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Inherited coding variants at the CDKN2A locus influence susceptibility to acute lymphoblastic leukaemia in children

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There is increasing evidence from genome-wide association studies for a strong inherited genetic basis of susceptibility to acute lymphoblastic leukaemia (ALL) in children, yet the effects of protein-coding variants on ALL risk have not been systematically evaluated. Here we show a missense variant in CDKN2A associated with the development of ALL at genome-wide significance (rs3731249, \( P = 9.4 \times 10^{-23} \), odds ratio = 2.23). Functional studies indicate that this hypomorphic variant results in reduced tumour suppressor function of p16INK4A, increases the susceptibility to leukaemic transformation of haematopoietic progenitor cells, and is preferentially retained in ALL tumour cells. Resequencing the CDKN2A–CDKN2B locus in 2,407 childhood ALL cases reveals 19 additional putative functional germline variants. These results provide direct functional evidence for the influence of inherited genetic variation on ALL risk, highlighting the important and complex roles of CDKN2A–CDKN2B tumour suppressors in leukaemogenesis.

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The risk of developing acute lymphoblastic leukaemia (ALL) is highest between 2 and 5 years after birth\textsuperscript{1,2}, with initiating sentinel somatic genomic lesions (for example, chromosomal translocations) detectable at the time of birth in many cases\textsuperscript{3,4}. This early disease onset suggests a strong inherited genetic basis for ALL susceptibility, and recent genome-wide association studies (GWAS) have discovered at least six risk loci: \textit{ARID5B, IKZF1, CEBPE, PIP4K2A-BMI1, GATA3} and \textit{CDKN2A–CDKN2B}\textsuperscript{5–10}. These ALL risk genes are directly involved in haematopoietic stem cell function, lymphocyte differentiation and development, and cell cycle regulation\textsuperscript{11–15}, several of which are also commonly targeted by somatic genomic lesions. In particular, the \textit{CDKN2A–CDKN2B} locus is one of the most frequently deleted genomic regions in childhood ALL with focal copy number loss in both B- and T-cell ALL\textsuperscript{14,16}.

The vast majority of variants examined in previous ALL GWAS are intronic or intergenic. Although it is now evident that non-coding variants related to disease traits are significantly over-represented in regulatory DNA and often function as modulators of local or distal gene transcription\textsuperscript{17,18}, questions also arise whether coding variants within ALL susceptibility genes might confer even greater effects on disease development. Moreover, a large number of low-frequency and rare-coding germline variants have been discovered by exome-sequencing efforts\textsuperscript{19}, but their contributions to ALL pathogenesis have yet to be examined systematically.

In the present study, we perform an exome-focused GWAS to systematically examine the impact of germline-coding variants on the development of ALL in children of European descent, experimentally explore the functional consequences of the genome-wide significant variant in the \textit{CDKN2A} gene, and comprehensively characterize coding variation at this locus by targeted resequencing.

**Results**

**Exome-focused GWAS of ALL susceptibility.** In the discovery GWAS, we genotyped 1,773 children with B-ALL and 10,448 non-ALL controls of European descent\textsuperscript{20,21} for 247,505 variants using the Illumina Infinium HumanExome array. Three loci with genome-wide significant association signals were observed: \textit{ARID5B} (10q21.2), \textit{IKZF1} (7q12.2) and \textit{CDKN2A} (9p21.3) (Fig. 1). Non-coding variants rs10821936 in \textit{ARID5B} and rs4132601 in \textit{IKZF1} showed the strongest association ($\text{P} = 9.9 \times 10^{-46}$ and $4.3 \times 10^{-37}$, the logistic regression test, respectively; Fig. 1 and Supplementary Table 1), confirming \textit{IKZF1} have been discovered by exome-sequencing efforts\textsuperscript{19}, but their large number of low-frequency and rare-coding germline variants confer even greater effects on disease development. Moreover, a large number of low-frequency and rare-coding germline variants have been discovered by exome-sequencing efforts\textsuperscript{19}, but their contributions to ALL pathogenesis have yet to be examined systematically.

The risk of developing ALL (CDKN2A–CDKN2B) locus is one of the most frequently deleted genomic regions in childhood ALL with focal copy number loss in both B- and T-cell ALL\textsuperscript{14,16}.

**Functional characterization of the rs3731249 variant.** To experimentally evaluate the effects of rs3731249 on ALL leukemogenesis, we directly compared the effect of wildtype versus variant allele p16\textsuperscript{INK4A} (p.148A versus p.148 T) on \textit{BCR–ABL1}-mediated leukaemic transformation \textit{in vitro}. We chose mouse haematopoietic progenitor Ba/f3 cell line because it is inherently p16\textsuperscript{INK4A}-defective due to methylation at the \textit{Ink4a-Arf} locus\textsuperscript{22}, and ectopic expression of \textit{BCR–ABL1} in Ba/f3 cells efficiently induces exogenous cytokine (interleukin 3 (IL3))-independent proliferation. Over-expression of wild-type p16\textsuperscript{INK4A} (p.148A) significantly inhibited leukaemic transformation by \textit{BCR–ABL1} (Fig. 2a, Supplementary Fig. 2), consistent with its role as a critical tumour suppressor in ALL. In contrast, Ba/f3 cells overexpressing variant p16\textsuperscript{INK4A} (p.148 T) were significantly more susceptible to \textit{BCR–ABL1} transformation measured by IL3-independent growth, suggesting that the p.148 T variant is likely hypomorphic with reduced tumour suppressor function. In Ba/f3 cells transfected with both variant and wild-type p16\textsuperscript{INK4A}, the relative ratio of the p.148 T (variant) to p.148A

![Figure 1: GWAS results of ALL susceptibility in European Americans.](image-url)
Gene (p16INK4A and p14ARF) in children with ALL, there was a trend for the increased leukaemia risk conferred by the variant allele at rs3731249. To further examine the potential susceptibility to ALL conferred by the rs3731249 in patients, we compared the genotype distribution in RNA and DNA from primary leukaemic blasts and matched germline samples from children with ALL (Fig. 2b). Of 15 cases with the heterozygous germline genotype at this SNP, six exhibited somatic deletion of one copy of CDKN2A, all of which retained the risk allele in tumour cells. Even in cases not affected by somatic copy number loss at this locus, the variant p16INK4A (c.442 T) was preferentially transcribed relative to wildtype (c.442C), with allele-biased expression ranging from 61 to 100% (Fig. 2b). Altogether, these results pointed to the possibility that cells carrying the hypomorphic risk allele at rs3731249 might have been enriched during leukemogenesis.

Targeted resequencing of CDKN2A and CDKN2B in childhood ALL. To comprehensively identify putative functional ALL susceptibility variants at this locus, we resequenced the coding region of the CDKN2A and CDKN2B genes in germline DNA from 2,407 childhood ALL cases (1,450 of which were also included in the discovery GWAS). In addition to rs3731249, we observed another 13 germline exonic variants in tumour suppressors p16INK4A and p14ARF encoded by the CDKN2A gene, 12 of which result in amino-acid sequence changes (Fig. 3, Supplementary Table 3). These missense variants were all singletons, except for the p.D125H variant in p16INK4A and the p.A121T variant in p14ARF observed in two and five cases, respectively. Five variants were predicted to be damaging based on combined annotation dependent depletions3 (CADD score > 13, Supplementary Table 3), and we did not observe germline insertions or deletions in CDKN2A in our ALL cohort. Comparing with 4,300 European American individuals from the NHLBI GO Exome Sequencing Project (ESP), there was a trend for a higher burden of rare missense variants in relative to controls the CDKN2A gene (p16INK4A and p14ARF) in children with ALL (0.71% versus 0.23%, P = 0.0045; Fisher’s exact test, Fig. 3). In addition, we identified six germline-coding variants in the adjacent CDKN2B gene in this cohort of children with ALL, although there was no significant over-representation compared with European controls in the ESP cohort (0.83% versus 0.79%, Fig. 3).

Discussion

Encoding three tumour suppressor proteins (p16INK4A, p14ARF and p15INK4B), the CDKN2A–CDKN2B locus at 9p21 is promiscuously associated with tumorigenesis and commonly

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**Table 1 | Genome-wide significant association and replication of novel coding B-ALL susceptibility variant at CDKN2A locus.**

<table>
<thead>
<tr>
<th>SNP ID</th>
<th>Chr</th>
<th>Position*</th>
<th>Gene</th>
<th>Alleles†</th>
<th>Genotype</th>
<th>GWAS series</th>
<th>Replication series</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs3731249</td>
<td>9</td>
<td>21970916</td>
<td>CDKN2A</td>
<td>C/T</td>
<td>TT</td>
<td>Case (N = 1,773)</td>
<td>Control (N = 10,441)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CT</td>
<td>8 (0.45%)</td>
<td>7 (0.07%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>224 (12.63%)</td>
<td>619 (5.93%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>357 (87.07%)</td>
<td>9,815 (94%)</td>
</tr>
</tbody>
</table>

Chr, chromosome; CI, confidence interval; GWAS, genome-wide association studies; OR, odds ratio; SNP, single-nucleotide polymorphism.

*Chromosomal localizations are based on hg19
†Bold denotes the allele that had a significantly higher frequency in children with B-ALL than in the non-ALL controls (that is, risk allele for B-ALL)
‡OR represents the increase in the risk of developing ALL for each copy of the risk allele compared with subjects who do not carry the risk allele; P-values and ORs were estimated by the logistic regression test.

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**Figure 2 | Functional characterization of ALL risk variant at rs3731249.** (a) Mouse haematopoietic progenitor cell Ba/f3 overexpressing wildtype, variant p16INK4A, or transfected with control vector was transduced with leukaemia oncogenic BCR-ABL1 fusion gene. Cell proliferation in the absence of cytokine IL3 was measured daily as an indicator of leukaemia oncogenic transformation. Ectopic expression of p16INK4A (p.148T, green) significantly potentiated leukaemic transformation by BCR-ABL1, compared with cells expressing wild-type p16INK4A (p.148A, blue), consistent with the association of this allele with susceptibility to ALL. Data represent the mean of three replicates ± s.e.m. (b) Allele-specific expression of p16INK4A in ALL blasts was determined by comparing the number of sequence reads for transcripts containing C or T alleles at rs3731249 (p16INK4A p.148A) significantly preferentially transcribed to wild-type p16INK4A (p.148T) in 15 childhood ALL cases with heterozygous genotype in the germline DNA at this SNP. Each dot represents an ALL case (red indicates cases with somatic deletion (loss of heterozygosity) and blue indicates cases without copy number change in tumour) and the line of identity indicate equal expression of both alleles. P-value was estimated by paired t-test based on the number of sequence reads for each allele.
targeted by somatic mutation, deletion and/or hypermethylation in various cancers. p16\textsuperscript{INK4A} and p15\textsuperscript{INK4B} are highly homologous inhibitors of cyclin-dependent kinase and function mainly as master regulators of cell cycle entry via the Rb-E2F signalling axis\textsuperscript{24}. Although also encoded by the CDKN2A gene, p14\textsuperscript{ARF} utilizes a completely different reading frame with distinct tumour suppression functions by inhibiting MDM2 and activating p53\textsuperscript{25}. Suppressed during normal haematopoiesis, p16\textsuperscript{INK4A} and p14\textsuperscript{ARF} expression is activated on oncogenic stimuli (for example, constitutive expression of BCR–ABL1 fusion) to trigger cell cycle exit (senescence) or apoptosis as a means of eliminating oncogene-stressed cells\textsuperscript{26}. In fact, the CDKN2A–CDKN2B locus is either bi- or monoallelicly deleted in 64% of BCR–ABL1-positive ALL cases and in 32–72% of T- or B-ALL cases without the BCR-ABL1 translocation, suggesting positive selection for either p16\textsuperscript{INK4A} or p14\textsuperscript{ARF} in oncogenic transformation compared with cells with the wild-type protein (Fig. 2a), pointing to rs3731249 as a possible functional variant directly contributing to the association with ALL risk. The structural basis of the hypomorphic effects of the p.A148T variant is unclear, since this residue is not directly involved in binding to CDK4 or CDK6\textsuperscript{27}. However, there was evidence that the variant p16\textsuperscript{INK4A} (p.148 T) is preferentially retained in the nucleus compared with the wild-type p16\textsuperscript{INK4A} (p.148A), compromising its ability to inhibit CDks in the cytoplasm\textsuperscript{28,29}. The relative contribution of p16\textsuperscript{INK4A} versus p14\textsuperscript{ARF} to ALL pathogenesis is not unequivocal because somatic deletions at this locus almost always lead to the loss of both genes. Although the rs3731249 variant also results in sequence changes of the 3'-UTR of the p14\textsuperscript{ARF} transcript, bioinformatic prediction did not identify any potential effects on mRNA stability or microRNA binding and no difference was observed in reporter gene transcription under the influence of 3'-UTR containing either the wildtype or variant allele at rs3731249 (Supplementary Fig. 4), suggesting minimal effects of this variant on p14\textsuperscript{ARF} transcription. Finally, rs3731249 is also observed in non-European populations, for example, there was a trend for a higher frequency of the risk allele in African American children with ALL than that in individuals from this racial background in the NHLBI ESP cohort (0.58% in 260 ALL cases versus 0.38% in 2,203 controls), although a much larger sample size is needed to rigorously examine the statistical significance of such differences. It should be noted that we and others previously showed that the
non-coding ALL risk variants (rs17756311 and rs3731217) at this locus had much stronger effects in European Americans than in other race/ethnic groups (35,36), suggesting potential racial differences in genetic susceptibility to ALL.

We subsequently identified additional coding variants in p16

\[ \text{INK4A} \], p14

\[ \text{ARF} \] and p15

\[ \text{INK4B} \] by resequencing, most of which were low frequency or rare. While there was a modest over-representation of potentially damaging coding variants in ALL cases compared with controls (Fig. 3), our data do not suggest that rare variants contribute substantially to the associations with ALL susceptibility observed at this locus. It should also be noted that the vast majority of coding variants within the CDKN2A gene affects only one of the two tumour suppressors (either p16

\[ \text{INK4A} \] or p14

\[ \text{ARF} \]). Interestingly, rs199880803 is the only variant that is located in the coding region of both p16

\[ \text{INK4A} \] and p14

\[ \text{ARF} \], resulting in an alanine-to-threonine change in p14

\[ \text{ARF} \] (p.A121T) with synonymous effect on p16

\[ \text{INK4A} \]. This is also the most frequent germline missense variant in p14

\[ \text{ARF} \] in our cohort and was over-represented in ALL compared with non-ALL controls (0.21% versus 0.046%, respectively, Fig. 3). The substitution of threonine in p14

\[ \text{ARF} \] adds a possible phosphorylation and phosphoprotein-binding FHA domain implicated in DNA damage response and cell cycling (31). Future studies are warranted to determine the exact consequences of this variant on p14

\[ \text{ARF} \] functions. To systemically evaluate the contribution of low frequency and rare-coding variants to ALL risk, we also performed genome-wide gene-level burden test but did not observe any genome-wide significant associations (Supplementary Table 4). Of the six known ALL risk loci, we noted two coding variants in CEBPE (rs141903485 and rs146580935, Supplementary Table 5) nominally associated with ALL susceptibility.

In conclusion, we comprehensively evaluated exonic genetic variations for association with ALL susceptibility and identified novel coding risk variants at the CDKN2A–CDKN2B locus that may directly affect tumour suppressor functions and potentiate leukaemic transformation. These results provided functional evidence for the inference of inherited genetic variants on ALL leukaemogenesis, further indicating that a continuum of genetic variations in both host and tumour genomes contribute to malignant transformation and cancer risk.

### Methods

#### Subjects and samples.

The discovery GWAS consisted of 1,773 childhood B-ALL cases and 10,488 non-ALL controls of European descent (>90% European genetic ancestry as estimated using STRUCTURE32,33). ALL cases were from the Children’s Oncology Group (COG) ALL20132 study (N = 1,277), the COG P9906 protocol (N = 115)34 and St Jude Total Therapy XIIIB and XV protocols (N = 381). Unrelated individuals of European descent from the Atherosclerosis Risk in Communities (ARIC) study20,21 were used as non-ALL controls because the prevalence of adult survivors of childhood ALL is less than 1 in 10,000 in the US. The replication series included 409 children with ALL from NOPHO ALL92, ALL2000 and ALL2008 protocols35,36 and 1,599 unrelated non-ALL controls from the Danish National Birth Cohort, a large population-based study in Denmark (52,543) in Holbæk and at random schools in Zealand, Denmark. ALL cases were selected only on the basis of sample availability, and we did not observe any statistically significant differences in demographic or clinical features of children included versus not included in this genetic study. We elected to focus on individuals of European descent to minimize population stratification.36

Germline DNA for cases was extracted from peripheral blood or bone marrow samples obtained during clinical remission (<5% ALL blasts by morphology). This study was approved by the Institutional Review Board at St Jude Children’s Research Hospital and COG member institutions and the Ethics Committee at the Danish Data Protection Agency, Region Zealand and the University Hospital Rigshospitalet, Denmark. Informed consent was obtained from parents, guardians, or patients, as appropriate.

#### Genotyping and quality control.

SNP genotyping was performed in germline DNA using the Illumina Infinium HumanExome Array v1.0 in the discovery GWAS, and using Illumina HumanCoreExome chip for the replication series.

Genotype calls (coded as 0, 1, and 2 for AA, AB and BB genotypes) were determined using the Illumina GenomStudio Software. For the ALL cases, samples for which genotypes were not determined at <99% SNP on the array were not included and were excluded from the analyses. Quality control procedures were performed for both samples and SNPs on the basis of call rate, minor allele frequency (MAF), and Hardy Weinberg equilibrium (Supplementary Fig. 5). Detailed quality control for the non-ALL controls from the ARIC study was performed at the University of Texas Health Science Center following establishing algorithms.37

We performed principal component analysis of cases and controls in the discovery GWAS to characterize population substructure (Supplementary Fig. 6).

#### Genome-wide analyses.

In the discovery GWAS, the association of each SNP individually with ALL susceptibility was tested by comparing the genotype frequency between ALL cases and non-ALL controls in logistic regression models, after adjusting for top 10 principal components to control for population stratification. Quantile–quantile (QQ) plots were constructed and indicated that the top 10 principal components explained only minimal inflation at the upper tail of the distribution (λ = 1.08, Supplementary Fig. 7). In the replication studies, we evaluated the novel genome-wide significant variant rs37312149, using the same logistic regression models. Multivariate logistic regression model including both rs3731217 and rs3731249 were tested to determine independent association signals at the CDKN2A locus in both discovery and replication series.

We also performed gene-level analyses to evaluate the aggregated effects of low-frequency variants on ALL susceptibility, using the SKAT test38. Missense, stop codon-altering and splice-site variants with MAF <5% were included. In total, 12,675 genes with at least two variants were tested.

#### CZEKNA2–CDKN2B resequencing and rare variant analyses.

Germline DNA from 2,407 children with ALL was used to create individual Illumina dual-indexed libraries. These libraries were pooled in sets of 96 and hybridized with a custom version of the Roche NimbleGen SeqCap EZ custom probes to capture the CDKN2A–CDKN2B region on 9p21. Quantitative PCR was used to determine the appropriate capture product titre necessary to efficiently populate an Illumina HiSeq 2000 flowcell for paired-end 2 × 101 bp sequencing. Each sequence pool of 96 samples was demultiplexed, with coverages of 20 × depth across >90% of the targeted regions for nearly all samples. Sequence reads in FASTQ format were filtered and aligned using the Bowtie aligner (0.1.10) and generated with Illumina recommend was called using the GATK pipeline version 3.1 (ref. 38). We compared the proportion of rare variant-carriers in ALL subjects (either homozygous or heterozygous) versus that in individuals of European descent in the ESP cohort (non-ALL controls), focusing on variants with MAF <1%. Statistical significance of the difference was estimated using Fisher’s exact test.

CDKN2A sequencing was also performed in matched germline and diagnostic ALL tumour DNA by Complete Genomics for all cases with available data, and in tumour RNA by RNA-seq. Details regarding sequencing, data analysis and coverage are available at ftp://calfdp.ncl.nih.gov/pub/bcc/target/ALL/Phase_II/sequence/WGS/CGI_TARGET_Pipeline_README.pdf, or as previously described39 (European Genome Phenome archive: EGAS00000100654).

#### Leukaemic transformation assay in Ba/F3 cells.

The full-length CDKN2A was purchased from GE Healthcare. The p14ARF variant (rs3731249) was introduced by site-directed mutagenesis (forward primer: 5’-TGGCCGGCATATGGCGG AAGGTCCCCGCTAGA-3’, reverse primer: 5’-TCTAGGGACCTTCCGCTGAC CTATGGGGGACGA-3’) and cloned into the cI20–IRE–GFP lentiviral vector, and lentiviral supernatants containing cl20–p16

\[ \text{INK4A} \] or cl20–p16

\[ \text{INK4A} \]–IRES–GFP were produced by transient transfection of the full-length CDKN2A–CDKN2B locus that may directly affect tumour suppressor functions and potentiate leukaemic transformation. These results provided functional evidence for the inference of inherited genetic variants on ALL leukaemogenesis, further indicating that a continuum of genetic variations in both host and tumour genomes contribute to malignant transformation and cancer risk.
For quantitative reverse transcription-PCR (qRT-PCR), total RNA was extracted using the RNeasy Micro kit (Qiagen) according to the manufacturer's protocol. Total RNA (500 ng) was reverse transcribed into cDNA using oligoT primers and the SuperScript III reverse transcriptase kit (Invitrogen). Quantitative real-time PCR was performed by using ABI Prism 7900HT detection system (Applied Biosystems) with FastStart SYBR Green master mix (Roche). Relative expression was calculated as a ratio of BCR-ABL1 to Hprt. Primer sequences of BCR-ABL1 and Hprt were as follows: BCR-ABL1 (forward: 5'-CTGGCCCAACCGATGGCCA-3'; reverse: 5'-CACCTGACCTGGATGCTCAA-3'); Hprt (forward: 5'-GAGCAGATCCTTGATCGTGTC-3'; reverse: 5'-TTCTTCCCTGTTGATGGAAAT-3'). To co-express rs3731249 variant and wild-type p16INK4a. Ba/F3 cells were transfected with equal molar cL20c-p16INK4a-1.48A-ires-GFP and cL20c-p16INK4a-2.148T-ires-3FV lentivirus and cells successfully transfected with both were selected by flow cytometry sorting for GFP/3FV double positivity. BCR-ABL1-mediated transformation was performed as described above. Genomic DNA and RNA samples were sorted at day 0, 2, 4, and 5 after H3 removal. p1.48A and p1.8 T transcript in RNA was quantified using allele-specific Tagman genotyping assay and normalized to allele ratio in matched DNA samples at respective time points. Each experiment was performed three times and each sample was assayed in triplicate.

Luciferase reporter assays. The p14INK4a 3'-UTR vector (3'-UTR for Human NM_005189.2) was placed downstream of luciferase reporter gene on the pExZ-MT01 backbone was generated from GeneCopoeia and the T variant at rs3731249 was introduced by site-directed mutagenesis (forward primer: 5'-CACATGGCCGC- ATAGCTGCAGTTCCGGACACATCC-3'; reverse primer: 5'-GGATGTCTGAGGACCTGACCAGATCCGATCCGATCGTGTC-3'). For reporter gene assay, 2.5 x 106 293 T cells cultured in 96-well plate were transiently transfected with 100 ng empty vector, variant, or wild-type p14INK4a 3'UTR constructs using Lipofectamine 2000 (Invitrogen). Firefly luciferase activities were measured 24 h after transfection by the Luciferase Assay System (Promega). The results were normalized against Renilla luciferase. Each reporter construct transfection was replicated at least three times, and each sample was assayed in triplicate.

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**Author contributions**


**Additional information**

**Accession codes.** The RNA-seq data have been deposited in European Genome Phenome archive under the accession codes EGAS00001000654.

**Supplementary Information** accompanies this paper at http://www.nature.com/naturecommunications

**Competing financial interests:** The authors declare no competing financial interests.

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