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6 hpa; (v) functional studies showing that the Y148S mutation leads to loss of function; (vi) DNA microarray data demonstrating disrupted Fgf signaling in *dob* (fig. S4A and table S2); and (vii) insensitivity of *dob* embryos to wild-type *fgf20a* mRNA overexpression (table S1). Thus, the early regenerative defect observed in *dob* results from Fgf20a dysfunction.

We have genetically identified a specific growth factor, Fgf20a, that is essential for initiating fin regeneration, regeneration epithelialization, and blastema formation. These findings provide a genetic foothold on the early signaling events of regeneration that will enable further identification of key regeneration genes. This information will broaden our understanding of regenerative mechanisms and may enable regenerative medicine.

Protein Synthesis upon Acute Nutrient Restriction Relies on Proteasome Function

Ramunas M. Vabulas* and F. Ulrich Hartl*

The mechanisms that protect mammalian cells against amino acid deprivation are only partially understood. We found that during an acute decrease in external amino acid supply, before up-regulation of the autophagosomal-lysosomal pathway, efficient translation was ensured by proteasomal protein degradation. Amino acids for the synthesis of new proteins were supplied by the degradation of preexisting proteins, whereas nascent and newly formed polypeptides remained largely protected from proteolysis. Proteasome inhibition during nutrient deprivation caused rapid amino acid depletion and marked impairment of translation. Thus, the proteasome plays a crucial role in cell survival after acute disruption of amino acid supply.

Protein biosynthesis in mammalian cells relies on the continuous uptake of essential amino acids from the environment. Acute amino acid restriction can occur in several physiological and pathophysiological conditions, such as after disruption of the trans-placental nutrient supply in neonates or during organ ischemia. Up-regulation of the autophagosomal-lysosomal pathway is known to provide free amino acids for protein synthesis under these nutrient stress conditions through the bulk degradation of cytoplasmic proteins and organelles (1, 2). However, this adaptation requires hours to become fully effective (2, 3), suggesting the existence of constitutive mechanisms that protect cells during short-term fluctuations in amino acid supply. Moreover, certain organs, such as the brain, are inefficient in up-regulating autophag-

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Materials and Methods

SOM Text

Figs. S1 to S4

Tables S1 and S2

References

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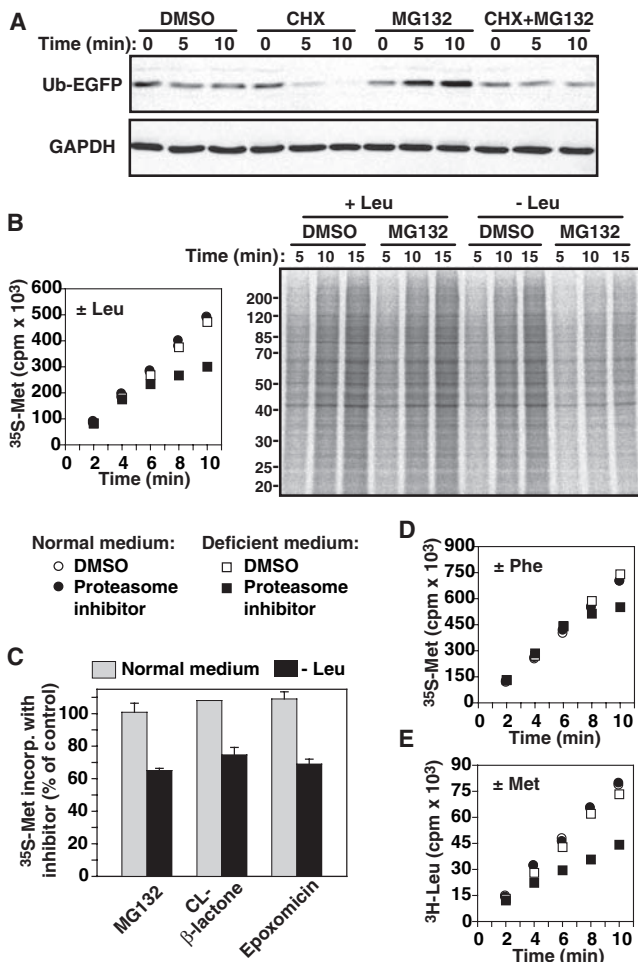
EGFP accumulated to a low steady-state level in transiently transfected cells, reflecting the equilibrium between its synthesis and degradation. As expected, upon inhibition of protein synthesis with cycloheximide (CHX), Ub-EGFP was degraded within minutes (Fig. 1A). In contrast, the addition of the proteasome inhibitor MG132 caused the virtually immediate accumulation of Ub-EGFP (Fig. 1A). To determine whether proteasome inhibition was complete, we analyzed the combined effect of MG132 and CHX. Inhibition of translation by CHX is known to be very rapid and efficient (10, 11). Thus, if MG132 were to block proteasome function only partially, the arrest of translation would lead to a decrease in Ub-EGFP level due to degradation. The simultaneous addition of CHX and MG132 instantaneously stabilized the Ub-EGFP reporter (Fig. 1A). Similar observations were made with the proteasome inhibitors clasto-lactacystin- β -lactone and epoxomicin (11). Thus, under the conditions chosen proteasome inhibition was immediate and essentially complete.

The effect of proteasome inhibition on translation was analyzed under conditions of acute amino acid restriction. The concentrations of the essential amino acids—leucine, phenylalanine, or methionine—were maintained in the range of normal adult plasma levels (12) or were reduced individually 100-fold to create insufficiency in external supply (13). Newly synthesized proteins were labeled with ^{35}S -methionine (^{35}S -Met), followed by cell lysis in SDS and precipitation of proteins with trichloroacetic acid (TCA). Proteasome inhibition with MG132, clasto-lactacystin- β -lactone or epoxomicin markedly impaired translation within 5 to 10 min, but only when cells were incubated in medium deficient in at least one essential amino acid (leucine or phenylalanine) (Fig. 1, B to D, and fig. S1, A and B). Similar results were obtained when cells were incubated in methionine-deficient medium with ^3H -leucine (^3H -Leu) as the tracer

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Fig. 1. Proteasome activity is required to sustain protein synthesis upon amino acid restriction. (A) Amounts of Ub-EGFP reporter protein were analyzed in transiently transfected HeLa cells at 0, 5, and 10 min after addition of dimethyl sulfoxide (DMSO) or 5 mM CHX, 100 μ M MG132 in DMSO, or CHX and MG132 combined by anti-GFP immunoblotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (B) Translation was followed by measuring the incorporation of 35 S-Met into TCA-insoluble material (left) or analyzing newly synthesized proteins by 10% SDS-PAGE and phosphorimaging (right). At time 0, 50 μ Ci/ml 35 S-Met and MG132 were added. Circles, normal medium (80 μ M Leu); squares, deficient medium (0.8 μ M Leu). Solid symbols, MG132 addition; open symbols, DMSO controls. (C) Translation was analyzed as in (B). In addition to MG132, 40 μ M clasto-lactacystin- β -lactone (CL- β -lactone) or 40 μ M epoxomicin were used, and translation was observed for 15 min (fig. S1, A and B). Protein synthesis is expressed in percentage of controls lacking inhibitor. Means \pm SD of three independent experiments are shown. Gray bars, normal medium; black bars, Leu-deficient medium. (D) Same as (B), except that Phe-sufficient (40 μ M) and Phe-deficient (0.4 μ M) media were used. (E) Same as (B), except that the amount of Met was varied from 20 μ M (normal medium) to 0.2 μ M (deficient medium) and labeling was with 100 μ Ci/ml 3 H-Leu. Representative results of at least three independent experiments are shown.



(Fig. 1E), except that proteasome inhibition affected translation earlier. These observations were reproduced in human embryonic kidney 293T cells (fig. S1C). Thus, the proteasome had a critical role in buffering the sudden disruption of the external amino acid supply, allowing translation to proceed normally.

The use of the specific proteasome inhibitors clasto-lactacystin- β -lactone and epoxomicin (14) (Fig. 1C and fig. S1, A and B) excluded an inhibition of lysosomal proteolysis as the cause of the observed reduction in translation. Moreover, translation was unimpaired when cells deficient in amino acids were treated with lysosomal inhibitors bafilomycin A or chloroquine (15, 16) (fig. S2A). In contrast, bestatin methyl ester, an inhibitor of the aminopeptidase hydrolyzing di- and tripeptides downstream of the proteasome (17, 18), caused a substantial inhibition of translation (fig. S2B), similar to proteasome inhibition. These results corroborate the critical role of the proteasome in sustaining translation upon acute amino acid restriction. In contrast, prolonged amino acid

starvation for several hours should induce the autophagosomal-lysosomal system (3), and this could reduce the dependence of protein synthesis on proteasome activity. Indeed, after 6 hours of amino acid starvation, the effect of proteasome inhibition on translation was substantially reduced (Fig. 2, A and B), suggesting that at this time lysosomal protein degradation contributed increasingly to providing amino acids. This adaptation was not seen when cells were treated with 3-methyladenine (3-MA), which prevents the formation of autophagosomes (19, 20). In cells treated with 3-MA, the addition of a proteasome inhibitor after 6 hours of prestarvation caused a reduction in translation similar to that in cells without prestarvation (Fig. 2C).

Lack of intracellular amino acids results in the accumulation of uncharged tRNAs and leads to the formation of active, phosphorylated general control nonderepressible 2 (GCN2) kinase (21, 22). As detected by a phospho-GCN2 antibody, GCN2 was activated upon shifting cells to leucine-deficient medium, but only when

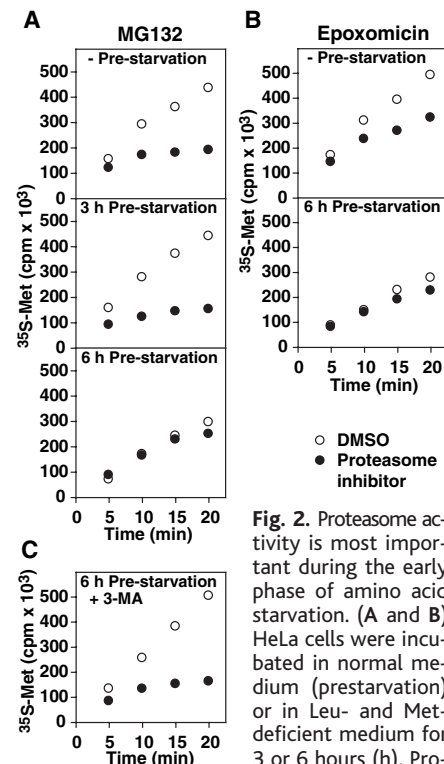


Fig. 2. Proteasome activity is most important during the early phase of amino acid starvation. (A and B) HeLa cells were incubated in normal medium (prestarvation) or in Leu- and Met-deficient medium for 3 or 6 hours (h). Protein translation was then analyzed in Leu-deficient medium by adding 50 μ M Ci/ml 35 S-Met with either DMSO (open symbols), MG132 (A), or epoxomicin (B) (solid symbols) (13). (C) Same as (A), except that 10 mM 3-MA was added at the beginning of prestarvation. DMSO (open symbols) or MG132 (solid symbols) was added during labeling. Representative results of at least three independent experiments are shown. cpm, counts per minute.

the proteasome was simultaneously inhibited (Fig. 3A). This is indicative of a physiologically relevant depletion of the intracellular leucine pool under these conditions. Efficient translation resumed rapidly upon re-addition of the lacking amino acid, even when the block of proteasome function was maintained (Fig. 3B). Amino acid analysis demonstrated directly that the proteasome supplied building blocks for protein synthesis. The addition of translation inhibitor (CHX) to cells growing in normal medium resulted in a measurable increase in intracellular leucine within 10 min (table S1). This effect was less pronounced in the presence of the proteasome inhibitor. In leucine-deficient medium, intracellular leucine was only detectable upon addition of CHX when the proteasome was not inhibited (table S1), indicating that combined amino acid deficiency and proteasome inhibition severely depleted the intracellular amino acid pool.

At least 30% of newly synthesized proteins are thought to be degraded by the proteasome during and immediately after translation, presumably reflecting a general inefficiency of protein biosynthesis and folding (7). How-

Fig. 3. Immediate cellular effects of amino acid restriction and proteasome inhibition. (A) Activation of GCN2 kinase in HeLa cells by reducing the medium concentration of Leu from 80 to 0.8 μ M and simultaneous proteasome inhibition. Activated GCN2 kinase (pGCN2) was detected by immunoblotting with an antibody to pGCN2. Asterisk, nonspecific band. Equal loading was confirmed with antibodies detecting GCN2 independent of its phosphorylation (GCN2). At time 0, 100 μ M MG132 or DMSO was added. (B) Translation was analyzed in Leu-deficient medium (–Leu) or Phe-deficient medium (–Phe). Incorporation of 35 S-Met into TCA-precipitable material was measured. 50 μ Ci/ml 35 S-Met was added either together with MG132 (solid symbols) or with DMSO (open symbols). After 10 min (dashed line), the respective lacking amino acid (triangles) or control amino acid (circles) (Phe in case of –Leu medium, Leu in case of –Phe medium) was added to normal concentration. aa, amino acid. Representative results of at least three independent experiments are shown.

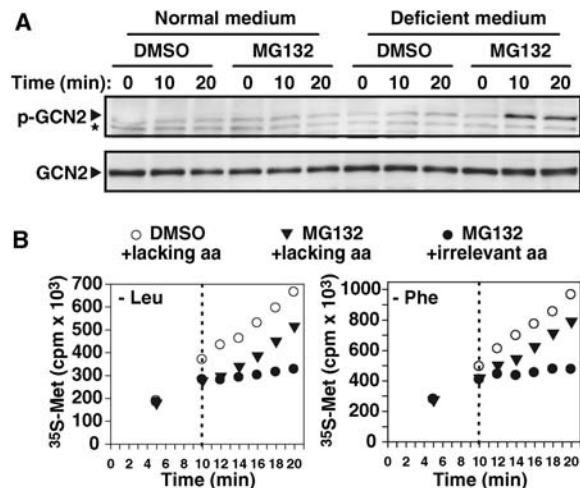
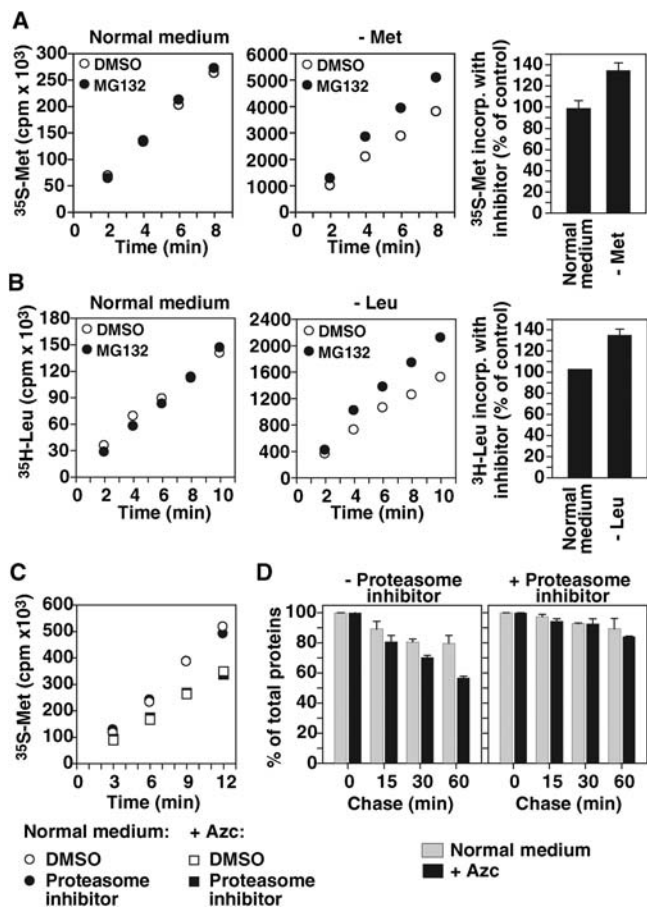


Fig. 4. Effect of proteasome activity on radiolabeling of newly synthesized proteins and degradation of faulty proteins. (A and B) Newly synthesized proteins in HeLa cells were labeled with 50 μ Ci/ml 35 S-Met in Met-deficient medium (A), or with 100 μ Ci/ml 3 H-Leu in Leu-deficient medium (B). Radioactivity of TCA-insoluble material was measured. MG132 (solid symbols) or DMSO (open symbols) were added with radioactive tracers at time 0. (Right) Protein synthesis after 8 min (A) or 10 min (B) of labeling is expressed in percentage of controls lacking inhibitor. Means + SD of three independent experiments are shown. (C) Labeling with 50 μ Ci/ml 35 S-Met in normal medium lacking nonessential amino acids. Circles, cells in normal medium; squares, cells preincubated for 15 min in 20 mM L-azetidine-2-carboxylic acid (Azc) before and during labeling. Open symbols, DMSO; solid symbols, MG132-containing cultures. cpm, counts per minute. (D) Proteins were labeled as in (C). Gray bars, control cells; black bars, Azc-treated cells.



After labeling for 10 min, a chase was performed with 20 mM unlabeled Met. Half of the cultures received 100 μ M MG132 and 10 μ M clasto-lactacystin- β -lactone during labeling and chase (+ proteasome inhibitor), and the other half received DMSO (– proteasome inhibitor). TCA-precipitable radioactivity at time 0 was set as 100%. Means + SD of three experiments are shown.

ever, our experiments revealed a notable degree of protection of newly made proteins against immediate degradation. In contrast to the reported observations, cells did not accu-

mulate an additional amount of newly synthesized, radiolabeled protein when proteasome function was blocked (Fig. 1, B to E). How can this discrepancy be explained?

For labeling, cells are usually preincubated in media lacking the respective nonradioactive amino acid to increase the incorporation of radiolabel into newly made proteins (7). Based on our findings, preincubation with a proteasome inhibitor (7) should enhance this effect as a result of severe intracellular amino acid depletion (table S1 and Fig. 3A). Indeed, the incorporation of 35 S-Met into newly synthesized protein increased more than 10-fold when labeling was performed in methionine-deficient medium (Fig. 4A). As predicted, proteasome inhibition during amino acid starvation resulted in a substantial further increase in incorporated radioactivity (Fig. 4A), even though the efficiency of translation was reduced (Fig. 1). This effect was independent of the specific ratio of labeled-to-unlabeled amino acid (fig. S3) and was equally observed when cells were labeled with 3 H-Leu in leucine-deficient medium (Fig. 4B). Double-labeling experiments with 3 H-Leu and 35 S-Met in methionine-deficient medium showed that proteasome inhibition increased the incorporation of 35 S-Met into equal amounts of newly synthesized, 3 H-Leu-labeled protein by twofold (fig. S4A). A threefold increase in 35 S-Met-tRNA was detected in cells after 15 min of proteasome inhibition, which would explain the increased incorporation of 35 S-Met into newly formed protein (fig. S4B). Thus, the higher incorporation of radiolabel observed upon proteasome inhibition in amino acid deficient medium was due to an increase in specific radioactivity of the intracellular amino acid pool, not to the stabilization of a large fraction of newly synthesized proteins. At most, only a few percent of total protein was rapidly degraded immediately upon translation in the cell types analyzed here.

To test whether translating polypeptides remain protected against proteasomal degradation even when unable to fold, we incubated cells in the presence of the proline analog L-azetidine-2-carboxylic acid (Azc). Whereas the addition of Azc resulted in a reduced incorporation of 35 S-Met, no notable additional accumulation of radiolabeled protein was detectable upon proteasome inhibition within 12 min (Fig. 4C). This suggested that removal by the proteasome of misfolded proteins containing Azc occurred only after a substantial lag period. To address this possibility, cells were labeled with 35 S-Met for 10 min, followed by a chase with excess unlabeled methionine. More than 40% of the proteins synthesized in the presence of Azc were degraded within 60 min and this effect was largely prevented by proteasome inhibition (Fig. 4D). The extent of protein degradation observed in normal medium (~20% over 60 min) is in agreement with previous studies demonstrating the proteasomal turnover of short-lived proteins (23, 24). Thus, when cells produce a substantial amount of protein chains

that are unable to fold correctly, the majority of these chains are not degraded during translation, but rather through a relatively slow, posttranslational process.

Our results provide evidence for a critical role of the proteasome in supplying amino acids for sustained protein synthesis. This function of the proteasome is most critical upon acute amino acid restriction, where the uninduced lysosomal system is unable to maintain a sufficient intracellular amino acid pool. Amino acids for translation are predominantly generated by the proteasomal degradation of preexisting proteins. Newly synthesized proteins are largely protected from degradation during and immediately after translation, both under normal conditions and upon amino acid starvation. Faulty proteins are predominantly degraded through a posttranslational process that is likely to involve a functional cooperation between molecular chaperones assisting in folding and the proteasome system (25, 26). The proteasome has a demonstrated capacity to degrade polypeptides during synthesis, provided they carry specialized N-terminal degradation signals (27), but this mechanism is likely to be more extensively used in cell reg-

ulation rather than during basic housekeeping processes.

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Supporting Online Material

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Materials and Methods
Figs. S1 to S4
Table S1

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Category-Specific Cortical Activity Precedes Retrieval During Memory Search

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Kenneth A. Norman^{2,3}

Here we describe a functional magnetic resonance imaging study of humans engaged in memory search during a free recall task. Patterns of cortical activity associated with the study of three categories of pictures (faces, locations, and objects) were identified by a pattern-classification algorithm. The algorithm was used to track the reappearance of these activity patterns during the recall period. The reappearance of a given category's activity pattern correlates with verbal recalls made from that category and precedes the recall event by several seconds. This result is consistent with the hypothesis that category-specific activity is cueing the memory system to retrieve studied items.

Human memory can be characterized as an elaborate network of stored representations (1, 2). Recalling a particular event involves reactivating the constellation of representations that was active during that event, a phenomenon that Tulving has referred to as "mental time travel" (3). One of the major

puzzles of human memory is how we enact this process of mental time travel. More concretely: When we are instructed to recall a particular event, how do we manage to select representations corresponding to that event, as opposed to representations from other events (4, 5)?

Several theorists have argued that recalling an event involves a process of contextual reinstatement (6, 7). When asked to recall memories of a certain type, a person activates knowledge about the general properties of those events and then uses this general knowledge to constrain the search for mem-

ories of the target events. For example, in trying to remember a trip to the zoo, a person could use their general knowledge of the kinds of animals that are typically found at zoos as a contextual cue for specific memories of seeing those animals. If specific details are recalled, these details can be used to further refine the retrieval cue, which leads to recall of additional details, and so on. Over time, the person continues to probe memory, and the set of representations that are active at recall increasingly comes to resemble the set of representations that were active during the targeted event. Whereas a number of behavioral memory studies have found evidence consistent with the contextual reinstatement hypothesis (8–10), this kind of evidence is necessarily indirect. We can infer (based on theoretical grounds) that the observed patterns of behavioral data arise from increased match between cues at test and stored memory traces, but these studies do not directly measure cue-trace match.

We used functional magnetic resonance imaging (fMRI) to more directly test the contextual reinstatement hypothesis. In neural terms, the contextual reinstatement hypothesis leads to a number of predictions. The most basic prediction is that, when subjects try to recall specific details from a particular episode or type of episode, the pattern of brain activity (during recall) will progressively come to resemble the pattern of activity that was present during the to-be-remembered episode. Furthermore, it should be possible to relate the reinstatement of

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