completely rescued mice of both strains from 60% killing doses of gamma irradiation (8 Gy for C57BL and 6 Gy for Balb/c). Significant protection was also seen at higher doses of irradiation that were lethal for control animals (Fig. 4A). PFTα-injected mice lost less weight than irradiated mice that were not pretreated with the drug (Fig. 4B). PFTα did not protect p53-null mice from lethal irradiation, which confirms that it acts through a p53-dependent mechanism in vivo (*13*).

Drug-mediated suppression of p53 results in the survival of cells that otherwise would be eliminated by p53 and that may increase the risk of new cancer development. In fact, p53-deficient mice are extremely sensitive to radiation-induced tumorigenesis (*14*). However, in our study, no tumors or any other pathological lesions were found in the group of 30 survivors rescued from lethal gamma irradiation by PFTα, even at 7 months after irradiation. Thus, temporary suppression of p53 appears to differ from p53 deficiency in terms of cancer predisposition.

To monitor PFTα activity at the tissue level, we compared the effect of gamma radiation on DNA synthesis in tissues in PFTα-treated and untreated mice using a ^{14}C-thymidine incorporation assay. ^{14}C labeling of skin, gut, and several other tissues was significantly decreased after gamma irradiation in $p53^{+/+}$ mice but not $p53^{-/-}$ mice, reflecting the p53 dependence of the effect. The radiation-induced decrease in ^{14}C-thymidine incorporation was less pronounced in PFTα-treated mice than in control irradiated animals, presumably reflecting PFTα inhibition of p53 (Fig. 4B). These results suggest that PFTα attenuates the p53-dependent block of DNA replication in rapidly proliferating tissues after whole-body gamma irradiation. Changes in thymidine incorporation correlated with the extent of apoptosis in the gut epithelium of gamma-irradiated mice. The extensive apoptosis observed in the crypts and villi of the small intestine was abrogated in mice treated with PFTα before irradiation (Fig. 4C).

Our results raise the possibility of using PFTα (or other compounds with similar activity) to reduce the side effects of radiation therapy or chemotherapy for human cancers that have lost functional p53. Because the effects of PFTα are p53-dependent, the compound should not affect the sensitivity of such tumors to treatment. In fact, i.p. injection of PFTα did not change the radiation response of p53-deficient tumor xenografts in $p53^{+/+}$ nude mice (*15*).

It is likely that suppression of p53-dependent apoptosis has already been successfully and broadly applied to cancer patients in the form of growth factors supplementing chemotherapy (*16*). The therapeutic effect of such supplements may be associated with their activity as survival factors suppressing p53-dependent apoptosis (*17*). PFTα can now be used to determine whether there are any other clinical situations in which p53 suppression might be desirable. These include heart and brain ischemia, which both result from local hypoxia, a potent activator of p53 (*18*). Systematic screening of synthetic and natural compounds may lead to the identification of additional p53 inhibitors that may protect tissue from the consequences of a variety of stresses.

References and Notes

1. T. M. Gottlieb and M. Oren, *Biochim. Biophys. Acta* **1287**, 77 (1996).
2. A. J. Levine *et al.*, *Br. J. Cancer* **69**, 409 (1994); R. J. Steele, A. M. Thompson, P. A. Hall, D. P. Lane, *Br. J. Surg.* **85**, 1460 (1998).
3. C. Cordon-Cardo, J. Sheinfeld, G. Dalbagni, *Semin. Surg. Oncol.* **13**, 319 (1997); S. W. Lowe *et al.*, *Science* **266**, 807 (1994); G. J. Deichman *et al.*, *Int. J. Cancer* **75**, 277 (1998); K. M. Dameron, O. V. Volpert, M. A. Tainsky, N. Bouck, *Science* **265**, 1582 (1994).
4. L. A. Donehower *et al.*, *Nature* **356**, 215 (1992); T. Jacks *et al.*, *Curr. Biol.* **4**, 1 (1994).
5. A. Rogel, M. Popliker, C. G. Webb, M. Oren, *Mol. Cell. Biol.* **5**, 2851 (1985); P. Schmidt, A. Lorenz, H. Hameister, M. Montenarh, *Development* **113**, 857 (1991); D. Schwartz, N. Goldfinger, V. Rotter, *Oncogene* **8**, 1487 (1993).
6. E. A. Komarova *et al.*, *EMBO J.* **16**, 1391 (1997).
7. J. H. Hendry, A. Adeeko, C. S. Potten, I. D. Morris, *Int. J. Radiat. Biol.* **70**, 677 (1996); M. Hasegawa, Y. Zhang, H. Niibe, N. H. Terry, M. L. Meistrich, *Radiat. Res.* **149**, 263 (1998); J. H. Hendry, W. B. Cai, S. A. Roberts, C. S. Potten, *ibid.* **148**, 254 (1997); V. A. Tron *et al.*, *Am. J. Pathol.* **153**, 579 (1998).
8. C. H. Westfal *et al.*, *Cancer Res.* **58**, 5637 (1998); C. H. Westfal *et al.*, *Nature Genet.* **16**, 397 (1997).
9. E. A. Komarova and A. V. Gudkov, *Semin. Cancer Biol.* **8**, 389 (1998).
10. P. G. Komarov *et al.*, data not shown. All the conclusions and observations obtained with UV have been confirmed with gamma irradiation and adriamycin to verify the generality of the observed phenomena.
11. S. W. Lowe, H. E. Ruley, T. Jacks, D. Housman, *Cell* **74**, 957 (1993).
12. V. S. Ossovskaya *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 10309 (1996).
13. Supplemental information is available at *Science* Online at www.sciencemag.org/feature/data/1041963.shl.
14. C. J. Kemp, T. Wheldon, A. Balmain, *Nature Genet.* **8**, 66 (1994).
15. E. A. Komarova and A. V. Gudkov, unpublished data. The effect of PFTα on tumor response to radiation was tested in nude mice bearing tumors formed by $p53^{-/-}$ *E1a*+*ras*-transformed MEF cells (line A4). In preliminary experiments, PFTα injection, which protected mice from radiation-induced killing, did not affect the dynamics of tumor regression and regrowth in irradiated animals.
16. E. M. Johnston and J. Crawford, *Semin. Oncol.* **25**, 552 (1998).
17. M. Bronchud, *Anticancer Drugs* 4, 127 (1993).
18. T. G. Graeber *et al.*, *Nature* **379**, 88 (1996).
19. M. V. Chernov and G. R. Stark, *Oncogene* **14**, 2503 (1997); M. V. Chernov, C. V. Ramana, V. V. Adler, G. R. Stark, *Proc. Natl. Acad. Sci. U.S.A.* **95**, 2284 (1998).
20. K. Okamoto and D. Beach, *EMBO J.* **13**, 4816 (1994); W. S. El-Deiry *et al.*, *Cell* **75**, 817 (1993); X. Wu, J. H. Bayle, D. Olson, A. J. Levine *Genes Dev.* **7**, 1126 (1993).

21. We thank A. Polinsky for his generous help and R. Davidson for helpful comments on the manuscript. Supported in part by grants from NIH (CA75179 and CA60730) and Quark Biotech.

20 May 1999; accepted 10 August 1999

A Role for the Proteasome in the Light Response of the Timeless Clock Protein

Nirinjini Naidoo, Wei Song, Melissa Hunter-Ensor,* Amita Sehgal†

The cyclic expression of the period (PER) and timeless (TIM) proteins is critical for the molecular circadian feedback loop in *Drosophila*. The entrainment by light of the circadian clock is mediated by a reduction in TIM levels. To elucidate the mechanism of this process, the sensitivity of TIM regulation by light was tested in an in vitro assay with inhibitors of candidate proteolytic pathways. The data suggested that TIM is degraded through a ubiquitin-proteasome mechanism. In addition, in cultures from third-instar larvae, TIM degradation was blocked specifically by inhibitors of proteasome activity. Degradation appeared to be preceded by tyrosine phosphorylation. Finally, TIM was ubiquitinated in response to light in cultured cells.

In organisms ranging from cyanobacteria to mammals, the endogenous circadian clock is a feedback loop composed of cycling gene products that control their own synthesis. Such autoregulation has been demonstrated for the *period* (*per*) and *timeless* (*tim*) genes in *Drosophila melanogaster*, the *frequency* (*frq*) gene in *Neurospora*, the *kai* gene cluster in cyanobacteria, and appears to be true of the mammalian *per* genes (*1*, *2*). In all cases, precisely timed negative feedback by the proteins results in rhythmic expression of the RNA.

Rhythmic feedback is effected largely

Howard Hughes Medical Institute, Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA.

*Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

†To whom correspondence should be addressed. E-mail: amita@mail.med.upenn.edu

through circadian control of protein levels. Thus, when protein levels are high, negative feedback occurs, resulting in low levels of RNA (*2*). Degradation of the proteins eliminates feedback and allows RNA levels to rise once again. Early disappearance of the protein (in the case of the *per*^{s} mutant) results in an earlier rise of RNA levels and a shortening of circadian period by ~5 hours (*3*). In addition, the accumulation of both PER and TIM proteins lags behind the rise of their RNAs by ~6 hours and, at least in the case of PER, is regulated at the level of protein stability rather than RNA translation (*4*, *5*). Phosphorylation of PER by the casein kinase encoded by the *double-time* gene renders it unstable in the absence of TIM (*6*). Finally, levels of the TIM protein, but not of the RNA, are reduced by light, a response that shifts the phase of the clock and apparently mediates resetting of behavioral rhythms (*7*–*12*).

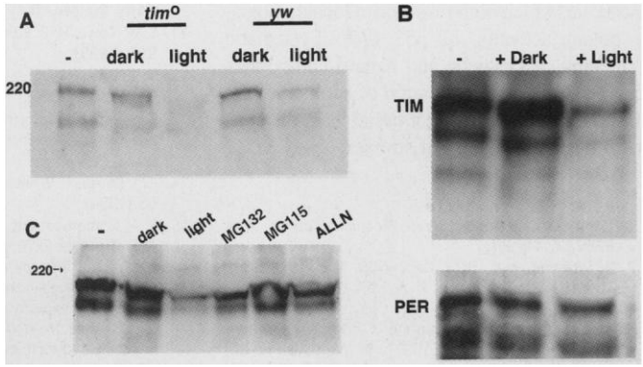

Fig. 1. Degradation of TIM by a light-induced activity. Dash indicates incubated without the addition of extract. Approximately 1 mg of head extract made from *yellow-white* (*yw*) flies collected at ZT19 (*36*) was incubated either with an antibody to TIM (UPR8) (*7*) or with an antibody to PER (UPR1) (*5*) overnight at 4°C. The immune complex was bound to protein G beads by incubation at 4°C for 1 hour and then washed in PBS. Head extract (*37*) from *yw* or *tim*^{0} flies collected at ZT20 either after a 1-hour pulse of light (light) or unpulsed (dark) was added to the immunoprecipitated TIM and incubated for 1 hour at 25°C. TIM levels were assayed by SDS–polyacrylamide gel electrophoresis and immunoblotting. We routinely detect two bands with this particular antibody to TIM. Based on data in the laboratory, we believe that they reflect products of alternatively spliced forms of TIM (*13*). They both respond to light. The variability of this assay is addressed in (*16*). (**A**) Degradation of TIM by a light-induced activity in *yw* and *tim*^{0} flies. (**B**) A light-treated *yw* extract that degraded TIM (upper panel) failed to degrade PER (lower panel). (**C**) Inhibition of degradation activity by inhibitors of the proteasome. These experiments were carried out as above, except for incubation of the light-treated extracts with the inhibitors MG115 (40 μM), MG132 (40 μM), and ALLN (40 μM) for 30 min at 4°C before their incubation with immunoprecipitated TIM. Light and dark control extracts had no inhibitors added.

To investigate the nature of the TIM light response, we developed an in vitro assay. We entrained flies to a 12 hour light/12 hour dark cycle and prepared head extracts from flies collected at either ZT (zeitgeber time) 20 or immediately after a 1-hour light pulse delivered at ZT 19 (ZT0 = lights on; ZT12 = lights off). These extracts were incubated with TIM protein immunoprecipitated from fly heads. After a 1-hour incubation at room temperature, we assayed TIM levels by protein immunoblots. Addition of the pulsed ex-

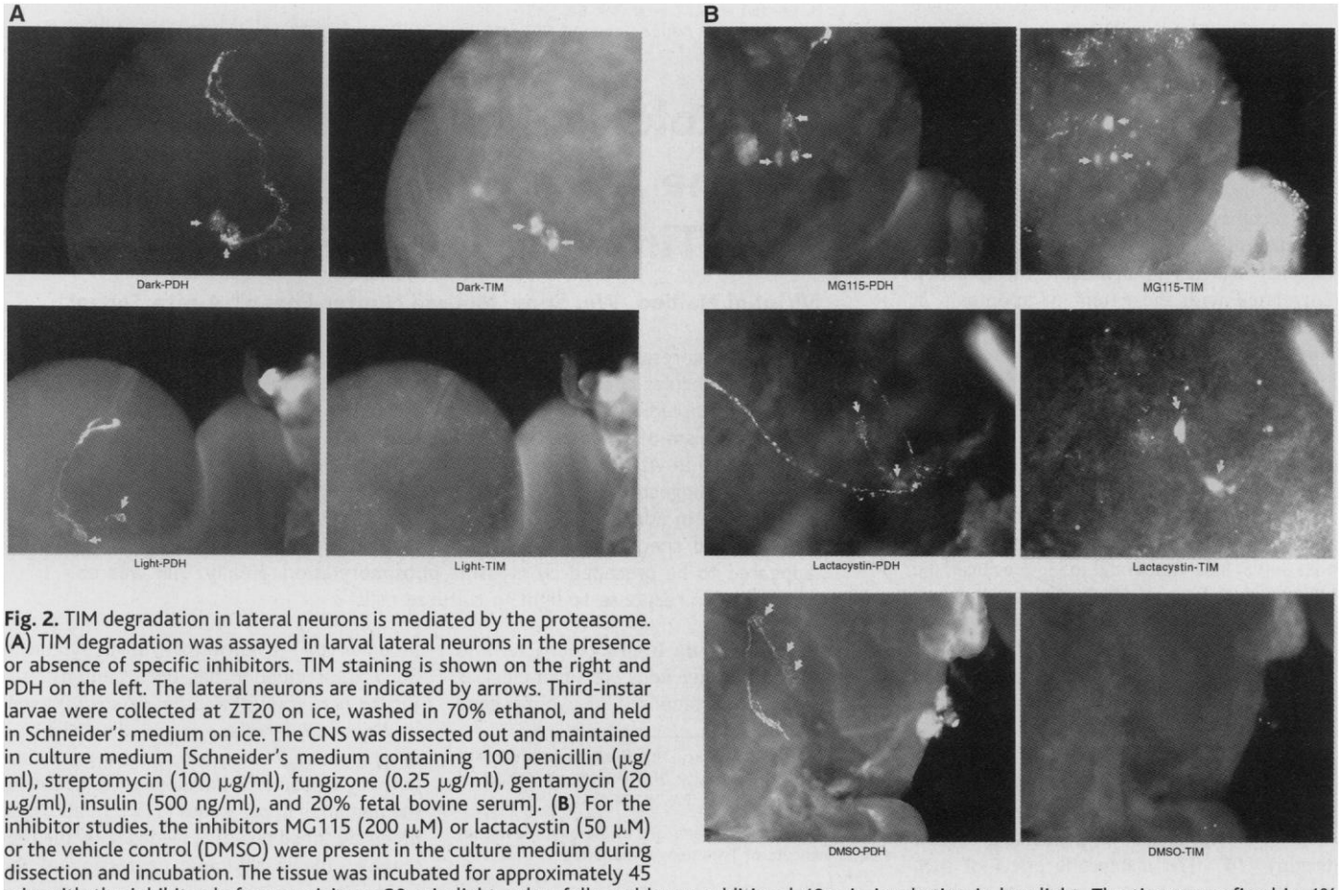

Fig. 2. TIM degradation in lateral neurons is mediated by the proteasome. (**A**) TIM degradation was assayed in larval lateral neurons in the presence or absence of specific inhibitors. TIM staining is shown on the right and PDH on the left. The lateral neurons are indicated by arrows. Third-instar larvae were collected at ZT20 on ice, washed in 70% ethanol, and held in Schneider's medium on ice. The CNS was dissected out and maintained in culture medium [Schneider's medium containing 100 penicillin (μg/ml), streptomycin (100 μg/ml), fungizone (0.25 μg/ml), gentamycin (20 μg/ml), insulin (500 ng/ml), and 20% fetal bovine serum]. (**B**) For the inhibitor studies, the inhibitors MG115 (200 μM) or lactacystin (50 μM) or the vehicle control (DMSO) were present in the culture medium during dissection and incubation. The tissue was incubated for approximately 45 min with the inhibitor before receiving a 20-min light pulse, followed by an additional 40-min incubation in low light. The tissue was fixed in 4% paraformaldehyde in PBS for 30 min before washing and staining with TIM and PDH antibodies (*38*).

tract reduced the TIM signal (Fig. 1A). Unpulsed head extract had no effect on the level of TIM, indicating that the reduction was light-specific. This light-induced reduction was also observed in tim^0 flies (which lack TIM protein) and was, in fact, routinely higher in these flies, which may suggest some down-regulation by the clock in wild-type flies. An immunoprecipitated PER substrate was not degraded by addition of the pulsed extract (Fig. 1B).

In order to determine the nature of the proteolytic activity, we assayed several general classes of protease inhibitors. Inhibitors of serine proteases [phenylmethylsulfonyl fluoride (PMSF) and aprotinin] and aspartate proteases (pepstatin) were not very effective in blocking TIM degradation (*13*). However, degradation was inhibited by the proteasomal inhibitors acetyl-leu-leu-norleucinal (ALLN), cbz-leu-leu-norvalinal (ZL_2NVaH or MG115) and cbz-leu-leu-leucinal (ZL_3H or MG132) (Fig. 1C). These peptide aldehydes strongly inhibit the chymotryptic activity of the eukaryotic 26*S* proteasome (*14*). TIM degradation was also blocked by bestatin, a metalloprotease inhibitor, and by leupeptin, which inhibits cysteine proteases and has some effects on other proteolytic systems, including the proteasome (*15*). The precise mechanism of action in this case is not known. Consistent with a role for the proteasome, we found that depletion of ubiquitin from the extract blocked TIM degradation (*13*).

Although the in vitro assay indicated a mechanism for TIM's response to light, its usefulness was limited by its variability (*16*). To verify the findings of this assay, we sought to develop an in vivo system. Thus, we used a primary culture assay in which we investigated the effect of two proteasomal inhibitors, lactacystin and MG115 on the TIM light response. Lactacystin, a microbial metabolite, is the most specific known naturally occuring inhibitor of the proteasome (*17*). It spontaneously hydrolyzes into clastolactacystin B lactone, which is the active species that reacts with the proteasome, inhibiting its chymotryptic and tryptic peptidase–like activity (*18*). MG115 (Fig. 1C) is a potent synthetic peptide aldehyde inhibitor with an inhibition constant (K_i) of 0.02 μM.

For our assay, we dissected the central nervous system (CNS) of third-instar larvae as described (*19*) and maintained them in culture medium for 1 hour. Some samples were exposed to a pulse of light for 20 min and were fixed at the end of the hour. Dark control samples were also incubated for an hour in the dark (Fig. 2). We then examined TIM expression in the lateral neurons (clock cells), which were located by costaining with an antibody to pigment-dispersing hormone (PDH) (Fig. 2). We observed strong TIM staining in lateral neurons of unpulsed tissue, but little to no TIM in CNS tissue that had received a light pulse (Fig. 2 and Table 1). The effect of inhibitors was tested by adding them to the culture medium at the start of the incubation. Tissue treated with lactacystin and MG115 before the light pulse revealed robust TIM staining in the lateral neurons. The strong inhibition by MG115 was consistent with a report that this is a much more effective inhibitor of proteolysis in intact cells than of in vitro hydrolysis of macromolecular substrates (*14*). The <100% block by lactacystin may reflect variable permeability or instability of the lactone metabolite. The dimethyl sulfoxide vehicle did not inhibit degradation. Treatment with PMSF, a serine protease inhibitor, also did not affect degradation (*13*).

Proteasomes are multicatalytic, multisubunit proteolytic complexes with highly conserved structures, and they play a key role in a variety of cellular processes, including the cell cycle, transcriptional regulation, removal of abnormal proteins from the cell, antigen presentation (*20*), and even in the turnover of a mammalian circadian-regulated protein (*21*). The TIM response to light is blocked specifically, in two different assays, by several inhibitors of the proteasome, which is important given that lactacystin, which was thought to affect only the proteasome (*17*), was recently shown to also act on a second multisubunit enzyme (*22*). Because the newly identified enzyme is insensitive to ALLN, it cannot account for the TIM response (see Fig. 1C). For the ubiquitin-proteasome system, proline glutamate serine threonine (PEST) re-

Table 1. Light-induced TIM degradation was assayed in lateral neurons in the presence of protease or kinase inhibitors. Each sample corresponds to lateral neurons from a single larval brain hemisphere (in some larvae both hemispheres were retained, but this was not always the case). *n* indicates the total number of samples from at least two experiments for each inhibitor (except PMSF and PD 98059, which were only tested once). Each experiment assayed three to five samples. "Residual" staining indicates samples in which staining was reduced but still detectable.

Inhibitor	Samples staining for TIM (%)			*n*
	Positive	Residual	Negative	
	Kinase inhibitors			
No Inhibitor (dark control)	100	0	0	14
No Inhibitor (light control)	0	10	90	19
Genistein (100 μg/ml)	100	0	0	10
Geinstein (25 μg/ml)	57	29	14	7
Genistein (12.5 μg/ml)	0	16	84	6
Staurosporin (1 μM)	0	0	100	7
Calphostin C (1 μM)	0	0	100	6
PD 98059 (100 μM)	0	0	100	4
	Protease inhibitors			
No Inhibitor (dark control)	100	0	0	37
No Inhibitor (light control)	0	11	89	36
ZI2NVa (200 μM)	100	0	0	15
Lactacystin (50–100 μM)	73	22	5	18
Bestatin (130–250 μM)	71	14	15	14
PMSF (1 mM)	0	0	100	3
DMSO (vehicle control)	0	12	88	8

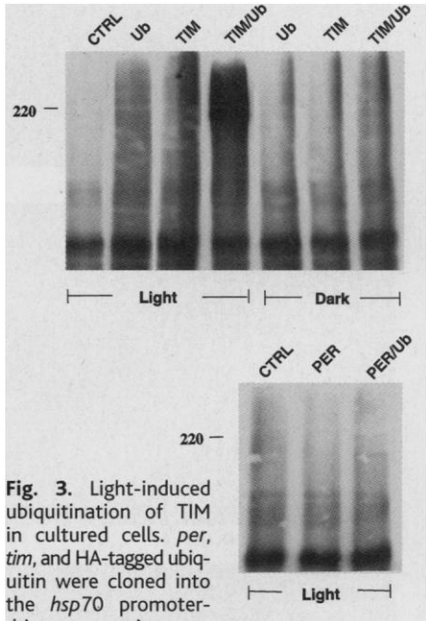

Fig. 3. Light-induced ubiquitination of TIM in cultured cells. *per*, *tim*, and HA-tagged ubiquitin were cloned into the *hsp70* promoter-driven expression vector pCasPer-hs, to form hs-*per*, hs-*tim*, and hs-Ub. *Drosophila* S2 cells were transfected by means of the calcium phosphate procedure, and expression was induced by heat shock (39). After 2 hours of light or dark treatment, the cells were harvested and lysed. TIM or PER was immunoprecipitated as described in Fig. 1. The protein immunoblots were probed with an antibody to HA (1:1000 dilution). The constructs used in each transfection are indicated above each lane. The number at the left of the panels indicates the position of the molecular weight marker.

gions sometimes serve as putative degradation/phosphorylation signals in the target molecule (*23*). The TIM protein sequence revealed the presence of seven PEST regions concentrated near the NH_2 and COOH termini.

Most cellular proteins that are degraded by the proteasome are ubiquitinated and then targeted to the proteasome (*24*). To determine whether TIM is ubiquitinated, which would also demonstrate that it is a direct target of the proteasome, we used a cell culture system. We expressed TIM and a hemagglutinin (HA)–tagged ubiquitin octamer (*25*) under heat shock control in *Drosophila* S2 cells. After a 30-min heat shock, cells were either maintained in the dark or treated with light for 2 hours, after which the cells were lysed and immunoprecipitates of TIM were probed with an antibody to HA. We found that TIM was ubiquitinated in response to light (Fig. 3). The effect was specific for TIM, because PER was not ubiquitinated with or without light

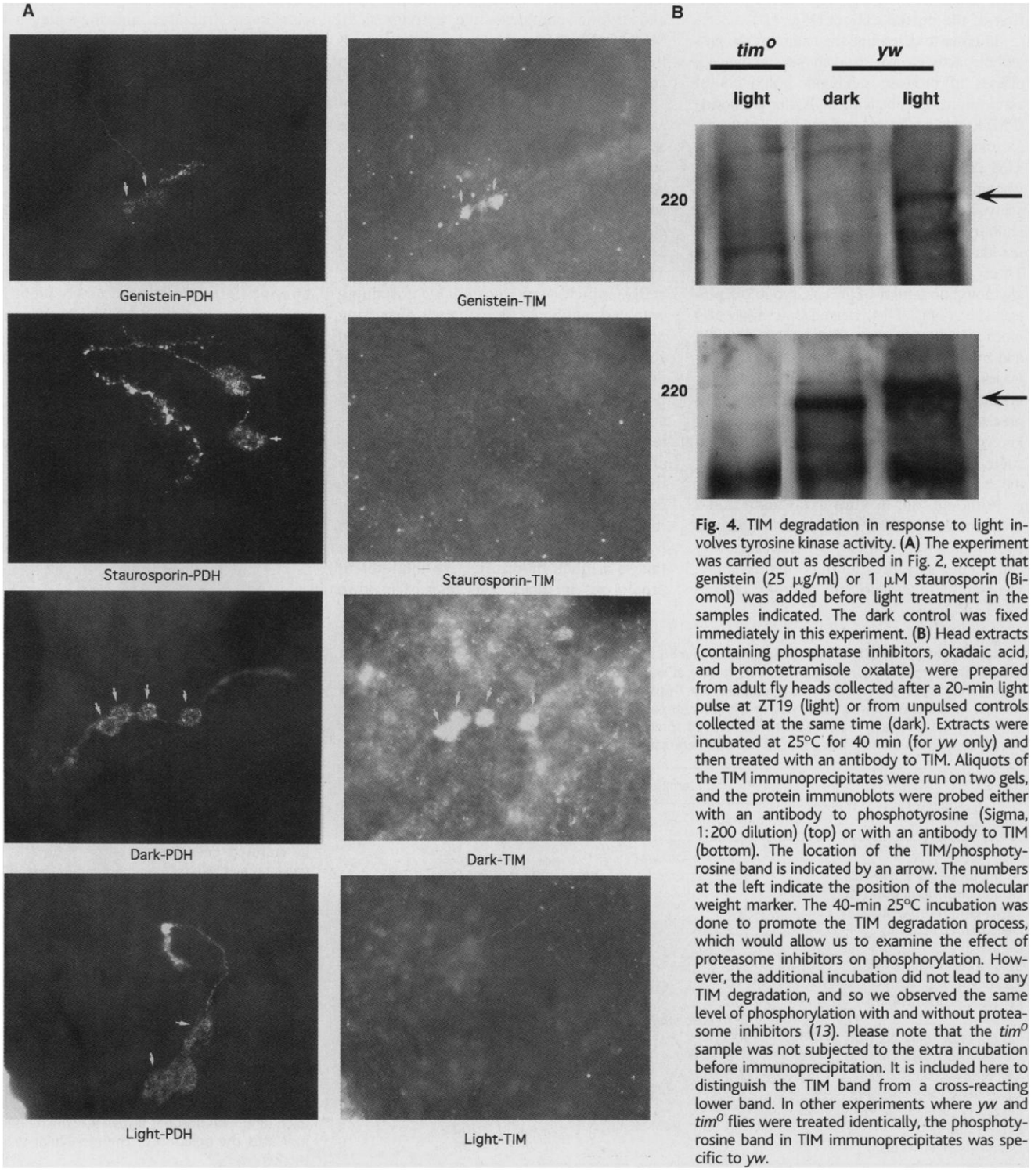

Fig. 4. TIM degradation in response to light involves tyrosine kinase activity. (**A**) The experiment was carried out as described in Fig. 2, except that genistein (25 μg/ml) or 1 μM staurosporin (Biomol) was added before light treatment in the samples indicated. The dark control was fixed immediately in this experiment. (**B**) Head extracts (containing phosphatase inhibitors, okadaic acid, and bromotetramisole oxalate) were prepared from adult fly heads collected after a 20-min light pulse at ZT19 (light) or from unpulsed controls collected at the same time (dark). Extracts were incubated at 25°C for 40 min (for *yw* only) and then treated with an antibody to TIM. Aliquots of the TIM immunoprecipitates were run on two gels, and the protein immunoblots were probed either with an antibody to phosphotyrosine (Sigma, 1:200 dilution) (top) or with an antibody to TIM (bottom). The location of the TIM/phosphotyrosine band is indicated by an arrow. The numbers at the left indicate the position of the molecular weight marker. The 40-min 25°C incubation was done to promote the TIM degradation process, which would allow us to examine the effect of proteasome inhibitors on phosphorylation. However, the additional incubation did not lead to any TIM degradation, and so we observed the same level of phosphorylation with and without proteasome inhibitors (*13*). Please note that the tim^0 sample was not subjected to the extra incubation before immunoprecipitation. It is included here to distinguish the TIM band from a cross-reacting lower band. In other experiments where *yw* and tim^0 flies were treated identically, the phosphotyrosine band in TIM immunoprecipitates was specific to *yw*.

treatment (Fig. 3). Extended light treatment also degraded TIM in these cells, and this degradation was inhibited by the proteasome inhibitor MG115 (*13*).

Although our data implicate a ubiquitin-proteasomal mechanism, they do not preclude a role for other proteolyic systems. In particular, bestatin is an effective blocker in the in vitro assay, which may indicate a role for a metalloprotease, in particular an aminopeptidase. The inhibitory effects of bestatin on this process could reflect a recently proposed proteasomal mechanism that involves a dipeptide pump (*26*) or, alternatively, preprocessing by a metalloprotease. The ErbB4 tyrosine kinase receptor is cleaved by a metalloprotease, then ubiquitinated and degraded by the proteasome (*27*).

As mentioned above, some cell cycle proteins are degraded via the proteasome pathway. In many cases, this degradation is preceded by a phosphorylation event (*28–30*). Moreover, PER degradation requires its phosphorylation by the casein kinase encoded by the *double-time* gene (*6*). To investigate a possible role for phosphorylation in the degradation of TIM, we examined the effect of several kinase inhibitors in the in vivo primary culture assay. The tyrosine kinase inhibitor genistein blocked the degradation of TIM in the lateral neurons after a pulse of light, whereas the serine-threonine inhibitors staurosporin and calphostin C and the MEK inhibitor PD98059 did not (Fig. 4A and Table 1) (*31–35*). These results suggest that tyrosine kinase activity precedes degradation of TIM. The concentrations of genistein that were effective in this assay (>50 μm) suggest a *c-src*–like kinase activity (*31*), although the concentration dependence must be interpreted with caution, because it could be a measure of permeability or drug stability.

To determine whether the tyrosine phosphorylation occured on TIM itself, we probed protein immunoblots of TIM immunoprecipitates with an antibody to phosphotyrosine. After 20 min of light treatment at ZT19, we were able to detect TIM with the antibody to phosphotyrosine (Fig. 4B). TIM in the "dark" samples was sometimes detected with this antibody but not consistently, which suggests that tyrosine phosphorylation of TIM is increased by light. The mobility of the TIM band in the light-treated sample was also reduced, presumably because of phosphorylation.

Together, our data indicate that the TIM response to light involves tyrosine phosphorylation and ubiquitination, followed by proteasomal degradation. An important question that arises from these studies is, what is the role of the proteasome pathway in free-running behavioral rhythms. Are the mechanisms that degrade TIM in response to light the same as those that degrade it in constant darkness, in which case light may serve only to further activate a process that is already under way. We propose that cyclic turnover of TIM under free-running conditions is mediated by phosphorylation, which targets it for degradation, perhaps by the proteasome. TIM is progressively phosphorylated throughout the night, and maximally phosphorylated forms are found just before the rapid decline of protein levels (*10*). From this point on, until the middle of the day, TIM levels remain low because of the low levels of RNA. As the repression of transcription is released, most likely because of the decrease in PER levels, RNA accumulates and protein also starts to accumulate, albeit slowly because it is still subject to phosphorylation and degradation. When the rate of TIM synthesis exceeds the rate of phosphorylation/degradation, higher levels of protein are observed, but as the phosphorylation program continues and RNA levels are reduced (because of negative feedback), levels of the protein drop off. Light could enhance TIM degradation by increasing TIM phosphorylation or increasing proteolytic activity in some manner (or both). This model would predict that the presence of light accelerates the falling phase of the protein and delays the rising phase, both through the same mechanism.

References and Notes

1. P. E. Hardin and A. Sehgal, in *Handbook of Behavioral State Control: Cellular and Molecular Mechanisms* (CRC Press, Boca Raton, FL, 1999), pp. 61–74.
2. J. C. Dunlap, *Cell* **96**, 271 (1999).
3. S. B. Marrus, H. Zeng, M. Rosbash, *EMBO J.* **15**, 6877 (1996).
4. M. E. Dembinska, R. Stanewsky, J. C. Hall, M. Rosbash, *J. Biol. Rhythms* **12**, 157 (1997).
5. Y. Chen, M. Hunter-Ensor, P. Schotland, A. Sehgal, *ibid.* **13**, 364 (1998).
6. J. L. Price *et al.*, *Cell* **94**, 83 (1998).
7. M. Hunter-Ensor, A. Ousley, A. Sehgal, *ibid.* **84**, 677 (1996).
8. C. Lee, V. Parikh, T. Itsukaichi, K. Bae, I. Edery, *Science* **271**, 1740 (1996).
9. M. P. Myers, K. Wager-Smith, A. Rothenflugh, M. W. Young, *ibid.*, p. 1736.
10. H. Zeng, Z. Qian, M. P. Myers, M. Rosbash, *Nature* **380**, 129 (1996).
11. Z. Yang, M. Emerson, H. S. Su, A. Sehgal, *Neuron* **21**, 215 (1998).
12. V. Suri, Q. Zuwei, J. C. Hall, M. Rosbash, *ibid.*, p. 225.
13. N. Naidoo, W. Song, M. Hunter-Ensor, A. Sehgal, data not shown.
14. K. L. Rock *et al.*, *Cell* **78**, 761 (1994).
15. E. Carafoli and M. Molinari, *Biochem. Biophys. Res. Commun.* **247**, 193 (1998).
16. In about 35% of the experiments, the pulsed extract did not display proteolytic activity or resulted in incomplete proteolysis, yielding a fragment with a molecular mass (MW) between 66 and 97 kD. We regarded complete or partial disappearance of the lower of two bands (which are recognized by the antibody to TIM) as positive. We usually also observed reduction in the upper band, which frequently shifted down in molecular mass.
17. G. Fenteany *et al.*, *Science* **268**, 726 (1995).
18. A. Craiu *et al.*, *J. Biol. Chem.* **272**, 13437 (1997).
19. M. Kaneko, C. Helfrich-Forster, J. C. Hall, *J. Neurosci.* **17**, 6745 (1997); I. F. Emery, J. M. Noveral, C. F. Jamison, K. K. Siwicki, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 4092 (1997).
20. W. Baumeister, J. Walz, F. Zuhl, E. Seemuller, *Cell* **92**, 367 (1998).
21. J. A. Gastel, P. H. Roseboom, P. A. Rinaldi, J. L. Weller, D. C. Klein, *Science* **279**, 1358 (1998).
22. E. Geier *et al.*, *ibid.* **283**, 978 (1999).
23. M. Rechsteiner and S. W. Rogers, *Trends Biochem. Sci.* **21**, 267 (1996).
24. C. Pickart, *FASEB J.* **11**, 1055 (1997).
25. M. Treier, L. M. Staszewski, D. Bohmann, *Cell* **78**, 787 (1994).
26. I. V. Davydov, D. Patra, A. Varshavsky, *Arch. Biochem. Biophys.* **357**, 317 (1998).
27. M. Vecchi and G. Carpenter, *J. Cell Biol.* **139**, 995 (1997).
28. R. J. Deshaies, V. Chau, M. W. Kirschner, *EMBO J.* **14**, 303 (1995).
29. J. Yaglom *et al.*, *Mol. Cell. Biol.* **15**, 731 (1995).
30. S. Lahav-Baratz, V. Sudakin, J. V. Ruderman, A. Hershko, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9303 (1995).
31. J. F. Geissler *et al.*, *J. Biol. Chem.* **265**, 22255 (1990).
32. T. Tamaoki *et al.*, *Biochem. Biophys. Res. Commun.* **135**, 397 (1986).
33. E. Kobayashi *et al.*, *J. Antibiotics* **42**, 1470 (1989).
34. D. Dudley, L. Pang, S. Decker, A. Bridges, A. Saltiel, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7686 (1995).
35. D. Alessi, A. Cuenda, P. Cohen, D. Dudley, A. Saltiel, *J. Biol. Chem.* **270**, 27489 (1995).
36. Heads from *yellow-white* flies collected at ZT19 or ZT20 were homogenized in IP buffer [10 mM Hepes, 150 mM NaCl, and 1 mM EDTA (pH 7.5)]. Triton X-100 was added to the lysate at a final concentration of 1% before incubation at 4°C for 30 min. The lysate was centrifuged at 5000 rpm. The protein concentration of the supernatant was determined before immunoprecipitation by means of the Bio-Rad assay.
37. The head extract was made by homogenizing fly heads in IP buffer, adding Triton X-100 to a final concentration of 1% and incubating the lysate at 4°C for 30 min with rotation. The lysate was centrifuged at 500 to 700 rpm at 4°C before use in the assay.
38. After light treatment, the tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min, rinsed in PBS containing 1% Triton for 2 periods of 20 min, and then blocked in PBS containing 0.5% Triton with 10% normal goat serum for 1 hour. Primary antibodies (1:1500 dilution of anti-TIM and a 1:10 000 dilution of rabbit anti-PDH) were incubated overnight at 4°C in blocking solution. The following day, the tissue was washed three times, for 1 hour each time, in rinse solution and incubated for 1 hour with a 1:500 dilution of fluorescein isothiocyanate–conjugated goat antibody to rabbit (Jackson Immunoresearch) and a 1:1000 dilution of Cy3-conjugated donkey antibody to rat (Jackson Immunoresearch). The tissue was again washed with rinse solution, then mounted with Vectastain H 1000 medium with 4′,6′-diamidino-2-phenylindole. Slides were analyzed with a Leica DMRE microscope.
39. L. Saez and M. W. Young, *Neuron* **17**, 911 (1996).

40. We thank C. Pickart and J. Field for critical reading of the manuscript and for many useful suggestions, L. Matthews and K. Siwicki for demonstrating the larval dissections, K. Hu for sharing results of her experiments, and other members of the laboratory for general advice. Supported by grants from NIH and NSF to A.S. M.H.E. was supported by an NIH training grant to the Behavioral Neuroscience program. A.S. is an assistant investigator in the Howard Hughes Medical Institute.

30 March 1999; accepted 3 August 1999