

# Furin as a novel pro-atherogenic gene

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# CAD: #1 Cause of Deaths Worldwide

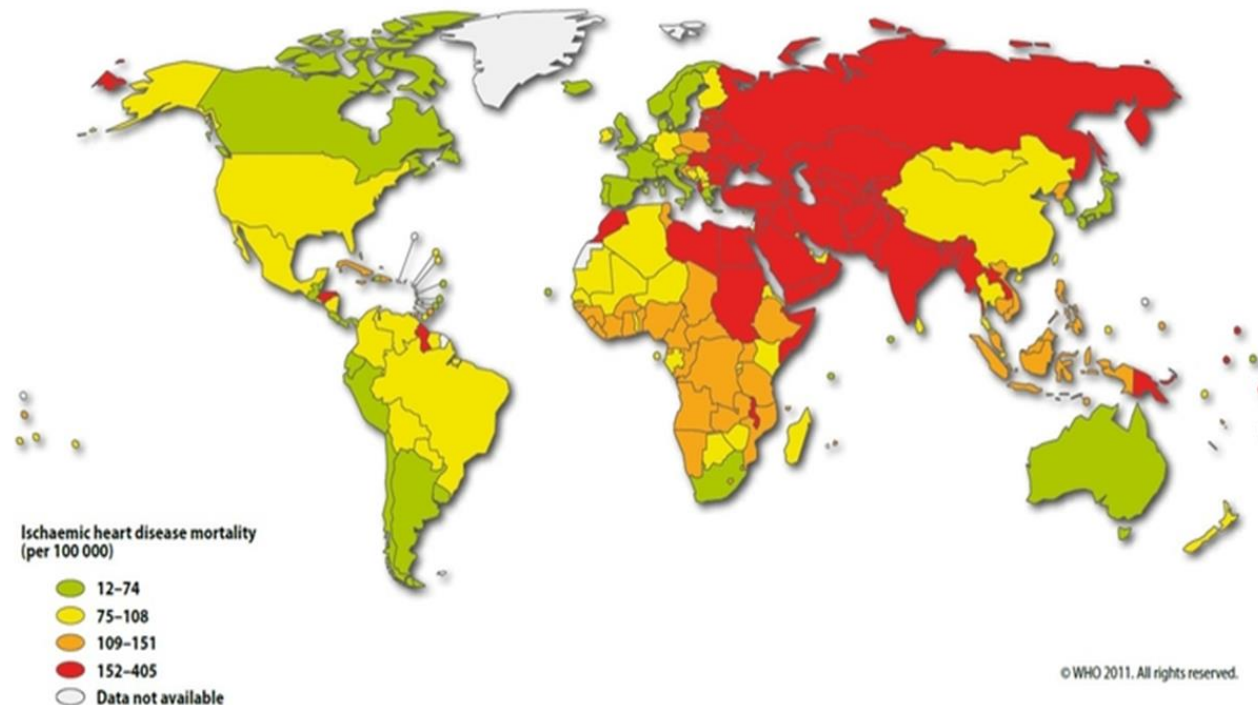
**17.5 million**

people die each year from CVDs, an estimated 31% of all deaths worldwide

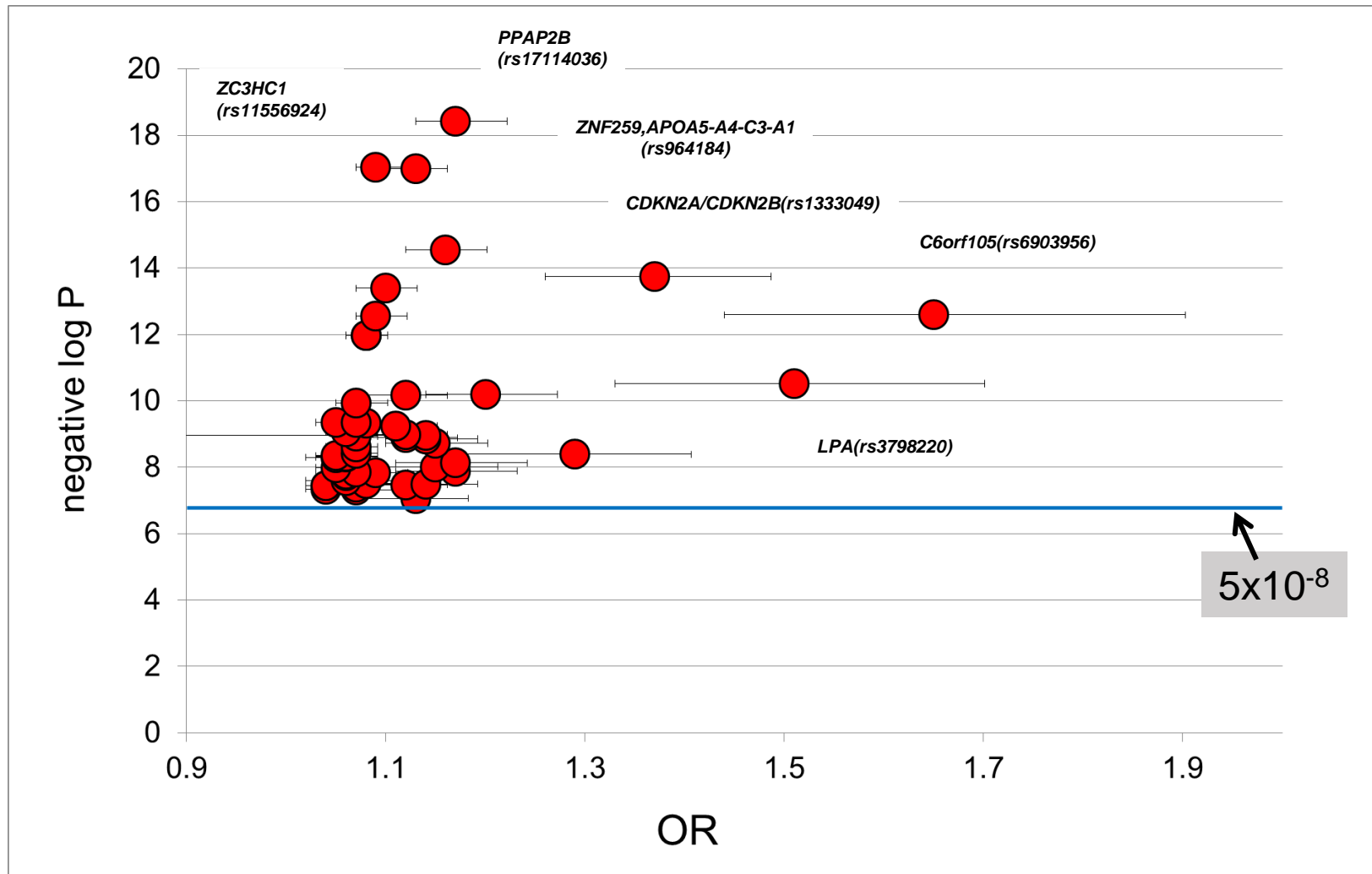
**80%**

of all CVD deaths are due to heart attacks and strokes

Figure 27 World map showing ischemic heart disease mortality rates (age standardized, per 100 000) (1).



# CAD is a heritable disease : Genome-wide significant loci associated with CAD ( $p < 5 \times 10^{-8}$ ) to date



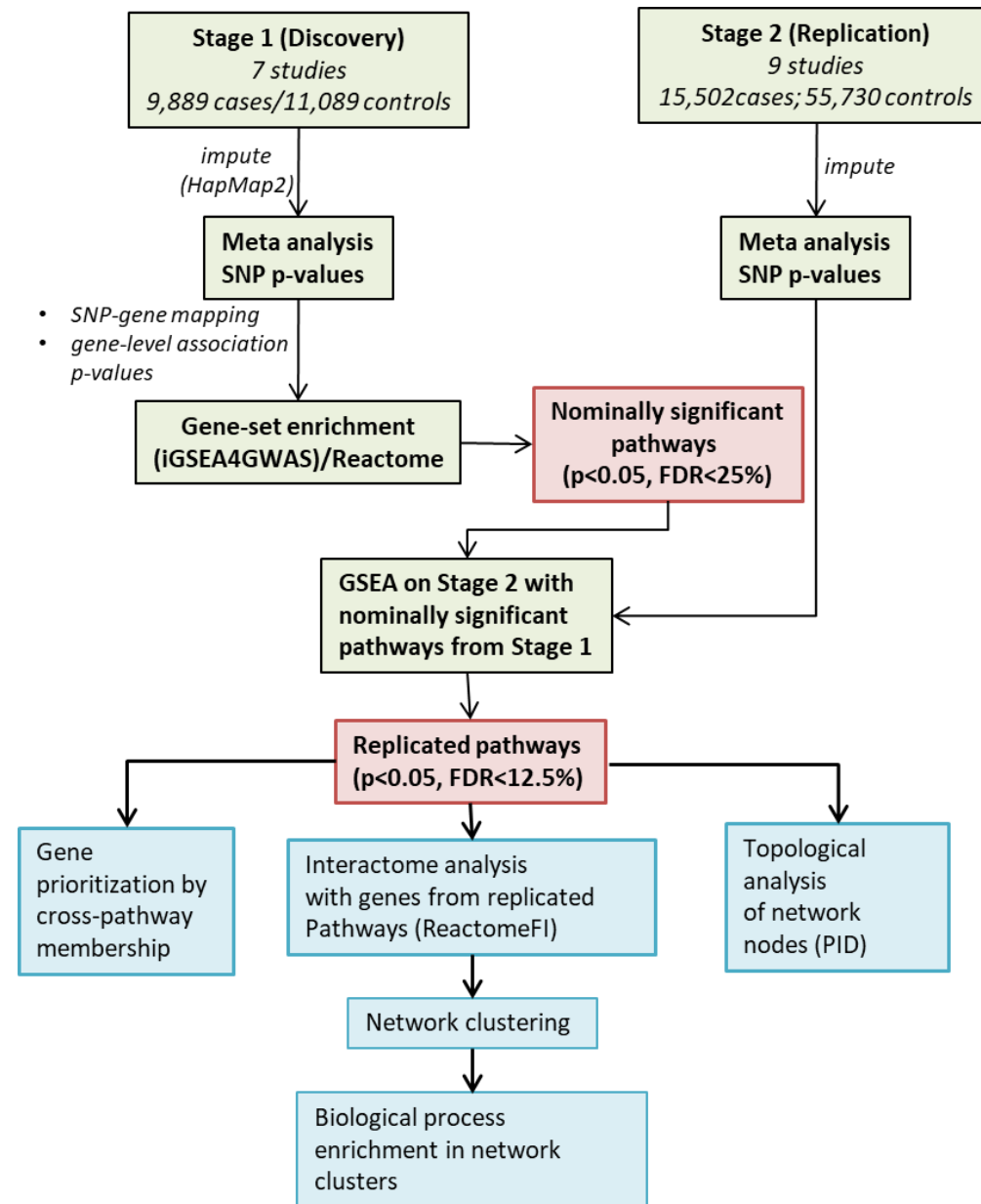
(Lieb and Vasan, 2013)

# Published Results

- Ghosh et al., *Arterioscler Thromb Vasc Biol.* 2015 Jul;35(7):1712-22. doi: 10.1161/ATVBAHA.115.305513. Epub 2015 May 14. PMID: 25977570; PMCID: PMC4841833.
- Yakala et al., *Arterioscler Thromb Vasc Biol.* 2019 Mar;39(3):387-401. doi: 10.1161/ATVBAHA.118.311903. PMID: 30651003; PMCID: PMC6393193

**Pathway  
Analysis of a  
large genotyped  
CAD  
case:control  
cohort  
(CardioGram  
consortium)**

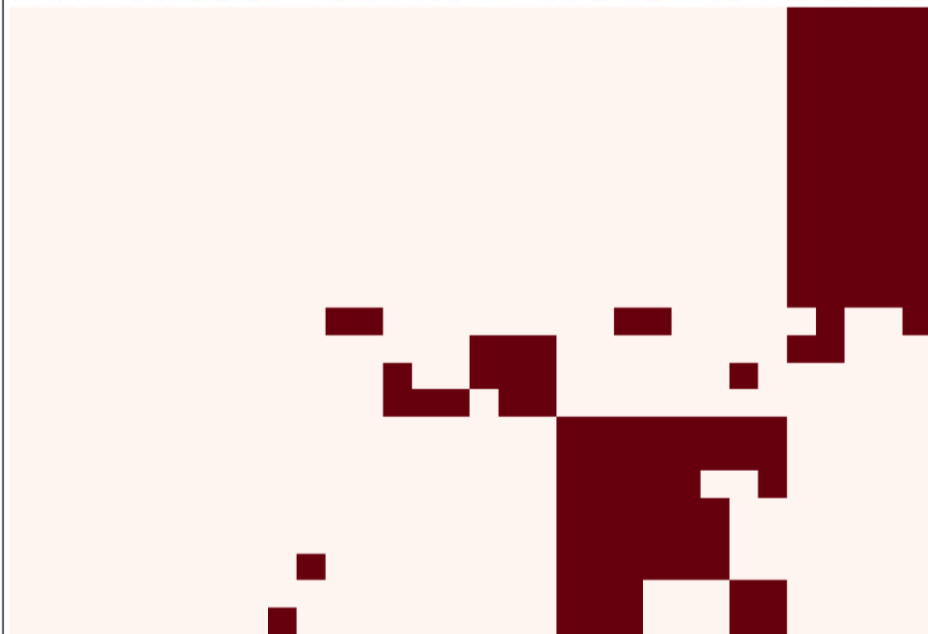
**Pathway  
Database:  
REACTOME**



*(Ghosh et al., 2015)*

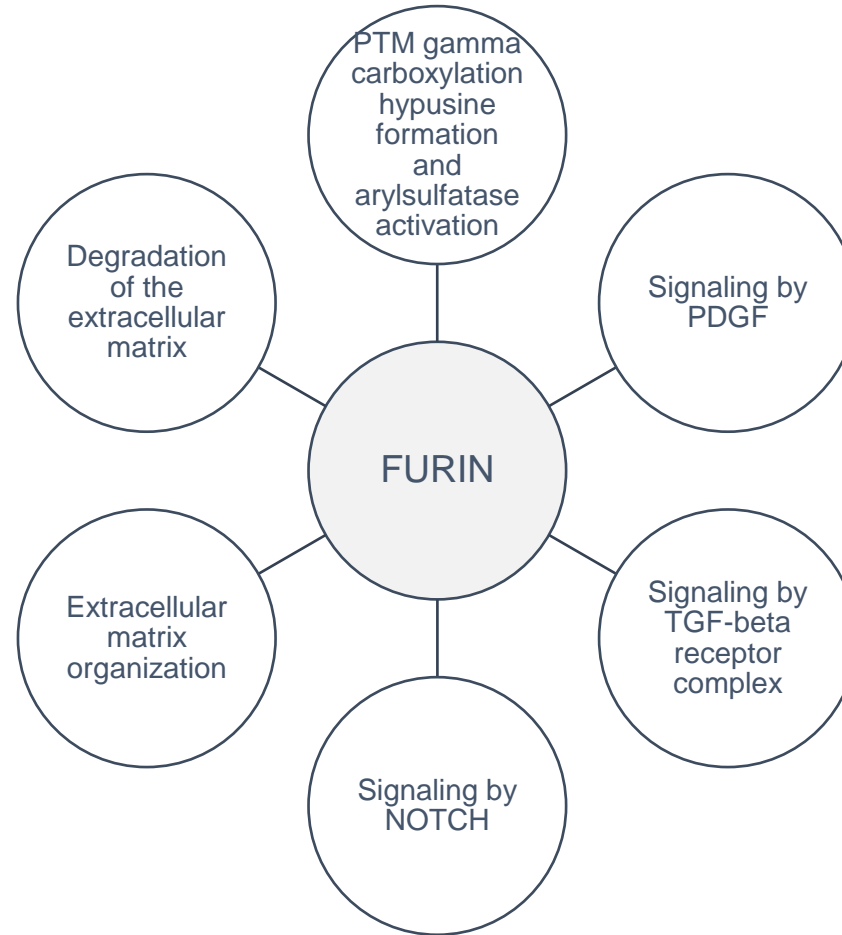
# Sharing of genes among CAD-associated pathways – FURIN associated with 6 pathways

CELL EXTRACELLULAR MATRIX INTERACTIONS  
 CRMP5 IN SEMA3A SIGNALING  
 G BETA GAMMA SIGNALING THROUGH PI3KGAMMA  
 INITIAL TRIGGERING OF COMPLEMENT  
 METABOLISM OF POLYAMINES  
 NUCLEAR RECEPTOR TRANSCRIPTION PATHWAY  
 ORGANIC CATION ANION ZWITTERION TRANSPORT  
 PI3K AKT ACTIVATION  
 SULFUR AMINO ACID METABOLISM  
 NOTCH HLH TRANSCRIPTION PATHWAY  
 TOLL RECEPTOR CASCADES  
 DEGRADATION OF THE EXTRACELLULAR MATRIX  
 PTM GAMMA CARBOXYLATION HYPUSINE FORMATION AND ARYL SULFATASE ACTIVA  
 CHYLOMICRON MEDIATED LIPID TRANSPORT  
 HEPARAN SULFATE HEPARIN HS GAG METABOLISM  
 HS GAG BIOSYNTHESIS  
 HDL MEDIATED LIPID TRANSPORT  
 LIPID DIGESTION MOBILIZATION AND TRANSPORT  
 LIPOPROTEIN METABOLISM  
 NOTCH1 INTRACELLULAR DOMAIN REGULATES TRANSCRIPTION  
 SIGNALING BY NOTCH1  
 SIGNALING BY NOTCH  
 SIGNALING BY TGF BETA RECEPTOR COMPLEX  
 TRANSCRIPTIONAL ACTIVITY OF SMAD2 SMAD3 SMAD4 HETEROTRIMER  
 SMAD2 SMAD3 SMAD4 HETEROTRIMER REGULATES TRANSCRIPTION  
 PPARA ACTIVATES GENE EXPRESSION  
 TRANSCRIPTIONAL REGULATION OF WHITE ADIPOCYTE DIFFERENTIATION  
 COLLAGEN FORMATION  
 EXTRACELLULAR MATRIX ORGANIZATION  
 NCAM SIGNALING FOR NEURITE OUT GROWTH  
 NCAM1 INTERACTIONS  
 SIGNALING BY PDGF



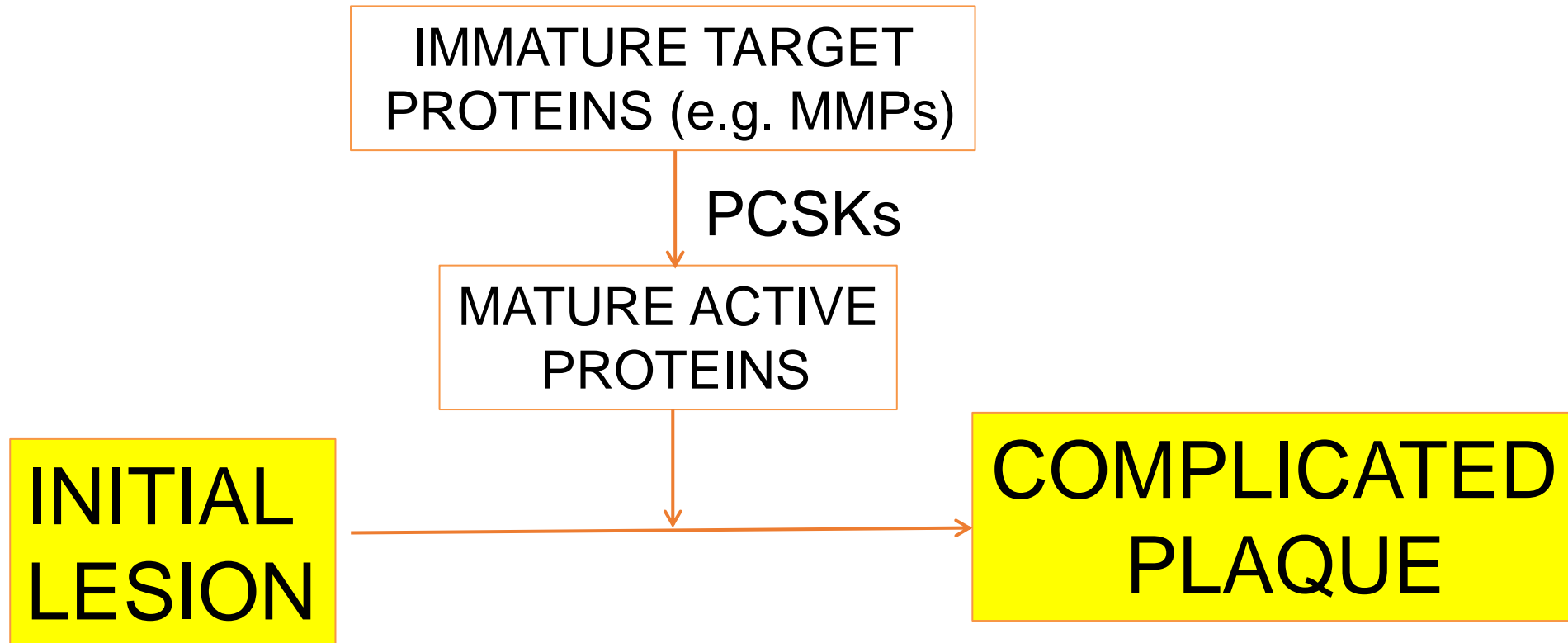
Gene	Index SNP	GWAS value	p
COL4A2	rs4773144	5.73023E-07	
COL4A1	rs12873154	0.000167841	
COL3A1	rs1914033	0.000818352	
COL9A1	rs9364078	0.001303269	
COL5A2	rs17166196	0.005286415	
COL1A2	rs2839112	0.010851699	
COL6A2	rs11103531	0.011018984	
COL5A1	rs1882436	0.016278631	
COL4A4	rs4663723	0.025022684	
COL6A3	rs6722825	0.029369868	
COL4A3	rs17514846	8.81276E-05	
FURIN	rs7819541	2.39805E-05	
BMP1	rs2075292	0.001093351	
APOA1	rs11689467	0.017288727	
SDC1	rs3736868	0.00085193	
CCNC	rs7987737	0.003019855	
CDK8	rs870844	0.001591388	
NCOR2	rs4733560	0.005534998	
MYC	rs7533676	0.021259365	
HDAC1	rs6554	0.029595178	
RPS27AP	rs6554	0.029595178	
TBL1XR1	rs6803575	0.002511815	
CREBBP	rs11076787	0.033028194	

# 6 CAD-associated pathways containing Furin



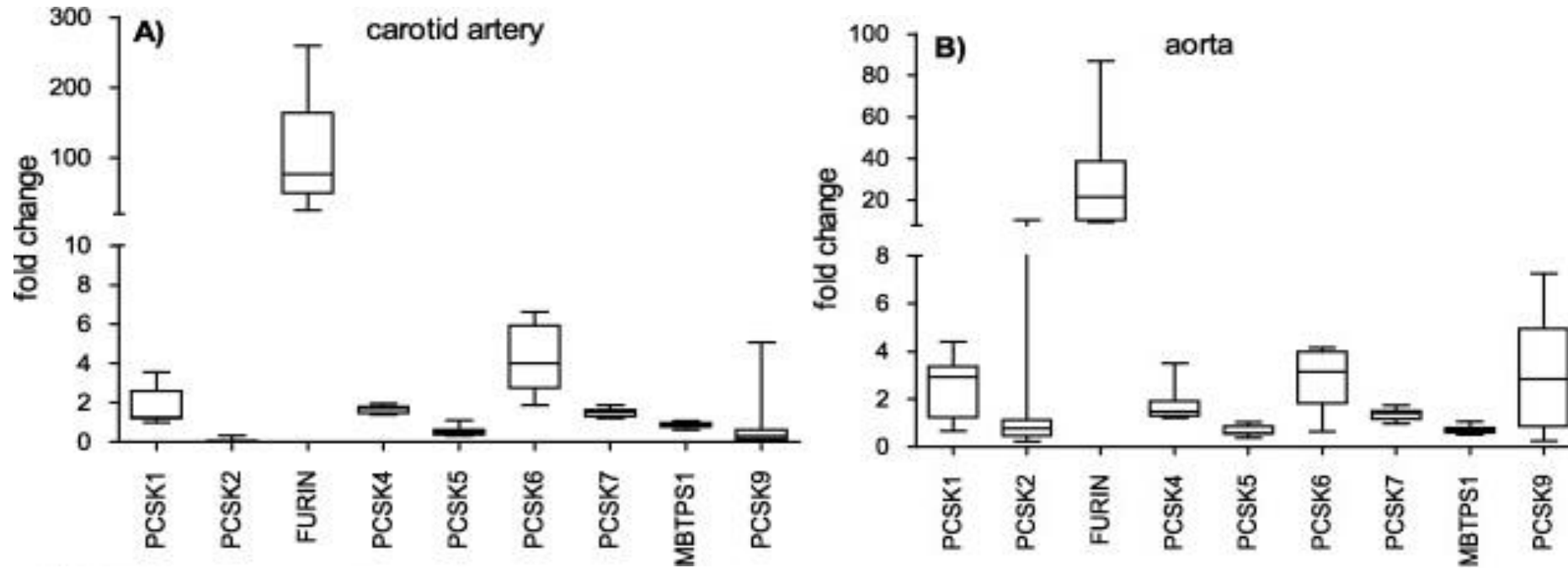
## FURIN : a proprotein convertase of the PCSK family

Proprotein convertase subtilisin/kexin (PCSK) enzymes are a family of nine proteases that cleave and convert their immature target proteins into biologically active forms.





# FURIN is highly expressed in Human atherosclerotic plaques



Furin is the major plaque-expressed proprotein convertase (RT-PCR)

# Furin: Target Validation in Cell Culture and Animal Models

## Cell-Based Studies

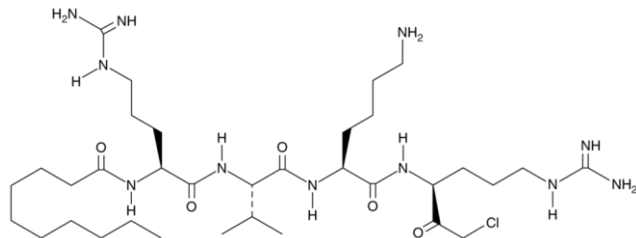
Vascular Smooth Muscle Cells

Vascular Endothelial Cells

Monocytes/Macrophages

Chemical/CRISPR Furin Inhibition

- Transmigration, adhesion, monolayer barrier function
- Adhesion
- MMP activation
- Inflammatory gene expression
- Lipid accumulation
- Monocyte → macrophage phenotype switching



Furin inhibitor dec-RVKR-CMK

## Animal Studies



ApoE -/-

Furin +/-

### Progression Study

Pre-treatment with Furin inhibitor followed by high-fat diet to see if atherosclerotic phenotype is abrogated.

- Lipid staining
- Lesion staining
- Enzyme activity assays
- qPCR

### Regression Study

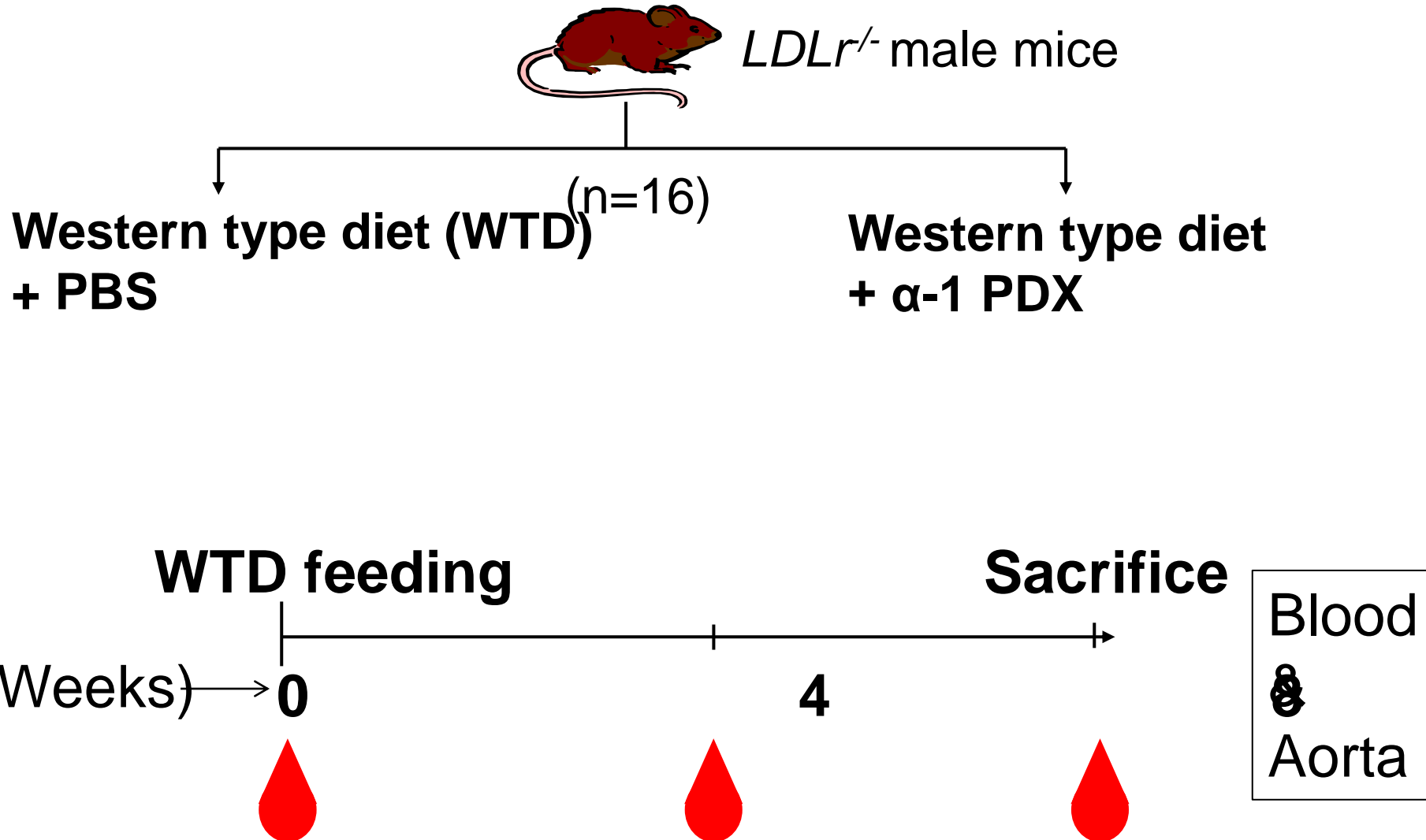
Initial high-fat diet followed by Furin inhibition to see if atherosclerotic phenotype can be reversed.

- Lipid staining
- Lesion staining
- Enzyme activity assays
- qPCR

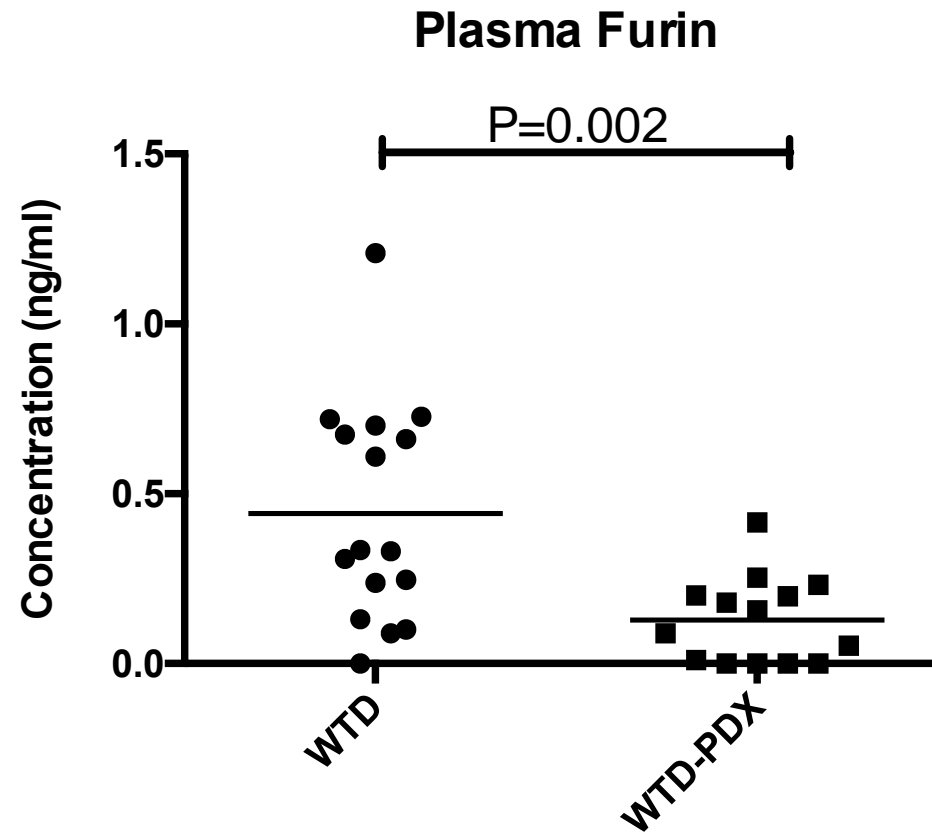
# Animal model studies

- **Atherosclerosis model**
  - Ldlr-/- mouse on atherogenic (HF/HC) diet
  - ApoE-/- mice on atherogenic (HF/HC) diet
- **Treatment**
  - PDX-1a (furin inhibitor) or PBS
- **Endpoints**
  - Atherosclerotic plaque related measures
  - Plasma lipids

# Experimental set-up

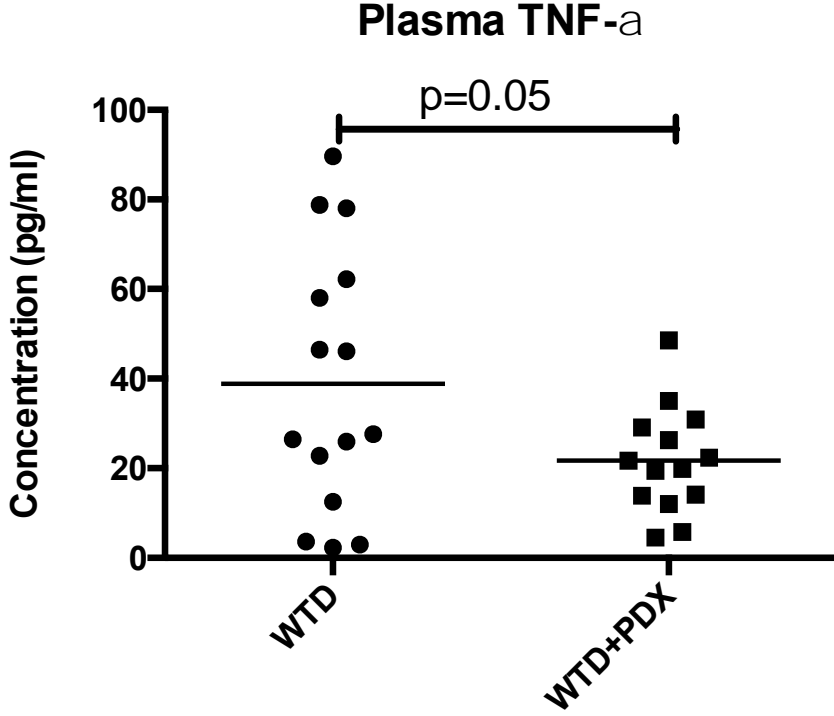
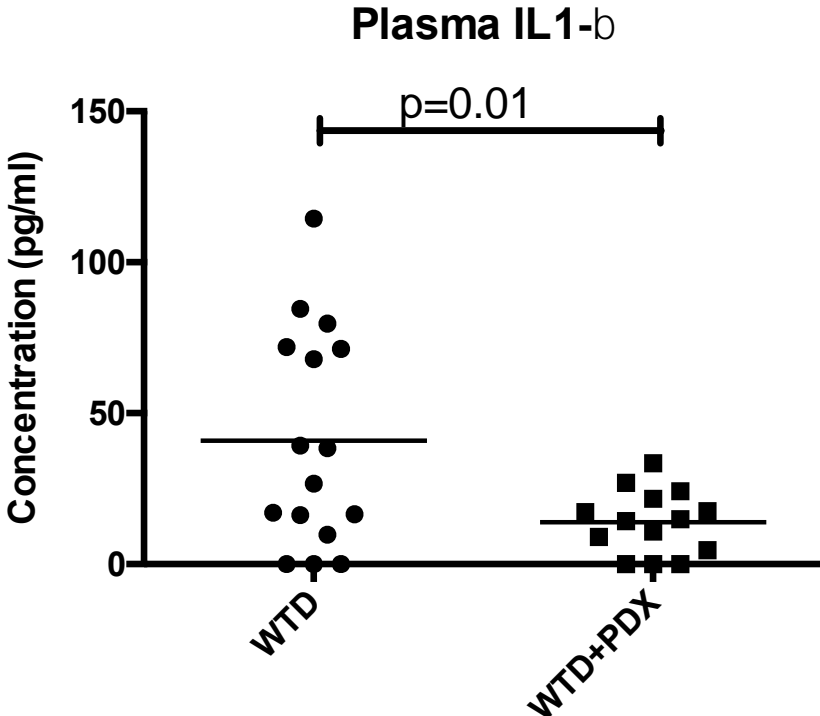


# Plasma Furin levels are significantly reduced in Furin inhibitor treated mice

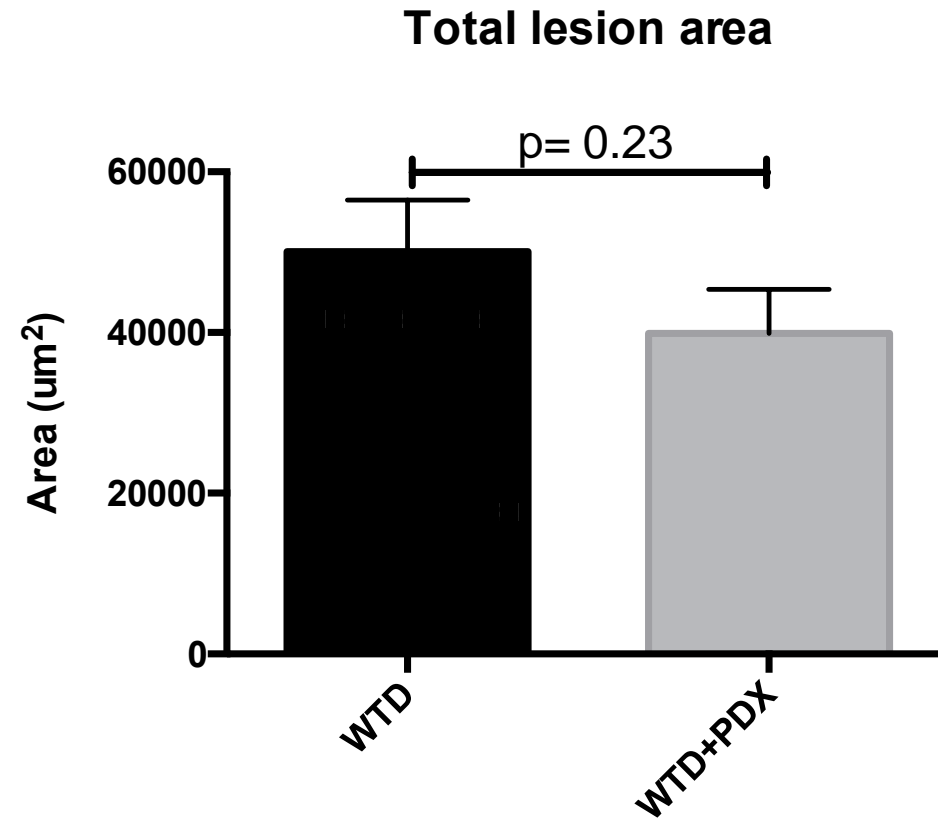
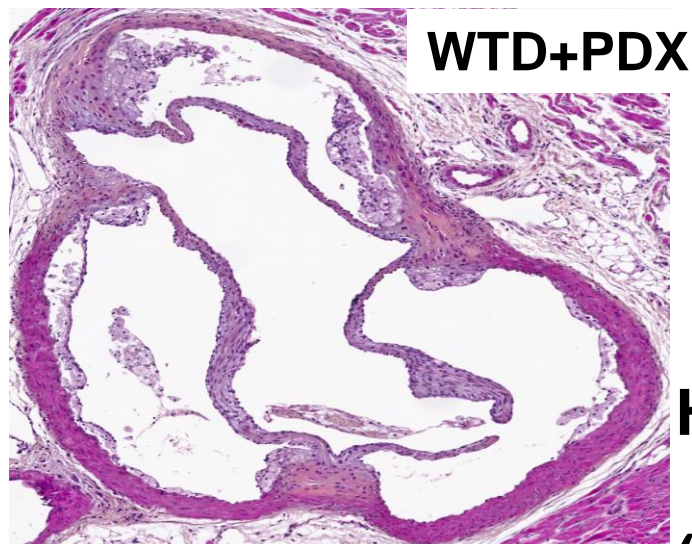
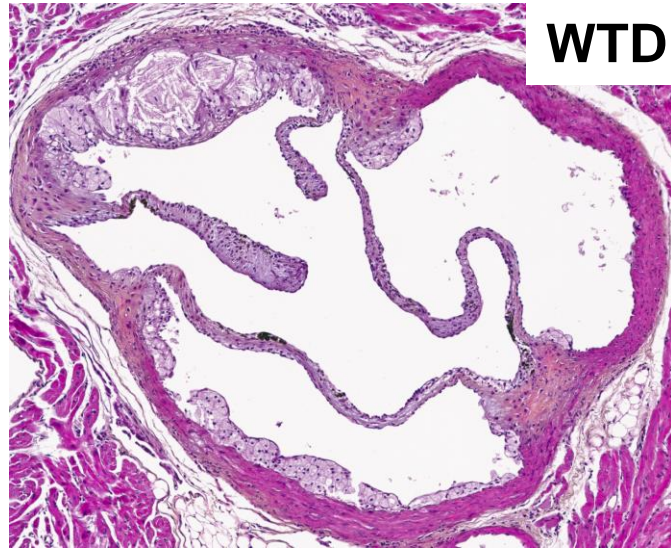


# Plasma inflammatory markers IL1- $\beta$ and TNF- $\alpha$ are significantly decreased in Furin inhibitor treated mice

N=16



# Furin inhibition reduces total atherosclerotic lesion area (NS)



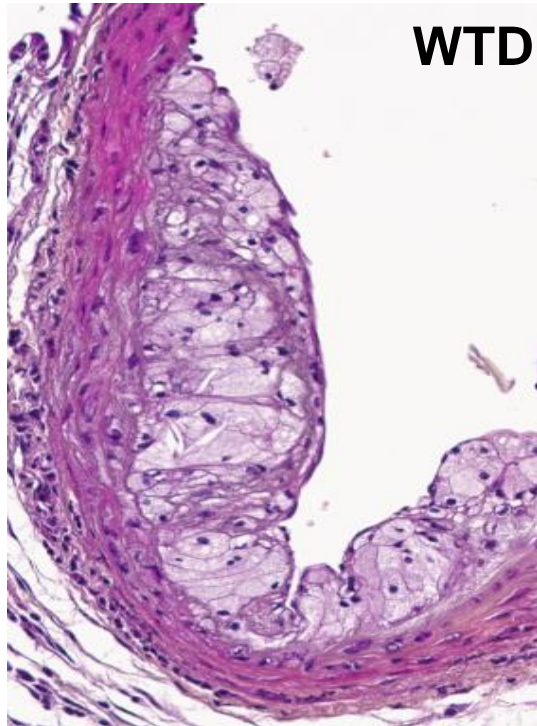
Haematoxylin Phloxine Saffron staining (HPS)

(Muscle cells- Pink stain; Collagen- light Yellow)

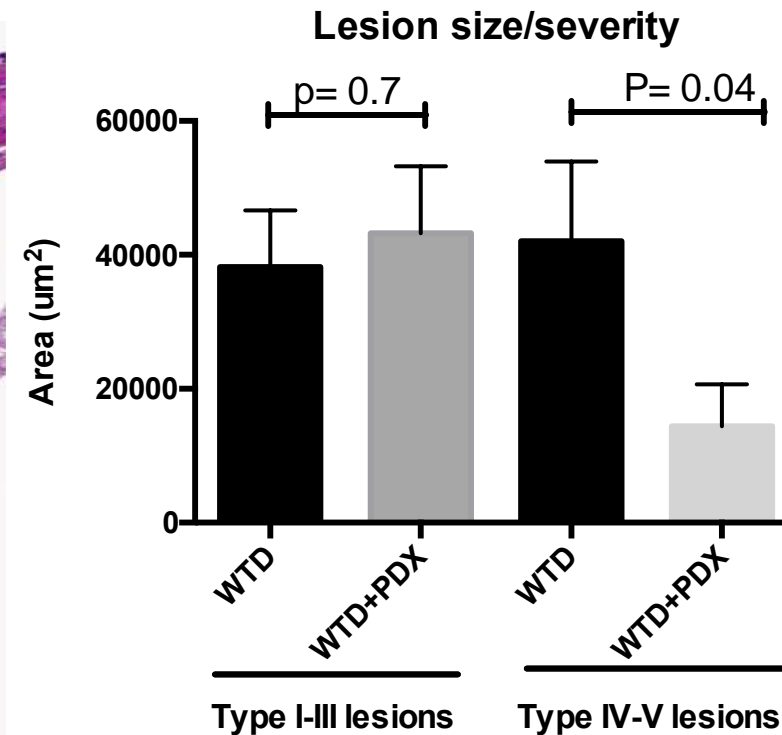
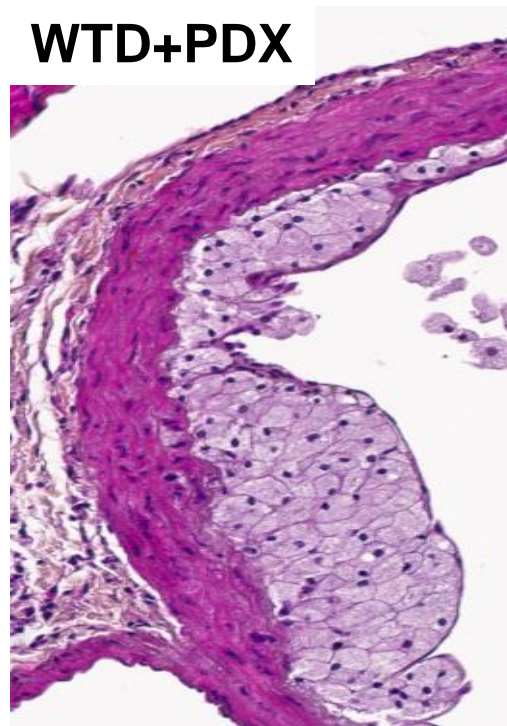
# Furin inhibition significantly reduces *severe* lesion area

N=15 each

Severe lesion



Mild lesion

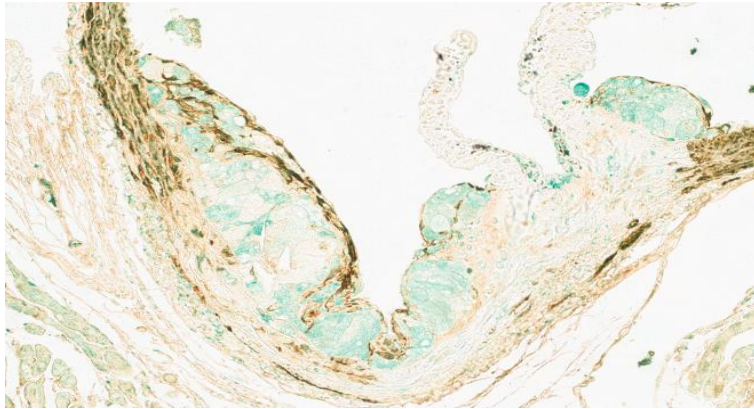




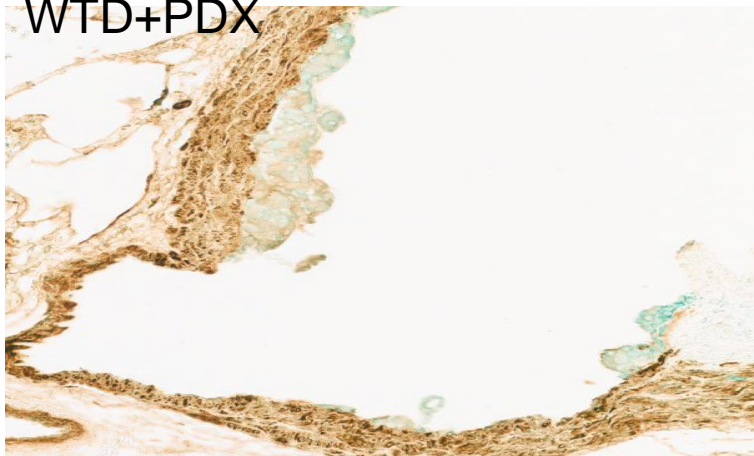
# Aortic lesions of Furin inhibitor treated mice have significant reductions in macrophage infiltration

N=15 each

WTD

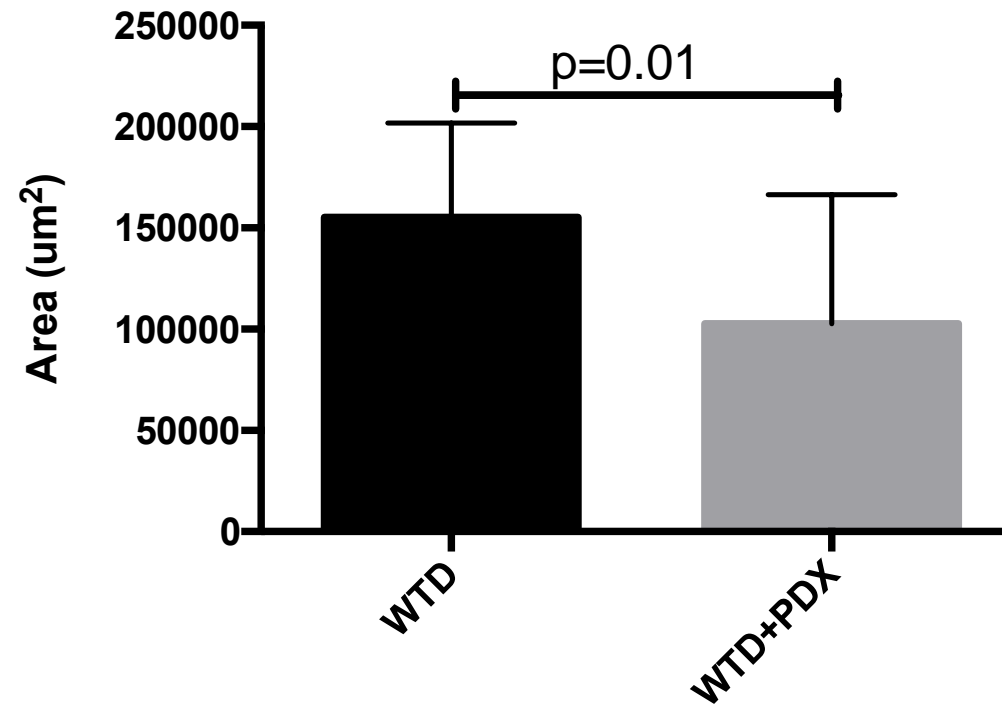


WTD+PDX

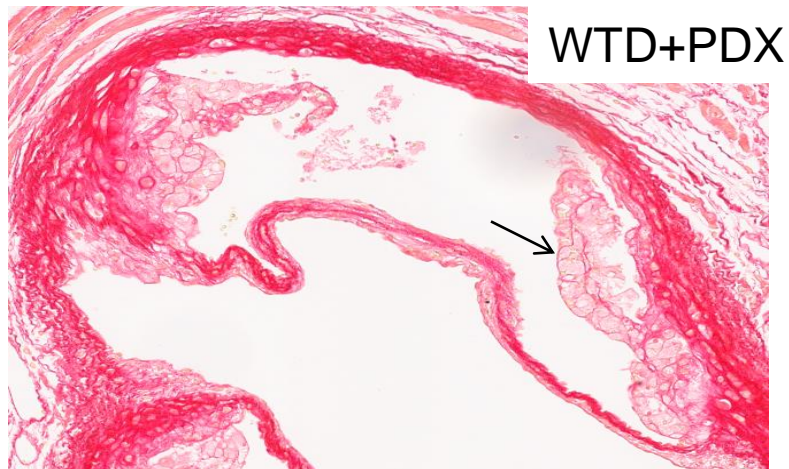
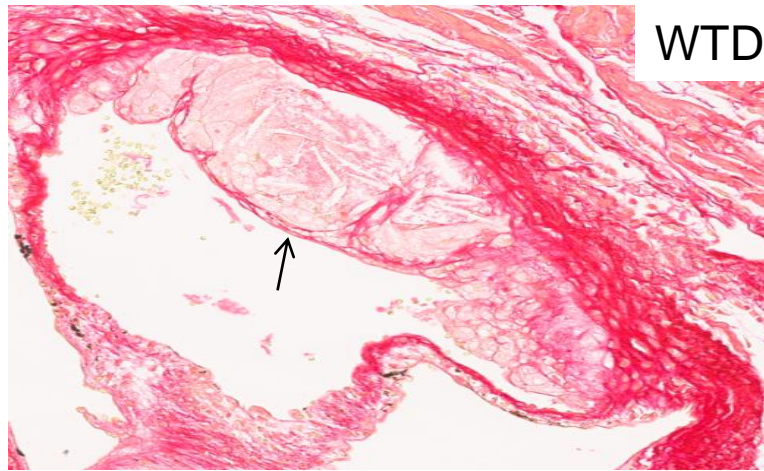


Macrophages- green stain

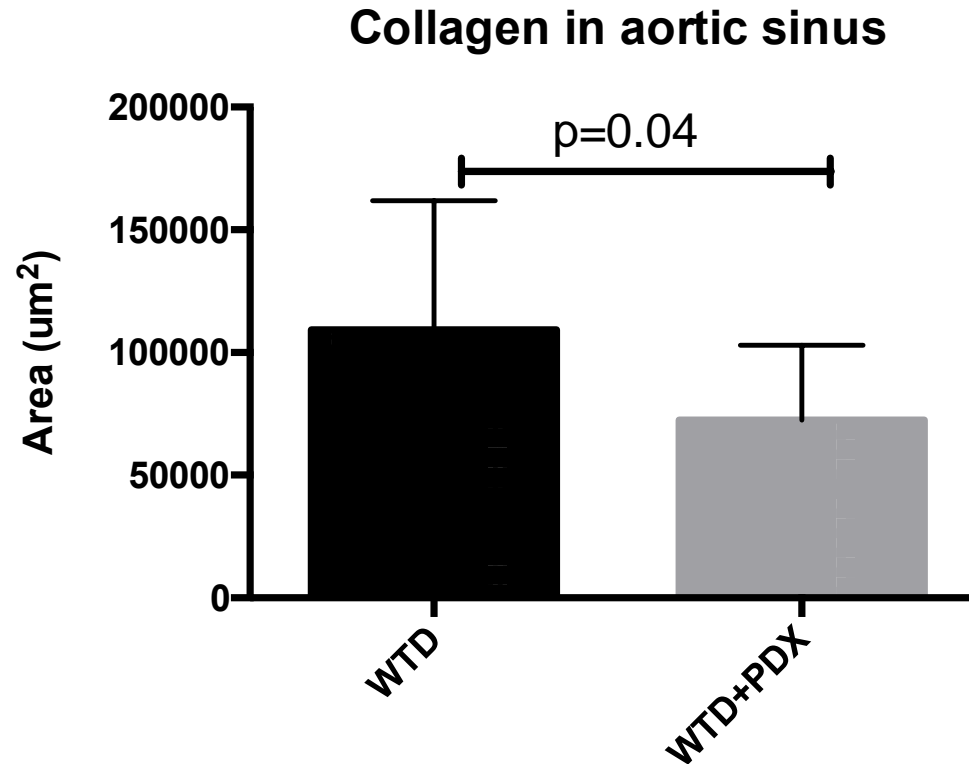
### Macrophages in aortic sinus



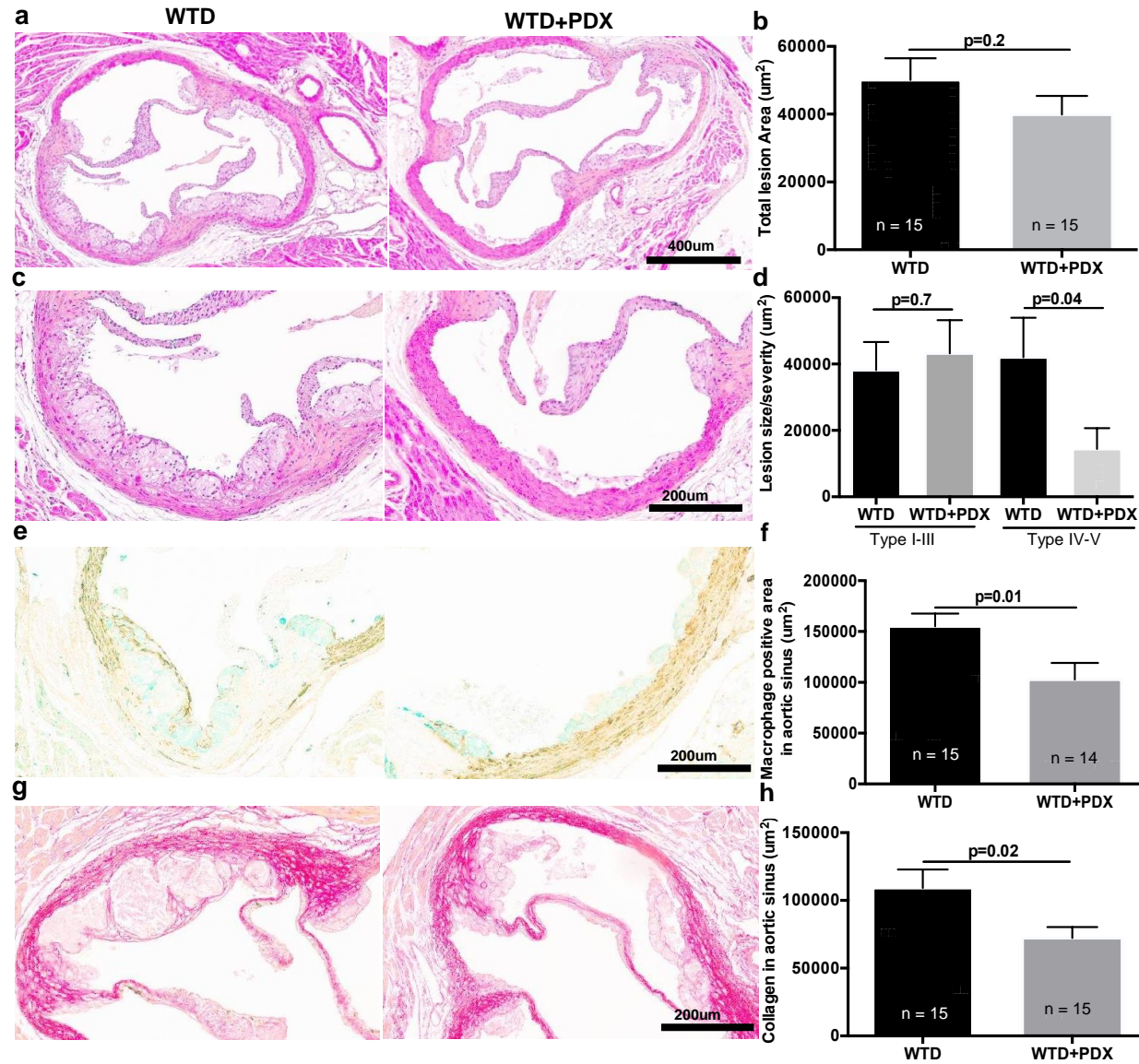
# Aortic sinus of Furin inhibitor treated mice show significantly reduced stenosis/collagen area



Red stain- collagen

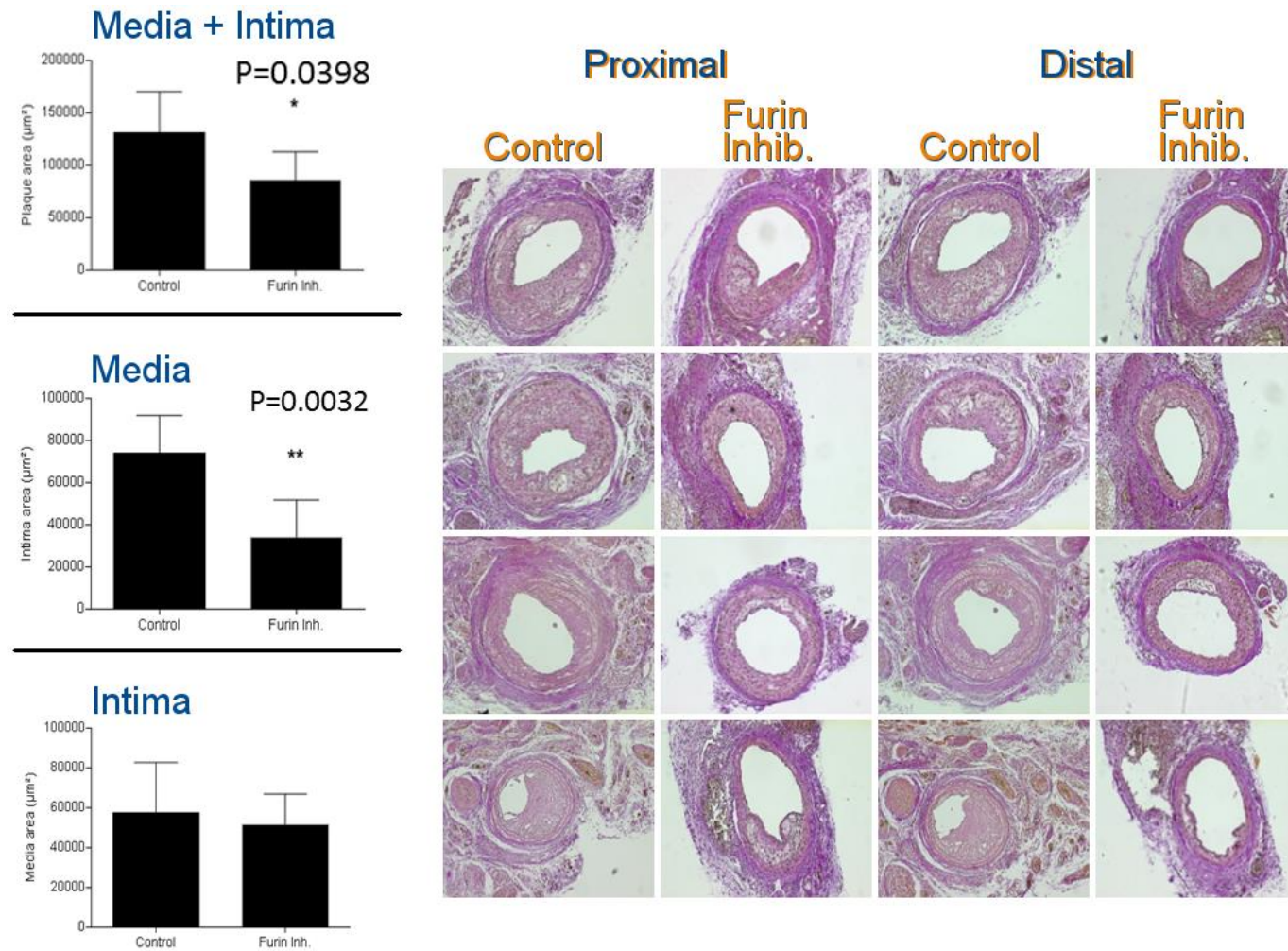


# Lower lesion complexity and severe atherosclerotic lesion size in FURIN inhibitor treated mice



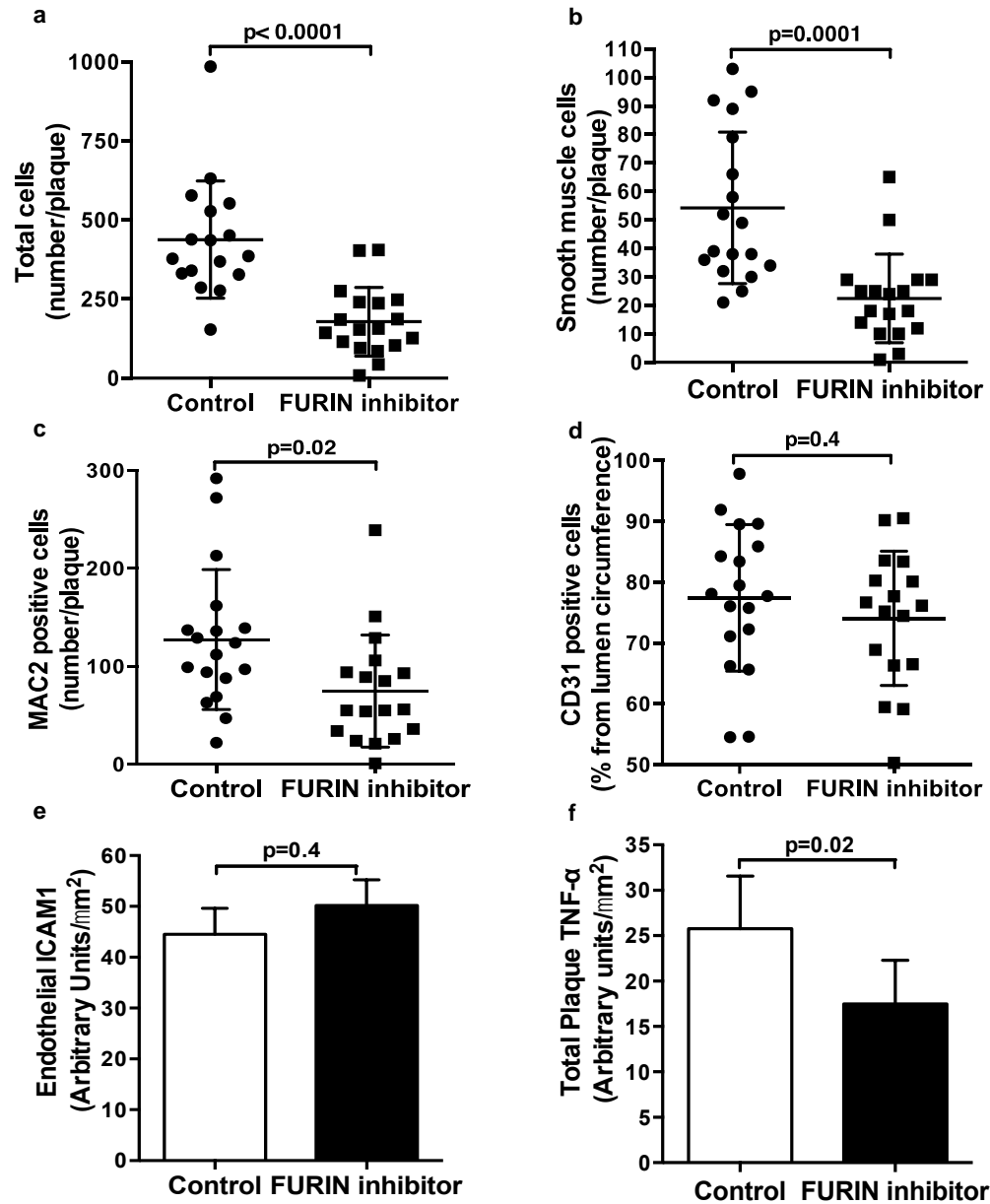
**Figure 2: Lower lesion complexity and severe atherosclerotic lesion size in FURIN inhibitor treated mice.** (a) Representative photomicrographs of aortic sinus after histological staining with hematoxylin-phloxine-saffron (200x). (b) A trend toward lower aortic sinus lesion area in FURIN inhibitor treated mice. (c) Representative photomicrographs of lesion severity in aortic sinus after histological staining with hematoxylin-phloxine-saffron (100x). (d) Significantly lower severe lesion area (type IV and V) in FURIN inhibitor treated mice. (e) Representative photomicrographs of macrophages (green) in aortic sinus (100x). (f) Significantly lower lesional macrophage area in FURIN inhibitor treated mice. (g) Representative photomicrographs of aortic root after histological staining with picrosirius red for collagen (100x). (h) Significantly lower collagen area in lesions of FURIN inhibitor treated mice. Groups are abbreviated as: *Ldlr*<sup>-/-</sup> mice fed Western type diet injected with PBS (WTD); *Ldlr*<sup>-/-</sup> mice fed Western type diet injected with the  $\alpha$ -1-PDX FURIN inhibitor (WTD+PDX). Values represent mean  $\pm$  SEM.

# Furin inhibition also reduces atherosclerosis in a second model: ApoE<sup>-/-</sup> mice ( $\alpha$ 1-PDX treatments)



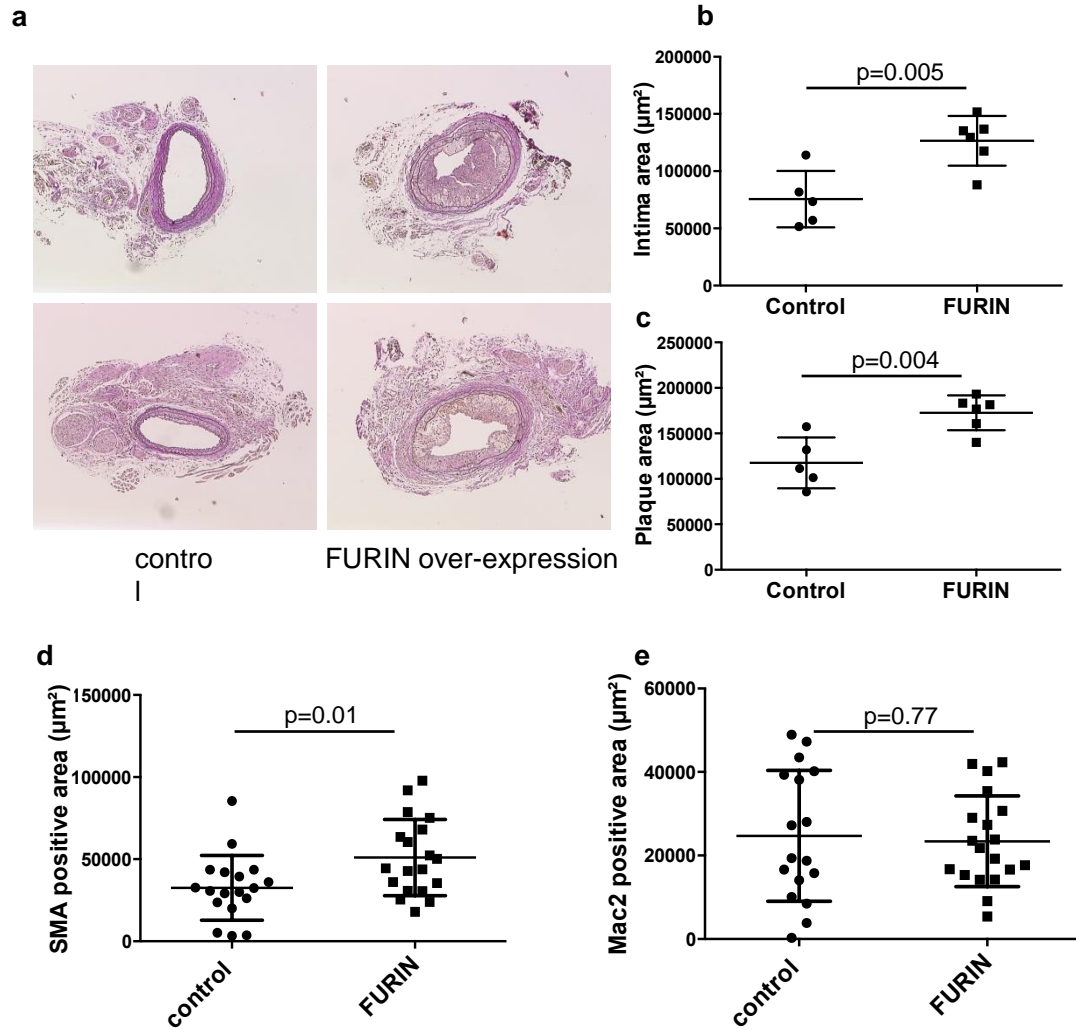
Mice were pretreated with Furin inhibitor for 1 week before induction of atherosclerosis, and treated for a further 2 weeks before sacrifice.

# FURIN inhibition reduces plaque complexity in a wire injury model of atherosclerosis



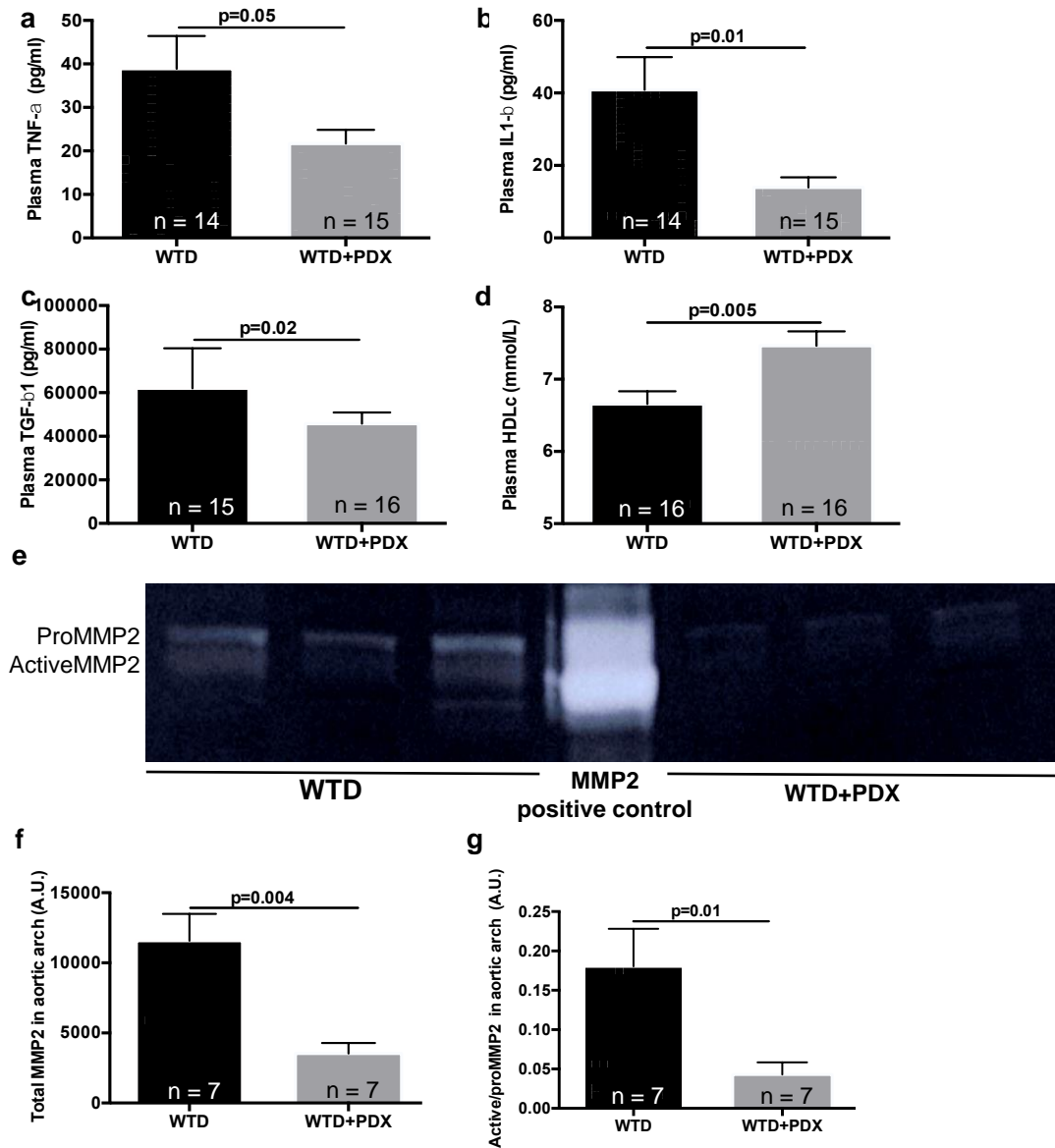
**Figure 5: FURIN inhibition reduces plaque complexity in a wire injury model of atherosclerosis.** *Apoe*<sup>-/-</sup> mice were fed a high-fat diet, treated with vehicle (control) or FURIN Inhibitor  $\alpha$ -1-PDX and subjected to wire-induced injury of the common carotid artery. **(a)** The total number of cells, **(b)** the number of smooth muscle cells, and **(c)** the number of MAC2 positive macrophages per plaque were all significantly lower in FURIN inhibitor treated mice. **(d)** No changes in CD31<sup>+</sup> endothelial cell numbers were observed. **(e)** Endothelial adhesion molecule ICAM1 levels were not changed in FURIN inhibitor treated mice. However, **(f)** vascular inflammatory cytokine TNF- $\alpha$  levels were significantly lower in plaques from FURIN inhibitor treated mice.

# FURIN over-expression increases neointimal plaque formation in a wire injury model of atherosclerosis



**Figure 6: FURIN over-expression increases neointimal plaque formation in a wire injury model of atherosclerosis.** *Apoe*<sup>-/-</sup> mice were fed a western-type diet, subjected to wire-induced injury of the common carotid artery, and treated with vehicle (n=5) or purified FURIN protein (n=6). **(a)** Representative photomicrographs of pentachrome-stained sections at 2 weeks after injury, and **(b)** significantly higher neointima and **(c)** plaque area in FURIN protein injected *Apoe*<sup>-/-</sup> mice. **(d)** Significantly increased smooth muscle cell area, and **(e)** no change in macrophage area in the lesions of FURIN over-expressing mice. Groups are abbreviated as: *Apoe*<sup>-/-</sup> mice (Control); *Apoe*<sup>-/-</sup> mice administered the purified FURIN protein (FURIN). Values represent mean  $\pm$  SEM.

# Lower plasma inflammatory markers, elevated plasma HDL cholesterol and lower MMP2 expression in aorta of FURIN inhibitor treated mice

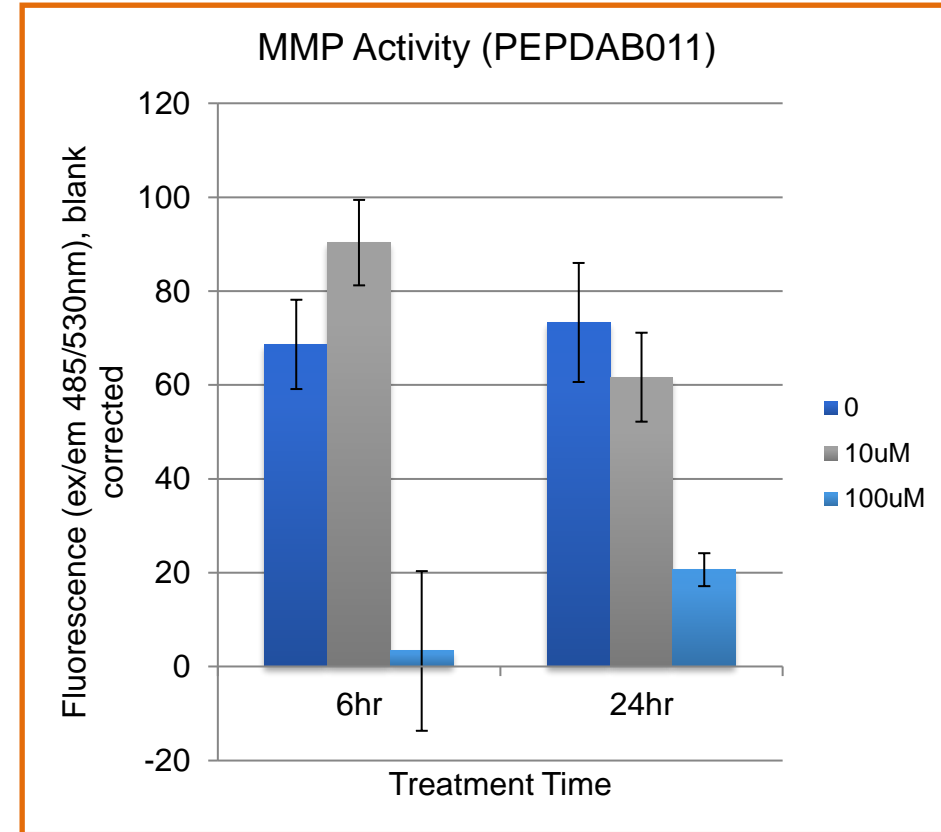
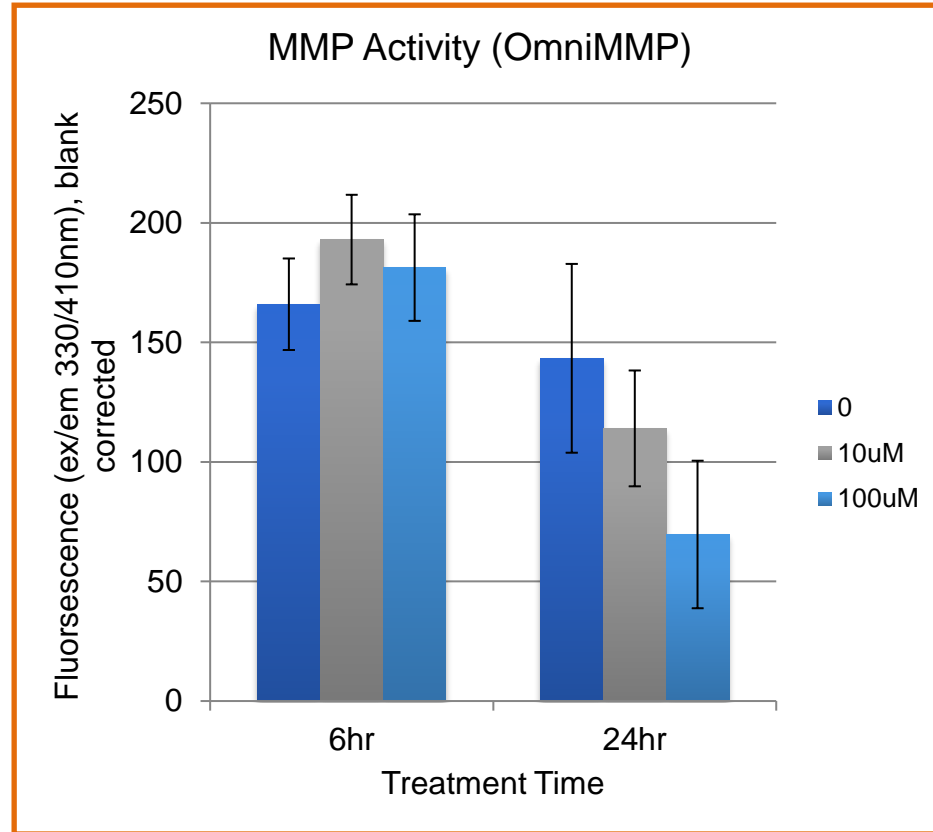


**Figure 3: Lower plasma inflammatory markers, elevated plasma HDL cholesterol and lower MMP2 expression in aorta of FURIN inhibitor treated mice.** Lower plasma levels of (a) TNF- $\alpha$ , (b) IL1- $\beta$ , (c) TGF- $\beta$ 1, and (d) elevated plasma HDL cholesterol levels in FURIN inhibitor treated mice. (e) Gelatin zymography in the aortic arch showing both the pro and active forms of MMP2. (f) Total MMP2 expression levels are significantly lower in the aortic arch of FURIN inhibitor treated mice (g) Significantly lower active MMP2/proMMP2 expression in the aortic arch of FURIN inhibitor treated mice. Groups are abbreviated as: *Ldlr*<sup>-/-</sup> mice fed Western type diet injected with PBS (WTD); *Ldlr*<sup>-/-</sup> mice fed Western type diet injected with the  $\alpha$ -1-PDX FURIN inhibitor (WTD+PDX). A.U.= Arbitrary Units. Values represent mean  $\pm$  SEM.

# Unpublished Results (cell-based assays)

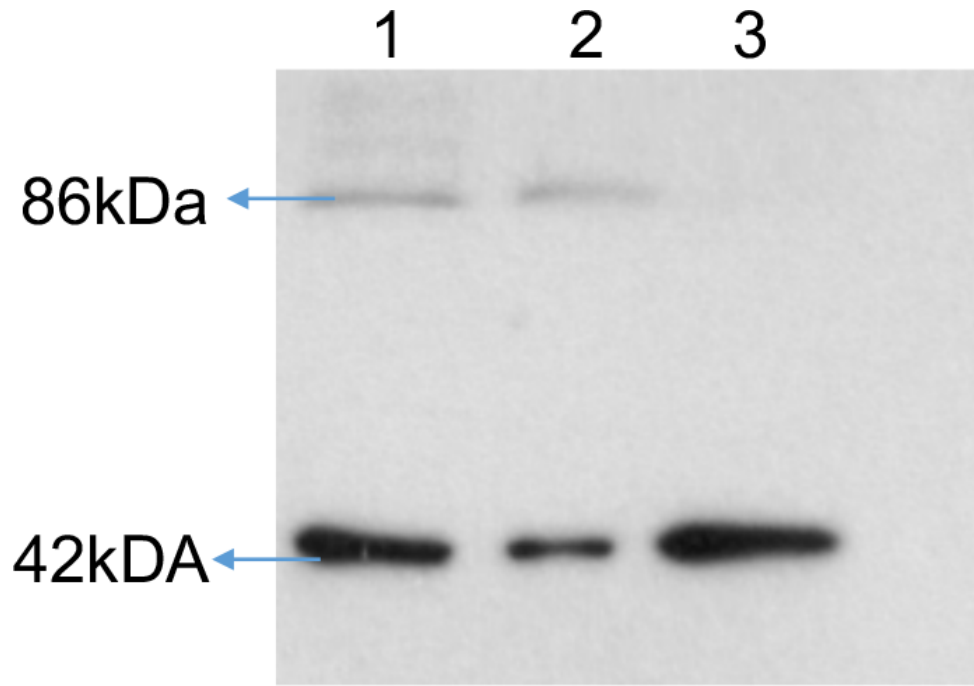


# Chemical inhibition of FURIN inhibits MMP activity in Human Coronary Artery Endothelial Cells



**Furin inhibitor : dec-RVKR-CMK**

**Generation of heterozygous and homozygous deletions of FURIN in U937 monocytes via CRISPR**



Legend:

Lane 1: U937 control

Lane 2: Furin Crispr 63 (Heterozygous)

Lane 3: Furin Crispr 180 (Homozygous)

Furin molecular weight: 86kDa

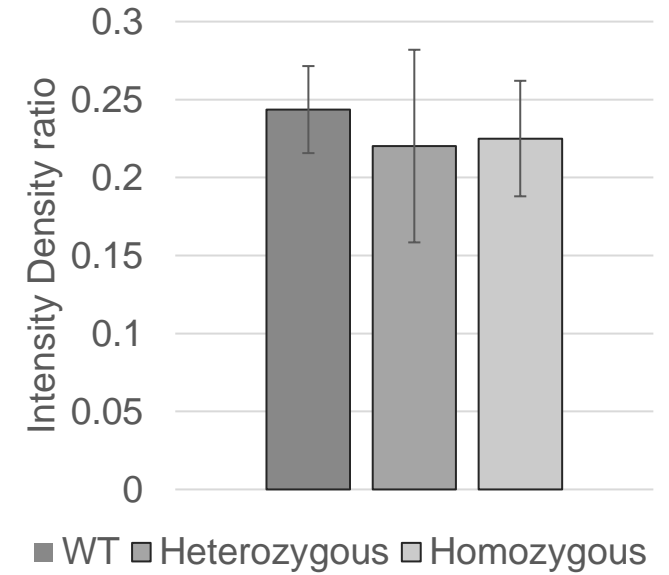
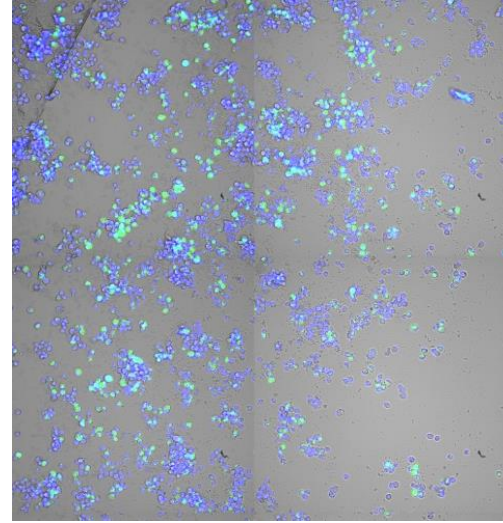
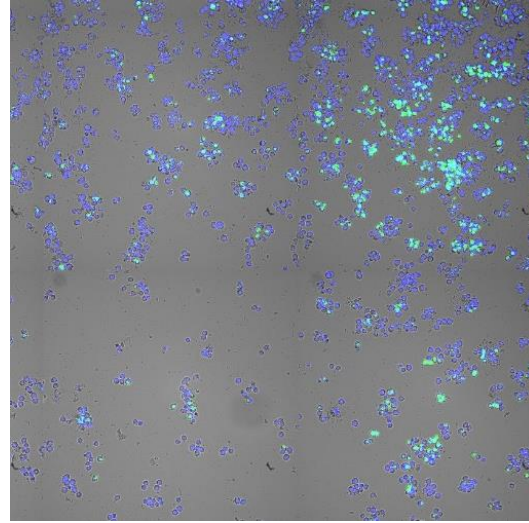
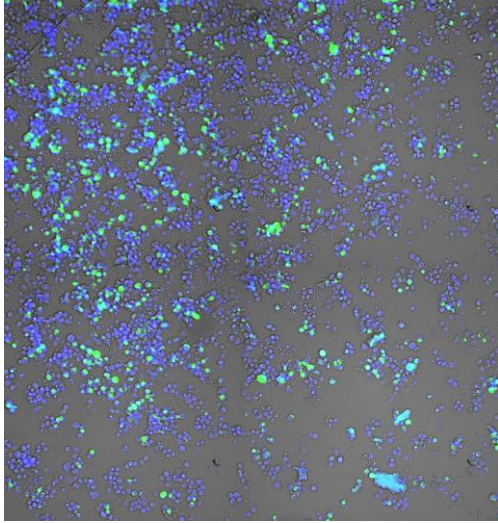
B-actin Molecular weight: 42kDa

# Effects of FURIN deletion on phagocytosis in U937 cells

WT

Hetero

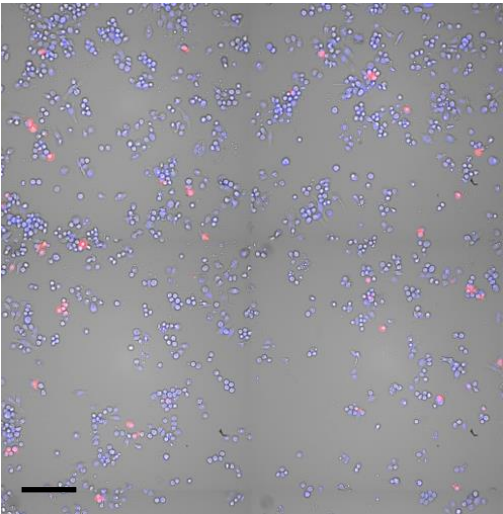
Homo



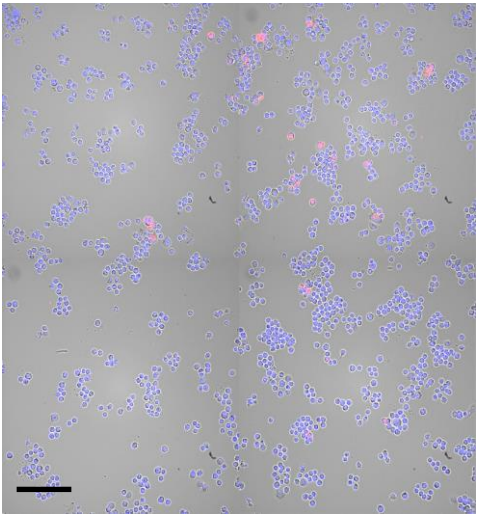
T-test	
WT / Hetero	WT / Homo
0.59429569	0.528096

# Effect of FURIN deletin on oxidized lipid uptake in U937 cells

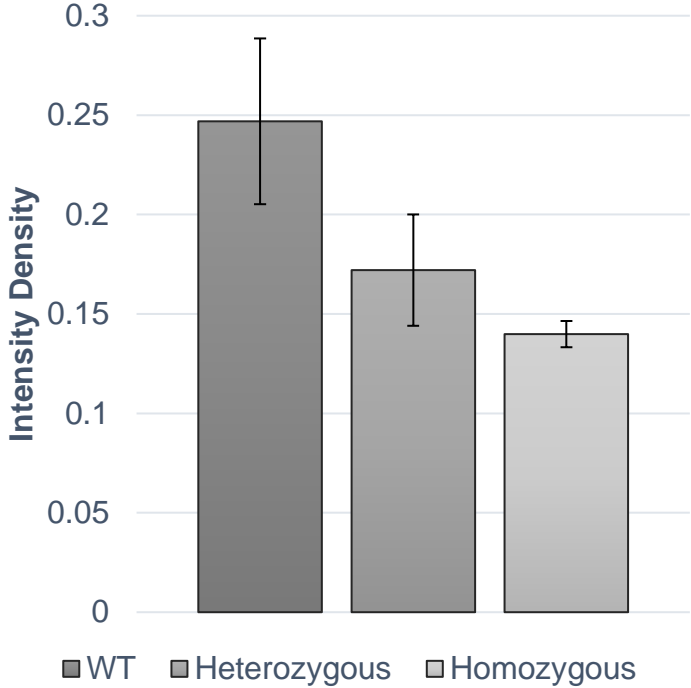
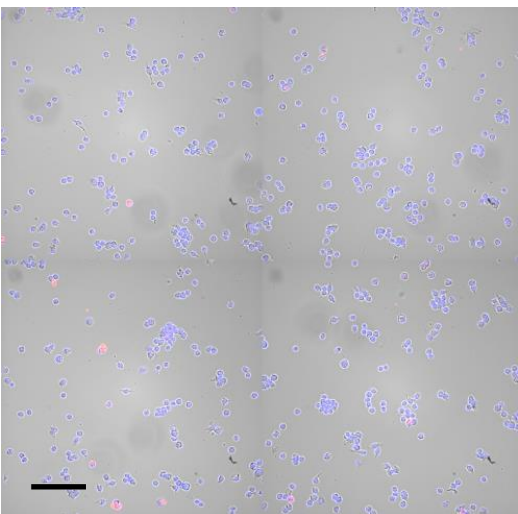
WT



Hetero

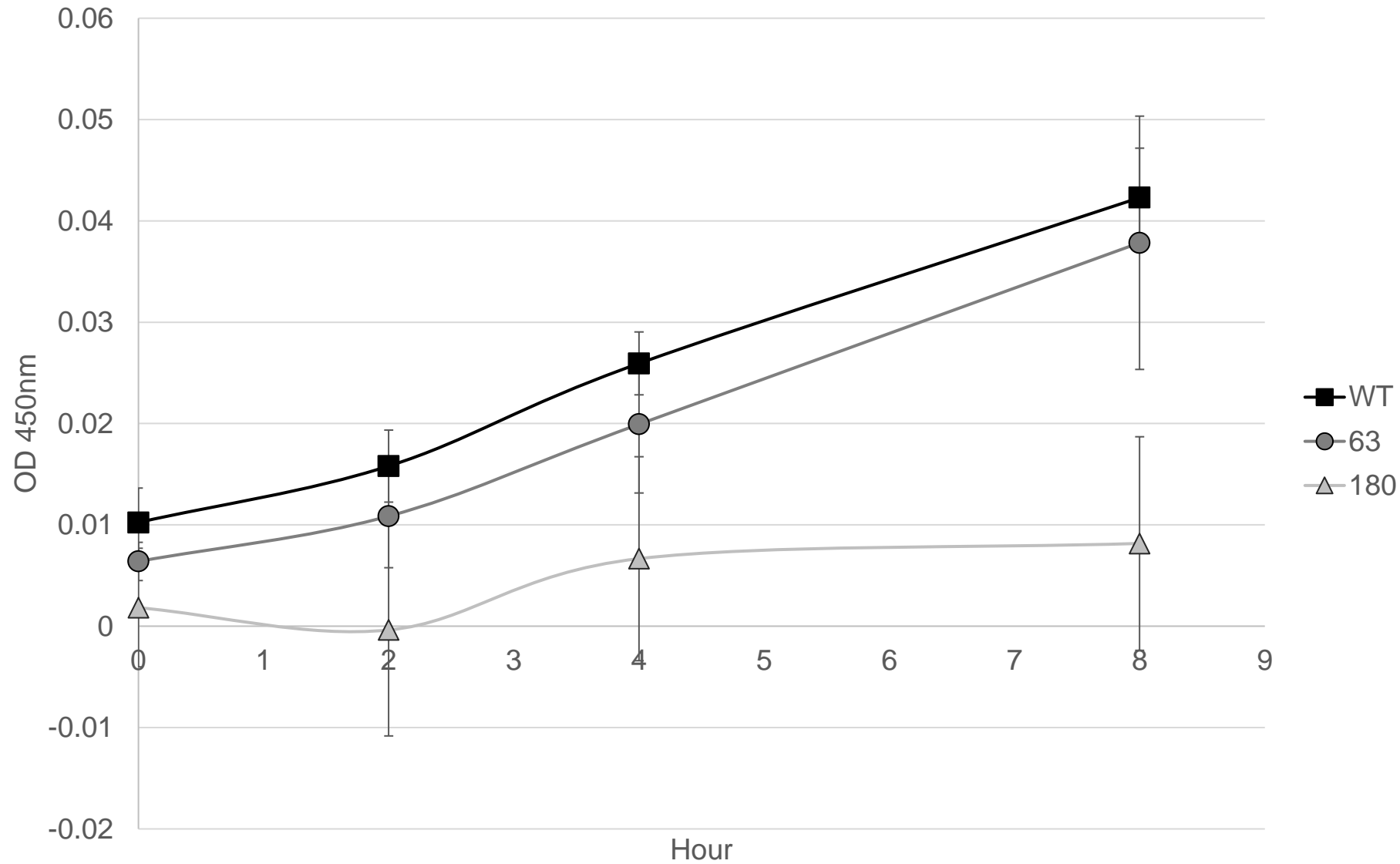


Homo



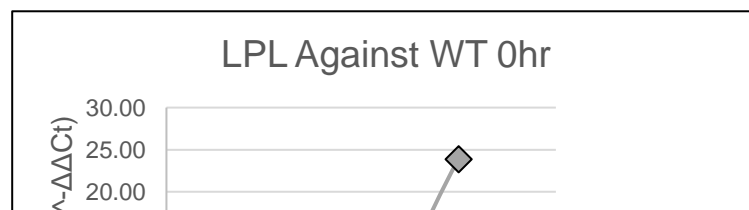
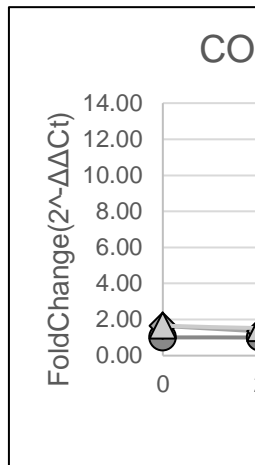
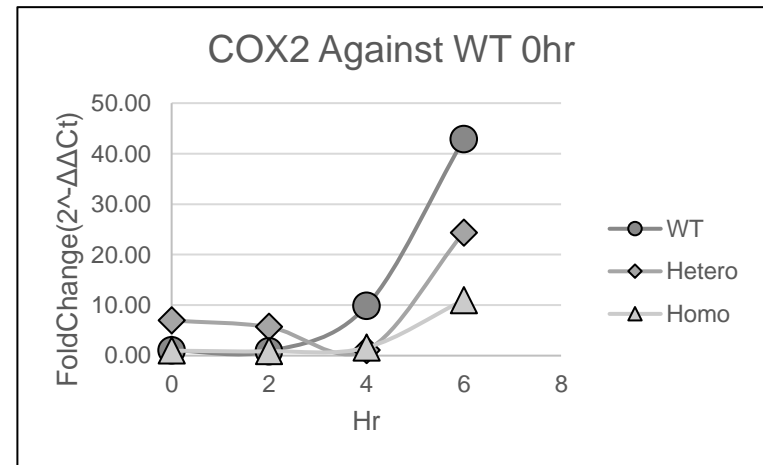
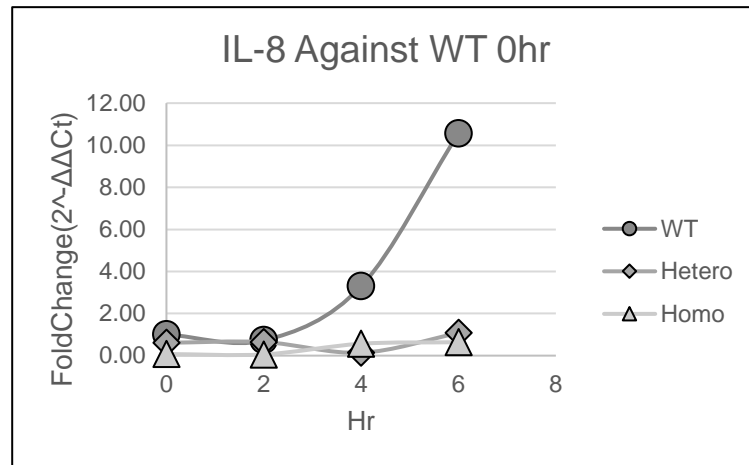
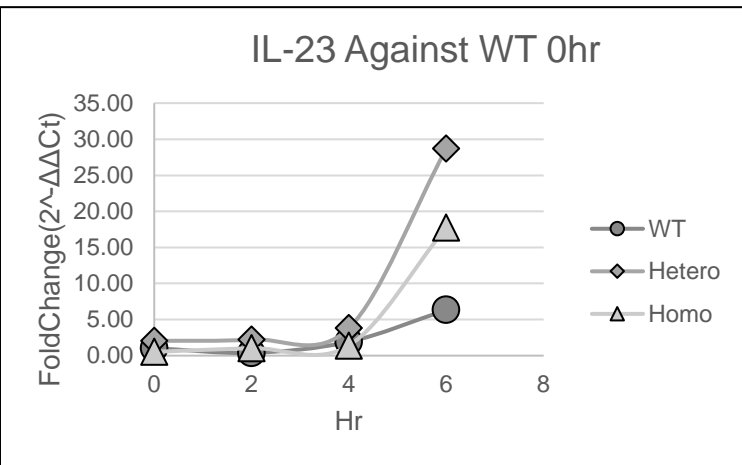
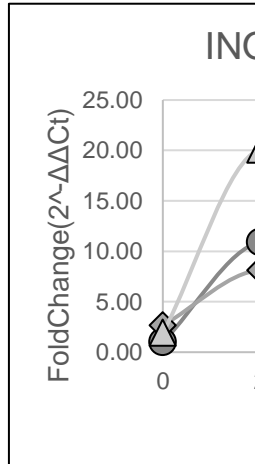
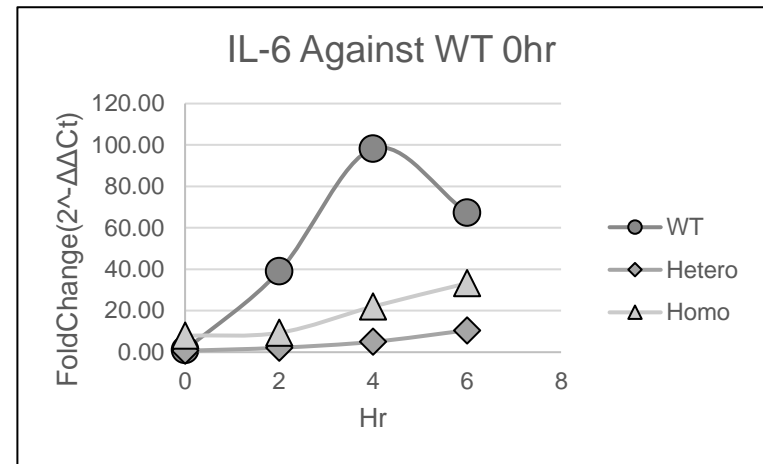
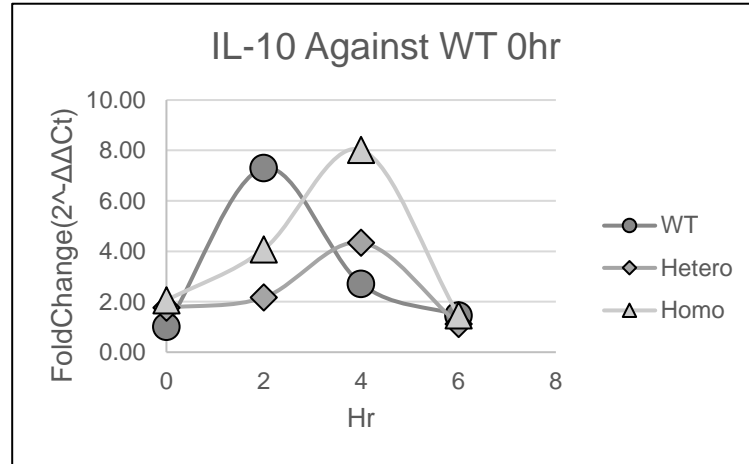
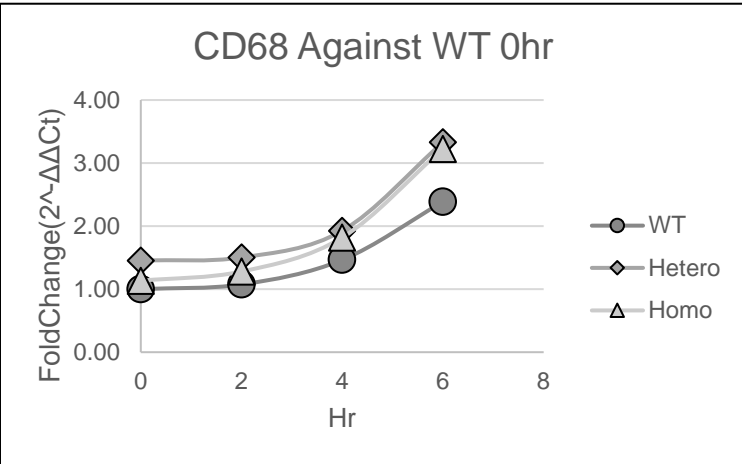
T test	
WT / 63	WT / 180
0.069827	0.043923

# Effect of FURIN deletion on transmigration of U937 cells



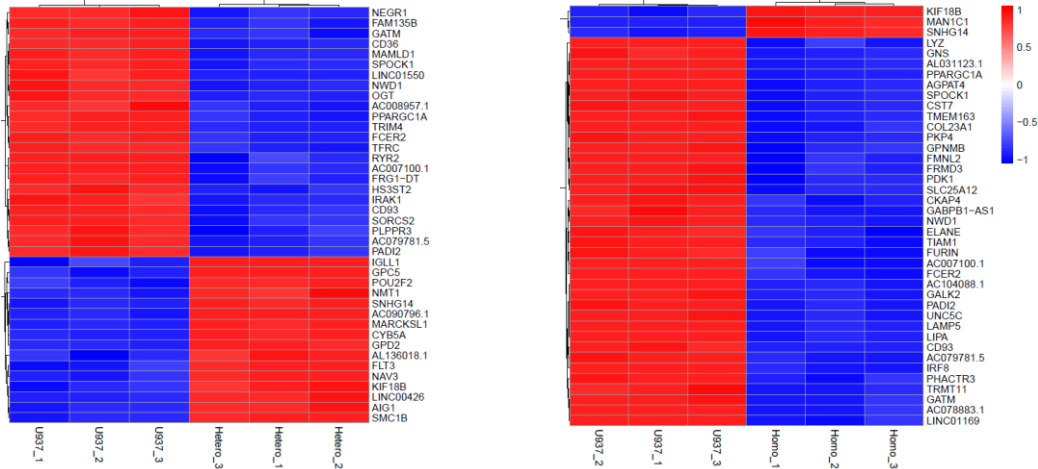
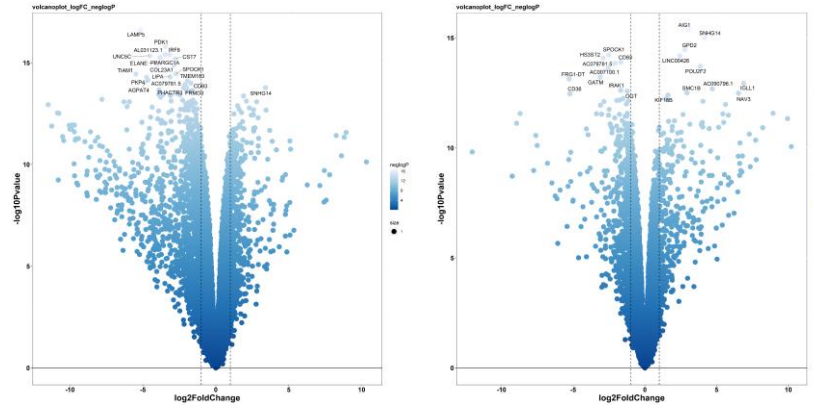
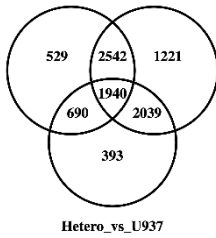
T-test		
	WT/ Hetero	WT/ Homo
0hr	0.182929	0.114937
2hr	0.248341	0.103412
4hr	0.263912	0.06935
8hr	0.610122	0.017041

# Effect of FURIN deletion on inflammatory gene expression in U937 cells

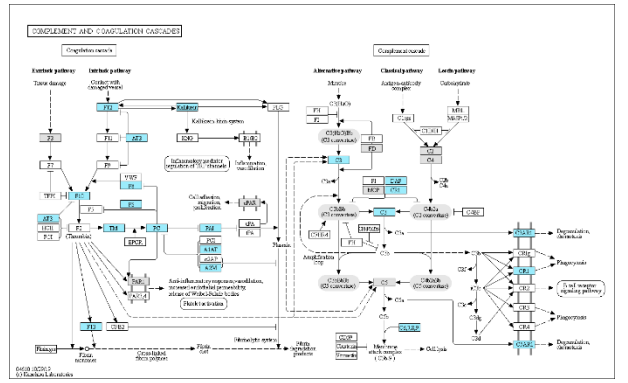
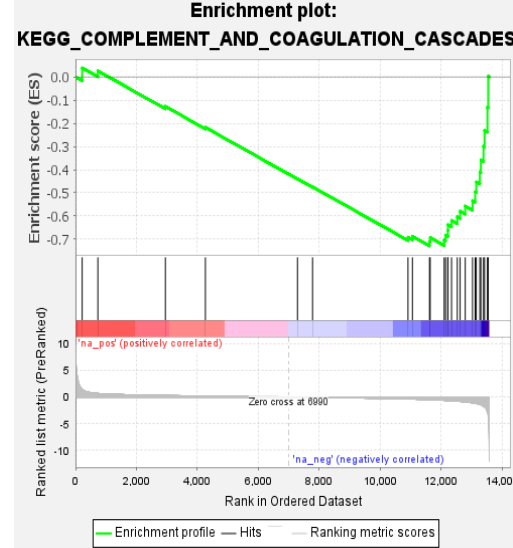


# Effect of FURIN deletion on gene and pathway expression in U937 cells

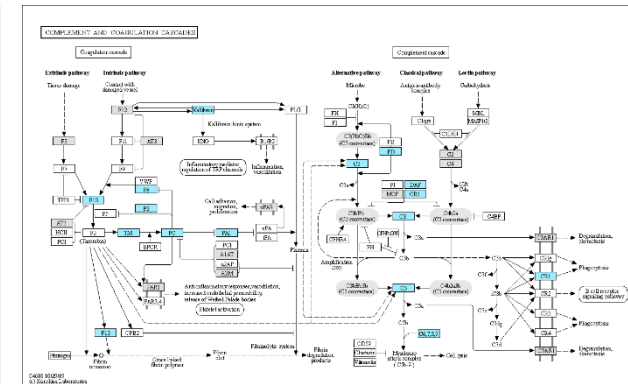
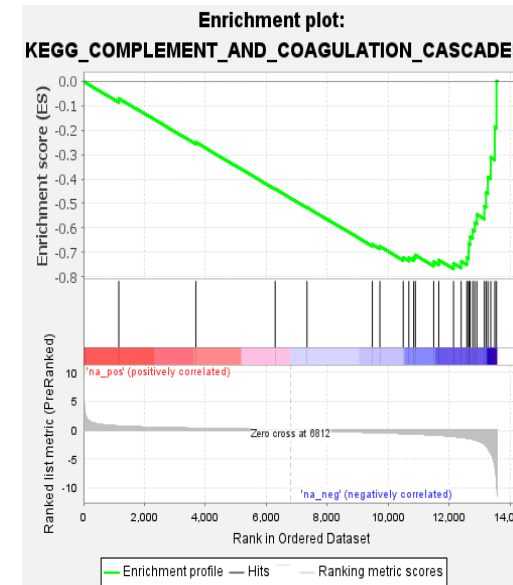
Homo\_vs\_Hetero Homo\_vs\_U937



## HeterovsU937



## HomovsU937



NAME	SIZE	NES	NOM p-val	FDR q-val	Comparison	Direction
KEGG_CARDIAC_MUSCLE_CONTRACTION	41	-1.8148402	0.00512821	0.07270604	HZ vs WT	dn_HZ
KEGG_COMPLEMENT_AND_COAGULATION_CASCADES	27	-1.9659134	0.00165837	0.02767488	HZ vs WT	dn_HZ
KEGG_GLYCINE_SERINE_AND_THREONINE_METABOLISM	19	-1.776342	0.00654665	0.09013652	HZ vs WT	dn_HZ
KEGG_NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY	50	-1.7752033	0.00326264	0.07335213	HZ vs WT	dn_HZ
KEGG_RETINOL_METABOLISM	13	-1.9057918	0.00168067	0.03158124	HZ vs WT	dn_HZ
GO_REGULATION_OF_MESENCHYMAL_CELL_PROLIFERATION	21	2.1335328	0	0.05423588	HZ vs WT	up_HZ
KEGG_COMPLEMENT_AND_COAGULATION_CASCADES	27	-1.8786367	0	0.00877457	NZ vs WT	dn_NZ
KEGG_HISTIDINE_METABOLISM	18	-1.8481181	0.00136426	0.01082356	NZ vs WT	dn_NZ
KEGG_SYSTEMIC_LUPUS_ERYTHEMATOSUS	25	-1.8060137	0	0.01712945	NZ vs WT	dn_NZ
KEGG_TRYPTOPHAN_METABOLISM	21	-1.7206078	0.01179554	0.06933154	NZ vs WT	dn_NZ
GO_HUMORAL_IMMUNE_RESPONSE_MEDIATED_BY_CIRCULATING_IMMUNOGLOBULIN	24	-1.909731	0	0.12325557	NZ vs WT	dn_NZ
GO_MODIFICATION_OF_POSTSYNAPTIC_STRUCTURE	13	-1.8803527	0	0.14710467	NZ vs WT	dn_NZ