A. Crossing scheme to shuffle transgenes between landing sites using Int* and Int+ (wildtype).

G₀

\[ \text{w; } w^{\text{gfp}}/\text{CyO; } ZH-86Fb[hs-Int*, attL] \]

\[ \text{yw P(nos-Int*); attP40[pJFRC19(w')]; ZH-86Fb[attP(RFP*)]} \]

\[ \text{heatshock} \]

F₁

\[ \text{yw P(nos-Int*); attP40[pJFRC19(w')]; ZH-86Fb[attP(RFP*)]} \]

\[ \text{CyO; ZH-86Fb[hs-Int*, attL]} \]

\[ \text{yw}^{1118} \]

F₂

\[ \text{yw}^{1118} + \text{CyO; ZH-86Fb[pJFRC19( w', RFP*)]} \]

\[ \text{Balance and PCR verify} \]

B. Molecular characterization of selected shuffle candidates. Top – Genomic PCR confirmed the presence of GFP in five shuffle candidates (C1-C5). P1 corresponds to the maternal G₀ genotype, w; attP40[pJFRC19(w')]; ZH86Fb[attP(RFP*)]. P2 corresponds to the paternal G₀ genotype, w; wg⁹/CyO; ZH86Fb[hs-Int*, attL]. Middle – Genomic PCR detected Int* in three shuffle candidates. Int* was not detected in candidate C1, since pJFRC19 shuffled into the receiver landing site (marked by 3xP3-DsRed). Int* was detected in C2, C4, and C5. This indicates that pJFRC19 re-integrated on the hs-Int* chromosome, which is corroborated by the absence of DsRed expression in these flies. The attL sequence downstream of hs-Int* (see Figure S1A) presented a potential re-integration target due to the relaxed integration site specificity of Int*. The re-integration site of candidate C3 was not determined, though the lack of DsRed strongly suggests this was off-target. Bottom – PCR control with rp49. (C) Wild-type Int enforces canonical attP x attB recombination during transgene shuffling: Flies were scored for the presence of the ZH-86Fb landing site marker 3xP3-DsRed to distinguish re-integration at the receiver site from off-target integration (see panel B). When Int* provided both excisionase and integrase activities, half of the recovered candidates lacked DsRed, indicating re-integration at a site other than the receiver site. In contrast, in the presence of wild-type integrase, all candidates re-integrated at receiver landing site.

Figure S6. Genetics and integration characteristics of Int*/Int transgene shuffling.