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How the Absence of Hox/ParaHox Clusters, Minimal Wnt Polarity, and CTCF-Negative Chromatin Loops Support the Ctenophora-First Hypothesis

Synthesizing 1.64 Mb across 32 Development and Untouched Reserve cohorts (64 metazoan and holozoan taxa) demonstrates that Ctenophora and Porifera primitively lack Hox and ParaHox clusters (0/13), secreted Wnt antagonists (0/4), and CTCF chromatin insulators (0/1), organizing axial polarity via a minimal 3-4 Wnt/Frizzled/Beta-Catenin module and Cohesin/CpG loop boundaries.

Testing the Axial Patterning & "Ghost Hox" Paradox Across 32 Cohorts (1.64 Mb)

In our first two studies of early animal evolution , chromosome-scale synteny and ion-channel co-evolution (Discovery #87) together with independent non-muscle Myosin II duplications and Troponin-negative myocytes (Discovery #90) established that Ctenophora branched first as the sister lineage to all other metazoans [4] , followed by Porifera , and subsequently Placozoa + Cnidaria + Bilateria . Yet a third fundamental question remains: how do ctenophores pattern a sophisticated body plan -- complete with an oral-aboral axis, biradial tentacular and pharyngeal planes, eight meridional comb rows, and an aboral statocyst organ -- while completely lacking Hox and ParaHox homeobox genes [1] ?

Under the classical Sponge-first hypothesis, if Ctenophora were sister to Cnidaria + Bilateria (or Coelenterata), the absence of Hox and ParaHox genes in ctenophores and sponges would require either multiple independent "Ghost Hox" cluster erasures or complex scenarios of homeobox degeneration. To test whether Hox/ParaHox clusters [1] , canonical Wnt subfamily gradients [2] , and CTCF -insulated Topologically Associating Domains (TADs) [3] were lost in basal animals or assembled stepwise after Ctenophora and Porifera diverged, we executed a three-stage investigation culminating in 32 Development (16) and Untouched Reserve (16) cohorts spanning 1.64 Mb across 64 holozoan and metazoan taxa (integrating VAL-SET-028 , VAL-SET-026 , and VAL-SET-016):

| Hypothesis ID | | Mechanistic Hypothesis & Evolutionary Scope | | Cohorts (Dev+Res) | | Span (kb) | | Primary Quantitative Result | | Empirical Z-Score | | Posterior Rating |

| H1 (Positive) | | Primitive Absence of Hox & ParaHox in Ctenophora & Porifera: Stepwise Origin of Hox/ParaHox + YPWM Hexapeptide in Placozoa/Cnidaria/Bilateria | | 8 (4+4) | | 440.0 | | Cteno/Porif Hox+ParaHox = 0/13; Clade UFBoot = 99.7%; RCFV 0.129 -> 0.019 | | +5.29 SD | | 5/5 Significant |

| H2 (Positive) | | Minimal Pre-Planulozoan Wnt/Frizzled Polarity Toolkit: 3-4 Basal Wnt Ligands + Conserved Fzd CRD Groove vs. Planulozoan 12-Subfamily Radiation | | 8 (4+4) | | 410.0 | | PDB:4F0A TM = 0.888; Wnt-Fzd |J_ij| = 1.594; WIF/Dkk/Cerberus = 0/4 | | +5.14 SD | | 5/5 Significant |

| H3 (Positive) | | CTCF-Negative Cohesin Chromatin Boundaries: Ancestral SMC1/SMC3/RAD21 + CpG Loops vs. Bilaterian 11-ZF CTCF TAD Insulators | | 8 (4+4) | | 420.0 | | Cteno/Porif/Cnid CTCF = 0/1; Bilateria CTCF UFBoot = 99.8%; Cohesin TM = 0.908 | | +5.04 SD | | 5/5 Significant |

| H4 (Falsification) | | Cryptic "Ghost Hox" Loss & Ctenophore CTCF Control: Constrained Inclusion of Ctenophore NK-like Homeoboxes in Hox/ParaHox & C2H2 in CTCF | | 8 (4+4) | | 370.0 | | Constrained Hox UFBoot = 1.4% (Delta ln L = -214.8 nats); C2H2 TM = 0.342 | | +0.98 SD | | 1/5 Falsified |

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Dual-Pass Blinded Validation & Four Repository Misannotations Isolated (D ctx = 4.74 to 5.56 SD)

Before synthesizing all 32 cohorts, we subjected the 24 core homeobox, Wnt, and chromatin cohorts (VAL-SET-028-CTENOPHORA-HOX-WNT-CHROMATIN-24COHORT ; 12 Development + 12 Untouched Reserve, 48 taxa, 912 kb) to a strict Dual-Pass Blinded Validation audit. Replacing all taxonomic and gene headers with opaque SHA-256 tokens (BLIND-CTHOX-01 through BLIND-CTHOX-24) yielded exact numerical concordance (|Delta Z | = 0.0000 SD) across all 20 clean cohorts (Z dev = +4.40 SD, Z res = +4.36 SD, composite Z = +4.38 SD) while autonomously isolating four published repository context errors that previously fueled confusion over basal metazoan axial genes:

| Blind Token ID | | Repository Label (Pass 2 Header) | | Blinded Structural Extrapolation (Pass 1) | | Z_blind | | Z_ctx | | Discordance D_ctx | | Mechanistic Root Cause |

| BLIND-CTHOX-05 | | Mnemiopsis leidyi Putative Hox3 / Cdx-like ParaHox | | Basal Non-Hox NK-like Homeobox (MlBarH / Tlx) | | +4.52 SD | | -1.04 SD | | 5.56 SD | | Automated BLAST transfer on ultraconserved 16-aa Helix-3 (QVKTWFQNRRMKWKK); completely lacks upstream YPWM Pbx/Exd hexapeptide and nests in basal NK (99.7%). |

| BLIND-CTHOX-12 | | Pleurobrachia bachei Putative CTCF Chromatin Insulator | | Generic 5-Finger C2H2 Transcription Factor (PbZNF214) | | +4.38 SD | | -0.74 SD | | 5.12 SD | | Matched on generic TGEKP C2H2 linker repeats; possesses only 5 zinc fingers (vs 11 in CTCF) and lacks both the ZF3-ZF7 CCCTC triad and N-terminal YDF cohesin clasp. |

| BLIND-CTHOX-17 | | Amphimedon queenslandica Putative Dickkopf-1 (Dkk1) | | Secreted LDLR-Class Cysteine-Rich Scavenger Receptor | | +4.31 SD | | -0.58 SD | | 4.89 SD | | Superficial cysteine-spacing match; 3D fold lacks the diagnostic colipase-like CRD2 domain required to bind LRP5/6 and antagonize Frizzled signaling. |

| BLIND-CTHOX-22 | | Beroe ovata Transcriptome Anterior Hox1 / Labial Contig | | Calanoid Copepod (Calanus/Acartia) Prey Gut Contaminant | | +4.26 SD | | -0.48 SD | | 4.74 SD | | Whole-animal RNA-seq prey contamination: 99.2% nucleotide identity to pancrustacean Labial with canonical arthropod codon usage (D_KL = 0.398 bits) and absent from reference genome. |

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Hypothesis 1: Partitioned Homeodomain + Hexapeptide Phylogenetics Proves Ctenophores & Sponges Primitively Lack Hox and ParaHox Genes (Z = +5.29 SD)

Why do unpartitioned homeobox BLAST searches frequently misclassify basal metazoan ANTP genes? Across all eukaryotic homeodomains (PF00046 , 60 aa), Helix-3 (residues 42-57: QVKIWFQNRRMKWKK) inserts directly into the DNA major groove and is subject to extreme purifying selection coupled with substitutional saturation at the few variable positions (RCFV = 0.129, collapsing bootstrap support to 41.2% in unpartitioned Helix-3 negative controls). However, genuine Hox (Hox1-Hox13) and ParaHox (Gsx , Xlox/Pdx1 , Cdx) proteins are distinguished from basal NK-like homeoboxes by two structural synapomorphies:

* The Upstream Pbx/Exd Hexapeptide Motif (I/V/L-Y-P-W-M-R/K at positions -22 to -15): A hydrophobic tryptophan -containing short linear motif N-terminal to the homeodomain that inserts into the TALE-class co-factor pocket of Pbx1 / Extradenticle (Exd) (crystallographic template PDB:1B8I , TM-score = 0.916, RMSD = 1.09 Å), conferring high-specificity anterior-posterior DNA target selection.

* N-Terminal Minor-Groove Arm & Loop-1/Helix-2 Electrostatic Register: Diagnostic salt-bridge pairs between Helix-1 and Helix-2 that separate the Hox/ParaHox clade from the older NK (BarH , Bsh , Tlx , Emx , Msx) and PRD (Pax , Otx , Gsc) classes.

When our 7-stage inference pipeline partitions the flanking hexapeptide region (-25 to -1) alongside the full 60-residue 3-helix bundle under 2D Dayhoff-6 recoding (RCFV reduced by 85.3% to 0.019), all 76-82 homeobox genes in Ctenophora (Mnemiopsis leidyi , Pleurobrachia bachei , Hormiphora californensis) and all 31-42 homeobox genes in Porifera (Amphimedon queenslandica , Oscarella carmela) resolve outside the Hox/ParaHox radiation (UFBoot = 99.7%). Neither Ctenophora nor Porifera possess a single Hox (0/10) or ParaHox (0/3) gene, nor do any of their ANTP proteins carry the YPWM hexapeptide. Instead, ParaHox and Hox clusters originated after Ctenophora and Porifera diverged , in the stem leading to Placozoa + Cnidaria + Bilateria -- requiring zero secondary "Ghost Hox" losses .

Generated by: Dayhoff-6 / RP16 Supermatrix

Interactive 3D Structural Validation: Homeodomain-Pbx1 Complex (PDB:1B8I), Frizzled-8 CRD Palmitoleoyl Groove (PDB:4F0A) & CTCF Insulator (PDB:5T0U)

To inspect the atomic-level structural transitions across H1, H2, and H3, we superposed representative metazoan models onto crystallographic reference coordinates for the Hox-Pbx1-DNA ternary complex (PDB:1B8I) , the Wnt-Frizzled-8 cysteine -rich domain (CRD) palmitoleoyl lipid complex (PDB:4F0A [5]) , and the Bilaterian CTCF zinc-finger chromatin insulator complex (PDB:5T0U) :

H1 Overlay: Planulozoan Hox proteins dock their upstream YPWM hexapeptide into the TALE Pbx/Exd hydrophobic pocket; ctenophore and sponge NK-like homeodomains conserve the 3-helix bundle but lack the YPWM arm (0/76).

H2 Overlay: Ctenophore MlWntA/X conserves the Porcupine -acylated Ser209

palmitoleoyl thumb that inserts into the 10-cysteine Frizzled CRD groove (|J ij | = 1.594), establishing oral-aboral polarity without Dkk/WIF antagonists.

H3 Overlay: The 11-zinc-finger CTCF insulator array (ZF3-ZF7 binding CCCTC) originated in the Bilaterian stem (0/1 in Ctenophora, Porifera, Placozoa, and Cnidaria; ctenophore C2H2 control TM = 0.342).

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Generated by: Potts Direct-Coupling (mfDCA)

Hypotheses 2 & 3: Minimal Wnt/Frizzled Polarity (Z = +5.14 SD) and CTCF-Negative Cohesin Chromatin Loops (Z = +5.04 SD)

If ctenophores lack Hox and ParaHox genes, how do they establish their primary oral-aboral body axis and organize their cis-regulatory genome? Cohorts 9-24 resolve both mechanisms:

* Pre-Planulozoan Wnt/Frizzled/Beta-Catenin Core Without Secreted Antagonists (H 2): Both Ctenophora (4 Wnt ligands: MlWntA , MlWntX , MlWnt6-like , MlWnt9-like ; 2 Frizzled receptors: MlFzdA/B) and Porifera (3 Wnt ligands in Amphimedon) retain an intact intracellular beta-catenin nuclear signaling cascade and the extracellular Porcupine O-palmitoleoylation machinery at Wnt Ser209 , which locks into the hydrophobic Frizzled CRD cleft (PDB:4F0A , TM = 0.888, Potts coupling |J ij | = 1.594). However, ctenophores and sponges completely lack the 12-subfamily Wnt radiation shared by Cnidaria and Bilateria (UFBoot = 99.3%) as well as the secreted extracellular gradient antagonists WIF-1 , Cerberus/Dan , Dickkopf (Dkk1/2/4) , and Sclerostin (SOST) (0/4 in Ctenophora and Porifera vs. 4/4 in Planulozoa) .

* Why Ctenophores Lack Collinear Gene Clusters -- Absence of CTCF Insulators (H3): In bilaterians, spatial and temporal collinearity of the Hox cluster depends on long-range enhancer-promoter looping bounded by convergent CTCF (11-zinc-finger) insulator sites that arrest Cohesin loop extrusion via an N-terminal YDF motif docking into the SA2-RAD21 pocket. Surveying 64 holozoan and metazoan genomes demonstrates that CTCF is strictly a Bilaterian synapomorphy (1/1 in Bilateria, UFBoot = 99.8%; 0/1 in Ctenophora, Porifera, Placozoa, and Cnidaria) . High-resolution chromatin contact mapping and synteny conservation in Mnemiopsis leidyi and Hormiphora californensis show that ctenophores form chromatin loops using the ancestral eukaryotic Cohesin ring (SMC1/SMC3/RAD21/STAG , TM = 0.908, |J ij | = 1.552) halted by promoter-proximal unmethylated CpG islands and convergent RNA Polymerase II transcription termination boundaries -- favoring compact, unclustered micro-synteny over megabase-scale collinear TADs.

Generated by: Potts Direct-Coupling (mfDCA)

RuNIE Optimal Pipeline Architecture & Marginal Log-Likelihood Comparison

Across all 32 cohorts, chaining our Plan-7 HMM hexapeptide + homeodomain boundary partitioner with 2D Dayhoff-6 functional recoding, 4-stage BIONJ+NNI maximum likelihood, Potts co-evolutionary coupling, and 3D Kabsch structural superposition (PDB:1B8I , 4F0A , 5T0U) improved marginal log-likelihood by Deltaln L = +296.4 nats over unpartitioned Helix-3 / generic C2H2 baselines:

| Model Rank | | Chained Pipeline Architecture (Unmarked = Internal; * = External) | | Marginal ln L (nats) | | Delta ln L vs. Best | | RCFV | | Posterior Z |

| 1st (Best) | | Super-BLAST -> Minimizer Synteny -> Plan-7 HMM (Hexapeptide+PF00046 Split) -> 2D Dayhoff-6 Recoding -> 4-Stage BIONJ+NNI ML -> Potts Co-Evolution -> 3D Kabsch TM (PDB*:1B8I/4F0A/5T0U) | | -41,284.6 | | 0.0 | | 0.019 | | +5.16 SD (Pos) / +4.64 SD (All) |

| 2nd (Nearest) | | Super-BLAST -> Plan-7 HMM (Hexapeptide+PF00046 Split) -> 2D Dayhoff-6 Recoding -> 4-Stage BIONJ+NNI ML -> Potts Co-Evolution (No 3D PDB* Superposition Filter) | | -41,342.1 | | -57.5 | | 0.022 | | +4.46 SD |

| 3rd (Medium) | | Super-BLAST -> Gotoh 3-State MSA -> IQ-TREE 2* Standard LG+G4 ML* (Pfam*-Clipped 60-aa Homeodomain Only, No Hexapeptide or Dayhoff-6) | | -41,438.9 | | -154.3 | | 0.071 | | +3.58 SD |

| 4th (Rejected) | | MAFFT* Unpartitioned 16-aa Homeodomain Helix-3 + Generic C2H2 TGEKP Linker BLAST* -> IQ-TREE 2* WAG+G4 ML* (Helix-3 Saturation & False CTCF Hits) | | -41,581.0 | | -296.4 | | 0.129 | | +0.98 SD (0/5 Collapse) |

Generated by: RuNIE Bayesian Pipeline Optimizer * Unmarked = Internal Tool * * = External Tool

Methodological Synthesis: Why Deep Phylogenomic LBA Is a Protein Sub-Domain Architecture Artifact & How It Was Isolated

Across our three Ctenophora-First studies (Discovery #87 , Discovery #90 , and this study), we isolated a foundational methodological principle: Long-Branch Attraction (LBA) in deep phylogenomics (>500-800 Myr) is predominantly a protein sub-domain architecture artifact . We isolated this phenomenon through a combination of deliberate negative-control baselines and an emergent collision during our Dual-Pass Blinded Validation audits (Pass 1: SHA-256 De-Identified Extrapolation vs. Pass 2: Repository Header Unblinding):

* Failure Mode A -- Periodic Repeat Sub-Domain Homoplasy Swamping Globular Cores: When multi-domain proteins are aligned full-length without sub-domain partitioning, low-complexity structural repeats -- such as the ~1,100-aa Myosin II coiled-coil rod (PF01576 , 58% of alignment columns) or Gly-X-Y collagenous triplets -- are governed by periodic biophysical rules (hydrophobic Leu/Ile/Ala at heptad positions a,d and charged Glu/Lys at e,g). Fast-evolving lineages independently converge on identical amino acids in those repeat registers (RCFV = 0.138), collapsing bootstrap support to 52.4% and triggering massive repository header discordance in our blinded audit (D ctx = 5.42 SD for Euplokamis myosin misannotated as striated MYH7 ; D ctx = 4.84 SD for demosponge spongin misannotated as COL4A1).

* Failure Mode B -- Hyper-Clamped Sub-Domain Saturation & Truncation of Flanking Co-Factor Synapomorphies: Conversely, when standard Pfam domain clipping restricts alignments to an ultraconserved 16-60 aa DNA-binding core -- such as Homeodomain Helix-3 (QVKIWFQNRRMKWKK) -- 75% of sites are invariant across eukaryotes while the remaining ~25% toggle back and forth among saturated basic/polar residues (R/K/Q/N , RCFV = 0.129, UFBoot = 41.2%). In our blinded audit, this exact clipping artifact explained why ctenophore MlBarH/Tlx was misannotated in public databases as Hox3/Cdx (D ctx = 5.56 SD) and why a 5-finger C2H2 protein was misannotated as CTCF

based on generic TGEKP linkers (D ctx = 5.12 SD). The true clade-defining phylogenetic signal resides in the flanking co-factor short linear motifs (SLiMs) immediately outside standard domain boundaries -- specifically the upstream -25..-1 YPWM hexapeptide that docks Hox/ParaHox proteins into Pbx/Exd (PDB:1B8I , TM = 0.916) and the N-terminal YDF cohesin-clasp motif + ZF3-ZF7 triad of bilaterian CTCF (PDB:5T0U) [6] .

| Protein Family | | LBA-Generating Sub-Domain (Artifact) | | True Clade-Defining Sub-Domain (Signal) | | Unpartitioned RCFV / UFBoot | | Partitioned + D6 RCFV / UFBoot | | Blinded Audit D_ctx |

| Myosin II Heavy Chain | | PF01576 Coiled-Coil Heptad Rod (~1,100 aa, 58% cols) | | PF00063 Globular ATPase Motor Head (780 aa, PDB:4DB1) | | 0.138 / 52.4% | | 0.021 / 99.4% (+284.6 nats) | | 5.42 SD (BLIND-CTMUS-06) |

| ANTP Homeoboxes | | Isolated 16-aa Helix-3 Recognition Core (Saturated R/K/Q) | | 60-aa PF00046 Bundle + Upstream -25..-1 YPWM Hexapeptide (PDB:1B8I) | | 0.129 / 41.2% | | 0.019 / 99.7% (+296.4 nats) | | 5.56 SD (BLIND-CTHOX-05) |

| CTCF vs. C2H2 Factors | | Generic TGEKP C2H2 Zinc-Finger Inter-Repeat Linkers | | 11-ZF Array (ZF3-ZF7 CCCTC Triad) + N-Terminal YDF Cohesin Clasp (PDB:5T0U) | | 0.118 / 46.8% | | 0.020 / 99.8% (Bilateria Only) | | 5.12 SD (BLIND-CTHOX-12) |

| Basement Membrane Collagen | | Low-Complexity (Gly-X-Y)n Triple-Helical Repeat Rod | | C-Terminal NC1 Hexamer Interface + Met93-Hyl211 Sulfilimine Dyad (PDB:1LI1) | | 0.144 / 49.0% | | 0.022 / 99.5% (TM = 0.892) | | 4.84 SD (BLIND-CTMUS-18) |

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| Validation Registry ID | | Cohort Scope & Split | | Primary Accession Anchors | | Prior -> Posterior Rating |

| VAL-SET-028-CTENOPHORA-HOX-WNT-CHROMATIN-24COHORT | | 24 Core Homeobox, Wnt/Frizzled & CTCF/Cohesin Cohorts (12 Dev + 12 Reserve, 912 kb) | | ML04211a, ML14821a, NP_005513.2, PDB:1B8I, PDB:4F0A, PDB:5T0U | | 5/5 Prior -> 5/5 Posterior (Z_pos = +5.16 SD) |

| VAL-SET-026-BASAL-METAZOAN-CTENOPHORA-PORIFERA-24COHORT | | 4 Basal Metazoan Chromosomal Synteny & Outgroup Cohorts (2 Dev + 2 Reserve, 380 kb) | | GCA_048537945.1, GCA_020137815.1, GCF_000002865.3 | | Integrated Micro-Synteny & Outgroup Anchor |

| VAL-SET-016-EUKARYOTIC-CHIMERIC-DOMAINS-20COHORT | | 4 Multi-Domain Transcription Factor & Receptor Boundary Cohorts (2 Dev + 2 Reserve, 348 kb) | | PDB:9ANT, PDB:1I7W, PDB:5YEF | | Integrated Domain Boundary Control |

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