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Dissecting functional regulatory convergence over 160 million years of therian evolution

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Understanding the molecular mechanisms underpinning convergent traits can provide insight into the predictability of the evolutionary process. The thylacine, an extinct marsupial carnivore, has been long known to have a very similar craniofacial morphology to that of eutherian canids, despite having diverged ≈ 160 million years ago. Using a massively parallel reporter assay (MPRA), we test the regulatory potential of a set of previously identified highly conserved craniofacial enhancers that showed convergent sequence acceleration in thylacine and wolf (TWARs). We compare orthologous sequences from six different taxa, including outgroup taxa with non-convergent craniofacial phenotypes (Tasmanian devil and giant panda) and ancestral reconstructions for marsupial and placental carnivores (Dasyuromorphia and Carnivora). Dense 10bp tiling allowed us to thoroughly examine features associated with activity, including percentage GC content and transcription factor binding motifs turnover. We identify marked conservation of levels and patterns of regulatory activity across all six taxa, as well as multiple cases of differentially active TWARs within each clade, with both thylacine

28 **and wolf driving functional divergence. However, evidence of convergent divergence**
29 **relevant to craniofacial biology is limited to a TWAR with reduced activity in both the**
30 **thylacine and wolf near the developmental gene *LMO4*. Ultimately our findings suggest**
31 **that shared gene regulatory potential is not a feature of these convergently accelerated**
32 **regions in the thylacine and wolf.**

33 Convergent evolution | thylacine | massively parallel reporter assay | craniofacial development

34 Introduction

35 Convergent evolution is the independent emergence of the same traits in multiple lineages or
 36 populations, possibly in response to shared selective pressures. Echolocation, for example, is
 37 thought to have independently originated in marine mammals and bats as an adaptation to their
 38 low visibility habitats (Liu et al., [2010](#); Parker et al., [2013](#)), with parallel amino-acid substitutions
 39 in the cochlear motor protein PRESTIN in these animals pointing to its key specialised role
 40 in enabling the phenotype (Choi, [2011](#); Li et al., [2008](#)). However, other traits, such as the
 41 development of winged structures in birds and bats, exhibit convergence at the phenotypic level,
 42 but not necessarily at the level of independent base substitutions, highlighting the flexibility
 43 of developmental toolkits and ontogenetic processes (Orkney et al., [2025](#)). Identifying and
 44 studying the genetic basis of convergent traits can therefore provide unique insight into the role
 45 of adaptation and constraint in trait evolution, and the repeatability of evolutionary processes.

46 The extinct marsupial thylacine (*Thylacinus cynocephalus*, also known as the Tasmanian tiger,
 47 and the Tasmanian dog by early European colonisers), displays high levels of morphological
 48 convergence with placental canids (Harris, [1808](#)), especially in the craniofacial region (Newton
 49 et al., [2021](#); Feigin et al., [2018](#))(Supplemental Figure 1). In particular, morphometric analyses
 50 have shown high feature overlap in craniodental structures (Wroe et al., [2007b](#); Goswami et al.,
 51 [2011](#); Feigin et al., [2018](#)). Although its specific predatory behaviours and diet are disputed, the
 52 thylacine is universally agreed to have been a hypercarnivore, like most placental canids (Jones
 53 et al., [1998](#); Wroe et al., [2007a](#); Rovinsky et al., [2021](#); Goswami et al., [2011](#)). This phenotypic
 54 convergence is thus hypothesized to be an adaptation to a shared feeding ecology requiring
 55 shearing of prey flesh.

56 We recently identified a set of 339 candidate *cis*-regulatory elements (CREs) active
 57 during murine craniofacial development that showed increased rates of substitution in
 58 the thylacine and the grey wolf (*Canis lupus*) relative to 61 vertebrate taxa. These
 59 thylacine-and-wolf-accelerated-regions, or TWARs (Feigin et al., [2019](#)), are promising candidates
 60 for explaining craniofacial convergence across these taxa, but their effects on craniofacial
 61 development cannot be directly tested in either species. Here we have employed a massively

62 parallel reporter assay (MPRA), to compare the gene regulatory potential of orthologs of
63 these TWAR elements from the thylacine, wolf, and four outgroup taxa (Inoue et al., [2015](#);
64 Gallego Romero et al., [2023](#)). We identify putative functional CREs and test for convergence
65 in two cell line models, mouse-derived cranial neural crest cells and preosteoblasts, chosen for
66 their relevance to craniofacial development.

Results

Designing an MPRA to compare marsupial and placental enhancer sequences

To investigate the genetic basis of convergent evolution in the thylacine and wolf, we performed an episomal MPRA testing the regulatory potential of 295 orthologous TWAR sequences from the two species, alongside orthologous sequences from two species with a non-convergent morphology and similar divergence times from the focal species, the Tasmanian devil, *Sarcophilus harrisi* (thylacine-Tasmanian devil TMRCA: 25-35 MYA (Duchêne et al., 2018)) and the giant panda, *Ailuropoda melanoleuca* (panda-wolf TMRCA = 40-50 MYA, (Hassanin et al., 2021)) and ancestral reconstructions of the two parent clades (Dasyuromorphia and Carnivora) (Figure 1A). Due to the lack of suitable marsupial and canid cell lines, we were limited to using murine cell lines in our experiment. We determined that cranial neural crest cells (CNCCs) and pre-osteoblast cells represented the tractable cell types to examine enhancer activity of our library, given we previously observed strong ontogenetic similarity and adult convergence in thylacine and wolf skull bones derived from fronto-nasal neural crest (NC) and mesodermal origins (Newton et al., 2021). Both cell types chosen possess the capacity to differentiate into osteoblasts and form calcified condensations (Wang et al., 1999; Mishina et al., 2014; Ishii et al., 2012). We thus also included the murine orthologous sequence of all tested TWARs to account for any effects driven by the mouse *trans*-regulatory environment.

TWAR lengths ranged from 84 to 1,001 bp; thus we tiled across elements longer than 170 bp with a 10 bp sliding window, enabling us to examine the regulatory potential of the whole TWAR at high resolution. However, because TWAR orthologs can display variable lengths and alignment, we note that tiles did not always map 1-to-1 across taxa. Our library consisted of 21,798 unique oligos (representing 2,757 distinct TWAR and control elements) (Supplemental Table S1). The synthesized library was tagged with a median of 337 barcodes/oligo (Supplemental Figure 2), assembled into an episomal vector, and transfected into 3× replicates per cell line. After sequencing and quality control, we found that $\approx 85\%$ of the library was captured in both cell lines (preosteoblasts: 18,510, CNCCs: 18,459) (Figure 1B), with 2,065 significantly active sequences (11.2% of tested) in preosteoblasts (log2 activity between -0.82 and 3.36) and 1,389 (7.5%) in

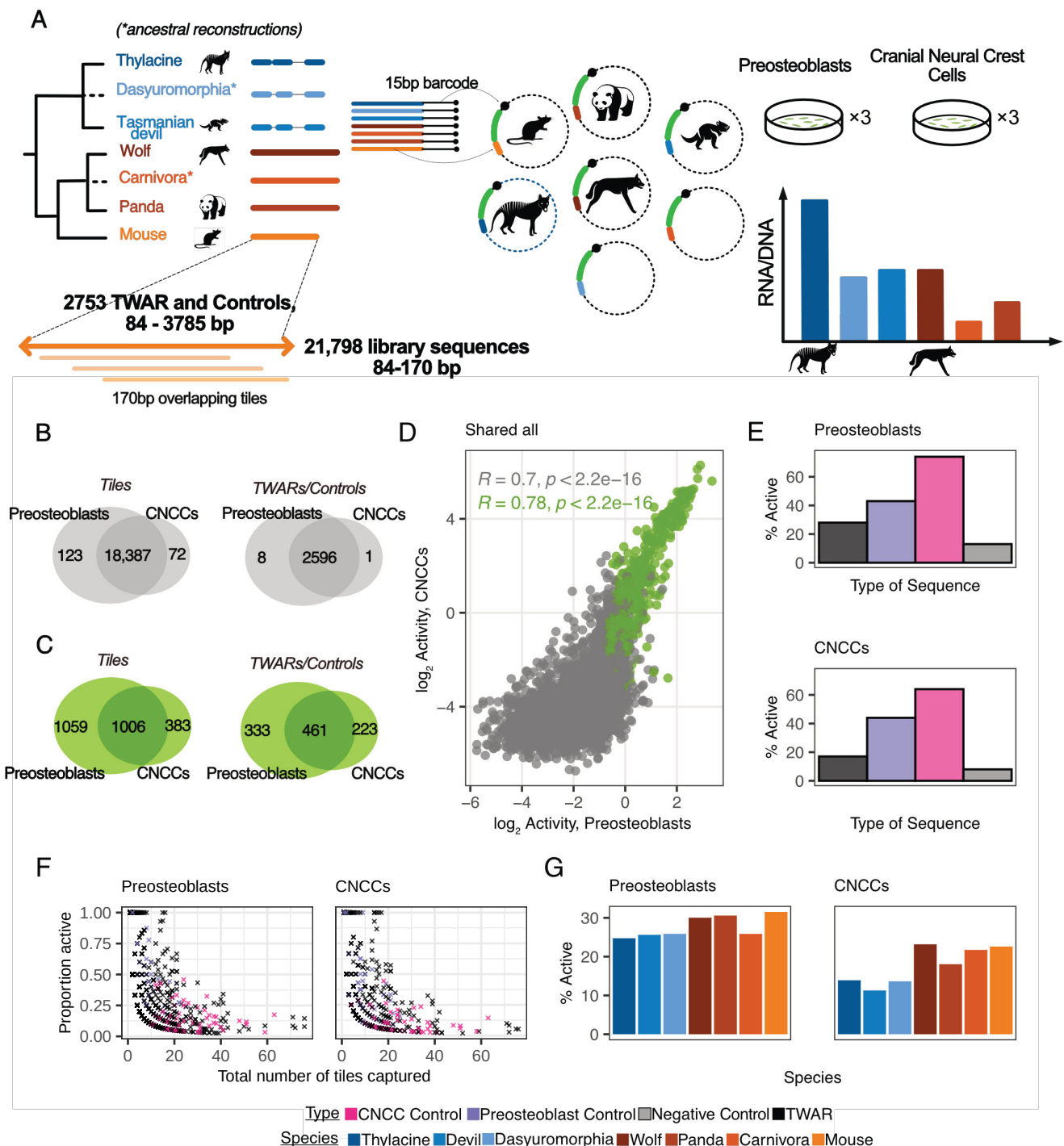


Figure 1. Performing an MPRA to test convergence of regulatory activity **A.** Orthologous TWAR sequences from seven different taxa — two test (thylacine and wolf), two outgroup (Tasmanian devil and panda), two ancestral (Dasyuromorphia and Carnivora) and one control (mouse) taxa were tested for activity in an episomal MPRA. Elements longer than 170 bp were covered with overlapping tiles in 10 bp steps. The cloned library was transfected into preosteoblasts and CNCCs. TWARs were then tested for convergent differential activity within each clade. **B.** Number of tiled library sequences and the number of TWARs and controls with at least 1 tile captured in each cell line. **C.** Number of active tiled library sequences and number of TWARs and controls with at least one active tile captured in each cell line. **D.** Correlation of $\log_2$ activity for library sequences in preosteoblasts and CNCCs (with significantly active library sequences in green). **E.** Proportion of TWARs and cell-line specific positive controls determined to be active in each cell line. **F.** Proportion of active tiles versus the total number of tiles captured for each multi-tiled TWAR and control sequence, for each cell line. **G.** Proportion of TWARs from each taxa determined to be active in each cell line.

95 CNCCs (log2 activity between -3.5 and 6.28) (Figure 1C). The lower range in activity values in
 96 the CNCCs is due to a technical artefact in the sequencing of mRNA from CNCCs (Supplemental
 97 Figure 3 and 4, see Methods); despite this, 72.4% (1006) of the library sequences active in
 98 CNCCs were also active in preosteoblasts, demonstrating high reproducibility. Spearman's
 99 correlation between activity values in the two cell lines was 0.70, increasing to 0.78 for shared
 100 active sequences (Figure 1D). The proportion of the negative controls that were active was
 101 lower than of the positive control and TWARs, and in every cell line replicate, the distribution
 102 of activity for these negative controls was significantly lower than those for the positive controls
 103 and the TWARs (Figure 1E, Supplemental Figure 5A and C, BH-adjusted pairwise *t*-tests results
 104 in Supplemental Table S2). It is worth noting that negative controls in MPRA experiments can
 105 sometimes display low levels of activity due to the stochastic nature of expression control and are
 106 best thought of as capturing the baseline activity of the minimal promoter in the reporter plasmid
 107 rather than a complete absence of activity.

108 Our overlapping tile design helped compensate for the loss of individual library sequences
 109 during cloning/transfection. By examining sequence coverage across complete TWAR and
 110 control sequences, we found that 2569 (98.7%) and 2563 (98.7%) have at least 80% coverage
 111 in preosteoblasts and CNCCs datasets, respectively (Supplemental Figure 6). We observed
 112 that regulatory potential is not uniform across tested sequences. For both TWARs and positive
 113 controls the overall region length was inversely correlated with the proportion of active tiles
 114 (Figure 1F). The mean and median number of active tiles for multi-tiled TWARs was 2.0 and
 115 2.6 in the preosteoblasts and 1.0 and 2.5 in the CNCCs. When focusing on the subset of active
 116 TWARs in each cell line for which i) all tiles were captured and ii) more than one tile was found
 117 active (146 in preosteoblasts and 78 in CNCCs), the median distance between active tiles was
 118 estimated to be 10 nt, i.e. most active tiles within this subset were neighbouring each other and
 119 shared most of their sequence (Supplemental Figure 7). These observations are consistent with
 120 a role for small transcription factor binding motifs in driving regulatory activity within promoter and
 121 enhancer regions. This also underlies the lack of activity in some of our cell-line specific positive
 122 controls; 83.9% of controls <170 bp were active, greater than the overall percentage of 48.7%
 123 that were active.

To assess the impact of the murine *trans*-regulatory environment, we compared TWAR activity for each of the test taxa with that of the orthologous mouse sequence. Activity values were not significantly different between the mouse and any of the other taxa in either cell line (BH-adjusted pairwise *t*-test results in [Supplemental Table S3](#), Supplemental Figure 5B and D). The percentage of active TWARs did not differ significantly among the placental taxa, with pairwise differences ranging from 0.9-5.6% in preosteoblasts and 0.5-4.3% in CNCCs (Figure 1G, BH-adjusted two-proportion tests in [Supplemental Table S4](#)). The mouse had the highest and the second-highest percentage of active TWARs in the preosteoblasts (31.5%) and CNCCs (21.5%, slightly lower than the 22% of active wolf TWARs) respectively and all placental taxa, excluding the Carnivora in preosteoblasts, had a greater percentage of active TWARs than the three marsupial taxa (pairwise differences ranging 4.1-6.8% in preosteoblasts and 4.0-11.3 % in CNCCs). This could indicate a slight placental bias arising from the placental *trans* environment; however, these differences were not statistically significant (two-proportion tests in [Supplemental Table S4](#)).

Genomic features underlying TWAR activity

To identify genomic features driving activity in our assay, we first used FIMO to scan TWARs for transcription factor binding motifs (TFBMs) from the HOCOMOCO Mouse Core v11, summarising all motifs inside a TWAR into a single metric that ranges from 0-1 and can be directly compared across all orthologs of a given TWAR (see Methods). Active TWARs have significantly higher aggregated FIMO scores than inactive TWARs (Figure 2A) (pairwise *t*-test, preosteoblasts *p*-value: 3.386×10^{-17} , CNCCs *p*-value: 4.794×10^{-17}). We also observed that active TWARs also had significantly higher percentage GC content, consistent with the well-known association of GC-rich DNA with open chromatin, promoters and broadly active enhancers (Fenouil et al., 2012; Colbran et al., 2017) (Figure 2B) (*t*-test preosteoblasts *p*-value: 3.027×10^{-10} , CNCCs *p*-value: 1.459×10^{-18}). Finally, we classified TWARs within 1 kb of a mouse (GRCm38) transcription start site as promoters and the rest as enhancers (26 and 271, respectively), to test for any differences in activity between distal and proximal elements. There was no enrichment for activity in proximal sequences relative to distal ones (Fisher's exact test, preosteoblasts *p*-value:

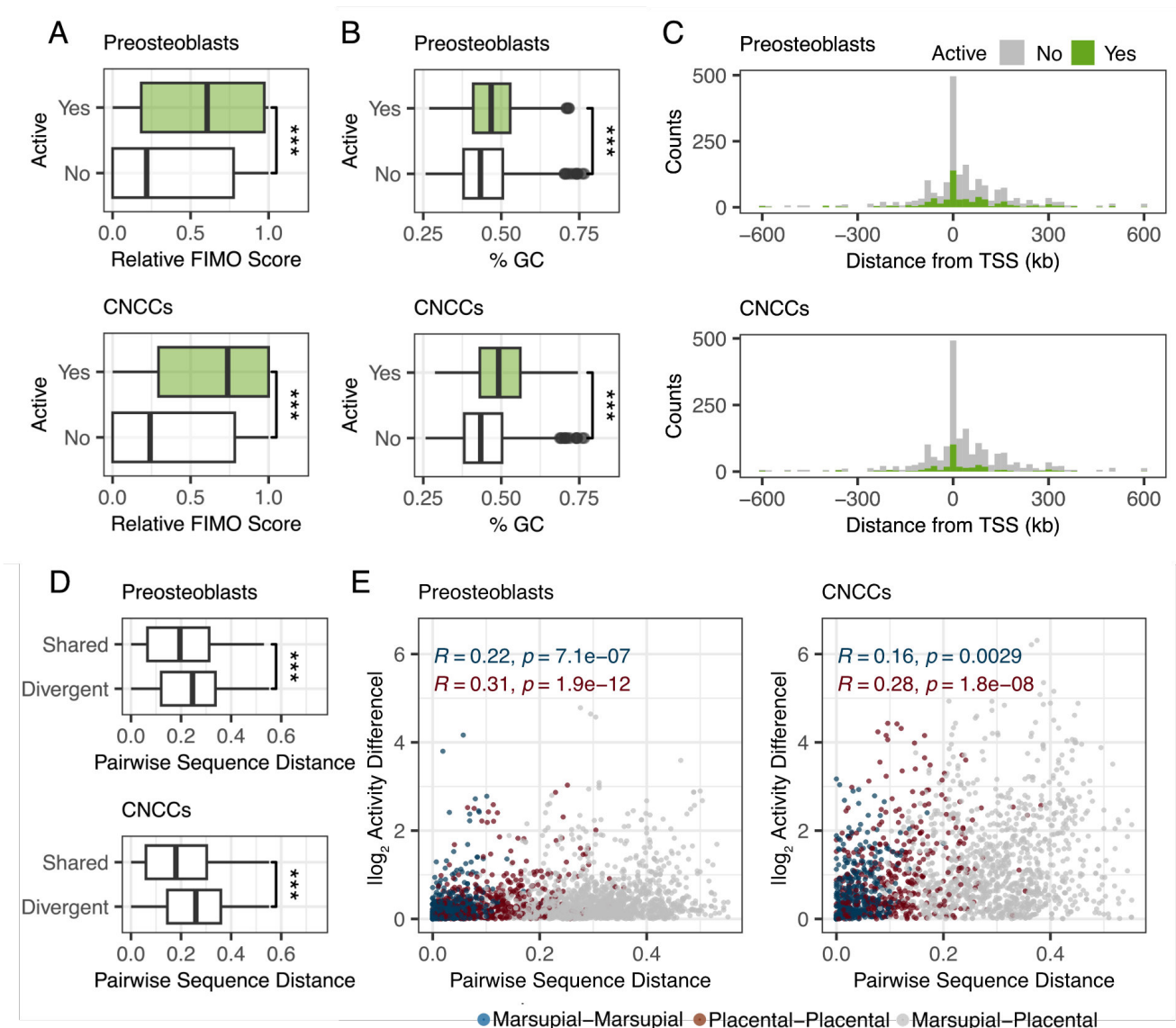


Figure 2. Examining sequence features driving activity of TWARs. Distribution of **A.** aggregated FIMO scores and **B.** Percentage GC content for active and non-active TWARs, for both cell lines. **C.** Distribution of active and non-active TWARs in cell lines by their distance from a transcription start site in the mouse genome. **D.** Distribution of pairwise sequence distances between each pair of TWAR orthologs that display shared or divergent activity. **E.** Correlation between pairwise sequence distance and absolute activity differences for each pair of TWAR orthologs.

0.447, CNCCs p -value: 0.454) (Figure 2C). Furthermore, there was only a weak association between the absolute distance from a transcription start site in mouse to the proportion of taxa in which a TWAR was determined to be active, in either cell line (see Methods).

We then examined the role of sequence divergence in influencing patterns of TWAR activity. Overall, 167 TWARs in preosteoblasts and 120 in the CNCCs were active in at least one of the six tested taxa. Of these 117 (70.0%) and 83 (56.5%), respectively, were active in more than 1 taxon (Supplemental Figure 8). Considering only TWARs with demonstrated activity

in at least one taxa, we observed significantly higher pairwise sequence distances between pairs of orthologous TWARs that show divergent activity (one active, one inactive) than pairs of orthologous TWARs for which activity was shared (both active or inactive) (Figure 2D) (pairwise *t*-test preosteoblasts *p*-value: 1.950×10^{-10} , CNCCs *p*-value: 1.440×10^{-19}). Differences in relative FIMO scores were also significantly larger between pairs of orthologous TWARs with divergent activity (Supplemental Figure 9) (*t*-test preosteoblasts *p*-value: 0.0004, CNCCs *p*-value: 4.630×10^{-15}). Spearman's ρ between pairwise sequence distance and absolute activity differences was 0.21 (*p*-value 1.86×10^{-33}) in preosteoblasts and 0.28 (*p*-value 7.41×10^{-26}) in CNCCs. We observed higher correlations for the placental-placental comparisons in both cell lines than the marsupial-marsupial comparisons (Figure 2E). This is likely due to the overall greater sequence similarity between the marsupial orthologs (mean pairwise sequence distance thylacine vs Tasmanian devil: 0.068, thylacine vs Dasyuromorphia: 0.015, Tasmanian devil vs Dasyuromorphia: 0.054, wolf vs panda: 0.166, wolf vs. Carnivora: 0.154 and panda vs Carnivora: 0.043) (Supplemental Figure 10).

Examining patterns of TWAR activity across taxa

Seventeen TWARs displayed significant activity across all six taxa in the preosteoblasts, with 6 being also universally active in CNCCs (Figure 3A, Supplemental Table S5 and Supplemental Table S6). Variation across taxa in TWAR lengths and thus tile numbers confounded 1:1 tile activity comparisons but activity-by-tile plots of all conserved elements are available as Supplemental File S1. The top-ranked TWAR by activity in all taxa was TWAR17.chr6; 85% of tiles in this TWAR were significantly active in all taxa. Our sliding window design identified a consistent pattern of decreasing activity when moving across tiles (5' to 3') in the TWAR (Figure 3B). Although only the first six panda and seven of the first eight Carnivora tiles were present in the final library, the orthologous mouse sequence had activity patterns nearly identical to those of the wolf, with a peak of activity in tile 12 (Wolf—Mouse Spearman's ρ preosteoblasts: 0.86, CNCCs: 0.91) (Supplemental Figure 11). Zooming in, our FIMO analyses identified a cluster of TFBMs within the first 70 bp of TWAR17.chr6 (Supplemental Figure 12), while the peak in activity in tile 12 for the wolf ortholog (spanning positions 111-280 in the sequence) overlapped

187 a placental-specific binding site for LYL1 (at positions 254-267). TWAR17.chr6 intersects with a
 188 super-enhancer in mouse embryonic stem cells for the neighbouring *ATF7IP* (Khan et al., 2016),
 189 known to inhibit premature differentiation of progenitor cells (including the preosteoblasts used
 190 in our study) (Hu et al., 2023; Tsusaka et al., 2020; Wu et al., 2023). Thus it is likely that the
 191 endogenous gene regulatory network that regulates *ATF7IP* expression is highly active in our cell
 192 lines and driving these activity patterns.

193 As originally defined, all TWARs overlapped H3K9ac and H3K27ac peaks in mouse
 194 craniofacial tissues collected at E11.5 in (Consortium et al., 2012)). Recently, we published a
 195 novel H3K27ac and H3K4me3 ChIP-seq dataset from craniofacial tissue of newborn fat-tailed
 196 dunnarts (*Sminthopsis crassicaudata*), a carnivorous dasyurid marsupial model species (Cook
 197 et al., 2025) that diverged from the thylacine 25-35 million years (Duchêne et al., 2018). Of
 198 the 17 universally active TWARs, 12, including TWAR17.chr6 overlapped or were adjacent to
 199 dunnart H3K27ac and/or H3K4me3 peaks, also confirming their activity in the craniofacial region
 200 of a developing dasyurid and their broad enhancer potential across therians.

201 Having established that our MPRA could distinguish TWARs with broad gene regulatory
 202 potential, we sought to establish its capacity to detect regulatory changes over long evolutionary
 203 distances. We identified TWARs with marsupial and placental-specific regulatory behaviour — 8
 204 TWARs in preosteoblasts and 1 in CNCCs, were exclusively active in the marsupial carnivores,
 205 and 6 TWARs in preosteoblasts and 8 in CNCCs (one overlapping) were exclusively active in
 206 the placental carnivores ([Supplemental Table S5](#), [Supplemental Table S6](#) and [Supplemental](#)
 207 [File S2](#)). Aggregated FIMO scores were higher in the marsupials for marsupial-active TWARs
 208 (mean difference marsupials—placentals 0.180, pairwise *t*-test *p*-value 0.055) and higher in
 209 placentals for the placental active TWARs (mean difference 0.128, *p*-value 0.128). While these
 210 differences were not significant overall, clustering elements by their aggregated FIMO scores
 211 highlights individual examples of clade-specific regulatory activity associated with clade-specific
 212 binding motifs (Figure 3C). For instance, TWAR10.chr11 displayed placental-specific activity in
 213 CNCCs (Figure 3D), with identical tile activity patterns across all three placental taxa. These
 214 correlated with the presence of TFBMs for SP/KLF family proteins in these sequences. Similarly,
 215 the first half of TWAR2.chr19 displayed marsupial-specific activity in the preosteoblasts (Figure

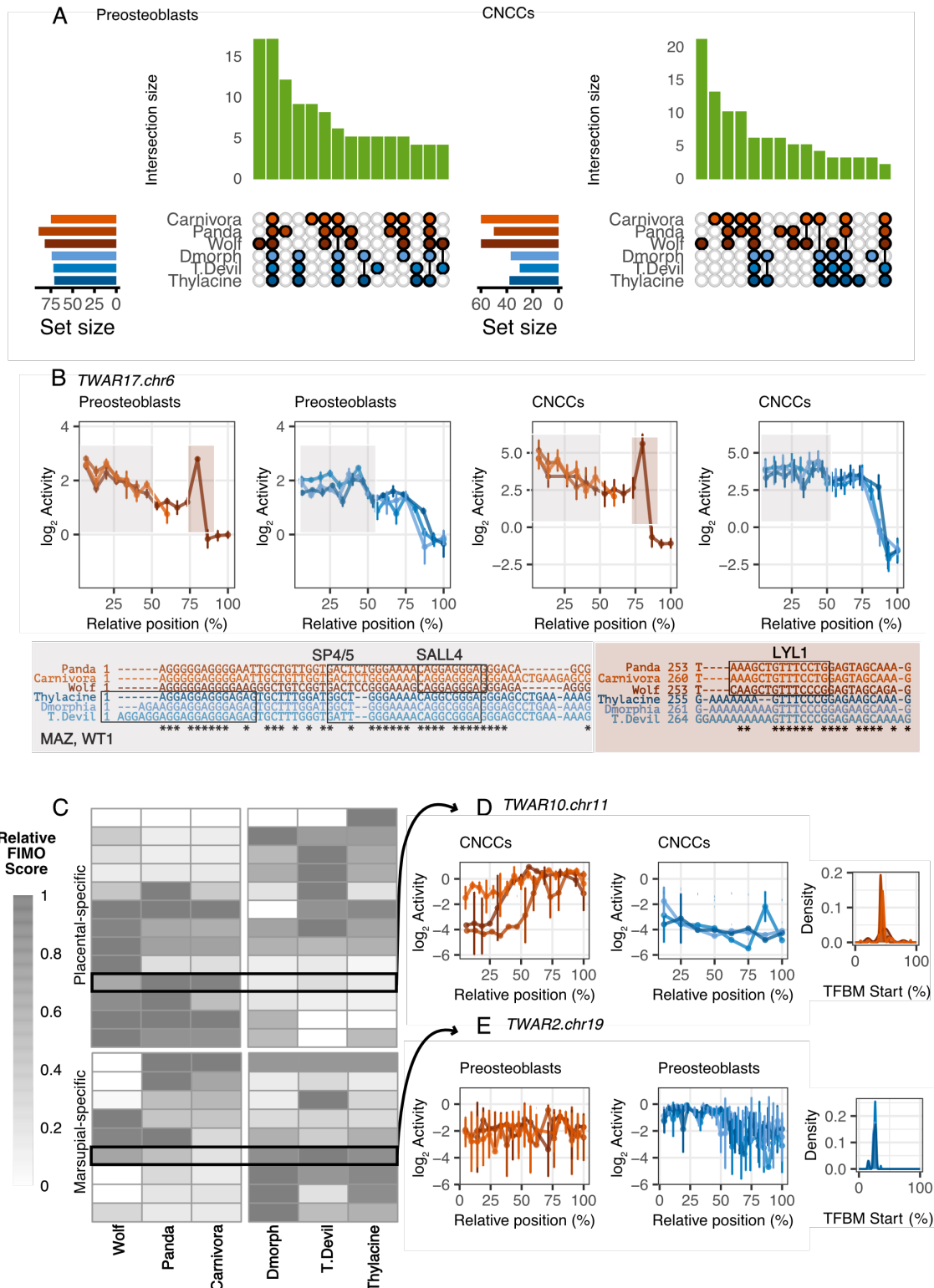


Figure 3. Functional conservation of activity across taxa. Dark blue: thylacine, medium blue: Tasmanian devil, lightblue: Dasyuromorphia; dark red: wolf, medium red: panda, light red: Carnivora. **A.** Upset plot (top 15 combinations) displaying overlap in active TWARs among the six focal taxa across both cell lines. **B.** Patterns of activity across tiles for *TWAR17.chr6* in marsupial and placental taxa. Due to variation in the number of tiles across taxa, tile number has been summarized as relative to the total number of tiles ("Relative position"). Boxes identify TFBMs motifs present in the sequences for this TWAR highlighted in the plots. **C.** Heatmap of relative FIMO scores for every TWAR with marsupial- or placental-specific activity in either cell line. **D.** Activity across tiles of the placental-active *TWAR10.chr11* for all taxa in the CNCCs, along with distribution of binding motifs across sequence length for the placentals. **E.** Activity across tiles of the marsupial-active *TWAR2.chr19* for all taxa in the preosteoblasts, along with distribution of binding motifs across sequence length specifically for the marsupials.

3E), consistent with a cluster of TFBMs for ELF5, ETV2, KLF15, MAZ, SP5 and SP1 in the region. TWAR2.chr19 was also distinguished by a stronger H3K27ac and H3K4me3 signal in the dunnart (which shares these motifs) than the mouse (Supplemental Figure 13). Across the clade-specific active TWARs, 5 (63%) marsupial-specific and 4 (31%) placental-specific TWARs overlapped or were adjacent to H3K27ac and/or H3K4me3 peaks in dunnart craniofacial tissue, confirming the ability of our MPRA to detect less conserved activity patterns.

Characterising differential TWAR activity in the thylacine and wolf

We then asked whether any TWARs exhibited activity patterns unique to the wolf or thylacine sequences. By assessing binary active/inactive trends, we found a distinct signal of unique behaviour for the wolf — 23 TWARs in preosteoblasts and 24 in CNCCs were either only active or inactive in this species (Supplemental Figure 8). To assess differential activity systematically, we applied the mpralm framework (Myint et al., 2019) (Methods). Given the large evolutionary and sequence distance separating our two focal species, these analyses were carried separately within each clade; additionally they were restricted to TWARs that were active in at least one species within the clade. We found that 33 TWARs (out of 110, 30.0%) in preosteoblasts and 11 TWARs in CNCCs (out of 50, 22.0%) were significantly differentially active (DA) (adjusted p -value < 0.05) and had a pairwise fold-change (FC) > 0.10 between the thylacine and Tasmanian devil and/or the thylacine and Dasyuromorphia (Figure 4A). Of these, 8 were shared between both cell lines, and 7/8 had identical directions of effect. An additional 18 TWARs in the preosteoblasts and none in the CNCCs were significantly differentially active but had FC < 0.10 relative to the Thylacine. In parallel, we investigated whether wolf TWAR activity differed from that of the panda and Carnivora, determining that, 40 (out of 134, 29.9%) TWARs were DA in preosteoblasts and 21 were DA in CNCCs (out of 103, 20.3%) with a FC > 0.10 ; 11 were shared and 10/11 had an identical direction of effect. Only 6 TWARs in the preosteoblasts and none in the CNCCs were differentially active with FC smaller than 0.10 relative to the wolf. Plots displaying log2 activity across species for every differentially active TWAR are available in [Supplemental File S3](#).

For both taxa in each cell line, we observe a positive linear correlation in log2 fold-changes in the focal species relative to the outgroup and the ancestor (Pearson's R 0.64-0.92; Figure 4B),

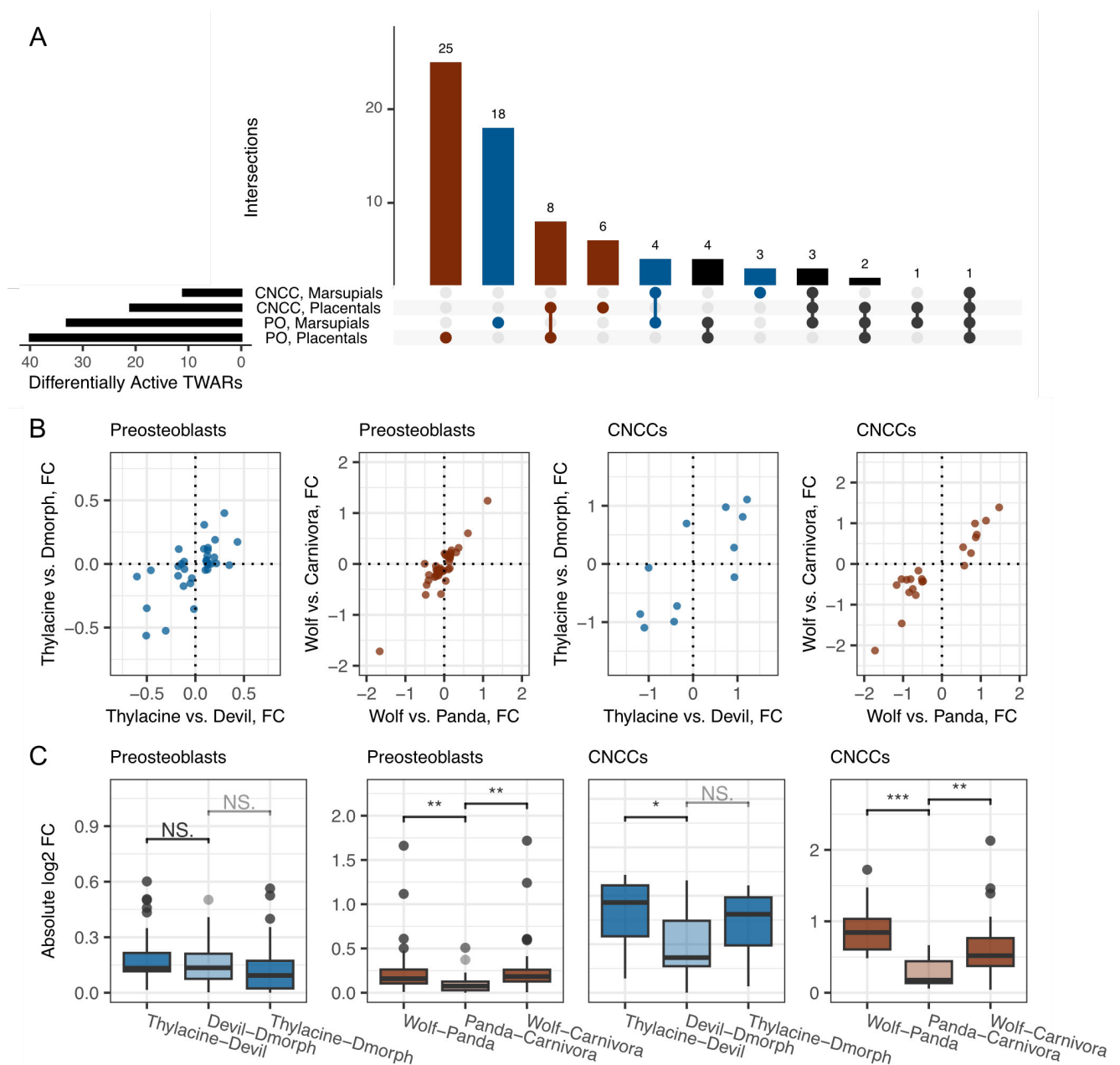


Figure 4. Differential activity of TWARs within the marsupials and placentals. **A.** Upset plot displaying overlap in TWARs found to be differentially active (DA) among the marsupials and the placentals in each cell line. **B.** Comparisons of activity differences between the test and outgroup taxa to the differences in activity between the test and ancestral taxa for marsupial and placental differentially active (DA) TWARs in both cell lines **C.** Distributions of activity differences between each pair of taxa for marsupial and placental DA TWARs in both cell lines.

indicating that the thylacine and wolf elements were driving differential activity within their clade. This was particularly evident among the placentals — absolute fold changes in activity between the wolf and the panda, and the wolf and Carnivora were significantly larger than those between the panda and Carnivora (Figure 4C) (pairwise *t*-test *p*-values, Wolf-Panda vs Panda-Carnivora, POs: 0.006, CNCCs: 6.32×10^{-8} ; Wolf-Carnivora vs Panda-Carnivora, POs: 0.003, CNCCs: 0.003). While a similar pattern of fold-change distribution was observed for thylacine, only one comparison was significant in the CNCCs (Thylacine-Devil vs Devil-Dasyuromorphia, *p*-value 0.044). This is possibly due to the greater overlap in sequence composition of the marsupial TWARs (Supplemental Figure 10). The highest pairwise log2 fold-change relative to the thylacine for each TWAR ranged between 0.103-0.602 in the preosteoblasts and 0.695-1.22 in the CNCCs; the highest pairwise fold-change relative to the wolf ranged between 0.105-1.72 in the preosteoblasts and 0.482-2.13 in the CNCCs. Fewer differentially active TWARs with smaller effect sizes were thus captured in CNCCs, which could be due to technical challenges associated with this cell line.

To identify associations between the presence of TFBMs and differential TWAR activity, we recalculated our FIMO scores for each TWAR relative to the most active ortholog separately for each clade. FIMO scores were overall comparable between DA TWARs than non-DA TWARs for both marsupials and placentals (Supplemental Figure 14). For placental differentially active TWARs, the direction of effect was significantly associated with differences in FIMO scores — TWARs with increased activity in the wolf relative to other placental taxa also have higher FIMO scores in the wolf than the other two taxa, and vice versa (Figure 5A) (pairwise *t*-test *p*-values Wolf-Panda 0.009; Wolf-Carnivora 0.005).

Clustering differentially active TWARs in each clade by their FIMO scores, we identified several examples for which direction of activity could be linked to variation in FIMO scores (Figure 5B). Taking advantage of the fact that our tiling design traverses TWARs 10bp at a time, we examine how activity patterns and binding motifs change across a TWAR's length at high resolution. For instance, TWAR27.chr3 was differentially active between marsupials (Figure 5C; BH-adjusted *p*-value preosteoblasts 2.44×10^{-11} , CNCCs 0.023), [Supplemental Table S7-Supplemental Table S10](#)). Both Tasmanian devil and Dasyuromorphia TWAR orthologs

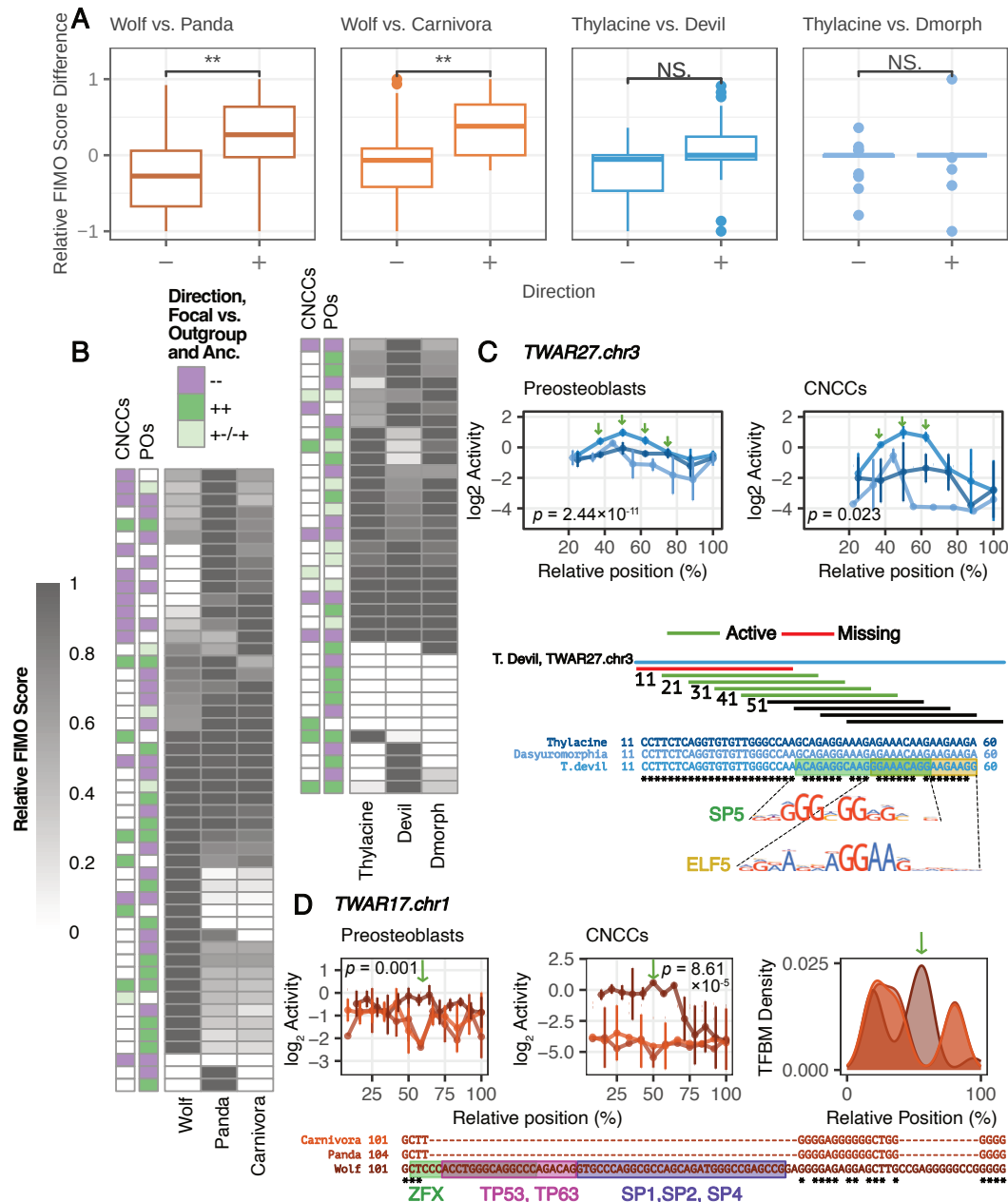


Figure 5. Transcription factor binding and differential activity. Dark blue: thylacine, medium blue: Tasmanian devil, lightblue: Dasyuromorphia; dark red: wolf, medium red: panda, light red: Carnivora. **A.** Differences in FIMO scores between the test species and the outgroup or ancestral taxon, for DA TWARs that have gained (+) or lost (-) activity in the focal species. **B.** Relative FIMO scores for differentially active (DA) TWARs in the marsupials and placentals for both cell lines. Direction of effect highlights if the thylacine/wolf (“test”) TWARs have gained activity relative to both outgroup (“O”) and ancestor (“A”) (+ +), lost activity relative to both outgroup and ancestor (- -) or have intermediate activity to that of the outgroup and ancestor (+ -). Note the concordance in direction for DA TWARs across cell lines. **C.** Activity across tiles of the differentially active TWAR27.chr3 for marsupial taxa; highlighted are the Tasmanian devil-specific binding motifs associated with Tasmanian devil-active tiles for this TWAR. **D.** Activity across tile in the preosteoblasts and CNCCs for the placental differentially active TWAR17.chr1, as well as density of TFBMs along the lengths of these sequences. The sequence alignments highlight the wolf-specific sequence containing clusters of binding motifs.

273 were more active than the thylacine one, with higher differences in activity between Tasmanian
 274 devil and thylacine (log2 FC preosteoblasts -0.602, CNCCs -1.00) than between the thylacine
 275 and *Dasyuromorphia* (log2 FC POs -0.099, CNCCs, -0.066). In the Tasmanian devil activity
 276 peaked between tiles 2-5; examining sequence covered uniquely by these tiles (11-60 nt) we
 277 found 3 fixed single nucleotides differences in the Tasmanian devil relative to the other marsupials,
 278 which span predicted TFBMs for ELF5, ETS2, ETV2, MAZ and SP5. Similarly, TWAR17.chr1,
 279 displayed increased regulatory potential in the wolf relative to other placentals (Figure 5D;
 280 preosteoblasts: BH-adjusted *p*-value 0.001; log2FC Wolf-Panda 0.032, Wolf-Carnivora 0.171.
 281 CNCCs: BH-adjusted *p*-value 8.61×10^{-5} ; log2FC Wolf-Panda 0.871, Wolf-Carnivora 0.651).
 282 Higher log2 activity was observed in wolf in tiles corresponding to unique sequence at positions
 283 105-159 nt of the wolf ortholog. This sequence does not align with other placental orthologs and
 284 contains a cluster of binding motifs, including for ZFX, TP53, TP63 and SP/KLF transcription
 285 factors (Figure 5D). These examples illustrate how combining evidence of activity and tiling
 286 patterns from the MPRA with sequence changes and FIMO results can provide granular insights
 287 into drivers of functional change within individual non-coding elements.

288 **Limited regulatory convergence of the thylacine and wolf accelerated regions**

289 Our initial hypothesis was that shared sequence acceleration of the TWARs for thylacine and
 290 wolf would be reflected in convergent patterns of regulatory activity. Ultimately however, only
 291 11 TWARs were differentially active in both the marsupials and placentals across the two cell
 292 lines (Figure 4A, [Supplemental Table S11](#); [Supplemental File S3](#)). Of these four agree in the
 293 direction of effect in the thylacine and the wolf, and TWAR27.chr3 is distinguished by its proximity
 294 to genes with known roles in craniofacial development. As discussed in the previous section,
 295 TWAR27.chr3 has significantly lower activity in the thylacine relative to the other two marsupials
 296 in both cell lines, an effect that co-localises with specific nucleotide changes (Figure 6A). A 123
 297 bp deletion in the wolf sequence also resulted in ablation of its regulatory potential, which is
 298 significantly lower than the other placentals in the CNCCs (Figure 6B and C, Supplemental Figure
 299 15; BH-adjusted *p*-value = 0.029; log2 FC Wolf-Panda = -0.843, Wolf-Carnivora = -0.699). The
 300 orthologous region to this TWAR in mouse is 97 kb downstream of *LMO4*, known to be expressed

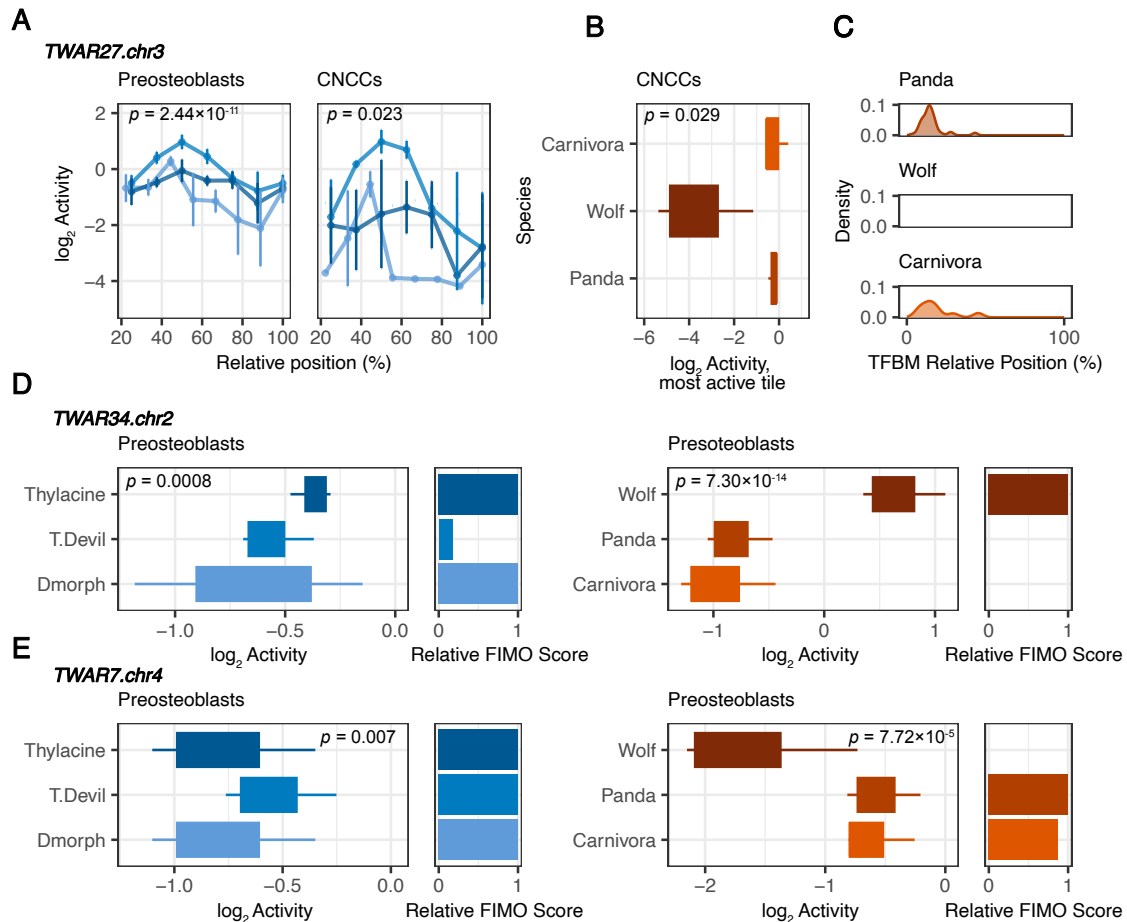


Figure 6. TWARs with convergent regulatory activity. Dark blue: thylacine, medium blue: Tasmanian devil, lightblue: Dasyuromorphia; dark red: wolf, medium red: panda, light red: Carnivora. **A.** Activity across tiles of the TWAR27.chr3 for marsupials (preosteoblasts and CNCCs). **B.** Activity of most active tile for each placental ortholog of TWAR27.chr3 in the CNCCs. **C.** Density of transcription factor binding motifs across the length of the placental orthologs of TWAR27.chr3. **D.** Activity in the preosteoblasts of the single-tiled TWAR34.chr2 for the marsupials and placentals, and the relative FIMO score (0-1) for each ortholog within a clade. **E.** Activity in the preosteoblasts of the single-tiled TWAR7.chr4 for the marsupials and placentals, and the relative FIMO score (0-1) for each ortholog within a clade.

in *pre-migratory* neural crest cells, regulating their formation (Ochoa et al., 2012; Kenny et al., 1998).

The single-tile TWAR34.chr2 (124.kb upstream *NUP35*) had significantly reduced activity in the thylacine (Figure 6D); preosteoblasts: BH-adjusted p -value = 0.0008; log₂ FC Thylacine-T.devil = 0.203, Thylacine-Dasyuromorphia = 0.189) and wolf (preosteoblasts: BH-adjusted p -value = 7.30×10^{-14} ; log₂ FC Wolf-Panda = 1.12, Wolf-Carnivora = 1.24). The wolf TWAR7.chr4 showed significantly lower regulatory potential (preosteoblasts: BH-adjusted p -value = p -value = 7.72×10^{-5}) than the panda (log₂ FC -0.216) and Carnivora (log₂ FC -0.245) orthologs (Figure 6E). This TWAR was also differentially active in the marsupials

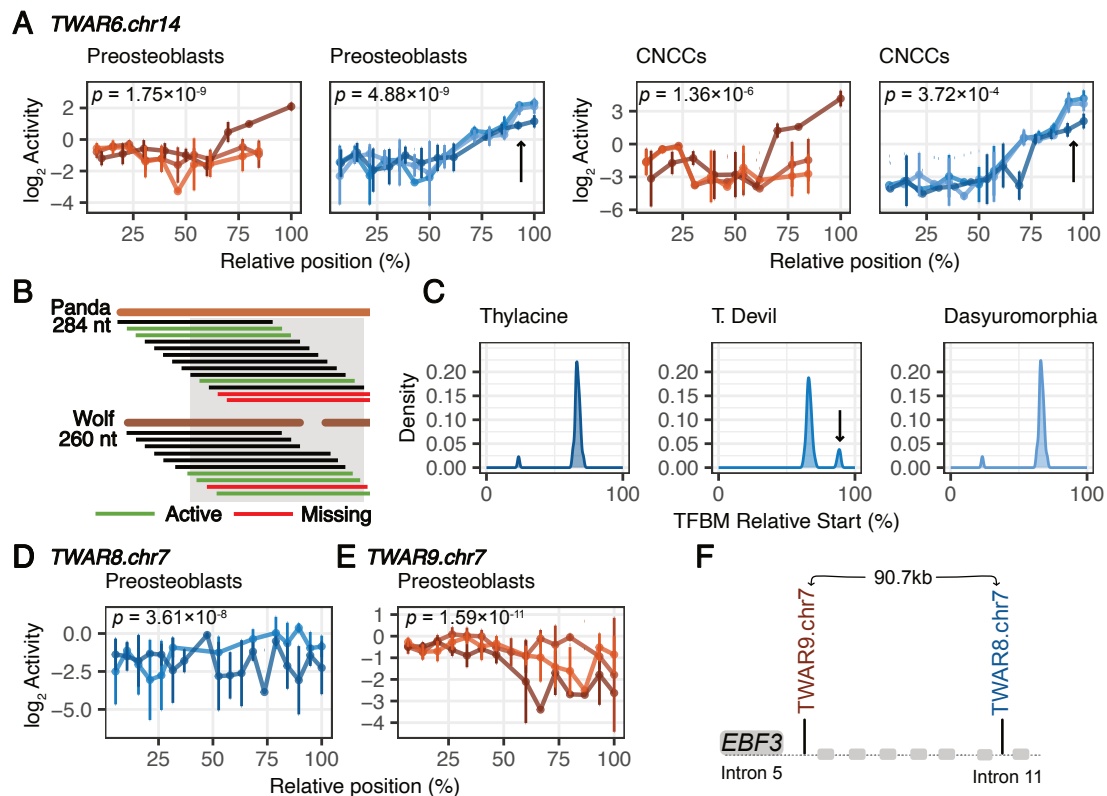


Figure 7. Regulatory convergence through non-identical changes. Dark blue: thylacine, medium blue: Tasmanian devil, lightblue: Dasyuromorphia; dark red: wolf, medium red: panda, light red: Carnivora. **A.** Activity across tiles of *TWAR6.chr14* for marsupials and placentals in each cell line. The arrow corresponds to tiles with increased activity in the Tasmanian devil which also have Tasmanian devil-specific binding motifs. **B.** The schematic compares the constitutive tiles of *TWAR6.chr14* for the wolf and panda; it describes how although we have lost the last two panda tiles for this *TWAR*, panda sequences orthologous to wolf tiles that have significant activity are indeed present in our dataset. **C.** Distribution of binding motifs for the marsupial taxa, with the Tasmanian devil-specific binding motif indicated by the arrow. **D.** Activity across tiles of *TWAR8.chr7* in the thylacine and Tasmanian devil. **E.** Activity across tiles of *TWAR9.chr7* for all of the placental taxa. **F.** Position of the *TWAR8.chr7* and *TWAR9.chr7* in the mouse genome in intron 11 and 5 of *EBF3*, respectively.

(BH-adjusted p -value = 0.007); the thylacine and Dasyuromorphia display lower activity relative to the Tasmanian devil ($\log_2$ FC Thylacine-T. Devil = -0.145, Dasyuromorphia-T. Devil = -0.145). *TWAR7.chr4* is 932bp downstream of the *SNX30* in mouse genome. While both *SNX30* and *NUP35* have crucial roles in cell processes (intracellular trafficking and forming the nuclear pore complex, respectively) (Antón et al., 2020; Chen et al., 2018), neither have been implicated in craniofacial biology.

TWAR6.chr14 was uniquely differentially active in each clade in both the preosteoblasts (Figure 7A and B; BH-adjusted p -value marsupials 4.88×10^{-9} ; placentals 1.75×10^{-9}) and CNCCs (BH-adjusted p -value marsupials 3.72×10^{-4} ; placentals 1.36×10^{-6}), although it had opposing directions of effect in the thylacine (reduced regulatory potential, preosteoblasts $\log_2$ FC Thylacine-T. Devil -0.500, Thylacine-Dasyuromorphia -0.348; CNCCs $\log_2$ FC Thylacine-T.

Devil -1.19, Thylacine-Dasyuromorphia -0.862) and wolf (gained regulatory potential, POs log2 FC Wolf-Panda 0.610, Wolf-Carnivora 0.603; CNCCs log2 FC Wolf-Panda 1.47 ; Wolf-Carnivora 1.39). These changes correlated to sequence features within each group; increased activity in the Tasmanian devil and Dasyuromorphia TWAR6.chr14 relative to the thylacine was limited to their last two tiles. We observe a cluster of sequence differences in the last 13bp of the Tasmanian devil and dasyuromorphia sequences, as well as TFBMs from the SP/KLF family that mapped to these positions only in the former (Figure 7C). Likewise, gain-of-activity in the wolf TWAR6.chr14 was associated to a 22bp deletion which introduces 2 unique binding motifs for ZBTB17 and ZNF143 absent in the other therian taxa (Supplemental Figure 17). TWAR6.chr14 is in the promoter region of *CHD8*, which is involved in the patterning and differentiation of cranial neural crest cells (Fan et al., 2021; Kasah et al., 2018; Kasah et al., 2018); as such, there is some evidence that this TWAR may have functional contribution to the thylacine-wolf convergent phenotype. However, the lack of concordance in direction of effect complicates interpretability across clades.

Given the limited evidence for convergence at individual loci, we finally turned to searching for overlaps in molecular functions or pathways amongst genes near the other marsupial and placental DA TWARs. We found one instance of potential regulatory convergence near a gene involved in craniofacial biology. TWAR8.chr7 had significantly reduced expression in the thylacine in the preosteoblasts, with absolute fold-change >0.10 relative to the Tasmanian devil (Figure 7D, Supplemental Figure 16A and C; BH-adjusted p -value = 3.61×10^{-8} ; log2 FC Thylacine-T. devil = -0.458, Thylacine-Dasyuromorphia = -0.050). TWAR9.chr7 had significantly reduced expression relative to the other two placentals, in both cell lines (Figure 7E, Supplemental Figure 16B,D and E); preosteoblasts: BH-adjusted p -value = 1.59×10^{-11} ; log2 FC Wolf-Panda = -0.393, Wolf-Carnivora = -0.216. CNCCs: BH-adjusted p -value = 0.002; log2 FC Wolf-Panda = -0.751, Wolf-Carnivora = -0.615). Both these TWARs are located in different introns of the gene *EBF3* (Figure 7F), which is highly-expressed during craniofacial development, particularly in the olfactory epithelium (Wang et al., 2004; Yankee et al., 2023). Mutations in this gene have also been associated with neurodevelopmental disorders involving facial pathologies and orofacial clefting (Cioffi et al., 2025; Harms et al., 2017).

350 Ultimately, the intersection of differentially active TWARs with similar regulatory potentials
351 between the marsupials and placentals was small, indicating limited role for nucleotide-level
352 genetic convergence in giving rise to the observed phenotypic similarities between the two taxa.

Discussion

We have used an MPRA to evaluate the activity of 295 conserved non-coding elements that display convergent sequence acceleration in the marsupial thylacine and the placental wolf. To our knowledge, this is the first high-throughput functional dissection of non-coding elements from lineages this distant (divergence time ≈ 160 MYA) (Bininda-Emonds et al., 2007). We observed substantial functional divergence and find that both thylacine and wolf sequences frequently drove differential activity within their respective clades. However, only 10 TWARs were differentially active in both the marsupials and placentals, and four had the same direction of effect in the thylacine and wolf. Of these, we find one loci near the key craniofacial developmental genes *LMO4* (TWAR27.chr3) as a possible candidate for further *in vivo* dissection.

We demonstrated how MPRA can be employed to ask complex evolutionary questions. Including outgroup and ancestral taxa facilitated our comparisons by controlling for sequence decay observed over longer divergence times. It also provides more conclusive evidence of gene regulatory activity specific to the focal species. Despite this separation and although the TWARS were defined by excess of sequence changes in the thylacine and wolf relative to other taxa, the function of promoters and enhancers can be robust to sequence turnover (Ludwig et al., 2000; Fisher et al., 2006; Snetkova et al., 2021). Correspondingly, 6.8% (17 out of the 250 TWARS captured in all taxa) of our TWARS displayed functional conservation of gene regulatory potential for all six marsupial and placental mammals in one or both cell lines. Furthermore, we identified for some TWARS conservation in patterns of regulatory activity across taxa, even with turnover of specific transcription-factor binding motifs. This supports higher-level evolutionary constraint restricting the order of motifs in non-coding elements, as the position of these can affect the strength and specificity of regulatory activity (Jindal et al., 2021; Reiter et al., 2023).

Largely however, patterns of conservation and divergence *were* significantly associated with sequence features, such as GC content and presence of transcription factor binding motifs, as in previous MPRA (Arnold et al., 2014; Whalen et al., 2022; Uebbing et al., 2021). For individual differentially active TWARS, we were able to identify overall differences in the strength in transcription factor binding (as summarised by the relative FIMO score) as well as for some

examples, specific nucleotide and motif changes that were underlying differences in regulatory function. A broader pattern was significant in the placentals, whereby increased and decreased activity in the wolf relative to the other taxa could be attributed to the gain and loss in the strength of binding respectively. This was not found among the marsupials however, partly because the greater sequence similarity between the marsupial sequences.

This sequence similarity in part also explains the lower differential activity we observed in the marsupials (36 DA TWARs across the two cell lines) than in the placentals (50 DA differentially active TWARs). In addition, our analyses suggest a limited impact of the murine *trans*-regulatory environment, with fewer TWARs active in marsupials than placentals, which could also obscure true signal. However, there are currently no validated craniofacial lineage cell lines from marsupials that would enable us to directly test this hypothesis. Our previous work has shown that the expression of craniofacial development genes is highly conserved during early development in the mouse and the marsupial fat-tailed dunnart (Cook et al., 2025), suggesting that the possibility of widespread rewiring of the developmental regulatory network is unlikely. Both the marsupial and placental taxa TWARs that are inactive in our results could also be active in other developmental stages, such as later ossification; alternatively, some sequences that we identify as active may be inactive *in vivo* due to being located in inaccessible chromatin.

Our study relied on a previously defined set of TWARs, which we found displayed lower sequence divergence amongst marsupials than amongst carnivores even when classified as accelerated. This reflects the challenges of studying an undersampled taxon, as the TWARs were derived from a 61-species whole genome alignment of vertebrates that contained 41 placentals and 4 marsupials (opossum, wallaby, Tasmanian devil and thylacine). Of these, only the Tasmanian devil is within the same order as the thylacine (Dasyuromorphia), whereas the alignment includes two representatives from Carnivora besides the wolf (Feigin et al., 2019). The undersampling of marsupials coupled with a placental-biased phylogeny could be overestimating sequence acceleration that is unique to the thylacine (Shukla et al., 2026) and explains the limited number of differences we observe within marsupials.

In conjunction with this, the low sequence and functional divergence in craniofacial non-coding elements among marsupials could be reflective of their unique biology. Unlike placentals,

410 marsupials give birth to highly altricial young that complete development *ex utero* in the maternal
 411 pouch. To enable the transition from newborn to suckling young, marsupials undergo accelerated
 412 development in both limbs and orofacial features (Smith, [1997](#); Smith, [2006](#); Cook et al., [2021](#)).
 413 This developmental requirement has been hypothesized to impose constraints on the evolution
 414 of early forming features in marsupials (Bennett et al., [2013](#); Goswami et al., [2016](#); Sears, [2004](#);
 415 Pevsner et al., [2022](#); Spiekman et al., [2017](#); Newton et al., [2021](#)). Though still subject to debate,
 416 this theory suggests that the craniofacial development machinery would also be more constrained
 417 in marsupials. Functional craniofacial non-coding elements may only tolerate sequence changes
 418 with no or subtle effects on regulatory activity which we do not have the power to capture in this
 419 assay.

420 Overall, we found that convergent regulatory activity was not widespread in the thylacine and
 421 wolf accelerated regions. This result aligns with previous findings on the lack of homoplastic
 422 substitutions in protein coding sequences between wolf and thylacine (Feigin et al., [2018](#)). Our
 423 work instead suggests that morphological convergence between the thylacine and wolf arises
 424 through distinct genetic changes, and demonstrate that shared acceleration in sequence may not
 425 be reflective of shared regulatory function. These results need to be considered with the caveats
 426 of our experimental design (including our murine cell lines) as well as the technical challenges
 427 with the cranial neural crest cells in which we identified fewer active and differentially active
 428 TWARs. Furthermore, as MPRA test sequences outside their native context and are limited by
 429 the length of sequences that can be synthesized and tested, the regulatory activity we identified
 430 may not be recapitulated *in vivo*. We also note that the novelty of our experimental design
 431 introduced unique challenges — for example, the variable lengths of orthologous sequences
 432 being compared — that are not perfectly resolved by existing analysis methods.

433 Nonetheless, taking into account the complexity of the gene regulatory architecture, our finding
 434 are not unprecedented. As non-coding elements are characterized by flexible organization and
 435 frequent redundancy (Spitz et al., [2012](#); Osterwalder et al., [2018](#)), identical genetic changes
 436 are not necessary to produce similar regulatory outcomes. Our decision to focus on TWARs that
 437 showed convergent evidence of acceleration was motivated by the desire to test the hypothesis of
 438 locus-level convergence; the flexibility of the regulatory architecture in craniofacial development

439 could facilitate the emergence of diverse paths to same trait. The intersecting thylacine and wolf
440 accelerated elements were only a fraction of the complete set of thylacine accelerated regions
441 (2.7%) and wolf accelerated regions (15.3%) defined by (Feigin et al., 2019). As suggested by
442 our finding of parallel changes near *EBF3*, a broader look at non-convergent, species-specific
443 regulatory elements with these massively parallel approaches could perhaps be more fruitful in
444 understanding the molecular origins of their convergent biology.

Methods

Library design

Sequence preparation We extracted all 339 previously identified TWARs from a 61-way whole genome alignment based on the mouse GRCm38 coordinates from (Feigin et al., 2019) using `maf_parse` (Hubisz et al., 2011). We obtained corresponding sequences for the thylacine, gray wolf, panda and Tasmanian devil with GNU `grep` for the species name and excluded elements if they were shorter than 90 bp in any taxa, as elements greater than this shared >80% of their sequence length between taxa. Furthermore, having a large range of sequence lengths in our oligosynthesis pool could skew reaction kinetics and artificially favour shorter sequences (Neumayr et al., 2019). This left a total of 295 TWARs to be tested.

Ancestral reconstruction We used tree files for Carnivora and Dasyuromorphia from [vertlife](http://vertlife.org/phylosubsets) (<http://vertlife.org/phylosubsets>) to generate independent tree models for each TWAR using the `phyloFit` utility from PHAST (Hubisz et al., 2011). We then applied PREQUEL (Hubisz et al., 2011) (`prequel i FASTA n s anc`) with the output FASTA parameter to generate the ancestral sequences for each TWAR for Carnivora and Dasyuromorphia. Species included in the ancestral reconstructions are listed in [Supplemental Table S12](#) and [Supplemental Table S13](#). For each reconstruction, we built a BLAST (Kent, 2002) database using the reconstructed sequences to compare sequence identity relative to the focal species. Fewer marsupial species (4) than placental taxa (11) were available as input for these reconstructions; consequently higher sequence sharing is observed between the Dasyuromorphia and the marsupial taxa (pairwise sequence identity 96% with thylacine and 94% with Tasmanian devil) than between Carnivora and the placental taxa (94.2% with the wolf and 85.4% with the panda).

MPRA controls We added mouse orthologs for each TWARs as controls for the murine cell lines used in our analysis, the MC3T3-E1 preosteoblasts (Wang et al., 1999) and O9-1 cranial neural crest cells (Ishii et al., 2012). To obtain a set of preosteoblast-specific positive controls, we first downloaded a dataset of ChIP peaks for H3K27ac in mouse craniofacial (nose, maxillary and mandibular) tissue at E13.5 and E15.5 (Brinkley et al., 2016). This was intersected with ChIP-seq peaks for *RUNX2*, a master regulator of osteoblast formation in MC3T3-E1 preosteoblasts cells

to obtain 194 overlapping regions (100-260 bp) (Wu et al., 2014). We further intersected the craniofacial H3K27ac ChIP-seq peaks with a DNase I hypersensitive site (DHS) sequencing dataset, also in MC3T3-E1 preosteoblasts cells, to get an additional 196 sequences (148-1,844 bp) (Tai et al., 2017). Lastly, we added 6 enhancer sequences (98-215 bp) found to be highly active in human primary fetal osteoblasts in a previous MPRA (Weiss et al., 2021). For our cranial neural crest-specific positive controls, we extracted elements from the VISTA enhancer database known to be active in the branchial arch, cranial nerves and or the facial mesenchyme at E11.5 (Visel et al., 2007). These were intersected with ChIP-seq peaks for KDM6A, involved in maintaining neural crest viability (Shpargel et al., 2017), as well as peaks for EP300 in O91 mouse neural crest cells (Attanasio et al., 2013). A set of 74 regions (196-3,785 bp in length) were found to be both strongly expressed in the craniofacial regions and intersecting with one or both of the ChIP-seq datasets. 170 bp scrambled sequences were generated as negative controls, and we used BLAT (Kent, 2002) and MEME FIMO (Grant et al., 2011) to confirm these sequences were not found in the mouse genome or predicted to have a transcription factor binding motif.

Tiling Given oligosynthesis constraints, we could only test 170bp of sequence at a time. When TWAR sequences were longer, we tiled across elements using a 10 bp sliding window, for a total of 22,110 tiles. Tiles can be identical when a TWAR is highly homologous across taxa; considering this redundancy, the library consisted of 21,798 unique tiles, of which 301 were shared between two species and 11 across three. This was carefully accounted for in our analysis.

Library amplification and plasmid assembly

PCR Amplification The full sequence library of 21,798 unique sequences flanked by shared 15 bp adapter sequences at either end (5' ACTGGCCGCTTGACG| CACTGCGGCTCCTGC 3') was synthesized by Twist Bioscience. We followed a 2-step amplification protocol, as recommended in Gordon et al. (2020). An initial 8-cycle PCR was first performed on the single-stranded oligo pool with the *F_adapter* and *R_adapter* primers, with the NEBNext Ultra II Q5 Master Mix (M0544, NEB) and the double-stranded library was cleaned up with 1.8× AMPure XP beads (A63881, Beckman Coulter). We subsequently performed parallel 8-cycle PCR reactions with *F_oligo_PCR*

501 and the degenerate *R_oligo_PCR*, to add a random 15 bp barcode to each oligo, as well as SfiI
 502 and BsiWI restriction sites and HiFi assembly sites for plasmid assembly. All primers used have
 503 been listed in [Supplemental Table S14](#).

504 *First cloning step* The purified barcoded library and the pMPRA1 plasmid (<https://www.addgene.org/49349/>, gifted by Tarjei Mikkelsen) (Melnikov et al., 2014) were both digested
 505 with the SfiI restriction enzyme (R0123S, NEB) for 2 hours at 50C. The pMPRA1 backbone was
 506 gel-extracted (T1020, NEB) while the oligo library was purified with a QIAquick PCR Purification
 507 Kit (28104, Qiagen) to minimize loss of library sequences. We then ligated the plasmid backbone
 508 and library overnight at 16C with T4 DNA ligase (M020S, NEB), using a molar insert:vector ratio
 509 of 1:3.
 510

511 The cloned library was transformed into competent bacterial *E. coli* cells (JM109, Promega)
 512 with heat-shock (42C for 2 minutes). The transformed bacteria were plated on Luria-Bertani (LB)
 513 agar plates containing 100 ug/mL of ampicillin (BP1760-5, Thermo Fisher Scientific) and cultured
 514 overnight. Bacterial dilutions (1:10, 1:100 and 1:1000) were also plated and used to estimate the
 515 library complexity. Bacterial colonies were collected from plates and the plasmids were extracted
 516 with the ZymoPure Plasmid Maxi-Prep Kit (D4202, Zymo Research). The oligo and tag region
 517 were amplified from the competent plasmid library in 12-cycle PCR reactions (NEBNext Ultra II
 518 Q5 Master Mix), purified (QIAquick PCR Purification Kit) and sent for NGS library preparation
 519 followed by amplicon sequencing (Illumina 2×150 bp) with Azenta Life Sciences. We performed
 520 this cloning step 3×, and sequenced each resulting library to a minimum depth of 100 million
 521 reads. The three cloned plasmid libraries were then combined for the following procedures.

522 *Second cloning step* We digested the oligo-pMPRA1 cloned plasmid with the BsiWI-HF
 523 restriction enzyme (R3553S, NEB) overnight at 37C, resulting in a linearised library with
 524 overhanging HiFi assembly sites. The reporter construct was synthesized as a gBlock™
 525 (Integrated DNA Technologies). This construct consisted of a minimal promoter in front of the
 526 open reading frame for the enhanced green fluorescent protein, with HiFi assembly overhangs
 527 flanking the sequence. We used the NEB HiFi DNA Assembly Master Mix (E2621S, NEB) to
 528 assemble the final plasmid, with a 1:2 ratio of vector to insert.

529 The reporter gene cloned plasmid was electroporated into 10-beta electrocompetent *E. coli*

cells (C3020K, NEB) with the recommended settings (2.0 kV, 200 Ω and 25 μ F) and plated in LB agar plates containing 100 ug/mL of ampicillin. They were then cultured overnight, along with a dilution plate (1:10, 1:100 and 1:1000). We used our oligo-tag sequencing results and Poisson modelling to estimate the number of individual bacterial colonies required to capture the oligo library with a sufficient number of barcodes (approximately $20\text{-}25 \times 10^6$ colonies). Bacterial cells were lifted from the cultured plates with LB, and the plasmid library was extracted with ZymoPure Plasmid GigaPrep Kit (D4202, Zymo Research).

Cell culture, transfection and harvesting

Preosteoblasts We cultured MC3T3-E1 Subclone 4 preosteoblasts (CRL-2593, ATCC) in MEM α (no ascorbic acid) (A1049001, Gibco) with 10% Fetal Bovine Serum (FBS) (FBSFR-S00JF, Scientifix). Prior to transfection, cells were seeded in 7×150 mm plates. Cells were disassociated with TrypLE (12605010, Thermo Scientific) for passaging and harvesting.

Cranial neural crest cells O9-1 cranial neural crest cells (SCC049, Merck) were cultured in Complete Embryonic Cell Media (15% FBS and LIF) (ES-101, Sigma-Aldrich) supplemented with 100 ug/mL of recombinant human bFGF (100-18B, PeproTech). Prior to transfection, cells were seeded in 4×150 mm plates coated with 5 mg/ml of Matrigel base membrane matrix (354234, Corning). Cells were disassociated with 0.25% Trypsin EDTA (25200072, Thermo Scientific) for passaging and harvesting.

Transfection At 60-70% confluence, we transfected 17.5 ug of the competent plasmid library into each dish (total $\approx 70\text{-}90$ million per replicate, per cell line) with the jetOptimus transfection reagent (101000006, Polyplus). A jetOptimus reagent to DNA ratio of 1.5 was used. The media was replaced 4 hours post-transfection.

Harvest For each replicate, we disassociated cells 24 hours post-transfection, re-suspended them in dPBS (14190136, Thermo Fisher Scientific), and centrifuged them for 5 minutes ($100\times g$ MC3T3-E1; $300\times g$ O9-1). The resulting pellets were re-suspended again in media and all the cell suspensions were combined. 10% of the total cell suspension was split equally into 2 microcentrifuge tubes, pelleted and stored at -80°C , for use in DNA extraction. The remainder of the cell suspension was centrifuged and stored at -80°C , for use in RNA extraction.

DNA and RNA extraction and library preparation

DNA extraction We extracted DNA with the Qiagen DNeasy Blood and Tissue Kit (69504, Qiagen), following the spin-column protocol for purification from animal cells, including the optional treatment with RNase A (19101, Qiagen) to obtain RNA-free genomic DNA.

RNA extraction and cDNA synthesis We extracted RNA with the Qiagen RNeasy Midi Kit (75144, Qiagen), following the protocol for the isolation of total RNA from animal cells, including the on-column DNase digestion. The eluted RNA was then treated again with Turbo DNase (AM1907, Thermo Fisher Scientific). We used an input of between 25-50 ug of RNA per replicate library for first-strand synthesis with Superscript IV (18090200, Thermo Fisher Scientific), with the reverse transcription primer (*Reverse_transcription_primer_R*). We additionally treated the first-strand synthesis product with 1 uL of RNase A (7000 units) (Qiagen, 19101) to remove excess leftover RNA.

Library amplification We performed qPCR reactions in triplicate per RNA and DNA library with SYBR Green 1 (628014, Thermo Fisher Scientific) to determine the number of cycles required for efficient amplification of the barcode sequence (15-17 cycles for gDNA libraries and 22-24 for cDNA libraries). The barcode region was then amplified in each gDNA and cDNA library with NEBNext Q5 Ultra II Master Mix and *GFP_F* and *Multipurpose_Buffer_R* primers. The amplicons were purified with the Qiagen PCR Minelute Kit (Qiagen, 28004) and sent for amplicon sequencing (Illumina 2×150 bp, >200M reads/library) with Azenta Life Sciences. All libraries were sequenced to a minimum depth of 200 million reads.

MPRA Data Analysis

Oligo-Barcode Association We used cutadapt (version 4.2) to trim sequencing adapters and filter the plasmid sequencing reads, keeping read pairs with Q>20 and length>75bp. The MPRAmatch pipeline developed by Ryan Tewhey (https://github.com/tewhey-lab/MPRA_oligo_barcode_pipeline/MPRAmatch.wdl) (Tewhey et al., 2016; Mouri et al., 2023) was used to associate barcodes with the oligos in our library. A custom Python script was used in place of `pull_barcodes.pl` to accommodate our library design.

Barcode counting cutadapt (version 4.2) was used to trim Illumina adapters from the barcode

sequencing reads from the cDNA and pDNA (plasmid DNA) libraries. Merged reads were then processed with the MPRAcount workflow from Ryan Tewhey (https://github.com/tewhey-lab/MPRA_oligo_barcode_pipeline/blob/master/MPRAcount.wdl) (Tewhey et al., 2016; Mouri et al., 2023). The pipeline maps the reads to the created oligo-barcode dictionary in the previous step, generating a table of raw barcode counts for each cDNA and pDNA library. A custom Python script was used in place of `make_counts.py`, to accommodate our library design. The number and percentage of reads that were successfully filtered and mapped is in **Supplemental Table S15**). We retained a lower read depth for the cDNA libraries of two of our cranial neural crest replicates; upon closer observation, sequences filtered out mapped to the mouse genome (GRCm38), and suggested the presence of excess residual endogenous RNA in these sequenced libraries.

Normalization, filtering and QC All analyses were performed in R (version 4.2.1) (R Core Team, 2022) unless otherwise stated. We normalized the raw count data by library size to get counts per million (CPM), followed by a log₂ transformation. We removed barcode observations with extremely low pDNA counts across replicates in each cell line, using the density distribution of the log₂ normalized pDNA to determine the cutoff at which read counts stabilized (preosteoblasts: -5.50; CNCCs: -5.75) (Supplemental Figure 18), as in (Uebbing et al., 2021). We further filtered out oligos with fewer than 10 barcodes in each cell line. Number of barcodes per oligo (per fragment, for tiled oligos) for every TWAR highlighted here have been provided in **Supplemental Table S16** and **Supplemental Table S17**. We mapped oligos passing all filters to the collapsed TWAR library with minimap2 (version 2.17) (Li, 2018), and used SAMtools (version 1.16) (Li et al., 2009) to calculate the percentage coverage of our TWAR elements (**Supplemental Table S18** and **Supplemental Table S19**). Of the 2,635 captured in the MC3T3-E1 cells (with at least 1 tile associated with 10 or more barcodes in the original library sequencing step) 2,569 TWARs had greater than 80% sequence coverage in the final dataset, 2,502 greater than 90% coverage and 2,135 had 100% coverage. Of the 2,629 captured TWARs in the O91-cells, 2,563 had greater than 80% coverage in the final dataset, 2,499 greater than 90% coverage and 2,130 100% coverage.

Activity calculation and testing We calculated activity across all barcode observations as the

ratio of normalized log2 cDNA CPM to normalized log2 pDNA CPM. We tested for statistically significant activity individually in each replicate. To do so, we applied a one-sided *t*-test per oligo (as in Uebbing et al. (2021)), comparing the distribution of activity across barcodes to the overall mean activity observed in a replicate. Per replicate, we applied a Benjamini-Hochberg multiple testing correction. For each cell line, we defined active oligos as those that passed a false discovery rate (FDR) of 5% in at least one of three replicates and 10% in at least two (Supplemental Table S20 and Supplemental Table S21). To summarize activity values for a library sequence we took the median activity across barcodes and averaged activity across the three replicates.

Activity analysis To compare activity distributions between TWARs, cell-line specific positive controls and negative controls, we first obtained the median log2 activity of the most active tile in each TWAR and control. We defined “active” TWARs and controls as having at least 1 active tile. In defining regions with marsupial- or placental-active TWARs, we required sequences to display good coverage (80% or higher) in at least 2 out of 3 species in the clade that was not active. GC content for each TWAR sequence was calculated with the seqinr package (version 4.2-36) in R. For each TWAR we determined the proportion of sites that differed between each pair of sequences (the pairwise sequence distance) with the ape package (version 5.8-1) in R (function `dist.dna()`, `model = "raw"`). Distance to nearest TSS in the mouse had been previously determined with GREAT in (Feigin et al., 2019) (Supplemental Table S22). We classified TWARs within 1kb of the a TSS as promoters, and the rest as enhancers.

Differential activity testing We used mpralm (Myint et al., 2019), which is based on the voom framework (Law et al., 2014) to test TWARs for regulatory activity. Aggregating DNA and RNA barcode counts across tiles for each TWAR, we then used the `mpralm()` command to model this count data and compute log2 activity ratios per TWAR. For the cranial neural crest cells replicates, because of the associated technical challenges, there were individual barcode sequences that displayed exceptionally high counts. Thus prior to aggregation of our raw counts, we performed an additional filtering step for these replicates, removing any outlier barcodes for an oligo if 1) it is the only barcode that has > 10,000 counts for that oligo and if 2) the oligo is not determined to be active by our initial activity *t*-test.

We extracted contrasts for each pair of marsupial and placental taxa. Taking only the subset of TWARs that were active in at least one of the three taxa and present in two of the three taxa — in the preosteoblasts, 110 and 134 TWARs for marsupials and placentals respectively; in the CNCCs, 50 and 103 TWARs for marsupials and placentals respectively — we re-computed the adjusted p -values using Benjamini-Hochberg correction. Differentially active TWARs were required to have an adjusted p -value < 0.05 and a pairwise log2 fold-change > 0.1 in either the focal-outgroup or the focal-ancestor comparison. We also required that differentially TWARs have $>80\%$ coverage in the focal species (thylacine or wolf) and $>90\%$ coverage in at least one of the other two taxa.

We also performed a second approach to differential activity testing, applying mpralm to the most active tile from each TWAR and applying the same filtering criteria as above. **Supplemental Table S23-Supplemental Table S26** contain results of differential activity testing from this approach, and **Supplemental Table S27** summarises the intersection of results between the two methods. As both approaches have merits — aggregating tiles will capture activity across the entire sequence context, however the activity of regulatory elements is known to be driven by a few clusters of short transcription factor binding motifs — we decided to report overall numbers from mpralm on aggregated counts across tiles but only describe examples found to be differentially active with both approaches.

Nearest gene analysis Feigin et al. (2018) had previously identified genes nearest to each TWAR in the mouse genome (GRCm38) with GREAT (McLean et al., 2010). Here we designated TWARs within 1000 bp of a TSS promoters and the remainder as enhancers. Per cell line, we applied a t -test comparing the absolute distance to a TSS for inactive and active TWARs (MC3T3: p -value = 0.451 ; O91: p -value = 0.020) A caveat of this test however was that we only had information for the mouse genome; as such each orthologous TWAR was assigned the same absolute distance to a TSS. We thus also applied a linear modelling approach, with absolute distance to a TSS as our explanatory variable and proportion of active orthologs for a TWAR as our response variable (MC3T3: p -value = 0.629 ; O91: p -value = 0.128).

Transcription Factor Motif Scan and Analysis We used FIMO (Grant et al., 2011) to scan our oligo library for non-redundant vertebrate transcription factor binding motifs from the

673 HOCOMOCO Core Mouse Collection (v11) (Kulakovskiy et al., 2018) (**Supplemental File S4**). A
674 q -value threshold of 0.10 was applied. When a single oligo was associated with multiple binding
675 motifs for the same TF, we retained the motif with highest binding score. We then counted the
676 number of unique transcription factor motifs discovered in each oligo, as well as the mean binding
677 score across these motifs. Multiplying the TFBM counts and the mean binding score, we obtained
678 a summary FIMO score. To compare FIMO scores for a TWAR across taxa (all six or within a
679 clade), we divided its summary score by the maximum possible score for an ortholog of that
680 TWAR.

681 *Marsupial craniofacial ChIP-seq* We used BLAST (Camacho et al., 2009) to identify
682 orthologous regions in the dunnart genome (Cook et al., 2025) using the Tasmanian devil
683 sequence for each TWAR. Uniquely mapped regions from Tasmanian devil to dunnart were then
684 blasted back to the dunnart genome to ensure reciprocal mapping. The dunnart coordinates for
685 each mapped TWAR were extracted and intersected with existing dunnart craniofacial ChIP-seq
686 peaks (Cook et al., 2025) using BEDTools intersect (Quinlan et al., 2010)

687

Data access

688

Raw sequencing reads have been deposited to the European Nucleotide Archive (ENA; <https://www.ebi.ac.uk/ena>) under accession number PRJEB90363. All analysis scripts are available

689

690

at <https://gitlab.unimelb.edu.au/igr-lab/tycnmpira> and as Supplemental Code.

691 **Competing interests statement**

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709 investigation, analysis and visualisation. LEC performed analysis of the dunnart ChIP-seq data.
710 NS, IGR and LEC prepared the original draft of the manuscript and DVM and AJP provided
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