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Differential association of *STK11* and *TP53* with *KRAS* mutation-associated gene expression, proliferation and immune surveillance in lung adenocarcinoma

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Abstract

While mutations in the *KRAS* oncogene are amongst the most prevalent in human cancer, there are few successful treatments to target these tumors. It is also likely that heterogeneity in *KRAS*-mutant tumor biology significantly contributes to the response to therapy. We hypothesized that presence of commonly co-occurring mutations in *STK11* and *TP53* tumor suppressors may represent a significant source of heterogeneity in *KRAS*-mutant tumors. To address this, we utilized a large cohort of resected tumors from 442 lung adenocarcinoma patients with data including annotation of prevalent driver mutations (*KRAS, EGFR)* and tumor suppressor mutations *(STK11 and TP53*), microarray-based gene expression and clinical covariates including overall survival (OS). Specifically, we determined impact of *STK11 and TP53* mutations on a new

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KRAS mutation-associated gene expression signature as well as previously defined signatures of tumor cell proliferation and immune surveillance responses. Interestingly, *STK11*, but not *TP53* mutations, were associated with highly elevated expression of *KRAS* mutation-associated genes. Mutations in *TP53* and *STK11* also impacted tumor biology regardless of *KRAS* status, with *TP53* strongly associated with enhanced proliferation and *STK11* with suppression of immune surveillance. These findings illustrate the remarkably distinct ways through which tumor suppressor mutations may contribute to heterogeneity in *KRAS*-mutant tumor biology. In addition, these studies point to novel associations between gene mutations and immune surveillance that could impact the response to immunotherapy.

Introduction

Lung cancer is the leading cause of cancer-related deaths worldwide. Despite therapeutic advances over the last several decades, the overall 5-year survival remains only 16% (1). Mutations in the *KRAS* gene occur frequently in non-small cell lung cancer (NSCLC), especially in adenocarcinoma (~30%) though less common in squamous cell carcinoma (about 7%) (2–4). Although mutationally activated *KRAS* tumors were originally identified in 1982 (5), to date there are no successful treatment strategies that target these mutations (3). However, key pathways activated by *KRAS* and mutation-associated vulnerabilities may be therapeutically targetable including the MEK, phosphoinositide 3-kinase (PI3K), GSK-3α and RAL/TBK1 pathways (6–11). Mutations in tumor suppressor genes *TP53* and *STK11* are also common in lung adenocarcinoma and often co-occur with *KRAS* mutations (2–4, 12). While much is known about tumor promotion mechanisms of *TP53*, less is known about *STK11* function and impact on disease progression and patient survival. The *STK11* gene encodes a serine/threonine protein kinase known as liver kinase β1 (LKB1) (13). The most common *STK11* mutations are deletion or inactivating mutations (14–21), which, along with murine studies provide strong evidence for a tumor suppressor function for this gene (19).

Recent studies have defined gene expression changes triggered by RAS (22–24). For example, a RAS signature associated with MEK pathway activation is also associated with sensitivity to MEK inhibitors (MEKi) (24). Gene expression studies have also defined signatures associated with enhanced tumor progression and reduced patient survival. Examples of such signatures include the malignancy risk signature reported by our group, which is rich in cell cycle regulating genes and therefore associated with highly proliferative tumors (25, 26). It is now well established that a functional immune system is crucial in controlling tumor growth (27–29). Consequently, T cell presence in tumors is associated with immune surveillance and improved patient survival (28, 30–35). It is important to note that benefit from immunotherapy, including T cell checkpoint blockade, is also commonly associated with high tumor expression of immuno-stimulatory genes and T cell infiltration (36–38). Thus, activation of key immune regulatory pathways such as JAK-STAT and NF-κB pathways (38–41) in tumor cells or tumor infiltrating non-malignant cells likely enhances the response to immunotherapy. We recently identified a gene expression signature of NF-κB regulated genes that is associated with an immune-active tumor microenvironment (42). The role and potential association between common lung cancer mutations and the

immune surveillance response has however not been investigated. The goal of studies described here was to better define molecular heterogeneity in *KRAS* mutant tumors, especially as it relates to effects of co-occurring mutations in *STK11* and *TP53* tumor suppressors in shaping *KRAS* mutant tumor biology, proliferative and immune surveillance responses in tumors.

Results and Discussion

Study population and prevalence of mutations

Study population characteristics and mutational status of the 442 adenocarcinoma lung cancer patients are summarized in Supplementary Table 1. Overall, the majority of patients were over 70 years of age (53.2%), female (54.3%), white (95.6%), self-reported ever smokers (91%), and early stage (stage I: 64%). In comparison of individual gene mutations to their wildtype counterpart, the overall prevalence was 34.8% for *KRAS*, 10.6% for *EGFR*, 15.3% for *STK11*, and 25.1% for *TP53* (Supplementary Table 1). Of the 442 tumors, 159 did not harbor a mutation in any of these four genes (Fig. 1a). Key variables including demographic and clinical information associated with mutations in these 4 genes are provided in Supplementary Table 1. As expected (4), *KRAS* and *EGFR* mutations were mutually exclusive while *STK11* mutations were significantly associated with *KRAS* mutations ($p<0.0001$) (Fig. 1a and Supplementary Table 1). In addition, co-occurrence of *STK11* and *TP53* mutations in *KRAS* mutant tumors was rare (n=4; Fig. 1a).

Overall the results of sequencing analysis yielded findings similar to those in the literature and in public datasets (COSMIC) (43). Briefly, *KRAS* alterations occurred at codon positions 12, 13 and 61 which are each well-characterized gain-of-function positions (43). The most common mutations in *EGFR* have also been well-characterized (3). Although the focus of the present study was not on *EGFR* mutations, we found 23 L858R point mutations and 21 in frame *indels* in codon 19 in this cohort. Consistent with a loss-of-function mutation pattern, deletions or inactivating mutations were commonly found in *TP53* and *STK11*. Criteria used to identify and remove germline variants in *TP53* and *STK11* are described in Supplementary Information which included filtering against the 1000 Genomes Project and the Exome Sequencing Project (ESP) dataset. In addition, we ensured that mutational events detected in these genes were previously reported in The Cancer Genome Atlas (TCGA) dataset, and if not, that they resulted in frame-shifted/truncated proteins (Supplementary Information). The role of *KRAS* mutations as a prognostic factor in NSCLC is presently unclear. However, a recent meta-analysis of 41 studies concluded that *KRAS* mutations are associated with poor prognosis in patients with NSCLC, especially in patients with adenocarcinoma and early stage NSCLCs (44). In the present cohort, we also found that *KRAS* mutations were associated with poor survival compared to wildtype among stage I patients (Fig. 1b). Conversely *EGFR* mutations were associated with significantly better OS compared to wildtype, while *STK11* and *TP53* were not significantly associated with OS (Fig. 1c–e).

Impact of STK11 and TP53 mutations on a novel KRAS mutation-associated gene expression signature

With the goal of determining impact of *STK11* and *TP53* mutations on *KRAS*-associated gene expression responses, we generated a *de novo* signature of differentially expressed genes in *KRAS* mutant versus *KRAS* wildtype tumors. We identified 58 probe sets encoding for 43 distinct genes that were differentially expressed in *KRAS* mutant tumors ($p < 0.05$ with a 1.5-fold change) (Supplementary Table 2 and Supplemental Information). Principal component analysis was used to evaluate activity of this signature as previously described (42). As expected, our *KRAS de novo* signature activity was highly elevated in *KRAS* mutant tumors (Fig. 2a; $p<0.0001$). Interestingly, activity of this signature was also substantially elevated in *STK11* (Fig. 2b; $p<0.0001$) but not in *TP53* mutant tumors (Fig. 2c; $p=0.832$). Importantly, signature activity was not only enhanced in $KRAS^{mut}/STK11^{mut}$ versus $KRAS^{mut}/STK11^{wt}$ tumors (Fig. 2d,e; $p=0.0015$), but also in $KRAS^{wt}/STK11^{mut}$ tumors compared to $KRAS^{wt}/STK11^{wt}$ tumors (Fig. 2d,e; $p=0.02$). Thus, *STK11* mutations not only further elevate expression of these genes in *KRAS* mutant tumors but can also independently increase their expression.

To provide independent validation of *KRAS* signature association with above gene mutations, we performed studies on TCGA dataset. The results shown here are based upon data generated by the TCGA Research Network at: http://cancergenome.nih.gov/. Normalized RNAseq data was utilized using this dataset of 483 lung adenocarcinoma. Importantly, not only $KRAS^{mut}$ (n=145) but also $STK11^{mut}$ (n=75) were very significantly associated with high *KRAS* signature activity (Supplemental Fig. 1; $p<0.0001$). In contrast, activity of this signature was not associated with TP53 mutations (Supplementary Fig. 1). Remarkably, and in complete concordance with our 442 dataset, *KRAS* signature activity was not only significantly enhanced in $KRAS^{mut}$ but also in $KRAS^{wt}/STK11^{mut}$ tumors compared to $KRAS^{wt}/STK11^{wt}$ tumors (Supplementary Fig. 1; $p=0.0015$). Therefore, mutations in *KRAS* and *STK11* are independently associated with upregulation of *KRAS* signature genes. To define the underlying biology of the *KRAS* signature, three analyses were performed using the following: Gene Ontology Biological process enrichment, GeneGO Pathway Map enrichment, and MSigDB pathway/signature enrichment. However, we were not able to reproducibly associate genes in this signature with a specific biological pathway. Nonetheless, several of these genes have been previously shown to be involved in RAS pathway function, including *DUSP4, RASGRF1/CDC25* and *HRASLS5.* Interestingly, *DUSP4* expression was also reported to be associated with *STK11* mutations (45), suggesting that mutations in *KRAS* and *STK11* may result in expression of at least some common genes.

Distinct association of TP53 mutations with tumor proliferative responses

Increased tumor cell proliferation is a main driver of malignancy and known to be strongly associated with poor patient survival in multiple tumor types (25, 26). We next determined whether proliferative responses were impacted by *KRAS* and tumor suppressor gene mutations. To this end, we used a previously defined malignancy risk (MR; gene list in Supplementary Table 3) signature that is significantly correlated with tumor cell proliferation (25, 26). Importantly, no significant difference in MR activity was observed in

KRAS mutant or *STK11* mutant tumors compared to wildtype tumors (Fig. 3a,b,d,e). In contrast, *TP53* mutations, either individually or with *KRAS* mutations, were significantly associated with higher MR activity (Fig. 3c,f,g). These findings therefore indicate that *TP53* and *STK11* tumor suppressor mutations have distinct association with tumor cell proliferation.

Suppression of immune surveillance in STK11 mutant tumors

T cell mediated immune surveillance is crucial in controlling tumor growth (27–29). We recently identified a gene expression signature of NF-κB regulated genes (gene list in Supplementary Table 3) that is associated with an immune-active tumor microenvironment and T cell presence (42). Using this signature, we next determined potential association of different mutations with an immune-active tumor microenvironment. Intriguingly, only *STK11* mutations were associated with significantly lower activity of this signature (Fig. 4a–c; $p<0.0001$). Furthermore, *STK11* mutations either individually or with *KRAS* mutations were strongly associated with lower NF-κB signature activity (Fig. 4d–e), while no such association was seen with *TP53* mutations (Fig. 4f–g). To determine more directly the impact of *STK11* mutations on T cell immune surveillance, we examined T cell infiltration by using T cell receptor α and β chain expression as previously described (42). Importantly, *STK11* mutations were the most significantly associated with reduced T cell presence in tumors (Fig. 4h; $p=0.002$), although *KRAS* and *TP53* also showed reduced T cell presence. Overall, these findings indicate that *TP53* and *STK11* tumor suppresser genes may promote tumor progression by different mechanisms: while *TP53* mutations lead to greater proliferative responses, *STK11* mutations appear to be associated with suppression of the tumor immune surveillance response. To the best of our knowledge, these findings provide amongst the first evidence of a potential association between a common cancer gene mutation and the immune surveillance response.

The primary goal of this study was to define molecular heterogeneity in *KRAS*-mutant tumors resulting from co-occurring *STK11* and *TP53* tumor suppressor mutations. Towards that goal, a key finding reported here is that *STK11* mutations can positively impact the activity of a novel *KRAS* mutation-associated gene expression signature. Thus, mutations in *STK11* may enhance KRAS associated signaling responses, both independently and concurrently with *KRAS* mutations. While the association of the *KRAS* signature with underlying tumor cell biology remains to be defined, our results suggest that *STK11* mutations may potentiate KRAS-induced signaling and gene expression responses that drive tumorigenesis. Indeed, mouse studies demonstrate acceleration of KRAS induced tumorigenesis and increased metastasis in the presence of concurrent *STK11* null mutations (19).

A key finding reported here is that tumors with *TP53* and *STK11* mutations are associated with distinct proliferative versus immune surveillance responses. Specifically, our results indicate that *TP53* mutations are strongly associated with enhanced tumor cell proliferation, a finding consistent with prior studies of this key tumor suppressor. In contrast, *STK11* mutations were not associated with differences in proliferation but strongly associated with suppression of the immune surveillance response. The relative lack of co-occurrence of

STK11 and *TP53* mutations is also noteworthy (Fig. 1a), and indicates that distinct tumor-promoting mechanisms resulting from these mutations dominate in different tumors. Immune suppression appears to be a specific feature of *STK11* mutations, which may enhance tumor progression in addition to activation of SRC-like kinases as reported previously (46). Since the response to immunotherapy is typically associated with an immune-active tumor microenvironment (36–38), our results further suggest that *STK11* mutant tumors may be less responsive to immunotherapy.

In conclusion, these findings not only provide novel insights into how *KRAS*-mutant tumor biology is shaped by co-occurring mutations, but may also provide insights for therapeutic targeting of lung cancers with distinct tumor suppressor mutations. Specifically, these studies illustrate the potentially significant effect that mutations in tumor suppressor genes could have on therapeutic strategies, especially immunotherapy. These findings also necessitate additional studies to understand specifically how *STK11*/LKB1 impacts *KRAS* mutation-associated gene expression as well as the tumor immune surveillance response. Interestingly, recent studies showed reduced PI3K pathway activity, including activity of NF-κB activating kinase PDK1, in *STK11* mutant human lung adenocarcinoma (45). Therefore, an interesting possibility is that *STK11* mutations directly impact activity of NF-κB and potentially other pathways involved in immune surveillance. Future studies should therefore be directed not only towards understanding mechanisms through which *STK11* mutations promote tumorigenesis through enhancement of KRAS induced responses but also by mediating suppression of immune surveillance. Finally, we believe that the extensive dataset described here will prove to be a valuable resource for cancer researchers, especially for interrogating gene expression networks prevalent in tumors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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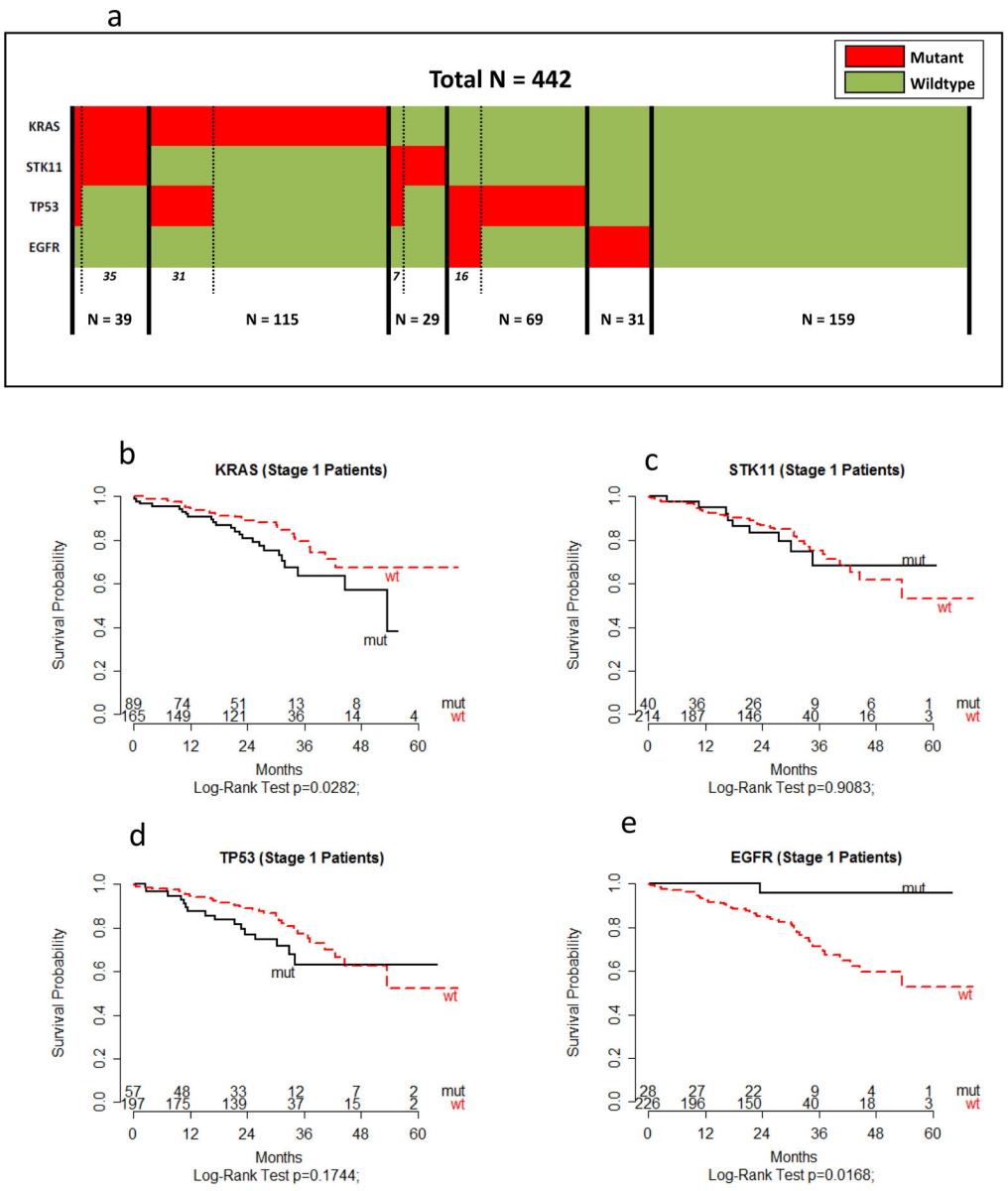

Fig. 1. Co-occurring and mutually exclusive mutations in *KRAS*, *STK11*, *TP53* and *EGFR* in 442 human lung adenocarcinoma samples

(a) Tumors with specific mutations are indicted in red while tumors without mutations are in green. To demonstrate co-occurring and exclusive mutations, the samples were sorted by *KRAS* mutations, then *SKT11* mutations, then *TP53* mutations, and then *EGFR* mutations. (b–e) Association of oncogene and tumor suppressor gene mutations with OS among stage I lung adenocarcinoma patients (n=265). Kaplan–Meier survival curves by mutation status of (b) *KRAS*, (c) *STK11*, (d) *TP53*, and (e) *EGFR* are shown. A two-sided log-rank test was used to assess statistically significant differences by mutational status. The number of patients at risk is listed below the survival curves. Additional methodology is provided in Supplemental Information.

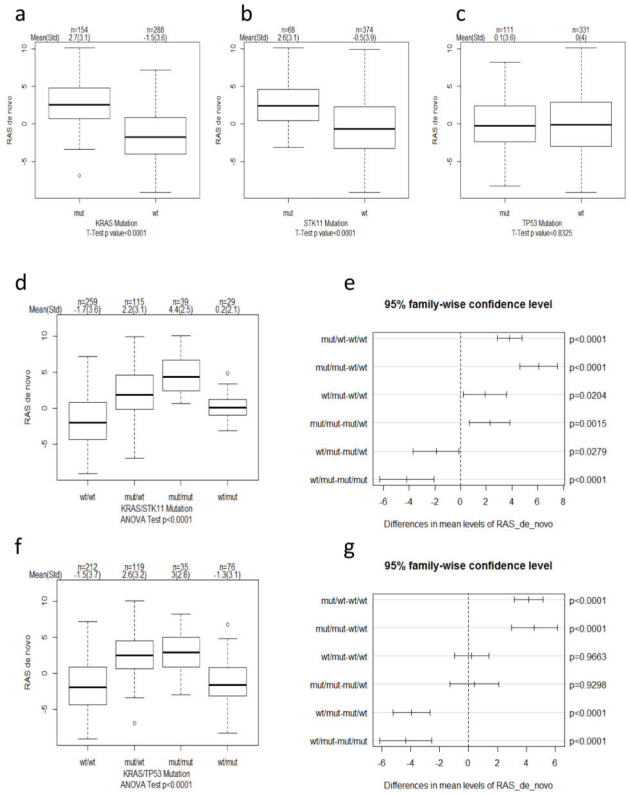

Fig. 2. Impact of *STK11* and *TP53* mutations on *KRAS* mutation-associated gene expression

(ac) Boxplots indicating *KRAS* mutation-associated (RAS de novo) signature activity (PC1) within each gene group (a) *KRAS*, (b) *STK11*, and (c) *TP53*. T-test was used to determine significance in difference in signature activity between mut and wt groups indicated. Sample size (n), mean and standard deviation (std) is indicated on top of each figure. (d) Boxplots and (e) pairwise comparison plots indicating RAS de novo signature activity in indicated co-occurring and exclusive mutations in *KRAS* and *STK11*. ANOVA was used to determine overall significant difference in RAS de novo signature activity among indicated groups and Tukey honest significant difference method was used to adjust for p value for pairwise comparison. (f) Boxplots and (g) pairwise comparison plots indicating RAS de novo signature activity in indicated co-occurring and exclusive mutations in *KRAS* and *TP53*. Additional methodology is provided in Supplemental Information.

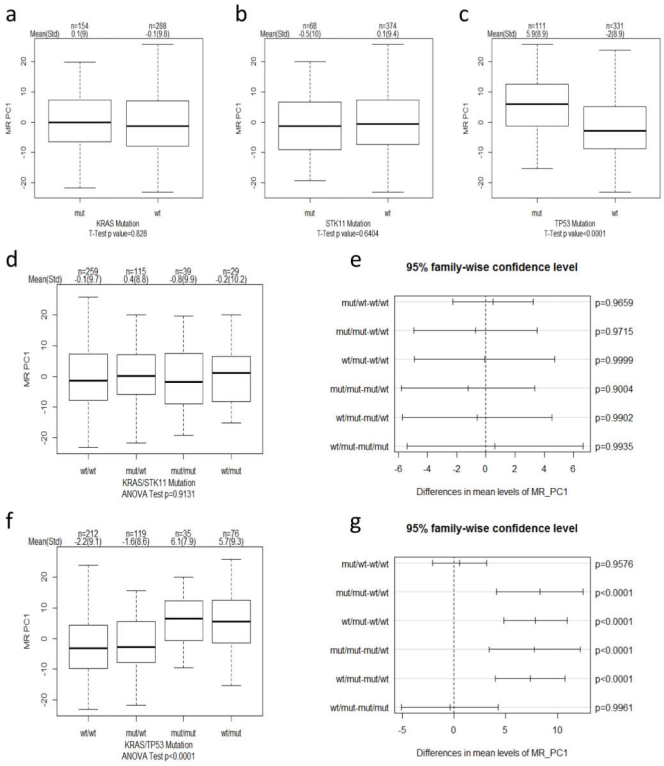

Fig. 3. Distinct association of *TP53* mutations with tumor proliferative responses

(a–b) Boxplots indicating MR signature activity (PC1) within each gene group (a) *KRAS*, (b) *STK11*, and (c) *TP53*. T-test was used to determine significance in difference in MR activity between mut and wt groups indicated. Sample size (n), mean and standard deviation (std) is indicated on top of each figure. (d) Boxplots and (e) pairwise comparison plots indicating MR signature activity in indicated co-occurring and exclusive mutations in *KRAS* and *STK11*. ANOVA was used to determine overall significant difference in MR activity among indicated groups and Tukey honest significant difference method was used to adjust for p value for pairwise comparison. (f) Boxplots and (g) pairwise comparison plots indicating MR signature activity in indicated co-occurring and exclusive mutations in *KRAS* and *TP53*.

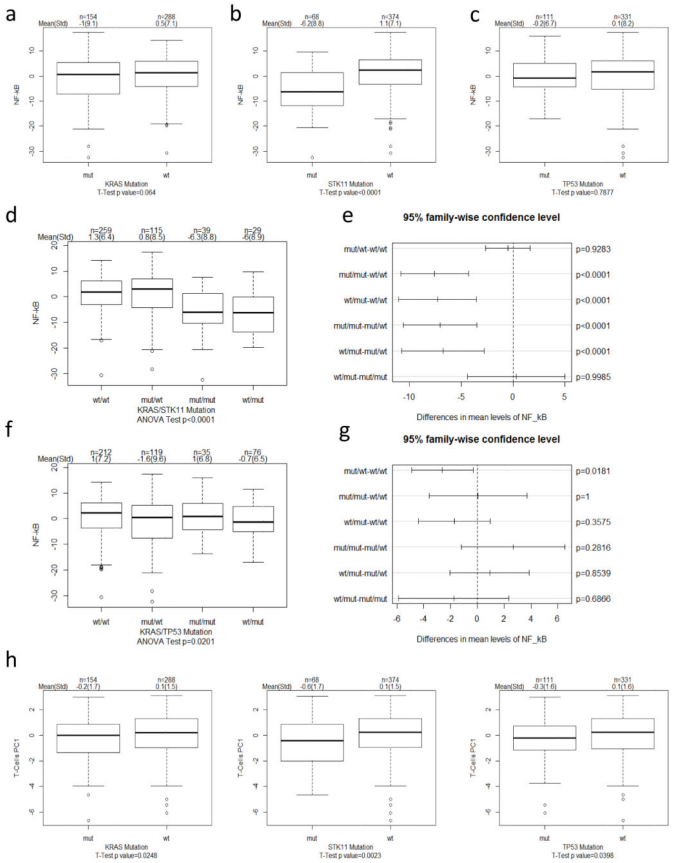

Fig. 4. Suppression of immune surveillance in *STK11* mutant tumors

(a–c) Boxplots indicating NF-κB signature activity (PC1) within each gene group (a) *KRAS*, (b) *STK11*, and (c) *TP53*. T-test was used to determine significance in difference in NF-κB activity between mut and wt groups indicated. Sample size (n), mean and standard deviation (std) is indicated on top of each figure. (d) Boxplots and (e) pairwise comparison plots indicating NF-κB signature activity in indicated co-occurring and exclusive mutations in *KRAS* and *STK11*. ANOVA was used to determine overall significant difference in NF-κB activity among indicated groups and Tukey honest significant difference method was used to adjust for p value for pairwise comparison. (f) Boxplots and (g) pairwise comparison plots indicating NF-κB signature activity in indicated co-occurring and exclusive mutations in *KRAS* and *TP53*. (h) Boxplots indicating TCR gene expression PC1 activity. T-test was used to determine significance in difference in TCR expression between indicated mut and wt groups.