Loss of Ewing sarcoma EWS allele promotes tumorigenesis by inducing chromosomal instability in zebrafish

Hyewon Park1,*, Richard Galbraith2,*, Thaddeus Turner2, Justin Mehojah1 & Mizuki Azuma1

The Ewing sarcoma family of tumors expresses aberrant EWSR1-ETS fusion genes that are derived from chromosomal translocation. Although these fusion genes are well characterized as transcription factors, their formation leaves a single EWS allele in the sarcoma cells, and the contribution that the loss of EWS makes towards disease pathogenesis is unknown. To address this question, we utilized zebrafish mutants for ewsa and tp53. The zebrafish tp53(M214K)w/m line and the ewsaM lines all displayed zero to low incidence of tumorigenesis. However, when the ewsa and tp53 mutant lines were crossed with each other, the incidence of tumorigenesis drastically increased. Furthermore, 27 hour post fertilization (hpf) MZ ewsam/m mutant embryos displayed a higher incidence of aberrant chromosome numbers and mitotic dysfunction compared to wildtype zebrafish embryos. Consistent with this finding, tumor samples obtained from ewsam/m;tp53w/m zebrafish displayed loss of heterozygosity (LOH) for the wildtype tp53 locus. These results suggest that wildtype Ewsa inhibits LOH induction, possibly by maintaining chromosomal stability. We propose that the loss of ewsa promotes tumorigenesis, and EWS deficiency may contribute to the pathogenesis of EWS-fusion-expressing sarcomas.

EWS (Ewing sarcoma region 1, EWSR1) was originally identified as part of a fusion gene with FLI1, in Ewing sarcoma cells1. Subsequent studies have shown that 90% of Ewing sarcoma tumors express the EWS/FLI1 fusion gene; the remainder express EWS-fusions with other ETS transcription factors: ERG, ETV1, ETV4, and FEV2-3. In addition to EWS-ETS fusion genes, EWS is fused to other transcription factors in different sarcomas: EWS/DDIT3 is expressed in myxoid liposarcoma, EWS/ATF1 or EWS/CREB is expressed in clear cell sarcoma, EWS/WT1 or EWS/ERG is expressed in desmoplastic round cell sarcoma, and EWS/NR4A3 is expressed in extraskeletal myxoid chondrosarcoma4. In all cases, the amino terminal domain of EWS is preserved in the fusion protein, and the most well-characterized function of the hybrid protein is induction of aberrant transcription leading to epigenetic deregulation and altered expression of target genes5–8. However, loss of one EWS allele is also a consequence of fusion gene formation and has been observed in EWS-associated sarcomas. The functional significance of loss of EWS on the pathogenesis of EWS-fusion associated sarcomas is unknown.

EWS is a multifunctional protein that regulates transcription and splicing, and as a result, it affects numerous biological processes, including cell differentiation. An example of this is the role EWS plays in the differentiation of brown adipocytes, through activation of BMP7 transcription9. EWS is also required for the differentiation of B cells10. Our previous study showed that EWS interacts with Sox9, a master regulator of chondrocyte differentiation, and promotes chondrogenesis by modulation of Sox9 transcriptional activity11. In addition, EWS regulates multiple phases of the cell cycle. This is demonstrated by the protein’s role in regulating the splicing of CYCLIN D1 mRNA in vitro12,13. We previously reported that EWS maintains mitotic integrity14. We subsequently demonstrated that EWS interaction regulates the relocation of Aurora B kinase to the midzone during late mitosis15. Compromised relocation of Aurora B kinase results in chromosomal instability (CIN) and aneuploidy, both hallmarks of cancer16.

1University of Kansas, Molecular Biosciences, Haworth Rm7031, 1200 Sunnyside Avenue, Lawrence KS 66045, USA.
2Lawrence Memorial Hospital, Department of Pathology, 325 Maine St, Lawrence KS 66044, USA. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to M.A. (email: azumam@ku.edu)
An animal model is a powerful approach for investigating the effect that loss of EWS has on transformation of normal cells into cancerous cells, because it enables the analysis of the molecular mechanism of transformation. The zebrafish is a well-established animal model for cancer research because of its suitability for live imaging, genetic analysis and drug screening. Previously, we reported on two zebrafish EWS orthologues, ewsa and ewsb, and demonstrated that both zebrafish Ewsa and Ewsb regulate mitosis. In this study, we utilized a zebrafish ewsa loss of function mutant to analyze the effect that ewsa mutation has on tumorigenesis. The zebrafish tp53(M214K) mutant, a well characterized cancer model, was utilized as a platform for studying tumor promotion. Berghmans et al. reported that homozygous tp53M214K/M214K (subsequently referred to as tp53tm/m) mutant zebrafish display a 28% incidence of tumorigenesis by 16.5 mpf, whereas no tumors were reported in heterozygous tp53tm/m zebrafish. In our study, we discovered that the zebrafish ewsa mutant promotes tumorigenesis in the tp53tm/m background by promoting loss of heterozygosity (LOH) of the wildtype tp53 locus (that was accompanied by the loss of tp53 wildtype allele), suggesting that Ewsa acts to suppress tumorigenesis. Furthermore, we demonstrate that Ewsa inhibits induction of CIN. Here, we propose a novel mechanism: loss of EWS contributes to pathogenesis in sarcomas expressing EWS-fusion genes.

Results

Loss of ewsa allele promotes tumorigenesis in tp53-mutation background zebrafish. Although EWS has an important role in regulating the cell cycle, and EWS-fusions in EWS-associated sarcomas result in the loss of one or both alleles of the gene, the contribution that this loss has on tumorigenesis is unknown. To address this question, we took advantage of an ewsa zebrafish mutant line that was originally generated using viral-insertion11. The insertion generated a premature stop codon at the seventh amino acid, creating a null mutant11.

Heterozygous ewsa/m zebrafish from this line were crossed to three genotypes (ewsa+/−, ewsa+/m, and ewsa/m/m) that were subsequently monitored for visible signs of tumor formation or other signs of disease from 3 months post fertilization (mpf) (the age at which the fish reach sexual maturity) to 26 mpf. We did not observe any tumors in the ewsa+/− (n = 41) or ewsa+/m (n = 21) background, and only one fish with the ewsa/m/m genotype (n = 135) developed a tumor between 3 to 26 mpf. Because tumorigenesis is a complex process requiring multiple mutations, and because 10–15% of Ewing sarcoma patients have mutations in the tumor suppressor gene TP53, we chose to utilize the zebrafish tp53 mutant to evaluate the synergistic effect of ewsa and tp53 mutations. We generated fish with six genotypes: in the heterozygous tp53+/m background: ewsa+/−:tp53+/m, ewsa+/m:tp53+/m, ewsa+/−:tp53+/m, and in the homozygous tp53+/m background: ewsa+/−:tp53+/+/m, ewsa+/m:tp53+/+/m, and ewsa+/−:tp53+/+/m. The fish were monitored, and after 10 mpf, some began to develop visible tumor-like masses (Fig. 1A). These fish were subsequently euthanized and tumors were diagnosed by histological analysis using Hematoxylin and Eosin (H&E) staining of sectioned tissue. Consistent with the original report, few heterozygous tp53+/m zebrafish with wildtype ewsa developed tumors; the incidence of tumorigenesis in ewsa+/−:tp53+/+/m zebrafish was 8% after 26 months of observation (n = 28) (Fig. 1B). However, zebrafish with heterozygous or homozygous ewsa mutations in the heterozygous tp53+/m background displayed a much higher tumor incidence. Zebrafish with the ewsa+/−:tp53+/m genotype had a tumor incidence of 33% (n = 46) (Fig. 1B). Zebrafish with the ewsa+/m:tp53+/m genotype displayed a tumorigenesis incidence of 23% (n = 39) (Fig. 1B). Heterozygous or homozygous ewsa mutations in the homozygous tp53+/m background also increased tumor incidence. While the tumor incidence in zebrafish with the ewsa+/−:tp53+/m genotype had a tumor incidence of 67% (n = 12), and those with the ewsa+/m:tp53+/m genotype had a tumor incidence of 58% (n = 19). These results are summarized in Table 1. The results suggest that loss of ewsa contributes to tumor formation, and Ewsa functions to suppress tp53-dependent tumorigenesis. Unexpectedly, tumor incidence was lower in homozygous ewsa/m/m zebrafish than in heterozygous ewsa+/m zebrafish when expressed in either the heterozygous tp53+/m or homozygous tp53+/m backgrounds. One potential explanation for this is that cells with homozygous ewsa+/m mutations have additional and unknown defects that result in cell death rather than transformation.

We also analyzed the age of tumor onset in fish from all genotypes. In the heterozygous tp53+/m background, ewsa+/−:tp53+/m zebrafish (20+/− 2 months, total fish n = 39) had an significantly earlier age of tumor onset than ewsa+/−:tp53+/m fish (23+/− 1 months, n = 28) (P = 0.04). The ewsa+/−:tp53+/m zebrafish (19+/− 4 months, n = 46) also had earlier onset of tumorigenesis than ewsa+/−:tp53+/m fish, but the difference was not significant. In the homozygous tp53+/m background, zebrafish with homozygous ewsa+/m mutations (16+/− 3 months, n = 19) also displayed an earlier age of tumor onset than fish with wildtype ewsa (17+/− 3 months, n = 48), but again, the difference was not significant. These results suggest that loss of ewsa contributes to tumor formation, and Ewsa suppresses tumorigenesis (Fig. 1C).

To address whether there is any tumor type specificity in the ewsa and tp53 mutants, H&E stained tumor samples were characterized based on cell morphology. The tumors were subcategorized into four types: 1. small round blue cell tumor (SRBCT); 2. malignant peripheral nerve sheath tumor (MPNST)-like tumors (which resemble the predominant tumors associated with tp53 mutation as described in the original publication by Berghmans, et al.); 3. tumors with rosette pattern (rosette-forming tumors); and 4. other tumors (monitored fish displayed a low incidence of unclassified neoplasm, squamous cell carcinoma and melanoma) (Fig. 1D)19,20. No correlation was found between zebrafish tumor type and tumor classification, including SRBCT and rosette-forming tumors that are tumor morphologies similar to those found in Ewing sarcoma (Table 1). These results suggest that the ewsa mutation does not specify cancer type. In summary, Ewsa has an inhibitory role in tp53-mutation dependent tumorigenesis, affecting both incidence and age at onset of tumor formation, but the ewsa mutation does not define the tumor type.

Loss of ewsa allele promotes chromosomal instability (CIN). Our previous studies showed that Ewsa in zebrafish, or EWS in human cell lines, regulates midzone formation during mitosis14,15. The structure known as the midzone forms midline consisted of central spindles between segregating chromosomes during anaphase21–23.
Aberrant midzone formation leads to failure of cell division, and induces aberrant chromosome distribution to daughter cells, in an event known as chromosomal instability (CIN)\(^2\). To address whether Ewsa regulates central spindle formation, maternal and zygotic ewsa mRNA was depleted from zebrafish embryos by incrossing homozygotic ewsam/w mutant zebrafish (Maternal-Zygotic (MZ) ewsam/w zebrafish). Immunohistochemistry using anti-α-tubulin antibody was performed on 27 hour post-fertilization (hpf) ewsw/w and MZ ewsam/w embryos. Mitosis was visualized in both wildtype and MZ ewsam/w embryos and the numbers of aberrant mitotic events were scored. Consistent with our previous results in knockdown experiments, (using morpholinos designed against ewsa mRNA), the MZ ewsam/w displayed a higher incidence of mitotic dysfunction compared to ewsw/w (Fig. 2A,B)\(^1\). Specifically, the incidence of spindle defects at anaphase and at metaphase was higher in cells from MZ ewsam/w embryos than in ewsw/w. The result suggests that Ewsa regulates mitosis, particularly central spindle formation.

Figure 1. Loss of ewsa promotes tumorigenesis. Total numbers of fish for each genotypes are: ewsw/w;tp53w/w (n = 41), ewsw/w;tp53m/w (n = 135), ewsw/m;tp53/w (n = 21), ewsw/w;tp53/w (n = 28), ewsw/m;tp53/m (n = 46), ewsa/m;tp53/m (n = 39), ewsw/w;tp53/m (n = 48), ewsa/m;tp53/m (n = 12), ewsa/m;tp53/m (n = 19). (A) Representative images of zebrafish. In images of ewsa and tp53mutants, tumors are indicated with an arrow (>). Scale bar: 1cm. (B) Tumor incidence is higher in ewsw/w;tp53/m and ewsa/m;tp53/m compared to ewsa/w;tp53/m, and in ewsw/w;tp53/m and ewsa/m;tp53/m compared to ewsa/w;tp53/m zebrafish. (C) Average age at tumor onset is lower in ewsw/w;tp53/m and ewsa/m;tp53/m compared to ewsa/w;tp53/m zebrafish, and in ewsw/w;tp53/m and ewsa/m;tp53/m compared to ewsa/w;tp53/m zebrafish. (D) Representative images of H&E staining of tumors observed in zebrafish. MPNST-like: malignant peripheral nerve sheath tumor-like; SRBCT: small round blue cell tumor.
It is known that mitotic dysfunction leads to uneven distribution of chromosomes between daughter cells. Therefore, chromosome numbers were quantified in cells obtained from 24 hpf ewsaw+ and MZ ewsaw+ embryos, using metaphase spreads (Fig. 2C). The majority of cells obtained from ewsaw+ embryos displayed the normal number of chromosomes (50 chromosomes). However, the MZ ewsaw+ mutants displayed a higher percentage of cells with aberrant chromosome numbers compared to wildtype zebrafish (Fig. 2D). Together with the evidence for defective central spindle formation in cells from MZ ewsaw+ embryos, these results suggest that Ewsa maintains chromosomal stability. Interestingly, despite the results that show a large percentage of cells with normal chromosome numbers at 27 hpf in MZ ewsaw+ mutants, these embryos survive to adulthood and the adults are fertile. How the embryo survives with this level of aneuploidy is unclear. One potential explanation is that the aneuploidy observed at 27 hpf is the result of CIN in a cell type that has less impact on the embryo's survival, possibly due to a low proliferation rate.

**Loss of ewsaw allele promotes Loss of Heterozygosity (LOH) of tp53 in tumor cells.** Homozygous tp53+/- zebrafish displayed a high incidence of tumors (35%, n = 48), while zebrafish with the heterozygous tp53+/+ genotype showed a low incidence of tumor development (8%, n = 28) over 26 mpf (Table 1)29. However, when we generated heterozygous tp53+/- zebrafish with homozygous ewsaw+ mutations, we observed a significant increase in tumor incidence (23%, n = 39) (Table 1). We hypothesized that loss of ewsaw may promote tumorigenesis by inducing LOH of the tp53 wildtype locus. To investigate LOH, we collected tumors obtained from three individual fish (that had been genotyped prior to tumor formation and identified as ewsaw+/+,tp53+/- from fin (non-tumor) DNA). The genotypes of DNA extracted from fins and tumors were assayed using Restriction Fragment Length Polymorphism (RFLP)29. The restriction enzyme MboII digests PCR products amplified from the tp53 mutation allele, but not from the tp53 wildtype allele29. This analysis demonstrated that all three fins were the heterozygous tp53+/+ genotype. Conversely, RFLP analysis of the tumors demonstrated loss of the tp53 wildtype allele, suggesting that the tumors underwent LOH for tp53 (Fig. 3A). LOH of the tp53 locus was verified by sequencing of the PCR product containing the mutation site (M214K). Sequencing verified that all three fin samples were tp53+/-, whereas all tumors lost the tp53 wildtype allele (Fig. 3B). From this data, it is unknown whether the tp53 mutant locus underwent duplication, or whether the tp53 wildtype locus was deleted. However, we conclude that loss of ewsaw leads to induction of tp53 LOH with loss of the tp53 wildtype locus. Our study suggests a novel mechanism for the pathogenesis of EWS fusion-associated sarcomas: loss of one EWS allele due to the formation of the EWS-fusion gene induces mitotic dysfunction accompanied by aberrant midzone formation, CIN, and promotion of tumorigenesis by inducing LOH. Loss of the ewsaw allele did not change the tumor type that developed (Table 1). Thus, it is likely that both loss of EWS and EWS-fusion expression are required for promotion of tumorigenesis, and determination of tumor type.

**Discussion**

Formation of EWS-fusion genes in Ewing sarcoma results in the loss of one or both wildtype EWS alleles in the sarcoma cells2,3,19. While most studies involving Ewing sarcoma have focused on the function of the EWS-fusion genes, there is little understanding of the role that loss of an EWS allele has on pathogenesis. To address this, use of an animal model is essential; therefore, we utilized the ewsaw zebrafish mutant. Although ewsaw+ and ewsaw- mutant zebrafish have no or a low incidence of tumors, heterozygous or homozygous ewsaw mutation accelerated tumorigenesis in the tp53+/- background. This result suggests that the loss of one or both alleles of ewsaw promotes tumorigenesis. We also discovered that mutation of both ewsaw alleles results in CIN. Consistent with this discovery, tumors that developed in ewsaw+/+,tp53+/- zebrafish underwent LOH for the wildtype tp53 allele. This is the first demonstration in an animal model of the role of Ewsa in the maintenance of chromosomal stability, and in suppressing tumorigenesis. Establishing double mutants for ewsaw and tp53 in zebrafish allowed us to compare large sample numbers of each genotype. These zebrafish lines will serve as a useful model in future studies to investigate the contribution of Ewsa haploinsufficiency towards the pathogenesis of EWS-fusion associated sarcomas.

Elucidating the mechanism of Ewsa-dependent LOH induction is another critical step in understanding the pathogenesis of Ewing sarcoma tumors. Our previous study demonstrated that zebrafish ewsaw knockdown induces chromosomal mis-segregation due to mitotic dysfunction. Chromosome exchange occurs during metaphase when sister chromatids are aligned. Because EWS knockdown alters the localization of Aurora B kinase during anaphase and metaphase, this may disrupt the critical orchestration of chromosome segregation, leading
to induction of LOH. Another possible explanation for LOH induction is that loss of ews\textsubscript{a} mitotic function leads to DNA damage, and DNA repair failure contributes to LOH. Previous studies have shown that EWS knockdown results in alternative splicing of DNA repair molecules (e.g. ABL1, CHEK2 and MAPK2). Therefore, it is possible that LOH is induced by compromising two separate functions of EWS: mitosis and splicing of DNA repair molecules. In this study, we utilized \(tp53\) zebrafish as a tool to analyze tumorigenesis, but it is likely that other tumor suppressor gene loci undergo LOH as well. Recent studies have shown the existence of common fragile sites that are regions of genomic instability and thus are “hot-spots” for recombination. These regions are enticing candidates for study to determine whether Ewsa/EWS regulates these loci to suppress tumorigenesis. In addition, it is also possible that the \(tp53\) wildtype locus was deleted due to the induction of CIN.

**Figure 2. Loss of ews\textsubscript{a} promotes chromosomal instability (CIN).** (A) Representative images of mitotic cells from 27 hours post fertilization (hpf) zebrafish embryos visualized by immunohistochemistry using an anti-\(\alpha\)-tubulin antibody (red) and DAPI (blue). (B) Percentages of cells with aberrant mitosis in zebrafish embryos at 27 hours post fertilization, as determined after immunohistochemistry using an anti-\(\alpha\)-tubulin antibody. Experiments were repeated three times. (\(n = 13\) to 60 mitosis from 3 to 6 embryos per genotype). *\(P < 0.05\). (C) Representative images of chromosomes obtained from metaphase spread of 24 hpf \(ews\textsubscript{a}\text{\textsubscript{w/w}}\) (top) and MZ \(ews\textsubscript{a}\text{\textsubscript{m/m}}\) (bottom) embryos. (D) Numbers of chromosomes per cell, and percentage of cells from \(ews\textsubscript{a}\text{\textsubscript{w/w}}\) (blue bars) and MZ \(ews\textsubscript{a}\text{\textsubscript{m/m}}\) (red bars) zebrafish embryos containing that number of chromosomes. The vast majority of \(ews\textsubscript{a}\text{\textsubscript{w/w}}\) cells contained the normal chromosome number (50) (blue), whereas chromosome numbers in MZ \(ews\textsubscript{a}\text{\textsubscript{m/m}}\) mutant zebrafish cells varied from 34 to 56 chromosomes per cell (red). \(P < 0.05\). Note that all samples were obtained from lines with wildtype \(tp53\text{\textsubscript{w/w}}\) background.
Mesenchymal stem cells are considered to be the cell of origin in Ewing sarcoma. Interestingly, most other EWS-fusion associated sarcomas develop in tissues that derive from mesenchymal cells. For this reason, the mesenchymal stem cell is a strong candidate to be the cell of origin for other EWS-fusion associated tumors. Our zebrafish model will allow us to investigate the cell of origin for EWS associated tumors, in a spatiotemporal manner, because both developmental and differentiation processes in the mesenchymal cell lineage are conserved between zebrafish and human. In addition, because the frequency of LOH induction may be increased in the tumor cell of origin, our zebrafish model will allow us to measure the frequency of LOH induction in various cell types, and this may provide a platform to elucidate the mechanism of LOH.

Finally, studies in which EWS/FLI1 was overexpressed in animal models suggest that expression of EWS/FLI1 alone is not sufficient to induce Ewing sarcoma. To achieve conditional expression of EWS/FLI1 in a mouse model, EWS/FLI1 transgenic mice were crossed to Prx1-Cre transgenic mice. The mice from these crosses did not develop tumors, but EWS/FLI1 did accelerate the formation of sarcomas in Tp53 deleted mice. Zebrafish with mosaic expression of human EWS/FLI1 had a very low incidence of tumor formation, but crossing these with a tp53 mutant increased the incidence of tumorigenesis. In addition, when EWS/FLI1-transduced mesenchymal stem cells were injected into mice, they developed fibrosarcoma. Together, these findings suggest that the pathogenesis of Ewing sarcoma may require both the expression of the EWS-fusion gene and the loss of an EWS allele. This hypothesis can be expanded to other EWS-fusion associated sarcomas as well, as loss of one wildtype allele is a common feature to all. In addition, we previously reported that expression of EWS/FLI1 leads to mitotic defects through dominant inhibition of EWS. Previous studies have shown that EWS/FLI1 and EWS biochemically interact. This interaction may further deplete the normal function of the remaining wildtype EWS allele. We previously demonstrated that EWS relocates the mitotic regulator Aurora B kinase to the midzone. The midzone is required for the maintenance of spindle architecture, spindle elongation, and cleavage furrow formation. Failure of Aurora B kinase to relocate to the midzone leads to defects in cytokinesis accompanied by uneven chromosome segregation, and the induction of aneuploidy. Therefore, it is possible that the high incidence of aberrant chromosome numbers observed in ewsa mutant zebrafish may be the result of mitotic defects due to the loss of Ewsa. All of this suggests that EWS haploinsufficiency may play a critical role in the tumorigenesis of EWS-fusion associated sarcomas.

Materials and Methods

Ethics statement. All protocols utilizing zebrafish were approved by the University of Kansas, Lawrence, Institutional Animal Care and Use Committee (IACUC) (Animal use statement: permit number #197–02). All methods were carried out in accordance with the relevant guidelines.

Zebrafish maintenance. The ewsa zebrafish mutant line was established and characterized in a previous report. The Oregon AB, ewsa, and tp53M214K zebrafish mutant lines were housed at 28 °C in an automated filtration system from Aquatic Habitats.

Histological analysis. Zebrafish were humanely euthanized, and tissues fixed with 4% paraformaldehyde at 4 °C overnight. The fixed samples were embedded, sectioned, and subjected to Hematoxilin and Eosin (H&E) staining using standard protocols.

Immunohistochemistry. Zebrafish 27 hpf embryos were fixed with 4% paraformaldehyde at 4 °C overnight, and subjected to immunohistochemistry using anti-α-tubulin antibody (Sigma) as described previously.
Metaphase chromosome spreads. Chorionic membranes from 24 hpf zebrafish embryos were removed. The embryos were euthanized with Tricaine mesylate, and rinsed twice in PBS. The embryos were then placed in 1.1% NaCitrate at room temperature for 8 min. The embryonic cells were dissociated using a syringe, and placed on ice for an additional 8 min, then were collected by centrifugation (3000 rpm for 5 min). The supernatant was replaced with methanol:acetic acid (3:1) and cells were incubated for 20 min at room temperature, and subsequently incubated at −20°C overnight. The cells were collected by centrifugation, resuspended in methanol:acetic acid (3:1) solution, and this suspension was dropped onto glass slides and the DNA was counterstained with DAPI.

Statistics. Error bars represents standard deviation (SD), and statistical confidence was determined to be at P < 0.05 by t-test (Figs 1C and 2B), or t-tests for normal distribution (Fig. 2D).

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M.A. wrote the main manuscript text. H.P., R.G., T.T. and J.M. prepared Figures 1–3 and Table 1. All authors reviewed the manuscript.

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