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LETTERS

Targeted deletion of the 9p21 non-coding coronary artery disease risk interval in mice

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Sequence polymorphisms in a 58-kilobase (kb) interval on chromosome 9p21 confer a markedly increased risk of coronary artery disease (CAD), the leading cause of death worldwide[1,2]. The variants have a substantial effect on the epidemiology of CAD and other life-threatening vascular conditions because nearly one-quarter of Caucasians are homozygous for risk alleles. However, the risk interval is devoid of protein-coding genes and the mechanism linking the region to CAD risk has remained enigmatic. Here we show that deletion of the orthologous 70-kb non-coding interval on mouse chromosome 4 affects cardiac expression of neighbouring genes, as well as proliferation properties of vascular cells. Chr4$^{\Delta 70kb/\Delta 70kb}$ mice are viable, but show increased mortality both during development and as adults. Cardiac expression of two genes near the non-coding interval, *Cdkn2a* and *Cdkn2b*, is severely reduced in chr4$^{\Delta 70kb/\Delta 70kb}$ mice, indicating that distant-acting gene regulatory functions are located in the non-coding CAD risk interval. Allele-specific expression of *Cdkn2b* transcripts in heterozygous mice showed that the deletion affects expression through a *cis*-acting mechanism. Primary cultures of chr4$^{\Delta 70kb/\Delta 70kb}$ aortic smooth muscle cells exhibited excessive proliferation and diminished senescence, a cellular phenotype consistent with accelerated CAD pathogenesis. Taken together, our results provide direct evidence that the CAD risk interval has a pivotal role in regulation of cardiac *Cdkn2a/b* expression, and suggest that this region affects CAD progression by altering the dynamics of vascular cell proliferation.

Each day, cardiovascular disease causes 2,400 deaths in the United States alone, more than cancer, accidents and diabetes combined[3]. The largest proportion of this mortality is due to CAD, which causes approximately 1 in every 5 deaths in the United States. CAD has a complex aetiology and there is strong evidence that both environmental and genetic factors are major determinants of disease risk[3]. However, identifying the genomic loci associated with increased CAD susceptibility has been a challenge, and most of the known risk loci explain only small proportions of CAD cases (for example, ref. 4). Genome-wide association studies have recently identified common sequence variants on human chromosome 9p21 that confer an increased risk for CAD and myocardial infarction[1,2]. These associations have been confirmed in several other cohorts[5–9] and were extended to other severe arterial diseases[10]. Even in homozygous individuals, the variants increase the relative risk of CAD only moderately by a factor of 1.3 to 2. However, because the risk alleles are very common, they contribute substantially to the epidemiology of CAD. Between 20% and 25% of Caucasians are homozygous for risk alleles, resulting in estimates of 10–31% population attributable risk, depending on cohort and cases considered[1,2].

Despite compelling genetic evidence for association, the mechanism by which 9p21 sequence polymorphisms confer an increased CAD risk is unknown, preventing the development of pharmacological or behavioural intervention strategies. The variants are not associated with established CAD risk factors such as plasma lipoprotein levels, hypertension or diabetes, suggesting that they influence CAD pathogenesis through a previously unappreciated pathway[1,2]. The CAD-associated single nucleotide polymorphisms (SNPs) are located within a 58-kb linkage disequilibrium block on chromosome 9p21.3 that does not contain any known protein-coding genes. Several expressed sequence tags of apparently non-coding transcripts, including a proposed long non-coding RNA, have been mapped to the risk interval, but their functional relevance remains elusive[11,12]. Gene expression studies in human peripheral blood cells and *in vitro* reporter assays have provided support for the notion that gene regulatory elements might be located within the risk interval, but there is conflicting evidence as to whether the CAD risk variants are associated with increased[13] or decreased[14] regulatory activity. More importantly, it is unclear whether altered gene regulation would result in cellular or physiological phenotypes that are relevant to CAD pathogenesis, highlighting the need to study the function of this non-coding interval in a suitable *in vivo* system.

To create a mouse model for investigating the function of the human 58-kb non-coding CAD risk interval, we sought to generate a severe (null) allele for this locus by its targeted removal from the mouse genome. Human–mouse orthology could be unambiguously established as 50% of the base pairs in the human region are alignable to mouse[15] and synteny with flanking genes is preserved (Fig. 1a–c). The mouse interval is 70 kb in size and thus 20% longer than the human orthologous region, partially due to increased repetitive sequence content. Owing to the large size of the interval of interest, its targeted deletion was accomplished through a sequential double-targeting strategy followed by Cre-mediated recombination (Fig. 1d and Supplementary Figs 1–3). Chr4$^{\Delta 70\,kb/\Delta 70\,kb}$ mice are viable and fertile. Although most of the live-born homozygous animals survive to weaning and beyond without obvious morphological or behavioural phenotypes, we did observe reduced embryonic, postnatal and adult survival due to the deletion (for details see Supplementary Material and Supplementary Fig. 4). Clinical analysis of adult animals at 7 months of age showed no significant general aberrations in urine and blood chemistry markers, differential blood cell counts, or histopathological appearance of internal organs including heart, liver, lung, kidney, spleen and gastrointestinal tract. However, in a larger cohort of chr4$^{\Delta 70kb/\Delta 70kb}$ mice on which gross necropsy was performed between 7 and 14 months of age (or at time of premature death), 9 out of 20 (45%) animals were found to have internal neoplasms or tumours of various types (see Supplementary Material),

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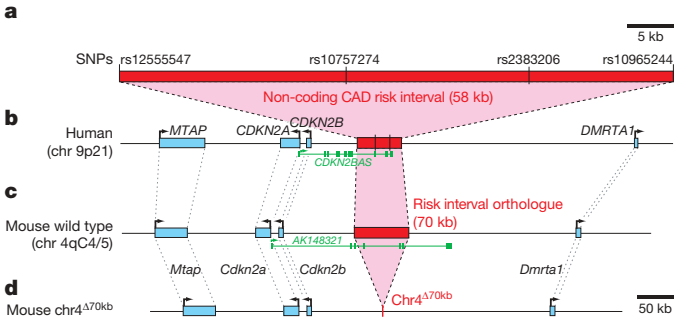

Figure 1 | Deletion of the non-coding region orthologous to the 58-kb CAD risk interval on human chromosome 9p21. a, Non-coding CAD risk interval with SNPs found to be most significantly associated with CAD in genome-wide association studies[1,2] and SNPs defining the boundaries of the linkage disequilibrium block. **b**, Overview of the human locus including neighbouring genes (blue; intron/exon structure not shown). A non-coding RNA of unknown function transcribed from this locus[12] is shown in green (*CDKN2BAS*, also known as *ANRIL*; GenBank accession NR_003529.3). **c**, Orthologous region on mouse chromosome 4. The exon structure of a non-coding transcript of unknown function, *AK148321*, is shown in green. **d**, Chr4$^{\Delta 70kb}$ after successful targeting and deletion of the 70-kb CAD risk interval.

compared to none in a cohort of age-matched wild-type controls ($P = 0.0012$, Fisher's exact test). In addition to this increased tumour incidence, both male and female chr4$^{\Delta 70kb/\Delta 70kb}$ mice that were fed standard mouse chow *ad libitum* gained weight significantly faster than wild-type controls, resulting in a 17% increased body mass by 30 weeks of age (Supplementary Fig. 5).

To investigate the effects of the deletion in more detail, we examined the possibility that the CAD risk interval is required for distant-acting regulation of gene expression. To compare messenger RNA expression levels of surrounding genes between wild-type and chr4$^{\Delta 70kb/\Delta 70kb}$ mice, we isolated mRNA from hearts and other adult mouse tissues and performed reverse transcription followed by quantitative PCR (qRT–PCR). In heart tissue, chr4$^{\Delta 70kb/\Delta 70kb}$ mice had substantially depressed expression levels of the neighbouring *Cdkn2a* and *Cdkn2b* genes, but no significant alteration in expression levels of two other neighbouring genes, *Mtap* and *Dmrta1* (Fig. 2a, b). Cardiac expression of *Cdkn2a* and *Cdkn2b* was more than tenfold decreased compared to wild-type controls. These results indicate that the CAD risk interval

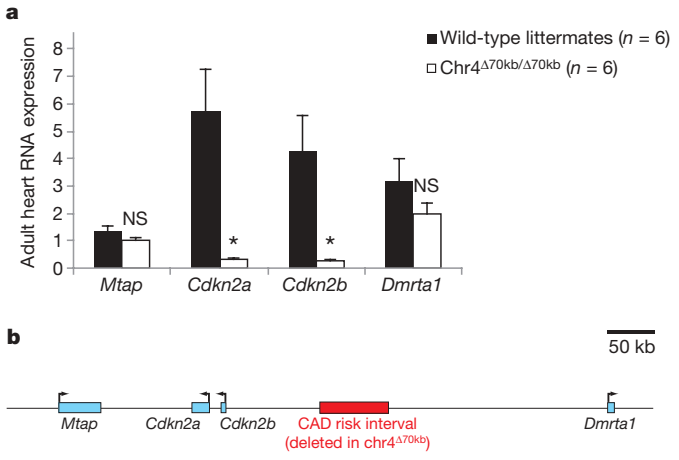

Figure 2 | Deletion of the CAD risk interval affects expression of neighbouring genes *Cdkn2a* and *Cdkn2b*. a, Expression levels of *Cdkn2a* (INK splice variant) and *Cdkn2b* are significantly reduced in heart tissue of chr4$^{\Delta 70kb/\Delta 70kb}$ mice. In contrast, expression of the neighbouring genes *Mtap* and *Dmrta1* is not significantly altered. Expression levels were normalized to the β-actin gene. NS, not significant. **b**, Overview of locus with locations of genes. Error bars are s.e.m.; *$P \leq 0.005$ (*t*-test, one-tailed).

is required for appropriate expression of *Cdkn2a* and *Cdkn2b* in the heart. Owing to the known roles of these genes in several disease-related pathways[16–19], these results support the possibility of a regulation-mediated mechanism by which the chr4$^{\Delta 70kb}$ deletion might affect cellular, physiological and pathological processes.

To test whether the observed regulatory effect on gene expression occurs through a *cis*- or *trans*-acting mechanism, we performed allele-specific expression analysis. We used for this purpose crosses of mice with the deletion linked to the *Cdkn2b* allele of the 129Sv strain (in which the deletion was originally created) and wild-type C57BL/6 strain mice. Strains C57BL/6 and 129Sv are distinguished by several transcribed SNPs in the *Cdkn2b* gene that can reveal quantitative differences in expression from the two alleles. Direct sequencing of the PCR product from tail genomic DNA confirmed that the mice were heterozygous for the expected SNPs and the two alleles were detected at the expected 1:1 ratio (Fig. 3a, b). As a control, in complementary DNA derived from tissues of C57BL/6 (wild type) × 129Sv (wild type), the two alleles were also expressed at indistinguishable levels, confirming the absence of general strain-specific differences in *Cdkn2b* expression levels (Fig. 3b). In contrast,

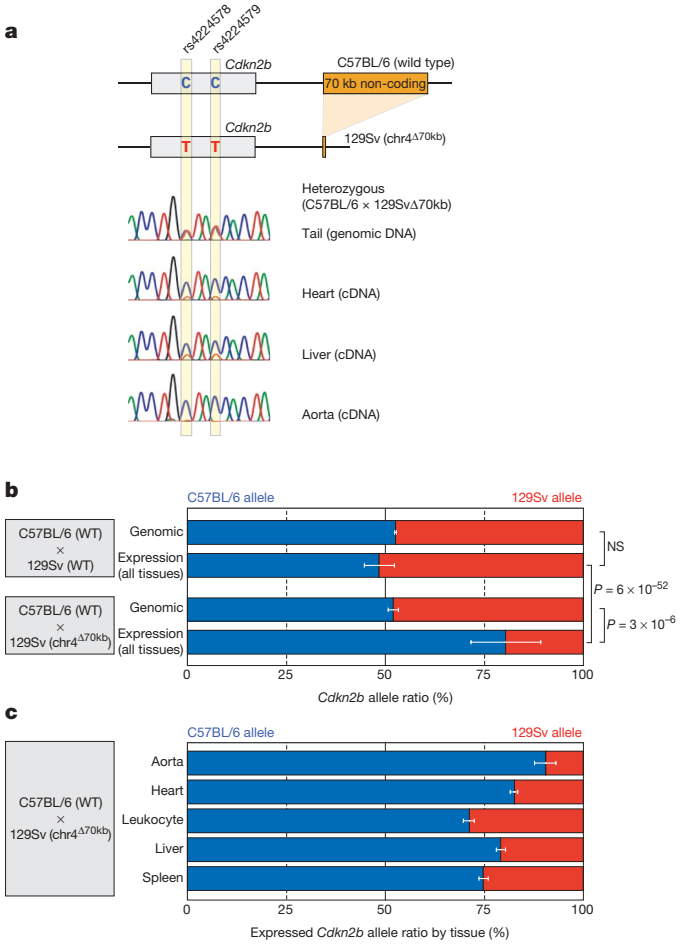

Figure 3 | Deletion of the CAD risk interval affects gene expression through a *cis*-regulatory mechanism. a, Two of seven transcribed SNPs that were used to distinguish expression of the C57BL/6 and 129Sv strain alleles of the *Cdkn2b* gene. The locus is not shown to scale. Representative electropherograms from direct Sanger sequencing of PCR product are shown. **b**, No differences between alleles are observed in genomic DNA or tissue-derived cDNA in chr4$^{+(C57BL/6)/+(129Sv)}$ mice or in genomic DNA of chr4$^{+(C57BL/6)/\Delta 70kb(129Sv)}$ mice. In contrast, in tissues of chr4$^{+(C57BL/6)/\Delta 70kb(129Sv)}$ mice, the C57BL/6 allele is expressed fourfold higher than the 129Sv allele (all tissues combined). WT, wild type. **c**, Allelic expression differences shown by individual tissues. Error bars denote s.d. (**b**) and s.e.m. (**c**). *P* values were determined by *t*-test, two-tailed.

direct sequencing of the RT–PCR product derived from RNA isolated from chr4$^{+(C57BL/6)/\Delta 70kb(129Sv)}$ hearts and other tissues showed that *Cdkn2b* was predominantly expressed from the wild-type C57BL/6 allele and expression from the chr4$^{\Delta 70kb}$ 129Sv allele was strongly diminished (Fig. 3b). Among five organs and cell types examined, the most severe allele-specific downregulation was observed for the heart and aorta (Fig. 3c). These results support the idea that the CAD risk interval controls gene expression in cardiac and other tissues through a distant-acting *cis*-regulatory mechanism.

The proteins encoded by *Cdkn2a*, *Cdkn2b* and other cyclin-dependent kinase inhibitor genes have been implicated in cellular phenotypes including regulation of proliferation and cellular senescence[16,18,19]. Given the severe effect of the chr4$^{\Delta 70kb}$ deletion on cardiac expression of *Cdkn2a* and *Cdkn2b*, we tested whether cell proliferation and senescence are affected in chr4$^{\Delta 70kb/\Delta 70kb}$ mice. To test for an effect on cell proliferation, we measured the proliferation rates of primary cultures of aortic smooth muscle cells (aSMCs) and mouse embryonic fibroblasts (MEFs) during early passages. In both cell types, cells derived from chr4$^{\Delta 70kb/\Delta 70kb}$ mice proliferated excessively compared to wild-type controls, with daily proliferation rates nearly twofold higher in aSMCs and nearly threefold higher in MEFs (Fig. 4a, b). During later passages of these primary cultures, wild-type aSMCs and MEFs became senescent, whereas chr4$^{\Delta 70kb/\Delta 70kb}$-derived cells that had been isolated and cultured under identical conditions continued to proliferate and did not show signs of senescence (Fig. 4c, d). These cellular phenotypes are consistent with known and proposed functions of Cdkn2a, Cdkn2b and other cyclin-dependent kinase inhibitors[16,18–20].

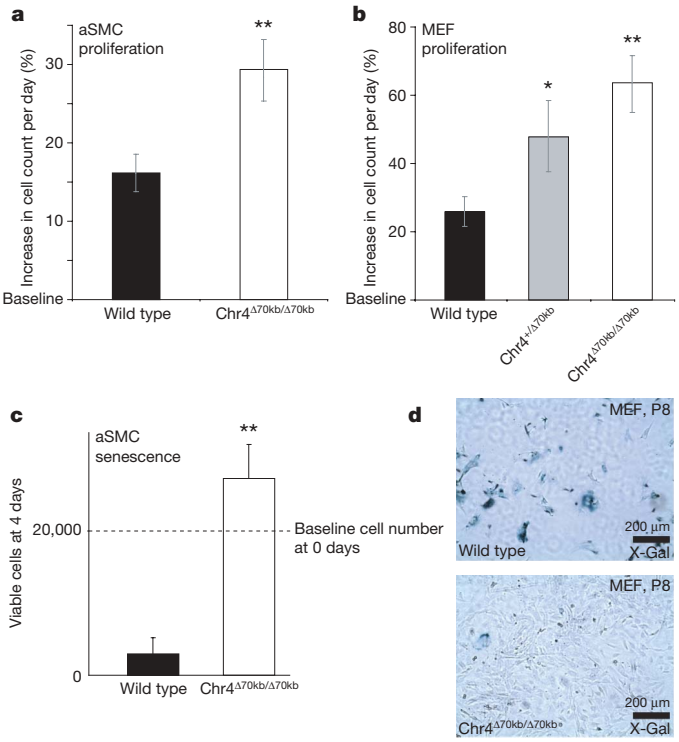

Figure 4 | Deletion of the CAD risk interval disrupts normal dynamics of cellular proliferation and senescence. **a**, Increased proliferation of primary aSMC cultures. **b**, Increased proliferation of primary MEF cultures. **c**, Failure of normal cellular senescence of primary aSMC cultures in late passages. Cells derived from wild-type and chr4$^{\Delta 70kb/\Delta 70kb}$ mice were grown to senescence under identical conditions, seeded at equal densities and cell counts were determined after 4 days. **d**, Chr4$^{\Delta 70kb/\Delta 70kb}$-derived MEFs fail to enter cellular senescence, as evident from absence of senescence staining by X-Gal in comparison to wild-type MEFs[30]. P, passage. Mean daily proliferation rates are shown in **a** and **b**, viable cell count at day 4 is shown in **c**. Error bars indicate s.e.m. $*P < 0.05$, $**P < 0.01$; *t*-test.

The risk interval affects human CAD through a mechanism that seems to be independent of plasma lipid levels and other known risk factors[1,2]. To study possible *in vivo* effects of the chr4$^{\Delta 70kb}$ allele on plasma lipids and the early stages of atherogenesis, we placed 40 chr4$^{\Delta 70kb/\Delta 70kb}$ mice and 40 wild-type controls in an isogenic 129Sv background on a high-fat, high-cholesterol ('western') diet for 20 weeks[21]. As expected, this diet caused substantial alterations in plasma lipid levels; however, no significant differences in this physiological response were observed between wild-type and chr4$^{\Delta 70kb/\Delta 70kb}$ mice (Supplementary Fig. 6; see Supplementary Material for details). Furthermore, we did not observe significant differences in fatty lesion formation (Supplementary Fig. 7). Nevertheless, the high-fat, high-cholesterol nutrition caused substantially increased mortality among chr4$^{\Delta 70kb/\Delta 70kb}$ mice compared to isogenic wild-type controls, indicating an overall increased susceptibility to detrimental effects of this noxious diet (Supplementary Fig. 8). Studies in complementary background strains and further genetic manipulations of the lipid metabolism in chr4$^{\Delta 70kb/\Delta 70kb}$ mice may be required to mimic the full course of human atherosclerosis[21–23]. Such data could also help to distinguish whether diet-induced increase in mortality is due to cardiovascular phenotypes other than the aortic fatty lesions examined here, or to extra-cardiovascular phenotypes that may be present in chr4$^{\Delta 70kb/\Delta 70kb}$ mice. Irrespective of the underlying aetiology, these observations indicate that the CAD risk interval is not required for maintenance of normal plasma lipid levels in mice, consistent with the observation that variation in the human interval influences CAD risk independent of altered lipid levels.

The chromosome 9p21 common haplotype linked to CAD represents an important but particularly puzzling risk interval, and the present study provides new insights about the *in vivo* function of this non-coding region. We have demonstrated that the precise orthologous mouse interval, despite its large distance from any protein-coding genes, is critically required for normal cardiac expression of two cell cycle inhibitor genes, *Cdkn2a* and *Cdkn2b*. These observations raise the question as to what type of underlying molecular mechanism mediates these regulatory effects. Subregions of the 58-kb non-coding risk interval increase transcriptional activity in cell-based *in vitro* transfection assays[13], but the location and function of distinct small enhancer sequences with relevant *in vivo* activities remains to be established (see Supplementary Material). Alternatively, our results are also consistent with models in which the non-coding interval shields the *Cdkn2a/b* genes from the influence of very distal negative regulatory elements, either by spacing effects or owing to presence of insulator elements. In contrast, any mechanism mediated by freely diffusible RNA molecules is not expected to result in the allele-selective regulation observed in chr4$^{\Delta 70kb/\Delta 70kb}$ mice (Fig. 3). Support for an RNA-mediated mechanism[11,12,24] is therefore restricted to models in which the transcriptional activity itself affects local chromatin state or in which RNA molecules remain tethered to the chromosome from which they are transcribed. We have also shown that the aberrations of *in vivo* expression of *Cdkn2a* and *Cdkn2b* coincide with abnormal regulation of vascular cell proliferation and senescence. These phenotypes are reminiscent of mouse models in which the *Cdkn2a/b* genes themselves have been deleted. Specifically, primary cultures of *Cdkn2a*- or *Cdkn2b*-deficient fibroblasts[16,19] and *Cdkn2a*-deficient aSMCs[25] show increased proliferation rates. Hence, a parsimonious explanation for the cellular phenotypes observed in chr4$^{\Delta 70kb/\Delta 70kb}$ mice is that the non-coding CAD risk interval affects vascular cell proliferation and senescence by modulating the expression levels of *Cdkn2a* and *Cdkn2b*. Altered proliferation rates of vascular cells resulting from genetic manipulation of other cyclin-dependent kinase inhibitors have been closely linked to the dynamics of CAD pathogenesis[26], and *Cdkn2a* deficiency causes altered vascular injury responses in a mouse model of CAD[25]. Moreover, sequence polymorphisms in the promoters of at least two other human cyclin-dependent kinase inhibitor genes have been implicated in increased

cardiovascular disease risk[27,28]. Thus, variation in distant-acting regulatory sequences required for cardiovascular expression of *CDKN2A* and *CDKN2B* provides a plausible mechanistic model for the increased CAD risk associated with the 9p21 region independently of lipid levels and other known risk factors.

METHODS SUMMARY

Targeted deletion of the 70-kb non-coding interval was performed by two sequential targeting steps, followed by Cre-mediated recombination (Supplementary Figs 1–3). See Supplementary Table 1 for primer sequences used for vector construction and genotyping. For quantitative real-time RT–PCR, total RNA was extracted from wild-type and knockout mouse tissues using Trizol Reagent (Invitrogen) following the manufacturer's instruction. Total RNA was treated with RNase-free DNase and first-strand cDNA was synthesized by standard methods. RT–PCR was performed by standard methods; primer sequences are provided in Supplementary Table 2. For allele-specific expression profiling, RNA extraction from tissues, DNase treatment and first-strand cDNA synthesis were done as described earlier. Genomic DNA was extracted from the tails of the same mice used for RNA extraction by standard methods. PCR products were generated using gene-specific primers containing M13 primer tags (M13 −40 forward or M13 −20 reverse) and Platinum Taq DNA Polymerase (Invitrogen). Primer sequences are provided in Supplementary Table 2. PCR products were gel purified using QiaGen MinElute Gel Extraction Kit (Qiagen) and sequenced using M13 primers. For proliferation and senescence assays, primary MEFs were isolated from embryonic day (E) 12.5–14.5 chr4$^{\Delta 70kb/\Delta 70kb}$, wild-type and heterozygous littermates. Each embryo was disaggregated in 0.25% trypsin, and fragments were cultured in DMEM with 10% FBS. Aortic smooth muscle cells were isolated from thoracic aorta of 4-week-old chr4$^{\Delta 70kb/\Delta 70kb}$ mice and wild-type littermates as previously described[29]. Cells were counted at each passage and re-cultured in constant concentrations (2×10^4 per well). For senescence assays, cells were grown to senescence (passage 12), trypsinized and plated at 2×10^4 per well on day 0. Cell counts were determined after 4 days. Senescence staining by X-Gal was done as previously described[30].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.V. and L.A.P. wrote the manuscript. All authors contributed to data collection and analysis and provided comments on the manuscript.

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METHODS

Targeted deletion of 70-kb non-coding interval. Two targeting vectors were generated for the deletion. See Supplementary Table 1 for primer sequences used for vector construction and genotyping. One targeting vector, containing a homologous region at the 5′ end of the region of interest (proximal to *Cdkn2a/b*), was in the ploxPneoTK-2 vector. The second targeting vector, containing a homologous region at the 3′ end of the region (distal from *Cdkn2a/b*), was in a ploxPhygTK vector backbone. Both ploxPneoTK-2 and ploxPhygTK were generated in this laboratory.

Homologous arms were generated by PCR from W4/129S6 ES cell genomic DNA. The PCR product of the 5′ homologous arm (with BamHI and EcoRI tags on primers) was cloned into ploxPneoTK-2 BamHI/EcoRI sites next to a *loxP* site, generating vector pCHD5′neo for 5′ targeting. The PCR product of the 3′-homologous arm (with NotI and BglII tags on primers) was cloned into ploxPhygTK NotI/BamHI sites at the 3′ side of the PGK terminator of the PGKtk cassette, generating targeting vector pCHD3′hyg for 3′ targeting.

Targeting vector pCHD5′neo was electroporated into W4/129S6 ES cells (Taconic Farms). Neomycin-resistant clones were picked, screened by PCR and confirmed by Southern hybridization (Supplementary Fig. 1). Successfully 5′-targeted clones were pooled and electroporated with the 3′ targeting vector pCHD3′hyg. Hygromycin-resistant clones were picked, screened by PCR and confirmed by Southern hybridization (Supplementary Fig. 2).

Double-targeted clones (Hyg^r/Neo^r (hygromycin- and neomycin-resistant); note that the two *loxP* sites could be in *cis* or in *trans*) were pooled together, expanded and electroporated with about 20 μg of Cre-recombinase-expressing plasmid pTURBO-Cre. When *loxP* sites were present *in cis*, the *loxP* bracketed sequence included: (1) the region chr4: 89,054,800–89,126,878 (mm9) to be deleted, and (2) the PGKhyg, PGKneo and HSV-tk cassettes, which can be deleted by Cre-recombinase-mediated *loxP* recombination (Supplementary Fig. 3). When *loxP* sites were present in *trans*, a translocation could be generated that resulted in one deleted and one duplicated allele of the region of interest. Embryonic stem (ES) cells that underwent *cis*-recombination and deletion were identified by selecting for neomycin or hygromycin sensitivity and negative selection of HSV-tk for 1-(2-deoxy-2-fluoro-b-D-arabinofuransyl)-5-iodouracil (FIAU) resistance. Cells surviving this selection were screened by PCR for the predicted deletion using a primer outside the deleted region and T7 primer within the vector backbone left on the chromosome after deletion. The predicted deletion was further confirmed by PCR using primers outside the deleted region, negative PCR of *neo* or *hyg* primers, and by Southern blot analysis using a probe outside the deletion.

Assessment of survival. To determine embryonic survival, embryos from timed pregnancies were dissected between E9.5 and E15.5. Embryos whose size and appearance was normal for the respective stage were considered as surviving and genotyped by PCR.

To determine the survival from birth to weaning, live-born pups resulting from wild type × wild type, $chr4^{+/\Delta 70kb} \times chr4^{+/\Delta 70kb}$ and $chr4^{\Delta 70kb/\Delta 70kb} \times chr4^{\Delta 70kb/\Delta 70kb}$ crosses were counted at postnatal day (P) 0 or 1 and the same litters were counted again at weaning at day P20 or P21. Any dead or missing pups were considered as having died between birth and weaning. Pups from heterozygous crosses were counted regardless of their genotype because missing pups could not generally be recovered for genotyping.

For adult survival analysis on standard chow and on high-fat diet, animals that were found dead, met euthanasia criteria, or had an expected remaining survival time of less than 7 days (based on progression of pre-mortal symptoms in animals that had previously died, which included severe weight loss and general inactivity) when euthanized for histological analysis were all considered as being in the 'animals that died' category. Tick marks in survival plots indicate 'censored' animals, which includes animals that were healthy when removed from the study for histological analysis. To exclude the possibility of bias in assessing euthanasia criteria, we also performed survival analysis for animals on the high-fat diet up to 120 days. All animals that died during this initial study phase were found dead and no animals were euthanized or removed for histological analysis before day 120. Consistent with the full study duration, a significantly increased mortality among $chr4^{\Delta 70kb/\Delta 70kb}$ animals on high-fat diet compared to wild-type controls was observed ($P = 0.006$, Kaplan–Meier log-ranked survival test).

High-fat diet, plasma lipid and aortic fatty lesion analysis. Deletion and wild-type mice at 6 weeks of age were fed with high-fat diet[21] containing about 15.8% fat, 1.25% cholesterol and 0.5% sodium cholate (Harlan, TD.88051). Mice were under the diet for 18–22 weeks. The control group was fed with chow diet containing about 6.5% fat (Labdiet, Formulab Diet). For plasma lipid analysis, mice were fasted overnight (approximately 15–17 h). Whole blood was collected into an EDTA Capillary Blood Collection tube (Fisher) by tail bleeding. Blood cells were removed by centrifugation at 4 °C. Clear plasma was transferred to a new tube and frozen at −80 °C until analysis. Plasma lipids were measured by standard assays at the Cincinnati Mouse Metabolic Phenotyping Center. Aortic fatty lesion analysis was done as previously described[31]. In brief, mouse hearts were excised, and the upper third, including the proximal aorta, was embedded in optimal cutting temperature (OCT) compound. Serial 10-μm thick cryosections were cut in the region extending from the appearance to the disappearance of the aortic valves. Sections were mounted on Superfrost (VWR) slides, fixed in 10% neutral buffered formalin vapour, and stained with Oil-Red O in PEG and Gill's III haematoxylin. Lesion areas were determined using a calibrated eyepiece at ×200 magnification.

Real-time RT–PCR. Total RNA was extracted from wild-type and knockout mouse tissues using Trizol Reagent (Invitrogen) following the manufacturer's instruction. Total RNA was treated with Promega RQ1 RNase-Free DNase. First-strand cDNA was synthesized using SuperScript First-Strand Synthesis System for RT–PCR (Invitrogen). Real-time RT–PCR was performed using the Applied Biosystems SYBR Green PCR Master Mix and run on a 7500 Fast Real-Time PCR System (Applied Biosystems). Primer Sequences are provided in Supplementary Table 2.

Allele-specific expression profiling. RNA extraction from tissues, DNase treatment and first-strand cDNA synthesis were done as described earlier. Genomic DNA was extracted from the tails of the same mice used for RNA extraction. Tails were digested overnight at 50 °C in a buffer containing 1% SDS and 100–200 μg ml^{-1} Proteinase K. The lysate was heated at 95–100 °C for 5 min, and diluted for PCR. PCR products were generated using gene-specific primers containing M13 primer tags (M13 −40 forward or M13 −20 reverse) and Platinum Taq DNA Polymerase (Invitrogen). Primer sequences are provided in Supplementary Table 2. PCR products were gel purified using QiaGen MinElute Gel Extraction Kit (Qiagen) and sequenced using M13 primers.

Histopathology, blood and urine analysis. Complete clinical blood chemistry profiles, haematological analysis, urine analysis, general histopathology, and histopathological analysis of neoplasms and tumours were performed at Charles River Research Animal Diagnostic Services.

Proliferation and senescence assays. Primary MEFs were isolated from E12.5–14.5 $chr4^{\Delta 70kb/\Delta 70kb}$, wild-type and heterozygous littermates. Each embryo was disaggregated in 0.25% trypsin, and fragments were cultured in DMEM with 10% FBS. Aortic smooth muscle cells were isolated from thoracic aorta of 4-week-old $chr4^{\Delta 70kb/\Delta 70kb}$ mice and wild-type littermates as previously described[29]. Cell counts were determined at each passage using a haemocytometer and re-cultured in constant concentrations (2×10^4 per well). Figure 4a shows mean daily proliferation rates over seven early passages in aSMC cultures derived from three animals per genotype. Figure 4b shows mean daily proliferation rates over four early passages of MEFs derived from four (heterozygous) to six (wild-type and homozygous) animals per genotype. For senescence assays, cells were grown to senescence, trypsinized and plated at 2×10^4 per well on day 0. Figure 4c shows senescence data from primary aSMC cultures in late passages (passage 12), derived from five animals per genotype. Cells were grown to senescence under identical conditions, seeded at equal densities and cell counts were determined after 4 days. Senescence staining by X-Gal in MEFs (Fig. 4d) was done as previously described[30] after eight passages.

31. Ueda, Y. *et al.* Relationship between expression levels and atherogenesis in scavenger receptor class B, type I transgenics. *J. Biol. Chem.* **275,** 20368–20373 (2000).