

ARTICLE ADDENDUM



## Do sodium channel proteolytic fragments regulate sodium channel expression?

Donatus O. Onwuli<sup>a,†</sup>, Laia Yañez-Bisbe<sup>b,†</sup>, Mel-lina Pinsach-Abuin<sup>b,c,d</sup>, Anna Tarradas<sup>b,c,d</sup>, Ramon Brugada<sup>b,c,d,e</sup>, John Greenman<sup>a</sup>, Sara Pagans<sup>b,c,d</sup>, and Pedro Beltran-Alvarez<sup>b</sup>

<sup>a</sup>Biomedical Sciences, School of Life Sciences, University of Hull, Kingston upon Hull, UK; <sup>b</sup>Cardiovascular Genetics Center, Institut d'Investigació Biomèdica de Girona (IDIBGI), University of Girona, Girona, Spain; <sup>c</sup>Medical Science Department, School of Medicine, University of Girona, Girona, Spain; <sup>d</sup>Centro de Investigación Biomédica en Red de Enfermedades Cardiovasculares (CIBERCV), Instituto de Salud Carlos III, Madrid, Spain; <sup>e</sup>Cardiology Service, Hospital Josep Trueta, Girona, Spain

### ABSTRACT

The cardiac voltage-gated sodium channel (gene: *SCN5A*, protein: Na<sub>v</sub>1.5) is responsible for the sodium current that initiates the cardiomyocyte action potential. Research into the mechanisms of *SCN5A* gene expression has gained momentum over the last few years. We have recently described the transcriptional regulation of *SCN5A* by GATA4 transcription factor. In this addendum to our study, we report our observations that 1) the linker between domains I and II (L<sub>DI-DII</sub>) of Na<sub>v</sub>1.5 contains a nuclear localization signal (residues 474–481) that is necessary to localize L<sub>DI-DII</sub> into the nucleus, and 2) nuclear L<sub>DI-DII</sub> activates the *SCN5A* promoter in gene reporter assays using cardiac-like H9c2 cells. Given that voltage-gated sodium channels are known targets of proteases such as calpain, we speculate that Na<sub>v</sub>1.5 degradation is signaled to the cell transcriptional machinery via nuclear localization of L<sub>DI-DII</sub> and subsequent stimulation of the *SCN5A* promoter.

### ARTICLE HISTORY

Received 26 May 2017  
Accepted 26 May 2017

### KEYWORDS

gene expression;  
mutagenesis; nuclear  
localization signal;  
transcription factor; voltage-  
gated sodium channel





## Introduction

Voltage-gated sodium channels are vital proteins in cardiac physiology. Upon changes in membrane potential, sodium channels open and enable the inward, depolarising sodium currents that underlie Phase 0 of the cardiomyocyte action potential.<sup>1</sup> Na<sub>v</sub>1.5, encoded by the *SCN5A* gene, is the pore-forming,  $\alpha$  subunit, of the cardiac voltage-gated sodium channel, and is necessary and sufficient to generate voltage-dependent, inward sodium currents. Na<sub>v</sub>1.5 is an essential protein and *Scn5A*  $-/-$  mice are not viable, while *Scn5A*  $+/-$  knockout mice display cardiac conduction defects and ventricular tachycardia.<sup>2</sup>

In humans, genetic variants in *SCN5A* have been linked to cardiac arrhythmias (atrial and ventricular fibrillation), sudden cardiac death syndromes (Brugada syndrome, long QT syndrome, sudden infant death syndrome) and other cardiac phenotypes (conduction defects, sick sinus syndrome).<sup>3</sup> We have recently shown that *SCN5A* expression is regulated by

the GATA4 transcription factor in the human heart.<sup>4</sup> Recent evidence suggests that abnormal *SCN5A* gene expression is associated with arrhythmogenic diseases (discussed in Tarradas *et al.* 2017),<sup>4</sup> and therefore the understanding of how *SCN5A* expression is controlled constitutes an important step forward in the field of cardiac diseases related to Na<sub>v</sub>1.5 dysfunction.

Na<sub>v</sub>1.5 is a large (2016 residues, *ca.* 220 KDa), hydrophobic, integral membrane protein that consists of 4 homologous domains (termed DI to DIV), joined by cytosolic interdomain linkers.<sup>1</sup> Great efforts over the past 7 y have shed light onto the structure of other voltage-gated sodium channels (mainly bacterial proteins),<sup>5,6</sup> and the first structure of an eukaryotic voltage-gated sodium channel  $\alpha$  subunit has recently been solved.<sup>7</sup> While these and other studies have provided invaluable insight into voltage-gating mechanisms and pore structure, the role and organization of cytosolic domains is less clear. The linker between

**CONTACT** Sara Pagans  [sara.pagans@udg.edu](mailto:sara.pagans@udg.edu)  Departament de Ciències Mèdiques, Universitat de Girona, C/ Emili Grahit, 77, 2n 17071 Girona, Spain; Pedro Beltran-Alvarez  [p.beltran-alvarez@hull.ac.uk](mailto:p.beltran-alvarez@hull.ac.uk)  University of Hull, Cottingham Road, Hull, HU6 7RX UK.

<sup>†</sup>These authors contributed equally as first authors.

Addendum to: Tarradas A, Pinsach-Abuin ML, Mackintosh C, Llorà-Batlle O, Pérez-Serra A, Batlle M, Pérez-Villa F, Zimmer T, Garcia-Bassets I, Brugada R, et al. Transcriptional regulation of the sodium channel gene (*SCN5A*) by GATA4 in human heart. *J Mol Cell Cardiol.* 2017; 102:74-82. <https://doi.org/10.1016/j.jmcc.2016.10.013>

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domains DI and DII ( $L_{DI-DII}$ ) of  $Na_v1.5$  contains 295 residues and has been of particular interest to us and other groups due to the fact that  $L_{DI-DII}$  undergoes post-translational modifications including phosphorylation and arginine methylation (for a recent review see<sup>8</sup>). Biochemical, genetic and electrophysiological studies suggest that  $L_{DI-DII}$  participates in the regulation of channel inactivation.<sup>9</sup>

Voltage-gated sodium channels have long been known to be regulated by proteases.<sup>10</sup> For example, calpain cleaves the brain isoform of the voltage-gated sodium channel,  $Na_v1.2$ , at  $L_{DI-DII}$ .<sup>11</sup> Calpain sodium channel fragments interact and localize at the plasma membrane hours after calpain activation,<sup>11</sup> suggesting that these fragments retain the protein-protein interactions that hold the sodium channel macromolecular complex together. However, there is currently no information on whether these fragments are subsequently degraded. A thought-provoking alternative is that sodium channel proteolysis creates new proteins with modified biologic activities. In this addendum, we further our understanding of *SCN5A* gene expression by showing that  $L_{DI-DII}$  contains a nuclear localization signal (NLS), localizes to the cell nucleus when expressed as an isolated domain in cardiac-like H9c2 cells, and increases *SCN5A* promoter activity *in vitro*.

## Results

### $L_{DI-DII}$ is predicted to be a target for calpain and contains an NLS

Previous reports have shown that  $L_{DI-DII}$  from  $Na_v1.2$  is a target for calpain. We used 2 published protease site prediction algorithms to search for possible calpain cleavage sites in the  $Na_v1.5$   $L_{DI-DII}$  sequence (residues 416–711).<sup>12,13</sup> Both algorithms predicted calpain cleavage after position E462. The other common hot spots for calpain cleavage in  $L_{DI-DII}$  were the regions

spanning residues 512–524, 573–579, and 630–644 (Fig. 1A).

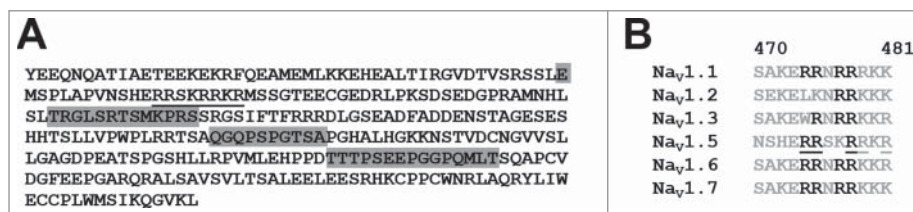
Inspection of the sodium channel  $L_{DI-DII}$  sequences revealed the presence of a conserved arginine-rich motif including residues 474–481 (Fig. 1A and B). We hypothesized that this motif could be a classical basic NLS. To test this hypothesis, we cloned the entire  $Na_v1.5$   $L_{DI-DII}$  as a FLAG-YFP fusion protein in a mammalian expression vector. We transfected this plasmid into H9c2 cells and observed  $L_{DI-DII}$  expression mainly in the nucleus (Fig. 2, left). When we mutated R474, R475, R478, R479 and R481 to K residues, the localization of the linker was no longer restricted to cell nuclei (Fig. 2, right). We repeated these transfections more than 10 times and reproducibly observed a similar fluorescence pattern.

### $L_{DI-DII}$ increases the activity of *SCN5A* promoter

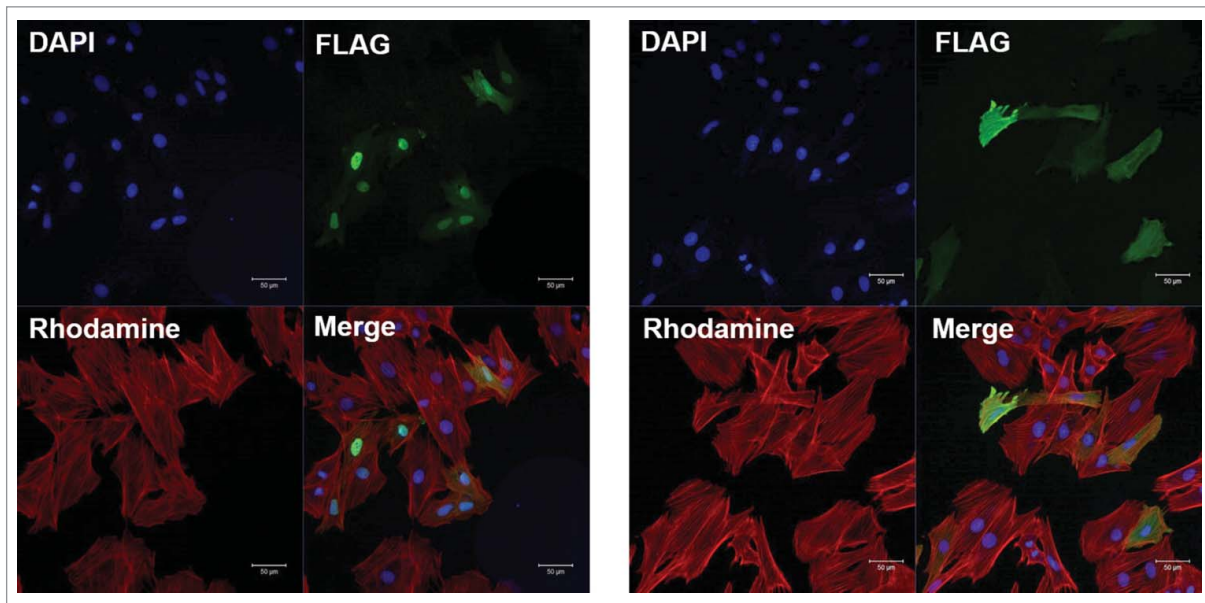
Why would an isolated cytosolic domain of a voltage-gated sodium channel localize to the cell nucleus? We asked whether  $L_{DI-DII}$  could modify *SCN5A* expression. We transfected H9c2 cells with a plasmid expressing luciferase under the control of the *SCN5A* promoter (“promoter A” in the original publication),<sup>4</sup> together with plasmids expressing either  $L_{DI-DII}$  with an intact NLS, or  $L_{DI-DII}$  with a mutated NLS (R474, R475, R478, R479 and R481 to K as above). Our luciferase assays showed that  $L_{DI-DII}$  with intact NLS, but not the mutated version, stimulated *SCN5A* promoter activity in a dose-dependent manner (Fig. 3).

## Discussion

In this addendum, we have built on our recently published investigation of *SCN5A* transcriptional regulation,<sup>4</sup> to identify another possible mechanism controlling *SCN5A* gene expression *i. e.*  $L_{DI-DII}$  stimulation of the *SCN5A* proximal promoter. We have



**Figure 1.** (A) Sequence of  $L_{DI-II}$  (residues 416–711 of  $Na_v1.5$ ) highlighting predicted calpain cleavage sites (E462, and regions 512–524, 573–579, and 630–644) as well as the putative NLS (underlined). (B) Sequence alignment of possible NLS in  $Na_v$  members ( $Na_v1.5$  numbering), highlighting conserved arginines (in black) and R residues 474, 475, 478, 479 and 481 (underlined).



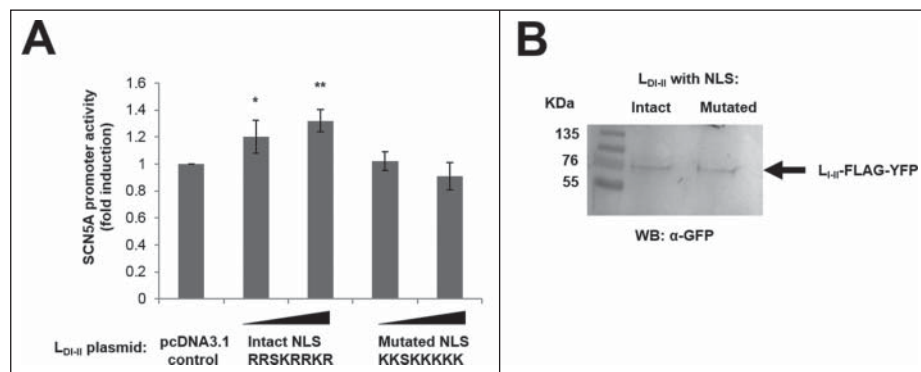
**Figure 2.** Representative confocal microscopy images of H9c2 cells transfected with  $L_{DI-II}$  including intact (left) and mutated (right) NLS. Scale bar corresponds to 50  $\mu\text{m}$ .

identified an NLS in  $L_{DI-DII}$  and provided evidence that nuclear  $L_{DI-DII}$  enhances *SCN5A* transcription. These new results should be regarded as preliminary at this stage given the nature of our cell and *in vitro* studies, but they raise the exciting possibility that *SCN5A* expression may be regulated by sodium channel proteolysis.

Proteolysis of sodium channel  $\beta$  subunits is known to regulate transcription of  $\alpha$  subunits such as  $\text{Na}_V1.1$ , the brain isoform of the voltage-gated sodium channel. The group of Kovacs, and others, demonstrated that 1) ADAM10 and BACE1 proteases cleave off the

extracellular domain of the  $\beta 2$  subunit; 2)  $\gamma$ -secretase releases the  $\beta 2$  intracellular domain, and 3) the  $\beta 2$  intracellular domain is internalised into the cell nucleus (by unknown mechanisms) and induces an increase in  $\text{Na}_V1.1$  mRNA and protein levels.<sup>14,15,16</sup> The  $\beta 1$  subunit is also a target for BACE1 *in vitro*,<sup>16</sup> and  $\beta 1$  silencing has recently been shown to result in decreased  $\text{Na}_V1.1$  mRNA (and protein) levels in cell models.<sup>17</sup>

NLS often consist of short arginine-rich sequences, and have been described in voltage-gated potassium channels, notably  $\text{K}_V10.1$ .<sup>18</sup> We have found that



**Figure 3.** (A) Luciferase experiments in H9c2 cells transfected with an *SCN5A* promoter-luciferase construct and increasing amounts (447 and 895 ng) of the indicated pcDNA3.1-based  $L_{DI-II}$  expression plasmids encoding for  $L_{DI-II}$  with intact and mutated NLS (residues 474–481). Luciferase values were normalized to renilla and are shown as fold induction relative to non-overexpressing control conditions (mean  $\pm$  SEM,  $n = 4$ ). Significance was examined by the t-test relative to control: \*  $p = 0.03$ , \*\*  $p = 0.001$ . (B) Representative Western blot showing expression of  $L_{DI-II}$  with intact and mutated NLS (895 ng plasmid each).

Na<sub>v</sub>1.5 L<sub>DI-DII</sub> localizes to the cell nucleus when expressed as an isolated domain in H9c2 cells, and that this nuclear localization is abrogated by mutation of 5 R residues to K in the sequence RRSKRKR spanning residues 474–481 of Na<sub>v</sub>1.5. To our knowledge, this is the first report of an NLS in  $\alpha$  subunits of voltage-gated sodium channels. Sequence alignment suggests that the NLS could be conserved among other members of the voltage-gated sodium channel family, including conservation of R474 and R475 in 4 and 5 members of the family, respectively, and an essential R478 (Na<sub>v</sub>1.5 numbering throughout, Fig. 1B). Taking together our new observations with previous reports, it is tempting to speculate that calpain (alone or in combination with other proteases) cleavage of  $\alpha$  subunits<sup>11,19</sup> creates L<sub>DI-DII</sub> fragments containing NLS, and that nuclear L<sub>DI-DII</sub> play a role in cardiac sodium channel transcriptional regulation, likely in combination with transcription factors.

Our approach was limited by the lack of information on precise calpain proteolytic site(s) within L<sub>DI-DII</sub>. Both algorithms used here predicted cleavage after E462, and we identified 3 possible calpain sites C-terminal to the NLS. Given these uncertainties, we decided to clone the complete 295-residue long L<sub>DI-DII</sub> domain and not smaller L<sub>DI-DII</sub> fragments. Mapping both calpain sites on L<sub>DI-DII</sub> experimentally and identifying the required sequences in L<sub>DI-DII</sub> necessary for activation of SCN5A transcription would greatly help design further experiments to understand the relevance of our results in more physiologic contexts. While acknowledging these weaknesses, this report raises the intriguing possibility that mutations and post-translational modifications of L<sub>DI-DII</sub> may control sodium channel activity not only at the electrophysiological level but also at the gene expression level. Bearing in mind that 1) the exact role of L<sub>DI-DII</sub> remains to be fully explored, 2) there are at least 63 disease-causing mutations in L<sub>DI-DII</sub>,<sup>20</sup> and 3) there are 15–20 phosphorylation sites,<sup>21,22</sup> as well as 3 arginine methylation sites (R513, R526 and R680)<sup>9</sup> in L<sub>DI-DII</sub>, our findings provide a new lens to look at the involvement of L<sub>DI-DII</sub> in Na<sub>v</sub>1.5 currents and arrhythmic diseases.

## Methods

### Cells and plasmids

Cardiac cells derived from embryonic rat ventricle (H9c2 cells) were maintained under standard cell

culture conditions. L<sub>DI-DII</sub> (residues 416–711) was amplified by PCR from a pCDNA3.1-based plasmid encoding for Na<sub>v</sub>1.5,<sup>9</sup> and cloned as a FLAG-YFP fusion into pCDNA3.1.<sup>23</sup> Site-directed mutagenesis was done using the Quikchange kit from Agilent Technologies, following the instructions of the manufacturer (<http://www.genomics.agilent.com/en/Site-Directed-Mutagenesis/QuikChange-Lightning/?cid=AG-PT-175&tabId=AG-PR-1162>). Sanger sequencing was performed in-house to ensure the introduction of the desired mutations. Cells were transfected using Lipofectamine 2000 (Thermo Fisher, <https://www.thermofisher.com/uk/en/home/brands/product-brand/lipofectamine/lipofectamine-2000.html>) and analyzed 24–48 h after transfection.

### Prediction of calpain sites in L<sub>DI-DII</sub>

We used recently published algorithms to predict calpain sites in L<sub>DI-DII</sub>. The FASTA sequence of L<sub>DI-DII</sub> was analyzed using the CaMPDB and GPS-CCD databases,<sup>12,13</sup> at score thresholds of 0.14 and 0.9, respectively.

### Confocal microscopy

Cells were fixed (4% paraformaldehyde) and permeabilised in 1% Triton-X100. We used an anti-FLAG antibody to detect L<sub>DI-DII</sub> (Sigma, <http://www.sigmaaldrich.com/catalog/product/sigma/f3165?lang=en&region=GB>), as well as fluorescent phalloidin to mark F-actin (Thermo Fisher, <https://www.thermofisher.com/order/catalog/product/A12379>) and DAPI for nuclear staining. Cells were visualised by confocal microscopy in a Zeiss LSM 710.

### Reporter assays

Luciferase / Renilla assays were performed as described in the original publication,<sup>4</sup> using L<sub>DI-DII</sub> plasmids. Results from 4 biologic replicates are reported here.

### Western blot

H9c2 cells were lysed in 1% NP-40 48 h after transfection. We used an anti-GFP antibody (Abcam, <http://www.abcam.com/gfp-antibody-chip-grade-ab290.html>) to detect L<sub>DI-DII</sub>-FLAG-YFP expression. Two biologic replicates were done.



## Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

## Acknowledgments

We thank Kath Bulmer for excellent technical assistance. The authors thank students who over the years have contributed to the present work, including David Roura and Oliver Taylor.

## Funding

DOO acknowledges Rivers state university of Science & Technology Port Harcourt Nigeria, and TETFund Nigeria (Academic staff Training and development unit) for funding. AT acknowledges a predoctoral fellowship from University of Girona (BR2012/47), MP-A a predoctoral fellowship from Generalitat de Catalunya (2014FI\_B 00586). This work was supported by the University of Hull, the Spanish Government (SAF2011-27627) and 7PM-PEOPLE Marie Curie International Reintegration Grant (PIRG07-GA-2010-268395).

## ORCID

Sara Pagans  <http://orcid.org/0000-0003-0772-6982>

Pedro Beltran-Alvarez  <http://orcid.org/0000-0001-8136-8963>

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