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Regulation of neurogenin stability by ubiquitin-mediated proteolysis

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Short title: ubiquitin-mediated proteolysis of neurogenin

SYNOPSIS

Neurogenin (NGN), a proneural basic helix loop helix transcription factor, plays a central role in promoting neuronal specification and differentiation in many regions of the central nervous system. NGN activity has been shown extensively to be controlled at the transcriptional level. However, in addition, recent experiments have indicated that the levels of NGN protein may also be regulated. Here, we demonstrate that NGN protein stability is regulated in both *Xenopus* embryos and P19 embryonal carcinoma cells, a mammalian neuronal model system. In both systems, NGN is a highly unstable protein that is poly-ubiquitinated for destruction by the proteasome. NGN binds to DNA in complex with its heterodimeric E protein partners E12 or E47. We see that NGN is stabilised by the presence of E12/E47. Moreover, NGN is phosphorylated, and mutation of a single threonine residue substantially reduces E12-mediated stabilisation of NGN. Thus, E-protein partner binding and phosphorylation events act together to stabilise NGN, promoting its accumulation when it can be active.

Keywords: degradation assay, neurogenin, proteasome, ubiquitin, *Xenopus*

INTRODUCTION

Cell cycle exit and differentiation of neurons is a tightly regulated process, involving the activity of an array of transcription factors, including the basic helix-loop-helix (bHLH) family of proneural proteins. One of the key molecules within this family is neurogenin, which is essential for neurogenesis in the brain, sensory ganglia and spinal cord [1, 8, 23]. In *Xenopus*, NGN-related 1, NGNR1, is the earliest marker of primary neuron differentiation [16], when the first neurons differentiate out of the neural plate. NGNR1 (hereafter referred to as NGN) is most closely homologous to mammalian NGN2, based on sequence and function, with mammalian NGN2 also found to be essential for development of the central nervous system [1].

The regulation of NGN expression and transcriptional activation has been extensively studied [16], but until recently, regulation of NGN protein levels has not been investigated. We previously showed that, in *Xenopus*, the cyclin-dependant kinase (cdk) inhibitor p27^{Xic1} promotes neurogenesis at least partially by stabilising NGN [30] and more recently, p27^{kip1} has been shown to stabilize mouse NGN2 [19], although interestingly the activity of these cdk inhibitors seems to be independent of their ability to regulate the cell cycle. Thus, NGN appears to be an unstable protein, although the mechanism regulating its stability is unknown.

One of the major pathways for degrading cellular proteins is the ubiquitin-proteasome system (UPS, reviewed in [18]). This form of regulated protein destruction requires the covalent attachment of the small protein ubiquitin to amino groups of the targeted substrates, usually onto lysine residues. Subsequent ubiquitin molecules are added to the first via lysines within ubiquitin itself to form a poly-ubiquitin chain. Poly-ubiquitinated proteins are then transported to the 26S proteasome and are degraded. A growing number of factors promoting cell proliferation or cell differentiation have been shown to be targeted by the UPS. In particular, the bHLH factor MyoD, a determinant of vertebrate muscle differentiation, is regulated by the UPS [27], as are the proliferative HLH proteins Id1, Id2 and Id3 [2]. However, evidence for the ubiquitin-mediated proteolysis of proneural proteins is limited [26] and a role for ubiquitin-mediated proteolysis in the control of NGN stability has not been investigated.

Here, using extracts from *Xenopus* eggs and embryos and the P19 cell line that is competent to differentiate into neurons, we demonstrate that the stability of NGN is regulated by ubiquitin-mediated proteolysis. NGN has a very short half-life in eggs, embryos and proliferating P19 cells. It is stabilised by inhibitors of the proteasome and by inhibitors of poly-ubiquitination. Moreover, poly-ubiquitinated forms of NGN are readily detected. NGN is thought to be transcriptionally active only when bound to its heterodimeric binding partner E protein (E12/E47). We show that the addition of E12/E47 significantly increases the half-life of NGN in both *Xenopus* extracts and P19 cells. Previously, E47 has been shown to regulate phosphorylation of the related bHLH proneural factor Mash1 on a specific Casein Kinase II-target site (CKII site), which also regulates Mash1 stability [31]. A similar CKII site is present in NGN, and we show that when it is mutated, E12 mediated stabilisation of NGN is severely impeded, indicating that phosphorylation state also regulates NGN levels. Thus, NGN protein has a very short half-life but this can be significantly lengthened by E12/E47, demonstrating a mechanism allowing NGN protein to accumulate specifically when it is competent to activate transcription.

MATERIALS AND METHODS

Plasmids

NGN [16] and *Xenopus* NeuroD [14] are cloned into pCS2. NGN T118A mutant was generated by site directed mutagenesis using the QuickChange® Multi Site-Directed Mutagenesis Kit (Stratagene) with NGN cloned into pCS2 as the template and the primer GAAGATGCCAAACTCGCCAAGATAGAGACCTTGCGC used. For bacterial expression, NGN was cloned into pET-30a. For mammalian studies, a hemagglutinin (HA) tag was added to NGN by PCR and cloned into pCS2 using the primers CGCGGATCCACCATGTT-CGTCAAATCTGAGACTCTGG and GCGAATTCTTAAGCGTAATCTGGAACATCGT-ATGGGTAGATACAGTCCCTGGCGAGG. *Xenopus* E12, mouse E47 and FLAG-ubiquitin constructs were described earlier [13, 22, 28].

Preparation of *Xenopus* egg and embryo extracts

Xenopus laevis eggs and neurula stage embryos were obtained by standard methods and staged according to Nieuwkoop & Faber [21]. Extracts were prepared as described previously [17] with minor modifications. De-jellied eggs or embryos were washed in chilled XB buffer (100mM KCl, 0.1mM CaCl₂, 1mM MgCl₂, 10mM potassium HEPES pH7.7, 50mM sucrose) and mixed with 10mg/μl cytochalasin B (Sigma). Eggs and embryos were packed for 1 min at 4°C at 100g and 400g respectively and then ruptured by centrifugation (5 min, 16,000g, 4°C). Out of the three phases formed, the intermediate cytoplasmic layer was removed and spun twice (5 min, 16,000g, 4°C). The extracts were supplemented with leupeptin, pepstatin and chymostatin (LPC), each at 10μg/ml, and 1/20th volume of energy mix (150mM creatine phosphate, 20mM ATP, 2mM EGTA, 20mM MgCl₂) and used immediately.

In vitro transcription and translation

NGN, NGN T118A and NeuroD were *in vitro* transcribed and translated (IVT) using ³⁵S-methionine (GE Healthcare) and the TnT® SP6 Quick Coupled Transcription/Translation System (Promega). GFP and E12 protein were obtained similarly using non-radiolabelled methionine.

***In vitro* degradation assays**

Degradation assays were performed as described earlier [9] with minor modifications. Extracts were supplemented with 100 μ g/ml cycloheximide (CHX, Sigma) and 1.25mg/ml ubiquitin (Sigma) or 10mg/ml methylated ubiquitin (BioMol). When indicated, 200 μ M MG132 (BioMol) was added for 5 min to the extracts before addition of NGN or NeuroD. ³⁵S-labelled proteins were mixed with extracts and incubated at 20°C. For degradation assays in the presence of E12, cold IVT E12 or GFP was added where indicated. Aliquots were taken at various time points and mixed with Laemmli sample buffer. Samples were denatured for 3 min at 95°C and separated on a 15% SDS-PAGE gel. Gels were dried and visualized by phosphorimaging and autoradiography.

***In vitro* ubiquitination assay**

Ubiquitination assay was conducted as described previously [25] with minor modifications. ³⁵S-labelled NGN was mixed with interphase egg or neurula stage embryo extract supplemented with 200 μ M MG132 and either 2.5mg/ml histidine-ubiquitin or 1.25mg/ml ubiquitin, and incubated at room temperature for 1h. The reaction was then diluted 10 fold in His buffer (100mM Tris pH 7.4, 1% NP40, 8M urea, 20mM imidazole, 600mM NaCl, 10% ethanol) and complemented with Complete Protease inhibitor (Roche) and 1 μ M Pepstatin before mixing with nickel NTA-agarose beads (Qiagen). After a 90 min incubation at room temperature, beads were washed several times with His buffer and proteins were eluted with Laemmli sample buffer. Samples were analysed as described above.

Ubiquitination assay of bacterially expressed NGN

NGN was cloned into pET-30a vector to allow the expression of a C-terminus 6xHis tag protein (NGN-His) in *Escherichia coli*. NGN-His was expressed in Rosetta cells and purified using the Ni-NTA agarose resin (Qiagen) according to the manufacturer's instructions. Since NGN-His is expressed in inclusion bodies, purification was performed in the presence of urea. Briefly, proteins were solubilised in N300 buffer (50mM Tris pH9.4, 500mM NaCl, 1mM PMSF, 10mM β -mercaptoethanol) supplemented with 8M urea. Ni-NTA agarose beads were added to the protein and incubated 1h at room temperature. Beads were washed successively with N300 buffer containing 4M urea, N300 buffer containing 1M urea, and finally transferred into XB buffer. NGN-His still bound to the beads was then incubated with

Xenopus activated egg extract together with ubiquitin (1.25mg/ml) and MG132 (700 μ M) for 1h at 20°C. NGN-His was then re-purify by first diluting the reactions 10 fold in a Urea buffer (100mM Tris pH 7.4, 1% IgePal CA-360 (Sigma), 8M urea, 20mM imidazole, and 600mM NaCl) and incubating at room temperature for 2h. Beads were washed several times with the Urea buffer and proteins were eluted with Laemmli sample buffer. Samples were analysed by Western blotting. Ubiquitinated NGN was detected using an anti Mono- and Poly-ubiquitinated conjugates antibody (FK2 clone, BioMol) and horseradish peroxidase (HRP) coupled anti-mouse antibody (GE Healthcare).

Cell culture and transfections

P19 cells were grown as previously described [24]. Transfections were performed on cells grown on poly-L-lysine (Sigma) coated plates with NGN-HA, pCS2 and/or E47 constructs using Lipofectamine2000 (Invitrogen). Degradation assays were performed 24h after transfection. Protein synthesis was blocked with 10 μ M CHX (T=0) and samples were collected over time. When indicated, 20 μ M MG132 was used. Cells were lysed in lysis buffer (50mM Tris pH7.5, 150mM NaCl, 1% NP40, Complete Protease inhibitor and 1 μ M Pepstatin) and subjected to Western blot using 20 μ g of total proteins. NGN protein was detected using an anti-HA antibody (Roche) and HRP-coupled anti-mouse antibody (GE Healthcare).

For immunoprecipitation assays, NGN-HA or untagged NGN was co-transfected with FLAG-Ubiquitin in P19 cells. 24h post-transfection, cells were incubated in the presence of 50 μ M MG132 for 1h and cell extracts were prepared in lysis buffer. NGN was immunoprecipitated in IP buffer (50mM Tris pH7.5, 150mM NaCl, 0.1% NP40, Complete Protease inhibitors and 1 μ M Pepstatin) using a HA-matrix (Roche) and purified by competition with the HA-peptide (Roche). Ubiquitinated proteins were immunoprecipitated using an anti-FLAG antibody (M2 clone, Sigma) and Protein-A sepharose (GE Healthcare). Purified proteins were subjected to Western blot using an HRP-coupled anti-HA antibody (Roche) or HRP-coupled anti-FLAG antibody (M2 clone, Sigma).

Phosphatase treatment

Lambda phosphatase (New England Biolabs) was used according to manufacturer's instructions. ³⁵S-labelled IVT NGN was incubated in the presence of E12 and egg extract for

1h and 200 μ M MG132 was added before treatment with lambda phosphatase. As control, the buffer alone with no enzyme was used. Samples were analysed as described previously. For P19 cells, NGN-HA and E47 were transfected into cells and 24h later, protein synthesis was blocked and samples were collected after 5 min. Analysis by Western blot was performed as described above.

RESULTS

NGN protein is highly unstable

Neurogenins, particularly *Xenopus* NGNR1 and mammalian NGN2, are crucially important proteins in development of the nervous system. However, despite this, very little is known about the regulation of NGN at the protein level. In recent years two studies have indicated that NGN is unstable [19, 30]. However, neither the half-life of the protein, nor the mechanism by which it is turned over has been investigated in detail.

Extracts from *Xenopus* eggs and embryos have been widely used to study proteolysis of several proteins [25]. We have already demonstrated that NGN protein stability is regulated in developing *Xenopus* embryos [30], so we have initially taken an extract approach to investigate NGN degradation biochemically. NGN was *in vitro* translated in reticulocyte lysate in the presence of ^{35}S -methionine (IVT ^{35}S -NGN) and incubated in extracts prepared from activated interphase *Xenopus* eggs or neurula stage embryos. Aliquots were removed at increasing times and the amount of NGN protein remaining was determined by SDS-PAGE, autoradiography and quantitative phosphorimaging analysis. NGN is highly unstable in interphase egg and embryo extracts, with a half-life of 21.9 ± 2.2 and 37.9 ± 4.0 minutes respectively (Figure 1A, B). Although the half-life of NGN in embryos is almost twice as long as in eggs, it seems likely that the mechanism of proteolysis is the same in both systems (see below). We wished to determine whether other proneural proteins were unstable in *Xenopus* eggs and embryos or whether NGN protein is degraded specifically. To this end, we measured the stability of the closely related proneural protein NeuroD, itself a transcriptional target of NGN [14]. Strikingly, in both egg and neurula-stage embryo extracts, NeuroD was remarkably stable, with half-lives of 190.7 ± 22.9 and 262.3 ± 11.4 minutes respectively (Figure 1A, B). From these results, it is clear that while NGN is rapidly degraded in both eggs and embryos, NeuroD is significantly more stable.

Covalent attachment of poly-ubiquitin chains is frequently used to target proteins for degradation. To detect such forms we incubated IVT ^{35}S -NGN in egg and embryo extracts for increasing times and performed a long autoradiographic exposure of the gel. A ladder of higher order forms of NGN, stretching up to the top of the gel, was observed that may correspond to poly-ubiquitinated forms of the protein (Figure 1C, D, asterisks).

Neurogenin is degraded by ubiquitin-mediated proteolysis

If NGN destruction requires poly-ubiquitination, it should be stabilised by the addition of methylated ubiquitin, a chemically modified form of ubiquitin that cannot form poly-ubiquitin chains [10]. Methylated ubiquitin was added to egg extracts in the presence of IVT ^{35}S -NGN, and aliquots removed for electrophoretic analysis at increasing times. Addition of methylated ubiquitin resulted in substantial stabilisation of NGN protein even up to 180 minutes (Figure 2A). Moreover, a prominent mono-ubiquitinated form running approximately 10 kDa slower by SDS-PAGE is clearly visible (arrow), again demonstrating that NGN protein is directly ubiquitinated.

Poly-ubiquitination is frequently used as a signal to direct proteins to the proteasome for degradation [4]. To investigate whether NGN is subject to proteasomal degradation, we performed degradation assays of IVT ^{35}S -NGN in the presence of a chemical inhibitor of the proteasome, MG132. When MG132 was added to egg extracts, NGN is significantly stabilised, with the half-life increasing from 21.9 ± 2.2 to 124.0 ± 19.1 minutes (Figure 2B). Moreover, a similar stabilising effect was seen in extracts from neurula stage embryos, extending the half-life from 37.9 ± 4.0 to 123.8 ± 14.6 minutes (Figure 2C), demonstrating that the mechanism of destruction is the same at these different developmental stages. A similar stabilisation was also seen with other proteasome inhibitors including MG262, vinyl-sulfone and epoxomicin (data not shown). Therefore, poly-ubiquitinated NGN is degraded by the proteasome.

To demonstrate conclusively that NGN is poly-ubiquitinated, we set out to isolate ubiquitin-bound forms of the NGN protein. To this end, we incubated IVT ^{35}S -NGN in egg extract in the presence of MG132 and histidine-tagged ubiquitin (His-Ub). Proteins covalently modified with His-Ub were subsequently isolated using NTA-agarose beads. To ascertain that only proteins covalently bound to ubiquitin would be pulled down, and since NGN is known to bind proteins that can themselves be poly-ubiquitinated [2, 12], a buffer containing 8M urea was used to wash the beads before elution. Purified proteins were then separated by SDS-PAGE for autoradiographic analysis. NGN is covalently associated with His-Ub and slower migrating forms that correspond to poly-ubiquitinated species are clearly visible (Figure 3A). Similar poly-ubiquitinated forms of NGN are generated when NGN is incubated with His-Ub in neurula stage embryo extracts (Figure 3B). In contrast, only very low background levels of associated protein is seen when non-his Ub was added. Similarly,

no radiolabelled proteins were purified when an IVT reaction containing the vector alone was added to extract in place of IVT ³⁵S-NGN (Figure 3A, B).

Another approach was taken to show directly that NGN is bound to ubiquitin. His-tagged NGN protein was expressed in bacteria, purified with NTA-agarose and incubated while still bound to the beads, with egg extract in the presence of MG132. NGN-his was then re-isolated and washed in buffer containing 8M urea before elution. Proteins were then separated by SDS-PAGE and Western blotting was performed using an antibody to detect ubiquitin. Poly-ubiquitin chains of different lengths are specifically detected after NGN-His has been passed through egg extract (Figure 3C). Thus, NGN is clearly poly-ubiquitinated and degraded by the proteasome in *Xenopus* eggs and embryos.

NGN is stabilised by its heterodimeric binding partner E12

We see that NGN is a highly unstable protein, subject to very rapid ubiquitin-mediated proteolysis, yet its transcriptional activity is vital to promote neurogenesis and repress gliogenesis in wide regions of the mammalian central nervous system [23]. To act as a transcription factor, NGN binds to DNA as a heterodimer with the ubiquitous splice products of the bHLH E2A gene, E12 and E47 [1, 23]. It has previously been shown that in some circumstances, the related bHLH proneural factor Mash1 can be stabilised by the presence of E47 [31]. We thus determine whether NGN is similarly stabilised by E12/E47. To this end, we incubated IVT ³⁵S-NGN, in the absence or presence of IVT unlabelled E12, in egg extract and took aliquots at increasing times for autoradiographic analysis. Co-incubation of NGN and E12 in egg extract led to a substantial stabilisation of NGN protein, with the half-life increasing from 21.9 ± 2.2 to 166.4 ± 19.9 minutes (Figure 4A). In contrast, co-incubation of NGN with GFP as control had no effect.

Interestingly, NGN that accumulated in the presence of E12 was significantly retarded in its electrophoretic migration compared to the protein incubated in the presence of GFP (Figure 4A, arrow). It has been shown that the destruction of some transcription factors by ubiquitin-mediated proteolysis, including those of other bHLH factors, such as MyoD, Myf5 and Mash1, is regulated by phosphorylation [6, 29, 31]. The mobility shift of NGN could thus be consistent with phosphorylation of a stable form of NGN. To confirm that this is the case, we treated extract containing E12 and NGN with lambda phosphatase. An increase in mobility of the higher order forms of NGN seen in the presence of E12 was

observed under these conditions, resulting in the loss of the upper band (Figure 4B), indicating that E12 does promote accumulation of hyperphosphorylated forms of NGN.

In the case of Mash1, a proneural factor related to NGN, it has been determined that the ubiquitous E protein E47 promotes Casein Kinase II (CKII)-dependent phosphorylation on serine 152 of Mash1 and that this modification increases Mash1 protein stability [31]. We performed a sequence alignment between Mash1 and NGN and identified a similar motif in NGN, located in the loop domain. In order to investigate the role of this site in modulating NGN stability, we used site directed mutagenesis to replace the potentially phosphorylated threonine (T118), with an alanine. Degradation assays were then performed using this mutant in the absence and presence of E12. Strikingly, we found that E12 promoted the accumulation of WT NGN but was less effective at inhibiting degradation of T118A NGN, while both proteins displayed similar stability in the absence of E12 (Figure 5). The half-lives in the absence of E12 are similar, 21.9 ± 2.2 and 23.3 ± 2.6 minutes for WT and T118A NGN respectively, but differed significantly in the presence of E12, 61.5 ± 5.3 minutes for T118A compared with 166.4 ± 19.9 minutes for WT. Interestingly, the slowest migrating form of NGN seen when WT NGN and E12 are co-incubated does not accumulate significantly when using the T118 mutant (compare Figure 4A, arrow, to Figure 5), indicating a differing phosphorylation status of the two proteins, hyperphosphorylation of NGN being potentially associated within enhanced stability.

NGN is unstable in P19 cells

The experiments described above demonstrate that NGN is a highly unstable protein in both *Xenopus* eggs and embryos. To assess whether the same is true in mammalian cells, we investigated NGN stability in P19 embryonal carcinoma cells, a cell line that is competent to differentiate into neurons under the influence of bHLH transcription factors [7]. Plasmid expressing a hemagglutinin (HA) tagged-NGN was transfected into P19 cells and at $T=0$, cycloheximide was added to stop ongoing protein synthesis. Samples were collected at increasing time and levels of NGN protein were monitored by Western blotting. Similar to our results in *Xenopus*, NGN is extremely unstable in P19 cells, having a half-life of just 17.1 ± 1.2 minutes (Figure 6A). Moreover, this degradation is mediated by the proteasome, as it is effectively blocked by addition of MG132. The short half-life exhibited by NGN might indicate why it is relatively poor at inducing neural differentiation of P19 cells relative

to other bHLH proneural genes tested [7].

To demonstrate that NGN is ubiquitinated in P19 cells, NGN-HA was co-transfected with a FLAG-tagged ubiquitin. Ubiquitinated proteins were then immunoprecipitated with an anti-FLAG antibody and subjected to Western blotting with anti-HA antibody. A ladder of proteins was clearly visible, indicating that NGN-HA is poly-ubiquitinated (Figure 6B). Similarly, when HA-tagged NGN was pull-down using an HA-matrix and subjected to Western blotting with anti-FLAG antibody to detect ubiquitin, a ladder of proteins of high molecular weight was observed (Figure 6B). This demonstrates clearly that NGN is poly-ubiquitinated in P19 cells.

Co-incubation with E12 in *Xenopus* egg extract leads to accumulation of hyperphosphorylated forms of NGN. To determine if a similar stabilisation occurs in mammalian cells, P19 cells were co-transfected with NGN and the splice isoform E47. The presence of E47 also results in a significant lengthening of the half-life of the NGN protein in P19 cells (Figure 6C). Again, distinct slower migrating forms of NGN accumulate, including a more slowly migrating form of NGN seen in the presence of E47, which is not seen in its absence (Figure 6C, arrow). Treatment with lambda phosphatase demonstrates that these slower migrating forms of NGN are indeed hyperphosphorylated (Figure 6D).

Therefore, NGN protein is similarly regulated in *Xenopus* eggs, embryos and P19 cells, where it is degraded by the UPS. Degradation is inhibited by co-expression of E12/E47 and this results in accumulation of hyperphosphorylated forms of the protein.

DISCUSSION

Over the past decade, it has become increasingly clear that a broad range of regulatory proteins including transcription factors are controlled at the levels of protein stability by the UPS [4-5]. Neural determination and differentiation are controlled by proneural proteins. While numerous studies have investigated their regulation at the level of gene expression, very little is known about regulation of the proneural proteins themselves. Here we demonstrate that one of the most prominent proneural proteins, NGN, is regulated at the level of protein stability.

NGN is a member of the bHLH class of transcription factors. Within this class, the most studied is the bHLH protein MyoD, which plays a pivotal role in regulating myogenesis. MyoD is degraded by the UPS [27], and is subject to more than one mode of ubiquitination [3], probably by several E3 ligases. However, pathways controlling its destruction are far from being fully understood. Moreover, the myogenic bHLH Myf5 is also degraded by the UPS but its regulation probably differs significantly from MyoD [6]. It is becoming clear that we will understand the regulation of destruction of each protein only by studying them on a case-by-case basis. Previously, only one proneural protein, Mash1, has been shown to be degraded following poly-ubiquitination [31] and, as with myogenic factors, the limited amount of data available makes it hard to draw general conclusions. Here we show that NGN protein has a short half-life, is poly-ubiquitinated and degraded by the proteasome in *Xenopus* eggs and embryos, and in mammalian P19 embryonal carcinoma cells. Interestingly, we show that a related bHLH protein NeuroD is stable in *Xenopus* eggs and embryos.

During development, NGN expression is transient and prolonged up-regulation by mRNA microinjection can result in apoptosis [32]. NeuroD is downstream of NGN and drives terminal differentiation of neurons [14]. Hence, it may be more desirable for the early factor NGN to have a short half-life, while the factor driving cell cycle exit and terminal differentiation, NeuroD, is more long-lived.

Poly-ubiquitination of many proteins targeted for destruction by the UPS is regulated by phosphorylation. This is certainly true of members of the bHLH family such as MyoD, Myf5 and E12 [6, 12, 20, 27]. MyoD and Myf5 stability is controlled by phosphorylation mediated by cyclin-dependent kinases and also appears to be cell cycle regulated [6, 15, 29].

We see that NGN is phosphorylated in both *Xenopus* and mammalian cells. Co-expression of NGN with E12/E47 leads to NGN stabilisation and results in accumulation of hyperphosphorylated forms of NGN, in both *Xenopus* eggs and P19 cells. The stability of the related proneural gene Mash1 is controlled by phosphorylation on a specific CKII site, an event that is itself promoted by binding to an E protein partner [31]. We identified a similar potential CKII site in NGN, T118. We found that a T118A mutant of NGN had a similar half-life to the WT protein on its own. However, the T118A mutant could no longer be stabilised by co-incubation with E12 and the usual associated hyperphosphorylated forms of NGN substantially reduced. This indicates that, like in the case of Mash1, E12 may promote phosphorylation on this residue, resulting in increased stabilisation of the complex. NeuroD is also phosphorylated in egg extract, but in this case is almost fully stable, and the functional significance of this phosphorylation is unclear.

The Neurogenin family of transcription factors are crucially important for neurogenesis. Indeed, levels of NGN protein have been experimentally manipulated in model systems with potential therapeutic implications. For instance, recovery from spinal cord injury in a rat model is significantly enhanced when grafted neural precursors have been supplied with ectopic NGN2 [11]. Here, we demonstrate for the first time that *Xenopus* NGN has a strikingly short half-life, both in *Xenopus* eggs and embryos, and in mammalian P19 cells that are capable of differentiating into neurons. In addition, the half-life of NGN is modulated by its heterodimeric binding partner E12/E47 and by phosphorylation. A detailed understanding both of the potentially multiple mechanisms that regulate NGN stability and identification of the molecules which can target NGN for ubiquitin-mediated proteolysis may not only be important for a thorough understanding of differentiation but may also have important therapeutic implications, if we are to potentiate mechanisms to promote neurogenesis formation *in vitro* and *in vivo*. This study is the first step towards this goal.

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FIGURE LEGENDS

Figure 1: NGN is a highly unstable protein.

Extracts were prepared from interphase eggs (A) or neurula stage embryos (B) and supplemented with IVT ³⁵S-NGN or NeuroD. Samples were taken at time points indicated and subjected to SDS-PAGE. Gels were analysed by autoradiography (left panels) and quantitative phosphorimaging analysis (right panels). (C, D) Long exposure of IVT ³⁵S-NGN in egg (C) and embryo (D) extracts separated by SDS-PAGE to reveal higher molecular weight forms of NGN (asterisks).

Figure 2: NGN is degraded by the ubiquitin proteasome system (UPS).

(A) Degradation assay of IVT ³⁵S-NGN was performed in interphase egg extracts in the presence of ubiquitin (Ub) or methylated ubiquitin (MeUb) and analysed by SDS-PAGE and autoradiography. Arrow indicates mono-ubiquitinated NGN. (B, C) Extracts were prepared from interphase eggs (B) or neurula stage embryos (C) and supplemented with IVT ³⁵S-NGN in the presence of the proteasome inhibitor MG132 (200 μM) or DMSO alone. Samples were taken at time points indicated and subjected to SDS-PAGE and subsequent autoradiography.

Figure 3: NGN is poly-ubiquitinated.

(A, B) IVT ³⁵S-NGN was incubated with interphase egg (A) or neurula stage embryo (B) extracts in the presence of ubiquitin (Ub) or histidine-ubiquitin (His-Ub) and ubiquitin-bound proteins were purified by affinity chromatography with NTA agarose. The empty vector (pCS2+) was used as control. Arrows indicate poly-ubiquitinated forms of NGN. (C) Bacterially expressed and purified NGN-His was incubated with interphase egg extracts in the presence of ubiquitin and MG132 and re-isolated before elution. Western blot was performed with an anti-ubiquitin antibody.

Figure 4: E12 stabilises NGN and promotes its phosphorylation.

(A) E12 protein was added to *Xenopus* interphase egg extracts and the rate of degradation of IVT ³⁵S-NGN was assayed relative to a control supplemented with GFP. Gels were analysed by autoradiography (left panels) and quantitative phosphorimaging analysis (right panel). (B) Lambda phosphatase treatment of NGN incubated with E12. Arrows in panel A and B

indicate a slower migrating form of NGN that accumulates in the presence of E12.

Figure 5: Reduced stability of a Casein Kinase II mutant of NGN in the presence of E12.

E12 protein was added to *Xenopus* interphase egg extracts and the rate of degradation of IVT ³⁵S-NGN T118A was assayed relative to a control supplemented with GFP. Gels were analysed by autoradiography (left panels) and quantitative phosphorimaging analysis (right panel).

Figure 6: NGN stability in P19 cells.

(A) HA tagged-NGN was transfected in P19 cells and after protein synthesis was inhibited with cycloheximide, samples were collected over-time and subjected to Western blotting with an anti-HA antibody. Control (CTL) corresponds to cells transfected with untagged NGN. Assay was performed in the presence or absence of 20 μ M MG132 to block proteasome activity. Quantification was performed by densitometry scanning.

(B) NGN-HA was co-transfected with FLAG-tagged ubiquitin and reciprocal immunoprecipitations were carried out. Ubiquitinated proteins were immunoprecipitated with an anti-FLAG antibody and subjected to Western blotting with anti-HA antibody (left panel). HA-tagged proteins were pull-down using an HA-matrix and subjected to Western blotting with anti-FLAG antibody (middle panel). 1/10th of the loading material was also subjected to Western blotting (right panel). Controls used were transfection of P19 cells with NGN-HA in the absence of FLAG-ubiquitin and untagged NGN in the presence of FLAG-ubiquitin.

(C) Stabilisation of NGN by E47. P19 cells were transfected with NGN-HA in the presence or absence of E47 and degradation assay was performed as described in A. Arrow indicates slower migrating form of NGN only seen in the presence of E47.

(D) Lambda phosphatase treatment of NGN transfected into the cells with E47.

FIGURE 1

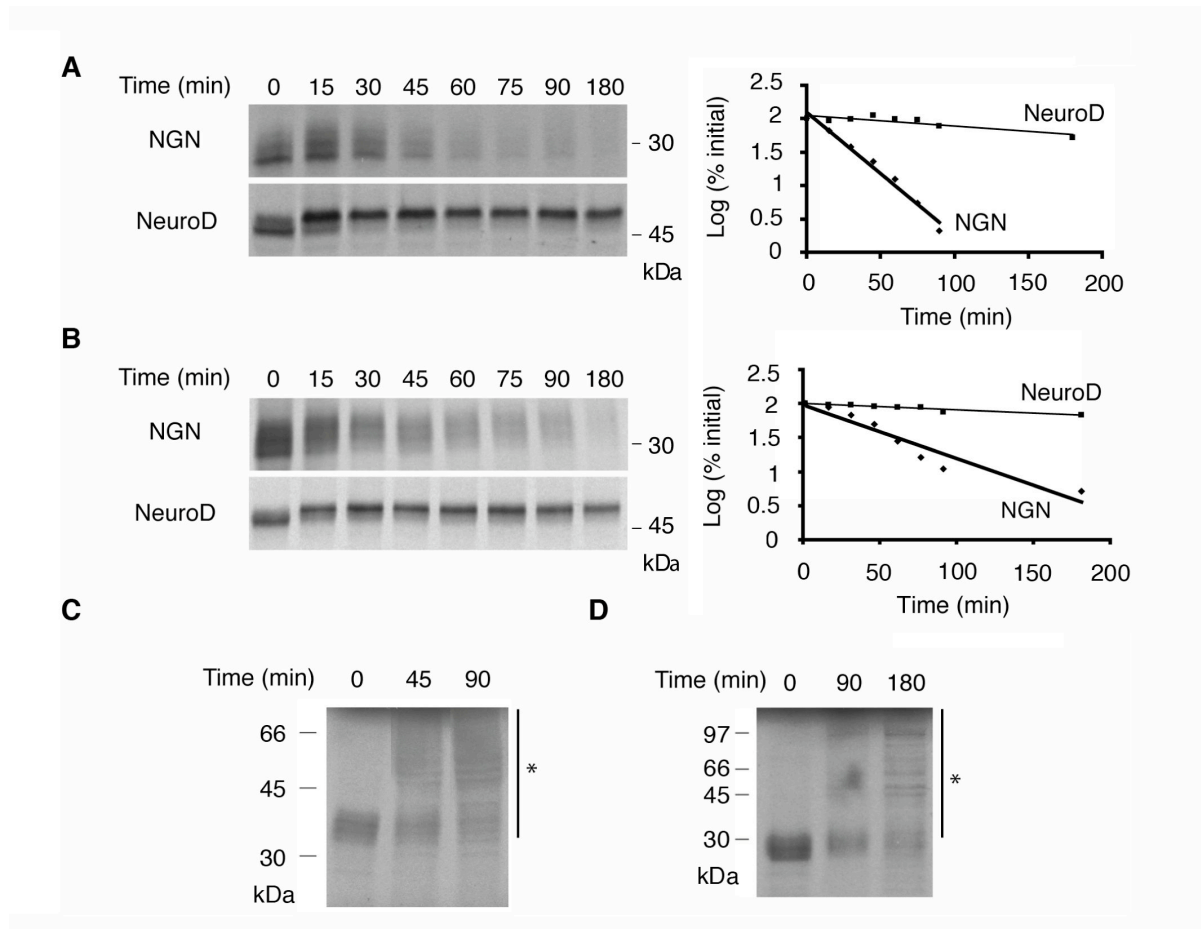


FIGURE 2

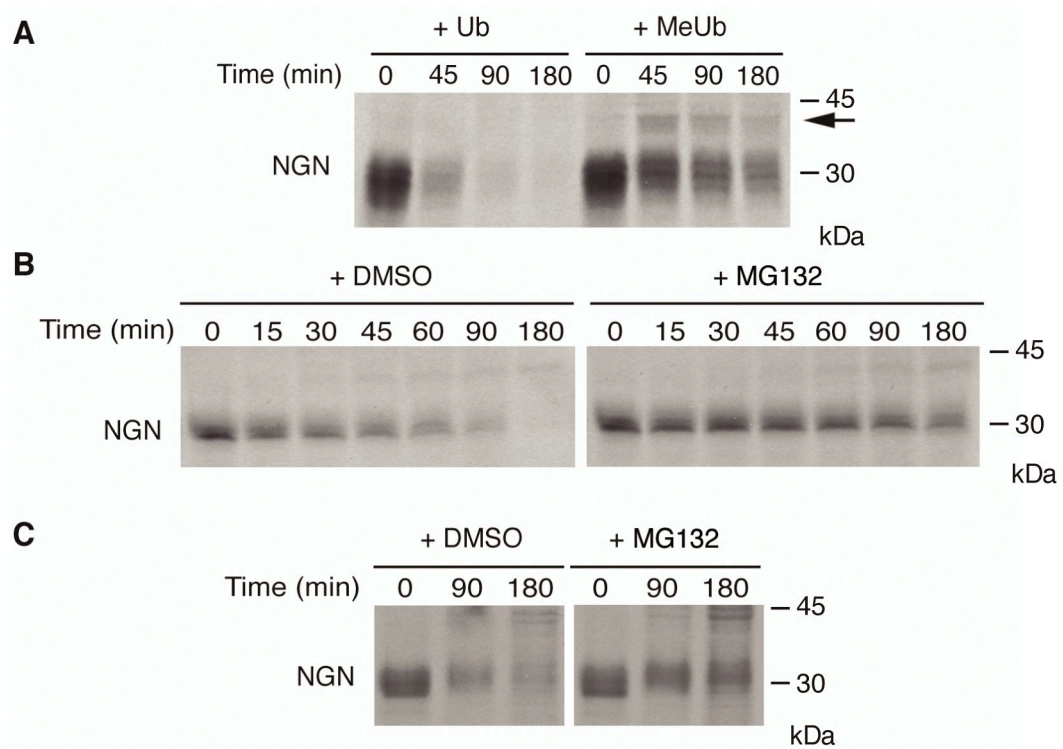


FIGURE 3

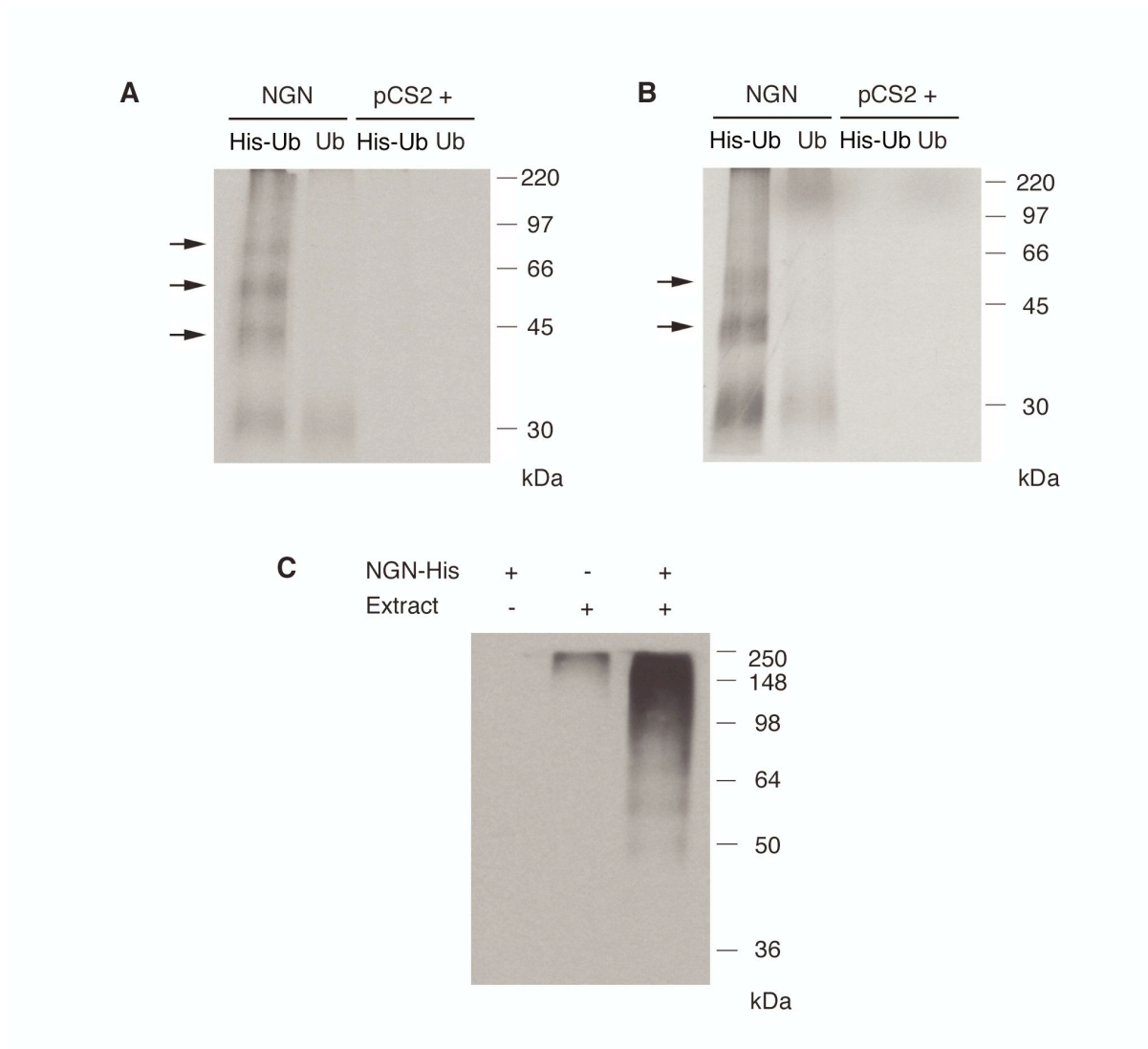


FIGURE 4

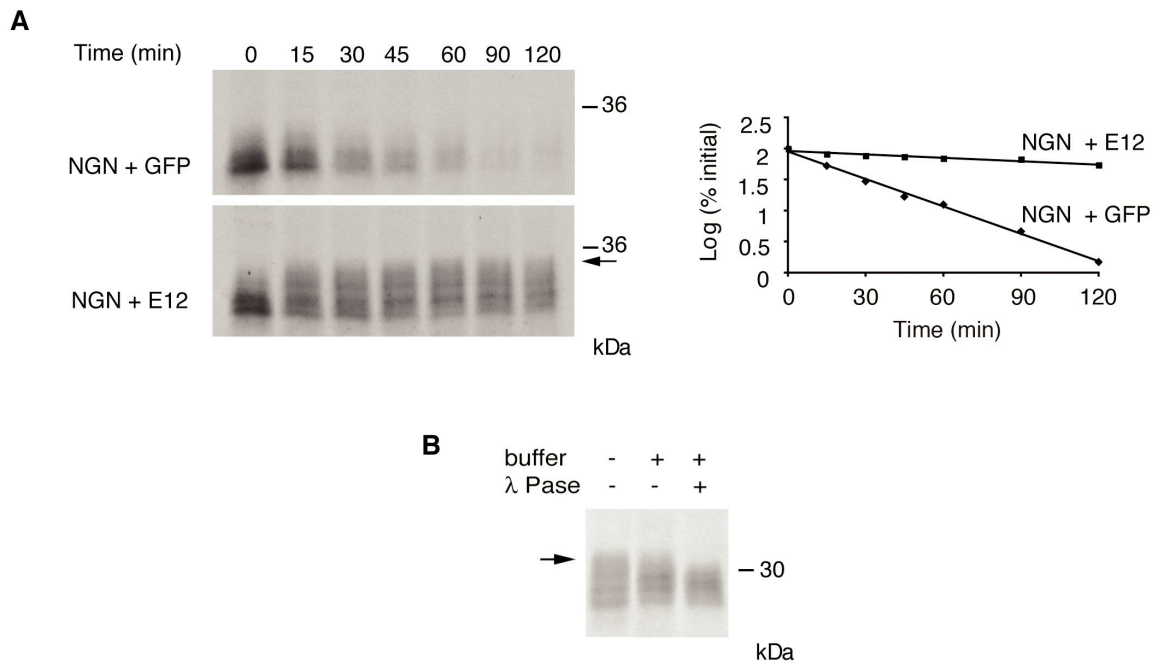


FIGURE 5

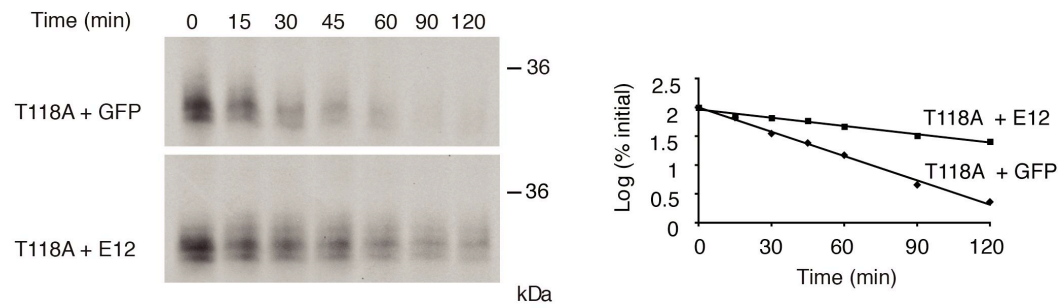


FIGURE 6

