



# Functional validation of variants in a diagnostic context

Frans Verheijen PhD

Biochemical Geneticist

*Department of Clinical Genetics*

*Erasmus Medical Center, Rotterdam, the Netherlands*

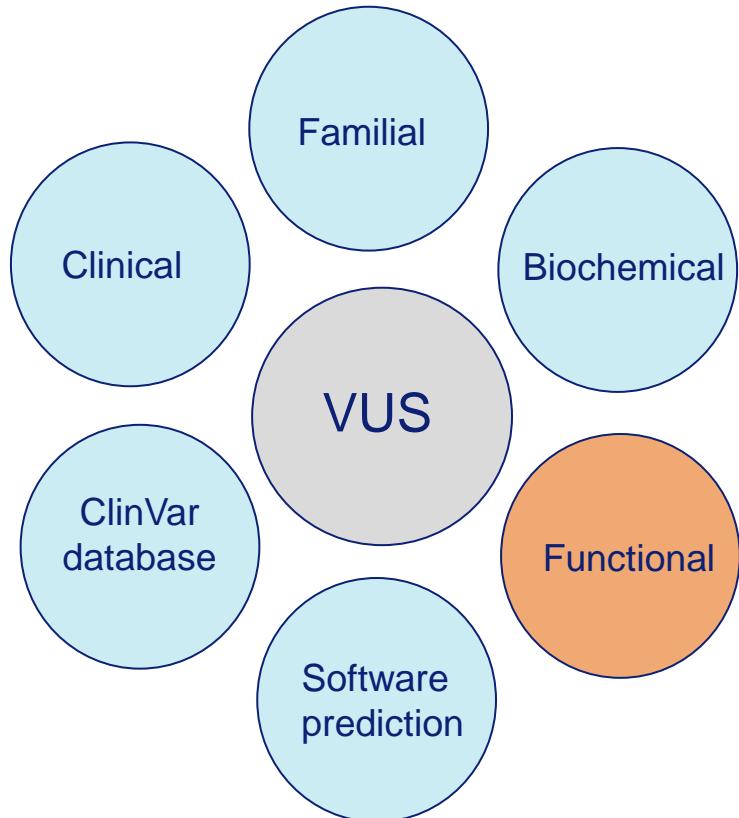
Course: Variant effect prediction 2-5 april 2019

Avans Hogeschool Breda

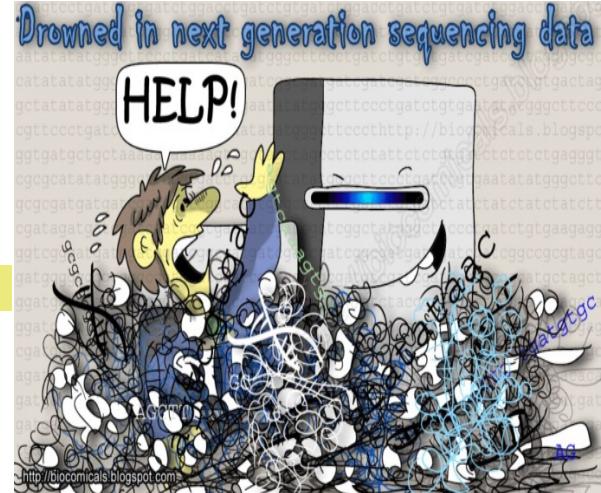
# Laboratory Diagnosis with NGS (WES, WGS)



NGS identifies many variants per person  
When do we call variant pathogenic?



Class	Clinical significance
1	Not pathogenic
2	Likely not pathogenic
3	Uncertain
4	Likely pathogenic
5	Pathogenic



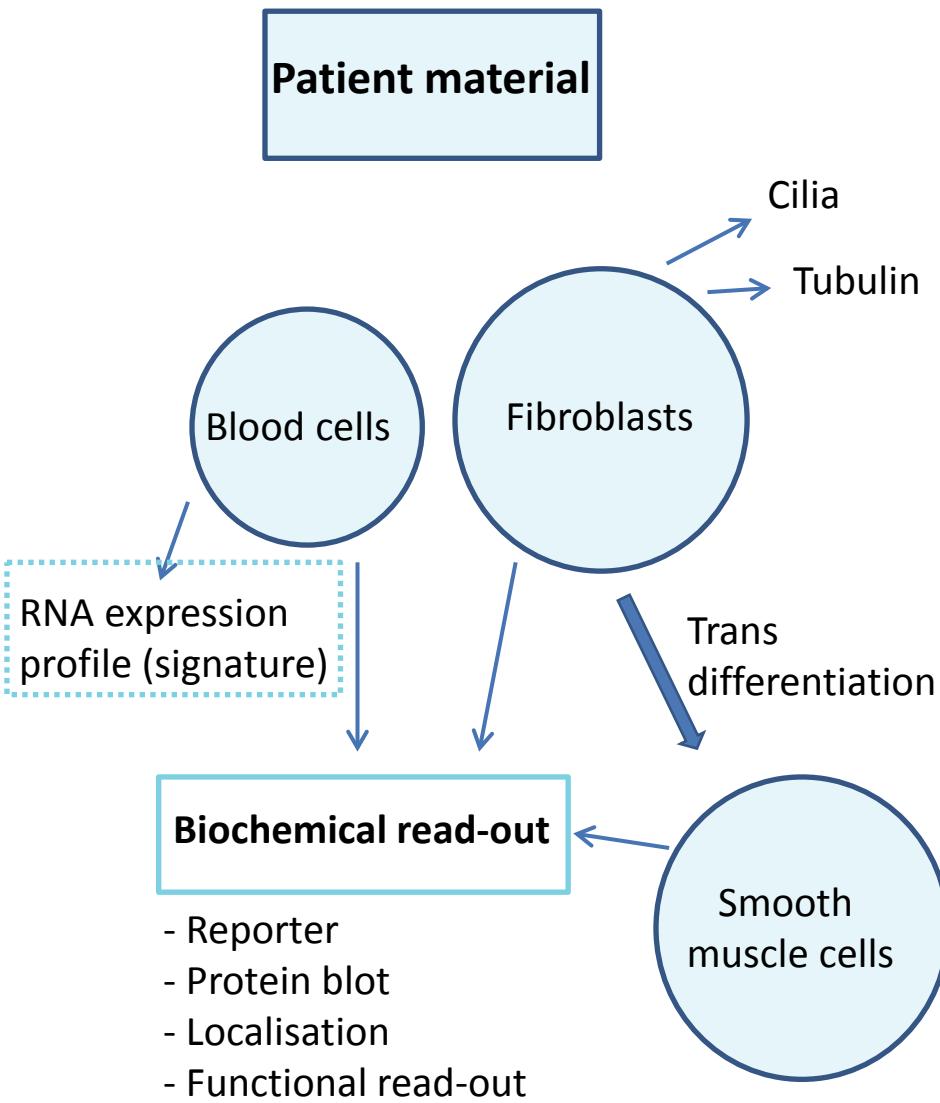
- Functional Genetics assists in VUS classification from class 3 > 4 or 5
- From **Research to Diagnostic** setting

Evaluation requires multidisciplinary approach

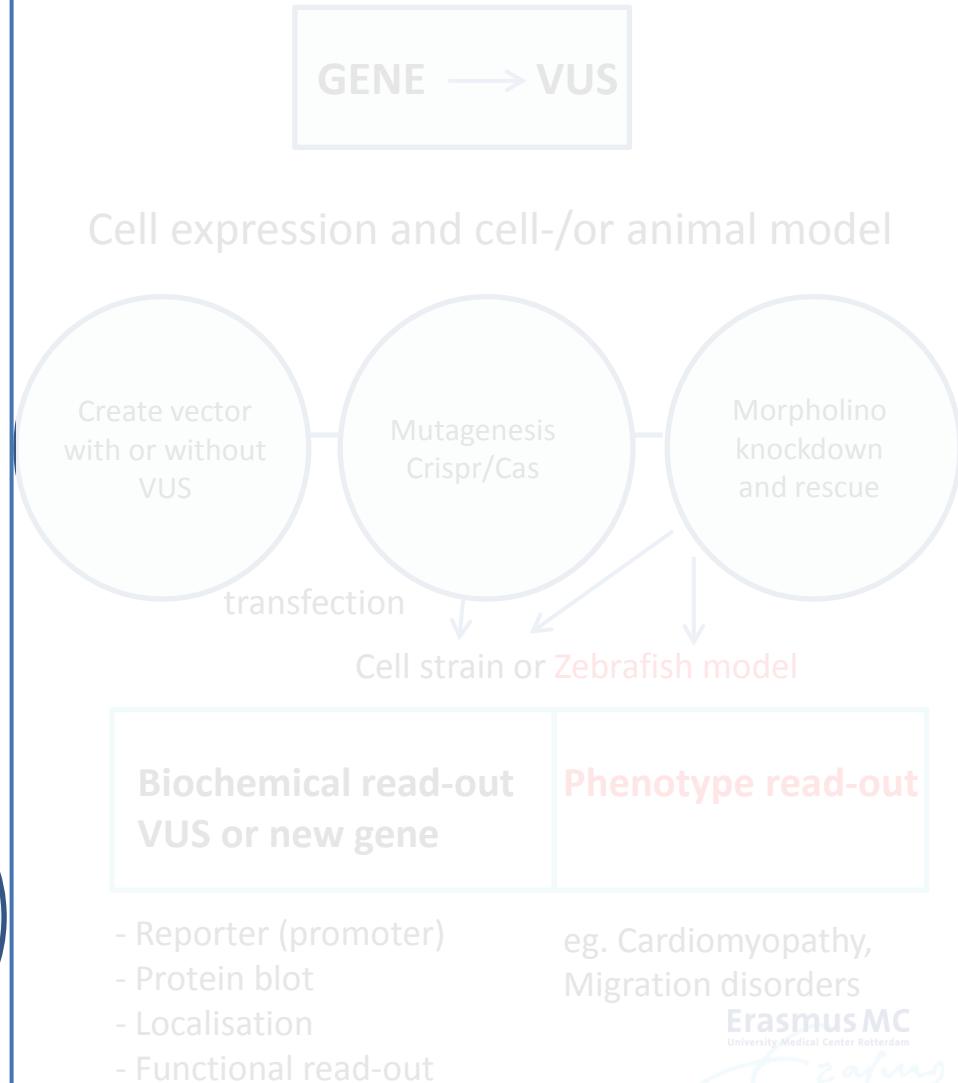
# Toolbox functional genetics, 3 parts



Indirect , screening



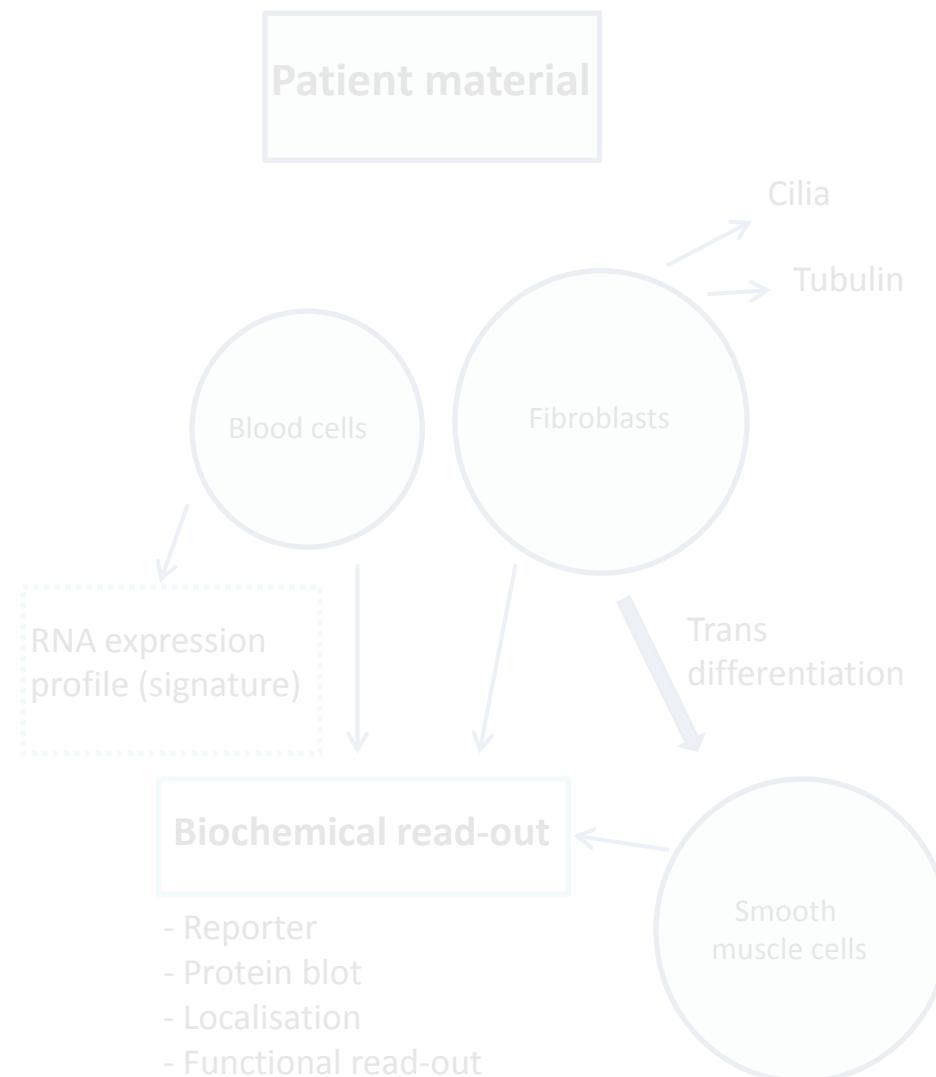
Direct variant classification



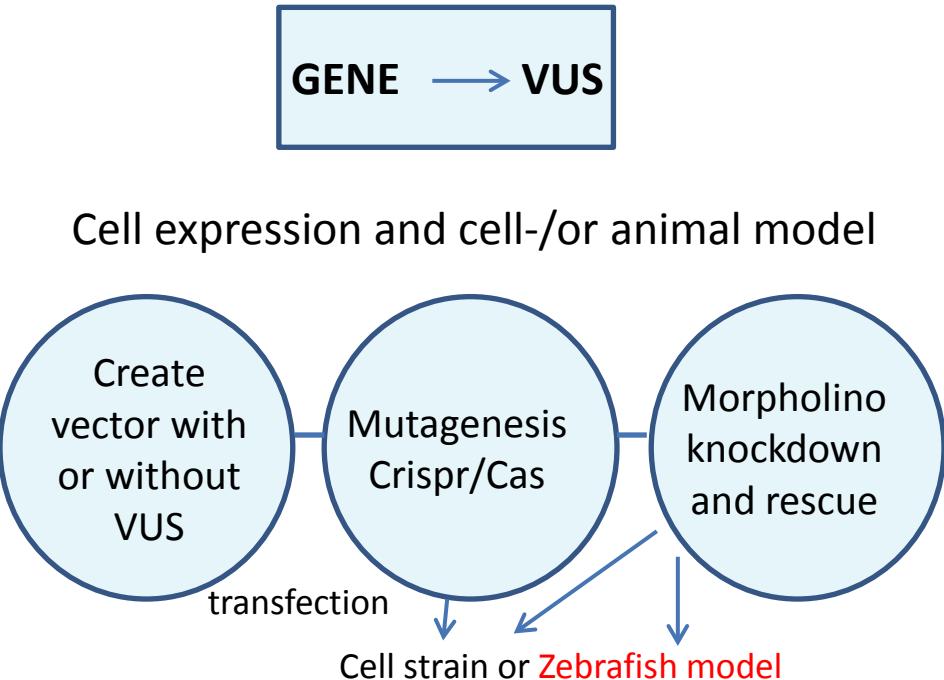
# Toolbox functional genetics



## Indirect , screening



## Direct variant classification



**Biochemical read-out  
VUS or new gene**

**Phenotype read-out**

- Reporter (promoter)
- Protein blot
- Localisation
- Functional read-out

eg. Cardiomyopathy,  
Migration disorders

**Erasmus MC**

University Medical Center Rotterdam

*Cazals*

# Diagnostic Functional Genetics requirements



- Which material, availability ?
- Relatively fast assay
- Simple read-out (biochemical, structural, mechanistic)
- Reproducibility
- Multi-purpose if possible
- Economical, not labor-intensive
- SOPS, diagnostic validated tests

## Three approaches:

### 1. Patient cells

-structural or protein function/expression

-before (screening) or after sequencing (confirmation)

### 2. Pathway approach with transfection, direct variant testing

### 3. Generate Phenocopy, animal model

# Functional validation: examples and future



TORopathies: TSC: TSC1, TSC2, megalencephaly: AKT3/TOR  
focal familiar epilepsy, (Cowdens, Peutz-Jeghers,...?)

RASopathies: Neurofibromatosis: NF1, Noonan

Lysosomal storage disorders: Enzyme deficiencies, biomarkers, Pompe disease

Ciliopathies: Screening test before WES or confirmation of effects of missense variants on cilia morphology/function in patient cells

NEW Disease modeling zebrafish: Heterotaxy disorders, cardiomyopathy

General Splice variants: confirm pathogenic effects RTPCR, RNASEQ, RNA expr.

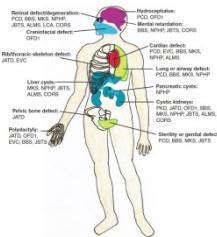
General Promotor mutants: Luciferase reporter assays

DNA-repair (CS, XP, AT, MMR)

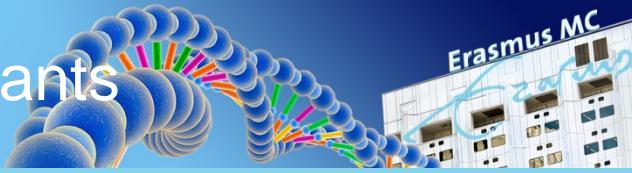
FraX: hair root test for expression

Interferonopathies

*In RED the examples  
that will be discussed*



# Functional analysis of *TSC1* and *TSC2* variants



## ***Tuberous Sclerosis Complex (TSC)***

### ***Autosomal dominant hamartoma syndrome***

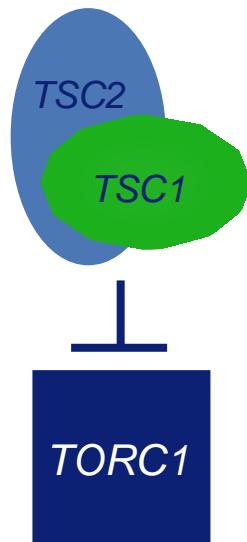
1/10 000 affected

***High penetrance***

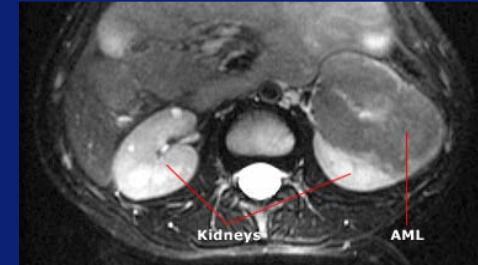
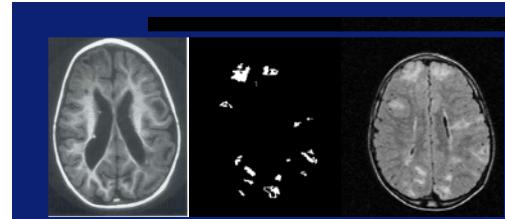
***Wide clinical variation***

*Inactivating TSC1 and TSC2*

*mutations*

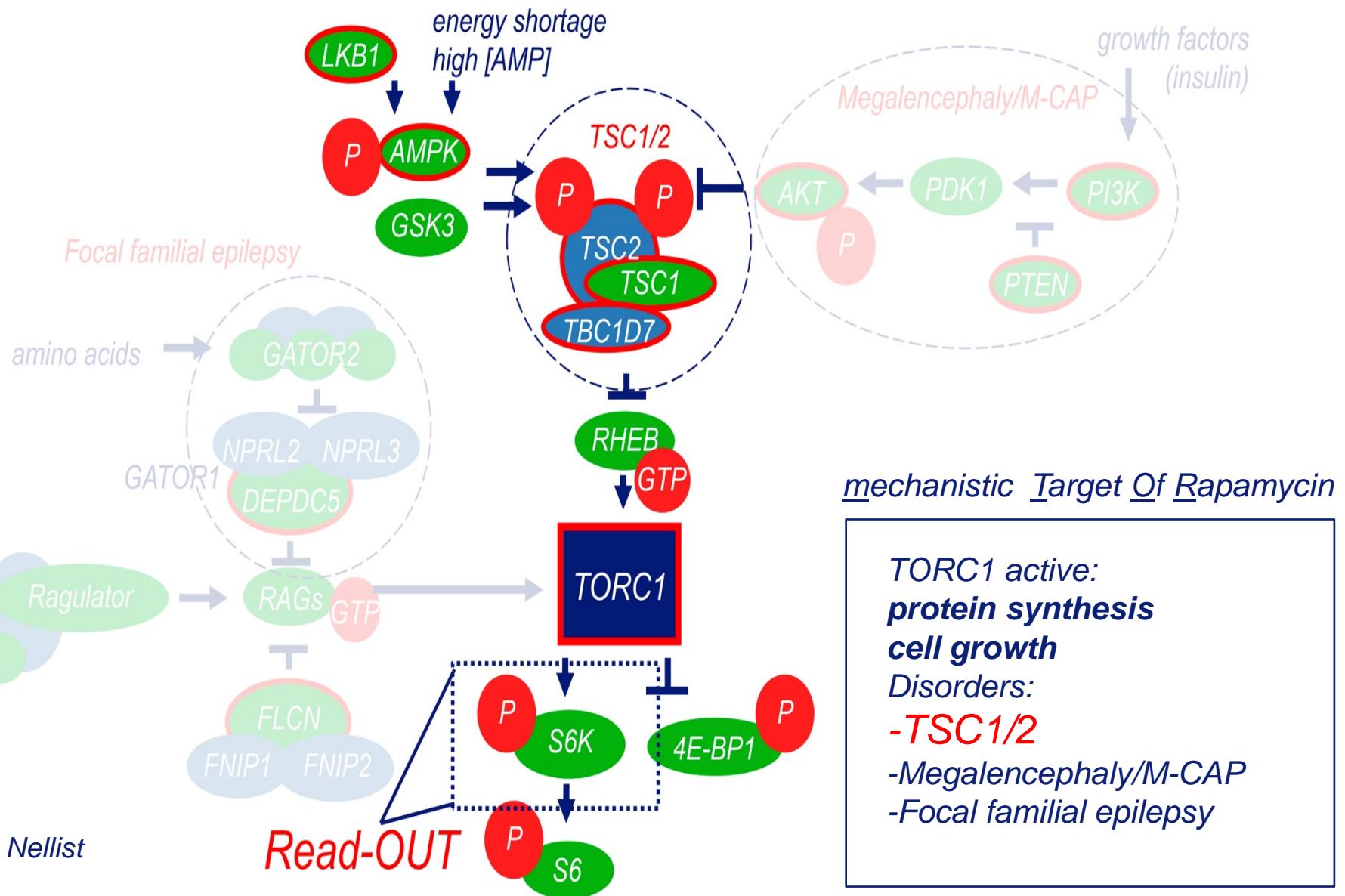
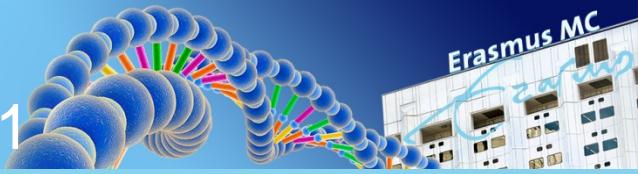


Mark Nellist

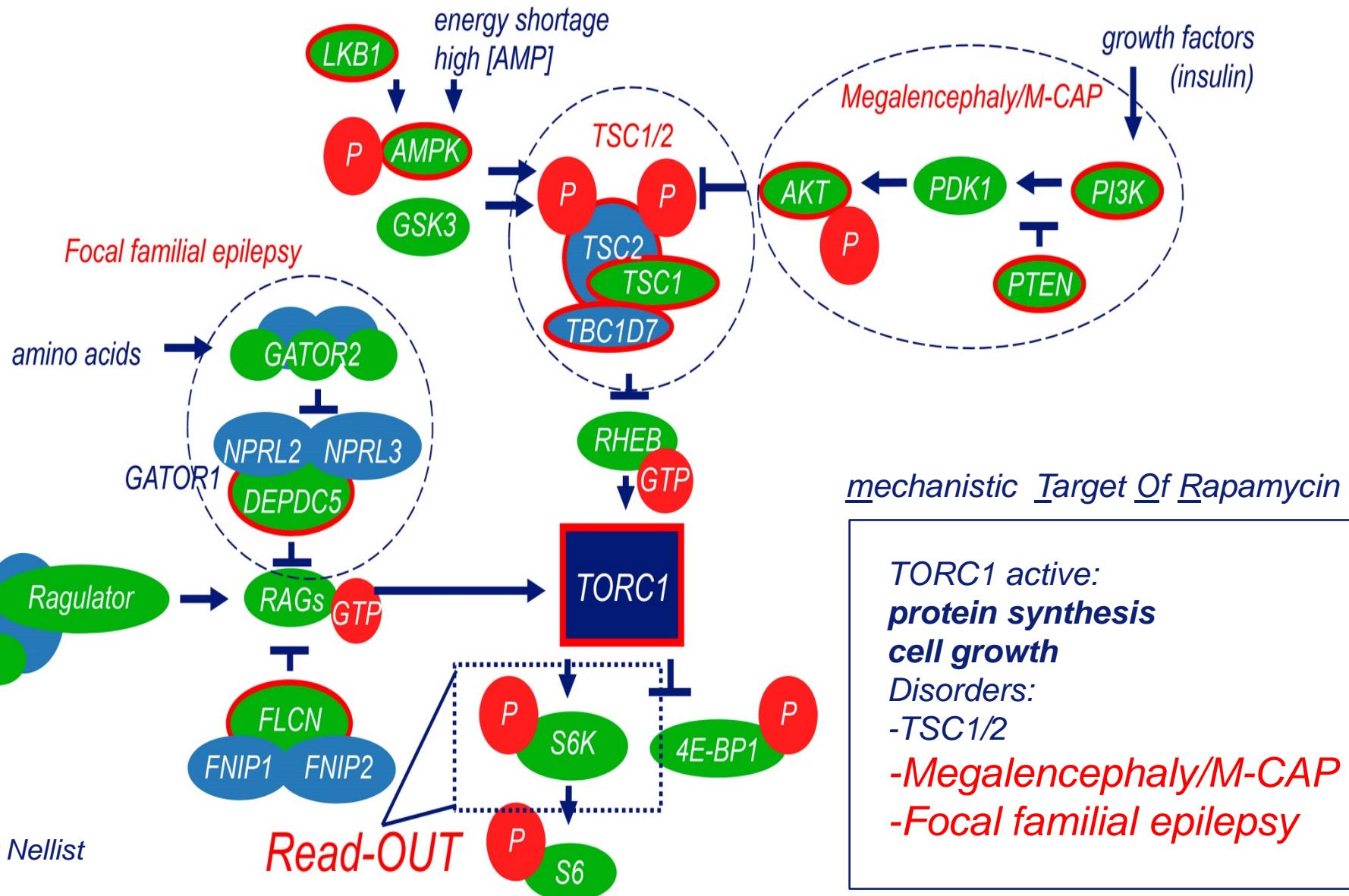


# Pathway approach

## Role of the TSC1-TSC2 complex in mTORC1



# Focal familial epilepsy (*DEPDC5*) and Megalencephaly (AKT3)



# Functional analysis of *TSC1* and *TSC2* variants: immunoblotting



Create variants  
(site-directed  
mutagenesis)



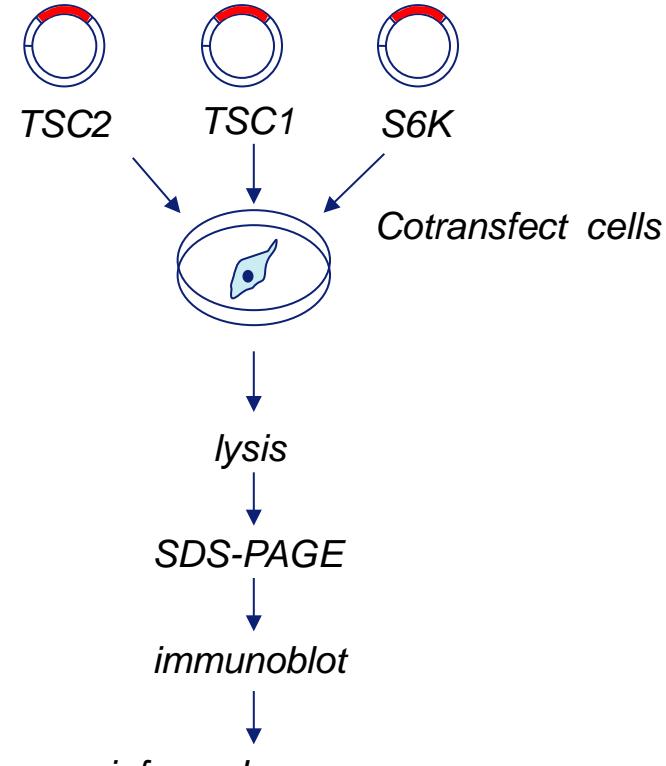
Check by  
sequencing



Transfect into cells  
(HEK293)



Effect on TORC1  
signaling  
(S6K-T389  
phosphorylation)

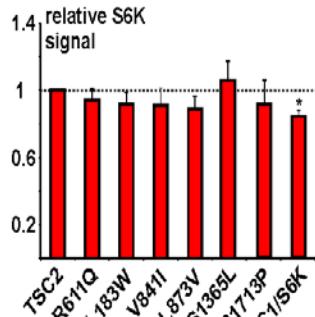
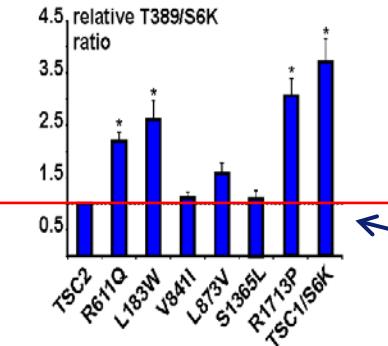
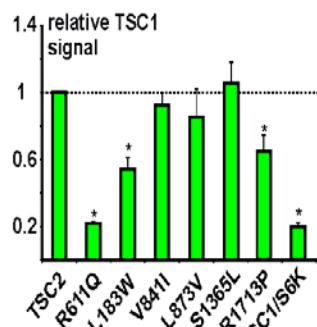
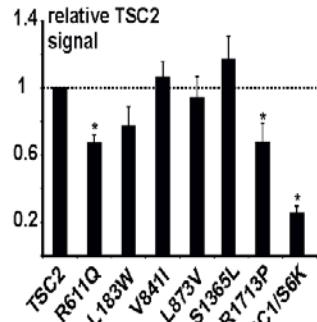
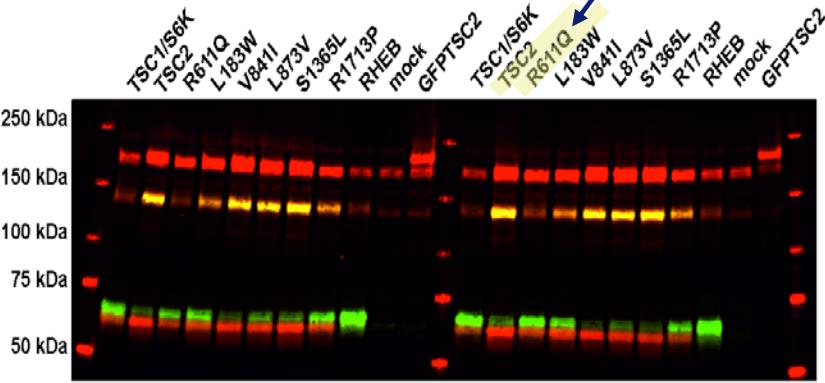


Constructs generate Flagged-Proteins and  
Specific antibodies against P and not P forms are used

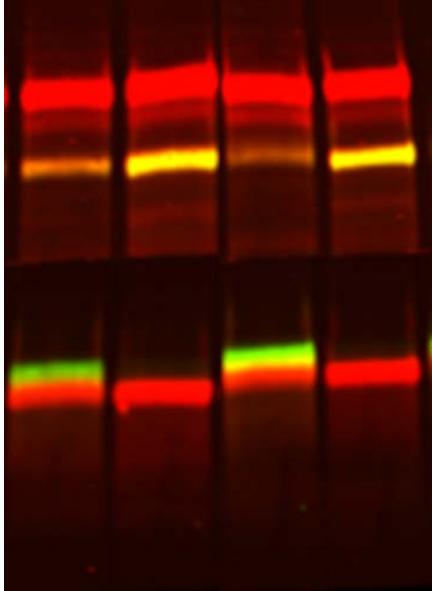
# Functional analysis of *TSC2* variants: immunoblot results



Known pathog. var. *TSC2* R611Q

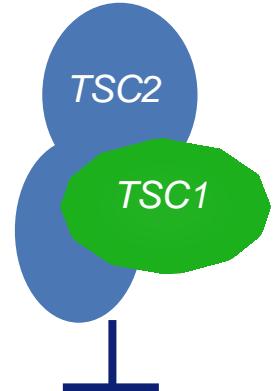


L180P  
M649L  
R611Q  
TSC2<sup>wt</sup>



TSC2  
TSC1

S6KP  
S6K

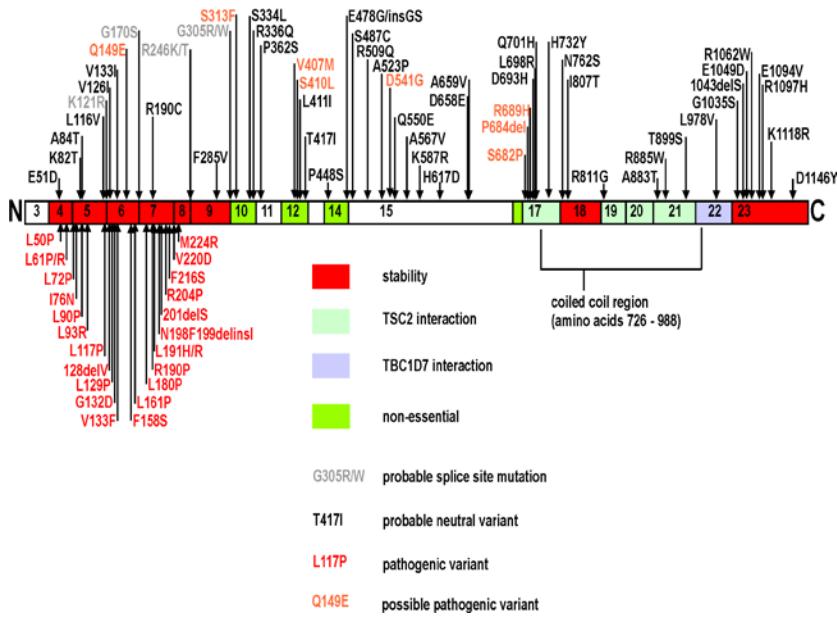


Marianne Hoogeveen-Westerveld  
Peter Elfferich

# Functional assay of *TSC1* and *TSC2* variants: overview

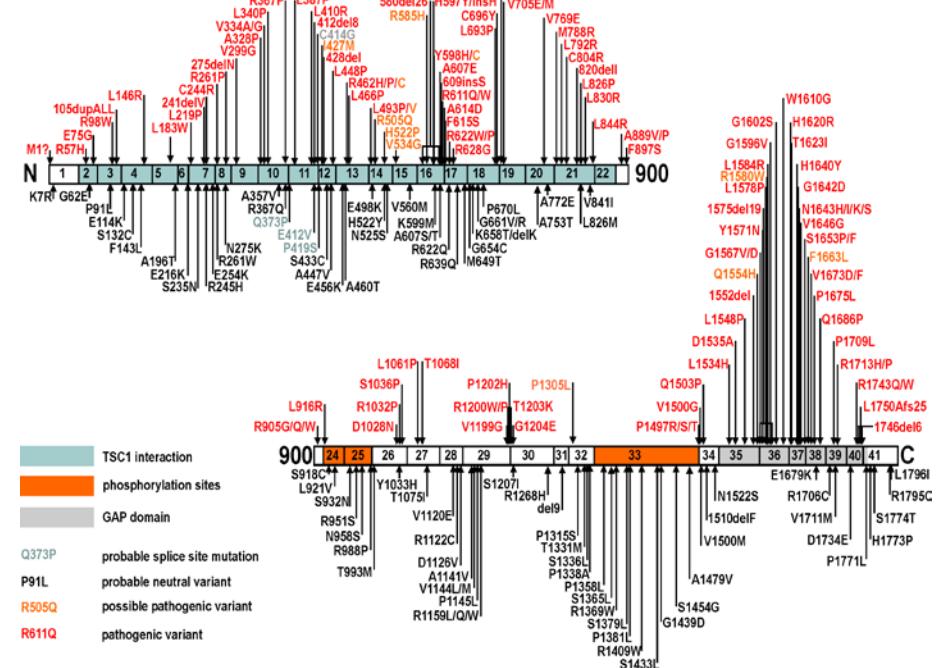


82 *TSC1* variants assessed



24 pathogenic (29%); 8 possibly pathogenic (10%); 44 no effect detected (54%); 6 putative splicing mutations (7%)

214 *TSC2* variants assessed

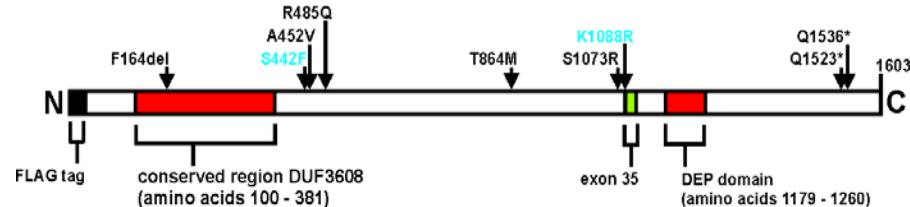


111 pathogenic (52%); 12 possibly pathogenic (6%); 87 no effect detected (41%); 4 putative splice mutations (2%)

# Functional analysis of *DEPDC5* and *AKT3* variants

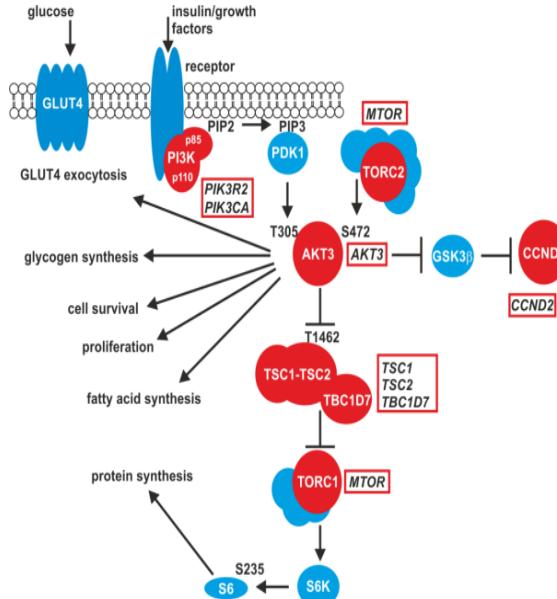


**Familial focal epilepsy with variable foci (FFEVF)  
Autosomal dominant epilepsy syndrome  
Inactivating *DEPDC5* mutations**



FFEVF/ovarian cancer:  
12 *DEPDC5* variants tested

**Megalencephaly  
Germ-line and somatic activating  
*AKT3* mutations  
*AKT3* is the brain-specific *AKT* isoform**



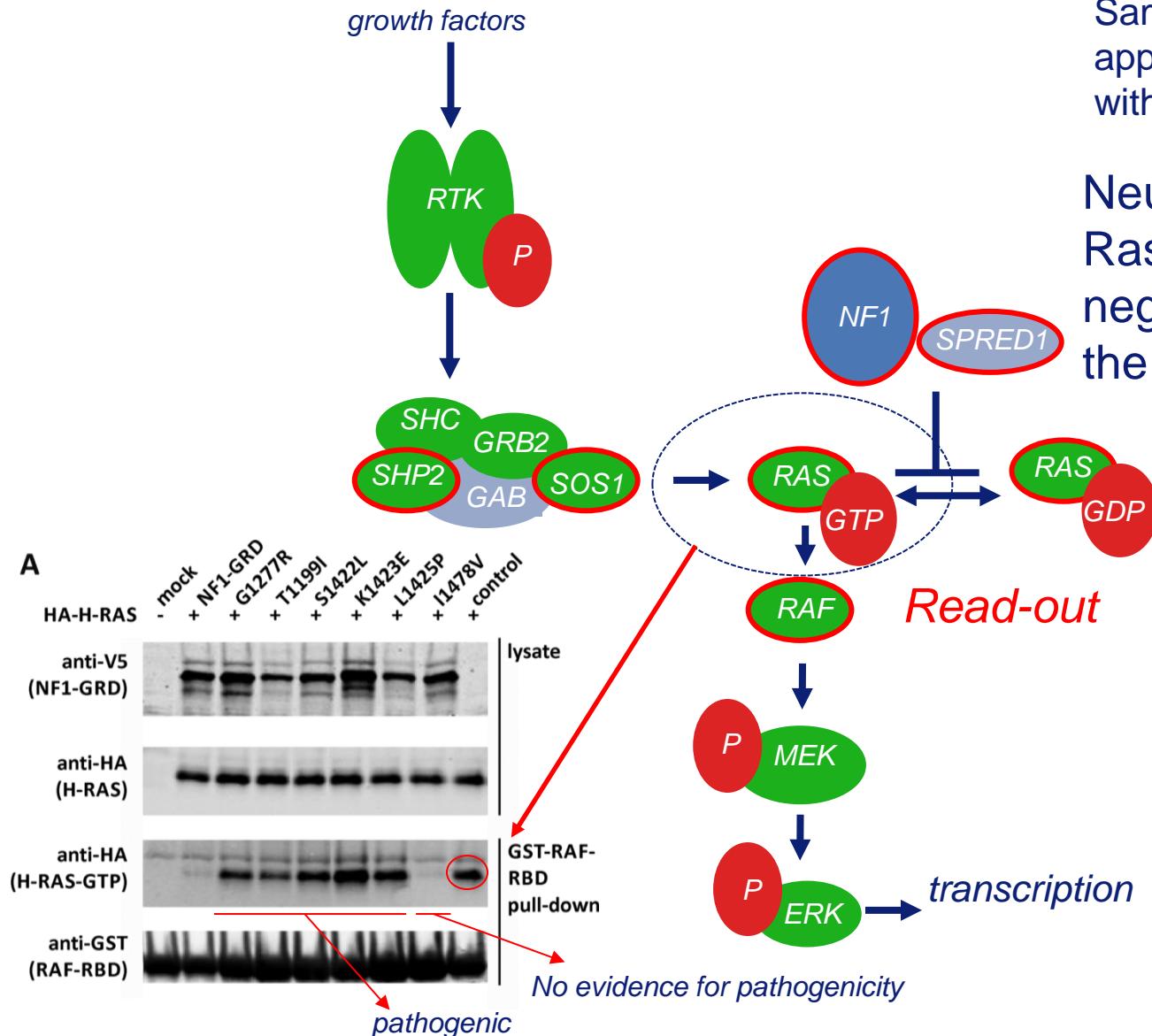
Megalencephaly:  
5 *AKT3* variants tested

# RAS signaling, Neurofibromatosis



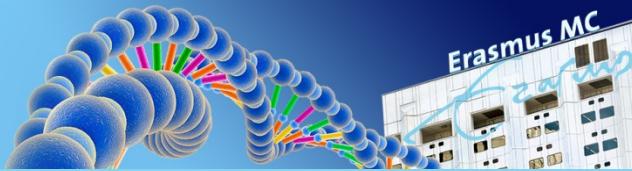
Same principle as TSC assay applied to another pathway with pull-down assay

Neurofibromin is a Ras-GTPase and is a negative regulator of the RAS pathway

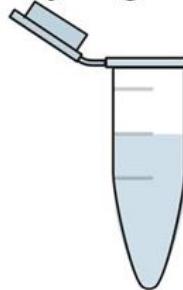


*Functional analysis of variants of uncertain clinical significance in components of the RAS signaling pathway*

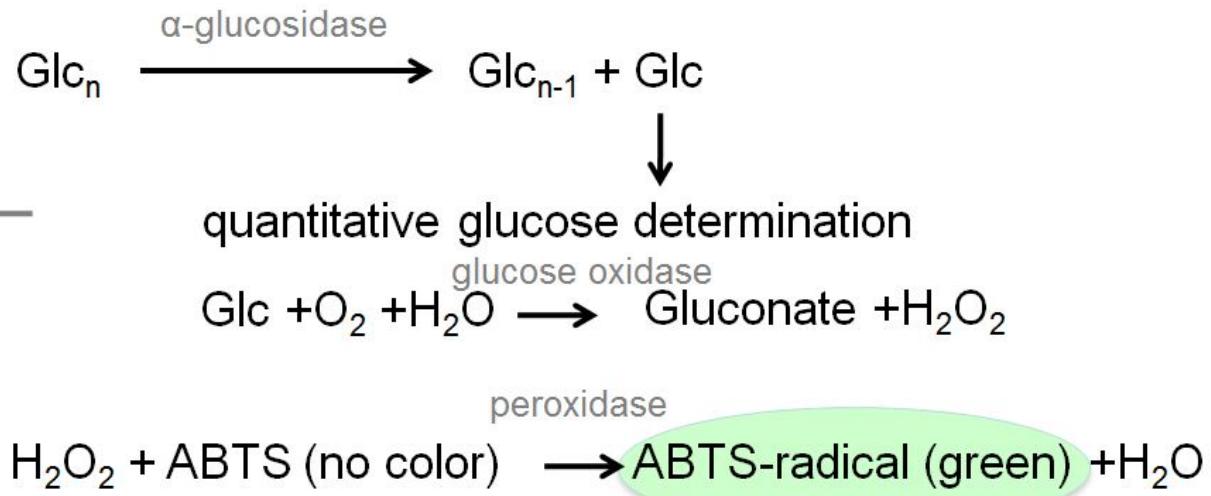
# Example : Lysosomal storage disorders Pompe disease, precision medicine



Glycogen Assay:



AGP reagents



(ABTS=2,2'-azino-di-[3-ethyl-benzthiazolinsulphonate])

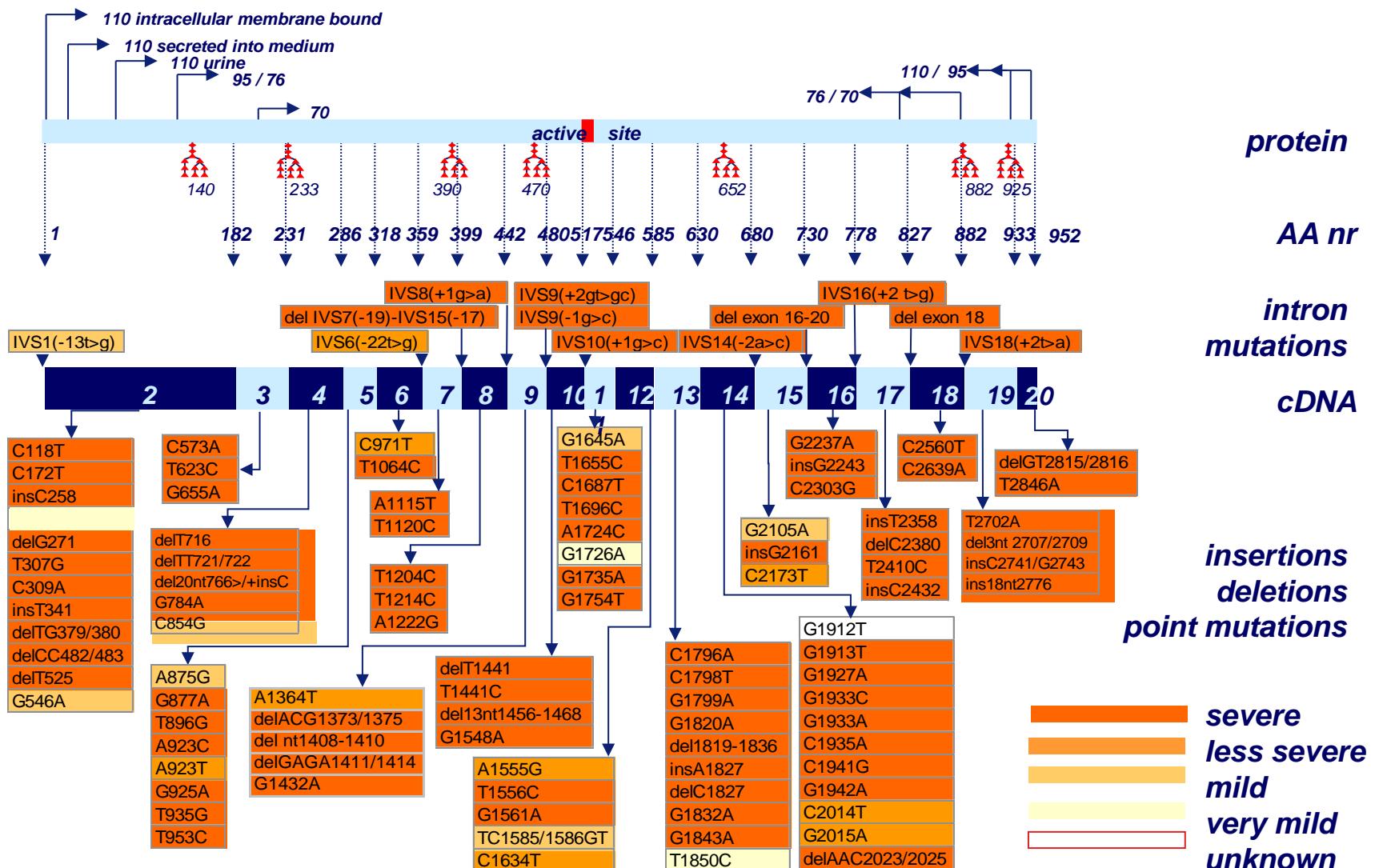
4MU-Glc Assay:



Measure fluorescence



# Functional analysis of GAA variants expression (>225 variants tested)



Marian Kroos, Arnold Reuser

Erasmus MC  
University Medical Center Rotterdam

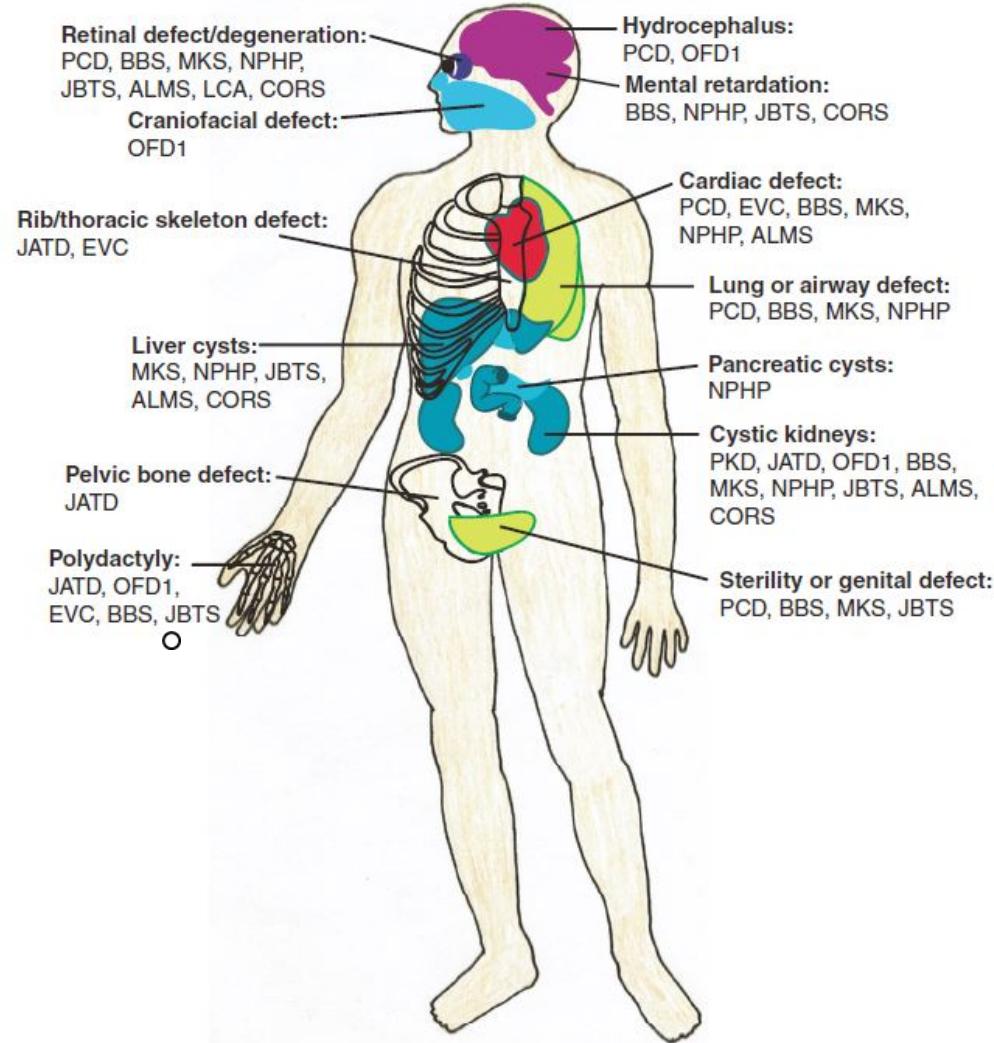
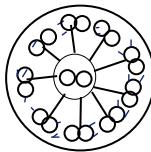
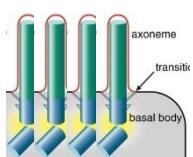
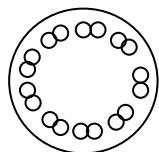
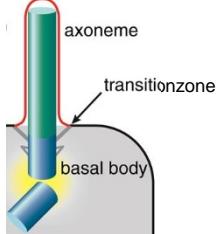
C2afus

# Structural mechanistic approach, Ciliopathies



Inherited developmental defects in the function of a specific cellular antenna-like organelle: the **cilium**, critical for numerous developmental pathways

2 cilia types: primary and motile



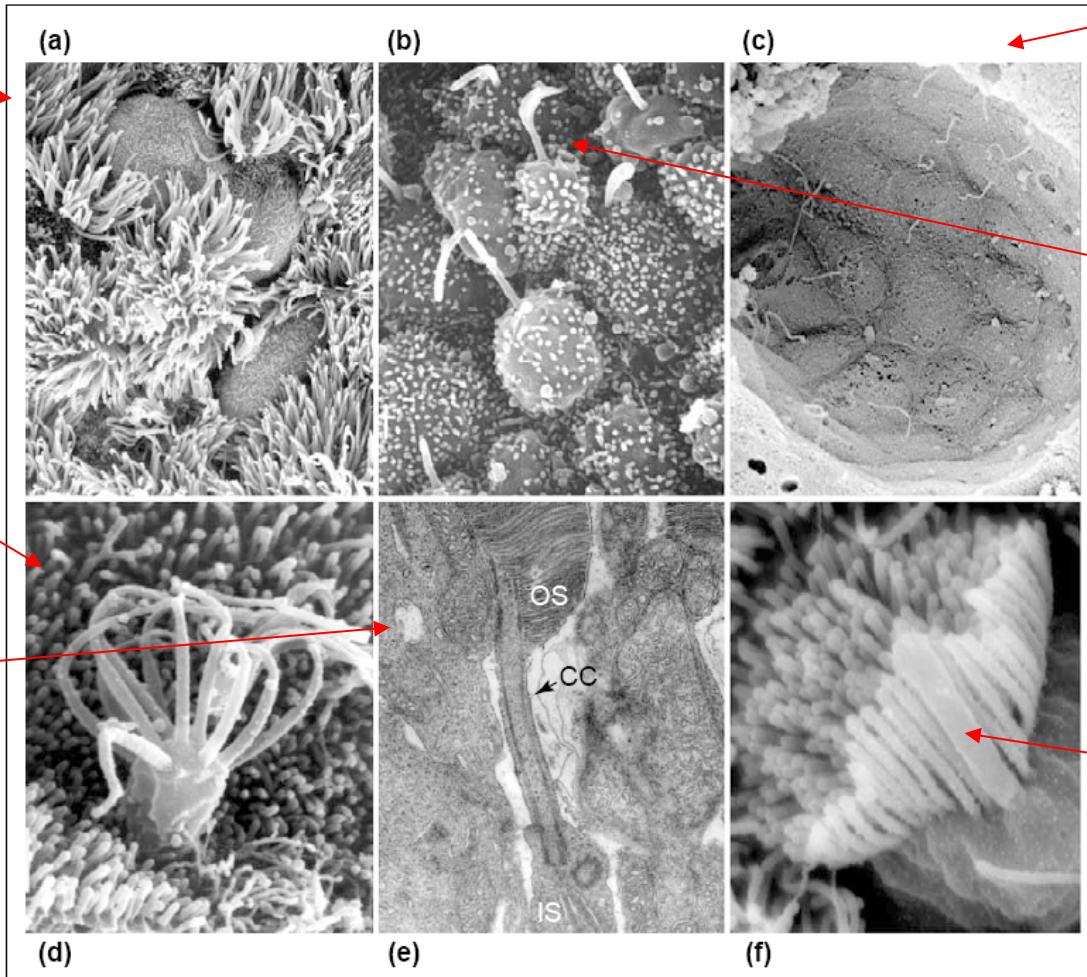
# Scanning EM images demonstrating cilia diversity



Airway epithelium,  
multiple cilia per cell

Olfactory epithelium,  
multiple cilia per cell

Rod photo-  
receptor cell with  
connecting cilium

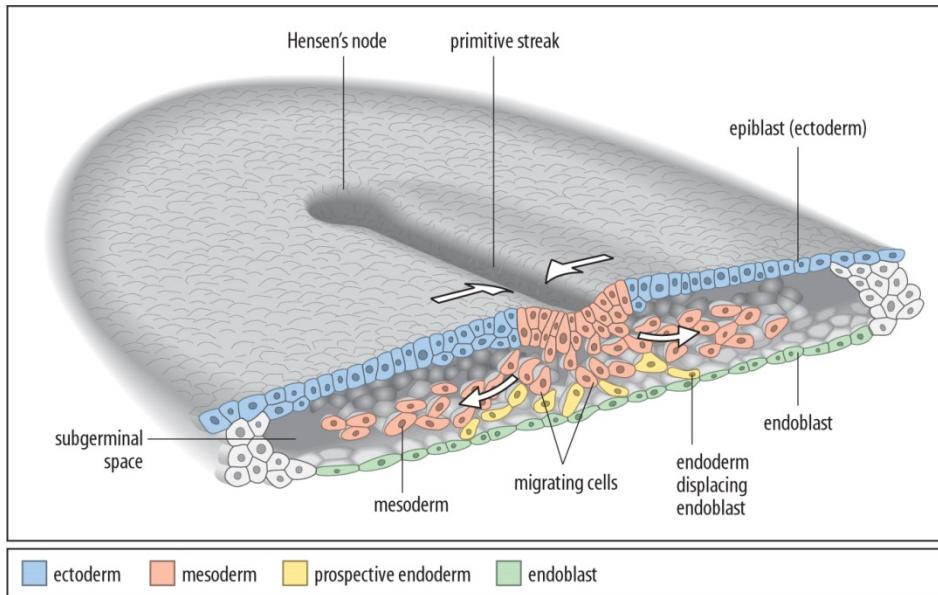
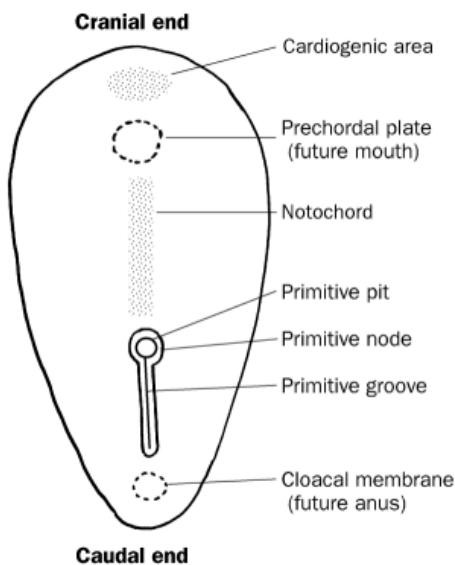
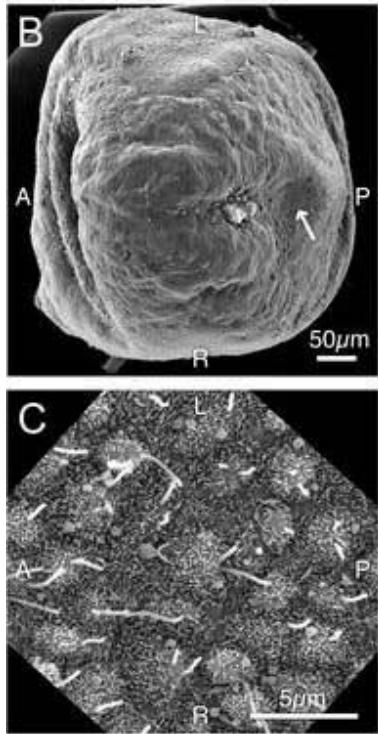
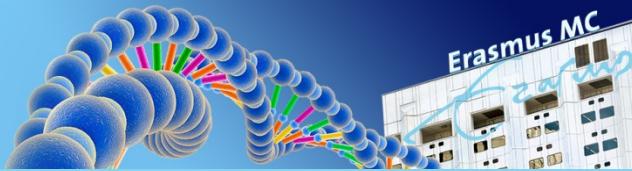


Renal tubuli epithelium,  
one cilium per cell

Node cilia embryonal,  
one cilium per cell  
important:**L/R symmetry**

Inner ear,  
kinocilium  
bundle, hair  
cells

# Nodal Cilia important for L/R asymmetry

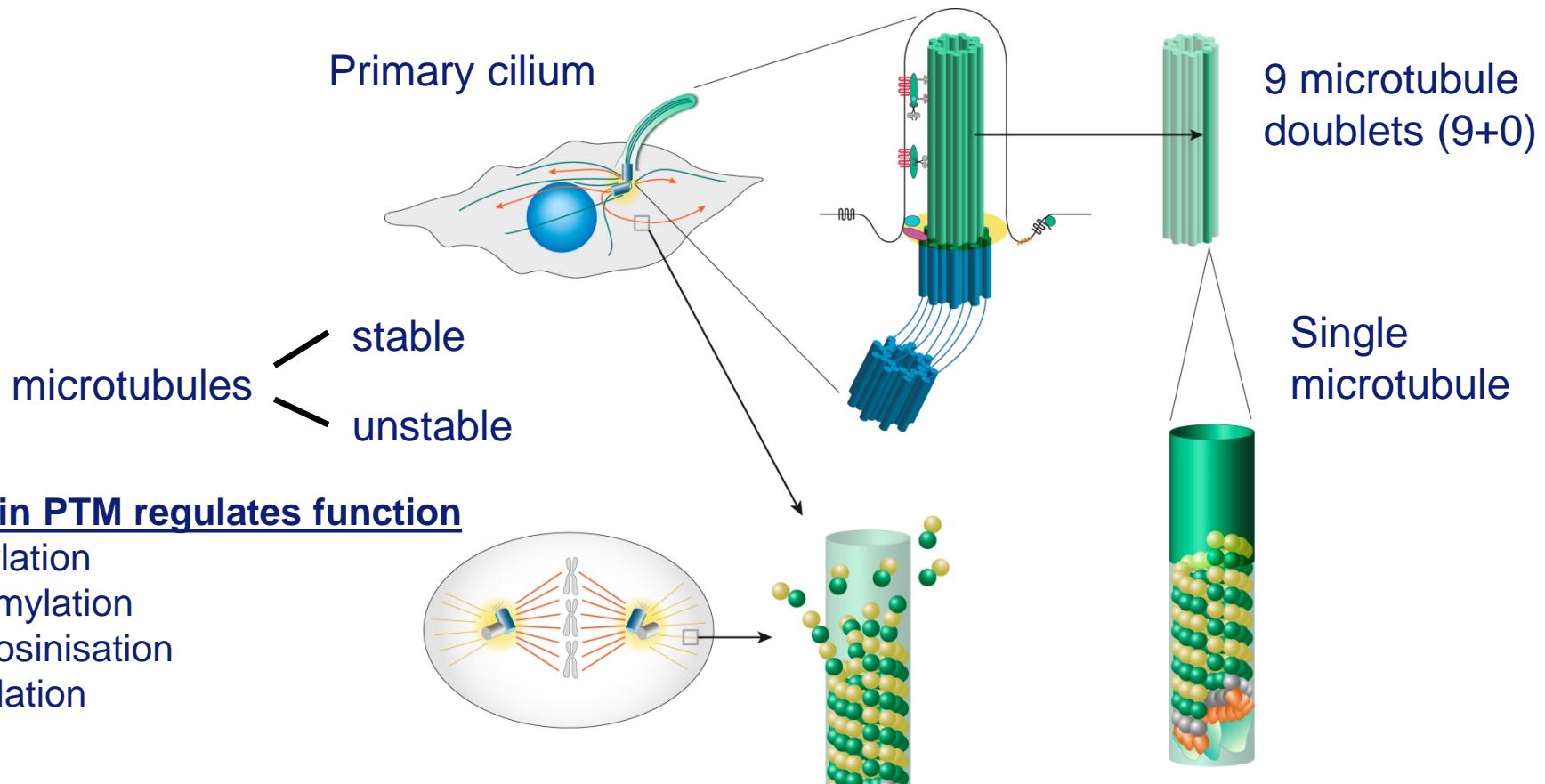


At the time of gastrulation, the mouse embryo (and the human embryo as well) is essentially a flat, two-layered sheet, with a groove in the middle called the primitive streak, and a dimple at the anterior end called Henson's node.

## Explanation for Ciliopathies with situs inversus (e.g. Kartagener syndrome)

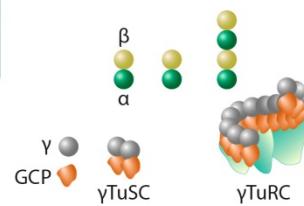
Nonaka S, Yoshioka S, Watanabe D, Ikeuchi S, Goto T, et al. (2005) De novo formation of left/right asymmetry by posterior tilt of nodal cilia. *PLoS Biol* 3(8): e268.

# Primary Cilia are made from tubulin microtubules



**Tubulin PTM regulates function**  
-acetylation  
-glutamylylation  
-detyrosinisation  
-glycylation

Microtubule with tubulin polymers synthesized from  $\alpha$ - and  $\beta$ - tubulin dimers on base of  $\gamma$ -tubulin

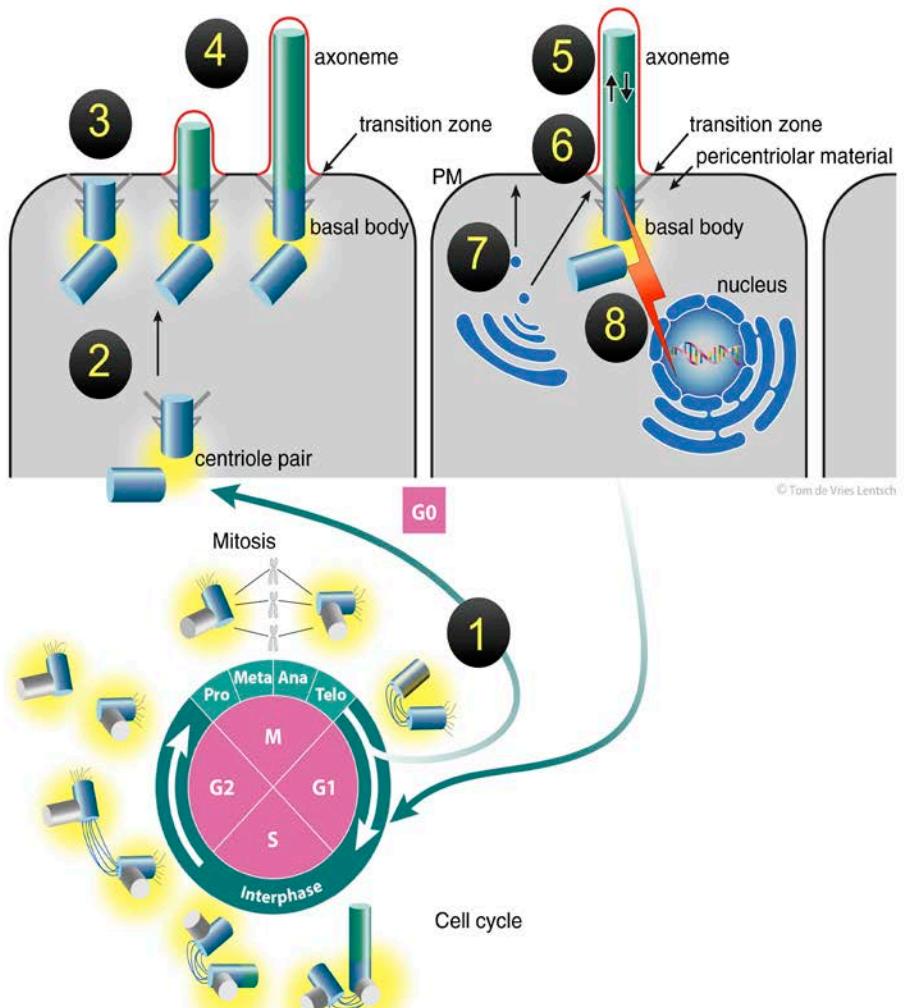


# Ciliogenesis starts from centrosomal tubulin



Primary Cilia are important sensory organelles

- Major signaling pathway during development goes via primary cilia **mammalian hedgehog signaling!**
  - **PDGFR $\alpha$  stimulated migration** of fibroblasts and stem cells
  - **Brain development**, cortex formation, migration?
- 
- Develop assays for these functions ?
  - Which cells? Cultured (patient) fibroblasts ?



# Primary Cilia structural test

Length and number, count >100 cells

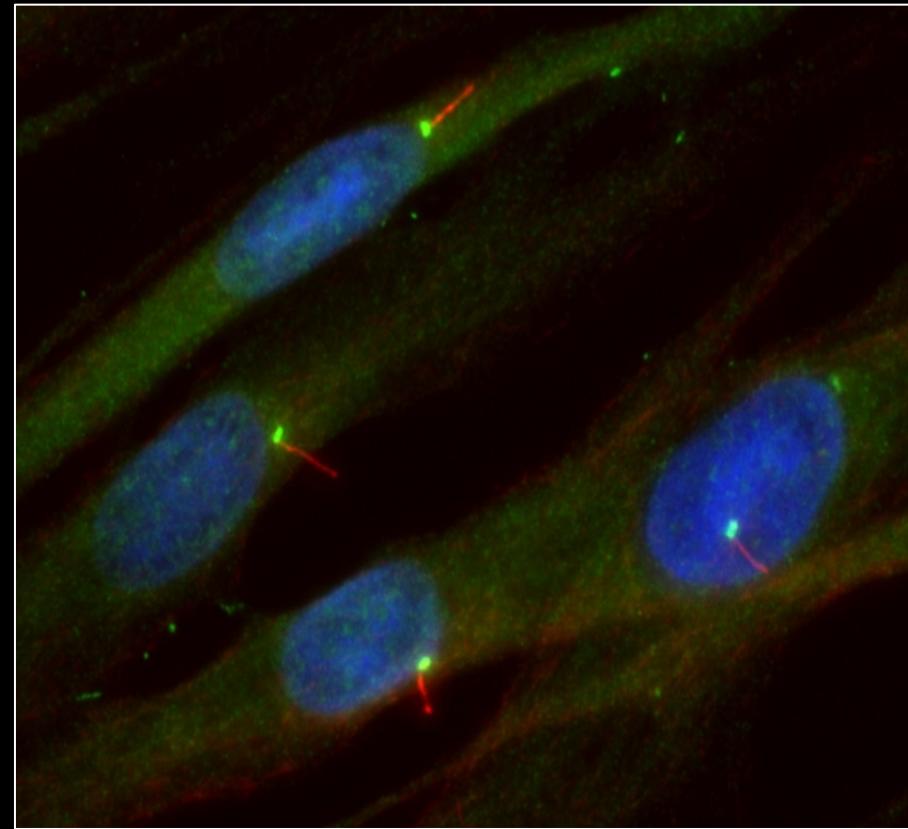
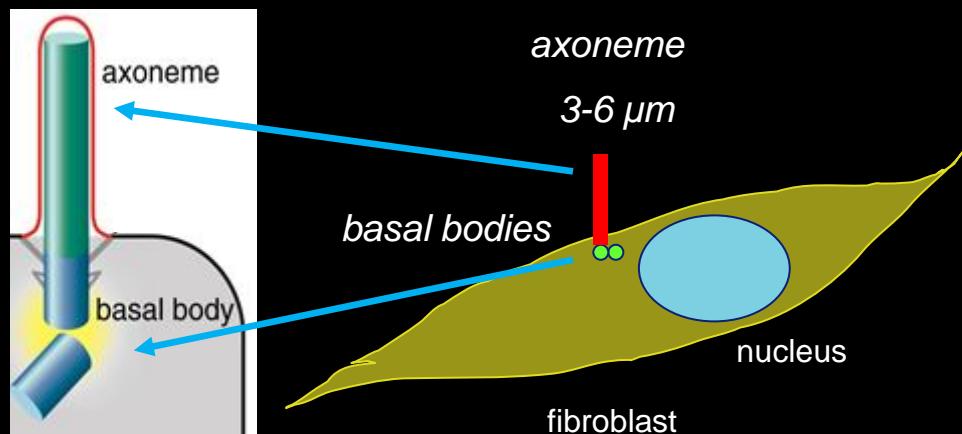


Cultured fibroblasts after starvation, staining with immunofluorescent antibodies

*Green*: anti- $\gamma$ -tubulin

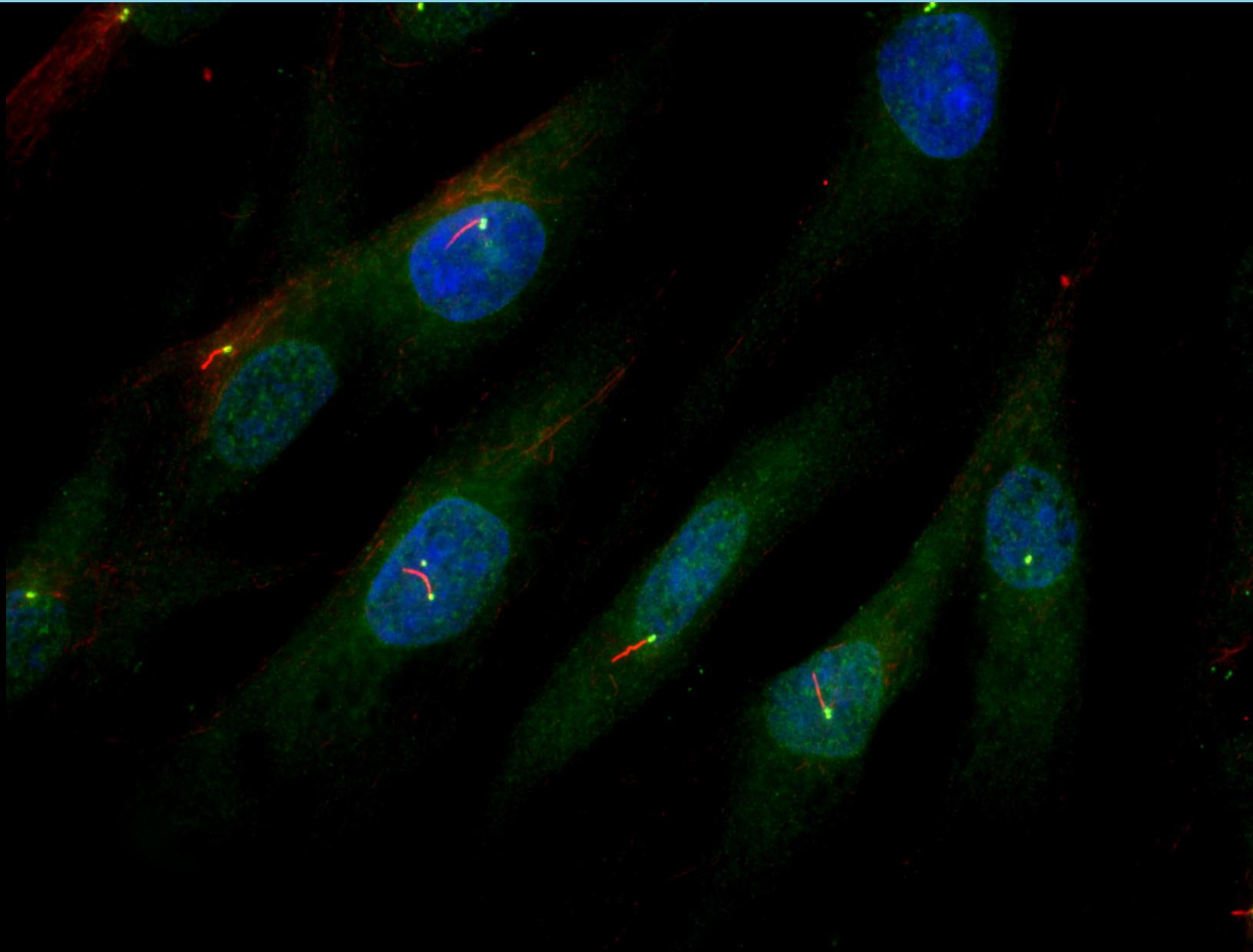
*Red*: anti-acetylated-tubulin

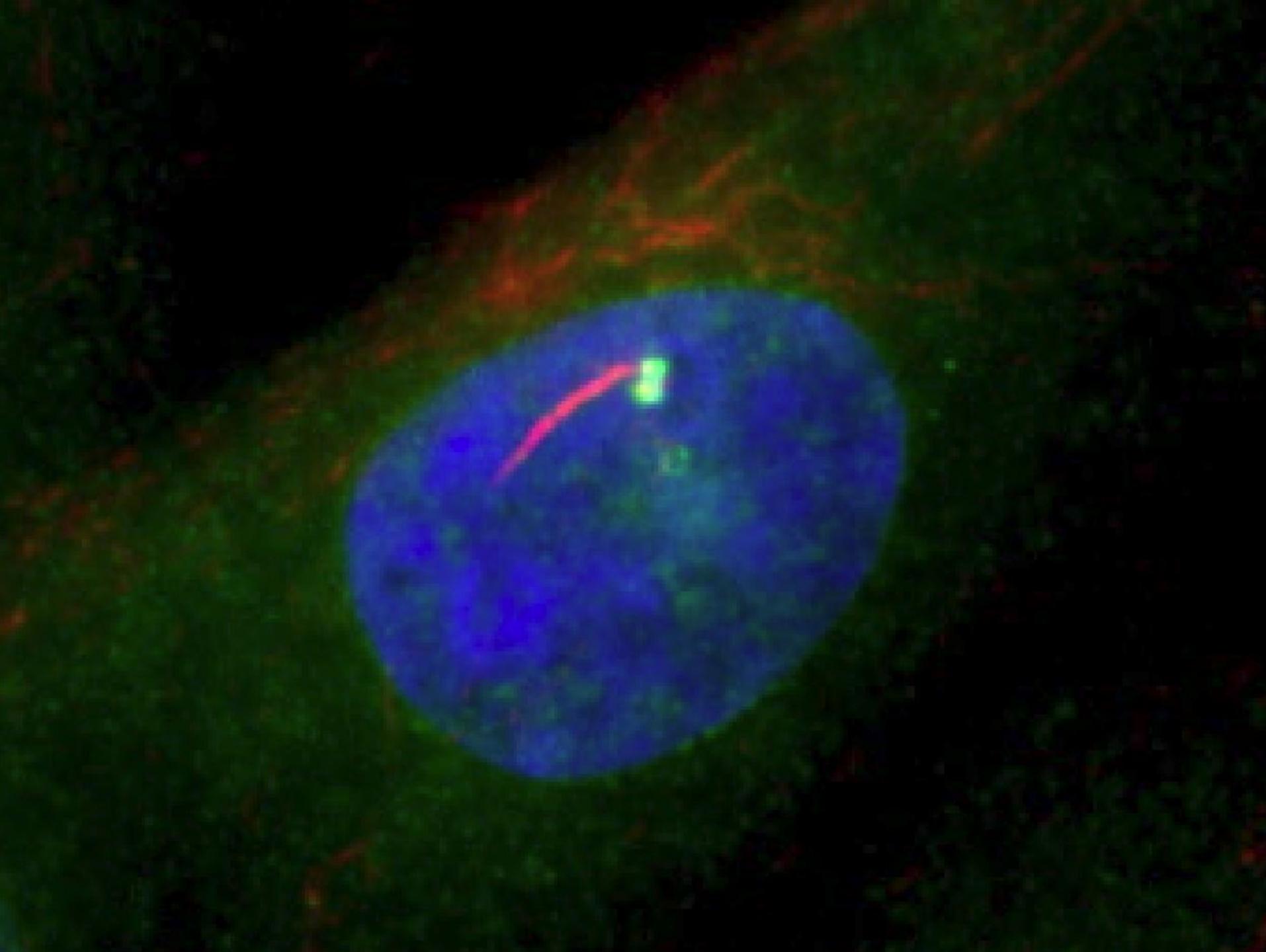
Primary cilium



Staining of primary cilia in control fibroblasts after 48 hrs serum starvation

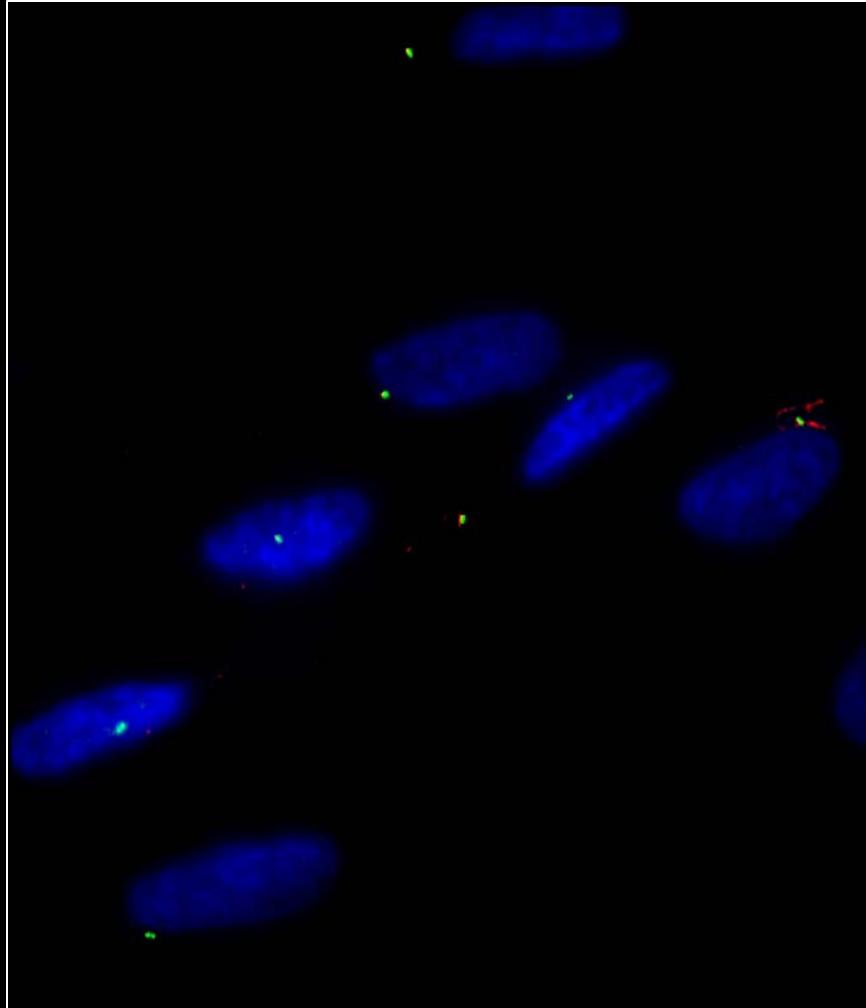
# Overview staining control fibroblasts



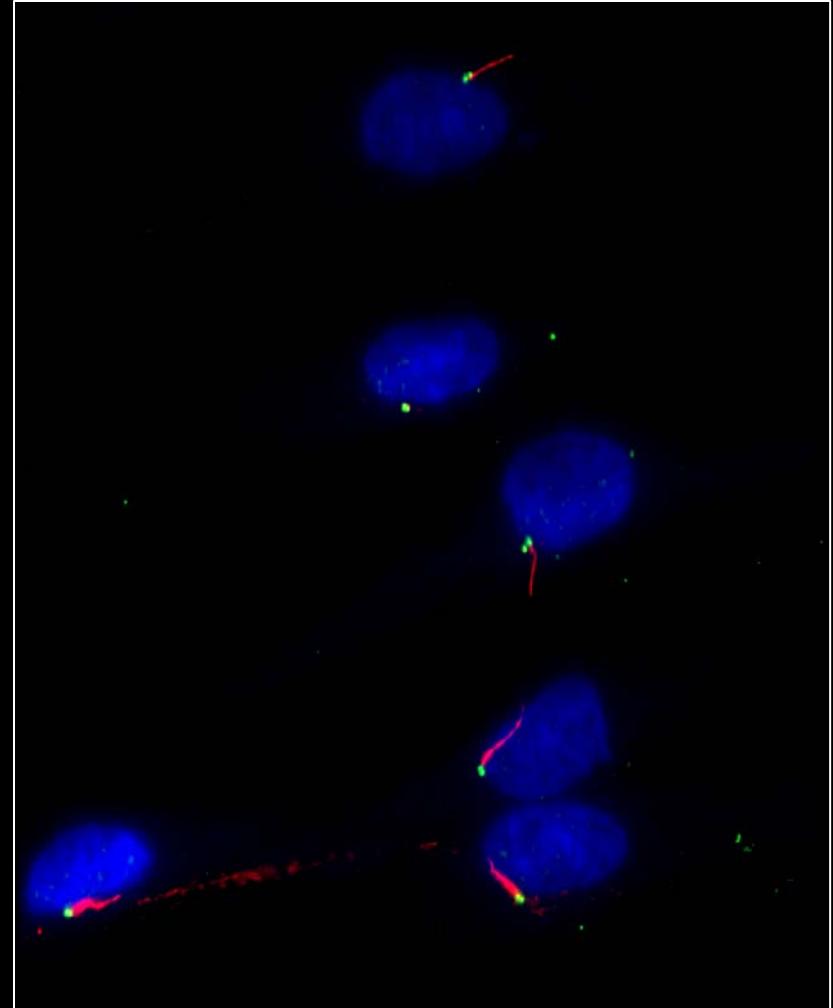


# Example staining patient and control fibroblasts

Joubert syndrome patient



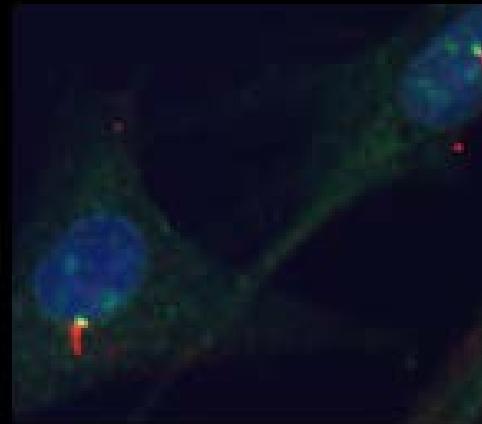
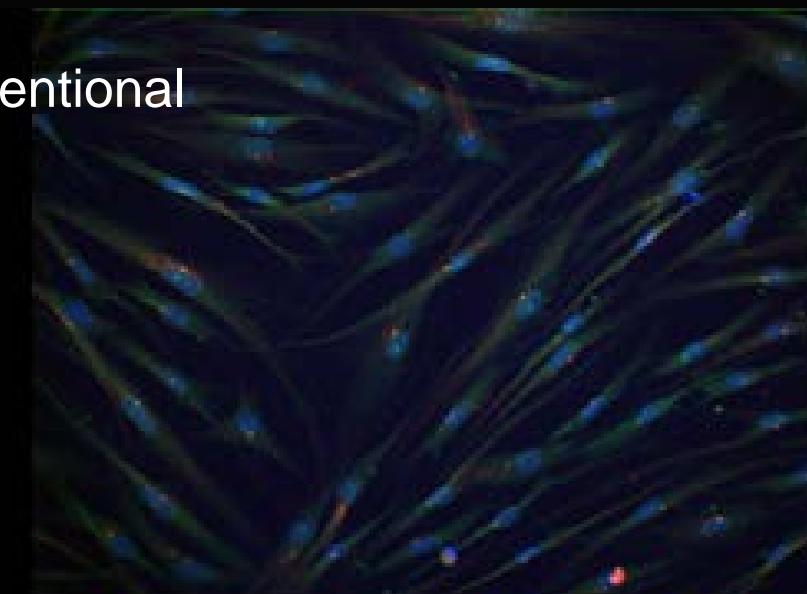
Control



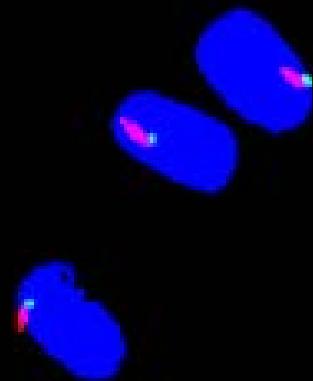
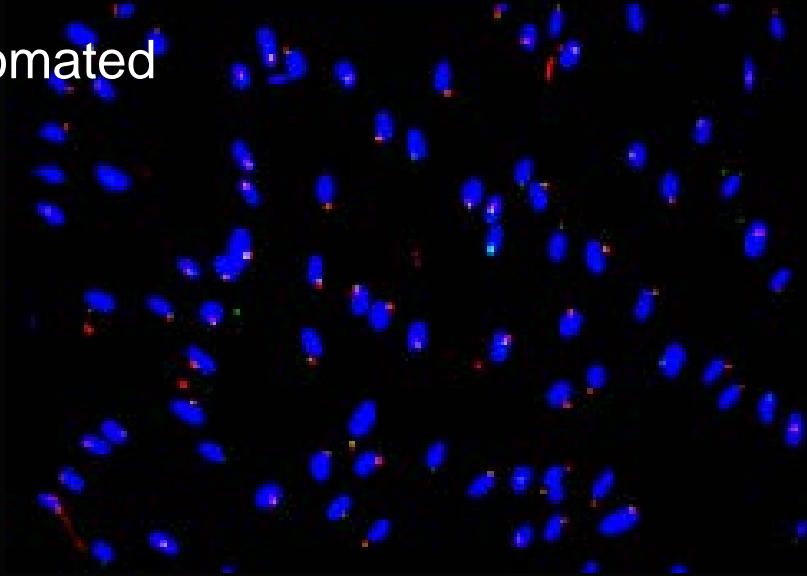
# Primary Cilia structural tests



conventional



automated



# Ciliary length investigation



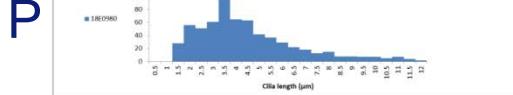
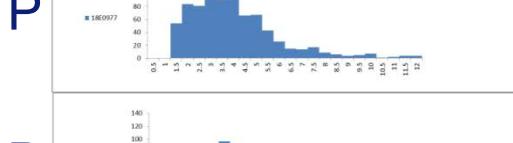
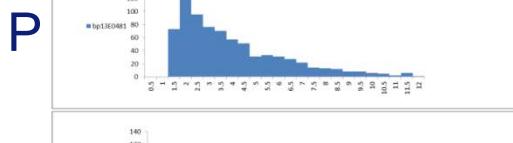
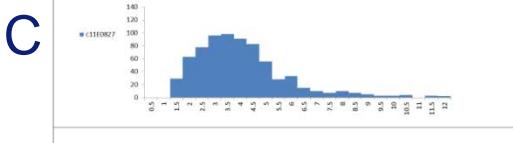
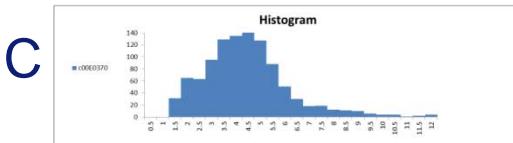
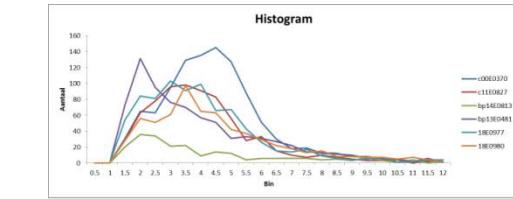
## Amount of Ciliated cells

Ciliated cells are determined on basis of fluorescence signal.

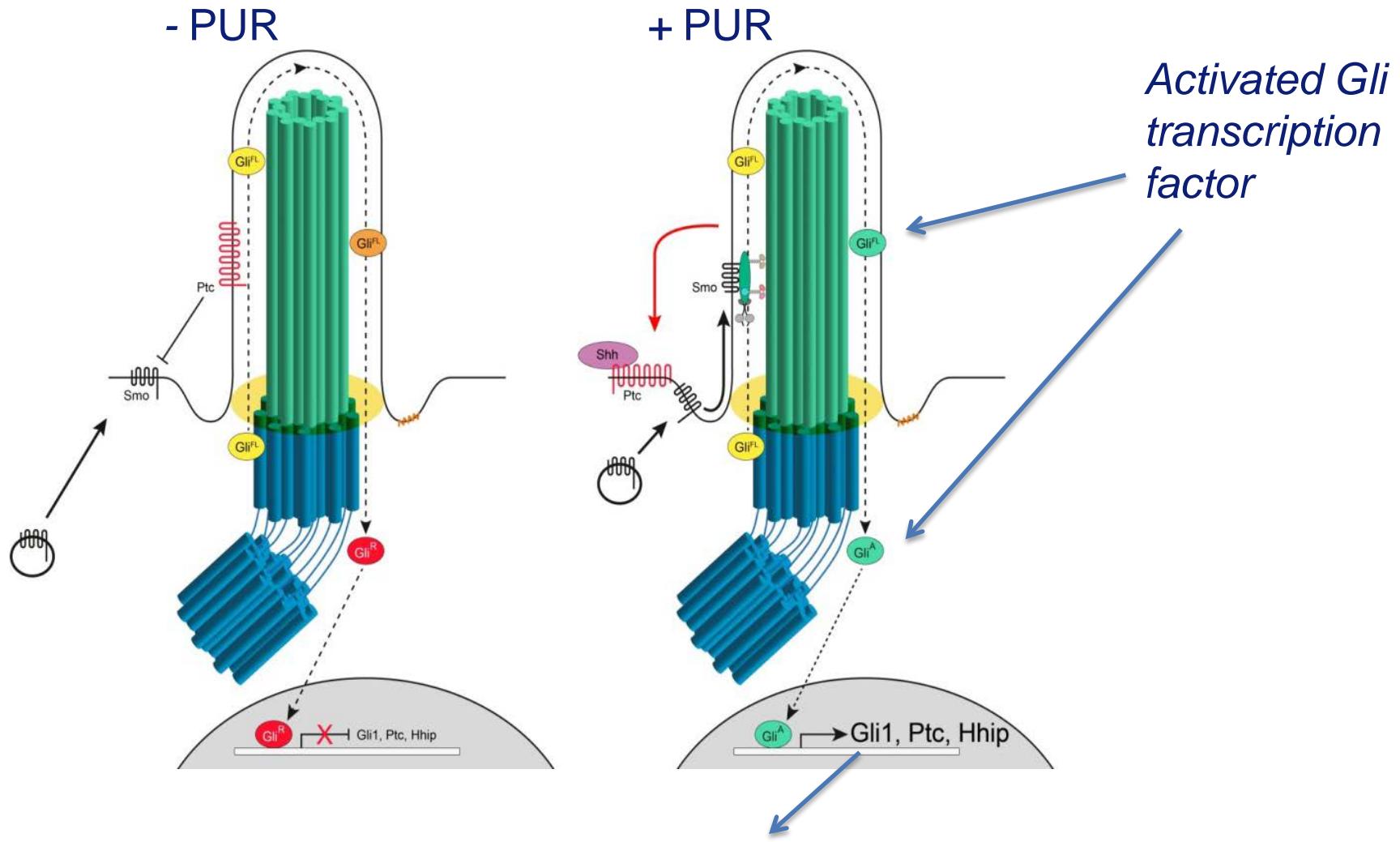
Signal intensity as a measure of ciliary length

Cilia type abnormalities:

- reduced amounts
- reduced ciliary length
- extended ciliary length



# Assay sonic hedgehog Gli-signaling pathway after stimulation with SMO agonist Purmorphamine

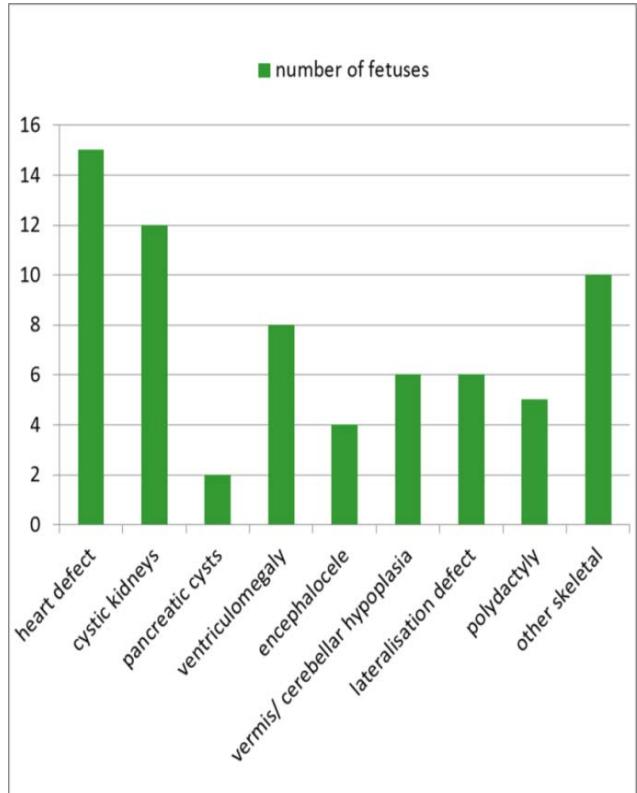


Increase in relative *Gli1* expression is  
measured with qRT-PCR

# Functional validation of severe ciliopathies in a cohort of terminated pregnancies



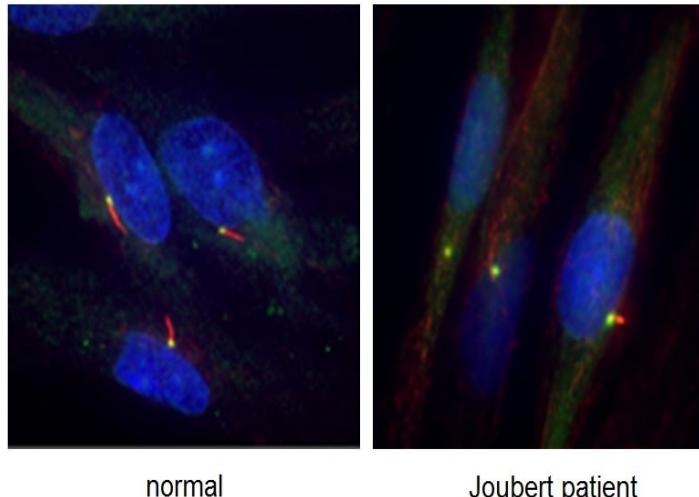
## 1. 48 TOP with congenital abnormalities (in the ciliopathy disorder spectrum)



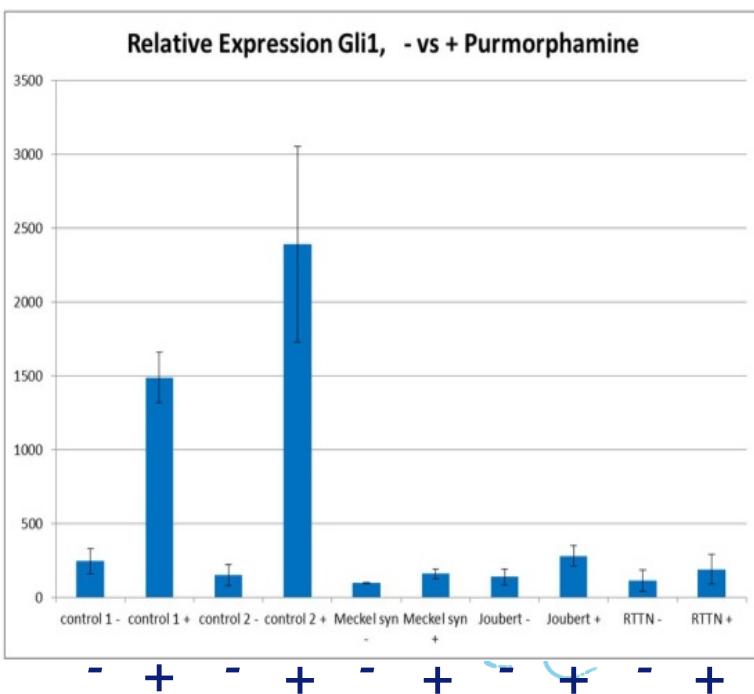
## 2. Structural and quantitative ciliary defects



**NGS  
ciliopathy  
panel**



## 3. Functional abnormalities of the sonic hedgehog signaling (Shh) pathway



# Ciliopathy genes and functions (NGS panel)

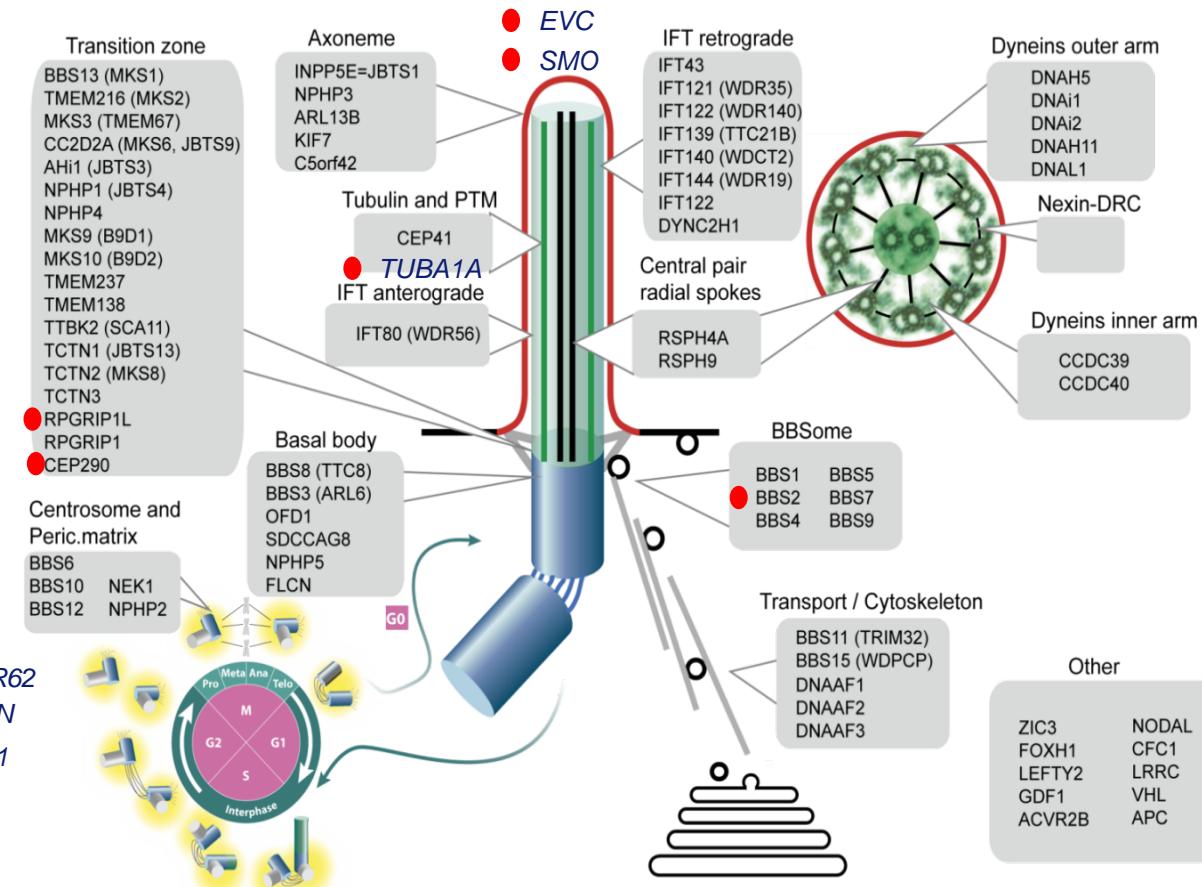


Diagnostic assays for these functions, when?

- Patient with unclear clinical diagnosis (phenotypic heterogeneity of ciliopathies)
- Prenatal cases with MCA suspected for ciliopathy
- Confirmation of molecular variant findings (VUS)

## Confirmed Gene Mutations

- *BBS2*
- *CEP290*
- *EVC*
- *RTTN*
- *RPGRIP1L*
- *WDR62*
- *EML1*
- *TUBA1A*
- *SMO*



Diagnostic assay: when?

- Patient with unclear clinical diagnosis (phenotypic heterogeneity)
- Prenatal cases with MCA suspected for ciliopathy
- Confirmation of molecular variant findings (VUS)

# Groningen patients SMO (smoothened) mutations pathogenic ? Request FU test



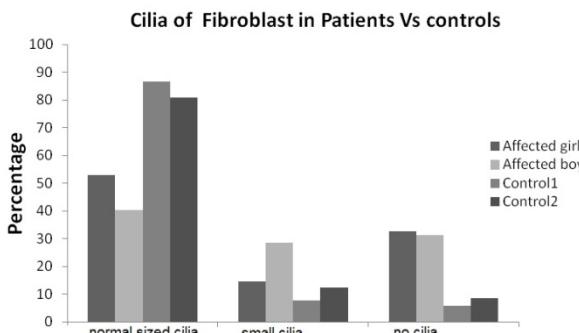
Mieke Kerstjens-Frederikse

Marlies Baardman, Yunia Sribudiani

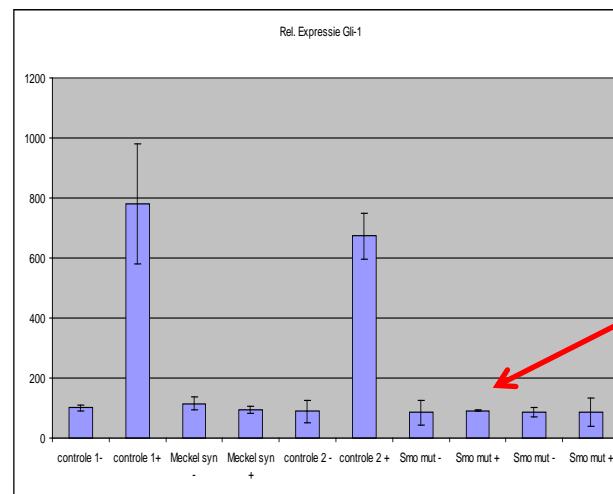
Robert Hofstra

-Twins, consanguineous, AVSD, large fontanel, postaxial polydactyly and skin syndactyly

-Homozygosity mapping in combination with exome sequencing: a novel homozygous missense mutation c.1725C>T (p.R575W) was detected in **SMO** on 7q32.3, a **member of the SHH-GLI signalling pathway**

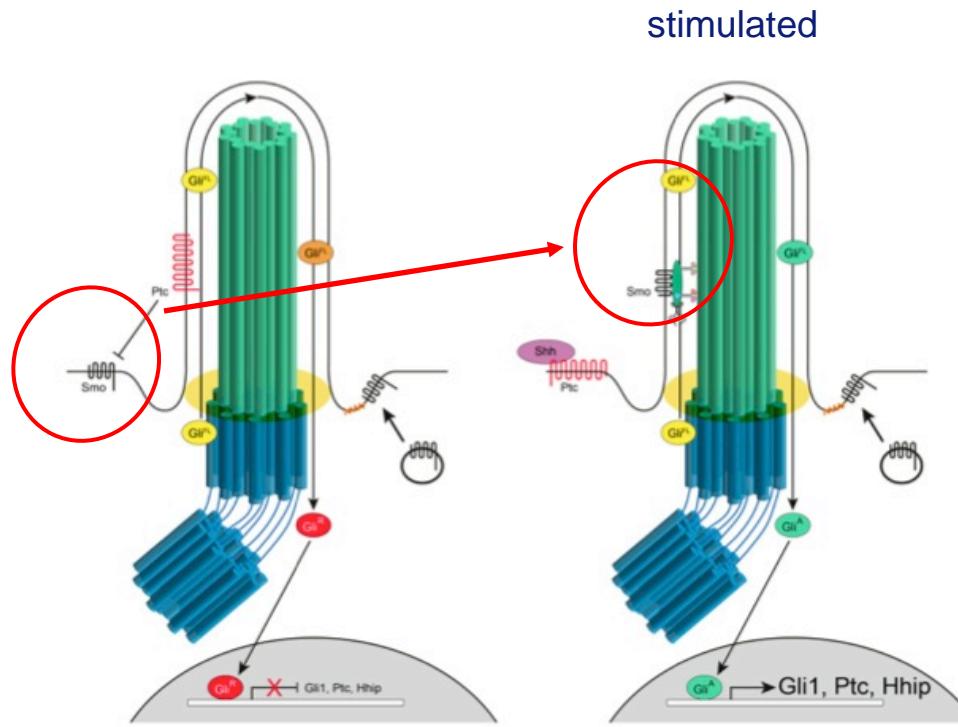


In our cilia staining test: Ciliary axonemes are formed , maybe slightly reduced in number

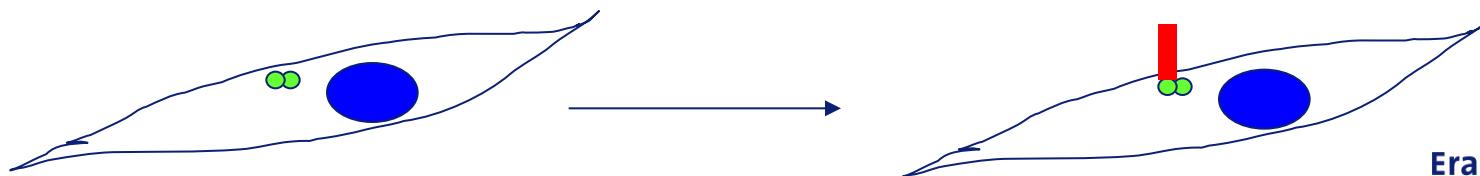


Shh signaling  
abnormal

# Smoothened (Smo) Ciliary Translocation test



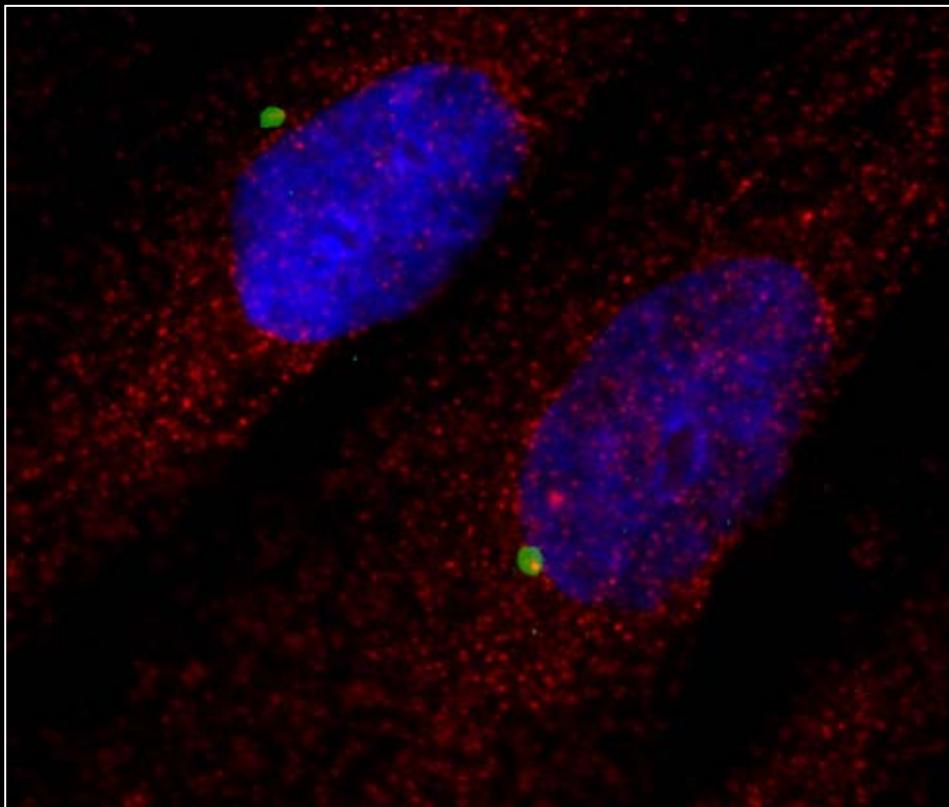
*Smo antibodies stain cilia (axoneme) in fibroblasts only after Purmorphamine or Shh stimulation*



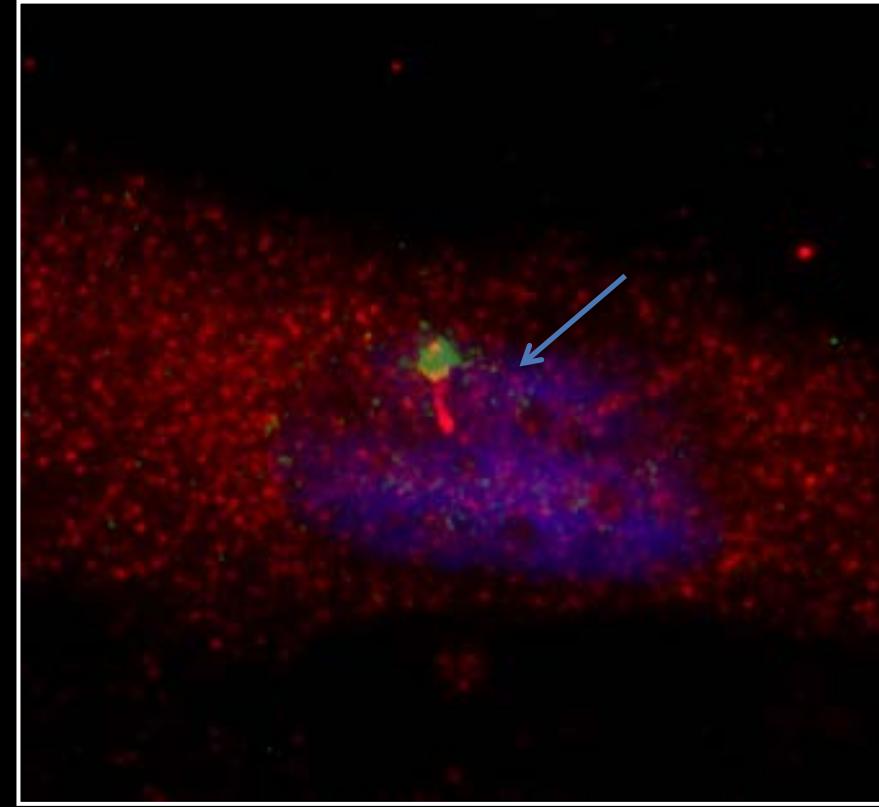
# Smo translocation to the cilium in control fibroblasts



anti-Smo (**RED**) & anti- $\gamma$ -Tubulin (**GREEN**)



$0 \mu\text{M}$  *Purmorphamine*

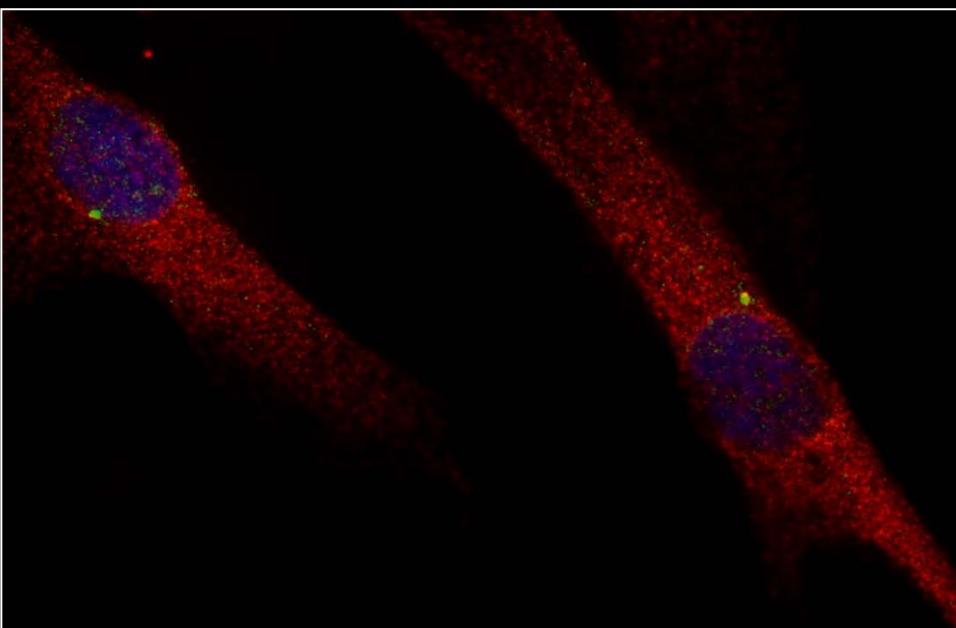


$2 \mu\text{M}$  *Purmorphamine*

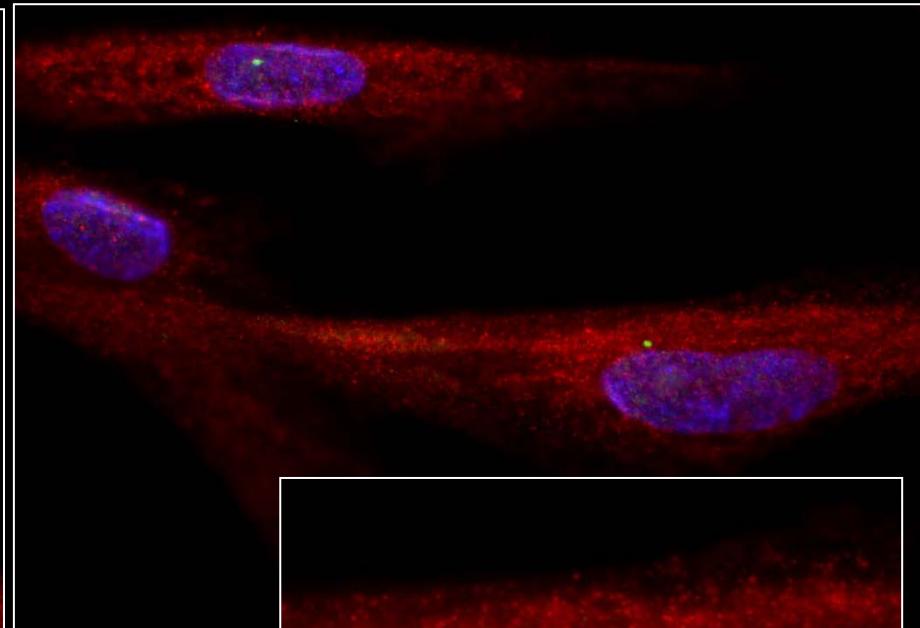
# Smo does not translocate to the cilium in Smo mutant fibroblasts



**anti-Smo (RED) & anti- $\gamma$ -Tubulin (GREEN)**



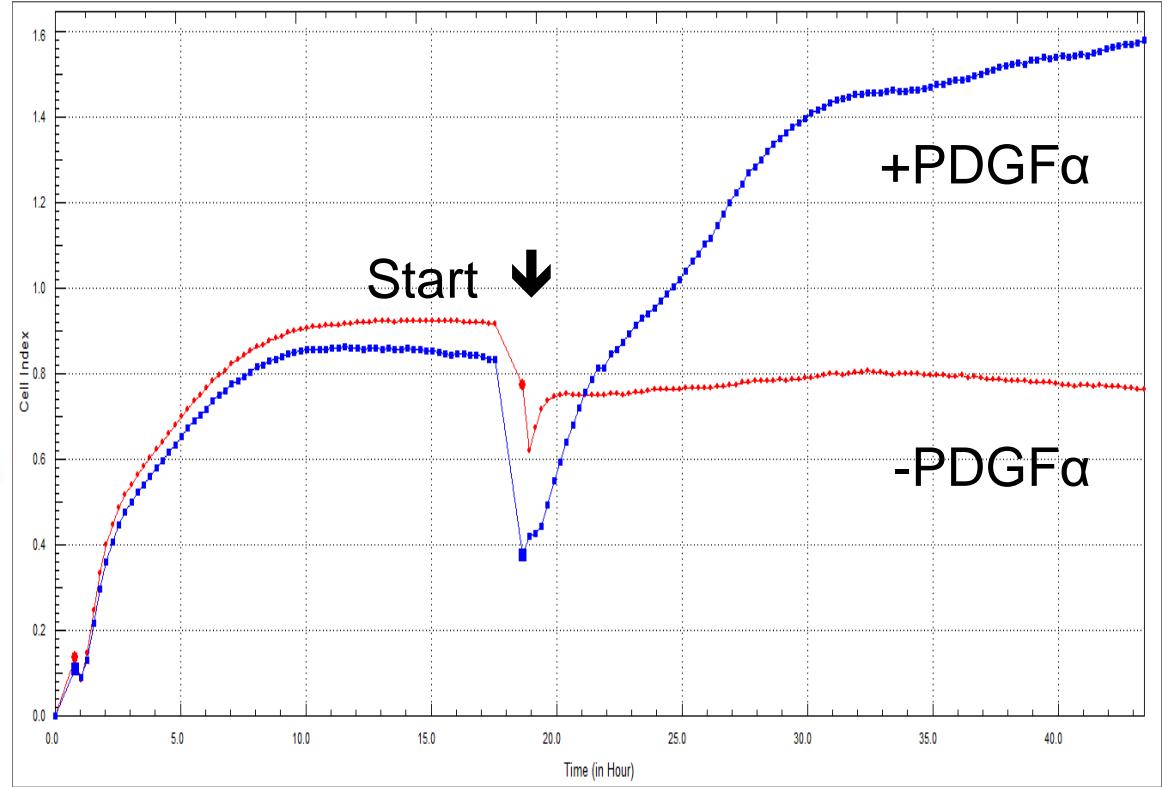
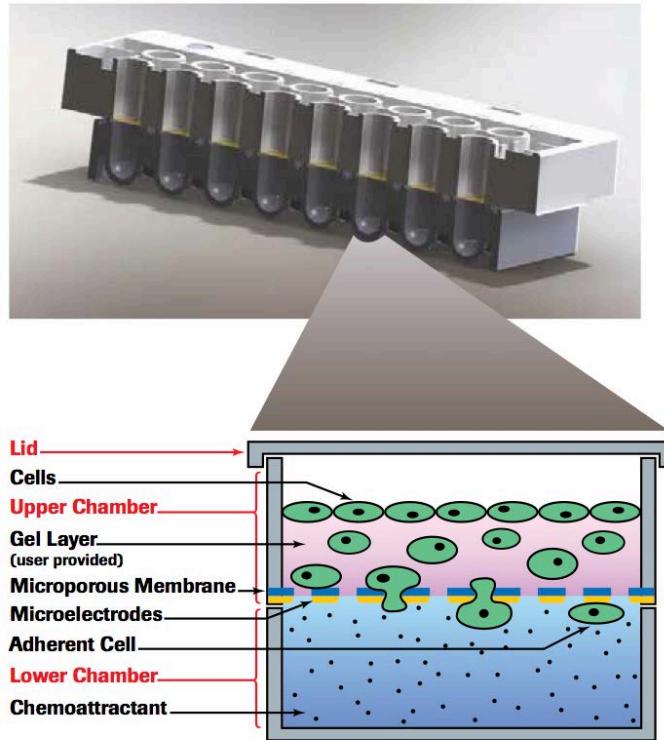
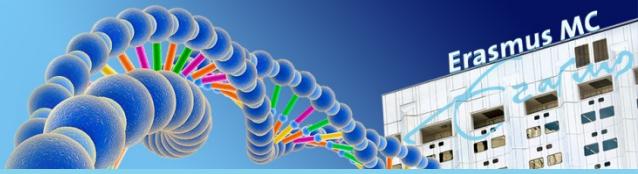
*0  $\mu$ M Purmorphamine*



*2  $\mu$ M Purmorphamine*

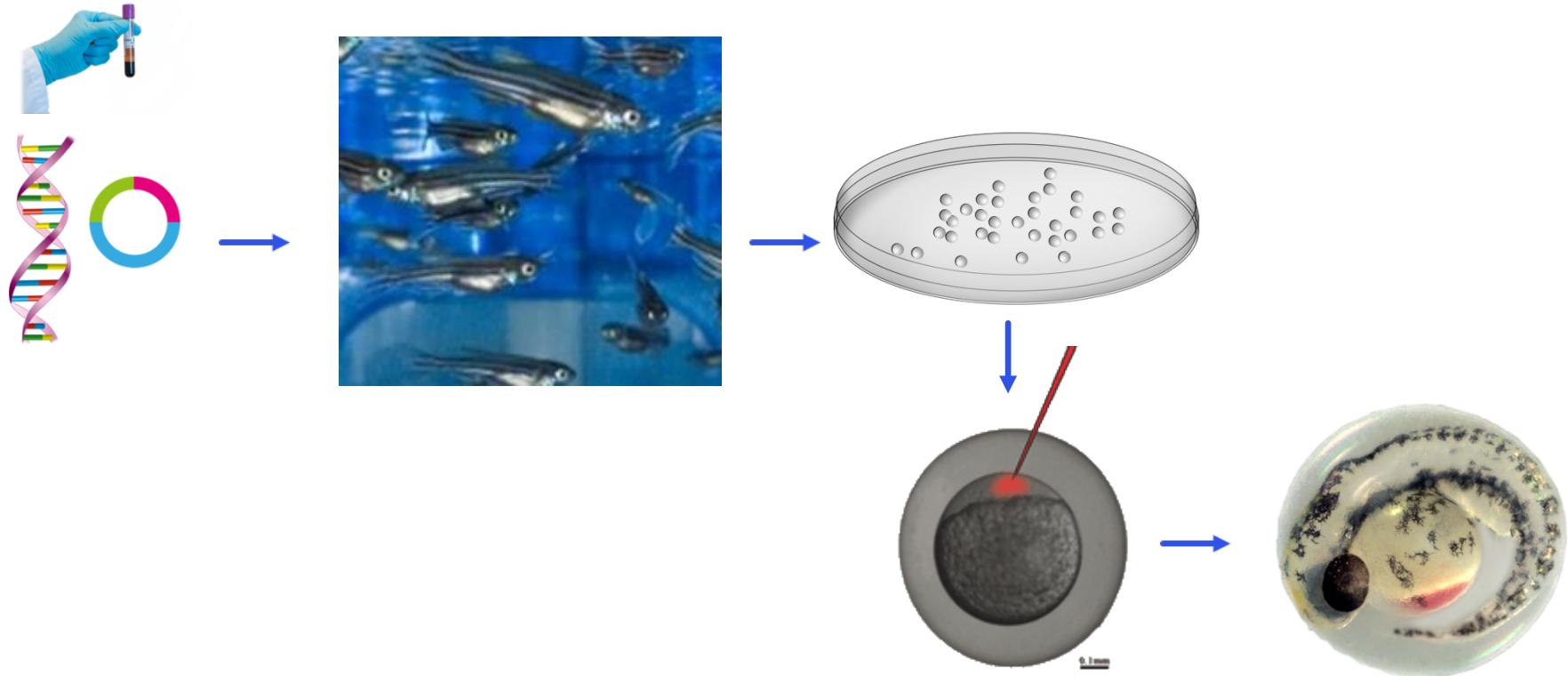
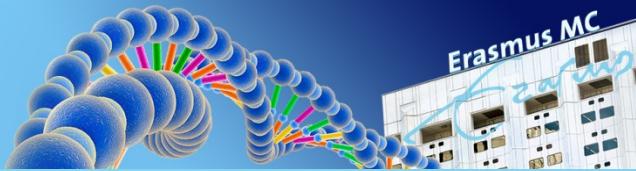
Ongoing new tests: chemotaxis

PDGF $\alpha$  stimulated migration of human fibroblasts



PDGF $\alpha$ -induced fibroblasts (controls) migration in serum-free medium, measured by change in impedance (xCELLigence RTCA DP Instrument for Flexible Real-Time Cell Monitoring.  
ACEA Biosciences

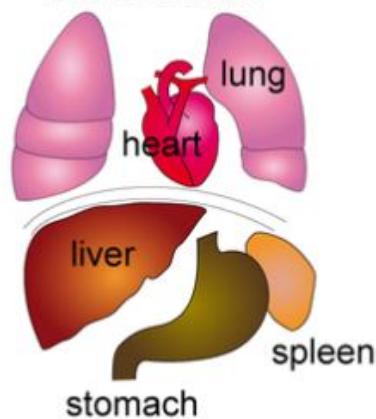
# Zebrafish and disease modeling



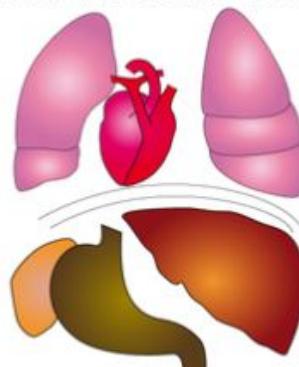
# Heterotaxy syndromes laterality disorders like PCD



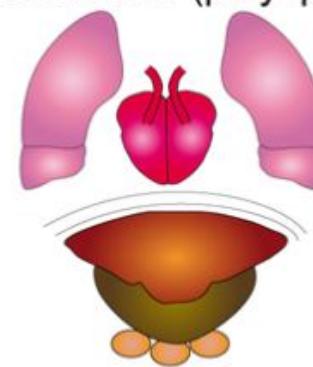
*situs solitus*



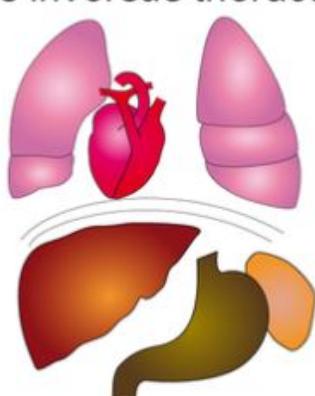
*situs inversus totalis*



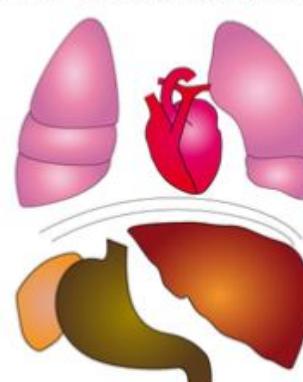
*left isomerism (polysplenia)*



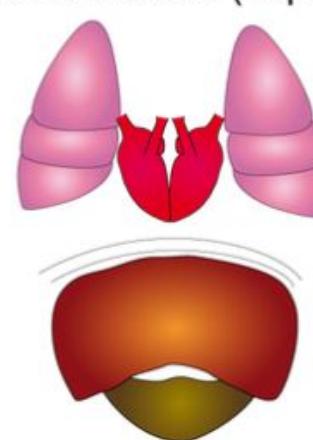
*situs inversus thoracalis*



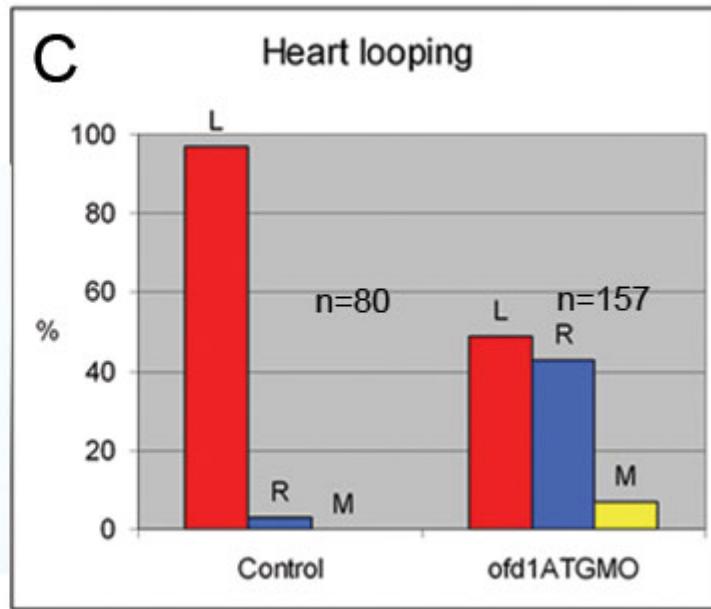
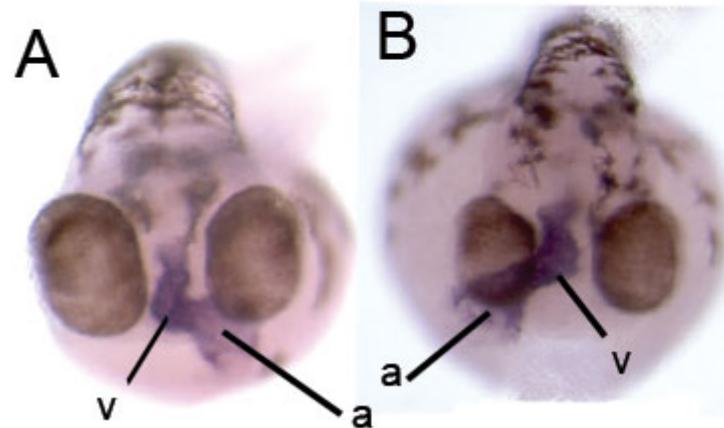
*situs inversus abdominalis*



*right isomerism (asplenia)*



# Example read-out



*Disrupted OFD1  
gene expression*

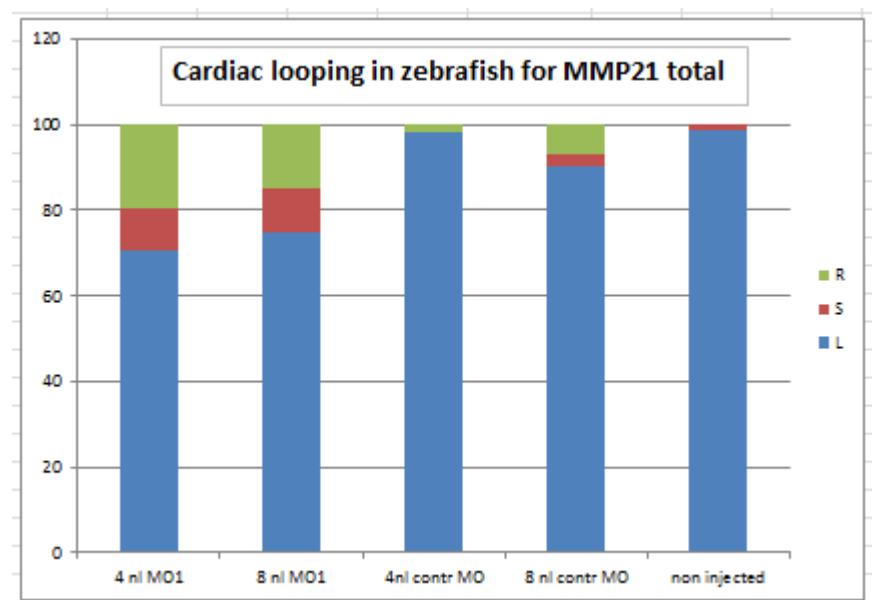
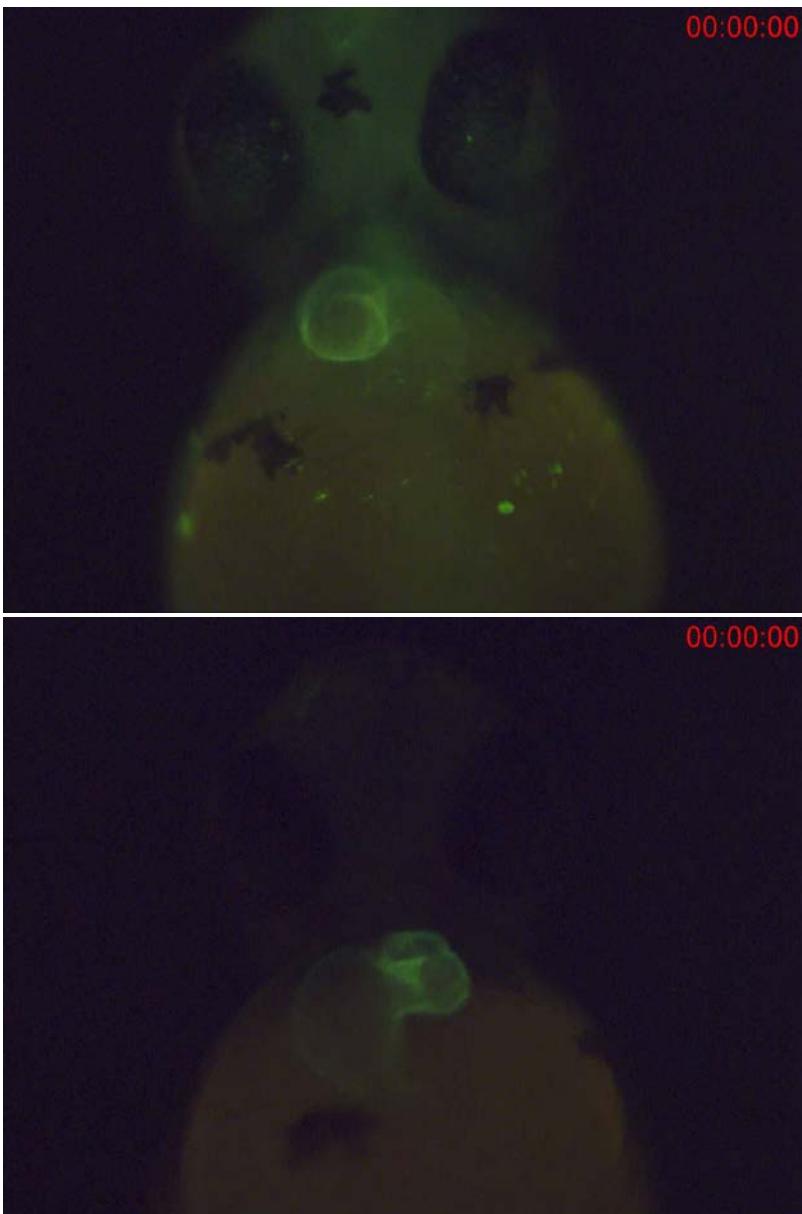
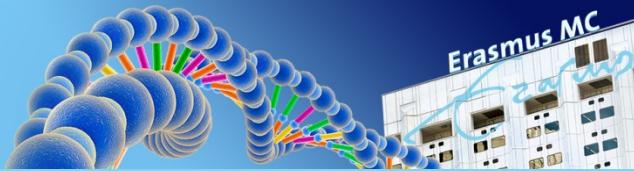
From:

Maria I. Ferrante, Leila Romio\*, Silvia Castro, John E. Collins,  
David A. Goulding, Derek L. Stemple, Adrian S. Woolf and  
Stephen W. Wilson (2009)

Convergent extension movements and ciliary function are  
mediated by *ofd1*, a zebrafish orthologue of the human oral-  
facial-digital type 1 syndrome gene

Human Molecular Genetics 2009 18(2):289-303

# Knock down MMP21 in zebrafish embryos



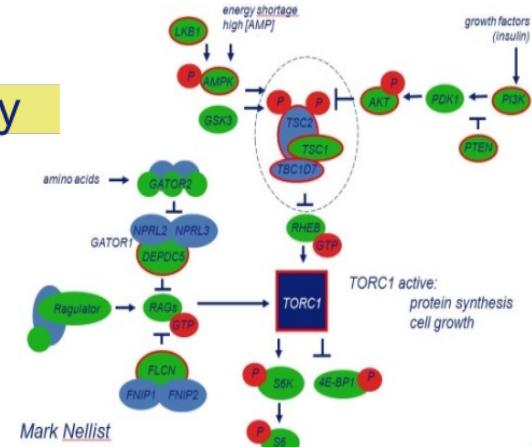
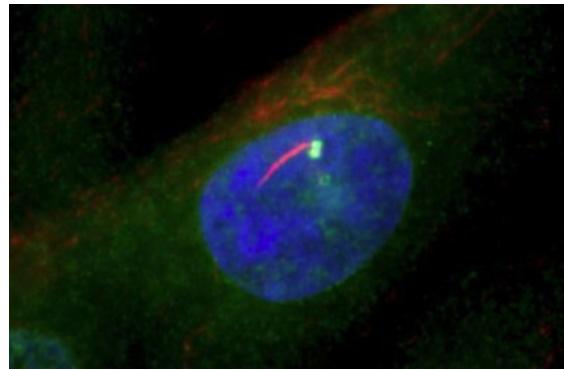
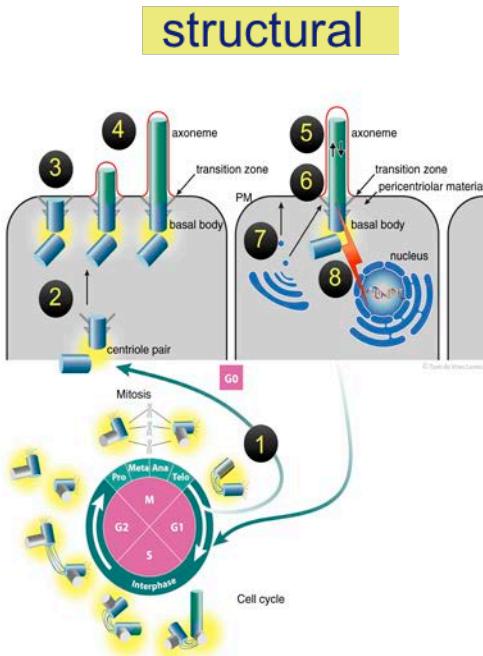
# Summary diagnostic functional genetics



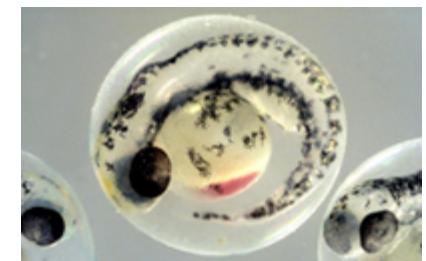
Development of diagnostic functional tests (in a multidisciplinary setting)

As screening tests and as validation for variants.  
In patient cells or cell lines after transfection and  
RNAi/ CRISPR/Cas

pathway



Mark Nellist



## Future

### Functional tests:

- Cancer genes, DNA-repair, MMR, BRCA1/2
- Cardiogenetics genes in progress
- Neurogenetics genes
- Zebrafish disease modeling
- iPSCs, differentiation, organoids

# Dept. of Clinical Genetics Functional Unit/ Enzyme Unit



website:

[https://www.erasmusmc.nl/klinische\\_genetica/verwijzer/info/diagn-functionele-genetica](https://www.erasmusmc.nl/klinische_genetica/verwijzer/info/diagn-functionele-genetica)

## Functional Unit

Marianne Hoogeveen-Westerveld  
Hannie Douven  
Leontine van Unen  
Peter Elfferich  
Marianne van der Sterre  
Jacqueline Boonman

## Enzyme Unit

Paul van den Berg  
Lidia Hussaarts  
Farah Sadeghi Niaraki

Frans Verheijen  
Mark Nellist  
Jasper Saris  
Kees Schoonderwoerd

Lies Hoefsloot  
Robert Hofstra

## Genome Diagnostics

Hennie Bruggenwirth  
Marjon van Slegtenhorst  
Rick van Minkelen  
Martina Wilke  
Ans vd Ouveland



## Counseling Neurogenetics Cardiogenetics

Grazia Mancini  
Karin Diderich  
Marieke Joosten  
Yolande van Bever  
Anneke Kievit  
Alice Brooks  
Marja Wessels  
Ingrid van de Laar  
Judith Verhagen  
Renske Oegema

## Research

Rachel Schot  
Herma Zondervan-van der Linde  
Tjakko van Ham  
Rob Willemsen

