Association of Sonic Hedgehog with the extracellular matrix requires its zinc-coordination center

Supplemental Figures

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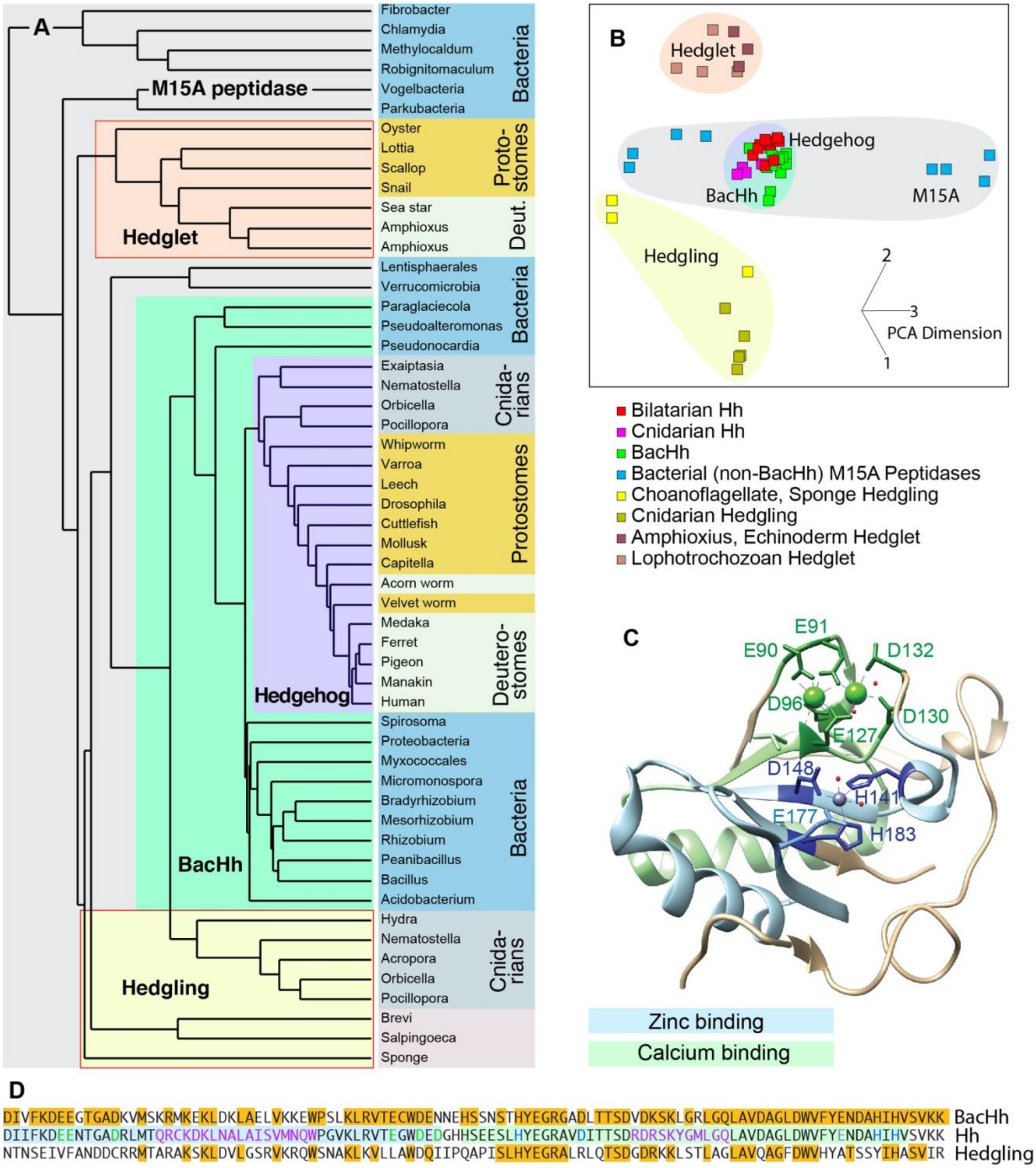
Short title:
The putative peptidase function of Shh

Supplementary Figure 1



Supplementary Figure 1. Sequence similarities between BacHhs, Hhs, Hedglings, and Hedglets.

A: a similarity tree of Hh sequences encompassing the calcium and zinc coordinating motifs. All Hhs are closely related and root from within the BacHhs. Hedglings (present in Choanoflagellates, Sponges, and Cnidarians) and Hedglets (present in Lophotrochozoans, Amphioxus and Echinoderms) form outgroups, but all are related to bacterial M15 peptidases. Organisms in the same phylum or clade are color coded, and common names of the organisms are used. The sequences used, their accession numbers, and numbering of aligned residues can be found in this supplemental file. Hedglings and Hedglets (red borders) are predicted to be unable to mediate catalysis. **B:** PCA plot of the tree presented in A. **C:** Structure of Shh with salient residues and domains indicated. **D:** Sequence lineup of the conserved domains of Mesorhizobium BacHh (ESZ55121.1), human Dhh (NP_066382.1), and Choanoflagellate Hedgling (XP_001749037.1). Identical residues are indicated in amber. Salient residues are color coded and color coordinated with C.

Supplementary Figure 1 Sequences. Below are the sequences used to generate the tree and PCA plot shown in Supplementary Figure 1. Accession number and stretch of residues used in the lineups are indicated.

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>Monosiga brevicollis, XP_001749037.1, 354-458
GLSFAPSSGNMYPNVVSARMASRLKVLANLVPRVFGESAAVLVLDAYRAAPLVAAEATLHNTGRAALLTVINVTASLDDELAALASVCADA
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>Human, BAA33523.2, 89-193
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>Mesorhizobium sp. L103C131B0, ESZ55121.1, 173-277
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>Pseudoalteromonas piratica, WP_040135141.1, 60-162
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>Spirosoma aerolatum, WP_080055297.1, 211-317
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>Drosophila melanogaster, NP_001034065.1, 144-247
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>Proteobacteria bacterium, PZN23661.1, 96-197
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>Robiginitomaculum sp., PHQ68463.1, 158-262
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>Rhizobium leguminosarum, WP_130783679.1, 310-413
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>Verrucomicrobia bacterium, HCF95878.1, 132-232
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>Paenibacillus sp. CAA11, WP_108465644.1, 1027-1131
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>Oryzias melastigma, ACL81248.1, 46-150
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>Capitella teleta, AAZ04357.1, 82-186
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>Acropora millepora, XP_029199742.1, 68-170
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>Euperipatoides kanangrensis, VDH80594.1, 88-190
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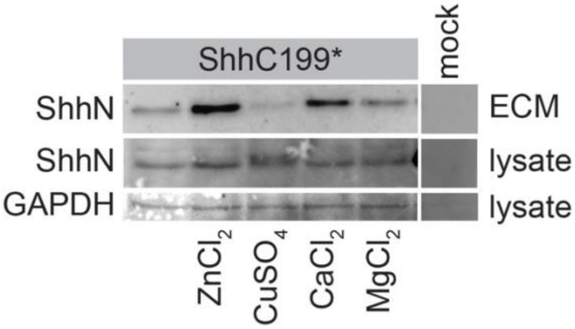
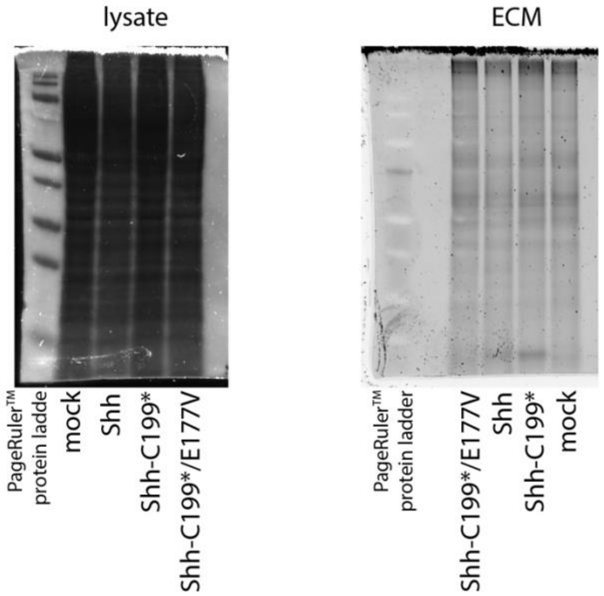
Supplementary Figure 2



Supplementary Figure 2: Association of Shh with the ECM is enhanced specifically in response to zinc. Western blot analysis of the lysate and ECM of Hek293t cells transfected with *Shh-C199** and cultured in medium containing 0.18 mM calcium and 5 µM zinc, 5 µM copper, 1.8 mM magnesium or 1.8 mM calcium as indicated.

Supplementary Figure 3: Uncropped and unedited gel and Western blots for Figure 1:

1A



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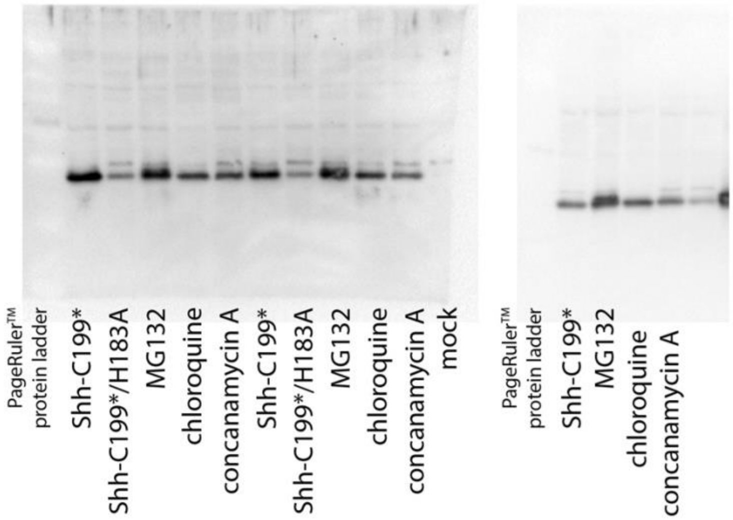

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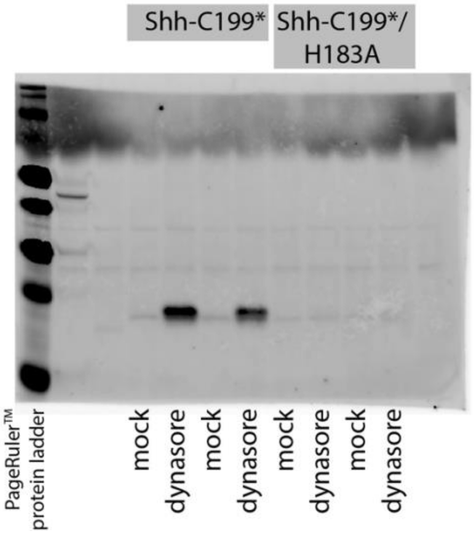
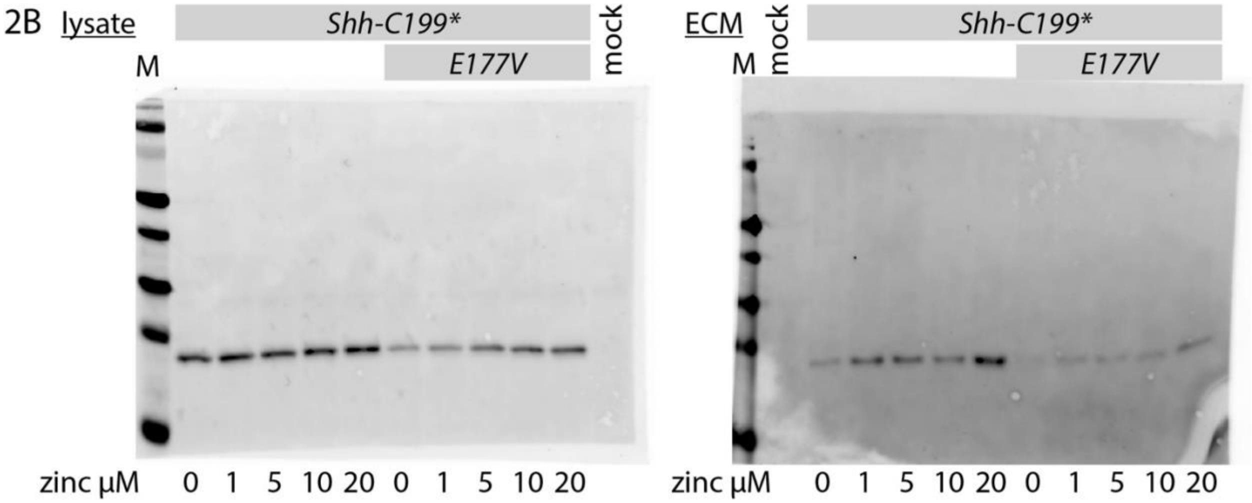


Supplementary Figure 3: Active ShhN associates with the extracellular matrix.

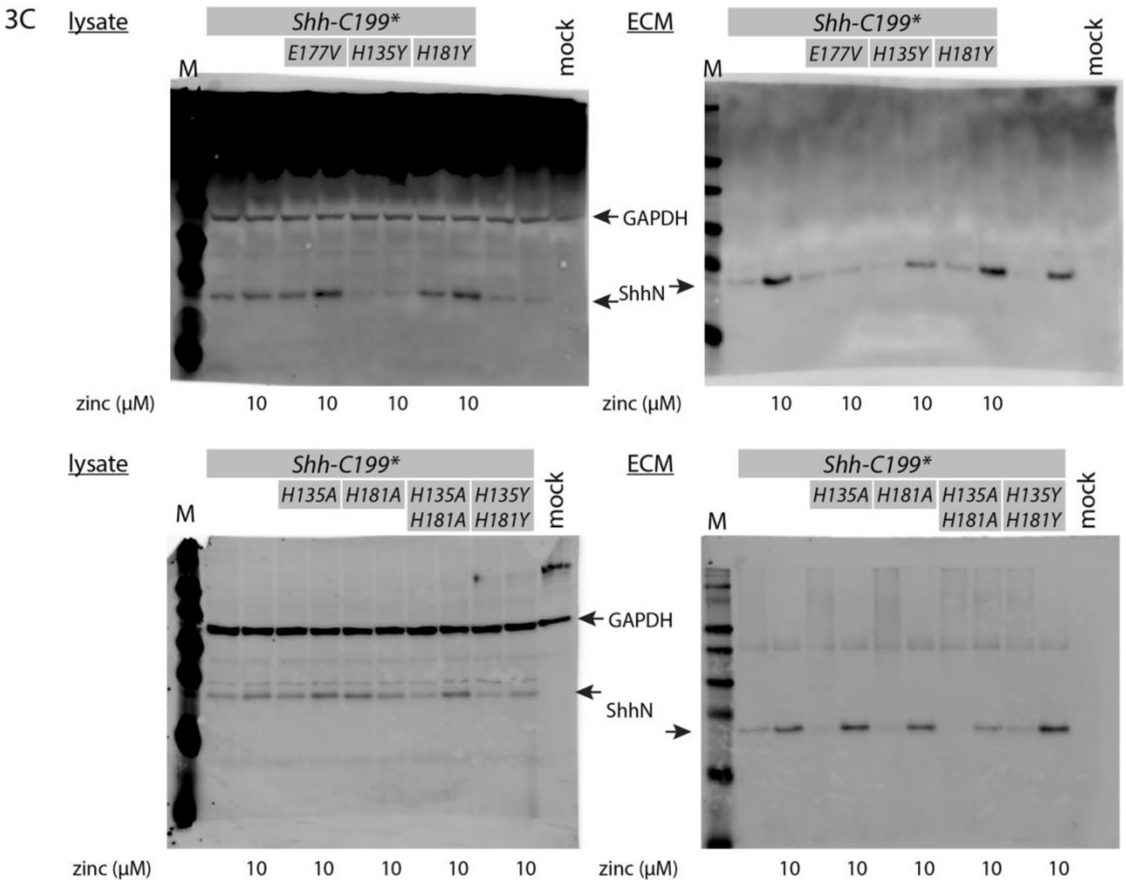
A: Lysate and ECM deposited by mock and *Shh-C199**-transfected Hek293t cells analyzed by SDS-PAGE and SYPRO-Ruby staining. ShhN is indicated. **E:** Western blot analysis of HEK293t cells transfected with the indicated Shh mutants. 100 nM MG-132 (proteasome inhibitor), 100 nM Chloroquine and 100 nM Concanamycin A (inhibitors of endosome acidification) were assessed for their ability to affect Shh accumulation. **F:** Western blot analysis of HEK293t cells transfected with the indicated Shh mutants, and the effects of the dynamin inhibitor Dynasore (50 µM) was assessed for its effect on Shh accumulation. M = (PageRuler pre-stained protein ladder)

Supplementary Figure 4: uncropped and unedited Western blots for Figure 2

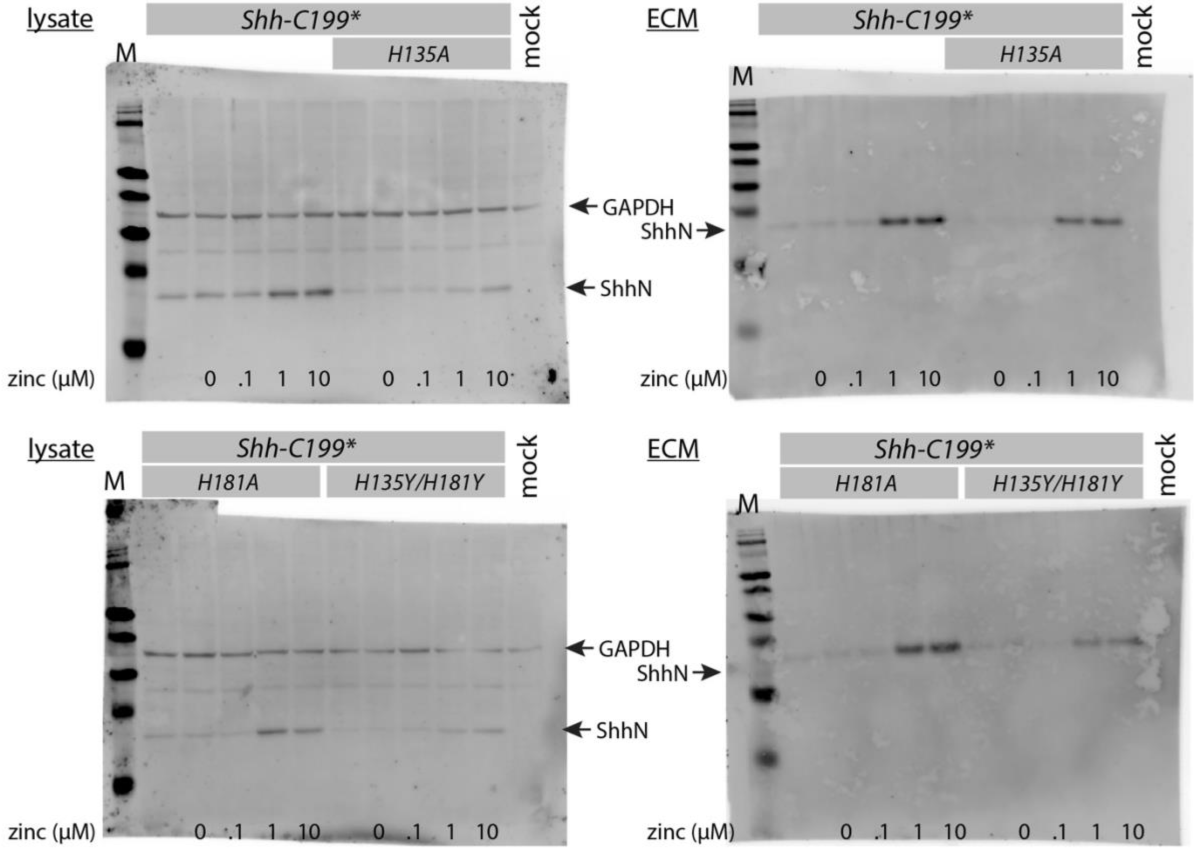


Supplementary Figure 4: ECM-association of ShhN requires zinc and the catalytic E177V residue. B: Western blot analysis of the lysate and ECM of HEK293t cells transfected with *Shh-C199** and *Shh-C199*/E177V* and cultured in DMEM containing 0.18 mM calcium and the indicated concentrations of zinc. M = (PageRuler pre-stained protein ladder)

Supplementary Figure 5: uncropped and unedited Western blots for Figure 3



3D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H135A
mock
M
GAPDH
ShhN
zinc (μM)
0 .1 1 10 0 .1 1 10
ECM
Shh-C199*
H135A
mock
M
ShhN
zinc (μM)
0 .1 1 10 0 .1 1 10
lysate
Shh-C199*
H181A
H135Y/H181Y
mock
M
GAPDH
ShhN
zinc (μM)
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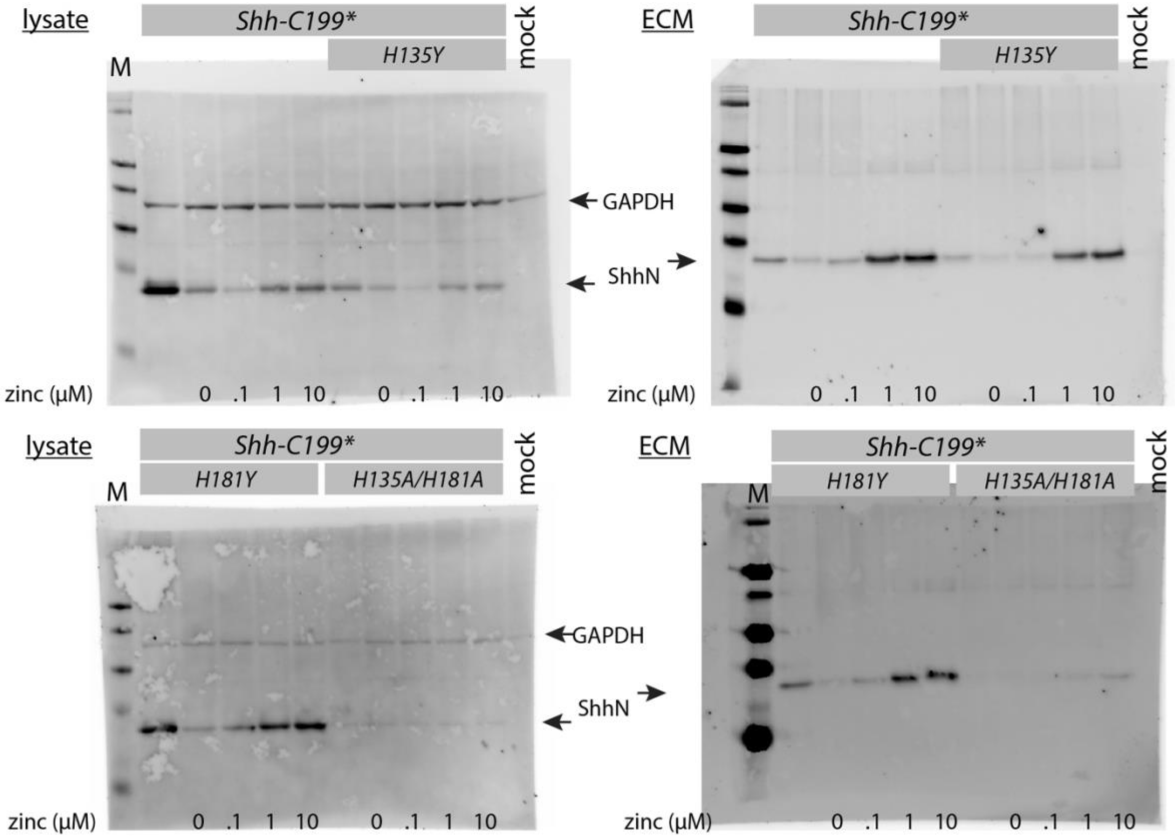
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GAPDH
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ECM
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H135Y
mock
ShhN
zinc (μM)
0 .1 1 10 0 .1 1 10
lysate
Shh-C199*
H181Y
H135A/H181A
mock
M
GAPDH
ShhN
zinc (μM)
0 .1 1 10 0 .1 1 10
ECM
Shh-C199*
H181Y
H135A/H181A
mock
M
zinc (μM)
0 .1 1 10 0 .1 1 10

Supplementary Figure 5: Stacking histidines of the zinc coordination center affect ECM association. C: The effect of mutations of the transition state-stabilizing residues H135 and H181 to alanine (A) or tyrosine (Y) on the zinc-dependent accumulation in the ECM was analyzed on a Western Blot of the extracted ECM from transfected HEK293t cells cultured in 0.18 mM calcium with or without 10 µM zinc. **D:** zinc dose-response analysis of H135 and H181 mutations assessed by Western blot of the lysate and ECM of HEK293t cells transfected with the indicated mutants and cultured in 0.18 mM calcium and increasing concentrations of zinc (0.1, 1, 10 µM). M = (PageRuler pre-stained protein ladder)

Supplementary Figure 6: uncropped and unedited Western blot for Figure 4

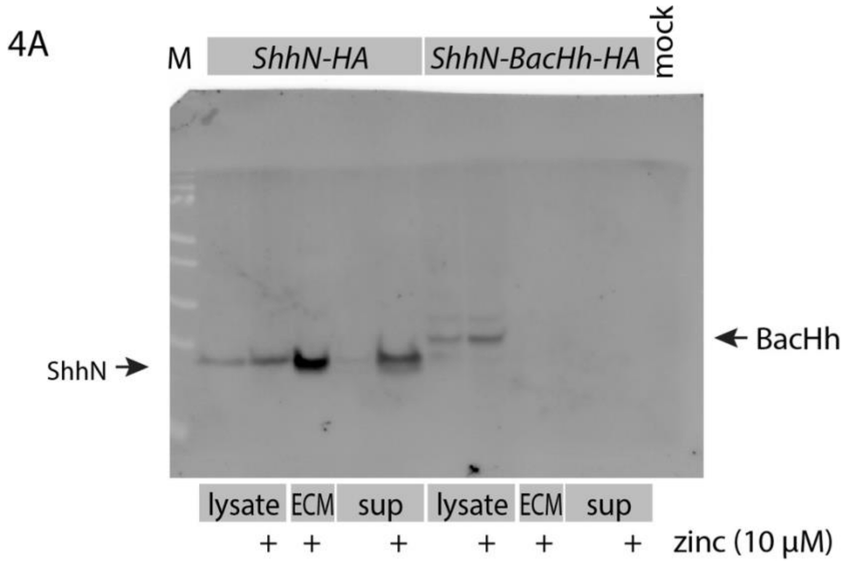

Figure S4: **The zinc-coordination domain of BacHh is not sufficient for association with the ECM. A:** Western Blot analysis of the lysate, ECM, and supernatant of *ShhN-HA* or *Shh-BacHh-HA* (diagrams) transfected HEK293t cultured in the indicated zinc concentrations.

M = (PageRuler pre-stained protein ladder)

Supplementary Figure 7, uncropped and unedited Western blots for Figure 5

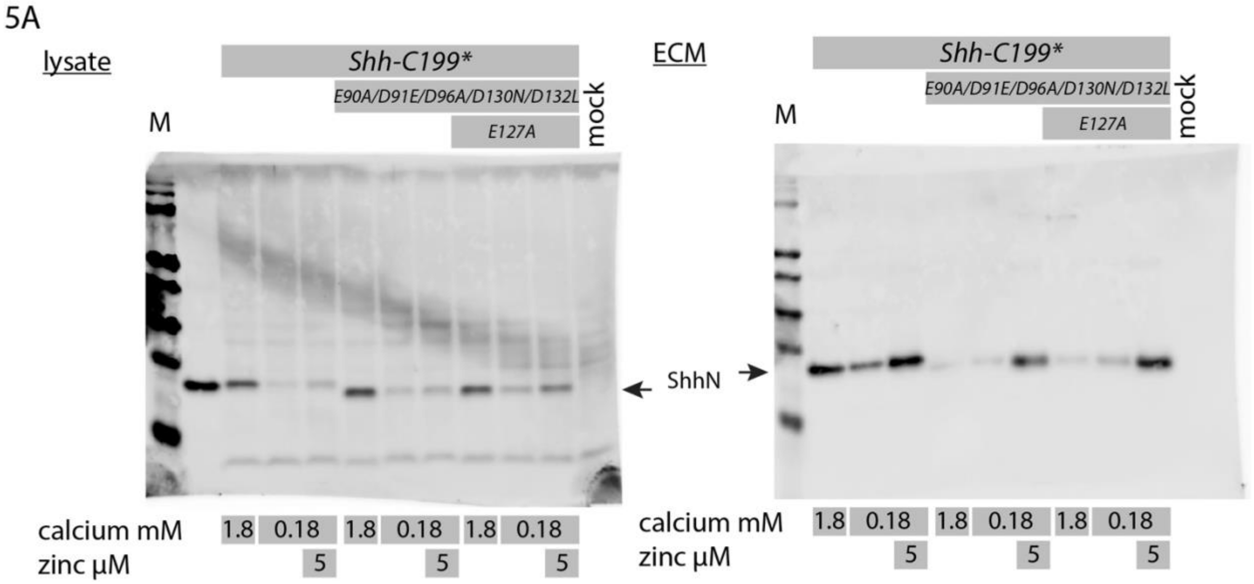

Figure 5: Calcium alters the sensitivity of Shh to zinc. **A:** Western blot analysis of lysates and ECM of HEK293t cells transfected with *Shh-C199** and *Shh-C199*/E90A/E91D/D96AD130N/D132L* , or *Shh-C199** and *Shh-C199*/E90A/E91D/D96A/E127A/D130N/D132L* (Ca^{Free}), cultured in the presence of 0.18 or 1.8 mM calcium, and in the absence or presence of 5 µM added zinc.

M = (PageRuler pre-stained protein ladder)

Supplementary Figure 8: uncropped and unedited Western blots for Supplementary Figure 2

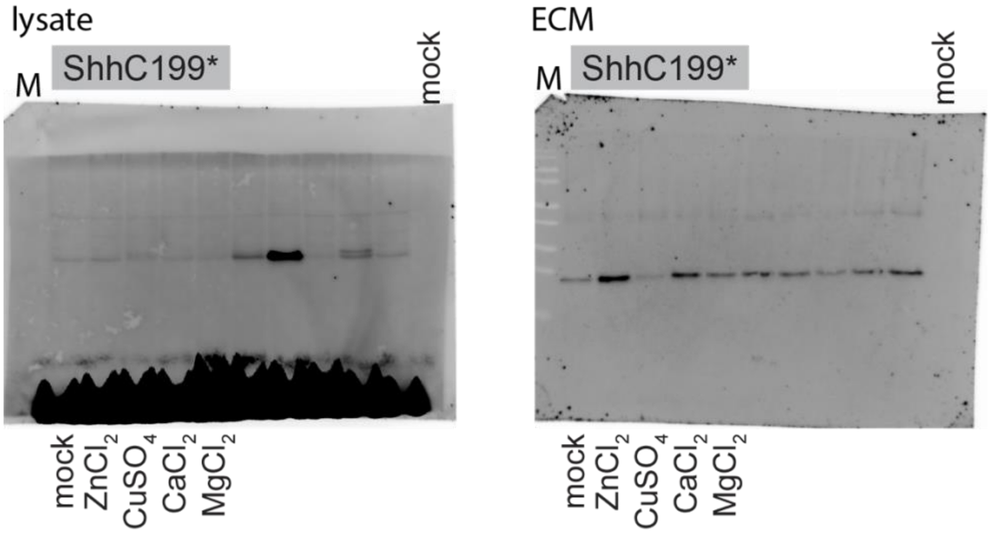

Figure S8: Association of Shh with the ECM is enhanced specifically in response to zinc. Western blot analysis of the lysate and ECM of Hek293t cells transfected with *Shh-C199** and cultured in medium containing 0.18 mM calcium and 5 µM zinc, 5 µM copper, 1.8 mM magnesium or 1.8 mM calcium as indicated.