Degradation of Serotonin N-Acetyltransferase, a Circadian Regulator, by the N-end Rule Pathway*

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Serotonin N-acetyltransferase (AANAT) converts serotonin to N-acetylserotonin (NAS), a distinct biological regulator and the immediate precursor of melatonin, a circulating hormone that influences circadian processes, including sleep. N-terminal sequences of AANAT enzymes vary among vertebrates. Mechanisms that regulate the levels of AANAT are incompletely understood. Previous findings were consistent with the possibility that AANAT may be controlled through its degradation by the N-end rule pathway. By expressing the rat and human AANATs and their mutants not only in mammalian cells but also in the yeast Saccharomyces cerevisiae, and by taking advantage of yeast genetics, we show here that two “complementary” forms of rat AANAT are targeted for degradation by two “complementary” branches of the N-end rule pathway. Specifically, the N-terminal acetylated (Nt-acetylated) Ac-AANAT is destroyed through the recognition of its Nt-acetylated N-terminal Met residue by the Ac/N-end rule pathway, whereas the non-Nt-acetylated AANAT is targeted by the Arg/N-end rule pathway, which recognizes the unacycated N-terminal Met-Leu sequence of rat AANAT. We also show, by constructing lysine-to-arginine mutants of rat AANAT, that its degradation is mediated by polyubiquitylation of its Lys residue(s). Human AANAT, whose N-terminal sequence differs from that of rodent AANATs, is longer-lived than its rat counterpart and appears to be refractory to degradation by the N-end rule pathway. Together, these and related results indicate both a major involvement of the N-end rule pathway in the control of rodent AANATs and substantial differences in the regulation of rodent and human AANATs that stem from differences in their N-terminal sequences.

Arylalkylamine N-acetyltransferase (AANAT)2 converts the neurotransmitter serotonin to N-acetylserotonin (NAS) (1–11). Regulatory functions of NAS include its activity as an agonist of TrkB, the receptor for the brain-derived neurotrophic factor (12–14). NAS is the immediate precursor of melatonin, a circulating hormone that regulates sleep and other circadian processes in vertebrates. Melatonin is also present in invertebrates, including insects, as well as in animals that lack recognizable neurons (1, 15–21). The functions of melatonin in mammals include the modulation of circadian rhythms in response to light–dark cycles. Melatonin also contributes to the control of seasonal physiology (20). These mechanisms are a part of a broader range of processes that involve distinct biological oscillators in all organisms (22–24). In the course of daily light–dark cycles, the activity of AANAT and the level of the ~23-kDa AANAT protein in the brain’s pineal gland are high during nocturnal periods and rapidly decrease following exposure to light (1, 20, 25).

In mammals, changes of AANAT levels in the brain’s pineal gland are controlled by the circadian oscillator in the suprachiasmatic nucleus of the hypothalamus. At night, axons of rodent superior cervical ganglion neurons release norepinephrine in the pineal gland, in response to circadian signals from the suprachiasmatic nucleus, thereby activating adrenergic receptors to increase intracellular Ca2+ and cAMP. The resulting phosphorylation of CREB, the cAMP-response element-binding protein, up-regulates the Aanat transcriptional promoter, which contains cAMP-response element sequence elements (1, 4, 25–27). Increases in cAMP also stimulate the site-specific phosphorylation of AANAT by PKA, thereby causing the binding of AANAT to 14–3–3 proteins. These transitions augment the enzymatic activity of AANAT and inhibit its degradation (1, 6, 7, 28). Although AANAT is predominantly expressed in the pineal gland and the retina, this enzyme is also present in cells of the gastrointestinal tract and apparently at other sites as well, including the hippocampus, the olfactory bulb, the cerebellum, and the spinal cord (12, 29, 30).

Melatonin is produced from NAS by hydroxyindole-O-methyltransferase. The levels of NAS, synthesized by AANAT, can constrain the rate of melatonin synthesis from NAS during light parts of daily cycles (when NAS levels are low) but appear

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2 The abbreviations used are: AANAT, arylalkylamine N-acetyltransferase; Ub, ubiquitin; NAS, N-acetylserotonin; CHX, cycloheximide; N-degron, an N-terminal degradation signal recognized by the N-end rule pathway; N-recognition, an E3 ubiquitin ligase that can recognize at least some N-degrons; CREB, cAMP-response element-binding protein; Nt-, Nt-termially/ N-terminal; MetAP, Met-aminopeptidase; LDS, lithium dodecyl sulfate; BisTris, 2-[bis(2-hydroxyethyl) amino]-2-(2-hydroxymethyl)propane-1,3-diol.

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not to be the rate-limiting step during dark parts of these cycles, when both AANAT and NAS levels are high (21). In rodents such as rats, the activity of AANAT starts to increase 3–4 h after the onset of darkness and begins to decrease before the onset of light in the morning (21, 31). Hence the importance of regulating the expression and the enzymatic activity of AANAT, because these parameters determine the levels of both NAS and melatonin.

The concentration of AANAT, a largely cytosolic enzyme (32), is controlled through both transcriptional and post-translational mechanisms (1, 11, 31, 33–35). In rodents, the onset of darkness leads to an increase in melatonin (requiring a preceding increase of NAS) after a lag period, whereas in sheep and primates, increases in melatonin occur rapidly upon the onset of darkness. The levels of Aanat mRNA in rodents can vary by >100-fold during light-dark cycles (10), whereas in non-rodent mammals such as, for example, sheep, these differences can be as low as ~2-fold, suggesting a major involvement of translational and/or post-translational AANAT regulation in latter cases (11, 36). In addition to variability in regulation at the level of AANAT-encoding mRNAs, AANAT is also unusual in the extent of variability of its N-terminal sequences during vertebrate evolution (Fig. 1C). In 2010, one of our laboratories showed that N-terminal residues of rat AANAT play a role in the control of its degradation, suggesting the involvement of the N-end rule pathway (37).

The N-end rule pathway is a set of intracellular proteolytic systems whose unifying feature is the ability to recognize and polyubiquitylate proteins containing N-terminal degradation signals (degrons) called N-degrons, thereby causing degradation of these proteins by the proteasome (Fig. 1, A and B) (38–46). Ubiquitin (Ub) ligases of the N-end rule pathway, called N-recognins, can recognize not only N-degrons but also specific internal degradation signals (47–50). The main determinant of an N-degron is a protein’s internal Lys residue(s). About 90% of human proteins are modified destabilizing N-terminal residues. About 90% of human proteins are modified destabilizing N-terminal residues (Fig. 1A) (41–43, 69). In the yeast Saccharomyces cerevisiae, the Arg/N-end rule pathway is mediated by the Ubr1 N-recognin, a 225-kDa RING-type E3 Ub ligase and a part of the targeting complex comprising the Ubr1-Rad6 and Ufd4-Ubc4/5 E2-E3 holoenzymes (41, 70). In multicellular eukaryotes, several E3 Ub ligases, including Ubr1, function as N-recognins of the Arg/N-end rule pathway (Fig. 1A).

In S. cerevisiae, the Ac/N-end rule pathway is mediated by at least the cytosolic/nuclear E3 Ub ligase Not4 and by Doa10, an endoplasmic reticulum membrane-embedded E3 (59). In mammalian cells, this pathway is mediated by at least the Tebp E3, which is sequologous (similar in sequence (71)) to yeast Doa10 (60). Human RGS2, a regulator of specific G proteins, is a short-lived substrate of the Ac/N-end rule pathway both in mammalian cells and in the heterologous setting of S. cerevisiae (60, 72, 73). In addition, the naturally occurring (blood pressure-elevating) human RGS2Q2L mutant (in which Gln at position 2 is replaced by Leu) is targeted for degradation by both the Ac/N-end rule pathway and the Arg/N-end rule pathway (60). The Ac/N-end rule pathway recognizes the Nt-acetylated Ac-RGS2Q2L (specifically its N-terminal Ac-Met residue), whereas the non-Nt-acetylated RGS2Q2L is targeted (through its N-terminal Met-Leu sequence) by the Arg/N-end rule pathway (Fig. 1A).

In the present work, we analyzed the proteasome-mediated degradation of rat AANAT (37), whose N-terminal sequence Met-Leu (Fig. 1C) is identical to that of the otherwise unrelated human RGS2Q2L protein (60). We also characterized human AANAT, which is highly sequologous (71) (84% identical) to rat AANAT but bears a different N-terminal sequence (Fig. 1C). Our analyses employed the previously helpful approach of dissecting degradation of a mammalian protein of interest not only...
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in a homologous (mammalian) setting but also in *S. cerevisiae* (60, 66), thereby making possible the use of yeast genetics.

We show here that two alternative versions of rat AANAT, its Nt-acetylated and non-Nt-acetylated forms, are targeted for degradation by the Ac/N-end rule pathway and the Arg/N-end rule pathway, respectively. In contrast, human AANAT, whose N-terminal sequence differs from that of rodent AANATs, is significantly longer-lived than its rat counterpart and appears to be largely refractory to degradation by the N-end rule pathway. Together, these and related results indicate both a major involvement of the N-end rule pathway in the control of rodent AANATs and substantial differences in the regulation of rodent and human AANATs that stem from differences in their N-terminal sequences.

**Results**

Wild-type Rat and Human AANATs and Their Mutants—The start (AUG) codon-encoded N-terminal Met residue of nascent proteins is cotranslationally cleaved off by ribosome-associated Met-aminopeptidases (MetAPs) if a residue at position 2, to be made N-terminal by the cleavage, is not larger than Val (41, 74). Thus, for example, the Met residue of the N-terminal sequence differs from that of rodent AANATs, is not Nt-acetylated in mammalian cells as well (59, 63).

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(cotranslationally cleaved off by MetAPs. The resulting N-terminal Pro is usually not Nt-acetylated in mammalian cells as well (59, 63).

Wild-type Rat and Human AANATs and Their Mutants—The start (AUG) codon-encoded N-terminal Met residue of nascent proteins is cotranslationally cleaved off by ribosome-associated Met-aminopeptidases (MetAPs) if a residue at position 2, to be made N-terminal by the cleavage, is not larger than Val (41, 74). Thus, for example, the Met residue of the N-terminal sequence differs from that of rodent AANATs, is not Nt-acetylated in mammalian cells as well (59, 63).

Default role of the Ac/N-end rule pathway because the latter does not recognize N-terminal Met residue in the Met-Leu-Ser sequence of wild-type rat AANAT (Fig. 1C) C-terminally tagged with a triple-FLAG epitope. It was used as a control for the wild-type human AANAT mutant described in item vii.

(v) (M)S\textsuperscript{ST}hAANAT\textsubscript{3f}, the wild-type human (M)S\textsuperscript{ST}hAANAT (Fig. 1C) C-terminally tagged with a triple-FLAG epitope.

(vi) (M)P\textsuperscript{ST}hAANAT\textsubscript{3f} in which the second-position Ser of the wild-type human (M)P\textsuperscript{ST}hAANAT\textsubscript{3f} (Fig. 1C) was replaced by the Pro residue, yielding (M)P\textsuperscript{ST}hAANAT\textsubscript{3f}. The N-terminal Met residue of either the wild-type human (M)P\textsuperscript{ST}hAANAT\textsubscript{3f} or the mutant (M)P\textsuperscript{ST}hAANAT\textsubscript{3f} would be cotranslationally removed by MetAPs (41, 74). However, in contrast to the resulting N-terminal Ser of wild-type (M)P\textsuperscript{ST}hAANAT\textsubscript{3f}, which would be expected to be cotranslationally Nt-acetylated, the N-terminal Pro of the mutant (M)P\textsuperscript{ST}hAANAT\textsubscript{3f} is not Nt-acetylated, at least in *S. cerevisiae*, and is usually not Nt-acetylated in mammalian cells as well (59, 63).

(vii) (M)L\textsuperscript{ST}hAANAT\textsubscript{3ha}, the otherwise wild-type triple-HA-tagged rat (M)L\textsuperscript{ST}hAANAT in which the Lys-8 residue was replaced by Arg.

(viii) (M)L\textsuperscript{ST}hAANAT\textsubscript{Kzero}, the otherwise wild-type triple-HA-tagged human (M)L\textsuperscript{ST}hAANAT in which each of its four Lys residues were replaced by Arg (Fig. 6B).

(ix) (M)L\textsuperscript{ST}hAANAT\textsubscript{Kzero}, the otherwise wild-type triple-HA-tagged human (M)L\textsuperscript{ST}hAANAT in which both of its Lys residues were replaced by Arg (Fig. 7, A and D).

Degradation of Rat AANAT by the *S. cerevisiae* N-end Rule Pathway—In cycloheximide (CHX) assays, a protein of interest is analyzed by immunoblotting as a function of time after the inhibition of translation by CHX (39, 59, 60). Both the wild-type rat (M)L\textsuperscript{ST}hAANAT\textsubscript{3f} (Fig. 1C) and its (M)NL\textsuperscript{ST}hAANAT\textsubscript{3f} mutant (see item ii above) were rapidly degraded in wild-type *S. cerevisiae*, with half-lives (t1/2) < 5 min (Fig. 2, B (lanes 3–10) and D). In striking contrast, (M)NL\textsuperscript{ST}hAANAT\textsubscript{3f} (see item iii above), produced from wild-type (M)L\textsuperscript{ST}hAANAT\textsubscript{3f} by deleting the Leu residue at position 2, was virtually completely stable under the same conditions, with a t1/2 > 1 h (Fig. 2, B (lanes 11–14) and D). Because the N-terminal Met residue in the Met-Leu-Ser sequence of the wild-type rat (M)L\textsuperscript{ST}hAANAT\textsubscript{3f} would be likely to be Nt-acetylated in *vivo* (59, 62, 63), the rapid degradation of (M)L\textsuperscript{ST}hAANAT\textsubscript{3f} (t1/2 < 5 min) (Fig. 2, B and D) suggested the involvement of the Ac/N-end rule pathway (Fig. 1A). To address this possibility, we constructed the non-Nt-acetylable (M)PL\textsuperscript{ST}hAANAT\textsubscript{3f} mutant (see item iv above). (This mutant would also not be expected to be targetable by the Arg/N-end rule pathway, because the latter does not recognize N-terminal Pro (40, 41).) Remarkably, (M)PL\textsuperscript{ST}hAANAT\textsubscript{3f} was long-lived in *S. cerevisiae* in comparison with the short-lived wild-type (M)L\textsuperscript{ST}hAANAT\textsubscript{3f} (t1/2 ~ 1 h versus t1/2 < 5 min, respectively), strongly suggesting (but not proving) a role for the Ac/N-end rule pathway in the degradation of the wild-type rat (M)L\textsuperscript{ST}hAANAT\textsubscript{3f} (Fig. 2, B (lanes 15–18) and D; also see below).

To further address the involvement of the Ac/N-end rule and Arg/N-end rule pathways (Fig. 1, A and B) in the degradation of wild-type (M)L\textsuperscript{ST}hAANAT\textsubscript{3f} we performed CHX-chases in *S. cerevisiae* mutants that lacked essential components of either
one or both of these pathways. The normally short-lived rat
\( ^{\text{Mls}}r\text{AANAT}_{3f} \) (T1/2 < 5 min; Fig. 2, C (lanes 2–5) and E) was partially stabilized in both \( \text{nna30}\Delta \) and \( \text{ubr1}\Delta \) mutants (Fig. 2, C (lanes 6–9 and 14–17) and E). S. cerevisiae \( \text{nna30}\Delta \) cells lack the cognate NatC Nt-acetylase whose substrates include proteins bearing the N-terminal Met-Leu sequence (59, 62, 63, 75, 76). S. cerevisiae \( \text{ubr1}\Delta \) cells lack Ubr1, the E3 Ub ligase (N-recognin) that is essential for the proteolytic activity of the Arg/N-end rule pathway (Fig. 1A) (40, 41, 59).

Crucially, the relative stabilization of \( ^{\text{Mls}}r\text{AANAT}_{3f} \) was dramatically higher in the double mutant \( \text{nna30}\Delta \text{ubr1}\Delta \) than in either one of the single mutants (Fig. 2, C (lanes 18–21) and E). These findings should be considered together with the conceptually independent result that the non-Nt-acetylatable (and...
also not targetable by the Arg/N-end rule pathway) (M)PLSrAANAT₃f mutant (see the preceding paragraph) was much longer-lived in wild-type S. cerevisiae than its wild-type MLSrAANAT₃f counterpart (Fig. 2, B (lanes 15–18 versus lanes 3–6) and D). Together, these sets of findings indicated that the wild-type rat MLSrAANAT₃f (its two alternative forms) was targeted for degradation by both the Ac/N-end rule pathway and the Arg/N-end rule pathway. Specifically, the Nt-acetylated Ac-MLSrAANAT₃f is destroyed through the recognition of its Nt-acetylated N-terminal Met residue by the Ac/N-end rule pathway (Fig. 1B), whereas the non-Nt-acetylated MLSrAANAT₃f is targeted by the “complementary” Arg/N-end rule pathway, which recognizes the unacetylated N-terminal Met-Leu sequence of MLSrAANAT₃f as the Met-H₉₀₂¹ motif (N-terminal Met followed by a bulky hydrophobic residue) (Fig. 1A).

This conclusion was in agreement with the finding that the rapid degradation of MLSrAANAT₃f in wild-type S. cerevisiae

![Diagram](https://example.com/diagram.png)
(t½ < 5 min) remained unchanged in naa10Δ cells, which lacked the non-cognate NatA Nt-acetylase (which does not Nt-acetylate N-terminal Met) (59, 76). Furthermore and also in agreement with the above conclusion, the degradation of MSLSrAANATΔ with double-mutant naa10Δ ubr1Δ cells proceeded at a rate similar to that in the single ubr1Δ mutant (Fig. 2, C (lanes 22–25) and E). Thus, a double-mutant background that nearly completely stabilizes the wild-type rat MSLSrAANATΔ must be naa30Δ ubr1Δ in that it should also lack the activity of the cognate NatC Nt-acetylase (Fig. 2, C (lanes 18–21) and E). In agreement with this conclusion, the ablation of NAA10, encoding the non-cognate NatA Nt-acetylase, did not have a synergistic effect on the rate of MSLSrAANATΔ degradation in double-mutant naa10Δ ubr1Δ cells (Fig. 2, C (lanes 22–25) and E).

Previous work has identified the endoplasmic reticulum membrane-embedded E3 Ub ligase Doa10 as one Ac/N-recognin of S. cerevisiae (Ac/N-end rule pathway (39, 59)). We found that the degradation of the wild-type rat MSLSrAANATΔ (t½ < 5 min in wild-type S. cerevisiae) was not significantly impaired in doa10Δ cells, in comparison with wild-type cells (Fig. 3, D and F). We did not examine, so far, the other yeast Ac/N-recognin, Not4 (see the Introduction) for its possible role in targeting the Nt-acetylated rat Ac-MSLSrAANATΔ in S. cerevisiae. We also do not know, thus far, whether Tbk4, the mammalian counterpart of the yeast Doa10 Ac/N-recognin (60), is involved in the degradation of rat MSLSrAANATΔ in a homologous (mammalian) setting.

Degradation of Rat AANAT Is Proteasome-dependent and Does Not Require Lys-8 — The demonstrated degradation of two forms of the wild-type rat MSLSrAANATΔ (Ac-MSLSrAANATΔ and its non-Nt-acetylated counterpart) by the two branches of the N-end rule pathway (Fig. 2) already implied the proteasome dependence of this degradation, given the known organization of the N-end rule pathway (Fig. 1, A and B). To verify this in S. cerevisiae by independent means, CHX-chases with rat MSLSrAANATΔ were carried out in wild-type versus pdr5Δ cells in either the presence or absence of the MG132 proteasome inhibitor. (Cells lacking the transmembrane transporter PDR5 are more sensitive to MG132 (77, 78).)

As expected, given the proteasome dependence of the N-end rule pathway (Fig. 1, A and B), the normally rapid degradation of the wild-type rat MSLSrAANATΔ was substantially inhibited in wild-type S. cerevisiae in the presence of 50 μM MG132 (Fig. 4, A and B). This degradation was inhibited even more strongly in mutant pdr5Δ cells under the same conditions, confirming the proteasome dependence of at least the bulk of MSLSrAANATΔ degradation (Fig. 4, A and B).

N-terminal regions of mammalian AANATs contain a highly conserved Lys residue (e.g. Lys-8 in rat AANAT and Lys-10 in human AANAT) that has been suggested as a potential ubiquitylation site and thus a determinant of the AANAT degron (33, 79–81). To address this possibility, CHX-chases were performed in S. cerevisiae with wild-type MSLSrAANATΔ and a mutant containing a Lys-8 → Arg mutation (MSLSrAANATΔKRR). Wild-type MSLSrAANATΔ and the mutant MSLSrAANATΔKRR were degraded at similar rates, indicating that the conserved lysine at this position (Lys-8 in rat MSLSrAANAT) is not, by itself, a significant determinant of MSLSrAANAT stability (Fig. 4C).

Degradation of Human AANAT in S. cerevisiae—Fig. 3 (A (lanes 6–9) and B) shows the results of a CHX-chase, in wild-type yeast, with the 207-position residue (not counting the tag) (M)SThAANATΔ, the wild-type human AANAT C-terminally tagged with a triple-FLAG epitope. Parentheses around the superscript’s N-terminal Met residue denote the fact that this Met is cotranslationally cleaved off by MetAPs. Whereas human (M)SThAANATΔ was relatively unstable in S. cerevisiae (t½ ~ 30 min), it was much longer-lived (including its higher zero-time, prechase level) than the wild-type rat MSLSrAANATΔ under the same conditions (Fig. 3, A and B).

As was also done with the wild-type rat MSLSrAANATΔ (Fig. 2C), we characterized the degradation of wild-type human (M)SThAANATΔ in S. cerevisiae strains deficient in specific components of the N-end rule pathway. The N-terminal Ser-Thr sequence of wild-type human (M)SThAANATΔ (after the cotranslational removal of the initially present N-terminal Met) made it likely that (M)SThAANATΔ was cotranslationally Nt-acetylated in vivo (59, 63). This modification of human (M)SThAANATΔ would make it a potential target for degradation by the Ac/N-end rule pathway (Fig. 1B). In contrast, the non-Nt-acetylated human (M)SThAANATΔ would not be expected to be recognized by either the Ac/N-end rule or the Arg/N-end rule pathway (Fig. 1A), in the latter case due to the absence of both a destabilizing N-terminal residue and a bulky hydrophilic (Φ) second residue. (A Met-Φ N-terminal motif, containing a second-position Φ residue, can act as an N-degron recognized by the Arg/N-end rule pathway (60, 82).

Indeed, the rate of degradation of human (M)SThAANATΔ in ubr1Δ S. cerevisiae, which lacked the Ub1 N-recognin and therefore lacked the Arg/N-end rule pathway (Fig. 1A), was similar to the rate of (M)SThAANATΔ degradation in wild-type cells (Fig. 3, C (lanes 1–4 versus lanes 9–12), D (lanes 2–5 versus lanes 14–17), E, and F). The rate of (M)SThAANATΔ degradation that was observed with wild-type S. cerevisiae was also not significantly changed in doa10Δ cells, which lacked one of two known Ac/N-recognins of the Ac/N-end rule pathway (Figs. 1B and 3, D (lanes 10–13 versus lanes 2–5) and F). Surprisingly, (M)SThAANATΔ was strongly destabilized in naa10Δ cells, which lacked the cognate Nt-acetylase for the N-terminal Ser residue (59, 62); similar results were obtained with double-mutant naa10Δ ubr1Δ cells (Fig. 3, C–F).

Given the much faster degradation of (M)SThAANATΔ in the absence of Naa10 (e.g. Fig. 3, C (lanes 1–4 versus lanes 5–8) and E (i.e. in cells that would be incapable of Nt-acetylating (M)SThAANATΔ), we also carried out CHX-chases, in wild-type S. cerevisiae, with (M)PThAANATΔ. In the latter mutant, the N-terminal Ser was replaced by the non-Nt-acetylable Pro residue. In contrast to wild-type (M)SThAANATΔ, in naa10Δ cells, in which the (non-Nt-acetylated) (M)SThAANATΔ became strikingly short-lived, the (non-Nt-acetylable) (M)PThAANAT mutant was found to be longer-lived, in wild-type cells, than the Nt-acetylatable wild-type (M)SThAANATΔ (Fig. 4, D and E). These results indicated that the observed accelerated degradation of the wild-type human
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In wild-type S. cerevisiae, the failure to Nt-acetylate (M)SThAANAT in the absence of the NatA Nt-acetylase was not caused by the failure to Nt-acetylate (M)SThAANAT in the absence of the NatA Nt-acetylase.

We do not understand the mechanistic cause of the reproducibly observed much faster degradation of human (M)SThAANAT in wild-type S. cerevisiae (Fig. 3, C–F). One possibility, which remains to be examined, is the apparent inhibition of the yeast Hsp90 chaperone system (centered on the S. cerevisiae Hsc82/Hsp82 proteins) in naa10Δ cells. If human (M)SThAANAT expressed in wild-type S. cerevisiae is protected (from degradation) client of the Hsc82/Hsp82 system, a failure of this protection in naa10Δ mutant cells would make (M)SThAANAT vulnerable to a currently unknown pathway of degradation.

3 J.-H. Oh and A. Varshavsky, unpublished data.

FIGURE 3. Degradation assays with the wild-type human (M)SThAANAT in S. cerevisiae. A, comparison of degradation rates of the wild-type rat MLSrAANATsf and the wild-type human (M)SThAANATsf in S. cerevisiae. Immunoblotting with antibody to actin was used as a loading control. Lane 1, wild-type S. cerevisiae were transformed with vector (V) alone (control). CHX-chases were performed at 30 °C for the indicated times in wild-type S. cerevisiae with the wild-type rat MLSrAANATsf (lanes 2–5) and with the wild-type human (M)SThAANATsf (lanes 6–9). The bands of AANAT and actin are indicated on the left. B, quantification of data in A, C, CHX-chases with the wild-type human (M)SThAANATsf in wild-type S. cerevisiae (lanes 1–4) and its mutants naa10Δ (lanes 5–8), ubr1Δ (lanes 9–12), and naa10Δ ubr1Δ (lanes 13–16). The bands of human (M)SThAANATsf and actin are indicated on the left.

D, lanes 1, wild-type S. cerevisiae were transformed with vector (V) alone (control). Shown are CHX-chases with the wild-type human (M)SThAANATsf in wild-type S. cerevisiae (lanes 2–5) and its mutants naa10Δ (lanes 6–9), doa10Δ (lanes 10–13), and ubr1Δ (lanes 14–17). The bands of human (M)SThAANATsf and actin are indicated on the left. E, quantification of data in C, F, quantification of data in D. All quantified CHX-chase assays were carried out at least three times and yielded results within 10% of the data shown.
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A proteolytic pathway in question would be distinct from the Arg/N-end rule pathway because (M)SThAANAT was equally short-lived in single-mutant naa10/H9004 and double-mutant naa10/H9004 ubr1/H9004 cells (Fig. 3, C–F). In sum, the above Hsp90-based mechanism (which remains to be addressed in the context of the (M)SThAANAT protein) might underlie the strongly accelerated degradation of (M)SThAANAT in naa10/S. cerevisiae.

Degradation of Rat AANAT in Human HEK293T Cells—To compare the relative rates of degradation of N-terminal mutants of rat AANAT in human HEK293T cells, the wild-type rat MLSrAANAT3f and its mutants (M)SMLSrAANAT3f, (M)ISrAANAT3f, and (M)PLSrAANAT3f (see items i–iv at the beginning of “Results”) were expressed in these cells using transient transfection. Preliminary experiments with wild-type MLSrAANAT3f encoded by a pcDNA3-based plasmid and expressed from the full-strength PCMV promoter indicated that the levels of expression of MLSrAANAT3f were high enough to saturate or nearly saturate pathways that targeted MLSrAANAT3f for degradation in HEK293T cells. Specifically, at those (high) levels of expression, wild-type rat MLSrAANAT3f either was stable or was degraded slowly (data not shown).

We addressed this problem by constructing a set of truncated derivatives of the original PCMVM promoter, aiming to attenuate its activity. The resulting nested set of truncated promoters, denoted as PCMVM1, PCMVM2, and PCMVM3, comprised progressively shortened versions of the original PCMVM. These truncations (Fig. 5A) were similar (although not identical) to a series of PCMVM truncations described earlier by...
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A

B

C

D

FIGURE 5. Weakened derivatives of the PCMV promoter in HEK293T cells and degradation assays using antibody to Nt-acetylated MLSrAANAT. A, a simplified diagram of the "wild-type" (unmodified) PCMV promoter in the cloning vector, with schematically illustrated binding sites for the NF-κB, SP1, and CREB transcriptional regulators. Two 5'-terminal truncations of wild-type PCMV, denoted as PCMV and PCMV1, are also indicated. B, lane 1, HEK293T cells were transformed with vector (V) alone (control). CHX-chases were performed at 37°C for the indicated times in HEK293T cells with the wild-type rat MLSrAANAT3f expressed either from the unmodified PCMV promoter (lanes 2–5), from the PCMV1 promoter (lanes 7–10), or from the PCMV promoter (lanes 12–15). Lanes 6, 10, and 16, same as lanes 5, 9, and 15 except that the MG132 proteasome inhibitor was present during 8-h CHX-chases in each case (see "Experimental Procedures" and "Results"). The bands of tubulin and MLSrAANAT3f are indicated on the left. C, quantification of data in B. Symbols shown separately and framed in black squares correspond to altered (increased) levels of test proteins in the presence of MG132. All quantified CHX-chase assays were carried out at least three times and yielded results within 10% of the data shown. D, immunoblotting with affinity-purified anti-AANAT antibody to Ac-MLSHPLKEPA peptide.}

Promega Inc. (83). Each of the PCMV1, PCMV2, and PCMV3 promoter variants was cloned into pcDNA3, replacing full-length PCMV.

The wild-type rat MLSrAANAT3f was cloned into each of the above pcDNA3-based plasmids, and CHX-chases were performed in HEK293T cells transiently transfected with these constructs. Expression of MLSrAANAT3f from the PCMV1 and PCMV2 promoters reduced time-zero levels of MLSrAANAT3f (measured by immunoblotting at the beginning of CHX-chase) by ~70% and by ~97%, respectively, in comparison with the unmodified PCMV promoter (Fig. 5, A–C). With the most truncated promoter, PCMV3, no MLSrAANAT3f could be detected at the same level of immunoblotting sensitivity (data not shown). The lower levels of MLSrAANAT3f expression that have been attained through the use of the PCMV1 and PCMV2 promoters resulted in a strongly increased rate of the post-translational degradation of MLSrAANAT3f (t1/2 < 2 h versus t1/2 > 8 h when MLSrAANAT3f was expressed from the full-length PCMV promoter; Fig. 5, A–C). Treatment of HEK293T cells with the proteasome inhibitor MG132 during the chase increased the levels of MLSrAANAT3f expressed from these promoters, thereby confirming that in each case, the bulk of degradation was proteasome-dependent (Fig. 5, A–C). The pcDNA3CMV1 plasmid, which contained PCMV1, one weakened version of the PCMV1 promoter, was chosen for experiments with HEK293T cells in this study.

In agreement with the relative metabolic stabilities of AANAT proteins expressed in S. cerevisiae (Fig. 1, A and B), the mutant rat MSLSrAANAT3f bearing a deletion of the Leu residue at position 2 of wild-type MLSrAANAT3f (Fig. 2A), was the longest-lived protein in the set of rat AANAT mutants examined in HEK293T cells (t1/2 > 4 h; Fig. 6, A (lanes 12–16) and C). The wild-type rat MLSrAANAT3f and its MSLSrAANAT3f mutant (see items i and ii at the beginning of "Results") were significantly shorter-lived in HEK293T cells than MSLSrAANAT3f with t1/2 ~ 1 h and t1/2 < 1 h, respectively, versus t1/2 > 4 h (Fig. 6, A (lanes 2–11) and C).

We expected, a priori, that MSLSrAANAT3f would initiate from the first encoded Met residue, particularly because of the inclusion of the Kozak “optimal” upstream sequence in the relevant plasmids used for expression of test AANAT proteins. We could verify that expectation through chase-degradation assays using an affinity-purified antibody against the Nt-acetylated N terminus of MLSrAANAT3f (Fig. 5D). We produced and purified this antibody as described under “Experimental Procedures.” As shown in Fig. 5D, immunoblotting of SDS-PAGE-fractionated samples containing MLSrAANAT3f or MSLSrAANAT3f with the anti-Ac-MLSrAANAT antibody generated a positive signal only in samples expressing MLSrAANAT3f. If a construct expressing MSLSrAANAT3f had initiated translation (to a significant extent) from the second Met residue, the resulting translated protein would have been MLSrAANAT3f. However, no signal was detected (using the above anti-Ac-MLSrAANAT antibody) with samples containing MSLSrAANAT3f (Fig. 5D), indicating negligible levels of translation initiation at the position 2 Met.

Although the mutant MSLSrAANAT3f was partially stabilized relative to wild-type MLSrAANAT3f, the former protein...
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was still significantly shorter-lived ($t_{1/2} \approx 2$ h) than the most stable (M)S1rAANAT$_{3f}$ mutant (Fig. 6, A (lanes 17–21) and C). Although N-terminal Pro is not Nt-acetylated (59, 63) (this makes N-terminal Pro-bearing proteins invulnerable to the Ac/N-end rule pathway), the N-terminal Pro residue might still be (weakly) recognized by the Arg/N-end rule pathway in some sequence contexts. Specifically, our SPOT-type, peptide-based binding assays (84) suggested that the Ubr1 N-recognin of the...
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S. cerevisiae Arg/N-end rule pathway (and, by inference, mammalian Ub1 and Ub2 as well) may weakly bind to N-terminal Pro when this residue is followed by a hydrophobic residue. Thus, one interpretation of the “residual” instability of the rat (M)PLSrAANAT3f mutant (in comparison with the longest-lived (M)SRrAANAT3f mutant) is that the (non-Nt-acetylated) N-terminal sequence Pro-Leu of (M)PLSrAANAT3f is weakly targeted by the Arg/N-end rule pathway, in contrast to the absence of targeting of the N-terminal Ser-Ile sequence of the (M)NIrAANAT3f mutant (Fig. 6, A and C; also see Fig. 2, B and D).

As mentioned above, we found that transient transfection-based expression of AANAT from the full-strength PCMV promoter could be high enough to result in saturation or near saturation of the relevant proteolytic pathways. Although the use of the considerably weaker PCMV promoter at least partially solved this problem (see above), additional studies would be required to make sure that pathway saturation effects were consistently negligible with longer-lived AANAT derivatives that were expressed from PCMV. That said, the preponderance of our data makes it nearly certain that the observed differences in time-zero (before chase) levels of AANAT proteins were caused largely by differences in the rates of their degradation.

The Bulk of Degradation of Rat AANAT Is Mediated by Polyubiquitylation of Its Lys Residue(s)—An earlier study, in which the wild-type rat (M)SrAANAT was apparently not stabilized by conversion of its Lys residues to Arg, was based on measurements of steady-state levels of (M)SrAANAT in HEK293 cells that had been treated or left untreated with the proteasome inhibitor MG132 (37). To address this issue more directly, we generated a triple-FLAG-tagged mutant, denoted (M)SThAANAT3f, of the wild-type rat (M)SrAANAT in which all four of its Lys residues were converted to Arg. (A FLAG-based epitope tag could not be employed in these experiments, because FLAG contains a Lys residue that could potentially serve as a target for polyubiquitylation.)

CHX-chases in HEK293T cells (performed as described above) with the wild-type (M)SrAANAT3f versus its lysine-lacking (M)SrAANAT3kzero mutant indicated that (M)SrAANAT3kzero was nearly completely stable during the chase (t1⁄2 ≈ 4 h), whereas the wild-type (M)SrAANAT3f was degraded, with t1⁄2 ≈ 2 h (Fig. 6, B and D). A treatment with MG132 during CHX-chase strongly stabilized the wild-type (M)SrAANAT3f, confirming that its degradation is proteasome-dependent (Fig. 6, B (left; lanes 2 and 6) and D). In contrast, the MG132 treatment during CHX-chase of the lysine-lacking (M)SrAANAT3kzero mutant produced at most a marginal stabilizing effect, suggesting that even if there is a small (residual) amount of lysine-independent degradation of (M)SrAANAT3kzero, such a degradation would still require the proteasome (Fig. 6, B (left; lanes 7 and 11) and D).

In a different approach to the same problem, HEK293T cells were transiently transfected with the pCDNA3 vector or with plasmids expressing either (M)SrAANAT3f or (M)SrAANAT3kzero. Cells were then treated for 6 h with either MG132 or an equivalent volume of DMSO (in which the stock solution of MG132 was made). Thereafter, cell extracts were immunoprecipitated with anti-HA magnetic beads, and the immunoprecipitates were fractionated by SDS-PAGE, followed by immunoblotting with anti-HA and anti-ubiquitin antibodies. Polyubiquitin chains (apparently linked to (M)SrAANAT3f could be readily detected in fractionated immunoprecipitates of extracts from cells that expressed (M)SrAANAT3kzero (but, crucially, not from cells that expressed an empty vector), and the levels of polyubiquitin chains were significantly increased in the presence of MG132 (Fig. 6F, lanes 1–4). In contrast, significantly lower amounts of polyubiquitin chains were observed in fractionated immunoprecipitates of extracts from cells that expressed the lysine-lacking (M)SrAANAT3kzero (Fig. 6F, lane 5). Moreover, the presence of MG132 did not increase the low levels of detected polyubiquitin chains (Fig. 6F, lane 5 versus lane 6). The latter result suggested that (residual) polyubiquitin chains observed in cells that expressed (M)SrAANAT3kzero may be of a kind (e.g. see Ref. 85) that do not contribute to the proteasome-dependent degradation of (M)SrAANAT3kzero. In agreement with this interpretation, the lysine-lacking (M)SrAANAT3kzero mutant was expressed at significantly higher steady-state levels than the wild-type rat (M)SrAANAT3f in the absence of MG132 (in DMSO-treated cells) (Fig. 6E, bottom), suggesting that the absence of Lys residues stabilized (M)SrAANAT3kzero against degradation throughout life histories of (M)SrAANAT3kzero molecules, including, possibly, their degradation during or immediately after their synthesis.

Degradation of Human AANAT in Human HEK293T Cells—Degradation of the wild-type human (M)SThAANAT3f and its N-terminal Pro residue-bearing mutant (M)PThAANAT3f (see items vi and vii at the beginning of “Results”) were examined by CHX-chases in HEK293T cells. In agreement with the findings in S. cerevisiae (Figs. 2 and 3), the wild-type human (M)SThAANAT3f was longer-lived than the wild-type rat (M)SrAANAT3f in HEK293T cells (compare Fig. 7A with Fig. 6A). Interestingly, the mutant human (M)PThAANAT3f was less stable than the wild-type human (M)SThAANAT3f during the course of a 2-h chase in HEK293T cells (Fig. 7, A and C).

We also asked whether the (relatively slow) degradation of the wild-type human (M)SThAANAT3f (Fig. 7, A and C) required both its lysine-dependent ubiquitylation and the proteasome. CHX-chases were carried out with (M)SThAANAT3f, and its lysine-lacking mutant (M)PThAANAT3kzero. The latter protein was stable during the chase (t1⁄2 ≈ 4 h), whereas wild-type (M)SThAANAT3f exhibited a weak but observable instability (Fig. 7, B and D). Treatment with the MG132 proteasome inhibitor increased the level of the wild-type human (M)SThAANAT3f during CHX-chase, confirming proteasome dependence of its (slow) degradation (Fig. 7, B (lanes 3 and 7) and D).

Discussion

The ~23-kDa AANAT (also called serotonin N-acetyltransferase) is apparently universal among animals and is present in plants as well. AANAT converts the neurotransmitter serotonin to NAS, a regulatory compound in its own right and the immediate precursor of melatonin, a circulating hormone that regulates sleep and other circadian processes in vertebrates. The levels of melatonin and NAS are modulated by oscillatory circadian circuits and also impact those circuits, in addition to

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4 J.-H. Oh, B. Wadas, and A. Varshavsky, unpublished data.
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AANAT enzymes from different species are highly sequologous (71) throughout their ORFs, the sequences of the first ~10 residues of AANAT tend to differ even among relatively closely related mammals, let alone other animals (Fig. 1C). This pattern of variation suggests a blend of adaptive (function-linked, natural selection-based) changes in N-terminal sequences of AANATs versus an unknown (extent-wise) but apparently strong flux of quasi-neutral, drift-mediated changes of these N-terminal sequences on evolutionary time scales.

In the present study, we approached the proteolysis-based regulation of rat and human AANATs (Fig. 1C) by expressing them and their mutants not only in mammalian cells but also in the yeast *S. cerevisiae*. This strategy made possible the use of yeast genetics to illuminate specific proteolytic pathways involved, in parallel with experiments in mammalian cells.

We found that the wild-type rat AANAT (MLSrAANAT) is targeted for degradation by two complementary branches of the N-end rule pathway. Specifically, we showed that the Nt-acetylated Ac-MLSrAANAT is destroyed through the recognition of its Nt-acetylated N-terminal Met residue by the Ac/N-end rule pathway, whereas the non-Nt-acetylated MLSrAANAT is targeted for degradation by the Arg/N-end rule pathway, which recognizes the unacetylated N-terminal Met-Leu sequence of MLSrAANAT.

The N-terminal sequence of the wild-type human (MISThAANAT ((Met)-Ser-Thr) is different from the Met-Leu-Ser sequence of rat MLSrAANAT (Fig. 1C). We found that the wild-type human (MISThAANAT is considerably longer-lived than its rat MLSrAANAT counterpart and does not appear to be an efficacious N-end rule substrate. Together, these and related results (Figs. 2–7) indicated both a major involvement of the N-end rule pathway in the control of rodent (specifically rat) AANAT and substantial differences in the regulation of rodent and human AANATs that stem from differences in their N-terminal sequences.

The observed co-targeting of the two “complementary” forms of the rat MLSrAANAT by the two “complementary” branches of the N-end rule pathway (Figs. 2 and 3) is in agreement with the earlier finding that the N-terminal region of MLSrAANAT, and specifically its Leu-2 residue, is an important determinant of the observed metabolic instability of this AANAT (37). The cited study also suggested that either the Lys residues of MLSrAANAT or its surface-exposed Cys residues were not essential for its proteasome-mediated degradation (37). In the present work, we compared the degradation of wild-type MLSrAANAT and its lysine-lacking MLSrAANAT + Kzero mutant using CHX-chases. Our results showed that the C-terminally triple-HA-tagged MLSrAANAT + Kzero mutant was completely stabilized in human HEK293T cells, in contrast to instability of wild-type MLSrAANAT, indicating that at least some Lys residues of MLSrAANAT were required for its degradation (Fig. 6, B and D).

A possible explanation of the above discrepancy stems from different choices of C-terminal epitope tags in the earlier and the present study. Specifically, Huang et al. (37) employed MLSrAANAT C-terminally tagged with c-Myc epitope (EQKLI-SEEDL), which contains a Lys residue. In the present work, we used a triple-HA tag, which lacks Lys residues (the sequence of other effects of these compounds (see the Introduction). Hence the importance of temporal control of the AANAT enzyme.

The expression of AANAT is regulated through both transcriptional and post-translational mechanisms. Details of these mechanisms differ among vertebrates. In addition, although...
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single HA is YPYDVPDYA). Previous analyses of N-degrons have shown that the targeting apparatus of the Arg/N-end rule pathway selects a substrate’s internal lysine as a polyubiquitylation site through a process in which different (spatially competing, conformationally mobile) Lys residues can be chosen stochastically, depending on their ability to become transiently close to the substrate-bound E2-E3 complex (41, 51, 86). Thus, the presence of a Lys residue in the C-terminal c-Myc epitope of the earlier study (37) might have rendered the otherwise lysine-lacking \textit{MLSrAANAT} mutant still targetable by the N-end rule pathway. Indeed, tellingly, our initial experiments with lysine-lacking mutants of \textit{MLSrAANAT} have employed, inadvertently, a lysine-containing C-terminal FLAG tag instead of the lysine-lacking HA tag. Remarkably, the otherwise lysine-lacking but FLAG-tagged \textit{MLSrAANAT$_{Th}$} mutant was found to be unstable (Figs. 6B (right) and 7E), in contrast to the actually (completely) lysine-lacking, HA-tagged \textit{MLSrAANAT$_{Th}$} mutant (Fig. 6, B (left) and D), in agreement with the above tag-based explanation.

In addition to polyubiquitylation and degradation, AANAT is also regulated through a site-specific phosphorylation, a modification that stimulates the binding of AANAT to at least one of the 14-3-3 chaperone-like proteins (they are encoded by a family of several 14-3-3 genes in mammals). This interaction with 14-3-3 both protects (phosphorylated) AANAT from degradation and enhances its catalytic activity. Rat, human, and other AANATs contain two previously mapped phosphorylation sites, Thr-29 and Ser-203 (position numbers refer to the sequence of rat \textit{MLSrAANAT}). Phosphorylation at Thr-29 is conserved in all examined mammalian AANATs (31). This phosphorylation, by PKA and/or by PKC, augments the interaction between AANAT and 14-3-3 both \textit{in vitro} and \textit{in vivo} (1, 5, 37). By expressing AANATs not only in mammalian cells but also in the heterologous setting of \textit{S. cerevisiae}, we could analyze the degradation of AANATs in the possible absence of phosphorylation at Thr-29 (or its equivalent) and also in the possible absence of AANAT interactions with 14-3-3 proteins. Whereas \textit{S. cerevisiae} has two 14-3-3 proteins (87), there is no evidence, so far, that bears on their binding, \textit{in vivo}, to a mammalian AANAT.

Our attempts to observe a phosphorylation-induced \textit{in vivo} binding of the wild-type rat \textit{MLSrAANAT} either to an endogenous yeast 14-3-3 protein or to the (overexpressed) human 14-3-3$_{\lambda}$ protein were unsuccessful so far, despite a variety of tried approaches.\textsuperscript{5} Nevertheless, the likely conceptual benefit of achieving a phosphorylation-inducible interaction between a mammalian AANAT and a specific 14-3-3 protein, in a setting of greatly reduced complexity of 14-3-3s (in comparison with the large set of their mammalian counterparts), justifies further work in this direction. 14-3-3 proteins are abundant, broadly expressed, multifunctional chaperone-like proteins that modulate catalytic activities of enzymes to which 14-3-3s bind and also regulate protein-protein interactions and subcellular targeting of specific proteins (88–90). It is possible, indeed likely, that 14-3-3 proteins may bind, in a regulated manner, not only to, for example, rat \textit{MLSrAANAT} but to other N-end rule substrates as well, thereby controlling the rates of degradation of such substrates in ways that remain to be explored.

Recently, the naturally occurring mutant human protein RGS2$^{Q2L}$ was shown to be a conditionally short-lived substrate of both the Ac/N-end rule pathway and the Arg/N-end rule pathway (60). This clinically relevant mutant of RGS2 (a negative regulator of specific G proteins) was identified in a cohort of hypertensive Japanese patients (91) (also see the Introduction). In the present study, we demonstrated that the otherwise unrelated rat \textit{MLSrAANAT} protein, whose N-terminal Met-Leu sequence is identical to that of RGS2$^{Q2L}$, is also targeted for degradation by both branches of the N-end rule pathway (Figs. 1 and 2). These results further expanded the set of identified mammalian N-end rule substrates of the Met-$\Phi$ type.

Interestingly, RGS2, an N-end rule substrate (60), has been identified as a physiologically relevant inhibitor of the AANAT-dependent melatonin production (92). Light-stimulated, noradrenephrine-induced increases in the levels and activity of AANAT are mediated by G-protein-coupled adrenergic receptors, whose activation increases intracellular cAMP, and subsequently the levels of AANAT as well, at least in part through an increased phosphorylation of AANAT and the resulting induction of its binding to protective 14-3-3 proteins (5, 28). While acting to increase the levels and activity of AANAT (which is required for the synthesis of NAS and melatonin), cAMP also acts as a negative feedback inhibitor of melatonin production through up-regulation of Rgs2 transcription. The resulting increase in RGS2 inhibits the activity of G proteins that are coupled to the noradrenergic-responsive receptor, a negative feedback circuit that acts to decrease cAMP levels (92).

AANAT results of the present study, together with the earlier evidence that RGS2 is both a conditionally short-lived N-end rule substrate and an (indirect) down-regulator of AANAT (5, 28) and (indirect) down-regulator of AANAT (60, 5, 92), indicate that the N-end rule pathway is a pleiotropic controller of circuits that either directly or indirectly modulate AANAT.

Previous studies of AANAT demonstrated that its transcriptional regulation can be different in different species, but it has been conjectured that mechanisms and regulation of AANAT degradation would be more conserved in evolution (11, 34–36). As shown in the present study, the rates of degradation of the human and rat AANAT proteins are quite different. Moreover, whereas rat \textit{MLSrAANAT} is destroyed by the N-end rule pathway (Figs. 2 and 6), human \textit{MSThAANAT} is substantially resistant to this pathway (Figs. 3 and 7). Nevertheless, if the identification of RGS2 as a feedback inhibitor of AANAT expression in the rat pineal gland (92) could also be shown to extend to human RGS2, the N-end rule pathway would be a regulator of AANAT in humans as well, albeit an indirect one.

An earlier study of human \textit{MSThAANAT} described its apparent degradation by the proteasome but did not detect its ubiquitylation (33). In that study, the instability of human \textit{MSThAANAT} was inferred from increases in its steady-state levels in the presence of a proteasome inhibitor. In the present work, we employed, in particular, CHX-chases to show that although human \textit{MSThAANAT} is degraded by the proteasome, it is substantially longer-lived than rat \textit{MLSrAANAT}.

\textsuperscript{5} B. Wadas and A. Varshavsky, unpublished data.
under the same conditions, either in mammalian cells or in *S. cerevisiae* (Figs. 2–7).

In sum, our results indicate both a major involvement of the N-end rule pathway in the control of rodent AANATs and substantial differences in the regulation of rodent and human AANATs that stem from differences in their N-terminal regions (Fig. 1C). What is the cause of the remarkable variability of N-terminal sequences of AANATs during mammalian (and, more generally, animal) evolution? Given the extent of variability (Fig. 1C), it is nearly certain that a substantial fraction of these changes resulted from a quasi-neutral, unselected genetic drift (93). At the same time, the broad and biologically relevant variation in detailed circadian rhythms among different animal species (due to specific ecological and physiological adaptations) and the role of AANAT in these rhythms suggest that the seeming randomness of the N-terminal sequences of AANATs (Fig. 1C) may be obscuring, so far, a set of adaptive (selected) changes in these sequences over evolutionary time scales.

### Experimental Procedures

**Yeast Strains, Media, and Genetic Techniques**—Standard yeast genetic techniques were used (94–96). *S. cerevisiae* strain BY297 was constructed by transforming the strain CHY345 (ubr1Δ::LEU2 in the strain background of BY4742) with a PCR-amplified DNA fragment that encoded the selection marker KanMX6 (making cells resistant to kanamycin) and was targeted to the 5′- and 3′-flanking regions of the *NAA10* gene, thereby replacing the ORF of *NAA10* with KanMX6 (Table 1). *S. cerevisiae* strain JOY487 was made by transforming BY4742 with a PCR-amplified DNA fragment that encoded the natNT2 marker (making cells resistant to nourseothricin) and was targeted to the 5′- and 3′-flanking regions of *PDR5*, thereby replacing the ORF of *PDR5* with natNT2 (Table 1). The resulting *pdr5Δ* cells were used in experiments that involved the MG132 proteasome inhibitor (Fig. 4, A and B). Other *S. cerevisiae* strains used in this study were constructed previously and are cited in Table 1. *S. cerevisiae* were transformed using the LiAc/PEG method (97). *S. cerevisiae* media included YPD medium, synthetic complete medium, and synthetic drop-out medium (95, 96).

**Plasmids, cDNAs, and Primers**—Turbo *Escherichia coli* (New England Biolabs) was used for cloning and maintaining plasmids. Phusion High-Fidelity DNA polymerase (New England Biolabs) was used for carrying out PCR. Nucleotide sequences of all plasmids were verified by DNA sequencing. The plasmids and PCR primers used in this study are described in Tables 2 and 3, respectively.

| Strains       | Relevant genotypes | Source or reference |
|---------------|--------------------|---------------------|
| BY4742        | *MATa his3-1 leu2-0 lys2-0 can1-100* | Open Biosystems     |
| BY15740       | *mga20::kanMX6*    | Open Biosystems     |
| BY10976       | *mga70::kanMX6*    | Open Biosystems     |
| BY17299       | *doa10::kanMX6*    | Open Biosystems     |
| CHY345        | *ubr1Δ::LEU2*     | Ref. 92             |
| CHY346        | *ubr1Δ::LEU2 doa10::kanMX6* | BY4742 This study |
| BWY29         | *mga30::kanMX6*    | This study          |
| CHY349        | *ubr1Δ::LEU2*     | Ref. 40             |
| JOY887        | *mga30::natNT2*   | This study          |

The low copy pbw105, pbw106, pbw107, and pbw209 plasmids expressed the wild-type rat *MLsrAANAT* (wild-type rat AANAT bearing the N-terminal sequence Met-Leu-Ser and C-terminally tagged with a triple-FLAG epitope) and its N-terminal mutants (MLsrAANAT*Δp*) and (MLsrAANAT*Δpp*) respectively, from the *P GAL1* promoter. To construct these plasmids, the rat Aanat ORF was amplified from pCISII-RAANAT (98), using primers BW2394, BW240, BW241, BW412, BW243, and BW244 (Table 3). The resulting PCR products were digested with EcoRI and HindIII and cloned in EcoRI/HindIII-cut pRS416GAL1.

The low copy pbw113 and pbw394 plasmids expressed the wild-type rat *MLsrAANAT* C-terminally tagged with the triple-FLAG epitope (MLsrAANAT*Δha*) and its Lys-8 → Arg mutant (MLsrAANAT*ΔhaR*) respectively, from the *P CLUP1* promoter. To construct these plasmids, the rat Aanat ORF was amplified from pCISII-RAANAT (98) using primers BW339, BW704, BW338, BW339, and BW340 (Table 3). The amplified DNA fragments were digested with EcoRI and HindIII and were ligated into EcoRI/HindIII-cut pRS313_CUP1 (59, 99).

The low copy pbw395 and pbw419 plasmids expressed the wild-type human (MLsrAANAT*Δh*) C-terminally tagged with a triple-FLAG epitope (MLsrAANAT*Δhha*) and its N-terminal mutant (MLsrAANAT*Δhpa*) respectively, from the *P GAL1* promoter. To construct these plasmids, the human *Aanat* ORF from Genscript clone OHu55705 (Genscript, Piscataway, NJ) was amplified using primers BW705, BW711, BW712, and BW244 or primers BW771, BW711, BW712, and BW244, respectively (Table 3). The PCR-amplified DNA fragments were digested with EcoRI and HindIII and were ligated into EcoRI/HindIII-cut pRS416GAL1 (99). All final DNA constructs were verified by DNA sequencing. See items i–x at the beginning of “Results” for information about rat and human AANAT notations.

For expression of the above AANAT-encoding ORFs in human HEK293T cells, these ORFs were cloned into a modified expression vector derived from pcDNA3. That (modified) vector, termed pcDNA3*CMVt1*, contained a truncated (weakened) version of the *P CMV* promoter, termed *P CMVt1*. The pcDNA3*CMVt1* plasmid was constructed by amplifying, using PCR, the 276-bp fragment that encompassed 104 bp at the 5′-end of the *P CMV* promoter region in pcDNA3, its T7 promoter site, and its multiple cloning site, using primers BW375 and BW376 (Table 3). The amplified DNA fragment was digested with NruI and XbaI and ligated into NruI/XbaI-cut pcDNA3, yielding the plasmid pbw173 (Table 2). Its truncated *P CMVt1* promoter retained several proximal promoter elements, including the consensus NFκB site, the CREB-binding site, and two SP1/SP3-binding sites, but lacked more distal enhancer elements. This resulted in a ~70% lower activity of the *P CMVt1* promoter in HEK293T cells, in comparison with *P CMV* (Fig. 5, A–C).

The pcDNA3*CMVt1*-based plasmids pbw206, pbw211, pbw212, and pbw213 expressed, in HEK293T cells, either the wild-type rat *MLsrAANAT* or its N-terminal mutants (MLsrAANAT*Δp*) and (MLsrAANAT*Δpp*) and (MLsrAANAT*Δhha*) from the *P CMVt1* promoter. To construct these plasmids, Aanat ORFs were amplified from the plasmids pbw105, pbw106, pbw107, and pbw209 plasmids. The amplified DNA fragments were digested with EcoRI and HindIII and were ligated into EcoRI/HindIII-cut pRS416GAL1.
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| Table 2 | Plasmids used in this study |
|----------|-----------------------------|
| Plasmid  | Description                  | Source or reference |
| pcDNA3   | rAANAT in pcDNA3            | Invitrogen          |
| pCiSH-rAANAT | pRS313 containing the P<sub>CMV</sub> promoter | Varshavsky laboratory collection |
| pRS313Cup1 | pRS313 containing the P<sub>Gal1</sub> promoter | Varshavsky laboratory collection |
| pRS416Gal1 | rAANAT<sub>3ha</sub> in pRS416 with P<sub>Gal1</sub> | Varshavsky laboratory collection |
| pBW105   | rAANAT<sub>3f</sub> in pRS416 with P<sub>GAL1</sub> | This study          |
| pBW106   | rAANAT<sub>3f</sub> in pRS416 with P<sub>GAL1</sub> | This study          |
| pBW107   | rAANAT<sub>3f</sub> in pRS416 with P<sub>GAL1</sub> | This study          |
| pBW135   | rAANAT<sub>3f</sub> in pRS416 with P<sub>GAL1</sub> | This study          |
| pBW173   | pcDNA3 (P<sub>CMV</sub>5/1)  | This study          |
| pBW206   | pcDNA3 (P<sub>CMV</sub>5/1)  | This study          |
| pBW207   | pcDNA3 (P<sub>CMV</sub>5/1)  | This study          |
| pBW394   | pcDNA3 (P<sub>CMV</sub>5/1)  | This study          |
| pBW395   | pcDNA3 (P<sub>CMV</sub>5/1)  | This study          |
| pBW419   | pcDNA3 (P<sub>CMV</sub>5/1)  | This study          |
| pBW466   | pcDNA3 (P<sub>CMV</sub>5/1)  | This study          |
| pBW467   | pcDNA3 (P<sub>CMV</sub>5/1)  | This study          |
| pBW477   | pcDNA3 (P<sub>CMV</sub>5/1)  | This study          |
| pBW478   | pcDNA3 (P<sub>CMV</sub>5/1)  | This study          |
| pBW481   | pcDNA3 (P<sub>CMV</sub>5/1)  | This study          |
| pBW482   | pcDNA3 (P<sub>CMV</sub>5/1)  | This study          |

Table 3 - PCR primers used in this study

| Primer | Sequence |
|--------|----------|
| BW239  | 5'-CCCTGAATTCACCACTGACCCTCA-3' |
| BW240  | 5'-CCCTGAATTCACCACTGACCCTCA-3' |
| BW241  | 5'-CCCTGAATTCACCACTGACCCTCA-3' |
| BW243  | 5'-CTCTGGATCAAGGCTACACCC-3'   |
| BW244  | 5'-CTCTGGATCAAGGCTACACCC-3'   |
| BW335  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW336  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW337  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW338  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW339  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW340  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW341  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW342  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW343  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW344  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW345  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW346  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW347  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW348  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW349  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW350  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW351  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW352  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW353  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW354  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW355  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW356  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW357  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW358  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW359  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW360  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW361  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW362  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW363  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW364  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW365  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW366  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW367  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW368  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW369  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW370  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW371  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW372  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW373  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW374  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW375  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW376  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW377  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW378  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW379  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW380  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW381  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW382  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW383  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW384  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW385  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW386  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW387  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW388  | 5'-TGGAATCTACGACCATCACCTCACC-3' |
| BW389  | 5'-TGGAATCTACGACCATCACCTCACC-3' |

pBW107, and pBW209 using primers BW352, BW353, BW354, BW413, and BW355, respectively (Table 3). The amplified DNA fragments were digested with EcoRI and XhoI and ligated in EcoRI/XhoI-cut pBW173 (Table 2). All pcDNA3-based constructs designed for expression in HEK293T cells contained the Kozak sequence 5'-ACC immediately upstream of the start codon.

pBW477, which expressed the MLS<sub>3</sub>AANAT<sub>3ha</sub> from the P<sub>CMV</sub>5/1 promoter, was constructed by using pBW135 as a PCR template and the primers BW352/BW340. The resulting PCR-amplified DNA fragment was digested with EcoRI and XhoI and ligated into EcoRI/XhoI-cut pBW173 (Table 2). pBW478 expressed MLS<sub>3</sub>AANAT<sub>3ha</sub> (i.e. the lysine-lacking derivative of MLS<sub>3</sub>AANAT<sub>3ha</sub>). It was constructed by using pBW135 as a PCR template to generate four separate DNA fragments containing lysine-to-arginine mutations. The primer pairs were 704/784, 785/786, 787/788, and 789/340 (Table 3). These fragments were then assembled into a contiguous ORF using PCR. The resulting DNA fragment, encoding lysine-to-arginine mutations at each of the four lysines contained in the wild-type rat MLS<sub>3</sub>AANAT<sub>3f</sub> was digested with EcoRI and XhoI, and ligated into EcoRI/XhoI-cut pBW173.

pBW466 and pBW467, the pcDNA3-based counterparts of pBW395 and pBW419 (Table 2) were generated by PCR using
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Cycloheximide Chase Assay in Mammalian Cell Lines—HEK293T cells were transfected with 4 μg of plasmid DNA per well (10-cm² surface area) in 6-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. 24 h after transfection, cells were treated with CHX (0.1 mg/ml) to initiate a chase. All cells from one well were collected at each indicated time point by washing cells quickly in ice-cold PBS and then rapidly scraping cells into 1.5-ml tubes, pelleting them by centrifugation at 4 °C (at 10,000 × g for 1 min), and snap-freezing the pellets in liquid nitrogen. Cells in these samples were later lysed by the addition of 0.2 ml of “mammalian lysis buffer” (1% Nonidet P-40, 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5) containing 1× Roche Complete Protease Inhibitor Mixture and brief sonication, followed by centrifugation at 11,200 × g for 10 min. Total protein concentration in the supernatants was measured by the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). 30 μg of total protein in a thus prepared extract in lithium dodecyl sulfate (LDS)-sample buffer (in a volume of 45 μl) were heated at 70 °C for 10 min, followed by LDS-PAGE on a 4–12% BisTris NuPAGE gel (Invitrogen) and subsequent transfer onto nitrocellulose membranes for immunoblotting.

Immunoblotting—Following electrophoresis, proteins separated by LDS-PAGE or SDS-PAGE, as indicated above, were electroblotted on nitrocellulose membranes by iBlot (Invitrogen; Program 3; 7-min transfer). Membranes were blocked and thereafter incubated with the appropriate primary antibody, followed by LI-COR IRDye-conjugated secondary antibodies. IRDye fluorescence was detected using an Odyssey 9120 system (LI-COR, Lincoln, NE), facilitating quantification of immunoblots. All quantification was done on LI-COR Odyssey software. Antibodies used include anti-FLAG mouse monoclonal antibody (clone M2; 1:2,000 dilution; Sigma-Aldrich), anti-α-tubulin mouse monoclonal antibody (clone B-5-1-2; 1:1,000 dilution; Sigma-Aldrich), anti-HA mouse monoclonal antibody (clone HA-7; 1:2,000 dilution; Sigma-Aldrich), anti-β-actin mouse monoclonal antibody Ab8224 (1:4,000 dilution; Abcam, Cambridge, UK), and anti-14-3-3ζ rabbit monoclonal antibody (1:4,000 dilution; Abcam), and anti-14-3-3ζ rabbit monoclonal antibody EPR6379 (1:100,000 dilution; Abcam).

Immunoprecipitation of Polyubiquitylated AANAT—HEK293T cells were transfected with 4 μg of plasmid DNA per well (10-cm² surface area) in 6-well plates using Lipofectamine-2000 (Invitrogen) according to the manufacturer’s protocol. 24 h after transfection, cells were treated with the proteasome inhibitor MG132 (final concentration of 10 μM; AG Scientific, San Diego, CA) or with an equivalent volume of DMSO in which the 10 mM stock solution of MG132 was made, as indicated. 6 h post-treatment, cells were collected by washing them quickly on a plate in ice-cold PBS and then rapidly scraping cells into 1.5-ml tubes and pelleting them by centrifugation at 4 °C at 10,000 × g for 1 min. Pellets were resuspended in 0.1 ml of buffer containing 1% SDS and 50 mM Tris (pH 7.5) and heated at 95 °C for 10 min. After boiling, samples were diluted with 1 ml of buffer TNN (0.5% Nonidet P-40, 0.25 M NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5) containing 1× Roche Complete Protease Inhibitor Mixture, 20 mM N-ethylmaleimide (Sigma-Aldrich), and 50 mM PR-619 (LifeSensors, Malvern, PA) as inhibitors of deubiquitylation. 25 μl of anti-HA magnetic beads

pBW395 and pBW419 as templates and the primers BW866/BW355 and BW868/BW355, respectively (Table 3). PCR-amplified DNA fragments were digested with EcoRI and XhoI and ligated into EcoRI/XhoI-cut pBW173 (Table 2).

Cell Culture—The HEK293T cell line (derived from human embryonic kidney cells) was obtained from American Type Culture Collection (ATCC, Manassas, VA) and was grown at 37 °C in 5% CO₂ in DMEM supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, CA) and penicillin/streptomycin (100 units/ml; HyClone). Cells were transfected using Lipofectamine-2000 (Invitrogen) according to the manufacturer’s protocol.

Antibody Specific for the Nt-acetylated Form of Rat AANAT (MLSrAANAT)—Unpurified rabbit polyclonal antiserum against the synthetic peptide Ac-MLSIHPLKPEAC were produced by Abgent (San Diego, CA) essentially as described for other peptide-mediated polyclonal antibodies produced in studies by the Varshavsky laboratory (e.g. see Refs. 59 and 66). The sequence of the Nt-acetylated peptide immunogen comprised the first 11 residues of MLSrAANAT (Fig. 1C; see below), followed by the C-terminal Cys residue that was used to conjugate the peptide to keyhole limpet hemocyanin carrier protein. The antiserum was affinity-purified in two sequential steps. First, the antibody sample was incubated with the C-terminally immobilized original immunogen Ac-MLSIHPLKPEAC. The peptide-bound fraction was eluted as described previously (59, 66). That fraction was thereafter “negatively” purified against the otherwise identical but non-Nt-acetylated immobilized MLSIHPKPEAC peptide (the peptides were immobilized using a sulphydryl-coupling resin (Abgent)), collecting, this time, the unbound fraction. The resulting antibody sample was used to detect, selectively, the Nt-acetylated form of rat AANAT (see “Results” and Fig. 5D). The fraction of once purified antibody that was bound to the non-Nt-acetylated MLSIHPKPEAC peptide was also eluted and retained and was used as an antibody that could recognize the N-terminal region of rat AANAT irrespective of its Nt-acetylation state.

Cycloheximide Chase Assay in S. cerevisiae—CHX-chase assays in S. cerevisiae were performed largely as described (39, 59). Briefly, S. cerevisiae cells were grown in overnight cultures, and expression of epitope-tagged test proteins was induced by the addition of 2% galactose (for plasmids containing the P₆₅L₆₅₁ promoter) or 0.1 mM CuSO₄ (for plasmids containing the P₆₅L₆₅₁ promoter (100)). 3 h post-induction, CHX was added to a final concentration of 0.1 mg/ml. Samples were collected at the indicated time points, centrifuged at 11,200 × g for 2 min to pellet the cells, and snap-frozen in liquid nitrogen. Proteins were extracted by resuspending the pellets in 1 ml of 0.2 M NaOH and incubating on ice for 20 min (101). Cells were then pelleted and resuspended in 50 μl of SUMEB loading buffer (1% SDS, 8 M urea, 10 mM EDTA, 0.01% bromphenol blue, 10 mM MOPS, pH 6.8), followed by heating at 95 °C for 10 min. Samples were then centrifuged at 11,200 × g for 5 min, followed by SDS-PAGE (with 10 μl loaded per well) using 4–12% NuPAGE gels (Invitrogen). Fractionated proteins were transferred onto nitrocellulose membranes for immunoblotting analyses (see below).
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were then added to each sample, and samples were incubated at 4 °C overnight, followed by three washes in TNN, 1 wash in 10 mM Tris–HCl, pH 8.5, and elution with 50 μl of 0.1 M glycine, pH 2.0, by incubation at room temperature for 10 min. After elution, the samples were neutralized and then heated at 70 °C in LDS-PAGE sample buffer, followed by LDS-PAGE on a 4–12% NuPAGE gel. Fractionated proteins were then electroblotted onto nitrocellulose membranes. The resulting membranes (containing transferred proteins) were autoclaved as described previously (102) before “downstream” immunoblotting procedures (containing transferred proteins) were autoclaved as described previously (102) before “downstream” immunoblotting procedures with either anti-HA or anti-ubiquitin antibodies to increase the sensitivity of detection of polyubiquitin chains.

Author Contributions—B. W., A. V., J. B., Z. H., J.-H. O., and C.-S. H. designed the experiments. B. W. performed the experiments, with participation by J.-H. O. B. W., A. V., and J. B. wrote the paper. All authors discussed the results and commented on the manuscript.

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