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Human-Chimpanzee Differences in a *FZD8* Enhancer Alter Cell-Cycle Dynamics in the Developing Neocortex

Graphical Abstract



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In Brief

This study reports the discovery of a human-accelerated enhancer of *Fzd8* functioning in brain development. Boyd et al. demonstrate species-specific activity differences and show that the human enhancer promotes a faster progenitor cell cycle and increased neocortical size. Enhancer sequence changes may contribute to unique features of the human brain.

Highlights

- Discovery of a human-accelerated enhancer functioning in the developing neocortex
- Compared to chimpanzee, human *HARE5* drives earlier and more robust brain expression
- The HARE5 locus physically contacts the core promoter of the WNT receptor, *Fzd8*
- *HARE5::Fzd8* mice have an accelerated neural progenitor cell cycle and enlarged brains

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Report

Human-Chimpanzee Differences in a *FZD8* Enhancer Alter Cell-Cycle Dynamics in the Developing Neocortex

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Summary

The human neocortex differs from that of other great apes in several notable regards, including altered cell cycle, prolonged corticogenesis, and increased size [1-5]. Although these evolutionary changes most likely contributed to the origin of distinctively human cognitive faculties, their genetic basis remains almost entirely unknown. Highly conserved non-coding regions showing rapid sequence changes along the human lineage are candidate loci for the development and evolution of uniquely human traits. Several studies have identified human-accelerated enhancers [6-14], but none have linked an expression difference to a specific organismal trait. Here we report the discovery of a human-accelerated regulatory enhancer (HARE5) of FZD8, a receptor of the Wnt pathway implicated in brain development and size [15, 16]. Using transgenic mice, we demonstrate dramatic differences in human and chimpanzee HARE5 activity, with human HARE5 driving early and robust expression at the onset of corticogenesis. Similar to HARE5 activity, FZD8 is expressed in neural progenitors of the developing neocortex [17–19]. Chromosome conformation capture assays reveal that HARE5 physically and specifically contacts the core Fzd8 promoter in the mouse embryonic neocortex. To assess the phenotypic consequences of HARE5 activity, we generated transgenic mice in which Fzd8 expression is under control of orthologous enhancers (Pt-HARE5::Fzd8 and Hs-HARE5::Fzd8). In comparison to Pt-HARE5::Fzd8, Hs-HARE5::Fzd8 mice showed marked acceleration of neural progenitor cell cycle and increased brain size. Changes in HARE5 function unique to humans thus alter the cell-cycle dynamics of a critical population of stem cells during corticogenesis and may underlie some distinctive anatomical features of the human brain.

Results

Identification of Human-Accelerated Enhancer Loci in the Developing Neocortex

The dramatic expansion of the neocortex during hominoid evolution is proposed to underlie the emergence of our uniquely human cognitive abilities [20-22], although strong genetic correlations between these traits have remained elusive [23]. The evolution of human cortical features, such as enlarged brain size, has been attributed to cellular changes including neuron number and neural progenitor cell cycle [1-5, 15]. However, the genetic basis for these traits, which so markedly distinguish humans from other primates, remains poorly understood. Mutations within regulatory elements have been proposed to play a significant role in the evolution of human-specific traits [24, 25]. Recent genomic studies support this notion and have collectively identified highly conserved non-coding regions that are rapidly evolving along the human lineage [6-10]. Of note, these human-accelerated non-coding loci are frequently located nearby genes implicated in brain development and function [11, 26, 27]. Together, these studies suggest that the evolution of human neocortical traits may have occurred through modification of cis-regulatory enhancers involved in brain development. Yet to date, just a handful of human-accelerated regions have been shown to function as forebrain enhancers [11-13], and none have been shown to impact neocortical expansion. Here we sought to discover human-accelerated regulatory loci important for corticogenesis in order to gain insights into the genetic basis for the evolution of uniquely human brain features.

We identified HARE5 from an in silico screen for rapidly evolving human non-coding regions predicted to function as developmental enhancers in the mammalian neocortex (Figures S1A and S1B, Table S1, and the Supplemental Experimental Procedures) [6-8, 28, 29]. Using a standard mouse transient transgenic assay [11, 14], we found that HARE5 reporter activity was robust in the lateral neocortex and dorso-lateral midbrain (15/15 embryos) (Figures 1A and S1C). HARE5 was prioritized due to this enhancer activity and its chromosomal location adjacent to Frizzled 8 (FZD8), a receptor for the Wnt signaling pathway that is implicated in neocortical development (Figure 1B) [15-18, 30, 31]. The Homo sapiens (Hs) HARE5 ortholog contains 16 changes compared to Pan troglodytes (Pt). Based on outgroup comparison, ten mutations were fixed on the human branch and six on the chimpanzee branch since the latest common ancestor (Figure 1B). A phylogenetic analysis of the 1.2 Kb HARE5 locus across several great-ape species revealed a longer branch for the Hs ortholog compared to that of Pt (Figure 1C). This is consistent with the original signature of positive selection detected in the human relative to chimpanzee lineage [7]. Analysis of predicted transcription factor binding sites across the HARE5 locus revealed differences, particularly at human-derived mutations, for key transcription factors relevant to corticogenesis (see Table S2) [32]. Together, these results support the prediction that Hs-HARE5 acquired unique enhancer activity since diverging from the common chimpanzee lineage.



Distinct Enhancer Activity of Human and Chimpanzee *HARE5* in the Developing Neocortex

We postulated that human and chimpanzee HARE5 might differentially regulate gene expression during corticogenesis. To test this, we generated independent stable mouse transgenic lines (Pt-HARE5::LacZ and Hs-HARE5::LacZ). Corticogenesis initiates at embryonic day 9.5 (E9.5) and continues to E18.5 [2]. At E9.5, both Pt-HARE5 and Hs-HARE5 enhancer activity were undetectable (Figures 2A-2C). However, within a half day of development at E10.0, Hs-HARE5 activity was rapidly and robustly upregulated in the lateral telencephalon (Figures 2E and 2F). In contrast, Pt-HARE5 activity in the E10.0 telencephalon was markedly weaker and was limited to more lateral regions (Figures 2D and 2F). This spatial difference in enhancer activity was sustained at E10.5, as evidenced by both whole-mount embryos and coronal brain sections (Figures 2G-2I and S2A-S2D). By E11.5, species-specific differences in HARE5-driven LacZ activity were still evident, although they were far less dramatic (Figures 2J-2L). These results indicate that HARE5 orthologs drive expression in the developing lateral telencephalon. However, relative to chimpanzee, the human enhancer has considerably earlier and robust activity during corticogenesis.

Having established spatial and temporal differences in chimpanzee and human HARE5 enhancer activity, we next sought a more sensitive and dynamic readout of HARE5 transcriptional activity. The LacZ protein is stable for at least 48 hr, whereas destabilized fluorescent proteins with PEST motifs are only stable for 2 hr post-translation [33]. We generated new stable transgenic mouse lines, Pt-HARE5:: tdTomato-PEST and Hs-HARE5::EGFP-PEST, and compared native fluorescence in embryos co-expressing the reporters (Figures 2M and 2N). Both orthologs drove enhancer activity in the E11.0 neocortex; however, Hs-HARE::EGFP was considerably brighter than Pt-HARE5::tdTomato, despite tdTomato having intrinsically brighter fluorescent emission than EGFP (Figures 2N-2T) [33]. This reporter difference was sustained at E12.5, though the chimpanzee enhancer remained active (Figures 2U–2AA and S2E–S2H). We quantified enhancer activity by qRT-PCR measurement of reporter transcript levels in E12.5 neocortices. Hs-HARE5::EGFP embryos showed 10- to

Figure 1. Identification of *Hs-HARE5* as a Human-Accelerated Neocortical Enhancer

(A) Representative E14.5 Hs-HARE5::LacZ embryo stained for β -galactosidase (LacZ) activity. Scale bars, 2 mm.

(B) Schematic of *Hs-HARE5* locus on human chromosome 10 (hg19). The 1,219-bp-long *HARE5* genomic locus with enhancer activity includes the original 619-bp human-accelerated sequence and flanking 5' and 3' sequences. Represented below is a PhastCons conservation track for the *HARE5* locus, shown with the region of high conservation (gray). Also shown are lineage-specific mutations for chimpanzee (six; arrows, above line) and human (ten; arrowheads, bottom), including one Denisovan (red) and one currently identified human polymorphism (blue). (C) Maximum-likelihood phylogenetic tree for the *HARE5* orthologous locus from five anthropoid primates.

See also Figure S1 and Table S1.

30-fold higher transcript levels than *Pt-HARE5::tdTomato* (Figure 2BB). Hence, multiple independent reporter lines (LacZ and fluorescent) demonstrate that, compared to chimpanzee *HARE5*, human *HARE5* drives dramatically higher enhancer activity in the telencephalon.

In the E10.5 telencephalon, the predominant neural progenitor populations are neuroepithelial cells, and by E12.5 these are replaced by radial glia (termed neural stem cells) [2]. At E10.5, both enhancers were active in the majority (about 75%) of Pax6-positive neuroepithelial cells and in some TuJ1-positive neurons (Figures S3I–S3U). At E12.5, reporter activity was highest in the ventricular zone (VZ) (Figure S2E– S2H), where radial glial cells reside. Thus, both human and chimpanzee *HARE5* enhancers are active in neural progenitors of the developing neocortex.

Chromosome Conformation Capture Detects HARE5-Fzd8 Interactions

Having established HARE5 activity within the lateral telencephalon, we next sought to identify the likely target gene. The most proximal gene, Hs-FZD8, is located 307,758 bp downstream from HARE5 and was an obvious candidate due to its expression in the developing human and mouse neocortex [17-19, 30, 31]. LacZ reporter activity and Fzd8 in situ hybridization showed similar expression patterns in E10.5 and E11.5 whole-mount embryos and neocortical sections (Figure S3; http://developingmouse.brain-map.org and http://www.emouseatlas.org) [31]. We used chromosome conformation capture (3C) assays [34] to test for physical association between endogenous mouse (Mm) HARE5 and the core Fzd8 promoter within E12.5 mouse neocortices (Figure 3A). In neocortices, we observed a strong peak of interaction between Mm-HARE5 and the proximal Fzd8 promoter compared to flanking loci (Figure 3B). In contrast, no interactions were evident between Mm-HARE5 and Fzd8 in agematched liver, which lacks detectable HARE5 activity and Fzd8 expression. These data indicate that HARE5 physically and specifically associates with the core Fzd8 promoter in the developing mouse neocortex. Given the cis-regulatory activity of HARE5 orthologs, we propose that HARE5 functions as a distal-acting enhancer of FZD8 during early human neocortical development.



Figure 2. Hs-HARE5 Activity Drives Robust, Early Enhancer Activity Relative to Pt-HARE5 during Corticogenesis

(A–L) Developmental time series of *Pt-HARE5::LacZ* (A, D, G, and J) and *Hs-HARE5::LacZ* (B, E, H, and K) reporter activity from stable transgenic lines. Representative images of LacZ stained embryos from lateral (top) and anterior (bottom) views are shown. Enhancer activity was qualitatively scored in the telencephalon, using the indicated scoring schema shown on the right, on a scale from no reporter activity (score 0) to full telencephalic activity (score 5) (C, F, I, and L). The number of embryos and independent transgenic lines analyzed for each stage is listed below. Embryos were scored blindly and independently by at least three individuals.

(M) Schematic of destabilized reporter constructs drawn to scale.

(N–AA) Representative embryos from dual reporter transgenic *Pt–HARE5::tdTomato*; *Hs–HARE5::EGFP* E11.0 (N–T) and E12.5 (U–AA) embryos detected by brightfield (N and U), and endogenous fluorescence for tdTomato (O, Q, S, V, X, and Z) and EGFP (P, R, T, W, Y, and AA) channels. Dotted lines demarcate dorsal neocortices of whole-mount embryos (N–P and U–W).

(Q, R, X, and Y) Coronal sections from mid-cortex (plane indicated by arrowheads in N and U) in tdTomato (Q and X) and EGFP (R and Y) channels.

(S, T, Z, and AA) High-magnification images of the lateral telencephalon for tdTomato (S and Z) and EGFP (T and AA). The number of embryos and lines for each analysis is listed beside (U). Endogenous fluorescence images were captured using identical exposure conditions.

(BB) Graph depicting log fold changes for qRT-PCR from E12.5 neocortices. Each data point is the average fold change for an individual *Hs-HARE5::EGFP* embryo relative to the aggregated average for all *Pt-HARE5::tdTomato* embryos. mRNA input levels were normalized to *Gapdh*. n = 4 technical replicates per embryo; n = 9 embryos from three transgenic lines from each genotype.

Scale bars represent 1 mm (A-K), 500 µm (N-P and U-W), 150 µm (Q, R, X, and Y), and 25 µm (S, T, Z, and AA). See also Figure S2 and Table S2.



Figure 3. 3C Analysis Showing that *HARE5* Physically Contacts the *Fzd8* Promoter

(A) Schematic of 3C protocol showing HARE5 and Fzd8 loci (black bars), with the indicated TaqMan probe (blue bar), test primers (black half arrows), and HindIII restriction sites (red lines).

(B) 3C assay of E12.5 mouse neocortices (blue dots) and liver control tissue (red dots). The dark vertical line indicates location of TaqMan probe and constant primer anchored within the *Mm*-HARE5 locus. The 0 position indicates ATG of *Fzd8* coding sequence. The graph depicts the relative frequency of interactions between *Mm*-HARE5 and six genomic locations. Error bars indicate the SD.

See also Figure S3.

Human HARE5 Accelerates Neural Progenitor Cell Cycle and Impacts Neocortical Size

We next assessed the functional consequences of chimpanzee and human HARE5 activities during corticogenesis. We generated new independent transgenic mouse lines in which Hs-HARE5 or Pt-HARE5 drove expression of a MYCtagged mouse Fzd8 coding sequence (Pt-HARE5::Fzd8 and Hs-HARE5::Fzd8; Figure 4A). Expression of MYC in embryonic neocortices was confirmed by western blot analysis (Figure S4A). We postulated that Fzd8 expression driven by the HARE5 enhancer would impact the cell-cycle state of neural progenitors based upon the following rationale. First, both Hs-HARE5 and Pt-HARE5 drive expression in neural progenitors. Second, modulation of Fzd8 levels impacts the neural progenitor cell cycle in the retina [18]. Third, overexpression of stabilized β -catenin, a Wnt signaling component downstream of Frizzled, induces an expanded and gyrencephalic brain and slows cell-cycle exit of neural stem cells in mice [15]. Fourth, cell-cycle length is critical for corticogenesis and is postulated as a likely mechanism for the evolutionary expansion of the primate neocortex [35, 36].

We measured the cell-cycle state of progenitors at E12.5, predicting that species-specific differences in *HARE5* activity would be evident within two days of onset of enhancer activity. At this stage, radial glial progenitors primarily undergo symmetric divisions to expand laterally, but a subset of these divide asymmetrically to produce excitatory neurons [2]. Quantification of G2/M phases using phospho-histone H3 (PH3) staining revealed a significant 1.3-fold increase in the proportion of total PH3-positive cells in *Hs-HARE5::Fzd8* brains relative to both *Pt-HARE5::Fzd8* and non-transgenic wild-type (WT) littermates (Figures 4B–4E). We also observed a trend toward more Pax6-positive radial glia in *Hs-HARE5::Fzd8* brains, with a significant increase relative to the WT (Figure S4B). These snapshot measurements indicate that at E12.5, *Hs-HARE5*-driven expression of *Fzd8* alters the proliferating population. More G2/M-positive progenitors may indicate a faster overall cell cycle with similar G2/M phases or, alternatively, an identical cell cycle with longer G2/M.

To help discriminate between these possibilities, we quantified cell-cycle duration at E12.5. We used a paradigm of 2 hr BrdU exposure and 30 min EdU exposure coupled with Ki67 staining, as previously described [37] (Figure 4F). Both WT and *Pt-HARE5::Fzd8* progenitors cycled for about 12 hr, as previously reported for this age [37, 38]. In contrast, *Hs-HARE5*-driven *Fzd8* expression significantly accelerated both the total cell cycle (to approximately 9.2 hr) and S phase, by 25% (Figures 4G–4J and Table S3). These cell-cycle differences correspond to a 23% shorter G1/G2/M duration (Tc-Ts) of *Hs-HARE5::Fzd8* progenitors compared to *Pt-HARE5::Fzd8* (p = 0.003). Thus, this functional analysis reveals that relative to both the WT and *Pt-HARE5::Fzd8*, human *HARE5*-directed expression of *Fzd8* accelerates neural progenitor cell cycle.

Increased proliferation of neural progenitors is frequently associated with changes in brain size. Therefore, we measured the cortical dimensions of transgenic E18.5 brains. Compared to Pt-HARE5::Fzd8 and WTs, the dorsal area of Hs-HARE5::Fzd8 cortices was significantly larger by 12% (Figures 4K-4O). Across five additional measurements, Hs-HARE5:: Fzd8 cortices were consistently larger than both Pt-HARE5:: Fzd8 and WTs (Figures S4F-S4H). As a larger cortical area could be due to increased cortical thickness or tangential length, we quantified these dimensions in sagittal and coronal sections (Figures 4P-4S). Hs-HARE5::Fzd8 brains were thinner than Pt-HARE5::Fzd8 and WT brains, although differences were only significant in comparison to the WT (Figure S4I). In contrast, compared to both Pt-HARE5::Fzd8 and WTs, Hs-HARE5::Fzd8 brains showed significantly longer tangential distance along the cortical VZ (Figure 4S). As seen in other mutants with longer tangential growth, Hs-HARE5::Fzd8 brains also showed enlarged ventricles. The increased tangential length phenotype is often associated with greater progenitor proliferation and larger cortical size, as evidenced in mouse embryonic brains mis-expressing β -catenin or FGF2 [15, 39]. These data indicate that tangential expansion is a likely contributing factor for the increased cortical area.

We predicted that faster progenitor proliferation would ultimately be associated with more neurons. To test this, we quantified the densities of FoxP1-positive neurons (mid-layers III-V), born between E13.5 and E16.5, and FoxP2 neurons (deep-layer VI), born around E12.5 (Figures 4T-4AA), within radial columns of E18.5 brains [40, 41]. Compared to chimpanzee, *Hs-HARE5::Fzd8* brains showed a significant 14% increase in the density of FoxP1 neurons, but no difference in FoxP2 neurons, nor any notable apoptosis. Thus, *Hs-HARE5::Fzd8* brains contain a higher density of neurons that are produced beginning around E13.5. Together, these data indicate that compared to *Pt-HARE5*, *Hs-HARE5* promotes



Figure 4. Hs-HARE5-Driven Expression of Fzd8 Accelerates the Cell Cycle of Neural Progenitors and Increases Neuron Number and Neocortical Size (A) Schematic of Pt-HARE5::Fzd8 and Hs-HARE5::Fzd8 constructs.

(B–I) Images of coronal sections from E12.5 WT littermate (B and G), *Pt-HARE::Fzd8* (C and H), and *Hs-HARE5::Fzd8* (D and I) transgenic cortices. Sections were stained for PH3 (green) and Hoechst (blue) (B–D) or BrdU (green) and EdU (red) (G–I). A graph of WT (white), *Pt-HARE::Fzd8* (gray), and *Hs-HARE5::Fzd8* (black) depicting percentage of all cells that are PH3-positive is shown in (E). The paradigm for analysis of cell-cycle length using double pulse of BrdU and EdU is shown in (F). Nucleotide analogs were injected at the indicated time points, and overall cell-cycle length (Tc) and S phase length (Ts) were calculated as shown.

(J) Graph of WT (white), Pt-HARE::Fzd8 (gray), and Hs-HARE5::Fzd8 (black) cell-cycle lengths of cycling progenitors.

(K–M) Whole-mount E18.5 brains from the indicated genotypes (n, number of brains examined). A dotted line was drawn on the WT cortex in (K) to indicate dorsal cortical area and was then superimposed on transgenic cortices in (L) and (M).

(N) Schematic cartoon representation of E18.5 brain with indicated regions of analyses for sagittal sections (P–S) and coronal sections (T–AA).

faster progenitor cell cycle, which is ultimately associated with increased Foxp1 excitatory neuron density, and overall larger cortical size.

Discussion

The neocortex expanded spectacularly during human evolution, giving rise to distinctively human anatomical and cognitive capabilities [1, 2, 20–22]. Yet to date, just a handful of genetic loci have been associated with human-specific brain traits [3, 5, 25], and none have been shown to functionally impact corticogenesis in an evolutionarily divergent fashion. In this study, we report the discovery of the first human-accelerated enhancer that functions in brain development. We demonstrate dramatic temporal and spatial differences in activity of human and chimpanzee enhancers of *FZD8* during early corticogenesis and show that these differences impact neural progenitor cell cycle and brain size. Our study suggests the intriguing hypothesis that evolutionary changes in *HARE5* sequence and activity contributed to the origin of unique features of the human brain.

The evolutionarily divergent activities of *HARE5* support a model proposed 16 years ago by Pasko Rakic: that species differences in progenitor proliferation may contribute to distinctions in brain size between humans and non-human primates [36]. The proposed radial unit hypothesis predicts that the number and proliferative capacity of progenitor cells drives the evolution of brain cytoarchitecture and explains species differences in neocortical size and structure. Indeed, both empirical and predicted measurements of the neural progenitor cell cycle reveal stark differences between humans, non-human primates, and mice [1, 36, 42]. In non-human primates, distinct G1 phase durations are associated with unique brain cytoarchitecture [35]. Moreover, genetic evidence strongly supports a causal link between neural stem cell proliferation and human brain size [43].

How might a faster cell cycle impact human brain size? We speculate that in the context of extended human corticogenesis and gestation, *HARE5* increases progenitor proliferation, which expands the progenitor pool during early corticogenesis. Increased progenitor expansion would ultimately produce more neurons and a larger neocortex. This could involve altering progenitor cell-cycle exit and/or the division state of progenitors from neurogenic to proliferative. In E14.5 mice, proliferating and neurogenic neural progenitors have distinct S phase durations [44]. Experimental shortening of the G1 phase in mice promotes proliferative divisions in lieu of neurogenic divisions, impacting neuron production [45, 46]. Our study implicates shorter G1 as a potential mechanism, as the Tc-Ts fraction was shorter in human transgenic brains. Follow-up studies of the *Hs-HARE5::Fzd8* mouse will clarify the detailed relationship between altered cell cycle and brain size and elucidate whether modifications in structural and behavioral traits exist.

We have shown that a key target gene of HARE5 activity in the neocortex is FZD8, which encodes a Wnt receptor. Given the neurogenesis roles of β -catenin and Lef/Tcf, it is likely that FZD8 acts via canonical Wnt signaling [16]. FZD8 expression in the neonatal human brain is highest in cortical areas at 9 weeks post-conception (http://brainspan.org) [19], when neural stem cells are rapidly expanding during early corticogenesis [2], but is markedly lower in non-cortical areas. The FZD8 expression pattern correlates strongly with the neural stem cell markers SOX2 and PAX6 (r > 0.90) [19, 47]. Hence, the pattern of HARE5 activity and FZD8 expression is consistent with a functional relationship in neural stem cell regulation in humans. Although chimpanzee expression data are not available, developing rhesus macaque (Macaca mulatta) neocortical data are (http://www.blueprintnhpatlas.org). Relative to ten common transcripts of human and macaque developing neocortices, FZD8 was more abundant in humans. As RNA expression data become available [48], it may become possible to more directly compare FZD8 levels in human and non-human primates.

In addition to its requirement for early mouse corticogenesis, Wnt signaling is implicated in human brain traits. In 2002, Chenn et al. showed that expression of stabilized β -catenin induced a larger, gyrencephalic phenotype reminiscent of the human brain [15]. However, evidence for the involvement of this pathway in human brain evolution has remained elusive until now. Our identification of *HARE5* highlights the transcriptional regulation of Wnt signaling components as a new avenue to explore for understanding the evolutionary origin of human-specific anatomical and cognitive traits. With the ability to identify regulatory elements active during development [49], we are now poised for the discovery of additional loci and pathways whose modification provided the underpinnings for the evolution of the human brain.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.01.041.

Author Contributions

J.L.B., G.A.W., and D.L.S. conceived the study and wrote the paper. J.L.B., S.L.S., J.P.R., L.-J.P., T.B., R.G., and D.L.S. performed and analyzed experiments.

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⁽O) Graph of WT (white), Pt-HARE::Fzd8 (gray), and Hs-HARE5::Fzd8 (black) dorsal cortical area measurements. Note that a 12% increase was seen in Hs-HARE5::Fzd8 cortical area.

⁽P–R) Sagittal E18.5 sections from brains of indicated genotypes. A line drawn on the WT cortex in (P) indicates ventricular length and was superimposed on transgenic cortices in (Q) and (R). Note no evidence of cortical gyrification was seen.

⁽S) Graph depicting ventricular length for indicated genotypes.

⁽T–V and X–Z) Coronal E18.5 sections from neocortices of indicated genotypes and stained for Foxp1 (T–V) and Foxp2 (X–Z). Note no significant apoptosis was observed.

⁽W and AA) Graphs depicting densities of Foxp1 (W) and Foxp2 (AA) neurons in radial columns of neocortical sections.

The following were analyzed for each genotype: for (B)–(E), n = 5 embryos each from three transgenic lines; for (F)–(J), five to seven embryos each from two to three transgenic lines; for (K)–(O), 16–57 embryos each from two to three transgenic lines; for (P)–(S), four to five embryos each (two to five sections per embryo) from two to three transgenic lines; and for (T)–(AA), five to six embryos each (two to four sections per embryo) from two to three transgenic lines; and for (T)–(AA), five to six embryos each (two to four sections per embryo) from two to three transgenic lines. All analyses were done blind to genotype. Error bars indicate the SD. *p < 0.05, **p < 0.01, ***p < 0.001. Scale bars represent 25 μ m (B–I), 1 mm (K–M), 500 μ m (P–R), and 100 μ m (T–Z). See also Figure S4 and Table S3.

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Current Biology Supplemental Information

Human-Chimpanzee Differences

in a FZD8 Enhancer Alter Cell-Cycle

Dynamics in the Developing Neocortex

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Supplemental Data





C Hs-HARE5::LacZ E14.5



Figure S1, Related to Figure 1. Screen for evolutionarily important enhancers. (A) Schematic workflow for identifying human-accelerated brain enhancers, as described in Supplemental Experimental Procedures. 106 unique putative neocortical enhancer loci with signatures of positive selection were identified from the screen (see also Table S1). (B) Images of E14.5 representative transient transgenic embryos for HARE1-6 assayed for β -galactosidase activity, and listed with relevant information. HARE1-6 were prioritized from the screen based on their proximity to genes known or predicted to be involved in corticogenesis. Selection scans are from following references (first author name listed): human-accelerated conserved noncoding sequence (Prabhakar) [S1]; accelerated conserved noncoding sequence (Bird) [S2]; and primate accelerated region (Lindblad-Toh) [S3]. Chip-Seg are from the following references: H3K4me1 [S4], P300 [S5]. HAREs1, 2, and 4 showed variable enhancer activity, whereas HAREs 3, 5, and 6 demonstrated more consistent enhancer activity. C) Images of E14.5 transient transgenic embryos for *Hs*-HARE5::*LacZ* assayed for β-galactosidase activity. Note HARE5 activity in the lateral forebrain and dorso-lateral midbrain, and to a lesser and more variable extent in the spinal cord and retina. Scale bars, 2mm.









Figure S2, Related to Figure 2. HARE5 activity in the lateral telencephalon. (A-C) Rostral to caudal coronal sections from E10.5 *Hs-HARE5::LacZ* brains depicting LacZ activity in the lateral telencephalon with a graded dorso-ventral boundary. (D) Comparison of rostro-caudal matched coronal sections from Hs-HARE5::LacZ and Pt-HARE5::LacZ E10.5 embryos. Arrows indicate boundary of uniform LacZ activity and arrowheads indicate boundary of graded LacZ activity. (E-H) Immunofluorescence of E12.5 coronal sections co-expressing *Pt-HARE5::tdTomato* and *Hs-HARE5::EGFP* at low magnification (E,F) and high-magnification along the lateral wall (G,H), and stained with anti-RFP (E, G) and anti-EGFP (F,H). td-Tomato was recognized by anti-RFP. Note both reporters are active in the VZ/SVZ of the lateral telencephalon. (I-U) Immunofluorescence of E10.5 coronal sections co-expressing Pt-HARE5::tdTomato (I-K; P-R) and Hs-HARE5::EGFP (L-N; S-U) and stained with anti-RFP (I,P), anti-EGFP (L,S), anti-Pax6 (J,K,M,N) or anti-TuJ1 (Q,R,T,U). Note both Hs-HARE5 and Pt-HARE5 activity are high in Pax6-positive neuroepithelial cells and are also evident in some TuJ1-positive neurons. (O) Graph depicting fraction of all Pax6-positive cells that costain for either RFP (*Pt-HARE5::tdTomato*) or EGFP (*Hs-HARE5::EGFP*). Error bars, s.d. ns, not significant. Scale bars, 100 µm (A-F), 20 µm (G,H), 25 µm (I-N, P-U).



Figure S3, Related to Figure 3. Hs-HARE5 activity and mouse Fzd8 mRNA expression. (A-E) E10.5 whole mount lateral views (A,B) and E11.5 sagittal sections (C-E) from Hs-HARE5::LacZ embryo stained for LacZ activity (A,C,E) or an in situ Fzd8 hybridization of mouse mRNA (B,D). In situ images are from www.emouseatlas.org [S6] (B), and http://developingmouse.brain-map.org (D). Note Hs-HARE5 and Mm-HARE5 share 51.1% sequence identity, as typical for highly conserved noncoding loci [S2, S3]. (E) High magnification image of Hs-HARE5::LacZ stained section in C. Scale bars, 1 mm (A-D) 590 µm (E).



Figure S4, Related to Figure 4. Analysis of *Pt-HARE5::Fzd8* and *Hs-HARE5::Fzd8* **brains.** (A) Representative western blots of cortices from *Pt-HARE5::Fzd8* and its control littermate (E11.5) and *Hs-HARE5::Fzd8* and its control littermate (E12.5). A

10% SDS-PAGE gel was probed for anti-MYC, and anti-Actin for a loading control. Note a major band of 75KDa is evident in both transgenic samples but not in control samples. No significant difference in MYC levels was detected between *Pt-HARE5::Fzd8* and *Hs-HARE5::Fzd8* samples in replicate westerns. (B) Graph depicting quantification of Pax6positive cells in control (white), *Pt-HARE5::Fzd8* (gray), and *Hs-HARE5::Fzd8* (black) brains. (C-E) Coronal E12.5 sections from indicated *Fzd8* transgenic lines, stained for Ki67. (F) Cartoon representation of E18.5 brains with indicated measurements for G,H shown. (G,H) Graphs depicting measurements of E18.5 whole mount brains of indicated genotypes. Note *Hs-HARE5::Fzd8* cortices trended to be larger across all measurements, with significance indicated. Included in the bars are the % increases above WT levels. (I) Graph depicting measurement of coronal sections (shown in Figure 4) from E18.5 neocortices of indicated genotypes. Scale bars, C-E, 25 µm. Error bars, s.d., *, *P* < 0.05, **, *P* < 0.01, *** *P*<0.001, ns, not significant.

Epigenetic	Epigenetic mark	P300	Pax6	Sox2	H3K4me1	H3K27ac
datasets	Tissue	E11.5	E12.5	E12.5	Neural	Neural
		forebrain	cortex	Neural	progenitors	progenitors
		[S5]	[S9]	stem	[S4]	[S11]
				cells		
				[S10]		
	# Loci (hg19)	2949	2433	8301	47,258	8,735
Human-	Prabhakar [S1]	10	0	2	24	4
accelerated	Bird [S2]	19	1	2	22	3
datasets	Pollard [S12]	1	0	0	1	0
	Lindblad-Toh	6	0	3	10	0
	(human-					
	accelerated)					
	[S3]					
	Lindblad-Toh	3	0	4	6	4
	(primate-					

	accelerated) [S3]					
Summary	Total	39	1	11	63	11
	epigenetic/HA intersections					

Table S1. Identification of human/primate-accelerated loci with enhancer marksfrom in silico screen.Related to Figure 1. Note 125 candidates overlapping wereidentified (106 are non-overlapping).

Table S2. Transcription factor prediction analysis. Related to Figure 2.

Hs-HARE5 gained binding sites for myc (#1,8,15), a positive regulator of neural stem cell proliferation [S7], and lost binding sites for myc repressors (#8,15), including 2 human-derived and 1 chimpanzee-derived mutations. *Hs-HARE5* lost binding sites for Lef/Tcf (#1,9), which mediates repressor activity in the absence of Wnt signaling, both in human-derived mutations [S8].

Genotype	Tc (hr)	Ts (hr)	Tc-Ts (hr)
WT	12.01 <u>+</u> 0.73	6.23 <u>+</u> 0.29	5.78 <u>+</u> 0.83
Pt	12.15 <u>+</u> 1.14	6.34 <u>+</u> 0.96	5.81 <u>+</u> 0.65
Hs	9.15 <u>+</u> 0.88	4.61 <u>+</u> 0.41	4.45 <u>+</u> 0.65
<i>P-value</i> Hs vs WT	1.5x10 ⁻⁴	2x10 ⁻⁵	0.016
<i>P-value</i> Hs vs Pt	1.4x10 ⁻⁴	0.001	0.003

Table S3. Analysis of cell-cycle kinetics in WT and transgenic E12.5 embryos.

Related to Figure 4.

Supplemental Experimental Procedures

Bioinformatics and in silico screen

We propose the term human-accelerated regulatory element (HARE) to denote loci that have been bioinformatically identified as rapidly evolving in humans and empirically demonstrated as in vivo enhancers. We identified putative HAREs from an in silico screen using the following approach (outlined in Figure S1A). Enhancer candidate loci were obtained from publicly available datasets containing empirically identified enhancers. These were ChIP-seq studies of mouse embryonic neocortical tissue or neural stem cells including: p300 [S5], PAX6 [S9], SOX2 [S10], H3K4me1 [S4] and H3K27ac [S11]. We then identified regions of overlap between these enhancer loci and human-accelerated noncoding regions derived from published datasets [S1-3, S12]. All human genomic intervals were then converted to hg19 with the Convert Genome Coordinates tool (v1.0.3) using default settings. Mouse genomic intervals (mm9) were converted to orthologous human coordinates (hg19) with the Extract Pairwise MAF blocks tool (1.0.1) using default settings. MAF blocks were then converted to the BED format using the Maf to BED tool (v.1.0.0) under default parameters. Intersections between human enhancer regions mapped from the mouse genome and human noncoding accelerated regions were determined using the Intersect tool (v.1.0.0) with at least a 1 bp of overlap. Phylogenetic analysis of the HARE5 locus was performed with MEGA (v5.2.1)[S13] on orthologous HARE5 loci obtained from the UCSC Genome Browser Convert to New Genome tool. The following HARE5 sequences were used for phylogenetic analysis and cloning: Homo sapiens: chr10:36238121-36239339 (hg19); Pan troglodytes: chr10:36445561-36446779 (panTro4); Gorilla gorilla: chr10:393994853940070 (gorGor3); *Pongo borneo*: chr10:37048530-37049744 (ponAbe2); *Macaca mulatta*: chr9:36159246-36160487 (rheMac2). We constructed a maximum likelihood tree using the Hasegawa-Kishino-Yano model and a gamma distribution of rates among sites with 5 discrete categories. We utilized the Galaxy Project platform for analyzing genomic datasets [S14].

Sub-cloning of transgenic constructs

For all reporter constructs, enhancer loci were amplified from human genomic DNA (GM12154) and chimpanzee (#5006007) genomic DNA using Physion High Fidelity Polymerase and directionally cloned into the Gateway entry vector pENTR/D-TOPO (Invitrogen) following the manufacturer's recommendations. DNA reporter constructs were generated containing the original human-accelerated locus, and flanking 5' and 3' sequences, which have been shown to have accessory regulatory motifs required for enhancer activity (total length of constructs, 1219 nt) [S15-17]. Clones were then subcloned into the minimal promoter hsp68::LacZ destination vector (kind gift of Len Pennacchio, Lawrence Berkeley National Laboratory). We generated new fluorescent reporter constructs by modifying the minimal promoter *hsp68*::3nls-*CFP-PEST* vector (generous gift from Jèròme Collignon, Universitè Paris-Diderot). The 3xNLS (nuclear localization sequence) was removed by first amplifying the CFP gene without the 3xNLS using primers engineered with BamHI (including a Kozak sequence and the first 5 codons of CFP) and a Blpl restriction site positioned upstream and downstream, respectively, of the reporter gene. The plasmid was then digested with BamHI and BlpI

restriction enzymes, which removed both the 3xNLS and *CFP* reporter, and re-ligated back together with the PCR amplified *CFP* gene lacking the 3xNLS. We subsequently replaced the *CFP* reporter gene with PCR amplified *tdTomato* and *EGFP* genes engineered with BamHI and BlpI restriction sites. Enhancer loci were subcloned into the multiple cloning site of either *hsp68::EGFP-PEST* or *hsp68::tdTomato-PEST*. Reporter constructs *Hs-HARE5-hsp68::EGFP-PEST* (linearized with XbaI, XhoI, and SaII-HF) and *Pt-HARE5-hsp68::tdTomato-PEST* (linearized with XhoI, DraIII, and BstB1) were purified by gel electrophoresis. We generated *Hs-HARE5::Fzd8* and *Pt-HARE5::Fzd8* constructs by first synthesizing mouse *Fzd8* cDNA downstream of the proximal promoter region of hsp68 flanked by XhoI and XbaI restriction sites (GenScript). This cassette was transferred to pCAG using XhoI/XbaI and *Hs/Pt-HARE5* was subcloned with XhoI and EcoNI. All DNA sequences used for generation of transgenic mice were fully sequenced.

Identification of putative transcription factor (TF) binding sites in HARE5

To identify potential TF binding sites, we used *in vitro* DNA binding specificity data generated using universal protein-binding microarray (PBM) assays [S18]. PBM data can be used to derive TF-DNA binding motifs, but in addition it yields measurements of TF binding specificity for all possible 8-bp sequences (8-mers), thus providing a more comprehensive view of TF binding preferences compared to DNA motifs. The relative binding preference of a TF for each 8-mer on universal PBMs is quantified by the PBM enrichment score (E-score) [S18, S19]. The E-score is a modified form of the Wilcoxon-Mann Whitney statistic and ranges from -0.5 (least favored sequence) to +0.5 (most

favored sequence), with values above 0.35 corresponding, in general, to sequencespecific DNA binding of the tested TF [S18]. We used 612 uPBM data sets for human and mouse TFs from the UniPROBE database and other resources [S20-23]. For each PBM data set, we scanned both the human and the chimpanzee enhancers to identify putative TF binding sites, defined as sites containing at least two consecutive 8-mers with E-score > 0.35, similarly to the procedure used in [S24]. Next, we focused on the specific mutations in the *HARE5* locus, and we identified TFs for which: 1) a putative binding site was predicted in only one of the two lineages, and 2) the difference in Escore between the human and the chimpanzee site was at least 0.2. The selected TFs were used in the analyses presented in the Table S3.

Mouse genetics and embryonic analysis

All experiments were performed in agreement with the relevant regulatory standards from the Division of Laboratory Animal Resources and IACUC at Duke University School of Medicine. DNA for pronuclear injection was prepared by digesting fully constructs with Xhol/Xbal/Sall-HF (NEB) and purified by electrophoresis (QiaEX II, Qiagen). All linearized constructs were submitted to the Duke Transgenic Mouse Facility for pronuclear injection into B6SJLF1/J strain blastocysts (F1 hybrid from C57BL/6J X SJL/J). For the initial screen transient transgenics were tested for enhancer activity using a standard enhancer assay for β -Galactosidase activity. This approach can capture spatial and temporal differences in enhancer function (see [S25, S26] for relevant discussion). For all transgenic analyses, following pronuclear injection 6SJLF1/J founders were backcrossed to C57BL/6J for subsequent analysis and breeding. The following transgenic lines were generated, with number of independent lines shown in parentheses: *Pt-HARE5::LacZ* (3), *Hs-HARE5::LacZ* (4), *Pt-HARE5::tdTomatoPEST* (3), *Hs-HARE5::EGFP-PEST* (3), *Pt-HARE5::Fzd8* (3), and *Hs-HARE5::Fzd8* (3). All analyses of enhancer LacZ activity, fluorescence activity, and functional analyses were performed blindly with respect to genotype, and in most cases by multiple investigators.

Imaging and immunofluorescence staining of transgenics

LacZ enhancer activity was detected as previously described [S15, S27]. Images of whole-mount LacZ or fluorescent embryos were obtained on a Leica M165 FC microscope using the Leica Application Suite software package (v.4.1.0). For sectioning LacZ embryos, previously fixed embryos were washed in 1X PBS, incubated overnight in 30% sucrose and then embedded in OCT. For sectioning all other embryos, mouse embryos were fixed in 4% paraformaldehyde for 2 hours at room temperature or overnight at 4°C and washed 3 x 5 min in 1X PBS solution before an overnight incubation with 30% sucrose at 4°C prior to OCT embedding. Tissue sections were cut 20 μ m thick on a cryostat and transferred to Super-Frost Plus slides (Thermo Scientific). Tissue sections were permeabilized with 0.25% triton-X100 in PBS, stained in primary antibodies for 2 hr. at room temperature or overnight at 4°C as previously described [S28] using the following antibodies: mouse anti-TuJ1 (Covance, 1:400), rabbit anti-Pax6 (Millipore, 1:1,000), mouse anti-Pax6 (DSHB, 1:64), mouse anti-MYC (Cell

Signaling, 1:200), rabbit anti-Ki67 (Abcam, 1:200), rabbit anti-RFP (Rockland, 1:400), rabbit anti-Foxp1 (Abcam, 1:200), rabbit anti-Foxp2 (Abcam, 1:1000), and chicken anti-EGFP (Abcam, 1:200). EdU stained sections were obtained using Click-iT EdU Alexa Fluor 594 Imaging Kit (Invitrogen) using manufacturer specifications. Fluorescent images were collected on a Zeiss Observer Z1 microscope using ApoTome optical sectioning. Images of native fluorescent proteins were acquired using the Zeiss 43 HE DsRed (Excitation: 545/15) and 38 HE Green Fluorescent (Excitation: 470/40) and Leica GFP3 ET (Excitation: 470/40) and DSR ET (Excitation: 545/30) filter sets.

RT-qPCR Analysis

RNA was extracted from microdissected E10.5 mouse neocortices stored in TRI reagent (Sigma, Cat #T9424) and stored at -80°C. RNA was extracted according to manufacturer recommendations. RNA samples were treated with DNasel and cDNA was synthesized with iScript cDNA Synthesis Kit (Bio-Rad, Cat # 170-8891). qPCR was performed using FastStart Universal SYBR Green Master (Rox) (Roche Applied Science Cat # 04913922001) or Fast SYBR Green Master Mix (Applied Biosystems, Cat # 4385612) using cycling conditions recommended by the manufacturer on an ABI StepOnePlus Real-Time PCR machine. Primers (see Table S4) for tdTomato and EGFP were designed using Primer3 (v.0.4.0) in order to produce similarly sized amplicons under identical cycling conditions. Similar amplification efficiencies for both primer sets were obtained.

Chromosomal Confirmation Capture (3C) Analysis

3C assays were performed as previously described [S29]. Embryonic neocortices and reference liver tissues were obtained from pools of 10-16 E12.5 embryos. Neocortical dissections included both the dorsal and ventral telencephalon. Tissue samples were dissociated in 0.125% collagenase type I shaking for 1-2 hr. at 37°C and fixed with 2% formaldehyde/10%FBS/PBS for 10 min at room temperature. Cell nuclei were prepared from aliquots of 10 million cells and stored at -80°C until chromatin preparation. Cell nuclei were digested with HindIII (New England BioLabs) overnight and re-ligated with T4 DNA ligase-HC (Promega) for 1-2 days. Control template DNA for guantification of 3C ligation products and normalization was generated from HindIII digested and religated BAC DNA covering the mouse HARE5 (RP23-137B19; CHORI), mFzd8 (RP23-292B21; CHORI), and Ercc3 (RP23-148C24; CHORI) internal control locus. Re-ligation events from chromatin and BAC DNA preparations were detected using TagMan Gene Expression Master Mix (Applied Biosystems) and double-dye labeled oligonucleotide probes (PrimeTime Integrated DNA Technologies). All reactions were performed in guadruplicate. Quantification of ligation events were determined from standard curves of re-ligated BAC DNA covering the locus of interest and normalized against the internal control *Ercc3* locus. Measurements are reported from at least four biological replicates.

Cell cycle analysis and quantitation

Analysis of cell-cycle kinetics was performed as previously described [S30, S31]. Briefly, pregnant dams were IP injected with BrdU at T=0 (70 μ g/g of body weight), EdU

(10 μ g/g of body weight) at T=1.5 hours, and sacrificed at T=2 hrs. Embryos were immediately fixed in 4% paraformaldehyde overnight, and embedded in OCT, and then cryosectioned. For detection of BrdU, samples were citrate boiled (Vector Laboratories, Cat H-3300) for at least 30 min at 98°C and then incubated 2 hrs with rat anti-BrdU (1:200, Abcam) followed by addition of secondary anti-rat (1:400, Molecular Probes). Detection of EdU was performed after secondary antibody incubation under conditions specified by the manufacturer (Life Technologies, Click-iT 594 or 647). Calculation of total cell cycle length (Tc) and length of S phase (Ts) were performed as follows: the portion of cells actively in the S-phase cells (S cells) was determined from the number of EdU+ cells, the leaving fraction (L cells) was determined by the number of BrdU+/EdUcells, the total number of proliferating cells (P cells) was determined by Ki67+ cells (1:100). Quantification of all cell cycle parameters were determined with at least 5 nonconsecutive slides stained for each marker and were counted blind to genotype using ImageJ software. Quantification of Foxp1 and Foxp2 neurons and Pax6 progenitors was performed using radial columns of coronal cortical sections counted with Image J software. For E18.5 brains, images of E18.5 embryos were captured using a Leica M165 FC microscope with the Leica Application Suite software package (v.4.1.0). Quantitation of tangential length (the distance between posterior and anterior boundaries of the ventricle in sagittal sections), cortical thickness (measured in coronal sections), and whole mount cortical measurements were performed using ImageJ (v1.48s). Measurements were made blind to genotype and in many cases by multiple investigators.

Western blotting

Cortical protein samples and Western blotting were performed as previously shown [S28], with the following modification. In order to resolve and optimally transfer the FZD8 membrane protein, gels were transferred overnight in cold at 16 volts, and samples in SDS-Page buffer were not boiled but instead warmed at room temperature for 2 hours. Blots were probed with mouse anti-Myc (Cell Signaling, 1:1000) and mouse anti-actin (Sigma, 1:200), recognized with appropriate secondary antibody (Amersham) and developed using ECL reagent (Pierce).

HARE Primers			
HARE1.F	CACCCCACTCACTCCACAAGCATC		
HARE1.R	GAATTGCCACTTCTCCACA		
HARE2.F	CACCTCTCCCCAGTTGGATAGAGTAA		
HARE2.R	ACCACACTTGAGGCTCTGGA		
HARE3.F	CACCTGACCAAGACAGAAGGGAAAA		
HARE3.R	ATTTGCTTGGAAAAAGAACCA		
HARE4.F	CACCGACATGCACTCTCCTCTCCTG		
HARE4.R	AGGGAGACTGATTTTCAAGCA		
HARE5 .F	CACCCACAGAGGTTGGGGCACA		
HARE5.R	CCAGTGGAAGGCGATAAGAG		
HARE6.F	CACCGGGAGAAGGAAAAACGAAGG		
HARE6.R	TATTGCTTGAATTGCCCAAAC		
Sub cloning Primers			
CFP.BamHI	ATTAGGATCCGCCACCATGGTGAGCAAGGG		
CFP.Blpl	AGCCATGGCTAAGCTTCTTGTACAGCTC		
td.tomato.BamHI	ATTAGGATCCAGCCACCATGGTGAGCAAGGG		
td.tomato.Blpl	GCATGATGCTAAGCTTGACGGTCCGCTTGTACA		
EGFP.BamHI	ATTAGGATCCAGCCACCATGGTGAGCAAGGGC		
EGFP.Blpl	CCATGGCTAAGCAATCTAGATCCGGTGGA		
RT-qPCR Primers			
tdTomato.F	ACCATCGTGGAACAGTACGAG		

tdTomato.R	CTTGAAGCGCATGAACTCTTT
EGFP.F	GAAGCAGCACGACTTCTTCAA
EGFP.R	AAGTCGATGCCCTTCAGCTC
GAPDH.R	ACCACAGTCCATGCCATCAC
GAPDH.R	CACCACCCTGTTGCTGTAGCC
3C Primers	
Ercc3.F	GCCCTCCCTGAAAATAAGGA
Ercc3.R	GACTTCTCACCTGGGCCTACA
Mm-HARE5 constant	AGTCTGAGCACCAAATTCAGG
Mm-Fzd8.Test1	TCCTTTCAGACACAAGCATAAG
Mm-Fzd8.Test2	GATGGATTTCCAGAGTGGTTG
Mm-Fzd8.Test3	TTGGCCTTTGTTCTACTTGAG
Mm-Fzd8.Test4	CAAAGAGAAGTTTGAACAAGCA
Mm-Fzd8.Test5	GGCACAGAAAAATGGAGAAAT
Mm-Fzd8.Test6	TTGACAGTGTCCTTTGCCTTA
3C TaqMan Probes	1
Mm-HARE5	AAATGAATTATTTTCCAAGTTGAATCA-BQH
Ercc3	AAAGCTTGCACCCTGCTTTAGTGGCC-BQH

 Table S4. List of primers used. Related to Experimental procedures.

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