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Igf2 pathway dependency of the *Trp53* developmental and tumour phenotypes

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Insulin-like growth factor 2 (IGF2) and the transformation related protein 53 (Trp53) are potent regulators of cell growth and metabolism in development and cancer. *In vitro* evidence suggests several mechanistic pathway interactions. Here, we tested whether loss of function of p53 leads to IGF2 ligand pathway dependency *in vivo*. Developmental lethality occurred in *p53* homozygote null mice that lacked the paternal expressed allele of imprinted *Igf2*. Further lethality due to post-natal lung haemorrhage occurred in female progeny with *Igf2* paternal null allele only if derived from double heterozygote null fathers, and was associated with a specific gene expression signature. Conditional deletion of $Igf2^{fl/fl}$ attenuated the rapid tumour onset promoted by homozygous deletion of $p53^{fl/fl}$. 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. *Igf2* genetic dependency of the *p53* null phenotype during development and tumour formation suggests that targeting the IGF2 pathway may be useful in the prevention and treatment of human tumours with a disrupted Trp53 pathway.

INTRODUCTION

The tumour suppressor gene *p53*, expressed throughout development and adult life, is frequently disrupted in human cancers (Olivier et al, 2002). DNA damage, oncogene activation, telomere erosion, nucleotide depletion and hypoxia, can activate p53 cellular responses including cell-cycle arrest, apoptosis, senescence and DNA repair (Menendez et al, 2009; Riley et al, 2008; Vousden & Prives, 2009). Degradation of transformation related protein (Trp53) is mediated primarily by the human double minute 2 (MDM2/4) ubiquitin E3 ligase which is itself a transcriptional target of Trp53 (Marine & Lozano, 2010). Individuals with Li-Fraumeni syndrome (LFS) develop early onset cancers, and in 30–50% of cases, the tumours are attributed to germ-line heterozygote mutation of *p53* with associated loss of heterozygosity (LOH) of the wild-type (WT) allele (Malkin, 2011; Srivastava et al, 1990). Modifiers alter the age of tumour onset in LFS. A single nucleotide polymorphism (SNP) of the MDM2 (SNP 309) gene promoter regulates the relative expression level of this negative regulator of p53 (Bond et al, 2004). SNP 309 associated gain of function of *MDM2/4* expression renders p53 functionally null, and reduces the age of tumour onset in LFS by 7–15 years (Bond et al, 2004). A modifier in *p53* itself (Trp53 codon 72) also reduces the age of tumour onset, but is more modest at 2–3 years (Fang et al, 2010). Screening of LFS patients for early onset tumours significantly impacts overall survival, such that identification of pathway modifiers of lethal tumour phenotypes have implications for both screening and prevention therapies (Villani et al, 2011).

Mice with homozygous disruption of *p53* ($p53^{-/-}$) are viable except for an exencephalic phenotype in females (Armstrong et al, 1995; Clarke et al, 1993; Donehower et al, 1992).

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Homozygote animals die within 6 months, due mainly to thymic lymphoma. *p53* heterozygosity markedly reduces lymphoma, sarcoma and epithelial tumour latency and mirrors the phenotype of LFS (Donehower et al, 1992; Jacks et al, 1994). Increased *p53* expression and conditional restoration of *p53* function resulted in reduced tumour frequency, senescence and tumour regression (Martins et al, 2006; Ventura et al, 2007; Xue et al, 2007). *p53* is frequently mutated at specific 'hotspot' bases in tumours. Mutations can lead to gain of function, as $p53^{R172H/+}$ and $p53^{R270H/+}$ can increase in carcinomas and osteosarcoma in the former, and carcinoma metastasis in the latter (Lang et al, 2004; Olive et al, 2004). Modelling of *Mdm2* SNP alleles (SNP 309G of *MDM2*) in the mouse has validated effects on tumour latency (Post et al, 2010). Stabilization of mutant p53 is also required in tumours, and is frequently associated with aggressive tumour phenotypes that can be induced by similar modifiers to those that regulate WT p53 (Suh et al, 2011). Thus modelling potential pathway modifiers of *p53* loss and gain of function are important intermediary steps in the validation of clinically important pathway targets for tumour treatment.

Evidence *in vitro* suggests convergence between IGF-PI3K-AKT-mTOR and ARF-MDM2-p53 pathways, mainly through promotion of IGF-PI3K-AKT-mTOR pathway activation following loss of p53 function. Interactions include; phosphorylation of MDM2 by Akt (Feng et al, 2004; Mayo & Donner, 2001), transcription of the ligand *IGF2* (Lee et al, 2000; Zhang et al, 1996, 1998) and regulation of the insulin-like growth factor receptor 1 (*IGF1R*; Ungewitter & Scrable, 2010; Werner et al, 1996). Insulin-like growth factor binding proteins (IGFBP-2, -3 and -7) are negative regulators of IGF bio-availability and are also p53 target genes (Buckbinder et al, 1995; Grimberg et al, 2006; Suzuki et al, 2010). Function of p53 may be mediated by Phlda3, a negative regulator of Akt (Brady et al, 2011), and via activation of PTEN (Stambolic et al, 2001), TSC2, AMP1 (Feng et al, 2007), sestrins 1 and 2 (Budanov & Karin, 2008) or REDD1 (Ellisen et al, 2002).

IGF2 mRNA over-expression increases ligand supply in common human cancers mainly due to loss of imprinting (LOI, bi-allelic expression; Foulstone et al, 2005; Ito et al, 2008). Binding of insulin-like growth factor 2 (IGF2) to IGF1R and isoform A of the insulin receptor (IR-A) transmits downstream signalling via IRS1 and IRS2 to both the PI3K-AKT-mTOR and RAS-RAF-MEK-ERK pathways (Chalhoub & Baker, 2009; Ulanet et al, 2010). Supply of IGF2 is also controlled via its sequestration by the mannose 6-phosphate/insulin-like growth factor receptor 2 (*Igf2r*; Foulstone et al, 2005; Ghosh et al, 2003; Zaccheo et al, 2006). *Igf2* expression is imprinted (maternal allele silenced) in the mouse, leading to paternal allele expression that is also highly regulated during embryonic and post-natal development (Baker et al, 1993). Targeted disruption of the paternal (p) allele of *Igf2* ($Igf2^{+m/-p}$) regulates whole organism growth (60% of the size of WT littermates; Burns & Hassan, 2001; DeChiara et al, 1990, 1991). Maternal (m) disruption of the imprinting control region (ICR) and the *H19* gene ($H19^{\Delta 13kb/+p}$) subsequently referred to as $H19^{-m/+p}$, results in bi-allelic *Igf2* expression and an overgrowth phenotype of 128% of the size of *WT* littermates (Leighton et al, 1995). Importantly, the $H19^{-m/+p}$ growth phenotype was rescued by $Igf2^{+m/-p}$, suggesting that the predominate growth effects were mediated by *Igf2*, rather than as a consequence of the disruption of either the maternal allele locus, *H19* non-coding RNA or the associated miR-675 (Gabory et al, 2009; Veronese et al, 2010; Yoshimizu et al, 2008). *Igf2* can be reactivated in mouse tumours by LOI, where it promotes tumour progression (Christofori et al, 1994, 1995). DNA hypomethylation also results in increased *Igf2* expression and tumour promotion (Gaudet et al, 2003; Linhart et al, 2007). Here we provide *in vivo* evidence of significant *Igf2* dependency of the *p53* null phenotype during development and tumour formation.

RESULTS

Igf2-dependent developmental lethality of *p53* null mice

Mice with varying allelic doses of *Igf2* and *p53* were combined. $Igf2^{+m/-p}$ are deficient in *Igf2* expression because of disruption of the expressed paternal allele associated with silencing (imprinting) of the maternal allele. $H19^{-m/+p}$ over-express *Igf2* because of disruption of imprinting of the maternal allele ICR leading to bi-allelic expression (p, paternal allele; m, maternal allele; +, intact allele; −, disrupted allele). These lines were first crossed with heterozygote $p53^{+/-}$ mice. The double heterozygote (e.g. $Igf2^{+m/-p}$, $p53^{+/-}$ or $H19^{-m/+p}$, $p53^{+/-}$) progeny were subsequently inter-crossed to generate combined genotypes including double homozygote alleles. As there are known strain-dependent phenotypic effects in *p53* null mice (Harvey et al, 1993), we utilized both the 129S2/J (129) and C57BL/6J (B6) genetic backgrounds backcrossed by >20 generations. We were unable to backcross $Igf2^{+m/-p}$ onto a B6 background, and so 129/B6 F2 hybrids were generated (see Supporting Information Table S1).

The initial observation was that litter sizes from the 129 double heterozygote inter-cross ($Igf2^{+m/-p}$, $p53^{+/-} \times Igf2^{+m/-p}$, $p53^{+/-}$) appeared significantly smaller, suggesting there was an overall loss of progeny (Fig 1A). This was only observed when breeding from double heterozygote mothers ($Igf2^{+m/-p}$, $p53^{+/-}$, Supporting Information Fig S1A and B) and appeared independent of the parental *Igf2* dose (Supporting Information Fig S2). The majority of implantation sites in double heterozygote ($Igf2^{+m/-p}$, $p53^{+/-}$) mothers appeared abnormal (Supporting Information Fig S1C). Haemorrhage was observed within the maternal decidua at implantation sites in double heterozygote ($Igf2^{+m/-p}$, $p53^{+/-}$) mothers, and was less frequently observed in either $p53^{+/-}$ or $Igf2^{+m/-p}$ heterozygote mothers (Supporting Information Fig S1C and D).

At birth, either $H19^{-m/+p}$, $Igf2^{+m/-p}$ or $p53^{+/-}$ alleles exhibited the expected Mendelian segregation (129, B6 and F1/F2, Supporting Information Tables S2 and S3). The exception, however, were the results from a 129 double heterozygote null inter-cross (e.g. $Igf2^{+m/-p}$, $p53^{+/-} \times Igf2^{+m/-p} \times p53^{+/-}$; Table 1). Evaluation of 297 mice from 34 litters led to three further observations.

First, genotypes associated with loss of function of the paternal expressed *Igf2* allele were 60% smaller from birth

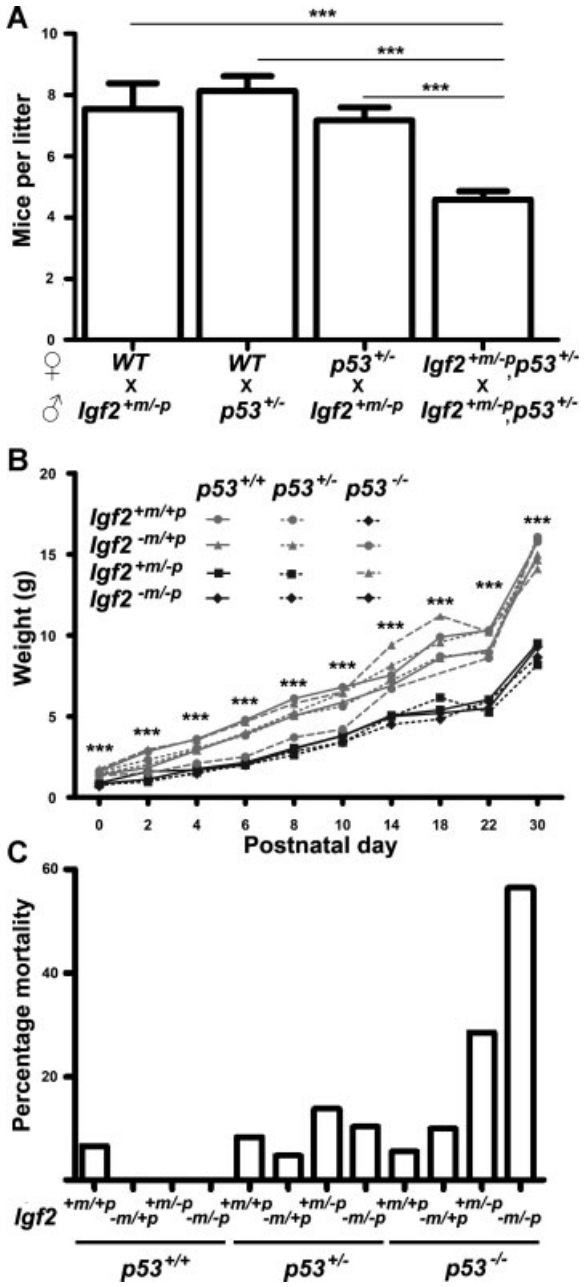

Figure 1. Combination of *Igf2* and *p53* null alleles results in developmental lethality independent of embryo growth.

A. Mean litter size at P0 was significantly reduced from $Igf2^{+m/-p}$, $p53^{+/-}$ inter-cross ($^{***}p < 0.0001$, $n = 67$ litters) relative to $Igf2^{+m/-p}$ mated with *WT* ($n = 11$ litters), $p53^{+/-}$ mated with *WT* ($n = 15$ litters) and $Igf2^{+m/-p}$ mated with $p53^{+/-}$ ($n = 36$ litters; one-way ANOVA, Tukey's multiple comparison).

B. Post-natal growth of progeny from a double heterozygote inter-cross (129, $Igf2^{+m/-p}$, $p53^{+/-}$). Mean weights of *WT* and $Igf2^{-m/+p}$ mice were significantly greater ($^{***}p < 0.0001$, one-way ANOVA, Tukey's multiple comparison) than $Igf2^{+m/-p}$ and $Igf2^{-m/-p}$ mice regardless of allelic dosage of *p53*.

C. Increased post-natal mortality by p30 in progeny null for both *Igf2* and *p53* ($Igf2^{+m/-p}$, $p53^{-/-}$, 2 of 7 and $Igf2^{-m/-p}$, $p53^{-/-}$, 5 of 9) relative to *WT* mice (1 of 15, $p = 0.037$, Fisher's exact test).

(Fig 1B). Second, pre-natal and post-natal lethality occurred in homozygote null *p53* combined with disruption of the paternal expressed allele of *Igf2* ($Igf2^{+m/-p}$, $p53^{-/-}$ and $Igf2^{-m/-p}$, $p53^{-/-}$ mice). The lethality was observed by birth (16 of 44 expected), but also continued during the post-natal period and was associated with no obvious developmental abnormalities (Fig 1C). Third, there was specific absence of $Igf2^{+m/-p}$ female progeny. Post-natal (P3.5) loss of $Igf2^{+m/-p}$ in both sexes was expected based on steroid responsive delayed lung development (Table 1; Silva et al, 2006). However, a deficit specific to females occurred in our case (Fig 2A and Table 2). If double heterozygote ($Igf2^{+m/-p}$, $p53^{+/-}$) males were crossed with either WT or $p53^{+/-}$ females (Table 2), we again failed to obtain any live $Igf2^{+m/-p}$ females. Thus, combined loss of alleles for both *p53* and *Igf2* in the paternal germ-line appeared incompatible with specific survival of subsequent $Igf2^{+m/-p}$, $p53^{+/+}$ female progeny. In contrast, double heterozygote ($Igf2^{+m/-p}$, $p53^{+/-}$) female littermates survived beyond post-natal day 10, indicating the lethality could be rescued by loss of an allele of *p53*. Morphological appearances of embryos appeared normal between E9.5 and E18.5, even though $Igf2^{+m/-p}$ embryos were smaller (Supporting Information Fig S3). Following delivery by caesarean section at E19.5, $Igf2^{+m/-p}$ females became increasingly cyanotic (Fig 2B). Analysis of organ growth revealed that the mean wet weights of lungs from $Igf2^{+m/-p}$ females were significantly reduced compared either to *WT*, $Igf2^{+m/-p}$ males or double heterozygote

Table 1. Abnormal Mendelian segregation of progeny from a $Igf2^{+m/-p}$, $p53^{+/-}$ inter-cross in 129J

Strain	WT	$Igf2^{+/-}$	$Igf2^{-/-}$	$p53^{+/-}$	$Igf2^{+/-}$, $p53^{+/-}$	$Igf2^{-/-}$, $p53^{+/-}$	$p53^{-/-}$	$Igf2^{+/-}$, $p53^{-/-}$	$Igf2^{-/-}$, $p53^{-/-}$	Total *p*-Value χ^2-Value
129	15	17	7	24	71	29	18	17	9	207
	(12.94)	(25.88)	(12.94)	(25.88)	(51.75)	(25.88)	(12.94)	(25.88)	(12.94)	$p < 0.02$
	0.329	3.044	2.725	0.136	7.161	0.377	1.981	3.044	1.198	19.995
129B6F2	7	22	5	19	33	12	8	13	7	126
	(7.88)	(15.75)	(7.88)	(15.75)	(31.5)	(15.75)	(7.88)	(15.75)	(7.88)	$p = NS$
	0.097	2.480	1.049	0.671	0.071	0.893	0.002	0.480	0.097	5.841

Progeny did not segregate according to a normal Mendelian distribution ($p < 0.02$, χ^2-test). In contrast, progeny from the 129B6F1 $Igf2^{+/-}$, $p53^{+/-}$ inter-cross had a normal Mendelian distribution. Observed (upper), expected (middle, in brackets) and χ^2 (lower).

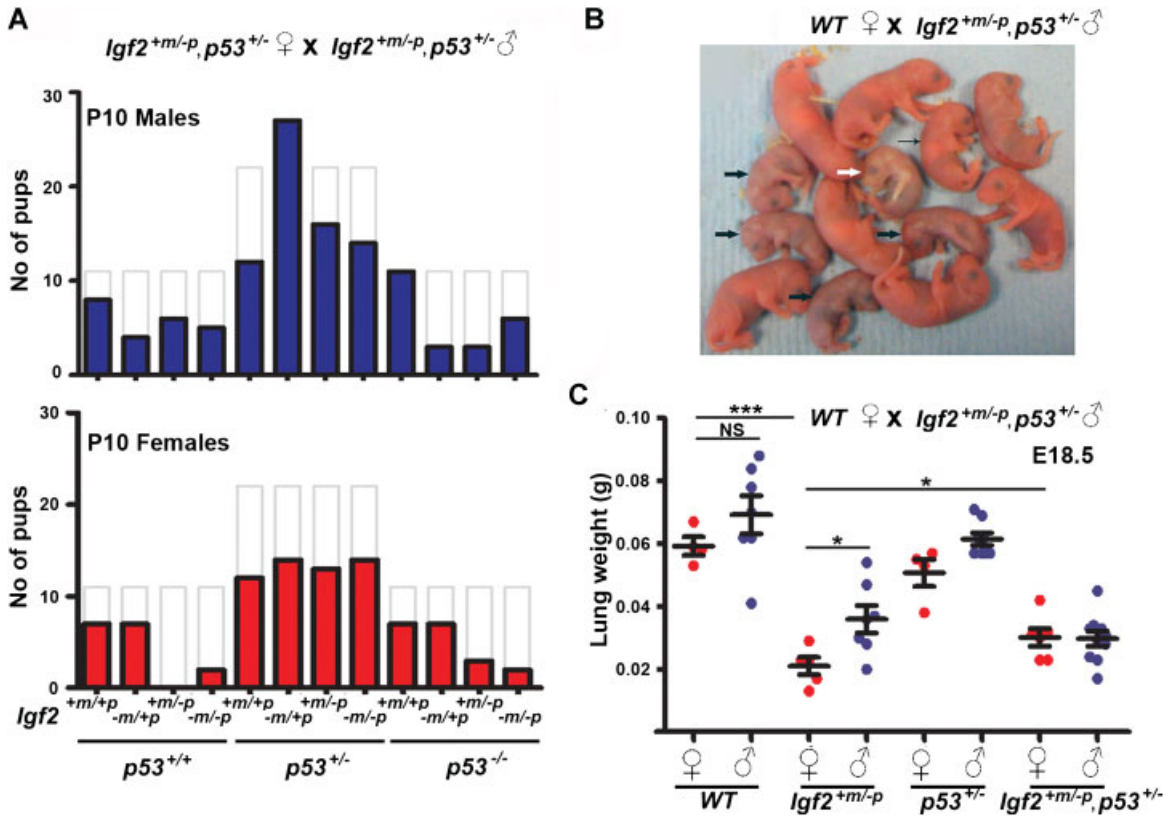

Figure 2. Lethality of female $Igf2^{+m/-p}$ progeny derived from $Igf2^{+m/-p}$, $p53^{+/-}$ fathers is associated with reduced lung growth.

A. Male ($n = 115$) and female ($n = 87$) progeny at P10 from the 129 $Igf2^{+m/-p}$, $p53^{+/-}$ inter-cross did not segregate according to the expected Mendelian distribution (see Table 1; $p < 0.05$) and were significantly different to expected numbers estimated from previous litter sizes (total expected numbers: males = 175, females = 175 based on mean litter size from earlier 129 breeding, $p < 0.0001$, χ^2-test). Solid bars, observed numbers, grey unfilled bars, expected numbers (see also Table 2).

B. Litter of 13 pups delivered by caesarean section at E19.5 from a *WT* female mated with $Igf2^{+m/-p}$, $p53^{+/-}$ male. One $Igf2^{+m/-p}$ female (white arrow) became increasingly cyanotic and died 1hr post-delivery. The remaining four $Igf2^{+m/-p}$ females (thick black arrows) were also cyanotic compared to their littermates (thin black arrow points to a representative $Igf2^{+m/-p}$ male).

C. Lung weights from E18.5 $Igf2^{+m/-p}$ female progeny derived from $Igf2^{+m/-p}$, $p53^{+/-}$ fathers were significantly lower than those from $Igf2^{+m/-p}$ male progeny ($^{*}p = 0.025$), WT female progeny ($^{***}p < 0.0001$) and $Igf2^{+m/-p}$, $p53^{+/-}$ female progeny ($^{*}p = 0.049$, two-tailed *t*-test, NS = not significant).

females ($Igf2^{+m/-p}$, $p53^{+/-}$, Fig 2C and Supporting Information Fig S3B and C).

$Igf2^{+m/-p}$ female specific lethality was associated with a gene expression signature and lung haemorrhage

We next investigated effects of paternal genotype on gene expression. Whole female embryos derived from *WT*, $Igf2^{+m/-p}$ or double heterozygote ($Igf2^{+m/-p}$, $p53^{+/-}$) fathers were obtained at E9.5 (Fig 3A). This time was chosen because it is known that *WT* and $Igf2^{+m/-p}$ embryos are of similar size at E9.5, and we wished to eliminate confounding effects from later differences in growth (Burns & Hassan, 2001). Illumina RNA expression profiles of all female progeny at E9.5 were then compared (Fig 3B). A specific gene expression signature was observed 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 Arrayexpress). Importantly, no effects were consistent with co-segregation of the gene expression signature with the *p53* null allele, suggesting the signature was a direct consequence of the combined paternal genotype rather than a segregating modifier allele. By filtering the initial signature of 1461 genes according to chromosomal location, we obtained 53 X-linked genes. These genes were distributed along the entire length of the X-chromosome and failed to show any differential expression patterns (data not shown).

We evaluated whether the gene lethality expression profiles represented a hierarchy of transcriptional regulation. *PScan* (Zambelli et al, 2009) determined transcription factors whose binding sites were statistically over-represented in the 50 most differentially expressed genes, including those located on the X-chromosome. A single X-linked transcription factor, *Zfx*, was

Table 2. Complete perinatal lethality of 129J $Igf2^{+m/-p}$ female progeny was only observed from females mated with 129J $Igf2^{+m/-p}$, $p53^{+/-}$ males.

Cross	Time-point	WT		$Igf2^{+m/-p}$		$p53^{+/-}$		$Igf2^{+m/-p}$, $p53^{+/-}$		$p53^{-/-}$		$Igf2^{+m/-p}$, $p53^{-/-}$		Total *p*-Value χ^2-Value
		♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	
WT ♀ x	P0	74	82	54	59	–	–	–	–	–	–	–	–	269
$Igf2^{+m/-p}$ ♂		(67)	(67)	(67)	(67)									$p = NS$
		0.67	3.23	2.61	1.01									7.54
	P3.5	66	77	32	35	–	–	–	–	–	–	–	–	210
		(53)	(53)	(53)	(53)									$p < 0.0001$
		0.35	11.4	8.00	5.83									28.74
$p53^{+/-}$ ♀ x	P10	20	18	13	18	16	22	13	17	–	–	–	–	137
$Igf2^{+m/-p}$ ♂		(17)	(17)	(17)	(17)	(17)	(17)	(17)	(17)					$p = NS$
		0.48	0.04	0.99	0.04	0.99	1.39	0.07	0.00					4.02
WT ♀ x	E18.5	7	3	7	4	6	1	6	3	–	–	–	–	37
$Igf2^{+m/-p}$,		(5)	(5)	(5)	(5)	(5)	(5)	(5)	(5)					$p = NS$
$p53^{+/-}$ ♂		1.22	0.57	1.22	0.08	0.48	2.84	0.48	0.57					7.32
	P0	9	9	5	0	8	8	9	2					50 $p < 0.05$
		(6.3)	(6.3)	(6.3)	(6.3)	(6.3)	(6.3)	(6.3)	(6.3)					14.00
		1.21	1.21	0.25	6.25	0.49	0.49	1.21	2.89					
	P10	6	12	4	0	6	13	6	5	–	–	–	–	52
		(6.5)	(6.5)	(6.5)	(6.5)	(6.5)	(6.5)	(6.5)	(6.5)					$p < 0.01$
		0.04	4.65	0.96	6.5	0.04	6.5	0.04	0.37					19.08
$p53^{+/-}$ ♀ x	E9.5	6	7	4	4	10	15	9	11	2	3	4	5	80
$Igf2^{+m/-p}$,		(5)	(5)	(5)	(5)	(10)	(10)	(10)	(10)	(5)	(5)	(5)	(5)	$p = NS$
$p53^{+/-}$ ♂		0.20	0.80	0.20	0.20	0.00	2.50	0.10	0.10	1.80	0.80	0.20	0.00	4.40
	E18.5	3	2	1	3	5	7	5	4	3	2	2	3	40
		(2.5)	(2.5)	(2.5)	(2.5)	(5)	(5)	(5)	(5)	(2.5)	(2.5)	(2.5)	(2.5)	$p = NS$
		0.10	0.10	0.90	0.10	0.00	0.80	0.00	0.20	0.10	0.10	0.10	0.10	1.40
	P10	10	8	7	0	15	12	10	10	2	3	1	1	75
		(4.7)	(4.7)	(4.7)	(4.7)	(9.4)	(9.4)	(9.4)	(9.4)	(4.7)	(4.7)	(4.7)	(4.7)	$p = NS$ †
		6.02	2.34	1.14	4.7	3.38	0.74	0.04	0.04	1.54	0.61	2.90	2.90	12.487

Significant deviation from the normal expected Mendelian ratio of $Igf2^{+m/-p}$ progeny from *WT* mothers at P3.5 in both females and males was detected, suggesting an effect of the paternal allele on survival. The $Igf2^{+m/-p}$ genotyped progeny derived from $Igf2^{+/-}$, $p53^{+/-}$ males were absent at P10 compared to E18.5, although the significance was not detected in the segregation of genotypes in progeny derived from $p53^{+/-}$ females (†).

a regulator of the gene sets, although there were no differences in *Zfx* mRNA levels (not shown; Cellot & Sauvageau, 2007; Luoh et al, 1997). We also identified *Igf2*-dependent genes in all females, with respect to the paternal genotype. A total of 41 *Igf2*-dependent genes, including *Igf2* itself, were obtained from this further analysis, with some apparent fold change differences dependent on paternal $p53^{+/-}$ status, suggesting that *Igf2* regulated genes may also be differentially modified by *p53*. Using *PScan* (Zambelli et al, 2009) to locate putative p53 binding sites in the promoters of the 50 most differentially expressed genes between progeny that were derived from double heterozygote fathers ($Igf2^{+m/-p}$, $p53^{+/-}$) and controls, we detected 48/50 potential p53 regulated genes. These were located in autosomes, without overlap with *Igf2* regulated genes.

We next focused on the *p53*-dependent rescue of lethality in $Igf2^{+m/-p}$ females. We reasoned that genes responsible for lethality would be differentially expressed in $Igf2^{+m/-p}$ female progeny of double heterozygote fathers ($Igf2^{+m/-p}$, $p53^{+/-}$), relative to the $Igf2^{+m/-p}$, $p53^{+/-}$ female littermates that survive. The expression levels of the lethality rescue genes would be expected to be closer to those in $Igf2^{+m/-p}$ embryos from $Igf2^{+m/-p}$ or WT fathers, i.e. that expression was normalized. The genes identified as significantly different in $Igf2^{+m/-p}$ females compared to $Igf2^{+m/-p}$ and $Igf2^{+m/-p}$, $p53^{+/-}$ embryos female littermates were then compared (Fig 3C). We obtained a signature that most likely represented the gene changes associated with $p53^{+/-}$ rescue. The E9.5 'lethality (rescue) signature' (Fig 3C) contained 23 genes including those encoding transcription factors (*Zmiz2*, *Tbx6*), endocrine regulators (*Sst*), endocytosis (*Necap2*), metabolic enzymes (*Pck2*, *Man1a*, *Pus7*) and the extracellular matrix protein, fibronectin (*Fn1*).

We noted that the relative expression levels of *Fn1* were high compared to the other genes (Fig 3C). Previous work had identified *Fn1* gain of function as a modifier of lung branching morphogenesis, associated with gain of cell–matrix adhesion relative to the reciprocal repression of E-cadherin cell–cell adherence (Sakai et al, 2003). Lung airspaces of the $Igf2^{+m/-p}$, $p53^{+/+}$ females were filled with blood suggesting that

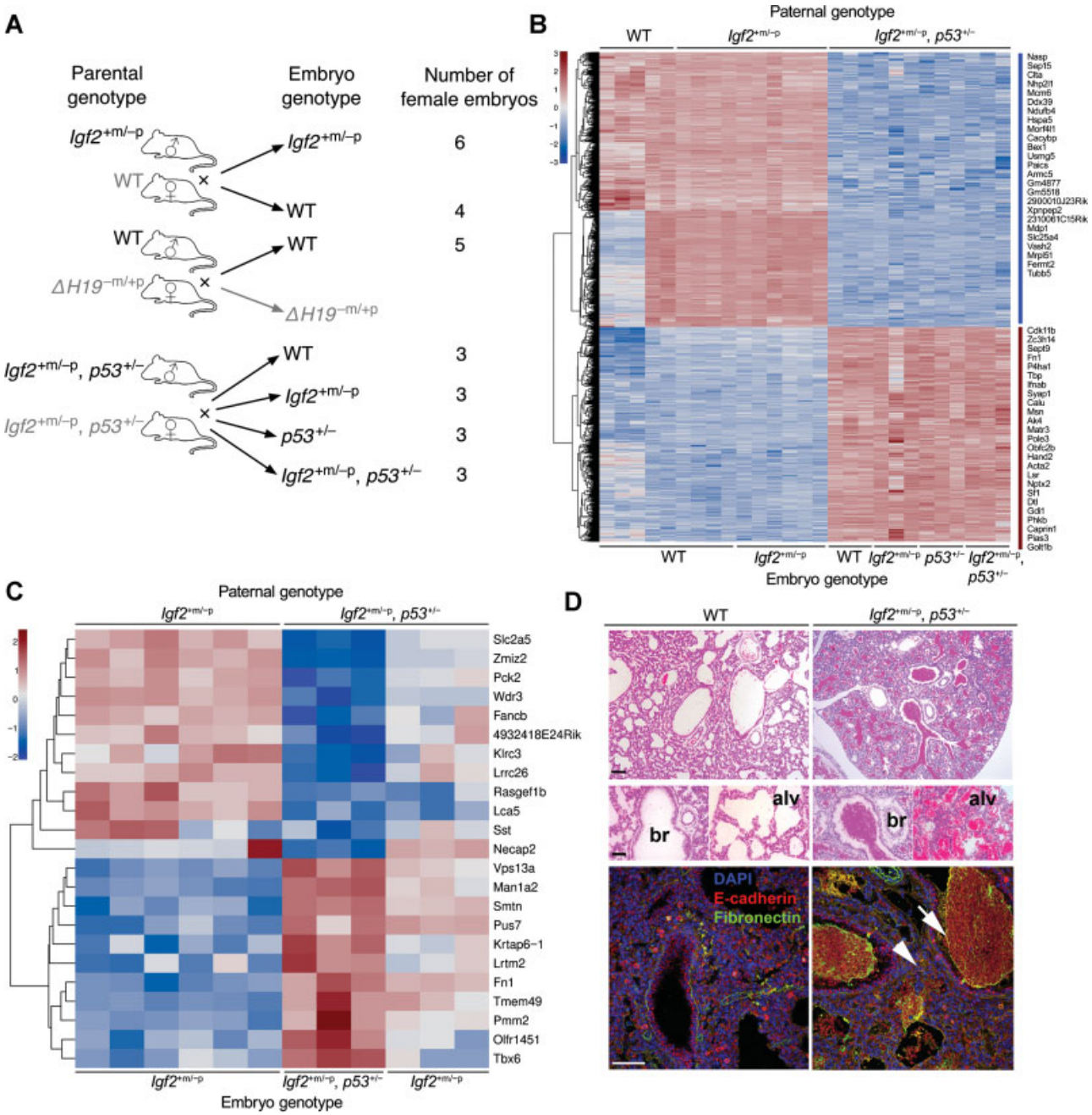

Figure 3. Paternal *p53* genotype alters embryonic gene expression and *Igf2*$^{+m/-p}$ female specific lethality.

A. Schema of embryonic RNA expression analysis. Three parental genotype pairs were crossed to yield a number of female embryos of specific genotype as illustrated.

B. Comparison of gene expression in E9.5 female embryos derived from *Igf2*$^{+m/-p}$ or WT fathers (WT and *Igf2*$^{+m/-p}$) with the expression in embryos from *Igf2*$^{+m/-p}$, *p53*$^{+/-}$ fathers (WT, *Igf2*$^{+m/-p}$, *p53*$^{+/-}$ and *Igf2*$^{+m/-p}$, *p53*$^{+/-}$). 1461 genes were significantly different in expression by SAM analysis and varied more than twofold, with most differentially expressed genes displayed in the heatmap.

C. Comparison of gene expression in *Igf2*$^{+m/-p}$ female embryos derived from *WT* fathers with those *Igf2*$^{+m/-p}$ and *Igf2*$^{+m/-p}$, *p53*$^{+/-}$embryos from *Igf2*$^{+m/-p}$, *p53*$^{+/-}$ fathers. 'Rescue' signature of 23 genes shown in the heatmap.

D. H & E-stained lungs from E19.5 *Igf2*$^{+m/-p}$ female embryos (low power above, bar 1 mm, high power 100 μm) were filled with blood in both bronchioles (br) alveolar air-spaces (alv) when compared to WT littermates. Confocal images showed there were no gross differences in lung structure labelled with E-cadherin, but fibronectin was predominantly present in blood within the airspaces (white arrow), and subjectively increased in small airway and alveolar regions (white arrow head). Bar = 100 μm.

haemorrhage was the likely cause of death (Fig 3D). Laser scanning confocal microscopy (LSCM) detected no obvious gross structural alterations in vessels and airways, neither in E-cadherin, Ki67 nor cleaved caspase-3 labelling (Fig 3D and not shown). Localization of fibronectin showed increased labelling in blood present in both large and small airways, presumably related to its known role in coagulation. However, an increase in fibronectin appeared in regions of the small airways and alveoli in $Igf2^{+m/-p}$, $p53^{+/+}$ females compared to female and male WT and $Igf2^{+m/-p}$, $p53^{+/-}$ littermates (Fig 3D and not shown).

Accelerated tumour formation in *p53* heterozygote females combined with loss of imprinting of *Igf2* ($H19^{-m/+p}$)

We next investigated dependencies of the *p53* and *Igf2* pathways during tumour development. Neither allelic loss nor gain of *Igf2* expression in WT ($p53^{+/+}$) and homozygote null ($p53^{-/-}$) mice had any effect on tumour latency and overall longevity to 18 months, even though the IGF and p53 pathways were both implicated in aging and longevity in the mouse (Donehower, 2002, 2009; Supporting Information Fig S4). Heterozygosity of *p53* ($p53^{+/-}$) combined with bi-allelic expression of *Igf2* (in this case associated with the 13 kb deletion of the *H19* gene and ICR $H19^{\Delta 13kb}$), resulted in accelerated tumour formation in B6, 129 or F2 backgrounds (Fig 4A–C). Interestingly, the effect on tumour progression also appeared unusually specific to females rather than males (Fig 4D). Control $p53^{+/-}$ females and males had similar tumour latencies (Fig 4D) indicating that the acceleration in tumour formation in females was primarily due to the consequences of Δ13 kb deletion on the *Igf2-H19* locus. The consequences of $H19^{-m/+p}$ include the expected bi-allelic expression of *Igf2*, loss of function of the *H19 ncRNA* and associated miR-675, and the potential up-regulation of miR-483 in the *Igf2* locus (Gabory et al, 2009).

A statistically significant alteration in tumour spectrum occurred in $p53^{+/-}$ mice with $H19^{-m/+p}$, with more tumours classified as carcinoma and sarcoma compared to $p53^{+/-}$ mice (Fig 4E, Supporting Information Fig S5). Inactivation of the remaining WT *p53* allele by LOH is normally required for tumour formation. We next determined the frequency of *p53* LOH, mutation and the presence of p53 protein by IHC in tumours and correlated this with latency and tumour spectrum in $H19^{-m/+p}$, $p53^{+/-}$ (Fig 5A; Supporting Information Table S4). Overall, 70% (21/30) of tumours from $p53^{+/-}$ littermates had either LOH or mutation of *p53* compared to 16.7% (2/12) of tumours in $H19^{-m/+p}$, $p53^{+/-}$ (Fig 5A). In carcinomas and sarcomas, and to a lesser extent in lymphoma, *p53* LOH frequency appeared independent of loss of the paternal *Igf2* allele (Fig 5B). In contrast, the untargeted (intact) *p53* allele was retained in sarcoma and carcinomas associated with bi-allelic expression of *Igf2* (Fig 5B), although this was independent of sex (not shown). High *Igf2* expression determined by quantitative PCR was particularly associated with an intact p53 allele in sarcomas that arose in $H19^{-m/+p}$, $p53^{+/-}$ mice, but was without consistent differences in Mdm2 localization (Supporting Information Figs S6 and S7). *p53* LOH was detected with equal frequency to intact alleles in all tumours with lower *Igf2* expression (Fig 5C, Supporting Information Fig S7). For the tumours that arose in $H19^{-m/+p}$, $p53^{+/-}$, we detected 1/10 with mutations in the p53 DNA-binding domain (Supporting Information Table S4). Our data suggested that bi-allelic expression of *Igf2* and the associated disruption of the *H19*

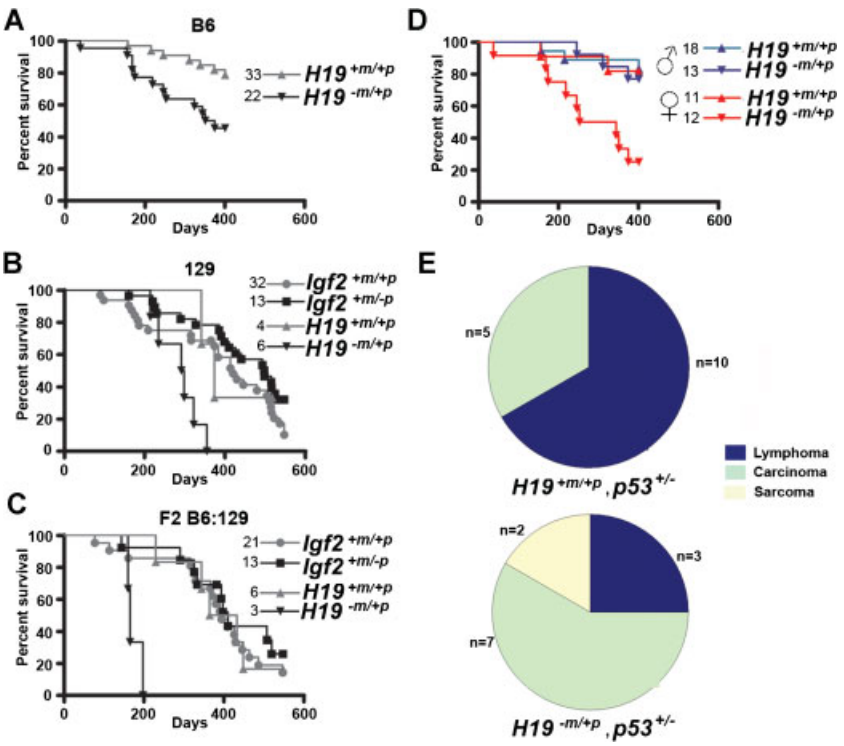

Figure 4. Bi-allelic *Igf2* expression accelerates tumour formation in $p53^{+/-}$ female mice, irrespective of strain.

- **A.** Survival of B6 $H19^{-m/+p}$, $p53^{+/-}$ mice was significantly reduced compared to $p53^{+/-}$ mice ($p = 0.0065$, log-rank test). Due to accelerated tumour latency the humane end point was brought forward to 400 days.
- **B.** Survival of 129 $H19^{-m/+p}$, $p53^{+/-}$ mice was significantly reduced compared to $Igf2^{+/-p}$, $p53^{+/-}$ ($p = 0.0001$), $Igf2^{+m/+p}$, $p53^{+/-}$ ($p = 0.008$) and $H19^{+m/+p}$, $p53^{+/-}$ mice ($p = 0.036$, log-rank test).
- **C.** Survival of F2 hybrid $H19^{-m/+p}$, $p53^{+/-}$ mice was significantly reduced compared to $Igf2^{+m/-p}$, $p53^{+/-}$ ($p = 0.0008$), $Igf2^{+m/+p}$, $p53^{+/-}$ ($p = 0.0017$) or $H19^{+m/+p}$, $p53^{+/-}$ mice ($p = 0.0018$, log-rank test).
- **D.** Tumour latency in B6 $H19^{-m/+p}$, $p53^{+/-}$ female mice was significantly reduced compared to $H19^{-m/+p}$, $p53^{+/-}$ males and $p53^{+/-}$ females and male mice ($p < 0.0007$, log-rank test).
- **E.** B6 $H19^{-m/+p}$, $p53^{+/-}$ mice had significantly more solid tumours (carcinomas and sarcomas) and fewer lymphomas than B6 $H19^{+m/+p}$, $p53^{+/-}$ mice ($p = 0.037$, Fisher's exact test).

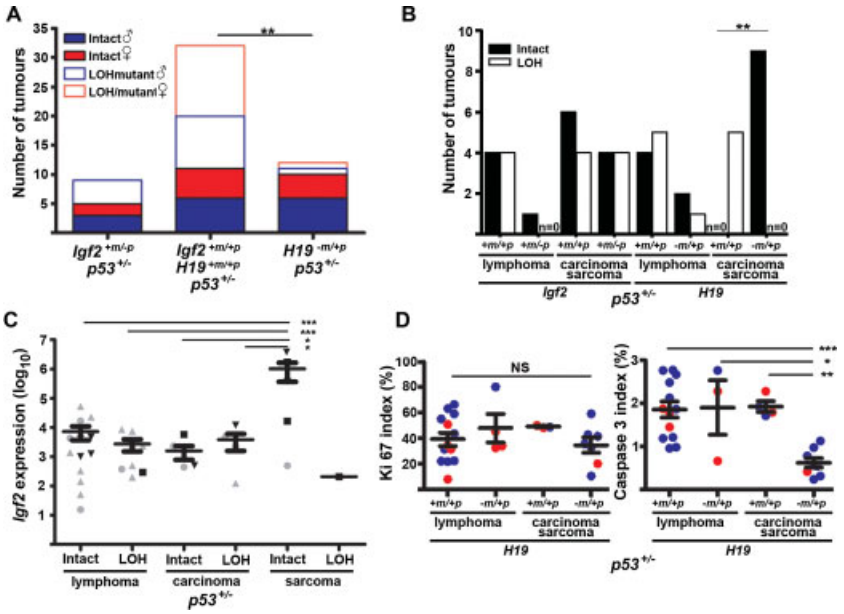

Figure 5. *p53* mutation, LOH and apoptosis in tumours from $p53^{+/-}$ mice with bi-allelic *Igf2* expression ($H19^{-m/+p}$).

A. Proportion of $p53^{+/-}$ tumours with LOH/mutation was significantly reduced in tumours from mice bi-allelic for *Igf2* ($H19^{-m/+p}$), compared to mice null for $Igf2^{+m/-p}$ and *WT* ($^{**}p < 0.002$, Fisher's exact test).

B. 100% of sarcomas and carcinomas from $H19^{-m/+p}$, $p53^{+/-}$ retain an intact *WT* p53 allele ($n = 9$), compared to 100% of LOH in the littermate $p53^{+/-}$ control mice ($n = 5$; $^{**}p = 0.0005$, Fisher's exact test)

C. Sarcomas with intact *WT* p53 ($n = 6$) had significantly higher *Igf2* mRNA expression than lymphomas and carcinomas, irrespective of p53 allelic status ($p < {}^{*}0.05$–$^{***}0.01$, one-way ANOVA, Tukey's multiple comparison). Sample annotation is as for genotypes in Fig 4A and B.

D. Solid tumours ($n = 8$) from $H19^{-m/+p}$, $p53^{+/-}$ mice had significantly fewer apoptotic cells, assessed by staining for cleaved caspase-3 (right) than solid tumours from $H19^{+m/+p}$, $p53^{+/-}$ mice ($n = 4$, $^{*}p = < 0.05$, $^{**}p = < 0.01$, $^{***}p < 0.001$, one-way ANOVA, Tukey's multiple comparison). Blue, male; Red, female.

locus may have reduced the selection pressure for inactivation of the remaining WT allele of *p53* mainly in carcinoma and sarcoma.

To determine whether proliferation and apoptosis were modified in the accelerated tumour formation from bi-allelic $H19^{-m/+p}$, $p53^{+/-}$ females, we performed immuno-labelling for Ki67 proliferative marker and cleaved caspase-3 apoptotic marker. In contrast to Ki67 labelling, cleaved caspase-3 labelling was significantly reduced in solid tumours (carcinoma and sarcoma) from $p53^{+/-}$ mice when combined with bi-allelic *Igf2* expression compared to tumours from mice with mono-allelic *Igf2* expression (Fig 5D). The correlation of cleaved caspase 3 labelling with *Igf2* expression approached significance (Supporting Information Fig S8). The cause of the sex-specific acceleration of tumour development in females still remains obscure. The effect may be either paternal or X-linked, as oestrogen receptor (ER-α) immuno-labelling appeared independent of sex, tumour histology or genotype (Supporting Information Fig S9).

Igf2 dependency of the rapid tumour onset in *p53* null using combined conditional alleles

We next utilized a new conditional allele for *Igf2* with loxp sites that flanked either side of exons 4–6 (Sandovici et al, in preparation), a conditional allele previously characterized for *p53*, and combined these with a conditional tamoxifen inducible Cre (ROSA26Cre; see Supporting Information Table S5). $Igf2^{fl/fl}$, $p53^{fl/fl}$ females were successfully bred with $Igf2^{fl/+}$, $p53^{fl/+}$, $R26CreER^{+/-}$ males on a C57BL6-FVBN background (Fig 6A). Injection of tamoxifen (1 mg) to mothers at E10.5 generated deletion alleles and the expected segregation of progeny (37 males and 28 females, Supporting Information Fig S10A). Non-quantitative PCR suggested that recombination was incomplete as most tissues retained the loxp flanked allele (Supporting Information Fig S10B). 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 non-recombined alleles. Following recombination *in utero*, males with deleted alleles ($p53^{\Delta/\Delta}$ or $p53^{+/\Delta}$) generated only seven progeny from 3 litters, with no subsequent transmission of the deleted (recombined) *p53* allele. By P30 the mean total body weights (BW) and that of the eviscerated carcass of $Igf2^{\Delta/\Delta}$, $p53^{\Delta/\Delta}$, $R26CreER^{+/-}$ progeny was significantly less than that of $Igf2^{fl/fl}$, $p53^{fl/fl}$ littermates, indicating that at least in these tissues *Igf2*-dependent functional growth effects could be detected (Fig 6B). Compared to other organs, the growth findings imply that *Igf2*-dependent total body and musculoskeletal growth may extend beyond E10.5 (Burns & Hassan, 2001).

Tamoxifen treatment of homozygous conditional null $Igf2^{\Delta/\Delta}$, $p53^{\Delta/\Delta}$ mice with ROSA 26 driven Cre ($R26CreER^{+/-}$), avoided the combined lethality observed using germ-line transmission to create $Igf2^{-m/-p}$, $p53^{-/-}$. Moreover, bi-allelic disruption of the *Igf2* allele eliminated the potential for tumour specific re-expression of the imprinted *Igf2* (silenced) maternal allele (LOI). Mice with conditional deletion of both alleles of *Igf2* and *p53* developed a longer tumour latency ($n = 14$, three lymphomas, four solid tumours, two unknown causes, no metastasis, median survival = 325 days) compared to mice with conditional deletion of *p53* but retention of the paternal *Igf2* allele ($n = 19$, 10 lymphomas, 1 solid tumour, 5 unknown causes, median survival = 206 days, Fig 6C). Importantly, the time-dependent onset of tumours mimicked that of the $p53^{-/-}$ germ-line modification (Supporting Information Fig S4). There was a significant shift in tumour spectrum of $Igf2^{\Delta m/+p}$, $p53^{\Delta/\Delta}$, $R26^{+/-}$ mice relative to $Igf2^{\Delta/\Delta}$, $p53^{\Delta/\Delta}$, $R26^{+/-}$ mice with a

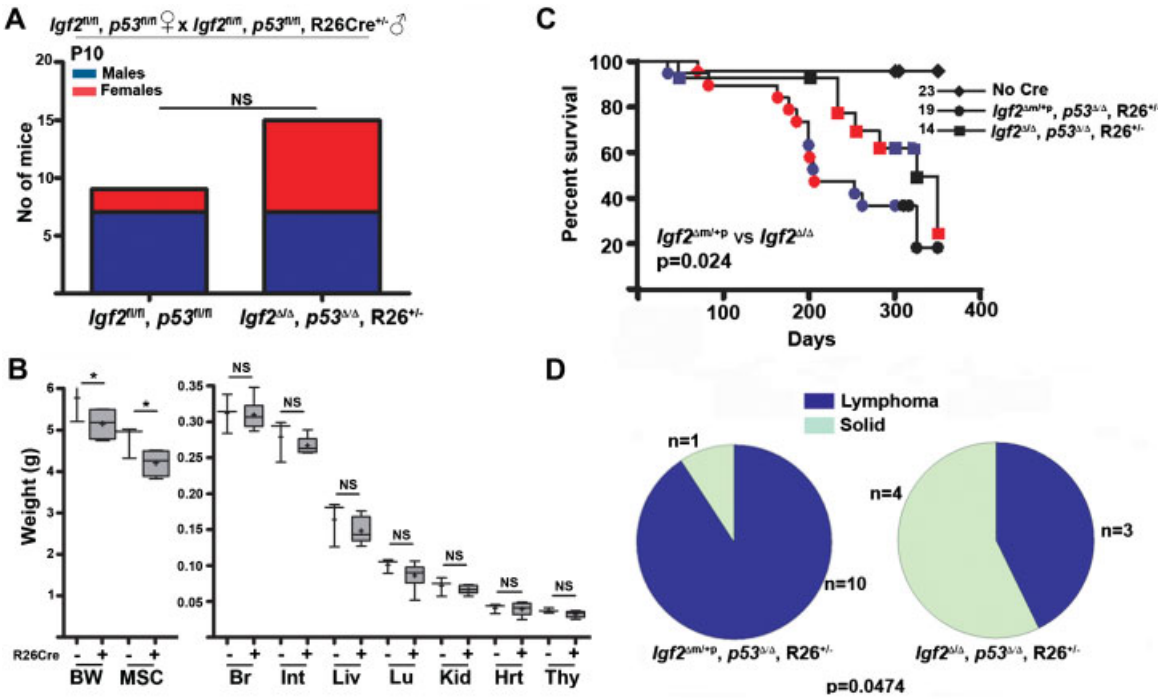

Figure 6. Decreased tumour latency and altered tumour spectrum following homozygous conditional deletion of both *Igf2* and *p53*.

A. Progeny from the homozygous conditional cross of $Igf2^{fl/fl}$, $p53^{fl/fl}$ females × $Igf2^{fl/fl}$, $p53^{fl/fl}$, $R26^{+/-}$ males segregated according to the expected Mendelian distribution at P10, regardless of sex.

B. Following maternal IP injection of tamoxifen at E10.5, BW and musculoskeletal weights (MS) of carcasses, were significantly less for $Igf2^{\Delta/\Delta}$, $p53^{\Delta/\Delta}$, $R26Cre^{+/-}$ mice ($n = 6$) compared to sham injected controls $Igf2^{fl/fl}$, $p53^{fl/fl}$, $R26Cre^{+/-}$ ($n = 3$, $^{*}p = 0.05$, unpaired *t*-test). No significant differences in the weight of viscera were detected; Br, brain, Int, small intestine; Liv, liver; Lu, lungs; Kid, kidney; Hrt, heart; Thy, thymus. Plot shows 95% of the range (whiskers), interquartile range (box), median (horizontal crossbar) and mean (cross).

C. Mice with homozygous conditional deletion of *Igf2* and *p53* ($Igf2^{\Delta/\Delta}$, $p53^{\Delta/\Delta}$, $R26^{+/-}$) developed tumours later and survived for longer (median survival = 325 days) than mice with an intact paternal allele of *Igf2* ($Igf2^{\Delta m/+p}$, $p53^{\Delta/\Delta}$, $R26^{+/-}$, median survival = 206 days, $p = 0.024$, log-rank test, blue, males; red, females).

D. $Igf2^{\Delta/\Delta}$, $p53^{\Delta/\Delta}$, $R26^{+/-}$ mice (right) developed more solid tumours than $Igf2^{\Delta m/+p}$, $p53^{\Delta/\Delta}$, $R26^{+/-}$ mice (left, 4 and 1, respectively), and fewer lymphomas (3 and 10, respectively, $p = 0.0474$, Fisher's exact test).

reduction in lymphoma development relative to carcinoma and sarcoma (Fig 6D). These results confirm that allelic dose of *Igf2* significantly reduced the onset of homozygote *p53* null tumour phenotype for all types of tumours.

DISCUSSION

Single cell type *in vitro* models tend to lack tissue specific auto-regulatory feedback loops, and so, although informative, significance for *in vivo* function may be limited (Feng & Levine, 2010). Here, we exploited the imprinting regulation of *Igf2*, a developmental and tumour growth factor, to both increase and decrease the allelic dosage of *Igf2* and ligand supply. An assumption was that genetic modifications altered *Igf2* expression alone. As in most mouse models, disruption of genomic regions can generate direct and indirect effects, with altered expression of microRNAs as an example. Loss of miR-675 in the *H19ncRNA* can promote tumour formation, as can an increase in miR-483 within the *Igf2* region (Gabory et al, 2009; Veronese et al, 2010; Yoshimizu et al, 2008). A second assumption is that alteration of IGF2 ligand supply leads to physiological activation of the pathway. The proportionate growth effects of the *Igf2* models support this assumption when compared to un-physiological expression derived from introduced transgenes. The resulting increase in supply of IGF2 would bind cell surface IGF1R and IR-A, to activate downstream signalling to all intact pathway components, and specific feedback mechanisms (Ulanet et al, 2010).

Igf2-dependent developmental lethality and an *Igf2*-dependent modification of tumour formation occurred in *p53* null mice. 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 (Christofori et al, 1994; Hassan & Howell, 2000; Ho et al, 2009; Wu et al, 2006). Thus, our observation that homozygote null p53 mice fail to survive to adulthood in the context of disruption of the paternal allele of *Igf2* suggests *Igf2* is required to rescue a *p53* loss of function-dependent lethality.

The specific lethality in $Igf2^{+m/-p}$ female progeny was genetically attributed to double heterozygote fathers. The paternal X-chromosome appears a prerequisite for lethality of the $Igf2^{+m/-p}$ females because of the survival of male $Igf2^{+m/-p}$ progeny. Female $Igf2^{+m/-p}$, $p53^{+/-}$ progeny also survived, albeit at lower than expected numbers, indicating that loss of one allele of *p53* from the genotype of the offspring was sufficient for a partial rescue of the female specific lethality. We speculate that potential rescue mechanisms might include interactions of p53 with a range of epigenetic and X-specific targets, e.g. DNA methyltransferases (*Dnmt*; Park et al, 2005; Peterson et al, 2003) and *Xist* expression (Panning & Jaenisch, 1996). In $Igf2^{-m/-p}$ mice, the canalicular phase of lung maturation during late gestation appeared delayed, leading to lungs having poorly organized alveoli with thick septae and reduced airspaces (Silva et al, 2006). Reduced exposure to maternal corticosteroids may have been responsible for the delayed lung development since progeny from $Igf2^{-m/-p}$ mothers treated with this glucocorticoid had lung architecture similar to that of WT controls (Silva et al, 2006). None of these effects were sex or female specific as in our case. Fibronectin (*Fn1*) is the major component of the extracellular matrix in the lung and excess supply promotes epithelial branching morphogenesis (Sakai et al, 2003). The ten-fold increase in expression of *Fn1* in $Igf2^{+m/-p}$, $p53^{+/+}$ female genotyped embryos destined to die, with lower levels in surviving $Igf2^{+m/-p}$, $p53^{+/-}$ littermates, appeared significant, yet we could not reliably infer the phenotype was *Fn1* dependent. Expression of *Man1a2* gene, which encodes golgi α1,2-mannosidase IB, was also elevated in $Igf2^{+m/-p}$, $p53^{+/+}$ female progeny. Homozygous knock-out of *Man1a2* produced a surprisingly severe lung phenotype that resulted in neonatal lethality (Tremblay et al, 2007). Importantly, $Man1a2^{-/-}$ mice died hours after birth from respiratory distress and alveolar haemorrhage, mirroring our phenotype even though in our case *Man1a2* appeared up-regulated (Tremblay et al, 2007).

Deletion of the *H19* locus (Leighton et al, 1995) is an imperfect model of LOI for *Igf2*. Importantly, we cannot exclude the possibility that some of the effects that we observe may be due to removal of *H19* non-coding RNA, as been demonstrated in the small intestine (Yoshimizu et al, 2008). *H19* long non-coding RNA has putative roles as a tumour suppressor (Yoshimizu et al, 2008) and regulator of embryonic growth (Gabory et al, 2009), mainly through loss of microRNA, miR-675, which is hosted by *H19* within its first exon (Cai & Cullen, 2007; Mineno et al, 2006). Thus the consequences of the 13 kb deletion in the $H19^{-m/+p}$ model we utilized may also have had *Igf2*-independent gain of function effects on the p53 pathway. However, our previous analysis of miRNA profiles from these *Igf2* models revealed numerous other miRNA changes, despite *Igf2* specific phenotypic effects in the context of *Pten* heterozygosity (Church et al, 2011).

The tumour-promoting effect of $H19^{-m/+p}$ appeared independent of the direct loss of the WT p53 allele (LOH) in carcinoma and sarcoma tumours. 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). It has been observed that anti-tumour effects can occur following re-introduction of a WT *p53* allele in *p53* null tumours (Martins et al, 2006; Ventura et al, 2007; Xue et al, 2007). Our data suggest that counteracting the inhibitory effects of the IGF2 pathway on the endogenous WT *p53* allele, may also lead to re-activation of the endogenous WT *p53* allele, and promote tumour suppression. In tumours with intact p53 alleles and *Igf2* over-expression, inhibiting the IGF2 signalling pathway may offer a therapeutic strategy. Conditional homozygous deletion of both *Igf2* and *p53* alleles slowed the development of tumours compared with the conditional deletion of *p53* alleles alone. Thus, *p53* loss of function in both development and tumour formation appears dependent on *Igf2*. The *Igf2* dependency of tumours that arise in the context of other models associated with p53 loss of function implicates *Igf2* as a significant factor that regulates the progression of tumours through regulation of apoptosis (Christofori et al, 1994, 1995; Lopez & Hanahan, 2002), more recently observed in a mouse model of high grade chondrosarcoma (Ho et al, 2009). In the chondrosarcoma model, a *p53*-dependent reduction of IGFBP levels may have accounted for a secondary increase in IGF2 ligand bio-availability, and hence dependence on *Igf2*. The dependency of tumour growth on the IGF pathway is further supported by observations in Li-Fraumeni associated tumours, as adrenal carcinoma and osteosarcoma have both been associated with dependency on IGF2, and are the basis of interventional clinical trials of agents that inhibit the activation of the IGF1 receptor (Avnet et al, 2009; Barlaskar et al, 2009). These data suggest that regulation of the IGF2 ligand in tumour susceptibility syndromes associated with *p53* (e.g. Li-Fraumeni, p53 and MDM2 associated SNPs) may be a potential strategy for tumour prevention. Finally, we cannot extend these observations to either gain of function mutants of *p53*, where partial, complete and altered pathway interactions may be evident (Hinkal et al, 2009). These issues require further genetic investigation during development and tumour formation.

MATERIALS AND METHODS

Animals and genotyping

Animal work was approved by University of Oxford ethics committee and UK Home Office. Mice [*Mus musculus* C57BL/6J (B6) and 129S2/J (129)] were maintained and genotyped as previously described (Hassan & Howell, 2000; Supporting Information Table S5, primers available on request). The new *Igf2* conditional allele has loxp sites flanking exons 4–6 and was maintained (>10 generations) on a C57Bl6 background (Sandovici et al in preparation). Seminal plugs at 9.00 am and conception was taken ±12 h. Weighed embryos were fixed in either 4% neutral buffered formalin (NBF) or RNA*later* (Qiagen, Crawley, UK). DNA from E9.5 embryo yolk sacs was extracted by TRI reagent (Applied Biosystems, Warrington, UK). To distinguish $Igf2^{+m/-p}$ from $Igf2^{-m/+p}$, embryos with reduced CR length and low *Igf2* expression with qPCR were designated as being $Igf2^{+m/-p}$. Recombination of conditional alleles by $R26CreER^{+/-}$ was with Tamoxifen (1 mg, 5% v/v ethanol and 95% v/v corn-oil, Sigma, Poole, UK) injected

The paper explained

PROBLEM:

The *p53* gene product is a transcription factor that controls the cells' response to genome toxins, such as radiation, and is a frequently mutated target in cancer. Inherited mutation of the *p53* gene can predispose humans to early onset cancers (Li-Fraumeni syndrome, LFS), and this effect can be reproduced in the mouse. When tumours form, the remaining wild-type (WT) allele is lost either through loss of genomic DNA in a process called 'loss of heterozygosity (LOH)', or by inhibition of p53. The pathways that modify the age of onset of LFS cancers are potential targets for screening and therapy, and such pathways may also be potential targets to restore function when p53 function is lost. Molecular interactions between p53 and a growth signalling pathway regulated by insulin-like growth factor (IGF) ligands have been observed in cell culture models. To date there has been no systematic analysis of the consequences of gain and loss of supply of the IGF2 ligand in the context of loss of p53 function in the whole organism.

RESULTS:

We addressed the problem by taking a comprehensive genetic approach in the mouse. We combined mice that lack supply of the IGF2 ligand (*Igf2* null) and mice with increased supply of *Igf2* (bi-allelic expressed alleles) with mice that were *p53* heterozygote and homozygote. If both these genes were homozygote null in all tissues, then embryonic development failed, suggesting an important pathway interaction. Interestingly, if the breeding male was heterozygote for both genes, only females with loss of the male derived *Igf2* allele developed a haemorrhagic lung syndrome, suggesting a more specific interaction. Importantly, the age of tumour onset was either reduced or increased if the supply of IGF2 was decreased and increased, respectively. When IGF2 supply was increased, we also observed that the WT p53 allele remained intact in an altered tumour spectrum towards carcinoma and sarcoma, suggesting the mechanism of suppression of p53 was independent of LOH in these tumours. Targeting IGF2 supply in this instance may reactivate the suppressed p53 and generate anti-tumour mechanisms.

IMPACT:

Screening patients with LFS can be a very effective method to detect early tumour formation and prevent development of lethal cancers. For some sporadic cancers, such as bone and soft tissue sarcoma that are frequently observed in LFS, defects in the p53 pathway have also been observed, suggesting that the sporadic development of p53-dependent cancers may in fact represent a spectrum of susceptibility ranging from germ-line mutation to pathway polymorphisms. To date, no therapeutic agents have been developed that modify the age of onset either in LFS tumours or in tumours (sarcoma) that arise in the context of p53 pathway modification rather than inherited gene mutation. Interruption of the IGF2 signaling pathway, for example by specific targeting of the ligand, may therefore be a useful mechanism to test the prevention of human tumours with a disrupted p53 pathway. Moreover, targeting IGF2 may be a treatment strategy in selected patients with tumours that arise in the context of p53 pathway disruption without LOH but with increased IGF2 pathway activity.

I.P. at E10.5. Humane end-point for tumour burden was set at 18 months. Animals were checked daily for palpable tumours and evidence of systemic decline (weight loss, lack of grooming, reduced activity and piloerection).

RNA extraction, reverse transcription and qPCR

Material stored at −80°C in RNA*later* (Applied Biosystems) were homogenized with either an Ultra-Turrax T8 (Ika Labortechnik, Staufen, Germany) or by a 25-gauge needle. A total nucleic acid isolation kit (Applied Biosystems) was used to extract RNA from formalin-fixed paraffin embedded (FFPE) tissue. RNA was quantified by NanoDrop 1000 (Thermo Fisher Scientific, Loughborough, UK). RT-PCR (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems) prior to quantitative PCR normalized to the expression of β-actin (primer sequences available on request).

Immunohistochemistry and microscopy

Paraffin-embedded tissues were sectioned and stained with haematoxylin and eosin (H & E; Hassan & Howell, 2000). Antibodies and antigen retrieval methods are available on request. Sections were blocked (1 h, 1.5% v/v) with goat or rabbit serum, or overnight using a Mouse-on-Mouse kit (Vector Laboratories, Peterborough, UK), and incubated with primary antibody either at 4°C (overnight) or at room temperature (RT, 1 h). Secondary labelling was with either biotinylated (Vectastain elite avidin–biotin complex kit, Vector Laboratories) or alexa-fluor 594-conjugated secondary antibodies and counterstained with haematoxylin or 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Digital images were acquired using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Welwyn Garden City, UK) and a Nuance MSI camera (CRi, Woburn, MA) and analysed using Nuance 2.4.10 software. The percentage of Ki67 or cleaved-caspase 3 positive cells was scored by counting at least 500–1000 cells from 4 to 5 fields *per* tumour.

Microarray gene expression profiling and bioinformatics

RNA from E9.5 embryos were hybridized to Illumina Mouse Whole Genome 6 v1.1 or v2 BeadChips (Illumina, San Diego, CA) by Cambridge Genomic Services (Cambridge, UK) or Wellcome Trust Centre for Human Genetics (Oxford, UK). Bioinformatics included R 2.12.0, (R Development Core Team, 2010) with microarray-specific packages obtained from the Bioconductor repository (Bioconductor 2.7, (Gentleman et al, 2004). Raw data were normalized with 'lumi'

(Du et al, 2008) and Significance Analysis of Microarrays (Tusher et al, 2001) with 'siggenes'. Mapping of Illumina identifiers to annotation terms was done with 'biomaRt' and data from the Ensembl BioMart database (European Bioinformatics Institute, Cambridge, UK).

LOH and mutation analysis

PCR for *p53* LOH with primers are described in Supporting Information Table S5. PCR products were purified with a Zymoclean Gel DNA Recovery Kit (Cambridge Bioscience, Cambridge, UK) and sequenced by GeneService (Oxford, UK).

Author contributions

Experiments were conceived and developed by ABH and VLH; VLH performed all experimental breeding except *Igf2* knockout backcrosses which were performed by CFG; VLH provided all of the experimental data, except array data analysis (DJB), histopathology diagnosis (FP), confocal imaging (CB) and some mouse genotyping (EC); IS and MC generated mice with *Igf2* conditional alleles utilized by VLH; The manuscript and figures were written by VLH, DJB and ABH, and all authors provided detailed comments.

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Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

For more information

P53 OMIM:
http://omim.org/entry/191170

IARC p53 database:
http://www-p53.iarc.fr/

IGF2 OMIM:
http://omim.org/entry/147470

Imprinting:
http://www.geneimprint.com/

Li-Fraumeni syndrome Orphanet:
http://www.orpha.net/

Li-Fraumeni support:
http://www.mdjunction.com/li-fraumeni-syndrome

LFS association:
http://www.lfsassociation.org/our-story/lfs-board/

Constancia lab:
http://www.mrl.ims.cam.ac.uk/staff/Constancia.php

Hassan lab:
http://www.path.ox.ac.uk/dirsci/cellbiol/hassan

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