Embryonic muscle splitting patterns reveal homologies of amniote forelimb muscles

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Limb muscles are remarkably complex and evolutionarily labile. Although their anatomy is of great interest for studies of the evolution of form and function, their homologies among major amniote clades have remained obscure. Studies of adult musculature are inconclusive owing to the highly derived morphology of modern amniote limbs but correspondences become increasingly evident earlier in ontogeny. We followed the embryonic development of forelimb musculature in representatives of six major amniote clades and found, contrary to current consensus, that these early splitting patterns are highly conserved across Amniota. Muscle mass cleavage patterns and topology are highly conserved in reptiles including birds, irrespective of their skeletal modifications: the avian flight apparatus results from slight early topological modifications that are exaggerated during ontogeny. Therian mammals, while conservative in their cleavage patterns, depart drastically from the ancestral amniote musculoskeletal organization in terms of topology. These topological changes occur through extension, translocation and displacement of muscle groups later in development. Overall, the simplicity underlying the apparent complexity of forelimb muscle development allows us to resolve conflicting hypotheses about homology and to trace the history of each individual forelimb muscle throughout the amniote radiations.

Limb muscles enable highly variable and diverse locomotor capabilities, perhaps making them more susceptible to evolutionary change than most other muscle groups. Different schemes for limb muscle homology have been proposed using various criteria, including innervation, topology and function (for a recent overview of the disparate nomenclature authors have applied to individual forelimb muscles see ref. 12). The ‘embryonic origin’ criterion of homology is often referenced but the vast majority of embryonic anatomical information for amniotes comes from a few studies on a handful of species, dating from the first half of the twentieth century and limited to the proximal portion of the arm13–21, as well as a handful of recent studies focusing on birds and mammals22–28. Far more is known about the cellular and molecular processes involved in muscle development: unlike the skeleton and connective tissue of the limbs, limb muscles derive from cells that arise from the dorsal portion of the somite, the dermomyotome, that delaminate, migrate and invade the limb bud. Here they form two opposing masses of differentiated myotubes. Each mass later cleaves, forming smaller divisions and subdivisions that eventually split into individualized recognizable muscles of the adult29,30. Muscles are referred to as being dorsal or ventral, on the basis of their putative embryonic origin and their topographic relationship with the nerves of the brachial plexus innervating them. Branches of the dorsal and ventral cords of the plexus typically innervate dorsal and ventral muscles, respectively31,32. This distinction has been used as a tool for further fine-tuning homology hypotheses on the basis of other criteria, with the idea that dorsal muscles of one species cannot be homologous to ventral muscles of another. Molecular markers can be used to distinguish between dorsal and ventral muscle precursors33,34; however, no known genes show patterns of expression specific to individual muscles derived from subsequent divisions. Embryonic assessments of homology must therefore centre on topology and cleavage pattern. Owing to assumptions regarding the embryonic origins and relationships among adult muscles, the evolutionary history of amniote forelimb musculature has been portrayed as involving a series of transitions or modifications, the more notable of these being an expansion of the deltoid musculature to form scapulohumeral muscles in reptiles and the humeroradial muscles in archosaurs11,32; a shift from ventral to dorsal of the supracoracoid musculature of mammals to form the ‘neomorphic’ muscles associated with the scapula33,34 and a shift of the dorsal hand muscles of mammals towards the forearm to become the distal forearm extensors of the digits6,11,12.

We followed the development of forelimb musculature in embryos of six species representing major clades of reptiles (birds, crocodilians, turtles and lizards) and therian mammals (marsupials and placentals) by means of whole-mount immunostaining, confocal imaging and three-dimensional (3D) volume segmentation (Fig. 1). We examined muscle development and cleavage by following the staining pattern of the MF-20 antibody (DSHB), targeting the myosin heavy chain protein expressed in mature myocytes—an antibody that has been used for the study of muscle development in amphibians and amniotes36–39. Previous studies have analysed the development of limb muscles by means of in situ hybridization of markers like MyoD32,34, which label more immature muscle progenitors like myoblasts and could allow visualization of earlier morphogenetic events. However, we find that the MF-20 antibody staining pattern shows the same anatomy as MyoD in situ in stages beyond HH28/E11 of birds and mouse, respectively (comparing with, for example, c, d and f–i of Fig. 1 from ref. 24); and that the earliest expression pattern of MyoD, labelling immature cells not stained by MF-20, shows the simplest anatomy of the opposing dorsal and ventral premuscles

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masses (a, b and e of Fig. 1 from ref. 24) but does not provide extra information regarding the splitting patterns that are the focus of this study. On the basis of this, MF-20 was used to label mature muscle cells in all stages of development and all species, even if other antibodies or molecular markers can provide more information regarding previous developmental processes, such as migration of somitic cells or the localization of premuscular progenitors.

Current consensus is that early muscle splitting patterns are different among the major amniote clades17-20 in the number of divisions emerging from the dorsal and ventral muscle masses and in the ultimate derivation of adult muscles from those early subdivisions. In contrast to this accepted notion of disparity, we found a common and stereotyped set of divisions across all Amniota (Fig. 2): the dorsal mass gives rise to one intrinsic (located within the arm) division and three extrinsic (located ‘outside’ of the arm) divisions. The extrinsic divisions include the deltoid division anteriorly, the latissimus division posteriorly and the subscapular division deep to the latissimus division. The ventral muscle mass gives rise to two extrinsic divisions—the anterior supracoracoideus division and posterior pectoral division—and one intrinsic division. Both intrinsic divisions later separate proximodistally into three subdivisions associated with the three developmental segments of the arm: proximal zeugopod (upper arm), intermediate stylopod (forearm) and distal autopod (wrist and hand). This contradicts the previous hypothesis of independent elbow and wrist forearm matrices6 (below). The intrinsic dorsal division forms, from proximal to distal, the triceps subdivision, extensor subdivision and hand extensor subdivision. The intrinsic ventral division forms the brachial subdivision, flexor subdivision and hand flexor subdivision. In all species studied, these conserved early muscle divisions produce six dorsal and five ventral anatomical units (Fig. 3). The only exception to this pattern appears to be the therian condition, in which the hand extensor subdivision is conspicuously absent. Therians therefore apparently lack dorsal hand muscles entirely, in contrast to current consensus that these muscles are present but shifted proximally along the forearm6,9. The highly conserved splitting patterns we found among arm muscles make it possible to determine most of their homologies across amniotes on the basis of development alone (Extended Data Figs. 1 and 2) and to challenge some assumed homologies and some proposed evolutionary transitions invoked to explain the origin of particular morphologies. Surprisingly, even turtles and birds, with their extremely modified anatomy, maintain the ancestral pattern of muscle division. Therian mammals have the most transformed forelimb musculature; however, their unique characters derive not from modification of these early cleavage patterns but from changes in position, translocation and extension of the resulting muscle divisions.

The shoulder and chest musculature

Although the pectoral girdles of turtles and crocodylians are differently and peculiarly modified from the superficially lizard-like ancestral reptilian skeletal architecture32,39,41, we find their pattern...
of muscle development is not different to that of geckoes, which are more anatomically conservative: ventrally, the pectoralis division forms the broad M. pectoralis, as previously described\(^{17,18}\) and anterior to it, the supracoracoideus division forms only M. supracoracoideus (Extended Data Fig. 3), in agreement with ref. \(^\text{17}\) but contradicting ref. \(^\text{18}\). Dorsally, the latissimus division gives rise to the large, fan-shaped M. latissimus dorsi and deep to it, the subscapular division divides into M. subscapularis and M. scapulo-humeralis, while the deltoid division cleaves early in development into two conspicuous lobes that become the clavicular and scapular division.
The cleavage of the dorsal mass into three divisions (latissimus, subscapular and deltid) in the proximal aspect of the arm was previously described in mammals and birds, although this last was ignored by Romer. Here we show that this is also the case in non-avian reptiles, contrary to the conclusions drawn by previous studies concerning lizards and turtles. Of the variations on this general pattern, the most remarkable is the development of ‘M. teres major’ from the latissimus division in crocodylians, as reported previously in turtles but not in lizards (see below for mammals). As described below, this means that turtles, crocodylians and birds all have divided latissimi, which may be a rare morphological apomorphy of the proposed clade Archelosauria.

In birds, the evolution of the flying apparatus resulted in a highly distinctive and modified pectoral girdle. The main ventral muscle of birds remains M. pectoralis. The supracoracoid division shifts from its original anterior position to lay deep to the pectoralis division and then extends posteriorly along the coracoid and sternum and anteriorly along the coracoid plate, where it curves at the level of the shoulder to sit along the proximal border of the humerus (Extended Data Fig. 3) in agreement with previous work. In contrast to this previous work, however, we find that this distal portion gives rise to M. tensor propatagialis, an avian muscle essential for the performance of sophisticated flight, which thus is not homologous to M. humeroradialis of crocodylians as was previously assumed (below). We find that in reptiles, including birds, muscles that have been proposed to be evolutionarily derived from the deltid division on the basis of adult anatomy (M. subcoracosapularis and M. scapulohumeralis posterior of ref. 11) are derived instead from the subscapular division. This is in agreement with some earlier embryological accounts (Extended Data Fig. 1).

The deltid division separates early into two muscle portions, as observed in most species studied. In non-avian reptiles, however, these portions generally associate with the scapula and clavicle and correspond to the Mm. deltoideus scapularis and clavicularis, respectively, while in birds they originate largely from the proximal humerus and have been referred to as Mm. deltoideus major and minor, respectively. Previous embryonic work misidentified the anterior portion, M. deltoideus minor, as M. tensor propatagialis, which forms anterior and deep to the deltid division from the extension of the supracoracoideus division, as described above. We find that in reptiles, including birds, muscles that have been proposed to be evolutionarily derived from the deltid division on the basis of adult anatomy (M. subcoracosapularis and M. scapulohumeralis posterior of ref. 11) are derived instead from the subscapular division. This is in agreement with some earlier embryological accounts (Extended Data Fig. 1). The scapula becomes thinner and longer, mirroring the evolutionary transition in bird-line
archosaurs, the derivatives of the avian subscapular division remain attached to its posterolateral surface, forming a large muscle accompanied by a smaller one. These correspond to M. subscapularis and M. scapulohumeralis posterior of other reptiles. In birds, these muscles have historically been called M. scapulohumeralis caudalis/posterior and M. scapulohumeralis cranialis, respectively. The so-called ‘M. subscapularis’ of birds is a small muscle originating on the proximal portion of the scapula, unlike M. subscapularis of other amniotes. Its homologies are unclear.

The most drastic transformation of shoulder and chest development pattern and anatomy among amniotes is observed in therian mammals. The coracoid is reduced to a small process fused to the scapula and the sternum is braced solely by ribs and the clavicle. The scapula is broad and its lateral surface is bisected by a ridge (the scapular spine), forming a supraspinous and infraspinous fossa. The supracoracoideus musculature has been suggested to have shifted either partially or entirely to the dorsal aspect of the scapula; and the pectoral musculature is regarded as broadly homologous to the reptilian pectoralis, having expanded over the chest region and separated into two main portions with several subdivisions of debated homologies. In contrast to that of reptiles, the pectoralis division of therians forms only M. pectoralis minor and the deeper portion of M. pectoralis major (in addition to the ‘skin muscle’ M. panniculus carnosus) while a large portion of M. pectoralis major (in addition to the ‘skin muscle’ M. panniculus carnosus) while a large portion of M. pectoralis major (in addition to the ‘skin muscle’ M. panniculus carnosus) (Extended Data Fig. 5) while a large portion of M. pectoralis major is in fact of supracoracoideus division origin, thus being non-homologous to the reptilian pectoralis it so resembles. The supracoracoideus division of therians extends dorsally and fully invades the lateral surface of the scapula, bifurcating around the sides of the scapular spine, giving rise to M. subscapularis and infraspinatus, occupying the supra and infraspinous fossae, respectively (Extended Data Fig. 5). On the ventral aspect of the shoulder the supracoracoideus division gives rise to the superficial portion of M. pectoralis major as noted above and the therian ‘M. deltoideus clavicularis’. Therefore, contrary to previous assumptions, the therian supracoracoideus division does not translocate dorsally but rather expands over the shoulder while still forming muscles of the chest. The deltoideus clavicularis, like those of other amniotes studied, separates into two muscular portions early in development; however, both remain associated with the scapula, probably owing to exclusion from the clavicle region by the precocious expansion of the supracoracoideus division. The most-anterior muscle, termed ‘M. deltoideus scapularis’, probably corresponds to the muscle portion that becomes the clavicular deltoïd in reptiles, while the posterior portion, termed M. teres minor, corresponds to that which becomes the scapular deltoïd of reptiles (Extended Data Fig. 6). M. teres minor was not observed in the short-tailed opossum; however, it is present in other marsupials and derives from the deltoïd division in a similar fashion to what we observe in the mouse. The subscapular division of therians expands occupying the broad medial surface of the scapula (forming M. subscapularis) and extends over its posterior edge to cover part of its posterolateral surface; this portion separates forming M. teres major (Extended Data Fig. 6). Note that the so-called teres major muscle of turtles and crocodilians derives from the latissimus division (Extended Data Fig. 4), while the mammalian homonym derives from the subscapular division, showing that these muscles are not truly homologues, contrary to previous assumptions.

The arm musculature

The triceps and biceps subdivisions form muscle complexes with origin on the girdles or proximal humerus. They respectively serve largely to extend and flex the forearm. We find the triceps subdivision in all amniotes extends well beyond the elbow along the posterior margin of the dorsal forearm, where it forms the posterior-most forearm extensor muscle. Although this muscle has received various names, its identity as a triceps division derivative and homology across all taxa is clear on the basis of our data (Extended Data Fig. 7). We also corroborate that M. dorsoepitrochlearis, an exclusively mammalian muscle, derives from the triceps and not from the latissimus division. The biceps division gives rise to the M. biceps brachii and Mm. coracobrachiales (Extended Data Fig. 8). While we find that M. brachialis of the Alligator also derives from the biceps division, it is not homologous to the brachialis muscles of other amniotes (below).

The forearm musculature was classically proposed (without embryological evidence) to derive from dorsal and ventral elbow and wrist matrices, respectively giving rise to muscles connecting the humerus to the antebrachium and the antebrachium to the hand. In contrast, we find that the extensor and flexor divisions cleave into three superficial and one deep lobes. Individual muscles derive from each lobe, instead of arising from proximal and distal forearm muscle divisions. The flexor division cleaves into three superficial lobes: one clearly associated with the ulna, another with the radius and a third, central lobe in between (Extended Data Fig. 9). The three extensor lobes, in contrast, are shifted anteriorly, as the ulnar region of the dorsal forearm is occupied by the distal extension of the triceps division mentioned earlier. Consequently, the posterior-most lobe is ‘central’, the middle lobe is radial and the most-anterior lobe extends proximally along the humerus. M. brachialis of mammals, birds, lizards and turtles was previously interpreted as being derived from the same region as M. biceps brachii; however, we find that this muscle derives from the anterior-most portion of the extensor lobe, as does the muscle called humeroradialis of crocodylians, which is homologous to the brachialis of other amniotes (Extended Data Fig. 8). The central lobes of both extensor and flexor divisions form the major muscles with actions on the digits or the whole hand (extensor digitorum and flexor digitorum) and, in some cases, secondary muscles that can associate with muscles from a neighbouring lobe, sharing a tendon or forming a muscle complex (Extended Data Fig. 9). The deep flexor lobe is present in all species and gives rise to a variable number and type of muscles. Its distal portion extends into the hand in reptiles, deep to the hand flexor division muscles, where it cleaves into variable individual units that aggregate into compound muscles (the ulnar and carpal heads of M. flexor digitorum longus in Alligator and palmar head of M. flexor digitorum longus in Paroedura, are examples).

The mammalian flexor division is slightly different from the reptilian one: instead of running parallel to each other, the superficial ulnar and radial lobes and their muscle fibres diverge with respect to the central lobe. This is possibly a consequence of the torsion of the radius associated with the change in elbow orientation that occurs in mammalian development and evolution. We find that the muscle referred to as M. flexor carpi radialis derives from the central lobe, making it homologous to the reptilian M. flexor digitorum longus, not to the identically named radial flexors of other amniotes as is the current consensus. Previous work has highlighted the possibility that muscles designated as M. palmaris longus are not homologous across amniotes. We find that the mammalian M. palmaris longus derives from the most-anterior portion of the ulnar lobe, confirming that it is not a homologue to the muscle so-named in turtles, which instead comes from the central lobe and is homologous to the M. flexor digitorum longus of other reptiles. M. epitrochlealconeus, a unique mammalian muscle, develops from the proximal end of the ulnar lobe in both Mus and Monodelphis, as has also been shown previously in humans.

The hand musculature

The muscles of the hand have generally been treated as an anatomical unit; however, during development they derive from two distinct regions. The most distal portions of the extensor and flexor divisions separate, forming the hand extensor (HE) and hand flexor (HF) subdivisions, respectively. Both regions separate into distinctive...
layers during development. Avian hand elements are greatly transformed\(^2\); accompanying this skeletal transformation, the hand musculature of birds is also highly simplified. This extreme reduction of the number of hand intrinsic muscles observed in birds does not derive from heavily reduced muscle precursors or absence of an entire division as seen in mammals (below). In early stages the precursor regions of these muscles look normal and it is later in development that both dorsal and ventral avian hand divisions fail to separate into the layers observed in other amniotes and ultimately form a considerably smaller number of superficial muscles. Trying to establish one-to-one homologies in this case, between a handful of individual muscles in birds and more than 50 in other reptiles, is extremely difficult. However, as the avian hands show an absence of stratification into layers in both dorsal and ventral muscle divisions, they are probably at least partially homologous to some of the most-superficial hand extensor and flexor muscles of other amniotes, as those superficial muscles are the first to differentiate in other species studied and the portion originating the deeper ones forms and individualizes later.

The hand extensor subdivision of non-avian reptiles separates distinctly into two layers, which form the Mm. extensores digitorum breves superficiales and Mm. extensor digitorum breves profundus, respectively. In contrast, the entire hand extensor subdivision of therians fails to develop and we find that therian hands bear no trace of the ancestral extensor musculature, contradicting the current hypothesis that these dorsal muscles shifted proximally to become extensors in the distal portion of the forearm\(^2\). All therian hand muscles derive from the hand flexor subdivision alone. The hand flexor subdivision of reptiles cleaves into separate layers (Extended Data Fig. 10). The most-superficial (ventral) layer appears to be itself stratified. Its more superficial portion gives rise to muscles of varying robusticity serving the first and fifth digits, as well as a variable number of muscles serving the other individual digits, while its deeper portion gives rise to muscles that have either been lumped together as part of the most superficially derived Mm. flexor digitorum breves superficiales or identified as separate muscles. Originating from the most-superficial portion, Mm. flexores digitorum breves superficiales of digits II–IV of the mouse undergo a radical translocation towards the proximal end of the forearm and fuse to form M. flexor digitorum superficiales as previously described\(^2\). We find that in perinatal stages of the short-tailed opossum these muscles also begin elongating towards the proximal forearm (Extended Data Fig. 10), indicating that this process might be ancestral for therian mammals. The therian M. lumbricales and crocodylian M. flexores digitorum breves intermedius and profundus appear to derive from the layer deep to this portion. The following, deeper, layers seem to become the reptilian M. flexores digitorum breves profundus or identified as separate muscles. Originating from the most-superficial portion, Mm. flexores digitorum breves superficiales of digits II–IV of the mouse undergo a radical translocation towards the proximal end of the forearm and fuse to form M. flexor digitorum superficiales as previously described\(^2\). We find that in perinatal stages of the short-tailed opossum these muscles also begin elongating towards the proximal forearm (Extended Data Fig. 10), indicating that this process might be ancestral for therian mammals. The therian M. lumbricales and crocodylian M. flexores digitorum breves intermedius and profundus appear to derive from the layer deep to this portion. The following, deeper, layers seem to become the reptilian M. lumbricales (possibly the therian M. contrahentes) and a deeper layer gives rise to Mm. interossei (the therian M. flexores breves profundi) (Extended Data Fig. 2). In perinatal stages of Monodelphis, we observed muscles derived from the hand flexor subdivision extending towards the dorsal aspect of the hand, squeezing in between the metacarpals (Extended Data Fig. 10), similar to the pattern described for M. flexores breves profundi of humans\(^4\). On the basis of this and the complete absence of dorsal muscle progenitors (hand extensor subdivision), the ventral and dorsal interossei/intern metacarpales seem to derive in their totality from the ventral musculature. In reptiles, we were not capable of observing the individualization or time of appearance of these deep muscles; thus, our data do not allow us to determine whether Mm. interossei dorsales in other amniotes also correspond to dorsally translocated ventral muscles or whether they are, in turn, dorsal muscles not homologous to the mammalian homonyms. As mentioned before, the ventral origin of all hand muscles of mammals, including those with a more dorsal location, and the complete absence of a dorsal hand extensor subdivision contradicts the notion that the dorsal hand musculature observed in reptiles migrated proximally into the dorsal forearm in mammal evolution\(^2\). Rather, this points to the possibility that independent muscles deriving from the extensor subdivision co-opted the tendinous framework left unused by the disappearance of the dorsal muscles of the hand, facilitated by the modular nature of tendon development as was shown by ref. \(^4\).

**Discussion**

Our data show that the muscular masses that give rise to the forelimb musculature cleave in a highly stereotyped pattern across amniotes, most similar to that described by Sullivan in chicken embryos\(^2\). The patterns previously described for non-avian reptiles (turtles and lizards) differ in greater measure from our own descriptions, probably owing more to the availability of material and the state of the art of the techniques used than to interspecific differences in development among turtles and lizards.

The development of the resulting divisions remains conservative in later stages of muscle division. Even the forelimb muscles of birds, which appear highly transformed, have largely arisen through simple stretching and translation of muscle divisions that look essentially like those of other reptiles. While the different adult anatomies exhibited by reptiles derive from these similar embryonic patterns, the origin of the unique adult musculature of therian mammals results from a rearrangement of the shoulder musculature, complete loss of the dorsal hand musculature and translocation and
reorganization of whole individual flexor muscles (Fig. 4)—all nevertheless achieved by modifying the stereotyped amniote trajectory. The adult anatomy of stem and early amniotes, including those ancestors of mammals, can be readily compared to that of non-avian reptiles, suggesting that the ancestral pattern of amniote forelimb muscle development was like that of reptiles and that the events observed in therian embryos represent unique therian or mammal developmental innovations leading to unique adult anatomies. Monotremes, whose embryos are not readily available, represent a mosaic of ‘reptilian’ and ‘therian’ musculoskeletal anatomy and information on their embryology could be vital to understand the developmental basis of mammal evolutionary history. Interestingly, the pattern of muscle development here described looks very different from that described for amphibian species, in which individual muscles seem to form without continuity with other muscles or derivation from a muscle mass. It is important to note that the cleavage pattern of muscle masses and even the distinctive morphology of individual muscles precede their association with bones. The association between muscle and bone is established well after the formation of the muscle and the

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**Fig. 5** | The developing brachial plexus and forelimb musculature in *A. mississippiensis*. The influence and instructing interactions of surrounding tissues is relevant for the developmental pattern of forelimb musculature. As observed in other species (not shown) the nerves of the brachial plexus (Spinal Nerves VII–XI in *Alligator*) extend in between the divisions of the forelimb developing musculature, being possibly even responsible or in part involved in the separation. In early stages, the proximal base of the dorsal cord separates the deep subscapular division from the rest of the dorsal muscle mass and, ventrally, the ventral intrinsic division is separated from the pectoralis and supracoracoideus divisions that remain together. In later stages, the nerves also extend to lie in between the separated deltoid, latissimus and dorsal intrinsic divisions dorsally and separating the pectoralis and supracoracoideus divisions. The correspondence between the passage of the nerves and the separation of the muscle masses is stronger in earlier stages and in older embryos the nerves associate less intimately with them, indicating that probably in later stages the separation of individual muscles relies on different cues than in the earlier cleavage steps. Scale bars, 500 μm.
cartilage predecessors of bones, as noted by ref. 46. A long-standing, implicit assumption is that homology of skeletal attachment sites is indicative of homology of muscles, an idea reformulated in more modern developmental terms as the muscle scaffold theory48.

Although skeletal elements as points of origin and attachment for muscles are important to function and, although attachments are often conserved, the early division of, for example, the deltoid division into two lobes relies neither on the presence of the clavicle nor the scapula; indeed, we find that muscles such as deltoids can become associated with different bones than in the ancestral condition while maintaining their topology relative to other muscles, as in mammals. This suggests that the notion that a muscle with the same embryonic origin but different attachments cannot be homologous is unfounded. Therefore, for instance, the clavicular deltoid of conservative amniotes, which is attached to the scapula, and the mammalian so-called ‘scapular deltoid’, which is not, appear nonetheless to be homologues. A view from early muscle development has thus allowed us to resolve the evolutionary history and identities of several problematic amniote arm muscles, including the homology of the archelosaurian teres major muscle, the embryonic origin of the avian tensor propatagii muscle, the complex rearrangement of the pectoral and supracoracoidus musculature in therian mammals, the identity of the crocodylian humeroradialis muscle and its homology to the brachialis muscle of other amniotes, the non-homology of muscles like M. teres major, flexor carpi radialis, palmaris longus and deltoideus claviclaris across amniotes.

The factors involved in the patterning and instruction of muscle cleavage, growth, attachment and individualization are not yet fully understood. Current evidence suggests that external cues from the developing connective tissue49–51, tendons52, vasculature53 and nerves54 provide information for the proper development of limb muscle architecture55. Interestingly, in the earliest stages of muscle mass splitting, the nerves of the brachial plexus are observed in tight connection with the developing muscle masses; they may in fact contribute to the physical separation of the divisions from each other (Fig. 5). Despite the dramatic variety of amniote locomotor anatomy, the initial steps of development and structural arrangement of the musculoskeletal elements have fundamental, and often highly conserved, influences on the adult anatomy of forelimb muscles. It is tempting to imagine the developing nervous system, extracellular connective tissue and tendinous system as agents involved in the cleavage of the early muscle masses, providing not only molecular cues but a physical scaffold exerting mechanical influence, directing and constraining muscle division patterns; however, more research is needed to understand how these developing anatomical structures can physically influence each other’s development. More research on these developmental processes across different clades is needed, also, to understand not only basic evolutionary correspondence but also the intricate interactions established among developing tissues and the role of those interactions as both effectors in development and constraints in evolution.

Methods
All methods were approved and complied with all ethical regulations of Yale University’s Office of Animal Research Support (OARS) IACUC protocol 2017-20153.

Embryo collection. Mouse (Mus musculus) timed matings were established between B6SJLF1/J males (obtained from the Jackson Laboratory) and Swiss Webster outbred females (obtained from Taconic BioSciences). Noon of the day of plug was recorded as embryonic E0.5. The uterus from pregnant females was isolated and embryos dissected into phosphate buffered saline (PBS). Grey short-tailed opossum (Monodelphis domestica) embryos were collected from a colony housed at Yale University. Males and females were paired and filmed to determine the time of implantation. Embryos were then incubated over three times for 15 min in PBS at 37 °C and then placed in a solution of sodium dodecyl sulphate (SDS) 4%, boric acid 200 mM, pH 8.5 on a rocker at 37 °C until they became transparent. Transparent embryos were washed twice in PBS at 37 °C for 30 min and four times in PBS (PBS + 1% Triton-X100, Sigma-Aldrich) for 1 h at room temperature. Embryos were then incubated overnight in a solution of 20% DMSO, glycine 0.3 M after which they were incubated overnight in a blocking solution of PBS 10% DMSO and 10% horse normal serum (HNS)56. Embryos were incubated in PBS 5% DMSO, 5% HNS, 0.1% sodium azide and primary antibodies against myosin heavy chain (MF-20, Developmental Studies Hybridoma Bank, DSHB) at 1:40 dilution or myosin heavy chain (MYH3, AB124205, ABCAM) 1:1,000 in some Monodelphis embryos, plus combinations of antibodies targeting neurofilament (3A10, DSHB) 1:50, collagen type II (II-II6B3, DSHB) or Sox9 (anti-Sox9, ABS535 ABCAM) 1:1,000 for 3–4 d on a rocker at 37 °C. After this, embryos were washed in PBS in 1 h at 37 °C and five times for 1 h in PBS at room temperature. Anti-mouse secondary antibodies (Alexa Fluor Goat anti-IgG1 488, Goat anti-IgG2b 555, Goat anti-rabbit 647 Invitrogen) were used at a dilution of 1:1,000 in PBS 5% DMSO, 5% HNS, 0.1% sodium azide and incubated for 3–4 d on a rocker at 37 °C inside a dark box. Embryos were then washed in PBS in 1 h at 37 °C and five times for 1 h in PBS at room temperature in a dark box. At least two (for Monodelphis) or three embryos were initially stained per each individual embryonic stage from around the forelimb paddle-stage to interdigital membrane thinning or disappearance stages, for each combination of antibodies. On examination of their anatomy, more embryos were stained in similar stages with intermediate external morphology to bridge the gaps of anatomical development progression when needed.

Imaging and processing. Immunostained embryos were transferred to RIMS (refractive index matching solution57) and allowed to settle for 3–4 d. After this, embryos were mounted on Clear Glass bottom dishes (PELCO) in liquid 1% low melting point agarose made on RIMS and left in the dark at room temperature to solidify. Embryos were then imaged using a Carl Zeiss LSM880 Confocal Microscope collecting multiple tiles of z stacks, according to the size of the embryo. Files were open with Fiji (ImageJ) (Fig. 1a–c) and each channel converted into .jpg file series to be imported as separated volumes into VGStudioMax 3.0 for digital segmentation where individual muscles or portions of muscles can be segmented using a combination of the volumetric brush and region-growing tools (Fig. 1de). No signal was excluded during the process and only objects that were obviously separately spaced were made into individual regions of interest. VGStudio segmentation is lossless in that the segments created using these tools are voxel objects instead of surfaces and therefore retain the full resolution, internal structure and gray-scale fidelity of the original data. Rendering was performed in VGStudio directly from the lossless volume objects such that the 3D images generated reflect the entirety of the regional fluorescent signal.

Muscles considered. A number of muscles (serratus muscles, M. levator scapularis, M. sternocoracoideus, M. costocoracoideus, M. trapezius (derived from the culliculus muscle), rhomboid muscles) attach to and effect their action on the pectoral girdle. However, they do not derive from the migratory cells forming the limb dorsal and ventral muscle masses but instead originate directly from the somites58–60 or the lateral plate mesoderm in the case of the culliculus muscle and its derivatives61. These muscles were not considered in this study, which only focuses on those muscles derived from the forelimb muscles masses.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Confocal files have been deposited in Dryad (https://doi.org/10.5061/dryad.r2280gb8d).
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**Author contributions**

D.S.-P. conceived the study, cared for and maintained the colony of *P. pictus*, collected eggs and embryos of all reptile species, performed immunostaining experiments and imaged the samples, processed the files and segmented them in VGStudio, created the figures and wrote the manuscript. M.E.V.-C. cared for and maintained the colony of *P. pictus*, collected eggs and embryos of *P. pictus*, bred and collected embryos of *M. domestica*, performed immunostaining experiments, imaged, processed and segmented in VGStudio all *M. domestica* samples. A.I. collected *S. odoratus* embryos. M.M.M. and R.R.B. bred and collected embryos of *M. musculus*. B.-A.S.B. provided logistical and financial support for the reptile colony, embryo collection, immunostaining, microscopic imaging and digital segmentation of the samples and helped write the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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### Dorsal Musculature

| Division | Lepidosauria | Testudines | Aves | Crocodylia | Theria |
|----------|-------------|------------|------|------------|-------|
|          | Russel and | Walker 1973 | Vanden Berge and | Meers 2003 | Diogo et al. 2009 |
|          | Bauer, 2008 |            | Zweers, 1993 | George and Berger, 1996 |       |

### Subscapular

|          | Subscapularis | Subscapularis | Scapulohumeralis | Scapulohumeralis | Subscapularis |
|----------|---------------|---------------|------------------|------------------|---------------|
|          | Subscapularis | Scapulohumeralis | Scapulohumeralis | Scapulohumeralis | Subscapularis |
|          | posterior     | cranialis     | posterior        | cranialis        |                |
|          |               |               |                  |                  |               |

### Latissimus

|          | Latissimus dorsi | Latissimus dorsi | Latissimus dorsi | Latissimus dorsi |
|----------|------------------|------------------|------------------|------------------|
|          | Teres major      | Teres major      | Latissimus dorsi | Latissimus dorsi |

### Deltoideus

|          | Deltoideus scapularis | Deltoideus major | Deltoideus minor | Deltoideus minor |
|----------|-----------------------|------------------|------------------|------------------|
|          | Deltoideus major      | Deltoideus major | Deltoideus minor | Deltoideus minor |
|          | Scapulohumeralis      |                  |                  |                  |
|          | anterior              |                  |                  |                  |

### Triceps

|          | Triceps complex      | Triceps complex  | Triceps brevis   | Triceps brevis   |
|----------|----------------------|------------------|------------------|------------------|
|          | Triceps brevis      | Triceps brevis   | Triceps brevis   | Triceps brevis   |
|          |                      |                  |                  |                  |

### Extensor

|          | Extensor carpi ulnaris | Extensor carpi ulnaris | Extensor carpi ulnaris | Extensor carpi ulnaris |
|----------|-------------------------|------------------------|------------------------|------------------------|
|          | Brachialis anticus     | Brachialis anticus    | Brachialis anticus    | Brachialis anticus    |
|          | Brachialis anticus     | Brachialis anticus    | Brachialis anticus    | Brachialis anticus    |

### Extended Data Fig. 1

See next page for caption.
Extended Data Fig. 1 | Homologies of the dorsal musculature of the forelimb in Amniota. Homologies of the dorsal musculature of the forelimb in Amniota. Muscles in individual rows are inferred to be homologous across clades based on their embryological origin and cleavage pattern from the divisions and subdivisions identified in the species studied. Rows containing more than one muscle per taxa reference cases in which the distinction of the muscles are not clear or individual portions of one muscle can be homologized to individual muscles of another group. Each muscle is named according to the nomenclature proposed by the authors listed under the clade names. Names proposed for reptilian musculature by Abdala and Diogo, 2010, are listed below if the nomenclature is not the same as in the other studies or they refer to a muscle complex.
| DIVISION | VENTRAL MUSCULATURE | PECTORAL | SUPRACORACOID | BRACHIAL | FLEXOR | HAND FLEXOR |
|----------|---------------------|----------|-------------|---------|--------|------------|
|          | Lepidosauria        | Pectoralis| Supracoracoideus | Coracobrachialis brevis | Pronator teres | Flexor digitorum brevis |
|          | Russet and Bauer, 2008 | Pectoralis| Supracoracoideus | Coracobrachialis brevis anterior | Flexor digitorum communis | Flexor digitorum brevis superciliaris |
|          | Walker 1973 | Pectoralis| Supracoracoideus | Coracobrachialis brevis ventralis | Flexor digitorum longus | Flexor digitorum brevis superciliaris |
|          | Vanden Berg and Zweers, 1993 | Pectoralis| Supracoracoideus | Coracobrachialis magnus | Flexor digitorum longus | Abductor digiti quinti |
|          | George and Berger, 1996 | Pectoralis| Supracoracoideus | Coracobrachialis posterior | Flexor digitorum longus | Abductor digiti minimi |
|          | Crocodylia Meers 2003 | Pectoralis| Supracoracoideus | Biceps brachii | Flexor digitorum longus | Flexor digitorum supercilii |
|          | Theria Diogo et al., 2009 | Pectoralis minor| Supracoracoideus | Biceps flexor superficialis | Flexor digitorum longus | Flexor digitorum brevis superficialis |
|          |                     | Pectoralis major profundus | Supracoracoideus | Biceps humeral head | Flexor digitorum brevis superficialis | Flexor digitorum brevis profundus |
|          |                     | Panniculus carneosus | Supracoracoideus | Brachialis | Flexor digitorum brevis superficialis | Lumbricales |
|          |                     | Infraespinatus | Supracoracoideus | Biceps short head | Flexor digitorum brevis superficialis | Contrahentes |
|          |                     | Supraspinatus | Supracoracoideus | | Flexor digitorum brevis superficialis | Lumbricales |
|          |                     |                     | Supracoracoideus | | Flexor digitorum brevis superficialis | Lumbricales |

Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Homologies of the ventral musculature of the forelimb in Amniota. Homologies of the ventral musculature of the forelimb in Amniota. Muscles in individual rows are inferred to be homologous across clades based on their embryological origin and cleavage pattern from the divisions and subdivisions identified in the species studied. Rows containing more than one muscle per taxa reference cases in which the distinction of the muscles are not clear or individual portions of one muscle can be homologized to individual muscles of another group. Each muscle is named according to the nomenclature proposed by the authors listed under the clade names. Names proposed for reptilian musculature by Abdala and Diogo, 2010, are listed below if the nomenclature is not the same as in the other studies or they refer to a muscle complex.
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Development of the forelimb musculature of reptiles, with focus on the ventral extrinsic musculature. Development of the forelimb musculature of reptiles, with focus on the ventral extrinsic musculature derived from the Pectoralis (dark green) and Supracoracoideus (light green) divisions. All views ventral, except Paroedura PO 22 that is lateral and Coturnix HH33-34 that is a medial view. The Supracoracoideus division usually lays anterior to the Pectoralis division; in Coturnix in turn, it has shifted deep to it and extends into the humerus forming a pronounced curve. The proximal portion forms M. supracoracoideus (empty light green arrow) while the distal portion, beyond the curve, forms M. tensor propatagialis (light green arrow). PD: Pectoralis division, sc: M. supracoracoideus, SCD: Supracoracoid division, tpp: M. tensor propatagialis. All scale bars are 500 µm.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Development of the forelimb musculature of reptiles, with focus on the dorsal extrinsic musculature. Development of the forelimb musculature of reptiles, with focus on the dorsal extrinsic musculature derived from the Latissimus (dark red), Deltoid (red) and Subscapular (magenta) divisions. All images show lateral view, except for the second row of Coturnix showing medial views of the forelimb musculature. The empty red arrows and the red arrows point at the development of the scapular and clavicular lobes of the Deltoid division respectively. In Sternotherus, like in some other turtles, the scapular portion does not form. The empty dark-red arrow points at the anterior lobe formed from the Latissimus division. In Alligator, this gives rise to the Teres major muscle (tm). In Chrisemys, as described by Walker 1947, this also gives rise to M. teres major. In Sternotherus, this muscle does not seem to develop, although in stage 16 an incipient anterior lobe of the Latissimus division, comparable to that of Alligator can be observed (tm?). In Coturnix, the Latissimus division divides (although slightly later) into two lobes, giving rise to the anterior (tm?) and posterior heads of the latissimus muscle. This sort of lobation is not observed in Paraedura and no comparable muscle develops in lizards. The empty magenta arrow points at M. subscapularis (M. scapulohumeralis caudalis of birds). dc: M. deltoideus clavicularis; DD: Deltoid division; ds: M. deltoideus scapularis; ld: M. latissimus dorsi; ldp: M. latissimus dorsi posterior; LD: Latissimus division; shc: M. scapulohumeralis caudalis; sbcc: M. subcoracoideus; sbsc: M. subscapularis; SSD: Subscapular division; tm: M. teres major; *: M. scapulohumeralis anterior. All scale bars are 500 μm.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Development of the forelimb musculature of therian mammals with focus on the ventral extrinsic musculature. Development of the forelimb musculature of therian mammals, Mus and Monodelphis, with focus on the ventral extrinsic musculature derived from the Pectoral (dark green) and Supracoracoideus (light green) divisions. The Pectoralis division originates *M. panniculus carnosus* (white bordered dark green arrow), *M. pectoralis minor* (empty dark green arrow) and the deep portion of *M. pectoralis major* (dark green arrow). The ventral portion of the Supracoracoideus division forms the superficial portion of *M. pectoralis major* (empty light green arrow) and the clavicular deltoid (light green arrow). The dorsal extension of the Supracoracoideus division invades the dorsal aspect of the scapula (*) and bifurcates around the scapular spine (**) originating *M. supraspinatus* (yellow bordered light green arrow) and *M. infraspinatus* (red bordered light green arrow). The upper series of mouse embryos depicts a medial view of stage E12.0, a lateral view of stage E12.5 and ventral views of E13.5 and 14.5. Bottom series of Monodelphis and Mus show the developing embryos with all muscle groups except for those deriving from the Pectoral and Supracoracoideus divisions removed, and the developing skeleton stained with either Sox9 or Col II antibodies. *M. panniculus carnosus* was removed from Monodelphis MC 33 and 33+ and Mus E14.5. dc: *M. deltoideus clavicularis*, isp: *M. infraspinatus*, pc: *M. panniculus carnosus*, PD: Pectoralis division, pmi: *M. pectoralis minor*, pmp: *M. pectoralis major profundus*, pms: *M. pectoralis major superficialis*, ssp: *M. supraspinatus*. All scale bars are 500 µm.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Development of the forelimb musculature of therian mammals with focus on the dorsal extrinsic musculature. Development of the forelimb musculature of therian mammals, Mus and Monodelphis, with focus on the dorsal extrinsic musculature derived from the Latissimus (dark red), Deltoid (bright red) and Subscapular (magenta) divisions. As in reptiles, the Deltoid division of mice cleaves early into two lobes, forming M. scapulodeltoid (red arrow) and M. teres minor (empty red arrow). In Monodelphis a Teres minor muscle was not observed. The posterior portion of the Subscapular division extends along the posterior margin and lateral surface of the scapula (*), forming M. teres major (empty magenta arrow). The upper series shows Mus embryos between E12.0 and 14.5, all in lateral view. The bottom series of Monodelphis and Mus show the development of the muscles and skeleton in lateral view. M. latissimus dorsi is not shown in mice E14.5. DD: Deltoid division, ds: M. deltoideus scapularis, ld: M. latissimus dorsi, LD: Latissimus division, ssc: M. subscapularis, SSD: Subscapular division, tm: M. teres major, tmi: M. teres minor. All scale bars are 500 µm.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Development of the forelimb musculature of amniotes, with focus on the dorsal intrinsic musculature. Development of the forelimb musculature of amniotes, with focus on the dorsal intrinsic musculature derived from the Triceps (light yellow) and Extensor (yellow) divisions. Note the distal extension of the Triceps division along the ulna, originating the posterior-most extensor muscle of the forearm (empty light-yellow arrow), and the humeral lobe of the Extensor division, extending proximally along the humerus (yellow arrow) and forming M. brachialis of lizards, turtles, birds and mammals, homologous to M. humeroradialis of crocodylians. Mm. dorsoepitrochlearis (light-yellow arrow) of mammals derives from the Triceps subdivision. c: central lobe (posterior-most lobe) of the Extensor division, dep: M. dorsoepitrochlearis, h: humeral lobe of the Extensor subdivision, r: radial lobe of the Extensor subdivision, u: ”ulnar” lobe of the forearm musculature, derived from the Triceps subdivision. All scale bars are 500 µm.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Development of the forelimb musculature of amniotes, with focus on the brachial muscles that act as flexors of the forearm.

Development of the forelimb musculature of amniotes, with focus on the brachial muscles that act as flexors of the forearm. From the Extensor division, on the dorsal forearm, a muscle extends proximally over the humerus (yellow arrow) forming M. brachialis of Paroedura, Sternotherus, Coturnix and Mus, homologous to M. humeroradialis of Alligator. On the other hand, the biceps muscle and the crocodylians M. brachialis derive from the Brachial subdivision (empty blue arrow). bi: M. biceps brachii, br: M. brachialis (note that the muscle termed “brachialis” derive from different origins than in the other species in Alligator), hr: M. humeroradialis (note that the humeroradialis muscle derives from the same portion of the Brachial subdivision than the brachialis muscles of the other species). All scale bars are 500 µm.
Extended Data Fig. 9 | See next page for caption.
Extended Data Fig. 9 | Development of the forelimb musculature of amniotes, with focus on the intrinsic musculature deriving from the Flexor division.

Development of the forelimb musculature of amniotes, with focus on the intrinsic musculature deriving from the Flexor division (light blue). The Flexor division forms a deep lobe, and superficially a central lobe (empty light-blue arrow), flanked by an ulnar lobe on the ulnar side of the forearm (bottom portion of each arm in the images) and a radial lobe on the radial side (top). Note that the central lobe of the Flexor subdivision (pointed at by the empty light-blue arrow and originating *M. flexor digitorum longus/communis*) is the source of the so-called *M. flexor carpi radialis* of mice, otherwise originated from the radial lobe in other species. *M. palmaris longus* of Sternotherus, derives from the central lobe too, while its homonym derives from the ulnar lobe in mice. c: central lobe of the Flexor subdivision, d: deep lobe of the Flexor subdivision, epa: *M. epitrochleoanconeus*, fcra: *M. flexor carpi radialis*, fcu: *M. flexor carpi ulnaris*, fdc: *M. flexor digitorum communis*, fdl: *M. flexor digitorum longus*, pal: *M. palmaris longus*, prop: *M. pronator profundus*, pros: *M. pronator superficialis*, prot: *M. pronator teres*, r: radial lobe of the Flexor subdivision, u: ulnar lobe of the Flexor subdivision. All scale bars are 500 µm.
Extended Data Fig. 10 | See next page for caption.
Extended Data Fig. 10 | Development of the forelimb musculature of amniotes, with focus on the ventral hand musculature deriving from the Hand flexor division. Development of the forelimb musculature of amniotes, with focus on the ventral hand musculature deriving from the Hand flexor division (dark blue). The hand flexor musculature derives from the distal portion of the Flexor division and stratifies into layers; a superficial one (white bordered blue arrow) forms two layers of muscles, usually grouped as the Mm. flexores digitorum breves. M. lumbricales (*) of the mouse seem to develop from this portion of the Hand flexor division. A deeper layer (light-blue bordered dark blue arrow), also stratified, forms the deepest sets of muscles, termed lumbricales and interossei dorsales and ventrales. In the mouse, the most-superficial set of hand flexor muscles (coloured white in E15.5) elongates and translocates proximally into the forearm to form M. flexor digitorum superficialis. Deriving from the HF, a group of muscles extends dorsally in between the metacarpals in Monodelphis stages MC 33+ and 34 (red bordered blue arrows), likely precursors of M. flexor digitorum breves profundi and/or M. intermetacarpales. Note that the Hand extensor musculature fails to develop as observed in a dorsal view of the mouse forearm in E14.5 and of the short-tailed opossum MC stages 33+ and 34. Also, both the dorsal and ventral hand musculature is conspicuously reduced in Coturnix, developing from a non-stratified muscle division and forming considerably fewer muscles than in other reptiles. DL: deep layer of the Hand flexor subdivision, fbp: M. flexores digitioum breves profundi, fdl: humeral head of M. flexor digitorum longus, fdlu: ulnar head of M. flexor digitorum longus, fds: M. flexor digitorum superficialis, SL: superficial layer of Hand flexor subdivision, *: M. lumbricales. All scale bars are 500µm.
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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Parameter              | Description                                                                 |
|------------------------|-----------------------------------------------------------------------------|
| Sample size            | At least 2 embryos per each of at least 5 distinct embryonic stages comparable to stages between HH25 to HH 36 of the chicken were initially used for each immunostaining experiment. Upon examination of their anatomy, more embryos were stained in similar stages with intermediate external morphology to bridge the gaps of anatomical development progression. |
| Data exclusions        | No data was excluded                                                        |
| Replication            | Multiple embryos in equivalent stages were immunostained with different combinations of antibodies, yielding similar results and staining patterns |
| Randomization          | Embryos were collected from different clutches of different females at different times of the year during different seasons |
| Blinding               | Each antibody labels very specific and particular structures in an easily recognizable pattern, so blinding was not possible nor necessary |

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| Clinical data                    |         |
| Dual use research of concern     |         |

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Antibodies used: MF-20, Developmental Studies Hybridoma Bank, DSHB, Neurofilament (3A10, DSHB), Collagen type II (II-II683, DSHB)

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- Cell line source(s): State the source of each cell line used.
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- Mycoplasma contamination: Confirm that all cell lines tested negative for mycoplasma contamination OR describe the results of the testing for mycoplasma contamination OR declare that the cell lines were not tested for mycoplasma contamination.
- Commonly misidentified lines (See ICLAC register): Name any commonly misidentified cell lines used in the study and provide a rationale for their use.
Palaeontology and Archaeology

Specimen provenance
Provide provenance information for specimens and describe permits that were obtained for the work (including the name of the issuing authority, the date of issue, and any identifying information). Permits should encompass collection and, where applicable, export.

Specimen deposition
Indicate where the specimens have been deposited to permit free access by other researchers.

Dating methods
If new dates are provided, describe how they were obtained (e.g. collection, storage, sample pretreatment and measurement), where they were obtained (i.e. lab name), the calibration program and the protocol for quality assurance OR state that no new dates are provided.

Ethics oversight
Identify the organization(s) that approved or provided guidance on the study protocol, OR state that no ethical approval or guidance was required and explain why not.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Eggs of quail (Coturnix japonica) and musk turtle (Sternotherus odoratus) were purchased to specialized farms. Mice (Mus musculus), Opossum (Monodelphis domestica) and pictus gecko (Paroedura pictus) embryos and/or eggs were collected from laboratory animals.

Wild animals
Alligator mississippiensis eggs were collected from the Rockefeller Wildlife Refuge, Louisiana, USA.

Field-collected samples
For laboratory work with field-collected samples, describe all relevant parameters such as housing, maintenance, temperature, photoperiod and end-of-experiment protocol OR state that the study did not involve samples collected from the field.

Ethics oversight
All protocols were approved by the Office of Animal Research Support under IACUC Protocol 2020-20153.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics
Describe the covariate-relevant population characteristics of the human research participants (e.g. age, gender, genotypic information, past and current diagnosis and treatment categories). If you filled out the behavioural & social sciences study design questions and have nothing to add here, write "See above."

Recruitment
Describe how participants were recruited. Outline any potential self-selection bias or other biases that may be present and how these are likely to impact results.

Ethics oversight
Identify the organization(s) that approved the study protocol.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Clinical data

Policy information about clinical studies
All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration
Provide the trial registration number from ClinicalTrials.gov or an equivalent agency.

Study protocol
Note where the full trial protocol can be accessed OR if not available, explain why.

Data collection
Describe the settings and locales of data collection, noting the time periods of recruitment and data collection.

Outcomes
Describe how you pre-defined primary and secondary outcome measures and how you assessed these measures.

Dual use research of concern

Policy information about dual use research of concern

Hazards
Could the accidental, deliberate or reckless misuse of agents or technologies generated in the work, or the application of information presented in the manuscript, pose a threat to:

- Public health
- National security
- Crops and/or livestock
- Ecosystems
- Any other significant area

Experiments of concern

Does the work involve any of these experiments of concern:

- Demonstrate how to render a vaccine ineffective
- Confer resistance to therapeutically useful antibiotics or antiviral agents
- Enhance the virulence of a pathogen or render a nonpathogen virulent
- Increase transmissibility of a pathogen
- Alter the host range of a pathogen
- Enable evasion of diagnostic/detection modalities
- Enable the weaponization of a biological agent or toxin
- Any other potentially harmful combination of experiments and agents

ChIP-seq

Data deposition

- Confirm that both raw and final processed data have been deposited in a public database such as GEO.
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

Data access links

- Provide a list of all files available in the database submission.

Files in database submission

- Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.

Genome browser session

(e.g. UCSC)

Methodology

| Replicates | Describe the experimental replicates, specifying number, type and replicate agreement. |
| Sequencing depth | Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end. |
| Antibodies | Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number. |
| Peak calling parameters | Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used. |
| Data quality | Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment. |
| Software | Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details. |
Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used.

Instrument
Identify the instrument used for data collection, specifying make and model number.

Software
Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details.

Cell population abundance
Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.

Gating strategy
Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between “positive” and “negative” staining cell populations are defined.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type
Indicate task or resting state; event-related or block design.

Design specifications
Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.

Behavioral performance measures
State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).

Acquisition

Imaging type(s)
Specify: functional, structural, diffusion, perfusion.

Field strength
Specify in Tesla

Sequence & imaging parameters
Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle.

Area of acquisition
State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined.

Diffusion MRI
- Used
- Not used

Preprocessing

Preprocessing software
Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.).

Normalization
If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization.

Normalization template
Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized.

Noise and artifact removal
Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration).
### Volume censoring

*Define your software and/or method and criteria for volume censoring, and state the extent of such censoring.*

### Statistical modeling & inference

#### Model type and settings

*Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects, drift or auto-correlation).*

#### Effect(s) tested

*Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used.*

#### Specify type of analysis:

- [ ] Whole brain
- [ ] ROI-based
- [ ] Both

#### Statistic type for inference

*(See Eklund et al. 2016)*

*Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods.*

#### Correction

*Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo).*

### Models & analysis

#### n/a

- [ ] Functional and/or effective connectivity
- [ ] Graph analysis
- [ ] Multivariate modeling or predictive analysis

#### Functional and/or effective connectivity

*Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information).*

#### Graph analysis

*Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.).*

#### Multivariate modeling and predictive analysis

*Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics.*