Subunits of the *Drosophila* Actin-Capping Protein Heterodimer Regulate Each Other at Multiple Levels

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Abstract

The actin-Capping Protein heterodimer, composed of the α and β subunits, is a master F-actin regulator. In addition to its role in many cellular processes, Capping Protein acts as a main tumor suppressor module in *Drosophila* and in humans, in part, by restricting the activity of Yorkie/YAP/TAZ oncogenes. We aimed in this report to understand how both subunits regulate each other *in vivo*. We show that the levels and capping activities of both subunits must be tightly regulated to control F-actin levels and consequently growth of the *Drosophila* wing. Overexpression of *capping protein* α and β decreases both F-actin levels and tissue growth, while expressing forms of Capping Protein that have dominant negative effects on F-actin promote tissue growth. Both subunits regulate each other’s protein levels. In addition, overexpressing one of the subunit in tissues knocked-down for the other increases the mRNA and protein levels of the subunit knocked-down and compensates for its loss. We propose that the ability of the α and β subunits to control each other’s levels assures that a pool of functional heterodimer is produced in sufficient quantities to restrict the development of tumor but not in excess to sustain normal tissue growth.

Introduction

The actin cytoskeleton controls numerous processes, including cell shape, mobility, division and intracellular transport. In normal cells, the actin cytoskeleton is tightly controlled to regulate these essential functions; however, it can be subverted by cancer cells and contributes to changes in cell growth, proliferation, stiffness, movement and invasiveness [1,2]. Moreover, alterations in the activity or expression of actin-binding proteins (ABPs) per se, have been linked to cancer initiation and progression [2,3,4,5,6].

Among these actin regulators, the actin Capping Protein (CP) heterodimer, composed of an α and a β subunit, appears to act as a main tumor suppressor module [7,8,9,10]. CP was named based on its ability to bind and cap actin filament barbed ends, inhibiting the addition and loss of actin monomers [11,12,13]. CP has homologs in nearly all eukaryotic cells, including vertebrates, invertebrates, plants, fungi, insects and protozoa [14]. *Drosophila* and organisms other than vertebrates have single genes encoding *capping protein* α (*cpa*) or β (*cpb*). In contrast, vertebrates contain two single genes encoding two α subunits (α1 and α2), and one single gene that produce two β isoforms (β1 and β2) through alternative splicing [15,16,17]. Although the amino acid sequences of the α and β subunits are not more similar to each other than they are to other ABPs, nor they share common sequences with other proteins, they have extremely similar secondary and tertiary structures [18]. When in complex, the heterodimer resembles a mushroom with the C-terminus of each subunit forming tentacles located on the top surface of the heterodimer [19,20]. *In vitro* analyses of chicken and budding yeast CP revealed that deletions or point mutations in either the α or β tentacles do not affect protein stability but reduce the capping affinity, while a complete removal of both tentacles fully abrogates the actin-binding activity [12,20]. Thus, CP appears to cap F-actin barbed ends via the independent interaction of both tentacles with actin. *In vivo*, a truncated form of *Drosophila* *cpa* deleted of the C-terminal 28 amino acids has no effect on F-actin when expressed alone but promotes F-actin accumulation when co-expressed with full length *cpb* [21]. Similarly, a chicken β subunit containing a point mutation changing a conserved leucine to arginine at position 262, which caps actin poorly, disrupts the early steps in myofibrillogenesis of cultured myotubes and the sarcomere of mouse heart [22,23,24].

In yeast and *Drosophila*, removing either *cpa* or *cpb* induces F-actin accumulation and identical phenotypes [25,26,27]. In the fly, CP is required for proper differentiation of adult bristles, survival of the adult retina, determination of the oocyte and cortical integrity of nurse cells in the egg chamber [27,28,29,30]. In addition, CP has a key role in restricting tissue growth. In the whole wing disc epithelium, CP-dependent F-actin regulation suppresses inappropriate tissue growth by inhibiting the activity of the Yorkie (Yki) oncogene, which mediates Hippo signalling activity [7,9]. This function is conserved, as the α1 subunit is also required to limit the activity of the Yki orthologs YAP and TAZ in mammary epithelial cells [31]. In addition, in the distal *Drosophila*...
wing disc epithelium, CP prevents JNK-mediated apoptosis or proliferation and counters the oncogenic ability of Src [9,21,32]. Furthermore, underexpression of the human β1 subunit correlates with cancer-related death and causes a significant increase in gastric cancer cell migration and invasion in vitro, whereas its overexpression has the opposite effect [10].

We aimed in this report to understand how both subunits regulate each other in vivo to control F-actin levels and tissue growth. We show that Cpa and Cpb stabilize each other’s protein levels and can stimulate the production of each other’s mRNA when the level of one of the subunit is reduced. Because overexpressing CP decreases F-actin levels and tissue growth, while expressing forms of CP mutated in their actin-binding domains has opposite effects, we propose that by regulating each other, Cpa and Cpb assure that a pool of functional CP heterodimer is produced in sufficient quantities to restrict tissue growth and therein prevent tumor development but not in excess to sustain proper tissue growth.

Materials and Methods

Molecular Biology
To generate UAS-cpb1262R, site-directed mutagenesis was performed on the plasmid UAS-cpb, using the QuickChange kit (Stratagene, #200519). The mutated plasmid was confirmed by sequencing and transgenic flies were generated by standard methods.

Fly strains and genetics

Fly stocks were UAS-cpa-IR25 or UAS-cpb-IR50 [21]; UAS-HA-cpa89E [32]; UAS-HA-cpb7 [36]; UAS-cpb-IR50E (Vienna Drosophila Research Center, VDRC; cpa107E/25); cpaM143 (FlyBase). To generate cpa mutant clones marked by the absence of GFP and expressing or not UAS-HA-cpa98E or UAS-HA-cpa436E or UAS-cpb7, FRT42D, cpa98E/CyoO or w; FRT42D, cpa98E/CyoO; UAS-HA-cpa436E/Tm6β or w; FRT42D, cpa98E/CyoO; UAS-HA-cpb436E/Tm6β or w; FRT42D, cpa107E; UAS-cpb7/CyoO or w, FRT42D, cpa107E; UAS-cpb7/CyoO or w, FRT42D, cpa107E, UAS-cpb7/CyoO males were crossed to y, w, hs, w, m, y, w, m, n, h, m, y, w, m, n; FRT42D, cpa107E to sustain proper tissue growth. We show that Cpa and Cpb stabilize each other’s protein levels and regulate each other in vivo to control F-actin levels and tissue growth. We show that Cpa and Cpb assure that a pool of functional CP heterodimer is produced in sufficient quantities to restrict tissue growth and therein prevent tumor development but not in excess to sustain proper tissue growth.

Results

Cpa and Cpb stabilize each other’s protein levels and accumulate at Adherens Junctions

To understand how Cpa and Cpb are regulated to restrict growth of Drosophila epithelia, we generated polyclonal antibodies to each CP subunit. In lyses from embryos expressing UAS-
mCD8-GFP under the control of the ubiquitous daughterless-Gal4 (da-Gal4) driver, the Cpa (Fig. 1A) and Cpb (Fig. 1B) antibodies revealed a band at around 32 and 31 kDa respectively by Western Blot. These signals were lost in embryonic extracts from homozygous cpa (Fig. 1A) or cpb mutants (Fig. 1B) respectively. Conversely, overexpressing full length cpa, tagged with HA (UAS-HA-cpa); Fig. 1A) or cpb (UAS-cpb), Fig. 1B) with da-Gal4, enhanced the anti-Cpa or anti-Cpb signals respectively. Similarly, Cpa levels were increased in wing disc lysates overexpressing HA-cpa under scallop-Gal4 control (sd>HA-cpa; Fig. 1C), while endogenous Cpa levels were similar to control sd-GFP lysates (Fig. 1D). Forcing cpa expression in this tissue also induced a significant increase in Cpb levels by Western Blot (Fig. 1D) but did not significantly affect endogenous Cpa levels (Fig. 1C). Cross-sections through wing disc epithelia expressing UAS-mCD8-GFP in the posterior compartment using the hedgehog-Gal4 (hh-Gal4) driver showed that Cpa (Fig. 1E”-E”’ and Cpb (Fig. 1F”-F”’) accumulated at the apical cell membrane and co-localized with components of Adherens Junctions, including the β-Catenin homolog Armadillo (Arm). Co-expressing cpa and mCD8-GFP in this domain strongly enhanced the anti-Cpb signals but did not affect Cpa levels (Fig. 2D”-D”). Conversely, hh>HA-cpa” wing disc epithelia displayed an apical localization of HA-Cpa, like endogenous Cpa (Fig. 1E”-E”), but no change in Cpb levels (Fig. 2E”-E”). Thus, the anti-Cpa and Cpb antibodies recognize specifically Cpa and Cpb respectively.

Strikingly, Cpa levels were strongly reduced not only in wing disc extracts expressing double-stranded RNAs (dsRNA) for cpa under sd-Gal4 control (sd>cpa-IR) but also in discs knocked-down for cpa (sd>cpb-IR; Fig. 1C). In the converse experiment, the amount of Cpa was also strongly reduced in both sd>cpb-IR and sd>cpa-IR wing disc extracts (Fig. 1D). Similarly, knocking down cpa (Fig. 1G”-G” and H-H”) or cpb (Fig. 1H”-H” and J-J”) in the posterior wing disc compartment with hh-Gal4 significantly reduced the apical accumulation of both Cpa and Cpb when compared to anterior compartments used as internal controls. Moreover, both Cpa and Cpb levels were also strongly reduced in lysates from first instar larvae homozygous mutant for cpa or cpb (Fig. S1A) and in clones mutant for cpa or cpb (Fig. S1B-B” to E–E”). To verify that the cpa dsRNA did not affect cpa mRNA and vice versa, we performed quantitative RT-PCR (qRT-PCR) experiments on wing imaginal discs knocked down for cpa or cpb. As expected, sd>cpa-IR or sd>cpb-IR wing discs showed a significant reduction of cpa (Fig. 1K, 2.3±0.45 folds) or cpb mRNA (Fig. 1L, 2.6±0.41 folds) levels respectively, relative to control sd>GFPI. However, cpa mRNA levels were not significantly affected by a reduction in cpa (Fig. 1K), nor were cpb mRNA levels reduced in wing discs knocked-down for cpa (Fig. 1L). Similarly, a reduction in cpa or cpb levels had no effect on cpa or cpb mRNA levels respectively, in first instar larvae expressing cpa-IR or cpb-IR under da-Gal4 control (Fig. S1F and G). Taken together, we conclude that Cpa and Cpb accumulate at apical cell membrane and enhance each other’s protein levels.

Cpa and Cpb levels are rate limited to form a functional heterodimer

The Capping Protein α and β subunits form a functional heterodimer, which caps F-actin barbed ends via the interaction of the α and β tentacles with actin (Fig. 1A and [11,12,13,20]). To confirm that the stabilization of Cpa and Cpb’s protein levels by each other promotes the formation of a functional heterodimer, we first tested if co-expressing cpa and HA-α-cpa would enhance the levels of both subunits by comparing the levels of HA-Cpa and Cpb when overexpressed alone or together, ensuring that each genetic combination contained the same number of UAS transgenes. Indeed, by Western Blot (Fig. 2B, P<0.0002) and in wing disc epithelia (Fig. 2 compare F”-F”’ with E”-E”), HA levels were strongly enhanced when HA-α-cpa was co-expressed with cpb. Similarly, the co-expression of HA-β-cpa and cpb strongly increased Cpb levels compared to wing disc lysates overexpressing cpb alone (Fig. 2C). Overexpressed HA-α-cpa and cpb appeared to form a functional heterodimer as their co-expression in the posterior wing disc compartment with hh-Gal4 decreased the apical F-actin ratio between both compartments compared to hh>HA-cpa control (Fig. 3F, P<0.0001). In contrast, overexpressing either HA-cpa or cpb alone has no effect on F-actin levels [21]. We conclude that the levels of endogenous Cpa and Cpb available are rate limited to form a functional heterodimer.

Forms of CP mutated in α or β tentacle counteract the ability of wild type CP to restrict F-actin accumulation

Surprisingly, expressing an HA-tagged form of Cpa deleted of the α tentacle (UAS-HA-cpa[Δα]) has no significant effect on F-actin when expressed alone [21] but triggered apical F-actin accumulation when co-expressed with cpb (Fig. 3F, P<0.00001) and [21], indicating that HA-Cpa[Δα] affects F-actin only in the presence of overexpressed cpb. We therefore tested if the co-expression of cpb would also enhance the levels of HA-Cpa[Δβ]. In contrast to full length HA-Cpa, which accumulated apically (Fig. 2E”-E”), HA-Cpa[Δβ] localized uniformly along the apical-basal axis in the posterior compartment of hh>HA-cpa[Δβ] wing discs (Fig. 2G–G”). Strikingly, co-expressing cpa not only strongly enhanced HA-Cpa[Δβ] levels as assessed by Western Blot (Fig. 2B, P<0.0002), but also relocalized HA-Cpa[Δβ] at the apical cell membrane (Fig. 2H–H”). Thus, forcing Cpa levels enhances the levels of HA-Cpa[Δβ] and promotes its apical localization.

The heterodimer formed between HA-Cpa[Δβ] and Cpb appears to have reduced capping activity and may be recruited to F-actin barbed ends, preventing the binding of wild type CP. If so, we would expect that a form of Cpb truncated of its β tentacle would also promote F-actin accumulation in the presence of endogenous CP. To test this possibility, we expressed a form of cpb mutated in the highly conserved Leucine 262 (UAS-cpb[ΔβL262R]), which has been proposed to directly interact with actin [12]. While overexpressing full length cpb had no significant effect on F-actin (Fig. 3 compare B–B” with A–A” and F, hh>cpb[ΔβL262R] wing discs accumulated apical F-actin in the posterior compartment (Fig. 3C–C” and F, P<0.0001). However, co-expressing full length HA-cpa in these tissues suppressed the apical F-actin accumulation due to the presence CpbL262R (Fig. 3D–D” and F, P<0.00001). Thus, forcing Cpa levels tethers the effects of CpbL262R on F-actin. In contrast, F-actin accumulation was strongly enhanced when cpbL262R was co-expressed with HA-cpa[Δβ] (Fig. 3E”-E” and F, P<0.00001). Moreover, CpbL262R, like full length Cpb, enhances HA-Cpa[Δβ] levels and triggered its relocalization to the apical cell membrane (Fig. 2I–I”). We conclude that forms of CP with reduced capping activity inhibit wild type CP to restrict F-actin accumulation, most likely by tethering barbed ends, preventing the recruitment of wild type CP.

CP and forms of CP with dominant negative effects on F-actin have opposite effects on tissue growth

Decreasing or increasing CP levels has opposite effects on F-actin levels (Fig. 3F and [25]). Because loss of CP induces overgrowth of the wing disc epithelium by promoting Yki activity [7,9], we asked of overexpressing cpa and cpb has an opposite effect on tissue growth. Indeed, overexpressing full length HA-cpa and cpb
in the wing primordium using the nubbin-Gal4 (nub-Gal4) driver significantly reduced the size of the adult wing (Fig. 4A, compare nub-GFP control wing in green to nub-cpa+, cpb+ wing in magenta and F, P<0.0151), but does not affect cell survival [21]. Thus, tight CP levels are critical to control tissue growth.

To determine if CP controls tissue growth via F-actin regulation, we analyzed the effect of expressing forms of cpa or cpb that have dominant negative effects on F-actin on wing growth. Expressing HA-cpaDABD and cpb (Fig. 4B and F, P<0.0001) or cpbL262R alone (Fig. 4C and F, P<0.0001) or combined with HA-cpaDABD (Fig. 4E and F, P<0.0001) under nub-Gal4 control, not
Figure 2. Increasing the levels of individual CP subunits alone has no effect on the endogenous levels of the other subunit, while co-expressing HA-cpa or HA-cpa<sup>ABD</sup> and cpb enhance synergistically the levels of both subunits. (A) model by which Cpa and Cpb cap F-actin barbed ends via the interaction of α and β tentacles with actin. (B) western blot on protein extracts from wing discs expressing UAS-mCD8-GFP (lane 1) or UAS-mCD8-GFP and UAS-HA-cpa<sup>B</sup> (lane 2) or UAS-HA-cpa<sup>D</sup> and UAS-cpb (lane 3) or UAS-mCD8-GFP and UAS-HA-cpa<sup>ABD</sup> (lane 4) or UAS-cpb<sup>B</sup> and UAS-cpb<sup>D</sup> (lane 5) under sd-Gal4 control, blotted with anti-HA (middle panel) and anti-H3 (lower panel). The means for lane 1 is 0, for lane 2 is 0.6250, for lane 3 is 2, for lane 4 is 0.0667, for lane 5 is 1.300. Error bars indicate s.e.m.. *P*, 0.0092 for comparison of lanes 2 and 3 and of lanes 4 and 5. (B) western blot on protein extracts from wing discs expressing UAS-mCD8-GFP (lane 1) or UAS-cpb<sup>B</sup> (lane 2) or UAS-cpb<sup>D</sup> and UAS-HA-cpa<sup>B</sup> (lane 3) under sd-Gal4 control, blotted with anti-Cpb (middle panel) and anti-H3 (lower panel). The upper panels in B and C represent a quantification of relative (B) HA or (C) Cpb intensity signals for each genetic combination, measured by 4 independent blots. The means for lane 1 is 0.1088, for lane 2 is 0.5699, for lane 3 is 0.7982. Error bars indicate s.e.m. *P*, 0.0182 for comparison of lanes 2 and 3. (D–I) optical cross sections through distal epithelia of third instar wing imaginal discs with apical sides up and posterior sides to the left in which hh-Gal4 drives (D–D′) UAS-cpb<sup>B</sup> and one copy of UAS-mCD8-GFP (green in D) or (E–E′) UAS-HA-cpa<sup>B</sup> and two copies of UAS-mCD8-GFP (green in E) or (F–F′) UAS-HA-cpa<sup>D</sup> and UAS-cpb<sup>B</sup> and one copy of UAS-mCD8-GFP (green in F) or (G–G′) UAS-HA-cpa<sup>ABD</sup> and two copies of UAS-mCD8-GFP (green in G) or (H–H′) UAS-cpb<sup>B</sup> and one copy of UAS-mCD8-GFP (green in H) or (I–I′) UAS-cpb<sup>B</sup> and one copy of UAS-mCD8-GFP (green in I). Discs are stained with anti-Cpb (Cyan blue) and (D–D′) anti-Cpa (magenta) or (E–E′) to I–I′ anti-HA (magenta), which reveals (E–E′) or (F–F′) HA-cpa<sup>B</sup> or (G–G′) to I–I′ HA-cpa<sup>ABD</sup> expression. The scale bars represent 15 μm. doi:10.1371/journal.pone.0096326.g002
mCD8-GFP (lane 5) or UAS-cpb\(^{L262R}\) and one copy of UAS-mCD8-GFP (lane 6) or UAS-cpb\(^{L262R}\) and one copy of UAS-mCD8-GFP (lane 7). The mean for lane 1 is 0.922 (n = 12) for lane 2 is 0.775 (n = 8), for lane 3 is 1.435 (n = 10), for lane 4 is 0.977 (n = 10), for lane 5 is 1.175 (n = 16), for lane 6 is 0.937 (n = 14), for 7 is 2.348 (n = 6). Error bars indicate s.e.m..

**Figure 3.** Overexpressing HA-cpa suppresses the apical F-actin accumulation of cpb\(^{-262R}\)-expressing wing discs, whereas HA-cpa\(^{AABD}\) expression has the opposite effect. (A–A′) standard confocal sections of the apical cell membrane of third instar wing imaginal discs with dorsal sides up and posterior sides to the left, expressing (A–A′) one copy of UAS-mCD8-GFP (green in A) or (B–B′) UAS-cpb\(^{L262R}\) and one copy of UAS-mCD8-GFP (green in B) or (C–C′) UAS-cpb\(^{L262R}\) and two copies of UAS-mCD8-GFP (green in C) or (D–D′) UAS-cpb\(^{L262R}\), UAS-HA-cpa\(^{IR}\) and one copy of UAS-mCD8-GFP (green in D) or (E–E′) UAS-cpb\(^{L262R}\), UAS-HA-cpa\(^{AABD}\) and one copy of UAS-mCD8-GFP (green in E) under hh-Gal4 control. Discs are stained with Phalloidin (white) to mark F-actin and (B–B′ to E–E′) anti-Cpb (cyan blue). The yellow lines outline the anterior-posterior compartment boundary. The scale bars represent 30 μm. (F) Mean intensity of the ratio of Phalloidin (lane 3) or UAS-cpb\(^{L262R}\) to lane 2 or UAS-cpb\(^{AABD}\) to lane 1 and UAS-cpb\(^{L262R}\) and two copies of UAS-cpb\(^{AABD}\) to lane 7. Error bars indicate s.e.m. *** indicate P<0.0001.

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Cpb compensates for a reduction in cpa by enhancing cpa mRNA levels and vice versa

Interestingly, co-expressing cpa with HA-cpa\(^{AABD}\) almost fully suppressed apoptosis of wing discs knocked-down for cpa (Fig. 6 compare B–B′ with A–A′ and D; P<0.0001). This effect could be due to the stabilization and apical relocalization of HA-Cpa\(^{AABD}\) when co-expressed with cpa (Fig. 2H–H′). However, apoptosis of sd>cpa-IR wing discs was also significantly suppressed by overexpressing cpa alone (Fig. 6C–C′ and D; P<0.0001). Conversely, expressing HA-cpa in tissues knocked-down for cpa (sd>cpa-IR) also prevented apoptosis (Fig. 7 compare B–B′ with A–A′ and G; P<0.0001).

To understand the mechanisms by which Cpa and Cpb compensate for each other’s function, we tested the effect of overexpressing cpa on Cpa levels in cpa-depleted tissues. As
nub quantification of relative wing size normalized to hairs on adult wings for the genotypes shown in A, B, C, D and E. (F) cpb7 for lane 1 is 1 (n = 32), for lane 2 is 0.9702 (n = 12), for lane 3 is 1.119 (n = 3), for lane 4 is 1.061 (n = 24), for lane 5 is 1.015 (n = 13), for lane 6 is 1.051 (n = 13). Error bars indicate s.e.m. P<0.015 for comparison of lanes 1 and 2. P<0.001 for comparison of lanes 1 and 3 or 4 or 6 and 2 for comparison of lane 4 and 5. doi:10.1371/journal.pone.0096326.g004

previously observed, by Western Blots, Cpa (Fig. 6F) and Cpb (Fig. 6G) levels were strongly reduced in wing disc extracts knocked-down for cpa. Forcing cpb levels in these tissues enhanced the levels of both Cpa (Fig. 6F and Fig. S2) and Cpb (Fig. 6G and Fig. S2). We quantified this effect by measuring the ratio of Cpa signals between the posterior and anterior compartments of hh>cpa-IR-expressing wing discs, in the presence or absence of overexpressed cpb. While in control hh>GFP tissues this ratio was 0.95, knocking down cpa reduced this ratio to 1.34 folds (Fig. 6H; P<0.0001). This effect was significantly alleviated by the overexpression of cpb (Fig. 6H; P<0.01). In contrast, overexpressing cpa in control hh>GFP wing discs did not affect Cpa levels (Fig. 6H), indicating that Cpb enhances Cpa levels only when cells contain reduced Cpa levels. By Western Blots, HA-cpa also enhanced both Cpa (Fig. 7D) and Cpb (Fig. 7E) levels when expressed in tissues knocked-down for cpb. Thus, Cpa compensates for a reduction in cpb by stimulating the production of Cpb, and vice versa.

Using qRT-PCR, we next analyzed if overexpressing either subunits affects the mRNA levels of the other. After normalization to the Rhgl2 transcript used as an internal control, we observed that whereas cpa (Fig. 6L, P<0.0027) but not cpb (Fig. 6K) mRNA levels were strongly reduced in wing discs knocked-down for cpa (sl<l-cpa-IR), forcing cph levels in these tissues fully restored cpa mRNA to wild type levels (Fig. 6L; P<0.0003). In contrast, in wing discs that contained endogenous cpa and cpb, overexpressing cpa, which strongly enhanced cph mRNA levels (Fig. 6L), had no significant effect on cpa mRNA levels (Fig. 6J). Thus, Cpb stimulates the production or stabilization of cpa mRNA only when Cpa levels are reduced. In the converse experiment, overexpressing HA-cpa in sl<l-cpb-depleted wing discs enhanced the levels of both cpa (Fig. 7F) and cph (Fig. 7H; P<0.0015) mRNA. However, in wing discs that contained endogenous cpa and cph, only cpa mRNA levels were strongly increased (Fig. 7G and I). The ability of Cpb to suppress apoptosis of cpa-depleted wing discs was due to the increase in cpa mRNA and protein levels as clones mutant for a cpa allele showed similar apoptotic levels in the absence or presence of overexpressing cpa (Fig. 6E). We conclude that Cpa compensates for a reduction in cpb by increasing cph mRNA levels and vice versa.

Discussion

Cpa and Cpb regulate each other at multiple levels

Our data argue that in Drosophila, different pools of Cpa and/or Cpb co-exist, and they regulate each other at various levels. One level of regulation involves their reciprocal stabilization of their protein levels. First, in Drosophila, like in yeast, the loss of one CP subunit reduces the protein levels of the other subunit ([26] and Fig. 1) but does not affect its mRNA levels (Fig. 1 and Fig. S1). Second, co-expressing cpa and cpb in Drosophila tissues enhances synergistically the levels of both subunits relative to the levels of each subunit overexpressed alone (Fig. 2). Third, large quantities of soluble active chicken CP can be produced in bacteria only when both subunits are co-expressed [40]. Cpa and Cpb may stabilize each other’s protein levels via direct protein-protein interactions [19]. The tight interaction between both subunits may prevent the recruitment of E3 ubiquitin ligases that would otherwise target individual CP subunits for degradation by the 26S proteasome. As an heterodimer, CP has been shown to bind

Figure 4. Overexpressing full length HA-cpa and cpb prevents wing growth, while ectopic expression of HA-cpaABD and/or cpb262R has the opposite effect. (A, B, C, D and E) merge between adult wings expressing in green UAS-mCD8GFP under nub-Gal4 control and in magenta (A) UAS-HA-cpaABD and UAS-cpb2 (B) UAS-HA-cpaABD and UAS-cpb7 (C) UAS-cpb1262R and UAS-HA-cpaABD or (E) UAS-cpb1262R and UAS-HA-cpaABD under nub-Gal4 control. (A’, B’, C’ D’ and E’) magnification of hairs on adult wings for the genotypes shown in A, B, C, D and E. (F) quantification of relative wing size normalized to nub-GFP control for nub-Gal4 driving UAS-mCD8-GFP (lane 1) or UAS-HA-cpaABD and UAS-cpb2 (lane 2) or UAS-HA-cpaABD and UAS-cpb7 (lane 3) or UAS-cpb1262R and one copy of UAS-mCD8-GFP (lane 4) or UAS-cpb262R and UAS-HA-cpaABD (lane 5) or UAS-cpb1262R and UAS-HA-cpaABD (lane 6). The mean for lane 1 is 1(mean = 32), for lane 2 is 0.9702 (n = 12), for lane 3 is 1.119 (n = 3), for lane 4 is 1.061 (n = 24), for lane 5 is 1.015 (n = 13), for lane 6 is 1.051 (n = 13). Error bars indicate s.e.m. P<0.015 for comparison of
to the fast polymerizing ends of actin filaments, preventing further addition of actin monomers [41,42] and to restrict F-actin accumulation in Drosophila tissues [25,27]. In addition, Cpa and Cpb appear to show some function on their own as overexpressing cpb rescues apoptosis of wing discs knocked-down for cpa and vice versa (Fig. 6 and 7). Overexpression of cpb alone is also sufficient to
Figure 6. Overexpressing \textit{cpb} in wing discs knocked-down for \textit{cpa}, restores \textit{cpa} mRNA and protein levels and suppresses apoptosis.

(A–A') standard confocal sections of third instar wing imaginal discs with dorsal sides up, expressing (A–A') UAS-\textit{cpa-IR}^{C10} and two copies of UAS-\textit{mCD8-GFP} (green in A) or (B–B') UAS-\textit{cpb-7} and UAS-\textit{mCD8-GFP} (green in B) under \textit{sd-Gal4} control. Discs are stained with anti-activated-Caspase 3 (magenta), which monitors DRONC activation and (B–B') Phalloidin (cyan blue in B) to underline wing disc shape. The scale bars represent 30 \textit{m}. (D) quantification of total C3 area per disc area for the genotypes \textit{sd-Gal4} \textit{UAS-HPA-\textit{cpa-IRC10}}, \textit{2XGFP} (lane 1); \textit{sd-Gal4} \textit{UAS-HPA-\textit{cpa-IRC10}}, \textit{HA-cpa} \textit{DABD} \textit{cpb7} (lane 2) and \textit{sd-Gal4} \textit{UAS-HPA-\textit{cpb7}}, \textit{1XGFP} (lane 3). The means for lane 1 is 92.4 (n = 23); for lane 2 is 10.61 (n = 19); for lane 3 is 32.9 (n = 20). Error bars indicate s.e.m. \(P, 0.0001\) for comparison of lane 1 and 2 or 3 or lane 2 and 3. (E) quantification of total C3 area per disc area for wing discs containing \textit{T155-flp; cpa107E} mutant clones (lane 1) or \textit{T155-flp; cpa107E} mutant clones expressing UAS-\textit{cpb7} (lane 2). The means for lane 1 is 10.80 (n = 26); for lane 2 is 13.77 (n = 20). n.s. indicates non-significant \(P\) value. (F and G) western blots on protein extracts from wing discs expressing two copies of UAS-\textit{mCD8-GFP} (lane 1) or UAS-\textit{cpb7} and one copy of UAS-\textit{mCD8-GFP} (lane 2) or UAS-\textit{cpa-IRC10} and two copies of UAS-\textit{mCD8-GFP} (lane 3) or UAS-\textit{cpa-IRC10} and UAS-\textit{cpb7} and one copy of UAS-\textit{mCD8-GFP} (lane 4). Panels derive from the same experiment shown in Figure 5E and F and blots were processed in parallel (see Figure S2 showing the whole experiment). (H) mean intensity of the ratio of Cpa signals between posterior and anterior wing compartments of \textit{hh-Gal4} driving two copies of UAS-\textit{mCD8-GFP} (lane 1) or UAS-\textit{cpb7} and one copy of UAS-\textit{mCD8-GFP} (lane 2) or UAS-\textit{cpa-IR}^{C10} and two copies of UAS-\textit{mCD8-GFP} (lane 3) or UAS-\textit{cpa-IR}^{C10} and UAS-\textit{cpb7} and one copy of UAS-\textit{mCD8-GFP} (lane 4). The mean for lane 1 is 0.959 (n = 15), for lane 2 is 0.970 (n = 20), for lane 3 is 0.716 (n = 21), for lane 4 is 0.776 (n = 20). Error bars indicate s.e.m., \(P, 0.0001\) for comparison of lanes 1 and 3 or 4 or \(P, 0.01\) for comparison of lanes 3 and 2. (I to L) graph of (I and J) \textit{cpa} or (K and L) \textit{cpb} mRNA levels measured by five independent qRT-PCR in wing imaginal discs expressing (I and K) two copies of UAS-\textit{mCD8-GFP} (lane 1) or UAS-\textit{cpa-IR}^{C10} and UAS-\textit{mCD8-GFP} (lane 2) or UAS-\textit{cpb7} and one copy of UAS-\textit{mCD8-GFP} (lane 3) or UAS-\textit{cpb-7} and UAS-\textit{mCD8-GFP} (lane 4). (I) the means for lane 1 is 1.084; for lane 2 is 0.4328; for lane 3 is 1.086. \(P, 0.0027\) for comparison of lane 1 with 2 or \(P, 0.0003\) for comparison of lane 2 with 3. (J) the means for lane 1 is 1.07; for lane 2 is 0.824; n.s. indicates non-significant \(P\) value. (K) the means for lane 1 is 0.621; for lane 2 is 0.503; for lane 3 is 3.735. \(P, 0.0001\) for comparison of lane 3 with 1 or 2. (L) the means for lane 1 is 0.292; for lane 2 is 1.961. \(P, 0.0001\) for comparison of lane 1 and 2. Error bars indicate s.e.m.

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enhance the retinal defects of flies knocked down for the Cbl-interacting protein cindr [43] and to rescue the migration and F-actin polarization defects of Drosophila border cells mutant for warts [44]. Because individual chicken CP subunits expressed in bacteria are mainly deposited into insoluble cytoplasmic inclusion bodies but can be renaturated as active heterodimers [45], individual subunit may exist in the cell as pools of insoluble monomers. The molecular mechanism by which individual CP subunit compensates for each other’s function remains to be determined. Several observations argue that this mechanism involves the production of the subunit knocked-down by the other subunit via an increase of its mRNA levels (Fig. 6 and 7). CP has been observed in the nuclei of chicken retinal and kidney epithelial cells in culture, in Madin-Darby canine kidney (MDCK) cells, in Xenopus laevis oocytes and bovine lens epithelial cells in culture [46,47]. Whether Cpa and Cpb influence each other’s transcription in the nucleus is an interesting possibility to be tested. The protein-mRNA feedbacks between Cpa and Cpb may guarantee that a pool of functional heterodimer is present to limit F-actin polymerization. However, a CP-dependent negative feedback mechanism must exist that restricts the production of CP in excess, as forcing the expression of one of the subunit in tissues that contain endogenous CP does not enhance the mRNA and protein levels of the other subunit (Fig. 6 and 7). Because the loss of one subunit has no effect on the mRNA levels of the other subunit (Fig. 1 and Fig. S1), the CP-dependent negative feedback may act by limiting the ability of individual subunits to stimulate the production of each other’s mRNAs. Thus, in addition to regulate each other’s protein levels, individual CP subunit stimulates each other’s mRNA production up to an optimal physiological threshold of functional heterodimers. Further experiments are necessary to elucidate the protein-mRNA feedbacks between Cpa and Cpb.
mRNA feedback loop mechanisms, which operate between both subunits.

**Capping activity of the CP heterodimer at actin filament barbed ends**

Our observations argue that in vivo the actin-binding domain of Cpa is not absolutely required to form a functional CP heterodimer, as HA-Cpa<sub>ABD</sub> partially compensates for the loss of endogenous Cpa (Fig. 5). Consistent with our observations, in actin assembly assays, a mutant form of the chicken α subunit that lacks the α tentacle is able to cap F-actin [12]. Nevertheless, the α tentacle may favor the interaction and therefore stabilization of the α subunit by the β. This possibility is consistent with the observation that HA-Cpa<sub>ABD</sub> is found in the cell at much lower levels than full length HA-Cpa (Fig. 2) despite both transgenes being inserted at the same locus in the fly genome and therefore likely expressed at similar levels [21]. Consistent with this hypothesis, Arginine 259 of the chicken α tentacle forms side-chain hydrogen bonds with three residues of the β subunit, all residues being conserved across isoforms and species [19]. Moreover, in vitro, a truncated form of the chicken α subunit, consisting only of the C-terminal domain, retains the ability to form a heterodimer [48]. The reduced ability of HA-Cpa<sub>ABD</sub> to interact with Cpb may explain its inability to fully suppress apoptosis of Cpa-depleted tissues (Fig. 5) and to affect F-actin levels when overexpressed alone [21]. However, several observations indicate that the α and β tentacles also enable full capping activity in vivo. First, in actin assembly assays, the C-termius of the chicken α and β1 subunits are required for high-affinity capping [12]. Second, in the presence of endogenous CP, stabilizing HA-Cpa<sub>ABD</sub> levels by forcing cpa expression does not reduce F-actin levels, as does overexpressed HA-ctpa/cpb, but instead, promotes F-actin accumulation (Fig. 3 and [21]). Third, replacing leucine 262 of the chicken β subunit has no effect on protein stability and global structure but decreases the capping affinity significantly [12,20]. Fourth, identical mutations in the β orthologs induces F-actin accumulation in Drosophila tissues (Fig. 3) and disrupts the sarcosome of mouse heart [24]. Thus, we propose that the heterodimers formed between HA-Cpa<sub>ABD</sub> and Cpb or between Cpb<sub>Δ262E</sub> and Cpa are recruited to F-actin barbed ends and cap actin filaments less efficiently than wild type CP. The low capping activity of the HA-Cpa<sub>ABD</sub>/Cpb heterodimer is sufficient to partially compensate for the loss of Cpa. However, in the presence of endogenous CP, the HA-Cpa<sub>ABD</sub>/Cpb heterodimers compete with wild type Cpa/Cpb heterodimers for binding the barbed ends of F-actin, which can lead to defects in F-actin.

**Tight regulation of CP levels is critical to control tissue growth**

CP appears to act as a gatekeeper, which limits the development of cancer-related processes. Loss of the α subunit promotes Yki/YAP/TAZ-dependent proliferation in Drosophila epithelia and in human cells [9,31], causes a significantly increase in gastric cancer cell migration and is associated with cancer-related death [10]. In contrast, increasing CP levels has opposite effects: it reduces tissue growth (Fig. 4) and prevents Src-mediated tumour development in Drosophila [21], and significantly restricts gastric cancer cell migration [10]. Several of our observations argue that the function of CP on tissue growth involves its F-actin capping activity. First expressing cpa<sup>Δ262E</sup>, which contains a single point mutation affecting the capping activity [23], induces F-actin accumulation (Fig. 3) and wing overgrowth (Fig. 4). Moreover, CP-dependent F-actin accumulation correlates with tissue overgrowth, whereas tissue undergrowth is associated with a CP-dependent reduction in F-actin (Fig. 3 and 4). Consistent with these observations, other actin regulators have been shown to control Yki/YAP/TAZ-dependent tissue growth [7,9,31]. Thus, a reduction or an increase of CP levels has deleterious consequences on tissue growth, implying that it must be tightly regulated. This may be achieved in part by the ability of Cpa and Cpb to stimulate or limit the production of each other in conditions of lower or higher CP levels respectively, assuring that a pool of functional CP heterodimer is produced in sufficient quantities in the cell to prevent cancer development but not in excess to sustain proper tissue growth.

**Supporting Information**

**Figure S1** Reducing cpa or cpb levels reduces both Cpa and Cpb protein levels. (A) western blot on protein extracts from first instar larvae, either white minus (lane 1) or homozygote mutant for cpa<sup>69E</sup> (lane 2) or homozygote mutant for cpa<sup>M145</sup> (lane 3), blotted with (upper panel) anti-Cpa (upper bands) and anti-Cpb (lower band) and (lower panel) anti-H3. (B–B' to E–E') standard confocal sections of third instar wing imaginal discs, containing (B–B' and C–C') T155-Gal4; UAS-Cpa<sup>69E</sup> induced mutant clones marked by the absence of GFP (green) or (D–D' and E–E') heat shocked-induced cpa<sup>M145</sup> mutant clones marked by the absence of GFP (green). Discs are stained with (A) anti-Cpa (upper panel) and anti-H3 (lower panel). The scale bars represent 15 μm. (F and G) graphs of (F) cpa or (G) cpb mRNA levels measured by three independent qRT-PCR in first instar larvae expressing UAS-mdcd8-GFP (lane 1) or UAS-cpa<sup>IR<sub>568</sub></sup> (lane 2) or UAS-cpb<sup>IR<sub>568</sub></sup> (lane 3) under da-Gal4 control. (F) The means for lane 1 is 7.04; for lane 2 is 1.13; for lane 3 is 5.91. Error bars indicate s.e.m. for comparison of lane 1 and 2. (F) The means for lane 1 is 1.97; for lane 2 is 1.96; for lane 3 is 0.46. Error bars indicate s.e.m. for comparison of lane 1 and 3. n.s. indicates non-significant P values. (TIF)

**Figure S2** Expressing HA-cpa or HA-cpa<sup>ABD</sup> or cpb in wing discs knocked down for cpa restores Cpa and Cpb levels. Western blots on protein extracts from wing discs expressing two copies of UAS-mdcd8-GFP (lane 1) or UAS-cpa<sup>IR<sub>568</sub></sup> and two copies of UAS-mdcd8-GFP (lane 2) or UAS-cpb<sup>IR<sub>568</sub></sup> and one copy of UAS-mdcd8-GFP (lane 3) or UAS-cpa<sup>IR<sub>568</sub></sup> and UAS-HA-cpa<sup>IR<sub>568</sub></sup> and one copy of UAS-mdcd8-GFP (lane 4) or UAS-cpb<sup>IR<sub>568</sub></sup> and UAS-HA-cpa<sup>ABD</sup>, which contains the last 28 amino acids of the Cpa C-terminus and one copy of UAS-mdcd8-GFP (lane 5) or UAS-cpa<sup>IR<sub>568</sub></sup> and UAS-cpb<sup>IR<sub>568</sub></sup> and one copy of UAS-mdcd8-GFP (lane 6) under sd-Gal4 control, blotted with (A) anti-Cpa (upper panel) and anti-H3 (lower panel) or (B) anti-Cpb (upper panel) and anti-H3 (lower panel). (TIF)

**Table S1** Intron-exon-specific primers used to quantify cpa, cpb and RpL32 mRNA levels by qRT-PCR. (DOCX)

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Author Contributions
Conceived and designed the experiments: ARA PG JLW FJ. Performed the experiments: ARA PG JLW FJ. Analyzed the data: ARA PG FJ. Wrote the paper: FJ.

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