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# Broad vertebrate conservation of Treg-associated molecular components constrains models of regulatory T-cell evolution

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**Abstract**

Regulatory T cells protect vertebrates from excessive immune responses, but the evolutionary origin of the canonical mammalian cell programme remains uncertain. We tested whether its history is explained by the appearance of new molecular components or by later organization of older immune machinery. A preregistered, duplication-aware analysis compared exact proteomes from 17 vertebrates, evaluated biological replication in public expression resources, and reanalyzed mammalian FOXP3 perturbation and promoter-accessibility data. Eight core suppressive-effector families occurred in a median 92.3% of 13 focal taxa, and all six represented suppressive mechanisms extended beyond mammals. However, broader Treg-dependency and immune-control families were similarly widespread, so genomic breadth was not Treg-specific. A confirmatory quantitative reanalysis recovered 84 stringent establishment/acquisition-associated genes but only one mature-maintenance-associated gene, supporting strong stage dependence of mammalian FOXP3 effects. Comparative cellular deployment could not be tested with adequate biological replication, direct promoter homology was sparse, strict promoter matching was non-estimable, and proximal accessibility showed little directional change. Vertebrates therefore conserve much of the molecular toolkit used by mammalian Tregs, but component conservation alone does not establish an ancestral Treg cell type or resolve when a dedicated regulatory programme emerged.

**Keywords:** regulatory T cell; FOXP3; vertebrate evolution; orthology; comparative genomics; chromatin

## 1 Introduction

Regulatory T cells (Tregs) suppress harmful immune activation and help maintain tolerance. In mammals, the canonical $CD4^+$ Treg state is closely associated with the transcription factor FOXP3 and uses multiple effector routes, including CTLA-4-dependent modulation of antigen-presenting cells, IL-10 and TGF-$\beta$ signalling, IL-35-associated subunits, extracellular nucleotide metabolism, and context-dependent cytolysis (Sakaguchi et al., 2008; Vignali et al., 2008; Josefowicz et al.,

2012; Shevach, 2009). These molecular functions are well established in mammals. Their evolutionary interpretation is much less straightforward.

Two observations have often been conflated. A gene family can predate the cell type in which it is now used, and a similar molecular marker can occur in cells that are not historically homologous. In other words, an ancient toolkit does not necessarily imply an ancient machine. Reconstructing a cell type therefore requires evidence about cellular deployment, regulatory integration, and function in addition to gene-family presence (Arendt et al., 2016; Mah and Dunn, 2024). This distinction is especially important for immune genes, which are frequently shared among activation, differentiation, stress, and effector programmes.

Evidence from teleosts already demonstrates that FOXP3 biology outside mammals is not merely hypothetical. Treg-like populations have been described in pufferfish and zebrafish (Wen et al., 2011; Kasheta et al., 2017); zebrafish studies have cloned foxp3 orthologues, linked them to immunoregulation, and shown inflammatory or lymphoproliferative phenotypes after genetic disruption (Quintana et al., 2010; Sugimoto et al., 2017). These experiments provide strong lineage-specific evidence. They do not, by themselves, establish that every component of the mammalian Treg programme had the same cellular deployment in the last common vertebrate ancestor.

Comparative genomic studies have proposed different histories. Sequence and structural analyses identified derived features of vertebrate Foxp3 (Andersen et al., 2012), while a broad survey of suppressive mechanisms argued for convergent assembly (Bhaumik et al., 2023). More recently, a bilaterian FoxP analysis placed the four gnathostome paralogues in the context of two vertebrate whole-genome duplications and proposed that FoxP3 immune specialization followed older FoxP functions (Tsai et al., 2025). These studies make paralogy and regulatory co-option central, not peripheral, to the evolutionary problem.

Mammalian chromatin studies supply a related caution. FOXP3 can use a pre-existing enhancer landscape (Samstein et al., 2012), Treg development depends on regulatory architecture established before FOXP3 expression (Kitagawa et al., 2017), and FOXP3 can reorganize or tune chromatin and trans-acting networks (van der Veeken et al., 2020; Liu et al., 2023). A recent inducible-degradation study further showed that FOXP3 requirements depend strongly on developmental and inflammatory context (Hu et al., 2025). Thus neither gene presence nor a single mature-cell assay is likely to capture the full evolutionary innovation.

Here we asked a deliberately narrower question: what can current comparative data establish about the molecular components used by canonical mammalian Tregs, and what remains beyond their reach? We integrated four evidence layers (Fig. 1): duplication-aware gene-family availability, source-native cellular deployment, mammalian FOXP3 dependence, and promoter sequence/accessibility. Components, controls, thresholds, uncertainty states, and stopping rules were fixed before the corresponding outcomes were inspected. Our central test was specificity: broad conservation of Treg-associated genes is informative only if it is interpreted relative to broader Treg dependencies and other immune programmes. The resulting analysis supports broad component conservation and mammalian FOXP3 stage dependence, while placing a firm ceiling on claims about cell-type ancestry.

# 2 Materials and methods

## 2.1 Study design and evidential boundaries

The project was organized as a sequence of prospectively gated analyses. Competing hypotheses were retained throughout: de novo addition of important vertebrate components, capture or individuation of an older immune programme, generic immune conservation, and regulatory or organizational specialization. No genomic observation was automatically promoted to a claim about cell identity or function. Similarly, mammalian perturbation evidence was not used to date evolutionary events, motif matches were not interpreted as protein occupancy, and a missing orthology call was not treated as biological absence.

Animals or independently resolved biological libraries were the units for cellular and perturbation inference. Cells, sequencing runs, lanes, and technical libraries were recorded separately. When nesting could not be established from source metadata, it remained unresolved. The prospective decision history and initial programme test are documented in Supplementary Methods 1, Supplementary Figs. S1 and S8, and Supplementary Tables S1 and S33; internal audit codes are not biological categories.

## 2.2 Molecular component and comparator sets

The primary suppressive-effector set contained eight mouse source genes grouped into six equal-weight mechanisms: *Ctla4*; *Il10*; *Tgfb1*; *Ebi3* and *Il12a*; *Entpd1* and *Nt5e*; and *Gzmb*. A broader sensitivity set did not replace the primary definition. A separate set of 19 source rows (17 unique families after mapping) represented experimentally supported mammalian dependencies affecting identity, differentiation, fitness, homeostasis, suppression, persistence, or adaptation. Activation, interferon, cytotoxic, B-cell, helper-T-cell, stress, cell-cycle, and housekeeping programmes provided comparators. Definitions, evidence grades, and overlaps are in Supplementary Methods 2 and Supplementary Tables S2–S4.

## 2.3 Exact proteomes and duplication-aware orthology

We used exact, versioned proteomes for 17 vertebrates. Thirteen focal taxa spanned cyclostomes, chondrichthyans, teleosts, amphibians, reptiles, birds, and mammals; four additional taxa strengthened phylogenetic placement. The longest translated isoform per locus was the primary representative-protein policy, with a source/canonical-isoform sensitivity. OrthoFinder 3.1.4 (Emms et al., 2026) generated the primary family coordinate. All one-to-many and many-to-many mappings were retained; no family was collapsed to a single best gene symbol.

The run assigned 342,702 of 353,926 proteins to 18,934 root families. Family confidence, orthology class, duplication complexity, copy number, reference risk, and external-database agreement were stored separately. Bounded cross-coordinate review assessed whether conclusions, rather than raw identifiers, were equivalent. Detailed proteome records, family maps, adjudications, and coordinate sensitivities are in Supplementary Methods 3, Supplementary Fig. S6, and Supplementary Tables S5, S6, S12, and S17.

## 2.4 Genomic representation, recovery, and specificity

For each source-compatible family and focal taxon, the primary layer recorded observed family presence, no observed member, or unresolved status. Occupancy was the fraction of 13 focal taxa with an observed compatible member. Taxa were not treated as statistical replicates; bootstrap summaries resampled families. Nonmammalian mechanism representation required defensible observations in more than three lineage bins.

A second recovery layer reviewed eligible non-observations using annotation, protein and profile searches, translated-genome evidence, locus integrity, and local synteny. Supported absence required all predefined reference and locus conditions. Reference-limited and synteny-unresolved cases remained distinct. Specificity was tested with prospectively matched family panels preserving copy class, orthology class, promoter eligibility, reference-quality stratum, overlap status, and paralog complexity. No post-outcome family reuse or relaxed structural match was allowed. Full family-by-taxon states and sensitivity analyses are in Supplementary Methods 4 and Supplementary Tables S7–S19.

## 2.5 Confirmatory reanalysis of mammalian FOXP3 perturbations

We reanalyzed processed mouse data associated with Hu et al. (2025) and related frozen perturbation contexts. Establishment/acquisition and mature-maintenance contexts were kept separate. A stringent response required false-discovery rate $\leq 0.05$, absolute $\log_2$ fold change $\geq 0.5$, same-direction support in at least two eligible contexts, and no strong opposite context. This analysis quantifies and checks the published stage-dependence conclusion; it is not presented as the discovery of that biological phenomenon. Complete context definitions and gene-level outputs are in Supplementary Methods 5, Supplementary Fig. S9, and Supplementary Table S34.

## 2.6 Comparative cellular-data feasibility

Public comparative expression resources were audited before project-gene expression was opened. An inferential endpoint required at least three resolved animals or biological libraries in each state, compatible count semantics, source-native labels, a defensible identifier map, and adequate cells or depth within every biological unit. Mammalian labels were not transferred to non-mammalian cells. Recent cross-species single-cell studies were used as context rather than substituted for missing replication (Jiao et al., 2024; Zhang et al., 2024; Huang et al., 2024; Wang et al., 2026). Dataset-specific gates are described in Supplementary Methods 6, Supplementary Fig. S2, and Supplementary Table S20.

## 2.7 Promoter sequence and accessibility

The promoter analysis separated gene-anchored comparison from direct nucleotide homology. Gene-anchored windows of $\pm 1$, $\pm 2$, and $\pm 5$ kb around annotated transcription start sites were summarized for GC fraction, CpG observed-to-expected ratio, callable fraction, and eligible transcription-factor-family motif burden. Direct sequence comparison additionally required reciprocal same-family mapping, unambiguous correspondence, and local synteny. JASPAR

2026 CORE vertebrate profiles (Ovek Baydar et al., 2026) were scanned at frozen relative-score thresholds after masking. Motif matches were treated as sequence-preference proxies, not binding or activity.

Human GSE118189 compared source-defined Regulatory_T-U and Effector_CD4pos_T-U libraries from three donors using a donor-blocked model. Deposited hg19 peaks were lifted to hg38 with minimum match 0.95 and unique mapping. Mouse GSE297597 was analyzed separately at day 3 (four mice per genotype) and day 7 (three per genotype). DESeq2 1.50.2 (Love et al., 2014) used complete model-tested promoter universes. A directional promoter required absolute $\log_2$ fold change $\geq 1$ and adjusted $P < 0.05$. Full thresholds, count filters, mapping rules, and sensitivities are in Supplementary Methods 7, Supplementary Tables S21–S30, and the Zenodo archive (Ghosh and Sinha, 2026).

# 3 Results

## 3.1 Core suppressive-effector families are broadly represented across vertebrates

The eight core suppressive-effector source genes resolved to eight primary families. Across the 13 focal taxa, median observed occupancy was 0.923 (mean 0.952; interquartile range 0.923–1.000). All six mechanisms had defensible representation outside mammals and in more than three nonmammalian lineage bins (Fig. 2A,B; Table 1). The categorical breadth result survived removal of each individual taxon and each individual family or mechanism.

These observations establish molecular availability, not conserved cell identity. In the detailed recovery layer, the core set contributed 110 annotated-presence, 11 search-negative or reference-limited, and 15 synteny-unresolved family-by-taxon cells among 136 observations. Targeted review did not convert unresolved cases into positive or supported-absence calls. In particular, low-quality cyclostome non-observations remained unavailable for absence inference. Core and broader-dependency family matrices, whole-genome recovery states, paralogs, and family-confidence restrictions remain visible in Supplementary Figs. S3–S5 and Supplementary Tables S7, S8, S10, and S11.

## 3.2 Broad genomic representation is not specific to Treg suppressive effectors

The 19 broader Treg-dependency rows resolved to 17 unique families with median occupancy 1.000 (mean 0.973), exceeding the descriptive median for the core suppressive-effector set. Activation, interferon, cytotoxic, B-cell, helper-T-cell, and housekeeping comparators were also broadly represented (Fig. 2C). Thus the strongest pattern was a broadly conserved immune toolkit, not a genomic signature unique to canonical mammalian Treg suppression.

The intended matched core-versus-broader-dependency test could not form a complete eight-family panel: two core matching slots competed for the same sole eligible comparator family, while the frozen design prohibited within-panel reuse. This produced zero panels rather than an effect estimate. Five immune comparator analyses produced 10,000 panels each, but none

supported greater breadth of the core set; cytotoxic families were similar and the remaining comparisons did not satisfy the joint occupancy-and-span rule. These are qualified specificity results, not evidence that all programmes have identical evolutionary histories (Supplementary Fig. S7; Supplementary Tables S14–S19).

### 3.3 Quantitative reanalysis confirms strong stage dependence of mammalian FOXP3 effects

Across the frozen same-direction rules, 67 positive and 17 negative genes were associated with Treg establishment or acquisition, compared with zero positive and one negative gene during mature maintenance (Fig. 3A; Table 1). Context-level differential-expression counts ranged from 145 in the thymic contrast to 985 in the infant contrast and 1,022 or 676 in lower-confidence reversible-acquisition strata; mature resting and activated bulk contrasts contained 30 and 23 genes at false-discovery rate below 0.05 (Fig. 3C).

The 84-versus-1 result exactly reproduced the frozen gene lists and was stable to predefined stage sensitivities. It quantitatively confirms, rather than newly discovers, the central conclusion of Hu et al. (2025): FOXP3 perturbation has a much larger transcriptional association during establishment or acquisition than during mature steady-state maintenance. The 84 genes are not asserted to be direct FOXP3 targets because the perturbations span multiple days, the contexts differ, two reversible-acquisition strata contain only two animals per state, and downstream adaptation may contribute. Promoter occupancy is shown only as descriptive context and does not convert the set into direct targets. Nor does the contrast date the evolutionary origin of FOXP3 function (Supplementary Figs. S9 and S10; Supplementary Table S34).

### 3.4 Public expression resources are insufficient for a replicated comparative deployment test

No pre-amniote dataset met the requirement for at least three resolved animals or biological libraries per eligible source-defined state. Several resources contained many cells or sequencing records, but these did not establish independent biological units. They were therefore retained as descriptive context, consistent with current cautions in cell-type phylogenetics (Mah and Dunn, 2024), and no early-to-late cellular trajectory was estimated.

Two later-lineage resources came closer but remained non-executable (Table 2; Supplementary Fig. S2). In chicken PRJEB88203, four animals each contributed CD25-high, CD25-low, and CD25-negative CD4 libraries. The exact processed Salmon count object and custom appended reference described by the source publication were not deposited. In human GSE201333 spleen, three donors and exact source-native labels were resolved, but 10X UMI counts and Smart-seq2 read counts were not interchangeable. The TSP2 target had 17 10X and 39 Smart-seq2 cells, below the frozen 50-cell rule within either platform; adding them would not make their count units comparable. This is a limitation of available inputs, not evidence that non-mammalian regulatory states are absent.

### 3.5 Direct promoter comparison is sparse and strict specificity matching is non-estimable

At the gene-anchored layer, 27,461 complete non-overlapping promoters were eligible at ±1 kb, 25,077 at ±2 kb, and 19,625 at ±5 kb (Fig. 4A). These windows permit same-gene summary features but do not imply base-level homology. Among 318,240 family-by-direction mapping records, only 4,941 met the direct-homology requirements; 935 were restricted, 124,020 supported gene anchoring only, and 188,344 remained mapping-unresolved (Fig. 4B). Distant-taxon comparisons were therefore dominated by gene anchoring rather than aligned noncoding sequence.

Strict matching then produced zero valid primary panels for the broader Treg dependencies and all eight control programmes at all three windows (Fig. 4C). Structural fields were not relaxed after seeing this outcome, and no favourable window was substituted. Consequently, raw differences in GC, CpG, callability, or motif burden cannot be attributed specifically to the Treg-associated core rather than family structure, reference quality, promoter eligibility, or paralog complexity. Comparative promoter specificity remains unresolved (Supplementary Tables S21–S23, S28, and S29).

### 3.6 Proximal promoter accessibility does not mirror the transcriptional stage contrast

For human GSE118189, 818,922 of 829,942 deposited peaks mapped uniquely from hg19 to hg38 (98.67%). Among 582 model-tested programme-associated promoters, 581 failed the joint directional threshold, one promoter assigned to *FCGR3A* was negative-directional, and none was positive-directional (Fig. 5A). In mouse GSE297597, 961 programme-associated promoters were tested at each time point, with no directional calls at day 3 or day 7 (Fig. 5B,C).

The contrast with the 84-versus-1 transcriptional result is informative but limited. The accessibility analyses covered proximal windows after multi-day perturbation, whereas Treg regulation includes distal enhancers, three-dimensional contacts, transcription-factor cooperation, and context-dependent chromatin states (Samstein et al., 2012; Kitagawa et al., 2017; Liu et al., 2023). These results therefore do not constitute a genome-wide chromatin null and do not contradict the broader regulatory conclusions of the source studies. They show only that the predefined proximal-promoter test did not reproduce the strong transcriptional asymmetry (Supplementary Tables S24–S27 and S30).

### 3.7 Integrated evidence constrains, but does not resolve, cell-type origin

The evidence map in Fig. 6 separates supported observations from their boundaries. Broad core-component conservation is supported; its Treg specificity is not. Mammalian FOXP3 stage dependence is supported as a confirmatory quantitative result; its evolutionary timing is not. Comparative cellular deployment and promoter specificity remain unresolved, and proximal accessibility is not supportive under the frozen thresholds.

The claim ceiling is therefore: vertebrates broadly conserve molecular components used by canonical mammalian regulatory T cells, but comparable breadth among other immune

programmes shows that genomic conservation alone is not evidence of Treg cell-type ancestry. Mammalian perturbation data additionally support a pronounced stage dependence of FOXP3-associated transcriptional effects. Available comparative cellular and proximal-regulatory data remain insufficient to determine when or by what regulatory process these conserved components became integrated into a dedicated regulatory T-cell programme (Table 3; Supplementary Tables S31 and S32).

# 4 Discussion

## 4.1 The conserved object is a molecular toolkit, not yet a reconstructed cell type

The most robust cross-species result is positive: every suppressive mechanism in the core set is represented beyond mammals, and the component families occur across most focal vertebrate genomes. This makes a simple model in which the mammalian Treg programme arose mainly through late invention of all its effector parts implausible. It instead directs attention toward how widely available immune components became coordinated.

That conclusion is intentionally narrower than cell-type ancestry. The equally broad representation of broader Treg dependencies and immune controls shows why specificity matters. CTLA-4-family signalling, cytokines, purinergic enzymes, and cytolytic machinery can participate in many immune states. Their presence provides evolutionary possibility; it does not identify the ancestral cell population in which they were used. The result is compatible with capture or individuation of an older programme, but also with independent recruitment from a generally conserved immune repertoire.

This framing complements rather than displaces experimental teleost work. Zebrafish foxp3 disruption and Treg-like-cell studies provide direct evidence for regulatory function in a specific osteichthyan lineage (Quintana et al., 2010; Sugimoto et al., 2017; Kasheta et al., 2017). The present analysis adds a cross-vertebrate, duplication-aware specificity test and explicit uncertainty accounting. It does not claim first discovery of FOXP3 outside mammals, the first fish Treg-like state, or the first evolutionary analysis of suppressive mechanisms.

## 4.2 FOXP3 stage dependence helps define the organizational problem

The confirmatory 84-versus-1 reanalysis reinforces a biological distinction that is easy to lose in a gene-presence narrative: a transcription factor can be crucial while a state is being established yet have a smaller steady-state requirement after the network has matured. This fits models in which FOXP3 works with pre-existing enhancers, stage-specific cofactors, and stabilizing epigenetic processes (Samstein et al., 2012; Kitagawa et al., 2017; Chorro et al., 2018; Hu et al., 2025). It also explains why the evolutionary problem cannot be reduced to asking when the *FOXP3* gene appeared.

The result remains mammalian. It does not show that the same stage dependency exists in fish, birds, or early-diverging vertebrates, and it does not identify which of the 84 genes are directly

bound. Comparative experiments would need matched developmental stages, acute perturbations, resolved animals, and measurements of both transcription and regulatory architecture.

### 4.3 Why the promoter analyses did not resolve regulatory rewiring

The promoter analysis was designed to separate two often-confused claims. A gene-anchored promoter window can be compared across taxa even when bases cannot be aligned, but sequence gain or loss requires defensible nucleotide homology. Only a small fraction of potential direction-specific maps satisfied the latter requirement. The strict control design then failed because the necessary structurally comparable families did not exist in sufficient combinations.

These failures are scientifically useful. Relaxing copy class, paralog complexity, or reference quality after viewing outcomes could create an attractive but uncalibrated answer. Preserving the failure instead shows that current assemblies and promoter coordinates cannot support a Treg-specific rewiring claim. The weak proximal-accessibility signal further narrows the claim without ruling out distal or three-dimensional regulation. Comparative enhancer maps, chromatin contacts, and binding data are more likely to resolve the hypothesis than additional unqualified promoter motif scans.

### 4.4 Public cellular data are growing faster than inferential comparability

Comparative single-cell atlases increasingly span vertebrate lineages (Jiao et al., 2024; Zhang et al., 2024; Huang et al., 2024; Wang et al., 2026), but cell number is not biological replication. Cross-species inference also depends on source-native annotation, comparable sampling, compatible count units, and explicit animal or donor nesting. The present gate failed because these requirements were not simultaneously met, not because relevant cells were necessarily missing.

The chicken resource is a concrete near-term opportunity. Its four-animal paired bulk design is appropriate, but reproducing its unpublished processed object would require approximately 60.7 GiB of compressed FASTQ for all 12 sorted libraries, reconstruction of the exact GRCg7b-plus-appended-transcript reference, and a newly approved analysis. A processed count object from the original authors remains preferable. The separate feasibility audit records this option without changing the current manuscript result.

### 4.5 Limitations and priorities

The study has five principal limitations. First, family occupancy across selected taxa is a descriptive breadth measure, not a phylogenetic ancestral-state reconstruction. Second, the core and comparator sets derive from mammalian experimental literature and may omit lineage-specific suppressive mechanisms. Third, deep paralogy and uneven reference quality restrict absence and copy-number claims. Fourth, no comparative cellular endpoint passed the replication and compatibility gates. Fifth, promoter windows are incomplete proxies for cis-regulatory architecture, and strict matching was non-estimable.

The decisive next study would collect matched source-defined immune populations from several individuals in strategically placed vertebrate lineages, combine functional suppression and

perturbation assays with transcriptomes and distal-regulatory maps, and retain all paralogues in a synteny-aware coordinate. Such evidence could distinguish conserved cellular deployment from independent recruitment of shared immune machinery.

## 5 Conclusion

Vertebrates broadly conserve molecular components used by canonical mammalian regulatory T cells. Because broader Treg-dependency and immune-control programmes are comparably widespread, this genomic breadth constrains models of de novo component invention but does not establish an ancestral Treg cell type. Mammalian perturbation data independently confirm pronounced stage dependence of FOXP3-associated transcription. The timing and regulatory mechanism by which the conserved components became integrated into a dedicated cell programme remain open questions that require replicated comparative functional and regulatory data.

## Data and code availability

The complete reproducibility materials, including LaTeX sources, code, frozen metadata, derived results, figures, machine-readable tables, environment records, and checksums, have been assembled for public release through Zenodo under the author-supplied DOI 10.5281/zenodo.22735270 (Ghosh and Sinha, 2026). The authors will confirm that the DOI resolves to the exact archived package before submission. Large public source data and third-party reference resources are not redistributed; accession numbers, versions, URLs, and acquisition instructions are included. No newly generated raw sequence data are reported.

## Author contributions

**Nabanita Ghosh:** Conceptualization, Methodology, Investigation, Project administration, Supervision, Validation, Writing—original draft, Writing—review and editing. **Krishnendu Sinha:** Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Visualization, Writing—original draft, Writing—review and editing. Both authors approved the manuscript and accept responsibility for its integrity.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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During preparation of this work, the authors used OpenAI ChatGPT and Codex to support project planning, code drafting and checking, literature-discovery assistance, editorial restructuring, and language refinement. The tools were not authors, did not make final biological decisions, and were not used as substitutes for scientific judgment. The human authors reviewed and verified the analyses, references, interpretations, and final text and take full responsibility for the content of the work.

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# Figures

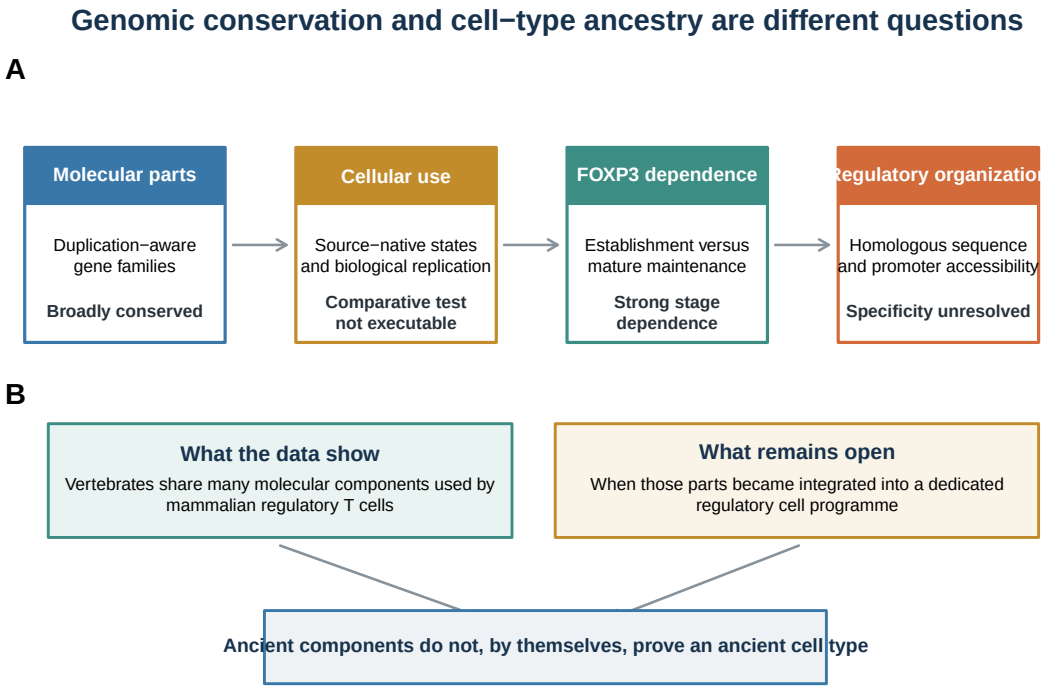



**Figure 1: Study logic and evidential boundaries.** (A) Four evidence layers were analyzed without treating one as a substitute for another. (B) Broad molecular conservation and reconstruction of cell-type ancestry are distinct conclusions. Arrows show analytical flow, not evolutionary chronology.

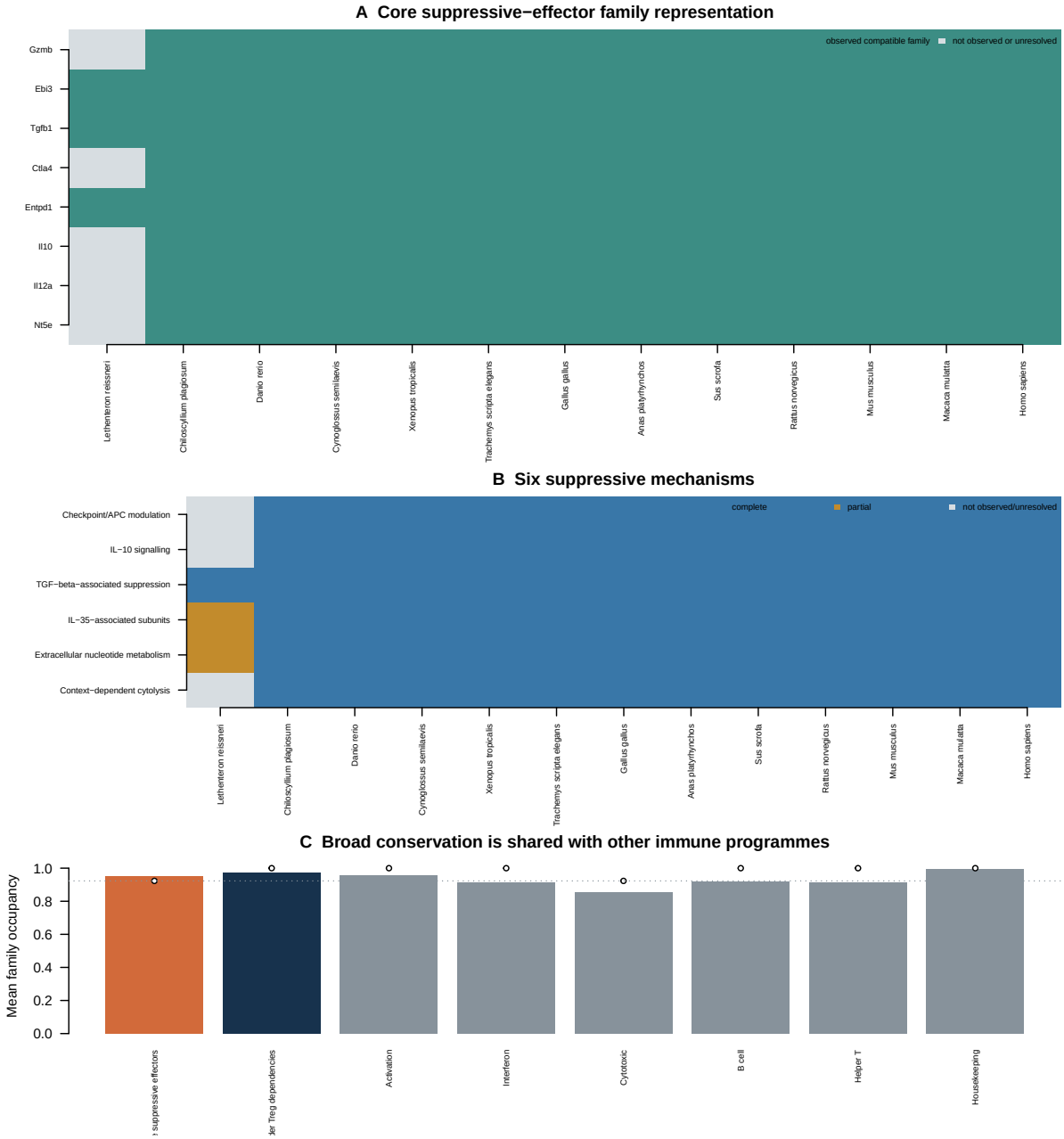



**Figure 2: Vertebrate genomic representation of Treg-associated and comparator molecular programmes.** (A) Observed source-compatible family members for eight core suppressive effectors across 13 focal taxa. Non-observation and unresolved evidence are combined visually but remain distinct in Supplementary Table S8. (B) Complete, partial, or unavailable representation of six equal-weight suppressive mechanisms. (C) Mean and median family occupancy for the core set, broader mammalian Treg dependencies, and immune controls. Families, not taxa, are the descriptive units.

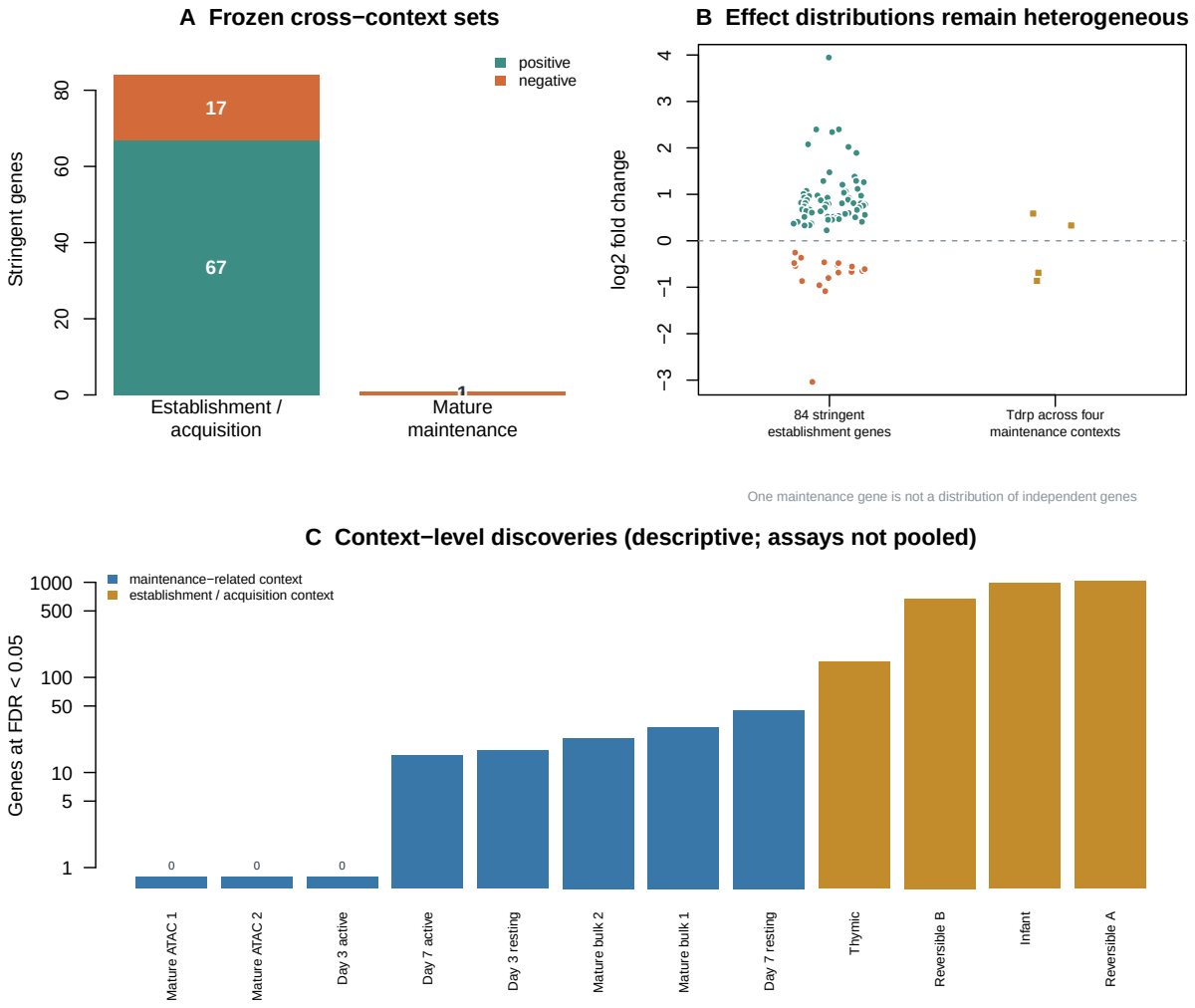



**Figure 3: Confirmatory reanalysis of mammalian FOXP3 stage dependence.** (A) Stringent same-direction counts for establishment/acquisition and mature maintenance. (B) Effect distributions for the 84 establishment/acquisition genes and the single maintenance gene. (C) Context-level differential-expression counts. Contexts are displayed descriptively and were not pooled as interchangeable replicates.

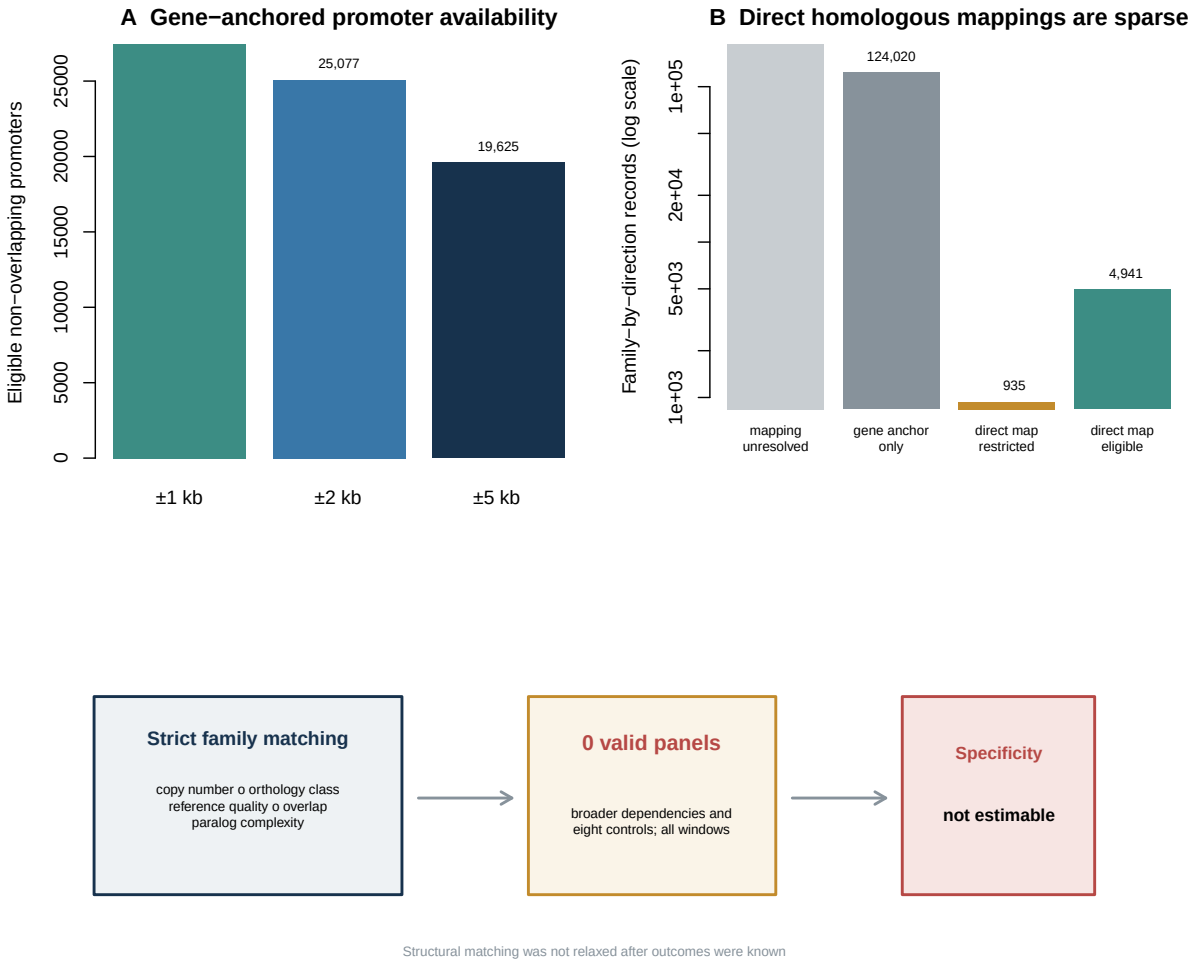



**Figure 4: Promoter availability and limits of comparative specificity testing.** (A) Eligible complete, non-overlapping gene-anchored promoters. (B) Direct homologous promoter comparison is available for only a small fraction of family-by-direction records. (C) Exact structural matching generated no valid panels for broader Treg dependencies or controls at any window, leaving programme specificity non-estimable.

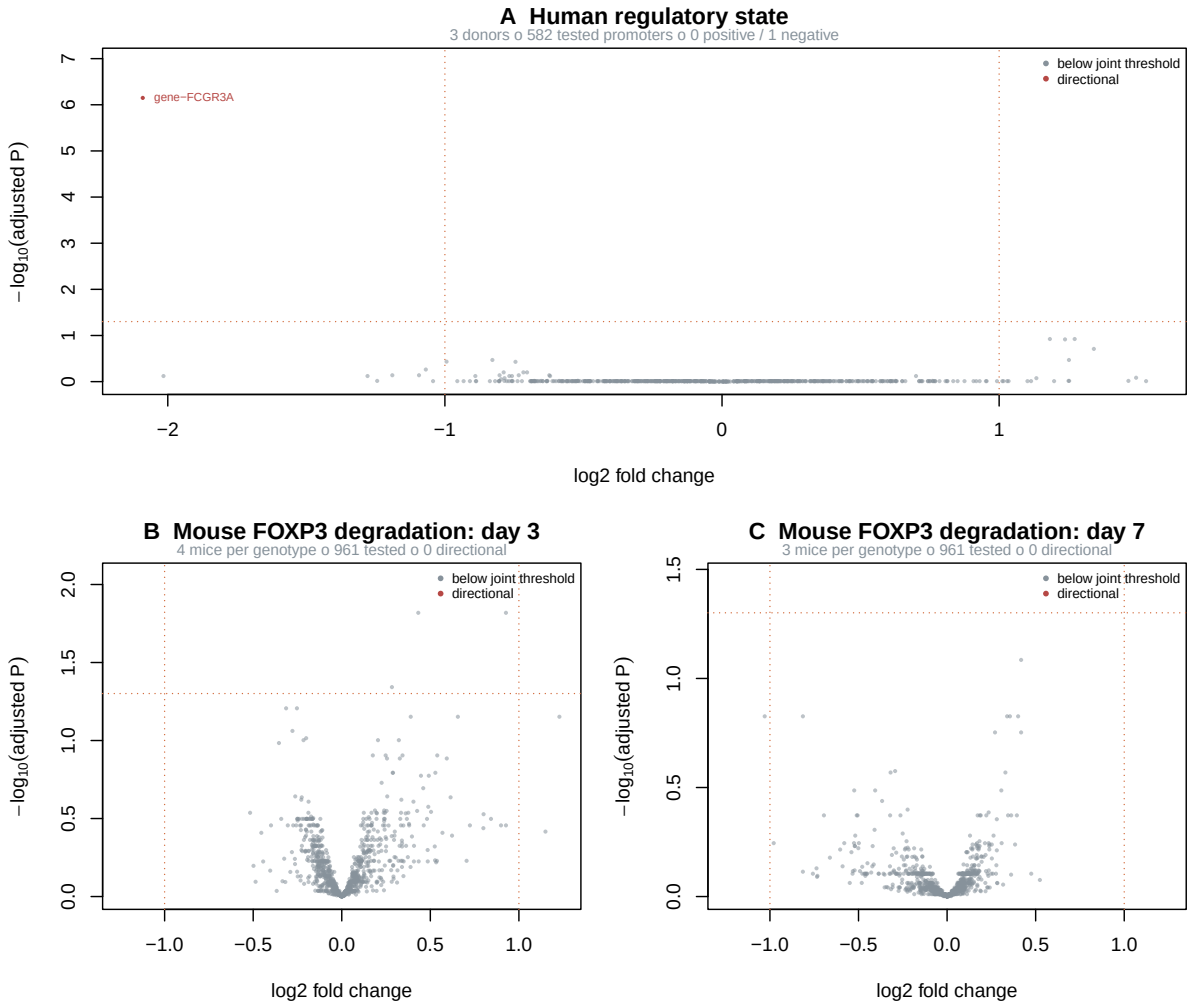



**Figure 5: Human and mouse proximal-promoter accessibility.** (A) Human Regulatory_T-U versus Effector_CD4pos_T-U effects across 582 tested promoters from three donors. (B,C) Mouse FOXP3-degradation effects across 961 tested promoters at day 3 and day 7. Dashed lines mark absolute $\log_2$ fold change 1 and adjusted $P = 0.05$.

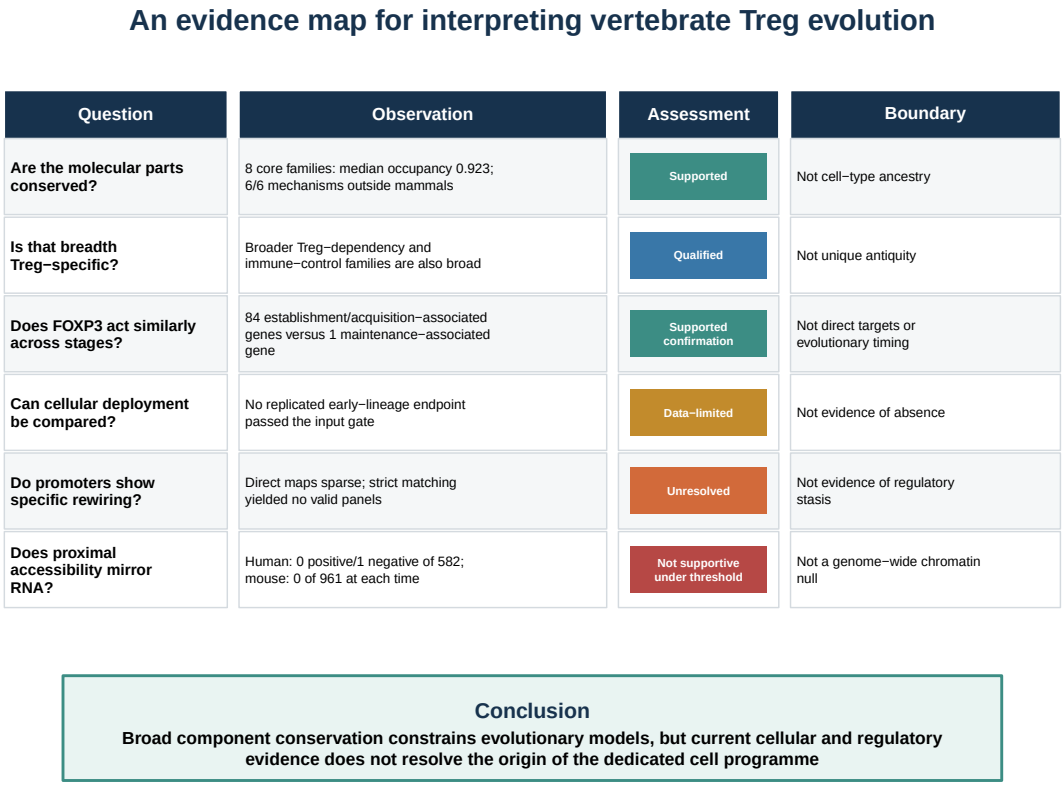



**Figure 6: Integrated evidence map.** Supported observations are separated from the inferences they cannot carry. Molecular conservation and mammalian FOXP3 stage dependence are supported; cellular deployment and promoter specificity remain data-limited or unresolved.

# Tables

**Table 1:** Evidence summary and interpretation boundaries.

| Question | Quantitative result | Supported conclusion | Not established |
|---|---|---|---|
| Molecular components | Eight core families; median occupancy 0.923; six of six mechanisms beyond mammals | Broad vertebrate availability | Ancestral Treg identity or conserved function |
| Specificity | Seventeen broader-dependency families; median occupancy 1.000; immune controls also broad | Conservation is shared with other immune programmes | Greater antiquity or uniqueness of the core set |
| FOXP3 stage dependence | 84 establishment or acquisition genes versus one maintenance gene | Confirmatory mammalian stage asymmetry | Direct targets or evolutionary timing |
| Cellular deployment | No comparative endpoint passed all replication and compatibility gates | Current public data are insufficient | Absence of non-mammalian regulatory states |
| Promoter specificity | Direct maps sparse; zero valid strict matched panels | Specificity is non-estimable | Regulatory stasis or loss |
| Proximal accessibility | Human: 0 positive/1 negative of 582; mouse: 0 of 961 per time point | Little directional proximal signal under frozen thresholds | Genome-wide chromatin null |

**Table 2:** Biological-replication and input-compatibility gate for comparative cellular resources.

| Resource | Biological units | Useful feature | Blocking limitation | Use |
|---|---|---|---|---|
| Pre-amniote resources | Mostly one or two animals or unresolved | Source-native immune annotations | No resource met the three-unit gate | Descriptive |
| Chicken PRJEB88203 | Four chickens; paired sorted libraries | Complete CD25-high/low/negative crosswalk | Exact processed Salmon object and reference absent | Not analyzed |
| Human GSE201333 spleen | Three donors | Exact source-native labels; raw-count layer | 10X and Smart-seq2 units differ; one target stratum below 50 cells | Not pooled |

**Table 3:** Integrated interpretive boundaries and decisive next evidence.

| **Observation** | **Status** | **Permitted claim** | **Required next evidence** |
|---|---|---|---|
| Core-family breadth | Supported | Vertebrates broadly conserve components used by mammalian Tregs | Source-native functional validation in deep lineages |
| Breadth versus controls | Qualified | Genomic breadth is not specific evidence of cell-type ancestry | Better-matched family sets and validated lineage functions |
| FOXP3 stage contrast | Confirmatory | Mammalian transcriptional effects are stage-dependent | Acute, stage-matched perturbation plus binding and distal chromatin |
| Cellular deployment | Unresolved | Public data do not resolve emergence of a dedicated programme | At least three animals per state with compatible counts |
| Promoter specificity | Unresolved | No Treg-specific promoter-rewiring conclusion | Dense homologous noncoding maps and matched controls |

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryinformation.pdf