**Supplementary Results**

The canonical insertion of a P-element into a target site is marked by the emergence of a direct duplication of an 8 bp sequence flanking the 5’ and 3’ end of the P-element point of incision (Position number marked in orange, **Figure 3A and 3B,** O’Hare and Rubin, 1983). Therefore, the substitution of one P-element (the KP element) with another (the StanEx P-element) could leave two sets of 8bp target site duplications when the excision - insertion occur in series, and only one set when the events occur concomitantly. To distinguish between these two models, we analyzed the flanking genomic sequence of the nine StanEx insertions at the 3R:14,356,561 site for direct repeats and compared these sequences to the direct repeat flanking the KP insertion. The only 8 bp direct repeat detected in all cases, KP as well as all StanEx insertions at 3R:14,356,561, was present already with the KP element in the StanEx[1] starter lineGCC CAA CC. No other additional sequence duplication was noted at this site in all StanEx insertions. The data therefore supports a model of concomitant excision of the KP combined with the insertion of the StanEx element.

The exact replacement of one P-element by another without the generation of an additional 8 bp target site duplication has been observed before (Williams, 1988, Heslip and Hodgetts, 1994, Gonzy-Treboul et al., 1995, de Navas et al., 2014). These events have been reported to occur frequently (4-30%) and were aptly named P-element replacement. While a preference towards conserved P-element directionality has been reported during P-element replacement (Gonzy-Treboul, 1995), inversions of direction of the replacing P-elements have been observed (Heslip and Hodgetts, 1994, De Navas, 2006). In this study, we report two out of the nine cases of P-element replacement with inversed direction, SE457 and RJ-3 **(Figure 3C).**

P-element replacement predicts the conservation of genomic sequences 5’ and 3’ of the insertion site. In some cases of P-element replacement, however, deletions, rearrangements and duplications of genomic sequences during P-element replacements have been reported (Gonzy-Treboul, 1995). To assess the status of adjacent genomic DNA, we sequenced the genomic DNA 5’ and 3’ of the breakpoint of the nine P-element replacements at 3R:14,356,561 (Methods). Out of the nine insertions, seven were conservative and did not show changes of 5’ or 3’ genomic sequence. Two lines, SE304 and SE405, did display alterations of genomic DNA 5’ to the insertion site. SE305 exhibits a nine base pair deletion of the 5’ 8bp direct repeat, and the first nucleotide of the 5’ end of the StanEx P-element. SE405 presents a 31 bp deletion of the 5’ P-element end and a further deletion/insertion of genomic sequences 5’ to the insertion site **(Suppl. Table 4)**.

One relevant question from these observations concerns the mobility of the resident KP element on chromosome III during the mobilization of the X chromosome StanEx1 P-element in our starter strain background (Kockel et al., 2016). To test if the KP element is co-mobilized in parallel to the StanEx1 P-element, we tested 29 non-NK7.1 StanEx insertions after hybrid dysgenesis for presence of the KP element at the *NK7.1*/*heatr2* site. We observed KP mobilization in approx. 50% of the cases (15/29), either by loss of the KP element (38%, 11/29) or by transposition of the KP element to a novel site other than *NK7.1*/*heatr2* (13.8%, 4/29).

To quantify the ratio of mobilization to re-integration of the KP-element during P-element replacement, we tested eight independent StanEx lines that replaced the KP at the *NK7.1*/*heatr2* locus. In 50% of these cases, we observe a loss of the KP element and in 50% of the cases re-integration of the KP at a site different from the *NK7.1*/*heatr2* locus. Noting the relatively small sample size (n=8), the frequency of re-integration of the replaced KP element is surprisingly high (50%) when compared to the frequency of KP element integration during a non-replacement mechanism (13.8%), especially considering that the genetic crossing scheme captures a single haplotype per mobilization event into each fly strain (the other chromosomes are balancers). It remains to be explored if mobilization of a P-element by means of replacement alters the mechanism of re-integration, explaining the observed disparity.