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Research Article

Depletion of ε-COP in the COPI Vesicular Coat Reduces Cleistothecium Production in *Aspergillus nidulans*

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Abstract We have previously isolated ε-COP, the α-COP interactor in COPI of *Aspergillus nidulans*, by yeast two-hybrid screening. To understand the function of ε-COP, the $aneA^+$ gene for ε-COP/AneA was deleted by homologous recombination using a gene-specific disruption cassette. Deletion of the ε-COP gene showed no detectable changes in vegetative growth or asexual development, but resulted in decrease in the production of the fruiting body, cleistothecium, under conditions favorable for sexual development. Unlike in the budding yeast *Saccharomyces cerevisiae*, in *A. nidulans,* over-expression of ε-COP did not rescue the thermo-sensitive growth defect of the α-COP mutant at 42°C. Together, these data show that ε-COP is not essential for viability, but it plays a role in fruiting body formation in *A. nidulans.*

Keywords α-COP, *Aspergillus nidulans*, Cleistothecium, ε-COP, Sexual development

Eukaryotic cells contain a collection of spatially separated internal organelles embedded in the cytoplasm. Communication between these internal organelles is mediated by trafficking events that are mainly accomplished by vesicular transport. These trafficking events facilitate the targeted delivery of newly synthesized proteins and lipids, uptake of extracellular cargo, and also play a role in regulatory processes [1]. The cargo recognized by coat proteins is stabilized, carried in the vesicle, and delivered to the target membrane. Among the various coat proteins identified to date, coatomer, the coat complex of COPI-coated vesicles composed of seven COPs (α-, β-, β′-, γ-, δ-, ε-, and ζ-COP) and the small ras-like GTPase, ADP ribosylation factor, mediates recycling of proteins in Golgi-to-endoplasmic reticulum (ER) interactions and within the Golgi complex [2, 3]

In yeast, all COPs, except ε-COP, are essential for cell viability. ε-COP stabilizes α-COP, and thus coatomer in the elevated temperatures [4]. In addition, α-, γ-, δ-, and ζ-COP mutants, referred to as *ret* mutants, show inability to retrieve proteins with a di-lysine motif (KKXX) [5-7]. α- and β′-COPs are involved in the recruitment of di-lysine-tagged proteins [6]. Ret1/α-COP of *Saccharomyces cerevisiae* is known to contribute to the maintenance of cell wall integrity and biogenesis by influencing glycosylation and localization of the secretome including cell wall proteins [8-12]. Quantitative proteomic analysis has revealed an association between calcineurin, a calcium-calmodulin-associated protein phosphatase, and ε-COP during high temperature stress in the human fungal pathogen *Cryptococcus neoformans* [13]. In *Aspergillus nidulans*, α-COP is essential for viability. However, like in yeast, a mutation in *A. nidulans* α-COP gene results in a temperature-sensitive defect in growth, cell-wall construction, and protein secretion [14, 15]. Interestingly, the temperature dependent-osmo-sensitive phenotype of the *S. cerevisiae* α-COP mutant is suppressed by introduction of the *A. nidulans* $sod^{VI}C$ (stabilization of disomy) gene for α-COP [15]. In addition, the C-terminus of the *A. nidulans* ε-COP, AneA, including tetratrico peptide repeat (TPR: $W_4G_8Y_{11}G_{15}Y_{17}A_{20}Y_{24}A_{27}P_{32}$) interacts with the C-terminus of α-COP [16]. These findings suggest that the action of the coatomer in filamentous fungi may be similar to that in yeast, but the function of ε-COP in conjunction with α-COP needs to be elucidated in *A. nidulans.*

In this study, to understand the function of ε-COP in *A.*

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nidulans during development, we performed phenotypic analyses of the *aneA* deletion mutant and α-COP mutant with over-expression of the gene encoding ε-COP, *aneA*$^+$.

MATERIALS AND METHODS

Strains and growth conditions. *Aspergillus nidulans* strains and plasmids used in this study are listed in Table 1. Plasmid amplification and transformation of *Escherichia coli* have been described previously [16]. Previously described growth conditions including those for inducing development were used for *A. nidulans* [17, 18]. Expression of *aneA*$^+$ via *niiA* promoter is induced by 0.6% sodium nitrate and repressed by 0.2% ammonium tartrate.

Construction of a disruption cassette. An *aneA*$^+$ disruption cassette (DC) was constructed according to methods described by Yu *et al.* [19]. Using genomic DNA prepared from *A. nidulans* FGSC A4, DNA fragments for the DC were amplified by PCR by using appropriate primer sets (AneA-A1/-A2, AneA-B1/-B2, and *argB*-For/-Rev). The complete DC was amplified by a nested-PCR primer set (AneA-C1/-C2), purified, and then used to transform the TJ1 strain. The primers used in this study are listed in Table 2.

Nucleic acid preparation, Northern blotting, and Southern blotting. Nucleic acid preparation and northern blot analysis were performed as described previously [17]. For Southern blotting, genomic DNA was treated with appropriate restriction enzymes and then separated on 1% agarose gels. The gels were washed with distilled water (DW) and soaked twice in a depurination solution (250 mM HCl) for 15 min. After the reaction, the gel was

Table 1. List of *Aspergillus nidulans* strains and plasmids used in this study

	Genotypes	Sources
Strains		
FGSC A4	*veA+*	FGSC
TJ1	*yA2; argB2; pyroA4; veA+*	S. K. Chae
SK880	*yA2; argB2; pyroA4; veA+; pILJ16 (argB*$^+$*)*	S. K. Chae
DEC	*yA2; argB2; pyroA4; veA+; aneAΔ::argB*	This study
PBR2	*pyrG89; yA2*	This Lab
B120	*pyrG89; yA2; sod*^{VI}*C1*	S. J. Assinder
B120A	*pyrG89; yA2; sod*^{VI}*C1;pRG3-AMA1-nii(p)::Pyr4*	This study
B120AE	*pyrG89; yA2; sod*^{VI}*C1;pRG3-AMA1-nii(p)::aneA::Pyr4*	This study
Plasmids		
pRG3-AMA1	*Amp*^{r}*;AMA1;Pyr4;niiA*	S. K. Chae
pAME	*Amp*^{r}*;AMA1;Pyr4;nii::aneA*	This study

FGSC, Fungal Genetics Stock Center.

Table 2. List of oligonucleotides used in this study

Oligo	Sequence (5′-3′)
aneA$^+$ disruption	
AneA-A1	GGC CAC GTT CAC CGC TAA
AneA-A2	**AGT CAA ATG AGG CCT CTA AAC** TGG TCA TGA CAA TGG GCT GTT TAG
AneA-B1	**AGC CAA GGT AGA TCC AGG CCT AAC ACA** AAT ATG ATT AGT CCT GTC
AneA-B2	GAC ACT CAA TCA CGG CGC
argB-For	GAC CAG TTT AGA GGC CTC
argB-Rev	GTG TTA GGC CTG GAT CTA
AneA-C1	AGG AAC CCA TTT CTG TTC GC
AneA-C2	GAG ATC CTC ACG GAG CTC AA
aneA$^+$ over-expression	
AneA-D1	CC**C CCG GGG** GAA TGG ATC CAT TCT CTG CAG
AneA-D2	TT**G CGG CCG C**AA TGA AGA AAC CTT GGC GGA GTA
Southern probe	
AneA-E1	CGG AGC TCC GCC ATT TTC TT
AneA-E2	CCC CAG ACG CCT CAA TAA AT
Northern probe	
AneA-F1	**GAA TTC** ATG GAT CCA TTC TCT GCA G
AneA-F2	**CTC GAG** ATC GAA AAA GTC ACT CTT CT

Bold and underlined letters indicate *argB* complement sequence.
Bold letters indicate restriction enzyme linker.

washed with DW again and then soaked in denaturation solution (1.0 M NaCl and 0.4 M NaOH) for 1 hr. The genomic DNA contained in the processed gel was transferred onto a Hybond-N$^+$ membrane (Amersham Biosciences, Pittsburg, PA, USA). Gene-specific probes were prepared from the PCR-generated fragments and were labeled using the ECL direct nucleic acid labeling system (Amersham Biosciences). The signals were visualized by exposing the membrane to an X-ray film.

RESULTS AND DISCUSSION

Effect of ε-COP depletion on growth and stress response. The DC for deletion of the *aneA*$^+$ gene was constructed and used to transform the recipient strain. Southern blot analysis of *Pst* I-digested genomic DNA from candidate strains with a PCR-amplified probe specific for the 5′-flanking region of *aneA*$^+$ confirmed the deletion of the chromosomal *aneA*$^+$ gene by revealing an expected band of 5.7 kb (Fig. 1). To investigate the function of *aneA*$^+$ in development of *A. nidulans*, the pattern of hyphal growth including radial growth, septation, and asexual sporulation were examined in one of the deletion mutant strains. However, we observed no differences between the wild-type and deletion mutant (data not shown). These results indicated that ε-COP is not essential for the viability of *A. nidulans*; these results are similar to those obtained in yeasts.

Recently, COPI proteins were reported to play roles in responses to ER stress and thermal stress in yeast [11, 13]. Here, we tested the effect of ε-COP deletion on sensitivity to environmental stresses caused by treatment of the fungi with several drugs that are known to exert adverse effects on cellular processes related to the stress response. No detectable changes were observed in the ε-COP-defective strain by treatment with drugs including inhibitors of cell-wall biosynthesis (calcofluor white, congo red, and caspofungin), N-glycosylation (tunicamycin), ergosterol biosynthesis (terbinafine), and glycerol biosynthesis (fludioxonil), an inducer of apoptosis (farnesol), and a Ca^{2+}

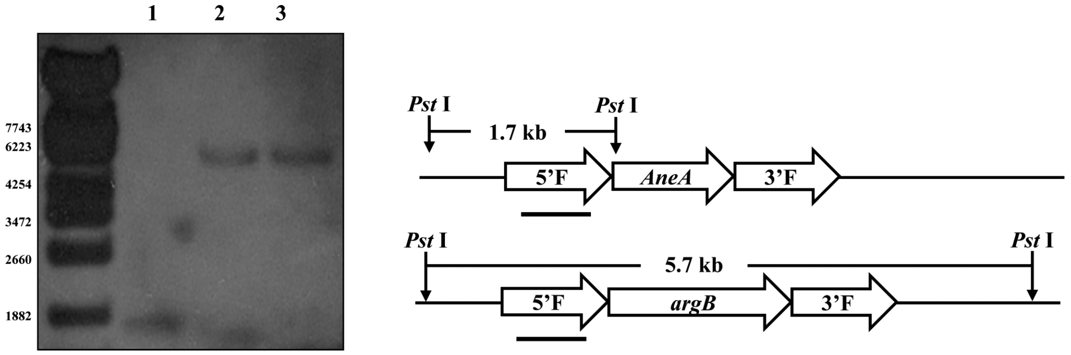

Fig. 1. Southern blot analysis of *aneA* deletion strains. The left panel shows the results from the recipient strain (lane 1) and *aneA*-deletion strains (lanes 2 and 3). The schematic diagrams in the right panel display the chromosome structures of the wild-type strain (upper) and the constructed *aneA*-deletion strain (lower). A cassette containing *argB* with a 5′-flanking region and a 3′-flanking region was integrated into the *aneA*$^+$ locus of the genome of *Aspergillus nidulans* by homologous recombination. The probe used for Southern blotting was located at the 5′-flanking region of *aneA*$^+$.

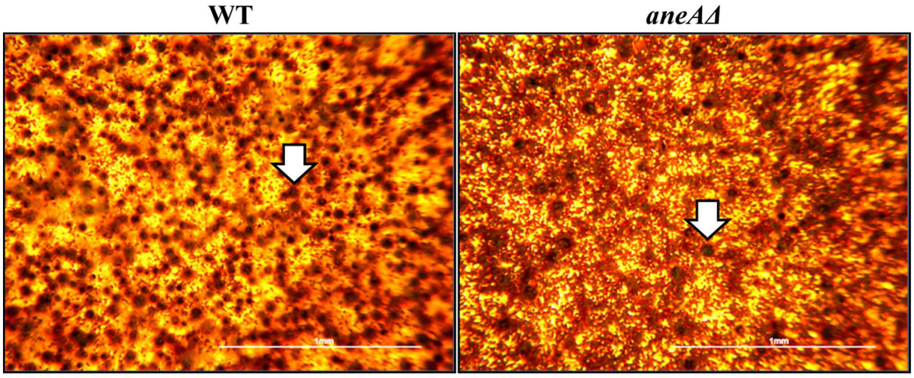

Fig. 2. Formation of the sexual reproductive organ (cleistothecium) of the isogenic wild-type (SK880) and *aneA*-deletion strain. Sexual reproduction was induced by incubating the cells on plates containing 1% glucose and 0.1% sodium nitrate under hypoxic and dark conditions. The cleistothecia are indicated by white arrows. WT and *ΔaneA* stand for wild-type and *aneA*-deletion strain, respectively.

Table 3. Effect of carbon sources on cleistothecium production

Carbon source	No. of cleistothecia/cm^2	
	WT	*ΔaneA*
1% Glucose + sealing	+++	+
1% Glucose	+++	−
2% Lactose	+++	+++

Average number of mature cleistothecia per cm^2 of 3 different area of a plate was calculated: –, < 1; +, 1~50; ++, 50~100; +++, > 100.

In all cases, plates were incubated under dark condition to enhance cleistothecium development.

chelator (EGTA). Thermal stress, which makes the ε-COP-defective yeast strain unviable [4], showed no effect on viability and vegetative growth in the ε-COP deletion strains (data not shown). Taken together, these results show that ε-COP in *A. nidulans* is neither essential for viability nor involved in stress responses unlike in yeast.

Effect of ε-COP depletion on development. In *A. nidulans*, a mutation in the $sod^{VI}C^{+}$ gene encoding α-COP is responsible for non-disjunction of the chromosome during cell division [20]. COPI depletion also results in the failure of cytokinesis and a reduction in the number of overlapping central spindle microtubules during meiotic divisions for spermatogenesis in *Drosophila* [21]. In the ε-COP-depleted (*ΔaneA*) *A. nidulans* strain, we observed no defects in asexual development (data not shown), but significant defects in sexual development (Fig. 2). Even after culturing in conditions favorable for cleistothecium development, with hypoxic treatment with 1% glucose in the dark [22], the *ΔaneA* strain showed more than 50% decrease of fruiting body formation (Table 3). In addition, in 1% glucose without hypoxic treatment, fruiting body formation was almost completely abolished by ε-COP depletion. In 2% lactose without hypoxic treatment, which predominantly induces the formation of fruiting bodies, ε-COP depletion showed no deleterious effect on fruiting body formation (Table 3). Because the effect of C-sources and hypoxic treatment was tested in the presence of 0.1% sodium nitrate as a nitrogen source, we tested 0.2% yeast extract, which preferentially induces sexual development, and found that the defect in fruiting body formation on 1% glucose without hypoxic treatment was recovered in *aneA* deletion strain (data not shown).

Although further experiments are required to determine the underlying mechanisms, our results suggested that ε-COP plays a role in sexual development in *A. nidulans* under certain environmental conditions, possibly by affecting cytokinesis and/or construction of ER-based spindle envelopes, as observed in fruit fly spermatogenesis [21].

Suppression of $sod^{VI}C1$ mutation by forced expression of $aneA^{+}$. Our previous study showed that the C-terminal domains of both α-COP and ε-COP are essential for their interaction, and that the N-terminal WD40 motif of α-COP and the TPR region of ε-COP are involved in controlling the interaction between these two COPs in *A. nidulans* [16]. We also reported that *A. nidulans* α-COP can substitute for *S. cerevisiae* α-COP functions *in vivo* [15]. In yeast, ε-COP is known to stabilize the thermo-sensitive α-COP mutation. Thus, over-expression of ε-COP confers viability to the α-COP mutant at elevated temperatures [4]. Therefore, we investigated whether the phenotype of the $sod^{VI}C1$ mutant was suppressed by over-expression of $aneA^{+}$ in *A. nidulans*. When a pRG3-AMA1 plasmid containing the $aneA^{+}$ gene encoding ε-COP was introduced into the $sod^{VI}C1$ mutant, over-expression of $aneA^{+}$ was confirmed by northern blot analysis (Fig. 3A). However, over-expression of ε-COP could not rescue the thermo-sensitive phenotype of the $sod^{VI}C1$ mutant at a non-permissive temperature (42°C)

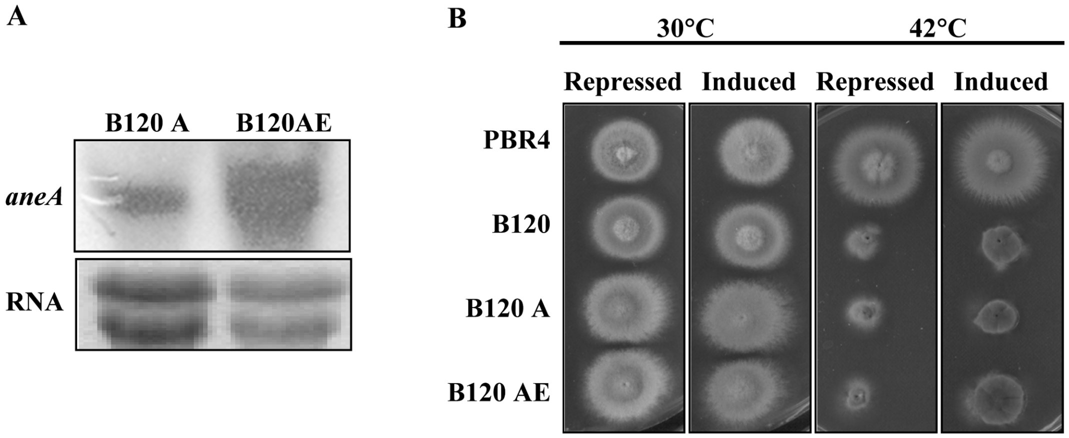

Fig. 3. Suppression of the temperature-dependent osmo-sensitive phenotype in the $sod^{VI}C1$ mutant. A, Expression of $aneA^{+}$ was confirmed by northern blot analysis; B, Growth patterns of wild type (PBR2) and the $sod^{VI}C1$ mutant (B120) with an empty vector (B120A) or the $aneA^{+}$ over-expression vector (B120AE) were observed at permissive (30°C) and non-permissive (42°C) temperatures.

(Fig. 3B). These results indicate that ε-COP is able to interact with α-COP [16], but does not stabilize α-COP at elevated temperatures unlike in yeast [4].

In summary, our results indicate that ε-COP in *A. nidulans* is not essential but related to formation of the sexual reproductive organ in response to particular environmental factor(s). It is worth highlighting that recent reports indicate that the function of the COPI complex (and its subunits) is not confined to intracellular vesicular trafficking. The COPI complex is also involved in other cellular events such as chromosome disjunction in *A. nidulans* [20], male development in chicken embryos [23], ER stress responses in yeast [11], association with calcineurin during heat stress [13], meiotic divisions for spermatogenesis in *Drosophila* [21], induction of productive autophagy and cellular survival [24], intercompartmental trafficking of specific RNAs in neuronal cells [25], and neurite outgrowth [26]. Although further studies are necessary to confirm the actual role of ε-COP (and/or COPI) in sexual development of fungi, our results open up interesting avenues for further studies on the function of the COPI complex.

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REFERENCES

1. Popoff V, Adolf F, Brügger B, Wieland F. COPI budding within the Golgi stack. Cold Spring Harb Perspect Biol 2011;3:a005231.
2. Kirchhausen T. Three ways to make a vesicle. Nat Rev Mol Cell Biol 2000;1:187-98.
3. Pellett PA, Dietrich F, Bewersdorf J, Rothman JE, Lavieu G. Inter-Golgi transport mediated by COPI-containing vesicles carrying small cargoes. Elife 2013;2:e01296.
4. Duden R, Kajikawa L, Wuestehube L, Schekman R. ε-COP is a structural component of coatomer that functions to stabilize α-COP. EMBO J 1998;17:985-95.
5. Cosson P, Démollière C, Hennecke S, Duden R, Letourneur F. δ- and ζ-COP, two coatomer subunits homologous to clathrin-associated proteins, are involved in ER retrieval. EMBO J 1996;15:1792-8.
6. Cosson P, Letourneur F. Coatomer interaction with di-lysine endoplasmic reticulum retention motifs. Science 1994;263:1629-31.
7. Letourneur F, Gaynor EC, Hennecke S, Démollière C, Duden R, Emr SD, Riezman H, Cosson P. Coatomer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. Cell 1994;79:1199-207.
8. Kim KH, Park HM. Enhanced secretion of cell wall bound enolase into culture medium by the *soo1-1* mutation of *Saccharomyces cerevisiae.* J Microbiol 2004;42:248-52.
9. Lee DW, Ahn GW, Kang HG, Park HM. Identification of a gene, *SOO1*, which complements osmo-sensitivity and defect in *in vitro* β-1,3-glucan synthase activity in *Saccharomyces cerevisiae*. Biochim Biophys Acta 1999;1450:145-54.
10. Lee DW, Kim KH, Chun SC, Park HM. Characterization of cell wall proteins from the *soo1-1/ret1-1* mutant of *Saccharomyces cerevisiae.* J Microbiol 2002;40:219-23.
11. Kim KH, Kim EK, Kim SJ, Park YH, Park HM. Effect of *Saccharomyces cerevisiae ret1-1* mutation on glycosylation and localization of the secretome. Mol Cells 2011;31:151-8.
12. Kim KH, Kim EK, Jeong KY, Park YH, Park HM. Effects of mutations in the WD40 domain of α-COP on its interaction with the COPI coatomer in *Saccharomyces cerevisiae.* J Microbiol 2012;50:256-62.
13. Kozubowski L, Thompson JW, Cardenas ME, Moseley MA, Heitman J. Association of calcineurin with the COPI protein Sec28 and the COPII protein Sec13 revealed by quantitative proteomics. PLoS One 2011;6:e25280.
14. Whittaker SL, Lunness P, Milward KJ, Doonan JH, Assinder SJ. *sod*^{VI}*C* is an α-COP-related gene which is essential for establishing and maintaining polarized growth in *Aspergillus nidulans*. Fungal Genet Biol 1999;26:236-52.
15. Lee HH, Park JS, Chae SK, Maeng PJ, Park HM. *Aspergillus nidulans sod*^{VI}*C1* mutation causes defects in cell wall biogenesis and protein secretion. FEMS Microbiol Lett 2002;208:253-7.
16. Song EJ, Kim KH, Lee HH, Park JS, Kang EH, Park HM. Analysis of protein domain for interaction between α-COP and ε-COP in *Aspergillus nidulans.* Kor J Mycol 2012;40:224-8.
17. Kang EH, Kim JA, Oh HW, Park HM. LAMMER kinase LkhA plays multiple roles in the vegetative growth and asexual and sexual development of *Aspergillus nidulans*. PLoS One 2013;8:e58762.
18. Lee JY, Kim LH, Kim HE, Park JS, Han KH, Han DM. A putative APSES transcription factor is necessary for normal growth and development of *Aspergillus nidulans.* J Microbiol 2013;51:800-6.
19. Yu JH, Hamari Z, Han KH, Seo JA, Reyes-Domínguez Y, Scazzocchio C. Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet Biol 2004;41:973-81.
20. Upshall A, Mortimore ID. Isolation of aneuploid-generating mutants of *Aspergillus nidulans*, one of which is defective in interphase of the cell cycle. Genetics 1984;108:107-21.
21. Kitazawa D, Yamaguchi M, Mori H, Inoue YH. COPI-mediated membrane trafficking is required for cytokinesis in *Drosophila* male meiotic divisions. J Cell Sci 2012;125(Pt 15):3649-60.
22. Han KH, Lee DB, Kim JH, Kim MS, Han KY, Kim WS, Park YS, Kim HB, Han DM. Environmental factors affecting development of *Aspergillus nidulans*. J Microbiol 2003;41:34-40.
23. Lin YP, Chen LR, Chen CF, Liou JF, Chen YL, Yang JR, Shiue YL. Identification of early transcripts related to male development in chicken embryos. Theriogenology 2010;74:1161-78.
24. Claerhout S, Dutta B, Bossuyt W, Zhang F, Nguyen-Charles C, Dennison JB, Yu Q, Yu S, Balázsi G, Lu Y, et al. Abortive autophagy induces endoplasmic reticulum stress and cell

death in cancer cells. PLoS One 2012;7:e39400.

25. Todd AG, Lin H, Ebert AD, Liu Y, Androphy EJ. COPI transport complexes bind to specific RNAs in neuronal cells. Hum Mol Genet 2013;22:729-36.
26. Custer SK, Todd AG, Singh NN, Androphy EJ. Dilysine motifs in exon 2b of SMN protein mediate binding to the COPI vesicle protein α-COP and neurite outgrowth in a cell culture model of spinal muscular atrophy. Hum Mol Genet 2013;22:4043-52.