RESEARCH ARTICLE

Genetic interaction implicates iRhom2 in the regulation of EGF receptor signalling in mice

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ABSTRACT

iRhoms are closely related to rhomboid intramembrane proteases but lack catalytic activity. In mammals iRhoms are known to regulate the trafficking of TACE, the protease that cleaves the membrane bound inflammatory cytokine TNF. We have mapped a spontaneously occurring mouse mutation with a loss of hair phenotype, curly bare (cub), to the Rhbdf2 locus, which encodes the iRhom2 protein. The cub deletion removes the first 268 amino acids of the iRhom2 protein but is not a loss of function. We have also identified a previously reported suppressor of cub, called Mcub (modifier of cub bare), and find it to be a loss of function allele of the amphiregulin gene (Areg). Amphiregulin is an activating ligand of the epidermal growth factor receptor (EGFR) that, like TNF, is released by TACE. Our results therefore imply a regulatory link between iRhoms and EGFR signalling in mammals. We have tested the model that the cub mutation leads to iRhom2 hyperactivity and consequently excess TACE processing of amphiregulin and elevated EGFR signalling. Our results do not support this hypothesis: we find that, compared to wild-type cells, cub mutant embryonic fibroblasts release less amphiregulin, and that the cub mutant form of iRhom2 is less able than wild type to bind to TACE and promote its maturation.

KEY WORDS: Rhomboid, iRhom, Rhbdf2, Amphiregulin, Mouse, TACE, ADAM17

INTRODUCTION

The earliest members of the rhomboid-like superfamily to be discovered were the rhomboid intramembrane serine proteases (Urban et al., 2001) which, by cleaving transmembrane serine helices and releasing soluble domains, have multiple functions in controlling signalling and other cellular functions (Urban and Dickey, 2011). More recently, the extent of the rhomboid-like superfamily has become clear, and this has highlighted that many members of this clan are predicted not to be active proteases (Freeman, 2014). The most studied non-protease rhomboid-like proteins are the iRhoms, which regulate trafficking and degradation of client proteins (Adrain et al., 2012; Maretzky et al., 2013; McIlwain et al., 2012; Siggs et al., 2012; Zettl et al., 2011). Despite intense recent scrutiny, there are still many aspects of the physiological role of iRhoms that are poorly understood.

Both in Drosophila and mammals, a genetic approach has underpinned our understanding of iRhom function: loss of function mutations in flies and mice have revealed the role of iRhoms in both ER associated degradation, and the control of trafficking of the metalloprotease TACE, the enzyme that releases active TNF and ligands of the EGF family (Adrain et al., 2012; McIlwain et al., 2012; Zettl et al., 2011). Beyond these simple loss of function alleles, disease mutations in humans have also been isolated. Several groups have shown that a rare familial hyperkeratosis and oesophageal cancer syndrome, tylosis, is caused by missense mutations in specific locations within the cytoplasmic N-terminus of iRhom2 (Blaydon et al., 2012; Saarinen et al., 2012); these have also been associated with ovarian cancer (Wojnarowicz et al., 2012). The molecular effect of these mutations is not yet clear, but they identify an important functional site in the iRhom2 protein, and there is recent evidence that they lead to increased release of EGF family ligands in keratinocytes (Brooke et al., 2014).

Johnson et al. reported a recessive mouse mutation on chromosome 11 with a hair-loss phenotype that they named curly bare (cub) (Johnson et al., 2003). The cub mutation mapped to an interval that included Rhbdf2, the gene encoding iRhom2. In the same work, it was shown that a second site dominant suppressor of the cub phenotype existed on chromosome 5; this was called Mcub for modifier of cub. One copy of the Mcub allele was sufficient to rescue the hair loss of cub/cub, although the coat of these rescued mice was wavy. Significantly, wavy coat phenotypes are characteristic of mutations in the EGFR pathway (Schneider et al., 2008).

Here we report that the cub mutation is a deletion of the N-terminal cytoplasmic domain of the Rhbdf2 gene, which encodes iRhom2. Mice with complete loss of iRhom2 have normal coats (Adrain et al., 2012; McIlwain et al., 2012), implying that the cub mutation is not a simple loss of function. We have also used whole genome sequencing to identify the modifier mutation on chromosome 5 as being a loss of function variant of the amphiregulin gene, Areg. Amphiregulin is a ligand for the EGFR, strengthening the case for functional links between iRhom2 and EGFR signalling. These results are consistent with two very recent reports, one of which also identifies the cub and Mcub mutations (Hosur et al., 2014), and the other which reports that a distinct but overlapping deletion in the cytoplasmic domain of iRhom2 leads to another mouse hair-loss phenotype (Liu et al., 2014). We have further explored the mechanistic implications of the links between iRhom2 and amphiregulin and we report here that the simple interpretation that the cub mutation is a gain of iRhom2 function, causing excess amphiregulin release, is not fully supported. The actual relationship between iRhom2, amphiregulin and EGFR signalling is more complex.
MATERIALS AND METHODS

Genome sequencing
Tail DNA from the cub/Mcub strain (003628 B6.Cg-Mcub cub/J) was obtained from the Jackson Laboratory, and subjected to whole-genome sequencing as described (Bull et al., 2013). Briefly, 100 bp paired-end libraries were prepared and sequenced on a single lane of an Illumina HiSeq 2000. Reads were aligned to the mouse reference genome (mm10) using Stampy (Lunter and Goodson, 2011) with BWA settings, and duplicate reads were identified and discarded using the MarkDuplicates tool (http://broadinstitute.github.io/picard). 94.6% of the reference genome was covered at least three times (as determined by BEDTools). Variants were called by Platypus version 0.1.9 (Rimmer et al., 2014), and annotated using ANNOVAR (Wang et al., 2010) with RefSeq and dbSNP 138 annotations.

All animal procedures in this manuscript were performed in compliance with the institutional animal care committee guidelines of the University of Oxford and according to protocols approved by the UK Home Office.

Antibodies
The following antibodies were used: Goat anti-mouse amphiregulin (R&D Systems, catalogue number AF989; 1:1000), Mouse anti-nonphospho-Tyr1173-EGFR (Millipore, catalogue number 05-484; 1:1000), mouse anti-beta-actin (Santa Cruz, catalogue number sc-47778; 1:200), rabbit anti-TACE/ADAM17 (Abcam, catalogue number ab39161; 1:2000), rabbit anti-HA (Santa-Cruz, catalogue number sc-805; 1:2000) and mouse anti-transferin receptor (Invitrogen, catalogue number 13-6800; 1:1000). Corresponding species-specific HRP-coupled secondary antibodies were used from Santa Cruz and Cell Signaling.

Molecular biology
pM6P.blast-GFP was a kind gift from Felix Randow (LMB, Cambridge). For pM6P.blast iRhom2-3xHA and pM6P.blast cub-3xHA, corresponding coding sequences were PCR amplified, the plasmid backbone was digested with restriction enzyme sites Ncol/Xhol and Ncol/NotI, respectively and PCR products were inserted using the InFusion cloning system (Clontech). All plasmids were verified by DNA sequencing.

Cell culture
Mouse embryonic fibroblasts (MEFs) were isolated from C57BL/6J NaCl, 50 mM Tris-HCl, pH 7.4, supplemented with 1 mM EDTA, 1 mM CaCl$_2$ and 1 mM MnCl$_2$) containing complete protease inhibitor cocktail (Roche) and 10 mM 1,10-phenanthroline, to prevent autocatalysis of TACE. Cells/lysates were then scraped and centrifuged at 20,000 × g. Protein concentration was then measured using BCA kit.

Lentiviral transduction of HEK293 cells
Using the pLex-based system (Thermo Scientific) for lentiviral production, HEK293 cells were transduced in the following way. Cells were plated on 6 cm plates one day before transfection, then transfected with 1 μg LTR plasmid containing iRhom2-3xHA or cub-3xHA, 0.7 μg pCMV delta8.91 (packaging plasmid) and 0.3 μg VSVG (envelope) in serum-free medium and PEI. After 24 hours, medium was removed and replaced with high-glucose DMEM, supplemented with 10% FCS, 100 μg/ml penicillin, and 100 μg/ml streptomycin. After 72 hours, medium was collected and centrifuged at 1,000 × g. Viral preparations were combined with 50 μg/ml polybrene and added to fresh HEK293 cells. After 24 hours, pLex-positive cells were washed and selected with 6 μg/ml puromycin for 7 days before assaying.

Immunoprecipitation and ConA enrichment
For ConA enrichment experiments, cells grown in 10 cm dishes were grown to ~80% confluency were washed twice in ice-cold PBS and then lysed for 10 minutes in TX-100 lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-Hcl, pH 7.4, supplemented with 1 mM EDTA, 1 mM CaCl$_2$ and 1 mM MnCl$_2$) containing complete protease inhibitor cocktail (Roche) and 10 mM 1,10-phenanthroline, to prevent autocatalysis of TACE. Cells/lysates were then scraped and centrifuged at 20,000 × g. Protein concentration was then measured using BCA kit.

For infection of target iRhom1;iRhom2 double knock-out (DKO) cells, viral supernatants were diluted 2-fold in fresh medium for transduction. Transduction was carried out in the presence of 50 μg/ml polybrene and a medium change was made 24 hours later. For selection of pM6P plasmids, cells were treated with 5 μg/ml blasticidin 48 hours later.

Lentiviral transduction of HEK293 cells
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SDS-PAGE and western blotting
Samples were typically electrophoresed at 200 V on 4–12% Bis-Tris gels (Invitrogen) until the dye front had migrated off the gel (approx.)
10–15 kDs). Gels were transferred onto PVDF membranes and blocked in PBS or TBS containing Tween 20 (0.05%) and 5% milk, before detection with the indicated primary antibodies and species-specific HRP-coupled secondary antibodies. Band visualisation was achieved with Enhanced Chemiluminescence (Amersham Biosciences) using X-ray film.

RESULTS AND DISCUSSION
Identification of the genes mutated in cub and Mcub mice
To identify the nature of the cub and Mcub mutations (Johnson et al., 2003), we sequenced the genome of a cub/cub;Mcub/Mcub double homozygote. cub arose spontaneously within an inbred colony of urogenital syndrome (us) mice at the Jackson Laboratory (a/a us/us), and has previously been mapped to a 75-gene interval between D11Mit214 and D11Mit303 (chr11:115130471–117238606) (Johnson et al., 2003). Interestingly, a similar phenotype, Uncovered, has also been mapped between D11Mit338 and D11Mit337 (chr11:115461783–118997798) (Shi et al., 2003). Although we detected no single nucleotide variants or short indels in the cub interval, a large deletion was present at the Rhbd2 locus (Fig. 1A). The Rhbd2<sup>cub</sup> deletion spanned 12,679 nucleotides (chr11:11664896–116617574, inclusive), which included exons 2–6. This deletion is predicted to result in the splicing of exon 1 to exon 7, the initiation of translation from an in-frame methionine codon in exon 8, and therefore the loss of the first 268 N-terminal residues, which include almost the entire cytoplasmic domain of iRhom2 (Fig. 1A).

Continuing our analysis of the cub/cub;Mcub/Mcub double homozygote, we sought to identify the Mcub mutation. From a total of 233,513 variants called across the genome, 240 were both homozygous and predicted to alter protein coding sense (i.e. nonsynonymous, stop, frameshift or splice variants) (Fig. 1B). Only one of these variants lay within the previously defined Mcub interval (chr5:75169841–97871790). This variant was a predicted critical splice donor mutation in the Areg gene (NM_009704:exon1:c.61+2T>G), and confirmed to be absent from CAST/EiJ, C3H/HeJ, and A/J inbred strains as established previously (Johnson et al., 2003). Given that the variant occurs after the first (coding) exon of Areg, we predicted that Areg<sup>Mcub</sup> would lead to intronic read-through, introduction of a premature termination codon in exon 2, and ultimately to nonsense-mediated decay (Fig. 1C).

These data establish that the hair defects seen in cub/cub homozygotes (Fig. 1D) are caused by a loss of the N-terminal cytoplasmic domain of iRhom2; that this mutation is not equivalent to a loss of function of iRhom2, which has a normal coat (Fig. 1E); and that the phenotype is largely suppressed by loss of one or both copies of the amphiregulin (Areg) gene. Our data agree with the recent identification of the cub and Mcub alleles by Hosur et al. (Hosur et al., 2014). Both reports are also consistent with the recent discovery of another hairless allele of Rhbd2, which is also caused by deletion of a significant part of the iRhom2 cytoplasmic domain (Liu et al., 2014).

Investigating the relationship between iRhom2 and amphiregulin
iRhos play an essential role in the maturation of TACE (also named ADAM17), the enzyme responsible for the release of active amphiregulin (Gschwind et al., 2003; Hinkle et al., 2004). Given that the cub mutation is suppressed by a reduction of amphiregulin, we speculated that there would be excessive amphiregulin release from cub mutant cells. This would imply that cub represents a gain of function in iRhom2, which would be consistent with it having a hair-loss phenotype not seen in iRhom2 null mice. We therefore investigated the effect that the cub mutation has on iRhom2 function. First, we compared the ability of MEFs from cub and WT embryos to shed amphiregulin. To our surprise, instead of increased release, cub cells showed strongly reduced levels (approx. 60% compared to WT cells) of amphiregulin in the medium (Fig. 2A,B).

Since the ADAM family of proteases exhibit stimulus dependent as well as constitutive shedding activity (Horiiuch et al., 2007), we separately examined the ability of cub mutant cells to promote the stimulated shedding of amphiregulin in a widely used assay (Zheng et al., 2002). MEFs were treated with PMA for one hour and the stimulated level of alkaline phosphatase (AP) tagged amphiregulin released into the medium was compared between WT and cub MEFs. The cub MEFs showed significantly reduced stimulated shedding of amphiregulin (Fig. 2C), demonstrating that both aspects of shedding activity (stimulated as well as constitutive) are diminished by the cub mutation. In comparison, there was no significant difference between WT and cub cells in their ability to shed AP-EGF, which does not depend on TACE for its release (Fig. 2D).

iRhom2 is an essential factor in the trafficking and maturation of TACE, so we analysed the effect of the cub mutation on the ability of iRhom2 to promote TACE maturation (Fig. 3A). Double knockout MEFs, lacking both iRhom2 and the related iRhom1 were stably transduced with either WT or the cub mutant form of iRhom2-HA. Relative levels of endogenous immature and mature TACE were compared, before and after ConA enrichment (to concentrate glycoproteins from lysates). Consistent with the reduced release of amphiregulin, MEFs transduced with the cub mutant form of iRhom2 had substantially less mature TACE than WT counterparts (Fig. 3A,B).

Finally, to test whether the reduced ability of the cub mutant form of iRhom2 to promote TACE maturation and amphiregulin shedding was caused by impaired binding between the cub mutant form of iRhom2 and its client, TACE, we stably expressed HA-tagged iRhom2 and the cub mutant form in HEK293 cells. Compared to WT iRhom2-HA, co-immunoprecipitation showed that the cub mutant form of iRhom2 showed greatly reduced binding to endogenous TACE (Fig. 4A). Analysis of levels of immature and mature TACE in these stable HEK293 cells revealed there is no major difference in TACE expression upon introduction of these constructs into the cell (Fig. 4B). These data demonstrate that the interaction between iRhom2 and TACE is strongly dimished by the cub mutation.

These results are consistent with each other but not with the model that the cub mutation causes a simple gain of function of iRhom2. In that case we would expect to see increased binding to and/or increased maturation of TACE, and elevated amphiregulin release. In each case our result is the opposite of that prediction. The genetics of cub, however, are complicated. First, we can rule out cub being a simple loss or reduction of iRhom2 function, since the complete loss of iRhom2 does not cause the coat defects seen in cub (compare Fig. 1D and Fig. 1E) (see also Adrain et al., 2012; McIlwain et al., 2012). Second, we can also rule out cub being a simple gain of function mutation because the hairless phenotype is fully rescued by a WT allele of iRhom2 but only somewhat rescued by a loss of function allele (compare Fig. 1F with Fig. 1E).
and Fig. 1G) (see also Hosur et al., 2014). In other words, addition of extra iRhom2 function (the WT allele in cub/+/ compared to cub/iRhom2 KO) rescues the effect of the cub mutation – which is inconsistent with cub being a gain of function. Therefore cub appears to be a complex allele that combines gain and loss of function characteristics. It is also worth noting Fig. 1. cub and M cub are mutations at the Rhbdf2 and Areg loci, respectively. (A) Coverage depth across the Rhbdf2 locus in cub/cub mice, and predicted translational consequences of the cub deletion on iRhom2 protein. (B) Filtering pipeline for variants detected after whole-genome sequencing of cub/cub;M cub DNA. (C) Identification and predicted consequences of the AregMcub variant. (D) The bald phenotype of an Rhbdf2cub/Rhbdf2cub mouse (Johnson et al., 2003). (E) Rhbdf2KO/Rhbdf2KO mouse has a WT coat (Adrain et al., 2012; McIlwain et al., 2012). (F) Rhbdf2cub is recessive: Rhbdf2cub/+ mouse has a WT coat (Johnson et al., 2003). (G) Rhbdf2cub/Rhbdf2KO mouse has sparse hair (Hosur et al., 2014). Mice in panels D–G are on a C57BL/6J background, except for Rhbdf2cub/Rhbdf2KO, which is on a mixed C57BL/6J/129 background.
amphiregulin production in both recent model proposed by Hosur et al., in which they show excess concede that, while their model that possible explanations for these significant discrepancies but keratinocytes (Hosur et al., 2014). We now discuss some of cub follicle-specific protein complex and that the One explanation to consider is that iRhom2 functions in a hair currently in a position to propose a well-supported alternative. noting that most cells express iRhom1 as well as iRhom2 and their function appears at least largely interchangeable (Christova et al., 2013), so even in cub homozygous mice, there remains WT iRhom function.

We note that our failure to detect excess amphiregulin release induced by the cub mutant form of iRhom2 disagrees with the recent model proposed by Hosur et al., in which they show excess amphiregulin production in both cub serum and the supernatant of cub keratinocytes (Hosur et al., 2014). We now discuss some of the possible explanations for these significant discrepancies but concede that, while their model that cub represents a gain of function is not well supported by current data, we are not currently in a position to propose a well-supported alternative. One explanation to consider is that iRhom2 functions in a hair follicle-specific protein complex and that the cub mutation causes a tissue specific dominant negative effect. Although this would formally be consistent with all current data, there is no evidence for such a complex and the idea is purely speculative.

A striking result reported by Hosur et al. is that amphiregulin mRNA expression is elevated 4-fold in cub skin (Hosur et al., 2014). This result could provide an additional or alternative explanation for the increased amphiregulin production in cub mutant cells that they observe: instead of the cub mutant form of iRhom2 directly triggering amphiregulin release, the effect could be indirect, via a transcriptional response.

We noticed that in cub mutant MEFs there was a dramatic down-regulation of EGFR levels (Fig 5A,B). This was also reported by Hosur et al. (Hosur et al., 2014). They interpreted this to be caused by increased endocytic degradation of cell surface receptor induced by the excess amphiregulin signalling. Indeed, EGFR levels are known to be tightly regulated by receptor activity (Wiley, 2003). However, this interpretation cannot easily be reconciled with our results since, in our case, the receptor downregulation occurs in cells with reduced, not increased, amphiregulin secretion.

Of relevance to this discussion, Brooke et al. have recently analysed disease mutations in the cytoplasmic domain of human iRhom2 (Brooke et al., 2014) and, reminiscent of the work of Hosur et al. (Hosur et al., 2014), they find that these mutations cause excess production of some EGF family ligands, including amphiregulin. But in contrast to Hosur et al., this was reported to be fully dependent on TACE activity. Hosur et al. are not explicit about which protease they believe to be responsible for elevated amphiregulin release in their experiments, but they find it to be insensitive to marimastat, an inhibitor of TACE and other ADAM proteases. They therefore conclude that the cub mutant form of iRhom2 promotes amphiregulin release by an ADAM protease-independent mechanism, a very different mechanistic conclusion to that of Brooke et al. (Brooke et al., 2014). In fact, Hosur et al. appear to favour the idea that there could be intrinsic proteolytic activity in the iRhom2 protein itself. This is a suggestion that is difficult to disprove formally but that would radically alter current models of iRhom function (Freeman, 2014): iRhom2 lacks all the necessary catalytic residues of an active rhomboid, indeed it has an activity-killing proline inserted at the site equivalent to a catalytic site in rhomboids. Moreover, it has never been found to cleave any potential substrate, and Hosur et al. do
not support their suggestion with direct evidence to counter these earlier studies. On the current balance of evidence, therefore, we do not think that the model proposed by Hosur et al. is likely to be correct.

Having tried to be explicit about where our work differs from that of Hosur et al. (Hosur et al., 2014) (or where we believe their interpretations are inconsistent with other existing data), we wish to point out that the experimental differences, primarily whether amphiregulin release is decreased or increased, may be due to differences in how the experiments were performed. For example, in our work, we have used embryonic fibroblasts derived from cub embryos; Hosur et al. focused mainly on keratinocytes. Since the cub phenotype affects specifically hair follicle cells, neither MEFs nor keratinocytes are fully appropriate model cells, although it is surprising that they appear to give such opposing results. It is worth noting that the Kelsell lab, when studying the tylosis mutations in human cells (Brooke et al., 2014), also used MEFs nor keratinocytes are fully appropriate model cells, leading to the production of a protein with most of its cytoplasmic domain deleted. We have also demonstrated that Mcub, the dominant suppressor of cub, is a loss of function mutation in the amphiregulin gene. These genetic data make a clear case for a functional relationship between iRhom2 function and EGFR signalling. This is certainly the case in Drosophila, where iRhom regulates EGFR signalling (Zettl et al., 2011). Importantly, though, in that case iRhom does not, as in mice, promote shedding but instead inhibits ligand production by degrading EGFR ligands as they pass through the secretory pathway. As noted above, we do not currently know the significance of these apparently profound differences between Drosophila and mammalian iRhom function. Our attempts to interpret mechanistically our current data to understand more about the role of iRhom2 in EGFR signalling in mice have failed to lead to a clear model. Contrary to the superficially attractive idea that cub is a gain of function allele of iRhom2, we find amphiregulin release to be reduced in cub MEFs, TACE maturation to be impaired, and the binding of the cub mutant form of iRhom2 to its client TACE also to be reduced.

Concluding remarks
Since our genetic identification of cub and Mcub exactly agrees with the recent report by Hosur et al. (Hosur et al., 2014), it is very surprising that our attempts to provide a mechanistic explanation differ so profoundly. While we disagree with many of their conclusions, we readily concede that we do not have a clear alternative picture. Further work will be needed to address these questions fully. We also want to emphasise that we do not rule out the possibility that amphiregulin overproduction is relevant to the cub phenotype. Indeed, the basic genetics supports this view, and other evidence, such as the ability of cub to enhance tumour growth in the ApcMin mouse model (Hosur et al., 2014), is consistent with the idea. However, the mechanistic explanation proposed by Hosur et al. is not easy to reconcile with current data. It is relevant to emphasise that we understand little about the cellular mechanisms by which iRhoms act. For example, there is no solid evidence for their physiological role in regulating ERAD in mammalian cells, despite it being an important function in Drosophila (Zettl et al., 2011). And if, in fact, iRhoms do have a role in mammalian ERAD, there is no understanding about how this relates to their function in forward trafficking of TACE.

We have shown that the cub mutation is a deletion predicted to cause the loss of 268 N-terminal amino acids of mouse iRhom2, leading to the production of a protein with most of its cytoplasmic domain deleted. We have also demonstrated that Mcub, the dominant suppressor of cub, is a loss of function mutation in the amphiregulin gene. These genetic data make a clear case for a functional relationship between iRhom2 function and EGFR signalling. This is certainly the case in Drosophila, where iRhom regulates EGFR signalling (Zettl et al., 2011). Importantly, though, in that case iRhom does not, as in mice, promote shedding but instead inhibits ligand production by degrading EGFR ligands as they pass through the secretory pathway. As noted above, we do not currently know the significance of these apparently profound differences between Drosophila and mammalian iRhom function. Our attempts to interpret mechanistically our current data to understand more about the role of iRhom2 in EGFR signalling in mice have failed to lead to a clear model. Contrary to the superficially attractive idea that cub is a gain of function allele of iRhom2, we find amphiregulin release to be reduced in cub MEFs, TACE maturation to be impaired, and the binding of the cub mutant form of iRhom2 to its client TACE also to be reduced.

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Competing interests
The authors have no competing interests to declare.

Author contributions
OMS, AG, HX and PB performed the experiments. OMS, AG, HX, PB, YC and MF designed the experiments. All authors participated in writing the manuscript.

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