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Igf2 pathway dependency of the Trp53 developmental and tumour phenotypes

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 09 January 2012

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from the three referees whom we asked to evaluate your manuscript.

You will see that they all find the topic of your study interesting but they all feel that the manuscript is difficult to follow and recommend extensive rewriting to improve clarity and add details, as recommended. Please make sure that all tables, Figures and Supplementary figures and tables provided are called for in the text. Please also make sure that all text embedded in Figures are readable, provide high quality figures. Should you be able to address these criticisms in full, and provide an easier manuscript to read and understand even for the non-specialist, we would be willing to consider a revised manuscript.

Please note that that it is our journal's policy to allow only a single round of revision, and that acceptance or rejection of the manuscript will therefore depend on the completeness of your response and the satisfaction of the referees with it.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions, except under exceptional circumstances in which a short extension is obtained from the editor. Also, the length of the revised manuscript may not exceed 60,000 characters (including spaces) and, including figures, the paper must ultimately fit onto optimally ten pages of the journal. You may consider including any peripheral data (but not methods in their entirety) in the form of Supplementary information.

I look forward to seeing a revised form of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

The paper provides a mouse genetic test of interactions between two factors that are frequent players in a spectrum of human tumours and, thus, provides an insight into cancer genetics using a highly pertinent in vivo model.

Referee #1 (Other Remarks):

The paper provides an extensive analysis of genetic interactions between Igf2 and Tp53 using mouse knockout models. There are a number of novel findings relevant to both mouse development and tumourigenesis. Given the importance of both factors individually in each of these processes, the paper should be of considerable interest to a broad scientific audience. This is a rigorous study and the major conclusions are sound, however, the manuscript in its current state is incredibly difficult to follow. Many of my detailed comments are aimed at improving the clarity of the paper.

Results (p8, paragraph 1), Text says no abnormalities of the maternal decidua were seen in p53+/- mothers, but a significant number are indicated in Figure S1D.

Results (P8, paragraph 2), text says "Morphological appearance of embryos was normal between E9.5 and E18.5 (Fig. S3). Should this refer to Fig. S4? The title of Fig. S4 indicates incorrectly that "Morphological and growth abnormalities were observed".

Results (P8, paragraph 3), it is not clear to me what is meant by "Illumina RNA expression profile specific to all female progeny was derived only when derived from double heterozygous fathers". Possibly linked to this, the text discusses comparison of embryos derived from males with Igf2+m/-p or WT with Igf2+m/-p, p53+/- genotypes, yet in Figure 3B the heatmap indicates only Igf2+m/-p and Igf2+m/-p, p53+/- paternal genotypes. Variation in the heatmap signature suggests that perhaps the first 3 embryo samples were derived from a WT father.

Results (P10, paragraph 2), reference to Fig. 2E should be to Fig. 3E.

Results (P11, paragraph 2), text mentions MDM2 but I can see no data on this or any context for it.

Results (P11, paragraph 2), the sentence beginning "For the former tumours..", it is ambiguous which tumours are being referred to and the reference to Table S5 presumably should be to Table S4.

Results (P12, paragraph 2), "variable recombination frequency" is inferred from PCR data, but is this properly quanitative? It is also difficult to assess the inability to "generate germ line transmission of floxed alleles". This statement indicates that none of the animals carrying the floxed deletion transmitted this to their offspring, but how many carriers and how many of their offspring were tested?

Results (P12, paragraph 2), why not show the data comparing mean weights of Igf2 Cre-deleted and floxed alleles?

Results (P12, paragraph 3), should the reference to Fig 5A be Fig. 6C?

Results (P13, paragraph 1), should the reference to Fig 5B be Fig. 6D?

Discussion (P14, paragraph 1), the sentence beginning "Moreover, activation of IGF1R..." does not make sense.

Discussion (P14, paragraph 2), the sentence containing "... confirming that the synergistic lethality phenotype with p53 appeared novel..." is also unclear.

Discussion (P15, paragraph 1), the sentence beginning "Rescue mechanisms..." needs further explanation. Are these established mechanisms or speculation?

Discussion (P15, paragraph 1), the sentence beginning "In both the lethality signature..." needs rewording.

Discussion (P16, paragraph 2), there is an unhealthy amount of "sidestepping" in the Discussion (it is not terribly clear what it means)?

Discussion (P16, paragraph 2), change "the latter model" to "the chondrosarcoma model".

Table 1, legend talks about litter size but this information is not evident in the table, which is a Chi-squared analysis of genotypes found in two heterozygote intercrosses. Although stated that the 129J intercross produced litters of a significantly reduced size, it is not clear what the comparison was (e.g. matings between 129J wild type animals?).

Figure S1, legend could be clearer. Title could better reflect the content of the figure. Also, in Panel D, p53+/- females are also shown as having significantly more abnormal implantation sites than controls, but this is not stated in the legend.

Table S1, legend inconsistent with the data. Legend says at the 5th generation there were 0 female and 1 male survivors at P3.5 while the data says 1 male and 2 female. Legend says there is a significant difference in numbers at P0 for generations F1-F3 while the data shows a difference only for generations F1 and F2.

Figure 3, In panel D it is not made clear what each image represents, e.g. genotype, magnification). It would also be useful to indicate some of the key features.

Figure 4. key includes "undifferentiated round cell" but none are present on the pie chart.

Table S4, the term "mutation of the WT p53 allele" is confusing, and could perhaps be changed to "mutation of the non-targeted p53 allele".

Table S5, under "Developmental phenotype", "none" or "none observed" should perhaps be changed to "normal" or "unchanged". Is the use of different phrases meant to indicate anything specific, e.g. "no phenotype" versus "none observed"? Why is there no references to exencephaly reported in p53 mutant mice?

Figure S2, legend refers to Panel C which is not labeled on the figure (p53 PCR data?).

Table S6, in the first column "sex" is not a gene, why not list Sry and Raspn (explain the purpose of these primers in the legend)?

Labelling of graphs, lettering is generally rather small and I found I had to zoom in quite a lot to distinguish, say, "H19+m" from "H19-m". Labelling will be very difficult to read in the typset paper.

Ordering of main and supplementary figures and tables, needs to be checked as I seemed to be flicking backwards and forwards more than may be necessary. It may be that not all figures and tables are referred to in the text (e.g. there is a reference to Fig. S5 on P10, then the next supporting figure referred to is S8 on P11, and then S10 on P12).

Referee #2 (Comments on Novelty/Model System):

This paper is difficult to read for non geneticists.

The work describes a very interesting observation with potential therapeutic implications.
Previous studies had already indicated an Igf2 dependency for tumorigenesis - this study clearly establishes this.
The model used is highly relevant - in vivo mouse models.

Referee #2 (Other Remarks):

In this manuscript, Haley et al. further establish the genetic link -using the mouse as a model system- between the p53 tumor suppressor and Igf2. They show that inactivation of the paternal expressed allele of imprinted Igf2 causes embryonic lethality on a p53-null background (and not on a p53-proficient background). Moreover, post-natal lethality - likely due to lung haemorrhage - occurred in Igf2 paternal null allele female progeny only if derived from double heterozygote null fathers. Importantly, conditional ablation of Igf2 significantly delayed tumorigenesis on a p53-null background. Bi-allelic expression of Igf2 accelerated tumorigenesis and alleviated/decreased the frequency of p53 LOH on a p53 heterozygous background - at least in sarcomas. Together the data highlight an Igf2 genetic dependency for embryonic development and tumorigenesis in absence of p53.

Al in all this solid genetic study provides evidence of a synthetic lethal interaction between Igf2 and p53 in vivo - even if this interaction appears to be to some extent gender and tumor type specific/dependent. Knowing that the activity of the p53 tumor suppressor pathway is compromised in virtually all human cancers, the clinical implications of this finding are potentially far reaching. Although there are a number of shortcomings and unanswered mechanistic questions in this study - because of the solid nature of this genetic study and its potential importance for cancer therapy- I recommend its publication in EMBO Molecular Medicine.

Minor comments:
-Abstract -please tune down the statement that bi-allelic Igf2 expression is associated with reduction in p53 LOH - this is only seen in sarcoma!

-I am not found of the first part of the first sentence of the introduction, which to my opinion does not mean very much-although correct. The p53 gene is indeed expressed during embryonic development but the gene is also expressed in all adult tissues and the protein is made but constitutively degraded unless undergoing stress...Do the authors mean that the p53 protein is made and functional in embryonic tissues as opposed to adult tissues - which is not correct? Please rephrase.

-Page 4 - Please use the correct nomenclature - MDM2 an MDM4 for the human proteins and Mdm2 and Mdm4 for the mouse proteins - and not (H)MDM2/4 which is really confusing.

-Page 4 Gain of function of (H)MDM2/4 renders p53 functionally null, and reduces the age of tumor onset in LFS by 7-15 years. Could you please provide a Reference behind this statement?

-Page 4 - Ventura et al., 2007 - two other studies should be cited here as published together with the Ventura et al paper - one from the Lowe (in Nature) and one from the Evan (in Cell) lab.

-Page 6, first lane - (60% wild-type littermates) ? replace by 60% of the size of wild-type littermates

-Throughout the text mir or miR675 should be written miR-675 (be consistent).
-last paragraph of results section
These data are described in Figure 6.
Please replace Figure 5A by Figure 6C
Please replace Figure 5B by Figure 6D
And describe figure 6A and 6B in the text.

-Discussion - last sentence of the first paragraph - please rephrase:
...isoform-A leads transfers signals...?

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Referee #3:

Igf2 pathway dependency of the Trp53 developmental and tumour phenotypes.

In this paper Haley and collaborators communicate to their readers the importance of the IGF signaling in p53 function. They used a mouse genetic approach to determine the role of Igf2 allelic dosage in p53 function during development and tumor formation. Their data provide evidence that there is an Igf2 dependent developmental lethality in the p53 null mice and an Igf2 -dependent modification of tumor formation in these null mice. Results from their experiments help them reach the conclusion that p53 null developmental and tumor phenotypes appear Igf2 dependent, suggesting the importance of the IGF signaling in fetal in development and tumor formation when the p53 pathway has been disrupted. The authors suggest that regulation of the IGF2 ligand in tumor susceptibility syndromes associated with p53 may be a potential strategy for tumor prevention.

The authors use appropriate experimental design and come to sound and interesting conclusions. This is an interesting manuscript that adds new data to our knowledge of the regulation of p53. I believe this is a good report worthy of publication in EMBO Molecular Medicine. However, I have some suggestions regarding points that should be addressed by the authors before the publication of this manuscript.

1. The text in the Results and Discussion sections of the manuscript should be revised so it is more clear and easier to read to a wider audience.

2. There should be a reduced number of supplemental figures. Having so many figures can overwhelm the reader and distract her/him from the important points from the paper.

3. The lettering in the Main Figures are very small and difficult to read, I would suggest to use larger fonts for easier reading. In Figure 3, I would suggest to either remove the heatmaps in panels B and D and add them to the supplemental figures (they are difficult to read because of their size) or make them bigger by removing Figures in panel A and C since this data is thoroughly explained in the results. I would also enlarge Figure 3E to appreciate the lung histology and immunohistochemistry results.

4. I would like to see the standard error bars in your bar graphs, particularly in Figures 1 and 2. In addition, it is not necessary to write P30 in the Fig 1B graph since it is written in the figure legend and define NS in Figure Legend 1C.

5. In Figure 4D, the x-axis in not labeled.

6. The authors performed Illumina microarray technology to try to find genes that were differentially expressed between E9.5 female WT and Igf2+m/-p or double Igf2+m/-p , p56+/- embryos. Although mentioned in the materials and methods and figure legend, it should be stated in the text (page 8 of the manuscript) that embryos for the experiments were collected on day 9.5 of gestation. It would be good to mention why this age was selected for this gene expression analysis and not another age.

7. The authors mention a ten-fold increase in expression of fibronectin (Fn1), but this gene is not found in the Microarray Table S1, where 50 of the most extreme fold changes in expression are found. Was this finding confirmed by RT-PCR or Q-PCR.
There are other differentially expressed genes mentioned in the text and tables, but were any of these genes verified by RT-PCR or Q-PCR? Table S7 only mentions qPCR probes for Igf2, H19, p53, YY2 and controls b-actin and GAPDH.

8. There was a 7 fold change in cdk11b gene (it appears as the largest fold difference between female embryos derived from Igf2+m/-p , p56+/- fathers and those derived from Igf2+m/-p or WT fathers, but this is never discussed in the text, I would like to see the importance of this finding explored a bit further.

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9. The authors found that there was an accelerated tumor formation in p53 heterozygote females crossed with H19-m/+p mice. In what tissues where these tumors observed? What was the method by which these tumors were detected?

1st Revision - Authors' Response 24 March 2012

Referee #1 (Comments on Novelty/Model System):

The paper provides a mouse genetic test of interactions between two factors that are frequent players in a spectrum of human tumours and, thus, provides an insight into cancer genetics using a highly pertinent in vivo model.

We agree with this comment.

Referee #1 (Other Remarks):

The paper provides an extensive analysis of genetic interactions between Igf2 and Tp53 using mouse knockout models. There are a number of novel findings relevant to both mouse development and tumourigenesis. Given the importance of both factors individually in each of these processes, the paper should be of considerable interest to a broad scientific audience. This is a rigorous study and the major conclusions are sound, however, the manuscript in its current state is incredibly difficult to follow. Many of my detailed comments are aimed at improving the clarity of the paper.

We thank this careful reviewer for their comments. We have corrected the issues raised with respect to the clarity of the paper.

1. *Results (p8, paragraph 1), Text says no abnormalities of the maternal decidua were seen in p53+/- mothers, but a significant number are indicated in Figure S1D.*

We have clarified this point in Page 7, paragraph 2, and in Figure S1C and D (note the change in annotation of FigS1D to indicate haemorrhagic change and the clarification in the legend). We have been explicit in stating that the abnormalities were predominantly due to decidua haemorrhage visualised on H and E sections, and their frequency quantified in Figure S1D. Although present in $p53^{+/-}$ mothers, the frequency was less than $Igf2^{+m/-p}$, $p53^{+/-}$ mothers.

2. *Results (P8, paragraph 2), text says "Morphological appearance of embryos was normal between E9.5 and E18.5 (Fig. S3). Should this refer to Fig. S4? The title of Fig. S4 indicates incorrectly "Morphological and growth abnormalities were observed".*

We have clarified this point (Page 8, paragraph 1) by explicitly stating "Morphological appearances of embryos appeared normal between E9.5 and E18.5, even though $Igf2^{+m/-p}$ embryos were smaller (Fig. S3). The title of the legend for Figure S3 (correctly assigned from S3 as the order of supplementary figures has changed) has also been corrected to "Normal gross morphology but reduced growth in 129 $Igf2^{+m/-p}$ female embryos".

3. *Results (P8, paragraph 3), it is not clear to me what is meant by "Illumina RNA expression profile specific to all female progeny was derived only when derived from double*

heterozygous fathers". Possibly linked to this, the text discusses comparison of embryos derived from males with Igf2+m/-p or WT with Igf2+m/-p, p53+/- genotypes, yet in Figure 3B the heatmap indicates only Igf2+m/-p and Igf2+m/-p, p53+/- paternal genotypes. Variation in the heatmap signature suggests that perhaps the first 3 embryo samples were derived from a WT father.

We have rewritten the poorly constructed sentence (now on P9, paragraph 1) that now reads; "Illumina RNA expression profiles specific to all female progeny at E9.5 were then compared (Fig 3A). A specific gene expression signature was then obtained in progeny that were derived from double heterozygote fathers (*Igf2*$^{+m/-p}$, *p53*$^{+/-}$) (Fig 3B). Greater than two-fold differences in gene expression were observed for 1461 genes (Fig. 3B lists the most differentially expressed genes, with the top 50 differentially expressed genes further annotated (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-1001; note all microarray data and tables of genes described are available from this Arrayexpress website).

We have altered Figure 3 and eliminated the Venn diagrams in A (also in line with Reviewer 3). This has been replaced with a simplified diagram of the origin on the embryos used for analysis that now links directly to Figure 3B, the larger heatmap. The three parental genotypes have been clarified in the upper line of Figure 3B (starting with *WT*, wildtype). The first 5 embryos were in fact derived from a WT father and this is now annotated.

4. *Results (P10, paragraph 2), reference to Fig. 2E should be to Fig. 3E.*

As a result of altered presentation of Figure 3, Fig 2E has been changed to Fig3D.

5. *Results (P11, paragraph 2), text mentions MDM2 but I can see no data on this or any context for it.*

We have clarified this point on Page 12, paragraph 2 by explicitly stating that we were correlating with MDM2 localisation results displayed in Fig S7.

6. *Results (P11, paragraph 2), the sentence beginning "For the former tumours.", it is ambiguous which tumours are being referred to and the reference to Table S5 presumably should be to Table S4.*

We have clarified that the sentence refers to H19$^{-m/+p}$, p53$^{+/-}$ mice, and this now reads on Page 12 paragraph 1; "For the tumours that arose in *H19*$^{-m/+p}$, *p53*$^{+/-}$, we only detected 1/10 with mutations in the p53 DNA-binding domain (Table S4)".

7. *Results (P12, paragraph 2), "variable recombination frequency" is inferred from PCR data, but is this properly quanitative? It is also difficult to assess the inability to "generate germ line transmission of floxed alleles". This statement indicates that none of the animals carrying the floxed deletion transmitted this to their offspring, but how many carriers and how many of their offspring were tested?*

The reviewer is correct, the PCR data is inferred and non-quantitative in Figure S10. We have adjusted the sentence on page 12 paragraph 3 to read; "Non-quantitative PCR suggested that recombination was incomplete as most tissues retained the loxp flanked allele (Fig. S10B)". In terms of germline transmission, we have added the next sentences; "We were unable to either conditionally modify both alleles prior to embryonic day 8, or to generate germ line transmission of deleted alleles. Continued breeding of males with *p53* loxp flanked alleles resulted in 73 mice from 5.6 litters with normal transmission of alleles. Following recombination in utero, males with deleted alleles (*p53*$^{\Delta/\Delta}$ or *p53*$^{+/\Delta}$) generated only 7 progeny from 3 litters, with no transmission of the deleted *p53* allele".

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8. *Results (P12, paragraph 2), why not show the data comparing mean weights of Igf2 Cre-deleted and floxed alleles?*

We have adjusted Figure 6B to show significant differences in body weights (BW) as well as musculoskeletal (MSC) and organ weights, and added this to the sentence in page 13 paragraph 1.

9. *Results (P12, paragraph 3), should the reference to Fig 5A be Fig. 6C?*

This has been corrected now on page 13 paragraph 2.

10. *Results (P13, paragraph 1), should the reference to Fig 5B be Fig. 6D?*

This has been corrected (paragraph 2).

11. *Discussion (P14, paragraph 1), the sentence beginning "Moreover, activation of IGF1R..." does not make sense.*

We have edited this paragraph and this sentence now reads; “The resulting increase in supply of IGF2 would lead to activation of cell surface IGF1R and Insulin receptor isoform-A, to activate downstream signalling to all intact pathway components, and specific feedback mechanisms.”

12. *Discussion (P14, paragraph 2), the sentence containing "... confirming that the synergistic lethality phenotype with p53 appeared novel..." is also unclear.*

We have reworded this sentence to make the point clearer; “When *Igf2*$^{+m/-p}$ has been previously combined with genetic models of tumour susceptibility, including several developmental genes, there has been little evidence for combined lethality with this allele, suggesting that the synergistic lethality phenotype we now describe with *p53* is novel.”

13. *Discussion (P15, paragraph 1), the sentence beginning "Rescue mechanisms..." needs further explanation. Are these established mechanisms or speculation?*

These mechanisms are speculations, and we have reworded this sentence (page 14, paragraph 3); “We speculate that potential rescue mechanisms might include interactions of p53 with a range of epigenetic and X-specific targets, e.g. DNA methyltransferases (Dnmts) (Park et al, 2005; Peterson et al, 2003) and Xist expression (Panning & Jaenisch, 1996).”

14. *Discussion (P15, paragraph 1), the sentence beginning "In both the lethality signature..." needs rewording.*

We have reworded this sentence and edited this paragraph. “Expression of *Man1a2* gene, which encodes Golgi α1,2-mannosidase IB, was also elevated in *Igf2*$^{+m/-p}$, *p53*$^{+/+}$ female progeny”.

15. *Discussion (P16, paragraph 2), there is an unhealthy amount of "sidestepping" in the Discussion (it is not terribly clear what it means)?*

The paragraph (page 16, paragraph 1) has been edited in light of these comments, avoiding the use of this term.

16. *Discussion (P16, paragraph 2), change "the latter model" to "the chondrosarcoma model".*

This has been changed (page 16, paragraph 1).

17. *Table 1, legend talks about litter size but this information is not evident in the table, which is a Chi-squared analysis of genotypes found in two heterozygote intercrosses. Although stated that the 129J intercross produced litters of a significantly reduced size, it is not clear what the comparison was (e.g. matings between 129J wild type animals?).*

We have edited Table 1 legend and quoted the data on litter sizes that is now added to Figure 1 (section Fig. 1A). The two legends cross-refer.

18. *Figure S1, legend could be clearer. Title could better reflect the content of the figure. Also, in Panel D, p53+/- females are also shown, as having significantly more abnormal implantation sites than controls, but this is not stated in the legend.*

These points have been corrected in the legend. The title of the figure is more explicit; "Reduced litter size derived from $Igf2^{+/-}$, $p53^{+/-}$ females was associated with decidual haemorrhage". This is line with point 1 above.

19. *Table S1, legend inconsistent with the data. Legend says at the 5th generation there were 0 female and 1 male survivors at P3.5 while the data says 1 male and 2 female. Legend says there is a significant difference in numbers at P0 for generations F1-F3 while the data shows a difference only for generations F1 and F2.*

We have documented the crosses by addition of a further column in the figure to reflect the N (generations) backcrossed. The confusion arises because of the repeat of one cross but on a different background. We hope this makes the figure clearer.

20. *Figure 3, In panel D it is not made clear what each image represents, e.g. genotype, magnification). It would also be useful to indicate some of the key features.*

We have corrected the annotation for Figure 3D. We have indicated the genotypes, the magnification, the bronchioles, alveoli and features on the confocal images. We have corrected the figure legend on page 27.

21. *Figure 4. key includes "undifferentiated round cell" but none are present on the pie chart.*

We have corrected this in Figure 4E, that now shows lymphoma, carcinoma and sarcoma classification only.

22. *Table S4, the term "mutation of the WT p53 allele" is confusing, and could perhaps be changed to "mutation of the non-targeted p53 allele".*

We agree and have corrected the title of the table and the tale annotation to indicated intact and mutated non-targeted alleles of p53.

23. *Table S5, under "Developmental phenotype", "none" or "none observed" should perhaps be changed to "normal" or "unchanged". Is the use of different phrases meant to indicate anything specific, e.g. "no phenotype" versus "none observed"? Why are there no references to exencephaly reported in p53 mutant mice?*

We have altered the table to state "unchanged" rather than none observed. We have now referred to the exencephaly phenotype in p53+/- females in the table.

24. *Figure S2, legend refers to Panel C which is not labeled on the figure (p53 PCR data?).*

We have renumbered this Figure as S10 in order to improve the continuity of the figures with the text. We have now eliminated reference to part C, and reworded part B (page 9 Supporting figures).

25. *Table S6, in the first column "sex" is not a gene, why not list Sry and Raspn (explain the purpose of these primers in the legend)?*

We have corrected "Sex" for "Sry" PCR, and explained the purpose of these primers.

26. *Labelling of graphs, lettering is generally rather small and I found I had to zoom in quite a lot to distinguish, say, "H19+m" from "H19-m". Labelling will be very difficult to read in the typset paper.*

We completely re-annotated Figures 1-6 and updated the size and clarity of the labelling in light of this and the other reviewers comments. In particular, the size and consistency of the superscripts, e.g. $Igf2^{+m/-p}$ and $H19^{-m/+p}$. The size for reproduction of the Figures will be important in the published version.

27. *Ordering of main and supplementary figures and tables, needs to be checked as I seemed to be flicking backwards and forwards more than may be necessary. It may be that not all figures and tables are referred to in the text (e.g. there is a reference to Fig. S5 on P10, then the next supporting figure referred to is S8 on P11, and then S10 on P12).*

We have reordered the supplementary data to be more consistent with the text, e.g. S2 moved to S10 in supplementary figures, and moving microarray data on line. We have ensured continuity of numbering of tables and figures with respect to their place in the text.

Referee #2 (Comments on Novelty/Model System):

This paper is difficult to read for non geneticists. The work describes a very interesting observation

with potential therapeutic implications. Previous studies had already indicated an Igf2 dependency for tumorigenesis - this study clearly establishes this. The model used is highly relevant - in vivo mouse models.

We thank this reviewer for these comments and have taken steps to improve the fluency of the paper by an extensive re-edit.

Referee #2 (Other Remarks):

In this manuscript, Haley et al. further establish the genetic link -using the mouse as a model system- between the p53 tumor suppressor and Igf2. They show that inactivation of the paternal expressed allele of imprinted Igf2 causes embryonic lethality on a p53-null background (and not on a p53-proficient background). Moreover, post-natal lethality - likely due to lung haemorrhage - occurred in Igf2 paternal null allele female progeny only if derived from double heterozygote null fathers. Importantly, conditional ablation of Igf2 significantly delayed tumorigenesis on a p53-null background. Bi-allelic expression of Igf2 accelerated tumorigenesis and alleviated/decreased the frequency of p53 LOH on a p53 heterozygous background - at least in sarcomas. Together the data highlight an Igf2 genetic dependency for embryonic development and tumorigenesis in absence of p53.

Al in all this solid genetic study provides evidence of a synthetic lethal interaction between Igf2 and p53 in vivo - even if this interaction appears to be to some extent gender and tumor type specific/dependent. Knowing that the activity of the p53 tumor suppressor pathway is compromised in virtually all human cancers, the clinical implications of this finding are potentially far reaching. Although there are a number of shortcomings and unanswered mechanistic questions in this study - because of the solid nature of this genetic study and its potential importance for cancer therapy- I recommend its publication in EMBO Molecular Medicine.

Minor comments:

1. *-Abstract -please tune down the statement that bi-allelic Igf2 expression is associated with reduction in p53 LOH - this is only seen in sarcoma!*

We have modified the abstract, and included the statement that reduced LOH was only seen in carcinoma and sarcoma (not in lymphoma, see Figure 5B). The wording of this sentence now reads "Accelerated carcinoma and sarcoma tumour formation in *p53*$^{+/-}$ females with bi-allelic *Igf2* expression was associated with reductions in *p53* loss of heterozygosity and apoptosis". The figure annotation and legend have been modified to make this data clearer. The text in the discussion (page 16, paragraph 2) has also been changed to indicate carcinoma and sarcoma are predominantly associated with retention of the non-targeted allele (Page 11, paragraph 2, line beginning, In carcinoma and sarcoma...).

2. *-I am not found of the first part of the first sentence of the introduction, which to my opinion does not mean very much-although correct. The p53 gene is indeed expressed during embryonic development but the gene is also expressed in all adult tissues and the protein is made but constitutively degraded unless undergoing stress...Do the authors mean that the p53 protein is made and functional in embryonic tissues as opposed to adult tissues - which is not correct? Please rephrase.*

We have reworded the first sentence in light of this correct comment to "The tumour suppressor gene p53, expressed throughout development and adult life, is frequently disrupted in human cancers".

3. *-Page 4 - Please use the correct nomenclature - MDM2 an MDM4 for the human proteins and Mdm2 and Mdm4 for the mouse proteins - and not (H)MDM2/4 which is really confusing.*

We apologise for this oversight and have changed the nomenclature (page 4-paragraph 1, page 5-paragraphs 1 and 2, page 12-paragraph 1, page 16-paragraph 1).

4. *-Page 4 Gain of function of (H)MDM2/4 renders p53 functionally null, and reduces the age of tumor onset in LFS by 7-15 years. Could you please provide a Reference behind this statement?*

The reference is Bond et al Cell 2004. We have added this to this section (page 4- paragraph 1).

5. *-Page 4 - Ventura et al., 2007 - two other studies should be cited here as published together with the Ventura et al paper - one from the Lowe (in Nature) and one from the Evan (in Cell) lab.*

We have added both of these references and thank the reviewer for pointing these out.

6. *-Page 6, first lane - (60% wild-type littermates)? Replace by 60% of the size of wild-type littermates*

We have made this change to the text.

7. *-Throughout the text mir or miR675 should be written miR-675 (be consistent).*

We have changed the text to be consistent with miR-675 and miR-483 (e.g. Page 6-paragraph 1, page 11- paragraph 1, page 14- paragraph 1.)

8. *-last paragraph of results section*

   These data are described in Figure 6. Please replace Figure 5A by Figure 6C. Please replace Figure 5B by Figure 6D And describe figure 6A and 6B in the text.

We have corrected the errors in this paragraph. We have described Figure 6A and 6 in the text.

-Discussion - last sentence of the first paragraph - please rephrase:

...isoform-A leads transfers signals...?

We have changed this sentence to; “The resulting increase in supply of IGF2 would lead to activation of cell surface IGF1R and Insulin receptor isoform-A, to activate downstream signalling to all intact pathway components, and specific feedback mechanisms.”

Referee #3:

Igf2 pathway dependency of the Trp53 developmental and tumour phenotypes.

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In this paper Haley and collaborators communicate to their readers the importance of the IGF signaling in p53 function. They used a mouse genetic approach to determine the role of Igf2 allelic dosage in p53 function during development and tumor formation. Their data provide evidence that there is an Igf2 dependent developmental lethality in the p53 null mice and an Igf2 -dependent modification of tumor formation in these null mice. Results from their experiments help them reach the conclusion that p53 null developmental and tumor phenotypes appear Igf2 dependent, suggesting the importance of the IGF signaling in fetal in development and tumor formation when the p53 pathway has been disrupted. The authors suggest that regulation of the IGF2 ligand in tumor susceptibility syndromes associated with p53 may be a potential strategy for tumor prevention.

The authors use appropriate experimental design and come to sound and interesting conclusions. This is an interesting manuscript that adds new data to our knowledge of the regulation of p53. I believe this is a good report worthy of publication in EMBO Molecular Medicine. However, I have some suggestions regarding points that should be addressed by the authors before the publication of this manuscript.

1. *The text in the Results and Discussion sections of the manuscript should be revised so it is more clear and easier to read to a wider audience.*

We have rewritten the introduction, results and discussion section in light of this and the other reviewers' comments. We hope that the manuscript is easier to follow and the links better to the figures and supplementary data.

2. There should be a reduced number of supplemental figures. Having so many figures can overwhelm the reader and distract her/him from the important points from the paper.

We agree with the reviewer.

We have removed Tables S6, S7 and S8 and changed the text in the Methods to state that this information is available on request (page 17/ 18- methods section).

We have registered the supplementary microarray data with Arrayexpress ((http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-1001). This is now referenced in the text.

We have changed the order of the supporting Figures so that they follow the text, but have retained these figures as we feel they do show additional important data relevant to the main figures.

3. The lettering in the Main Figures are very small and difficult to read, I would suggest to use larger fonts for easier reading. In Figure 3, I would suggest to either remove the heatmaps in panels B and D and add them to the supplemental figures (they are difficult to read because of their size) or make them bigger by removing Figures in panel A and C since this data is thoroughly explained in the results. I would also enlarge Figure 3E to appreciate the lung histology and immunohistochemistry results.

We have completely represented this figure in the light of these comments. We have retained the heatmaps as they provide summaries for the expression data, and introduced a simpler part of the figure (Fig 3A) that explains the origins of the embryo samples and associated breeding (parental and embryo genotypes). We have also improved the annotation of Fig3D in line with this and other reviewers comments.

4. I would like to see the standard error bars in your bar graphs, particularly in Figures 1 and 2. In addition, it is not necessary to write P30 in the Fig 1B graph since it is written in the figure legend and define NS in Figure Legend 1C.

We have included error bars in all the figures that do not present absolute data. For example, Figure 1A has error bars, but Figure 1 does not because of the complexity they would generate in presenting growth data, Figure 1C, 2A, 5A, 5B and 6A are categorical data. Figure 2c has error bars. All the rest of the Figures have error bars as would normally be expected.

We have removed P30 from Fig 1C (was Fig1B) and have defined NS as non-significant in the legend.

5. In Figure 4D, the x-axis in not labeled.

This is now labelled.

6. The authors performed Illumina microarray technology to try to find genes that were differentially expressed between E9.5 female WT and Igf2+m/-p or double Igf2+m/-p , p56+/- embryos. Although mentioned in the materials and methods and figure legend, it should be stated in the text (page 8 of the manuscript) that embryos for the experiments were collected on day 9.5 of gestation. It would be good to mention why this age was selected for this gene expression analysis and not another age.

We have clearly stated in the text that embryos were obtained at E9.5 (page 8- paragraph 2) and added the sentence “This time was chosen because it is known that WT and $Igf2^{+m/-p}$ embryos are of similar size at this time, and we wished to eliminate confounding effects from later differences in growth”.

7. The authors mention a ten-fold increase in expression of fibronectin (Fn1), but this gene is not found in the Microarray Table S1, where 50 of the most extreme fold changes in expression are found. Was this finding confirmed by RT-PCR or Q-PCR.

There are other differentially expressed genes mentioned in the text and tables, but were any of these genes verified by RT-PCR or Q-PCR? Table S7 only mentions qPCR probes for Igf2, H19, p53, YY2 and controls b-actin and GAPDH.

The gene for fibronectin was only identified when the gene expression of the littermate embryos from double heterozygote fathers were compared. This analysis aimed to determine why only embryos with $Igf2^{+m/-p}$ died, whereas their female littermates with the genotype $Igf2^{+m/-p}$, $p53^{+/-}$ survived. It was only then that we detected the Fn1 gene as one that changed in line with the rescue of the lethal phenotype. This gene did not appear in the extreme fold changes group as these changes would have been present in all of these littermates. We have confirmed the Fn1 gene expression to be raised by Q-PCR, but several other candidates were not raised and more variable. We have not shown this data as we only had enough RNA for 2 embryos for comparison, and the the data is not fully convincing.

8. There was a 7 fold change in cdk11b gene (it appears as the largest fold difference between female embryos derived from Igf2+m/-p , p56+/- fathers and those derived from Igf2+m/-p or WT fathers, but this is never discussed in the text, I would like to see the importance of this finding explored a bit further.

We have not shown this data as we only had enough RNA remaining from two E9.5 embryos for a comparison derived from an *Igf2*$^{+m/-p}$ father, and so the data is not fully convincing. We are planning to pursue this finding and other observations in further experiments beyond the scope of this paper which is already wide ranging.

9. The authors found that there was an accelerated tumor formation in p53 heterozygote females crossed with H19-m/+p mice. In what tissues where these tumors observed? What was the method by which these tumors were detected?

The tumours were observed in the tissues listed in Figure S5. Most of the tumours in females were an excess of lymphomas, sarcomas (tumours of soft tissues in some cases involving underlying bone) and GI carcinomas. We state in the methods “Animals were checked daily for palpable tumours and evidence of systemic decline (weight loss, lack of grooming, reduced activity, piloerection)”.

We thank the reviewers for excellent and helpful comments that have greatly improved the text and presentation of this paper. We thank you for your consideration of this revised version for publication in EMBO Molecular Medicine.

2nd Editorial Decision 18 April 2012

Thank you for the submission of your revised manuscript to EMBO Molecular Medicine. We have now received the enclosed reports from the referees that were asked to re-assess it. As you will see the reviewers are now supportive and I am pleased to inform you that we will be able to accept your manuscript pending the following final amendments:

Please reply to the comments of referee #1 and modify the manuscript adequately

Please submit your revised manuscript within two weeks. I look forward to seeing a revised form of your manuscript as soon as possible.

I look forward to reading a new revised version of your manuscript as soon as possible.

Yours sincerely,

Editor
EMBO Molecular Medicine

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

The paper is a rigorous study of an important in vivo model that presents a number of significant novel findings.

Referee #1 (Other Remarks):

The paper is a rigorous study of an important in vivo model that presents a number of significant novel findings. All of my previous comments have been satisfactorily addressed and the clarity of presentation is much improved. I have only a few minor points to be considered:

Introduction (p3, paragraph 1), "of MDM2 promoter" should read "of the MDM2 gene promoter" or "of the MDM2 promoter".
Use of italics for gene symbols needs to be checked throughout, e.g. p4 "Mdm2" (paragraph 1) should be italicized whereas "IGFBP" (paragraph 2) is presently in italics but should not.

Results (p8, line 1), reference to Fig. 3A should I think be Fig. 3B.

Results (p10, line 5), the two references need to be put into context (how they relate to Fig. 4D), or they can be removed and this can be explained in the Discussion.

Results (p10, paragraph 2), It is not clear what is meant by "p52 LOH was detected in all tumours with lower Igf2 expression (Fig. 5C)". The figure shows that many of the tumours with the lowest Igf2 expression have an intact p53 allele.

Results (p11, penultimate line), should this read "with no subsequent transmission of the deleted p53 allele"? If so, were all 7 progeny tested or transmission and to what extent?

Discussion (p12 opening lines, should this read "...and so, although informative, significance for in vivo function may be limited"?

Referee #2 (Comments on Novelty/Model System):

The study is based on the use of a highly relevant mouse model.

The authors have made significant efforts in making the paper more accessible to non-geneticists. It reads much better now.

Referee #2 (Other Remarks):

The authors have adequately addressed all my concerns and criticisms.

This is a very interesting study which should be published in EMBO MM.

2nd Revision - Authors' Response 24 April 2012

Referee #1 (Comments on Novelty/Model System):

The paper is a rigorous study of an important in vivo model that presents a number of significant novel findings.

Referee #1 (Other Remarks):

The paper is a rigorous study of an important in vivo model that presents a number of significant novel findings. All of my previous comments have been satisfactorily addressed and the clarity of presentation is much improved.

Thank you for these comments and the very helpful and detailed review.

I have only a few minor points to be considered:

1. *Introduction (p3, paragraph 1), "of MDM2 promoter" should read "of the MDM2 gene promoter" or "of the MDM2 promoter".*

This has been amended to read “of the MDM2 gene promoter”.

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2. *Use of italics for gene symbols needs to be checked throughout, e.g. p4 "Mdm2" (paragraph 1) should be italicized whereas "IGFBP" (paragraph 2) is presently in italics but should not.*

The paper has been checked throughout and amended appropriately, and these two points have been addressed.

3. *Results (p8, line 1), reference to Fig. 3A should I think be Fig. 3B.*

Correct, this has been amended.

4. *Results (p10, line 5), the two references need to be put into context (how they relate to Fig. 4D), or they can be removed and this can be explained in the Discussion.*

We have removed these two references and placed them in the discussion. The wording in the discussion is now "We cannot however explain the female dependency of this phenotype, except to propose that it may depend on either the X chromosome, a strain modifier or via early hormonal changes (Harvey et al, 1993; Jacks et al, 1994)".

5. *Results (p10, paragraph 2), It is not clear what is meant by "p52 LOH was detected in all tumours with lower Igf2 expression (Fig. 5C)". The figure shows that many of the tumours with the lowest Igf2 expression have an intact p53 allele.*

The reviewer is correct in this point. We have clarified this sentence to read. "*p53* LOH was detected with equal frequency to intact alleles in all tumours with lower *Igf2* expression".

6. *Results (p11, penultimate line), should this read "with no subsequent transmission of the deleted p53 allele"? If so, were all 7 progeny tested or transmission and to what extent?*

None of the 7 progeny had a deleted (recombined) p53 allele when genotyped. We have altered this sentence. "....with no subsequent transmission of the deleted (recombined) *p53* allele".

7. *Discussion (p12 opening lines, should this read "...and so, although informative, significance for in vivo function may be limited"?*

We are happy to reword this sentence as suggested.

Referee #2 (Comments on Novelty/Model System):

The study is based on the use of a highly relevant mouse model.

The authors have made significant efforts in making the paper more accessible to non-geneticists. It reads much better now.

Referee #2 (Other Remarks):

The authors have adequately addressed all my concerns and criticisms. This is a very interesting study which should be published in EMBO MM.

Thank you for both comments and the helpful initial review.

Referee #1 (Comments on Novelty/Model System):

The paper provides a mouse genetic test of interactions between two factors that are frequent players in a spectrum of human tumours and, thus, provides an insight into cancer genetics using a highly pertinent in vivo model.

We agree with this comment.

We thank the reviewers for excellent and helpful comments that have greatly improved the text and presentation of this paper. We thank you for your acceptance of this revised version for publication in EMBO Molecular Medicine.