**Supplementary Figure 1.** Exclusion list effectiveness. (a) The average % protein sequence coverage from 3 tagless control experiments before, during (preliminary) and after the refined (final) exclusion list implementation. (b) m/z values used to exclude selected common contaminants.

**Supplementary Figure 2.** ProteinCenter™ workflow for data analysis of multiple MS runs and generation of contaminant lists.

**Supplementary Figure 3.** Protein Interaction confidence scoring scheme for 4 or 6 datasets. Proteins are assigned a score based on the number of appearances in multiple replicates with higher weighting towards proteins identified with different affinity purification methods compared to those found multiple times by a single method.

**Supplementary Figure 4.** Pilot data for selecting suitable affinity tags.
(a) Immunoblot of the two HN6- or HAT-StrepII tagged Mago proteins purified by Probond resin and visualized with anti-GFP antibody. (b & c) GFP fluorescence in S2 cells with the StrepII with either HAT (b) or HN6 (c) tagged Mago protein. Arrows indicate aggregates in the HN6-tagged cells. (d) Coomassie stained gel to show GFP-StrepII tagged Mago protein (*) and confirmed binding partner, Y14, indicated by arrows. (e) Construct used to generate GFP-StrepII tagged Mago protein in S2 cells using a Metallothionein (MT) promoter.

**Supplementary Figure 5.** Protein tag construct.
A combination of P-element and *PiggyBac* vectors with splice donor and acceptor sites is used to insert the FLAG, *Strep*II and YFP coding tags into the *Drosophila melanogaster* genome.

**Supplementary Figure 6.** Qualitative comparison of parallel versus tandem affinity purifications using an equal starting amount of tagged FLW. (a) Immunoblot showing, for each purification, an equal volume (10μl) of the final eluate (left) and bait remaining on the resin (right) (resin boiled in 10μl sample buffer and loaded) all detected by anti GFP. Starting soluble extract (S10) on the far right. (b) Mapping the bait peptides generated from parallel and tandem pulldowns highlighting the need for parallel approaches to obtain maximal coverage. Colours correlate to pulldown method highlighted in (c). (c) Mass Spec and interaction analysis data of FLW interactors following the four different purification methods. The numbers of ‘validated interactors’ are the those in our list that have been previously reported and those in parenthesis are published interactors from others’ affinity purifications. (d & e) Venn diagrams to show the overlap of FLAG (F) and *Strep*II (S) parallel purifications and tandem (FS and SF) before (d) and after (e) removal of controls demonstrating that parallel methods generate more hits and more overlap, many of which were observed in public data. FDR= Protein false discovery rate. For seven other baits tested yield for serial purifications were undetectable by immunoblot and Mass Spec and therefore data is not shown.

**Supplementary Figure 7.** Analysis of tagged proteins with insertions at different introns to show that the functioning of this protein is not altered and that an interaction partner
(exd) is specifically binding. (a) Venn diagrams to show the overlap of proteins identified in both FLAG and Strep pulldowns from the three differently tagged lines (CPTI -2852, -1356, -2808). Differences in detected interacting proteins may be due to isoform specificity. The 20%Contam_list indicates known contaminating interacting proteins present in >20% of all datasets (See Supplementary Table 5). (b) Position of tag insert determined by pcr to show the protein isoforms that are likely to be tagged. Coloured boxes indicate the isoforms detected by Mass Spectrometry. Each parallel pulldown was performed twice.

**Supplementary Figure 8.** Identification of multiple isoforms of a bait protein. (a) Mascot protein summary to show 3 different isoforms of Rtnl-1 in an affinity purified complex sample. (b) Alignment of the unique N-terminal portions with the observed unique peptides identified in red and corresponding ions scores (right). Underlined K indicates the C-terminus of first identified peptide where more than one sequential peptide appears.

**Supplementary Figure 9.** Growth of contaminant lists showing a steady increase in multiple protein hits and a gradual increase in single protein hits for the FLAG- and STREPomes that approaches saturation, The negative control data from groups of 5 pulldown experiments were merged and the proteins counted. Successions of further 5 pulldowns were added and the protein appearances counted. For the BEADome, all negative control data were merged and proteins occurring in more than one Strep
experiment and FLAG experiment were counted and the frequency calculated. Each list contains 25 sets of negative control data.

**Supplementary Table 1.** The LC gradient used to elute peptides from the affinity purification complexes.

**Supplementary Table 2.** Mascot searched MS data of six tagged protein baits. Summary of the Mascot data from seven fly lines expressing six different sized YFP tagged proteins as listed. The Mascot scores, number of peptides (including No. of Bold Red hits), and the derived percentage of protein sequence coverage are listed.

**Supplementary Tables 3a-c.** Protein contaminants comprising the (a) FLAGome, (b) STREPome and (c) BEADome.

**Supplementary Tables 4a-f.** Lists of complex proteins with FlyMine validation for (a) FLW, (b) SGG, (c) RTNL-1, (d) FAS2(A&B), (e) SEMA-1a, and (f) DP.

**Supplementary Table 5.** The TRAPome; Protein contaminants common to FLAG and StrepII-tagged bait protein purifications.