

# A hyperactive *piggyBac* transposase for mammalian applications

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DNA transposons have been widely used for transgenesis and insertional mutagenesis in various organisms. Among the transposons active in mammalian cells, the moth-derived transposon *piggyBac* is most promising with its highly efficient transposition, large cargo capacity, and precise repair of the donor site. Here we report the generation of a hyperactive *piggyBac* transposase. The active transposition of *piggyBac* in multiple organisms allowed us to screen a transposase mutant library in yeast for hyperactive mutants and then to test candidates in mouse ES cells. We isolated 18 hyperactive mutants in yeast, among which five were also hyperactive in mammalian cells. By combining all mutations, a total of 7 aa substitutions, into a single reading frame, we generated a unique hyperactive *piggyBac* transposase with 17-fold and ninefold increases in excision and integration, respectively. We showed its applicability by demonstrating an increased efficiency of generation of transgene-free mouse induced pluripotent stem cells. We also analyzed whether this hyperactive *piggyBac* transposase affects the genomic integrity of the host cells. The frequency of footprints left by the hyperactive *piggyBac* transposase was as low as WT transposase (~1%) and we found no evidence that the expression of the transposase affects genomic integrity. This hyperactive *piggyBac* transposase expands the utility of the *piggyBac* transposon for applications in mammalian genetics and gene therapy.

reprogramming | gene correction

DNA transposons are genetic elements that can mobilize from one location to another in the host genome. These have been used as laboratory tools for transgenesis and insertional mutagenesis in a wide range of model organisms such as *Drosophila* (1, 2), *Caenorhabditis elegans* (3, 4), and plants (5). However, their application to mammalian genetics had been hampered because of the lack of active transposons in mammals. Approximately a decade ago, the first active DNA transposon in mammals, *Sleeping Beauty*, was reconstructed from fossilized transposon sequences found in the salmonid genome (6). This pioneer work has greatly expanded the repertoire of tools for mammalian genetics. Germline transposition has accelerated the generation of mutant mice and rats (7–11), and somatic transposition has opened up numerous possibilities to conduct forward genetic screens in vivo such as cancer gene discovery in solid tumors (12–15). Furthermore, DNA transposons hold great promise for gene therapy as nonviral vehicles (16). Since the generation of the *Sleeping Beauty* transposon, a number of transposons from different families have been reported to show active transposition in mammalian cells. Among them, the *piggyBac* transposon isolated from cabbage looper moth *Trichoplusia ni* is most promising because of a variety of unique characteristics, namely exhibiting the most efficient transposition in mammalian cells, the ability of the transposase to form functional protein fusions, large cargo capacity, and traceless excision, i.e., its excision restores the donor site to its pretransposon state and leaves no trace of transposon insertion (17–20). Taking advantage of these unique characteristics, we have recently demonstrated the generation of factor-free mouse induced pluripotent stem (iPS) cells (21).

The DNA transposon system consists of two components: a DNA element flanked by two terminal inverted repeats (IRs) and a transposase that catalyzes the transposon's mobilization by a "cut-and-paste" mechanism. The transposases first bind to the IRs, then excise the DNA segment flanked by the IRs from the genome (i.e., cut) and finally reintegrate the segment into a new location (i.e., paste). Thus, engineering the transposase is central to increasing the transposition efficiency. This has been successfully applied to the *Sleeping Beauty* transposon system. The most recent version of the *Sleeping Beauty* transposase (SB100) shows a marked hyperactivity compared with the original transposase (22). We have previously demonstrated that a mammalian codon-optimized version of the *piggyBac* transposase (PBase) mediates more efficient transposition than the original insect version, a 20-fold increase in "plasmid-to-genome" transposition (20), and elevated rates of chromosomal transposition (23).

Here we report the generation of a hyperactive mutant of PBase. We first established a yeast assay to efficiently screen for mutations in the PBase, which give rise to hyperactivity. Individual candidate mutants were then verified for their transposition activity in mouse ES cells. Finally, we combined all the mutations into one sequence and generated a hyperactive PBase (hyPBase), which shows more than 10-fold higher rates of transposition than the WT mammalian codon-optimized PBase (mPBase).

## Results

**Isolation of hyPBase Mutants in *S. cerevisiae*.** We have previously reported that the insect *piggyBac* element can transpose in *Saccharomyces cerevisiae* and described assays for both transposon excision and integration (24). We have now used the excision assay to isolate hyperactive transposase mutants (Fig. 1A). In our two-plasmid *piggyBac* excision system in a yeast *URA3<sup>-</sup>* strain, the transposon donor plasmid carries a *URA3::actin* intron cassette (25) containing a 2.1-kb mini-*piggyBac* transposon in the actin intron. The actin intron::mini-*piggyBac* segment is too large to be spliced and thus the strain is a uracil auxotroph. Upon excision of the mini-*piggyBac* element, however, the intron can be spliced, reverting the strain to uracil prototrophy. Thus, measuring the frequency of reversion to ura<sup>+</sup> is a convenient assay for transposition. The transposase is supplied by a second plasmid containing the PBase gene under the galactose-inducible control of the *GALS* promoter (26).

We generated mutant pools of PBase DNA by error-prone PCR and introduced the pooled DNA into a yeast expression vector by

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The authors declare no conflict of interest.

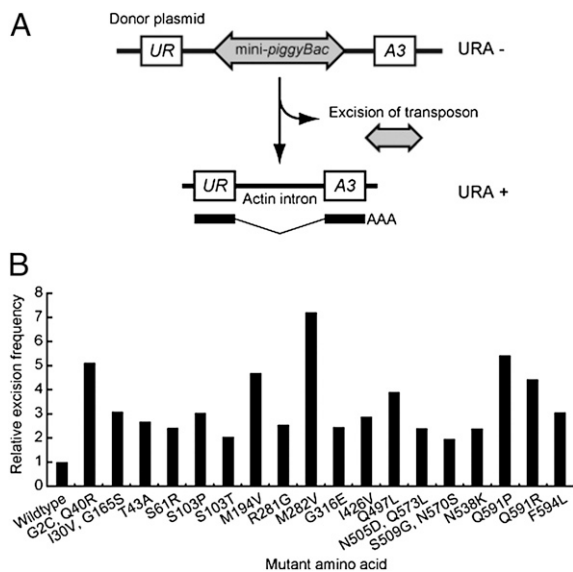
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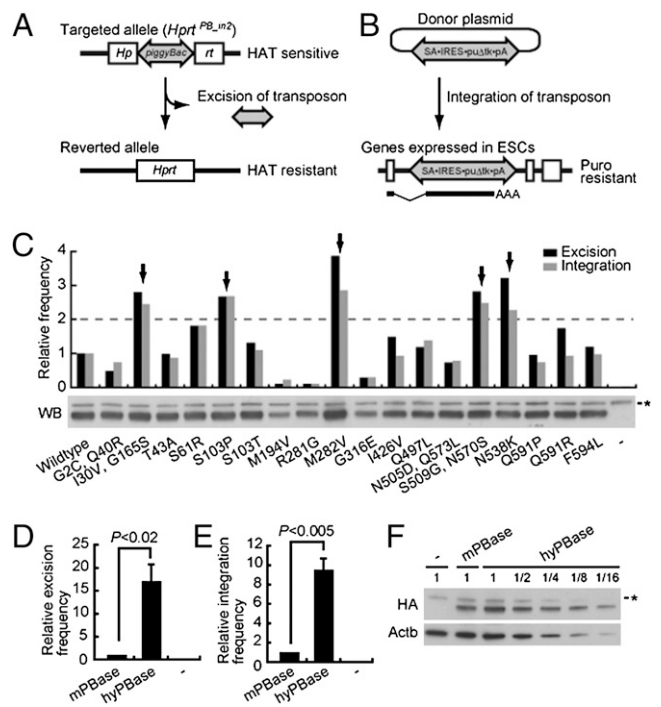


**Fig. 1.** Hyperactive mutant screening in yeast. (A) Schematic representation of the excision assay in yeast. The *URA3* gene is separated by the actin intron containing the mini-*piggyBac* transposon, which completely disturbs the normal splicing. After excision, the actin intron is spliced out normally and the *URA3* gene is restored. (B) Relative activities of hypBase mutants in yeast. These values are the median of assays of 10 colonies of WT and each mutant type performed in glucose, i.e., without induction of the GAL5 promoter. The absolute value of transposition promoted by WT transposase was  $4.7 \times 10^{-4}$  ura<sup>+</sup> cells/total cells.

homologous recombination. The mutant library was subsequently screened using the ura<sup>+</sup> reversion assay and the PBase mutants that gave rise to the higher number of ura<sup>+</sup> revertants than the WT PBase were isolated. In the initial screen, we examined 10,000 transformants and obtained approximately 200 candidates. The excision frequencies of these candidates were then analyzed quantitatively and we consequently isolated 18 hyperactive mutants, which showed two- to sevenfold increases in the excision activity (Fig. 1B).

**hypBase in Mammalian Cells.** We next investigated whether the PBase mutants isolated in yeast could also show elevated transposition in mammalian cells. The coding sequence of each mutant was transferred from the yeast expression vector to a CMV promoter-based mammalian expression vector. To find bona fide hyperactive mutants, we measured both excision and integration frequencies in mouse ES cells as illustrated in Fig. 2A and B, respectively. In the excision assay (Fig. 2A), we used an ES cell line that has a *piggyBac* transposon targeted into intron 2 of the *Hprt* gene (Fig. S14). The transposon carries a splice acceptor element, which disrupts the expression of the *Hprt* gene. The targeted ES cells are thus sensitive to HAT. When the transposon jumps out from the donor site, *Hprt* expression is restored and ES cells become resistant to HAT. In the integration assay (Fig. 2B), we cotransfected WT ES cells with the transposase expression vector and a transposon carrying a gene-trap cassette. When the transposon integrates into genes expressed in ES cells, the puromycin resistant gene is expressed; thereby ES cells become resistant to puromycin.

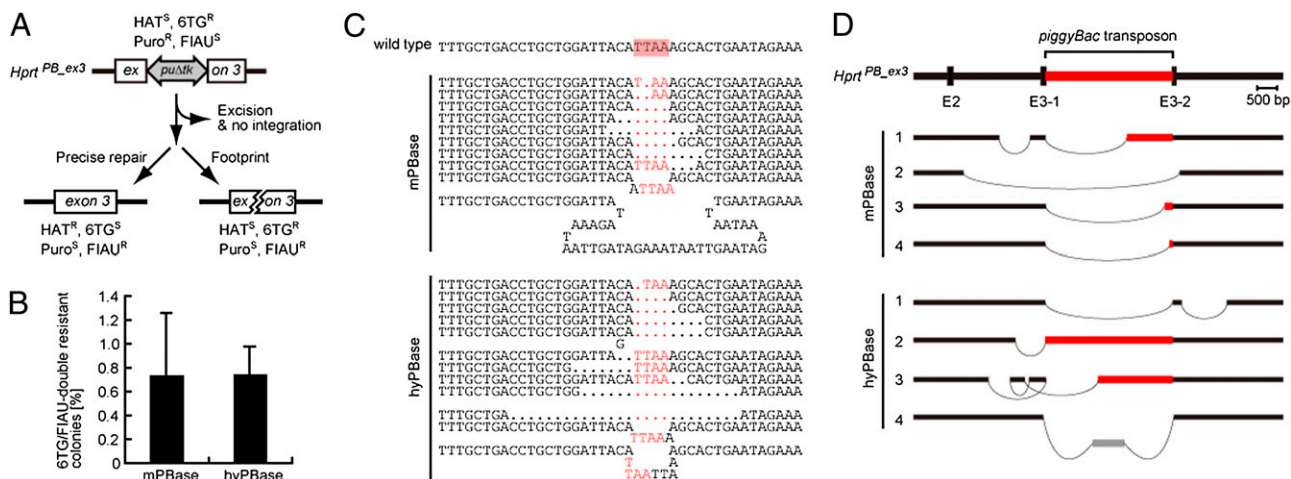
The activity of the 18 candidate mutants and the WT PBase are shown in Fig. 2C. Contrary to the results in the yeast assay, 13 mutants showed similar or weaker activity in ES cells in both excision and integration compared with the WT PBase. For instance, the second-strongest mutant in yeast (Q591P) did not show any significant increase in transposition in ES cells. The fourth strongest mutant in yeast (M194V) had very low activity in ES cells. Nevertheless, five mutants, namely I30V/G165S, S103P, M282V,



**Fig. 2.** Comparison of the transposase mutants and generation of hyperactive transposase in mouse ES cells. (A) Schematic representation of the excision assay in ES cells. The *Hprt* gene is disrupted by the *piggyBac* transposon carrying a gene-trap unit; thus, the ES cells are sensitive to HAT. When the transposon jumps out, the *Hprt* gene is restored, making cells resistant to HAT. (B) Schematic representation of the integration assay in ES cells. The *piggyBac* transposon vector carrying a gene-trap cassette was cotransfected into WT ES cells together with a transposase expression vector. When the transposon jumps into an active gene, the puromycin resistant gene is expressed; thus cells become resistant to puromycin. White boxes, exons; SA, splice acceptor site; IRES, internal ribosome entry site; *puΔtk*, the puromycin-resistant gene fused with the herpes simplex virus *thymidine kinase* gene; pA, poly-adenylation signal. (C) Relative excision and integration activities of the hyperactive mutant in ES cells (Upper) and protein expression of HA-tagged PBase and mutants in 293T cells (Lower). As a control, a WT transposase with the original insect-derived coding sequence was used. The mutants indicated by arrows were combined for the generation of the hyperactive transposase. Representative data are shown. Asterisk marks nonspecific band. (D and E) Comparison of hypBase with the WT transposase (mPBase) in excision (D) and integration (E) assays. Data are shown as mean  $\pm$  SD ( $n = 3$ ). (F) Western blot analysis showing expression of HA-tagged mPBase and hypBase in ES cells. Dilution factors are shown on the top of gel picture.  $\beta$ -Actin was used for loading controls. Asterisk marks nonspecific band.

S509G/N570S, and N538K, showed more than a twofold increase in activity in both the excision and integration assays. The fold changes of these mutants were similar between excision and integration, suggesting that the mutations mainly enhanced the excision reaction and the integration reaction remained unaffected. These outcomes could be caused by altered levels of protein expression. We assessed this by using Western blot analysis of HA-tagged mutant Pbases (Fig. 2C, Lower). Decreased transposition activities in the M194V, R281G, and G316E mutants were associated with marked reduction of their protein levels. The strongest mutant in both yeast and ES cells (M282V) had a slightly increased level of protein. The rest of the mutants showed similar protein levels to the WT PBase. Steady-state protein levels were thus correlated with the transposition activity in some, but not all, mutants. The mutants with unchanged protein levels most likely affected the mechanism and/or kinetics of transposition.

We then investigated whether combinations of these mutations can synergistically enhance the transposition efficiency.



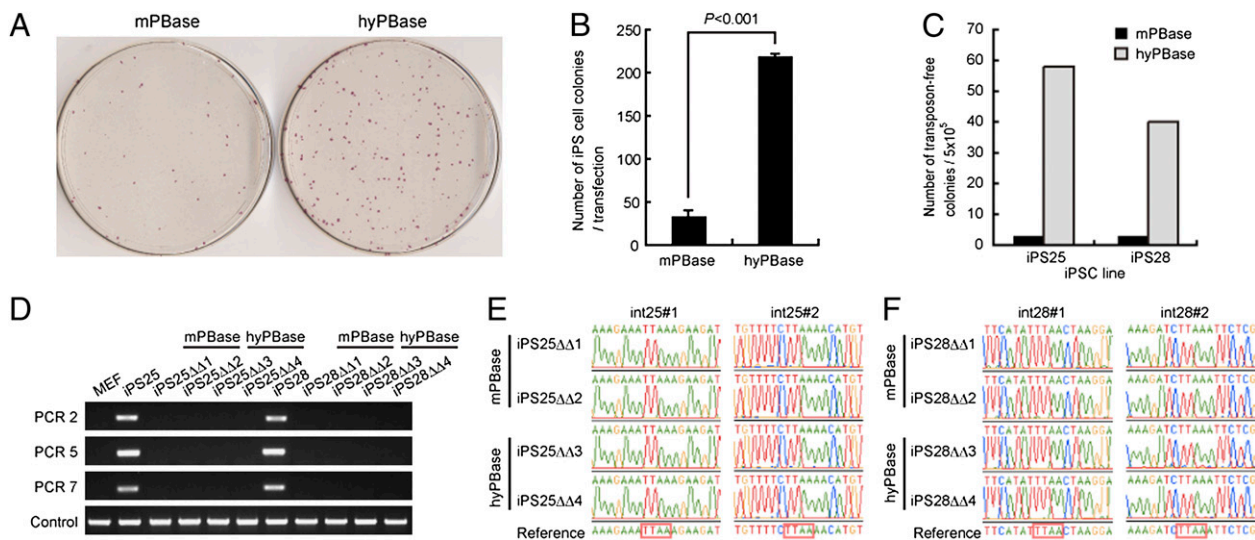
**Fig. 3.** Analysis of the *piggyBac* excision-induced mutations. (A) Schematic representation of isolation of cells with footprints. In *Hprt*<sup>PB-ex3</sup> ES cells, the *Hprt* gene is disrupted by targeted insertion of the *piggyBac* transposon carrying the *puΔtk* cassette into a TTA site in exon 3. When the excision site is precisely repaired, exon 3 is reconstructed and thus the *Hprt* gene is restored. If a footprint is generated, then the *Hprt* is permanently disrupted. Cells with footprints can be selected by FIAU and 6TG double selection. (B) Frequencies of *Hprt*-deficient colonies after transposon excision. Data are shown as mean ± SD (*n* = 6 for mPBBase, *n* = 3 for hyPBBase). (C) Sequences of the footprints generated after mPBBase- or hyPBBase-mediated excision. The donor TTA site is highlighted in red. (D) The *piggyBac* excision-induced genomic alterations. Most of these carry broken transposons (the remaining part of the transposon is shown in red). A 413-bp unrelated sequence (shown in gray) was inserted in hyPBBase clone 4. Redundant clones are not shown. Exon 3 with the transposon insertion is shown as E3-1 and E3-2. Red box indicates the *piggyBac* transposon; black boxes are exons; thin lines are the regions deleted.

Given that certain mutations might negatively affect each other, we tested several combinations of two (I30V/G165S plus M282V, M282V plus N538K, I30V/G165S plus N538K), three (I30V/G165S, M282V, and N538K), and four (I30V/G165S, M282V, N538K, and S103P or I30V/G165S, M282V, N538K, and S509G/N570S) mutations, as well as the combination of all five mutants. To maximize the expression level of the transposase, we introduced these mutations into the coding sequence of the mPBBase (20) by site-directed mutagenesis. All combinations tested showed synergistic enhancement (Fig. S2). As a result, the combination of all five mutants, i.e., a mutant with 7 aa substitutions, showed the highest activity in both the excision and integration assays with 17-fold and ninefold enhancement, respectively, compared with the mPBBase (Fig. 2D and E). The protein expression level of this mutant was approximately three times higher than mPBBase in ES cells (Fig. 2F). We thus successfully generated a hyPBBase for use in mammalian systems and termed it hyPBBase.

**hyPBBase Performs Traceless Excision.** One of the unique characteristics of the *piggyBac* transposon system is its traceless excision. One possibility, however, was that the hyperactive transposase may lose this important property by altering the molecular mechanism of transposon excision. To address this possibility, we measured the frequencies of footprint mutations upon mPBBase- and hyPBBase-induced excision. A large number of the *piggyBac* excisions are required for quantitative measurement of excision-induced mutation frequency and detection of rearrangements. We therefore generated a reporter ES cell line, in which a *piggyBac* transposon carrying the *PGK-puΔtk* cassette was targeted into a TTA site in exon 3 of the *Hprt* gene (Fig. S1B). This ES cell line is HAT-sensitive and 6-thioguanine (6TG)-resistant as a result of the inactivation of the *Hprt*. If the transposon is excised precisely, the *Hprt* gene is restored and the cells become HAT-resistant and 6TG-sensitive (Fig. 3A). On the contrary, if the transposon excision leaves a footprint, the *Hprt* gene is permanently inactivated; hence the cells remain resistant to 6TG (Fig. 3A). To select for cells that had mobilized the transposon and had left a footprint at the donor locus without reintegration, we applied 5-iodo-1-(2'-deoxy-2'-fluoro-β-D-arabinofuranosyl) uracil (FIAU)/6TG double se-

lection (Fig. 3A). Of the total of 3,478 excision events induced by mPBBase, 26 *Hprt*-deficient colonies were identified (mean mutation frequency, 0.74 ± 0.51%; *n* = 6; Fig. 3B and Table S1). hyPBBase showed a footprint frequency similar to mPBBase: 18 footprints of the total of 2,441 excisions (mean mutation frequency, 0.74 ± 0.23%; *n* = 3; Fig. 3B and Table S1). PCR and sequencing analyses revealed that the majority of *Hprt* mutations were caused by microinsertions or microdeletions (81% and 69% of mPBBase- and hyPBBase-induced footprints, respectively; Fig. 3C). We did not observe any unique pattern in the type of microinsertions/deletions between mPBBase and hyPBBase. Five mPBBase excisions and eight hyPBBase excisions generated slightly bigger insertions or deletions (Fig. 3D). The insertions were mostly caused by imperfect excision of the transposon, leaving a part of the transposon behind in exon 3. The biggest deletion, approximately 3 kb, was detected in a clone with an mPBBase-induced excision (Fig. 3D, mPBBase clone 2). In some clones, additional deletions were found in the introns flanking the donor site in exon 3. One clone from a hyPBBase-induced excision had small rearrangements (Fig. 3D; hyPBBase clone 3). As we did not observe spontaneous mutations in a mock control, these insertions and deletions were caused by the transposase expression. Thus, both mPBBase- and hyPBBase-mediated excisions generate footprints with a very low frequency. hyPBBase possesses an increased transposition activity while maintaining the unique property of predominantly traceless excision.

**hyPBBase Can Improve the Generation of Transgene-Free iPS Cells.** We have previously demonstrated reprogramming of mouse embryonic fibroblasts (MEFs) into pluripotent stem cells using a *piggyBac* transposon carrying a 2A-peptide-linked *Oct4* (also known as *Pou5f1*), *Sox2*, *Klf4*, *Myc*, and *Lin28* transgene (21). The traceless excision property of the *piggyBac* transposon enabled us to generate transgene-free iPS cells while maintaining an unaltered genome. Mouse iPS cells generated with this *piggyBac* transposon are genuinely pluripotent and can contribute to all somatic cell types and germ cells in chimeric mice. To investigate whether hyPBBase can increase reprogramming efficiency, we conducted transposon-based reprogramming of primary MEFs. As shown in Fig. 4A and B, the number of alkaline phosphatase-positive colonies was in-



**Fig. 4.** Improved generation of transgene-free iPSC cells using the hyPBBase. (A) A representative image of alkaline phosphatase staining of iPSC cell colonies generated using WT (Left) and hyperactive (Right) PBBase. (B) Numbers of iPSC cell colonies obtained from transfection of MEFs with 100 ng transposon and 100 ng transposase in a 12-well plate. Data are shown as mean  $\pm$  SD ( $n = 3$ ) (C) Number of transgene-free iPSC cell colonies generated by mPBBase or hyPBBase. Both primary iPSC cell lines (iPS25 and iPS28) have two transposon integrations. Representative data are shown. (D) PCR analysis showing transposon removal and no evidence of random integration of plasmids. (E) Precise repair of the excised site. All clones examined possess intact genomic sequences. The transposon donor sites are highlighted in red.

creased eightfold by using hyPBBase compared with the mPBBase, indicating that the hyPBBase enhanced reprogramming efficiency.

To generate factor-free iPSC cells, the integrated transposons need to be excised from the iPSC cell genome. In a previous study (21), the *piggyBac* excision efficiency was approximately  $1 \times 10^{-5}$  per cell, which was three orders of magnitude lower than the excision frequency at the *Hprt* locus. To test if hyPBBase could be a useful tool for transposon removal from iPSC cells, we transfected two primary iPSC cell lines (iPS25 and iPS28) (21) with mPBBase and hyPBBase and subjected the transfected cells to FIAU selection. As shown in Fig. 4C, hyPBBase removed the transposons approximately 20 times more efficiently than mPBBase. PCR analyses showed that all clones analyzed had lost the transposon and did not have random integrations of the PBBase expression vector (Fig. 4D). Importantly, we did not detect any footprint mutations at the primary transposon integration sites in both mPBBase- and hyPBBase-mediated excisions, showing the generation of transgene-free iPSC cells by the hyPBBase (Fig. 4E and F).

**PBBase Does Not Affect Genomic Integrity.** Transposases catalyze transposon excision from the host genome by introducing double-stranded breaks (DSBs) at both ends of the transposon. In principle, expression of the transposase may cause genomic instability as a result of DSBs at cryptic sites in the genome. To investigate this possibility, we conducted comparative genomic hybridization (CGH) on genomic DNA isolated from PBBase-exposed cells by using an Agilent 244K mouse genome array. We first compared two primary iPSC cell lines (iPS25 and iPS28; both carry two copies of the transposon; ref. 21) with 10 transposon-free iPSC cell lines derived from them following mPBBase- or hyPBBase-mediated excision. The results, summarized in Table S2, confirmed that the primary iPSC cell lines do not have any abnormalities compared with the donor MEFs. Half the transposon-free iPSC cell lines did not show any aberrations within the limits of CGH resolution, indicating that they possess unaltered genomes. The remaining half the cell lines, however, had deletions and/or amplifications ranging from 30 to 300 kb and one line, 25 $\Delta\Delta$ 1, had a large rearrangement on chromosome 8. None of the changes were linked to the original transposon integration sites. These genomic alterations could be caused by continuous transposition before trans-

poson loss or the transposase directly, or arise spontaneously during culture. As donor site deletions caused by transposon excision accounted for just 0.8% of total excision events and their sizes were less than 5 kb (Fig. 3), the genomic alterations found in transposon-free iPSC cell lines do not seem to be mediated by transposon excision. We assessed this further by isolating 15 transposon-free iPSC cell lines using hyPBBase from the iPS25 primary line and we compared these with 25 subclones from the same primary line using CGH analysis. Genomic alterations were found in 20% of transposon-free iPSC cell lines (3 of 15 lines) and 20% of subclones (5 of 25 lines; Table 1). Therefore, we did not find a statistically significant difference in genomic abnormality in hyPBBase-treated versus untreated iPSC cells (iPS25-hyPBBase, four of 17; iPS25-subclone, 5 of 25;  $P = 0.75$ , Fisher exact test).

We carried out two additional assays to investigate if PBBase itself can induce significant damage to the host genome. In the first assay, we investigated *Hprt* mutation frequency upon transposase exposure. WT ES cells were transfected with iPBase, mPBBase, or hyPBBase expression vectors ( $\sim 90\%$  transfection efficiency), cultured for 6 d, and subsequently subjected to 6TG selection. We observed a few *Hprt*-deficient colonies from iPBase and mPBBase-transfected cells, but none in the control (GFP-transfected) or hyPBBase-transfected cells (Table S3). The colonies that did appear occurred at background rates but none appeared following treatment with hyPBBase.

In the second assay, we analyzed DSB levels after transient expression of PBBase by using two surrogates, H2AX phosphorylation and foci as well as sister chromatid exchange (SCE). In these experiments, we expressed PBBase by mRNA transfection, which also can induce efficient transposition (Fig. S3A and B). First we analyzed H2AX phosphorylation, a biomarker of DNA damage (27), by Western blotting. All types of PBBase-transfected cells showed similar levels of H2AX phosphorylation compared with GFP-transfected and nontreated cells (Fig. S3C).  $\gamma$ H2AX foci in transfected NIH 3T3 analyzed by immunofluorescence were unaffected by PBBase expression (Fig. S3D and E). Last, we measured SCEs in PBBase-transfected ES cells. SCE is used as a cytogenetic biomarker to examine mutagenicity of chemical agents (28). SCEs are also induced by endonucleases (29). We did not observe any differences between nontreated and PBBase-

**Table 1. CGH analysis of iPS cell lines**

iPS cell line	Transposase	Aberration	Aberration type	Chr.	Size, kb	Found in other lines	No. of genes
iPS cell lines subcloned from iP525 (five abnormal lines of 25 lines analyzed)							
25-sub6	NA	Yes	1-copy deletion	4	58.3	No	2
25-sub8	NA	Yes	1-copy gain	5	524.8	No	8
			1-copy deletion	15	32.9	No	0
25-sub9	NA	Yes	1-copy deletion	6	1,847.6	Yes	0
25-sub16	NA	Yes	1-copy deletion	6	1,847.6	Yes	0
25-sub18	NA	Yes	1-copy deletion	5	92.8	No	0
Transposon-free iPS cell lines derived from iP525 (three abnormal lines of 15 lines analyzed)							
25-hy4	hyPBase	Yes	1-copy deletion	4	64.0	No	2
25-hy7	hyPBase	Yes	1-copy deletion	9	105.9	No	2
25-hy12	hyPBase	Yes	1-copy deletion	10	73.3	No	4

treated ES cells (Fig. S3 F and G). These results suggest that PBase itself does not seem to induce genomic instability.

Taken together, genomic alterations found in transposon-free iPS cells were highly likely to be acquired during culture and were not caused by expression of transposases.

### Discussion

In this study, we developed a hyperactive version of the *piggyBac* transposase and demonstrated its use to improve the efficiency of generation of the transgene-free iPS cells. We have also investigated in detail whether the use of *piggyBac* transposon and transposase cause genomic instability.

Two lines of evidence suggest that *piggyBac* transposition may be a host factor-independent reaction: first, its transposition is highly efficient in a wide range of organisms such as yeast (24), insects (30), planarian (31), the malaria parasite (32), and mammals (17); second, transposition can be reconstituted in vitro by using purified PBase and DNA elements (24). These characteristics of the *piggyBac* transposon allowed us to first screen a large number of transposase mutants in yeast and then test the candidates in mammals. Based on this strategy, we found five hyperactive mutants in ES cells among 10,000 mutants screened initially in yeast. As our mutant library in yeast was not saturated, more hyperactive mutants might be identified with this screening system in the future.

Despite the host-factor independence of *piggyBac* transposition, three fourths of the hyperactive mutants in yeast did not show hyperactivity in ES cells. There may be two factors contributing to this observation. First, species-specific factors may modulate the transposition positively or negatively. For example, the *Sleeping Beauty* transposase directly interacts with Miz-1 transcription factor (33) and HMGB1 (34), leading to the enhancement of transposition. Alternatively, epigenetic modifications may influence transposition frequency. The *Sleeping Beauty* transposase has an affinity for heterochromatic regions; hence, repressive epigenetic modification of the *Sleeping Beauty* transposon can increase the transposition frequency (35, 36). Although no transposase–host factor interaction in the *piggyBac* transposon system has been reported, if they do occur, PBase mutations might influence these interactions in a species-specific manner. Another possibility is that the reaction temperature affects catalytic activity and/or protein stability as mammalian cells are grown at very different temperatures from yeast. The activity of a yeast protein, FLP recombinase, was improved for mammalian use by increasing its stability at higher temperatures (37). Although the mechanism of how each mutation modulates transposition frequency remains to be determined, the mutants with different transposition efficiency in yeast and mammalian cell lines could be useful tools for further investigation of transposon biology.

DNA transposons are very useful as nonviral vehicles for genome engineering, which may be used for gene therapy. However,

safety concerns, especially genotoxicity, must be fully assessed. We have measured two aspects of the *piggyBac* transposon system: excision-induced genomic alterations at the donor site and a genome-wide assessment of transposase-induced genomic instability. Excision-induced genomic alterations are detected in 0.8% of excision events by mPBase and hyPBase transposases, which is much lower than the previously reported footprint frequency (~5–10%) (19). This difference might reflect the excision loci assayed or the number of excision events analyzed. Among excision-induced mutations, we found genomic alterations up to 5 kb from the donor site at very low frequency (~0.2% of excision events). These types of excision-induced mutations have not been described in previous reports (19). It is well recognized that mobilization of other types of DNA transposons can cause larger genomic rearrangements including insertion, deletion, duplication, inversion, and translocations, particularly by the *P*-element (38) and *Ac/Ds* elements (39–41) in *Drosophila* and *Maize*, respectively. In mice, transposition of *Sleeping Beauty* from a concatemer donor site can cause deletions and/or inversions of megabases of sequence as well as translocations (42). We did not observe such large and complex alterations following *piggyBac* excision. Although our results do not rule out the possibility of larger genomic alterations occurring, in general *piggyBac* excision from a single-copy donor site does not significantly affect genomic integrity.

A second concern is whether the *piggyBac* transposase itself causes genomic instability at sites other than a donor locus. To address this issue, we performed CGH analysis and compared 17 transposon-free iPS cells generated by hyPBase transfection and 25 subclones not exposed to hyPBase. Although we found that several cell lines carried deletions or amplifications, there were no obvious differences between these two groups in the nature of the detectable genomic aberrations such as their size, gain or loss of sequence, and the frequency with which they occurred. Moreover, we previously reported that mouse ES cells are genetically heterogeneous as a result of copy number change, with 30% to 40% of single cell subclones exhibiting copy number change (43). These results strongly suggest that the genomic alterations in the transposon-free iPS cells arose spontaneously and were not caused by PBase expression; hence, the hyPBase can mediate more efficient transposition without compromising the genomic integrity. Nevertheless, it might be worth noting that transposons can jump multiple times before they are integrated or lost and thus there is a small probability of footprint mutations. These mutations would be too small to be detected by currently available genome-wide methods. Whole-genome sequencing would have the resolution to detect such changes, and this might be considered before clinical use of iPS cells. Although we believe footprint mutations occur rarely, the development of transposases that can catalyze only excision but not integration or small compounds that can inhibit

only integration reaction might eliminate the chance of generating footprint mutations in applications of transposon removal.

The *piggyBac* transposase is amenable to modification; for instance, terminal fusion with activity modulator domains such as ERT2 and Gal4 DNA binding domains have been reported (18, 20, 44), and in this study we have used HA-tagged versions. These modifications allow us to regulate transposition in a spatiotemporal manner. In vivo applications of such engineered transposases are useful to address a variety of biological questions. This hyperactive *piggyBac* transposon system will further expand the use of transposons as tools for genome engineering such as insertional mutagenesis and gene therapy.

## Materials and Methods

**Plasmid Construction.** To construct mammalian expression vectors of the mutant *piggyBac* transposase, the BamHI-XhoI fragments containing the

mutant sequences in the yeast expression vector pGALS were transferred into the BamHI-XhoI site of pcDNA3 (Invitrogen). The point mutations were introduced into pCMV-mPBase (20) by site-directed mutagenesis to generate hyperactive transposases. The hyPBBase expression vector (pCMV-hyPBBase) is available upon request to the Sanger Institute Archives (<http://www.sanger.ac.uk/technology/clonerequests/>).

Construction of the targeting vectors, mutant screening in yeast, ES cell experiment, iPSC cell reprogramming, transposon excision in iPSC cells, and CGH analysis are described in *SI Materials and Methods*. Primer sequences are shown in Table S4.

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