RESEARCH ARTICLE

SUMOylation of Pax7 is essential for neural crest and muscle development

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Abstract Regulatory transcription factors of the Pax family play fundamental roles in the function of multipotent cells during vertebrate development, post-natal regeneration, and cancer. Pax7 and its homologue Pax3 are important players in neural crest and muscle development. Both genes are coexpressed in various tissues and are thought to provide similar, but not identical, functions. The mechanisms that allow specific regulation of Pax7 remain largely unknown. Here, we report for the first time that Pax7 is regulated by SUMOylation. We identify the interaction of Pax7 with Ubc9, the SUMO conjugating enzyme, and reveal that SUMOylation machinery is enriched in neural crest precursors and plays a critical role in NC development. We demonstrate that Pax7 becomes SUMOylated and identify an essential role for lysine 85 (K85) in Pax7-SUMOylation. Despite high conservation surrounding K85 amongst Pax genes, we were unable to identify SUMOylation of other Pax proteins tested, including Pax3. Using a non-SUMOylatable Pax7 variant (K85 X R), we demonstrate that SUMOylation is essential for the function of Pax7 in neural crest development, C2C12 myogenic differentiation, and transcriptional transactivation. Our study provides new mechanistic insight into the molecular regulation of Pax7's function by SUMOylation in neural crest and muscle development.

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Introduction

Pax genes emerged from the landmark study by Christiane Nüslein-Volhard and Eric Weischaus that identified genes essential for development [1]. Pax proteins contribute to embryonic development, organogenesis, and adult tissue homeostasis and regeneration. Furthermore, the expression of some *Pax* genes is essential for the growth and survival of certain cancers [2–4]. Despite these fundamental roles in mammalian tissues, the regulation of Pax proteins is not well understood.

Pax proteins contain a distinguishing paired box [5] that binds DNA [6] through two distinctive domains [7–9]. The family of 9 vertebrate Pax genes has been organized into four subgroups based on additional structural features, and their members often display overlapping expression and function [10]. Pax3 and Pax7, which form the subfamily III, display highly homologous N-terminus regions containing a paired domain, octapeptide domain, and a paired type-homeodomain. This N-terminus domain mediates DNA binding properties, and therefore it has been suggested that Pax3 and Pax7 target similar genes.

Both Pax3 and Pax7 are expressed and function in the developing central nervous system, neural crest (NC), and muscle progenitors. Pax3 mutations in mice and humans lead to various NC defects and in some cases myogenic deficiencies. Pax7 is required for NC development in mouse and

chick [11, 12], and plays a pivotal role in adult muscle homeostasis and regeneration [13–18]. Pax3 and Pax7 mimic each other's function in most of these circumstances; yet, important divergent functions between these Pax genes have been identified (reviewed in [19]).

Molecularly, few differences have been noted between Pax3 and Pax7. Pax3, but not Pax7, is regulated by mono-ubiquitination of the C-terminus transactivation domain [20]. In addition, differential binding of interacting partners may lead to distinct functions of Pax3 and Pax7 [2, 19, 21].

To further characterize the mechanism of action of Pax7-specific functions, we sought to identify interacting partners for Pax7. Here, we identified the interaction of Pax7 with the E2 SUMO-conjugating enzyme, Ubc9. Our data demonstrate that Pax7, but not Pax3, is regulated by SUMOylation. We further demonstrate that SUMOylation of Pax7 is essential for neural crest development in chick embryos and for the inhibitory roles of Pax7 in C2C12-muscle differentiation. These data implicate post-translational modification of Pax7 as an essential mechanism for the regulation of Pax7 during development of multiple tissues.

Materials and methods

Yeast two-hybrid screening

All vectors, yeast strains, reagents, and methods were derived from the MATCHMAKER Two-Hybrid System (Clontech, Palo Alto, CA, USA). PCR-generated chick Pax7 full length was inserted into pGBKT7 (EcoRI and BamHI) in-frame with the DNA binding domain of GAL4 at its 3′ end, to generate pGBKT7-Pax7. Screening was performed using the Pax7-Gal4 DNA binding domain fusion protein expressed from pGBKT7-Pax7 as the bait. A stage 8 chick whole embryo cDNA library was constructed and screened according to the manual. Yeast AH109 was co-transformed with pGBKT7-Pax7, chick whole embryo library cDNA, and linearized pGADT7-Rec, using a high-efficiency lithium acetate/poly-ethylene glycol method. Positive colonies were selected on SD/-Ade/-His/-Leu/-Trp/+8 mM 3-AT plates and assayed for β-galactosidase activity. Secondary screens were performed in a similar manner to minimize false positives, and the bait was expelled through saturation growth in SD-Leu. Positive interacting colonies were recovered, sequenced, and matched to known sequences using BLAST (National Centre for Biotechnology Information, NCBI).

GST pull

Expression of the GST-fusion proteins was induced in BL21 bacteria (Stratagene) with isopropyl-D-galactoside for 3 h at 37 °C. Bacterial lysates were harvested and purified on Sepharose 4B glutathione beads (Amersham Pharmacia Biotech). Bead-immobilized GST-tagged proteins were incubated with the bacterial lysates expressing HA-tagged Pax7 binding proteins for 3 h, washed four times in lysis buffer supplemented with a protease inhibitor mixture, and 1 mM DTT, solubilized in $2\times$ sample buffer, and subjected to western blot analysis.

Cell culture and transfection

293T cells and Murine C2C12 myoblasts were grown in DMEM, 10 % FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5 % CO_2. C2C12 differentiation was induced on 90 % confluent cultures with differentiation medium (DMEM, 2 % horse serum, 4 mM L-glutamine) and analyzed after 96 h. Lipofectamine 2000 (Invitrogen) was used for transient transfection, and stable Pax7-expressing cells were selected using G418 at 1,000 μg/ml.

Electrophoretic mobility shift assay (EMSA)

293T cells were transfected with pCIG-Pax7 or pCIG-Pax7^{K85R} for 48 h to prepare nuclear extracts (Nuclear and Cytoplasmic Extraction Kit; Pierce Biotech, Rockford, IL, USA). Pax binding oligonucleotides were labeled using the Biotin 30-End DNA Labeling Kit (Pierce Biotech), and LightShift Chemi-luminescent EMSA Kit TM (Pierce Biotech) was used. Briefly, 20 fmol biotin-labeled probes were incubated with cell nuclear extract, 50 μg/ml poly (dI–dC), 5 % glycerol, 0.05 % NP-40, 5 mM MgCl2 in binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM KCl, 1 mM DTT) for 20 min at room temperature. As required, 1 μg FLAG antibody (F1804; Sigma–Aldrich) was added and incubated for additional 30 min at room temperature. Samples were run in 6 % polyacrylamide DNA retardation gels and transferred to a positively charged nylon membrane (Immobilon-NY+ ; Millipore, Bedford, MA, USA). After UV crosslinking, detection was performed with streptavidin–horseradish perioxidase (HRP)-antibody (Pierce Biotech).

SUMO conjugation assay

293T cells, transiently transfected with Pax7-Myc or empty vector (pcDNA), Ubc9 and HA-SUMO-1 (48 h) lysed in immunoprecipitation buffer 1 (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5 % (w/v) sodium deoxycholate, 1 % NP40, 0.1 % SDS, 20 mM NEM, 10 μg/mL leupeptin, 10 μg/mL pepstatin A and 10 μg/mL aprotinin), were sonicated and clarified by centrifugation. Lysates were incubated with an anti-Myc monoclonal antibody to immunoprecipitate tagged Pax7 followed by

SDS-PAGE and immunoblotting with anti-HA (HA-SUMO-1).

Western blots

Protein extracts were run on SDS–polyacrylamide gels and electrophoretically transferred to PDVF membranes (Schleicher & Schuell, Dassel, Germany), blocked with 5 % nonfat milk in Tris-buffered saline containing 0.1 % Tween 20 and incubated with each primary antibody overnight at 4 °C. Finally, blots were incubated with HRP-linked secondary antibody (cell signaling) and monitored for chemiluminescence (ECL kit; GE Healthcare).

Mutagenesis

Pax7 and Pax3K for R substitutions were generated with Quikchange II XL site-directed mutagenesis (Stratagene) using pCDNA3.1A-Pax7 or Pax3 as a template, and confirmed by DNA sequencing.

Morpholino electroporation

Morpholinos (Gene Tools) were used at 0.48 μM in 3 % sucrose with 1 μg of DNA (pCIG) as carrier and fast green, injected and electroporated [22] (5 square electroporation pulses of 5 mV of 50 ms with 100 ms rest) into St 4 embryos immobilized in filter paper rings. Embryos were EC-cultured [23] overnight and analyzed by in situ hybridization or immunofluorescence.

In situ hybridization

Whole mount in situ hybridization was performed as previously described [24].

Immunofluorescence, microscopy and imaging

Immunofluorescence was performed according to standard protocols [25]. Briefly, embryos were fixed in 4 % PF, rinsed and washed (3 × 60 min) in PBT (PBS, 2 mg/ml BSA, and 0.1 % Tween), and blocked overnight in PBT+ 10 % FBS. Incubation with primary and secondary antibodies was performed at 4 °C overnight. Isotype specific Alexa secondary Abs were used at 1:2,000. Dissections were performed on Nikon stereo-microscopes, and images were acquired with a Spot SE camera and software using a Nikon Eclipse 80i microscope, and processed with Photoshop.

Q-rt-pcr

RNA (Trizol; Agilent Technologies-Stratagene) was used to generate cDNA (AMV reverse transcriptase; Promega, San Luis Obispo, CA, USA) with poly-dT primers. RT-qPCR was performed on a iQ5 Real Time PCR Detection System (Bio-Rad).

Sequences and antibodies

Morpholino target	Target site relative to start coden	Sequence
Ubc9-morpholino-NM_204265.	-4 thru +21	ACTTAGAGCTATGCCAGACATATTC
Ubc9-5MM morpholinos		ACTTAcAGgTATcCCAGAgATAaTC
Pax7-morpholino-NM_205065.1	-44 thru-22	TCCGTGCGGAGCGGGTCACCCC
Pax7- 5MM morpholinos		TCgGTcCGGAGccGGTgACaCCC

EMSA probes	
P6CON	TGGAATTCAGGAAAAATTTTCACGCTTGAGTTCACAGCTCGAGTA
P3OPT	TGGTGGTCACGCCTCATTGAATATTA
P2	GATCCTGAGTCTAATTGATTACTGTACAGG
P1/2	GATCCTGAGTCTAATTGAGCGTCTGTAC
PHO	GATTTCTTCCAATTAGTCACGCTTGAGTG

| Name	Dilution	Catalogue #	Name of company
Anti-HA ms IgG2b	(1:1,000)	3992-200	BioVision
Anti-Myc ms IgG2a	(1:1,000)	sc-47694	Santa Cruz
Anti-DDK (Flag) ms IgG1b	(1:1,000)	TA50011	ORIGENE
Anti-mouse IgG HRP-linked	(1:10,000)	NXA931	GE Healthcare
Anti-Ubc9 Rb	(1:400)	sc-10759	Santa Cruz
Anti-SUMO-1 Rb	(1:200)	sc-9060	Santa Cruz
Anti-Pax7 ms IgG1	(1:100)	Pax7	DSHB (University of Iowa)
Light meromy ms IgG2b	(1:100)	MF20	DSHB (University of Iowa)
Myosin S1 ms IgG2a	(1:100)	A4.1025	DSHB (University of Iowa)
Anti-Sox2 gt	(1:300)	AF2018	R&D
Anti-Ap2 ms IgG2b	(1:10)	3B5	DSHB (University of Iowa)
Anti-Caspase 3 Rb	(1:500)	aa163-175	R&D
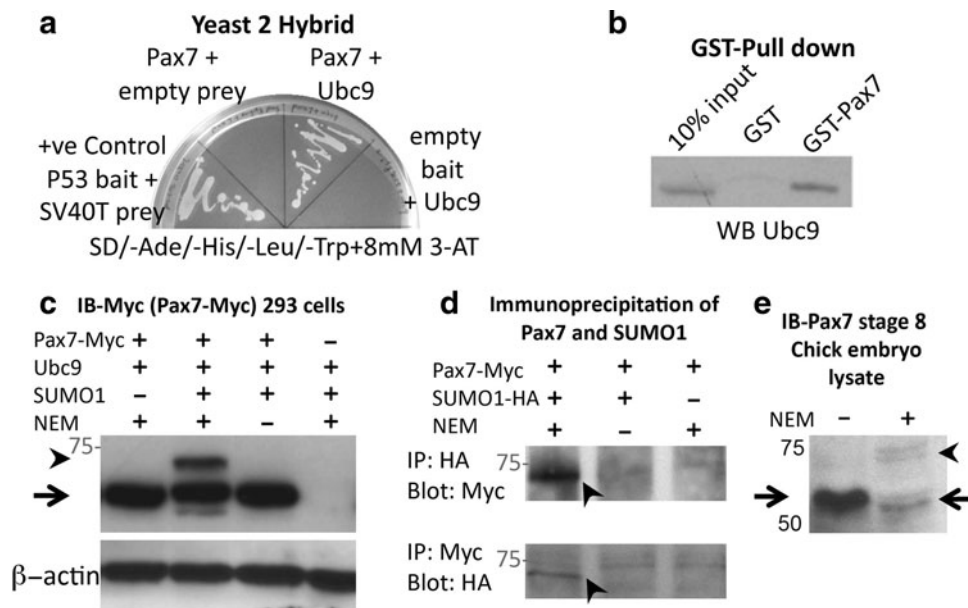
Fig. 1 Pax7 interacts with Ubc9, and is SUMOylated. **a** Pax7 interaction with Ubc9 identified by yeast two-hybrid screen, and **b** verified with full-length Ubc9 by GST-Pax7 pull-down. **c** Western blot (Myc antibody) of SUMO-conjugation assays in 293T cells cotransfected with Pax7-Myc, Ubc9, and HA-SUMO1, lysed with/without NEM, a SUMOylation-stabilizing compound; *Arrow* Pax7, *arrowhead* SUMOylated Pax7. **d** Co-immunoprecipitation of Pax7 and SUMO from 293T cells cotransfected with Pax7-Myc, Ubc9, and HA-SUMO1, lysed in the presence of NEM. *Top panel* anti-HA IP followed by anti-Myc WB, reversed in *bottom panel*. **e** Endogenous Pax7 SUMOylation observed by WB of stage 8 chick embryos lysed with/without NEM, using an anti-Pax7 antibody, *Arrow* indicates Pax7, *arrowhead* SUMOylated Pax7

Results

Pax7 interacts with Ubc9 and is SUMOylated

To improve our understanding of the molecular mechanisms that regulate Pax7 function, we sought to identify interacting proteins. To this end, we carried out a yeast two-hybrid screen using Pax7 as bait against a chick cDNA library. We identified Ubc9, the only E2 SUMO-conjugating enzyme [26], as a Pax7-interacting protein (Fig. 1a). GST pull-down experiments showed that full-length Ubc9 specifically interacts with Pax7-GST, and not with GST alone (Fig. 1b).

Since Ubc9 is critical for the conjugation of small ubiquitin-like modifier (SUMO) motifs to target proteins in a dynamic and reversible fashion [27, 28], we explored the possibility that Pax7 is SUMOylated by performing SUMO-conjugation experiments. We cotransfected 293T cells with myc-tagged Pax7 (Pax7-Myc), Ubc9, and SUMO1. Western blot analysis revealed the appearance of a species of Pax7 with slower mobility (≈ 70 kDa). This band requires the expression of SUMO1, and the presence of the sulfhydry-blocking agent *N*-ethylmaleimide (NEM) that stabilizes SUMOylated conjugates [29] (Fig. 1c). This slower mobility band of Pax7 is therefore thought to correspond to SUMOylated-Pax7.

To confirm that Pax7 is SUMOylated, we performed reciprocal co-immunoprecipitation assays in 293T cells. Blotting against the HA tag on SUMO-1 (SUMO1-HA) following immunoprecipitation of Pax7-Myc, or the reverse experiment, confirmed the association between Pax7 and SUMO1 (Fig. 1d). To investigate if Pax7 is SUMOylated in vivo, we performed western blot analysis of total protein extracts from stage 8 chick embryos [31]. This revealed additional slower mobility Pax7-isoforms (≈ 70 kDa) in the presence of NEM that correspond to stabilized SUMO-conjugated Pax7 (Fig. 1e). These results demonstrate that Pax7 interacts with Ubc9; that this interaction leads to the SUMOylation of Pax7, and that Pax7 is SUMOylated in vivo in chick embryos.

Ubc9, SUMO1 and Pax7 are coexpressed by prospective neural crest

In chick embryos, Pax7 expression starts after gastrulation, marking NC precursors in the neural plate border, and is later maintained in the neural folds [12, 30]. To ascertain if Pax7 and Ubc9 could interact in vivo during NC development, we analyzed the expression of these proteins in chick embryos. While in situ hybridization on stages 4–6 [31] revealed a broad expression of *Ubc9* mRNA throughout the embryo, at stage 8 we identified an
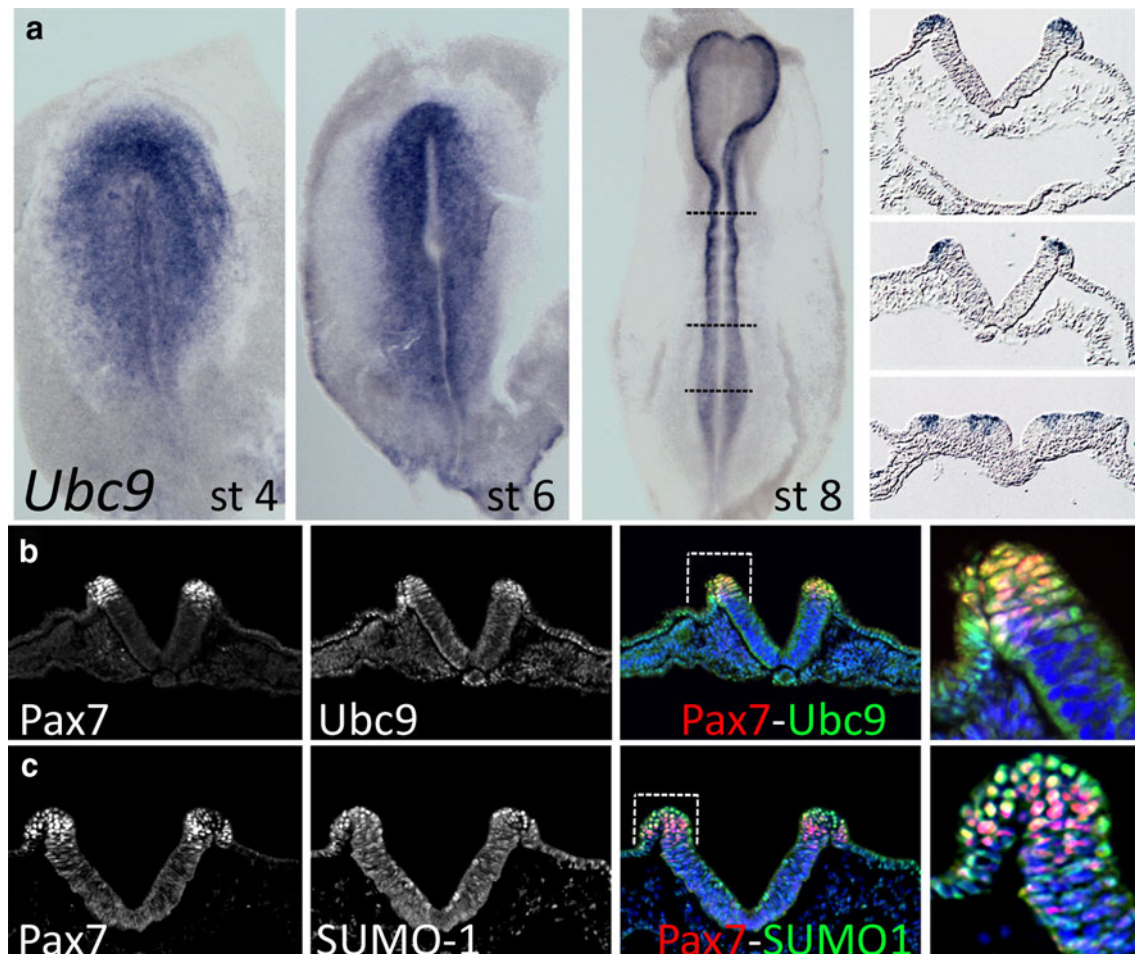
Fig. 2 Ubc9 and SUMO-1 co-localizes with Pax7 in NC precursors at the neural folds. **a** *Ubc9* expression (in situ hybridization) in chick embryos at early stages (*st*). Sections at the indicated level of the st 8 embryo are shown on right. **b** Ubc9 and **c** SUMO1 proteins are coexpressed with Pax7 in NC precursors of the neural fold of st 8 embryos. *Framed regions* of merged images are shown at higher magnification

enrichment of *Ubc9* in the neural folds (Fig. 2a). Furthermore, immunofluorescence analysis of sections of chick embryos demonstrated that neural fold cells coexpress Pax7 with Ubc9 (Fig. 2b) and SUMO-1 (Fig. 2c). These studies demonstrate that Pax7, Ubc9, and SUMO1 are coexpressed in NC precursors, and reveal an unexpected enrichment of SUMOylation machinery in these precursors.

**The SUMO pathway is crucial for neural crest development**

Genetic and biochemical studies have linked SUMOylation to craniofacial defects [32], such as cleft lip/palate ([33–36], although see [37, 38]) and heart [39] malformations. Craniofacial and heart structures receive important contributions from NC, and several NC-related transcription factors are also SUMOylated [34]. Our observed unexpected enrichment of Ubc9 expression in the neural

folds, suggests that perhaps the SUMOylation pathway may be required during early NC development.

To test this hypothesis, we used sequence-specific morpholinos (Mo) to reduce Ubc9 levels in chick embryos. Unilateral electroporation of morpholinos provide an internal control in the untreated contralateral side. A 5-mismatch Ubc9-control morpholino (5MM-Mo) had minimal or no effect on NC development as indicated by the normal mRNA expression of the NC markers *Snail2* (1 out of 9 embryos displayed abnormal pattern, 1/9), *Sox9* (1/10), and *FoxD3* (1/8) (Fig. 3a, c, e). Conversely, Ubc9-Mo led to reduced expression of mRNA encoding *Snail2* (7/9), *Sox9* (5/6), and *FoxD3* (6/10) in the anterior neural folds (Fig. 3b, d, f).

To further analyze the effects of Ubc9-Mo treatment, we monitored the expression of earlier markers associated with the neural plate (Sox2) or its border (Pax7, AP2), as well as the apoptosis marker Caspase 3 (Casp3). To afford the study of multiple markers by immunofluorescence in a

Fig. 3 SUMO pathway is required for neural crest development. Unilateral electroporation of 5 mismatch control morpholinos have no effect on the expression of NC markers *Snail2* (**a**), *Sox9* (**c**), and *FoxD3* (**e**), while Ubc9-morpholino downregulates their expression (*Snail2* (**b**), *Sox9* (**d**), and *FoxD3* (**f**). *Insets* show the localization of morpholinos (*green*) before in situ hybridization. *Right panels* show higher-magnification of framed regions, *arrows* point to untreated internal control signal and *brackets* demarcate treated areas. The number of embryos with altered expression over the total of embryos treated indicated in *right bottom corner*. Representative sections from embryos electroporated with control-Mo (**g–i**) and Ubc9-Mo (**j–l**), showing *FoxD3* in situ hybridization (**g**, **j**), and immunofluorescence for Pax7 and Sox2 (**h**, **k**), and Pax6, Sox2 and Ap2α (**l**, **j**). While Ubc9-Mo treatment reduced *FoxD3* in the unilaterally treated neural fold (**j**), no effects were noted for the earlier neural crest markers Pax7 and Ap2, nor for the neural marker Sox2 (**k**, **l**)
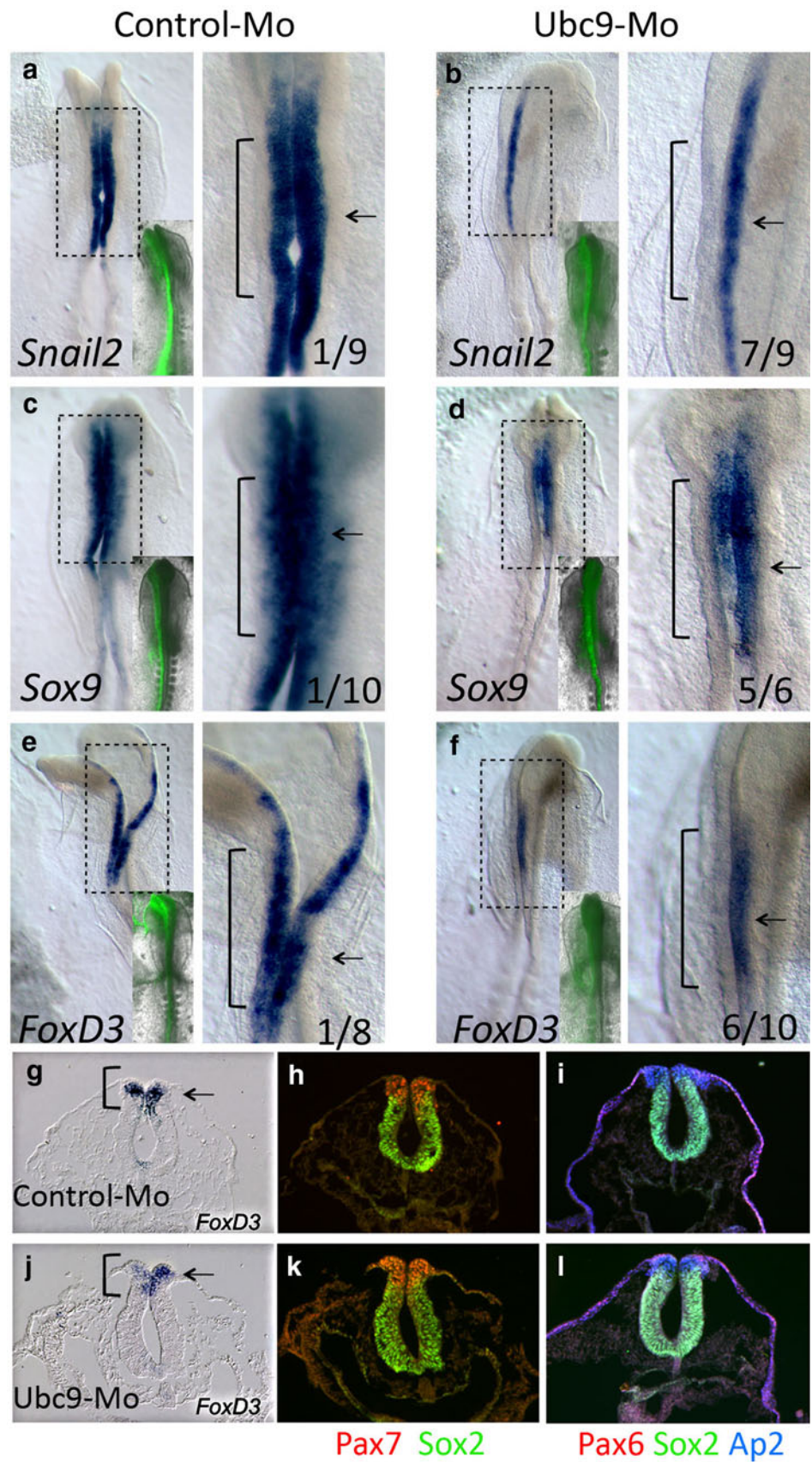
restricted region, we generated sets of sequential sections from Ubc9-Mo treated embryos ($n = 14$) and 5MM-Mo control embryos ($n = 4$) that had previously been processed for in situ hybridization (*Snail2*, *Sox9*, or *FoxD3*). We acquired images of 4–6 sections per embryo and analyzed the specific signal for each marker counterstained with DAPI to visualize nuclei. Embryos electroporated with control-Mo displayed similar expression of Pax7, AP2, and Sox2 in treated and contralateral neural folds. Similarly, the majority of embryos treated with Ubc9-MO (9/14) display even expression of Pax7 and AP2 in both neural folds despite clear depletion of in situ hybridization signal for neural crest specifiers in the treated side (Fig. 3g–l). In 3/14 embryos, a unilateral moderate reduction in Pax7 and AP2 signal was noted in the Ubc9-Mo-treated neural fold, while in 2 other embryos, a spatially restricted depletion of both markers was found (data not shown). To analyze possible changes in neural tissue, we monitored Sox2 expression, as well as the width and length of the forming neural tube. Our results suggest that neural development was not affected, as Sox2 expression and neural tube dimensions were not significantly associated with Ubc9-Mo treatment and changes in expression of any of the neural crest markers analyzed (12/14; Fig. 3g–l).

Finally, we sought to establish if the reduced Ubc9 triggered by Ubc9-Mo treatment could be associated with increased apoptosis, which in turn could explain the reduced signal of NC specifiers (*Snail2*, *Sox9*, and *FoxD3*). To this end, images of sections immunostained with Caspase 3, Sox2, and Pax7 were processed with Photoshop and ImageJ to score the number of cells with positive Casp3 signal in the forming neural tube and neural folds. In Ubc9-Mo-treated embryos, we noted an average of 5.5 Casp3+ cells in treated neural folds and 4.3 in the contralateral not treated side. In the forming neural tube (excluding the neural fold territory defined by the expression of Pax7), we observed an average of 6.37 Csp3+ cells in the treated side versus 6.34 in the contralateral side. The different values observed in treated and control sides of the neural folds and forming neural tube were analyzed using a *t* test and found to be not statistically significant (data not shown).

Because Ubc9 is required for the expression of NC transcription factors like *Snail2* prior to emigration, but is not required for the expression of earlier markers like Pax7 and AP2, we suggest that SUMOylation is required for NC development after initial specification.

SUMOylation of Pax7 requires Lysine 85

SUMO conjugation occurs at an acceptor lysine, often found within a consensus sequence ψ-Lys-X-Glu/Asp

(ψ = large hydrophobic amino acid, often isoleucine or valine), consensus-variations, or their inverted arrangement [28, 40]. Identification of SUMOylation aceptor sites is regularly achieved by performing a mild substitution of the acceptor lysine (K), for an arginine (R), rendering the molecule "unSUMOylatable". Putative SUMOylation sites for Pax7 were suggested by two different software applications [SUOplot^{TM} Prediction (Abgent) included K139, K161, K164, and K483, and SUMOsp 2 software [41] suggested K164]; however, Pax7-SUMOylation was not disrupted when KxR substitutions where performed in any of these positions. Because several molecules are known to undergo SUMOylation in non-consensus sites, we tested possible SUMOylation acceptor sites throughout Pax7 by generating KxR substitutions, and performing SUMOconjugation assays in the presence of NEM. From 22 substitutions, K85R was the only one that resulted in a loss of the slower migrating form of Pax7 (Fig. 4a). This strongly suggests that Pax7 is SUMOylated at K85, within a non-consensus SUMOylation sequence. Importantly, K85 resides in the α3 helix of the PAI subdomain of the PAIRED domain (Fig. 4b), a region strongly conserved amongst all Pax genes (Fig. 4c). This suggested the possibility that other Pax genes could be SUMOylated at the same position. We therefore tested full-length sequences of at least one member of each Pax-subfamily in SUMO conjugation assays. Unlike Pax7, neither Pax2, Pax3, Pax6, nor Pax9 were SUMOylated (Fig. 4c).

Pax7 protein structure is essential for its SUMOylation

To further characterize the requirements for Pax7-SUMOylation, we performed conjugation assays with Pax7 deletion constructs (Fig. 4d). We generated Myc-tagged forms of Pax7 lacking 64 or 124 amino acids in the C-terminus (ΔC′-64 and ΔC′-124). We also generated forms of Pax7 lacking the whole transactivation domain (ΔTAD), the transactivation domain and the homeodomain (ΔHD), transactivation homeo and octapeptide domains (ΔOP), and one in which only the paired domain was removed (ΔPD). As expected, the ΔPD construct could not be SUMOylated. Surprisingly, however, all the other Pax7 deletion constructs, which retain a full paired domain with the acceptor K85, failed to undergo SUMOylation (Fig. 4d). Therefore, it seems that, in addition to K85, the general Pax7 protein structure is critical for Pax7-SUMOylation.

Pax7 transactivation domain confers SUMOylation capabilities to Pax3

Pax3 and Pax7 share high homology at the paired, octapeptide, and homeo domains, while they differ significantly at the transactivation domain. To test the possibility that

Fig. 4 Pax7 requires K85 and additional structural features for its SUMOylation. **a** Mapping the SUMOylation acceptor site of Pax7 through SUMO-conjugation assays in 293T cells cotransfected with Ubc9, SUMO1, and a Pax7 variant with a single K to R substitution, and carried out in the presence of NEM. The position of the substituted K is indicated at the *top of each lane*. *Arrow* indicates Pax7, *arrowhead* SUMOylated Pax7, *short arrows* K85. **b** Diagram of Pax7 domains and detail of the PAI subdomain, *arrow* indicates acceptor K85. **c** Sequence around Pax7-K85 is highly conserved amongst other Pax genes. Representatives from other Pax subfamilies were subjected to SUMO-conjugation assays; however, despite homology surrounding the putative Pax7-accepotor site, no other Pax was SUMOylated. **d** Structural features of Pax7 are essential for its SUMOylation. All Pax7 deletion constructs tested lack SUMOylation capacity in our conjugation assays. *PD* paired, *OP* octapeptide, *HD* homeo, *TA* transactivation domains. **e** Exchange of the transactivation domain of Pax7 confers SUMOylability to the N-terminus of Pax3 under SUMO-conjugation assays. *PD* paired, *OP* octapeptide, *HD* homeo, *TA* transactivation domain
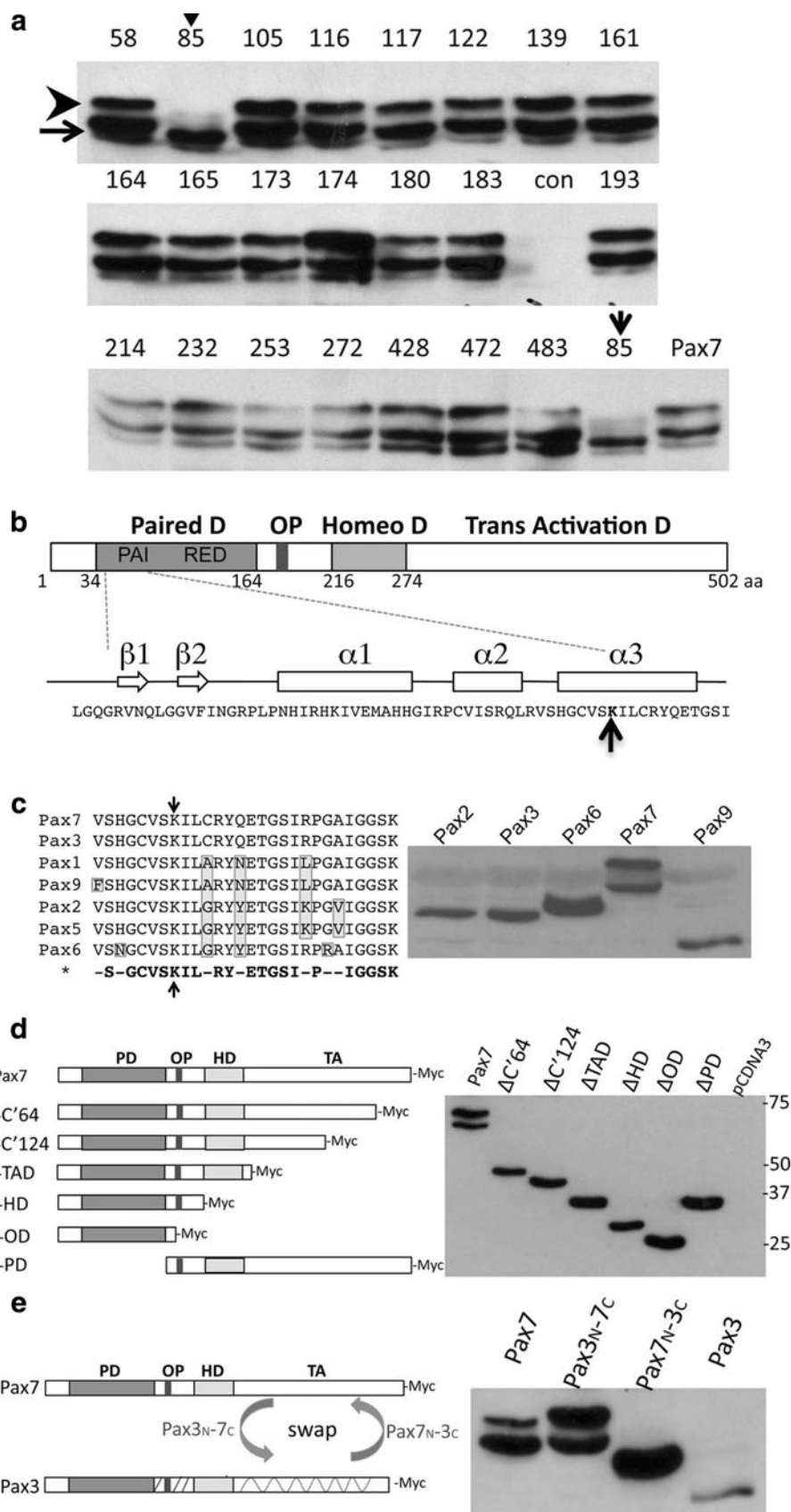
the SUMOylation machinery relies on Pax7's-transactivation domain, we exchanged these domains in Pax3 and Pax7. Remarkably, Pax3$_N$-7$_C$ (Pax3 N-terminus (paired, octapeptide, and homeo domains) with Pax7 C-terminus (transactivation domain)) was efficiently SUMOylated, while the Pax7$_N$-3$_C$ was not (Fig. 4d). These results demonstrate that the transactivation domain of Pax7 confers SUMOylation capabilities to the N-terminus of Pax3 and that it is essential for the SUMOylation of Pax7.

Pax7-SUMOylation is dispensable for its DNA binding activity

Given the location of the acceptor K85 in the conserved PAI DNA binding domain, we decided to investigate whether SUMOylation of Pax7 is necessary for its DNA binding ability. To this end, we compared the capacity of flag-tagged forms of Pax7 and the mutant Pax7 that cannot be SUMOylated, Pax7^{K85R}, to bind Pax-specific DNA sequences in electrophoretic mobility shift assays (EMSA). Probes included paired (P3OPT)-, homeodomain (P1/2)-, and dual paired-homeodomain (PH0)-specific sequences (Fig. 5). Both Pax7-flag and Pax7^{K85R}-flag formed clear complexes with all labeled probes, and were disrupted by a specific flag antibody (Fig. 5). Similar results were obtained with two additional probes targeted by paired and paired-type homeodomain factors (P6CON and P2; data not shown). As an additional test, we attempted to supershift the Pax7-DNA complexes with an anti-SUMO antibody. However, we were never able to show binding under these conditions (data not shown), suggesting that SUMOylated Pax7 does not bind to DNA. Alternatively,
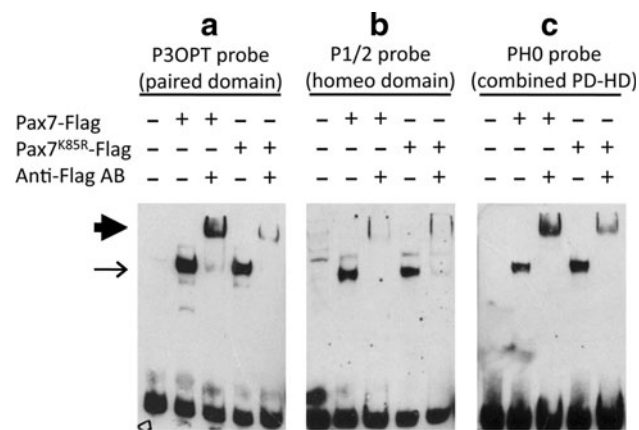

Fig. 5 Pax7 SUMOylation is dispensable for DNA binding. DNA binding capacity of Pax7 and the unSUMOylatable form (Pax7 K85R) measured in electrophoretic mobility shift assay (EMSA). FLAG-tagged Pax7 and FLAG-tagged Pax7^{K85R} bind to: **a** paired domain-, **b** homeodomain-, and **c** combined PD–HD-recognition sequences in EMSA assays. All oligos formed a complex with Pax7 and Pax7^{K85R} (*thin arrow*), which were disrupted by Flag antibody leading to slower mobility complexes (*thick arrow*)

the interaction between SUMOylated Pax7 and DNA might be beyond our detection capabilities, either because of a short life span and/or low quantity. In a second effort to test if SUMOylated Pax7 is able to bind target sequences, we performed EMSA experiments similar to the ones described above, but in which NEM and control extracts were used. However, the inclusion of NEM prevented the formation of DNA–protein complexes of both Pax7-flag and Pax7^{K85R}-flag (data not shown).

Our results demonstrate that Pax7^{K85R} is capable of binding Pax consensus sequences, and that SUMOylation is dispensable for this function.

SUMOylation of Pax7 is required for early neural crest formation

To examine the specific involvement of Pax7-SUMOylation in NC development, we interrogated the capacity of Pax7 and its unSUMOylatable form (Pax7^{K85R}) to rescue NC deficiencies seen in embryos electroporated unilaterally with a Pax7-Mo. We previously showed that knockdown of Pax7 via sequence specific morpholino oligonucleotides (Pax7-Mo) compromises the expression of neural crest markers like Snail2, Sox9, Sox10, and HNK-1 [12]. Since the target sequence for the Pax7-Mo is not present in our constructs overexpressing Pax7 or Pax7^{K85R}, translation of these molecules will not be altered by the presence of the Pax7-Mo.

Electroporation of Pax7-Mo and a control vector causes a downregulation of *Snail2* (9/11), *Sox9* (6/8), and *FoxD3* (5/6) expression (Fig. 6a). When a full-length Pax7 construct is coelectroporated with the Pax7-Mo, this phenotype is rescued and only a few embryos display defects in the expression of *Snail2* (2/10), *Sox9* (1/9), and *FoxD3* (0/11) (Fig. 6b). Importantly, coelectroporation of a Pax7^{K85R} construct with Pax7-Mo is unable to rescue expression of these markers, and several of the embryos display reduced expression for *Snail2* (6/10), *Sox9* (6/7), and *FoxD3* (7/10) (Fig. 6c). To further address the role of SUMOylated Pax7, we generated a SUMO1-Pax7^{K85R} fusion construct, attaching SUMO-1 to the carboxy terminus of Pax7^{K85R}, and performed rescue experiments for the Pax7-Mo phenotype on *Sox9*. Our results suggest that the SUMO-1-Pax7^{K85R} fusion is able to partially rescue the *Sox9* phenotype because only 40 % (4/10) of embryos receiving Pax7-Mo and SUMO1-Pax7^{K85R} fusion displayed a defective *Sox9* pattern (data not shown). The 40 % phenotype obtained with SUMO1-Pax7^{K85R} is halfway from the 11 % obtained with wild-type Pax7, and the more than 75 % observed with pCIG control or Pax7^{K85R}. These results demonstrate that K85 is required for Pax7's function in NC development, and strongly suggest an essential role for Pax7-SUMOylation during this process.
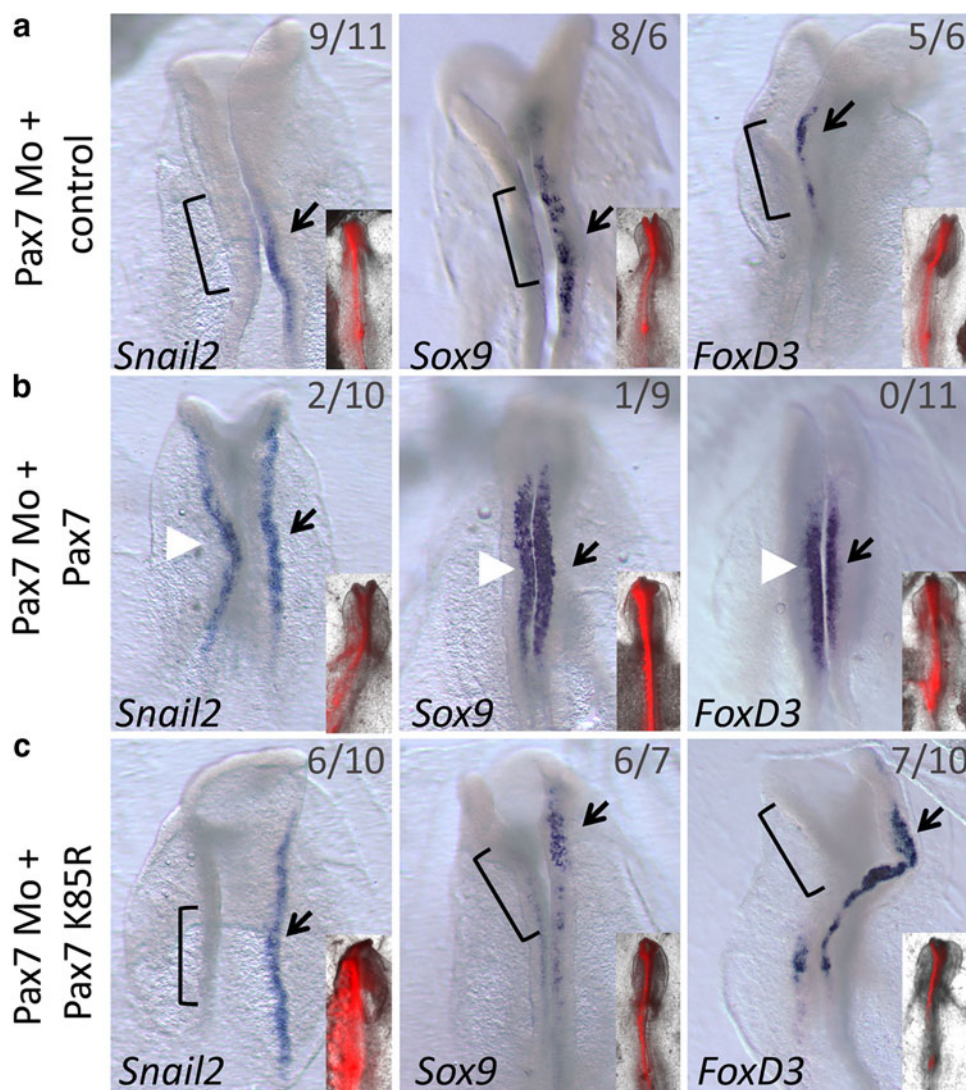
Fig. 6 SUMOylation of Pax7 is required for its function in early neural crest formation. A Pax-7 morphant-rescue assay was performed to assess the effect of Pax7-SUMOylation on neural crest development. **a** Unilateral electroporation of Pax7-Mo and control empty vector (pCIG) downregulates the expression of the NC markers *Snail2* (*left*), *Sox9* (*middle*), and *FoxD3* (*right*). **b** Coelectroporation with a Pax7 construct not targeted by the Mo rescues the expression of *Snail2*, *Sox9*, and *FoxD3* (*white arrowhead*); **c** instead, Pax7[K85R] co-electroporation is unable to rescue. *Insets* display electroporated areas (*red*) before in situ hybridization. *Arrows* point to untreated internal control signal and *brackets* highlight treated areas. The fraction of embryos showing inhibition of expression is shown in the *top right* of each image

Pax7-SUMOylation is necessary to prevent C2C12 myogenic differentiation

In addition to its role in NC development, Pax7 plays a critical role in myogenesis. It is expressed in muscle stem cell progenitors, is necessary for the survival and activation of adult satellite cells, and prevents their premature differentiation [13–18, 42–49]. To investigate the possible role of Pax7-SUMOylation in myogenic differentiation, we generated stable C2C12 myoblast cell lines expressing a vehicle control, Pax7, or Pax7[K85R]. Control C2C12 cells transfected with an empty vector readily generate myotubes when induced in low serum media and express Myosin

heavy chain components (Fig. 7a, b). As previously shown by other groups [44–49], overexpression of Pax7 led to a dramatic delay in myogenesis and neither myotubes nor myosin components (Myosin S1 domain) were detected (Fig. 7a, b). In contrast, overexpression of Pax7[K85R] failed to prevent C2C12 myogenic differentiation (Fig. 7a, b).

Pax7-SUMOylation is necessary for transactivation during myogenic differentiation

To determine if Pax7-SUMOylation is required for expression of known Pax7 target genes [46, 48], we analyzed mRNA expression levels via real time PCR in C2C12
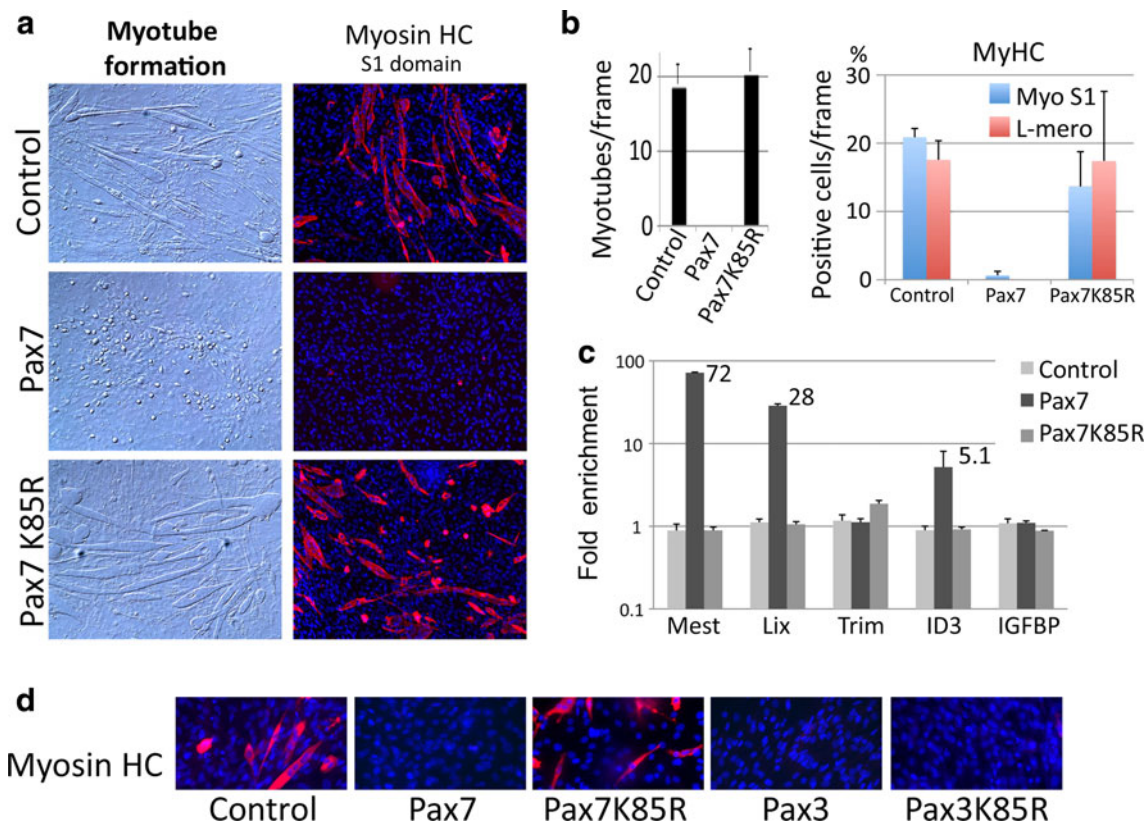
Fig. 7 SUMOylation of Pax7 is required in C2C12 to delay myogenic differentiation and to transactivate Pax7 responsive genes. **a** Myogenic differentiation of control C2C12 cells permanently transfected with empty vector (pCDNA) is seen after 4 days under low serum conditions. C2C12 cells permanently transfected with Pax7 display a severe delay or inhibition of myotube formation and myosin heavy chain expression. Instead, C2C12 cells permanently transfected with Pax7^{K85R} fail to inhibit myogenic differentiation and differentiate similar to control cells. Myosin heavy chain *red*, DAPI *blue*. **b** Graphs of myotube formation and myosin expression. Quantification of myotubes was performed in 3 independent 10× frames per sample. Percentage of DAPI stained nuclei positive for MyHC markers was performed in 5 independent frames of 50^2 μm. **c**,

Q-RT-PCR analysis of Pax7 responsive genes in C2C12 cells permanently transfected with an empty control (pcDNA3.1A), Pax7 or Pax7^{K85R}. Expression of *Lix1*, *Mest* and *ID3* is activated in C2C12 myoblasts expressing Pax7, but not in those expressing Pax7^{K85R}. Expression was normalized to *GAPDH* transcript levels and is shown relative to the empty vector (pcDNA3.1A) control. **d** K85 is critical for Pax7, but not for Pax3 inhibition of C2C12 myogenic differentiation. C2C12 control line permanently transfected with pCDNA empty vector, undergoes myogenic differentiation. Lines permanently transfected with Pax7, Pax3, and Pax3^{K85R} all inhibit differentiation. Instead, Pax7^{K85R} fails to inhibit and cells differentiate as in control samples

cells transfected with Pax7 or Pax7^{K85R}. While Trim and IGFBP were unresponsive to Pax7, ID3—a direct target of Pax7 [48]—as well as Mest, and Lix were activated in C2C12 cells stably expressing Pax7, but not Pax7^{K85R} (Fig. 7c). These results demonstrate that K85 is essential for Pax7's function during myogenic differentiation, including target gene expression, and strongly support the need for Pax7-SUMOylation during myogenesis.

K85 is essential for Pax7- but not for Pax3- inhibiton of C2C12 differentiation

Our results demonstrate that the mild substitution of K85 for R in Pax7 dramatically changes Pax7 properties. Pax7K85R can not be SUMOylated, and appears to lack function in neural crest development, myogenic target

transactivation, and myogenic differentiation in C2C12 cells. We suggest that the lack of function of this variant is due to its defective SUMOylation. An alternative explanation could be that the K85R substitution may abrogate Pax function regardless of the SUMOyation status. Pax3 performs similar functions to Pax7 and shares this K85, but does not seem to be modified by SUMOylation. Therefore, Pax3 offers an excellent opportunity to test the role of K85 in a SUMO-independent context. To assess this alternative explanation, we took advantage of the robust C2C12 myogenic differentiation assay in which Pax3, like Pax7, is known to inhibit C2C12 myogenic differentiation. We first generated two additional stable C2C12 myoblast cell lines expressing Pax3 or Pax3^{K85R}. Then, we performed differentiation assays with controls (C2C12 alone, and C2C12 with pCDNA empty vector), Pax7, Pax7^{K85R}, Pax3, and

Pax3^{K85R}. In agreement with previous reports and our own results, cells transfected with both controls readily differentiate and express myosin heavy chain antigens. Instead, C2C12 cell permanently transfected with either Pax3 or Pax7 display a clear delay or inhibition of differentiation. Similarly, C2C12 cells transfected with the Pax3^{K85R} display a delay/inhibition of differentiation as effective as when transfected with Pax3 or Pax7, suggesting that the K85R substitution did not alter Pax3 function. Instead, C2C12 cells transfected with the Pax7^{K85R} differentiate like the controls.

These results demonstrate that the substitution of K85R alters the function of Pax7 and not of Pax3, further supporting our thesis that the lack of function in Pax7^{K85R} is due to its defective SUMOylation.

Discussion

We previously identified Pax7 as a critical component and early marker of neural crest in the chick embryo [12]. In the mouse, Pax7 is also involved in cranial neural crest development [11]. And Pax7-progenitor cells have been recently shown to contribute to multiple neural crest derivatives, including olfactory ensheathing cells in the olfactory epithelium [50, 51]. In humans, as in model organisms, Pax7 is expressed in the dorsal neural tube and in early migratory neural crest cells of young human embryos [25]. However, Pax7 role in muscle progenitors has attracted considerably more attention (reviewed in [52]). Several signaling pathways are known to regulate Pax7 expression, particularly during early NC development [12, 53–56]. Yet, the molecular mechanisms controlling Pax7 function are not well understood. Here, we provide several lines of evidence exposing the interaction between Pax7 and Ubc9, leading to the SUMOylation of Pax7. We identified and verified the interaction between Pax7 and Ubc9 through a yeast two-hybrid screen and GST pull-down assays. Conjugation and co-immunoprecipitation assays confirm interaction and concomitant Pax7-SUMOylation. We also exposed the coexpression of Ubc9 and SUMO1 with Pax7 in the neural folds of stage 8 chick embryos, a region containing neural crest precursors, and provide in vivo evidence that Pax7 is SUMOylated in similarly staged embryos. Other transcription factors that influence neural crest development have been shown to be SUMOylated, such as Msx1, Msx2, Sox9, Sox10, Tbx22, and the TGFß transducer SMAD4 [34, 57], but direct evidence of the requirement of Ubc9 or SUMOylation during early stages of NC development had not been reported. Using morpholino-mediated knockdown of Ubc9, we were unable to identify effects on neural fate (Sox2) or early neural plate border markers (Ap2 and Pax7); instead, we demonstrate the requirement of Ubc9 and therefore

SUMOylation for expression of later neural crest markers (Snail2, Sox9, and FoxD3), exposing a critical role for SUMOylation in early neural crest development. Additionally, we identified the requirement of K85 for Pax7 SUMOylation, and suggest that this lysine is most likely the acceptor site on Pax7; however, definitive proof awaits mass spectrometry analysis.

It was surprising that, from representatives of all Pax subfamilies tested using our conjugation assay, only Pax7 was SUMOylated. This suggests that the acceptor site and the surrounding conserved sequences shared with other Pax genes are not sufficient to direct SUMOylation. Importantly, recent reports have identified the SUMOylation of other Pax proteins; however, in neither case has the paired domain been involved. Murine Pax8 (no avian form reported) is SUMOylated at its transactivation domain, and a variant of Pax6 lacking its paired domain is SUMOylated in a variant-specific region [58, 59].

Further analysis of the molecular requirements for SUMOylation of Pax7 revealed that the integrity of protein structure and the transactivation domain are critical for Pax7-SUMOylation. Pax3 and Pax7 differ more on their C-terminus transactivation domain, and, unlike Pax7, Pax3 is not SUMOylated. When exchanged, the C-terminus of Pax7 confered SUMOylation capabilities to the N-terminus of Pax3, and, instead, the N-terminus of Pax7 lost its SUMOylation capabilities when fused to the C-terminus of Pax3. Therefore, the C-terminus of Pax7 may provide a specific structure enabling required contacts between SUMOylation machinery and Pax7's paired domain, perhaps operating as a docking site for SUMOylation machinery, bringing it closer to a putative acceptor site (like K85). Interestingly, differential regulation between Pax3 and Pax7 has been reported to rely on the C-terminus [20]. These results pinpoint critical differences between Pax3 and Pax7 that aid in explaining noted disparate functions and/or regulation between these paralogous, often coexpresed proteins [15, 16, 20, 46, 49].

We took advantage of the unSUMOylatable form of Pax7, Pax7^{K85R}, to assess the roles of Pax7-SUMOylation. Our data show that, unlike Pax7, Pax7^{K85R} is unable to rescue the neural crest phenotype generated by Pax7-morpholino treatment. These results strongly suggest that Pax7-SUMOylation is critical for NC development. We further demonstrate that Pax7-SUMOylation is also critical in a different context, the C2C12 myogenic paradigm. Pax7 is known to block C2C12 myogenic differentiation [44–47, 49], and now we show that the unSUMOylateble form of Pax7, Pax7^{K85R}, is unable to perform this function, and to transactivate known myogenic targets of Pax7 in C2C12 cells. Importantly, Pax3 also inhibits C2C12 differentiation; however, the substitution of K85 for R in Pax3 (Pax^{3K85R}) retains full inhibitory activity in C2C12

differentiation. These data strongly suggest that Pax7's inhibitory role in myogenic differentiation relies on its SUMOylation. Future research should address the SUMOylation status of Pax7 in quiescent satellite cells, activated satellite cells, and differentiating progenitors as well as the possible involvement of Pax7-SUMOylation in differential target transactivation.

In summary, our study is the first to identify posttranslational modifications of Pax7 that regulate its function in development through a mechanism not shared by Pax3. Here, we have uncovered the SUMOylation of Pax7, and have provided robust evidence that this modification is necessary for Pax7 functions in NC and C2C12 myogenic differentiation. Molecularly, Pax7 SUMOylation offers a wide spectrum of possibilities for novel partners, targets, and effects. Assessing the relevance of Pax7-SUMOylation in neurocristopathies and muscle diseases is an attractive opportunity that could entail the design of novel diagnostic and therapeutic tools.

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