lead citrate, then examined in a Jeol 1200EX electron microscope. The number of adherent and intracellular infected erythrocytes and the number of phagosomes containing pigment granules were counted in each sample in thin sections of 100 randomly selected dendritic cells.

Monoclonal antibodies and flow cytometry. The following monoclonal antibodies directed against the respective human surface markers were used: CD3, clone OKT3; HLA A,B,C, clone W6/32; CD14, clone Tük4; CD54, clone 6.5B5; CD19, clone HD37 (DAKO); CD36, clone 89; CD80, clone BB1; CD40, clone LOB7/6; CD86, clone BU63; HLA DR, clone BF-1 (Serotec); CD83, clone HB15a (a gift from T. F. Tedder)[15]. Dendritic-cell staining was performed as described[15] and stained cells were analysed using a flow cytometer (Becton Dickinson).

T-cell proliferation assays. Total T cells (allogeneic MLR) or $CD4^+$ T cells (primary T-cell responses) were purified using a Cellect column (TCS). For the allogeneic MLR, dendritic cells were added in increasing numbers (156 to 10,000) to 1×10^5 T cells in triplicate and incubated for 5 days. T cells were pulsed with 0.5 μCi ^{3}H-thymidine per well for the last 18 h of the culture. For antigen-specific T-cell responses, 1×10^6 dendritic cells were incubated with medium alone, with 1×10^8 infected erythrocytes or with parasite lysate corresponding to 1×10^8 infected erythrocytes for 18 h and then pulsed with antigen as specified (10 μg ml^{-1} parasite lysate, 30 μg ml^{-1} KLH, 0.025 μM AChR-α: amino-acids 3–181, 1 μM AChR-α: amino acids 145–163). Subsequently, dendritic cells were matured with LPS for a further 48 h and purified by sedimentation through Lymphoprep to remove parasite debris and dead cells. For primary T-cell responses, 1×10^5 dendritic cells were cultured with 1.5×10^6 autologous $CD4^+$ T cells. From days 4 to 8 of culture, 50-μl aliquots were taken in triplicate and pulsed with 0.5 μCi ^{3}H-thymidine per well for 8 h[18]. For secondary T-cell responses of antigen-specific T-cell lines, 1×10^5 T cells were incubated in triplicate with 1×10^4 autologous dendritic cells for 4 days. For antigen-specific T-cell responses of clone TB-2, increasing numbers of MHC class-II-matched dendritic cells were incubated with 3×10^4 T cells for 72 h. Proliferation was measured in all assays by adding 0.5 μCi ^{3}H-thymidine per well for the last 8 h of culture. Antigen-specific T-cell lines were generated in parallel with the primary T-cell responses from peripheral blood mononuclear cells according to standard procedures.

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Eph receptors and ephrins restrict cell intermingling and communication

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Eph proteins are receptors with tyrosine-kinase activity which, with their ephrin ligands, mediate contact-dependent cell interactions[1] that are implicated in the repulsion mechanisms that guide migrating cells and neuronal growth cones to specific destinations[2,3]. Ephrin-B proteins have conserved cytoplasmic tyrosine residues that are phosphorylated upon interaction with an EphB receptor[4,5], and may transduce signals that regulate a cellular response[6]. Because Eph receptors and ephrins have complementary expression in many tissues during embryogenesis[7], bidirectional activation of Eph receptors and ephrin-B proteins could occur at interfaces of their expression domains, for example at segment boundaries in the vertebrate hindbrain. Previous work[8,9] has implicated Eph receptors and ephrin-B proteins in the restriction of cell intermingling between hindbrain segments[10]. We therefore analysed whether complementary expression of Eph receptors and ephrins restricts cell intermingling, and whether this requires bidirectional or unidirectional signalling. Here we report that bidirectional but not unidirectional signalling restricts the intermingling of adjacent cell populations, whereas unidirectional activation is sufficient to restrict cell communication through gap junctions. These results reveal that Eph receptors and ephrins regulate two aspects of cell behaviour that can stabilize a distinct identity of adjacent cell populations.

Ephrins fall into two structural classes with different binding specificities: the ephrin-A ligands anchored with glycosyl phosphatidylinositol bind to the EphA class of receptors, whereas the transmembrane ephrin-B proteins bind to EphB receptors[7,11]. An exception is EphA4, which binds ephrin-B2 as well as ephrin-A ligands[7]. Because truncated EphA4 or EphB receptors activate ephrin-B proteins as well as blocking Eph receptors, it is not possible to use these reagents to manipulate unidirectional and bidirectional signalling at interfaces of endogenous receptor and ephrin expression.

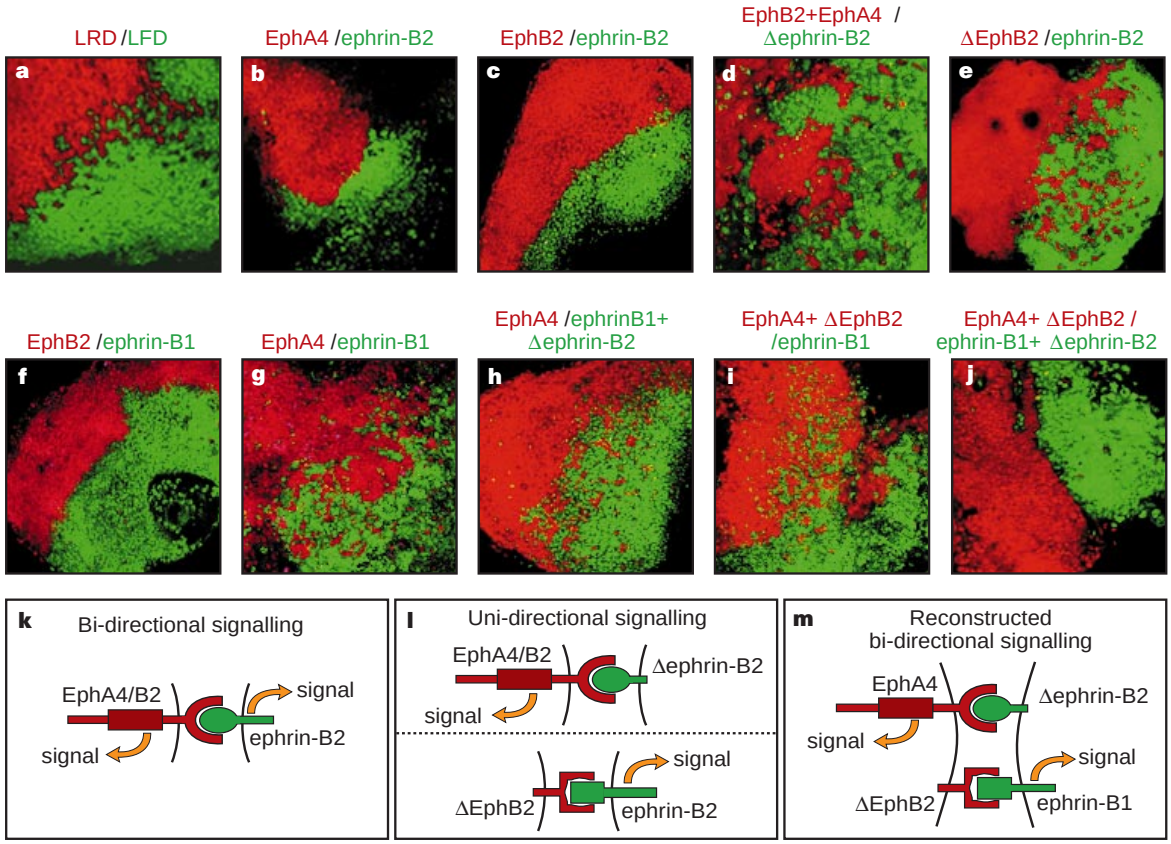

Figure 1 Bidirectional but not unidirectional signalling restricts cell intermingling. Animal caps that were labelled with rhodamine dextran (LRD) or fluorescein dextran (LFD) were juxtaposed and co-injected with RNA encoding Eph receptor or ephrin-B, respectively. After overnight culture, serial confocal sections of the fluorescent tracers were visualized. The following reagents were expressed in adjacent animal caps. **a**, Control assay: LRD/LFD only. **b**, **c**, Bidirectional signalling: **b**, EphA4/ephrin-B2; **c**, EphB2/ephrin-B2. **d**, **e**, Unidirectional signalling: **d**, EphA4 + EphB2/truncated ephrin-B2 (ΔephrinB2); **e**, truncated EphB2 (ΔEphB2)/ephrin-B2. **f**–**j**, Controls and experiments to reconstruct bidirectional signalling: **f**, EphB2/ephrin-B1; **g**, EphA4/ephrin-B1; **h**, EphA4/ephrin-B1 + truncated ephrin-B2; **i**, EphA4 + truncated EphB2/ephrin-B1; **j**, reconstructed bidirectional signalling: EphA4 + truncated EphB2/ephrin-B1 + truncated ephrin-B2. **k**, Diagram depicting bidirectional activation of Eph receptor and ephrin-B protein. **l**, Diagram depicting unidirectional activation of Eph receptor by truncated ephrin-B2, or ephrin-B by truncated EphB2. **m**, Diagram depicting the combination of unidirectional activation used to reconstruct bidirectional signalling.

To circumvent this, we established an assay in which an Eph receptor and an ephrin are expressed in adjacent cell populations and the amount of cell intermingling determined. Zebrafish embryos at the one-cell stage are injected with fluorescent lineage tracer and then animal caps are dissected at the 1,000-cell stage. Upon juxtaposition of two animal caps, one labelled with rhodamine dextran and the other with fluorescein dextran, they rapidly adhere, and this aggregate is cultured overnight. Confocal microscopy revealed that intermingling occurs between uninjected control animal caps (Fig. 1a).

To test whether the interaction of Eph receptor and ephrin can restrict cell intermingling, we co-injected lineage tracer and RNA such that ephrin-B2 is expressed in one animal cap and EphB2 and/or EphA4 in the other. In all combinations, only a few cells crossed into the adjacent territory and a clear border was visible (Fig. 1b, c). In contrast, extensive cell intermingling occurred if the Eph receptor was omitted from one population, or ephrin from the other (data not shown). These results show that cell intermingling is restricted by the interaction of exogenous Eph receptor and ephrin-B2 at the interface of two cell populations.

To test whether the restriction of cell intermingling requires bidirectional activation (shown in Fig. 1k), we used truncated versions of ephrin-B2 and EphB2 lacking the intracellular domain, including tyrosine residues implicated in signal transduction, but which can act as ligands to activate phosphorylation of full-length Eph receptor or ephrin-B, respectively[9]. Juxtaposition of, for example, cells expressing EphB2 with cells expressing truncated ephrin-B2 should therefore lead to unidirectional signalling into receptor-expressing cells (Fig. 1l). We found that after unidirectional signalling through Eph receptor (Fig. 1d) or ephrin-B (Fig. 1e), there is extensive cell intermingling between the two cell populations. To quantify the amount of cell intermingling, we counted the number of single labelled cells present in the adjacent territory in serial confocal sections (Fig. 2). Compared with uninjected controls, cell intermingling was significantly reduced by bidirectional signalling, but not by unidirectional signalling. These data indicate that unidirectional signalling in either direction is not sufficient, and bidirectional signalling is required to restrict cell intermingling.

A potential problem is raised by the discovery that Eph-receptor phosphorylation does not always correlate with a biological response, and that higher-order clustering of ephrin is required for functional activation of receptor[12]. Because clustering could involve interaction of the intracellular domain of ephrins with cytoplasmic proteins[13], it is possible that truncated ephrin does not fully activate Eph receptors. Therefore we tested whether truncated EphB2 and ephrin-B2 each elicit the relevant biological response. We devised a modified assay in which these reagents are used to reconstruct bidirectional signalling between cell populations from unidirectional signals. We took advantage of the finding that EphB2 binds ephrin-B1, whereas EphA4 binds ephrin-B2, but not ephrin-B1 (ref. 7). We found that restrictions to cell mixing

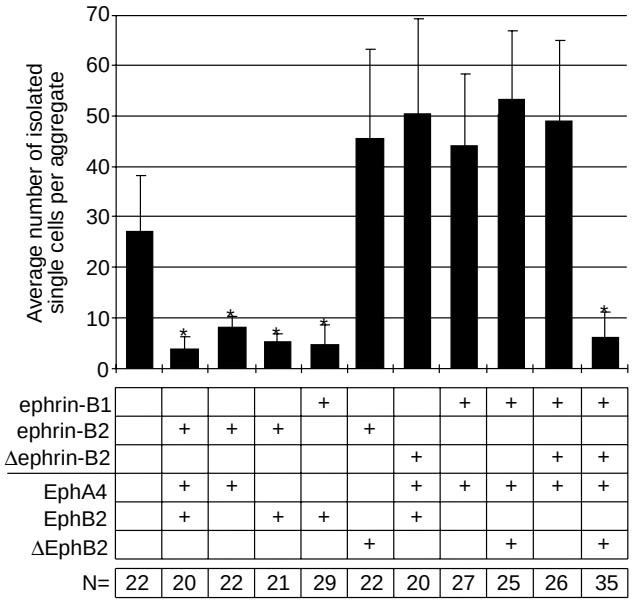

Figure 2 Quantification of cell intermingling. The bars indicate the average number of isolated single cells appearing in the adjacent territory for the indicated combinations of Eph receptors in one animal cap (below the line) and ephrin in the other (above the line). *N*, number of aggregates analysed. Intermingling is significantly reduced compared with uninjected controls ($P < 0.01$; indicated by asterisks) in combinations that give bidirectional activation of Eph receptor and ephrin-B. More intermingling compared with uninjected controls occurs ($P < 0.01$) when, for example, one cell population expresses full-length Eph receptor, and the other truncated ephrin. This could result from some autoactivation of full-length Eph receptor or ephrin-B that decreases cell–cell adhesion and thus increases the amount of cell movement.

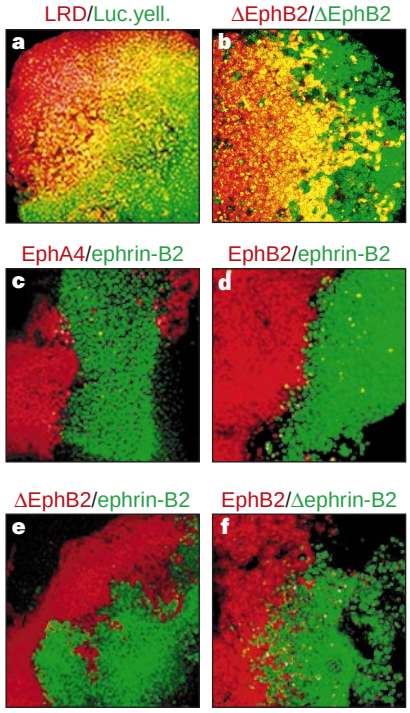

Figure 3 Activation of Eph receptor or ephrin blocks communication through gap junctions. Assays were carried out as described in Fig. 1, except that receptor-expressing embryos were labelled with LRD and ligand-expressing embryos were labelled with Lucifer Yellow (Luc.yell.), which gives a green signal on confocal microscopy. **a**, **b**, Control assays: **a**, LRD/Luc.yell.; **b**, truncated EphB2 (LRD)/truncated EphB2 (Luc.yell.). **c**, **d**, Bidirectional signalling: **c**, EphA4/ephrin-B2; **d**, EphB2/ephrin-B2. **e**, **f**, Unidirectional signalling: **e**, activation of ephrin-B2 by truncated EphB2; **f**, activation of EphB2 by truncated ephrin-B2.

correlate with these binding specificities, as EphB2 in combination with ephrin-B1 restricts cell intermingling, whereas EphA4 in combination with ephrin-B1 does not (Figs 1f, g, 2). Furthermore, intermingling occurs in the combination of (EphA4)/(ephrin-B1 + truncated ephrin-B2) (Figs 1h, 2), indicating that EphA4 does not activate ephrin-B1 indirectly through a heterodimer of ephrin-B1 and truncated ephrin-B2. Similarly, intermingling occurs in the combination of (ephrin-B1)/(EphA4 + truncated EphB2) (Figs 1i, 2), which argues against ephrin-B1 activating EphA4 through receptor heterodimerization. We could therefore reconstruct bidirectional signalling so that in one direction EphA4 is activated by truncated ephrin-B2, and in the other ephrin-B1 is activated by truncated EphB2 (Fig. 1m). In this situation, cell intermingling is restricted (Fig. 1j; quantification in Fig. 2). This result confirms that bidirectional signalling between two cell populations restricts their intermingling, but unidirectional signalling does not.

Correlations between sites of interaction between Eph receptors and ephrin in the hindbrain and somites[2,3,14,15] and disruptions to cell communication through gap junctions[16,17] led us to speculate that Eph receptors might regulate the formation of gap junctions. Gap junctions form by assembly of connexin proteins into intercellular channels that allow passage of molecules with a relative molecular mass below 1,200 ($M_r < 1.2K$)[18,19], and can be detected by the ability of Lucifer Yellow to diffuse through these channels. We juxtaposed one animal cap that was labelled with Lucifer Yellow (green in the confocal image), and another that was labelled with rhodamine dextran (red fluorescence). In the absence of co-injected reagents, Lucifer Yellow transfers into rhodamine dextran-labelled cells (the overlap leading to a yellow signal; Fig. 3a), indicating that gap junctions have formed between the cell populations. In contrast, when EphA4 or EphB2 were expressed in one animal cap and ephrin-B2 in the other, Lucifer Yellow did not diffuse between the cell populations (Fig. 3c, d). This result indicates that bidirectional signalling blocks the formation of gap junctions, so we next tested the effect of unidirectional signalling. In control experiments, we found that expression of truncated EphB2 in adjacent animal caps did not restrict formation of gap junctions (Fig. 3b). In contrast, after unidirectional activation of ephrin-B2 by truncated EphB2 (Fig. 3e) or of EphB2 by truncated ephrin-B2 (Fig. 3f), Lucifer Yellow did not transfer into rhodamine-labelled cells, despite intermingling of the cell populations. Formation of gap junctions was also reduced after activation of ephrin-B2 with clustered soluble EphB1-Fc, but this reagent was less effective than membrane-bound truncated receptor (data not shown).

Based on these results, and evidence that Eph receptors and ephrins regulate repulsion or de-adhesion responses[2,3], we propose a model to account for the requirement for bidirectional signalling to restrict intermingling of adjacent cell populations, whereas unidirectional activation is sufficient to restrict communication by means of gap junctions (Fig. 4). At the interface of cells expressing Eph receptor and cells expressing ephrin-B, bidirectional activation leads to a mutual repulsion or de-adhesion that restricts the movement of each cell population into the other. In contrast, unidirectional signalling will repel one population, but the cells expressing truncated Eph receptor or ephrin are not themselves repelled and can invade adjacent territory, leading to intermingling. However, repulsion of only one of the two cell populations is sufficient to prevent stable cell–cell contacts required for assembly of gap junctions. After mosaic ectopic expression of truncated ephrin-B2 in zebrafish embryos, the expressing cells sort to the boundaries of rhombomeres r3/r5 (which express EphA4 and EphB receptors), indicating that unidirectional activation can restrict cells

to a specific region within segments[9]. This outcome, which is distinct from that observed with the animal-cap assay, could result from several differences between the systems. For example, in the hindbrain, stable boundaries are formed at which interactions between Eph receptors and ephrin occur, and r3/r5 cells expressing exogenous ephrin-B2 may sort because they have similar adhesive properties as boundary cells[9].

There is much evidence that differential expression of cell adhesion molecules and the preferential association of cells with similar adhesive properties can establish and maintain organized cellular patterns during development[20,21]. Although Eph receptor activation is required to prevent cell intermingling between hindbrain segments[8,9], this restriction may also require cell adhesion molecules[22]. Our findings reveal that complementary expression of Eph receptors and ephrins is sufficient to restrict cell intermingling, and that this can be accomplished without differential co-expression of exogenous adhesion molecules. However, Eph receptors and ephrins may regulate the function of cell adhesion molecules[23], leading to a de-adhesion of cells at boundaries. Alternatively, or in addition, activation may trigger cell repulsion responses involving localized collapse of the actin cytoskeleton[24]. It is therefore likely that Eph receptors and ephrins have parallel or cooperative roles with cell adhesion systems in restricting cell intermingling during development.

Our results indicate that interfaces of endogenous Eph receptor and ephrin-B expression may also restrict formation of gap junctions. Communication by means of gap junctions has been implicated in tissue patterning and the regulation of cell proliferation and differentiation[18,19,25,26], and it is believed to allow passage of regulatory molecules so that a coordinate response occurs in cells connected by gap junctions. In the hindbrain, rhombomere boundaries are barriers to the spread of signals that regulate regional identity, and this correlates with the absence of gap junctions[27]. Similarly, formation of gap junctions is restricted at segment boundaries in insects[28]. Several mechanisms could underlie such restrictions[18,19], including disassembly of connexins regulated by diffusible growth factors, and the interdependence of cell–cell adhesion and gap junction assembly. The regulation of gap junction formation by Eph receptors and ephrins enables a restriction of communication across boundaries, or even between intermingled cell populations. Thus, our findings indicate that interactions between Eph receptors and ephrin regulate two mechanisms—the restriction of cell intermingling and communication—that may stabilize a distinct identity or behaviour of adjacent cell populations. □

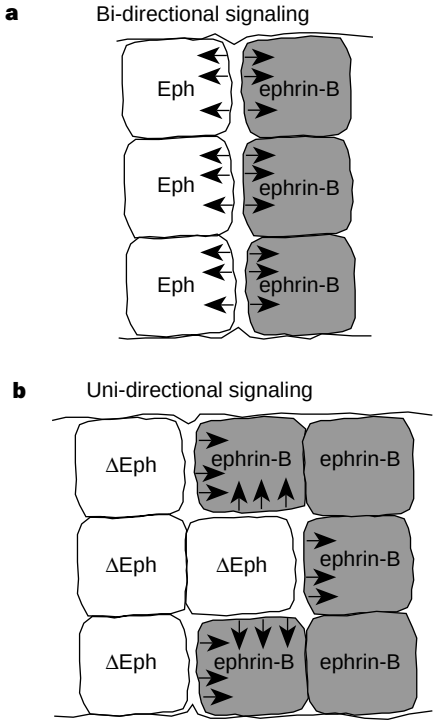

Figure 4 Model of restriction of cell intermingling and communication through gap junctions. **a**, Bidirectional activation leads to local de-adhesion and/or repulsion (indicated by arrows) in both the Eph-receptor- and ephrin-expressing cells at the interface of their expression domains. This prevents invasion of each cell population into the other, thus restricting cell intermingling. In addition, the absence of stable cell contacts (indicated by spaces between cells) disrupts the formation of gap junctions. **b**, Unidirectional activation leads to repulsion of cells expressing ephrin-B, but not of cells expressing truncated Eph receptor. This allows invasion of cells expressing truncated Eph receptor into ephrin-expressing territory, but repulsion of one cell population is sufficient to disrupt the formation of gap junctions. A similar situation occurs when cells expressing Eph-receptor and truncated ephrin-B are juxtaposed.

Methods

Animal cap assay for cell intermingling. Between 20 and 100 pg RNA encoding Eph receptor or ephrin (constructs described in ref. 9) was microinjected into one-cell zebrafish embryos as described[8], together with rhodamine dextran (LRD), fluorescein dextran (LFD) or Lucifer Yellow. At the 1,000-cell stage, embryos were dechorionated and animal caps were dissected. Upon juxtaposing animal caps, they adhere within several minutes to form an aggregate. Each aggregate was mounted under a coverslip and cultivated overnight in L15 medium containing 10% fetal calf serum. The aggregates were then fixed in 4% paraformaldehyde and equilibrated in 70% glycerol. Serial optical sections of the fluorescent tracers were visualized using a Leica confocal microscope. The images were displayed using NIH Image and processed with Adobe Photoshop software.

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DNA-dependent protein kinase is not required for the p53-dependent response to DNA damage

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Damage to DNA in the cell activates the tumour-suppressor protein p53 (ref. 1), and failure of this activation leads to genetic instability and a predisposition to cancer. It is therefore crucial to understand the signal transduction mechanisms that connect DNA damage with p53 activation. The enzyme known as DNA-dependent protein kinase (DNA-PK) has been proposed to be an essential activator of p53 (refs 2, 3), but the evidence for its involvement in this pathway is controversial[3,4]. We now show that the p53 response is fully functional in primary mouse embryonic fibroblasts lacking DNA-PK: irradiation-induced DNA damage in these defective fibroblasts induces a normal response of p53 accumulation, phosphorylation of a p53 serine residue at position 15, nuclear localization and binding to DNA of p53. The upregulation of p53-target genes and cell-cycle arrest also occur normally. The DNA-PK-deficient cell line SCGR11 contains a homozygous mutation in the DNA-binding domain of p53, which may explain the defective response by p53 reported in this line[3]. Our results indicate that DNA-PK activity is not required for cells to mount a p53-dependent response to DNA damage.

DNA-PK$^{-/-}$ primary mouse embryonic fibroblasts (MEFs) were derived from a mouse harbouring a targeted disruption of the catalytic subunit of DNA-PK which causes loss of the kinase activity[5]. The phenotype of these MEFs is radiosensitive, as are the phenotypes of SCID (severe combined immunodeficient) mice and of high-passage-number transformed lines derived from these mice, such as SCGR11 (Fig. 1a). Although it is unclear whether there is a residual DNA-PK function in SCID-derived cells, DNA-PK$^{-/-}$ MEFs have no DNA-PK activity or any detectable DNA-PK protein by western blotting[5]. If DNA-PK were required for p53-mediated cell-cycle arrest in response to DNA damage, then DNA damage should not induce a cell-cycle arrest in DNA-PK$^{-/-}$ cells. However, like wild-type cells, irradiated DNA-PK$^{-/-}$ MEFs accumulate in G1 phase as a result of cell-cycle arrest (Fig. 1b); we also found that irradiated DNA-PK$^{-/-}$ MEFs have a higher G1/S ratio than DNA/PK$^{+/+}$ or +/– cells irradiated with the same dose, probably because of the persistence of unrepaired double-stranded DNA breaks. DNA-PK$^{-/-}$ MEFs also undergo arrest of the cell cycle in response to the antimetabolite PALA (*n*-(phosphonacetyl)-L-aspartate) or the microtubule-depolymerizing agent colcemid (data not shown). In contrast, p53$^{-/-}$ MEFs and SCGR11 cells continue to cycle after DNA damage (Fig. 1b).

We next investigated the stabilization of p53 and its mobilization to the nucleus after different types of DNA damage. Using indirect immunofluorescence to track the subcellular localization of p53 in wild-type and DNA-PK$^{-/-}$ MEFs, we found similar rates of nuclear accumulation and a comparable duration of nuclear localization after γ-irradiation or treatment with actinomycin D in both cell types (Fig. 2A, and data not shown). By contrast, SCGR11 cells contained large amounts of nuclear p53 even in untreated cells, which is indicative of the presence of mutant, stable p53 (Fig. 2A).

p53 is phosphorylated at highly conserved amino-terminal serine residues in response to DNA damage[2,6]. Phosphorylation of p53 at serine 15 reportedly blocks binding of the p53 inhibitor *MDM2*, preventing *MDM2*-mediated degradation of p53. This stabilized p53 protein can then transactivate its target genes[2,6]. The structurally related kinases ATM, ATR and DNA-PK all phosphorylate p53 at Ser 15 *in vitro*[7,8]. However, phosphorylation of Ser 15 and accumulation of p53, monitored by immunoprecipitation after either ultraviolet or γ-irradiation, occurred to the same extent in DNA-PK$^{-/-}$ and wild-type MEFs (Fig. 2B, C, and data not shown). We conclude that DNA-PK is therefore dispensible for the nuclear accumulation of p53, phosphorylation of Ser 15, and stabilization of p53 that occur in response to DNA damage.

Once in the nucleus, p53 binds to specific DNA sequences in promoters and enhancers of genes that are involved in cell-cycle arrest and apoptosis[1]. It has been proposed that p53 must be

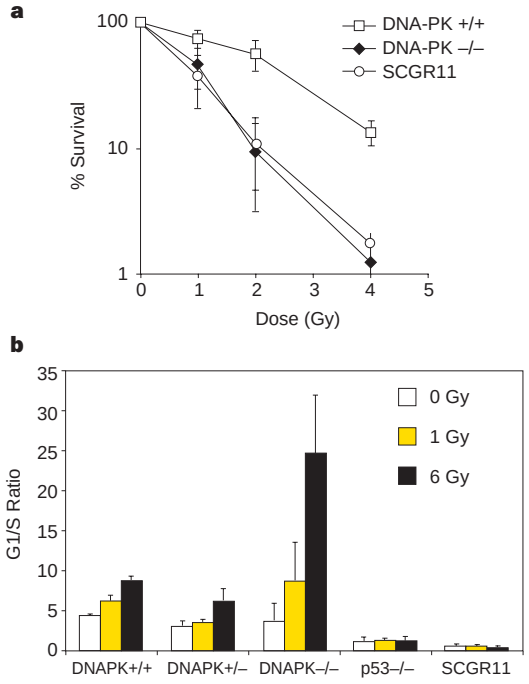

Figure 1 Cell-cycle arrest response of DNA-PK$^{-/-}$ MEFs following irradiation. **a**, DNA-PK$^{-/-}$ cells show a level of radiosensitivity that is identical to that of SCGR11 cells. The results represent the mean ± s.d. of 3 independent experiments. **b**, Using dual-parameter microfluorimetry, G1, S or G2/M phase was assessed on >10,000 collected cells per sample. Fluorescence-activated cell sorting (FACS) analysis was performed at least twice for each treatment and the results represent the mean of the G1/S phase ratio ± s.d.