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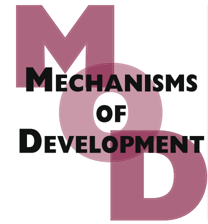

Klhl31 is associated with skeletal myogenesis and its expression is regulated by myogenic signals and Myf-5

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ABSTRACT

Klhl31 is an orthologue of *Drosophila Kelch* and belongs to a family of Kelch-like proteins in vertebrates. Members of this family contain multiple protein domains, including an amino-terminal *b*road complex/*t*ram-track/*b*ric-a-brac (BTB) or *po*xvirus and *z*inc finger (POZ) domain, carboxy-terminal Kelch repeats and a central linker region. We show that *Klhl31* is highly expressed in the developing heart, the somite myotome and later in differentiated skeletal muscle and the myocardium. In developing somites expression of *Klhl31* was initiated in the epaxial domain of the myotome, shortly after the skeletal muscle specific bHLH transcription factor, *MyoD*, was first expressed. *Klhl31* remained expressed in skeletal muscle throughout embryonic and fetal development. Tissue ablations and rescue experiments that regulate myogenesis also govern expression of *Klhl31* expression in somites. In particular, axial tissues, neural tube, floor plate and notochord, and surface ectoderm, provide combinatorial cues for myogenesis and the appropriate expression of *Klhl31*. We show that a combination of myogenic signals, Shh and either Wnt-1 or Wnt-6, are sufficient for *Klhl31* expression in the dorsal somite. Furthermore, ectopic expression of *Myf-5* led to expression of *Klhl31* in the developing neural tube, indicating that *Klhl31* is a novel and integral part of vertebrate myogenesis.

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1. Introduction

Somites are transient mesodermal structures that arise from the unsegmented paraxial mesoderm during vertebrate segmentation. They are arranged in pairs on either side of the neural tube/notochord. Initially somites consist of columnar epithelial cells arranged around a central cavity (the somitocoel) and surrounded by extracellular matrix that forms a basement membrane. The dorsal somite (the dermomyotome) is divided into a dorsomedial part, which differentiates into epaxial back muscle, and a ventrolateral part, which contains the precursor cells of the hypaxial muscles of the ventral body wall and skeletal muscle of the limb. The dermomyotome generates the cells of the myotome in successive waves of growth and differentiation (Gros et al., 2004). The ventral somite contains the chondrogenic precursor cells of the vertebral body, pedicles and ribs (Christ et al., 2004; Scaal and Christ, 2004).

Work from our lab and others showed that tissues surrounding the somite provide signals that promote the differentiation of the various cell lineages (Brent and Tabin, 2002). The factors derived from the axial midline structures, neural

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tube, floor plate and notochord, that pattern the somite along the dorsoventral axis include Wnt molecules, in particular Wnt-1 and Wnt-3a, and Sonic hedgehog (Shh) (Borycki et al., 2000; Münsterberg et al., 1995; Schmidt et al., 2000). In addition, the surface ectoderm has been shown to provide extrinsic cues important for somite epithelialization, including Wnt-6 (Buckingham, 2001; Geetha-Loganathan et al., 2006; Schubert et al., 2002).

Kelch and Kelch-like proteins are also known as BTB-BACK-Kelch (BBK) proteins, based on their characteristic domain structure. They are composed of a BTB/POZ domain, a BACK domain and a carboxy-terminal region containing four to seven Kelch motifs (Supplemental Fig. 1). BTB/POZ domains can act as substrate specific adaptors for protein ubiquitination in Cullin based E3 ubiquitin-ligase complexes. The conserved Kelch repeats generate a propeller comprised of a number of β-sheets (Bork and Doolittle, 1994). The similarity between the repeats ranges from 25% to 50% identity (Xue and Cooley, 1993). Finally, the BACK domain may act to position the Kelch propeller and its bound substrate in Cul3-E3 complexes (Stogios et al., 2005; Stogios and Prive, 2004).

The prototype of the Kelch-family in *Drosophila* is required to maintain actin organization in ovarian ring canals. Kelch mutant egg chambers have severely disorganized ring canal actin, leading to a defect in cytoplasm transport and the production of small sterile eggs (Xue and Cooley, 1993). The functions of some Kelch-like proteins in vertebrates have been characterized. For example, KLHL12 interacts with Cullin-E3-ubiquitin ligases and targets disheveled for degradation, thus negatively regulating Wnt signaling in cells and embryos (Angers et al., 2006). Other family members seem to affect the cytoskeleton. For example, Muskelin, a more distantly related and widely expressed protein, causes alterations in the adhesive behavior and cytoskeletal organization in C2C12 myoblast (Adams et al., 1998). KLHL20 interacts with F-actin, and in MDCK cells it is concentrated at cell–cell contact sites where it may be involved in Rac1-induced actin organization (Hara et al., 2004).

The function of *Klhl31* during embryo development has not been studied. Experiments in cell culture suggest that KLHL31 protein may act as a new transcriptional repressor in MAPK/JNK signaling (Yu et al., 2008), and the expression of *Klhl31* in skeletal and cardiac muscle in zebrafish (Wu and Gong, 2004) and chicken embryos (this report), and conserved synteny across a number of species suggest an important and highly conserved function for this protein (Wu and Gong, 2004).

We examined the spatio-temporal expression of *Klhl31* during embryogenesis. *Klhl31* transcripts were highly restricted and detected in cardiac progenitors, the developing heart and the myocardium of the left ventricle and atrium. *Klhl31* was strongly expressed during skeletal myogenesis. Transcripts were first detected in the epaxial myotome of developing somites. In differentiating myotomes, *Klhl31* colocalized with *MyoD* and *Klhl31* remained expressed in all skeletal muscles until at least E10 (HH36). Next, we investigated the association of Klhl31 with skeletal myogenesis. We show that signals known to pattern the dorsal somite and to induce the expression of skeletal muscle specific bHLH transcription factors also regulate *Klhl31* expression. Tissue ablations followed by rescue experiments indicate that members of the Wnt family, Wnt-1 and Wnt-6, and Shh are involved in the initiation of *Klhl31* expression in developing somites. Finally, ectopic expression of Myf-5, but not myogenin, in the neural tube resulted in ectopic expression of *Klhl31* transcripts, demonstrating that *Klhl31* is intimately linked to skeletal myogenesis and is regulated by myogenic signals and Myf-5.

2. Results

2.1. *Identification of Klhl31 full-length coding sequence and phylogenetic comparison*

Initial expression analyses were performed using ChEST997C15 obtained from MRC/BBSRC genome resources (Boardman et al., 2002). This EST corresponded to a partial sequence of a predicted chicken gene encoding a novel protein with a BTB/POZ domain. Further analyses using the Ensembl Genome Browser (www.ensembl.org) revealed that ChEST997 C15 corresponds to the chicken orthologue of human KLHL31. Sequence alignments showed that Klhl31 protein is highly conserved in vertebrates (Supplemental Fig. 1A and B), with particularly strong amino acid identity across species in the BTB/POZ domain and the Kelch-repeats (Supplemental Fig. 1A). Phylogenetic comparison of chicken Klhl31 with other chicken Kelch-like orthologues demonstrated that Klhl31 is most closely related to Klhl14, Klhl9 and Klhl26 (Supplemental Fig. 1C). Importantly, the functions of many of these proteins have not been described to date.

2.2. *Developmental expression of Klhl31 is closely linked with cardiac and skeletal myogenesis*

To investigate the spatio-temporal expression of *Klhl31* during chicken embryogenesis we used whole-mount in situ hybridization followed by cryosections, as described previously (Schmidt et al., 2000; Smith et al., 2005). *Klhl31* transcripts were first detected at Hamburger–Hamilton (HH) stage 8 (Hamburger and Hamilton, 1992) in the mesoderm of the anterior intestinal portal (Fig. 1A–A′′). By HH10 *Klhl31* was expressed throughout the heart and was faintly detected in anterior somites adjacent to the neural tube (Fig. 1B–B′′′). As somites matured, *Klhl31* transcripts were specifically expressed in the myotome (Fig. 1C–C′′′ and D–D′′). From HH27 *Klhl31* was expressed in fore- and hind-limb buds and sections showed that transcripts were restricted to the developing dorsal and ventral muscle masses (Fig. 1D and D′′). *Klhl31* was also detected in the hypoglossal chord, the branchial arches and some eye muscles (Fig. 1D). In older embryos, *Klhl31* expression was specifically found in the myocardium of the heart, in differentiating skeletal muscles in the limb buds (Fig. 1E–E′′, K, K′, L and L′), and in axial muscles of the back (Fig. 1I) and neck (Fig. 1J and J′).

2.3. *Klhl31 is expressed after myogenic commitment*

Because of the close correlation of *Klhl31* with skeletal myogenesis we next established the timing of expression relative to the onset of *MyoD* expression. *MyoD* is one of

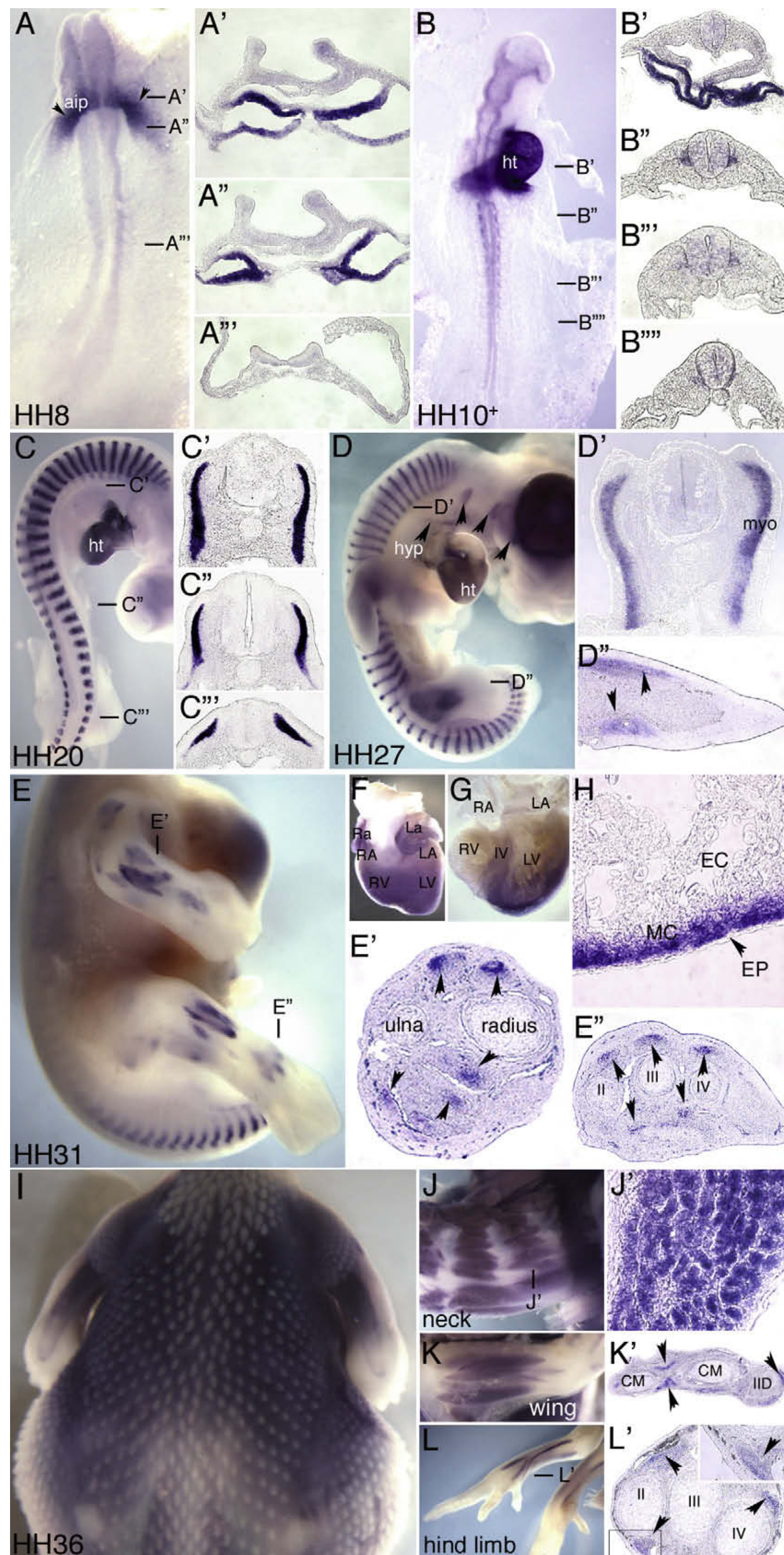

Fig. 1 – *Klhl31* is expressed in skeletal and cardiac muscle. In situ hybridization with *Klhl31* antisense RNA probes was performed in whole mount followed by cryosections. Hamburger–Hamilton stages are indicated on each panel. The planes of sections are indicated on the respective whole mount photograph. aip, anterior intestinal portal, ht, heart; myo, myotome; EC, endocardium; MC, myocardium; EP, epicardium; Ra, right auricle; RA, right atrium; La, left auricle; LA, left atrium; RV, right ventricle; IV, interventricular septum; LV, left ventricle; CM, carpometacarpus; IID, second digit; II, III, IV, metatarsal bones; arrow in (H) myocardium; arrows in E′, E′′, K′, L′ skeletal muscles.

the first muscle regulatory factors (MRFs) detected in skeletal muscle progenitors in the dorsomedial lip of the myotome in developing chicken somites. Comparing stage-matched embryos, we found that *Klhl31* expression was first detected in the dorsomedial somite adjacent to the neural tube, similar to *MyoD* (Fig. 2C). However, it lagged behind the expression of *MyoD* by approximately 6–7 h (Fig. 2). At HH11 or HH13 *MyoD* transcripts were detected in 10 or 17 somites, respectively and only two to three most recently formed epithelial somites did not express detectable amounts of transcripts (Fig. 2E and M). In contrast, *Klhl31* transcripts were seen in only 6 or 12 somites at HH11 and HH13 respectively and could not be detected in seven most recently formed somites (Fig. 2A and I).

Sections showed that *Klhl31* transcripts localized to the medial epithelial somites and then correlated closely with the expanding myotome (Fig. 2B–D and J–L). In older embryos, *Klhl31* and *MyoD* expression overlapped in the differentiated myotome (2R-U, W-Z). Expression was never detected in the dermomyotome or the sclerotome.

2.4. *Redundant signals from the neural tube and the surface ectoderm are involved in initiating Klhl31 expression in the developing myotome*

We performed tissue ablations in order to determine the origin of extrinsic cues necessary for the initiation of *Klhl31* expression in the developing myotome. Prior to the onset of *Klhl31* expression, we separated the surface ectoderm from the underlying presegmented mesoderm at HH11–12 with tantalum foil to create an impermeable barrier (Fig. 3A and B). After 24 h incubation, somites had formed and in situ hybridization showed that somite patterning had occurred normally on the operated side and on the control side. *Pax-1* transcripts were expressed in the sclerotome in the ventral somite, and *Klhl31* expression had been activated in the myotome more dorsally. Next, we removed the dorsal neural tube leaving the floor plate, notochord and surface ectoderm in place (Fig. 3C and D). This resulted in mesenchymal cells fusing across the midline and occasionally in fused myotomes (not shown). In all cases, dorsal somite patterning and *Klhl31* expression was not affected. Next, we removed both surface ectoderm and dorsal neural tube and inserted an impermeable barrier (Fig. 3E and F). This led to complete loss of myotome formation and *Klhl31* expression, suggesting that these tissues provide cues that are important for the initiation *Klhl31* expression concomitantly with myogenesis. This result is consistent with the fact that there are redundant signals derived from surface ectoderm and neural tube, which are required for dorsal somite patterning, dermomyotome epithelialization and activation of myogenesis. *Pax-1* remained expressed in the mesenchymal sclerotome owing to the presence of Sonic hedgehog (Shh) from the notochord (Fan et al., 1995).

To examine whether axial midline tissues are required for the maintenance of *Klhl31* or sclerotomal gene expression we removed neural tube and notochord (Fig. 3G and H) adjacent to somites IX–XII (of a HH12 embryo) and replaced them with a pellet of RatB1a fibroblasts. These somites already expressed Klhl31 transcripts (Fig. 2). After 24 h incubation *Klhl31* and *Pax-1* transcripts were still present. To determine whether signals from the surface ectoderm were required we ablated axial midline tissues and surface ectoderm. This led to the loss of *Klhl31* but not *Pax-1* transcripts on one side (Fig. 3I and J).

2.5. *Wnt-1 and surface ectoderm are not sufficient for myogenesis or Klhl31 expression, both require additional cues from axial midline tissues*

The neural tube and surface ectoderm are known to provide Wnt signals important for somite epithelialization and patterning. Thus we investigated whether Wnt signals could be sufficient for the activation of *Klhl31* expression in the developing myotome. One half of the neural tube was removed next to epithelial somites II–IV at HH stages 11–12, before *Klhl31* was expressed (see Figs. 1 and 2). Grafting a large cell pellet (approximately 150 μm in diameter) of Rat-B1a fibroblasts into the gap prevented the onset of *Klhl31* expression on the operated side (Fig. 4B). The concomitant loss of *Pax-1* in the ventral somite indicated that Shh signals from the ventral midline were not able to diffuse that far. Therefore, this result showed that surface ectoderm alone was not sufficient to initiate *Klhl31* expression. To determine whether Wnt-1 is able to substitute for the dorsal neural tube to rescue the initiation of *Klhl31* expression in the presence of the surface ectoderm, we implanted a large pellet of RatB1a-Wnt-1 fibroblasts (Fig. 4D). A pellet of this size has a barrier effect and prevents signals from the ventral midline (notochord, floor plate) from reaching the somite as illustrated in Fig. 4B. Expression of *Klhl31* or *MyoD* was not observed in this scenario (Fig. 4D and H) suggesting that Wnt-1 plus surface ectoderm are not sufficient to induce myogenesis and thus *Klhl31*. Conversely, when a small cell pellet (approximately 60 μm) was implanted between the axial midline tissues and paraxial mesoderm, *Klhl31* and markers of ventral somite patterning (*Pax-1*) were expressed (Fig. 4E and F). This was consistent with a combinatorial role for the ventral midline tissues, notochord and floor plate and neural tube, surface ectoderm for the initiation of *Klhl31* expression and is reminiscent of the activation of skeletal myogenesis in response to signals from these tissues (Fig. 4G–J) (Münsterberg et al., 1995; Münsterberg and Lassar, 1995). In all ablations, the opposite side served as internal control.

2.6. *Sonic hedgehog from the ventral midline is able to restore Klhl31 expression in the presence of the surface ectoderm*

Next we examined the potential role of Sonic hedgehog (Shh), which is expressed in the notochord and floor plate, for the activation of *Klhl31* expression and myogenesis. As shown before removal of half the neural tube followed by implantation of a large control cell pellet (DF1-RCAS-GFP) prevented expression of *Klhl31* transcripts (Fig. 5A and B). However, grafting a large pellet of DF-1 cells transfected with an avian retrovirus expressing Shh (RCAS-Shh) restored somite patterning and expression of *Klhl31*, *Pax-3* (Fig. 5C and D) and *MyoD* (not shown). This demonstrated that, whilst the

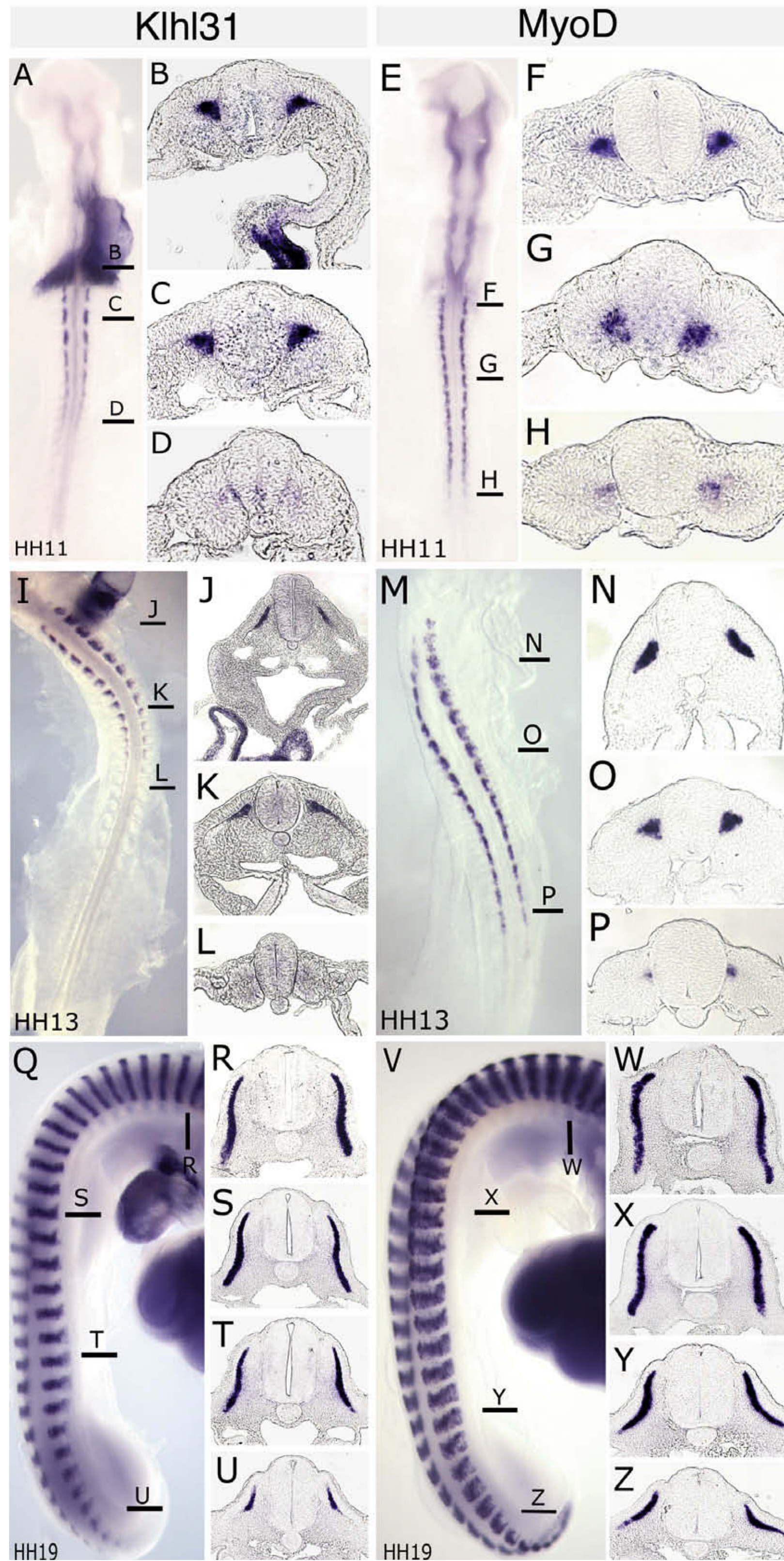

Fig. 2 – Onset of *Klhl31* expression in the epaxial myotome follows myogenic commitment. Stage-matched embryos were hybridized with probes detecting *Klhl31* or *MyoD* as indicated. Hamburger–Hamilton stages are indicated on each panel. The planes of sections are indicated on the respective whole mount photograph. *Klhl31* was first detected in the dorsomedial somite (C and D). At HH11 (A–H) and HH13 (I–P) *Klhl31* transcripts were detected in more anterior somites when compared to *MyoD* transcripts. (Q–Z) In older embryos *Klhl31* expression overlapped with that of *MyoD* and was restricted to the differentiating myotome.

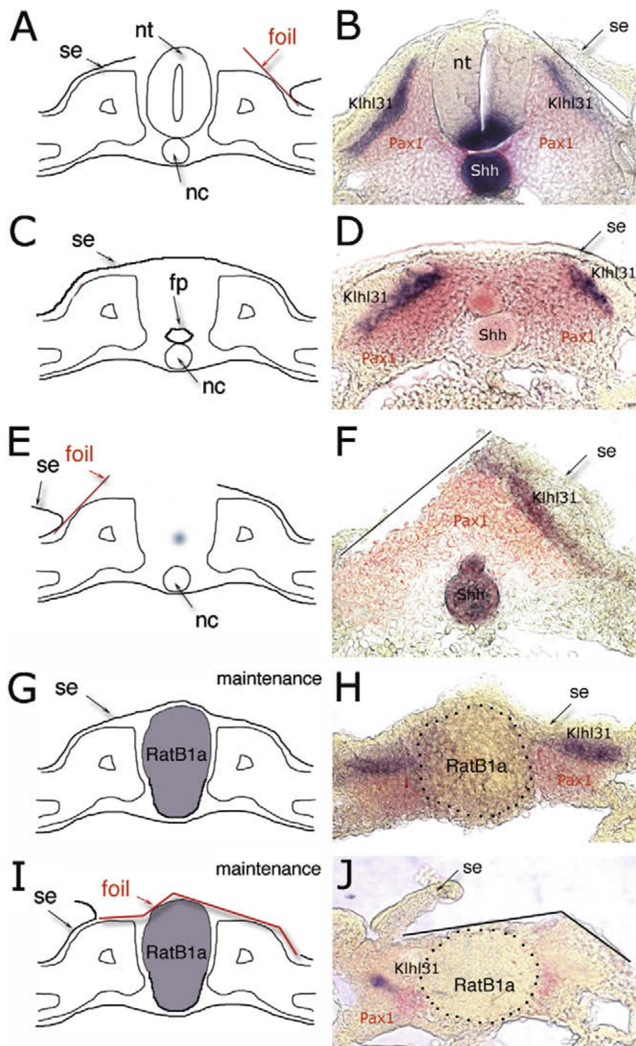

Fig. 3 – The neural tube and surface ectoderm are involved in the activation of *Klhl31* expression but are not required for maintenance. Tissue ablations were performed as indicated in the schematic drawings (A, C and E). Whole mount in situ hybridization were performed and cryosectioned. (B, D and F) Probes have non-overlapping expression patterns and were developed with NBT/BCIP (purple) or with Fast Red (red). (A and B) Separation of the surface ectoderm from the somite by tantalum foil (*n* = 12), (C and D) removal of the dorsal neural tube (*n* = 14) did not result in loss of *Klhl31* transcripts. (E and F) However, after removal of surface ectoderm and neural tube the epithelial dermomyotome and myotome did not form and *Klhl31* expression was lost (*n* = 10) whilst sclerotome cells expressing *Pax-1* were not affected. (G and H) Removal of midline tissues adjacent to older somites of HH11–12 embryos did not affect the maintenance of *Klhl31* or *Pax-1* expression (*n* = 11). (I and J) However, removal of midline tissues and surface ectoderm affected the maintenance of *Klhl31* expression but not expression of *Pax-1* (*n* = 10). nc, notochord; nt, neural tube; se, surface ectoderm.

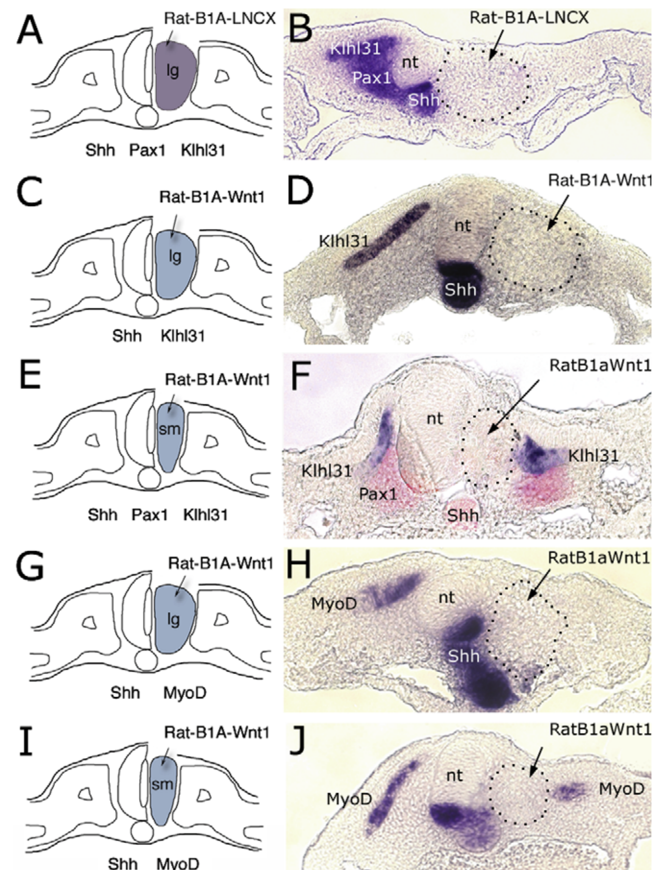

Fig. 4 – Wnt-1 and surface ectoderm are not sufficient for the activation of *Klhl31* expression, additional signals from the ventral midline tissues are required. One half of the neural tube was removed next to somites II–IV, before the onset of *Klhl31* expression. Cell pellets were grafted into the gap. The opposite side served as internal control. (A and B) A large pellet of RatB1a fibroblasts formed a physical barrier and *Klhl31* expression was lost on the operated side, indicating that signal(s) from the midline structures are essential for *Klhl31* activation (*n* = 21). (C and D) A large pellet of Wnt-1-expressing fibroblasts was not able to restore *Klhl31* expression (*n* = 22). (G and H) The loss of *Klhl31* correlated with loss of *MyoD*. The grafting of small pellets of Wnt-1 cells permitted expression of (E and F) *Klhl31* (*n* = 10) and (I and J) *MyoD* (*n* = 9) on the operated side. (E and F) The expression of *Pax-1* under these conditions showed that a small cell pellet did not prevent ventral midline signals (Shh) from reaching the somite. Implanted cell pellets are indicated by a stippled line. lg, large; nt, neural tube; sm, small.

notochord on its own (i.e. in the absence of the surface ectoderm) is not sufficient (Fig. 3E and F), Shh expressing cells could substitute for the ventral midline tissues and restore the initiation of *Klhl31* expression in the myotome in the presence of the surface ectoderm. To exclude the possibility that Wnt signals derived from the remaining half of the neural tube were diffusing across to the opposite side, we also replaced all of the midline tissues with Shh expressing cells (Fig. 5E and F). As before in the presence of the surface ectoderm this was sufficient for myotome formation and thus *Klhl31* expression.

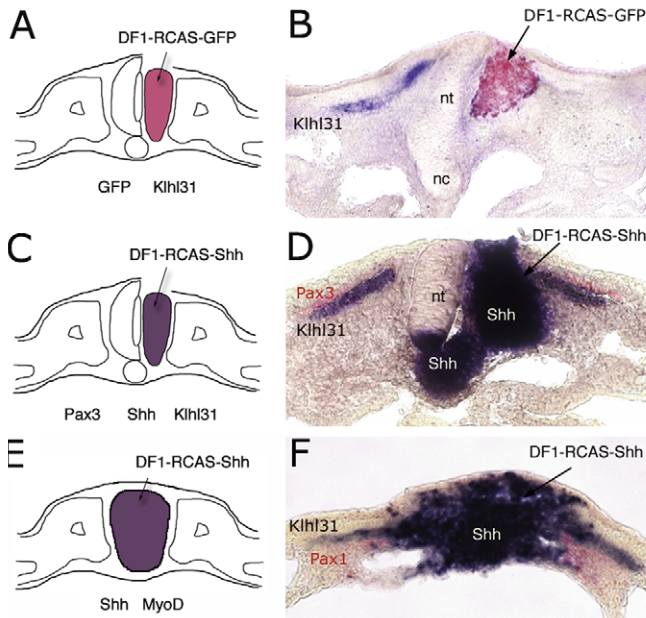

Fig. 5 – Shh can rescue *Klhl31* expression in the presence of surface ectoderm after neural tube/notochord removal. (A and B) In situ hybridization and sections showed that *Klhl31* expression was lost on the operated side, after removal of half of the neural tube and implantation of a large control cell pellet (DF-1 cells expressing RCAS GFP) (*n* = 16). (C and D) When half the neural tube was replaced by cells expressing RCAS-Shh, dorsal somite patterning and the activation of *Klhl31* expression was restored (*n* = 11). (E and F) Complete removal of axial midline tissues followed by implanting Shh expressing cells restored *Klhl31* expressing in the presence of surface ectoderm (*n* = 12). (B and D) Probes for *Klhl31* and *Shh* were developed with NBT/BCIP (purple), and probes for (B) *GFP*, (D) *Pax-3* and (F) *Pax-1* were developed with Fast Red (red). nc, notochord; nt, neural tube.

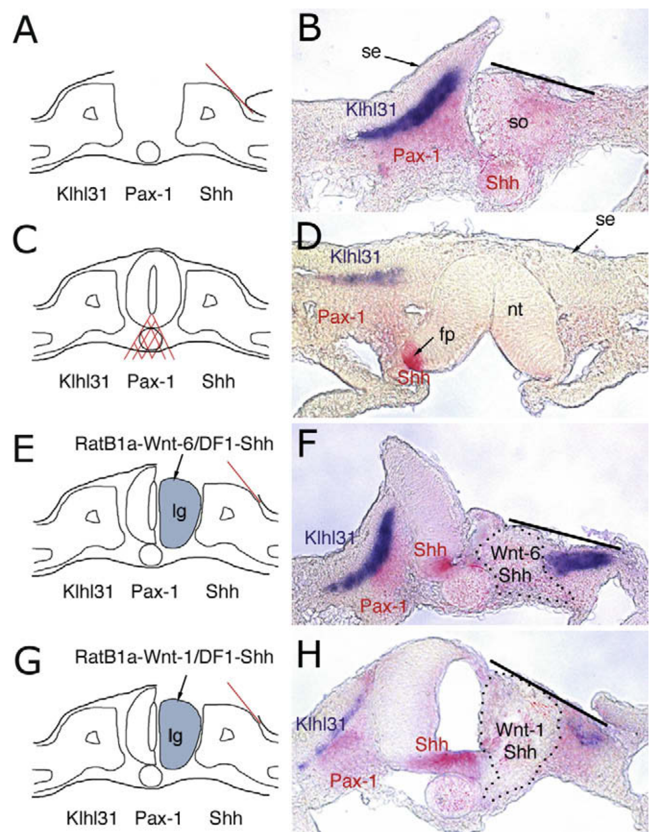

Fig. 6 – Shh together with Wnt-6 or Wnt-1 can rescue *Klhl31* expression in the dorsal somite after tissue ablation. (A and B) In situ hybridization and sections showed that *Klhl31* expression was lost on the operated side, after removal of the neural tube and surface ectoderm. *Pax-1* remained expressed in the presence of the notochord/Shh (*n* = 10). (C and D) *Klhl31* expression was lost on the operated side, after removal of the notochord and floor plate (*n* = 10). (E and F) When a large mixed cell pellet containing DF1-RCAS-Shh and RatB1a-Wnt-6 cells was implanted, dorsal somite patterning and the activation of *Klhl31* expression was restored in the absence of surface ectoderm (*n* = 13). (G and H) When a large mixed cell pellet containing DF1-RCAS-Shh and RatB1a-Wnt-1 cells was implanted, dorsal somite patterning and the activation of *Klhl31* expression was restored in the absence of surface ectoderm (*n* = 12). (B, D, F and H) *Klhl31* probe was developed with NBT/BCIP (purple), and probes for *Pax-1* and *Shh* were developed with Fast Red. Implanted cell pellets are indicated by a stippled line and position of tantalum foil is indicated by a solid line. fp, floor plate; lg, large; nt, neural tube; se, surface ectoderm.

2.7. Sonic hedgehog together with Wnt-1 or Wnt-6 mediates the activation of Klhl31 expression in the myotome

The above tissue ablation experiments suggested a role for neural tube and surface ectoderm as well as ventral midline tissues, notochord and floor plate, for correct dorsoventral somite patterning and activation of *Klhl31* expression in the myotome. We showed that neither of these tissues on their own were able to induce a myotome and thus *Klhl31* expression. The notochord was not sufficient to activate *Klhl31* expression in the absence of neural tube and surface ectoderm (Figs. 3E, F and 6A, B). Similarly, the dorsal neural tube and surface ectoderm was not sufficient to activate *Klhl31* expression in the absence of the notochord and floor plate (Fig. 6C and D). These tissues secrete Wnt and Shh molecules, which are known to affect dorsoventral somite patterning and cell fates. Therefore, we determined whether Wnt-1 or Wnt-6, expressed in dorsal neural tube and surface ectoderm respectively, together with Shh can substitute in the absence of the inductive tissues and mediate the formation of the myotome and the initiation of *Klhl31* expression. The surface ectoderm and half of the dorsal neural tube were removed and an impermeable barrier (tantalum foil) was inserted. In addition, a large mixed cell pellet containing Shh and Wnt-6, or Shh and Wnt-1 expressing cells was implanted between the axial midline and the paraxial mesoderm (Fig. 6E–H). Embryos were harvested after 24 h incubation and in situ hybridization showed that *Klhl31* expression was induced on the operated side, suggesting that Wnt-6 or Wnt-1 together with Shh can substitute for the ablated tissues.

2.8. *Ectopic expression of Myf-5 but not myogenin in the neural tube results in expression of Klhl31*

Because the timing of Klhl31 expression closely followed the induction of myogenesis and the loss of *Klhl31* transcripts was linked to the loss of myotome formation, we asked whether myogenic regulatory factors (MRFs) were able to activate *Klhl31* expression (Fig. 7). Expression vectors encoding Myf-5 or myogenin and an IRES-GFP were electroporated into the neural tube as described (Sweetman et al., 2008). Embryos were incubated for 48 h and in situ hybridization with probes detecting GFP demonstrated successful targeting of MRF expression vectors to the neural tube. Detection of *Klhl31* showed that Myf-5 (Fig. 7A and C) but not myogenin (Fig. 7B and D) led to the ectopic expression of *Klhl31* in the developing neural tube.

3. Discussion

Here, we provide the first characterization of a vertebrate orthologue of *Klhl31* during amniote development. Sequence comparison showed that *Klhl31* is a highly conserved member of the family of Kelch-like proteins with a typical domain structure (Supplemental Fig. 1). Biochemical analyses have shown that the BTB domain may be involved in transcriptional repression (Yu et al., 2008), however, the molecular functions of *Klhl31* and the cellular processes it may regulate are currently unknown.

The specific expression of *Klhl31* transcripts in the early heart and in developing myoblasts shortly after their commitment to this fate suggest an important role during skeletal and cardiac myogenesis (Fig. 1). In particular, we show here that *Klhl31* expression is intimately linked to skeletal myogenesis in developing somites and is initiated just after *MyoD*

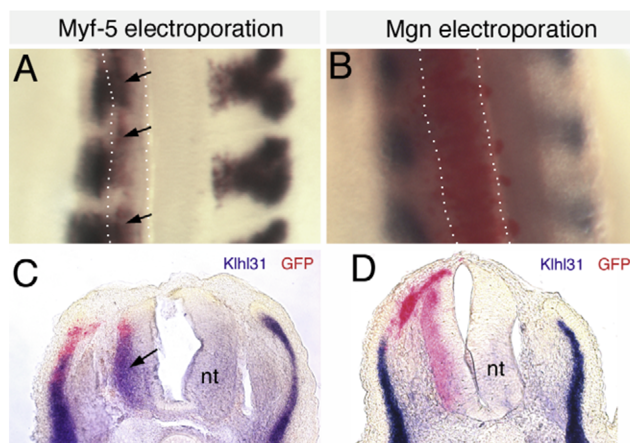

Fig. 7 – Targeted expression of *Myf-5* but not *myogenin* in the neural tube led to ectopic expression of *Klhl31*. (A and B) Dorsal views of whole mount embryos and (C and D) sectioned samples hybridized with probes detecting *GFP* (red) and *Klhl31* (purple) show that ectopic *Klhl31* expression was detected in embryos electroporated with *Myf-5* (A and C, black arrows, *n* = 20) but not with *myogenin* (B and D, *n* = 18). nt, neural tube; dotted white lines in (A and B) indicate the electroporated half of the neural tube.

(Fig. 2). Expression remains confined to skeletal muscles and the myocardium of the heart in late gestation embryos suggesting possible functions in both, the early phases of myogenic commitment and during the later muscle differentiation programme.

To determine which inductive tissues and molecular signals are involved in the regulation of *Klhl31* expression, we performed a series of tissue ablation experiments. The chicken embryo is particularly suitable for this type of approach due to the ease with which it can be accessed throughout development. These ablations combined with barrier insertion showed that the ventral midline tissues, floor plate and notochord, together with the surface ectoderm and dorsal neural tube provide signals important for the activation of *Klhl31* expression during embryogenesis. Notochord and neural tube (Fig. 3B) or notochord/floor plate and surface ectoderm (Fig. 3D) were sufficient to activate *Klhl31* expression. Notochord alone (Figs. 3F and 6B), surface ectoderm alone (Figs. 4B, D, H and 5B) or surface ectoderm and dorsal neural tube (Fig. 6D) were not sufficient. This is consistent with previous data from many laboratories, which demonstrated a requirement for all these tissues for dorsal somite patterning and myogenic differentiation (Borycki et al., 1997; Dietrich et al., 1998; Münsterberg and Lassar, 1995; Pownall et al., 1996, 2002). Interestingly, a surface ectoderm derived signal seems to be required for maintenance of *Klhl31* expression (Fig. 3J), whilst *Pax-1* continues to be expressed in the absence of the floor plate/notochord for at least 24 h (Fig. 3H and J).

We have previously demonstrated that Shh secreted from the notochord and floor plate together with Wnt signaling molecules derived from the neural tube and notochord are important for the activation of myogenesis in somite explants (Münsterberg et al., 1995). These observations were extended and confirmed *in vivo* in amniote embryos (Borycki et al., 1999; Marcelle et al., 1997; McDermott et al., 2005; Tajbakhsh et al., 1998). We also demonstrated that β-catenin is specifically expressed in the epaxial myotome together with Lef/TCF transcription factors (Schmidt et al., 2004) suggesting that the Wnt mediated signal acts through β-catenin. This was further supported by our finding that β-catenin expression in developing somites is regulated by myogenic signals (Schmidt et al., 2000). Following the characterization of crucial enhancer elements in mice (Summerbell et al., 2000) it has been shown that *Myf-5* is a direct target of Wnt/β-catenin (Borello et al., 2006). Furthermore, its full activation requires a cooperative interaction between the canonical Wnt and the Shh/Gli pathways in muscle progenitor cells (Borello et al., 2006).

We show here that the specific expression of *Klhl31* in skeletal muscles is regulated by myogenic signals, highly reminiscent of what we have previously found for β-catenin (Schmidt et al., 2000). In particular, a combination of Wnt-1 or Wnt-6 with Shh was able to rescue the ablation or blocking of inductive tissues and mediate myotome formation and initiation of *Klhl31* expression in myogenic cells (Fig. 6F and H). The activity of Wnt-1 and Wnt-6 is consistent with their expression in the dorsal neural tube and the surface ectoderm respectively (Hollyday et al., 1995; Schubert et al., 2002). Following tissue ablations, *Klhl31* expression was lost concomitantly with the loss of the

myotome itself suggesting that Klhl31 is a novel, integral component of vertebrate myogenesis and that it may be down-stream of the myogenic regulatory factors (MRFs). This was confirmed by electroporation of Myf-5 into the neural tube, which has been demonstrated to activate the myogenic programme ectopically (Delfini and Duprez, 2004; Sweetman et al., 2008) and led to expression of *Klhl31*. Interestingly, targeted mis-expression of myogenin did not result in *Klhl31* expression in the neural tube even though it can often mimic the activity of Myf-5 (Delfini and Duprez, 2004; Sweetman et al., 2008). It will be important to determine whether Myf-5 directly activates *Klhl31*, however, this will need further investigation.

4. Experimental procedures

4.1. *Cloning of chicken Klhl31 homologues*

The full-length coding region for chicken *Klhl31* was amplified by PCR from cDNA prepared from stage 8–14 chicken embryos using standard molecular biology protocols (Münsterberg and Lassar, 1995). Primers were designed using predicted *Klhl31* sequences for the chicken orthologue (ENSGALG00000016309) derived from the Ensembl Genome Browser (www.ensembl.org). *Klhl31* primer sequences were: Klhl31-Not1 forward primer: 5′- GCGGCCGCATGGCACCTAA GAAGAAGAAC-3′ and Klhl31-EcoR1-HA tag reserve primer: 5′-GAATTCTCAAGCGTAATCTGGAACATC GTATGGGTAAATA CTGACTGGTACAGAAG-3′. The reverse primer included a haemagglutonin tag. PCR products were cloned into pGEM-T (Promega) and sequenced.

4.2. *Whole-mount in situ hybridization, cryosections and photography*

Fertilized eggs were incubated at 37 °C until the desired stage of development was reached (Hamburger and Hamilton, 1992). The embryos were collected into DEPC treated PBS, cleaned and fixed overnight at 4 °C in 4% paraformaldehyde, then dehydrated through ascending grades of PTW/methanol washes and stored in methanol at −20 °C. Antisense digoxigenin-labelled riboprobe corresponding to the full-length coding sequence for chicken *Klhl31* was synthesized using linearized pGEM-T plasmid and Sp6 polymerase. Shh, Pax-1, Pax-3 and MyoD plasmids were kind gifts from Randy Johnson, Rudi Balling, Martin Goulding and Cliff Tabin. Whole-mount in situ hybridization was performed as previously described (Schmidt et al., 2000). After in situ hybridization, the embryos were fixed in 4% paraformaldehyde overnight and photographed. For cryosectioning, embryos were embedded in OCT (Tissuetec) and 20 μm sections were cut, collected on TESPA coated slides, washed with PTW, coverslipped with Entellan (Merck, Germany) and examined on 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 using Axiovision. Images were imported into Adobe Photoshop for labelling.

4.3. *Tissue ablations, grafting of cell pellets and implantation of impermeable barriers*

Embryos were incubated to the desired stage (HH11–HH12), eggs were opened from its broad end using blunt forceps. Surgical manipulations were performed under a stereo dissecting microscope using flame-sharpened tungsten needles. To examine effects on initiation of *Klhl31* expression tissues were removed adjacent to at least three epithelial somites (somite stage II–IV/V). To examine the requirement for the maintenance of *Klhl31* expression axial midline tissues were removed adjacent to somites IX–XII of HH12 embryos. One to two drops of Dispase (1 mg/ml) were added to facilitate tissue removal. Wnt-1, Shh, Wnt-1/Shh, Wnt-6 or Wnt-6/Shh expressing cell pellets were used as a source for signaling molecules and/or as a physical barrier. The parental cell lines, RatB1a-LNCX and DF1-RCAS-GFP, were used as controls. Cell pellets were picked up with a pipette and placed into the operated site using tungsten needles, large cell pellets were 100–150 μm in diameter, small cell pellets were 40–60 μm in diameter. After separation of surface ectoderm from the paraxial mesoderm impermeable tantalum foil (thickness = 0.0075 mm) was implanted to prevent reattachment of the ectoderm (Alvares et al., 2003; Dietrich et al., 1997). Embryos were incubated for 24 h after microsurgery and then harvested and processed for in situ.

4.4. *Cell culture and transfection*

RatB1a fibroblasts expressing Wnt-1 or Wnt-6 and DF-1 cells infected with RCAS-Shh or RCAS-GFP were cultured in DMEM (GIBCO Paisley UK) containing 10% fetal calf serum. RatB1a fibroblasts were grown in presence of G418 (250 μg/ml) as described (Münsterberg et al., 1995). When the cells became confluent they were trypsinized and transferred to a 35 mm bacterial dish with fresh medium. After overnight incubation cells formed aggregates in suspension, which were used for grafting. Mixed cell aggregates were generated by seeding a 1:1 mixture of Wnt-1/Shh or Wnt-6/Shh expressing cells in bacterial dishes.

4.5. *Electroporation of neural tube*

The expression vectors and electroporation procedure used were as described previously (Sweetman et al., 2008).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2009.07.006.

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