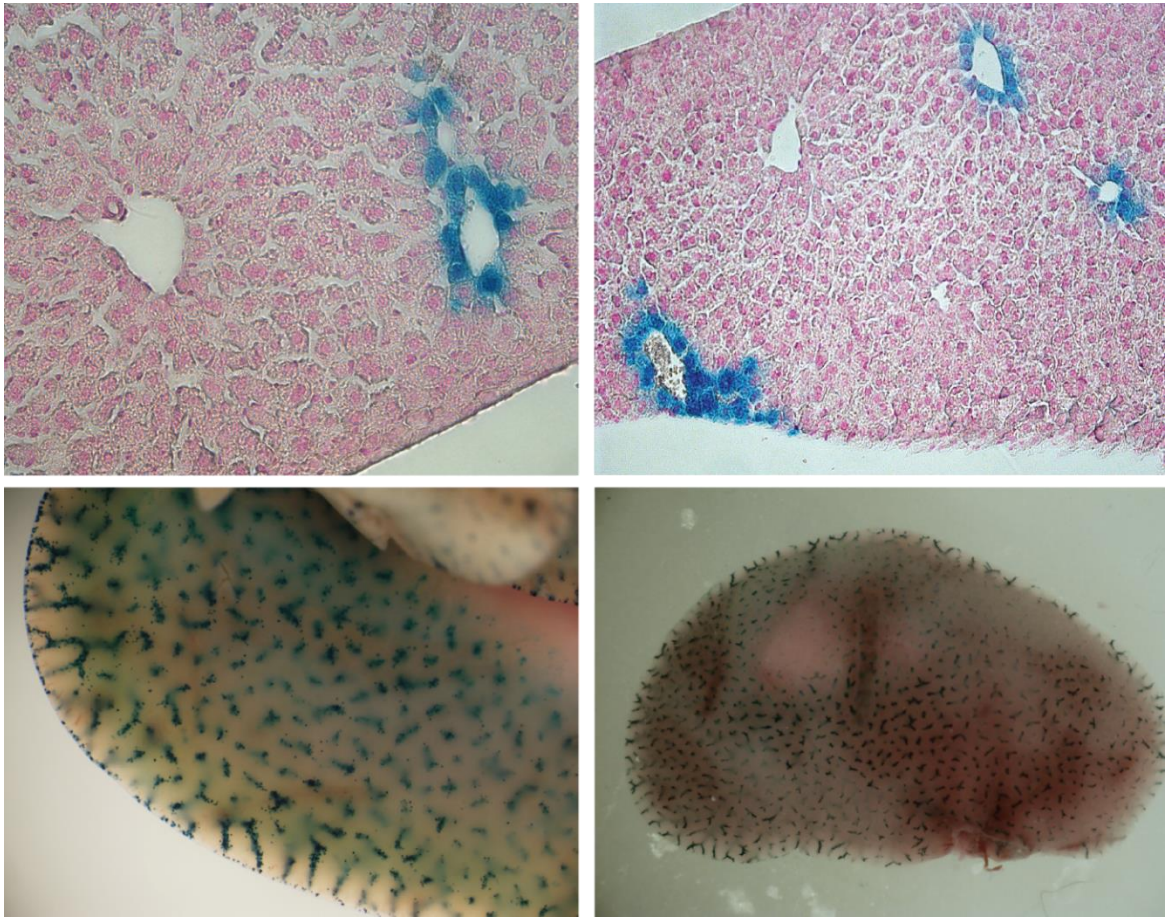
Both Nkd1-CreERT2 and Troy-CreERT2 transgenes are expressed in pericentral hepatocytes

ROSA-LacZ/**TROY**-CreERT2 ROSA-LacZ/**NKD1**-CreERT2



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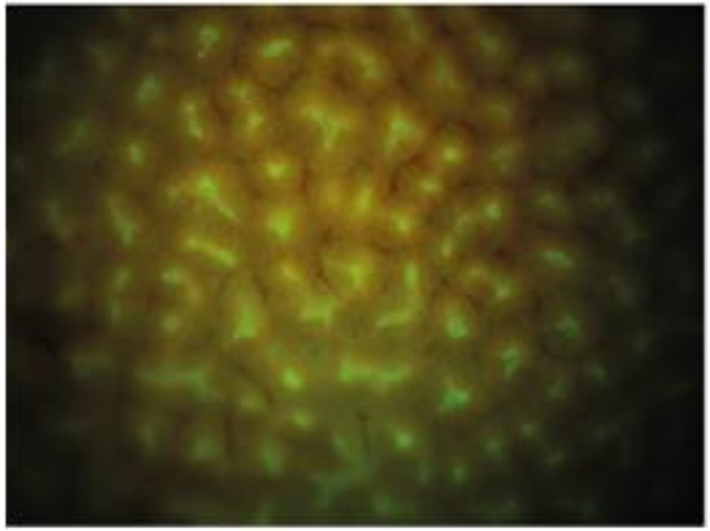
Microarray profiling of EYFP+ pericentral hepatocytes

ROSA-EYFP/NKD1-CreERT2



ID	ENTREZ	ENSEMBL	KEGG	EC	SYMBOL	BioGPS	GENENAME	logFC	p-value	q-value	A	R
ILMN_1216149	14864	•	• • •	•	Gstm3	•	glutathione S-transferase, mu 3	4.92	0.00019	0.02	9.95	5.03
ILMN_1251449	14863	•	• • •	•	Gstm2	•	glutathione S-transferase, mu 2	4.73	0.0035	0.047	10.9	6.19
ILMN_2789650	13009	•			Csrp3	•	cysteine and glycine-rich protein 3	4.64	0.00032	0.022	9.41	4.78
ILMN_2792485	218793	•	• •	•	Ube2e2	•	ubiquitin-conjugating enzyme E2E 2	4.56	0.0014	0.034	10.3	5.71
ILMN_3139875	26897	•	•	•	Acot1	•	acyl-CoA thioesterase 1	4.44	0.0022	0.04	10.3	5.87
ILMN_1258442	26876	•	• • • • • • •	•	Adh4	•	alcohol dehydrogenase 4 (class II), pi polypeptide	4.22	0.00082	0.028	9.33	5.12
ILMN_1232081	109624	•	•		Cald1	•	caldesmon 1	4.16	0.0017	0.036	9.48	5.32
ILMN_2810624	246277	•	• •	•	Csad	•	cysteine sulfinic acid decarboxylase	4.15	0.0016	0.035	9.66	5.51
ILMN_2624854	14863	•	• • •	•	Gstm2	•	glutathione S-transferase, mu 2	4.14	0.0032	0.045	11.1	6.94
ILMN_1231553	71911	•	• • •	•	Bdh1	•	3-hydroxybutyrate dehydrogenase, type 1	4.14	0.0011	0.03	11	6.82
ILMN_2789651	13009	•			Csrp3	•	cysteine and glycine-rich protein 3	4.12	0.00026	0.022	8.88	4.76
ILMN_2619565	83702	•	• • • •	•	Akr1c6	•	aldo-keto reductase family 1, member C6	4.09	0.003	0.044	10.3	6.18
ILMN_3106592	53901	•			Rcan2	•	regulator of calcineurin 2	3.98	1.2e-05	0.011	8.48	4.5
ILMN_3160292	432720	•			Akr1c19	•	aldo-keto reductase family 1, member C19	3.98	0.00014	0.018	9.89	5.91
ILMN_2862470	14863	•	• • •	•	Gstm2	•	glutathione S-transferase, mu 2	3.96	0.0019	0.037	9.7	5.75
ILMN_1215139	93673	•			Cml2	•	camello-like 2	3.96	0.0038	0.049	9.48	5.52
ILMN_3126277	114301	•			Palmd	•	palmdelphin	3.94	0.00024	0.021	9.06	5.12
ILMN_2921215	105387	•			Akr1c14	•	aldo-keto reductase family 1, member C14	3.92	0.00037	0.023	9.77	5.84
ILMN_2838308	14261	•	•	•	Fmo1	•	flavin containing monooxygenase 1	3.89	6e-04	0.026	9.34	5.45

- liver perfusion with colagenase/hyalurinodase
- cell isolation, FACS sorting
- RNA isolation, miroarray profiling

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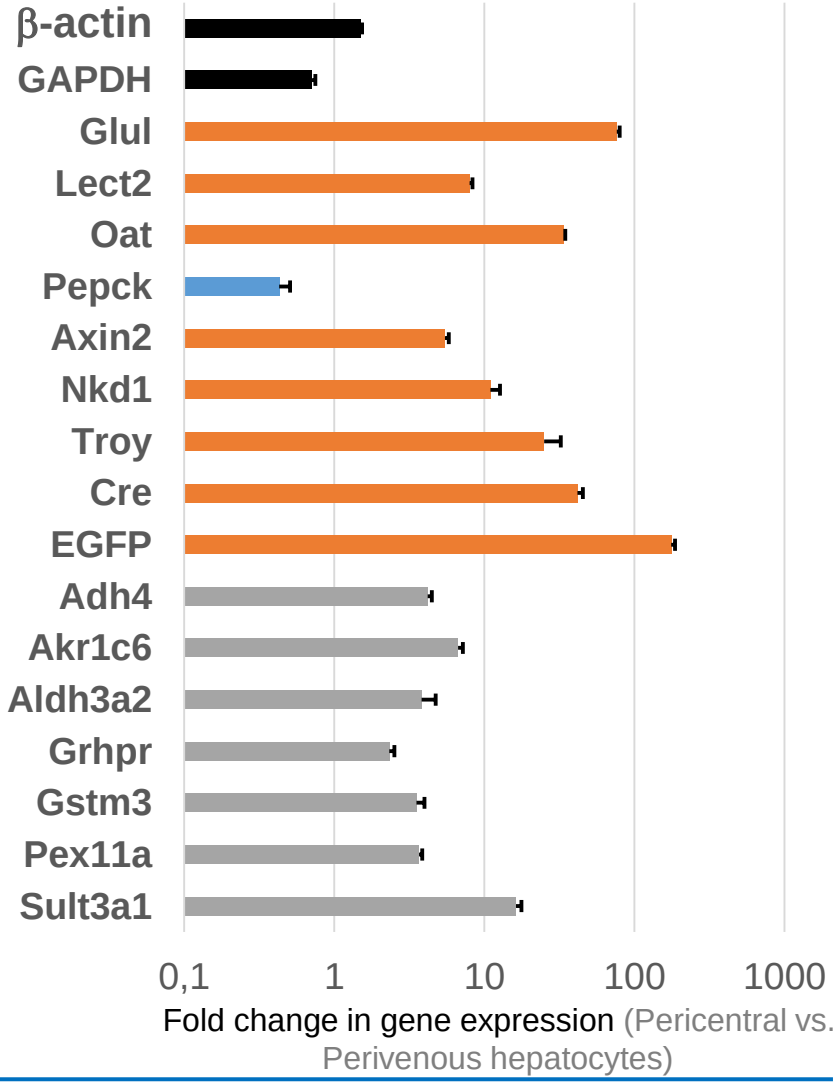
- qRT PCR confirms pericentral zonation of microarray detected genes

housekeeping gene
gene expected to be pericentrally zonated
gene expected to be perivenously zonated
newly identified pericentally zonated gene



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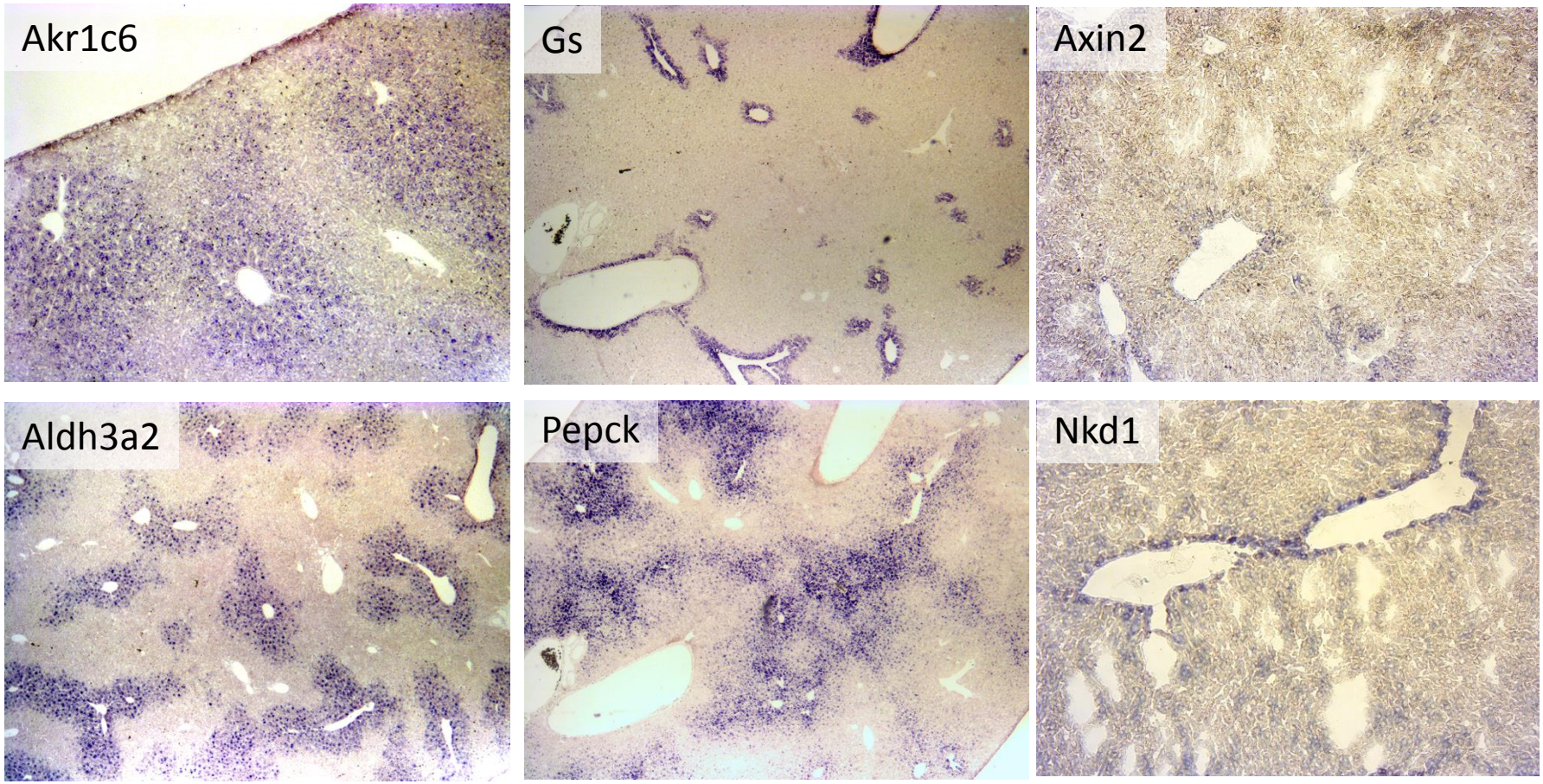
ISH shows zonated expression of Akr1c6 and Aldh3a2



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Liver: Conclusions II

- Wnt responsive Cre recombinases Troy-CreERT2 and Nkd1-CreER2 represent unique markers of othervise undistinguishable pericentral hepatocytes
- Adh4, Akr1c6, Aldh3a2, Grhpr, Gstm3, Pex11a and Sult3a1 are newly identified pericentally expressed genes
- N-Myc Troy^{+} cells can be potentially sorted directly via N-Myc

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Czech Centre for Phenogenomics

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Thank you for your attention!

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