Cytoskeletal architecture and cell motility remain unperturbed in mouse embryonic fibroblasts from *Plk3* knockout mice

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Abstract

Polo-like kinase 3 (Plk3) is best known for its involvement in cell cycle checkpoint regulation following exposure to cytotoxicants or induction of DNA damage. Yet, Plk3 has also been implicated in roles beyond those of cellular responses to DNA damage. Here, we have investigated the proposition, suggested by the Plk literature, that Plk3 regulates cytoskeletal architecture and cell functions mediated by the cytoskeleton. To this end, we have assayed mouse embryonic fibroblasts (MEFs) generated from both *Plk3* knockout and wild-type mice. In particular, we asked whether Plk3 is involved in actin fiber and microtubule integrity, cell migration, cell attachment, and/or cell invasion. Our results demonstrate that functional Plk3 is not critical for the regulation of cytoskeletal integrity, cell morphology, cell adhesion, or motility in MEFs.

Keywords: Polo-like kinase, plk3, cytoskeleton, cell migration, cell motility, and attachment

Experimental Biology and Medicine 2016; 241: 603–610. DOI: 10.1177/1535370216629010

Introduction

The discovery of *polo*, the first member of the polo-like kinase (Plk) gene family to be described, was made over 27 years ago.¹ Mutation of the *polo* locus in *Drosophila melanogaster* led to mitotic abnormalities, which subsequently were determined to be the result of mutation in a gene encoding a kinase also designated as Polo.^{1,2} Since then, this evolutionarily conserved family of kinases has been expanded and currently includes five members in humans that are now designated Plk1 through Plk5.^{3–6} Functionally, members of the Plk family are known to regulate progression through the cell cycle and to coordinate mitosis.^{7–10} Plk1, the human homolog of *Polo*, has received the majority of attention as it possesses a primary role in driving cancer cell proliferation.¹¹ The Plk family member that is the focus of this study, polo-like kinase 3 (Plk3), regulates cell cycle progression in response to multiple stressors, by participating in the activation of the G1/S checkpoint, mitotic spindle assembly checkpoint, Golgi fragmentation, and apoptotic signaling.^{12–16}

However, Plk3 may possess cellular functions separate from its role in safeguarding cell cycle progression and cell division and these potential functions remain largely underexplored. Our study investigates one such suggested Plk3 function. Previous publications have shown that Plk3 and the other Plks associate with cytoskeletal components and play a role in regulating cytoskeletal dynamics.^{17–19}

The ability of cells to continuously reorganize the cytoskeleton is vital to the growth of cells and proper execution of cell division. During mitosis, the centrosome serves as the command center for this continuous reorganization.²⁰ All of the Plk family members except Plk5 are reported to localize to the centrosome during mitosis. Once present at the centrosome, they collectively function to ensure the proper duplication of centrioles, attachment of spindle poles, alignment of chromosomes, and separation of sister chromatids.^{21–24} The Plks help orchestrate these processes through phosphorylation of numerous centrosomal proteins including direct modification of cytoskeletal components.^{8,21} Many of the same structural proteins and signaling pathways involved in organization and coordination of the cytoskeleton during mitosis also regulate the structure and dynamics of the cytoskeleton in non-dividing cells.^{19,25,26}

More directly suggesting a possible role in cytoskeletal dynamics, Plk3 has been shown to phosphorylate β -tubulin although the physiological consequences of this phosphorylation are currently unknown.¹⁸ In addition, Plk3 has been demonstrated to co-localize with F-actin and its overexpression disrupts actin polymerization and results in cell rounding and eventually induces apoptosis.²⁷ Another report suggests that Plk3 expression is induced in macrophages upon cell adhesion and shows that Plk3 interacts with the integrin regulator, calcium and integrin binding protein

1 (CIB1).²⁸ Subsequently, CIB1 was reported to be a negative regulator of both Plk3 kinase activity and cell migration.^{29,30} In aggregate, these findings suggest that Plk3 has a role in cytoskeletal regulation; however, causal relationships between Plk3 and cytoskeletal organization have remained elusive. The intent of the work described here was to establish whether loss of Plk3 is sufficient to perturb cytoskeletal organization or interfere with functions dependent on cytoskeletal signaling, such as cell migration, adhesion, and invasion. For this purpose, we examined mouse embryo fibroblasts (MEFs) from mice in which the *Plk3* gene has been inactivated by deletion of its promoter and first six exons.¹⁴ In addition, Plk3 has been shown to be down-regulated in several cancer subtypes suggesting a tumor suppressor function.^{16,31,32} Establishing whether or not Plk3 contributes to the regulation of cytoskeletal organization impacts our understanding of cell migration and invasion and ultimately tumor cell metastasis.

Materials and methods

Cell culture

Cell culture media and reagents were purchased from Life Technologies (Carlsbad, CA). Cells were grown in Dulbecco's Modified Eagle Medium supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 0.1 mM non-essential amino acids, and 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂. MEFs were generated from E13.5 to E14.5 embryos. Pregnant mice were euthanized by exposure to concentrated CO₂ followed by cervical dislocation to guarantee non-recovery. All efforts were made to minimize animal suffering. This work was conducted in strict accordance with the regulations established by Laboratory Animal Management Services at the University of Cincinnati. The protocol was approved by University of Cincinnati Institutional Animal Care and Use Committee (Protocol Number: 06-08-28-02). All MEFs used for experiments were fourth passage or earlier and all experiments were passage matched. Prior to experiments, MEFs were thawed from liquid nitrogen, plated and allowed to recover overnight. The following day cells were counted by hemocytometer and plated at equal cell number.

Genotyping and qPCR

Genomic DNA from mouse tail clips or ear punches was isolated using QIAamp DNA Mini Kit (Qiagen, Valencia, CA) and subjected to standard PCR. For genotyping *Plk3* knockout mice, the primer sequences were 5'-AAAC CACCTGTGTTGGTGATGTGC-3' and 5'-AGCTAGCTTGG CTGGACGTAAAC-3' for the wild-type allele and 5'-TTTC CTGGAGCTCTGTAGCCGAAA-3' and 5'-ACACCCATC TGTGCCATACACTCA-3' for the insert in the *Plk3* knockout mice (IDT, San Jose, CA). For qPCR, total RNA was extracted from wild-type and *Plk3*-null MEFs with a commercially available magnetic mRNA isolation kit (Life Technologies, Carlsbad, CA). Taqman probes against *Plk3* and GAPDH (Life Technologies) were used to set-up qPCR

reactions according to Life Technologies' suggested protocol.

Immunofluorescence

MEFs were grown on coverslips prior to fixation with 4% paraformaldehyde in PBS for 20 min. Cells were washed twice with PBS, permeabilized and blocked with blocking buffer (10% goat serum, 1% BSA, and 1% Triton X-100). Cells were incubated with a primary antibody against β -tubulin at 1/500 (AbCam, Cambridge, UK) in blocking buffer for 1 h at room temperature or overnight at 4°C. Alexa Fluor 546 conjugated secondary antibodies (Life Technologies) were diluted 1:2000 in buffer and added to cells for 1 h at room temperature in the dark. F-actin filaments were stained with Alexa Fluor 488-conjugated phalloidin (Life Technologies) using a 1:500 dilution for 1 h at room temperature in the dark. DNA was stained with 1x DAPI (Sigma-Aldrich) and coverslips were mounted onto slides using Fluoromount G (Southern Biotech, Birmingham, AL). Cells were imaged by fluorescence microscopy (Zeiss) and captured with Axiovision software.

Multinucleation assay

Coverslips were fixed and stained for β -tubulin and DAPI as described above. Fifteen fields were imaged for both wild-type and *Plk3*-null MEFs. Mononucleated and multinucleated MEFs were manually scored. The percentage of multinucleated cells was calculated by dividing the total number of cells with two or more nuclei by the total number of cells counted. The experiment was repeated in duplicate and in each trial at least 500 cells were counted per cell type.

Migration assay

The IncuCyte Zoom (Essen Bioscience, Ann Arbor, MI) an automated live-cell imager was set-up and used according to the manufacturer's instructions. Prior to migration experiments, the detection software was calibrated specifically for MEFs to ensure accurate distinction of cells from empty space. MEFs (2.5×10^4 cells/well) were plated into 96-well plates approximately 24 h prior to wounding. Cells were 95–100% confluent at time of wounding. Scratch wounds ($\sim 200 \mu\text{m}$) were made using the accompanying WoundMakerTM (Essen Bioscience). Plates were immediately placed into the IncuCyte Zoom and images were recorded every 15 min for 48 h. Experiments were conducted at 37°C and 5% CO₂. The software incorporated into the IncuCyte Zoom was used to analyze the images and determine the rate of cell migration graphed as the percentage of wound confluence. Each data point graphed represents the mean of the 48 wells recorded per cell type.

Woundless migration assay

Cells (2×10^6) were plated in 60 mm cultures dishes printed with the vertically patterned polylactic acid-linker-polyethylene glycol (PLA-L-PEG) polymer. The cells attach to the polymer free spaces and grow to confluence (24–48 h). Subsequently, culture plates were exposed to low dose UV

light to cleave the polymer from the cell attachment resistant PLA-L-PEG to attachment permissive PLA. The cells were manually imaged every 4 h using a charge-coupled device (CCD) camera attached to a microscope (Nikon, TE-2000S) until the 200 μ m gap was closed. Patterned scratches in the bottom of the cell culture plates were used to ensure each of the same fields was captured at each of the time points. The images were individually analyzed using ImageJ to determine percent gap confluence and graphed as the average gap confluence for the 15 fields imaged for each cell type.

Attachment assays

For assessing cell attachment to fibronectin and collagen, precoated plates were purchased from BD Biosciences (San Jose, CA). MEFs were counted and plated into individual wells of 12-well plates at 3×10^5 cells/well. After 4 h, the media was aspirated, wells were gently washed with $1 \times$ PBS, and the attached cells were fixed with 4% paraformaldehyde. Cell attachment was measured by counting cells in three randomly selected fields per well. The counting was blinded such that the cell type in each well was not known until after the data were collected. The data were graphed as the average number of cells attached per well. Each data point represents a single well and a minimum of 12 wells was counted for each cell type.

Invasion assay

For matrigel invasion assays, precoated matrigel plates were purchased from Millipore. MEFs were counted and plated at 1×10^6 cells/well into 0.1% serum-containing media in the upper chamber of the 12-well plates with 10% serum-containing media added to the bottom well to induce invasion. After 20 h, the media was aspirated and MEFs attached to the underside of the filter were fixed and stained using a Kwik-diff kit (Thermo Scientific, Waltham, MA). Filters were removed from the well with a scalpel, affixed to microscope slides using immersion oil, and imaged. Five randomly selected fields were counted per well. The data were graphed as the average number of cells attached per well. A total of 12 wells were counted for each cell type.

RNA sequencing

A commercially available magnetic mRNA isolation kit (Life Technologies) was used for the polyA RNA (including mRNA and polyA lncRNA) purification. A total of 1 μ g

total RNA was used as the input. An Apollo 324 system (WaferGen, Fremont, CA) was used and runs PrepX PolyA script for the automatic polyA RNA isolation. Using the same system, the isolated RNA was RNase III fragmented, adaptor ligated, converted into cDNA with Superscript III reverse transcriptase (Life Technologies), and subjected to automatic purification using Agencourt AMPure XP beads (Beckman Coulter, Indianapolis, IN). Next, using the universal (SR) and index-specific primer with a limited PCR cycle number (~ 13), sample-specific index was added to each ligated cDNA sample and the amplified library was enriched by AMPure XP bead purification with a final elution volume of 16 μ L. To check the quality and yield of the purified library, 1 μ L of library was analyzed by Bioanalyzer (Agilent, Santa Clara, CA) using a DNA high sensitivity chip. To accurately quantify the library concentration for the clustering, the library was diluted $1:10^4$ in dilution buffer (10 mM Tris-HCl, pH 8.0 with 0.05% Tween 20), and qPCR measured by Kapa Library Quantification kit (Kapabiosystem Woburn, MA) using ABI's 9700HT real-time PCR system (Lifetech). The raw data (transcript counts) were transformed into Reads Per Kilobase of transcript per Million mapped reads (RPKM) tables for the purpose of comparative analysis of gene expression.

Results

Validation of the *Plk3* knockout model

Initially, we used genomic PCR to verify that the deleted region of the *Plk3* gene was absent from the *Plk3* knockout mice (Figure 1(a)). To ask whether or not *Plk3*-null cells have an altered phenotype, we derived MEFs from the *Plk3* knockout animals. In addition, we confirmed that *Plk3* mRNA expression was not present in the *Plk3*-null MEFs by qPCR, as no amplification of *Plk3* was detectable (Figure 1(b)). These cells were then utilized as a model system to assay for potential *Plk3* function relating to cytoskeletal organization, cell motility, adhesion, and invasion.

Plk3-null MEFs display normal cytoskeletal organization and cytokinesis

Based on reports that *Plk3* interacted with or co-localized with elements of the cytoskeleton, we asked whether the absence of *Plk3* might have an overt effect on cytoskeletal organization. We therefore stained *Plk3*-null and wild-type MEFs with antibody to β -tubulin to assess the gross microtubule architecture. Randomized images of the stained cells assessed in blinded fashion did not distinguish any

Figure 1 Validation of *Plk3* targeting and absence of *Plk3* RNA from knockout animals: (a) PCR-based genotyping of genomic DNA isolated from wild-type, *Plk3* knockout, and *Plk3* heterozygous mice. (b) qPCR from total RNA showing *Plk3* mRNA and *Gapdh* mRNA expression in wild-type and *Plk3*-null MEFs. Values represent the average number of PCR cycles to reach the threshold for RNA detection (n = 3). No detectable amplification of *Plk3* occurred in the *Plk3*-null MEFs

Figure 2 Loss of Plk3 does not visibly disrupt the arrangement of β -tubulin or F-actin in MEFs. (a) Representative images showing immunofluorescent staining for β -tubulin (green, upper panels) and F-actin (red, lower panels). Cell nuclei were also stained with DAPI (blue). (b) Multinucleation assay: Quantification of interphase cells with two or more nuclei in wild-type and Plk3-null MEFs. (A color version of this figure is available in the online journal.)

differences in staining patterns between the isogenic MEFs (Figure 2(a), top panels). When cells were stained with phalloidin to examine F-actin architecture, there was also no apparent difference in F-actin organization. (Figure 2(a), lower panels). Repeated attempts to stain the cells by immunofluorescence using antibody to Plk3 were unsuccessful since all of the commercially available anti-Plk3 antibodies tested lacked specificity and gave non-reproducible staining patterns. Lastly, Plk3 has been reported to play an important role in cytokinesis,^{23,33} a process in which regulation of the cytoskeleton is critical and which is required for proper division of a parent cell into two daughter cells. We therefore scored the cell populations for cells with multiple nuclei, an indicator of compromised cytokinesis. MEFs were stained for β -tubulin and with the nuclear stain DAPI. The cells were imaged, and the number of interphase cells with two or more nuclei was manually counted as a marker of abnormal cytokinesis. Rounded cells were not included in the count to eliminate cells actively undergoing cell division. As shown in Figure 2(b), there was no significant difference between the Plk3-null MEFs and wild-type MEFs in the percentage of cells with multiple nuclei. Collectively, our results showed there are no differences in cytoskeletal organization and morphology between Plk3-null and wild-type MEFs.

Plk3-null MEFs exhibit normal cell migration properties

As another measure of cytoskeletal function, we asked whether Plk3 plays a role in cell migration. To this end, we assayed cell migration by two different methodologies. First, we used the IncuCyte Zoom, an automated live cell imager with high-throughput capabilities and built-in data analysis. Cells were seeded into 96-well plates at equal number and allowed to grow to confluence overnight. The Essen Bioscience WoundMakerTM, an accessory for the IncuCyte ZOOM[®] (Essen Bioscience), was utilized to create wounds of a standardized width. Cells were imaged every 15 min for at least 48 h and the percentage of wound confluence was analyzed at intermediate times and at the conclusion of the experiment. By this assay, both wild-type and Plk3-null MEFs closed the wound at between 20 and 24 h (Figure 3(a)). These results are also graphed as the average wound confluence of each of 48 independent

wells per cell type (Figure 3(b)). Based on these studies it appears that there is no difference in the rate of wound closure between wild-type and Plk3-null MEFs, suggesting that Plk3 does not participate in migration signaling.

Since the previous assay is based on generating a wound, we employed a second approach which enables measurements of cell migration while avoiding the killing or damaging of cells at the edge of the wound during its production. The method involves printing cell culture plates with a light-cleavable polymer that prevents cell attachment. By carefully controlling the positioning of the polymer, it can be used to create patterned areas onto which the cells can be seeded. In this case, the polymer was used to create strips of a defined width and MEFs were seeded into the area between strips. When the cell-resistant polymer is exposed to low dosage UV light, the polymer is cleaved leaving behind PLA; a material that is permissive for cell attachment, allowing the cells to migrate into the previously restricted area and the rate of migration to be measured. Briefly, cells were seeded into the patterned area for 24–48 h prior to beginning the assay followed by exposure to low dose UV light ($t=0$ h). The cells were manually imaged every 4 h, until the gap was closed. Both wild-type and Plk3-null cells migrated into the gap at a similar rate (Figure 3(c)). Multiple fields were imaged per experiment, the percentage confluence of each gap within the individual fields was quantified using ImageJ software, and the results were graphed as the average confluence of all fields at each time point. Again, there was no significant difference in the rate of gap closure between the wild-type and Plk3-null MEFs (Figure 3(d)). Thus, based on data from two very different methodologies, we conclude that absence of Plk3 does not alter MEF migration under the conditions used here.

Effect of Plk3 loss on cell attachment and invasion

Regulation of Plk3 kinase activity by CIB1, a known mediator of integrin signaling, suggests that Plk3 might participate in regulation of cell attachment.²⁹ Therefore, the capacity of Plk3-null and Plk3 proficient MEFs to attach to collagen-coated and fibronectin-coated culture plates was compared. The Plk3-null MEFs showed a slight but significant decrease in the percentage of cells attached to

Figure 3 Loss of Plk3 does not alter the rate of cell migration in MEFs. (a) Representative images showing the migration of wild-type and Plk3-null MEFs using a standard scratch wound assay. The wound area not populated with cells is pseudo-colored. (b) Graph of average wound confluence for all wells for standard scratch wound assay. (c) Representative images showing the migration of wild-type and Plk3-null MEFs using a novel woundless migration assay. (d) Graph of average wound confluence for all image fields for woundless migration assay. (A color version of this figure is available in the online journal.)

Figure 4 The absence of Plk3 does not significantly alter cell attachment efficacy or invasion in MEFs. (a) and (b) Graphs comparing the means of the average number of Plk3-null or wild-type MEFs attached per well to collagen IV and fibronectin-coated plates, respectively. (c) Matrigel invasion assay: Graphs comparing the means of the average number of invasive Plk3-null or wild-type MEFs per well after 20 h

collagen-coated plates compared with their wild-type counterparts (Figure 4(a)); however, it is unlikely such a minor difference is biological relevant. Both wild-type and Plk3-null MEFs attached equally well to fibronectin-coated wells (Figure 4(b)). Thus, Plk3 may play a minor role, if any, in the attachment of MEFs during cell-to-matrix adhesion. As metastasis is a hallmark of cancers and Plk3 is a suspected tumor suppressor,^{31,32,34} we asked whether loss of Plk3 would alter the invasiveness of MEFs. Cells were interrogated using a standard matrigel invasion assay. The

Plk3-null MEFs were equally invasive as the wild-type cells (Figure 4(c)), suggesting that loss of Plk3 neither facilitates nor impairs the invasive potential of these cells.

Plk2 is expressed at high levels in MEFs and may compensate for the absence of Plk3

Given that loss of Plk3 did not discernibly alter the phenotype of mice or MEFs, we asked whether expression of compensatory genes might provide an explanation. We

Figure 5 Relative levels of mRNAs transcript for the Plk family. In the absence of a discernible phenotype in Plk3-null MEFs, RNA sequencing was performed to determine whether another Plk family member could potentially be compensating for loss of Plk3. The graph shows the standardized level of mRNA for each polio-like kinase family member in MEFs and Gapdh as a control

therefore performed RNA-Seq on mRNA from wild-type and Plk3-null MEFs. Importantly, the low level of Plk3 mRNA in Plk3-null MEFs is most likely artifactual and likely due to misalignment of transcript belonging to some of the other Plks. This is supported by the total absence of Plk3 when the same cDNA library from Plk3-null MEFs was tested by qPCR (Figure 1(b)). Interestingly, the mRNA level of Plk2 was significantly higher than that of all the other members of the Plk family (8–10-fold over Plk1 and Plk4) and about 30-fold higher than the level of Plk3 transcript in wild-type MEFs (Figure 5). Future experiments should consider testing the consequence of combined loss of Plk2 and Plk3 to investigate the existence and extent of compensatory signaling between the two kinases.

Discussion

The data presented argue that the absence of Plk3 in MEFs has little, if any, effect on cytoskeletal organization, cell adhesion, migration, or invasion. This finding is surprising given the reports of associations between Plk3 and elements of the cytoskeleton. Perturbation of microtubule integrity has been ascribed to dysregulated Plk3,^{33,35} and Plk3 has been localized to centrosomes, particularly during cell division.^{23,29} Plk3 clearly co-immunoprecipitates with CIB1, a protein linked to the inhibition of Plk3 activity.^{28,33} Furthermore, CIB1 has been reported to regulate microtubule organization in coordination with Plk3 and has been implicated as a regulator of the p21-activated kinases that contribute to cytoskeletal rearrangement and cell migration.³⁰ Further, adhesion of monocytes to a substrate increased levels of Plk3 mRNA, consistent with possible CIB1 involvement.²⁸ However, whether there is a causal relationship between elevation of Plk3 and monocyte adhesion is unclear. Targeting Plk3 with an shRNA in T47D cells also led to multinucleation, possibly due to compromised microtubule architecture and defective cytokinesis.³⁶

Although the cumulative literature suggests the involvement of Plk3 in the dynamics of cytoskeletal organization,

adhesion, migration, and invasion, in retrospect the expectation that absence of Plk3 in MEFs would compromise these activities may have been overly optimistic. The fact that mice lacking Plk3 have normal fecundity and display normal development over their lifetime suggests that the complete absence of Plk3 has no overt deleterious effects on developmental or *in vivo* cellular effects. This proposition is supported by our RNA-seq data showing that the level of Plk3 mRNA is relatively low compared to the whole genome set. It is also possible that no altered phenotype was observed because Plk3 is solely a stress response kinase and MEFs were not subjected to an appropriate challenge.

Alternatively, the *in vivo* environment may be responsible for the differences in observable phenotypes in experiments performed with cells that had been in culture for multiple generations compared with early passage MEFs that are not long removed from the *in vivo* context. The absence of an abnormal phenotype in Plk3-null MEFs may also be due to the Plk3 possessing tissue or cell-type specific functions that are not manifest in MEFs. For example, the previously mentioned role for Plk3 in macrophage adhesion may be cell-type specific.²⁸ In hepatocytes, Plk3 functions in the unfolded protein response by facilitating the resolution of ER stress.³⁷ In neurons, Plk3 is suggested to regulate synaptic plasticity.³⁸ Whereas, in corneal epithelial cells, Plk3 responds to osmotic stress by phosphorylating the transcription factors ATF-2 and c-jun to induce apoptosis.^{39,40}

Another plausible explanation for the absence of an aberrant phenotype in Plk3-null mice and cells, frequently alluded to in the literature, is that Plk2 functionally compensates for the loss of Plk3. Like Plk3, Plk2 can act as a stress response gene which functions to activate checkpoint arrest following cell damage.⁴¹ In addition, the consensus phosphorylation sites for Plk2 and Plk3 are highly similar, with both kinases preferring acidophilic residues at the same locations surrounding the targeted serine/threonine.^{18,42} This is supported by data showing that both kinases share a number of phosphorylation targets.¹⁸

Our results suggest that Plk3 alone is not required for regulation of the cytoskeleton in adherent, non-mitotic MEFs. Nevertheless, the data provide insight into the functionality of Plk3, suggesting that its interactions with cytoskeletal components and CIB1 are not directly involved in regulation of cell morphology or motility. These findings further provide direction to future studies, allowing research to focus on alternative possibilities when investigating a role for Plk3 in cytoskeletal regulation. Defining the consequences of loss of Plk3 function could lead to an improved understanding of its downregulation in some cancers and could aid in the development of clinically efficacious inhibitors for Plks.

Authors' contributions: Conceived and designed the experiments: DRM, KSM, CCH, and PJS. Performed the experiments: DRM and KSM. Analyzed the data: DRM, KSM, CCH, and PJS. Contributed reagents/materials/analysis tools: DRM, KSM, CCH, and PJS. Wrote the manuscript: DRM and PJS.

ACKNOWLEDGEMENTS

This work was funded by NIH grants awarded to Dr Stambrook (R01ES016625) and Dr Ho (R01EB010043 and R01GM112017) and by a NIEHS training grant awarded to Dr Stambrook (T32 ES07250). The authors would like to acknowledge Michael Conley of Essen Bioscience for providing technical assistance for the IncuCyte Zoom. Also, thank you to The Genomics, Epigenomics, and Sequencing Core at the University of Cincinnati for completing the RNA sequencing and to the laboratory of Dr Medvedovic at the University of Cincinnati for formatting the RNA-sequencing data. The authors would also like to thank Elisia Tichy, PhD and Enerlyn Lozada, PhD for their helpful feedback in experimental design and revising the manuscript prior to submission.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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(Received September 10, 2015, Accepted January 4, 2016)