AtHDA15 attenuates COP1 *via* transcriptional quiescence, direct binding, and sub-compartmentalization during photomorphogenesis

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SHORT TITLE: Mechanisms for HDA15 attenuation of COP1

Abstract

Light is an essential environmental cue that determines the overall growth and development of plants. However, the molecular mechanisms underpinning the light signaling network are obscured by the epigenetic machinery where reversible acetylation and deacetylation play crucial roles in modulating light-regulated gene expression. In this paper, we demonstrate that HDA15 represses COP1, the master switch in the light signaling network, by deacetylation, protein interaction, and sub-compartmentalization. *hda15* T-DNA mutant lines exhibited light hyposensitivity with significantly reduced HY5 and PIF3 transcript levels leading to long-hypocotyl phenotypes in the dark while its overexpression exhibited elevated HY5 transcripts and short hypocotyl phenotypes. *In vivo* and *in vitro* binding assays further show that HDA15 directly interacts with COP1 inside the nucleus modulating COP1's repressive activities. Crossing *hda15-t*27 with *cop1-4* mutants resulted in short-hypocotyl and dwarfed phenotypes, reminiscent of *cop1-4* mutants suggesting COP1 is epistatic to HDA15. Although light signals the nucleocytoplasmic shuttling of HDA15, the presence of COP1 triggers its nuclear localization. A working model is presented elucidating the concerted interplay between HDA15 and COP1 under light and dark conditions.

Keywords: histone deacetylases, photomorphogenesis, protein interaction, light signal transduction, gene expression regulation

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Introduction

Plants adapt extensively to the dynamic changes in their light environment as they are critically dependent on light for photosynthesis, growth, and development. In general, light induces massive changes in a plant's transcriptional activity resulting in distinct expression profiles during seedling development (Jiao et al., 2007; Ma et al., 2013). Approximately 15% of these transcriptional changes have been attributed to histone modifications, specifically the acetylation in H3K9K27, which coincides with the upregulation of key photomorphogenesis gene expression, such as HY5 and its homolog, HYH (Charron et al., 2009). H3K9ac is one of the most characterized epigenetic marks invariably correlated with transcriptional activation in all the species studied (Ausin et al., 2004; Kurdistani et al., 2004; Schübeler et al., 2004; Benhamed et al., 2008; Guo et al., 2008; Charron et al., 2009; Zhou et al., 2009; Schenke et al., 2014; Liu et al., 2017). However, most of these light-induced pathways remain obscure, while little is understood about histone deacetylases' involvement in regulating these genes.

Studies on histone active marks such as H3K9K27ac have been limited to positive photomorphogenic regulators such as CABs, LHBIB1, LHCA1, HYH and HY5 and its downstream targets CHS, IAA3, RBCS-1a (Benhamed et al., 2006; Guo et al., 2008; Charron et al., 2009). Among the 12 members of the RPD3/HDA1 superfamily in *Arabidopsis*, HDA19, HDA6, and HDA15 have been ascribed active roles in light-regulated gene expression. Based on studies, HDA19 and HDA6 play a negative regulatory role by repressing some of these light-regulated genes (Benhamed et al., 2006; Tessadori et al., 2009). On the other hand, HDA15 has recently been shown to have a regulatory role in photomorphogenesis. As human Class II HDACs are known to undergo nucleocytoplasmic shuttling, we previously demonstrated that HDA15 shuttles to the nucleus under light (Alinsug et al., 2012). Moreover, phytochrome

80 interacting factor 3 (PIF3) associates with HDA15 repressing chlorophyll biosynthesis and
81 photosynthesis in etiolated seedlings (Liu et al., 2013). Similarly, PIF1 recruits HDA15 at the
82 promoter sites repressing the transcription of light-responsive genes involved in multiple
83 hormonal signaling pathways and cellular processes in germinating seeds in the dark (Gu et al.,
84 2017). Furthermore, HDA15 forms a complex with nuclear factor homologs (NF-YC)
85 functioning as a transcriptional co-repressor via deacetylation inhibiting hypocotyl elongation in
86 photomorphogenesis (Tang et al., 2017). A recent study describes that long hypocotyl 5 (HY5)
87 protein interacts with HDA15 repressing hypocotyl cell elongation during photomorphogenesis
88 (Zhao et al., 2019).

89 HY5 and PIFs are key transcription factors playing crucial regulatory roles in
90 photomorphogenesis and skotomorphogenesis, respectively. Along with the light signaling
91 network, both HY5 and PIFs are repressed by COP1, an E3 ubiquitin ligase that targets both
92 photoreceptors HY5 and HYH for ubiquitination and proteasome degradation in the dark
93 (Osterlund et al., 2000). However, the molecular interplay between COP1, HY5, PIFs, and
94 HDA15 remains unclear. We do not know how or when HDA15 precisely interjects within the
95 light response molecular network. As epigenetic regulation further adds complexity to the light
96 signaling pathway, we attempt to explore the multiple roles HDA15 plays during
97 photomorphogenesis. In this paper, we demonstrate that HDA15 represses COP1, the master
98 switch in the light signaling network, by transcriptional silencing via promoter deacetylation,
99 protein-protein association, and sub-compartmentalization. A working model is presented
100 elucidating the interplay of the multiple actions exerted by HDA15 on COP1 under light and
101 dark conditions.
102

Materials & Methods

Plant Material

Seeds of T-DNA knockout lines *hda5-t24* (SALK_030624), *hda15-t27* (SALK_004027C), and *hda18-t* (SALK_006938C) in Col-0 background were purchased from ABRC and screened via PCR using specific primers. Homozygous mutant plants with positive T-DNA inserts were grown under long day and assessed for the expression of the corresponding gene compared to wildtype via RT- PCR using specifically designed primers. Seeds of homozygous T-DNA knockout lines were then collected and further used for this study. To establish the double mutant line, we crossed the homozygous *hda15-t27* mutant with the *cop1-4* mutant. The resultant F2 lines were then finally screened by PCR.

Protoplast PEG Transfection

Leaves of 3-week old Col-0 wildtype, T2 transgenic lines, and mutant lines *phyA, phyB, cry1/cry2,* and *cop1-4* were used for protoplast transient expression assay. Isolation of Arabidopsis mesophyll protoplasts and PEG transfection were done as described previously (Yoo et al., 2007) with some modifications. Mesophyll protoplasts were isolated from 3-week old Col-0 plants and Arabidopsis PSB-D cell lines. Twenty µg of HDA15-YFP/GFP fusion plasmid and VirD2-NLS, a nuclear marker, were co-transfected into protoplasts using polyethylene glycol (PEG) solution (0.4 g/ml PEG 4000, 0.8 M mannitol, 125 mM CaCl2). After incubation for 5-15 min at room temperature, the protoplasts were washed and resuspended in W5 solution (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 2 mM MES at pH5.7), and further incubated under white light for 16–24 h before imaging using Leica SP5 confocal microscope. For the localization of HDA15 in different light treatments, transfected protoplasts were

125 incubated under white light for 18 h then transferred to E30LEDL3 growth chambers (Percival
126 Scientific) with far-red, red, and blue light-emitting diode sources for 3 h. Low light intensities
127 used as treatment were measured at 2.77 µmol m^{-2} s^{-2} (FR), 1.77 µmol m^{-2} s^{-2} (R), and 3.84 µmol
128 m^{-2} s^{-2} (B).

129 **Light treatments and hypocotyl measurements**

130 Arabidopsis seeds were surface sterilized, cold treated for 3-4 days and sown on 1/2 MS
131 plates with 0.1% sucrose then subjected to different light treatments at 21ºC for 5-7 consecutive
132 days. White light intensity was approximately 130 µmol $m^{-2}s^{-1}$. Plated seeds were incubated in
133 E30LEDL3 colored light chambers (Percival Scientific) with the corresponding low (L) and high
134 (H) wavelength measurements as follows: far-red at 18.065 (H) and 2.77 (L) µmol m^{-2} s^{-1}, red
135 light at 22.938 (H) and 1.77 (L) µmol m^{-2} s^{-1}, and blue light at 11.095 (H) and 3.84 (L) µmol m^{-2}
136 s^{-1}, respectively. On the other hand, dark treated seedlings were wrapped in foil and placed
137 inside a dark growth chamber. Hypocotyl length was measured using UTHSCA Image Tool
138 using 30-50 seedlings for each line and further analyzed for statistical tests.

139 **Total RNA extraction & qRT-PCR**

140 Gene expression was initially assessed by semi-quantitative RT-PCR. Total RNA was
141 extracted from plant samples weighing 0.25 to 0.3 g using TRIZOL reagent (Invitrogen).
142 Oligo(dT) primed reverse transcription of first-strand cDNA synthesis was carried out with 7 mg
143 total RNA using Super ScriptTM III (Invitrogen). Equal volumes of each first-strand reaction
144 were amplified with gene-specific primer pairs. Thermocycling conditions were 95ºC for 4 mins
145 followed by 30 cycles of 95ºC for 30 s, 55–60ºC for 30 s, and 74ºC for 1–2 min. cDNAs
146 obtained from RT were then used as templates to run real-time PCR. The following components
147 were added to a reaction tube: 9 µL of iQ SYBR Green Supermix solution (Bio-Rad), 1 µL of 5

148 mM specific primers, and 8 μL of the diluted template. Thermocycling conditions were as
149 follows: 95ºC for 3 min followed by 40 cycles of 95ºC for 30 s, 60ºC for 30 s, and 72ºC for 20 s,
150 with a melting curve detected at 95ºC for 1 min, 55ºC for 1 min, and detection of the denature
151 time from 55ºC to 95ºC. Each sample was quantified in triplicate and normalized using
152 Ubiquitin10 as the internal control. Gene-specific primer pairs were designed and are available
153 upon request.

154 **Chromatin Immunoprecipitation (ChIP) assay**

155 ChIP assay was performed as described (Gendrel et al., 2005). Chromatin extracts were
156 prepared from light treated 4-day old seedlings treated with formaldehyde. The chromatin was
157 sheared to an average length of 500 bp by sonication and immunoprecipitated with anti-
158 acetylated histone H3K9K14 (catalog no. 06-599; Millipore). The DNA cross-linked to
159 immunoprecipitated proteins was analyzed by real-time PCR. Relative enrichments of various
160 regions of COP1 over Col-0 were calculated after normalization to Ubiquitin10. Each of the
161 immunoprecipitations was tested and analyzed in triplicates, and each sample was quantified at
162 least three times.

163 **Protein Extraction and Gel-Blot Analysis**

164 Total protein extraction was conducted as described (Hsieh et al., 2000). Four-day old
165 seedlings of wild type Col-0, all mutant, and overexpression lines were continuously grown
166 under different light treatments and duration. After the treatment, the seedlings were harvested
167 and frozen immediately for protein extraction. One hundred micrograms of total protein were
168 separated on a 12% SDS-polyacrylamide gel, blotted on immuno-blot PVDF (Bio-Rad), and
169 detected using anti-HY5, anti-COP1, and anti-RPN6 specific antibodies.

170 **Protein-Protein Interaction**

171 For the bifluorescence complementation (BiFC) assay, HDA15 and COP1 coding
172 sequences were cloned into the pCR8/GW/TOPO vector and used for recombination into its
173 corresponding destination vectors, pEG201-N-YFP and pEG202-C-YFP, using LR mix.
174 Kanamycin was used for bacterial selection. Purified plasmids were then analyzed for DNA
175 sequencing for confirmation and further used for protoplast PEG transfection and imaging.

176 Pull-down assay was carried out as described (Yang et al., 2008) with some
177 modifications. For GST pull-down, GST and GST-HDA15 recombinant proteins were incubated
178 in 30 mL of GST resin with binding buffer (50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 0.25%
179 Triton X-100, and 35 mM β-mercaptoethanol) for two h at 4ºC. The binding reaction was
180 washed three times with the binding buffer, and then the COP1-His recombinant protein was
181 added and incubated for an additional two h at 4ºC. For His pull-down, His and COP1-His
182 recombinant proteins were incubated in 30 mL of His resin in a phosphate buffer (10 mM
183 Na2HPO4, 10 mM NaH2PO4, 500 mM NaCl, and 10 mM imidazole) for two h at 4ºC, the
184 binding reaction was washed three times with the phosphate buffer, and then GST-HDA15
185 recombinant protein was added and incubated for an additional two h at 4ºC. After extensive
186 washing (for at least eight times), the "pulled-down" proteins were eluted by boiling, separated
187 by 10% SDS-PAGE, and detected by western blotting using anti-His, anti-GST, or anti-COP1
188 specific antibody.

189 **Bioinformatics analysis**

190 The protein sequences of AtHDA15, COP1, and HY5 (ACCN #: Q8GXJ1, P43254, and
191 O24646) were retrieved from the NCBI's protein sequence repository database
192 (www.ncbi.nlm.nih.gov/). Putative protein ubiquitylation sites were predicted by UbPred (Kumar

193 and Kumar, 2019), BDM-PUB (Li et al., 2009), and Ubi-Site (Akimov et al., 2018). ProtParam
194 server (http://us.expasy.org/tools/protparam.html) was used to compute the instability
195 index (Guruprasad et al., 1990).

196 **Statistical analysis**

197 Statistical analyses were conducted using SigmaStat 11 software. One-way ANOVA
198 using the Holm-Sidak method was performed for multiple pairwise comparisons. Similarly,
199 Dunnette's method was conducted for multiple comparisons using WT as control. However,
200 Kruskal-Wallis One-way ANOVA on Ranks was used to analyze data that failed to pass the
201 Normality test. Significance testing was set at an α of 0.05 or 0.01. Graphs were presented in
202 mean values with error bars set from its standard deviation.

203

204

Results

1. Class II HDA mutant lines display light hyposensitivity.

T-DNA SALK lines of Class II histone deacetylases were screened, analyzed, and grown under various light conditions where long hypocotyl phenotypes were initially observed. To determine the maximum phenotypic expression of the mutant lines, we exposed the plants to varying light fluence rates, light intensities, and durations. Seeds of knockout lines were grown under white light and dark treatment for seven consecutive days. Class II mutant lines were likewise grown under continuous far-red, red, and blue light at high and low light intensities for five consecutive days. Hypocotyl lengths were measured on the 3rd, 5th, and 7th days (Figure 1).

Results indicate that loss-of-function lines of HDA5, HDA15, and HDA18 were generally normal under white light treatment. However, in the absence of light, we observed significantly longer hypocotyls starting on the 3rd day for *hda15-t27* and 4th day for *hda5-t24* and *hda18-t*. The hyper-skotomorphogenic phenotype exhibited by Class II HDA mutants after four days of dark treatment was noticeably pronounced as those displayed by the *phyA* and *phyB* mutants. This behavior suggests that HDA15 and HDA18 may act downstream of the phytochrome signaling pathway.

Furthermore, light hyposensitive phenotypes of T-DNA knockout mutants were significantly prominent at low light intensities after 3 to 4 days of continuous light treatment. Under far-red, red, and blue light treatments, four days of constant light treatment not only induced long hypocotyls but also yielded bigger, more developed open cotyledons with longer petioles indicating that Class II histone deacetylases might act as positive photomorphogenic regulators. Hypocotyl length of mutant lines eventually leveled off with the wildtype on the fifth

227 day of light treatment, indicating that these histone deacetylases could be active at the early
228 stages of growth and development.

229 All in all, among all the Class II HDAs tested, *hda15-t27* mutants had the most prominent
230 hyposensitive phenotype in both light and dark conditions. Thus, this study focused on
231 elucidating the function of HDA15 in photomorphogenesis.

232 **2. HDA15 targets the deacetylation of COP1 promoter**

233 Like the long-hypocotyl phenotype displayed by the *hy5* mutant, we hypothesize that
234 HDA15 acts as a positive regulator of photomorphogenesis. To determine if HDA15 is indeed
235 involved in photomorphogenesis, expression levels of downstream positive regulators of
236 photomorphogenesis were assessed *via* RT-PCR and qPCR. As presented in Figure 2, among the
237 key positive regulators, which are known to be targeted by acetylation upon light exposure,
238 include HY5, LHB1B1, LHCA1, RBCS-1a, and 1b, CAB1, and CAB2 (Guo et al., 2008;
239 Charron et al., 2009). However, most of these genes, including HY5, were significantly reduced
240 in all the light treatments, which explain the long-hypocotyl phenotype suggesting that a negative
241 regulator catapulted the downregulation of these genes, possibly targeted by HDA15.

242 To further assess the regulatory role of HDA15, mRNA expression profiles of key
243 regulators of photomorphogenesis in *hda15* mutant and overexpression lines were conducted
244 (Figure 3). Compared to white light and far-red light treated plants, dark-treatment has eminently
245 amplified HY5 transcripts levels up to 7-folds in HDA15 overexpression lines. Concomitantly,
246 mRNA levels of COP1 were significantly elevated 3-folds in *hda15* mutants, while its
247 overexpression leads to COP1's extreme downregulation compared to wild type. ChIP assay
248 using anti-H3K9K14ac further reveals that the promoter and start sites of COP1 were highly

249 acetylated in the absence of HDA15 in dark-treated plants. The comparative acetylation profiles
250 suggest that HDA15 targets the deacetylation of COP1 in the dark.

251 **3. Light signals HDA15 nuclear localization, dark treatment induces cytoplasmic**
252 **translocation**

253 Hypocotyls of 4-day old HDA15-GFP transformants were used to determine its
254 localization (Figure 4). Under white light conditions, HDA15 remained to be prominently
255 nuclear. When grown under far-red light, HDA15-GFP concentrated in the nucleus, which is
256 similar to blue and red light treated lines. Consequently, seedlings grown in the dark exhibited
257 strong signals at the cytoplasmic area, although small quantities were still detected at the
258 nucleus. Similar results were observed when HDA15-GFP were transiently expressed in
259 protoplasts under various light treatment.

260 As mammalian Class II HDAs undergo nucleocytoplasmic shuttling, there is a possibility
261 that HDA15 may exhibit similar functional regulatory mechanisms. To illustrate whether
262 HDA15 undergoes nucleocytoplasmic shuttling, we determined the response of transfected
263 protoplasts after successive incubation under white light overnight, dark treatment, and light re-
264 exposure. As shown in Figure 5, HDA15-GFP transfected protoplasts exhibited nuclear
265 localization after 18 hours of white light incubation. Further dark treatment for 3 hours elicited
266 partial cytoplasmic translocation of HDA15-GFP. After one hour, re-exposure of the protoplasts
267 to white light led to its complete nuclear import. The observed response demonstrates that light
268 indeed drives the nucleocytoplasmic shuttling of HDA15.

269 **4. COP1 triggers the nuclear localization of HDA15**

270 Scanning through the amino acid sequence of HDA15, it contains a type par4 nuclear
271 localization signal (NLS) near the amino end, two overlapping bipartite NLS near the carboxyl

end, and a nuclear export signal (NES) near the C terminal half. Our previous study has indicated that these signals navigate the subcellular compartmentalization of HDA15, and that light, in general, drives the nucleocytoplasmic shuttling of HDA15 (Alinsug et al., 2012). Albeit the exact mechanisms controlling its NLS and NES signals upon light exposure remains obscure. Thus, we speculated that the only way for HDA15 to detect light is through the photoreceptors; their inactivation upon the termination of light may have initiated HDA15's nuclear export.

To test this hypothesis, HDA15-GFP/YFP was transiently expressed in protoplasts of mutant photoreceptors *phyA, phyB*, and *cry1xcry2*. As shown in Figure 6, all these mutant photoreceptor protoplasts displayed nuclear localization of HDA15 suggesting that the nuclear import of HDA15 is not dependent on light quality nor controlled singly by the phytochrome/cryptochrome signaling cascades, but implicates the involvement of all photoreceptors. The downstream target of all four photoreceptors, COP1, appears to have a significant impact on HDA15's nuclear localization, where *cop1-4* mutant protoplasts exhibited speckled distribution of HDA15 in the cytoplasm immediately near the vicinity of the nucleus. Overall, our results suggested that the activation of photoreceptors did not trigger HDA15 nuclear localization. Instead, the shuttling observed was likely attributed to the presence of COP1.

5. HDA15 directly interacts with COP1 inside the nucleus

The human Class II HDAC6 has been well studied to catalyze non-histone proteins such as α-tubulin, cortactin, and HSP90, as well as, bind to ubiquitinated proteins inhibiting their proteosomal degradation via its zinc finger ubiquitin-binding protein (ZnF-UBP) domain (Hook et al., 2002; Kovacs et al., 2005; Luxton and Gundersen, 2007; Valenzuela-Fernandez et al., 2008). Although the corresponding plant ortholog of HDAC6 is unclear, HDA15 contains a

295 RanBP zinc finger analogous to ZnF-UBP of HDAC6. To determine if HDA15 can associate
296 with non-histone proteins such as COP1, pull-down and BiFC assays were conducted (Figure 7).
297 Direct binding between HDA15 and COP1 was illustrated via pull-down assay using anti-His,
298 anti-GST, and anti-COP1 antibodies where HDA15 and COP1 were fused with GST- and His-
299 tags, respectively. This interaction was further confirmed *in vivo* using BiFC demonstrating their
300 direct binding inside the nucleus both under white light and dark conditions. Although it has
301 been consistently demonstrated that HDA15 is nuclear under all the light treatments, the
302 abrogation of COP1 in *cop1-4* mutant protoplasts renders it cytoplasmically speckled.

303 **6. HDA15 positively regulates photomorphogenesis by repressing COP1**

304 It has long been hypothesized that COP1 may recruit deacetylases or chromatin
305 remodeling factors that can repress transcription from target promoters (Holm and Deng, 1999).
306 If HDA15 functions as a co-repressor of COP1, then the mutant lines of HDA15 should exhibit
307 *cop1*-like phenotypes. On the contrary, *hda15-t27* lines manifested light hyposensitivity similar
308 to *hy5* mutants with down-regulated levels of HY5 and other positive regulators of
309 photomorphogenesis. Thus, HDA15 may indirectly regulate HY5 by repressing COP1, making
310 it a positive regulator of photomorphogenesis.

311 To establish the genetic relationship between HDA15 and COP1, we generated the
312 double mutant *cop1-4xhda15-t27*. As shown in Figure 8, the cross between *hda15-t27* and *cop1-*
313 *4* mutants exhibited short hypocotyl phenotypes under white light and dark treatments and
314 dwarfed phenotypes in 3-week old plants all reminiscent of *cop1-4* mutants. These responses
315 suggest that COP1 is epistatic to HDA15. Therefore, HDA15 targets the repression of COP1 via
316 direct binding, thereby attenuating its capacity to ubiquitinate HY5 and its targets.

317 **7. Ubiquitinated sites of HY5 and COP1 are less stable than HDA15**

318 Considering that COP1 is an E3 ligase, the binding of HDA15 with COP1 raises pertinent
319 questions as to whether COP1 ubiquitinates HDA15 or HDA15 represses COP1. To address
320 this, bioinformatics analysis using three algorithms, namely: Ub-Pred, BDM-PUB, and UbiSite,
321 were used to predict ubiquitination sites comparing HY5, COP1, and HDA15. Based on the
322 algorithms' consensus, as presented in Table 1, HY5 was found to contain five residues that are
323 actively targeted for ubiquitination, rendering it the most unstable among the three, with
324 instability index of 68.21. COP1 follows HY5 with an instability index of 47.13, with four
325 consensus residues prone to ubiquitination. On the other hand, it appears that HDA15 is the
326 most stable among the three proteins with an instability index of 37.11, noting that it only has
327 two residues as prospective sites for ubiquitination. This bioinformatics analysis hence reaffirms
328 our prior notion that HY5 is targeted for ubiquitination by COP1 in the dark during
329 skotomorphogenesis. With HDA15 being more stable than COP1, the direct interaction between
330 HDA15 and COP1 leads us to conclude that HDA15 represses COP1.
331
332

333 **Discussion**

334 Histone active marks such as H3K9K27ac signals transcriptional activation whereby light
335 induces the acetylation of positive photomorphogenic regulators functioning as chlorophyll a/b-
336 binding protein, peroxidase, citrate synthase, RBCS small chain 1B, ribosomal protein, PSII-
337 light-harvesting complex, HYH, HY5 and its downstream targets CHS, IAA3, RBCS-1a during
338 photomorphogenesis (Benhamed et al., 2006; Guo et al., 2008; Charron et al., 2009). On the
339 other hand, histone deacetylation stimulates chromatin compaction triggering transcriptional
340 quiescence. Among the RPD3/HDA1-like superfamily of HDAs, Class I histone deacetylases
341 HDA6 and HDA19 display negative regulatory roles in photomorphogenesis. Together with
342 PHYB, Tessadori *et al.* have identified HDA6 as a regulator of light-induced chromatin
343 condensation (Tessadori et al., 2009). Similarly, HDA19 is suspected of repressing many light-
344 regulated genes by deacetylating H3K9K14 at the transcriptional start site of the phyA locus
345 (Benhamed et al., 2006; Jang et al., 2011).

346 On the other hand, it appears that Class II HDAs play a positive regulatory role in the
347 light signaling network with HDA15 repressing COP1 at its locus and modulating its activities
348 via direct interaction. As HDA5 and HDA18 also exhibit long-hypocotyl phenotypes at varying
349 light treatments, it remains a conundrum of how these and other Class II HDAs function during
350 photomorphogenesis. These findings fine-tune our prior understanding of the role of histone
351 modifications in light-regulated gene expression. Light not only induces the acetylation of key
352 positive regulators of photomorphogenesis but also renders the simultaneous repression of vital
353 negative regulators such as COP1 via direct interaction. Taken together, our results indicated
354 that histone acetyltransferases and deacetylases act as multi-functional and potent chromatin-
355 modifying enzymes during photomorphogenesis.

356 As light signals the nucleocytoplasmic shuttling of HDA15, its nuclear import is
357 primarily dependent on COP1. Since HDA15 have contrasting localization with COP1 in the
358 presence and absence of light, it is yet to be determined if a concentration gradient on either of
359 the proteins influences the movement of the other, assuming these enzymes are not degraded; or,
360 if a dominant ligand induces the degradation of the other. It would be interesting to unravel the
361 molecular underpinnings of the opposing nucleocytoplasmic translocation of HDA15 & COP1
362 upon light and dark signaling cues. Although a significant amount of HDA15 translocates into
363 the cytoplasm, this nucleocytoplasmic shuttling suggests a functional regulatory mechanism for
364 deactivation both as a histone deacetylase and a direct repressor of COP1. As HDA15 have been
365 demonstrated to shuttle in and out of the nucleus with a functionally active nuclear export signal
366 and two nuclear localization signals, the direct association of HDA15 and COP1 may also
367 implicate the possibility of HDA15 acting as a chaperone to transport COP1 to the cytoplasm as
368 it lacks a nuclear export signal.

369 While other mutant Class II HDAs also manifest light hyposensitivity, only HDA15 is
370 driven by light to shuttle in and out of the nucleus. The environmental cue or stress that would
371 signal the nuclear import of the other Class II HDAs and their functional characterization remain
372 to be investigated as most of them have been observed to be cytoplasmic (Alinsug et al., 2012;
373 Tran et al., 2012).

374 Histone deacetylases constitute a family of nuclear enzymes known primarily for their
375 role in transcriptional repression. Class II HDAs, in particular, are known to be versatile
376 regulators catalyzing the deacetylation of both histones and non-histone proteins. The
377 mammalian HDAC6 modulates a wide variety of cellular activities involving complex
378 interactions with α-tubulin, cortactin, HSP90, and other proteins (Matthias et al., 2008;

379 Valenzuela-Fernandez et al., 2008). Similarly, HDAC1 complexes with MDM2, an E3 ubiquitin
380 ligase, deacetylate p53, and promote its ubiquitination (Ito et al., 2002). In the case of
381 AtHDA15, it attenuates COP1 modulating its repressive activities via direct interaction,
382 subcellular compartmentalization, and chromatin compaction at its transcriptional start site.
383 Based on our results, this versatile regulator not only functions as a traditional histone
384 deacetylase silencing key regulators of photomorphogenesis, specifically COP1, but it also
385 directly interacts with COP1 likely attenuating its E3 ligase activities inside the nucleus.

386 The specific molecular mechanisms for the degradation and/or cytoplasmic export of
387 COP1 in the nucleus under light conditions remain obscure. The nuclear interaction of HDA15
388 with COP1 under light conditions may support the notion that COP1's depletion may be
389 enhanced by the binding of HDA15 to induce auto-ubiquitination. However, based on the
390 Western blot, the mutant nor overexpression lines of HDA15 neither influenced COP1's protein
391 levels. Instead, HDA15 attenuates the E3 ligase activity of COP1 that prevents the
392 ubiquitination of HY5. This action explains the downregulation of HY5 protein concentrations
393 in *hda15* lines and the elevated levels of the protein in HDA15-OE lines.

394 On a similar note, in the dark, where COP1 is active and nuclear, partial concentrations of
395 HDA15 are inactivated via cytoplasmic importation. HDA15 in the nucleus may modulate the
396 activities of COP1 and target the deacetylation of its transcriptional start site. Presented in
397 Figure 9 is a working model depicting the sub-compartmentalization and modularized activities
398 of COP1 and HDA15 under light and dark conditions.

399 In light, COP1 is mainly cytoplasmic, although some trace amounts are left inside the
400 nucleus. When HDA15 shuttles into the nucleus, it binds to COP1 and conceivably leads to the
401 abrogation of HY5's ubiquitination regulating photomorphogenesis. In the dark, contrarily,

402 COP1 exclusively localizes inside the nucleus and targets the ubiquitination of HY5 to advance
403 skotomorphogenesis. While the majority of HDA15 translocates into the cytoplasm, partial
404 amounts left in the nucleus target the deacetylation of the transcriptional start site of COP1 locus
405 and/or interact with COP1 to modulate its repressive activities. The molecular switches in
406 apportioning the exact amounts of HDA15 to remain active inside the nucleus while others are
407 deactivated inducing their nuclear export remains elusive and requires further investigation.

408 We also attempted to determine the binding sites of the two repressors by generating
409 different truncated constructs of COP1 and HDA15 containing various combinations of potential
410 protein-binding domains. Surprisingly, only the full-length HDA15 was able to bind the full-
411 length COP1 suggesting strict, proper protein conformation for such interaction.

412 Previous studies on the conserved domains of RPD3/HDA1-like superfamily show that
413 Zn finger domains are imprinted on several Class II HDAs (Pandey et al., 2002; Alinsug et al.,
414 2009). The monocot ortholog of AtHDA15 in maize, ZmHDA110, has a C3HC4 type RING Zn
415 finger that binds with ubiquitin while the ZnF-UBP of the mammalian Class II HDAC6 binds to
416 ubiquitin specifically at the C termini diglycine motif catalyzed by a deubiquitinase, ataxin-3
417 (Ouyang et al., 2012). RanBP-ZnF in HDA15 may similarly interact with ubiquitin, such as
418 those found in Npl4 (Alam et al., 2004). Based on sequence analyses on Zn fingers conducted
419 by Wang *et al.*, the molecules have highly conserved cysteine residues and other key motifs in
420 binding ubiquitin (Wang et al., 2003). As illustrated in Figure 10, sequence comparison between
421 HDA15's RanBP2-ZnF to the human Np14 (NZF) reveals the presence of the two canonical
422 highly-conserved cysteine residues and a key ubiquitin-binding motif, Thr_{95}. Considering the
423 RING-ZnF domain of COP1 catalyzes the transfer of ubiquitin units from E2 transferases to

424 target proteins such as HY5, we speculate that ubiquitin may constitute a recognition site for
425 HDA15 to identify COP1 as an active E3 ligase.

426 Nevertheless, unlike HDAC6, which binds and transports ubiquitinated proteins to
427 aggresomes, the binding of HDA15 to COP1 undertakes a regulatory role via the attenuation of
428 the latter's E3 ligase activity. Based on bioinformatics analysis comparing the ubiquitination
429 sites of HY5, COP1, and HDA15, it is predicted that HDA15 is more stable than COP1. As
430 expected, HY5 is the least stable making it more prone to ubiquitination. Still, questions remain
431 on the exact nature of binding of HDA15 to ubiquitin and/or polyubiquitin, as well as the
432 properties of the molecular switches downstream of the HDA15-COP1 complex.

433

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441

442 **Author Contribution**

443 MVA conceptualized the study, screened and generated double mutant lines for phenotypes,
444 conducted light experiments, ChIP and BiFC assay, and wrote the manuscript. CD performed
445 pull down assay, mRNA extraction, bioinformatics analyses, and wrote a portion of the
446 manuscript.

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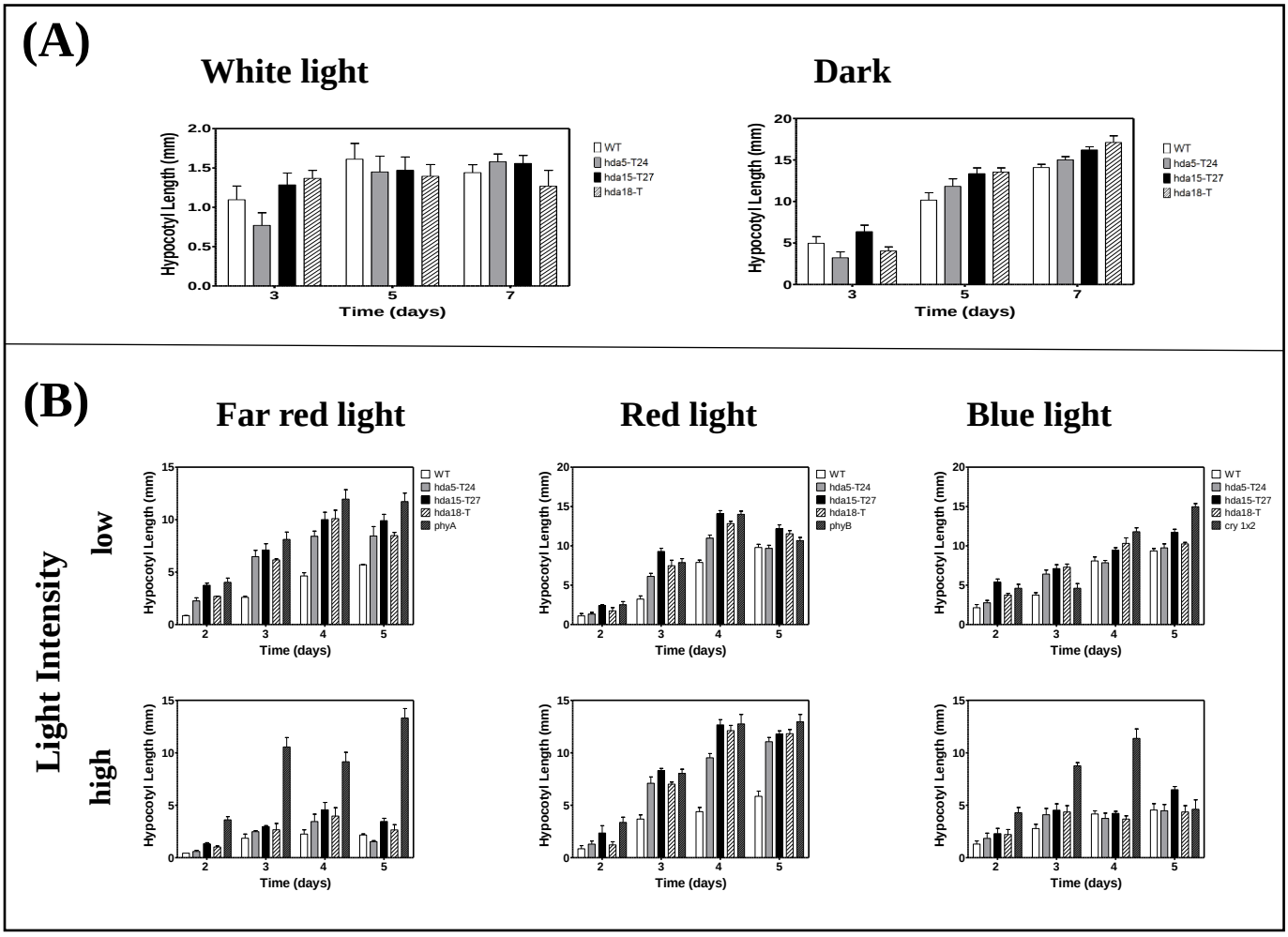

Figure 1. Light hyposensitivity in Class II HDAs (A) Hypocotyl lengths of WT, *hda5*, *hda15* and *hda18* mutants under dark and white light were measured for 7 consecutive days. (B) Hypocotyl lengths of Class II HDA mutants under far red, red and blue light treatments at different light intensities were observed for 5 days. Light intensity treatments were designated as low (L, upper panel) or high (H, lower panel): far red, H at 18.065 and L at 2.77 μmol m^{-2} s^{-1}; red light, H at 22.938 and L at 1.77 μmol m^{-2} s^{-1}; and blue light, H at 11.095 and L at 3.84 μmol m^{-2} s^{-1}. Data presented are mean values with error bars set from its standard deviation.

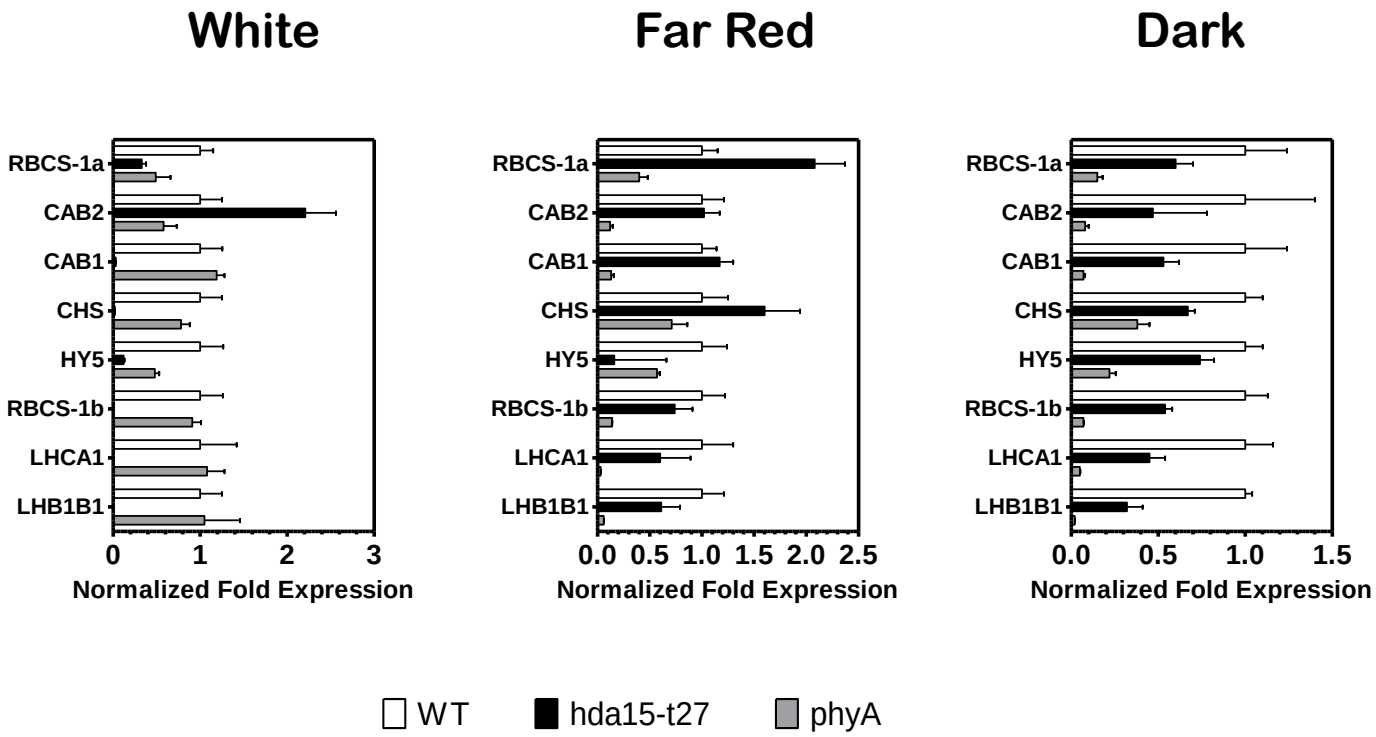

Figure 2. Down-regulation of positive regulators to photomorphogenesis in AtHDA15 knockout lines. mRNA expression levels of positive regulators of photomorphogenesis were assessed in *A. thaliana* exposed to (A) white light, (B) far-red light, and (C) the dark. Expression levels reflected were normalized to ubiquitin.

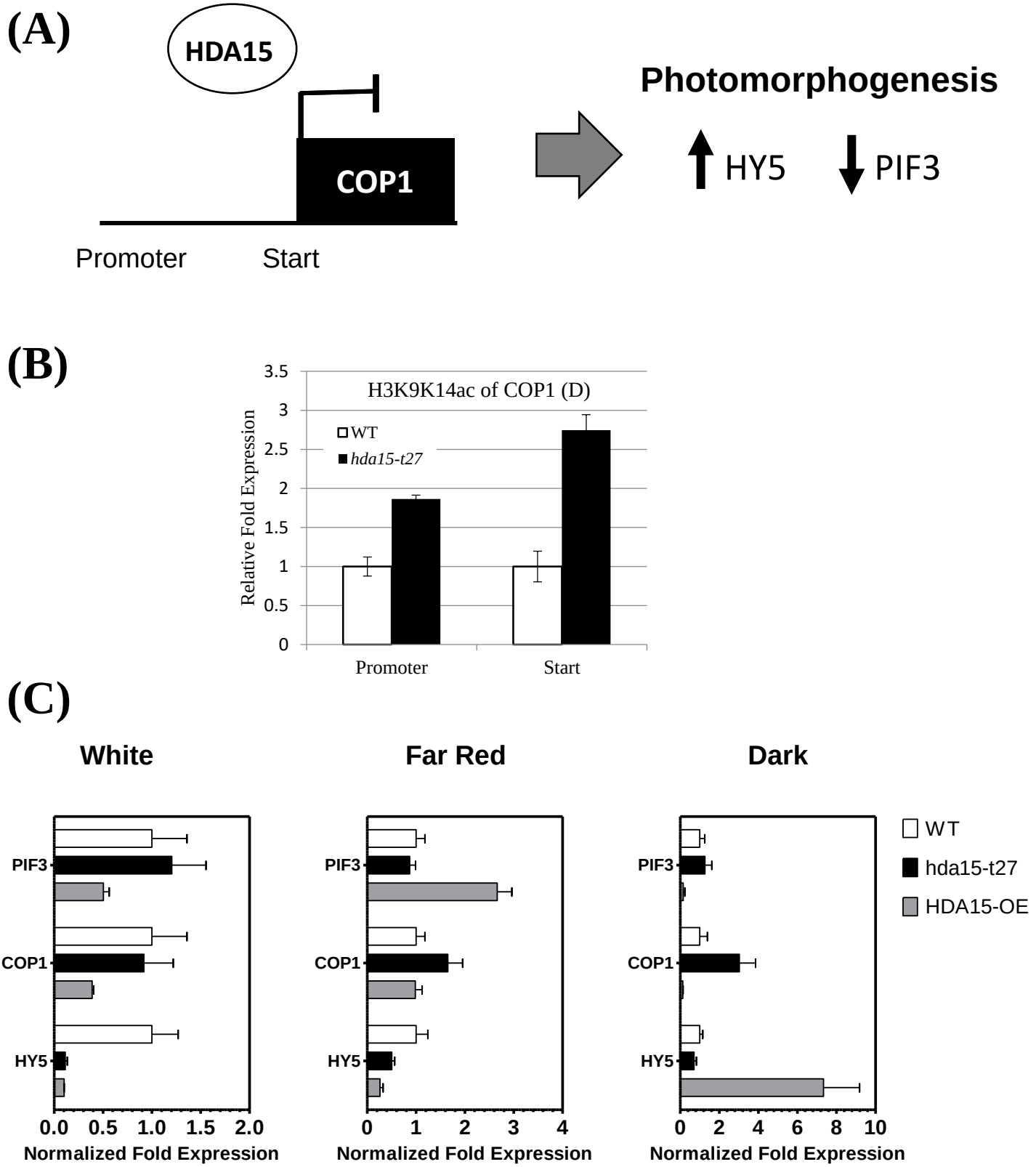

Figure 3. mRNA expression profiles of key regulators of photomorphogenesis in HDA15 mutant and overexpression lines. (A) Schematic diagram of repressive action of HDA15 on COP1 locus. (B) ChIP assay using anti-H3K9K14ac further reveals that the promoter & start sites of COP1 were highly acetylated in the absence of HDA15 in dark-treated plants. (C) Compared to white light and far red light treated plants, dark-treatment has eminently amplified HY5 transcripts in HDA15 overexpression lines. Concomitantly, mRNA levels of COP1 were significantly elevated in *hda15* mutants while its overexpression lead to COP1's extreme downregulation.

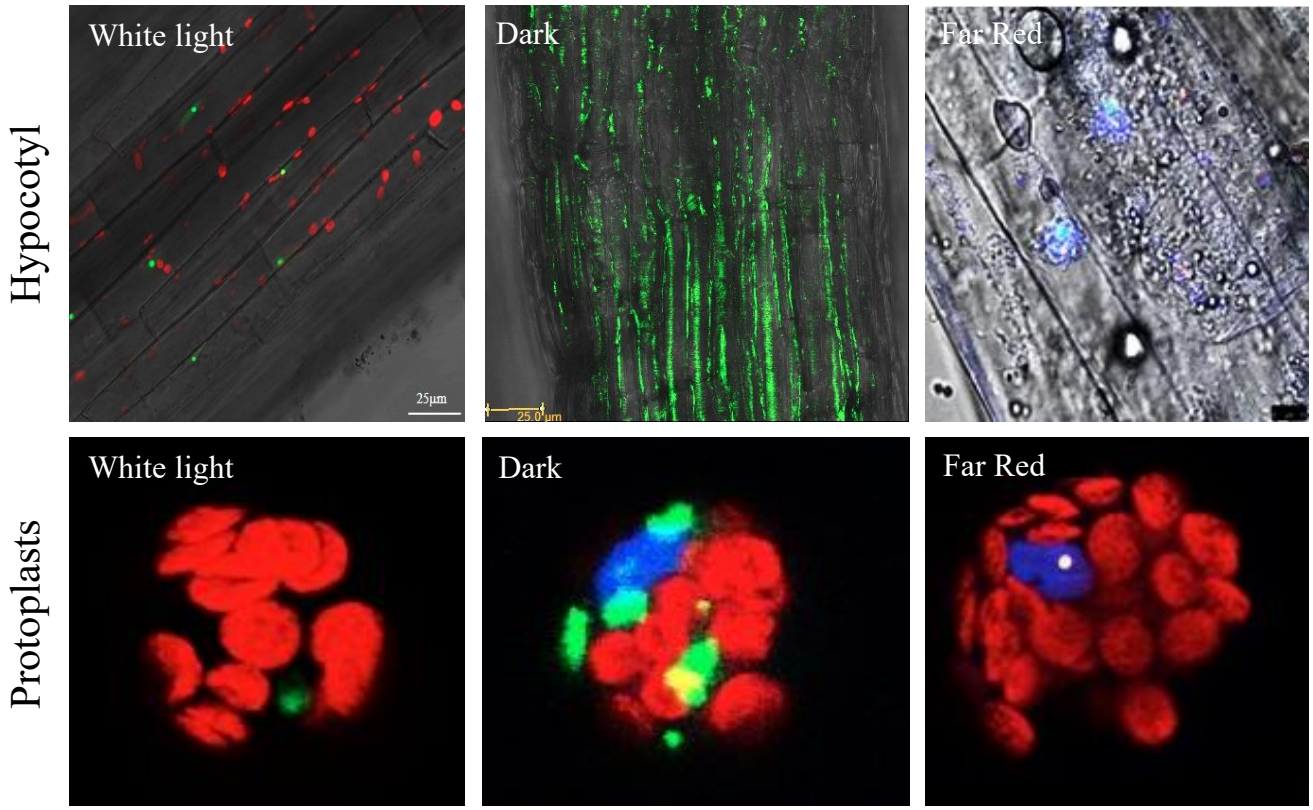

Figure 4. Subcellular localization of AtHDA15 under different light radiance with wildtype and mutant photoreceptors. Localization HDA15-GFP/YFP was localized in the nuclei of hypocotyls and protoplasts of transgenic lines grown under white light and different colored light treatments of A. thaliana with wild-type photoreceptors. Note: In dark treated plants, HDA15 has a pan-cytoplasmic distribution. Hoechst was used as a nuclear marker.

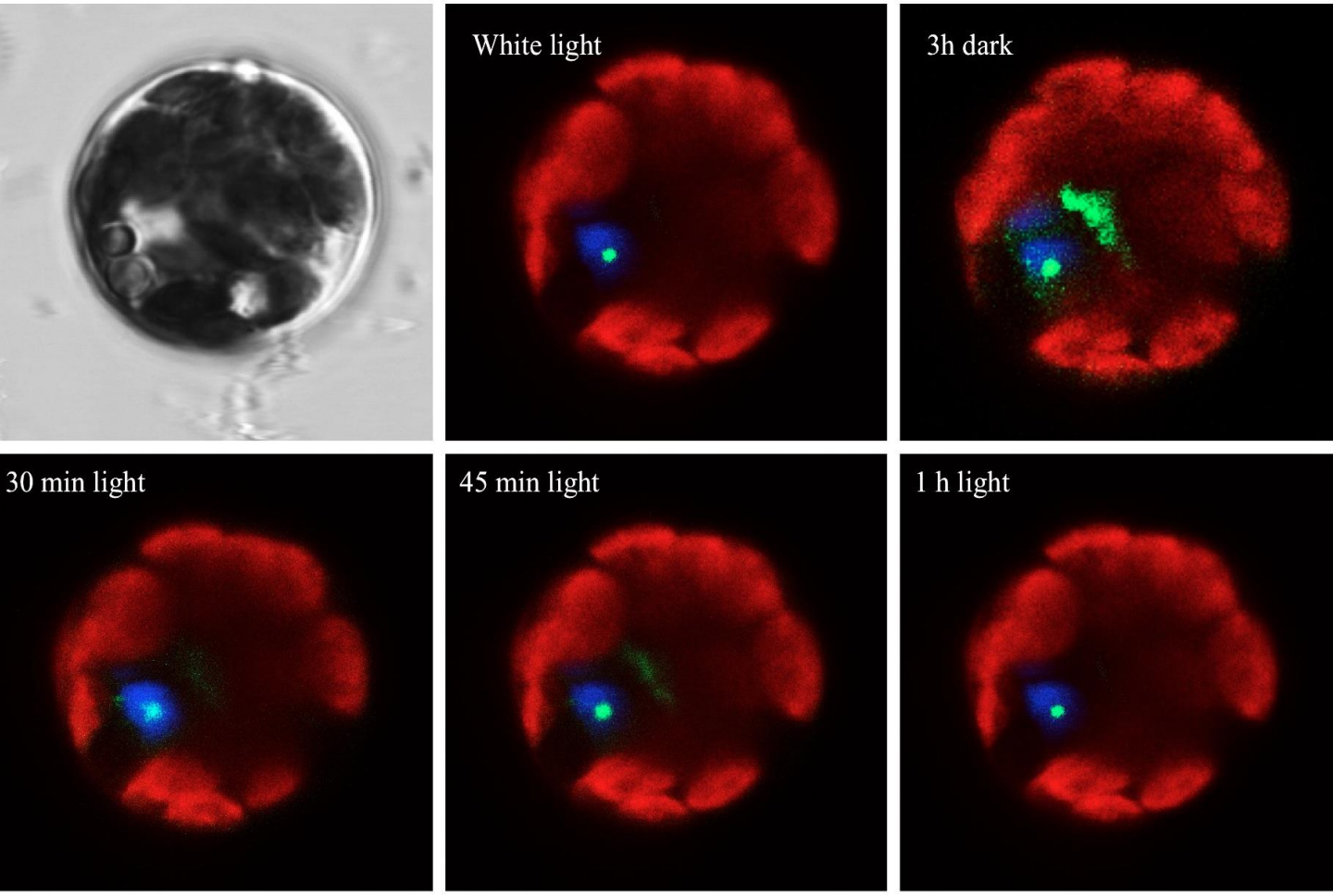

Figure 5. Time-course monitoring of nucleocytoplasmic shuttling and pan-cytoplasmic scattering of HDA15. Transfected protoplasts were incubated under white light overnight then covered with foil for 3 hours and re-exposed to white light. HDA15-GFP transfected protoplasts exhibited nuclear localization after 18h of white light incubation. Further dark treatment for 3h elicited partial cytoplasmic translocation of HDA15-GFP. Re-exposition to white light after 1h lead to its complete nuclear import. VirD2NLS was co-transfected as nuclear marker (blue).

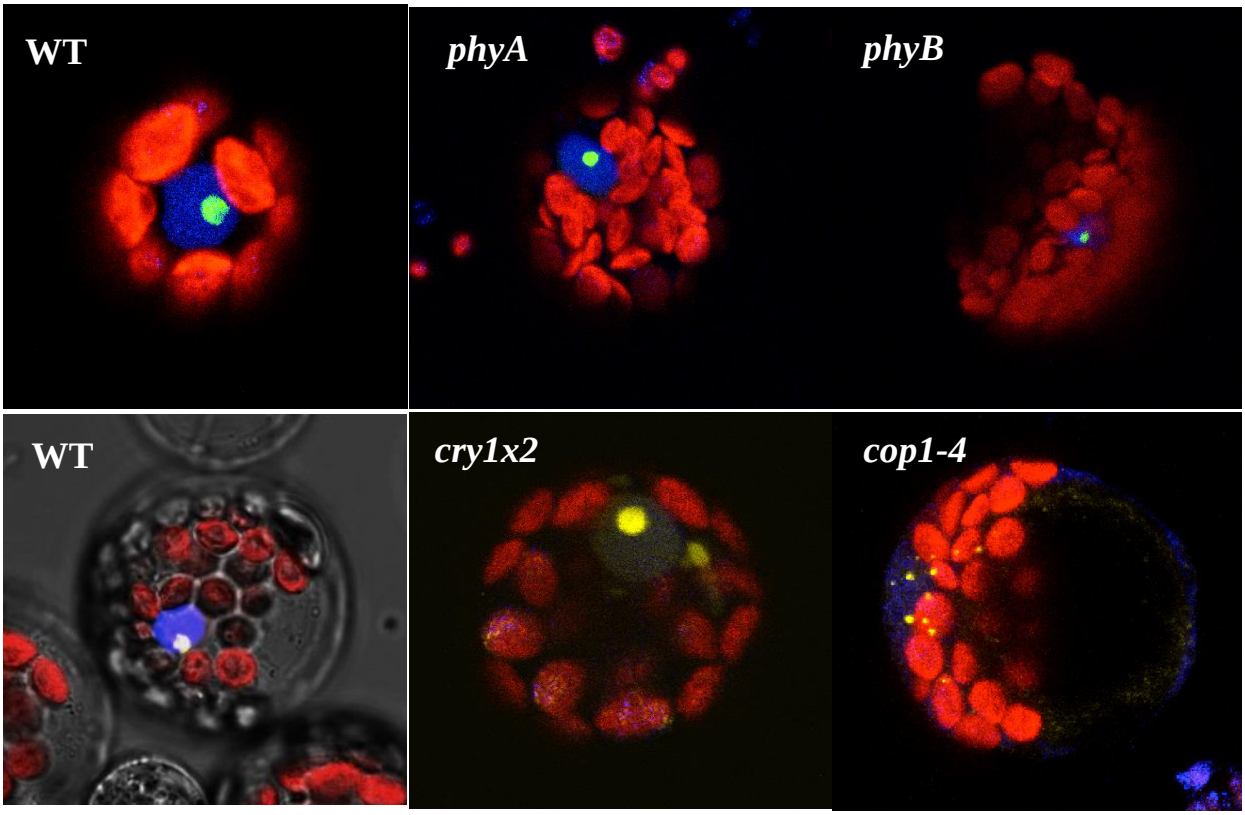

Figure 6. COP1 activates nuclear localization of HDA15. HDA15 localization in protoplasts of mutant photoreceptors were all nuclear. However, when transiently expressed in *cop1-4* mutant protoplasts, HDA15 exhibited cytoplasmic localization. VirD2-NLS was co-transfected as nuclear marker (blue).

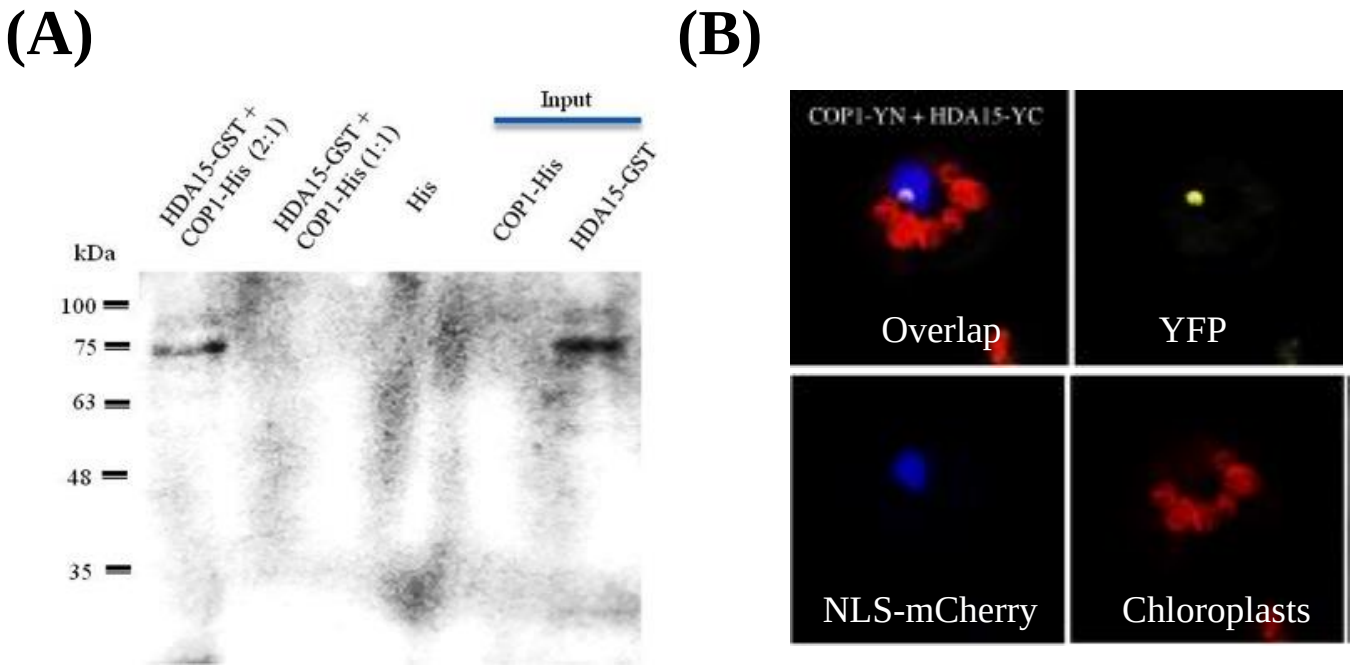

Figure 7. Binding to COP1 is associated with the nucleocytoplasmic shutting of AtHDA15. **(A)** *In vitro* and **(B)** i*n vivo* interaction between COP1 and AtHDA15 indicates their direct binding inside the nucleus. AtHDA15-COP1 interaction was further confirmed via pull down assay using anti-GST antibody where HDA15 was fused with GST and COP1 with His tags. Interaction of COP1 and AtHDA15 in isolated protoplasts was visualized 18–24 hr after transfection using bifluorescence complementation assay under laser confocal microscopy. AtHDA15 and COP1 genes were recombined with N-YFP and C-YFP. VirD2-NLS was used as nuclear marker.

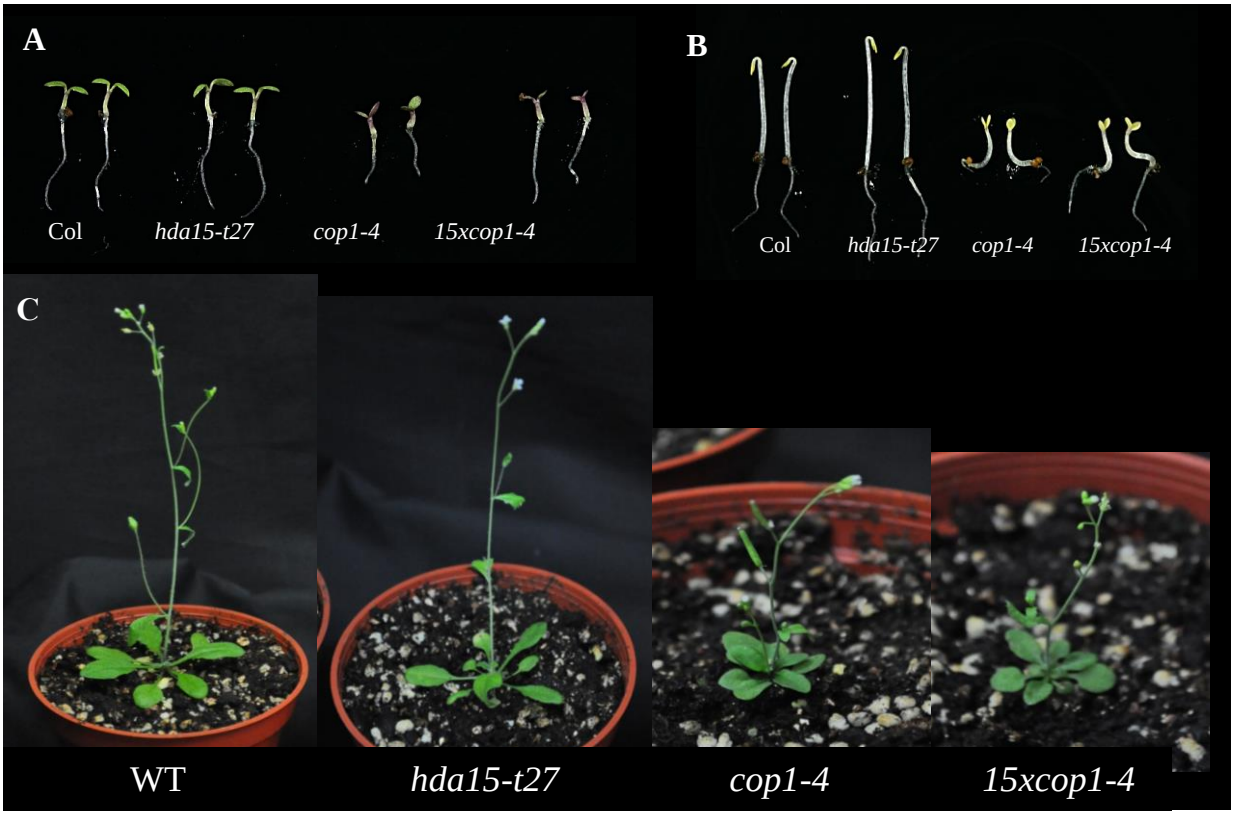

Figure 8. *hda15-t27xcop1-4* phenotype. The cross between *hda15-t27* and *cop1-4* mutants exhibited short hypocotyl phenotypes under white light (A) and dark (B) treatment for 4 days and dwarfed phenotypes in 3-week old plants (C) which are reminiscent of *cop1-4* mutants.

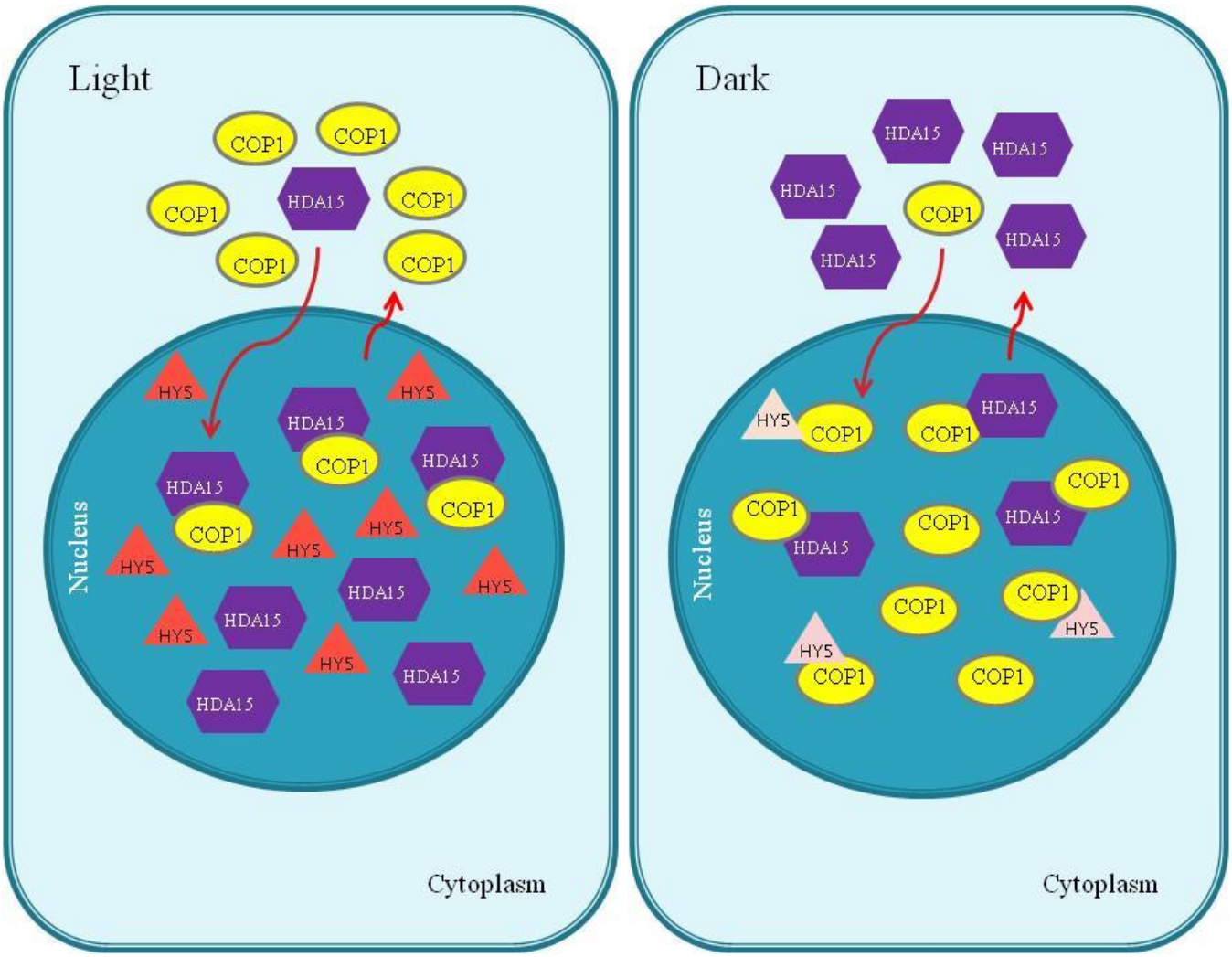

Figure 9. Working hypothesis illustrating sub-compartmentalization and modular activities of COP1 and HDA15 under light and dark conditions. In the presence of light, COP1 is cytoplasmic with some trace amounts left inside the nucleus. On the contrary, HDA15 concentrates inside the nucleus binding to nuclear COP1, which prevents the ubiquitination of HY5 thereby positively regulating photomorphogenesis. In the dark, however, COP1 exclusively localizes inside the nucleus targeting the ubiquitination of HY5 proceeding to skotomorphogenesis. On the other hand, majority of HDA15 translocates into the cytoplasm with some minimal amounts left in the nucleus. HDA15 directly associates with COP1 to modulate its repressive activities and targets its deacetylation at the promoter and start sites leading to transcriptional quiescence.

NZF	$_{3}$TSAMWACQHCTFMNQPGTGHCEMCSLPRT$_{31}$
RanBP2	KEGQWDCSACLVQNEGSSTKCAACQNPRK
HDA15-RanBP2	$_{86}$FVKWCCVNCTMSNPGDMVHCCICGEHKES$_{115}$

Figure 10. Sequence comparison between Zn fingers in human Npl4 (NZF), wild type RanBP2 which has minimal Ubq binding, and RanBP2 in AtHDA15. Conserved residues in NZF was defined by Wang *et al* (2003) where blue letters indicate semi-conserved residues, green cysteine residues are highly conserved and contact Ubq, and orange residues as key motifs in binding Ubq.

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