SCIENCE
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8 April 1977, Volume 196, No. 4286

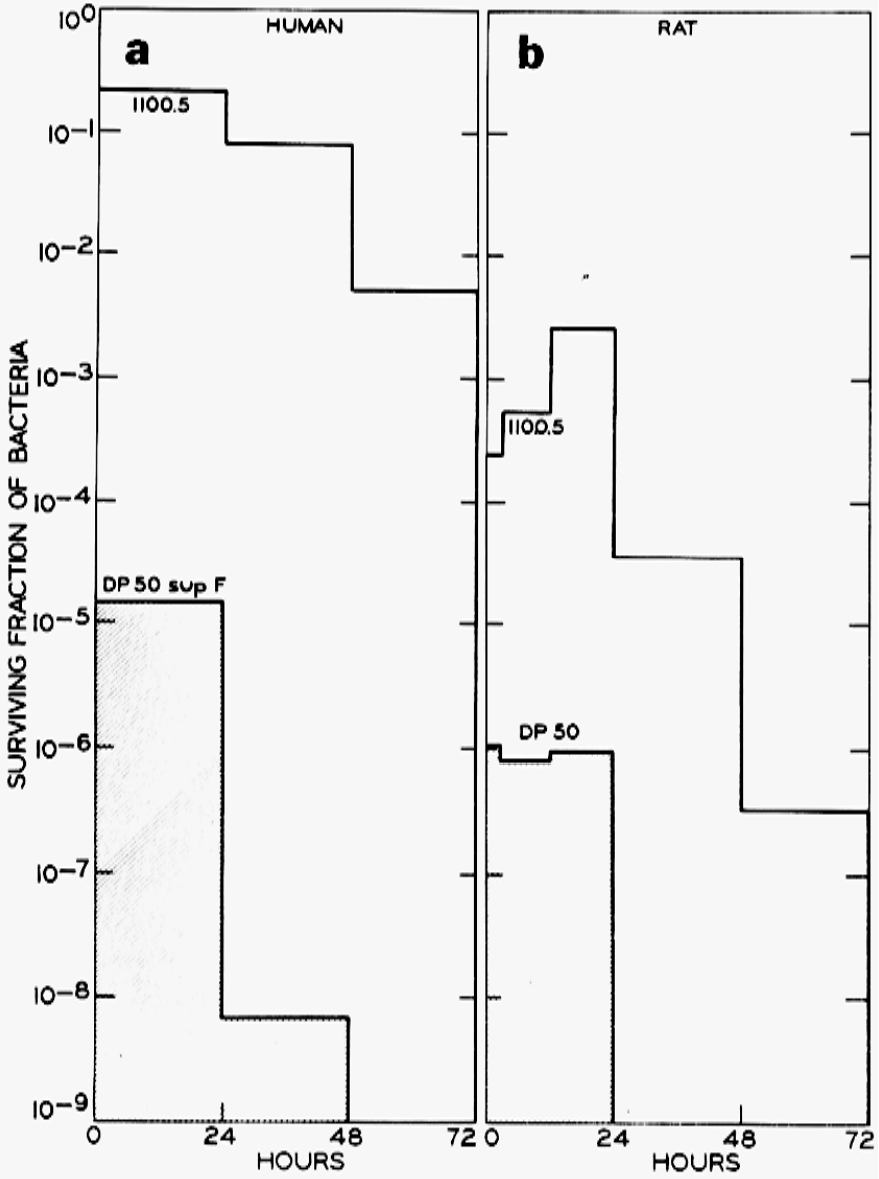


Fig. 4. Survival of host and vector in natural environments. Survival i
debilitated laboratory hosts DP50 and DP50SupF and, for comparison
strains are resistant to nalidixic acid, and titers can be determined on e
free Casamino acids and containing nalidixic acid (100 μg/ml). Such plat
yeast extract, and permit discrimination between Gal$^+$ (1100.5) and Gal
9.4×10^8 1100.5 was fed in 250 ml of milk to three humans. Bar heights

Background

1) ACE inhibitors slow the progession of nephropathy in diabetics.

2) The common D allele of the ACE gene is associated with higher levels of ACE, and is a risk factor for diabetic nephropathy.

3) In mice, a modest genetic increase in ACE expression causes a decrease in the levels of its substrates but does not change the levels of its products.

4) Mice with higher ACE levels develop more severe nephropathy than wildtype when made diabetic with STZ.

5) Since bradykinin is an active substrate of ACE, we predicted that Akita diabetic mice lacking the kidney receptor (B2R) for bradykinin would develop nephropathy more severe than when only diabetic.

6) This prediction was confirmed experimentally in B2R/Akita diabetic mice.

7) The phenotypes of these mice led us to hypothesize that a major cause of diabetic complications is mitochondrial damage.

8) Our three specific aims are intended to unravel the roles in this overall process of:

(i) The two bradykinin receptors.

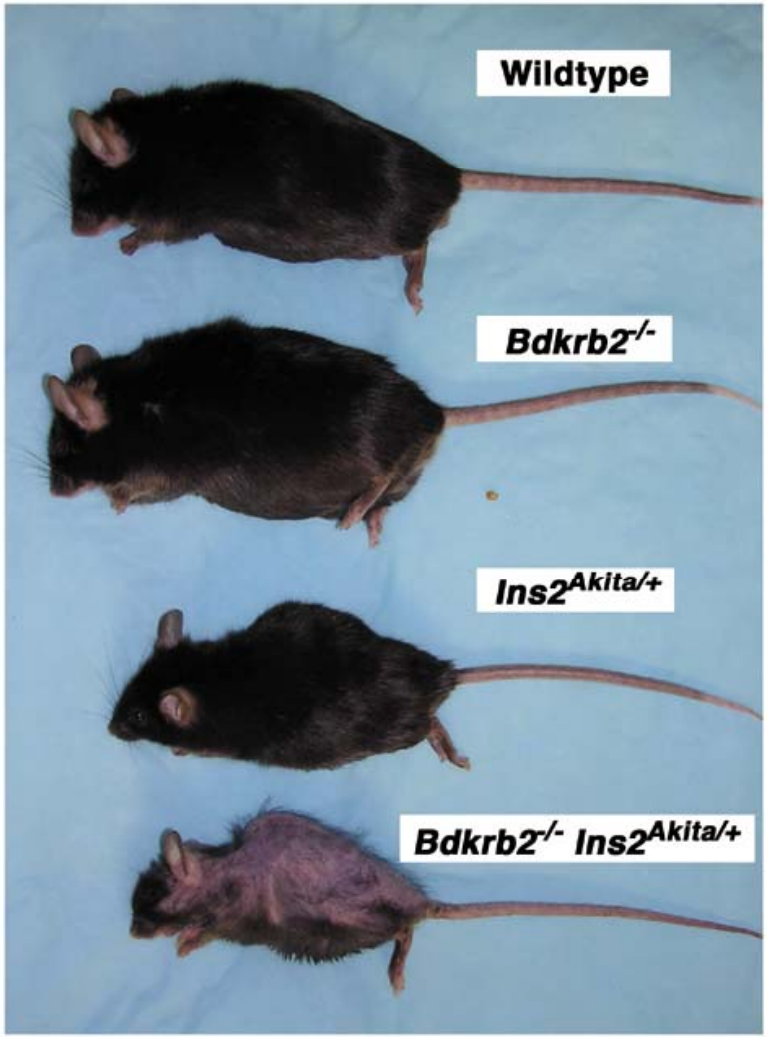
(ii) Endothelial nitric oxide synthase.

(iii) Mitochondrial mutations.

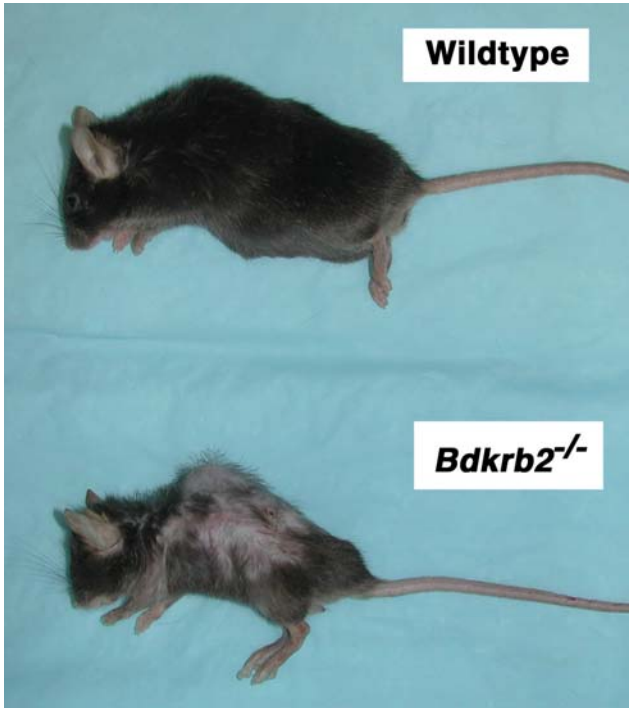
Masao Kakoki

General appearance of male B2R-deficient and Akita mice

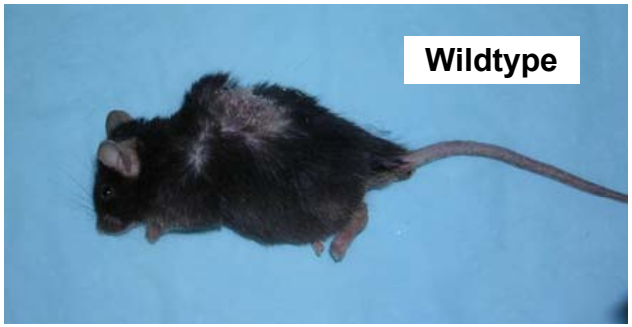
1-year-old mice



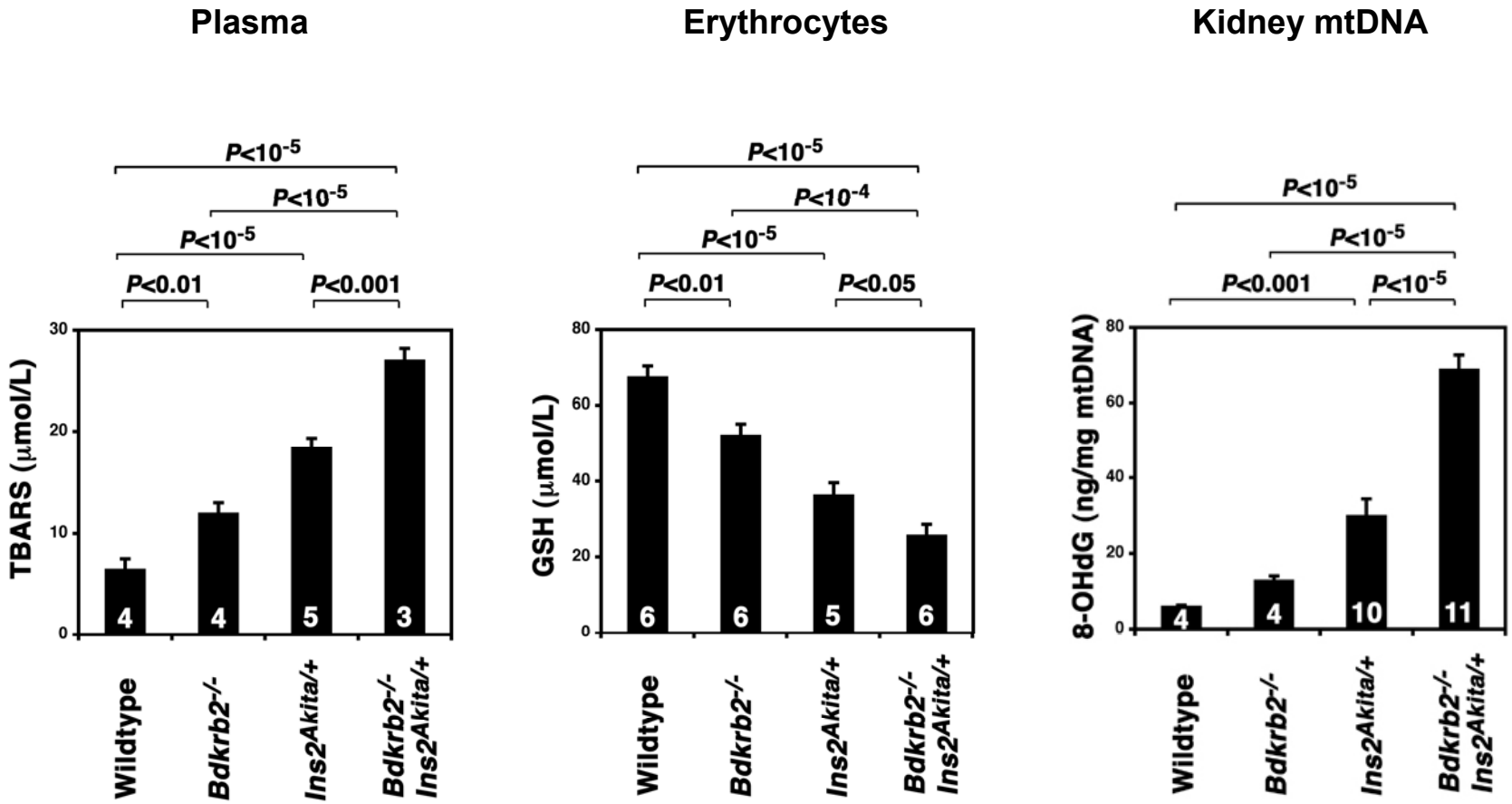
2-year-old mice



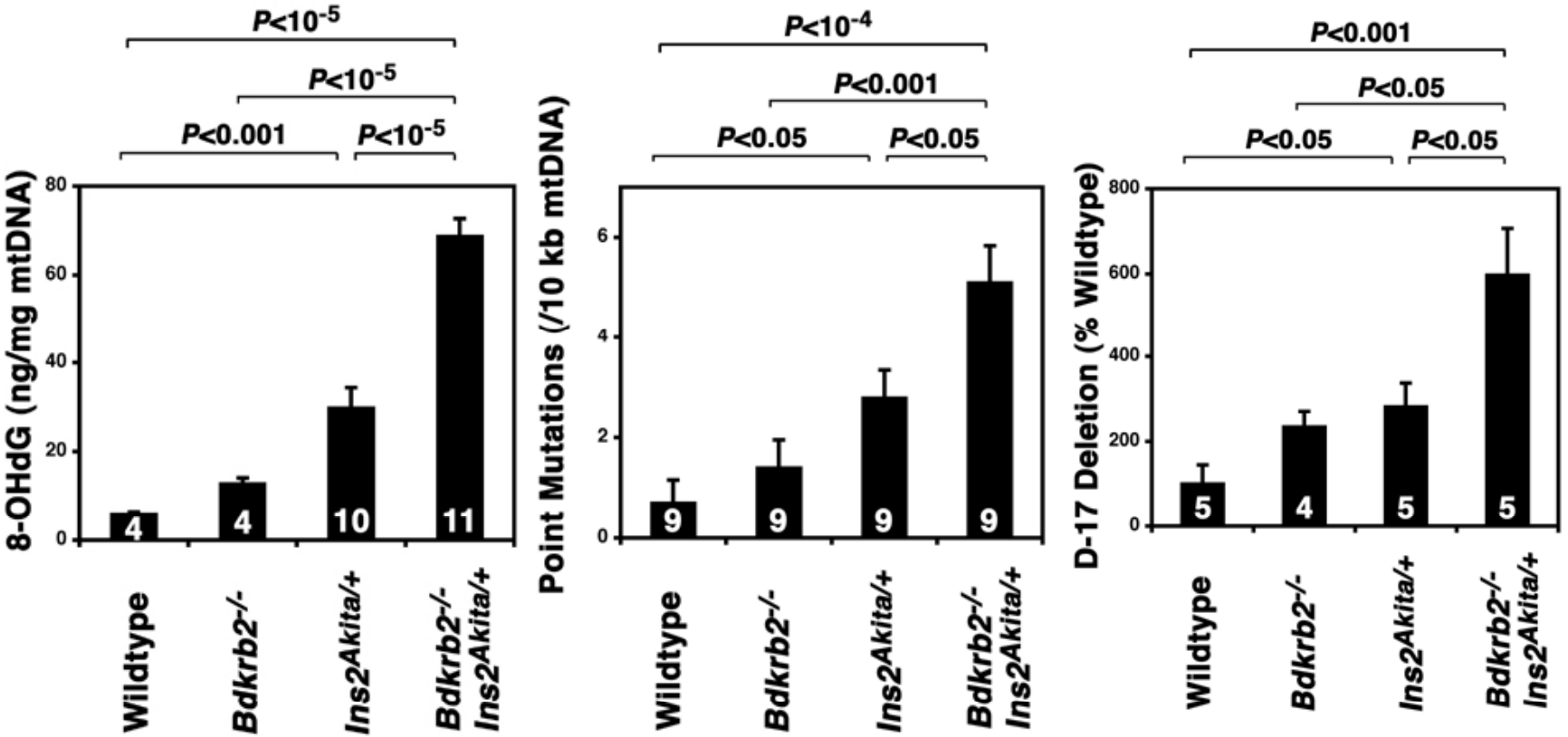
3-year-old mouse



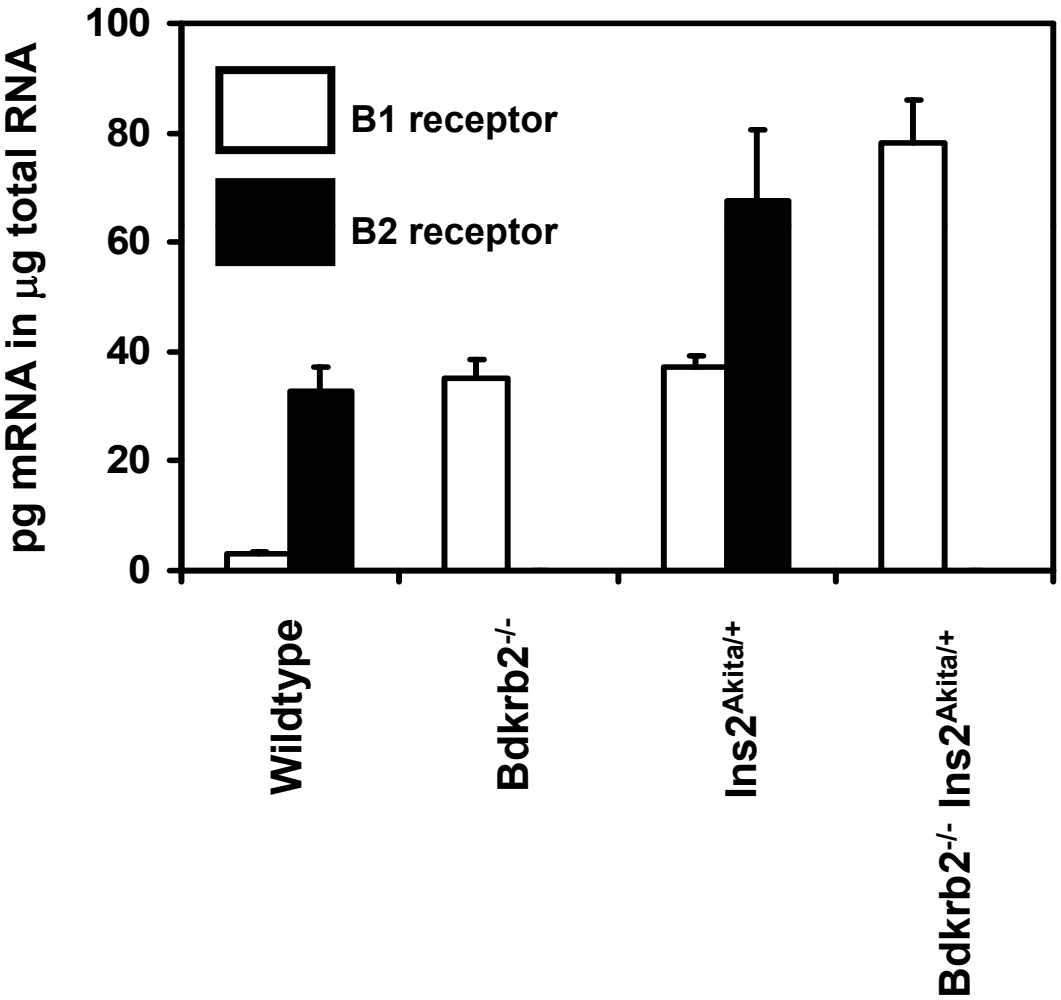
Oxidative Stress in B2R-null and Akita diabetic mice



mtDNA damages in the kidney of 12-month-old male mice



mRNA in the kidney of the four types of animals



Specific aim 1 will determine the effect on diabetic complications of eliminating both bradykinin receptors

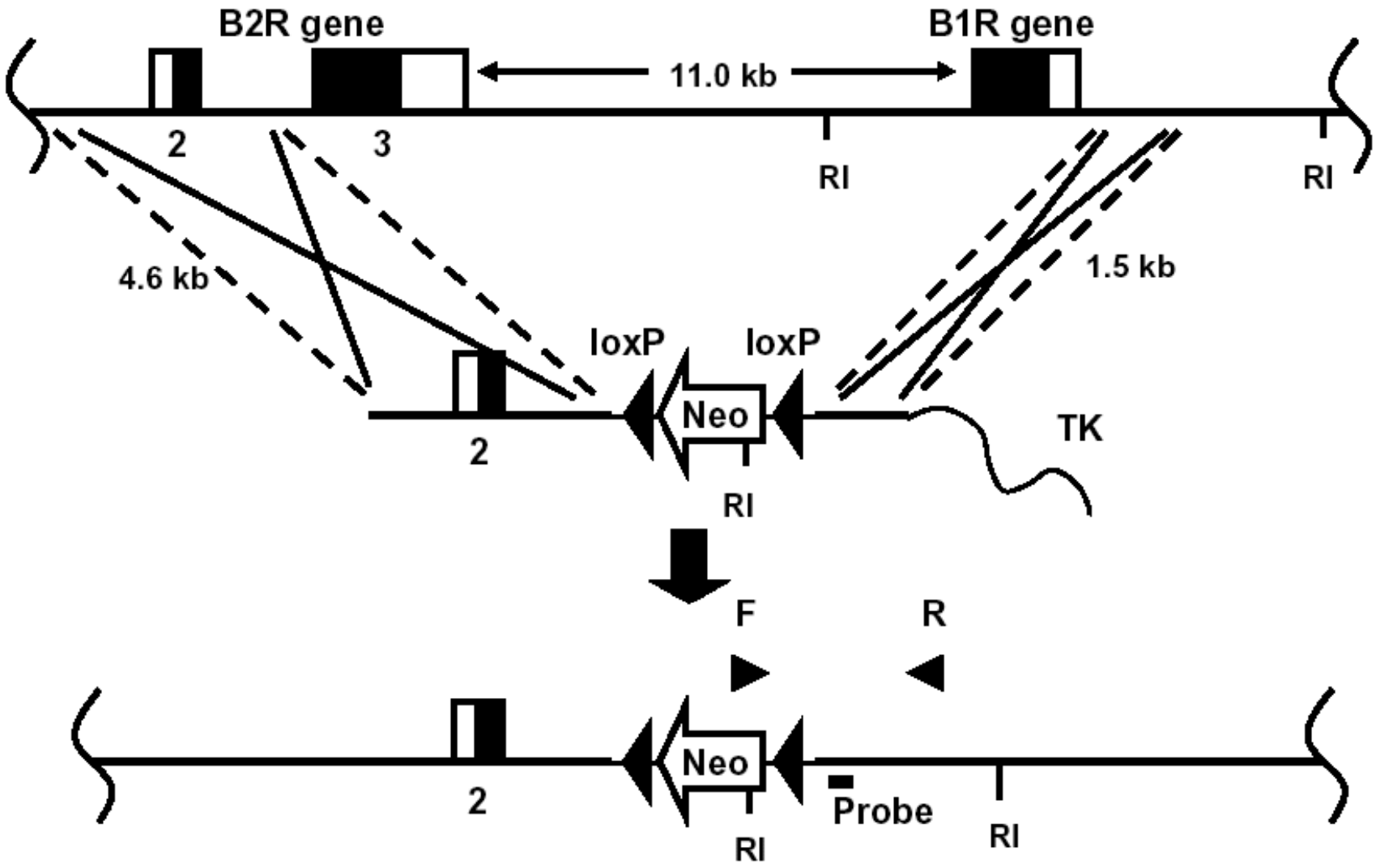
Specific aim 1a: Global KO (bdkrb2/bdkrb1 ^{tm1Mki})

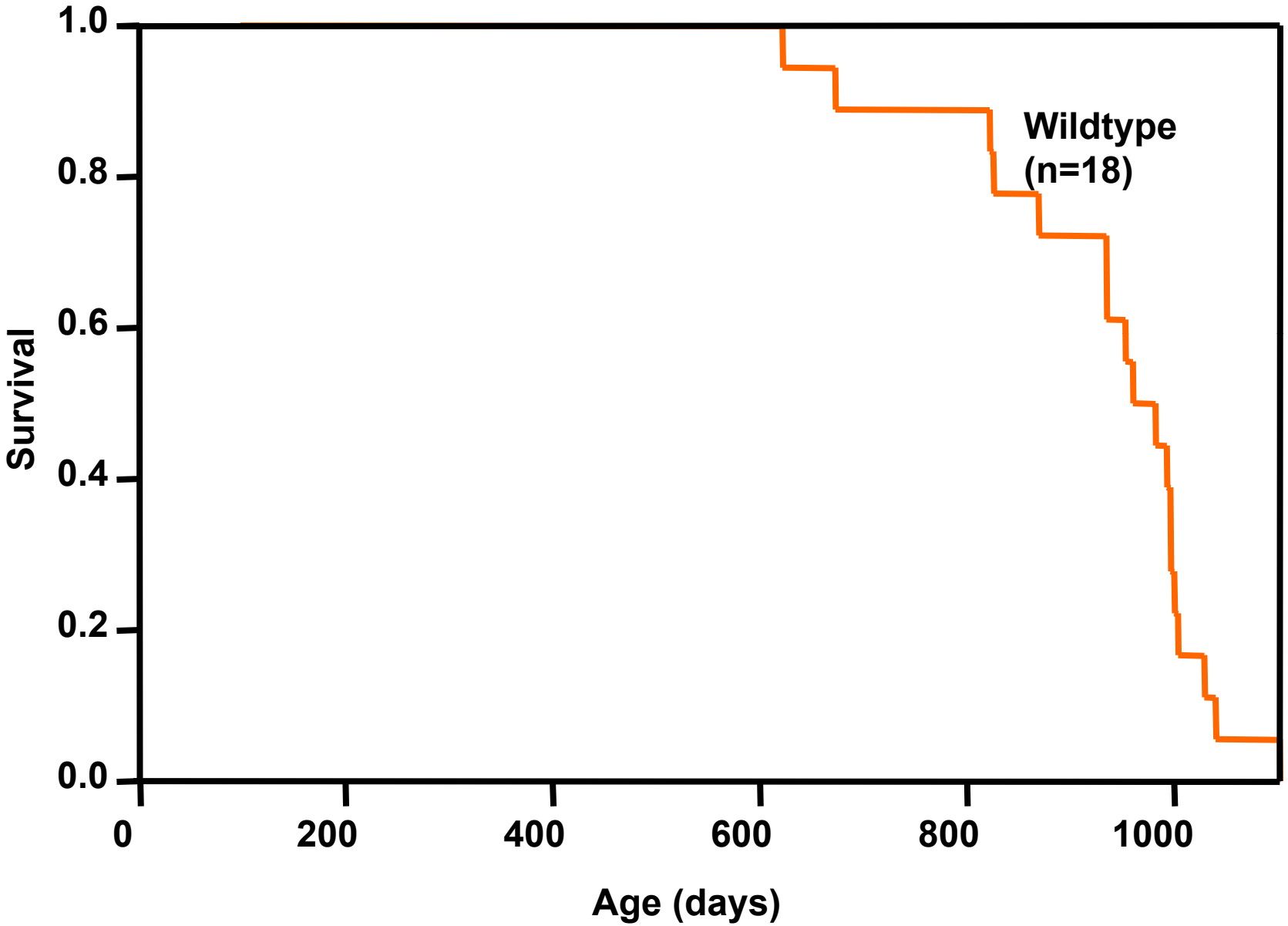
We are analyzing the phenotypes of
B1RB2R global KO mice
with or without Akita mutation in Ins2 gene (Ins2^{Akita}).

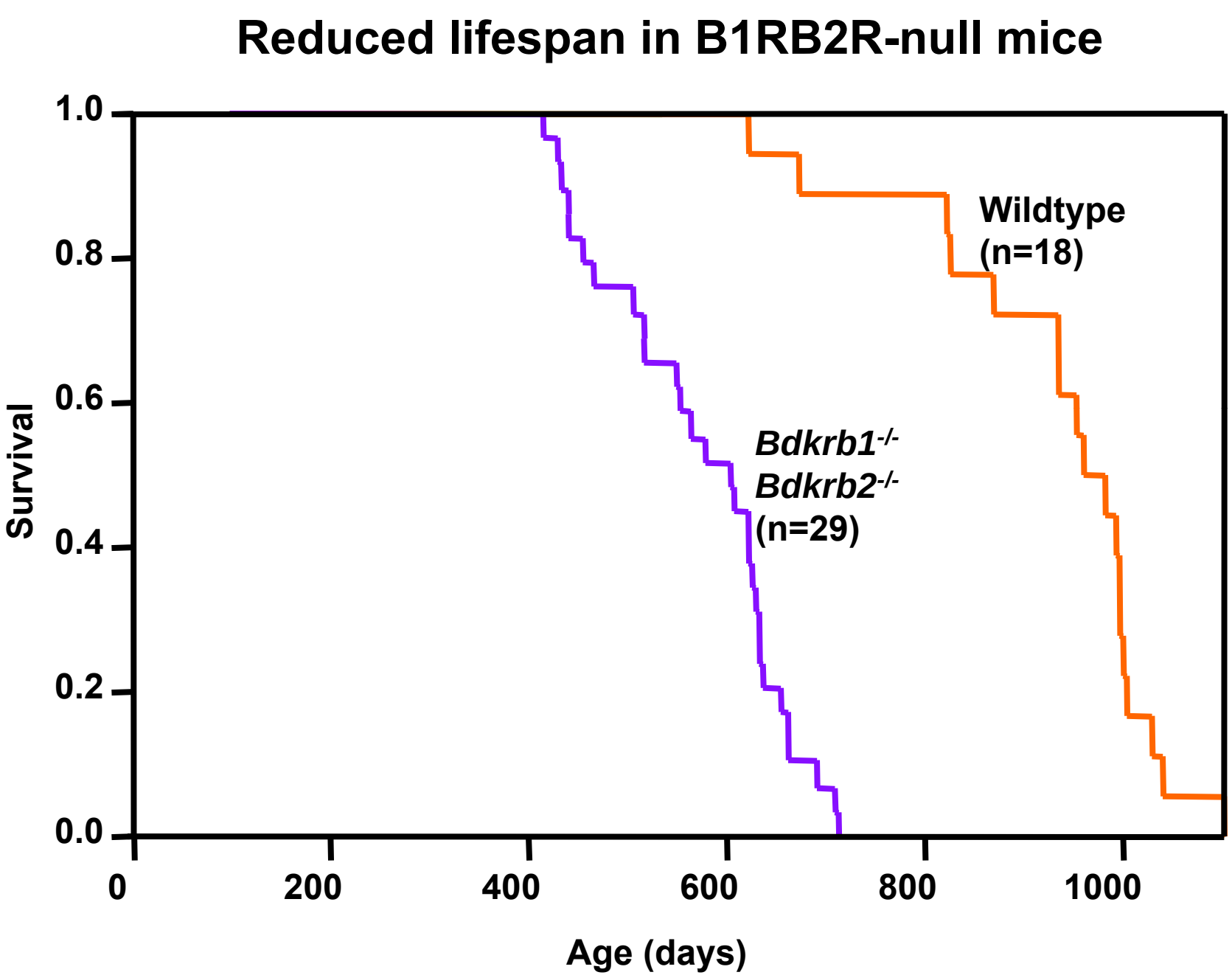
Specific aim 1b: Conditional KO

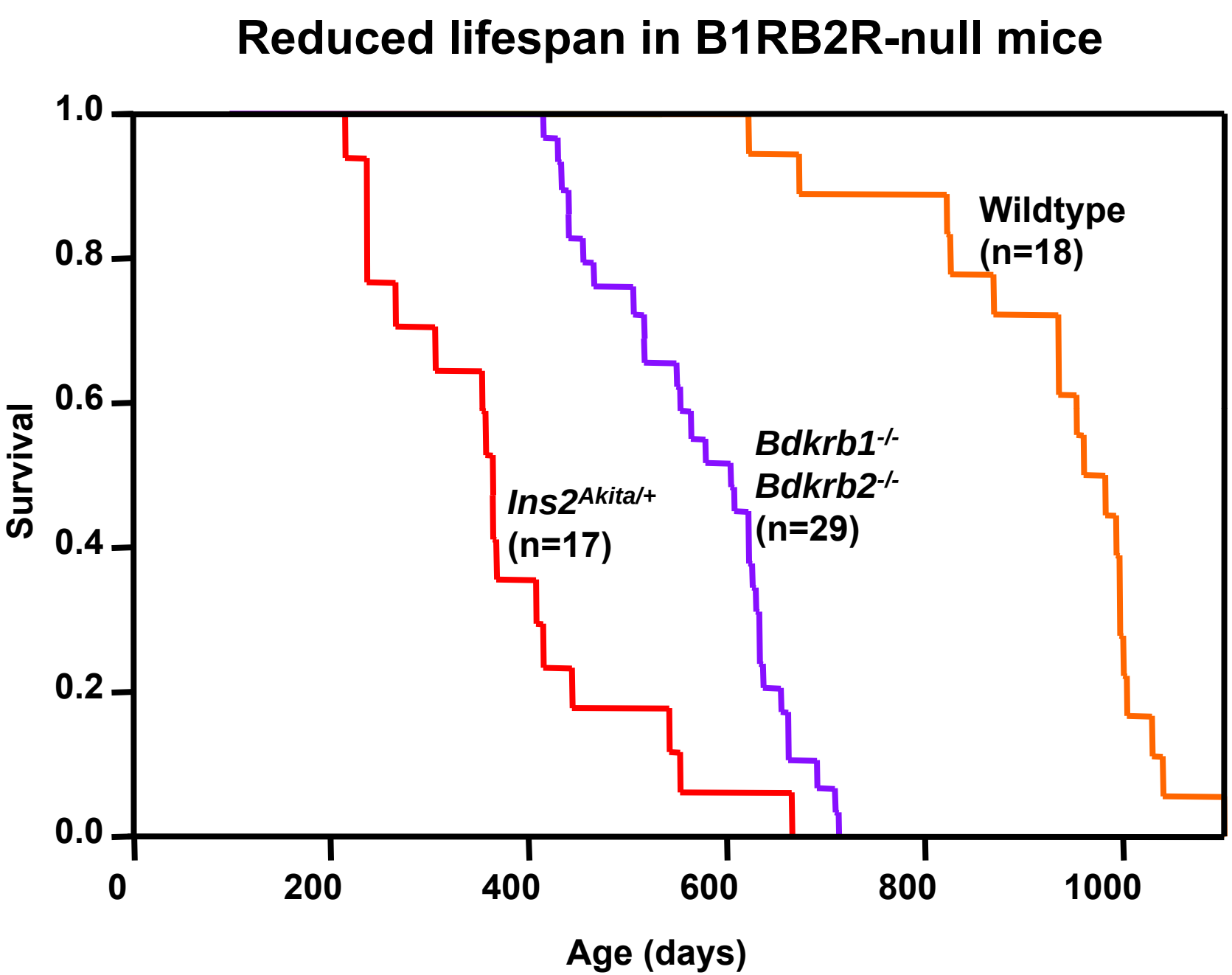
Targeting vector is under construction.

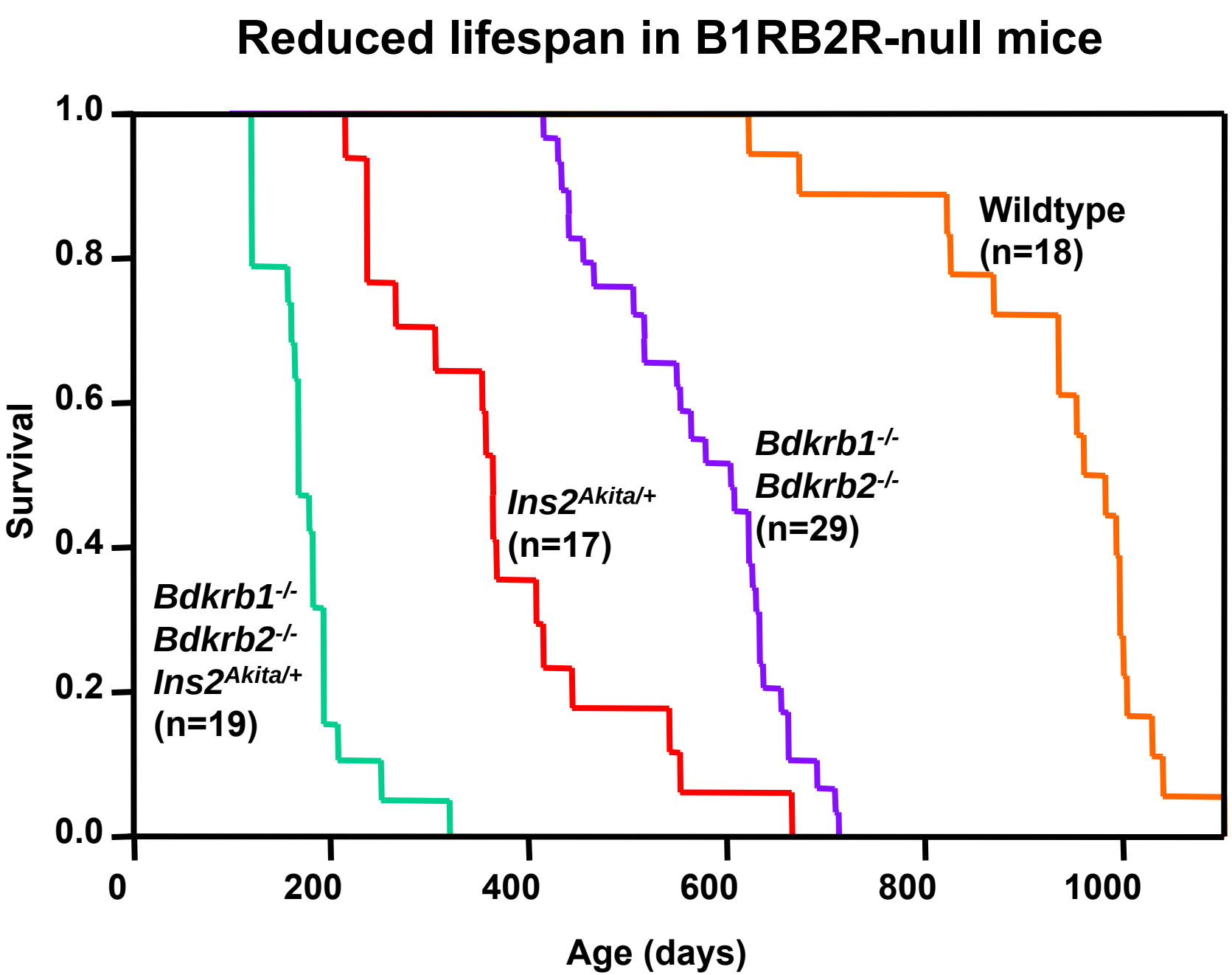
Disruption of both B1 and B2 receptor genes (Global KO)




Reduced lifespan in B1RB2R-null mice
1.0
0.8
0.6
0.4
0.2
0.0
Survival
0
200
400
600
800
1000
Age (days)
Wildtype
(n=18)


Reduced lifespan in B1RB2R-null mice
1.0
0.8
0.6
0.4
0.2
0.0
Survival
0
200
400
600
800
1000
Age (days)
Wildtype
(n=18)
Bdkrb1-/-
Bdkrb2-/-
(n=29)


Reduced lifespan in B1RB2R-null mice
1.0
0.8
0.6
0.4
0.2
0.0
Survival
0
200
400
600
800
1000
Age (days)
Wildtype
(n=18)
Bdkrb1-/-
Bdkrb2-/-
(n=29)
Ins2Akita/+
(n=17)


Reduced lifespan in B1RB2R-null mice
Survival
1.0
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0.6
0.4
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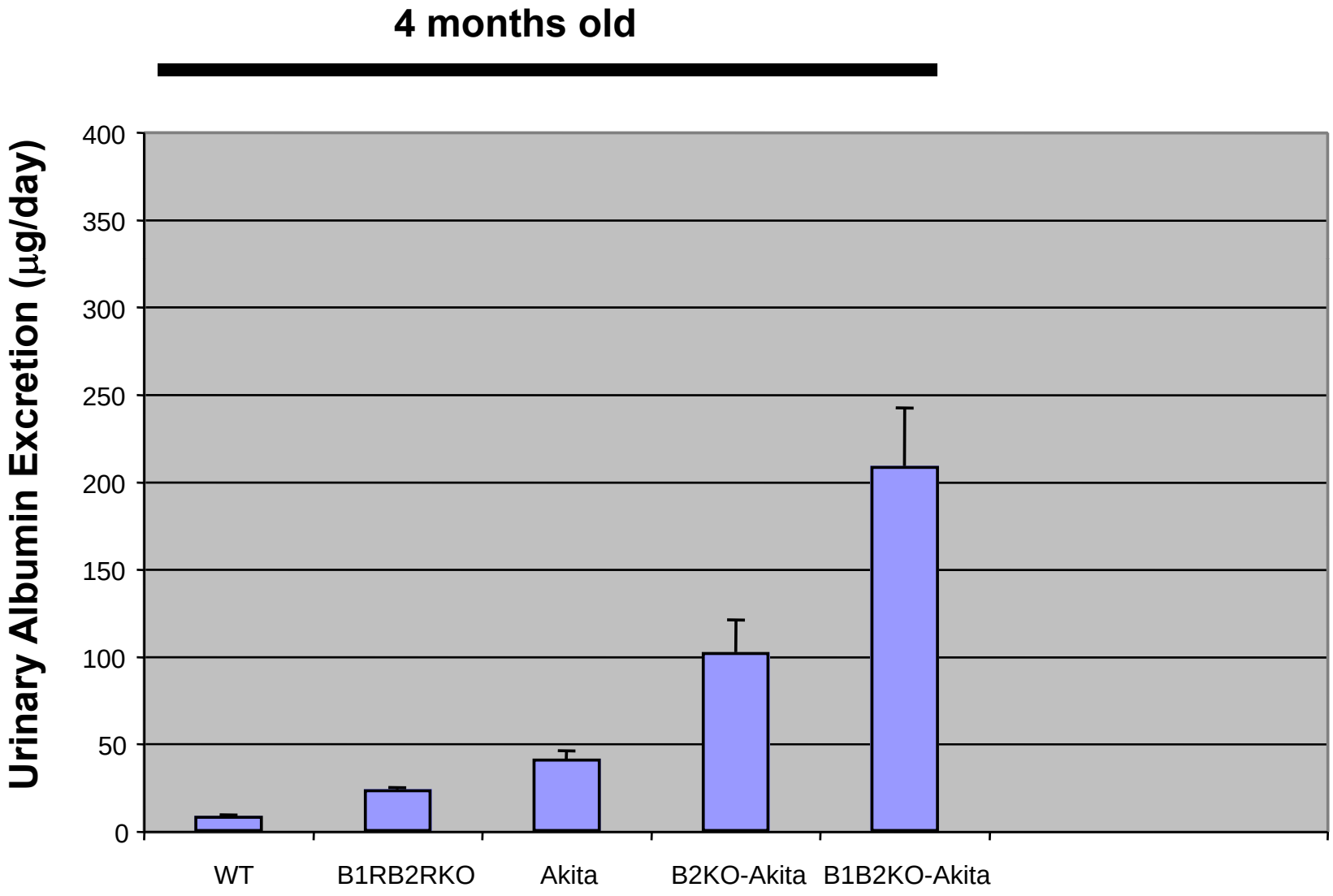
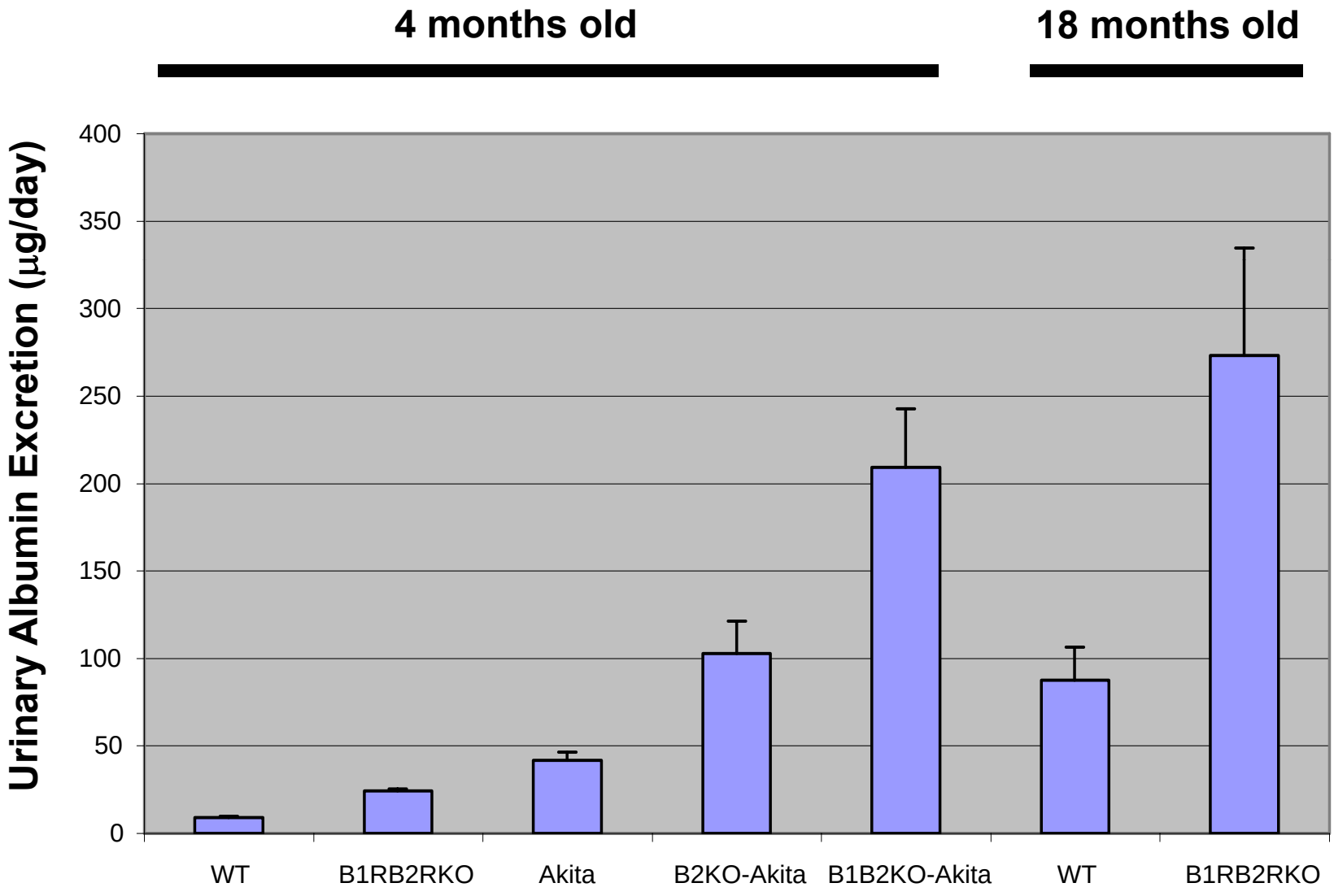
0.0
0
200
400
600
800
1000
Age (days)
Wildtype
(n=18)
Bdkrb1-/-
Bdkrb2-/-
(n=29)
Ins2Akita/+
(n=17)
Bdkrb1-/-
Bdkrb2-/-
Ins2Akita/+
(n=19)

Albuminuria depends on the presence of diabetes, bradykinin receptors and senescence

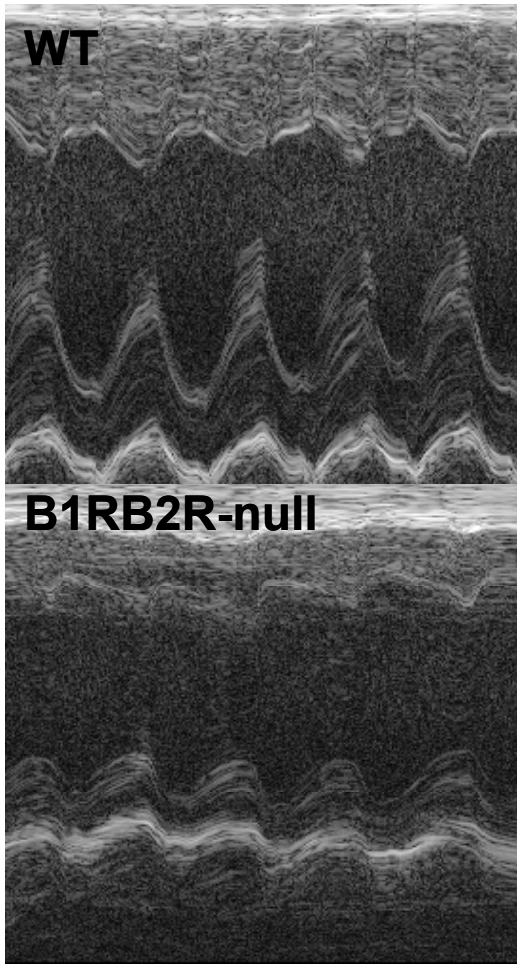


Albuminuria depends on the presence of diabetes, bradykinin receptors and senescence

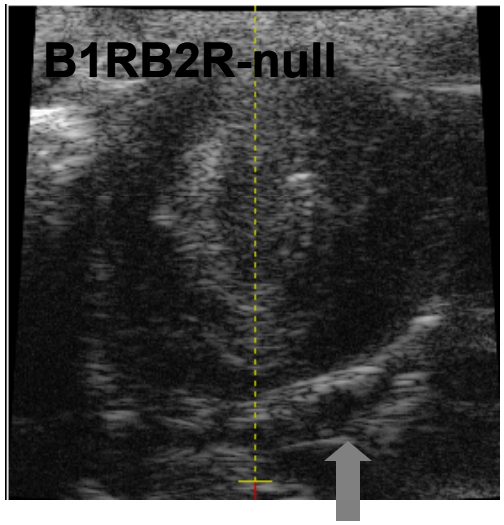


Impaired cardiac function in 1.5-yr-old B1RB2R-null mice

M-Mode

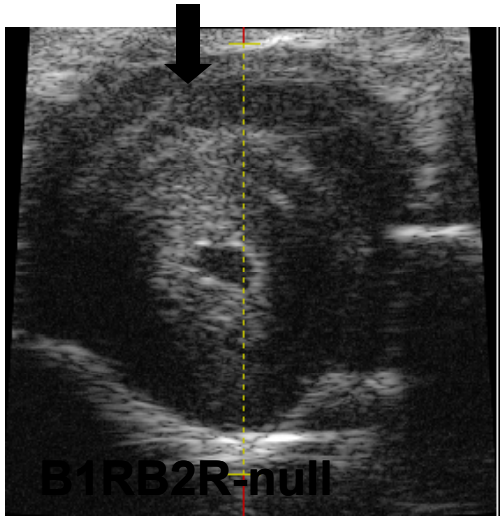


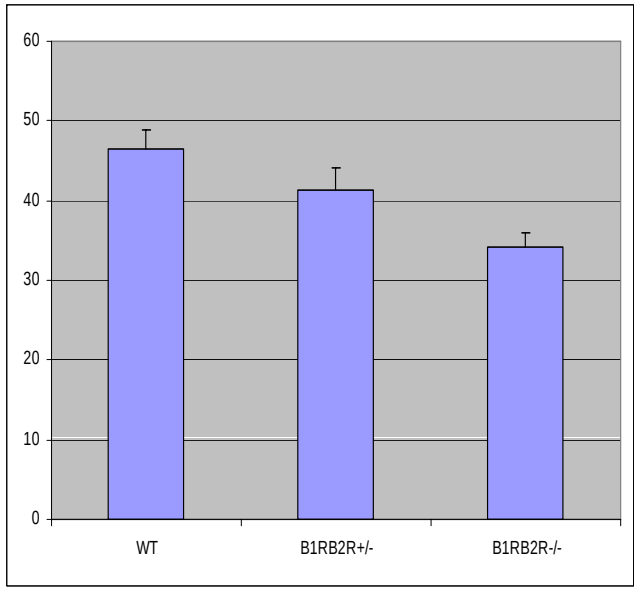
B-Mode

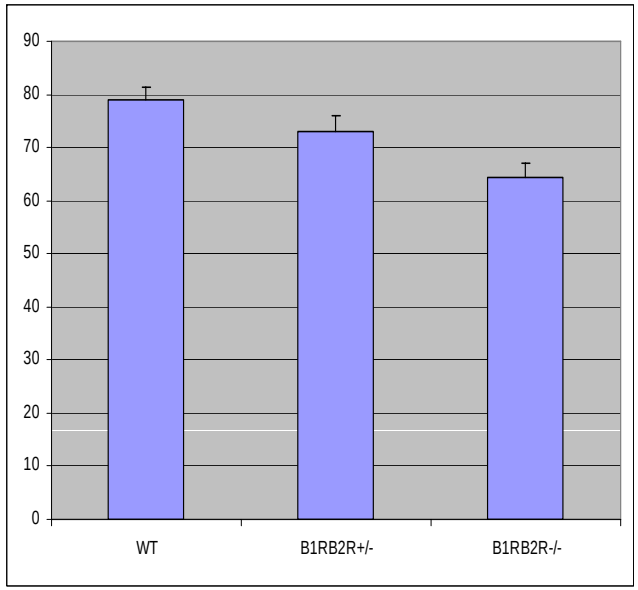


Pericardiac effusion

Right ventricular dilatation



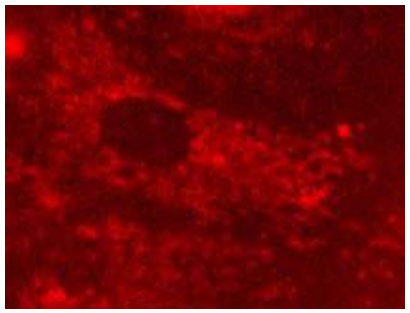




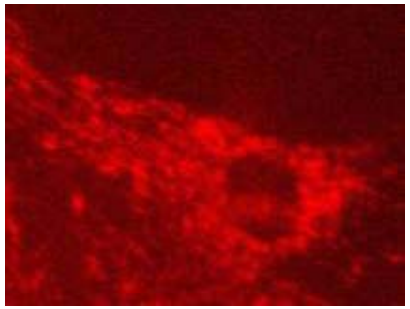
Enhanced oxidative metabolism and premature growth arrest in B1RB2R-null MEFs

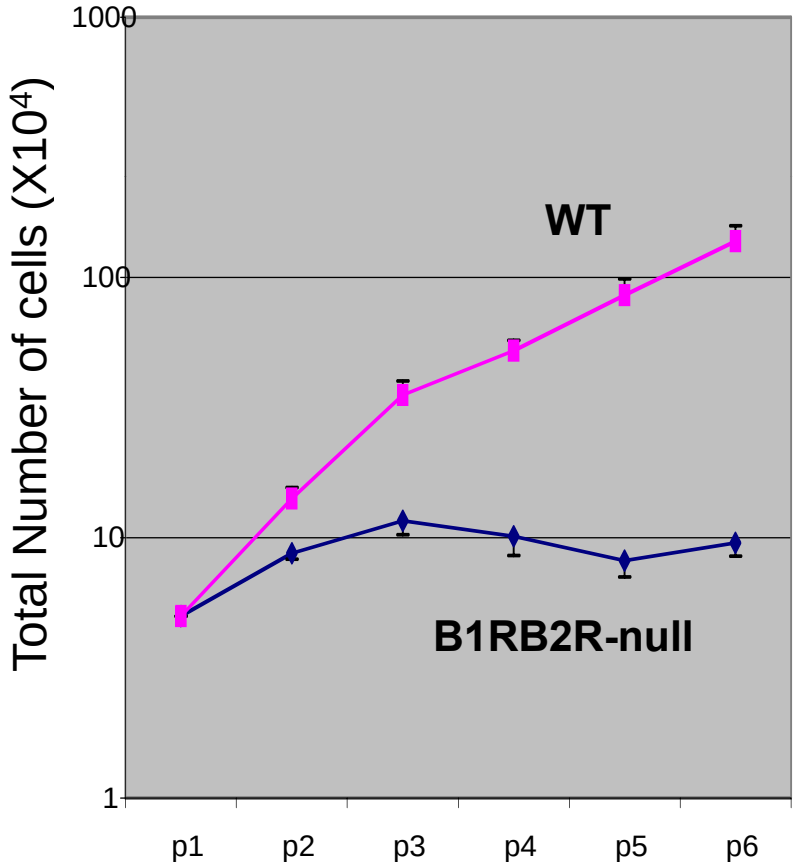
MitoTracker Red CM-H_2XRos Fluorescence

WT (passage 3)



B1RB2R-null (passage 3)





Summary

(1) Absence of the two receptors for bradykinin (B1R and B2R) leads to a less life span than WT mice with and without Akita diabetes.

(2) Urinary albumin excretion increases by diabetes, absence of B1R and B2R and aging.

(3) Absence of B1R and B2R leads to reduced cardiac function in aged mice.

(4) B1RB2R-null MEFs have premature replicative senescence associated with enhanced oxidative metabolism.

Future Directions

(1) Completing conditional B1RB2R-null construct

(2) Collaborative studies of cardiac and neuronal function in B1RB2R-null and/or Akita mice

(3) Studying mechanisms for premature senescence using B1RB2R-null embryonic fibroblasts

(4) Studying male-female differences in the phenotypes of the B1RB2R-null and/or Akita mice

Nobuyuki Takahashi

Specific Aim 2: To investigate the relationship between glomerular damage and mtDNA mutations in eNOS-/- diabetic mice.

Lack of eNOS exacerbates DN?

Role of a high fat diet?

Mechanisms?

eNOS deficiency exacerbates DN.

Zhao, Breyer, Harris et al. JASN 2006

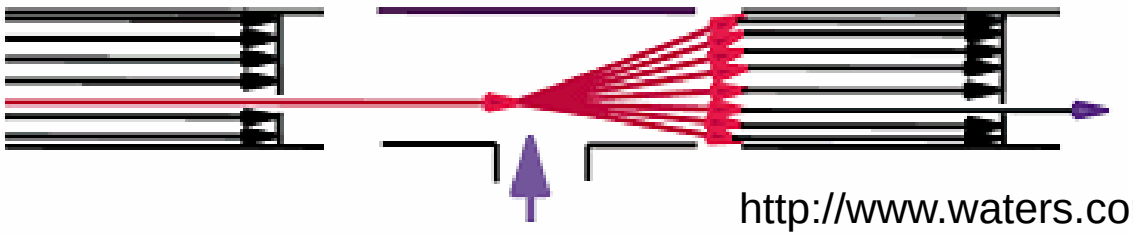
Nakagawa et al. JASN 2006

Kanetsuna, Breyer, Harris et al. AJPath 2007

8 groups of mice used for experiments (all C57BL/6)

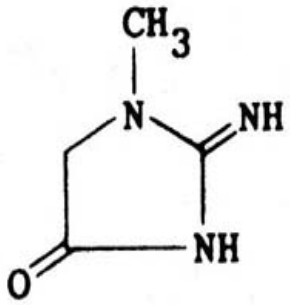
diet	diabetes (low dose STZ)	genotype	
normal chow (NC) (17% cal fat)	non-DM	WT (NN)	NN NC
		eNOS-/- (nn)	nn NC
	DM	WT	NN NC DM
		eNOS-/-	nn NC DM
high fat (HF) diet (42% cal fat)	non-DM	WT	NN HF
		eNOS-/-	nn HF
	DM	WT	NN HF DM
		eNOS-/-	nn HF DM

LC-MS/MS for creatinine measurement

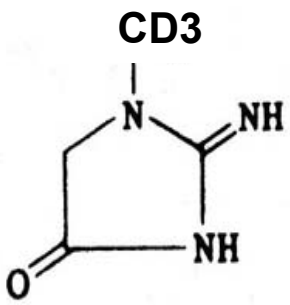


http://www.waters.com

collision gas



MW=114



MW=117

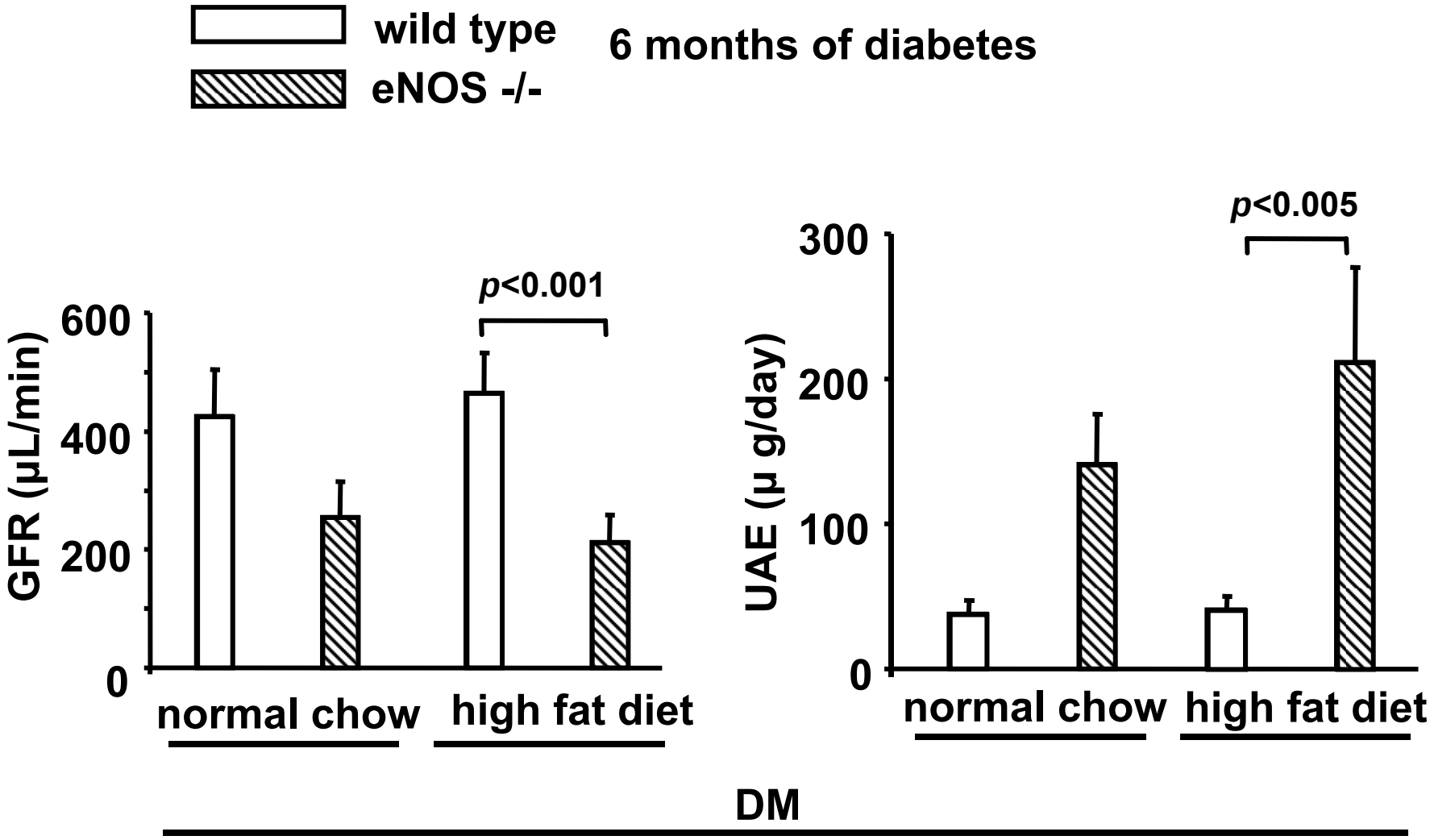
Cre m/z=114 m/z 114 → 44

Cre-D3 m/z=117 m/z 117 → 47

background ↓

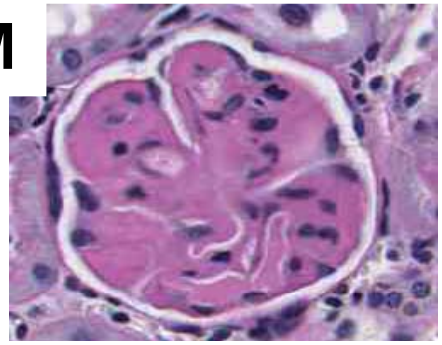
LC-MS/MS is 6 times more sensitive than HPLC, specific, simple, fast, and inexpensive, and requires small sample volumes (10 μl). Takahashi et al. KI 2007, 71: 266

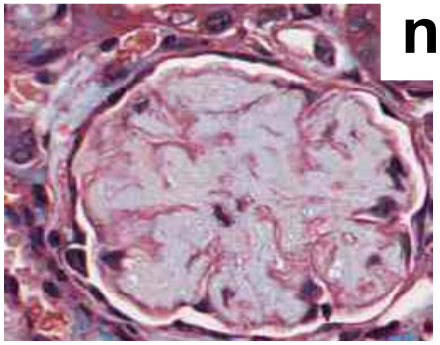
GFR (creatinine clearance) and urinary albumin excretion



Severe glomerulosclerosis & tubulointerstitial fibrosis in nn DM mice (normal chow, NC)

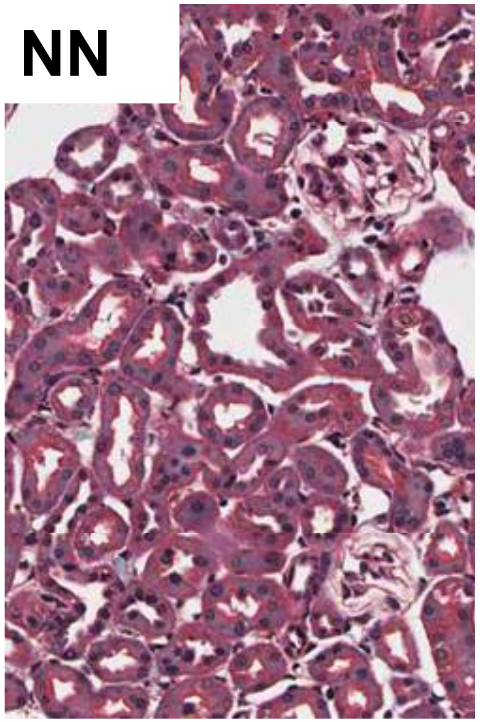
nn DM



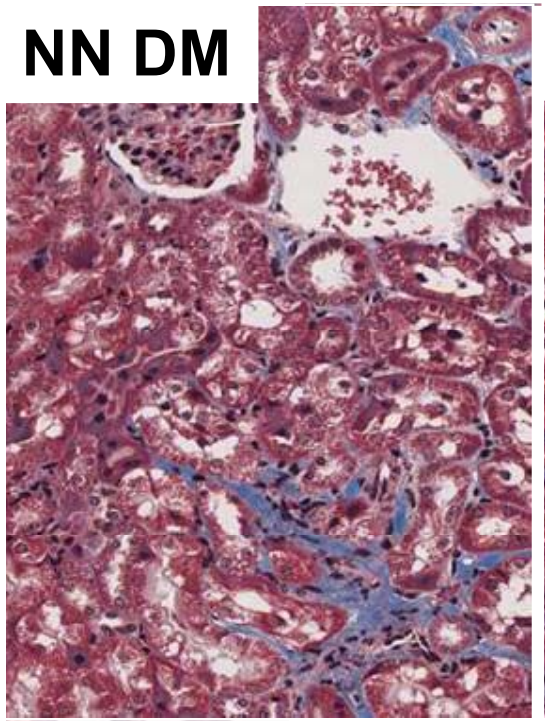


nn DM

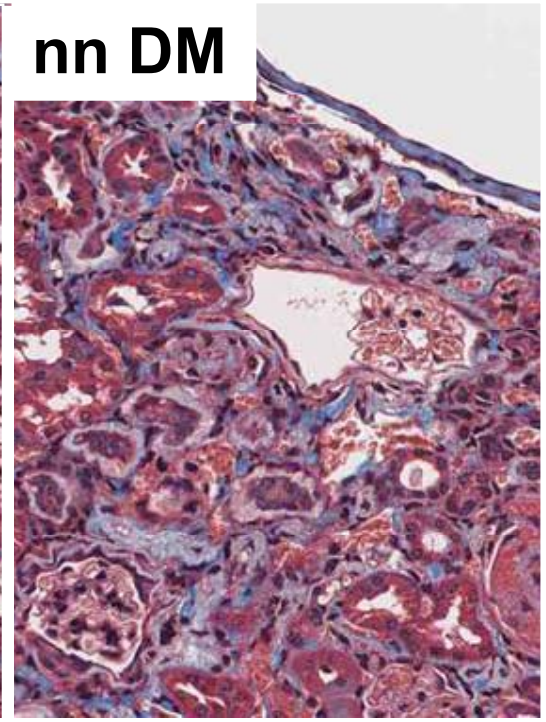
NN



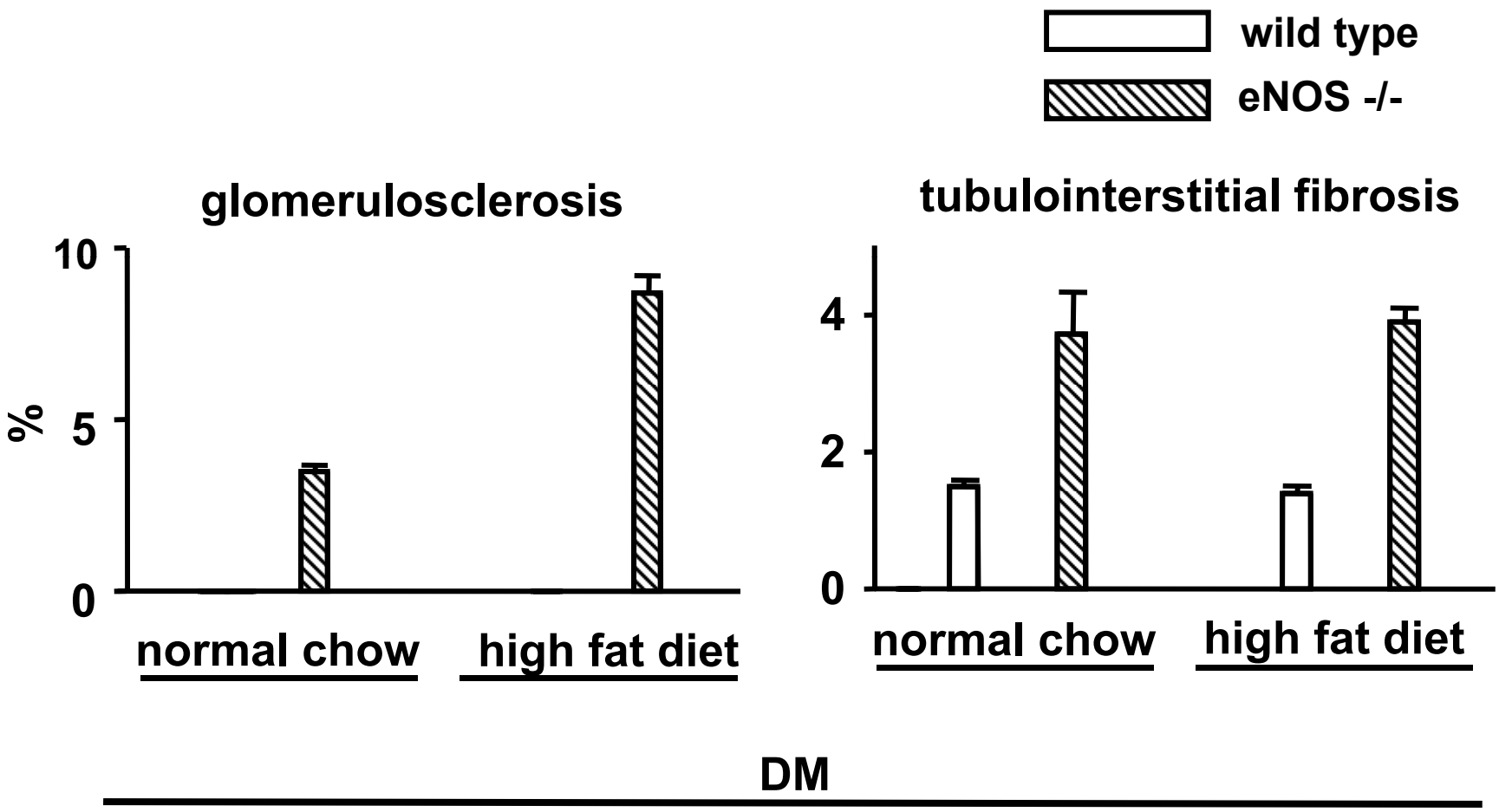
NN DM



nn DM



Sclerosis & fibrosis in DM mice

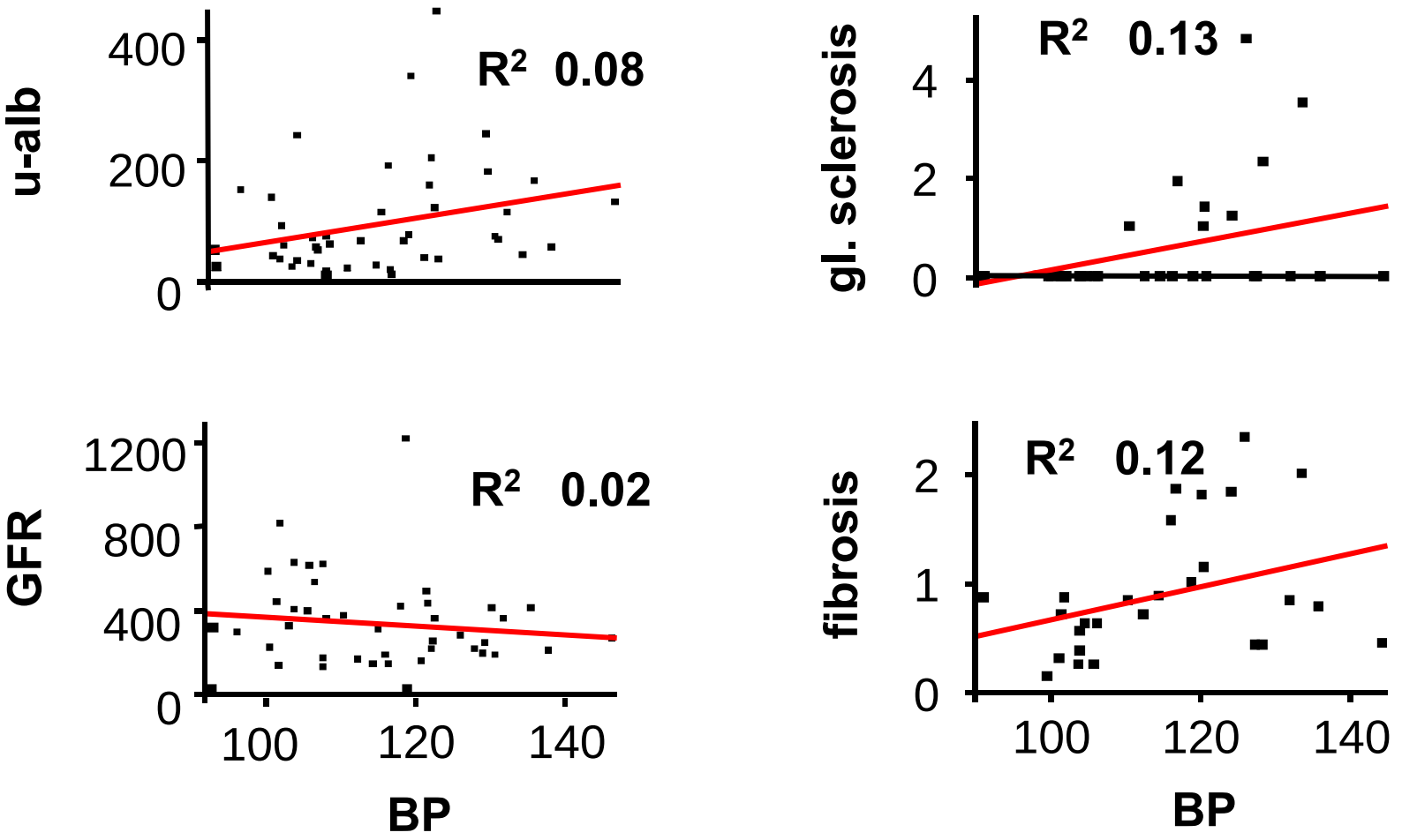


Why lack of eNOS exacerbates DN?

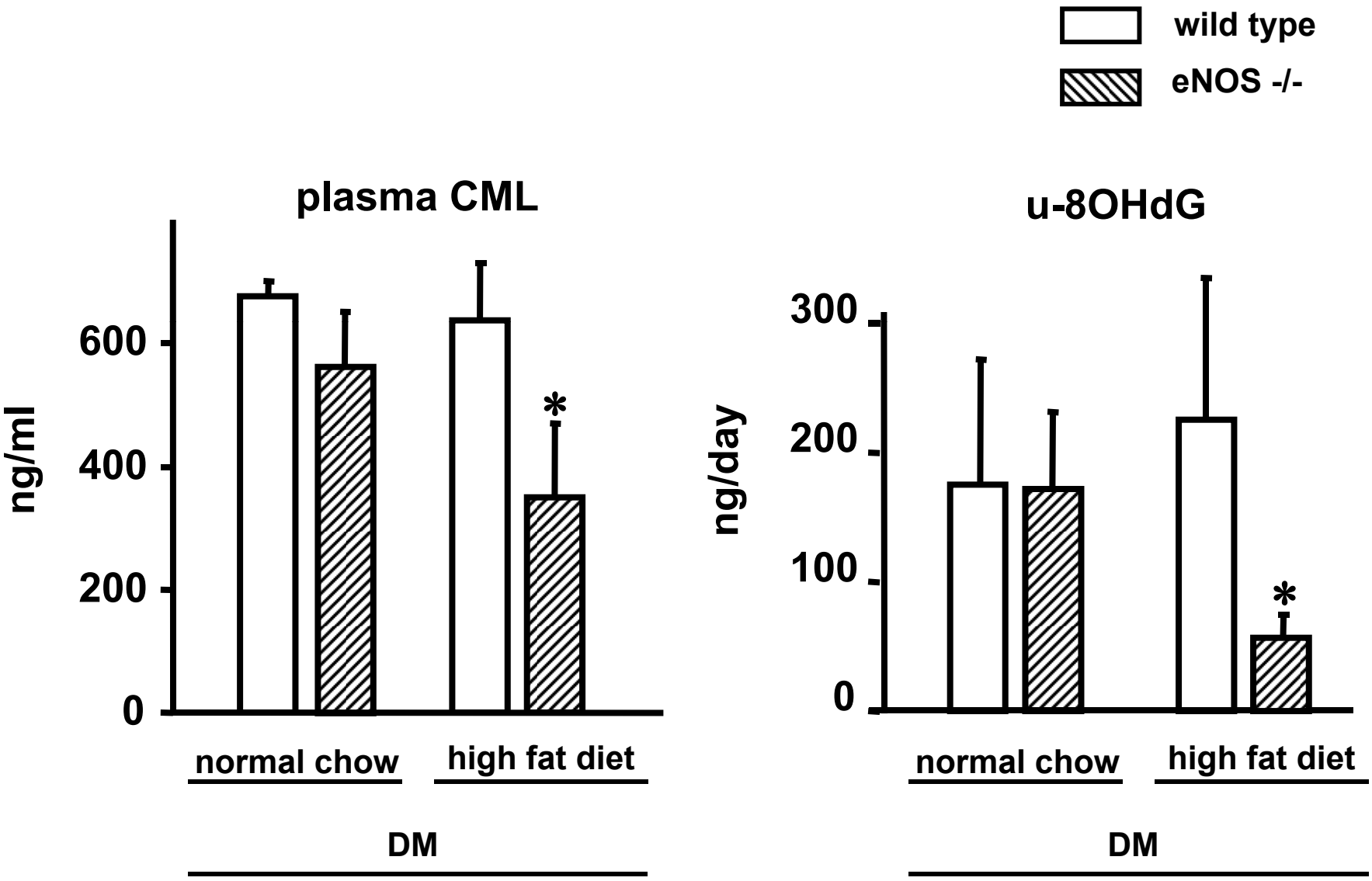
Physiological role of NO:

1. **vasodilator (BP)**
2. **anti-oxidant**
3. **anti-coagulation**
4. **inhibition of leukocyte adhesion**

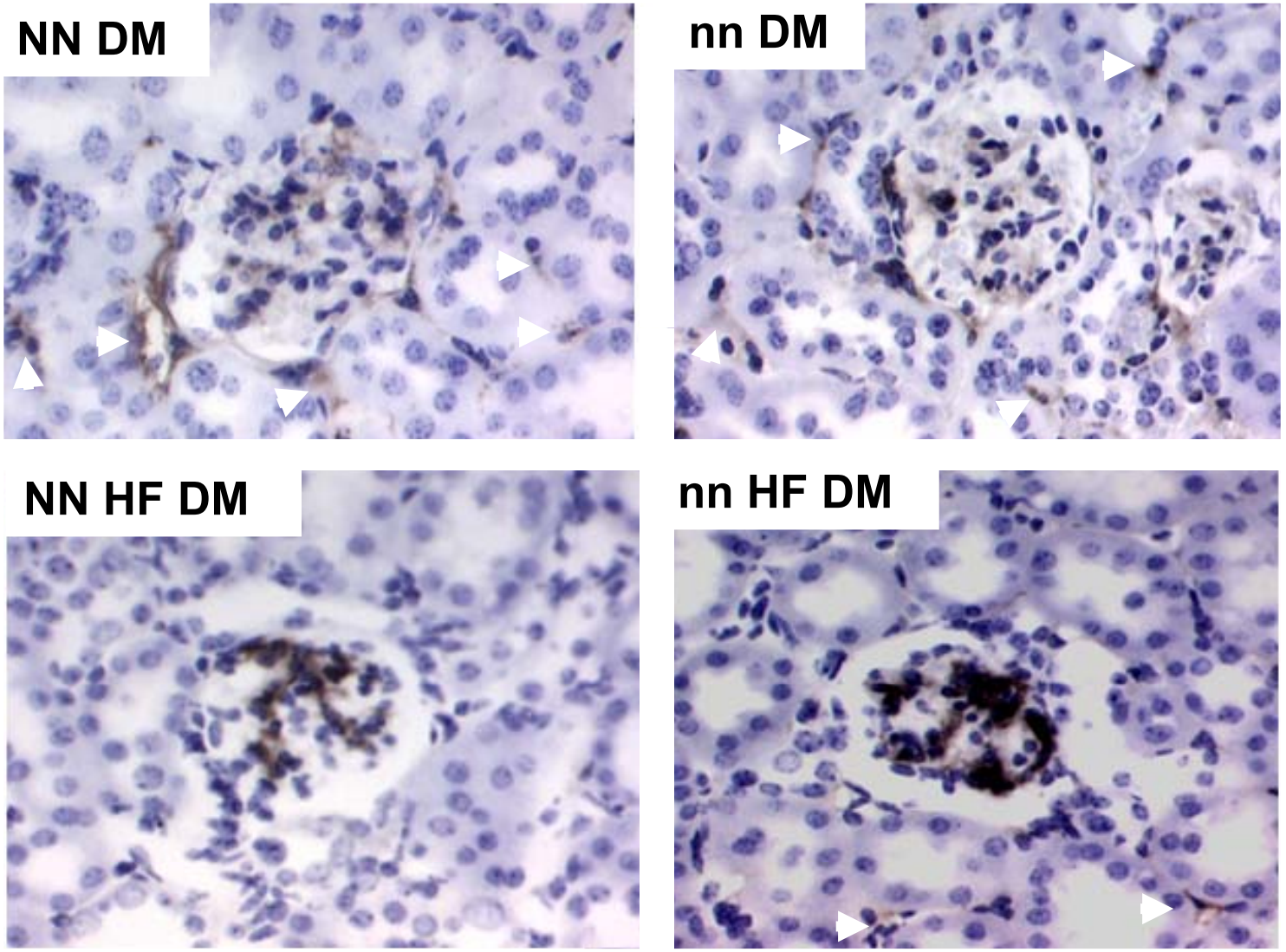
BP does not explain much of the variation of DN.



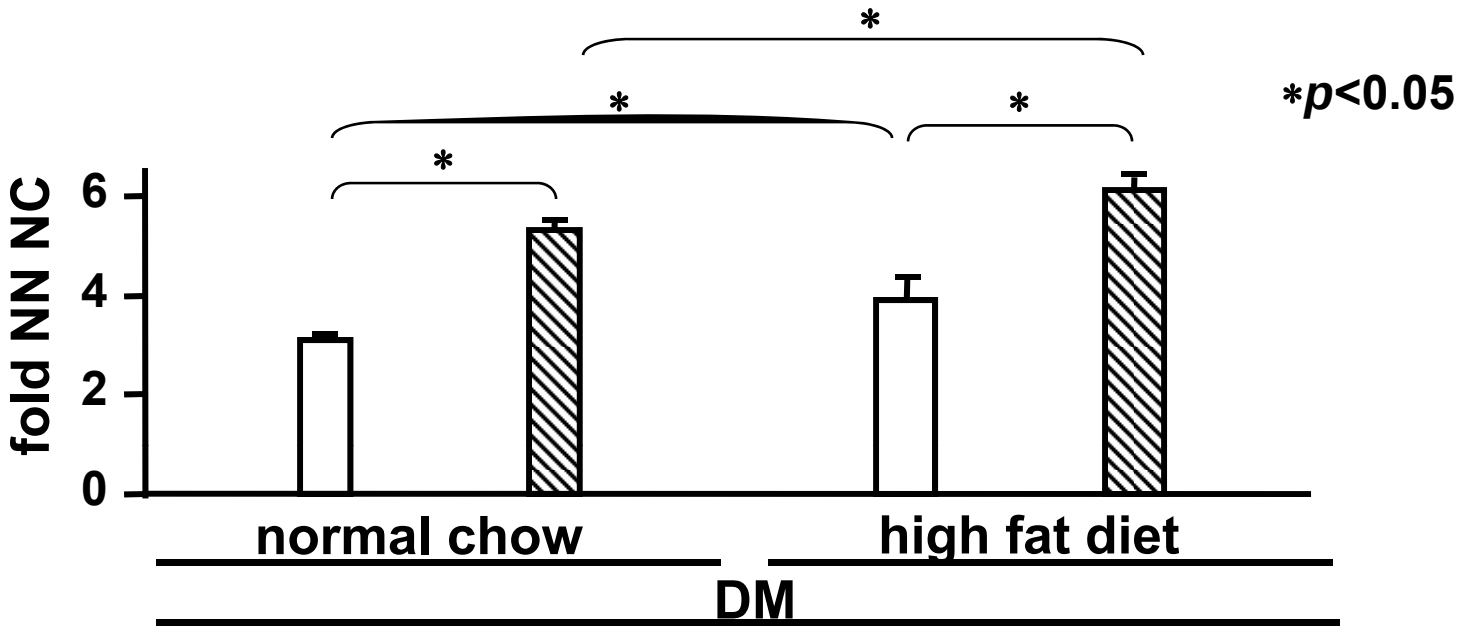
Oxidative stress of nn DM is not higher than that of NN DM.

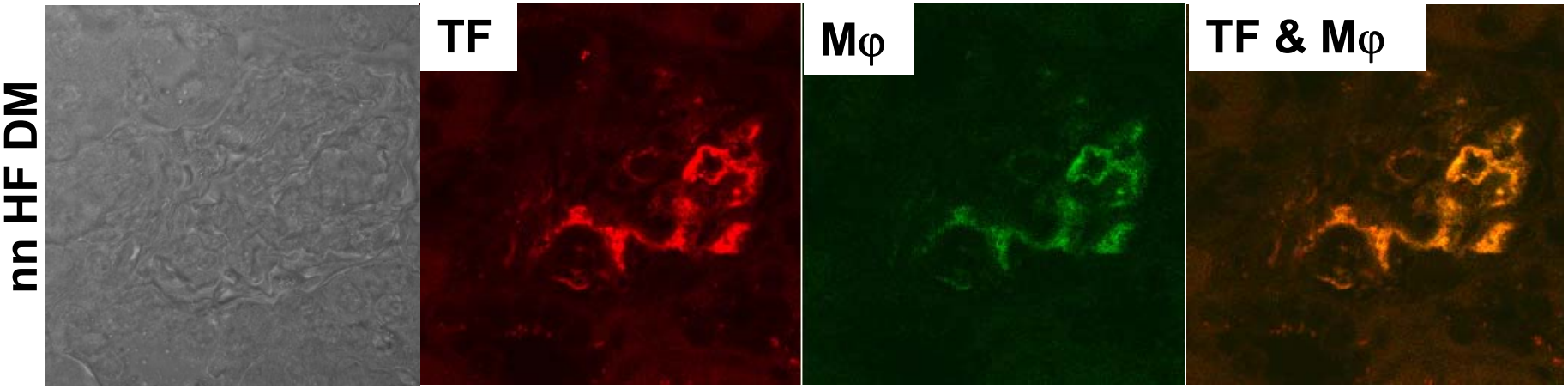


Most abundant fibrin in nn HF DM glomeruli



DM, nn, and HF all Increase Renal Tissue Factor

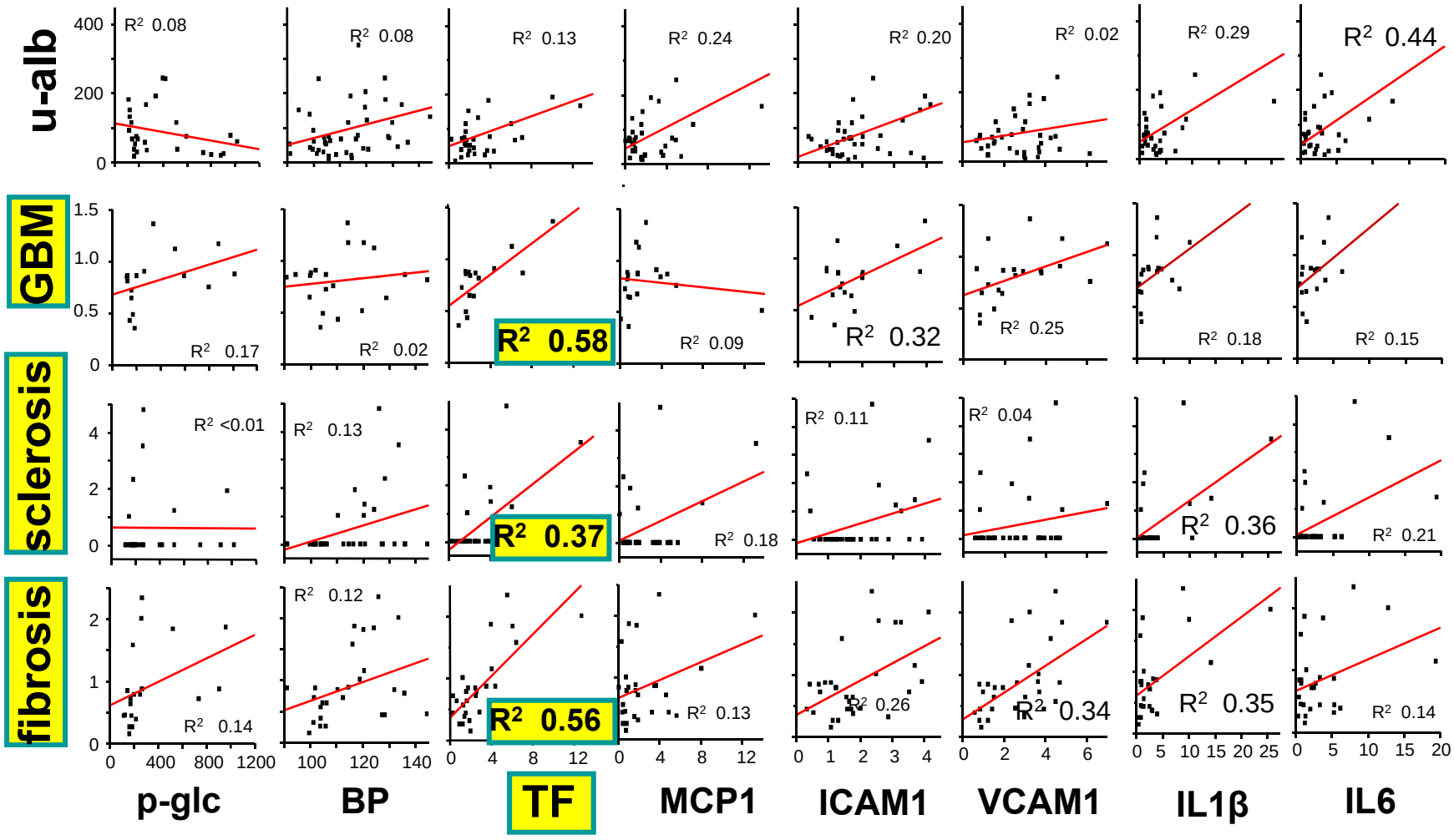




Rat anti-mouse TF moAb from Dr. Daniel Kirchhofer (Genetech)

Mφ: MOMA-2

DN correlates best with TF.



Summary & Conclusion

1. Lack of eNOS and HF additively exacerbate DN without further increasing oxidative stress.

2. Lack of eNOS and HF increase fibrin and TF in diabetic glomeruli.

3. TF is highly expressed in glomerular Mφ, and correlates well with DN.

Current & future direction

Is TF pathogenic??

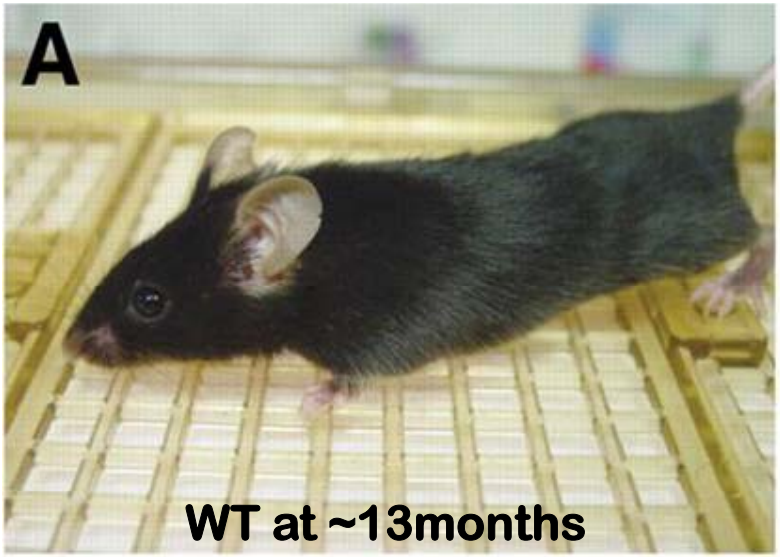
1. **To delete TF only in Mono-Mφ of eNOS-/- DM mice by transplanting bone marrow of [CD11b-Cre]-TF floxed mice to diabetic eNOS-/- mice.**

2. **To inactivate NF-kB pathway only in Mono-M φ of eNOS-/- DM mice by transplanting bone marrow of [CD11b-Cre]-Ikk2 floxed mice to diabetic eNOS-/- mice.**

Raymond Fox

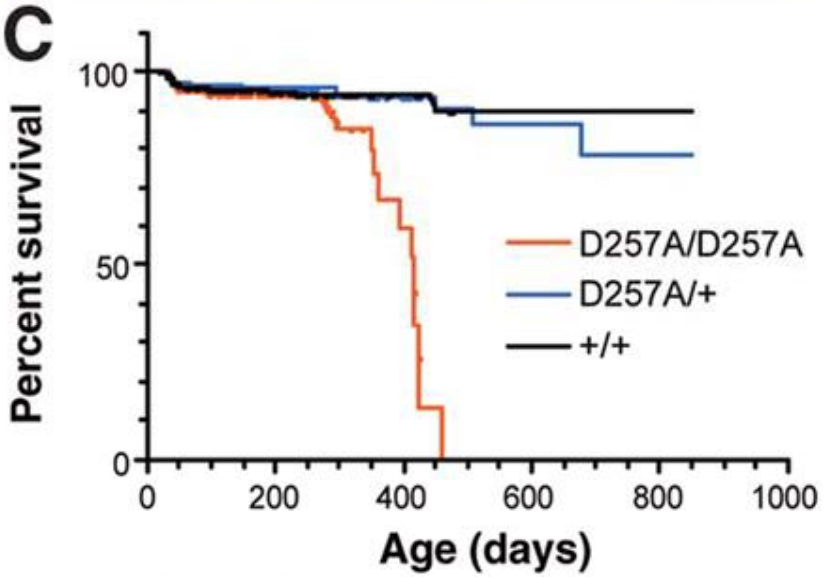
Specific Aim 3.1 : To determine if mutations in mitochondrial DNA enhance organ damage due to diabetes in the absence of increased oxidative stress.

Polg $^{(D257A / D257A)}$ Mice



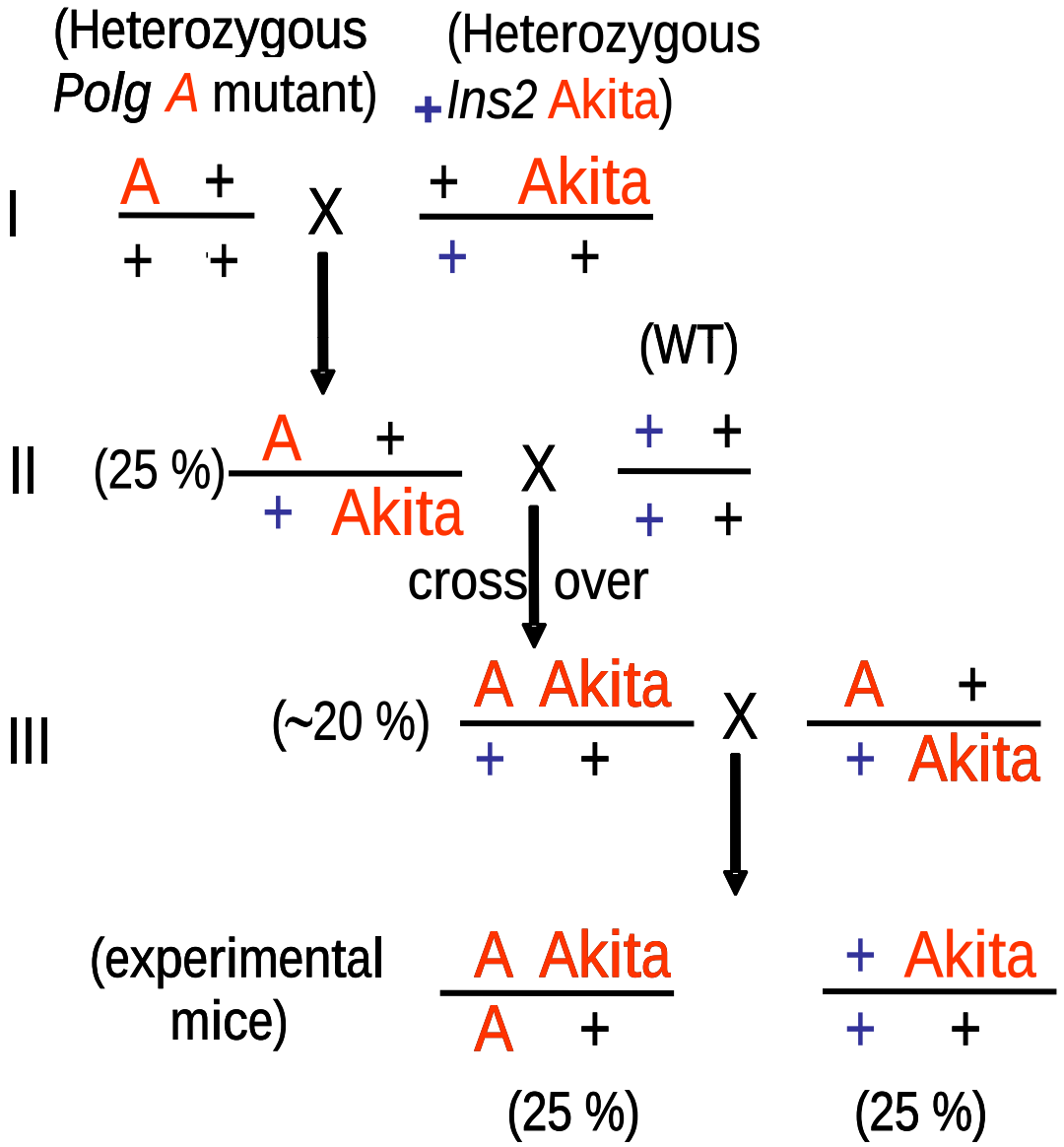
Polg $^{(D257A / D257A)}$ mice.

- Graying hair, hair loss, kyphosis.
- Oxidative stress not increased.
- Apoptosis increased.
- Lifespan reduced.



Greg Kujoth, et al Science (2005) 309: 481-484

Breeding Scheme



Alleles

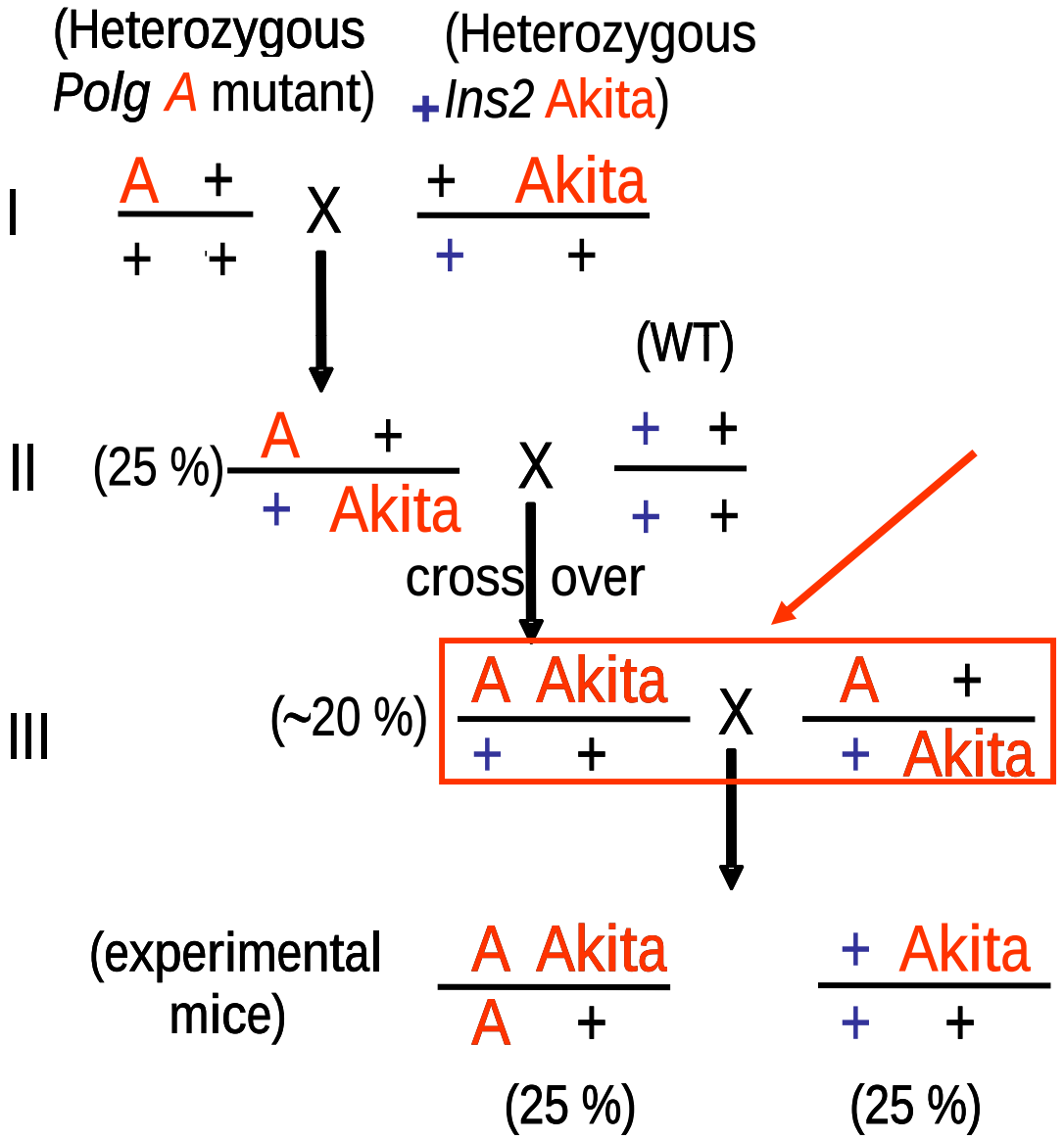
A = *Polg (256A)*

+ = WT

Akita = *Ins2 (86Y)*

Ray Fox

Breeding Scheme



Alleles

A = *Polg (256A)*

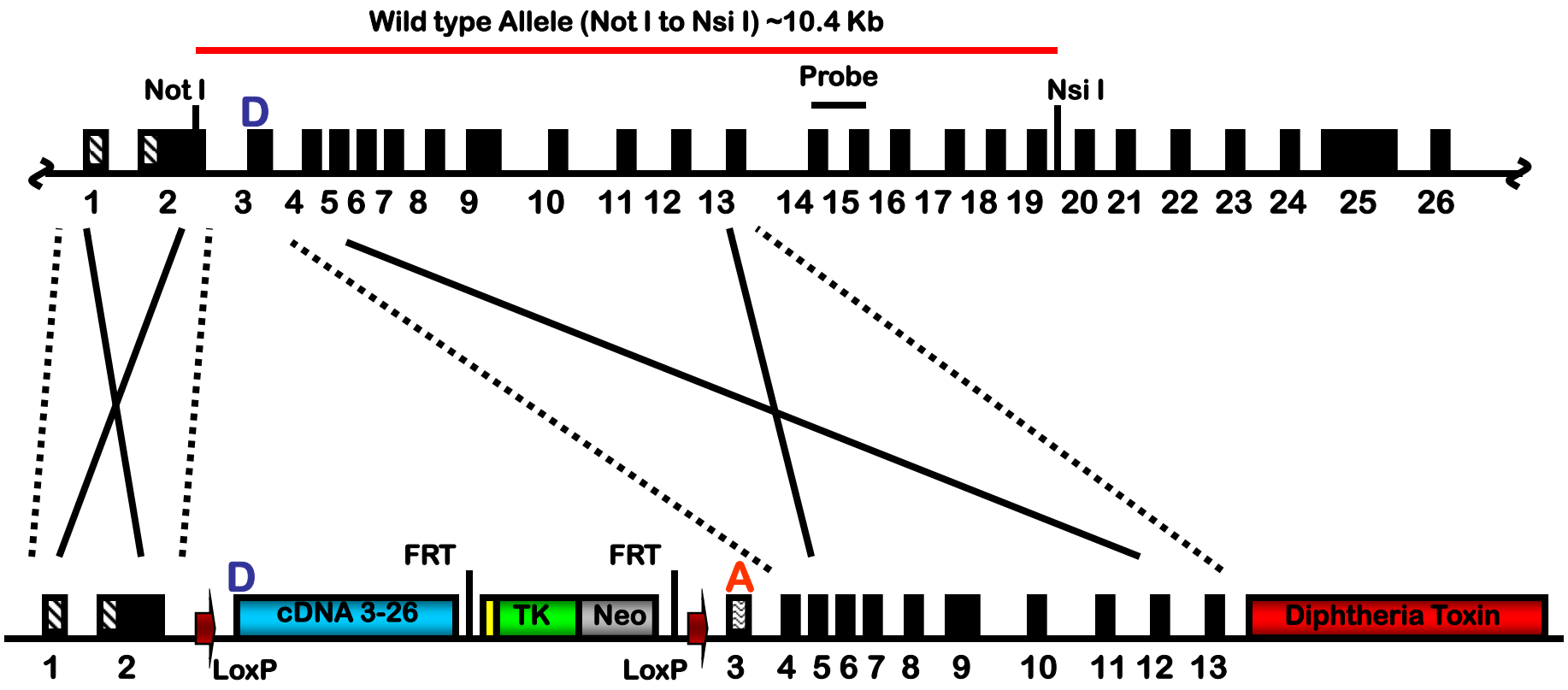
+ = WT

Akita = *Ins2 (86Y)*

Ray Fox

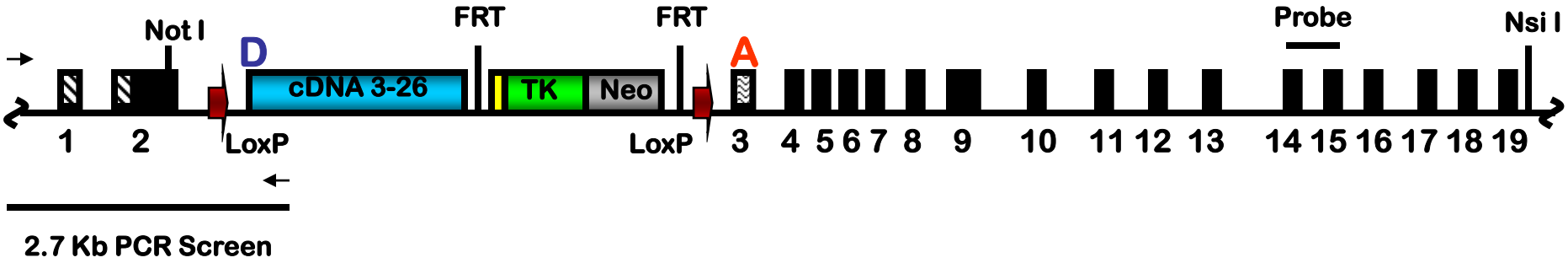
Specific Aim 3.2 : To identify tissues that exacerbate diabetic complications when *Polg* $^{(D257A/D257A)}$ is expressed

Conditional *Polg*^{D257A} Targeting



Ray Fox

Targeted Conditional *Polg* $^{(D257A)}$ Allele



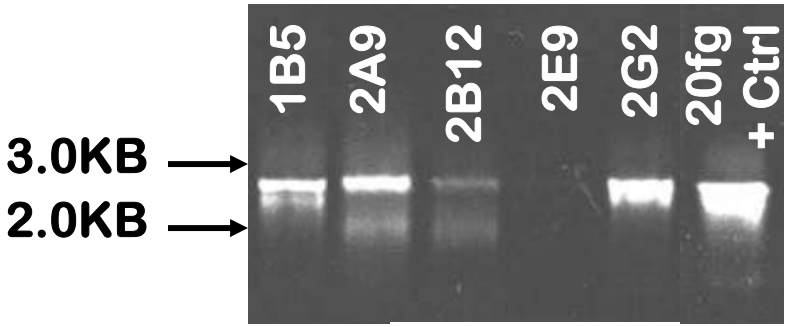
Targeted Allele (Not I to Nsi I) ~13.6 Kb

Tissue Specific
Cre treatment

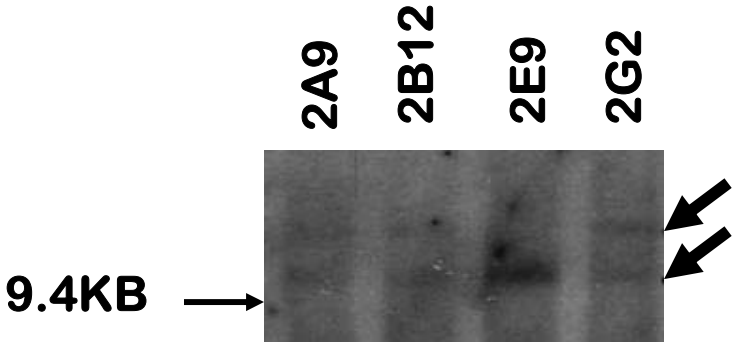
Ray Fox

Targeting the Conditional Allele in Embryonic Stem Cells

A. PCR for ES cell targeting



B. Southern Blot Confirmation



Top Arrow indicates the targeted conditional *Polg* allele (13.6 KB)

Bottom arrow indicates the wild type *Polg* allele (10.7 KB)

C. Real Time PCR Results for Chimeras

Sample ID	Neo	ß-actin	δ Ct	Genotype	Note
310	30.47	28.94	1.53	Neo+	*Postive chimera*
311	40	27.98	12.02	WT	
328	28.82	28.09	0.73	Neo+	*Positive chimera*
329	40	28.13	11.87	WT	
334	40	28.59	11.41	WT	
Ctrl	40	30.91	9.09	WT	
Ctrl	29.28	32.44	-3.16	Neo+	One copy Neo (Germline)

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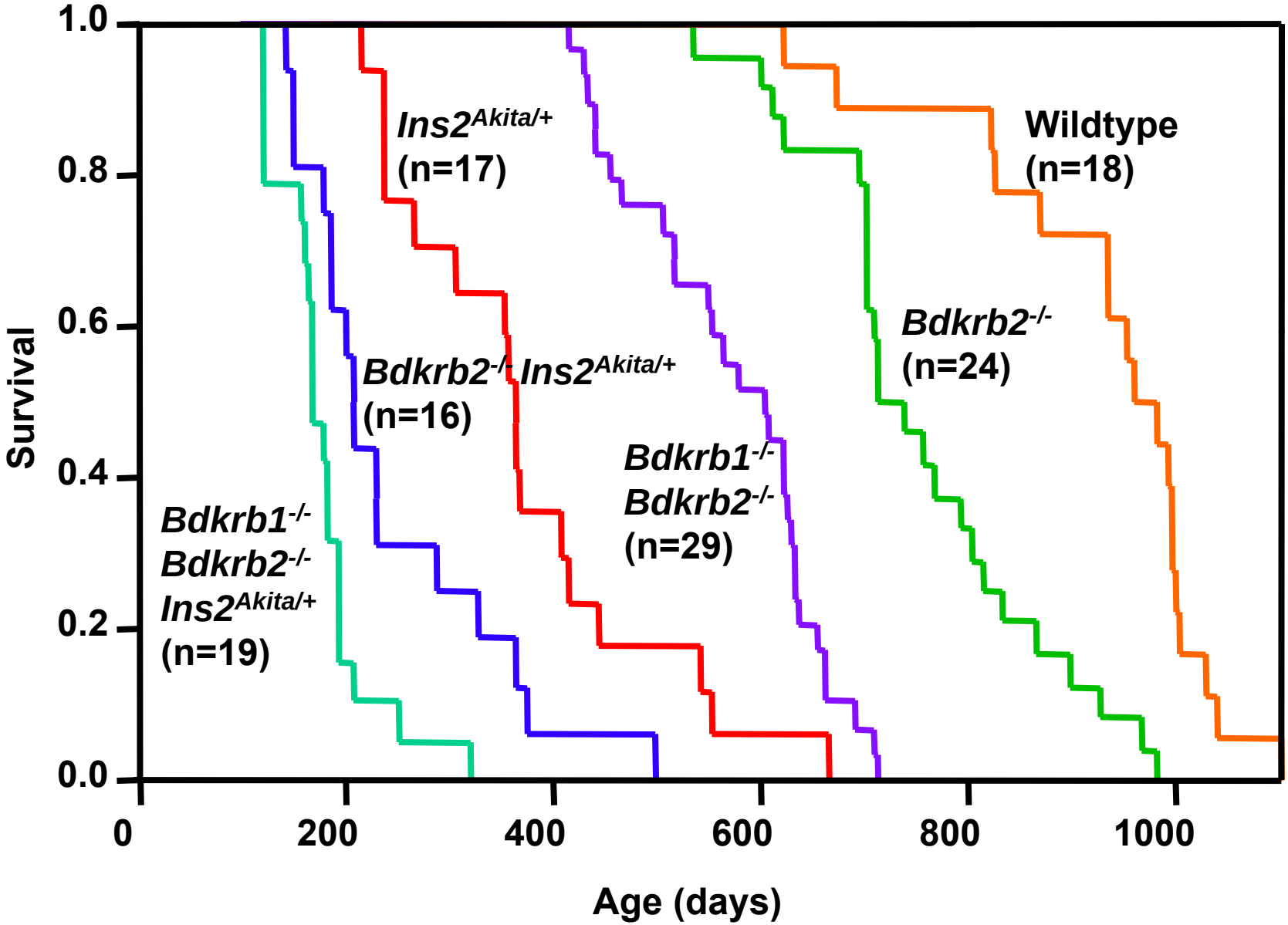
Reduced lifespan in B1RB2R-null mice
Survival
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
1000
Age (days)
Ins2Akita/+
(n=17)
Wildtype
(n=18)
Bdkrb2-/-
(n=24)
Bdkrb2-/- Ins2Akita/+
(n=16)
Bdkrb1-/-
Bdkrb2-/-
(n=29)
Bdkrb1-/-
Bdkrb2-/-
Ins2Akita/+
(n=19)