COGNITIVE ENDOPHENOTYPES OF MODERN AND EXTINCT HOMININS ASSOCIATED WITH *NTNG* GENE PARALOGS

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ABSTRACT

A pair of gene paralogs, *NTNG1* and *NTNG2*, contributes to the Intellectual Quotient (IQ) test scores in a complementary manner. Single nucleotide polymorphisms (SNPs) of *NTNG1* are associated with attenuated verbal comprehension (VC) or processing speed (PS) while *NTNG2* SNPs affect working memory (WM), and perceptual organization (PO) forming cognitive endophenotypes in healthy and schizophrenia (SCZ)-affected human subjects. Regions of interest (ROIs), defined as 21 nucleotide (nu) long loci embedding the IQ-affecting mutation alleles, underwent dramatic evolutionary changes from mice through primates to hominin gene orthologs, and resulted in accelerated evolution of VC and WM/PO affecting ROIs. Mutation alleles associated with the higher VC and WM IQ scores are found in genomes of extinct hominins of Neolithic times, however, lower WM scores associated allele is detectable in Mesolithic hunters genomes. Protein sequence of *NTNG1* is 100% conserved among the archaic and modern extinct hominins while *NTNG2* underwent a recent selection sweep encoding a primate-specific S371A/V (~50,000 yrs BC), and a modern human (5,300 yrs BC) T346A substitutions. Hereby, we show that a 500 mln yrs old genomic duplication of a synapse primordial gene of an urochordate provided a substrate for further synapse elaborations and its ultimate capacitive expansion of what evolved into a vertebrate cognitive superior complexity – intelligence.

INTRODUCTION

In 1970 S.Ohno ingeniously proposed that new gene function can result from a gene duplication and following it gene paralogs SF (**Ohno, 1970**). Two gene paralogs, *NTNG1* and *NTNG2*, expressed predominantly in the brain (**Nakashiba et al., 2002**), and encoding Netrin-G1 and Netrin-G2 proteins, respectively, are localised pre-synaptically and segregate in a non-overlapping manner into distinct neuronal circuits (**Nishimura-Akiyoshi et al., 2007; Matsukawa et al., 2014**). They are related to the netrin family of axonal guidance cues (**Sun et al., 2011**) but differ in that they attach to the axonal membrane via a glycosylphosphatidylinositol (GPI) link (**Yu et al., 2013**; **Sevcsik et al., 2015**), a known lipid raft-associated membrane signaling cascade organiser (**Klotzsch and Schutz, 2013; Yu et al., 2013**). The first evolutionary precursor of *NTNG* as a single gene copy can be located in the genome of a primitive vertebrate tunicate/urochordate *Ciona intestinalis* (sea squirt, ENSCING00000024925), reported to be the first organism with the neural crest primordials (**Abitua et al., 2012**), multipotent brain progenitor cells (**Stolfi et al., 2015**), and neurogenic placodes, facilitating the transition from pelagic invertebrate life style to a predatory vertebrate (**Abitua et al., 2015**). The dramatic expansion of human cerebral cortex over the course of evolution (**Wise, 2008**; **Preuss, 2012**; **Geschwind and Rakic, 2013**; **Belmonte et al., 2015**) had provided new niches for accommodating either *de novo* or advancing pre-existed cognitive features and culminating in the positively selected human cognitive functions (**Joshi et al., 2015**).

IQ tests are a surrogate measure of general human cognitive ability characterising intelligence. They are often administered as WAIS-III/IV (**Wechsler, 1958**) and represent a cumulative score of 4 cognitive indices: VC, WM, PO and PS frequently referred as "cognitive domains" (**Deary et al., 2006**). IQ has been validated by factor analyses (**Glascher et al., 2009**), and a common factor (correlate) influencing each of them frequently

70 referred as *g*, or "general intelligence", proposed by Spearman in 1904 (**Spearman, 1904**),
71 and recently challenged as the only existing correlate (**Hampshire et al., 2012**). IQ is
72 affected by several mental disorders including schizophrenia (SCZ) characterized by severe
73 cognitive deficits in WM and behavioral flexibility and resulting in low performance in
74 several cognitive tests (**Forbes et al., 2009; Leeson et al., 2009**; **Barnett et al., 2010**). Since
75 both *NTNG* paralogs have been reported associated with SCZ (**Fukasawa et al., 2004**; **Aoki-**
76 **Suzuki et al., 2005**; **JSSLG et al., 2005**; **Eastwood and Harrison, 2008**; **Ohtsuki et al.,**
77 **2008**; **Zakharyan et al., 2011**; **Zhu et al., 2011**; **Ayalew et al., 2012**; **Wilcox and Quadri,**
78 **2014**) we investigated whether these gene paralogs contribute to human intelligence by
79 assessing the IQ of human carriers for the *NTNG1* and *NTNG2* SNP alleles against non-
80 carriers with and without SCZ.
81

81 RESULTS

82 Several *NTNG1* and *NTNG2* SNPs have been previously reported to be associated with SCZ
83 (e.g. **Aoki-Suzuki et al., 2005**; **Ohtsuki et al., 2008**}. We have found that out of 11 SNPs
84 tested, five affect the IQ scores and composite domains in human subjects (**Figure 1**,
85 **Supplementary Table 1** (**ST1**)). SCZ patients carrying rs2218404 T allele (**Figure 1A-1**) of
86 *NTNG1* (T/G and T/T genotypes, N = 25 patients) compared with G/G genotype (N = 36
87 patients) demonstrated attenuated full-scale IQ (FIQ, ANCOVA $p = 0.0057$ (F = 7.80)), and
88 VIQ ($p = 0.0033$ (F = 8.87)). VC domain score was the main contributor to the VIQ decline
89 ($p = 0.0050$ (F = 8.08), **Figure 1B-1**), with low scores across all parameters except
90 comprehension (CH): vocabulary (Vc, $p = 0.020$ (F = 5.49)), similarities (SiM, $p = 0.041$ (F
91 = 4.23)), and information (IF, $p = 0.0067$ (F = 7.50), **Figure 1B-1** lower panel). Thus, a point
92 mutation in *NTNG1*, rs2218404, is associated with low VC affecting the VIQ via the
93 attenuated Vc, SiM and IF subscores. The next *NTNG1* SNP found to affect IQ was rs96501,
94 with attenuated PS in healthy human subjects C allele carriers (N = 45) vs T/T genotypes (N
95 = 98, $p = 0.028$ (F = 4.89), **Figure 1B-1**) with no effect on SCZ patients. The contributing
96 affecting score was symbol search (SS, $p = 0.053$ (F = 3.79), **Figure 1B-1**, lower panel) with
97 digit symbol coding (DSC) being also attenuated but non-significantly ($p = 0.12$ (F = 2.40)).
98 Three other SNPs mapped to *NTNG2* (**Figure 1A-2**) have been also shown to affect IQ.
99 Healthy carriers of the *NTNG2* SNP rs1105684 A allele (N = 49) showed a lower FIQ ($p =$
100 0.018 (F = 5.70)), VIQ ($p = 0.029$ (F = 4.90)), and PIQ ($p = 0.048$ (F = 3.99)) when
101 compared with the T/T genotypes (N = 96, **Figure 1B-2**). To check for a potential dosage-
102 dependent effect of a mutation allele on IQ score, *NTNG2* SNP rs2149171 SCZ and healthy
103 human subject cohorts were each split on 3 genotypes, respectively: C/C (N = 14 and 39),
104 C/T (N = 29 and 73), and T/T (N = 15 and 30). The presence of the C allele as a single copy
105 (C/T genotype) was strongly associated with a prominent attenuation in the IQ scores of SCZ

106 patients and was essentially identical to that produced by the C/C genotype when both are
107 compared to the T allele carriers (FIQ: $p = 0.014$ (F = 4.35); VIQ: $p = 0.029$ (F = 3.60); PIQ:
108 $p = 0.035$ (F = 3.42), **Figure 1B-2**). If in case of *NTNG1* located SNP rs2218404 the lower
109 VIQ score was contributed mainly by the decreased VC domain scores for Vc, SiM, and IF
110 (**Figure 1B-1**), in the case of *NTNG2* located rs2149171 the CH and WM domain scores
111 were responsible for the VIQ decline in C allele carriers ($p = 0.012$ (F = 4.54) for CH and $p =$
112 0.040 (F = 3.27) for WM (N = 12 (C/C); N = 25 (C/T) and N = 14 (T/T)). Similarly to VIQ,
113 where CH of rs2149171 complements the cognitive endophenotype produced by the T-allele
114 of rs2218404, the PIQ attenuated score in the case of rs2149171 was due to lower PO score
115 ($p = 0.050$ (F = 3.04), **Figure 1B-2**) in the C allele carriers. The third *NTNG2* located SNP
116 found to affect the human IQ was rs2274855 (**Figure 1A-2**) with a cognitive endophenotype
117 associated with the A-allele presence in SCZ patients (N = 33) vs G/G genotypes (N = 26)
118 and resembling that of the described above C-allele of rs2149171. Accordingly, the
119 attenuated scores were: FIQ ($p = 0.012$ (F = 6.44)), VIQ ($p = 0.018$ (F = 5.70)), and PIQ ($p =$
120 0.036 (F = 4.46), **Figure 1B-2**). Similarly to rs2149171 the lower VIQ score was due to
121 declined CH ($p = 0.035$ (F = 4.49)) but unaffected VC that is contrary (complementary) to the
122 rs2218404 endophenotype (**Figure 1B-1**). WM was robustly affected by the A-allele
123 presence (N = 29; $p = 0.023$ (F = 5.28)) contributed by the low DS score ($p = 0.026$ (F =
124 5.04)) with LNS and AM being unaffected (**Figure 1B-2**, lower panel). The observed PO
125 score was comprised by the declined matrix reasoning (MR, $p = 0.038$ (F = 4.34)), block
126 design (BD, $p = 0.041$ (F = 4.23)) with picture completion being unchanged (**Figure 1B-2**,
127 lower panel). Thus, all three aforementioned *NTNG2* SNPs affect the VIQ and PIQ in human
128 subjects with the first one contributed by the CH subscore and WM and the latter by the PO
129 score (**Figure 1C**). Contrary to this, the *NTNG1* located SNP (rs2218404) affects the VIQ
130 through the lower Vc, SiM and IF scores and affecting the VC domain scores. Another SNP,

131 rs96501, affects PS domain, though in healthy subjects only. It can be concluded that both
132 genes (as paralogs) contribute to the cognitive scoring produced upon the implemented IQ
133 testing but in a cognitive domain-complementary manner, *NTNG1* is responsible for the VC
134 and PS in human while *NTNG2* for the WM and PO domain scores (**Figure 1C**).

135 The robust link observed between a single SNP and affected cognitive domain IQ
136 score (**Figure 1**) can be explained by some global dramatic perturbations caused by the
137 presence of a mutated allele and/or a functinal importance of its context-dependent
138 positioning on the gene (**Figure 2A** and **3A**). To determine a potential significance of the
139 SNP alleles' epistatic environment we compared the nucleotide (nu) sequence within the
140 immediate vicinity of a SNP allele positioning (50 nu upstream and downstream) in mice,
141 primates and modern human. We compared all 11 SNPs used for the IQ screening and plotted
142 the identity percent as a function of distance from the mutated allele position (**Figure 2B** and
143 **3B**, see **Supplementary Materials** = **SM**). We found that the identity percent distribution
144 over the analysed areas of ±50 nu is not uniform and displays a SNP allele position-centred
145 dramatic evolutionary changes pointing towards a potential functional significance of the
146 immediate vicinity of a SNP as short as ±10 nu and not further, referred from here and
147 beyond as a Region Of Interest (ROI) for each specific SNP allele. We calculated the rates of
148 evolutionary changes for each ROI as a percent identity change over the lapsed mln yrs of
149 evolution (**Figure 2C and 3C**). Among the 6 *NTNG1*-located SNP ROIs three of them
150 display accelerated rates of evolution from marmoset to chimpanzee (rs2218404, rs628117,
151 rs96501) when compared to the mouse-marmoset rates, and rs2218404 (affecting VC in
152 human subjects) additionally demonstrates an accelerated rate of evolution on the
153 chimpanzee to human path (**Figure 2C**). As for the *NTNG2* located ROIs (**Figure 3-B**),
154 rs1105684 is remarkably consistent at displaying high evolutionary rates around 0.8 and,
155 together with rs2274855, both have identical rates at the mouse-marmoset and chimpanzee-

156 human paths, but differ dramatically at the marmoset-chimpanzee point (0.8 vs 0,
157 respectively). rs2274855 is the only *NTNG2*-nested SNP ROI which underwent an AE from
158 chimpanzee to human. Next we compared the DNA sequences of all 11 ROIs across mice,
159 primates and extinct hominins *NTNG* gene paralogs (see **ST2** for the datasets sources used
160 for the genes reconstruction). T-allele of rs2218404 is detectable in marmoset and in mouse
161 its position corresponds to adenosine (**Figure 2D**). G-alelle (associated with a higher VC
162 score comparing to the human T-allele carriers) is found in Mesolithic hunter Loschbour
163 (8,000 BC) but not in another ancient hunter Motala12 and is also present in other two
164 hominins belonging to the Neolithic period, Iceman and Eskimos (5,300 and 4,000 yrs BC,
165 respectively). rs628117 is the only *NTNG1*-related mutation near vicinity of which (±50 nu)
166 is located an intra-hominins (Es, Ice, Lo) mutation (**Figure 2B**, low left). The next, PS-
167 affecting, ROI of rs96501 displays an intricate path of T-allele evolution (associated with a
168 higher PS score) being anciently conserved from mice to primates but later substituted on the
169 less efficient (in terms of the generated IQ scores) C allele in Neanderthals, later again
170 replaced by the T allele in Mesolithic hunters and coming back during the Neolithic times
171 (**Figure 2D**). The first *NTNG2* SNP rs1105684 is located at the beginning of the gene and
172 affects WM in healthy human subjects (**Figure 3A**). The origin of the T-allele is evolutionary
173 bound to marmoset since its position in mice is occupied by another pyrimidine base C
174 (**Figure 3D**). Next on the gene are two SNP alleles for rs7851893 and rs3824574 which do
175 not affect IQ and similarly to rs1105684 are surrounded by highly conserved ROIs not only
176 in hominins and chimpansee (100% identity) but also in marmoset (except 1 mutation for
177 rs7851893). rs2149171 ROI (affecting the WM and PO scores) similarly to rs3824574 is
178 100% conserved across the all species (including the mouse) except the allele itself. The
179 attenuating IQ C-allele position is occupied in mice genome by T but present in Iceman and
180 Eskimos genes. A distinct evolutionary path is taken by another cognitive endophenotype-

181 associated and affecting WM and PO scores A-allele of rs2274855 and its ROI (**Figure 3D**).
182 Its position in mice is likely to be occupied by the C pyrimidine base (the software places a
183 blank instead of it) which is gradually substituted on purine G in chimpanzee and misplaced
184 by the lower IQ score-associated A-allele in Mesolithic hominins. And 20 nu downstream of
185 the centre of ROI is located a modern human-specific point mutation translated into the
186 T346A protein substitution (as described below).

187 The distinct picture of evolutionary changes among the *NTNG1* and *NTNG2* nested
188 SNPs has prompted us to compare evolutionary rates for the full-length proteins encoded by
189 these gene paralogs, Netrin-G1 and Netrin-G2, respectively (**Nakashiba et al., 2000 and**
190 **2002**). Netrin-G1 undergoes only few changes in its amino acid (aa) composition with the
191 maximum calculated rate of evolution reaching 0.05 when mice and marmoset proteins are
192 compared, 0.01 among the primates, and 0.03 between chimpanzee and human due to a
193 single point mutation A81S (**Figure 2E** and **SM:** Netrin-G1), absent in other primates.
194 Netrin-G2 evolves 2.8 times faster between mouse and marmoset than its paralog Netrin-G1
195 and continues evolving with a steady rate of 0.05 from primates to human (**Figure 3E** and
196 **SM:** Netrin-G2). We have also reconstructed both proteins from ancient (Neanderthals,
197 Paleolithic time) and extinct hominins (Mesolithic and Neolithic times) and compared them
198 with primates' and mice' Netrin-G orthologs (**Figure 2F** and **3F)**. Netrin-G1 is a highly
199 conserved protein among the primates and hominins (**Figure 3F**). As for Netrin-G2, a
200 mutation shared among the Neanderthals' and Mesolithic genomes, primates and mice
201 (T346A) is absent in the Neolithic Iceman and modern human (the signal for Motala3,
202 Motala1 and MezmayskayaNea is not clear due to low sequence coverage). Primates
203 (marmoset and chimpanzee) share another mutation (S371A/V) preserved in mouse and
204 absent in hominins (further details can be found in the **SM:** Results).
205

205 DISCUSSION

206 ***NTNG* paralog SNPs and associated cognitive endophenotypes of human subjects.**
207 Shortcomings of cognitive and information processing are key features of SCZ diagnosis
208 (**APA, 2013**). They are manifested as impairments in PO, WM, VC and PS (see **Yoon et al.,**
209 **2014** for references) frequently reported as attenuated scores upon IQ tests implementation.
210 SCZ patients carrying a mutation allele for one of *NTNG* gene paralog SNPs form cognitive
211 endophenotypes affecting the IQ scores (**Figure 1C**). The given endophenotypic groups
212 comprise from subjects with either affected VIQ (via attenuated VC by *NTNG1* rs2218404 or
213 WM by *NTNG2* rs2149171 and rs2274855) or affected PIQ (via attenuated PO by *NTNG2*
214 rs2149171 and rs2274855). In two extra cases PS is affected by rs96501 of *NTNG1* and WM
215 by rs1105684 of *NTNG2* (**Figure 1B-1** and **B-2**, respectively) but in healthy human subjects.
216 Such intriguing non-overlapping effect on the IQ domains prompts us to conclude that *NTNG*
217 paralogs complement each other function and represent an example of how a synapse-
218 expressed genes affect the human cognitive abilities, perhaps through the precision of
219 neuronal connectivity perturbations and concomitant miswirings. The observed phenomena
220 of the affected WM is the most striking due to its multifaceted constructive nature (**Frydecka**
221 **et al., 2014**) underlying many, if not all, cognitive tasks such as comprehension, reasoning
222 and learning (**Baddeley, 1992**) and historically introduced by Baddeley as the reading span
223 test (**Mackintosh, 2011**). Lack of the localization effect of *NTNG2* SNP mutation alleles, all
224 three are located in different parts of the gene (**Figure 1A-2**) but associated with identical
225 endophenotype (**Figure 1B-2**), points to a uniform nature of the *NTNG2* function distribution
226 over the entire gene. An obviously non-coding nature of all five IQ-affecting alleles
227 (rs2149171 despite being exon 4-located encodes a silent F246F mutation) corroborates an
228 idea that anthropoid trait-associated loci lie outside coding protein areas (**del Rosario et al.,**
229 **2014; Kellis et al., 2014**) and hints towards a potential of these alleles to perturb genes

230 regulatory functions, e.g. mRNA splicing, affecting downstream located pivotally functional
231 *NTNG* elements such as Ukd-domain encoding exons 6 and 7 or a unique Netrin-Gs trait –
232 GPI-link. Alternatively, or simultaneously, the *NTNG* SNP alleles may be embedded into an
233 epistatic network of other genes influencing human cognitive traits (**Hemani et al., 2014**).
234 However since it is usual for a SNP effect to be estimated using an additive model (assuming
235 either independent and cumulative single contribution) to the mean of a trait with the small
236 effect size the power to detect the epistatic environment drastically declines. Contrary to the
237 genetic associations with gene expression having large effect sizes (**Hemani et al., 2014**),
238 cognitive trait-associated effect sizes are reportedly small (**Plomin and Deary, 2015**), e.g.
239 the largest effect sizes of the variance of intelligence scores accounted for only 0.2%
240 (**Benyamin et al., 2014**), 0.5% on GWA studies of 1,583 adolescence (**Desrivieres et al.,**
241 **2015**) or was predicted to be ~1% on 3,511 adults (**Davies et al., 2011**). Another GWAS of
242 educational attainment (sharing a moderate correlate with intelligence), which included
243 126,559 individuals, reports on just 1% of the variance but only 0.02% in a replication
244 sample (**Rietveld et al., 2013**). Our data support the preexisted conclusion that human
245 cognitive traits modalities are not described by statistically large effect sizes.
246 **Evolutionary elaborations of the embedding IQ-affecting mutations loci.** Eleven
247 previously published SCZ-associated SNPs were tested for their effect on IQ performance of
248 human subjects and 5 of them were found to be associated with attenuated IQ cognitive
249 endophenotypes (**Figure 1B-1** and **B-2**). ROIs of 3 of them (rs2218404, rs1373336 and
250 rs2274855) underwent an AE from chimpanzee to human when compared to New World
251 monkeys to apes (marmoset-chimpanzee) path (**Figure 2C** and 3**C**). Two of them affect IQ in
252 humans (rs2218404 – VC and rs2274855 – WM, **Figure 1B-1** and **B-2**) while being located
253 within the vicinity of exon 5 (2,275 nu downstream and 15 nu upstream, respectively) – a
254 part of the lowest percent identity coding DNA among the *NTNG* gene paralogs (**Prosselkov**

255 **et al., in press**). Presence of the evolutionary accelerated regions within the *NTNG* genes
256 non-coding areas underscores them as contributors to the human-specific traits along with
257 other genes (**Prabhakar et al., 2006**). However, not only ROIs of the IQ-affecting alleles but
258 the alleles themselves demonstrate several unique evolutionary features (see **SM:** Discussion).
259 To understand evolutionary forces driving the emergence of cognitive endophenotype-
260 associated alleles we have deduced a set of rules outlined as follows. 1. An alternative
261 (mutated) allele evolutionary appearance coincides with the lack of any other mutations
262 within ROI (a conserved island rule); 2. positioning of the future mutation often represents a
263 turning point of dramatic changes of an allele ROI (e.g. as seen in marmoset: rs2218404 (50-
264 90%), rs628117 (30-80%), rs96501 (100-40%)); 3. an AE of ROI often precedes the
265 emergence of a mutation allele (e.g. rs2218404: chimpanzee to human (k = 1.59), appearance
266 of "G" in Loschbour; rs628117: marmoset to chimpanzee (k = 1.10), appearance of "T" in
267 AltaiNea; rs96501: marmoset to chimpanzee (k = 0.83), appearance of "C" in AltaiNea;
268 rs2274855: chimpanzee to human (k = 0.79), appearance of "A" in Motala12; 4. low identity
269 percent (equivalent to subsequent substantial evolutionary changes) among the evolutionary
270 species within the allele surrounding proximity of as long as $\pm$50 nu is not sufficient for the
271 future mutated allele significance as a cognitive endophenotype determinant (as deduced by
272 the IQ score) as seen for the rs1373336, rs1444042, rs4915045 and rs7851893 (none of them
273 are IQ-affecting, though associated with SCZ, despite showing (very) low identity in mice).
274 Rather some dramatic changes within the allele's immediate proximity of $\pm$10 nu (defined as
275 a ROI) preceded or followed by more stringently conserved DNA are necessary (the
276 conserved island perturbation rule). Currently we are unable to state that the IQ-associated
277 alleles ROIs are regulatory loci and an important source of evolutionary innovation
278 (**Rubinstein and de Souza, 2013**) but they may be the smallest functional blocks of a strong
279 positive selection exerts its action upon similarly to the 20–30 nu clusters of strongly

280 conserved non-coding elements (CNEs), transcription factor binding sites (TFBS), RNA
281 splicing and editing motifs (**Harmston et al., 2013**).
282 **Extinct hominins and IQ-associated mutation alleles.** Availability of archaic genomes
283 allows excavation for the advantageous alleles that modern humans acquired from archaic
284 extinct hominins such as Neandertals and Denisovans who used to live 230,000-30,000 years
285 ago (Middle/Upper Paleolithic, Old Stone Age) defined by distinct morphological features
286 (**Meyer et al., 2012**), and from modern extinct humans (hunters, farmers) from Mesolithic
287 (Middle Stone Age, ~10,000 yrs BC, **Lazaridis et al., 2014**) and Neolithic (New Stone Age,
288 ~5,000 BC, **Keller et al., 2012**; **Rasmussen et al., 2010**) periods. Though exhibiting several
289 anatomical features, making archaic hominins different from the modern human, there are
290 studies challenging the idea that reserve symbolism and abstract thinking was an exclusive
291 prerogative of modern human (**Appenzeller, 2013**; **Wong, 2015**). The time Neanderthals
292 used to live in is thought to be associated with the onset of cognitive fluidity involving the
293 capacity to draw analogies (early paintings), to combine concepts (making tools) and to adapt
294 ideas for new contexts (**Gabora and Russon, 2011**). Wynn and Coolidge believe that
295 evolution of WM was central to the evolution of human cognitive traits consisted from few
296 genetic mutations that led to "enhanced WM" 200,000-40,000 BC (**Balter, 2010**). Our work
297 partially supports this idea showing the perseverance of higher WM score-associated alleles
298 across the hominins such as T of rs1105684 and G of rs2274855 (**Figure 3D**) but a Neolithic
299 appearance of rs2149171 T in Iceman previously found only in mice genome supporting the
300 conclusion made by **Crabtree (2013**) that modern humans as species "are surprisingly
301 intellectually fragile and perhaps reached a peak 2,000–6,000 years ago". Mesolithic period
302 has been always considered as a key gate for the evolution of human languages (**Haak et al.,**
303 **2015**) with our data showing that rs2218404 G-allele associated with a higher VC score
304 (**Figure 1B-1**) emerges for the first time in the Loschbour hunter *NTNG1* gene (**Figure 3D**).

305 VC as a part of abstract symbol usage is associated with the global network efficiency (as a
306 part of fronto-parietal network in **Song et al., 2008**; **Glascher et al., 2009**), and global
307 communication and intellectual performance (**Pamplona et al., 2015**). From this point of
308 view it is not surprising that appearance of the G allele in Loschbour and Iceman genomes
309 coincides with the presence of PS enhancing rs96501 T allele (**Figure 3D**). A wealth of data
310 has been collected characterising possible look and health status of archaic hominins and
311 modern but extinct humans (for ref. see **Sarkissian et al., 2015** and **SM:** Discussion). Based
312 on our own data we may also speculate that the extinct hominins may have had a lower VC
313 comparing to us, and consequently, Neanderthals were unlikely able for a semantic
314 communication due to a global network inefficiency VC is associated with; they have had
315 similar to us PS (if they had lived beyond the Mesolithic period), and were likely to have had
316 identical to modern human WM, corroborating the advanced evolutionary nature of this
317 important human cognitive domain of a limited capacity and associated with intelligence.
318
319 CONCLUSION
320 Evolution of a novel function relies on enhanced genetic robustness through functional
321 redundancy potentially provided by a gene duplication event. Further evolutionary outcome
322 depends on the substrate availability (undergoing its own evolution) upon which the novel
323 function(s) exerts its action. Nature does not create but tinkers to perfection provided to it
324 material exploring available evolutionary tools. Half a billion years ago a gene duplication
325 event had provided a plethora of such substrate thus converting the evolution itself into a
326 "Creator" of new functions. Here we have described how a pair of twin genes got themselves
327 involved into the human cognitive functioning believed to be emerged in a primordial state in
328 primitive vertebrates prior to the first recorded gene duplication. Subsequent process of the
329 function specialisation made *NTNG* paralogs to subfunctionalise into distinct cognitive

330 domains in a complementary manner (**Prosselkov et al., in press**).

331

MATERIALS AND METHODS

Ethics statement. This study was performed in accordance with the World Medical Association's Declaration of Helsinki and approved by the Osaka University Research Ethics Committee. A written informed consent was obtained from all subjects after the procedures had been fully explained.

Subjects. The procedures were performed as per established protocols at Osaka University as described previously (**Ohi et al., 2012**). The subjects consisted from 339 patients with SCZ and 716 healthy controls. The sex ratio did not differ significantly between the groups, but the mean age was significantly different. The subjects were all biologically unrelated Japanese and recruited from both outpatient and inpatient units at Osaka University Hospital and other psychiatric hospitals. Each patient with SCZ had been diagnosed by at least two trained psychiatrists based on unstructured clinical interviews, according to the criteria of the DSM-IV (**APA, 2013**). In case if the diagnosis of the two trained psychiatrists was discordant, it was resolved through the further negotiations on both specialist opinions. In case of unresolved diagnostic disputes, the patient was omitted from the study. Psychiatrically healthy controls were recruited through local advertisements and were evaluated by means of unstructured interviews to exclude individuals with current or past contact with psychiatric services, those who experienced psychiatric medications, or who were not Japanese. Controls for family history of a CD, such as SCZ, BD, or major depressive disorder were not included. Ethnicity was determined by self-report and was not confirmed by genetic analyses. Additionally, subjects were excluded from this study if they had neurologic or medical conditions that could have potentially affect their central nervous system, such as atypical headaches, head trauma with loss of consciousness, chronic lung disease, kidney disease, chronic hepatic disease, thyroid disease, active cancer, cerebrovascular disease, epilepsy, seizures, substance abuse related disorders, or mental retardation.

356 **SNPs selection, genotyping, and genomic sequencing.** This study was designed to examine
357 the association of SCZ patients cognitive performance (through WAIS-III implementation,
358 **Wechsler, 1958**) with *NTNG* genes. Venous blood was collected from the subjects. Genomic
359 DNA was extracted from the whole blood using standard procedures. The SNPs (**Fukasawa**
360 **et al., 2004**; **Aoki-Suzuki et al., 2005**; **JSSLG et al., 2005**; **Eastwood and Harrison, 2008**;
361 **Ohtsuki et al., 2008**; **Zakharyan et al., 2011**; **Zhu et al., 2011**; **Ayalew et al., 2012**; **Wilcox**
362 **and Quadri, 2014**) were genotyped using the TaqMan allelic discrimination assay (Applied
363 Biosystems, Foster City, CA). No deviations from the Hardy-Weinberg equilibrium in the
364 examined SNPs were detected ($p > 0.05$).
365 **Statistical analysis.** The effects of the diagnosis, genotype and their interaction on cognitive
366 performances in the WAIS were analyzed by two-way analyses of covariance (ANCOVA).
367 Diagnosis and genotype statuses were included in the model as independent variables (**ST1**).
368 FIQ and each WAIS subscale score (VIQ, PIQ, VC, PO, WM, PS, Vc, SiM, IF, CH, AM, DS,
369 LNS, PC, BD, and MR) were included as dependent variables. Sex, age and years of
370 education were treated as covariates, as they were possible confounding factors. All p values
371 are two tailed, and statistical significance was defined as $*p < 0.05$ and $**p < 0.01$.
372 **Identity percent calculations and the definition of ROIs.** The complete procedure is
373 described in the **Figure 4** legend. Stretcher (**McWilliam et al., 2013**) was used for the
374 alignments (the default values were: gap penalty – 16 (DNA) and 12 (protein), and the extend
375 penalty – 4 (DNA) and 2 (protein)), for the percent identity calculations and evolutionary
376 rates. A ROI was selected as a minimal area surrounding a SNP mutation allele incorporating
377 the outmost evolutionary dramatic changes.
378 **Mice, primates and hominins *NTNG* paralogs DNA and encoded aa sequences**
379 **reconstruction.** Genomes for mouse (GRC38.p3) and marmoset (C_jacchus3.2.1) were from
380 Ensemble. Since chimpanzee's genome is based only on a single individual (CHIMP2.1.4,

Clint) and contains several questionable information we have reconstructed a consensus genome sequence for both *NTNG* genes based on 25 primate sequences of *Pan troglodytes* (**Prado-Martinez et al., 2013**). All datasets used for the *NTNG* paralogs DNA and encoded by them proteins reconstruction are listed in **ST2**. For details refer to **SM**.

SUPPLEMENTARY MATERIALS (**SM**)

Contain additional Results and Discussion, Supplementary Methods (ancient and primate genomes reconstructions), and Supplementary Tables (**ST1** and **ST2**) as a single compiled pdf file. Also included are human Netrin-G1 and Netrin-G2 alignments, as well as 101 nu alignments for all 11 ROIs across the all analysed species.

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COMPETING INTERESTS

Authors would like to express a lack of any competing interests associated with the work.

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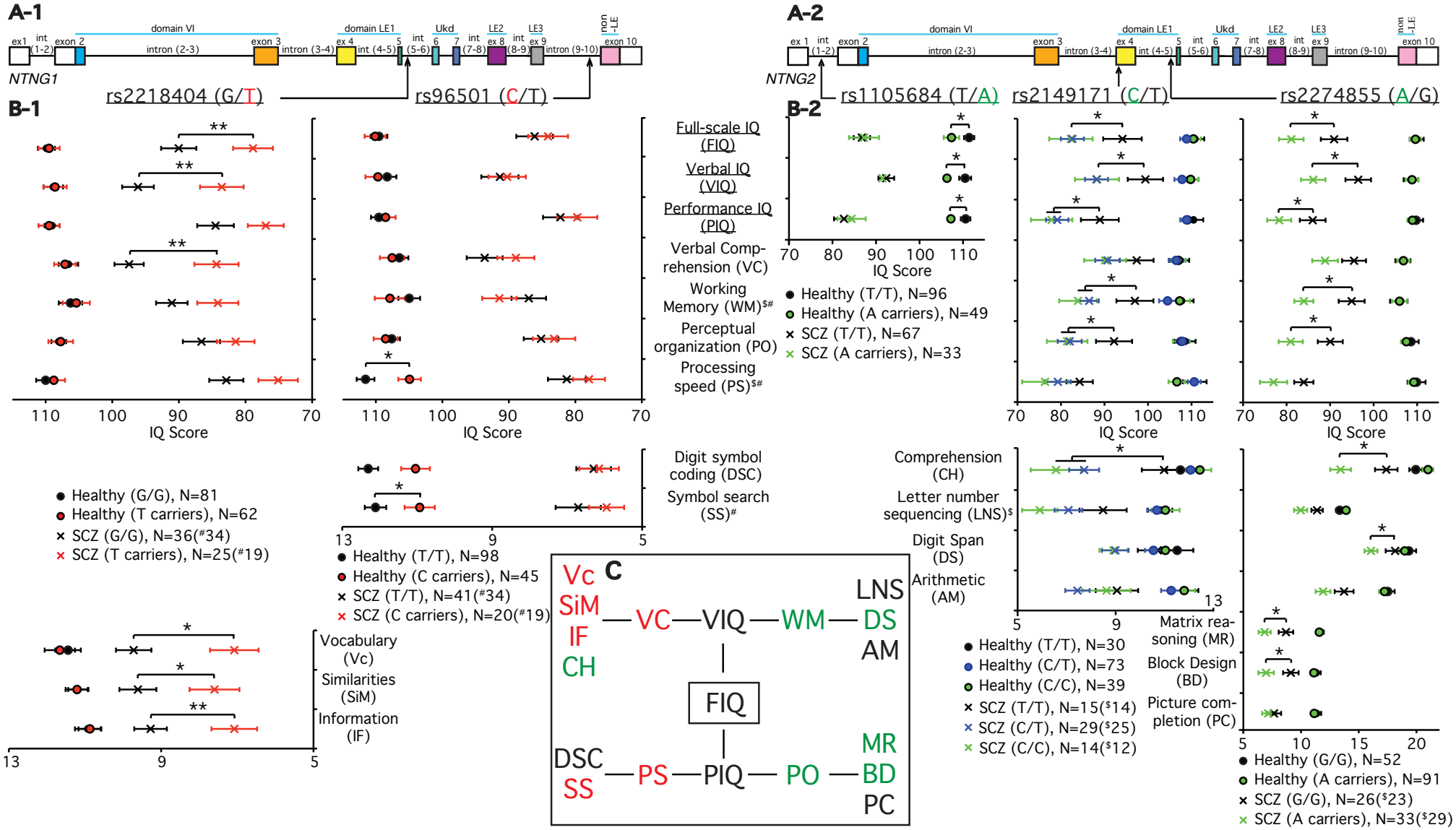

Figure 1. Complementary effect of *NTNG* paralog SNPs on IQ cognitive domains of human subjects as measured by WAIS-III. (**A-1, A-2**) *NTNG1* and *NTNG2* gene structures with the SNPs location indicated. (**B-1, B-2, C**) Affected cognitive domains and scores. Red highlights *NTNG1* and green (or blue, in case of heterozygosity) – *NTNG2* located alleles associated with the attenuated IQ scores. The data are presented as a mean±SEM. *$p<0.05$,**$p<0.01$ (two ways ANCOVA (sex, education, and age as co-variates)). The number of human subjects is indicated as N. For statistical and diagnosis details refer to **ST1.**

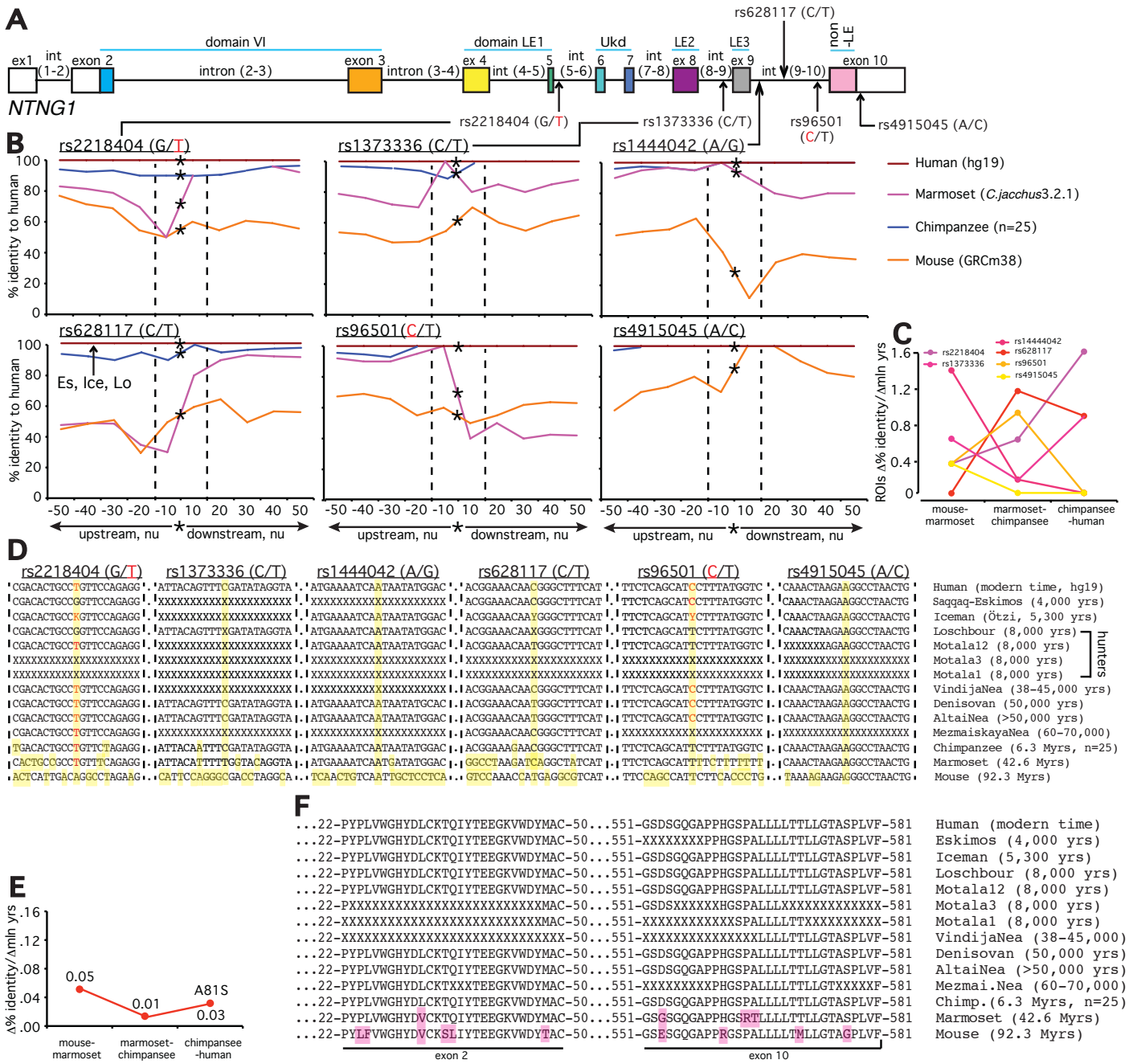
A
NTNG1
ex1
int (1-2)
exon 2
domain VI
intron (2-3)
exon 3
intron (3-4)
domain LE1
ex 4
int (4-5)
5
int (5-6)
Ukd
6
7
int (7-8)
LE2
ex 8
int (8-9)
LE3
ex 9
int (9-10)
non -LE
exon 10
rs628117 (C/T)
rs2218404 (G/T)
rs1373336 (C/T)
rs96501 (C/T)
rs4915045 (A/C)
B
rs2218404 (G/T)
rs1373336 (C/T)
rs1444042 (A/G)
rs628117 (C/T)
rs96501(C/T)
rs4915045 (A/C)
% identity to human
Es, Ice, Lo
Human (hg19)
Marmoset (C.jacchus3.2.1)
Chimpanzee (n=25)
Mouse (GRCm38)
upstream, nu
downstream, nu
C
ROIs Δ% identity/Δmln yrs
rs2218404
rs1373336
rs14444042
rs628117
rs96501
rs4915045
mouse-marmoset
marmoset-chimpansee
chimpansee-human
D
rs2218404 (G/T)
rs1373336 (C/T)
rs1444042 (A/G)
rs628117 (C/T)
rs96501 (C/T)
rs4915045 (A/C)
Human (modern time, hg19)
Saqqaq-Eskimos (4,000 yrs)
Iceman (Ötzi, 5,300 yrs)
Loschbour (8,000 yrs)
Motala12 (8,000 yrs)
Motala3 (8,000 yrs)
Motala1 (8,000 yrs)
hunters
VindijaNea (38-45,000 yrs)
Denisovan (50,000 yrs)
AltaiNea (>50,000 yrs)
MezmaiskayaNea (60-70,000)
Chimpanzee (6.3 Myrs, n=25)
Marmoset (42.6 Myrs)
Mouse (92.3 Myrs)
E
Δ% identity/Δmln yrs
0.05
0.01
A81S
0.03
mouse-marmoset
marmoset-chimpansee
chimpansee-human
F
...22-PYPLVWGHYDLCKTQIYTEEGKVWDYMAC-50...551-GSDSGQGAPPHGSPALLLLTTLLGTASPLVF-581 Human (modern time)
...22-PYPLVWGHYDLCKTQIYTEEGKVWDYMAC-50...551-XXXXXXXXPPHGSPALLLLTTLLGTASPLVF-581 Eskimos (4,000 yrs)
...22-PYPLVWGHYDLCKTQIYTEEGKVWDYMAC-50...551-GSDSGQGAPPHGSPALLLLTTLLGTASPLVF-581 Iceman (5,300 yrs)
...22-PYPLVWGHYDLCKTQIYTEEGKVWDYMAC-50...551-GSDSGQGAPPHGSPALLLLTTLLGTASPLVF-581 Loschbour (8,000 yrs)
...22-PYPLVWGHYDLCKTQIYTEEGKVWDYMAC-50...551-GSDSGQGAPPHGSPALLLLTTLLGTASPLVF-581 Motala12 (8,000 yrs)
...22-PXXXXXXXXXXXXXXXXXXXXXXXXXMAC-50...551-GSDSXXXXXXHGSPALLLXXXXXXXXXXXXX-581 Motala3 (8,000 yrs)
...22-XXXXXXXXXXXXXXXXXXXXXXXXXXMAC-50...551-XXXXXXXXXXXXXSPALLLLTTXXXXXXXXXX-581 Motala1 (8,000 yrs)
...22-XXXXXXXXXXXXXXXXXXXXXXXXXXXX-50...551-XXXXXXXXXXXXXXXXLLLLTTLLGTASPLVF-581 VindijaNea (38-45,000)
...22-PYPLVWGHYDLCKTQIYTEEGKVWDYMAC-50...551-GSDSGQGAPPHGSPALLLLTTLLGTASPLVF-581 Denisovan (50,000 yrs)
...22-PYPLVWGHYDLCKTQIYTEEGKVWDYMAC-50...551-GSDSGQGAPPHGSPALLLLTTLLGTASPLVF-581 AltaiNea (>50,000 yrs)
...22-PYPLVWGHYDLCKTXXXTEEGKVWDYMAC-50...551-XXXXXXXXXPPHGSPALLLLTTLLGTXXXXXF-581 Mezmai.Nea (60-70,000)
...22-PYPLVWGHYDLCKTQIYTEEGKVWDYMAC-50...551-GSDSGQGAPPHGSPALLLLTTLLGTASPLVF-581 Chimp.(6.3 Myrs, n=25)
...22-PYPLVWGHYDVCKTQIYTEEGKVWDYMAC-50...551-GSGSGQGAPPHGSRTLLLLTTLLGTASPLVF-581 Marmoset (42.6 Myrs)
...22-PYLFVWGHYDVCKSLIYTEEGKVWDYTAC-50...551-GSESGQGAPPRGSPALLLLTMLLGTAGPLVF-581 Mouse (92.3 Myrs)
exon 2
exon 10

Figure 2.

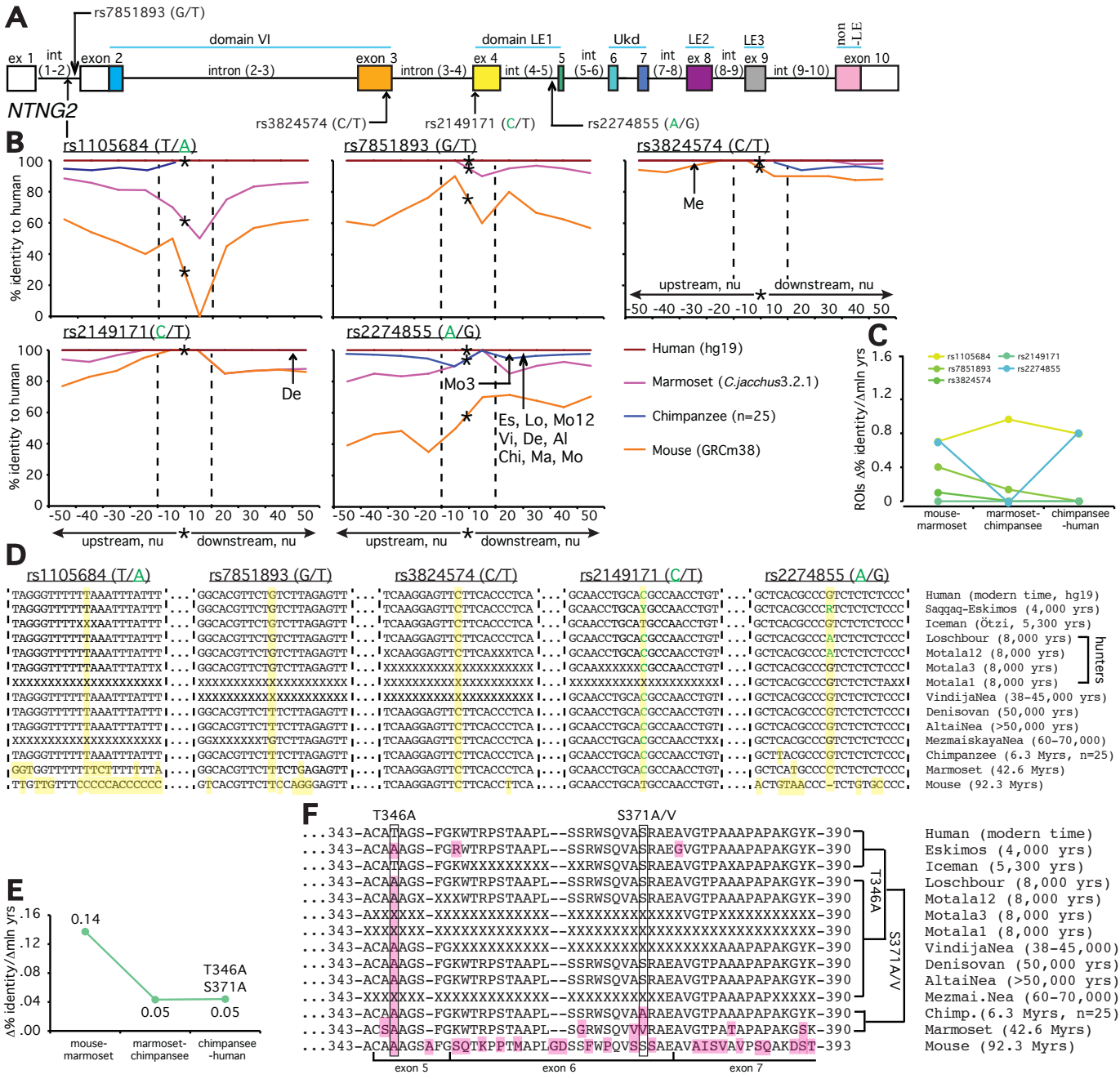
A
rs7851893 (G/T)
domain VI
domain LE1
Ukd
LE2
LE3
non -LE
ex 1
int (1-2)
exon 2
intron (2-3)
exon 3
intron (3-4)
ex 4
int (4-5)
5
int (5-6)
6
7
int (7-8)
ex 8
int (8-9)
ex 9
int (9-10)
exon 10
NTNG2
rs3824574 (C/T)
rs2149171 (C/T)
rs2274855 (A/G)
B
rs1105684 (T/A)
rs7851893 (G/T)
rs3824574 (C/T)
% identity to human
Me
upstream, nu
downstream, nu
rs2149171(C/T)
rs2274855 (A/G)
De
Mo3
Es, Lo, Mo12
Vi, De, Al
Chi, Ma, Mo
Human (hg19)
Marmoset (C.jacchus3.2.1)
Chimpanzee (n=25)
Mouse (GRCm38)
C
ROIs Δ% identity/Δmln yrs
rs1105684
rs7851893
rs3824574
rs2149171
rs2274855
mouse-marmoset
marmoset-chimpansee
chimpansee-human
D
rs1105684 (T/A)
rs7851893 (G/T)
rs3824574 (C/T)
rs2149171 (C/T)
rs2274855 (A/G)
Human (modern time, hg19)
Saqqaq-Eskimos (4,000 yrs)
Iceman (Ötzi, 5,300 yrs)
Loschbour (8,000 yrs)
Motala12 (8,000 yrs)
Motala3 (8,000 yrs)
Motala1 (8,000 yrs)
hunters
VindijaNea (38-45,000 yrs)
Denisovan (50,000 yrs)
AltaiNea (>50,000 yrs)
MezmaiskayaNea (60-70,000)
Chimpanzee (6.3 Myrs, n=25)
Marmoset (42.6 Myrs)
Mouse (92.3 Myrs)
E
Δ% identity/Δmln yrs
0.14
0.05
0.05
T346A
S371A
mouse-marmoset
marmoset-chimpansee
chimpansee-human
F
T346A
S371A/V
Human (modern time)
Eskimos (4,000 yrs)
Iceman (5,300 yrs)
Loschbour (8,000 yrs)
Motala12 (8,000 yrs)
Motala3 (8,000 yrs)
Motala1 (8,000 yrs)
VindijaNea (38-45,000)
Denisovan (50,000 yrs)
AltaiNea (>50,000 yrs)
Mezmai.Nea (60-70,000)
Chimp.(6.3 Myrs, n=25)
Marmoset (42.6 Myrs)
Mouse (92.3 Myrs)
exon 5
exon 6
exon 7

Figure 3.

Figures 2 and 3. Accelerated evolution (AE) and definition of a ROI of *NTNG* loci embedding associated with the cognitive endophenotype alleles. Hominins and primate-specific protein amino acid substitutions. (**A**) *NTNG1* and *NTNG2* SNPs' gene locations. Alleles associated with the lower IQ scores are shown either in red (*NTNG1*) or green (*NTNG2*). (**B**) Calculated identity percent of primates and mice to human gene loci as a function of distance from the position of a mutated allele (denoted as a star). Comparison was done in a stepwise manner as ±10 nu to the maximum of ±50 nu using Stretcher (http://www.ebi.ac.uk/Tools/psa, **McWilliam et al., 2013**). The areas were compared based on the positioning relative to the point mutation without any manual curation for "the best-fit" alignment, but as per the algorithm output only. Due to low level of identity for mouse and marmoset the initial search of the mutation allele positioning was done aligning against the human corresponding full-length intron, and then second time against the obtained 101 nu query ("-50 nu-SNP+50 nu"). Two dashed lines define an area of -10 nu to +10 nu from the mutation allele position. This area (21 nu in total) is defined as a ROI of the given mutation allele of a representing SNP. An arrow indicates a position of extinct hominins-specific mutations (see below). (**C**) Evolutionary rates for the ROIs calculated as a percent identity change relative to the hg19 over the mln of years of evolution. The spectrum color reflects the mutations' positioning order on a gene, as purple-yellow for *NTNG1*, and yellow-blue for *NTNG2*. (**D**) ROIs DNA sequences across hominins, primates and mice loci. The extinct and ancient hominin's *NTNG* paralogs were reconstructed from the available datasets (see **SM**): Saqqaq-Eskimos (Es: **Rasmussen et al., 2010**); Iceman (Ice, **Keller et al., 2012**); Hunters (Loschbour (Lo), Motala12 (Mo12), Motala3 (Mo3), Motala1: **Lazaridis et al., 2014**); VindijaNea (Vi: **Green et al., 2010**); Denisovan (De), AltaiNea (Al) and MezmaiskayaNea (Me): **Prüfer et al., 2013**; chimpanzee (Chi: reconstructed from **Prado-Martinez et al., 2013**, n=25 animals). Non-available sequences due to poor reads quality are denoted as X. Ensemble was used for the initial *NTNG* paralogs retrieval in marmoset (Ma) and mouse (Mo). Yellow (vertical strip) denotes the position of the SNP-related mutation allele and non-matched to human substitutions. (**E**) Evolution rates for the proteins encoded by the *NTNG1* (Netrin-G1) and *NTNG2* (Netrin-G2). (**F**) Amino acid changes for Netrin-Gs across hominins, primates and mice. For Netrin-G1, there are no common mutations among primates, hominins and modern human. Es contains 5 point mutations absent in other hominins; Chi contains 1 mutation outside the depicted area (A81S); all 4 mutations for marmoset are shown, and 10 more extra mutations for mouse are not shown. For Netrin-G2, all hominins except Ice (relative to hg19) carry a T346A mutation (exon 5-located and known as rs4962173), also detectable in primates and mouse. Chi's Netrin-G2 differs from all hominins by S371A mutation (exon 6 - nested) and present in marmoset as S371V. Non-matched to hg19 amino acids are highlighted as pink. Refer to **SM** for the full alignments.