

1 KRAB Zinc Finger Proteins coordinate across evolutionary time scales to battle
2 retroelements

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25 **KRAB Zinc Finger Proteins (KZNFs) are the largest and fastest evolving family of**
26 **human transcription factors^{1,2}. The evolution of this protein family is closely**
27 **linked to the tempo of retrotransposable element (RTE) invasions, with specific**
28 **KZNF family members demonstrated to transcriptionally repress specific families**
29 **of RTEs^{3,4}. The competing selective pressures between RTEs and the KZNFs**
30 **results in evolutionary arms races whereby KZNFs evolve to recognize RTEs,**
31 **while RTEs evolve to escape KZNF recognition⁵. Evolutionary analyses of the**
32 **primate-specific RTE family L1PA and two of its KZNF binders, ZNF93 and**
33 **ZNF649, reveal specific nucleotide and amino changes consistent with an arms**
34 **race scenario. Our results suggest a model whereby ZNF649 and ZNF93 worked**
35 **together to target independent motifs within the L1PA RTE lineage. L1PA**
36 **elements eventually escaped the concerted action of this KZNF “team” over ~30**
37 **million years through two distinct mechanisms: a slow accumulation of point**
38 **mutations in the ZNF649 binding site and a rapid, massive deletion of the entire**
39 **ZNF93 binding site.**

40

41 KZNFs repress RTEs by recruiting the co-factor Kap1 (Trim28) which then recruits a
42 variety of repressive factors that establish heterochromatin^{1,6}. We reasoned that KZNFs
43 expressed highly in the pluripotent stem cell (PSC) state were likely to be involved in arms
44 race scenarios as the pluripotent state is a high stakes evolutionary battleground since
45 RTEs that successfully retrotranspose in this state are inherited by all daughter cells
46 including the germ line⁷. We further narrowed our investigation to the L1PA RTE lineage,
47 which contains the only active, autonomous RTE family (L1HS elements) in humans, and

48 is therefore likely to experience high evolutionary pressure for repression⁸. Recent ChIP-
49 SEQ studies have revealed many KZNFs that bind L1PA families (Extended Data Fig 1),
50 although these studies were performed in an artificial overexpression context in 293T
51 cells^{4,9}. In order to analyze which of these binders might be important for repression in
52 the PSC context, we mapped KZNF ChIP-SEQ data to consensus repeat elements via
53 the UCSC Repeat Browser (*MH, in preparation*), and then correlated Repeat Browser
54 “meta-peaks” with Kap1 ChIP-SEQ “meta-peaks” from PSCs (Fig 1A). This analysis
55 identified two KZNFs, ZNF649 and ZNF93 which are highly expressed in the PSC state
56 (Extended Data Fig 1), as responsible for the majority of Kap1 recruit on L1PA elements
57 in the human PSC context. We previously identified ZNF93 as an important repressor of
58 L1PA elements in hPSCs, and traced its binding site to a 129-bp region that was deleted
59 in the youngest (L1PA2, L1HS) L1PA families⁵. Interestingly, ZNF649 recognition of L1PA
60 elements is strongest on L1PA6-L1PA4 elements, appears to weaken in younger
61 elements (L1PA3-L1PA2), and is unable to bind L1HS elements (Fig 1A). Additionally,
62 the correlation between Kap1 binding and ZNF649 and ZNF93 binding also varies across
63 each L1PA family, suggesting that ZNF93 is most effective on L1PA4 and L1PA3, while
64 ZNF649 was most active on older L1PA5 elements but slowly lost its efficacy (Fig 1A).
65 However, the DNA changes that allowed L1PA escape from ZNF649 require more
66 complex analysis than the ZNF93 case.

67

68 In order to determine how L1HS escaped ZNF649 binding, we compared the sequences¹⁰
69 (centered around our Repeat Browser meta-summits) of L1PA elements with ChIP-SEQ

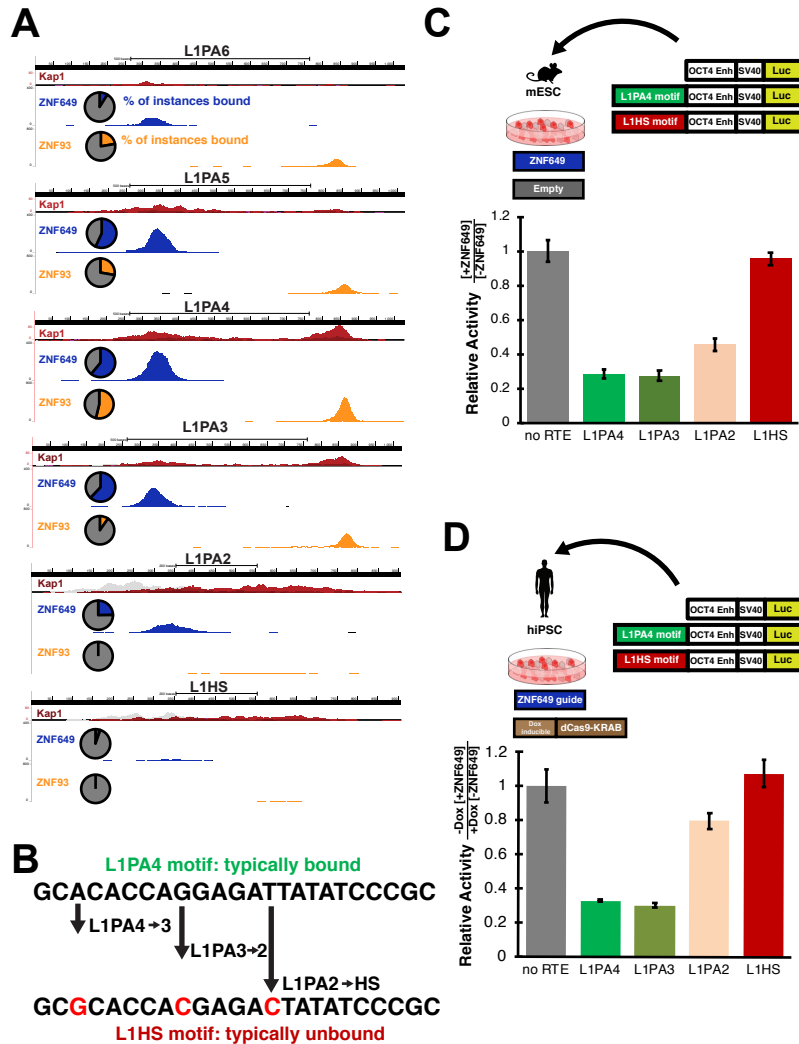


Figure 1: ZNF649 recognizes a sequence-specific motif in L1PA elements. A) Repeat Browser Analysis of ZNF649 (blue), ZNF93 (gold), Kap1 (red/grey; independent replicates) on L1PA elements. Pie charts show the percentages of each L1PA family that are independently bound by ZNF649 (blue slices) and ZNF93 (yellow slices). B) Discriminative analysis of the sequence under the ZNF649 binding site reveals a recognition motif that acquires 3 sequential point mutations in younger L1PA families. C) Reporter assay in mouse embryonic stem cells (mESC) in which a reporter containing the ZNF649 binding site in each of the L1PA sequences are tested in the presence or absence of ectopic ZNF649. Bars represent the relative activity of the reporter in the presence of ZNF649 normalized compared to an empty vector. D) Analogous experiment in CRISPRi hiPSCs expressing guides targeting ZNF649 and inducible dCas9-KRAB. Induction with dox depletes endogenous ZNF649. Data represents reporter activity in the knockdown condition normalized by the uninduced endogenous condition (all activities normalized to no RTE control). All error bars represent standard deviations of four biological replicates.

70 submits versus that of L1PA elements without submits. This analysis revealed two
 71 adjacent motifs that together form one long putative ZNF649 recognition sequence (Fig
 72 1B). To test if this sequence was sufficient for ZNF649 binding, we transfected a reporter

73 plasmid with the ZNF649 binding site cloned upstream of a luciferase gene in the
74 presence or absence of human ZNF649 in mouse embryonic stem cells (mESCs) which
75 are free of other primate-specific repressive elements⁵. ZNF649 specifically repressed
76 this sequence, validating it as a bona fide recognition motif (Fig 1B). We then examined
77 the evolution of this motif in the L1PA families which appear to escape ZNF649
78 recognition. The ZNF649 recognition sequence accumulated three mutations that
79 flourished in younger families over the last 18 million years (Fig 1B). We tested the effect
80 of each of these mutations (representing L1PA3, L1PA2 and L1HS-like states) and
81 observed complete loss of repression in the L1HS-like state and an intermediate
82 phenotype in the L1PA2-like state. To confirm that our reporter system accurately
83 recapitulated ZNF649 action within a human PSC, we performed CRISPRi knockdowns
84 of ZNF649 in human iPSCs¹¹ and repeated our reporter assay. These experiments
85 matched our mESC results, with the reporter repressed by endogenous ZNF649 but
86 expressed in the knockdown context (Fig 1C).

87

88 Interestingly, the ZNF649 recognition motif contains a TATA sequence¹², an important
89 recognition motif for the transcription factor TBP. Furthermore, this TATA sequence
90 arose in a subset of the L1PA6 lineage through a dramatic rearrangement of the L1PA 5'
91 UTR that also deleted an old TATA sequence present in ancient elements (Fig 2A). While
92 only a minority of L1PA6 elements contained this new arrangement of the 5'UTR, almost
93 all instances of younger L1PA elements are configured in this manner suggesting a
94 comparative fitness advantage (Fig 2A). Furthermore, mutation of the TATA sequence
95 results in a complete loss of ZNF649-mediated repression (Fig 2B), demonstrating a

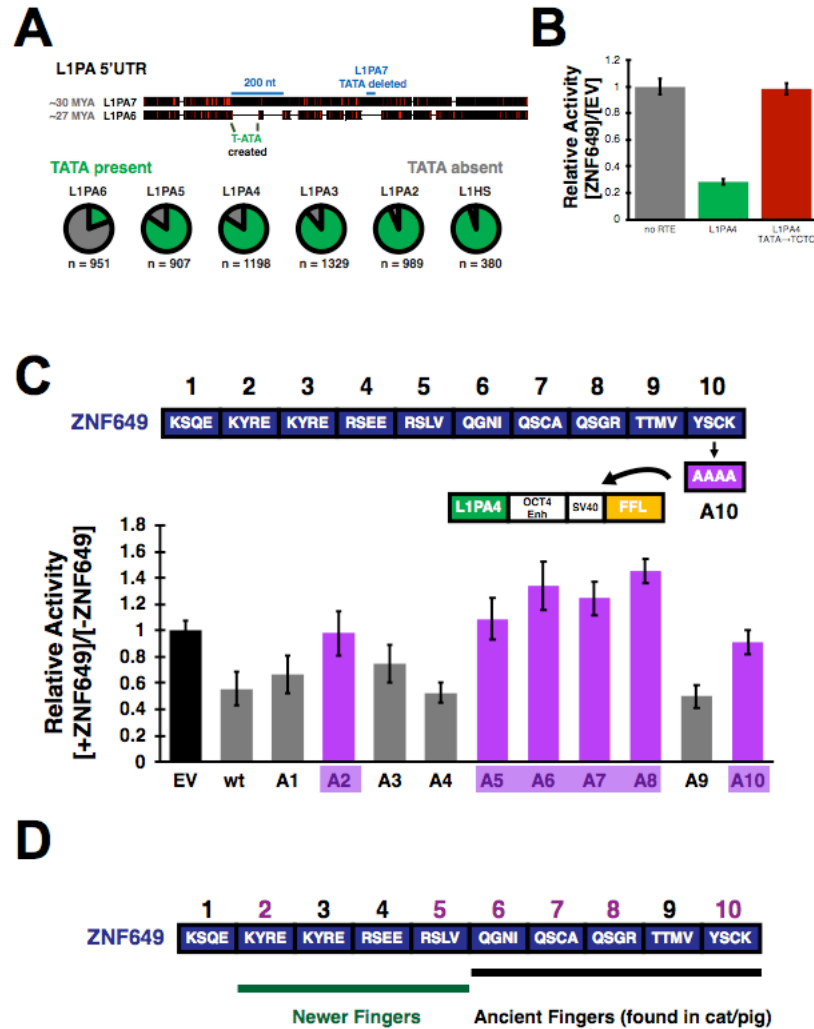


Figure 2: Evolution of L1PA families and ZNF649. A) Consensus L1PA7 sequence aligned to consensus L1PA6 sequence. Black coloring indicates conservation, red indicates variation. (Below) Pie charts representing the number of instances of each L1PA family that have a perfectly conserved “TATA” sequence (green slice) in the new 5’ configuration. B) mESC reporter assay measuring ZNF649’s ability to repress when the highly conserved TATA sequence is mutated. C) Testing of finger mutations in the mESC reporter assay. Shown is the relative activity of each single finger mutant (A1-A10) compared to wild type (wt). Purple coloring indicates mutants that show no repression. D) Cartoon representation of ancient and modern fingers of ZNF649 overlaid with reporter data from C).

96 potential mutational route for L1PA escape from ZNF649. However, mutations in this site
 97 are rare indicating that L1PA elements have a competing selective pressure (presumably
 98 to maintain transcription factor binding to initiate transcription) to avoid this route.
 99
 100 In order to understand how ZNF649 evolved to repress these elements, we synthesized

101 10 ZNF649 mutants (one for each ZNF domain) with each mutant designed to ablate the
102 binding activity of a single finger. Canonically each ZNF within a KNZF recognizes three
103 nucleotides of double stranded DNA via four specific DNA contact residues, typically
104 (though not always) amino acids -1,2,3 and 6 relative to the ZNF helix^{13,14} (Extended Data
105 Fig 2); therefore, we mutated all four canonical DNA contact residues to alanine in each
106 construct and tested each mutant's ability to repress the L1PA4 luciferase reporter.
107 Mutations to fingers 2,5,6,7 and 8 led to a loss of repression, indicating their importance
108 in L1PA recognition. (Fig 2C). We then traced the evolutionary history of ZNF649, which
109 is found across Eutheria, making it over 100 million years old. Determining true orthologs
110 of KZNFs is challenging given their rapid evolutionary rates, high sequence similarity,
111 propensity for duplication, and potential for gene conversion. By focusing on individual
112 ZNF domains we reconstructed ancestral states for ZNF649 in the primate lineage
113 (*Armstrong et al., in preparation*). L1PA6 elements with the modern 5'UTR configuration
114 are found only in Old World and not New World monkeys, meaning that ZNF649 must
115 have evolved to battle these RTEs within the last 30 million years. Fingers 2 and 5,
116 identified by our mutational analyses as being critical for L1PA repression, occur in a part
117 of the gene that appears to have evolved more recently (*Armstrong et al, in preparation*).
118 Fingers 6, 7, and 8 which are also critical for binding, have more ancient roots, clearly
119 matching fingers in distantly related species such as pigs and cats (Fig 2D, Extended
120 Data 3). These fingers are bioinformatically predicted to bind the TATA sequence¹⁵ which
121 may suggest an ancient gene regulatory role for ZNF649 at a TATA sequence; ZNF649
122 may have then been repurposed to target the TATA sequence of L1PA6 and younger
123 elements upon RTE invasion, which resulted in the acquisition of fingers 2 and 5.

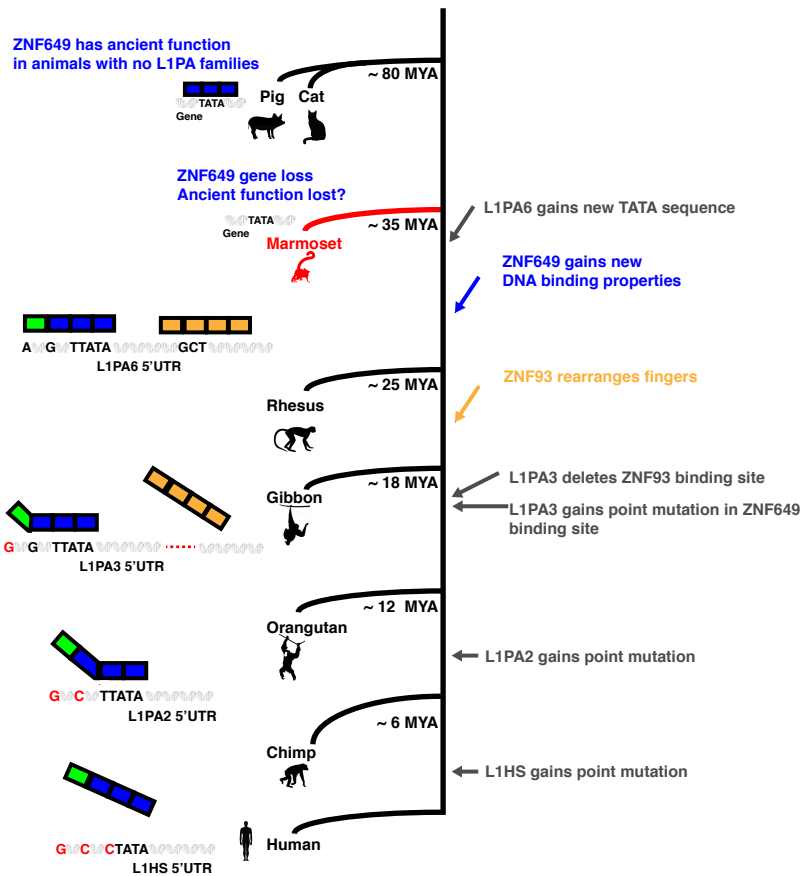


Figure 3: Model for L1PA arms races with ZNF649 and ZNF93. (Top) ZNF649 (blue) is found in cats and pigs where it presumably regulates cellular genes as these animals have no L1PA6 elements. Marmosets (red) lose ZNF649, and presumably any ancient ZNF649 function. After the marmoset divergence, L1PA6 elements invade the primate lineage leading to ZNF649 and ZNF93 evolution that results in both KZNFs binding and repressing the RTE together. L1PA3 elements subsequently acquire the deletion of the entire ZNF93 binding site and a point mutation that loosens the ZNF649 binding site and is eventually followed by two successive mutations in L1PA2 and L1HS elements that allow complete escape from ZNF649 binding.

124

125 Together these data suggest a model whereby L1PA elements rearranged their 5'UTRs

126 ~30 million years ago - perhaps to escape repression from an unknown ancient KZNF.

127 ZNF649 quickly adapted to bind L1PA elements at the new TATA sequence by gaining

128 new fingers, followed by rearrangements in ZNF93 that allowed it to repress L1PA4

129 elements. When L1PA3 elements responded by deleting the entire ZNF93 binding site,
130 ZNF649 was left to battle L1PA elements on its own – a battle that it then lost as mutations
131 accumulated to generate the active L1HS state (Fig 3).

132

133 These results illustrate novel mechanisms of evolutionary innovation, whereby a host
134 genome rapidly evolves unique “teams” of KZNFs with distinct DNA binding abilities in
135 order to repress RTEs. Some team members such as ZNF649 target essential portions
136 of the RTE, with RTE escape requiring gradual point mutational paths on which a ZNF’s
137 grip slowly “loosens” finger by finger; other team members, such as ZNF93, play
138 supporting roles and target non-essential portions of the RTE that are rapidly escaped via
139 a single event in which the ZNF completely “drops” the TE. Furthermore, ZNF649 may
140 have been itself repurposed to battle L1PA, as previous literature demonstrates that it
141 binds and regulates genes in highly conserved and cellular growth factor pathways¹⁶,
142 possibly explaining its role in species that never faced L1PA6 elements. This repurposing
143 demonstrates evolution’s exquisite ability to reuse existing cellular tools, and suggests
144 that these genetic conflicts can compel ancient regulatory networks to evolve at the tempo
145 of an arms race, which can then drive the creation of species-specific regulatory
146 networks^{17–19}.

147

148 **Methods**

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150 **UCSC Repeat Browser Analysis**

151 We mapped Kap1 and KZNF ChIP-SEQ data to the UCSC Repeat Browser as

152 previously described. To calculate the percentages of each element bound, we filtered
153 all L1PA7-2 and L1HS elements annotated in Repeat Masker to only full length
154 elements (size limits of 5500-7500 nt) and used bedtools to intersect these instances
155 with ChIP-SEQ datasets.

156

157 **Determination of Motifs**

158 To perform discriminate analysis of motifs, we extracted 140 nt centered around the
159 Repeat Browser meta-summit for every L1PA6, L1PA5, L1PA4, L1PA3, L1PA2 and
160 L1HS element. We then used DREME¹⁰ to perform a discriminative analysis on these
161 sequences using as our positive dataset all full-length genomic instances with ChIP-
162 SEQ summits on them (bound), and the remaining instances as our negative set
163 (unbound). We performed these comparisons for each L1PA family individually as well
164 as a grouping of all L1PA6-HS elements together to confirm our predicted motif.

165

166 **mESC Reporter Assay**

167 In order to generate luciferase constructs containing ZNF649 binding sites, we first
168 synthesized a 201 bp region centered around our Repeat Browser metapeak on the
169 L1PA4 consensus and cloned it into a Kpn I digested pgl-cp FFL vector (previously
170 described⁵, map on Addgene) to create pglcp-SV40 ZNF649 L1PA4. We then used
171 primers containing the appropriate point mutations to create pglcp-SV40 ZNF649
172 L1PA4-1mut (“L1PA3”), pglcp-SV40 ZNF649 L1PA4-2mut (“L1PA2”), and pglcp-SV40
173 ZNF649 L1PA4-3mut (“L1HS”). All maps will be provided on Addgene upon publication.
174 To test the activity of each construct we plated E14 mESC at 200,000 cells/mL in a 24-

175 well plate coated with 1% Porcine Gelatin. Cells were transfected 24 hours later with
176 100 ng of pCAG ZNF649 or pCAG Empty Vector with 20 ng of L1PA firefly luciferase
177 reporter and 2 ng renilla luciferase. 24 hours later, cells were washed 2x in PBS and
178 lysed for 15 min in 100 ul passive lysis buffer and 90 ul was analyzed per
179 manufacturer's instructions (Promega) on a Perkin Elmer 1420 Luminescence Counter.

180

181 **hiPSC Reporter Assay**

182 To generate hiPSC knockdown lines for ZNF649, we designed guides downstream of
183 the ZNF649 TSS using the CRISPOR track on the UCSC Genome Browser. Two
184 separate guides with high efficacy and specificity were cloned into p783ZG, a modified
185 version of MP783 (kind gift, S. Carpenter) in which the Puromycin-t2A-mCherry
186 resistance gene was replaced by a Zeocin-t2a-GFP. Gen1C iPSC lines were then
187 transfected with 1 ug of guide plasmid and selected at 50 ug/mL Zeocin for 2 weeks.
188 The resulting stable populations were used with transient reporter. Briefly, two separate
189 plates of the stable cell line pools were plated at 50,000 cells/cm² and grown in 50
190 ug/mL zeocin (with one plate receiving 1 ug/mL dox). After two days dox-induced and
191 uninduced cells were plated at 35,000 cells/well in separate Matrigel coated 24-well
192 plates and transfected with 200 ng of the appropriate RTE reporter construct (as
193 described above), 2 ng Nanoluc (Promega) and 2 ul Lipofectamine 2000 (Invitrogen).
194 24 hours later, cells were washed 2x in PBS and lysed for 15 min in 100 ul passive lysis
195 buffer and analyzed per manufacturer's instructions (Promega) on a Perkin Elmer 1420
196 Luminescence Counter.

197

198 **Generation and Testing of Alanine ZNF Mutants**

199 We synthesized (Twist Biosciences) 10 codon optimized inserts (to break repetitive
200 structure) containing an HA-tagged ZNF649 coding sequence, as well as the
201 corresponding mutations to simultaneously ablate all DNA contact residues in a single
202 finger in ZNF649. In addition to 10 independent single-finger mutants (1A, 2A, etc.), we
203 synthesized a wild-type control. All constructs were tested on the “L1PA4” construct
204 (ZNF649 binding site intact) in our mESC assay as described above.

205

206 **Reconstruction of ZNF649 Evolutionary History**

207 The evolutionary history of ZNF649 was determined by syntenic and domain-based
208 analyses as described in *Armstrong et al. in prep.* Briefly, we defined a high-quality
209 syntenic locus encompassing ZNF649

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211 **References**

- 212 1. Yang, P., Wang, Y. & Macfarlan, T. S. The Role of KRAB-ZFPs in Transposable
213 Element Repression and Mammalian Evolution. *Trends Genet.* **33**, 871–881
214 (2017).
- 215 2. Huntley, S. *et al.* A comprehensive catalog of human KRAB-associated zinc finger
216 genes: insights into the evolutionary history of a large family of transcriptional
217 repressors. *Genome Res.* **16**, 669–77 (2006).
- 218 3. Thomas, J. H. & Schneider, S. Coevolution of retroelements and tandem zinc
219 finger genes. *Genome Res.* **21**, 1800–12 (2011).
- 220 4. Imbeault, M., Helleboid, P.-Y. & Trono, D. KRAB zinc-finger proteins contribute to

- 221 the evolution of gene regulatory networks. *Nature* **543**, 550–554 (2017).
- 222 5. Jacobs, F. M. J. *et al.* An evolutionary arms race between KRAB zinc-finger
223 genes ZNF91/93 and SVA/L1 retrotransposons. *Nature* **516**, 242–5 (2014).
- 224 6. Wolf, D. & Goff, S. P. Embryonic stem cells use ZFP809 to silence retroviral
225 DNAs. *Nature* (2009). doi:10.1038/nature07844
- 226 7. Gerdes, P., Richardson, S. R., Mager, D. L. & Faulkner, G. J. Transposable
227 elements in the mammalian embryo: pioneers surviving through stealth and
228 service. *Genome Biol.* **17**, 100 (2016).
- 229 8. Khan, H., Smit, A. & Boissinot, S. Molecular evolution and tempo of amplification
230 of human LINE-1 retrotransposons since the origin of primates. *Genome Res.* **16**,
231 78–87 (2006).
- 232 9. Schmitges, F. W. *et al.* Multiparameter functional diversity of human C2H2 zinc
233 finger proteins. *Genome Res.* **26**, 1742–1752 (2016).
- 234 10. Bailey, T. L. DREME: motif discovery in transcription factor ChIP-seq data.
235 *Bioinformatics* **27**, 1653–9 (2011).
- 236 11. Mandegar, M. A. *et al.* CRISPR Interference Efficiently Induces Specific and
237 Reversible Gene Silencing in Human iPSCs. *Cell Stem Cell* **18**, 541–553 (2016).
- 238 12. Comai, L., Tanese, N. & Tjian, R. The TATA-binding protein and associated
239 factors are integral components of the RNA polymerase I transcription factor, SL1.
240 *Cell* **68**, 965–76 (1992).
- 241 13. Garton, M. *et al.* A structural approach reveals how neighbouring C2H2 zinc
242 fingers influence DNA binding specificity. *Nucleic Acids Res.* **43**, 9147–57 (2015).
- 243 14. Patel, A. *et al.* DNA Conformation Induces Adaptable Binding by Tandem Zinc

- 244 Finger Proteins. *Cell* **173**, 221–233.e12 (2018).
- 245 15. Najafabadi, H. S., Albu, M. & Hughes, T. R. Identification of C2H2-ZF binding
246 preferences from ChIP-seq data using RCADE. *Bioinformatics* **31**, 2879–81
247 (2015).
- 248 16. Yang, H. *et al.* ZNF649, a novel Kruppel type zinc-finger protein, functions as a
249 transcriptional suppressor. *Biochem. Biophys. Res. Commun.* **333**, 206–215
250 (2005).
- 251 17. Yang, P. *et al.* A placental growth factor is silenced in mouse embryos by the zinc
252 finger protein ZFP568. *Science (80-.)*. **356**, 757–759 (2017).
- 253 18. Ecco, G. *et al.* Transposable Elements and Their KRAB-ZFP Controllers Regulate
254 Gene Expression in Adult Tissues. *Dev. Cell* **36**, 611–623 (2016).
- 255 19. Trono, D. Transposable Elements, Polydactyl Proteins, and the Genesis of
256 Human-Specific Transcription Networks. *Cold Spring Harb. Symp. Quant. Biol.*
257 (2016). doi:10.1101/sqb.2015.80.027573
- 258 20. Persikov, A. V. & Singh, M. De novo prediction of DNA-binding specificities for
259 Cys2His2 zinc finger proteins. *Nucleic Acids Res.* **42**, 97–108 (2014).

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267

268 **Author Contributions**

269 JDF, MH, and TT performed Repeat Browser analysis with assistance from SK. JDF,

270 JA, BP, and DH analyzed the evolutionary history of ZNF649. JDF, KT, JG, NF, JP and

271 PA performed experiments and analyzed data. JDF, SRS, and DH conceived of the

272 project and designed the experiments and analysis.

273

274 **Author Information**

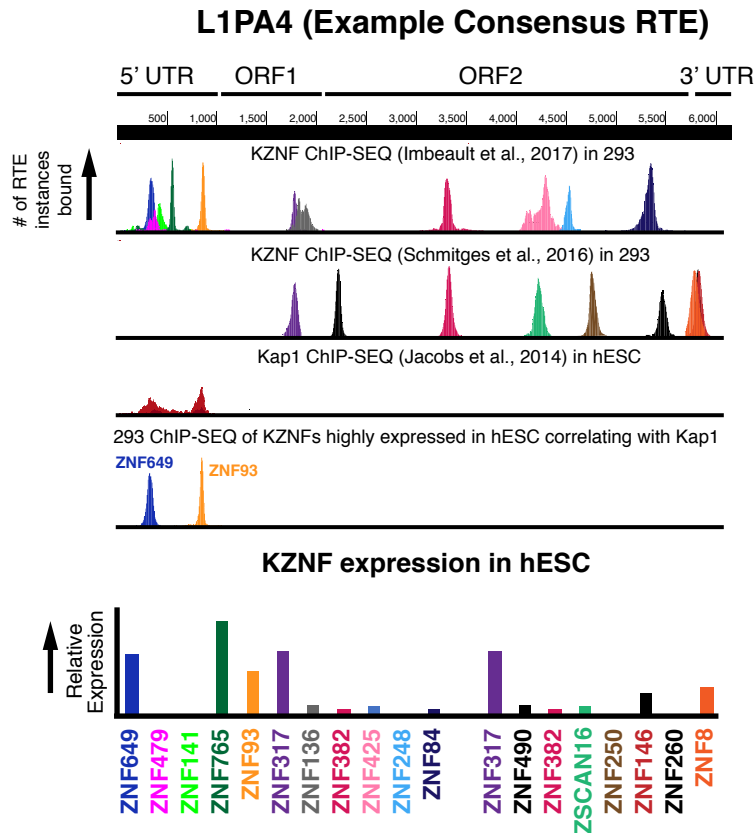
275 No competing interests. Correspondence and request for material should be addressed

276 to SRS and DH.

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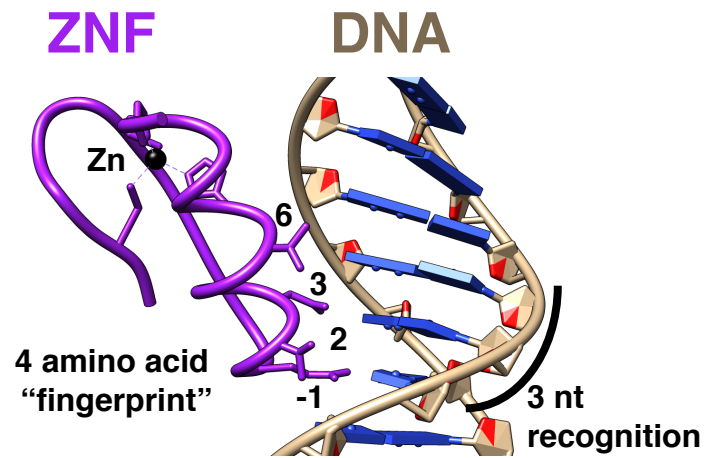
Supplementary Information

Extended Data Fig 1

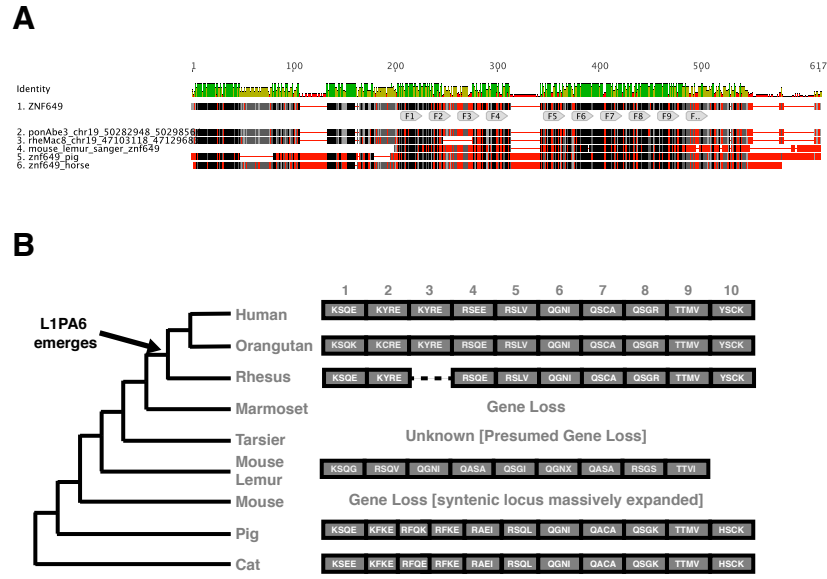


Extended Data Figure 1: Mapping of all KZNF ChIP-SEQ to L1PA elements on the Repeat Browser (L1PA4 consensus shown as an example) shows many candidate KZNFs that might bind and repress these RTEs. However only ZNF649 and ZNF93 correlate with Kap1 binding and have high expression in pluripotent stem cells.

Extended Data Fig 2



Extended Data Figure 2: Canonical model for ZNF recognition on DNA. Shown here is the crystal structure (1G2F) of Zif268 bound to DNA. Numbered residues are in reference to the start of the helix (-1,2,3,6) and traditional make base-specific contacts to the DNA (four amino acid "fingerprint").



Extended Data Figure 3: A) Alignment of ZNF orthologs as identified by syntenic analysis and resequencing of genomic DNA (mouse lemur). Fingers 1-10 (as defined in human ZNF649) are labeled on the alignment. B) Cartoon representation of ZNF orthologs reduced to the DNA binding residues of each ZNF (four amino acid “fingerprint”).