The transcription factor MAFF regulates an atherosclerosis relevant network connecting inflammation and cholesterol metabolism

von Scheidt – Identification of MAFF as a regulator of the LDLR

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Total word count

12,349 (Manuscript only: 4,606)

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Abstract

Background – Coronary artery disease (CAD) is a multifactorial condition with both genetic and exogenous causes. The contribution of tissue specific functional networks to the development of atherosclerosis remains largely unclear. The aim of this study was to identify and characterise central regulators and networks leading to atherosclerosis.

Methods – Based on several hundred genes known to affect atherosclerosis risk in mouse (as demonstrated in knock-out models) and human (as shown by genome-wide association studies (GWAS)) liver gene regulatory networks were modeled. The hierarchical order and regulatory directions of genes within the network were based on Bayesian prediction models as well as experimental studies including chromatin immunoprecipitation DNA-Sequencing (ChIP-Seq), ChIP mass spectrometry (ChIP-MS), overexpression, siRNA knockdown in mouse and human liver cells, and knockout mouse experiments. Bioinformatics and correlation analyses were used to clarify associations between central genes and CAD phenotypes in both human and mouse.

Results – The transcription factor *MAFF* interacted as a key driver of a liver network with three human genes at CAD GWAS loci and eleven atherosclerotic murine genes. Most importantly, expression levels of the low-density lipoprotein receptor (*LDLR*) gene correlated with *MAFF* in 600 CAD patients undergoing bypass surgery (STARNET) and a hybrid mouse diversity panel involving 105 different inbred mouse strains. Molecular mechanisms of *MAFF* were tested under non-inflammatory conditions showing a positive correlation between *MAFF* and *LDLR in vitro* and *in vivo*. Interestingly, after LPS stimulation (inflammatory conditions) an inverse correlation between *MAFF* and *LDLR in vitro* and *in vivo* was observed. ChIP-MS revealed that the human CAD GWAS candidate *BACH1* assists *MAFF* in the presence of LPS

stimulation with respective heterodimers binding at the MAF recognition element (MARE) of the *LDLR* promoter to transcriptionally downregulate *LDLR* expression.

Conclusion – The transcription factor *MAFF* was identified as a novel central regulator of an atherosclerosis/CAD relevant liver network. *MAFF* triggered context specific expression of *LDLR* and other genes known to affect CAD risk. Our results suggest that *MAFF* is a missing link between inflammation, lipid and lipoprotein metabolism and a possible treatment target.

Keywords

Atherosclerosis, BACH1, coronary artery disease, inflammation, key driver analysis, LDLR, lipid metabolism, MAFF, network modeling

Translational Perspective

What is new?

- Our study identified the transcription factor *MAFF* as key driver gene in a liver-specific network involving several genes with established, genome-wide significant association to coronary artery disease (CAD).
- *MAFF* regulated context-specifically expression of *LDLR* in experimental model systems and human individuals.
- *MAFF* induced *LDLR* expression under non-inflammatory conditions. After LPS stimulation *MAFF* downregulated *LDLR* expression via heterodimerisation with *BACH1* and binding at the maf-recognition element (MARE) – also known as stress-responsive element – in the promoter of *LDLR*.

What are the clinical implications?

- Cholesterol metabolism and inflammation represent two major causes of CAD. Here we identified a transcriptional regulator (*MAFF*) to differentially affect the major determinant of cholesterol levels (*LDLR*) dependent on the inflammatory state.
- Further studies of the transcription factor *MAFF*, its interaction partners and downstream cascades might generate new therapeutic targets to treat hypercholesterolemia, inflammation and reduce CAD risk.

Introduction

Coronary artery disease (CAD), a globally leading cause of death,[1, 2] is brought about by atherosclerosis of the epicardial arteries, which is prompted by a multifactorial interplay of genetic and lifestyle factors.[3] From a functional point of view, the mechanisms which result in CAD can be grouped into different pathways or networks.[4-7] A systematic analysis of genes identified by genome-wide association studies (GWAS) of CAD patients and genetic mouse models of atherosclerosis revealed a strong concordance of relevant networks and pathways for atherosclerosis between the two species.[8] However, characterization and regulation of these functional networks is far from being complete.[9] Central mechanisms for atherosclerosis are the disturbance of cholesterol metabolism and inflammation, which – like CAD – have multifactorial etiologies.[10-12] Clinical as well as epidemiological trials and Mendelian randomization studies confirmed that elevation of plasma cholesterol and increased inflammation promote the progression of atherosclerosis and CAD[13-16], whereas reduction of plasma cholesterol levels and inflammatory processes lowered significantly the subsequent risk of cardiovascular events.[10, 17-19] Both, cholesterol metabolism and inflammatory responses are largely orchestrated in the liver. Therefore, the analysis focused on hepatic tissue to further elucidate regulatory gene networks involved in atherosclerosis.

Methods

Data used in this study are available in persistent repositories. Human data from STARNET are accessible through the Database of Genotypes and Phenotypes (dbGAP). Mouse data from HMDP are accessible through the Mouse Phenome Database (MPD). All experimental data supporting the findings of this study can be requested from qualified researchers at the German Heart Center Munich from the corresponding author. An expanded and detailed materials and

methods section is provided in the supplement section (**Expanded Methods**). Human and mouse candidate genes were retrieved from the literature.[8, 20-30] Gene-gene relations of atherosclerotic genes were retrieved utilising Bayesian gene regulatory networks derived from previous expression analyses on human and mouse tissues as described.[31-36] The key driver analysis (KDA) was based on an established algorithm to identify central regulators of atherosclerosis relevant networks.[31, 37-40] ChIP-Seq experiments of *MAFF* on human HepG2 cells were performed as described.[41-44] Binding capacities of *MAFF* and heterodimerisation partners were confirmed using ChIP-Seq data. siRNA experiments targeting liver genes were performed in cultured AML12 murine and Hep3b human liver cells. *Maff* overexpression experiments were performed in AML12 cells. ChIP-MS was performed to identify *MAFF* binding partners. Molecular docking of heterodimerisation partners was assessed based on lowest free energy.[45-48] The STARNET study was conducted in accordance with the provisions of the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice guidelines. The protocol was approved by an independent ethics committee and all patients provided written informed consent. All animal studies in mice followed the guidelines of the Animal Care and Use Committees of the University of California Los Angeles. The approach is graphically summarised in **Figure 1**.

Results

Bioinformatics identification of the MAFF network

The bioinformatics approach was based on a comprehensive search for mouse genes that have been previously found to affect the manifestation of atherosclerosis in genetically engineered mouse models as well as human chromosomal loci significantly associated with CAD in GWAS and the respective annotation of responsible genes at these loci.[20-30] Specifically, 244 human

CAD GWAS candidate genes (**Supplemental Table 1**) and 827 mouse atherosclerosis genes (**Supplemental Table 2**) were used to model gene regulatory networks using a key driver analysis.[49] Liver Bayesian network models composed from multiple published genetic and gene expression datasets were constructed to retrieve gene-gene regulatory relationships in each dataset,[31-36] followed by summarising the individual liver networks into a union liver Bayesian network (**Expanded Methods**). The mouse atherosclerosis genes and human CAD GWAS genes were then mapped separately to the liver Bayesian network model to retrieve subnetworks (specific parts of the global Bayesian liver network) of disease genes and to predict key regulatory genes in these subnetworks. Several subnetworks enriched for known atherosclerosis or CAD associated genes were identified. **Figure 2** displays the interconnected top 10 liver subnetworks containing mouse atherosclerosis or human CAD genes. **Table 1** lists these subnetworks in mouse and human ranked by fold enrichment of disease genes in each subnetwork. Based on mouse atherosclerosis genes, the top five key driver genes of the regulatory networks were *Maff, Il1b, Ccl7, Atf3* and *Cxcl10*. With the exception of *Maff* these genes have already been shown to have significant effects on atherosclerosis in mouse models, which may serve as positive control for this approach.[50-53] Regarding the human CAD genes, the top ranked key driver gene *ALDH2* is known to reside at a CAD GWAS locus. The key driver *SERPINE1*, which is also part of the *MAFF* network, shares several atherosclerosis relevant genes with *MAFF*.

The top ranked liver subnetwork, over-represented with both mouse atherosclerosis and human CAD candidate genes, was predicted to be orchestrated by *MAFF/Maff*, which interacts with 24 atherosclerosis related genes. A number of these genes are known to be associated with lipid metabolism and others with inflammation, and eleven genes (*Atf3, Epha2, Gdf15, Ldlr, Nr4a3, Phlda1, Serpine1, Tnfaip3, Tnfrsf12a, Trib1* and *Zfp36*) were found to affect atherosclerosis in

genetically engineered mouse models.[53-63] On the human side, the *MAFF* interacting genes *LDLR*, *MCL1* and *TRIB1* reside at genome-wide significant CAD GWAS loci.

MAFF is a member of the MAF family, which consists of large and small MAF proteins. Large MAFs possess a transactivation domain and modulate regulatory processes. Small MAFs are lacking a transactivation domain and are therefore classified as transcriptional repressors.[64] *MAFF*, a small MAF, is a basic region leucine zipper (bZIP)-type transcription factor composed of a DNA binding domain and a leucine zipper domain necessary for dimerisation. *MAFF* can mediate both transcriptional activation or repression by forming heterodimers with other bZIP transcription factors. But the precise mechanism by which *MAFF* forms specific dimers, and therefore induces or represses specific target genes is currently not well established.

Prediction of regulatory directionality in the MAFF/Maff network

Individual liver Bayesian network models were constructed using multiple genetic and gene expression datasets from mouse and human studies and combined the networks into one union liver network (**Expanded Methods**). As the directionality between two genes in a network might differ across studies due to different environmental perturbations and physiological states, the dominant direction that is supported by more datasets was taken as the directionality between two genes (**Figure 2**).

Based on this data *ATF3, TRIB1, SERPINE1, FOSL2,* and *ZFP36* were predicted to be upstream of *MAFF*, i.e. these genes appear to affect regulation of the transcription factor. All other genes of interest in the context of atherosclerosis (*ARID5B, CLCF1, CREM, CXCL8, DUSP5, EPHA2, FOXP1, GDF15, LDLR, MCL1, NAV2, NR4A3, PHLDA1, PPPLRL15A, SLC20A1, TGFBI,*

TNFAIP3, TNFRSF12A and *TSC22D1*) were predicted to be downstream of *MAFF* and therefore likely to be regulated by the transcription factor *MAFF*.

Confirmation of Maff coexpression with lipid metabolism and inflammation processes in mouse atherosclerosis models

The Hybrid Mouse Diversity Panel (HMDP) is a set of 105 different inbred mouse strains, which were studied under different dietary conditions and different genetic backgrounds.[65, 66] Ldl and Vldl cholesterol levels increased from chow diet over high-fat diet to the atherogenic transgenic mice on high-fat diet (26mg/dl vs. 42mg/dl, vs. 92mg/dl; $p<0.001$). In transgenic mice inflammation associated factors like *Il1b*, *Il6* and *Tnfa* were also upregulated.

On regular chow (Pearson's r=0.30, p=9.88e-07) and high-fat diet (Pearson's r=0.35, p=1.78e-07) positive correlations between *Maff* and *Ldlr* were detected. By contrast, in mice on high-fat diet with transgenic expression of human APOE-Leiden and cholesteryl ester transfer protein (*CETP*), causing increased hyperlipidemia and inflammation, significant inverse correlations between *Maff* and *Ldlr* were observed (Pearson's r=-0.27, p=4.65e-05). Correlations between *Maff* and its network interaction partners are summarised in **Supplemental Table 3**. Also, associations between *Maff* and various molecular and biochemical phenotypes were studied, showing significant correlations with atherosclerosis related traits under non-inflammatory conditions (mice on chow and high-fat diet): total cholesterol (Pearson's r=0.31, p=7.90e-4), unesterified cholesterol (Pearson's r=0.38, p=2.22e-3), body weight (Pearson's r=0.32, p=1.54e-3); and inflammatory conditions (atherogenic): aortic lesion area (as a measure of atherosclerotic lesions) (Pearson's r=-0.37, p=1.34e-3), *Il6*-levels (element of the inflammasome axis; Pearson's r=0.45, p=5.24e-6), *Tnfa*-levels (as a measure of inflammation; Pearson's r=0.33, p=1.23e-3) and *Mcp1*-levels (recruiting monocytes, memory T cells and

dendritic cells to the sites of inflammation; Pearson's r=0.33, p=1.09e-3). Furthermore, the density of absolute values of Pearson correlation efficient r was assessed between gene pairs of the *Maff* network as a parameter of network gene coexpression or connection activity and the network coexpression activity between HMDP panels (chow vs. high fat vs. atherogenic) was compared. Overall, significantly increased gene-gene coexpression from chow diet over high-fat diet to the transgenic group was identified (p<0.01) (**Supplemental Figure 1**). The gradual elevation of coexpression activity of the *Maff* network along with the accompanying increases in cholesterol levels from low-atherogenic to high-atherogenic conditions suggests a context-specific role of *Maff* in the regulation of the liver gene network and LDL cholesterol.

Confirmation of MAFF coexpression with CAD and related processes in human liver

To study the effects of *MAFF* in humans with CAD phenotype data from the Stockholm-Tartu Atherosclerosis Reverse Network Engineering Task (**STARNET**) were used. STARNET provides RNA-sequencing data from different tissues of 600 CAD patients undergoing coronary artery bypass graft (CABG) surgery. All patients gave written informed consent to donate tissue samples prior to CABG surgery.[9] Based on liver samples in STARNET, a strong positive correlation between expression levels of *MAFF* and *LDLR* (Pearson's r=0.57, p=4.7e-49) was detected (**Figure 3a**). Studying the *MAFF* network expression values, 22 out of the 24 predicted neighbouring genes were found to be significantly correlated to *MAFF* expression (**Supplemental Table 1**).

MAFF expression was also associated with several cardiometabolic traits in the STARNET database. Importantly, *MAFF* expression was inversely associated with the SYNTAX-Score I – a measure of CAD severity (Pearson's r=-0.1; p<0.01). Weight (Pearson's r=-0.19; p=4.4e-6) and BMI (Pearson's r=-0.15; p=2.90e-4) were found to be significantly inversely correlated to

MAFF expression, whereas *MAFF* expression was positively correlated with hsCRP (Perason's r=0.1; p<0.01). Notably, *MAFF* expression values were higher in women (p=5.5e-3) (**Figure 3b**), such that it might be of interest that 9 *MAFF* interacting genes in **Supplemental Table 3** (*ATF3*, *EPHA2*, *FOXP1*, *GDF15*, *SERPINE1*, *SLC20A1*, *TGFBI*, *TNFRSF12A* and *ZFP36*) have been recognised as sexually dimorphic in mice.[34] We note that the phenotypic correlations between *MAFF* and phenotypes other than CAD were weak in this human CAD cohort compared to the genetically defined mouse populations. This could be explained by low phenotypic variability or medication in the CAD cohort.

Summarising the results from the STARNET cohort, lower levels of *MAFF* expression were correlated with I) lower levels of *LDLR* expression (less capacity to lower circulating LDL cholesterol), II) higher risk for complex and severe CAD, and III) male gender.

Coexpedia[67], an open tool for exploring biomedical hypotheses via coexpression analyses, revealed in 467 different studies by gene set analysis the most relevant biological correlates of *MAFF* to be *LDLR* mediated cholesterol biosynthetic process (p=1.40e-23), negative regulation of apoptotic process (p=8.47e-7), and inflammatory response (p=3.28e-5). Further, gene set analysis of disease ontology highlighted several CAD relevant traits and risk factors. *MAFF* expression was associated with arthritis (p=1.86e-20), ischemia (p=7.76e-12), myocardial infarction (p=1.37e-11), atherosclerosis (p=4.14e-10) and coronary heart disease (p=1.74e-8). On risk factor level hypertension (p=3.15e-12), obesity (p=9.38e-12), diabetes mellitus (p=1.44e-9), kidney disease (p=9.49e-9) and hypercholesterolemia (p=7.32e-5) showed significant association with *MAFF*.

In vitro validation of MAFF/Maff regulatory capacities

To confirm the role of *MAFF* orchestrating the predicted regulatory liver network of CAD genes Hep3b and AML12 were studied representing human and mouse hepatocyte cell lines, respectively. siRNA-knockdown (KD) of *MAFF* in human cells and *Maff* in mouse cells showed consistent, significant reductions of the *LDLR*/*Ldlr* expression ($p<0.001$) (**Figure 4a+b**) (**Expanded Methods**), as well as consistent and significant perturbations of other neighbouring genes. *MAFF/Maff*-KD caused significant upregulation of *EPHA2/Epha2, GDF15/Gdf15* and *TNFAIP3/Tnfaip3*, in contrast to *LDLR/Ldlr* downregulation in both species. These results are in line with the predictions of the Bayesian networks in that these genes are downstream of *MAFF/Maff* (**Supplemental Figure 2**).

Knockdown of *MAFF/Maff* did not perturb expression levels of *Trib1* in AML12 cells and only slightly decreased expression values in human Hep3b cells. By contrast, siRNA knockdown of *TRIB1/Trib1* led to decreased levels of *MAFF/Maff* in both cell lines ($p<0.05$), suggesting that the known CAD risk gene *TRIB1* might be upstream acting as a regulator of *MAFF*/*Maff* expression levels, which was consistent with the Bayesian modeling.

Further, the effect of *Maff* overexpression was investigated using plasmid DNA transfection in mouse AML12 cells and revealed a significant upregulation of the *Ldlr* expression ($p=0.002$) (**Figure 4c**). Based on these silencing and overexpression experiments in the absence of inflammatory stimuli, *Maff* and *Ldlr* expression was found to be positively correlated *in vitro*.

Ldlr reduction in Maff-/- mouse models

To explore *in vivo* the effects of *MAFF* on the expression levels of *Ldlr*, an inbred *Maff*-/- mouse model on C57BL/6-background was employed. Homozygous null mice are viable and fertile and show no obvious functional deficiencies. Liver samples from *Maff*-/-, *Maff*+/- and wildtype

(WT) mice, all fed with chow diet, were collected. Expectedly, circulating cholesterol levels were low in mice lacking a pro-atherosclerotic background (e.g. Apoe-/-, Ldlr-/-). *Maff*-/- mice showed no significant differences on serum cholesterol levels compared to WT mice. Significant decrease of *Ldlr* expression levels in *Maff*-/- mice was observed compared to WT mice (p=0.028) (**Figure 4d**) and lower amounts of *Ldlr* protein were confirmed by Western blot analysis in *Maff*-/- compared to WT mice (p=0.010). This is in line with the *in vitro* findings. There was no significant difference between the groups and heterozygous *Maff* mice.

Context specific influence on MAFF and LDLR in the presence of LPS stimulation

Maff has been described to be a context specific transcription factor.[64] Pro-inflammatory lipopolysaccharide (LPS) was used intraperitoneally as a strong inductor of acute systemic inflammation in male *Maff* WT and knockout (KO) mice. A significant induction of *Maff* expression levels (p<0.001) was detected in WT animals six hours after LPS treatment. *Maff* mRNA was not detectable in *Maff*-/- mice. In addition to changes in *Maff* levels, there was a significant decrease of *Ldlr* expression levels in the WT group (p<0.001), but no significant change in *Maff*-/- mice, suggesting that *Maff* and its network are sensitive to inflammatory processes and that *Maff* is involved in inflammation induced suppression of *Ldlr* (**Figure 4e-f**). Circulating Ldl/Vldl cholesterol levels were non-significantly elevated in *Maff* WT mice compared to LPS treated *Maff* WT mice (66.1mg/dl vs. 61.4mg/dl, p=0.24) six hours after LPS injection. Further, expression of the pro-inflammatory cytokine *Tnfa* was assessed in *Maff* WT and *Maff*-/- mice with and without LPS treatment. *Tnfa* expression was significantly upregulated in *Maff* WT and *Maff*-/- mice after LPS stimulation (p<0.001). However, comparing *Tnfa* expression between both groups 6 hours after LPS stimulation, upregulation of *Tnfa* expression was found to be significantly increased in *Maff* WT mice (p=0.048) (**Figure**

4g). Under inflammatory conditions – using LPS stimulation – *Maff* and *Ldlr* expression was found to be inversely correlated in *Maff* WT mice.

Identification of the regulatory MAFF binding site

Next, the regulatory role of *MAFF* as a transcription factor was investigated in the network. Potential binding sites of *MAFF* were assessed using Chromatin-Immuno-Precipitation-DNA-Sequencing (ChIP-Seq) data on human HepG2 cells as studied in the ENCODE project.[68] Computational analysis revealed a Leucine-Zipper binding motif to be enriched in binding elements of gene members of the *MAFF* network (20 of 24 genes, Fisher's exact test, fold change=4.03, p=3.2e-34). Moreover, multiple *MAFF* binding sites were identified in the *LDLR* gene, including the promoter region (**Figure 5**). These results suggest that MAFF has the potential to bind to the promoter regions of the network member genes and regulate their expression.

MAFF binding partners in homeostasis and inflammation

To further elucidate the role of *MAFF/Maff* and its binding partners in homeostasis and inflammation Chromatin Immunoprecipitation followed by mass spectrometry (ChIP-MS) was performed in Hep3b and AML12 liver cells. Both cell lines were treated with either vehicle PBS or LPS (10ng/ml) for 48h with and without *MAFF/Maff* siRNA knockdown.

MAFF/Maff siRNA knockdown and LPS stimulation identified *BACH1*/*Bach1* as relevant transcriptional interaction partner of *MAFF/Maff* (**Figure 6**). Specifically, LPS induction determined *BACH1/Bach1* as a robust *MAFF/Maff* interactor in both cell lines. On expression level, LPS stimulation led to a significant increase of *BACH1*/*Bach1* expression in human Hep3b (p=0.006) and mouse AML12 cells (p=0.001) compared to controls (vehicle) (**Figure**

7a+b). *BACH1*/*Bach1* is a known repressor of the MARE – also known as stress-responsive element – and downregulates transcription (**Figure 8**).[69] Of note, *BACH1* is a human CAD GWAS candidate gene and the underlying mechanism of how *BACH1* contributes to CAD is unclear.[70]

Also the heterochromatin markers *Trim28* (*Kap1*) and *Cbx3*, as well as the RNA related factors *Dhx40*, *Srrm1* and *Srsf2/5* were enriched in *Maff* ChIP-MS of LPS stimulated AML12 mouse liver cells. A clear reduction of the enrichment signal in response to *Maff* knockdown indicated that these proteins are specific *Maff* interactors (**Figure 6c+d**). *Maff* interactors and their individual enrichment patterns under different conditions are summarised after normalisation in **Figure 7c**.

Further, it was studied if activating transcription factors bind with *MAFF*/*Maff* under basal conditions and in the presence of LPS stimulation in both cell lines. No enrichment of activating transcription factors was identified comparing both conditions.

Verification of MAFF-BACH1 heterodimers binding at the LDLR promotor

Based on the findings of the ChIP-MS approach in human Hep3b cells, human ChIP-Seq data was used to validate the binding capacities of the *MAFF* heterodimerisation partners at the *LDLR* promoter. Three dimensional structures of heterodimers were modeled (**Expanded Methods)** and confirmed the preferred binding of the *MAFF-BACH1* complex at the *LDLR* promoter as measured by the lowest free energy (p=9.5e-05) (**Supplemental Figure 3**).

Discussion

Gene regulatory network modelling based on hundreds of genes known to affect atherosclerosis retrieved a dense liver network highly enriched for mouse atherosclerosis and human CAD GWAS candidate genes. Notably, the network – centered at the transcription factor *MAFF* – contains *LDLR* and inflammatory genes, which are all implicated to play causal roles in coronary artery disease. Prediction by Bayesian models and experimental studies clarified the hierarchical order and regulatory direction of the genes within the network as well as the central role of *MAFF* as a regulating element.

Under non-inflammatory conditions a positive correlation between *MAFF* and *LDLR* expression *in vitro* and *in vivo* was identified, whereas inflammatory conditions led to inverse correlation between *MAFF* and *LDLR* expression. Most importantly, after acute induction of systemic inflammation via LPS stimulation, we observed that binding of *MAFF-BACH1* heterodimers at the MARE in the *LDLR* promoter region significantly downregulated *LDLR* expression (**Figure 8**).

BACH1 resides at a genome-wide significant CAD GWAS locus and the underlying mechanism how *BACH1* contributes to CAD progression is hitherto unknown.[70] Based on pathway analyses *BACH1* has been described to be involved in vascular remodeling, proliferation and transcriptional regulation.[71] We speculate that *BACH1* promotes atherosclerosis based on the downregulation of the *LDLR* via *MAFF*.

The transcriptome studies in mouse models and human samples as well as *in vitro* and *in vivo* *MAFF* perturbation experiments provided evidence supporting a context-specific role of *MAFF* in the regulation of *LDLR* as well as other genes. *In vivo* data from STARNET, including liver tissue samples from a large set of CAD patients undergoing bypass surgery showed a significant positive correlation between expression of the transcription factor *MAFF* and the *LDLR*. Lower

expression values of *MAFF* were found in men and were significantly correlated with more complex and severe coronary artery lesions as measured by the Syntax Score I. Likewise, a positive correlation between *Maff* and *Ldlr* was found in mice on chow and high-fat diets in HMDP. In mice with transgenic expression of human *APOE-Leiden* and *CETP*, which display a significant increase of inflammatory cytokines, showed in contrast a significant inverse correlation between *Maff* and the *Ldlr*. These results support a context-specific relationship between the expression of *Maff* and *Ldlr*.

Substantial convergence of tissue-specific regulatory mechanisms was observed in the *MAFF/Maff* liver specific network between human and mouse. The finding that knockdown of *Maff in vitro* in a mouse hepatocyte cell line led to reduced expression of the *Ldlr* was validated in a human *in vitro* model, highlighting similar interaction patterns between *MAFF* and *LDLR* in human and mouse. Besides *LDLR*, the other experimentally examined neighbouring genes within the *MAFF* network (*EPHA2*, *GDF15, TNFAIP3, and TRIB1*) also showed convergent regulation in human and mouse liver cell lines. The *in vivo* data in *Maff* knockout mice strongly supported this assumption. Additionally, the presence of MAFF binding motifs in the *LDLR* gene region and 19 out of the overall 24 additional predicted neighbouring genes using human ChIP-Seq data were identified. However, under baseline conditions it was not achievable to identify the molecular partners effecting *LDLR*/*Ldlr* downregulation following *MAFF*/*Maff* silencing, in contrast to inflammatory conditions. Nevertheless, *Maff* overexpression in AML12 cells led to significant upregulation of the *Ldlr* under non-inflammatory conditions.

MAFF was also found to be sensitive to environmental stimuli (e.g. high-fat diet, inflammation). Treatment with lipopolysaccharides markedly increased inflammatory cytokine levels in the liver of wildtype mice, as measured by *Il1b*, *Il6* and *Tnfa* levels and led to an excessive upregulation of *Maff* that was accompanied by reduced *Ldlr* expression. The same pattern of

elevated inflammatory mediators and inverse correlation of *Maff* and *Ldlr* expression levels has been identified in the HMDP in mice with transgenic implementation of human *APOE-Leiden* and *CETP*. This is in line with findings in the literature, that cholesterol accumulation in cells of genetically modified mouse models triggers the inflammasome and results in elevated release of inflammatory mediators such as *Il1b*, *Il6* and *Tnfa*.[72]

Indeed, inflammation and perturbation of cholesterol levels coincide in several human conditions, including rheumatoid arthritis (RA), which goes along with an increased risk of cardiovascular events.[73-77] Recently, Fernández-Ortiz and colleagues studied lipid metabolism in different stages of RA disease activity and observed that total cholesterol, LDL cholesterol and oxidized LDL cholesterol were slightly elevated in the low disease activity group, whereas high disease activity individuals showed distinct reduction of total cholesterol, HDL cholesterol and LDL cholesterol along a drastic increase of pro-atherosclerotic oxidized LDL cholesterol.[78] These alterations are mirrored by a gene set analysis of disease ontology[67] focused on *MAFF* expression revealing its association with arthritis, LDLR expression, atherosclerosis and myocardial infarction risk. Thus, our offer an explanation on that *MAFF* could be a link between inflammation, lipid and lipoprotein metabolism and cardiovascular disease.

Limitations

Bioinformatics analyses on key drivers of liver gene expression may be further enriched in the future as the numbers of loci showing significant signals in human CAD GWAS and mouse candidate genes will increase within the next years. However, our previous network modeling of CAD GWAS loci demonstrated that network predictions are relatively insensitive to changes in the number of loci included, supporting the robustness of the overall patterns of disease pathways and networks.[79] In this study, a *MAFF* centered regulatory network was revealed involving multiple atherosclerosis related genes in mouse and human, with profound effects of

MAFF on downstream targets, and its regulation by inflammatory mediators. The molecular mechanisms affecting *MAFF* expression as well as *MAFF* downstream targets remain inadequately understood. Specifically, the molecular partners of *MAFF* executing transcriptional activation of *LDLR* under non-inflammatory conditions remain to be elusive. By contrast, *BACH1* was identified to physically interact with *MAFF* in the presence of LPS stimulation. While we provided multiple layers of evidence placing *MAFF* in the center of a top ranked liver-specific key-driver network linking lipid metabolism and inflammation, further investigations should aim to identify potential mechanisms relevant for cardiovascular risk via regulation of *MAFF*, *LDLR* and downstream pathways. Of specific interest would be the elucidation of the molecular model explaining *MAFF*-related *LDLR* induction and subsequent atheroprotective effects. In this respect our findings may be a starting point for a better elucidation of interactions between inflammatory processes, hypercholesterolemia and CAD risk.

In conclusion

The transcription factor *MAFF* is the key driver of a liver specific network which includes a large number of genes known to affect atherosclerosis in mouse and human. Depending on the underlying context (non-inflammatory vs. inflammatory) *MAFF* is able to mediate activation or repression of gene expression.[80, 81] Under non-inflammatory conditions *MAFF* appears to induce *LDLR* expression. In the presence of LPS stimulation, heterodimerisation of *MAFF* with the CAD GWAS candidate and transcriptional regulator *BACH1*[82] binding at the MARE in the promoter region of the *LDLR* led to downregulation of *LDLR* expression. It appears that both, the degree of inflammation (none vs. excessive) and expression values of *MAFF* modulate these processes. Our experiments in different model systems and human samples demonstrate a direct

connection between *MAFF* and the *LDLR* and also revealed significant changes in this relationship under inflammatory conditions (**Figure 8**).

Acknowledgements

MVS reported funding by the Clinician Scientist Excellence Program of the German Center for Cardiovascular Research (DZHK), the German Society of Cardiology (DGK) and the German Heart Foundation (Deutsche Herzstiftung e.V.). This work was supported by grants from the Fondation Leducq (CADgenomics: Understanding CAD Genes, 12CVD02), the German Federal Ministry of Education and Research (BMBF) within the framework of ERA-NET on Cardiovascular Disease, Joint Transnational Call 2017 (ERA-CVD: grant JTC2017_21-040), within the framework of target validation (BlockCAD: 16GW0198K), within the framework of the e:Med research and funding concept (AbCD-Net: grant 01ZX1706C and e:AtheroSysMed, grant 01ZX1313A-2014). Further from the Deutsche Forschungsgemeinschaft (DFG) directly (SA 1668/5-1) and as part of the Sonderforschungsbereich CRC 1123 (B2), and additionally from the German Heart Research Foundation [F/28/17] (HS). Maff work is supported in TQdeAV and PAE. labs by NIH grant DK102559. Bioinformatics work was supported by the American Heart Association Postdoctoral Fellowship (YZ) and NIH grants DK104363 (XY). The STARNET study was supported by the University of Tartu (SP1GVARENG), the Estonian Research Council (ETF grant 8853), the Astra-Zeneca Translational Science Centre-Karolinska Institutet (a joint research program in translational science), Clinical Gene Networks AB (CGN) as an SME of the FP6/FP7 EU-funded integrated project CVgenes@target (HEALTH-F2-2013-601456), the Leducq transatlantic networks, CAD Genomics, the American Heart Association (A14SFRN20840000, the National Institutes of Health (NIH NHLBI R01HL125863; NIH NHLBI R01HL71207; R01AG050986; NIH NHLBI K23HL111339; NIH NHLBI K08HL111330) (JB). In vitro and HMDP work was supported by NIH grants HL138196 (MMS) HL28481, HL30568, HL144651, HL147883 and DK117850 (AJL).

Author contributions

MVS drafted the manuscript and performed the literature search of mouse genes. YZ performed the bioinformatics modeling. *In vitro* experiments have been performed by MVS, NC and TV. TV contributed with KO mouse models. MY and PAE supported the Maff-/- mouse model. MW and MM supported the ChIP-MS approach. AJL provided *in vivo* data from HMDP. OF, AR, JK and JB contributed with human data from STARNET. SP performed the MAFF binding approach. XY supervised the bioinformatics analysis. All authors participated in the analyses of the data and critically reviewed the manuscript, written by MVS, YZ, AJL, XY and HS.

Disclosures

None.

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Figures

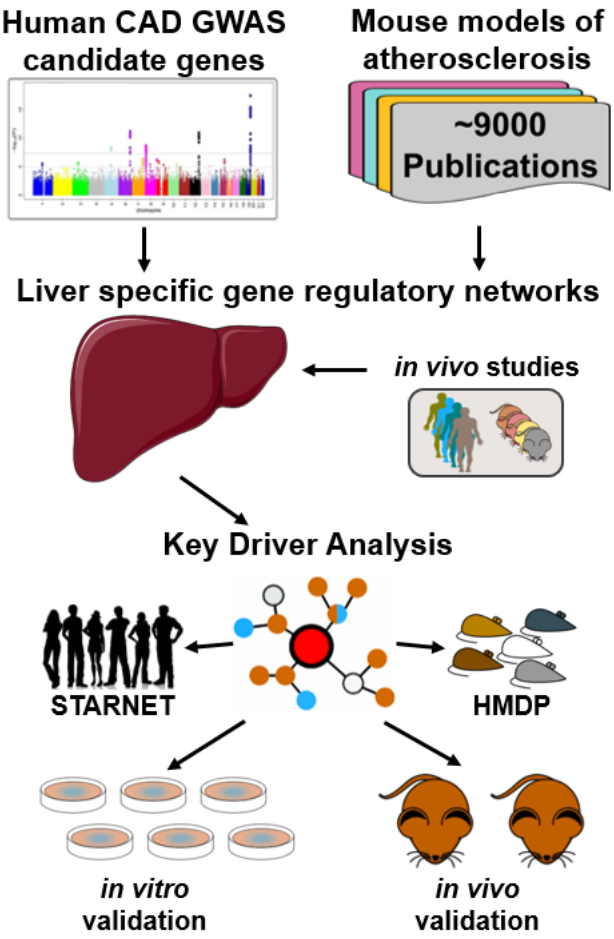


Figure 1. Study workflow: Human and mouse atherosclerosis candidate genes were used to first model liver specific regulatory networks and second decipher key driver genes of gene regulatory networks in both species. Prediction of bioinformatics modeling was validated in human and mouse genetic studies as well as in in vitro and in vivo experiments. CAD: Coronary artery disease; GWAS: Genome wide association study.

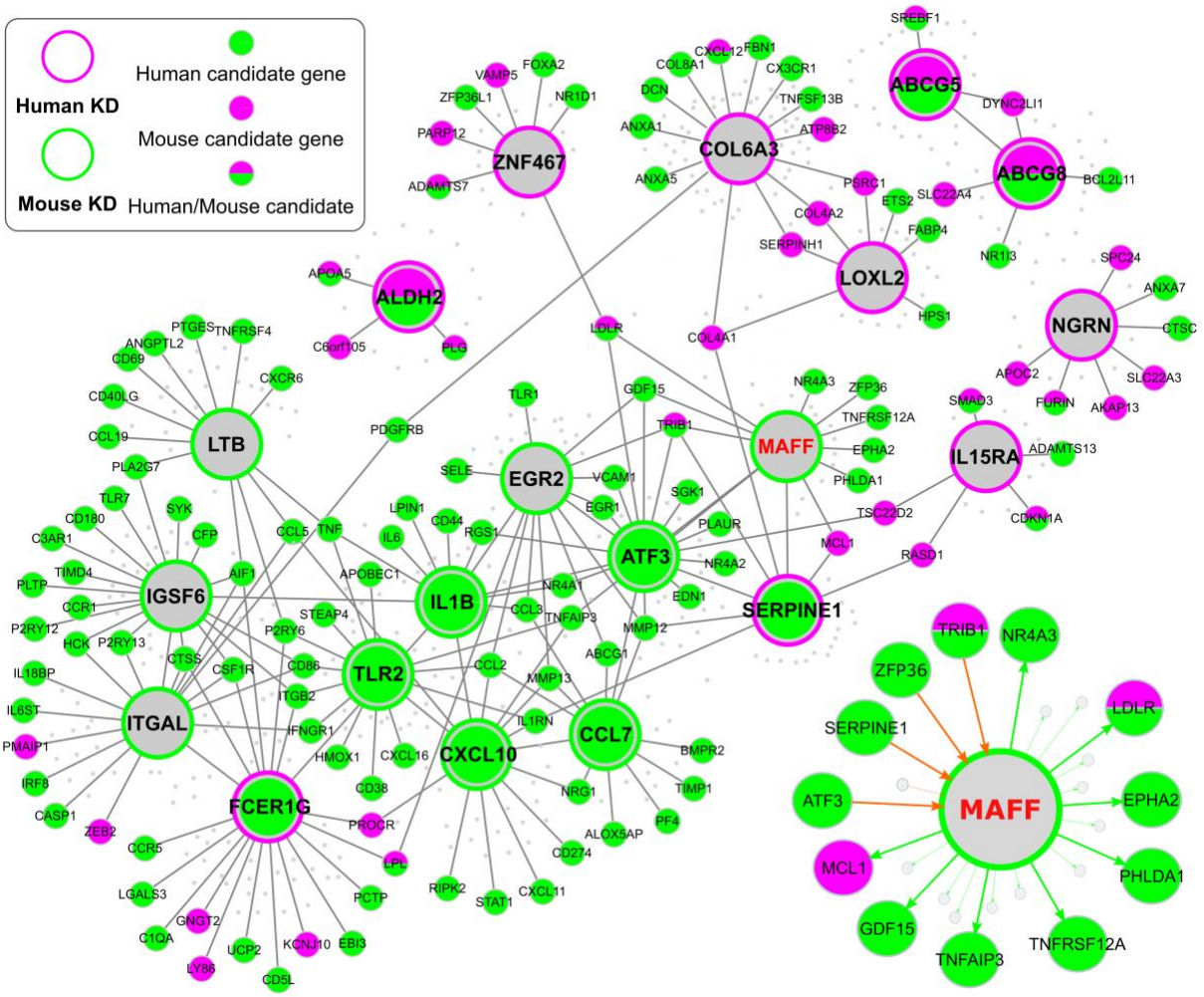


Figure 2. Liver specific regulatory subnetworks and their key driver genes. The key driver analysis was performed on human and mouse networks respectively and the architecture of the illustrated network is based on both, mouse and human data. Key drivers are depicted as the largest nodes in the networks. All genes highlighted in solid green have already been studied to have a significant effect on atherosclerosis in genetically engineered mouse models. Human CAD GWAS candidate genes are highlighted in magenta. Key driver genes in grey need to be validated. Genes with both colors have an effect on atherosclerosis/CAD in human and mouse. Lower right: The MAFF network is the top ranked key driver gene network based on mouse data and closely connected to other human key driver subnetworks. Directionality between genes was based on the consensus of directional predictions from Bayesian networks constructed from different datasets, with the directionality predicted by the majority of studies shown. Red arrows indicate genes that are predicted to regulate MAFF, whereas green arrows indicate genes that are predicted to be regulated by the transcription factor MAFF. CAD: coronary artery disease; GWAS: Genome wide association study; Human KD: Human key driver gene; Mouse KD: Mouse key driver gene; MAFF: v-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog F.

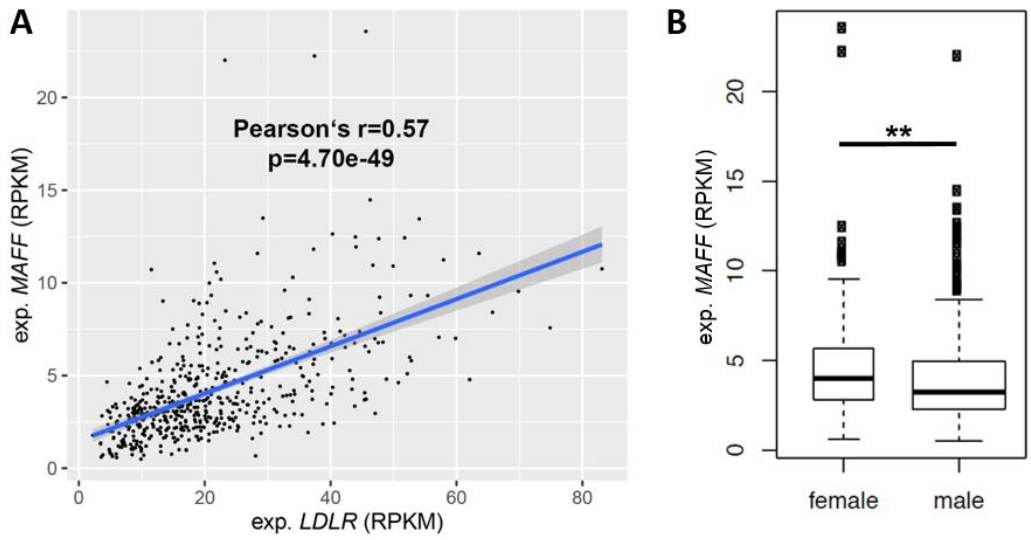


Figure 3. Correlation of expression levels of MAFF in human liver samples from the Stockholm-Tartu Atherosclerosis Reverse Network Engineering Task (STARNET) with A: LDLR and B: Sex. ** indicates p<0.01. LDLR: low-density lipoprotein receptor; MAFF: v-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog F; RPKM: Reads per kilobase million.

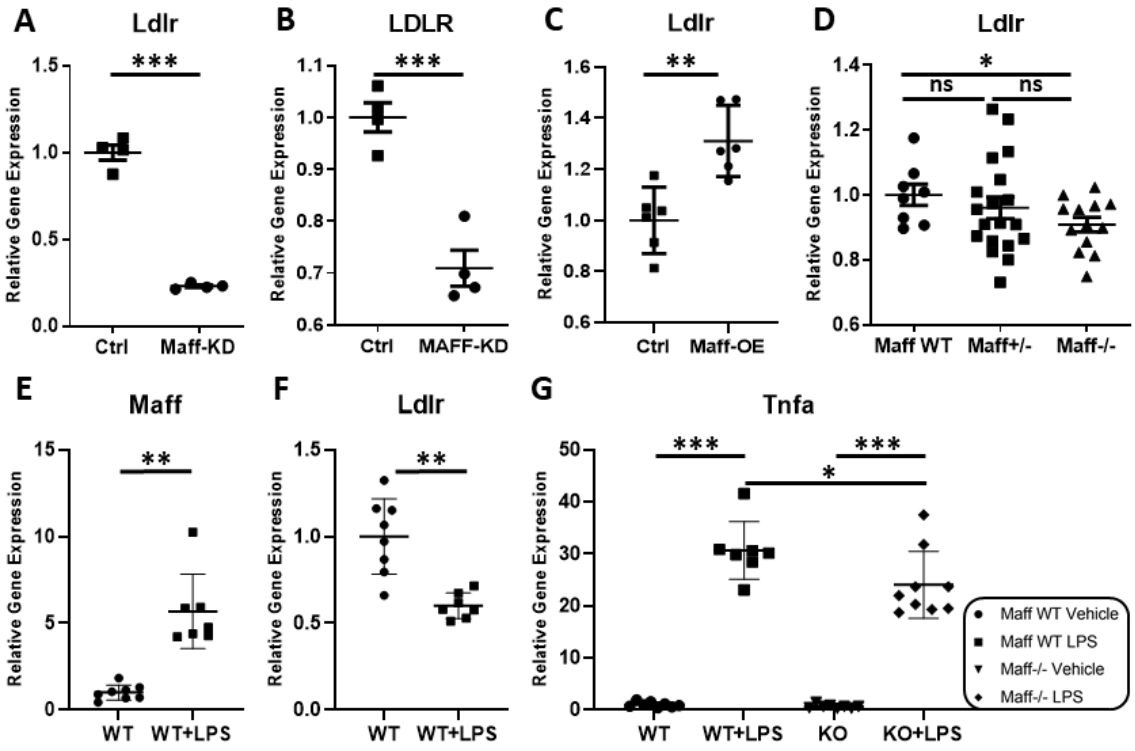


Figure 4. A: In vitro results of Ldlr expression after siRNA-knockdown of Maff compared to controls (vehicle) in mouse AML12 liver cells. B: In vitro results of LDLR expression after siRNA-knockdown of MAFF compared to controls (vehicle) in human Hep3b liver cells. C: In vitro results of Ldlr expression cells after Maff overexpression compared to controls (vehicle) in mouse AML12. D: In vivo results of Ldlr expression in Maff-/- mice compared to Maff+/- and WT mice. E: In vivo results of Maff expression in Maff WT mice 6 hours after LPS stimulation compared to controls (vehicle). F: In vivo results of Ldlr expression in Maff WT mice 6 hours after LPS stimulation compared to controls (vehicle). G: In vivo results of Tnfa expression in Maff WT and Maff-/- mice 6 hours after LPS stimulation compared to controls (vehicle). *** indicates $p<0.001$, ** indicates $p<0.01$, * indicates $p<0.05$, ns indicates non-significant. Bonferroni correction was applied for multiple comparison. Ctrl: control group; KD: knockdown; LDLR: low-density lipoprotein receptor; LPS: lipopolysaccharide; MAFF: v-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog F; OE: overexpression; WT: wildtype.

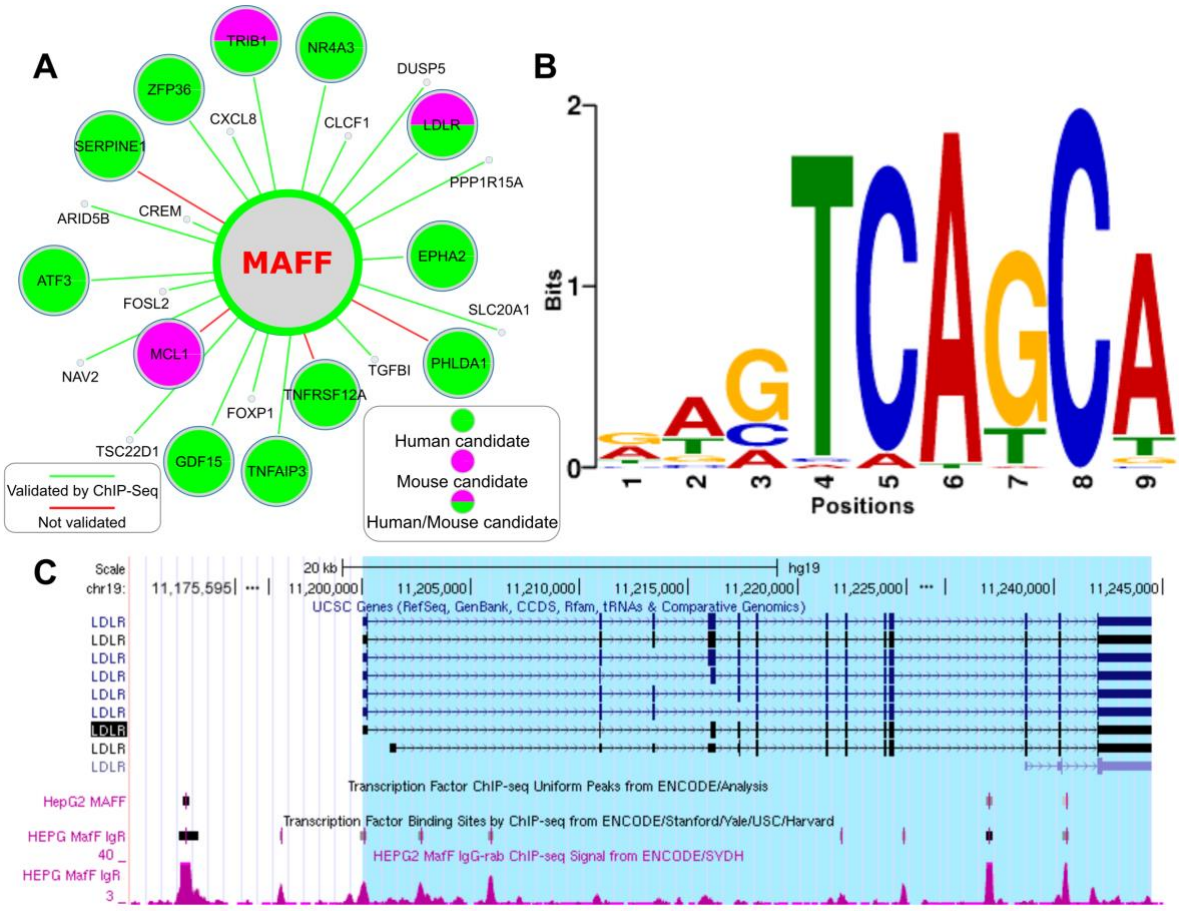


Figure 5. A: ChIP-seq data of human HepG2 cells supports potential binding of MAFF to genes in the MAFF subnetwork. Green edges indicate a binding motif was shared in the selected network genes, whereas red edges indicate that no known shared binding motif was found in the particular network genes. A matching binding motif was found in 20 out of 24 predicted interaction partners of MAFF. B: The matching motif among the MAFF network genes, which agrees with the previously known MAFF binding motif. The matching motif was identified using publically available ChIP-Seq data of the MAFF gene in human HepG2 cells from ENCODE. The height of the letter represents the frequency of the observed nucleotide in that position. C: Presence of the MAFF binding motif (small boxes below) upstream and within the LDLR gene (highlighted in blue).

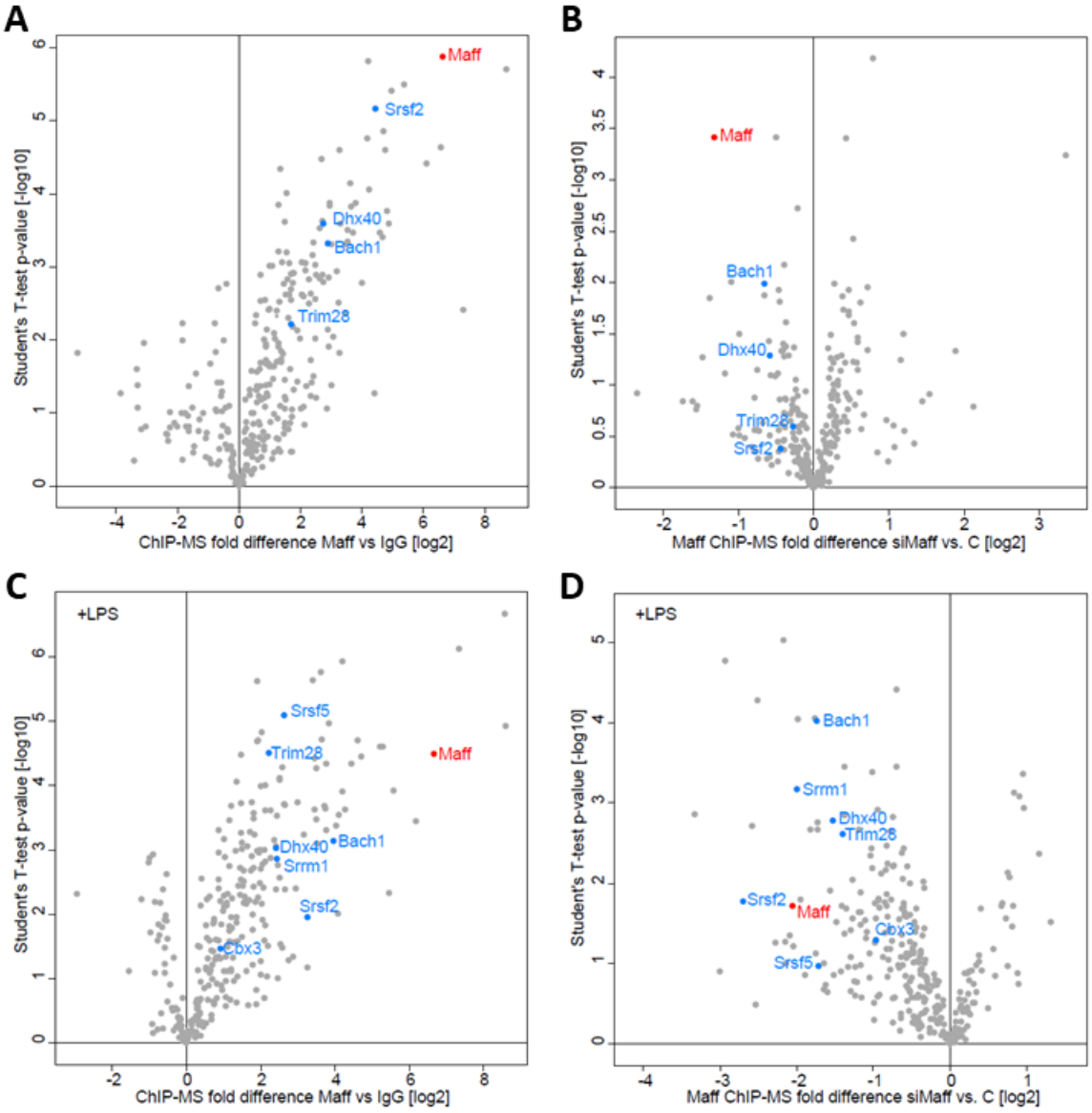


Figure 6. Volcano plot of the p-values (y-axis) vs. the log2 protein abundance differences (x-axis) of Maff binding partners in AML12 cells identified by ChIP-MS under (A) homeostatic conditions, (B) after Maff siRNA knockdown (which led to a 91% decrease on protein level), (C) LPS stimulation and (D) Maff siRNA knockdown in combination with LPS stimulation. Significant Maff interaction partners were highlighted in blue. Enrichment of binding partners is provided as fold difference compared to negative control (IgG) in panel A and C and compared to control (Maff WT) after siRNA knockdown in panel B and D. C: control (Maff WT); IgG: nonspecific IgG served as negative control.

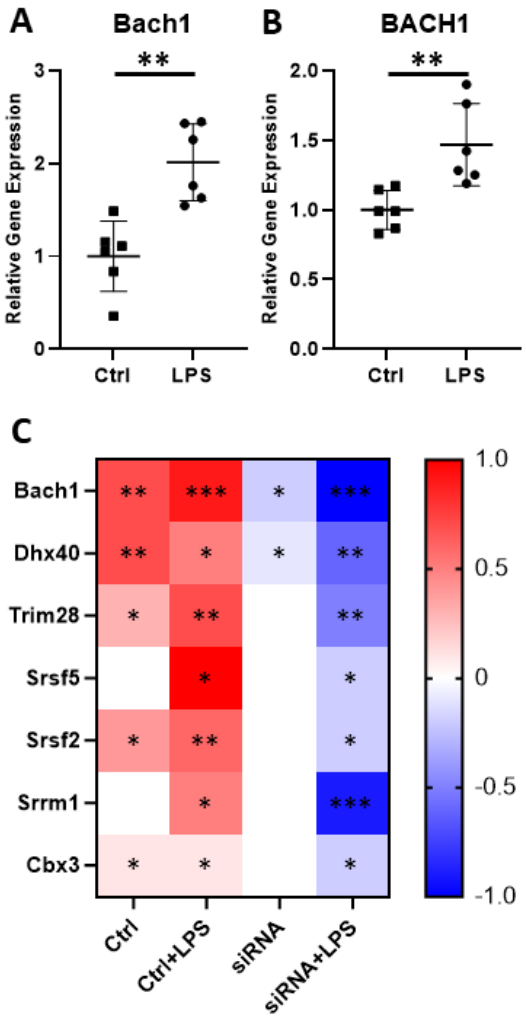


Figure 7. LPS stimulation led to a significant increase of BACH1/Bach1 expression in mouse AML12 cells (A) and human Hep3b cells (B) compared to controls (vehicle). (C) The heat map of z-scored Maff ChIP-MS visualises LFQ intensities of selected Maff interactors in extracts from AML12 cells under homeostatic conditions (Ctrl), LPS stimulation (Ctrl+LPS), after Maff siRNA knockdown (siRNA) and after Maff siRNA knockdown in combination with LPS stimulation (siRNA+LPS). Provided are adjusted p-values. *** indicates $p<0.001$, ** indicates $p<0.01$, * indicates $p<0.05$.

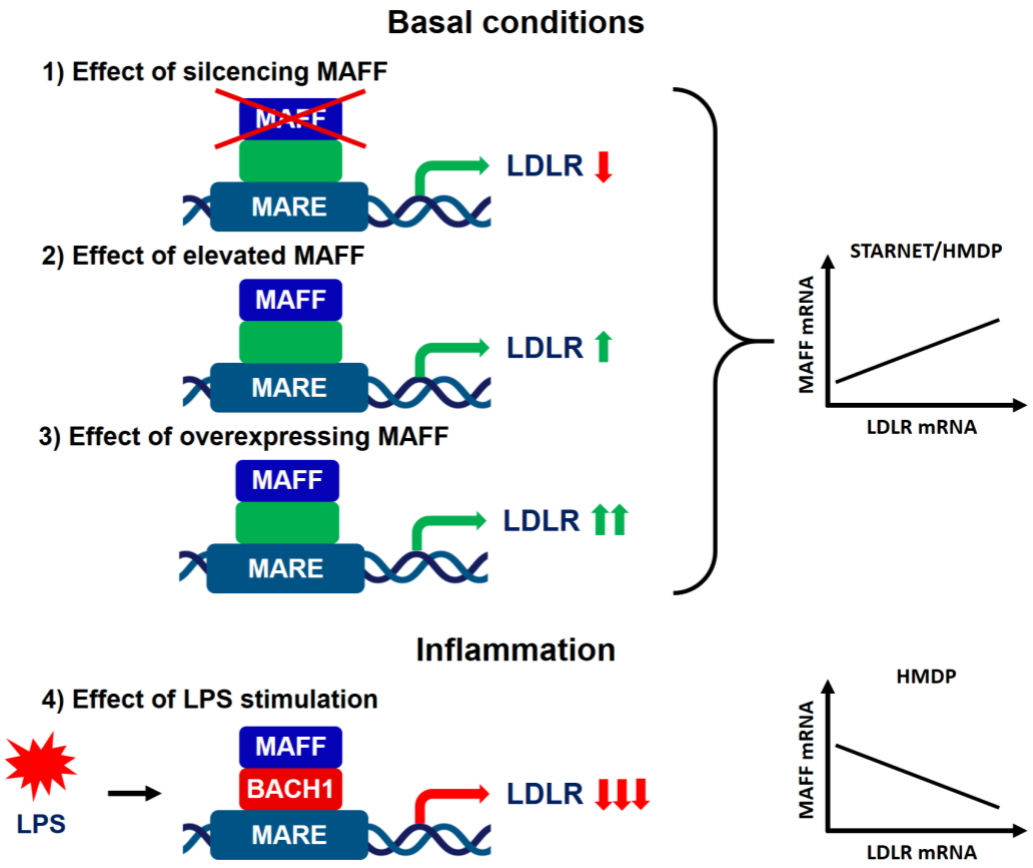


Figure 8. The role of the transcription factor MAFF in activation or repression of the LDLR is based on heterodimerisation partners and environmental conditions. MAFF heterodimers bind at the MAF recognition element (MARE) of the LDLR promoter and execute regulation of the LDLR. Under basal conditions 1) MAFF knockdown/knockout led to reduced LDLR expression, 2) elevated MAFF expression was correlated with higher expression of LDLR in a human CAD cohort (STARNET) and in wildtype mice of the hybrid mouse diversity panel (HMDP), 3) Overexpression of Maff using plasmid DNA transfection led to increased Ldlr expression in vitro. In the presence of LPS stimulation MAFF-BACH1 heterodimers result in downregulation of the LDLR in vivo. HMDP mice on atherogenic background (transgenic expression of human APOE-Leiden and cholesteryl ester transfer protein (CETP)) showed increased inflammation and revealed that elevated Maff expression correlates with lower Ldlr expression. BACH1: BTB domain and CNC Homolog 1; MAFF: v-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog F; MARE: Maf recognition element; LDLR: low-density lipoprotein receptor; LPS: lipopolysaccharide.

Table 1. Listed are the top 10 key driver genes detected in human and mouse liver networks based on the bioinformatics approach. Several genes have already been studied and confirmed with regard to atherosclerosis/CAD. FDR: False discovery rate.

Species	Key Driver Gene	Studied effect on atherosclerosis	Network size (Genes)	Atherosclerosis associated genes	FDR	Fold Enrichment
Mouse	***Maff***	**no**	**25**	**11**	**1.50E-05**	**19.08**
	Il1b	yes	27	11	1.50E-05	19.08
	Ccl7	yes	34	12	1.17E-05	16.53
	Atf3	yes	50	15	3.89E-06	14.05
	Cxcl10	yes	46	13	1.26E-05	13.23
	Egr2	no	50	14	7.79E-06	13.11
	Igsf6	no	66	18	1.49E-06	12.77
	Ltb	no	44	12	2.45E-05	12.77
	Itgal	no	52	14	8.81E-06	12.61
	Tlr2	yes	57	15	5.90E-06	12.32
Human	*ALDH2*	yes	19	4	1.10E-04	31.29
	IL15RA	no	20	4	1.50E-04	29.72
	ZNF467	no	22	4	2.70E-04	27.02
	NGRN	no	32	5	1.87E-07	23.22
	LOXL2	no	28	4	1.15E-03	21.23
	ABCG8	yes	30	4	1.73E-03	19.82
	ABCG5	yes	33	4	3.00E-03	18.01
	SERPINE1	no	34	4	3.55E-03	17.48
	COL6A3	no	69	6	1.22E-07	12.92
	FCER1G	no	81	5	3.16E-03	9.17

Supplemental Materials Appendix

Supplemental Table 1. List of 244 human CAD candidate genes based on annotations of 169 known significant and suggestive human CAD GWAS loci.

Supplemental Table 2. List of 827 mouse atherosclerosis genes as defined by a significant effect on atherosclerotic lesion size or composition when perturbed in a mouse model.

Supplemental Table 3. Correlation analysis of network genes in HMDP and STARNET based on Pearson correlation coefficient.

Supplemental Figure 1. Comparison of pair-wise correlations among all *Maff* network genes according to their expression values in three HMDP populations. Large and bright circles represent strong correlations. A: Mice on chow diet. B: Mice on high-fat diet. C: Mice on high-fat diet with transgenic implementation of human *APOE-Leiden* and *CETP*.

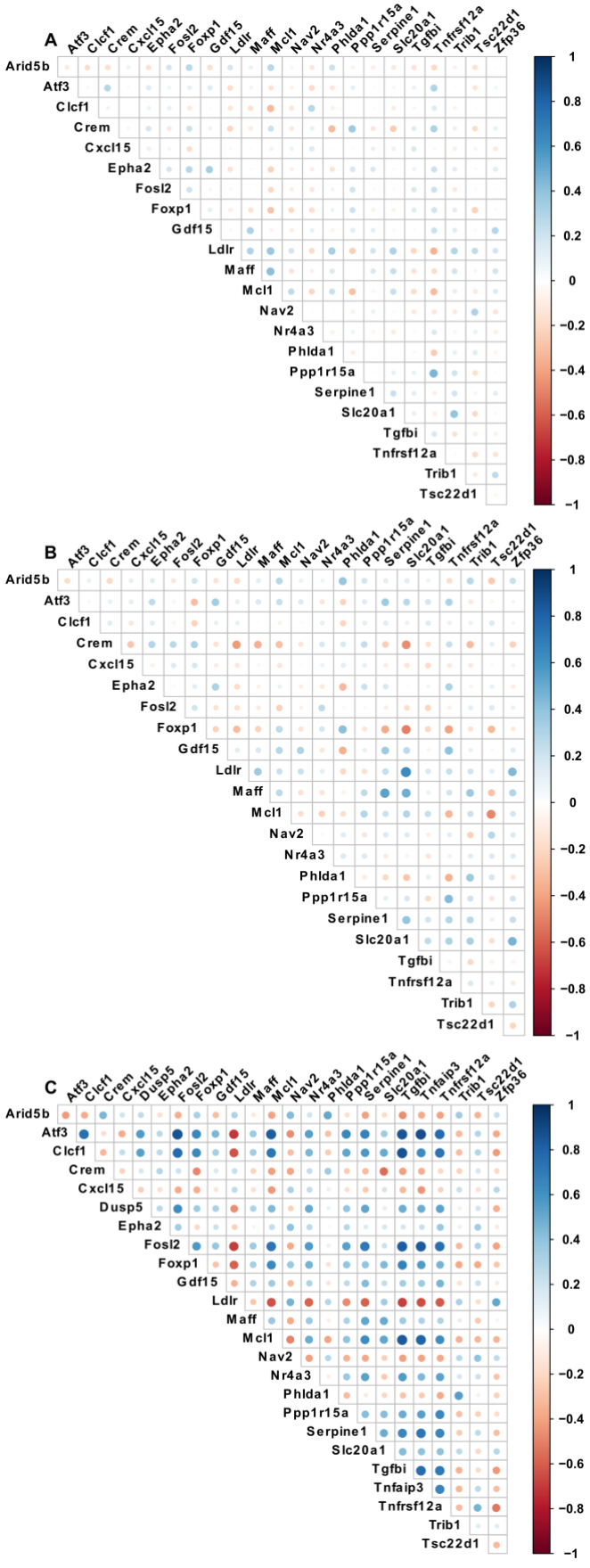
Supplemental Figure 2. A: In vitro results of MAFF neighbour gene expression after siRNA knockdown of *MAFF* in human Hep3b liver cells and B: In vitro results of Maff neighbour gene expression after siRNA knockdown of *Maff* in mouse AML12 liver cells. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$. Ctrl: control group; KD: knockdown; LDLR: low-density lipoprotein receptor; MAFF: v-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog F.

Supplemental Figure 3. Three-dimensional binding of the heterodimerised transcription factor *MAFF* (blue) and the transcriptional repressor *BACH1* (red) at the MARE of the *LDLR* promoter in the presence of LPS stimulation. BACH1: BTB domain and CNC Homolog 1; MAFF: v-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog F; MARE: Maf recognition element; LDLR: low-density lipoprotein receptor; LPS: lipopolysaccharide.

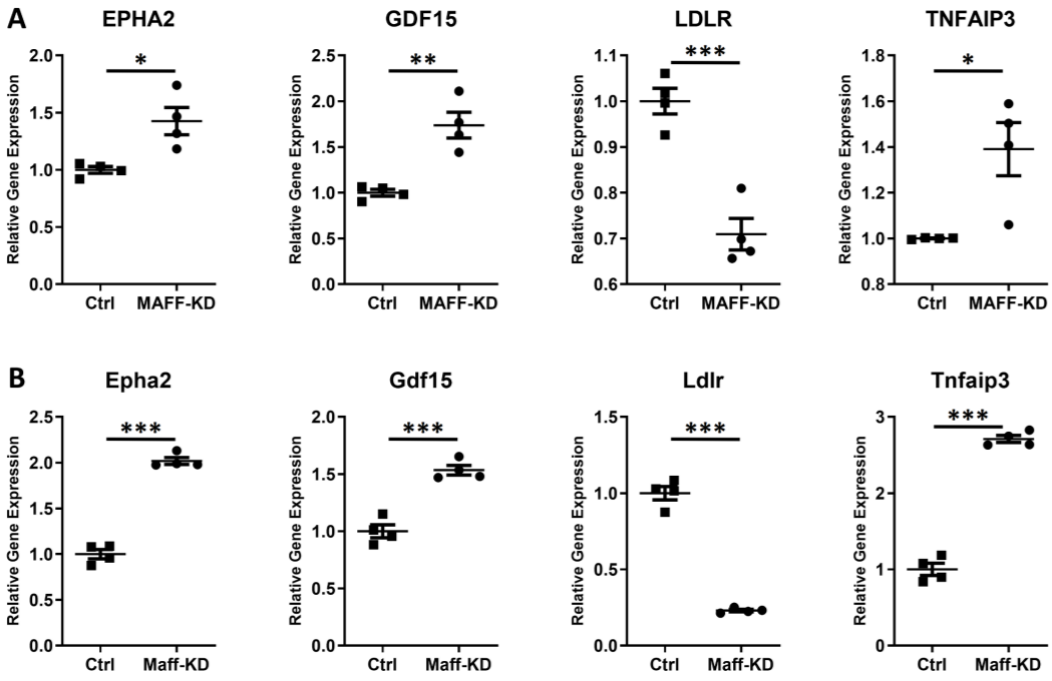
Supplemental Table 3. Correlation between *Maff/MAFF* and its network genes under different conditions. HMDP mice on chow diet, high-fat diet and with transgenic implementation of human *APOE-Leiden* and *CETP* (left). On the right, human data from the STARNET cohort.

	Mouse data (HMDP)										Human data		
	Chow - both sexes		High-fat - female		High-fat - male		Transgenic - female		Transgenic - male		STARNET - both sexes		
Gene	**Pearson's R**	**p-value**	**Pearson's R**	**p-value**	**Pearson's R**	**p-value**	**Pearson's R**	**p-value**	**Pearson's R**	**p-value**	**Pearson's R**	**p-value**	**Gene**
Arid5b	0.10	1.30E-01	0.08	2.31E-01	0.13	5.87E-02	-0.12	7.33E-02	0.09	3.79E-01	0.04	3.05E-01	*ARID5B*
Atf3	0.06	3.24E-01	0.17	1.70E-02	0.09	1.78E-01	0.26	7.31E-05	0.37	1.58E-04	0.66	2.26E-68	*ATF3*
Clcf1	-0.17	6.67E-03	0.01	8.50E-01	-0.01	8.39E-01	0.26	7.66E-05	0.34	4.35E-04	0.43	1.54E-26	*CLCF1*
Crem	-0.10	1.01E-01	-0.27	1.07E-04	-0.35	4.89E-08	-0.22	8.67E-04	-0.21	3.29E-02	0.35	2.97E-17	*CREM*
Dusp5	n/a	n/a	n/a	n/a	n/a	n/a	0.09	2.00E-01	0.31	1.57E-03	0.62	5.74E-59	*DUSP5*
Epha2	0.03	6.44E-01	-0.08	2.33E-01	-0.08	2.23E-01	-0.06	3.89E-01	-0.07	4.92E-01	0.62	2.99E-59	*EPHA2*
Fosl2	0.01	8.68E-01	0.00	9.51E-01	-0.11	8.88E-02	0.30	5.72E-06	0.37	1.36E-04	0.54	3.54E-42	*FOSL2*
Foxp1	-0.15	1.56E-02	-0.07	3.05E-01	-0.23	5.95E-04	0.26	7.38E-05	0.33	6.36E-04	-0.03	4.43E-01	*FOXP1*
Gdf15	0.31	4.14E-07	0.17	1.74E-02	0.16	1.46E-02	0.09	1.91E-01	0.31	1.54E-03	0.57	2.88E-48	*GDF15*
Ldlr	0.30	9.88E-07	0.35	1.78E-07	0.30	2.90E-06	-0.27	4.65E-05	-0.19	4.98E-02	0.57	4.70E-49	*LDLR*
Mcl1	0.42	5.30E-12	0.27	9.79E-05	0.25	1.62E-04	0.37	1.35E-08	0.35	3.82E-04	0.47	4.86E-31	*MCL1*
Nav2	-0.14	2.50E-02	-0.16	2.16E-02	0.03	7.06E-01	-0.38	9.23E-09	-0.26	7.24E-03	0.31	2.03E-13	*NAV2*
Nr4a3	-0.08	2.00E-01	-0.06	3.61E-01	-0.14	3.38E-02	0.22	1.10E-03	0.37	1.44E-04	0.35	5.81E-17	*NR4A3*
Phlda1	0.16	1.21E-02	-0.07	3.07E-01	0.00	9.53E-01	-0.09	1.83E-01	-0.03	7.83E-01	0.48	8.69E-33	*PHLDA1*
Ppp1r15a	0.00	9.70E-01	0.10	1.56E-01	0.26	7.86E-05	0.32	1.91E-06	0.28	4.25E-03	0.67	2.05E-72	*PPP1R15A*
Serpine1	0.17	7.92E-03	0.42	2.01E-10	0.54	1.40E-18	0.53	4.63E-17	0.37	1.57E-04	0.43	1.54E-26	*SERPINE1*
Slc20a1	0.23	1.73E-04	0.40	3.28E-09	0.49	5.78E-15	0.50	1.44E-15	0.24	1.59E-02	0.41	2.66E-23	*SLC20A1*
Tgfbi	-0.16	1.27E-02	0.02	7.54E-01	0.09	1.55E-01	0.31	3.97E-06	0.35	2.54E-04	0.31	1.22E-13	*TGFBI*
Tnfaip3	n/a	n/a	n/a	n/a	n/a	n/a	0.32	1.81E-06	0.27	6.00E-03	0.46	6.02E-30	*TNFAIP3*
Tnfrsf12a	-0.18	3.11E-03	0.13	5.55E-02	0.19	3.79E-03	0.30	4.52E-06	0.27	6.26E-03	0.62	5.74E-59	*TNFRSF12A*
Trib1	0.20	1.50E-03	0.31	5.93E-06	0.37	1.03E-08	0.11	8.98E-02	0.08	4.13E-01	0.4	3.49E-22	*TRIB1*
Tsc22d1	-0.06	3.35E-01	-0.29	2.90E-05	-0.11	9.25E-02	-0.18	6.88E-03	-0.26	7.57E-03	0.48	8.35E-33	*TSC22D1*
Zfp36	0.20	1.75E-03	0.30	1.40E-05	0.18	5.70E-03	0.05	4.19E-01	0.07	4.85E-01	0.6	3.50E-54	*ZFP36*

Supplemental Figure 1.



Supplemental Figure 2.



Supplemental Figure 3.