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# Complex domain interactions regulate stability and activity of closely related proneural transcription factors

Gary S. McDowell [1], Laura J.A. Hardwick, Anna Philpott *

*Department of Oncology, University of Cambridge, Hutchison/MRC Research Centre, Cambridge Biomedical Campus, Cambridge CB2 0XZ, UK*

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ABSTRACT

Characterising post-translational regulation of key transcriptional activators is crucial for understanding how cell division and differentiation are coordinated in developing organisms and cycling cells. One important mode of protein post-translational control is by regulation of half-life via ubiquitin-mediated proteolysis. Two key basic Helix-Loop-Helix transcription factors, Neurogenin 2 (Ngn2) and NeuroD, play central roles in development of the central nervous system but despite their homology, Ngn2 is a highly unstable protein whilst NeuroD is, by comparison, very stable. The basis for and the consequences of the difference in stability of these two structurally and functionally related proteins has not been explored. Here we see that ubiquitylation alone does not determine Ngn2 or NeuroD stability. By making chimeric proteins, we see that the N-terminus of NeuroD in particular has a stabilising effect, whilst despite their high levels of homology, the most conserved bHLH domains of these proneural proteins alone can confer significant changes in protein stability. Despite widely differing stabilities of Ngn2, NeuroD and the chimeric proteins composed of domains of both, there is little correlation between protein half-life and ability to drive neuronal differentiation. Therefore, we conclude that despite significant homology between Ngn2 and NeuroD, the regulation of their stability differs markedly and moreover, stability/instability of the proteins is not a direct correlate of their activity.



## 1. Introduction

Basic Helix-Loop-Helix (bHLH) transcription factors play a central role in cell fate and differentiation in a wide variety of tissues, often by acting as master regulators coordinating expression of multiple downstream targets [1]. Tissue-specific class II bHLH proteins contain a DNA-binding basic domain, followed by two α-helices separated by a loop, and flanked either side by regions of poorly defined structure [2]. Structure and function studies have shown that these transcriptional regulators act as heterodimers with the ubiquitously expressed class I bHLH E2A gene products E12 or E47; the Helix-Loop-Helix (HLH) domain mediates heterodimerisation whilst the basic region binds to a consensus E-box DNA motif in the promoter region of target genes [3,4].

One member of this family, Neurogenin 2 (Ngn2), acts as a master regulator of neurogenesis in regions of the central nervous system [5]. Ngn2 is essential for neuronal differentiation during primary neurogenesis in the *Xenopus* frog embryo [6] and induction of ectopic neurons in *Xenopus* by Ngn2 has been widely used to study Ngn2 function [7–9]. Differentiation of these primary neurons also absolutely requires activity of an additional related bHLH transcription factor, NeuroD [10]. In *Xenopus*, it has been shown that Ngn2 both upregulates NeuroD expression in a unidirectional cascade, and functions in parallel with NeuroD, activating a large number of common target genes required for primary neurogenesis [11]. Yet even with their structural and functional similarities, the half-life of these proteins differs significantly [12]. The basis for this difference and its functional consequences have not been investigated.

Transcription factors tend to be highly unstable proteins degraded by the Ubiquitin–Proteasome System (UPS) [13]. To target proteins for destruction, Ubiquitin (Ub) is activated and covalently fused to a specific substrate protein at electron-rich sites (usually lysines, reviewed in [14]). Ubiquitylation can be repeated to build up a chain of at least 4 Ub moieties that then targets the substrate to the 26S proteasome [15]. Using energy from ATP

*Abbreviations:* bHLH, basic Helix-Loop-Helix; Ngn2, Neurogenin 2; UPS, Ubiquitin–Proteasome System; Ub, ubiquitin; ANOVA, analysis of variance; UD, unfolded domain.

* Corresponding author. Fax: +44 1223 336902.
*E-mail address:* ap113@cam.ac.uk (A. Philpott).
[1] Present address: Center for Regenerative and Developmental Biology, Department of Biology, Tufts University, 200 Boston Avenue, Medford, MA 02155, USA.

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hydrolysis, ubiquitylated proteins are then unfolded from an unfolding initiation site [16] and cleaved into small peptides. This regulation generally results in highly dynamic protein levels, which adjust in response to intrinsic and extrinsic controls.

We have previously shown that Ngn2 is rapidly degraded by the UPS, whereas NeuroD is stable under similar conditions [12]. Unusually, this rapid degradation of Ngn2 is brought about by both canonical ubiquitylation on lysine residues, and non-canonical ubiquitylation on cysteines, serines and threonines [17,18]. The structural aspects of NeuroD and Ngn2 that confer stability/instability have not been explored, and whether differences in stability relate to differences in ubiquitylation, or whether they relate to differences in destruction of ubiquitylated proteins is yet to be determined. Moreover, the relationship between proneural protein half-life and ability to activate downstream target activation and drive neurogenesis remains unknown.

In this study we compare the roles of protein structure and ubiquitylation in regulating Ngn2 and NeuroD stability and activity by undertaking a domain-swap analysis between the two proteins. We show that similarly structured proteins do not necessarily exhibit similar biochemical properties with respect to ubiquitylation and degradation. Furthermore, we show that there is poor correlation between protein half-life and protein activity *in vivo*.

## 2. Materials and methods

### 2.1. Cloning

Point-mutant constructs were made by site-directed mutagenesis (Stratagene) and cloned into pCS2+ as described previously [12,17] using standard methods.

#### 2.1.1. Unfolded domains

Unfolded domain constructs were a kind gift of Andreas Matouschek [19]. The domains were fused to the N- and C-termini of NeuroD using the Gateway® cloning system (Invitrogen). **NeuroD-UD**: NeuroD DNA was amplified by PCR between attB1 and att5Br sites: Forward ATGACCAAATCGTATGGAGAGAATGG, Reverse TTAATCATGAAAGATGGCATTTAGCTGG. UD DNA was amplified between attB5 and attB2 sites: Forward ATGCTAAAATACAAACCTTTAC, Reverse TTATTCAGCGGGCGAAAATC. **UD-NeuroD**: NeuroD DNA was amplified by PCR between attB5 and attB2 sites: Forward ATGACCAAATCGTATGGAGAG, Reverse ATCATGAAAGATGGCATTTAGC. UD DNA was amplified between attB1 and att5Br sites: Forward ATGCTAAAATACAAACCTTTAC, Reverse TTCAGCGGGCGAAAATCTTTTG.

#### 2.1.2. Domain-swaps

Domain-swapped mutants were produced using primers containing Ngn2 fused to NeuroD sequence, so that there was no artificial linker between the domains of the proteins. The PCR products of the N-terminal portion of the domain-swap were used as the forward primers in a second PCR reaction, using a plasmid encoding the other protein as the vector. The primers at the extreme N- and C-termini of the final domain-swapped product lie between BamHI and XhoI restriction sites, with a Kozak sequence before the initiation site.

#### 2.1.3. Primer sequences (where primers overlap, the Ngn2 sequence is in bold)

**N-Ngn/BC-NeuroD**, Ngn2 portion: Forward: ATGGTGCTGCTCAAGTG, Reverse: **TAAAGATCAAGAAGACC**AGACGCATGAAGGCAAA; N-Ngn/BC-NeuroD full protein: Forward: Ngn2 portion, Reverse: TTAATCATGAAAGAT.

**NB-Ngn/C-NeuroD**, Ngn2 portion: Forward: ATGGTGCTGCTCAAGTG, Reverse: **TTAGCGAAACTTTGCGC**TCCGGCAAAAGCCCAGA; NB-Ngn/C-NeuroD full protein: Forward: Ngn2 portion, Reverse: TTAATCATGAAAGAT.

**N-Ngn/BC-NeuroD** full protein: Forward: Ngn2 portion, Reverse: TTAATCATGAAAGAT. N-NeuroD/BC-Ngn, NeuroD portion: Forward: ATGACCAAATCGTATGGA, Reverse: TGGAGCGATTTAAAGTG**CGGCGCGTTAAAGCTAA**; N-NeuroD/BC-Ngn full protein: Forward: NeuroD portion, Reverse: TCAAATGAAAGCGCT.

**NB-NeuroD/C-Ngn**, NeuroD portion: Forward: ATGACCAAATCGTATGGA, Reverse: TTTCTGAGATTTTAAGG**CTTGGCGACCCAGTGCA**; NB-NeuroD/C-Ngn full protein: Forward: NeuroD portion, Reverse: TCAAATGAAAGCGCT.

For NgnNDNgn and NDNgnND proteins, the domain-swapped plasmids above were used as vectors for the PCR reaction of the C-terminal portion of the protein e.g. for NgnNDNgn the N-terminal Ngn2 PCR product (Forward: ATGGTGCTGCTCAAGTG, Reverse: **TAAAGATCAAGAAGACC**AGACGCATGAAGGCAAA) was used as the forward primer and the reverse primer was TTAATCATGAAAGAT, using NB-NeuroD/C-Ngn as the vector.

### 2.2. In Vitro Translation

TNT® SP6 quick coupled transcription/translation system (Promega), with $^{35}$S-methionine (GE Healthcare), was carried out according to the manufacturer's instructions.

### 2.3. Xenopus extracts

Activated interphase egg extracts [12], mitotic egg extracts [17] and neurula embryo extracts [18] were prepared as described previously.

### 2.4. Degradation assays

Degradation assays were performed as described previously [17].

### 2.5. Ubiquitylation assays

Ubiquitylation assays were performed as described previously [18].

### 2.6. Clustal W2 analysis

Clustal W2 analysis was carried out to align protein sequences [20].

### 2.7. Xenopus laevis embryos

Acquisition of *Xenopus laevis* embryos, preparation and injection of synthetic mRNA, staging of embryos and *in situ* hybridisation and qPCR were conducted as described previously [7,21].

### 2.8. Multiple comparison testing

Multiple comparison tests were carried out on the $\log_2$-transformed ratios of protein half-lives compared to wild type. Analysis was carried out with MATLAB® by one-way analysis of variance (ANOVA) followed by a multiple comparison test using the statistical output of the ANOVA. Statistical significance of the differences between the means was determined using a critical level of alpha of 0.05.