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Kras regulatory elements and exon 4A determine mutation specificity in lung cancer

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Abstract

*KRAS*is the most frequently mutated*ras*family member in lung carcinomas[1,2], whereas*HRAS*mutations are common in tumours from stratified epithelia such as bladder or skin (www.sanger.ac.uk/genetics/CGP/cosmic/). Using a mouse model (*HrasKI*)[3] in which the*Hras*coding sequence was inserted into the*Kras*locus, we demonstrate that specificity for*Kras*mutations in lung and*Hras* mutations in skin tumours is determined by local regulatory elements in the target*ras*genes. We further show that, while*Kras-4A*is dispensable for mouse development[4,5], it is necessary both for lung carcinogenesis*in vivo*and for the previously reported[6,7] inhibitory effect of wild-type (*WT*)*Kras*on the transforming properties of the mutant allele. Kras-4A expression is detected in a sub-population of normal lung epithelial cells, but at very low levels in lung tumours, suggesting a role in tumour initiation rather than in tumour maintenance. The two Kras isoforms undergo different post-translational modifications[8], therefore these findings can have important implications for the design of therapeutic strategies for inhibiting oncogenic Kras activity in the prevention and treatment of cancer.

Kras deficiency in mice leads to embryonic lethality[4,5], thus it has not been possible to determine whether the selection for *Kras* mutations in lung tumours reflects a specific oncogenic function that is required for lung carcinogenesis and cannot be compensated for by mutant *Hras* or *Nras*. The *HrasKI* mouse provides a viable model that completely lacks *Kras* expression[3] (Fig. 1), but carries a “knock-in” *Hras* cDNA expressed under the control of *Kras* regulatory elements. We reasoned that *HrasKI* mice should be resistant to lung tumour development if *Kras* protein is essential for lung carcinogenesis. To control for the possibility of structural alterations affecting experimental outcomes, we used *KrasKI* mice in which the coding sequence corresponding to the *Kras-4B* isoform was knocked back into the *Kras* locus in the exact manner used to generate the *HrasKI* mouse[3]. *HrasKI* mice expressed elevated levels of Hras protein, consistent with the increase in *Hras* gene dosage from two copies to four in these animals (Fig. 1). *KrasKI* mice express similar levels of Kras protein as *WT* mice, as expected since *Kras-4B* is the major splice isoform[9,10]. Levels of Akt and Erk activation in these animals were similar to *WT* mice (Fig. 1). There was also no difference in levels of p38α, recently shown to affect *ras* mediated lung carcinogenesis[11]. The elevated level of Hras

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therefore does not affect the major downstream effector pathways relevant to cancer development.

We treated *HrasKI* homozygous and heterozygous mice and *WT* littermates with urethane and sacrificed the animals 20 weeks later for analysis. Surprisingly, *HrasKI* homozygous mice developed ~10 fold more lung tumours than *WT* littermate controls (44.8 ± 11.7 vs. 4.7 ± 2.9 (Fig. 2)). The number of lung tumours in *HrasKI* heterozygous mice (29.9 ± 5.8) was intermediate between those of *WT* and *HrasKI* homozygous animals. While previous studies showed that activating mutations in *Kras* are important in lung cancer development[2,7,12,13], our findings demonstrate that *Kras* is clearly dispensable for lung carcinogenesis.

The observation that lung tumour multiplicity correlates with the number of *HrasKI* alleles suggests that this allele is the target for mutations in *HrasKI* mice. The majority of lung tumours (21 of 27) from *WT* mice contain activating mutations in *Kras*, frequently at codon 61 but also at 12 or 13, as previously described[2,7] (Supplementary Table 1). Of 35 lung tumours from *HrasKI* homozygous mice analyzed, 32 (91%) have mutations in the *HrasKI* allele. In heterozygous *HrasKI* mice, the vast majority of lung tumours (>90%) have a mutation in the *HrasKI* allele, with mutations in the endogenous *Kras* gene occurring in less than 10% of the tumours ($p = 1.6 \times 10^{-22}$, Fisher's exact test). In all cases, mutation in the *HrasKI* allele involves a CAA>CTA transversion at codon 61, which is the signature mutation in the *Hras* gene in mouse skin tumours induced by the two stage chemical carcinogenesis protocol[14]. However, we never found the endogenous *Hras* gene to be mutated in urethane induced lung tumours. The occurrence of *Hras* mutations in lung tumours therefore is completely dependent on *Hras* being expressed from the *Kras* locus.

We further pursued the specificity of *ras* gene mutations in lung and skin tumours by inducing both tumour types in the same *HrasKI* homozygous mice. A single dose of urethane was injected intraperitoneally to induce lung tumours, followed by a single topical treatment on the skin with urethane and subsequent biweekly application of tetradecanoyl-phorbol acetate (TPA) to induce papilloma development. All lung tumours (n = 28) analyzed have the CAA>CTA mutation in the *HrasKI* allele. Of the papillomas (n = 20) developed in these same mice, 15 (75%) carried the CAA>CTA mutation in the endogenous *Hras* gene on chromosome 7, consistent with previous studies[14,15]. Of the remaining 5 papillomas, 4 (20%) contained the CAA>CTA mutation in the *HrasKI* allele. Mutations in the endogenous *Kras* gene are rarely seen in *WT* mice, but the frequency is increased in *Hras*-/- mice where no endogenous *Hras* target is available[16]. Regulatory elements in *Kras* are therefore capable of directing expression to at least a subset of target cells within the epidermis, but the overall preference is strongly in favour of *Hras*. These results indicate that the mechanisms underlying K*ras* mutation selection in lung cancer and *Hras* in skin cancer involve cis-acting regulatory elements specific to each gene rather than functional differences between the encoded proteins. Previous studies have demonstrated the existence of multiple sequence variants in the mouse *Kras* gene that may potentially affect regulation of expression[17], and further studies would be required to identify the specific element(s) involved.

As with other *Kras* models of lung tumour development[12,13], *HrasKI* mice developed papillary adenomas (Fig. 3a), but also significantly more mixed adenomas (Fig. 3b) and solid adenomas (Fig. 3c), sometimes with intrabronchiolar extension (Fig. 3c, inset). A significant percentage of the solid adenomas contain cells with epithelioid morphology (Fig. 3d, e) - large, plump cells with eccentrically located nuclei and abundant cytoplasm ranging from finely granular and eosinophilic to microvesicular and amphophilic. Positive cytokeratin 8/18 staining (Fig. 3f) and negative PAS staining (Fig. 3g) demonstrated that the vast majority of these cells were epithelioid, non-mucinous tumour cells. These solid epithelioid tumours

stained positively for SP-C (Fig. 3h) and negatively for CCA/CC10 (Fig. 3i), like tumours from urethane treated *WT* mice and other mouse models of lung cancer[12,13,18].

Both *Kras-4A* and *Kras-4B* splice variants are present in normal lungs, but *Kras-4B* is clearly the more abundant isoform[9,10]. However, *KrasKI* homozygous mice that express only *Kras-4B* proved to be highly resistant to urethane-induced lung tumour formation (Fig. 4a). Of 10 *KrasKI* homozygous mice examined, 8 did not develop any surface lung tumours. Two mice developed 1 and 2 small tumours each, but none of the tumours contained a mutation in the *KrasKI* allele, nor in the endogenous *Hras* or *Nras* genes. Consistent with our findings, mice with a genomic knockout of exon 4A and therefore able to express only *Kras-4B* displayed a dramatic decrease in lung tumor number following treatment with N-methyl-N-nitrosourea[19].

Tumours from mice heterozygous for the *KrasKI* allele were also positive for SPC and negative for CCA/CC10 (Supplementary Fig. 1). *Kras* mutations were present in 24 of 33 tumours (73%) from *KrasKI* heterozygous mice, but in all 24 cases the mutation occurred in the endogenous *Kras* gene (which retains both *Kras-4A* and *Kras-4B*) and not in the *KrasKI* allele (which expresses exclusively *Kras-4B*) ($p = 6.2 \times 10^{-14}$, Fisher's exact test). These findings are consistent with the resistant phenotype of *KrasKI* homozygous mice, and indicate that oncogenic mutations involving only *Kras-4B* do not provide the necessary advantage required for tumourigenesis. Since both *Kras-4A* and *Kras-4B* transcripts are present in lungs of mice containing the intact *Kras* gene[9,10], our results suggest that the transforming effect of *Kras* mutations during lung carcinogenesis *in vivo* are mediated primarily through the activity of the Kras-4A protein.

Kras-4A and Kras-4B isoforms differ only at their carboxyl termini, resulting in differences in post-translational modifications[8]. Kras-4A and Hras proteins undergo similar post-translational modifications and therefore are localized within similar microdomains in the membrane, distinct from those involving Kras-4B[8,20]. The similarities of Kras-4A and Hras in this respect correlate with their ability to induce lung tumours in *WT* and *HrasKI* mice, respectively. *In vitro* studies also demonstrated that mutant *Kras-4A* and *Hras* are more potent oncogenes than mutant Kras-4B[21,22].

The *WT ras* alleles in tumours that contain *ras* mutations are frequently deleted, and/or the mutant allele is amplified[23-25]. The possibility of a tumour suppressor function for WT Ras proteins was supported by *in vitro* functional assays[26] and *in vivo* genetic studies involving genetically engineered mice[6,7]. These studies demonstrated that WT Kras can potently suppress the activity of oncogenic Kras to regulate lung tumour development[6,7]. We found that *KrasKI* heterozygous mice surprisingly developed ~2-fold more lung tumours than *WT* mice (Fig. 4a). The observation, together with results from our mutational analysis, suggested that the *KrasKI* allele may be less efficient in this suppressor activity. To further test this idea, *KrasKI* heterozygous mice were bred with $Kras^{LA2}$ mice[13], which undergo spontaneous recombination to activate *Kras* and do not require carcinogen treatment. The genotypes tested included animals with one $Kras^{LA2}$ allele and either a *WT Kras* gene or a *WTKrasKI* allele. $Kras^{LA2}$ mice carrying a *KrasKI* allele developed significantly more lung tumours than those that are able to express *Kras-4A* isoform from the *WT Kras* gene (Fig. 4b). Interestingly, the *HrasKI* allele is also more effective than the *KrasKI* allele in suppressing tumor multiplicity in $Kras^{LA2}$ mice, indicating that it can substitute for *Kras-4A* in suppressor activity *in vivo*. While *in vitro* work showed that overexpressed WT *Kras-4B* has inhibitory effects, our collective *in vivo* findings suggest that efficient suppression of oncogenic Kras activity requires an intact *WT Kras* locus that is capable of generating the *Kras-4A* isoform.

Studies of human tumours have shown that *Kras-4B* is the more abundant splice variant, and in fact that levels of *Kras-4A* may be even further reduced compared to *Kras-4B* in tumors carrying *Kras* mutations[27]. This is in apparent contradiction to our genetic evidence for a major role of the *Kras-4A* isoform in lung carcinogenesis. We therefore investigated the localization of *Kras-4A* expression in normal lung and lung tumours. Using an antibody specific for Kras-4A, expression was detected in a sub-population of Clara cells throughout the bronchial tree, including at the bronchio-alveolar junctions (Fig. 5a, b). Similar sections from mice homozygous for the *KrasKI* allele (Fig. 5c) or the *HrasKI* allele (not shown), both of which lack *Kras-4A*, were negative. Antibodies against Kras-4B yielded non-specific signals, as positive staining was observed even in sections from *HrasKI* homozygous mice that completely lack *Kras* expression (data not shown). The bronchio-alveolar junctions have been reported to contain the putative bronchio-alveolar stem cells (BASCs)[28], but Kras-4A positive cells are certainly more abundant than BASCs. Sections of tumours from *WT* mice however showed almost no detectable signal for Kras-4A protein (Figure 5d), although low level expression was detected at the RNA level, as previously described for human cancers[27]. These data, taken together with the genetic evidence presented above, suggest that *Kras-4A* is required for tumor initiation, but may not be necessary for maintenance of tumour growth. It is possible that *Kras-4A* expression may affect the stem cell fate decision leading to the adenocarcinoma lineage, but further detailed studies will be required to elucidate the relationship between *Kras* isoform expression and lung stem cells. Interestingly, differential roles for *Kras-4A* and *-4B* have previously been proposed in embryonic stem cell differentiation[9], but there are presently no data on adult stem cells.

Inhibitors targeting specific Ras post-translational events, in particular farnesylation, have not been successful in clinical trials, the reasons for which are unclear. Nevertheless, *in vitro* studies as well as mouse models strongly suggest that the activity of oncogenic Ras proteins can be effectively inhibited through manipulation of their carboxyl domains[8,20]. The identification of Kras-4A, and not Kras-4B as previously thought, as the main mediator of both the oncogenic activity of mutant Kras and the suppressor activity of WT Kras provides new opportunities for designing and testing novel targeted therapies.

Methods

Mouse breeding and Carcinogenesis

The *HrasKI* and *KrasKI* alleles were originally generated, and maintained in a *129/Sv* background. Animals heterozygous for the *HrasKI* or *KrasKI* allele were intercrossed to generate *WT*, *KI* heterozygous or *KI* homozygous animals. Genotyping of mice was performed on DNA extracted from tail clippings (see Supplementary Table 2 for PCR primer sequences). Chemical carcinogenesis of the lungs and skin was performed as previously described[2,7,14,15]. $Kras^{LA2}$ mice (on *FVB/N* background) were bred with *KrasKI* heterozygous mice, and all mice that inherited the $Kras^{LA2}$ were selected for analysis. Animals were sacrificed at 4 months of age for analysis.

All mouse experiments were approved by the University of California, San Francisco Laboratory Animal Resource Center.

Mutational Analysis

Lung tumours were dissected from fixed lungs under a dissecting microscope, and genomic DNA was purified by phenol/chloroform extraction following proteinase K digestion. Mutations were detected by sequencing of PCR products corresponding to the endogenous *Kras*, *HrasKI*, *KrasKI* fragments containing the mutation hotspots at codon 12, 13 or 61 (Supplementary Table 2 for primer sequences). Since mutations in the *HrasKI* and *Hras* were

found by sequencing to be exclusively the CAA-CTA alteration at codon 61, we took advantage of the *XbaI* digestion assay to detect this mutation[29].

Immunoblotting

Protein lysates of lung tissues were prepared as previously described[30]. Immunoblots were probed with antibodies against Kras (F234 and C-19), Hras (C-20), Nras (C-20), and Erk1 (C-16) from Santa Cruz Biotechnology; phospho-p44/p42 Map Kinase, Akt, and αp38 from Cell Signaling; β-actin from Sigma; and phospho-Akt from Dr. David Stokoe (UCSF).

Immunohistochemistry

Sections were deparaffinized and antigen retrieval was performed in 10mM sodium citrate solution (pH 6). Endogenous peroxidase was quenched with a 3% H_2O_2 solution (Sigma) for 10 min at room temperature, and blocking was performed with 5% goat serum (Zymed) diluted in PBS containing 0.1% Tween-20 for 1h at room temperature. Overnight incubation with the primary antibodies against CCA/CC10 (a gift from Dr. A. Mukherjee, NICHD/NIH), SP-C (Chemicon), and Kras-4A (Santa Cruz Biotechnology) was performed at 4 °C. After washing in PBS containing 0.1% Tween-20 (PBS-T) for 3× 5min sections were incubated with a biotinylated secondary antibody anti rabbit (Vector) at a dilution of 1:200 for 1h at room temperature. Sections were washed with PBS-T, incubated in Vectastain ABC reagent (Vector) according to the manufacturer's instructions, and developed using DAB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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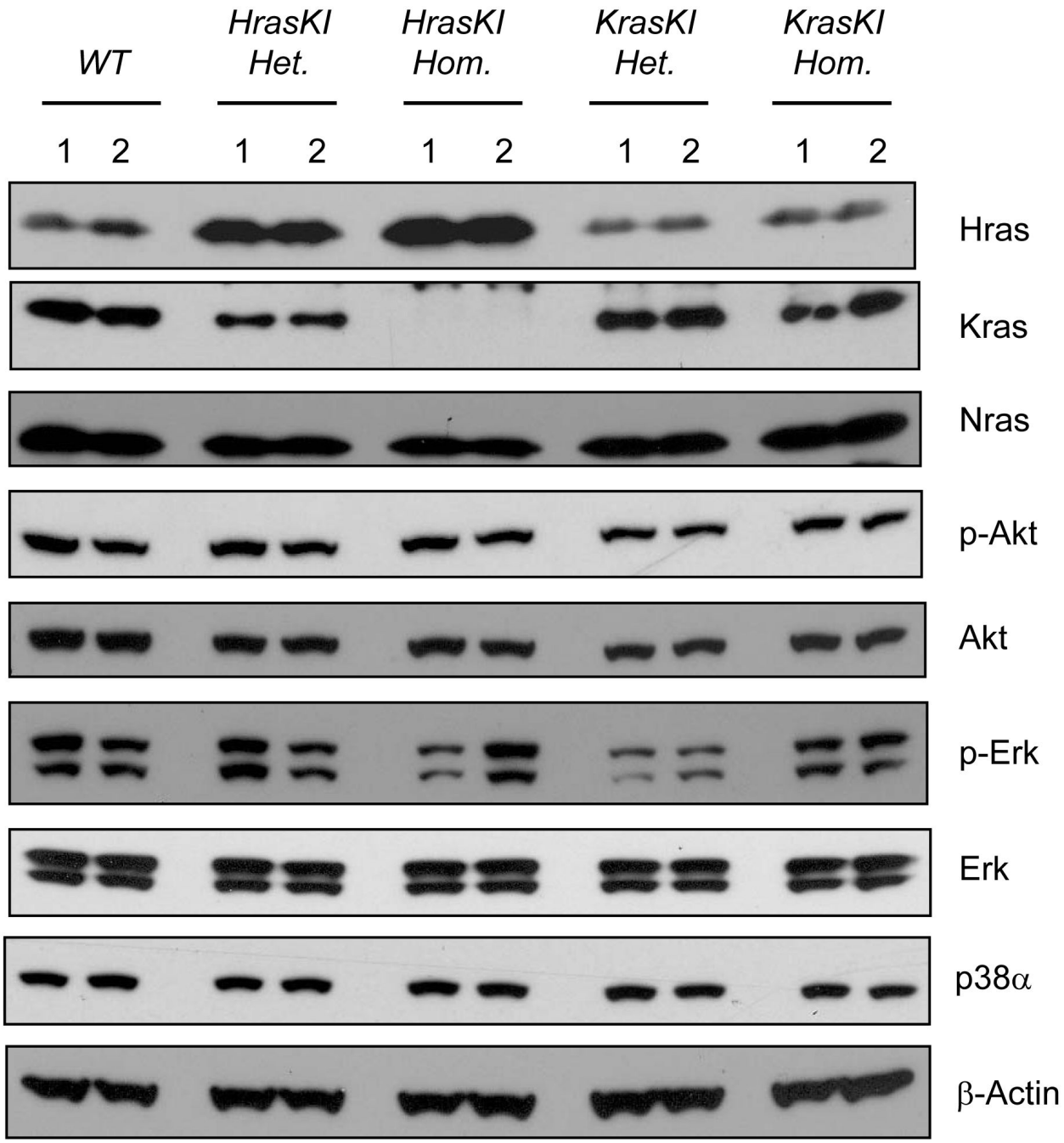

Figure 1. Ras protein levels and effects on downstream signaling effectors in *HrasKI* and *KrasKI* mice

Analysis of Ras protein levels in lungs of untreated mice showed elevated levels of Hras protein and complete absence of Kras protein in *HrasKI* homozygous mice. Mice homozygous and heterozygous for the *KrasKI* allele express similar Kras protein levels to *WT* mice. No apparent differences in activation of Akt and Erk, as measured by protein phosphorylation, and in level of p38α were observed among mice of the indicated genotypes.

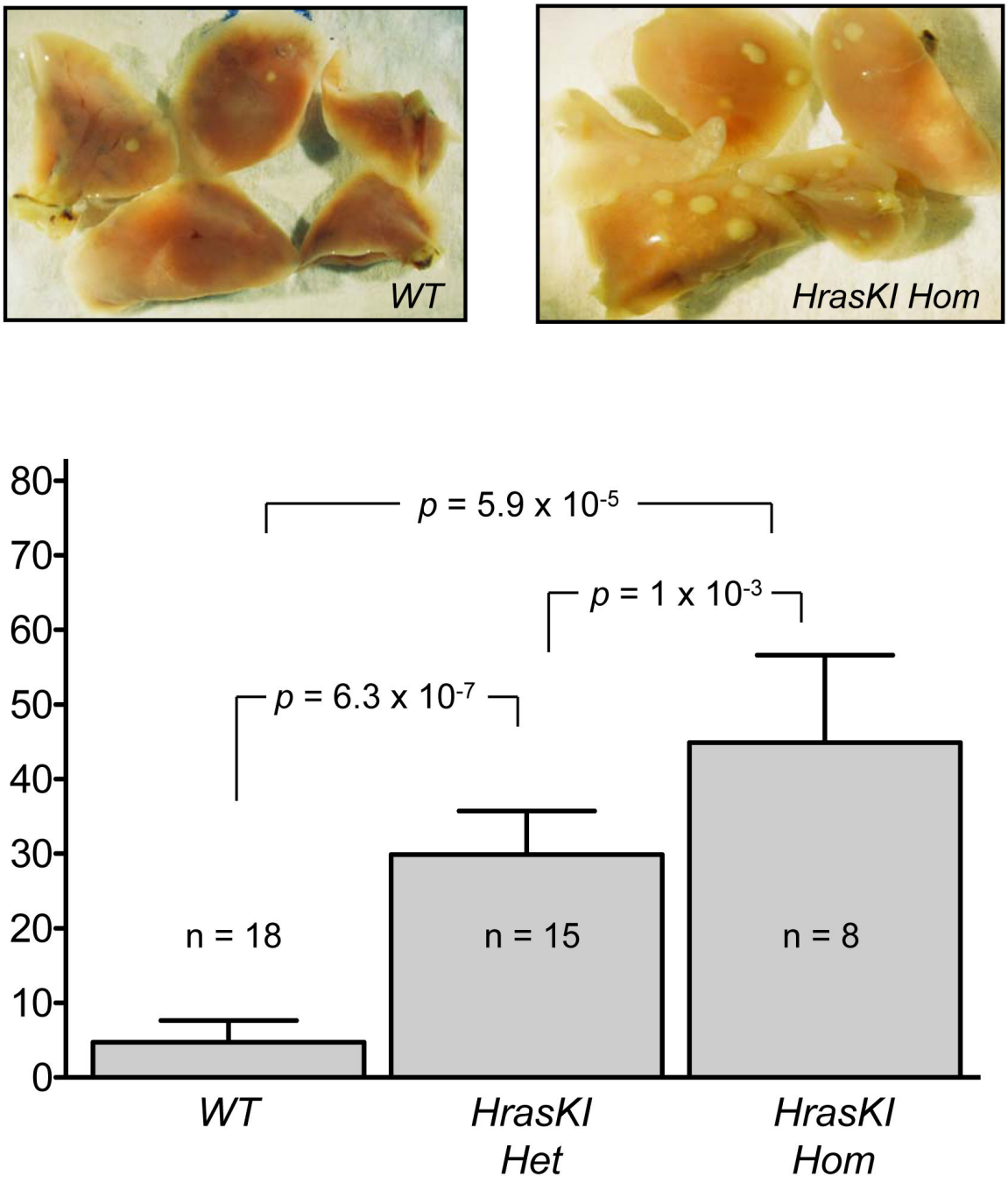

Figure 2. *HrasKI* mice are highly susceptible to urethane induced lung tumours

HrasKI homozygous mice developed significantly more lung tumours than *WT* littermates. The increase in lung tumour number correlated with the number of *HrasKI* alleles. Error bars indicate standard deviations, and statistics were performed using the Wilcoxon Mann-Whitney test.

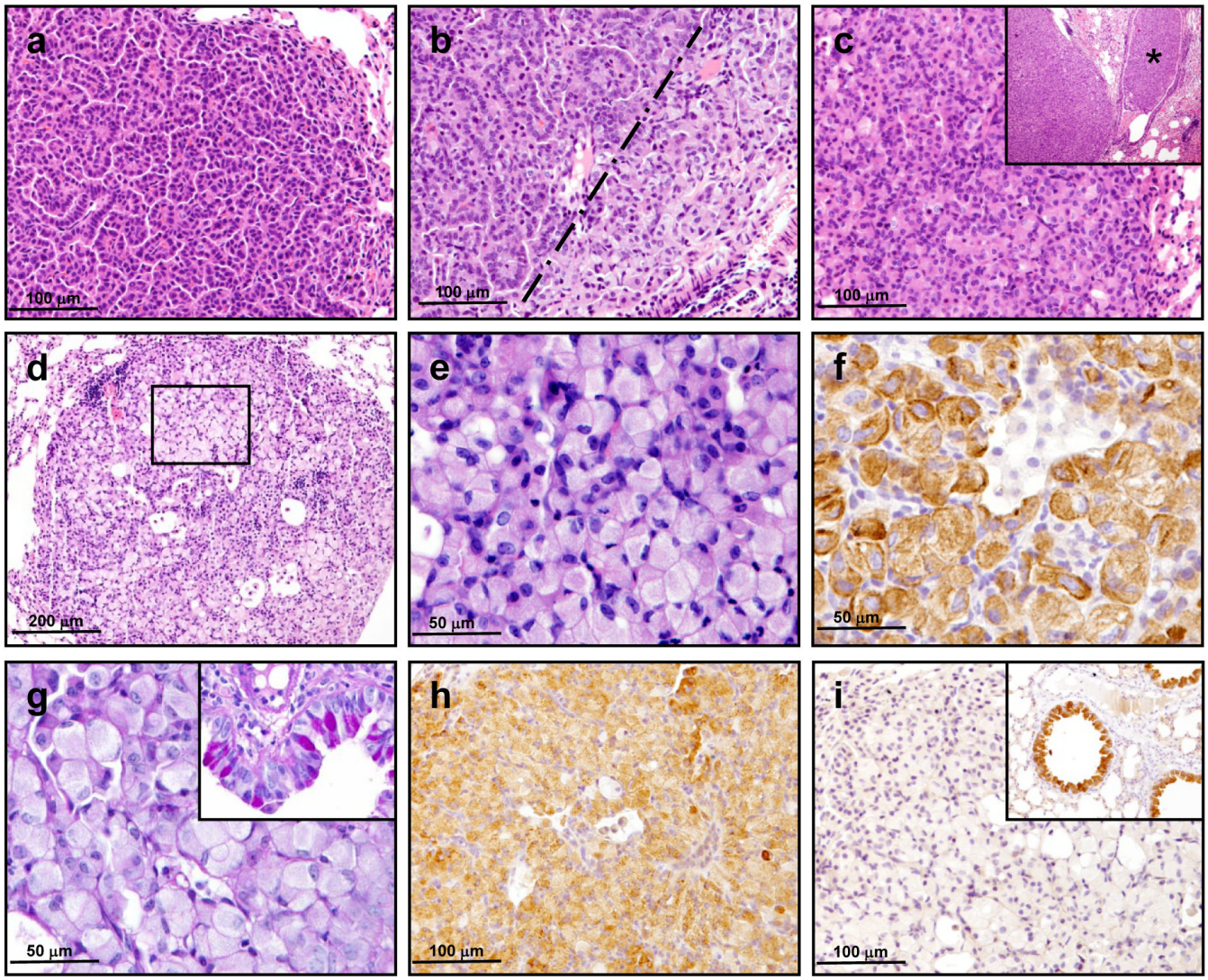

Figure 3. Lung tumours from *HrasKI* mice display papillary, solid and mixed growth patterns, and contain cells with an epithelioid morphology

WT and *HrasKI* mice developed a spectrum of papillary (**a**), mixed (**b**) and solid adenomas (**c**). Whereas *WT* animals predominantly developed papillary adenomas with occasional mixed and solid tumours, the *HrasKI* mice tended to develop more mixed and solid adenomas, sometimes with prominent intrabronchiolar extension (**c**, inset, marked by asterisk). The dashed line in (**b**) marks the boundary between the papillary (left) and solid (right) portions of the mixed adenoma. Several solid adenomas from *HrasKI* mice contained epithelioid cells (**d,** inset enlarged in **e**) which stained positive for (**f**) cytokeratin 8/18 and negative for PAS (**g**). Occasional macrophage clusters (**f,** keratin-negative cells) were also present in the epithelioid adenomas. Goblet cell metaplasia served as a positive control for PAS/mucin (**g,** inset). These adenomas displayed features of alveolar type II pneumocytes like their papillary counterparts (not shown) as they stain positively for SPC (**h**) and negatively for CCA/CC10 (**i**). The adjacent bronchiolar epithelium stained positive for CCA/CC10 as expected (**i,** inset).

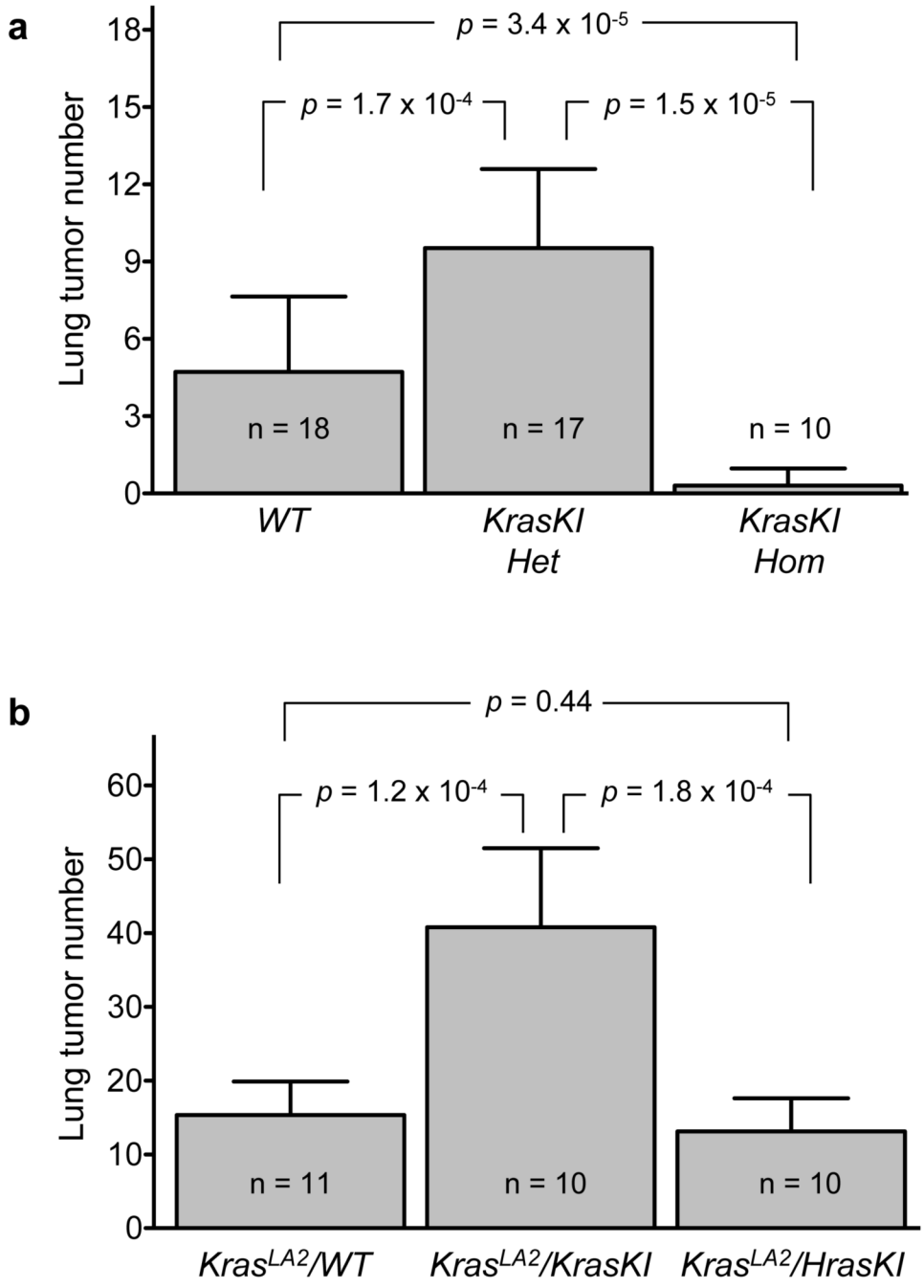

Figure 4. The *KrasKI* allele renders mice resistant to urethane induced lung carcinogenesis, and is deficient in suppression of lung tumour development in *Kras*^{LA2} animals

(**a**) *KrasKI* homozygous mice were almost free of lung tumours, while *KrasKI* heterozygous mice remained susceptible to lung carcinogenesis, and developed significantly more lung tumours than *WT* mice. Mutations in these tumours were found exclusively in the endogenous *Kras* gene and not the *KrasKI* allele. (**b**) *Kras*^{LA2} mice harboring the *KrasKI* allele, which generates only the 4B isoform, developed almost 3-fold more lung tumours than those carrying an intact *Kras* gene capable of generating both 4A and 4B isoforms. The *HrasKI* allele in *Kras*^{LA2} displayed an inhibitory effect on tumor number similar to *WT* mice. Error bars indicate standard deviations, and statistics were performed using the Wilcoxon Mann-Whitney test.

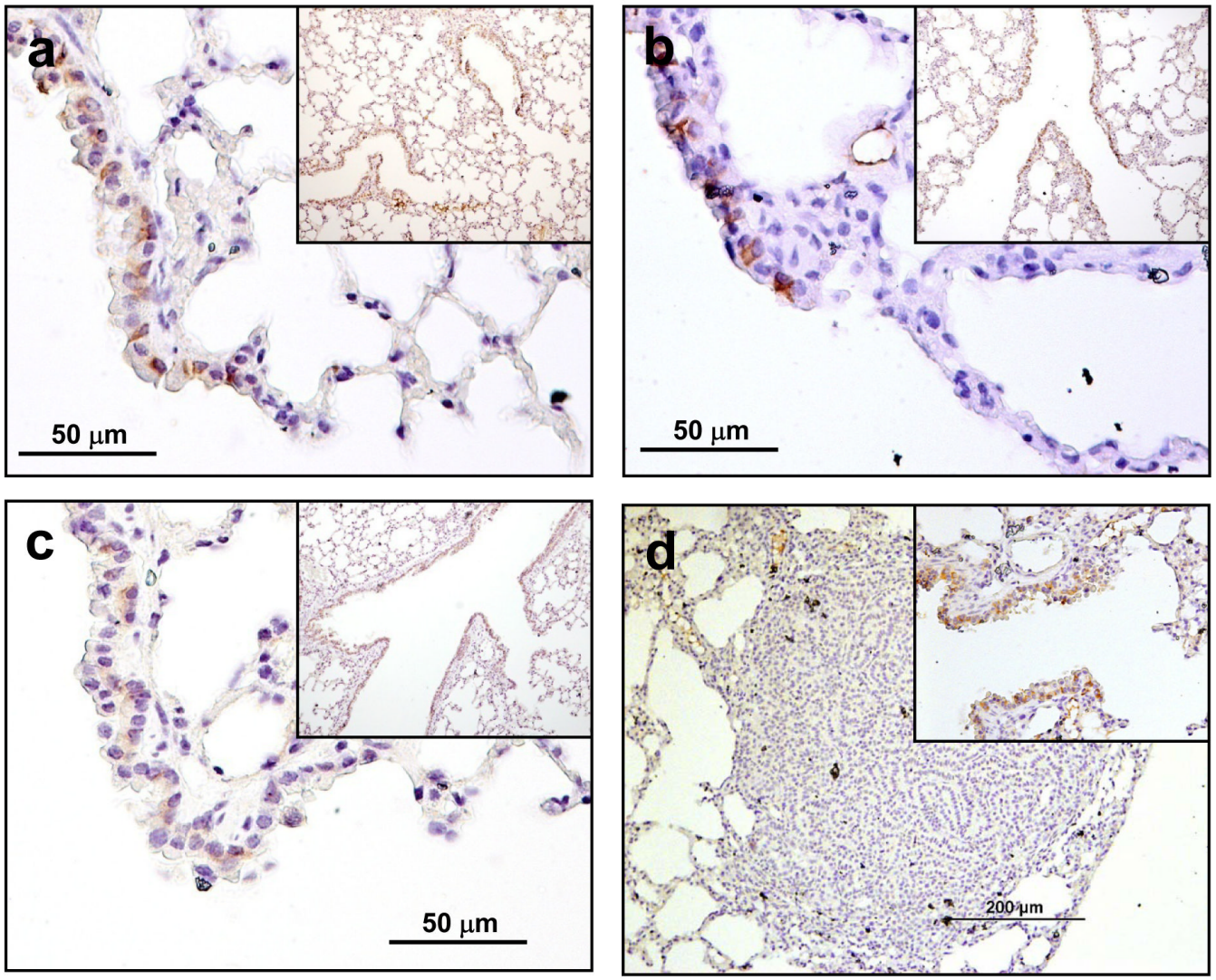

Figure 5. *Kras4A* is expressed in normal lung epithelium but not significantly in tumours

Kras-4A staining was observed in a sub-population of Clara cells in *WT129/Sv* (**a**) and *FVB/N* mice (**b**) but absent in homozygous *KrasKI* mice (expressing only *Kras-4B*) (**c**). The staining was observed throughout the bronchial tree, terminating at bronchio-alveolar duct junctions, locations of the BASCs. In contrast, papillary adenomas from *129/S*v mice do not show significant staining for Kras4A (**d**), although adjacent bronchial structure showed positive staining (**d,** inset).