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FGFR-related gene *nou-darake* restricts brain tissues to the head region of planarians

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The study of planarian regeneration may help us to understand how we can rebuild organs and tissues after injury, disease or ageing[1]. The robust regenerative abilities of planarians are based upon a population of totipotent stem cells (neoblasts)[2–4], and among the organs regenerated by these animals is a well-organized central nervous system[5,6]. In recent years, methodologies such as whole-mount *in situ* hybridizations and double-stranded RNA have been extended to planarians with the aim of unravelling the molecular basis of their regenerative capacities[7–11]. Here we report the identification and characterization of *nou-darake* (*ndk*), a gene encoding a fibroblast growth factor receptor (FGFR)-like molecule specifically expressed in the head region of the planarian *Dugesia japonica*. Loss of function of *ndk* by RNA interference results in the induction of ectopic brain tissues throughout the body. This ectopic brain formation was suppressed by inhibition of two planarian FGFR homologues (*FGFR1* and *FGFR2*). Additionally, *ndk* inhibits FGF signalling in *Xenopus* embryos. The data suggest that *ndk* may modulate FGF signalling in stem cells to restrict brain tissues to the head region of planarians.

Planarians have a well-organized and molecularly complex[12,13] central nervous system (CNS), which, in *D. japonica*, consists of two lobes that connect at their most anterior ends to form an inverted U-shaped brain. Each lobe is connected to a ventral nerve cord that traverses the animal along its anterior–posterior axis, and 9 lateral branches project away from each lobe towards the periphery of the head[5]. In order to identify and characterize molecules involved in the process of brain regeneration in planarians, we prepared microarrays containing 1,640 non-redundant transcripts derived from a head complementary DNA library (M.N. and K.M., unpublished results). Here we present the characterization of one such gene, which we have named "*nou-darake*" ("brains everywhere", in Japanese); the phenotype was observed in specimens of *D. japonica* that had been injected with double-stranded RNA (dsRNA).

The gene *ndk* is highly and specifically expressed in the head in both brain and non-brain tissues (Fig. 1a). During regeneration, *ndk* expression is first detected 24 h after amputation only in anterior blastemal cells, including the new brain primordium[6]. Sequence analyses reveal that *ndk* codes for a putative transmembrane protein with two extracellular immunoglobulin-like domains related to FGF receptors (Figs 1b and 2), but significantly divergent from the IgG domains found in two planarian FGF-receptor homologues recently isolated[14]. But NDK lacks the cytoplasmic kinase domains characteristic of this receptor family (Fig. 1b). Instead, the cytoplasmic domain of NDK is short, rich in serine residues, and has no significant homology to known sequences. When the entire deduced amino-acid sequence of *ndk* is considered, the highest similarity is found to the human *FGF receptor-like 1* (*FGFRL1*) gene[15].

We analysed the function of *ndk* in planarians by RNA interference (RNAi)[10,16]. Animals were injected with *ndk* dsRNA and subsequently amputated (see Methods). Silencing of *ndk* expression in dsRNA-injected animals was confirmed by whole-mount *in situ* hybridizations (Fig. 3a–d). Seven days after amputation, ectopic eyes began to differentiate in dorso-posterior regions of the body. After 15 d of regeneration, 94% of the injected animals (108/115) formed ectopic eyes (Fig. 3h), consisting of both pigment (Fig. 3h, arrows) and visual cells with projecting axons (Fig. 3i, j, arrowheads). In all of the injected animals (115/115), we also found ectopic brain tissues differentiating in posterior regions of the planarian body (Fig. 3j, l, n, p; arrows). Ectopic brain cells express specific genes of both lateral branches (Fig. 3n) and the central region (Fig. 3p) of the brain. In trunk pieces allowed to regenerate

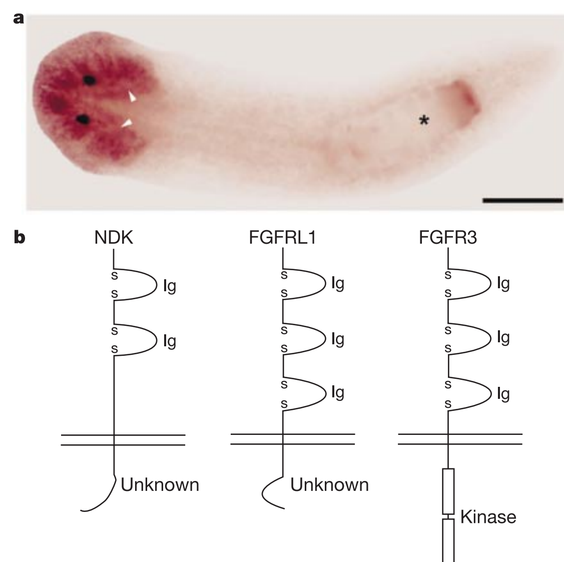

Figure 1 Expression pattern of *ndk* and predicted structure. **a**, *ndk* is expressed in the head region in both the inverted U-shaped brain (arrowheads) and non-brain cells. Signal detected in the pharynx (asterisk) is probably a non-specific signal due to trapped probe. Anterior to the left. Scale bar, 1 mm. **b**, Two extracellular IgG domains are predicted in NDK.

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bipolarly, we observed both a posterior expansion of the regenerated brain tissues, and the *de novo* appearance of brain cells in areas near or within the posterior blastema (Fig. 3l, n).

In animals injected with *ndk* dsRNA, the early stages of regeneration are normal and new brain tissues are restricted to the blastema; at day 4–5 of regeneration, however, brain-like structures differentiate outside the blastema, and as regeneration proceeds the ectopic brain gradually expands into more posterior regions (Supplementary Fig. 1). The new pharynx appears normally in the new central region in regenerating head and tail fragments, suggesting that the ectopic appearance of brain cells in posterior regions does not disturb the relative proportions of the planarian body (Supplementary Fig. 1). Differentiation of ectopic brain tissues and extra eyes were also observed in intact, non-regenerating animals after injection of *ndk* dsRNA (Fig. 4b, d, f). To test whether only the brain or the whole anterior region is expanded, we analysed the expression of clone 0821_HN (unknown gene), which is specifically expressed in the head periphery (Fig. 4e). After *ndk* dsRNA injection, the expression pattern of this gene is not changed (Fig. 4f), suggesting that the head region is not expanded. These results suggest that ectopic brain formation can occur without disturbing head–trunk identities. Planarian neoblasts can be specifically eliminated by X-ray irradiation, resulting in the death of the animals after a few weeks[11,14]. In irradiated animals that had been injected with *ndk* dsRNA, ectopic brain formation was completely absent or significantly reduced (data not shown), clearly implicating neoblasts in ectopic brain formation.

Because of the structural similarities between NDK and FGFRs, we wondered whether *ndk* might be modulating a putative planarian FGF pathway. The recent identification of two FGFR homologues in *D. japonica* and their expression in both brain and neoblasts indicate that FGF-like signalling pathways may be operating in planarians[14]. The function of these genes, however, remains unknown, as no detectable abnormality was found after RNAi experiments[14]. In double *ndk/FGFR1* and *ndk/FGFR2* dsRNA-injected animals, ectopic brain tissues could be detected, although at a level slightly lower when compared to *ndk*-dsRNA-injected

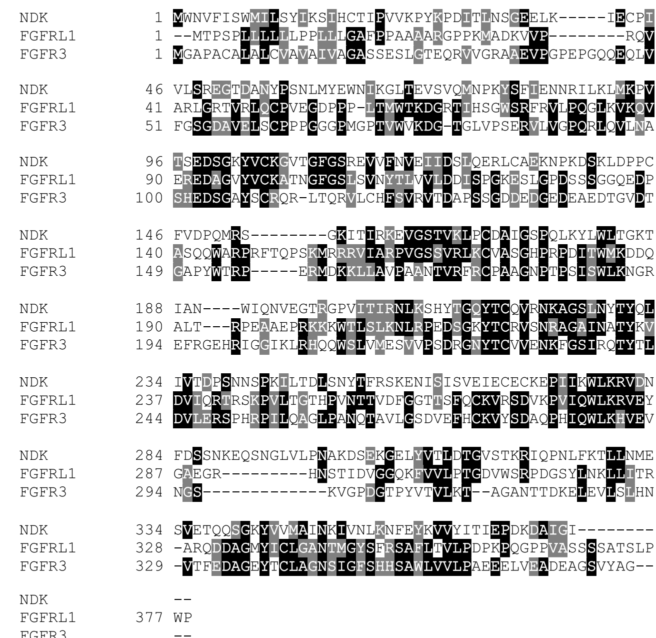

Figure 2 Sequence comparison between the extracellular domains of NDK and the proteins encoded by human *FGFRL1* and *FGFR3* genes. Identical or similar amino-acid residues are indicated in black or grey, respectively.

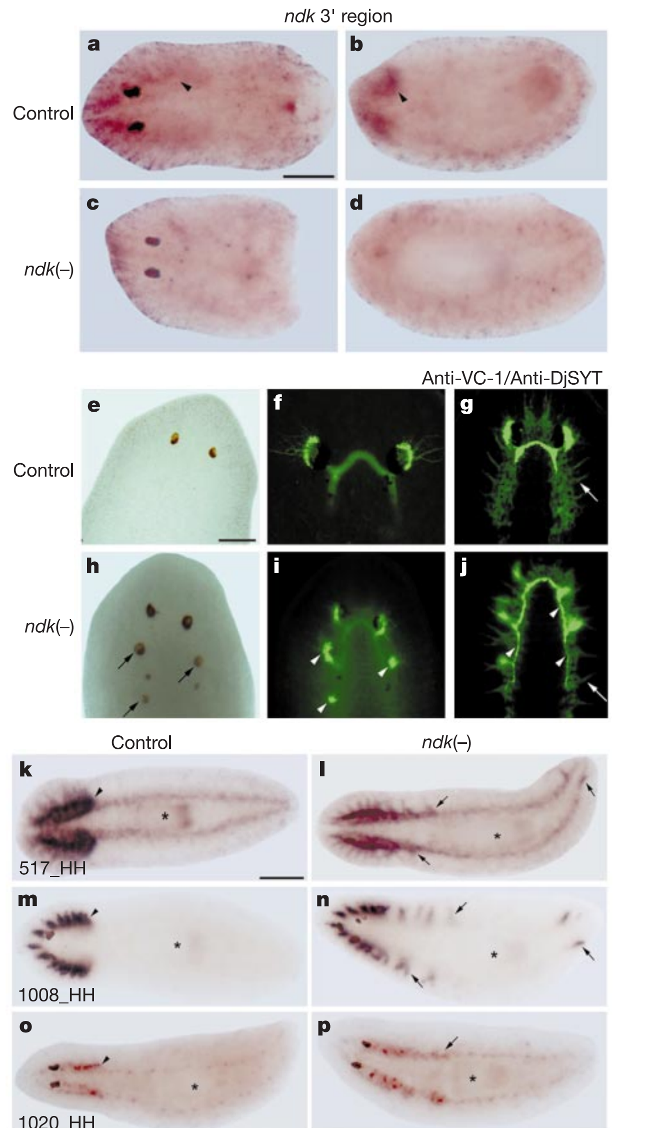

Figure 3 Effects of *ndk*-dsRNA injections during regeneration. **a**–**d**, Such injections silence the *ndk* endogenous expression. In control animals, *ndk* is expressed in the brain (arrowheads) of both head (**a**) and trunk (**b**) pieces after 3 days of regeneration. No expression, in contrast, is detected in *ndk*-dsRNA-injected head (**c**) and trunk (**d**) regenerants. **e**–**j**, Ectopic eyes appear in injected animals. Bright field view of control (**e**) and *ndk*-dsRNA-injected animals (**h**) after 16 days of regeneration. In dsRNA-injected animals, ectopic eyes differentiate in posterior regions. (**h**, arrows). Double whole-mount immunostaining with anti-DjSYT[27] and anti-VC-1 (ref. 28) antibodies on control (**f**, **g**) and injected animals (**i**, **j**). Ectopic visual cells detected by anti-VC-1 are dorsally located (arrowheads in **i**), and project visual axons to more ventral positions (arrowheads in **j**). Animals injected with *ndk*-dsRNA also show an abnormal regenerated brain after immunostaining with anti-DjSYT. In control animals, a normal brain is regenerated (**g**, pale green; arrow points to lateral branches). In injected animals, brain-like tissues appear more posterior (arrow in **j**). Ectopic brain is detected for different neural-specific markers in animals that have been regenerating for 15 days. In control animals, trunk pieces regenerate a normal brain, as shown after *in situ* hybridization for 517HH (**k**, protein tyrosine phosphatase receptor homologue and pan-neurally expressed), 1008HH (**m**, glutamate receptor homologue and expressed in brain lateral branches), and 1020HH (**o**, unknown and expressed in the central spongy region of the brain). Arrowheads in **k**, **m** and **o** point to the posterior end of the regenerated brain. In *ndk*-dsRNA-injected animals, unambiguous brain expansion is detected for the same 3 neural markers (**l**, **n**, **p**, respectively). Arrows in **l**, **n** and **p** point to ectopic brain structures. In **k**–**p**, asterisks mark the pharynx. In **a**–**d** and **k**–**p**, anterior, to the left; in **e**–**j**, anterior is to the top. Scale bars: **a**–**d**, 0.3 mm; **e** and **h**, 0.4 mm; and **k**–**p**, 1 mm.

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animals (Fig. 5). In triple-injected animals, however, no ectopic brain tissues were detected in posterior body regions in any of the regenerating trunk and tail fragments (17/17) (Fig. 5h). In 5/5 of triple-injected regenerating head fragments, some level of ectopic brain tissues could be detected (Fig. 5g). These results suggest that *ndk* negatively regulates the FGFR signalling in the trunk, which are required for the ectopic brain formation.

Because it is not yet possible to perform gain-of-function analyses in planarians, we carried out *ndk* messenger RNA injection experiments in *Xenopus* embryos in order to test whether or not *ndk* could modulate FGF signalling pathways, as well as to analyse the degree of functional conservation on FGF signalling. Injection of *ndk* mRNA in dorsal blastomeres arrested gastrulation movements, leading to open yolk plug phenotypes (data not shown) that are indistinguishable from those obtained by the introduction of the dominant-negative FGF receptor (XFD)[17]. Examination of the expression pattern of the early mesoderm marker *brachyury* (*Xenopus bra*) uncovered a suppression of *bra* expression (blue) by NDK as well as XFD in the region where the nuclear β-galactosidase lineage tracer is present (visualized in red; Fig. 6a). We also found that the effects of *ndk* are still observed when both its intracellular and transmembrane regions were deleted (Fig. 6a), indicating that the extracellular domain of NDK retains the inhibitory activity against the *bra* expression[18].

As the structural features of NDK suggest that this protein may be interacting with FGF signalling, and FGF is known to induce *bra* expression in *Xenopus* animal cap explants[19], we next examined whether NDK could inhibit *bra* expression by FGF. Basic FGF (bFGF) treatment induces *bra* expression in animal caps, and this expression is inhibited by *ndk* in a fashion that is similar to XFD[20] (Fig. 6b). In addition, we have so far been unable to co-immuno-precipitate Flag-tagged eFGF after expression of myc-tagged NDK. These results do not exclude the possibility that NDK might modulate FGF signalling by a regulatory mechanism involving the interaction of signalling receptors with a pseudoreceptor, in a similar fashion to the inhibition of TGF-β signalling by the pseudoreceptor BAMBI[21]. Recently, an inhibitory molecule of FGF signalling, named Sef, has been identified[22,23]. Whereas its intracellular domain is required for inhibiting FGF signalling, NDK can inhibit FGF signalling when lacking its intracellular domain (Fig. 6a). Thus, *ndk* may be a new modulator of the FGF signalling pathway.

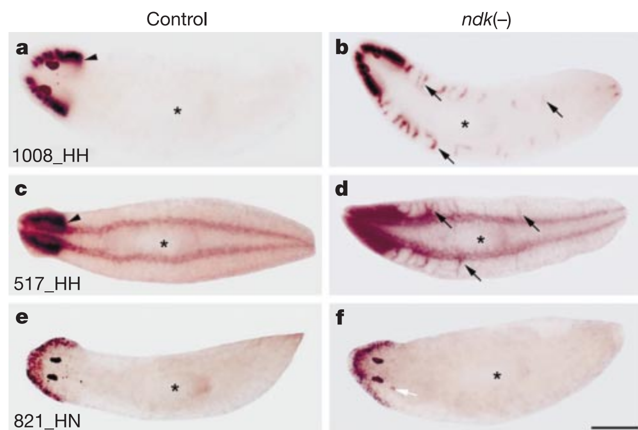

Figure 4 Brain expansion in intact animals. Ten days post-injection, ectopic brain structures (arrows in **b**, **d**) are detected by *in situ* hybridization with clones 1008_HH (**b**) and 517_HH (**d**). Arrowheads in **a**–**c** indicate the posterior border of the brain in control animals. Clone 821_HN is expressed in the head periphery of intact and control animals (**e**). This head marker is not expanded posteriorly in *ndk*-dsRNA-injected animals (**f**), even though they show ectopic eyes (white arrow in **f**), which suggest that brain expansion has taken place in them. In all figures, anterior is to the left. Scale bar, 1 mm.

How could the silencing of a gene specifically expressed in the planarian head lead to the differentiation of brain-like tissues throughout the body in a non-autonomous cell manner? Sequence analyses, *in situ* hybridization data, RNAi experiments and mRNA injections in *Xenopus* show that NDK has potential FGF binding domains (Fig. 2), is expressed in the head of planarians (Fig. 1a), restricts brain differentiation to the planarian head region (Figs 3 and 4), and is capable of inhibiting FGF signalling (Fig. 6). Then, one simple model would be that NDK may restrict the diffusion range from a putative source of brain-inducing factors (FGF or FGF-like molecules) in the planarian head to the rest of the body. Loss of function of *ndk*, therefore, would allow these factors to diffuse to posterior regions, and interact with FGF receptors outside of the head region, thus triggering ectopic brain formation. Our observation of a gradual brain expansion to more posterior regions in dsRNA-injected animals supports this idea. As these hypothetical brain-inducing factors must diffuse distances of several millimetres between the planarian head and the posterior regions where the ectopic brain is formed, diffusion rates as well as the role of extracellular matrix components[24] during this process should be analysed.

Even though we have yet to identify FGF-like ligands in planarians, *ndk* provides strong molecular evidence for the existence of a brain-inducing circuit based on the FGF signalling pathway. Whereas antagonists of BMP4 are believed to be the main neural inducers in vertebrates, recent work has suggested that FGFs are important in neural formation and patterning. For instance, BMP antagonists do not induce neural tissues in the presence of dominant-negative FGF receptors in *Xenopus*[25]. In addition, studies in chicken have shown that neural induction by BMP antagonists requires FGF signalling[26], suggesting that FGFs, as in planarians, may be essential for neural tissue formation in the vertebrates. The

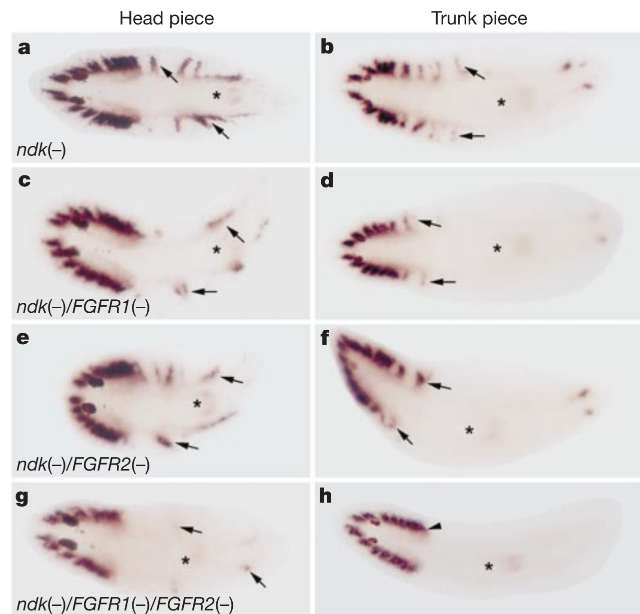

Figure 5 Effects of *ndk*- and *FGFR*-dsRNA injections on regenerating head (**a**, **c**, **e**, **g**) and trunk (**b**, **d**, **f**, **h**) fragments. In *ndk/FGFR1* (**c**, **d**) and *ndk/FGFR2* (**e**, **f**) double dsRNA-injected samples ectopic brain tissues appear in posterior regions, although the levels of ectopic brain in the trunks appear slightly lower when compared to *ndk*-dsRNA-injected animals (**b**). In *ndk/FGFR1/FGFR2* (**g**, **h**) triple dsRNA-injected animals, no ectopic brain tissues are detected in regenerating trunks (**h**), but low level of brain expansion occurs in regenerating heads (**g**). All samples correspond to 15-day regenerants. Arrows indicate ectopic brain tissues. Arrowhead in **h** indicates the posterior border of the brain. Asterisks mark the pharynx. Anterior to the left.

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fact that planarian *ndk* can functionally inhibit *Xenopus bra* activation during *Xenopus* gastrulation raises the possibility that the vertebrate homologue of *ndk* may be modulating FGF signalling. Further studies will be required to understand the extent of the involvement of *ndk* in vertebrate organogenesis—in particular, neurogenesis. □

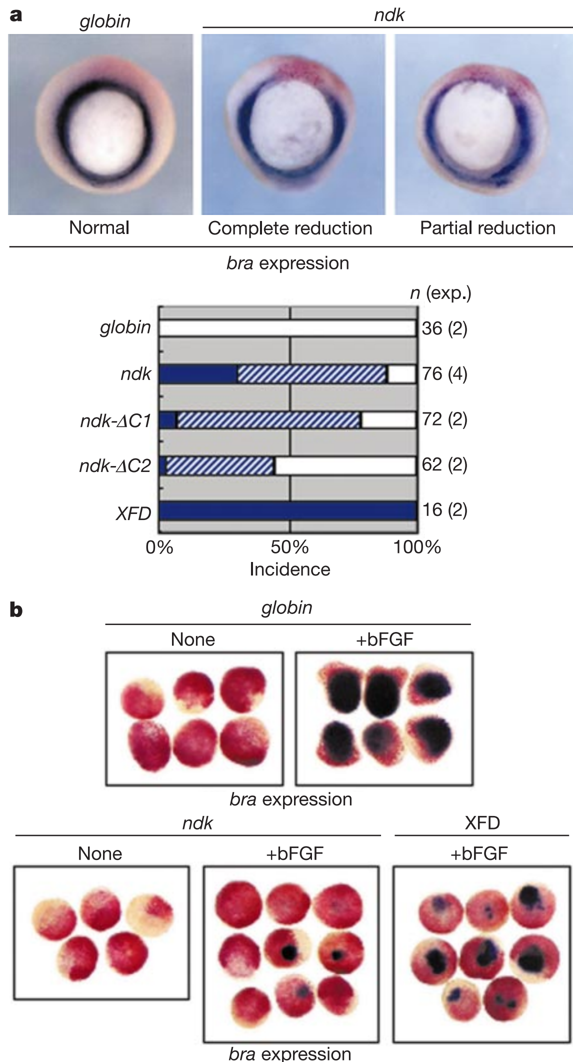

Figure 6 Effects of *ndk* mRNA injections into *Xenopus* embryos. **a**, *ndk* inhibits *Xenopus bra* expression in whole embryos. Embryos were injected with mRNA for *ndk* and nuclear β-galactlsidase (nβ-gal) as a tracer into the dorsal marginal zone at the 4-cell stage, fixed at the gastrula stage (stage 11), stained with Red-gal as a substrate for nβ-gal, and analysed by whole-mount *in situ* hybridization for *bra*. Levels of *bra* expression in the region where nβ-gal was stained in red are categorized into normal expression (open bar) and complete (filled bar) or partial (striped bar) reduction as indicated in the middle three bars. *n*, number of embryos analysed; (exp.), number of experiments. Note that deletion of both intracellular domain (*ndk-ΔC1*) and transmembrane domain (*ndk-ΔC2*) reduces the activity. Amounts of mRNA (pg per embryo): *globin* (negative control) and *ndk* constructs, 500–1,000. **b**, *ndk* inhibits *bra* expression in animal caps treated with bFGF. Embryos were injected with mRNA for *globin*, *ndk*, or XFD into the animal pole region at the 2-cell stage. Animal caps were dissected at the blastula stage (stages 8.5 to 9), treated with 100 ng ml^{-1} bFGF until the sibling embryos reached stage 11, and assayed for nβ-gal staining and *bra* expression by whole-mount *in situ* hybridization. Only animal caps stained in red by nβ-gal were scored. Amounts of mRNA (pg per embryo): *globin* (negative control) and *ndk*, 1,000; XFD, 200.

Methods

Organisms

We used a clonal strain of the planarian, *D. japonica*, obtained from the Irima river, Gifu prefecture, Japan, established in Watanabe's laboratory in the Himeji Institute of Technology. Intact animals were cultured in autoclaved tap water at 21 °C, and they were starved for 2 weeks before being used. For all experiments planarians 4–7 mm in length were used.

Whole-mount *in situ* hybridization

Planarians that had been starved for two weeks were used. Animals were treated with 2% HCl for 5 min at 4 °C and then fixed in Carnoy's solution (ethanol:chlorform:acetic acid at 6:3:1, respectively) for 2 h at 4 °C. Hybridization was carried out using 20 ng ml^{-1} of digoxygenin (DIG)-labelled riboprobes, as previously described[5,12].

Synthesis of dsRNA

This was done basically as previously described[10,16]. pBluescriptII SK + containing a cDNA insert of 2,483 base pairs (bp) including a putative full-length open reading frame (ORF) of *ndk* was digested with *Sac*I or *Kpn*I to synthesize antisense (T7) or sense (T3) RNAs, respectively. After RNA synthesis, the samples were digested with DNaseI for 20 min at 37 °C; they were then pooled and extracted with phenol/chloroform. The RNAs were denatured for 20 min at 65 °C, and annealed for 40 min at 37 °C. After ethanol precipitation, dsRNA was resuspended in H_2O that had been treated with diethyl pyrocarbonate (DEPC). Electrophoretical mobilities of dsRNA and single-strand RNA were assessed in 1.5% agarose gels. For double and triple-gene injections, dsRNAs were individually synthesized and then mixed, precipitated with ethanol and resuspended in 10 μl DEPC-treated H_2O.

Analysis of endogenous expression of *ndk* after dsRNA injection

Clone 721HH containing *ndk* was digested with *Eco*RI to generate two non-overlapping fragments of about 1 kb (5′ region) and 1.4 kb (3′ region). The 1-kb fragment was recloned in *Eco*R1 site dephosphorylated pBluescript SK + and the 1.4-kb fragment was self-ligated. After subcloning, the 1-kb fragment was digested with *Sac*I or *Kpn*I to synthesize sense (T7) or antisense (T3) RNA, respectively for dsRNA synthesis. The 1.4-kb fragment was digested with *Sac*I to synthesize antisense (T7) for the DIG-RNA probe.

Microinjection and amputation

Intact planarians were injected with dsRNA three times (32 nl per injection) for three consecutive days using a Drummond Scientific (Broomall) Nanoject injector[10]. Control animals were injected with H_2O. Two–three hours after the last injections, animals were amputated at different levels along the antero–posterior axis using sterile surgical blades (Keisei Medical Industrial Co.). Three kinds of regenerating fragments were obtained after amputation: head fragments capable of regenerating new central and tail regions; trunk fragments able to regenerate new head and tail regions; and tail fragments capable of regenerating a new head and central regions. During the regeneration process, the samples were maintained at 21 °C.

Immunofluorescence

This was carried out as previously described[10]. Intact and regenerating planarians were treated with 2% HCl for 5 min at 4 °C and then fixed in Carnoy's solution for 3 h at 4 °C. The samples were rinsed in 100% methanol, and bleached overnight at room temperature in 6% H_2O_2 in methanol. After bleaching, the samples were rehydrated through a graded methanol series (75%, 50% and 25% in PBS-0.3% Triton X-100) for 15 min each, and rinsed in PBS containing 0.3% triton X-100 (PBST; Sigma). After blocking for 2 h in PBST containing 0.25% BSA (PBSTB; Sigma) samples were incubated in PBSTB at room temperature for 14–16 h with the following primary antibodies: a polyclonal antibody against planarian synaptotagmin (SYT)[27], and a monoclonal antibody against visual cells (VC-1)[28] diluted 1:2,000 and 1:10,000, respectively. After washing in PBSTB for 6–8 h with several changes of the medium, the samples were incubated in secondary anti-mouse Alexa 488 diluted 1:400 in PBSTB, overnight at room temperature. Finally, the samples were washed for several hours in PBST, mounted in DAKO fluorescent mounting medium and observed with a Olympus BX62 microscope.

X-ray irradiation

Intact planarians were irradiated using a Softex B-4 X-ray source operating at 18 kV, 5 mA as previously described[14]. Four days after irradiation, the animals were injected with *ndk* dsRNA as described above. Twelve days after injections, they were fixed for *in situ* hybridization experiments.

mRNA injection experiments with *Xenopus* embryos

Fertilization, manipulation and mRNA injection of *Xenopus* embryos were as previously described[29]. The entire or truncated coding region of *Dugesia ndk* was amplified by PCR and inserted into the *Cla*I and *Xba*I sites of pCS2 + to construct pCS2 + ndk, pCS2 + *ndk-ΔC1* (amino acids 1–408), and pCS2 + *ndk-ΔC2* (amino acids 1–362). Whole-mount *in situ* hybridization was performed with *Xenopus bra* probe[20] as previously described[30].

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1. Pearson, H. The regeneration gap. *Nature* **414,** 388–390 (2001).
2. Baguñà, J., Saló, E. & Auladell, C. Regeneration and pattern formation in planarians. III. Evidence that neoblasts are totipotent stem cells and the source of blastema cells. *Development* **107,** 77–86 (1989).
3. Agata, K. & Watanabe, K. Molecular and cellular aspects of planarian regeneration. *Semin. Cell Dev. Biol.* **10,** 77–83 (1999).
4. Newmark, P. A. & Sánchez Alvarado, A. Not your father's planarian: a classic model enters the era of functional genomics. *Nature Rev. Genet.* **3,** 210–219 (2002).
5. Agata, K. *et al.* Structure of the planarian nervous system (CNS) revealed by neuronal cell markers. *Zool. Sci.* **15,** 433–440 (1998).
6. Cebrià, F. *et al.* Dissecting planarian CNS regeneration by the expression of neural-specific genes. *Dev. Growth Differ.* **44,** 135–146 (2002).
7. Kato, K., Orii, H., Watanabe, K. & Agata, K. The role of dorsoventral interaction in the onset of planarian regeneration. *Development* **126,** 1031–1040 (1999).
8. Kobayashi, C., Nogi, T., Watanabe, K. & Agata, K. Ectopic pharynxes arise by regional reorganization after anterior/posterior chimera in planarians. *Mech. Dev.* **89,** 25–34 (1999).
9. Orii, H. *et al.* The planarian HOM/HOX homeobox genes (Plox) expressed along the anteroposterior axis. *Dev. Biol.* **210,** 456–468 (1999).
10. Sánchez Alvarado, A. & Newmark, P. A. Double-stranded RNA specifically disrupts gene expression during planarian regeneration. *Proc. Natl Acad. Sci. USA* **96,** 5049–5054 (1999).
11. Shibata, N. *et al.* Expression of *vasa(vas)*-related genes in germline cells and totipotent somatic stem cells of planarians. *Dev. Biol.* **206,** 73–87 (1999).
12. Umesono, Y., Watanabe, K. & Agata, K. Distinct structural domains in the planarian brain defined by the expression of evolutionarily conserved homeobox genes. *Dev. Genes Evol.* **209,** 31–39 (1999).
13. Cebrià, F. *et al.* The expression neural-specific genes reveals the structural and molecular complexity of the planarian central nervous system. *Mech. Dev.* **116,** 199–204 (2002).
14. Ogawa, K. *et al.* Planarian FGFR homologues expressed in stem cells and cephalic ganglions. *Dev. Growth Differ.* **44,** 191–204 (2002).
15. Wiedermann, M. & Trueb, B. Characterization of a novel protein (FGFRL1) from human cartilage related to FGF receptors. *Genomics* **69,** 275–279 (2000).
16. Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391,** 806–811 (1998).
17. Amaya, E., Musci, T. J. & Kirschner, M. W. Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. *Cell* **66,** 257–270 (1991).
18. Conlon, F. L. & Smith, J. C. Interference with brachyury function inhibits convergent extension, causes apoptosis, and reveals separate requirements in the FGF and activin signalling pathways. *Dev. Biol.* **213,** 85–100 (1999).
19. Smith, J. C., Price, B. M., Green, J. B., Weigel, D. & Herrmann, B. G. Expression of a *Xenopus* homolog of Brachyury (T) is an immediate-early response to mesoderm induction. *Cell* **67,** 79–87 (1991).
20. Isaacs, H. V., Pownall, M. E. & Slack, J. M. eFGF regulates Xbra expression during *Xenopus* gastrulation. *EMBO J.* **13,** 4469–4481 (1994).
21. Onichtchouk, D. *et al.* Silencing of TGF-β signalling by the pseudoreceptor BAMBI. *Nature* **401,** 480–485 (1999).
22. Tsang, M., Friesel, R., Kudoh, T. & Dawid, I. B. Identification of Sef, a novel modulator of FGF signaling. *Nature Cell Biol.* **4,** 165–169 (2002).
23. Fürthauser, M., Lin, W., Ang, S. L., Thisse, B. & Thisse, C. Sef is a feedback-induced antagonist of Ras/MAPK-mediated FGF signalling. *Nature Cell Biol.* **4,** 170–174 (2002).
24. Dowd, C. J., Cooney, C. L. & Nugent, M. A. Heparan sulfate mediates bFGF transport through basement membrane by diffusion with rapid reversible binding. *J. Biol. Chem.* **8,** 5236–5244 (1999).
25. Launay, C., Fromentoux, V., Shi, D. L. & Boucaut, J. C. A truncated FGF receptor blocks neural induction by endogenous *Xenopus* inducers. *Development* **122,** 869–880 (1996).
26. Streit, A., Berliner, A. J., Papanayotou, C., Sirulnik, A. & Stern, C. D. Initiation of neural induction by FGF signalling before gastrulation. *Nature* **406,** 74–78 (2000).
27. Tazaki, A. *et al.* Neural network in planarian revealed by an antibody against planarian synaptotagmin homologue. *Biochem. Biophys. Res. Commun.* **260,** 426–432 (1999).
28. Sakai, F., Agata, K., Orii, H. & Watanabe, K. Organization and regeneration ability of spontaneous supernumerary eyes in planarians—Eye regeneration field and pathway selection by optic nerves. *Zool. Sci.* **17,** 375–381 (2000).
29. Taira, M., Otani, H., Saint-Jeannet, J. P. & Dawid, I. B. Role of the LIM class homeodomain protein Xlim-1 in neural and muscle induction by the Spemann organizer in *Xenopus*. *Nature* **372,** 677–679 (1994).
30. Harland, R. M. in *Methods in Cell Biology* (eds Kay, B. K. & Peng, H. B.) **36,** 685–695 (Academic, San Diego, 1991).

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The polycomb group protein EZH2 is involved in progression of prostate cancer

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Prostate cancer is a leading cause of cancer-related death in males and is second only to lung cancer. Although effective surgical and radiation treatments exist for clinically localized prostate cancer, metastatic prostate cancer remains essentially incurable. Here we show, through gene expression profiling[1], that the polycomb group protein enhancer of zeste homolog 2 (EZH2)[2,3] is over-

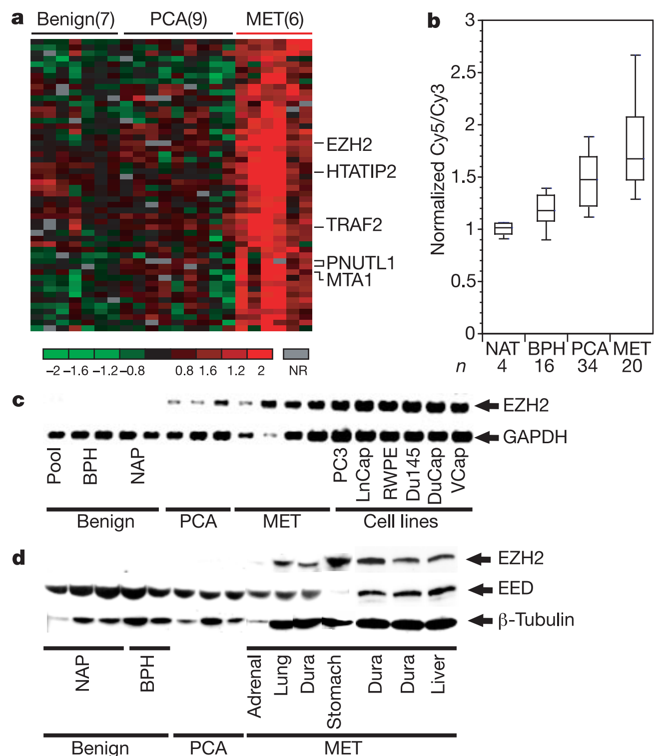

Figure 1 Overexpression of EZH2 in metastatic hormone-refractory prostate cancer (MET). **a**, Cluster diagram depicting genes that distinguish MET from clinically localized prostate cancer (PCA). Genes upregulated in METs relative to prostate cancer are shown. Red and green represent upregulatiom and downregulation, respectively, relative to the median of the reference pool. Grey represents technically inadequate or missing data, and black represents equal expression relative to the reference sample. **b**, DNA microarray analysis of prostate cancer shows upregulation of EZH2 in METs. BPH, benign prostatic hyperplasia; NAT, normal adjacent prostate tissue. **c**, RT–PCR analysis of the EZH2 transcript in prostate tissue and cell lines. **d**, Increased expression of EZH2 protein in METs. Immunoblot analysis of EZH2 and EED in prostate tissue extracts. The site of metastasis is indicated.

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