Here we report a genome-wide association study for non-pathological pinna morphology in over 5,000 Latin Americans. We find genome-wide significant association at seven genomic regions affecting: lobe size and attachment, folding of antihelix, helix rolling, ear protrusion and antitragus size (linear regression $P$ values $2 \times 10^{-8}$ to $3 \times 10^{-14}$). Four traits are associated with a functional variant in the Ectodysplasin A receptor ($EDAR$) gene, a key regulator of embryonic skin appendage development. We confirm expression of $Edar$ in the developing mouse ear and that $Edar$-deficient mice have an abnormally shaped pinna. Two traits are associated with SNPs in a region overlapping the T-Box Protein 15 ($TBX15$) gene, a major determinant of mouse skeletal development. Strongest association in this region is observed for SNP rs17023457 located in an evolutionarily conserved binding site for the transcription factor Cartilage paired-class homeoprotein 1 (CART1), and we confirm that rs17023457 alters $in vitro$ binding of CART1.
The human pinna is made up of a piece of cartilage covered with skin and attached to the skull by ligaments, muscles and fibrous tissue. This cartilage does not extend into the ear lobe, which consists mostly of areolar and adipose tissue. A range of disorders affecting human pinna development have been described, occurring in isolation or as part of complex syndromes with multiple affected organs1–2. There is also great non-pathological variation between humans in pinna shape and size, and this variation has been reported to be influenced by age, sex and ethnicity3–5. The study of rare, familial microtia cases (a disorder characterized by a small, abnormally shaped pinna) has established that mutations in the HOXA2 gene can severely impact pinna development6. However, no genetic variants influencing normal pinna morphology have yet been reported. Here we aimed at identifying such variants by performing a genome-wide association study (GWAS) in a large sample of Latin American individuals with no pinna abnormalities. We identified seven loci with genome-wide significant association to variation in various pinna features, including several strong candidate genes with known developmental effects. We provide follow-up experimental evidence supporting the pinna morphology associations for two gene regions (which include the Ectodysplasin A receptor (EDAR) and the T-Box Protein 15 (TBX15) genes).

Results
Our study sample is part of the CANDELA (Consortium for the Analysis of the Diversity and Evolution of Latin America; http://www.ucl.ac.uk/silva/candela) cohort collected in five Latin American countries (Brazil, Colombia, Chile, Mexico and Peru) for the study of the genetics of physical appearance7. This sample consists of individuals of both sexes (median age 22 years), with a mixed African, European and Native American genetic background (Supplementary Table 1). Using facial photographs, we performed a qualitative assessment (on a three-point-ordered categorical scale; Fig. 1; Supplementary Figs 1 and 2) of 10 pinna traits in 5,062 individuals: ear protrusion, lobe size, lobe attachment, tragus size, antitragus size, helix rolling, folding of antihelix, crus helix expression, superior crus of antihelix expression and Darwin’s tubercle.

Variation in the pinna traits examined. The trait scores show a weak-to-moderate correlation between them, and with age, sex, body mass index (BMI) and genetic ancestry (Supplementary Table 2). Most noticeably, lobe attachment shows a moderate and significant (permutation P value <0.001) negative correlation with lobe size (r = −0.49). Significant but weaker positive correlations were observed for folding of antihelix with helix rolling (r = 0.25) and with superior crus of antihelix expression (r = 0.23), as well as between ear protrusion and helix rolling (r = 0.16).

Individuals were genotyped on Illumina’s Omni Express BeadChip. After quality control, 671,038 single-nucleotide polymorphisms (SNPs) and 4,919 individuals were retained for further analyses. Autosomal admixture proportions for the full sample were estimated as: 53% European, 43% Native American and 4% African (Supplementary Fig. 3). On the basis of a kinship matrix derived from the genome-wide SNP data8, we estimated narrow-sense heritability using GCTA9 and found moderate and significant values for all traits, with the highest heritability observed for ear protrusion (61%) and the lowest for tragus size (25%) (Supplementary Table 3). Similar heritabilities have been calculated for a range of facial traits using family data10,11.

Primary association analyses. For the primary genome-wide association tests, we applied multivariate linear regression, as implemented in PLINK12, using an additive genetic model adjusting for: age, sex, height, BMI and the first five principal components (PCs) calculated from SNP data. The resulting statistics showed no evidence of residual population stratification for any of the traits (Supplementary Fig. 4). To account for the possibility of cryptic relatedness between individuals, we also performed genome-wide association tests using random-effects mixed linear regression models (using FastMM13) and obtained similar results as in the PLINK analyses (Supplementary Table 4). Six of the traits examined showed genome-wide significant association (linear regression P values <5 × 10−8) with SNPs in at least one of the seven genomic regions (Fig. 1; Table 1). A global false discovery rate test across all traits and SNPs identified the same significantly associated regions (Supplementary Table 5). Lobe size is associated with SNPs in four regions (2q12.3, 2q31.1, 3q23 and 6q24.2); three traits show association with two regions: lobe attachment (2q12.3 and 2q31.1), helix rolling (2q12.3 and 4q31.3) and antihelix folding (1p12 and 18q21.2). The remaining two traits show association with a single region: ear protrusion (2q12.3) and antitragus size (1p12). Since the traits examined show some correlation between them, the associations detected are likely not fully independent. Most noticeably, the moderate negative correlation observed between lobe attachment and size is consistent with SNPs at the same two loci (2q12.3 and 2q31.1) showing significant association with both traits. Suggestive association with lobe attachment is also observed for 6q24.2, which is significantly associated with lobe size (Table 1).

Secondary analyses. Since correlations between traits could have a shared underlying genetic basis, we performed a multivariate analysis combining all phenotypes in a single regression model (using MULTIPHEN14). This analysis identified the same set of regions as in the independent regression tests, but no additional associated regions (Supplementary Fig. 5). We also examined the association signals for all index SNPs (Table 1) in each country separately and combined results as a meta-analysis using METAL15. For each association, the effects were in the same direction in all countries, the variability of effect size across countries reflecting sample size (Fig. 2; Supplementary Table 6; Supplementary Fig. 6a). There was no significant evidence of effect size heterogeneity across countries for any of the associations. A full genome-wide meta-analysis did not reveal additional regions showing significant association with pinna morphology (Supplementary Fig. 6b). The seven index SNPs of Table 1 provide modest phenotypic prediction accuracy (Supplementary Table 7). The fraction of the phenotypic variance explained by these SNPs is small relative to the heritability estimates (Supplementary Tables 3 and 7), suggesting a polygenic architecture for these traits beyond that captured by the genome-wide significance threshold used.

Features of associated regions. The genomic regions showing genome-wide significant association have features with independent evidence suggestive of an involvement in pinna development. This evidence is particularly strong for the regions in 2q12.3 and 1p12, and we followed-up these two regions with additional experiments.

2q12.3. Includes SNPs associated with four traits (lobe size, lobe attachment, helix rolling and ear protrusion; Table 1). These SNPs extend over ~500 kb and show substantial linkage disequilibrium (LD; Fig. 3b). Strongest association with all four traits was found for SNP rs3827760 (Table 1), and conditioning on this SNP abolishes the signal of association at other SNPs in the region (Supplementary Fig. 8a). Marker rs3827760 leads to a functional p.Val370Ala substitution in the intracellular death
domain of EDAR. This residue affects the interaction with the EDAR-binding death domain adapter protein EDARADD; the derived EDAR allele encodes a protein with higher activity than the ancestral one. EDAR signalling acts during prenatal development to specify the location, size and shape of ectodermal appendages, such as hair follicles, teeth and glands. The p.Val370Ala variant has been associated with characteristic tooth morphologies, hair type and sweat gland density in East Asians, where this allele is present at high frequency while being nearly absent in European or African populations (Supplementary Table 8). Consistent with these effects in humans, mice expressing EDAR370A, or with increased EDAR function, show thickened and straightened hair fibres.

We examined Edar expression in the developing mouse embryo (Fig. 4a). The structure of the pinna is defined prenatally in both mouse and human, in mouse primarily between gestation days 13 and 16 (ref. 2). Using whole-mount in situ hybridization, we confirmed Edar expression along the distal margin of the developing pinna (Fig. 4a), in addition to the well-characterized expression in the developing hair follicles. Edar expression at the distal margin of the embryonic pinna may aid in determining its growth and expansion, thus influencing the ultimate form adopted by the ear. To assess the functional significance of this Edar expression during pinna development, we examined postnatal pinna morphology in Edar mouse mutants. The Edar<sup>dlj</sup> (ref. 26) and Edar<sup>Tg951</sup> (ref. 16) mouse lines have a loss and a gain of Edar function, respectively (see Methods). At 2 weeks of age, the pinna of homozygous Edar<sup>dlj/dlj</sup> mutant and heterozygous Edar<sup>dlj/+</sup> littermates, wild-type mice and Edar<sup>Tg951</sup> mice. Landmark coordinate PC1, capturing 69% of the variation in shape, reflects mainly a change in the extent of helix rolling of the mouse pinna (Fig. 4e; Supplementary Fig. 10d), consistent with one of the effects we observed of EDAR variants on human pinna shape. The Edar<sup>Tg951</sup> does not display a detectable change in helix rolling likely due to the fact that the wild-type mouse pinna does not have a prominent helix roll, thus hampering the detection of any further pinna flattening that might be caused by increased Edar function.
1p12. SNPs in this region show genome-wide association with antihelix folding and antitragus size. This region extends over ~800 kb and overlaps the gene encoding transcription factor TBX15 (Fig. 3a), a key regulator of cartilaginous and skeletal development in the mouse37. A spontaneous Tbx15 mouse mutant (called ‘droopy ears’), is characterized by altered positioning, projection and shape of the pinnae28,29.

In humans, mutations of TBX15 result in Cousin syndrome, a disorder characterized by craniofacial dysmorphism, including a dysplastic pinna30. Strongest association in this region was observed for intergenic SNP rs17023457 (P value 2 × 10^{-8} for antitragus size and 1 × 10^{-11} for antihelix folding Table 1) and conditioning on this SNP abolishes the signal of craniofacial and cartilage abnormalities in the mouse31. Mutations of which have been shown to result in a range of craniofacial and cartilage abnormalities in the mouse32,33.

In Supplementary Fig. 11a, mutations of which have been shown to result in a range of craniofacial and cartilage abnormalities in the mouse32,33. Another interesting candidate gene in the vicinity is that encoding the forkhead box transcription factor CART1 (cartilage-paired-class homeoprotein 1), mutations of which have been shown to result in a range of craniofacial and cartilage abnormalities in the mouse32,33.

Other regions. For the other regions showing genome-wide association, there is currently less compelling information suggestive of a mechanism explaining their effect on pinna morphology, and we did not attempt their experimental follow-up. SNPs in 3q23 are associated with lobe size, with strongest association being seen for intergenic SNP rs10212419 (P value 3 × 10^{-14}) in a region with substantial LD over ~500 kb (Fig. 3d). Intriguingly, considering that the ear lobe is made up mainly of loose connective tissue, intergenic SNPs in this region have been strongly associated with keloid formation32, an exaggerated skin wound-healing reaction characterized by excessive deposition of extracellular matrix and collagen fibres. Some highly penetrant mutations across this genomic region have also been found to result in alterations of craniofacial development involving pinna morphology.

To assess the potential for rs17023457 to alter DNA–protein interactions involving the CART1-binding site, electrophoretic mobility shift assays were performed using nuclear extracts from a CART1-expressing cell line (Huh7). Double-stranded oligonucleotides containing the rs17023457 T allele demonstrated binding of a nuclear protein, this binding being undetectable for oligonucleotides carrying the derived C allele (Supplementary Fig. 11b). Binding was eliminated upon prior incubation with excess unlabelled CART1 consensus sequence DNA, confirming the specificity of the assay and supporting the ability of the sequence containing the T allele to bind CART1 in vitro. Reporter constructs containing either the rs17023457 T or C alleles driving the expression of the luciferase gene, showed a decreased expression of 36 and 22% in constructs with the C allele when the genomic sequence was positioned in the forward and reverse orientation, respectively (Supplementary Fig. 11b). These data indicate that CART1, or other nuclear DNA-binding proteins with the same sequence specificity, is able to bind to an enhancer that includes rs17023457, with variation at this SNP determining whether binding and full transactivation occurs or not. The implications of these observations for the in vivo regulation of TBX15 expression remain to be established.

Table 1 | Chromosomal location and \(-\log_{10}(P)\) for index SNPs showing strongest genome-wide association to pinna traits.

| Region | SNP | Nearest gene | EP | AS | FoA | HR | LA | LS | TS | CHE | DT | SCoAE |
|--------|-----|--------------|----|----|-----|----|----|----|----|-----|----|-------|
| 1p12   | rs17023457 | CART1 binding site | 2.37 | 7.76 | 10.94 | 1.12 | 0.97 | 0.16 | 0.27 | 1.62 | 0.31 | 1.26 |
| 2q12.3 | rs3827760 | EDAR | 10.00 | 0.13 | 1.92 | 12.00 | 8.66 | 11.85 | 4.33 | 0.63 | 0.59 | 0.13 |
| 2q3.1 | rs2080401 | SP5 | 0.16 | 0.40 | 0.41 | 0.49 | 11.03 | 9.90 | 1.13 | 0.50 | 0.25 | 0.03 |
| 3q23   | rs10212419 | MPRS22 | 0.08 | 0.27 | 0.06 | 1.26 | 1.41 | 13.49 | 1.98 | 0.08 | 2.59 | 0.15 |
| 4q1.3  | rs1960918 | LRBA | 0.42 | 3.16 | 1.13 | 7.64 | 0.88 | 1.81 | 2.96 | 0.46 | 0.70 | 0.15 |
| 6q4.2  | rs263156 | LOC153910 | 0.31 | 0.13 | 0.99 | 1.75 | 5.66 | 12.61 | 0.57 | 0.04 | 2.18 | 0.78 |
| 18q2.1 | rs1619249 | LOC100287225 | 1.22 | 0.27 | 7.99 | 1.22 | 0.01 | 0.92 | 0.92 | 1.48 | 0.33 | 0.09 |

SNP, single-nucleotide polymorphism. Trait acronyms (Fig. 1): A, antitragus size; CHE, crus helix expression; DT, Darwin’s tubercle; EP, ear protrusion; FoA, folding of antihelix; HR, helix rolling; LA, lobe attachment; LS, lobe size; SCoAE, superior crus of antihelix expression; TS, tragus size.

Genome-wide significant values (–log10(P) > 7.3) are highlighted in the darkest shade of red. Below this significance threshold, intensity of colour background is proportional to –log10(P) value.
rolling; Fig. 3e) and rs1619249 in 18q21.2 (P value $1 \times 10^{-8}$ for antihelix folding; Fig. 3g). Intronic marker rs1960918 is in an LD region of $\sim 400$ kb overlapping the LRBA gene, whose product (LPS-responsive vesicle trafficking, beach and anchor containing) is known to be involved in coupling signal transduction, vesicle trafficking and immunodeficiency,

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Figure 2 | Meta-analysis of significant genome-wide associations. Effect sizes (in each country sample and in a combined meta-analysis) for the index SNPs and their associated traits (Table 1). Regression coefficients (x axis) estimated in each country are shown as blue boxes (box size indicating sample size). Red diamonds indicate effect sizes estimated in the meta-analysis. Horizontal bars indicate s.e. Results for all the SNPs and traits shown in Table 1 are provided in Supplementary Fig. 6A. The two alleles at each SNP are shown in brackets with effect size referring to the allele in the numerator.
**Figure 3 | LocusZoom and linkage disequilibrium plots of significantly associated genetic regions.** Plots of the seven genomic regions showing genome-wide significant association to pinna traits (Table 1). For regions showing association with several pinna traits, we present here only results for the trait with strongest association (plots for the other associated traits are presented in Supplementary Fig. 7). Association results from a multivariate linear regression model (on a − log10 P scale; left y axis) are shown for SNPs ~500 kb on either side of the index SNP (that is, the SNP with the smallest P value, purple diamond; Table 1) with the marker (dot) colour indicating the strength of LD ($r^2$) between the index SNP and that SNP in the 1000 Genomes AMR data set. Local recombination rate in the AMR data is shown as a continuous blue line (scale on the right y axis). Genes in each region, their intron–exon structure, direction of transcription and genomic coordinates (in Mb, using the NCBI human genome sequence, Build 37, as reference) are shown at the bottom. Plots were produced with LocusZoom. Below each region we also show an LD heatmap (using $D^r$, ranging from red indicating $D^r = 1$ to white indicating $D^r = 0$) produced using Haploview. Note that the location of SNPs on the LD heatmap can be shifted relative to the regional display on top of it.
with no obvious functional connection to pinna development. Intergenic SNP rs1619249 is in an LD region of about 300 kb, the closest candidate being LOC100287225 (about 100 kb from rs1619249), a hypothetical gene of unknown function.

**Discussion**

In conclusion, we have identified the first genetic variants influencing normal variation in human pinna morphology. It will be important to evaluate further the role of these regions in ear development and its disorders. Since pinna morphology in
mammals shows extensive evidence of evolutionary adaptation, particularly in relation to thermoregulation, acoustic perception and sound localization14–15, it will be interesting to examine whether variation in these genomic regions relates to adaptive models, as implemented in FastLMM13. In this approach, the GRM again specifies linear regression results, we also performed a GWAS using mixed-effects regression.

Methods

Study subjects. In total, 5,062 volunteers from 5 Latin American countries (Brazil, Chile, Colombia, Mexico and Peru) part of the CANDELA consortium sample (http://www.uc.lac.us/can/canela/), were included in this study. Ethics approval was obtained from: Universidad Nacional Autónoma de México (Mexico), Universidad de Antioquia (Colombia), Universidad Perúana Cayetano Heredia (Peru), Universidad de Tarapacá (Chile), Universidade Federal do Rio Grande do Sul (Brazil) and University College London (UK). All participants provided written informed consent. Blood samples were collected by a certified phlebotomist and DNA extracted following standard laboratory procedures. Five digital photographs of the face: left side (—90°), left angle (—45°), frontal (0°), right angle (45°) and right side (90°) were taken from ~1.5 m at eye level using a Nikon D90 camera fitted with a Nikkor 50 mm fixed focal length lens. Other phenotypes including height, weight, BMI, age and sex were also recorded for each participant.

Pinna phenotyping. Right side, right angle and frontal photographs were used to score 10 pinna traits. These were (Fig. 1): ear protrusion, lobe size, lobe attachment, tragus size, antitragus size, helix rolling, folding of antihelix, crus helix expression, superior crus of antihelix expression and Darwin’s tubercle. Each trait was scored as an ordered categorical variable, with 0 being the lowest level of expression of the trait and 2 the highest (Supplementary Table 1). Software to assist scoring and all photographs was developed in MATLAB8. Intraclass correlation coefficients calculated by double-scoring photographs of 100 subjects indicate a moderate-to-high intra- and inter-rater reliability of the trait scores (Supplementary Table 9). Photographs for all the volunteers were scored by the same rater (G.R.).

DNA genotyping and quality control. DNA samples from participants were genotyped using the HumanOmniExpress-85 A and B arrays (Supplementary Fig. 1). Software to assist scoring and all photographs was developed in MATLAB8. Intraclass correlation coefficients calculated by double-scoring photographs of 100 subjects indicate a moderate-to-high intra- and inter-rater reliability of the trait scores (Supplementary Table 9). Photographs for all the volunteers were scored by the same rater (G.R.).

Statistical analyses. P values for Pearson correlation coefficients were obtained by permutation. All regressions were performed using an additive multivariate linear or logistic model, providing P values that are obtained from the standard t-statistic derived from their regression coefficients. Narrow-sense heritability (defined as the phenotypic variance explained by a genetic relatedness matrix, GRM, computed from the LD-pruned set of markers with minor allele frequency B0.01, 1% SDS containing 750 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl2 and 0.1% Tween 20.

Whole-mount in situ hybridization of mouse embryos. Embryos were fixed in 4% paraformaldehyde in PBS at 4°C overnight, dehydrated to methanol, rehydrated, treated with proteinase K, refixed and hybridized to a digoxigenin-labelled cRNA covering the entire Edar transcript (AF160502). Hybridization was performed in 50% formamide, 750 mM NaCl, 75 mM Na-Cacodylate, 1% SDS containing 50 µg ml-1 yeast RNA and 50 µg ml-1 heparin at 65°C overnight. After washing and blocking of embryos in 10% sheep serum, the signal was detected using 1/2,000 sheep anti-digoxigenin conjugated to alkaline phosphatase (sheep anti-digoxigenin alkaline phosphatase antibody; 11093274910, Roche Applied). Animals were used to remove the effects of translation, rotation and scaling8. After dehydration and BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine) staining in 100 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl2 and 0.1% Tween 20.

Mouse pinna phenotyping. Overall, pinna shape variation was examined using geometric morphometric techniques2–12. Twenty-seven landmarks and semilandmarks (depicted in Supplementary Fig. 8) were digitized, scaled and superimposed in TPSDig and TPSUtil (http://life.bio.sunysb.edu/morph/) routine was used to allow semilandmarks to slide so as to minimize bending energy. Reliability of pinna landmarking was evaluated by scoring the same landmarks by a second rater and examining the variation (Supplementary Table 10). Generalized procrustes analysis was used to remove the effects of size and weight on pinna shape. Photographs of superior and lateral views of the head were taken and were blinded prior to landmarking for analysis. A scale was included in the photographs for calibration. Landmark homology across specimens was achieved by controlling the head orientation in all photos (for example, by placing the sagittal plane of the head orthogonal to the anterior–posterior axis of the camera lens). This fixed orientation avoids rolling, heading and pitching rotations and guarantees the coplanarity of landmarks placed on the pinnae (see below).

Human tissue culture for the CART1-binding assay. The human hematopoietic Huh7 (ECACC, UK) cell line was grown in high-glucose DMEM (PAA) supplemented with 2 mM l-glutamine and 10% fetal bovine serum and maintained in 5% CO2 at 37°C. Nuclear extracts were obtained from Huh7 cells using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific) as described in the manual, with the addition of Complete Protease Inhibitor (Roche, UK) to buffers CER I and NER I.
Electrophoretic mobility shift assay. Probe sequences: rs17023475 T→G
rs17023475 T forward 5′-ACTAATCTACCACTACCTCTTTGAGA-3′
s17023475 T reverse 5′-TGGTCTGCAAGGAAGGTGATTGATTGTG-3′
s17023475 C forward 5′-ACTAATCTACCACTACCTCTTTGAGA-3′
s17023475 C reverse 5′-TGGTCTGCAAGGAAGGTGATTGATTGTG-3′

Probes were labelled using the Biotin 3′-End DNA Labelling Kit (Pierce) as described in the manufacturer’s manual. Binding reactions consisted of 2 μl 10× binding buffer (100 mM Tris, 500 mM KCl, pH 7.5), 1 μg p(DC)-biotin, 200 fmol biotin-labelled DNA, made to a total of 20 μl with H2O and incubated at 25 °C for 30 min, followed by the addition of 5× loading buffer. Competition reactions were performed with 30 min on ice, prior to addition of labelled probes, using 100× CARTI cold-compettitor oligonucleotides. Samples were run on 6% polyacrylamide gel, electrophoresed for 150 min at 120 V.

Luciferase reporter assay. Luciferase reporter vectors were based on the pGL3 Promoter vector (Promega), with a 872-bp genomic sequence surrounding rs17023475 (Supplementary Fig. 11c) inserted upstream of the SV40 promoter in both orientations using the following primers designed for the InFusion PCR Cloning kit (Clontech):

Forward Ear1FF: 5′-TCCTAGTATGTTACCATCAGTTCAACCTGTGAAGAACGAG-3′
Ear1FR: 5′-GATGCGCATCCAGGTCAGAATTCAAGACGTCCTGGCCAAA-3′

Reverse Ear2R2: 5′-GATCCGCCTGTCGACGGATCTACGGGCTGGAGGAC-3′
Ear2RR: 5′-TCATCTGATGATACCGCTACGAGGCAATGCATCACCAAGTCCAAA-3′

Site-directed mutagenesis was performed using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) as detailed in the manual, using the following oligonucleotide sequences (designed for reverse strands):

Primer name; primer sequence (5′–3′)
SDMFFC 5′-ATGATCCGAAAGGATTGTTAGTGGTGCTGATCAT-3′
SDMERC 5′-ATGATCCGAAAGGATTGTTAGTGGTGCTGATCAT-3′
SDMFFC 5′-GACCACTAATCAACGACCCTCTTGAGAATACGCT-3′
SDMGRF 5′-GTGGAAGTCGTATTGCCAAAGGATTGTTAGTGGTGCTGATCAT-3′

All constructs were verified by direct sequencing.

Huh7 cells were seeded at a density of 2 x 10^4 well per 96-well plate and grown to confluence overnight in the appropriate media (described above). Cells were transfected with 250 ng pGL3 reporter construct, with 10 ng pRLTK as a loading control, using Lipofectamine 2000 (Invitrogen) as described in the manual. Media was replaced 24 h after transfection, with serum-containing media described above, and the cells left for 2 days before harvesting. Cells were lysed using Passive Lysis Buffer (Promega) and luciferase expression was determined using a Dual Luciferase Reporter Assay System (Promega), and measured in the Tropix TR717 Microplate Luminometer (PE Applied Biosystems, UK). For each construct used, the transfection assay was performed using 12 wells of a 96-well plate and the mean luciferase reading reported. The experiment was repeated in triplicate using fresh vector preparations. Analysis of variance was performed to construct used, the transfection assay was performed using 12 wells of a 96-well plate and the mean luciferase reading reported. The experiment was repeated in triplicate using fresh vector preparations. Analysis of variance was performed to account the variability across replicates and wells.

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Author contributions
K.A., A.I.P.S., M.Q.-S., J.R., M.C., M.F.B., R.G.-J., D.H., D.B. and A.R.-L. conceived and designed the study. K.A., V.A.-A., C.J., W.A., M.F., M.P., G.M.P., R.B.L., J.G.-V., H.V.-R., T.H., V.R., C.C.S.d.C., V.G., M.H., V.V. and D.H. contributed reagents/material. G.R., A.I.P.S., E.K., J.P., M.Q.-S., M.C., D.H., K.A., V.A.-A., C.J., W.A., M.F., M.P., G.M.P., R.B.L., J.G.-V., H.V.-R., T.H., V.R., C.C.S.d.C., V.G., M.H. and V.V. performed experiments. K.A., A.I.P.S., M.Q.-S., M.C., D.H. and A.R.-L. analysed data. V.A.-A., J.G.-V., C.G., G.P., L.S.-F., F.M.S., M.-C.B., S.C.-Q., F.R., G.B., R.C., J.R., M.C., K.A., S.E.H., R.G.-J., D.H., D.B. and A.R.-L. supervised research/PI. K.A. and A.R.-L. wrote the manuscript. A.I.P.S., C.G., G.P., S.C.-Q., J.R., M.C., M.F.B., R.G.-J., D.H. and D.B. critically revised the manuscript.

Additional information

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