SUPPLEMENTAL MATERIAL

Title: NFκB Activity Regulates Cell Type and Context Specific Susceptibility to Calcification in the Aortic Valve

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Major Resources Tables

Animals (in vivo studies)

Mouse strain name	Vendor or Source	Background Strain	Other Information
C57BL/6J	Jackson Laboratory: 000664	C57BL/6J	Wildtype Back 6 strain used for backcrossing other mouse lines
B6.129S7-Ldlr^{tm1Her}/J	Jackson Laboratory: 002207	C57BL/6J	Ldlr^{tm1Her} targeted knockout for low density lipoprotein receptor
B6;129S4-Gt(ROSA)26Sor^{tm1Sor}/J	Jackson Laboratory: 003309	C57BL/6J	Gtrosa26^{tm1Sor} targeted mutation of lacZ gene flanked by loxP sites
Ikbkb^{tm2Mka}	Michael Karin (University of California, San Diego)	C57BL/6J	LoxP sites insertion flanking exon 3, ATP binding site of catalytic domain
Nfatc1$^{tm1.1(cre)Bz}$	Bin Zhou (Albert Einstein College of Medicine, Bronx, NY)	C57BL/6J	Nfatc1^{Cre}, IRES-cre cDNA sequence inserted into 3' UTR of Nfatc1 gene.
Tg(Nfatc1c-cre)1Bz	Bin Zhou (Albert Einstein College of Medicine, Bronx, NY)	C57BL/6J	Nfatc1^{enCre}, nuclear localized cre recombinase sequence inserted between HSP68 minimal promoter and Nfatc1 intron 1 fragment (Nfatc1 enhancer).

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration
mouse anti-human CD31	Cell Signaling Technology	3528	1:200

rabbit anti-human NFkB p65	Cell Signaling Technology	8242	1:400
mouse anti-human αSMA	Abcam	ab7817	10 ug/ml (1:100)
rabbit anti-human VCAM-1	Cell Signaling Technology	32653	1:100
rabbit anti-human NFkB p65	Cell Signaling Technology	8242	1:400
rabbit anti-human ki67	Abcam	ab15580	10 ug/ml (1:100)
rabbit anti-mouse CD31	Abcam	ab28364	1:50
rabbit anti-human IKKβ	Abcam	ab124957	10 ug/ml (1:100)
rabbit anti-human VE-Cadherin	Cell Signaling Technology	2500	1:200
mouse anti-human Runx2	Abcam	ab76956	10 ug/ml (1:100)
mouse-anti human Osteocalcin	Abcam	ab13420	10 ug/ml (1:100)

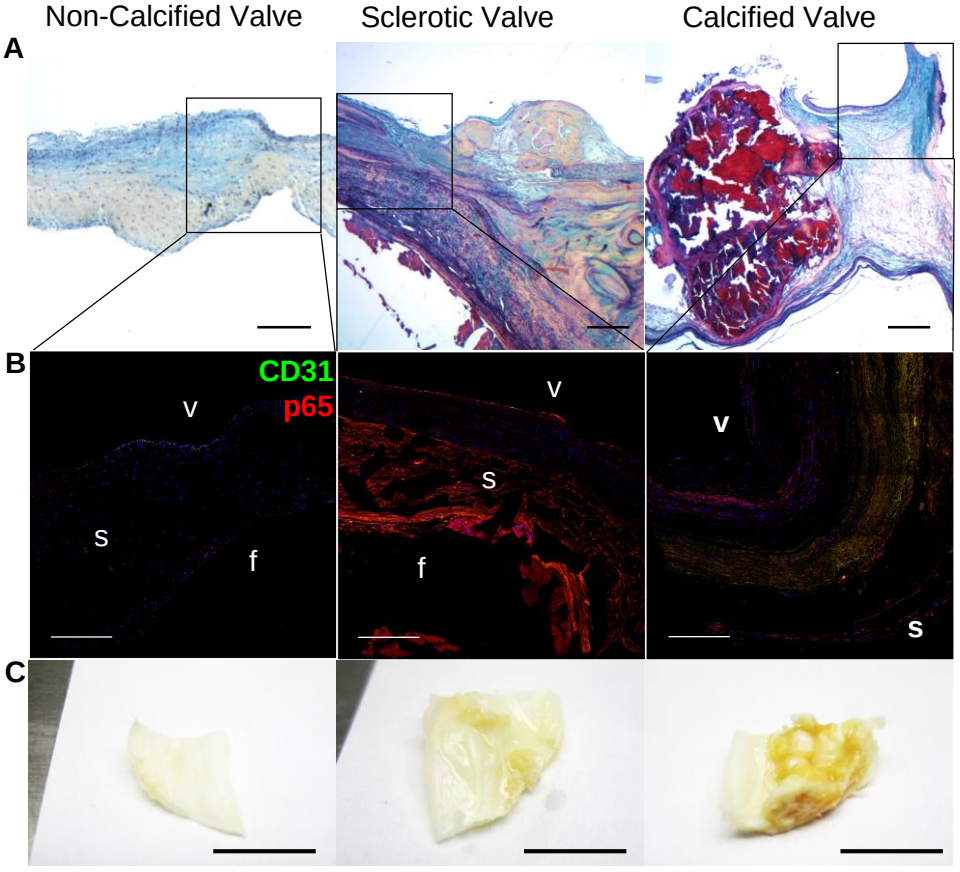
qRT-PCR Primers

Gene	Forward Primer	Reverse Primer	Ascension Number
18S	TAGAGGGACAAGTGGCGT	AATGGGGTTCAACGGGTT	NR_046261.1
ACTA2	CAGCCAGGATGTGTGAAGAA	TCACCCCCTGATGTCTAGGA	NM_001164650.1
Osteocalcin	CTCCAGCCACAACATCCTTT	TGGCCTCCAGCACTGTTTAT	NM_001164004.1
PECAM1	ATCTGCATCTCGTGGGAAGT	GAGCTGAAGTGTCAGCAGGA	NM_213907.1
RUNX2	GCACTACCCAGCCACCTTTA	TATGGAGTGCTGCTGGTCTG	XM_005666074.3
SNAI1	GCCCAACTACAGCGAGCTAC	CCAGGAGAGAGTCCCAGATG	XM_021077961.1
VE-Cadherin	CGTGGTGGAAACACAAGATG	TGTGTACCTGGTCTGGGTGA	NM_001001649.2

Cultured Cells

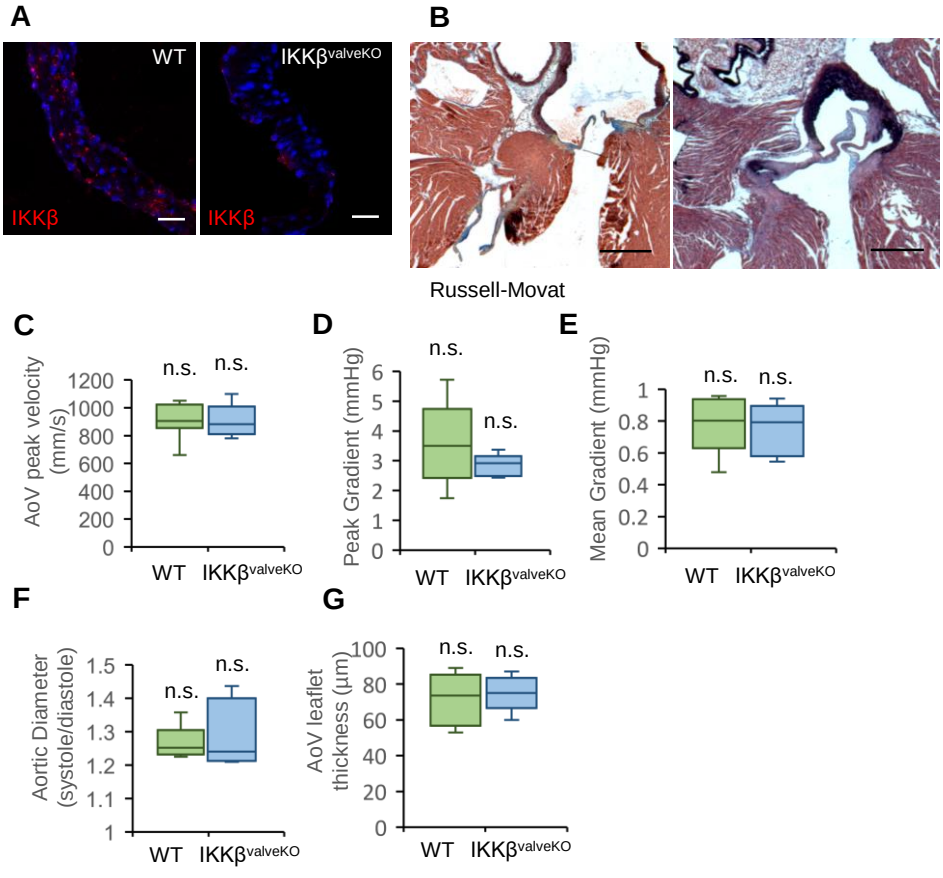
Name	Vendor or Source	Sex (F, M, or unknown)
Porcine Aortic Valve Endothelial Cells (PAVEC)	Primary cell isolation (Shirk Meats, Dundee, NY)	Male
Porcine Aortic Valve Interstitial Cells (PAVIC)	Primary cell isolation (Shirk Meats, Dundee, NY)	Male

Supplementary Figure SI.



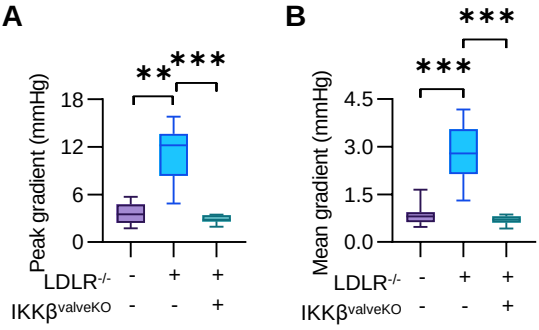
Supplementary Figure SI. A. Russell-Movat staining of non-calcified, sclerotic, and calcified/stenosed human aortic valves. Scale bar is 200 um. B. Sister section immunofluorescence stain for NFkB p65 and CD31. Letters indicate trilaminar region classifications of valve for subsequent analysis (v: ventricularis, s: spongiosa, f: fibrosa). Scale bar is 200 um. C. Macroscopic images of fixed non-calcified, sclerotic, and calcified/stenosed human aortic valves. Scale bar is 1 cm.

Supplementary Figure SII.



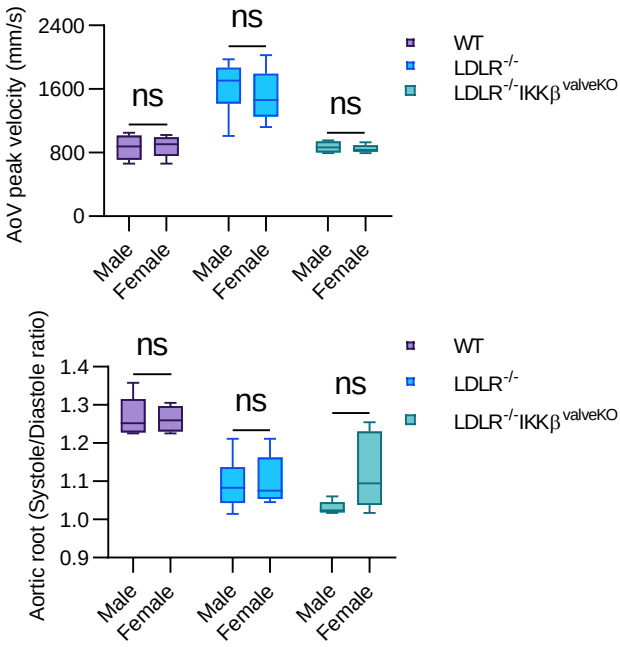
Supplementary Figure SII. A. Immunoflorescence staining of IKKβ expression of 5 month-old WT and IKKβ^{valveKO} mouse valves, scale bar is 20 um. B. Russell-Movat staining of 5 month-old WT and IKKβ^{valveKO} (Nfatc1^{Cre};IIKKβ$^{flox/flox}$) mouse hearts, magnified at aortic valve region. Scale bar is 0.5 mm. C-G. Echocardiographic measurements of peak velocity, peak gradient, and mean gradient through aortic valve, no difference between groups (n > 5). E. Measurement of aortic root diameter from echocardiographic M-mode with comparison between systolic and diastolic ratio, no difference between groups (n > 5). F. Thickness of the AoV leaflet, measured from EKV. No difference between groups. (n > 5). Error bars indicate SEM.

Supplementary Figure SIII.



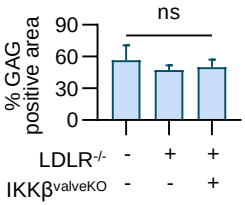
Supplementary Figure SIII. Echocardiographic measurement of Peak gradient (A) and Mean gradient (B) using the average peak from > three cardiac cycles for each mouse (WT: n=11 LDLR$^{-/-}$: n=17, LDLR-/-IKKB^{valveKO}: n=11). Asterisks indicate statistically significance between groups (*: $p < 0.05$, **: $p < 0.005$, ***: $p < 0.0005$).

Supplementary Figure SIV.



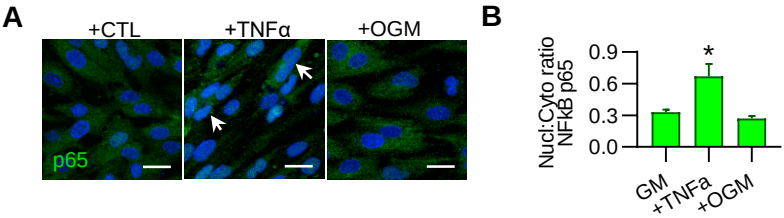
Supplementary Figure SIV. Comparison of Sex-specific echocardiographic parameters of transgenic mouse lines. (WT: n=6 male, 5 female LDLR-/-: n=10 male 7 female, LDLR-/-IKKBvalveKO: n=6 male 5 female)

Supplementary Figure SV.



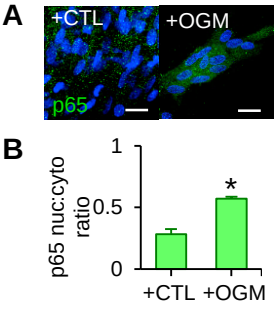
Supplementary SV. A. Glycosaminoglycan (GAG) content according to Russell-Movat stain (blue). n = 5. B. Valve area, calculated from the maximum valve area observed in each sample and averaged across all animals in each group (no significant differences). n = 5. Asterisks indicate statistically significant groups (*: $p < 0.05$). Error bars indicate SEM.

Supplementary Figure SVI.



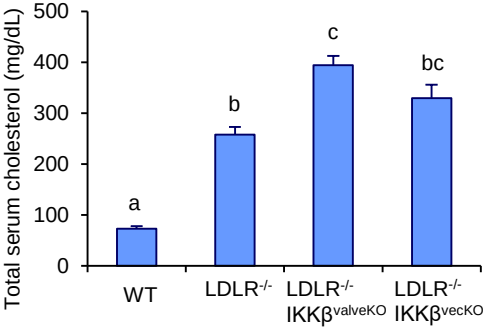
Supplementary Figure SVI. A. Representative images of p65 expression in 48 hour VEC on 3D hydrogels treated with TNFα or OGM. White arrows indicate cells with nuclear-positive signal. Scale bar is 20μm. B. Quantification of p65 nuclear translocation in VEC using the ratio of nuclear to cytosolic p65 protein (nuc:cyto ratio). n = 4. Asterisks indicate statistically significant groups (*: $p < 0.05$).

Supplementary Figure SVII.



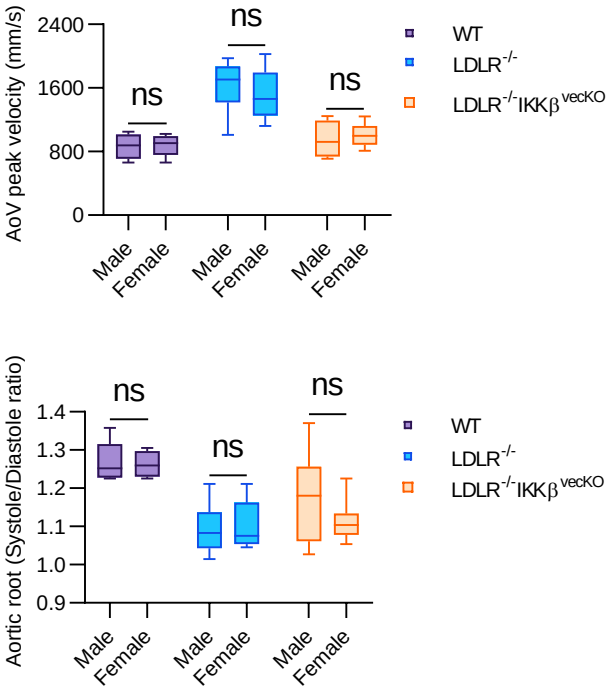
Supplementary Figure SVII. A. p65 expression in 14-day VIC in 3D hydrogels treated with CTL or OGM. Scale bar is 20μm. B. Quantification of p65 nuclear translocation in VIC using the ratio of nuclear to cytosolic p65 protein (nuc:cyto ratio). n = 4.

Supplementary Figure SVIII.



Supplementary Figure SVIII. Total serum cholesterol in each group, collected at 5 months of age (n = 5). Groups that do not share letters are statistically significant, $p < 0.05$. Error bars indicate SEM.

Supplementary Figure SIX.



Supplementary Figure SIX. Comparison of Sex-specific echocardiographic parameters of transgenic mouse lines. (WT: n=6 male, 5 female LDLR-/-: n=10 male 7 female, LDLR-/-IKKBvalveKO: n=6 male 9 female)