Do sodium channel proteolytic fragments regulate sodium channel expression?

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Abstract

The cardiac voltage-gated sodium channel (gene: *SCN5A*, protein: $\mathrm{Na_V1.5}$) 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 ($L_{DI\text{-}DII}$) of $\mathrm{Na_V1.5}$ contains a nuclear localisation signal (residues 474-481) that is necessary to localise $L_{DI\text{-}DII}$ into the nucleus, and 2) nuclear $L_{DI\text{-}DII}$ 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 $\mathrm{Na_V1.5}$ degradation is signalled to the cell transcriptional machinery *via* nuclear localisation of $L_{DI\text{-}DII}$ and subsequent stimulation of the *SCN5A* promoter.

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.[1] $\mathrm{Na_V1.5}$, encoded by the *SCN5A* gene, is the pore-forming, α subunit, of the cardiac voltage-gated sodium channel, and is necessary and sufficient to generate voltage-dependent, inward sodium currents. $\mathrm{Na_V1.5}$ is an essential protein and *Scn5A* -/- mice are not viable, while *Scn5A* +/- knockout mice display cardiac conduction defects and ventricular tachycardia.[2]

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).[3] We have recently shown that *SCN5A* expression is regulated by the

GATA4 transcription factor in the human heart.[4] Recent evidence suggests that abnormal *SCN5A* gene expression is associated with arrhythmogenic diseases (discussed in Tarradas *et al*. 2017),[4] and therefore the understanding of how *SCN5A* expression is controlled constitutes an important step forwards in the field of cardiac diseases related to $Na_V1.5$ dysfunction.

$Na_V1.5$ is a large (2016 residues, *ca*. 220 KDa), hydrophobic, integral membrane protein that consists of four homologous domains (termed DI to DIV), joined by cytosolic interdomain linkers.[1] Great efforts over the past 7 years have shed light onto the structure of other voltage-gated sodium channels (mainly bacterial proteins)[5, 6] and the first structure of an eukaryotic voltage-gated sodium channel α subunit has recently been solved.[7] While these and other studies have provided invaluable insight into voltage-gating mechanisms and pore structure, the role and organisation of cytosolic domains is less clear. The linker between domains DI and DII ($L_{DI\text{-}DII}$) of $Na_V1.5$ contains 295 residues and has been of particular interest to us and other groups due to the fact that $L_{DI\text{-}DII}$ undergoes post-translational modifications including phosphorylation and arginine methylation (for a recent review see ref. 8). Biochemical, genetic and electrophysiological studies suggest that $L_{DI\text{-}DII}$ participates in the regulation of channel inactivation.[9]

Voltage-gated sodium channels have long been known to be regulated by proteases.[10] For example, calpain cleaves the brain isoform of the voltage-gated sodium channel, $Na_V1.2$, at $L_{DI\text{-}DII}$.[11] Calpain sodium channel fragments interact and localise at the plasma membrane hours after calpain activation,[11] 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 biological activities. In this addendum, we further our understanding of SCN5A gene expression by showing that $L_{DI\text{-}DII}$ contains a nuclear localisation signal (NLS), localises to the cell nucleus when expressed as an isolated domain in cardiac-like H9c2 cells, and increases *SCN5A* promoter activity *in vitro*.

Results

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

Previous reports have shown that $L_{DI\text{-}DII}$ from $Na_V1.2$ is a target for calpain. We used two published protease site prediction algorithms to search for possible calpain cleavage sites in the $Na_V1.5$ $L_{DI\text{-}DII}$ sequence (residues 416-711).[12, 13] Both algorithms predicted calpain cleavage after position E462. The other common hot spots for calpain cleavage in $L_{DI\text{-}DII}$ were the regions spanning residues 512-524, 573-579, and 630-644 (Figure 1A).

Inspection of the sodium channel $L_{DI\text{-}DII}$ sequences revealed the presence of a conserved arginine-rich motif including residues 474-481 (Figures 1A and 1B). We hypothesised that this motif could be a classical basic NLS. To test this hypothesis, we cloned the entire $Na_V1.5$ $L_{DI\text{-}DII}$ as a FLAG-YFP fusion protein in a mammalian expression vector. We transfected this plasmid into H9c2 cells and observed $L_{DI\text{-}DII}$ expression mainly in the nucleus (Figure 2, left). When we mutated R474, R475, R478, R479 and R481 to K residues, the localisation of the linker was no longer restricted to cell nuclei (Figure 2, right). We repeated these transfections more than 10 times and reproducibly observed a similar fluorescence pattern.

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

Why would an isolated cytosolic domain of a voltage-gated sodium channel localise to the cell nucleus? We asked whether $L_{DI\text{-}DII}$ 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),[4] together with plasmids expressing either $L_{DI\text{-}DII}$

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 (Figure 3).

Discussion

In this addendum, we have built on our recently published investigation of *SCN5A* transcriptional regulation,[4] to identify another possible mechanism controlling *SCN5A* gene expression *i.e.* L_{DI-DII} stimulation of the *SCN5A* proximal promoter. We have 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 β subunits is known to regulate transcription of α subunits such as $Na_V1.1$, 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 β2 subunit; 2) γ-secretase releases the β2 intracellular domain, and 3) the β2 intracellular domain is internalised into the cell nucleus (by unknown mechanisms) and induces an increase in $Na_V1.1$ mRNA and protein levels.[14, 15, 16] The β1 subunit is also a target for BACE1 *in vitro*,[16] and β1 silencing has recently been shown to result in decreased $Na_V1.1$ mRNA (and protein) levels in cell models.[17]

NLS often consist of short arginine-rich sequences, and have been described in voltage-gated potassium channels, notably $K_V10.1$.[18] We have found that $Na_V1.5$ L_{DI-DII} localises to the cell nucleus when expressed as an isolated domain in H9c2 cells, and that this nuclear localisation is abrogated by mutation of five R residues to K in the sequence RRSKRRKR spanning residues 474-481 of $Na_V1.5$. To our knowledge, this is the first report of an NLS in α 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 four and five members of the family, respectively, and an essential R478 ($Na_V1.5$ numbering throughout, Figure 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 α subunits[11, 19] creates L_{DI-DII} fragments containing NLS, and that nuclear L_{DI-DII} 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_{DI-DII}. 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_{DI-DII} domain and not smaller L_{DI-DII} fragments. Mapping both calpain sites on L_{DI-DII} experimentally and identifying the required sequences in L_{DI-DII} necessary for activation of *SCN5A* transcription would greatly help design further experiments to understand the relevance of our results in more physiological contexts. While acknowledging these weaknesses, this report raises the intriguing possibility that mutations and post-translational modifications of L_{DI-DII} 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_{DI-DII} remains to be fully explored, 2) there are at least 63 disease-causing mutations in L_{DI-DII},[20] and 3) there are 15-20 phosphorylation sites,[21, 22] as well as 3 arginine methylation sites (R513, R526 and R680)[9] in L_{DI-DII}, our findings provide a new lens to look at the involvement of L_{DI-DII} in $Na_V1.5$ currents and arrhythmogenic diseases.

Methods

Cells and plasmids

Cardiac cells derived from embryonic rat ventricle (H9c2 cells) were maintained under standard cell culture conditions. $L_{DI\text{-}DII}$ (residues 416-711) was amplified by PCR from a pCDNA3.1-based plasmid encoding for $Na_V1.5$,[9] and cloned as a FLAG-YFP fusion into pCDNA3.1.[23] 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 analysed 24-48 h after transfection.

Prediction of calpain sites in $L_{DI\text{-}DII}$

We used recently published algorithms to predict calpain sites in $L_{DI\text{-}DII.}$ The FASTA sequence of $L_{DI\text{-}DII}$ was analysed using the CaMPDB and GPS-CCD databases,[12, 13] 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 $L_{DI\text{-}DII}$ (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,[4] using $L_{DI\text{-}DII}$ plasmids. Results from 4 biological 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 $L_{DI\text{-}DII}$-FLAG-YFP expression. Two biological replicates were done.

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Disclosure statement

The authors have declared no conflict of interest.

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Legends

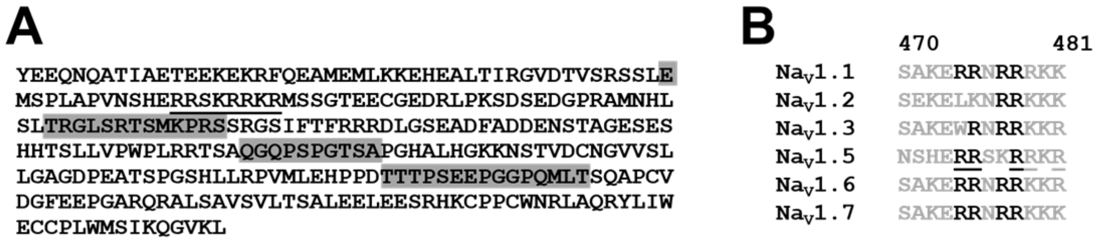
Figure 1. A. Sequence of $L_{DI\text{-}II}$ (residues 416-711 of $Na_V1.5$) highlighting predicted calpain cleavage sites (E462, and regions 512-524, 573-579, and 630-644) as well as the putative NLS (underlined). **B.** Sequence alignment of possible NLS in Na_V members ($Na_V1.5$ numbering), highlighting 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 $L_{DI\text{-}II}$ including intact (left) and mutated (right) NLS. Scale bar corresponds to 50 µm.

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 $L_{DI\text{-}II}$ expression plasmids encoding for $L_{DI\text{-}II}$ 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 ± 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 $L_{DI\text{-}II}$ with intact and mutated NLS (895 ng plasmid each).

Figures

Figure 1



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	470 481
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$Na_V1.2$	SEKELKNRRKKK
$Na_V1.3$	SAKEWRNRRKKR
$Na_V1.5$	NSHERRSKRRKR
$Na_V1.6$	SAKERRNRRKKR
$Na_V1.7$	SAKERRNRRKKK

Figure 2

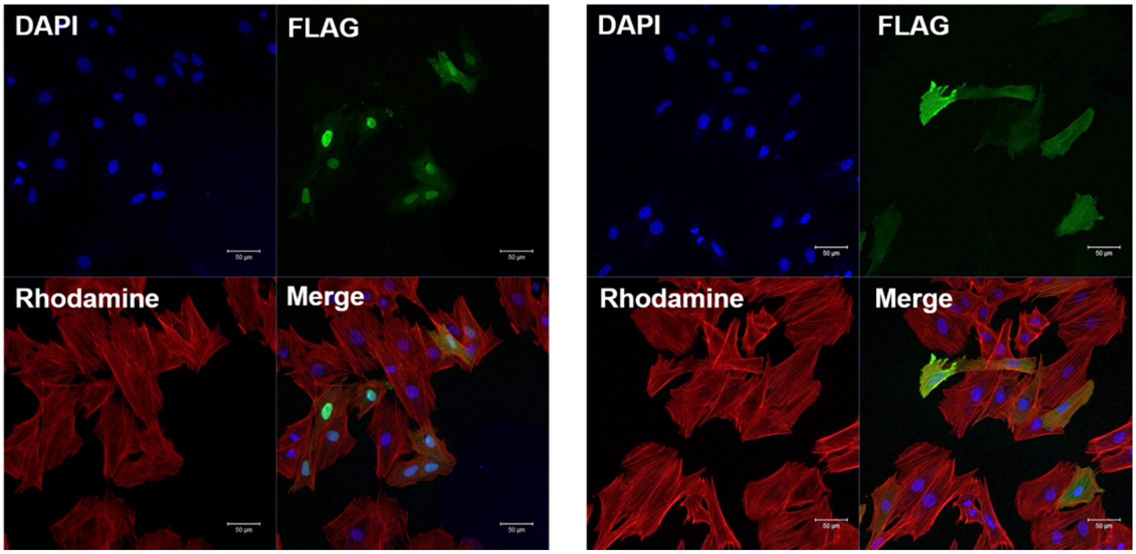
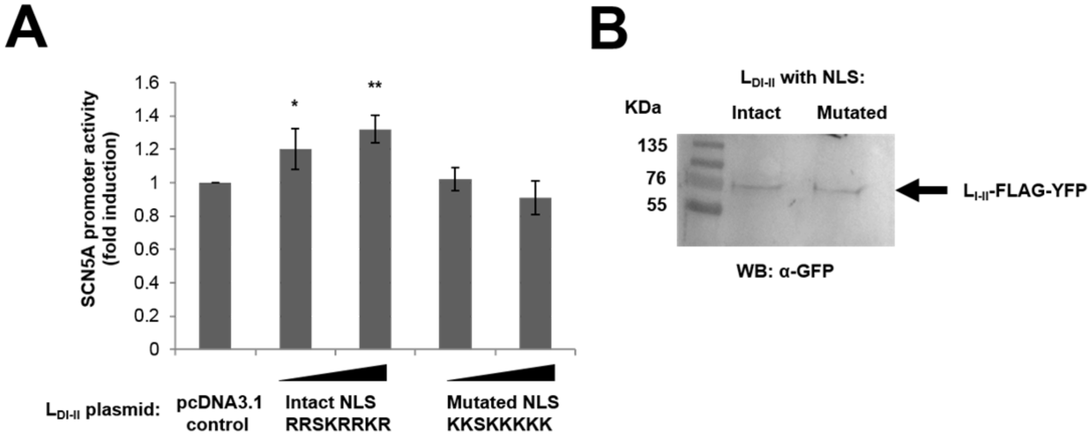


Figure 3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1.4
1.2
1
0.8
0.6
0.4
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0
*
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pcDNA3.1 control
Intact NLS RRSKRRKR
Mutated NLS KKSKKKKK
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L_{DI-II} with NLS:
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Mutated
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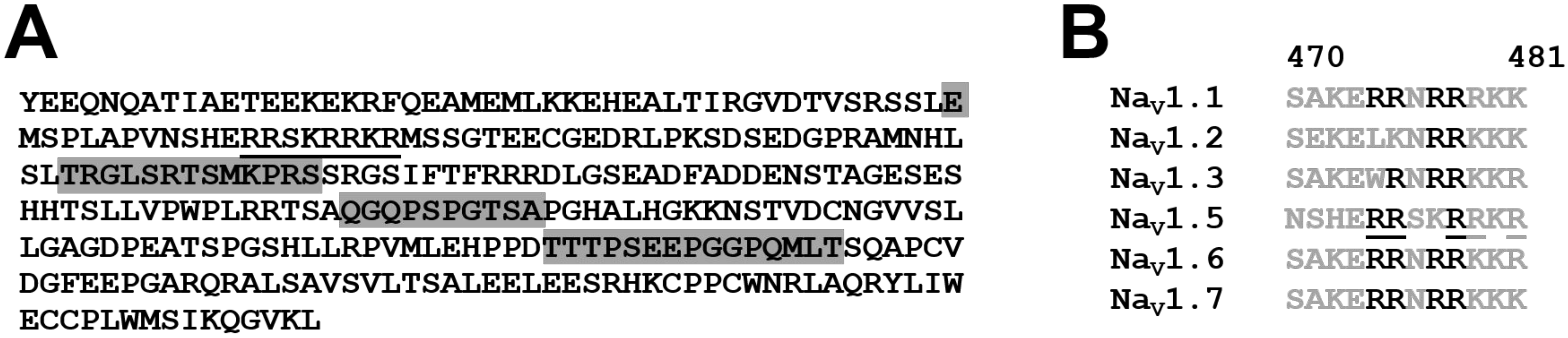
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B
470
481
Na$_V$1.1 SAKERRNRRRKK
Na$_V$1.2 SEKELKNRRKKK
Na$_V$1.3 SAKEWRNRRKKR
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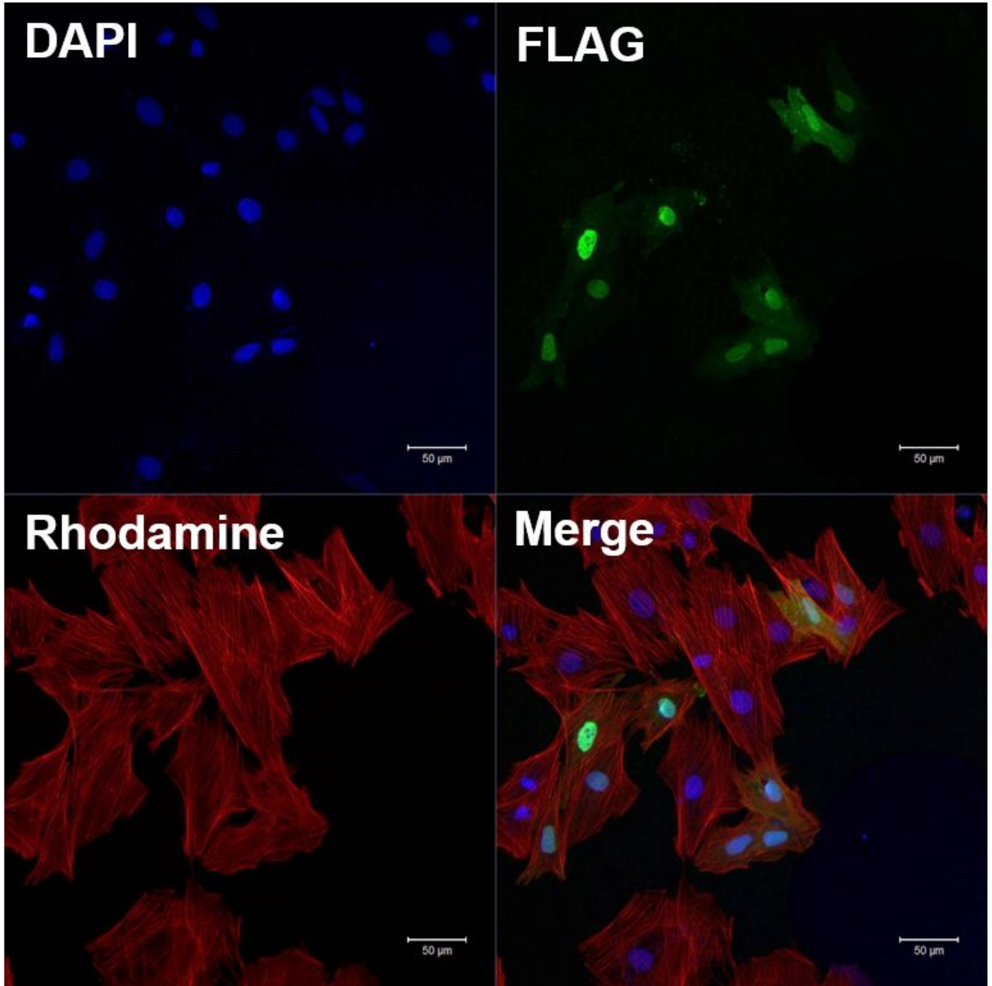
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FLAG
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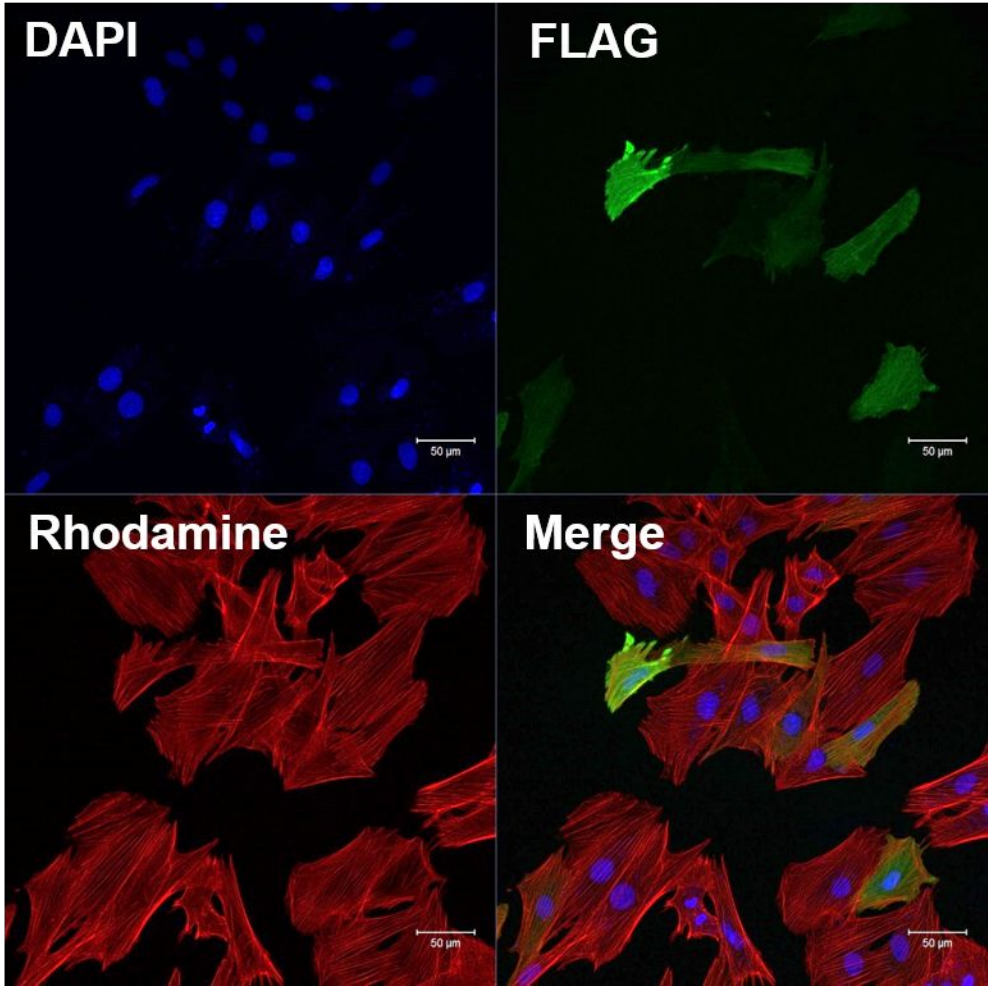
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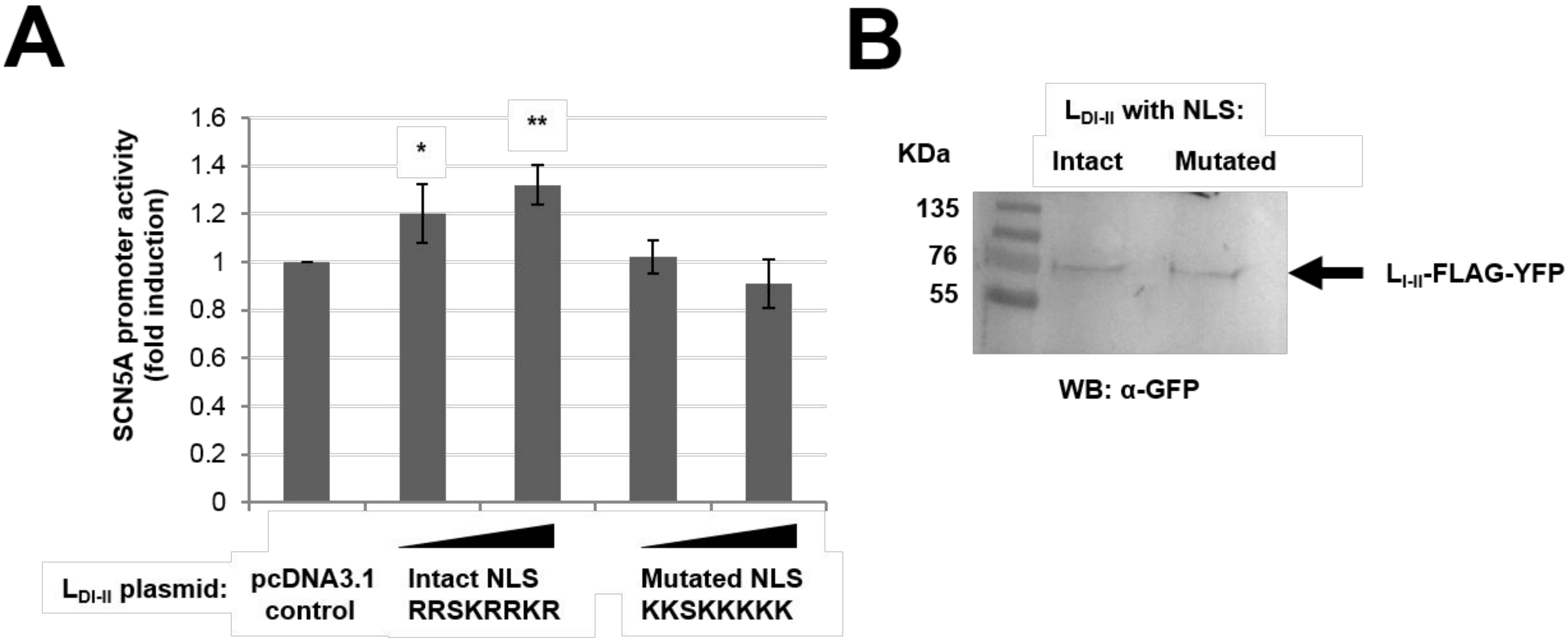
A
SCN5A promoter activity (fold induction)
1.6
1.4
1.2
1
0.8
0.6
0.4
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0
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**
L$_{DI-II}$ plasmid:
pcDNA3.1 control
Intact NLS RRSKRRKR
Mutated NLS KKSKKKKK
B
L$_{DI-II}$ with NLS:
Intact
Mutated
KDa
135
76
55
L$_{I-II}$-FLAG-YFP
WB: α-GFP