



Klhl31 attenuates β -catenin dependent Wnt signaling and regulates embryo myogenesis



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ABSTRACT

Klhl31 is a member of the Kelch-like family in vertebrates, which are characterized by an amino-terminal broad complex tram-track, bric-a-brac/poxvirus and zinc finger (BTB/POZ) domain, carboxy-terminal Kelch repeats and a central linker region (Back domain). In developing somites *Klhl31* is highly expressed in the myotome downstream of myogenic regulators (MRF), and it remains expressed in differentiated skeletal muscle. *In vivo* gain- and loss-of-function approaches in chick embryos reveal a role of Klhl31 in skeletal myogenesis. Targeted mis-expression of Klhl31 led to a reduced size of dermomyotome and myotome as indicated by detection of relevant myogenic markers, Pax3, Myf5, myogenin and myosin heavy chain (MF20). The knock-down of Klhl31 in developing somites, using antisense morpholinos (MO), led to an expansion of Pax3, Myf5, MyoD and myogenin expression domains and an increase in the number of mitotic cells in the dermomyotome and myotome. The mechanism underlying this phenotype was examined using complementary approaches, which show that Klhl31 interferes with β -catenin dependent Wnt signaling. Klhl31 reduced the Wnt-mediated activation of a luciferase reporter in cultured cells. Furthermore, Klhl31 attenuated secondary axis formation in *Xenopus* embryos in response to Wnt1 or β -catenin. Klhl31 mis-expression in the developing neural tube affected its dorso-ventral patterning and led to reduced dermomyotome and myotome size. Co-transfection of a Wnt3a expression vector with Klhl31 in somites or in the neural tube rescued the phenotype and restored the size of dermomyotome and myotome. Thus, Klhl31 is a novel modulator of canonical Wnt signaling, important for vertebrate myogenesis. We propose that Klhl31 acts in the myotome to support cell cycle withdrawal and differentiation.

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Introduction

In vertebrate embryos, segmentation of the paraxial mesoderm generates paired somites on either side of the neural tube. Initially, the somite consists of columnar epithelial cells with a central cavity (the somitocoel) and is surrounded by extracellular matrix that forms a basement membrane. Upon differentiation, the dorsal part of the somite, the dermomyotome, will generate the myotome, which gives rise to epaxial back muscle and the hypaxial muscles of the ventral

body wall and the limbs. The ventral somite undergoes an epithelial to mesenchymal transition to form the sclerotome, which is divided into a ventromedial part containing chondrogenic precursor cells of the vertebral body, pedicles and proximal ribs, and a ventrolateral part giving rise to the main portion of the ribs (Brand-Saberi et al., 1996; Christ et al., 2004; Scaal and Christ, 2004).

Wnts are a large family of highly conserved glycoproteins, which activate a number of signaling pathways involved in many cellular processes including the control of gene expression, cell proliferation, cell adhesion, cell polarity and cell fate (Anastas and Moon, 2013; Moon et al., 2002). Wnt signaling pathways are crucial during embryo myogenesis and during muscle regeneration and repair (von Maltzahn et al., 2012a, 2012b). Wnt signaling via the canonical/ β -catenin-dependent pathway leads to nuclear localization of β -catenin and activation of *Lef/Tcf* transcription factors. In developing somites and neural tube, activation of Wnt signaling increases proliferation (Dickinson et al., 1994; Galli et al., 2004; Megason

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and McMahon, 2002). Manipulation of Wnt signaling in the neural tube affects the differentiation of adjacent somites and evidence suggests that Wnt ligands secreted by the dorsal neural tube are delivered to somites by migrating neural crest cells, which express the heparan sulfate proteoglycan GPC4 (Serralbo and Marcelle, 2014). Overexpression of Wnt3a in half of the neural tube leads to an increase in the number of dorsal/dermomyotomal cells and a marked enlargement of the myotome (Galli et al., 2004). Consistent with this finding, retroviral mediated delivery of constitutively active Lef1 results in an increase in the numbers of mitotic cells in the dermomyotome and myotome. This was also observed with active forms of Pitx2. In contrast, dominant negative forms of these transcription factors inhibit myogenesis (Abu-Elmagd et al., 2010) and in mice the loss of Pitx2c modulates Pax3 expression and the number of proliferating myoblasts (Lozano-Velasco et al., 2011). This work suggests an interaction between Pitx2 and β -catenin/Lef1 dependent Wnt signaling and an effect on the proliferation of early myoblasts.

Wnt signaling is essential for the induction of Pax3 and Pax7 expression in myogenic progenitors and in the dorsal neural tube (Alvarez-Medina et al., 2008). Pax3 can activate myogenic markers Myf5 and MyoD in neural tissue (Maroto et al., 1997), it induces proliferation of myogenic cells (Mennerich et al., 1998) and inhibits their terminal differentiation (Epstein et al., 1995). The negative post-transcriptional regulation of Pax3 by microRNAs, including miR-206 and miR-27, is important to regulate the balance between proliferation and differentiation of myoblasts (Goljanek-Whysall et al., 2011; Crist et al., 2009; Lozano-Velasco et al., 2011).

Kelch-like proteins are also known as BTB-BACK-Kelch (BBK) proteins, based on their characteristic domain structure. They are composed of an amino-terminal broad complex tram-track, bric-a-brac/poxvirus and zinc finger (BTB/POZ) domain, a BACK domain and a carboxy-terminal region containing four to seven Kelch motifs (Stogios and Prive, 2004). The conserved Kelch repeats generate a propeller comprised of a number of β -sheets, likely interaction domains for multiple partners (Angers et al., 2006; Gray et al., 2009; Rondou et al., 2008). Kelch-like proteins have diverse cellular and biological roles, and several associate with the actin cytoskeleton, regulate cell adhesion and morphology (Gray et al., 2009; Greenberg et al., 2008; Hara et al., 2004), gene expression (Cullinan et al., 2004; Yu et al., 2008), cell signaling (Angers et al., 2006) and cell division (Maerki et al., 2009; Sumara et al., 2007). Many Kelch-like and BTB containing proteins function as adapter proteins and effect the recruitment of E3 ubiquitin ligase complexes to their substrate, leading to its degradation by 26S proteasomes (Angers et al., 2006; Perez-Torrado et al., 2006; Pintard et al., 2003; Sumara et al., 2007). While the BTB domain is implicated as substrate specific adapter for protein ubiquitinylation, the Kelch-domain is presumed to act as a substrate recognition module and the BACK domain is speculated to position the Kelch-motif β -propeller and its substrate in the Cul3 E3 complexes (Furukawa et al., 2003; Geyer et al., 2003; Pintard et al., 2003; Prag and Adams, 2003; Stogios et al., 2005; Xu et al., 2003). Specific interaction partners for Khlh31 have not yet been identified.

Interestingly, many Kelch-like family members are implicated in skeletal muscle disease (Gupta and Beggs, 2014). However, not much is known about the role of Khlh31, although it was found highly expressed in human embryonic skeletal muscle and heart tissues (Yu et al., 2008). This pattern is conserved in chick embryos, where Khlh31 is strongly expressed in the somite myotome and the developing heart and later in striated myofibers and in the myocardium (Abou-Elhamd et al., 2009). During embryo development, Khlh31 expression becomes upregulated in developing somites after myogenic commitment in response to Wnt and Shh signals, and ectopically in the neural tube after electroporation of Myf5 (Abou-Elhamd et al., 2009).

Here, we investigate Khlh31 function during somite myogenesis. We used targeted mis-expression by electroporation of Khlh31 full-length protein. This led to the reduced size of dermomyotome and myotome. Interestingly, the same result was observed following ectopic expression of Khlh31 in the neural tube, suggesting an indirect effect on the adjacent somite. Gain-of-function experiments were complemented by morpholino (MO) mediated knock-down (KD) of Khlh31 in the myotome. Phenotypic effects were investigated using dermomyotomal and myogenic markers. Khlh31-MOs led to a thickening of the dermomyotome and myotome, and we show that this correlated with an increase in the number of mitotic cells. To determine the molecular mechanism by which Khlh31 exerts its function, we tested the hypothesis that Khlh31 affects Wnt signaling. This was confirmed using two independent approaches, a luciferase reporter assay (TOP-flash) and an axis duplication assay in *Xenopus laevis* embryos. Both suggested that Khlh31 attenuates β -catenin dependent Wnt signaling. Rescue experiments, using expression of secreted Wnt3a, indicate that this is the mechanism by which Khlh31 exerts its effects on somite differentiation. Thus, we identify Khlh31 as a novel modulator of Wnt signaling and propose that this is important for regulating the balance between proliferation and differentiation in embryonic myoblasts.

Experimental procedures

Injection and electroporation into neural tube and somites

Fertilized eggs were incubated at 37 °C until the desired stage of development was reached (Hamburger and Hamilton, 1992). Expression constructs or MOs were injected into the posterior six somites of HH14–15 embryos and embryos were electroporated using six 10 ms pulses of 60 V. Expression constructs were injected into the neural tube of HH14–15 embryos and embryos were electroporated using four 50 ms pulses of 25 V. The concentration used for expression constructs was 2000 ng/ μ l. Embryos were harvested after 24 or 48 h for analyses, at least 3 embryos were examined per experimental condition and marker gene.

Whole-mount in situ hybridization, cryosections and photography

Embryos were collected into DEPC treated PBS, cleaned and fixed in 4% paraformaldehyde overnight at 4 °C. Whole mount *in situ* hybridization was performed as previously described (Schmidt et al., 2004). For cryosectioning, embryos were embedded in OCT (Tissuetec) and 20 μ m sections were collected on TESPAs coated slides, washed with PTW, coverslipped with Entellan (Merck, Germany) and examined using an Axioplan microscope (Zeiss). Whole mount embryos were photographed on a Zeiss SV11 dissecting microscope with a Micropublisher 3.5 camera and acquisition software. Sections were photographed on an Axiovert (Zeiss) using Axiovision software. Montages of images were created and labeled using Adobe Photoshop. Analysis of area percentage from *in situ* hybridization and immunohistochemistry was performed using ImageJ.

DNA constructs

The full length coding sequence for Khlh31 was initially cloned as described by Abou-Elhamd et al. (2009). Khlh31 was subcloned into the pCA β -IRES-GFP vector using EcoRI and NotI restriction enzymes. pCIG-mWnt3a-IRES-GFP was kindly provided by Elisa Marti (Alvarez-Medina et al., 2008). Cloning of cWnt3a into the pCA β -IRES-GFP vector was described by Yue et al. (2008) and

constitutively active (*ca*)- β -catenin was described by Guger and Gumbiner (1995).

Transfections and luciferase reporter assay

HEK293 cells were seeded into 24 well plates at 2×10^5 cells/well, transfection was carried out using Lipofectamine™ 2000 (Invitrogen, UK) according to manufacturer's instructions. The following amounts of plasmid were used: 100 ng pCA β -Wnt3a-IRES-GFP, 10 ng pCA β -Klh131-IRES-GFP, 10 ng Super 8x TOP-flash or 10 ng of Super 8x FOP-flash (kindly provided by Randy Moon), co-transfected pRLTK (3 ng) served as internal control. pCA β -IRES-GFP was used as a negative control and for normalization. Experiments included triplicate samples and were performed three times. Dual-luciferase reporter assays were performed according to manufacturer's instructions (Promega). Luciferase activity was normalized to Renilla activity and to GFP-only control. The mean of three transfections was taken for comparison of different constructs. Statistical analysis was performed by Student's *t*-test.

X. laevis embryo manipulations and microinjections

All experiments were performed in compliance with the relevant laws and institutional guidelines at the University of East Anglia. *X. laevis* eggs were fertilized *in vitro* and incubated in 0.1X MMR solution and de-jellied using 2% L-cysteine (Fluka) as described by Garcia-Morales et al. (2009). Embryos were staged according to the normal table of Niewkoop and Faber. Capped RNAs coding for Wnt1, *ca*- β -catenin and Klhl31 were synthesized using SP6 mMACHINE™ kit (Ambion). Microinjection was performed into the ventral marginal zone of four-cell stage embryos and carried out in 3% MMR containing 3% Ficoll PM400 (Sigma). The amounts injected were as follows: Wnt1 0.4 pg, *ca*- β -catenin 5 pg, Klhl31 between 0.25 and 5 ng, as indicated. Embryos were left to grow in 0.1X MMR at 22 °C until stage 39–40 and fixed for 1 h at room temperature in MEMFA. Injections were performed in three independent experiments; statistical analysis was done using the Mann–Whitney test.

Immunohistochemistry

Immunohistochemistry was performed as described (Abu-Elmagd et al., 2010). Sections were incubated overnight at 4 °C with primary antibodies at the following concentrations: Pax3, Pax7 and MF20 (1:100, Developmental Studies Hybridoma Bank, University of Iowa), anti-rabbit KLHL31 (8 μ g/ml, Abcam), anti-rabbit phospho-histone H3 (1 mg/ml). Secondary antibody was anti-rabbit Alexa Fluor 568 (Invitrogen) 1 mg/ml in 10% goat serum/PBS. DAPI was used in a concentration of 0.1 mg/ml in PBS. Sections were visualized using an AxioScope microscope using Axiovision software (Zeiss, Germany). Images were imported into Adobe Photoshop for labeling. The data was analyzed using ANOVA and *post-hoc* by Scheffe test using SPSS version 17.

Morpholino injections

Antisense morpholino oligonucleotides (MO) were designed and synthesized by Gene Tools and were FITC-labeled. Klhl31 MO: 5'-TCACGTTCTTCT AGGTGCCAT-3' targets the ATG start codon and 5' UTR. Klhl31 splice MO: 5'-ACAAACAACAACGAGTTACCTGCA-3'. Standard control morpholino: 5'-CCTCTTACCTCAGTTACAATTATATA-3'. MOs (1 μ M) were injected into epithelial somites and electroporated. After 48 h incubation the MO was observed using a GFP filter or visualized using an alkaline phosphatase coupled anti-fluorescein antibody (Roche) with Fast red (Sigma) as a substrate.

Results

Targeted mis-expression of Klhl31 protein in somites affects dermomyotome and myotome size

Within developing somites, Klhl31 expression is detected shortly after the first expression of myogenic regulatory factors (MRFs) and is restricted to the early and late myotome (Supplementary Fig. S1; Abou-Elhamd et al., 2009). To investigate the effect of Klhl31 on myogenesis, embryos were electroporated into epithelial somites II–V at stage HH14 with plasmids encoding full length Klhl31 ($n=4$). GFP was expressed from the same vector backbone. Embryos were analyzed after 24 h and cryosectioned for immunohistochemistry detecting Pax3 or myosin heavy chain (MF20), GFP and DAPI (Fig. 1). We also used double whole mount *in situ* hybridization with GFP probes and probes detecting *Myf5* or *Myogenin* (Supplementary Fig. S2, $n=9$, $n=15$). This showed that the dermomyotome and the myotome were smaller on the side electroporated with Klhl31 plasmid compared to the contralateral, non-electroporated side (Fig. 1A, and B). Measurements using ImageJ illustrate the reduced size of the dermomyotome and myotome. The size reduction was reproducible but variable, which is most likely due to variations in experimental parameters, such as for example transfection efficiency.

Morpholino mediated knock-down of Klhl31 increases the size of the dermomyotome and myotome

To assess the requirement for Klhl31 in developing somites, we used antisense morpholinos (MOs) to knock-down protein. A

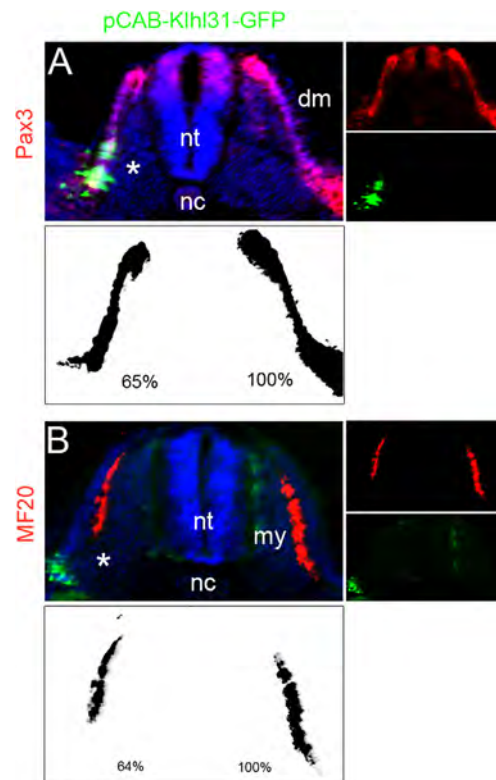


Fig. 1. Klhl31 misexpression in somites leads to decrease in size of dermomyotome and myotome. pCAB-Klh131-GFP was electroporated into HH14 epithelial somites, the electroporated side is indicated with an asterisk (*). Contralateral somites serve as internal control. After 24 h embryos were fixed, sectioned and immunostained for Pax3 (A) and MF20 (B) as indicated, nuclei were stained with DAPI (blue). ImageJ was used to measure dermomyotome and myotome size. The area positive for Pax3 staining was decreased by 35% and MF20 staining was decreased by 36%. Neural tube, nt; notochord, nc; dermomyotome, dm; and myotome, my.

translation-blocking or splice-blocking MO directed against Khlh31 was introduced by electroporation into epithelial somites I–V at stage HH14. The embryos were harvested after 48 h and analyzed. Efficiency of Khlh31 MOs was established by immunostaining on sections using a Khlh31 antibody. Khlh31 protein was reduced on the MO-injected side when compared to the non-injected side (Supplementary Fig. S3, $n=5$ for each type of MO). MO electroporation tends to be mosaic; however, clear effects on protein expression were seen with both the translation and splice blocking MO. Control MO had no effect on Khlh31 expression or on the expression of myogenic markers Pax3 ($n=8$), MyoD ($n=7$), Myf5 ($n=9$) or myogenin ($n=6$) (Fig. 2A–D).

We next examined the effect of Khlh31 knock-down on somite patterning and differentiation. Following *in situ* hybridization and immunostaining for the FITC-labeled MO, we found that knock-down of Khlh31 using translation-blocking MO (Fig. 2E–H) or splice-blocking MO (Fig. 2I–L) led to an increased thickness of the dermomyotome and myotome. This was measured using ImageJ to illustrate the expanded expression domains for the dermomyotomal marker, Pax3 (Fig. 2E and I; $n=11$, $n=10$), and myogenic markers, MyoD (Fig. 2F and J, $n=12$, $n=13$), Myf5 (Fig. 2G and K, $n=14$, $n=12$) and Myogenin (Fig. 2H and L, $n=10$, $n=11$) on the injected side when compared with the non-injected side, or with control MO injected embryos (Fig. 2A–D). Effects observed were variable. This is expected and due to experimental variations. For this reason it is difficult to compare between embryos; however comparisons of electroporated and non-electroporated sides showed clear phenotypes, which tended to be less prominent with the splice-MO compared to the translation blocking MO.

Knock-down of Khlh31 results in increased cell proliferation in the dermomyotome and myotome

Some Kelch-like family members play important roles during mitosis (Maerki et al., 2009; Sumara et al., 2007). To investigate whether the increased dermomyotome and myotome thickness following knock-down of Khlh31 was due to an increase in the number of proliferating cells, we performed immunostaining for phosphor-histone H3, a marker of mitotic cells. Nuclei in the dermomyotome and myotome were stained with DAPI. The number of phosphor-histone H3 positive cells in the dermomyotome and myotome was counted on injected and non-injected sides of embryos electroporated with Khlh31 translation-blocking MO or Khlh31 splice-blocking MO or control MO. In each case 41 sections from nine embryos were counted (three to seven sections per embryo). The mean number of phosphor-histone H3 positive cells on the injected and non-injected sides was calculated. Statistical analysis showed that knock-down of Khlh31 with Khlh31 MOs led to a 1.6-fold ($P < 0.01$, student's *t*-test) increase in the number of cells in mitosis on the injected side compared to the non-injected side (Fig. 3I–P, and Q). Control MO injected embryos did not show a significant difference between the two sides (Fig. 3A–H, and Q).

Khlh31 negatively regulates Wnt-induced expression of a TOP-flash reporter downstream of β -catenin

The canonical Wnt signaling pathway is vital in myogenesis. This is consistent with the expression of β -catenin and Lef1 in the dorso-medial lip of the dermomyotome (Schmidt et al., 2004, 2000) and the effects of canonical Wnt signaling via Lef1 on the size of the myotome (Galli et al., 2004; Abu-Elmagd et al., 2010). Interestingly KLHL12, another Kelch-like family member, was shown to downregulate Wnt signaling by recruiting Dsh for ubiquitylation and degradation (Angers et al., 2006). We therefore asked whether Khlh31 can modulate β -catenin dependent Wnt signaling. We examined

activation of a TOP-flash reporter, containing multiple Lef/Tcf binding sites (Super8xTOP-flash), in HEK 293 cells, which were co-transfected with pCA β -Wnt3a-IRES-GFP and the reporter, with or without pCA β -Khlh31-IRES-GFP plasmid (Fig. 4A). Renilla luciferase vector served as internal control and Super8xFOP flash, in which the Lef/Tcf binding sites are mutated, was used as a negative control. The Wnt3a induced activation of luciferase expression in the TOP-flash reporter was reduced to 64% in the presence of pCA β -Khlh31-IRES-GFP (Fig. 4A, $P < 0.01$).

To determine where in the Wnt pathway Khlh31 might interfere, we co-transfected β -catenin with pCA β -Khlh31-IRES-GFP and with TOP-flash or FOP-flash reporter and internal Renilla control, in HEK 293 cells. This showed that β -catenin dependent activation of TOP-flash was reduced to 78% by Khlh31 ($P < 0.01$), indicating that Khlh31 affects Wnt signaling downstream of β -catenin.

*Khlh31 interferes with the Wnt/ β -catenin mediated induction of a secondary axis in *X. laevis* embryos*

Induction of a secondary dorsal axis from ventral blastomeres is a functional readout for Wnt activity *in vivo* (Kuhl and Pandur, 2008). To corroborate the results with luciferase reporters, we investigated whether Khlh31 could interfere with Wnt-induced secondary axis formation in *Xenopus* embryos. Capped RNA was generated for Khlh31 and injected together with Wnt1 RNA into the ventral marginal zone of four-cell stage embryos. Embryos were examined when they reached stage 39–40 (Fig. 4C–E). Injection of Wnt1 RNA (0.4 μ g) led to complete axis duplication (cad) in 90.2% of embryos and 9.8% showed partial axis duplication (pad). Co-injection of 1 ng Khlh31 did not significantly affect the ability of Wnt1 to induce a secondary axis and resulted in similar proportions of embryos with complete (86.6%) or partial axis duplication (2.2%) (Fig. 4D; Supplementary Table 1). However, co-injection of Khlh31 at 2 ng significantly inhibited the effect of Wnt1 ($P < 0.01$) and a smaller proportion of embryos displayed complete axis duplication (35.7%), with more embryos displaying partial axis duplication (51.4%) and some embryos, which were phenotypically normal (10%). Injection of 5 ng of Khlh31 RNA did not further enhance the ability of Khlh31 to block Wnt1 mediated induction of a secondary axis (Fig. 4D). Control GFP RNA was injected and all embryos appeared normal (data not shown).

Overexpression of β -catenin in *Xenopus* ventral blastomeres leads to axis duplication (Funayama et al., 1995; Guger and Gumbiner, 1995). Because Khlh31 can modulate activation of a TOP-flash reporter in response to β -catenin (Fig. 4B), we examined whether it affected β -catenin-mediated axis duplication. Capped RNA of constitutively active (*ca*) β -catenin (Guger and Gumbiner, 1995) and Khlh31 was co-injected in the ventral marginal zone of four-cell stage embryos (Fig. 4E). As expected, overexpression of β -catenin (5 μ g) led to complete axis duplication in 97% of embryos, 1.5% had partial axis duplication and 1.5% developed abnormally. Co-injection of Khlh31 (250 μ g) significantly ($P < 0.01$) inhibited the effect of β -catenin and only 56% of embryos developed with a completely duplicated axis, 33% with a partially duplicated axis and a small proportion of embryos was normal (5.7%). The ability of Khlh31 to counteract β -catenin was enhanced when using 500 μ g of Khlh31 RNA. In 40% of embryos we observed complete axis duplication, 35% had partial axis duplication and 20% were normal (Fig. 4E; Supplementary Table 1).

Targeted mis-expression of Khlh31 in the dorsal neural tube affects the adjacent somite

Neural tube derived Wnt signals affect myogenesis in adjacent somites (Dickinson et al., 1994; Galli et al., 2004; Megason and McMahan, 2002; Serralbo and Marcelle, 2014). Therefore we next

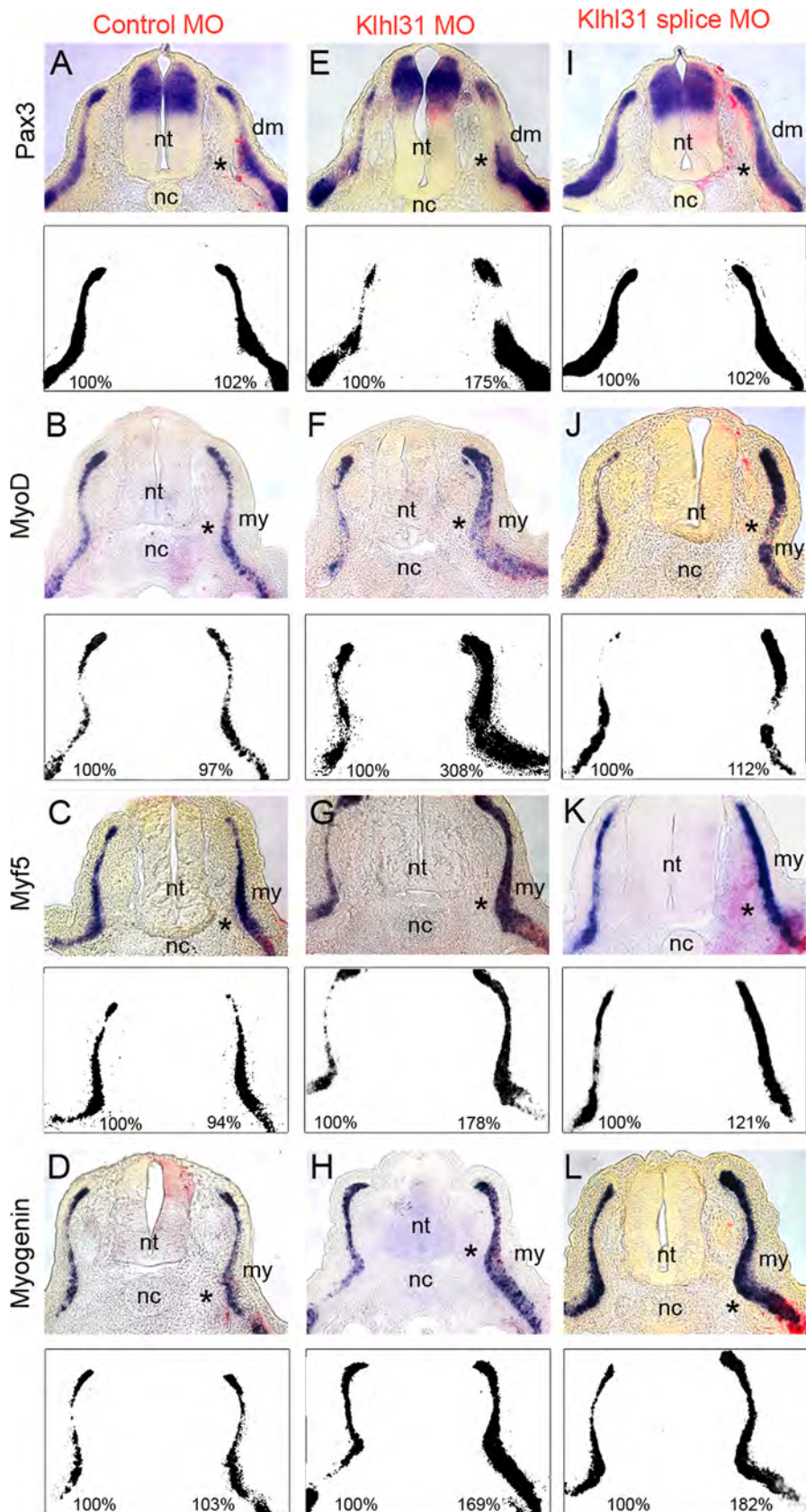


Fig. 2. Morpholino-mediated knock-down of Khl31 results in formation of a thickened myotome. Control morpholino (A–D), translation start site morpholino Khl31 MO (E–H) or a splice morpholino (I–L) was electroporated into somites, the electroporated side is indicated with an asterisk (*). Contralateral somites serve as internal control. After 48 h embryos were subjected to *in situ* hybridization with Pax3, MyoD, Myf5 or myogenin (purple), as indicated. MOs were detected using anti-FITC antibody coupled to alkaline phosphatase (red). Some Fast red signal is lost after sectioning procedure. A thickened dermomyotome and myotome are evident after Khl31 knock-down. Area of expression is presented below each panel. Neural tube, nt; notochord, nc; dermomyotome, dm; and myotome, my.

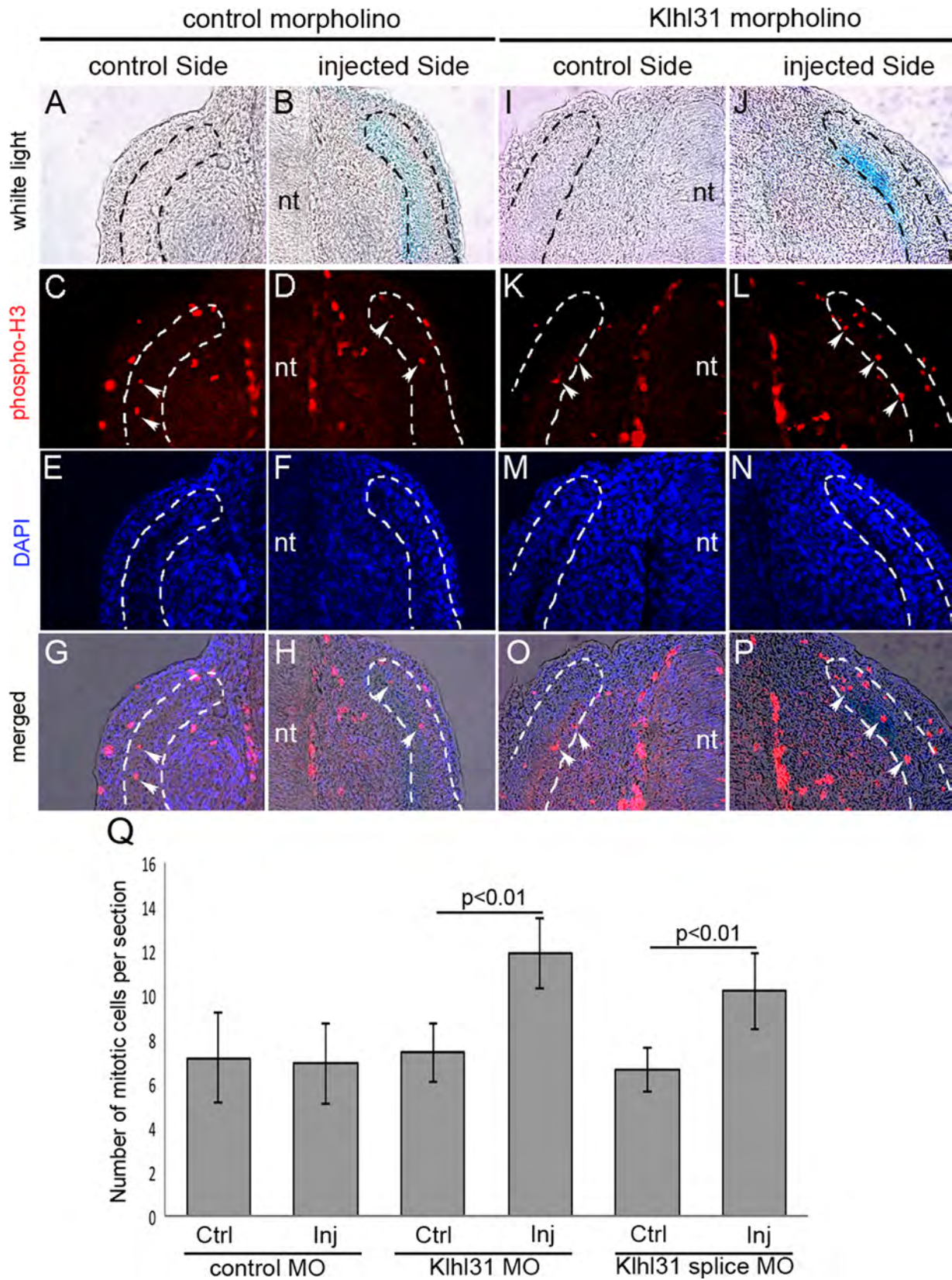


Fig. 3. Khlh31 knock-down in somites results in an increased number of mitotic cells in the dermomyotome and myotome. Morpholinos (MOs) were electroporated into somites, and detected using anti-FITC alkaline phosphatase coupled antibody and BCIP (turquoise) on cryosections (A, B, I, J). Immunolabeling for phosphor-histone H3 reveals mitotic cells in the dermomyotome and myotome, indicated by white arrowheads (C, D, K, L). DAPI staining shows all nuclei (E, F, M, N), overlay is shown in (G, H, O, P). Dotted line indicates outline of dermomyotome and myotome. (Q) Quantification of phosphor-histone H3 positive cells in different conditions, as indicated, knock-down of Khlh31 using MOs led to an increased number of cells in mitosis ($P < 0.01$). Neural tube, nt.

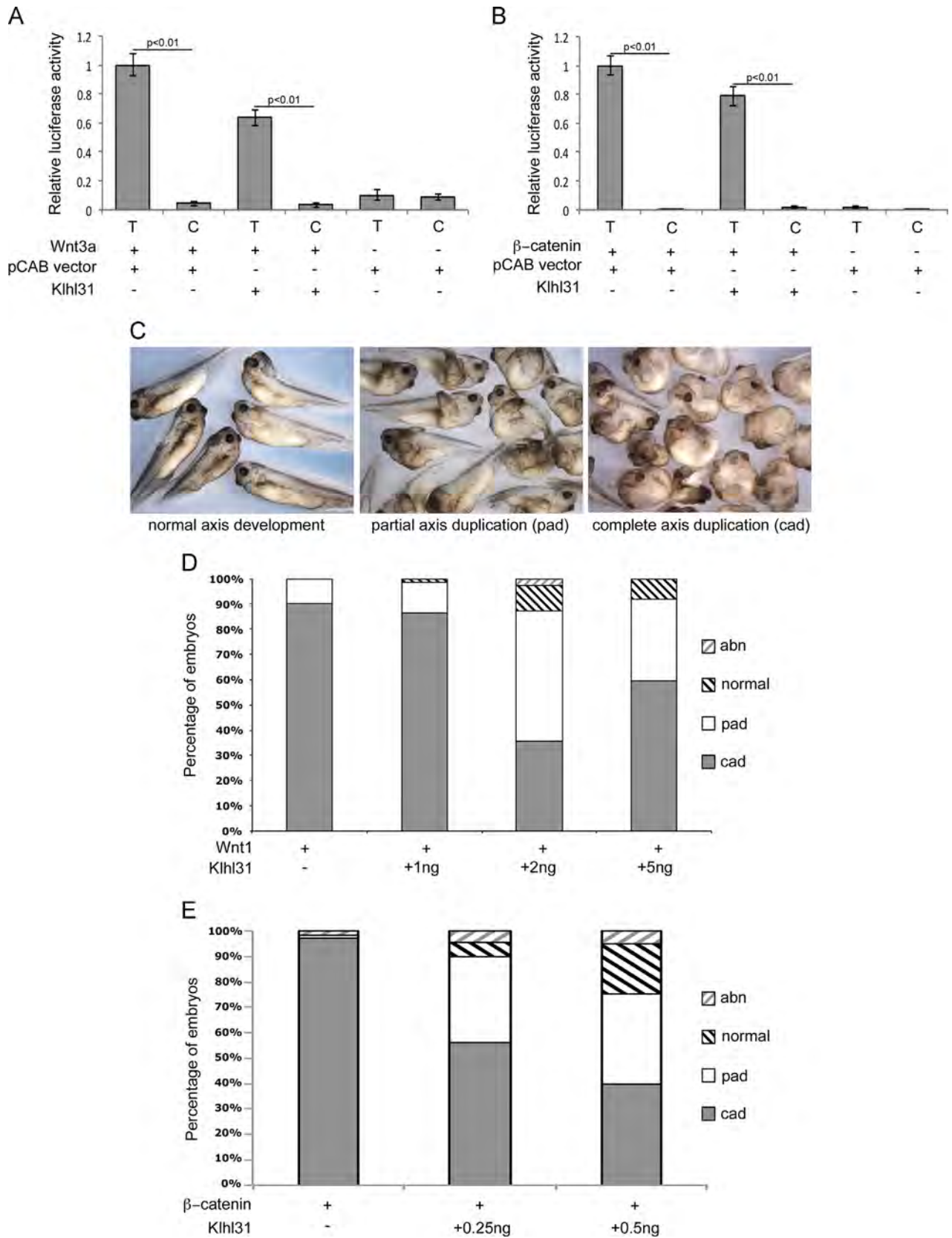


Fig. 4. Khl31 interferes with β -catenin dependent Wnt signaling. (A, B) HEK293 cells were transfected with Super8Topflash (T) or Super8Fopflash control vector (C), together with either Wnt3a (A) or β -catenin (B) or control vector. pCAB vector alone or pCAB vector expressing Khl31 was co-transfected as indicated. Luciferase activity was measured, counts were normalized to Renilla and relative values are plotted ($P < 0.01$). (C) Examples of tadpoles with normal axis development, or with partial (pad) or complete axis duplication (cad) induced by activation of canonical Wnt signaling in ventral blastomeres. (D) Percentage of embryos with normal axis, partial or complete axis duplication, after injection of Wnt1 RNA into ventral blastomeres with increasing amounts of Khl31 (D). (E) Percentage of embryos with normal axis, partial or complete axis duplication, after injection of β -catenin RNA into ventral blastomeres with increasing amounts of Khl31 as indicated. Khl31 proteins attenuated the effect of Wnt1 or β -catenin and greater proportions of normal embryos or embryos with partial axis duplication were observed. Numbers of embryos injected are shown in [Supplementary Table 1](#).

examined whether ectopic expression of Klhl31 in the dorsal neural tube would interfere with somite differentiation. Plasmid encoding Klhl31 and GFP protein from the same backbone was electroporated into the neural tube at HH14 and embryos were examined after 24 h (Fig. 5, $n=5$) or 48 h (Supplementary Fig. S4, $n=5$). Immunohistochemistry for Pax3 and Pax7 was used to assess effects on the dermomyotome and the dorsal neural tube, and detection of myosin heavy chain using MF20 antibody revealed effects on the myotome. After 48 h, the ventral expansion of dorsal neural tube markers, Pax3 and Pax7, was reduced consistent with a negative effect of Klhl31 on Wnt signaling (Supplementary Fig. S4). Quantification using ImageJ showed that the dermomyotome and myotome were reduced in size in somites adjacent to the neural tube half that was expressing ectopic Klhl31 (Fig. 5; Supplementary Fig. S4). This suggests that interference with Wnt signaling in the neural tube indirectly affects somite differentiation.

Co-expression of Wnt3a with Klhl31 rescues the negative effects on dermomyotome and myotome size

Given the negative modulation of Wnt signaling by Klhl31, we next asked whether expression of the secreted ligand Wnt3a could rescue the effects observed after Klhl31 mis-expression. Expression plasmids encoding Klhl31 or Wnt3a were co-electroporated either in the dorsal neural tube or in epithelial somites (Fig. 6, $n=3$, $n=3$). Embryos were analyzed after 24 h and cryosections were immunostained using Pax3 or MF20 antibodies and dermomyotome and myotome size was measured using ImageJ. This showed that dermomyotomes and myotomes were no longer reduced in size, but were comparable to the non-electroporated

side or slightly larger. Thus, Wnt3a expression counteracts the negative effects of Klhl31 on the Wnt/ β -catenin signaling pathway.

Discussion

Kelch-like proteins have diverse biological functions in vertebrates. Here we characterize the role of Klhl31 in developing somites. We showed previously that Wnt signaling in combination with Shh is responsible for activation of Klhl31 expression in the myotome where it is expressed downstream of MRFs (Abou-Elhamd et al., 2009). Striated muscle specific expression of Klhl31 is conserved between Zebrafish, chick and human (Yu et al., 2008; Abou-Elhamd et al., 2009). Despite its striking expression pattern in the developing myotome (Supplementary Fig. S1), Klhl31 function in early myogenesis has not been investigated.

We show here that Klhl31 attenuates canonical Wnt signaling downstream of β -catenin, both *in vitro* and *in vivo* (Fig. 4). The negative effects of Klhl31 on the activation of a TOP-flash luciferase reporter (Fig. 4A and B), or on the inhibition of Wnt1 induced secondary axis induction (Fig. 4C and D), were subtle but statistically significant. This suggests that Klhl31 modulates levels of Wnt signaling and this may be mediated through regulation of protein stability and turnover. The functional domains of Kelch-like proteins are highly conserved. Structural information suggests that the Kelch repeats generate interaction domains for multiple partners, while the BTB domain acts as substrate specific adapter for protein ubiquitinylation via E3 ubiquitin ligase complexes. The BACK domain may be involved in positioning (Stogios et al., 2005; Stogios and Prive, 2004). We speculate that Klhl31 facilitates the ubiquitinylation of an as yet unknown substrate that is important for the β -catenin dependent Wnt pathway. Precedence for this idea comes from the related protein, Klhl12, which is recruited to disheveled in a Wnt-dependent manner. This interaction promotes disheveled poly-ubiquitinylation and degradation (Angers et al., 2006). It is unlikely that Klhl31 affects disheveled as it interfered with canonical Wnt signaling downstream of β -catenin (Fig. 4). It will be interesting to see whether the Klhl31-induced phenotype can be rescued by Lef-1/Tcf overexpression and, more importantly, to identify interacting proteins in an unbiased fashion. This will also enable a better characterization of the functional domains of Klhl31.

Overexpression of Klhl31 in somites (Fig. 1) resulted in a reduction of dermomyotome and myotome size. This phenotype was rescued by Wnt3a expression (Fig. 6), consistent with the interpretation that the effects of Klhl31 on dermomyotome and myotome size are mediated by inhibition of Wnt signaling. Interestingly expression of Klhl31 in the neural tube resulted in a similar somite phenotype (Fig. 5), which could also be rescued by co-electroporation of Wnt3a (Fig. 6). The dorso-ventral patterning of the neural tube itself was altered by electroporation of Klhl31, specifically the expression domains of dorsal markers, Pax3 and Pax7, were reduced (Supplementary Fig. S4). This is again consistent with the idea that Wnt signaling, which promotes neural tube dorsalization (Alvarez-Medina et al., 2008), was antagonized within the neural tube; however, the finding that adjacent somites were affected implies additional indirect effects. It is not clear at present what this indirect mechanism might entail, but it could for example involve the reduced production of Wnt by the dorsal neural tube. Neural tube derived Wnt signals have been shown to affect proliferation in adjacent somites (Galli et al., 2004). Overexpression of Wnt also increases proliferation in the developing neural tube (Dickinson et al., 1994; Megason and McMahon, 2002).

Detection of GFP showed that neural crest cells were also transfected after dorsal neural tube electroporation (Figs. 5 and 6). Migrating neural crest cells (NCCs) have been shown to be important for myogenesis (Kalcheim, 2011). Specifically,

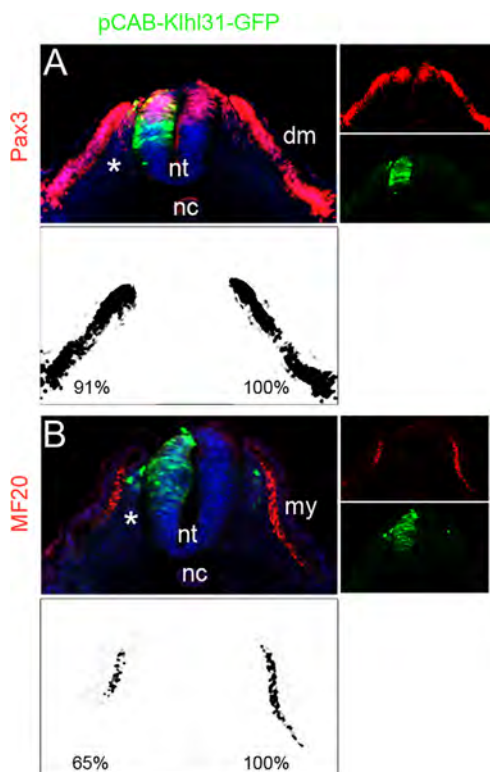


Fig. 5. Misexpression of Klhl31 in the neural also leads to decrease in size of dermomyotome and myotome. pCAB-Klhl31-IRES-GFP (green) was electroporated into the dorsal neural tube of HH14 embryos, the electroporated side is indicated with an asterisk (*). After 24 h embryos were fixed, sectioned and immunostained for Pax3 (A) and MF20 (B) as indicated, nuclei were stained with DAPI (blue). Area of expression is presented below each panel. Neural tube, nt; notochord, nc; dermomyotome, dm; and myotome, my.

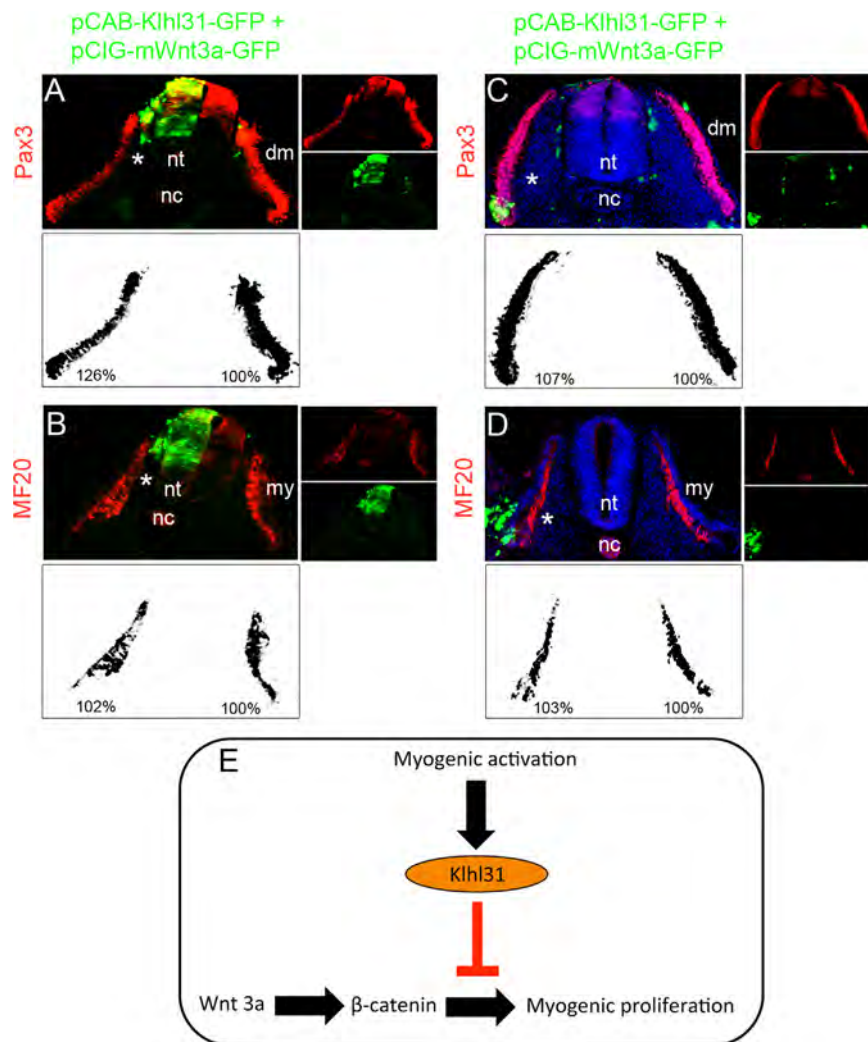


Fig. 6. Co-expression of Wnt3a with Khlh31 in neural tube and somites rescues the phenotype and restores dermomyotome and myotome size. pCAB-Klh31-IRES-GFP was co-electroporated with pCIG-mWnt3a-IRES-GFP into the neural tube (A, B) and somites (C, D) of HH14 embryos, the electroporated side is indicated with an asterisk (*). The non-transfected neural tube half and contralateral somites serve as internal control. After 24 h embryos were fixed, sectioned and immunostained for Pax3 (A, C) and MF20 (B, D) as indicated in red, nuclei were stained with DAPI (blue). Area of expression is presented below each panel. (E) The proposed role of Khlh31 after myogenic activation is the attenuation of the response to Wnt/ β -catenin signaling in myogenic cells. Neural tube, nt; notochord, nc; dermomyotome, dm; and myotome, my.

dorsally migrating NCC signal *via* delta-like1 (DLL1) to transiently activate Notch signaling in myogenic cells in the dermomyotomal lip, whereas ventrally migrating NCCs express neuregulin1 (Nrg1), which activates the ErbB3 receptor in the central and hypaxial myotome (Rios et al., 2011; Van Ho et al., 2011). These interactions regulate the differentiation of Pax7 positive progenitors and thus the eventual size of the myotome. Migrating NCCs are also implicated in delivering Wnt protein to pattern somites at a distance (Serralbo and Marcelle, 2014). We show here that Wnt3a transfection could rescue the indirect effects on somites of Khlh31 expression in the neural tube and NCCs (Fig. 6A, and B). We speculate therefore that in this scenario Khlh31 may affect the production or display of myogenic ligands such as Wnt3a, but also potentially DLL1 and/or neuregulin1.

Interestingly, filopodia-like protrusions, which invade the sub-epidermal space, have recently been characterized on dermomyotomal cells (Sagar et al., 2015). It is reasonable to assume that filopodia are similarly involved in contacting migrating NCCs to receive myogenic stimuli. Our experiments suggest that Khlh31 may affect the ability of dorsally migrating NCCs to promote somite myogenesis (Fig. 5).

It was also recently reported that manipulation of BRE expression (=Brain-and-Reproductive-Expression) had indirect effects

on adjacent tissues. BRE is an adapter protein involved in stress responses, DNA repair and maintenance of stemness. Both over-expression and silencing of BRE in the neural tube indirectly affected the size of adjacent somites (Wang et al., 2015) and evidence suggests that BRE activates BMP/Smad signaling and NCC migration.

Klh31 is first detected in the early myotome (Supplementary Fig. S1). However, we cannot exclude the possibility that Khlh31 is expressed in myogenic progenitors within the dermomyotome, at a level that is not detectable by *in situ* hybridization. Given the expression pattern and the finding that Khlh31 is upregulated by MRFs (Abou-Elhamd et al., 2009), the main function of Khlh31 is likely to be in committed myoblasts. Its detailed mechanism of action within myoblasts remains to be determined, but we propose that Khlh31 regulates the balance between myoblast proliferation and differentiation by attenuating Wnt signaling (Fig. 6E). Consistent with this, we show that MO-mediated knock-down of Khlh31 led to a thickened myotome (Figs. 2 and 3). Interestingly, Khlh31 knock-down also led to increased size of the dermomyotome and we measured a small but significant increase in the numbers of mitotic cells in the region encompassing the dermomyotome and myotome (Fig. 3, 1.6 fold, $P < 0.01$). This implies an indirect effect of Khlh31 knock-down in the myotome on the dermomyotome that is currently not understood, but

may involve mechanisms similar to those discussed above for the neural tube and NCCs. The overall effect of *Klhl31* loss-of-function is enhanced proliferation and myogenesis. This involves direct mechanisms within the myotome and indirect mechanisms on the dermo-myotome, both of which may include the de-repression of Wnt signaling. In order to determine the underlying molecular effectors that mediate these effects, it will be necessary to identify proteins that interact with *Klhl31* in developing muscle.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2015.02.024>.

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