# CRISPR Genome Editing 1

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[Delivered to **Bio3950 – Biotechnology** – May 6th & 7th 2024. Edited by SM Carr]

# Lectures

**Monday May 6th**

1) CRISPR locus in Bacteria
   - Role in adaptive immunity

2) inducing CRISPR mutations in the zebrafish genome
   - *atp11a* mutants

- Heteroduplex mobility assays for genotyping CRISPR mutations

**Tuesday May 7**

1)The discovery CRIPSR
   - Nobel Prize and patents

2) CRIPSR based medicines

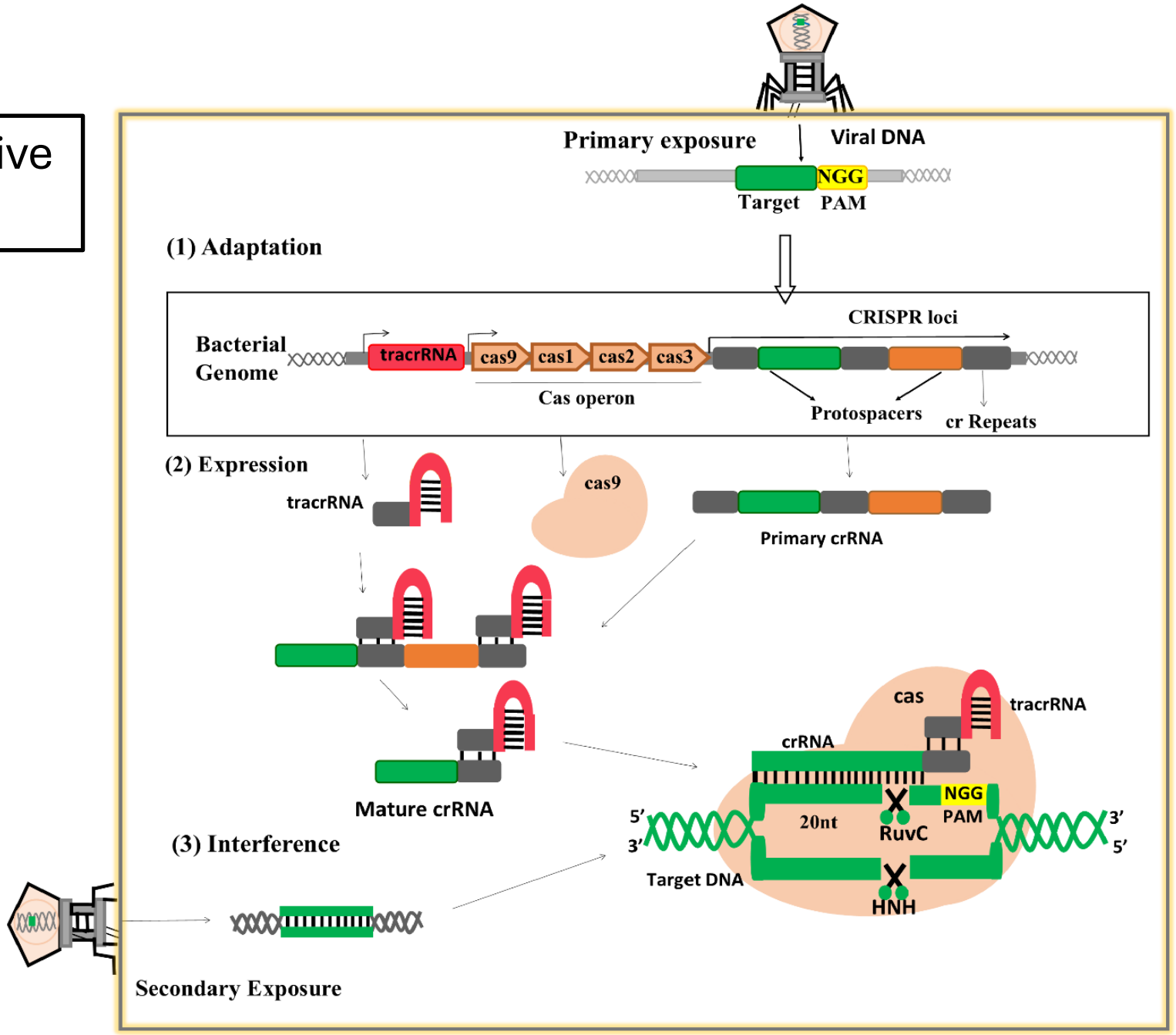
   a cure for sickle cell anemia and B-Thallasamia

   phase III trials for Transthyretin Amyloidosis

# CRISPR/CAS9- What is it?

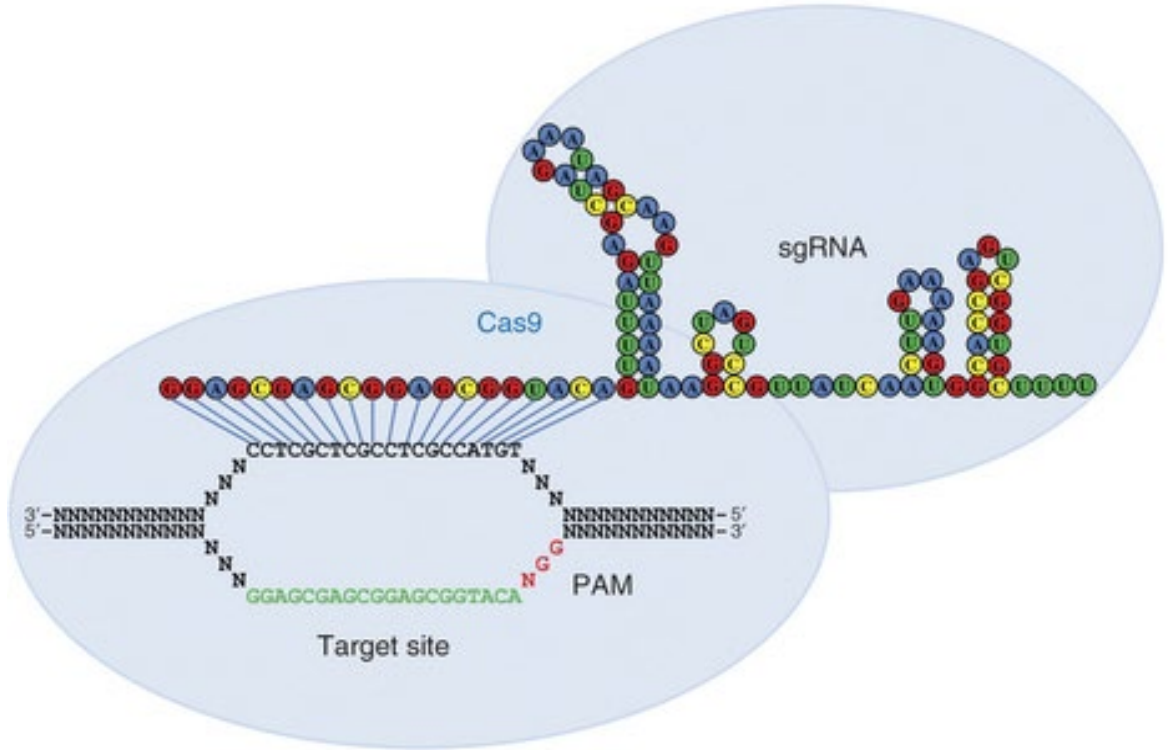
- **C**lustered **R**egulatory **I**nterspaced **S**hort **P**alindromic **R**epeats.
- **RNA** mediated adaptive defense system in bacteria that protects from invading phages.
- **CRISPR/**CAS **operon** contains genes encoding CAS9 nuclease.
- Creates **targeted double-stranded breaks** in invading phage **DNA** to protect from infection.
- **Genome Editing**: Double-stranded breaks allows introduction of foreign **DNA** gene segments with specific mutations through **error-prone non-homologous end joining process**.

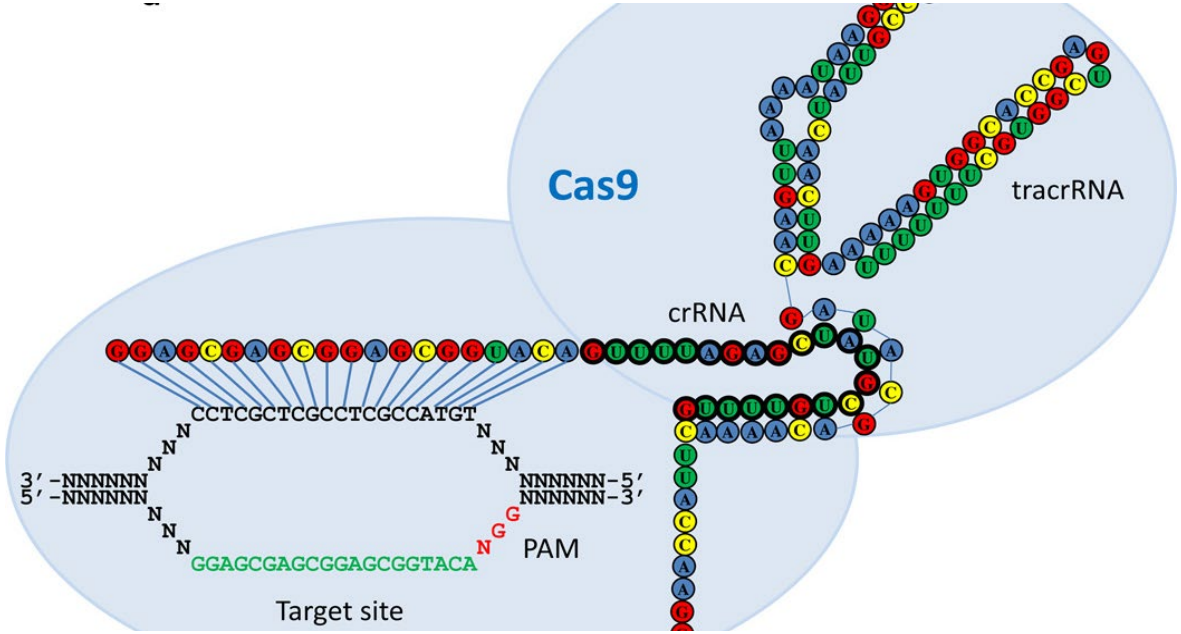
# CRISPR/CAS 9 system of adaptive Immunity in bacteria



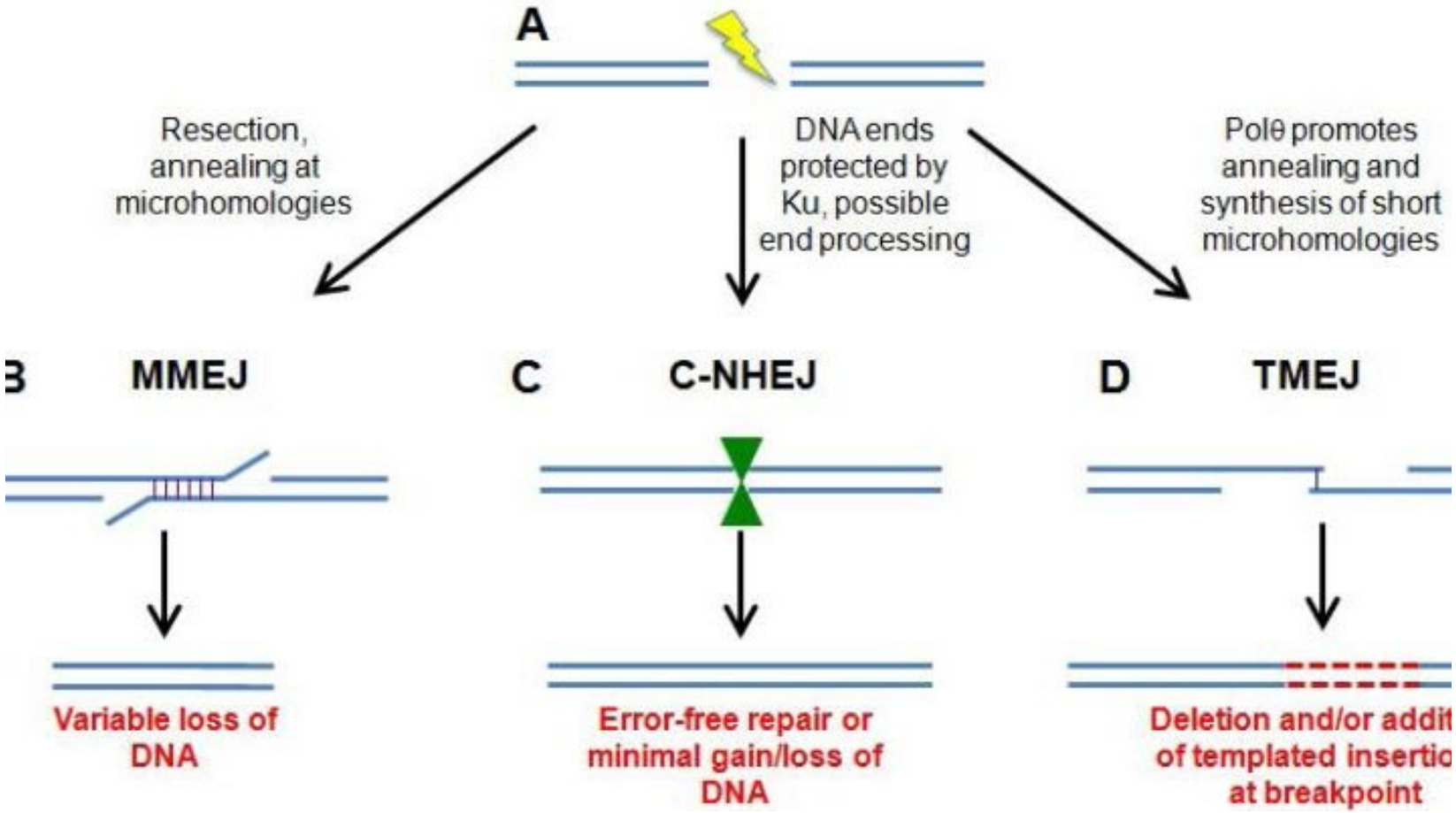

Farooq et a., 2018

CRISPR Genome editing



# Non-homologous end joining




Rogers et al., 2014

# Creation of **loss-of-function** atp11a mutants

- atp11a gene linked to **Sensorineural Hearing Loss** in four families.
- First described family from Newfoundland (Pater et al., 2022).
  - Link conventional **Pedigree Analysis** to **molecular biology**
- **Severe Covid19**
  - **G**enome-**W**ide **A**ssociation **S**tudies (**GWAS**)

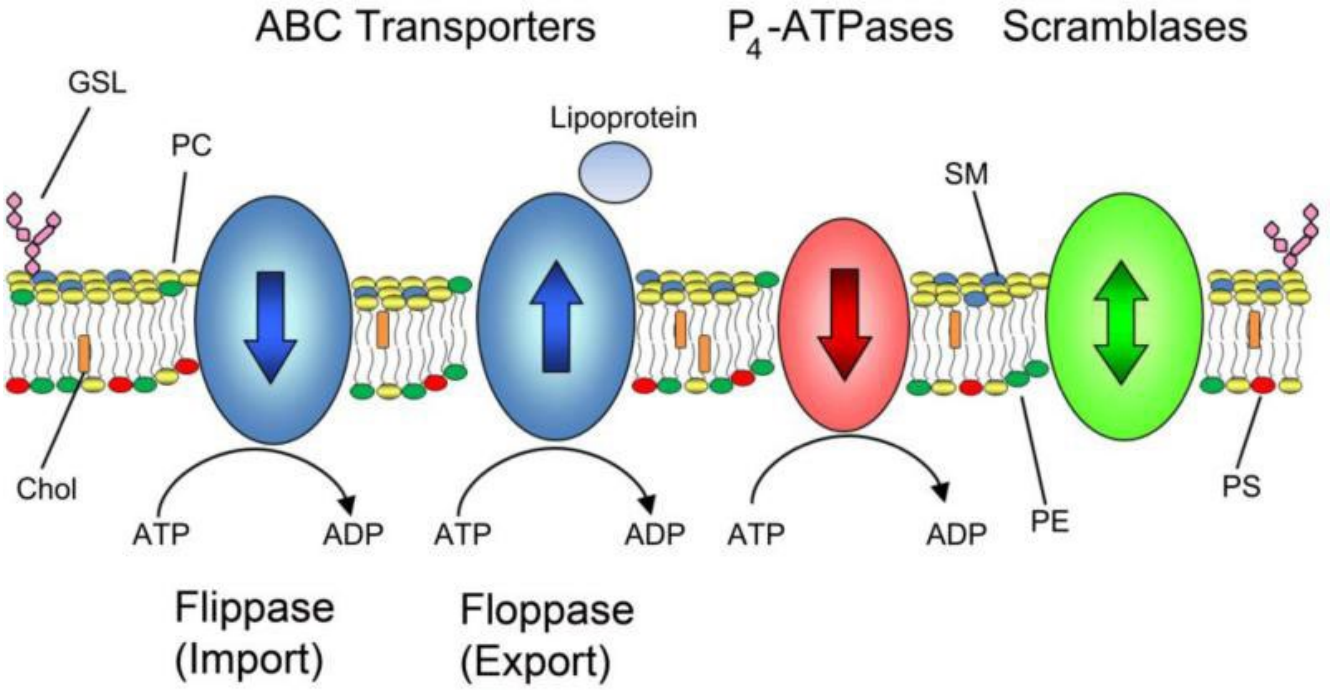
# Phospholipid flippases, floppases & scramblases

atp11a**- A P4-Atpase**

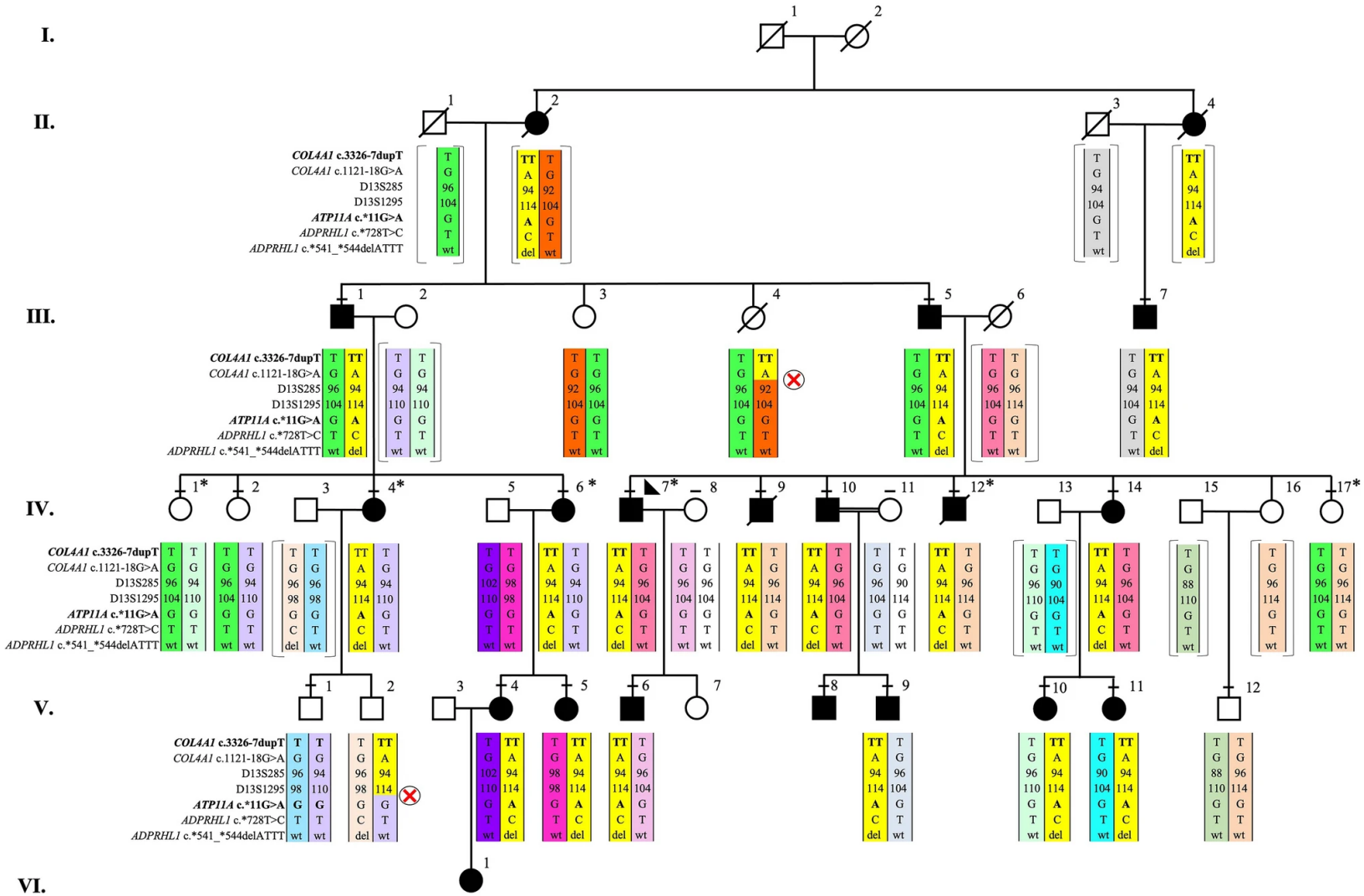
Flips **phosphatidylserine** (PtdSer) % **Phosphatidylethanolamine** (PtdEtn) from outer cell membrane to inner.

**Membrane asymmetry  Required for:**

Membrane curvature
Blood coagulation
Apoptosis / phagocytosis

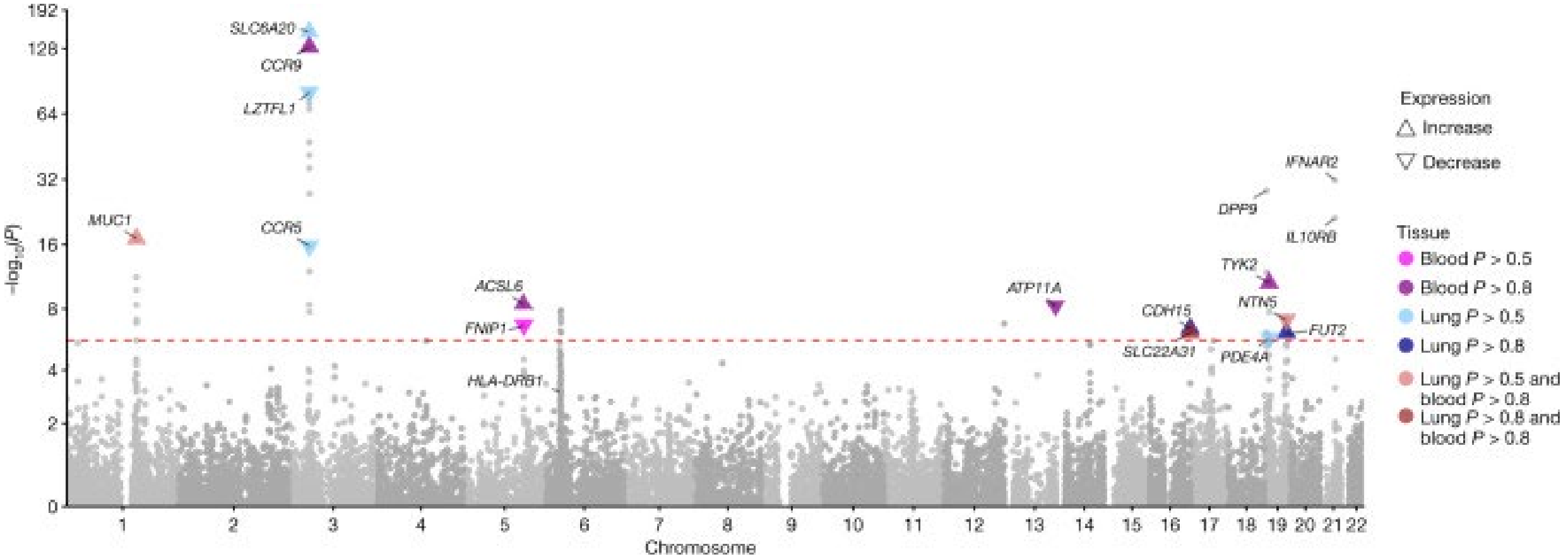


# Pedigree of hearing loss in a Newfoundland family




Pater et al., 2022

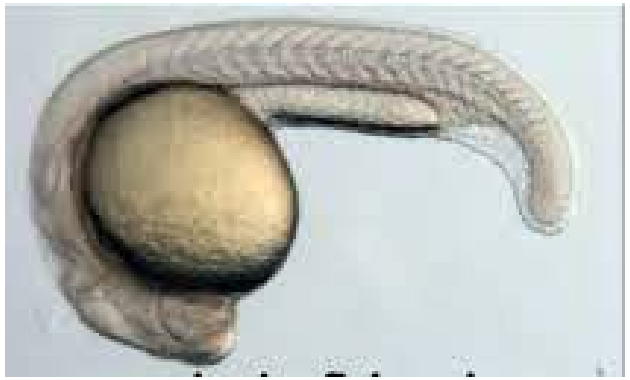
# ATP11A association with **severe Covid19**




Kousathanas et al., 2022

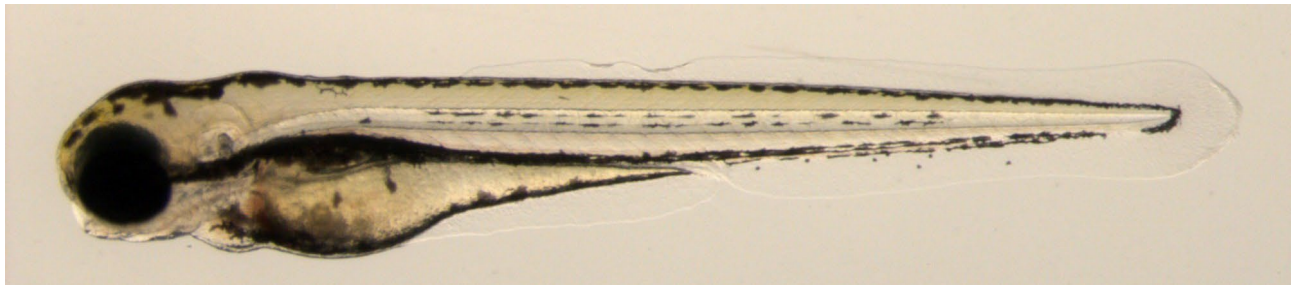
# **Zebrafish** as a Model Organism

84% of Genes in OMIM have at least one zebrafish homolog



24 hpf

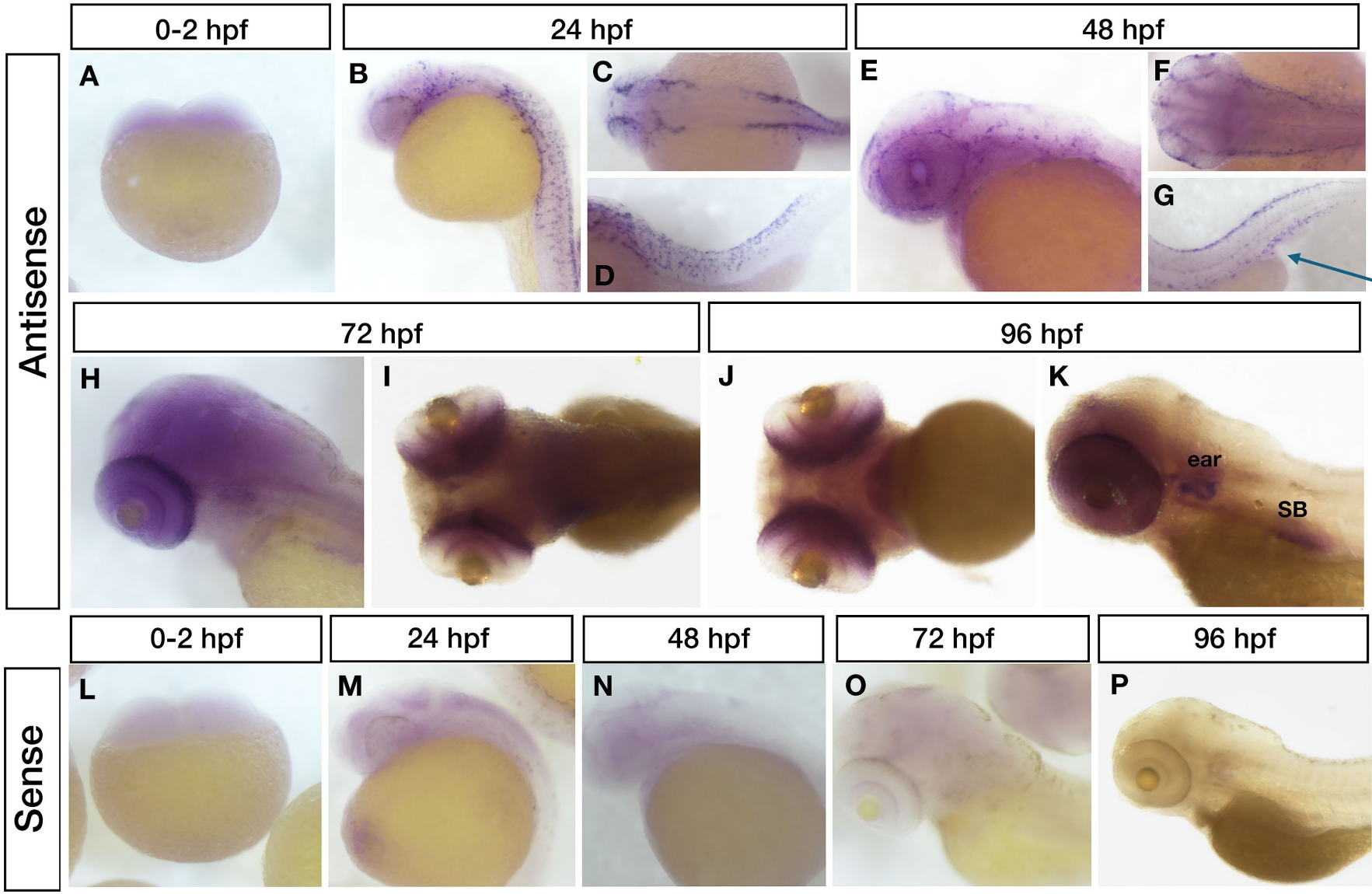
48 hpf



96 hpf

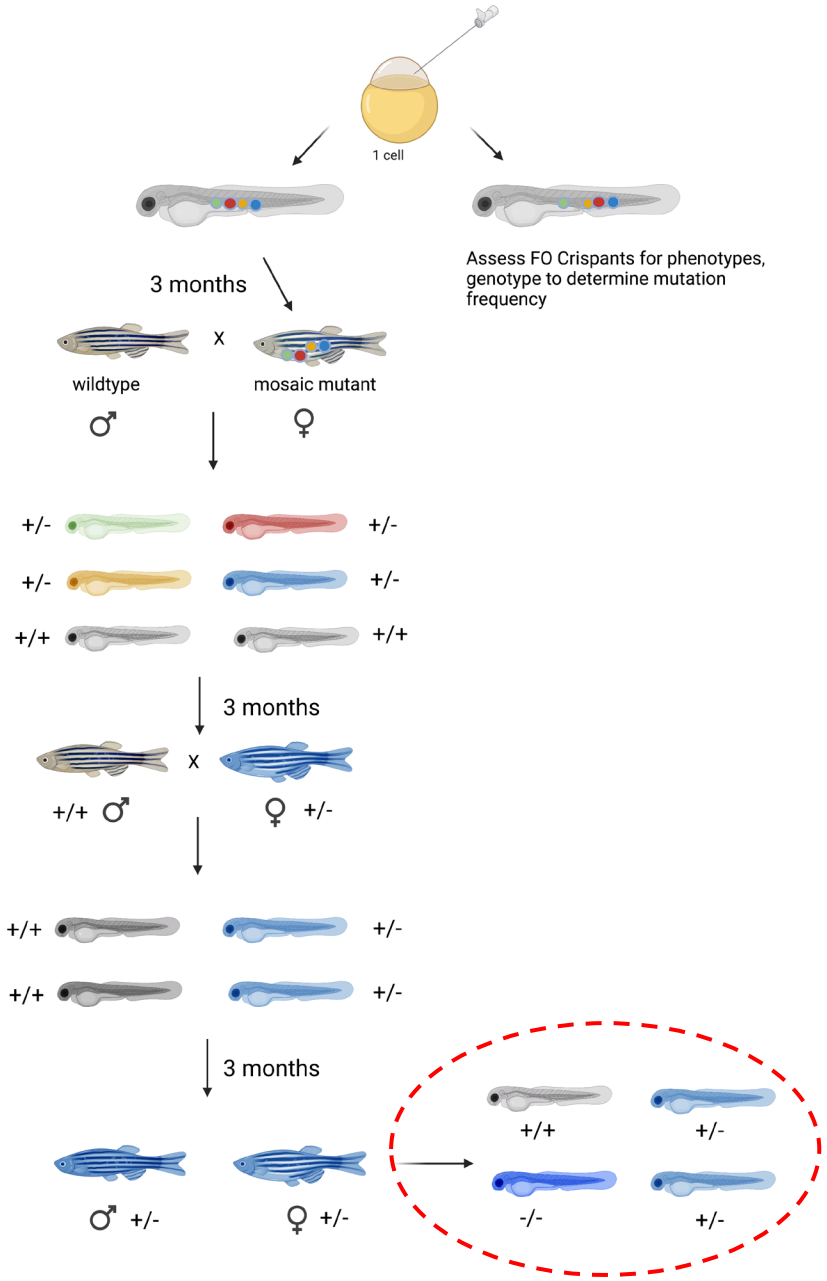
3 months, sexually mature

# Is atp11a expressed in Zebrafish ear & immune cells?




Hawkey-Noble et al., 2020

# Creation of Zebrafish atp11a loss-of-function mutants




Insert **CRISPR-induced indel** mutations into various zebrafish eggs.

**P**: Cross **mosaic mutants** with **wildtype**,

Raise them.

Inspect adults for desired atp11a phenotype.
Genotype of **blue** mutant shows **7 bp** deletion (**7BPD**):

**F1**: Backcross candidate fish to wildtype:
expect **1:1** ratio of (**+/+**) : (**+/-**)

**F2**: Cross (**+/-**) fish:
expect **1:2:1** of (**+/+**):(**+/-**):(**-/-**)
Proceed with fish (**-/-**)

# Design **CRISPR** gRNA to atp11a Exon 17

AAATGTTTTAAACAGAGAATAGAGGTAGTGCAATGATTCCCAGTATGTGTAAAAAAAGTG
CTATTAAATGCATTAAAACTTGTAACTTGTAGTAAACAAATAAATAAATACATATCATAT
GGTCTACTGTTTTGAATTAATAGCTGTTATAGATTTATTGAAGGAAGTTTTTTGTATAGT
TGCTAATTATTTTATTCTTCAATTGTTTGTTTGCTCTAG**GCGAGTACTACTTGTTCTGTA**
**AAGGTGCCGATTCCTCTGTTTTCCCACGGGTGGTTTCTGGGAAGGTGGAGCAGGTCAGAG**
**CGCGAGTCGAGCACAACGCTGTG**GTGAGCAGAAATTGGAAACATCTTCATCTTCTTTCTT
ATTTTAGCAGAGCTATTTATAGGTCACAAAAGAACCTTCCTACATCTTGATGAAGAATAA
AAAAAGCTCCCCCCAAATGTTTGCTTATGCAACTTTTGTTTAGCTTGAGAACCATCCTGA

**Yellow** : **Primers** for amplicon generation
**Beige** : **Exon17** sequence
**Red** : **Protospacer** adjacent motif

Blue: **gRNA** sequence
Underlined: Site of **7 bp** deletion (**7BPD**)

# Atp11a Open Reading Frame (**ORF**)

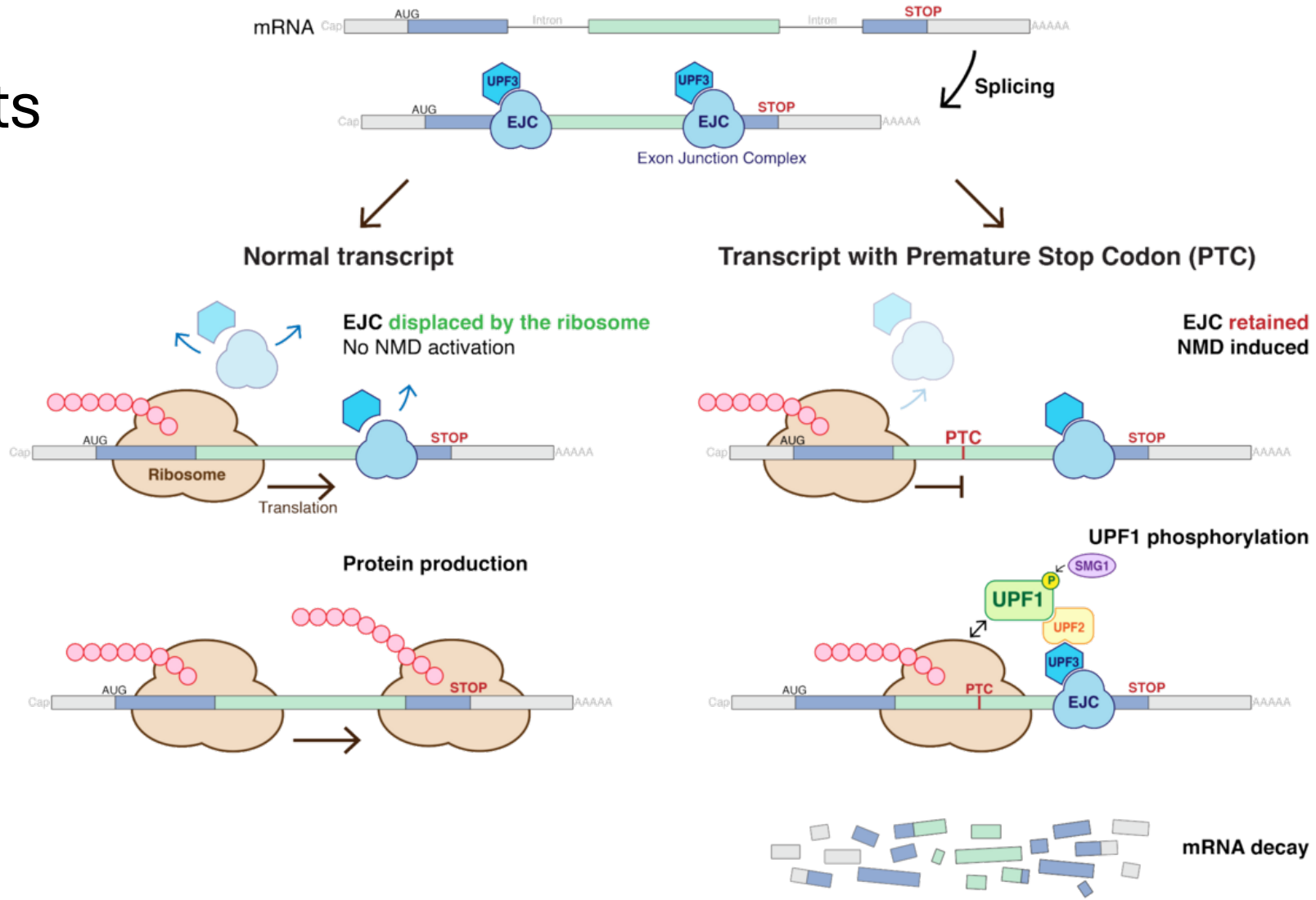
MDFSTIRNLITRYCTGEENWVDSRTVYIGHKEPPPGTEAYIPQRFPDNRIVSSKYTFWNF
IPKNLFEQFRRIANFYFLIIFLVQLIIDTPTSPMTSGLPLFFVITVTAIKQGYEDWIRHK
ADNSVNQCPVHIVQHGKVVRKQSQKLRVGDIVQVKENETFPCDLILLSTSREDGTCFVTT
ASLDGESSHKTYYAVQDTKAFSTAEEVDTLHATIECEQPQPDLYKFVGRINIYLDRDEPI
ARPLGSENLLLRGATLKNTEYIHAVAIYTGMETKMALNYQSKSQKRSAVEKSMNAYLIVY
LCILISKALINTVLKYVWQADPNRDEPWYNQRTESERQRHVLIRAFTDFLAFMVLFNYII
PVSMYVTVEMQKFLGSYFILWDDDMFDEEVGERPLVNTSDLNEELGQVEYVFTDKTGTLT
ENNMELRECCVDGHVYVPHAICNGQILPGAAGMDMIDSSPGVEGKEREELFFRALCLCHT
VQVKEEETVDGIKRGIHQGKATSFYISSSPDEVALVEGMKRLGFTYLRLKDSHMEILNRE
DEMERFELLDVLNFDSVRRRMSVIVRSN**SGEYYL**FCKGADSSVFPRVVSGKVEQVRARVE
HNAVEGLRTLCVAYKRLSQEEYEETCRLLTSAKLALQERDKKLAEAYDVIEKDFILLGAT
AVEDRLQDKAADTIESLHKAGIKVWVLTGDKMETAAATCYASKLFHRNTQILELTTKRTE
EQSLHDVLFDLSRTVLRQHGSMTRDTFSGLSGDYQDYGLIIDGATLSAVLKPTQDATSNS
GNYKEIFLEICRNCSAVLCCRMAPLQKAQIVKLIKASKEHPITLAIGDGANDVSMILEAH
VGIGIMGKEGRQAARNSDYAITKFKHLKKMLLVHGHYYYIRIAELVQYFFYKNVCFIFPQ
FLYQFFCGFSQQPLYDTAYLTLYNISFTSLPILLYSLMEQHINMDILKRDPSLYRDIAKN
SLLTWPTFIYWTFLGVFDAVVFFFGAFFLFDNTTFTSNGQLMATNTQMMFGNWTFGTLVF
TVLVFTVTLKLALDTHYWTWINHFVIWGSLLFYVIFSLLWGGIIWPFLNYQRMYYVFMQM
LSSGPAWLSIILLIIVSLLPDVLKKVLCRALWPTTTERIQNADKLYKGHLSEFSPLTSLH
APPARKHERRGNERQNHAHRRTNWCCLCANLLSRNTP*

# Atp11a

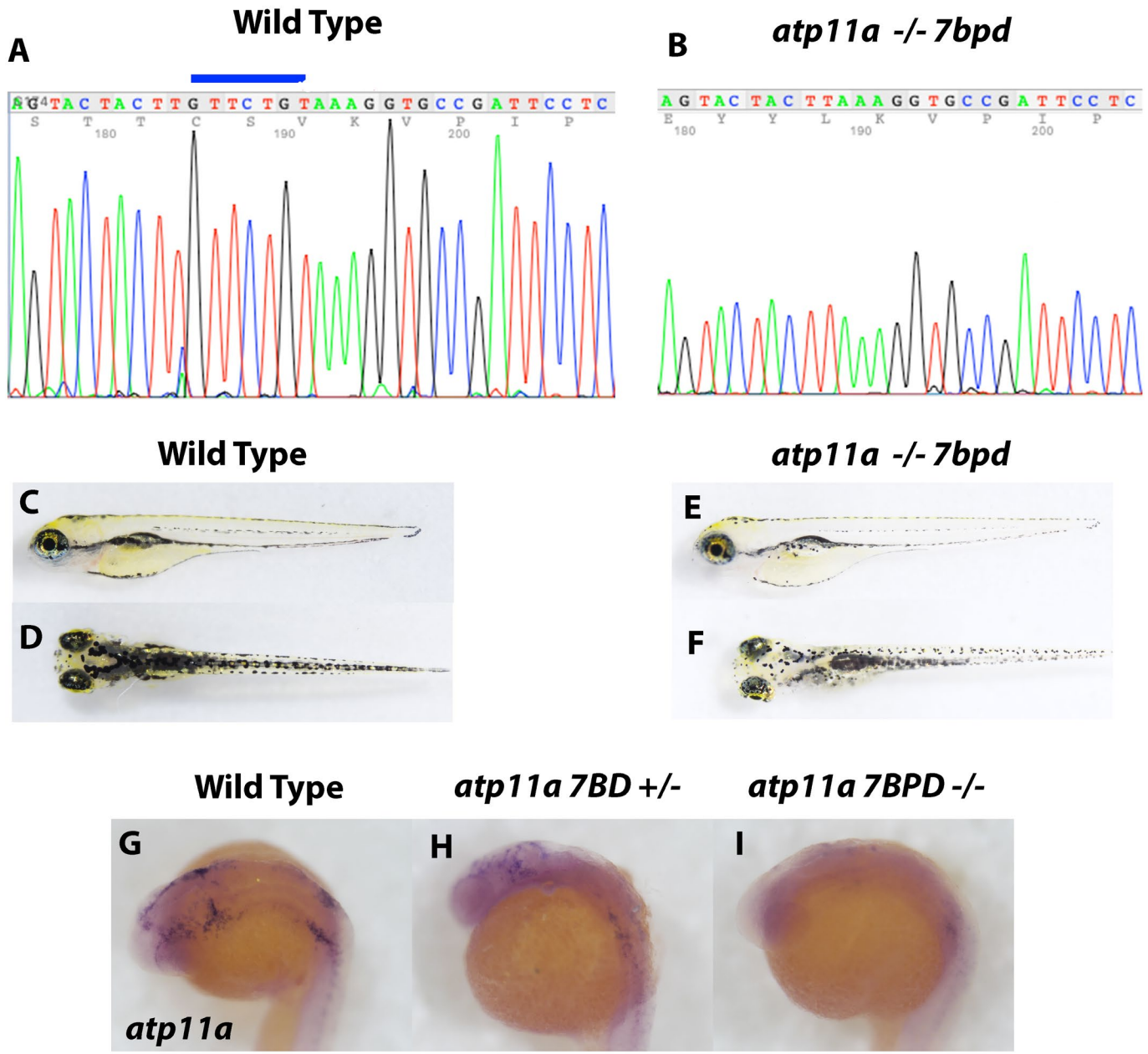
**7BPD** deletion =>
missense &
termination
mutations

MDFSTIRNLITRYCTGEENWVDSRTVYIGHKEPPPGTEAYIPQRFPDNRIVSSKYTFWNF
IPKNLFEQFRRIANFYFLIIFLVQLIIDTPTSPMTSGLPLFFVITVTAIKQGYEDWIRHK
ADNSVNQCPVHIVQHGKVVRKQSQKLRVGDIVQVKENETFPCDLILLSTSREDGTCFVTT
ASLDGESSHKTYYAVQDTKAFSTAEEVDTLHATIECEQPQPDLYKFVGRINIYLDRDEPI
ARPLGSENLLLRGATLKNTEYIHAVAIYTGMETKMALNYQSKSQKRSAVEKSMNAYLIVY
LCILISKALINTVLKYVWQADPNRDEPWYNQRTESERQRHVLIRAFTDFLAFMVLFNYII
PVSMYVTVEMQKFLGSYFILWDDDMFDEEVGERPLVNTSDLNEELGQVEYVFTDKTGTLT
ENNMELRECCVDGHVYVPHAICNGQILPGAAGMDMIDSSPGVEGKEREELFFRALCLCHT
VQVKEEETVDGIKRGIHQGKATSFYISSSPDEVALVEGMKRLGFTYLRLKDSHMEILNRE
DEMERFELLDVLNFDSVRRRMSVIVRSN**SGEYYL**KVPIPLFSHGWFLGRWSRSERESSTT
LWKVYGPSVWLIRDCLRRSMKRRVVCSPALN***LCRSATRNWLKLTMSLKRISSCWEPRLW
KIGCRIKLPTPLSPSIRLALKFGF*QETKWRRRQPHATLASCSIAIHKSWN*RPSGQKSK
VSTMCCST*AGPF*GNTAA*PETLSQGSPVITRTMV*S*MERHCQLC*SRHRTQPAIVET
IRRFSWRSAGTAALYSAVAWHLYKKHRLLS**KHQKSTPSPWPSGTEPMTSA*FWKLTWA
*VSWVKRDVRRLVTATMQLPSSNT*RRCCWFMDTTITSESLSWSSISSIRTSASSSLSFS
ISSSVASLSSHCTTQHI*PYTISALRLCPSCCTV*WSSTSTWTS*NGTPLSTEILPRIPS
*RGPPSSTGRFWGSSMRWFSSLVLSSFSTTQPSPATDS*WPPTHR*CLETGLLALLCSLS
WCSPSH*SLHWTHITGRGSTILSYGALCFSMSSSPCSGEASFGLSSTIRGCTTCSCRCCR
VVLRGSVLFCSLSSVCCLMC*RKCCAELCGPQPLREYRMQISYIRATCRSSPR*PPSMLH
LRGNTKDVATNARTTPTAEQTGVAFVQTYYQETLR

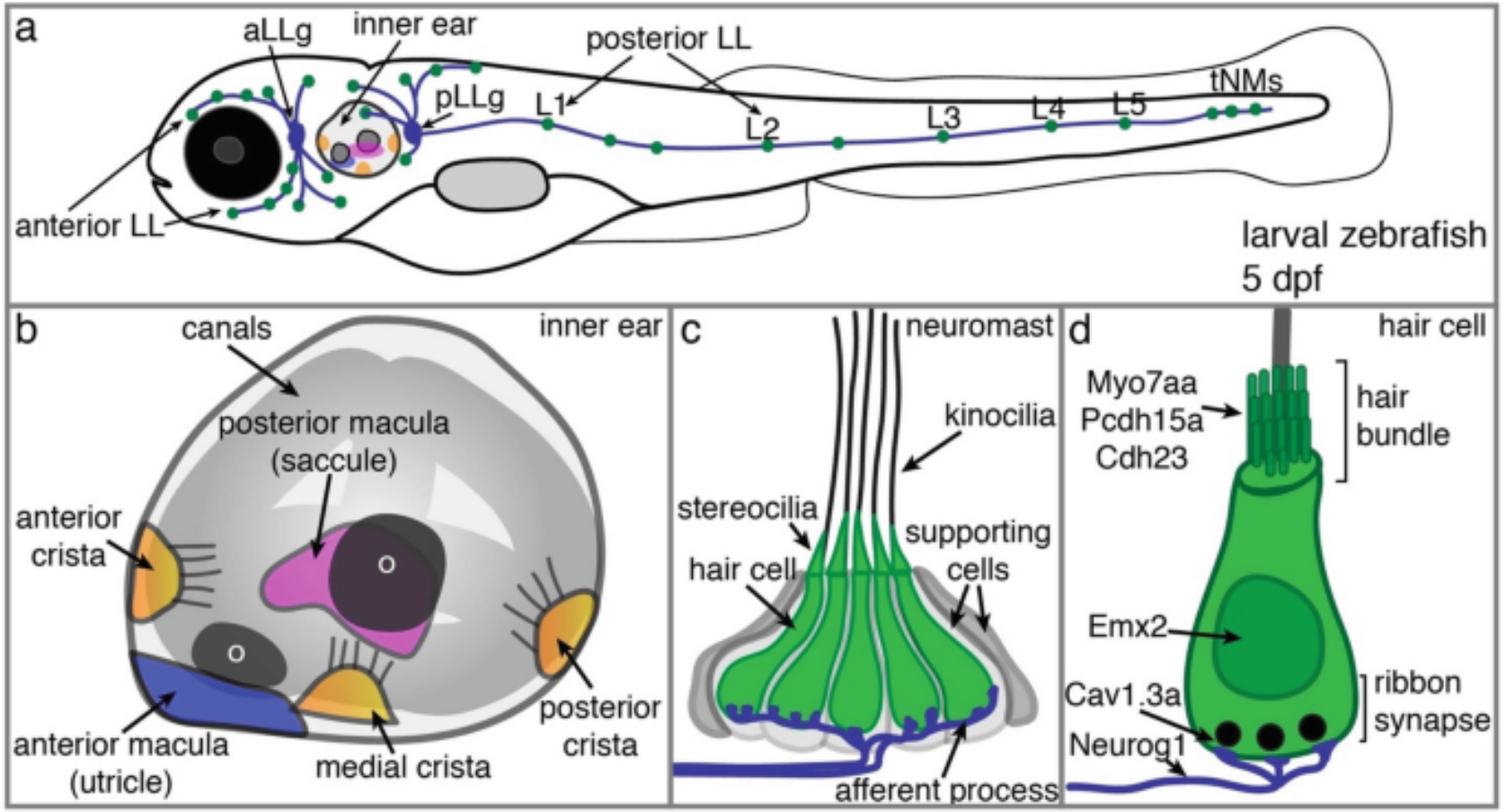
**Nonsense** mutants mediate decay of **mRNA** transcripts



Zebrafish atp11a **7BPD** loss-of-function mutants

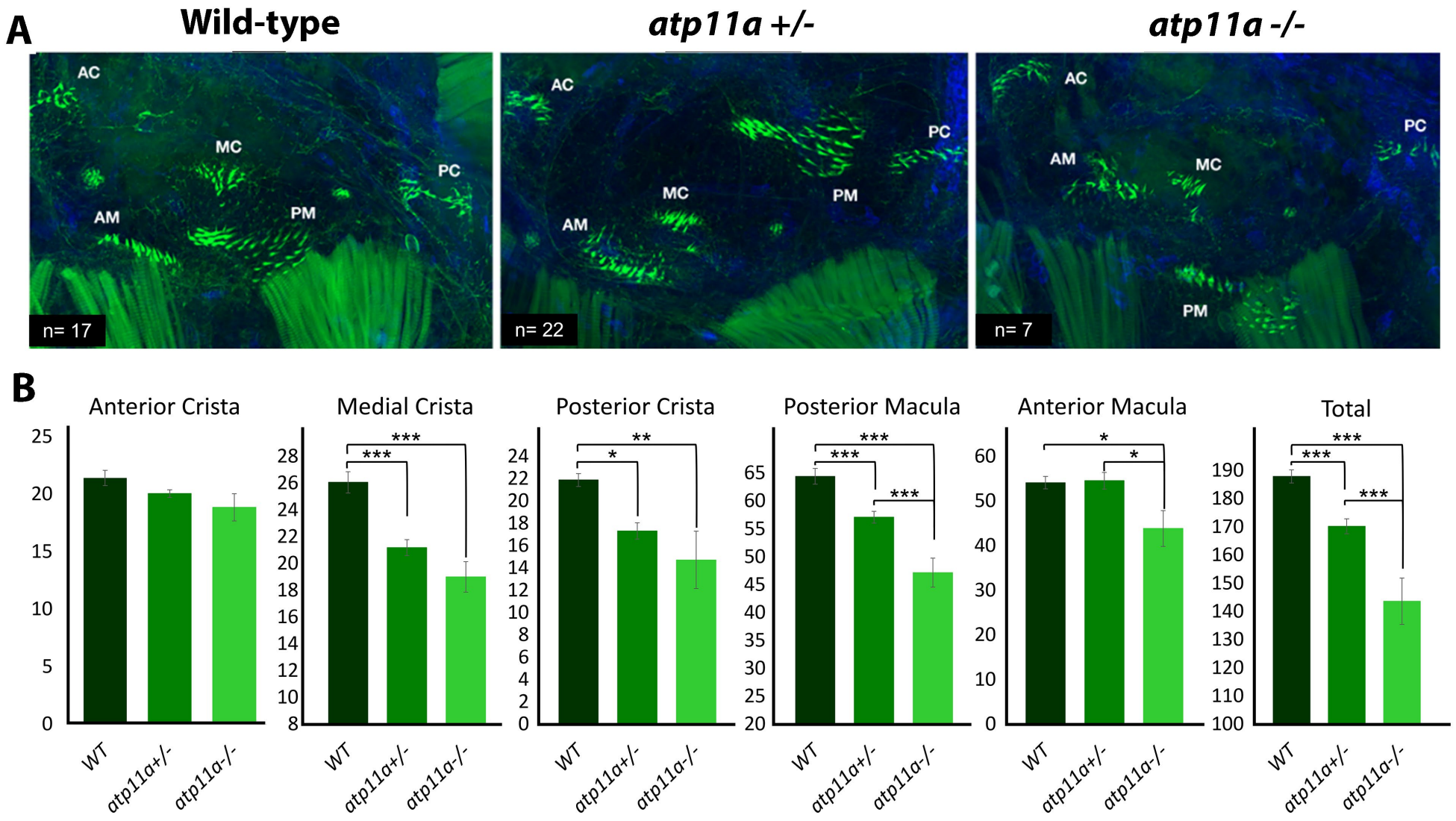


# The zebrafish ear- Model for Deafness research?



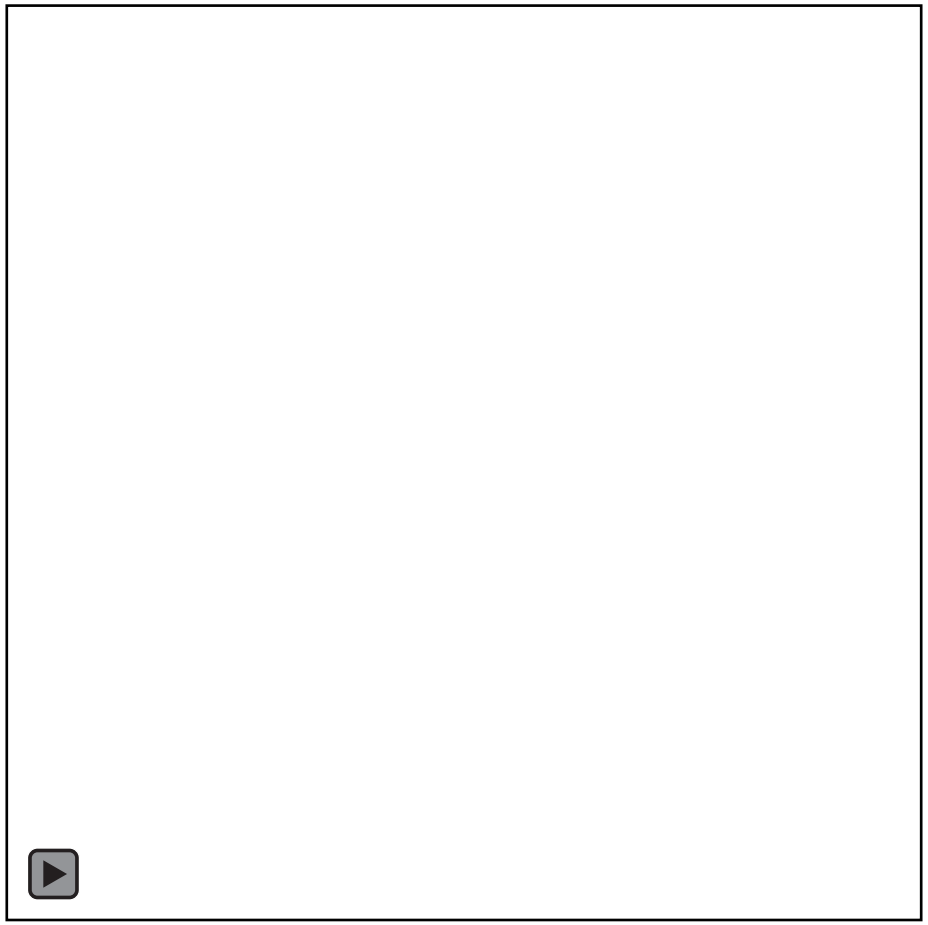

Hussain et al., 2022

# atp11a mutants have a loss of ear cell cilia



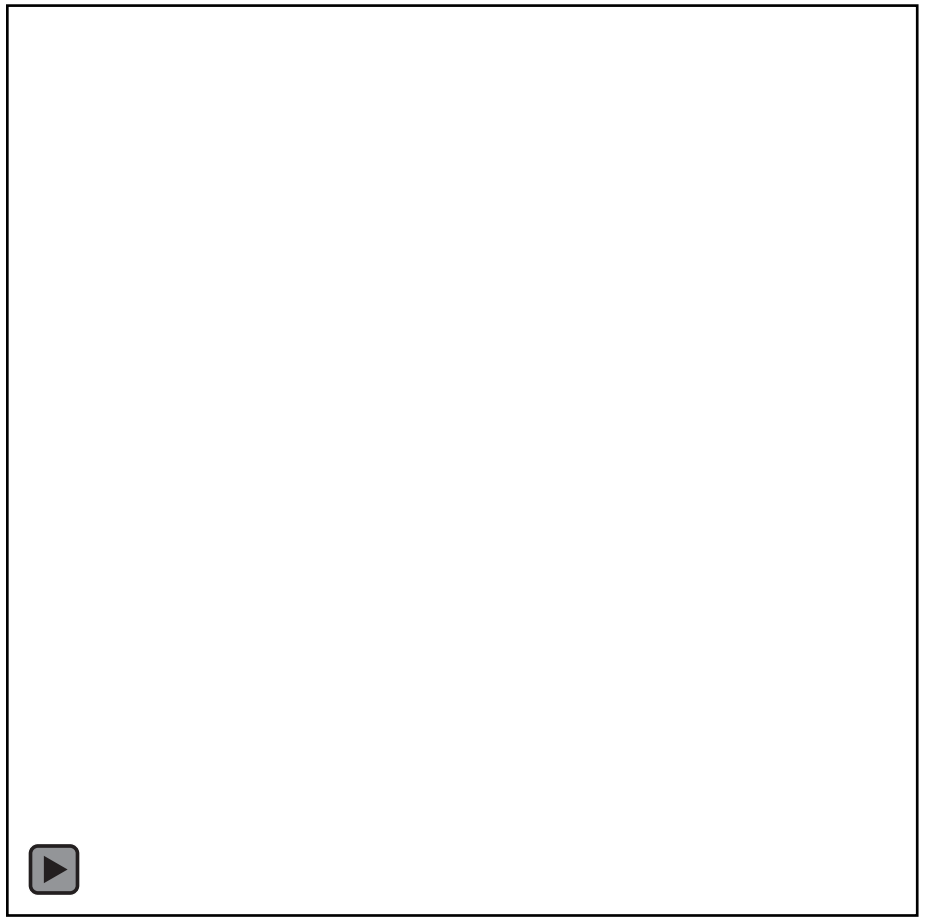
# Mutation of atp11a causes increases clotting when exposed to SARSCoV2 Spike Protein

Wild Type



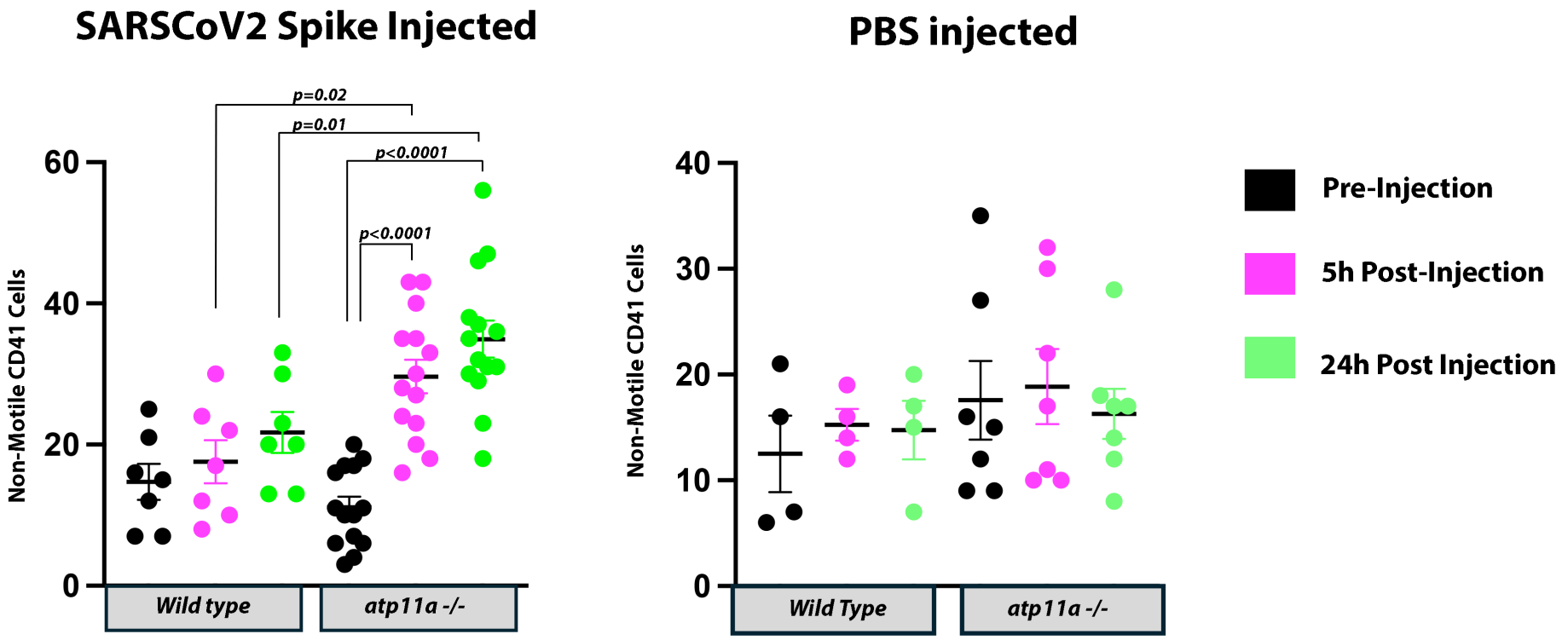
5 hours post -injection

*atp11a -/-*

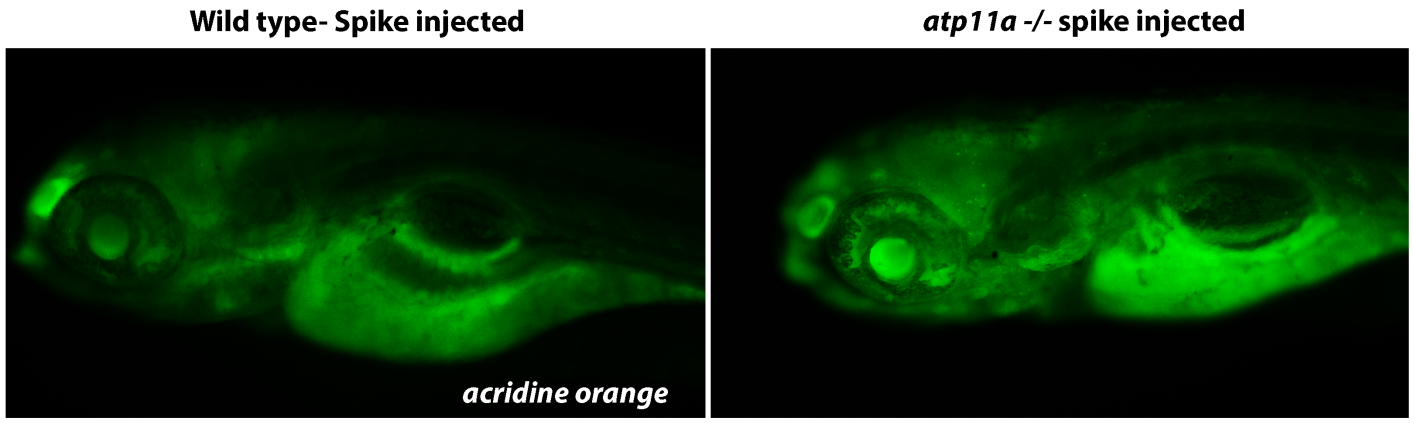


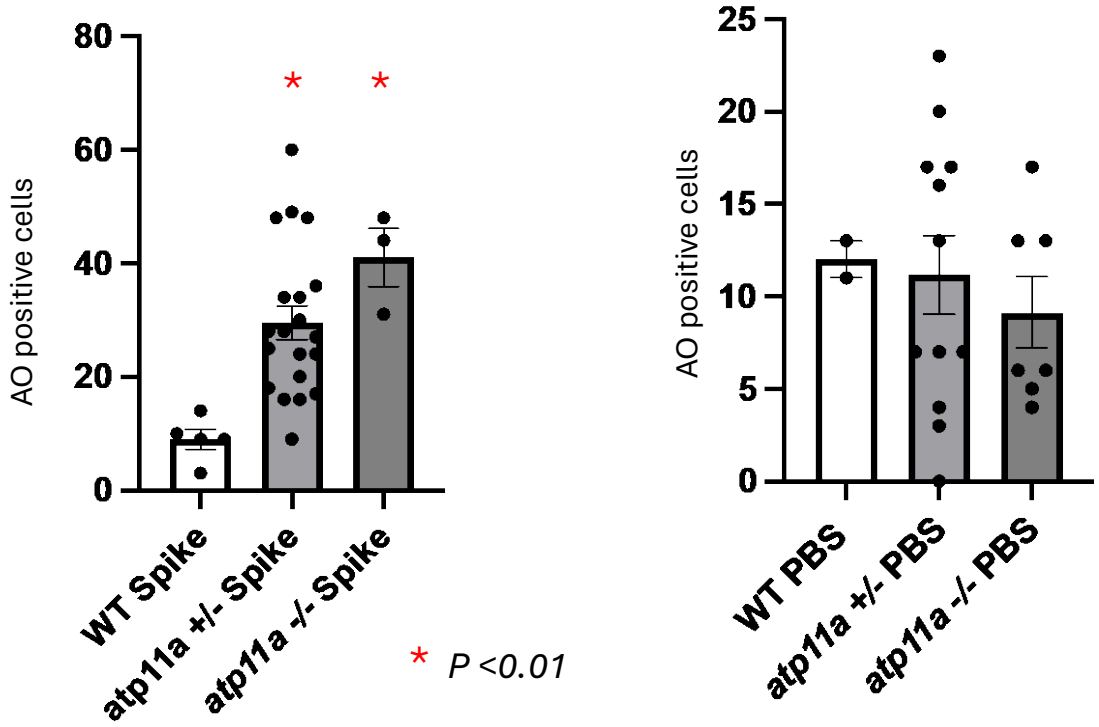
5 hours post injection

# Mutation of atp11a increases clotting when exposed to **SARSCoV2** Spike Protein



Increased cell death in atp11a mutants in response to Spike Protein injection



# Heteroduplex mobility Assay

- Assays performed on larvae are blind to genotype.
  - Genotype obtained *only after* data collection & quantification.

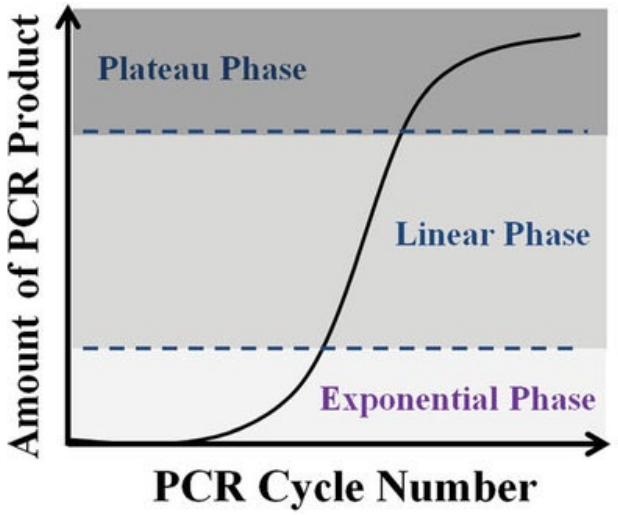
- **Sanger Sequencing** *could* identify genotype, *but*.
  - Expensive & time consuming: $3-4 per sample.

- **100's** of embryos / larvae genotyped every month

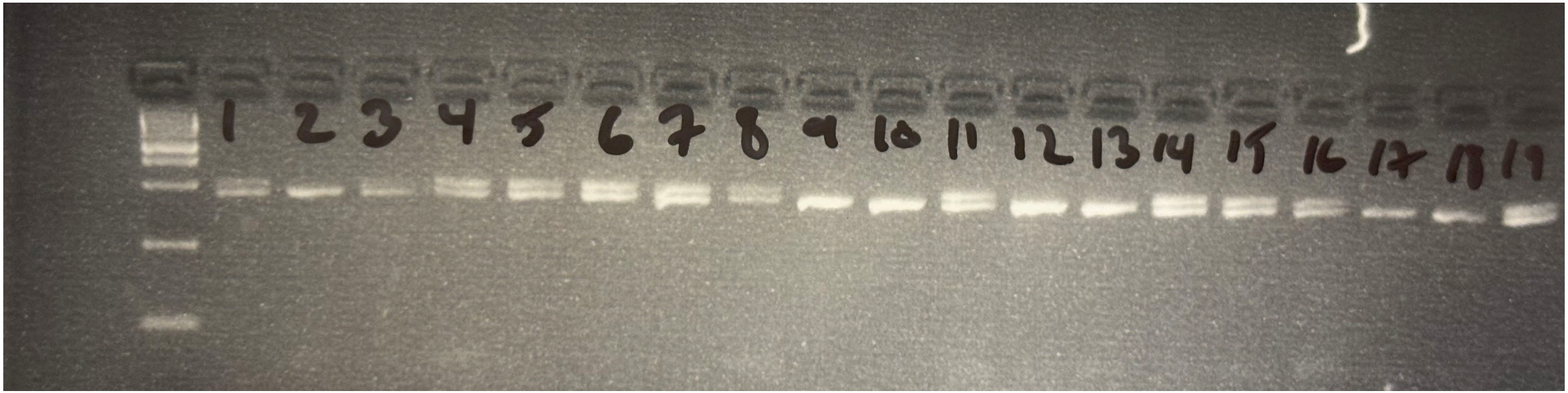
- **Heteroduplex assay**: fast, cheap (<$1) method for genotyping

# Heteroduplex mobility

- **PCR** generates **amplicons** that include mutated area of gene (~485 bp)
- In last few **PCR** cycles, reagents are limiting: not all molecules amplify.
- **PCR** amplicons, first **melted** then **cooled**, can **reanneal** to other amplicon strands in **heterozygous** larvae.
  - **Wildtype** strands form heteroduplexes with **7BPD** strands in heterozygotes
  - **Wildtype** & **7BPD** form homoduplexes with themselves in homozygotes.
- **Heteroduplexes** migrate through gel **more slowly** that **homoduplexes**.
  - Heterozygote embryos / larvae show **two** bands on a gel
  - Homozygous **wildtype** or homozygous **7BPD** larvae have **one** band
  - "Single band" on gel does *not* identify *which* homozygote



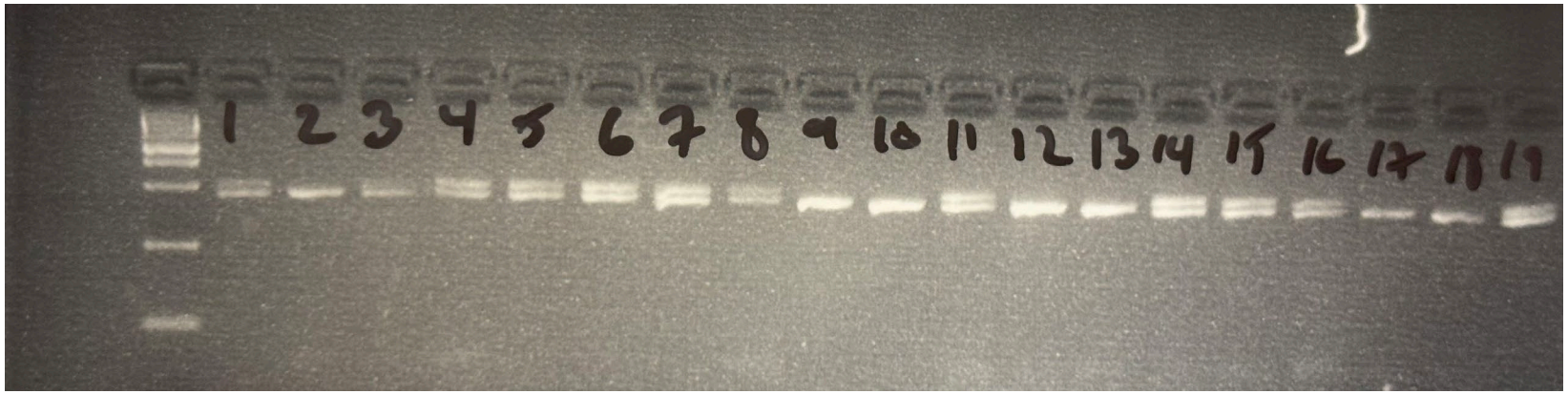
# Heteroduplex assay- identification of heterozygotes



# Discrimination of Wildtype & Mutant homozygote larvae

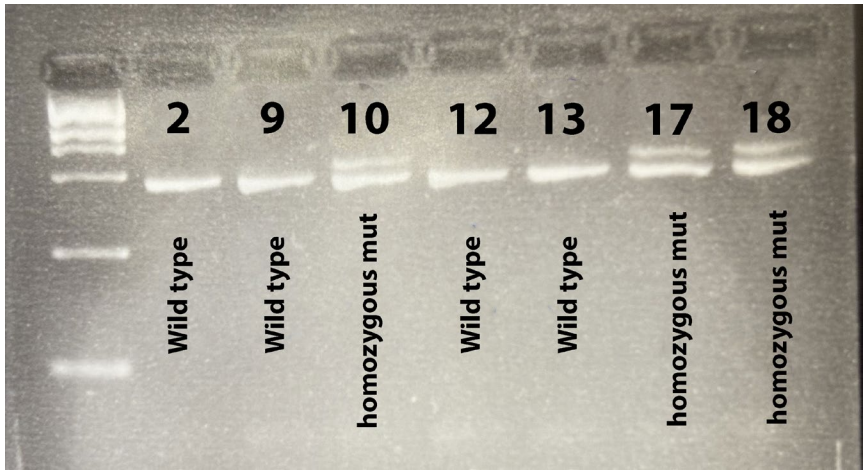
- Mix amplicons with single bands on previous gel with Control (Wildtype) **DNA**.
- Heat **DNA** to melt, then cool, and reanneal, as before
- Wildtype **experimental DNA** mixed with Wildtype **control DNA**, can only form homoduplexes: a **single band** is again seen on the gel.
- **7BPD experimental DNA** mixed with Wildtype **control DNA**, can form either **homoduplexes** with itself, or **heteroduplexes** with the wildtype control: **two bands** are seen on the gel.
- The two experiments taken together identify the genotype of each larva

# Identification of Wildtype & 7BPD homozygotes




1st agarose gel

Mix **single band samples** with known wildtype DNA
Melt (95$^0$C), re-anneal (~50$^0$C)




2$^{nd}$ agarose gel