

**Mitochondrial DNA polymerase gamma is essential for mammalian embryogenesis**

Nicole Hance and Mats I. Ekstrand and Aleksandra Trifunovic\*

Department of Medical Nutrition and Department of Biosciences at Novum, Karolinska Institutet, Stockholm, Sweden

\*Corresponding author:

E-mail: [Aleksandra.Trifunovic@mednut.ki.se](mailto:Aleksandra.Trifunovic@mednut.ki.se)

Tel : +46 8 5858 3726

Fax : +46 8 779 5383

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## Abstract

Mitochondrial DNA polymerase gamma (Polg) is a heterodimeric enzyme containing a Pol I-like catalytic core (PolgA) and an accessory subunit (PolgB). Mutations in *POLGA*, affecting the stability of mtDNA, have been identified in several human pathologies such as progressive external ophthalmoplegia (PEO) and Alpers' syndrome. Extensive literature shows mitochondrial toxicity effects nucleoside analogue reverse transcriptase inhibitors (NRTIs) used in the treatment of HIV and chronic hepatitis B as a consequence of an inhibitory effect on Polg. We have previously shown that mice with an error-prone version of PolgA accumulate higher levels of somatic mtDNA mutations resulting in a premature aging phenotype. In the present paper we demonstrate PolgA deficiency in mouse embryos causes an early developmental arrest between embryonic day 7.5-8.5 associated with severe mtDNA depletion. Heterozygous knockout mice have half the wild-type levels of *PolgA* transcripts and a slight reduction in mtDNA levels, but develop normally. Surprisingly, amounts of *PolgA* transcripts in heterozygous knockout mice are increased in response to artificially elevated mtDNA copy number, revealing a possible regulatory link between mtDNA maintenance and *PolgA* expression. Our results show that Polg indeed is the only DNA polymerase capable of maintaining mtDNA in mammalian mitochondria. Furthermore, presence of Polg is absolutely essential for the organogenesis during mammalian embryonic development.

## Introduction

Fifteen distinct cellular DNA polymerases have been identified in mammalian cells but only four of these are devoted to DNA replication while the rest are devoted to DNA repair and specialized DNA synthetic processes that contribute substantially to the maintenance of genetic integrity (1). Most of these enzymes are involved in nuclear DNA repair and replication whilst DNA polymerase  $\gamma$  (Polg) remains the only DNA polymerase found in mitochondria (for recent review see (2)). As such, Polg is proposed to be uniquely responsible for all DNA synthetic reactions including both replication and repair of mitochondrial DNA (mtDNA) (2).

Mitochondria are the sole organelles in animal cells that contain their own DNA. Individual cells have between 1,000-10,000 copies of the mitochondrial genome (mtDNA) with the more energy consuming tissues, such as heart, having proportionally higher amounts. Replication of mammalian mitochondrial DNA (mtDNA) is under relaxed control as there is no known mechanism to ensure that each molecule is replicated only once per cell cycle. Polg is not believed to directly regulate mtDNA levels, as overexpression of *Polg* in cell lines does not result in a corresponding increase in mtDNA copy number (3). The enzyme contains a Pol I-like catalytic core (PolgA), similar to the bacteriophage T7 DNA polymerase, in a heterodimeric complex with an accessory subunit (PolgB) (4-6). This accessory subunit substantially increases both the catalytic activity and the processivity of the enzyme (6). Interaction of the catalytic and accessory subunits is through multiple contact sites and it has been proposed that these contacts may enhance DNA binding and also increase the nucleotide binding of the holoenzyme (7, 8). Furthermore, Polg exhibits a high fidelity in nucleotide selection and incorporation as well as high processivity during replication (9, 10). High fidelity of the Polg is partly due to the 3'-5' exonuclease activity of the catalytic subunit. It has

been shown that the exonuclease activity of Polg contributes several hundred-fold to error avoidance *in vivo* (8). We recently described a knock-in mouse model that expresses an error-prone version of Polg (mtDNA mutator mice) (11). This mouse strain exhibits a dramatic decrease in exonuclease activity whilst Polg polymerase activity is preserved. Abolished exonuclease activity leads to a three- to five-fold increase in somatic mtDNA mutations that, in turn, cause a progressive respiratory chain deficiency and premature aging phenotypes (11). These results offer direct genetic evidence that the collective effect of a variety of somatic mtDNA mutations can cause aging.

Polg is unique among the cellular replicative DNA polymerases as it is highly sensitive to inhibition by nucleoside analogue reverse transcriptase inhibitors (NRTIs) used in treatment of HIV and chronic hepatitis B and C infections (12, 13). Highly active antiviral therapy (HAART) that includes NRTIs has changed AIDS from a lethal illness to a chronic disease. Unfortunately, it was quickly seen that significant mitochondrial toxicity due to HAART was an important clinical entity, and has since been shown that it is primarily a consequence of Polg inhibition. Pyrimidine NRTIs, such as AZT, fialuridine (FIAU) and others, cause cardiac dysfunction, hepatic failure, skeletal myopathy, lactic acidosis with defective mtDNA replication, mtDNA depletion and altered mitochondrial ultrastructure (for recent review see (13)).

Mutations in *POLGA*, the gene encoding the catalytic subunit of human mtDNA polymerase has been associated with a number of mitochondrial disorders that affect the stability of mtDNA (14, 15). These mutations can lead to both mtDNA deletion and mtDNA depletion syndromes. Most common and best known are the autosomal dominant (ad) or autosomal recessive (ar) forms of familial progressive external ophthalmoplegia (PEO) (15-17). Additional clinical presentations like autosomal

recessive sensory atactic neuropathy with dysarthria and ophthalmoplegia (SANDO) have been associated with *POLGA* mutations (18). Recently, different *POLGA* mutations causing depletion of liver mtDNA have been reported in Alpers' hepatophatic poliodystrophy (19, 20). Furthermore, dominant *POLGA* mutations have shown to cause a severe multisystem disorder including Parkinsonism and premature menopause, which are not typical of mitochondrial disease (21). Finally, specific polymorphisms in the *POLGA* gene have been associated with male infertility (22).

The involvement of Polg in a number of different human pathologies, its complex role in mtDNA maintenance and the still ongoing debate about the number of DNA polymerases in mitochondria, makes it a very interesting target for further analysis.

In the present study we investigated the role of Polg in mammalian development by creating PolgA deficient mice. We generated a transgenic mouse model lacking PolgA through homologous recombination in embryonic stem (ES) cells. Loss of Polg in mice leads to developmental arrest at around embryonic day (E) 7.5 associated with severe mtDNA depletion. Mice heterozygous for the *PolgA* null allele have only half the wild-type levels of *PolgA* transcripts. However, these heterozygous *PolgA* knockouts appear completely normal and have close to wild-type mtDNA copy number. Interestingly, mice with artificially elevated mtDNA levels show a corresponding up-regulation of *PolgA* transcripts, suggesting a link between mtDNA copy number and *PolgA* expression.

## Results

### Generation of *PolgA* knockout mice

The gene encoding *PolgA* was disrupted in the mouse by utilizing the *cre-loxP* recombination system. *PolgA<sup>loxP/loxP</sup>* mice were mated with animals containing a  $\beta$ -actin *cre*-transgene that ubiquitously expresses *cre*-recombinase in the pre-implantation embryo (figure 1a). Resulting *PolgA<sup>+/-</sup>* offspring were identified by Southern blot analysis detecting the *cre*-mediated excision of exon III from one allele of these mice (figure 1b). Deletion of *PolgA* exon III results in a frameshift mutation leading to a stop codon early into exon IV. The severely truncated mRNA lacks virtually all the active domains of *PolgA*, and therefore we do not expect any active protein to be derived from this allele.

### *PolgA* knockout is embryonic lethal

In order to completely knockout *PolgA* in the mouse, *PolgA<sup>+/-</sup>* mice were intercrossed. The resulting genotype distribution from this cross can be seen in table 2. No homozygous knockout (*PolgA<sup>-/-</sup>*) pups were born, PCR genotyping (figure 1c) was therefore carried out on embryos at different stages of development. Results showed that *PolgA<sup>-/-</sup>* embryos died between embryonic days (E) 7.5 - 8.5. Wild-type and *PolgA<sup>+/-</sup>* embryos (figure 2a and data not shown) at E8.5 were morphologically indistinguishable from each other, while *PolgA<sup>-/-</sup>* embryos (figure 2b) were generally smaller and much less developed. Enzyme histochemical staining showed a severe respiratory chain dysfunction. There was no cytochrome c oxidase activity (catalytic subunit encoded by mtDNA), whereas normal succinate dehydrogenase activity was found (all subunits encoded by the nuclear genes) (figure 2c). Southern blots were

performed on total DNA isolated from embryos to confirm that the respiratory chain dysfunction was the result of mtDNA depletion due to a loss of PolgA. Results showed there was no difference in mtDNA levels between wild-type and *PolgA*<sup>+/-</sup> embryos, whilst the *PolgA*<sup>-/-</sup> embryos contained barely detectable levels of mtDNA (figure 2d).

### **Normal mtDNA levels in *PolgA*<sup>+/-</sup> mice**

*PolgA*<sup>+/-</sup> mice were born at the expected Mendelian ratio, developed normally, and displayed no obvious phenotype even at 18 months of age. TaqMan PCR was performed on cDNA generated from kidney total RNA to confirm that expression from the remaining *PolgA* allele was not up-regulated in *PolgA*<sup>+/-</sup> mice. Relative levels of *PolgA* mRNA were reduced by approximately one half in *PolgA*<sup>+/-</sup> mice compared with age-matched controls (figure 3a). These results are in accordance with previous reports that *PolgA* is constitutively expressed at similar levels in different tissues (3). We failed to quantify PolgA protein levels in both total cell and purified mitochondrial extracts by Western blot analysis (data not shown). Available antibodies were raised against human POLGA and although they show cross reaction with the mouse protein they were not sensitive enough for quantification by Western blot.

Since a total loss of PolgA resulted in an almost complete depletion of mtDNA in embryos we looked at mtDNA levels in adult *PolgA*<sup>+/-</sup> mice. Total DNA isolated from heart, muscle and liver was analysed by Southern blot and the mtDNA levels were found to be comparable in *PolgA*<sup>+/-</sup> and wild-type mice (figure 3b). As Southern blots showed a tendency towards reduced mtDNA levels in *PolgA*<sup>+/-</sup> mice, the analysis was repeated on the same DNA samples using real-time PCR. Again, we observed the same trend of slightly lower mtDNA levels, a finding that was now significant in heart tissue (figure 3c).

### **TFAM overexpression increases mtDNA levels in *PolgA*<sup>+/-</sup> mice**

Recently, it was demonstrated that overexpression of the human TFAM protein in mice leads to an increase of mtDNA copy number of up to 60% (23). Since mtDNA levels were normal in *PolgA*<sup>+/-</sup> mice we decided to cross them with TFAM overexpressors (*TFAM*<sup>OE</sup>) and study if *PolgA* expression was sufficient to allow for a similar increase in mtDNA. Total DNA was isolated from mice of three genotypes obtained from this cross (*PolgA*<sup>+/+</sup>; *PolgA*<sup>+/+</sup>,*TFAM*<sup>OE</sup> and *PolgA*<sup>+/-</sup>,*TFAM*<sup>OE</sup>) and analysed by both Southern blot and real-time PCR to determine levels of mtDNA. Both methods confirmed that overexpression of human *TFAM* indeed increases mtDNA levels (~70% above wild-type) and also showed that *PolgA*<sup>+/-</sup>,*TFAM*<sup>OE</sup> mice were just as capable of raising mtDNA levels as *PolgA*<sup>+/+</sup>,*TFAM*<sup>OE</sup> mice (figure 4a, b). *PolgA* mRNA levels were determined by TaqMan PCR analysis of cDNA generated from total RNA isolated from mice of the three genotypes mentioned above. Surprisingly, we detected an increase in *PolgA* transcripts in both *PolgA*<sup>+/+</sup>,*TFAM*<sup>OE</sup> and *PolgA*<sup>+/-</sup>,*TFAM*<sup>OE</sup> mice (figure 4c). The values observed in *PolgA*<sup>+/-</sup>,*TFAM*<sup>OE</sup> animals are equivalent to *PolgA* steady-state transcript levels in wild-type animals and are almost double those seen in the *PolgA*<sup>+/-</sup> animals (figure 4c).



## Discussion

We have shown that PolgA is absolutely essential for mammalian embryonic development and mtDNA maintenance. Homozygous disruption of the mouse *PolgA* gene leads to embryonic death at the time of late gastrulation and before early organogenesis. Loss of PolgA coincides with a dramatic decrease in mtDNA levels, with *PolgA* null embryos having almost no detectable mtDNA at E8.5. *PolgA* null embryos at E8.5 are much smaller than wild-type embryos and have a severe respiratory chain deficiency, as shown by an absence of cytochrome *c* oxidase activity, whose catalytic subunits are encoded by mtDNA.

Furthermore, the data presented directly shows the necessity of mtDNA replication during early mouse embryogenesis. Previous mouse models have shown that mtDNA maintenance is critical during embryogenesis, however, here we have described the first mouse model where only mtDNA replication has been disrupted (24, 25). The importance of replication and not only mtDNA transcription and maintenance in early mammalian embryogenesis was somewhat underestimated as it is believed that during embryogenesis mtDNA transcription starts as early as the two-cell stage, whilst mtDNA replication could be dispensable until organogenesis occurs (26-28). This idea was further supported by the finding that in *Xenopus laevis* embryos, mtDNA replication begins at the heartbeat stage emphasizing the need for mature mitochondria at the time the circulatory system become available (29, 30). Furthermore, mice lacking mitochondrial transcription factor A (*Tfam*<sup>-/-</sup>) or ribonuclease H (*Rnaseh*<sup>-/-</sup>) die around E8.5 showing signs of organogenesis, although impaired and unfinished (24, 25). Common problems in *Tfam*<sup>-/-</sup> and *Rnaseh*<sup>-/-</sup> embryos include cardiovascular malformations, and neuronal tube defects (24, 25). We here show that knockout of

*PolgA* has a lethal phenotype comparable to those previous mouse models. Furthermore, *PolgA*<sup>-/-</sup> embryos seem to be more severely affected, as they are stalled at the egg-cylinder stage and there is a lack of any signs of organogenesis occurring. Therefore, our results show that early mouse organogenesis critically depends on active mtDNA replication and not just maintenance of mtDNA. Although, we cannot rule out that the more severe phenotype may partly be a consequence of the different genetic backgrounds between these mouse models or perhaps the higher maternal contribution of both Tfam and Rnase H in comparison to Polg, the difference in developmental arrest between embryos of these three knockout models makes it unlikely.

Our results show that partial PolgA deficiency in heterozygous animals (*PolgA*<sup>+/-</sup>) is not compensated for by an increased transcription of the *PolgA* gene. This fits with the idea that *PolgA* expression is not tightly regulated as it is constitutively expressed in tissues even in the absence of mtDNA (31). Steady-state levels of *PolgA* transcripts have previously been shown not to correlate with mtDNA content in different tissues and indeed, overexpression of the *PolgA* gene did not change mtDNA content in cell lines (3).

Our findings show that *PolgA*<sup>+/-</sup> animals have a slight reduction in mtDNA levels. A study of animals heterozygous for the Tfam null mutation has demonstrated that a 35% decrease in mtDNA levels does not have any effect on mitochondrial function (24). Hence, it is no surprise that the 15% reduction in mtDNA levels in *PolgA*<sup>+/-</sup> mice does not cause any phenotype. Increased transcription and mitochondrial RNA stability are believed to have the potential to compensate for decreased mtDNA copy number and preserve the overall mitochondrial protein demands (24, 32).

In this study we also show that increased mtDNA copy number leads to increased expression of *PolgA*. We chose to increase mtDNA copy number in *PolgA*<sup>+/-</sup> mice by

overexpressing the TFAM protein. Overexpression of TFAM leads to an overall increase in mtDNA copy number by ~70% in both wild-type and *PolgA*<sup>+/-</sup> animals. Our results demonstrate that up-regulation of mtDNA levels leads to an increase in steady-state levels of *PolgA* transcripts. Although it is generally believed that *PolgA* expression is not regulated by the rate of mitochondrial biogenesis, there are some known cases where an increase in PolgA is required. Firstly, levels of *PolgA* transcripts coincide with cellular mtDNA copy number changes in *Xenopus laevis* during vitellogenesis (4). Secondly, a simultaneous increase in both Polg and cytochrome *c* activity was observed immediately before an increase in ATP levels, as a response to increased energy demands, in the early S phase of the cell cycle (33). Finally, a consistent and direct relationship between mtDNA copy number, mtRNA content, respiratory chain capacity and Polg activity was observed in murine, bovine and rabbit hearts (34).

Our results suggest that there is an active feedback mechanism that increases *PolgA* expression as a result of an overall increase in TFAM and mtDNA levels. This further supports the idea that TFAM is a major regulatory factor in determining mtDNA levels within mitochondria as it has been shown that mtDNA copy number mirrors the amounts of TFAM within cells (23, 24). Others have failed to detect changes in *PolgA* expression levels coinciding with increasing amounts of mtDNA, following chronic motor nerve stimulation in rabbit muscle. However, the authors found that the 3.5-fold increase in mtDNA levels was paralleled by a comparable increase in Polg activity (3, 34). One possible explanation for the feedback mechanism could be activation of *PolgA* expression via NRF1, a transcription factor driving *Tfam* expression as well as a number of other nuclear-encoded mitochondrial genes (35). It has been shown that the *PolgA* promoter region has a DNA element potentially recognized by NRF1 (36). The

importance of this NRF1-like element in *PolgA* transcription regulation is further supported by the finding that mutations in this site significantly reduce activity of the *PolgA* promoter in Schneider cells (37).

In conclusion, this study emphasizes the role of PolgA as a key player in mtDNA maintenance that is absolutely necessary for mtDNA replication from an early stage in embryogenesis. Our work also provides evidence that *PolgA* expression can be induced as a consequence of increased demand for replication of mtDNA. However, the minor reduction in mtDNA levels observed in *PolgA*<sup>+/-</sup> mice appears not to be significant enough to induce a compensatory induction of *PolgA* expression. Finally, our results put an end to the long-standing debate of whether mtDNA polymerase gamma is the sole polymerase capable of replicating and maintaining mtDNA in the cell.

## Materials and Methods

### Matings and genotyping of transgenic animals.

The generation of mice with a *loxP*-flanked *PolgA* -allele (figure 1) have previously been described (11). *PolgA* knockout animals are genotyped with both Southern blot and PCR analysis. For Southern blot analyses, ~5 µg of genomic DNA was digested with *BglII* and blotted to Hybond-C+ (Amersham) membrane using standard procedures. The membranes were hybridized with a 2.7kb probe spanning exons 11-14 (figure 1a and 1b) labeled with [ $\alpha$ -<sup>32</sup>P]dCTP (3,000 Ci/mmol; Amersham), and visualized by autoradiography with standard procedures.

PCR genotyping was performed by multiplex PCR with primers F1 (5'-ttt gcc aag ttc taa ttc ca -3'), F2 (5'-gga tgg gca gga aca gtt ag -3') and R1 (5'- ctg cca ttc acc ttc ccc-3'). Cycling conditions were: 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 57°C for 45 sec, 72°C for 30 sec, with final extension at 72°C for 7 min. The PCR reaction designed in this way can give two differently sized products corresponding to the wild-type fragment of 658 bp and the knockout fragment of 471 bp.

Heterozygous *PolgA* knockout animals (*PolgA*<sup>+/-</sup>) were crossed with transgenic animals overexpressing TFAM (*TFAM*<sup>OE</sup>; previously called PAC19) (23) generating *PolgA*<sup>+/-</sup>, *TFAM*<sup>OE</sup>. From the same cross we generated two control groups of animals used in our experiments: *PolgA*<sup>+/+</sup> and *PolgA*<sup>+/+</sup>, *TFAM*<sup>OE</sup>. Double transgenic animals were genotyped to detect the PAC transgene (23) and *PolgA*<sup>+/-</sup> allele as described above. Animal studies were approved by the animal welfare ethics committee and performed in compliance with Swedish law.

### Enzyme histochemistry

Enzyme histochemical analyses of succinate dehydrogenase (SDH) and cytochrome c oxidase (COX) activities were performed on 14 µm cryostat sections of fresh frozen

wild-type and *PolgA*<sup>+/-</sup> embryos as described previously (24).

### **Southern and Western blot analyses**

DNA was prepared by SDS and proteinase K treatment as previously described (38). The DNA was purified by phenol:chloroform extraction, precipitated with ethanol and dissolved in TE pH 8.0. Total DNA (5 µg) from heart, liver and skeletal muscle of adult mice (>10 weeks of age) and from whole E8.5–E9.5 embryos was digested overnight with the *Sac I* restriction enzyme, precipitated with 0.2M NaCl and ethanol and separated on 0.8% agarose gels. Separated DNA was transferred to nitrocellulose membranes by Southern blotting. The membranes were probed with [ $\alpha$ -<sup>32</sup>P]-dCTP random-labeled murine total mtDNA or a *CoxI* DNA fragment. Phosphorimager analyses were used to assess total mtDNA levels normalized to the nuclear 18S rRNA gene. Two-sided, unpaired *t*-tests were used to assess statistical significance.

Total liver homogenate and mitochondrial preparations were suspended in protein buffer [equal parts of suspension buffer (100mM NaCl, 10mM Tris–HCl pH 7.6, 1mM EDTA pH 8.0, 1% Aprotinin, 1% PMSF) and loading buffer (100mM Tris–HCl pH 6.8, 4% SDS, 20% glycerol, 200mM DTT)]. Each sample was boiled for 10 min, sonicated and the supernatants containing the proteins were collected after centrifugation for 10 min at 10 000 *g*. We used 1:100 – 1:2000 dilutions of the Ab-4 cocktail antibody consisting of antibodies raised against the C- and N-terminal domains of POLGA (Lab Vision). Also, we used 1:2000- 1:8000 dilution of the CD-7 antibody, raised against the C-terminal POLGA domain, as recommended by William C. Copeland. Western blot analyses to determine protein levels of mouse Polg were performed as described (24).

### **Real-time PCR to quantify mtDNA**

DNA primers to detect *CoxI* and  $\beta$ -*actin* were designed to give a maximum amplicon

length of 150bp. *β-actin* forward primer 5'-TGTTCCCTTCCACAGGGTGT-3' and reverse primer 5'-TCCCAGTTGGTAACAATGCCA-3'. *CoxI* forward primer 5'-GCCCCAGATATAGCATTCCC-3' and reverse primer 5'-GTTTCATCCTGTTTCCTGCTCC-3'. The 25µl PCR reaction mixture contained 1x Platinum SYBR Green qPCR SuperMix UDG (Invitrogen), 500nM each primer, and ~1ng of total genomic DNA. Real-time PCR conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C. Fluorescent signal intensities were recorded and analyzed during PCR in an ABI Prism 7700 Sequence Detector system (Applied Biosystems) using the SDS (Ver.1.91) software. The threshold cycle ( $C_T$ ) is the cycle at which a significant increase in the reaction product is first detected from the increasing fluorescent signal. Thus, the  $C_T$  values within the linear exponential phase were used to measure the original DNA template copy numbers from a standard curve generated from five 10-fold dilutions of either pure mtDNA (*CoxI*) or pure nuclear DNA (*β-actin*). Relative values for *CoxI* and *β-actin* were compared within each sample to generate a ratio which is the relative level of mtDNA in that sample.

### **RNA isolation and TaqMan PCR**

Isolation of total RNA from mouse kidney was carried out following the Rneasy midi-kit protocol (Qiagen). Reverse transcription was performed using 5µg of total RNA and Superscript reverse transcriptase (Invitrogen). Amplification was performed with the ABI PRISM 7700 Sequence Detector (Applied Biosystems) with a two-step PCR protocol (preincubation for 10 min at 95°C followed by 40 cycles at 95°C for 15 sec and at 60°C for 1 min). All primers and probes were designed using Primer Express software (Applied Biosystems) avoiding contamination of the genomic DNA

amplification by positioning one of the primers over the exon/intron boundary (table 1). The *PolgA* probe was labelled with FAM at the 5' end as reporter dye and TAMRA at the 3' end as quencher dye. The 18S probe was labelled with VIC at the 5' end as reporter dye and TAMRA at the 3' end as quencher dye. Relative quantification was performed using the standard curve method where curves were constructed using five 10-fold dilutions of the standard sample. Standards were run in triplicates with 18S being the endogenous control. Relative amounts of *PolgA* and endogenous control were calculated for one sample, and the results were used to calculate the final amount of *PolgA* in that sample as a ratio between the two relative amounts.



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## References:

1. Burgers, P.M., Koonin, E.V., Bruford, E., Blanco, L., Burtis, K.C., Christman, M.F., Copeland, W.C., Friedberg, E.C., Hanaoka, F., Hinkle, D.C. *et al.* (2001) Eukaryotic DNA polymerases: proposal for a revised nomenclature. *J. Biol. Chem.*, **276**, 43487-43490.
2. Kaguni, L.S. (2004) DNA polymerase gamma, the mitochondrial replicase. *Annu. Rev. Biochem.*, **73**, 293-320.
3. Schultz, R.A., Swoap, S.J., McDaniel, L.D., Zhang, B., Koon, E.C., Garry, D.J., Li, K. and Williams, R.S. (1998) Differential expression of mitochondrial DNA replication factors in mammalian tissues. *J. Biol. Chem.*, **273**, 3447-3451.
4. Ye, F., Carrodegua, J.A. and Bogenhagen, D.F. (1996) The gamma subfamily of DNA polymerases: cloning of a developmentally regulated cDNA encoding *Xenopus laevis* mitochondrial DNA polymerase gamma. *Nucleic Acids Res.*, **24**, 1481-1488.
5. Lecrenier, N., Van Der Bruggen, P. and Foury, F. (1997) Mitochondrial DNA polymerases from yeast to man: a new family of polymerases. *Gene*, **185**, 147-152.
6. Carrodegua, J.A., Pinz, K.G. and Bogenhagen, D.F. (2002) DNA binding properties of human pol gammaB. *J. Biol. Chem.*, **277**, 50008-50014.
7. Fan, L. and Kaguni, L.S. (2001) Multiple regions of subunit interaction in *Drosophila* mitochondrial DNA polymerase: three functional domains in the accessory subunit. *Biochemistry*, **40**, 4780-4791.
8. Vanderstraeten, S., Van den Brule, S., Hu, J. and Foury, F. (1998) The role of 3'-5' exonucleolytic proofreading and mismatch repair in yeast mitochondrial DNA error avoidance. *J. Biol. Chem.*, **273**, 23690-23697.
9. Farr, C.L., Wang, Y. and Kaguni, L.S. (1999) Functional interactions of mitochondrial DNA polymerase and single-stranded DNA-binding protein. Template-primer DNA binding and initiation and elongation of DNA strand synthesis. *J. Biol. Chem.*, **274**, 14779-14785.
10. Lim, S.E., Longley, M.J. and Copeland, W.C. (1999) The mitochondrial p55 accessory subunit of human DNA polymerase gamma enhances DNA binding, promotes processive DNA synthesis, and confers N-ethylmaleimide resistance. *J. Biol. Chem.*, **274**, 38197-38203.
11. Trifunovic, A., Wredenberg, A., Falkenberg, M., Spelbrink, J.N., Rovio, A.T., Bruder, C.E., Bohlooly, Y.M., Gidlöf, S., Oldfors, A., Wibom, R. *et al.* (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature*, **429**, 417-423.
12. Lewis, W., Simpson, J.F. and Meyer, R.R. (1994) Cardiac mitochondrial DNA polymerase-gamma is inhibited competitively and noncompetitively by phosphorylated zidovudine. *Circ. Res.*, **74**, 344-348.
13. Lewis, W., Day, B.J. and Copeland, W.C. (2003) Mitochondrial toxicity of NRTI antiviral drugs: an integrated cellular perspective. *Nat. Rev. Drug. Discov.*, **2**, 812-822.
14. Suomalainen, A. and Kaukonen, J. (2001) Diseases caused by nuclear genes affecting mtDNA stability. *Am. J. Med. Genet.*, **106**, 53-61.
15. Van Goethem, G., Dermaut, B., Lofgren, A., Martin, J.J. and Van Broeckhoven, C. (2001) Mutation of POLG is associated with progressive external

- ophthalmoplegia characterized by mtDNA deletions. *Nat. Genet.*, **28**, 211-212.
16. Lamantea, E., Tiranti, V., Bordoni, A., Toscano, A., Bono, F., Servidei, S., Papadimitriou, A., Spelbrink, H., Silvestri, L., Casari, G. *et al.* (2002) Mutations of mitochondrial DNA polymerase gammaA are a frequent cause of autosomal dominant or recessive progressive external ophthalmoplegia. *Ann. Neurol.*, **52**, 211-219.
  17. Agostino, A., Valletta, L., Chinnery, P.F., Ferrari, G., Carrara, F., Taylor, R.W., Schaefer, A.M., Turnbull, D.M., Tiranti, V. and Zeviani, M. (2003) Mutations of ANT1, Twinkle, and POLG1 in sporadic progressive external ophthalmoplegia (PEO). *Neurology*, **60**, 1354-1356.
  18. Van Goethem, G., Schwartz, M., Lofgren, A., Dermaut, B., Van Broeckhoven, C. and Vissing, J. (2003) Novel POLG mutations in progressive external ophthalmoplegia mimicking mitochondrial neurogastrointestinal encephalomyopathy. *Eur. J. Hum. Genet.*, **11**, 547-549.
  19. Naviaux, R.K. and Nguyen, K.V. (2004) POLG mutations associated with Alpers' syndrome and mitochondrial DNA depletion. *Ann. Neurol.*, **55**, 706-712.
  20. Ferrari, G., Lamantea, E., Donati, A., Filosto, M., Briem, E., Carrara, F., Parini, R., Simonati, A., Santer, R. and Zeviani, M. (2005) Infantile hepatocerebral syndromes associated with mutations in the mitochondrial DNA polymerase-gammaA. *Brain*.
  21. Luoma, P., Melberg, A., Rinne, J.O., Kaukonen, J.A., Nupponen, N.N., Chalmers, R.M., Oldfors, A., Rautakorpi, I., Peltonen, L., Majamaa, K. *et al.* (2004) Parkinsonism, premature menopause, and mitochondrial DNA polymerase gamma mutations: clinical and molecular genetic study. *Lancet*, **364**, 875-882.
  22. Rovio, A.T., Marchington, D.R., Donat, S., Schuppe, H.C., Abel, J., Fritsche, E., Elliott, D.J., Laippala, P., Ahola, A.L., McNay, D. *et al.* (2001) Mutations at the mitochondrial DNA polymerase (POLG) locus associated with male infertility. *Nat. Genet.*, **29**, 261-262.
  23. Ekstrand, M.I., Falkenberg, M., Rantanen, A., Park, C.B., Gaspari, M., Hultenby, K., Rustin, P., Gustafsson, C.M. and Larsson, N.G. (2004) Mitochondrial transcription factor A regulates mtDNA copy number in mammals. *Hum. Mol. Genet.*, **13**, 935-944.
  24. Larsson, N.G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G.S. and Clayton, D.A. (1998) Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat. Genet.*, **18**, 231-236.
  25. Cerritelli, S.M., Frolova, E.G., Feng, C., Grinberg, A., Love, P.E. and Crouch, R.J. (2003) Failure to produce mitochondrial DNA results in embryonic lethality in Rnaseh1 null mice. *Mol. Cell*, **11**, 807-815.
  26. Ebert, K.M., Liem, H. and Hecht, N.B. (1988) Mitochondrial DNA in the mouse preimplantation embryo. *J. Reprod. Fertil.*, **82**, 145-149.
  27. Piko, L. and Taylor, K.D. (1987) Amounts of mitochondrial DNA and abundance of some mitochondrial gene transcripts in early mouse embryos. *Dev. Biol.*, **123**, 364-374.
  28. Piko, L. and Matsumoto, L. (1976) Number of mitochondria and some properties of mitochondrial DNA in the mouse egg. *Dev. Biol.*, **49**, 1-10.
  29. Dawid, I.B. and Chase, J.W. (1972) Mitochondrial RNA in *Xenopus laevis*. II. Molecular weights and other physical properties of mitochondrial ribosomal and 4 s RNA. *J. Mol. Biol.*, **63**, 217-231.
  30. Chase, J.W. and Dawid, I.B. (1972) Biogenesis of mitochondria during *Xenopus*

- laevis development. *Dev. Biol.*, **27**, 504-518.
31. Davis, A.F., Ropp, P.A., Clayton, D.A. and Copeland, W.C. (1996) Mitochondrial DNA polymerase gamma is expressed and translated in the absence of mitochondrial DNA maintenance and replication. *Nucleic Acids Res.*, **24**, 2753-2759.
  32. Wredenberg, A., Wibom, R., Wilhelmsson, H., Graff, C., Wiener, H.H., Burden, S.J., Oldfors, A., Westerblad, H. and Larsson, N.G. (2002) Increased mitochondrial mass in mitochondrial myopathy mice. *Proc. Natl. Acad. Sci. U. S. A.*, **99**, 15066-15071.
  33. Hardt, N., De Kegel, D., Vanheule, L., Villani, G. and Spadari, S. (1980) DNA polymerase gamma, cytochrome c oxidase and mitochondrial integrity in rabbit spleen lymphocytes stimulated with concanavalin A. *Exp. Cell. Res.*, **127**, 269-276.
  34. Annex, B.H. and Williams, R.S. (1990) Mitochondrial DNA structure and expression in specialized subtypes of mammalian striated muscle. *Mol. Cell. Biol.*, **10**, 5671-5678.
  35. Virbasius, J.V. and Scarpulla, R.C. (1994) Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis. *Proc. Natl. Acad. Sci. U. S. A.*, **91**, 1309-1313.
  36. Scarpulla, R.C. (1997) Nuclear control of respiratory chain expression in mammalian cells. *J. Bioenerg. Biomembr.*, **29**, 109-119.
  37. Wu, Z., Puigserver, P., Andersson, U., Zhang, C., Adelmant, G., Mootha, V., Troy, A., Cinti, S., Lowell, B., Scarpulla, R.C. *et al.* (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell*, **98**, 115-124.
  38. Wang, J., Wilhelmsson, H., Graff, C., Li, H., Oldfors, A., Rustin, P., Bruning, J.C., Kahn, C.R., Clayton, D.A., Barsh, G.S. *et al.* (1999) Dilated cardiomyopathy and atrioventricular conduction blocks induced by heart-specific inactivation of mitochondrial DNA gene expression. *Nat. Genet.*, **21**, 133-137.

**Figure 1** Creation of *PolgA*<sup>+/-</sup> animals (A) Mice that were heterozygous for the recombinant *PolgA* allele (*PolgA*<sup>+/*mut*Neo</sup>) were mated to  $\beta$ -actin *cre*-mice to excise the *loxP*-flanked exon 3 and generate mice heterozygous for the *PolgA* knockout allele (*PolgA*<sup>+/-</sup>). (B) Southern blot analysis to detect *PolgA* alleles in wild-type (+/+), heterozygous knockout (+/-) and heterozygous *loxP*-flanked (+/*loxP*) mice. Removal of the *PolgA* exon 3 changed the restriction pattern obtained after *BglI* digestion, and resulted in the fragment of ~9.0 kb instead of ~10.5 kb. (C) A multiplex PCR was developed to simplify genotyping of the *PolgA* locus. PCR genotyping with three primers results in a wild type allele (+) fragment of 658bp and a knockout allele (-) fragment of 471bp.

**Figure 2.** Morphology of (A) wild-type (+/+) and (B) *PolgA*<sup>-/-</sup> (-/-) embryos at E8.5 (C) Enzyme histochemical double staining for COX and SDH activities Cells with both COX and SDH activity appear brown, whereas cells with deficient COX activity appear pale blue. (D) Phosphorimager quantification of mtDNA levels. Southern blots of DNA isolated from embryos were probed for mtDNA and normalized to 18S nuclear DNA. Heterozygous knockout embryos (*PolgA*<sup>+/-</sup>) are indicated by +/- . The mtDNA levels are indicated as percentage of the mean levels in wild-type animals. *Statistical analysis:* All bars indicate mean  $\pm$  SD. Statistical significance from two-sided *t*-tests is indicated by stars \*\*\* *P*<0.001.

**Figure 3.** Levels of *PolgA* transcripts and mtDNA in wild-type (+/+) and heterozygous knockout (+/-) mice. (A) TaqMan PCR quantification of *PolgA* mRNA levels normalized to 18S transcripts. (B) Phosphorimager quantification of mtDNA levels in heart, liver and skeletal muscle DNA. Southern blots were probed for mtDNA (*CoxI*) and normalized to 18S nuclear DNA. The levels are indicated as percentage of the mean levels in wild-type animals. (C) Real-time PCR analysis to determine mtDNA

levels in heart, liver and skeletal muscle. Values were normalized to levels of the nuclear actin gene and are indicated as percentage of the mean levels in wild-type animals. *Statistical analysis*: All bars indicate mean  $\pm$  SD. Statistical significance from two-sided *t*-tests is indicated by stars \*  $P < 0.05$ .

**Figure 4.** *PolgA* transcript levels and mtDNA copy number in *PolgA* wild-type (+/+), heterozygous *PolgA* knockout (+/-), heterozygous knockout *PolgA* also overexpressing *TFAM* (+/-,TFAM<sup>OE</sup>) and *PolgA* wild-type overexpressing *TFAM* (+/+,TFAM<sup>OE</sup>) mice. **(A)** Phosphorimager quantification of mtDNA levels. Southern blots of liver DNA were probed for mtDNA, normalized to 18S nuclear DNA. The mtDNA levels are indicated as percentage of the mean levels in wild-type animals. **(B)** Real-time PCR analysis to determine mtDNA levels. Values were normalized to the nuclear actin gene and are indicated as percentage of the mean levels in wild-type animals. **(C)** TaqMan PCR quantification of *PolgA* mRNA levels normalized to 18S transcripts. *Statistical analysis*: All bars indicate  $\pm$  SD. Statistical significances from two-sided *t*-tests are indicated by stars \*  $P < 0.05$ ; \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$ .

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**Table 1.** Primers and probes for TaqMan PCR

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Gene	5' primer	3' primer	Probe
<i>PolgA</i>	GCAGGATGGGC	GCATCCGGGAG	AGATACTGTTCC
	AGGAACA	TCCTGAA	CTGATCTT
<i>18S</i>	CGGCTACCACATC	GCTGGAATTAC	TGCTGGCACCAGAC
	CAAGGAA	CGCGGCT	TTGCCCTC

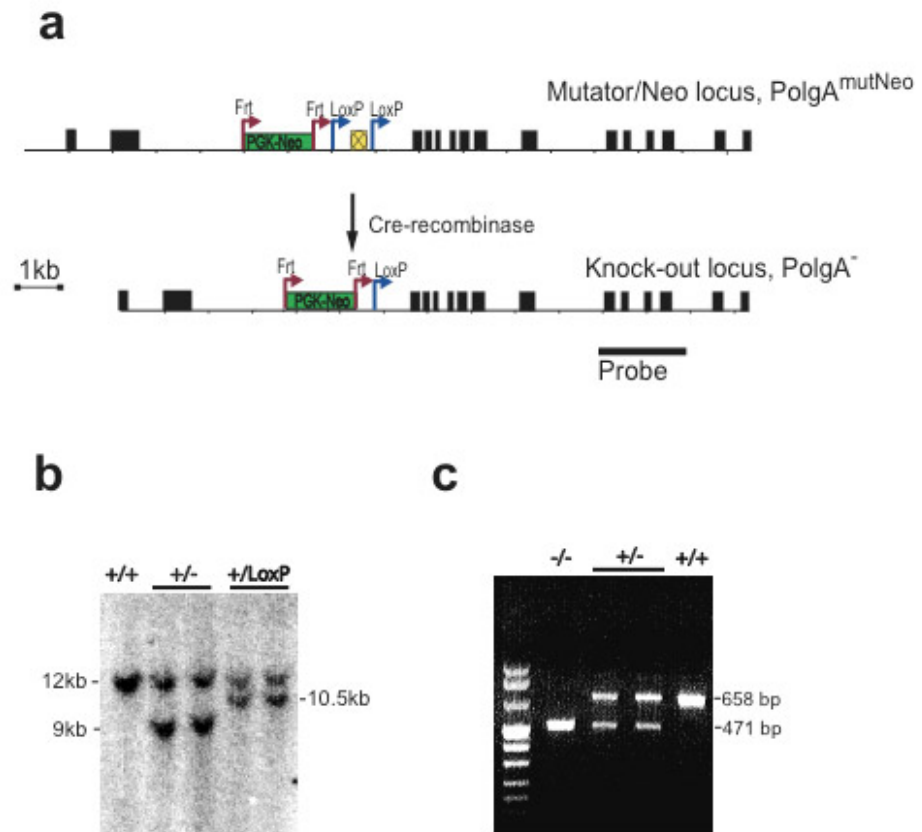
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**Table 2.** Genotyping of newborn pups and staged embryos obtained by intercrossing *PolgA*<sup>+/-</sup> mice

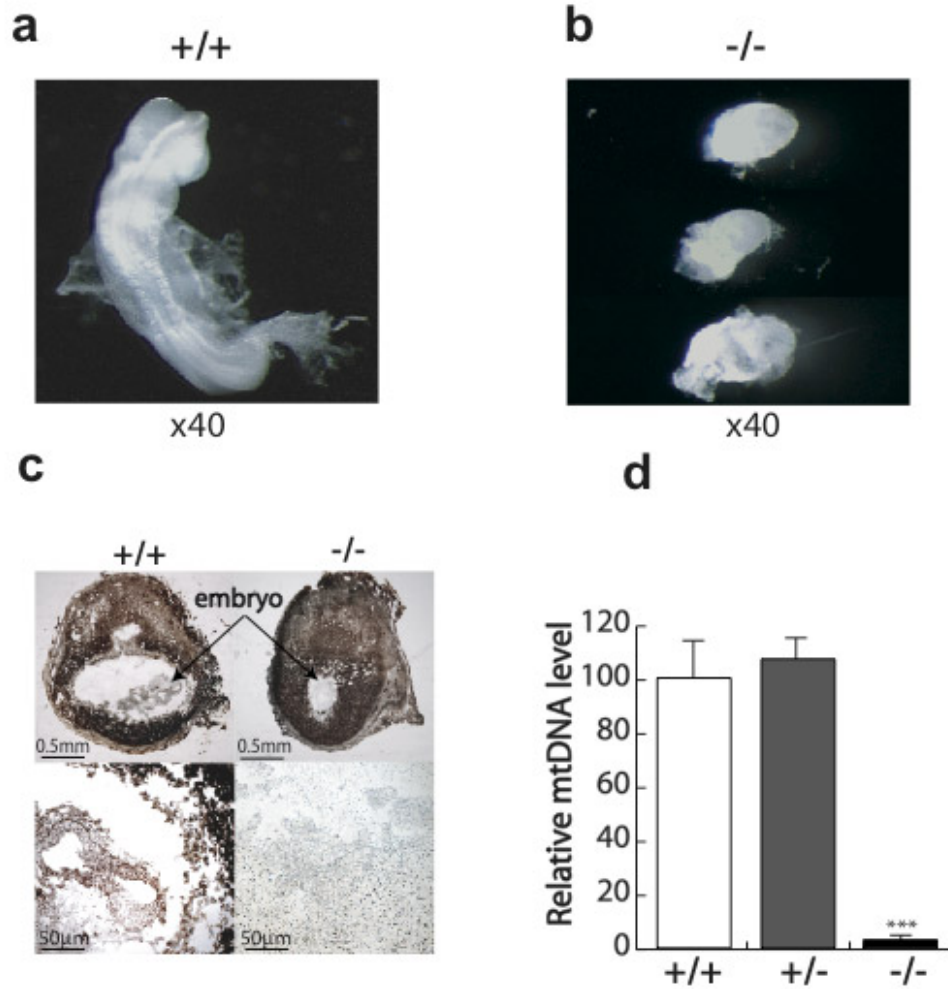
Stage	Total (tot. lit.)	+/+	+/-	-/-
<b>Newborn</b>	<b>182 (22)</b>	60	122	0
<b>E10.5</b>	<b>12 (2)</b>	4	7	1*
<b>E9.5</b>	<b>17 (2)</b>	5	9	3*
<b>E8.5</b>	<b>69 (7)</b>	17	36	16**
<b>E7.5</b>	<b>17 (2)</b>	4	9	4

\*resorbed embryos; \*\*1/4 of embryos resorbed

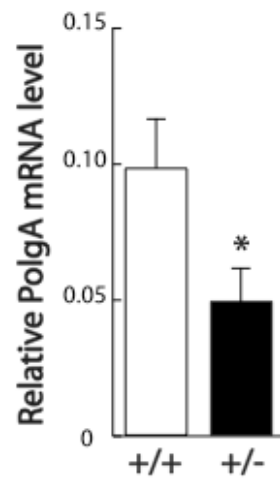
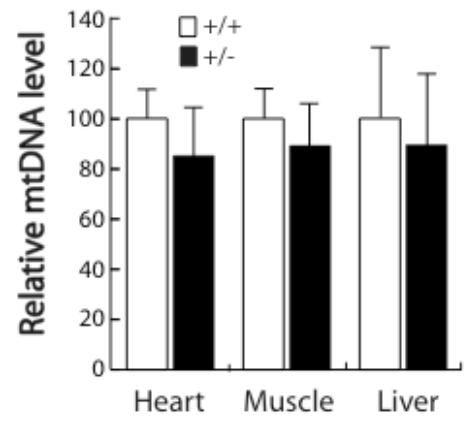
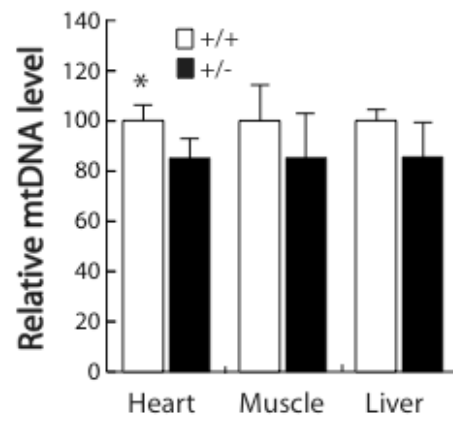




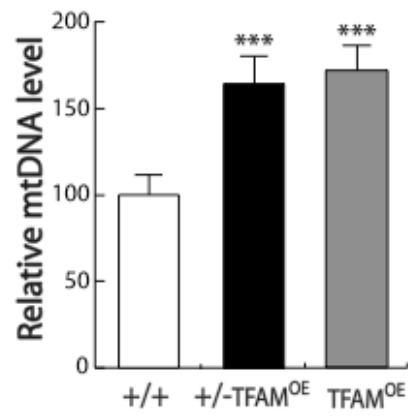
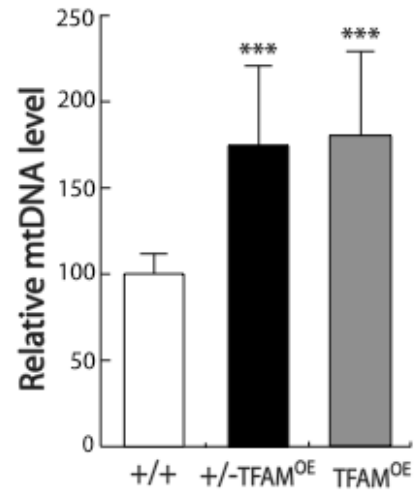
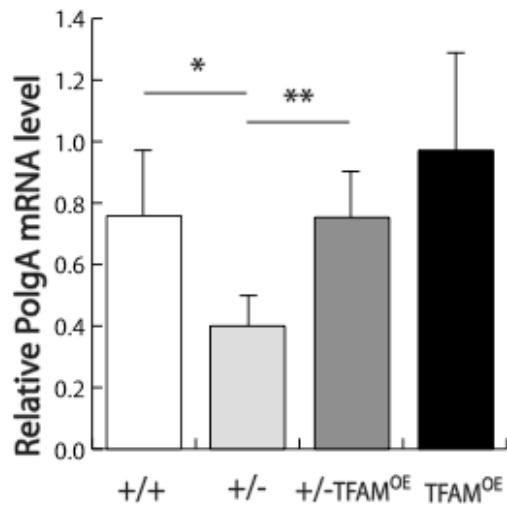
Hance *et al.* Figure 1



Hance *et al.* Figure 2

**a****b****c**

Hance *et al.* Figure 3

**a****b****c**

Hance *et al.* Figure 4