

Evolutionary Origin of the Turtle Shell

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Summary

The origin of the turtle shell has perplexed biologists for more than two centuries [1]. It was not until *Odontochelys semitestacea* [2] was discovered, however, that the fossil and developmental data [3–8] could be synthesized into a model [9] of shell assembly that makes predictions for the as-yet unestablished history of the turtle stem group. We build on this model by integrating novel data for *Eunotosaurus africanus*—a Late Guadalupian (~260 mya) [10] Permian reptile inferred to be an early stem turtle [11]. *Eunotosaurus* expresses a number of relevant characters, including a reduced number of elongate trunk vertebrae (nine), nine pairs of T-shaped ribs, inferred loss of intercostal muscles, reorganization of respiratory muscles to the ventral side of the ribs, (sub)dermal outgrowth of bone from the developing perichondral collar of the ribs, and paired gastralia that lack both lateral and median elements. These features conform to the predicted sequence of character acquisition and provide further support that *E. africanus*, *O. semitestacea*, and *Proganochelys quenstedti* represent successive divergences from the turtle stem lineage. The initial transformations of the model thus occurred by the Middle Permian, which is congruent with molecular-based divergence estimates [12–15] for the lineage, and remain viable whether turtles originated inside or outside crown Diapsida.

Results

Unlike the bony covering of other “shelled” amniotes (e.g., anguillid lizards, ankylosaur dinosaurs, armadillos, and placodonts), the carapace of turtles is not simply a composite of dermal ossifications but rather integrates outgrowths of intramembranous bone from the perichondrium of the developing ribs and thoracolumbar vertebrae [3–8]. Whether the origin of the turtle shell also involved melding of overlying osteoderms (composite model) or not (de novo model) was vigorously

debated throughout the 20th and early 21st centuries (see [16]) with support falling largely along disciplinary lines [3–9, 17–25]. Paleontological explanations relied heavily on the composite model [19–25], but their efficacy was hampered by the large morphological gap separating the earliest, fully shelled turtles (e.g., *Proganochelys quenstedti* [26]) from all other known groups. In contrast, developmental biologists promoted the de novo model and viewed the lack of clear transitional fossils as support for a rapid evolution of the shell, perhaps coincident with the appearance of a bone morphogenetic protein (BMP) developmental pathway critical to shell construction in modern turtles [3–9, 27]. The lack of osteoderms in the recently discovered stem turtle *Odontochelys semitestacea* [2] strongly supports the de novo model of shell origination and liberates the paleontological search for the even deeper history of the turtle stem from its previously self-imposed constraint of osteoderm-bearing forms. For example, the long-recognized similarities between turtles and the osteoderm-free, Late Guadalupian Permian reptile *Eunotosaurus africanus* (Figure 1 and Figure S1 available online) [11, 23, 24, 28, 29] can now be viewed as homologous, with *Eunotosaurus* lying just deep to *Odontochelys* on the turtle stem [11]. Our study tests the role of *Eunotosaurus* in understanding the origin of turtles and their shell by determining whether its morphology conforms to the predictions of the Kuratani et al. [9] model of turtle shell evolution and development. To this end, we employ new specimens and novel data sources (including rib histology; Figures 1, 2, and 3 and the Supplemental Experimental Procedures) in a phylogenetic analysis of shell-related features (Figure 4 and the Supplemental Experimental Procedures). We summarize our results by expanding the Kuratani et al. [9] model to include the turtle stem deep to *Odontochelys*.

Morphology

GM 86/341 is the only specimen of *Eunotosaurus africanus* with a complete cervical and trunk vertebral series (see section I of the Supplemental Experimental Procedures for a complete list of the material examined). Nine elongated trunk vertebrae and nine pairs of dorsal ribs are clearly present, which differs from the traditional reconstruction of ten trunk vertebrae and ten pairs of ribs [23, 30, 31]. A distinct change in vertebral length, neural spine shape, and rib morphology occurs between presacral vertebrae 6 and 7 (Figures 1A–1C and S1A–S1C). We argue this is the transition between the cervical and trunk regions (sensu [32]). The cervical vertebrae are short with a bulbous neural spine [24]. They have long, slender ribs that are round in cross-section and unexpanded distally. Cervical rib 6 (dorsal rib 1 of other authors) is long and mostly round in cross-section except for a small, middle portion that is distinctly broadened. Thus, there are six, not five, cervical vertebrae and nine, not ten, dorsal vertebrae with nine pairs of distinctly broadened dorsal ribs. The dorsal ribs are T-shaped in cross-section and contact each other for most of their length. The first eight pairs are oriented slightly posteriorly, whereas the last pair projects slightly anteriorly. Unlike other specimens (USNM 23099, SAM 4328, and BMNH 4949), the last pair of ribs in GM 86/341 articulates with, but is not

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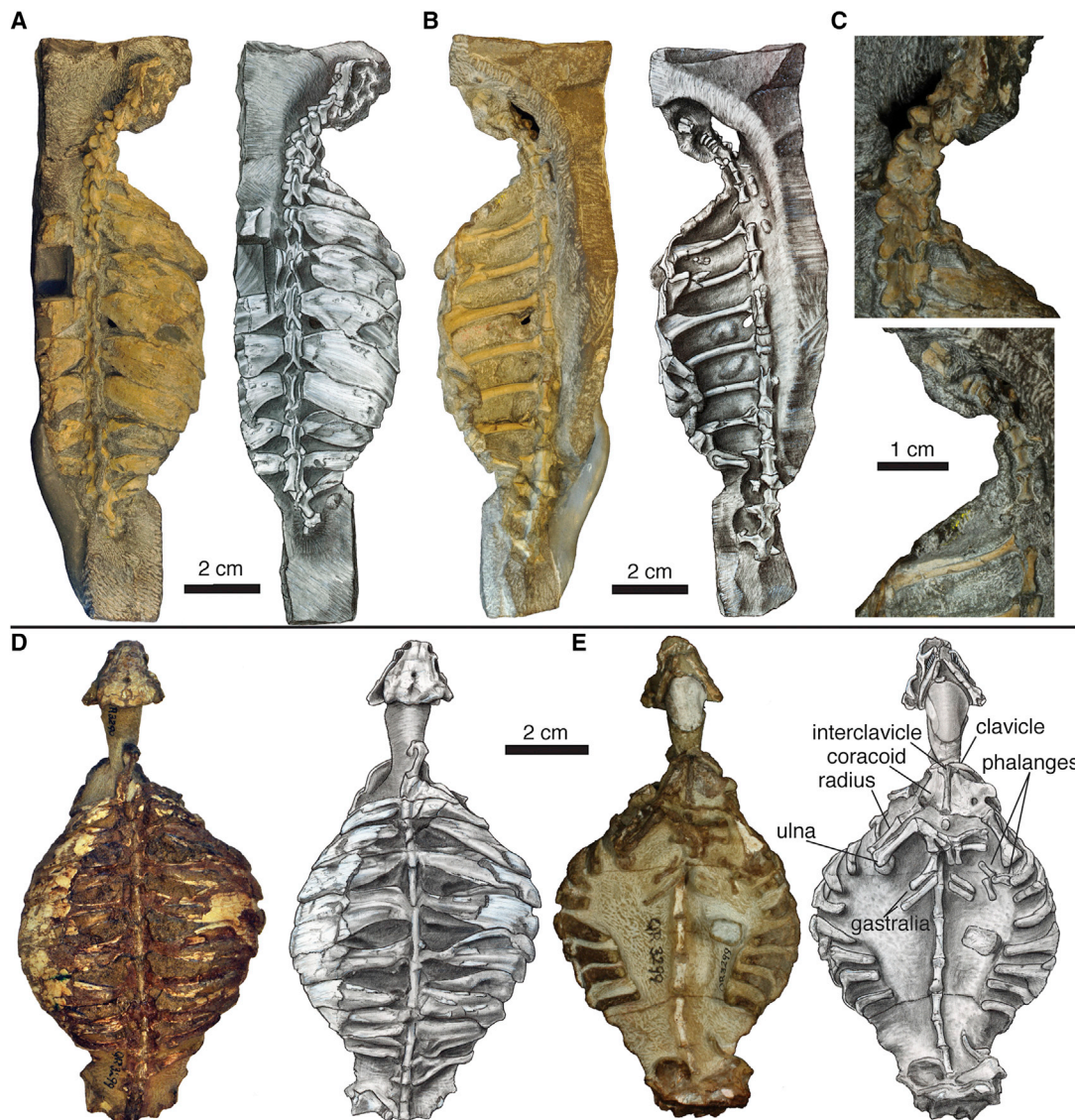


Figure 1. Newly Described *Eunotosaurus africanus* Material

(A) Photograph (left) and illustration (right) of GM 86/341 in dorsal view.

(B) Photograph (left) and illustration (right) of GM 86/341 in ventral view.

(C) Close-up photographs of the neck region of GM 86/341 in dorsal (top) and ventral (bottom) views showing differences between cervical (short centra with bulbous neural spines, and elongate ribs) and dorsal (greatly elongate centra, with long neural spines, and anteroposterior broadened ribs) vertebrae.

(D) Photograph (left) and illustration (right) of QR 3299 in dorsal view.

(E) Photograph (left) and illustration (right) of QR 3299 in ventral view.

See also [Figure S1](#) for red/blue stereophotographs of each specimen.

fused to, the corresponding vertebra. That this condition reflects size/age variation is supported by the smaller size of GM 86/341.

Several specimens preserve a complete, articulated shoulder girdle (AM 5999, NMQR 3299, GM 777, SAM K 1133, and SAM K 7909). The scapula is situated vertically and rostral to the dorsal ribcage ([Figures S1D and S1E](#)). The clavicles are slender elements with a distinct dorsal process ([Figure S1E](#)). A slender cleithrum, which was hypothesized by Cox [23], is confirmed. Its ventral end contacts the dorsal tip of the clavicle ([Figure S1E](#)). Paired gastralia lacking lateral and medial element(s) are preserved in NMQR 3299 ([Figures 1F and S1C](#)) and BP/1/7024.

Microanatomy and Histology

The dorsal ribs of *Eunotosaurus africanus* ([Figures 2B, 2F, and S2](#)) and *Proganochelys quenstedti* ([Figure 2G](#)) are T-shaped in cross-section (longitudinal or parasagittal section in reference to the body axis) proximally, with a broadened surface and a rounded visceral aspect that is arranged vertically ([Figure 2B](#)). In both taxa, the vertical portion of the “T” includes a gentle bulge distally ([Figures 2F and 2G](#); see [33]).

In GM 86/341, the anterior-most part of the broadened part of the rib deviates ventrally and tapers to a sharp edge so that the outer bone surface is straight to slightly concave. The posterior part does not deviate ventrally but maintains a constant thickness, and it ends in an upturned blunt tip such

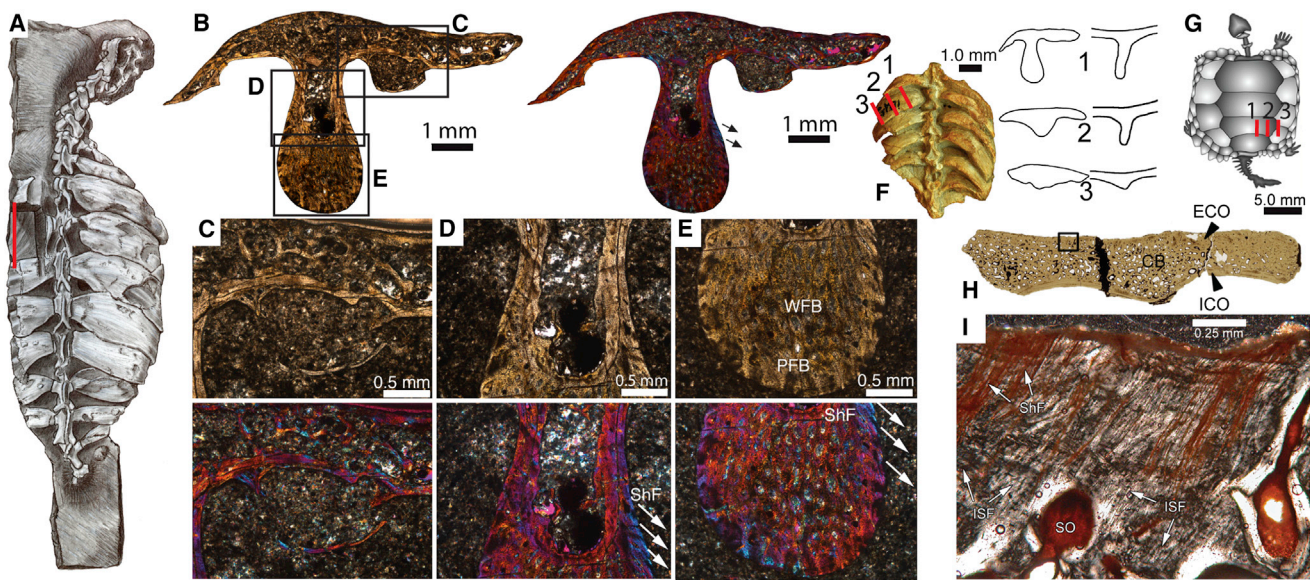


Figure 2. Histological Data from the Ribs of *Eunotosaurus africanus* and *Proganochelys quenstedti*

(A) Illustration of *Eunotosaurus* (GM 86/341) showing where the left third dorsal rib was sectioned histologically (red line).

(B) Histological section in normal (left) and polarized (right) light showing the T shape of the rib in cross-section (see “1” in F, which shows the approximate place the histological section was taken as compared to where the section was taken for *Proganochelys*). Black arrows indicate the presence and orientation of Sharpey’s fibers.

(C–I) Images in (C), (D) and (E) are seen in normal transmitted (upper) and cross-polarized light using a lambda compensator (lower). Image in (H) is seen in normal transmitted and the one in (I) is seen in cross-polarized light.

(C) Close-up view of the posterior diploe portion of the T-shaped rib. Thin external and internal compact layers frame interior cancellous bone, which is composed of thin trabeculae. Note the thin ring-like structure at internal (visceral) surface of the rib.

(D) Close-up view of the midshaft region of the rib. Note central ovoid cavity surrounded by periosteal parallel-fibered bone (PFB). Sharpey’s fibers (ShF) are present in the posterior part of this region (blue colors). White arrows indicate the orientation of insertion of the ShF.

(E) Close-up of the drop-shaped bulge, which consists internally of highly vascularized woven bone tissue (WFB) and externally of PFB. Sharpey’s fibers (white arrows) are absent from the anterior and ventral parts of the bulge. White arrows indicate presence and orientation of Sharpey’s fibers.

(F and G) *Eunotosaurus* specimen (F, left; GM 71) showing the change in cross section of the rib (F, right) as you move distally compared to the change in cross section of the rib/costal morphology (G, left) of *Proganochelys* (G, right).

(H) Histological section of *Proganochelys* (MB.R. 3449.2) taken from the right costal 7? at approximately level three (see the corresponding number in G).

(I) Close-up view of the external cortex, which is composed mainly of interwoven structural fibers (ISF). Numerous parallel Sharpey’s fibers insert into the bone tissue at high angles (ShF). A few scattered secondary osteons (SO) are visible.

See also [Figure S2](#).

that the internal (visceral) bone surface is slightly convex. A tapering anterior portion cannot be confirmed in NHM PV R 4949, but its posterior portion is either horizontal or slightly downturned due to its more posterior position in the trunk. Three phases of bone deposition, an initial phase and two successive phases, are recognizable (see section II of the [Supplemental Experimental Procedures](#)). No interwoven dermal structural fibers or remnants of cartilage are apparent anywhere in the sectioned rib.

Although of similar overall size, both sectioned *Eunotosaurus* ribs differ somewhat in cortical bone thickness, as well as in internal trabecular thickness and arrangement, with NHM PV R 4949 appearing more robust overall. In this specimen, growth marks are visible in the cortical bone as light and dark banding in normal transmitted light, but histological details (e.g., Sharpey’s fibers) are otherwise not well preserved. The source of these variations is unclear.

Three discrete phases of bone deposition are visible, an initial and two successive phases (see the [Supplemental Experimental Procedures](#) for a detailed histological description). In the initial phase, a central large cavity (representing the rib primordium) gets encased by parallel-fibered bone (PFB). In the next phase, thin sheets of anteriorly and posteriorly extending bone are added dorsally and interior trabeculae

spread out from the bone around the central ovoid cavity, thus creating a diploe structure. In the final phase, the drop-shaped bulge of the ventral shaft of the T-shaped rib is deposited laterally and ventrally to the initial tube structure. During growth, inconspicuous Sharpey’s fibers are locally present only at the posterior margin of the ventral shaft.

Phylogenetic Analysis

Fifteen morphological characters and one taxon, the shelled *Sinosauropsphargis yunguiensis*, were added to the morphological data set of Lyson et al. [11] ([Supplemental Experimental Procedures](#), sections III–VI). In turn, this data set is that of deBraga and Rieppel [34], with Li et al.’s [2] addition of *Odontochelys semitestacea* and one morphological character and Lyson et al.’s [11] addition of *Eunotosaurus africanus* and *Proganochelys quenstedti* and seven morphological characters. Two most parsimonious trees (confidence interval [CI] = 0.3333, retention index = 0.6785, consistency index = 0.2262) with 723 steps were obtained ([Figure S3](#)). In addition, we performed a Bayesian phylogenetic analysis, and the resulting topology is identical to that of the parsimony analysis. There is strong support for a turtle + *Eunotosaurus* clade (bootstrap = 69% and posterior probability = 95%).












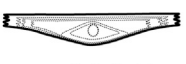
A		circular cartilaginous rib primordia (based on extant tetrapod embryos)		ovoid (?) cartilaginous rib primordia		circular cartilaginous rib primordia
B		development of periost; no periosteal outgrowths into adjacent subdermal tissue		development of periost; initial outgrowth of bone spiculae from rib periost into adjacent subdermal tissue		development of periost; initial outgrowth of bone spiculae from rib periost into adjacent dermal tissue
C		continued deposition of periosteal bone layers (rib shape can differ depending on taxon); development of medullary cavity		formation of dorsal (horizontal) blade of T-shaped diploe part of rib through periosteal and appositional bone growth		early formation of costal around the initial rib through incorporation of preformed dermal tissue structures indicates switch from periosteal to metaplastic ossification (early costal shape can differ depending of taxon)
D		continued deposition of periosteal bone layers until skeletal maturity is reached (rib shape can differ depending on taxon); increase in diameter of medullary cavity usually in equilibrium with new periosteal bone formation		subsequent formation of visceral (vertical) drop-shaped bulge of rib; adjacent ribs overlap and abutt each other but lack sutures; earlier growth stages of the dorsal blade part of rib are completely remodelled		growth of costal plate through metaplastic ossification; continued growth throughout ontogeny enabled through sutures; depending on the species, traces of rib and subsequent costal plate growth stages can get lost through remodelling processes (costal shape can differ depending of taxon)

Figure 3. Comparative Rib Development in Amniotes

The first stage of development is similar in the three groups (left, generalized amniote; middle, *Eunotosaurus africanus*; and right, generalized turtle. (Sub) dermal outgrowth of bone from the perichondral/periosteal collar of the developing rib is a developmental feature shared by *Eunotosaurus* and turtles. *Eunotosaurus* exhibits subsequent stages of rib development that we interpret as autapomorphic. See also Figure S2.

Discussion

Eunotosaurus, which previously was excluded from global analyses of amniote relationships, was recently recovered as sister to undisputed turtles [11]. Likewise, the addition of turtles to the latest “parareptile” data set (i.e., [35]) yields a *Eunotosaurus* + turtle clade [11]. The previously undescribed *Eunotosaurus* material provides compelling additional morphological support for a privileged relationship with turtles.

Strengthening Support for a Turtle-*Eunotosaurus* Clade

Our examination of previously undescribed specimens of *Eunotosaurus*, as well as the comparative histology of *Eunotosaurus* ribs, strengthens support for *Eunotosaurus* as both a stem turtle and a critical transitional form in the evolution of the turtle body plan. Gross morphological features related to the shell that are shared between *Eunotosaurus* and turtles now include trunk vertebrae reduced to nine (shared with *Odontochelys*), nine pairs of broadened dorsal ribs (shared with *Odontochelys semitestacea* and *Proganochelys quenstedti*), elongation of trunk vertebrae with length exceeding width by four times or more (shared with all undisputed stem and crown turtles), cross-section of ribs T-shaped proximally developing into a gentle ventral bulge distally (shared with several early turtles, including *O. semitestacea* [2], *P. quenstedti* [26], and *Palaeochersis talampayensis* [36]), and paired gastralia that do not overlap medially and lack both a central medial element and lateral element(s) (shared with all turtles and parareptiles in which gastralia are preserved, and lacking in all diapsids that have a central medial and/or lateral element(s); e.g., [37]). Each of these characters optimizes as unambiguous synapomorphies of *Eunotosaurus* and undisputed turtles among reptiles. In addition, the cartilaginous rib primordium of *Eunotosaurus* changes position in relation to the developing T-shaped rib, which is also the case in the developing costals of hard-shelled turtles [38] (Figure S2). In both *Eunotosaurus* and hard-shelled turtles, the proximal end of the primordial rib cartilage is situated more dorsally, whereas distally it becomes progressively ventral, finally touching the internal cortical bone (NHM PV R 4949; Figure S2). It should be noted that this change in position of the primordial rib cartilage is not seen in the trionychid *Apalone ferox* (Figure S2), in which the primordial rib cartilage maintains a fairly constant position. This appears to be an autapomorphy for soft-shelled turtles.

The morphology of *Eunotosaurus* is consistent with the explicit prediction of Kuratani et al. [9] that the early stages of the turtle shell, prior to the emergence of *Odontochelys*,

was marked by a vertical scapula positioned rostral to the ribcage. This condition is expressed in *Eunotosaurus*, in contrast to some other putative turtle sister groups, which have a scapula dorsal to the ribcage (see [39]). Furthermore, the gross morphology of *Eunotosaurus* only differs from undisputed stem turtles, such as *Odontochelys*, in sharing fewer derived characters with crown turtles, as would be expected for an earlier member of the turtle stem. For example, *Eunotosaurus* lacks the derived conditions of neural plates, a hypoischium, and a co-ossified plastron (though all of the bones that form the plastron are present in *Eunotosaurus*).

The rib histology of *Eunotosaurus* provides further support for a sister-group relationship with undisputed turtles (Figures 2, 3, and S2). The nature of the bone tissue found in the horizontal flange and the shaft region of the rib is compatible with periosteal and appositional bone growth (“Zuwachsknochen” sensu [40]). The histological data indicate three phases of bone morphogenesis in *Eunotosaurus* (Figure 3). Periosteal bone is first deposited around a circular to ovoid cartilaginous rib primordium and is then followed by successive resorption of the cartilage tissue. This phase is found in all amniotes examined thus far. Second, outgrowth of bone trabeculae and bony sheets forms the dorsally broadened, horizontal flange part of the rib. Such outgrowth of dermal bone from the developing perichondral/periosteal collar of the rib is a unique synapomorphy (CI = 1.00) of *Eunotosaurus* and turtles. Finally, the visceral portion of the rib is reinforced by successive deposition of parallel fibered periosteal and woven endosteal bone, and remodeling processes of the dorsal diploe structure set in. This last phase of bone development differs profoundly from all other amniotes examined and is considered an autapomorphy of *Eunotosaurus*. There is no evidence of metaplastic ossification at any stage of rib formation, unlike in undisputed turtles in which the costals incorporate interwoven structural fibers (i.e., metaplastically ossified integumentary layer [33, 41]). However, this stage of costal development normally occurs late in development, mostly posthatchling [5]. Thus, the absence of this feature might be expected deep in the turtle stem, especially if the feature evolved through terminal addition at some point between the divergence of *Eunotosaurus* and *Proganochelys*.

The local presence of Sharpey’s fibers at only the posterior part of the ventral shaft in both the first and second successive phase of bone deposition is here interpreted as an insertion of respiratory or locomotory muscles (or associated ligaments) into the rib, which stayed active throughout the ontogenetic timeframe recorded in the thin sections. The Sharpey’s fibers are not considered indicative of intercostal muscles because they are only found on the posterior portion of the rib, and

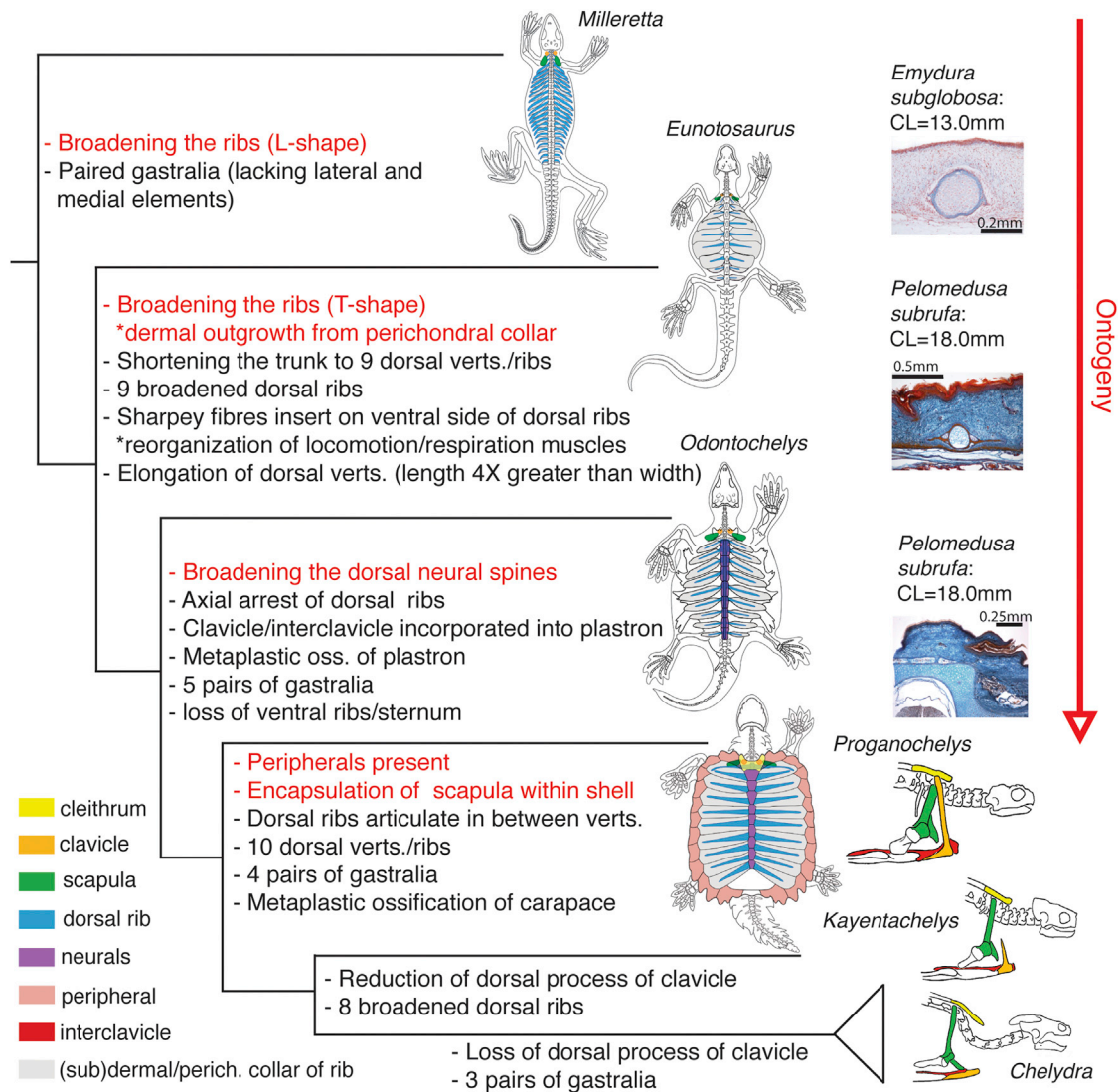


Figure 4. Evolutionary Developmental Model for the Origin of the Turtle Shell

Results of a phylogenetic analysis of shelled reptiles and characters important in constructing a shell are plotted against the ontogeny of pleurodire turtles. Thin sections through turtle embryos show the initial outgrowth of (sub)dermal bone through the costals first (carapace length [CL] = 13.0 mm in the pleurodire *Emydura subglobosa*) and then the neurals (CL = 18.0 mm in the pleurodire *Pelomedusa subrufa*). The timing of ontogenetic transformations of those features (in red) important in the construction of the shell (i.e., the number of dorsal vertebrae or ribs does not change through ontogeny) is congruent with the phylogenetic transformation of those same features based on our recovered tree topology. Our model makes explicit morphological and histological predictions for the lineage prior to the most recent common ancestor of *Eunosaurus africanus* and turtles that are met by the morphology found in *Milleretta rubidgei*. Numbers above each node represent bootstrap frequencies obtained in the phylogenetic analysis. See section VIII of the [Supplemental Experimental Procedures](#) for justification for each reconstruction. See also [Figures S3](#) and [S4](#).

intercostal muscles normally insert on both the anterior and posterior portion of the ribs (Figure S2). Furthermore, even in animals with anteroposterior broadened ribs and poorly developed intercostal musculature (e.g., the mammal *Cyclopes didactylus*; Figure S2), Sharpey's fibers are still present on both the anterior and posterior surfaces of the rib. Thus, the restriction of Sharpey's fibers to the posterior side of the rib is an unambiguous synapomorphy (CI = 1.00) shared only with turtles. We interpret these fibers to reflect not an intercostal muscle per se, but rather a muscle used in locomotion or more directly in respiration. Given the observation that the dermis in turtles completely ossifies, numerous muscles involved in locomotion (e.g., *m. testocoracoideus*) and respiration (e.g., *m. diaphragmaticus* and *m. transversus*

abdominis) are obliged to insert on the ventral portion of the ribs. This differs from other amniote ribs, in which intercostal muscles extend between the ribs, with no muscles inserting on the ventral face of the rib [42, 43] (Figure S2).

Expansion of the Kuratani Model

Given the inference of *Eunosaurus* as the sister to turtles in phylogenetic analyses of both amniotes and "parareptiles" [11], the numerous unique morphologic and developmental synapomorphies it shares with turtles, and its congruence with the transformational model outlined by Kuratani et al. [9], we here incorporate *Eunosaurus* into an expanded evolutionary developmental model for the origination of the turtle shell. Kuratani et al. [9] used the timing of development

of key shell features to predict ancestral morphologies and secondarily place fossils within this developmental framework (Supplemental Experimental Procedures, section VII). We extend this model to include the developmental timing of a more comprehensive list of individual characters that are essential to building a turtle shell (Figure 4). Outgrowth of membrane bone from the perichondral/periosteal collar of the developing rib occurs first, followed by outgrowth of bone from the neural spines of the trunk vertebrae, and finally by the acquisition of peripheral bones and the encapsulation of the scapula within the shell [7, 8] (Figure 4). The phylogenetic sequence exhibited by *Eunotosaurus* (broadened ribs T-shaped in cross-section, dermal outgrowth of bone from the perichondral/periosteal collar of the rib, and reorganization of locomotion and respiratory muscles), *Odontochelys* (broadened neurals), and *Proganochelys* (acquisition of peripherals and encapsulation of scapula within shell) is consistent with the evolutionary developmental model. This expanded model pulls the initial transformations of the turtle shell back to at least the late Middle Permian. But, this is still younger than all recent molecular-based divergence estimates for Pan-Testudines [12, 13] and thus is viable irrespective of whether turtles originated inside or outside of crown Diapsida. To be clear, our phylogenetic analysis does recover the *Eunotosaurus* + turtle clade outside Diapsida, which places it in conflict with molecular based topologies [12–15].

Our model for the origin of the turtle shell makes a number of morphological and histological predictions for stem turtles that existed or diverged prior to the most recent common ancestor of *Eunotosaurus* and extant turtles. For example, based on the early development of outgrowth of membrane bone from the ribs of extant turtles (e.g., [38]), the model predicts that earlier stem turtles had slightly broadened ribs with some intramembranous outgrowth of bone from the perichondral/periosteal collar of the rib. Based on the inference that the unique abdominal muscle ventilation system of turtles [44], in which the muscles attach to the ventral portion of the carapace [45], arose from a basal amniote with costal ventilation [46], the model also predicts that early stem turtles likely had both intercostal muscles (unlike *Eunotosaurus*) and muscles beginning to insert on the ventral side of the trunk/dorsal ribs. Histological data for *Milleretta rubidgei* has yet to be obtained, but this moderately broad-ribbed species, inferred in phylogenetic analyses to have diverged from the turtle stem earlier than *Eunotosaurus* [11, 30, 47], meets many of these morphological predictions (Figure 4). The dorsal ribs of *Milleretta* display an intermediate condition between the rod-like ribs found in stem amniotes (i.e., *Limnoscelis paludis*) and the broad ribs found in *Eunotosaurus* and *Odontochelys*. We look forward to testing the predictions of the expanded model through further study of this taxon.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.cub.2013.05.003>.

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