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Transcription activity is required for p53-dependent tumor suppression

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Abstract

As a transcription factor, the critical tumor suppressor p53 directly regulates the transcription of hundreds of genes, leading to cell cycle arrest, apoptosis, cellular senescence and differentiation. While it has been assumed that p53 transcription activity is critical for tumor suppression, this assumption has been increasingly contested by recent findings of transcription-independent roles of p53 in apoptosis as well as the findings that none of the mutant mice lacking important p53 transcription targets are cancer prone. Based on previous findings that p53 transcription activity is abolished in $p53^{QS}$ (Leu25Trp26 to Gln25Ser26) knock-in mouse cells after DNA damage, to determine the importance of transcription activity of p53 in tumor suppression, we generated a knock-in mice that can conditionally express $p53^{QS}$ protein in a Cre-dependent manner. By breeding the knock-in mice with Lck-Cre transgenic mice that specifically express Cre in thymocytes, we demonstrate that p53-dependent suppression of thymic lymphomas is abolished in thymocytes expressing high levels of $p53^{QS}$ protein. In addition, $p53^{QS}$ protein is accumulated in some of the thymic tumors. Therefore, p53 transcription activity induced by DNA damage is required for tumor suppression. Together with the findings that disruption of various p53-dependent functions individually fails to promote cancer, our findings indicate that various transcription-dependent functions of p53 must collaborate to efficiently suppress tumorigenesis.

Keywords

p53; transcription activity; tumor suppression; metastasis

As the guardian of the genome, tumor suppressor p53 plays multiple roles in both somatic and stem cells to maintain genetic stability, including cell cycle arrest that allows time for the repair of DNA damage, apoptosis and senescence that prevent the cells with damaged genome from replicating, cellular differentiation that eliminates the stem cells with damaged DNA from the self-renewing pool (Ko and Prives, 1996; Michael and Oren, 2002; Xu, 2005). Structural and functional analyses of p53 indicate that it is a transcription factor with a sequence-specific DNA binding domain in the central region, transcriptional activation domains at the N-terminus, and a tetramerization domain at the C-terminus (Ko and Prives, 1996). p53 directly regulates the expression of hundreds of genes that play important roles in p53-dependent functions (Wei *et al.*, 2006). For example, p21 and 14-3-3σ are required for mediating p53-dependent cell cycle G_1/S and G_2/M cell cycle checkpoints respectively (Oren, 2003). Puma is required for p53-dependent apoptosis after DNA damage (Oren, 2003). Plasminogen activator inhibitor-1 is required for p53-dependent replicative senescence (Kortlever *et al.*, 2006). p53 can induce the differentiation of embryonic stem (ES) cells after DNA damage by

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directly suppressing the expression of Nanog, which is required for the self-renewal of ES cells (Lin *et al.*, 2005).

While it is clear that the transcription activity is important for p53-dependent functions in response to various stresses, the importance of p53 transcription activity in tumor suppression remains to be established. In this context, while p53-dependent cell cycle G_1/S arrest after DNA damage is abolished in $p21^{-/-}$ mice, these mice are genetically stable and are not cancer prone (Brugarolas *et al.*, 1995; Deng *et al.*, 1995). Despite the abolishment of p53-dependent apoptosis, Puma-deficient mice are not cancer prone (Jeffers *et al.*, 2003; Villunger *et al.*, 2003). In addition, the importance of p53 transcription activity in tumor suppression is contested by recent studies that have identified transcription-independent roles of p53 in apoptosis and tumor suppression (Schuler and Green, 2005).

To determine the importance of p53-dependent transcription in apoptosis, others and us took the advantage of two missense mutations (Leu22Trp23 to Gln22Ser23) at the N-terminus of human p53 that abolish the transcriptional activities of p53 to independently establish the $p53^{QS}$ (Leu25Ser26 of mouse p53 to Gln25Ser26) knock-in ES cells and mice (Chao *et al.*, 2000; Johnson *et al.*, 2005; Lin *et al.*, 1994). While transcription-independent role of p53 in apoptosis remains intact in $p53^{QS}$ knock-in cells (Chipuk *et al.*, 2004), p53-dependent transcription and apoptosis are abolished in $p53^{QS}$ knock-in cells after DNA damage (Chao et al. 2000; Johnson et al. 2005). Therefore, p53-dependent transcription is critical for p53-dependent apoptosis after DNA damage.

To determine the physiological importance of p53-dependent transcription in tumor suppression, we introduced the $p53^{QS\text{-}Neo}$ allele into mouse germline (Fig. 1a). The detail of the targeting construct and strategy to generate $p53^{QS\text{-}Neo}$ allele in ES cells was described by us previously (Chao *et al.*, 2000). It is known that the PGK-Neo cassette in the targeted allele suppresses the expression of the targeted gene (Chao *et al.*, 2000;Inlay *et al.*, 2002), the $p53^{QS\text{-}Neo}$ mice were bred with CMV-Cre mice that express Cre at the zygote stage and have been routinely used to excise the LoxP-flanked PGK-Neo gene from various p53 knock-in mice (Chao *et al.*, 2003;Chao *et al.*, 2006a;Chao *et al.*, 2006b;Song *et al.*, 2007). Similarly to previous findings in an independently established $p53^{QS}$ mice that normal expression of $p53^{QS}$ allele leads to embryonic lethality (Johnson *et al.*, 2005), no offsprings harboring the PGK-Neo-deleted allele could be found from the breeding of $p53^{QS\text{-}Neo}$ and CMV-Cre mice.

$p53^{-/-}$ mice mostly develop thymic lymphomas, indicating the critical roles of p53 in tumor suppression in mouse thymocytes (Donehower *et al.*, 1992; Jacks *et al.*, 1994). Therefore, to determine the physiological importance of p53 transcription activity in tumor suppression, $p53^{QS\text{-}Neo}$ mice were bred with Lck-Cre transgenic mice that express Cre specifically in the thymocytes. $p53^{QS\text{-}Neo}$Lck-Cre^{+} mice, denoted $p53^{QSL}$ mice, were born with expected mendelian ratio and could mature into adulthood. When assayed by PCR, the PGK-Neo gene was deleted from the targeted alleles in the thymocytes of all homozygous $p53^{QSL}$ mice analyzed (>20 mice), indicating that Lck-Cre transgene expresses sufficient Cre enzyme in thymocytes of $p53^{QSL}$ mice for efficient LoxP/Cre-mediated deletion of the PGK-neo gene. Consistent with previous findings that QS mutation disrupts the interaction between p53 and Mdm2 leading to p53 stabilization (Chao *et al.*, 2000; Lin *et al.*, 1994), significantly higher p53 protein levels were detected specifically in the thymocytes derived from $p53^{QSL}$ mice than those from Lck-Cre^{+} control mice (Fig. 1b, c, d). In addition, the expression of p53 is specifically silenced in the thymocytes of Lck-$Cre^{+}p53^{LoxP/LoxP}$ mice (Figure 1b, c, d). Similarly to what was observed in other cell types (Chao *et al.*, 2000; Johnson *et al.*, 2005), while DNA damage agents such as ionizing radiation (IR) and doxorubicin significantly induce the protein levels of p53 in the Lck-Cre^{+} thymocytes, they did not increase the p53 protein levels in $p53^{QSL}$ thymocytes (Fig. 1b, c). In addition, p53 was constitutively phosphorylated

at Ser18 in $p53^{QSL}$ thymocytes, suggesting the chronic activation of DNA damage responses responsible for p53 phosphorylation in these thymocytes (Fig. 1b). In summary, p53 is constitutively stable in $p53^{QSL}$ thymocytes. Despite the greatly increased protein levels of p53, p53-dependent induction of target genes was abolished in $p53^{QSL}$ thymocytes after IR, confirming that p53-dependent transcription is essentially abolished in $p53^{QSL}$ thymocytes after DNA damage (Fig. 1e). Consistent with this finding, similar to that in $p53^{-/-}$ mice, p53-dependent apoptosis after IR is abolished in $p53^{QSL}$ thymocytes (Fig. 1f).

To determine the contribution of p53 transcription activity to tumor suppression, the tumorigenesis in $p53^{QSL}$ mice were monitored. As controls, conditional p53 knockout mice ($p53^{LoxP/LoxP}$) were bred with Lck-Cre^{+} mice to specifically eliminate p53 in the thymocytes (Jonkers *et al.*, 2001). As expected, none of the Lck-$Cre^{+}p53^{+/+}$ control mice died of tumors within 50 weeks of age (Fig. 2a). The $p53^{LoxP/LoxP}$Lck-Cre^{+} mice were all succumbed to thymic lymphomas by 50 weeks of age, further supporting the critical roles of p53 in suppressing thymic lymphomas (Fig. 2a, b). $p53^{QSL}$ mice uniformly died of tumors by 50 weeks of age, and the tumors are predominantly thymic and peripheral lymphomas (Fig. 2a, b). Since there is no statistically significant difference in the survival between $p53^{LoxP/LoxP}$Lck-Cre^{+} and $p53^{QSL}$ mice, we concluded that p53-dependent tumor suppression is abolished in $p53^{QSL}$ thymocytes.

Previous studies have suggested that $p53^{QS}$ protein retains some transcription activity in response to non-genotoxic stresses (Johnson *et al.*, 2005). If $p53^{QS}$ protein still retains significant tumor suppression activities, the expression of $p53^{QS}$ protein should be selected against in the tumors developed in $p53^{QSL}$ mice. However, p53 protein was accumulated to high levels in some of the thymic lymphomas of $p53^{QSL}$ mice (Fig. 3a). Sequencing analysis of the p53 gene in two of these tumors indicate that only QS mutations but no other mutations are present in the p53 gene. The basal levels of p53-dependent transcription of target genes were reduced in the thymic tumor cells when compared with that in pre-tumor $p53^{QSL}$ thymocytes, indicating that the remnant transcription activity of $p53^{QS}$ is selected against in $p53^{QSL}$ thymic tumor cells as seen in most human cancer cells that harbor wild type p53 (Fig. 3b). Similarly to thymic tumors observed in $p53^{-/-}$ mice (Donehower *et al.*, 1992; Jacks *et al.*, 1994), none of thymic tumors in $p53^{LoxP/LoxP}$Lck-Cre^{+} mice exhibited metastatic phenotypes to invade other tissues. However, about 40% of thymic lymphomas detected in $p53^{QSL}$ mice were metastatic and invaded other tissues (Fig. 2b, 3c). In addition, peripheral lymphomas were frequently detected in $p53^{QSL}$ mice (Fig. 2b). Therefore, similarly to what has been observed in p53 gain of function mutant knock-in mice, the constitutively stable $p53^{QS}$ protein could behave like a gain-of-function p53 cancer mutant to promote metastasis by disrupting the functions of other proteins (Lang *et al.*, 2004; Olive *et al.*, 2004; Song *et al.*, 2007).

With recent discovery of the transcription-independent roles of p53 in apoptosis and tumor suppression (Moll *et al.*, 2005), the requirement of p53-dependent transcription in tumor suppression has been challenged. Since transcription-independent roles of p53 are retained by $p53^{QS}$ in both transfected cells and knock-in cells (Chipuk *et al.*, 2004), our findings provide definitive evidence that p53 transcription activity induced by DNA damage is required for p53-dependent tumor suppression. Together with the findings that the disruption of a single p53-dependent function does not lead to increased tumorigenesis, our findings support the conclusion that these p53-dependent functions cooperate to suppress tumorigenesis *in vivo*.

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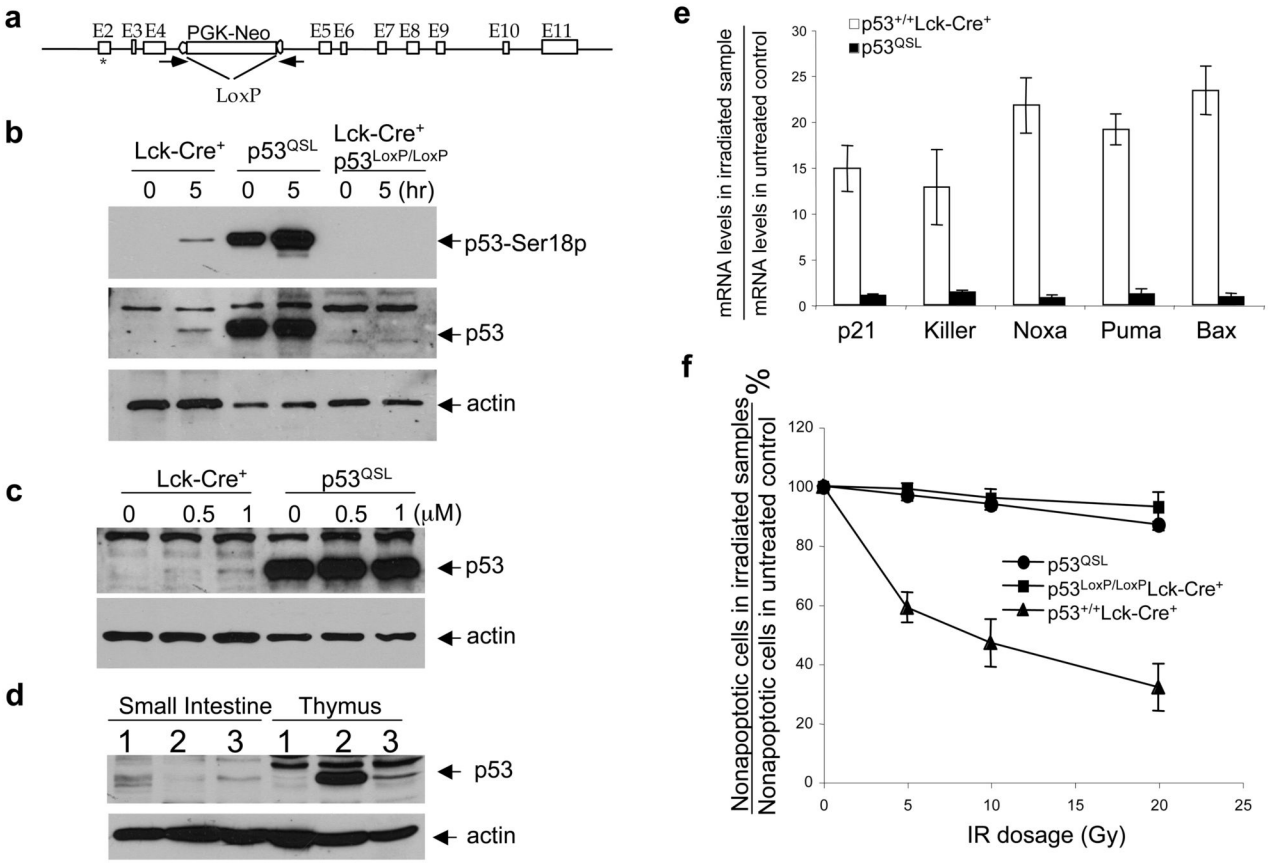
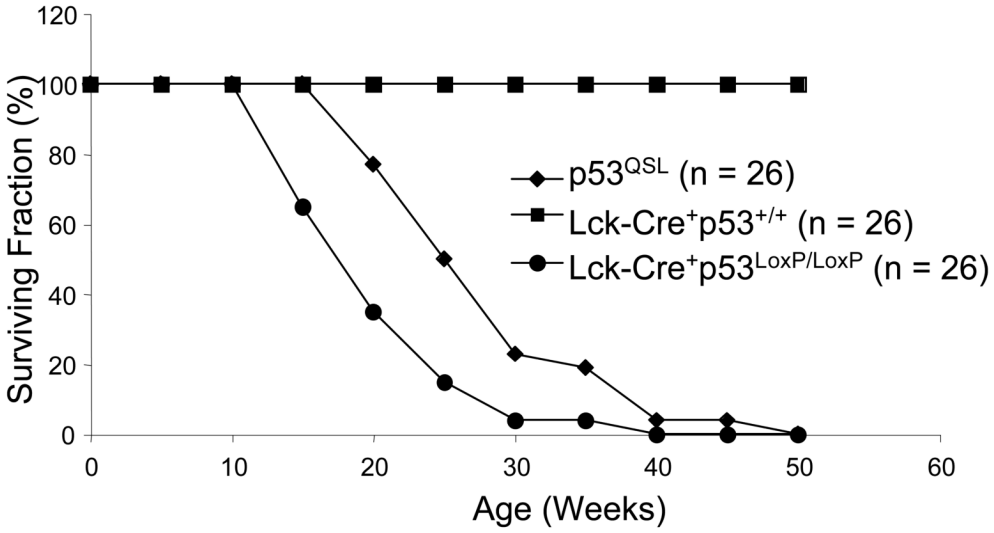


Figure 1.

Expression and function of p53 in the thymocytes of $p53^{QSL}$ mice. (**a**) The $p53^{QS\text{-}Neo}$ knock-in allele. The exons are represented by open boxes. PCR primers to screen for the LoxP/Cre-mediated deletion are indicated by arrowheads. The QS mutations are indicated by an asterisk. The generation of the knock-in ES cells were described previously (Chao *et al.*, 2000). (**b**) Expression of p53 protein in the thymocytes of Lck-Cre^{+}, $p53^{QSL}$ and Lck-$Cre^{+}p53^{LoxP/LoxP}$ mice before and 5 hours after IR (10Gy). Lck-Cre^{+} mice express the Cre enzyme specifically in the thymocytes but not in other cell types (Lee *et al.*, 2001). In $p53^{LoxP/LoxP}$ mice, the exons 2–10 of the p53 gene are flanked by LoxP sites and can be deleted from the genome in a Cre-dependent manner (Jonkers *et al.*, 2001). Protein extracts from 2×10^{6} thymocytes were resolved on 10% SDS PAGE gel and transferred to nitrocellulose membrane, which was probed with a polyclonal phosphor-specific antibody against p53 phosphorylated at Ser18 (Cell Signaling Technology, Danvers, MA) or ploclonal antibody against p53 or β-actin (Santa Cruz Biotechnology). The membrane was subsequently probed with a horseradish peroxidase-conjugated secondary antibody and developed with Supersignal Pico reagents (Thermo Scientific, Rockford, IL). (**c**) Induction of p53 protein levels in Lck-Cre^{+} and $p53^{QSL}$ thymocytes 8 hours after treatment with increasing concentration of doxorubicin. (**d**) Expression of p53 protein in the small intestine and thymus of Lck-Cre^{+}, $p53^{QSL}$ and Lck-$Cre^{+}p53^{LoxP/LoxP}$ mice 1 hour after whole body IR (10Gy). No p53 protein can be detected in the brain and liver of these mice of all genotypes by Western blotting (data not shown). (**e**) Induction of p53 target genes in Lck-Cre^{+} and $p53^{QSL}$ thymocytes 5 hours after 10Gy of IR. Total RNA from thymocytes was isolated using Trizol (Invitrogen, Carlsbad, CA) and RNAeasy Mini Kit (Qiagen, Valencia, CA), reverse-transcribed using Superscript II RT (Invitrogen). Real-time PCR was performed with an AjBI Prism 7000 (Applied Biosystems,

Foster City, CA) with Power SyberGreen PCR Master Mix (Applied Biosystems, Foster City, CA). The PCR conditions were: 10 min at 95°C, 40 cycles of 15 sec at 95°C and 1 min at 60° C. The average Ct value for each gene was determined from triplicate reactions and normalized with the levels of GAPDH as previously described (Chao *et al.*, 2006a). The primers were described previously (Chao *et al.*, 2006a; Lin *et al.*, 2005). Mean value from three independent experiments are presented with standard derivation. (**f**) p53-dependent apoptosis of Lck-Cre^{+}, p53^{QSL} and Lck-Cre^{+}p53$^{LoxP/LoxP}$ thymocytes 10 hours after increasing dosages of IR. Single cell suspension of thymocytes derived from 4-week-old mice was cultured in DMEM supplemented with 5% FBS and 25 mM HEPES at pH 7 at a density of 10^{6} cells/ml. The cells were irradiated and the percentage of apoptotic cells was analyzed 10 hr later by staining with Annexin V-FITC as described (Chao *et al.*, 2006a).

a



b

Tumor Type	$p53^{QSL}$	$p53^{LoxP/LoxP}$Lck-Cre^{+}	P value
Thymic only	12 (46%)	26 (100%)	P < 0.0001
Thymic, spread to ribcage Thymic, spread to lymph nodes	3 (12%) 5 (19%)	0 (0%) 0 (0%)	P = 0.004
Peripheral lymphomas only	6 (23%)	0 (0%)	P = 0.02

Figure 2.

Tumorigenesis of $p53^{QSL}$ mice. (**a**) Survival curve of Lck-Cre^{+}, $p53^{LoxP/LoxP}$Lck-Cre^{+} and $p53^{QSL}$ mice. N shows the number of mice monitored. The P value for the difference of survival between $p53^{LoxP/LoxP}$Lck-Cre^{+} and $p53^{QSL}$ mice is 0.46. (**b**) Tumor spectrum of $p53^{LoxP/LoxP}$Lck-Cre^{+} and $p53^{QSL}$ mice. The p values are shown.

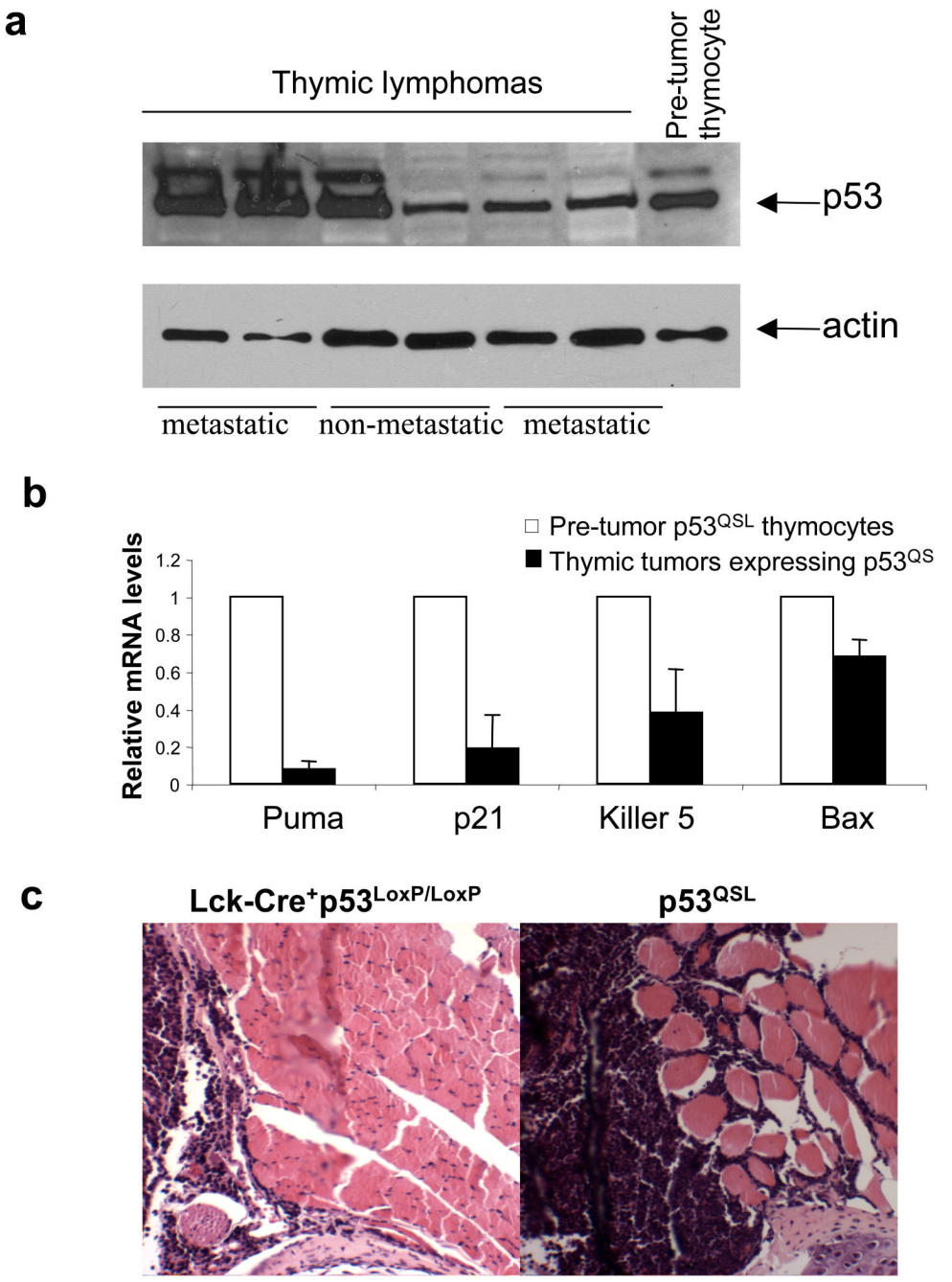


Figure 3.

Metastatic phenotypes of thymic tumors in $p53^{QSL}$ mice. (**a**) p53 protein is accumulated to high levels in some of the thymic tumors derived from $p53^{QSL}$ mice. The metastatic and non-metastatic tumors are indicated. (**b**) The relative expression of p53 target genes in the tumors expressing $p53^{QS}$ versus in pre-tumor $p53^{QSL}$ thymocytes. The mRNA levels of each gene were determined by quantitative real time PCR and standardized by the mRNA levels of GAPDH. Mean value from three tumors are presented with standard derivation. (**c**) Representative histological image of one metastatic thymic tumor in $p53^{QSL}$ mice that invade the skeleton muscles of the rib cage as well as the one from $p53^{LoxP/LoxP}$Lck-Cre^{+} mice. Tumor samples were fixed in 10% buffered formalin, embedded in paraffin and sliced. All sections

were stained with hematoxylin and eosin for histological assessment as previous described (Chao *et al.*, 2006a).