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Dp1 Is Largely Dispensable for Embryonic Development

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E2F/DP complexes activate or repress the transcription of E2F target genes, depending on the association of a pRB family member, thereby regulating cell cycle progression. Whereas the E2F family consists of seven members, the DP family contains only two (*Dp1* and *Dp2*), *Dp1* being the more highly expressed member. In contrast to the inactivation of individual E2F family members, we have recently demonstrated that loss of *Dp1* results in embryonic lethality by embryonic day 12.5 (E12.5) due to the failure of extraembryonic lineages to develop and replicate DNA properly. To bypass this placental requirement and search for roles of *Dp1* in the embryo proper, we generated *Dp1*-deficient embryonic stem (ES) cells that carry the *ROSA26-LacZ* marker and injected them into wild-type blastocysts to construct *Dp1*-deficient chimeras. Surprisingly, we recovered mid- to late gestational embryos (E12.5 to E17.5), in which the *Dp1*-deficient ES cells contributed strongly to most chimeric tissues as judged by X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining and Western blotting. Importantly, the abundance of DP2 protein does not increase and the expression of an array of cell cycle genes is virtually unchanged in *Dp1*-deficient ES cells or chimeric E15.5 tissues with the absence of *Dp1*. Thus, *Dp1* is largely dispensable for embryonic development, despite the absolute extraembryonic requirement for *Dp1*, which is highly reminiscent of the restricted roles for *Rb* and cyclins E1/E2 in vivo.

The retinoblastoma tumor suppressor (pRB) and its family members (p107 and p130) control cell cycle progression to a large part by repressing the E2F/DP transcription factor family, whose targets include genes involved in DNA replication, cyclin-dependent kinase activation, apoptosis, checkpoints, and mitosis (22, 26). The E2F/DP transcription factor family is composed of seven E2F members (E2F1 to -7) and two DP family members (DP1 and -2). All known E2F1-6 functions, except pRB family member binding, are totally dependent on the prior heterodimerization with a DP family member, and thus the DP family is thought to be required for E2F-mediated gene activation and repression by pRB family members.

To understand the role of E2F/DP complexes in vivo, we and others have constructed mutant mice lacking one or more of these family members. Six of the genes encoding E2F family members have been inactivated in mice, leading to a wide range of tissue-specific phenotypes. *E2f1* deficiency leads to increased tumor predisposition and tissue atrophy (4, 27). When combined with *E2f1* deficiency, *E2f2* deficiency causes abnormal maturation of multiple hematopoietic lineages (11), similar to what is observed with *E2f4* deficiency alone (8, 19). *E2f3* deficiency results in reduced viability and congestive heart failure (9). Since the combined deficiency of *E2f1*, *E2f2*, and *E2f3* prevents the proliferation of primary mouse embryonic fibroblasts, E2F/DP complexes involving these family members are required for normal development (25). Loss of *E2f5* or *E2f6* results in highly restricted phenotypes, abnormal choroid plexus function, and homeotic transformations of the axial skeleton, respectively (12, 21).

In sharp contrast to the milder phenotypes resulting from the inactivation of the *E2f* family members, we have demonstrated that loss of *Dp1* results in the death of all embryos prior to embryonic day 12.5 (E12.5) (10). *Dp1* deficiency greatly impairs the development of trophectoderm-derived tissues, resulting in placental insufficiency, which secondarily leads to embryonic death. Loss of *Dp1* severely compromises DNA replication in the ectoplacental cone and the extent of endoreduplication occurring in trophoblast giant cells, results that are consistent with the known roles of E2F/DP complexes in stimulating S-phase entry and completion. The inability of DP2 to compensate for the loss of DP1 is likely due to the low level of expression of DP2 in the extraembryonic compartment. The severity of the *Dp1*-deficient defect (early lethality and its complete penetrance) is consistent with the observations that *Dp1* is the most highly expressed family member and activation and repression by E2F/DP complexes depends completely on the presence of the DP proteins.

To define the roles of *Dp1* in the embryo proper, we first generated *Dp1*-deficient embryonic stem (ES) cell lines carrying the *ROSA26* promoter–*LacZ* reporter (5), which allowed us to follow the contribution of *Dp1*-deficient cells into embryonic tissues by staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). To bypass the extraembryonic requirement for *Dp1*, these *Dp1*-deficient ES cells were injected into wild-type blastocysts to allow the development of the *Dp1*-deficient chimeric embryos. Surprisingly, these experiments have demonstrated that, in most tissues, *Dp1* is dispensable for normal development.

MATERIALS AND METHODS

Generation of *Dp1*-deficient; *ROSA26-LacZ* ES cells. $Dp1^{+/-}$ mice were mated to *ROSA26* mice in which a *LacZ* gene is integrated at the *ROSA26* locus (5).

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Then, *Dp1*$^{+/-}$; *ROSA26-LacZ* males were mated to *Dp1*$^{+/-}$ females, and blastocysts of all possible genotypes were isolated at E3.5. ES cell lines were generated from blastocyst outgrowths as previously described (16, 20). Briefly, the blastocysts were cultured with gamma-irradiated feeders in ES cell-Dulbecco modified Eagle medium (16) for 5 days. The inner cell masses were picked, treated with trypsin and replated for another 6 to 7 days. Undifferentiated ES cell colonies were picked, treated with trypsin, expanded, and frozen.

Genomic PCR genotyping. Genotyping at the *Dp1* locus was performed by using genomic PCR in combined reactions as previously described with the common primer L75 and the unique primers L78 and L79 (10).

The presence or absence of the *LacZ* reporter at the *ROSA26* locus was detected by genomic PCR with the common L221 primer and the unique primers R316 and L238, which are specific to the wild-type *ROSA26* locus or the *LacZ* transgene, respectively. To improve the reliability of the *ROSA26* genotyping assay, we sequenced the *ROSA26* locus and designed the L221 ([5′-CTTGTGA TCCGCCTCGGAGTATT-3′]) and L238 ([5′-CGCGCCGCTGTAAAGTGTT ACGT-3′]) primers to shorten the length of the PCR products to 186 bp (wild-type allele) and 400 bp (*LacZ* trapped allele). A description of the R316 primer ([5′-GGAGCGGGAGAAATGGATATG-3′]) was previously published (28). Amplification was performed with annealing at 54°C for 34 cycles and then visualized on a 1.6% agarose gel with ethidium bromide.

Generation and implantation of *Dp1*-deficient chimeric embryos. Wild-type C57BL/6 females were mated to C57BL/6 stud males, and vaginal plugs were detected the next morning (0.5 days postcoitum). The uterine horns of these pregnant C57BL/6 donor females were flushed with M2 medium to collect blastocysts (at 3.5 days postcoitum). Logarithmically growing ES cells with a minimum of gamma-irradiated embryonic feeders were treated with trypsin and resuspended in ES-DMEM, supplemented with HEPES (pH 7.0). Blastocysts were then injected with 10 to 15 ES cells per blastocyst. Manipulated blastocysts were allowed to recover in M2 medium at 37°C in a CO_2 incubator and were then reimplanted into the uterine horns of pseudopregnant CD-1 recipient females as described previously (16). After various times of additional gestation, the pregnant recipients were sacrificed, and chimeric embryos were dissected away from deciduas and placenta and then processed for either whole-mount analysis or cryosectioning. All animals were maintained in a pathogen-free, barrier facility with the oversight of the institutional veterinarian and Columbia's Institutional Animal Care and Use Committee.

X-Gal staining of ES cells, whole-mount embryos, and cryosections. For staining ES cell colonies, feeder layer-bound colonies were rinsed in phosphate-buffered saline (PBS), fixed briefly in 4% paraformaldehyde, rinsed in PBS again, and then incubated in X-Gal staining solution [20 mM $MgCl_2$, 0.2% NP-40 (Calbiochem), 50 mM $K_3Fe(CN)_6$, 50 mM $K_4Fe(CN)_6$, and 1 mg of X-Gal (LabScientific, Inc.)/ml in PBS] overnight at 37°C to detect β-galactosidase activity.

For staining of whole-mount embryos, embryos were collected from pregnant recipient females, fixed in 10% buffered formalin (hemisected for embryos older than E12.5), rinsed in PBS, and incubated in staining solution overnight at 30°C. For cryosections, embryos were collected, fixed in 2% paraformaldehyde in PBS for several hours, hemisected, fixed longer, and then rinsed in PBS and equilibrated in 18% sucrose in PBS overnight at 4°C before sectioning on positively charged slides. The embryonic cryosections (10 μm) were then incubated overnight in staining solution at 30°C, counterstained in Nuclear Fast Red (Vector Laboratories), dehydrated through a graded series of methanol, and mounted with Permount.

Western blotting of ES cell lysates and *Dp1*-deficient chimeric organs. ES cell lines were grown without feeders for several days and then treated with trypsin, and the cell pellets were collected and lysed in 2× Laemmli buffer with two rounds of sonication and boiling. Microdissected organs were collected and frozen from two *Dp1*-deficient chimeric embryos (29A and 29C) and two *Dp1*$^{+/+}$ C57BL/6 embryos at E15.5. For Fig. 4A, organs were lysed with 2× Laemmli buffer by multiple rounds of sonication and boiling. For Fig. 4B and C , organs were lysed in TRIzol (Invitrogen), which allowed the recovery of RNA and protein according to the manufacturer's instructions. Total protein was solubilized in 2× Laemmli buffer with sonication and boiling. An approximately equal amount of total protein (estimated by Coomassie blue staining for each lysate) was separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis and then transferred to Immobilon-P. Monoclonal antibodies to DP1 (1DP06/TFD10 [Labvision]) and DP2 (BC-2 [Sigma] and G-12 [Santa Cruz]) were used at 1 to 2 μg/ml and visualized with a horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G antibody (Amersham). A rabbit polyclonal antibody against actin (Sigma) was used at a 1:500 dilution and visualized with a horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G antibody. The blots were developed with an ECL kit (Amersham) and exposed to autoradiographic film.

FACS and cell cycle array analysis. ES cell lines were grown logarithmically with gamma-irradiated feeders and treated with trypsin, and then feeder cells were depleted by temporarily plating prior to pelleting of the ES cells. A portion of each ES cell line was used for fluorescence-activated cell sorting (FACS) analysis after staining with propidium iodide to measure DNA content according to standard protocols.

For array analysis on ES cells, total RNA was extracted from another portion of each ES cell line with TRIzol (Invitrogen) and ca. 3 μg of RNA was reverse transcribed and labeled with [α-^{32}P]dCTP (according to manufacturer instructions). For array analysis on E15.5 organs, total RNA was extracted from the embryonic brains and kidneys dissected from a *Dp1*-deficient chimera (29C) and a wild-type embryo (*ROSA26-LacZ*; *Dp1*$^{+/+}$) with TRIzol, and ca. 5 μg of RNA was reverse transcribed and labeled with [α-^{32}P]dCTP (according to manufacturer instructions). Hybridization of labeled RNAs was performed by using a GEArray Q series mouse cell cycle gene array (Superarray). Array filters were hybridized overnight at 60°C, washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS and then 0.1× SSC–0.5% SDS at 60°C, and exposed to autoradiographic film. (A list of individual cDNAs spotted and their positions is available at http://www.superarray.com.)

RESULTS

Isolation of *Dp1*-deficient; *ROSA26-LacZ* ES cell lines. With the eventual goal of determining how well *Dp1*-deficient ES cells contribute to embryonic development, we first generated *Dp1*-deficient ES cell lines that carry the widely expressed *ROSA26-LacZ* marker, so that embryonic tissues derived from *Dp1*-deficient ES cells would stain blue in the presence of X-Gal. To do this, we crossed *Dp1*$^{+/-}$ animals with the *ROSA26-LacZ* promoter trap line (5) and then mated *Dp1*$^{+/-}$; *ROSA26-LacZ* males to *Dp1*$^{+/-}$ females and harvested the blastocysts (E3.5). These blastocysts were cultured on embryonic feeder layers to promote blastocyst outgrowth (16, 20). After 5 days, the outgrowths were treated with trypsin and replated onto embryonic feeder layers and, after 7 more days, undifferentiated ES cell colonies were picked, expanded, and frozen. A portion of each colony was genotyped at the *Dp1* locus (Fig. 1A) and at the *ROSA26* locus (Fig. 1B), and another portion was used for X-Gal staining (Fig. 1C). In this way, we isolated three *Dp1*-deficient; *ROSA26-LacZ* ES cell lines (2B1, 8G1, and 8G2) and three *Dp1*$^{+/+}$; *ROSA26-LacZ* ES cell lines (1C1, 5F4, and 9C2).

To determine whether the loss of *Dp1* changed the expression of DP2 protein, we performed Western blot analysis on the three *Dp1*-deficient; *ROSA26-LacZ* ES cell lines and the three *Dp1*$^{+/+}$; *ROSA26-LacZ* ES cell lines. As expected, DP1 protein is not detectable in the three *Dp1*-deficient lines, whereas there is abundant DP1 expression in the *Dp1*$^{+/+}$ lines (Fig. 1D, top panel). DP2 protein is detectable in all ES cell lines, but there is no appreciable change of DP2 expression observed with the absence of DP1 protein, with actin as a loading control (Fig. 1D, middle and bottom panels).

***Dp1* is largely dispensable for embryonic development.** To determine how well the *Dp1*-deficient; *ROSA26-LacZ* ES cell lines contribute to embryonic development, we constructed chimeric embryos, in which wild-type placentas allowed us to bypass the extraembryonic requirement for *Dp1*. To do this, *Dp1*-deficient; *ROSA26-LacZ* ES cells were injected into wild-type donor blastocysts (E3.5) and then reimplanted into the uterine horns of pseudopregnant recipient mice. Chimeric embryos were recovered after various additional lengths of gestation (10 to 15 days) to allow the *Dp1*-deficient cells to con-

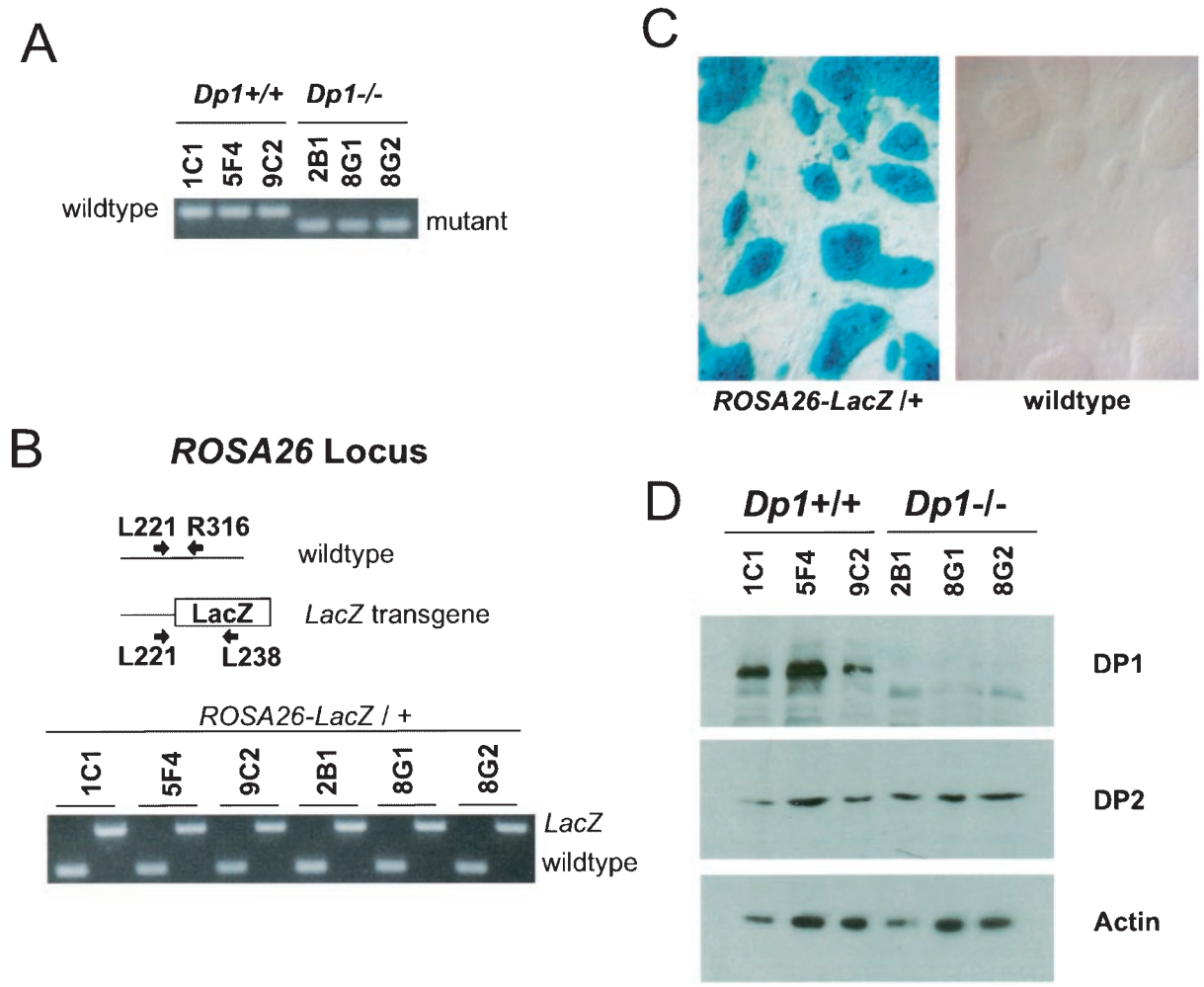

FIG. 1. Characterization of *Dp1*-deficient; *ROSA26-LacZ* ES cell lines. Three wild-type (1C1, 5F4, and 9C2) and three *Dp1*-deficient (2B1, 8G1, and 8G2) ES cell lines were generated from blastocysts outgrowths by successive subculturing trypsin-treated fragments, followed by the expansion of undifferentiated ES colonies. (A) Portions of the expanded colonies were genotyped at the *Dp1* locus with agarose gels to separate the genomic PCR products. (B) Similarly, the presence of one copy of the *LacZ* gene at the *ROSA26* locus was verified in each line by using the genomic PCR scheme (top panel) to detect the wild-type *ROSA26* allele and the integrated *LacZ* transgene (using the common primer L221 and the unique primers R316 and L238) as visualized by agarose electrophoresis (bottom panel). (C) β-Galactosidase activity as measured by blue staining in the presence of X-Gal was demonstrated in one of these ES cell lines carrying the *ROSA26-LacZ* (left panel). Another ES cell line, which does not carry the *ROSA26-LacZ* marker and did not stain positively with X-Gal, is shown for comparison (right panel). (D) Western blotting was performed on lysates from all six ES cell lines to assess the expression of DP1 and DP2. Monoclonal antibodies specific to DP1 (clone 1DP06/TFD10, top panel) or DP2 (clone G-12, middle panel) were used to show that the *Dp1*-deficient lines do not express DP1 and do not upregulate DP2. Actin (polyclonal antiactin, bottom panel) was used as a loading control.

tribute to the normal development of all embryonic tissues. Chimeric embryos were then fixed briefly and subjected to whole-mount analysis with X-Gal staining to visualize the incorporation of the *Dp1*-deficient; *ROSA26-LacZ* cells into embryonic tissues, as judged by the extent of blue coloration in the embryo. As a positive control, *ROSA26-LacZ* embryos were stained with X-Gal in parallel, which leads to entirely blue embryos. As a negative control, uninjected sibling embryos were stained with X-Gal alongside the chimeric embryos. In addition, the *Dp1*-deficient status of the injected cells was confirmed by regenotyping the unused portion of the ES cells from the day of injection.

Surprisingly, *Dp1*-deficient chimeric embryos at E12.5 were dark blue, indicating that the *Dp1*-deficient; *ROSA26-LacZ* cells successfully contributed to embryonic development (Fig. 2A). This is in sharp contrast to the failed development of *Dp1*-deficient embryos, which are severely stunted in growth, delayed in development or dead at E11.5 (10). All three *Dp1*-deficient ES cell lines (2G1, 8G1, and 8G2) contributed well to E12.5 chimeric embryos. This result prompted us to check later gestational embryos by using whole mount X-Gal staining to determine whether *Dp1*-deficient ES cells could contribute strongly at these time points.

Consistent with our result at E12.5, the exterior and interior surfaces of hemisected *Dp1*-deficient chimeric embryos at E15.5 and E17.5 (from multiple injections of the 2B1 line) were dark blue in the presence of X-Gal, signifying that *Dp1*-deficient ES cells contribute highly to most embryonic organs,

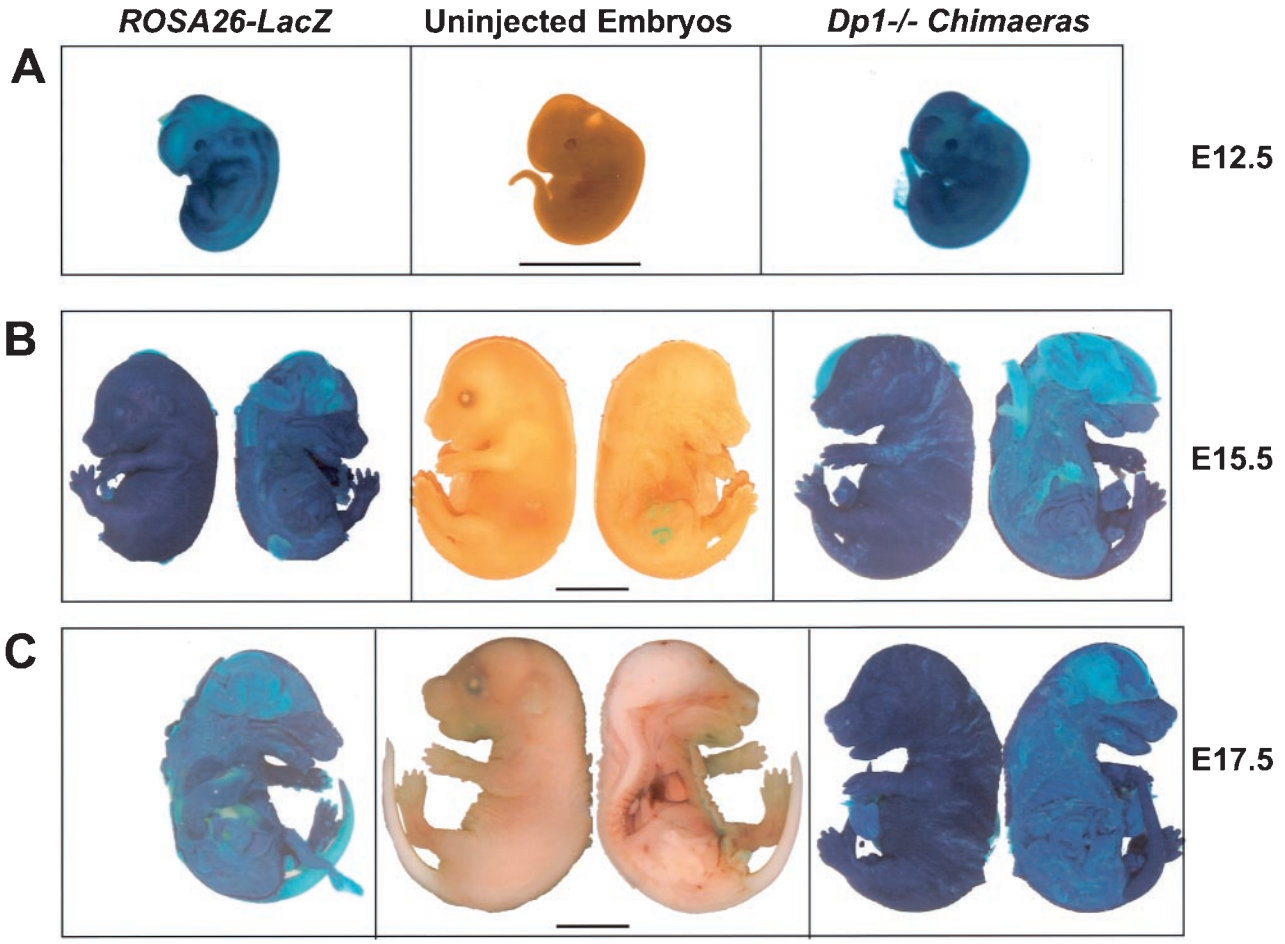

FIG. 2. *Dp1* is largely dispensable for development of the embryo proper. (A) *Dp1*-deficient chimeric embryos recovered at E12.5 from the injection of the 2B1 line at E12.5 are shown after X-Gal staining. For comparison, a *ROSA26-LacZ* embryo and an uninjected wild-type embryo recovered at E12.5 are also displayed after whole-mount embryo staining with X-Gal. (B) The exterior and interior surfaces of hemisected *Dp1*-deficient chimeric embryos at E15.5 are shown for the 2B1 line after X-Gal staining. For comparison, hemisected *ROSA26-LacZ* embryos and uninjected wild-type embryos recovered at these time points are also displayed after whole-mount embryo staining with X-Gal. (C) The exterior and interior surfaces of hemisected *Dp1*-deficient chimeric embryos at E17.5 are shown for the 2B1 line after X-Gal staining. For comparison, hemisected *ROSA26-LacZ* embryos and uninjected wild-type embryos recovered at these time points are also displayed after whole-mount embryo staining with X-Gal. Because of technical difficulties, we do not have an external image of a *ROSA26-LacZ* embryo at E17.5. In panels B and C, the exterior surface is seen in left-facing embryos, whereas the interior surface is seen in right-facing embryos. *Dp1* deficiency does not prevent the recovery of high contribution chimeras.

even late in development (Fig. 2B and C for E15.5 and E17.5, respectively). Similar results were obtained with two *Dp1*-deficient lines (2B1 and 8G1) at E14.5 (data not shown). The developing brain consistently appeared light blue in the *Dp1*-deficient chimeras; however, the brain also appeared light blue in *ROSA26-LacZ* embryos stained with X-Gal as positive controls. Whole-mount X-Gal staining clearly demonstrated that *Dp1*-deficient ES cells are capable of forming most embryonic organs.

***Dp1*-deficient ES cells are pluripotent.** To understand how effectively *Dp1*-deficient; *ROSA26-LacZ* ES cells contribute to tissues late in gestation, we performed X-Gal staining on cryosections from *Dp1*-deficient chimeric embryos at E15.5 and E17.5 to visualize the cytoplasmic deposition of β-galactosidase in *ROSA26-LacZ*-marked cells. To visualize both the wild-type and *ROSA26-LacZ*-marked cells, we counterstained these sections with nuclear fast red. Most tissues showed a predominance of *ROSA26-LacZ*-marked *Dp1*-deficient cells, with ample contribution of these cells to all histological cell types and morphological structures in these tissues. Tissues arising from all three germ layers (i.e., endoderm, ectoderm, and mesoderm) are well represented on the list of tissues in which *Dp1*-deficient; *ROSA26-LacZ* cells are found in abundance. A sampling of such chimeric tissues at E17.5, including the eye, heart, tongue, kidney, lung, and intestine, is shown in Fig. 3A to F (upper panels). Additional chimeric tissues at E15.5, including the thymus, liver, skin, pancreas, cartilage, and dorsal root ganglion, are shown in Fig. 3G to L (upper panels). The equivalent cryosections from *ROSA26-LacZ* embryos at E17.5 and E15.5 were stained with X-Gal for comparison (Fig. 3A to L, lower panels).

A small subset of tissues consistently appeared negative in X-Gal-stained cryosections from *Dp1*-deficient chimeric embryos at all time points examined. These are subregions of the

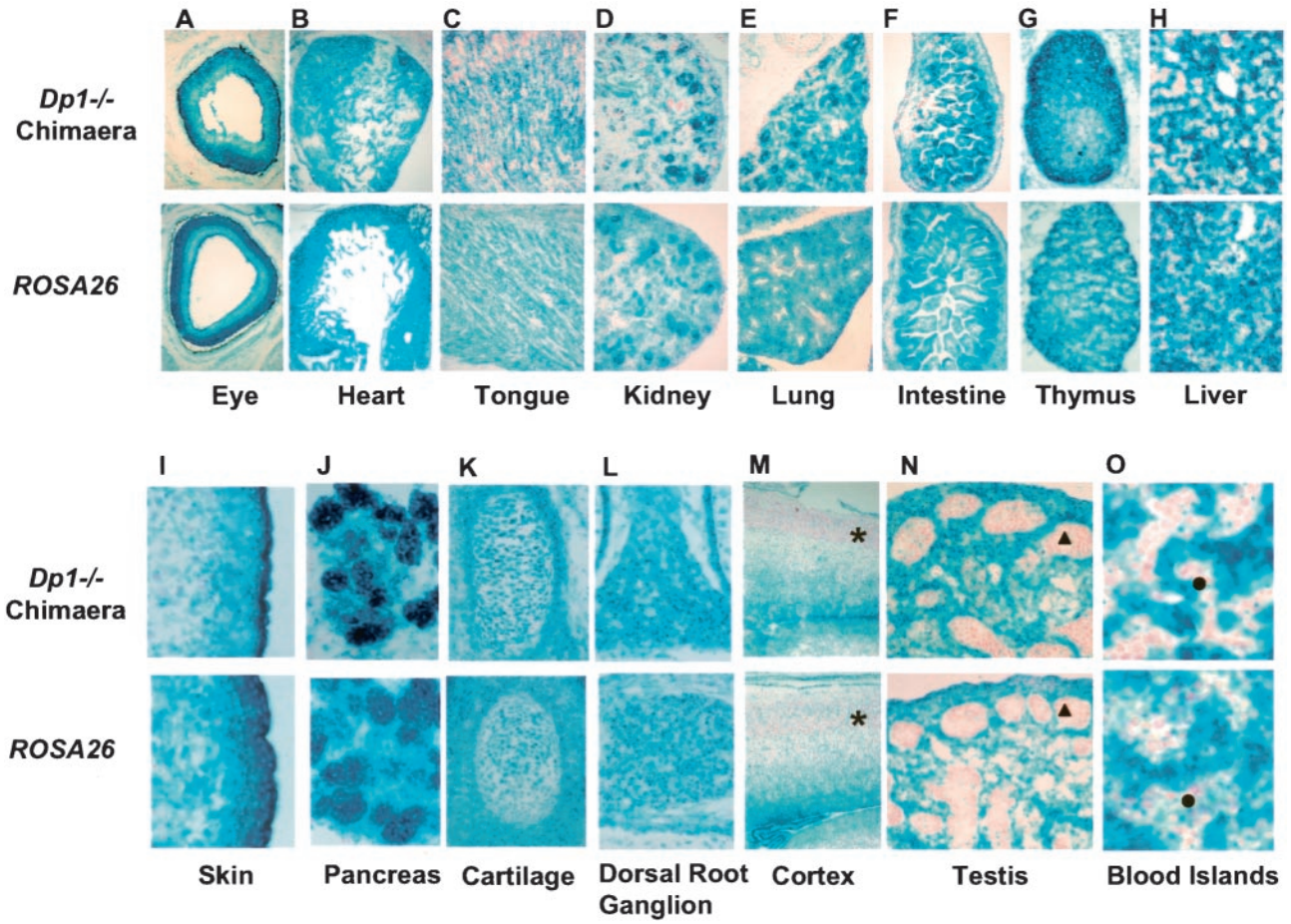

FIG. 3. *Dp1*-deficient ES cells are pluripotent. Cryosections from *Dp1*-deficient chimeras were stained with X-Gal to detect the cytoplasmically located β-galactosidase in *Dp1*-deficient; *ROSA26-LacZ* cells and counterstained with nuclear fast red to visualize all cells in the section. The vast majority of *Dp1*-deficient chimeric tissues consists of *LacZ*-expressing, *Dp1*-deficient cells, as judged by comparing the *LacZ* expression pattern to that in equivalent *ROSA26-LacZ* cryosections. A sampling of these X-Gal positively stained tissues from *Dp1*-deficient chimeras are shown at E17.5 (A to F, top row) and at E15.5(G to L, top row). Tissues: A, eye; B, heart; C, tongue (skeletal muscle); D, kidney; E, lung; F, intestine; G, thymus; H, fetal liver (parenchymal hepatocytes); I, skin; J, pancreas; K, cartilage; L, dorsal root ganglion. Equivalent X-Gal-stained cryosections from *ROSA26-LacZ* embryos are shown for comparison (lower row in panels A to F for E17.5 and in panels G to L for E15.5). However, a subset of cell types cannot be assessed with X-Gal staining since *LacZ* expression is not seen even in the *ROSA26-LacZ* control embryo cryosections (upper row of panels M to O for *Dp1*-deficient chimeras and lower row of panels M to O for *ROSA26-LacZ* embryos). Cell types and regions which cannot be scored (predominantly pink regions) are the neopallial layer (denoted by an asterisk) of the cortex near the lateral ventricle at E17.5 (M), male germ cells (indicated by a triangle) in the seminiferous tubules of the developing testis at E15.5 (N), and hematopoietic blood islands (denoted by the black circle) in the fetal liver shown at E15.5 (O). Clearly, *Dp1*-deficient ES cells are pluripotent and contribute to the development of most tissues and cell types.

developing central nervous system (e.g., neopallial cortex, midbrain, and spinal cord), germ cells in the developing testis, and hematopoietic blood islands in the fetal liver (three of which are shown in Fig. 3M to O, upper panels). However, these same regions are the ones in which we observe poor staining with X-Gal in *ROSA26-LacZ* embryos (Fig. 3M to O, lower panels), suggesting that the *ROSA26* promoter does not drive expression in these cell types. Thus, except for these isolated regions, our cryosection analysis demonstrates that *Dp1*-deficient ES cells are pluripotent, contributing well to most tissues and cell types.

DP2 is not increased in *Dp1*-deficient tissues. To begin to understand how *Dp1*-deficient tissues are able to proliferate and develop normally until E15.5 to E17.5, we reasoned that perhaps the expression of DP2 protein is increased to compensate for the loss of *Dp1*. Although we did not observe a change of DP2 expression in *Dp1*-deficient ES cells (Fig. 1D), we thought it possible that the differentiation of *Dp1*-deficient ES cells into various tissues could induce a change in DP2 expression.

To test this notion, we microdissected two sets of tissues from wild-type embryos and two high-contribution *Dp1*-deficient; *ROSA26-LacZ* chimeric embryos (29A and 29C) at E15.5. We then performed Western blot analysis for DP1 and DP2 with specific monoclonal antibodies (Fig. 4). First, we compared the expression of DP family members in three tissues (heart, lung, and intestine) that showed a high contribution of *Dp1*-deficient cells relative to the total population, as judged by the proportion of X-Gal-stained cells in cryosections (Fig. 4A). As expected, we saw little if any expression of DP1

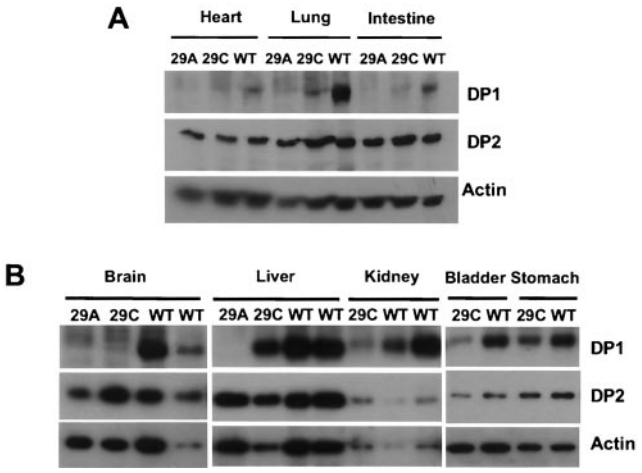

FIG. 4. Levels of DP2 are not increased with loss of *Dp1*. Two wild-type embryo and two hemisected *Dp1*-chimeric embryos (29A and 29C) at E15.5 were microdissected to isolate embryonic organs. Lysates from these tissues were used for Western blot analysis with monoclonal antibodies to DP1 (1DP06/TFD10, upper panel) and DP2 (BC-2 and G-12, middle panel) and probed for actin (lower panel) to normalize protein loading. For panel A, we tested three tissues (heart, lung, and intestine) to which *Dp1*-deficient ES cells contributed highly in these chimeras, as judged by X-Gal staining of the remaining half of the embryo. For panel B, we tested five tissues to which *Dp1*-deficient ES cells showed either a high contribution (kidney), a partial contribution (brain and liver), or an undetermined contribution (bladder and stomach), as judged by X-Gal staining of the remaining half of the embryo. All tissues tested from both *Dp1*-deficient chimeras, with the exception of fetal liver, exhibited greatly reduced levels of DP1 protein, indicating that the *Dp1*-deficient cells contributed well to these tissues. For fetal liver, only one of the *Dp1*-deficient chimeras (29A) displayed greatly reduced levels of DP1 protein, a finding indicative of a strong contribution by *Dp1*-deficient cells. Importantly, there is no consistent increase in DP2 levels observed with loss of *Dp1* even in highly developed embryonic tissues at late gestation.

protein in chimeric heart, lung, or intestine in contrast to the expression of DP1 protein in matched wild-type tissues (Fig. 4A, upper panel). Second, we compared the expression of DP family members in five tissues that showed either a high contribution (kidney), patchy contribution (brain and liver), or an undetermined contribution (stomach and bladder) of *Dp1*-deficient cells relative to the total population, as judged by X-Gal staining in cryosections (Fig. 4B). We observed much less expression of DP1 protein in chimeric brain, kidney, bladder, and stomach compared to the expression of DP1 protein in matched wild-type tissues (Fig. 4B, upper panel). We also observed much less expression of DP1 protein in liver from one of two chimeras examined (29A versus 29C). These Western blotting results suggest that *Dp1*-deficient cells contribute well even to organs that show uneven X-Gal staining in our cryosection analysis (i.e., brain and fetal liver).

Consistent with our result in *Dp1*-deficient ES cells (Fig. 1D), we observed no consistent upregulation of DP2 in *Dp1*-deficient chimeric tissues with actin as a loading control, regardless of the level of DP1 expression in the equivalent wild-type tissue (Fig. 4A and B, middle and lower panels). Thus, the normal development of *Dp1*-deficient chimeric tissues occurs without obvious compensation in the expression of DP2 protein.

Cell cycle gene expression is unchanged with loss of *Dp1*. To understand our result that *Dp1* deficiency did not compromise embryonic development in most tissues, we determined whether or not *Dp1* deficiency changed the expression profile of cell cycle-related genes, many of which are known E2F/DP target genes. To do this, our matched *Dp1*-deficient ES cell line (2B1), which was used for the bulk of our chimeric analysis, and the wild-type ES cell line (5F4) were grown logarithmically and used for an array analysis, in which the expression of 96 cell cycle-related genes was examined, including cyclins, cyclin-dependent kinases and their inhibitors, E2F/DP, and pRB family members, as well as replication and repair factors. FACS analysis was performed on these ES cell cultures to ensure that both populations were proliferating similarly (73 to 75% in S phase [Fig. 5A]). The gamma-irradiated embryonic fibroblast feeder cell population necessary to maintain the undifferentiated growth of ES cell colonies was removed prior to both the FACS analysis and array analysis.

Although the loss of *Dp1* expression is easily apparent in this ES cell array analysis, the vast majority of cell cycle-related mRNAs tested did not change with *Dp1*-deficiency, including *Dp2* (Fig. 5B, upper panels; see green boxed region for *Dp1* and *Dp2*, the second and third spots from the left). Only very moderate changes were seen in the expression of any other mRNA, as judged by comparing the abundance of internal loading controls, such as GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and actin (Fig. 5B, lower panels). Thus, loss of *Dp1* does not compromise the pattern of cell cycle gene expression in highly proliferative ES cells.

To determine whether loss of *Dp1* compromises the pattern of cell cycle gene expression in more differentiated tissues, we conducted the same array analysis with kidney and brain isolated from an E15.5 *Dp1*-deficient chimera or an E15.5 wild-type (*ROSA26-LacZ*) embryo. In either the kidney or the brain, *Dp1* deficiency did not alter the overall pattern of cell cycle gene expression from that seen in counterpart wild-type organs (Fig. 5C, kidney, and D, brain). This occurs despite these embryonic tissues being highly proliferative, as judged by the expression of PCNA and Ki67. We note that both the wild-type kidney and brain express little *Dp1* mRNA, although we have shown that these wild-type tissues clearly express detectable levels of DP1 protein. In summary, loss of *Dp1* in embryonic tissues that have differentiated substantially from their ES cell origin does not change the expression pattern of genes, many of which are E2F targets.

Taken together, the normal development of high contribution *Dp1*-deficient chimeras and the minor changes in gene expression of cell cycle-related mRNAs in *Dp1*-deficient ES cells and *Dp1*-deficient chimeric tissues demonstrate that, surprisingly, the loss of *Dp1* is tolerated very well in the embryonic compartment, in contrast to the severe defect we observed previously in the extraembryonic compartment with *Dp1* deficiency.

DISCUSSION

Clearly, we have demonstrated that *Dp1* is largely dispensable for the development of most tissues in the embryo. Our Western blot analysis has demonstrated that even two of the tissues that could not be evaluated with X-Gal staining are

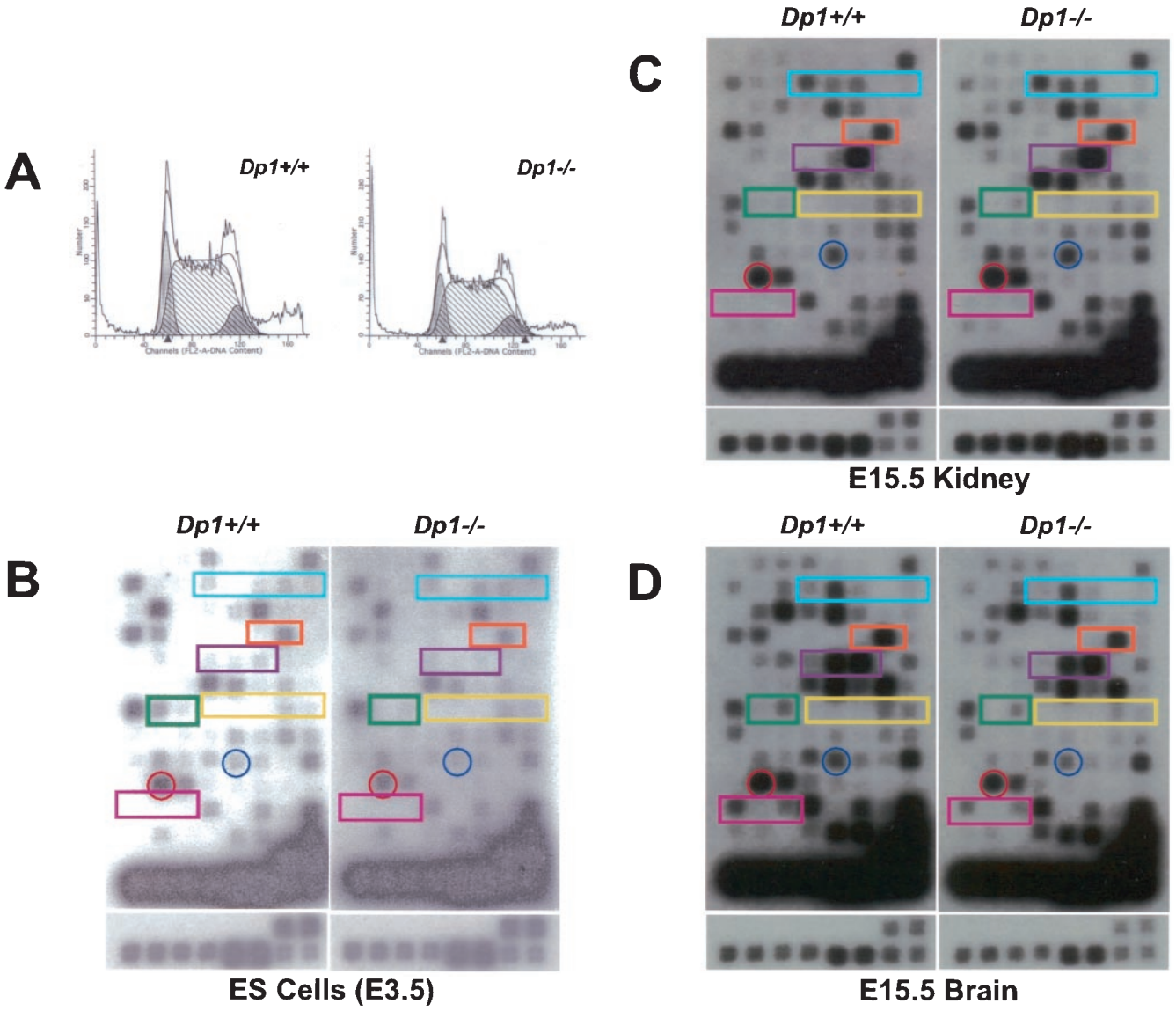

FIG. 5. Unchanged expression of cell cycle genes with the loss of *Dp1*. Three different RNA sources were analyzed by using arrays to determine whether *Dp1* deficiency changed the expression pattern of known cell cycle genes. These included ES cells that we established from E3.5 blastocysts and two organs (kidney and brain) from E15.5 embryos. (A) First, wild-type (5F4) and *Dp1*-deficient (2B1) ES cell lines were grown logarithmically, depleted of feeder cells and used for FACS analysis, for which ES cells were labeled with propidium iodide to measure DNA content. Both the $Dp1^{+/+}$ and $Dp1^{-/-}$ cultures yielded similar FACS profiles (73 to 75% S phase), indicating that they were proliferating similarly. (B) For array analysis of ES cell lines, aliquots of logarithmically grown cells prepared for panel A were used to isolate total RNA, which was then reverse transcribed and used to probe arrays that contain 96 cell cycle cDNAs. (C and D) For array analysis of E15.5 kidney (C) and E15.5 brain (D), total RNA was isolated and then reverse transcribed and used to probe identical arrays. For each set of arrays, a shorter exposure (lower panels) of the bottom two rows of the arrays, which contain internal controls, show that equal levels of starting RNA were used from the wild-type and *Dp1*-deficient sources. Boxed regions in the upper panels of B, C, and D indicate the positions of G1 cyclins (light blue), G1 cyclin-dependent kinases (orange), Cip/Kip inhibitors (purple), the DP family (green, *Dp1* on the left and *Dp2* on the right), the E2F family (yellow), and Rb family members (pink). Proliferation markers are indicated by circles (PCNA in red and Ki67 in blue). (The identity of all individually spotted cDNAs can be found at http://www.superarray.com.) In panel B, the absent expression of *Dp1* is clearly apparent in the *Dp1*-deficient (2B1) ES cell line, while *Dp2* expression remains unchanged in the absence of *Dp1*. In panels C and D, expression of *Dp1* is not apparent even in the wild-type tissue, although ample DP1 protein is observed in these tissues in Fig. 4B. Nevertheless, expression of *Dp2* remains unchanged in the absence of *Dp1*. In all array comparisons, the expression of the vast majority of cell cycle mRNAs remains unchanged with the loss of *Dp1*.

clearly composed of *Dp1*-deficient cells. Still, it is possible that a small subset of tissues that could not be evaluated may require *Dp1* for development. We are testing this possibility directly by generating a conditional knockout allele for *Dp1*, in which these specific tissues may be targeted by using Cre/loxP-mediated recombination.

The normal development of the majority of tissues in the *Dp1*-deficient chimeric embryos until late gestation (E15.5 to

E17.5) is surprising, particularly because the loss of *Dp1* so completely impairs the development of the extraembryonic compartment by E8.5 (10). In the trophectoderm lineage, loss of *Dp1* lowers the number of precursor cells and inhibits the rate of DNA replication in extraembryonic cell types arising from the trophectoderm. The latter effect is likely due to the known role of E2F/DP complexes in the induction of factors important for the initiation and also the execution of S phase. Extrapolating this mechanism to the embryonic compartment, we had expected to identify numerous tissues in which the loss of *Dp1* compromised proliferation and, thus, normal development. However, loss of *Dp1* is apparently well tolerated in the embryo once the extraembryonic requirement for *Dp1* is bypassed. Consistent with this result is our previous observation that morphologically abnormal *Dp1*-deficient embryos at E8.5 showed comparable levels of bromodeoxyuridine incorporation in the soma as wild-type sibling embryos despite the clear failure of bromodeoxyuridine incorporation in the extraembryonic compartments (10).

Four possibilities for *Dp1* function in the embryo proper are suggested by the normal development of the majority of tissues in *Dp1*-deficient chimeras until late gestation, despite the death of purely *Dp1*-deficient embryos before mid-gestation. First, *Dp1* may have no role in the embryo proper. This seemed unlikely given the high levels of detectable DP1 protein and RNA in embryonic tissues and its demonstrated role in DNA replication in the extraembryonic tissues (10) but is now a possibility. Second, E2F/DP family activity may not be necessary for normal development in the embryo proper. This is also unlikely given the detectable levels of DP1 and DP2 in embryonic tissues, the known role of E2F/DP complexes in the G_1/S transition, and the demonstrated requirement of E2F1-3 for proliferation of primary mouse embryonic fibroblasts (25). Furthermore, we detected no obvious change in the expression of cell cycle-related mRNAs (many of which are E2F/DP target genes) with *Dp1* deficiency (Fig. 5), suggesting that E2F/DP function is still optimal in highly proliferating *Dp1*-deficient ES cells, which we have clearly demonstrated are pluripotent, and in differentiated embryonic tissues. However, loss of *Dp1* might be tolerated by compromising both transcriptionally active and repressive E2F/DP complexes simultaneously, in a manner analogous to that in *Drosophila*, where a single E2F/DP complex (dE2F1/dDP) activates transcription and a single E2F/DP complex (dE2F2/dDP) represses transcription. Although loss of dE2F1 results in abnormal development, the simultaneous inactivation of both dE2F1 and dE2F2 leads to normal development at least until the late pupal stage (6). In addition, loss of dE2F1 leads to larval lethality and growth retardation, but loss of dDP leads to later pupal lethality with no growth retardation (20.a), suggesting that loss of activating and repressive dE2F/dDP complexes does not completely block embryonic development.

Third, DP2 may compensate for the loss of *Dp1* in the embryo proper subsequent to its inactivation. An increased level of DP2 in *Dp1*-deficient cells or tissues also would have supported the notion that DP2 replaced DP1 function; however, the static levels of DP2 protein and *Dp2* RNA that we detected in the absence of *Dp1* actually does not argue against any of the three possibilities. A search of the NCBI mouse genome database does not reveal the existence of any additional DP family members that might have suggested another route of compensation. Therefore, the unchanged levels of *Dp2* mRNA and DP2 protein in the absence of *Dp1* suggests that a low level of DP2 expression is sufficient for maintaining E2F/DP activity and thus proliferation in the embryo proper. The simultaneous inactivation of *Dp1* and *Dp2* in the embryo proper will test this hypothesis directly, and such experiments with the conditional knockout allele of *Dp1* are under way.

The fourth and last possibility is that *Dp1* is required for the correct development of embryonic tissues, but this requirement is masked in the *Dp1*-deficient chimeras by the presence of a small population of wild-type embryonic cells that rescue chimeric development in a non-cell-autonomous manner. Conditional inactivation of *Dp1* throughout the embryo proper at the epiblast stage by using Cre/loxP-mediated recombination or the construction of tetraploid embryos with *Dp1*-deficient ES cells will address this possibility.

Interestingly, conditional inactivation of *Rb* and tetraploid rescue experiments have recently been used to demonstrate that *Rb*-deficiency results in increased apoptosis and embryonic lethality by compromising the development of the placenta in mid-gestation (2, 24). Once this placental defect involving hyper-proliferation of labyrinthine trophoblast cells is bypassed, surprisingly normal development occurs in most embryonic tissues despite *Rb* deficiency (14, 23). Conditional inactivation of *Rb* specifically compromises the development of the brain, muscle, and lens in the embryo, sites where ectopic proliferation is observed (3, 13, 15). Furthermore, the simultaneous inactivation of cyclins E1 and E2 results in mid-gestational embryonic death, due to abnormal development of the placenta (7, 18). In this case, bypassing the endoreduplication defect of trophoblast giant cells in the placenta through tetraploid rescue experiments results in the normal development of late-gestational cyclin E1/2-deficient embryos. In contrast to the placental requirement for *Dp1*, *Rb*, or cyclin E1/E2, the loss of *Cdk2* has recently been shown to be dispensable for normal embryonic development, although it is required for meiosis and fertility (1, 17).

Thus, there are striking requirements for *Dp1*, *Rb*, and cyclins E1/2 in the extraembryonic compartment, whereas the requirements for these genes in the embryo proper are really quite limited. Understanding the nature of the requirements for these key cell cycle regulators specifically in the trophectoderm-derived lineages will be the focus of further study. Finally, discerning how the embryo develops so well in the absence of these cell cycle regulators is of great interest and will undoubtedly require a revision of current cell cycle paradigms.

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