

**Arterial Medial Calcification through Enhanced small Extracellular Vesicle Release in  
Smooth Muscle-Specific *Asah1* Gene Knockout Mice**

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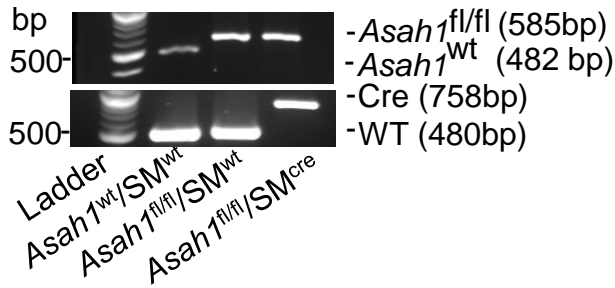
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Running title: Arterial medial calcification in SM-specific *Asah1* KO mice

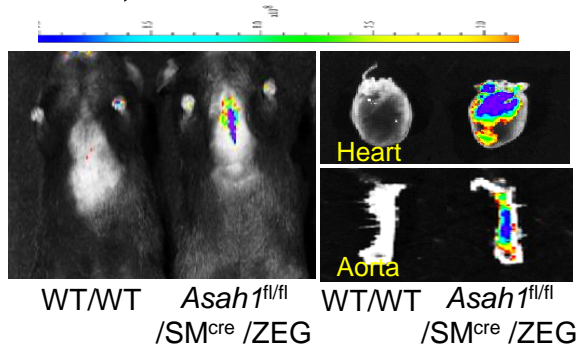
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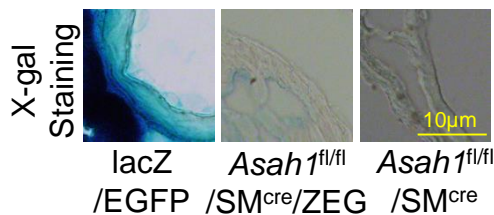
A



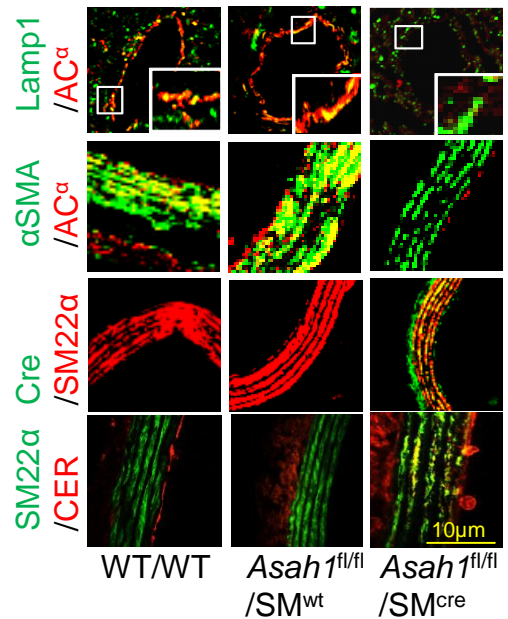
B



C

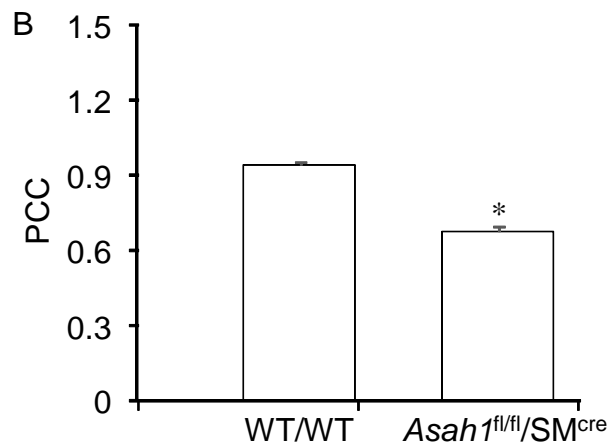
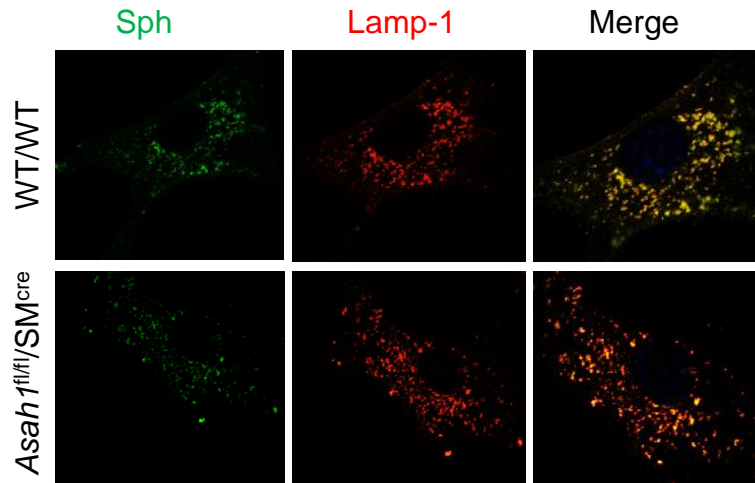


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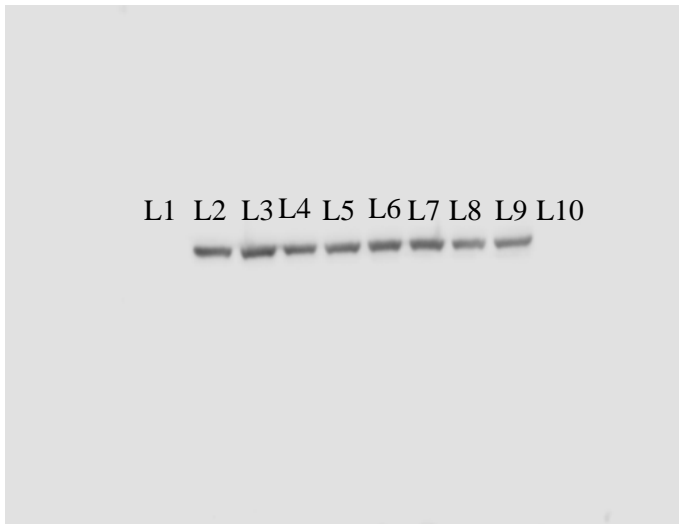


Supplementary Fig. S1 Characterization of SM-specific *Asah1* knockout (KO) mice. **A.** *Asah1*<sup>fl/fl</sup>/*SM*<sup>Cre</sup> has 2 positive PCR products including 758 bp for Cre and 585 bp for floxed *Asah1* gene. *Asah1*<sup>fl/fl</sup>/*SM*<sup>wt</sup> mice had positive floxed *Asah1* gene (585 bp), but no Cre (758 bp). Deletion of the *Asah1* was verified by PCR analysis. **B.** Cre-mediated SM-specific recombination was validated by breeding the *Asah1*<sup>fl/fl</sup>/*SM*<sup>Cre</sup> mice with Z/EG reporter mice, *in vivo* and *ex vivo* imaging of the offspring showed GFP expression in the aorta and coronary arteries of heart. **C.** Representative X-gal staining in the coronary arterial wall of mice. **D.** Representative fluorescent confocal microscopic images showed no co-localization of AC-α protein (red) with Lamp-1 (green, as a lysosome protein marker) in coronary arterial wall or α-SMA (green, as SMC marker) in aortic wall was observed in *Asah1*<sup>fl/fl</sup>/*SM*<sup>Cre</sup> compared to their littermates (*Asah1*<sup>fl/fl</sup>/*SM*<sup>wt</sup> and WT/WT). In contrast, Cre was only detected in aortic SMCs of *Asah1*<sup>fl/fl</sup>/*SM*<sup>Cre</sup> as shown in colocalization of Cre (green) vs. SM22-α (red, SMC marker). CER was markedly increased SMCs of *Asah1*<sup>fl/fl</sup>/*SM*<sup>Cre</sup> as shown in colocalization of CER (red) vs. SM22-α (green, SMC marker). Yellow spots or patches in the overlaid images were defined as colocalization of AC-α, CER & Cre with smooth muscle cell markers and Lamp-1 in arterial wall. SM (smooth muscle cell): AC-α: acid ceramidase-α, CER: ceramide, Cre: cre recombinase protein, α-SMA: α-smooth muscle cell actin (smooth muscle cell marker), Lamp-1: lysosomal associated membrane protein-1: SM22-α: smooth muscle cell marker: SM (smooth muscle cell). (n=3)

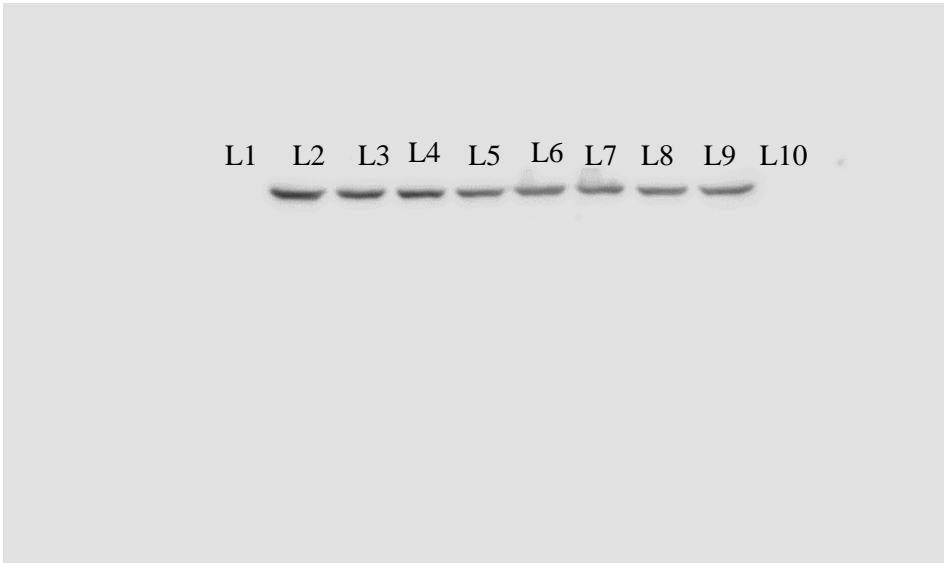
A Sph vs Lamp-1



Supplementary Fig. S2 **A**. Representative confocal images showed colocalization of Sph (green) and Lamp-1(red), and also sphingosine staining in *Asah1*<sup>fl/fl</sup>/SM<sup>Cre</sup> CASMCs was much lower as compared to WT type cells **B**. Bar graph shows *Ac* gene deletion significantly decreased colocalization of Sph/Lamp-1 in *Asah1*<sup>fl/fl</sup>/SM<sup>Cre</sup> CASMCs, n=3. \* P < 0.05 vs. WT/WT group. Lamp-1: lysosomal associated membrane protein-1; Sph: Sphingosine.

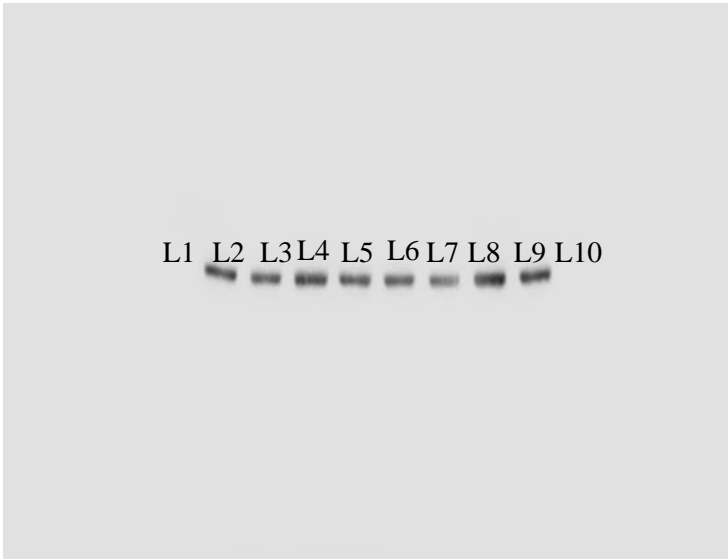


Original blot of beta-actin (OSP) Fig 5

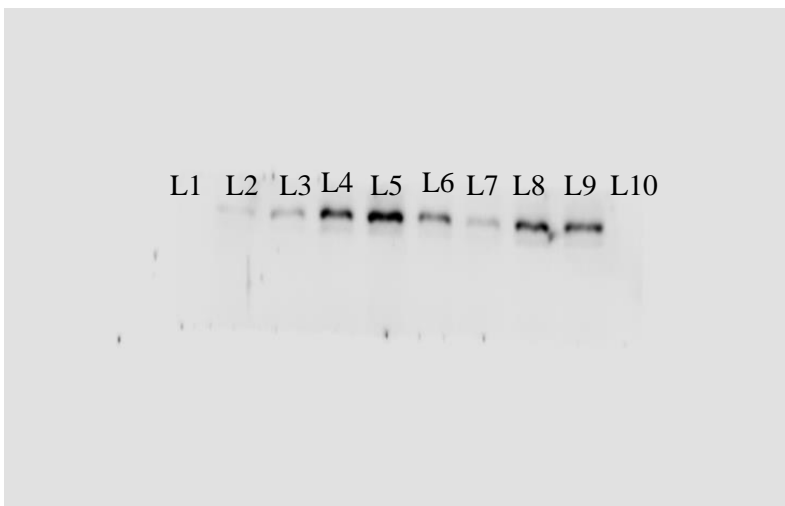


- L1-Ladder
- L2-WT Vehl
- L3-WT Pi
- L4-Asah<sup>fl/fl</sup>SM<sup>cre</sup> Vehl
- L5-Asah<sup>fl/fl</sup>SM<sup>cre</sup> P<sub>i</sub>
- L6-WTVehl
- L7-WTP<sub>i</sub>
- L8-Asah<sup>fl/fl</sup>SM<sup>cre</sup> Vehl
- L9-Asah<sup>fl/fl</sup>SM<sup>cre</sup> P<sub>i</sub>
- L10-Ladder

Original blot of beta-actin (RUNX2) Fig 5

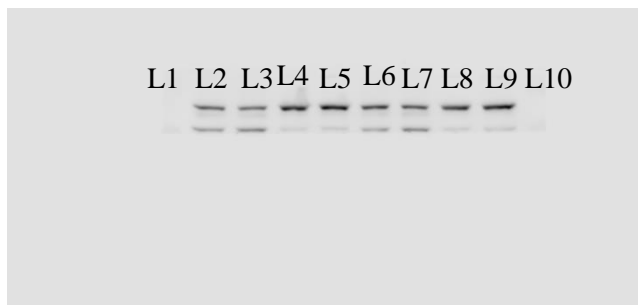


Original blot of beta-actin (SM22 alpha) Fig 5

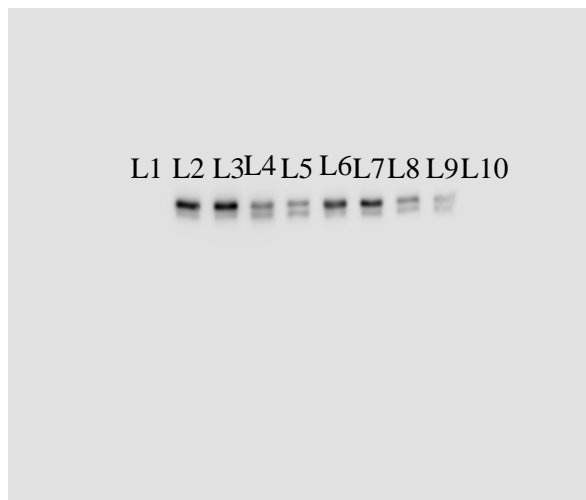


Original blot of OSP Fig 5

- L1-Ladder
- L2-WT Vehl
- L3-WT Pi
- L4-Asah<sup>fl/fl</sup>SM<sup>cre</sup> Vehl
- L5-Asah<sup>fl/fl</sup>SM<sup>cre</sup> P<sub>i</sub>
- L6-WTVehl
- L7-WTP<sub>i</sub>
- L8-Asah<sup>fl/fl</sup>SM<sup>cre</sup> Vehl
- L9-Asah<sup>fl/fl</sup>SM<sup>cre</sup> P<sub>i</sub>
- L10-Ladder



Original blot of RUNX2 Fig 5



Original blot of SM22 alpha Fig 5