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Supplementary Materials for

The Akt1-eNOS Axis Illustrates the Specificity of Kinase-Substrate Relationships in Vivo

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Published 4 August 2009, *Sci. Signal.* **2**, ra41 (2009)
DOI: 10.1126/scisignal.2000343

This PDF file includes:

Fig. S1 Generation of S1176D/Akt1$^{-/-}$ and S1176A/Akt1$^{-/-}$ mice.
Fig. S2. NO production in mouse lung endothelial cells.
Fig. S3. eNOS phosphorylation in tissue after hind-limb ischemia.
Fig. S4. PGC-1α–dependent pathways are not involved in ischemic recovery.
Fig. S5. NO-dependent stabilization of the HIF cascade requires PHD activity.

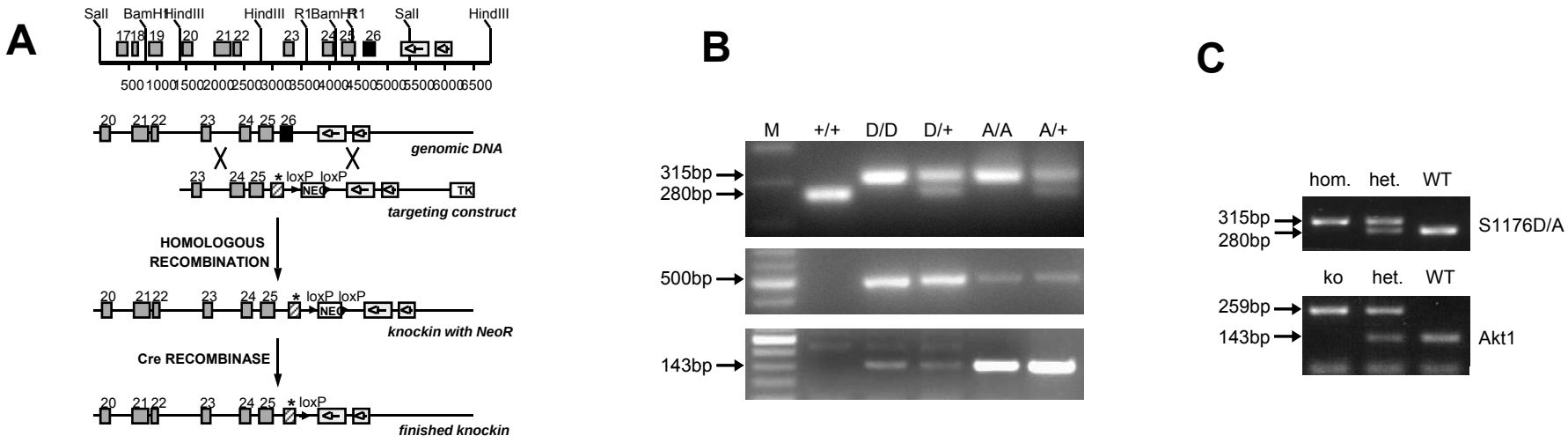

Fig. S1 Generation of S1176D/Akt1$^{-/-}$ and S1176A/Akt1$^{-/-}$ mice.

(A) Cloning strategy for the generation of S1176D and S1176A mice. Restriction map of genomic eNOS clone that includes exons 17 to 26 is shown. The targeting construct includes the mutation (hatched exon 26, *), a neomycin resistance gene (NEO) flanked by loxP sites (black triangles), and a thymidine kinase gene (TK). Homologous recombination (indicated by crossed lines) replaces the region surrounding exon 26 with the mutated exon, as well as the NEO gene flanked by loxP sites. Treatment with Cre recombinase by mating the chimeric mice with EIIa-Cre mice results in excision of the NEO gene, and one residual loxP site. **(B)** Genotyping of S1176D and S1176A mice. Three genotyping protocols are available. The first one (top panel) indicates the presence of the knock-in cassette with a band at 315bp and the presence of the WT gene with a band at 280bp. To distinguish S1176D and S1176A two further protocols were used: One resulting in a band at 500bp when the S1176D insert is present (middle panel) and one resulting in a band at 300bp when the S1176A insert is present (bottom panel). Primer sequences and genotyping protocols are described in Supplementary Materials and Methods. **(C)** Genotyping of S1176D/Akt1$^{-/-}$ and S1176A/Akt1$^{-/-}$ mice. Top panel shows genotyping for the eNOS knock-in insert as in (B). Bottom panel shows genotyping for Akt1$^{-/-}$. Abbreviations are homozygous (hom), heterozygous (het) and knockout (ko).

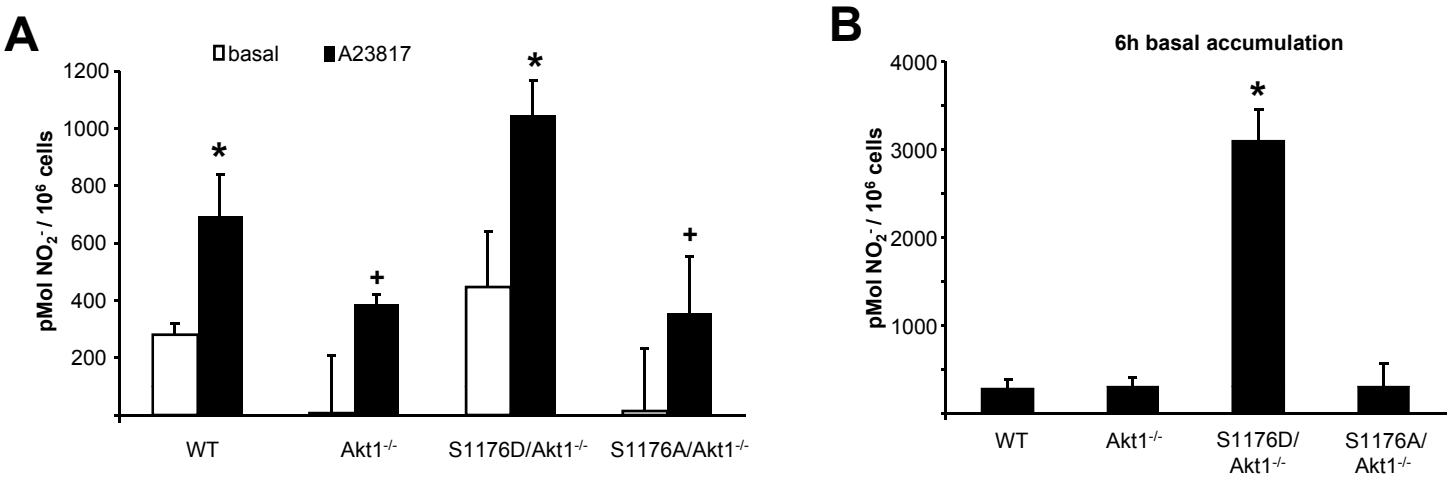

Fig. S2. Nitric oxide production in mouse lung endothelial cells.

Mouse lung endothelial cells (MLEC) were isolated, immortalized, and subcultured as described (*1*). **(A)** Nitrite amount in supernatant of confluent MLEC treated with (black bars) or without (white bars) the calcium ionophore A23187 (1 μM) for 30 min were analyzed using a Sievers NO Chemiluminescence Analyzer. **(B)** Nitrite accumulation over 6h in supernatants of confluent MLEC under basal conditions was analyzed. (All panels: n=4; error bars indicate S.E.M; *$p<0.05$ compared to basal and + $p<0.05$ compared to WT using 2 way ANOVA.

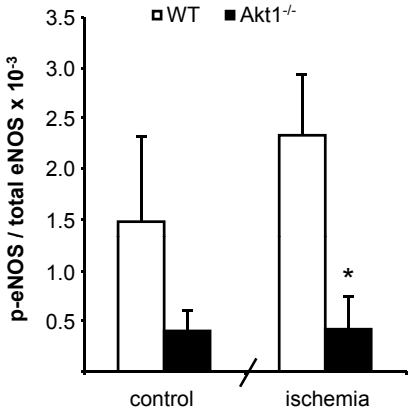

Fig. S3. eNOS phosphorylation in tissue following hindlimb ischemia.
WT and Akt1$^{-/-}$ mice (n=3 mice per group) were subjected to hindlimb ischemia for 12hrs. After ischemia, mice were perfused with cold PBS including phosphatase inhibitors and gastrocnemic muscles of the contralateral and ischemic limbs were harvested and snap frozen. Homogenized lysates were probed for p1176eNOS, total eNOS by Western blot and blots were analyzed by densitometry. *$p \leq 0.05$ compared to ischemic WT mice using an unpaired Student's t-test.

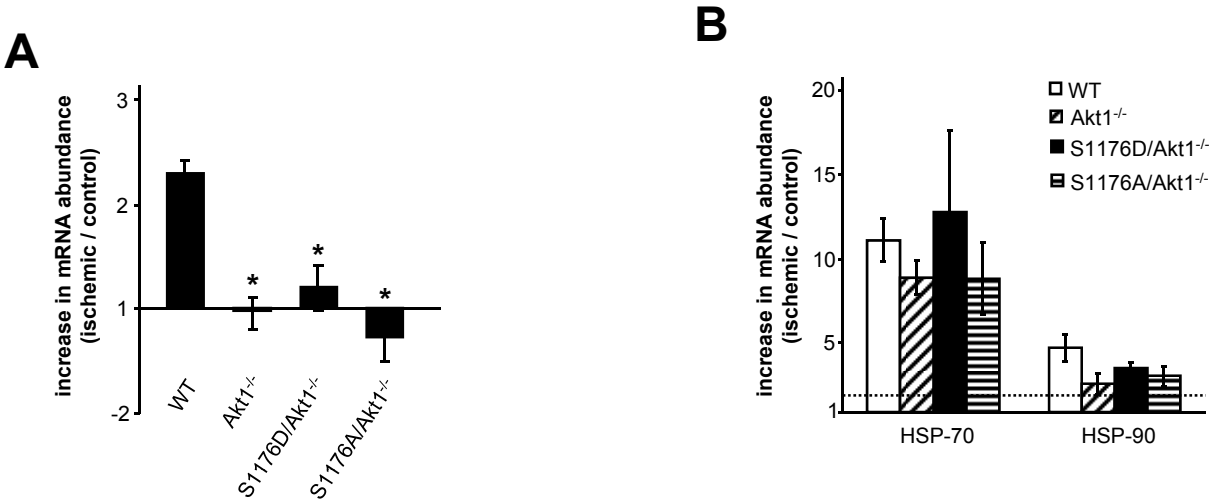

Fig. S 4. PGC-1α-dependent pathways are not involved in ischemic recovery.
(A) Expression of PGC-1α mRNA following hindlimb ischemia was quantified by qPCR **(B)** Increases in HSP-70 and HSP-90 mRNA after hindlimb ischemia were quantified by qPCR. (In all panels: N=3 mice in duplicates; error bars indicate s.e.m.; $p<0.05$ against WT). $P<0.05$ compared to WT mice.

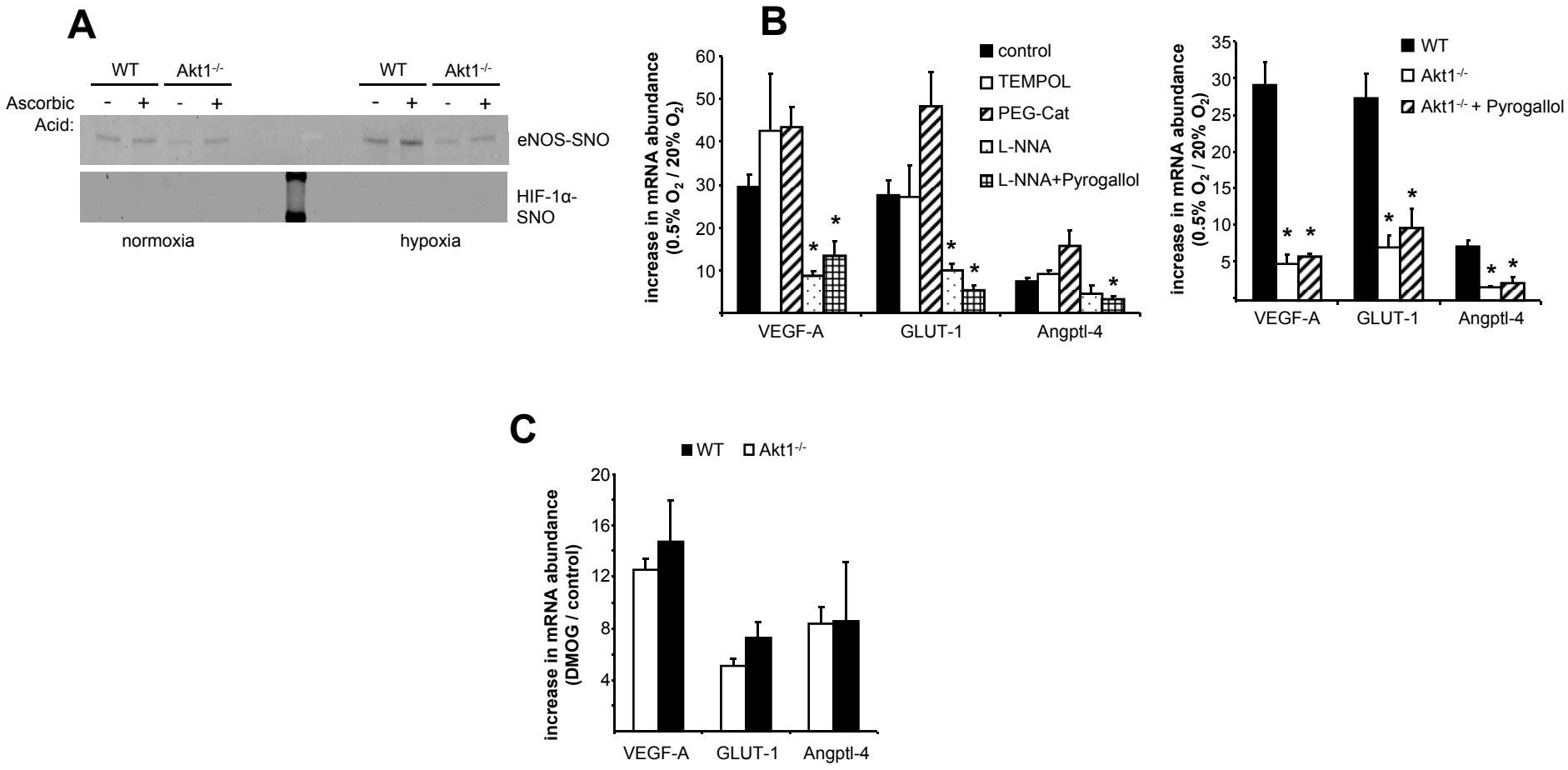

Fig. S 5. NO-dependent stabilization of the HIF cascade requires PHD activity.

(A) Nitrosylation of HIF-1α and eNOS under normoxia or hypoxia (3h, 0.5% O_2) in WT and Akt1$^{-/-}$ MLEC were analyzed by biotin switch experiments. **(B)** HIF-1α-responsive genes in WT (left panel) and Akt1$^{-/-}$ (right panel) MLEC in response to hypoxia (12h, 0.5% O_2) in the presence of ROS scavengers TEMPOL (5mM) or PEG-CAT(300 U/ml) and superoxide donor pyrogallol (250μM) was quantified by qPCR. **(C)** Expression of HIF-1α responsive genes in WT and Akt$^{-/-}$ MLEC in the presence of DMOG (1mM, 6h) was quantified by qPCR.
(In panels B+C: n=3 in duplicates; Error bars indicate S.E.M.; panel B left: *p<0.05 against control; panel B right: *p<0.05 against WT using two way ANOVA.