Published online 7 July 2021

Nucleic Acids Research, 2021, Vol. 49, No. 14 **8135–8144**
https://doi.org/10.1093/nar/gkab578

Cognate restriction of transposition by *piggyBac*-like proteins

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Received October 28, 2020; Revised June 16, 2021; Editorial Decision June 17, 2021; Accepted June 26, 2021

ABSTRACT

Mobile genetic elements have been harnessed for gene transfer for a wide variety of applications including generation of stable cell lines, recombinant protein production, creation of transgenic animals, and engineering cell and gene therapy products. The *piggyBac* transposon family includes transposase or transposase-like proteins from a variety of species including insect, bat and human. Recently, human *piggyBac* transposable element derived 5 (PGBD5) protein was reported to be able to transpose *piggyBac* transposons in human cells raising possible safety concerns for *piggyBac*-mediated gene transfer applications. We evaluated three *piggyBac*-like proteins across species including *piggyBac* (insect), *piggyBat* (bat) and PGBD5 (human) for their ability to mobilize *piggyBac* transposons in human cells. We observed a lack of cross-species transposition activity. *piggyBac* and *piggyBat* activity was restricted to their cognate transposons. PGBD5 was unable to mobilize *piggyBac* transposons based on excision, colony count and plasmid rescue analysis, and it was unable to bind *piggyBac* terminal repeats. Within the *piggyBac* family, we observed a lack of cross-species activity and found that PGBD5 was unable to bind, excise or integrate *piggyBac* transposons in human cells. Transposition activity appears restricted within species within the *piggyBac* family of mobile genetic elements.

INTRODUCTION

DNA transposons are mobile genetic elements accounting for 3% of genomic space in the human genome (1). The *piggyBac* family of transposons are found across species including but not limited to *piggyBac* from the cabbage looper moth (*Trichoplusiani ni*) (2,3), *piggyBat* from the little brown bat (*Myotis lucifugus*) (4) and PGBD5 (*piggyBac* transposable element derived 5) from humans (*Homo sapiens*) (5). The *piggyBac* transposon system has been shown to have activity in human cells and has been harnessed for a variety of applications including generating stable cell lines, transgenic animals, recombinant protein production, induced pluripotent stem cells (iPSCs) and cell therapy products (6–12).

piggyBat has demonstrated similar activity to *piggyBac* in mammalian cells (4). More recently, PGBD5 has been reported to be able to mobilize *piggyBac* transposons in human cells leading to identification of putative PGBD5 target sites within the human genome that can be mobilized, and PGBD5 appears to be linked to human cancer (13,14). The possibility that PGBD5 can transpose *piggyBac* transposons raises questions in using *piggyBac* for therapy and other applications and its importance has been reviewed by others (15).

In general, transposase-transposon interactions are very precise requiring highly specific protein sequence interaction with cognate DNA inverted terminal repeat elements (ITRs) (16,17). For instance, the *hAT* family *TcBuster* transposon from the red flour beetle (*Tribolium castaneum*) exhibited transposition activity within human cells (18,19). However, *TcBuster* transposase-related human proteins Buster1, Buster3 and SCAND3 were unable to mobilize *TcBuster* transposons in human cells (18). We previously evaluated several *piggyBac*-ITR-like sequences within the human genome and observed an inability of insect *piggyBac* transposase to mobilize such sequences (20).

Given the recent report by Henssen *et al*. reporting genomic DNA transposition by human PGBD5 (13), we sought to evaluate the potential for cross-species transposition activity within the *piggyBac* family. As *piggyBac* is being considered and used for preclinical and therapeutic applications (21–24), we evaluated whether *piggyBat* and

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PGBD5 could mobilize *piggyBac* transposons in human cells.

MATERIALS AND METHODS

Protein alignment

Protein alignment was performed using Clone Manager 9 Professional Edition software. Global protein alignment was performed using a BLOSUM 62 scoring matrix with the *piggyBac* transposase as the reference protein.

Plasmid constructs

pCMV-SB, pCMV-PB, pCMV-HA-PB and pTpB have been described previously (6). pCMV-m7pB (HyPBase) has been described previously (25). pCMV-pBat and pTpBat were generated by subcloning the *piggyBat* transposase cDNA and *piggyBat* ITRs kindly provided by Dr Nancy Craig (4) into pCMV-PB and pTpB, respectively. PGBD5 plasmids were obtained from Addgene including pINDUCER21-PGBD5 (#78121) and pRecLV103-GFP-PGBD5 (#65409) (13). PB-EF1-NEO was purchased from Systems Biosciences (Palo Alto, CA) as described by Henssen *et al*. (13). pRec-GFP-PB was generated by subcloning PB in place of PGBD5 in pRecLV103-GFP-PGBD5. pCMV-HA-PGBD5 vectors were generated by subcloning PGBD5 in place of PB in pCMV-HA-PB (6). pCMV-HA-NHE3 has been described previously (26). pCMV-GFP has been described previously (20). μpTpB was generated by using PCR to shorten the *piggyBac* ITRs to 39 bp (left end, LE) and 67 bp (right end, RE) to correspond to those lengths in PB-EF1-NEO. iμpTpB (i, inverted) has the same ITR lengths but with the RE ITR sequence on the 5′ end and the LE ITR sequence on the 3′ end thereby being in the same orientation at PB-EF1-NEO. pLE-Luc (LE IR in front of luciferase) and prLE-Luc (flipped orientation of LE IR in front of luciferase) plasmids have been previously described (20). VPR-HAPB and VPR-HAPGDB5-v2 plasmids were constructed through In-Fusion cloning in which the VPR cDNA fragment was PCR amplified from the PB-TRE-dCas9-VPR plasmid (Addgene, #63800) and inserted into the N-terminus of the cDNAs in pCMV-HA-PB and pCMV-HA-PGBD5v2, respectively. Standard molecular biology techniques were used throughout, and all DNA sequences were confirmed using sanger DNA sequencing.

Cell culture and transfection

HEK293 or HT-1080 cells were seeded at a density of 100 000 cells per well in a six-well plate and transfected with 2 μg of total plasmid DNA, containing 1 μg of transposon and 1 μg of transposase plasmid DNA unless otherwise indicated using Lipofectamine 2000 (Life Technologies, CA, United States), according to manufacturer's instructions and in order to attempt to replicate experiments by Henssen *et al*. ((13). Cells were trypsinized and re-plated for functional assays 24 h later.

Excision assay

Excision assay analysis was performed as described by Henssen *et al*. and by us previously (6,13). Plasmid DNA was recovered from transfected cells 24 h after transfection and subjected to excision PCR analysis using primers described in the Supplementary Table S1. PCR products were visualized using agarose gel electrophoresis. Excision bands were excised and *piggyBac* transposition was confirmed via DNA sequencing as described previously (6).

Colony count assay

Two days after transfection, 10 000 cells were replated on 10-cm dishes in growth media plus G418 (1 mg/ml) and selected for 2 weeks. Dishes were then fixed, stained with methylene blue and counted as described previously (6).

Plasmid rescue and mapping of transposon integration sites

We performed plasmid rescue of transposon integration sites as described previously (6). Briefly, HEK293 or HT-1080 cells were transfected as above for colony count analysis with pCMV-PB, pCMV-HA-PGBD5v1, or pCMV-HA-PGBD5v2 and pTpB. After one day of transfection, cells were split to 100 mm dishes and selected with 1 mg/ml of G418 for 2 weeks or longer for PGBD5 transfected cells. Selected cells were harvested for genomic DNA preparation using DNeasy Blood and Tissue kit (Qiagen, Germantown, MD). Ten micrograms of genomic DNA was digested with *Ahd* I and *Bsa* I (all enzymes from NEB, Ipswich, MA) then dephosphorylated using antarctic phosphatase to remove unintegrated pTpB plasmid. DNA was digested with one of three combinations of restriction enzymes with overlapping ends that do not cut within the transposon segment of pTpB being *Nhe* I/*Xba* I, *Acc65* I/*BsrG* I or *Xho* I/*Sal* I. Digested genomic DNA was ligated using T4 ligase and DH10B *Escherichia coli* were transformed by electroporation and subsequently plated on LB-agar with kanamycin for selection. Kanamycin-resistant colonies were replica plated on LB-ampicillin plates. Colonies that grew in the presence of kanamycin but not in the presence of ampicillin (the pTpB backbone harbors ampicillin resistance) were presumed to represent possible transposon integrations. We isolated plasmid DNA and performed sequencing using several primers that reads through the ITR elements of the pTpB transposon (Supplementary Table S2). We used the UC Santa Cruz BLAT genome web-browser (human, December 2013 assembly (GRCh38/hg38)) to map integration sites in the human genome. We used ∼35 bp of high-quality sequence starting at the first genomic junction for BLAT searches.

Quantitation of transposon copy number

qPCR for transposon ITR and RNAse P copy number was performed as described previously (18,27). Briefly, HEK293 or HT-1080 cells were transfected and selected as described above. After a minimum of 2 weeks of selection, genomic DNA was isolated as above. Fifty nanograms of genomic DNA was used to amplify the *piggyBac* left (5′) IR (forward, 5′-CTAAATAGCGCGAATCCGTC-3′; reverse, 5′-TCATTTTGACTCACGCGG-3′) or RNase P (forward, 5′-AGATTTGGACCTGCGAGCG-3′; reverse, 5′-GAGC GGCTGTCTCCACAAGT-3′). pTpB and RNase P plasmids were serially diluted to generate standard curves for

analysis to quantitively determine the copies of *piggyBac* IR per copy of RNase P recovered from stably gene-modified cells.

Western blot

Whole cell lysates were prepared in RIPA buffer (Sigma, St. Louis, MO) supplemented with mammalian protease inhibitor mix (Sigma) and phosphatase inhibitor mix (PhosStop, Roche, Indianapolis, IN) and protein concentration was determined by BCA. Lysates were normalized with RIPA buffer to equal concentrations and prepared for electrophoresis by adding 1× NuPAGE LDS Sample Buffer and 1× NuPAGE Reducing Agent (Invitrogen, Carlsbad CA) and heating 70°C for 10 min. Equal volumes were run on 4–12% NuPage Bis-Tris gels with MOPS buffer followed by transfer to nitrocellulose for immunoblotting. Blots were incubated in rat anti-HA (clone 3F10, Roche) at 1:1000, and mouse anti-βactin (Novus Biologicals, Centennial, CO, # NB600-501) 1:10K 4°C overnight. IRD-labeled secondary antibodies (LICOR, Lincoln, NE) 800 goat anti-rat and 680RD goat anti-mouse were each used at 1:15 000 for detection with an Odyssey Infrared Imaging System (LICOR). TBS-based Odyssey blocking buffer and TBS with 0.1% Tween were used for all antibody dilutions and washing.

Luciferase assay of protein-DNA interaction

HT-1080 cells were seeded to six-well plates at 4 $\times 10^5$ cells/well one day before transfection. Cells were transfected with 1 μg of LE-Luc and 1 μg of VPR-HA-PB, VPR-HA-PGBD5-v2, or pCMV-eGFP plasmids using X-tremeGENE 9 DNA transfection regent according to the manufacturer's instructions (Roche). Twenty-four hours after transfection, 6 μl of 30 mg/ml of D-Luciferin (PerkinElmer, Pittsburgh, PA) was added to cells. Images were captured using IVIS imaging (Perkin Elmer) after 10 min of addition of luciferin.

Statistical analysis

All comparisons of >2 samples involved one-way analysis of variance followed by Dunnett's multiple comparison post-test comparing to the control.

RESULTS

PGBD5 lacks the C-terminal cysteine-rich domain of *piggyBac* family members

The *piggyBac* transposase contains a C-terminal cysteine-rich domain (CRD) that is essential for interaction with *piggyBac* ITRs leading to DNA cleavage and transposition (28). Both *piggyBac* and *piggyBat* contain this CRD and have been shown to transpose their cognate transposons in mammalian cells (28). We aligned the two known versions of PGBD5 (NM_024554.3 (v1) and the more recent update NM_001258311.2 (v2)) with *piggyBac* and found no evidence of a CRD that could mediate interaction with *piggyBac* ITRs (Figure 1). Therefore, based on protein sequence analysis *in silico*, PGBD5 appears to lack the known CRD within *piggyBac* family members necessary for transposition.

Cognate restriction of *piggyBac* transposition

Although PGBD5 appeared to lack the necessary CRD for transposition, PGBD5 could contain protein–DNA interaction motifs that enable transposition through a different mechanism. Therefore, we compared the ability of *piggyBac, piggyBat* and PGBD5 to mobilize *piggyBac* transposons in human cells using standard assays for detecting transposition in transfected cells with a typical *piggyBac* transposon, pTpB (Figure 2A) (6). We first used an excision assay to evaluate the ability of the respective transposase or transposase-like proteins to excise a *piggyBac* transposon from a plasmid transfected into HEK293 cells (2,3). PCR was used to evaluate for end-joining of the plasmid construct with an appropriately sized PCR product indicating transposon excision. We used the pRecLV103-GFP-PGBD5 (PGBD5v1) plasmid to express PGBD5 in HEK293 cells in attempts to replicate the data from Henssen *et al*. (13). We found that both *piggyBac* and HyPBase (hyperactive *piggyBac*, (29)) were able to excise a transfected *piggyBac* transposon; however, the negative control *s*leeping beauty transposase*, piggyBat* and PGBD5 demonstrated no detectable excision of a *piggyBac* transposon (Figure 2B). DNA sequencing confirmed precise excision and TTAA site reconstitution after isolation of PCR bands from *piggyBac*- and HyPBase-transfected cells. We also used colony count analysis as a proxy for measuring transposase activity wherein an antibiotic resistance transposon is integrated into transfected cells via transposition. The colony count assay thereby provides a quantitative readout of transposition by enabling measurement of antibiotic resistant colonies of cells resultant from transfection and transposition. Transposase activity can be limited by overproduction inhibition or transposase–transposase interaction at high concentration (30,31). Therefore, we evaluated the potential for PGBD5 to mobilize *piggyBac* transposons at a variety of transposon-to-transposase DNA ratios. Despite using 1:1, 9:1 and 1:9 ratios, we observed a lack of evidence of colony formation using PGBD5 in HEK293 cells (Figure 2C). We subsequently used qPCR to quantitate the number of transposon integrations in cells comparing the copies of the *piggyBac* left (5′) ITR per copy of the RNAse P gene. Although we found multiple copies of the *piggyBac* transposon when transfected with *piggyBac* transposase, we found that PGBD5 was not statistically different than no transposase (pUC) control (Figure 2D). Colony count analysis of a *piggyBat* transposon harboring neomycin resistance (pTpBat) revealed that only *piggyBat* transposase, and not *piggyBac* or PGBD5, was able to confer transposition of *piggyBat* transposons in human HEK293 cells (Figure 3). Therefore, only *piggyBac* can excise *piggyBac* transposons and neither *piggyBac* nor PGBD5 can excise or integrate *piggyBat* transposons. Additionally, PGBD5 showed a lack of evidence of excision or integration of *piggyBac* transposons even when evaluated at multiple transposon-to-transposase ratios.

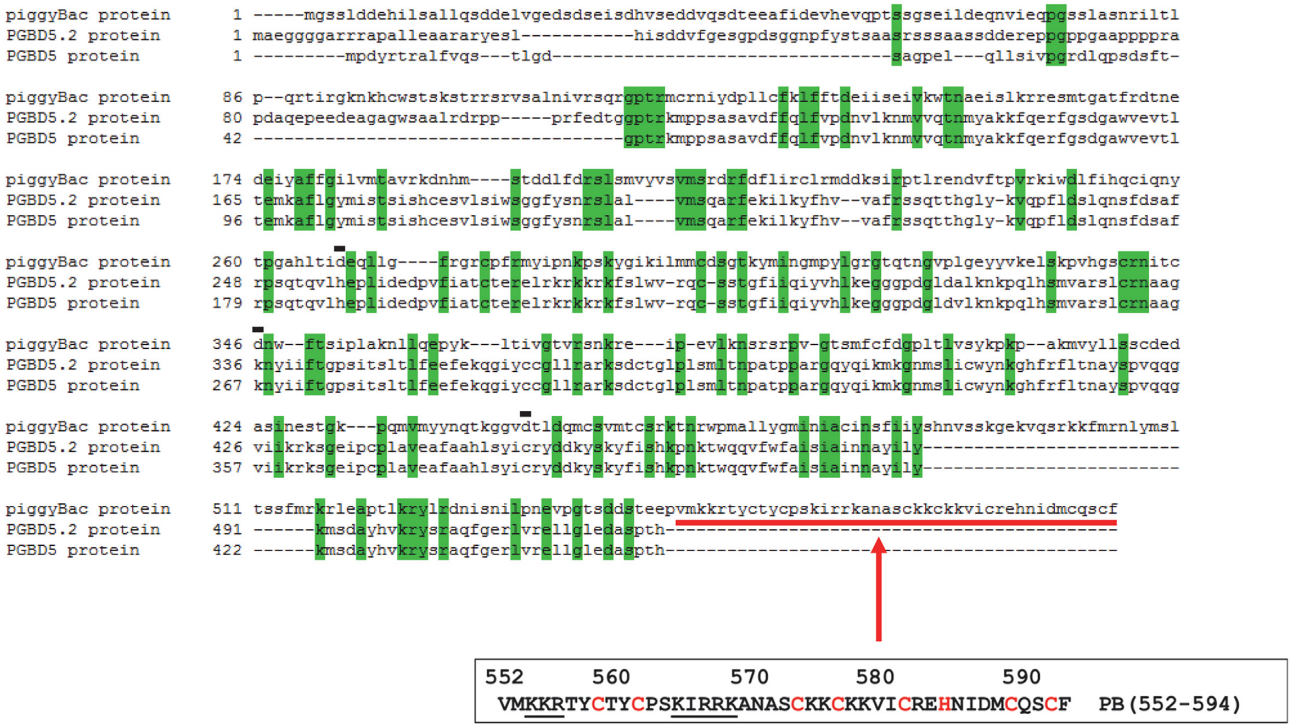

Figure 1. Alignment of *piggyBac* and PGBD5 protein sequences as described in the Materials and Methods section. Identical amino acids are highlighted in green. The DDD catalytic motif of *piggyBac* is marked with black bars above the amino acids. The CRD of *piggyBac* is underlined in red and then expanded to demonstrate the bipartite nuclear localization sequence (underlined black) and cystine residues of the CRD (highlighted red). PGBD5, version 1; PGBD5.2, version 2.

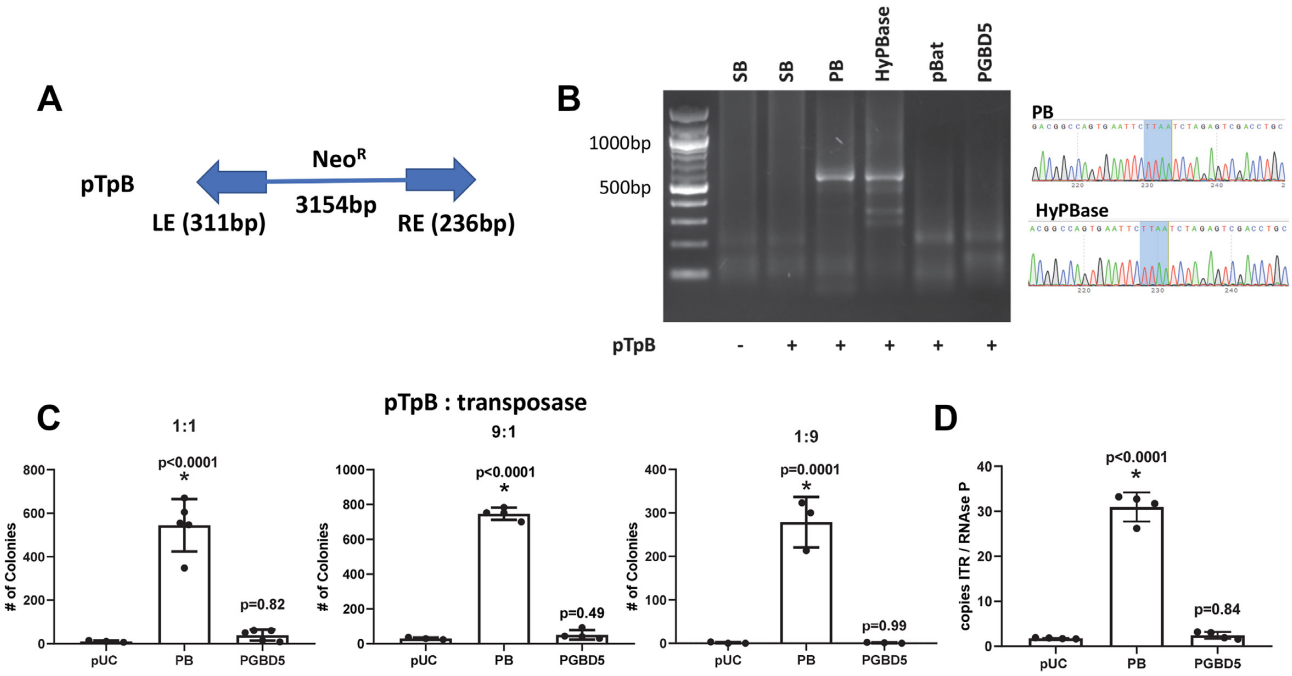

Figure 2. The pTpB *piggyBac* transposon is only excised and integrated by *piggyBac* in human cells. (**A**) Schematic of pTpB transposon conferring neomycin resistance. (**B**) Excision assay using pCMV-SB, -PB, -HyPBase, -pBat or -PGBD5 (pRecLV103-GFP-PGBD5) to express the putative transposase in HEK293 cells transfected with pTpB. The expected excision product is 539 bp. Shown is representative of three independent experiments. (**C**) Colony count analysis of various transposon:transposase (1 μg:1 μg, 1.8 μg:200 ng or 200 ng:1.8 μg pTpB:transposase) ratios in HEK293 cells using pUC (negative control) or PB/PGBD5; $N = 3 \pm$ SD; *, $P < 0.05$. (**D**) qPCR of copies of *piggyBac* ITR/RNAse P of 1 μg:1 μg pTpB:transposase stably transfected HEK293 cells; $N = 2$ (in triplicate) $\pm$ SD; *, $P < 0.05$ compared to pUC control.

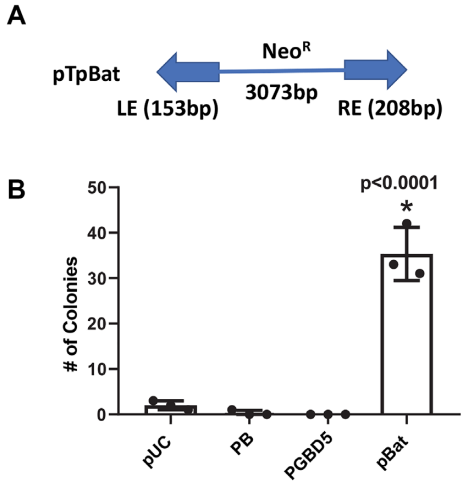

Figure 3. *piggyBat* transposons are only integrated by *piggyBat* in human cells. (**A**) Schematic of pTpBat transposon conferring neomycin resistance. (**B**) Colony count analysis measuring integration efficiency of pTpBat in HEK293 cells by pUC (negative control), pCMV-PB, pRecLV103-GFP-PGBD5, or pCMV-pBat; $N = 3 \pm$ SD; *, $P < 0.05$.

Verification of *piggyBac* family member protein expression while exhibiting cognate restricted transposition

In the above experiments, we used a standard *piggyBac* transposon (pTpB, (6)) containing ITRs of 311 (left end [LE] or 5′,) and 236 (right end [RE] or 3′,) bp in length (Figure 2A). The *piggyBac* transposon used by Henssen *et al*. PB-EF1-NEO used shorter ITRs of 67 and 39 bp but flipped in orientation such that the 67 bp ITR is derived from the RE but used on the 5′ end and the 39 bp is derived from the LE and used on the 3′ end (Figure 4A) (13). Additionally, the 67 bp ITR used by Henssen *et al*. contains a G to A point mutation. Given the differences between the *piggyBac* transposon used by Henssen *et al*. (PB-EF1-NEO) and the one we used above (pTpB, Figure 2), we repeated excision assays and colony counts using PB-EF1-NEO to attempt to reproduce their results.

Additionally, the vectors used by Henssen *et al*. contained GFP fused to PGBD5 (13). We therefore generated a GFP-*piggyBac* vector. As we have used hemagglutinin (HA)-tagged *piggyBac* to detect transposase expression in the past (6), we generated an HA tagged version of PGBD5 in its two protein versions. These constructs enabled us to detect transfection and expression using GFP fluorescence as well as full-length protein expression via western blot analysis.

Although we could detect GFP expression in transfected cells using GFP, GFP-PB, GFP-PGBD5v1 and GFP-PGBD5v2 (Figure 4B), we only observed excision of PB-EF1-NEO when using *piggyBac* and not PGBD5 (Figure 4C). Evaluation of our HA-tagged constructs revealed appropriate expression of *piggyBac* and PGBD5 (Figure 5); however, only HA-*piggyBac* resulted in excision of PB-EF1-NEO (Figure 4C). Given the shorter and flipped ITRs in PB-EF1-NEO compared to pTpB, we performed colony count analysis. To correspond to the shorter ITRs of PB-EF1-NEO used by Henssen *et al*., we shortened the *piggyBac* ITRs of pTpB to 39 bp (LE) and 67 bp (RE) to correspond to those lengths in PB-EF1-NEO, thereby creating μpTpB that is also described in recent structural analysis of *piggyBac* (32). iμpTpB (i, inverted) has the same ITR lengths but with the RE ITR sequence on the 5′ end and the LE ITR sequence on the 3′ end thereby being in the same orientation at PB-EF1-NEO. This allowed us to compare shortened ITRs to full-length ITRs transposing the same promoter-Neo^R cassette as in pTpB (Figure 6A). We found that only *piggyBac* was capable of transposition of PB-EF1-NEO in HT-1080 cells (Figure 6B). Although μpTpB resulted in fewer colonies than pTpB, only *piggyBac* resulted in measurable colonies whereas PGBD5 was not different than no transposase control (Figure 6C). Even though pTpB exhibited increased colonies with *piggyBac*, thereby demonstrating increased sensitivity of detecting transposition when compared to μpTpB, we found PGBD5 to be no different than no transposase control when co-transfected with pTpB. We used qPCR to quantitate the number of *piggyBac* transposons in stably transfected HT-1080 cells. Although we observed *piggyBac* transposase mediated integration, PGBD5 was no different than no transposase control (Figure 6D). Therefore, despite effective transfection (GFP fluorescence) and expression (western analysis) of PGBD5, we observed a lack of evidence for PGBD5-mediated transposition of *piggyBac* transposons using full-length or shortened ITRs.

Recovery of *piggyBac* but not PGBD5 mediated integrations in human cells

We next used a proven method for recovering transposon integrations. We chose to use plasmid rescue as it allows recovery of the full-length transposon fragment with the potential for sequencing from both transposon ends (Figure 7). Plasmid rescue involves no PCR amplification and therefore is not subject to potential PCR artifacts or ligation of PCR-amplified products. The pTpB transposon harbors kanamycin/neomycin resistance and a p15A origin of replication with ampicillin resistance and a pUC origin of replication outside of the transposon fragments. Our plasmid rescue method therefore allowed us to select for kanamycin resistant/ampicillin sensitive bacterial colonies for plasmid isolation and DNA sequencing. Using plasmid rescue, we analyzed 103 *piggyBac*-mediated integration events. For 43 of those, we attempted to sequence from both transposon ends and found 36 genomic and 5 inter-plasmid integrations with 2 not mappable. All integrations contained full-length ITRs with *bona fide* TTAA ends correlating with a TTAA site in the human genome or plasmid, even the ones no mappable due to integration into genomic repeats (Table 1 and Supplementary Table S3). For the other 60 events, we sequenced from one of the transposon ends and found 51 genomic and 7 inter-plasmid integrations with 2 not mappable within genomic repeats. Again, all integrations demonstrated a full-length ITR and occurred at a TTAA site in the human genome or plasmid with the exception of one ATAA site in the human genome. Therefore, using plasmid rescue, we observed 92% transposon integration recovery mapped to specific sites when *piggyBac* transposase was used (Table 1). Our results are consistent with what we and others have reported previously (6,33). We next evaluated 114 PGBD5-mediated plasmid rescue

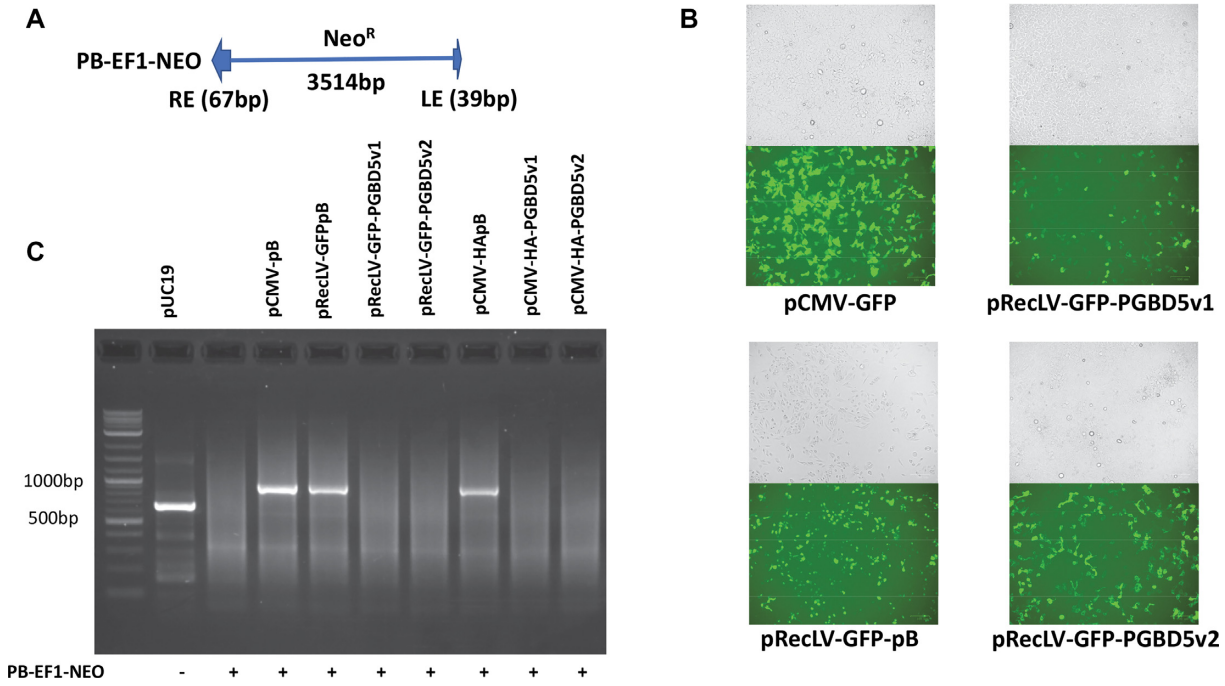

Figure 4. The PB-EF1-NEO *piggyBac* transposon is only excised by *piggyBac* in human cells. (**A**) Schematic of PB-EF1-NEO transposon with shortened ITRs in a flipped orientation compared to pTpB. (**B**) GFP expression confirmed using a ZOE fluorescent microscope after transfection of various plasmid vectors. Phase contrast images are placed above the GFP images. (**C**) Excision PCR analysis of various transposase constructs co-transfected with PB-EF1-NEO in human HT-1080 cells. The pUC (negative control) band results from primer binding to the pUC plasmid backbone. The expected PCR product from *piggyBac*-mediated excision is 926 bp. Shown is representative of three independent experiments.

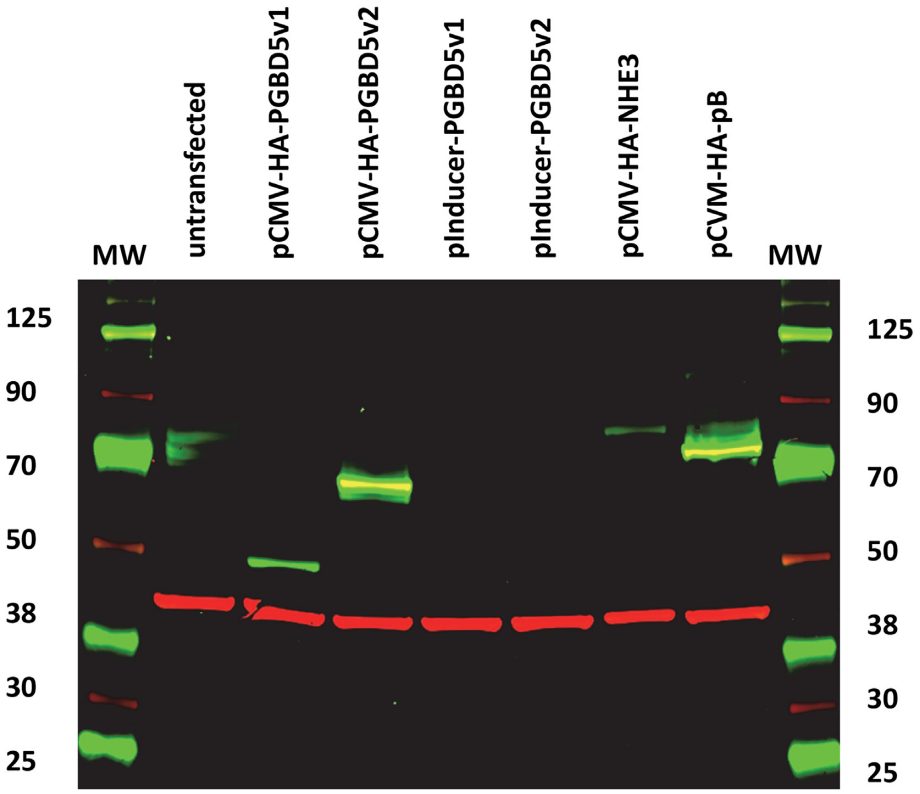

Figure 5. Western blot analysis confirms correct expression of *piggyBac* and PGBD5 in HEK293 cells. HA-PGBD5v1 and v2 have expected molecular weight of 52 and 58 kDa, respectively. The pInducer plasmids lack an HA tag and serve as negative control. HA-tagged (sodium hydrogen exchanger 3) NHE3 and PB serve as positive controls. Blot demonstrates HA (green) with B actin (red) serving as a loading control confirming equal total protein input for each lane.

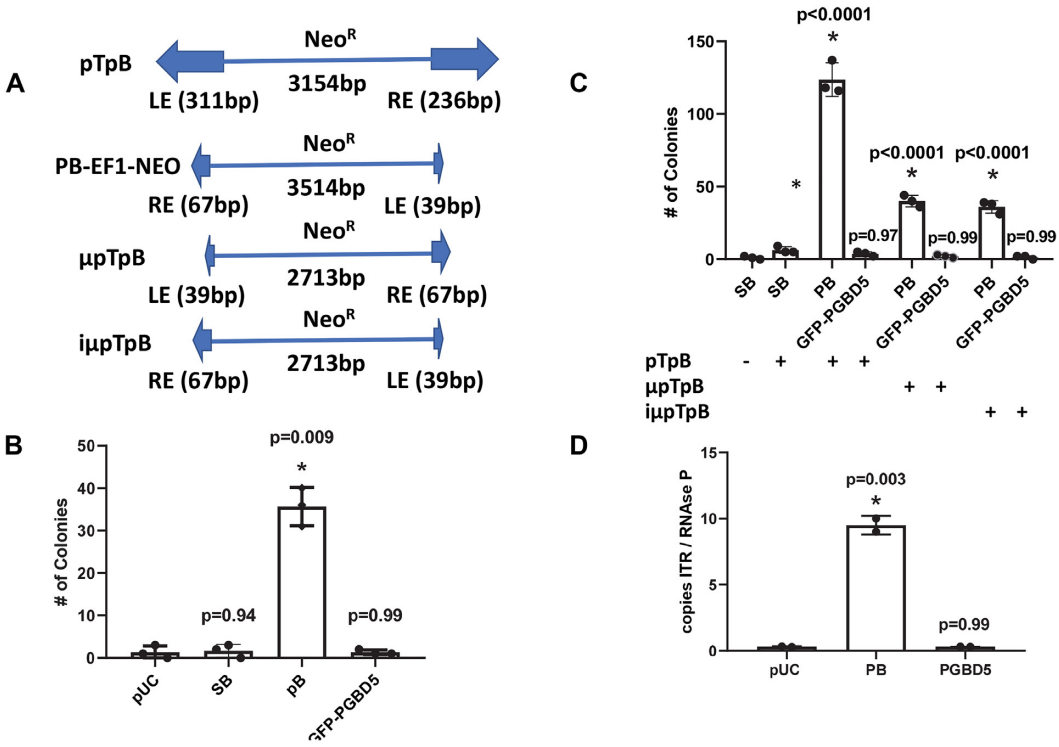

Figure 6. *piggyBac*, and not PGBD5, integrates PB-EF1-NEO, μpTpB and iμpTpB (i, inverted) transposon vectors. (**A**) Schematic of μpTpB with shortened ITRs, and iμpTpB with shortened ITRs in flipped orientation (to correspond to PB-EF1-NEO), conferring neomycin resistance. (**B**) Colony count analysis of G418 resistance in HT-1080 cells transfected with PB-EF1-NEO and pUC, pCMV-SB, pCMV-pB, or pRecLV103-GFP-PGBD5; $N = 3 \pm$ SD; *, $P < 0.05$. (**C**) Colony count analysis after transfection of the various transposon and transposase vectors into HT-1080 cells and subsequently selected with G418; $N = 3 \pm$ SD; *, $P < 0.05$. (**D**) qPCR of copies of *piggyBac* ITR/RNAse P of 1 μg:1 μg pTpB:transposase stably transfected HT-1080 cells; $N = 2$ (in triplicate) $\pm$ SD; *, $P < 0.05$ compared to pUC control.

colonies. We were unable to recover any integrations into the human genome with the sequencing primers used (Table 1 and Supplementary Table S2). We observed no transposon breakpoints with neighboring genomic DNA at the end of the ITR or other sites in the transposon plasmid. We only recovered transposon plasmid DNA flanking the ITRs. Therefore, we recovered no full-length ITRs with a terminal TTAA sites neighboring genomic DNA from any plasmid rescue analyzed after PGBD5 transfection with the pTpB transposon.

piggyBac but not PGBD5 binds ITRs in human cells

As mentioned above, the CRD domain of *piggyBac* is known to mediate protein–DNA interaction between the transposase and ITRs (28). We created an assay to evaluate the ability of *piggyBac* family member proteins to bind *piggyBac* ITRs by fusing the respective proteins to a VPR activation domain, which contains VP64, p65 and Rta activation domains (34). We used a luciferase reporter construct wherein we had cloned the 311 bp LE (left end) ITR in forward or reverse orientation upstream of luciferase (20). Binding of the transposase-VPR fusion protein to the ITR sequence would be expected to result in luciferase expression (Figure 8A). *piggyBac*, but not PGBD5v2, demonstrated measurable luciferase activity indicating that only *piggyBac* and not PGBD5 bound the cognate *piggyBac* ITR (Figure 8B and C). Therefore, not only does PGBD5 appear to lack the CRD necessary for protein–*piggyBac* ITR DNA interaction, PGBD5 does not bind the *piggyBac* ITR sequence based on our luciferase reporter readout of transposase–ITR interaction that confirmed *piggyBac*–ITR interaction.

DISCUSSION

Mobile genetic elements are common, contribute to genomic diversity, and may be linked to certain forms of cancer (35,36). Transposons can be harnessed for genomic DNA insertion of transgenes for a variety of applications. Some transposons have even been used for clinical trials in humans for cell and gene therapy applications (37). Much investigative groundwork must be laid in consideration of using DNA transposons, or any vector system, for therapeutic or other applications.

If proteins exist in humans that can re-mobilize therapeutically inserted DNA elements, then genomic rearrangements and subsequent genotoxicity could result (15,38,39). Therefore, it is imperative to investigate such a possibility. For transposon systems like *TcBuster*, human Buster-like proteins appear incapable of mobilizing *TcBuster* transposons (18). PGBD5 has been implicated as one such protein with transposase-like activity not only capable of mobilizing *piggyBac* transposons but also leading to genomic rearrangements in human cells linked to cancer (13,14).

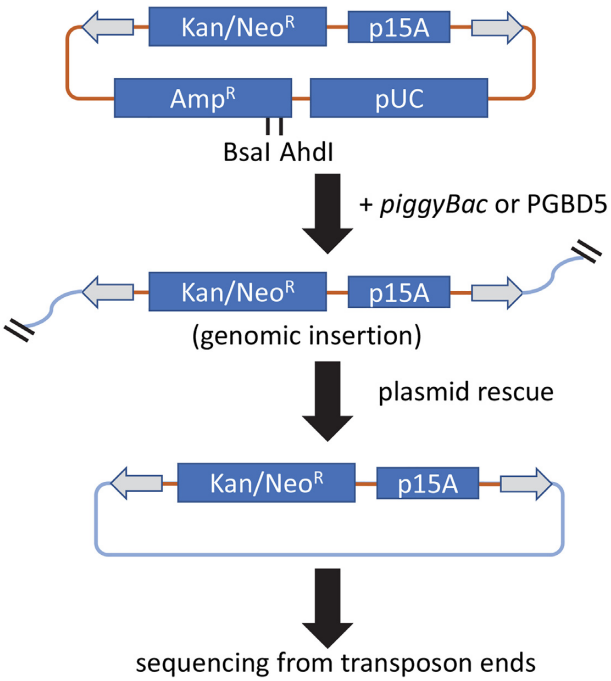

Figure 7. Plasmid rescue of transposon integration sites in the genomes of human cells. Plasmid rescue was performed as described in the Materials and Methods section. The pTpB transposon harbors kanamycin/neomycin resistance and a p15A origin of replication within the transposon. If the transposon segment (light gray arrows, ITRs; brown line, plasmid backbone) integrates into the genome (light blue line), plasmid rescue can be used to recover the genomic DNA (light blue) neighboring the transposon segment. Plasmids can then be sequenced to determine integration sites within the human genome.

We evaluated three *piggyBac*-like family members for their ability to mobilize *piggyBac* transposons. In doing so, we evaluated *piggyBac*-like proteins from three different species. We found that *piggyBac* was selectively capable of mobilizing *piggyBac,* and *piggyBat* was similarly restricted to *piggyBat*. We found that PGBD5 was incapable of mobilizing either *piggyBac* or *piggyBat*. Lack of PGBD5 activity on *piggyBac* transposon was confirmed using different transposon vector designs despite confirmation of transfection and protein expression in two different human cell types. Based on sequence analysis of PGBD5, it would not be predicted to bind *piggyBac* ITRs and this lack of binding was confirmed in an in-cell assay of transposase–ITR interaction to corroborate excision and colony count transposition assays.

Recently, Helou *et al*. studied the activity of the *piggyBac* transposase to that with deletion of the CRD (PB-1–558) and deletion with an added nuclear localization sequence (NLS) (PB.NLS-1–558), as the NLS in *piggyBac* is thought to overlap with the CRD (40). They concluded that the CRD is not required for *piggyBac*-mediated transposition. However, the CRD deleted *piggyBac* with an additional NLS (PB.NLS-1–558) demonstrated at least at 10-fold reduction in integration efficiency as well as loss of fidelity in transposition with proper target site duplication and full-length ITRs only found in 19% of sequenced integration sites compared to 96.7% with *piggyBac* (40). In some of the colony count analysis done by Helou *et al*., PB.NLS-1–558 did not appear to be significantly different than no transposase controls. PB.NLS-1–558 appears to perhaps work more like a nuclease by cutting DNA rather than a transposase. No experiments were performed to determine if the PB.NLS-1–558 variant lacking the CRD could bind *piggyBac* ITRs. Therefore, we conclude that the CRD is necessary for *bona fide piggyBac*-mediated transposition with high fidelity and efficiency.

In the same paper by Helou *et al*., they looked at transposon junction break points recovered from integrations in cells for both mouse and human PGBD5 (40). PGBD5 appeared to have even less evidence for *bona fide piggyBac*-mediated transposition with sequencing confirming <5% having LE (5′) ITRs and apparent target site duplication (40). Sequencing of transposon insertions performed by Helou *et al*. and Henssen *et al*. involved PCR-based amplification of transposon ends and next generation sequencing (NGS) (13,40). Henssen *et al*. reported analysis of 66 integration sites after biotinylated primer selection of transposon insertions for PGBD5 (13). We chose to use plasmid rescue as an alternative methodology because it does not involve PCR amplification and it allows recovery of the full-length transposon segment. Our analysis revealed a lack of evidence for PGBD5-mediated transposition despite easily recovering *piggyBac*-mediated integration events. Although plasmid rescue is presumably less sensitive than PCR-amplification and NGS of insertion sites, it is not subject to PCR-amplification and ligation of PCR products. Our inability to plasmid rescue PGBD5 integrations resultant from *bona fide* transposition is consistent with our excision, colony count and qPCR of transposon integration results.

The NGS analysis of possible PGBD5 insertions by Helou *et al*. is inconsistent with the excision assay reported in Henssen *et al*. If PGBD5 leads to transposon breakage with poor fidelity, a single PCR band with TTAA site reconstitution would not be expected as reported by Henssen *et al*. (13). The NGS analysis of possible PGBD5-mediated insertions by Helou *et al*. is also inconsistent with the insertion site analysis reported by Henssen *et al*. Henssen *et al*. reported 65 out of 66 transposon junctions occurred with a TTAA and transposon ends, whereas Helou *et al*. reported much less fidelity being <5% for the left end analyzed (13,40).

Helou *et al*. 'normalized' their colony counts based on what they perceive to be 'cytotoxicity' mediated by the transposase (40). They report this based on colony count reduction compared to transfected GFP in place of transposase. No other cytotoxicity analysis was offered. The colony count reduction, or reduced integration rate of the antibiotic selection cassette, could also be impacted by overexpressed proteins cutting the antibiotic resistance/transposon segment with poor fidelity thereby disrupting expression of antibiotic resistance needed for colony growth.

We evaluated possible transposition mediated by PGBD5 in human cells using excision assays, colony count analysis, qPCR of transposon insertions, plasmid rescue of genomic integrations and analysis of possible binding to ITRs within cells. We found either no evidence of transposition (excision

Table 1. Plasmid rescue of transposon integration sites in the human genome

	Recovered	Transposon insertion into genome	Transposon insertion into plasmid	Recovered both sides of transposon with integration	Not mappable	% transposon recovery with mappable integration
piggyBac	43	34	5	41	4	
	60	49	7	n/a	4	
total:	**103**	**83**	**12**			**92.23%**
PGBD5	114	0	0	n/a		
total:	**114**	**0**	**0**			**0%**

Plasmid rescue was used to recover possible transposon integration sites from stably transfected human cells. 95/103 *piggyBac*-mediated integration sites were mappable to genomic or plasmid sites with recovery of both sides of the transposon in 41 (out of 43 where we attempted recovery of both sides) of those with *bona fide* TTAA target site duplication. The other 60 *piggyBac* recovered sites involved sequencing from only one of the transposon ends. We attempted plasmid rescue of PGBD5-mediated transposon integrations and recovered zero genomic integration events with full-length ITRs with a terminal TTAA and neighboring genomic DNA. Only transposon plasmid sequence flanking the terminal repeat was recovered.

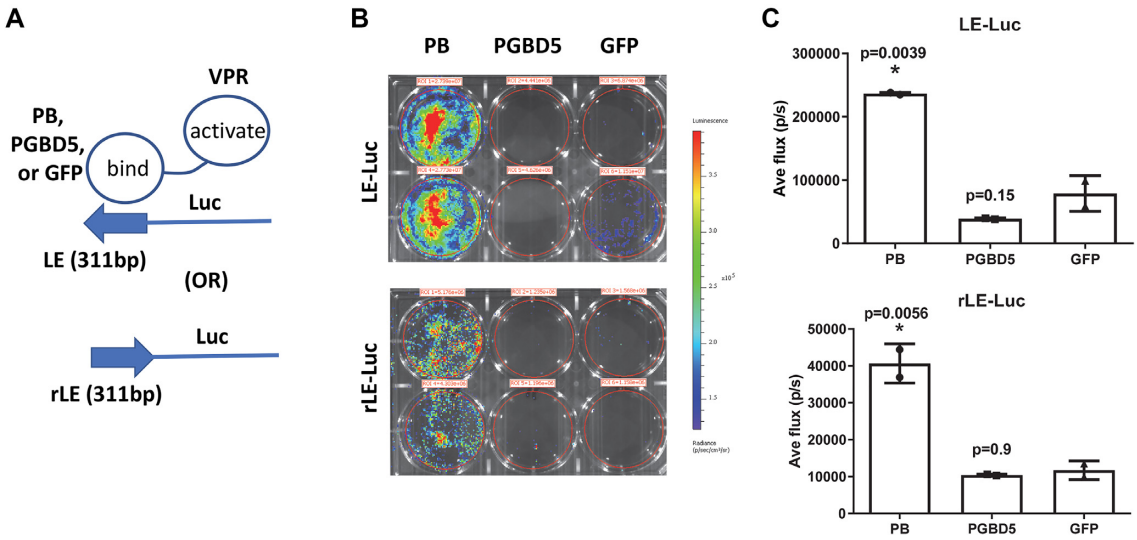

Figure 8. *piggyBac*, but not PGBD5, binds the *piggyBac* ITR using reporter readout of protein-DNA interaction in transfected cells. (**A**) Schematic demonstrating that transposase (PB (+ control), GFP (− control) or PGBD5 fused to a VPR activation domain. If binding to the LE (left end) or flipped LE (rLE, reverse LE) ITR occurs, luciferase is expressed and can be measured by IVIS imaging and quantified. (**B**) IVIS imaging of transfected cells. (**C**) Photons/s (p/s) readout of transposase ITR interaction; $N = 3 \pm$ SD. *, $P < 0.05$ compared to GFP control.

and plasmid rescue assays) or no difference from no transposase controls (colony count, qPCR of transposon copy number and ITR-binding analysis). Could PGBD5 work like a nuclease, and not a transposase with fidelity? It remains to be determined if this possible activity is due to overexpression of PGBD5 in heterologous cells. Nonetheless, we disagree with previous studies by Henssen *et al*. and reviewed by Ivics stating that PGBD5 is working like a bona fide *piggyBac* transposase (14,15).

We conclude that *piggyBac* family member transposition activity is species restricted to cognate ITR sequences, likely due to the high specificity of protein–DNA interactions. We also conclude that PGBD5 is incapable of mobilizing *piggyBac* transposons in a canonical manner. Our findings may have implications for not only application and therapeutic potential of *piggyBac* but also for PGBD5 in linkage to and underlying mechanisms of cancer that were based on PGBD5′s presumed ability to work as a transposase.

DATA AVAILABILITY

All primary data is available from the authors upon request.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

piggyBat transposase and transposon vectors were kindly provided by Dr Nancy Craig (Johns Hopkins University).

FUNDING

U.S. Department of Veterans Affairs [BX004528]; National Institute of Diabetes and Digestive and Kidney Diseases [DK093660]. Funding for open access charge: U.S. Department of Veterans Affairs [BX004528]; National Institute of Diabetes and Digestive and Kidney Diseases [DK093660].
Conflict of interest statement. None declared.

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