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Kremen is required for neural crest induction in *Xenopus* and promotes LRP6-mediated Wnt signaling

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Kremen 1 and 2 (Krm1/2) are transmembrane receptors for Wnt antagonists of the Dickkopf (Dkk) family and function by inhibiting the Wnt co-receptors LRP5/6. Here we show that Krm2 functions independently from Dkks during neural crest (NC) induction in *Xenopus*. *Krm2* is co-expressed with, and regulated by, canonical Wnts. *Krm2* is differentially expressed in the NC, and morpholino-mediated Krm2 knockdown inhibits NC induction, which is mimicked by LRP6 depletion. Conversely, *krm2* overexpression induces ectopic NC. Kremens bind to LRP6, promote its cell-surface localization and stimulate LRP6 signaling. Furthermore, Krm2 knockdown specifically reduces LRP6 protein levels in NC explants. The results indicate that in the absence of Dkks, Kremens activate Wnt/β-catenin signaling through LRP6.

KEY WORDS: Dkk, Kremen, LRP6, Mesd, Slug, Sox10, Wnt, *Xenopus*

INTRODUCTION

The canonical Wnt/β-catenin signaling cascade is an evolutionarily conserved and ancient pathway playing major roles during animal development, e.g. in axis formation, anteroposterior (AP) patterning and neural crest formation (Moon et al., 1997; Wodarz and Nusse, 1998). Misregulation of Wnt signaling is implicated in cancer formation (Bienz and Clevers, 2000). During Wnt/β-catenin signaling, Wnt glycoproteins bind to Frizzled receptors and LRP5/6 co-receptors, and activate a complex signaling cascade to stabilize β-catenin, a transcriptional co-activator (Wodarz and Nusse, 1998; Nusse, 2005).

LRP5/6 proteins are essential components for Wnt/β-catenin signal transduction, and their inactivation phenocopies loss of Wnt signaling in vertebrates and invertebrates (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). LRP5/6 proteins are members of the low-density lipoprotein receptor family (LDLR), transmembrane receptors characterized by an extracellular domain containing LDLR repeats and YWTD β-propeller/EGF modules (He et al., 2004). The YWTD β-propeller/EGF modules are cysteine-rich domains of around 35 kDa, which require specific folding and maturation assistance in the endoplasmic reticulum (ER), mediated by the chaperone Mesd (Culi and Mann, 2003; Hsieh et al., 2003; Culi et al., 2004). Mesd is a resident protein of the ER that binds to and promotes cell surface localization of LRP5/6 by reducing receptor aggregation (Hsieh et al., 2003).

Wnt/LRP5/6 signaling is antagonized by Dickkopf (Dkk) proteins, which bind and inhibit LRP5/6 (Bafico et al., 2001; Mao et al., 2001; Semenov et al., 2001). During early vertebrate development, Dkk1 is expressed in anterior endomesoderm and plays an important role in AP patterning by inhibiting Wnt/LRP6 signaling in the head organizer and prechordal plate (Glinka et al., 1998; Hashimoto et al., 2000; Shinya et al., 2000; Mukhopadhyay et al., 2001).

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Other Dkk receptors are the transmembrane proteins Kremen1 and 2 (Krm1 and 2, collectively termed Krms). Dkk1 forms a ternary complex with Krm1 or 2 and LRP6, which is cleared from the cell surface, thereby shutting down Wnt signal transduction (Mao et al., 2002). Krms are evolutionary conserved in vertebrates and are differentially expressed during embryonic development in mouse and frog (Nakamura et al., 2001; Davidson et al., 2002). In *Xenopus*, Krms and Dkk1 are co-expressed in the prechordal plate and functionally cooperate in Wnt inhibition to regulate AP patterning of the central nervous system (CNS) (Davidson et al., 2002). Recently, it was shown that Krm1 is required for formation of thymic architecture in mice by acting as Wnt inhibitor (Osada et al., 2006).

While it is established that Krms and Dkk1 cooperate in Wnt inhibition during *Xenopus* AP patterning, it is unknown whether Krms play other roles during early development. In *Xenopus* embryos, *krm2* is expressed in various regions that do not overlap with *dkk1* expression domains (Davidson et al., 2002). We therefore investigated the possibility of Dkk1-independent functions of Krm2.

Here we report that Krm2 plays a Dkk1-independent role in neural crest (NC) formation. *Krm2* is positively regulated by zygotic Wnt signaling and is required for NC formation. We show that in the absence of Dkk1, Krms stimulate Wnt signaling and promote, via direct binding, cell-surface localization of LRP6. Furthermore, Krm2 knockdown specifically reduces LRP6 protein levels in NC cells. The results suggest that Krms act as inhibitor or activator of Wnt signaling, dependent on the presence or absence of Dkk, respectively.

MATERIALS AND METHODS

Embryos and explants

In vitro fertilization, embryo culture, staging and microinjection were carried out as described (Gawantka et al., 1995). Preparation of mRNA for *Xenopus* injections was carried out in the presence of cap analog using the MegaScript in vitro transcription kit (Ambion), according to the manufacturer's instructions. Animal cap explants and conjugates were prepared as described (Villanueva et al., 2002).

In situ hybridization and RT-PCR

Whole-mount in situ hybridizations were carried out essentially as described (Gawantka et al., 1995). For lineage tracing, *lacZ* mRNA (250 pg per blastomere) was co-injected and β-galactosidase staining was performed as described (Sive et al., 2000), using blue (X-Gal) or red (Magenta-Gal)

substrate. RT-PCR assays were carried out in the exponential phase of amplification and with primers as described [*H4* (Niehrs et al., 1994); *chordin* (Sasai et al., 1994); *XWnt8* (Christian et al., 1991); *Xhox3* (Ruiz i Altaba and Melton, 1989)]. Other primers were: *Xenopus krm2* (forward, GGAACCAGACCACACAGCACTTG; reverse, CCGCCTCCACAC-CTGCATACT) and *Xenopus brachyury* (forward, CACAGTTCA-TAGCAGTGACCG; reverse, TTCTGTGAGTGTACGGACTGG).

Morpholino antisense oligonucleotides

Krm2 MO-1 targeting the ATG start codon of *Xkrm2* was as described (Davidson et al., 2002); Krm2 MO-2 [targets 5′ untranslated region (UTR) of *Xkrm2*]: ATCCTCACATGAAGACGTGCTGGAA; LRP6 MO [targets 5′ UTR of *X. laevis* (AF508961) and *X. tropicalis* (CX889920) *LRP6*]: CCCCGGCTTCTCCGCTCCGACCCCT. Control morpholino: standard control morpholino oligo designed by Gene Tools, LLC.

Explant immunostaining experiments

For neural crest immunostaining experiments, two-cell stage embryos were injected with 7.5 and 12.5 ng CoMO or Krm2 MO-2, 0.2, 2 and 5 ng LRP6 MO, or 1.5, 3 and 5 ng Krm2 MO-1 per blastomere, respectively (Fig. 7B). Anterior NC explants were prepared from neurulae by peeling off the epidermal layer before dissection with eyebrow knives. Immunostaining procedures were essentially carried out as described (Unterseher et al., 2004), and embryos were mounted in Mowiol. Primary antibodies used were rabbit anti-LRP6 T1479 (dilution 1: 50) (Davidson et al., 2005) and mouse anti-β1-integrin (dilution 1:10) (8C8, DSHB). Secondary antibodies were goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 546 (Molecular Probes). Nuclei were counterstained with Hoechst. Explants were examined on a confocal laser-scanning microscope (Nikon C1Si). For statistics, all images were transformed into monochrome RGB images (red for Alexa 546, green for Alexa 488, blue for Hoechst) and processed using ImageJ. For each neural crest explant, fluorescence intensity was measured in red (R) and green (G) channels at three random plasma membrane positions and averaged. The average R/G signal intensity of all explants per sample was determined. LRP6 protein levels were classified as unchanged when R/G <1.5, as moderately reduced (+) when R/G=1.5-3, or as strongly reduced (++) when R/G >3.

Cell culture and luciferase reporter gene assays

HEK293T cells were maintained in DMEM supplemented with 1% L-Glutamine, 1% PEN-STREP, and 10% FCS and grown at 10% CO_2. Luciferase reporter gene assays were carried out in triplicates in 96-well plates using Promega's Dual-Luciferase Reporter Assay System as described (Wu et al., 2000). In all experiments a total of 50 ng DNA per well was transfected; 1 ng pCMV-SPORT6-*mesd* (mouse) or 5 ng pCS2-*Xkrm1* DNA were used as indicated. For Wnt reporter assays transfected DNAs per well were: 10 ng TOPFLASH and 1 ng pTK-Renilla reporter plasmids; 12 ng (Fig. 4B) or 24 ng (Fig. 4A,D) human *LRP6*; 2 ng mouse *frizzled8* (*fz8*); 0.25 ng human *dishevelled1* (*dvl1*); 0.5 ng human *LRP6ΔE1-4* (Mao et al., 2001); 5 ng/3 ng mouse *wnt1*/human *LRP6* (Fig. 4D); 0.5 ng *Xdkk1*.

For BMP responsive reporter assays transfected DNAs per well were: 20 ng BREx4-E1b-dLuc plasmid [modified from Hata et al. (Hata et al., 2000)] and 1 ng pTK-Renilla; 10 ng pcDNA3.1-*BMP4* as indicated.

After transfection, cells were grown for 48 hours, then lysed in 50 μl passive lysis buffer (Promega) per well. Luciferase activity was normalized against Renilla activity.

Co-immunoprecipitation assays, in vitro binding assays, Endoglycosidase H treatment and cell surface biotinylation

For co-immunoprecipitation (Co-IP) assays, HEK293T cells were transfected in 10 cm plates with 0.1 μg pCS2-*krm1*-V5 or pCS2-*krm2*-V5 (both mouse) together with 1.5 μg pCS2-flag-*LRP6*, 0.1 μg pCS2-flag-*LDLRΔC* (both human), 0.5 μg pCS2-flag-*XFLRT3* (*Xenopus* fibronectin leucine-rich transmembrane protein 3) or 1.5 μg empty vector pCS2 using Fugene6 (Roche). After 48 hours, cells were washed in PBS and lysed in NP-40 buffer containing 150 mM NaCl, 50 mM Tris pH 7.4, 7.5% glycerol, 1 mM EDTA, 1 mM β-mercaptoethanol, 25 mM NaF, one protease inhibitor cocktail tablet/25 ml (Roche) and 0.8% NP-40. Lysates were subjected to Co-IP with anti-flag antibody beads (Sigma) overnight at 4°C. Co-IPs were washed with NP-40 buffer and analyzed by SDS-PAGE and western blotting.

In vitro binding assays were carried out essentially as described (Cruciat et al., 2006). Recombinant proteins were produced as conditioned media by transient transfection of HEK293T cells with pCS2-*krm1ΔTMC*-V5, pCS2-*krm2ΔTMC*-V5, pCS2-V5-*dkk3* (all mouse) or pCS2-myc-*LRP6ΔTMC* (human) in serum-free media (Optimem I, Gibco). Media were concentrated about 50-fold using Centricon Plus-20 filters (Millipore). Equal amounts of V5-tagged proteins were resuspended in NP-40 buffer containing 0.2% (w/v) NP-40 and incubated with anti-V5 antibody beads (Sigma) under gentle shaking overnight at 4°C. IPs were washed and incubated for 5 hours with media containing pCS2-myc-*LRP6ΔTMC*. Co-IPs were washed again and analyzed by SDS-PAGE and western blotting.

For deglycosylation of LRP6, HEK293T cells were grown in 10 cm plates and transfected with 1 μg flag-*LRP6* together with 0.4 μg pCS2-myc-*dkk3*, pCMV-SPORT6-*mesd*, pCS2-*krm2*-V5 (all mouse) or empty pCS2. After 2 days, cells were washed in Hank's buffer and resuspended in 1 ml hypotonic buffer containing 1 mM EDTA, 5 mM HEPES, pH 7.5, 0.1 mM PMSF and one protease inhibitor cocktail tablet per 25 ml. Samples were dounced 25 times, and after removal of cell debris by centrifugation at 2500 rpm (1050 ***g***), membranes were pelleted by centrifugation at 30,000 rpm (40,300 ***g***). Pellets were lysed in NP-40 buffer (without NaF) containing 2% NP-40 and subjected to EndoH (Roche) treatment (0.25 U/ml) in 100 mM NaOAc, pH 5.5, for 30 minutes at 37°C. Samples were analyzed by SDS-PAGE and western blotting.

For cell surface biotinylation, HEK293T cells were transfected in 6 cm dishes with 0.25 μg pCS2-flag-*LRP6* together with 0.1 μg pCS2-myc-*dkk3*, pCMV-SPORT6-*mesd* or pCS2-*krm2*-V5, or pCS2-flag-*NME* (human). After 2 days, cells were surface biotinylated by using 0.5 mg/ml sulpho-NHS-LC-biotin (Pierce) according to the manufacturer. After preparation of membranes as described above, samples were immunoprecipitated with anti-flag antibody beads and analyzed by SDS-PAGE and western blotting.

RESULTS

Xkrm2 is co-expressed with and regulated by Wnts

We previously reported that *Xkrm1* and *2* are co-expressed with *dkk1* in the *Xenopus* prechordal plate during mid-neurula stages, where they cooperate to regulate AP CNS patterning (Davidson et al., 2002). However, *krm2* showed additional expression in regions devoid of *dkk1* transcripts. At gastrula stages *Xkrm2* expression occurred in the ventrolateral marginal zone of the embryo (Fig. 1A). During neurula stages *krm2* was expressed in the lateral neural plate, overlapping with the neural crest marker *slug* (Fig. 1B). Furthermore, *krm2* was co-expressed with Wnt genes such as *Wnt8* and *Wnt3a* (Fig. 1A), raising the possibility that *krm2* expression is regulated by Wnt signaling. Indeed, local inhibition of Wnt signaling by injection of dominant-negative *XWnt8* mRNA abolished the expression of *krm2* in the marginal zone (Fig. 1C,E). Conversely, injection of *Wnt8* and *Wnt3a* DNA led to ectopic expression of *krm2* (Fig. 1D,E).

Furthermore, we analyzed the effect of Wnt and BMP pathway activation on *krm2* expression in animal caps. In control caps *krm2* was expressed at moderate levels. *Wnt8* and *Wnt3a* mRNA injections increased *krm2* expression, whereas *BMP4* mRNA had no effect (Fig. 1F). LiCl treatment of early embryos, which leads to dorsoanteriorization, downregulated *krm2* expression along with *Wnt8* and other ventrolaterally expressed genes (Fig. 1G, and C.H. and C.N., unpublished).

We conclude that: (1) at gastrula stages *krm2* is expressed and regulated like a classical ventrolateral gene; (2) *krm2* is co-expressed with Wnts and regulated by zygotic Wnt signaling; (3) at neurula stages *krm2* shows differential expression in the neural crest.

Overexpression of *krm2* induces NC markers and NC-derived structures

To study potential Dkk1-independent roles of Krm2, we first analyzed gain-of-function effects. Localized injection of *krm2* mRNA into prospective anterior embryonic regions induced ectopic

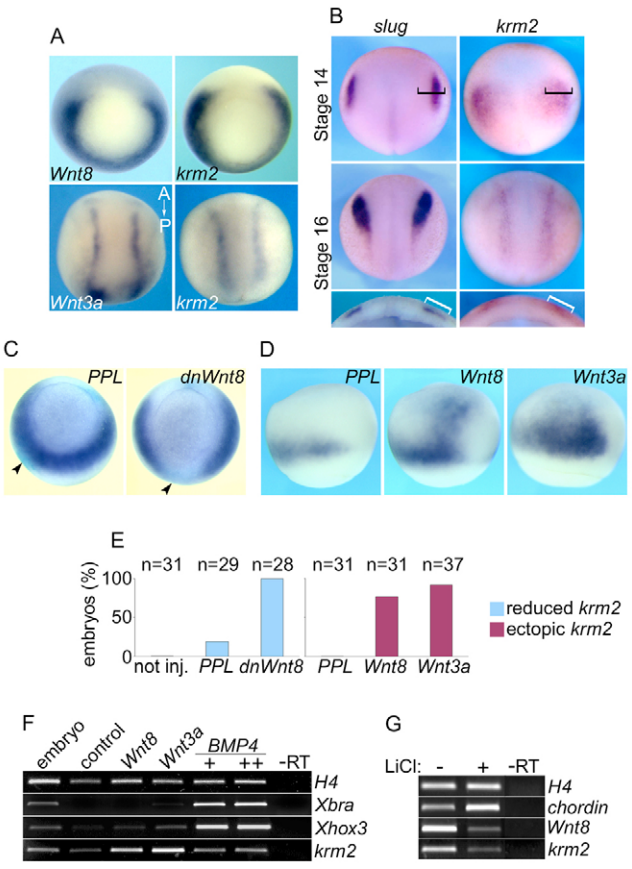

Fig. 1. In *Xenopus*, *Krm2* is regulated by Wnt signaling and expressed in the NC region. (A-D) Whole-mount in situ hybridizations. (**A**) Comparison of *Xkrm2*, *XWnt8* and *XWnt3a* expression patterns in gastrula and early neurula stage embryos. Top: gastrula stage. Vegetal view, dorsal is up. Bottom: neurula stage. Dorsal view, anterior is up. (**B**) Comparison of *Xkrm2* and *slug* expression patterns at the indicated stages. Dorsal view, anterior is up. Lowermost panel: view of frontally cut stage 16 embryos, dorsal is up. Brackets indicate overlapping expression domains of *krm2* and *slug*. (**C-E**) Effect of Wnt pathway perturbations on *krm2* expression. (C) Embryos at the 32-cell stage were injected equatorially in two opposite blastomeres with 1 ng *PPL* or *dnWnt8* and 250 pg *lacZ* mRNA and analyzed at gastrula stage. Arrowheads indicate β-gal lineage tracer staining (blue). Vegetal view, dorsal is up. (D) Embryos at the four-cell stage were injected animally with 200 pg pCS-*PPL* or pCSKA-*Wnt8* DNA or 100 pg pCS-*Wnt3a* DNA in one blastomere and analyzed at gastrula stage. Lateral view, dorsal to the right. (E) Statistical overview of experiments shown in C and D. (**F**) Embryos at the four- to eight-cell stage were injected animally with 100 pg *XWnt8* or *Wnt3a* mRNA, or 1 or 2 ng *BMP4* mRNA. Animal caps were cut at stage 8-9, cultured until stage 20 equivalent and were analyzed by RT-PCR for expression of the indicated genes. (**G**) Embryos at the 32-cell stage were treated with 120 mM LiCl for 50 minutes, cultured until stage 11.5 and analyzed by RT-PCR. Histone H4 was used for normalization. –RT, minus reverse transcriptase control.

cement glands (Fig. 2A, arrowhead) and retinal pigment epithelium (Fig. 2B, arrowhead). Ventral injection of *krm2* mRNA led to induction of protrusions containing melanocytes and fin-like structures (Fig. 2C, arrowhead). Widespread *krm2* overexpression led to strongly hyper-pigmented embryos due to overproduction of melanocytes (Fig. 2E). Embryos frequently also showed eye and tail defects (not shown).

Melanocytes are NC derivatives, which in *Xenopus* are characteristically overproduced when NC regulators such as *slug* or *sox10* are overexpressed (LaBonne and Bronner-Fraser, 1998; Aoki et al., 2003; Honore et al., 2003). Indeed, Krm2 overexpression affected NC markers in a region-specific fashion: posterior *krm2* mRNA injection led to expansion of *slug* and *sox10* (Fig. 2J,L, black arrowheads). By contrast, anterior *krm2* injection reduced *slug* and *sox10* expression (Fig. 2K,M, black arrowheads). Thus, the region of *krm2* expression determines its effect on NC markers (summarized in Fig. 2N). This may be explained by the interaction of anteriorly overexpressed Krm2 with Dkk1, to inhibit Wnt signaling and NC induction. In posterior regions, where Dkk1 is absent, Krm2 had a stimulatory effect on NC development. Furthermore, *krm2* mRNA injection can also induce ectopic expression of the NC marker *sox10* (Fig. 2O,P, arrowheads).

Morpholino-mediated knockdown of Krm2 inhibits NC formation

As *krm2* is expressed in the prospective NC region and is sufficient to induce NC tissue, we next asked if it is also required for NC development. We made use of the previously characterized morpholino antisense oligonucleotide targeting the ATG codon of *Xkrm2* (MO-1) (Davidson et al., 2002). Besides the previously described microcephaly, we found that Krm2 MO-1 injection strongly reduced the pigmentation of embryos (Fig. 3A). Co-injection of V5-*Xkrm2* mRNA (Davidson et al., 2002), a construct lacking the MO-binding site, partially restored the pigmentation (Fig. 3A), showing the specificity of the morpholino. Furthermore, Krm2 MO-1 inhibited *slug* expression (Fig. 3B,C). This is also the case for a second Krm2 MO (Krm2 MO-2), which targets the 5′ UTR of *krm2* (Fig. 3B,C), thus corroborating the specificity of the NC inhibition.

To analyze if Krm2 is required specifically in the ectoderm for NC formation, or in the underlying, inducing mesoderm, we combined Krm2 MO-1-injected animal caps with uninjected dorsolateral marginal zones (DLMZs) and analyzed NC induction. DLMZs induced *slug* in control caps in 62% of cases (n=42). *Slug* induction was reduced upon injection of Krm2 MO-1 into the responding ectoderm (38%, n=24). By contrast, injection of Krm2 MO-1 into DLMZs did not affect *slug* induction in animal caps (86%, n=13) (Fig. 3D). These results indicate that Krm2 is required directly in the ectoderm for NC formation, where it is also expressed.

Morpholino-mediated knockdown of LRP6 inhibits NC formation

It is well established that canonical Wnt signaling is required for NC induction (LaBonne and Bronner-Fraser, 1999; Wu et al., 2003; Barembaum and Bronner-Fraser, 2005). In *Xenopus*, overexpression of Wnts or Wnt receptors, as well as downstream signaling components, can all induce NC. Conversely, inhibition of Wnts, Wnt receptors and β-catenin blocks NC induction (Yanfeng et al., 2003; Barembaum and Bronner-Fraser, 2005; Wu et al., 2005; Abu-Elmagd et al., 2006).

As Krm2 is a negative Wnt modulator and is itself Wnt-regulated, we therefore hypothesized that Krm2 may also play a positive role in Wnt/LRP6-mediated NC induction. Hence, we tested whether LRP6 knockdown mimics Krm2 loss-of-function during NC development.

Injection of an LRP6 MO targeting both *X. laevis* and *X. tropicalis* genes resulted in strongly anteriorized embryos with enlarged cement gland, shorter and ventrally bent tail and triangular body shape (Fig. 3E). This phenotype closely mimics the anteriorization induced by overexpression of the LRP6 antagonist *dkk1* (Fig. 3E). Of note, a

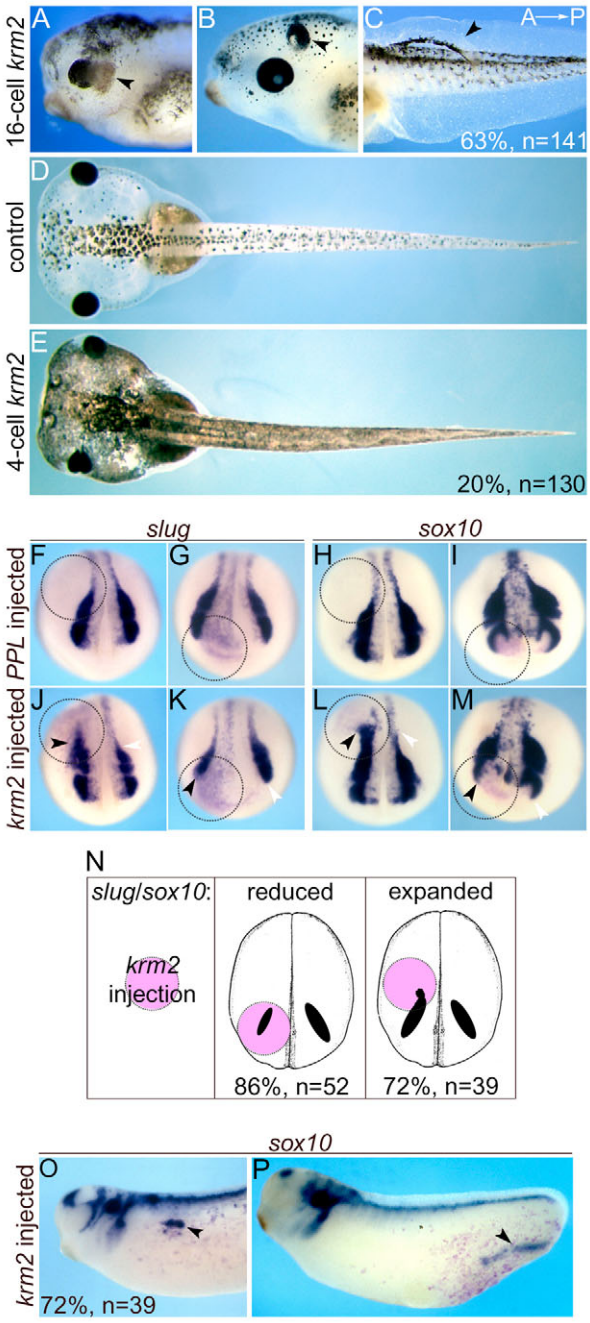

Fig. 2. *Krm2* overexpression induces NC-derived structures and NC markers in *Xenopus*. (**A-E**) Phenotypic analysis of *krm2* overexpression. Anterior to the left. (A-C) Embryos at the 16-cell stage were injected in a single animal (A,B) or ventral equatorial (C) blastomere with 400 pg *krm2* mRNA and photographed at tadpole stages. Arrowheads indicate ectopic pigment-containing structures. (D) Uninjected control embryo at tadpole stage. (E) Embryos at the four-cell stage were injected equatorially in both dorsal blastomeres with 400 pg *krm2* mRNA each. (**F-M**) Neurula stage embryos, shown in anterior view. Embryos were injected with 400 pg *PPL* (F-I) or *krm2* (J-M) mRNA into one ventral equatorial blastomere at the 16-cell stage (F,H,J,L) or one dorsal animal blastomere at the 8-16-cell stage (G,I,K,M). In situ hybridizations were performed using *slug* and *sox10* probes as indicated. β-gal lineage tracer is stained in red. Circles indicate lineage-tracer-positive cells. Black and white arrowheads indicate altered and control marker gene expression, respectively. (**N**) Scheme and statistical overview of experiment shown in F-M. Red area indicates region of *krm2*-injected cells. (**O**,**P**) Embryos were injected as in C and *sox10* expression was analyzed at tailbud stage by whole-mount in situ hybridization. Arrowheads indicate ectopic *sox10* expression. Co-injected *lacZ* was used as lineage tracer (red).

morphologically highly sensitive structure to injection of low doses of LRP6 MO is the dorsal fin (see Fig. S1 in the supplementary material). The LRP6 MO phenotype was fully rescued by co-injecting human *LRP6* mRNA (Fig. 3Ec,e), confirming specificity of the MO. Furthermore, injection of LRP6 MO in *X. tropicalis* embryos resulted in the same phenotype as in *X. laevis* (Fig. 3F).

Similarly to Krm2 MO, LRP6 MO inhibited *slug* expression (Fig. 3G). Thus, MO-mediated knockdown of both LRP6 and its modulator Krm2 result in inhibition of NC formation.

Krms stimulate LRP6-mediated Wnt signaling in HEK293T cells

The results obtained so far suggest that Krm2, besides its well-established role in Wnt inhibition, may also, in the absence of Dkk1, positively regulate Wnt signaling. Previously we found no effect of *krm1* or *2* in Wnt responsive reporter assays triggered by transfection of *wnt/fz* or *wnt/fz/LRP6* in HEK293T cells. We now find that *Xkrm1*, but also *Xkrm2*, significantly stimulated Wnt signaling when co-transfected with *LRP6* alone, whereas no effect was observed with *frizzled8* or *dishevelled* (Fig. 4A and see Fig. S2 in the supplementary material). We conclude that Krms can promote LRP6-mediated signaling.

One possibility for how Krm may stimulate LRP6 signaling is by promoting its trafficking or stability. We therefore compared its effects to Mesd, an LRP6 chaperone, which also stimulates LRP6-mediated Wnt signaling (Culi and Mann, 2003; Hsieh et al., 2003) (Fig. 4A). As previously shown, Mesd is required for maturation of β-propeller/EGF modules of LDLR family members (Culi et al., 2004). Consequently, *mesd* transfection did not promote signaling stimulated by LRP6ΔE1-4, a constitutive active LRP6 construct with truncated extracellular domain (Fig. 4A). In these experiments, *LRP6ΔE1-4* was intentionally transfected at low amounts to sensitize the reporter assay for synergistic effect. By contrast, *krm1* does moderately stimulate LRP6ΔE1-4-mediated signaling (Fig. 4A). Co-transfection of *krm1* and *mesd* shows a merely additive effect (Fig. 4B), suggesting that these genes do not functionally interact.

To test the specificity of the Wnt promoting effect of *krm1*, we analyzed a BMP responsive reporter and found it unaffected (Fig. 4C).

We next analyzed the effect of *Xkrm1* and *mesd* on Wnt signaling in the presence of a very low amount of *dkk1*, which alone was insufficient to block LRP6 signaling. As reported (Mao et al., 2002; Li et al., 2005), co-transfection of *dkk1* with *Xkrm1*, but not *mesd*, reduces the *LRP6* signal to background levels (Fig. 4D). When Wnt1 was co-transfected additionally, the same result was observed.

Taken together, these data indicate a context-dependent role of Krms in Wnt signaling. Together with Dkk1, Krms inhibit Wnt/LRP6 signaling; however, in the absence of Dkk1, Krms promote LRP6 signaling.

Krms bind to LRP6

We previously reported that Dkk1 binds to both Kremen and LRP6, thereby bridging the two receptors in a ternary complex, which is then removed from the cell surface (Mao et al., 2001; Mao et al.,

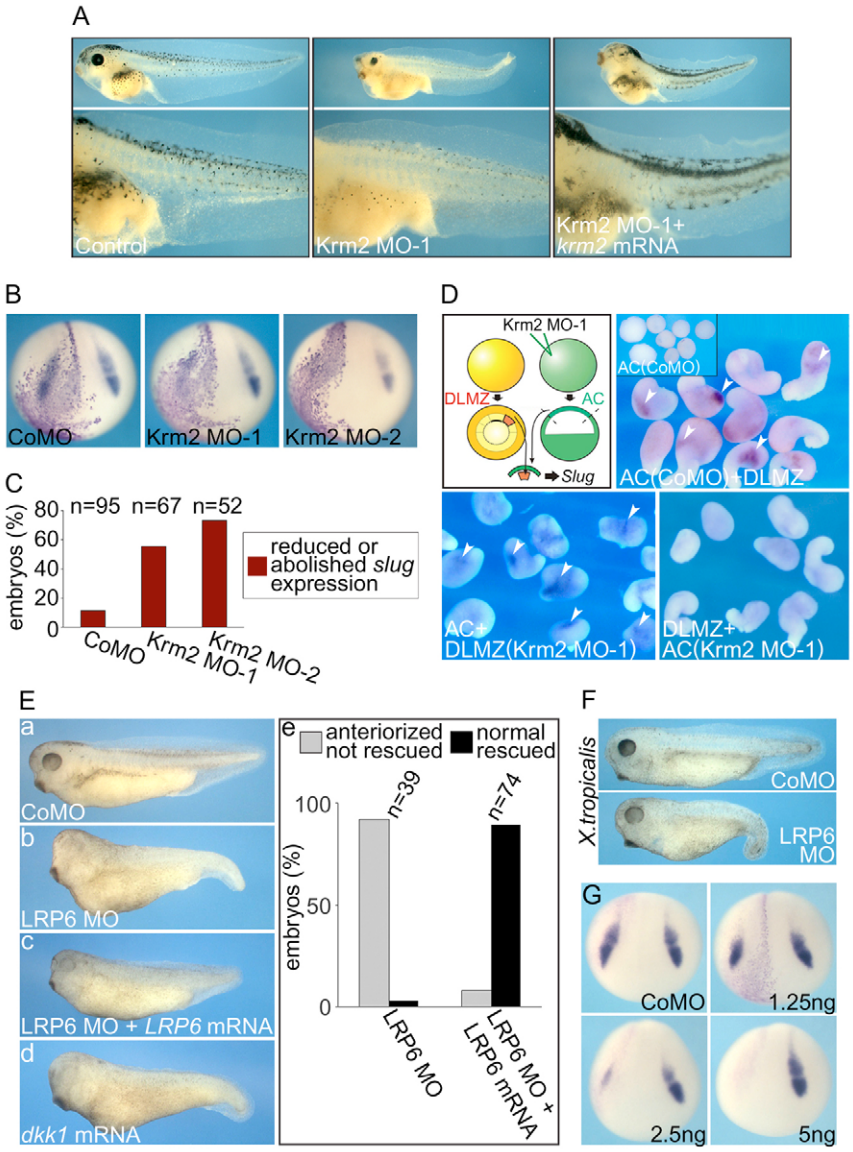

Fig. 3. Krm2 and LRP6 are required for NC induction in *Xenopus*. (**A-D**) Morpholino-mediated knockdown of *krm2* expression. (A) Embryos at the eight-cell stage were injected animally with 10 ng Krm2 MO-1 plus either 0.8 ng *PPL* (middle panel) or V5-*Xkrm2* mRNA (right panel), respectively. Control, *PPL* mRNA injected. Normal or hyper-pigmentation was observed in 9% (*n*=35) of (Krm2 MO-1 + *PPL*) and in 44% (*n*=27) of (Krm2 MO-1 + V5-*Xkrm2*) mRNA injected embryos, respectively. (B) Embryos at the eight-cell stage were injected animally with 10 ng Krm2 MO-1 or 20 ng Krm2 MO-2 or control MO (CoMO), respectively. Shown are whole-mount in situ hybridizations for *slug* expression at neurula stage in anterior view. β-gal lineage tracer is stained in red. (C) Statistical overview of MO injection experiment in B. (D) Top left: diagram of experiment. Two-cell-stage embryos were injected animally with 7.5 ng CoMO or Krm2 MO-1, and animal caps (ACs) were explanted at stage 8-9 and combined with dorsolateral marginal zones (DLMZs) of uninjected or Krm2 MO-1 (7.5 ng) injected gastrula-stage embryos. Conjugates were assayed at stage 20 for *slug* expression by in situ hybridization. Top right: conjugates of DLMZs and CoMO-injected caps. Arrowheads indicate *slug* expression. Inset: ACs injected with CoMO and processed for *slug* expression. Bottom left: conjugates of ACs and Krm2 MO-1 injected DLMZs. Arrowheads indicate *slug* expression. Bottom right: conjugates of DLMZs and Krm2 MO-1 injected ACs. (**E**,**F**) Phenotypic analysis of LRP6 MO in *X. laevis* (E) and *X. tropicalis* (F). All embryos were injected equatorially at the two-cell stage. (E) (a) Injection of 5 ng CoMO. (b,c) Co-injection of 5 ng LRP6 MO and either 1 ng control (*PPL*) (b) or 400-600 pg human *LRP6* mRNA (c). (e) Statistical overview of a-c. (d) Injection of 20 pg *dkk1* mRNA. (F) *Xenopus tropicalis* embryos injected with 1.25 ng CoMO or LRP6 MO show the displayed phenotypes at frequencies of 89%, *n*=38, upper panel and 98%, *n*=44, lower panel. (**G**) Two-cell-stage *X. laevis* embryos were injected equatorially in one blastomere with 5 ng CoMO or increasing LRP6 MO doses as indicated. Neurula stage embryos were processed for *slug* expression by in situ hybridization and are shown in anterior view. β-gal lineage tracer is stained in red.

2002). As our results indicate that Krms can also function without Dkk1, we tested whether they might bind directly to LRP6, in the absence of Dkk1. We used HEK293T cells, which express very low levels of *dkk1* (our unpublished observations). In co-immunoprecipitation experiments Krm1 and 2 were specifically precipitated with LRP6 (Fig. 5A,A′, lane 2), but not with the control transmembrane proteins FLRT3 and LDLRΔC (Fig. 5A,A′, lanes 3,4). This was also the case in Co-IPs with added anti-Dkk1

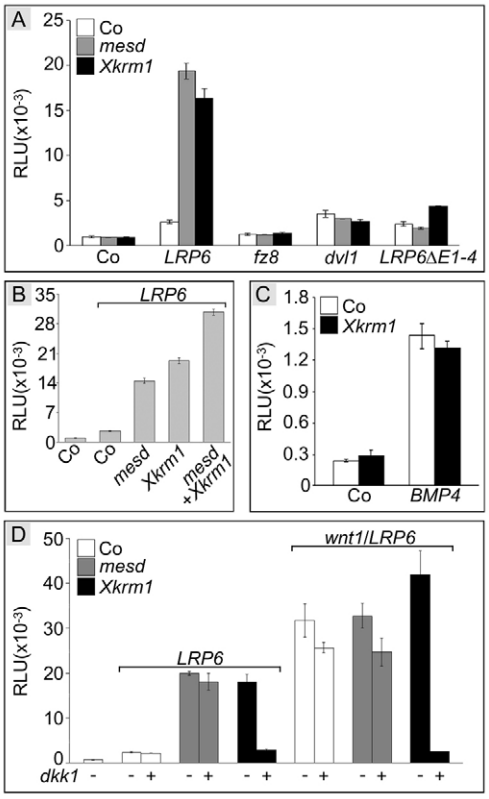

Fig. 4. Krm promotes LRP6-mediated Wnt signaling. (**A-D**) TOPFLASH luciferase reporter assays in HEK293T cells. Co, empty pCS2 vector. (A) XKrm1 cooperates specifically with LRP6. (B) Co-transfection of *mesd* and *Xkrm1*. (C) BMP luciferase reporter assay in HEK293T cells. (D) Effect of XKrm1 on LRP6 signaling is Dkk1-dependent. RLU, relative light units.

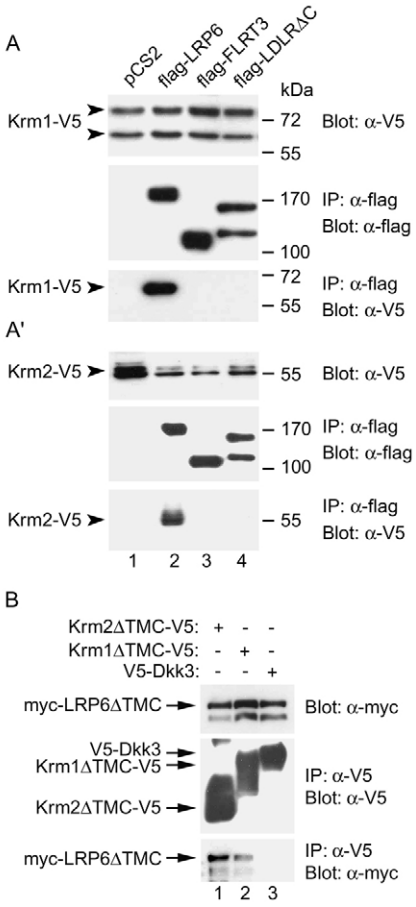

Fig. 5. Krm binds to LRP6 specifically and directly. (**A**,**A′**) Co-IP assays of HEK293T cell lysates transfected with *krm1-V5* (A) or *krm2-V5* (A′) and the indicated constructs. Co-IPs were performed with anti-flag antibody and analyzed by SDS-PAGE and western blotting. (**B**) In vitro binding assay with secreted recombinant proteins as indicated. IPs were performed with anti-V5 antibody and analyzed by SDS-PAGE and western blotting. Upper panel, protein expression. Middle and lower panels, IPs.

antibody, to block any endogenous Dkk1 protein (C.M.C. and C.N., unpublished). To corroborate the directness of binding and to demonstrate extracellular interaction we also performed in vitro binding assays using secreted, recombinant proteins, which show that LRP6 co-precipitates with both Krm1 and 2, but not Dkk3 (Fig. 5B). We conclude that Krm1 and 2 specifically and directly bind to LRP6.

Krm2 promotes cell-surface localization of LRP6

To explore the possibility that Krm may influence protein expression or intracellular transport of LRP6, we performed co-transfection experiments. In western blot analysis of transfected HEK293T cell lysates, LRP6 was detected as an upper and a lower band, which are thought to correspond to the mature cell surface (ma), and immature cytoplasmic (im) forms of LRP6 (Fig. 6A, lane 1) (Hsieh et al., 2003). We confirmed this by treatment with Endoglycosidase H (EndoH), which cleaves immature glycans that have not yet traversed the Golgi apparatus. Only the lower band was EndoH-sensitive and downshifted due to deglycosylation (dg) (Fig. 6A, lane 2).

Co-transfection of *mesd* increased mature LRP6, consistent with it being a reported chaperone (Culi and Mann, 2003; Hsieh et al., 2003) (Fig. 6A, lanes 5,6). Co-transfection of *krm2* had a very similar effect on LRP6. Mature LRP6 increased at the expense of the immature form (Fig. 6A, lanes 7,8), the total amount of LRP6 protein being mostly unaffected. Co-transfection of empty vector (Fig. 6A, lanes 1,2) or *dkk3* (Fig. 6A, lanes 3,4), a gene not affecting the Wnt pathway (Niehrs, 2006), had no effect on LRP6 protein expression.

To corroborate this finding, we monitored plasma membrane levels of LRP6 by cell surface biotinylation. Following co-transfection with *krm2*, cell surface levels of LRP6 were increased, while the total LRP6 was mostly unaffected (Fig. 6B). This effect mimicked *mesd* co-transfection (Fig. 6B). The cytoplasmic protein Nucleoside diphosphate kinase A (NME1) serves as control and was not biotinylated (Fig. 6B′). These data indicate that Krm2 promotes cell-surface localization of the Wnt co-receptor LRP6.

Krm2 knockdown reduces LRP6 protein levels in the NC

Our embryological data indicate a requirement of Krm2 for NC induction, and the cell culture data suggest that this may be due to a role of Krm2 promoting LRP6 cell-surface localization and thus Wnt signaling. To corroborate that it acts on LRP6 in vivo, we analyzed by immunofluorescence microscopy whether Krm2 is required for the cell-surface localization of endogenous LRP6 in NC

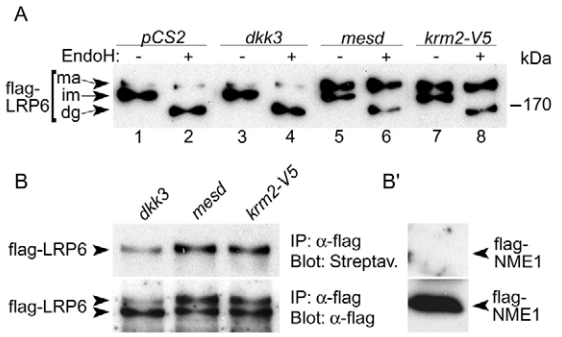

Fig. 6. Krm increases cell-surface levels of LRP6. (**A**) Western blot analysis of HEK293T cells transfected with flag-*LRP6* and the indicated constructs. Samples were treated with EndoH as indicated. Arrows indicate the EndoH-resistant mature and EndoH-sensitive immature forms of LRP6. (**B**) Cell surface biotinylation assay. HEK293T cells were co-transfected with flag-*LRP6* and the indicated constructs. After cell surface biotinylation, cell lysates were immunoprecipitated with anti-flag antibody and subjected to SDS-PAGE and western blot analysis. Membranes were probed with streptavidin-HRP to detect plasma membrane LRP6 (upper panel) and anti-flag antibody to detect total LRP6 (lower panel). (**B′**) The cytoplasmic protein NME1 is not biotinylated (upper panel). Lower panel: Total NME1 protein. dg, deglycosylated form; im, EndoH-sensitive immature form; ma, EndoH-resistant mature form.

explants. We used an antibody against the intracellular LRP6 domain, which is likely to recognize both mature and immature forms of the protein (Davidson et al., 2005) (data not shown).

In NC cells, LRP6 was predominantly localized at the plasma membrane (Fig. 7A). This signal was abolished following LRP6 MO injection, attesting to the antibody specificity. Injections of Krm2 MO-1 or Krm2 MO-2 both reduced LRP6 levels in a concentration-dependent manner, without affecting β1-Integrin levels (Fig. 7A,B). Unlike in NC explants, in animal caps Krm2 MO injection did not affect LRP6 protein levels (Fig. 7A), indicating that Krm2 is specifically required to maintain LRP6 protein levels in NC.

DISCUSSION

Krm2 is required for NC induction

The Wnt signaling pathway is reiteratively used during NC induction, delamination, migration and differentiation (Kalcheim and Burstyn-Cohen, 2005; Raible and Ragland, 2005; Taneyhill and Bronner-Fraser, 2005). During NC induction BMP and Wnt signaling are thought to act together to specify NC fate. In *Xenopus*, overexpression of various Wnts leads to NC induction (Saint-Jeannet et al., 1997; Chang and Hemmati-Brivanlou, 1998; LaBonne and Bronner-Fraser, 1998). Conversely, inhibition of Wnt signaling, e.g. by dominant-negative versions of Wnt1/8, Tcf-3 and LRP6 (Dorsky et al., 1998; LaBonne and Bronner-Fraser, 1998; Tamai et al., 2000; Garcia-Castro et al., 2002), or MO-mediated depletion of Frizzled3/7 or β-catenin (Deardorff et al., 2001; Wu et al., 2005; Abu-Elmagd et al., 2006), blocks NC induction.

Krm2 is differentially expressed in the NC precursors and is required for NC formation. As Krm2 is both regulated by Wnts and promotes LRP6 activity, this suggests that in the context of NC induction Krm2 functions by promoting Wnt signaling. Consistent with this, depletion of LRP6 inhibited NC induction similarly to Krm2 knockdown. Incidentally, this is the first evidence indicating that LRP6 acts non-redundantly from LRP5 in NC formation in *Xenopus*.

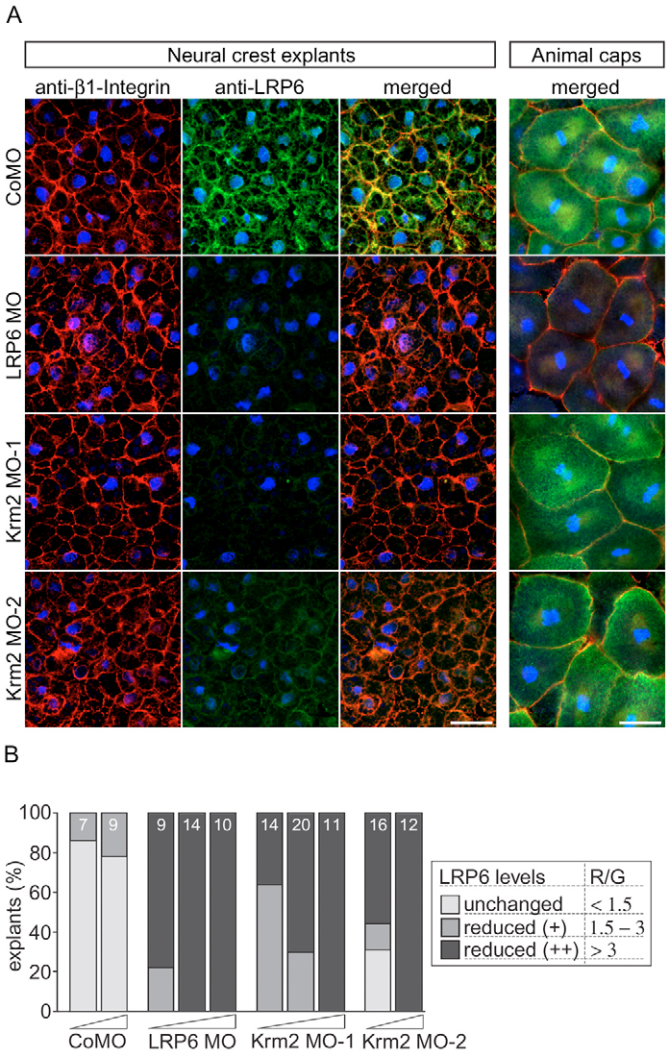

Fig. 7. LRP6 protein is reduced in neural crest of Krm2 morphants. (**A**) Confocal immunofluorescence analysis of *Xenopus* NC and animal cap (AC) explants. For NC explants, two-cell-stage embryos were injected with 2 ng LRP6 MO, 3 ng Krm2 MO-1 or 7.5 ng Krm2 MO-2 or CoMO per blastomere. Explants were dissected at stage 14-15, immunostained with anti-β1-Integrin and anti-LRP6 antibodies, and analyzed by confocal laser scanning microscopy. For AC explants, eight-cell-stage embryos were injected animally with 0.025 ng LRP6 MO, 4 ng Krm2 MO-1 or 15 ng Krm2 MO-2 or CoMO. Explants were dissected at stage 9-10 and analyzed like NC explants. (**B**) Quantification of relative LRP6 protein levels. The graph shows the ratio of β1-Integrin (R; red in A) to LRP6 (G; green in A) signal intensities. Numbers in bars indicate the number of analyzed explants. Hoechst-stained nuclei are blue. Scale bar: 50 μm.

The finding that anteriorly overexpressed Krm2 inhibited, while posterior overexpression enhanced, NC formation suggests a dual Krm2 action. Anteriorly overexpressed Krm2 probably cooperates with Dkk1 in Wnt inhibition, while in posterior regions Krm2 on its own promotes Wnt signaling. This dual activity is also supported by our cell culture experiments.

We cannot exclude the possibility that Krm2 may act also on other pathways that are involved in NC formation, e.g. BMP signaling. However, in cell culture reporter assays BMP signaling was unaffected by Krms.

Krm1 and *2* show differential expression at all stages of development both in *Xenopus* and mouse (Nakamura et al., 2001; Davidson et al., 2002), and analyses of their developmental role will need to take into account a possible positive action on Wnt signaling. Interestingly, based on EST expression data, *KRM2* mRNA is significantly upregulated in several human cancers, e.g. brain, testis, kidney and gastrointestinal tract tumors (http://cgap.nci.nih.gov/), and it is a Wnt target (this study), raising the possibility that Krm2 may be a tumor associated gene or may even have an oncogenic (or tumor suppressive) role.

Krms promote LRP6-mediated Wnt signaling

One surprising result of this study is that Krms not only act as negative, but also as positive, Wnt modulators, providing an explanation for their role in Wnt-mediated NC induction. Krm2 overexpression promoted cell surface localization of LRP6 in cultured cells; in embryos, Krm2 was required to maintain LRP6 protein levels in NC. Taken together, these data raise the possibility that Krm2 regulates LRP6 intracellular transport and turnover.

This is reminiscent of the LRP6 chaperone Mesd, which promotes LRP6 folding and cell surface localization (Culi and Mann, 2003; Hsieh et al., 2003) (this study). Do Krms then act as ER chaperones of LRP6? Three lines of evidence argue against this: (1) Unlike Mesd, Krms are not ER-resident (Culi and Mann, 2003; Hsieh et al., 2003; Cruciat et al., 2006); (2) artificially ER-trapped Krm2 has a strongly reduced effect on LRP6 surface localization compared to wild type Krm2 (C.H. and C.N., unpublished), indicating that a subcellular localization other than in the ER is required for Krm to exert its full effect on LRP6; (3) Krms can, albeit weakly, promote Wnt signaling induced by LRP6ΔE1-4 (see Fig. 4A), a construct lacking all four β-propeller/EGF regions, which are the target of Mesd (Culi et al., 2004). LRP6ΔE1-4, in contrast to full-length LRP6, is predominantly cell surface localized (C.H. and C.N., unpublished) (Cong et al., 2004), indicating that this construct does not require support in trafficking.

What, then, could be the mechanism of action of Krms? Krms may attenuate LRP6 endocytosis and degradation, thus promoting cell surface localization of LRP6. This would be the reverse of Krm action in the presence of Dkk1, which induces rapid LRP6 internalization (Mao et al., 2002). Krms may therefore be context-dependent endocytosis regulators of LRP6. Indeed, a hallmark of LDLR family members is their regulated endocytosis (Howell and Herz, 2001; May et al., 2003; Schneider and Nimpf, 2003).

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/23/4255/DC1

References

Abu-Elmagd, M., Garcia-Morales, C. and Wheeler, G. N. (2006). Frizzled7 mediates canonical Wnt signaling in neural crest induction. *Dev. Biol.* **298**, 285-298.

Aoki, Y., Saint-Germain, N., Gyda, M., Magner-Fink, E., Lee, Y. H., Credidio, C. and Saint-Jeannet, J. P. (2003). Sox10 regulates the development of neural crest-derived melanocytes in Xenopus. *Dev. Biol.* **259**, 19-33.

Bafico, A., Liu, G., Yaniv, A., Gazit, A. and Aaronson, S. A. (2001). Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nat. Cell Biol.* **3**, 683-686.

Barembaum, M. and Bronner-Fraser, M. (2005). Early steps in neural crest specification. *Semin. Cell Dev. Biol.* **16**, 642-646.

Bienz, M. and Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. *Cell* **103**, 311-320.

Chang, C. and Hemmati-Brivanlou, A. (1998). Neural crest induction by Xwnt7B in Xenopus. *Dev. Biol.* **194**, 129-134.

Christian, J. L., McMahon, J. A., McMahon, A. P. and Moon, R. T. (1991). Xwnt-8, a Xenopus Wnt-1/int-1-related gene responsive to mesoderm-inducing growth factors, may play a role in ventral mesodermal patterning during embryogenesis. *Development* **111**, 1045-1055.

Cong, F., Schweizer, L. and Varmus, H. (2004). Wnt signals across the plasma membrane to activate the beta-catenin pathway by forming oligomers containing its receptors, Frizzled and LRP. *Development* **131**, 5103-5115.

Cruciat, C. M., Hassler, C. and Niehrs, C. (2006). The MRH protein Erlectin is a member of the endoplasmic reticulum synexpression group and functions in N-glycan recognition. *J. Biol. Chem.* **281**, 12986-12993.

Culi, J. and Mann, R. S. (2003). Boca, an endoplasmic reticulum protein required for wingless signaling and trafficking of LDL receptor family members in Drosophila. *Cell* **112**, 343-354.

Culi, J., Springer, T. A. and Mann, R. S. (2004). Boca-dependent maturation of beta-propeller/EGF modules in low-density lipoprotein receptor proteins. *EMBO J.* **23**, 1372-1380.

Davidson, G., Mao, B., del Barco Barrantes, I. and Niehrs, C. (2002). Kremen proteins interact with Dickkopf1 to regulate anteroposterior CNS patterning. *Development* **129**, 5587-5596.

Davidson, G., Wu, W., Shen, J., Bilic, J., Fenger, U., Stannek, P., Glinka, A. and Niehrs, C. (2005). Casein kinase 1 gamma couples Wnt receptor activation to cytoplasmic signal transduction. *Nature* **438**, 867-872.

Deardorff, M. A., Tan, C., Saint-Jeannet, J. P. and Klein, P. S. (2001). A role for frizzled 3 in neural crest development. *Development* **128**, 3655-3663.

Dorsky, R. I., Moon, R. T. and Raible, D. W. (1998). Control of neural crest cell fate by the Wnt signalling pathway. *Nature* **396**, 370-373.

Garcia-Castro, M. I., Marcelle, C. and Bronner-Fraser, M. (2002). Ectodermal Wnt function as a neural crest inducer. *Science* **297**, 848-851.

Gawantka, V., Delius, H., Hirschfeld, K., Blumenstock, C. and Niehrs, C. (1995). Antagonizing the Spemann organizer: role of the homeobox gene Xvent-1. *EMBO J.* **14**, 6268-6279.

Glinka, A., Wu, W., Delius, H., Monaghan, A. P., Blumenstock, C. and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**, 357-362.

Hashimoto, H., Itoh, M., Yamanaka, Y., Yamashita, S., Shimizu, T., Solnica-Krezel, L., Hibi, M. and Hirano, T. (2000). Zebrafish Dkk1 functions in forebrain specification and axial mesendoderm formation. *Dev. Biol.* **217**, 138-152.

Hata, A., Seoane, J., Lagna, G., Montalvo, E., Hemmati-Brivanlou, A. and Massague, J. (2000). OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways. *Cell* **100**, 229-240.

He, X., Semenov, M., Tamai, K. and Zeng, X. (2004). LDL receptor-related proteins 5 and 6 in Wnt/beta-catenin signaling: arrows point the way. *Development* **131**, 1663-1677.

Honore, S. M., Aybar, M. J. and Mayor, R. (2003). Sox10 is required for the early development of the prospective neural crest in Xenopus embryos. *Dev. Biol.* **260**, 79-96.

Howell, B. W. and Herz, J. (2001). The LDL receptor gene family: signaling functions during development. *Curr. Opin. Neurobiol.* **11**, 74-81.

Hsieh, J. C., Lee, L., Zhang, L., Wefer, S., Brown, K., DeRossi, C., Wines, M. E., Rosenquist, T. and Holdener, B. C. (2003). Mesd encodes an LRP5/6 chaperone essential for specification of mouse embryonic polarity. *Cell* **112**, 355-367.

Kalcheim, C. and Burstyn-Cohen, T. (2005). Early stages of neural crest ontogeny: formation and regulation of cell delamination. *Int. J. Dev. Biol.* **49**, 105-116.

LaBonne, C. and Bronner-Fraser, M. (1998). Neural crest induction in Xenopus: evidence for a two-signal model. *Development* **125**, 2403-2414.

LaBonne, C. and Bronner-Fraser, M. (1999). Molecular mechanisms of neural crest formation. *Annu. Rev. Cell Dev. Biol.* **15**, 81-112.

Li, Y., Chen, J., Lu, W., McCormick, L. M., Wang, J. and Bu, G. (2005). Mesd binds to mature LDL-receptor-related protein-6 and antagonizes ligand binding. *J. Cell Sci.* **118**, 5305-5314.

Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A. and Niehrs, C. (2001). LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. *Nature* **411**, 321-325.

Mao, B., Wu, W., Davidson, G., Marhold, J., Li, M., Mechler, B. M., Delius, H., Hoppe, D., Stannek, P., Walter, C. et al. (2002). Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. *Nature* **417**, 664-667.

May, P., Bock, H. H. and Herz, J. (2003). Integration of endocytosis and signal transduction by lipoprotein receptors. *Sci. STKE* **2003**, PE12.

Moon, R. T., Brown, J. D. and Torres, M. (1997). WNTs modulate cell fate and behavior during vertebrate development. *Trends Genet.* **13**, 157-162.

Mukhopadhyay, M., Shtrom, S., Rodriguez-Esteban, C., Chen, L., Tsukui, T., Gomer, L., Dorward, D. W., Glinka, A., Grinberg, A., Huang, S. P. et al. (2001). Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. *Dev. Cell* **1**, 423-434.

Nakamura, T., Aoki, S., Kitajima, K., Takahashi, T., Matsumoto, K. and

Nakamura, T. (2001). Molecular cloning and characterization of Kremen, a novel kringle-containing transmembrane protein. *Biochim. Biophys. Acta* **1518**, 63-72.
Niehrs, C. (2006). Function and biological roles of the Dickkopf family of Wnt modulators. *Oncogene* **25**, 7469-7481.
Niehrs, C., Steinbeisser, H. and De Robertis, E. M. (1994). Mesodermal patterning by a gradient of the vertebrate homeobox gene goosecoid. *Science* **263**, 817-820.
Nusse, R. (2005). Wnt signaling in disease and in development. *Cell Res.* **15**, 28-32.
Osada, M., Ito, E., Fermin, H. A., Vazquez-Cintron, E., Venkatesh, T., Friedel, R. H. and Pezzano, M. (2006). The Wnt signaling antagonist Kremen1 is required for development of thymic architecture. *Clin. Dev. Immunol.* **13**, 299-319.
Pinson, K. I., Brennan, J., Monkley, S., Avery, B. J. and Skarnes, W. C. (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* **407**, 535-538.
Raible, D. W. and Ragland, J. W. (2005). Reiterated Wnt and BMP signals in neural crest development. *Semin. Cell Dev. Biol.* **16**, 673-682.
Ruiz i Altaba, A. and Melton, D. A. (1989). Involvement of the Xenopus homeobox gene Xhox3 in pattern formation along the anterior-posterior axis. *Cell* **57**, 317-326.
Saint-Jeannet, J. P., He, X., Varmus, H. E. and Dawid, I. B. (1997). Regulation of dorsal fate in the neuraxis by Wnt-1 and Wnt-3a. *Proc. Natl. Acad. Sci. USA* **94**, 13713-13718.
Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L. K. and De Robertis, E. M. (1994). Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* **79**, 779-790.
Schneider, W. J. and Nimpf, J. (2003). LDL receptor relatives at the crossroad of endocytosis and signaling. *Cell. Mol. Life Sci.* **60**, 892-903.
Semenov, M. V., Tamai, K., Brott, B. K., Kuhl, M., Sokol, S. and He, X. (2001). Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. *Curr. Biol.* **11**, 951-961.
Shinya, M., Eschbach, C., Clark, M., Lehrach, H. and Furutani-Seiki, M. (2000). Zebrafish Dkk1, induced by the pre-MBT Wnt signaling, is secreted from the prechordal plate and patterns the anterior neural plate. *Mech. Dev.* **98**, 3-17.
Sive, H. L., Grainger, R. M. and Harland, R. M. (2000). Fate mapping and lineage labeling. In *Early Development of Xenopus laevis: A Laboratory Manual* (ed. S. Curtis and M. Cozza), pp. 143-170. New York: Cold Spring Harbor Laboratory Press.
Tamai, K., Semenov, M., Kato, Y., Spokony, R., Liu, C., Katsuyama, Y., Hess, F., Saint-Jeannet, J. P. and He, X. (2000). LDL-receptor-related proteins in Wnt signal transduction. *Nature* **407**, 530-535.
Taneyhill, L. A. and Bronner-Fraser, M. (2005). Recycling signals in the neural crest. *J. Biol.* **4**, 10.
Unterseher, F., Hefele, J. A., Giehl, K., De Robertis, E. M., Wedlich, D. and Schambony, A. (2004). Paraxial protocadherin coordinates cell polarity during convergent extension via Rho A and JNK. *EMBO J.* **23**, 3259-3269.
Villanueva, S., Glavic, A., Ruiz, P. and Mayor, R. (2002). Posteriorization by FGF, Wnt, and retinoic acid is required for neural crest induction. *Dev. Biol.* **241**, 289-301.
Wehrli, M., Dougan, S. T., Caldwell, K., O'Keefe, L., Schwartz, S., Vaizel-Ohayon, D., Schejter, E., Tomlinson, A. and DiNardo, S. (2000). arrow encodes an LDL-receptor-related protein essential for Wingless signalling. *Nature* **407**, 527-530.
Wodarz, A. and Nusse, R. (1998). Mechanisms of Wnt signaling in development. *Annu. Rev. Cell Dev. Biol.* **14**, 59-88.
Wu, J., Saint-Jeannet, J. P. and Klein, P. S. (2003). Wnt-frizzled signaling in neural crest formation. *Trends Neurosci.* **26**, 40-45.
Wu, J., Yang, J. and Klein, P. S. (2005). Neural crest induction by the canonical Wnt pathway can be dissociated from anterior-posterior neural patterning in Xenopus. *Dev. Biol.* **279**, 220-232.
Wu, W., Glinka, A., Delius, H. and Niehrs, C. (2000). Mutual antagonism between dickkopf1 and dickkopf2 regulates Wnt/beta-catenin signalling. *Curr. Biol.* **10**, 1611-1614.
Yanfeng, W., Saint-Jeannet, J. P. and Klein, P. S. (2003). Wnt-frizzled signaling in the induction and differentiation of the neural crest. *BioEssays* **25**, 317-325.