Supplemental Material for

ATAXIA TELANGIECTASIA MUTATED (ATM)-MEDIATED DNA DAMAGE RESPONSE IN OXIDATIVE STRESS-INDUCED VASCULAR ENDOTHELIAL CELL SENESCENCE

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Running head: ATM mediates endothelial cell senescence

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Supplemental Experimental Procedures

siRNA

Small interference RNA (siRNA) constructs were obtained as Silencer Select Validated siRNA from Applied Biosystems/Ambion (CA, USA); Akt siRNA (sense: 5'-GCGUGACCAUGAACGAGUUtt-3'; antisense: 5'-AACUCGUUCAUGGUCACGCgg-3'); p53 siRNA (sense: 5'-GUAAUCUACUGGGACGGAAtt-3'; antisense: 5'-UUCCGUCCCAGUAGAUUACca-3'); p21CIP1 siRNA (sense: 5'-CAAGGAGUCAGACAUUUUAtt -3'; antisense: 5'-UAAAAUGUCUGACUCCUUGtt-3'); and negative control #1 siRNA.

Antibodies

Normal mouse and rabbit IgG antibodies were purchased from Santa Cruz Biotechnology (CA, USA).

Supplemental Figure Legends

Supplemental Fig. 1. Four higher magnification photographs of representative cells shown in Fig. 1D, 1E, 1F and 1G to better show nuclear foci. *A*, *B*, *C & D*. Immunofluorescence analysis for (A) 53BP1 (green), (B) phosphorylated ATM (S1981) (red), (C) total ATM (red) and (D) p21 (red) in etoposide- or H_2O_2-treated HUVECs. Hoechst 33258 was used as nuclear stain (blue). Scale bar, 5 μm.

Supplemental Fig. 2. Higher magnification photographs of representative cells in Fig. 3A to better show nuclear foci (cells were treated with 100 μM H_2O_2 for 30 min in the absence or presence of NAC or KU-55933). Immunofluorescence analysis of the effects of antioxidant (NAC) and ATM inhibitor (KU55933) on phosphorylated ATM (red). Hoechst 33258 was used as nuclear stain (blue). Scale bar, 5 μm.

Supplemental Fig. 3. Immunofluorescence analysis for 53BP1 and γ-H2AX-S139 in H_2O_2-treated HUVECs. Hoechst 33258 was used as nuclear stain (blue). Expression of 53BP1 and γ-H2AX-S139 labeled by green fluorescence in HUVECs was significantly increased with nuclear

foci formation after H_2O_2 treatment. Scale bar, 5 μm.

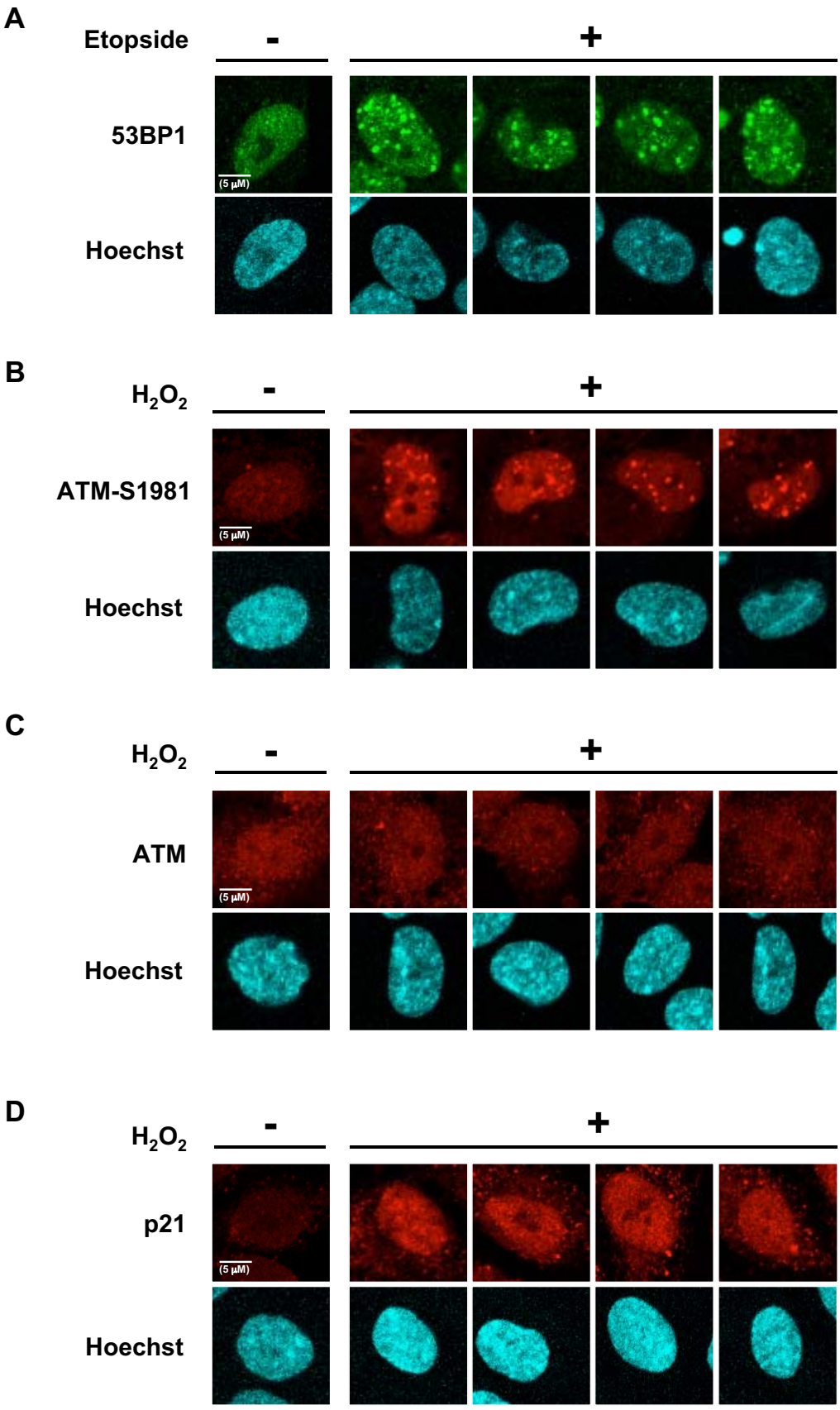
Supplemental Fig. 4. Effects of silencing (RNAi) of signaling molecules in the Akt/p53/p21 pathway. *A*. Western blot analysis of the effects of siRNA against Akt on expression of total Akt, p53 phosphorylation (S15) and p21 induction. *B*. Western blot analysis of the effects of siRNA against p53 on expression of total p53 and p21 induction. *C*. Western blot analysis of the effects of siRNA against p21 on expression of p21 induction. $1.5x10^5$ cells/well were transfected with siRNA against Akt, p53 and p21 for 72h followed by incubation with 100 μM H_2O_2 for 30 min in (A, B) or 3h in (C). GAPDH was used as loading control. Values are mean ± s.e.m (n=3). **P*<0.05 versus cells transfected with the same concentration of negative control siRNA. Representative blots are shown in the left panel while corresponding quantitation is shown in the right panels. Reagent only: cells transfected with Lipofectamine 2000 alone.

Supplemental Fig. 5. Effects of abrogation of gene expression by siRNA against Akt, p53 or p21 in oxidative stress-induced endothelial senescence. *A*. Staining of SA-β-gal activity in cells silenced for Akt, p53 or p21. *B*. Quantitation of percentage of SA-β-gal-positive cells in cells silenced for Akt, p53 or p21. Values are mean ± s.e.m (n=3). **P*<0.05 versus cells transfected with the same concentration of negative control siRNA (lanes 14, 15 or lane 16, respectively) (n=3 each). Original magnification, ×100. Scale bar, 200 μm. Reagent only: cells transfected with Lipofectamine 2000 alone. Silencing of Akt, p53 or p21 by siRNA suppressed increase in SA-β-gal-positive cells induced by H_2O_2.

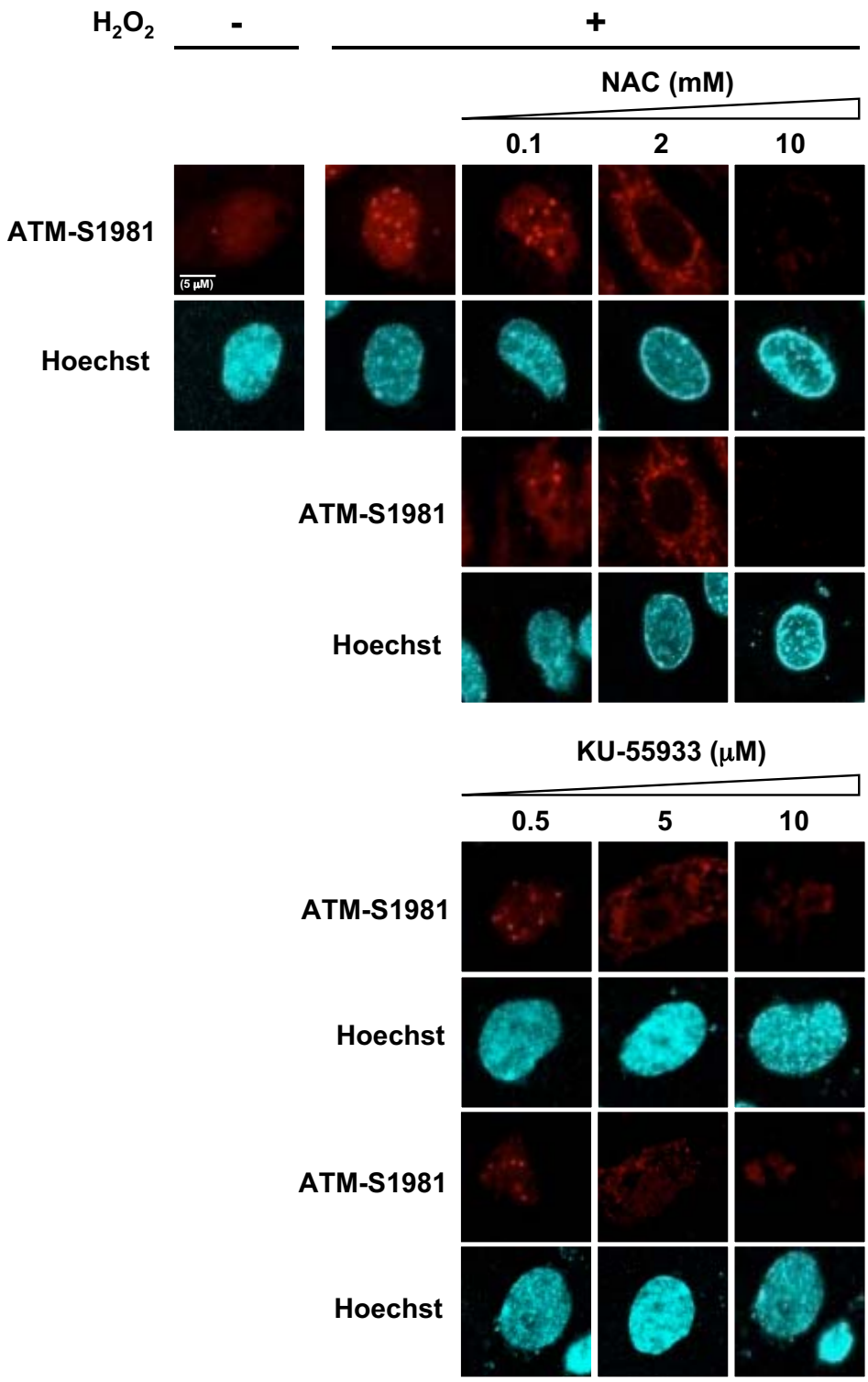
Supplemental Fig. 6. Immunostaining for von Willebrand factor, p21 or p16 in the thoracic aortas of STZ-diabetic ATM knockout mice. Six respective ATM+/+ (Wild), ATM+/- (Hetero), ATM-/- (Homo) mice were used. *A*. Immunostaining for von Willebrand factor, an endothelial cell marker, in the thoracic aortas (brown). Normal rabbit IgG antibody was used as negative control. Arrows indicate positive staining in the endothelium. *B*. Immunostaining for p21 and p16 (brown). Normal mouse IgG antibody was used as negative control. Scale bar, 50 μm, respectively.

Supplemental Fig. 7. *A, B*. Western blot analysis of ATM expression in thoracic aortas (A) and kidneys (B) of ATM knockout mice (wild-type, and heterozygous- and homozygous-knockout mice, n=3, respectively).

Supplemental Fig.1



Supplemental Fig. 2



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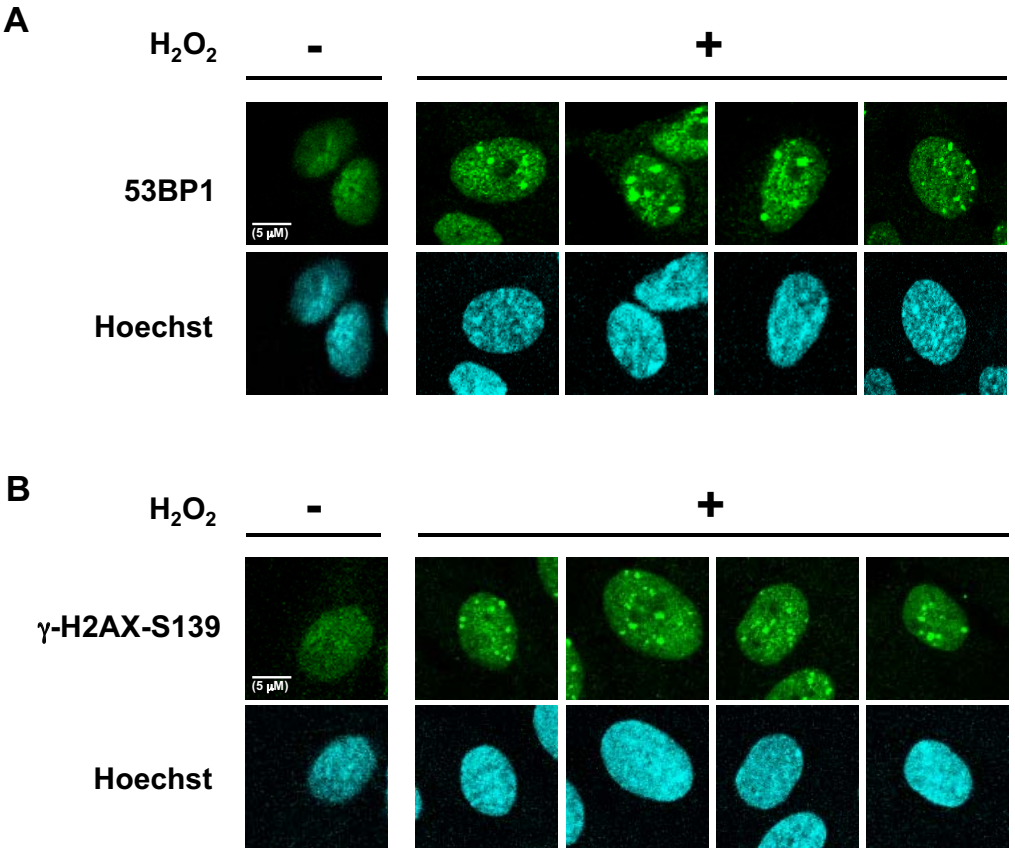

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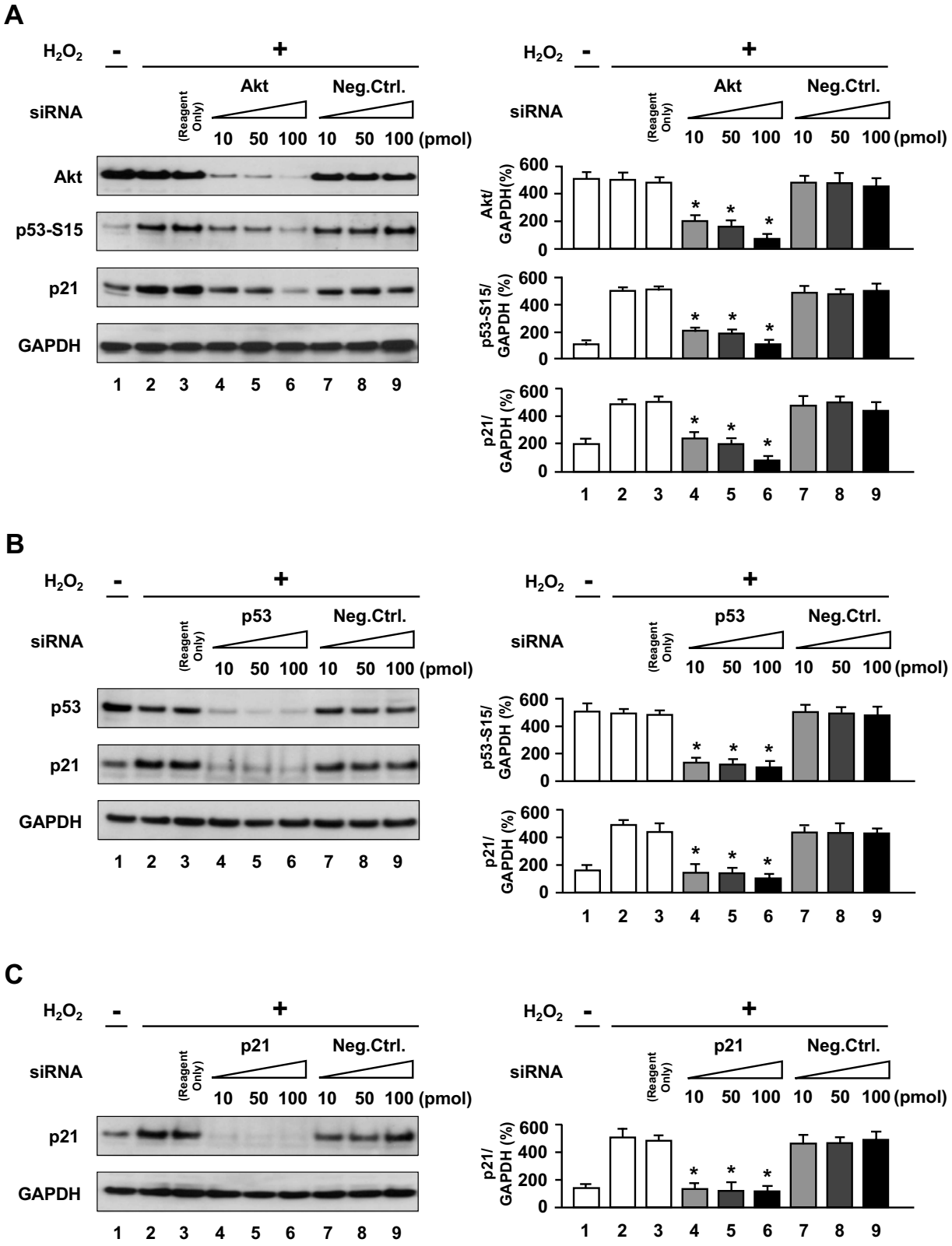

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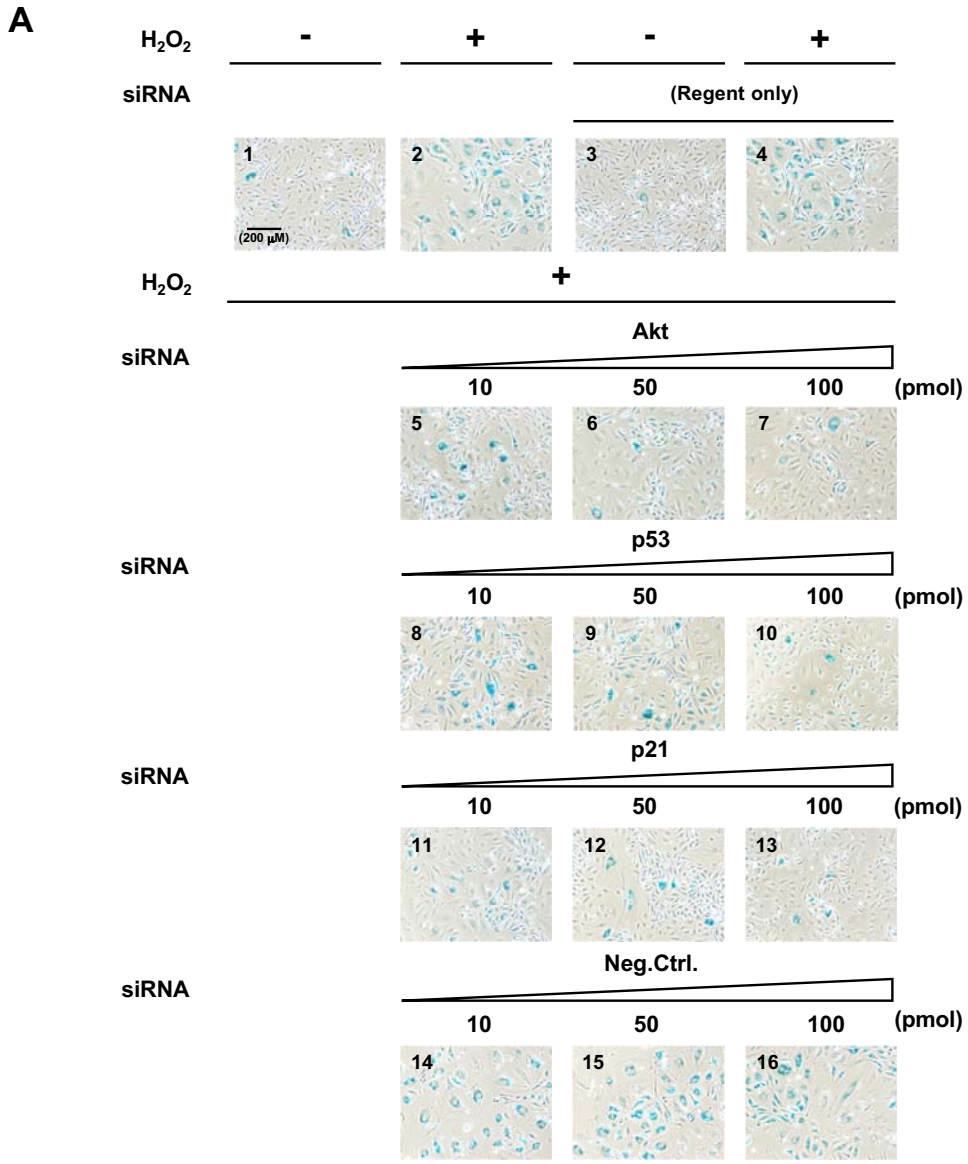

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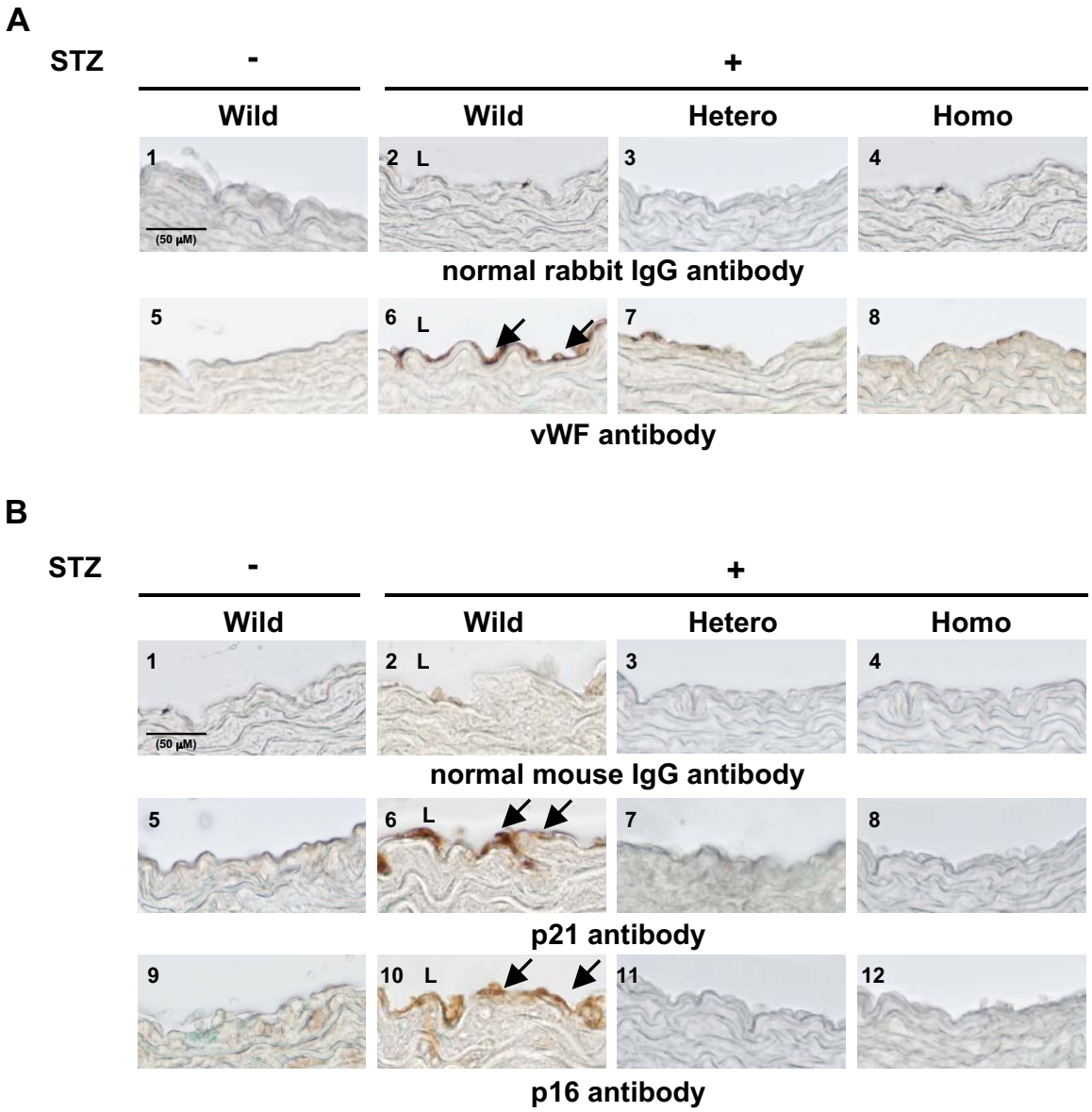

Supplemental Fig. 7