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Aging Cell
Primary research paper
(ACE-12-0084R1)
August 16, 2012

Crosstalk between arginase-II and S6K1 in vascular endothelial inflammation and aging

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Supporting Information listing

Supplementary figure legends

Fig. S1: Senescent endothelial cells exhibit enhanced Arg-II expression and activity. (A) SA-β-gal staining in young (Y) and senescent (S) HUVECs. **(B)** Immunoblotting analysis of senescence markers p53-S15, p53, and $p21^{Cip1}$ levels, and endothelial inflammation markers VCAM1 and ICAM1 expression. Tubulin served as protein loading control. **(C)** Immunoblotting analysis of Arg-II protein levels and arginase activity. ***p<0.005 vs Y group. Scale bar = 0.2 mm.

Fig. S2: Overexpression of Arg-II gene in young cells induces eNOS uncoupling, endothelial senescence, and inflammation. Young endothelial cells were transduced with empty rAd/CMV vector as control (con) or rAd/CMV-Arg-II to overexpress Arg-II. **(A)** Immunoblotting analysis of Arg-II overexpression. **(B)** DHE staining for detection of $O_2^{\cdot -}$ and DAF-2DA staining for detection of NO, and effect of the eNOS inhibitor L-NAME (1 mmol/L, 1 hour). Bar graphs show quantifications of DHE and DAF-2DA signals. **(C)** SA-β-gal staining. Bar graphs show quantifications of percentage of SA-β-gal positive cells. **(D)** Immunoblotting analysis of senescence markers p53-S15, p53, and $p21^{Cip1}$ levels, and endothelial inflammation markers VCAM1 and ICAM1 expression. Tubulin serves as loading control. Bar graphs show quantifications of the markers. **p<0.01, ***p<0.005 vs control; †p<0.05 vs Arg-II. Scale bar = 0.2 mm.

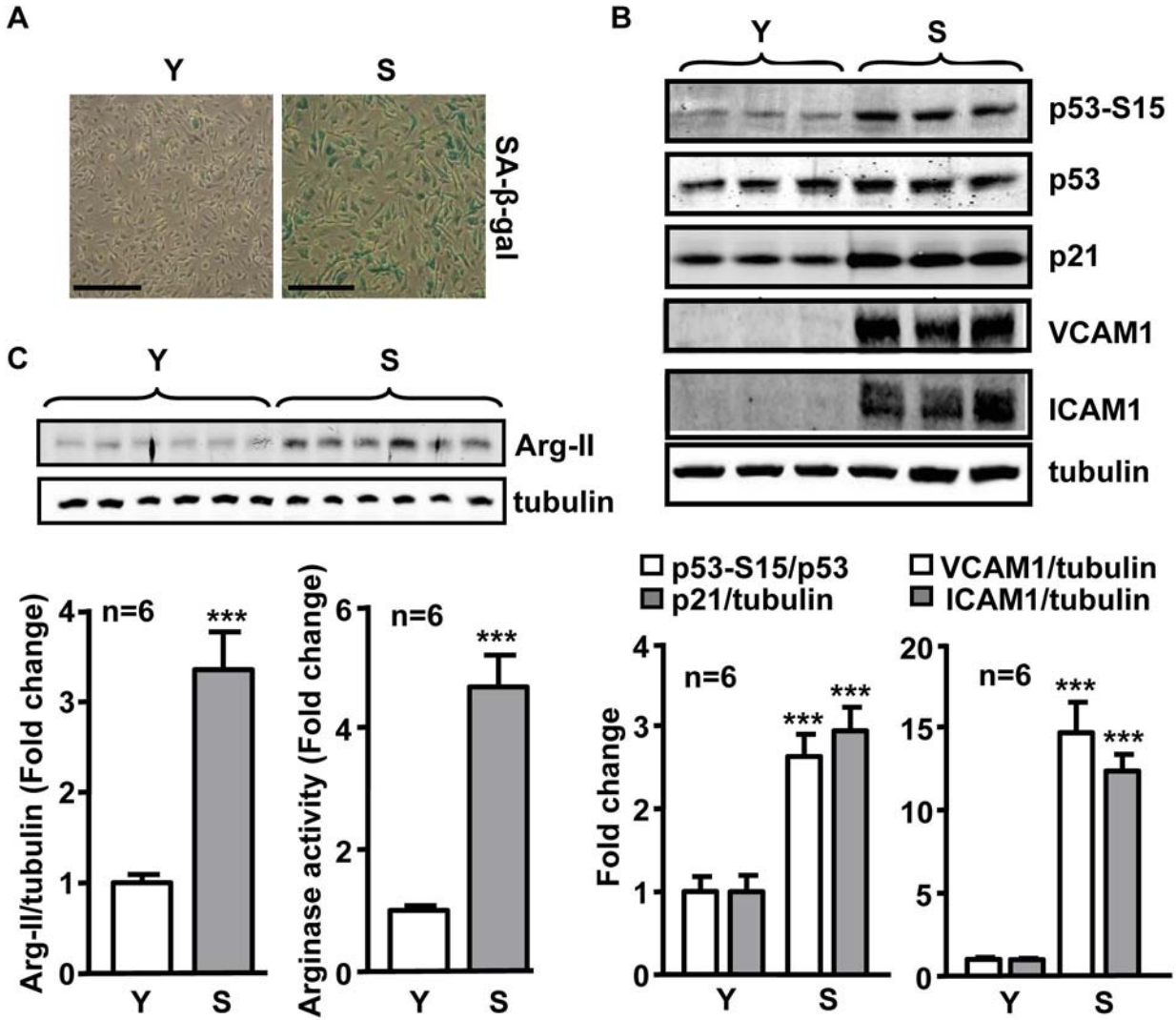
Fig. S3: Enzymatic activity dependence of the Arg-II-promoted endothelial aging in young cells. The young endothelial cells were transduced with empty rAd/CMV vector as control (con), rAd/CMV-Arg-II to overexpress wild type Arg-II or with rAd/CMV-Arg-II (H160F) to overexpress the inactive Arg-II mutant. **(A)** Immunoblotting analysis of Arg-II overexpression. **(B)** DHE staining for detection of $O_2^{\cdot -}$ and DAF-2DA staining for detection of NO. Bar graphs show quantifications of DHE and DAF-2DA signals. **(C)** SA-β-gal staining. Bar graphs show quantifications of percentage of SA-β-gal positive cells. **(D)** Immunoblotting analysis of p53-S15, p53, $p21^{Cip1}$ levels, VCAM1, and ICAM1 expression.

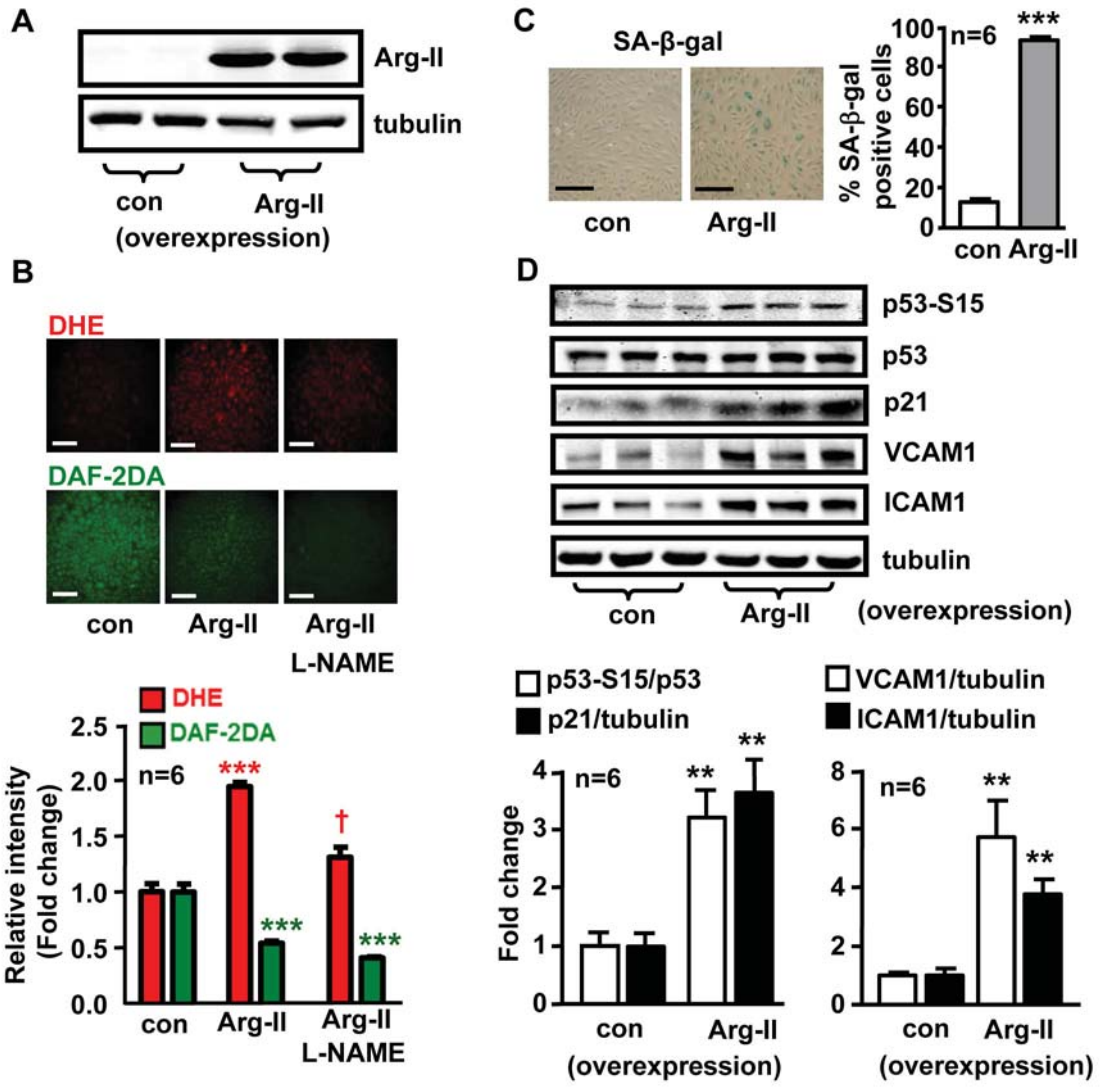
Tubulin serves as loading control. Bar graphs show quantifications of the markers. *p<0.05, **p<0.01, ***p<0.005 vs control; ††<0.01, †††p<0.005 vs Arg-II. Scale bar = 0.2 mm.

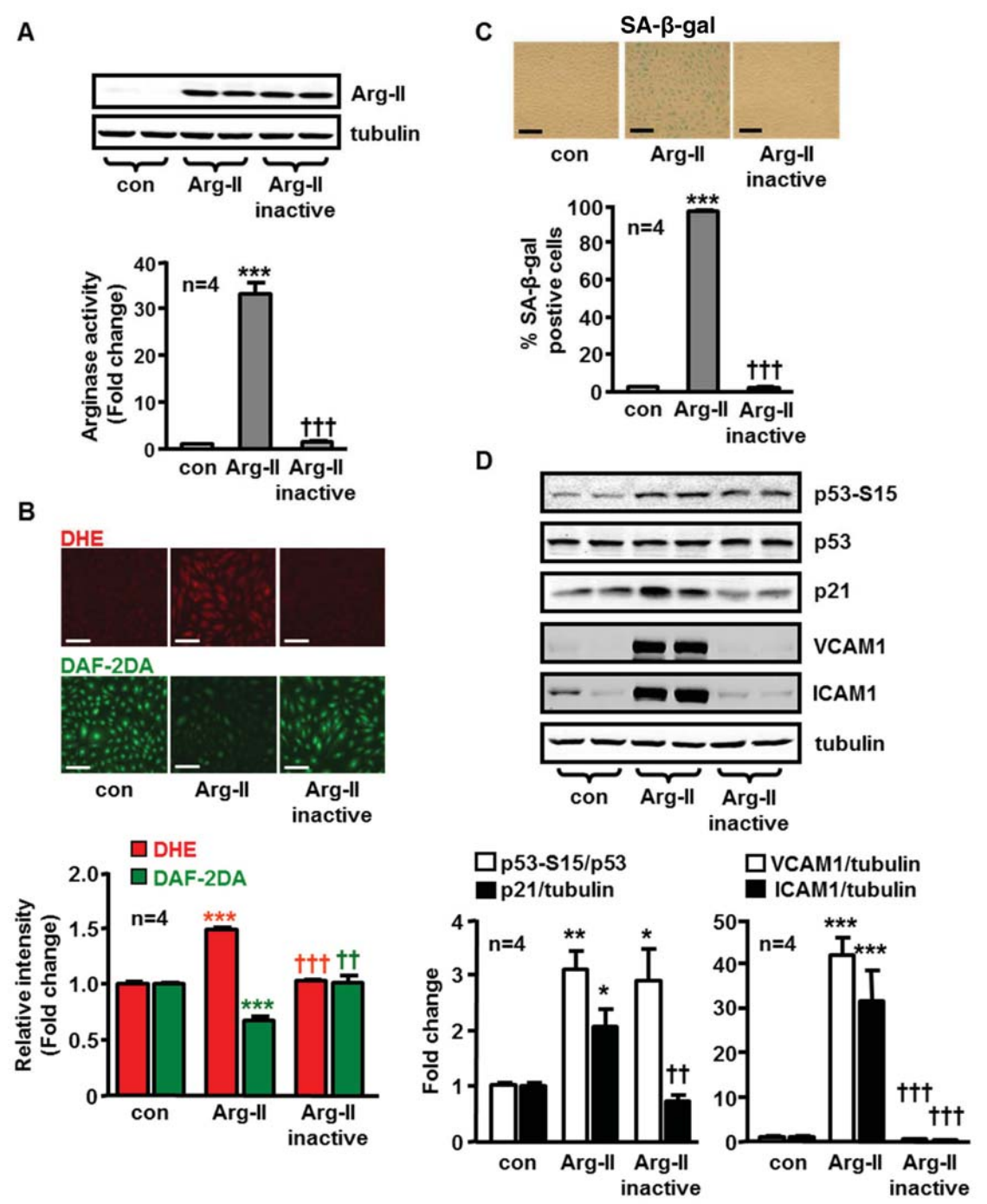
Fig. S4: Silencing Arg-II prevents S6K1-induced Arg-II expression and arginase activity. Young HUVECs were first transduced either with rAd/U6-LacZ^{shRNA} as control or rAd/U6-Arg-II^{shRNA}. Four days post transduction with rAd/U6-shRNA, cells were then transduced either with rAd/CMV as control (con) or rAd/CMV-HA-S6K1ca (a constitutively active S6K1 mutant). Experiments were performed on day two post 2nd transduction. Cells were serum starved with 0.2% FCS-RPMI 12 hours prior to experiments. Blots above reveal the immunoblotting analysis of HA-tagged S6K1ca and Arg-II with anti-HA and anti-Arg-II antibody, respectively. Bar graphs below show quantifications of Arg-II/tubulin protein level, arginase activity, and Arg-II/GAPDH mRNA levels as analysed by qRT-PCR. ***p<0.005 vs control; †††p<0.005 vs S6K1ca.

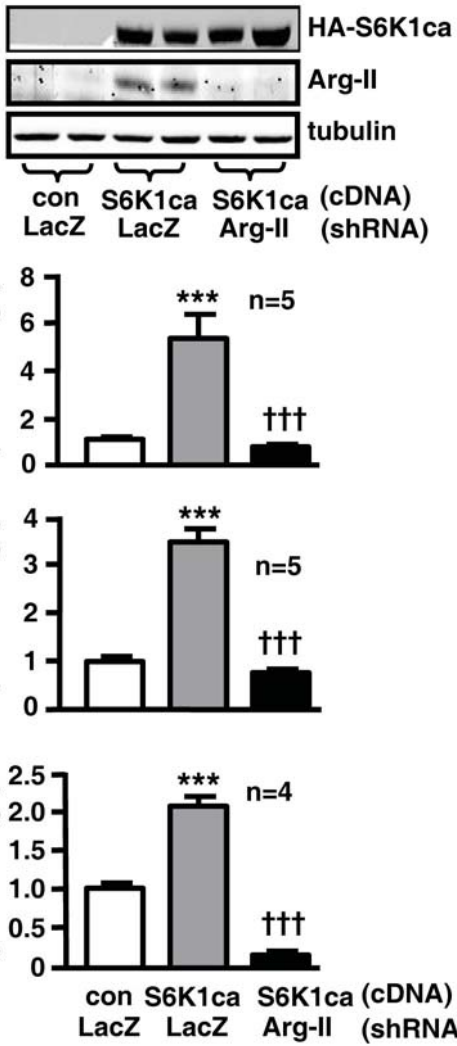
Figure S5: Deficiency in Arg-II gene in mice (Arg-II$^{-/-}$) improves eNOS function in aging. (**A**) Immunofluorescence confocal microscopy showing *en face* detection of aortic endothelial $O_2^{\cdot-}$ (DHE staining) and NO production (DAF-2DA staining) in young (2–3 months) and old (23–24 months) WT and Arg-II$^{-/-}$ mice followed by counterstaining with DAPI for endothelial nuclei. (**B**) Bar graphs show quantifications of DHE and DAF-2DA signals. **p<0.01, ***p<0.005 vs young WT mice; †p<0.05, †††p<0.005 vs old WT mice. Scale bar = 100 μm.

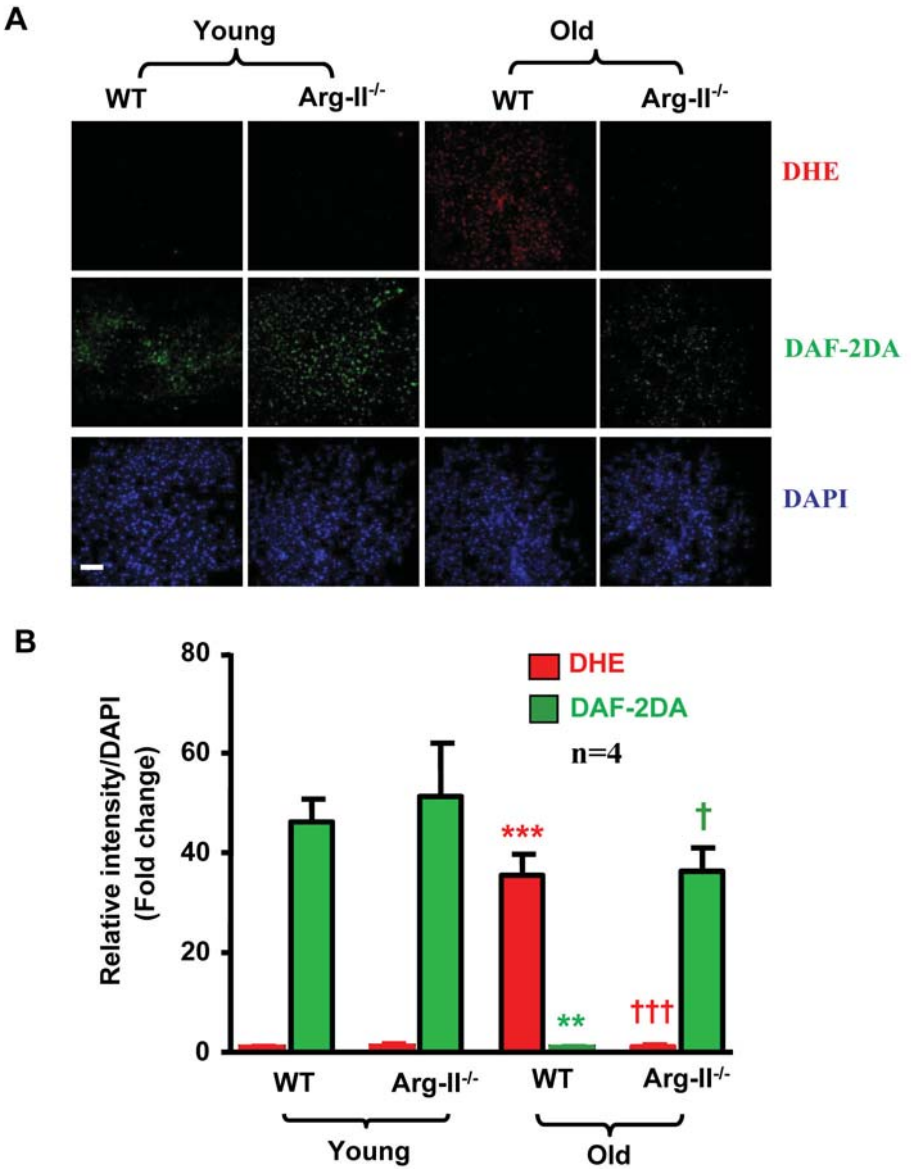
Fig. S6: S6K1 activity in aging is positively regulated by Arg-II. Immunoblotting analysis of S6-S235/S236 (p-S6) and total S6 in **(A)** aortas of young (2–3 months) and old (23–24 months) WT and Arg-II$^{-/-}$ mice and **(B)** senescent endothelial cells transduced with LacZ^{shRNA} as control or Arg-II^{shRNA}. Bar graphs below show quantifications of the above experiments. **p<0.01, ***p<0.005 vs young WT mice or LacZ^{shRNA} group; †††p<0.005 vs old WT mice. **(C)** Scheme illustrating mutual positive crosstalk between S6K1 and Arg-II in endothelial aging.

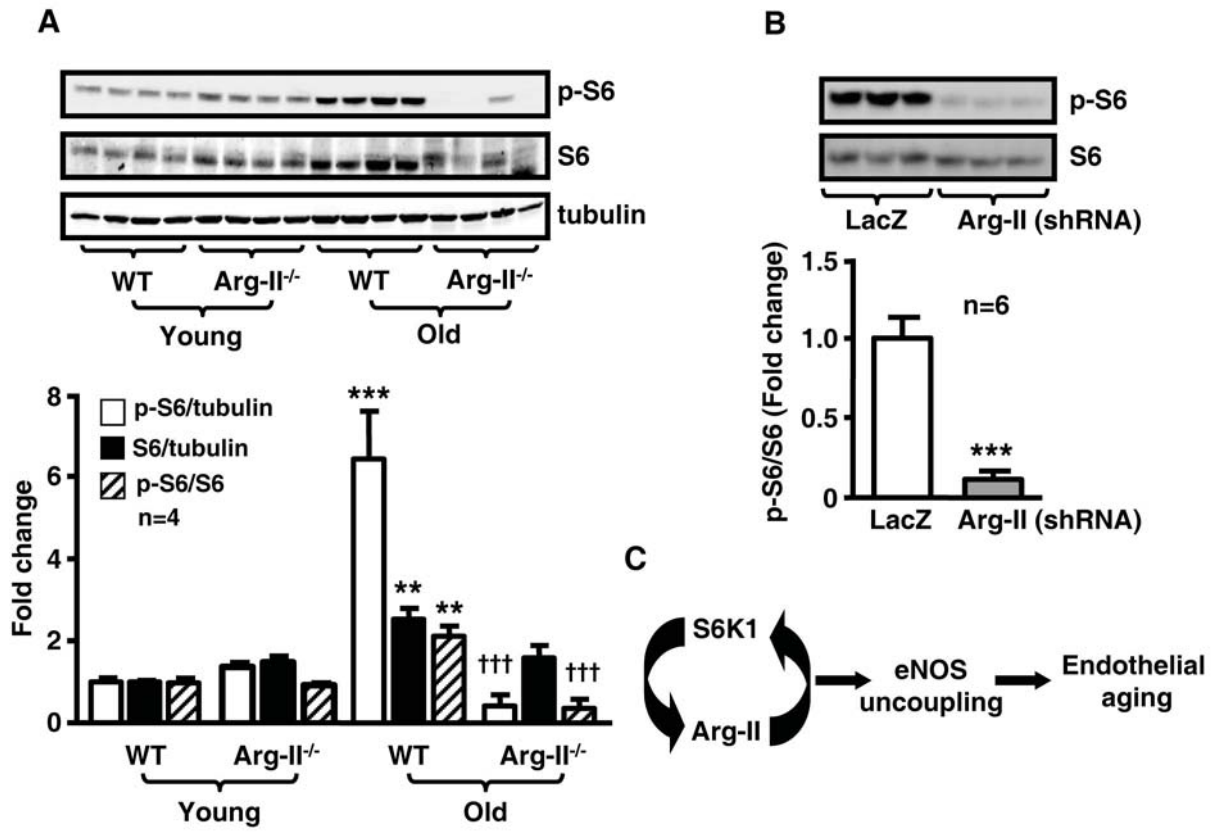

A
Y
S
SA-β-gal
B
Y
S
p53-S15
p53
p21
VCAM1
ICAM1
tubulin
C
Y
S
Arg-II
tubulin
Arg-II/tubulin (Fold change)
n=6
***
Y
S
Arginase activity (Fold change)
n=6
***
Y
S
p53-S15/p53
p21/tubulin
VCAM1/tubulin
ICAM1/tubulin
Fold change
n=6
***
***
Y
S
n=6
***
***
Y
S


A
Arg-II
tubulin
con
(overexpression)
Arg-II
B
DHE
DAF-2DA
con
Arg-II
Arg-II
L-NAME
DHE
DAF-2DA
n=6
***
***
†
***
Relative intensity
(Fold change)
2.5
2.0
1.5
1.0
0.5
0.0
con
Arg-II
Arg-II
L-NAME
C
SA-β-gal
con
Arg-II
n=6
***
% SA-β-gal
positive cells
100
80
60
40
20
0
con
Arg-II
D
p53-S15
p53
p21
VCAM1
ICAM1
tubulin
con
Arg-II
(overexpression)
p53-S15/p53
p21/tubulin
n=6
**
**
Fold change
4
3
2
1
0
con
Arg-II
(overexpression)
VCAM1/tubulin
ICAM1/tubulin
n=6
**
**
8
6
4
2
0
con
Arg-II
(overexpression)


A
Arg-II
tubulin
con
Arg-II
Arg-II inactive
Arginase activity (Fold change)
n=4
***
†††
B
DHE
DAF-2DA
con
Arg-II
Arg-II inactive
DHE
DAF-2DA
Relative intensity (Fold change)
n=4
***
***
†††
††
C
SA-β-gal
% SA-β-gal postive cells
n=4
***
†††
D
p53-S15
p53
p21
VCAM1
ICAM1
tubulin
p53-S15/p53
p21/tubulin
VCAM1/tubulin
ICAM1/tubulin
Fold change
n=4
**
*
*
*
††
***
***
†††
†††


HA-S6K1ca
Arg-II
tubulin
con LacZ
S6K1ca LacZ
S6K1ca Arg-II
(cDNA)
(shRNA)
Arg-II/tubulin (Fold change)
***
n=5
†††
Arginase activity (Fold change)
***
n=5
†††
Arg-II/GAPDH mRNA (Fold change)
***
n=4
†††
con LacZ
S6K1ca LacZ
S6K1ca Arg-II
(cDNA)
(shRNA)


A
Young
Old
WT
Arg-II-/-
WT
Arg-II-/-
DHE
DAF-2DA
DAPI
B
Relative intensity/DAPI
(Fold change)
80
60
40
20
0
DHE
DAF-2DA
n=4
***
**
†††
†
WT
Arg-II-/-
WT
Arg-II-/-
Young
Old


A
p-S6
S6
tubulin
WT
Arg-II-/-
WT
Arg-II-/-
Young
Old
p-S6/tubulin
S6/tubulin
p-S6/S6
n=4
Fold change
8
6
4
2
0
***
**
**
†††
†††
WT
Arg-II-/-
WT
Arg-II-/-
Young
Old
B
p-S6
S6
LacZ
Arg-II (shRNA)
p-S6/S6 (Fold change)
1.5
1.0
0.5
0
n=6
***
LacZ
Arg-II (shRNA)
C
S6K1
Arg-II
eNOS uncoupling
Endothelial aging