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Co-occurring genomic alterations in non-small cell lung cancer biology and therapy

Ferdinandos Skoulidis[1], **John V. Heymach**[1]

[1]Department of Thoracic and Head and Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX, 77030

Abstract

The impressive clinical activity of small molecule receptor tyrosine kinase inhibitors (TKIs) for oncogene-addicted subgroups of non-small cell lung cancer (NSCLC) [for example those driven by activating mutations in the gene encoding epidermal growth factor receptor (*EGFR*) or rearrangements in the genes encoding the receptor tyrosine kinases anaplastic lymphoma kinase (*ALK*), ROS proto-oncogene 1 (*ROS1*), and rearranged during transfection (*RET*)] has established an oncogene-centric molecular classification paradigm in this disease. However, recent studies have revealed considerable phenotypic diversity downstream of tumor-initiating oncogenes. Co-occurring genomic alterations, particularly in tumor suppressor genes such as *TP53* and *LKB1* (also known as *STK11*), have emerged as core determinants of the molecular and clinical heterogeneity of oncogene-driven lung cancer subgroups through their effects on both tumor cell-intrinsic and non-cell-autonomous cancer hallmarks. In this review, we discuss the impact of co-mutations on the pathogenesis, biology, micro-environmental interactions, and therapeutic vulnerabilities of NSCLC and assess the challenges and opportunities that co-mutations present for personalized anti-cancer therapy, as well as the expanding field of precision immunotherapy.

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Co-occurring genomic alterations contribute to the heterogeneity of driver oncogene-defined non-small cell lung cancer (NSCLC) subgroups. This Review discusses the effects of co-mutations on the pathogenesis, biology, microenvironmental interactions, and therapeutic vulnerabilities of NSCLC.

Correspondence should be addressed to: Ferdinandos Skoulidis, M.D., Ph.D., Assistant Professor, Department of Thoracic and Head and Neck Medical Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, Telephone: 7137456764, fskoulidis@mdanderson.org.

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Competing interests

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Introduction

The identification in 2004 of activating oncogenic mutations in the tyrosine kinase domain of epidermal growth factor receptor (EGFR) in a subset of patients with non-small cell lung cancer (NSCLC) that exhibited dramatic clinical responses to the first-generation EGFR tyrosine kinase inhibitor (TKI) gefitinib launched the field of targeted therapy in NSCLC and reinforced the concept of oncogene addiction as a pillar of modern cancer therapeutics[1, 2]. Subsequent discovery of *ALK* re-arrangements in 2007[3] in 3–7% of NSCLC expanded the spectrum of targetable genomic alterations in this disease[4]. Since then, several additional driver events with robust transforming potential have been reported, including oncogenic *ROS1*[5], *RET*[6], *NTRK1*[7] and *NRG1*[8] fusions, oncogenic somatic mutations in *BRAF* (V600E and non-V600E)[9–11], intragenic insertions in *ERBB2* (also known as *HER2*)[12] and exon 14 skipping mutations in the *MET* proto-oncogene[13–15]. Pivotal clinical studies established the superiority of molecularly targeted therapy compared with platinum-doublet chemotherapy **[G]** for *EGFR*-mutant, *ALK*-rearranged and *ROS1*-rearranged NSCLC and led to the FDA approval of several first, second and third-generation small molecule inhibitors of mutant oncoproteins[16–32]. The robust clinical activity of these targeted agents, coupled with the apparent mutual exclusivity of strong oncogenic drivers in NSCLC, cemented a driver oncogene-centric paradigm in NSCLC oncogenesis and molecular classification. This prevailing model, commonly represented graphically as an "oncogenic pie chart", constitutes the bedrock of NSCLC clinical practice and the framework that underpins the design and implementation of a generation of precision oncology clinical trials aimed at matching patients with available targeted therapies based on identification of a single genomic driver event (Figure 1). However, accumulating evidence points towards the existence of substantial clinical heterogeneity within oncogenic-driver defined NSCLC subgroups that is currently incompletely accounted for by the single oncogenic driver model. In this review, we discuss the emerging role of co-occurring genomic alterations as major determinants of both tumor cell-intrinsic as well as non-cell-autonomous cancer hallmark traits, including their impact on the composition of the tumor microenvironment and response to systemic anti-cancer therapies.

Diversity in driver-defined subgroups.

There is mounting evidence that substantial molecular and clinical heterogeneity exists within oncogenic driver-defined subgroups of NSCLC ("intra-driver heterogeneity"). Despite known associations between certain NSCLC oncogenic subtypes and distinct tumor histopathologic features or growth patterns, NSCLCs driven by the same dominant oncogenic alteration can vary considerably in their histological appearance and immunohistochemical profile. For example, *KRAS*-mutant lung adenocarcinomas (LUADs) demonstrate dual propensity towards either solid growth pattern with positivity for the NKX2–1 homeobox transcription factor (also known as TTF1) or, alternatively, invasive mucinous adenocarcinoma histology and corresponding lack of NKX2–1 expression[33]. At the molecular level, considerable efforts have focused on segregating LUAD into molecular subtypes on the basis of multi-dimensional molecular profiling, coupled with unsupervised clustering computational approaches[34]. Enrichment for specific oncogenic drivers has been

observed within distinct subtypes, however cluster membership typically transcends initiating oncogenes, thus providing further evidence for intra-driver molecular diversity[34].

Most importantly, overwhelming evidence indicates that intra-driver molecular diversity translates into heterogeneous clinical behavior and variable sensitivity to anticancer therapies. Across clinical trials of first-line targeted therapy for oncogene-addicted subgroups of NSCLC, rates of objective response **[G]** typically range between 50% and 83% and complete responses **[G]** are rare; in addition, some patients exhibit *de novo* resistance[16–26, 28–32, 35]. Even more variable are duration of response to targeted therapy, progression-free survival and overall survival[16–26, 28–32, 35]. Phenotypic variability and therapeutic response heterogeneity are particularly evident within *KRAS*-mutant LUAD. The pervasive diversity of this oncogenotype was aptly demonstrated in a study that applied affinity propagation clustering analysis[36] to mRNA expression data from 106 genomically-annotated NSCLC cell lines; strikingly, variation in mRNA expression within *KRAS*-mutant NSCLC cell lines was equivalent to that observed across the entire cell line panel[37]. Inter- and intra-driver heterogeneity are also evident following treatment with inhibitors of the immune checkpoint molecules PD-1 or PD-L1, with only ~20% of unselected NSCLC patients deriving durable clinical benefit[38–43].

What are the molecular underpinnings of this remarkable intra-driver heterogeneity in NSCLC? In many cases, divergent clinical behavior can be directly attributed to the distinct effects of individual oncogenic alleles. Multiple studies have affirmed the favorable prognostic impact of exon 19 *EGFR* deletions compared with exon 21 L858R amino acid substitution, although the molecular basis for this association has not been conclusively determined[44, 45]. Furthermore, *EGFR* exon 20 in-frame insertion mutants are recalcitrant to all currently FDA-approved EGFR TKIs due to insertion-imposed steric hindrance of the drug binding pocket, but exhibit sensitivity to poziotinib - a smaller and more flexible inhibitor - *in vitro* and *in vivo*[46, 47]. Among *ALK*-rearranged NSCLC, both the fusion partner as well as *EML4-ALK* fusion variantshave been considered candidate modifiers of transforming potential and response to ALK TKIs[48, 49]. For example, the PRKAR1A-ALK fusion was consistently demonstrated to be less sensitive to first, second and third generation ALK TKIs[48]. In addition, *EML4-ALK* variant 3 was associated with more frequent secondary resistance mutations (including the G1202R solvent front mutation) compared to *EML4-ALK* variant 1 and, consequently, longer progression-free survival with the 3rd generation ALK inhibitor lorlatinib, that is active against the $EML4\text{-}ALK^{G1202R}$ mutation[49]. Similarly, in *RET*-rearranged NSCLC, non-*KIF5B-RET* fusions have been associated with significantly higher response rates to RXDX-105 (a RET and BRAF inhibitor) but not to the potent and selective RET inhibitor LOXO-292[50]. Finally, distinct *KRAS* mutant alleles differentially engage downstream effectors with $KRAS^{G12C}$ or $KRAS^{G12V}$ preferentially activating RALA or RALB signaling and $KRAS^{G12D}$ triggering increased PI3K–AKT and MAPK/ERKpathway activation[51]. Currently, the prognostic and predictive utility of *KRAS* alleles in NSCLC remains unclear but is likely to increase in view of the ongoing clinical development of covalent, direct $KRAS^{G12C}$ inhibitors[52–56]. Nonetheless, distinct types of somatic mutations or gene rearrangements appear to only partially account for intra-driver heterogeneity because marked differences in biological behavior can also be observed

between NSCLC that bear identical oncogenic alterations in driver genes. Taken together, these studies challenge the single-oncogene paradigm in NSCLC by unveiling multiple layers of heterogeneity within oncogenic subgroups that can only partially be attributed to the driver oncoprotein itself.

Co-occurring genomic alterations

The compendium of co-occurring genomic alterations in NSCLC are potentially more impactful than distinct mutations in oncogenic drivers with regard to determining tumor heterogeneity. LUADs and lung squamous cell carcinomas (LUSCs) are characterized by a high average number of somatic mutations per Mb in comparison to many other tumor types[57]. Although passenger mutations account for the largest fraction of this mutational burden, combinations of somatic mutations in *bona fide* cancer driver genes are identified in the majority of LUAD and a substantial fraction of LUSC, even when next generation sequencing platforms are limited to the evaluation of pre-defined sets of cancer-relevant genes. Importantly, large-scale profiling studies utilizing either whole exome sequencing or broad targeted sequencing panels in NSCLC tumors have revealed multiple non-random patterns of co-occurring or mutually exclusive mutations, which typically vary depending on the particular oncogenic driver mutation[15, 34, 58–62]. From an evolutionary standpoint, co-selection of oncogenic alterations implies functional co-operation that converges on improved fitness, whereas mutual exclusivity indicates redundancy (potentially manifesting as soft exclusivity) or antagonism (resulting in more strict patterns of mutual exclusivity due to deleterious effects of the combined alterations)[63, 64]. Thus, from its inception, NSCLC develops through a network of evolving genetic interactions that collectively determine cancer hallmark traits[65, 66]. This further suggests that early oncogenic events may channel tumor evolution towards distinct trajectories and influence the likelihood of positive or negative selection of subsequent genomic alterations. It is important to note, however, that even genomic alterations that do not show statistically significant patterns of co-occurrence may still have important interactions biologically. For example, mutations in *TP53* (which encodes p53) are under-represented among *KRAS*-mutant LUAD compared to other oncogene-driven subgroups, yet p53 inactivation is common and impactful in *KRAS*-mutant LUAD[67–71]. The importance of co-mutations as mediators of diverse NSCLC phenotypes has only recently attracted focus and their functional impact remains largely uncaptured within current molecular stratification frameworks.

Knowledge of the clinical context is paramount when evaluating the functional importance as well as prevalence of co-occurring genomic alterations. In particular, it is critical to distinguish between early-stage, surgically resected tumors and locally advanced or metastatic disease because several distinct patterns of co-mutations are enriched in metastatic disease, likely reflecting acquisition of traits that promote tumor progression and metastatic dissemination[72, 73]. In addition, selective pressure imposed by previous anticancer therapy can substantially influence patterns of co-mutations; therefore, detailed knowledge of prior therapeutic exposures is critical for accurate interpretation and understanding of the functional effect of co-mutation patterns.

Determination of the clonal or sub-clonal nature as well as timing of individual co-alterations may also provide important information regarding their contributions to different stages of carcinogenesis and impact on therapeutic response. Early clonal events are more likely to impact core cancer hallmarks that are critical for tumor initiation. In addition, targeting clonal events is more likely to yield sustained responses; although both clonal and sub-clonal events can contribute to clinical resistance, clonal events are more likely to result in primary resistance. In the landmark TRACERx study, multiregion sequencing of 100 early-stage NSCLCs provided a measure of the extent of clonal driver events (1–18 in LUAD and 1–14 in LUSC) and sub-clonal driver events (0–10 in LUAD and 0–12 in LUSC) and established a catalog of clonal alterations[74]. Furthermore, evidence from other tumor types supports the notion that within a network of epistatic oncogenic interactions the chronology – or order – of individual genomic alterations can impart distinct phenotypic outcomes. For example in myeloproliferative disorders, the order in which mutations in *JAK2* and *TET2* arise in hematopoietic stem and progenitor cells can affect age of disease onset, influence the likelihood of the disease manifesting as polycythemia vera versus essential thrombocythemia and result in different propensities for development of thrombosis[75, 76].

Finally, when assessing patterns of co-occurring events in human NSCLC it is important to also consider the impact of mutational processes and immune selection. Several distinct mutational signatures sculpt the genome of NSCLC – including signatures of tobacco exposure and APOBEC-mediated cytidine deamination[57]. Certain recurrent oncogenic mutations, for example classical mutations in *PIK3CA* (which encodes a catalytic subunit of PI3K), occur within APOBEC deaminase trinucleotide motifs and are enriched in tumors with a high APOBEC mutational footprint[77]. While the full extent to which mutational processes account for unique combinations of somatic genomic alterations in NSCLC is currently unknown, there is evidence that APOBEC-mediated mutagenesis fuels sub-clonal diversification and branched evolution[78, 79]. Furthermore, tumor genomes can be shaped by immunosurveillance through early elimination of clones that present strong antigenic neo-peptides. For example, the major histocompatibility complex (MHC) class I genotype of individual patients was demonstrated to impose restrictions on the tumor mutational landscape and predict for selection of distinct driver mutations[80]. Such immunoediting likely influences patterns of co-mutations in NSCLC and these associations warrant further study. On the other hand, imposition of a cold tumor immune microenvironment **[G]** as a result of tumor cell-intrinsic processes may relax immune selection and result in a more diverse spectrum of co-mutations.

Co-mutations within LUAD subgroups

KRAS-mutant LUAD

Activating mutations in *KRAS* are the most prevalent oncogenic driver event in both early-stage and metastatic LUAD, occurring in 25–32% of tumors[34, 59, 60, 62, 67]. As noted previously, *KRAS*-mutant LUADs are intrinsically heterogeneous in their biology and clinical behavior. We previously identified three robust and reproducible transcriptomic sub-groups of *KRAS*-mutant LUAD by applying non-negative matrix factorization consensus

clustering[81–83] to RNASeq data from 68 tumors from The Cancer Genome Atlas (TCGA) dataset[67]. Remarkably, superimposition of somatic genetic alterations of key tumor suppressor genes revealed non-overlapping patterns of co-occurring genomic alterations in the three subgroups: one subgroup was dominated by co-occurring *TP53* alterations (thereafter referred to as KP), whereas co-mutations or genomic loss in *LKB1* (also known as *STK11*) were a hallmark of the second cluster (referred to as KL), that was further enriched in somatic mutations in *KEAP1* and *ATM*. Bi-allelic inactivation of the *CDKN2A/CDKN2B* locus was significantly enriched in the third cluster (referred to as KC), that was defined by lack of NKX2–1 expression. Notably, distinct *KRAS* alleles were not differentially distributed between the three clusters – with the exception of enrichment for $KRAS^{G12D}$ in the KC subgroup in some cohorts. These findings established co-mutations as major determinants of the molecular diversity of *KRAS*-mutant LUAD.

Landmark large scale sequencing studies have established a census of major *KRAS* co-mutations in both early-stage and advanced LUAD[15, 34, 59, 62]. The significance of co-occurrence for individual pairs of genetic alterations varies depending on the size of the clinical cohort, the number of possible interactions that are surveyed and the sequencing platform. However, co-mutations in a set of core genes including *LKB1*, *KEAP1*, *ATM* and *RBM10* are consistently enriched in *KRAS*-mutant LUAD (Figure 2). Additional significantly co-altered genes reported in some studies include *PTPRD*, *U2AF1*, *POLE*, *NTRK3* and *LRP1B*. Mutations in *TP53* and inactivation of *CDKN2A, CDKN2B* or combined *CDKN2A/CDKN2B* loss due to bi-allelic deletion, are common and functionally relevant co-alterations, although they are not enriched in *KRAS*-mutant compared to other oncogene-driven subgroups. Mutations in other established drivers within the receptor tyrosine kinase-RAS-RAF network including *EGFR*, *ERBB2*, *BRAF*, *NF1*, as well as *ALK*, *ROS1* and *RET* rearrangementsare largely non-overlapping with *KRAS*, although the strength of their negative association varies depending on the individual gene.

Insights from genetically engineered mouse models and cell line studies have been pivotal towards elucidating the phenotypic sequelae of the most prominent *KRAS* co-mutations in NSCLC. Somatic deletion of *Lkb1* is insufficient for initiation of lung carcinogenesis in mice as a singular event but dramatically accelerates $Kras^{G12D}$-driven carcinogenesis and fosters early metastatic dissemination[84]. In addition, loss of *Lkb1* results in epigenetic reprogramming and an expanded tumor histological repertoire, with high incidence of squamous or adenosquamous carcinomas, in agreement with data supporting enrichment of *LKB1* mutations in human adenosquamous NSCLC[84–86]. Mechanistically, trans-differentiation **[G]** is mediated by LKB1 loss-triggered down-regulation of the Polycomb Repressive Complex 2 (PRC2) subunit EED and relief of PRC2-mediated repression of squamous differentiation genes[86]. Epigenetic reprogramming upon *LKB1* loss in *KRAS*-mutant cells is further fueled by a metabolic network, that promotes increased flux of glucose-derived carbon towards serine biosynthesis and the methionine salvage pathway, bolstering synthesis of S-adenosyl methionine (SAM), a critical substrate for DNA methylation[87]. Generation of SAM via this pathway, coupled with up-regulation of DNA methyl-transferases, underpins an increase in global levels of CpG methylation in KL cells that is critical for tumor maintenance. Furthermore, KL NSCLC cells exhibit increased

dependence on dTTP synthesis and rely on an unorthodox pathway of pyrimidine biosynthesis that utilizes mitochondrially generated carbamoyl phosphate[88, 89]. The unique metabolic phenotypes associated with combined expression of oncogenic *KRAS* and *LKB1* inactivation – but not with mutations in either gene alone - may at least partially explain their preferential co-occurrence in human NSCLC.

Intricately linked with LKB1 inactivation is loss of KEAP1, an adaptor protein that mediates ubiquitination and proteasomal degradation of NRF2, a key transcription factor in cellular antioxidant, metabolic, cyto-protective, and anti-inflammatory pathways[90]. Somatic mutations in *LKB1* and *KEAP1* significantly co-occur with mutant *KRAS* and with each other in NSCLC[15, 59, 63, 91]. In the conditional $Kras^{G12D/+};Trp53^{Fl/FL}$ mouse model, loss of *Keap1* increases both tumor burden and the percent of high-grade lesions, pointing towards roles in both tumor initiation and progression[92]. At the cellular level KEAP1 loss results in increased cellular proliferation *in vivo* and an altered metabolic profile characterized by increased glucose-derived carbon flux towards the pentose phosphate and serine–glycine biosynthetic pathways[93–95] with enhanced dependence on glutaminolysis for tricarboxylic acid (TCA) cycle anaplerosis[92], a dependence that is further enhanced by co-occurring *LKB1* alterations[96]. Thus, NRF2- mediated metabolic reprogramming and regulation of redox homeostasis likely underpin the strong co-selection of *KEAP1* with *LKB1* and *KRAS* mutations in NSCLC at least partially because oncogenic KRAS itself promotes oxidative stress and anabolic metabolism and because KL NSCLC cells depend on the pentose phosphate pathway for NAPDH generation and for detoxification of reactive oxygen species because these cells have defective fatty acid oxidation[94, 97, 98]. This suggests that up-regulation of a NRF2-driven transcriptional program may represent a bottleneck in the evolution of LKB1-deficient NSCLC. Potentiation of cellular anabolic, antioxidant and detoxification pathways collectively support the aggressive clinical phenotype of *KEAP1*-mutant NSCLC that is concordant with its role as an independent negative prognostic indicator[92, 99].

Loss of p53 or expression of either dominant negative or dominant gain-of-function *Trp53* mutants also co-operate with oncogenic *Kras* to induce LUADs with shortened latency and increased metastatic proclivity, although these tumors are less aggressive than those with *Lkb1* loss[69–71, 84]. Notably, the selective pressure for p53 inactivation is most critical in aggressive, high-grade lesions that exhibit high levels of ERK signaling, whereas engagement of p53-mediated signaling is minimal in low-grade adenomas, thus highlighting stage and signal intensity - dependent patterns of co-operativity[100–102]. This notion is further supported by identification of *TP53* mutational inactivation as a clonal and predominantly early event in established NSCLC that precedes genome doubling and subsequent branched evolution[74, 103].

Mutations in *ATM*, encoding an apical kinase in the DNA damage response pathway, also significantly co-occur with mutant *KRAS*. In murine models the impact of *Atm* inactivation on *Kras*-driven lung carcinogenesis is context-dependent and varies according to the functional status of p53[104]. In a p53-proficient setting, bi-allelic loss of *Atm* is tolerated but does not promote $Kras^{G12D}$-initiated neoplasia. In contrast, complete *Atm* inactivation is incompatible with cellular viability in the context of $Kras^{G12D}$ expression and bi-allelic

Trp53 inactivation, suggesting that excessive DNA damage in this context removes incipient cancer cells from the proliferative pool. Interestingly, *Kras*-driven lung carcinogenesis is accelerated by incomplete *Atm* loss in a p53 deficient settingThus, data from genetically engineered mouse models (GEMMs) point towards a context-dependent, conditional haplo-insufficient role for *Atm* loss in $Kras^{G12D}$-driven lung tumorigenesis. Selection against complete ATM inactivation may explain the mutual exclusivity of *ATM* and *TP53* mutations in human LUAD as well as the enrichment of *ATM* mutations in the KL subgroup[67]. However, *ATM* haplo-insufficiency has not been convincingly established yet in human LUAD, where there is evidence for complete lack of ATM expression by immunohistochemistry in a significant proportion of LUAD[105].

Intriguingly, analysis of patterns of co-occurrence and mutual exclusivity between a set of 505 pre-selected candidate functional genomic events in 6456 tumors from the Pan-Cancer TCGA Dataset using a novel algorithmic approach (SELECT algorithm) identified somatic mutations in *RBM10* as the top-scoring *KRAS* co-occurrence motif in both NSCLC and colorectal adenocarcinoma[63]. *RBM10* encodes a splicing regulator that is involved in cellular growth control via regulation of NOTCH signaling[106, 107]. *In vivo* depletion of *Rbm10* in mice using CRISPR/Cas9- mediated gene editing concurrently with activation of endogenous oncogenic $Kras^{G12D}$ confers a modest fitness advantage that is lost when *Trp53* or *Lkb1* are also inactivated[108]. The precise phenotypic consequences of RBM10 inactivation in NSCLC and the mechanisms that underpin its oncogenic cooperation with *KRAS* remain incompletely understood.

Somatic genomic alterations in *CDKN2A* [encoding the p16 and $p14^{ARF}$ ($p19^{ARF}$ in the mouse) tumor suppressors] and *CDKN2B* (encoding p15) are observed in ~20% and ~12% of metastatic *KRAS*-mutant NSCLC respectively and bi-allelic loss of the *CDKN2A/CDKN2B* locus is a hallmark of the KC subgroup[15, 67]. KC tumors are characterized by lack of NKX2–1 expression and frequent activation of a gastrointestinal transcriptional program (manifesting histologically as invasive mucinous carcinoma in some cases), enrichment for the $Kras^{G12D}$ mutation and poor prognosis. Several of these features are recapitulated in mice where endogenous expression of oncogenic $Kras^{G12D}$ is coupled with bi-allelic deletion of *Cdkn2a/Cdkn2b*, resulting in concurrent abrogation of p16, $p19^{ARF}$ and p15. Both isolated *Cdkn2a* loss (leading to inactivation of p16 and $p19^{ARF}$) as well as combined *Cdkn2a/Cdkn2b* inactivation accelerate $Kras^{G12D}$-driven lung carcinogenesis and promote loco-regional metastatic spread but combined loss of p16, $p19^{ARF}$ and p15 elicits a more marked phenotype than *Cdkn2a* loss alone with enhanced cellular proliferation, frequent loss of NKX2–1 expression and up-regulation of the embryonal protein HMGA2, increased burden of poorly differentiated, high-grade tumors, enhanced metastatic proclivity and curtailed survival[109]. Mechanistically, loss of NKX2–1 unleashes a hepatocyte nuclear factor 4-alpha (HNF4A)-driven gastric differentiation program whereas concomitant loss of HNF4A promotes de-repression of HMGA2[110].

EGFR-mutant LUAD

Although *EGFR* –mutant tumors represent the prototypical oncogene-addicted LUAD subgroup that spearheaded adoption of the single-driver model, the overwhelming majority

of *EGFR*-mutant lung tumors harbor one or more co-mutations, even when the analysis is limited to pre-defined sets of cancer-relevant genes within established panels (FoundationOne, Guardant360, MSK-IMPACT)[15, 59, 72, 73]. The spectrum of enriched genomic co-alterations in advanced *EGFR*-mutant LUAD is dominated by recurrent mutations in a core set of genes including *TP53* (54.6% % - 64.6%), *RB1* (9.6%−10.33%), *CTNNB1* (which encodes β-catenin; 5.3%−9.6%), and *PIK3CA* (9%−12.4%) as well as amplifications involving *EGFR* itself (22% - 25.5%), *NKX2–1* (12.2% - 16.7%), *CDK4* (7% −10%), *CDK6* and *CCNE1*[15, 72, 73] (Figure 2). The spectrum and prevalence of co-mutations does not appear to vary depending on the specific initiating *EGFR* mutation and is similar across the three most common subtypes (*EGFR* exon 19 deletion, $EGFR^{L858R}$ and *EGFR* exon 20 insertions)[47]. Prior therapy is associated with increased average number of co-alterations. Mutations in *PIK3CA* and *CTNNB1* are more frequent in advanced stage tumors compared with early stage LUADs, pointing towards functional roles in malignant progression and metastasis, whereas alterations in *TP53* (62.5%), *RB1* (9.5 −12.5%) and *NKX2–1* (12.5%) appear to occur with comparable frequencies in early- and advanced-stage tumors[15, 34, 72, 73].

Somatic mutations in *TP53* represent by far the most prevalent co-alteration in *EGFR*-mutant LUAD (54.6%−64.5%) and their clinical significance has been evaluated in several studies. *TP53* mutations are mostly truncal events (present in all geographically distinct segments of the tumor) that occur early during tumor evolution and prior to whole genome doubling, and are frequently accompanied by truncal loss of heterozygosity at the *TP53* locus, indicating strong selective pressure for complete *TP53* inactivation in early stage LUADs[103]. Furthermore, tumors bearing co-mutations in *TP53* exhibit higher degrees of copy number genomic instability (aneuploidy), and a higher somatic mutation burden, both on the trunk and in the branches of the tumor phylogenetic tree[103]. Therefore, *TP53* co-mutations impact the natural history of *EGFR*-mutant NSCLC at least partially by allowing tolerance of a greater degree of genomic instability that results in both larger numbers of co-occurring truncal drivers as well as late sub-clonal diversification with focal emergence of high amplitude amplifications and deletions in mediators of therapeutic resistance[103]. In keeping with a more complex genomic landscape and a larger burden of clonal or sub-clonal co-drivers, multiple clinical studies have identified *TP53* co-alterations as a negative prognostic marker in *EGFR*-mutant LUAD and a consistent predictor of worse clinical outcomes following EGFR TKI therapy[72, 73, 111–116].

Mutational inactivation of *RB1* is a clonal and early genetic event in 9.5%−12.5% of *EGFR*-mutant LUAD[72, 73, 103, 117]. The majority of *RB1*-mutant tumors also harbor *TP53* co-alterations, underscoring the critical contributions of these archetypal tumor suppressor genes to cell cycle control. *TP53* and *RB1* co-mutations mark the earliest ancestors of *EGFR*-mutant LUAD that transform to small cell carcinoma following exposure to EGFR TKIs and dramatically increase the risk of small cell transformation, although loss of RB1 is insufficient to directly induce neuroendocrine trans-differentiation.[117–119]. Alterations in other regulators of G1/S cell cycle transition including amplification of *CDK4*, *CDK6* and *CCNE1* are prevalent and appear to be enriched in tumors that express the $EGFR^{T790M}$ gatekeeper mutation that confers TKI resistance, although data regarding their preferential occurrence in *EGFR*-mutant compared to *EGFR* wild-type LUAD are less consistent

between studies[73]. Similarly, genomic alterations –most commonly deletion events - in the *CDKN2A* and *CDKN2B* genes are observed in ~24.6% and 20.2% of *EGFR*-mutant tumors and these alterations are typically truncal, further underscoring the significance of G1/S checkpoint dysregulation in the early stages of lung carcinogenesis driven by mutant *EGFR*[72, 73, 103].

Activating mutations in *CTNNB1* represent one of the most consistently co-selected alterations in *EGFR*-mutant LUAD across different studies. *CTNNB1* mutations are rare in early-stage *EGFR*-mutant LUAD (1.8% in the TCGA cohort) but their prevalence increases in late-stage tumors (5.3% - 9.6%), in agreement with earlier studies that identified a central role for WNT signaling in LUAD metastasis and experimental data demonstrating increased invasive potential of *EGFR* and *CTNNB1* co-mutated NSCLC cells *in vitro*[15, 34, 72, 73, 120, 121]. However, in a mouse LUAD model driven by compound $Egfr^{L858R/T790M}$ mutations genetic deletion of *Ctnnb1* reduced tumor burden indicating non-redundant functions in tumor initiation[122]. Interestingly, mutations in *CTNNB1* have been reported to occur more frequently in LUAD with the $EGFR^{T790M}$ mutation following exposure to first or second generation EGFR TKIs suggesting enhanced genetic interaction in this setting[73]. Mutant EGFR has further been shown to directly tyrosine phosphorylate β-catenin resulting in its stabilization and nuclear accumulation[120].

PIK3CA mutations, including classical kinase (H1074R and H1074L) and helical (E545K and E542K) domain mutations are observed in 9%−12.4% of advanced stage *EGFR*-mutant LUAD and, like *CTNNB1* mutations, are encountered preferentially in advanced-stage tumors[15, 34, 72, 73]. *In vitro*, co-occurring *PIK3CA* mutations promote cellular invasion and migration whereas *in vivo* they are associated with worse overall survival in some studies but do not appear to impact response rates and progression-free survival with first or second line EGFR TKI therapy[73, 115, 123].

NKX2–1 amplification is significantly enriched in *EGFR*-mutant LUAD and constitutes a classical example of a context-dependent genetic interaction. In mouse models of *Kras*-mutant LUAD, *Nkx2–1* loss fosters metastasis and *Nkx2–1* haplo-insufficiency promotes both initiation and progression of invasive mucinous adenocarcinomas[124]; therefore, in this genomic background *Nkx2–1* functions as a tumor suppressor gene. In contrast, hemizygous *Nkx2–1* loss suppresses $Egfr^{L858R}$-driven lung carcinogenesis, indicating that sustained NKX2–1 expression is essential for tumor initiation downstream of mutant *Egfr*[125]. Mechanistically, NKX2–1 transactivates the receptor ROR1, which directly binds to EGFR and sustains EGFR–ERBB3 heterodimerization, ERBB3 phosphorylation and pro-survival PI3K–AKT signaling; in addition, ROR1 can interact with and phosphorylate SRC, providing a parallel pathway to AKT activation[126]. Thus, the function of *NKX2–1* as a lineage survival oncogene in *EGFR*-mutant NSCLC provides a plausible explanation for its preferential amplification in this oncogenic subgroup.

ALK, *ROS1*, *RET* and other oncogenic fusion-driven molecular subgroups

Recent studies have also begun to shed light on the co-mutation landscape and genomic architecture of LUAD driven by oncogenic fusions[15, 127], although the clinical significance of co-alterations in this setting is less well characterized. Interestingly, advanced-stage *ALK*-

rearrangement -positive LUAD are enriched in somatic alterations in *CDKN2A* (32.5%) and *CDKN2B* (26.5%), but are less likely to harbor *TP53* alterations (23.8%–26.5%) compared with other driver subgroups[15, 128]. *TP53* co-mutations promote genomic instability and are an independent negative prognostic factor in *ALK*-re-arrangement-positive LUAD, regardless of the type of systemic therapy used[128–130]. The prevalence of additional co-mutations in this group is low,[128] and both the rarity of co-drivers and strong addiction to the ALK fusion oncoprotein may account for the long progression-free survival observed in patients with *ALK*-rearrangements with the potent and selective second and third generation ALK inhibitors alectinib, brigatinib and lorlatinib[23, 26, 27]. Similarly to LUAD with *ALK* fusions, both *RET* and *ROS1* fusion-positive LUAD are characterized by high rates of concurrent *CDKN2A* loss (29.8% and 30.4% for *RET* and *ROS1*-rearranged tumors respectively) and *CDKN2B* loss (25% and 17.7% respectively) and relative paucity of *TP53* mutations, although the frequency of *TP53* mutations appears to be somewhat higher compared to *ALK*-rearranged tumors (34.6%–45.5% for *RET*-and 45.6% for *ROS1*-rearranged tumors)[15, 131]. The key finding that *TP53* somatic mutations are underrepresented across LUAD driven by different oncogenic fusions was validated in a subsequent study that further identified frequent bi-allelic *SETD2* deletions in this group[127]. The functional consequence of these associations is currently incompletely understood. The co-alteration spectrum of LUAD driven by *NRG1* or *NTRK1* fusion events has not been elucidated to date.

Other oncogenic subgroups.

A distinct pattern of co-occurring alterations is observed in LUAD driven by *MET* exon 14 skipping mutations. Specifically, these tumors are characterized by highly significant enrichment of *MDM2* and *CDK4* amplification (41.6%) compared with other driver oncogenes, as well as amplification of *MET* itself[15]. In contrast, mutations in *TP53* (33.57%) are under-represented, whereas loss of *CDKN2A* (24.1%) and *CDKN2B* (17.5%) occur with similar frequencies to that in the overall population of patients with NSCLC[15]. The spectrum of co-occurring alterations in *BRAF*-mutant NSCLC mirrors the background frequency of alterations in *TP53* (53.3%), *LKB1* (16.2%), *ATM* (5.8%), *NF1* (6.9%), *PIK3CA* (6.6%), *KEAP1* (6.6%), *MYC* (10.8%), *NKX2–1* (7.3%), although alterations in *RB1*, *MDM2*, *CDKN2A* (16.6%) and *CDKN2B* (11.2%) are less frequent within this molecular subgroup[15]. Finally, patients with *ERBB2*-mutant NSCLC exhibit preferential amplification of *NKX2–1* (19.4%) and *ERBB2* itself (14.4%) as well as frequent mutations in *RB1* (8.9%), but the frequencies of co-mutations in *TP53* (51.7%), *CDKN2A* (27.2%), *CDKN2B* (17.2%), *PIK3CA* (5%), *CTNNB1* (4.4%) and *MDM2* amplification (7.2%) are similar to that observed in the overall population of patients with NSCLC[15].

Effects on the immune microenvironment

In addition to their impact on cell-autonomous cancer hallmarks, co-mutations can also shape the NSCLC microenvironment and determine its immune contexture (Figure 3). Inactivating *LKB1* genomic alterations, present in ~25% of *KRAS*-mutant LUAD, have emerged as a major driver of the cold, non-T cell-inflamed microenvironment in NSCLC, characterized by paucity of infiltrating CD3+, CD4+ and CD8+ T-cells and low tumor cell

expression of PD-L1, despite intermediate to high tumor mutational burden (TMB)[67, 68, 132–134]. These findings are recapitulated in the *Kras*$^{LSL\text{-}G12D/+}$; *Lkb1*$^{Fl/Fl}$ GEMM, where Cre-mediated *Lkb1* ablation triggers marked influx of tumor-associated neutrophils with T cell suppressive properties including increased expression of Arginase 1 (ARG1) and Interleukin 10 (IL-10)[135]. Mechanistically, *Lkb1* loss in this model results in altered tumor cytokine milieu with increased expression of interleukin 1β (IL-1β), IL-6, CXCL7 and G-CSF that foster myeloid cell recruitment[135]. In addition, LKB1 inactivation induces epigenetic repression of *STING* (also known as *TMEM173*), thus promoting insensitivity to cytosolic dsDNA accumulation[136]. Silencing of *STING* in this context is triggered by enhanced activity of the EZH2 and DNMT1 methyltransferases due – at least in part- to increased production of SAM through diversion of glucose towards the serine biosynthetic pathway in LKB1-deficient cells[87, 136]. Increased expression of MYC has also been observed following LKB1 loss[137] and may provide an additional mechanistic clue to the immune inert phenotype of LKB10-deficient NSCLC because IL-23 and CCL9-mediated inflammation and exclusion of B cells, T cells and NK cells have been reported to underpin the strong oncogenic cooperation between KRAS and MYC in lung cancer pathogenesis[138]. Finally, LKB1 inactivation has also been reported to impinge on non-immune components of the microenvironment of *Kras*^{G12D}-mutant mouse tumors, including increased collagen deposition as a result of elevated lysyl oxidase (LOX) expression and effects on angiogenesis[139, 140].

Inactivating mutations in *KEAP1* have also been associated with an altered NSCLC immune microenvironment[134]. In a conditional GEMM of LUAD (*Keap1*$^{Fl/Fl}$;*Pten*$^{Fl/Fl}$), co-deletion of *Keap1* and *Pten* resulted in immunologically cold tumors, akin to *Lkb1*-mutant NSCLC[141]. Interestingly, NRF2 was recently identified as a negative regulator of STING expression via effects on *STING* mRNA stability[142] thus suggesting a tantalizing mechanistic connection between the effects of KEAP1 and LKB1 inactivation that warrants further study. Additional immune phenotypes may be uniquely associated with *KEAP1* loss; for example, increased peri-tumoral accumulation of natural killer (NK) cells in *KEAP1*-mutant tumors was reported in a cohort of surgically resected early-stage LUAD[132].

Finally, *TP53* co-mutations are associated with an inflamed tumor immune microenvironment and increased tumor cell PD-L1 expression in *KRAS*-mutant NSCLC and GEMMs. This is at least in part due to activation of the nuclear factor κB (NF-κB) pathway driven by p53 loss, as well as increased tolerance of a higher mutational burden that may ostensibly result in enhanced immunogenicity due to increased neoantigen load[143, 144, 145, 146].

The impact of co-mutations on other oncogene-driven subgroups of NSCLC, including those driven by *EGFR* mutations, *ALK*, *ROS1* and *RET* translocations, as well as *ERBB2* and *MET exon 14* skipping mutations has not hitherto been determined and represents an area of active investigation. This will be particularly pertinent for *BRAF*-mutant NSCLCs, which are characterized by high tumor cell PD-L1 expression and more favorable clinical response to PD-1 and PD-L1 inhibitors[147, 148].

Effects on drug sensitivity

Large-scale efforts aimed at linking tumor genomic alterations with sensitivity to cytotoxic and targeted therapies have uncovered a wealth of pharmacogenomic interactions in NSCLC and other cancer types[149–152]. These seminal high-throughput studies yielded multiple novel associations but also highlighted challenges in therapeutic response modeling that underscore the genomic complexity and biological heterogeneity of cancer. Interestingly, logic models – generated using the LOBICO ("Logic Optimization for Binary Input to Continuous Output"computational approach- that combine multiple input features such as mutations in cancer genes, gene fusions, recurrent copy number aberrations and binarized pathway activity scores (derived from gene expression profiling outperform single-gene models for prediction of drug sensitivity[153, 154]. Thus, co-occurring alterations can function as robust, and in many settings more precise, biomarkers of therapeutic response than single-gene predictors.

Chemical and genetic screens in panels of molecularly annotated NSCLC cell lines as well as candidate target approaches have uncovered several *KRAS* co-mutation-driven molecular dependencies and collateral vulnerabilities. KL NSCLC cell lines are characterized by unique sensitivity to depletion of multiple components of the coatomer 1 (COPI) complex and pharmacological inhibition of lysosomal acidification (for example by exposure to bafilomycin A) as a result of critical dependence on lysosomal macromolecule degradation for supply of TCA cycle substrates[155]. Other studies have linked LKB1 loss with enhanced sensitivity to energetic stress triggered by the biguanides metformin and phenformin[156] or the combination of phenformin with the mTOR inhibitor MLN0128[157], as well as to endoplasmic reticulum stress induced by 2-deoxy-D-glucose[158]. Enhanced dependence on nucleotide (and especially dTTP) synthesis further underpins the selective sensitivity of KL cells to deoxythymidylate kinase (DTYMK) depletion and to combined treatment with gemcitabine - a deoxycytidine analog that inhibits DNA synthesis and further depletes dNTP pools by targeting ribonucleotide reductase[159]- and CHK1 inhibitors, that abrogate the CHK1-mediated checkpoint response to replicative stress[160, 161]. Furthermore, LKB1 deficient cells are selectively vulnerable to inhibition of the ATP1A1 Na+/K+-ATPase by cardiac glycosides[162] and to several structurally distinct inhibitors of the HSP90 family of molecular chaperones[67]. While some of these vulnerabilities are associated with LKB1 inactivation irrespective of concurrent *KRAS* mutations, others, such as addiction to lysosomal enzymatic degradation, appear to be specific to the KL oncogenotype and thus represent *de facto* co-mutation-dependent vulnerabilities. In contrast, KL lung tumors exhibit resistance to MEK inhibitors in mouse models and LKB1 deficiency by immunohistochemistry is associated with lack of benefit from the addition of the vascular endothelial growth factor A (VEGFA) inhibitor bevacizumab to platinum doublet chemotherapy[163, 164]. Beyond the KL genotype, *KRAS* and *KEAP1* co-altered NSCLC cells display chemically tractable selective dependence on GLUT8-mediated uptake for effective diversion of glucose towards the serine biosynthetic pathway[165] and rely on glutaminolysis for TCA cycle anaplerosis; thus, they are selectively sensitive to glutaminase inhibition in both cell line and mouse models[92]. In agreement with the role of NRF2 as a transactivator of antioxidant as well as phase II detoxifying and cytoprotective enzymes that mediate

resistance to cytotoxic chemotherapy, *KEAP1* co-mutations are associated with resistance to multiple inhibitors of oncogenic kinases within the receptor tyrosine kinase–MAPK pathway *in vitro*[166] and significantly worse clinical outcomes with platinum-doublet chemotherapy in *KRAS*-mutant LUAD[99] or, as shown in preliminary data, with chemo-immunotherapy using pemetrexed-carboplatin (or cisplatin) plus the PD-1 antibody pembrolizumab in non-squamous NSCLC[167]. Finally, *ATM* co-alterations increase the sensitivity of *KRAS* and *BRAF*-mutant NSCLC cell lines to MEK inhibitor-induced apoptosis and genetic deletion of *Atm* is associated with increased sensitivity to poly(ADP-ribose) polymerase (PARP) and ATR inhibitors as well as to radiation therapy in mouse models of *Kras*-mutant LUAD[168, 169]. Notably, despite apparent mutual exclusivity between classical activating mutations in RAS pathway genes, oncogenic co-operativity has been observed between atypical, weakly activating mutations[170]. In this setting signaling inputs from multiple co-altered RAS pathway genes coordinately contribute towards thresholds of oncogenic activity that are critical for transformation and tumor maintenance but also bestow therapeutic vulnerabilities; for example, co-mutations in *NF1* and *RASA1*, encoding two critical RAS pathway GTPase-activating proteins (GAPs), drive addiction to the MEK–ERK signaling axis and confer enhanced sensitivity to MEK inhibitors in a subset of both LUAD and LUSC[171, 172].

Of particular relevance is the impact of co-occurring alterations on clinical outcomes with EGFR and ALK TKIs as well as other targeted therapies. *TP53* co-mutations have consistently been associated with shorter progression-free survival following upfront treatment with 1st or 2nd generation EGFR TKIs and there is further evidence that they adversely impact clinical outcomes with the third generation, mutant-selective EGFR TKIs, in patients whose tumors have acquired the $EGFR^{T790M}$ gatekeeper mutation[72, 115]. In a study of 200 *EGFR*-mutant patients with extensive molecular profiling at baseline, pre-existing *MET* (present in 2% of cases) or *ERBB2* (4% prevalence) amplification were also associated with significantly shorter progression-free survival with first-line 1st or 2nd generation EGFR TKI therapy, whereas among patients with acquired $EGFR^{T790M}$ mutation from a distinct cohort, co-mutations in *RB1* and *PTEN* and amplification of *MDM2* were independently associated with worse progression-free survival following treatment with 3rd generation EGFR TKIs[72, 173]. Co-alterations in *BRAF*, *CDKN2A*, *CDKN2B*, fibroblast growth factor receptor 3 (*FGFR3*) and amplification of *MET* and *EGFR* itself were all enriched in patients with acquired resistance to EGFR TKIs compared to pre-treatment tumors, indicating roles in mediating the drug resistant phenotype. Interestingly, co-mutations in *PIK3CA* don't impact response to first, second or third generation EGFR TKIs[113, 123, 174]. Importantly, co-occurring clonal alterations in both *TP53* and *RB1*, present in ~9 % of *EGFR*-mutant LUAD at baseline, substantially increase the risk of transformation to small cell carcinoma upon treatment with EGFR TKI; therefore co-occurring alterations can affect not only the likelihood and duration of response to targeted therapy but also impact mechanisms of acquired resistance[117]. It is currently unknown whether the likelihood of acquisition of an $EGFR^{T790M}$ secondary resistance mutation can also be influenced by the co-mutation status of the tumor.

In keeping with their prominent role in shaping tumor immunobiology and immune contexture, co-occurring genomic alterations can further impact clinical response to immune

checkpoint inhibitors. This is particularly evident in *KRAS*-mutant LUAD. Inactivating somatic mutations in *LKB1*, present in ~25% of *KRAS*-mutant LUAD, have emerged as a major genomic driver of primary resistance to PD-1 and PD-L1 inhibition, despite KL LUAD harboring intermediate to high TMB[68]. Importantly, the negative impact of *LKB1* genomic alterations on clinical outcomes with anti-PD1 or anti-PD-L1 therapy extends to PD-L1 positive tumors[68]. Therefore, somatic genomic alterations may represent independent predictors of clinical outcomes with immune checkpoint inhibitors, in addition to previously established markers such as PD-L1 expression and TMB. *De novo* resistance to immune checkpoint blockade following *LKB1* loss is further associated with primary resistance to combined anti-PD-1 and anti-CTLA4 therapy with nivolumab and ipilimumab[175]. In contrast, *KRAS*-mutant tumors bearing co-mutations in *TP53* exhibit high rates of clinical response to PD-1 axis immunotherapy and markedly improved progression-free and overall survival compared to KL[68]. In addition to *LKB1*, co-mutations in *KEAP1* have also been implicated in *de novo* resistance to PD-1 blockade[99] and both *LKB1* and *KEAP1* are associated with inferior clinical outcomes with chemo-immunotherapy with pemetrexed-carboplatin (or cisplatin)-pembrolizumab, particularly among PD-L1-positive and TMB-high tumors[167]. In this context, double *LKB1;KEAP1* mutant tumors exhibita particularly recalcitrant clinical response phenotype[167]. Finally, mutations in *PTEN* have also been nominated as a candidate driver of primary resistance to immune checkpoint inhibition in NSCLC, in agreement with similar reports in melanoma[175, 176].

Conclusions and perspectives.

As our understanding of the genomic landscape of NSCLC deepens, broad tumor genomic profiling becomes increasingly accessible and our therapeutic armamentarium continues to evolve, there is growing appreciation that the current single oncogenic driver model fails to adequately capture the clinical complexity of NSCLC and warrants revision. Co-occurring genomic alterations in oncogenic drivers and tumor suppressor genes have emerged as major tenets of the molecular diversity of NSCLC. Antecedent knowledge of a key set of major co-mutations may therefore allow more granular insights into NSCLC biology; facilitate development of improved clinical response prediction algorithms; anticipate and forestall the emergence of acquired resistance; and enable development of novel, highly personalized therapeutic approaches in the next wave of precision oncology clinical trials. Based on accumulated and emerging evidence we propose a next-generation, dynamic model for the molecular classification of NSCLC that encompasses the molecular and clinical diversity affected by co-mutations (Figure 4). Immediate priorities and challenges for the future are to catalog, functionalize and systematically evaluate the therapeutic utility of the full spectrum of co-occurring alterations in NSCLC and, simultaneously, to expeditiously translate the most robust and critical insights into more precise therapeutic strategies that yield improved clinical outcomes for NSCLC patients. These tasks will require novel computational tools and high throughput *in vivo* platforms as well as large, prospectively assembled collaborative clinical datasets and efficient and flexible umbrella clinical trial **[G]** designs.

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Glossary

Platinum-doublet chemotherapy
Cisplatin or carboplatin-based combinations with a second chemotherapeutic agent, most commonly pemetrexed (LUAD), taxanes (LUAD or LUSC) and gemcitabine (LUSC)

Objective response
Measurable decrease in tumor burden of a predefined amount in response to therapy

Complete responses
The disappearance of all signs of cancer in response to treatment, including both target and non-target lesions (with reduction of all lymph nodes to <10mm in short axis), without emergence of any new lesions

A less strict pattern of mutual exclusivity in which combinations of somatic mutations in different genes occu

Cold tumor immune microenvironment
Tumor microenvironment characterized by lack or paucity of infiltrating T cells

Trans-differentiation
Conversion of one differentiated somatic cell type to another without passage through an intermediate pluripotent or progenitor cell state

Umbrella clinical trial
A clinical trial that assesses multiple targeted therapeutic strategies in a single cancer type

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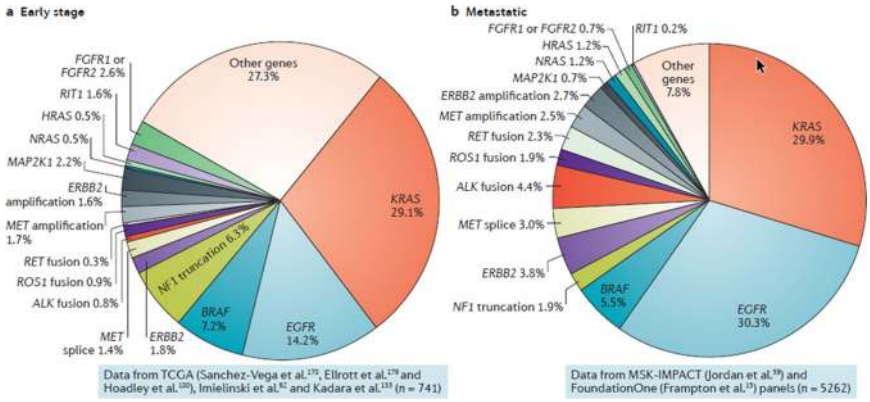

Figure 1. Single oncogenic driver paradigm of lung adenocarcinoma molecular classification.

The dominant contemporary model of non-small cell lung cancer pathogenesis and molecular classification is based on identification of single and largely non-overlapping oncogenic driver events. Oncogenic pie charts are presented for early-stage (a) and metastatic (b) lung adenocarcinomas (LUADs). The prevalence of individual genomic alterations in early-stage disease is based on combined analysis of whole exome sequencing data from the PanCancer Atlas cohort of The Cancer Genome Atlas (TCGA) (n=785)[177–179], as well as the cohorts reported by Imielinski et al (n=148)[62] and Kadara et al (n=108)[132], following exclusion of patients with stage 4 disease (n=741 patients in total). The prevalence of *MET* splice site alterations, *MET* amplification, *ERBB2* amplification, *HRAS* and *NRAS* mutations as well as *ALK*, *ROS1* and *RET* fusions was based on data from the TCGA and Imielinski cohorts only. Oncogenic driver alterations in advanced or metastatic LUAD (encompassing both treatment-naïve patients as well as patients that received prior anti-cancer therapies) are based on next-generation sequencing of pre-defined panels of cancer-relevant genes from patients treated at Memorial Sloan Kettering Cancer Center (N=860,MSK-IMPACT panel[59]) and samples referred to Foundation Medicine (n=4402,FoundationOne panel[15]) (n=5262 patients with advanced/metastatic LUAD in total). The prevalence of alterations in *NF1*, *NRAS*, *HRAS*, *MAP2K1*, *FGFR1/2* and *RIT1* is based on data from MSK-IMPACT only. It is notable that although the prevalence of oncogenic *KRAS* mutations is similar in both early and advanced stage LUADs the frequency of other driver alterations (for example truncating *NF1* mutations) differs substantially depending on the disease stage. The increased prevalence of *EGFR* mutations in the metastatic dataset may partially reflect referral bias. Data were visualized and downloaded from the open source web program cBioPortal[180, 181] or curated from the scientific literature.

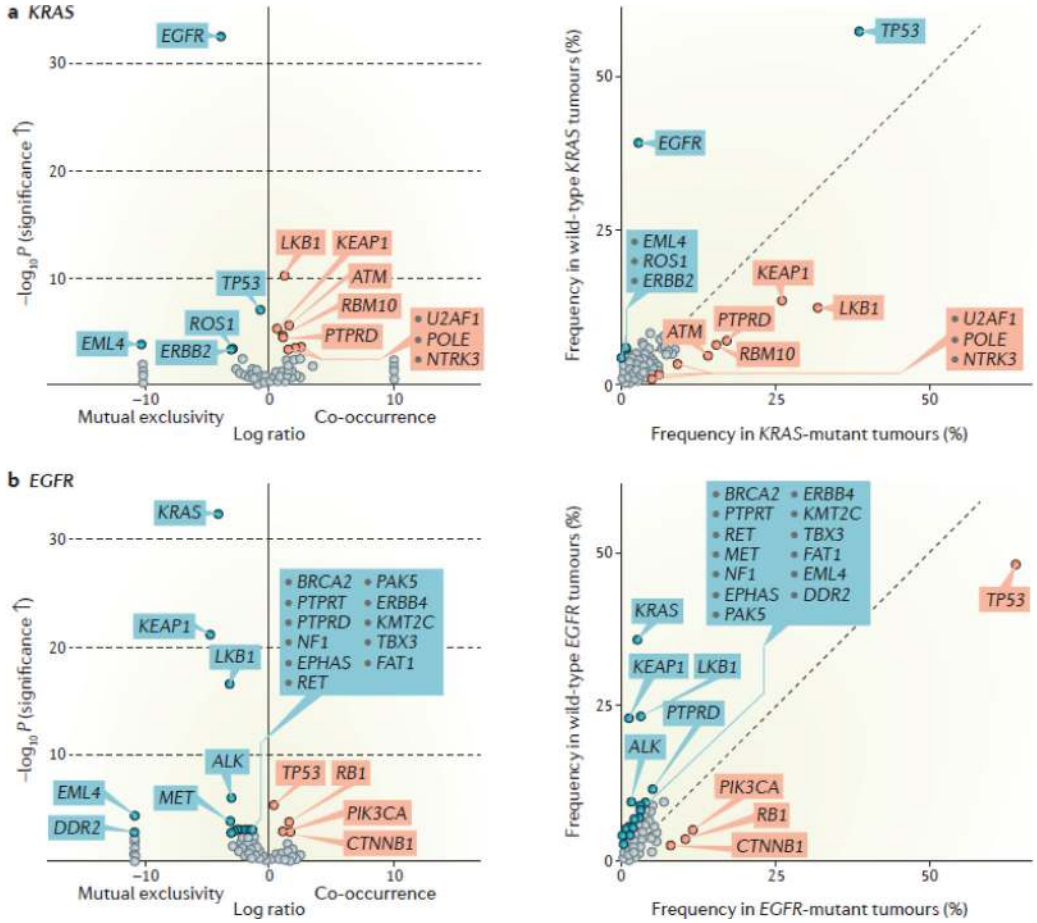

Figure 2. Spectrum of major co-occurring genomic alterations in *KRAS*- and *EGFR*-mutant lung adenocarcinoma.

Volcano plots (left graphs) summarizing enrichment of individual co-alterations in: *KRAS*-mutant compared with *KRAS*-wild-type LUADs (a) and *EGFR*-mutant compared with *EGFR*-wild-type LUADs (b). The magnitude of co-mutation enrichment is indicated on the *x-axis* and is expressed as $\log_2$ (% in *KRAS*-mutant / % in *KRAS*-wild-type) or $\log_2$ (% in *EGFR*-mutant / % in *EGFR*-wild-type) respectively, whereas the statistical significance of the association is plotted on the *y-axis* and is expressed as $-\log_{10}$P value (derived from a Fisher's exact test). Significantly enriched co-mutations based on a q value <0.05 (derived from Benjamini-Hochberg procedure[182]) are highlighted in red, whereas under-represented genomic events are highlighted in blue. The prevalence of each co-alteration in *KRAS*-mutant and *KRAS*-wild-type groups (or *EGFR*-mutant and *EGFR*-wild-type groups) is shown in the adjacent frequency plots (right graphs of parts a and b). Targeted next generation sequencing-based molecular profiling (MSK-IMPACT platform) from 860 patients with metastatic LUAD treated at Memorial Sloan Kettering Cancer Center were included in this enrichment analysis that was performed using the cBioPortal web program[180, 181]. Oncogene-driver specific, non-random patterns of co-occurring alterations in key tumor suppressor genes are evident for both *KRAS*-mutant and *EGFR*-mutant tumors.

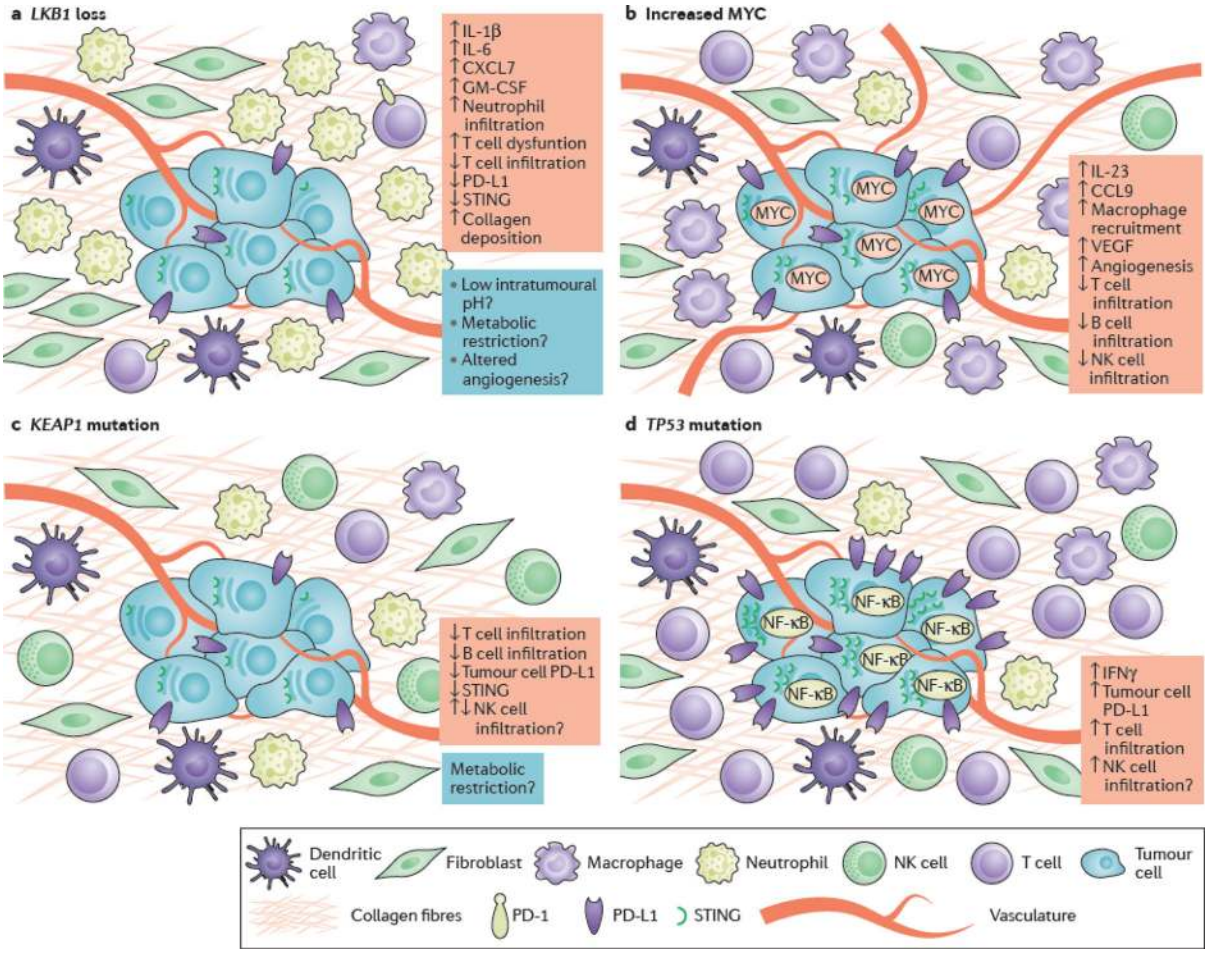

Figure 3. Impact of co-mutations on the microenvironment of *KRAS*-mutant lung adenocarcinoma.

Schematic representation of co-mutation-associated changes in the immune and non-immune microenvironment of *KRAS*-mutant lung adenocarcinoma (LUAD). (a) *LKB1* inactivation promotes epigenetic suppression of *STING* and insensitivity to cytosolic DNA that accumulates in the cytoplasm of *KRAS*- and *LKB1*-mutant (KL) cells due to dysfunctional mitochondria[136]. KL tumors are further characterized by a pro-inflammatory cytokine milieu with accumulation of immunosuppressive neutrophils, marked paucity of CD4+ and CD8+ T-cells and evidence of T-cell exhaustion[68, 135]. The potential contributions of immune cell metabolic restriction, altered angiogenesis and acidification of the tumor microenvironment (highlighted in blue) to the immune-inert phenotype of KL tumors remain as yet unexplored, but represent plausible directions for future study. (b) MYC fosters immune evasion of murine $Kras^{G12D-}$ driven LUADs through IL-23- mediated expulsion of T, B and NK cells and CCL9-mediated macrophage recruitment and secretion of immunosuppressive VEGF[138]. (c) *KEAP1* mutations, which frequently co-occur with mutations in *LKB1*, particularly in the context of *KRAS*-mutant LUAD, have also been associated with low intra-tumoral density of infiltrating T- and B- lymphocytes, although the possible role of *KEAP1* loss on NK cell infiltration remains unclear[141]. Stabilization of NRF2 as a result of KEAP1 inactivation may further promote reduced expression of STING

through post-transcriptional regulation[142]. (d) Finally, somatic *TP53* mutations have been shown to mediate NF-κB pathway activation in *Kras*-mutant murine models of LUAD[146]. Although *TP53* mutations have been associated with reduced production of chemokines required for the recruitment of NK and T cells in some models and human tumors, in the context of *KRAS*-mutant LUAD *TP53* co-alterations promote an inflamed tumor immune microenvironment with increased production of interferon γ (IFNγ) and increased expression of PD-L1 on the surface of tumor cells[67, 68, 186].

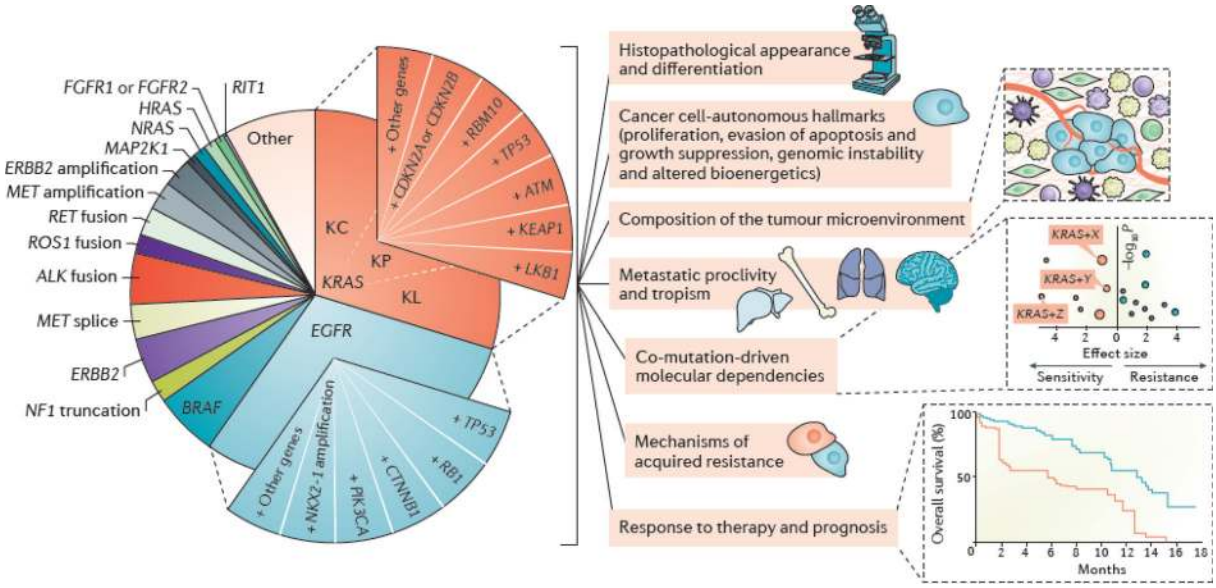

Figure 4. Next-generation model for the molecular stratification of lung adenocarcinoma.

Oncogenic subgroups of lung adenocarcinoma (LUAD) are divided into smaller subsets on the basis of key co-occurring genomic alterations. Co-mutations constitute major determinants of tumor molecular diversity and can impact both tumor cell-autonomous and non-cell-autonomous cancer hallmarks; determine prognosis; predict response to systemic therapies and influence mechanisms of innate and acquired resistance. For simplicity, only *KRAS* and *EGFR* co-alterations are depicted graphically. For *KRAS*-mutant LUADs the previously identified KL, KP, and KC transcriptome-based subgroups are also indicated[67]; co-mutations in *LKB1*, *KEAP1* and *ATM* are significantly enriched in the KL subgroup, whereas co-occurring alterations in *TP53* and bi-allelic inactivation of *CDKN2A/CDKN2B* are hallmarks of the KP and KC subgroups respectively. Co-mutations in *RBM10* don't appear to exhibit predilection for any of the three *KRAS* transcriptomic subgroups. It should therefore be noted that several of the reported co-alterations within oncogene-defined groups are not mutually exclusive. Although co-mutation-defined cohorts are represented as slices of equal size, both the spectrum and prevalence of individual co-mutations evolve according to disease stage, prior treatment exposures, immune editing and the mutational processes that are operational at each stage of carcinogenesis.