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CSF1R mutations in hereditary diffuse leukoencephalopathy with spheroids are loss of function

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Hereditary diffuse leukoencephalopathy with spheroids (HDLS) in humans is a rare autosomal dominant disease characterized by giant neuroaxonal swellings (spheroids) within the CNS white matter. Symptoms are variable and can include personality and behavioural changes. Patients with this disease have mutations in the protein kinase domain of the colony-stimulating factor 1 receptor (*CSF1R*) which is a tyrosine kinase receptor essential for microglia development. We investigated the effects of these mutations on Csf1r signalling using a factor dependent cell line. Corresponding mutant forms of murine Csf1r were expressed on the cell surface at normal levels, and bound CSF1, but were not able to sustain cell proliferation. Since Csf1r signaling requires receptor dimerization initiated by CSF1 binding, the data suggest a mechanism for phenotypic dominance of the mutant allele in HDLS.

Hereditary diffuse leukoencephalopathy with spheroids (HDLS) is an autosomal-dominant neurodegenerative condition with rather variable penetrance. A recent study identified mutations in the colony-stimulating factor 1 receptor (*CSF1R*) in multiple families with this disorder[1]. Subsequently, CSF1R mutations were identified in a related disorder termed pigmented orthochromatic leukodystrophy[2]. CSF1R signalling is required for the generation of the majority of mature macrophages[3], including the microglia of the brain[4]. CSF1-dependent microglial activation has been implicated in neural damage in a model of Charcot-Marie-Tooth disease[5]. The authors of the recent report suggest that the causal mutations in CSF1R result in a loss of function, although there was no evidence of altered CSF1R levels or phosphorylation state in blood or brain samples from HDLS patients. Conversely, *in vitro* analysis of transfected HeLa cells resulted in no detectable autophosphorylation in three HDLS CSF1R mutations. The authors suggest that the presence of wild type CSF1R in the heterozygous individuals as the cause of this discrepancy[1]. Heterozygous mutation of *Csf1r* in mice does not generate any phenotype, suggesting that haploinsufficiency is an unlikely explanation for the dominant inheritance in HDLS. An alternative suggestion was that in patients the products of the mutant allele might assemble into heterodimers with wild-type protein and have a dominant negative effect.

CSF1R is a type III receptor tyrosine kinase belonging to the platelet-derived growth factor (PDGF) receptor family whose members include PDGF-α and –β, the FMS-like tyrosine kinase 3 (FLT3) and the receptor for stem cell factor (c-KIT)[6]. These proteins have similar structures consisting of five immunoglobulin-like domains, a transmembrane domain, a juxtamembrane domain (JM) and a protein kinase domain divided in two by an insert domain (KID)[7]. Protein kinase domains are structurally conserved and as key regulators of most cellular pathways are frequently associated with disease and are often oncogenic[8]. Mutations in the kinase domains of PDGF-α and c-KIT result in increased receptor dimerization leading to gastrointestinal tumours and mastocytosis (reviewed in[9]) whilst FLT3 gain of function mutations are often found in acute myeloid leukemia[10]. Overexpression of CSF1R has been reported in a number of diseases including myeloid malignancies[11].

CSF1R, like many related tyrosine kinase receptors, exists in an autoinhibited state, stabilized by the JM domain[12,13]. Upon activation, the receptor dimerizes which results in autophosphorylation of a number of tyrosine residues in the intracellular domain and leads to recruitment of signalling molecules and ultimately internalization of the receptor. Yu et al[14]. generated a CSF1R in which all 6 major tyrosines involved in signalling were replaced by phenylalanine. Restoration of Y807 (Y809 in human) produced a receptor that was able to support ligand independent proliferation in a factor dependent cell line[15]. Three recent HDLS case reports have found additional mutations; K793T[16], A781V[17] and R782H[18]. R782, in the catalytic loop, binds to Y809 in the autoinhibited CSF1R[12].

In this study, we chose four HDLS mutations and created expression plasmids introducing the corresponding mutation in murine *Csf1r*. All mutated residues are highly conserved and are located in the protein kinase (PTK) domain. In addition to these, we examined four other mutations. We included a mutation (K584E) in the conserved N terminal region of the PTK domain that has not previously been implicated in autoinhibition as well as a mutation in the activation loop (R814P). As a positive control, a kinase-defective receptor, K614R, with a mutation in the ATP-binding site was created[19]. In addition, we created a double mutation within the catalytic site (V661I/T663A). The mutant proteins were expressed in IL-3-dependent Ba/F3 cells. Although these cells were originally referred to as pro-B cells, they express the myeloid-specific F4/80 and CD11b antigens and may therefore be an appropriate model system in which to investigate CSF1 signalling[20]. We report that the mutations identified in HDLS[1] as well as the K614R mutant were unable to sustain growth in CSF1. They were nevertheless expressed on the cell surface at the same level as the wild-type receptor and could be internalized in response to addition of CSF1. Csf1r signalling was intact in R814P, V661I/T663A and K584E whilst the latter two mutations displayed varying degrees of constitutive activity. We thus confirm that mutations of CSF1R in HDLS are loss of function and that the use of the Ba/F3 factor dependent cell line is an invaluable tool for assessing the effects of further Csf1r mutations *in vitro*.

Results

The protein kinase domain of human and mouse CSF1R is highly conserved. In order to examine the CSF1R mutations found in HDLS the equivalent murine mutations were produced. An overlay of the protein kinase domains of human and mouse CSF1R emphasizes the highly conserved nature of this protein (Figure 1). The kinase insert domain is not required for kinase activity[21] and the highlighted mutations did not affect the structure of the protein kinase domain when modeled in YASARA (data not shown).

Csf1r mutants found in HDLS do not signal when expressed in Ba/F3 cells. Rademakers and colleagues[1] suggested that the CSF1R mutations are effectively gain of function, producing dominant negative repressors. To identify the nature of the mutations found in HDLS, equivalent murine Csf1r proteins were expressed in factor dependent Ba/F3 cells. Expression of the wild-type receptor generated cells that could survive and proliferate in CSF1 (Figure 2). FACS analysis confirmed surface expression of Csf1r in these wild-type receptor expressing cells (Figure 3A). Conversely, the four mutations reported by Rademakers et al[1] all produced cells that were unable to survive in CSF1 (Figure 2) and therefore the four HDLS mutations were cultured in IL-3. Because it was not possible to select for CSF1 dependence, not all cells were positive, but those that were demonstrated the same level of surface receptor as cells expressing wild-type receptor (Figure 3C).

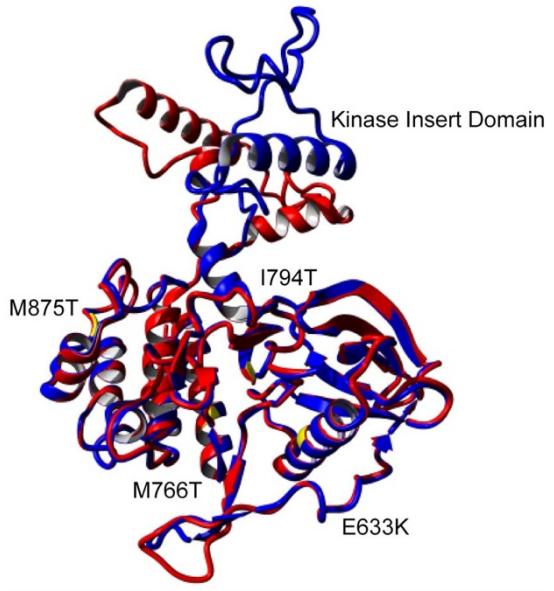

Figure 1 | Overlay of the murine (red) and human (blue) CSF1R autoinhibited kinase domain shows significant sequence homology. YASARA-predicted proteins show a sequence similarity of 95.74% and RMSD of 0.365 Å. Locations of the HDLS mutations[1] are shown in yellow.

Functional signaling depends on the mutation site within Csf1r. We examined four other Csf1r mutations: K584E, V661I/T663A, R814P and K614R, with the latter serving as a positive control for loss of kinase activity. Like the HDLS mutants, the K614R mutant receptor could not survive in CSF1 alone in Ba/F3 cells (Figure 2), yet still expressed Csf1r on the surface (Figure 3B). To further validate the hypothesis that conserved amino acids within the catalytic site are important for autoinhibition, we produced a double mutation within the active site (V661I/T663A) that was not predicted to impact kinase activity. This double mutant demonstrated a small but statistically significant amount of constitutive activity in promoting survival and proliferation of Ba/F3 cells in the absence of growth factors (Figure 2). K584E was constitutively active in Ba/F3 cells (Figure 2) and expressed Csf1r on the surface (Figure 3B) whilst Ba/F3 cells expressing the activation loop mutation (R814P) remained CSF1-dependent (Figure 2).

HDLS mutant Csf1r can bind CSF1, dimerize, and internalize. Upon binding of its ligand, (Csf1 or IL-34) the Csf1 receptor dimerizes and following autophosphorylation of intracellular tyrosine residues the receptor is internalized and eventually degraded[22]. We have shown that the mutations in HDLS are unable to signal via Csf1r in the presence of CSF1 yet still express Csf1r on the surface. To confirm that loss of receptor kinase activity does not necessarily affect receptor downregulation[23] we co-treated the factor dependent mutants with CSF1. In each case, addition of CSF1 down-regulated surface CSF1R (Figure 3C).

Discussion

Csf1r regulates the proliferation, differentiation and survival of cells of the mononuclear phagocyte lineage, which include the microglia in the brain[4]. In this study we chose four CSF1R mutations identified in HDLS as well as a kinase defective mutation (K614R), a highly conserved lysine mutation (K584E), an activation loop mutation (R814P) and a double mutation (V661I/T663A) within the catalytic site of the Csf1r protein kinase domain and created the equivalent murine mutations.

The IL3-dependent Ba/F3 cell line[24] was used to test the biological activity of mutant receptors. When wild type Csf1r is introduced into these cells, survival can be maintained in the presence of CSF1 alone. Autophosphorylation of Csf1r dimers in response to ligand binding initiates recruitment of and activation of downstream signaling molecules such Src[15,25], Grb2[26], STAT proteins[27] and PI3 kinase[28]. Rademakers and colleagues[1,2] found that CSF1 did not induce autophosphorylation of the 3 HDLS mutant CSF1R (E633K, M766T, and M875T) suggesting that they were kinase-dead, similar to K614A, which fails to exhibit tyrosine phosphorylation or kinase activity[14].

In keeping with this view, Ba/F3 expressing any of the HDLS mutations (E631K, M764T, I792T and M873T) were unable to survive in CSF1. Nevertheless, the mutant proteins were on the cell surface at similar levels to the wild type protein and were removed from the surface in response to CSF1 (Figure 3), consistent with previous evidence that receptor internalization does not require CSF1R kinase activity[29]. These data demonstrate clearly that the

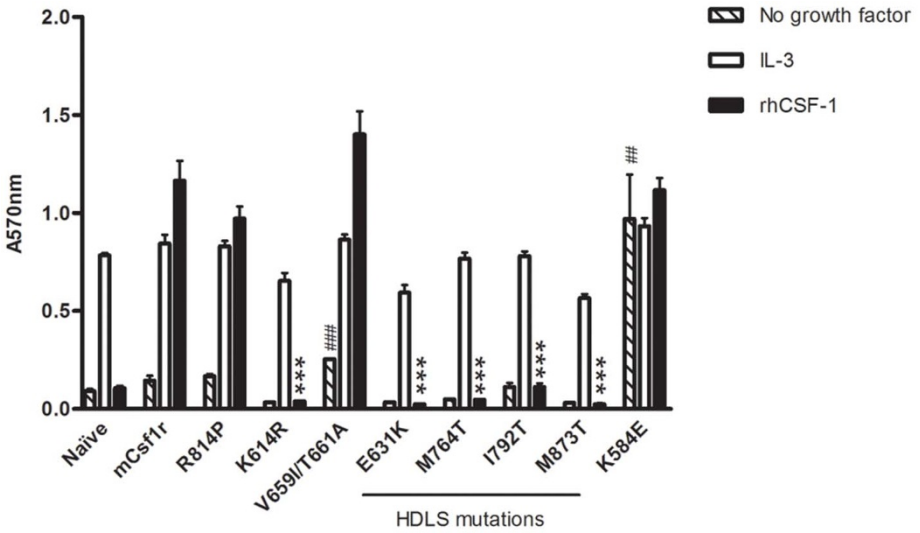

Figure 2 | Mutations in the intracellular domain of mCsf1r resulted in either loss of Csf1r signalling or promoted factor independent survival in Ba/F3 cells. Untransfected Ba/F3 (naïve) or Ba/F3 cells expressing wild-type or mutant mCsf1r were cultured alone or in either IL-3 or rhCSF1. The mean of 3 experiments + SEM is shown. P values were <0.0001 and <0.002 when mutants were compared to wild-type mCsf1r growing in rhCSF1 (*) or in no growth factor (#) respectively.

mutant receptors can form dimers and can bind CSF1. Hence, in heterozygous individuals, 75% of ligand-receptor complexes would be either mutant dimers, or wild-type mutant heterodimers. The consequence would be a 75% reduction in the formation of active CSF1-CSF1R dimers competent to signal upon addition of the ligand. The inactive dimers are nevertheless internalized and degraded, so there is no possibility to recycle the wild-type proteins into active complexes.

The intracellular domain of Csf1r is highly-conserved across species. Our data suggest that there are many other mutations that could

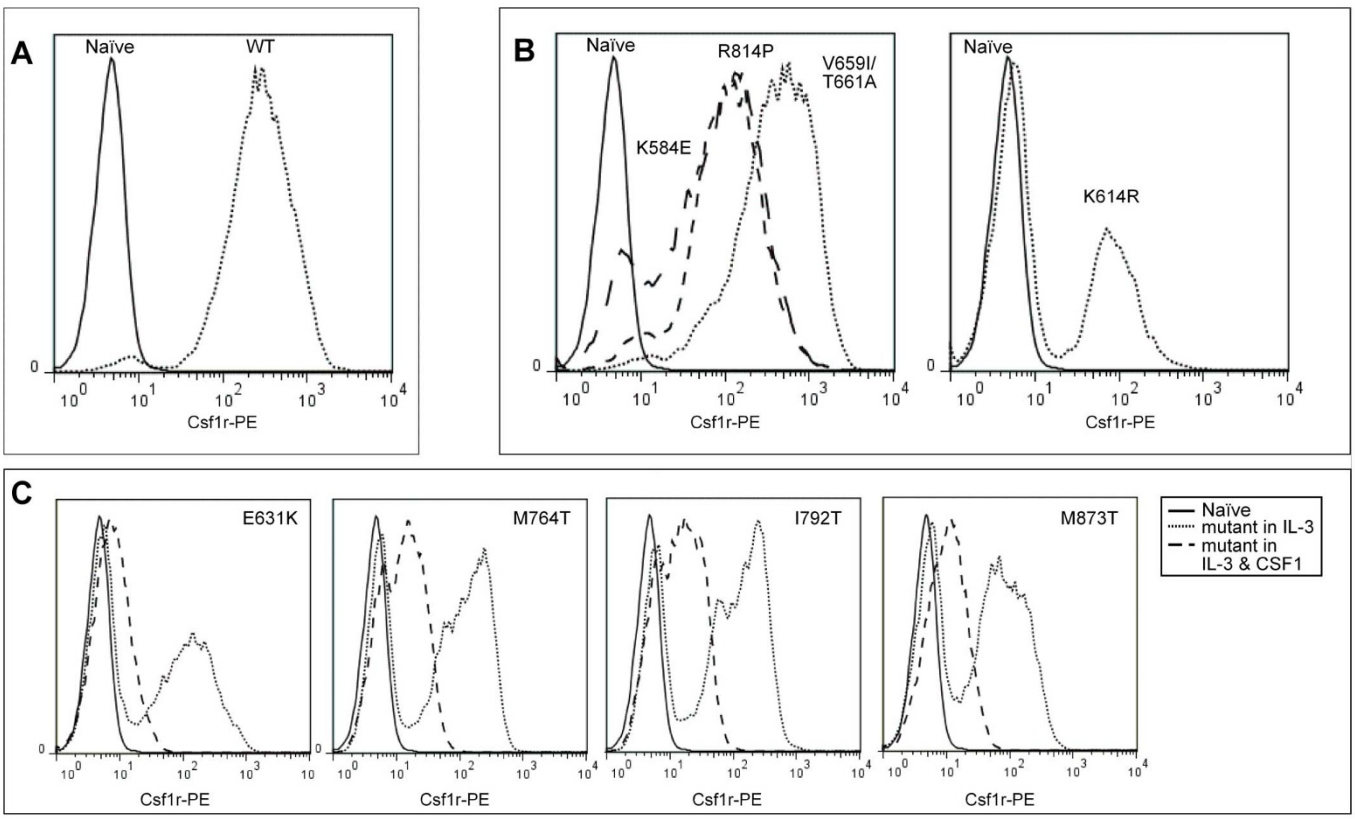

Figure 3 | Csf1r expression in mCsf1r Ba/F3 cells. (A) Flow cytometry analysis of Csf1r expression on naive and wild-type mCsf1r Ba/F3 cells cultured in IL-3 or rhCSF1, respectively. (B) Left: Expression of Csf1r on naïve cells cultured in IL-3 and on mutant cells capable of growth in rhCSF1; K584E, R814P and V659I/T661A. Right: Expression of Csf1r on naïve and K614R mutant cells cultured in IL-3. (C) Expression of Csf1r on mCsf1r mutant cells cultured in IL-3 alone or both IL-3 and rhCSF1 compared to naïve cells. All cells were gated on live cells as determined by negative staining for propidium iodide.

Table I | Mutagenesis primers

Human Mutation	Murine Mutation	Mutagenesis primers (5′)
K586E	K584E	AACAACCTGCAGTTTGGT**G**AGACTCTAGGAGCCG
K616R	K614R	N/A - supplied by Taconic
E633K*	E631K	AGAAGGAGGCCCTGATGTCA**A**AGCTGAAGATCATG
V661I/T663A	V659I/T661A	N/A - supplied by Taconic
M766T*	M764T	CAAGTGGCTCAGGGCA**C**GGCCTTCCTTG
I794T*	I792T	CCAGCGGACATGTGGC**C**AAGA**C**TGGGGACTTTG
M875T*	M873T	TGGTGAAGGATGGATACCAAA**C**GGCCCAGCCTG
R816P	R814P	CAAGGGCAATGCCC**C**CCTGCCTGTAAAGT

*mutations from[1].

act in a dominant manner. In the autoinhibited CSF1R domain, E633 forms a salt bridge with the invariant amino acid K616[12]. Because E633K was identified in HDLS patients, we generated a mutant receptor in which K616 was mutated to arginine (K614R in mice) to further test the importance of this interaction. The K616R mutant is known to have reduced *in vitro* kinase activity[14]. Like the known HDLS mutants it was unable to survive in CSF1, highlighting the importance of the E633-K616 interaction in the autoinhibited CSF1R. Rademakers and colleagues[1] also identified two splice site variants amongst the HDLS patients that generate in-frame deletions of Exon 13 or Exon 18. Exon 13 is very highly conserved across species, even in birds and fish. We tested K584E, a charge reversal of an invariant amino acid within the exon 13-encoded region. This mutation generated a constitutively-active receptor that could produce growth factor independent Ba/F3 cells. Previous studies used another factor dependent cell line to identify activating mutations in exon 18 of CSF1R. R802V was characterized, which is equivalent to a known activating mutant in c-kit, a receptor tyrosine kinase (RTK) that is a member of the same RTK subfamily as Csf1r[30]. The R802V variant caused constitutive activation, and associated receptor internalization and degradation[31]. Mutation of Asp814 in the phosphotransferase domain of murine c-kit, has been shown to produce factor independent growth[32]. This amino acid is conserved in Csf1r. Unexpectedly, this mutation had no effect on function; the mutant receptor was able to sustain CSF1-dependent growth. We hypothesize that a hydrophobic amino acid substitution would have resulted in an activating mutation. Morley and colleagues found that substitution of human A802 with a polar residue could not transform FDC-P1 cells[31]. Conserved amino acids within the catalytic site of Csf1r are considered to be important for autoinhibition. We produced a double mutation within the active site, V661I/T663A. Cells expressing the mutant grew in CSF1 but also displayed a small, but statistically significant level of constitutive activity in the absence of growth factors. T663 has been identified as a 'Gatekeeper Residue,' an amino acid located in a kinase active site which confers selectivity for binding nucleotides. Mutation of gatekeeper residues in kinases have been shown to result in autoactivation due to enhanced phosphorylation[33].

The intracellular domain of the CSF1 receptor is highly conserved across species, and indeed is closely-related to other receptor protein tyrosine kinases[6]. The crystal structure of the autoinhibited kinase domain revealed a very extensive interface between the JM domain and the catalytic loop. Remarkably, the variation table for the *CSF1R* gene in Ensembl identifies $>$ 200 non-synonymous variants with minor allele frequencies of 1/1000 or more, many affecting conserved amino acids in the intracellular domain. It appears likely that other mutations in the receptor will be linked to more subtle microglial defects, and perhaps to other macrophage-related pathologies. We have demonstrated that mutations corresponding to those in HDLS are required for the function of the mouse Csf1r. Csf1r signaling in mice is known to be necessary for the generation of microglia[4] and there is some evidence that the receptor may contribute directly to neuronal homeostasis[34,35]. The generation of the HDLS mutations in the mouse germ line via ES cell mutagenesis may therefore generate subtle hypomorphs and provide insight into neuroprotective roles of Csf1r in the brain.

Methods

Cell culture. Untransfected Ba/F3 (naïve) or Ba/F3 cells expressing wild-type or mutant murine *Csf1r* were maintained in RPMI 1640 containing 10% FCS, 25 U/ml penicillin, 25 μg/ml streptomycin and 2 mM GlutaMAX™ supplemented with either 5% conditioned media from x63-IL-3 cells[36] or 10^4 U/ml (100 ng/ml) recombinant human CSF1 (rhCSF1, a gift from Chiron, USA).

Plasmid construction and transfection. The wild type murine *Csf1r* and the mutations K614R and V659I/T661A were amplified from plasmids provided by Taconic using the following primers F: ACCATGGAGTTGGGGCCT and R: GCAGAACTGGTAGTTGTTAGGCTG. The receptor sequences were subcloned into pEF6/V5-His TOPO (Invitrogen). Murine mutations were prepared from the wild type *Csf1r* pEF6/V5-His construct using Agilent's QuikChange II XL Site-Directed Mutagenesis kit according to instructions. The mutagenic primers are listed in Table I with mutated nucleotides in bold and underlined. All clones were sequence verified.

For generation of cells expressing wild-type or mutant mCsf1r, 5×10^6 Ba/F3 cells were electroporated (1 pulse, 300 V, 975 μF, GenePulser, Bio-Rad) with 10 μg pEF6 in 250 μl complete medium. Stable transfectants were selected in 10 μg/ml Blasticidin (Invitrogen).

Cell viability assays. 2×10^4 cells/well of a 96-well plate were plated in complete medium either without growth factors, with x63-IL-3 conditioned media or 10^4 U/ml (100 ng/ml) rhCSF1 and incubated at 37°C, 5% CO_2 for 72 h. MTT stock solution (5 mg/ml in PBS) was added directly to growth medium at a concentration of 0.5 mg/ml and the plate was incubated at 37°C for 3 h. Solubilization of tetrazolium salt was achieved with a solution of 10% SDS/50% isopropanol/0.01 M HCl at 37°C overnight. The plates were read at 570 nm with a reference wavelength of 650 nm.

FACS analysis. Cells capable of growth in rhCSF1 (wild-type Csf1r, K584E, R814P and V659I/T661A) were starved of rhCSF1 24 hours prior to FACS analysis to allow for cell surface expression of the receptor. Cells unable to grow in rhCSF1 (K614R and the four HDLS mutants) were cultured in IL-3 prior to FACS analysis. Live cells were stained for Csf1r expression with anti-Mouse CD115 (c-fms) PE (eBioscience) according to standard protocols and analysed on a FACS Calibur (BD). Dead cells were excluded with propidium iodide staining (1 μg/ml). For analysis of cell surface Csf1r downregulation, HDLS mutants were cultured in IL-3 and were also co-treated with rhCSF1 for 4 h prior to analysis.

Protein visualization. YASARA (http://www.yasara.org/) was used to predict the structure of the murine (aa540–917) and human (aa542–919) autoinhibited kinase domain based on the human crystallized structure (PDB ID code 2OGV) reported by Walter and colleagues[12]. Our models include the kinase insert domain which was omitted from 2OGV. Both proteins were aligned using MUSTANG[37].

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Author contributions

D.A.H. designed the project with contributions from C.P., K.A.S., K.B. and H.K. C.P., K.A.S. and D.A.H. wrote the manuscript and all authors reviewed the manuscript. C.P. prepared figures 1–2 and K.A.S prepared figure 3. C.P. and K.A.S. performed the experiments. K.B. and H.K. provided reagents and D.A.H. provided funding and supervised the project.

Additional information

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