Uncoupling of S phase and mitosis in cardiomyocytes and hepatocytes lacking the winged-helix transcription factor Trident

Wouter Korver*†‡, Marco W. Schilham*†§, Petra Moerer*, Maurice J. van den Hoff¶, Klaas Dam*, Wouter H. Lamers¶, René H. Medema¥ and Hans Clevers*

In order to maintain a stable karyotype, the eukaryotic cell cycle is coordinated such that only one round of S phase precedes each mitosis, and mitosis is not initiated until DNA replication is completed. Several checkpoints and regulatory proteins have been defined in lower eukaryotes that govern this coordination, but little is known about the proteins that are involved in mammalian cells. Previously, we have shown that the winged-helix transcription factor Trident – also known as HFH-11, FKL16 and WIN [1–3] – is exclusively expressed in cycling cells and is phosphorylated during mitosis [1,4]. The cellular function of Trident has yet to be described, however. Here, we have shown that disruption of the *Trident* gene in mice resulted in postnatal death, most probably because of circulatory failure. Histological analysis of *Trident*$^{-/-}$ embryos from embryonic day 10 (E10) onwards revealed a specific, characteristic defect in the developing myocardium. The orientation of the myocytes was highly irregular and the nuclei of these disorganized cardiomyocytes were clearly polyploid with up to a 50-fold increase in DNA content. Polyploidy was also observed in embryonic hepatocytes. Our results indicate that expression of *Trident* is required to prevent multiple rounds of S phase in the heart and the liver. Trident therefore appears to have a role in preventing DNA re-replication during the G2 and M phases.

Addresses: *Department of Immunology, University Hospital Utrecht, P.O. Box 85500, 3508 GA, Utrecht, The Netherlands. ¶Department of Anatomy and Embryology, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ, Amsterdam, The Netherlands. ¥Department of Hematology, University Hospital Utrecht, P.O. Box 85500, 3508 GA, Utrecht, The Netherlands.

Present addresses: ‡DNAX Research Institute, 901 California Avenue, Palo Alto, California 94304, USA. §Department of Pediatrics, University Hospital Leiden, P.O. Box 9600, 2300 RC, Leiden, The Netherlands.

†W.K. and M.W.S. contributed equally to this work.

Correspondence: Hans Clevers
E-mail: h.clevers@lab.azu.nl

Received: **17 September 1998**
Revised: **23 October 1998**
Accepted: **28 October 1998**

Published: **23 November 1998**

Current Biology 1998, 8:1327–1330
http://biomednet.com/elecref/0960982200801327

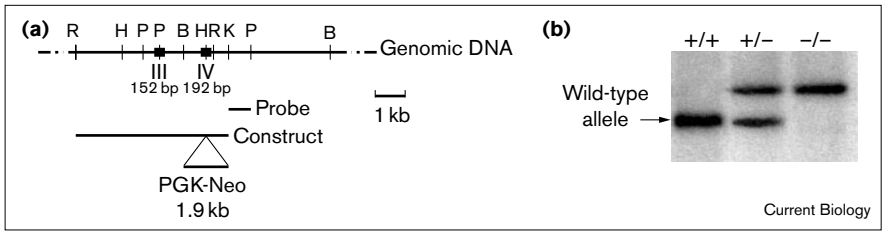
Results and discussion

In order to assess the function of Trident *in vivo*, we inactivated the *Trident* locus by means of gene targeting in embryonic stem (ES) cells (Figure 1). When litters derived from heterozygous crosses were analyzed at weaning, no homozygous mice were observed. Analysis of timed pregnancies revealed that the homozygous mutants died in the perinatal period (Table 1) and were usually found dead. The occasional live mutants that could be observed, however, were gasping and succumbed to apparent suffocation. Histological analysis of neonates revealed that their respiratory tracts were normal, but their hearts were dilated. The compact myocardium was significantly thinner (Figure 2a,b). At higher magnification, it was noted that the orientation of the myocytes was highly irregular and, most strikingly, the nuclei of these unstructured cardiomyocytes were clearly enlarged, suggesting polyploidy (Figure 2c,d). The nuclei of the myocytes in the outer wall of the compact myocardium of *Trident*$^{-/-}$ animals were of a similar size as those of their heterozygous and wild-type littermates, but the size of the nuclei increased dramatically as myocytes progressed closer to the ventricular cavity. Polyploidy was therefore most pronounced in the trabeculae. No clear difference was seen between the right ventricle and the left. Polyploidy was also present in the atria where, as in the ventricles, it was mainly seen in the trabeculae (Figure 2e,f).

To confirm the nuclear polyploidy of neonatal cardiomyocytes, the DNA content was visualized using 4,6-diamidino-2-phenylindole (DAPI) staining (Figure 3). By quantification of the intensity of DAPI fluorescence, we found that the enlarged nuclei of the cardiomyocytes from the *Trident*$^{-/-}$ littermates showed striking increases in DNA content of up to 50-fold (Figure 3b). As expected, no polyploidy was observed in the hearts of control *Trident*$^{+/-}$ animals (Figure 3a). The only other organ that appeared to be affected by a mutation in the *Trident* gene was the liver. Nuclei of increased size were observed in hepatocytes from *Trident*$^{-/-}$ embryos (Figure 2g,h). The number of polyploid cells in livers from neonatal *Trident*$^{-/-}$ animals was determined to be 5.5-fold higher than in their heterozygous littermates (Table 2). Premature development of polyploidy was not seen elsewhere in the newborn animals.

Trident$^{-/-}$ embryos were analyzed at E10, E13 and E16 to determine at which stage of development polyploidy developed in heart and liver. At E10, the differences

Figure 1



Targeted disruption of the *Trident* gene. **(a)** Structure of the *Trident* gene and the construct used for homologous recombination. The 152 bp exon III and the 192 bp exon IV (nomenclature as for the human gene [13]) are indicated. The probe used for genotype analysis is also shown. Restriction sites: B, *Bam*HI; H, *Hin*cII; K, *Kpn*I; P, *Pst*I; R, *Eco*RI. **(b)** Identification of *Trident*$^{-/-}$ embryos by Southern blotting. Examples of wild-type, heterozygous and homozygous mutant embryos are shown. A 1.5 kb *Bam*HI–*Kpn*I genomic fragment of the *Trident* gene was cloned and the PGK-Neo cassette (1.9 kb) was inserted (in opposite reading orientation) into a *Hin*cII site in exon IV of the murine *Trident* gene disrupting the sequence encoding the DNA-binding domain. A 3.6 kb *Eco*RI–*Bam*HI genomic fragment (containing another *Hin*cII site) was ligated back to the *neo*-containing fragment to restore the original configuration. ES cells from day E14 embryos were electroporated, selected in G418-containing medium and 160 colonies were screened for the correct targeting event, using the 0.8 kb *Kpn*I–*Pst*I flanking region as a probe for a Southern blot of genomic DNA digested with *Bam*HI. The frequency of homologous recombination was 1 in 40 clones. Targeted ES cell clones were injected in C57BL/6 blastocysts. Chimeras generated with one of the targeted cell lines transmitted the mutation to their offspring. Mice were routinely genotyped by Southern blotting of *Bam*HI-digested tail DNA, and hybridization with the *Kpn*I–*Pst*I flanking probe.

observed in homozygous mutants compared with heterozygous and wild-type embryos were minimal (see Supplementary material published with this paper on the internet), although increased numbers of polyploid cells were already detectable in the *Trident*$^{-/-}$ animals. Polyploidy in the cardiac trabeculae of homozygous animals was seen clearly at E13, and the distribution of polyploid cells in the developing heart and liver was similar to that seen in the neonates (see Supplementary material).

The number of polyploid hepatocellular nuclei in the livers of E13, E16 and neonatal *Trident*$^{-/-}$ animals was also increased (Table 2). The relative amount of polyploid cells in the liver of E13 mutant embryos was more than 10-fold higher than that found in heterozygous control embryos, while that in E16 and neonatal animals was 3.9-fold higher. This indicates that either the number of diploid cells increases faster or that polyploid cells are selectively cleared from the liver during embryogenesis. Assuming a Poisson distribution of the number of large nuclei in the heterozygotes, the number of large nuclei in *Trident*$^{-/-}$ animals is highly significant ($p < 0.001$).

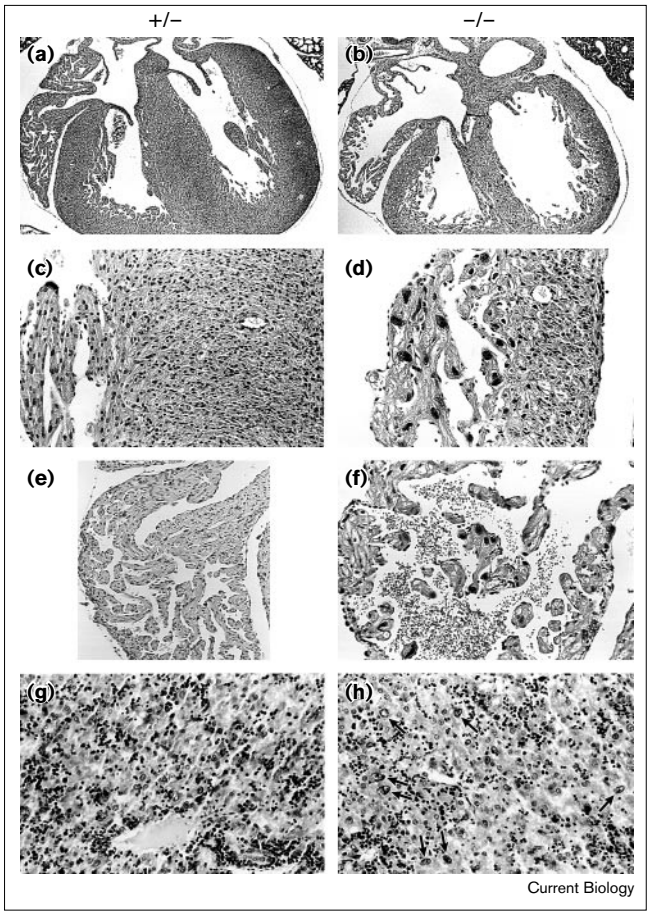
Cardiomyocytes and hepatocytes are known to become polyploid in wild-type animals as well, but this occurs much later in ontogeny. Mitotic polyploidization (up to a DNA content of 8N) of murine cardiomyocytes occurs predominantly during the first postnatal week [5], but it does not begin in the liver until after weaning [6]. Adult mouse liver consists of hepatocytes with DNA contents ranging from 2N to 128N. Typically, over 80% of cardiomyocytes and hepatocytes in the normal adult animal will be polyploid, with the majority of cells having a 4N DNA content. Moreover, most of these cells are binucleate [5,6]. Clearly, the polyploidization seen in the embryonal cardiomyocytes of the *Trident*$^{-/-}$ mice is different from the normal polyploidization, not only because it occurs at an earlier stage in development, but also because the extent of polyploidization is much greater. In addition, we have never observed binucleate cardiomyocytes in the *Trident*$^{-/-}$ mice. Moreover, the polyploid hepatocytes that arise early (at around E13) in the embryonal liver of *Trident*$^{-/-}$ mice seem to disappear again later (by E16), suggesting that these cells are unstable, which is in contrast to normal polyploidization where the percentage of polyploid cells continues to rise [6]. Therefore, the observed phenotype does not seem to be caused by premature polyploidization, but rather by a primary cell-cycle perturbation. The natural capacity of cardiomyocytes and hepatocytes to become polyploid might be important for the phenotype observed in the *Trident*$^{-/-}$ mice, however, and could explain the tissue specificity of this effect.

Table 1

Genotypes of embryos and mice generated from *Trident*$^{+/-}$ matings.

Age	Number	+/+ (%)	+/– (%)	–/– (%)
E12	30	9 (30)	14 (47)	7 (23)
E13	23	6 (26)	11 (48)	6 (26)
E14	27	4 (15)	18 (67)	5 (18)
E15	28	4 (14)	17 (61)	7 (25)
E16	34	11 (32)	15 (44)	8 (24)
E17	22	7 (32)	10 (45)	5 (23)
E18	39	8 (21)	20 (51)	11 (28)
E19	35	14 (40)	14 (40)	7 (20)
Neonate	77	24 (31)	44 (57)	9 (12)
4 weeks	84	35 (42)	49 (58)	0 (0)
Total	399	122 (31)	212 (53)	65 (16)

Figure 2

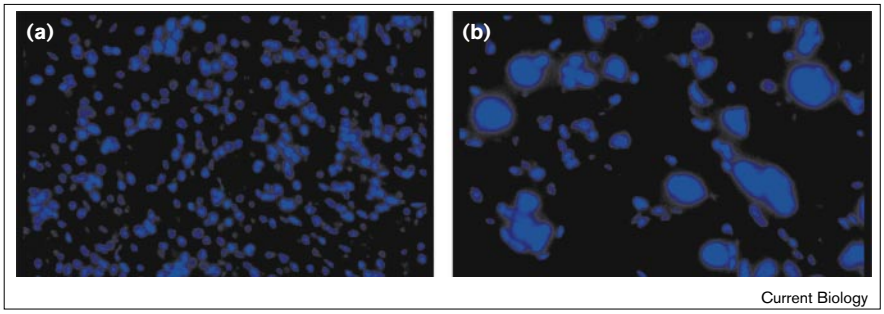


(a–f) Cardiac and **(g,h)** hepatic phenotypes of *Trident*$^{-/-}$ and *Trident*$^{+/-}$ neonates. Transverse sections of heterozygous (a,c,e,g) and the homozygous (b,d,f,h) mutants are shown. Higher magnifications of the left ventricle and the atrium are shown in (c,d) and (e,f), respectively. Note the hypoplastic myocardial wall and the highly irregular orientation of the myocytes in the *Trident*$^{-/-}$ animal. Also, the diameter of the subepicardial myocyte nuclei is comparable in *Trident*$^{-/-}$ and *Trident*$^{+/-}$ animals, but the size of the subendocardial and trabecular myocyte nuclei of both ventricle and atrium is far bigger in the *Trident*$^{-/-}$ animal than in the *Trident*$^{+/-}$ animal. In the liver, many nuclei of hepatocytes (arrows in h) are much larger in *Trident*$^{-/-}$ than in *Trident*$^{+/-}$ animals, but the erythropoietic nuclei are similar in size. Neonates were fixed in 4% formaldehyde overnight. After another overnight step in 70% ethanol, the embryos were routinely embedded in paraffin and 6 μm sections were stained with haematoxylin and eosin.

Our data indicate that the gross nuclear abnormalities in the cardiomyocytes and hepatocytes are the result of over-replication of DNA. This could arise through a perturbation of normal cell-cycle controls involved in initiation of DNA replication. Alternatively, these cells could have lost a checkpoint that would normally prevent re-initiation of DNA synthesis prior to mitosis. If so, this would suggest that Trident is required for the functionality of this cell-cycle checkpoint. Whether the polyploidization in the *Trident*$^{-/-}$ mice occurs by lack of a checkpoint or by a cell-cycle perturbation is not clear at present; however, our data clearly show that Trident plays a role in the coupling of S and M phase in certain tissues.

DNA re-replication is normally prevented because the origins of replication have lost the pre-replicative complexes during the first round of replication [7]. Formation of these pre-replicative complexes is inhibited during S, G2 and part of M phase by the action of cyclin-dependent kinases (Cdks) [8,9]. Re-replication can be induced if cells are depleted of Cdk activity in S or G2 [10–12]. The re-replication described in this paper could arise by a similar mechanism. Trident could be required for the formation of an M-phase-promoting cyclin–Cdk complex in the affected tissues. In this respect, it is interesting to note that putative DNA-binding sites for Trident have been found in evolutionarily conserved regions of the *cdc2* gene promoter [13]. Also, expression of *Trident* is induced at the onset of S phase and the protein is phosphorylated in M phase [1,14]. These characteristics could all point to a role for Trident in upregulating the transcription of M-phase cyclins or Cdks whose expression is induced in S-phase and shut off in anaphase.

Figure 3



DAPI staining of **(a)** *Trident*$^{+/-}$ and **(b)** *Trident*$^{-/-}$ neonatal hearts to determine relative DNA content and presence of polyploidy. Neonates were fixed in 4% formaldehyde overnight, followed by fixation in 70% ethanol. The embryos were then embedded in paraffin and sections were incubated in PBS containing 0.1 μg/ml DAPI for 30 min. Digital images of DAPI fluorescence were recorded with a META systems camera using fixed exposure times (Altlussheim, Germany). Relative DNA content was subsequently determined by quantification of intensity of fluorescence of individual nuclei using the META systems software. DNA content of polyploid cells in the *Trident*$^{-/-}$ cardiomyocytes was consistently increased 30–50-fold compared with diploid nuclei within the same images as well as compared with cells from a *Trident*$^{+/-}$ embryonic heart.

Table 2

***Trident*$^{-/-}$ embryos contain increased numbers of polyploid hepatocytes.**

Age	+/–	–/–	Fold difference
E13	19	261	13.7
E16	28	110	3.9
Neonate	24	132	5.5

The number of polyploid hepatocyte nuclei in *Trident*$^{-/-}$ and *Trident*$^{+/-}$ E13, E16 and neonatal livers is shown. The excess of polyploid hepatocytes in E13, E16 and neonate livers of *Trident*$^{-/-}$ mice was determined according to Abercrombie's equation [17]: NV = NA/D, where NV represents the number of polyploid hepatocytes per volume fraction; NA, the number of polyploid hepatocytes per section; and D, the average diameter of hepatocellular nuclei. It is clear from the equation that, if D in the polyploid populations of the *Trident*$^{-/-}$ and *Trident*$^{+/-}$ groups is similar, NA will be a fair estimate of NV. As simultaneously processed tissue sections were compared, the number of polyploid hepatocytes in *Trident*$^{-/-}$ mice and *Trident*$^{+/-}$ littermates was determined. To that end, six random photographs of either *Trident*$^{+/-}$ or *Trident*$^{-/-}$ embryos per age group were used to count polyploid nuclei. Care was taken not to include the typical multinodular nuclei of megakaryocytes.

Uncoupling of S and M phase has also been observed in cells lacking the Cdk inhibitor p21^{waf1}. In response to DNA damage, cells lacking p21^{waf1} arrest in G2 but then undergo additional rounds of S phase without mitosis [15]. Therefore, p21^{waf1} seems to have a role in the checkpoint that normally blocks DNA re-replication during G2. As putative Trident-binding sites have been identified in the promoter region of the p21^{waf1} gene, one could hypothesize that the phenotype of *Trident*$^{-/-}$ animals is due to the absence of p21^{waf1} expression. Mice lacking p21^{waf1} expression, however, do not have a similar phenotype as the *Trident*$^{-/-}$ mice, indicating that absence of p21^{waf1} cannot fully explain the observed polyploidization in the *Trident*$^{-/-}$ mice. Also, we have been unable to induce polyploidization in *Trident*$^{-/-}$ mouse embryo fibroblasts under conditions that cause re-replication in *p21$^{waf1-/-}$* cells, indicating that the function of Trident is different from the cell-cycle regulators that have been characterized to date.

The data presented here suggest that Trident has a role in preventing re-initiation of DNA replication before mitosis is initiated. Phosphorylation of Trident in M phase could inactivate this function of Trident and allow assembly of new pre-replicative complexes. In this way, activation of the mitotic Cdks would not only trigger mitosis, but would allow the next round of DNA replication by inactivation of Trident. Interestingly, another member of the forkhead/winged-helix family of transcription factors, Ches1 (checkpoint suppressor 1), was recently shown to control a G2 checkpoint in yeast [16]. This suggests that the coordination of cell-cycle events by winged-helix proteins might be a more general phenomenon.

Supplementary material

Histological analysis of cardiomyocytes and hepatocytes from embryos at E10 and E13 is published with this paper on the internet.

Acknowledgements

The authors thank the members of the Clevers lab for discussions and support. This study was financially supported by a PIONIER grant from the Dutch scientific organization NWO. M.J.v.d.H. is supported by a grant from the Netherlands Heart Foundation (M96.002).

References

1. Korver W, Roose J, Clevers H: **The winged-helix transcription factor Trident is expressed in cycling cells.** *Nucleic Acids Res* 1997, **25:**1715-1719.
2. Yao K-M, Sha M, Lu Z, Wong GG: **Molecular analysis of a novel winged-helix protein, WIN.** *J Biol Chem* 1997, **272:**19827-19836.
3. Ye H, Kelly TF, Samadani U, Lim L, Rubio S, Overdier DG, *et al.*: **Hepatocyte nuclear factor 3/fork head homolog 11 is expressed in proliferating epithelial and mesenchymal cells of embryonic and adult tissues.** *Mol Cell Biol* 1997, **17:**1626-1641.
4. Korver W, Roose J, Wilson A, Clevers H: **The winged-helix transcription factor Trident is expressed in actively dividing lymphocytes.** *Immunobiol* 1997, **198:**157-161.
5. Brodsky WY, Arefyeva AM, Uryvaeva IV: **Mitotic polyploidization of mouse heart myocytes during the first postnatal week.** *Cell Tissue Res* 1980, **210:**133-144.
6. Gerhard H, Schultze B, Maurer W: **Cell classes and ploidy distribution of isolated mouse liver cells.** *Exp Cell Res* 1971 **69:**223-226.
7. Stillman B: **Cell cycle control of DNA replication.** *Science* 1997, **274:**1659-1664.
8. Dahmann C, Diffley JFX, Nasmyth K: **S-phase promoting cyclin dependent kinases prevent re-replication by inhibiting the transition of replication origins to a pre-replicative state.** *Curr Biol* 1995, **5:**1257-1269.
9. Broek D, Bartlett R, Crawford K, Nurse P: **Involvement of p34cdc2 in establishing the dependency of S phase in mitosis.** *Nature* 1991, **349:**388-393.
10. Hayles J, Fisher D, Woollard A, Nurse P: **Temporal order of S phase and mitosis in fission yeast is determined by the state of the p34cdc2-mitotic B cyclin complex.** *Cell* 1994, **78:**813-822.
11. Hall LL, Th'ng JP, Guo XW, Teplitz RL, Bradbury EM: **A brief staurosporine treatment of mitotic cells triggers premature exit from mitosis and polyploid cell formation.** *Cancer Res* 1996, **56:**3551-3559.
12. Yang Q, Bassel-Duby R, Williams RS: **Transient expression of a winged-helix protein, MNF-β, during myogenesis.** *Mol Cell Biol* 1997, **17:**5236-5243.
13. Korver W, Roose J, Heinen K, Olde Weghuis D, de Bruin D, Geurts van Kessel A, Clevers H: **The human TRIDENT/HFH-11/FKHL16 gene: structure, localization and promoter characterization.** *Genomics* 1997, **46:**435-442.
14. Westendorf JM, Rao PN, Gerace L: **Cloning of cDNAs for M-phase phosphoproteins recognized by the MPM2 monoclonal antibody and determination of the phosphorylated epitope.** *Proc Natl Acad Sci USA* 1994, **91:**714-718.
15. Waldman T, Lengauer C, Kinzler KW, Vogelstein B: **Uncoupling of S phase from mitosis induced by anticancer agents in cells lacking p21.** *Nature* 1996, **381:**713-716.
16. Pati D, Keller C, Groudine M, Plon SE: **Reconstitution of a MEC1-independent checkpoint in yeast by a novel human fork head cDNA.** *Mol Cell Biol* 1997, **17:**3037-3046.
17. Weibel ER: **Morphometry of the human lung: state of the art after two decades.** *Bull Physiopathol Respir* 1979, **15:**999-1013.

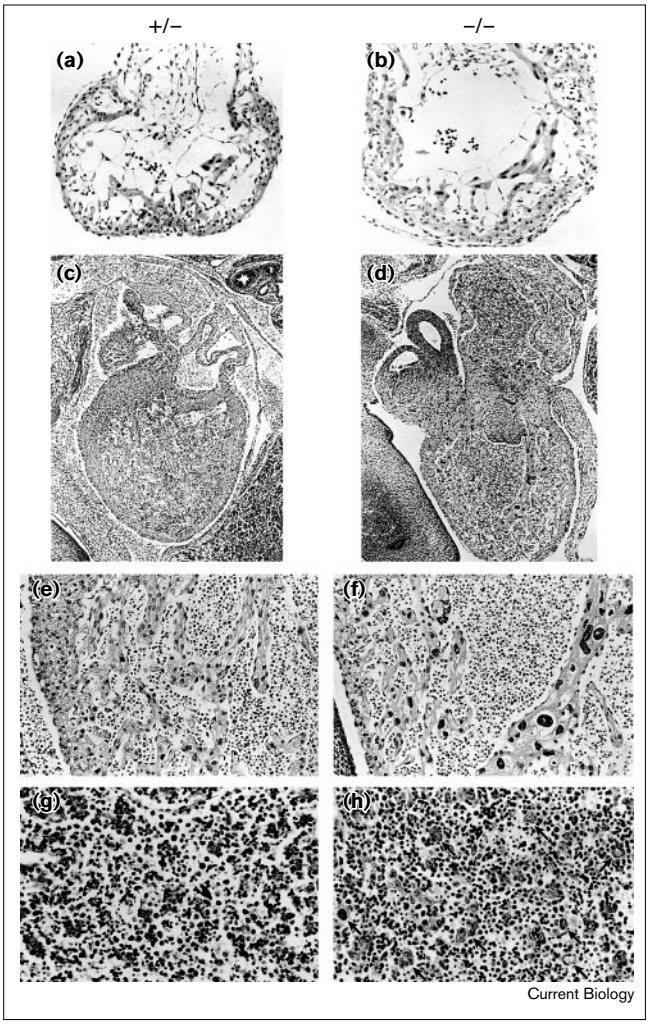
Supplementary material

Uncoupling of S phase and mitosis in cardiomyocytes and hepatocytes lacking the winged-helix transcription factor Trident

Wouter Korver, Marco W. Schilham, Petra Moerer, Maurice J. van den Hoff, Klaas Dam, Wouter H. Lamers, René H. Medema and Hans Clevers

Current Biology 23 November 1998, **8**:1327–1330

Figure S1



(a–f) Cardiac and **(g,h)** hepatic phenotypes of *Trident*$^{+/-}$ and *Trident*$^{-/-}$ embryos at (a,b) E10 and (c–h) E13. Sagittal sections of (a,c,e,g) heterozygous and (b,d,f,h) homozygous mutants are shown. Higher magnifications of the E13 hearts in (c,d) are shown in (e,f). Note the similar diameter of the subepicardial myocyte nuclei in *Trident*$^{-/-}$ and *Trident*$^{+/-}$ E13 embryos, and the much larger diameter of the trabecular myocyte nuclei in both ventricle and atrium of *Trident*$^{-/-}$ animals when compared with the *Trident*$^{+/-}$ animals. In the liver, the majority of hepatocyte nuclei (arrows) are much larger in *Trident*$^{-/-}$ embryos than in *Trident*$^{+/-}$ embryos, but the erythropoietic nuclei are of a similar size.