piggyBac transposase tools for genome engineering

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Edited by Richard A. Young, Massachusetts Institute of Technology, Cambridge, MA, and approved May 1, 2013 (received for review December 30, 2012)

The transposon *piggyBac* is being used increasingly for genetic studies. Here, we describe modified versions of *piggyBac* transposase that have potentially wide-ranging applications, such as reversible transgenesis and modified targeting of insertions. *piggyBac* is distinguished by its ability to excise precisely, restoring the donor site to its pretransposon state. This characteristic makes *piggyBac* useful for reversible transgenesis, a potentially valuable feature when generating induced pluripotent stem cells without permanent alterations to genomic sequence. To avoid further genome modification following *piggyBac* excision by reintegration, we generated an excision competent/integration defective (Exc^+Int^-) transposase. Our findings also suggest the position of a target DNA–transposase interaction. Another goal of genome engineering is to develop reagents that can guide transgenes to preferred genomic regions. Others have shown that *piggyBac* transposase can be active when fused to a heterologous DNA-binding domain. An Exc^+Int^- transposase, the intrinsic targeting of which is defective, might also be a useful intermediate in generating a transposase whose integration activity could be rescued and redirected by fusion to a site-specific DNA-binding domain. We show that fusion to two designed zinc finger proteins rescued the Int^- phenotype. Successful guided transgene integration into genomic DNA would have broad applications to gene therapy and molecular genetics. Thus, an Exc^+Int^- transposase is a potentially useful reagent for genome engineering and provides insight into the mechanism of transposase–target DNA interaction.

GULOP | ROSA26 | protein-DNA interaction | induced pluripotent stem cell production

DNA "cut-and-paste" transposable elements are important tools for genome engineering, such as insertional mutagenesis and transgenesis. Research with the DNA transposon *Sleeping Beauty*, a "resurrected" transposon, has pioneered the use of DNA transposons in mammalian cells (1, 2). *piggyBac* is also a DNA transposon and a promising alternative to *Sleeping Beauty*. *piggyBac*, originally isolated from the cabbage looper moth *Trichoplusia ni* genome (3), has a large cargo size (4), is highly active in many cell types, and mediates long-term expression in mammalian cells in vivo (5–10). *piggyBac* is also distinguished by its ability to excise precisely (11), thus restoring the donor site to its pretransposon insertion sequence.

Because it can excise precisely, *piggyBac* is especially useful if a transgene is only transiently required. Transient integration and expression of transcription factors are important approaches to generate transgene-free induced pluripotent stem cells (iPSCs) (12, 13) as well as directed differentiation of specific cell types for both research and clinical use. Removal of the transgenes is key for potential therapeutic applications of iPSCs. *piggyBac* has been used as a vector for reversible integration; however, reintegration of the transposon catalyzed by *piggyBac* (PB) transposase occurs in 40–50% of cells (14). To generate iPSCs without any genetic change, a PB mutant, which can promote only excision (Exc^+) and not integration (Int^-), would be a useful tool. Here we performed site-directed mutagenesis of the PB catalytic domain and isolated an excision competent/integration defective (Exc^+Int^-) PB. We also find that introduction of the Exc^+Int^- mutations into a hyperactive version of PB (5, 8, 15) yields an Exc^+Int^- transposase whose excision frequency is five- to six-fold higher than that of wild-type PB. We speculate that the changed amino acids in the Exc^+Int^- mutant are likely positions of interaction between the transposase and the target DNA.

Modification of nonviral vector systems to alter native integration patterns in a predictable manner would provide molecular biological tools with relevance to diverse applications, including therapeutics for genetic disease and ex vivo stem cell manipulation. *piggyBac* inserts at target sites with the sequence TTAA but displays little selectivity for particular regions of the genome other than a modest preference for regions of DNase I sensitivity (16, 17). A useful modification of PB would be to be able to guide integrations to safe harbor sites. Others have shown that PB is distinguished by its ability to remain active when fused to a DNA binding domain and that such fusions can bias insertion toward cognate sites (10, 16, 18). Notably, Wilson and colleagues (19) fused a zinc finger protein (ZFP) targeted to the upstream promoter region of the cell-cycle checkpoint kinase 2 protein-coding gene *CHEK2* to the native insect-derived *piggyBac* (iPB) transposase. The ZFP–iPB fusions exhibited comparable transposition activity to unmodified transposase and bound the target site in their study. However, no

Significance

DNA transposons that translocate by excision from a donor site and insertion into a target site are often used for genome engineering by insertional mutagenesis and transgenesis. The *piggyBac* element is especially useful because it can excise precisely from an insertion site, restoring the site to its pretransposon state. Precise excision is particularly useful when transient transgenesis is needed, for example, in the transient introduction of transcription factors for induced pluripotent stem cell production. We have used mutagenesis to generate an Excision$^+$ Integration$^-$ transposase that allows *piggyBac* excision without potentially harmful reintegration. These mutations likely lie in a target DNA-binding domain.

Author contributions: X.L., E.R.B., P.B.M., P.L.S., and N.L.C. designed research; X.L., E.R.B., and A.L.C. performed research; J.D.S. contributed new reagents/analytic tools; X.L., N.M., T.B., J.S., S.J.W., J.K.J., P.B.M., F.D.B., P.L.S., and N.L.C. analyzed data; and X.L., F.D.B., P.L.S., and N.L.C. wrote the paper.

Conflict of interest statement: J.K.J. has a financial interest in Transposagen Biopharmaceuticals. J.K.J.'s interests were reviewed and are managed by Massachusetts General Hospital and Partners HealthCare in accordance with their conflict of interest policies.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1305987110/-/DCSupplemental.

integration events were recovered at the native site in HEK293 cells, presumably due to the paucity of TTAA sites within the target region. Indeed, this hypothesis is supported with subsequent experiments demonstrating a significant increase in integration recovery near a *CHEK2* target site engineered with 12 TTAA sites. Stringent target-site selection in the genome has not been demonstrated with any transposon system, including *piggyBac* or *Sleeping Beauty* (20).

In this study we analyzed the effects of targeting by the Exc^+Int^- PB upon fusion to several ZFPs designed to recognize putative safe harbor regions. Importantly, we observed that N-terminal fusion of heterologous ZFPs rescued the integration defect. No significant targeting to the ZFP-binding sites, however, was observed. We conclude that an Exc^+Int^- *piggyBac* transposase provides a tool for use in genome engineering.

Results

Design and in Vivo Analysis of Exc^+Int^- Mutants. Like many transposase and integrase enzymes (21), the catalytic core of PB contains three aspartic acids that are necessary for catalytic activity: D268, D346, and D447 (22, 23) (Fig. 1). Interspersed near these required residues are 15 arginines and lysines that are highly conserved among many *piggyBac* proteins (22, 23) (Fig. S1). We hypothesized that a subset of these positively charged amino acids are involved in the transposase–target DNA interaction. To identify the contributions of these amino acids for transposition, we performed scanning alanine mutagenesis of the basic residues in the core domain, as well as of two additional residues (S351 and K356) (Fig. 1), which are comparable to positions in HIV-1 integrase where mutations alter HIV target joining (24).

The catalytic domain of a transposase has two functions: excision from the donor DNA and integration into the target DNA. As such, the alanine substitutions introduced into the catalytic domain may alter excision, integration, both, or neither. To discern the phenotype of the mutants, we assayed their excision activity by PCR amplification of repaired donor backbone in HEK293 cells by following cotransfection of (*i*) a donor plasmid, which carries a *piggyBac* transposon, and (*ii*) a plasmid expressing wild-type or mutant PB (Fig. 2*A*). As expected, a repaired plasmid fragment length of 278 bp was readily detectable with wild-type PB, indicating an excision positive (Exc^+) phenotype. The following mutants were Exc^+: R245A, R275A/R277A, R315A, R341A, S351E, K356E, R372A/K375A, R388A, K409A/K412A, and K432A (Fig. 2*B*). By contrast, several other mutants—K287A, K287A/K290A, and R460A/K461A—had less rejoined donor backbone, which might reflect decreased excision (Exc^-) and/or decreased donor backbone repair (Fig. 2*B*).

We next determined if the Exc^+ mutants retain the capacity to catalyze integration using a colony formation assay. A *piggyBac* transposon expressing a blasticidin resistance gene was cotransfected into HeLa cells along with a wild-type or mutant PB expression plasmid. Subsequent blasticidin resistance of the HeLa cells indicates that the transposase is active for integration. Transfection of wild-type transposase and the transposase mutants R315A, R341A, and K356E each resulted in abundant blasticidin-resistant colonies, indicating that they are integration competent (Int^+) (Fig. 2*C*). The single mutants R372A or K375A are also Int^+ (Fig. S2). In contrast, transposase mutants R245A, R275A/R277A, S351E, R372A/K375A, and R388A had low integration activity similar to the no transposase (−Tps) control (Fig. 2*C*), indicating that they are integration-defective (Int^-). Thus, these five mutants are categorized as candidate Exc^+Int^- mutants.

In Vitro Analysis of the Exc^+Int^- Candidates. To further analyze the PB Exc^+Int^- candidates, we assayed their transposition activities in a cell-free assay using transposase purified from *Escherichia coli* (22). *piggyBac* transposition, starting with excision, initiates with a nick at the 3′ end of the transposon, followed by the attack of the newly exposed end on the complementary strand, four nucleotides within the donor DNA that releases the flanking donor DNA and a TTAA hairpin-containing transposon end. This hairpin is then opened by the transposase, re-exposing the 3′OH transposon end that attacks the target DNA and leaving TTAA attached to the 5′ transposon end.

To analyze double-strand breaks at the ends of the transposon, we end-labeled a linear substrate DNA containing a *piggyBac* element. Cleavage at the transposon ends releases the labeled DNA fragments that flank the transposon in the donor site (Fig. 3*A*). This assay reveals that mutant PBs R245A, R275A/R277A, R341A, and R372A/K375A promote double-strand breaks at the transposon ends at levels similar to wild-type PB; i.e., they are Exc^+ (Fig. 3*B*). In contrast, the excision activity of the transposase mutants R388A and S351E was markedly reduced.

The hairpin opening and target-joining activity of the purified Exc^+Int^- candidates was determined by analysis of an end-labeled *piggyBac* end hairpin (Fig. 3*C*). Upon incubation with transposase, the hairpin is opened and the transposon ends can join to a target plasmid DNA. We find that mutants R245A, R275A/R277A, R341A, and R372A/K375A open terminal hairpins with activity comparable to wild-type PB (Fig. 3*D*). Mutant transposases S351E and R388A, however, had diminished hairpin opening and target-joining activity. Only one mutant among the Exc^+ and hairpin opening-competent transposases, R372A/K375A, was defective in target joining. These results suggest that the PB mutant R372A/K375A ($PB^{R372A/K375A}$) was the best candidate for focused studies that separate the excision and integration transposase functions.

Analysis of $PB^{R372A/K375A}$ Transposase in Mammalian Cells. To quantitatively characterize $PB^{R372A/K375A}$ transposition in cells, we assayed the excision activity in a genetically manipulated HEK293 cell line (8). Briefly, *Tol2* transposition was used to stably introduce a gene cassette carrying a cycle 3 GFP gene (Invitrogen) interrupted by a *piggyBac* transposon, termed *GFP::PB*, into the genome. Following transient transfection of a plasmid expressing PB, the *piggyBac* transposon is precisely excised, the *GFP* gene is restored, and the resulting GFP signal can be measured by FACS analysis. We found that the excision activity of $PB^{R372A/K375A}$ is about 30–50% that of wild-type PB (Fig. 4). This observation prompted us to search for "suppressor" (i.e., excision-hyperactive) mutations that would increase the excision activity of $PB^{R372A/K375A}$ but maintain its Int^- phenotype.

Fig. 1. Catalytic core amino acids selected for mutagenesis. The catalytic core of PB contains 3 requisite aspartic acids (blue). Fifteen conserved arginines and lysines (red) and two conserved amino acids important for HIV integration (green), which are targets of mutagenesis, are indicated.

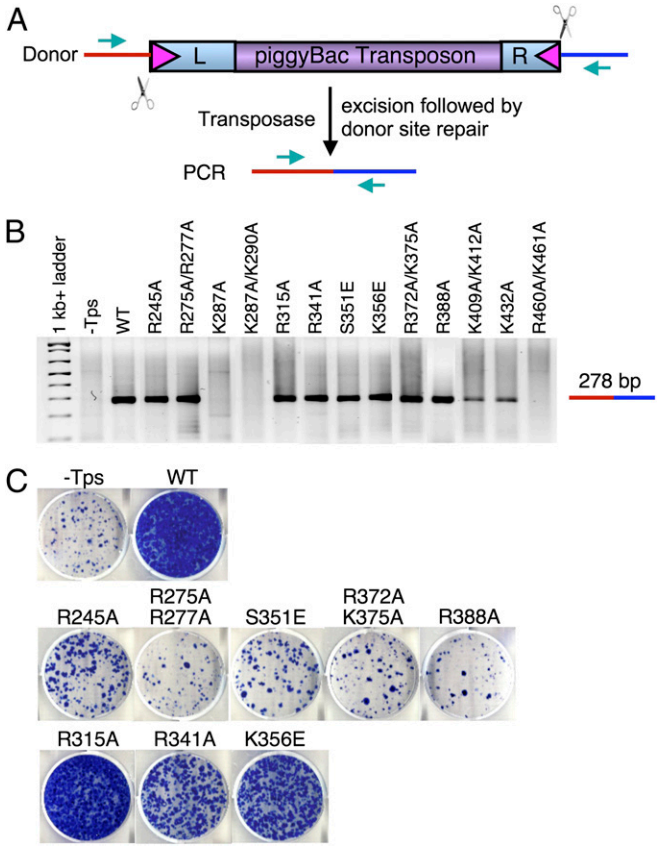

Fig. 2. Excision and integration assays for screening mutant *piggyBac* transposases in mammalian cells. (*A*) Schematic of transposase-catalyzed transposon excision from a donor plasmid followed by donor-site repair. PCR product of 278 bp indicates precise excision from the donor and appropriate repair. (*B*) HEK293 cells were transiently transfected with both the transposon donor plasmid and the indicated mutant PB plasmids. A no-transposase (–Tps) negative control and a wild-type PB (WT) positive control are indicated. Cells were lysed, plasmids were recovered, and PCR was performed using the indicated primers to detect donor plasmid from which the transposon had been excised and the backbone repaired. (*C*) For colony formation assays, HeLa cells were transiently cotransfected with a transposon expressing blasticidinR and the indicated mutant PB. Cells were selected for blasticidin resistance and stained with methylene blue to identify viable cell colonies.

Screening in *Saccharomyces cerevisiae* for $PB^{R372A/K375A}$ Mutants That Are Hyperactive for Excision. We previously reported that *piggyBac* transposes efficiently in *S. cerevisiae* (22). We used this genetically tractable system to isolate excision-hyperactive mutants of $PB^{R372A/K375A}$. Similarly to the HEK293 *GFP::PB* assay described above, we used a fluorescence assay in yeast, in which PB promotes the precise excision of a *piggyBac* transposon from a fluorescent reporter gene mCherry, termed mCherry::*PB* (8). In this system, colonies are screened for increased mCherry expression to reflect increased excision. As expected, the –Tps negative control did not result in increased fluorescence, whereas mCherry-positive yeast were readily observed in the presence of wild-type PB (Fig. 4*A*).

Consistent with the observations in mammalian cells, the excision promoted by $PB^{R372A/K375A}$ in *S. cerevisiae* was reduced compared with wild-type PB (Fig. 4*A*). To identify mutations that would increase excision activity, we generated a $PB^{R372A/K375A}$ mutant library by error-prone PCR mutagenesis and introduced it into the mCherry::*PB* strain by homologous recombination with a gapped p*GALS* PB plasmid. After transformation, single colonies were pin-replicated to SC (synthetic complete)–Trp–Ura+galactose plates and grown for 3 d, and mCherry fluorescence was analyzed using a Typhoon scanner or a fluorescence microscope.

We screened ~6,000 colonies from three separate mutagenesis libraries and isolated about 30 hyperactive candidates that showed increased fluorescence relative to $PB^{R372A/K375A}$, some of which were indeed hyperactive upon retesting (L15P, D19N, E45G, S103P*, R189K, M194V*, N384T, M413V, D450N, T560A, M589V, S17G/K102E, S31P/T164A, H33Y/N571S*, E44K/K334R, C97R/T242I; Fig. 4*A*). A subset of these mutations (indicated by *) was also isolated in our previously reported screen for increased excision hyperactives of PB in yeast (15). We then measured excision activity of the $PB^{R372A/K375A}$ mutants in mammalian HEK293 *GFP::PB* cells using the GFP excision assay (Fig. 4*B*). We found the highest excision activity with the single amino acid changes M194V or D450N. Notably, M194V was previously identified in our yeast screen for hyperactive mutants of wild-type PB although it was not hyperactive in mammalian cells (15). D450 is near D447, one of the requisite catalytic core amino acids (22, 23), and is highly conserved among *piggyBac* proteins (Fig. S1).

Characterization of $PB^{M194V/R372A/K375A}$ and $PB^{R372A/K375A/D450N}$ in Vitro. To confirm the increased excision activity and diminished integration activity of $PB^{M194V/R372A/K375A}$ and $PB^{R372A/K375A/D450N}$, we purified transposase protein from *E. coli* and examined the double-strand break excision activity (Fig. 5*A*) and hairpin opening/target-joining activity (Fig. 5*B*) as described above. Both $PB^{M194V/R372A/K375A}$ and $PB^{R372A/K375A/D450N}$ had excision (Fig. 5*A*) and hairpin opening (Fig. 5*B*) activity at levels similar to or higher than wild type or the hyperactive mutant iPB7. iPB7 differs from wild type at seven amino acid positions (5, 8, 15). Both mutants $PB^{M194V/R372A/K375A}$ and $PB^{R372A/K375A/D450N}$ had diminished target joining, suggesting that the M194V or D450N mutations did not revert to the integration-defective phenotype (Fig. 5*B*). These data support the view that $PB^{M194V/R372A/K375A}$ and $PB^{R372A/K375A/D450N}$ are hyperactive for excision but are integration-defective.

Excision and Integration Activities of $PB^{M194V/R372A/K375A}$ and $PB^{R372A/K375A/D450N}$ in Mammalian Cells. Using $PB^{M194V/R372A/K375A}$ and $PB^{R372A/K375A/D450N}$, we performed *GFP::PB* excision assays in HEK293 cells, screening for increased GFP as described above. Both $PB^{M194V/R372A/K375A}$ and $PB^{R372A/K375A/D450N}$ had excision activities greater than $PB^{R372A/K375A}$ and similar to wild-type PB (Fig. 6*A*). Combining the M194V and D450N mutations as in $PB^{M194V/R372A/K375A/D450N}$ did not lead to any further increase in activity; rather, the frequency was decreased to below that of $PB^{R372A/K375A}$.

When the M194V/R372A/K375A and R372A/K375A/D450N mutations were introduced into the hyperactive iPB7 mutant background, excision activities for all mutants increased, and the effect of the hyperactive M194V and D450N mutations was more pronounced (approximately five- to sixfold) (Fig. 6*A*). Combination of the M194V and D450N mutations in the $iPB7^{R372A/K375A}$ backbone, however, resulted in excision activities decreasing to wild-type PB levels.

To confirm that the M194V and D450N mutations did not revert the integration defect conferred by the R372A/K375A mutations in the iPB7 background, we analyzed their activity using a colony formation assay in HeLa cells as described above. We observed that $iPB7^{M194V/R372A/K375A}$ and $iPB7^{R372A/K375A/D450N}$ had very low integration activity, similar to that of the –Tps control (Fig. 6*B*). Thus, they are iPB7 $Exc^{+}Int^{-}$ mutants that are hyperactive for excision but defective for integration, i.e., iPB7 $Exc^{+hyper}Int^{-}$ transposases. Paradoxically, the M194V and D450N mutations increase the excision activity of $PB^{R372A/K375A}$ and $iPB7^{R372A/K375A}$ yet decrease the excision activity of PB and iPB7 in mammalian cells (Fig. S3).

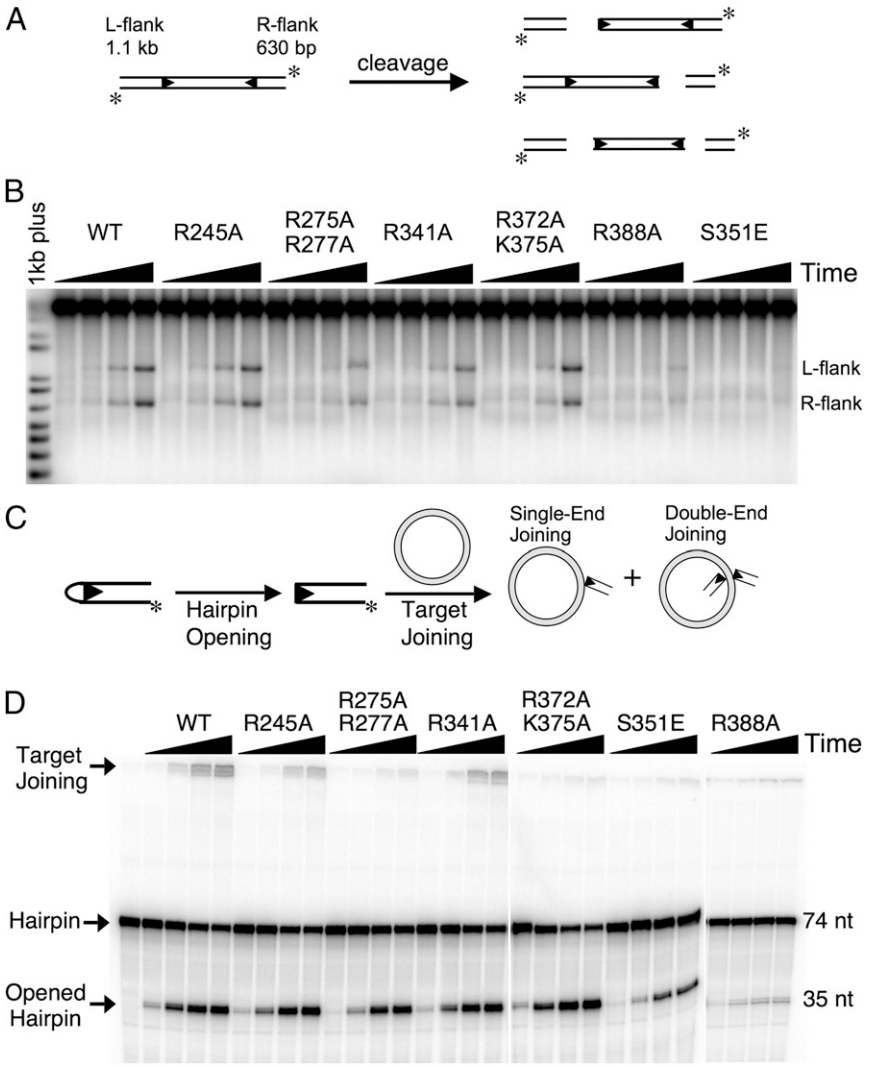

Fig. 3. In vitro cleavage, hairpin opening, and target-joining assays of mutant *piggyBac* transposases. (*A*) Linearized transposon donor plasmid DNA was end-labeled (*) at both the 3′ end of the left (L) and right (R) end flanking sides. Cleavage at the appropriate *piggyBac* transposon terminal repeat (triangles) sites results in products of the indicated sizes: left flank cleaved (1.1 kb), right flank cleaved (630 bp), or both flanks cleaved. (*B*) Linearized donor plasmid DNA was incubated with the indicated wild-type or mutant PB for increasing amounts of time (1, 5, 25, and 125 min). The cleavage events of the L-end, R-end, or both ends are discerned by electrophoresis on an agarose gel. (*C*) Schematic representation of the hairpin opening and target-joining assay. A 74-nt hairpin DNA containing 35 bp of the *piggyBac* left end including the 13-bp terminal inverted repeat (triangle) and the TTAA hairpin was end-labeled (*) at the 5′ end. Appropriate cleavage of the 4-bp terminal hairpin results in a 35-nt opened hairpin product on a denaturing gel. Incubation of the opened hairpin with PB and a circular DNA target 2.7-kb plasmid results in single-end or double-end joining as indicated. (*D*) End-labeled hairpin DNA was incubated with the indicated wild-type or mutant PB for increasing amounts of time (1, 3, 10, and 20 min), and products were discerned by electrophoresis on a denaturing gel.

Exc$^+$Int$^-$ *piggyBac* Transposases Promote Precise Excision. One reason for isolating Exc$^+$Int$^-$ PBs was to avoid element reintegration during transient transgenesis. Another goal for transient transgenesis is restoration of the transposon donor site to its original pretransposon sequence. We evaluated precise excision by the Exc$^+$Int$^-$ transposases in yeast by determining the frequency at which the transposases promoted imprecise excision from the *CAN1* gene, the arginine transporter. If cells are *CAN1*, they are sensitive to the arginine analog canavanine. Inactivation of *CAN1* results in canavanine resistance (Can^R). We found that, following transposon excision, the imprecise excision frequencies of the Exc$^+$Int$^-$ mutants scored by measuring the frequency of Can^R upon excision in yeast are less than 0.3%, similar to wild-type PB (Table S1).

Fusion of iPB7 and iPB7 Exc^{+hyper}Int$^-$ Mutants to Designer Zinc Finger Proteins That Bind to Putative Safe Harbor Regions of the Human Genome. Another motivation for generating the Exc$^+$Int$^-$ PBs was to ask if their integration activity could be restored and redirected to selected genomic regions by fusion of designed ZFPs with engineered specificities to the transposase. We chose to fuse the Exc^{+hyper}Int$^-$ iPB7s to ZFPs designed to target binding sites in putative safe harbor loci, the human *ROSA26* gene, or the human L-gulono-γ-lactone oxidase (*GULOP*) pseudogene. Construction and analysis of the binding activity of the ROSA26 and GULOP ZFPs is described in Figs. S4–S6 in *SI Materials and Methods*.

We made fusion proteins linking the ROSA26 or GULOP ZFPs with a 15-amino-acid linker to the N terminus of iPB7, the Exc^{+hyper}Int$^-$ transposase iPB7$^{R372A/K375A/D450N}$, and iPB7$^{R372A/D450N}$, a transposase lacking the K375A mutation that was recovered during plasmid construction (Fig. 7). We used colony formation assays to test the integration activity of our chimeric transposases. Cotransfection into HeLa cells of the GULOP–iPB7 and the ROSA26–iPB7 fusion constructs with a transposon plasmid containing a puromycin resistance selectable marker resulted in about

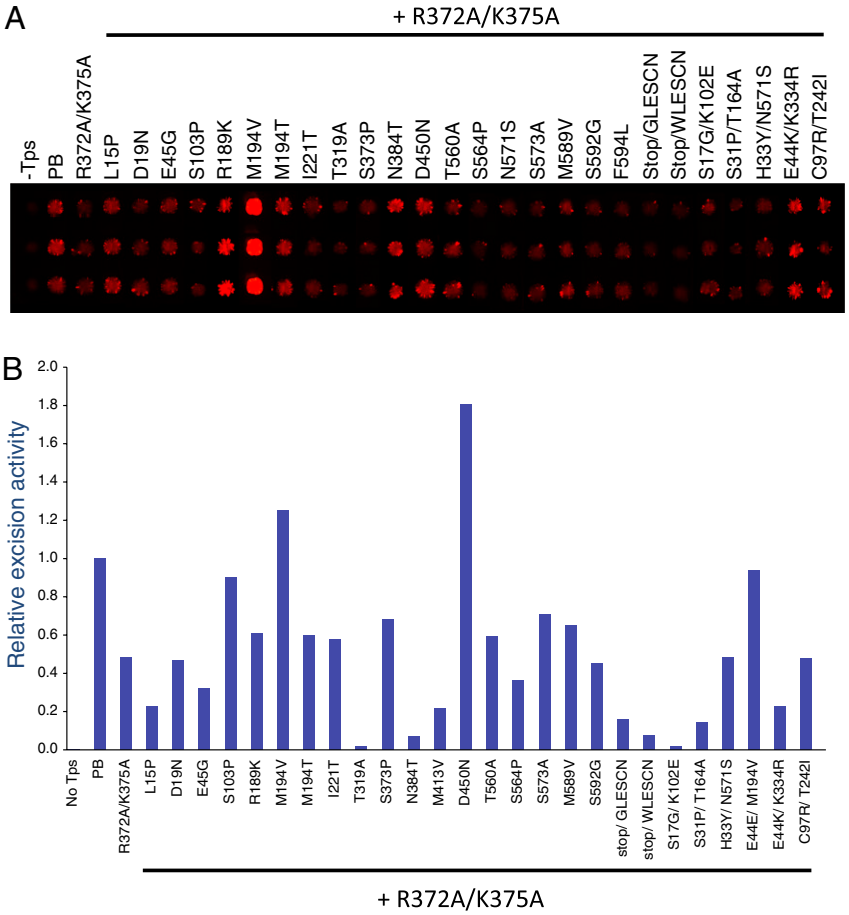

Fig. 4. Mutagenesis screen for hyperactive excision variants of $PB^{R372A/K375A}$ in yeast and mammalian cells. (*A*) Error-prone PCR was used to generate $PB^{R372A/K375A}$ transposase variants. The *piggyBac* transposon donor plasmid, mCherry::*PB-TnKanMX/URA3*, and the $PB^{R372A/K375A}$ transposase variant library were introduced to yeast and grown on selective media. mCherry fluorescence intensity indicates relative precise excision frequency. The corresponding genotypes and phenotypes of the potential hyperactive excision variants are indicated. (*B*) Promising variants identified in the yeast-based excision assay were retested in a mammalian cell-based excision assay. The indicated $PB^{R372A/K375A}$ variants were transiently transfected into HEK293 *GFP::PB* cells. The precise excision of the *piggyBac* transposon from the genome results in a repaired GFP expression cassette. The frequency of excision is indicated by GFP fluorescence intensity and determined by FACS analysis. No transposase (–Tps), wild-type PB, and $PB^{R372A/K375A}$ transposase were included as controls in both assays.

80–100% of the number of drug-resistant colonies compared with those of the unmodified iPB7 control. Fusion of the GULOP ZFP to the $Exc^{+hyper}Int^{-}$ transposase $iPB7^{R372A/K375A/D450N}$ did not restore integration activity.

Notably, however, we did see restoration of integration activity with a ZFP fusion in two cases. The integration activity of $iPB7^{R372A/D450N}$, a transposase lacking the K375A mutation was less than 10% of iPB7. Fusion of both the ROSA26 and GULOP ZFPs to this integration-defective transposase mutant $iPB7^{R372A/D450N}$ did restore integration activity (Fig. 7). These results support the hypothesis that engineered ZFPs fused to the $iPB7^{R372A/D450N}$ can restore the DNA target-binding function. Comparison of the integration patterns of the ZFP fusion and the ZFP fusion transposases (Table S2 and Fig. S7) did not, however, reveal discernible differences.

Discussion

Several properties make *piggyBac* a particularly useful tool for genetic manipulation. It is active in many cell types, including mammalian cells (9, 14, 25), and is distinguished by its ability to excise precisely (11), restoring the donor site to its preinsertion sequence. In addition, PB and iPB7 remain active when fused to heterologous DNA-binding domains. We have manipulated the target recognition and joining properties of the PB to create a $Exc^{+hyper}Int^{-}$ PB that probes the *piggyBac* transposition mechanism and is a useful tool for genome engineering.

Here, we have isolated mutants that will be valuable for reversible transgenesis. Several highly conserved acidic residues (D268, D346, and D467) in PB are essential for DNA breakage and joining (22, 23). We previously suggested that they lie on an RNase H-like fold as is found in other transposases and retroviral integrases (21). Such active-site domains must also contain amino acids that interact with the DNA substrates of transposition. As a strategy to identify amino acids that interact with the target DNA, we substituted highly conserved basic amino acids in the catalytic domain to alanine. We also mutated two other amino acids that correspond to residues in HIV integrase that influence target joining (24). Most of these mutations had no effect on transposition or resulted in the loss of both excision and integration activity. However, one mutant, $PB^{R372A/K375A}$, had only slightly decreased excision activity but was highly defective in integration in mammalian cells. These amino acids are good candidates for interacting with target DNA, and future studies of their mechanism will provide insights into transposase structure and function.

We used two strategies to improve the modestly decreased excision activity of the integration-defective $PB^{R372A/K375A}$ mutant. First, we used a screen in yeast to identify two additional mutations, M194V and D450N, that each increased the excision

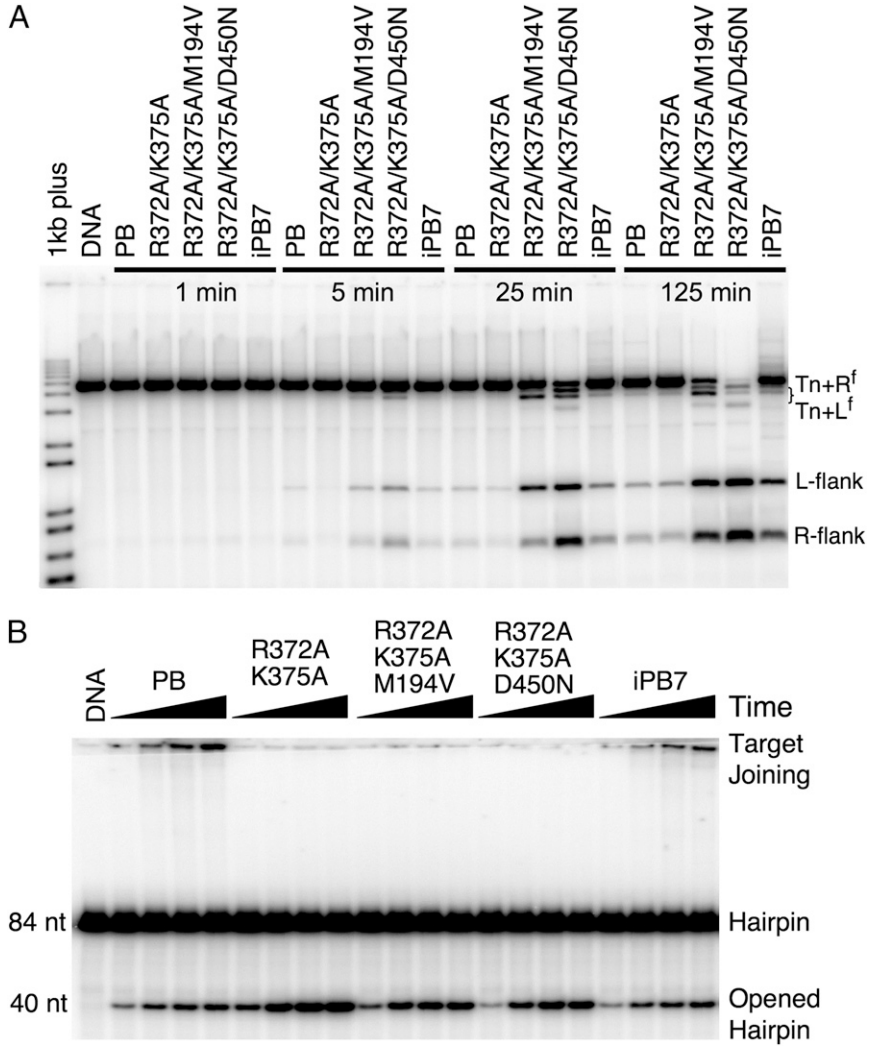

Fig. 5. In vitro cleavage, hairpin-opening, and target-joining assays of Exc^{+hyper}Int^{-} mutant transposases. (*A*) Linearized donor plasmid DNA was end-labeled and incubated with the indicated transposase mutant for increasing amounts of time (1, 5, 25, or 125 min) as shown. The cleavage events of the L-end, R-end, or both ends are discerned by electrophoresis on an agarose gel. The single cleavage products—transposon plus the right flank (Tn+R^{f}) and transposon plus the left flank (Tn+L^{f})—are indicated. (*B*) A 84-nt hairpin DNA containing 40 bp of the *piggyBac* transposon left end including the 13-bp terminal inverted repeat (triangles) and the TTAA hairpin was end-labeled at the 5′ end. Labeled hairpin was incubated with the indicated PB mutant and a circular DNA target for increasing amounts of time (1, 5, 25, and 125 min), and the products were discerned by electrophoresis on a denaturing gel. Appropriate cleavage of the 4-bp terminal hairpin results in a 40-nt opened hairpin fragment and target joining to pUC19 (2.7 kb). Wild-type PB, PB$^{R372A/K375A}$, and iPB7 were included as controls in both assays.

activity of PB$^{R372A/K375A}$ to levels above the excision frequency of wild-type PB. We also tested the effect of the M194V/R372A/K375A and R372A/K375A/D450N mutations on a hyperactive version of the transposase (iPB7) (5, 8, 15) that contains seven changed amino acids. We found that iPB7$^{M194V/R372A/K375A}$ and iPB7$^{R372A/K375A/D450N}$ had increased excision activity but were still integration-defective.

The mechanism by which the M194V and D450N mutations suppress the excision defect imposed by R352A/K375A remains to be determined. M194V is located in the conserved N terminus of PB, upstream of the catalytic domain, the function of which is not yet known (23). In a screen for hyperactive PB mutants in yeast, we also isolated a M194V mutant but found that it is not hyperactive in mammalian cells (15). D450 is highly conserved among *piggyBac*s (22, 23) and is closely positioned to D447, which is essential for DNA breakage and joining and is part of the RNase H-based motif of conserved acidic amino acids.

One reason to isolate the Exc^{+hyper}Int^{-} PB was to facilitate reversible transgenesis without harmful transposon reintegration. Another was to facilitate construction of transposases that direct insertion to chosen specific sites. Studies show that the *Sleeping Beauty* transposase is not very amenable to this strategy; for example, addition of a DNA-binding domain either ablates or greatly diminishes *Sleeping Beauty* transposase activity (10). Hyperactive *Sleeping Beauty* enzymes can be active as a fusion ZFP transposase; however, precise genomic site-directed integration has not been achieved using such methods (20). PB is still highly active when fused to a heterologous DNA-binding domain at its amino terminus (10). We speculated that decreasing the intrinsic targeting activity would make target-site selection more dependent upon a heterologous DNA-binding domain in a fusion protein.

Thus, we fused the Exc^{+hyper}Int^{-} PB to two designed ZFPs that bind to the human GULOP and ROSA26 region that we hypothesized would be safe harbors for PB integration. Despite the significant increase in the frequency of integration with GULOP-

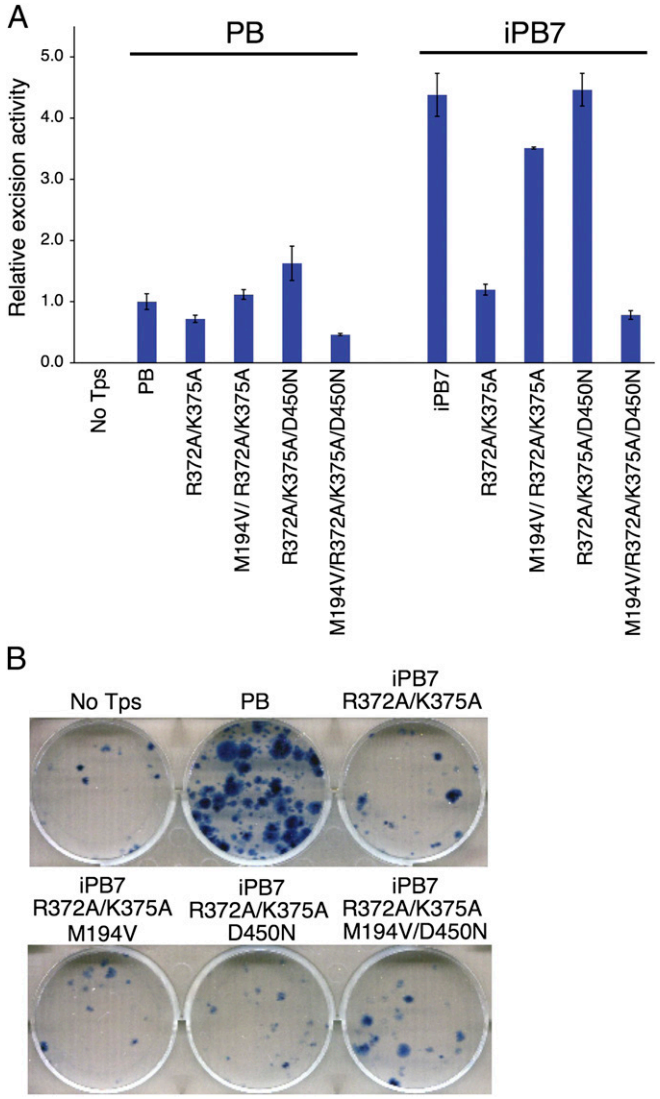

Fig. 6. Excision and colony formation assays of $Exc^{+hyper}Int^{-}$ mutant transposases. (*A*) The indicated wild-type *piggyBac* (*Left*) or iPB7 (*Right*) transposase mutants were transiently transfected into HEK293 GFP::PB cells. The frequency of excision is indicated by GFP fluorescence intensity, determined by FACS analysis, and normalized to the wild-type PB control. –Tps and $PB^{R372A/K375A}$ transposase were included as controls. (*B*) For colony formation assays, HeLa cells were transiently cotransfected with a transposon expressing $blasticidin^{r}$ and the indicated mutant iPB7. Cells were selected for blasticidin resistance and stained with methylene blue to identify viable cell colonies.

$iPB7^{R372A/D450N}$ and ROSA26-$iPB7^{R372A/D450N}$ compared with $iPB7^{R372A/D450N}$, there was no change in the genome-wide patterns of element insertion nor did we observe increased integration near the *ROSA26* and *GULOP* target sites. It is possible that the DNA target-site binding activity of PB engages additional domains in the protein. DNA targeting activity may also be mediated by a zinc finger Really Interesting New Gene (RING) domain located in the C terminus of PB (22, 23). Efficient site-directed targeting using engineered ZFP–PB fusions may require additional modifications to the transposase. Perhaps alteration of the C terminus may aid in this pursuit. The finding that fusion of the heterologous DNA-binding domain suppresses the integration defect of $iPB7^{R372A/D450N}$ suggests that $Exc^{+}Int^{-}$ PBs are good platforms for continued studies.

Unlike other known DNA transposons, PB excises precisely, restoring the donor site to its pretransposon sequence (11) and allowing reversible transgenesis (12, 15, 26). Thus, *piggyBac* can be exceptionally useful in generating iPSCs without a permanent DNA sequence change. The genes for the necessary transcription factors for iPSC generation are integrated into the target genome using a *piggyBac* transposon as the vector that can serve as a long-term source of transcription factors. Once iPSC cell transformation has occurred, however, the *piggyBac* vector can then be re-exposed to transposase and excised by its natural "precise excision" pathway, restoring the genome to its pretransposon state. One potential hazard, however, is that the excised transposon may reintegrate. Wild-type PB mediates reintegration about 40–60% of the time after excision (14) and thus is a significant hazard. Such reintegration, however, will not occur with $Exc^{+hyper}Int^{-}$ transposase, proving a safer pathway for genome engineering.

Combining the technologies of ZFP engineering and transposase enzymatic biology presents an opportunity to create tools for genomic engineering. These tools have the potential to improve the safety and utility of delivery vehicles for therapeutic genes, both in vivo and ex vivo.

Materials and Methods

***piggyBac* Transposase Mammalian Expression Vector Constructs.** The *piggyBac* transposase helper plasmid pXL-PB-EI for mammalian expression contains a Flag-tag fused to the N terminus of PB in pcDNA3.1/myc-HisA (Invitrogen). The *piggyBac* ORF was amplified using the primer *N*-Flag-PB-5f [GC*GA-ATTC*GCCACCATGGACTACAAGGACGACGATGACAAG atgggtagttctttagacgatgag (EcoRI, italic; GCCACC, Kozak sequence; Flag tag, uppercase; *piggyBac*, lowercase)] and primer PB-4r [CAG*GCGGCCGC*tcagaaacaactttggcacatatcaatattatg (NotI, italic; *piggyBac*, lowercase)], digested with EcoRI and NotI, and cloned into EcoRI- and NotI-digested pcDNA3.1/myc-HisA vector. The initial $Exc^{+}Int^{-}$ mutant candidates were obtained by site-directed mutagenesis. All hyperactive excision candidates obtained by screening in yeast plasmids were recloned into pcDNA3.1/myc-HisA for testing in mammalian cells. The PB ORFS were PCR amplified with primers PB-f GA*GGTACC*GCCACCatgggtagttctttagacgatgag (KpnI, Italic; *piggyBac*, lower case) and PB-4r, digested by KpnI and NotI and cloned into the plasmid digested with KpnI and NotI.

Mammalian Transposon Donor Plasmid. The transposon donor plasmid pXL-*PB*-D-*GFP*/BlasticidinR (Bsd) containing a transposon used for excision and integration assays of the $Exc^{+}Int^{-}$ candidates, as well as the PB integration libraries, has been previously described (13, 17).

Mammalian Cell Culture. HEK293 and HeLa cells were cultured in DMEM supplemented with 5% (vol/vol) FBS.

Donor Repair Excision Assay in Mammalian Cells. HEK293 cells (2×10^6) were transfected with 8 μg transposon donor plasmid pXL-*PB*-D-*GFP*/*Bsd* and 2 μg *piggyBac* transposase helper plasmid pXL-PB-EI or the $Exc^{+}Int^{-}$ candidate mutants with FuGENE 6 transfection reagent (Roche Applied Science). Three days later, cells were harvested and the helper, donor, and repaired donor plasmids were recovered by the Hirt method (27). The plasmid DNA was used as a PCR template with two primers against the DNA sequence flanking the transposon in the donor plasmids pbexl2 (GGAACAGGAGAGCGCACGAG) and pbexr2 (GAGAGTGCACCATATATGCGGTG), and the products were displayed on a 1% agarose gel in 1× tris/borate/EDTA buffer (TBE).

Mammalian Colony Formation Assay for Integration. HeLa cells ($3–5 \times 10^5$) were transfected with 1.6 μg transposon donor plasmid pXL-*PB*-D-*GFP*/*Bsd* and 0.4 μg pXL-PB-EI helper PB and $Exc^{+}Int^{-}$ mutant transposase plasmids with FuGENE 6 transfection reagent (Roche Applied Science). Two days later, cells were trypsinized and diluted, and growth continued in DMEM containing 3.5 μg/mL of blasticidin for 18–21 d. The medium was changed every 2–3 d. Surviving colonies were fixed with 4% (wt/vol) paraformaldehyde, stained with 0.2% methylene blue, and counted.

***piggyBac* Transposase Purification.** PB wild type and selected $Exc^{+}Int^{-}$ mutant transposase proteins were expressed in *E. coli* BL21 codon plus/DE3 RIL (Stratagene) using the pET22b vector (Novagen) with a C-terminal His-tag and purified by chromatography on a Ni^{2+} column as previously described (22).

In Vitro Cleavage of Linear Donor Plasmid DNA. The donor plasmid pXL-*PB*-D-*GFP*/*Bsd* was linearized by digestion with Psp1046I and 3′ end-labeled with

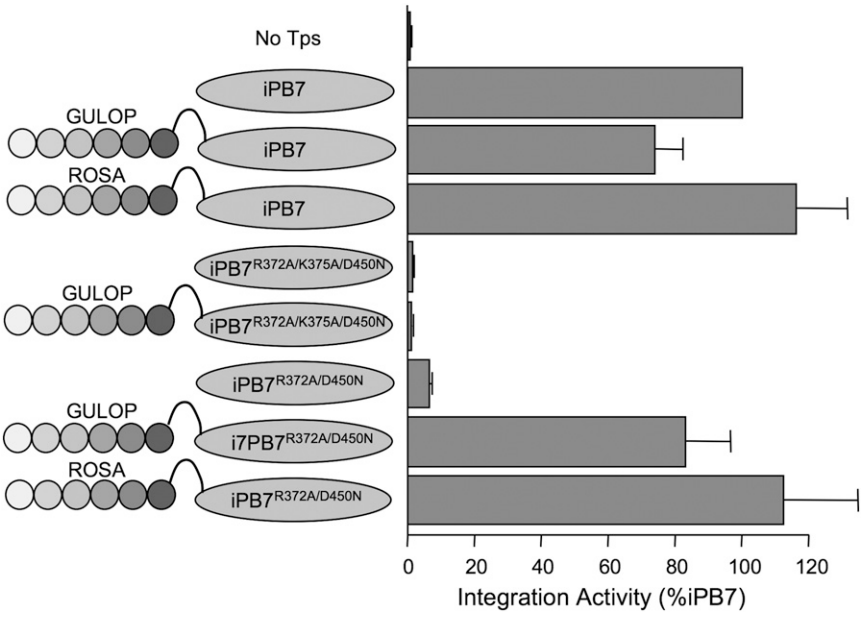

Fig. 7. Colony formation assays of ZFP–transposase fusions. Schematic representations of the iPB7 transposase and ZFP–iPB7 fusion constructs are shown. Six-finger ZFPs (circles) designed to bind genomic sites in human *ROSA26* or *GULOP* were fused to the N terminus of iPB7. A *piggyBac* transposon plasmid carrying the puromycin resistance gene was cotransfected into HeLa cells with the indicated transposase construct. Cells were selected for 10–14 d with 0.5 mg/mL puromycin, stained with methylene blue, counted, and normalized to the unmodified iPB7 control.

[α^{32}P]dCTP. Ten-microliter reactions were performed as previously described (22) and stopped at 1, 5, 25, and 125 min by addition of 500 mM EDTA to 40 mM; 10% (wt/vol) SDS was added to a final concentration of 1.2% (wt/vol), and the reactions were incubated at 65 °C for 20 min. Samples were displayed on a 1% (wt/vol) agarose gel in 1× TBE. The gel was dried, exposed to a PhosphorImager screen, and analyzed by Imagequant software.

In Vitro Hairpin Opening and Strand Transfer. Hairpin-forming oligonucleotides (74 or 84 nt) containing *piggyBac* left end sequences with a central TTAA were synthesized (Integrated DNA Technologoes), PAGE gel-purified, and labeled at the 5′ end with [γ-^{32}P]ATP. Ten-microliter reactions were performed as previously described (22), stopped at times indicated in the figure legends by addition of 500 mM EDTA to 40 mM. Samples were spin-dried, resuspended in 2× (vol/vol) STOP solution (United States Biologicals), boiled for 5 min, chilled on ice, and displayed on a 16% acrylamide–7 M urea–1× TBE denaturing acrylamide gel. The gel was dried, exposed to a PhosphorImager screen, scanned on a PhosphorImager (GE Healthcare), and analyzed by Imagequant software.

Excision Assay in Yeast Using a Fluorescent Reporter. This assay was previously described by Burnight et al. (8). Briefly, the transposon donor plasmid mCherry::*PB-TnKanMX* with *URA*3 on the plasmid backbone does not express the mCherry fluorescent protein because of the *piggyBac* transposon insertion. Precise excision of the *piggyBac* element restores the mCherry ORF, leading to colony fluorescence that can be detected with a Typhoon scanner or a fluorescence microscope (Leica; M165FC). In this work, the *piggyBac* element was *PB-TnKanMX*. The yeast strain was BY4727 (28) *MAT***a** *his3Δ200 leu2Δ*O *lys2Δ*O *met5Δ*O *trp1Δ63 ura3Δ*O. The wild-type and R372A/K375A mutants of *piggyBac* transposase ORFs were cloned into the XmaI and XhoI sites of the p414*GALS* vector (29).

Isolation of Hyperactive Derivatives of the Reduced Excision Activity of PB$^{R372A/K375A}$. We mutagenized the PB$^{R372A/K375A}$ ORF by error-prone PCR in the presence of Mn^{2+} with primers from the p414*GALS* promoter and *CYC1* terminator region, using p414*GALS* PB$^{R372A/K375A}$ as template. The resulting library was introduced into a linearized p414*GALS TRP1* vector digested with XmaI and XhoI by homologous recombination in yeast BY4727 containing the mCherry::*PB-TnKanMX URA3* donor plasmid. Transformant colonies were selected on synthetic complete (SC) plates lacking tryptophan and uracil (SC–Trp–Ura).

To identify Exc^{+}Int^{-} mutants, more than 6,000 individual transformants were picked in 96-well SC–Trp–Ura + 2% (wt/vol) galactose plates. We scored transposition by looking for increased fluorescence over 1–3 d, and 30 hyperactive candidates were found in the primary screen. Yeast plasmid DNAs were isolated, and the mutant PB$^{R372A/K375A}$ ORFs were sequenced and reassayed, yielding M194V and D450N as the best suppressors.

Mammalian *GFP* Excision Assay. This system was previously described by Burnight et al. (8). Briefly, a *GFP* gene containing the *piggyBac* transposon *GFP*::*PB* was introduced into the genome of HEK293 cells. No GFP is detectable because of the *piggyBac* insertion in *GFP*. Excision occurs in the presence of transposase introduced by transfection with wild-type or various mutant plasmids and FuGENE 6 transfection reagent. Four days later, cells were harvested and FACS-analyzed with FACSCalibur (BD Biosciences) to measure the percentage of GFP^{+} cells in the entire cell population. Data were analyzed with FlowJo 8.5.3. GFP^{+} cells were counted against total cells, and the number of GFP^{+} cells from wild-type PB was used to normalize the readout of various mutant PBs.

Construction of *can1*::*PBTn-KanMX/URA3*. A *piggybac* transposon carrying KanMX and *URA3* was constructed by PCR amplification of *PBTnKanMX* from mCherry::*PBTnKanMX* with Pbl-f7 (CAG *GGGCCC* TTAACCCTAGAAAGATAGTCTGC; ApaI) and Pbr-r4 (CAG *GGCGCC* TTAACCCTAGAAAGATAATCATATTG; NarI) and cloned into pCMV/Zeo digested with ApaI+NarI to give pCMV/*PB-TnKanMX*. A *URA3* cassette was PCR-amplified from a *piggyBac* integration plasmid (22) with Ura-kset-r (CGTACCCAATTCGCCCTATAGTG) and Ura-kset-f (TCGCGCGTTTCGGTGATGAC) and cloned into pCR2.1 (Invitrogen) to create pCR2.1-*URA3*. The *URA3* cassette was excised from pCR2.1-*URA3* by digestion with EcoRI and inserted into the EcoRI site of the pCMV/*PB-TnKanMX* site, which is located at the 3′ end of the KanMX cassette, to give pCMV/*PBTn-KanMX/URA3*. To insert *PBTn-KanMX/URA3* in the *CAN1* gene at TTAA 263–266, the *PB-Tn-KanMX/URA3* cassette was PCR-amplified with primers CAN1+PBL (GAAGATGAAGGAGAAGTACAGAACGCTGAAGTGAAGAGAGAGCttaaccctagaaagatagtctgcg; *CAN1* homology in uppercase; *PB-Tn KanMX/URA3* and flanking TSD TTAA in lowercase) and *CAN1*+PBR (CCAATAGTACCACCAAGGGCAATCATACCAATATGTCTTTGCttaaccctagaaagataatcatattgtgac; *CAN1* in uppercase; *PB-Tn KanMX/URA3* and flanking TSD TTAA in lowercase). To insert *PB-Tn KanMX/URA3* into *CAN1*, the PCR product was gel-purified with QIAquick Gel Extraction Kit (Qiagen) and transformed into BY4727, selecting transformants on SC–URA-Arg+canavanine+G418 plates.

Assay of Imprecise *piggyBac* Excision from *can1*::*PBTn-KanMX/URA3*. *can1*::*PBTn-Kan/URA3* is Can^{R} because of the *piggyBac* insertion in the arginine transporter *CAN1* and is Ura^{+} (5-FOA^{S}) because of *URA3* in the *piggyBac*

transposon. p414*GALS*-piggyBac transposase plasmids expressing PB, $PB^{R372A/K375A}$, $PB^{R372A/K375A}$, $PB^{M194V/R72A/K375A/D450N}$, and iPB7 transposase were transformed into *can1::PBTn-Kan/URA3* by selection on SC–Trp–Ura-Arg+Can+G418 plates and then restreaked on SC–Trp-Arg+Can+ 2% (wt/vol) galactose to induce transposase expression. Colonies were resuspended in water and plated on SC−Trp-Arg+Can+5-FOA to measure the number of cells that underwent imprecise excision—i.e., were Can^R without element reintegration—and on SC–Trp+5-FOA to measure cells that underwent excision without transposon reintegration. The frequency of imprecise excision is calculated as the ratio of the number of cells that underwent imprecise excision without element reintegration to the number of cells that underwent excision without transposon reintegration. It should be noted that this underestimates the imprecise excision frequency for transposases that are Int^+ because imprecise excision events in cells in which reintegration occurs (about 40–60% of excisions) are not considered.

Designed ZFP–Transposase Fusion Plasmid Constructs. The ZFP–iPB7 chimeric transposase plasmids were constructed by inserting each of the ZFP-linker cDNAs into the EcoRI/XbaI sites upstream of the iPB7 sequence (5, 15). The ZFPs and transposases are separated by the amino acid linker sequence EFGGGGSGGGGSGGGGSQF. The described ZFP–iPB7 mutants were constructed by mutating different combinations of three amino acids (R372A, K375A, and/or D450N) via site-directed mutagenesis (Quikchange Site-directed Mutagenesis Kit, Stratagene).

Mammalian Transposition Assays Using a Puromycin-Resistant Transposon with the ROSA26 and GULOP ZFP Fusions to *piggyBac*. A total of 400 ng of *pXL-Bac PB-GFP/Puro* transposon plasmid (8) alone or with 400 ng of the indicated transposase plasmids was transfected into HeLa cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and cultured under puromycin selection (0.5 μg/mL) for 2 wk. Following selection, puromycin-resistent colonies were fixed with 4% (wt/vol) paraformaldehyde, stained with methylene blue, and counted.

ACKNOWLEDGMENTS. We thank Dr. Rupak Mitra and Dr. Courtney Busch for many discussions about *piggyBac*; Benjamin Brett, Dr. Todd Scheetz, and Dr. Tom Bair for their expert consultation; and Maureen R. Regan for technical assistance. This work was supported by a grant from the Maryland Stem Cell Research Fund (to N.L.C.). N.L.C. is an investigator of the Howard Hughes Medical Institute. This work was supported by Grants AI 052845 and AI082020 (to F.D.B.). J.D.S. was supported by National Institutes of Health (NIH) Grant T32 CA009216. J.K.J. was supported by NIH Director's Pioneer Award DP1 GM105378 and by NIH Grant R01 GM088040. This work was supported by the NIH Grant R01 HL-105821 (to P.L.S. and J.K.J.) and partially supported by the University of Iowa Center for Gene Therapy (NIH P30 DK-54759).

1. Yant SR, et al. (2000) Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. *Nat Genet* 25(1):35–41.
2. Izsvák Z, Hackett PB, Cooper LJ, Ivics Z (2010) Translating Sleeping Beauty transposition into cellular therapies: Victories and challenges. *Bioessays* 32(9):756–767.
3. Cary LC, et al. (1989) Transposon mutagenesis of baculoviruses: Analysis of Trichoplusia ni transposon IFP2 insertions within the FP-locus of nuclear polyhedrosis viruses. *Virology* 172(1):156–169.
4. Li MA, et al. (2011) Mobilization of giant piggyBac transposons in the mouse genome. *Nucleic Acids Res* 39(22):e148.
5. Doherty JE, et al. (2012) Hyperactive piggyBac gene transfer in human cells and in vivo. *Hum Gene Ther* 23(3):311–320.
6. Nakanishi H, Higuchi Y, Kawakami S, Yamashita F, Hashida M (2010) piggyBac transposon-mediated long-term gene expression in mice. *Mol Ther* 18(4):707–714.
7. Saridey SK, et al. (2009) PiggyBac transposon-based inducible gene expression in vivo after somatic cell gene transfer. *Mol Ther* 17(12):2115–2120.
8. Burnight ER, et al. (2012) A hyperactive transposase promotes persistent gene transfer of a piggyBac DNA transposon. *Mol Ther Nucleic Acids* 1:e50.
9. Ding S, et al. (2005) Efficient transposition of the piggyBac (PB) transposon in mammalian cells and mice. *Cell* 122(3):473–483.
10. Wu SC, et al. (2006) piggyBac is a flexible and highly active transposon as compared to sleeping beauty, Tol2, and Mos1 in mammalian cells. *Proc Natl Acad Sci USA* 103(41):15008–15013.
11. Elick TA, Bauser CA, Fraser MJ (1996) Excision of the *piggyBac* transposable element *in vitro* is a precise event that is enhanced by the expression of its encoded transposase. *Genetica* 98(1):33–41.
12. Woltjen K, et al. (2009) piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* 458(7239):766–770.
13. Yusa K, Rad R, Takeda J, Bradley A (2009) Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. *Nat Methods* 6(5):363–369.
14. Wang W, et al. (2008) Chromosomal transposition of PiggyBac in mouse embryonic stem cells. *Proc Natl Acad Sci USA* 105(27):9290–9295.
15. Yusa K, Zhou L, Li MA, Bradley A, Craig NL (2011) A hyperactive piggyBac transposase for mammalian applications. *Proc Natl Acad Sci USA* 108(4):1531–1536.
16. Wang H, Mayhew D, Chen X, Johnston M, Mitra RD (2012) "Calling cards" for DNA-binding proteins in mammalian cells. *Genetics* 190(3):941–949.
17. Li X, et al. (2013) A resurrected mammalian hAT transposable element and a closely related insect element are highly active in human cell culture. *Proc Natl Acad Sci USA* 110(6):478–487.
18. Owens JB, et al. (2012) Chimeric piggyBac transposases for genomic targeting in human cells. *Nucleic Acids Res* 40(14):6978–6991.
19. Kettlun C, Galvan DL, George AL, Jr., Kaja A, Wilson MH (2011) Manipulating piggyBac transposon chromosomal integration site selection in human cells. *Mol Ther* 19(9):1636–1644.
20. Voigt K, et al. (2012) Retargeting sleeping beauty transposon insertions by engineered zinc finger DNA-binding domains. *Mol Ther* 20(10):1852–1862.
21. Dyda F, Chandler M, Hickman AB (2012) The emerging diversity of transpososome architectures. *Q Rev Biophys* 45(4):493–521.
22. Mitra R, Fain-Thornton J, Craig NL (2008) piggyBac can bypass DNA synthesis during cut and paste transposition. *EMBO J* 27(7):1097–1109.
23. Keith JH, Schaeper CA, Fraser TS, Fraser MJ, Jr. (2008) Mutational analysis of highly conserved aspartate residues essential to the catalytic core of the piggyBac transposase. *BMC Mol Biol* 9:73.
24. Harper AL, Skinner LM, Sudol M, Katzman M (2001) Use of patient-derived human immunodeficiency virus type 1 integrases to identify a protein residue that affects target site selection. *J Virol* 75(16):7756–7762.
25. Di Matteo M, et al. (2012) PiggyBac toolbox. *Methods Mol Biol* 859:241–254.
26. Rad R, et al. (2010) PiggyBac transposon mutagenesis: A tool for cancer gene discovery in mice. *Science* 330(6007):1104–1107.
27. Hirt B (1967) Selective extraction of polyoma DNA from infected mouse cell cultures. *J Mol Biol* 26(2):365–369.
28. Brachmann CB, et al. (1998) Designer deletion strains derived from Saccharomyces cerevisiae S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14(2):115–132.
29. Mumberg D, Müller R, Funk M (1994) Regulatable promoters of Saccharomyces cerevisiae: Comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res* 22(25):5767–5768.