Loss of PICH Results in Chromosomal Instability, p53 Activation, and Embryonic Lethality

Albers, Eliene; Sbroggiò, Mauro; Pladevall-Morera, David; Bizard, Anna H.; Avram, Alexandra; Gonzalez, Patricia; Martin-Gonzalez, Javier; Hickson, Ian D.; Lopez-Contreras, Andres J.

Published in:
Cell Reports

DOI:
10.1016/j.celrep.2018.08.071

Publication date:
2018

Document Version
Publisher’s PDF, also known as Version of record

Citation for published version (APA):
Loss of PICH Results in Chromosomal Instability, p53 Activation, and Embryonic Lethality

Graphical Abstract

Highlights

- *Pich* is essential for embryonic development
- *Pich* KO embryos exhibit DNA damage, p53 activation, and apoptosis
- *Pich* heterozygous mice are born at sub-Mendelian ratios
- *Pich*-deficient MEF are resistant to RASV12/E1A-induced transformation

Authors

Eliene Albers, Mauro Sbroggiò, David Pladevall-Morera, ..., Javier Martin-Gonzalez, Ian D. Hickson, Andres J. Lopez-Contreras

Correspondence

ajlopez@sund.ku.dk

In Brief

Albers et al. show that PICH is essential for mouse embryonic development and that PICH deficiency limits oncogenic-induced cellular transformation. These findings suggest that PICH activity is critical during events requiring rapid cell proliferation such as embryonic development and tumorigenesis.

Albers et al., 2018, Cell Reports 24, 3274–3284

September 18, 2018 © 2018 The Author(s).
https://doi.org/10.1016/j.celrep.2018.08.071
Loss of PICH Results in Chromosomal Instability, p53 Activation, and Embryonic Lethality

Eliene Albers,1,4 Mauro Sbroggiò,1,4 David Pladevall-Morera,1 Anna H. Bizard,1 Alexandra Avram,1 Patricia Gonzalez,2 Javier Martin-Gonzalez,3 Ian D. Hickson,1 and Andres J. Lopez-Contreras1,5,*

1Department of Cellular and Molecular Medicine, Center for Chromosome Stability and Center for Healthy Aging, University of Copenhagen, Copenhagen 2200, Denmark
2Histopathology Core Unit, Spanish National Cancer Research Centre, Madrid 28029, Spain
3Transgenic Core Facility, Department of Experimental Medicine, University of Copenhagen, Copenhagen 2200, Denmark
4These authors contributed equally
5Lead Contact
*Correspondence: ajlopez@sund.ku.dk
https://doi.org/10.1016/j.celrep.2018.08.071

SUMMARY

PICH is a DNA translocase necessary for the resolution of ultrafine anaphase DNA bridges and to ensure the fidelity of chromosomal segregation. Here, we report the generation of an animal model deficient for PICH that allowed us to investigate its physiological relevance. Pich KO mice lose viability during embryonic development due to a global accumulation of DNA damage. However, despite the presence of chromosomal instability, extensive p53 activation, and increased apoptosis throughout the embryo, Pich KO embryos survive until day 12.5 of embryonic development. The absence of p53 failed to improve the viability of the Pich KO embryos, suggesting that the observed developmental defects are not solely due to p53-induced apoptosis. Moreover, Pich-deficient mouse embryonic fibroblasts exhibit chromosomal instability and are resistant to RAsV12/E1A-induced transformation. Overall, our data indicate that PICH is essential to preserve chromosomal integrity in rapidly proliferating cells and is therefore critical during embryonic development and tumorigenesis.

INTRODUCTION

Chromosomal stability is challenged each time a cell undergoes DNA replication and segregates its genetic material to the daughter cells. Many factors have evolved to safeguard genomic integrity during these processes and to promote the fidelity of chromosome transmission. The resolution of interlinked sister chromatids (e.g., due to unfinished DNA replication, DNA repair intermediates, DNA catenation) that persist from S phase must occur before or during the anaphase of mitosis for successful sister chromatid disjunction to occur (Mankouri et al., 2013). In rare cases, these sister chromatid entanglements may persist until late anaphase, whereupon they require several factors for their resolution. Among these factors is PICH (PLK1-interacting checkpoint helicase), which, despite its importance in mitosis, is poorly characterized. PICH, also known as ERCC6L, was discovered as a PLK1 interacting protein (Baumann et al., 2007). Based on its protein sequence and the results of small interfering RNA (siRNA)-mediated depletion experiments, PICH was initially defined as a DNA helicase and mitotic checkpoint protein (Baumann et al., 2007). However, subsequent studies revealed that PICH lacks helicase activity and does not regulate the spindle assembly checkpoint (Hübner et al., 2010). Instead, PICH has double-stranded DNA (dsDNA) translocase activity and binds preferentially to DNA under tension in vitro (Siebricher et al., 2013).

PICH has an unusual pattern of subcellular localization. It localizes in the cytosol during interphase and is recruited to chromatin after nuclear envelope breakdown in prometaphase. During anaphase, PICH decorates so-called ultrafine anaphase DNA bridges (UFBs) (Baumann et al., 2007; Chan et al., 2007). These mitotic DNA bridges can be found in most cells and are thought to consist of unresolved DNA replication intermediates and DNA catenanes that link the separating sister chromatids. UFBs usually arise from repetitive sequences such as telomeres, rDNA, and centromeres, or from common fragile sites (CFSs) (Nielsen and Hickson, 2016). PICH contributes to the resolution of UFBs, most likely by stimulating the catalytic activity of the TOP2a topoisomerase (Nielsen et al., 2015; Rouzeau et al., 2012). As a consequence, the lack of PICH leads to DNA segregation defects that result in the formation of micronuclei and aneuploidy (Hengeveld et al., 2015; Nielsen et al., 2015). Nevertheless, PICH is not essential for the viability of chicken DT40 or human cancer cell lines, although in both cases its depletion causes extensive genomic instability (Hengeveld et al., 2015; Nielsen et al., 2015).

Given the mitotic segregation defects observed in PICH-deficient cells, it is likely that PICH deficiency would have detrimental consequences in vivo; however, to date, there are no reports of animal models lacking functional PICH. Here, we have generated
Figure 1. Pich KO Leads to Embryonic Lethality in Mice
(A) Gene-targeting strategy for the generation of Pich KO mice (see Experimental Procedures for details). ORF, open reading frame. (B) Morphology of WT and Pich KO embryos at 10.5–13.5 days post-coitum (DPC). Pich KO embryos are smaller than WT embryos and die at approximately 12.5 DPC.

(legend continued on next page)
a Pich knockout (KO) mouse model to investigate the relevance of PICH at the organismal level in mammals. Our results show that PICH is essential for embryonic development. Pich KO embryos and Pich KO mouse embryonic fibroblasts (MEFs) show high levels of genomic instability, which is incompatible with a sustained viability.

RESULTS

Generation of a Pich Conditional KO Mouse Model
The mouse Pich gene is located on the X chromosome and consists of two exons: a short exon 1 (204 bp) and a long exon 2 (4,039 bp) that encodes most of the protein. To study the consequences of PICH deficiency in vivo, we generated a conditional Pich KO mouse model in which the deletion of the Pich gene could be induced by the expression of Cre recombinase. We targeted this locus in mouse embryonic stem cells (mESCs) using a standard recombination strategy with a targeting construct containing a neomycin cassette for selection and two long homology arms for efficient recombination. We targeted exon 2 to ensure that most of the PICH protein-coding region would be excised upon Lox recombination (Figure 1A). Due to the large size of exon 2, inserting the LoxP sites in such a way as to flank the entire length of the exon would compromise the efficiency of Cre-mediated recombination. Therefore, one LoxP site was positioned inside the 3′ UTR of exon 2 (Figure 1A). Using this strategy, we successfully generated Pich conditional KO (Pichlox/lox) mESC (Figures S1A and S1B). Following that, we generated male chimeras by microinjecting Pichlox/lox mESC into WT embryos that were able to transmit the Pichlox allele to their progeny. Pichlox males and Pichlox/lox and Pichlox/lox females were born at expected Mendelian ratios and did not show any obvious phenotypic abnormalities.

Pich KO Mice Die during Embryonic Development
After removal of the neomycin cassette via a cross with Flippase mice, Pichlox/lox females were crossed with UQ-CMV-Cre transgenic males to generate mice harboring a Pich KO allele. Like Pich, the UQ-CMV-Cre allele is located on the X chromosome and was therefore transmitted only to females. The deletion of the Lox-targeted locus was efficient, and Pich heterozygous (Het) females were obtained. To generate Pich KO mice, Pich Het females were crossed with wild-type (WT) males. A total of 50% of the male offspring from this breeding would be expected to be Pich KOs. However, all of the males born were found to be WT for Pich (Table 1), indicating that PICH is essential for embryonic development. More important, Pich Het females were born, indicating that the Pich KO gene is transmitted to the offspring. However, Pich Het females were born at sub-Mendelian ratios; 40% of the females were Pich Het, whereas 60% were WT (Table 1).

To characterize the phenotype of the targeted mice, we examined embryos obtained from Pich Het females crossed with WT males at different developmental stages. Pich KO male embryos were obtained at 10.5, 11.5, 12.5, and 13.5 days post-coitum (DPC), although at a lower proportion than expected (Table 2). These results indicate that most Pich KO embryos lose viability at or before 13.5 days of embryonic development. It should be noted that Pich Het female embryos were observed at expected Mendelian ratios (Table 2). Next, we examined the morphology of Pich KO embryos. Although Pich KO embryos exhibited normal gross morphology at 10.5 DPC, they were smaller than their WT or Pich Het counterparts (Figures 1B and 1C). This difference in size became more evident after 11.5 DPC. We did not observe any obvious tissue-specific alteration, but rather a general developmental arrest incompatible with viability, which was particularly evident in the few Pich KO embryos found at 12.5 and 13.5 DPC (Figure 1B). At these stages Pich Het embryos were indistinguishable from WT littermates in terms of size and morphology.

We confirmed the absence of PICH protein in Pich KO embryos by immunohistochemistry (IHC) (Figure 1D). PICH is located in the cytosol of cells in interphase, and its presence on chromatin is restricted to mitosis. As expected, PICH IHC revealed a predominantly cytosolic pattern of PICH localization in interphase cells. In WT and Het embryos, PICH was detected in most embryonic tissues (Figures 1D, S1D, S2A, and S3A).

Pich KO Embryos Show a Global Increase in DNA Damage and Apoptosis
To characterize the phenotype of the Pich KO embryos, we performed IHC analysis for factors involved in genome stabilization or DNA damage signaling. Because PICH promotes accurate chromosome segregation, we examined whether the loss of PICH led to chromosomal instability. We detected an increase in the proportion of cells that are positive for γH2AX, a marker of DNA damage, in 10.5 DPC Pich KO embryos (Figures 2A, 2D, and S1E). Evidence of DNA damage was detectable in all of the tissues in the Pich KO embryos, but it was particularly evident in some areas, including the embryonic brain. Next, we assessed whether activation of the DNA damage response in Pich KO embryos could also lead to p53 stabilization. We observed a high number of p53 positive cells in Pich KO embryos at day 10.5 DPC and onward (Figures 2B, 2D, S1F, and S2C). Moreover, we observed an increase in the number of apoptotic cells in Pich KO embryos, as determined by IHC staining for

<table>
<thead>
<tr>
<th>Male Mice</th>
<th>Female Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pich*</td>
<td>Pich*</td>
</tr>
<tr>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Pich**</td>
<td>Pich**</td>
</tr>
<tr>
<td>Observed</td>
<td>Expected</td>
</tr>
<tr>
<td>12</td>
<td>108</td>
</tr>
<tr>
<td>132</td>
<td>84</td>
</tr>
<tr>
<td>108</td>
<td>108</td>
</tr>
</tbody>
</table>

*We stopped genotyping males from this strain after finding that Pich KO males were not born, while we continued genotyping more females.
cleaved caspase-3 (Figures 2C, 2D, S1G, S2D, and S3D). These data indicate that the lack of PICH gives rise to DNA damage and p53 activation throughout the whole embryo, which ultimately leads to increased apoptosis and failure of the embryo to successfully complete development.

**Pich KO MEF Exhibit Mitotic Defects and Impaired Proliferative Capacity**

To further characterize the cause of embryonic lethality in the Pich KO mice, we generated MEFs from WT and Pich KO embryos at 10.5 and 11.5 DPC. We confirmed the absence of PICH expression in the KO MEFs by western blotting (Figure 3A). Pich KO MEFs proliferated at a lower rate than their WT counterparts (Figures 3B, S4A, and S4B) and entered into a senescent state after only three to four passages in culture, as determined by senescence-associated beta-galactosidase staining (Figures 3C and 3D). At early passages, Pich KO MEF incorporated 5-ethyl-2'-deoxyuridine (EdU) poorly, indicating a very limited replicative capacity, and also showed a greater tendency to accumulate in the G2 cell-cycle phase as compared to WT cells (Figures 3E and 3F). The loss of PICH resulted in various phenotypes associated with genome instability, including increased levels of 53BP1 nuclear foci (Figures 4A and 4B), micronucleus formation (Figures 4A, 4C, and S4C), and polyploidy (Figures 3E and 3F). These results confirm that PICH-deficient cells have chromosomal segregation defects that result in the activation of the DNA damage response (DDR) and a reduction in proliferative capacity.

**Pich Heterozygous Females Are Born at Sub-Mendelian Ratios**

As mentioned above, Pich Het females are born at sub-Mendelian proportions (Table 1). Of 216 females obtained, 132 were WT and 84 were Pich Het. Given that the proportion of Pich Het female embryos at 12.5 DPC was Mendelian (Table 2) and that we genotyped these mice at 4 weeks of age, ~20% of Pich Het females died either during the latter stages of embryonic development or perinatally. We believe that this lethality is most likely explained by the random X chromosome inactivation that affects the remaining Pich WT allele. Pich Het embryos showed a mosaic pattern of PICH expression that was compatible with random chromosome X inactivation (Figures 1D and S1D). However, we cannot rule out that the Pich gene escapes from full inactivation, as has been reported for Pich in mESCs (Marks et al., 2015), and low levels of PICH may still be expressed in all of the cells. The penetrance of the phenotype was variable, similar to human X-linked disorders. While ~20% of Pich Het females died, the rest were born and did not show any obvious abnormalities, as reflected in their similar appearance and weight as compared to WT littermates (Figures S4E and S4F). Pich Het females exhibited reduced fertility and they produced fewer pups (4.6 ± 2.3) than did WT females (15.8 ± 2.9) during a 14-week period (Figure S4G). Of note, two of the Pich Het females studied did not produce any viable pups (Figure S4G).

The Pich Het embryos analyzed at 10.5, 11.5, and 12.5 DPC were similar in size and morphology to their WT littermates (Figure 1C). Pich Het MEFs proliferated at a slightly lower rate than their WT counterparts (Figure 3B). Pich Het MEFs exhibited a mild but significant increase in the number of 53BP1 foci and micronuclei as compared to their WT counterparts (Figures 4B and 4C), indicative of genomic instability.

**Loss of p53 Does Not Rescue Pich KO Embryonic Lethality**

We observed increased p53 stabilization in Pich KO embryos (Figures 2B, S1F, S2C, and S3C). p53 stabilization leads to the induction of apoptosis and senescence (Haupt et al., 2003; Itahana et al., 2001), which could contribute to the embryonic lethality that is observed in Pich KO embryos. To investigate this possibility, we crossed our Pich KO model with Trp53 KO mice (Jacks et al., 1994). The loss of p53 has been demonstrated to rescue the lethality of other mouse models with defective DNA repair, such as Brca1- and DNA Ligase IV-deficient mice (Frank et al., 2000; Xu et al., 2001). However, no viable Pich KO mice were born in the absence of Trp53 or in a Trp53 heterozygous background (Table 3). In addition, we did not observe a rescue in the phenotype of Pich KO embryos at 10.5 days of embryonic development in the absence of Trp53 (Table S1; Figure S5). These data suggest that p53-dependent apoptosis and senescence are unlikely to be the sole drivers of Pich KO embryonic lethality.

To further characterize the interaction between these two genes, we derived WT and Pich KO mESCs from our mouse model. These cell lines were infected with retroviruses expressing either control small hairpin RNA (shRNA) or a previously validated Trp53 shRNA (Murga et al., 2009) (Figure S6A). We could not perform these experiments in MEFs due to the reduced proliferative capacity of Pich KO MEFs. The number of cells with high levels of γH2AX or more than two 53BP1 foci per nucleus were increased in Pich KO mESCs as compared to WT mESCs. Both of these phenotypes were slightly increased upon knockdown of Trp53 (Figures S6B and S6C).

In agreement with the above, rather than providing a rescue, our data indicate that the proportion of successful viable births in Pich Het females was further reduced in a Trp53 null background; only one Pich+/–;Trp53−/− female was born out of seven expected (Table 3). This observation suggests that a partial synthetic lethal interaction between these two genes may occur in a scenario with reduced levels of PICH.

**In Silico Analysis of the Impact of PICH Status on Human Cancer**

Given the relevance of PICH during embryonic development, we wanted to address the potential impact of PICH in

<table>
<thead>
<tr>
<th>Table 2: Genotypes of Embryos from Crosses between Pich+/- Females and WT Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPC</td>
</tr>
<tr>
<td>-----</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>10.5</td>
</tr>
<tr>
<td>11.5</td>
</tr>
<tr>
<td>12.5</td>
</tr>
<tr>
<td>13.5</td>
</tr>
</tbody>
</table>
tumorigenesis, which is another scenario of intense cell proliferation. We used the Xena browser (https://xena.ucsc.edu) and The Cancer Genome Atlas (TCGA) Pan-Cancer (PANCAN) data-set to analyze a potential correlation between PICH expression levels and the presence of mutations in TP53 and RB1, two of the tumor suppressor genes involved in cell-cycle regulation that are most frequently mutated in human cancers. Only truncating mutations (frameshift deletion or insertions and nonsense
Figure 3. Characterization of Pich KO MEFs
(A) Western blotting confirming the absence of PICH protein in extracts from two Pich KO MEF cell lines as compared to WT MEF cell lines. Vinculin was used as the loading control.

(legend continued on next page)
mutations) were analyzed to disregard the potential gain of function mutations. PICH expression was significantly higher in cancer patient samples with truncating mutations in TPS3 or in RB1 as compared to other samples without mutations in either of these genes (Figures S7A–S7D). Kaplan-Meier analysis showed that low PICH expression correlated with a better overall survival in cancer patients (Figure S7E). In addition, cancer type-specific survival was analyzed using the Kaplan-Meier Plotter tool (kmplot.com) (Györfy et al., 2010), showing that lower PICH expression correlates with better overall survival in breast and lung cancer patients (Figures S7F and S7G).

**PICH Is Required for RAS/E1A-Induced MEF Transformation and Growth**

To investigate the requirement for PICH in cellular transformation in vitro, we performed classical MEF transformation assays by transducing Pich KO and WT MEFs with RASV12/E1A constructs. This transformation assay combines Ras activation and the effect of the viral oncoprotein E1A, which overrides the G1/S checkpoint by sequestering RB1 protein (Bandara and La Thangue, 1991). We found that Pich KO MEFs were not able to form colonies following the expression of these proteins (Figures 4D, 4E, and S4D), indicating that RASV12/E1A transformation is insufficient to override the proliferative defects of Pich KO primary MEFs. Pich Het MEFs were also refractory to transformation, giving rise to a fewer number of colonies as compared to WT MEFs (Figures 4D, 4E, and S4D), despite the fact that primary Pich Het MEFs were only slightly affected in terms of proliferation as compared to WT counterparts (Figure 3B). These in vitro data, together with our in silico analyses, suggest that PICH activity is important to sustain the proliferation of certain types of transformed or cancerous cells.

**DISCUSSION**

In this study, we report a PICH-deficient mouse model, which has allowed us to investigate the role of PICH during normal mammalian development. That Pich is located on the X chromosome and has an unusual, two-exon gene structure (with a short exon 1 and a long exon 2) probably limited the generation of genetic models in the past.

Our results reveal that PICH is essential for embryonic development. Pich KO embryos exhibit genome instability that leads to generalized apoptosis, developmental arrest, and embryonic lethality. This genome instability is observed throughout the entire embryo. Thus, we believe that PICH function is important at the cellular level and particularly during embryonic development, when the rate of cell proliferation is very high. The penetrance of the lethal phenotype in the embryos is variable, with some KO embryos reaching day 13.5 (albeit with evidence of extensive cell death). A similar variability has been reported for other models that accumulate genomic instability, such as the Atr-Seckel (Murga et al., 2009) or Brca1−/−11 models (Xu et al., 2001). We believe that this variability likely reflects a stochastic degree of DNA damage accumulation and repair.

Pich Het females also exhibited a variable phenotype, ranging from perinatal lethality to the absence of any obvious defects. In principle, these Het females should be mosaic for Pich expression due to the random inactivation of one of the X chromosomes in female cells. However, and similar to several X-linked disorders, a bias in X chromosome inactivation (i.e., preferentially targeting the KO allele) could occur. We have analyzed PICH expression in four Pich Het embryos, observing a mosaic pattern that is compatible with random inactivation (Figures 1D and S1D). However, we cannot rule out an incomplete silencing of the WT Pich gene on the inactivated X chromosome, an observation that has been reported for Pich in mESCs (Marks et al., 2015). Further research will be required to understand the degree of Pich inactivation in adult mice and to investigate whether Pich Het females develop any age-associated phenotypes due to a progressive accumulation of DNA damage.

We observed a stabilization of p53 in Pich KO embryos, which is likely a consequence of the chronic activation of a DDR. In some mouse models with genomic instability, the activation of p53-dependent apoptosis and senescence programs accounts for the lethality observed during embryonic development (Frank et al., 2000; Xu et al., 2001). However, the inactivation of Trp53 did not rescue the lethality caused by PICH deficiency. We believe that the extensive genomic alterations accumulated during the first 12 days of embryonic development in Pich KO embryos are incompatible with viability. Furthermore, this is most likely attributable to deleterious genome alterations rather than to chronic DDR activation and the subsequent p53-induced apoptosis.

We investigated the genetic interaction between Pich and Trp53 in further detail and did not observe a significant rescue or potentiation of the phenotype of Pich KO embryos at 10.5 DPC in the absence of Trp53.

Of particular interest, we found that Pich Het females were born at a much-reduced frequency than expected in a Trp53 null background (only one was found of seven expected), suggesting that a partial synthetic lethal interaction between these genes may actually occur in a scenario with reduced levels of PICH. Consistent with this, we have found that PICH expression is significantly higher in cancer patient samples containing TPS3 mutations.
Figure 4. Genomic Instability and RASV12/E1A Transformation in Pich KO MEFs

(A) Representative images of immunofluorescence staining for 53BP1 in WT and Pich KO MEFs at early passages. Arrows indicate micronuclei, the number of which is increased in Pich KO MEFs.

(B) Quantification of cells with 53BP1 foci by high-content microscopy. Data correspond to three WT, one Pich Het, and two Pich KO MEF cell lines. Three wells were counted per cell line, with >1,000 cells for each three wells combined. Means and SDs are indicated.

(C) Quantification of cells with micronuclei. Images were acquired by an automated microscope and micronuclei were manually scored by two independent researchers. Data correspond to technical triplicates of three WT, one Pich Het, and two Pich KO MEF cell lines, counting three wells per cell line and at least 500 cells per sample. Means and SDs are indicated.

(legend continued on next page)
Table 3. Genotypes of Pups from Pich<sup>+/−</sup>:Trp53<sup>+/−</sup> Females and Trp53<sup>+/−</sup> Males

<table>
<thead>
<tr>
<th>Male Progeny</th>
<th>Pich&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Trp53&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>Trp53&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Pich&lt;sup&gt;−&lt;/sup&gt;</th>
<th>Trp53&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>Trp53&lt;sup&gt;+/+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>4</td>
<td>26</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Expected</td>
<td>7</td>
<td>15</td>
<td>7</td>
<td>7</td>
<td>15</td>
<td>7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Female Progeny</th>
<th>Pich&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>Trp53&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>Trp53&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Pich&lt;sup&gt;−&lt;/sup&gt;</th>
<th>Trp53&lt;sup&gt;+/−&lt;/sup&gt;</th>
<th>Trp53&lt;sup&gt;+/+&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>10</td>
<td>23</td>
<td>8</td>
<td>11</td>
<td>21</td>
<td>1</td>
</tr>
<tr>
<td>Expected</td>
<td>7</td>
<td>15</td>
<td>7</td>
<td>7</td>
<td>15</td>
<td>7</td>
</tr>
</tbody>
</table>

See also Table S1 and Figures S5, S6, and S7.

truncating mutations as compared to samples without mutations in TP53 (Figures S7A and S7B). This correlation is compatible with the idea that higher PICH expression may be required to sustain growth in tumors that have lost TP53. Further experimental research will be required to understand the functional relevance of these correlations. We believe that the Pich Het mESC is a relevant model for this purpose, particularly using cell differentiation protocols that promote X chromosome inactivation. In addition, our in silico analyses revealed that lower PICH expression correlated with an overall better survival across a wide range of cancer patient samples (TCGA Pan-Cancer dataset).

In line with the above, we observed that PICH is essential to sustain proliferation in transformed MEFs. In our studies, the use of an RasV12/E1A transformation assay combines Ras pathway activation with the expression of the viral oncoprotein E1A, which suppresses the G1/S checkpoint by sequestering RB1. We have also observed a correlation between high PICH expression levels and the presence of RB1 mutations in human cancer samples. One plausible explanation for these correlations is that PICH activity is important for tumors with altered cell-cycle checkpoints caused by mutations in tumor suppressor genes such as TP53 and RB1. Recent data derived from the use of siRNAs and shRNAs against PICH in breast and kidney cancer cell lines also support the idea that PICH is important for the proliferation of cancerous cells (Pu et al., 2017). Our findings are consistent with PICH being of critical importance for intense bouts of cell proliferation such as those during embryonic development and cancer. We believe that these findings open new avenues to investigate whether a strategy based on targeting PICH could provide a potential benefit for cancer patients.

EXPERIMENTAL PROCEDURES

Mouse Model Generation

The targeting construct for the Pich (Ercc8l) gene was generated by Gene Bridges (Heidelberg, Germany). It was linearized and incorporated into mESCs by targeted homologous recombination in collaboration with the Core Facility for Transgenic Mice at the University of Copenhagen. ESC clones containing the Pich<sup>lox(neo)</sup> allele were identified by PCR using primers Pich_F1 and Pich_R1 (Figures 1A, S1A, and S1B; see also sequences below). The expression of the allele was confirmed by RT-PCR (Albers et al., 2017) using primers Pich_F3 and Pich_R3 (data not shown; see also primers below). Pich<sup>lox(neo)</sup> ESCs were injected in mouse morulae, which were then transferred to pseudo-pregnant female mice to generate chimeras. The chimeras were then crossed with FLP-transgenic females (JAX strain 003800, Jackson Laboratory) (Rodriguez et al., 2000) and produced Pich<sup>lox(neo)</sup> female progeny. We generated Pich<sup>+/−</sup> females by crossing Pich<sup>+/−</sup> females with UQ-CMV-Cre males (JAX strain 006054) (Schwenk et al., 1995). The Pichcko strain has a mixed 129S2/C57BL/6N background. The Trp53 KO strain has been described previously (JAX strain 002101) (Jacks et al., 1994). Mice were housed at the animal facility of the Department of Experimental Medicine at the University of Copenhagen and the research was monitored by the Institutional Animal Care and Use Committee. All of the mouse work was performed in compliance with Danish and European regulations.

Mouse Genotyping

Mice were genotyped using three primers—two surrounding the loxP site in exon 2 of the Ercc8l gene, Pich_F1 (5′-CATGCTTGTCTCCTCCAGC-3′) and Pich_R1 (5′-GCTTAACGAACTAGGGCCT-3′), and one upstream of exon 1, Pich_F2 (5′-AAAGCCCCAACTACGTGCG-3′) (Figure 1A). A 390-bp WT fragment and a 493-bp Pich<sup>lox(neo)</sup> fragment were amplified using primers Pich_F1 and Pich_R1. The Pich KO allele was amplified by the Pich_R1 and Pich_F2 primer and generated a 220-bp fragment. RT-PCR was performed using primers Pich_F3 (5′-GTCTCCTTTCCTGGGCTCTC-3′) and Pich_R3 (5′-AGGCTACAGTGGCACCAGAA-3′).

Other primers used for genotyping were Cre (gCre5: 5′-TGTTTCCTCCGCA GAACCTGAG-3′ and gCre3: 5′-GACCTGTATTGGCACGTTACC-3′), Ripplase (FLP_F: 5′-CCATTCATTGCGGGTGATCG-3′ and FLP_R: 5′-GCTA CTGGGAGACTCAGTGG-3′), Trp53 WT allele (KM057: 5′-GTGGTCCTTTCCTGGGCTCTC-3′) and Trp53 KO allele (KM055: 5′-GTGGAGAGGGAGAAAAATGT GCCAGCC-3′ and KM054: 5′-TTACCGAGGCCCTGCGCTGATG-3′).

Embryo Characterization

Female mice were sacrificed between 10.5 and 13.5 DPC and embryos were kept in PBS and dissected. Yolk sac DNA was used for genotyping. Embryos were genotyped using the Pich_F1, Pich_F2, and Pich_R1 primers described in Mouse Genotyping. Embryo gender was determined by genomic PCR of
Xist and SRY using the following primers: Xist_F (5'-GCTTGTCTTCTGTTACTCCAGTGTCTTG-G), Xist_R (5'-ATTCTGACCTATTGAGGAA), SRY_F (5'-GCATTATGTTGCGTCTCAG), and SRY_R (5'-CCAGTCTGCTCTGATGTA-G). Images were taken with an Olympus SC50 camera (Olympus) and a Leica M125 stereomicroscope (Leica Microsystems) before embryo fixation.

Embryos at 10.5 DPC were obtained by in vitro fertilization of oocytes from superovulated Pich+/+; TriPS3−/− females with sperm from TriPS3−/− males and transferred to pseudopregnant recipient mothers. These procedures were performed following CARD/Infrafrontier protocols (https://www.infrafrontier.eu/knowledgebase/protocols/cryopreservation-protocols).

IHC

The IHC analyses were performed in collaboration with the Histopathology Core Unit at the Spanish National Cancer Research Centre (CNIO, Madrid, Spain). Mouse embryos were fixed in 4% neutral buffered formalin (Sigma-Aldrich), paraffin embedded, and processed according to standard procedures. For different staining methods, slides were deparaffinized in xylene and samples were rehydrated through graded concentrations of ethanol in water. Consecutive 3-μm sections were stained with H&E, and for IHC, an automated immunostaining platform was used (Ventana Discovery XT, Roche Diagnostics, or Autostainer Link, Dako). Antigen retrieval was performed with high or low (CC1m or RibCC, Roche; PICH, Dako) pH buffer, following standard procedures. The primary antibodies used were 53BP1 (Novus Biologicals 100-304A2) and β-galactosidase (SA-gal) using the Senescence Cells Histochemical Staining Kit (Sigma-Aldrich CS0030).

MEF Transformation Assay

Early passage MEFS were transduced with a pBabe-RAS(E1A) retroviral vector (gift from M. Barbacid, CNIO, Madrid, Spain) or a pBabe empty vector as a control according to standard procedures. Two days after transduction, cells were selected with 2 μg/mL puromycin for 2 days. After selection, 10,000 cells were seeded on 10-cm Petri dishes and stained with crystal violet after 2 weeks.

mESC Transduction

mESCs were obtained from in vitro fertilization of Pich+/+: oocytes with WT spermatocytes, performed at the Core Facility for Transgenic Mice at the University of Copenhagen. For the transduction with retroviral vectors encoding control and p53 shRNA previously validated (Murga et al., 2009), 300,000 cells were transduced in suspension with 1 mL retroviral supernatant and 1 μg/mL polybrene. After selection with 1 μg/mL puromycin, cells were seeded on gelatin-coated μCLEAR 96-well plates for immunofluorescence and high-content microscopy (see above).

Statistical Analysis

Statistical significance was determined with the unpaired t test in GraphPad Prism 7. p values are indicated above the graphs: *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p ≤ 0.0001.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.08.071.

ACKNOWLEDGMENTS

This work was funded by the Danish National Research Foundation (DNRF115), the Danish Council for Independent Research (Sapere Aude, DFF-Starting Grant 2014, DFF-4004-00185), the Danish Cancer Society (KBVU-2014-R09-A6031 and KBVU-2017_R167-A11063), the European Research Council (ERC-2015-STG-679068), and the Lundbeck Foundation (R218-2016-415). We thank Toyota Fonden and Læge Sofus Carl Emil Friis og hustru Olga Doris Fond for funding the acquisition of the high-content microscope used in this study. We thank Dr. Hocine Marikouri and Dr. Berta L. Sanchez-Laorden for critical reading and editing of the manuscript. We thank María Gómez and Zaira Vega from the CNIO Histopathology Core Unit for technical support. We also thank the Core Facility for Transgenic Mice and the Department of Experimental Medicine at the University of Copenhagen.


