Cooperative actions of p21^{WAF1} and p53 induce Slug protein degradation and suppress cell invasion

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Abstract

How the p53 transcription factor/tumor suppressor inhibits cell invasion is poorly understood. We demonstrate that this function of p53 requires its direct interaction with p21^{WAF1}, a transcriptional target of p53, and that both p21 and p53 bind to Slug, which promotes cell invasion. Functional studies reveal that p21 and p53 cooperate to facilitate Mdm2-dependent Slug degradation and that this p53 function is mimicked by p53^{R273H}, a mutant lacking trans-activating activity. These actions of p21 and p53 are induced by γ -irradiation of cells and also operate *in vivo*. This is the first study to elucidate a mechanism involving p53 and p21 cooperation.

Keywords Cancer; invasion; p21; p53; Slug

Subject Categories Autophagy & Cell Death; Cell Adhesion, Polarity & Cytoskeleton

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Introduction

The initiation of cancer metastasis requires an increase in the migratory and invasive capabilities of cancer cells [1]. These properties of cancer cells can be down-regulated by p53 [2], a tumor suppressor that is lost or mutated in > 50% of human tumors [3]. However, the molecular mechanism underlying p53-mediated suppression of cancer cell invasion remains poorly understood. Elucidating this mechanism is critical for understanding metastatic process and developing new therapeutic strategies for cancer.

Because p53 is a transcription factor, most studies investigating the mechanism of action of p53 have focused on its transcriptional targets. Consequently, the list of targets that are induced by p53 and, in turn, support p53 functions has been growing [4]. However, p53 might not always regulate cellular functions by acting as a transcription factor. For example, p53 induces Slug protein degradation

through a transcription-independent mechanism [2]. Slug is a member of the Snail family of transcription factors that is up-regulated in various types of cancer [1]. Because Slug up-regulation can increase the invasive and metastatic potential of cancer cells, identifying the cellular factors that regulate Slug levels could enhance our understanding of metastatic processes. One such cellular factor is Mdm2, an E3 ligase that binds to and ubiquitinates Slug and leads to its proteasome-dependent degradation. Recently, p53 was shown to bind Slug together with Mdm2 and form a p53–Mdm2–Slug complex, which facilitates Mdm2-dependent Slug ubiquitination and degradation [2]. Thus, p53 might exert its tumor-suppressing functions through both transcription-dependent and transcription-independent mechanisms.

A widely recognized transcriptional target of p53 is p21^{WAF1}, which acts as a downstream mediator of various p53 functions, such as the induction of cell growth arrest and senescence [4]. Therefore, we investigated whether p21 is also involved in mediating p53-dependent suppression of cell invasion. Our results demonstrated that p21 can suppress cell invasion. However, p21 did not merely act as a downstream effector of p53: it formed a complex with p53 to suppress cell invasion. We show that Slug is bound by both p53 and p21 and that Mdm2-dependent degradation of Slug requires not only p53 but also p21. Moreover, we provide evidence that this novel cooperative action of p53 and p21 is elicited in response to external stimuli and also operates *in vivo*.

Results and Discussion

p53 and p21 require each other for suppressing cell invasion

To investigate whether p21 is involved in the invasion-suppressing activity of p53, we compared H460 (p53^{wt}) and H1299 (p53^{null}) lung cancer cells. Invasion assays revealed that H1299 cells were more invasive than H460 cells (Supplementary Fig S1). Whereas the invasiveness of H460 cells was enhanced following siRNA-mediated knockdown of p53 (Fig 1A and Supplementary

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Figure 1. p53 and p21 require each other to reduce Slug protein levels and cellular invasiveness.

- A H460 cells were transfected with pcDNA3 or pcDNA3-p21. Where indicated, the cells were additionally treated with control or p53 siRNA. After 24 h, levels of p53 and p21 were compared by Western blotting. β -actin was the loading control. Cellular invasiveness was compared using Matrigel-coated filters. *n*, the number of biological replicates. ns, not significant.
- B The experiments were performed as in (A) using H1299 cells transfected with the indicated expression vectors and siRNAs.
- C H460 cells were transfected with 2 sets of siRNAs targeting p53 or p21. Western blotting was performed using the indicated antibodies.
- D The experiments were performed as in (A) using H1299 and H1299-p53 cells. Where indicated, cells were transfected with pcDNA3-p21 or p21 siRNA.

Source data are available online for this figure.

Fig S2A), the invasiveness of H1299 cells was diminished when exogenous p53 was introduced into the cells (Fig 1B). These results confirmed that p53 suppressed cell invasion in this system. The invasiveness of H460 cells was also increased and decreased following p21 knockdown (Supplementary Fig S2B) and overexpression (Fig 1A), respectively, which indicated that p21 can suppress cell invasion. To examine whether p53 suppressed invasion in a p21-dependent manner, we expressed p53 in H1299 cells and down-regulated p21 expression by using its siRNA. Under these conditions, p53 overexpression failed to suppress cell invasion (Fig 1B), suggesting that p53 requires p21 to suppress cell invasion. If p21 acts as a downstream effector of p53 function, it should be able to suppress cell invasion even in the absence of p53. However, p21 overexpression did not markedly affect the invasiveness of either H1299 cells (Fig 1B) or p53-knockdown H460 cells (Fig 1A): although cell invasion decreased slightly after p21 overexpression, the effect was not statistically significant. This suggests that p21 requires p53 to suppress cell invasion.

Collectively, these data suggest that p53 and p21 require each other to suppress cell invasion.

Both p53 and p21 are required for down-regulating Slug protein

This finding raised the possibility that p53 and p21 suppress cell invasion by acting on a common target. A potential target is Slug, a key promoter of cell invasion [1]. siRNA-mediated knockdown of Slug consistently reduced the invasiveness of H460 cells (Supplementary Fig S2C) and also abolished the cell invasion induced by p53 or p21 knockdown (Supplementary Fig S3), suggesting that p53 and p21 suppress cell invasion by acting on Slug. Because p53 can reduce Slug protein levels [2], we investigated whether p21 also influences Slug expression. First, we confirmed that p53 lowers Slug protein levels in our system: knocking down p53 increased Slug levels in H460 cells (Fig 1C). Similar results were obtained after knocking down p21, suggesting that p21 also reduces Slug protein levels. Moreover, control cells and cells treated with the p21 siRNA expressed similar amounts of p53, suggesting that p53 alone cannot lower Slug levels and requires p21 to perform this function. To confirm this, we expressed p53 in H1299 cells, and this resulted in a decrease in Slug levels (Fig 1D); however, Slug levels in the H1299-p53 cells were restored to nearly original levels when p21 accumulation was prevented using the p21 siRNA. These results bolster the view that p21 is required for p53-induced Slug down-regulation. Interestingly, p21 overexpression decreased Slug protein levels in H1299-p53 cells but not H1299 cells, indicating that p21 requires p53 to lower Slug levels. Collectively, these data indicate that both p53 and p21 are required for reducing Slug protein levels.

p21 promotes Mdm2-dependent Slug ubiquitination and degradation

RT-PCR and real-time PCR analyses revealed that, in contrast to Slug protein levels, Slug mRNA levels were not markedly altered by knocking down p53 or p21 in H460 cells and expressing p53 in H1299 cells (Supplementary Fig S4). This suggests that p21 regulates Slug expression at the protein level. Because p53 promotes Mdm2-dependent Slug ubiquitination and degradation [2], we investigated whether p21 is also involved in this process. In H460 cells, p21 overexpression reduced the stability of Slug protein in the presence of cycloheximide, a translation inhibitor (Fig 2A); by contrast, p21 failed to destabilize Slug in H1299 cells, suggesting that p21 reduces Slug protein stability only in the presence of p53. Moreover, p21 overexpression failed to lower Slug protein levels in H1299-p53 cells treated with an Mdm2-targeting siRNA or with MG132, a proteasome inhibitor (Fig 2B). This suggested that p21 reduces Slug levels through an Mdm2- and proteasome activity-dependent mechanism, consistent with the possibility that p21 promotes Slug ubiquitination. Supporting this possibility, p21 overexpression increased the levels of ubiquitinated Slug in H1299-p53 cells, but this effect was abolished when Mdm2 was knocked down (Fig 2C). Furthermore, p21 knockdown consistently reduced Slug ubiquitination levels in H1299-p53 cells (Fig 2D), but Slug ubiquitination levels in H1299 cells were not markedly altered by p21 overexpression or knockdown, suggesting that p21 requires p53 to promote Slug

Figure 2. p21 promotes Mdm2-dependent Slug ubiquitination and degradation.

- A H460 and H1299 cells transfected with pcDNA3 or pcDNA3-p21 were treated with cycloheximide (10 μ M) for the indicated periods. Slug levels were compared by Western blotting. The plot shows the best-fit curves of relative Slug levels in each transfectant.
- B H1299-p53 cells transfected with pcDNA3 or pcDNA3-p21 were treated with Mdm2 siRNA for 40 h or MG132 (10 μ M) for 5 h. Western blotting was performed for Slug, p21, and Mdm2.
- C H1299-p53 cells transfected with the indicated combinations of expression vectors encoding HA-tagged ubiquitin, Flag-tagged Slug, p21, and the Mdm2 siRNA were treated with MG132 for 5 h. Cell lysates were prepared and immunoprecipitated with anti-Flag-agarose beads. The precipitated proteins and input controls were analyzed by Western blotting.
- D The experiments were performed using H1299 and H1299-p53 cells transfected with the indicated expression vectors and siRNAs.

Source data are available online for this figure.

ubiquitination. Collectively, these findings suggest that p21 is required for the p53/Mdm2-dependent ubiquitination and degradation of Slug protein.

p21 forms a complex with p53, Slug, and Mdm2

Our results raised the possibility that p21 is an additional component of the previously reported p53–Slug–Mdm2 complex [2]. Co-immunoprecipitation studies performed using cell lysates (Fig 3A) and recombinant proteins (Supplementary Fig S5A and B) revealed that p21 co-precipitated with p53, Slug, and Mdm2. Because p21 and Mdm2 can interact directly [5], we examined the binding of recombinant proteins to determine whether p21 also directly interacts with p53 and Slug: p21 co-precipitated with both Slug and p53 (Supplementary Fig S5C, left and middle), indicating that p21 interacts directly with these proteins. To define the protein sites involved

in mediating these interactions, we created various deletion mutants of p21, Slug, and p53 (Supplementary Fig S6). Deletion of the N-terminal 9 residues of Slug or the C-terminal 37 residues of p53 resulted in a substantial or complete loss of the ability of these proteins to bind p21 (Fig 3B), suggesting that Slug and p53 bind to p21 through their N- and C-terminal regions, respectively. Conversely, removing the C-terminal 12 residues of p21 (p21^{ΔC12}) abolished the binding of p21 to both Slug and p53 (Fig 3C), suggesting that the C-terminal region of p21 mediates its interaction with Slug and p53.

If p21 concurrently forms a complex with Slug and p53, distinct regions of p21 should interact with Slug and p53. To test this, we scrutinized the C-terminal 12 residues of p21, which contains 2 phosphorylatable serine residues, at positions 153 and 160 [6–8]. A point mutation of serine¹⁵³ to aspartic acid (S153D), a phosphorylation-mimetic residue, greatly reduced the ability of p21 to interact with p53, but exerted a considerably weaker effect on p21 binding to Slug (Fig 3D). By contrast, an S160D mutation interfered with p21 binding to Slug but not p53. These differential effects suggest that serine¹⁵³ and serine¹⁶⁰ of p21 preferentially support binding to p53 and Slug, respectively. However, these interactions were not markedly altered when the 2 serines were mutated to alanines (S153A, S160A), suggesting that the negative charge associated with the phosphorylation of these serine residues inhibits the interactions of p21 with Slug and p53. Notably, the finding that distinct residues of p21 mediate binding to Slug and p53 supported the possibility that these 3 components form a complex concurrently, which was directly demonstrated using a 2-step co-precipitation assay (Fig 3E).

Formation of a p21–p53–Slug complex is required for reducing Slug levels, cellular invasiveness, and intravasation

We tested whether the formation of a complex containing p21, p53, and Slug is required for down-regulating Slug levels and cellular invasiveness. In contrast to p21^{wt}, p21^{ΔC12}, which binds neither Slug nor p53, failed to reduce Slug levels and cellular invasiveness upon introduction into H460 cells (Fig 4A). Similar results were obtained using p21^{S153D} and p21^{S160D}, which do not efficiently bind p53 and Slug, respectively. However, p21^{S153A} and p21^{S160A} efficiently reduced Slug levels and cellular invasiveness. Collectively, these results support the notion that p21 binds to p53 and Slug to induce Mdm2-dependent Slug degradation and thereby suppress cell invasion.

To investigate whether p21 inhibits cell invasion *in vivo*, we used a mouse model for comparing the intravasation potential of cancer cells, a property that depends on their invasive activity [1]. We established xenograft tumors in mice by using GFP-expressing H460 cells and then monitored the appearance of tumor cells in mouse blood. Whereas the size of xenograft tumors was not significantly influenced by the expression of p53 shRNA, p21^{wt}, or p21 mutants in H460 cells (Fig 4B, top), the number of circulating tumor cells was increased following p53 knockdown (Fig 4B, bottom). This confirmed that p53 can suppress cancer cell intravasation in this system. By contrast, the number of circulating tumor cells was decreased following p21^{wt} overexpression. This effect was not observed when p21^{ΔC12}, p21^{S153D}, or p21^{S160D} were used, suggesting that p21 suppresses cancer cell intravasation by binding

Figure 3. p21 directly binds to Slug and p53.

A H1299-p53 cells transfected with p21 or Flag-tagged-p21 expression vectors were treated with MG132 for 5 h. Cell lysates were prepared and immunoprecipitated with anti-Flag beads. The precipitated proteins and input controls were analyzed by Western blotting.

B, C Full-length and deletion mutants of Slug, p53, and p21 were labeled with ³⁵S and incubated with GST, GST-p21, GST-Slug, or GST-p53 in the indicated combinations. Pull-downs were performed with glutathione-conjugated beads, and the precipitated proteins were analyzed by SDS-PAGE and autoradiography. The numbers correspond to the relative intensity of the bands on the right.

D The experiments were performed using p21 wild-type and point mutants. The graphs show the relative intensity of the bands on the right.

E ³⁵S-labeled Slug and p21 were incubated with GST or GST-p53 and then subjected to pull-downs using glutathione beads, followed by SDS-PAGE and autoradiography (GST pull-down). The precipitated proteins were eluted using glutathione, immunoprecipitated with anti-p21 or control IgG, and analyzed using SDS-PAGE and autoradiography (2nd IP).

Source data are available online for this figure.

to p53 and Slug and implies that the p21–p53–Slug–Mdm2 system operates *in vivo*.

Slug degradation is regulated by extracellular stimuli

Because p53 responds to numerous extracellular stimuli [4], we investigated whether Slug degradation is regulated by cell-signaling processes. We expressed Flag–Slug in H460 cells and γ -irradiated the cells. This treatment increased levels of p53 and its transcriptional targets p21 and Mdm2 (Fig 4C). γ -Irradiation consistently reduced Flag–Slug protein levels, but this effect was prevented when we knocked down p21, p53, or Mdm2. This suggests that p21, p53, and Mdm2 are involved in γ -irradiation-induced down-regulation of Slug. Thus, external stimuli appear to induce the cooperative action of p53 and p21 and thereby trigger Mdm2-dependent Slug degradation.

p21 can cooperate with a p53 mutant

We investigated whether p21 cooperates with a p53 mutant. R273H is a naturally occurring mutation that abolishes the DNA binding

and thus the trans-activating activity of p53 [3]. Therefore, in contrast to p53^{wt} expression, p53^{R273H} expression in H1299 cells failed to induce p21 and Mdm2 (Fig 4D, left). Slug protein levels and cellular invasiveness were not markedly altered after expressing p53^{R273H}, but decreased when p53^{R273H} and p21 were co-expressed. Consistent with this, p53^{R273H} and p21 co-expression elevated Slug ubiquitination to considerably higher levels than p53^{R273H} expression alone did (Fig 4D, right). These results provide support for the ability of p53^{R273H} to cooperate with p21 in promoting Slug ubiquitination/degradation and thus suppressing cell invasion. Our data also suggest that p53 can contribute to tumor suppression even after the loss of its trans-activating activity. However, our results are in contrast with a previous report that shRNA-mediated knockdown of endogenous p53^{R273H} in SW620 colorectal cells increases Mdm2 levels and decreases Slug levels [2], which suggested that p53^{R273H} increases Slug levels by down-regulating Mdm2 levels. The reason for the discrepancy between this and our results remains unclear, but the inability of p53^{R273H} expression to markedly alter Mdm2 levels under our experimental conditions (Fig 4D, left) might reflect a difference between experimental settings.

Figure 4. The interaction of p21 with p53 and Slug is required for Slug down-regulation and suppression of cell invasion and intravasation.

A H460 cells were transfected with wild-type and mutant p21 expression vectors. Slug protein levels and cellular invasiveness were analyzed.

B GFP-expressing H460 cells were additionally transfected with one of these expression vectors: control, p53 shRNA, p21^{WT}, p21^{ΔC12}, p21^{S153D}, or p21^{S160D}. The cells were implanted into mice and allowed to form xenograft tumors. Top: Tumor diameters were measured, and volumes were calculated. The mouse photo was taken using a Nikon Coolpix L100 digital camera, and the green (GFP) images of mice were taken under blue light by using the IF550 emission filter (Olympus). Bottom: After 2 weeks, blood was obtained from mice and analyzed by confocal microscopy. Circulating tumor cells were identified as GFP- and DAPI-positive cells (arrow). Graphs show the tumor-cell/DAPI-positive-cell ratio in each blood sample.

C H460 transfectants stably expressing Flag-Slug were transfected with control, p53-, p21-, or Mdm2-targeting siRNAs. After 48 h, the cells were irradiated with a sublethal dose (1 Gy) and incubated for an additional 4 h. Cell lysates were analyzed by Western blotting.

D Left: H1299 cells were transfected with expression vectors encoding p53^{WT}, p53^{R273H}, and p21. Invasion and Western blotting assays were performed. Right: Transfection was performed using the indicated expression vectors. After treating cells with MG132 for 5 h, cell lysates were prepared and immunoprecipitated with anti-Flag beads. The precipitated proteins and input controls were analyzed by Western blotting.

E Schematic model of the suppression of cell invasion through the cooperative actions of p53 and p21. p53 induces p21. These proteins together form a complex with Slug and Mdm2 that facilitates Mdm2-dependent Slug ubiquitination and degradation. Consequently, cellular invasiveness is reduced.

Source data are available online for this figure.

We have shown here that p21 cooperates with p53 to suppress cancer cell invasion and intravasation. This cooperation might be clinically relevant: previous analyses of patient samples showed that

patients with mutation in either p53 or p21 exhibited greater cancer progression and diminished survival rates than patients with p53^{WT} and p21 [9]. Similarly, both p53 and p21 are required for sustaining

cell cycle arrest after DNA damage, although the underlying mechanism is unknown [10]. In our system, p53 and p21 appear to form a complex with Slug and Mdm2, in which the 4 molecules could be in direct contact with each other. This hypothetical model has been proposed based on our finding that p21 directly interacts with p53 and Slug and on previous reports of direct interactions between p21 and Mdm2 [5] and p53 and Mdm2 [11]. We further confirmed that p53 and Slug can interact directly (Supplementary Fig S5C, right). Formation of the p21–p53–Slug–Mdm2 complex might promote Mdm2-dependent Slug ubiquitination and degradation, thereby reducing cellular invasiveness (Fig 4E). To our knowledge, this is the first report to propose a molecular mechanism involving a cooperative action of p53 and p21. Identifying molecules besides Slug that bind to both p53 and p21 could be critical for understanding the mechanisms underlying the cooperation between p53 and p21 in other cellular functions. Thus, our findings advance our understanding of metastatic processes and provide new insights into the mechanisms of action of p53 and p21.

Materials and Methods

Materials, cell cultures, mutagenesis, and transfection are described in Supplementary Methods.

Invasion assay

Cells were seeded onto Matrigel-coated Transwell chambers (BD Biosciences) and analyzed for their invasiveness as described [12].

Western blot analysis

Cells were lysed. Equal amounts of proteins were resolved using SDS-PAGE, electrotransferred to Immobilon membranes (Millipore), and probed using the specified antibodies and an ECL detection system (Pierce) [12].

Co-immunoprecipitation assay

Cells transfected with pFLAG-C1 or pcDNA3 vectors encoding p21 were treated with 10 μ M MG132 (Sigma) for 5 h and lysed in a buffer containing 20 mM Tris–HCl (pH 7.4), 100 mM NaCl, 0.5% NP-40, 50 mM NaF, 0.1 mM Na_3VO_4 , 30 mM $\text{Na}_4\text{O}_7\text{P}_2 \cdot 10\text{H}_2\text{O}$, and a proteinase-inhibitor cocktail (Sigma). The lysates were immunoprecipitated with anti-Flag-agarose beads (Sigma). The proteins bound to beads were eluted using a Flag epitope (300 μ g/mL; Sigma) and subjected to Western blotting with the indicated antibodies.

In vitro binding assay

pGEX3-p6 vectors encoding p53, p21, and Slug were introduced into the *E. coli* BL21 strain. These GST-tagged proteins were purified by using glutathione-Sepharose beads (GE Healthcare) according to the manufacturer's protocols. ^{35}S -labeled recombinant proteins were prepared by cloning the genes into pCITE-4a(+) vectors and expressing them *in vitro* in the presence of ^{35}S -methionine (Perkin Elmer) by using the TNT Quick Coupled Transcription/Translation Systems (Promega). The ^{35}S -labeled proteins were mixed with GST or

GST-tagged proteins in a buffer containing 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM DTT, 1% NP-40, and 1 mM PMSF. The mixtures were subjected to pull-downs performed using glutathione-Sepharose beads. The proteins bound to beads were eluted using 100 mM glutathione and analyzed using SDS-PAGE and autoradiography. The intensities of autoradiogram bands were compared using the ImageJ program (<http://rsbweb.nih.gov/ij/plugins/track/track.html>).

Two-step co-immunoprecipitation assay

GST-p53 proteins were incubated with ^{35}S -labeled p21 and Slug proteins and then pulled down and eluted using glutathione-Sepharose beads and glutathione, respectively. The eluted proteins were immunoprecipitated using the anti-p21 antibody and Protein G Sepharose beads and analyzed using SDS-PAGE and autoradiography.

Ubiquitination assay

Cells (2×10^6) co-transfected with expression vectors encoding Flag–Slug and HA-ubiquitin were treated with 10 μ M MG132 for 5 h and lysed in 50 μ l of a buffer containing 150 mM Tris–HCl (pH 7.8), 1% SDS, and 30% glycerol. After adding 1 ml of a buffer containing 20 mM Tris–HCl (pH 7.8), 150 mM NaCl, 0.2% Triton X-100, and a proteinase-inhibitor cocktail, cell lysates were immunoprecipitated with anti-Flag-agarose beads; the precipitated proteins were eluted by boiling and analyzed by Western blotting using anti-HA and anti-Flag antibodies.

Intravasation assay

Animal protocols were approved by our Institutional Animal Care and Use Committee. H460 cells transfected with the pEGFP-C1 vector were selected using G418 sulfate (1 mg/mL) and were additionally transfected with the pSUPERIOR.puro vector or the vector encoding a p53 shRNA. Alternatively, a second transfection step was performed using pFLAG-C1 vectors encoding p21 wild type or mutants. All transfectants were selected a second time using puromycin (500 ng/mL). To generate xenograft tumors, the transfectants (5×10^6) were subcutaneously injected into the flanks of 6-week-old female BALB/cAnNCrj-nu/nu mice (Charles River). Tumor volumes were calculated as described [12]. After 2 weeks, mice were anesthetized, blood was obtained through cardiac puncture, and 0.2 mL of blood was mixed with 4 mL of RBC lysis buffer (Intron Biotech). Cells were collected through centrifugation ($350 \times g$, 5 min), resuspended in PBS, stained with DAPI, and then analyzed by confocal microscopy. Circulating tumor cells were identified as GFP- and DAPI-positive cells.

Statistical analysis

Means and standard deviations were obtained from biological replicates of the experiments shown in graphs. Statistical significance was defined as $P < 0.05$, which was determined using Student's *t*-tests or one-way ANOVA (GraphPad software).

Supplementary information for this article is available online:

<http://embor.embopress.org>

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Author contributions

JK performed all the experiments. SB, SA, EMK, and SGH assisted with protein–protein interaction assays. JKP assisted with animal studies. WJK contributed conceptual insights. HDU supervised the project and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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