Arl3 rod-specific knockout displays RP-like photoreceptor degeneration (#421 - C0192)

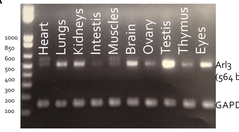
Christin Hanke[1,2], Houbin Zhang[2,3], Cecilia D. Gerstner[2], Jeanne M. Frederick[2], Wolfgang Baehr[2]

[1]Physikalische Biochemie, Universität Potsdam, Karl-Liebknecht-Strasse 24-25, D-14476 Potsdam, Germany, [2]Department of Ophthalmology, University of Utah, 65 Mario Capecchi Drive, Salt Lake City, UT-84132, USA , [3]Sichian Academy of Medical Sciences and Sichuan People's Hospital, Chengdu, Sichuan, China

Arl3 protein (ADP-ribosylation-factor-like 3 protein)

A: Arl3 protein is a small GTPase switching between its GDP-bound (inactive) and GTP-bound (active) conformation. Ubiquitously expressed as 182 aa long protein (figure **A**), Arl3 is involved in membrane associated vesicular and intracellular trafficking. Transgenic knockouts of Arl3 exhibit typical ciliopathy manifestations characterized by cysts in the kidney, liver and pancreas and photoreceptor degeneration (Schrick et al, *Amercl of Path*, 2006). The mice were 'very sick' and died within 3 weeks after birth. So far, Arl3 has not been identified as a causal locus in any human ciliopathy.

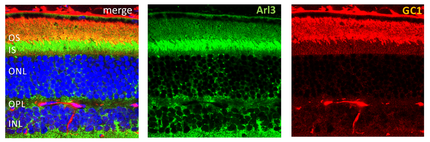
A



B

B: An unknown GEF catalyzes GDP/GTP exchange to generate active Arl3-GTP. Arl3 hydrolyzes GTP very slowly. GTP hydrolysis is activated by a GTPase Activating Protein (GAP). The GAP for Arl3 protein is *retinitis pigmentosa* 2 gene (RP2) accelerating GTP hydrolysis around 90.000 X (Veltel et al, *Nature*, 2008).

C

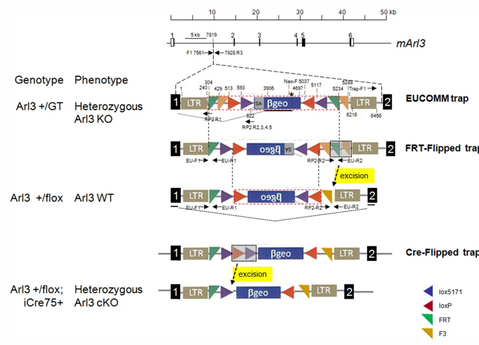


C: Arl3 protein is predominantly localized in the inner segment and connecting cilium in wildtype photoreceptor cells as shown in figure **C**. Arl3 is also expressed in the inner retina. Preabsorption of Arl3 antibody with recombinant protein shows no signal for Arl3 in wildtype mouse retina.

Generating the Arl3 conditional knockout mouse model

D: Breeding heterozygous transgenic knockout mice ($Arl3^{+/GT}$) never produced homozygous knockout litters, indicating the germline Arl3 homozygous knockout is embryonic lethal. To study the function of Arl3 in photoreceptors, a rod-specific knockout was generated ($Arl3^{floxflox;iCre75+}$) by breeding heterozygous germline knockout mice with FLP mice, followed by mating floxed mice with iCre75+ transgenic mice.

D



A gene trap vector in mouse chromosome 19 leads to splicing of exon 1 to the splice acceptor (SA) causing early termination of *arl3* transcription.

1. Trapped allele (mutagenic)

2. Transient step (Inversion of FRT-sites by FLP recombinase)

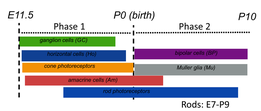
3. Conditional allele (floxed; non-mutagenic)

4. Transient step (Re-inversion of loxP-sites by Cre recombinase)

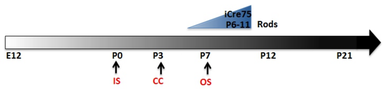
5. Re-inverted trapped allele (mutagenic)

E-I: Retina development has two phases (http://www.nap.edu/). Initially, ganglion cells, horizontal cells, amacrine cells and cones differentiate, followed by bipolar and Müller glia cells. Rods develop between E7-P9.
E-II: The arrow represents the development of rod photoreceptors in mice. Mouse is a rod-dominant species - just 3 % of the photoreceptors are cones. Rods start to differentiate at E7 (phase 1) and are fully developed by P9 (phase 2). The inner segment (IS) exists at P0, the connecting cilium (CC) starts to extend at P_3, and the nascent outer segment (OS) appears by P7.

E-I:

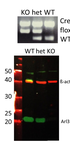


E-II:

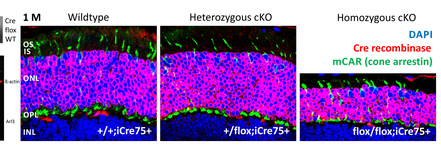


F-I: Genotype analysis shows amplification of Cre, floxed and wildtype alleles representing wildtype (+/+; iCre75+), heterozygous (+/flox; iCre75+) and homozygous rod-specific knockout mice (flox/flox; iCre75+). Expression level of Arl3 protein in homozygous knockout mice is highly reduced in Western Blot analysis due to complete photoreceptor degeneration.

F-I:



F-II:

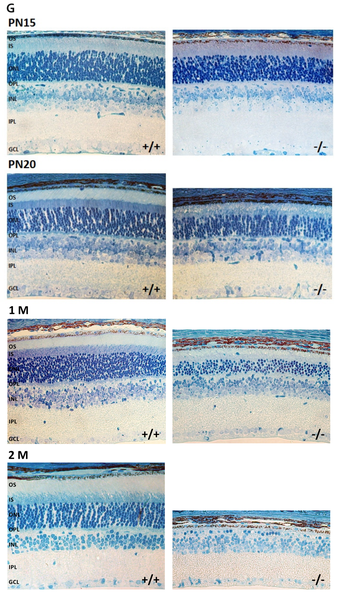


F-II: iCre75+ expression in mouse retina was tested with Cre-specific antibody. As shown in figure F-II , Cre recombinase is expressed evenly in retinas of wildtype, heterozygous knockout and homozygous knockout mice. Cone-arrestin labelling with mCAR antibody reveals no Cre expression in cones, just in rods. Progression of photoreceptor degeneration is severe in one month-old $Arl3^{floxflox;iCre75+}$ mice.

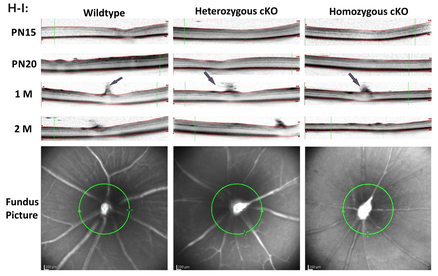
Rod degeneration and retina thinning

G: Histology reveals rapidly progressing rod degeneration in rod-specific Arl3 knockout mice. In PN15 mice, all three nuclear layers are apparent in the rod-specific knockout with rod outer segments (ROS) being minimally shorter. ROS are moderately shortened at PN20. In 1 month-old conditional knockout mice, only 4-5 nuclear rows of the outer nuclear layer (ONL) remain and the ROS/RIS (rod inner segment) are extremely shortened compared to wildtype littermate mice. In 2 months-old mutant mice, the ONL consists of only one nuclear row and photoreceptor OS and IS are absent; the inner nuclear layer (INL) and ganglion cell layer (GCL) are present.

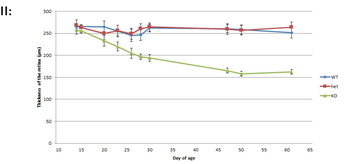
G



H-I:



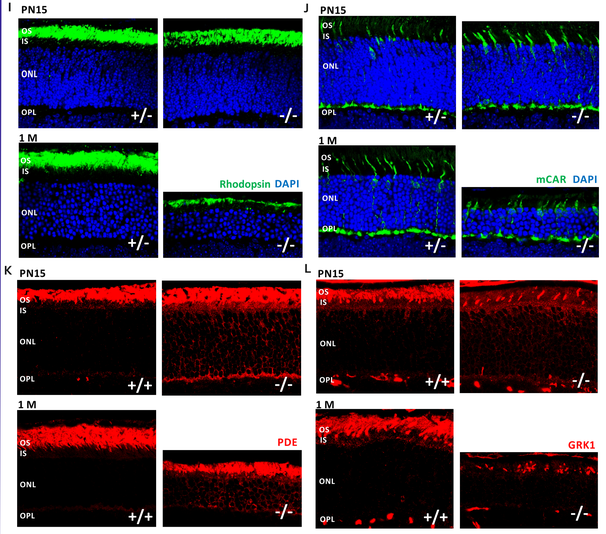
H-II:



H-I: Optical coherence tomography (OCT) reveals fast thinning of $Arl3^{floxflox;iCre75+}$ retina. At PN15 homozygous rod-specific knockout mice have retina thicknesses comparable to wildtype littermates. As shown in the diagram underneath (figure **H-II**), retina thinning proceeds quickly after PN15. Severe progression of thinning is visible in retinas of PN20 and 1 month-old mutant mice. In 2 month-old knockout mice, the retina averages ~ 100 µm thinner than wildtype. Heterozygous knockout mice behave like wildtype mice with no difference in retina thickness. The arrow shows the optic nerve.

Thanks to Judd Cahoon, U of Utah, for technical assistance!

Rhodopsin traffics normally, PDE is mislocalized , GRK1 is reduced



I: Immunohistochemistry shows the trafficking/localization of several peripheral membrane-associated and transmembrane proteins in absence of Arl3 protein in photoreceptors. As seen in figure I, rhodopsin-labeling shows normal trafficking in mutant mice comparable to wildtype and heterozygous knockout mice. Rhodopsin disappears almost completely in 2 months-old mutant mice due to photoreceptor degeneration (data not shown).

J: Labeling frozen sections with cone-specific arrestin (mCAR) antibody shows that PN15 mutant cone inner and outer segments are normally developed. Cones start to degenerate in 1 month-old rod-specific knockout mice, with COS shorter relative to those of wildtype and heterozygous knockout mice. Defect of cones is secondary to rod degeneration. In 2 months-old mutant mice, remaining cells of the ONL are cones (data not shown).

K: MOE-labelling shows localization of the enzyme cGMP-specific phosphodiesterase (cGMP-PDE). In PN15-old $Arl3^{floxflox;iCre75+}$ mice, PDE traffics mainly to the rod outer segment (ROS), but it also mislocalizes into the ONL. The amount of PDE is highly reduced in 1 month-old mutant mice due to rod degeneration (shown in figure K), whereas PDE is still present in the cone outer segment. PDE is absent in 2 month-old mutant mice due to photoreceptor degeneration (data not shown).

L: In PN15 old mutant mice the amount of rhodopsin kinase (GRK1) is highly reduced in ROS due to its mistrafficking in absence of Arl3 protein. Therefor Arl3 is important for correct transport of prenylated GRK1. GRK1 persists in cones in 1 month-old rod-specific knockout mice, but disappears after cones have degenerated at 2 months (data not shown).

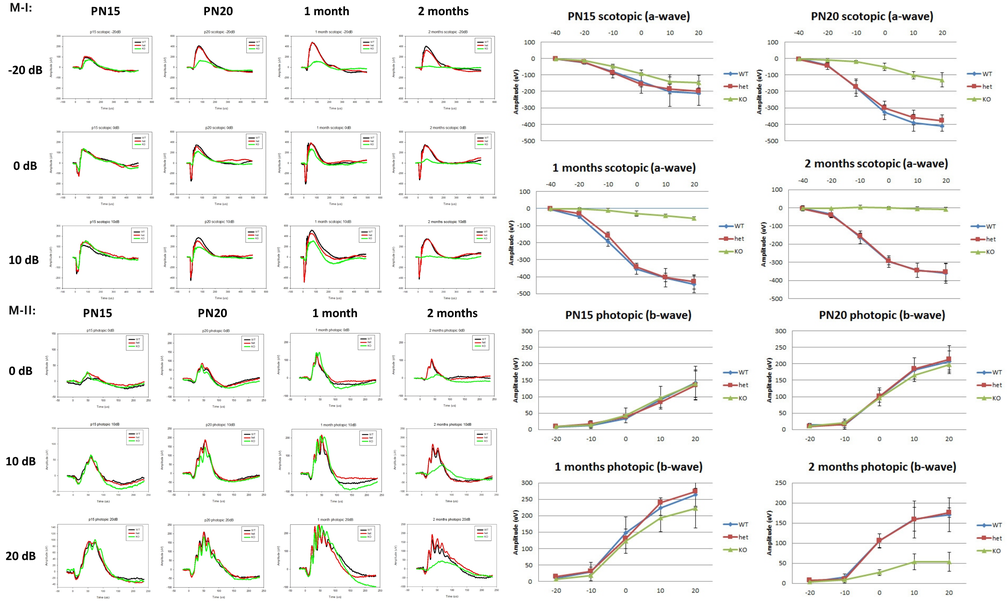
Abstract

Arf-like protein 3 (Arl3) is a small GTPase interacting with lipid-binding proteins. We generated rod-specific Arl3 knockouts to elucidate the role of Arl3 in transport of photoreceptor membrane-associated proteins.
Chimeras containing a gene trap in intron 1 of the *arl3* gene were generated at the University of Utah core using a EUCOMM cell line. Breeding with FLP mice followed by mating with iCre75+ transgenic mice generated rod-specific knockouts. Photoreceptor function and morphology were analyzed by ERG and immunohistochemistry. An Arl3-specific polyclonal antibody was generated using full-length recombinant Arl3 polypeptide expressed in bacteria.
Immunoblots of wildtype retina lysates identified a 20 kDa protein, which was reduced significantly in two months-old $Arl3^{floxflox;iCre75+}$ retina. Immunohistochemistry revealed Arl3 localizes predominantly in wildtype photoreceptor inner segments and connecting cilium. Arl3 immunoreactivity was specifically absent in homozygous rod knockouts, while still present in cones and inner retina. Scotopic and photopic ERGs of PN15 rod knockout and wildtype mice revealed comparable a- and b-wave amplitudes suggesting normal photoreceptor development. At PN15, knockout mice show slightly shorter ROS. At PN20, scotopic ERG a-wave amplitudes were reduced (70-80 %) but the photopic ERG was unaffected. One month-old $Arl3^{floxflox;iCre75+}$ mice showed 80-90% reduction in a-wave amplitude with only 4-5 rows of nuclei surviving in the ONL. In retinas of two month-old knockout mice, scotopic ERGs were extinguished and cone ERGs were highly attenuated. OCT confirmed the rapid loss of photoreceptors in the homozygous rod knockout starting at PN15. Although the $Arl3^{floxflox;iCre75+}$ retina fundi appeared comparable to wildtype, one month-old mutant retina was on average 100 µm thinner than its wildtype counterpart. Immunohistochemistry performed using retina sections of PN15 and one month-old knockout mice revealed that rhodopsin transport is normal; rhodopsin was undetectable in two month-old conditional knockout mice due to complete photoreceptor degeneration. Rod PDE6 and GRK1 mislocalized suggesting trafficking defects.
Rod-specific knockout of Arl3 revealed a rapidly-progressing photoreceptor degeneration. Rod-specific knockout mice were blind at two months of age. Outer segment development appeared to be unimpaired by Arl3 deletion and rod photoreceptor function was normal at P14.

No scotopic and highly reduced photopic ERGs in 2 months-old Arl3 cKO mice

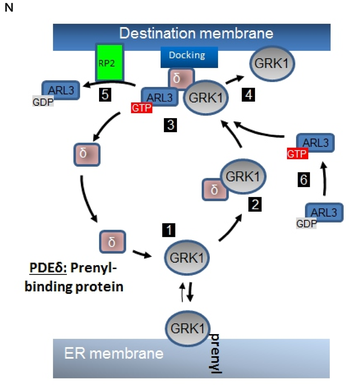
M-I: The function of the mutant retina was evaluated with eletroretinography (ERG). At PN15, the a-wave amplitude is almost normal for the homozygous rod-specific knockout mouse indicating that the ROS are formed. However, the photoreceptor degeneration started at this time point as suggested by the reduction in the a-wave amplitude. A much more severe effect in the reduction of scotopic ERG response is visible in PN20 old mutant mice relative to the wildtype; the a-wave amplitude is "almost flat" in 1 month-old mice. In 2 months-old mutant mice, no scotopic ERG response is detectable. Heterozygous knockout mice behave like wildtype mice.
M-II: The b-wave amplitudes of homozygous mutant, heterozygous mutant and wildtype mice are normal in PN15- and PN20-old animals. Cones start to degenerate in 1 month-old knockout mice evidenced by reduction in the b-wave amplitude. In 2 months-old knockout mice, the b-wave amplitude is significantly reduced, whereas heterozygous knockout mice behave as wildtype mice.

M-I:



M-II:

N



Model: How does GRK1 traffic from the ER membrane to its destination membrane?

N: GRK1 dissociates from ER and is "snatched" by PDEδ (function as GDI-like solubilization factor, GSF). The soluble complex GRK1-PDEδ combines with Arl3-GTP. The ternary complex docks to the destination membrane. Next, ArL3-GTP (function as Guanine nucleotide dissociation inhibitor (GDI) displacement factor, GDF) forces PDEδ to release its cargo (GRK1). GRK1 binds to the destination membrane (e.g. TGN vesicle loaded with rhodopsin). Arl3-GTP dissociates from PDEδ. RP2 (function as GTPase activating protein, GAP) catalyzes GTP hydrolysis at Arl3 protein. Arl3-GDP is converted to Arl3-GTP through unknown Guanine nucleotide exchange factor (GEF). The trafficking cycle starts over again (Zhang et al, unpublished data).

Conclusion: A conditional Arl3 knockout mouse model ($Arl3^{floxflox;iCre75+}$) was used to study the function of Arl3 protein in photoreceptors. Although outer segments are formed, retina degeneration starts as early as PN15. Rod degeneration is followed by cone degeneration, and thus simulates a *Retinitis pigmentosa* (RP)-like phenotype. Arl3 deletion in rods has no effect on trafficking of the transmembrane protein rhodopsin. Peripheral, membrane-associated photoreceptor proteins, e.g. prenylated proteins PDE and GRK1, are either mislocalized or down-regulated. In 2 months-old knockouts, these proteins disappear due to photoreceptor degeneration and the mice are completely blind (no scotopic ERG; highly reduced photopic ERG). Severe phenotype of Arl3 conditional knockout in rods will be rescued with sc pAAV2/8 virus gene replacement.

email: hanke.christin@gmx.de; wbaehr@hsc.utah.edu ARVO, Orlando, Florida USA, 2014.