23. Wathey, J. C., Nass, M. M. & Lester, H. A. Numerical reconstruction of the quantal event at nicotinic synapses. *Biophys. J.* **27,** 145–164 (1979).
24. Kumins, N. H., Hunt, J., Gamelli, R. L. & Filkins, J. P. Partial hepatoectomy reduces the endotoxin-induced peak circulating level of tumor necrosis factor in rats. *Shock* **5,** 385–388 (1996).
25. Gregory, S. H. & Wing, E. J. Neutrophil-Kupffer-cell interaction in host defenses to systemic infections. *Immunol. Today* **19,** 507–510 (1998).
26. Tracey, K. J. *et al.* Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature* **330,** 662–664 (1987).
27. Madretsma, G. S. *et al.* Nicotine inhibits the in vitro production of interleukin 2 and tumour necrosis factor-alpha by human mononuclear cells. *Immunopharmacology* **35,** 47–51 (1996).
28. van Dijk, A. P. *et al.* Transdermal nicotine inhibits interleukin 2 synthesis by mononuclear cells derived from healthy volunteers. *Eur. J. Clin. Invest.* **28,** 664–671 (1998).

Acknowledgements

We thank D. Nardi for help in ELISA assays and D. Prieto for administrative assistance.

Correspondence and requests for materials should be addressed to L.V.B. (e-mail: lborovikova@mindspring.com) or K.J.T. (e-mail: kjtracey@sprynet.com).

Targeted destabilization of HY5 during light-regulated development of *Arabidopsis*

Mark T. Osterlund, Christian S. Hardtke, Ning Wei & Xing Wang Deng

Department of Molecular, Cellular and Developmental Biology, Yale University, PO Box 208104, 165 Prospect Street, OML 301, New Haven, Connecticut 06520-8104, USA

Arabidopsis **seedlings display contrasting developmental patterns depending on the ambient light. Seedlings grown in the light develop photomorphogenically, characterized by short hypocotyls and expanded green cotyledons. In contrast, seedlings grown in darkness become etiolated, with elongated hypocotyls and closed cotyledons on an apical hook. Light signals, perceived by multiple photoreceptors and transduced to downstream regulators, dictate the extent of photomorphogenic development in a quantitative manner. Two key downstream components, COP1 and HY5, act antagonistically in regulating seedling development[1]. HY5 is a bZIP transcription factor that binds directly to the promoters of light-inducible genes, promoting their expression and photomorphogenic development[2,3]. COP1 is a RING-finger protein with WD-40 repeats whose nuclear abundance is negatively regulated by light[4,5]. COP1 interacts directly with HY5 in the nucleus to regulate its activity negatively[1]. Here we show that the abundance of HY5 is directly correlated with the extent of photomorphogenic development, and that the COP1–HY5 interaction may specifically target HY5 for proteasome-mediated degradation in the nucleus.**

To characterize the nuclear HY5 protein[2,3] biochemically, we produced rabbit polyclonal antibodies (anti-HY5) against recombinant HY5 protein. Western blot analyses of total protein extracts from light-grown wild-type seedlings revealed that anti-HY5 recognizes a protein band at an apparent relative molecular mass of 30,000 (M_r 30K) (Fig. 1a). The apparently large size of HY5 relative to its predicted size of 18K (ref. 2) is largely due to abnormal migration of the HY5 protein in our polyacrylamide gel system (data not shown). The presence of a 30K band in wild-type seedlings but not in the three *hy5* mutant alleles examined indicates that it is the endogenous *Arabidopsis* HY5.

Quantitative western blot analyses indicated that HY5 is 15–20 times more abundant in seedlings grown in white light than in those grown in the dark (Fig. 1b). This difference is observed in green tissues (cotyledon and hypocotyl) as well as in the non-photosynthetic roots (data not shown). We examined HY5 levels during light–dark transitions. Wild-type seedlings were grown in continuous light or darkness for four days and then transferred to the opposite light condition for 5, 10, 15 or 20 h. A western blot of the protein extracts from these seedlings revealed that although changes in the abundance of HY5 occur within 5 h, a full day of light or darkness is required for HY5 to reach its maximum or minimum, respectively (Fig. 1c).

Consistent with a previous report[2], HY5 messenger RNA levels

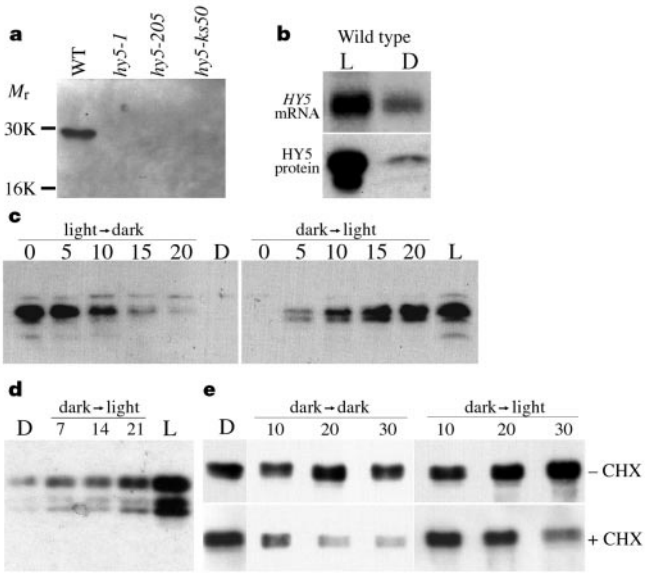

Figure 1 HY5 expression is regulated by light at both the mRNA and protein levels. **a**, A western blot of wild-type (WT), *hy5-1*, *hy5-205* and *hy5-ks50* seedling extracts using anti-HY5. **b**, A *HY5* northern blot and an anti-HY5 western blot of light- and dark-grown seedlings (L, light; D, dark). **c**, Anti-HY5 western blots from seedlings grown in continuous light (L) or darkness (D) for 4 d and then transferred to the opposite light condition for 5, 10, 15 or 20 h. **d**, An anti-HY5 western blot of 35SHY5 seedlings grown in continuous darkness for 4 d and then transferred to light for 0 (D), 7, 14 or 21 h. Seedlings grown in continuous light were included as a positive control (L). **e**, Anti-HY5 western blots of 35SHY5 seedlings treated with cycloheximide (+CHX) or buffer only (–CHX). Seedlings were grown in continuous darkness for four days before treatment and then transferred to light or darkness. Protein extracts were made 0, 10, 20 and 30 h after the addition of cycloheximide.

showed only a two-to-threefold difference between light- and dark-grown seedlings (Fig. 1b). This is considerably less than the 15–20-fold difference seen in HY5 protein levels. Therefore the mRNA levels could contribute to the differential accumulation of the HY5 protein, but translational or post-translational regulation may be primarily responsible for regulating the abundance of HY5. This conclusion is supported by the observation that HY5 exhibits a similar light-induced accumulation in HY5-overexpressing (35SHY5) transgenic seedlings (Fig. 1d). This is particularly convincing considering the greater than 50-fold excess in the total HY5 mRNA and its lack of light regulation in the transgenic lines (data not shown).

We used cycloheximide, a cytoplasmic protein-synthesis inhibitor, to determine whether protein degradation or protein synthesis is the key regulatory step for the differential abundance of HY5 in the light and dark. Four-day-old 35SHY5 seedlings grown in darkness were transferred to liquid cultures containing cycloheximide. These cultures were then placed in white light or darkness for variable lengths of time, after which the abundance of HY5 was determined. For reasons not yet clear, the HY5 protein increased more slowly and to a lesser extent in seedlings grown in liquid culture (Fig. 1e, top left) than in seedlings grown on plates (Fig. 1d). Although the amount of HY5 decreases within 30 h in both light and darkness in the presence of cycloheximide, the rate of decrease is significantly greater in darkness (Fig. 1e, bottom). As 100 μM cycloheximide effectively blocks new protein synthesis (data not shown), the reduced levels of HY5 must be due to the degradation of pre-existing protein. Therefore, our result indicates that HY5 is degraded at a greater rate in darkness than in light.

To reveal which cellular mechanism is responsible for HY5 degradation, we developed a cell-free assay based on a reported degradation system[6]. In this assay, HY5 is completely degraded within one hour (Fig. 2a). This degradation is greatly enhanced by the presence of exogenous ATP (data not shown) and can be prevented by four distinct proteasome-specific inhibitors (Fig. 2b). However, general protease inhibitors (leupeptin, PMSF) had little or no effect on the degradation of HY5 in this assay. As the control proteins[7] showed little or no degradation in the extract during the assay, the degradation of HY5 was specific. Thus, we conclude that the proteasome is largely responsible for the degradation of HY5.

Eleven pleiotropic *COP/DET/FUS* loci are required to mediate repression of photomorphogenic development[8,9]. Of these, at least six COP/DET/FUS proteins are components of the COP9 signalosome, a multi-subunit complex that is similar to the lid subcomplex of the 19S proteasome regulatory particle[10–13]. This structural similarity, together with the reported interaction between subunits of the COP9 signalosome and the proteasome[7], may indicate that the COP9 signalosome has a direct or regulatory role in proteasome-mediated degradation. Therefore we examined the light regulation of HY5 in representative mutants of all available pleiotropic *COP/DET/FUS* loci. Although there was still a two-to-threefold difference in HY5 mRNA levels between light- and dark-grown mutant seedlings (Fig. 3 and ref. 2), HY5 protein accumulated to similar levels in both light and darkness in all mutants examined (Fig. 3). Furthermore, *in vitro* assays using extract from the weak *cop1-6* mutant seedlings showed a greatly reduced rate of proteasome-mediated degradation of HY5 protein (data not shown). Therefore, all defined pleiotropic COP/DET/FUS proteins are essential for the light-regulated instability of HY5 that is mediated by the proteasome.

The accumulation of or reduction in HY5 during light–dark transitions occurred over the same timescale as the redistribution of COP1 between the cytoplasm and the nucleus[14]. As nuclear COP1 interacts with HY5 and negatively regulates its activity[15], COP1 may directly regulate HY5 stability. As COP1 activity and nuclear abundance are controlled by a wide spectrum of light through several photoreceptors[16], we first examined whether the same set of photoreceptors is also involved in regulating the abundance of HY5. To determine their effects on the accumulation of HY5, photoreceptor mutant and overexpression lines were grown in various wavelengths of light and the abundance of HY5 was determined (Fig. 4). As expected, phytochrome B[17], phytochrome A[18] and the cryptochromes (CRY1 and CRY2)[19,20] are the primary photoreceptors responsible for mediating the accumulation of HY5 under specific light. CRY1 and CRY2 are largely redundant and are responsible for the accumulation of HY5 in blue light (Fig. 4c). In red light, phyB is primarily responsible for the accumulation of HY5, although phyA has a minor role (Fig. 4a). Interestingly, phyA and the two cryptochromes all affect the abundance of HY5 in far-red light (Fig. 4b). In all cases, the abundance of HY5 directly correlates with the degree of photomorphogenic development. There is also a strong correlation between the effects of each photoreceptor on the nuclear exclusion of COP1 (ref. 16) and HY5 abundance.

To provide further evidence that nuclear COP1 is involved in regulating HY5 abundance, we determined the levels of HY5 in wild-type seedlings grown in various intensities of continuous white light. Figure 5a shows that in high-intensity light, seedlings display typical photomorphogenic phenotypes and HY5 accumulates to high levels. As the intensity decreases, HY5 also decreases and seedlings become more etiolated with longer hypocotyls and smaller cotyledons. Increasing light intensities result in a proportional decrease in the nuclear abundance of COP1 (refs 5, 14). Thus the

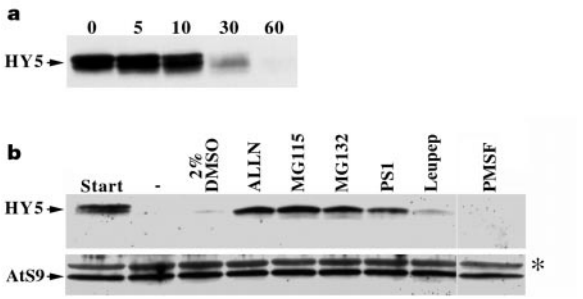

Figure 2 HY5 degradation is mediated by the proteasome pathway. **a**, Cell-free degradation of HY5 is completed within 1 h in a buffer system supporting the proteasome pathway[6,30]. **b**, Cell-free degradation of HY5 in the presence of different inhibitors. Extracts were treated with nothing (–), 2% DMSO (solvent for the inhibitors), 40 μM of one of the proteasome inhibitors ALLN, MG115, MG132 or PS1, 40 μM leupeptin or 4 mM PMSF. After 2 h of incubation at room temperature, the HY5 levels were examined by western blotting. An unrelated control (AtS9) and a crossreacting band (asterisk) that are unaffected by proteasome degradation in the same extracts are shown as loading controls.

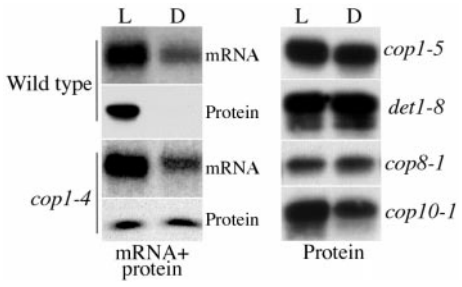

Figure 3 The pleiotropic *COP/DET/FUS* genes are essential for mediating light control of HY5 protein degradation but not its mRNA accumulation. Representative *HY5* northern and western blots of wild-type, *cop1-4*, *cop1-5*, *det1-8*, *cop8-1* and *cop10-1* mutants grown in continuous light and darkness. All pleiotropic *cop/det/fus* mutants from the eleven available loci were examined and failed to reduce the abundance of HY5 in darkness. The blots were exposed for variable times for optimal results.

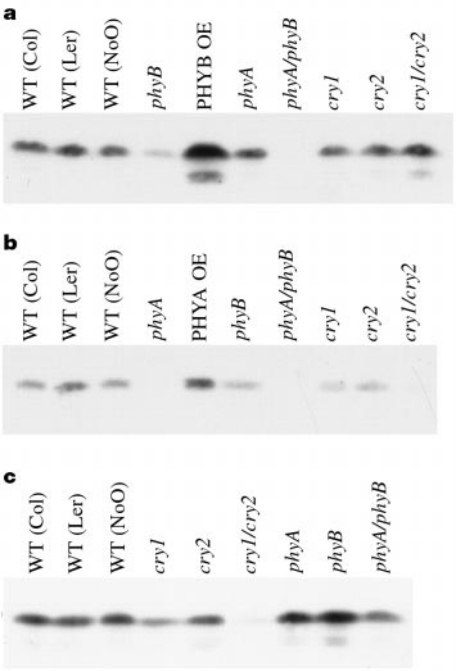

Figure 4 Several photoreceptors mediate the accumulation of HY5 under distinct wavelengths of light. **a–c**, Anti-HY5 western blots of seedlings grown in continuous red light (**a**), continuous far-red light (**b**) and continuous blue light (**c**). The seedlings include wild type (three ecotypes), *cry1*, *cry2*, a *cry1/cry2* double mutant, *phyA*, *phyB*, a *phyA/phyB* double mutant, a PHYB overexpression line (PHYBOE, red light only) and a PHYA overexpression line (PHYAOE, far-red light only).

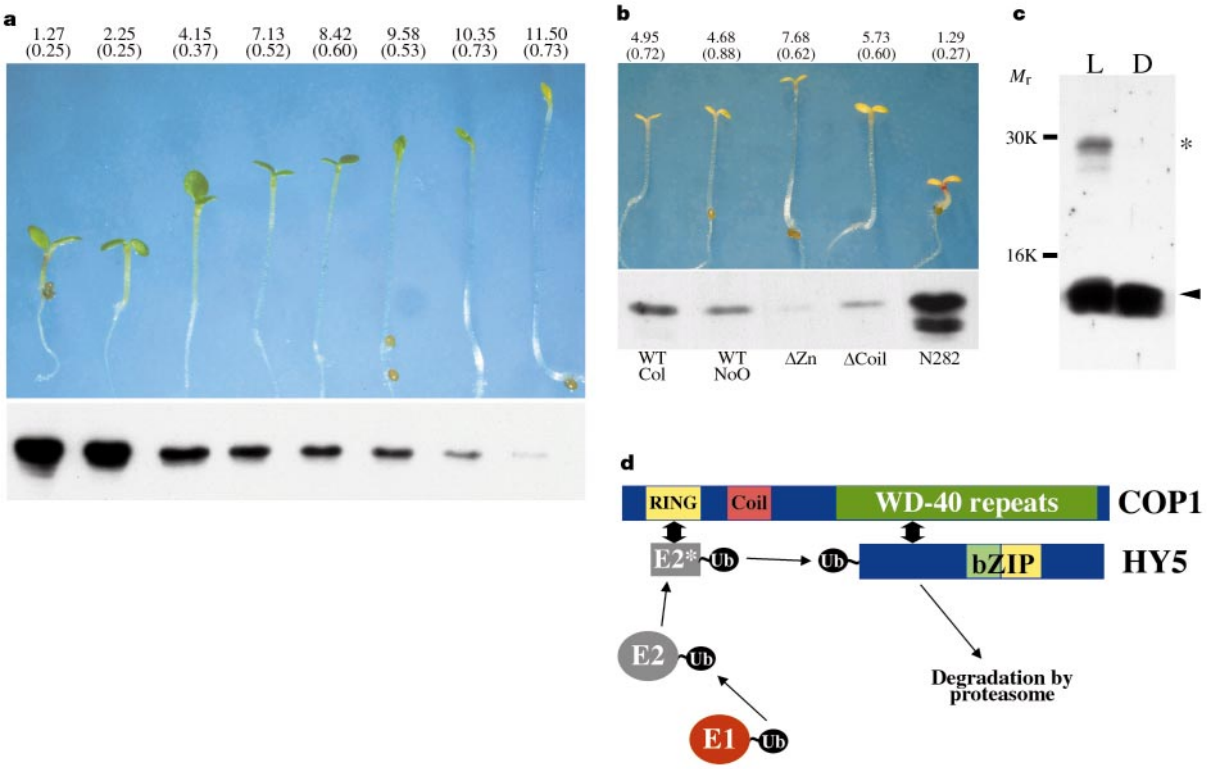

Figure 5 The light regulation of HY5 is dependent on the nuclear abundance of COP1 and its ability to interact with COP1. **a**, An anti-HY5 western blot of wild-type seedlings grown in varying light intensities (left to right: 156, 70, 30, 10, 5, 2.5, 0.6 and 0.3 μmol m^{-2} s^{-1}). Representative seedlings are shown above the western blot with the average hypocotyl lengths (s.d. in parentheses). **b**, An anti-HY5 western blot of wild-type (Columbia and No-0 ecotypes), ΔZn, ΔCoil and N282 seedlings grown in continuous far-red light. Representative seedlings are shown above the western blot with the average hypocotyl lengths (s.d. in parentheses). **c**, An anti-HY5 western blot of light- and dark-grown seedlings expressing truncated HY5 (ΔN77). Asterisk, wild-type HY5; arrowhead, ΔN77. **d**, Proposed role of COP1 in targeting HY5 degradation. Here, COP1 acts as an ubiquitin-protein ligase (E3) that recruits an E2 and HY5 through distinct interacting domains (RING-finger motif and WD-40 repeat domain), mediating the ubiquitination of HY5 and its subsequent degradation by the proteasome.

nuclear abundance of COP1 is inversely correlated with the cellular abundance of HY5, which is exclusively localized within the nucleus[2,3].

To test whether the COP1–HY5 interaction is required for the observed degradation of HY5, we examined several transgenic lines expressing truncated versions of COP1 (Fig. 5b). HY5 interacts with COP1 through the WD-40 repeat domain[1,21]. The overexpression of a truncated form of COP1 that lacks the WD-40 repeat domain (N282) results in a hyper-photomorphogenic phenotype[21,22]. In contrast, overexpression of domain-deletion constructs of COP1 that lack the RING-finger domain (ΔZn) or coiled-coil domain (ΔCoil) results in elongated hypocotyls[21]. The effects of the transgenes were most evident when seedlings were grown in far-red light (Fig. 5b, top). The abundance of HY5 in these transgenic lines was strictly correlated with the observed extent of photomorphogenic development. When grown in the same far-red light condition, both ΔZn and ΔCoil had reduced levels of HY5 protein relative to wild type, whereas N282 showed a marked increase in HY5 (Fig. 5b, bottom).

We also examined the light-regulated accumulation of a truncated form of HY5 (ΔN77). ΔN77 lacks the COP1-interaction domain but still promotes photomorphogenic development, causing a hyper-photomorphogenic phenotype[1]. As shown in Fig. 5c, the ΔN77 protein is not light-regulated and accumulates to similar levels in both light and darkness. In the same seedlings, the endogenous wild-type HY5 protein exhibits consistent light-regulated accumulation. This indicates that the amino-terminal portion of HY5, which contains the COP1-interacting domain, may be responsible for targeting HY5 for degradation. Those results support the conclusion that the interaction between COP1 and HY5 is necessary and essential for the degradation of HY5.

Our results show that the cellular abundance of HY5 is light regulated and is directly correlated with the degree of photomorphogenic development of the seedling. This regulation is primarily controlled at the level of protein degradation, probably mediated by the proteasome pathway. The data indicate that *Arabidopsis* COP1, a light-inactivatable repressor of photomorphogenesis, interacts directly with HY5 within the nucleus, resulting in degradation of HY5. Light may inactivate COP1, reducing its nuclear abundance and allowing the accumulation of HY5. Therefore, COP1 may directly target HY5 for proteasome-mediated degradation.

There is evidence that many RING-finger proteins can act as ubiquitin–protein ligases (E3) to target proteins for degradation by recruiting ubiquitin-conjugating enzymes (E2) and transferring the polyubiquitin from E2 to the targeted proteins[23,24]. COP1 has both a RING-finger motif and a WD-40 repeat domain that can interact with several protein targets including HY5 (refs 15, 21). Thus it is not unreasonable to consider COP1, either alone or with other proteins, acting in a similar manner to an E3 ubiquitin–protein ligase. Specifically, nuclear COP1 may recruit an E2 ubiquitin-conjugating enzyme, targeting HY5 and other substrates for ubiquitination and subsequent degradation by the proteasome (Fig. 5d). The fact that all other pleiotropic COP/DET/FUS proteins are required for the light-regulated degradation of HY5 and that many of these proteins are part of the COP9 signalosome[25] indicates that all COP/DET/FUS proteins may have either a direct or a regulatory role in this proteasome-mediated degradation. □

Methods

Plant material and light conditions

The wild type used was of the Wassilewskija (WS) ecotype, unless otherwise indicated. The *cop/det/fus* mutants used have been described[25]. The photoreceptor single and double mutants used were *phyA-201*, *phyB-5*, *phyA-201/phyB-5*, *cry1-304*, *cry2-1* and *cry1-304/cry2-1* (refs 17–19, 24, 26, 27). The PHYA overexpression lines (PHYAOE) and the PHYB overexpression lines (PHYBOE) were as described[28,29].

For all light shift experiments, seedlings were grown in continuous light or darkness for at least 4 d. Seedlings were then transferred to the opposite light condition for the designated length of time, such that all seedlings were the same age when protein was extracted. Unless otherwise stated, the white light intensity used was 156 $\mu mol\,m^{-2}\,s^{-1}$. The colour light growth chambers (Percival Scientific E-30LED2/3) have intensities of 33.5 $\mu mol\,m^{-2}\,s^{-1}$ for blue light, 111.5 $\mu mol\,m^{-2}\,s^{-1}$ for red light and 191.7 $\mu mol\,m^{-2}\,s^{-1}$ for far-red light.

HY5 antibody and western analysis

We generated polyclonal HY5 antibodies by cloning the *HY5* complementary DNA into the pet28a expression vector (Novagen). His_6-tagged HY5 was injected into rabbits at three-week intervals. HY5 antibody was purified from rabbit serum using GST–HY5 coupled to an *N*-hydroxy-succinimide-activated affinity column (Pharmacia Biotech).

All seedlings grown in the designated light conditions were 4–5 d old when protein was extracted. In most cases, 50 seedlings were ground in 50 μl of grinding buffer (400 mM sucrose, 50 mM Tris pH 7.5, 2.5 mM EDTA and 10 mM PMSF) and centrifuged, and equal amounts of extract were added to equal gel-loading buffer. Equal volumes of extract were loaded on the gels and western blots were performed.

Northern analysis

We isolated RNA from seedlings using the RNeasy kit (Qiagen). Northern blots were prepared using RNA from equal numbers of seedlings probed with radioactively labelled HY5 DNA fragments according to standard procedures.

Cycloheximide experiments

We grew 35SHY5 seedlings on germination media[22] plates for 4 d in continuous darkness. After 4 d, seedlings were transferred to liquid culture (with the same germination medium lacking agar) where cycloheximide (100 μM) or buffer only (control) was added. The cultures were placed in the light or in the darkness and seedlings were removed 0, 10, 20 and 30 h after cycloheximide was added. Protein extracts were made by grinding seedlings in 100 μl of grinding buffer. The protein concentration was determined by Bradford assay and equal amounts of protein were loaded on an SDS–polyacrylamide gel.

Cell-free degradation assay

Five-day-old light-grown seedlings were ground in liquid nitrogen and resuspended in a buffer (25 mM Tris pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 10 mM NaCl and 10 mM ATP) modified according to ref. 30. Cell debris was pelleted by centrifugation and equal amounts of extract were transferred to individual tubes. For inhibitor studies, we incubated the extract aliquots at room temperature for 2 h in the presence of the respective supplements (Calbiochem), and reactions were stopped by adding an equal volume of 2× protein gel-loading buffer. Equal amounts of sample were then analysed by western blot.

Received 1 November 1999; accepted 14 March 2000.

1. Ang, L. H. *et al.* Molecular interaction between COP1 and HY5 defines a regulatory switch for light control of *Arabidopsis* development. *Mol. Cell* **1,** 213–222 (1998).
2. Oyama, T. *et al.* The *Arabidopsis HY5* gene encodes a bZIP protein that regulates stimulus-induced development of root and hypocotyl. *Genes Dev.* **11,** 2983–2995 (1997).
3. Chattopadhyay, S. *et al.* Arabidopsis bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. *Plant Cell* **10,** 673–683 (1998).
4. Deng, X. W. *et al.* COP1, an *Arabidopsis* regulatory gene, encodes a protein with both a Zinc-binding motif and a Gβ homologous domain. *Cell* **71,** 791–801 (1992).
5. von Arnim, A. G. & Deng, X. W. Light inactivation of *Arabidopsis* photomorphogenic repressor COP1 involves a cell-specific regulation of its nucleocytoplasmic partitioning. *Cell* **79,** 1035–1045 (1994).
6. Naidoo, N., Song, W., Hunter-Ensor, M. & Sehgal, A. A role for the proteasome in the light response of the timeless clock protein. *Science* **285,** 1737–1741 (1999).
7. Kwok, S. F. *et al.* Characterization of two subunits of *Arabidopsis* 19S proteasome regulatory complex and its possible interaction with the COP9 complex. *J. Mol. Biol.* **285,** 85–95 (1999).
8. Deng, X. W. *et al. cop1*: a regulatory locus involved in light-controlled development and gene expression in *Arabidopsis*. *Genes Dev.* **5,** 1172–1182 (1992).
9. Kwok, S. F. *et al.* A complement of ten essential and pleiotropic *Arabidopsis COP/DET/FUS* genes is necessary for repression of photomorphogenis in darkness. *Plant Physiol.* **110,** 731–742 (1996).
10. Karniol, B. *et al.* The *Arabidopsis* homologue of an eIF3 complex subunit associates with the COP9 complex. *FEBS Lett.* **439,** 173–179 (1998).
11. Staub, J. M. *et al.* Evidence for FUS6 as a component of the nuclear-localized COP9 complex in *Arabidopsis*. *Plant Cell* **8,** 2047–2056 (1996).
12. Wei, N. *et al. Arabidopsis* COP9 is a component of a novel signaling complex mediating light control of development. *Cell* **78,** 117–124 (1994).
13. Serino, G. *et al. Arabidopsis cop8* and *fus4* mutations define the same locus that encodes subunit 4 of the COP9 signalosome. *Plant Cell* **11,** 1967–1980 (1999).
14. von Arnim, A. G. Genetic and developmental control of nuclear accumulation of COP1, a repressor of photomorphogenesis in *Arabidopsis*. *Plant Physiol.* **114,** 779–788 (1997).
15. Osterlund, M. T. *et al.* The role of COP1 in repression of *Arabidopsis* photomorphogenic development. *Trends Cell Biol.* **9,** 113–118 (1999).
16. Osterlund, M. T. & Deng, X. W. Multiple photoreceptors mediate the light-induced reduction of GUS-COP1 from *Arabidopsis* hypocotyl nuclei. *Plant J.* **16,** 201–208 (1998).
17. Reed, J. W. *et al.* Mutations in the gene for the red/far-red light receptor phytochrome B alter cell elongation and physiological responses throughout *Arabidopsis* development. *Plant Cell* **5,** 147–157 (1993).
18. Whitelam, G. C. *et al.* Phytochrome A null mutants of *Arabidopsis* display a wild-type phenotype in white light. *Plant Cell* **5,** 757–768 (1993).
19. Ahmad, M. & Cashmore, A. R. *HY4* gene of *A. thaliana* encodes a protein with characteristics of a blue-light photoreceptor. *Nature* **366,** 162–166 (1993).
20. Lin, C. *et al.* Enhancement of blue-light sensitivity of *Arabidopsis* seedlings by a blue light receptor cryptochrome 2. *Proc. Natl Acad. Sci. USA* **95,** 2686–2690 (1998).

21. Tori, K. U. *et al.* Functional dissection of *Arabidopsis* COP1 reveals specific roles of its three structural modules in light control of seedling development. *EMBO J.* **17,** 5577–5587 (1998).
22. McNellis, T. W. *et al.* Expression of an N terminal fragment of COP1 confers dominant-negative suppression of seedling photomorphogenic development in transgenic *Arabidopsis*. *Plant Cell* **8,** 1491–1503 (1996).
23. Joazeiro, C. A. P. *et al.* The tyrosine kinase negative regulator c-Cbl as a RING-type E2-dependent ubiquitin-protein ligase. *Science* **8,** 309–312 (1999).
24. Lorick, K. L. *et al.* RING fingers mediate ubiquitin-conjugating enzyme (E2)-dependent ubiquitination. *Proc. Natl Acad. Sci. USA* **96,** 11364–11369 (1999).
25. Wei, N. & Deng, X. W. Making sense of the COP9 signalosome. *Trends Genet.* **15,** 98–103 (1999).
26. Reed, J. W. *et al.* Phytochrome A and phytochrome B have overlapping but distinct function in *Arabidopsis* development. *Plant Physiol.* **104,** 1139–1149 (1991).
27. Ahmad, M. *et al.* Cryptochrome blue-light photoreceptors of *Arabidopsis* implicated in phototropism. *Nature* **16,** 720–723 (1994).
28. Wagner, D. *et al.* Overexpression of phytochrome B induces a short hypocotyl phenotype in transgenic *Arabidopsis*. *Plant Cell*, **3,** 1275–1288 (1992).
29. Boylan, M. T. & Quail, P. H. Phytochrome A overexpression inhibits hypocotyl elongation in transgenic *Arabidopsis*. *Proc. Natl Acad. Sci. USA* **88,** 10806–10810 (1991).
30. Koegl, M. *et al.* A novel ubiquitination factor, E4 is involved in multiubiquitin chain assembly. *Cell* **96,** 635–644 (1999).

Acknowledgements

We thank R. Fry and H. Wang for reading and commenting on this manuscript, and A. Cashmore, C. Lin, P. Quail and G. Whitelam for providing *Arabidopsis* photoreceptor mutant strains. Our work was supported by grants from NIH (to X.W.D.), USDA (to N.W.) and the Human Frontiers Science Program Organization. X.W.D. is an NSF Presidential Faculty Fellow, M.T.O. is an NIH and Dept of Education predoctoral trainee and C.S.H. is a Human Frontier Science Program Organization postdoctoral fellow.

Correspondence and requests for materials should be addressed to X.W.D. (e-mail: xingwang.deng@yale.edu).

Genomic rearrangement in *NEMO* impairs NF-κB activation and is a cause of incontinentia pigmenti

The International Incontinentia Pigmenti (IP) Consortium

* *A full list of authors appears at the end of the paper*

Familial incontinentia pigmenti (IP; MIM 308310) is a genodermatosis that segregates as an X-linked dominant disorder and is usually lethal prenatally in males. In affected females it causes highly variable abnormalities of the skin, hair, nails, teeth, eyes and central nervous system. The prominent skin signs occur in four classic cutaneous stages: perinatal inflammatory vesicles, verrucous patches, a distinctive pattern of hyperpigmentation and dermal scarring[1]. Cells expressing the mutated X chromosome are eliminated selectively around the time of birth, so females with IP exhibit extremely skewed X-inactivation[2]. The reasons for cell death in females and *in utero* lethality in males are unknown. The locus for IP has been linked genetically to the *factor VIII* gene in Xq28 (ref. 3). The gene for NEMO (NF-κB essential modulator)/IKKγ (IκB kinase-γ) has been mapped to a position 200 kilobases proximal to the *factor VIII* locus[4]. NEMO is required for the activation of the transcription factor NF-κB and is therefore central to many immune, inflammatory and apoptotic pathways[5–9]. Here we show that most cases of IP are due to mutations of this locus and that a new genomic rearrangement accounts for 80% of new mutations. As a consequence, NF-κB activation is defective in IP cells.

The extreme skewing of X-inactivation observed in IP patients has implications for screening of candidate genes. Complementary DNA prepared from females with IP will not contain the mutated allele of the disease locus, so screening of candidate genes for mutation must be performed with genomic DNA. We used two approaches to screen *NEMO*: (1) generation and sequencing of fibroblast cDNA from extremely rare cases that express only the mutated X chromosome (two male cases and one female abortus, see Methods); and (2) resolution of the *NEMO* genomic structure for mutational analysis of individual exons. Reverse transcriptase polymerase chain reaction (RT–PCR) from messenger RNA derived from skin fibroblasts from four affected fetuses (D, K, IP85m and G) and controls (C1 and C2) was conducted with *NEMO*-specific primers (Fig. 1). Male samples D and IP85m have only one X chromosome. Female sample K is unusual in that contrary X-inactivation skewing has resulted in expression of only the mutated (IP) X chromosome. Affected female G expresses both Xs, as expected for a fetal IP case that has not yet undergone selective elimination of affected cells.

Amplification between primers located in *NEMO* exons 2 and 3 (exon organization in Fig. 2) for fetuses K, D and G produced a product corresponding to the 5′ end of *NEMO* cDNA. However, amplification between exons 2 and 4 produced the predicted fragment from fetus G and control RNAs but not from fetuses D and K. Thus, the 3′ end of the *NEMO* cDNA is absent in fetuses K and D. This could not be explained by loss of individual exons because we could amplify all 10 coding exons from genomic DNA (not shown). These results indicate that a mutation that disturbs mRNA production may exist between primers R4 and R1 in exons 3 and 4, respectively.

In a sample from another affected male (IP85m), RT–PCR across the entire coding region of *NEMO* was successful and the products

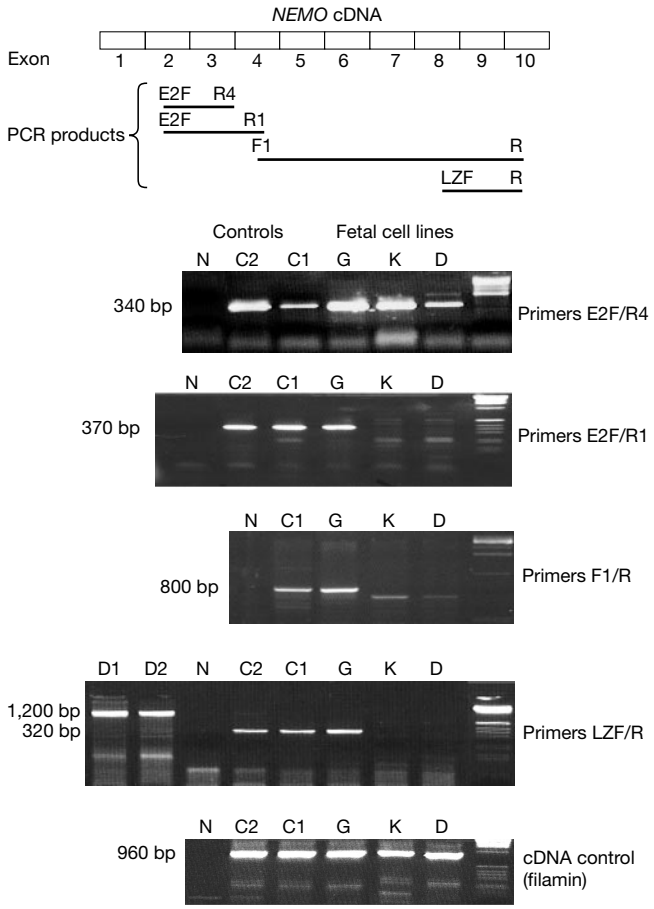

Figure 1 Amplification by RT–PCR of *NEMO* cDNA from IP patient cell lines. The positions of PCR products relative to NEMO exons are shown at the top. Amplification is from fetal cDNA samples G, K and D, cDNA from healthy donors (C1 and C2) and control DNA (D1 and D2). PCR of filamin cDNA from all samples acted as a control for RNA integrity. Primer sequences are given in Methods.