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RESEARCH ARTICLE

The secreted protein augurin is a novel modulator of canonical Wnt signalling involved in osteoblast differentiation

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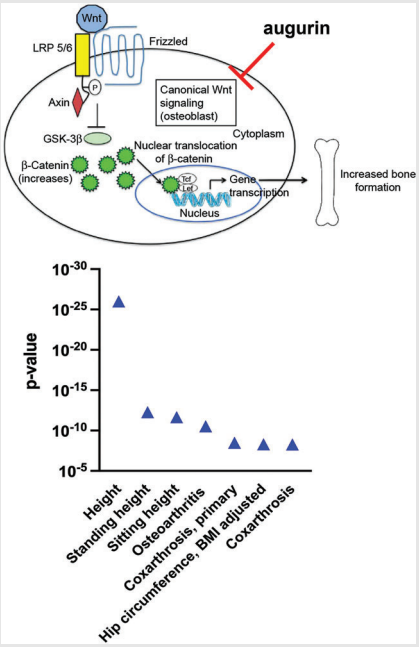
Graphical Abstract



- The secreted protein augurin is a novel inhibitor of canonical Wnt signalling
- Inactivation of the gene encoding augurin (*Ecrg4*) produced increased osteogenic differentiation of mouse calvarial osteoblasts
- Genome-wide association studies showed that an intronic *ECRG4* variant (rs66989638) is associated with height and osteoarthritis
- Augurin is a new potential drug target to modulate Wnt signalling

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The secreted protein augurin is a novel modulator of canonical Wnt signalling involved in osteoblast differentiation

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Abstract

Background: *ECRG4/C2ORF40* is a tumour suppressor gene downregulated in several cancer types, which encodes the secreted protein augurin. A wide number of functions in health and disease have been assigned to augurin, but the signalling pathways it regulates are still poorly characterized. Augurin expression is strongly upregulated during in vitro differentiation of neonatal mouse osteoblasts.

Methods: In vitro differentiation assays of calvarial osteoblasts isolated from *Ecrg4* -/- and wild-type mice; transient transfection assays using reporters activated by Wnt signalling and other signal transduction pathways; Real-time quantitative polymerase chain reaction for measurement of gene expression; protein expression in Chinese hamster ovary cells and *Escherichia coli*; in situ binding assays of proteins expressed as fusions to alkaline phosphatase with cells expressing various membrane receptors.

Results: Osteoblasts from *Ecrg4* -/- mice have an accelerated differentiation compared to wild-type and upregulation of Wnt markers. Augurin is a specific repressor of Wnt-stimulated transcriptional activity, both when coexpressed together with the reporter and when added to the culture medium as a soluble protein. We confirmed the previously described binding of augurin to LOX1, a scavenger receptor, but an inhibitor of this molecule did not impair augurin repression of Wnt-stimulated transcription specifically. Genome-wide association studies showed an association of *ECRG4* genomic variation with body height and osteoarthritis.

Conclusions: Our study sheds new light on the wide spectrum of functions previously ascribed to augurin in brain function, stem cell biology,

inflammation/immunity and cancer. Furthermore, our discovery paves the way to further characterization of the mechanisms involved in augurin repression of Wnt signalling and the development of agonists and antagonists for this protein, which have a wide array of potential applications in the clinic.

KEYWORDS
bone, peptides, Wnt signalling

1 | INTRODUCTION

Peptide hormones are processed and secreted by small proteins that signal via membrane receptors and play important roles in virtually every aspect of physiology. In an effort to identify new peptide hormones in the human genome by bioinformatic methods, Mirabeau et al. described augurin, a 148 aa. protein containing a single prohormone cleavage site which is conserved from mammals to fish (83% identity between humans and mice).[1] Augurin is encoded by the human *C2ORF40*/*ECRG4* gene and by *1500015O10Rik/Ecrg4* in the mouse. The full-length protein can undergo post-translational processing[2] (Figure S1A). Remarkably, *ECRG4* expression is downregulated in the great majority of cancer types compared to the corresponding normal tissues and has the characteristics of a tumour suppressor gene[3] (Figure S1B). Augurin is expressed in several tissues and a variety of biological functions have been ascribed to it, with roles in the regulation of cell proliferation, stem cell renewal, inflammation and injury.[4] On the cell surface, augurin has been shown to bind to LOX1 and to other scavenger receptors, which are membrane proteins that bind a variety of unrelated ligands.[5] However, the mechanisms of the biological effects of augurin and the signalling pathways it regulates are still poorly characterized.

Here we show that augurin modulates the canonical Wnt pathway, a key signalling pathway during development and in tissue homeostasis. These findings open new avenues in our understanding of augurin function in health and disease.

2 | MATERIALS AND METHODS

2.1 | Cell culture

MC3T3-E1 subclone 4 cells (ATCC CRL-2593) were cultured in Minimum Essential Medium Alpha (MEMα, Gibco #A1049001) with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine and 1 mM sodium pyruvate without ascorbic acid, supplemented with penicillin-streptomycin (pen/strep) and fetal bovine serum (FBS) to a final concentration of 10%. L (ATCC CRL-2648), HEK293T and COS1 cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco #11995-065) supplemented with pen/strep and FBS to a final concentration of 10%. L-Wnt3a cells (ATCC CRL-2647) were cultured in the same medium with the addition of 0.4 mg/ml G418. Chinese hamster ovary (CHO) cells were cultured in MEMα medium (Gibco #22571-020) supplemented with pen/strep, 2 mM L-glutamine, 0.1 mM non-essential amino acids and 10% FBS.

2.2 | DNA clones

pCMV6-Entry *ECRG4*/augurin human tagged ORF clone (#RC206239) and pCMV6-Kan/Neo *1500015O10Rik*/augurin mouse untagged clone (#MC200116) were purchased from Origene. The protein convertase/furin consensus sites R67A/K69A human and mouse augurin mutants were obtained from the above-mentioned expression vectors by site-directed mutagenesis using the QuikChange kit (Stratagene) according to the manufacturer's instructions. Mouse augurin deletion constructs were PCR-generated from pCMV6-Kan/Neo *1500015O10Rik*/augurin to encode amino acids 1–29 (mAug STOP 30), 1–59 (mAug STOP 60), 1–89 (mAug STOP 90). Mutations were introduced into pCMV6-Kan/Neo *1500015O10Rik*/augurin by the QuickChange mutagenesis system (Stratagene). Mutagenic primer sequences are available upon request. To obtain human and mouse augurin alkaline phosphatase (AP) fusion constructs the augurin G31-Y148 fragments were excised from the Origene clones (see above) with *AgeI* and *XbaI* and cloned in frame with the carboxy terminus of the AP ORF inserted into a pCS2+ vector (pCS2+ NCHD Secreted Flag AP5; a kind gift of B. Reversade). All constructs were sequenced to confirm their identities. The expression vectors pcDNA 3.1+ DKK1-AP were a kind gift of C. Niehrs, and the AP-hRSPO1 fusion construct was a kind gift of A. Glinka. The expression vector coding for HA-hLGR4 was a kind gift from X. Gong. The TOPFLASH

(#21-170) and FOPFLASH (#21-169) reporter plasmids were purchased from Merck/Millipore. The pcDNA3.3 LRP5 (#115907), pcDNA6-N-3xFlag-LRP6 (#123595) and p1242 3x-KB-L (#26699) plasmids were obtained from Addgene. The LOX1 (OLR1) human-tagged ORF clone (#RC204704) was purchased from Origene. The p5xSFRE luciferase reporter was a kind gift of D. McDonnell, p5 × 17mer globin luc was previously described,[6] pCRE luc (#219076) was purchased from Agilent, phRL-CMV (E6271) from Promega.

2.3 | Conditioned media and AP fusion proteins production

Conditioned media containing human or mouse augurin were prepared by transfecting CHO cells (500.000 cells/well in 6 well-plates) with Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions with wild-type (WT) or mutant constructs. The day after transfection cells were washed twice with Dulbecco's phosphate-buffered saline (D-PBS; Gibco #14190250), and then MEMα medium without serum was added for a further 48 h prior to conditioned media collection. For L and L-Wnt3a conditioned media, cells were plated in culture medium (without G418 for L-Wnt3a cells) in 10 cm tissue culture dishes and grown for 4 days (approximately to confluency). The medium was then collected and sterile filtered (first batch). Then, a fresh culture medium was added and cells were cultured for another 3 days. The medium was then collected and sterile filtered (second batch). The first and the second batches of medium were mixed 1:1. Protein expression was verified by Western blot analysis of conditioned media using an anti-C2ORF40 antibody (Sigma-Aldrich #HPA008546).

For the production of AP fusion proteins, unfused AP, hAug-AP, mAug-AP and DKK1-AP were prepared by transfecting HEK293T cells (500 000 cells/well in 6-well plates) with Trans-IT 293 Transfection Reagent (Mirus) according to the manufacturer's instructions. After 48 h of transfection, the old medium was replaced by a fresh one, that was conditioned for 4 days. Once the medium was collected, the debris was eliminated by centrifugation at 12 000 g in a benchtop centrifuge and the supernatant was buffered with 10 mM HEPES, pH 7. RSPO1-AP conditioned medium was produced from HEK293T cells (3×10^6 in a 10 cm dish) transfected with Trans-IT 293 Transfection Reagent (Mirus) according to the manufacturer's instructions. After 24 h the medium was collected, sterile filtered and replaced with fresh complete medium. The day after a second batch was collected, sterile filtered and mixed with the first batch (1:1). AP activity was measured as follows. One ml of supernatant was incubated at 65°C for 10 min to heat inactivate the endogenous AP activity. After centrifugation at maximum speed for 5 min, the supernatant was collected and an equal amount of BM purple (Roche #11442074001), a chromogenic substrate for AP, was added. AP activity was measured in a 96-well plate by the change in absorbance at 405 nm using a microplate reader. The conditioned media obtained by the described procedures were stored at 4°C for 6 months.

2.4 | Mouse recombinant augurin production

The mouse cDNA sequence corresponding to amino acids 31–148 of the *1500015O10Rik*/augurin ORF was cloned into the bacterial expression vector pET15b between restriction sites *NdeI* and *BamHI*. BL21DE3 *Escherichia coli* were transformed with this expression vector, cultured and induced to express the protein, which was subsequently purified to homogeneity by ion exchange and gel filtration chromatography.

2.5 | Immunohistochemistry

Adrenal glands were dissected from adult mice, paraformaldehyde-fixed and embedded in paraffin using standard protocols, as previously described.[7] Augurin was detected by the anti-C20RF40 rabbit polyclonal antibody (Sigma-Aldrich #HPA008546) diluted 1:100 and revealed using the EnVision+ System-HRP Labelled Polymer Anti-Rabbit (Dako #K4002). Sections were counterstained with hematoxylin-eosin, mounted and observed using a Leica microscope.

2.6 | Immunoblots

The expression of mAug in the supernatants of CHO cells transfected with EV or mouse augurin expression vector was verified by Western blot according to the protocol provided by Invitrogen for SDS polyacrylamide gel electrophoresis using tricine mini gels. Briefly, 50 μl (from a total volume of 6 ml) of EV- or mAug-conditioned media were mixed with 2X Tricine SDS sample buffer (Invitrogen #LC1676) and 10X NuPage Reducing Agent (Invitrogen #2148880). Samples were heated at 85°C for 2 min and proteins were separated on 10%–20% SDS polyacrylamide Tricine gels (Invitrogen #EC6625BOX) for 90 min at 125 V using an XCELL Surelock mini-gel running tank. Proteins were transferred to a polyvinylidene fluoride membrane. The anti-C20RF40 rabbit polyclonal antibody (Sigma-Aldrich #HPA008546) diluted 1:1000 and the

anti-GAPDH mouse monoclonal antibody (6C5) (Sigma-Aldrich #CB1001) diluted 1:2000 were used for augurin and GAPDH detection, respectively. Signals were revealed by the ECL Plus chemiluminescence system (GE Healthcare #RPN2232).

2.7 | Transient transfection assays

MC3T3-E1 cells were plated in 96-well plates (5000 cells/well) in a complete culture medium. The day after the cells were co-transfected with Lipofectamine 3000 (Invitrogen), according to the manufacturer's instructions, with 50 ng of the reporter plasmid TOPFLASH (to monitor Tcf/beta-catenin transcriptional activity) or its negative control FOPFLASH and 50 ng of empty vector (EV) or human or mouse WT or R67A/K69A mutant augurin expression vectors or mouse augurin deletion constructs (mAug STOP 30, mAug STOP 60 and mAug STOP 90) plus 1 ng of the *Renilla* luciferase reporter phRL-CMV. For dose-response assays, cells were transfected with different amounts of WT mAug expression vectors (10, 25, 40 and 50 ng). Alternatively, cells were co-transfected with 50 ng of the reporters 5xSFRE, pCRE-luc or 5 × 17 mer globin luciferase reporters and 50 ng of EV or human or mouse WT or R67A/K69A augurin expression vectors plus 1 ng of phRL-CMV. Before adding the transfection mix to the cells, the culture medium was aspirated and replaced with a mix (1:1) of L-Wnt3a/RSPO1 conditioned media to stimulate TOPFLASH reporter activity or with L-medium alone as a control. The same protocol was employed when the effects of increasing doses of mAug recombinant protein were evaluated on the activity of TOPFLASH, CRE luc, 5 × 17 mer globin and p1242 3x-KB-L reporters. For experiments employing conditioned media, cells were co-transfected with 100 ng of FOPFLASH or TOPFLASH or 5xSFRE or CRE luc or 5 × 17 mer globin reporters and 1 ng of phRL-CMV. Before the addition of the transfection mix, the culture medium was replaced by a mix (1:1:1) of L-Wnt3a/RSPO1/mAug conditioned media. For all the described protocols after 48 h of transfection, the signal of Firefly luciferase was measured by the Luciferase assay system (Promega #E1500), whereas the Renilla luciferase signal was measured by the Renilla Luciferase assay system (Promega #E2810). Luciferase assays were realized using a Luminoskan Ascent (Thermo Labsystems) luminometer. Results were normalized by the Renilla signal. To assess a possible effect of the scavenger receptor pan inhibitor poly(I) on the TOPFLASH reporter activity, cells were co-transfected with 100 ng of TOPFLASH reporter and 1 ng of phRL-CMV. The day after they were pretreated for 1 h with 2.5 μg/ml poly(I) or the inactive poly(C), then control medium or mAug conditioned medium supplemented with vehicle or with 2.5 μg/ml poly(I) or poly(C) were added. Cells were incubated with those media for 48 h prior to Firefly and Renilla luciferase signals measurement as described above.

2.8 | In situ binding experiments

COS1 cells (150 000 cells/well in 24-well plates) were transfected with Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions with EV or LRP6 or LRP5 or LRG4 or LOX1 expression vectors. After 48 h of transfection, cells were washed with HBAH buffer (Hanks' balanced salt solution, bovine serum albumin [0.5 mg/ml], 0.1% [w/v] NaN_3, 20 mM HEPES [pH 7.0]), then incubated with unfused AP or hAug-AP or mAug-AP or DKK1-AP or RSPO1-AP for 90 min at room temperature (RT) with shaking. The AP fusion protein solutions were removed and cells were washed six times with cold HBAH. For each wash, cells were incubated with HBAH for 5 min on a platform shaker. Cells were then incubated in a 65°C preheated oven. After 100 min, the medium was aspirated and the AP BM purple substrate (see above) added, then cells were incubated at RT wrapped in aluminium foil, staining being monitored periodically against a white background under a dissecting microscope. Colour development required different times (from 1 h to overnight) to be visible, depending on the condition. The reaction was stopped by washing the plate with D-PBS. Cells were stored in D-PBS, 10 mM EDTA at 4°C in the dark.

2.9 | Generation of mice null for the *1500015O10Rik/Ecrg4* gene

A *1500015O10Rik/Ecrg4* mutant mouse strain carrying a "knockout-first" conditional allele (tm1a) was generated in the C57BL6/N genetic background. It contains an IRES-lacZ trapping cassette and a floxed promoter-driven neo cassette inserted into the second intron of the gene, disrupting its function. Crossing of those mice with mice expressing the Cre recombinase under the control of a ubiquitous promoter resulted in the deletion of the promoter-driven selection cassette and floxed exon of the tm1a allele to generate a lacZ tagged allele (tm1b).

2.10 | Isolation of osteoblasts from neonatal mouse calvaria and osteogenic differentiation protocol

Mouse experimentation protocols were performed in accordance with institutional and international

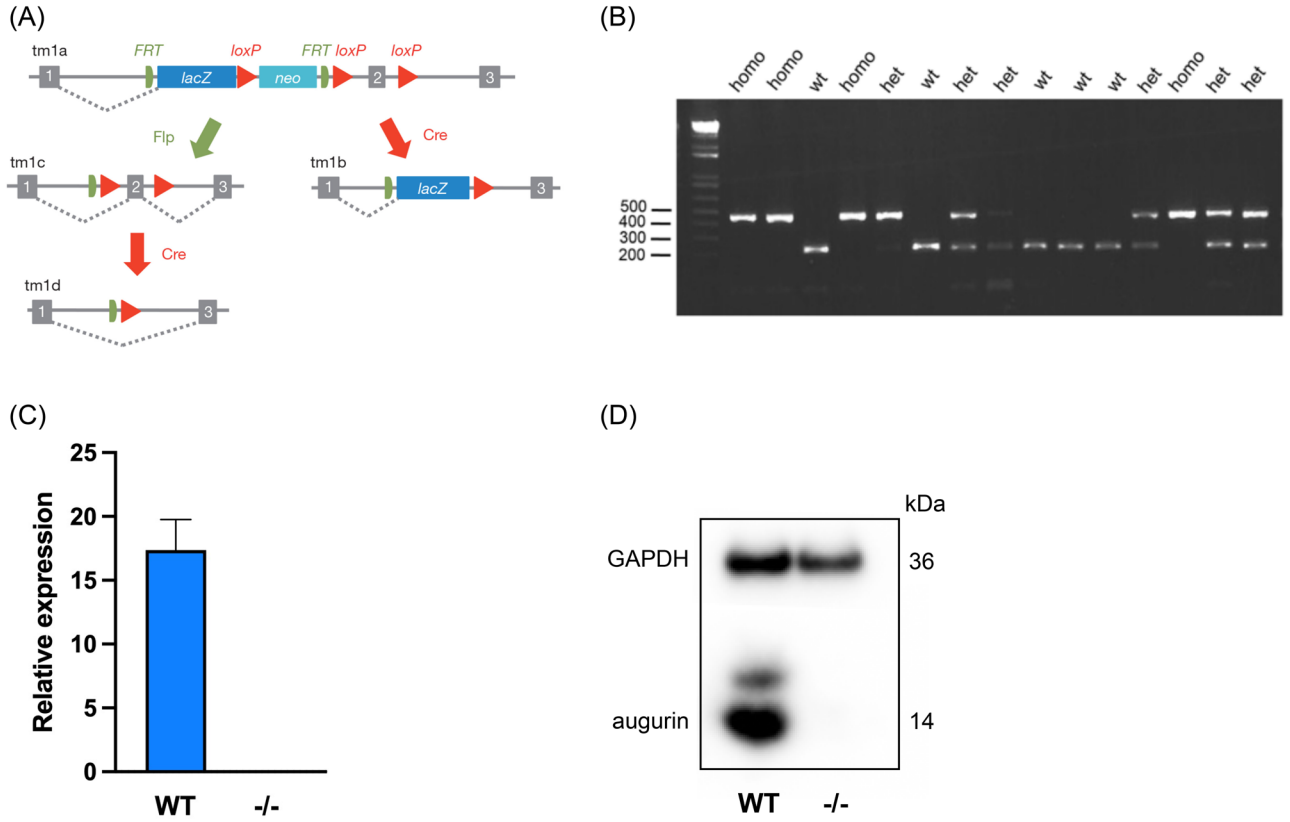

FIGURE 1 **Generation of *Ecrg4* null mice**. (A) Diagram showing the gene invalidation strategy. (B) Mouse tail PCR genotyping shows the different band patterns for -/- (homo), +/- (het) and wild-type (WT) animals. (C) *Ecrg4* expression measured by real-time quantitative polymerase chain reaction (RT-qPCR) in osteoblast cultures from WT and -/- mice. (D) Immunoblot showing augurin expression in lysates of osteoblast cultures from WT and -/- mice

guidelines and were approved by the local ethics committee (CIEPAL-AZUR) and by the Ministry of Research (#15167-201805021259809 v2). Four to six days old neonate WT or *Ecrg4* -/- mice were euthanized by CO_2 inhalation followed by decapitation. The dissection was carried out under a sterile hood. The heads were sprayed with 70% ethanol and placed in sterile cold D-PBS. The skin was removed by sterile instruments from the skull, calvariae were removed and placed into sterile cold D-PBS. Harvested calvariae were then placed in sterile digestion solution (2 mg/ml 260 U/mg collagenase II [Sigma-Aldrich] dissolved in MEMα) and incubated at 37°C, with 70–80 rpm shaking for 20 min. Four digests were performed, the first two being discarded. For the remaining two digests an equal volume of sterile plating medium (MEMα with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine, 1 mM sodium pyruvate, pen/strep and 10% FBS) was added and then samples were stored on ice. The fractions were combined, filtered through a 70-μm sterile strainer and counted, then plated into 12 well plates at 50 000 cells/1 ml complete medium. After 7 days, confluent cells (Day 0) were placed in a sterile differentiation medium (MEMα with ribonucleosides, deoxyribonucleosides, 2 mM L-glutamine, 1 mM sodium pyruvate, pen-strep and 10% FBS plus 50 ug/ml ascorbic acid and 10 mM beta-glycerophosphate). Thereafter, every 72 h the differentiation medium was replaced with a fresh medium. At 5, 14, 21 and 34 days (D5, D14, D21 and D34) after the beginning of the differentiation protocol RNA was extracted from cells to monitor the expression of osteogenic markers (see below). For analysis of mineralization, osteoblast cultures were washed with PBS, fixed with 10% formaldehyde, washed with distilled water, stained with 2% Alizarin Red S (Sigma-Aldrich) solution for 45 min at room temperature in the dark and then washed four times with distilled water. Staining was quantified by ImageJ and normalized by the culture surface area. Three independent experiments were performed, each one in duplicate.

2.11 | Mouse body length measurement

Anaesthetized mice were placed on a disinfected ruler so that their noses were aligned with the zero position, the bodies were gently pressed against the ruler and

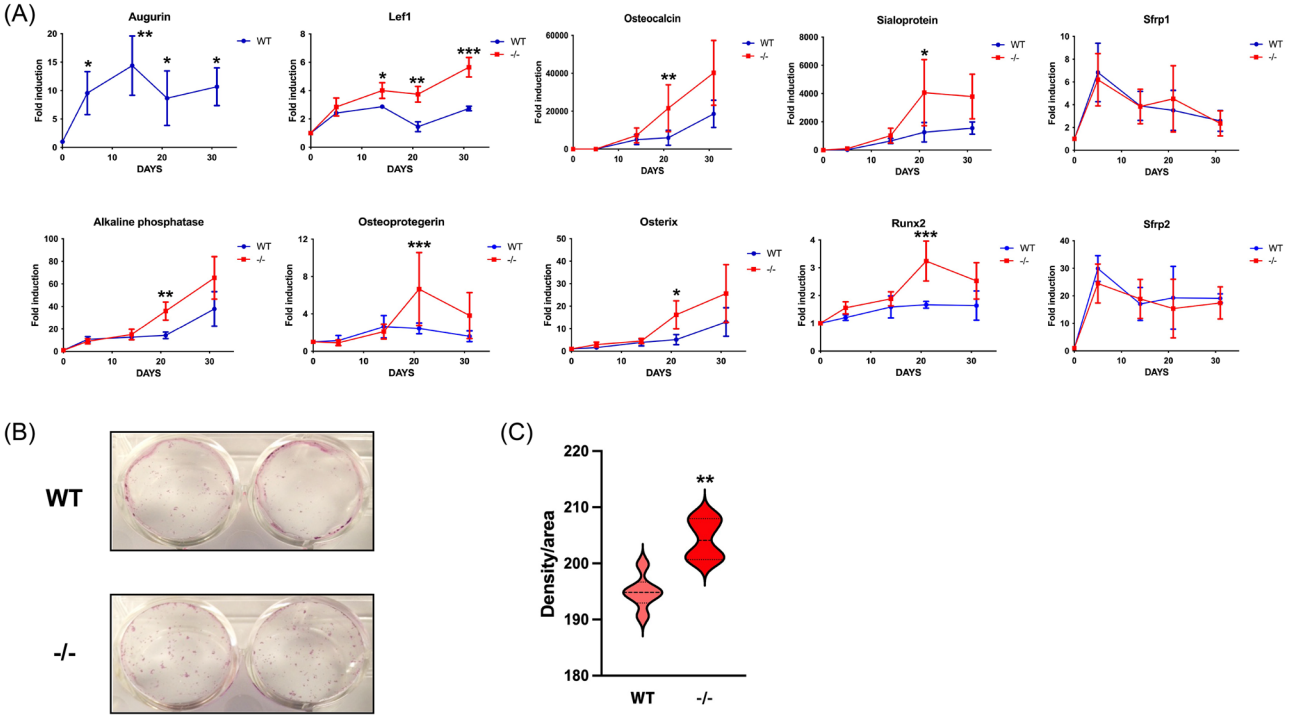

FIGURE 2 **Accelerated osteogenic differentiation of *Ecrg4* -/- osteoblasts**. (A) Osteoblast differentiation marker gene expression during the time course of osteogenic differentiation of osteoblasts isolated from wild-type (WT) (blue) and *Ecrg4* -/- (red) neonatal mice measured by real-time quantitative polymerase chain reaction (RT-qPCR). $n = 3–5$. $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$, REST analysis. (B) Alizarin Red staining of WT and *Ecrg4* -/- osteoblasts at day 21 of differentiation. The staining adjacent to the side of the well is artifactual. (C) Alizarin Red staining quantification at day 31 of differentiation in WT and *Ecrg4* -/- osteoblasts. Results are expressed in optical density units normalized by the area of osteoblast culture. $n = 3$, $^{**}p < .01$, Mann-Whitney test

the tails were pulled to ensure that the spines reached their full length. Body length was measured starting from the nose (0 cm) to the beginning of the tail. Accuracy was within 0.1 cm. (https://www.mousephenotype.org/impress/ProcedureInfo?procID=346).

2.12 | Gene expression analysis

Total RNA at fixed timepoints of osteogenic differentiation (D0, D5, D14, D21 and D34) from primary calvarial osteoblasts or upon 6, 12, 24, 48 and 72 h of MC3T3-E1 incubation with a 1:1 mix of L-Wnt3a/RSPO1 conditioned medium was isolated using the RNeasy Mini-Kit (Qiagen #74004) and subjected to genomic DNA decontamination by Turbo DNA-free kit (Invitrogen #AM1907) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using Superscript IV (Invitrogen #18090050) reverse transcriptase. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed using the SYBR Green I dye assay (Roche #04707516001) on a LightCycler 480 (Roche Applied Science) instrument using TATA-binding protein (Tbp) as a reference transcript. Primer sequences employed were as follows: *Lef1*: 5′- GAC GAG CAC TTT TCT CCG GG-3′ (forward) and 5′-TGG GGT GAT CTG TCC AAC GC-3′ (reverse); *Ibsp* (sialoprotein): 5′-AAG CAG CAC CGT TGA GTA TGG-3′ (forward) and 5′-CCT TGT AGT AGC TGT ATT CGT CCT C-3′ (reverse); *Alpl* (AP): 5′-GGG ACG AAT CTC AGG GTA CA -3′(forward) and 5′-AGT AAC TGG GGT CTC TCT CTT T-3′ (reverse); *Bglap* (osteocalcin): 5′-GCA ATA AGG TAG TGA ACA GAC TCC-3′ (forward) and 5′-GTT TGT AGG CGG TCT TCA AGC-3′ (reverse); *Tnfrsf11b* (osteoprotegerin): 5′-CAG AGC GAA ACA CAG TTT G-3′ (forward) and 5′-CAC ACA GGG TGA CAT CTA TTC-3′; *Sp7* (Osterix): 5′-AGC GAC CAC TTG AGC AAA CAT-3′ (forward) and 5′-GCG GCT GAT TGG CTT CTT TC-3′ (reverse); *Runx2*: 5′-CCT GAA CTC TGC ACC AAG TCC T-3′ (forward) and 5′-TCA TCT GGC TCA GAT AGG AGG G-3′ (reverse); *Ecrg4*: 5′-GGC ATA AGT GGA AAC AAA CTC A-3′ (forward) and 5′-TGC TGT GTT CTC GGC TAC AG-3′ (reverse); *Axin2*: 5′- TGG GGA GTA AGA AAC AGC TCC-3′ (forward) and 5′-CCA GCT CCA GTT TCA GTT TCT C-3′ (reverse); *Tbp*: 5′-AGG CCA GAC CCC ACA ACT-3′ (forward) and 5′-GGG TGG TGC CTG GCA A-3′ (reverse). Data are expressed as the mean±SEM of at least three independent experiments, each one performed in duplicate.

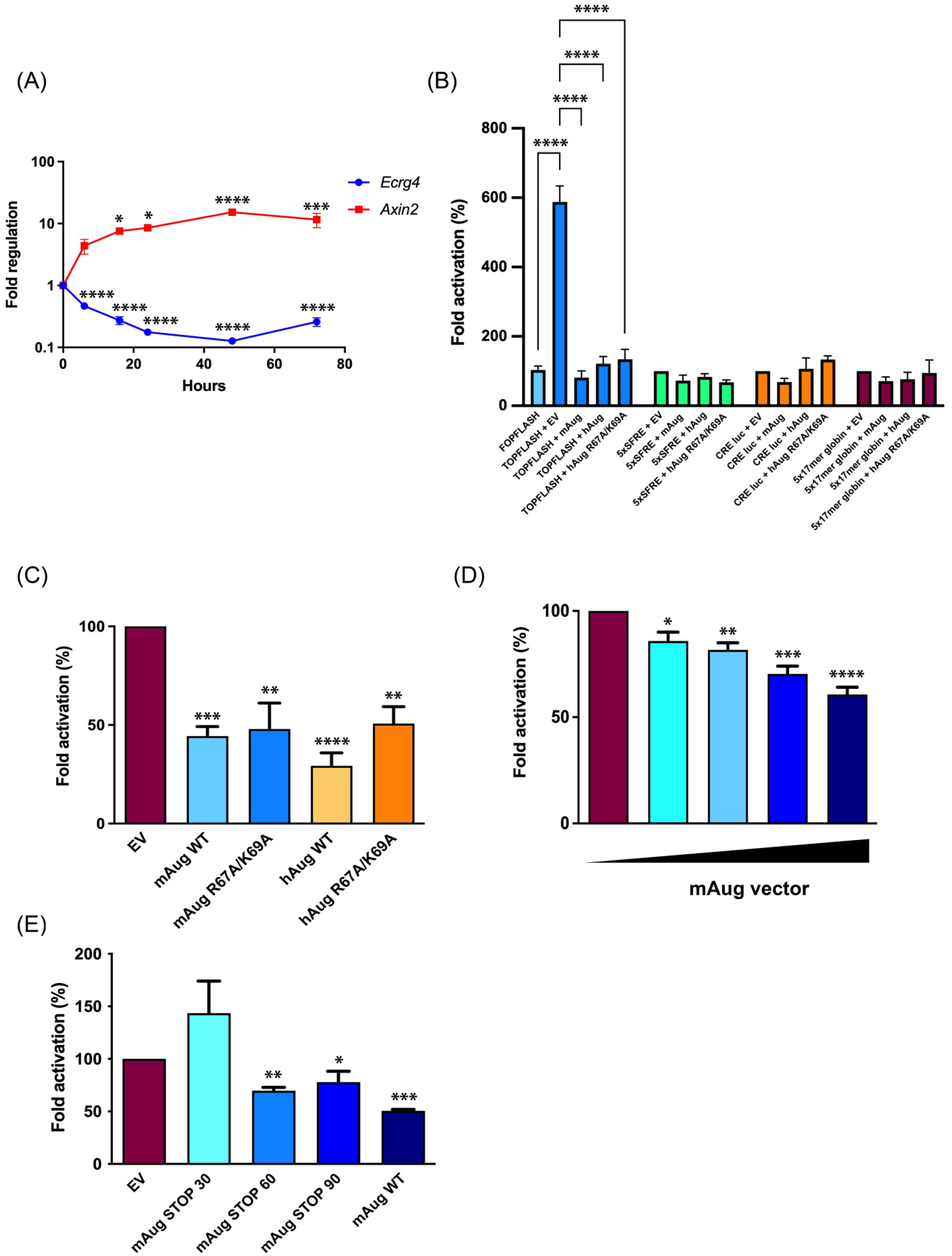

FIGURE 3 **Augurin is a specific inhibitor of Wnt-stimulated transcriptional activity**. (A) Time course of *Ecrg4* (blue) and *Axin2* (red) expression after stimulation of MC3T3-E1 cells with Wnt3a and RSPO1. Mean ± SEM is shown. $n = 3$. (B) Mouse (mAug) and human (hAug) and the hAug R67A/K69A specifically repress the activity of Wnt-stimulated TOPFLASH reporter but not the unrelated 5xSFRE, CRE

2.13 | Statistical analyses

One-way ANOVA and Mann-Whitney test were performed using GraphPad Prism v.9.4. RT-qPCR data were analyzed using the REST software.[8]

3 | RESULTS

We identified *1500015O10Rik/Ecrg4* as a gene differentially expressed in the adrenal gland of *Kcnk3* mutant mice, a model of sexually dimorphic primary hyperaldosteronism.[7] In the rat[9] and mouse adrenal gland, augurin is specifically localized in the cortex *zona glomerulosa*, an area of active Wnt signalling[10] (Figure S2A). Remarkably, *Ecrg4* is one of the most significantly repressed genes in a mouse model of Wnt signalling downregulation in the adrenal gland.[11] These data led to the hypothesis that augurin may be involved in the Wnt signalling pathway.

3.1 | *Ecrg4* regulates osteoblast differentiation in vitro

Ecrg4 expression is strongly increased during the process of osteoblast differentiation in vitro (Figure S2B), following similar kinetics to the well-known Wnt modulators *Sfrp1* and *Sfrp2* (Figure S3A,B). In differentiating osteoblasts, *Ecrg4* expression is highly correlated (>0.9) to the expression of both *Sfrp1* and *Sfrp2* (Figure S3C). In bone, mutations in genes encoding Wnt signalling regulators are associated with bone mass alterations, with activation of the pathway leading to increased, and inhibition leading to decreased, bone mass and strength.[12] We generated *Ecrg4* null mice (Figure 1) and compared the differentiation of calvarial osteoblasts isolated from *Ecrg4* -/- and wild-type (WT) osteoblasts. The *Lef1* Wnt target gene was upregulated at earlier times and at higher levels in *Ecrg4* -/- osteoblasts compared to WT, similarly to the transcripts encoding several bone differentiation markers [*Bglap* (osteocalcin), *Ibsp* (sialoprotein), *Alpl* (AP), *Tnfrsf11b* (osteoprotegerin), *Sp7* (Osterix), *Runx2*] (Figure 2A). No alteration in the kinetics of *Sfrp1* and *Sfrp2* expression during osteogenic differentiation was detected in *Ecrg4* -/- osteoblasts compared to WT (Figure 2A). Analysis of mineralization showed the increased intensity of Alizarin Red staining in *Ecrg4* -/- osteoblasts compared to WT during (Figure 2B) and at the end of the osteogenic differentiation protocol (Figure 2C). These data are consistent with augurin acting as an inhibitor of Wnt signalling in osteoblasts.

3.2 | Augurin is a specific negative modulator of Wnt-dependent transcription

Ecrg4 is itself a negative Wnt target gene, being markedly downregulated by treatment of osteoblast-like mouse MC3T3-E1 cells with Wnt3a and RSPO1. By contrast, the positive Wnt target gene *Axin2* was strongly upregulated by this treatment (Figure 3A). In transient transfection assays in MC3T3-E1 cells, both mouse (mAug) and human augurin (hAug) significantly repressed activity of the Wnt-responsive TOPFLASH promoter stimulated with Wnt3a/RSPO1 (Figure 6B). Mutation of a putative furin cleavage site (R67A/K69A) in both mAug and hAug had no effect on the repression of TOPFLASH (Figure 3B,C). In contrast, mAug and hAug had no effect on the activity of other unrelated reporters (5xSFRE [nuclear receptor response element]; CRE luc [cAMP response element]; 5 × 17 mer globin [yeast GAL4 response element]) (Figure 3B). mAug repression of Wnt-stimulated TOPFLASH activity was dose-dependent, reaching significance at a dose as low as 10 ng of the mAug expression vector (Figure 3D). Transfection of a mAug construct encoding only the signal peptide (aa. 1–29) of the secreted protein did not repress Wnt-stimulated TOPFLASH activity, while repression was already efficient by a construct encoding mAug aa. 1–59 (Figure 3E). Importantly, mAug also specifically repressed Wnt-stimulated TOPFLASH when expressed both as a secreted protein in CHO cells (Figure 4A) and as a recombinant protein in *E. coli* (Figure 4B).

luc and 5 × 17 mer globin reporters in MC3T3-E1 cells. EV, cells transfected with empty vector. For TOPFLASH, luciferase activity values were normalized by the activity of the control reporter FOPFLASH, while for the other reporters they were normalized by luciferase activity in cells transfected with EV. $n = 3$–5. (C) Effect of transfection with empty vector (EV) and plasmids encoding wild-type (WT) and R67A/K69A mutant mouse and human augurin. $n = 4$. (D) Dose-dependent repression of Wnt-stimulated TOPFLASH activity by increasing doses (10, 25, 40 and 50 ng) of mAug expression plasmid. The plasmid amount in each transfected sample was kept constant by the addition of varying quantities of empty vectors. $n = 3$. (E) MC3T3-E1 cells were transfected with the TOPFLASH reporter and plasmids encoding WT mAug or different C-terminally truncated mutants (STOP 30, aa. 1–29; STOP 60, aa. 1–59; STOP 90, aa. 1–89). $n = 6$. For all transfection experiments, mean ± SEM is shown *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$, one-way ANOVA

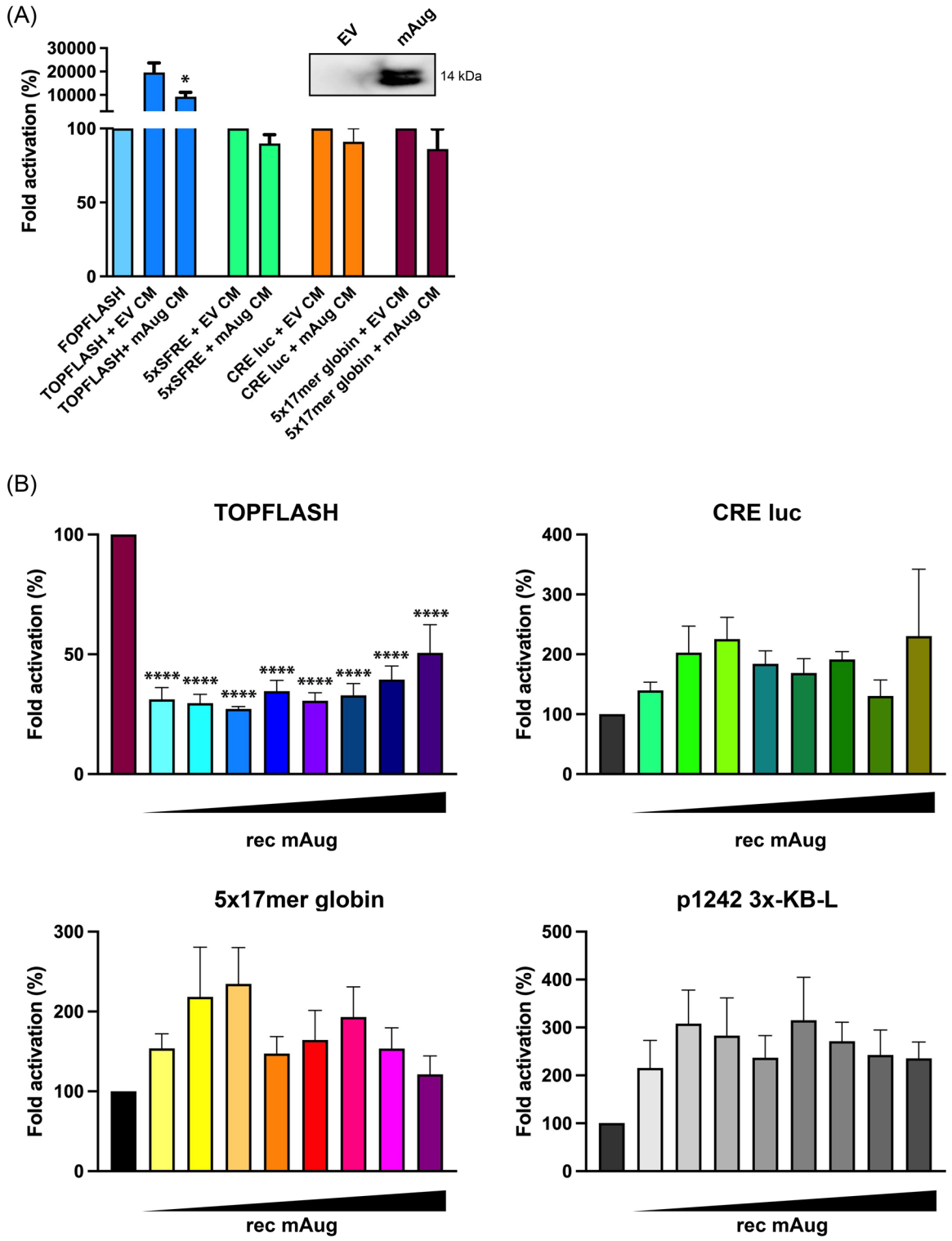

FIGURE 4 **Augurin specifically represses Wnt-stimulated transcriptional activity as a secreted protein**. (A) Repression of TOPFLASH activity in MC3T3-E1 cells by conditioned medium (CM) from CHO cells transfected with empty vector (EV) or an expression vector encoding mAug. Augurin expression in CMs is shown in the inset by Western blot. mAug-containing CM did not repress the activity of 5xSFRE, CRE luc and 5 × 17 mer globin reporters. For TOPFLASH, luciferase activity values were normalized by the activity of the control reporter FOPFLASH, while for the other reporters they were normalized by luciferase activity in cells treated with EV CM. $n = 3$. (B) Effect of treatment with increasing doses of recombinant mAug (0.13, 1.28, 12.8, 128 nM - 1.3, 2.1, 4.2 and 6.4 nM) on the activity of the TOPFLASH, CRE luc, 5 × 17 mer globin and p1242 3x-KB-L reporters, respectively, in transfected MC3T3-E1 cells.n = 3-6. For all experiments mean ± SEM is shown. $^{**}p < .01$; $^{***}p < .001$; $^{****}p < .0001$, one-way ANOVA

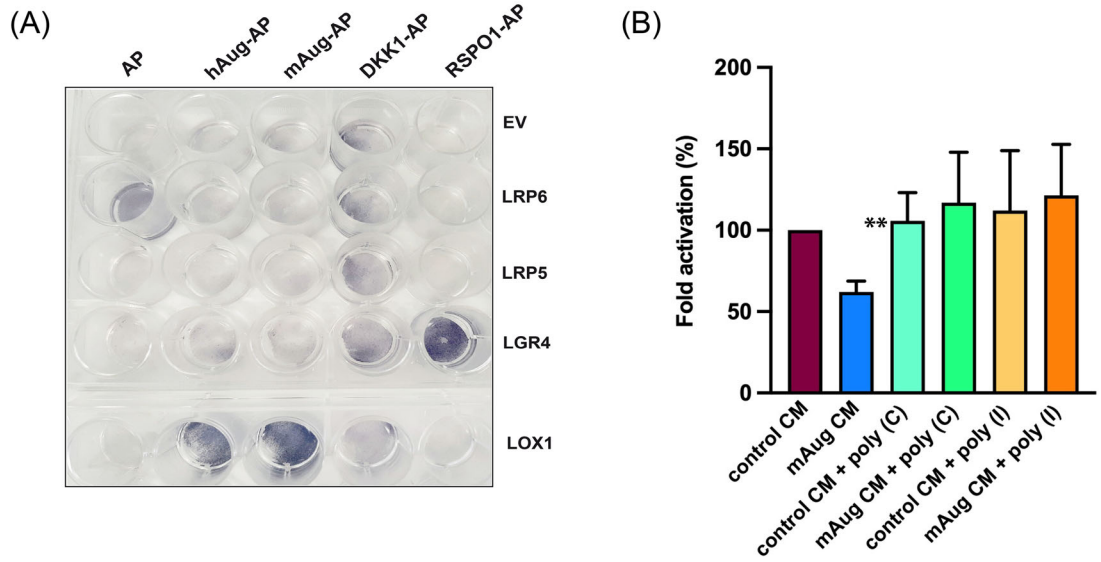

FIGURE 5 Repression of Wnt-stimulated transcriptional activity by augurin is not mediated by its binding to scavenger receptors. (A) Binding of alkaline phosphatase (AP) (negative control) and AP-fusion proteins (hAug-AP, mAug-AP, DKK1-AP and RSPO1-AP) to COS1 cells transfected with empty vector (EV) or expression vectors encoding LRP5, LRP6, LGR4 or LOX1. Binding is shown by the development of blue colour after washing. Background binding is present in all samples for the DKK1-AP protein, while RSPO1-AP is specifically bound to LGR4. hAug and mAug specifically bound LOX1, as previously shown.[5] (B) Both Poly(I) and poly(C) inhibited Wnt-stimulated TOPFLASH repression by conditioned medium (CM) containing mAug. $n = 3$. For all transfection experiments, mean±SEM is shown. *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$, one-way ANOVA

3.3 | The effect of augurin on the Wnt pathway is not mediated by scavenger receptors

Augurin was reported to bind to LOX1 and other members of the scavenger receptor family.[5] We confirmed the binding of both mAug and hAug, expressed as AP fusion proteins, to LOX1, while they did not bind to the Wnt and R-spondin coreceptors LRP5, LRP6 and LGR4 (Figure 5A). However, Wnt-stimulated TOPFLASH repression by a conditioned medium containing mAug was inhibited both by poly(I), a specific inhibitor of scavenger receptors, and poly(C), which does not bind to scavenger receptors[5] (Figure 5B). These data suggest that Wnt signalling inhibition by augurin is not mediated through its binding to scavenger receptors.

3.4 | Association of *ECRG4* genomic variation with body height and osteoarthritis

Detailed skeletal phenotyping did not show any significant difference in bone mass, mineralization or strength between *Ecgr4* -/- and WT mice, either in basal conditions or after challenge (ovariectomy) (data not shown), possibly due to compensatory mechanisms. However, *Ecrg4* -/- female mice have increased body length compared to WT (Figure 6A). Furthermore, genome-wide association studies (GWAS) have shown that the intronic rs66989638 variant (Figure 6B), which is a quantitative trait locus for *ECRG4* expression in multiple tissues (Figure 6C), is significantly associated with body height in a European population[13] (Figure 6D). Body length determination is a complex phenotypic trait, which is influenced not only by determinants related to bone and cartilage physiology but also by systemic (metabolic and endocrine) factors. Remarkably, augurin expression is highly upregulated during chondrogenic differentiation, while it is decreased in osteoarthritic cartilage compared to normal tissue.[14] Tight regulation of Wnt signalling has an essential role in joint structure and function[15] and the same rs66989638 *ECRG4* variant is also associated with osteoarthritis in GWAS[16] (Figure 6D).

4 | DISCUSSION

Wnt signalling is a key developmental pathway present in all Metazoan clades and also an important regulator of stem cell and tissue homeostasis in the adult. Canonical Wnt signalling functions by regulating the translocation of beta-catenin from the cell membrane to the nucleus, where it controls key gene expression programs through interaction with Tcf/Lef and other families of transcription factors. Wnts can also act through non-canonical pathways

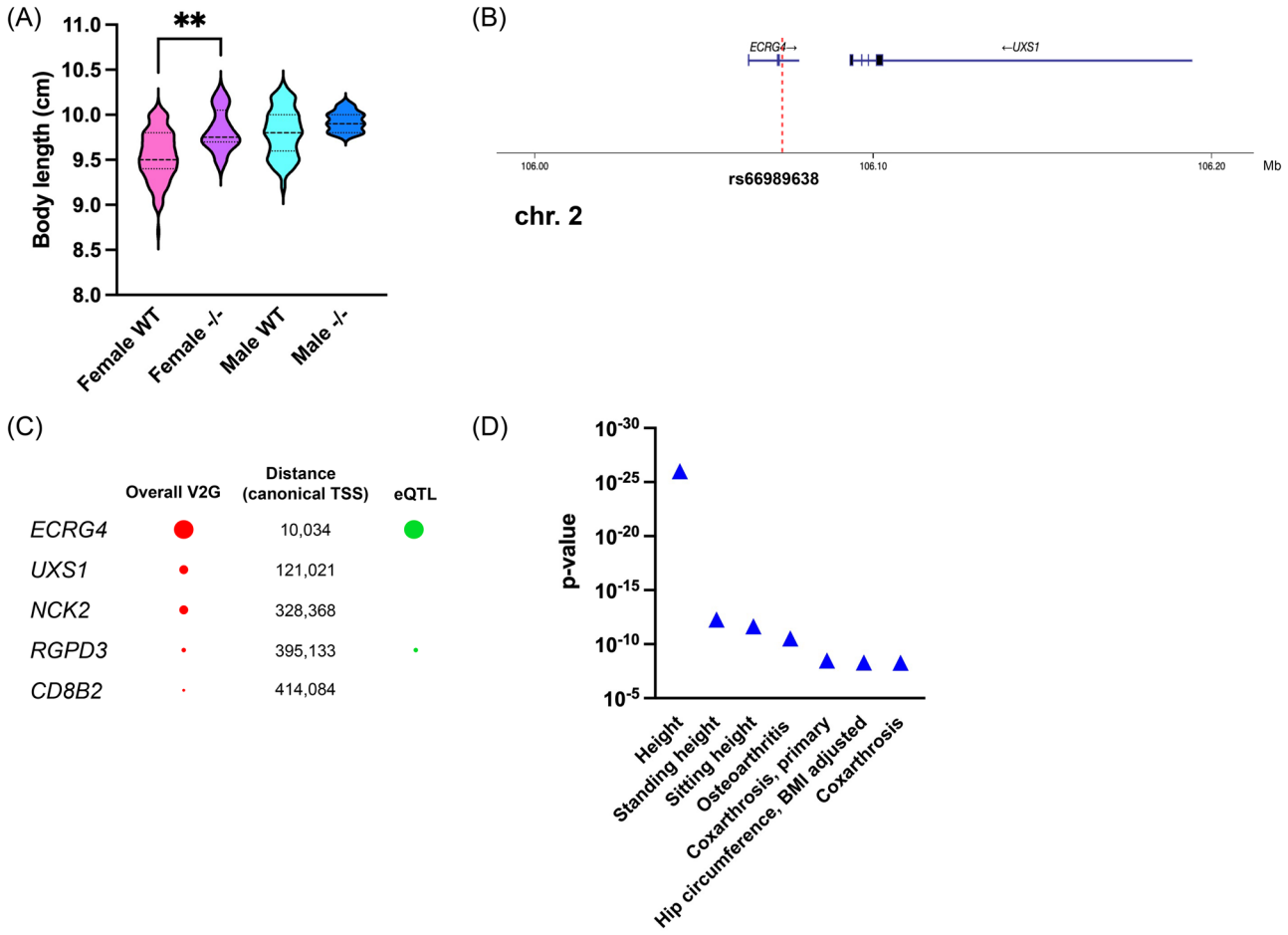

FIGURE 6 **Genetic associations of *Ecrg4*/*ECRG4* in mice and humans**. (A) The body length of wild-type (WT) and *Ecrg4* -/- mice of both sexes. $n = 135$, WT females; $n = 8$, *Ecrg4* -/- females; $n = 127$, WT males; $n = 7$ *Ecrg4* -/- males. $^{**}p < .01$, one-way ANOVA. Data from https://www.mousephenotype.org/data/charts?accession = MGI:1926146&allele_accession_id = MGI:5766758&zygosity = homozygote&parameter_stable_id = IMPC_DXA_006_001&pipeline_stable_id = ICS_001&procedure_stable_id = IMPC_DXA_001&parameter_stable_id = IMPC_DXA_006_001&phenotyping_center = ICS. (B) Genomic location of rs66989638 in an intron of the *ECRG4* gene on chromosome 2. (C) Table showing the influence of the rs66989638 variant on the expression of neighbouring genes (quantitative trait locus [eQTL]). Overall V2G (variant-to-gene) is an index of the probability that a variant influences the expression of a given gene, based on several parameters, including eQTL and chromatin structure data. The distance of the variant from the respective gene canonical transcription start site (TSS) is indicated in base pairs. (D) Phenotypic traits associated with rs66989638 in genome-wide association studies (GWAS) with a p-value reaching genome-wide significance ($p < 5 \times 10^{-8}$). From https://genetics.opentargets.org/variant/2_106073280_G_A

that do not involve beta-catenin activation but implicate small GTPases/JNK kinase and intracellular calcium. Dysregulation of Wnt signalling is the basis of many human disorders and a leading cause of cancer.[17,18]

Here we have shown a novel function for the secreted protein augurin as a canonical Wnt signalling inhibitor and its involvement in osteoblast differentiation. Calvarial osteoblasts isolated from *Ecrg4* -/- mice have an accelerated osteogenic differentiation in vitro, which is consistent with Wnt pathway upregulation induced by loss of augurin expression. We have shown that the interaction of augurin with its previously described LOX1 membrane receptors most likely does not mediate its inhibition of Wnt-stimulated transcriptional regulation (Figure 5). Previous studies showed that augurin can either activate or repress NF-κB signalling depending on the cell type.[5,19] Both positive and negative crosstalk between the NF-κB and Wnt signalling pathways are known to occur[20] and the possibility then exists that the effects of augurin on Wnt signalling in osteoblastic cells may be mediated by modulation of NF-κB activity. However, we observed no modulation of the activity of a transfected NF-κB reporter by treatment of MC3T3-E1 cells with recombinant augurin (Figure 4B). These results suggest that in those cells augurin represses Wnt-stimulated transcription independently from NF-κB. However, further experiments are needed to identify the mechanisms of Wnt signalling repression by augurin in osteoblastic cells.

The involvement of the Wnt pathway in regulating bone differentiation is well known, as shown by genetic syndromes characterized by alterations in bone mass and/or mineralization, which are caused by mutations in genes acting as positive or negative regulators of the Wnt pathway, and by GWAS studies.[12] Augurin can then be considered as another actor in the complex interplay of Wnt activators and repressors regulating bone and cartilage physiology. It is possible, therefore, that alterations in bone and cartilage function may underlie the traits associated with the gene encoding augurin, both in mice and in humans. In addition, given the expression pattern of *ECRG4* in many tissues under physiological conditions and its alterations in disease, the molecular function of augurin as a canonical Wnt signalling inhibitor may underpin several of its reported effects on cancer cell proliferation and tumour growth,[3,21] cell senescence,[22] stem cell renewal[2,23] and inflammation/response to tissue injury.[24,25]

5 | CONCLUSIONS

We have shown here that augurin is a negative modulator of canonical Wnt signalling and that its genetic inactivation induces an accelerated osteogenic differentiation of calvarial osteoblasts in vitro. Our study sheds new light on the wide spectrum of functions previously attributed to augurin in the brain, stem cell biology, inflammation/immunity and cancer. Furthermore, our discovery paves the way to further characterization of the mechanisms involved in augurin repression of Wnt signalling and the development of agonists and antagonists for this protein, which has a wide array of potential applications in the clinic.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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