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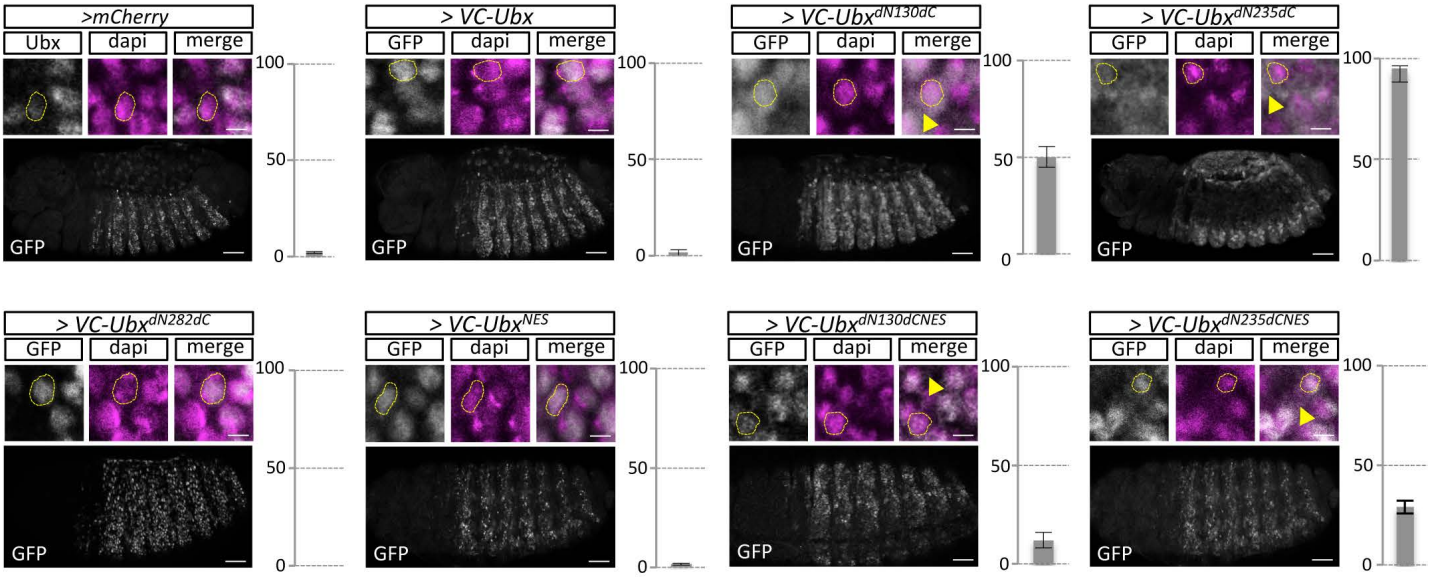

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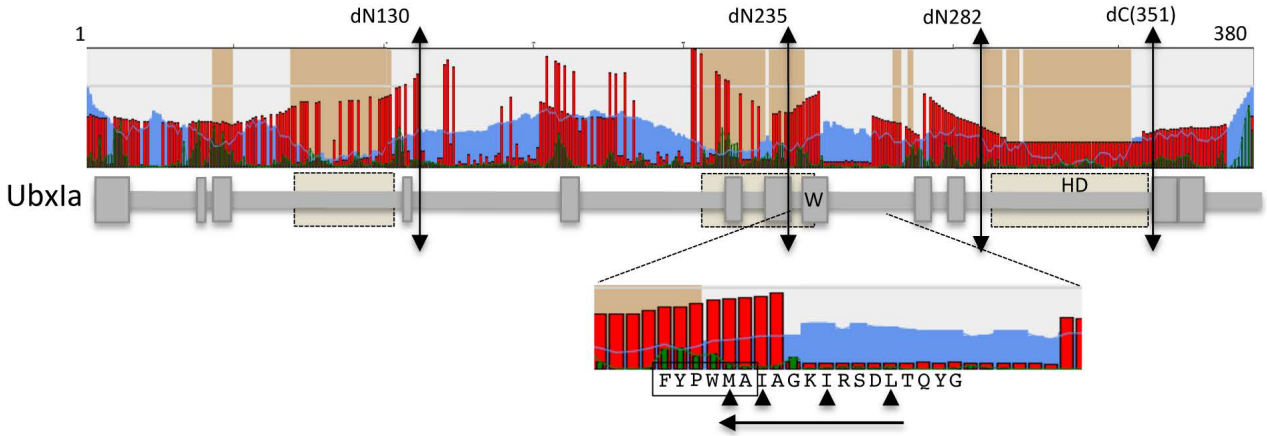

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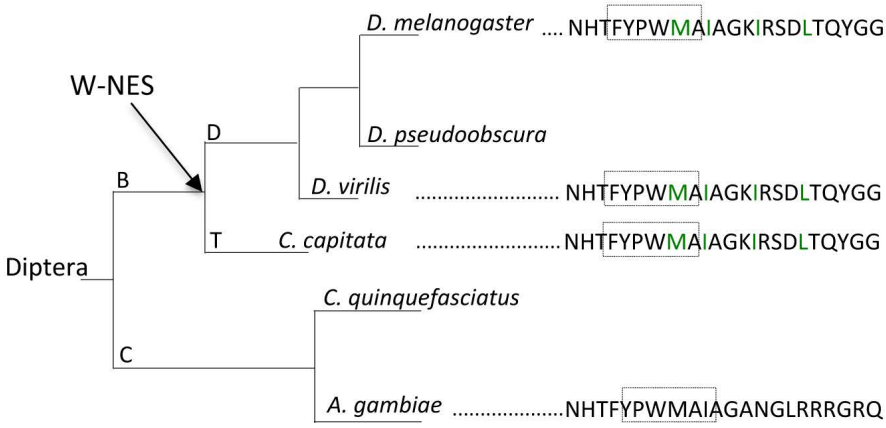


Figure S1. N-terminal deletions induce Ubx cytoplasmic localization in the embryo. A. Immunostaining of endogenous Ubx and VC-Ubx constructs (green), as indicated. VC-Ubx constructs were expressed with an *Ubx-Gal4* driver and revealed with an anti-GFP antibody recognizing the VC epitope (gray), as previously described (27). Immunostainings were co-revealed with dapi (magenta) to stain nuclei. Scale bars are for 40µm. Enlargements are shown above each illustrative confocal acquisition of the embryo. Scale bars are for 4µm. Graphs indicate the percentage of Ubx expressing cells with a staining in the cytoplasm. Bars represent mean ± SD of acquisitions from five different embryos from at least three independent experiments. Yellow arrowheads depict the cytoplasmic localization. Note that the VC-Ubx^{dN130dC} and VC-Ubx^{dN235dC} construct are respectively found in the cytoplasm of half or almost all expressing cells. **B.** Schematic representation of UbxIa as shown in Fig. 1. Enlargement shows the W-motif containing region with the putative inverted NES (arrow). Arrowheads indicate the core hydrophobic residues of the putative NES. **C.** Evolutionary tree of Diptera, showing that the non-canonical NES (highlighted in green) is only conserved in Ubx proteins of fruit flies (Tephritidae (T) and Drosophilidae (D) branches) from the Brachycera sub-order. This NES is not present in Ubx proteins from the Culicidae (C) family (mosquitos).

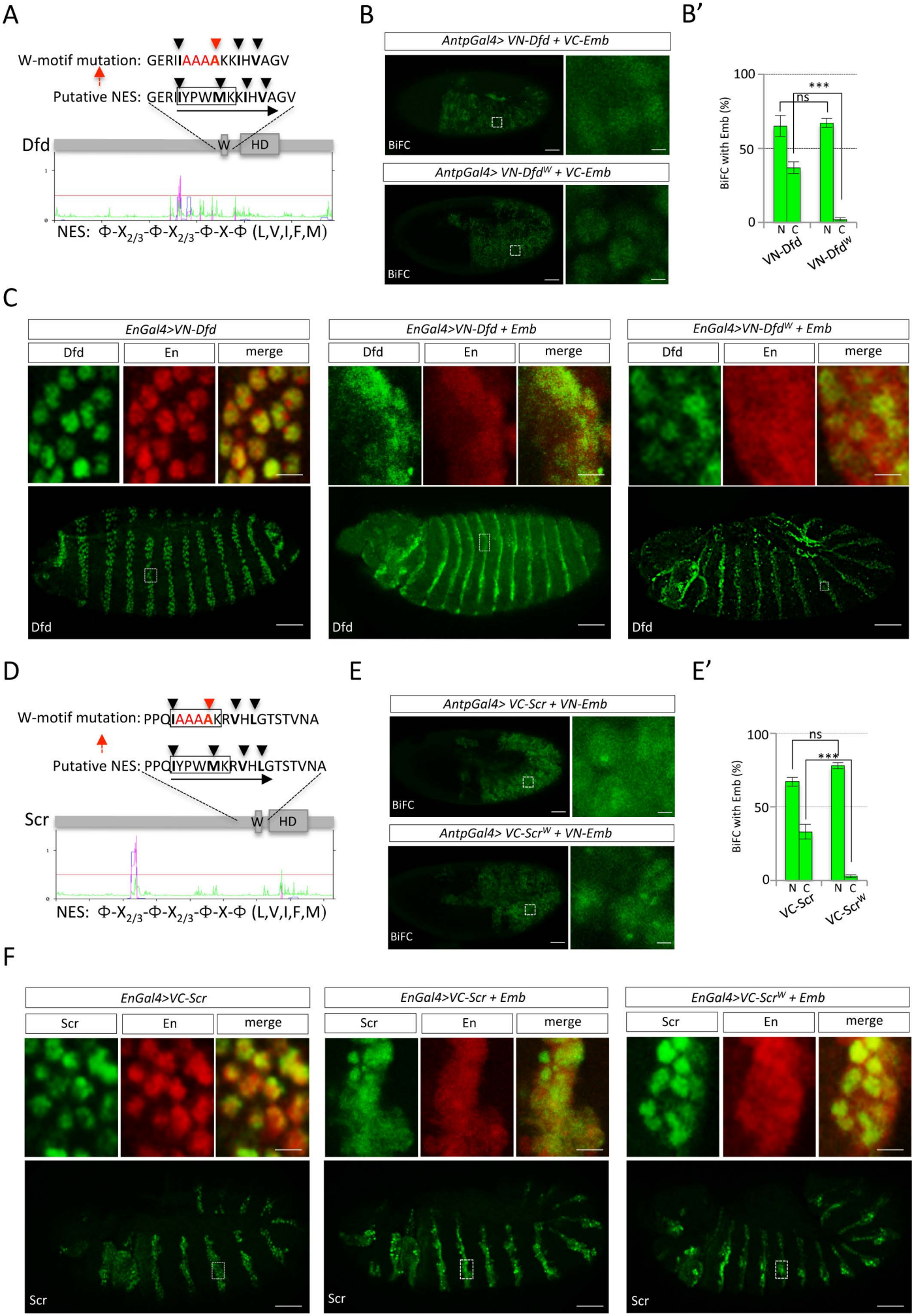
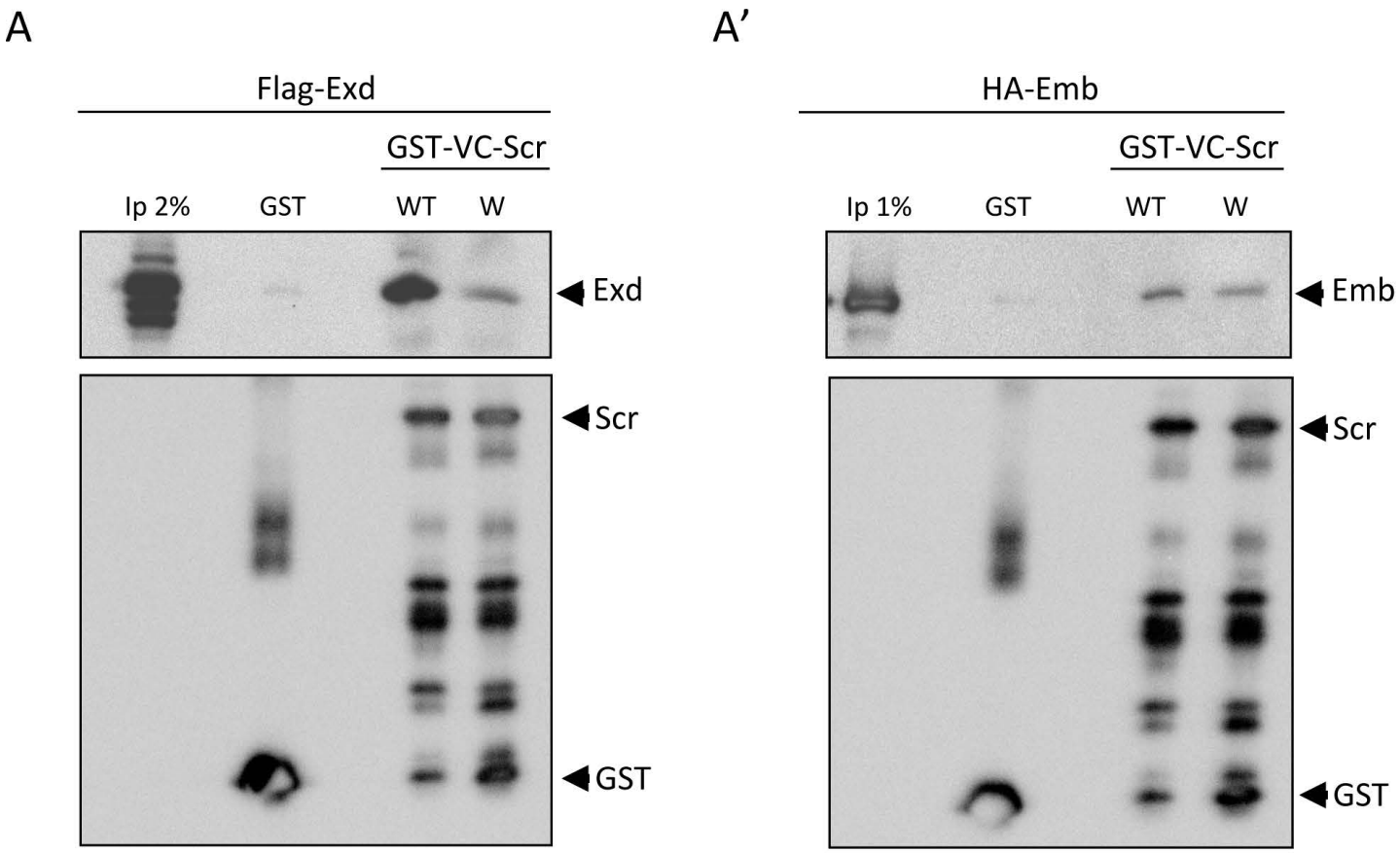
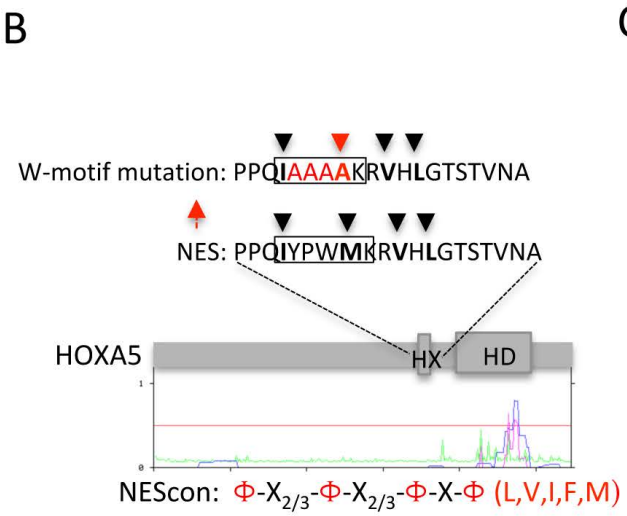


Figure S2. The W-containing motif is part of an unconventional NES in two other *Drosophila* Hox proteins. A. Protein sequence of the W-containing motif surrounding region in Deformed (Dfd). Black arrowheads indicate hydrophobic residues that could participate to the NES. Black arrow shows the orientation of the putative NES. The classical W-containing motif mutation (highlighted in red) affects one core hydrophobic residue of the putative NES. The graph below illustrates predicted NES with NetNES, taking into account the consensus repartition of hydrophobic residues (indicated below the graph). NetNES predicts a putative NES with a good confidence score in the region upstream of the HX (magenta peak). **B-B'.** BiFC between wild type or mutated W-containing motif of VN-Dfd fusion protein with VC-Emb in the live *Drosophila* embryo, as indicated. Fusion proteins are expressed with the *Antennapedia (Antp)-Gal4* driver. Scale bars are for 60µm (full embryo) or 3µm (enlargement). Graphs on the right (B') show the statistical quantification of BiFC signal in the nucleus (N) and cytoplasm (C) of epidermal cells of stage 10 embryos. The mutation of the W-containing motif affects the Dfd-Emb interaction only in the cytoplasm. BiFC was evaluated by t-test (***p<0,001 and ns=non significant). **C.** Genetic interaction between Dfd and Emb. The co-expression of cold HA-Emb triggers Dfd in the cytoplasm except if the W-containing motif is mutated. Magnification of protein expression in the nucleus and cytoplasm is shown below each embryo. This effects was significant (p<0,001 by t-test). Scale bars are for 40µm (full embryo) or 5µm (enlargement). **D.** Protein sequence of the W-containing motif surrounding region in Sex combs reduced (Scr). Black arrowheads indicate hydrophobic residues that could participate to the NES. Black arrow shows the orientation of the putative NES. The classical mutation of the W-containing motif (highlighted in red) affects a core hydrophobic residue of this unconventional NES. The graph below illustrates predicted NES with NetNES, taking into account the consensus distribution of hydrophobic residues (indicated below the graph). NetNES predicts a putative NES with a good confidence score in the region upstream of the W-containing motif (magenta peak). **E-E'.** BiFC between wild type or mutated W-containing motif of VC-Scr fusion protein with VN-Emb, as indicated. Fusion proteins are expressed with the *Antennapedia (Antp)-Gal4* driver. Scale bars are for 60µm (full embryo) or 3µm

(enlargement). Graphs on the right show the statistical quantification of BiFC signal in the nucleus (N) and cytoplasm (C) of epidermal cells of stage 10 live embryos. The W-containing motif mutation affects the Scr-Emb interaction only in the cytoplasm. BiFC was evaluated by t-test (***p<0,001 and ns=non significant). **F.** Genetic interaction between Scr and Emb. The co-expression of cold HA-Emb triggers Scr in the cytoplasm except if the W-containing motif is mutated. Magnification of protein expression in the nucleus and cytoplasm is shown below each embryo. This effects was significant (p<0,001 by t-test). Scale bars are for 40µm (full embryo) or 5µm (enlargement).





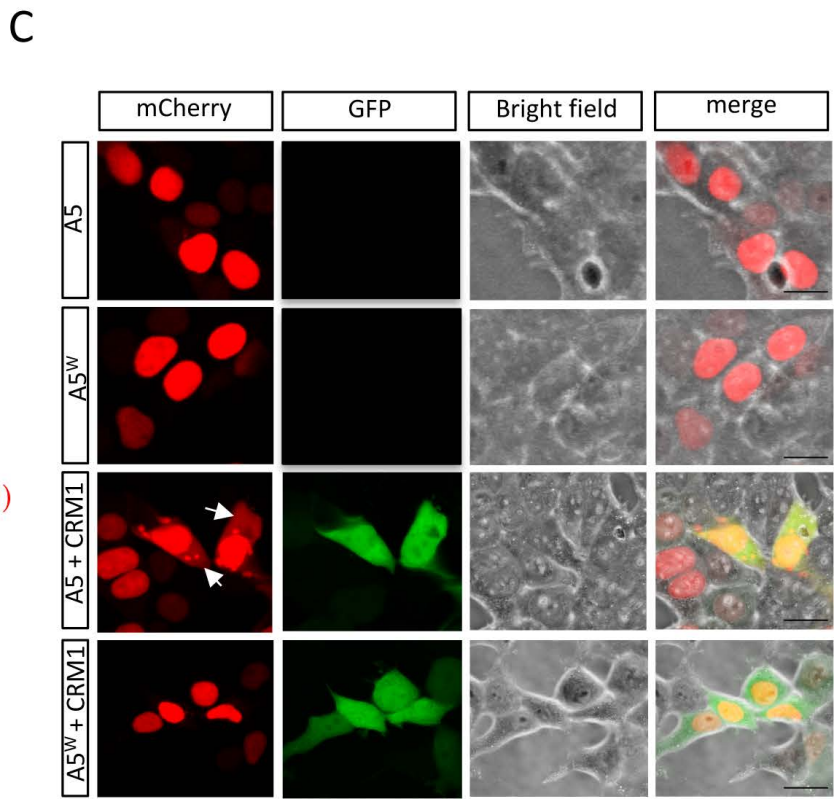
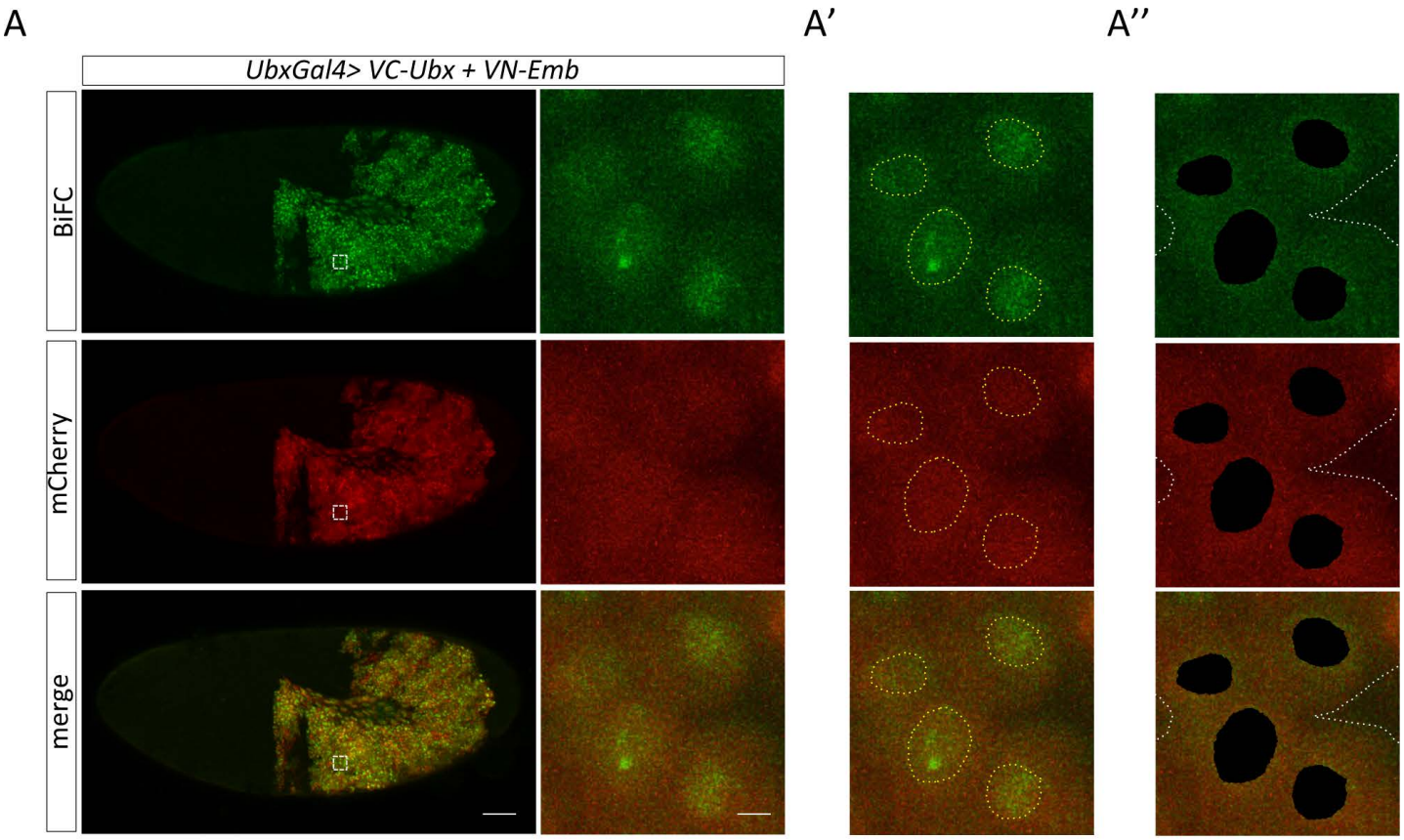


Figure S3. The W-containing motif is part of an unconventional NES in other Hox proteins. A-A'. Immunoblots of GST-pull-down assay using *in vitro* produced GST-fused derivatives (GST alone; GST-Scr-wild type, WT; GST-Scr-W-containing motif mutated, HX) and whole S2R+ cell extracts expressing Flag-Exd (C) or HA-Emb (C'). HA antibody was used to recognize HA-Emb. Flag antibody was used to recognize Flag-Exd. Inputs (Ip) are systematically loaded, as indicated (first lane of each gel). Pull-down assays showed that Scr-WT and Scr-W could interact with Exd and Emb (lanes 3-4). Quantification of interactions relative to GST-Scr signal indicates that the interaction between Emb and Scr-W is decrease of 30% on average when compared to the Scr-WT form. In contrast, the W-containing motif mutation affected 90% of the interaction with Exd. Quantification was calculated with ANOVA from four independent experiments. **B.** Sequence of the putative non-canonical NES overlapping with the W-containing motif of HOXA5. The W-containing motif mutation is indicated (red). Prediction of NES with NetNES is shown below the schematized protein (with one canonical NES in the HD). **C.** Wild type and mutated W-containing motif HOXA5-mCherry proteins are localized in the nucleus upon transfection in HEK cells. Co-expression of CRM1 induced cytoplasmic localization of wild type but not mutated W-containing motif of HOXA5-mCherry. This effect was observed in 100% of co-transfected cells. Cells expressing CRM1 are recognized by the GFP that is fused downstream of CRM1 (see Materials and Methods). Scale bars are for 10μm.



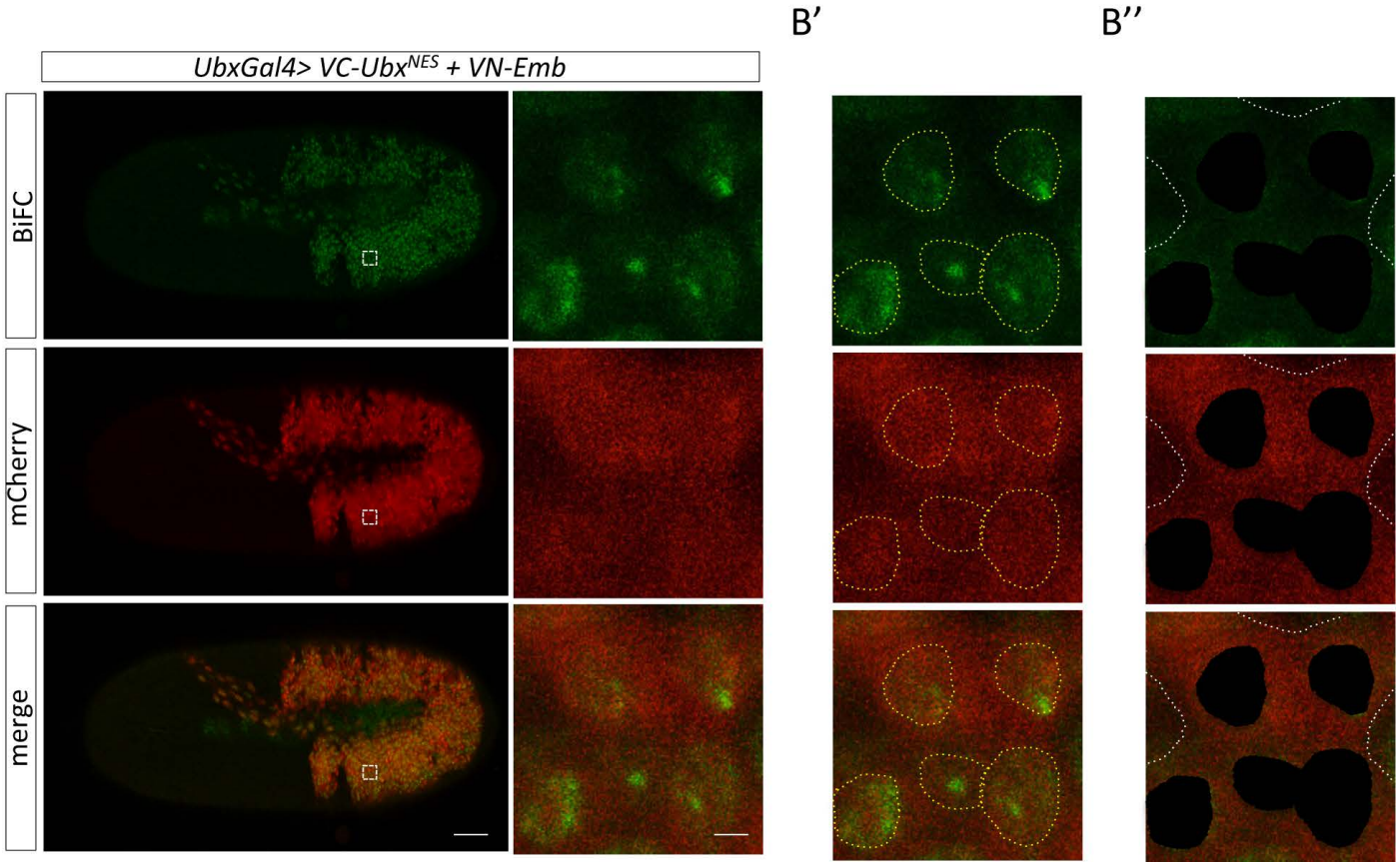


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Figure S4. Quantification of BiFC between VC-Ubx and VN-Emb or VC-Ubx^{NES} and VN-Emb in the nuclear or cytoplasmic compartment of epidermal expressing cells of live embryos. **A.** BiFC (green) between VC-Ubx and VN-Emb expressed with the mCherry reporter (red) in a stage 10 live embryo. Enlargement on few expressing cells (white-dotted square) is shown on the right. **A'.** Illustrative calque used in Fiji to quantify BiFC specifically in nuclei (yellow-dotted circles). The calque is applied based on a threshold that allows discarding weak BiFC signals of the cytoplasm and the background. **A".** Illustrative calque used in Fiji to quantify BiFC specifically in the cytoplasm of expressing cells. A calque is applied to remove the specific BiFC signal of nuclei by using the same threshold as in A' (black-filled circles). An additional calque is applied to remove non-expressing cells, based on a threshold that allows discarding the background in the mCherry channel (white-dotted lines). BiFC is then quantified in the remaining mCherry-positive space of the field, which corresponds to the cytoplasm of expressing cells. **B.** BiFC (green) between VC-Ubx mutated in the NES and VN-Emb expressed with the mCherry reporter (red) in a stage 10 live embryo. Enlargement on few expressing cells (white-dotted square) is shown on the right. **B'-B".** Calques are defined as described in A'-A" for quantifying BiFC in the nucleus or cytoplasm of expressing cells. Scale bars are for 60μm (full embryo) or 3μm (enlargement).

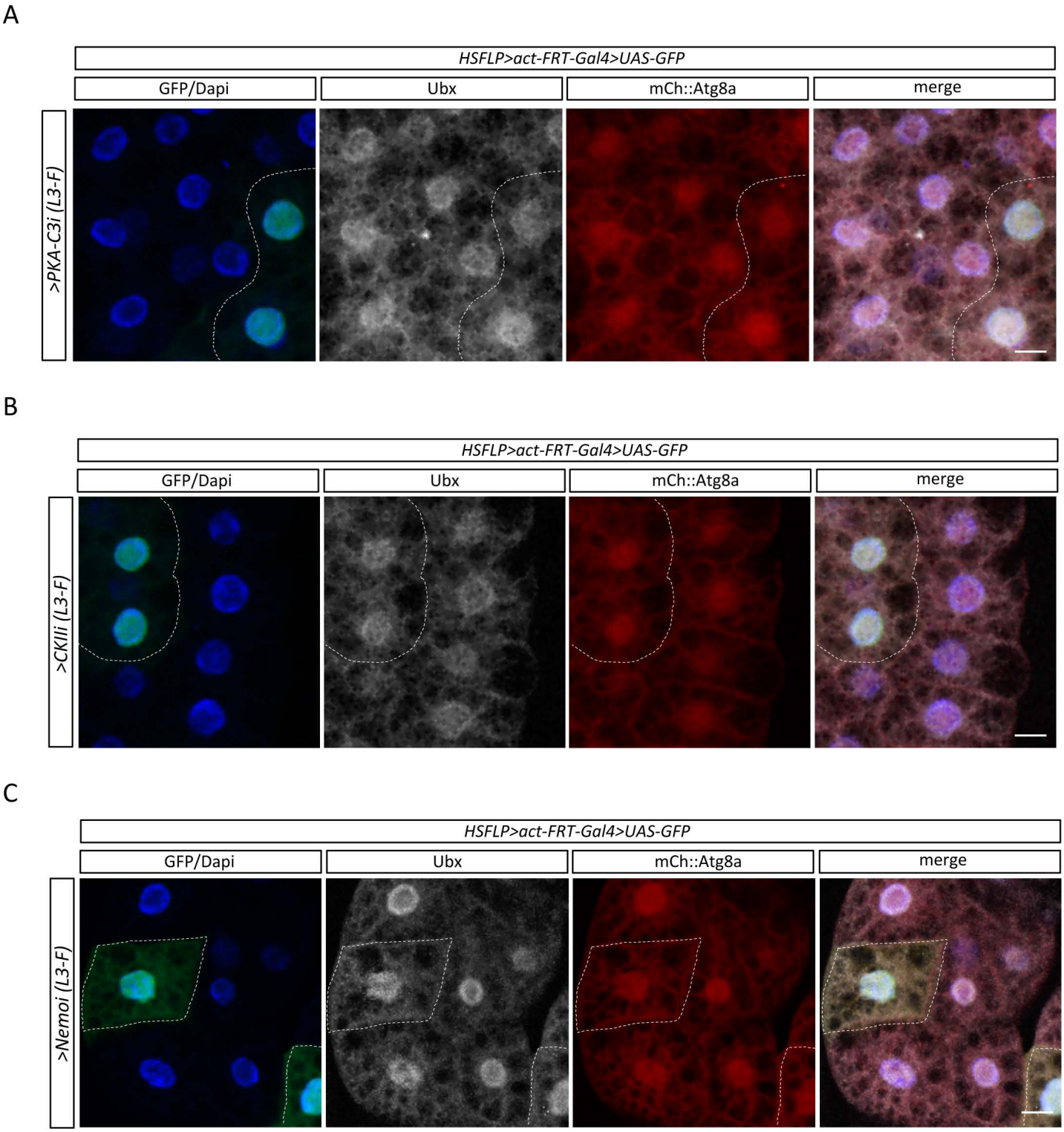

Figure S5. Inhibition of kinases affects neither the nuclear localization of Ubx nor the autophagy repression in the fat body of L3-F stage larva. A. Inhibition of the protein kinase PKA-C3, as indicated. **B.** Inhibition of the protein kinase CKII, as indicated. **C.** Inhibition of the protein kinase Nemo, as indicated. Scale bars are for 10μm.