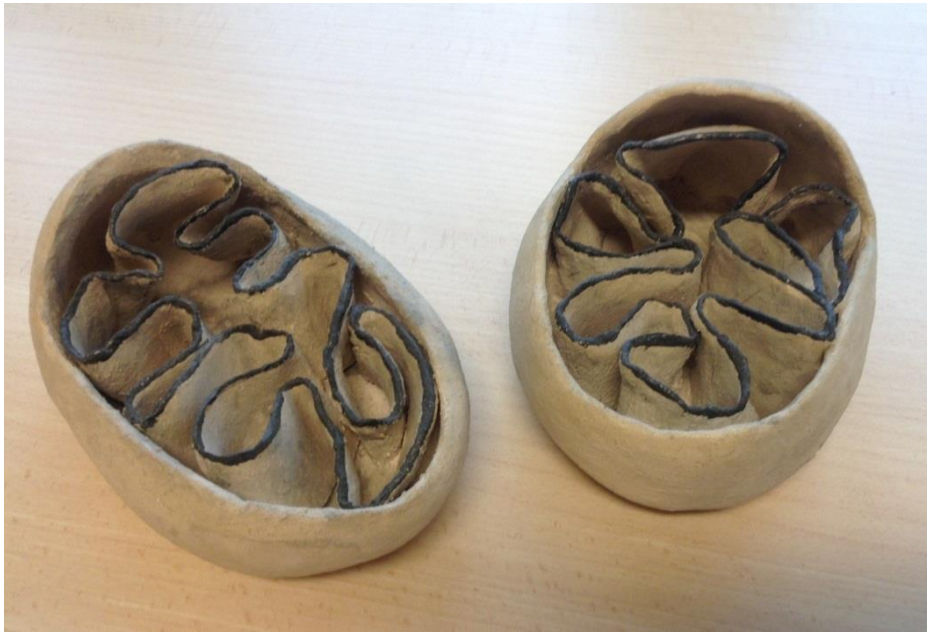

Quatorze
Juillet
à Maestricht
Freedom
is never
Free

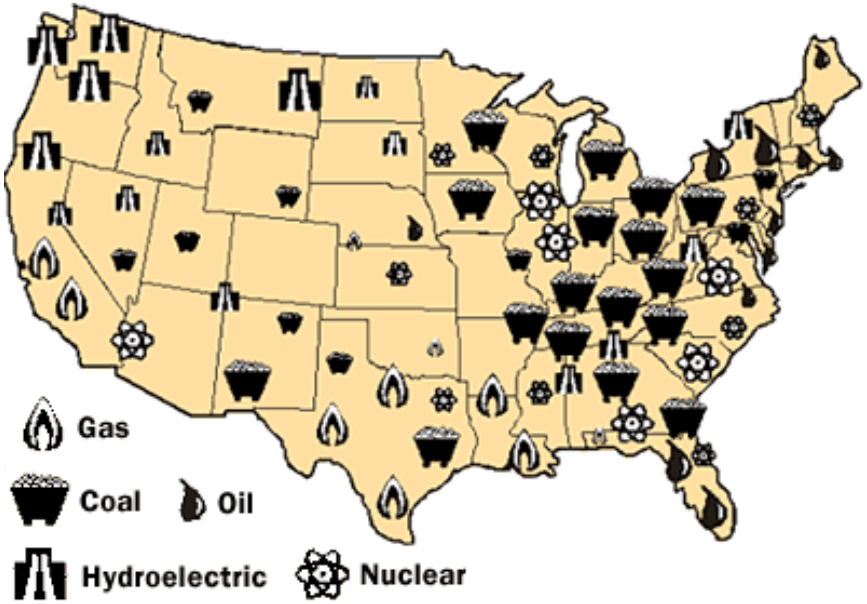
Preventing the transmission of mitochondrial DNA diseases

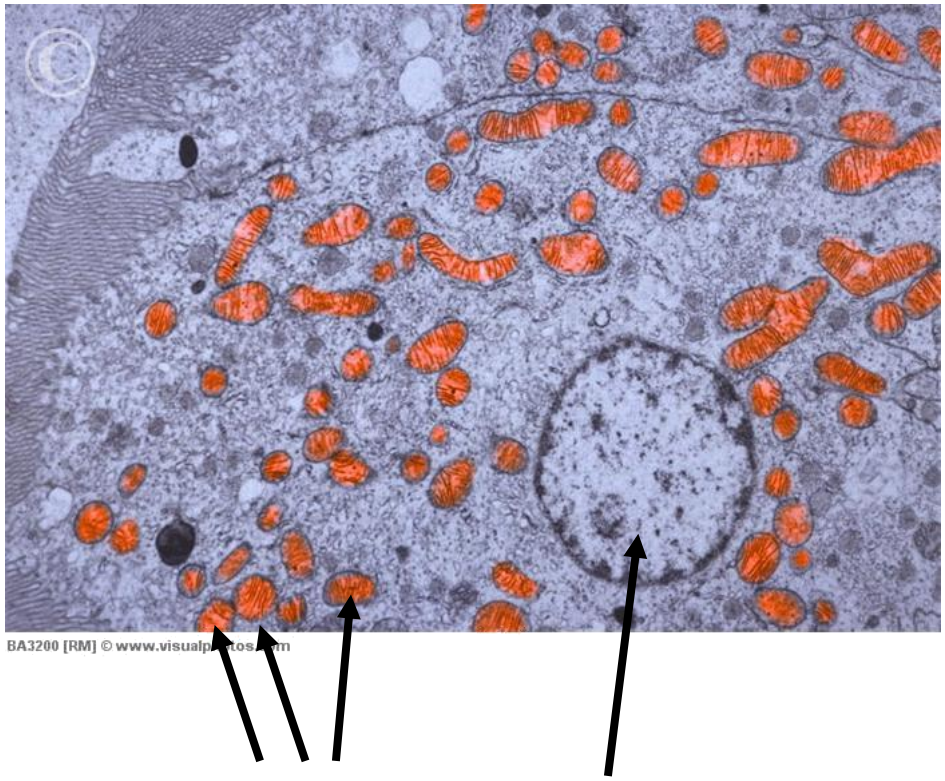


Hubert Smeets

Professor in Clinical Genomics with a focus on Mitochondrial Disorders
Research School GROW and CARIM
Maastricht University Medical Center, NL
bert.smeets@maastrichtuniversity.nl

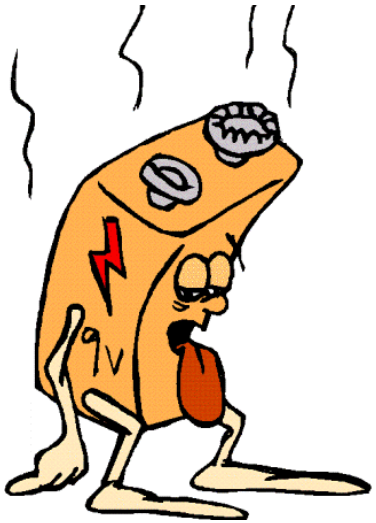
Mitochondria: Power plants of the cell

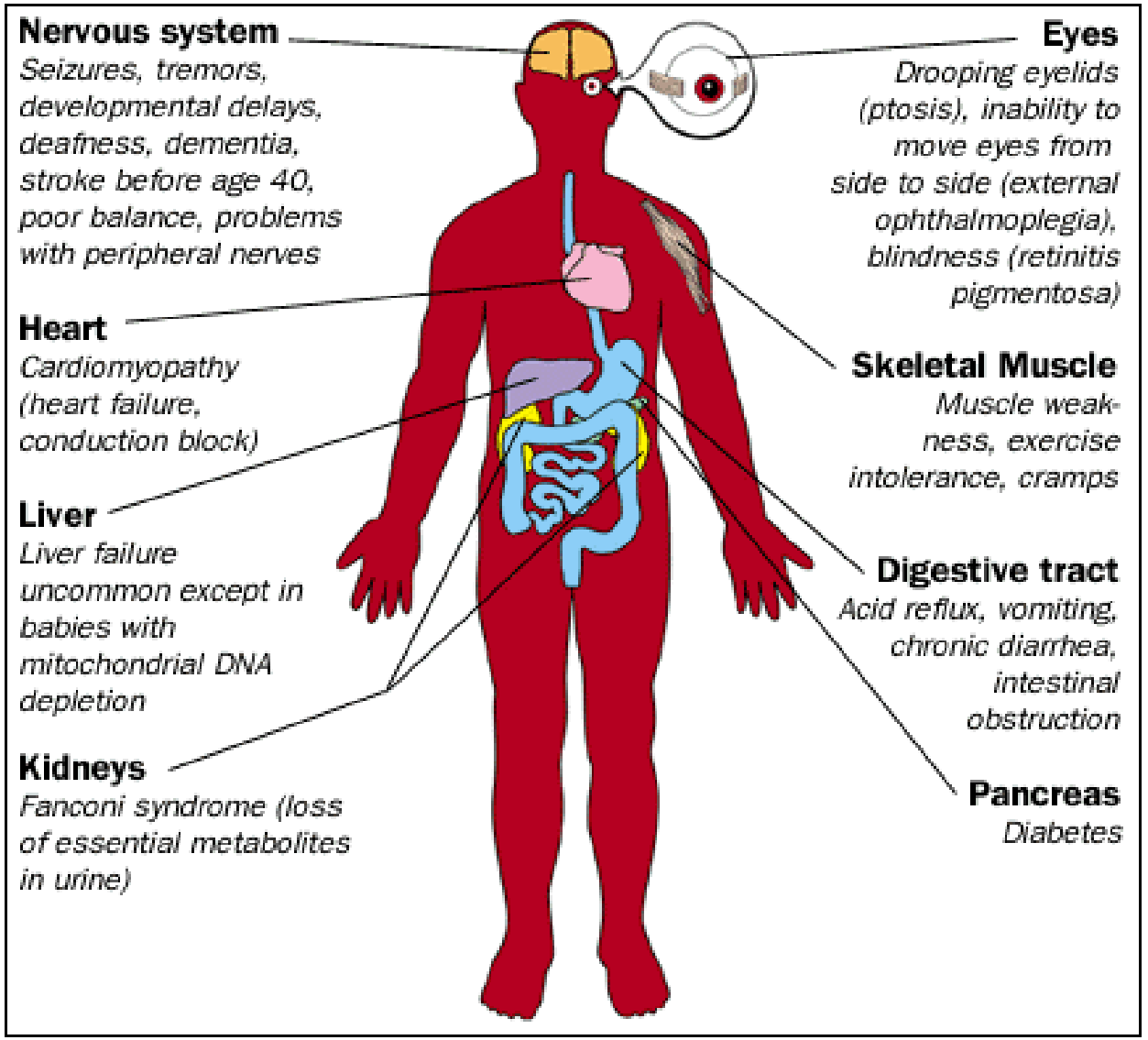




Mitochondrial disease:

General or local power failure

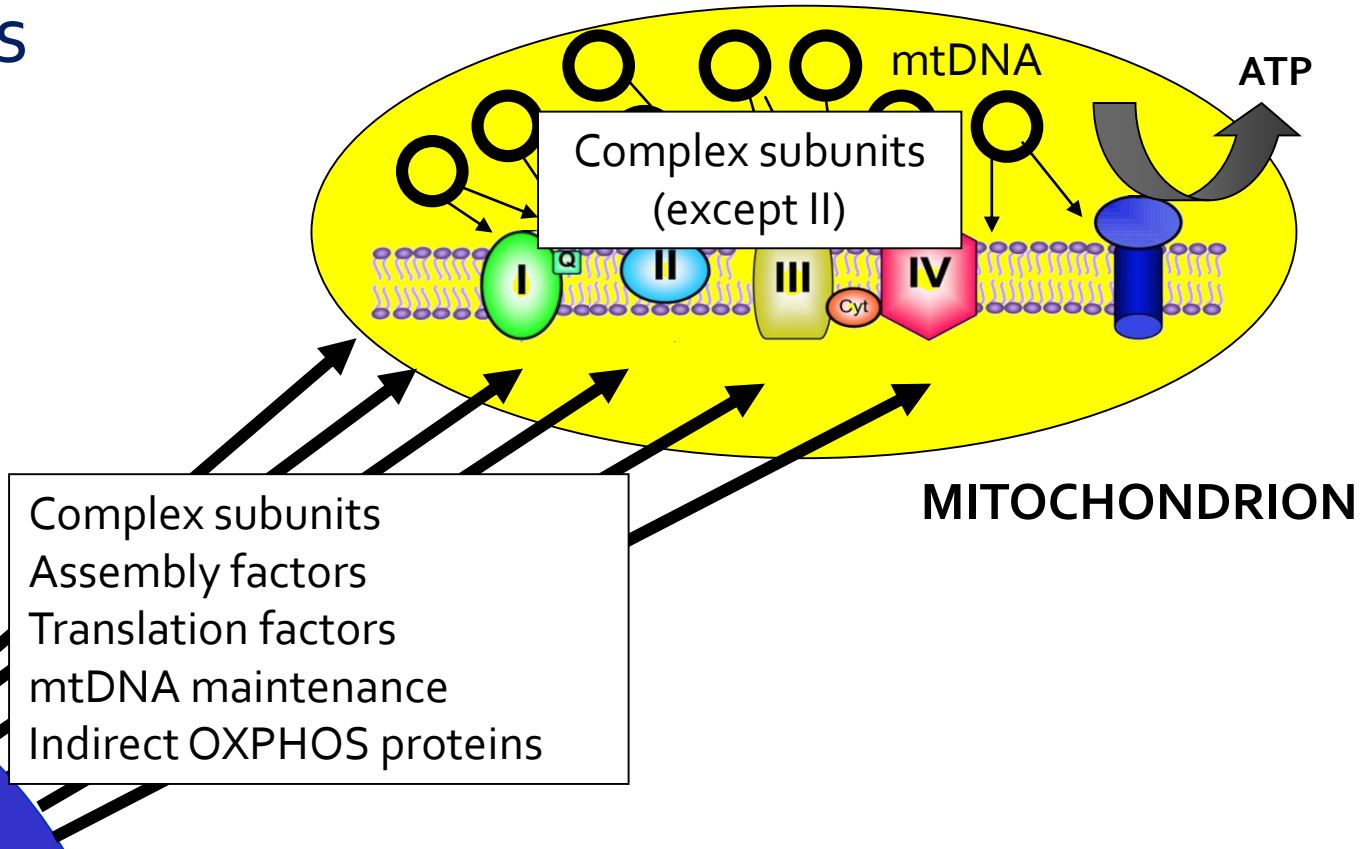




www.thesophiestory.co.uk/medical/symptoms-of-mitochondrial-disease.aspx

Maternal Inheritance

37 genes



NUCLEUS

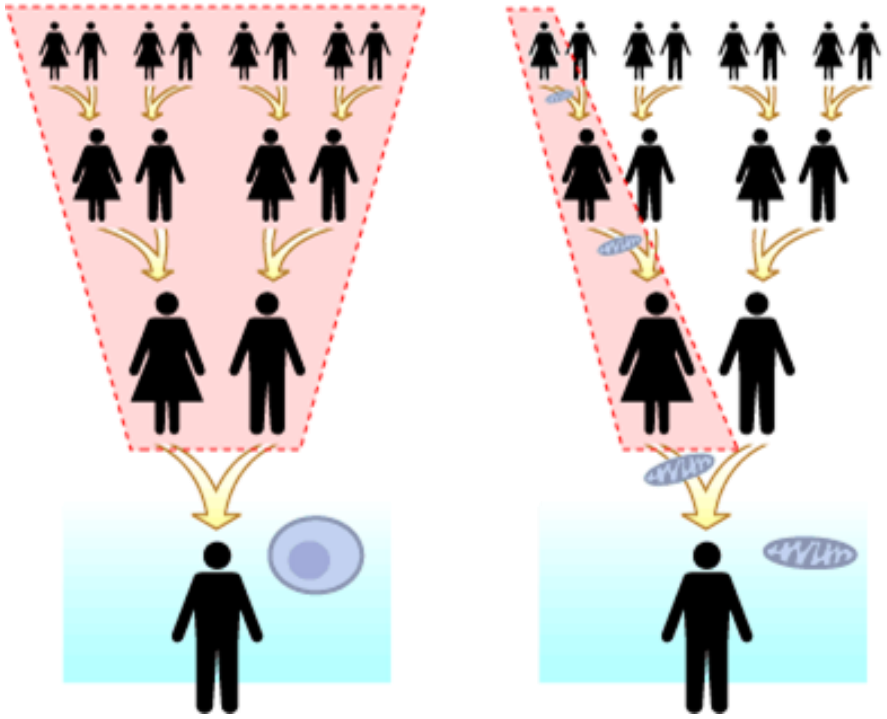
Mendelian Inheritance

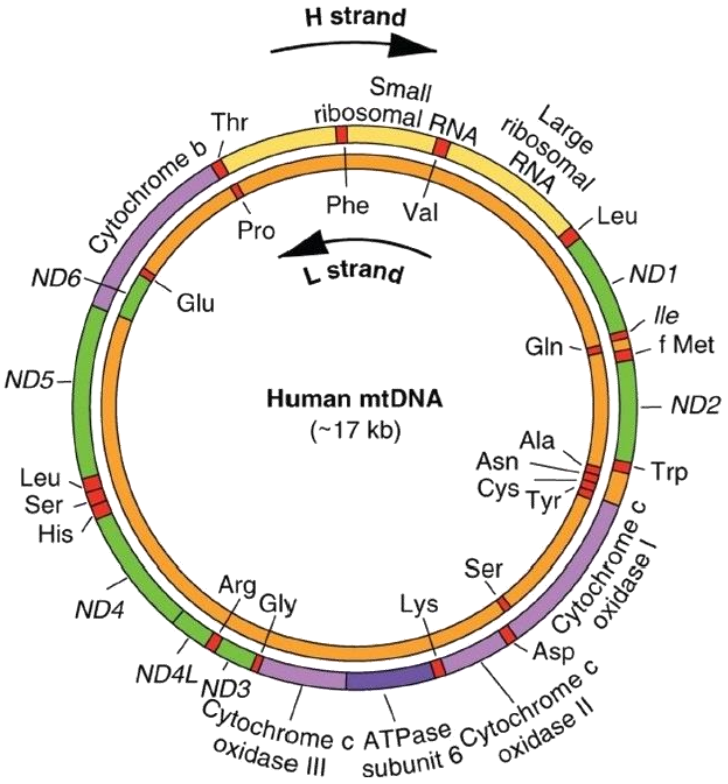
>1500 genes

Mitochondrial inheritance/mitochondrial DNA

Nuclear DNA is inherited from all ancestors

Mitochondrial DNA is inherited from a single lineage





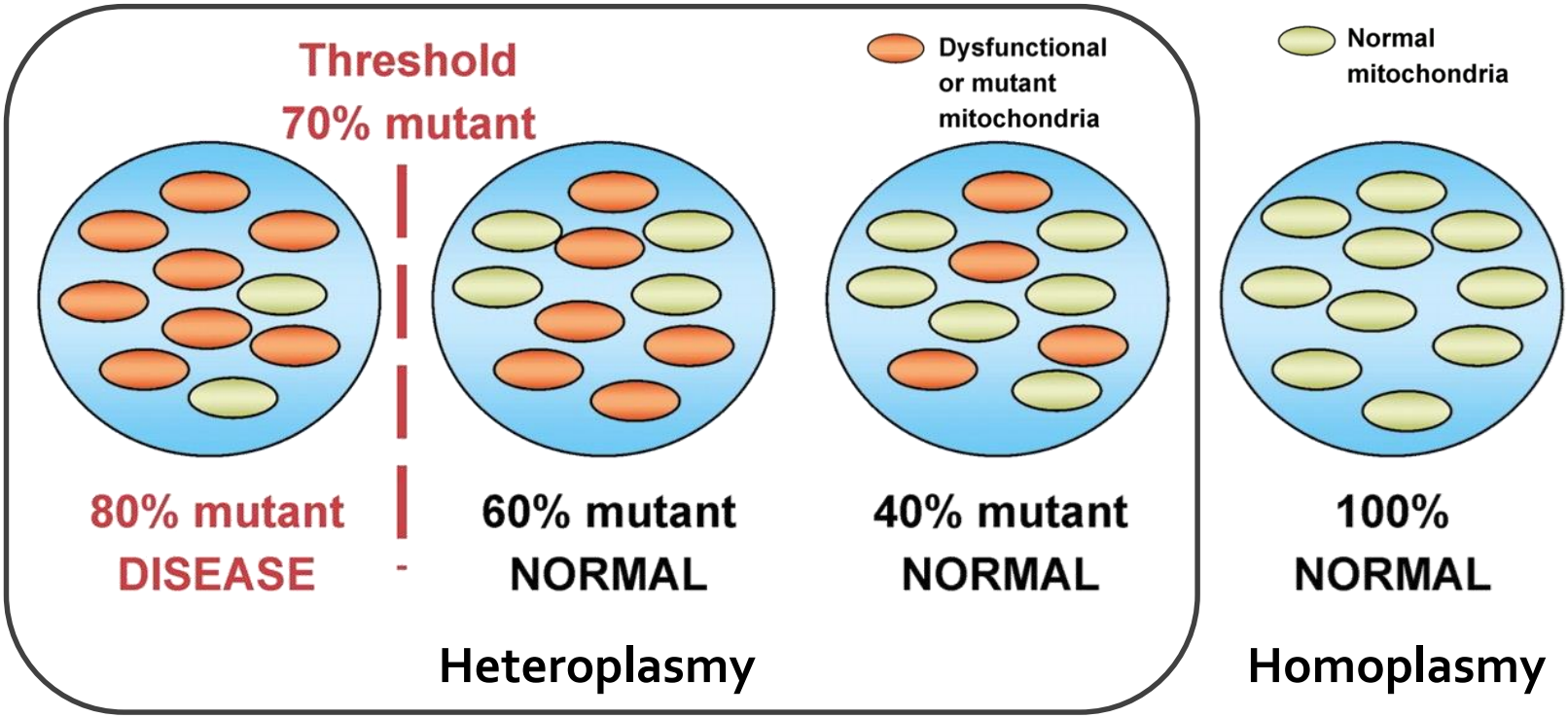
16.569 nucleotides

- Many deletions
- >159 disease-causing point mutations

Frequency mtDNA disease: 1 in 5,000

Frequency mtDNA mutations: 1 in 250-400 (in low percentage)

Threshold of expression mtDNA diseases



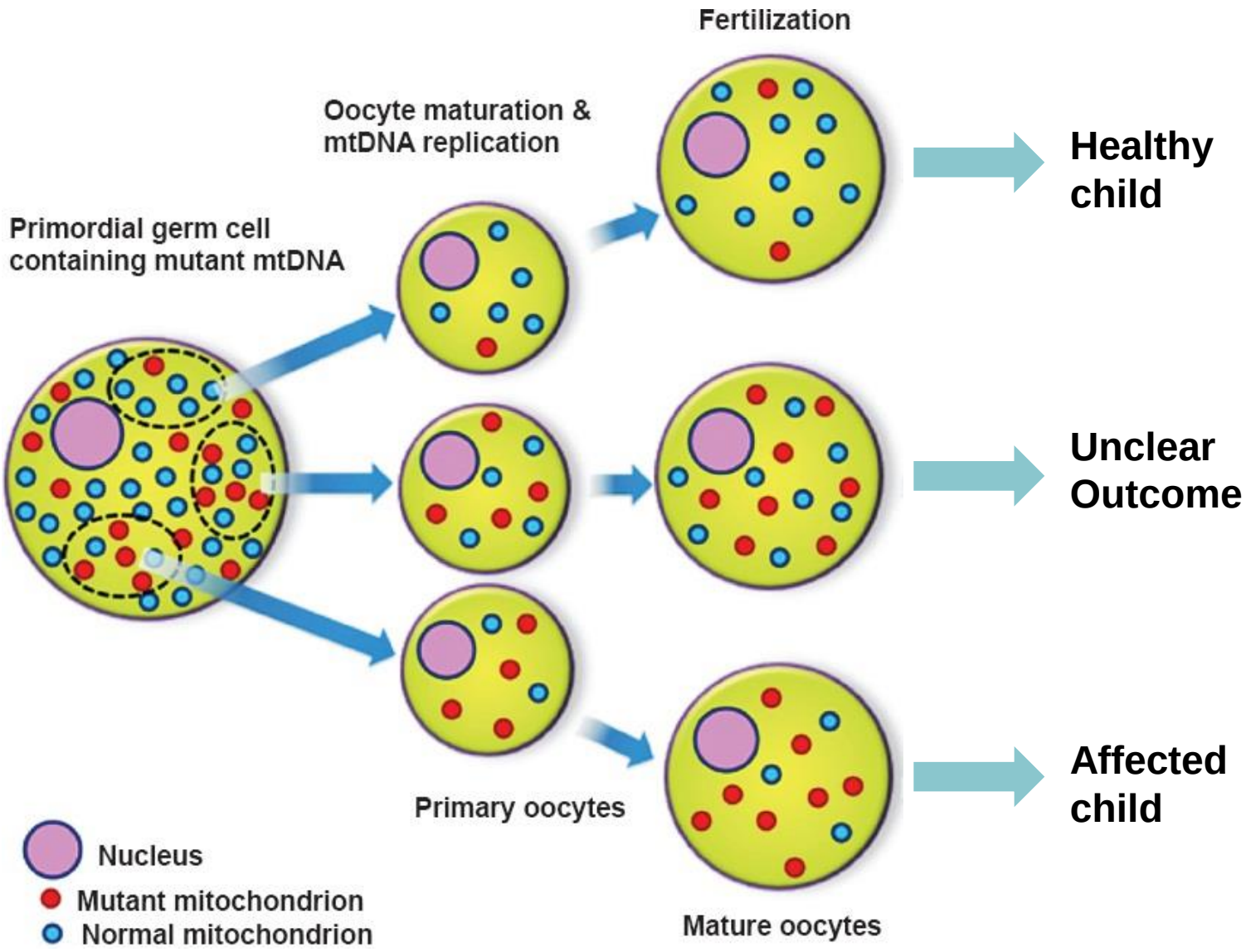
Threshold varies among tissues

Mutation percentage can change in time

Relation mutation percentage clinical symptoms often not straightforward

Most pathogenic mutation leading to severe disease are heteroplasmic
Homoplasmic pathogenic mutations exist (LHON mutations), but severe, life-threatening homoplasmic mutations are rare

Mitochondrial transmission bottleneck



Preventing the transmission of mitochondrial DNA disease

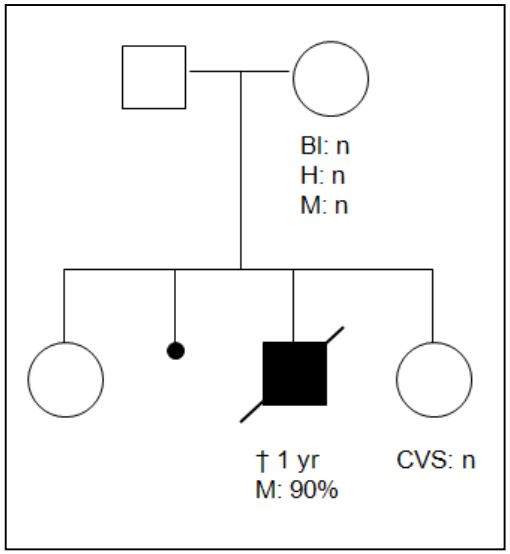
1. Selecting the good guys (healthy oocyte/embryo)

- Oocyte donation
  - homo/heteroplasmic mutations
- Prenatal diagnosis
  - some heteroplasmic/de novo mutations
  - not reliable for most inherited heteroplasmic mutations
  - interpretation problematic
- Preimplantation Genetic Diagnosis
  - all heteroplasmic mutations

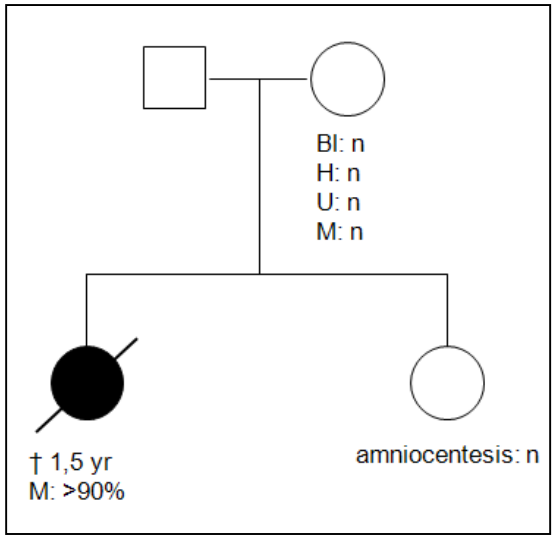
2. Kicking out the bad guys (exchange/correct faulty mitochondria)

- Spindle-chromosomal Complex Transfer, Pronuclear Transfer, Polar Body Genome Transfer
- Genome editing
- Homo/heteroplasmic mutations
- Under development

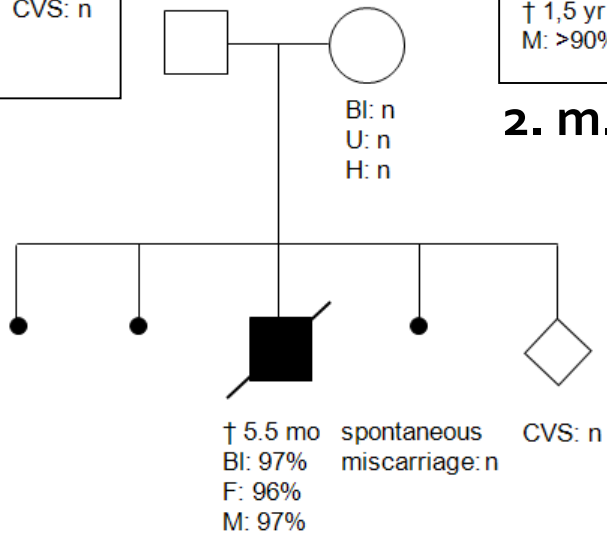
Prenatal diagnosis for *de novo* mtDNA mutations



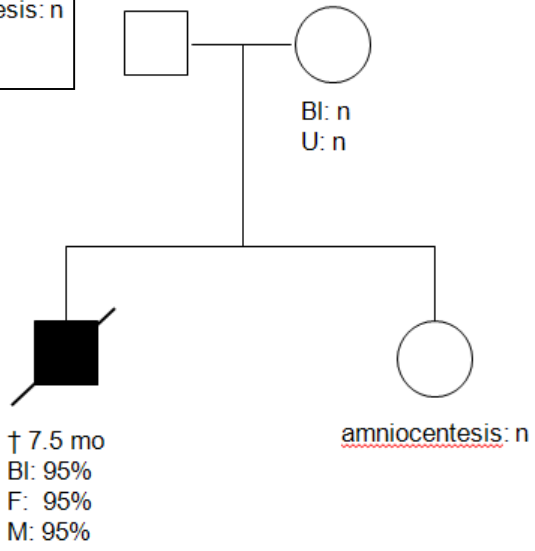
1. m.8993T>G



2. m.5556G>A



3. m.8993T>G



4. m.8969G>A

Recurrence risk and frequency *de novo* mutations in mtDNA diseases

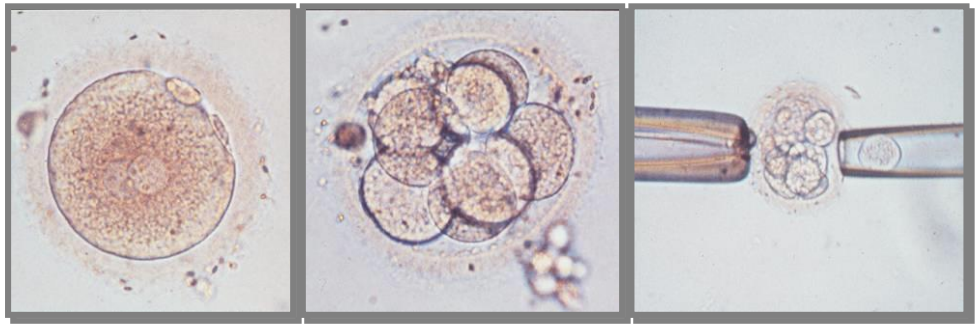
Recurrence risk in case of *de novo* mutations is low

- *De novo* mutations: defined by the absence of the mutation in different tissues of the mother of an mtDNA patient
- Often counselled incorrectly based on mutation load in patient and not on absence mutation in mother
- Chances of having another child without the mutation high, though germline mosaicism exists (14 cases followed by PND and/or PGD – 12 only wt offspring, 2 germ line mosaicism – m.9176T>C)
- PND for confirmation or reassurance

***De novo* mtDNA mutations are frequent**

- 23.5% of the (likely) pathogenic mtDNA mutations are *de novo* (own data)
- 109 *de novo* cases reported in families in literature: absence of the mtDNA mutation in 64 siblings of individuals with a presumed *de novo* mtDNA mutation
- Generally not tested after birth for ethical reasons

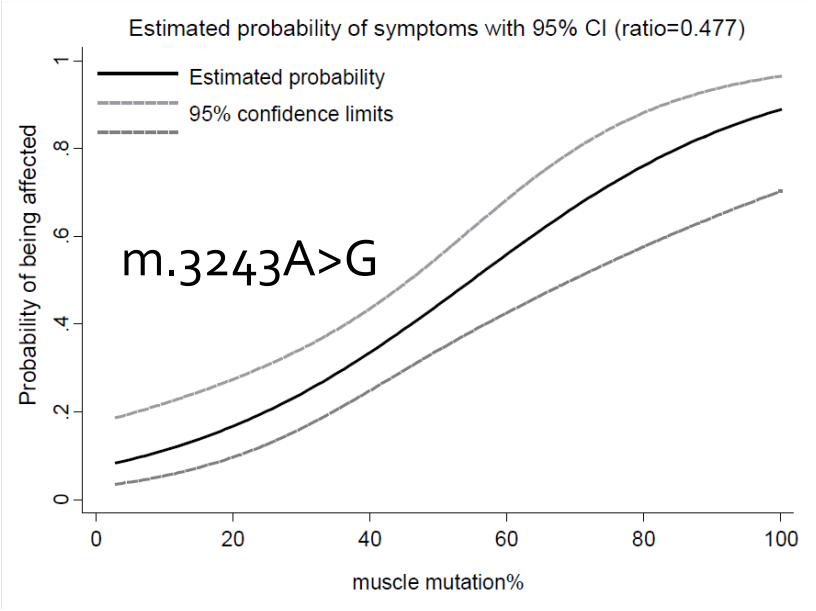
Is Preimplantation Genetic Diagnosis (PGD) a better option for recurrent mtDNA mutations?

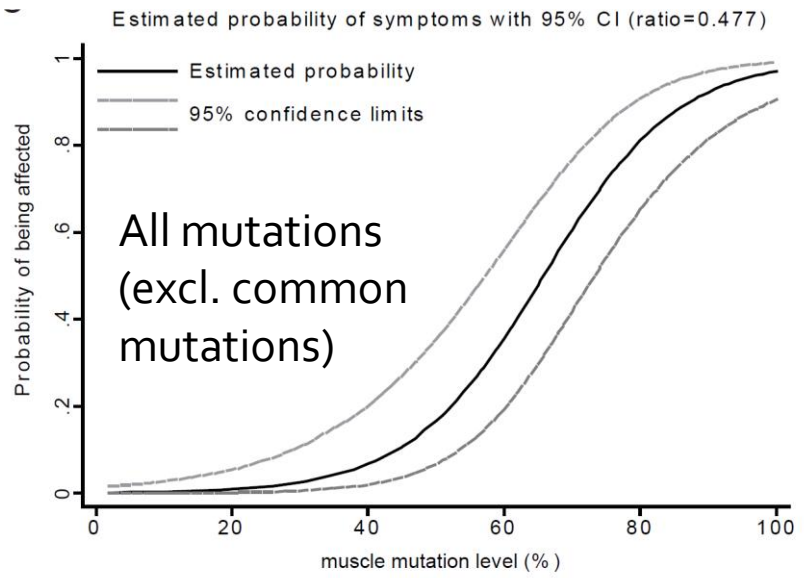


Selection embryos with mutation load below threshold expression, but:

- Only heteroplasmic mutations (main group of severe mutations)
- What is the threshold? (many private mutations)
- Is it reliable? (mutation load blastomere representative?)
- Does a carrier have such embryos?

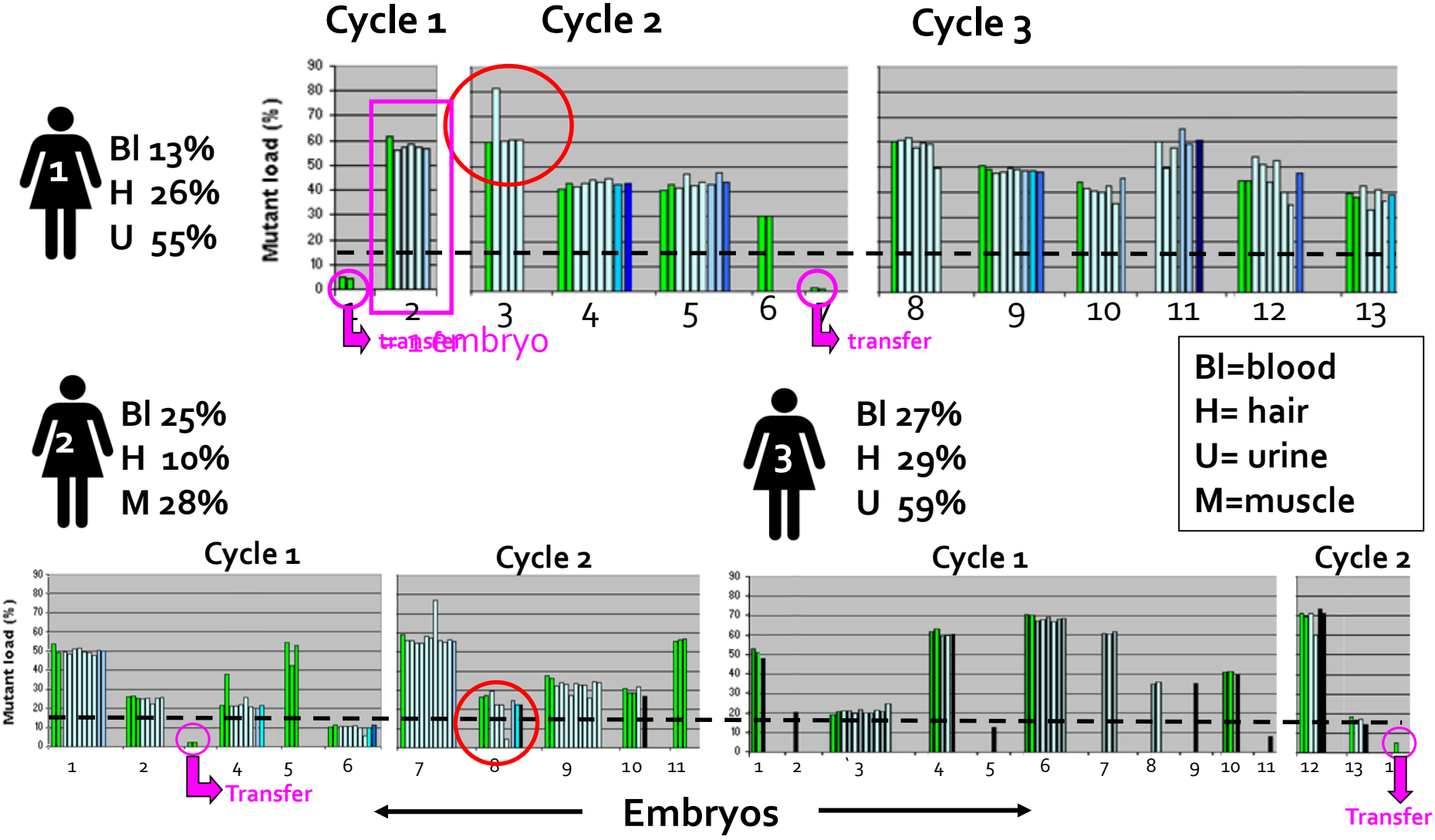
The likelihood of being affected as a function of mtDNA mutation load in muscle





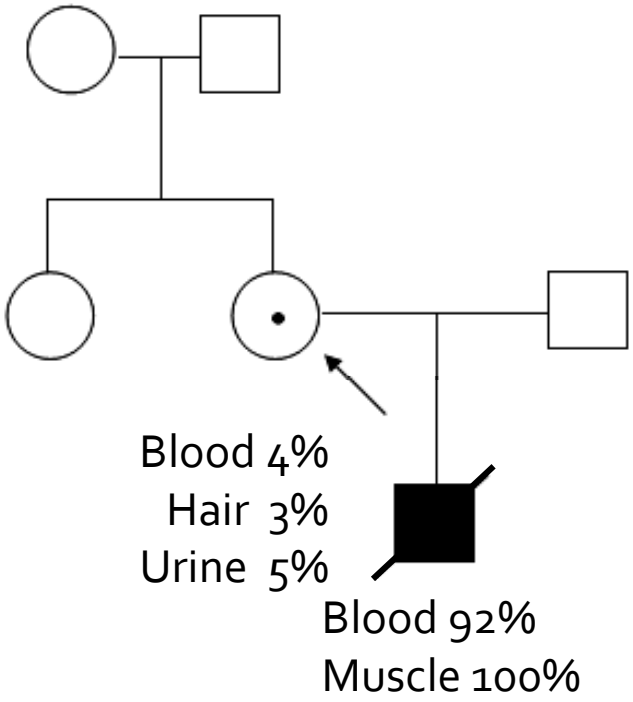
- For few common mutations a mutation-specific threshold can be determined
- For all other rare or private mutations (>99%) a general threshold defined (159 mutations, 327 pedigrees)
- At mutant level ≤ 18% → P(unaffected) ≥ 95% irrespective of mutation
- **Opens up PGD for all heteroplasmic mutations**

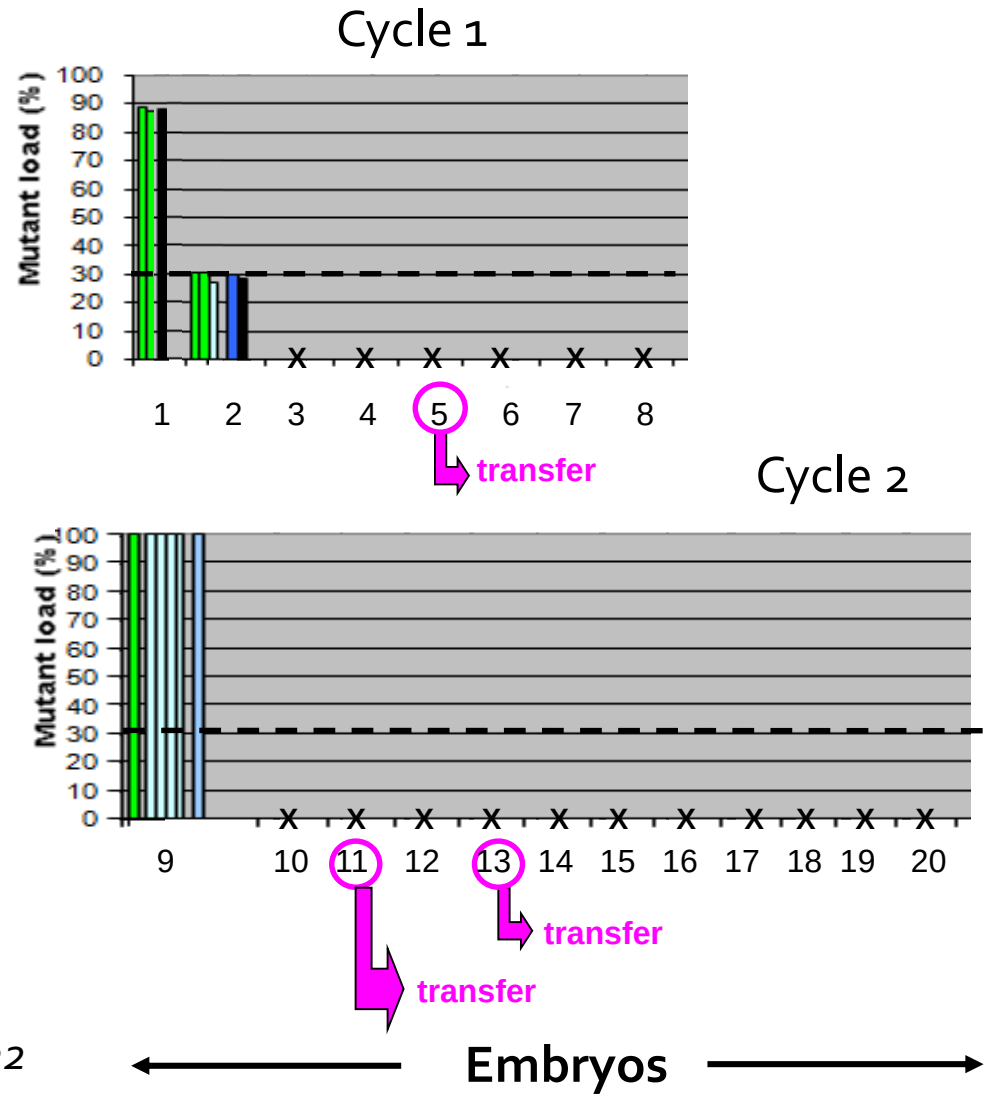
Interblastomere differences m.3243A>G



Interblastomere differences m.8993T>G

Leigh syndrome





Sallevelt et al. J Med Genet 50:125-132

Overview PGD for mtDNA disorders in Maastricht

- m.8993T>G
- m.3243A>G
- m.8344A>G
- m.14487T>C
  - private mutation

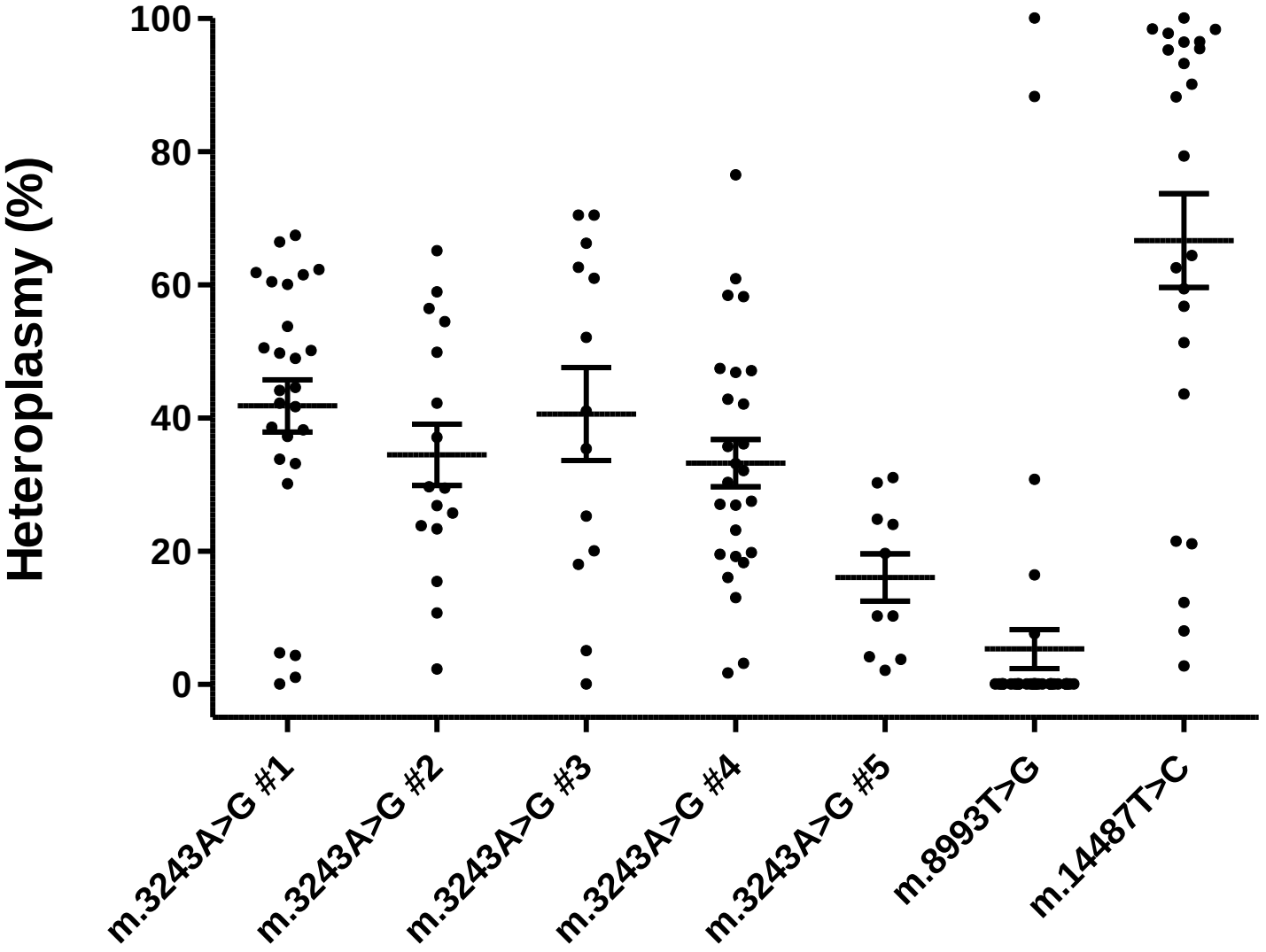
Mitochondrial disorders PGD cycles performed						
June 2015	Leigh/NARP (m.8993T>C/G)	MELAS (m.3243A>G)	MERRF (m.8344A>G)	Leigh (14487T>C)	POLG en MELAS (m.3243A>G)	Total
Couples	1	5	1	1	1	9
Cycles	4	11	1	2	2	20
Cancelled	0	0	0	0	1	1
Cycles on thawed embryos	0	0	0	0	0	0
Cycles to OR	4	11	1	2	1	19
Female mean age	32.38	32.61	35.33	41.36	31.69	33.58
Infertile	0	2	0	0	0	2
ICSI	4	11	1	2	1	19
Cancelled after OR	0	0	0	0	0	0
Cycles with analysis	4	11	1	2	1	19
COC	51	124	6	29	13	223
Inseminated	44	103	6	29	12	194
Fertilised (2PN)	28	63	4	23	6	124
Biopsied	28	63	4	23	6	124
Successfully biopsied	26	63	3	23	6	121
Diagnosed	25	58	3	23	6	115
Transferable	16	11	1	2	4	34
Transferred	5	7	1	2	1	16
Cycles to ET	4	7	1	1	1	14
Frozen	3	2	0	1	2	8
HCG positive	2	1	0	1	1	5
FHB positive	2	1	0	1	1	5
% FHB per OR	50	9	0	50	100	26
% FHB per ET	50	14	0	100	100	36

How far will Preimplantation Genetic Diagnosis in mtDNA disease bring us?

- Carriers of **all** heteroplasmic mtDNA mutations have a fair chance of having healthy offspring by applying PGD
- PGD is **technically safe and reliable** (no polar bodies)
- Estimating a **"safe" cut-off mutation percentage** at which the risk of being affected is acceptably low (risk reduction strategy)
- Based on limited PGD cycles for specific mutations we expect that **most mtDNA mutation carriers will have oocytes below this threshold**
- Exact cut-off mutation percentage determined by **case-by-case counselling**
- Selection of male embryos (sex analysis) could definitely eliminate mtDNA disease in future generations (ethical issue)
- **Trophectoderm biopsy** performed to test m.324A>G in 2 cases, 1 together with Y-chromosome, the other currently debated (most likely technical issue)

Treff et al. Fertil Steril 2014; Mitalipov et al. Cell Rep, 2014; Stefann et al. Cell Rep 2014

Mutation load distribution in PGD oocytes, zygotes and blastomeres

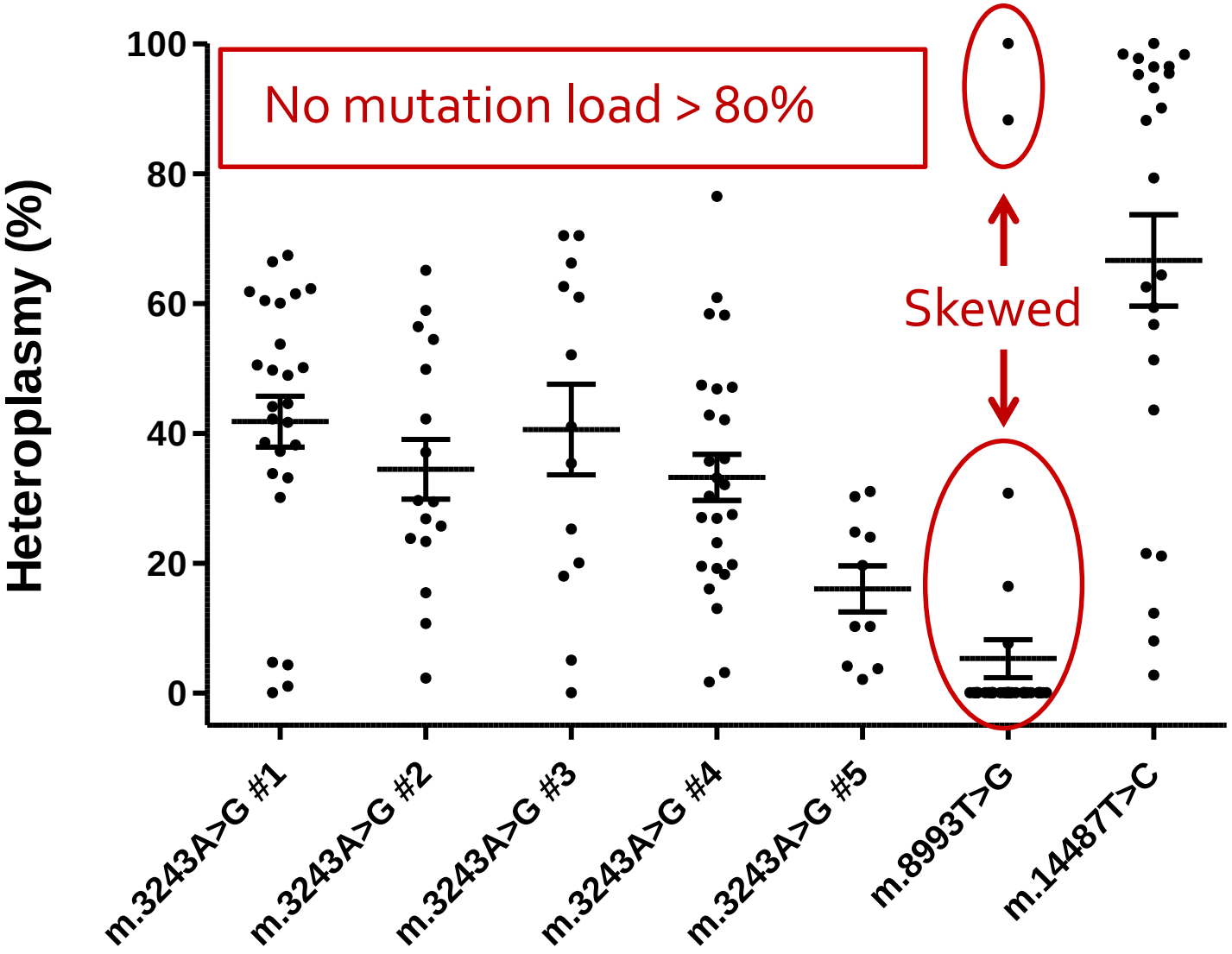


Bottleneck sizes for m.3243A>G, m.8993T>G and m.14487T>C mutation carriers

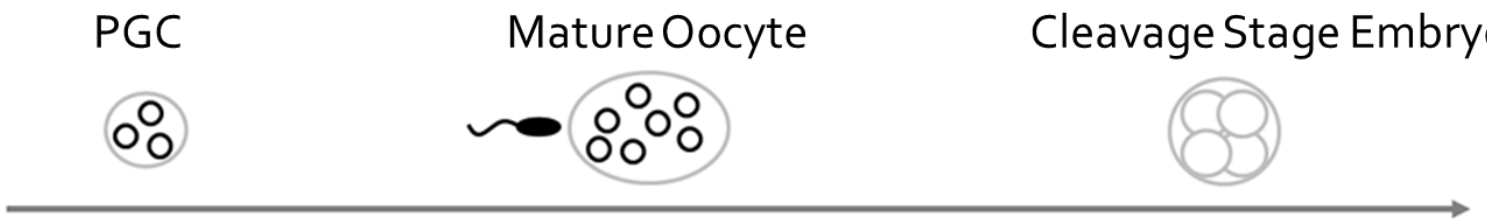
Carrier	n	p_o, heteroplasmy in samples (Average ± SEM)	Effective bottleneck size (N_{eff}) (value [95% CI])
m.3242A>G #1	26	0.42 ± 0.04	83 [50-159]
m.3242A>G #2	16	0.34 ± 0.05	94 [50-233]
m.3242A>G #3	13	0.41 ± 0.07	49 [24-117]
m.3242A>G #4	26	0.33 ± 0.04	92 [55-173]
m.3242A>G #5	10	0.16 ± 0.04	152 [69-473]
m.8993T>G	46	0.05 ± 0.03	10 [4-57]
m.14487T>C	23	0.67 ± 0.07	21 [13-38]

Bottleneck sizes calculated on the assumption of genetic drift only

Mutation load distribution in PGD oocytes, zygotes and blastomeres

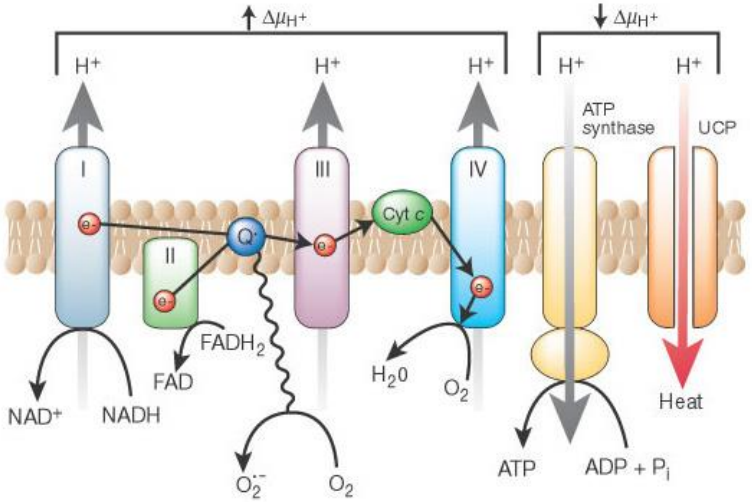


Selection on OXPHOS function in oogenesis



Metabolic Dependency

Mitochondrial Membrane Potential (MMP)



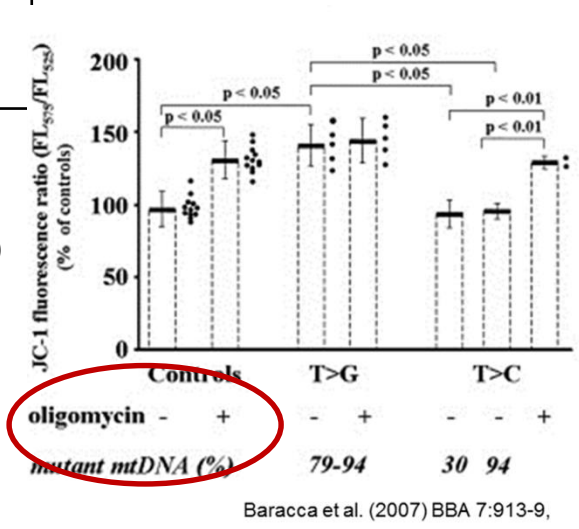
Most mutations:

- Reduced OXPHOS function
- Reduced MMP

But:

- Differences between mutations exist
- Mutation loads are involved as well

Bottleneck, genetic drift and selection define mtDNA mutation distribution in oocytes

Segregational mechanism	**m.3243A>G**	**m.8993T>G**	**m.14487T>C**
Genetic drift	+		+
Selection OXPHOS			
- ATP production	>80% drops to 0%		No major effect
- OXPHOS assembly	>90% lost (CI)		CI affected
- Membrane potential	Strongly reduced		No effect

Baracca et al. (2007) BBA 7:913-9,

Random, but no mutation loads >80% (negative selection)

Random, but positive selected for high mutation loads

Random, no selection

Preventing the transmission of mitochondrial DNA disease

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- Oocyte donation
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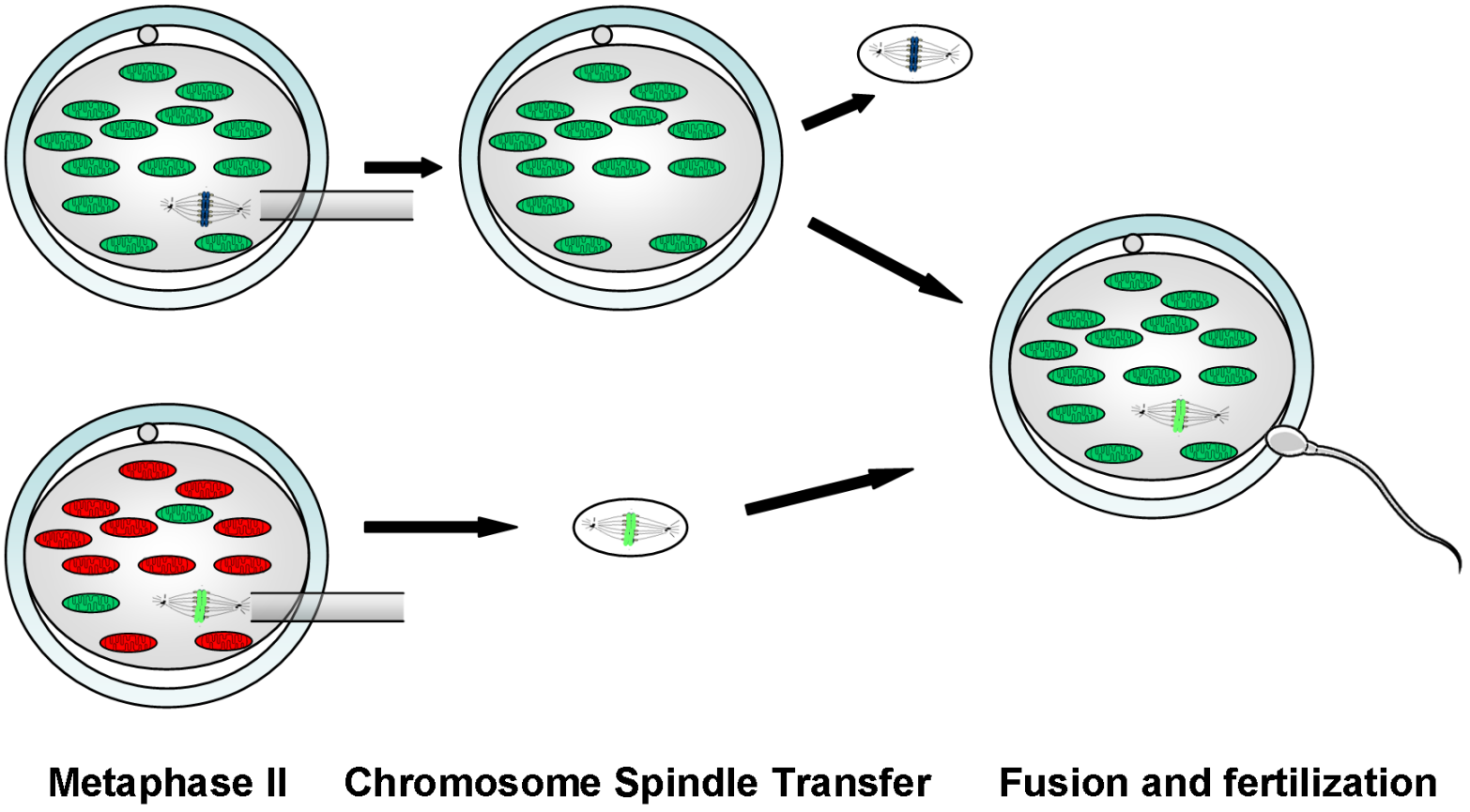
2. Kicking out the bad guys (exchange/correct faulty mitochondria)

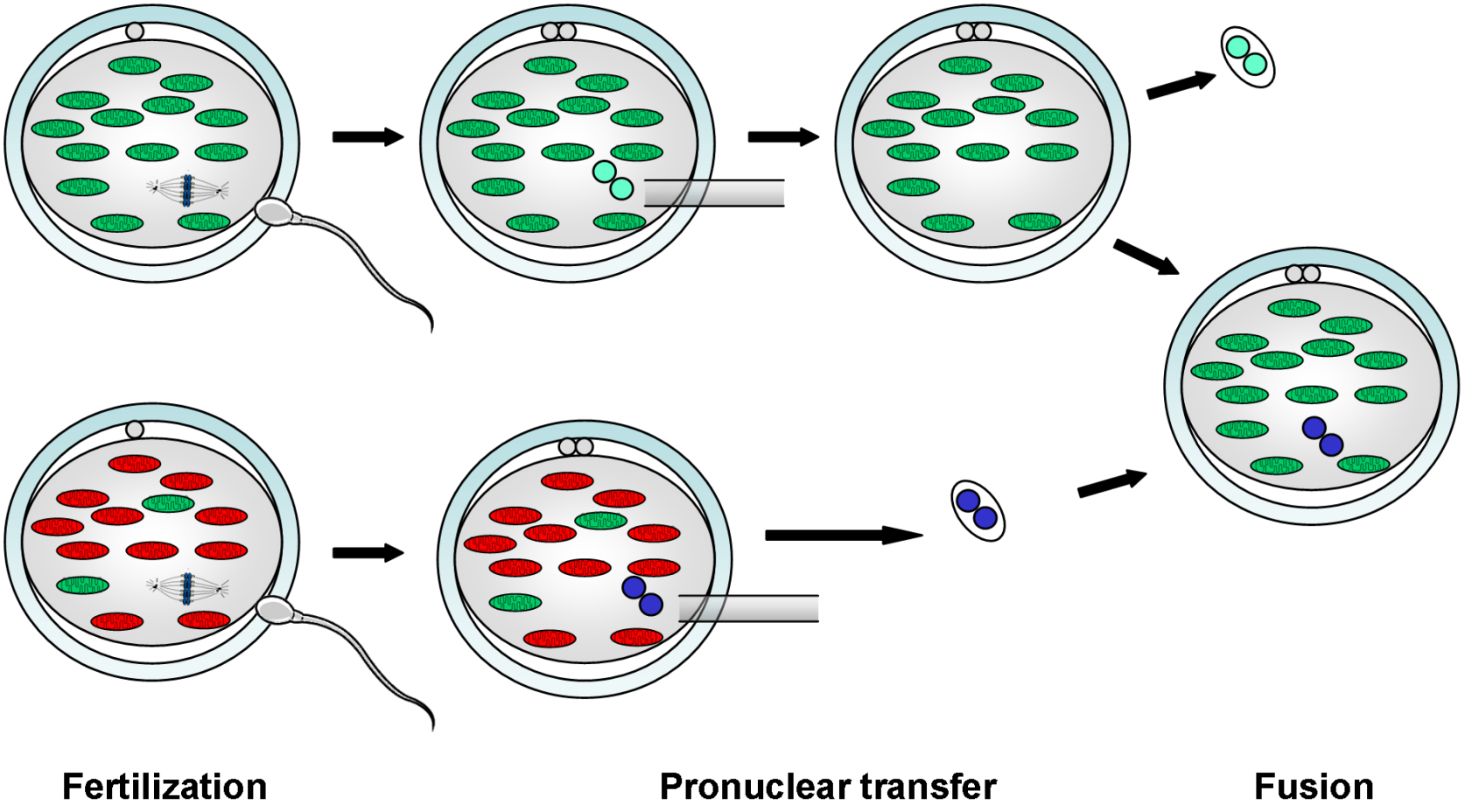
- Spindle-chromosomal Complex Transfer, Pronuclear Transfer, Polar Body Genome Transfer - mitochondrial donation
- Genome editing
- Homo/heteroplasmic mutations
- Under development

Chromosome Spindle Transfer



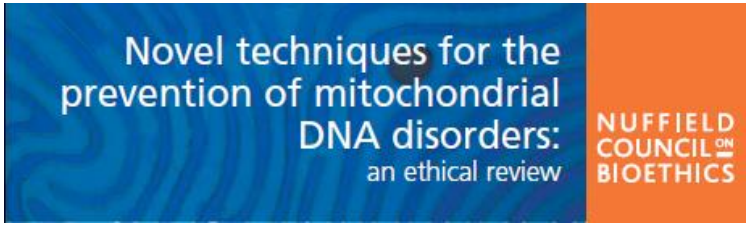
Tachibana et al. (2009) Nature 461: 367-372; Tachibana et al. (2013) Nature 493:627-631; Paull et al. (2013) Nature 493:632-637

Pronuclear Transfer



Craven et al. Nature (2010) 465:82-5

Ethical Issues concerning nuclear Transfer Technologies



Ethical considerations:

- Implications for identity
- Germline therapy
- Introduction of novel techniques and follow-up
- Parentage of the child (genetic contribution third party)
- Status of the mitochondrial donor
- Implications for wider society and future generations (creating boys)

Conclusions and issues for future consideration:

- Treatment as part of a research trial (safety issues - specialized centres)
- Regulation: follow-up (central register)
- Parentage of the child (no 'third parent' or 'second mother')
- Regulation: status of the mitochondrial donor (identity not required)
- Further issues for discussion (germline therapy)

Bredenoord et al. J Med Ethics (2011) 37:97-100
Report Nuffield Council on Bioethics 2012

Nuclear transplantation or mitochondrial donation approved in the UK

United Kingdom

"In a historic debate, the House of Commons voted by 382 to 128 – a majority of 254 – to allow mitochondrial donation for severe mitochondrial diseases through a controversial amendment to the 2008 Human Fertilisation and Embryology Act. They approved the regulation in spite of some critics warning it was a step towards creating "three-parent" designer babies." The Guardian, February 3, 2015

United States of America

Food and Drug Administration decides tha additional investigations are required to demonstrate safety, which is expected to take 2 more years.

Asia

Already performed to treat female infertility. Triplet pregnancy, 1 foetus aborted , 2 others died because of complications at birth

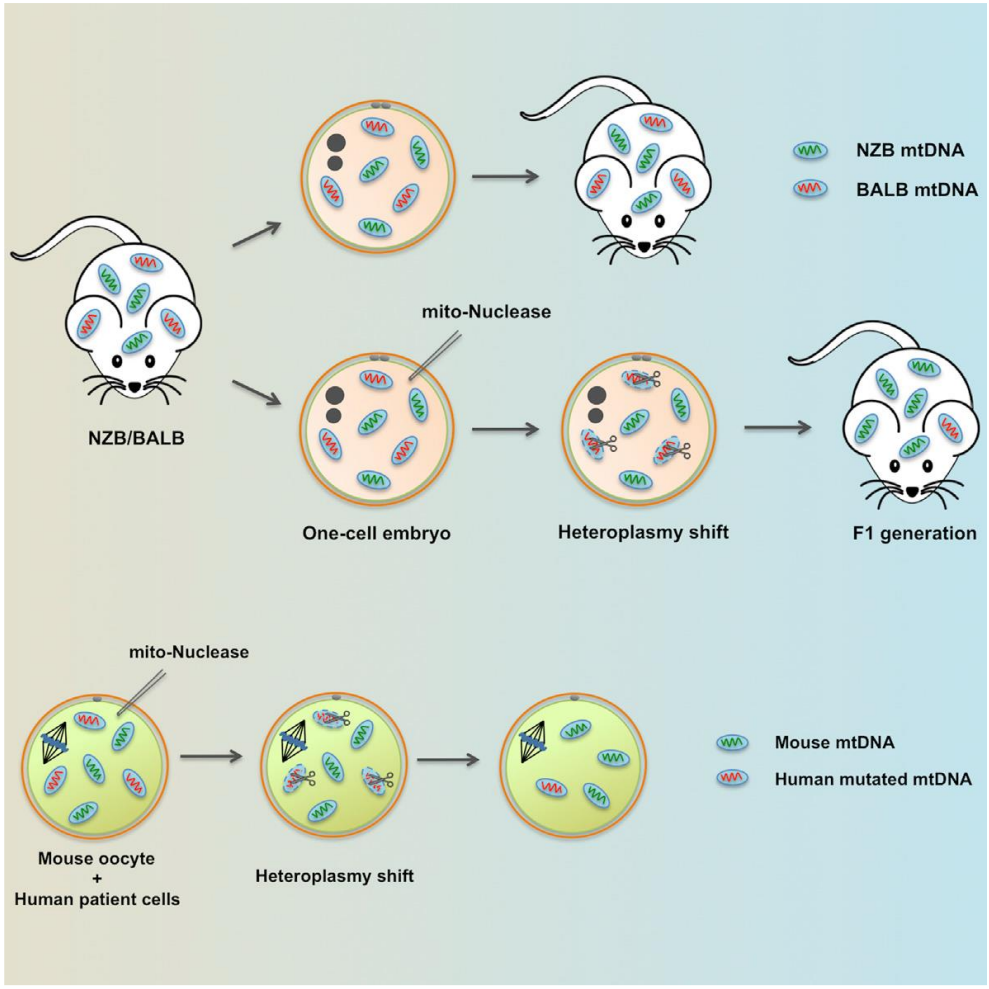
Netherlands

Positive advice Health Council , when safety has been demonstrated (March 20, 2001)
Treatment allowed under Dutch legislation (June 20, 2002)
However, research with embryos is forbidden, making safety studies on human embryos impossible

How far will nuclear Transfer in mtDNA Disease bring us?

- Spindle, Pronuclear and Polar Body GenomeTransfer are capable of generating (almost) mtDNA mutation-free embryos
- The minimal amount of mtDNA carry-over is unlikely to cause disease and is primarily wild-type mtDNA (MMP selection)
- In primates, mice, (abnormally) and fertilized oocytes the methods seem safe, but issues remain (long term effects, epigenetic issues)
- All methods can be used for heteroplasmic and homoplasmic mutations
- The clinical safety of the methods will be tested in the UK by the first clinical trial
- Require sufficient donor oocytes or zygotes (vitrification possible)

New Approach: Genome editing Break-down mutated mtDNA



Nucleases can cleave and reduce the mutation load of specific mtDNA mutations in germ cells of mice

Reducing the mutation load prevents transmission to offspring in mice

Technology also works in human oocytes

Promising, but still experimental:,
- Reduction mutation load not sufficient for clinical applications
- Safety not yet demonstrated

Reddy et al. (2015) Cell 161:459-69

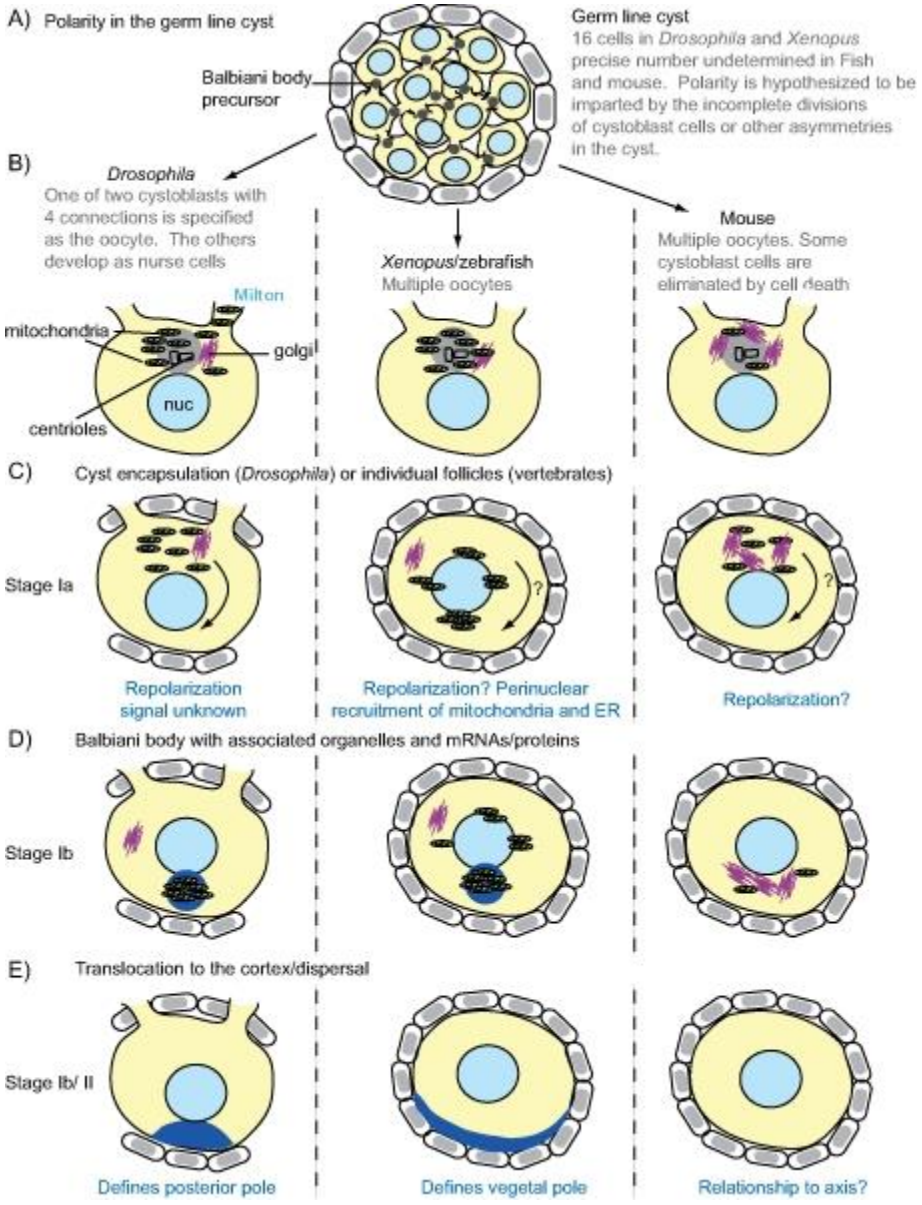
Towards a Future without mitochondrial DNA Disease

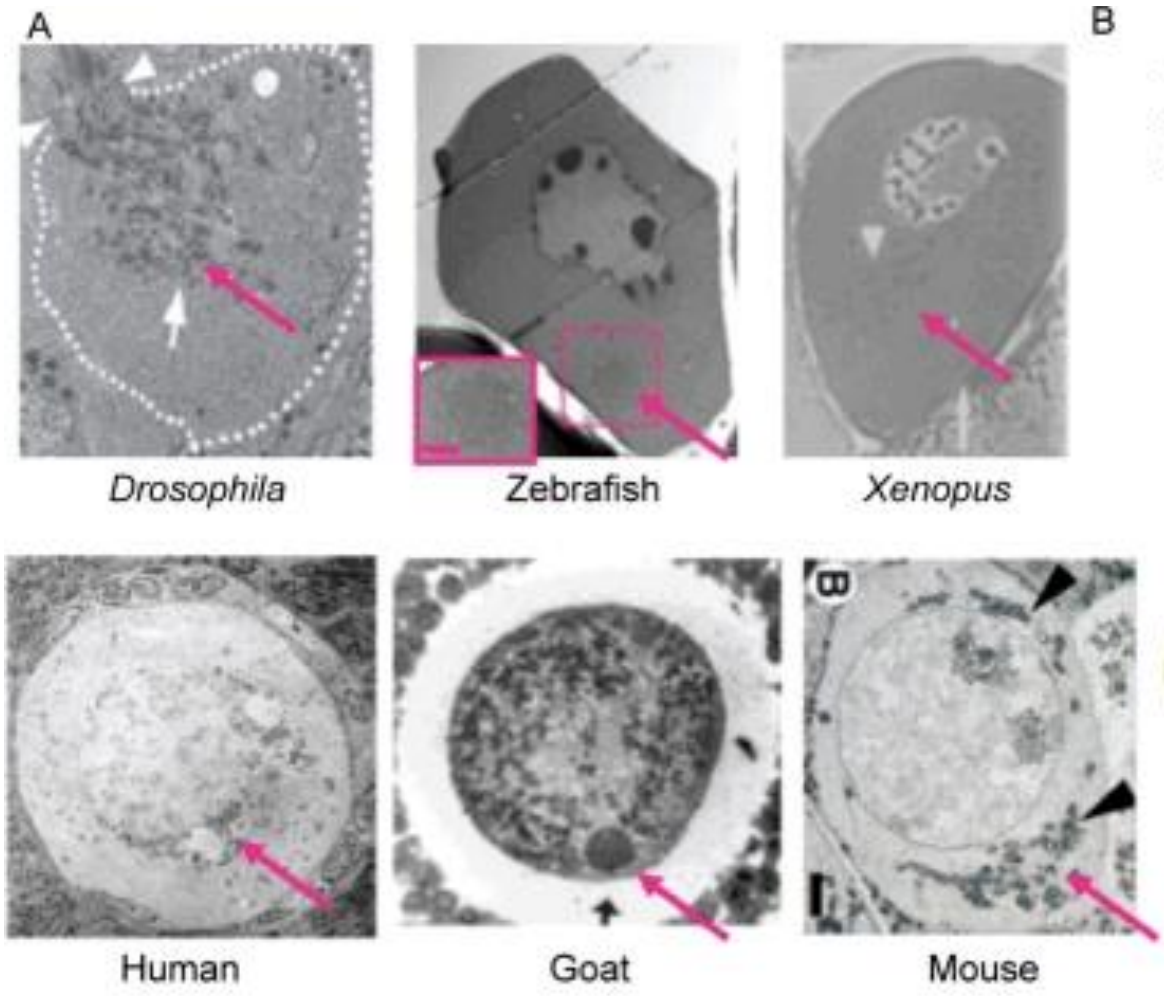
1. **The transmission of mtDNA disease can be effectively stopped** by:
   - **Prenatal Diagnosis**: *de novo* mutations, some recurrent mutations
   - **Preimplantation Genetic Diagnosis**: heteroplasmic mutations
   - Both methods are safe with a small residual risk based on heteroplasmy level of embryo/foetus
2. **Future options are nuclear transfer or genome editing technologies**:
   - **Spindle Transfer**: homoplasmic and heteroplasmic mutations
   - **Pronuclear Transfer**: homoplasmic and heteroplasmic mutations
   - **Polar Body Genome Transfer**: homoplasmic and heteroplasmic mutations
   - **Genome Editing**: homoplasmic and heteroplasmic mutations
   - Residual risk based on carry-over seems low
   - Safety of the methods needs to be demonstrated in clinical trial
   - Ethical issues need to be settled
3. **Therapy development is still fundamental** as mtDNA disease occurs *de novo* in 1 in 10.000 (not prevented by any of the methods above)

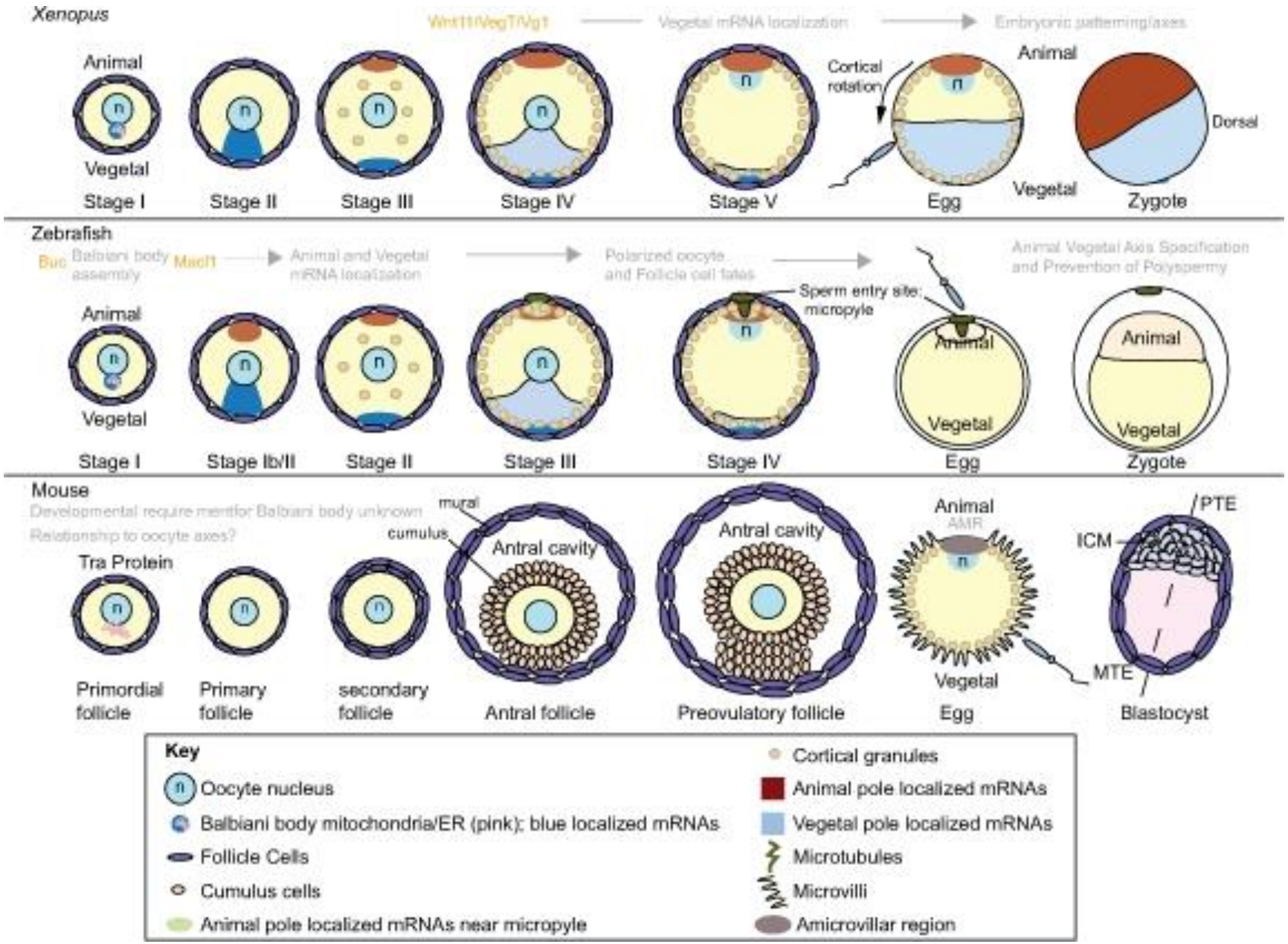
Collaborators and Support

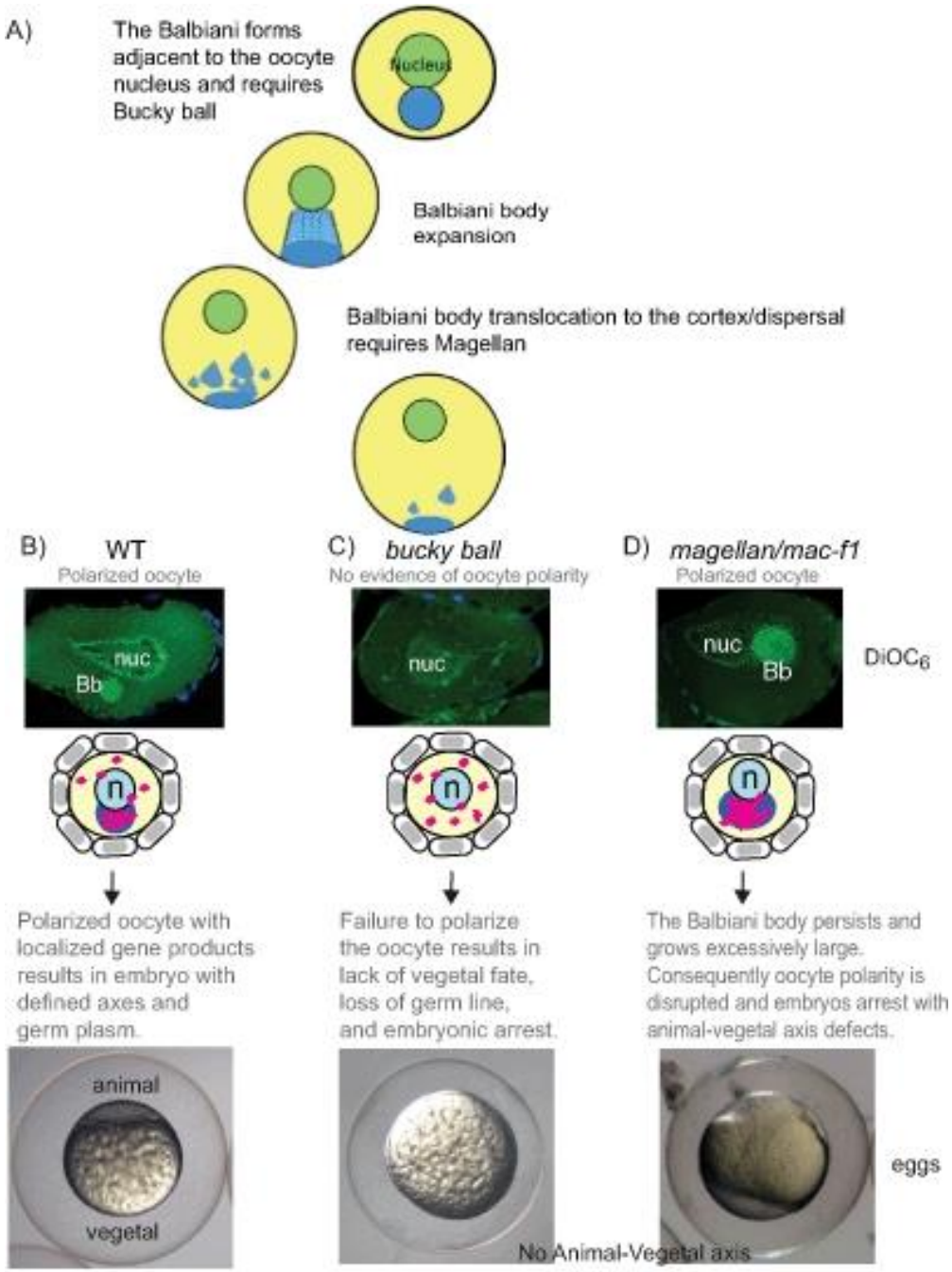
PGD Dream Team Maastricht

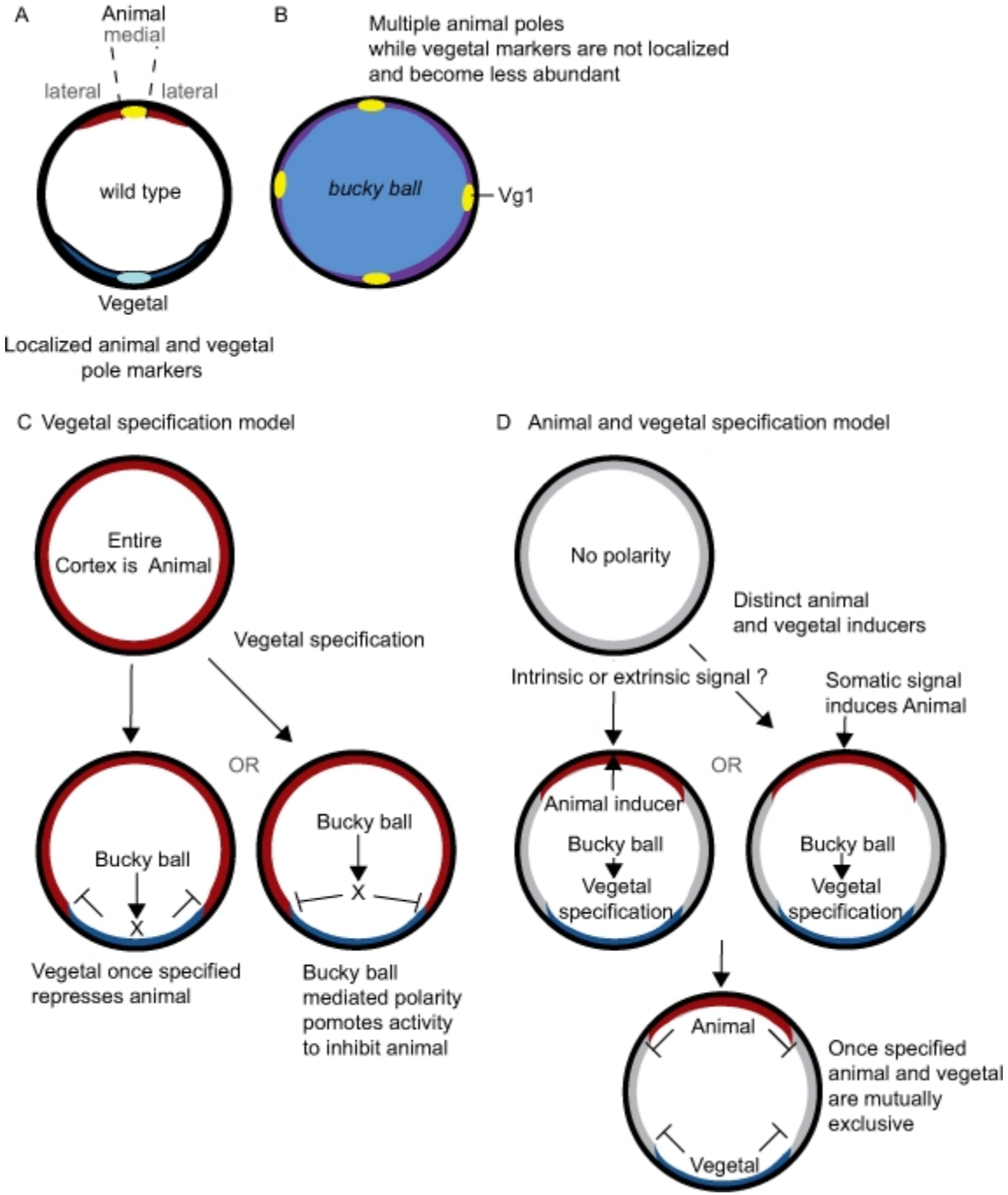
Suzanne Sallevelt, UM
Mike Gerards, UM
Alexandra Hendrickx, UM
Jos Dreesen, UM
Bianca van den Bosch, UM
Bert Smeets, UM
Jörgen Bierau, UM
Christine de Die, azM
Jo Vanoevelen, azM
Auke Otten, UM
TomTheunissen, UM
Rick Kamps, UM
Minh Nguyen, UM
Fons Stassen, azM
Florence van Tienen, UM
An Voets, UM
René de Coo, ErasmusMC
Kees Schoonderwoerd, ErasmusMC
Marc Muller, GIGA, Liège
Mary Winandy, GIGA, Liège
Dave Samuels, Vanderbilt University, Nashville
Referring neurologists, gynecologists and clinical geneticists
Illustrations Maurice van Opdorp †

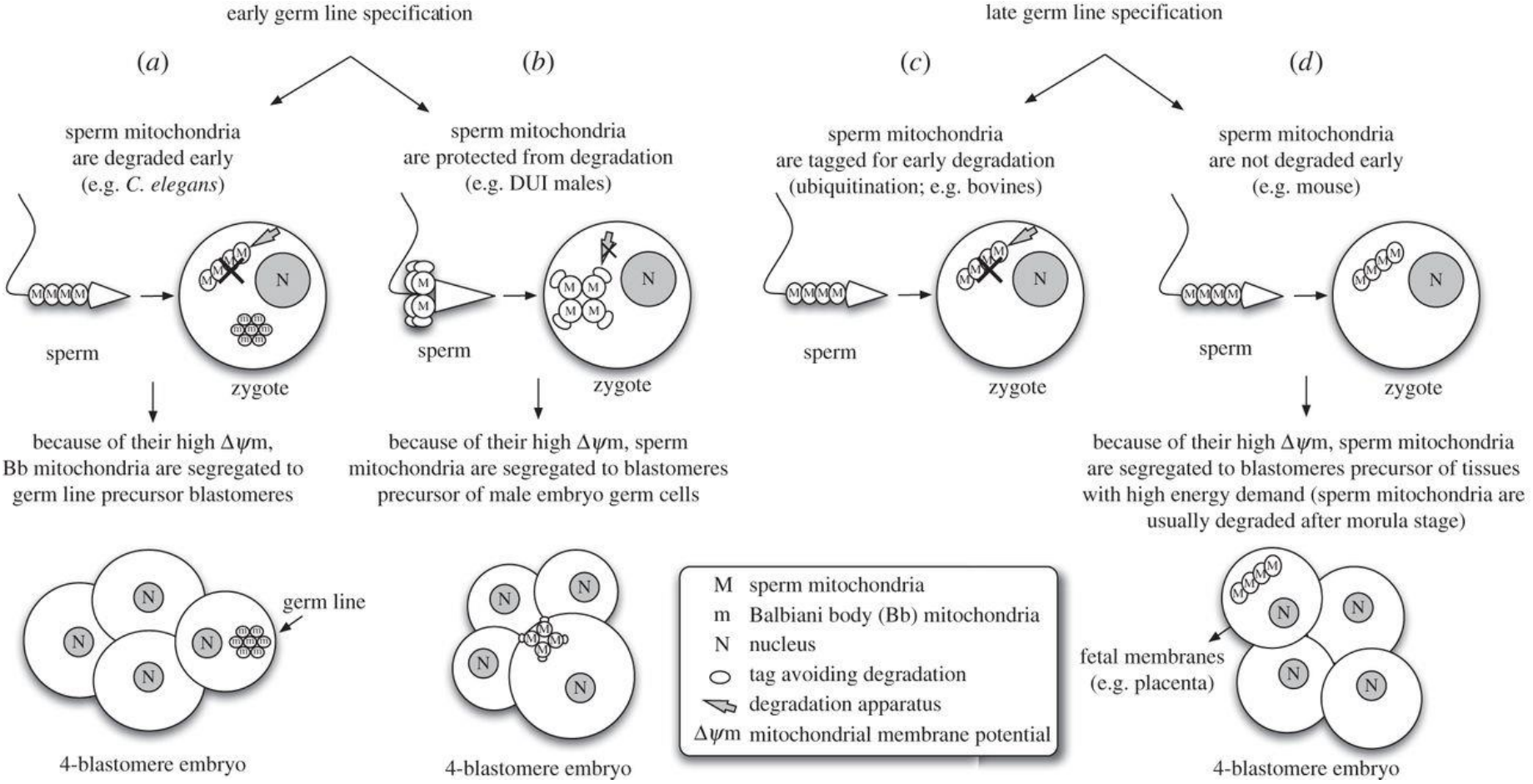

A) Polarity in the germ line cyst
Balbiani body precursor
Germ line cyst
16 cells in Drosophila and Xenopus precise number undetermined in Fish and mouse. Polarity is hypothesized to be imparted by the incomplete divisions of cystoblast cells or other asymmetries in the cyst.
B)
Drosophila
One of two cystoblasts with 4 connections is specified as the oocyte. The others develop as nurse cells
Xenopus/zebrafish
Multiple oocytes
Mouse
Multiple oocytes. Some cystoblast cells are eliminated by cell death
Milton
mitochondria
golgi
nuc
centrioles
C) Cyst encapsulation (Drosophila) or individual follicles (vertebrates)
Stage Ia
?
?
Repolarization signal unknown
Repolarization? Perinuclear recruitment of mitochondria and ER
Repolarization?
D) Balbiani body with associated organelles and mRNAs/proteins
Stage Ib
E) Translocation to the cortex/dispersal
Stage Ib/ II
Defines posterior pole
Defines vegetal pole
Relationship to axis?


A
Drosophila
Zebrafish
Xenopus
Human
Goat
Mouse
B
The Balbiani forms adjacent to the oocyte nucleus
Nucleus
Balbiani body expansion
Balbiani body dispersal


Xenopus
Wnt11/VegT/Vg1
Vegetal mRNA localization
Embryonic patterning/axes
Animal
Vegetal
Stage I
Stage II
Stage III
Stage IV
Stage V
Cortical rotation
Egg
Dorsal
Zygote
Zebrafish
Buc
Balbiani body assembly
Mach
Animal and Vegetal mRNA localization
Polarized oocyte and Follicle cell fates
Animal Vegetal Axis Specification and Prevention of Polyspermy
Sperm entry site: micropyle
Stage Ib/II
Mouse
Developmental require meritor Balbiani body unknown
Relationship to oocyte axes?
Tra Protein
mural
cumulus
Antral cavity
AMR
PTE
ICM
MTE
Primordial follicle
Primary follicle
secondary follicle
Antral follicle
Preovulatory follicle
Blastocyst
Key
Oocyte nucleus
Balbiani body mitochondria/ER (pink); blue localized mRNAs
Follicle Cells
Cumulus cells
Animal pole localized mRNAs near micropyle
Cortical granules
Animal pole localized mRNAs
Vegetal pole localized mRNAs
Microtubules
Microvilli
Amicrovillar region


A)
The Balbiani forms adjacent to the oocyte nucleus and requires Bucky ball
Nucleus
Balbiani body expansion
Balbiani body translocation to the cortex/dispersal requires Magellan
B) WT
Polarized oocyte
nuc
Bb
n
Polarized oocyte with localized gene products results in embryo with defined axes and germ plasm.
C) bucky ball
No evidence of oocyte polarity
nuc
n
Failure to polarize the oocyte results in lack of vegetal fate, loss of germ line, and embryonic arrest.
D) magellan/mac-f1
Polarized oocyte
nuc
Bb
n
The Balbiani body persists and grows excessively large. Consequently oocyte polarity is disrupted and embryos arrest with animal-vegetal axis defects.
DiOC6
animal
vegetal
No Animal-Vegetal axis
eggs


A
Animal
medial
lateral
lateral
wild type
Vegetal
Localized animal and vegetal
pole markers
B
Multiple animal poles
while vegetal markers are not localized
and become less abundant
bucky ball
Vg1
C Vegetal specification model
Entire
Cortex is Animal
Vegetal specification
OR
Bucky ball
X
Vegetal once specified
represses animal
Bucky ball
X
Bucky ball
mediated polarity
pomotes activity
to inhibit animal
D Animal and vegetal specification model
No polarity
Distinct animal
and vegetal inducers
Intrinsic or extrinsic signal ?
Somatic signal
induces Animal
OR
Animal inducer
Bucky ball
Vegetal
specification
Bucky ball
Vegetal
specification
Animal
Vegetal
Once specified
animal and vegetal
are mutually
exclusive


early germ line specification
late germ line specification
(a)
(b)
(c)
(d)
sperm mitochondria are degraded early (e.g. C. elegans)
sperm mitochondria are protected from degradation (e.g. DUI males)
sperm mitochondria are tagged for early degradation (ubiquitination; e.g. bovines)
sperm mitochondria are not degraded early (e.g. mouse)
sperm
zygote
because of their high Δψm, Bb mitochondria are segregated to germ line precursor blastomeres
because of their high Δψm, sperm mitochondria are segregated to blastomeres precursor of male embryo germ cells
because of their high Δψm, sperm mitochondria are segregated to blastomeres precursor of tissues with high energy demand (sperm mitochondria are usually degraded after morula stage)
germ line
4-blastomere embryo
M sperm mitochondria
m Balbiani body (Bb) mitochondria
N nucleus
tag avoiding degradation
degradation apparatus
Δψm mitochondrial membrane potential
fetal membranes (e.g. placenta)

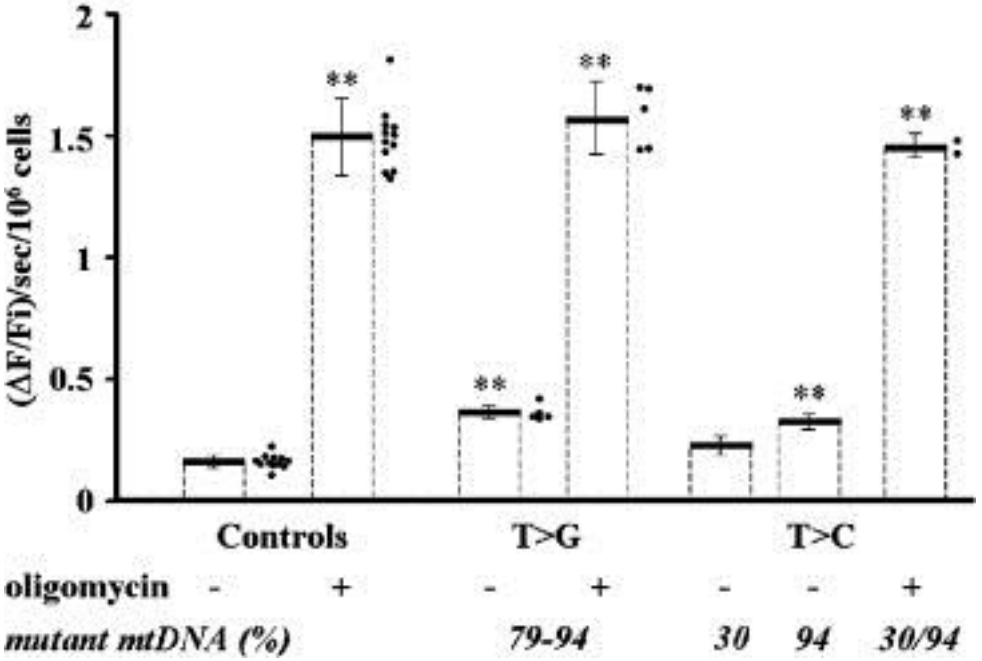


Fig. 3. Mitochondrial membrane potential in digitonin-permeabilized lymphocytes of individuals harbouring the mtDNA 8993T > C/G mutation. (ΔF/Fi/sec) is an expression of the decay rate of RH-123 fluorescence, strictly related with ΔΨm [20]. Mean ± SD of thr...

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Biochemical phenotypes associated with the mitochondrial ATP6 gene mutations at nt8993

Biochimica et Biophysica Acta (BBA) - Bioenergetics, Volume 1767, Issue 7, 2007, 913–919

http://dx.doi.org/10.1016/j.bbabio.2007.05.005

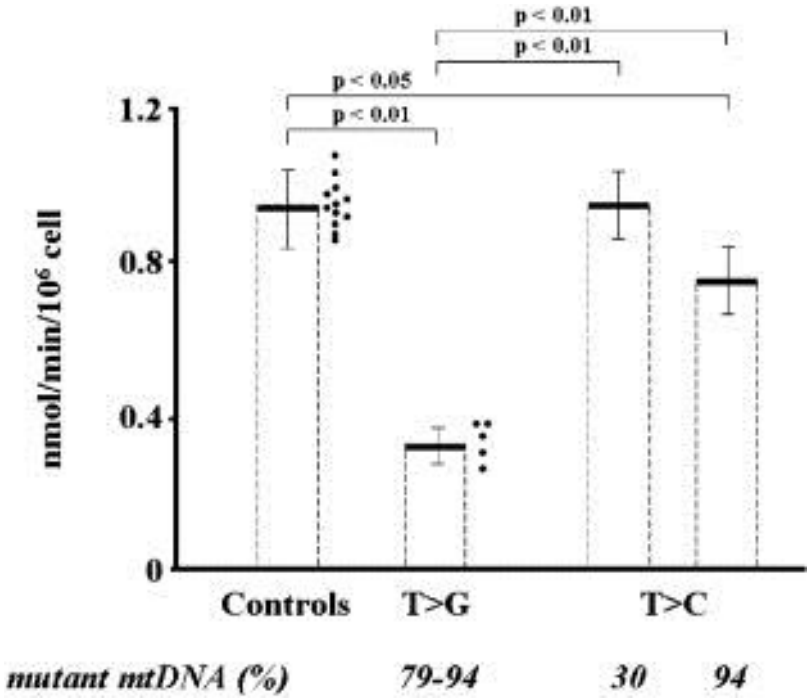

Fig. 2. Rate of ATP synthesis by permeabilized 8993T > C/G lymphocytes energized with succinate. Data reported for individuals harbouring the 8993T > C mutation are presented as mean ± SD of three determinations on each lymphocyte preparation, whereas me...

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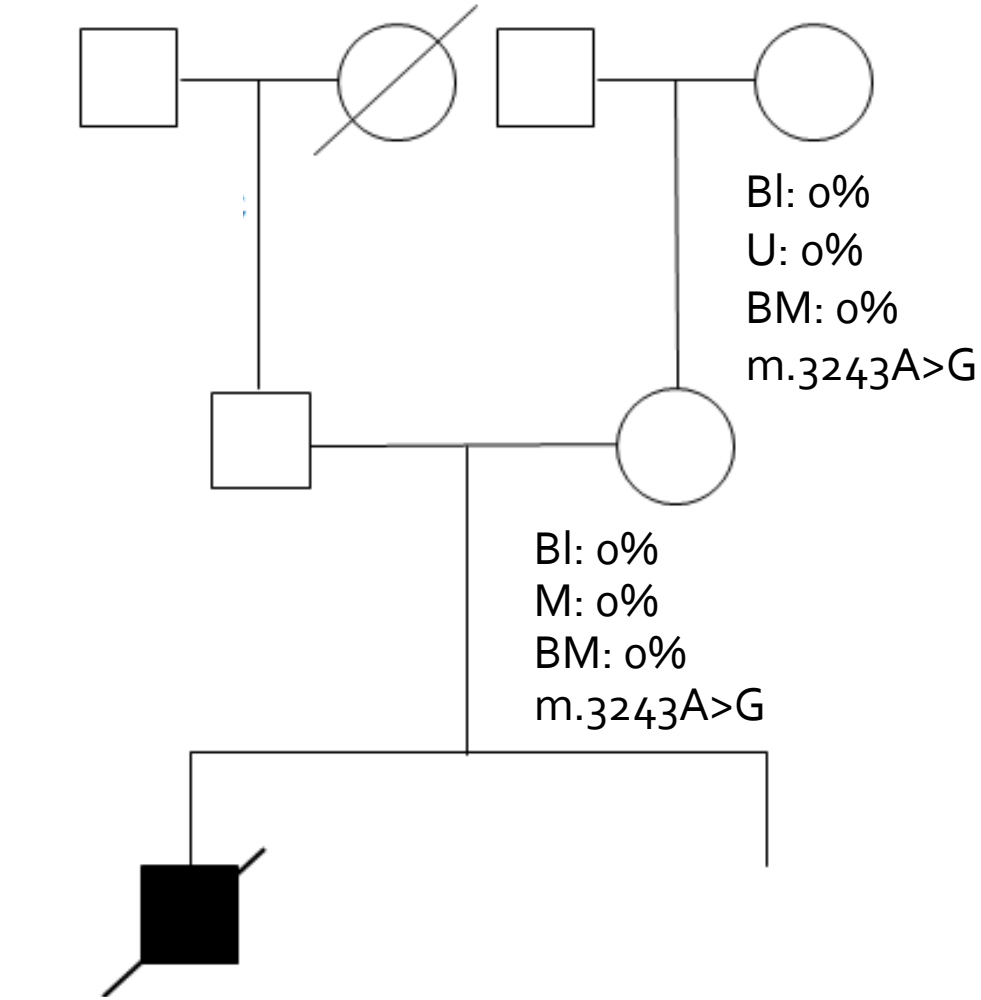
14 Cases of *de novo* mtDNA mutations followed PND and/or PGD in a subsequent pregnancy

Gene	Mutation	Mutation load(s) in tested tissue(s) of index patient	Mutation load(s) in tested tissues of (maternal) relative(s)
ATP6	m.8993T>G	90% (M)	Mother: n (Bl, H, M), pregnancy: n (CVS)
tRNA(Tryp)	m.5556G>A	>90% (M)	Mother: n (Bl, H, U, M), pregnancy: n (amniocentesis)
ATP6	m.8969G>A	95% (Bl, F, M)	Mother: n (Bl, U); pregnancy: n (amniocentesis)
ATP6	m.8993T>G	97% (Bl, M), 96% (F)	Mother: n (Bl, U, H); 2 pregnancies: n (abortion material), n (CVS)
tRNA(Leu(UUR))	m.3243A>G	13% (Bl), 12% (M), 17% (F), 16% (U), 14% (BM)	Mother: n (Bl, M, BM); 11 oocytes/embryos in PGD cycle: n
ND3	m.10158T>C	85% (M)	Mother: n (Bl); pregnancy: n (CVS and amniocentesis)
ATP6	m.8993T>G	90% (Bl)	Mother: n (Bl); 2 pregnancies: n (CVS and amniocentesis)
ND5	m.13513G>A	89% (M), 80% (Bl)	Mother: n (Bl, U), pregnancy: n (amniocentesis) Postpartum analysis of this sister: n (cord blood, Bl)
ATP6	m.9176T>C	99% (in "all tissues examined", unspecified)	Mother: n (Bl, BM, U, 15 oocytes), 40% (2 oocytes together), ≤5% (1 oocyte); pregnancy: n (CVS)
ND3	m.10198C>T	100% (M, heart, liver, brain)	Mother: n (Bl, U, H); pregnancy: n (CVS and amniocentesis)
tRNA(Ser(UCN))	m.7453G>A	100% (M)	Mother: n (Bl); pregnancy: n (CVS)
tRNA(Leu(UUR))	m.3243A>G	?	Mother: n (Bl, U, BM); pregnancy: n (CVS)
ATP6	m.9176T>C	97% (Bl, M)	Mother: n (Bl, U), pregnancy: 98% (CVS), 6 PGD embryos: n; second spontaneous pregnancy: 8% (CVS)
ND6	m.14453G>A	?	Mother: n (tissues unknown); pregnancy: n (CVS)



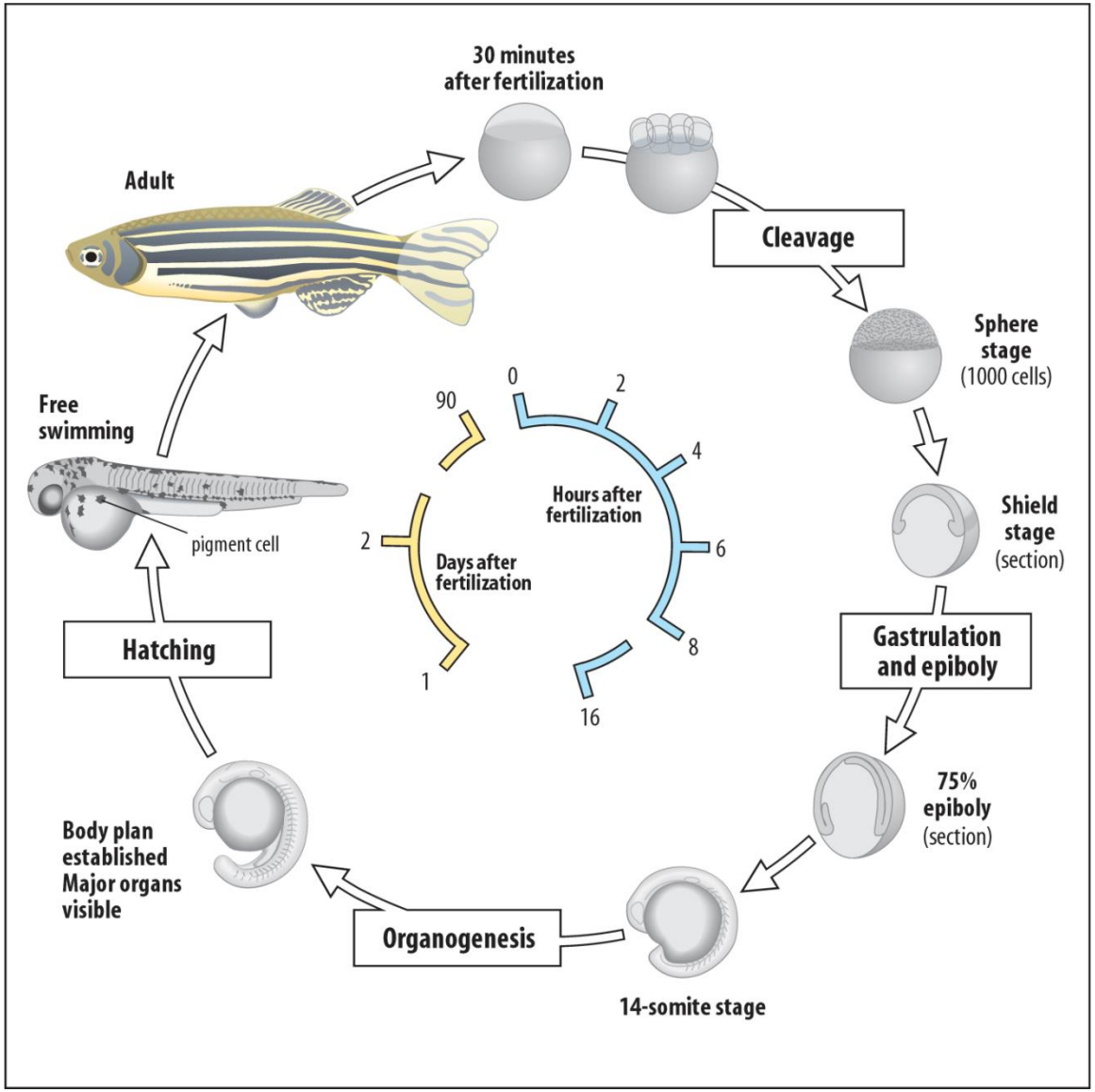
Not all mtDNA mutation are causative!!

- Patient: severe, infantile-onset clinical presentation with feeding problems, hypotonia, psychomotor retardation and epilepsy
- Low percentage m.3243A>G
- Can not explain phenotype
- Additional POLG mutation
- PGD for both POLG mutations and m.3243A>G on 2 separate blastomeres

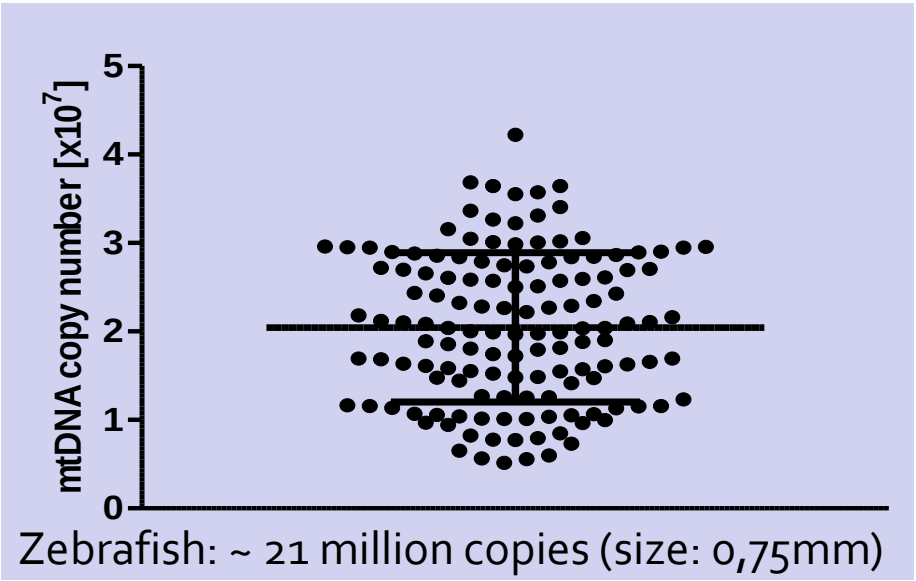
Zebrafish: Model for mtDNA segregation

Zebrafish (*Danio rerio*)

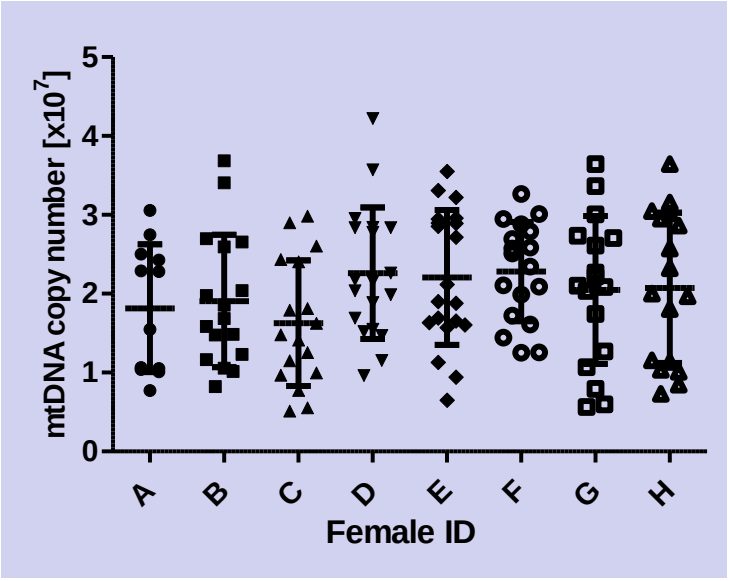
- rely on many of the same organs as humans
- optical clarity during development (*in vivo* assays)
- rapid development
- high number of offspring (cheap in breeding and keeping)
- easy genetic manipulation
- highly suitable for large scale intervention studies



MtDNA copy number in zebrafish oocytes



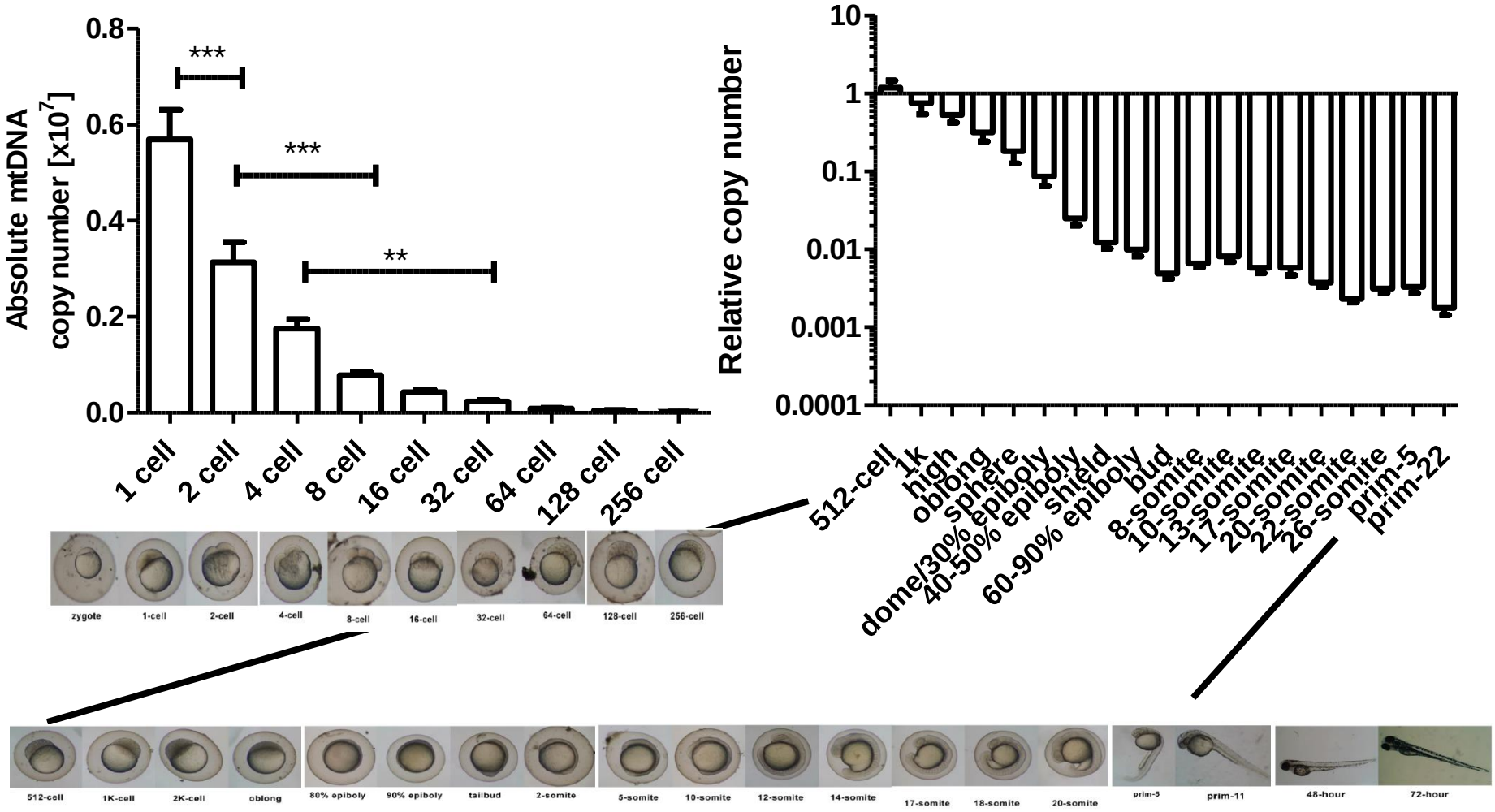
Zebrafish: ~ 21 million copies (size: 0,75mm)



Salmon: ~ 3 billion copies (size: 4.5mm)
Bovine, sheep, pigs: 300,000 – 1 million copies (size:<0.15m)
Human, mice, rats: 100.000-300.000 copies

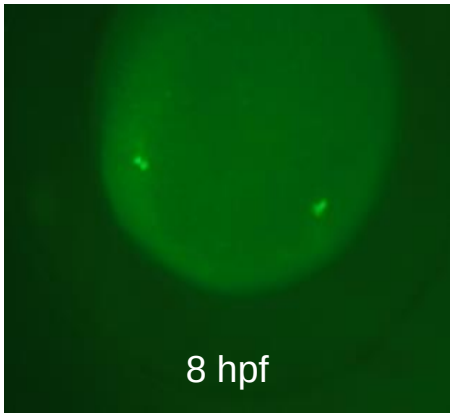
- Copy number might relate to pattern/speed implantation (absent, centric, eccentric/interstitial)
- Copy number correlates with size oocyte (mtDNA copy number per unit of volume seems equal across species)
- Selection against low mtDNA copy number zebrafish (<5 million copies)
- Low variation mean mtDNA copy among individual fish
- High intra-individual variation mtDNA copy number across oocytes individual fish

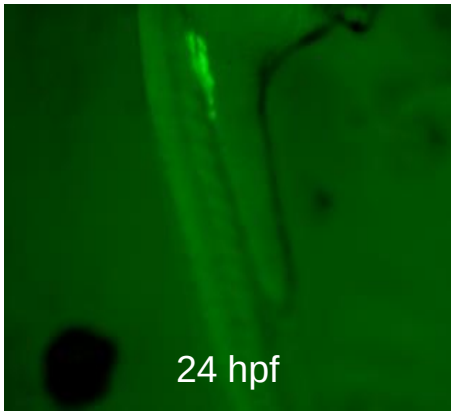
MtDNA bottleneck in zebrafish embryos

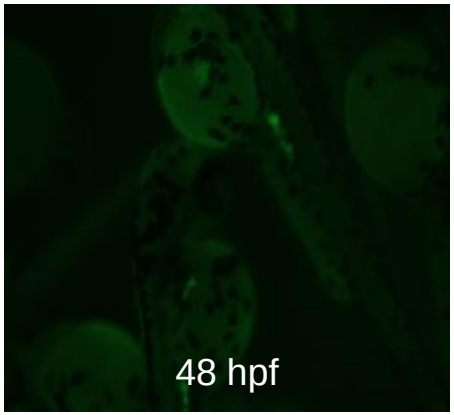


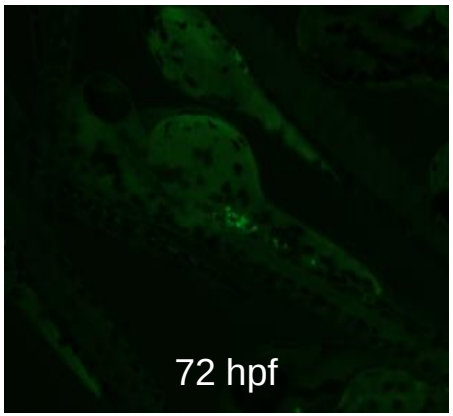
No mtDNA replication until early somitogenesis

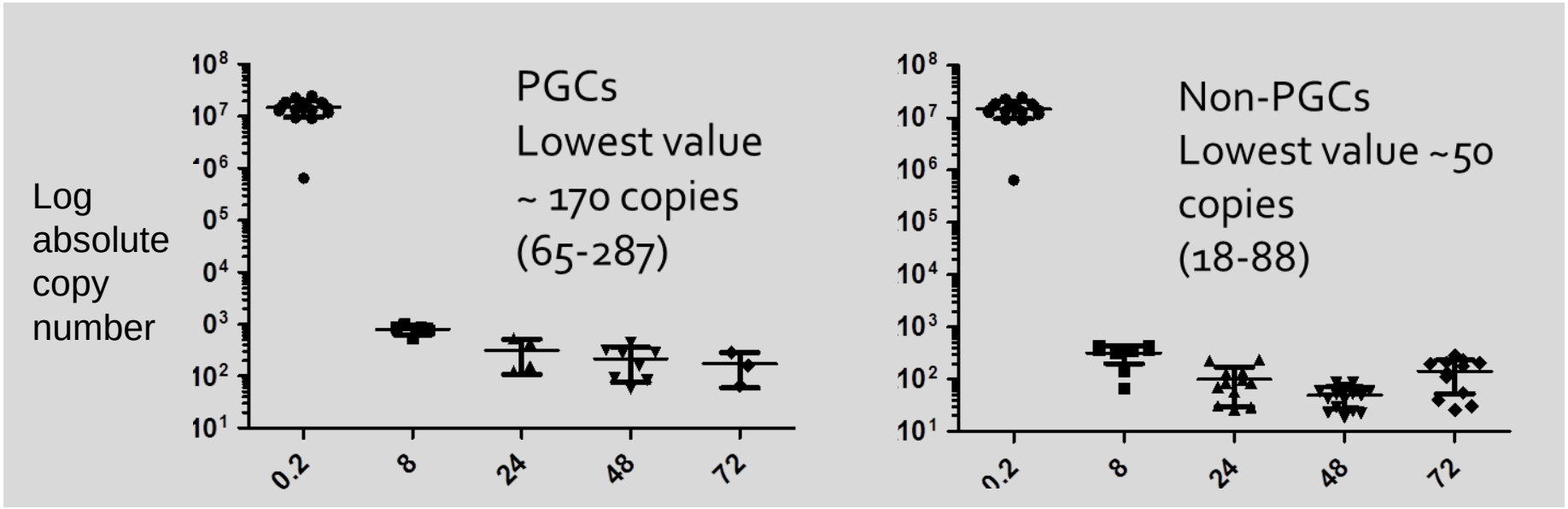
Isolation of PGCs/non-PGCs from zebrafish embryos with FACS-sorting (*nanos3*)





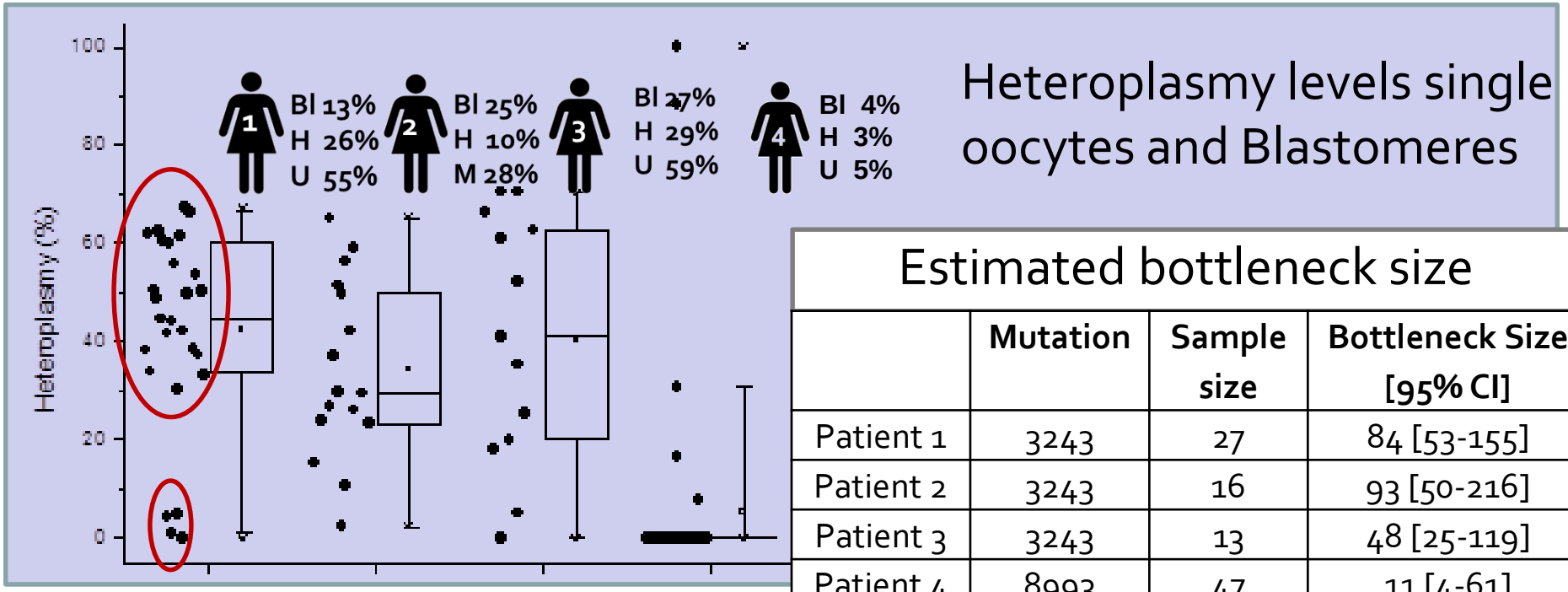






High variation in all stages of development

Heteroplasmy level and bottleneck size carriers m.3243A>G and m.8993T>G



Heteroplasmy levels single oocytes and Blastomeres

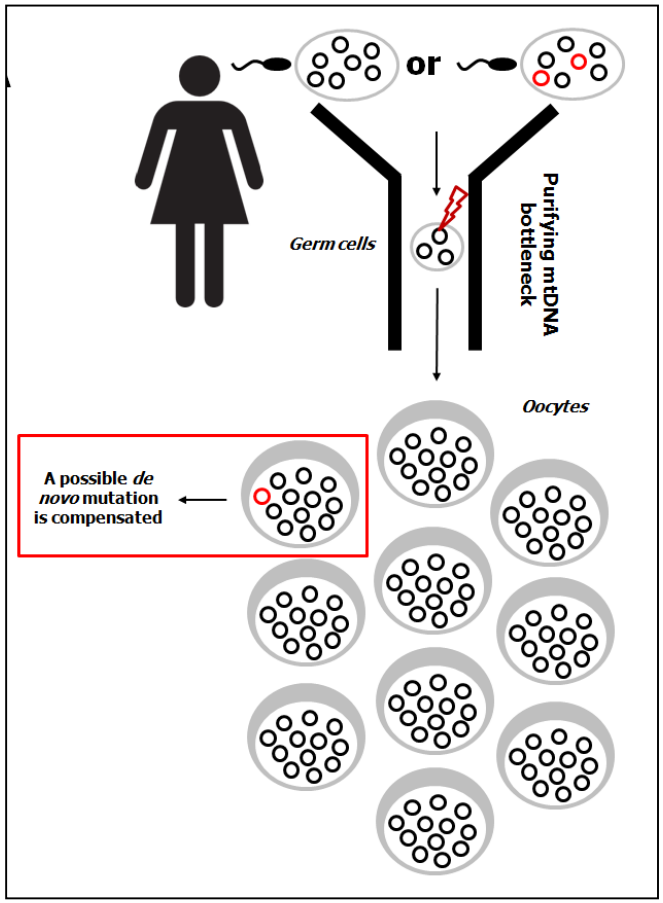
Estimated bottleneck size

	Mutation	Sample size	Bottleneck Size [95% CI]
Patient 1	3243	27	84 [53-155]
Patient 2	3243	16	93 [50-216]
Patient 3	3243	13	48 [25-119]
Patient 4	8993	47	11 [4-61]

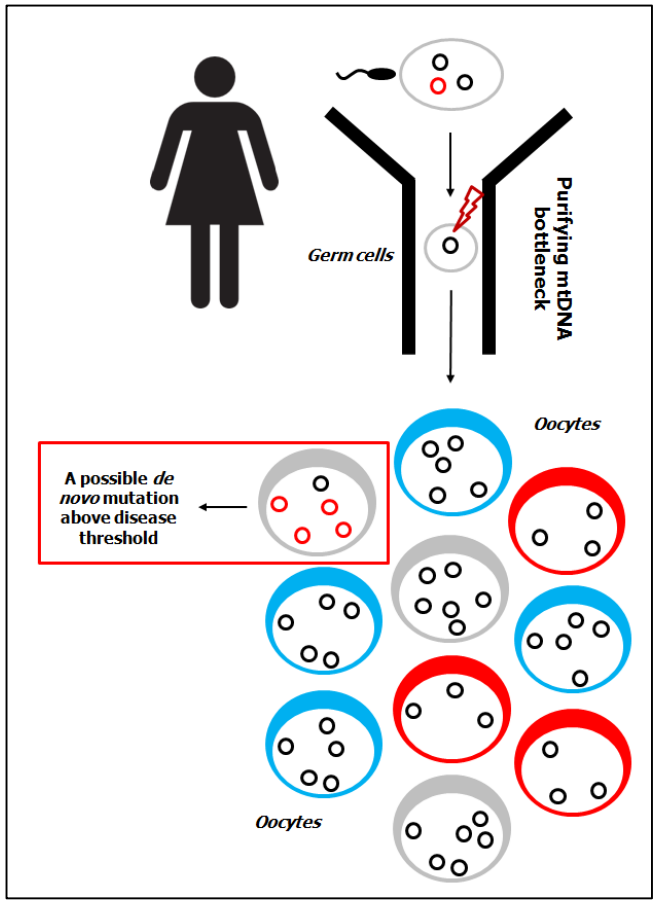
Reported bottleneck sizes (mostly indirect calculations):

- ~173 copies humans (82 m.3243A>G oocytes, Brown et al. 2001)
- 30-35 copies humans (Rebolledo-Jaramillo, et al. 2014)
- 65-287 copies PGC, 18-88 copies non-PGC zebrafish
- 65-163 copies cows (Rand et al. 1986)
- 80-88 salmons (Wolff et al. 2011)
- 87-395 crickets (Rand et al. 1986)

MtDNA disease:
Causes, segregation, reproductive options (1)

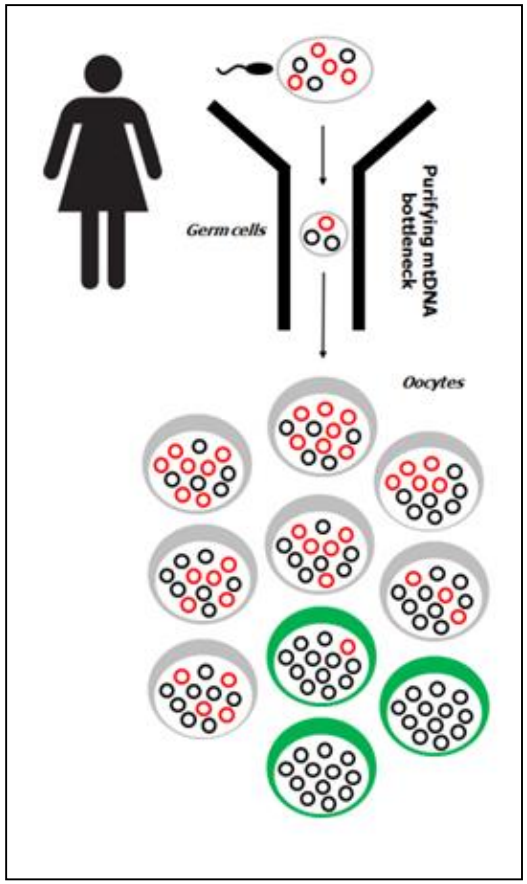


De novo mutation compensated
Not causative

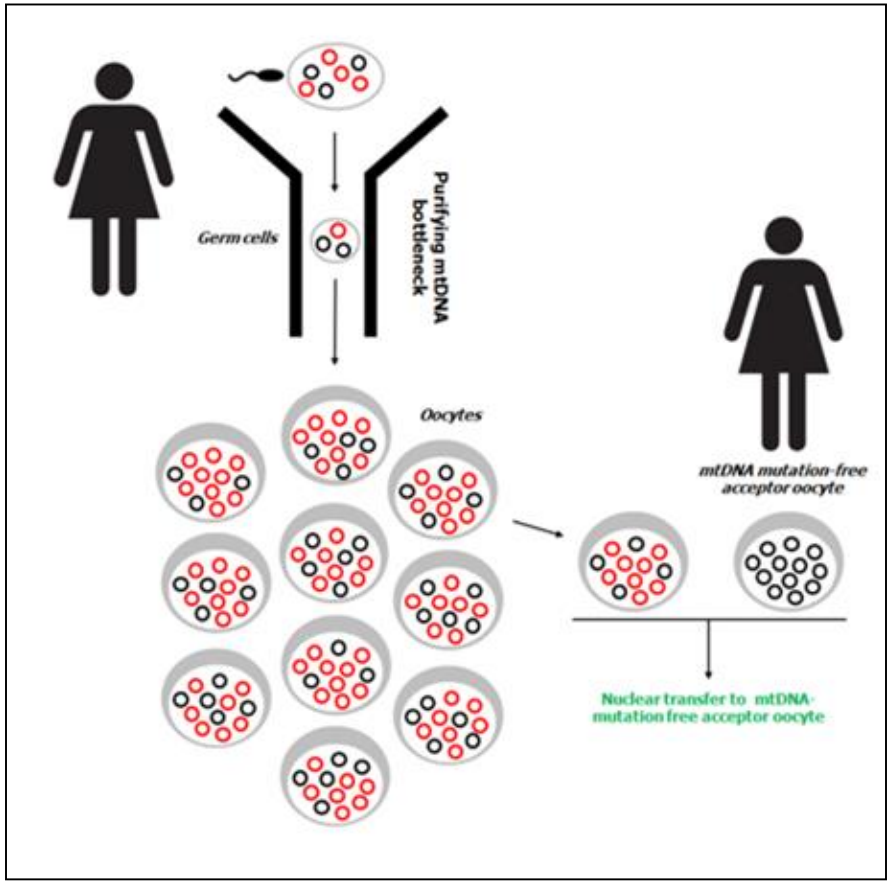


De novo mutation above threshold
Causative, low recurrence risk
PND in subsequent pregnancy

MtDNA disease: Causes, segregation, reproductive options (2)



Inherited heteroplasmic mtDNA mutations
Oocytes with mutation load below threshold
PGD can be offered



Inherited hetero/homoplasmic mutations
No oocytes with mutation load below threshold
In future nuclear transfer offered (UK)

Conclusions

1. **The transmission of mtDNA disease can be effectively stopped** by:
   - Prenatal Diagnosis: *de novo* mutations, some recurrent mutations
   - Preimplantation Genetic Diagnosis: heteroplasmic mutations
   - Both methods are safe with a small residual risk based on heteroplasmy level of embryo/foetus
2. **Future options are nuclear transfer technologies**
3. **Therapy development is still fundamental** as mtDNA disease occurs *de novo* in 1 in 10.000 (not prevented by any of the methods above)
4. **Zebrafish models shed further light on:**
   - Mechanism of the bottleneck (evolutionary highly conserved)
   - Relation mtDNA copy number and size oocyte/implementation pattern and speed
   - Difference between PGCs and non-PGCs
   - Intra-individual variation and de novo mutation risk
5. **Current studies**
   - Sequencing mtDNA oocytes zebrafish (*de novo* mutations)
   - Induced heteroplasmy mtDNA in zebrafish oocytes (bottleneck)
   - Gene expression analysis (block mtDNA replication -TFAM knockdown)