Supporting Information Text S1. Identification of mutations and removal of background mutations.

We generated three isogenic $\gamma COP^{P\{1ArB\}A383.2M3}$ lines and found that these three lines were all homozygous viable (although in average only 31% of the expected homozygous flies were eclosing) and weakly fertile (egg-laying activity was lower than observed for wild type; data not shown). In addition, the flies were smaller and lacked the posterior cross-vein (data not shown).

In our $P$-element remobilization experiment we started with all three different isogenic $P\{1ArB\}A383.2M3$ lines and set up an initial 844 crosses, with single males, harboring both the $P\{1ArB\}A383.2M3$ insertion and the $P$-element transposase (Figure 1A). Finally, the $P$-element remobilization experiment yielded 1108 stably balanced excision chromosomes, which had lost the $ry^+$ marker and therefore likely the $P\{1ArB\}$. Of these lines, 277 were lethal, the others were homozygous viable and crossveinless. Since $\gamma COP$ had been found to be an essential gene in other organisms [30], we expected a *Drosophila* $\gamma COP$ null mutant to be embryonic lethal; thus, novel $\gamma COP$ alleles should be found among the lethal lines. By screening through a large number of these embryonic lethal lines using a PCR assay, we identified a few $\gamma COP$ mutants harboring deletions (deletions 6, 8 and 10; Figure 1B-C, E; Materials and Methods). In order to identify more and larger deletions, we were looking for suitable lines to be used in complementation assays: We used a jump-out deletion of the distal neighboring gene *pygo* [34], $Df(3R)pygo^{11-4}$, which also deletes parts of the $\gamma COP$ gene (Figure 1E), a *pygo* allele, *pygo* 130, which specifically affects the *pygo* locus [34-35] and an independent $\gamma COP$ allele, which had become available in the meantime, called *kg06383* (Flybase). By testing the remaining 144 embryonic lethal excision lines in complementation assays with *pygo* 130, $Df(3R)pygo^{11-3}$ and $\gamma COP^{kg06383}$ and our PCR-based assay, we identified additional $\gamma COP$ and $\gamma COP$ *pygo* double mutants (lines 5, 12, 577 and 677
Sequence analysis revealed the deletion breakpoints (Materials and Methods): In the case of 5, 12, 6, 8 and 677, a few base pairs of the 5’P inverted repeat sequence and in the case of deletion 6 also a few base pairs of unknown origin had stayed behind after the imprecise excision of the P{IArB}. In \(\gamma\text{COP}^5\) 474 bp of the \(\gamma\text{COP}\) gene are deleted, in \(\gamma\text{COP}^{12}\) 682 bp, in \(\gamma\text{COP}^6\) 1045 bp, and in \(\gamma\text{COP}^8\) 1072 bp. In \(\gamma\text{COP}^{10}\), sequences 5’ of the known transcription start site, and sequences of the \(\gamma\text{COP}\) gene including both translation start sites (of \(\gamma\text{COP-RA}\) and \(\text{RB}\)) are deleted, in total 1900 bp (Figure 1E; Supporting Figure 1). Sequences 5’ of the known transcription start site are also deleted in \(Df(3R)\gamma\text{COP}^{577}\). In addition, \(Df(3R)\gamma\text{COP}^{577}\) and \(Df(3R)\gamma\text{COP}^{677}\) not only remove the entire \(\gamma\text{COP}\) transcription unit, but also parts of the 3’ end of the distal neighboring gene \(\text{pygo}\) ([34-35]; Fig. 1E). In total 5405 bp are deleted in \(Df(3R)\gamma\text{COP}^{677}\) and 6209 bp are deleted in \(Df(3R)\gamma\text{COP}^{577}\) (Figure 1E).

We were also analyzing the homozygous viable lines, to find evidence that the \(P\)-element had precisely excised in these lines. Indeed by the single fly PCR method using primer pairs flanking the original \(P\)-element insertion (e.g. cop14 and cop2rev (Materials and Methods)), we revealed that in 51 of 93 (51/93) homozygous viable lines, the \(P\)-element had precisely excised. In only one line, which unfortunately was lost, a tiny deletion was detected. In another 10 lines, part of the \(P\)-element seemed to have remained in place as the PCR signal was bigger than in wild type; sequencing a few of these lines confirmed that only internal \(P\)-element deletions were present (data not shown). The remaining 31 lines were not further analyzed. This initial PCR analysis confirmed that in most homozygous viable lines the \(P\)-element had excised precisely or only small parts of it had stayed in place (and only the \(\text{ry}^+\) marker was lost). Sequencing two of these excision alleles confirmed that indeed the entire \(P\)-element was precisely removed (data not shown). However, these lines were still crossveinless (cv) and to our big surprise were
female sterile (fs). Crossing these excision lines to the lethal lines showed that most isolated lines were female sterile and crossveinless. This suggested to us that the crossveinless phenotype was due to a second mutation present in the original stock and that a third mutation was induced through the jump-out procedure. We considered this likely due to a second transposable element present in the background, which was not seen by our in situ hybridization experiment to polytene salivary gland chromosomes, using a ry probe [33]. In summary, not only $\gamma$COP deletion alleles but also $\gamma$COP-unassociated female sterile or potentially also lethal hits on chromosome 3 could be present in the lethal excision lines.

In complementation assays with the newly available independent $\gamma$COP allele $kg06383$ (Flybase), we found that only 81 of the 144 of the lethal lines, as well as our PCR-identified $\gamma$COP deletions, did not complement $kg06383$ and thus represent $\gamma$COP alleles. The others seemed to be lethal due to second hits present on chromosome 3, which were not associated with the $\gamma$COP locus. There is also no complementation observed between the $\gamma$COP$^{P(lArB)A383.2M3}$ allele and $kg06383$ or the other newly identified $\gamma$COP deletions, indicating that the $\gamma$COP$^{P(lArB)A383.2M3}$ allele is indeed a hypomorphic $\gamma$COP allele (data not shown).

The presence of background mutations could severely disturb a functional analysis of $\gamma$COP. Therefore, the background mutations (fs and cv) were removed. We mapped them roughly by analyzing meiotic recombination events between the multiply marked rucuca chromosome (Materials and Methods, data not shown) and the $P(lArB)A383.2M3$ and found the cv phenotype proximal to e and distal to ry (where two known cv mutations map (cv-c and cv-d (Flybase)) and the fs mutation on 3L. Then, we removed the background mutations by replacing all segments of 3L and all sequences proximal to e$^\delta$ with an isogenic rucuca chromosome. This resulted in lines like e.g. ru$^{l}$ h$^{l}$ th$^{l}$ st$^{l}$ cu$^{l}$ sr$^{l}$ e$^{l}$ $\gamma$COP$^{10}$. Furthermore, we removed most of the
*rucuca* makers again by recombination with either an isogenic FRT82B or another isogenic chromosome 3. In this way, lines like e.g. *FRT82B sr¹ eγCOP¹⁰* or *FRT82B eγCOP¹⁰* were obtained. Subsequently, these lines were tested in complementation assays to verify the absence of both the background *fs* and the *cv* mutation and used in our further analysis. Although we have replace almost the entire third chromosome, we can a priori not know whether potential lethal mutations have also been present or and if so, been removed, for they could principally be located distal to *ebony*. Therefore, rescue experiments were performed to prove that indeed no other lethal mutation than the one in *γCOP* was present on these chromosomes (Figure 2).