



Transcriptional regulation of *Atp2b2* determines the severity of AHL in C57BL/6J Mice

Thesis Defense
Rebecca Minich
December 9, 2015

Talk Outline

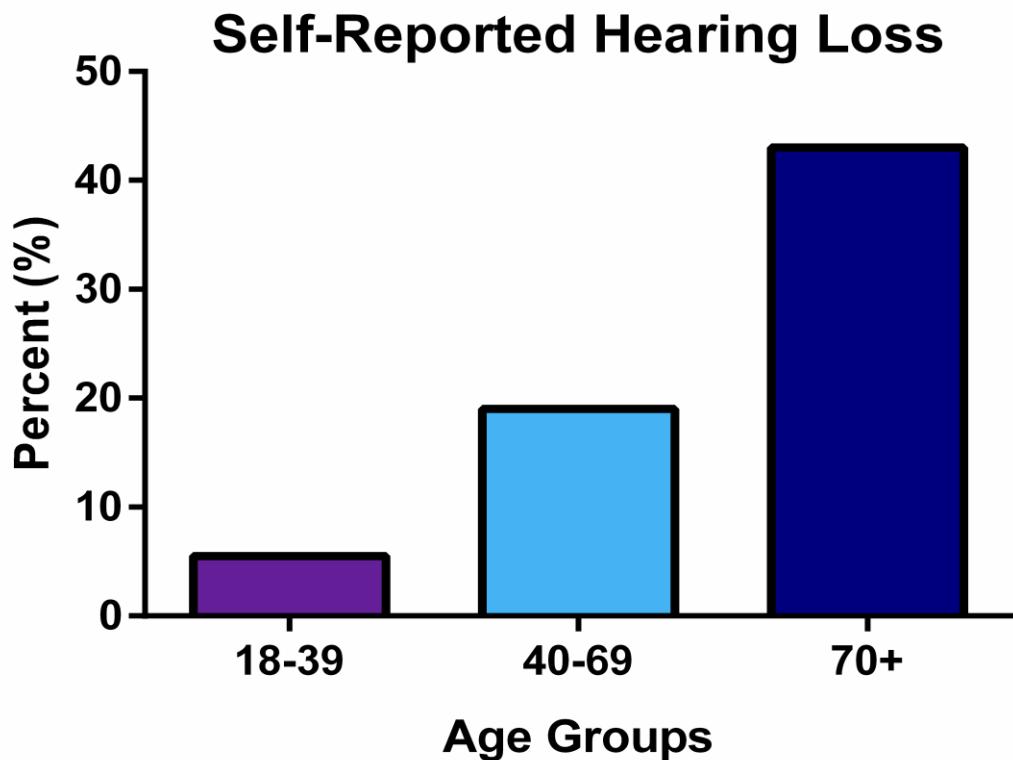
1. Introduction
2. *Atp2b2* downregulation in B6 mice
3. α *Atp2b2* promoter characterization
4. Long non-coding RNA (LncRNA)
5. Characterization of *Atp2b2* congenic mice

How prevalent is hearing loss?

360 million people
worldwide have
disabling hearing loss,
most are adults.



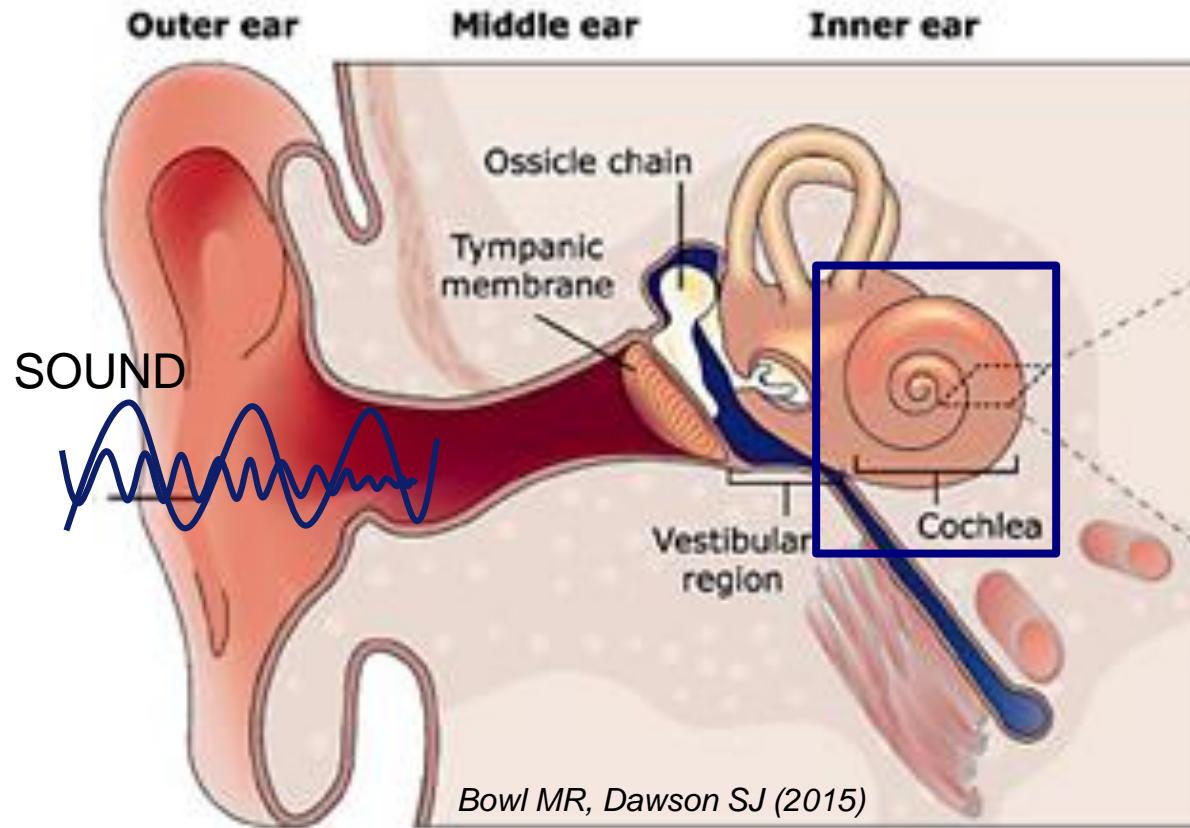
The Burden of Age-Related Hearing Loss (AHL)



*“Nearly half of those older than 75 have difficulty hearing...[making it] hard to understand and ...respond to warnings...hard to enjoy talking with family and friends, leading to feelings of isolation.”**

Causes of AHL: Mammalian Auditory Transduction

Sound Waves Mechanical Electrochemical



*"There are many causes of age-related hearing loss. Most commonly, it arises from changes in the inner ear..."**

AHL is caused by environmental and **genetic factors**.

Genetics of AHL: A Mouse Model

Locus	Gene	Protein	Function ^a	Human HL ^b
Ahl1	<i>Cdh23</i>	Cadherin 23	Component of the tip link connecting hair cell stereocilia	USH1D, DFNB12
Ahl2				
Ahl3				
Ahl4	<i>Cs</i>	Citrate synthase	Rate-limiting step in citric acid cycle	
Ahl5	<i>Gipc3</i>	GIPC PDZ domain-containing family, member 3	Scaffolding protein involved in hair cell transduction	DFNB15, DFNB95
Ahl6				
Ahl8	<i>Fscn2</i>	Fascin homologue 2, actin bundling protein, retinal	Actin cross-linker in hair cell stereocilia	
ApoE	<i>ApoE</i>	Apolipoprotein E	Lipid metabolism	
Chrnb2	<i>Chrnb2</i>	Cholinergic receptor, nicotinic, $\beta 2$	Nicotinic acetylcholine receptor	
DRASIC	<i>Asic3</i>	Acid-sensing (proton-gated) ion channel 3	Mechanoreceptor channel	
Gpx1	<i>Gpx1</i>	Glutathione peroxidase 1	Anti-oxidant enzyme	
mt-Tr	<i>mt-Tr</i>	Mitochondrially encoded tRNA arginine	tRNA arginine of mitochondria	
Pcdh15	<i>Pcdh15</i>	Protocadherin 15	Component of the tip link connecting hair cell stereocilia	USH1F
Pmca2	<i>Pmca2</i>	Plasma membrane calcium ATPase 2	Calcium transporter	Modifier of DFNB12
Polg1	<i>Polg1</i>	Polymerase (DNA directed), γ	Polymerase of mtDNA	
Pou3F4	<i>Pou3F4</i>	POU domain, class 3, transcription factor 4	Transcription factor	DFN3
Sod1	<i>Sod1</i>	Superoxide dismutase 1, soluble	Destroys free radicals	
Trpv4	<i>Trpv4</i>	Transient receptor potential cation channel, subfamily V, member 4	Mechanosensitive cation channel	

Well characterized hearing phenotypes in inbred mouse strains

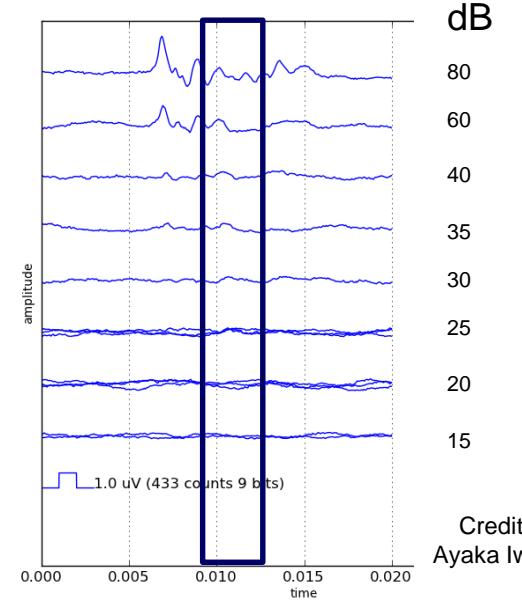
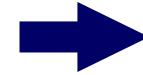
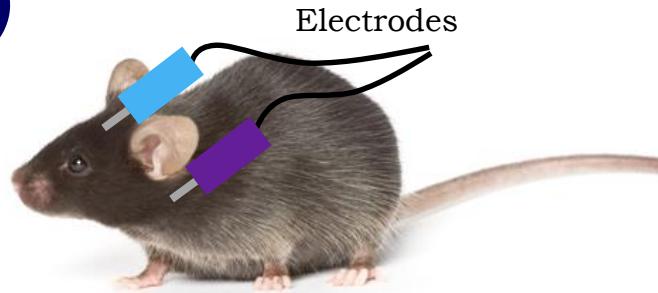


CBA/CaJ (CB)
Standard good hearing strain



C57BL/6J (B6)
AHL from mutation in *Cadherin 23*

Auditory Evoked Brainstem Responses

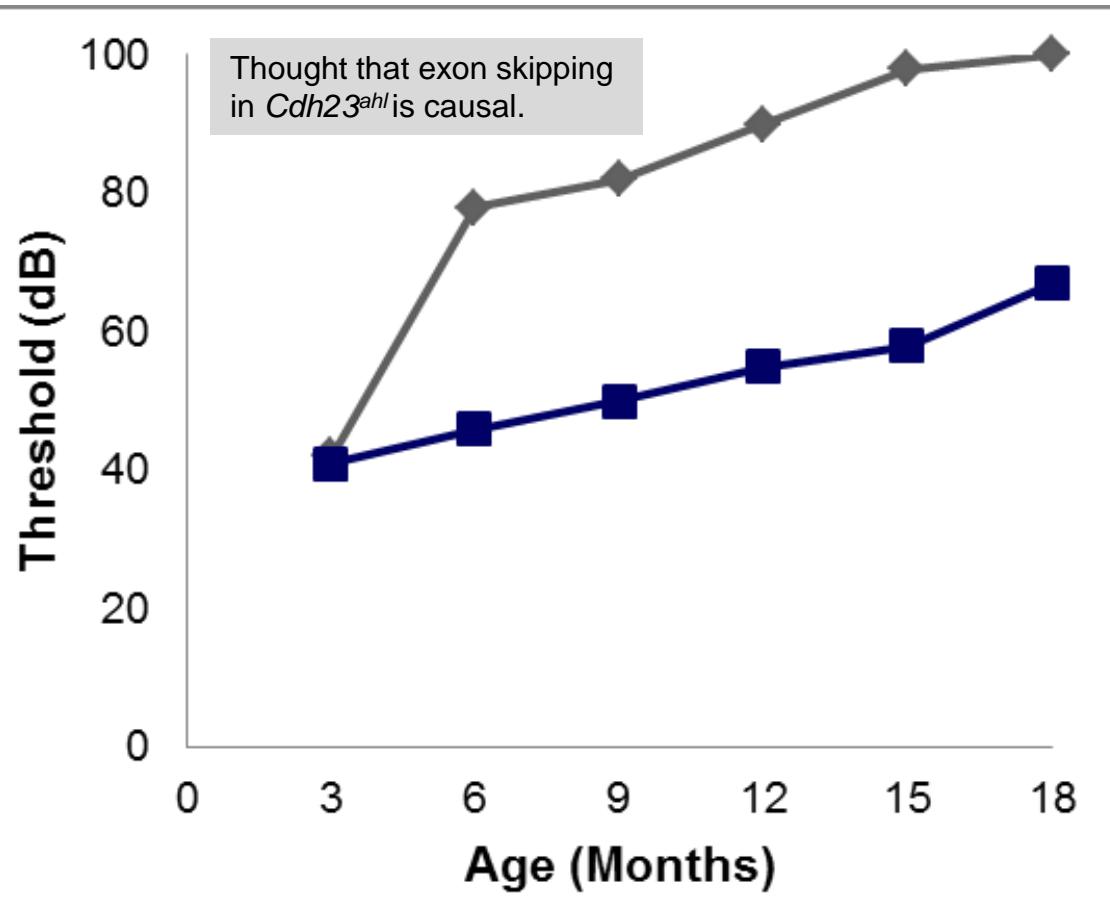


ABRs:

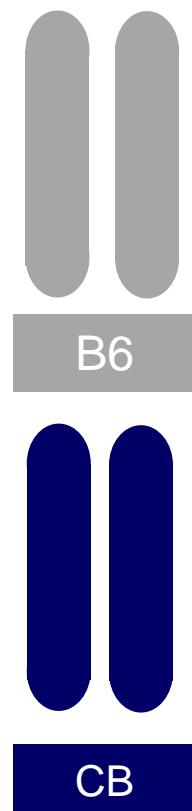
1. Insert electrode subcutaneously into mouse.
2. Expose mouse to frequencies (5.6 kHz to 40 kHz) at decreasing intensities (80 dB to 15 dB).
3. Threshold is the lowest intensity that produces a **repeatable** waveform.

Hearing in Inbred Strains

Thresholds at 32 kHz

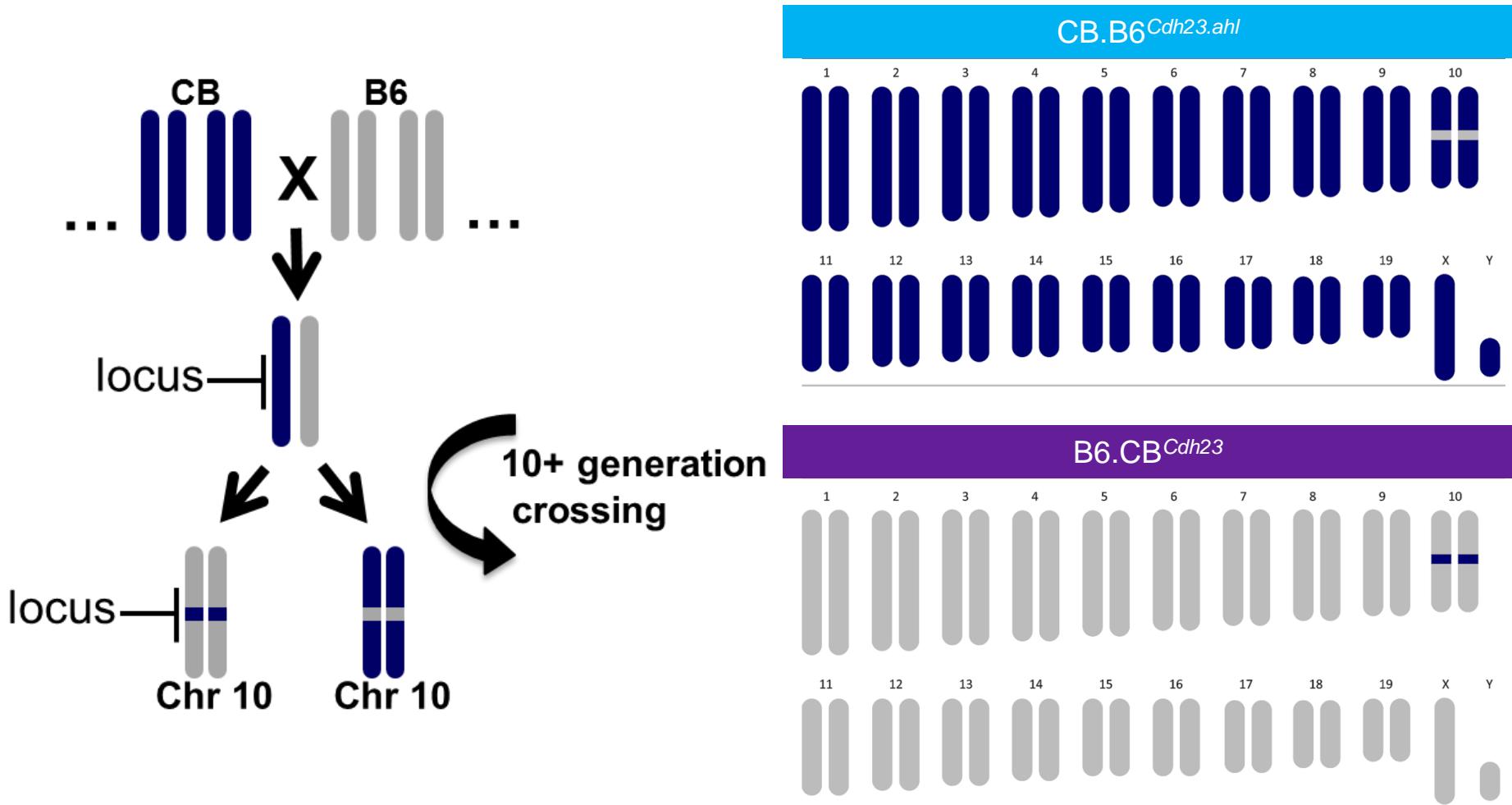


Chr 10 contains *Cdh23^{ahl}*



Jackson Labs (JAX)

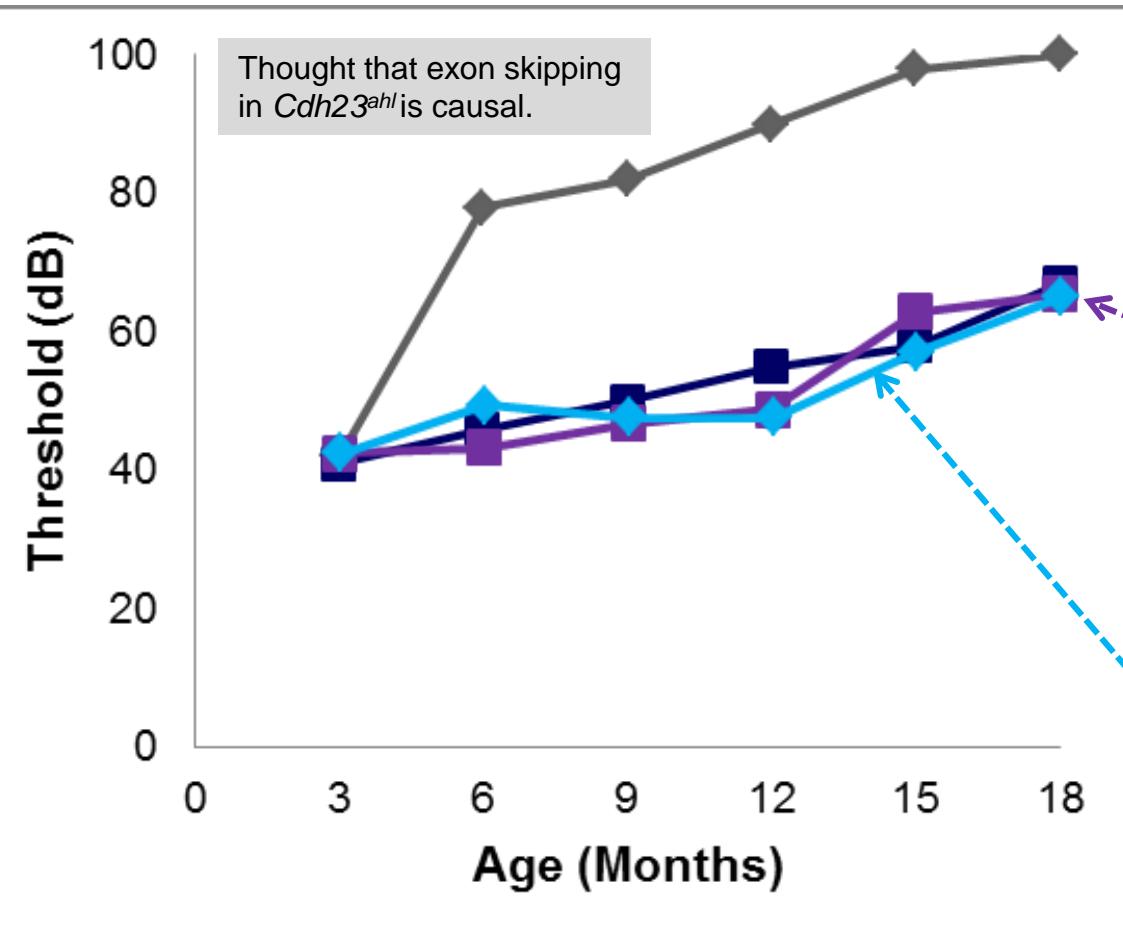
Cdh23 Congenic Mouse



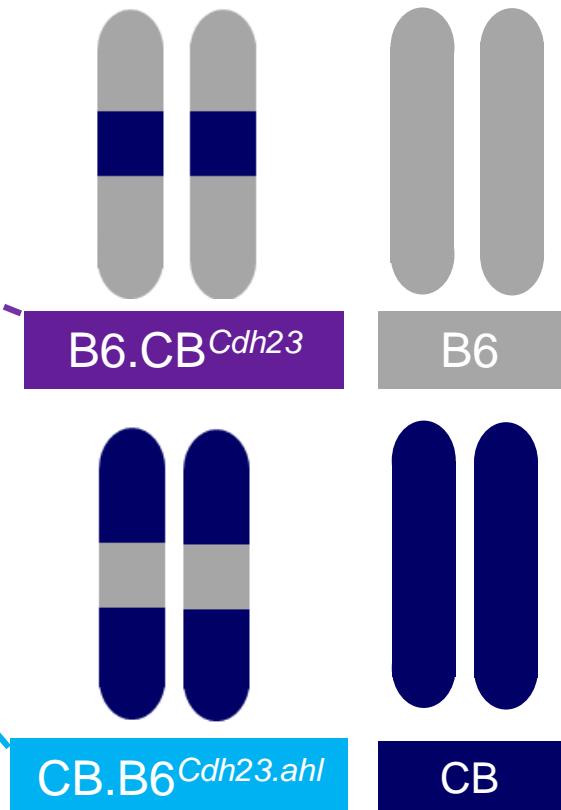
Schematic adapted from Braulio Peguero (2014)

ABRs for Congenic mice from JAX

Thresholds at 32 kHz



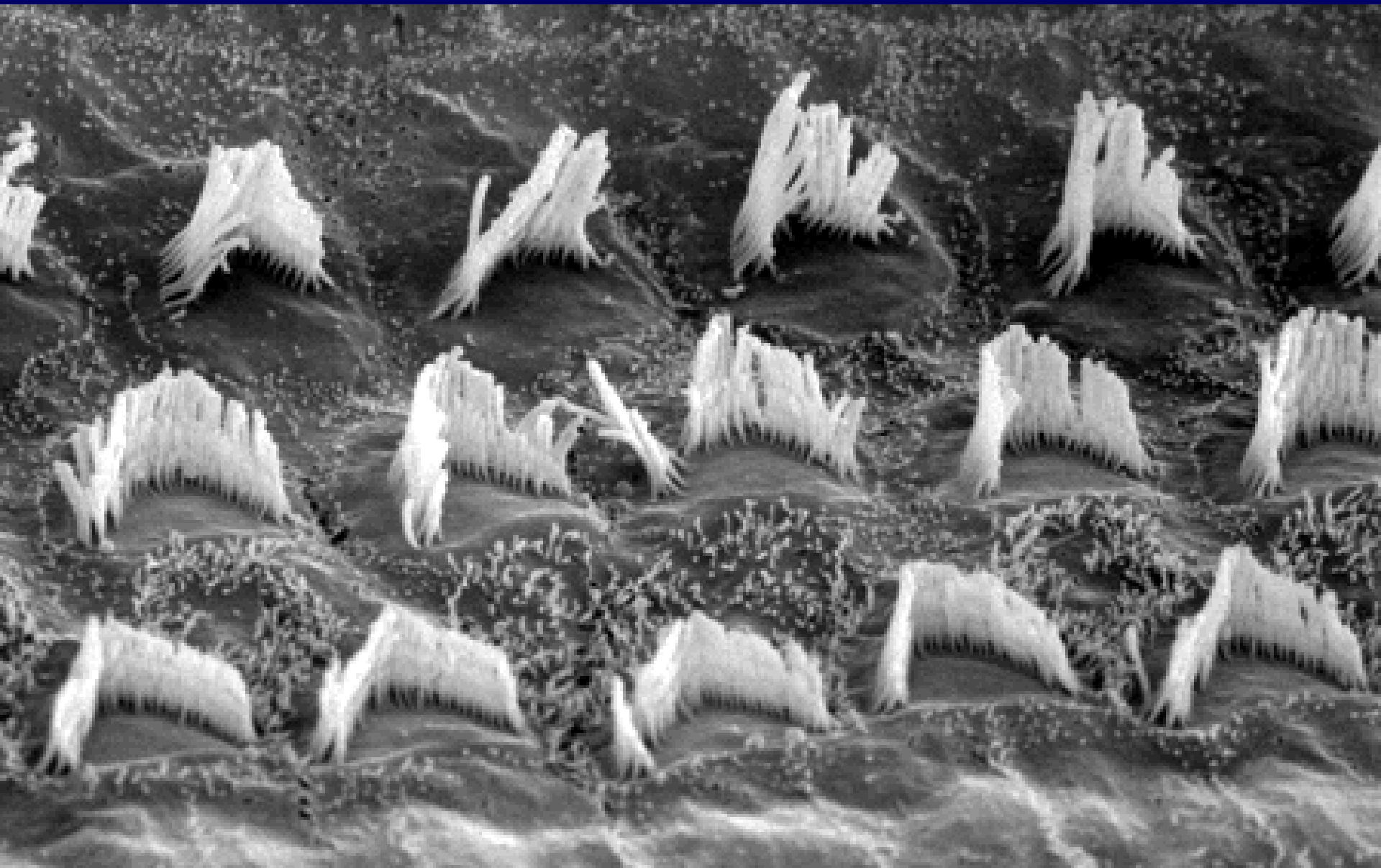
Chr 10 contains *Cdh23^{ahl}*



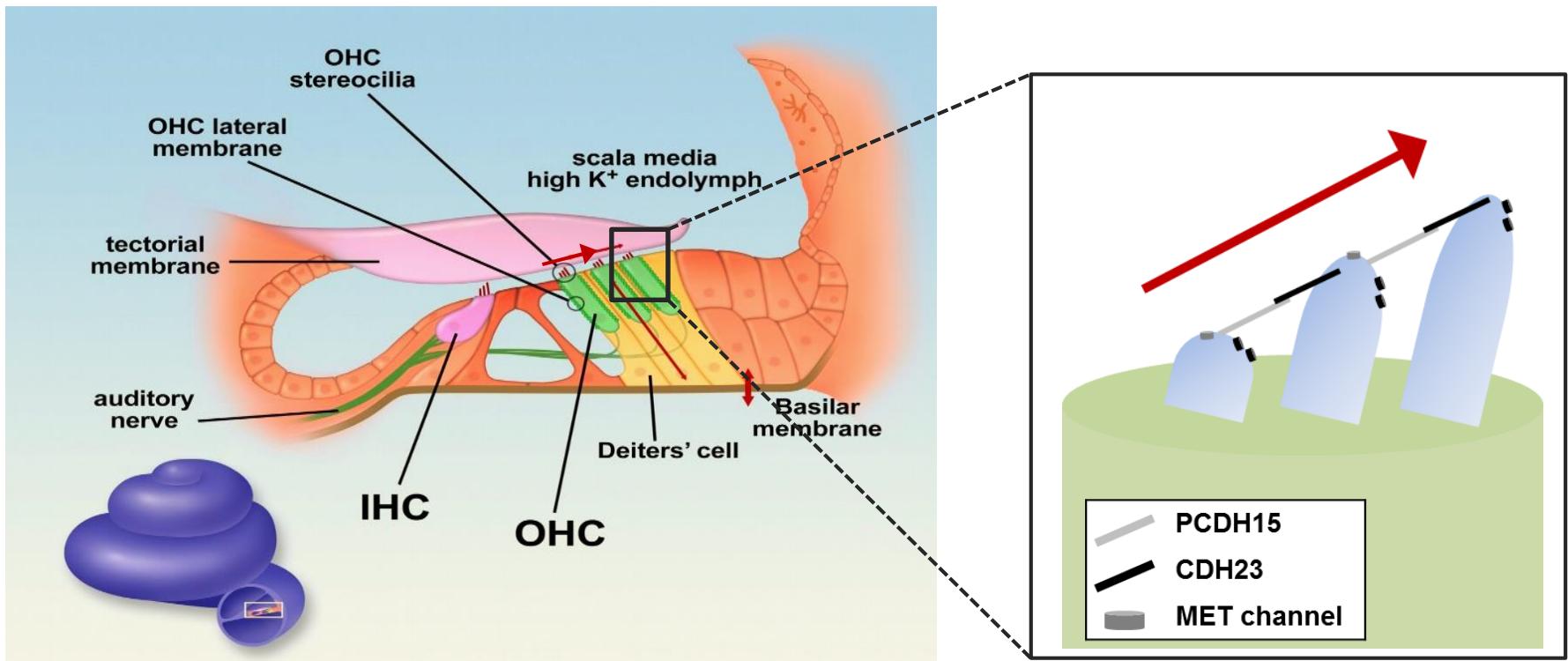
Adapted from Kane, KL et al. (2011)

Cdh23^{ahl} alone does not cause AHL in B6.

Which gene is interacting with *Cdh23*?

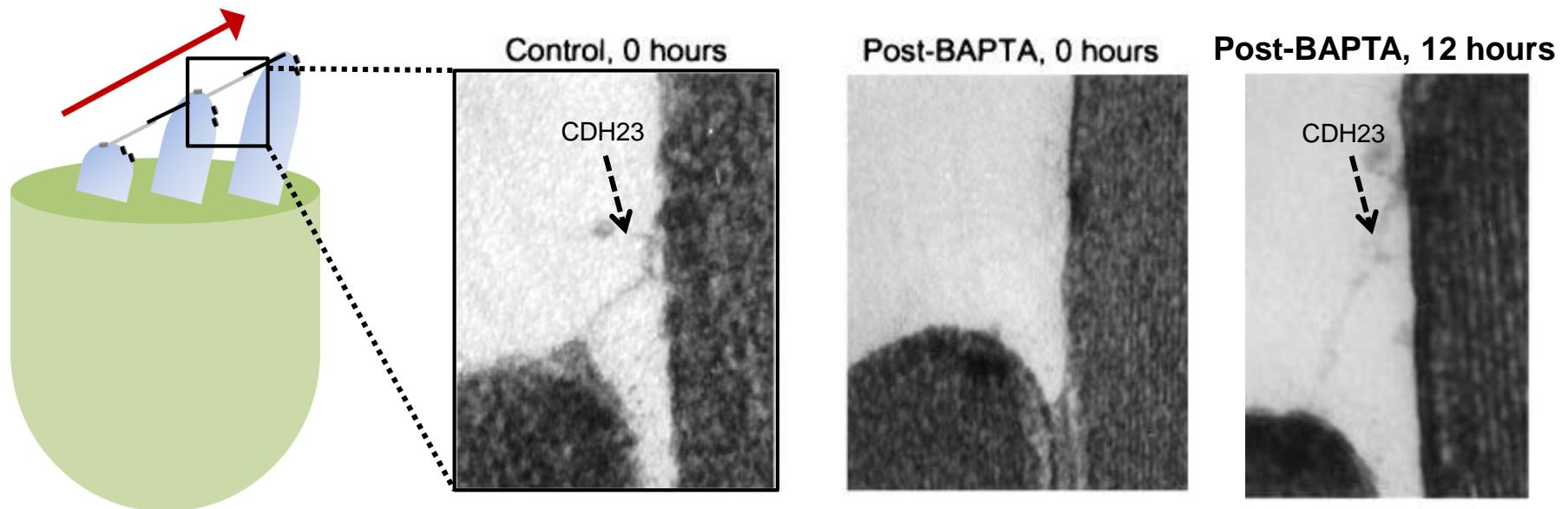


Mechanotransduction: CDH23 is required for auditory transduction



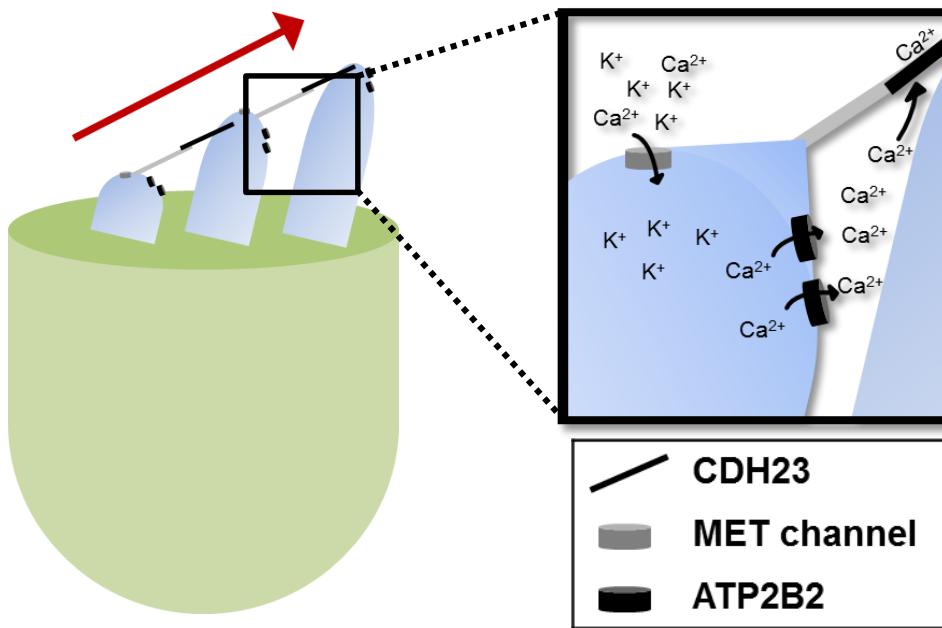
- Sound causes fluid motion in the inner ear leading to deflection of the stereocilia activating MET channels.
- Tip links, composed of Protocadherin 15 (PCDH15) and **CDH23**, connect adjacent stereocilia and are important for synchronized deflection of stereocilia and activation of MET channels.

Ca^{2+} and CDH23



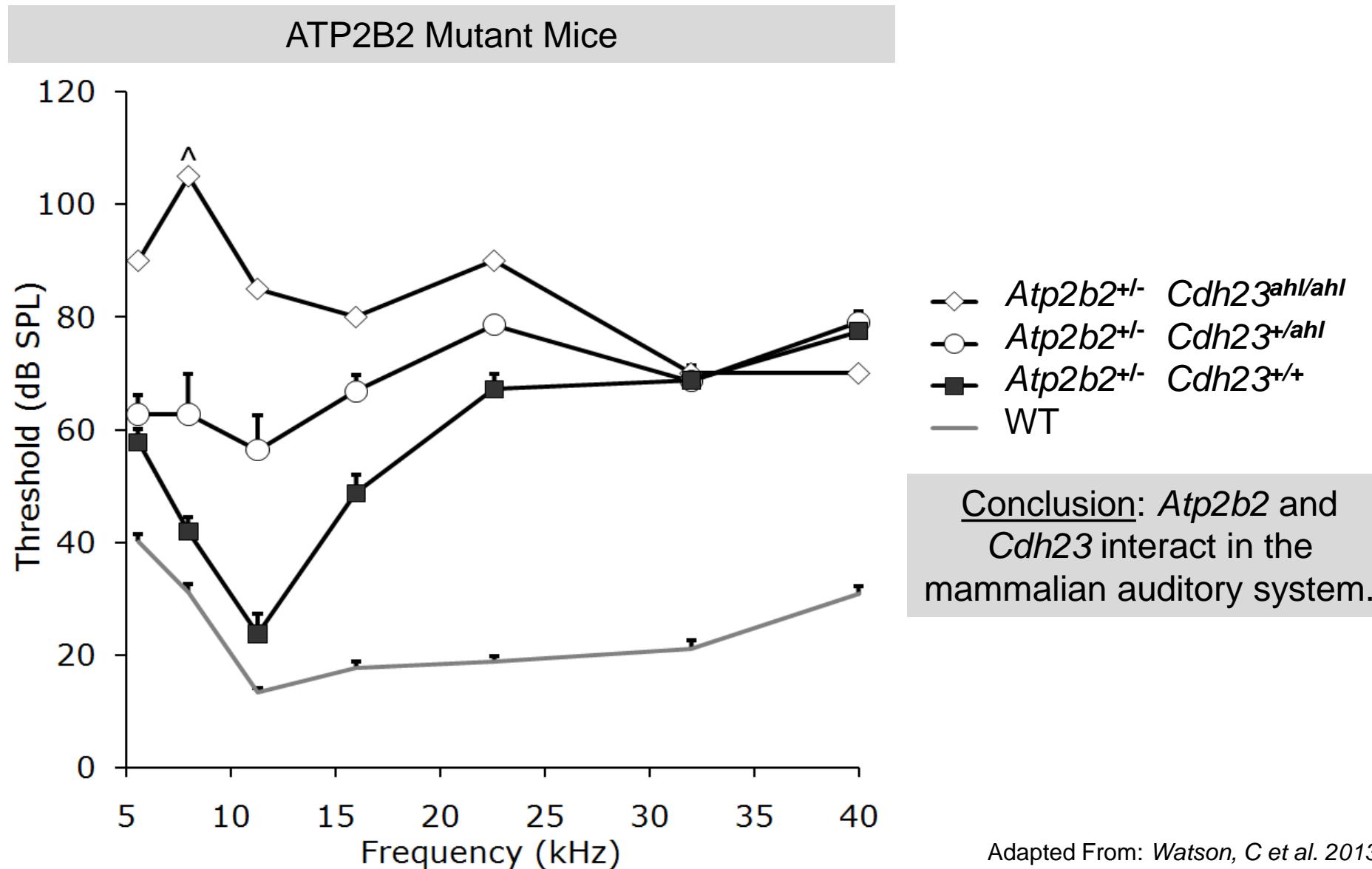
Ca^{2+} is necessary to maintain the structural integrity of the tip-links composed of PCDH15 and **CDH23**.

Atp2b2 (ATP2B2): An Important Ca^{2+} Regulator in the Stereocilia



- Plasma Membrane Calcium ATPase2 (ATP2B2) uses energy to pump calcium out of the cell against its electrochemical gradient.
- Tight regulation of *Atp2b2* is necessary for auditory transduction.
- **ATP2B2 helps maintain Ca^{2+} concentration in the endolymph which bathes the tip-links.**

Known interaction of ATP2B2 and CDH23



Is *Atp2b2* misregulated in B6 mice?



Total *Atp2b2* Expression in Hybrid



CBA/CaJ (CB)
Standard good hearing strain

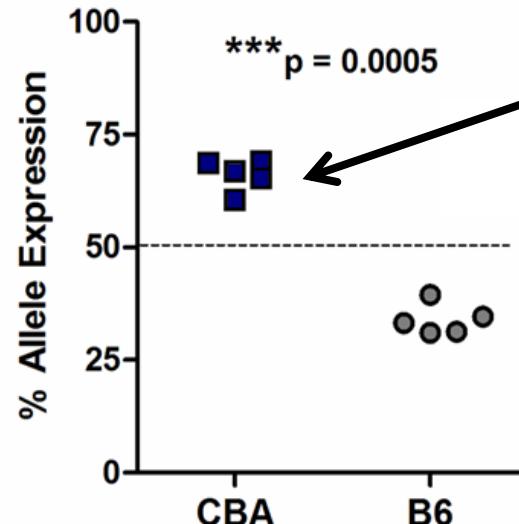


C57BL/6J (B6)
AHL from mutation in *Cadherin 23*



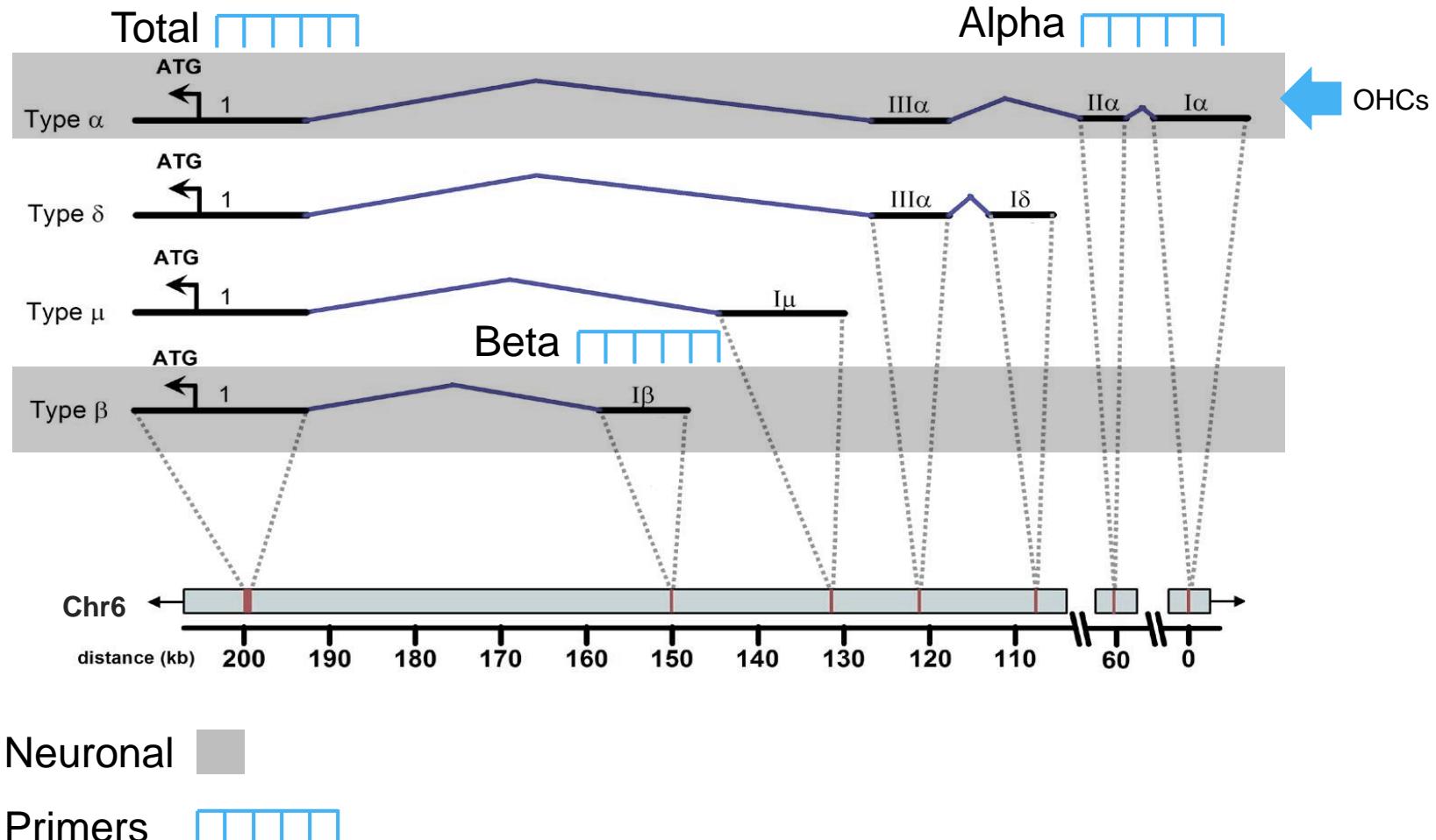
Atp2b2

Atp2b2

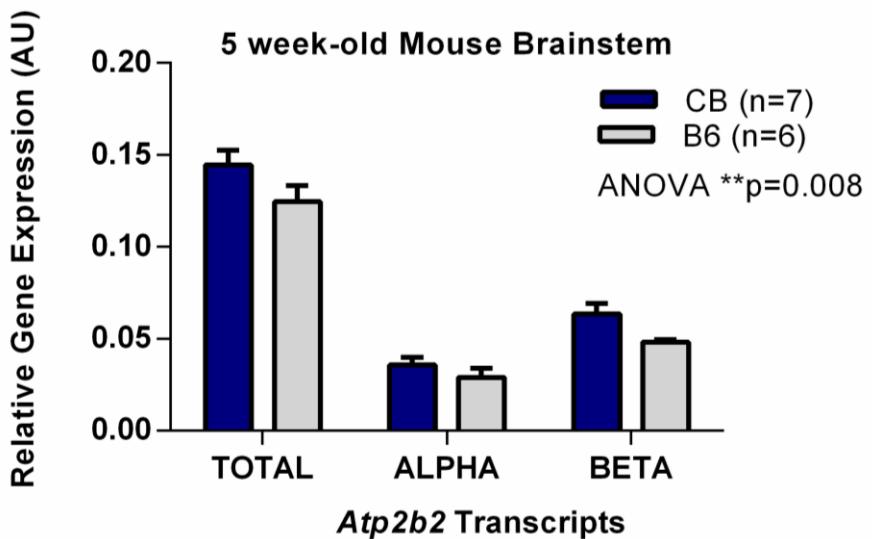
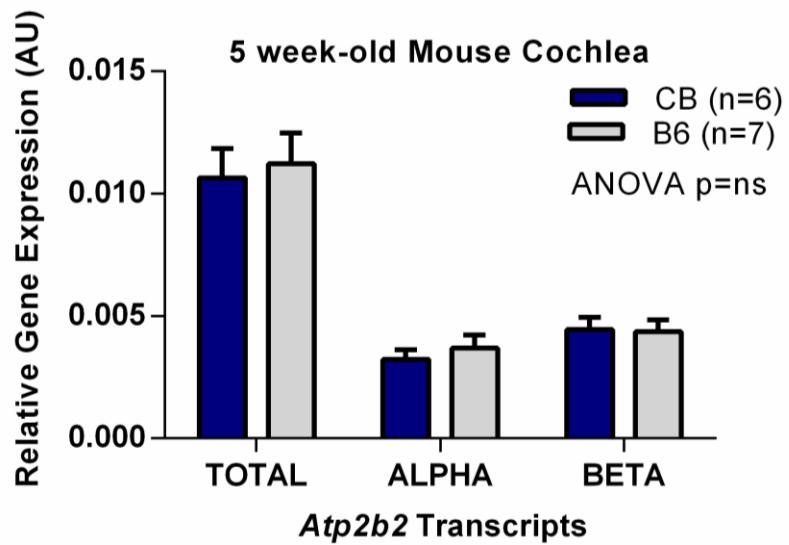


The allelic discrimination revealed that the hybrid mice have more CBA/CaJ *Atp2b2* transcript than C57BL/6J *Atp2b2* transcript.

Transcripts Found in the Brain and Cochlea

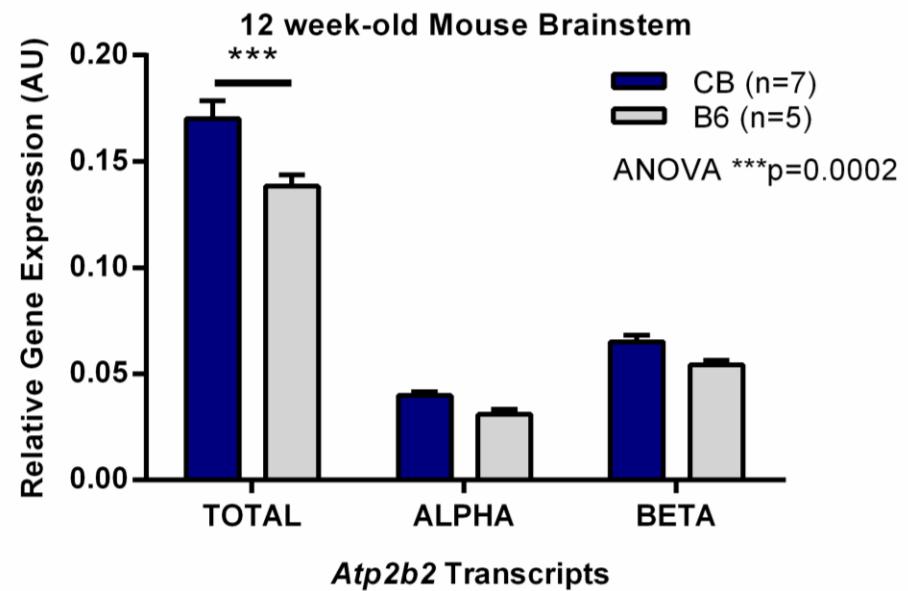
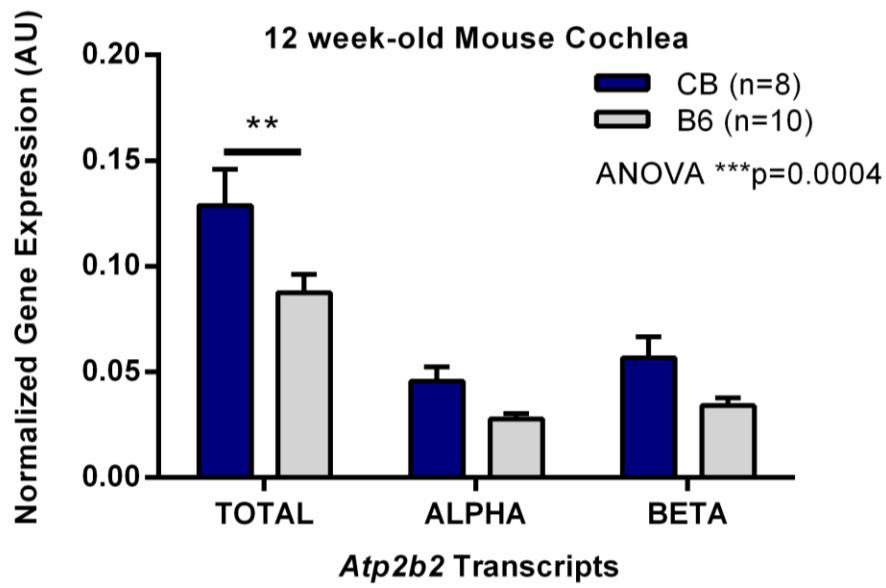


qPCR: Differential *Atp2b2* Expression in 5wk Brainstem



Brainstem qPCR data agrees with brainstem allelic discrimination data at 5 weeks of age.

qPCR: *Atp2b2* is Downregulated in 12 week-old Tissues



At 12 weeks of age, *Atp2b2* transcript is downregulated in whole cochlea and brainstem.

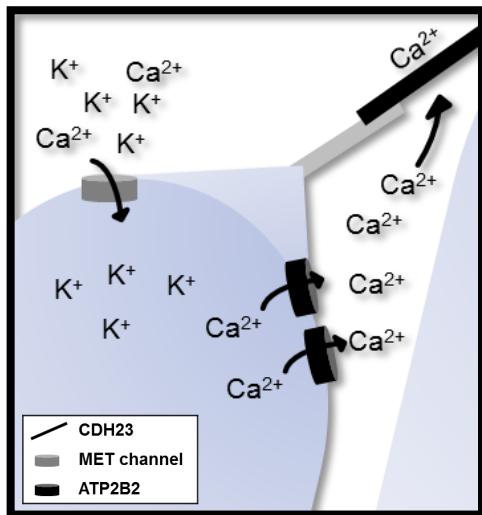
Conclusions

- *Atp2b2* transcript is downregulated in B6 brainstem tissue at 5 weeks of age and in both brainstem and cochlea tissue at 12 weeks of age.
- This recapitulates findings from the allelic discrimination suggesting that B6 has weaker allelic expression than CB.

Cdh23 and *Atp2b2* digenic interaction model



Normal CDH23 and ATP2B2 interaction

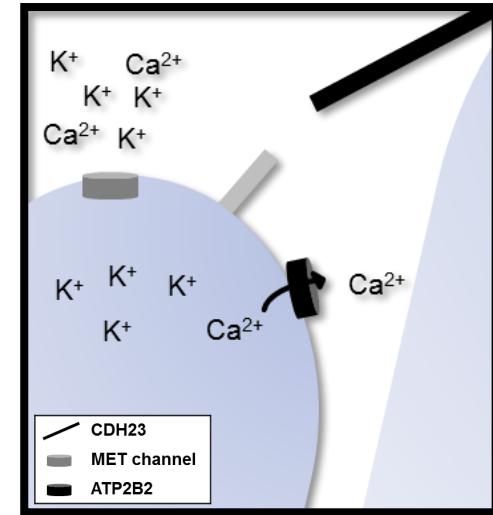


Normal function in Stereocilia of CB mice:

- ATP2B2 maintains endolymph Ca²⁺ levels.
- Tip-links maintain structural integrity.



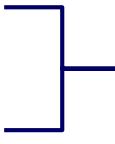
CDH23 and ATP2B2 Interaction in B6



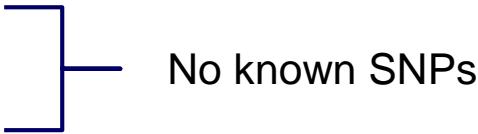
Atp2b2 downregulation in B6 mice:

- Decreased endolymph Ca²⁺ → tip-link degradation and loss of mechanotransduction.
- Increased intracellular Ca²⁺ causes metabolic stress → hair cell death.

What is the mechanism of *Atp2b2* misregulation in B6 mice?

1. Exon SNPs (1 non-synonymous SNP in CB)
 2. Splice site SNPs
 3. Untranslated Regions
 4. Proximal Promoter
 5. Long non-coding RNA (LncRNA)
- 
- No known SNPs

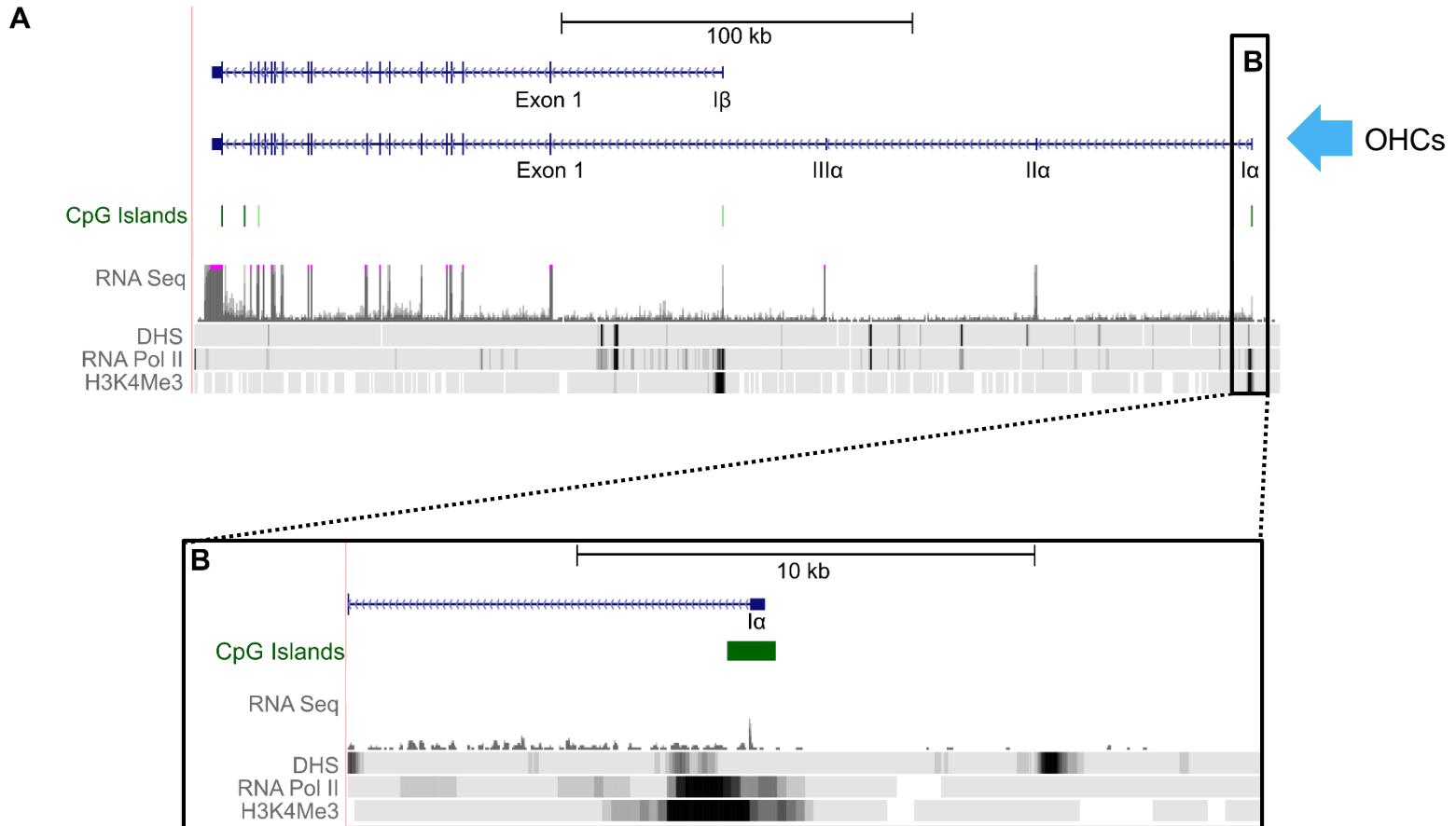
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Where is the $\alpha Atp2b2$ Promoter?

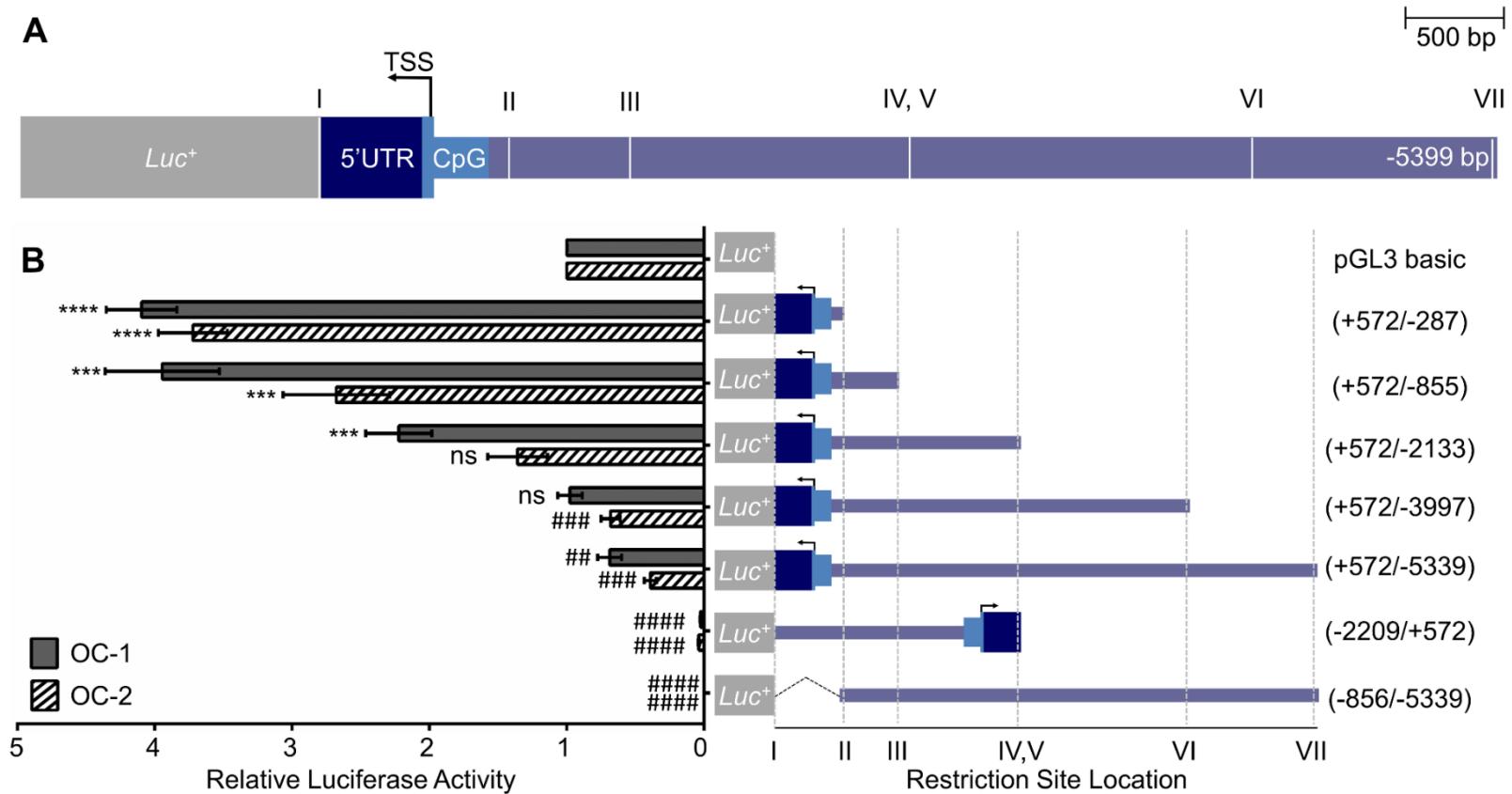


Atp2b2 Multiple Promoters



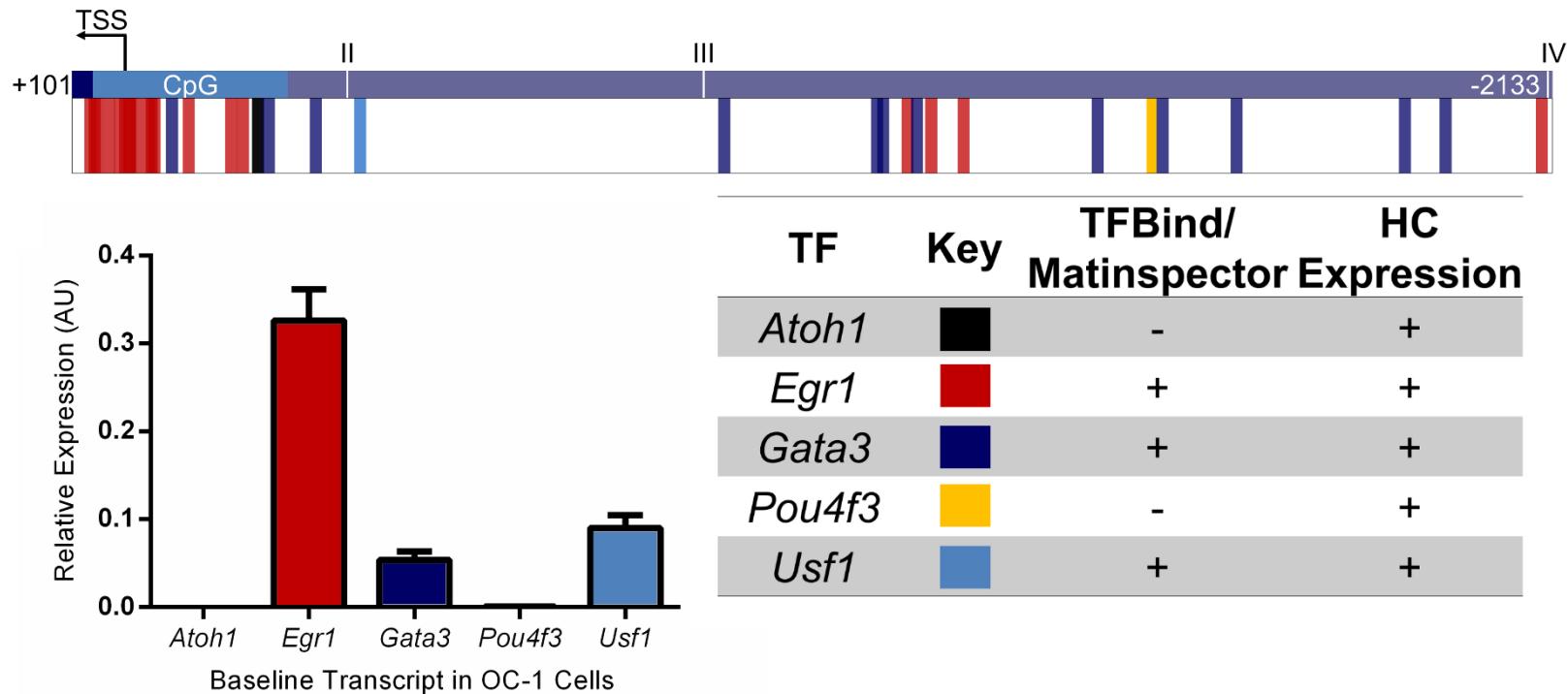
α and β *Atp2b2* have unique promoters. The α *Atp2b2* promoter is likely located directly upstream of the transcriptional start site (TSS)

CpG island is Key Component of Promoter



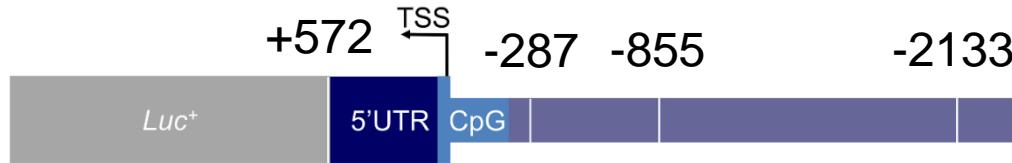
The CpG Island and 5'UTR contain the main α Atp2b2 promoter elements

Predicted Transcription Factor (TF) Binding sites in α Atp2b2 Promoter

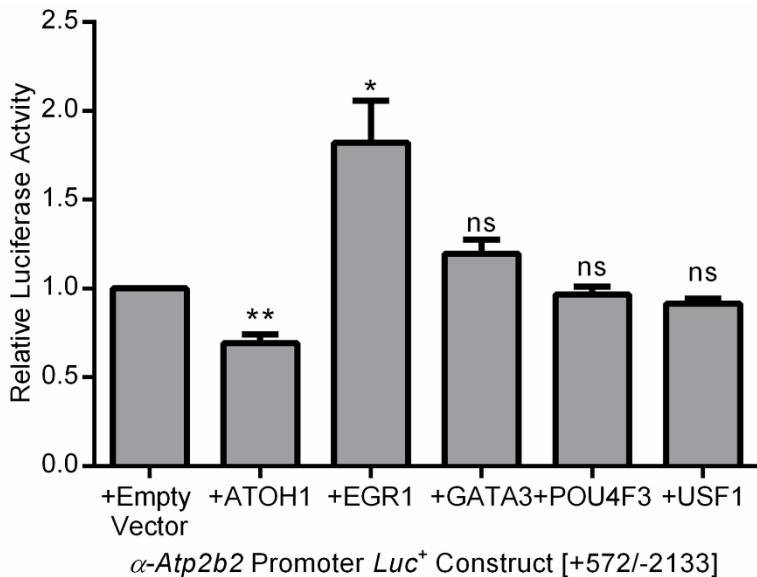


These genes are involved in hair cell development and maintenance, synaptic plasticity, regeneration and tissue repair.

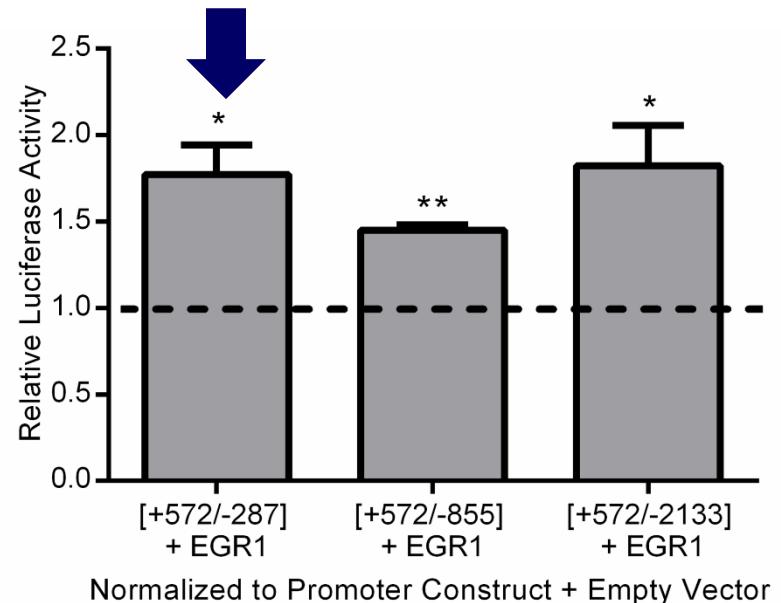
EGR1 and ATOH1 Modulate the α Atp2b2 Promoter



TF Effect on Promoter Activity



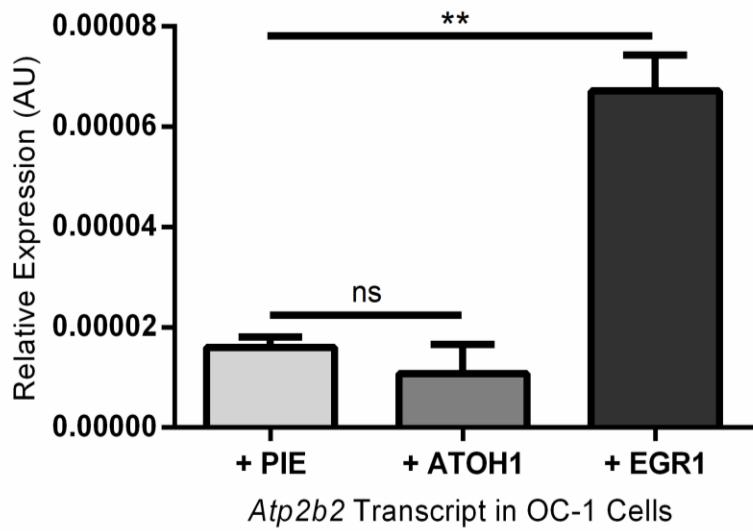
EGR1 Binding Site Location



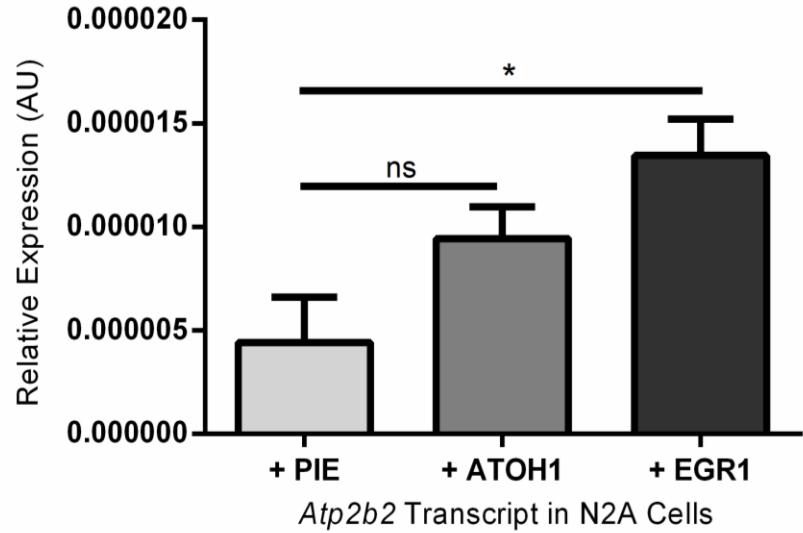
EGR1 activates the promoter, ATOH1 moderately inhibits it.
EGR1 likely binds in the CpG Island

EGR1 Increases Endogenous *Atp2b2* Expression

Atp2b2 Transcript in OC-1



Atp2b2 Transcript in N2A



EGR1 increases *Atp2b2* transcript in OC-1 and N2A cells.

Promoter Probe Binds Protein Specifically

EGR1 Probes	Sequence
[-71 to -98]	GCC CGA <u>GGG GAG CGG GGG AGG AGA GAG C</u>
Mutant [-71 to -98] (Mutant)	GCC CGA <u>AGG TAG CAG GGT AGG AGA GAG C</u>

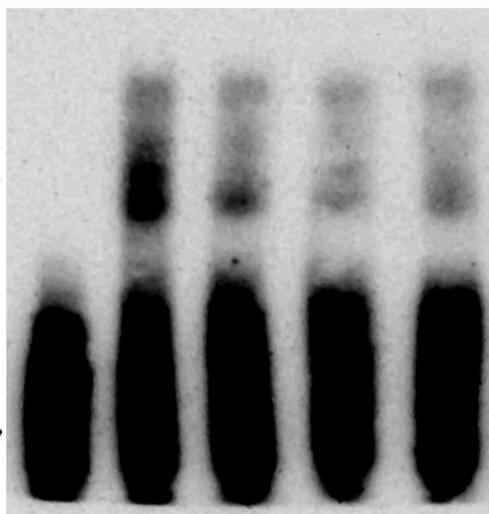
B

Egr1 [-71 to -98]

Biotin Probe	+	+	+	+	+
Nuc. Extract	-	+	+	+	+
Competition	-	-	200x	400x	800x

Shift ►

Free Probe ▶



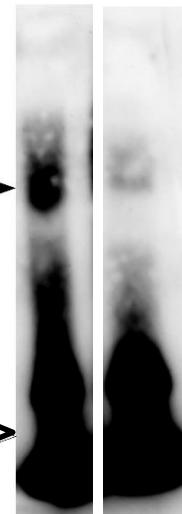
C

Egr1 [-71 to -98]
Mutant

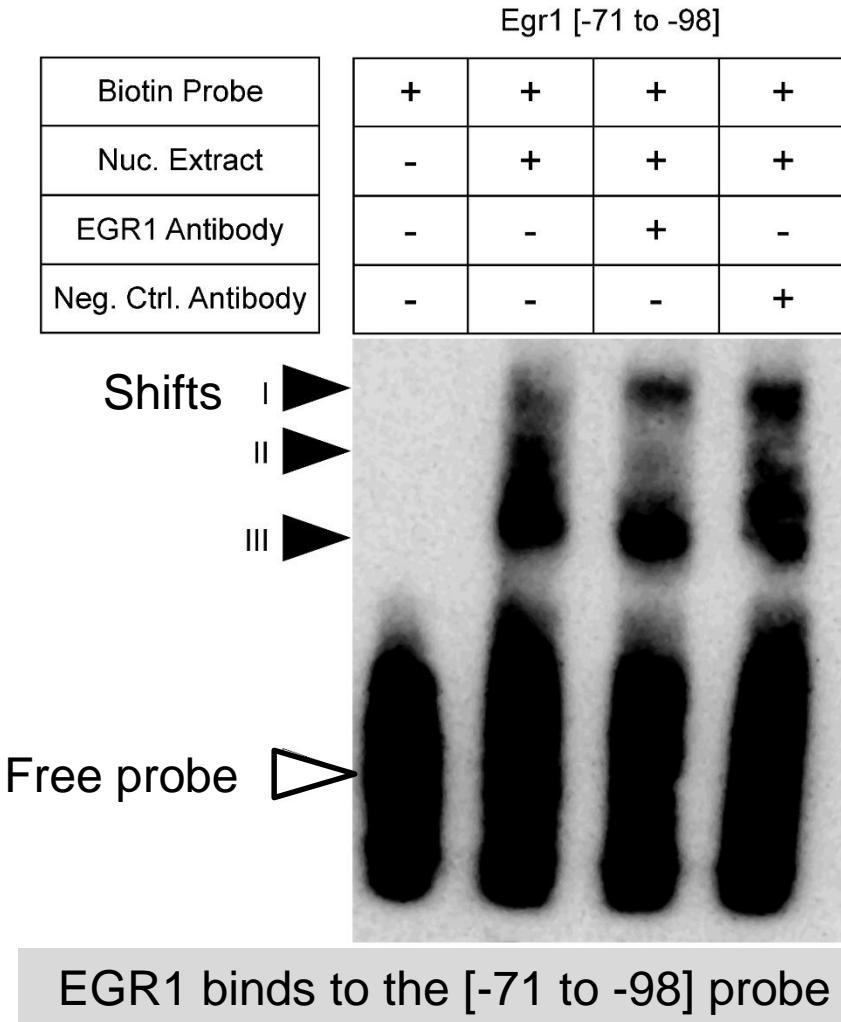
Biotin Probe	+	+
Nuc. Extract	+	+

Shift ►

Free Probe ▶



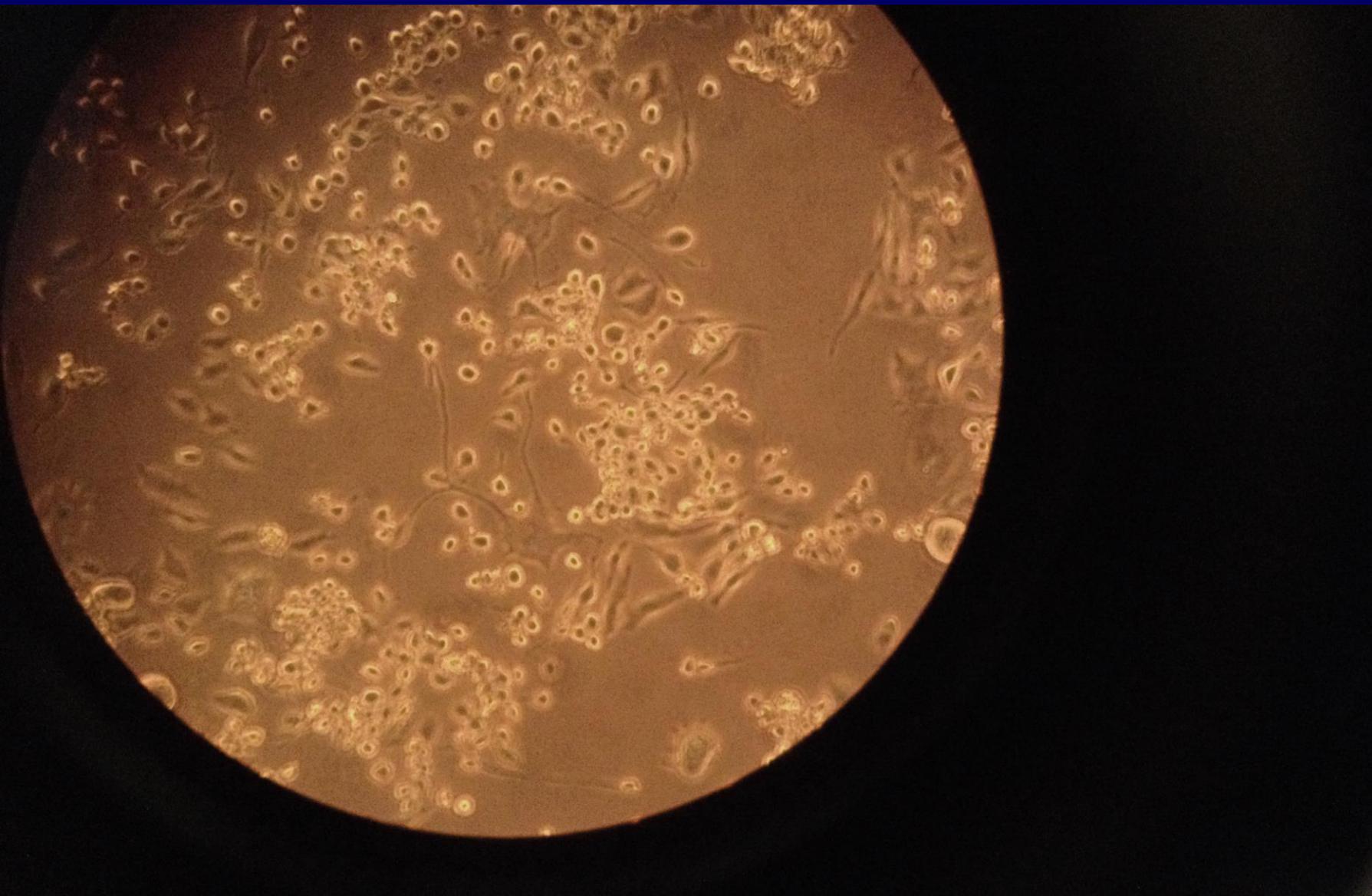
Supershift Identifies Binding of Egr1



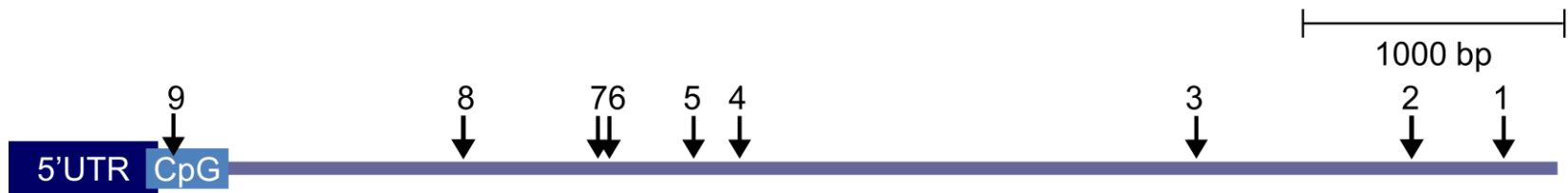
Conclusions

- $\alpha Atp2b2$ has a unique promoter and the CpG island is required for activity.
- ATOH1 and EGR1 modulate the $\alpha Atp2b2$ promoter.
- EGR1 increases endogenous $Atp2b2$ transcript.
- EGR1 binds to and activates the promoter at a site 71 bases upstream of the $\alpha Atp2b2$ TSS.

Is the $\alpha Atp2b2$ promoter ‘weaker’ in B6 mice?

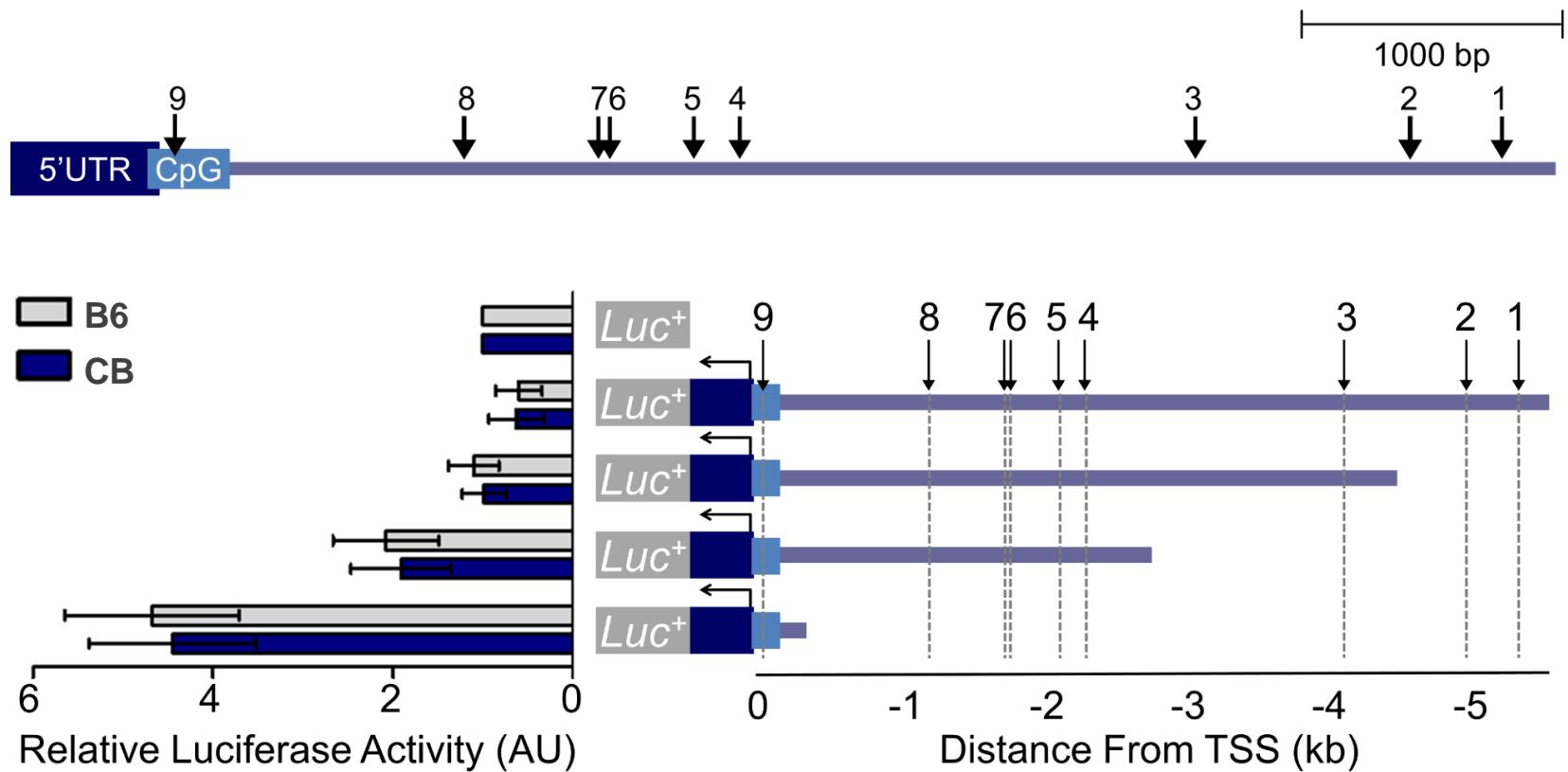


SNPs Affecting TF Binding Sites



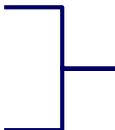
SNP#	TSS	RS number	B6	CB	Predicted binding
1	-5227	(RM)	G	A	Gata2 (CBA) AP2 (B6)
2	-4809	rs30659916	C	T	None
3	-3944	rs30608672	G	C	TLX, HOXC8, VAX1 (CBA)
4	-2188	rs29921976	T	A	TBPF, MAF1 (B6)
5	-1957	rs29969841	G	A	BSX, DLX (CBA) MAF1 (B6)
6	-1593	rs29975662	T	C	MAF1(B6)
7	-1588	rs30956043	C	T	MAF1 (B6)
8	-1192	rs30561839	G	A	none
9	-111	(RM)	G	T	SRF, STAT1, MYB (CBA) AP4 (B6)

No Strain Difference in Full Promoter

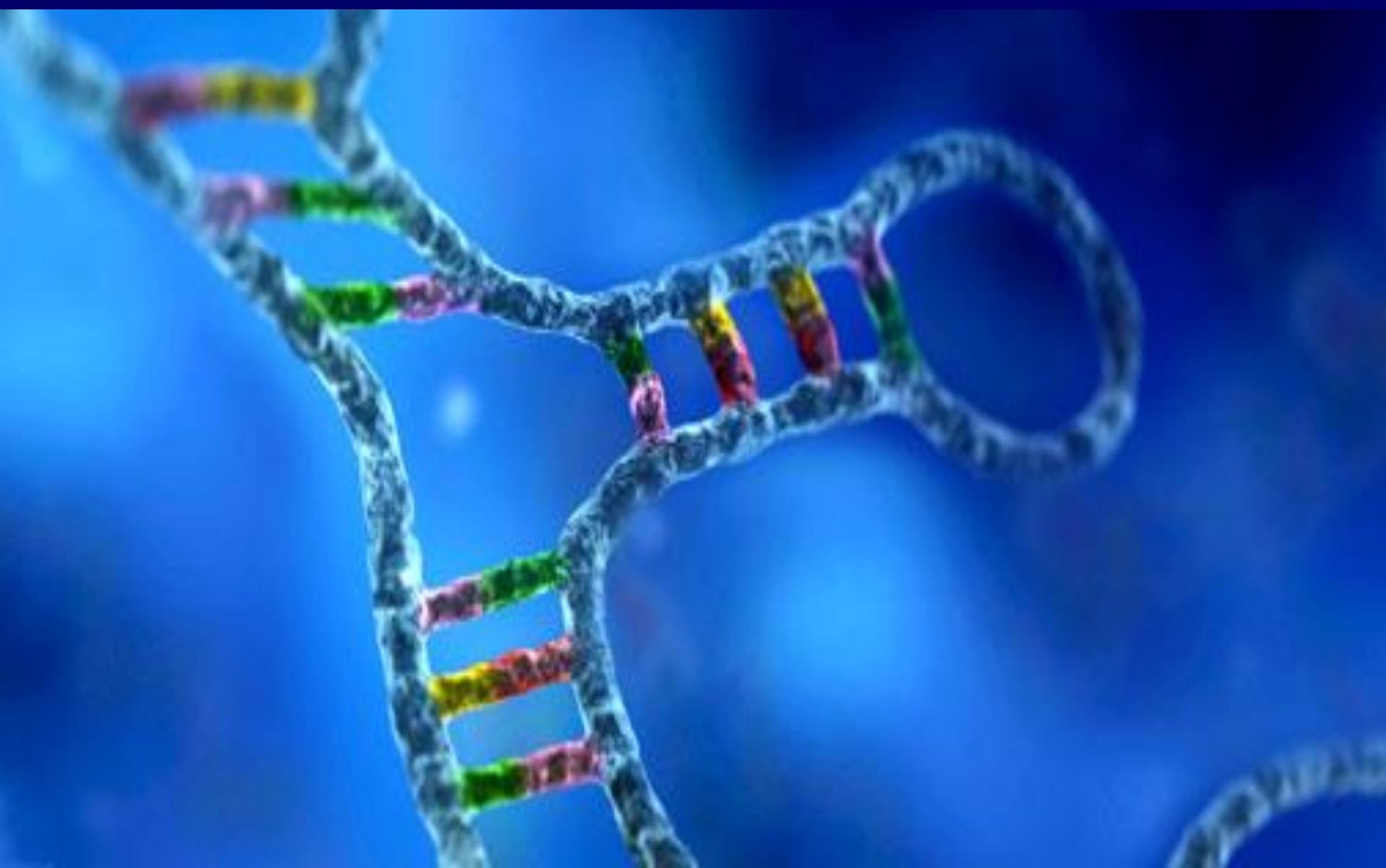


No difference in *Luc⁺* activity is seen between CB and B6 promoters.

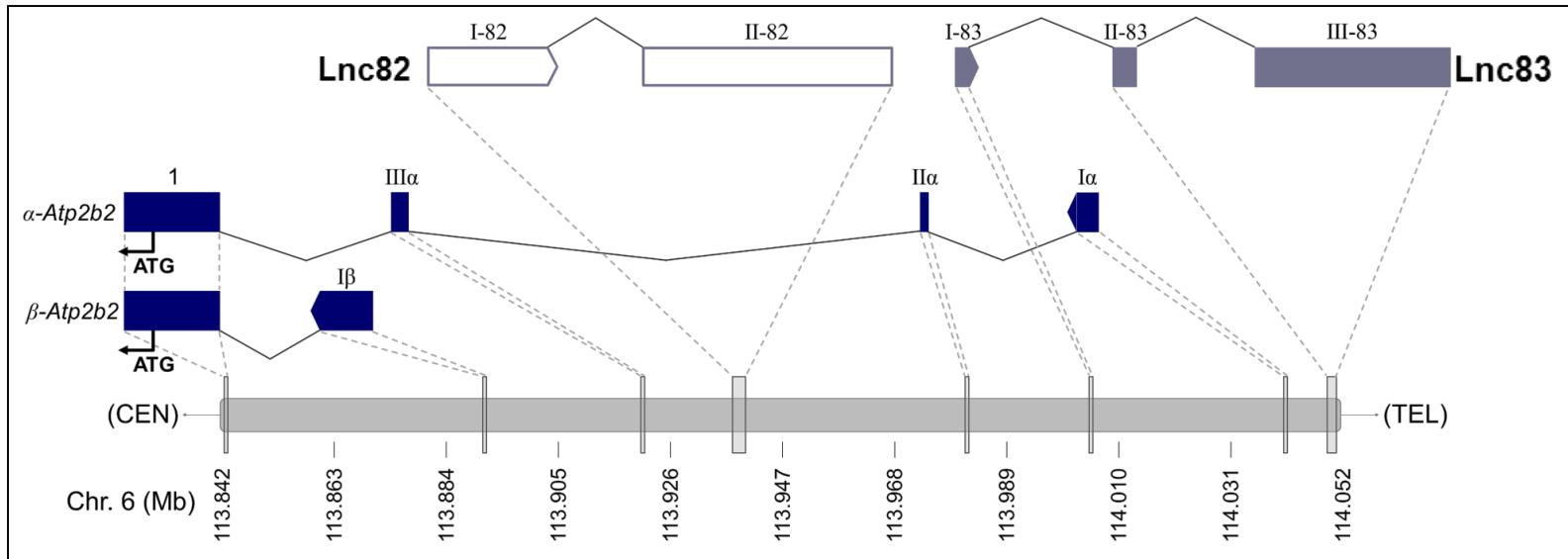
What is the mechanism of *Atp2b2* misregulation in B6 mice?

1. ~~Exon SNPs~~ (1 non-synonymous SNP in CB)
2. ~~Splice site SNPs~~  No known SNPs
3. ~~Untranslated Regions~~ 
4. ~~Proximal Promoter~~  No difference detected
5. Long non-coding RNA (LncRNA)

Do lncRNAs modulate *Atp2b2* expression?



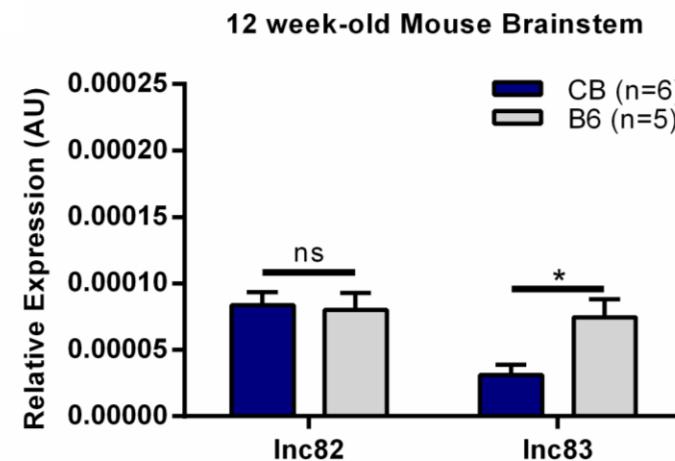
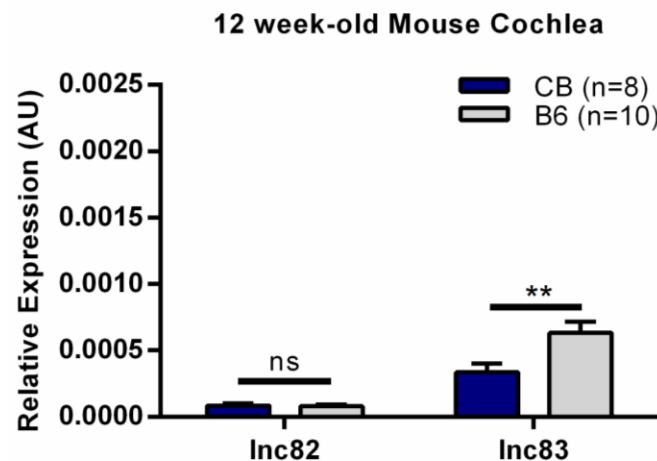
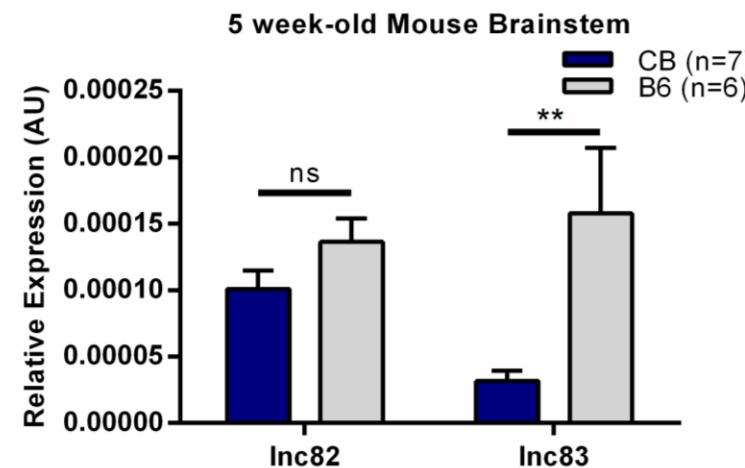
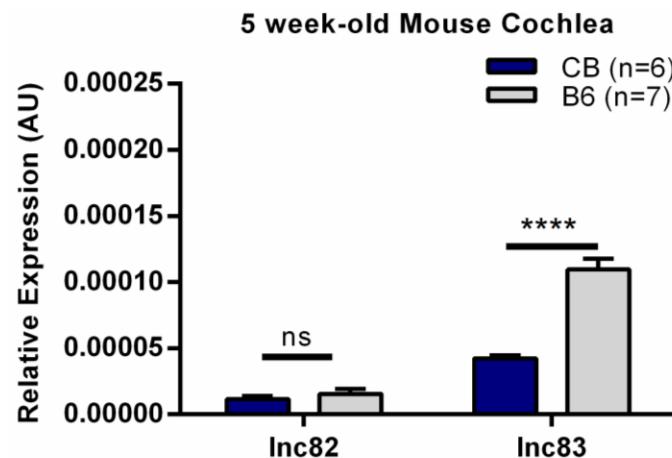
LncRNA in *Atp2b2*



LncRNA:

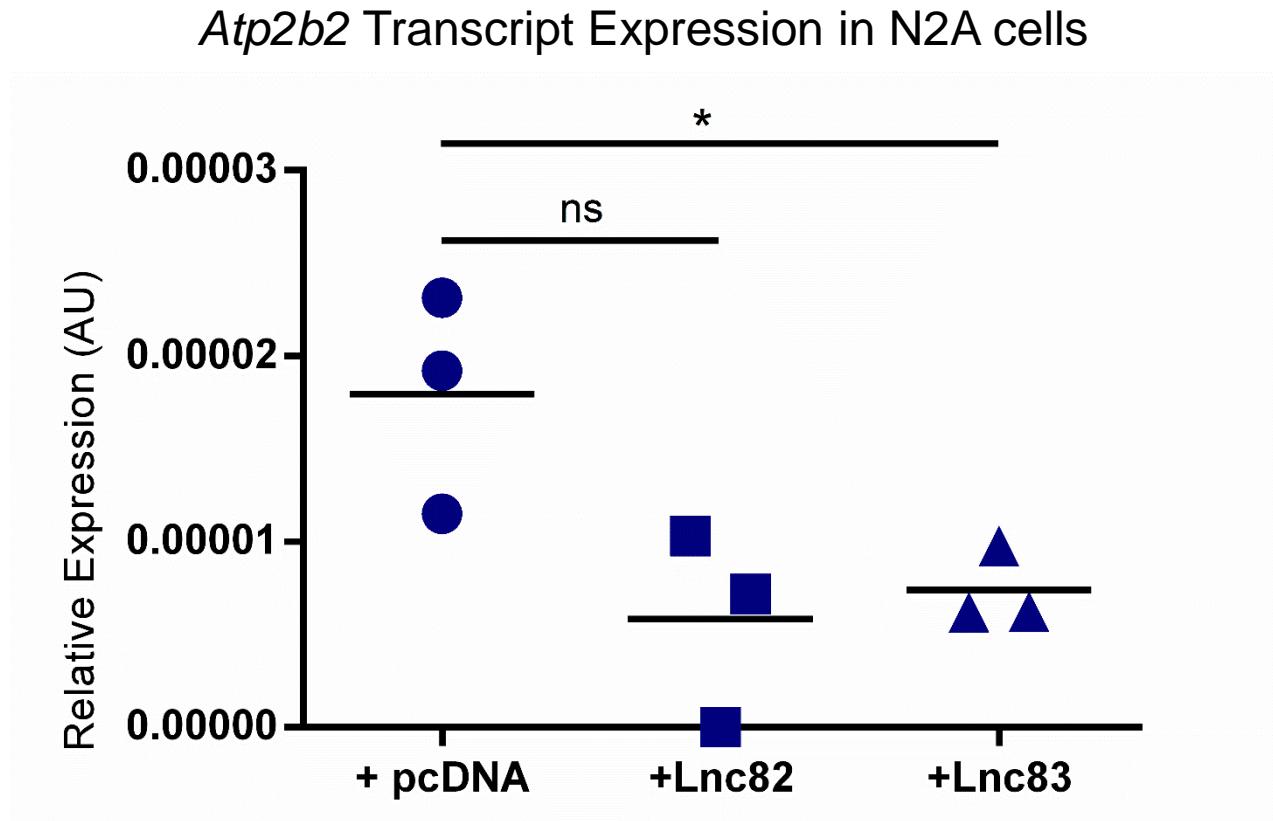
- Can act as transcriptional regulators.
- Coded on the opposite strand of gene (complementarity to *Atp2b2*).
- SNPs are found between CB and B6 in these regions (exons of *Lnc82*, 'promoter' for *Lnc83*).

LncRNA in Mouse Brainstem and Cochlea



Lnc83 is upregulated in brainstem and cochlea of B6 mice.

LncRNA and Endogenous *Atp2b2* Expression



Lnc83 inhibits *Atp2b2* transcript expression in N2A Cells

Conclusions

- Lnc83 is selectively upregulated in brainstem and cochlea of B6 mice.
- Overexpression of Lnc83 in N2A cells leads to a significant decrease in *Atp2b2* transcript levels.

Does misregulation of *Atp2b2* and *Inc83* effect hearing in B6 mice?



What we know so far:



CBA/CaJ (CB)

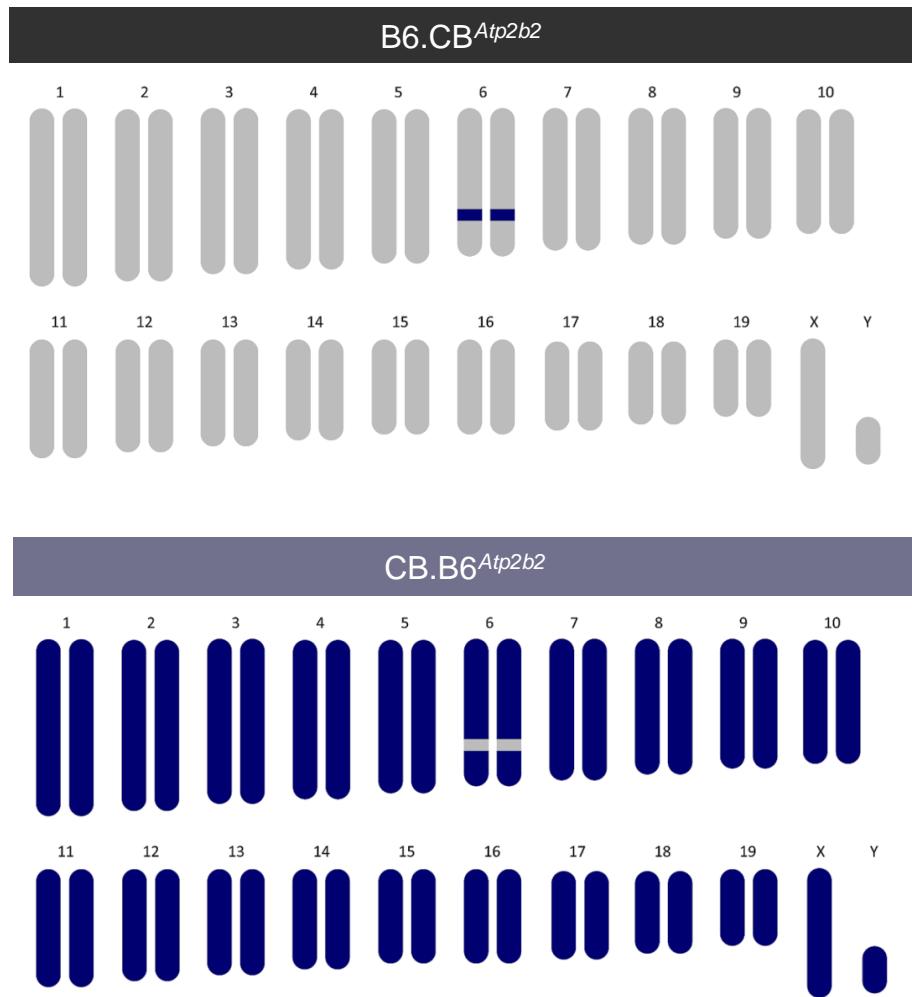
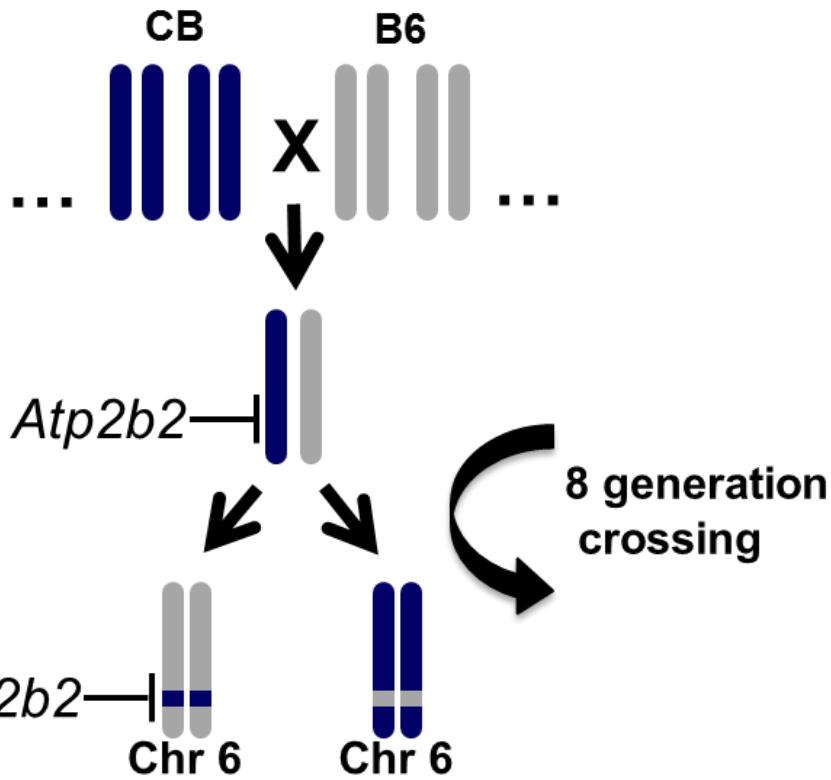


C57BL/6J (B6)

- **Standard good hearing strain**
- Normal *Atp2b2* transcript expression
- Normal *Inc83* expression

- AHL in part due to *Cdh23^{ahl}*
- Decreased *Atp2b2* transcript expression
- Increased *Inc83* expression

Tempel Lab Congenic: Karyotype



Atp2b2 Congene Predictions

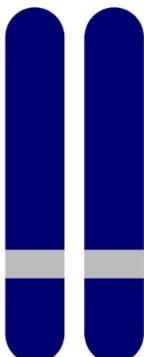
Chromosome 6



B6.CB^{*Atp2b2*}

We expect this strain to be more 'CB like'

- Exhibits improved hearing thresholds
- Normal *Atp2b2* transcript expression
- Normal Inc83 expression



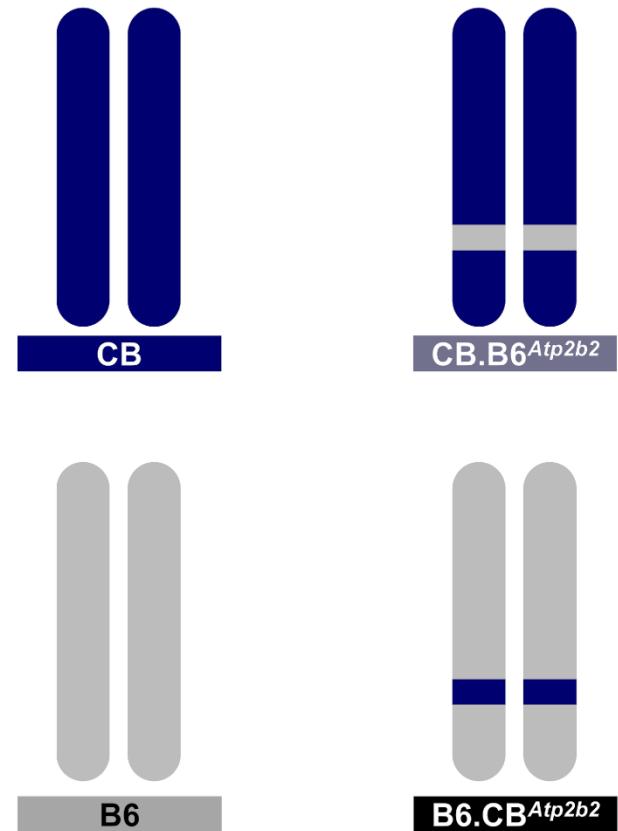
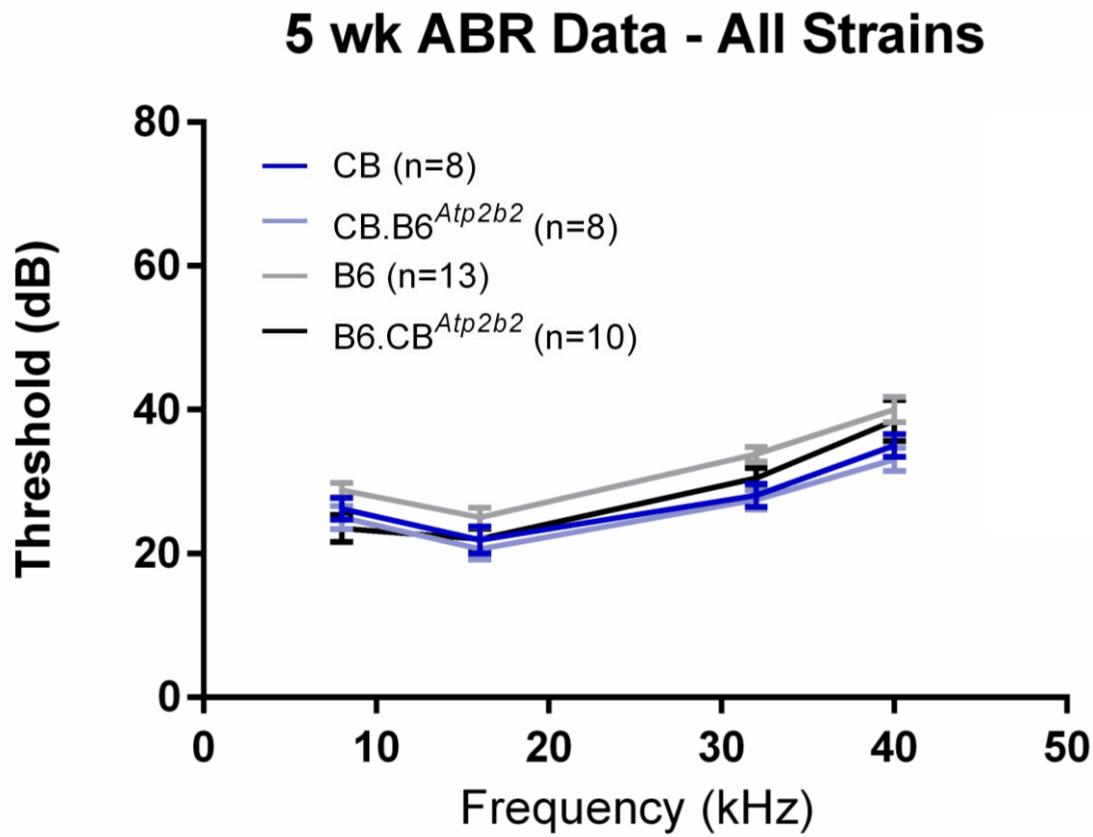
CB.B6^{*Atp2b2*}

We expect this strain to be more 'B6 like'

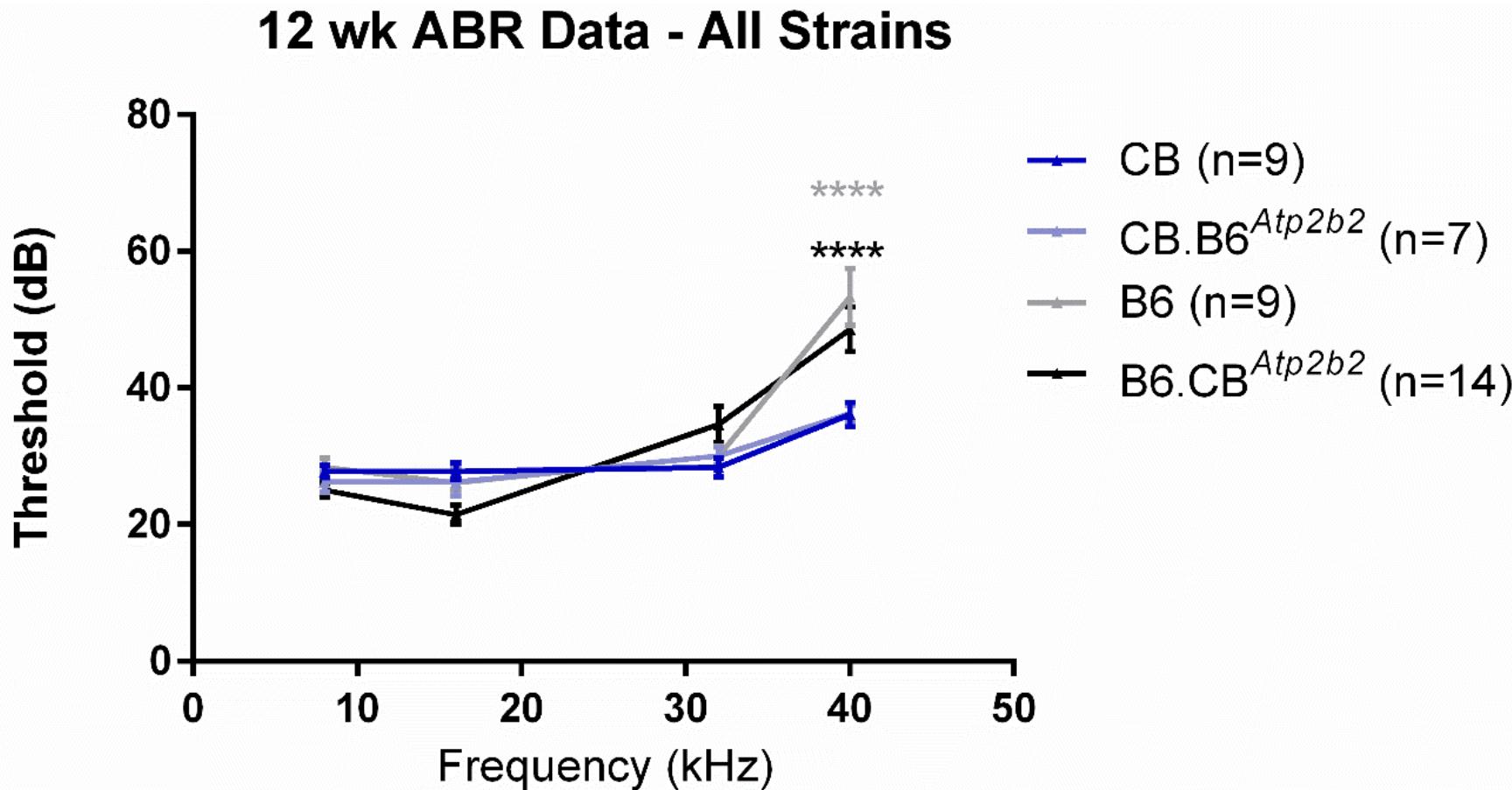
- Exhibits hearing loss
- Decreased *Atp2b2* transcript expression
- Increased Inc83 expression

All Strains Have Normal Hearing at 5wks

Tempel Congenes: Chromosome 6

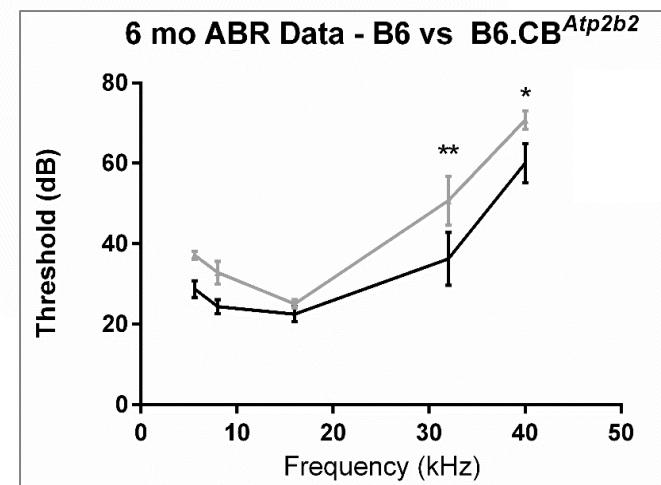
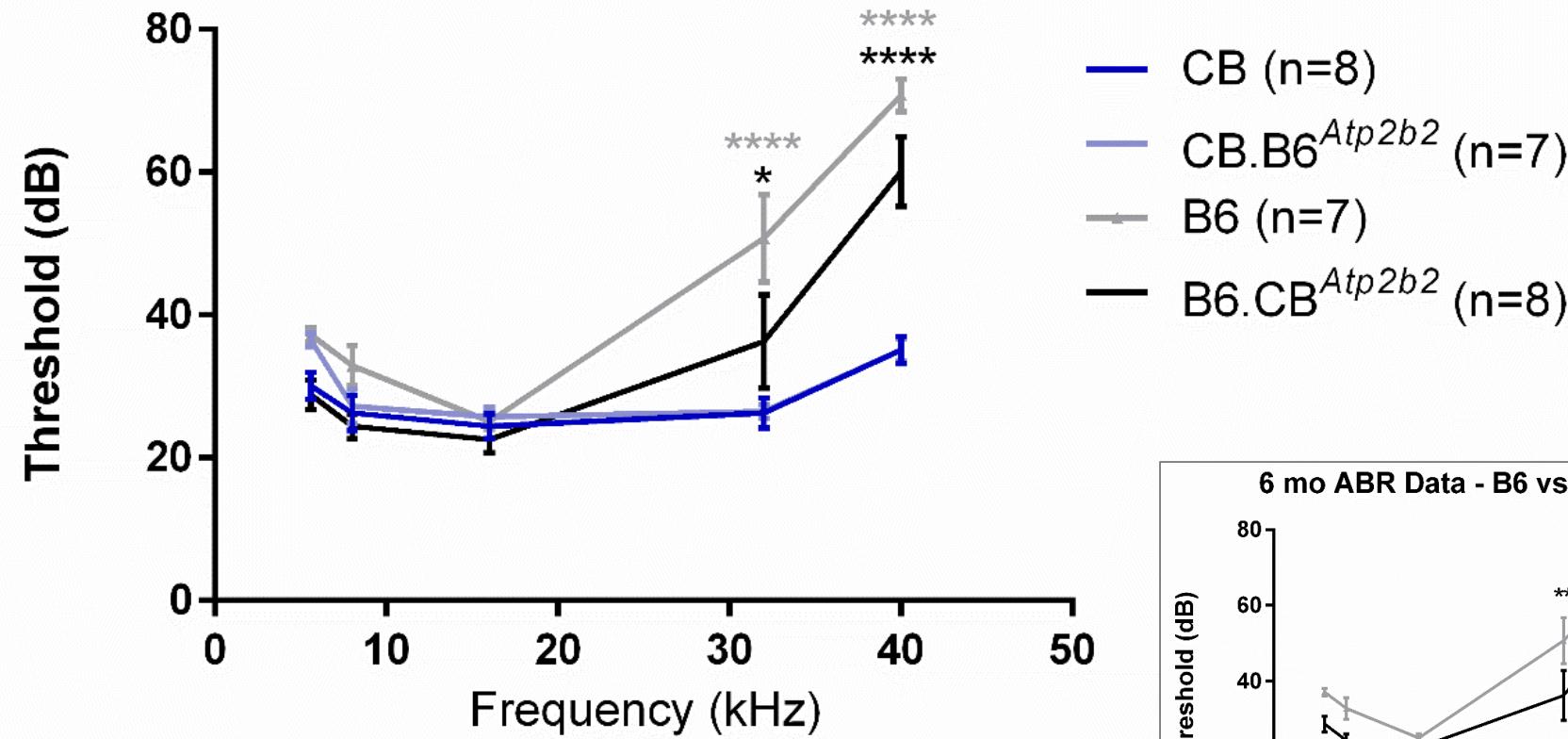


B6 Mice have Elevated Thresholds at 12wks

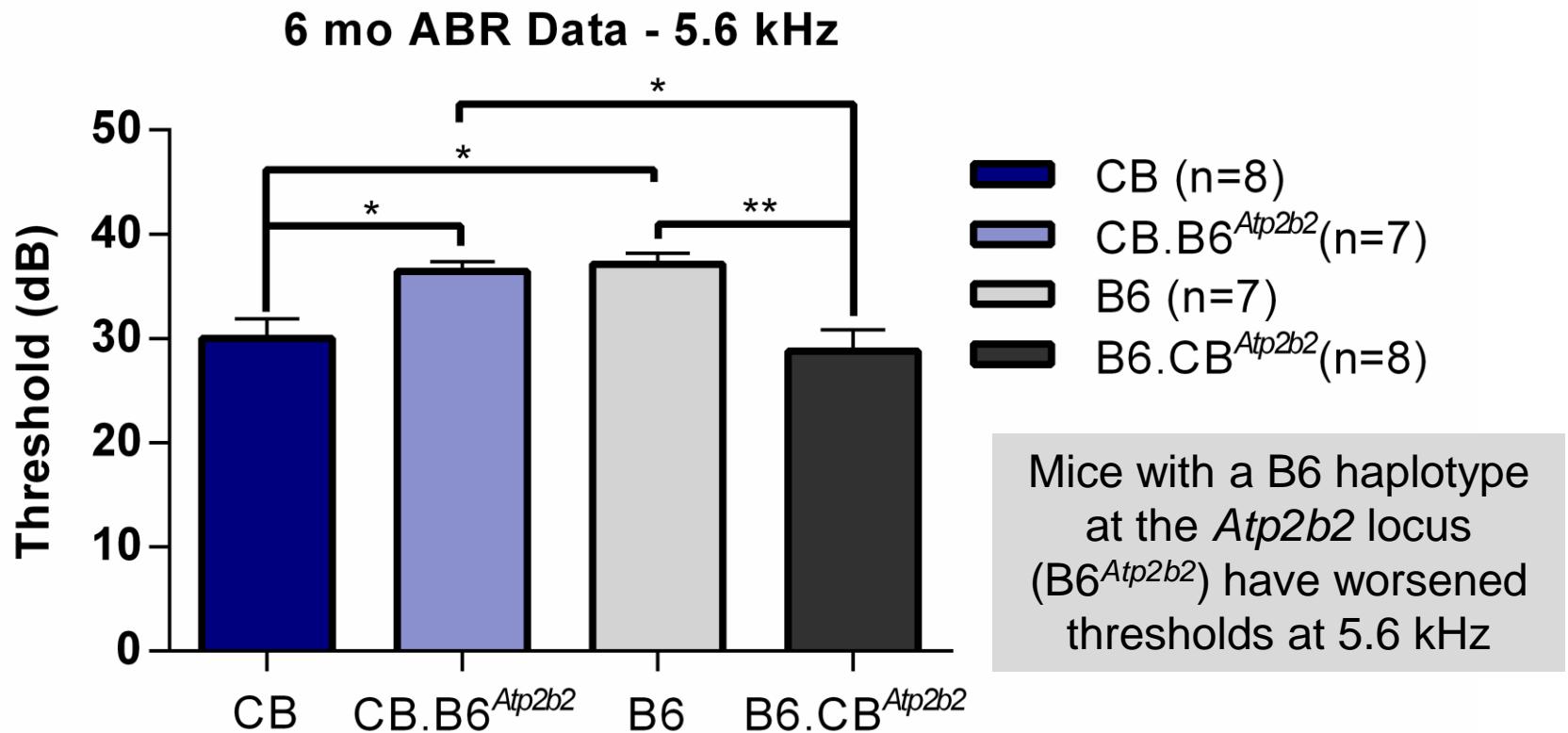


B6.CB^{Atp2b2} Exhibits a Partial Rescue Phenotype

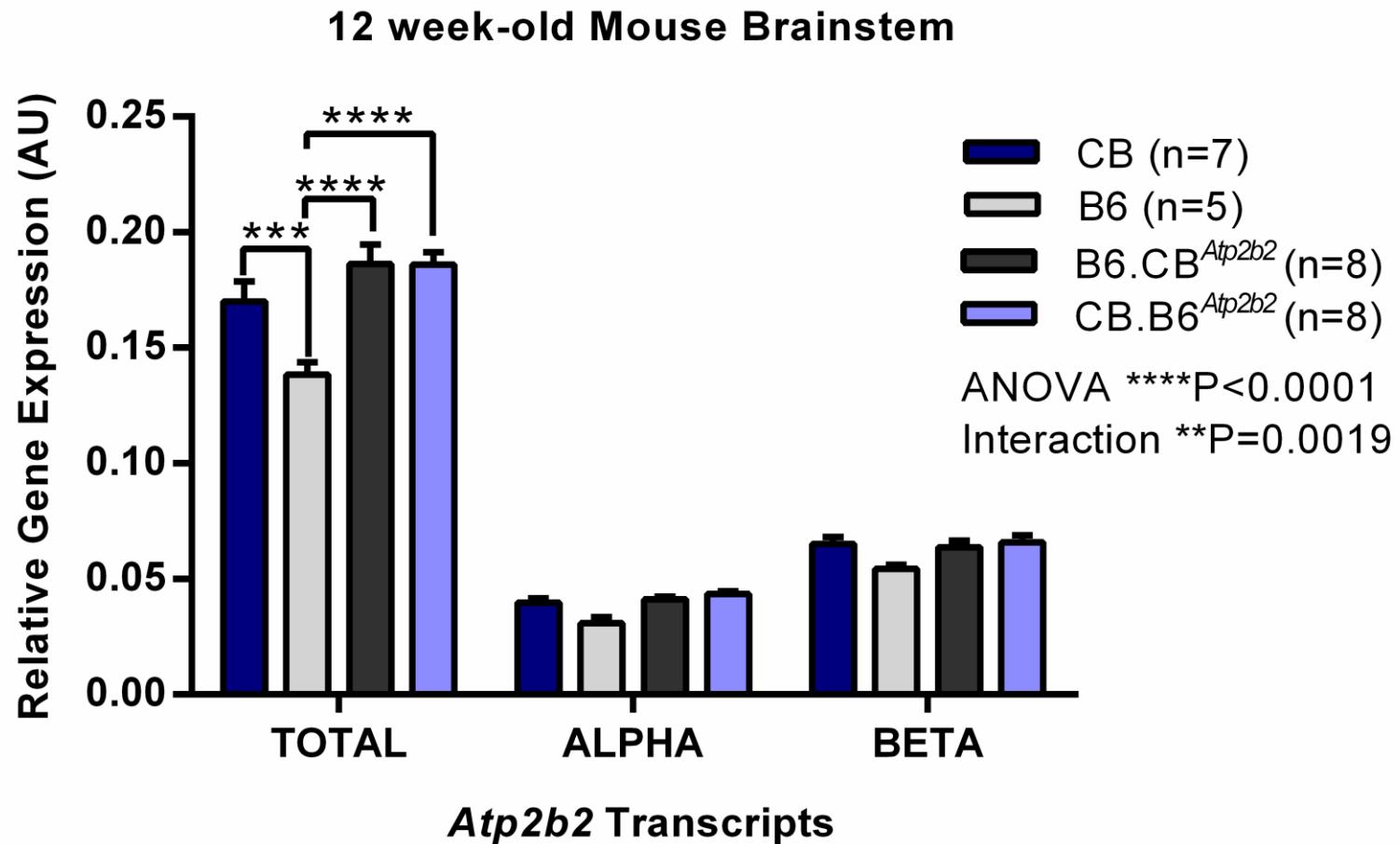
6 mo ABR Data - All Strains



6 Month Thresholds at 5.6 kHz

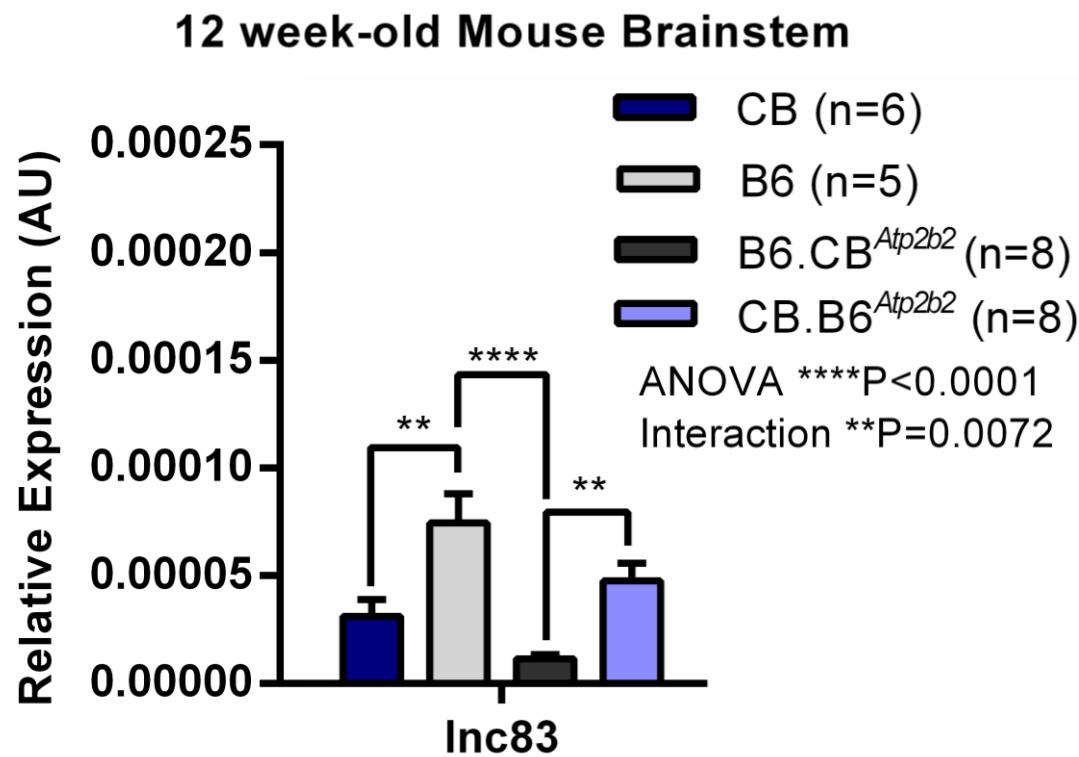


Atp2b2 Transcript Levels in All Strains



CB^{Atp2b2} locus rescues downregulation of *Atp2b2* in B6 Mice

Lnc83 Expression in All Strains



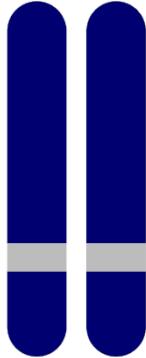
B6^{Atp2b2} locus increases expression of Lnc83

Atp2b2 Congene Conclusions



B6.CB^{*Atp2b2*}

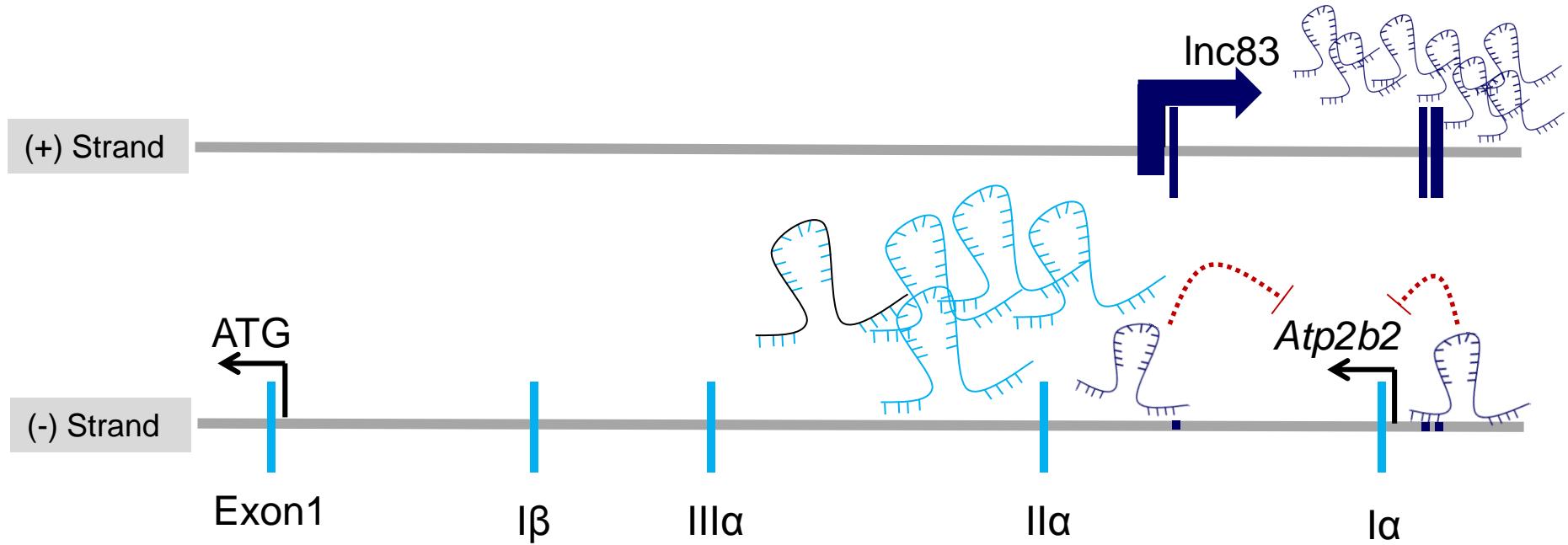
- Exhibits better hearing than B6 inbred strain **at 6 months**
- Normal *Atp2b2* transcript expression
- Normal Inc83 expression



CB.B6^{*Atp2b2*}

- Exhibits **some** hearing loss at **5.6 kHz**
- Normal** *Atp2b2* transcript expression
- Increased Inc83 expression

How does lncRNA Modulate *Atp2b2*?



Proposed Mechanisms:

1. Lnc83 Interferes with *Atp2b2* transcription (via protein repressor or physical obstruction).
2. Lnc83 Interferes with processing of transcript (i.e. splicing)

More tests are needed to determine the function of Inc83 in mouse.

Thesis conclusions:

- The promoter of $\alpha Atp2b2$ was identified and requires the CpG island.
 - EGR1 modulates the promoter and increases expression of $Atp2b2$ transcript.
 - EGR1 binds to the promoter in the CpG island.
-
- $Atp2b2$ transcript is misregulated in B6 mouse brainstem (at 5 and 12 weeks of age) and cochlea tissue (at 12 weeks of age).
 - Lnc83 is upregulated in B6 brainstem and cochlea.
 - Lnc83 overexpression in N2A cells inhibits $Atp2b2$ transcript.
-
- Congenes reveal intermediate auditory phenotypes.
 - The B6 $Atp2b2$ locus causes increased Lnc83 expression but was not sufficient to decrease $Atp2b2$ transcript levels in 12wk brainstem.
 - The CB $Atp2b2$ locus rescued misregulation of $Atp2b2$ and Lnc83 in B6.

AHL is a complex multigenic disorder in B6 mice and the $Atp2b2$ gene (via Lnc83) likely contributes to this phenotype.

Future Directions:

- Test hearing of *Atp2b2* congenes at later ages.
- Measure *Atp2b2* and *Inc83* expression in cochlea tissue and in old mice.
- Breed and test hearing of digenic (*Atp2b2* and *Cdh23*) congene mice.
- Study the function and mechanism of *Inc83*.

Thank You

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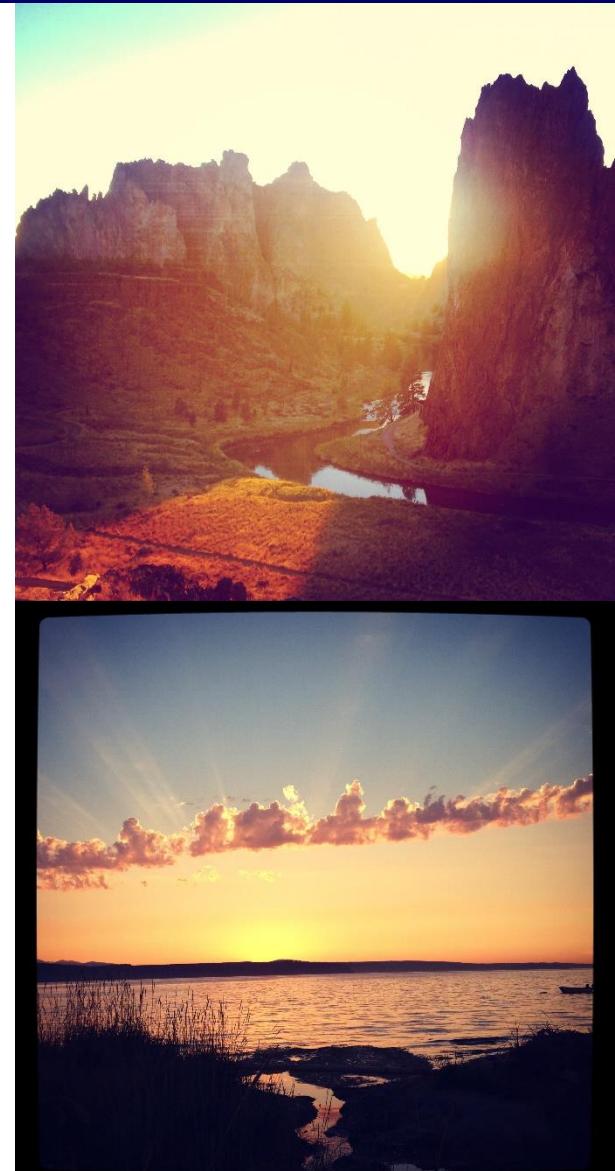
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Thank you for listening!