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 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 trophoderm-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 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<sup>−/−</sup> mice were mated to ROSA26 mice in which a LacZ gene is integrated at the ROSA26 locus (5).
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 Dpl 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 expression 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′-CTTGTGATGCCGCTTGAGATATT-3′]) and L238 ([5′-CGCCGGCTGGTAAAGTGTTACGGT-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′-GGAACCGGAGAATATGATAGT-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 collected and the uterine horns of these females were allowed to recover in M2 medium at 37°C for 3 days. Blastocysts (at 3.5 days postcoitum). Logarithmically growing ES cells with a transgene were grown without feeders for several days and then treated with trypsin, replated onto embryonic feeder layers and, after 7 more days, undifferentiated ES cell colonies were picked, expanded, and frozen.

**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 reporter at the Dpl 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 placental allows 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-
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, confirming that Dp1-deficient ES cells contribute highly to most embryonic organs.
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...
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 \textit{ROSA26-LacZ} embryos (Fig. 3M to O, lower row of panels M to O for \textit{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, \textit{Dp1}-deficient ES cells are pluripotent and contribute to the development of most tissues and cell types.

DP2 is not increased in \textit{Dp1}-deficient tissues. To begin to understand how \textit{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 \textit{Dp1}. Although we did not observe a change of DP2 expression in \textit{Dp1}-deficient ES cells (Fig. 1D), we thought it possible that the differentiation of \textit{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 \textit{Dp1}-deficient; \textit{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 \textit{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...
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, we 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
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

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 (F4) 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.
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 trophoderm lineage, loss of Dp1 lowers the number of precursor cells and inhibits the rate of DNA replication in extraembryonic cell types arising from the trophoderm. 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 G1/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 (20a), 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 trophoblastic 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 trophoderm-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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