

1 **Title:** A novel surfactant protein is associated with extrapulmonary respiration in
2 lungless salamanders

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4 **Short title:** Novel surfactant protein in salamanders

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21 **Keywords:** Salamander, respiration, gene duplication, molecular evolution,
22 surfactant, plethodontid

23 **Abstract:** Numerous physiological and morphological adaptations were achieved
24 during the transition to lungless respiration following evolutionary lung loss in
25 plethodontid salamanders, including those that enable efficient gas exchange
26 across extrapulmonary tissue. However, the molecular basis of these adaptations
27 is unknown. Here we show that lungless salamanders express in the skin and
28 buccal cavity—the principal sites of respiratory gas exchange in these species—
29 a novel paralog of the gene Surfactant-Associated Protein C (SFTPC), which is a
30 critical component of pulmonary surfactant expressed exclusively in the lung in
31 other vertebrates. The paralogous gene appears to be found only in
32 salamanders, but, similar to SFTPC, in lunged salamanders it is expressed only
33 in the lung. This heterotopic gene expression, combined with predictions from
34 structural modeling and respiratory tissue ultrastructure, suggest that lungless
35 salamanders produce pulmonary surfactant-like secretions outside the lungs and
36 that the novel paralog of SFTPC might facilitate extrapulmonary respiration in the
37 absence of lungs. Heterotopic expression of the SFTPC paralog may have
38 contributed to the remarkable evolutionary radiation of lungless salamanders,
39 which account for more than two thirds of urodele species alive today.

40 **Introduction:** Most amphibians must confront the challenges of respiring both in
41 water and on land. To do so, they utilize numerous gas exchange surfaces
42 including the lungs, gills, integument and buccopharyngeal mucosa, which are
43 employed to varying extents depending on species and life-history stage. In adult
44 salamanders, for example, the integument may be responsible for 50% or more
45 of oxygen uptake [1,2]. Lability in sites of gas exchange is especially critical for
46 metamorphosing species, which face different respiratory demands in air and
47 water. Little is known, however, about the molecular mechanisms that enable the
48 ontogenetic and evolutionary transitions from aquatic to aerial respiration. The
49 mechanism of aerial respiration is even more enigmatic in lungless species,
50 which rely entirely on extrapulmonary sites of respiration [1–3]. The family
51 Plethodontidae includes more than two thirds of all living salamander species;
52 most are fully terrestrial, and all adults lack lungs. Respiration takes place solely
53 across the integument and buccopharyngeal mucosa, and also across the gills in
54 aquatic larval forms, when present. Lunglessness is not unique to
55 plethodontids—it has evolved several times in other amphibians, including
56 salamanders, frogs and caecilians [4]—but its adaptive significance is unresolved
57 [5,6].

58

59 How lungless salamanders are able to satisfy metabolic demands for oxygen is a
60 topic of considerable interest. In theory, lunglessness limits thermal tolerance
61 and maximum body size, yet lungless salamanders paradoxically occupy diverse
62 thermal environments and attain relatively large body sizes. Plethodontids

63 possess highly vascularized skin and buccopharyngeal mucosa, which may
64 compensate for the loss of pulmonary respiration [1,7–9]. The buccopharyngeal
65 membranes, in particular, may function as an adaptive respiratory surface that
66 facilitates gas exchange, as evidenced by increased oscillation of the floor of the
67 buccal cavity under hypoxia, high temperature, or activity, which presumably
68 serves to draw more air into the mouth [1,7,10]. Selection for efficient
69 extrapulmonary respiration may have played a major role in the adaptive
70 radiation of terrestrial plethodontids [1]. Indeed, the evolution of highly efficient
71 cutaneous and buccopharyngeal respiration is believed to have freed
72 plethodontids from the ontogenetic and functional constraints associated with the
73 use of a buccal pump for pulmonary ventilation, thereby enabling them to occupy
74 diverse habitats and evolve ballistic tongue projection [2,11,12].

75

76 To identify the molecular adaptations that might facilitate lungless respiration, we
77 investigated the expression of a crucial pulmonary surfactant-associated protein
78 in plethodontid salamanders. Proper lung function requires pulmonary surfactant,
79 a complex and evolutionarily variable mix of molecules that facilitate mucous
80 spreading and lung compliance and improve oxygen diffusion [13–15].

81 Surfactant-associated protein C (SFTPC) is a hydrophobic protein found in
82 pulmonary surfactant that localizes to the lung's air-liquid interface. It reduces
83 surface tension by aiding the adsorption and distribution of lipids within
84 pulmonary surfactant and specifically enhances oxygen diffusion [14,16,17].
85 SFTPC also regulates production and turnover of phosphatidylcholine, a major

86 component of pulmonary surfactant, and it limits the thickness of the hypophase,
87 the liquid layer that lines the lung's inner surface [18–20]. Mucous layer thickness
88 greatly impacts gas exchange between the environment and the blood supply
89 [21]. Additionally, oxygen uptake is enhanced by the presence of surfactant in the
90 hypophase, likely due to convective effects that facilitate mixing of oxygen and
91 mucous or increased rate of oxygen trafficking [15,22]. The expression of SFTPC
92 is highly conserved among tetrapods: all species evaluated previously express
93 SFTPC exclusively in the lungs [23–25] (and Supplementary Text).

94

95 **Results and Discussion:** Despite lacking lungs as adults, plethodontid
96 salamanders express SFTPC (Fig. S1). Due to the consistent restriction of
97 SFTPC expression to the lungs and to SFTPC's conserved role across tetrapods,
98 we compared SFTPC expression between lungless and lunged salamanders.
99 Surprisingly, several species of salamanders express two transcripts with high
100 sequence identity to annotated SFTPC sequences (Fig. S1a, b). Both transcripts
101 exclusively match SFTPC within the NCBI nucleotide collection database, but
102 one possesses higher sequence similarity to amniote and frog SFTPC. We
103 denote the transcript with lower similarity SFTPC-like. We did not find SFTPC-
104 like within vertebrate genomes or transcriptomes outside of salamanders.
105 Phylogenetic analyses support the hypothesis that SFTPC-like represents a
106 previously undescribed salamander-specific paralog of the highly conserved
107 lung-specific gene, SFTPC (Figs. S1, S3; Supplementary Text). SFTPC-like may

108 maintain or partially maintain the characteristic hydrophobic alpha-helical
109 configuration of the SFTPC mature peptide [16] (Fig. S1c).
110
111 Numerous studies of tetrapods localize SFTPC exclusively to the lungs [23–25].
112 SFTPC and SFTPC-like in the lunged salamander *Ambystoma mexicanum*
113 match this highly conserved pattern (Fig. 1). As visualized by *in situ* hybridization,
114 SFTPC and SFTPC-like staining is only observed in the extremely squamous
115 alveolar epithelial cells lining the lungs and trachea (Figs. 1, S5, S6). SFTPC-like
116 expression, however, is lower than SFTPC and the genes are expressed at
117 different times: SFTPC-like is low or non-existent before hatching, whereas
118 SFTPC is expressed beginning in embryos immediately following the formation of
119 the laryngotracheal groove, a ventral outpocketing of the foregut that precedes
120 lung outgrowth (Fig. 1a, b), and continuing into adulthood (Fig. 1d).
121
122 In contrast, SFTPC-like is expressed dynamically in lungless salamanders. In
123 embryos and early larvae of *Desmognathus fuscus*, a metamorphosing species,
124 SFTPC-like is expressed throughout much of the integument, with reduced
125 staining on the dorsal (internal) surface of the operculum (gill covering) and in the
126 limbs (Fig. 2a–h). Expression begins to diminish in the integument immediately
127 before metamorphosis, but at the same time it expands to the buccopharyngeal
128 mucosa (oral epithelium) (Fig. 2i–l). Integumentary expression at this stage is
129 patchy: remaining SFTPC-like-positive cells are displaced towards the apical
130 surface and display an irregular morphology (Fig. 2i, k). Cessation of

131 integumentary expression of SFTPC-like coincides with several metamorphic
132 transitions, but especially molting [26], when the integument is profoundly
133 remodeled from a simple stratified epithelium to a thickened pseudostratified
134 tissue rich in acinous glands (Fig. S4, Fig. 2i, k).

135

136 Immediately following metamorphosis, expression is absent from the integument
137 and restricted to the buccopharyngeal mucosa (Fig. 2m–r). In adults, SFTPC-like
138 is expressed in the buccal cavity and adjacent pharynx (Fig. 2m–r): it is confined
139 to oral mucosa, with a strict boundary at the marginal teeth. SFTPC-like is not
140 expressed along the vomerine bones (near the internal nares) or in the dorsal
141 midline of the tongue, although it is strongly expressed along the tongue margin
142 (Fig. 2m, o). Additional data from immunohistochemistry and mass spectroscopy
143 are needed to determine if SFTPC-like transcripts are translated and whether this
144 protein is then processed and secreted in a similar fashion to SFTPC in the lung.

145

146 Unlike SFTPC-like, SFTPC is expressed at an extremely low level in embryos of
147 lungless species, which develop a transient lung rudiment [27]. The SFTPC
148 transcript detected from transcriptome sequencing of the lung rudiment of
149 *Plethodon cinereus* (Supplemental Data File 1) could not be cloned from either *P.*
150 *cinereus* or *D. fuscus*, nor was it found in adult plethodontid transcriptomes. This
151 suggests that SFTPC expression is inhibited by lung loss in lungless species,
152 with the exception of SFTPC expression at certain embryonic stages in the
153 presumptive lung region.

154

155 The larval integument of *Desmognathus fuscus* displays pronounced secretory
156 activity (Fig. 3a–d). The outer layer—the stratum corneum—is a protective layer
157 composed of anuclear keratinized cells. It displays an extremely high level of
158 secretory activity evidenced by the near universal distribution of bilamellar
159 secretory vesicles along its superficial surface (Fig. 3a, “SV”). Just basal to the
160 stratum corneum is a layer of secretory cells, which are heavily vacuolated at
161 their apical extent. The stratum corneum and the layer of secretory cells are not
162 separated by a cell membrane, but a dark and consistent division appears
163 between them (Fig. 3b). Bilamellar secretory vesicles virtually identical to those
164 observed on the stratum corneum of *D. fuscus* are secreted from alveolar
165 epithelial cells in the lung of *Ambystoma mexicanum* but are not present in its
166 integument (Fig. 3e, g, h; Fig. S6, “SV”).

167

168 Pulmonary surfactant is trafficked in lamellar bodies in alveolar epithelial cells.
169 These lamellar bodies are distinct from other ultrastructural lamellar elements in
170 that they are large in diameter and spherical in shape [28–30]. Furthermore,
171 these lamellar bodies are distinct from the bilamellar structures identified on the
172 surface of the integument, in that they have many lamellae. The larval
173 integument of *D. fuscus* contains large (0.5–0.75 μm diameter), spherical
174 lamellar bodies (Fig. 3c, d, “LB”) that closely resemble those found in alveolar
175 epithelial cells, which otherwise are known only from the lungs of other
176 tetrapods.. Secretory activity and the presence of these distinctive lamellar

177 bodies strongly suggest that *D. fuscus* produces surfactant in extrapulmonary
178 sites of gas exchange, which correspond to sites of SFTPC-like expression.
179
180 Despite the lung- and trachea-specific expression of SFTPC and SFTPC-like in
181 *A. mexicanum* revealed by *in situ* hybridization and the numerous reports of lung-
182 specific expression of SFTPC in mammals (Supplementary text), recently
183 published transcriptomes of *A. mexicanum* purport to map low numbers of
184 SFTPC and SFTPC-like reads to several tissues, including blood vessels, bone,
185 heart, regenerating limbs, and mixed stages of whole embryos [31]. Therefore,
186 SFTPC and SFTPC-like may not be entirely lung-specific transcripts in lunged
187 salamanders. Additional studies are needed, however, to evaluate the alternative
188 interpretation that contamination, mapping or assembly issues might have
189 yielded spuriously mapped reads.

190
191 Passive diffusion across a tissue layer, as occurs during cutaneous respiration, is
192 a function of several variables. These include the size (area) of the surface over
193 which gas exchange occurs and the thickness of the barrier between the
194 underlying blood supply and the environment [3]. Barrier thickness depends
195 mainly on the distance between the environment and the blood supply, but also
196 on the thickness of the mucous layer between the environment and respiratory
197 tissue. Diffusivity of mucus is about 30% lower than water [21]. Reduction of
198 surface tension by pulmonary surfactant helps maintain a thin layer of airway
199 surface liquid [20] and increases convection within the mucous, which together

200 result in increased oxygen uptake [22]. SFTPC indirectly influences mucous layer
201 thickness in the lung by regulating the production of phosphatidylcholine [18].
202 Pulmonary surfactant aids oxygen transport across the air-liquid interface, and
203 hydrophobic surfactant proteins increase the rate of oxygen diffusion twofold over
204 surfactant lipid alone [15,17]. SFTPC-like may facilitate respiration through a
205 reduction of effective barrier thickness or an increase in diffusivity of the mucus
206 layer. Pulmonary surfactant also plays non-respiratory roles, including facilitation
207 of mucus spreading, innate immune defense, anti-edema agent, hydrostatic gas
208 exchange, and preventing adhesion of lung surfaces when the lungs deflate
209 [13,32,33]. It is possible that extrapulmonary surfactant produced in
210 *Desmognathus fuscus* is performing one or more of these functions instead of, or
211 in addition to, facilitating gas exchange.

212

213 Spatial and temporal expression of SFTPC-like and the ultrastructure of the
214 associated integument suggest that, following SFTPC-gene duplication, SFTPC-
215 like became neofunctionalized for extrapulmonary respiration in lungless
216 salamanders. Sequence and structural conservation of SFTPC-like and SFTPC
217 suggests that the two proteins function similarly (Fig. S1). However, to confirm
218 neofunctionalization requires a more detailed functional characterization of
219 SFTPC-like. This may include determining with increased phylogenetic and
220 technical precision how expression of SFTPC-like has evolved in salamanders,
221 proteomic characterization of skin and buccopharyngeal secretions, and

222 assessment of whether SFTPC-like displays surface activity or aids gas
223 exchange.

224

225 In metamorphosing species such as *Desmognathus fuscus*, respiratory and fluid-
226 retention demands shift dramatically upon the transition from aquatic to terrestrial
227 habitats [1]. Cutaneous water loss becomes a critical liability, but reduced skin
228 permeability hinders cutaneous gas exchange. To counter this limitation,
229 terrestrial plethodontids show increased reliance on buccopharyngeal respiration
230 [1]. Ontogenetic shift of SFTPC-like from the skin to the buccopharyngeal cavity
231 during metamorphosis (Fig. 2) correlates with the transition from aquatic to aerial
232 respiration; SFTPC-like is expressed at the preferential sites of gas exchange at
233 each life-history stage [1]. However, it is also possible that instead of playing a
234 direct role in facilitating gas exchange, extrapulmonary surfactant balances fluid
235 retention and respiratory demands by aiding fluid uptake from the mucus. Such a
236 role would be consistent with the proposed anti-edema properties of surfactant
237 within the lungs [13].

238

239 Gene duplication is increasingly recognized as a driving force of evolutionary
240 innovation [34]. While gene duplication does not always lead to functional
241 divergence [35,36], regulatory changes in duplicated genes, such as altered
242 expression sites, may enable the evolution of novel traits in individual lineages.
243 Many studies of the evolutionary phenomenon of adaptive radiation have
244 emphasized morphological traits whose appearance in particular lineages

245 promote phylogenetic and ecological diversification [37]. We propose that such
246 morphological traits, or key adaptations, work in concert with novel and
247 functionally significant molecular features to enhance evolutionary success, and
248 that such instances of concerted evolution are more widespread than is currently
249 recognized.

250

251 In plethodontid salamanders, it is possible that the combination of morphological
252 adaptations [7,9,38] and novel deployment of a critical surfactant protein enables
253 efficient extrapulmonary respiration via the buccopharynx and integument.
254 Conserved expression of SFTPC-like in lunged salamanders relative to SFTPC
255 may be due to dosage-sharing between SFTPC and SFTPC-like, which
256 constrains tolerable mutations in SFTPC-like gene regulation [35]. Indeed, lung
257 loss may have resulted in relaxed stabilizing selection for SFTPC-like gene
258 regulation, thereby enabling the evolution of novel expression patterns. Greater
259 understanding of the evolution and function of SFTPC-like in additional
260 salamander species will yield a more complete picture of the evolution and
261 consequences of lung loss, while functional studies of SFTPC-like promise to
262 reveal whether this novel pulmonary surfactant protein plays similar roles to
263 SFTPC or has potential therapeutic applications. Given the convergent evolution
264 of lung loss in several amphibian lineages, it will be interesting to investigate the
265 molecular physiology of other lungless taxa. Neofunctionalization of SFTPC-like
266 may represent an additional mechanism by which plethodontid salamanders
267 have become one of the most speciose and geographically widespread clades of

268 vertebrates on earth, despite the theoretical limitations on thermal tolerance and
269 body size imposed by lunglessness.

270

271 **Materials and Methods:**

272 **Animal husbandry**

273 All animal protocols were reviewed and approved by Harvard's Institutional
274 Animal Care and use Committee (protocol 99-09). *Desmognathus fuscus*
275 (Northern dusky salamander) embryos were field-collected from the following two
276 localities under Massachusetts Department of Fish and Wildlife collection permits
277 080.11SCRA (2012), 027.13SCRA (2013), 083.14SCRA (2014), and
278 022.15SCRA (2015) and appropriate local permits: Ashfield, Mass. (42.483111, -
279 72.761263) and Mass Audubon Wachusett Meadows Preserve (42.450922, -
280 71.913009). Adults were collected from Willard Brook State Forest (42.671606, -
281 71.776156). *Plethodon cinereus* (Eastern red-backed salamander) embryos were
282 field collected from Willard Brook State Forest (42.671606, -71.776156).
283 *Desmognathus fuscus* embryos were maintained at 15°C in 0.1x Marc's Modified
284 Ringer solution (MMR; 0.01 M NaCl, 0.2 mM KCl, 0.1 mM MgSO₄, 0.2 mM CaCl₂,
285 0.5 mM HEPES pH 7.4). Following hatching, larvae were fed *Artemia* spp. and
286 maintained at 17–20°C until they metamorphosed at approximately seven
287 months post-hatching. Older larvae were hand-fed blood worms. Embryos and
288 larvae were sampled at intermediate stages from embryogenesis until 3–5 days
289 post-metamorphosis and fixed overnight in MEMFA (0.1 M MOPS pH 7.4, 2 mM
290 EGTA, 1 mM MgSO₄, 3.7% formaldehyde) at 4°C, then dehydrated and stored at

291 -20°C in 100% methanol. Adults were fixed in a similar manner immediately upon
292 collection.

293 *Ambystoma mexicanum* (Mexican axolotl) embryos were obtained from the
294 *Ambystoma* Genetic Stock Center, University of Kentucky, and maintained in
295 20% Holtfreter solution at 17°C. Larvae were raised similarly to larval
296 *Desmognathus fuscus*. Fixation was performed as described above.

297 *Ambystoma mexicanum* were staged according to [39,40]. *Desmognathus fuscus*
298 embryos were staged using a staging table derived for *Plethodon cinereus*, as
299 their developmental timing and morphology are grossly similar [41].

300 **PCR**

301 Embryonic cDNA from *Desmognathus fuscus*, *Plethodon cinereus*, and
302 *Ambystoma mexicanum* was used to clone the gene SFTPC-like. RNA was
303 isolated from homogenates of whole animals at a variety of embryonic stages
304 using TRIzol Reagent (Invitrogen/Thermo Fisher Scientific, Grand Island, N.Y.)
305 and reverse-transcribed to cDNA using iScript reverse transcriptase (BioRad,
306 Hercules, Calif.). The gene SFTPC was cloned from *A. mexicanum* and repeated
307 attempts were made to clone SFTPC from plethodontids. Degenerate and non-
308 degenerate PCR primers were used (Table S1).

309 Primers were designed based on alignment of SFTPC sequences from
310 *Desmognathus fuscus*, *Xenopus laevis*, *X. tropicalis*, *Anolis carolinensis*,
311 *Neovison vison*, *Bos taurus*, *Monodelphis domesticus*, and *Homo sapiens*. All

312 sequences but one were obtained from GenBank; the *D. fuscus* sequence was
313 kindly provided by Dr. David Weisrock.

314 **Transcriptome assembly**

315 Transcriptomes for *Plethodon cinereus* and *Ambystoma mexicanum* were
316 prepared from microdissected tissue from pharyngeal endoderm and mesoderm
317 of embryos and from the lung of a juvenile *A. mexicanum*. Total RNA was utilized
318 for library preparation by using the IntegenX PrepX RNA-Seq Library Kit
319 (IntegenX, Pleasanton, Calif.) on an Apollo 324 robotic sample preparation
320 system (WaferGen Biosystems, Fremont, Calif.), closely following kit instructions.
321 Agencourt Ampure XP beads were used for magnetic purification steps
322 (Beckman Coulter, Indianapolis, Ind.). Beads were kept at room temperature for
323 15 min before starting block setup. Beads were added last to the block, after a
324 30-sec vortex to fully resuspend them.

325 Following cDNA synthesis, the concentration of samples was assessed by using
326 a Qubit 1.0 fluorometer (Invitrogen/Thermo Fisher Scientific, Grand Island, N.Y.),
327 high-sensitivity dsDNA reagents (Molecular Probes/Thermo Fisher Scientific,
328 Grand Island, N.Y.), and Qubit Assay Tubes (Invitrogen). Samples were diluted
329 to 20 µg/ml and then sheared on a Covaris S220 Focused ultrasonicator
330 (Covaris, Woburn, Mass.) using a 72-sec protocol and targeting 220/320 bp
331 output. TapeStation HS D1K tape (Agilent) was used to examine sheared DNA
332 for optimal size range. BIOO Scientific NEXTflex DNA barcodes (#514102,
333 Austin, Tex.) were diluted to 5 µm and used in the IntegenX PrepX DNA Library
334 ILM prep kit (#P003279, Pleasanton, Calif.). Library prep was performed on the

335 Apollo 324 using the kit manufacturer's precise instructions. 5- μ l aliquots of ~2-
336 μ g/ml samples were then subjected to four PCR amplification cycles by using
337 NEB Next Master Mix (#M0541S, NEB, Ipswich, Mass.) and NEXTflex Primer
338 Mix (BIOO Scientific) and the following cycle conditions: denaturation at 98°C for
339 120 sec; 5 cycles of 98°C for 30 sec, 6°C for 30 sec, and 72°C for 60 sec; and
340 final extension for 5 min at 72°C. The Agilent Apollo 324 was used for cleanup of
341 PCR samples using the built-in PCR cleanup protocol and Agencourt Ampure XP
342 beads. Libraries were analyzed with Qubit 1.0, TapeStation and qPCR to assess
343 library concentration, size and quality. Samples were each diluted to 0.29 nM
344 concentration and then pooled. Two lanes of 2 x 150 bp Illumina HiSeq 2500
345 Rapid Run RNA-sequencing (Illumina, San Diego, Calif.) yielded a total 232.4
346 reads that passed filter.

347 Sequenced reads were trimmed with Trimmomatic [42] and concatenated.
348 Ribosomal rRNA reads were removed by using Bowtie [43] and a custom
349 database of known rRNA sequences for each species. Transcriptomes were
350 assembled *de novo* using Trinity [44,45]. BLAST databases were created from
351 the *de novo* assemblies and used to identify SFTPC and SFTPC-like sequences.

352 **SFTPC phylogeny**

353 SFTPC sequences were identified through BLASTX, TBLASTN and TBLASTX
354 searches of sequenced transcriptomes from this study (*Plethodon cinereus* and
355 *Ambystoma mexicanum*) as well as from the transcriptomes listed in Table S2.

356 Sequence identifiers and corresponding sequence data are provided as
357 Supplemental Data File 1.

358 SFTPC sequences from annotated genomes were also taken from NCBI and
359 ENSEMBL (Supplemental Data File 1). Outgroup proteins were selected based
360 on previous phylogenies of SFTPC [46,47]. Predicted amino acid sequences
361 were generated from all nucleotide sequences. Multiple sequence alignment was
362 performed using PRANK [48]; resulting alignments were visually inspected
363 (Supplemental Data File 2). ProtTest was used to identify an appropriate amino
364 acid substitution model [49]. The optimal amino acid substitution model was
365 JTT+G [50], as judged by AIC. Subsequently, 95% maximum clade credibility
366 gene trees were reconstructed in MrBayes (v3.2.6) [51] using Markov chain
367 Monte Carlo analysis with one million generations sampled every 100
368 generations and a relative burn-in of 25%. Convergence of the posterior
369 probabilities was assessed by examining output statistics, including the potential
370 scale reduction factor, which equaled or exceeded 1.000.

371 A phylogeny was also constructed in RAxML (v8.2.9) [52] by using 1000
372 bootstrap replicates and the aforementioned amino acid substitution model. Tree
373 topology was concordant with the Bayesian tree generated in Mr. Bayes.

374 JalView was used to generate the multiple sequence alignment image [53].

375 PHYLOGEN [54] was used to estimate gene duplication of SFTPC. A guide tree
376 was constructed using NCBI taxonomy for major groups and [55] for amphibian
377 relationships (Fig. S2). Topology optimization was not used.

378 ***In situ* hybridization**

379 Embryos were fixed overnight in 4% paraformaldehyde (PFA) or MEMFA at 4°C,
380 dehydrated and stored in 70% or 100% MeOH at -20°C. Wholemount mRNA *in*
381 *situ* hybridization (ISH) was performed by rehydrating samples, which were then
382 treated with 5–10 µg/ml proteinase K for 30–60 min, washed with PBTw (137 mM
383 NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ and 0.2% Tween-20),
384 post-fixed in 4% PFA, washed with PBTw, and pre-hybridized in hybridization
385 buffer for 2 hr at 65°C (hybridization buffer: 50% formamide, 5x SSC, 0.1 mg/ml
386 heparin, 1x Denhardt solution, 0.01% CHAPS, 0.2 mg/ml tRNA and 0.1% Tween-
387 20; all solutions were RNase-free). DIG-labeled riboprobes were diluted
388 approximately 1:40 in hybridization buffer, denatured at 85°C for 10 min, and
389 then added to specimens. Hybridization was carried out overnight at 65°C.
390 Posthybridization washes were performed with a solution of 50% formamide, 5x
391 SSC and 0.2% Tween-20 at 65°C for eight changes of 30 min each. Specimens
392 were washed with maleic acid buffer plus 0.2% Tween-20 (MABT) prior to
393 blocking and antibody incubation. Antibody block solution included 20% heat-
394 inactivated sheep serum and 2% blocking reagent (Roche, Penzberg, Germany)
395 in MABT. Samples were incubated overnight at 4°C with 1:2500 anti-DIG-AP Fab
396 fragments (Roche) diluted in blocking solution. Extensive washes with MABT
397 were performed prior to color development using BM-Purple (Roche) or
398 NBT/BCIP (Sigma, St. Louis, Mo.). Color development occurred over several
399 hours. Embryos were then embedded for cryosectioning at 14–16 µm thickness.
400 Photographs were taken using a Leica DMRE microscope (Wetzlar, Germany)
401 equipped with a QImaging Retiga 2000r camera and a QImaging RGB slider

402 (Model: RGB-HM-S-IR; Surrey, Canada) and Volocity 6.0 software (PerkinElmer,
403 Waltham, Mass.).

404 **Structure models**

405 An experimentally determined protein data bank (PDB) model for porcine SFTPC
406 [16] was downloaded from the Research Collaboratory for Structural
407 Bioinformatics PDB. The secondary and supersecondary structure of
408 *Desmognathus fuscus* SFTPC-like was predicted using Quark *Ab initio* Protein
409 Structure Prediction [56]. The N- and C-terminal extent of the *Desmognathus*
410 SFTPC-like sequence was chosen based on alignment with mature forms of
411 SFTPC found in mammals. Protein structure prediction was also performed with
412 I-TASSER [57].

413 A structure model for *D. fuscus* SFTPC-like was also predicted in SWISS-
414 MODEL [58] using the molecular structure derived by Johansson et al. [16] as a
415 template. The PDB files for each SFTPC-like model were imported into PyMol
416 (Schrödinger) and aligned with the SFTPC model to graphically illustrate
417 structural similarities.

418 **Transmission electron microscopy**

419 Two 24-mm (total length) *Desmognathus fuscus* larvae were euthanized and
420 decapitated. Specimens were then dissected in fixative (2.5% glutaraldehyde and
421 2% paraformaldehyde in 0.1 M HEPES; the aldehydes were free of alcohol
422 stabilizers). The head was cut into three 1-mm sagittal sections. An 18-cm adult
423 *Ambystoma mexicanum* was euthanized and then dissected in fixative. Samples

424 of the gular skin from the ventral head, the oral epithelium and the lungs were
425 trimmed to 1-mm-thick pieces in fixative and fixed as above.

426 The samples were left in fixative for 3 d and then washed twice quickly with 0.1 M
427 HEPES and three times for 5 min each with Milli-Q H₂O (mqH₂O). Next, samples
428 were fixed for 24 hr at 4°C in aqueous 1% osmium tetroxide, followed by five
429 washes in mqH₂O for 5 min each. Subsequently, specimens were stained with
430 2% uranyl acetate (EMS, Hatfield, Pa.) overnight at 4°C, then washed two times
431 for 5 min each with mqH₂O. Specimens were dehydrated with 5-min washes of
432 50%, 70% and 95% ethanol, followed by three 10-min washes with 100%
433 ethanol, then two quick rinses with propylene oxide (PO). Specimens were
434 embedded in Embed 812 resin (EMS) formulated to medium hardness by rinsing
435 30 min each in 1:1 PO to Embed 812, 1:2 PO to Embed 812, then 60 min in 1:4
436 PO to Embed 812. Specimens were then transferred to 100% Embed 812 and
437 incubated overnight at room temperature, followed by two subsequent changes
438 of Embed 812, over a total embedding time of 48 hr. Samples were then
439 positioned in molds and placed at 60°C for 3 d to polymerize.

440 Sectioning was performed on a Leica UCT ultramicrotome, using glass knives for
441 trimming blocks and generating semi-thin (1 µm) sections, and a DiATOME
442 diamond knife for generating thin sections of approximately 60–100 nm thickness
443 (target thickness: 80 nm). Sections were flattened with chloroform vapor,
444 transferred onto precoated Formvar/carbon 200 mesh copper grids (#01803F,
445 Ted Pella, Redding, Calif.), and dried on filter paper.

446 Grids were imaged with an FEI Tecnai G2 series F20 transmission electron
447 microscope (Hillsboro, Ore.) at 80 kV using a Gatan CCD camera and Gatan
448 Digital Micrograph Software (Pleasanton, Calif.).

449 **Data Availability**

450 All data are available as Supplemental Data Files 1 and 2.

451

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460

461 **Author contributions:** Z.R.L. cloned SFTPC and SFTPC-like from *A.*
462 *mexicanum* and *P. cinereus*, generated and analyzed transcriptomes, performed
463 phylogenetic and structural analyses, performed TEM, collected and raised
464 animals, generated gene expression data and participated in writing the
465 manuscript. J.A.D. cloned SFTPC-like from *D. fuscus* and assisted with
466 characterizing the expression of SFTPC-like. J.H. participated in the data
467 analyses and writing of the manuscript.

468

469 **Competing interests:** The authors declare that they have no competing financial
470 interests.

471

472 **References:**

- 473 1. Whitford WG, Hutchison VH. 1965 Gas exchange in salamanders. *Physiol.*
474 *Zool.* **38**, 228–242.
- 475 2. Gatz RN, Crawford EC, Piiper J. 1974 Respiratory properties of the blood
476 of a lungless and gill-less salamander, *Desmognathus fuscus*. *Respir.*
477 *Physiol.* **20**, 33–41.
- 478 3. Feder ME, Burggren WW. 1985 Cutaneous gas exchange in vertebrates:
479 design, patterns, control and implications. *Biol. Rev. Camb. Philos. Soc.*
480 **60**, 1–45.
- 481 4. Bickford D, Iskandar D, Barlian A. 2008 A lungless frog discovered on
482 Borneo. *Curr. Biol.* **18**, 374–375.
- 483 5. Wilder IW, Dunn E. 1920 The correlation of lunglessness in salamanders
484 with a mountain brook habitat. *Copeia* **1920**, 63–68.
- 485 6. Reagan NL, Verrell PA. 1991 The evolution of plethodontid salamanders:
486 did terrestrial mating facilitate lunglessness? *Am. Nat.* **138**, 1307–1313.
- 487 7. Czopek J. 1965 Quantitative studies on the morphology of respiratory
488 surfaces in amphibians. *Acta Anat. (Basel)*. **62**, 296–323.
- 489 8. Seelye A. 1906 Circulatory and respiratory systems of *Desmognathus*
490 *fusca*. *Proc. Bost. Soc. Nat. Hist.* **32**, 335–357.
- 491 9. Barrows AI. 1900 Respiration of *Desmognathus*. *Anat. Anz.* **18**, 461–464.

- 492 10. Sheafor EA, Wood SC, Tattersall GJ. 2000 The effect of graded hypoxia on
493 the metabolic rate and buccal activity of a lungless salamander
494 (*Desmognathus fuscus*). *J. Exp. Biol.* **203**, 3785–3793.
- 495 11. Lombard RE, Wake DB. 1986 Tongue evolution in the lungless
496 salamanders, family Plethodontidae IV. Phylogeny of plethodontid
497 salamanders and the evolution of feeding dynamics. *Syst. Zool.* **35**, 532–
498 551.
- 499 12. Wake DB. 1982 Functional and developmental constraints and
500 opportunities in the evolution of feeding systems in urodeles. In
501 *Environmental Adaptation and Evolution* (eds D Mossakowski, G Roth), pp.
502 51–66. Stuttgart: Gustav Fisher.
- 503 13. Daniels CB, Orgeig S, Smits AW. 1995 The evolution of the vertebrate
504 pulmonary surfactant system. *Physiol. Zool.* **68**, 539–566.
- 505 14. Whitsett JA, Weaver TE. 2002 Hydrophobic surfactant proteins in lung
506 function and disease. *N. Engl. J. Med.* **347**, 2141–2148.
- 507 15. Olmeda B, Villén L, Cruz A, Orellana G, Perez-Gil J. 2010 Pulmonary
508 surfactant layers accelerate O₂ diffusion through the air-water interface.
509 *Biochim. Biophys. Acta - Biomembr.* **1798**, 1281–1284.
- 510 16. Johansson J, Szyperski T, Curstedt T, Wüthrich K. 1994 The NMR
511 structure of the pulmonary surfactant-associated polypeptide SP-C in an
512 apolar solvent contains a valyl-rich alpha-helix. *Biochemistry* **33**, 6015–
513 6023.
- 514 17. Hafner D, Germann PG, Hauschke D, Kilian U. 1999 Effects of early

- 515 treatment with rSP-C surfactant on oxygenation and histology in rats with
516 acute lung injury. *Pulm. Pharmacol. Ther.* **12**, 193–201.
- 517 18. Rice WR, Sarin VK, Fox JL, Baatz J, Wert S, Whitsett JA. 1989 Surfactant
518 peptides stimulate uptake of phosphatidylcholine by isolated cells. *Biochim.*
519 *Biophys. Acta* **1006**, 237–245.
- 520 19. Weaver TE, Whitsett JA. 1991 Function and regulation of expression of
521 pulmonary surfactant-associated proteins. *Biochem. J.* **273**, 249–264.
- 522 20. Guyton AC, Moffatt DS, Adiar TH. 1984 Role of alveolar surface tension in
523 transepithelial movement of fluid. In *Pulmonary Surfactant* (eds B
524 Robertson, L Van Golde, J Batenburg), pp. 171–185. Amsterdam: Elsevier
525 Science Publishers.
- 526 21. Ultsch GR, Gros G. 1979 Mucus as a diffusion barrier to oxygen: Possible
527 role in O₂ uptake at low pH in carp (*Cyprinus carpio*) gills. *Comp. Biochem.*
528 *Physiol. Part A Physiol.* **62**, 685–689.
- 529 22. Sosnowski T, Gradon L, Skoczek M, Drozdiel H. 1998 Experimental
530 evaluation of importance of the pulmonary surfactant for oxygen transfer
531 rate in human lungs. *Int. J. Occup. Saf. Ergon.* **4**, 391–409.
- 532 23. Bourbon JR, Chailley-Heu B. 2001 Surfactant proteins in the digestive
533 tract, mesentery, and other organs: evolutionary significance. *Comp.*
534 *Biochem. Physiol. A, Mol. Integr. Physiol.* **129**, 151–161.
- 535 24. Fisher JH, Shannon JM, Hofmann T, Mason RJ. 1989 Nucleotide and
536 deduced amino acid sequence of the hydrophobic surfactant protein SP-C
537 from rat: expression in alveolar type II cells and homology with SP-C from

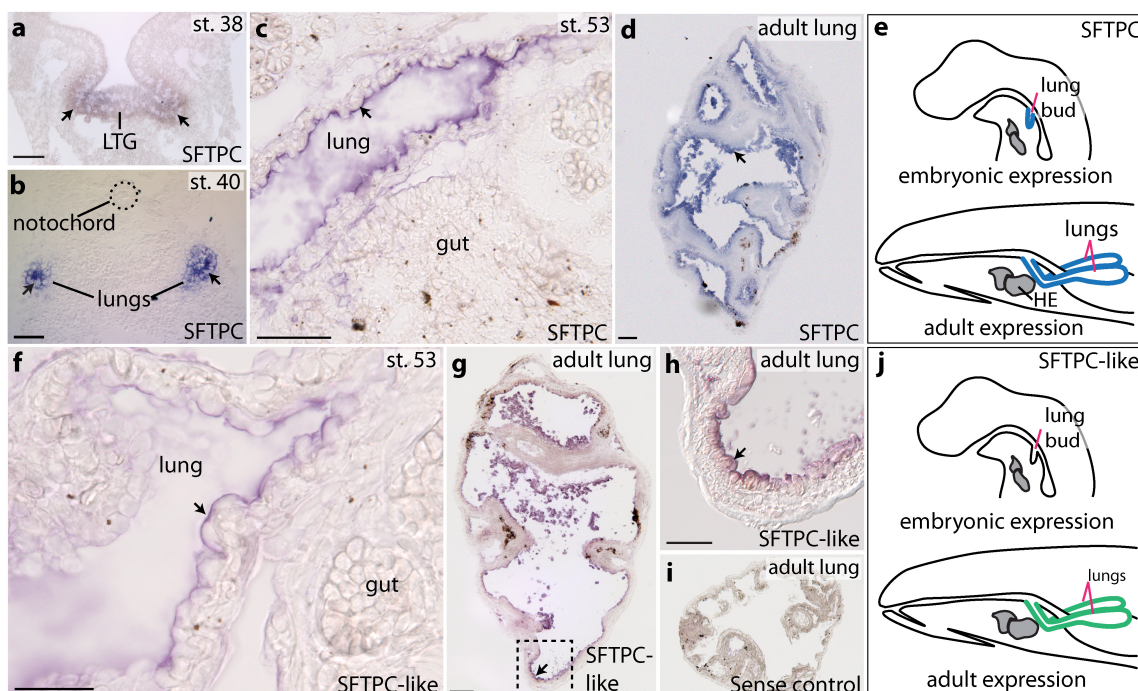
- 538 other species. *Biochim. Biophys. Acta* **995**, 225–30.
- 539 25. Wert SE, Glasser SW, Korfhagen TR, Whitsett JA. 1993 Transcriptional
540 elements from the human SP-C gene direct expression in the primordial
541 respiratory epithelium of transgenic mice. *Dev. Biol.* **156**, 426–443.
- 542 26. Wilder IW. 1925 *The Morphology of Amphibian Metamorphosis*.
543 Northampton: Smith College.
- 544 27. Lewis ZR. 2016 Causes and Consequences of Lung Loss in Salamanders.
545 PhD Dissertation. Harvard University.
- 546 28. Schmitz G, Müller G. 1991 Structure and function of lamellar bodies, lipid-
547 protein complexes involved in storage and secretion of cellular lipids. *J.*
548 *Lipid Res.* **32**, 1539–1570.
- 549 29. Matoltsy AG, Bednarz JA. 1975 Lamellar bodies of the turtle epidermis. *J.*
550 *Ultrastructure Res.* **53**, 128–132.
- 551 30. Grayson S, Johnson-Winegar AG, Wintroub BU, Isseroff RR, Epstein EH,
552 Elias PM. 1985 Lamellar body-enriched fractions from neonatal mice:
553 preparative techniques and partial characterization. *J. Invest. Dermatol.* **85**,
554 289–294.
- 555 31. Bryant DM *et al.* 2017 A tissue-mapped axolotl de novo transcriptome
556 enables identification of limb regeneration factors. *Cell Rep.* **18**, 762–776.
- 557 32. Daniels CB, Orgeig S, Wilsen J, Nicholas TE. 1994 Pulmonary-type
558 surfactants in the lungs of terrestrial and aquatic amphibians. *Respir.*
559 *Physiol.* **95**, 249–258.
- 560 33. Cass AN, Servetnick MD, McCune AR. 2013 Expression of a lung

- 561 developmental cassette in the adult and developing zebrafish swimbladder.
562 *Evol. Dev.* **132**, 119–132.
- 563 34. Conant GC, Wolfe KH. 2008 Turning a hobby into a job: How duplicated
564 genes find new functions. *Nat. Rev. Genet.* **9**, 938–950.
- 565 35. Lan X, Pritchard JK. 2016 Coregulation of tandem duplicate genes slows
566 evolution of subfunctionalization in mammals. *Science* **352**, 1009–13.
- 567 36. Nehrt NL, Clark WT, Radivojac P, Hahn MW. 2011 Testing the ortholog
568 conjecture with comparative functional genomic data from mammals. *PLoS*
569 *Comput. Biol.* **7**, e1002073.
- 570 37. Hunter JP. 1998 Key innovations and the ecology of macroevolution.
571 *Trends Ecol. Evol.* **13**, 31–36.
- 572 38. Lewis ZR, Hanken J. 2017 Convergent evolutionary reduction of atrial
573 septation in lungless salamanders. *J. Anat.* **230**, 16–29.
- 574 39. Bordzilovskaya N, Dettlaff T, Duhon S, Malacinski G. 1989 Developmental-
575 stage series of axolotl embryos. In *Developmental Biology of the Axolotl*
576 (eds J Armstrong, G Malacinski), pp. 201–219. Oxford: Oxford University
577 Press.
- 578 40. Nye HLD, Cameron JA, Chernoff EAG, Stocum DL. 2003 Extending the
579 table of stages of normal development of the axolotl: limb development.
580 *Dev. Dyn.* **226**, 555–60.
- 581 41. Kerney R. 2011 Embryonic staging table for a direct-developing
582 salamander, *Plethodon cinereus* (Plethodontidae). *Anat. Rec.* **294**, 1796–
583 808.

- 584 42. Bolger AM, Lohse M, Usadel B. 2014 Trimmomatic: a flexible trimmer for
585 Illumina sequence data. *Bioinformatics* **30**, 2114–2120.
- 586 43. Langmead B, Trapnell C, Pop M, Salzberg SL. 2009 Ultrafast and memory-
587 efficient alignment of short DNA sequences to the human genome.
588 *Genome Biol.* **10**, R25.
- 589 44. Grabherr MG *et al.* 2011 Full-length transcriptome assembly from RNA-
590 Seq data without a reference genome. *Nat. Biotechnol.* **29**, 644–652.
- 591 45. Haas BJ *et al.* 2013 De novo transcript sequence reconstruction from RNA-
592 seq using the Trinity platform for reference generation and analysis. *Nat.*
593 *Protoc.* **8**, 1494–512.
- 594 46. Hughes AL. 2007 Evolution of the lung surfactant proteins in birds and
595 mammals. *Immunogenetics* **59**, 565–72.
- 596 47. Hedlund J, Johansson J, Persson B. 2009 BRICHOS - a superfamily of
597 multidomain proteins with diverse functions. *BMC Res. Notes* **2**, 180.
- 598 48. Löytynoja A, Goldman N. 2010 webPRANK: a phylogeny-aware multiple
599 sequence aligner with interactive alignment browser. *BMC Bioinformatics*
600 **11**, 579.
- 601 49. Abascal F, Zardoya R, Posada D. 2005 ProtTest: selection of best-fit
602 models of protein evolution. *Bioinformatics* **21**, 2104–2105.
- 603 50. Jones DT, Taylor WR, Thornton JM. 1992 The rapid generation of mutation
604 data matrices from protein sequences. *Bioinformatics* **8**, 275–282.
- 605 51. Ronquist F *et al.* 2012 MrBayes 3.2: Efficient Bayesian phylogenetic
606 inference and model choice across a large model space. *Syst. Biol.* **61**,

- 607 539–542.
- 608 52. Stamatakis A. 2006 RAxML-VI-HPC: maximum likelihood-based
609 phylogenetic analyses with thousands of taxa and mixed models.
610 *Bioinformatics* **22**, 2688–90.
- 611 53. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. 2009
612 Jalview Version 2—a multiple sequence alignment editor and analysis
613 workbench. *Bioinformatics* **25**, 1189–1191.
- 614 54. Boussau B, Szollosi GJ, Duret L, Gouy M, Tannier E, Daubin V. 2013
615 Genome-scale coestimation of species and gene trees. *Genome Res.* **23**,
616 323–330.
- 617 55. Pyron RA, Wiens JJ. 2011 A large-scale phylogeny of Amphibia including
618 over 2,800 species, and a revised classification of extant frogs,
619 salamanders, and caecilians. *Mol. Phylogenet. Evol.* **61**, 543–583.
- 620 56. Xu D, Zhang Y. 2012 Ab initio protein structure assembly using continuous
621 structure fragments and optimized knowledge-based force field. *Proteins*
622 **80**, 1715–1735.
- 623 57. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. 2015 The I-TASSER
624 Suite: protein structure and function prediction. *Nat. Methods* **12**, 7–8.
- 625 58. Arnold K, Bordoli L, Kopp J, Schwede T. 2006 The SWISS-MODEL
626 workspace: a web-based environment for protein structure homology
627 modelling. *Bioinformatics* **22**, 195–201.
- 628

629 **Figures:**



630

631 **Figure 1. Expression patterns of Surfactant-Associated Protein C (SFTPC)**

632 **and SFTPC-like in a lunged salamander, *Ambystoma mexicanum*,**

633 **visualized with antisense wholemount *in-situ* hybridization.** Arrows point to

634 representative regions of expression. SFTPC is expressed in the embryonic

635 laryngotracheal groove (LTG) **a**, or lungs **b–d**, of all stages examined between

636 embryo (st. 40), juvenile (st. 53) and adult. **f–h**, SFTPC-like is also expressed

637 specifically in the lung in juveniles and adults, but at a lower level than SFTPC; it

638 is not expressed in embryos. **h**, Boxed region in **g**. **i**, Negative (sense) control run

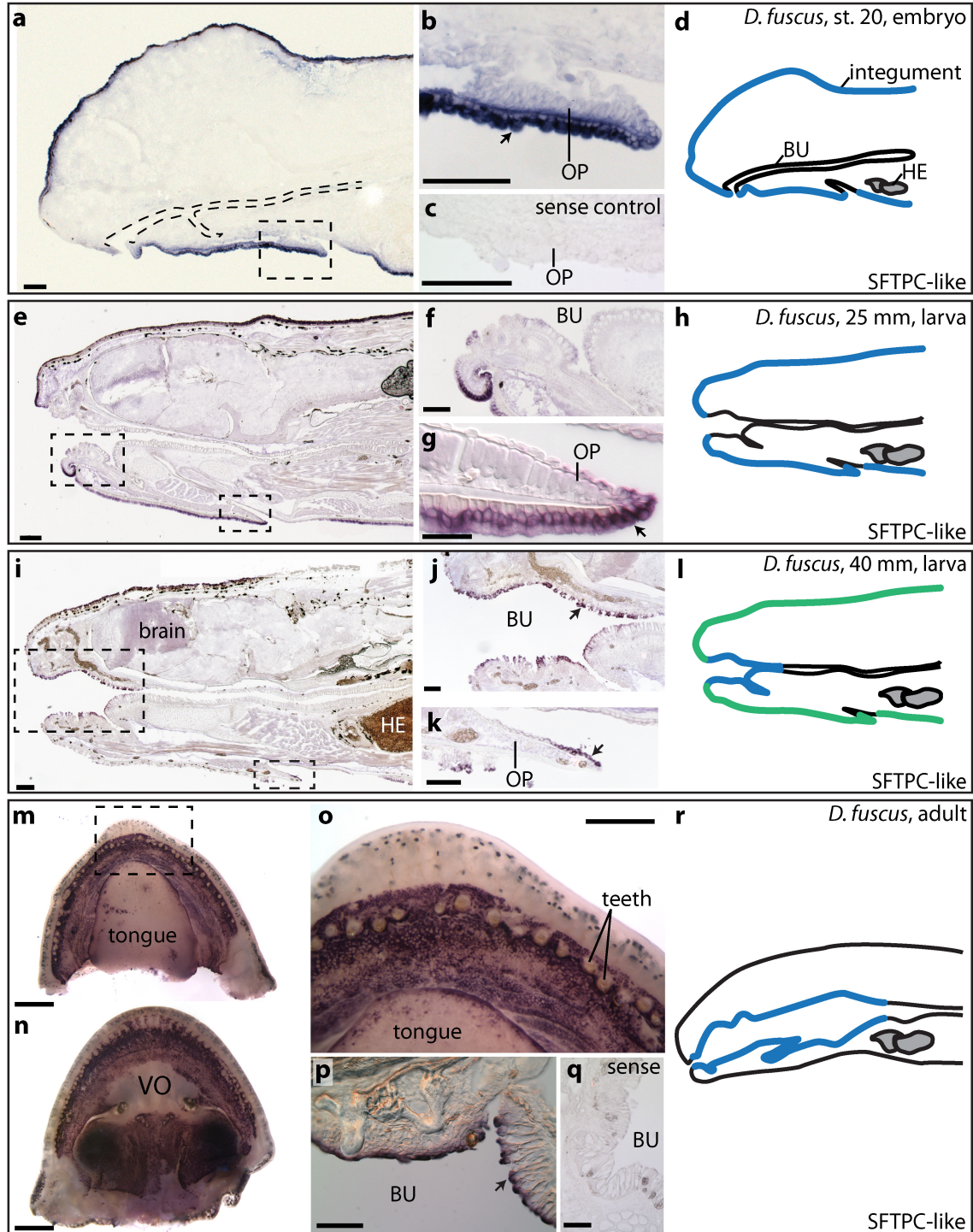
639 in parallel with SFTPC-like. **e, j**, Schematic sagittal views summarize the

640 expression sites of SFTPC and SFTPC-like. Blue regions denote high

641 expression; green indicates lower level. **a, b, d** and **g–i** depict transverse

642 sections; **c** and **f** are sagittal sections, anterior to the left. Scale bars: 100 μm.

643 Additional abbreviation: HE, heart.



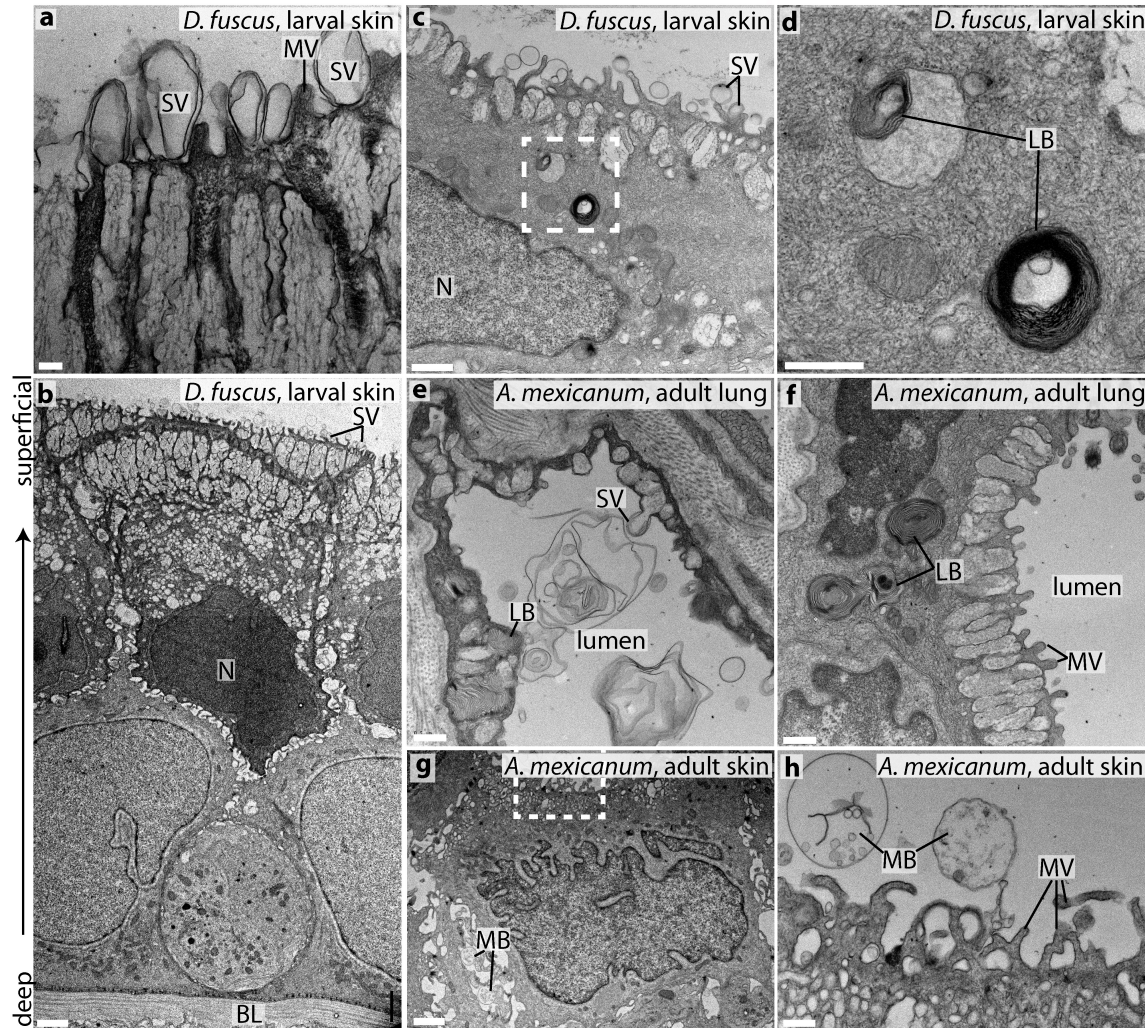
644

645 **Figure 2. Expression of Surfactant-Associated Protein C-like (SFTPC-like)**

646 **in the lungless salamander *Desmognathus fuscus*, visualized with**

647 **antisense wholemount *in-situ* hybridization. Arrows point to representative**

648 regions of expression. **a–c**, In embryos, SFTPC-like is expressed in the
649 integument. **b**, Boxed region in **a**. **c**, Negative (sense) control. **e–g**, SFTPC-like is
650 expressed in the larval integument, 25 mm total length. **i–k**, SFTPC-like in a larva
651 just prior to metamorphosis, 40 mm total length. Expression has declined in the
652 integument but is now present in the buccopharyngeal mucosa (BU). **m–q**, Adult
653 expression of SFTPC-like is confined to the buccopharyngeal cavity. **d, h, l, r**,
654 Schematic sagittal views summarize the expression sites of SFTPC-like. Blue
655 regions correspond to high expression; green indicates lower level. **a–l** are
656 sagittal sections, anterior to the left; **p** and **q** are transverse sections. Scale bars:
657 **a–c, e, f, i–k, p, q**, 100 μm ; **g**, 50 μm ; **m, n**, 1 mm; **o**, 0.5 mm. Additional
658 abbreviations: HE, heart; OP, opercular covering; VO, vomer.



659

660 **Figure 3. Secretory activity and lamellar bodies in the larval integument of**
661 **lungless *Desmognathus fuscus* resemble those in the lung of *Ambystoma***
662 ***mexicanum*. a–d, Transmission electron micrographs of a 24-mm *D. fuscus*. a,**
663 **The superficial (apical) surface is covered with secretory vesicles (SV), which**
664 **emerge from columnar vacuolar structures, and is interspersed with microvilli**
665 **(MV). b, Sagittal section through the epidermis; the superficial surface points**
666 **upwards. c,d, Lamellar bodies (LB), indicative of surfactant production, are**
667 **visible in the integument. The boxed region in c is enlarged in d. e,f, Lamellar**
668 **bodies and secretory vesicles in the distal portion of the lung of an adult A.**

669 *mexicanum*; transverse section. **g,h**, Transverse section of gular integument of
670 adult *A. mexicanum*. The boxed region in **g** is enlarged in **h**. Multivesicular
671 bodies (MB) are visible in extracellular spaces in **g** and external to the skin in **h**,
672 but there is no indication of active secretion of vesicles. The integument does not
673 play a pronounced secretory role in this species. Additional abbreviations: BL,
674 basal lamina; N, nucleus. Scale bars: **a**, 200 nm; **b, g**, 2 μ m; **c**, 1 μ m; **d, e, f, h**,
675 500 nm.

1 **Supporting Information**

2

3 **Supplemental Text**

4

5 **SFTPC expression specificity**

6 The expression pattern of SFTPC is highly conserved: all tetrapods express
7 SFTPC exclusively in the lungs [1–9]. In anamniotes, SFTPC is expressed
8 throughout the lung [5,6,10], whereas in mammals it is confined to alveolar type II
9 cells. Four reports cite expression of SFTPC outside of the lungs in humans, but
10 each report has methodological problems, including possible contamination, that
11 may make such claims unreliable. Mo et al. (2006) report SFTPC in human fetal
12 and adult skin [11]. This claim, however, relies on immunohistochemical data
13 obtained with an antibody that may yield spurious labeling, and on RT-PCR data
14 that was not followed up with sequencing. RT-PCR is subject to contamination
15 and mispriming. Bräuer et al. (2009) report SFTPC expression in submandibular
16 and parotid glands based on RT-PCR and western immunoblots [12]. The
17 western blots reveal a protein of the expected size of the SFTPC pro-protein, and
18 RT-PCR of focal cDNA is performed alongside a positive control, but there is no
19 follow-up sequencing. Schicht et al. (2015) report SFTPC in saliva from human
20 patients based on western blots and ELISA [13]. The antibody used to detect
21 SFTPC is not identified, however, and the isolated band is at 16 kDa while
22 SFTPC proprotein is 21 kDa and mature SFTPC 3.7 kDa [14]. Additionally, saliva
23 may be subject to contamination by surfactant produced in the lungs. Finally,
24 Schob et al. (2013) report SFTPC expression in central nervous system tissue
25 and cerebrospinal fluid based on RT-PCR, but they fail to rule out the possibility
26 of genomic DNA contamination [15]. Their western blots also fail to demonstrate
27 a band at the expected size for SFTPC given the antibody they employed, and
28 the authors express confusion about how mRNA for SFTPC could be present in
29 cerebrospinal fluid. In sum, there are problems with all recent studies that cite
30 extrapulmonary expression of SFTPC in humans. At the same time, numerous
31 studies in mammals and frogs, including ISH and reporter knockins, have failed
32 to demonstrate extrapulmonary expression of SFTPC. For instance, a mouse line
33 with Cre recombinase knocked in to the SFTPC locus fails to label cells outside
34 of the lung when crossed to a reporter line [16]. Nevertheless, it remains possible
35 that humans and perhaps other animals endogenously express SFTPC outside
36 of the lungs. Optimally, *in situ* hybridization and transcriptome sequencing should
37 be used to validate the human results presented above.

38

39

40 **Evidence for Duplication of SFTPC**

41

42 Both SFTPC and SFTPC-like diverge within exonic regions, but not according to
43 putative splice boundaries (Fig. S1a), which indicates that SFTPC-like is not an
44 isoform of SFTPC. While SFTPC-like is divergent from SFTPC sequences, it is
45 not an ortholog of a closely related BRICHOS domain-containing gene (Fig.
46 S1b). We found SFTPC-like expressed in ten species of lunged and lungless
47 salamanders (Fig. S1a, b; Supplemental Data File 1), most of which also express
48 SFTPC. SFTPC-like is not found in genomes or transcriptomes of any other
49 tetrapods, or even other vertebrates.

50

51 The most parsimonious explanation for the corresponding gene tree is that the
52 tetrapod ortholog of SFTPC was duplicated in the salamander lineage following
53 its divergence from frogs, followed by substantial sequence divergence between
54 SFTPC and SFTPC-like (Fig. S1b). Low statistical support at the SFTPC-like
55 node should be interpreted as a polytomy, and long-branch attraction likely has
56 caused an artifactual affiliation between coelacanth and lungfish SFTPC
57 orthologs and salamander SFTPC-like. We applied PHYLOG [17] to explicitly
58 test for gene duplications of SFTPC. Given a guide tree with known phylogenetic
59 relationships (Fig. S2), PHYLOG predicts that SFTPC-like arose by gene
60 duplication (Fig. S3). SFTPC-like has been meiotically mapped to linkage group
61 6 in *Ambystoma mexicanum*, a lunged salamander, and is located within a region
62 syntenic to human chromosome 15 [18]. SFTPC-like and SFTPC have been
63 assembled to two separate genomic scaffolds from *A. mexicanum* [19],
64 supporting SFTPC-like's origin via gene duplication.

65 **Supporting Information Tables:**

66

67 **Table S1. Primers used to clone SFTPC and SFTPC-like from *Ambystoma***
 68 ***mexicanum*, *Desmognathus fuscus* and *Plethodon cinereus*.**

Gene	Species	Forward	Reverse
SFTPC	<i>A. mexicanum</i>	5'-CAC ACA GAR AMG ATT TTC CAG ATG-3'	5'-CGT CTT GTC CAT TTT TGT KAB GTA GCA-3'
SFTPC-like	<i>A. mexicanum</i>	5'-AAG ATG GAA ACC GGC AGC AAG C-3'	5'-CGT CTT GTC CAT TTT TGT KAB GTA GCA-3'
SFTPC-like	<i>D. fuscus</i>	5'-AAG ATG GAA ACC GGC AGC AAG C-3'	5'-AGT ATT GGA AGC GGT CTG GGT G-3'
SFTPC-like	<i>P. cinereus</i>	5'-AAG ATG GAA ACC GGC AGC AAG C-3'	5'-GGT GTA GTC ATA GAC CAC-3'

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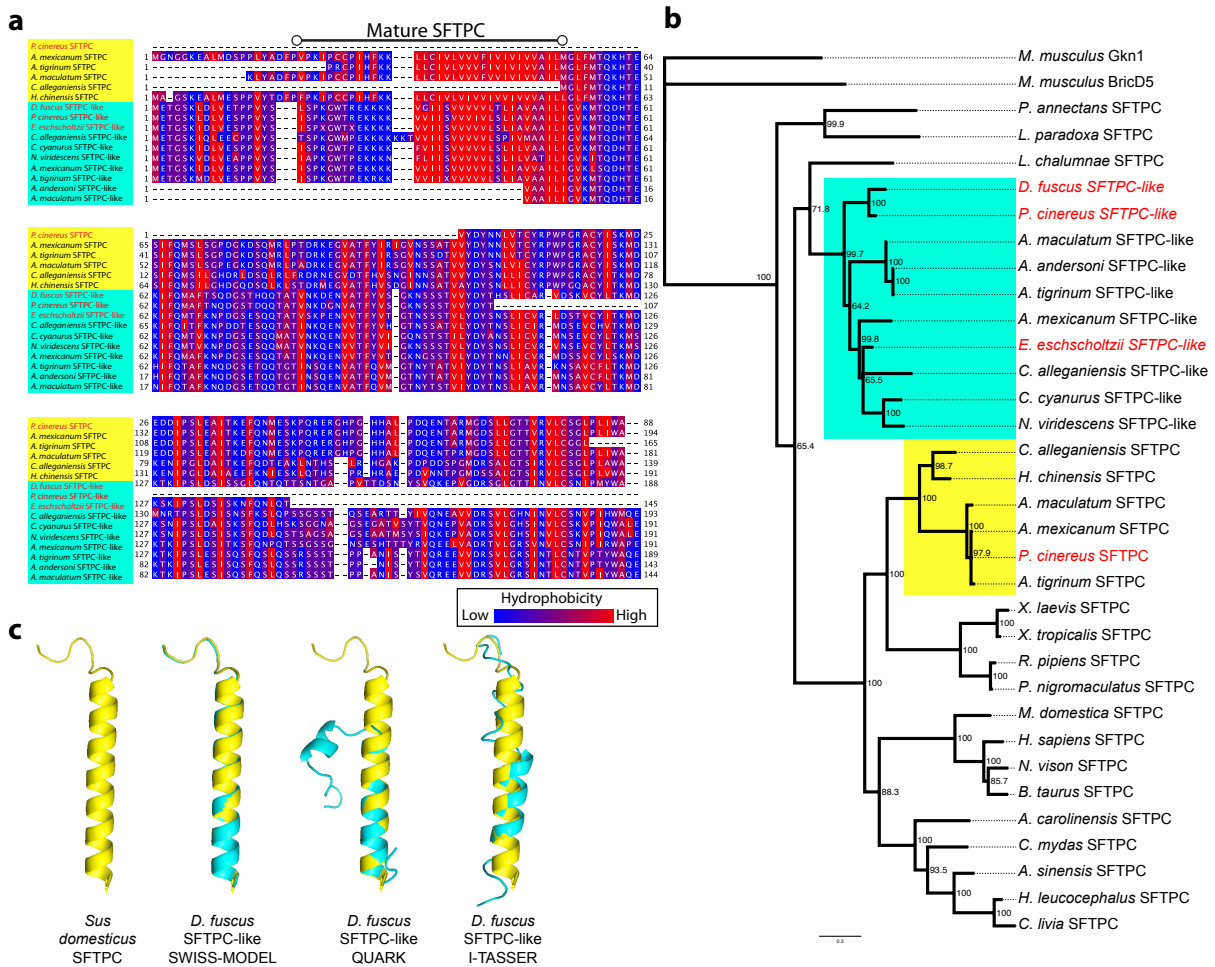
71 **Table S2. Transcriptomes used to identify SFTPC and SFTPC-like.**

Species	Transcriptome Source
<u>Salamanders</u>	
<i>Ambystoma andersoni</i>	Ryan Woodcock and Randal Voss
<i>Ambystoma mexicanum</i>	Present study
<i>Ambystoma tigrinum</i>	Ryan Woodcock and Randal Voss
<i>Ambystoma tigrinum</i>	[20]
<i>Cryptobranchus alleganiensis bishopi</i>	David Weisrock and Paul Hime
<i>Cynops cyanurus</i>	David Weisrock and Paul Hime
<i>Desmognathus fuscus</i>	David Weisrock and Justin Kratovil
<i>Ensatina eschscholtzii</i>	Rachel Mueller [21]
<i>Hynobius chinensis</i>	[22], reassembled by Paul Hime
<i>Hynobius retardatus</i>	[23]
<i>Notophthalmus viridescens</i>	[24]
<i>Plethodon cinereus</i>	Present study
<u>Frogs</u>	
<i>Pelophylax nigromaculatus</i>	[25]
<i>Rana (Lithobates) pipiens</i>	[26]
<u>Dipnoi</u>	
<i>Lepidosiren paradoxa</i>	Igor Schneider [27]
<i>Protopterus annectans</i>	Chris Amemiya [27]

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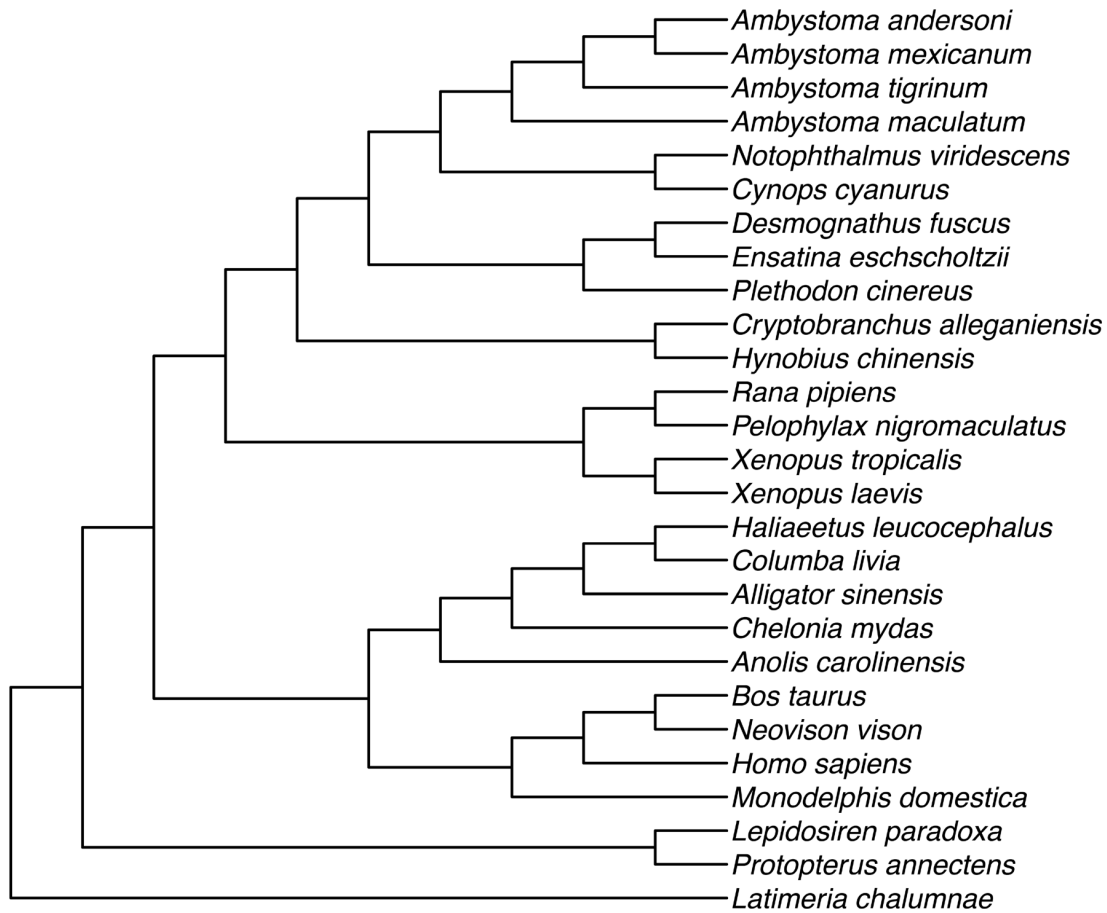
73 **Supporting Information Figures:**

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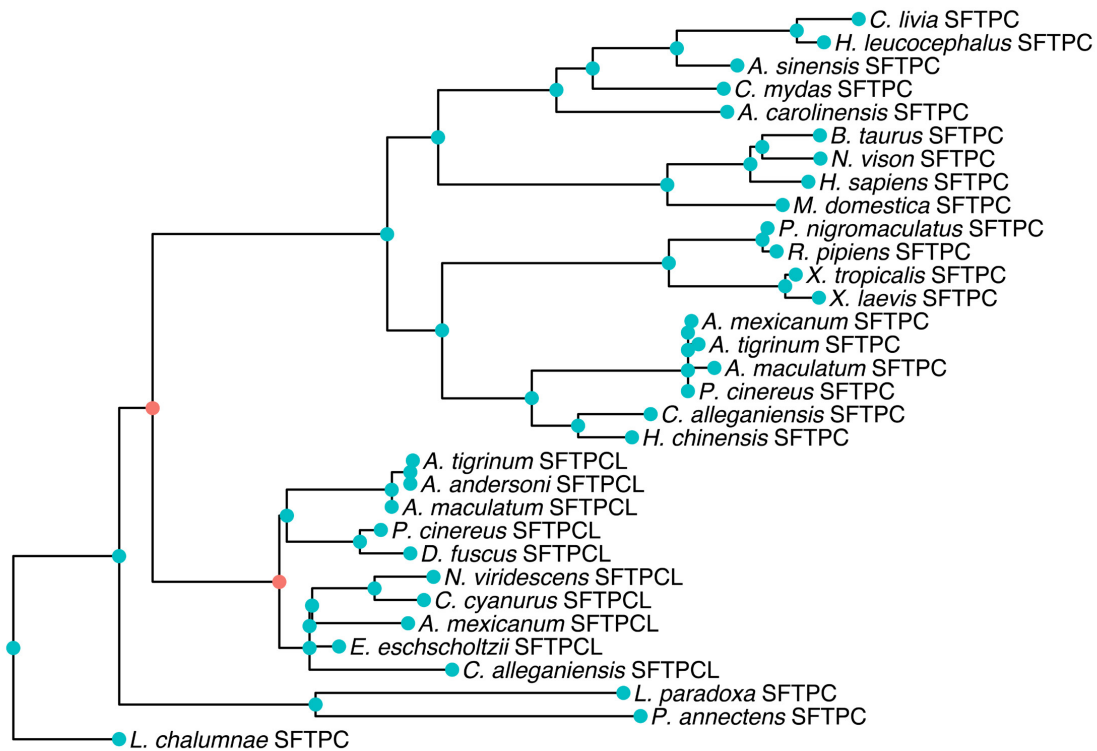
76 **Figure S1. A novel form of Surfactant-Associated Protein C (SFTPC) is**
 77 **expressed in several species of salamanders. a,** Amino acid alignment of
 78 SFTPC (yellow) and SFTPC-like (cyan) sequences reveals conservation of
 79 hydrophobic residues within the mature peptide domain. Full species names and
 80 accession numbers are listed in Supplemental Data File 1. Lungless
 81 (plethodontid) species are in red font. **b,** Bayesian 95% maximum clade
 82 credibility tree for SFTPC reveals SFTPC-like transcripts in 10 species of
 83 salamanders. SFTPC-like is not a related ortholog because it is nested within the
 84 SFTPC phylogeny. SFTPC-like is not found in any genome or transcriptome
 85 outside of salamanders. Node values are posterior probabilities; scale bar is
 86 expected changes per site. **c,** Predicted secondary structure of SFTPC-like from
 87 *Desmognathus fuscus*. SFTPC-like structure predictions (cyan) utilizing SWISS-
 88 MODEL [28], QUARK *Ab initio* predictions [29] and I-TASSER [30] are aligned
 89 with the resolved SFTPC mature peptide (yellow) [31].



90

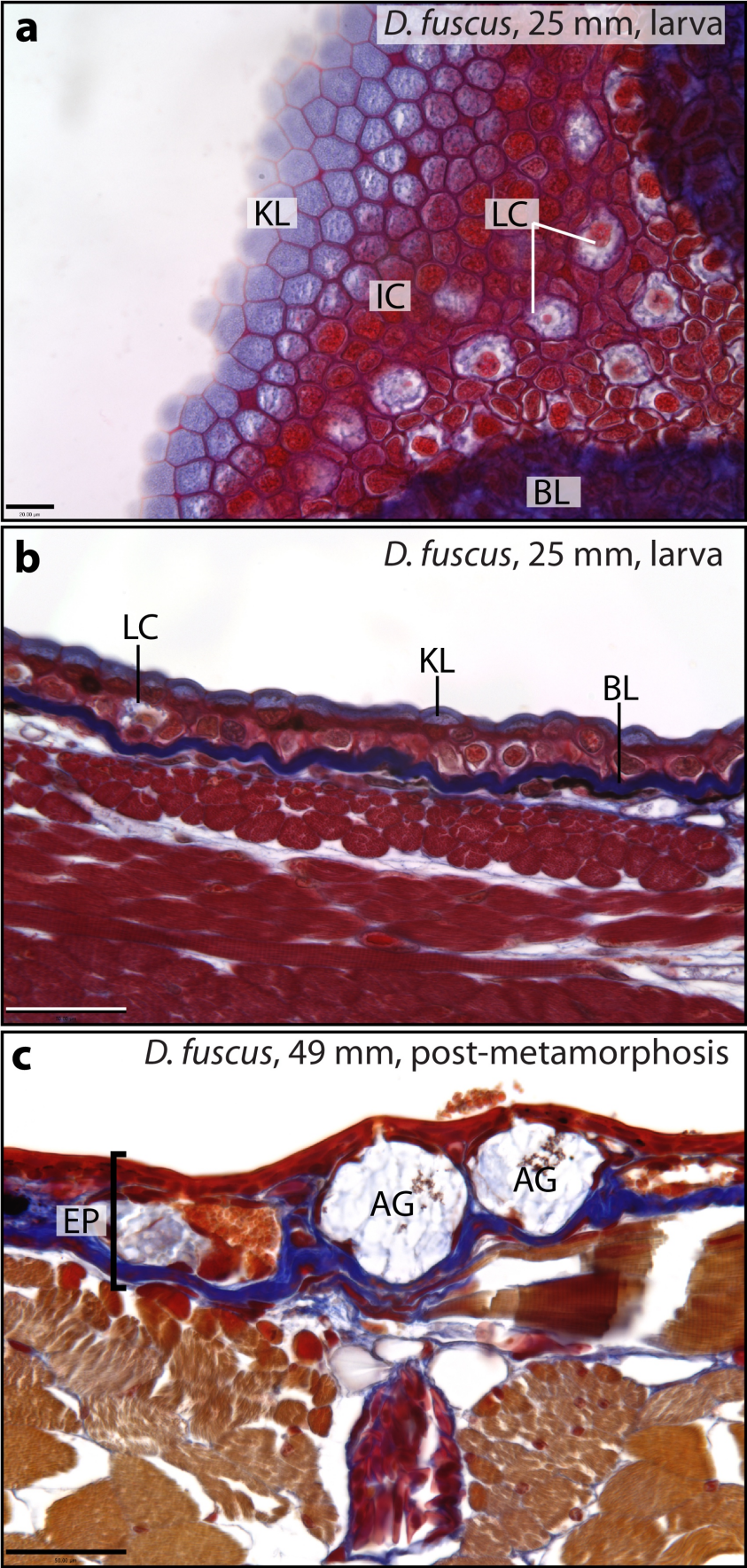
91 **Figure S2. Guide tree for PHYLOG. NCBI taxonomy was used to generate the**

92 **tree, combined with Pyron and Wiens (2011) [32] for amphibian taxonomy.**



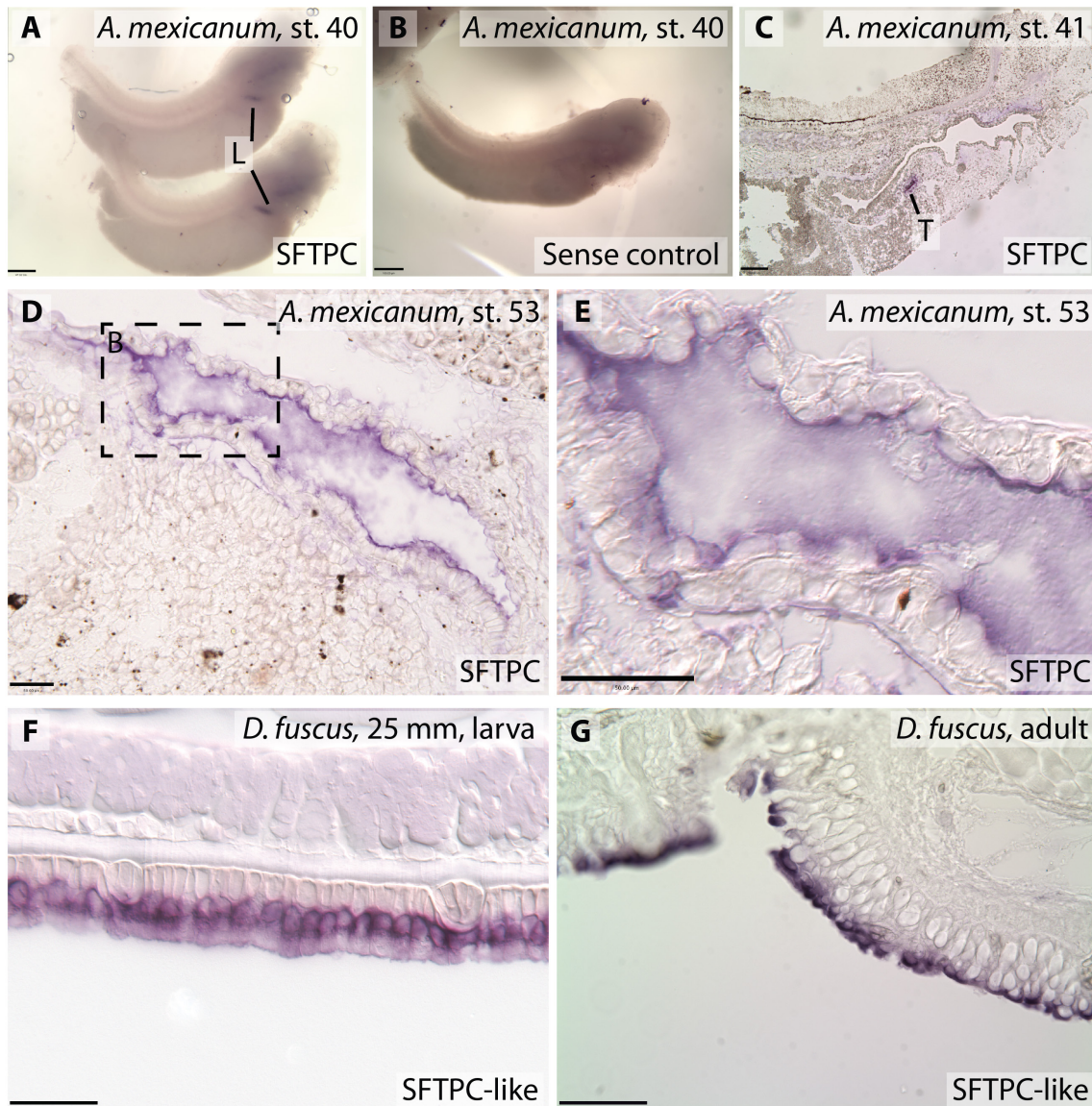
93

94 **Figure S3. Gene duplications predicted by PHYLOGENETIC ANALYSIS.** The two nodes where
95 a gene duplication event is predicted are colored red. Blue nodes indicate
96 divergence due to speciation events. PHYLOGENETIC ANALYSIS predicts that SFTPC-like
97 (SFTPCL) originated due to gene duplication. A second duplication event of
98 SFTPC-like is predicted in salamanders. While most species of salamanders
99 appear to express only one form of SFTPC-like, several species express SFTPC-
100 like transcripts with slight sequence differences, as noted in Supplemental Data
101 File 1. Further work is needed to determine if these sequence differences can be
102 attributed to further duplications of SFTPC-like or are the result of assembly error
103 or alternative splicing. Only one sequence per species was selected for
104 phylogenetic analysis. Complete species names are provided in Figure S2 and
105 Table S2.



107 **Figure S4. Histology of the integument in *Desmognathus fuscus* before and**
108 **after metamorphosis. a,** Tangential section through the gular region of a larva
109 shows the layers of the integument (from left to right): flattened, cuticle-like
110 keratinized layer (KL); an inner cell layer (IC); large cuboidal Leydig cells (LC)
111 intermingled with capillaries and other supporting cells; basal lamina (BL). **b,**
112 Sagittal section from the abdominal region of a larva. **c,** Transverse section from
113 a recently metamorphosed specimen showing the acinous glands (AG) and a
114 greatly thickened epidermis (EP). Mallory's trichrome stain. Scale bars: **a,** 20 μm ;
115 **b,c,** 50 μm .
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119 **Figure S5. Additional images of SFTPC and SFTPC-like expression**

120 **patterns.** a, Wholemount embryos of *Ambystoma mexicanum* display SFTPC

121 expression specific to the lungs (L). b, SFTPC sense control at the same stage

122 shows no lung expression. c, Midsagittal section of *A. mexicanum* embryo

123 stained for SFTPC shows expression in the trachea (T), but no expression in the

124 integument or buccopharynx. d, e, SFTPC expression in *Ambystoma mexicanum*

125 lung is confined to the squamous epithelial cells lining the lumen. e is an

126 enlargement of the boxed region in d. f, SFTPC-like expression in

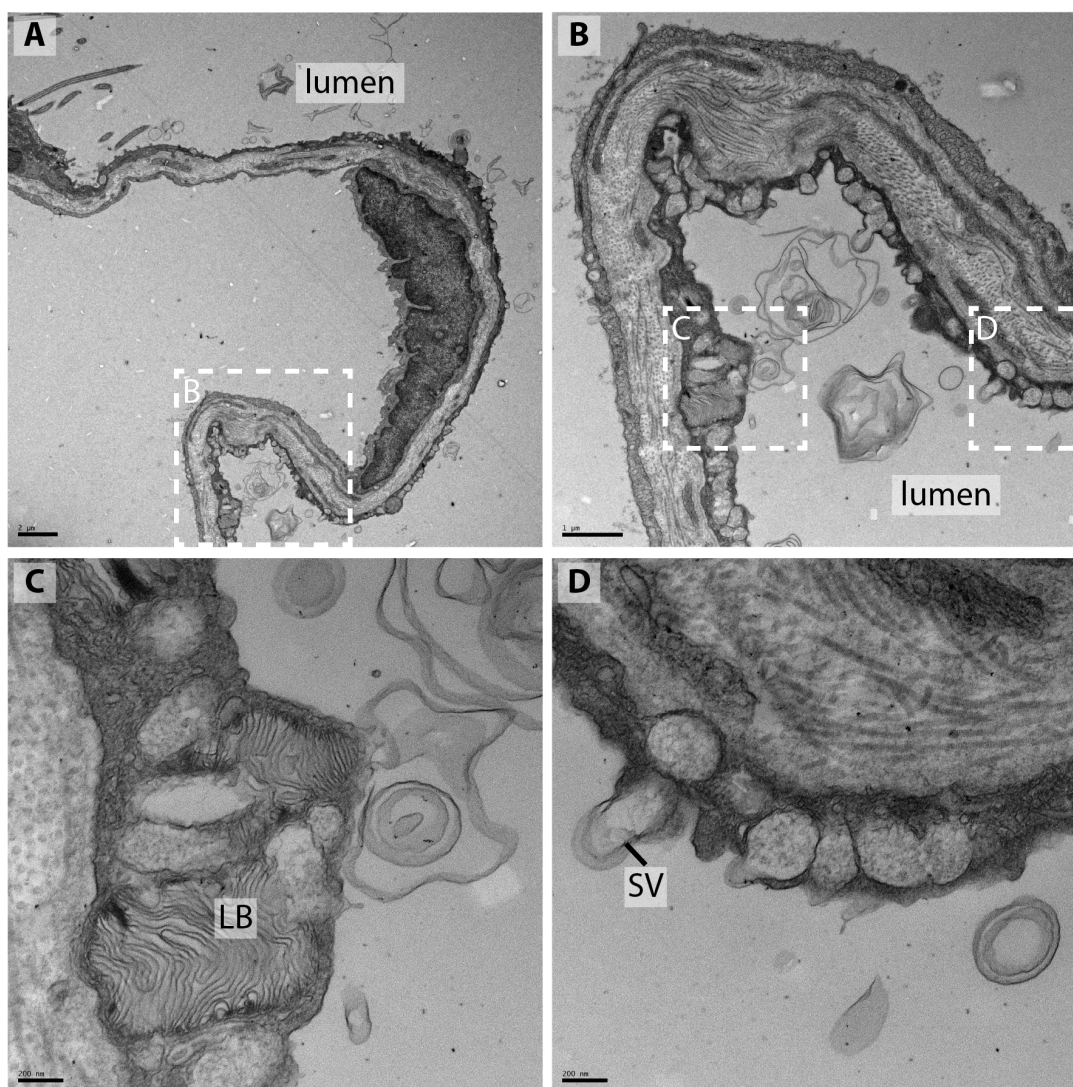
127 *Desmognathus fuscus* integument is confined to the apical cellular layer. g,

128 SFTPC-like expression in adult *D. fuscus* buccal epithelium. Scale bars: d, e, f, 50

129 μm; a, b, c, g 100 μm.

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Figure S6. Ultrastructure of alveolar epithelial cells in adult *Ambystoma mexicanum*. **a**, low magnification view of the pulmonary epithelium. The lumen of the lung is to the right. **b**, enlargement of boxed area in **a**. **c,d**, enlargement of boxed regions in **b** show lamellar bodies (LB) and secretory vesicles (SV). Scale bars: **a**, 2 μm; **b**, 1 μm; **c,d**, 200 nm.

Captions for Supplemental Data Files:

Supplemental Data File 1: Excel spreadsheet with all sequence data used for the study.

Supplemental Data File 2: A FASTA amino acid alignment used to generate the gene tree.

146 **Supporting Information References:**

147

- 148 1. Bourbon JR, Chailley-Heu B. 2001 Surfactant proteins in the digestive
149 tract, mesentery, and other organs: evolutionary significance. *Comp.*
150 *Biochem. Physiol. A, Mol. Integr. Physiol.* **129**, 151–161.
- 151 2. Fisher JH, Shannon JM, Hofmann T, Mason RJ. 1989 Nucleotide and
152 deduced amino acid sequence of the hydrophobic surfactant protein SP-C
153 from rat: expression in alveolar type II cells and homology with SP-C from
154 other species. *Biochim. Biophys. Acta* **995**, 225–30.
- 155 3. Glasser SW, Burhans MS, Eszterhas SK, Bruno MD, Korfhagen TR. 2000
156 Human SP-C gene sequences that confer lung epithelium-specific
157 expression in transgenic mice. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **278**,
158 L933-45.
- 159 4. Wert SE, Glasser SW, Korfhagen TR, Whitsett JA. 1993 Transcriptional
160 elements from the human SP-C gene direct expression in the primordial
161 respiratory epithelium of transgenic mice. *Dev. Biol.* **156**, 426–443.
- 162 5. Rankin SA *et al.* 2015 A molecular atlas of *Xenopus* respiratory system
163 development. *Dev. Dyn.* **244**, 69–85.
- 164 6. Hyatt BA, Resnik ER, Johnson NS, Lohr JL, Cornfield DN. 2007 Lung
165 specific developmental expression of the *Xenopus laevis* surfactant protein
166 C and B genes. *Gene Expr. Patterns* **7**, 8–14.
- 167 7. Weaver TE, Whitsett JA. 1991 Function and regulation of expression of
168 pulmonary surfactant-associated proteins. *Biochem. J.* **273**, 249–264.
- 169 8. Wohlford-Lenane CL, Durham PL, Snyder JM. 1992 Localization of
170 Surfactant-associated Protein C (SP-C) mRNA in fetal rabbit lung tissue by
171 in situ hybridization. *Am. J. Respir. Cell Mol. Biol.* **6**, 225–234.
- 172 9. Yin A, Winata CL, Korzh S, Korzh V, Gong Z. 2010 Expression of
173 components of Wnt and Hedgehog pathways in different tissue layers
174 during lung development in *Xenopus laevis*. *Gene Expr. Patterns* **10**, 338–
175 44.
- 176 10. Rankin SA, Gallas AL, Neto A, Gómez-Skarmeta JL, Zorn AM. 2012
177 Suppression of Bmp4 signaling by the zinc-finger repressors Osr1 and
178 Osr2 is required for Wnt/ β -catenin-mediated lung specification in *Xenopus*.
179 *Development* **139**, 3010–20.
- 180 11. Mo YK, Kankavi O, Masci PP, Mellick GD, Whitehouse MW, Boyle GM,
181 Parsons PG, Roberts MS, Cross SE. 2006 Surfactant protein expression in
182 human skin: evidence and implications. *J. Invest. Dermatol.* **127**, 381–386.
- 183 12. Bräuer L, Möschter S, Beileke S, Jäger K, Garreis F, Paulsen FP. 2009
184 Human parotid and submandibular glands express and secrete surfactant
185 proteins A, B, C and D. *Histochem. Cell Biol.* **132**, 331–8.
- 186 13. Schicht M *et al.* 2015 The distribution of human surfactant protein within
187 the oral cavity and their role during infectious disease of the gingiva. *Ann.*
188 *Anat.* **199**, 92–7.
- 189 14. Beers MF, Mulugeta S. 2005 Surfactant protein C biosynthesis and its
190 emerging role in conformational lung disease. *Annu. Rev. Physiol.* **67**,
191 663–696.

- 192 15. Schob S, Schicht M, Sel S, Stiller D, Kekulé A, Paulsen F, Maronde E,
193 Bräuer L. 2013 The detection of surfactant proteins A, B, C and D in the
194 human brain and their regulation in cerebral infarction, autoimmune
195 conditions and infections of the CNS. *PLoS One* **8**, e74412.
- 196 16. Barkauskas CE, Cronce MJ, Rackley CR, Bowie EJ, Keene DR, Stripp BR,
197 Randell SH, Noble PW, Hogan BLM. 2013 Type 2 alveolar cells are stem
198 cells in adult lung. *J. Clin. Invest.* **123**, 3025–36.
- 199 17. Boussau B, Szollosi GJ, Duret L, Gouy M, Tannier E, Daubin V. 2013
200 Genome-scale coestimation of species and gene trees. *Genome Res.* **23**,
201 323–330.
- 202 18. Voss SR, Kump DK, Putta S, Pauly N, Reynolds A, Henry RJ, Basa S,
203 Walker JA, Smith JJ. 2011 Origin of amphibian and avian chromosomes by
204 fission, fusion, and retention of ancestral chromosomes. *Genome Res.* **21**,
205 1306–1312.
- 206 19. Nowoshilow S *et al.* 2018 The axolotl genome and the evolution of key
207 tissue formation regulators. *Nature* **554**, 50–55.
- 208 20. Doyle JM, Siegmund G, Ruhl JD, Eo SH, Hale MC, Marra NJ, Waser PM,
209 Dewoody JA. 2013 Microsatellite analyses across three diverse vertebrate
210 transcriptomes. *Genome* **56**, 407–414.
- 211 21. Mohlhenrich ER, Mueller RL. 2016 Genetic drift and mutational hazard in
212 the evolution of salamander genomic gigantism. *Evolution (N. Y.)*. **70**,
213 2865–2878.
- 214 22. Che R, Sun Y, Wang R, Xu T. 2014 Transcriptomic analysis of endangered
215 Chinese salamander: Identification of immune, sex and reproduction-
216 related genes and genetic markers. *PLoS One* **9**, e87940.
- 217 23. Matsunami M, Kitano J, Kishida O, Michimae H, Miura T, Nishimura K.
218 2015 Transcriptome analysis of predator- and prey-induced phenotypic
219 plasticity in the Hokkaido salamander (*Hynobius retardatus*). *Mol. Ecol.* **24**,
220 3064–3076.
- 221 24. Abdullayev I, Kirkham M, Bjorklund SK, Simon A, Sandberg R. 2013 A
222 reference transcriptome and inferred proteome for the salamander
223 *Notophthalmus viridescens*. *Exp. Cell Res.* **319**, 1187–1197.
- 224 25. Huang L, Li J, Anboukaria H, Luo Z, Zhao M, Wu H. 2016 Comparative
225 transcriptome analyses of seven anurans reveal functions and adaptations
226 of amphibian skin. *Sci. Rep.* **6**, 24069.
- 227 26. Christenson MK *et al.* 2014 De novo assembly and analysis of the
228 Northern leopard frog *Rana pipiens* transcriptome. *J. Genomics* **2**, 141–9.
- 229 27. Amemiya CT *et al.* 2013 The African coelacanth genome provides insights
230 into tetrapod evolution. *Nature* **496**, 311–316.
- 231 28. Arnold K, Bordoli L, Kopp J, Schwede T. 2006 The SWISS-MODEL
232 workspace: a web-based environment for protein structure homology
233 modelling. *Bioinformatics* **22**, 195–201.
- 234 29. Xu D, Zhang Y. 2012 Ab initio protein structure assembly using continuous
235 structure fragments and optimized knowledge-based force field. *Proteins*
236 **80**, 1715–1735.
- 237 30. Yang J, Yan R, Roy A, Xu D, Poisson J, Zhang Y. 2015 The I-TASSER

- 238 Suite: protein structure and function prediction. *Nat. Methods* **12**, 7–8.
239 31. Johansson J, Szyperski T, Curstedt T, Wüthrich K. 1994 The NMR
240 structure of the pulmonary surfactant-associated polypeptide SP-C in an
241 apolar solvent contains a valyl-rich alpha-helix. *Biochemistry* **33**, 6015–
242 6023.
243 32. Pyron RA, Wiens JJ. 2011 A large-scale phylogeny of Amphibia including
244 over 2,800 species, and a revised classification of extant frogs,
245 salamanders, and caecilians. *Mol. Phylogenet. Evol.* **61**, 543–583.
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