BMP4-dependent expression of *Xenopus* Grainyhead-like 1 is essential for epidermal differentiation

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Summary

Morphogen-dependent epidermal-specific transacting factors have not been defined in vertebrates. We demonstrate that a member of the grainyhead transcription factor family, Grainyhead-like 1 (*XGrhl1*) is essential for ectodermal ontogeny in *Xenopus laevis*. Expression of this factor is restricted to epidermal cells. Moreover, *XGrhl1* is regulated by the BMP4 signaling cascade. Disruption of *XGrhl1* activity in vivo results in a severe defect in terminal epidermal differentiation, with inhibition of *XK81A1* epidermal keratin gene expression, a key target of BMP4 signaling. Furthermore, transcription of the *XK81A1* gene is modulated directly by binding of XGRHL1 to a promoter-localized binding motif that is essential for high-level expression. These results establish a novel developmental role for *XGrhl1* as a crucial tissue-specific regulator of vertebrate epidermal differentiation.

Key words: *Xenopus*, Grainyhead-like 1, BMP

Introduction

The pre-metamorphic tadpole, like the early mammalian fetus, uses an epidermal bi-layer to protect itself against environmental insults (Fuchs, 1998; Nieuwkoop and Farber, 1994). Study of these simple epithelial sheets has led to the delineation of key mechanisms necessary for epidermal specification, differentiation and stratification (Fuchs and Raghavan, 2002; Koster et al., 2004; Nieuwkoop and Farber, 1994; Porter and Lane, 2003; Sasai and De Robertis, 1997). Extracellular transforming growth factor β (TGFβ)-like ligands play crucial regulatory roles in these events (Altmann and Brivanlou, 2001; Botchkarev, 2003). For example, BMP4 is essential for epidermal specification of naïve ectodermal cells in the *Xenopus* embryo. Conversely, low or absent BMP4 activity results in neural specification (Wilson and Hemmati-Brivanlou, 1995). This gradient is established across the developing *Xenopus* embryo by the Spemann organizer, a small group of cells localized initially in the dorsal lip of the blastopore (Sasai and De Robertis, 1997; Spemann and Mangold, 1924). This region expresses a series of extracellular inhibitors of BMP4 signaling, including *chordin* or *noggin*, which are essential for appropriate development of the epidermis and central nervous system, as well as the dorsal mesoderm and gut (Lamb et al., 1993; Piccolo et al., 1996; Sasai et al., 1994; Smith and Harland, 1992). Thus, endogenous or ectopic expression of BMP4 ligand-binding antagonists results in neural specification of ectodermal cells (Sasai et al., 1994; Smith and Harland, 1992; Zimmerman et al., 1996). Conversely, inhibition of BMP antagonist activity, or enforced BMP4 expression, results in epidermal differentiation (Munoz-Sanjuan and Brivanlou, 2002; Wilson et al., 1997; Wilson and Hemmati-Brivanlou, 1995).

The importance of the BMP4 signaling has resulted in the identification of two major types of effector molecules, downstream of the BMP4 receptor, that modulate epidermal-specific gene expression. Activation of Smad signal transducers, and expression of the 'immediate early response' (IER) transacting factors *Msx1* and *XVent2*, occurs with BMP4 binding to its cognate receptor (Onichtchouk et al., 1996; Suzuki et al., 1997b; Wilson et al., 1997). In naïve ectodermal cells, ectopic expression of these factors (like BMP4) induces an epidermal fate with concomitant repression of neurogenesis. IER factors, in turn, modulate transcription of a second set of widely expressed *trans*-acting factor genes, including *XAP-2* and *Dlx-3*, which have a narrower range of action in ectodermal cells, activating epidermal gene expression with concomitant repression of the neural gene expression (Feledy et al., 1999b; Luo et al., 2001b; Luo et al., 2002; Snape et al., 1991).

Although a plethora of genes activated by this deceptively simple signaling cascade have been identified, it remains unclear how epidermal-specific gene expression is achieved. Examination of the regulatory sequences of several mammalian epidermal-specific structural genes implicate a combinatorial network of ubiquitous factors, such as *Ap1* or *Sp1*, and tissue-restricted proteins, such as *Ap2* or *Dlx3*, that bind promoter/enhancer elements in a context-specific manner to facilitate appropriate high-level expression (Byrne, 1997;

Crish et al., 2002; Kaufman et al., 2003; Ng et al., 2000; Presland et al., 2001; Sinha et al., 2000; Sinha and Fuchs, 2001). However, several tissue-specific binding activities have been reported, suggesting that, as yet, unidentified regulators of vertebrate epidermal differentiation await characterization.

This hypothesis is supported by *Drosophila* mutagenesis screens, numerous tissue-specific gene loci being identified that influence ectodermal differentiation (Juergens et al., 1984; Nusslein-Volhard et al., 1994; Wieschaus et al., 1984). One of these, grainyhead or *grh* (also known as *NTF-1* or *Elf1*), has an ectodermal-restricted pattern of expression (Bray et al., 1989; Dynlacht et al., 1989; Uv et al., 1997). Loss of *grh* function results in ectodermal defects including flimsy cuticles, abnormal head structures and a 'blimp' phenotype (Attardi et al., 1993; Bray and Kafatos, 1991; Ostrowski et al., 2002). Recent studies suggest an evolutionary conservation of *grh*-like function. Cuticular defects occur with disruption of *ceGrh*, an ectodermal-restricted, *C. elegans* ortholog of *grh* (Venkatesan et al., 2003). Moreover, we, and others, have identified three mammalian orthologs of *grh,* grainyhead-like factors 1, 2 and 3 (Grhl1-3), which share an ectodermally restricted pattern of expression and a high degree of identity in the DNA binding and dimerization domains (Huang and Miller, 2000; Kudryavtseva et al., 2003; Ting et al., 2003b; Wilanowski et al., 2002).

In this report, we provide the first evidence for a critical role of a vertebrate grainyhead-like factor, *Xenopus* grainyhead-like 1 (*XGrhl1*) in epidermal ontogeny. This factor is regulated in an epidermal-specific BMP4-dependent manner, modulating expression of epidermal-specific gene expression. Concordant with these observations, disruption of *XGrhl1* activity results in defective epidermal differentiation. Moreover, we identify a XGRHL1-dependent target gene, epidermal keratin (*XK81A1*) (Dawid et al., 1985; Jonas et al., 1985), and show that a crucial 5′ regulatory motif of the *XK81A1* gene, which is necessary for high level promoter activity, binds XGRHL1 directly. Together, these observations provide key mechanistic insights into the role of an epidermal-specific transacting factor in vertebrate development.

Materials and methods

Cloning *Xenopus* grainyhead-like 1 cDNA

Xenopus cDNA libraries (stages 11, 17 and 42, a gift from Dr M. King) were screened under low stringency using a ^{32}P-labeled human Grhl1 cDNA probe (Wilanowski et al., 2002). A full-length *Xenopus* ortholog of Grhl1 was identified, designated *Xenopus* grainyhead-like 1 (*XGrhl1*) (GenBank Accession Number, AY591750).

Xenopus embryo manipulations

Wild-type and albino *Xenopus* (NASCO) eggs were fertilized in vitro, dejellied, cultured using standard methods (Sive et al., 2000) and staged according to Nieuwkoop's normal table of development (Nieuwkoop and Farber, 1994). Microinjection, lineage tracing and animal cap assays were performed as described (Luo et al., 2002; Parvin et al., 1995; Sargent et al., 1986; Smith and Harland, 1991; Wilson and Hemmati-Brivanlou, 1995). Isolation of superficial and deep ectodermal layers was performed as described with minor modification (Chalmers et al., 2002).

Expression analysis and probes

Whole-mount in situ hybridization was performed using albino *Xenopus* embryos (Harland, 1991; Hemmati-Brivanlou et al., 1990). Digoxigenin-labeled antisense RNA probes were generated from *XGrhl1* full-length cDNA. Other probes used were *XK81A1*, *BMP4*, *XAP2* and *Dlx3* (Feledy et al., 1999a; Jonas et al., 1985; Luo et al., 2002; Mariani and Harland, 1998). β-Galactosidase activity was used as an injection tracer (Sive et al., 2000) using the substrates XGal (blue) or Red-Gal (Research Organics, Cleveland, OH). For histological analysis, embryos were embedded in paraffin. RNA extractions, first-strand cDNA synthesis and PCR were carried out as previously described (Kelley et al., 1994; Mead et al., 1996). All assays were performed in the linear range.

RT-PCR and primers

RNA extractions, first-strand cDNA synthesis and PCR were carried out as previously described (Kelley et al., 1994; Mead et al., 1996). Primers for *ODC*, *XK81A1* keratin, *α-actin*, *NCAM*, *geminin*, *XBra*, *GATA2*, *Sox3*, *BMP4*, *Msx1*, *XOtx2*, *XAG1*, *XZic3*, *XNrp1*, *XVent-2*, *HIS4* and *EDD* have been reported previously (Kroll et al., 1998; Sasai and de Robertis, 1997; Wilson et al., 1997) (http://www.xenbase.org/methods/RT-PCR.html). Other primers for PCR included: *XGrhl1* (5′-TGACCACCGCCTTCAGTGCT-3′ and 5′-CCTTGGCTGCCCTGACATTG-3′ amplify a 444 bp fragment at 56°C, 25 cycles), *PCNA* (5′-CGATCAGACGGCTTTGACAC-3′ and 5′-CTCCGCTCGCAGAGAACTTT-3′ amplify a 364 bp fragment at 56°C, 25 cycles), *P63* (5′-CATGCCCAATCCAAATCAAA-3′ and 5′-CATCTGCCTTGCGGTCTCT-3′ amplify a 444 bp fragment at 56°C, 25 cycles), *ESR-1* (5′-GGATTACAAGCAAGGGTTC-3′ and 5′-TCCCATAGGATAACGTTCAT-3′ amplify a 378 bp fragment at 54°C, 28 cycles), *XNotch1* (5′-TGCCTTCCAATCTTACGC-3′ and 5′-AGGGCAGTGTTTTAGGTCAA-3′ amplify a 428 bp fragment at 54°C, 27 cycles), *XDelta1* (5′-CTGTCCCCCTGGCTACATT-3′ and 5′-CCCTCACACAGACAACCACA-3′ amplify a 305 bp fragment at 56°C 25 cycles), *Dlx3* (5′-GCTTGTGGGCAACGAG-3′ and 5′-CTGCGTCTGAGTGAGTCCTA-3′ amplify a 292 bp fragment at 56°C, 25 cycles), *Dlx5* (5′-ATTCTCCCCAGTCTCCAGTG-3′ and 5′-GATAGTGTCCCCAGTTGCGC-3′ amplify a 425 bp fragment at 55°C, 25 cycles), *XAP2* (5′-CGGGTATGTGTGCGAAACAG-3′ and 5′-GGCGGGAGACCAATAGAGAA-3′ amplify a 445 bp fragment at 56°C, 25 cycles) and *ESR6e* (5′-GGCACAGGGCAATACTGGT-3′ and 5′-CCCCACTTGGCATTATGTTC-3′ amplify a 400 bp fragment at 55°C, 27 cycles). PCR conditions were determined for each primer set to ensure that amplification was within a linear range.

Plasmid construction

A 3.4 kb *XGrhl1* cDNA was subcloned into pRN3 and pCS2+ (kind gifts from P. Lemaire and D. Turner, respectively) to create RN3-*XGrhl1* and pCS2+*XGrhl1*. The plasmid RN3-*Δ227XGrhl1* or RN3-EGFP*Δ227XGrhl1* were generated from RN3-*XGrhl1* by replacing *Bgl*II/*Eco*RI fragment with an adaptor (annealing 5′-GATCTGAGAGCATCATGGCG-3′ and 5′-AATTCGCCATGATGCTCTCA-3′) or PCR amplified fragment of the EGFP cDNA.

Synthetic RNA and antisense morpholino (MO) oligonucleotides

Synthetic RNA was made from linearized plasmid DNA with the mMessage mMachine in vitro transcription kits (Ambion, TX). The RNA yield was quantitated by spectrophotometer and its integrity checked by gel electrophoresis. The following plasmids were digested and incubated with the appropriate RNA polymerase: RN3-*XGrhl1* (*Sfi*I, T3), RN3-*Δ227XGrhl1* (*Sfi*I, T3), RN3-EGFP1*Δ227XGrhl1* (*Sfi*I, T3), pSP64T-nucβGal (*Xho*I, SP6), pSP64T-*BMP4* (*Bam*HI, SP6), pSP64T-activin βB (*Eco*RI, SP6), pCS2+*XMAD* (*Not*I, SP6), pSP64T-*tBR* (*Eco*RI, SP6), pCS2MT-*Ngem* (*Not*I, SP6) and pCS2+*noggin* (*Not*I, SP6), RN3-*XVent2* (*Pst*I, T3) (Huber et al., 1998; Huber et al., 2001; Kroll et al., 1998; Maeno et al., 1996; Onichtchouk et al., 1996; Smith and Harland, 1992). MOs were obtained from GeneTools, *XGrhl1*-MO2 having the sequence 5′-GTCGTAGTCT-

TGTGTCATGATGCTC-3′, and a company-supplied control morpholino (CMO) serving as a control.

GST chromatography, electrophoretic mobility shift analysis and luciferase reporter assays

Protein:protein interaction assays using GST chimeric factors and electrophoretic mobility shift assays (EMSA) were performed as previously described (Jane et al., 1995).

Blastomeres at the four-cell stage were co-injected with 10 nl of RNA encoding *Δ227Grhl1* mutant and the KP487 reporter construct. pRL-TK, a construct in which the thymidine kinase promoter was linked to the renilla luciferase cDNA (30 pg/embryo), was injected to control for injection efficiency. After injection, the embryos were cultured to stage 11. Ten embryos were collected and lysed in 1× lysis buffer (20 µl/embryo). Luciferase activity was determined in 10 µl of the supernatant, according to the manufacturer's instructions and quantified with a Model TD-20/20 luminometer (Turner Designs). Relative firefly luciferase activity (RLU) was normalized with Renilla luciferase activity in cellular lysates.

Results

A *Xenopus* ortholog of grainyhead is expressed exclusively in non-neuronal ectoderm

To explore the role of grainyhead-like factors in vertebrate embryogenesis, we cloned full-length cDNAs encoding orthologs of *grh* from staged *Xenopus* embryo cDNA libraries. Three unique transcripts were identified which share a high degree of identity/similarity with *Drosophila grh*, a *C. elegans* grainyhead-like ortholog, and the murine and human Grhl species, particularly in regions defined previously as mediating DNA binding and dimerization (Uv et al., 1994; Venkatesan et al., 2003; Wilanowski et al., 2002). *Xenopus Grainyhead- like 1* (*XGrhl1*), the subject of this report, has the highest level of homology with Drosophila *grh* (see Fig. S1 in the supplementary material).

Consistent with *Drosophila grh* expression (Bray et al., 1989; Dynlacht et al., 1989; Uv et al., 1997), *XGrhl1* is expressed throughout ontogeny. Maternally derived transcripts (stage 2-8) are replaced by zygotic expression after the mid-blastula transition (stage 9+), which persists throughout embryogenesis (Fig. 1A). Dissection of the germ layers at stage 11 revealed that *XGrhl1* transcripts were restricted, like other epidermal-restricted genes, *ESR6e* and *XK81A1* keratin, to the superficial (non-neuronal) layer of the ectoderm at mid-gastrulation (Fig. 1B) (Chalmers et al., 2002; Deblandre et al., 1999; Jonas et al., 1985). By contrast, unlike *Drosophila grh*, or the neuronal-specific *xNotch*, *xDelta* and *ESR1* genes, *XGrhl1* was neither expressed in the neuroectodermal layer, nor the mesoderm or endoderm at any time point (Fig. 1B; data not shown).

In situ hybridization analysis of staged embryos extended these studies significantly. After fertilization, *XGrhl1* transcripts were localized to the blastomeres of the animal pole only (Fig. 2A-D), becoming restricted to the non-neuronal ectoderm of the embryo with progression through gastrulation and neurulation (Fig. 2E-L). Histological sections confirmed this pattern. *XGrhl1* expression was observed only in the most superficial cellular layer of the embryo, detectable transcripts being absent from the neural plate (Fig. 2M, arrowhead). At later stages, *XGrhl1* transcripts are restricted to the epidermis (Fig. 2P-R), in an identical pattern to *XK81A1* (Jonas et al., 1985) (Fig. 2N,O). We conclude that *XGrhl1* is a unique marker of superficial non-neuronal ectoderm.

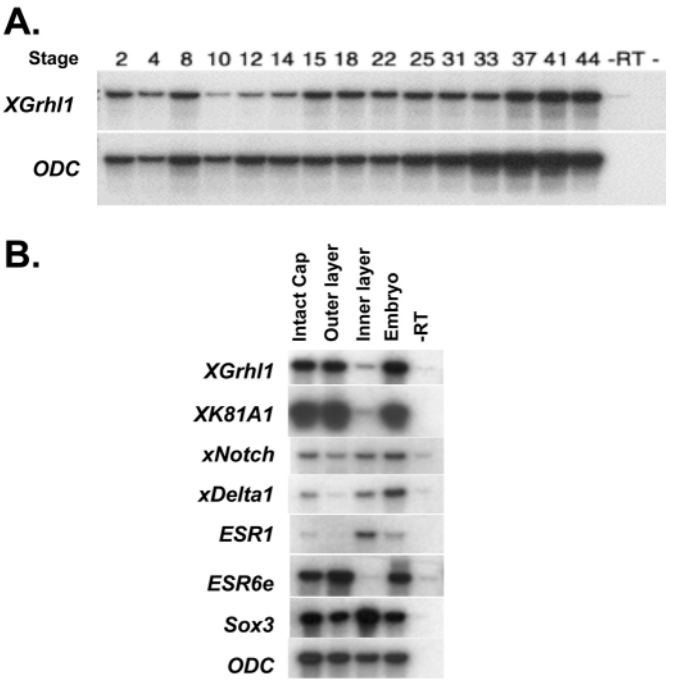

Fig. 1. Expression of *XGrhl1* is restricted to the ectodermal layer. (A) RT-PCR analysis of *XGrhl1* expression in staged embryos during development (n=5). Ornithine decarboxylase (ODC) gene expression was used as a control for RNA concentration. –RT, no reverse transcriptase; –, no RNA. (B) *XGrhl1* expression is restricted to the superficial epidermal layer of the developing embryo. RT-PCR analysis of *XGrhl1* and developmental gene expression in different ectodermal tissues at stage 11 of *Xenopus* development.

Antagonists of BMP4 signaling inhibit *XGrhl1* expression

The highly restricted pattern of *XGrhl1* expression during ontogeny, coupled with the documented role of BMP4 signaling in *Xenopus* epidermal differentiation, suggested a potential functional interaction between these factors. To determine whether an active BMP4 signaling pathway was necessary for *XGrhl1* expression, we assessed the results of expression of a dominant-negative truncated BMP4-specific receptor (*tBR*), the BMP antagonist *noggin*, or the BMP-inhibitory transacting factor *geminin*, on *XGrhl1* transcription. Consistent with previously reported effects of neuralization of ectodermal cells expressing these factors ectopically (Kroll et al., 1998; Lamb et al., 1993; Suzuki et al., 1997b), we observed upregulation of *NCAM* transcripts in explanted animal caps of embryos microinjected with *tBR, noggin* or *geminin* (Fig. 3A-B; data not shown). Coincidentally, we observed a dose-dependent reduction in *XK81A1* and *XGrhl1* expression.

Similar effects were observed in the context of the whole embryo. After co-injection of one blastomere at the four-cell stage with appropriate transcripts and a β-galactosidase (β-gal) mRNA, embryos were allowed to develop to late gastrulation and assayed for *XGrhl1* expression by whole-mount in situ hybridization. Epidermal progeny of blastomeres injected with

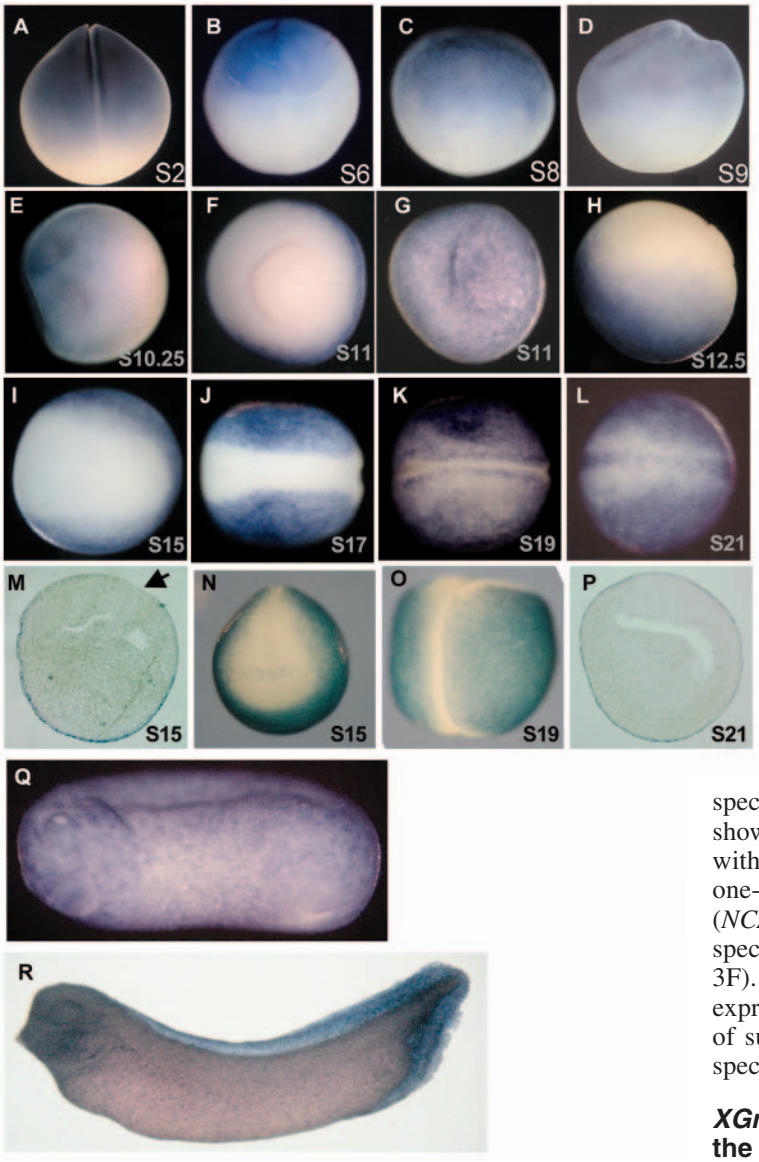

Fig. 2. Expression of *XGrhl1* is restricted to tissues with an epidermal fate. Whole-mount in situ hybridization analysis of *XGrhl1* expression in staged embryos. Maternal *XGrhl1* transcripts are detected in the animal pole of early cleavage (A), blastula (B-D) and gastrula (E-H) stage embryos. Embryos are in a lateral orientation except for F (dorsal) and G (ventral). Zygotic expression is observed in presumptive epidermis through neurulation (I-L; dorsal orientation), tailbud and swimming tadpole stages (Q and R respectively; lateral orientation, anterior towards the left). Transverse embryonic section at stage 15 (M) demonstrates *XGrhl1* expression (blue stain) in the presumptive epidermis, this stain being absent from the neural plate (arrowhead). By stage 21 (P), the neural plate is closed and covered by epidermis. Epidermal keratin (*XK81A1*) and zygotic *XGrhl1* have a similar pattern of expression (N,O; anterior and dorsal orientation, respectively).

geminin (*n*=19) or *noggin* (*n*=23) showed a complete loss of *XGrhl1* expression (Fig. 3C). By contrast, embryos injected with RNA encoding *xVent-2* (*n*=19), an IER component of the BMP4 pathway, or BMP4 (*n*=20), failed to show any defect in *XGrhl1* expression.

To assess whether the specificity of these effects was related to inhibition of BMP4 signaling directly, we examined the outcome of co-injection of *tBR* and increasing amounts of *XMad1* RNA. Enforced expression of *XMad1*, a BMP4-specific signal transduction molecule, is sufficient to activate the BMP4-dependent signaling cascade, reversing *tBR*-induced neuroectodermal specification (Wilson et al., 1997), as demonstrated by downregulation of *NCAM* expression (Fig. 3D). *XAG* expression, a marker of the cement gland whose expression is modulated by the relative concentration of BMP4, was also repressed (Gammill and Sive, 2000). By contrast, *XK81A1* and *XGrhl1* expression was restored to levels observed in caps derived from uninjected embryos. We observed a similar outcome with co-injection of *noggin* and *XMad1* (data not shown). Thus, *XGrhl1* expression is dependent on activation of the epidermal differentiation program, is modulated by the BMP4-signaling pathway, and is repressed by regulatory factors that induce neuralization.

These observations raised the possibility that *XGrhl1* alone may specify epidermal fate and/or inhibit neural specification. To address this hypothesis, we co-injected increasing amounts of XGrhl- and β-gal-encoding RNA into animal pole blastomeres, observing no effect on epidermal (top) or neural (bottom) specification at any stage of ontogeny (Fig. 3E; data not shown). Furthermore, co-injection of *XGrhl1*-expressing RNA with either *tBR* (Fig. 3F) or *noggin* (data not shown) at the one-cell stage failed to suppress neural gene expression (*NCAM*, *Sox3* or *Nrp1),* or rescue known BMP4 epidermal-specific targets including *XK81A1* in animal cap explants (Fig. 3F). Taken together, our studies demonstrate that *XGrhl1* expression is activated by BMP4 signaling, but in the absence of such a cue, enforced *XGrhl1* expression is insufficient to specify an epidermal fate.

XGrhl1 can modulate downstream components of the BMP4 signaling pathway

Establishment of the relative position of *XGrhl1* in the BMP4 signaling cascade is complicated by the high concentrations of endogenous BMP4 protein present in ectodermal explants. To circumvent this problem, embryos were isolated at stage 9, and dissociated in Ca^{2+}- and Mg^{2+}-free buffer (Fig. 4A) (Sargent et al., 1986; Wilson and Hemmati-Brivanlou, 1995). After washing, to remove endogenous extracellular factors, re-aggregated cells were incubated with recombinant BMP4 or media alone until control embryos reached the mid-neurula stage. In the absence of BMP4, cap cells had a neural fate, expressing *NCAM,* but failing to express either the *XK81A1* and *ESR6e* epidermal-specific markers, or *XGrhl1* mRNA, as assayed by semi-quantitative RT-PCR (Fig. 4B). By contrast, increasing amounts of BMP4 induced a significant increase in *XK81A1* and *ESR6e* expression, and a concomitant decrease in *NCAM* expression. Moreover, epidermal marker expression was associated with a BMP4-dependent induction of *XGrhl1* transcription. Concomitantly, induction of the *Dlx3*, *Dlx5* and *XAP2* transacting factors was observed in BMP4-treated cells. Given that these factors are known downstream components of the BMP4 signaling cascade,

these results suggest strongly that *XGrhl1* is modulated by BMP4 directly.

New protein synthesis is not required for *xVent2* expression, an IER gene transcribed soon after BMP4 binding to its cognate receptor, as shown by its resistance to cycloheximide-mediated (CHX) inhibition (Onichtchouk et al., 1996). By contrast, a second group of BMP4-responsive genes, including *Dlx3* and *XAP2,* cannot be induced in the presence of CHX (Luo et al., 2001a; Luo et al., 2002; Luo et al., 2001b). Studies of *XGrhl1* expression demonstrated that, unlike *xVent2,* this

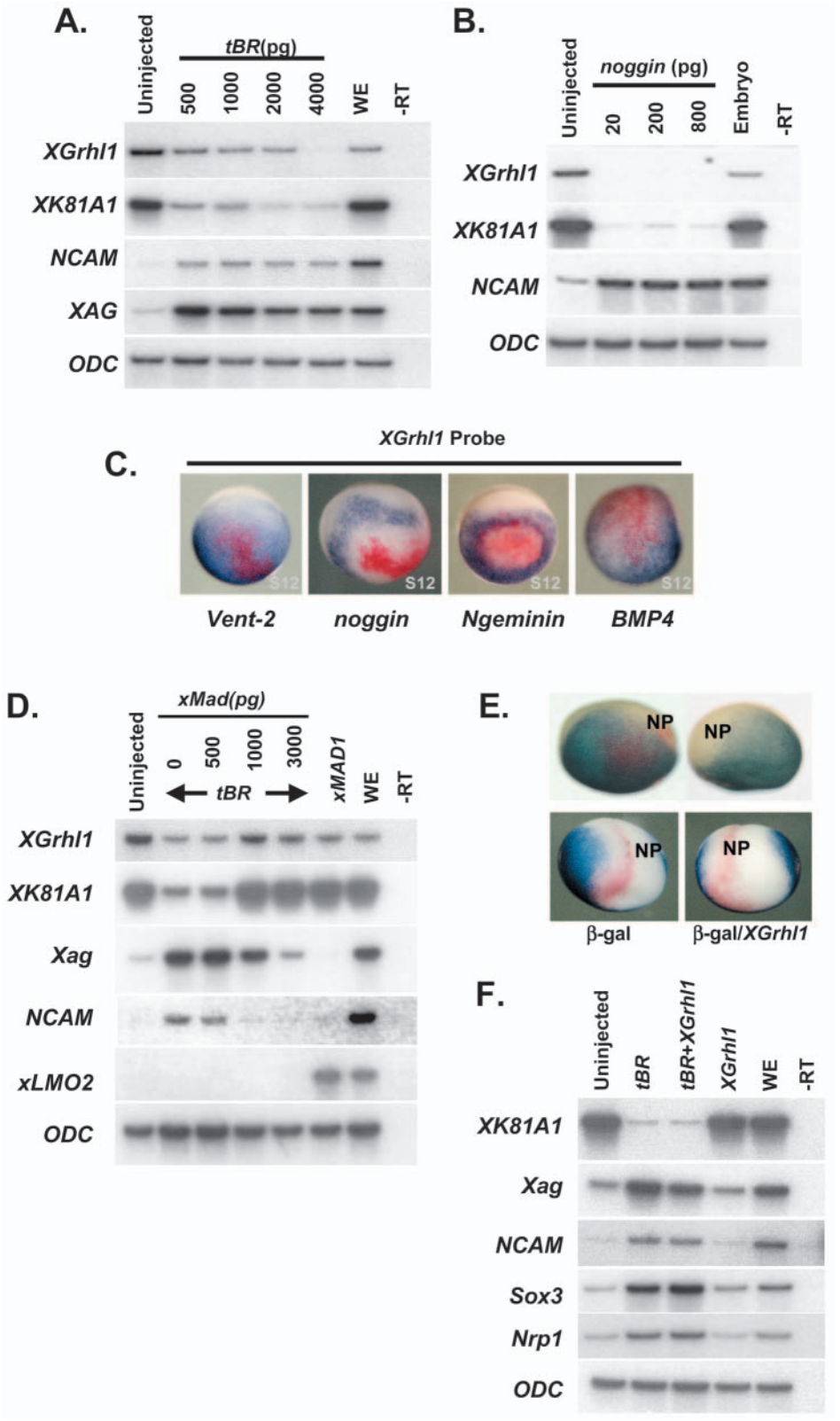

Fig. 3. *XGrhl1* expression is dependent on the BMP4 signaling pathway. Microinjection of a dominant-negative BMP receptor mutant (*tBR*) results in a decrease in *XGrhl1* and *XK81A1* expression with a concomitant increase in transcripts encoding the *NCAM* neural marker. *ODC* was used as a control for RNA recovery. Uninjected, uninjected cap; WE, whole embryo. (A) Enforced expression of the BMP4 antagonist *noggin* represses both *XGrhl1* and *XK81A1* expression. (B) RT-PCR analysis of animal pole explants at stage 21 injected at the one-cell stage with *noggin* mRNA. (C) Factors antagonizing BMP4 signaling block *XGrhl1* expression in vivo. In situ hybridization analysis (blue) for *XGrhl1* in embryos injected with neuralizing [*noggin* (600 pg), *Ngeminin* (1 ng)] or epidermal-inducing factors [*XVent-2* (400 pg), *BMP4* (1 ng)]. Embryos were co-injected with β-galactosidase mRNA (50 pg; stained red) for lineage tracing. *Vent-2* and *geminin*, anteroventral view; *noggin*, lateral view; *BMP4*, ventral view. (D) Co-injection of *xMad1* rescues *XGrhl1* expression in *tBR*-expressing explants. RT-PCR analysis of animal pole explants injected at the one-cell stage with either *tBR* alone (2 ng), or *tBR* with increasing concentrations of *xMAD1* encoding RNA. Induction of *XLMO2* indicates functional *XMad1* transcripts (Mead et al., 2001). (E) Ectopic expression of *XGrhl1* does not alter epidermal specification or neuralization in vivo. In situ hybridization for *XK81A1* expression (blue) of stage 14 embryos injected at the one- to four-cell stage in the animal pole with *XGrhl1*-encoding transcripts (4 ng). β-Galactosidase (red stain) was used as a lineage tracer. The upper panels illustrate representative embryos injected in blastomeres with an epidermal fate (lateral orientation); lower panels are representative of blastomeres with a neural fate (anterior orientation). (F) Co-injection of *XGrhl1* transcripts does not rescue *tBR*-induced neuralization. RT-PCR analysis of animal cap explants injected at the one-cell stage with either *tBR* (2 ng) alone or *tBR* with *XGrhl1* encoding RNA (4 ng).

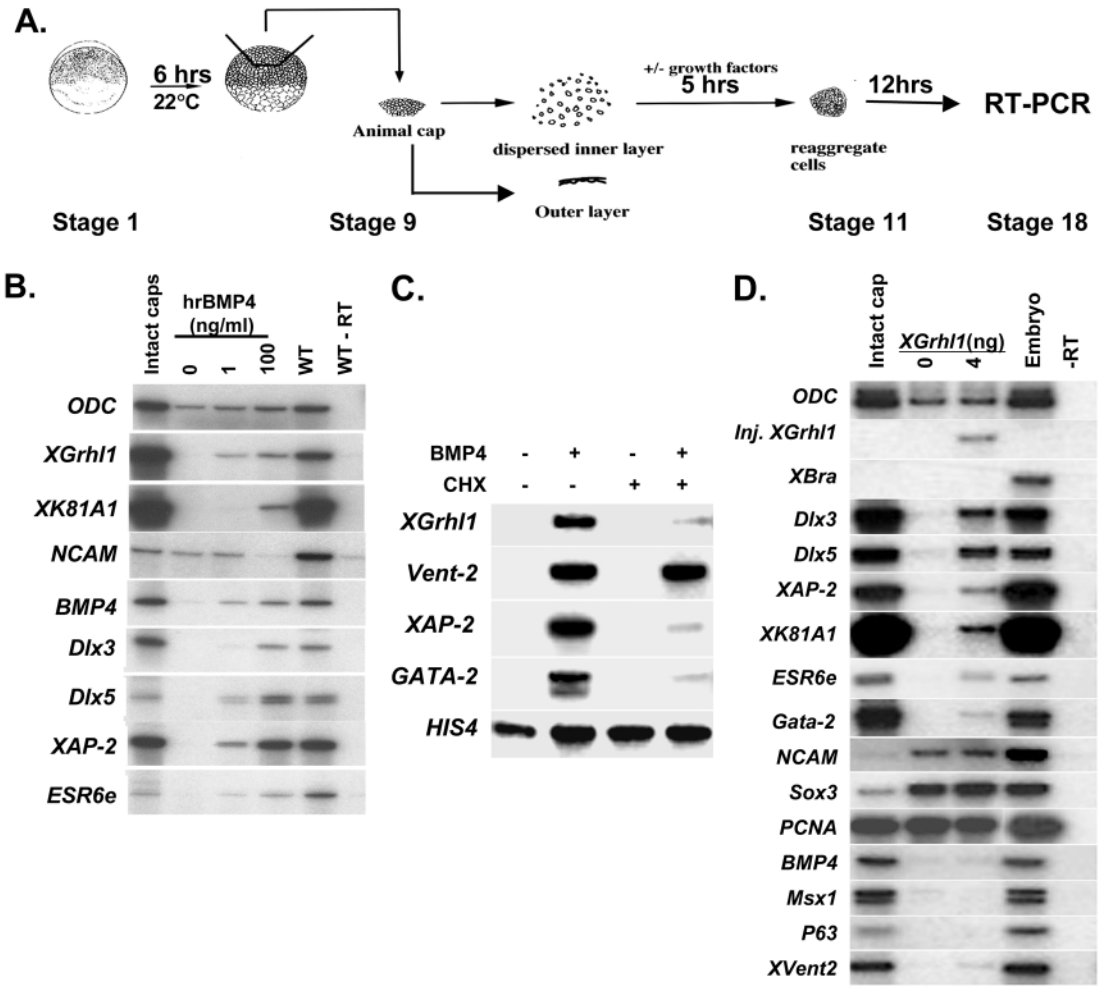

Fig. 4. *XGrhl1* is downstream of the BMP4 receptor, and can modulate endogenous BMP4-responsive targets. (A) Dissociated animal cap assays were performed as indicated in the schematic. Dispersed animal pole cells were incubated in increasing doses of recombinant human BMP4 (hrBMP4) (B,C) or one-cell embryos were injected with *XGrhl1* mRNA (D), allowed to develop to stage 9, and animal pole explants were dissected, dispersed and allowed to re-aggregate. Aggregates were allowed to mature until stage 18, harvested, RNA prepared and assayed by semi-quantitative RT-PCR. (B) Exposure of dissociated ectodermal cells to hrBMP4 results in an increase in epidermal-specific gene expression, including *XGrhl1* and *XK81A1*. (C) *XGrhl1* is not an immediate early response gene. Dissociated caps were incubated in BMP4 in the presence/absence of the protein synthesis inhibitor cycloheximide (10 μg/ml; CHX). (D) Ectopic expression of *XGrhl1* in dispersed cap cells results in upregulation of epidermal-specific gene expression.

factor had a similar pattern of response to BMP4 as *XAP2* and *GATA2* in the presence of CHX (Fig. 4B).

To localize the relative position of *XGrhl1* in the BMP4 signaling pathway, we micro-injected fertilized eggs with *XGrhl1* encoding RNA and assessed its effect(s) in the dissociated cap assay. Induction of BMP4, or of the IER genes, *Msx1*, *xVent2* or *p63* was not observed (Fig. 4C), consistent with the absence of BMP4 stimulus, and a lack of effect of *XGrhl1* on expression of these factors (Bakkers et al., 2002; Wilson and Hemmati-Brivanlou, 1995). By contrast, not only was *XK81A1* induced by *XGrhl1*, but upregulation of *Dlx3*, *Dlx5*, *XAP2* and *ESR6e* expression was also observed. Our results strongly support the conclusion that *XGrhl1* is induced by BMP4 signaling, and is located downstream of the IER genes, but upstream of structural genes, such as XK81A1, that are necessary for epidermal differentiation.

XGrhl1 function/expression is required for epidermal differentiation in the developing embryo

Our results are consistent with a model in which *XGrhl1* expression modulates epidermal differentiation predominantly at a stage subsequent to commitment of ectodermal cells to an epidermal fate. To elucidate further the role of this factor in epidermal differentiation, we identified a specific dominant-negative form of *XGrhl1*, guided in part by previous observations of a *Drosophila* dominant-negative mutant. The fly factor, Δ447*grh*, lacked a 447 amino acid N-terminal activation domain, dimerized with the wild-type protein and blocked GRH function (Attardi et al., 1993). The structurally homologous mutant of *XGrhl1* tested here, Δ227XGRHL1, lacks a N-terminal activation domain encoding the first 227 amino acids, dimerizes with wild-type XGRHL1 and has comparable binding affinity to XGRHLl for a consensus *grh*-binding motif (see Fig. S2 in the supplementary material). In

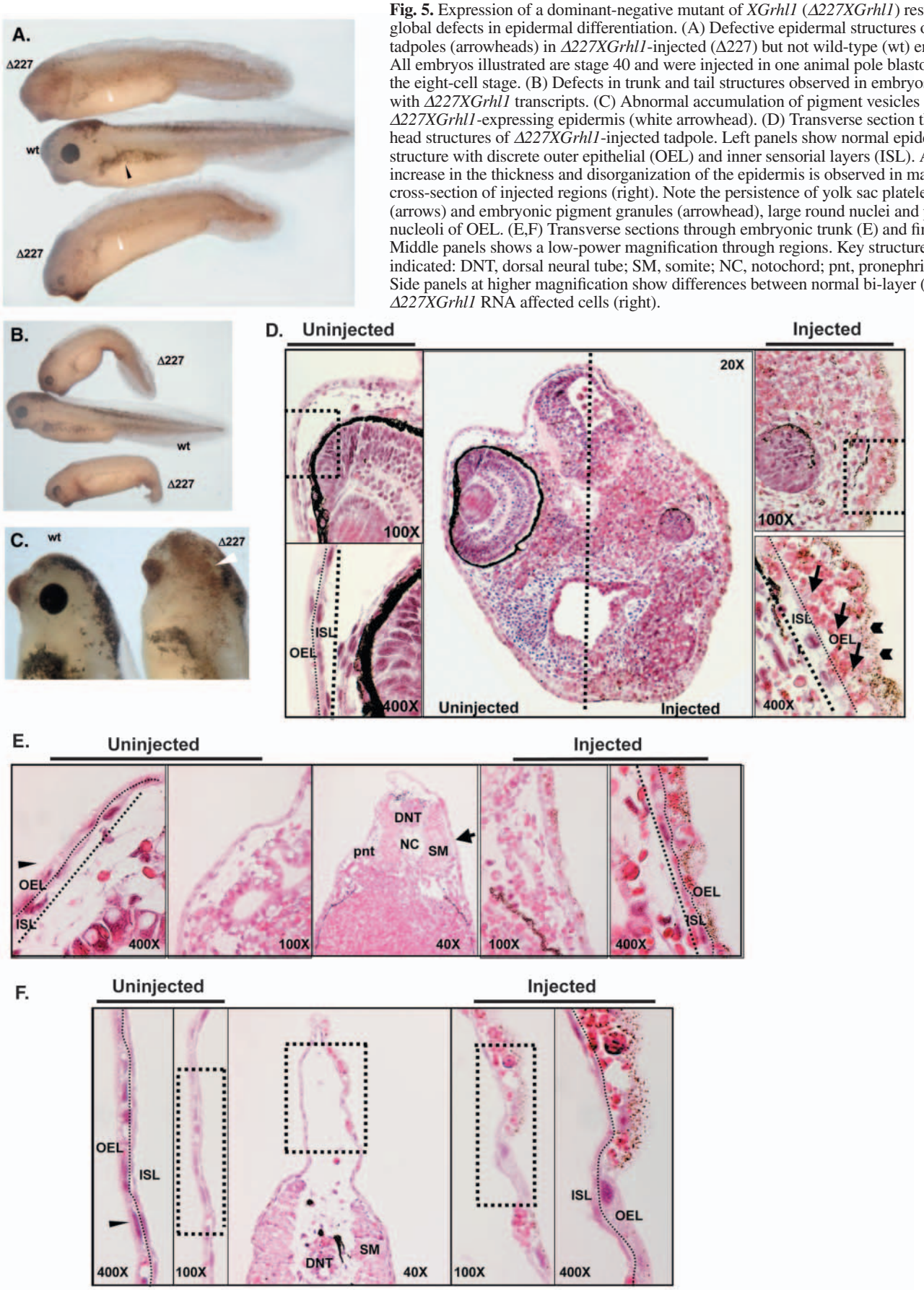

Fig. 5. Expression of a dominant-negative mutant of *XGrhl1* (*Δ227XGrhl1*) results in global defects in epidermal differentiation. (A) Defective epidermal structures observed in tadpoles (arrowheads) in *Δ227XGrhl1*-injected (Δ227) but not wild-type (wt) embryos. All embryos illustrated are stage 40 and were injected in one animal pole blastomere at the eight-cell stage. (B) Defects in trunk and tail structures observed in embryos injected with *Δ227XGrhl1* transcripts. (C) Abnormal accumulation of pigment vesicles in *Δ227XGrhl1*-expressing epidermis (white arrowhead). (D) Transverse section through head structures of *Δ227XGrhl1*-injected tadpole. Left panels show normal epidermal structure with discrete outer epithelial (OEL) and inner sensorial layers (ISL). A marked increase in the thickness and disorganization of the epidermis is observed in magnified cross-section of injected regions (right). Note the persistence of yolk sac platelets (arrows) and embryonic pigment granules (arrowhead), large round nuclei and prominent nucleoli of OEL. (E,F) Transverse sections through embryonic trunk (E) and fin (F). Middle panels shows a low-power magnification through regions. Key structures are indicated: DNT, dorsal neural tube; SM, somite; NC, notochord; pnt, pronephric duct. Side panels at higher magnification show differences between normal bi-layer (left) and *Δ227XGrhl1* RNA affected cells (right).

addition, both *Δ447grh* and *Δ227XGrhl1* transcripts inhibited *XGrhl1*-induced *XK81A1*, *Dlx3*, *Dlx5* and *XAP2* expression specifically in dissociated cell explant assays (see Fig. S2 in the supplementary material).

Microinjection of *Δ227XGrhl1* encoding mRNA (1 ng) into animal pole blastomeres at the two- to 16-cell stage resulted in normal gastrulation and neurulation of *Δ227XGrhl1*/β-gal injected embryos (stages 1-25; data not shown). By contrast, the effect of *Δ227XGrhl1* expression on non-neuronal ectoderm at later timepoints was profound, with gross macroscopic distortion of pre-larval epidermal differentiation (Fig. 5A-C; stages 35-40). We observed a consistent loss of specialized surface structures and a failure of evolution of the normal pigmentation pattern in injected ectoderm. Head and neck structures were defective, with absence of eye and otic placodes, failure of formation of the stomatodeal anlage and lack of melanization (Fig. 5A,B). Trunk/tailbud structures were also abnormal, with frequent misshapen embryos and loss of appropriate fin formation (Fig. 5B). Furthermore, there was apparent persistence of embryonic pigment at stage 40 on the injected side alone, which is more characteristic of earlier stages of pre-larval development (Fig. 5A,C; arrows). By contrast, *Δ227XGrhl1* transcripts, even at high concentrations, had no macroscopic effect when injected into blastomeres with a neural or mesendodermal fate, specificity being confirmed by rescue of *Δ227XGrhl1*-mediated defects by co-injection of XGrhl1 transcripts (data not shown).

Histological examination confirmed a primary *Δ227XGrhl1*-

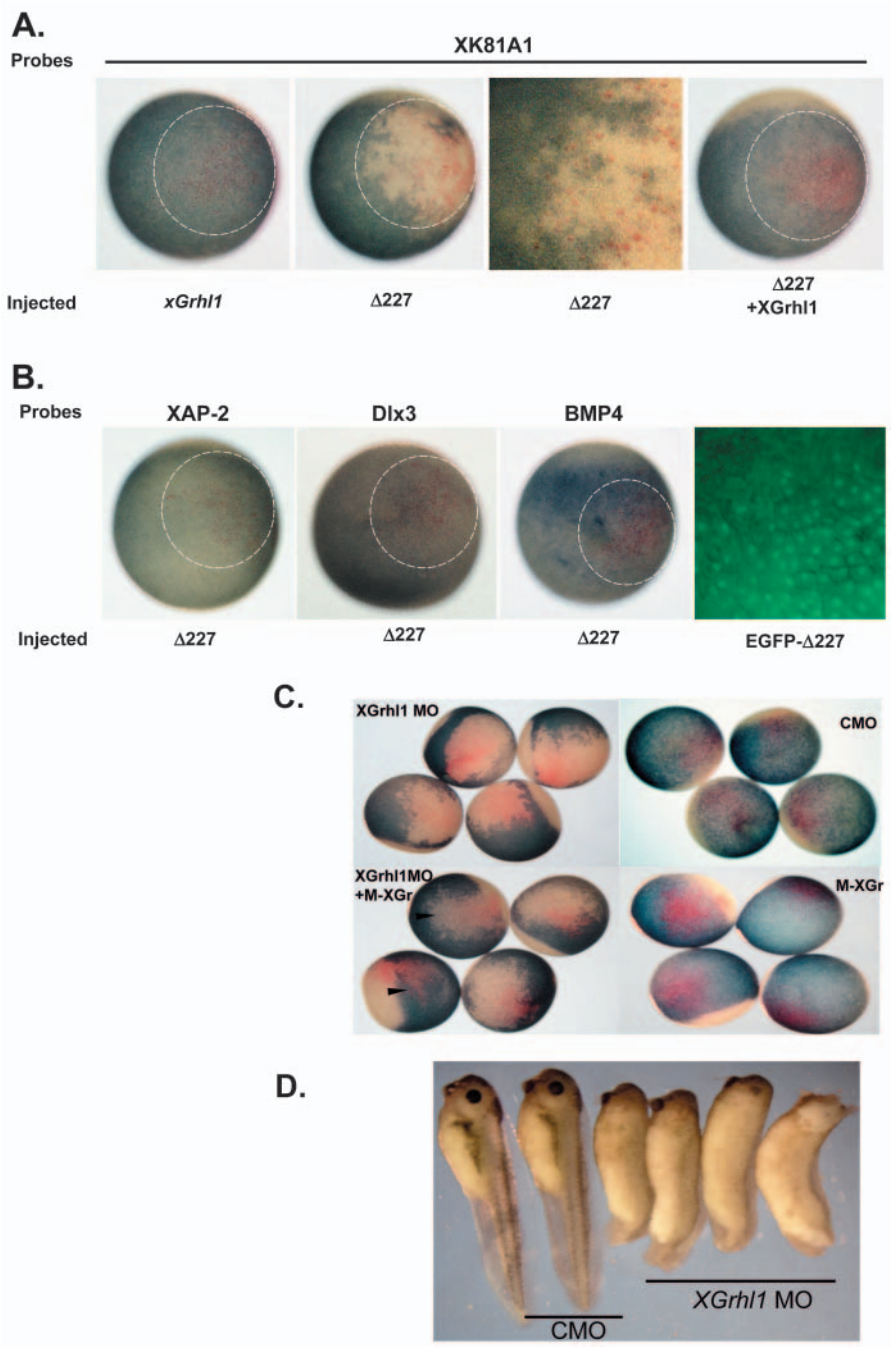

Fig. 6. Inhibition of X*Grhl1* activity or expression blocks *XK81A1* keratin expression in vivo. (A) Expression of *Δ227XGrhl1* blocks endogenous *XK81A1* expression specifically. Embryos were injected in one animal blastomere at the four-cell stage with *XGrhl1* (2 ng) and/or *Δ227XGrhl1* (1 ng)-encoding transcripts. In situ hybridization for *XK81A1* expression (blue stain) was performed on stage 14 embryos. β-Galactosidase, a lineage tracer, stained red. The broken white lines delineate areas of *Δ227XGrhl1* expression. (B) *Δ227XGrhl1*-encoding transcripts (1 ng) do not block expression of other BMP4 signaling pathway components. The product of a factor chimera [*Δ227XGrhl1* sequences linked in frame with enhanced green fluorescent protein cDNA (EGFP)] was detected in nuclei of transfected cells (extreme right panel), consistent with appropriate nuclear localization. (C) Injection of a *XGrhl1*-targeted MO blocks endogenous *XK81A1* keratin gene expression specifically. Embryos were injected into one animal blastomere at the four-cell stage with *XGrhl1MO±M-XGr* (a MO-resistant *XGRhl1*-expressing RNA transcript) (upper left). In situ hybridization for *XK81A1* expression (blue stain) and a β-galactosidase lineage tracer (red) was performed on stage 14 embryos. The *XGrhl1*-MO mediated block in *XK81A1* gene expression is rescued partially by co-expression of *M-XGr* mRNA (lower left). Coincident blue and red staining is indicated (arrowheads). A control morpholino (CMO, upper right) or *M-XGr* alone (lower right) failed to affect normal development. (D) Injection of *XGrhl1*-targeted MO induces an epidermal defect in maturing tadpole specifically. Defects in head and trunk structures representative of those observed in embryos injected with *XGrhl1*-specific MO in one animal pole blastomere at the eight-cell stage are shown. Epidermal and pigment changes in head and trunk are observed in *XGrhl1MO*-injected embryos (when compared with CMO-injected embryos) that are identical to those seen with the *Δ227XGrhl1*-expressing mutants in Fig. 5.

Table 1. Specific inhibition of endogenous *XK81A1* expression by *Δ227XGrhl1*

mRNA injected	*n*	Number and percentage of embryos affected		
		Unaffected	Reduced (25-90%)	Reduced (>90%)
β-Gal	18	18 (100%)	0	0
Δ227XGrhl1	26	0	10 (38%)	16 (62%)
Δ227XGrhl1 + *XGrhl1*	33	1 (3%)	30 (90%)	2 (6%)
XGrhl1	17	17 (100%)	0	0

Embryos were injected together with the appropriate mRNA and 50 pg of β-gal-encoding transcripts in one animal blastomere at the four-cell stage. Embryos were harvested at stage 14, stained with red-gal to identify injected progeny, and then whole-mount in situ hybridization was performed with an antisense-labeled *XK81A1* specific probe. The data represent one of two independent experiments.

induced block in epidermal differentiation, most strikingly at stage 40. The outer epithelial layer (OEL) or periderm on the uninjected side consisted of columnar cells with small nuclei and a few apically distributed cytoplasmic yolk platelets (Fig. 5D). Expression of *Δ227XGrhl1* resulted in poorly differentiated peridermal cells with large nuclei, prominent nucleoli, the presence of cytoplasmic yolk platelets and a pancellular distribution of embryonic pigment granules more characteristic of earlier less differentiated stages of *Xenopus* ontogeny (Nieuwkoop and Farber, 1994). By contrast, the underlying flattened inner sensorial layer (ISL) was similar on both sides. Similar changes in the epidermis were observed in *Δ227XGrhl1*-injected blastomeres, which localized to the trunk and fin regions (Fig. 5E,F), confirming the generalized effects of *Δ227XGrhl1* expression on epidermal differentiation.

Additional features were evident from histological analysis of embryos in which blastomeres with a non-neuronal ectodermal fate were injected with *Δ227XGrhl1*-encoding RNA transcripts. First, disorganization of specialized epidermal structures including otic and optic placode formation was observed with consequent failure of lens structure development in the latter instance (Fig. 5C; data not shown). Coincident with the latter changes, significant regression of the neuroretinal structures was observed, with a reduction of the surrounding pigment layer. Second, structures deep to the epidermis showed significant changes, including disorganized head and trunk mesenchyme, and alteration in the neural tube and the stomodeal anlage. By contrast, histology of embryos in which *Δ227XGrhl1* transcripts were injected into blastomeres with a mesoendodermal fate was normal (data not shown). These results indicate that loss of XGRHL1 function results in a specific primary alteration in epidermal differentiation in the maturing *Xenopus* embryo, with apparent loss of epidermal inductive signals to underlying structures.

XGrhl1 function/expression is required for appropriate epidermal keratin expression

Architectural changes in the OEL occurring with *Δ227XGrhl1* expression, coupled with the modulation of expression of *XK81A1, ESR6e* and other epidermal-restricted factors in explanted cells, support the contention that *XGrhl1* activity is necessary for appropriate epidermal differentiation subsequent to commitment. To test this idea further, we asked if *XGrhl1* activity was required for epidermal structural gene expression in vivo. We chose to focus our efforts on *XK81A1* expression, given the central role of keratins in structural and morphogenetic events in vertebrate epidermal cells (Fuchs and Raghavan, 2002; Porter and Lane, 2003). Transcripts encoding *Δ227XGrhl1,* co-injected with β-gal into one blastomere at the four-cell stage, resulted in complete loss of *XK81A1* expression in the progeny of injected blastomeres when assayed at stage 14 (Fig. 6A; data not shown). This effect was reversed with co-injection of wild type *XGrhl1*, confirming the specificity of *Δ227XGrhl1* activity (Fig. 6A; Table 1). By contrast, *Δ227XGrhl1* had no effect on *XAP2*, *Dlx3* or BMP4 expression (Fig. 6B). A green fluorescent protein/*Δ227XGrhl1* chimera gave a similar result and confirmed that the dominant-negative factor, like the wild-type protein was localized to the nucleus of injected blastomere progeny (Fig. 6B; data not shown).

To corroborate these observations, embryos were injected with a *XGrhl1*-specific antisense morpholino (*XGrhl1*MO; see Fig. S3 in the supplementary material). An identical phenotype to *Δ227XGrhl1* was observed with loss of *XK81A1* expression with co-injection of a *XGrhl1*MO and transcripts encoding β-gal into one blastomere of four-cell stage embryos (Fig. 6C). By contrast, a control MO (CMO) had no effect on *XK81A1* expression. *XK81A1* expression was rescued partially by co-injection of *XGrhl1*MO with *M-XGrhl1* transcripts, the latter encoding a synthetic isoform of *XGrhl1* which is partially resistant to *XGRhl1*MO (see Fig. S3 in the supplementary material; Fig. 6D and Table 2). Embryos injected with *XGrhl1*-specific MOs and followed until later time points (stages 35-40) had a similar phenotypic defect in epidermal differentiation to that observed with *Δ227XGrhl1* (Fig. 6E). Together, these studies strengthen significantly our model of a key role for *XGrhl1* in modulating epidermal differentiation and *XK81A1* expression specifically.

An *XGrhl1*-binding site in the *XK81A1* promoter is required for maximal transcriptional activation

To determine whether *XGrhl1* regulates *XK81A1* transcription directly, we examined DNA sequences directly upstream of the *XK81A1*-coding region using a previously defined consensus *Drosophila GRH* binding motif (Huang et al., 1995; Shirra and Hansen, 1998). A sequence centered at ~200 bp upstream of the transcriptional start site shares considerable nucleotide identity (56%) with the invertebrate factor binding region and binds recombinant XGRHL1 specifically (Fig. 7A; see Fig. S4 in the supplementary material). Mutagenesis of this sequence identified nucleotides that, when altered, ablated XGRHL1 binding (designated M2; see Fig. S4 in the supplementary material). Interestingly, this region also encodes an adjacent *XAP2*-binding motif, which has been implicated previously as being essential for *XK81A1* expression (Snape et al., 1991). To test the in vivo relevance of in vitro XGRHL1 binding, firefly luciferase reporter cassettes linked to *XK81A1* promoter fragments with specific mutations were introduced into *Xenopus* embryos (Fig. 7B). Similar activity was observed with

Table 2. Inhibition of XK81A1 expression by a *XGrhl1*-specific morpholino (MO1)

mRNA injected	*n*	Embryos (%) with alteration in *XK81A1* expression		
		Unaffected (0-20%)	Reduced (20-90%)	Absent (>90%)
XGrhl1 MO1	71	3	10	87
CMO	60	88	8	0
M-XGr	53	91	9	0
XGrhl1 MO1 + *M-XGr*	74	17	39	43

Embryos were injected together with the appropriate MO (40 ng)±mRNA (2 ng) and 50 pg of β-gal-encoding transcripts in one animal blastomere at the four-cell stage. Embryos were harvested at stage 14, stained with red-gal to identify injected progeny, and then whole-mount in situ hybridization was performed with an antisense-labeled *XK81A1*-specific probe. The data represent one of two independent experiments.

the full-length promoter, and a promoter truncated to –276 bp relative to the transcriptional initiation site (compare KP487 and KP276). However, deletion of a region between –215 and –101 bp (–113KP487) resulted in a greater than 50% reduction in reporter activity. Ablation of *XAP2* binding (M157E) or the *XGrhl1*-binding motif (M2) resulted in a 50% reduction in reporter gene activity. Mutation of both motifs failed to show a further decrease in luciferase expression (M2M157E).

To confirm the importance of XGRHL1 binding for *XK81A1* transcription, we assessed the functional consequences of inhibition of *XGrhl1* activity on *XK81A1* promoter (KP487) function. Co-injection of *Δ227XGrhl1* transcripts with the KP487 reporter construct resulted in a 50-60% reduction in luciferase reporter activity (Fig. 7C). By contrast, Δ227*XGrhl1* transcripts failed to affect M2 reporter gene activity,

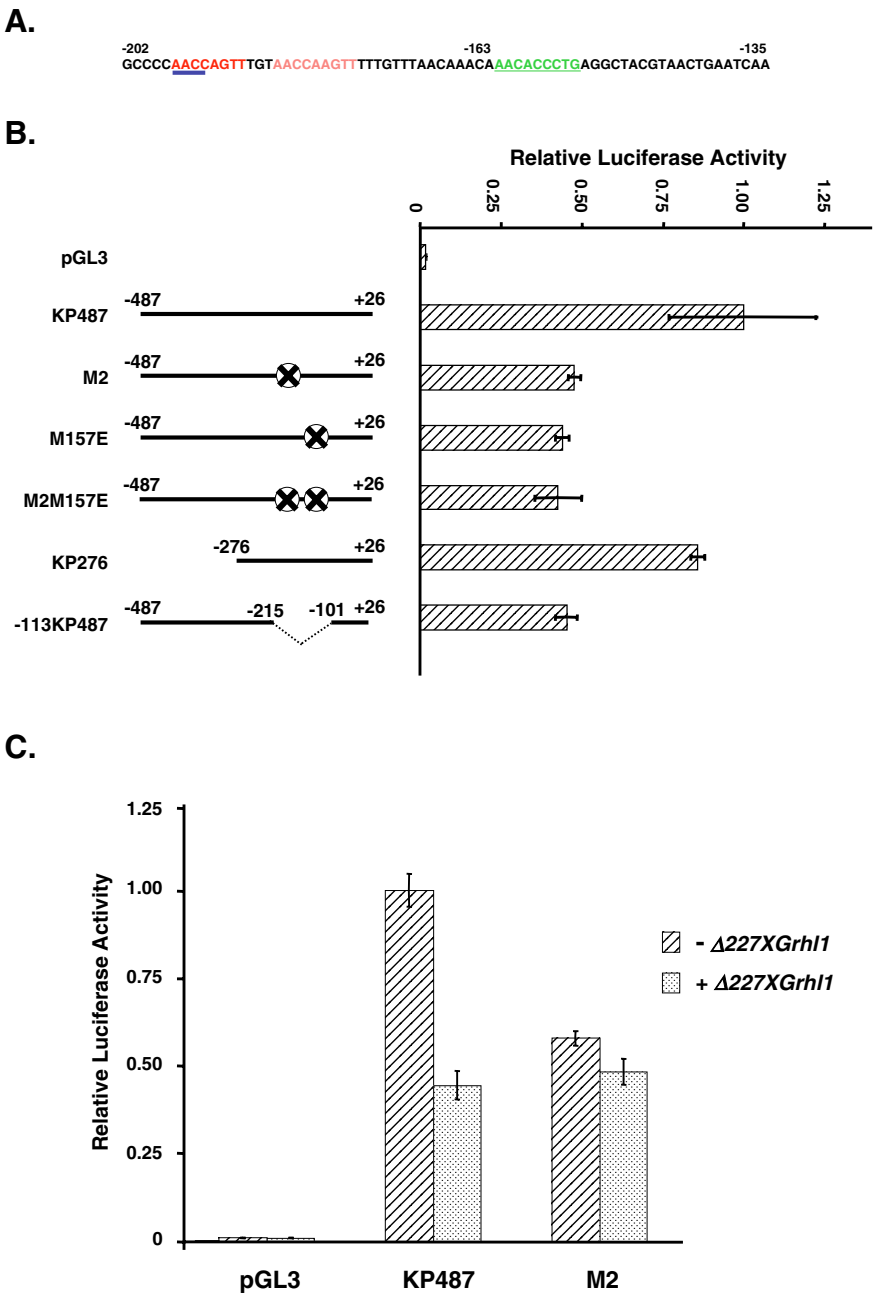

Fig. 7. *XGrhl1* modulates *XK81A1* keratin promoter activity specifically. (A) A region upstream of the XK81A1 transcriptional start site (red) has significant homology with a *Drosophila* GRH consensus sequence. Green type indicates a previously defined *XAP-2*-binding sequence. Mutation of a 4 bp motif (blue line; M2) blocks XGRHL1 binding. (B) Loss of the –200 XGRHL1-binding motif results in a significant defect in *XK81A1* promoter activity. Whole embryo reporter assays were performed using *XK81A1* keratin promoter sequences linked in cis to the firefly luciferase gene. All values were standardized to the full-length wild-type promoter sequence (arbitrary value of 1). KP487, previously defined XK81A1 promoter; M157E, deletion of previously defined AP-2-binding motif; M2, mutation of the putative XGRHL1-binding site; M2M157E, AP-2/XGRHL1 double mutant; –113KP487, deletion of promoter region with epidermal-specific activity. (C) Expression of *Δ227XGrhl1* blocks XK81A1 promoter activity. The full-length *XK81A1* luciferase reporter construct (KP487), a XK81A1 reporter construct in which the XGRHL1-binding site was mutated (M2), or a luciferase only control (pGL3) was co-injected into animal pole blastomeres at the four-cell stage with or without *Δ227XGrhl1*-encoding transcripts. Luciferase reporter assays were performed as described in B. All values were standardized with respect to the full-length wild-type promoter sequence (arbitrary value of 1).

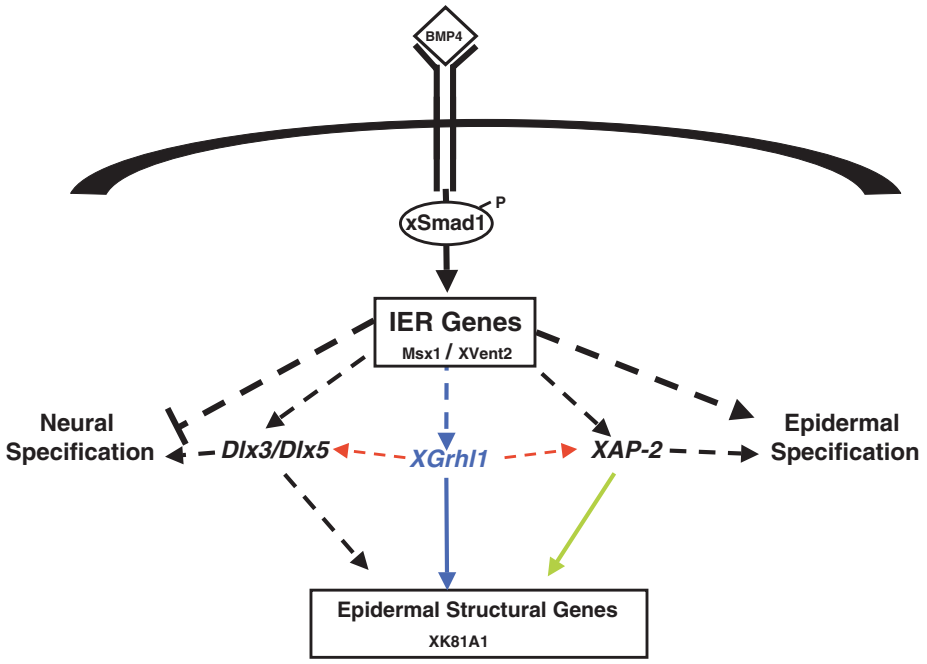

Fig. 8. *XGrhl1*, with *XAP-2*, modulates BMP4-dependent epidermal structural gene expression. BMP4-dependent phosphorylation of XMad1 induces IER gene expression directly (unbroken arrow). After an unknown number of intermediary steps (broken arrow), these factors activate *Dlx3*, *Dlx5*, *AP-2* and *XGrhl1* gene expression. Blue arrows indicate newly identified tissue-specific components of the BMP4-signaling cascade. XGRHL1 and AP-2, cooperatively, activate structural gene expression directly (blue and green unbroken arrows). The molecular mechanism(s) by which Dlx3/Dlx5 facilitates gene activation in this context are currently unclear (broken arrow). *XGrhl1* also stimulates AP-2 and Dlx3/Dlx5 gene expression (broken red arrows). For simplicity, all BMP4-mediated events are not shown.

when compared with embryos injected with the M2-driven luciferase construct alone. Collectively, these results demonstrate that the XGRHL1-binding motif is required for *XK81A1* promoter activity.

Discussion

Intact BMP4 signaling is necessary for differentiation of the embryonic *Xenopus* epidermis (Baker and Harland, 1997). Our studies demonstrate that *XGrhl1* is a downstream epidermal-specific target of this pathway. This observation varies significantly from that reported in *Drosophila,* in which *grh* expression modulates BMP4 activity (Huang et al., 1995). This evolutionary divergence raises three questions: (1) is *XGrhl1* necessary for BMP4-dependent epidermal specification; (2) if not, what is the role of *XGrhl1* in the pathway; (3) is *XGrhl1* involved in other epidermal-specific signaling events?

Ectopic expression of BMP4 or of IER factors, such as *Xmad1*, result in epidermal re-specification in cellular progeny of blastomeres with a neural fate (Wilson et al., 1997). Similarly, co-expression of IER factors and BMP antagonists/inhibitors induces epidermal specification in injected ectodermal cells, with coincident repression of neural gene expression (Feledy et al., 1999a; Suzuki et al., 1997a; Suzuki et al., 1997b). Given the temporal pattern of endogenous expression, we expected a similar outcome with enforced expression of *XGrhl1*. Differing sharply from the effects of IER factors, ectopic expression of *XGrhl1* failed to induce epidermal specification. These observations suggest that *XGrhl1* activity is dispensable for this process, a conclusion supported by the inability of injection of *Δ227XGrhl1*-encoding transcripts or *XGrhl1*-specific MOs to affect germ layer specification.

Our studies suggest an alternate model, *XGrhl1* functioning downstream of the IER factors in the BMP-signaling cascade (Fig. 8). In this context, *AP2* and *Dlx*-like factors have been shown previously to be essential for appropriate epidermal differentiation (Fuchs and Raghavan, 2002; Luo et al., 2002; Panganiban and Rubenstein, 2002). However, it remains unclear how these factors achieve tissue specificity given their wider pattern of gene expression (Luo et al., 2001b; Luo et al., 2002). We show that induction of *XK81A1* is dependent on appropriate *XGrhl1* function. Like *Dlx3*, expression of *XGrhl1* does not induce expression of the epidermal structural gene *XK81A1* in the absence of a functional BMP4 pathway, suggesting that morphogen-induced expression of other factors is necessary. One candidate may be *AP2,* given its ability to rescue the epidermal defect induced by tBR expression in a similar manner to IER regulatory factors (Luo et al., 2001b; Suzuki et al., 1997b; Wilson et al., 1997). Furthermore, like *XGrhl1, AP2* fails to repress expression of pan-neural gene markers, a divergence from the effects of IER factor expression (Luo et al., 2002). These observations, together with our studies of the *XK81A1* promoter, indicate that *XGrhl1* functions predominantly downstream of the IER factors in the BMP4 signaling cascade. Furthermore, our studies of the XK81A1 promoter demonstrate that both *AP2* and XGRHL1 are required for *XK81A1* expression (see below). Thus, we suggest that characterization of the expression of *XGrhl1* and its mechanism of action represents a significant new insight into the regulation of BMP4-responsive epidermal-specific targets, this tissue-specific factor modulating structural gene expression in concert with the more widely expressed regulator *AP2* during terminal differentiation.

Intriguingly, *XGrhl1* induces *AP2* and *Dlx3* expression in dissociated animal explant cells, consistent with models in which positive feedback loops initiated by downstream regulatory factors stabilize and/or potentiate the decision to undergo a specific cellular fate (Ferrell, 2002; Green, 2002). In addition, this positive 'epidermal' loop underscores emerging evidence of a complex crosstalk occurring between regulatory molecules within vertebrate epidermal differentiation programs (Fuchs and Raghavan, 2002). Recent studies have implicated *Drosophila* GRH as a target of the FGF, Notch and Wnt/*Wingless* (*Wg*)

signaling pathways (Furriols and Bray, 2001; Hemphala et al., 2003; Lee and Adler, 2004), suggesting that the expression and function of *XGrhl1* may represent the sum of these inputs. In this context, the effects of XGRHL1 on the transcription of the bHLH factor *ESR6e*, an ectodermal target of Notch and BMP4 signaling, may be instructive (Chalmers et al., 2002; Deblandre et al., 1999). *ESR6e* expression is restricted to the epidermis predominantly, although it is also observed in cells of the neural plate. The coincident expression of *ESR6e* and *XGrhl1* expression, and the induction of *ESR6e* by ectopic expression of *XGrhl1* (Fig. 1C; Fig. 4C) suggest a functional link. Indeed, the lack of ciliated cells in *Δ227XGrhl1*-expressing epidermis (Fig. 5 and data not shown), coupled with the role of *ESR6e* in Notch-mediated specification and migration of ciliated cells into the *Xenopus* periderm (Deblandre et al., 1999) suggest that *XGrhl1* may be involved in this process.

Our observations demonstrating a severe defect in end-stage epidermal differentiation induced by perturbation of XGRHL1 function raise the issue of whether this defect is related to loss of *XK81A1* expression alone, or to a more generalizable defect in expression of genes of the epidermal program. The latter model is supported by several observations. Although the effect of a knock down of *XK81A1* expression on *Xenopus* development has not been described, studies of dysregulation of keratin K14, its murine homolog, suggest otherwise. Animals homozygous for K14 disruption exhibit a similar increase in keratinocyte fragility that resembles the *Δ227XGrhl1*-induced phenotype (Lloyd et al., 1995). Conversely, these mice are viable at parturition, have appropriate differentiation of embryonic and adult epidermal keratinocytes, and have no evidence of a secondary defect in underlying mesendodermal tissues. A second line of evidence supporting a more generalized epidermal defect is provided by the observation that enforced co-expression of *XK81A1* in *Δ227XGrhl1*-expressing embryos (or in embryos injected with XGrhl1-MOs) failed to reverse the phenotype (J.T., unpublished). Collectively, these results suggest that *XGrhl1* modulates transcription of a range of epidermal-specific genes.

Additional lines of evidence support this conclusion. First, comparison of *Drosophila* and *Xenopus* Grhl-defective phenotypes confirm an evolutionary conservation of Grhl factor function during epidermal ontogeny. Thus, the failure of appropriate epidermal differentiation, abnormal head structures and defective specialized epidermal structures characteristic of *Δ227XGrhl1* expression mirrors the defects observed with expression of a structurally similar mutant, Δ447*grh*, in *Drosophila*. The mutant fly phenotype includes cuticlar defects, a ‘grainyhead’ phenotype and deficits in hooks, mouth pieces and wing structures (Attardi et al., 1993; Bray and Kafatos, 1991; Lee and Adler, 2004). Interestingly, injection of Δ447*grh* into *Xenopus* blastomeres with an epidermal fate at the eight- to 16-cell stage results in a similar outcome to that observed with *Δ227XGrhl1* (data not shown). Second, we observed *XGrhl1*-mediated expression of other epidermal-restricted differentiation factors, including *AP2*, *Dlx3* and *ESR6e* in ectodermal cells (Fig. 4). As discussed above, this suggests a complex requirement for *XGrhl1* in the expression of these genes.

What are the identities and functions of other *XGrhl1*-dependent genes? The failure to resorb cytoplasmic yolk platelets and assume the flattened morphology of the mature peridermal cell suggests that *XGrhl1* may modulate repression of the primitive epidermal gene program and/or modulate morphogenetic changes in cell shape. The latter hypothesis is supported by observations demonstrating a role for *Drosophila grh* in changes in tracheal cell shape and the epidermal-specific failure of murine neural tube closure observed with mouse Grhl3 deficiency (Hemphala et al., 2003; Ting et al., 2003a). Given our demonstration of a conservation of Grhl function, other Grhl targets may be provided by recent characterization of orthologs of *Drosophila* blimp phenotypes similar to *grh* (Ostrowski et al., 2002). Preliminary analysis has identified several genes epistatic to *GRH*, including cadherins and adhesion molecules (Lee and Adler, 2004). Indeed, some of these molecules are also involved in the development of specialized epidermal appendages. Given the apparent evolutionary conservation of Grhl function, it will be important to determine the functional role of vertebrate orthologs of *XGrhl1*, particularly its role in the stratification of the adult epidermis, as similar mechanisms are operative in the embryonic epidermis and the basal layer of the adult skin (Byrne et al., 1994; Koster et al., 2004; Nieuwkoop and Farber, 1994).

Vertebrate promoter/enhancer regulatory elements of type I (and II) keratins, and other structural genes contain functionally important motifs for the non-epidermal specific AP2, AP1 and Sp1 DNA-binding factors, amongst others (Byrne and Fuchs, 1993; Jonas et al., 1989; Kaufman et al., 2002; Leask et al., 1990; Leask et al., 1991; Sinha et al., 2000; Sinha and Fuchs, 2001; Snape et al., 1990; Snape et al., 1991). The expression patterns of these factors suggest that non-epidermal specific factors interact in a combinatorial manner, potentially recruiting keratinocytic-specific co-regulators to modulate appropriate expression (Fuchs and Raghavan, 2002). Several mammalian keratinocyte-specific promoter/enhancer-binding activities have been identified recently, although detailed characterization is awaited (Kaufman et al., 2002; Sinha et al., 2000; Sinha and Fuchs, 2001). Our studies alter this model significantly. We demonstrate clearly the molecular basis by which binding of a novel epidermal-specific factor, XGRHL1, is essential for high-level transcription in epidermal cells. Interestingly, preliminary exploration of murine K14 sequences, as well as *Xenopus AP2* and *Dlx3* promoters, identified similar Grhl binding motifs (J.T., unpublished).

Adjacent to the XGRHL1 site is a previously defined AP2 motif crucial for maximal promoter activity (Snape et al., 1990; Snape et al., 1991). Our studies suggest a functional interaction between these factors (Fig. 7D,E). *Drosophila GRH* interacts with $dTAF_{II}110$ (Attardi and Tjian, 1993; Dynlacht et al., 1989; Dynlacht et al., 1991), a component of the TFIID TATA-binding complex providing a structural basis for the exploration of the molecular mechanism(s) of keratin gene transcription. Interestingly, $hTAF_{II}130$, the ortholog of $dTAF_{II}110$, interacts with the co-regulator CBP (Nakajima et al., 1998), the latter co-factor being required for AP2-mediated gene activation (Braganca et al., 2003). Together, these observations suggest the existence of a molecular ‘bridge’ between the DNA-bound transacting factors and the transcriptional initiation complex which may explain the requirement for binding of both XGRHL1 and AP2 for maximal promoter function. It will be important to confirm these relationships in future studies.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/5/1021/DC1

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A.

Supplementary Figure 1
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B.

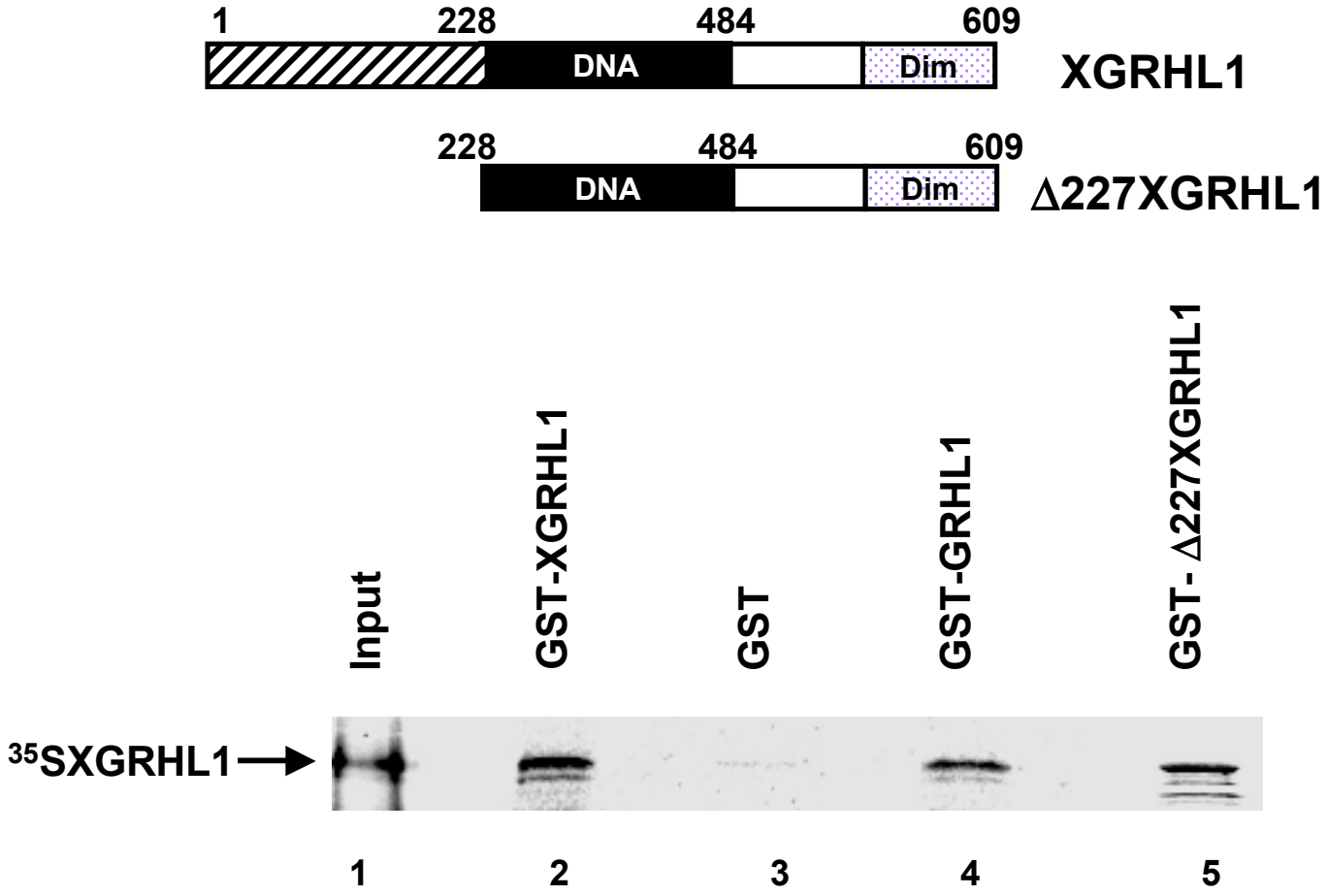
Full Length Factor	hGRHL1	mGRHL1	dGRH	ceGRH
Identity (%)	82	82	34	34
Similarity(%)	87	87	42	44

DNA binding domain	hGRHL1	mGRHL1	dGRH	ceGRH
Identity(%)	96	99	57	57
Similarity(%)	99	97	67	69

Dimer domain	hGRHL1	mGRHL1	dGRH	ceGRH
Identity(%)	85	85	41	38
Similarity(%)	93	92	54	56

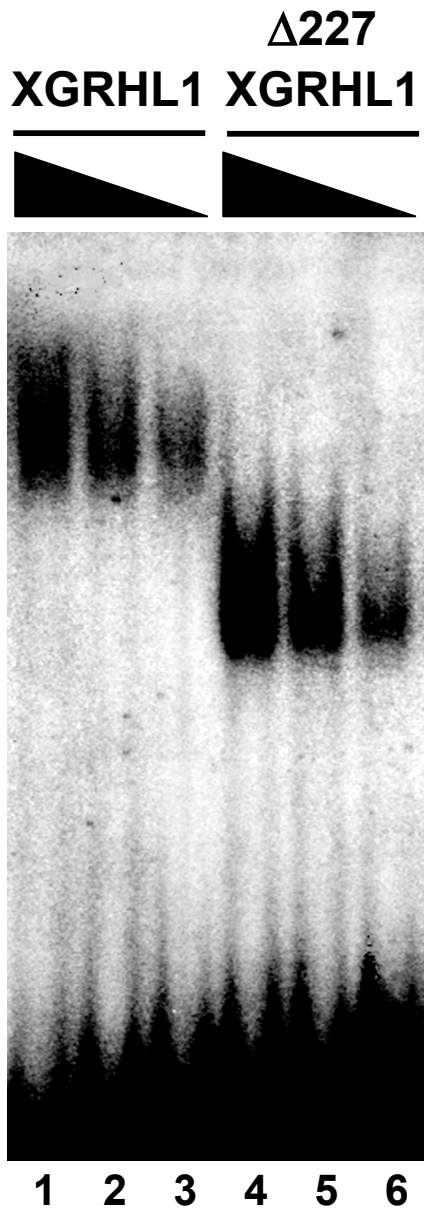
Supplementary Figure 1
Tao et al.

A.

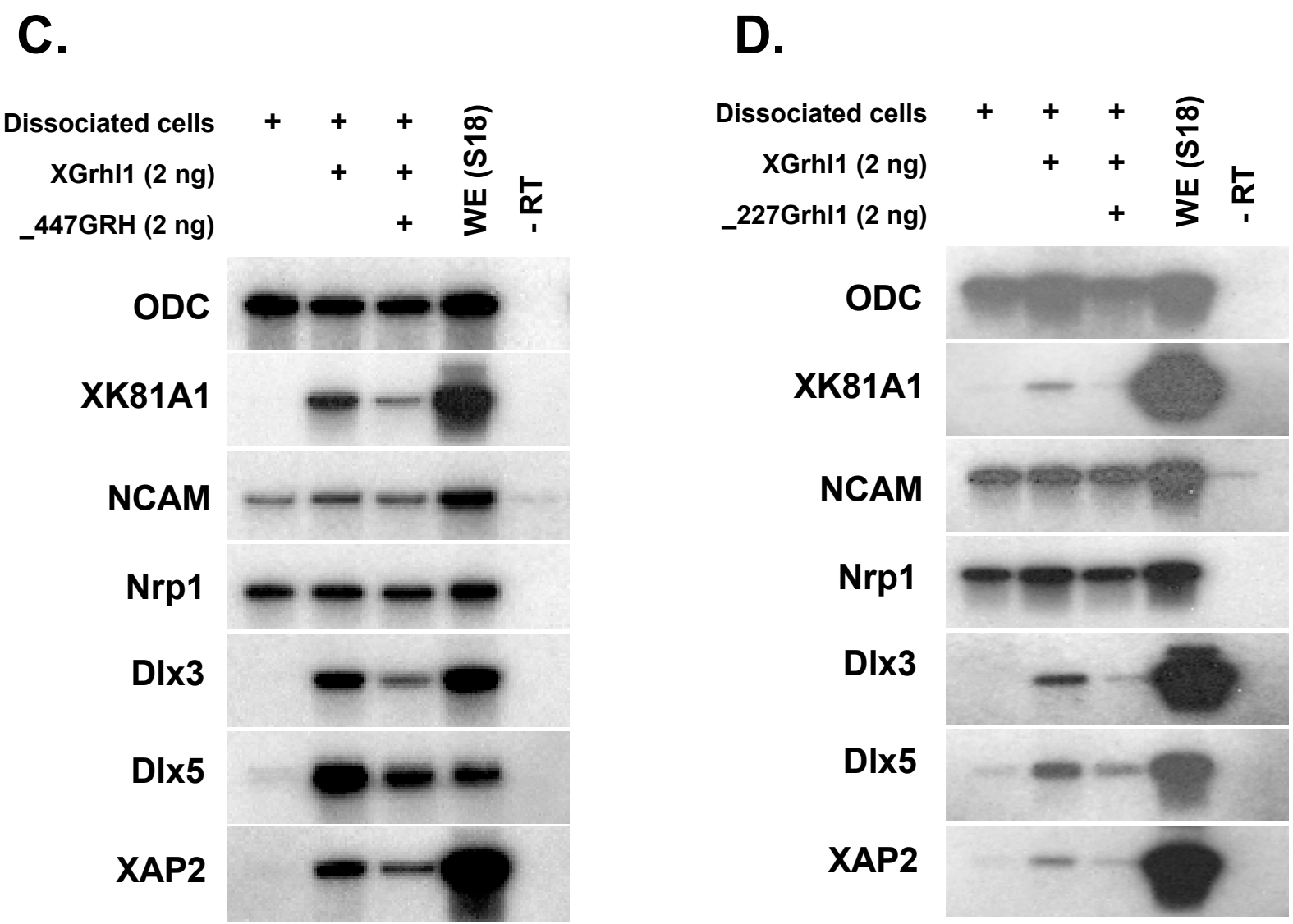


Supplementary Figure 2
Tao et al.

B.



Supplementary Figure 2
Tao et al.



Supplementary Figure 2
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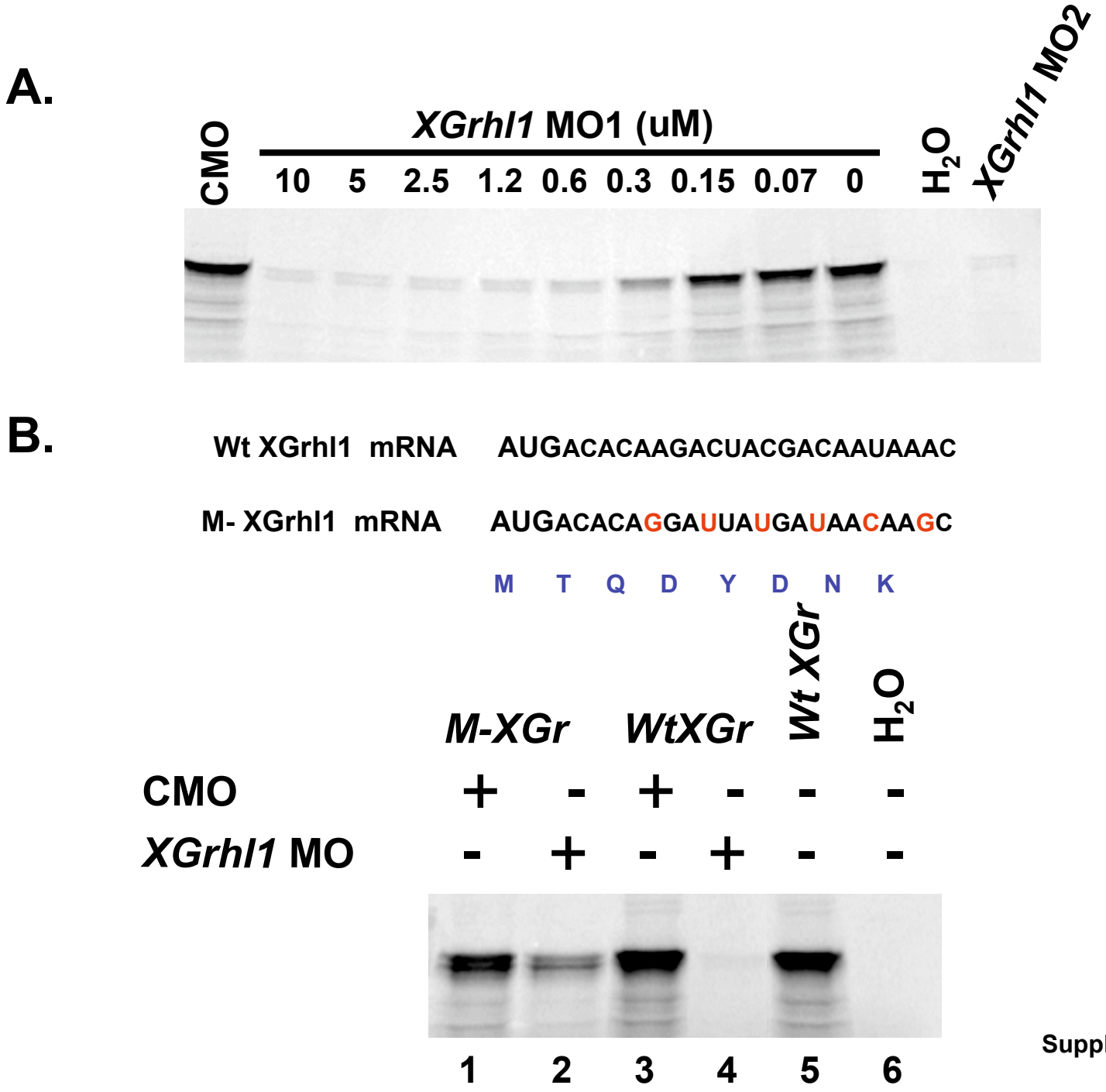
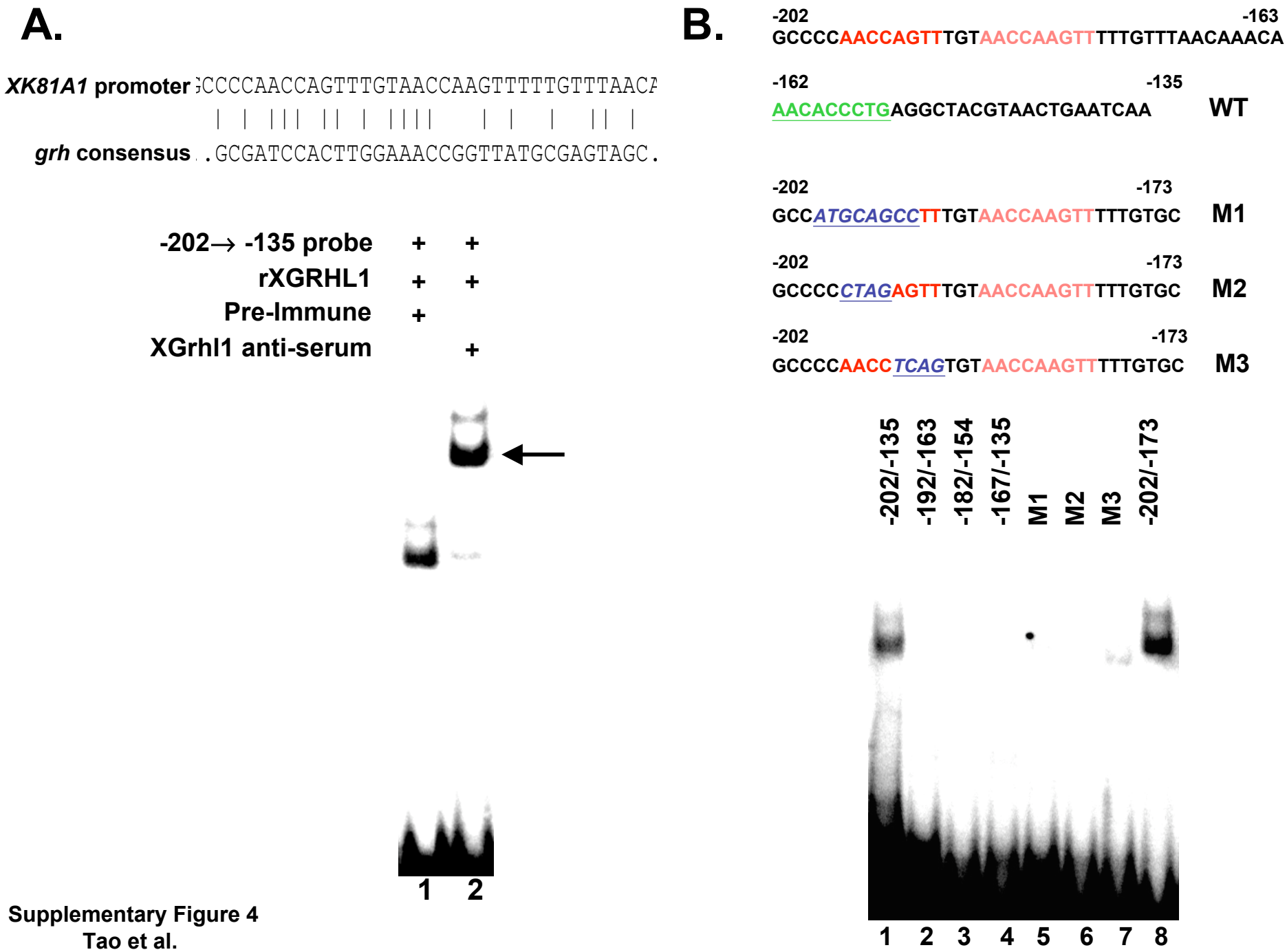
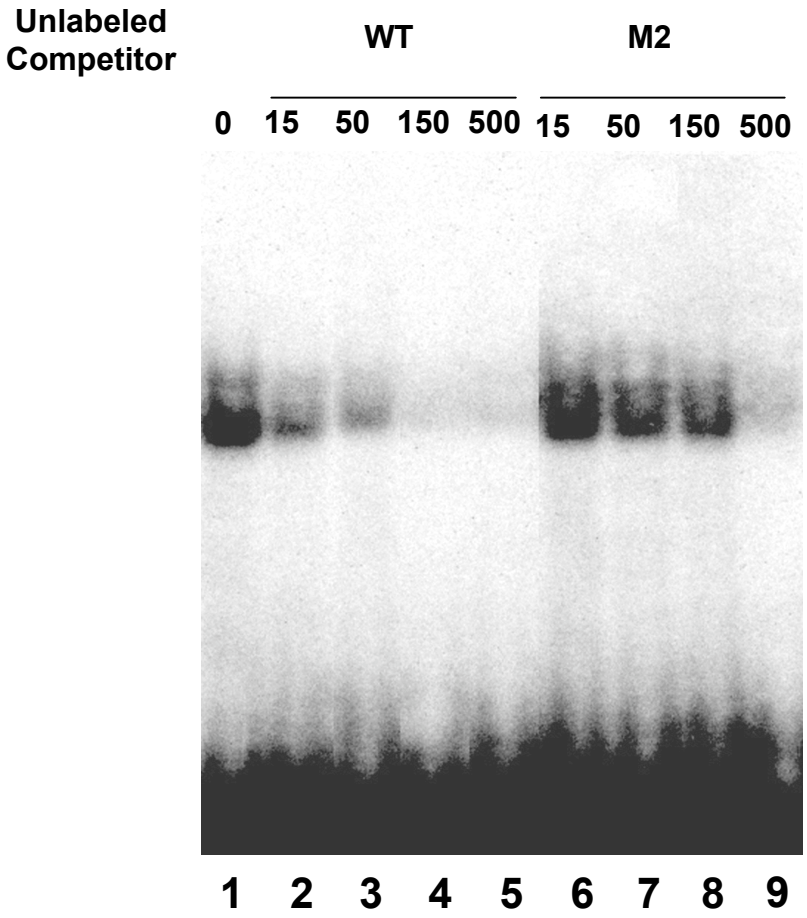


Supplementary Figure 3
Tao et al.



Supplementary Figure 4
Tao et al.

C.



Supplementary Figure 4
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