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Molecular cloning and characterization of *dullard*: a novel gene required for neural development

Reiko Satow,[a] Te-chuan Chan,[b] and Makoto Asashima[a,b,*]

[a] *Department of Biological Sciences, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8654, Japan*
[b] *Department of Life Sciences (Biology), Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan*

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Abstract

In a screen for genes expressed in neural tissues and pronephroi, we isolated a novel gene, named *dullard*. *Dullard* protein contains the C-terminal conserved domain of NLI-IF (Nuclear LIM Interactor-Interacting Factor), a protein whose function is not yet characterized. *Dullard* mRNA was maternally derived and localized to the animal hemisphere. At neurula stages, the expression was in neural regions and subsequently localized to neural tissues, branchial arches, and pronephroi. Using antisense morpholino oligonucleotide-mediated inhibition, we showed that *dullard* was required for neural development. The translational knock-down of *dullard* resulted in failure of neural tube development and the embryos consequently showed a reduction of head development. Expression of neural marker genes in *dullard*-inhibited embryos was also suppressed. These results suggest that *dullard* is necessary for neural development.

Keywords: *Xenopus laevis*; Neural development; Morpholino; *Dullard*; Neural tube closure; Pronephros

Neural development consists of several steps, including neural induction, patterning, and differentiation. In amphibians, neural induction occurs on the dorsal ectoderm, whereas the epidermis forms on the ventral ectoderm. In early studies, transplantation experiments showed that the organizer region can induce neural fate from the surrounding dorsal ectoderm at gastrula stages [1,2]. In amphibians, the mechanism by which the organizer region induces neural fate was studied using both whole embryos and in vitro cultures of explants [3–5]. Follistatin, noggin and chordin were found to be expressed in the organizer region of *Xenopus* embryos and to induce neural markers in animal cap explants by directly binding to and antagonizing the function of bone morphogenetic proteins (BMPs) [6–8]. Recently, Wnt and FGF were revealed to repress BMP signaling by inhibiting *Bmp* expression and be required for neural induction [6,8–13]. Thus, neural induction depends on down-regulation of BMP signaling at both the transcriptional and post-translational levels. Several genes, such as, *Xiro* [13,14], *SoxD* [15], *Zic1* and *Zic3* [16–19], *XSIP1* [20,21], and *Geminin* [22], which promote neural fate after neural induction in early to mid-gastrulation, have been recently identified. However, their direct downstream targets have not yet been identified and relationships between them that may contribute to neural fate determination remain unknown.

In this report, we introduce a novel factor necessary for neural development. This factor is named *dullard*, as low sensitivity to stimulus was observed in *dullard*-inhibited embryos. *Dullard* mRNA is maternally derived and localized to the animal hemisphere. At neurula stages, the expression is restricted to neural regions and subsequently localized to neural tissues, branchial arches, and pronephroi. The translational knock-down of this factor by antisense morpholino oligonucleotide resulted in failure of neural tissue development. Thus, we suggest that *dullard* is a new regulatory factor of neural tissue in development.

* Corresponding author. Fax: +81-3-5454-4330.
E-mail address: asashi@bio.c.u-tokyo.ac.jp (M. Asashima).

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Materials and methods

Eggs and embryos. Xenopus eggs were obtained by injecting adult males and females with 600 U human chorionic gonadotropin (Gestoron; Denka Seiyaku). Staging of embryos was according to Nieuwkoop and Faber [23]. The jelly coat was removed with Steinberg's solution containing 4.5% cysteine hydrochloride (pH 7.8).

cDNA library screening. mRNA was extracted from lateral plate regions of stage 14–20 *Xenopus* embryos and a cDNA library was constructed according to the procedure described in the Lambda Zap II Library Kit (Stratagene). Whole mount in situ hybridization screening was performed to isolate neural region- and pronephros-specific genes. In vivo excision of the pBluescript SK-*dullard* phagemid from the lambda zap II vector was performed according to the protocol.

Plasmid construction. Dullard was cloned into the *Stu*I site of the pCS2+ vector (pCS2+-*dullard*). To construct d5n-*dullard*, the ORF sequence of *dullard* was amplified by PCR using 5′-TGCAGGATCC ATGATGcGgACaCCtGGtCTCCT-3′ (small letters indicate modifications) and 5′-CTTAGAGCGTCCAACATCGG-3′ primers. The PCR product was then digested with *Bam*HI and *Bgl*II and cloned into the equivalent sites of the pCS2+-*dullard* construct.

RT-PCR. Total RNA was extracted from *Xenopus* embryos. First-strand cDNA was synthesized using oligo-(dT) primer from 1 μg total RNA. One-tenth of the cDNA was used as a template for RT-PCR. The primers used were: *dullard* forward; 5′-AACCGCCTCAGTCA GGTGAAAC-3′ and *dullard* reverse; 5′-CCCTCCCCATAAAGT GTCCAAG-3′. ODC primers were as described previously [24].

Whole mount in situ hybridization. Whole mount in situ hybridization was performed according to [25]. Antisense digoxigenin-labeled RNA probes were generated by in vitro transcription (MEGASCRIPT Kit; Ambion). For injected embryos, β-galactosidase staining was performed. The β-galactosidase mRNA-injected embryos were fixed in MEMFA (0.1 M MOPS, 2 mM EGTA, 1 mM $MgSO_4$, and 3.7% formaldehyde, pH = 7.4) for 30 min at room temperature, assayed for β-galactosidase activity by Red-Gal (Research Organics) substrates, and refixed in MEMFA for 1 h at room temperature and whole mount in situ hybridization was performed.

Microinjection and histological examination. Eggs were fertilized in vitro, dejellied with 1% sodium thioglycolate solution, and microinjection was performed in Steinberg's solution containing 5% Ficoll. Capped RNAs were synthesized by in vitro transcription (mMESSAGE mMACHINE Kit; Ambion). For sectioning, injected embryos were fixed in MEMFA for 3 h, dehydrated through a graded series of ethanol, transferred to xylene, embedded in paraffin, and sectioned at 8 μm.

Antisense morpholino oligonucleotide. Antisense *dullard* morpholino oligonucleotide (*dullard* Mo: 5′-CGAGGAGCCCTGGAGTCCGCA TCAT-3′) was designed to block translation of *dullard* mRNA (Gene Tools, LLC).

Results and discussion

Isolation and sequence analysis of the novel gene, dullard

A cDNA library was made from lateral regions of *Xenopus laevis* stage 14–20 embryos. Whole mount in situ hybridization screening was performed and a neural region- and pronephros-specific novel gene termed *dullard* (after the phenotype of *dullard* Mo-injected embryos) was isolated (GenBank Accession No. AB084264). The ORF was 732 bp with the predicted protein containing 244 amino acids. Sequence analysis of the full-length cDNA showed *dullard* has no functionally known obvious motif nor domain. A BLASTP search performed with the *dullard* amino acid sequence revealed the existence of homologs from nematode (F45E12.1 gene product), fly (CG1696 gene product),

Fig. 1. Amino acid sequence alignments of *dullard* with related proteins obtained from GenBank. *Dullard* shares 92% sequence identity with both a human protein and a mouse protein, 68% sequence identity with a fly protein, and 62% sequence identity with a nematode protein. *Dullard* also shares 64% sequence identity with the C-terminal conserved domain of NLI-IF at the C-terminal region (amino acids 64–236).

mouse (unknown), and human (hypothetical protein) (Fig. 1). This high degree of amino acid conservation suggests that these proteins may have an essential function. The protein with known function displaying the highest sequence homology with *dullard* was the Nuclear LIM Interactor-Interacting Factor (NLI-IF) from *Gallus gallus*. *Dullard* and NLI-IF have a conserved C-terminal region (Fig. 1), which suggests that the C-terminal conserved domain is responsible for a highly conserved cellular function. But the function of this conserved domain could not be predicted as functional analysis of NLI-IF is yet to be reported.

Temporal and spatial distribution of dullard

The temporal expression patterns of *dullard* during *Xenopus* embryogenesis were analyzed by RT-PCR. Similar levels of *dullard* transcripts were observed from eggs through to early *Xenopus* development (Fig. 2A). Whole mount in situ hybridization analysis revealed the localization of *dullard* transcripts. *Dullard* mRNA was localized to the animal hemisphere of the embryo before the gastrula stage. The expression of *dullard* became restricted to the neural region as gastrulation proceeded and subsequently localized to the neural tissues, branchial arches, and pronephroi at the tail-bud stages (Figs. 2B–I).

Overexpression of dullard leads to apoptosis in early Xenopus development

To investigate the function of *dullard* in *Xenopus* development, we first examined effects of overexpression. Injection of ectopic *dullard* mRNA resulted in the injected cells being sorted out from the inner of embryos and the injected embryos showing white mottled aspects at the gastrula stages, which is characteristic of apoptosis in *Xenopus*. This effect was rescued by co-injection of human *Bcl-2* (Figs. 2J and K). Furthermore, TUNEL staining was performed and TUNEL positive cells were observed in *dullard* mRNA injected embryos (data not shown). These data confirmed that overexpression of *dullard* led to apoptosis. In *Xenopus laevis*, the apoptotic program is known to be under tight developmental control and widespread apoptosis at stage 10.5 is observed in embryos treated with agents before the mid-blastula transition (MBT) [26,27]. We also examined microinjection of *dullard* mRNA at levels low enough such that no cells were sorted out from the inner of embryos (less than 70 pg), but no obvious phenotype was observed.

As *dullard* has the C-terminal conserved domain of NLI-IF, we investigated the relationship between *dullard*, *Xldb1* (LIM-domain-binding protein 1), and *Xlim1*. Co-injection of *Xlim1* and *Xldb1* in the ventral side of embryos is known to induce a partial secondary axis in *Xenopus* [28]. *Dullard* was co-injected with *Xlim1* and *Xldb1*, but no obvious change of their ability was observed (data not shown). So we next examined a loss-of-function experiment.

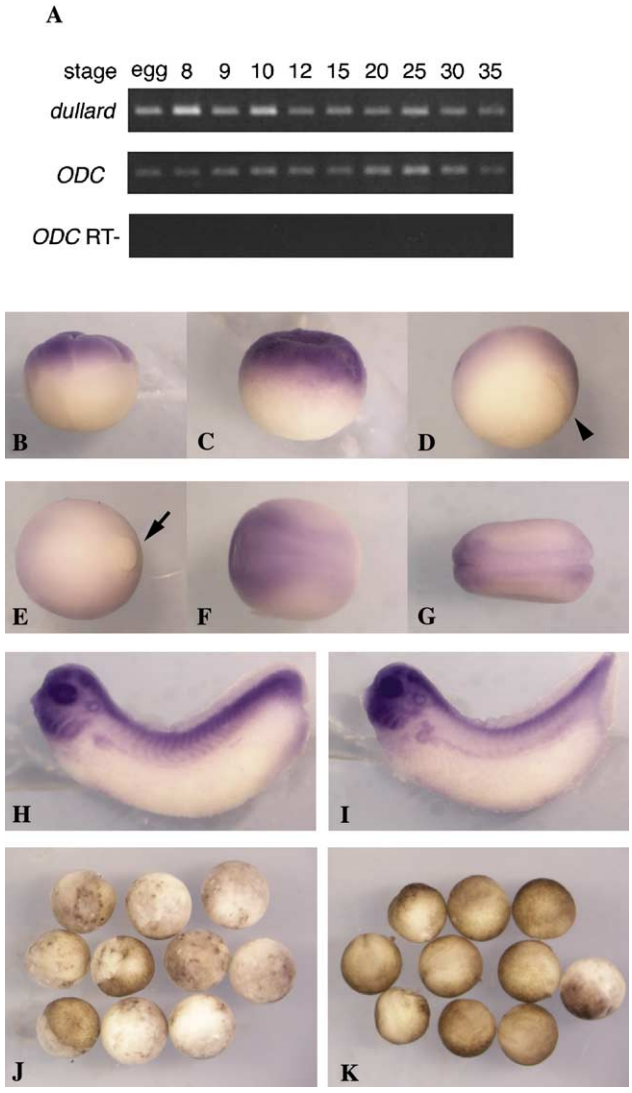

Fig. 2. (A–I) Temporal and spatial expression patterns of *dullard*. (A) RT-PCR analysis was performed at the various stages indicated above the lanes. From egg to stage 35, similar levels of transcript were present. (B–I) Whole mount in situ hybridization analysis of *dullard* expression. (B–D) The transcripts localized to the animal hemisphere of the embryo before the gastrula stage. (B) Eight-cell stage, lateral view. (C) Stage 8, lateral view. (D) Stage 10.5, ventro-lateral view. (E–G) As gastrulation proceeded, expression became restricted to the neural region. (E) Stage 12, lateral view. (F) Stage 15, dorsal view. (G) Stage 20, dorsal view. (H, I) Expression subsequently localized to the neural tissues, branchial arches, and pronephroi at the tail-bud stages. (H) Stage 28, lateral view. (I) Stage 33, lateral view. Animal pole is towards the top (B–D) and anterior is to the left (E–I). The arrowhead in (D) and arrow in (E) indicate the dorsal lip and yolk plug, respectively. (J, K) Overexpression of *dullard* caused apoptosis. (J) *Dullard* mRNA (100 pg) was injected in one blastomere of 2-cell embryos. White mottled aspects were observed (95%, $n = 40$). (K) Embryonic death caused by overexpression of *dullard* was rescued by co-injection of h*Bcl-2* mRNA (200 pg) (5% of embryos exhibited white mottled aspects, $n = 40$).

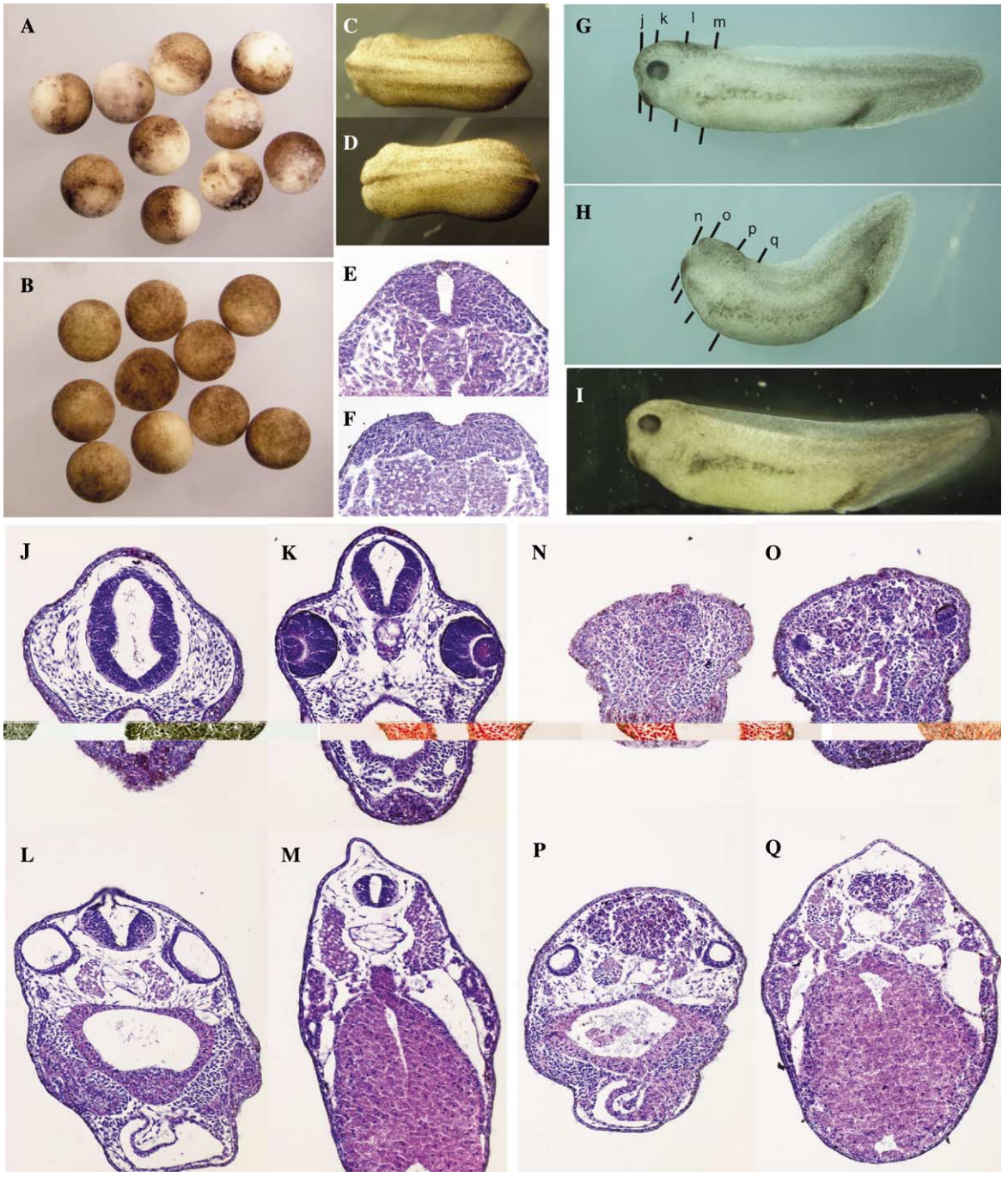

Fig. 3. Antisense *dullard* morpholino oligonucleotide (*dullard* Mo) blocked *dullard* mRNA function and injection of the *dullard* Mo caused failure of neural tube closure and disorganization of neural tissue. (A) *Dullard* mRNA (200 pg), injected embryos show white mottled aspects, characteristic of apoptosis. (B) This effect was blocked by co-injection of *dullard* Mo (40 ng). (C–Q) *Dullard* Mo (40 ng/embryo) was injected in dorsal animal blastomeres of 8-cell stage embryos and the embryos raised until stage 24 or 35. (C) Control embryo at stage 24. (D) *Dullard* Mo (40 ng), injected embryo at stage 24 shows failure of neural tube closure. (E, F) Transverse sections of stage 24 control and *dullard* Mo-injected embryos, respectively. In the *dullard* Mo-injected embryo, the neural tube fails to close while other structures are normal. (G) Control embryo at stage 35. (H) *Dullard* Mo (40 ng), injected embryo at stage 35 shows a reduction of head development and poor axis formation. (I) These effects were rescued by co-injection of d5n-*dullard* mRNA. (J–Q) Transverse sections of the control (J–M) or *dullard* Mo-injected (N–Q) embryo at stage 35 at the sites indicated with small letters on the whole embryos. The transverse section through the forebrain of a *dullard* Mo-injected embryo compared to that of a control embryo shows the absence of head structures (J, N). The transverse section through the midbrain of a *dullard* Mo-injected embryo compared to that of a control embryo shows no or poor eye formation and disorganization of the midbrain (K, O). The transverse sections through the hindbrain and spinal cord of the *dullard* Mo-injected embryo compared to that of a control embryo show disorganization of brain and neural tube, while other structures around them appear unaffected (L, M, P, Q).

Table 1
Effects on head development of embryos injected with *dullard* Mo and embryos co-injected with *dullard* Mo and d5n-*dullard* mRNA

Head development	*Dullard* Mo ($n = 67$) (%)	*Dullard* Mo + d5n mRNA ($n = 67$) (%)	Control morpholino ($n = 45$) (%)	Control (uninjected) ($n = 77$) (%)
Normal	4	48	96	97
+	43	42	4	3
++	43	9	0	0
+++	10	2	0	0

+: Reduction of forehead; ++: severe defect of head development as shown in Fig. 3F; +++: almost no head.

Translational inhibition of dullard causes failure of neural development

The microinjection assay of *dullard* mRNA did not provide sufficient information to elucidate the function of *dullard* in *Xenopus* development. Next we performed a loss-of-function experiment by using an antisense morpholino oligonucleotide. This approach depends on blocking translation of the *dullard* mRNA by the antisense morpholino oligonucleotide [29,30]. When the antisense *dullard* morpholino oligonucleotide (*dullard* Mo) was co-injected with *dullard* mRNA, apoptosis caused by overexpression of *dullard* mRNA was prevented (Figs. 3A and B). This result confirmed the efficiency of inhibition mediated by *dullard* Mo.

Dullard Mo-injected embryos showed severe defects in neural development. Embryos at the neurula stage typically failed in neural fold fusion (Fig. 3D) and consequently showed a reduction of head development (Fig. 3H, Table 1). The transverse section of a stage 24

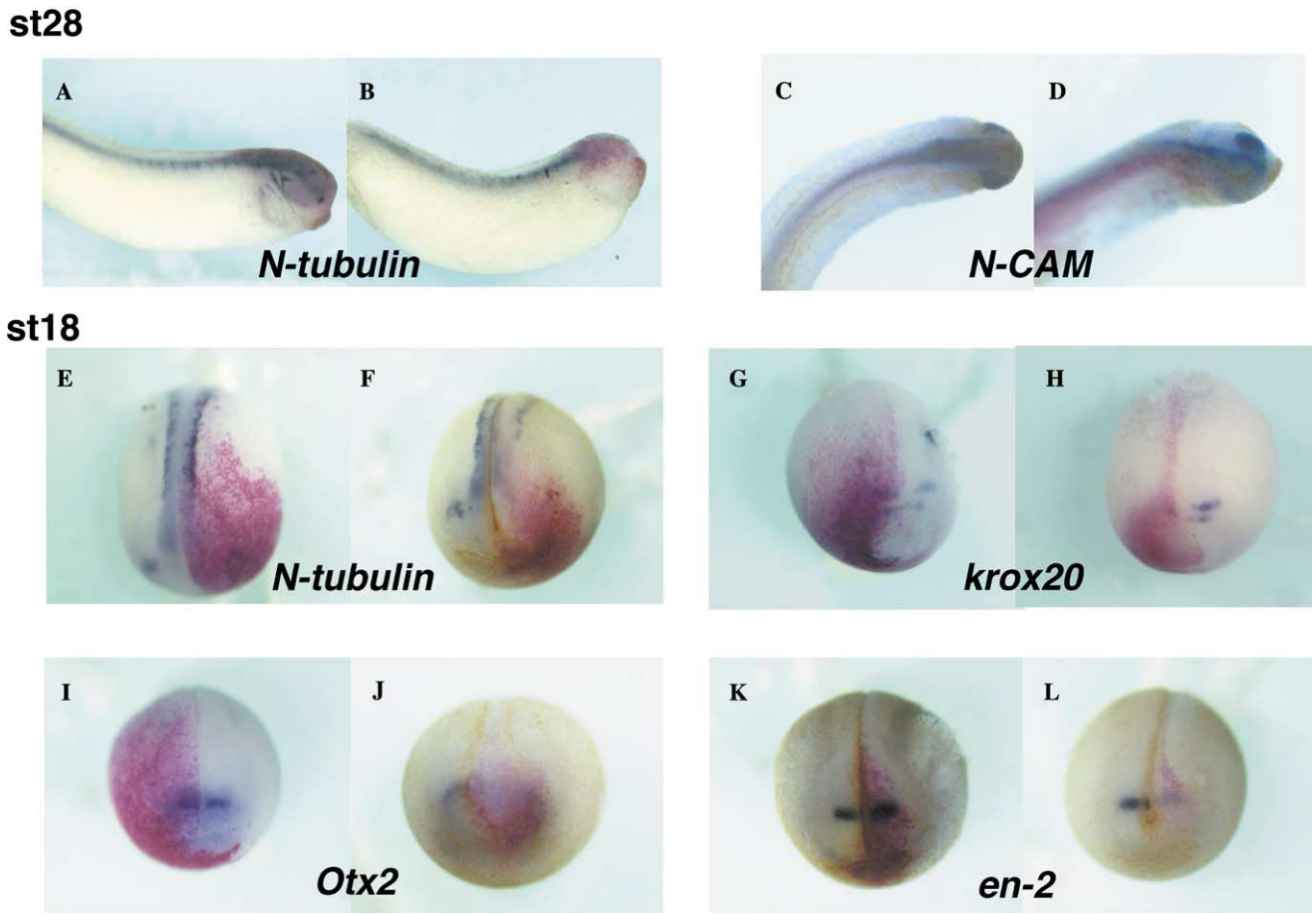

Fig. 4. Microinjection of *dullard* Mo suppressed expression of neural markers. Control morpholino oligonucleotide (A, C, E, G, I, K) or *dullard* Mo (B, D, F, H, J, L) (40 ng) together with lacZ mRNA (200 pg) as a tracer was injected into a dorsal animal blastomere of 8-cell embryos. Embryos were harvested at the early tail-bud stage (A–D) or the neurula stage (remaining panels) and analyzed by whole mount in situ hybridization with the indicated probes. In the *dullard* Mo-injected side of the embryo, expression of these markers is downregulated (B, D, F, H, J, L), while no change is observed in the control morpholino oligonucleotide-injected embryos (A, C, E, G, I, K). (A and B) lateral view, (C–F) dorsal view, (G–L) anterior view.

embryo clearly shows that neural tube closure was defective, while notochord, somites, and other structures developed normally (Fig. 3F). The effect of *dullard* Mo in later neural development was also analyzed. The transverse sections of the stage 35 embryo through the head regions showed complete failure of forehead development, no or poor eye formation, and disorganization of midbrain, hindbrain, and spinal cord. Development of other tissues such as notochord and otic vesicles was not inhibited (Figs. 3N–Q). These defects could be rescued by co-injection of *dullard* mRNA (70 pg), where 5-base pairs within the *dullard* Mo target sequence have been modified (d5n-*dullard*) so that translation can no longer be blocked by *dullard* Mo (Fig. 3I, Table 1). This suggests that the defects were caused by *dullard* Mo-specific blocking of *dullard* mRNA.

The observed phenotypes induced by injection of *dullard* Mo suggest that *dullard* Mo interferes with neural developmental processes. To analyze these effects in more detail, whole mount in situ hybridization analysis for several neural marker genes was performed as described in Fig. 4. We observed that the expression of neuronal marker *N-tubulin* (73%, $n = 15$) [31] and pan-neural marker *N-CAM* (96%, $n = 25$) [32] was suppressed in the *dullard* Mo-injected side of embryos at stage 28 (Figs. 4A–D). To determine whether the suppression was due to the secondary effect of failure of neural tube closure, the expression of neural marker genes in neurula was also examined. The expression of *N-tubulin* (100%, $n = 13$), forebrain and midbrain marker *otx-2* (95%, $n = 44$) [33,34], midbrain/hindbrain marker *en-2* (79%, $n = 24$) [35], and presumptive rhombomere 3 and 5 marker *krox20* (94%, $n = 35$) [36] was suppressed in the *dullard* Mo-injected side of embryos but not in control morpholino oligonucleotide-injected embryos (Figs. 4E–L). These results indicate that *dullard* is necessary for the induction of these neural markers and neural differentiation.

In this report, we isolated and characterized the novel gene *dullard* which was expressed in neural tissues and pronephroi in early *Xenopus* development. *Dullard* has the conserved C-terminal domain of NLI-IF, but contains no functionally known motif nor domain. *Dullard* shares 92% sequence identity with both the human and mouse homologs. This high degree of conservation may indicate the basic and functional importance of this protein. The result of the *dullard*-misexpression experiment might suggest that the fine balance of *dullard* in *Xenopus* embryos regulates a basic cellular behavior for survival. However in *Xenopus*, agents that cause cellular damage or inhibit normal cell cycle progression (such as transcription inhibitors, translation inhibitors, and mitosis blocking agents) applied to embryos before the MBT lead to widespread apoptosis at stage 10.5 [26,27,37]. So whether *dullard* is a direct effector of apoptosis or causes apoptosis indirectly due to cell damage is not clear.

We investigated *dullard* function by translational knock-down using an antisense morpholino oligonucleotide. Inhibition of *dullard* caused failure of neural tube closure and a reduction of head development and the expression of neural marker genes was suppressed at the tail-bud stage and also at the neurula stage. The defect in early neural differentiation suggests that the loss of neural differentiation at the later stage is not caused by the failure of neural tube closure. Taken together the expression pattern of *dullard* (*dullard* transcripts are maternally observed and localize through the entire neural tissue), loss of *dullard* function may affect early neural developmental events before neurulation and consequently cause loss of ability to respond to signals for neural patterning. In this work, we show that *dullard* is a novel factor required for neural developmental events. Although the mechanism has not been elucidated and further analysis is necessary, *dullard* expression throughout early development might suggest the multi-functional importance of *dullard* in *Xenopus* neural development.

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