Supporting Information

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SI Text

Fig. S1: Schematic Representation of the Strategy Used to Generate Estrogen Receptor-α Activation Function 2 Null Mutants Mice. Mouse estrogen receptor-α (mERα) genomic clones were isolated by screening a 129/Sv ES cell DNA library with an mERα cDNA probe (nucleotides 177–2007) (1). A genomic 16-kb NotI fragment, containing the mERα exons 7 and 8 sequences, was identified with the cDNA probe encoding amino acids 539–552 (nucleotides 1805–1846). A motif essential for the transcriptional activation function 2 (AF2) present in the E region of ERα (exon 8) has been previously characterized as an amphipathic α-helix, and its main features are conserved between transcriptionally active members of the nuclear receptor family (2, 3). We generated the targeting vector XhoI-KpnI encoding the amino acids LLLEMLD motif (nucleotides 1817–1837 and amino acids 543–549) (1). We isolated and subcloned the EcoRI fragment into the Bluescript II plasmid and performed the deletion of the LLLEMLD motif by PCR-based site-directed mutagenesis using primers P1 (5′-ctctacaacatgaaatgcaagaacgttgtgcccctctatgacgcccaccgccttcatgccccagccagtcgcatgggagtg-3′) and P2 (5′-cactcccatgcgactggctggggcatgaaggcggtgggcgtcatagaggggcacaacgttcttgcatttcatgttgtagag-3′; nucleotides 1775–1816 and 1838–1876 and amino acids 529–542 and 550–562) (1). We reconstituted the XhoI-KpnI targeting vector and checked the success of the mutation by sequencing. The TKneo cassette from pHR56^{-} (4) was cloned into the PshAI site. The 8-kb XhoI-KpnI fragment of the targeting vector was electropored into 129/SvPas H1 ES cells (5), and G418 neomycin-resistant clones were expanded (6). ES cells containing a targeted ERαAF2 allele were identified by Southern blot analysis of BamHI- or XhoI-digested ES cell genomic DNA using 5′ (P5′: 5′-atgaattcgagggcaagtggagttgagag-3′ and 5′-taaagcttttccttggagggacagagaga-3′) and 3′ (P3′: 5′-atgaattctggtaaagaatccagtgggat-3′ and 5′-taaagcttgcctactacggagactgaaac-3′) external probes. The expected sizes of DNA fragments are given in the table. Targeted ES cells were injected into C57BL/6 blastocysts and implanted into pseudopregnant mice hosts of the same strain. Chimeric males were obtained that transmitted the mutation through crosses with C57BL/6 females, yielding heterozygous ERαAF-2$^{+/-}$ mice. Then, ERαAF-2$^{+/-}$ mice were bred with homozygous CMV-Cre transgenic mice (7) to delete the selectable marker. Inbreeding of ERαAF-2$^{+/-}$ mice yielded ERαAF-2$^{-/-}$ (also designated as ERαAF2^{0} or ERαAF-2 KO) mice homozygous for the deletion of the transactivating function 2 core motif. Genotyping on tail biopsy DNA was performed by PCR using primers A1 (5′-atgaattcttaataggtttaaaaaatgact-3′) and A2 (5′-gaatccctttgcctgttccc-3′). The size of the A1-A2 fragment from the WT allele is 355 bp, and the size of the fragment from the ERαAF2^{0} allele is 313 bp.

Male and female *ERalphaAF2^{0}* are infertile, and *ERalphaAF2$^{+/-}$* mice were used as parental progenitors. The litter sizes from this cross are normal, and offspring are produced with equal sex frequency.

Fig. S2: ERαAF1 Activity Is Preserved in the ERαAF2^{0} Mutant. HepG2 cells were maintained in DMEM (Sigma-Aldrich) supplemented with 10% FCS (Biowest) and antibiotics (Sigma-Aldrich) at 37 °C in 5% CO_2. Transfections were carried out using jetPEI reagent according to manufacturer's instructions (Polyplus). One day before transfection, cells were plated in 24-well plates at 50% confluence. One hour before transfection, the medium was replaced with phenol red-free DMEM (Sigma-Aldrich) containing 2.5% charcoal-stripped FCS (Biowest). Transfection was carried out with 100 ng C3-LUC reporter gene, 100 ng CMV-βGal internal control, and 50 ng pCR, pCR-ERα, pC-ERαAF1^{0} (pCR hERα46) (8), or pC-ERαAF2^{0} expression vectors. After an overnight incubation, cells were treated for 24 h with 4-hydroxytamoxifen (2 μM) or vehicle (control). Cells were then harvested, and luciferase and β-galactosidase assays were performed as previously described (8).

Fig. S3: ERαAF2 Is Necessary for the Effect of Exogenous E2 on Atherosclerosis Prevention in 18-Wk-Old Mice. Four-week-old ovariectomized *ERαAF2$^{+/+}$LDL-r$^{-/-}$* and *ERαAF2^{0} LDL-r$^{-/-}$* mice were given either placebo or 17β-estradiol (E2; 80 μg/kg per d for 12 wk) and were switched to atherogenic diet from the age of 6–18 wk. For immunohistochemical staining of macrophages, a rat polyclonal anti-CD68 antibody (117–5521; AbCys) was used. Collagen fibers were stained with Sirius red. Quantification (mean ± SEM) was performed for CD68 and Sirius red-positive staining areas at the aortic sinus. A two-way ANOVA factor indicated an interaction between E2 treatment and genotype ($^{***}P < 0.0001$), and the protective action of E2 was present only in *ERαAF2$^{+/+}$ LDL-r$^{-/-}$* mice ($^{***}P < 0.0001$).

Table S1: FGF2 and Osteopontin Are Absolutely Necessary for the Accelerative Effect of E2 on Endothelial Healing but Dispensable for the E2 Atheroprotective Effect. Mice were ovariectomized at 4 wk and implanted or not with s.c. pellets releasing E2 (9, 10) at 0.1 mg for a 60-d release (i.e., 80 μg kg^{-1}d^{-1}; Innovative Research of America). The results are expressed as means ± SEM.

E2 treatment affects uterine weight and fatty streak lesion size in 24-wk-old osteopontin (OPN)$^{-/-}$*apoE*$^{-/-}$ and *FGF2*$^{-/-}$*apoE*$^{-/-}$ mice.

E2 treatment affects uterine weight and endothelialized area on *OPN*$^{-/-}$ and *FGF2*$^{-/-}$ mice. E2 treatment was initiated 2 wk before carotid injury and continued until the sacrifice.

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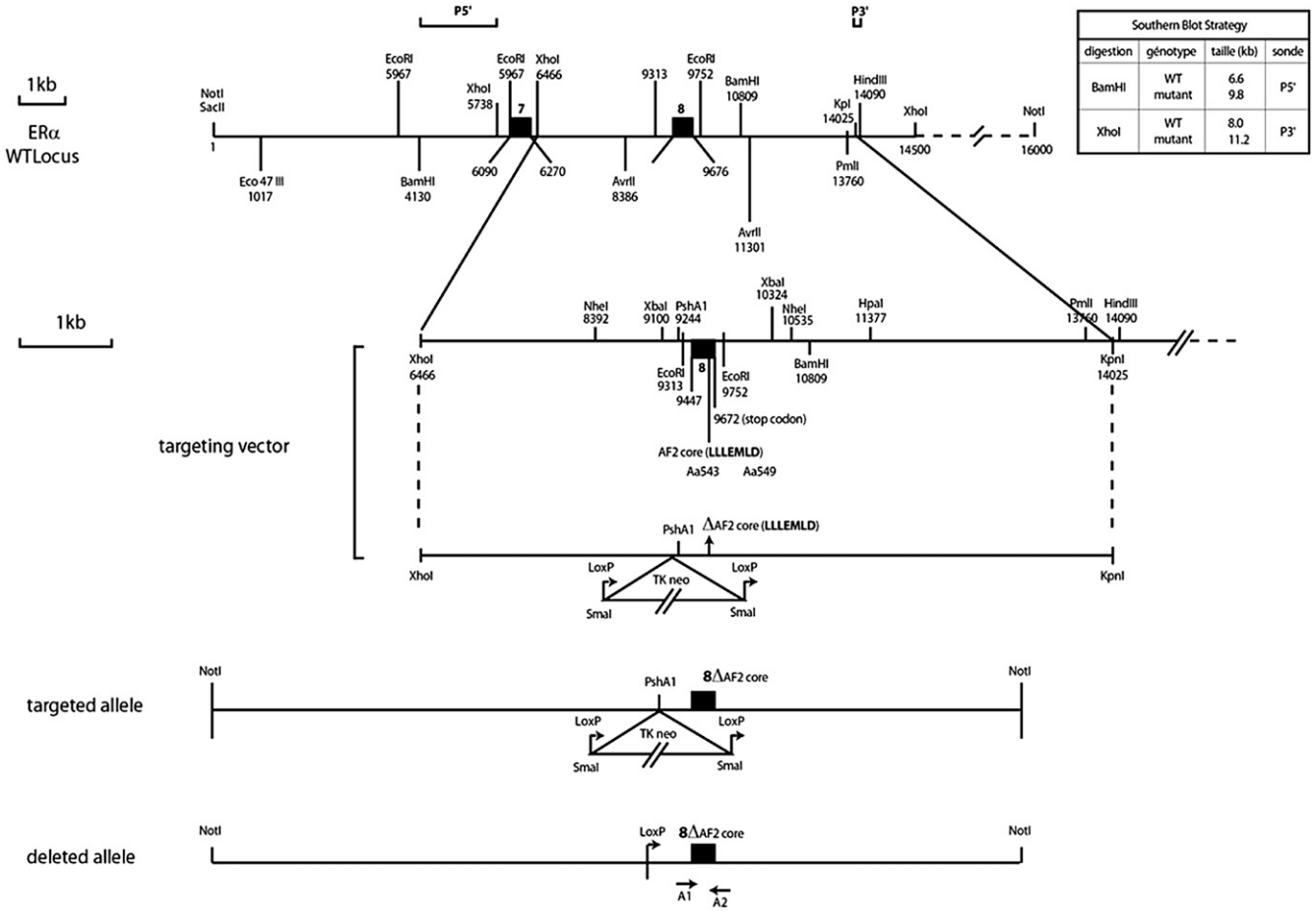

Fig. S1. Schematic representation of the strategy used to generate estrogen receptor-α activation function 2 null mutants mice.

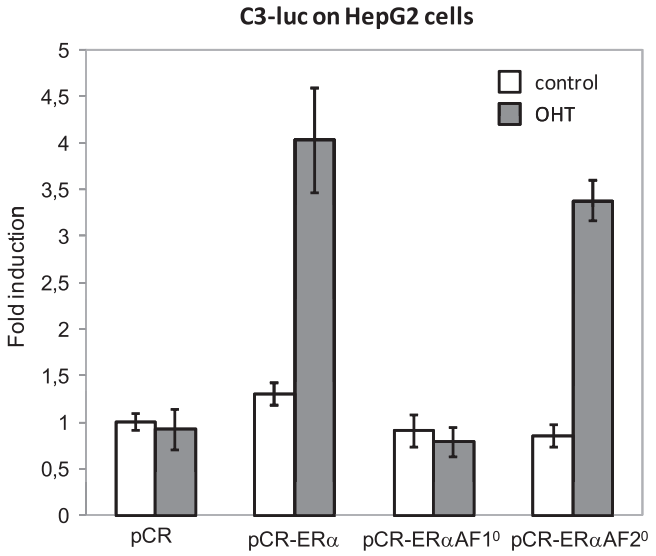

Fig. S2. ERαAF1 activity is preserved in the ERαAF2^0 mutant.

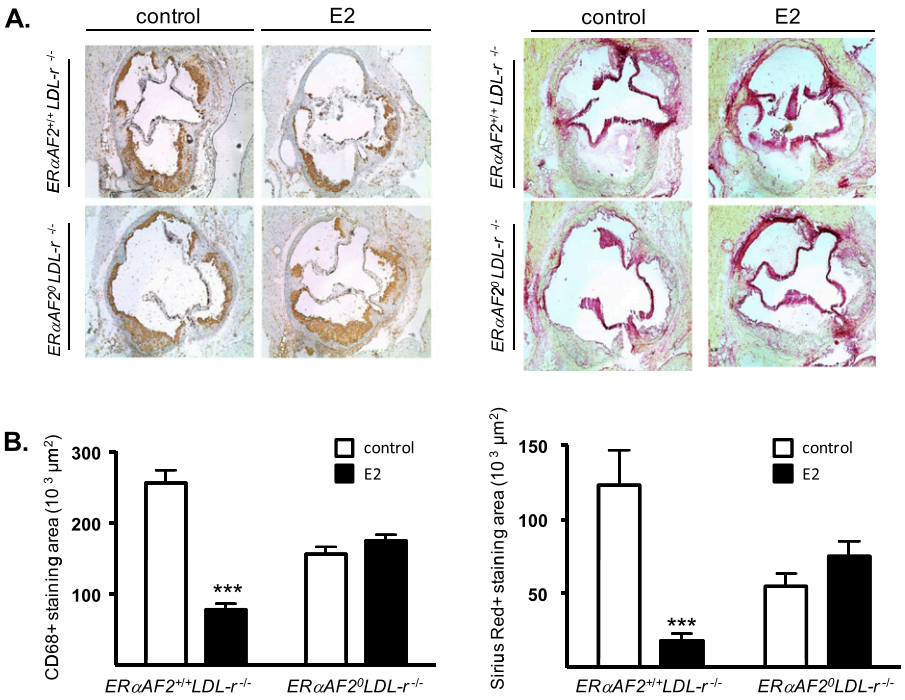

Fig. S3. ERαAF2 is necessary for the effect of exogenous E2 on atherosclerosis prevention in 18-wk-old mice.

Table S1. FGF2 and osteopontin are absolutely necessary for the accelerative effect of E2 on endothelial healing but dispensable for the E2 atheroprotective effect

	Placebo	E2	Placebo	E2
	OPN$^{-/-}$*apoE*$^{-/-}$		*FGF2*$^{-/-}$*apoE*$^{-/-}$	
Uterine weight (mg)	<5	123 ± 17*	<5	182 ± 37*
Percent atherosclerotic lesion vs. placebo	100 ± 12	46.6 ± 8.3*	100 ± 9.3	39.9 ± 9.9*
	OPN$^{-/-}$		*FGF2*$^{-/-}$	
Uterine weight (mg)	<5	148 ± 13*	<5	141 ± 12*
Endothelialized area (percent of carotid area)	25.0 ± 2.9	26.1 ± 1.9	22.8 ± 1.9	21.7 ± 2.0