RESEARCH ARTICLE

Figures and figure supplements

Structural rather than catalytic role for mitochondrial respiratory chain supercomplexes

Michele Brischigliaro *et al*.

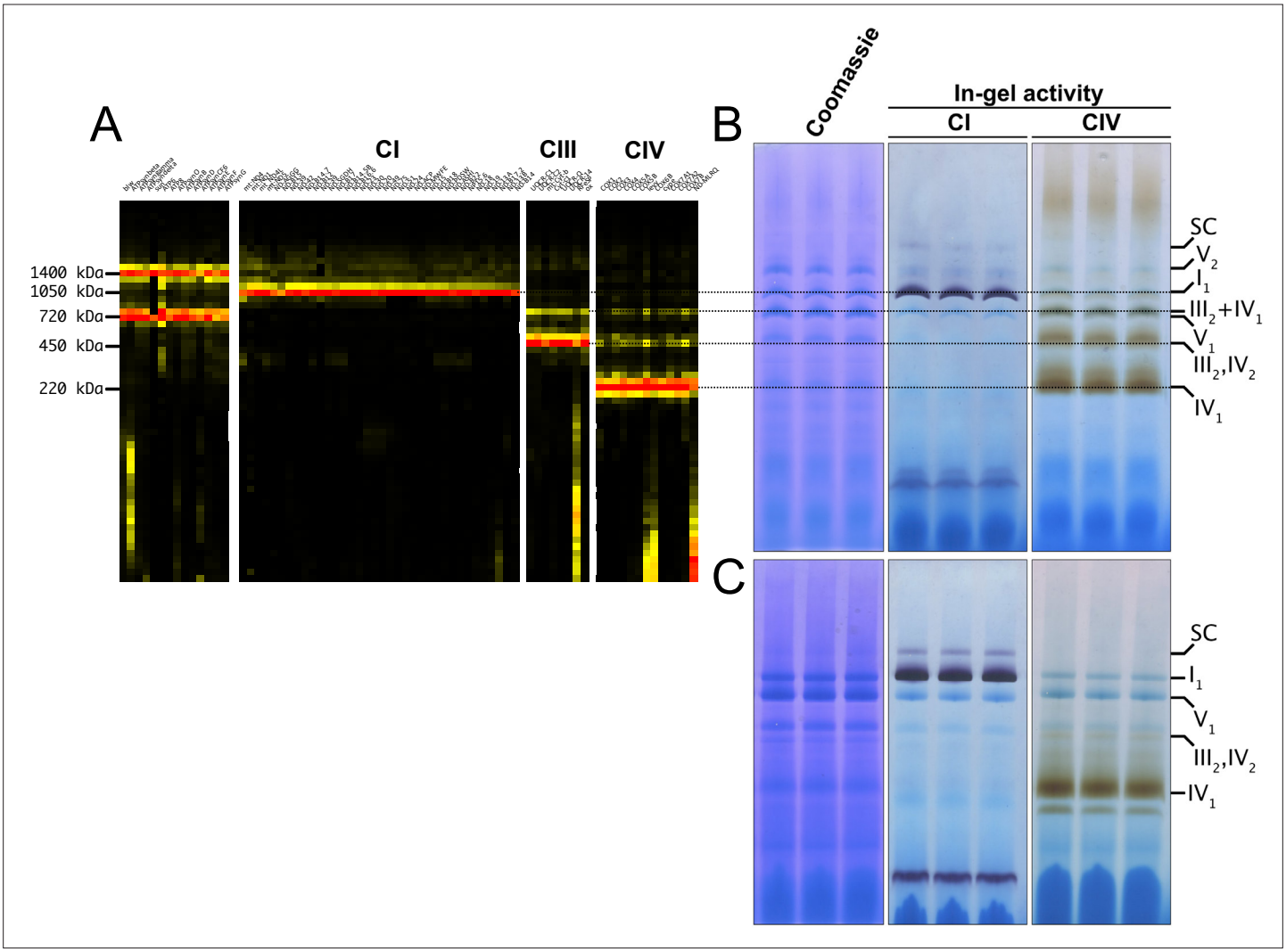

Figure 1. *D. melanogaster* mitochondrial respiratory chain does not rely on SC formation under physiological conditions. (**A**) Complexome profiling of wild-type *D. melanogaster* mitochondria. Heatmaps show relative abundance of MRC subunits belonging to complex I (CI), complex II (CII), complex III_2 (CIII), and complex IV (CIV). Color scale of normalized peptide intensities are 0 (black), 96th percentile (yellow) and 1 (red). (**B**) BN-PAGE separation of mitochondria from wild-type *D. melanogaster* solubilized with digitonin. Native gels were either stained with Coomassie R250 or analyzed by in-gel activity (IGA) for complex I (CI) and complex IV (CIV). (**C**) BN-PAGE separation of mitochondria from wild-type *D. melanogaster* solubilized with dodecylmaltoside (DDM). Native gels were either stained with Coomassie R250 or analyzed by in-gel activity assay (IGA) for complex I (CI) and complex IV (CIV).

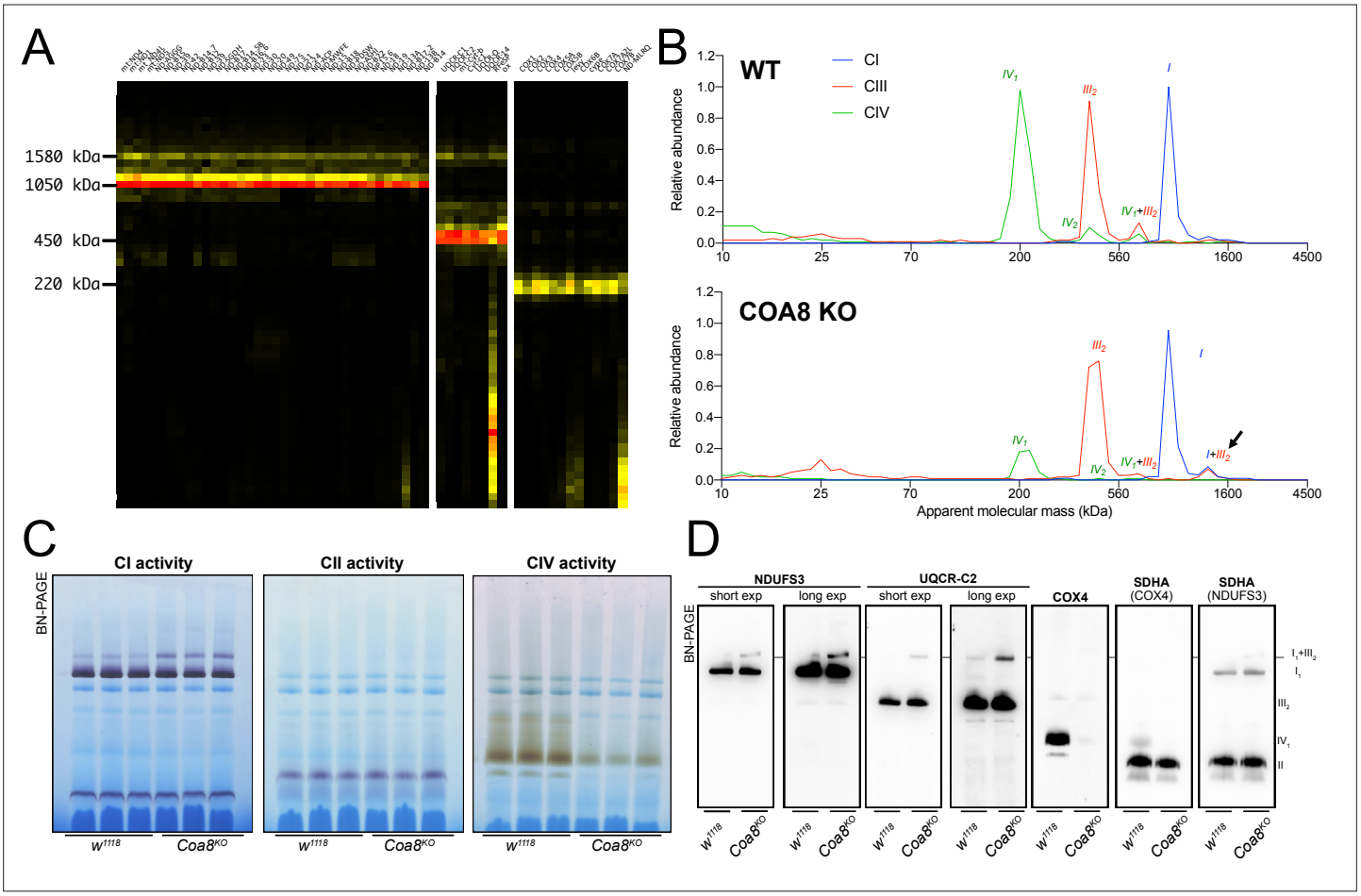

Figure 2. Severely perturbed CIV assembly results in increased formation of SC I_1III_2. (**A**) Complexome profiling of Coa8 KO *D. melanogaster* mitochondria. Heatmaps show relative abundance of MRC subunits belonging to complex I (CI), complex II (CII), complex III_2 (CIII) and complex IV (CIV). Color scale of normalized peptide intensities are 0 (black), 96° percentile (yellow) and 1 (red). (**B**) Average MS profiles depicted as relative abundance of MRC enzymes in natively separated complexes from wild-type (top) and Coa8 KO (bottom) fly mitochondria. Profiles of complexes I, III_2 and V (CI, CIII and CV) are plotted as average peptide intensity of the specific subunits identified by MS for each complex vs. apparent molecular weight. The increase in the relative abundance of SC I_1III_2 in Coa8 KO mitochondria is indicated by a black arrow. (**C**) In gel-activity assays for MRC complex I (CI), complex II (CII), and complex IV (CIV) in DDM-solubilized mitochondria from wild-type (w^{1118}) and Coa8 KO ($Coa8^{KO}$) flies. (**D**) BN-PAGE, western blot immunodetection of MRC complexes from a pool of three control wild-type (w^{1118}) and three Coa8 KO ($Coa8^{KO}$) fly mitochondria preparations, using antibodies against specific subunits: anti-UQCRC2 (complex III), anti-NDUFS3 (complex I), anti-COX4 (complex IV), and anti-SDHA (complex II).

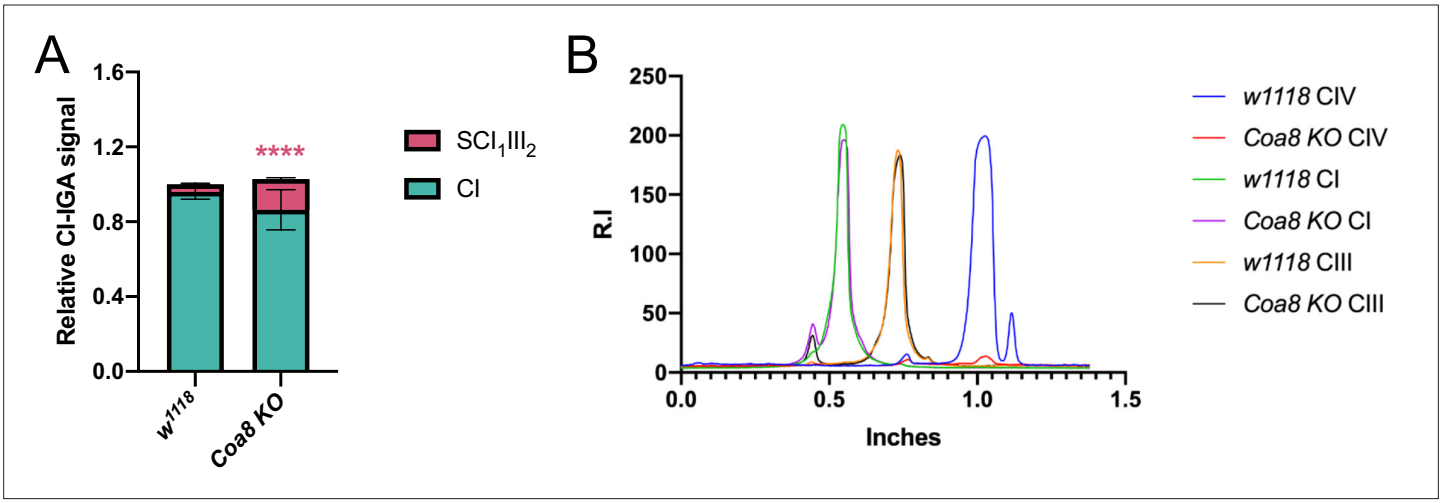

Figure 2—figure supplement 1. Quantification of complex and supercomplex bands in *Coa8*^{KO} fly mitochondria. (**A**) The signal intensity of the bands from CI- and CII-in gel activity assays (IGA) in mitochondria from the *Coa8*^{KO} strain, and its corresponding control, were quantified using the Gel analyzer 19.1 software. The graphs show the relative signal intensity of each CI-reactive band normalized to the intensity of the CII-reactive band from the same sample (n=3 biological replicates for each genotype, pairwise comparisons by unpaired Student's t test ****p≤0.0001). The total relative intensity of all the CI-containing bands in the control samples was set to 1. Green bars = data corresponding to free complex I; red bars and stars = data corresponding to supercomplex containing CI and $CIII_2$. (**B**) Western blot signal intensity profiles obtained with Fiji (ImageJ) applying the same ROI (region of interest) in the BNGE lanes of *Coa8*^{KO} in comparison with the corresponding control. Relative intensity (R.I.) of the bands along the lane length (in inches) was plotted. High molecular weights are on the left and low molecular weights are on the right.

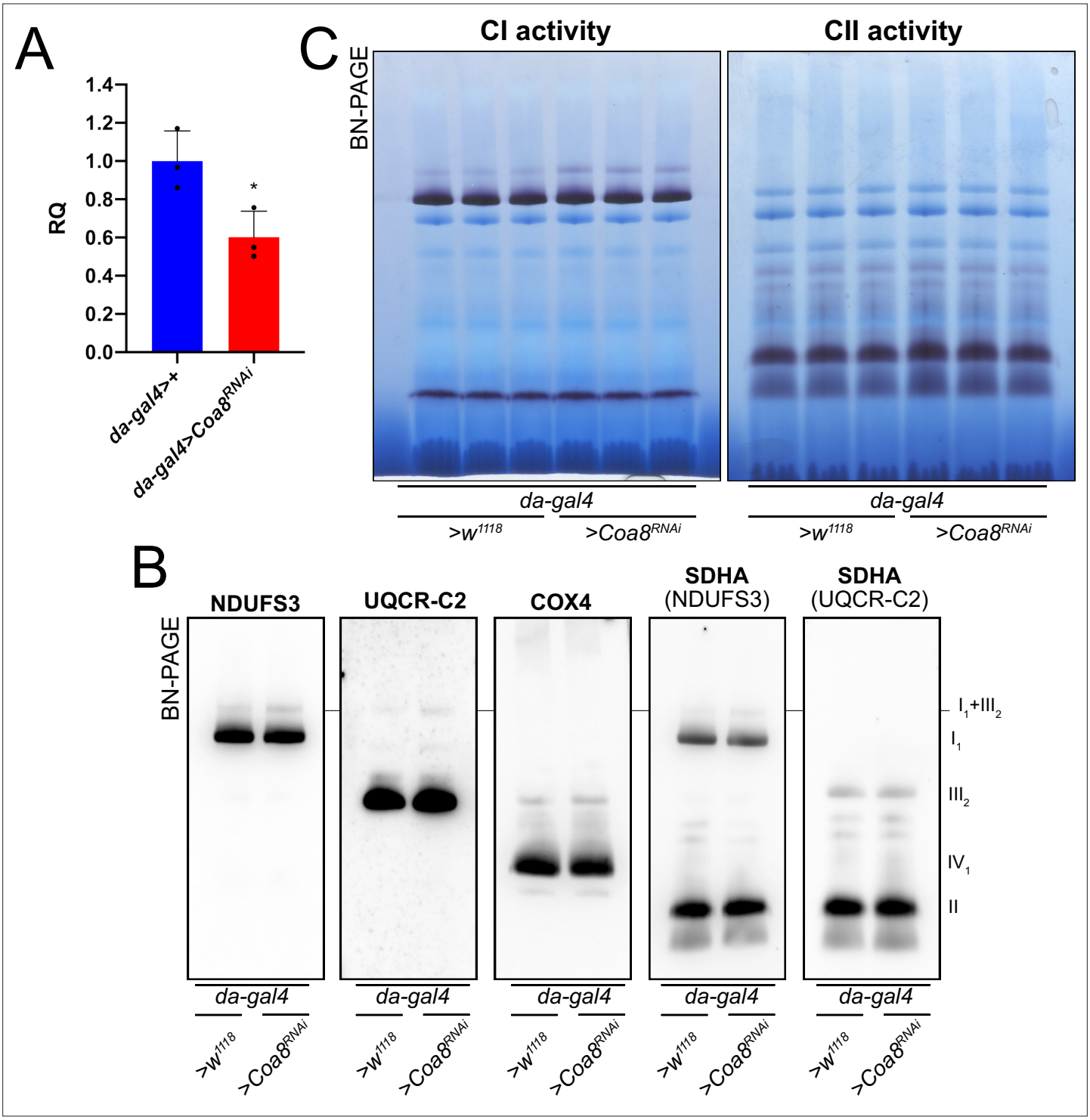

Figure 3. Mildly perturbed CIV assembly results in increased formation of SC I_1III_2. (**A**) Relative quantification (RQ) of *Coa8* mRNA expression in control (*da-gal4>+*) and *Coa8* KD (*da-gal4 >Coa8* RNAi) flies measured by qPCR. Data are plotted as mean ± SD (n = 3 biological replicates, Student's t test *p ≤ 0.05). (**B**) In gel-activity assays for MRC complex I (CI), complex II (CII) and complex IV (CIV) in DDM-solubilized mitochondria from control (*da-gal4>+*) and *Coa8* KD (*da-gal4 >Coa8* RNAi) flies. (**C**) BN-PAGE, western blot immunodetection of MRC complexes from a pool of three control (*da-gal4>+*) and three Coa8 KD (*da-gal4 >Coa8* RNAi) fly mitochondria samples, using antibodies against specific subunits: anti-UQCRC2 (complex III), anti-NDUFS3 (complex I), anti-COX4 (complex IV), and anti-SDHA (complex II).

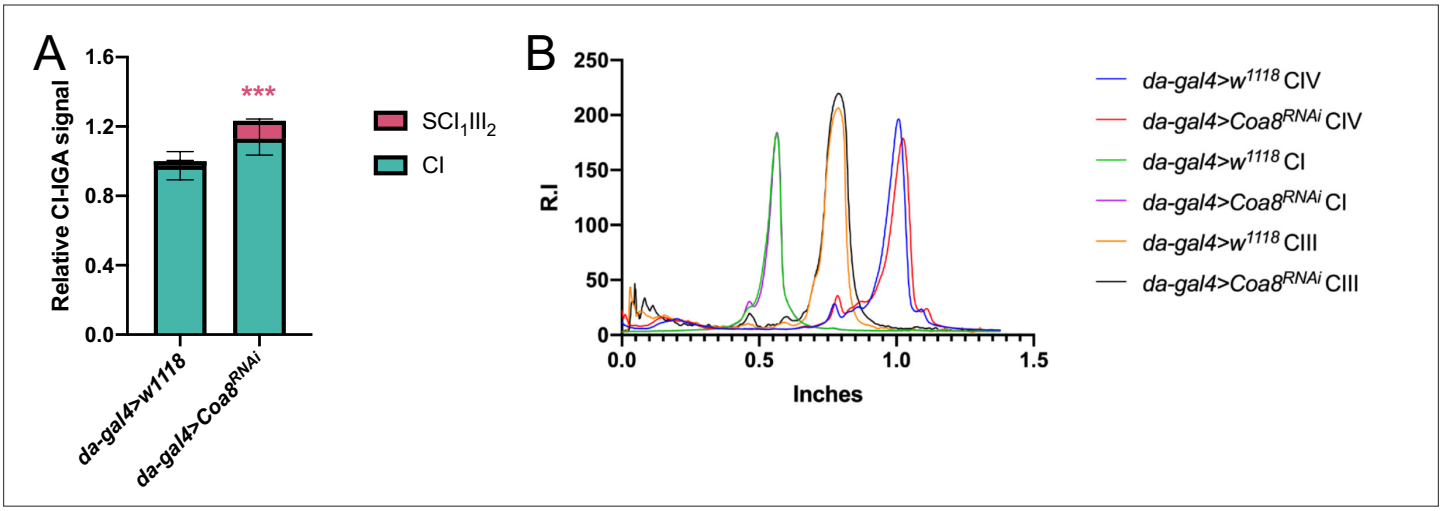

Figure 3—figure supplement 1. Quantification of complex and supercomplex bands in *Coa8* KD fly mitochondria. (**A**) The signal intensity of the bands from CI- and CII-in gel activity assays (IGA) in mitochondria from the mild *Coa8*^{RNAi} strain, and its corresponding control, were quantified using the Gel analyzer 19.1 software. The graphs show the relative signal intensity of each CI-reactive band normalized to the intensity of the CII-reactive band from the same sample (n=3 biological replicates for each genotype, pairwise comparisons by unpaired Student's t test ***$P \leq 0.001$). The total relative intensity of all the CI-containing bands in the control samples was set to 1. Green bars = data corresponding to free complex I; red bars and stars = data corresponding to supercomplex containing CI and $CIII_2$. (**B**) Western Blot signal intensity profiles obtained with Fiji (ImageJ) applying the same ROI (region of interest) in the BNGE lanes of *Coa8*^{RNAi} in comparison with the corresponding control. Relative intensity (R.I.) of the bands along the lane length (in inches) was plotted. High molecular weights are on the left and low molecular weights are on the right.

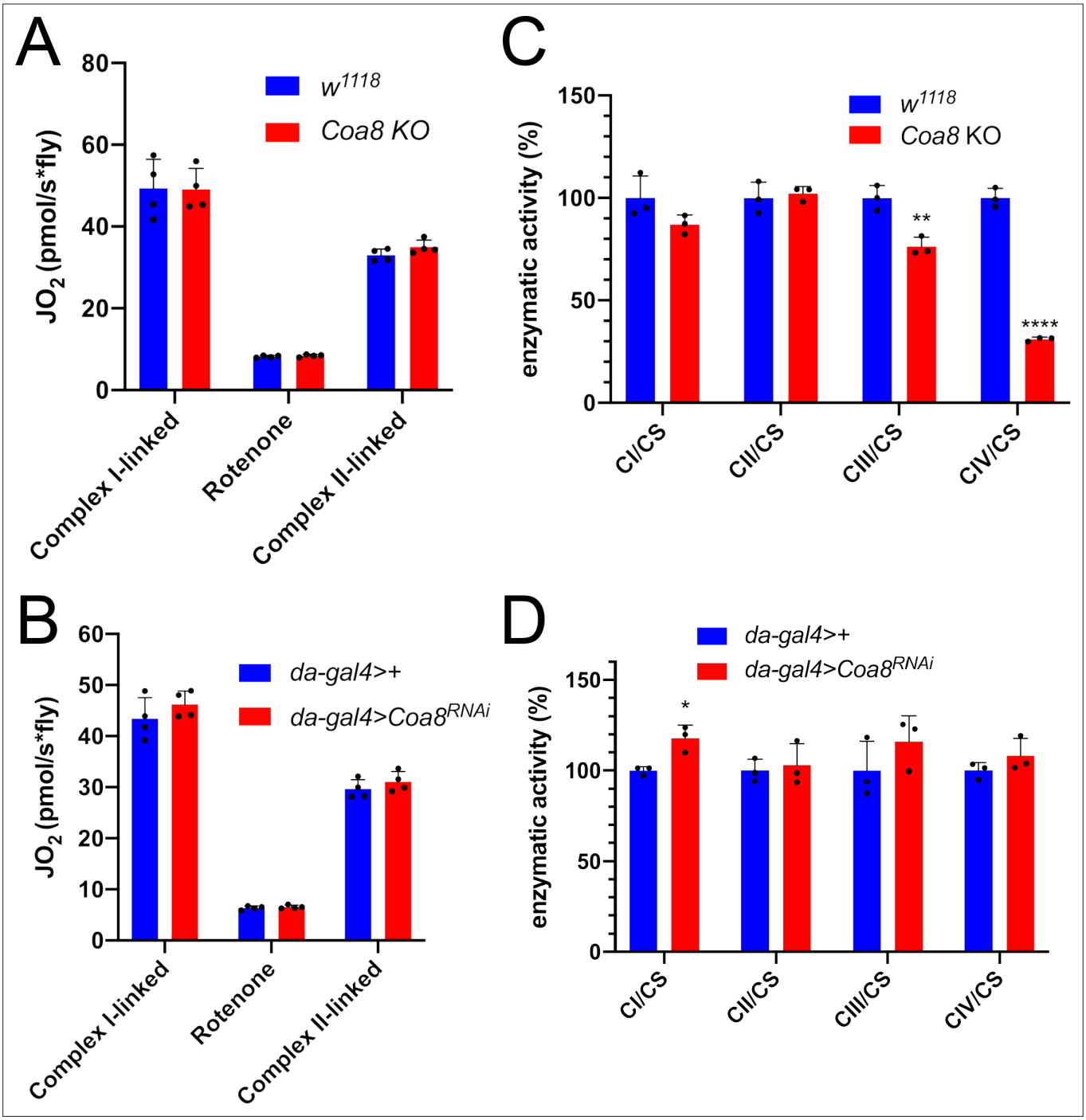

Figure 4. Enhanced formation of SC I_1III_2 does not result in increased respiration. (**A–B**) High-resolution respirometry (HRR) analyses of whole-fly homogenates. Respiration is represented by oxygen flux (JO_2) measured by oxygen consumption rates (OCR – pmol/s*fly). OCR have been measured via substrate-driven respiration under saturating concentrations of substrates inducing either complex I (CI) or complex II (CII) -linked respiration. Rotenone was used to block CI-linked respiration before measuring CII-linked respiration. HRR was performed on (**A**) *Coa8* KO and (**B**) *Coa8* KD fly homogenates compared to relative controls. Data are plotted as mean ± SD (n = 4 biological replicates). (**C–D**) Kinetic enzyme activity of individual MRC complexes in (**C**) *Coa8* KO and (**D**) *Coa8* KD compared with the relative control individuals, normalized by citrate synthase (CS) activity. Data are plotted as mean ± SD (n = 3 biological replicates, pairwise comparisons by unpaired Student's t test *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001).

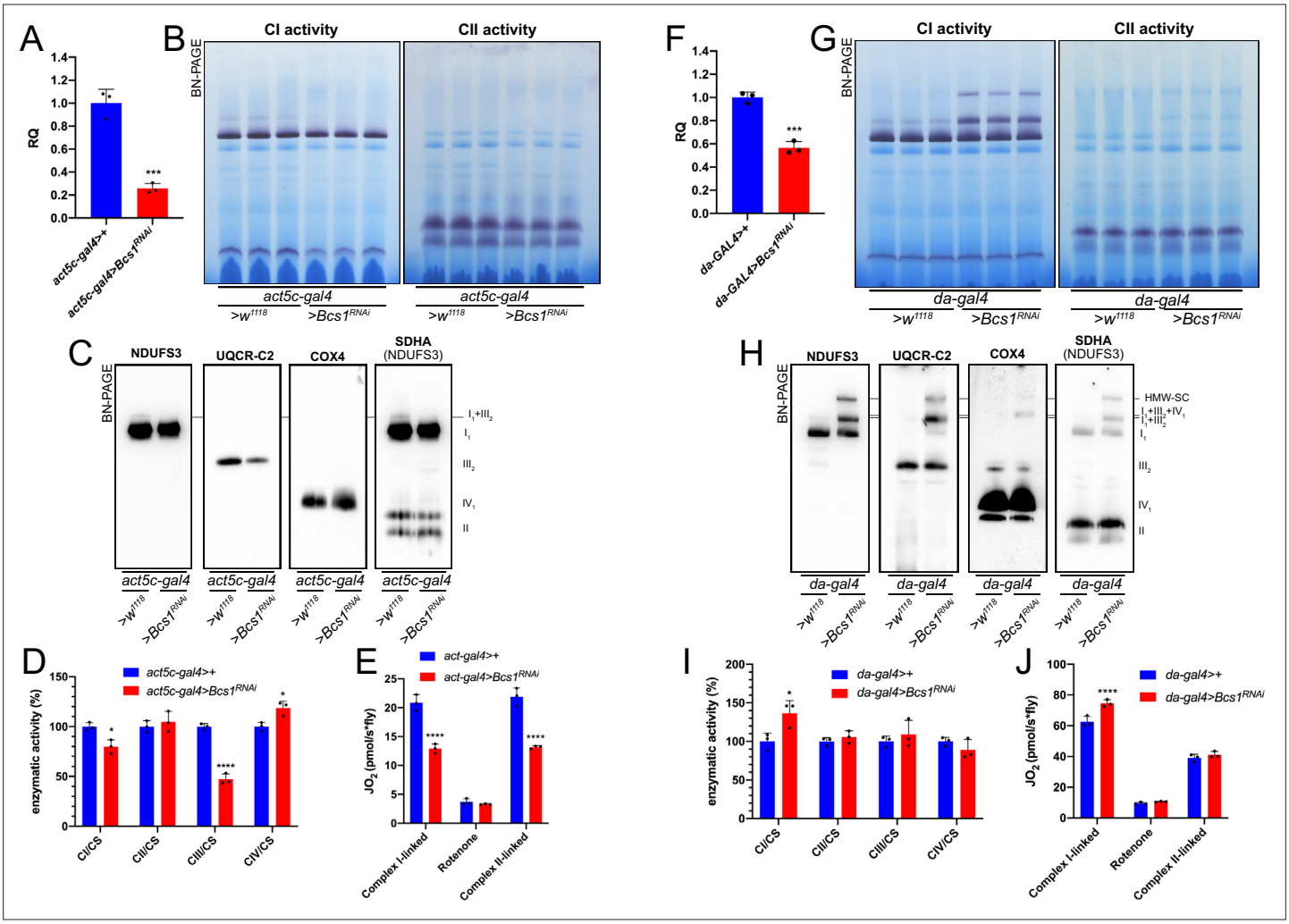

Figure 5. Mild perturbation of $CIII_2$ biogenesis enhances SC formation in *D. melanogaster*. (**A**) Relative quantification (RQ) of *Bcs1* mRNA expression in control (*act5c-gal4>+*) and *Bcs1* KD (*act5c-gal4>Bcs1* RNAi) larvae measured by qPCR. Data are plotted as mean ± SD (n = 3 biological replicates, Student's t test ***p ≤ 0.001). (**B**) In gel-activity assays for MRC complex I (CI), complex II (CII) and complex IV (CIV) in DDM-solubilized mitochondria from control (*act5c-gal4>+*) and *Bcs1* KD (*act5c-gal4>Bcs1* RNAi) larvae. (**C**) BN-PAGE, western blot immunodetection of MRC complexes from a pool of three control (*act5c-gal4>+*) and three *Bcs1* KD (*act5c-gal4>Bcs1* RNAi) larvae mitochondria samples, using antibodies against specific subunits: anti-UQCRC2 (complex III), anti-NDUFS3 (complex I), anti-COX4 (complex IV) and anti-SDHA (complex II). (**D**) Kinetic enzyme activity of individual MRC complexes in control (*act5c-gal4>+*) and *Bcs1* KD (*act5c-gal4>Bcs1* RNAi) larvae mitochondria normalized by citrate synthase (CS) activity. Data are plotted as mean ± SD (n = 3 biological replicates, pairwise comparisons by unpaired Student's t test, *p ≤ 0.05, ****p ≤ 0.0001). (**E**) High-resolution respirometry (HRR) analyses of whole-fly homogenates. Respiration is represented by oxygen flux (JO_2) measured by oxygen consumption rates (OCR – pmol/s*fly). OCR have been measured via substrate-driven respiration under saturating concentrations of substrates inducing either complex I (CI) or complex II (CII) -linked respiration. Rotenone was used to block CI-linked respiration before measuring CII-linked respiration. HRR was performed on control (*act5c-gal4>+*) and *Bcs1* KD (*act5c-gal4>Bcs1* RNAi) homogenates compared to relative controls. Data are plotted as mean ± SD (n = 3 biological replicates, two-way ANOVA with Sidak's multiple comparisons, ****p ≤ 0.0001). (**F**) Relative quantification (RQ) of *Bcs1* mRNA expression in control (*da-gal4>+*) and *Bcs1* KD (*da-gal4 >Bcs1* RNAi) larvae measured by qPCR. Data are plotted as mean ± SD (n = 3 biological replicates, Student's t test ***p ≤ 0.001). (**G**) In gel-activity assays for MRC complex I (CI), complex II (CII) and complex IV (CIV) in DDM-solubilized mitochondria from control (*da-gal4>+*) and *Bcs1* KD (*da-gal4 >Bcs1* RNAi) larvae. (**H**) BN-PAGE, western blot immunodetection of MRC complexes from a pool of three control (*da-gal4>+*) and three *Bcs1* KD (*da-gal4 >Bcs1* RNAi) larvae mitochondria samples using antibodies against specific subunits: anti-UQCRC2 (complex III), anti-NDUFS3 (complex I), anti-COX4 (complex IV), and anti-SDHA (complex II). HWM-SC: high molecular weight supercomplex. (**I**) Kinetic enzyme activity of individual MRC complexes in control (*da-gal4>+*) and *Bcs1* KD (*da-gal4 >Bcs1* RNAi) larvae mitochondria normalized by citrate synthase (CS) activity. Data are plotted as mean ± SD (n = 3 biological replicates, pairwise comparisons by unpaired Student's t test, *p ≤ 0.05). (**J**) High-resolution respirometric (HRR) analyses of whole-fly homogenates. Respiration is represented by oxygen flux (JO_2) measured by oxygen consumption rates (OCR - pmol/s*fly). OCR have been measured via substrate-driven respiration under saturating concentrations of substrates inducing either complex I (CI) or complex II (CII) -linked respiration. Rotenone was used to block CI-linked respiration before measuring CII-linked respiration. HRR was performed on control (*act5c-gal4>+*) and *Bcs1* KD (*act5c-gal4>Bcs1* RNAi) homogenates compared to relative controls. Data are plotted as mean ± SD (n = 3 biological replicates, two-way ANOVA with Sidak's multiple comparisons, ****p ≤ 0.0001).

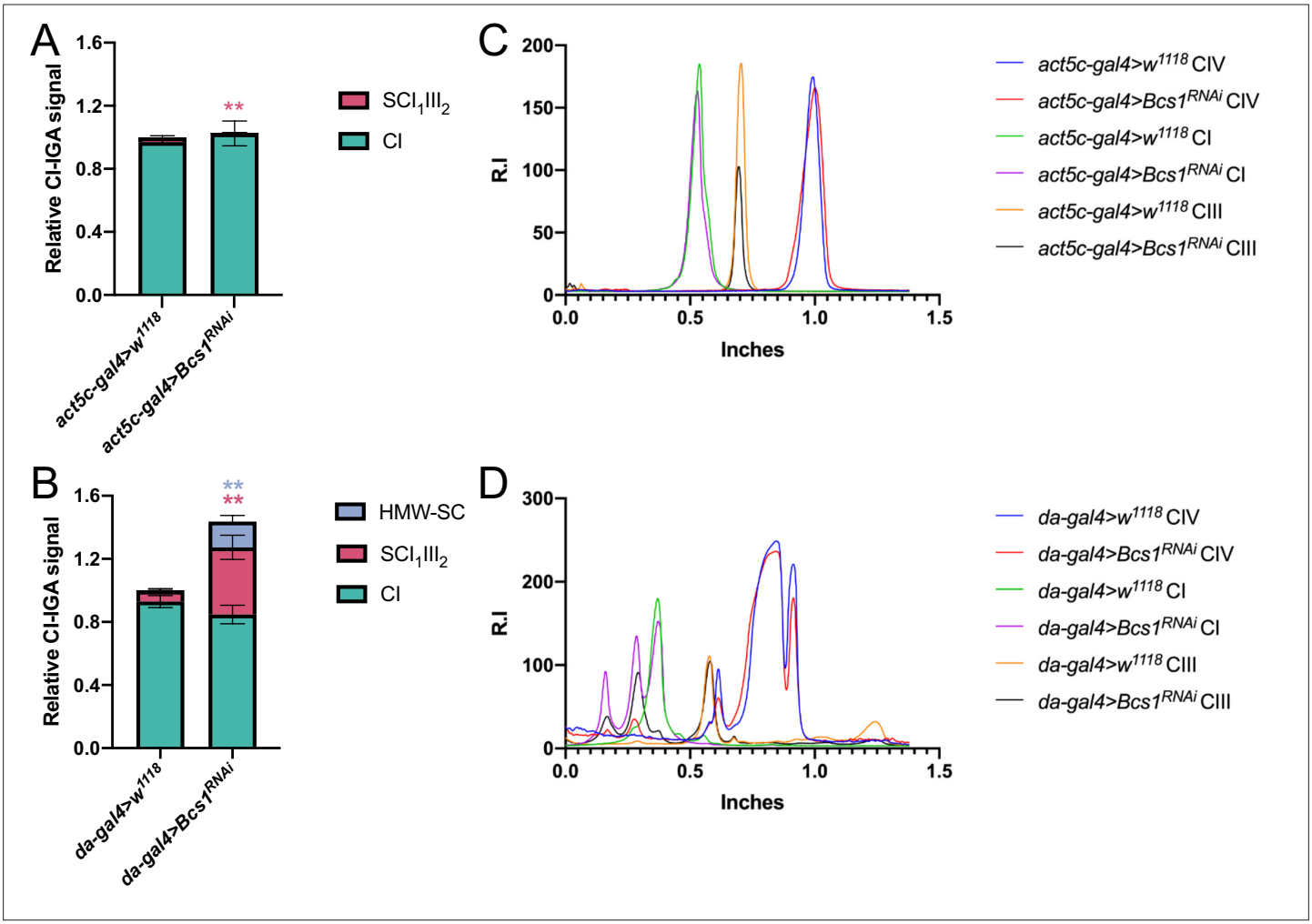

Figure 5—figure supplement 1. Quantification of complex and supercomplex bands in *Bcs1* KD fly mitochondria. The signal intensity of the bands from CI- and CII-in gel activity assays (IGA) of the mutated fly strains: (**A**) strong $Bcs1^{RNAi}$ and (**B**) mild $Bcs1^{RNAi}$ and their corresponding controls were quantified using the Gel analyzer 19.1 software. The graphs show the relative signal intensity of each CI-reactive band normalized to the intensity of the CII-reactive band from the same sample (n=3 biological replicates for each genotype, pairwise comparisons by unpaired Student's t test **p≤0.01). The total relative intensity of all the CI-containing bands in the control samples was set to 1. Green bars = data corresponding to free complex I; red bars and stars = data corresponding to supercomplex containing CI and $CIII_2$ (and CIV in the case of the mild Bcs1 KD); blue bars and stars = data corresponding to supercomplex containing CI, $CIII_2$ and CIV. HMW-SC: high molecular weight supercomplex of unknown stoichiometry containing CI, $CIII_2$, and CIV. (**C**) and (**D**) Western Blot signal intensity profiles obtained with Fiji (ImageJ) applying the same ROI (region of interest) in the BNGE lanes of strong $Bcs1^{RNAi}$ and mild $Bcs1^{RNAi}$, respectively, in comparison with their corresponding controls. Relative intensity (R.I.) of the bands along the lane length (in inches) was plotted. High molecular weights are on the left and low molecular weights are on the right.

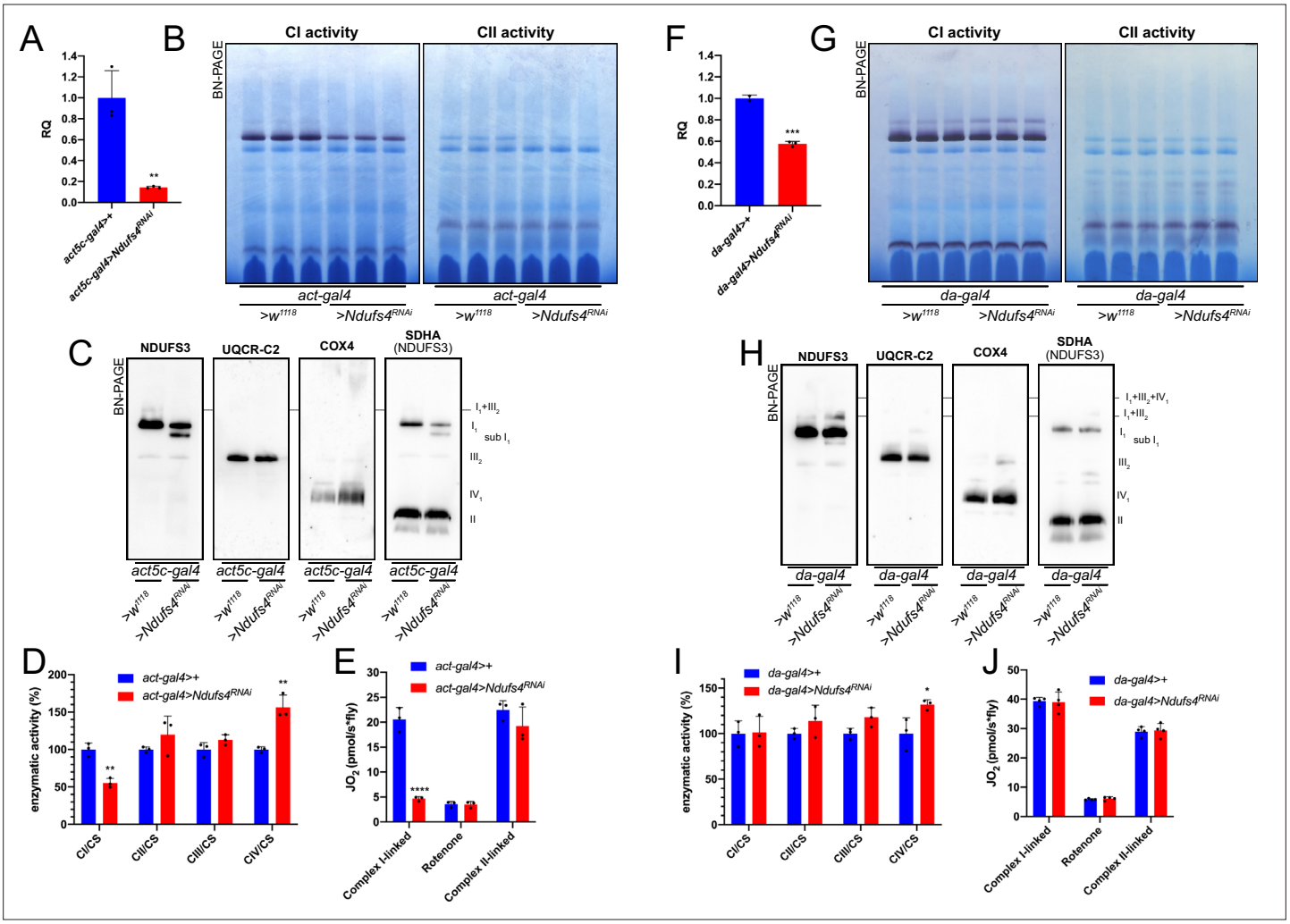

Figure 6. Mild perturbation of CI biogenesis enhances SC formation in *D. melanogaster*. (**A**) Relative quantification (RQ) of *Ndufs4* mRNA expression in control (*act5c-gal4>+*) and *Ndufs4* KD (*act5c-gal4>Ndufs4* RNAi) larvae measured by qPCR. Data are plotted as mean ± SD (n = 3 biological replicates, Student's t test **p ≤ 0.01). (**B**) In gel-activity assays for MRC complex I (CI), complex II (CII) and complex IV (CIV) in DDM-solubilized mitochondria from control (*act5c-gal4>+*) and *Ndufs4* KD (*act5c-gal4>Ndufs4* RNAi) larvae. (**C**) BN-PAGE, western blot immunodetection of MRC complexes from a pool of three control (*act5c-gal4>+*) and three *Ndufs4* KD (*act5c-gal4>Ndufs4* RNAi) larvae mitochondria samples, using antibodies against specific subunits: anti-UQCRC2 (complex III), anti-NDUFS3 (complex I), anti-COX4 (complex IV) and anti-SDHA (complex II). (**D**) Kinetic enzyme activity of individual MRC complexes in control (*act5c-gal4>+*) and *Ndufs4* KD (*act5c-gal4>^{RNAi}*) larvae mitochondria normalized by citrate synthase (CS) activity. Data are plotted as mean ± SD (n = 3 biological replicates, pairwise comparisons by unpaired Student's t test, **p ≤ 0.01). (**E**) High-resolution respirometry (HRR) analyses of whole-fly homogenates. Respiration is represented by oxygen flux (JO_2) measured by oxygen consumption rates (OCR – pmol/s*fly). OCR have been measured via substrate-driven respiration under saturating concentrations of substrates inducing either complex I (CI) or complex II (CII) -linked respiration. Rotenone was used to block CI-linked respiration before measuring CII-linked respiration. HRR was performed on control (*act5c-gal4>+*) and *Ndufs4* KD (*act5c-gal4>Ndufs4* RNAi) homogenates compared to relative controls. Data are plotted as mean ± SD (n = 3 biological replicates, two-way ANOVA with Sidak's multiple comparisons, ****p ≤ 0.0001). (**F**) Relative quantification (RQ) of *Ndufs4* mRNA expression in control (*da-gal4>+*) and *Ndufs4* KD (*da-gal4 >Ndufs4* RNAi) larvae measured by qPCR. Data are plotted as mean ± SD (n = 3 biological replicates, Student's t test ***p ≤ 0.001). (**G**) In gel-activity assays for MRC complex I (CI), complex II (CII) and complex IV (CIV) in DDM-solubilized mitochondria from control (*da-gal4>+*) and *Ndufs4* KD (*da-gal4 >Ndufs4* RNAi) larvae. (**H**) BN-PAGE, western blot immunodetection of MRC complexes from a pool of three control (*da-gal4>+*) and three *Ndufs4* KD (*da-gal4 >Ndufs4* RNAi) larvae mitochondria samples, using antibodies against specific subunits: anti-UQCRC2 (complex III), anti-NDUFS3 (complex I), anti-COX4 (complex IV) and anti-SDHA (complex II). (**I**) Kinetic enzyme activity of individual MRC complexes in control (*da-gal4>+*) and *Ndufs4* KD (*da-gal4 >Ndufs4* RNAi) larvae mitochondria normalized by citrate synthase (CS) activity. Data are plotted as mean ± SD (n = 3 biological replicates, pairwise comparisons by unpaired Student's t test, *p ≤ 0.05). (**J**) High-resolution respirometry (HRR) analyses of whole-fly homogenates. Respiration is represented by oxygen flux (JO_2) measured by oxygen consumption rates (OCR - pmol/s*fly). OCR have been measured via substrate-driven respiration under saturating concentrations of substrates inducing either complex I (CI) or complex II (CII) -linked respiration. Rotenone was used to block CI-linked respiration before measuring CII-linked respiration. HRR was performed on control (*act5c-gal4>+*) and *Ndufs4* KD (*act5c-gal4>^{RNAi}*) homogenates compared to relative controls. Data are plotted as mean ± SD (n = 4 biological replicates, two-way ANOVA with Sidak's multiple comparisons).

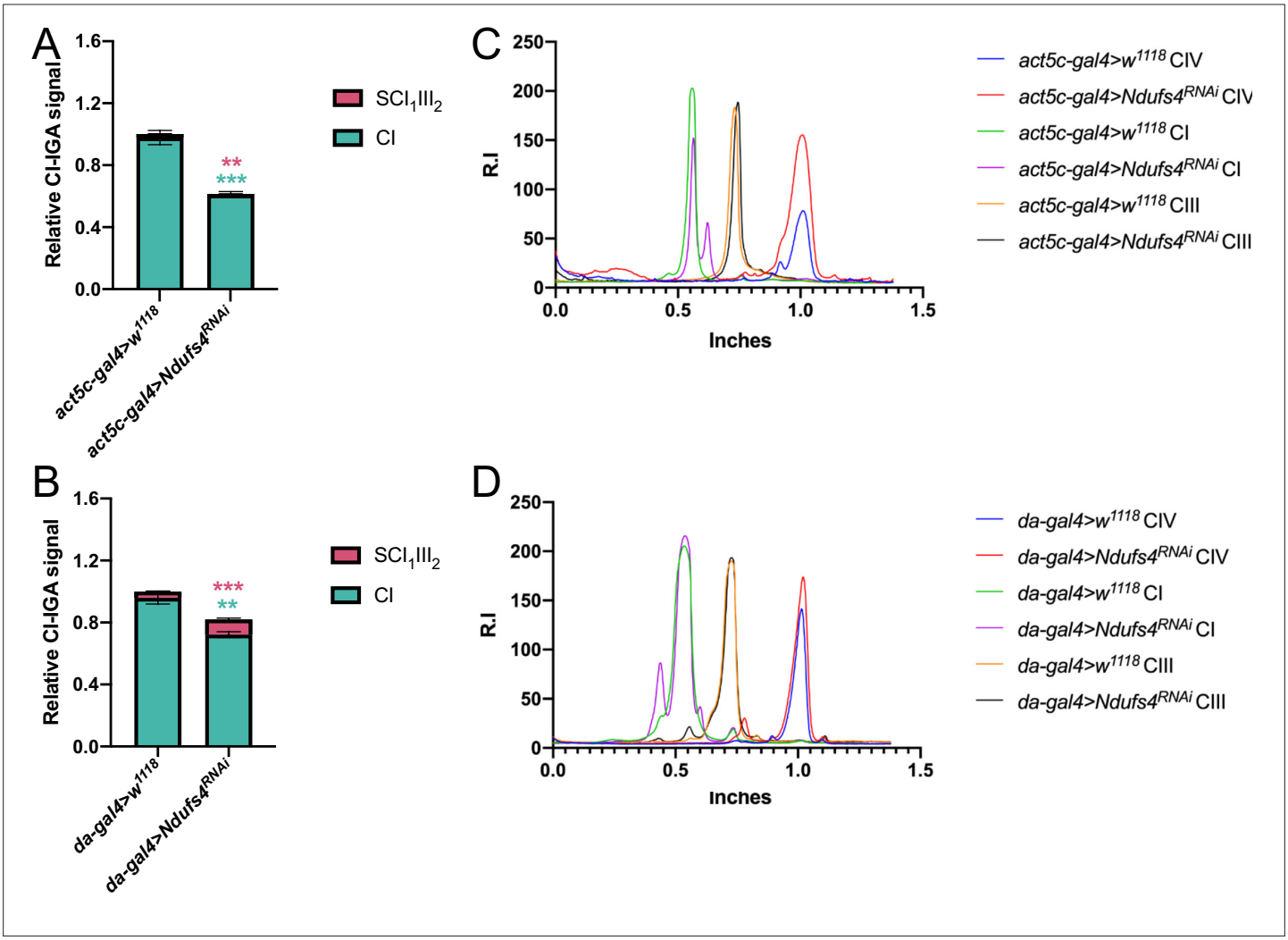

Figure 6—figure supplement 1. Quantification of complex and supercomplex bands in *Ndufs4* KD fly mitochondria. The signal intensity of the bands from CI- and CII-in gel activity assays (IGA) of the mutated fly strains: (**A**) strong *Ndufs4*^{RNAi} and (**B**) mild *Ndufs4*^{RNAi} and their corresponding controls were quantified using the Gel analyzer 19.1 software. The graphs show the relative signal intensity of each CI-reactive band normalized to the intensity of the CII-reactive band from the same sample (n=3 biological replicates for each genotype, pairwise comparisons by unpaired Student's t test **p≤0.01, ***p≤0.001). The total relative intensity of all the CI-containing bands in the control samples was set to 1. Green bars and stars = data corresponding to free complex I; red bars and stars = data corresponding to supercomplex containing CI and $CIII_2$. (**C**) and (**D**) Western Blot signal intensity profiles obtained with Fiji (ImageJ) applying the same ROI (region of interest) in the BNGE lanes of strong *Ndufs4*^{RNAi} and mild *Ndufs4*^{RNAi}, respectively, in comparison with their corresponding controls. Relative intensity (R.I.) of the bands along the lane length (in inches) was plotted. High molecular weights are on the left and low molecular weights are on the right.