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Review

Conquering oncogenic KRAS and its bypass mechanisms

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Abstract

Aberrant activation of KRAS signaling is common in cancer, which has catalyzed heroic drug development efforts to target KRAS directly or its downstream signaling effectors. Recent works have yielded novel small molecule drugs with promising preclinical and clinical activities. Yet, no matter how a cancer is addicted to a specific target – cancer's genetic and biological plasticity fashions a variety of resistance mechanisms as a fait accompli, limiting clinical benefit of targeted interventions. Knowledge of these mechanisms may inform combination strategies to attack both oncogenic KRAS and subsequent bypass mechanisms.

Key words: KRAS, RAF, MEK, targeted therapy resistance, cancer

Introduction

The oncogenic function of *RAS* was first discovered in 1982, when three groups independently found that genomic DNA from cancerous cells contains homologues of the viral *ras* genes (see review [1]). Later, *HRAS* and *KRAS* were discovered in several carcinogen or radiation-induced mouse cancer models. In 1984, *KRAS* G12R mutation was identified first in human lung cancer. Soon after, frequent KRAS G12 mutations were confirmed in various human cancers including pancreatic ductal adenocarcinoma (PDAC), colorectal cancer (CRC) and non-small cell lung cancer (NSCLC). The genetically engineered mouse (GEM) models generated since late 1980s further support the oncogenic role of *RAS* in tumor initiation and maintenance.

RAS proteins are small GTPases that are in either active GTP-bound state or inactive GDP-bound state regulated by large multi-domain protein complexes – guanine nucleotide exchange-factors (GEFs) including SOS family members and GTPase-activating proteins (GAPs) such as NF1 [2]. While GAPs facilitate RAS GTPase to hydrolyze GTP into GDP leading to RAS inactivation, GEFs dislodge bound GDP for GTP to activate RAS. Importantly, the regulation of RAS by GEFs and GAPs depends not only on upstream signal inputs such as receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs) and integrins, but also by their proximal localization on cell membrane. Aberrant RAS hyperactivation can be achieved by gain-of-function mutations of RTKs that assemble several scaffolding proteins to stimulate RasGEFs, loss-of-function mutations of RasGAPs, and RAS oncogenic mutations. In contrast to wildtype protein, oncogenic RAS mutants have impaired GTPase activity, such as the two mutation hot spots glycine 12 and 13 (G12/13) and glutamine 61 (Q61), that keeps RAS in a constitutively active state [3-6]. Other factors that regulate RAS signaling transduction include posttranslational modifications and RAS dimerization [7]. Though dissociation of mutant KRAS homodimer results in tumor suppression, blocking heterodimerization between wildtype and mutant KRAS accelerates cancer cell growth in lung cancer models [8].

Oncogenic KRAS regulates almost all the cancer hallmarks. KRAS promotes Cyclin D1-dependent cell proliferation, suppresses apoptosis by promoting the expression of BCL-2 family proteins, rewires cancer metabolism covering glycolysis, glutaminolysis, macropinocytosis, mitophagy, redox balance, and macromolecule biosynthesis of amino acids, nucleotides and fatty acids (see review [9]), remodels tumor microenvironment (TME) to facilitate tumor growth [10-13], and protects tumor cells from immune surveillance [10, 14-17].

The KRAS-RAF-MEK cascade

KRAS mediates mitogenic signal transduction from cell surface receptors to intracellular effectors and pathways, including RAF-MAPK, PI3K-AKT, RalGEF-Ral-TBK1, TIAM1-Rac, PLCε-PKC, AF6, RIN1 and RASSF proteins (see review [18-20]). Upon binding of ligands to these RTKs (e.g., EGFR, FGFR, PDGFR, IGFR, etc.), RasGEFs activate KRAS, which recruits Raf proteins (A-raf, B-raf and C-Raf) to cell membrane, and thus leading to Raf activation [21, 22]. C-raf is the first identified *bona fide* KRAS effector. As serine/threonine protein kinases, Raf proteins phosphorylate mitogen/extracellular protein kinases (MEK1 and MEK2), subsequently leading to the phosphorylation of the extracellular signal-regulated kinases (ERK1 and ERK2) by MEK at threonine and tyrosine residues. Phosphorylated ERK1 and ERK2 translocate to nucleus to activate transcription factors such as ternary complex factors (TCFs), MYC and AP-1 that promote cell cycle progression. In addition, ERK has over 200 targets contributing to cell survival, growth, metabolism and mobility (see review [23]). Due to the central role in regulating fundamental cellular processes, the RTK-RAS-MAPK pathway is the most frequently altered signaling pathway in 46% of all cancer types in the Cancer Genome Atlas (TCGA) datasets, and KRAS is mutated in 9% cancer cases with high frequency in PDAC (72%), genomically stable CRC (69%), and NSCLC (33%) [24].

The regulation of KRAS-RAF-MEK signaling pathway has been extensively studied in cancer and significant progress has been made in targeting the cascade. This review will introduce current progress in inhibitors and novel therapeutic approaches targeting KRAS, RAF and MEK, summarize resistance mechanisms and discuss strategies to overcome resistance.

Targeting KRAS-RAF-MEK signaling

KRAS inhibition

Efforts in chemically targeting KRAS were largely unsuccessful in the past 30 years due to, but not limited to, its picomolar affinity for GTP/GDP that restricts the usage of GTP analogues to compete binding with the catalytic domain [25, 26], the lack of known allosteric regulatory sites that increases the difficultness in exploring drug docking pockets [27], and the alternative post-translational modifications of KRAS to ensure correct membrane localization such as geranylgeranylation that escapes the blockade of farnesyl transferase [28]. Inhibition of wild type KRAS also raises concerns about potential adverse events (AEs) regarding the importance of KRAS signaling in normal cells. Thus, targeting specific KRAS mutants would be ideal, and, in fact, great progress has been made in recent years (**Table 1**).

KRAS small molecule inhibitors. A breakthrough came in 2013, when the Shokat group exploited the KRAS G12C mutation by a disulphide-fragment-based "tethering" screening against KRAS-GDP and discovered the first covalent inhibitor specifically relied on KRAS G12C mutation and GDP-bound inactive state [29]. A new pocket under the effector binding switch-II region of KRAS G12C facilitates the inhibitor binding that causes the GDP-favorable structure remodeling. Inspired by the pioneering work, high affinity KRAS G12C inhibitors (G12Ci) are developed by several groups [30-34], which share a common trapping mechanism that locks KRAS G12C in a constitutively inactive state [35]. The first-in-class G12Ci with clinical activity are listed in **Table 1**, among which sotorasib (a.k.a LUMAKRAS or AMG 510) received FDA approval recently.

The phase 1 clinical trial data of sotorasib shows 32.2%-37.6% objective response rate (ORR) and improved median progression-free survival (PFS) of 6.3-6.8 months as compared with 2.5-4 months under standard of care (SOC) in heavily pretreated NSCLC patients, while CRC patients only exhibits 7.1% ORR and median PFS of 4 months as compared with 1.9-2.1 months under SOC [36, 37]. Similarly, NSCLC patients respond to G12Ci adagrasib (MRTX849) better than CRC patients (43% versus 25% ORR). Thus, understanding the different tumor responses to G12Ci in individuals with the same cancer type or with distinct cancers are needed to expand clinical applications and enhance the anti-tumor effect of G12Ci. In addition, to increase the efficacy, clinical trials evaluating combinations of G12Ci with chemotherapy reagents, immune checkpoint blockade such as PD-L1/PD-1 antibodies, and inhibitors of SHP2, tyrosine kinases and MEK are ongoing. These treatment regimens are designed to either enhance the KRAS vertical pathway inhibition or leverage the increased T cell infiltration upon G12Ci treatment, which are discussed in the next section. Other KRAS inhibitors (KRASi) under pre-clinical evaluation

include G12Ci BI 1823911, G12D inhibitors MRTX1133 and BI-KRASG12D1, RAS G12C(ON) inhibitor RMC-629 and pan-RAS (ON) inhibitor RMC-6236. In contrast to current G12Ci, RMC-629 and RMC-6236 interact with and inhibit active RAS by forming a RAS-inhibitor-chaperone protein tri-complex, which may overcome resistance induced by active residue RAS.

Exosome-delivered KRAS siRNA. Compared to artificial carriers such as liposomes, natural carrier exosomes express CD47 immunoglobulin that avoids phagocytosis by macrophages to prolong the half-life of delivered drugs [38]. Recent pre-clinical studies revealed that fibroblasts-derived exosomes loaded with G12D siRNA (iExosomes) efficiently attenuate PDAC tumor growth and extend tumor bearing mouse survival [38, 39]. The efficacy of the iExosomes is currently being evaluated in a phase 1 clinical trial. However, AE and "off-target" concerns remain to be addressed since exosome-delivered reporter was also observed in liver and spleen [39].

mRNA vaccines. Though the first *in vivo* test was reported in 1990, mRNA vaccine has become a feasible and promising therapeutic strategy in recent years as the instability, high immunogenicity and inefficient mRNA delivery *in vivo* have been improved [40]. The KRAS mRNA vaccine V941 (mRNA-5671) co-developed by Moderna and Merck is in phase 1 clinical trials. Lipid nanoparticle encapsulated mRNA vaccines encoding mutant KRAS neoepitopes (G12D, G12V, G13D and G12C) are taken up and translated in antigen presenting cells, and then presented by MHC molecules on cell surface. However, KRAS mutant cancer cells may still evade neoantigen-specific T cell immune surveillance by suppressing antigen presentation machinery [41-43] and inflammatory response in TME [16, 43].

Table 1. List of therapeutic approaches targeting KRAS-RAF-MEK in clinical trials and FDA-approved

Therapy	Direct target	Cancer type	Trial phase and combinations	Clinical trial ID#	Sponsor
AMG 510 (sotorasib)	KRAS G12C	$KRAS^{G12C}$ mutant NSCLC	Ph3	NCT04303780	Amgen
		$KRAS^{G12C}$ mutant advanced cancers	Ph1/2	NCT03600883	
			Ph1; with inhibitors targeting PD1, MEK, SHP2, pan-ErbB, PD-L1 or EGFR, or with chemotherapeutic regimen	NCT04185883	
			Ph1	NCT04380753	
MRTX849 (adagrasib)	KRAS G12C	$KRAS^{G12C}$ mutant advanced cancers	Ph1/2; with TNO155 (SHP2 inhibitor)	NCT04330664	Mirati
			Ph1/2; monotherapy and with pembrolizumab, cetuximab or afatinib	NCT03785249	
JNJ-74699157	KRAS G12C	$KRAS^{G12C}$ mutant advanced cancers	Ph1	NCT04006301	Janssen
LY3499446	KRAS G12C	$KRAS^{G12C}$ mutant advanced cancers	Ph1/2; monotherapy and with abemaciclib, cetuximab, erlotinib or docetaxel	NCT04165031	Eli Lilly
iExosomes	KRAS G12D	$KRAS^{G12D}$ mutant mPDAC	Ph1	NCT03608631	MDACC
V941 (mRNA-5671)	mutant KRAS	KRAS mutant mNSCLC, mCRC or mPDAC	Ph1; monotherapy and with pembrolizumab	NCT03948763	Merck
anti-KRAS G12D mTCR PBL	KRAS G12D	$KRAS^{G12D}$ mutant cancers	Ph1/2	NCT03745326	NCI
Anti-KRAS G12V mTCR PBL	KRAS G12V	$KRAS^{G12V}$ mutant cancers	Ph1/2	NCT03190941	NCI
BI 1701963	SOS1	KRAS mutant cancers	Ph1; monotherapy and with trametinib	NCT04111458	Boehringer Ingelheim
Rigosertib	RBD domain	KRAS mutant advanced NSCLC (first line treatment refractory)	Ph1/2; with nivolumab	NCT04263090	Onconova
Vemurafenib	BRAF V600E	$BRAF^{V600E}$ mutant metastatic melanoma	Approved	/	Genentech
Dabrafenib	BRAF V600E	$BRAF^{V600E/K}$ mutant metastatic melanoma	Approved; monotherapy and with trametinib	/	GlaxoSmithKline
Encorafenib	BRAF V600E	$BRAF^{V600E/K}$ mutant metastatic melanoma and mCRC	Approved; with binimetinib (melanoma) or with cetuximab (CRC)	/	Novartis
LXH254	RAF and RAF dimer	MAPK pathway altered advanced cancers	Ph1	NCT02607813	Novartis
		metastatic melanoma	Ph2; with LTT462, trametinib or ribociclib	NCT04417621	
		KRAS or BRAF mutant NSCLC and NRAS mutant melanoma	Ph1; with LTT462, trametinib or ribociclib	NCT02974725	
PLX8394	BRAF dimer	BRAF mutant advanced cancers	Ph1/2	NCT02428712	Plexxikon
Lifirafenib (BGB-283)	BRAF V600E and EGFR	Advanced or treatment refractory cancers	Ph1/2; with PD-0325901	NCT03905148	BeiGene
Trametinib (GSK1120212)	MEK1/2	$BRAF^{V600E/K}$ mutant metastatic melanoma	Approved; monotherapy and with dabrafenib	/	GlaxoSmithKline
Cobimetinib (GDC-0973)	MEK1	$BRAF^{V600E/K}$ mutant metastatic melanoma	Approved; with vemurafenib	/	Genentech
Binimetinib (ARRY-162)	MEK	$BRAF^{V600E/K}$ mutant metastatic melanoma	Approved; with encorafenib	/	Array
Mirdametinib (PD-0325901)	MEK1/2	KRAS mutant advanced NSCLC	Ph1/2; with dacomitinib	NCT02039336	Pfizer

Ph, Phase; mPDAC, mNSCLC and mCRC, metastatic PDAC, NSCLC and CRC.

Anti-KRAS T cell transfer. Adaptive cell therapies, such as tumor-infiltrated lymphocytes (TILs) therapy, engineered T cell receptor (TCR) therapy and chimeric antigen receptor T cell therapy, have made impressive progress in melanoma, sarcoma and B cell lymphomas in the last decade [44-47]. HLA-A*11:01-restricted human KRAS G12D or G12V-reactive TCRs are identified in immunized HLA-A*11:01 transgenic mice. Peripheral blood lymphocytes (PBL) transduced with these TCRs can recognize several HLA-A*11:01-positive KRAS mutant human PDAC cell lines and suppress xenograft tumor growth [48]. Based on these results, phase 1 clinical trials utilizing autologous PBLs transduced with HLA-A*11:01-restricted murine TCR (mTCR) recognizing human KRAS G12D or G12V are ongoing now. A promising clinical result from a TILs transfer therapy in 2016 showed that infusion of TILs specifically against KRAS G12D in a metastatic CRC patient resulted in tumor regression in all seven metastatic lung lesions for 40 days, followed by one lesion progression after 9 months [49]. Four distinct TCRs were identified that recognize two KRAS G12D neoantigens, a 9mer and a 10mer, which are all restricted by human leukocyte antigen HLA-C*08:02 [49, 50]. Importantly, both KRAS G12D and HLA-C*08:02 are required to generate the recognizable neoantigens [50]. Though not as common as HLA-A*11:01, 2.5-10% KRAS G12D patients have HLA-C*08:02 allele [51], who would potentially benefit from the TCR therapy.

KRAS antibody. While traditional antibodies target extracellular proteins, a new type of antibodies is internalized via endocytosis and subsequent endosomal escape to target intracellular proteins [52, 53]. The second generation of cell permeable RAS antibody in human IgG1 format (pan-RAS iMab, inRas37) showing favorable PK *in vivo* with half-life of 3.5 days, only interacts with ATP-bound active RAS mutants but not wild type RAS, attenuates ERK and AKT signaling and suppresses tumor growth in several KRAS mutant (G12D, G12V, G13D, etc.) xenograft models [52]. Whether the KRAS antibody will be effective in cancer patients needs to be explored.

Targeting RBD domain of RAS effectors. RAS effector proteins interact with RAS through RAS-binding domain (RBD). Rigosertib is a RAS mimetic that interacts with RBDs of RAF, PI3K and Ral-GDS to interrupt their RAS binding [54]. Rigosertib could effectively block RAS-driven fibroblast cell transformation and suppress xenograft tumor growth [54]. The efficacy of rigosertib and nivolumab is evaluated in a phase 1 clinical trial in KRAS mutant NSCLC patients with advanced disease as a second-line treatment.

RAF inhibitors

Though wildtype RAF inhibitors show limited anti-tumor activity in KRAS or BRAF mutant cancers in clinical trials, ATP-competitive RAF inhibitors that selectively target BRAF V600E monomers such as vemurafenib, dabrafenib and encorafenib increase clinical benefit, but they often paradoxically activate ERK signaling by transactivation of the other protomer in RAF dimers, which eventually results in drug resistance. Combination therapy of BRAF V600E inhibitor with MEK or EGFR inhibitors enhances the efficacy compared to monotherapy and prolongs the overall survival of CRC and melanoma patients [55-58]. In addition, the next-generation RAF inhibitors can block BRAF monomers and dimers via two distinct mechanisms. BRAF inhibitors such as LY3009120, BGB-283 and LXH254 equipotently inhibit both protomers in RAF dimers without paradoxical activation of ERK [59, 60]. Alternatively, RAF inhibitors including PLX7904 and PLX8394 can disrupt BRAF homo- or hetero-dimers as "paradox breakers", but not CRAF dimers [61]. Though these inhibitors show great efficacy in preclinical models, clinical efficacy varies. BGB-283 and LXH254 monotherapy resulted in objective responses in BRAF mutant patients in phase 1 clinical trials [62, 63], while LY3009120 or PLX8394 monotherapy lacked responses [64, 65]. Combined PLX8394 and cobicistat treatment is promising that 42% and 65% PR are observed in BRAF V600E CRC and glioma patients, respectively [65]. Taken together, whether the next-generation BRAF inhibitors perform better and exhibit broader anti-tumor responses than the first-generation inhibitors need further clinical evaluation.

MEK inhibitors

MEK inhibitors trametinib, cobimetinib, binimetinib and selumetinib are the four FDA-approved drugs to date. Additionally, there are more than a dozen MEK inhibitors in clinical trials and about 8 inhibitors under preclinical development [66]. Most of the MEK inhibitors are with high specificity and strong binding affinity to MEK kinase because they dock in an allosteric pocket near catalytic site that causes conformational changes and subsequent blockage of kinase activity. The fact that MEK inhibitors only have modest clinical efficacy in RAS mutant cancers compared to BRAF mutant cancers is mainly due to the relief of ERK-mediated negative feedback and the BRAF, CRAF-dependent reactivation of MEK [67, 68]. Thus, combined inhibition of MEK and their upstream kinases (e.g., RAF, RAS, EGFR, etc.) are expected to enhance MAPK signaling inhibition and show better clinical efficacy

than monotherapy in RAS mutant cancers, as did in clinical trials now.

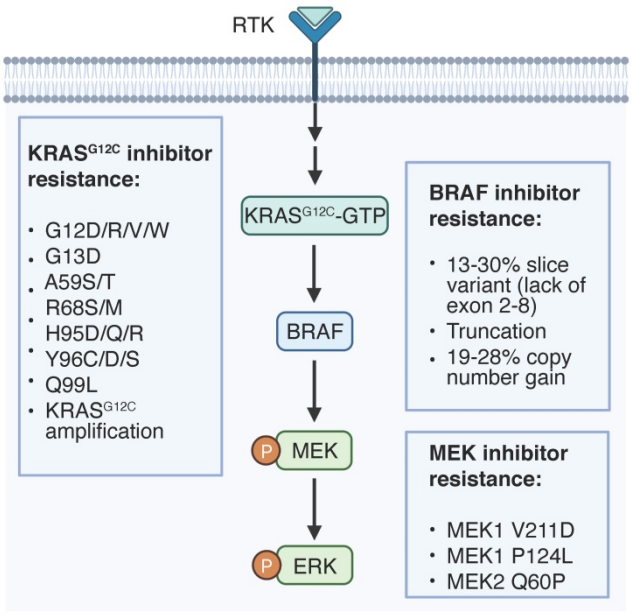

Figure 1. On-target resistance mechanisms to RAS signaling inhibition. Genetic alternations of targeted proteins that regulate therapy resistance are listed.

Resistance mechanisms to KRAS-RAF-MEK signaling inhibition

Given the recent development of agents targeting Kras pathways and their clinical application, it is paramount to understand the various therapeutic resistance mechanisms and develop better agents to overcome them. Resistance mechanisms to KRAS-RAF-MEK signaling inhibition include genetic alterations of targeted oncogenes (**Figure 1**) and adaptive activation of alternative regulators or signaling pathways in tumor cell intrinsic and extrinsic manners (**Figure 2** and **Table 2**).

On-target resistance

Second-site mutations

Site-mutations could confer resistance to G12Ci [69-72] and allosteric MEK inhibitors (MEKi) [73, 74]. Resistance mutations in KRAS (G12C/V, G13D, Y96D and Q61L/R/K) have been detected in cell-free DNA of a NSCLC patient post G12Ci adagrasib treatment [70]. A larger study evaluating adagrasib in 38 patients also identified several KRAS mutations (G12D/R/V/W, G13D, Q61H, R68S, H95D/Q/R, Y96C) associated with drug resistance [71]. Similarly,

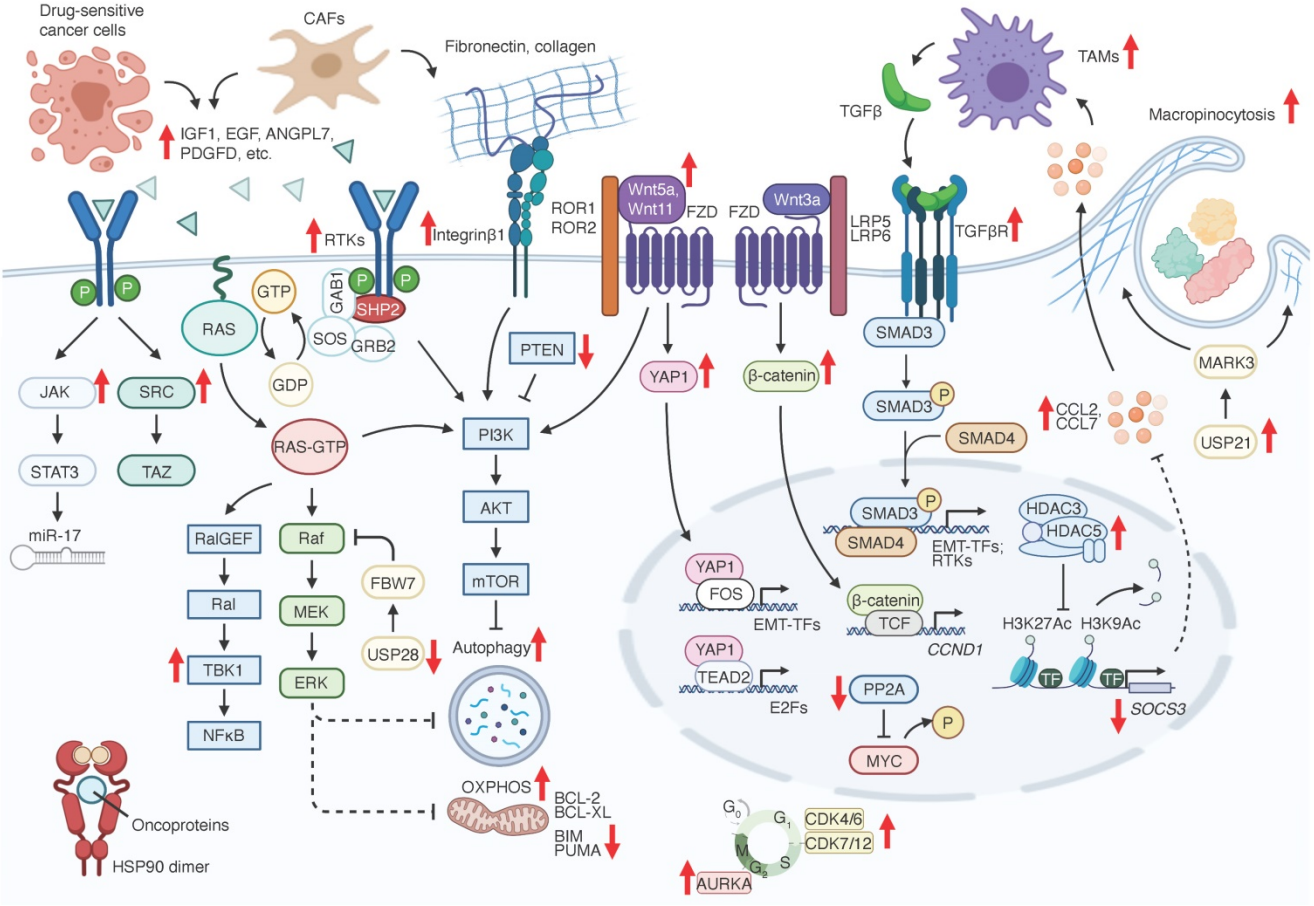

Figure 2. Adaptive resistance mechanisms to RAS signaling inhibition. Bold red arrowheads indicate major factors that either promote (up) or suppress (down) KRAS signaling inhibition resistance.

a mutation screening in lung cancer cells revealed second-site KRAS mutations that confer resistance to sotorasib and adagrasib (Y96D/S), sotorasib only (G13D, R68M, A59S and A59T) and adagrasib only (Q99L) [69]. Mechanistically, switch II pocket mutations (R68S, H95D/Q/R and Y96C/D/S) interferes the binding of G12Ci to inactive KRAS, while a RAS G12C(ON) inhibitor or dual inhibition of SOS1 and MEK1 may overcome the G12Ci resistance [69, 70].

Table 2. Adaptive resistance mechanisms to RAS-RAF-MEK signaling inhibition

Regulator	Role	Agent	Cancer type	Mechanism	References
AURKA	Pro	G12Ci	$KRAS^{G12C}$ mutant NSCLC	Induces adaptive KRAS signaling reactivation	[158]
autophagy	Pro	G12Ci; MEKi; ERKi	KRAS mutant PDAC, NRAS mutant melanoma, BRAF mutant CRC	Pro-survival	[162, 188, 189]
AXL	Pro	MEKi	KRAS mutant PDAC	Activates PI3K/AKT/mTOR pathway	[121]
BCL-XL/ BCL-2	Pro	MEKi+PI3Ki	KRAS mutant NSCLC	Pro-survival	[194]
BET	Pro	dual MEKi and TBKi; MEKi; BRAFi	KRAS mutant NSCLC, CRC, TNBC, MM and PDAC; $BRAF^{V600E}$ mutant melanoma	Required for treatment-induced chromatin remodeling	[105, 106, 108, 109, 159]
CCL2	Pro	KRASi	KRAS mutant PDAC	Recruits TGFβ-secreting M2-like macrophages to support KRAS independent tumor growth	[102]
CDK4/6	Pro	G12Ci	$KRAS^{G12C}$ cancers	Required for tumor cell growth	[33, 34, 118, 143]
CDK7/12	Pro	BRAFi, MEKi	KRAS mutant NSCLC and gastric cancer, BRAF mutant melanoma	Required for treatment induced transcriptional and epigenetic remodeling	[163]
chemotherapy	Anti	MEKi+CDK4/6 i	KRAS mutant PDAC	Causes cell death	[199]
COT/TPL2	Pro	RAFi; MEKi	$BRAF^{V600E}$ mutant melanoma	Activates MAPK independent of RAF	[166]
CRAF	Pro	BRAFi	BRAF mutant melanoma and CRC	Supports transactivation of RAS-MAPK signaling	[138]
ERK	Pro	RAFi, BRAFi+MEKi, BRAFi+EGFRi	BRAF mutant CRC, KRAS mutant PDAC	Promotes resistance	[86, 145, 214]
FAK	Pro	BRAFi and MEKi	$BRAF^{V600E}$ mutant melanoma	Required for cell proliferation	[159]
FGFR	Pro	G12Ci; MEKi	KRAS mutant cancers	Promotes reactivation of MAPK signaling	[118, 126]
HAT1	Anti	dual BRAFi and MEKi	$BRAF^{V600E}$ mutant melanoma	Loss of HAT1 upregulates MAPK via IGF1R	[96]
HDAC5	Pro	MEKi; KRASi	KRAS mutant PDAC	Suppresses Socs3 to reprogram chemokine expression	[102]
HDAC family	Pro	MEKi	PDAC, uveal melanoma and CRC	Required for treatment-induced upregulation of pAKT and YAP1	[98-100]
HER family	Pro	G12Ci; BRAFi; MEKi	KRAS or BRAF mutant CRC, thyroid cancer, NSCLC, LGSC and melanoma	Promotes reactivation of MAPK and AKT-mTOR pathways	[33, 34, 113-120, 122, 123, 158, 215]
HSP90	Pro	G12Ci; BRAFi; MEKi	RAS or RAF mutant MM and NSCLC	Amplifies MAPK signaling	[143, 182, 183]
IGF1R	Pro	G12Ci; BRAFi; MEKi	KRAS or BRAF mutant CRC, NSCLC, melanoma	Activates PI3K pathway	[131-135]
JUN	Pro	BRAFi and MEKi	$BRAF^{V600E}$ mutant melanoma	Required for cell proliferation	[159]
MEK	Pro	G12Ci; BRAFi	$BRAF^{V600E}$ mutant melanoma, $KRAS^{G12C}$ mutant cancers	Required for cell growth	[34, 210, 213]
MET	Pro	BRAFi	$BRAF^{V600E}$ mutant ATC	Promotes MAPK reactivation	[139]
NRAS	Pro	RAFi+EGFRi	$BRAF^{V600E}$ mutant CRC	Promotes RAF dimerization and activates ERK	[88]
OXPHOS	Pro	KRASi	KRAS mutant PDAC	Adaptive energenic metabolism	[162]
P-TEFb complex	Pro	MEKi	TNBC	Upregulates RTKs by promoting *de novo* enhancer formation	[107]
PD-L1	Pro	MEKi+CDK4/6i	KRAS mutant PDAC	Causes exhaustion of infiltrated CD8+ T cells	[199]
PDGFRα	Pro	RAFi; MEKi	$BRAF^{V600E}$ mutant melanoma, KRAS mutant PDAC	Activates JAK/STAT3	[124, 125]
PI3K-AKT-mTOR	Pro	G12Ci; BRAFi; MEKi	RAS or RAF mutant cancers	Supports drug resistant cell growth, infiltration and metastasis; supports drug sensitive cell survival	[33, 34, 110, 121, 143, 161, 168, 170, 198, 201, 216]
PP2A	Anti	MEKi	KRAS mutant NSCLC	Suppresses MAP3K2	[157]
SETD5	Pro	MEKi	KRAS mutant PDAC	Suppresses cytochrome P450 and glutathione metabolism pathways	[101]
SHOC2	Pro	RAFi; MEKi	KRAS mutant PDAC and NSCLC, BRAF mutant CRC	Required for growth factor-mediated RAS signaling activation	[111, 138]
SHP2	Pro	G12Ci; BRAFi; MEKi	BRAF mutant CRC, KRAS mutant cancers	Mediates RTK (e.g., EGFR)-induced RAS/MAPK reactivation	[33, 34, 112, 118, 142, 143, 148-150, 158]
Src family	Pro	BRAFi, MEKi	KRAS mutant cancers, $BRAF^{V600E}$ melanoma	Promotes cancer cell survival and proliferation	[159, 165]
STAT3	Pro	MEKi	NSCLC	Pro-survival	[172, 173]
SUZ12	Anti	MEKi	MPNST	Amplifies Ras-driven transcription	[1]
TBK1	Pro	MEKi	NRAS mutant melanoma, KRAS mutant NSCLC	Activates STAT3 by inducing autocrine IL-6 and CCL5	[154, 155]
TGFβ	Pro	KRASi; MEKi	KRAS mutant NSCLC	Induces EMT and activates PI3K through FGFR1	[102, 127]
USP28	Anti	MEKi	$BRAF^{V600E}$ melanoma	Stabilizes FBW7 to degrade BRAF	[184]
WNT5A	Pro	BRAFi	BRAF mutant melanoma	Activates PI3K/AKT	[174]
YAP1	Pro	KRASi; RAFi; MEKi; dual MEKi and EGFRi	BRAF or RAS mutant PDAC, NSCLC, melanoma, CRC and thyroid cancer	Activates cell cycle and DNA replication; regulates EMT; suppresses pro-apoptotic genes	[178-181]
ZEB1	Pro	MEKi	KRAS mutant NSCLC	Induces EMT and promotes MAPK-independent cell proliferation	[196]
β-catenin	Pro	MEKi	KRAS mutant CRC	Pro-survival	[176]
β1 integrin	Pro	MEKi	KRAS mutant PDAC	Pro-survival	[200]

Pro, pro-resistance; anti, anti-resistance; ATC, anaplastic thyroid cancer.

Resistance-related mutations of MEK1 favor hydrophobic drug docking pocket to directly interrupt drug interaction (e.g., V211D), or the N-terminal negative regulatory helix (helix A) to upregulate intrinsic MEK1 kinase activity (e.g. P124L). MEK1 P124L mutation was observed in a metastatic tumor biopsy from a drug resistant melanoma patient upon MEKi AZD6244 treatment, while dual inhibition of MEK and BRAF can overcome the acquired resistance [73]. MEK1 V221D mutation, which is localized within the arylamine binding pocket, emerged in a CRC patient following the treatment with MEKi binimetinib and anti-EGFR antibody panitumumab [74]. MEK1 V211D reduces drug affinity of all the available allosteric MEKi, leading to MEK1 hyperactivation, but it is sensitive to an ATP-competitive MEKi MAP855 [74]. MEK2 Q60P mutation was identified as a resistance driver in 3 out of 5 BRAF mutant melanoma patients under dual inhibition of MEK and BRAF, while ERK inhibitors can attenuate the elevated ERK signaling and reverse the refractory phenotype [75]. Collectively, second site mutations are predominant resistance mechanisms that require follow up targeted genomic sequencing for cancer patients under such regimes.

Splice variants

An internal splice variant of BRAF V600E, which lacks exons 2-8, a region encoding RBD, is recurrently observed in treatment refractory melanoma patients to RAF inhibitor (RAFi) at a frequency of 13-30% [76-80]. In comparison to full length protein, the truncated BRAF V600E tends to form homodimer and activates MAPK signaling regardless of RAFi [76, 81], blocking of which can abolish persistent ERK activation and sensitize tumor cells to RAFi. Alternatively, S729 phosphorylation in truncated BRAF V600E enhances the interaction with MEK1/2 to confer RAFi resistance [79], thus MEKi combination may overcome it. In-frame insertion of three residues on αC-β4 loop of BRAF in cancers assembles a large hydrophobic network that involves in R-spine, which impairs BRAF dimerization and MEK inhibitor association [82]. Thus, these splice variants are sensitive to MEK inhibitors albeit resistant to BRAF inhibitors. In-frame deletions of the β3-αC loop of MEK1 can also enhance MEK1 homodimerization, enforce ERK activation and drive resistance to MEK inhibitors [81].

Amplification

High-level amplification of KRAS G12C allele has been observed in 2 out of 17 ctDNA samples from cancer patients who are refractory to adagrasib [71]. Whole exome sequencing of drug resistant tumors revealed the recurrent copy number gain of BRAF V600E at a frequency of 19%-28% as a common resistant mechanism to RAFi in melanoma, which hyperactivates ERK signaling independent of CRAF [80, 83-85]. Similarly, acquired focal BRAF V600E amplification was found in a BRAF mutant CRC patient whose disease progressed to triple inhibition of RAF, EGFR and MEK [86]. Importantly, these non-responder tumor cells are still addicted to KRAS-RAF-MAPK cascade and sensitive to inhibitors targeting the downstream factor ERK.

Off-target resistance

Genetic alterations in vertical RTK-RAS-MAPK pathway

KRAS inhibition resistance. Recent clinical data reveal acquired resistance mechanisms of adagrasib included MET amplification, activating mutations in NRAS (Q61K and G13R), MRAS (Q71R), BRAF (V600E and G596R) and MAP2K1 (K57T/N), oncogenic fusions involving ALK, RET, BRAF, RAF1, and FGFR3, and loss-of-function mutations in NF1 and PTEN [71, 72], all converging on RAS signaling pathway reactivation.

RAF inhibition resistance. Off-target MAPK vertical pathway alterations account for 52% BRAFi resistance in 71 melanoma patients, including NRAS mutations (G12D/R, G13R, and Q61K/R/L) at 18%, KRAS mutations (G12C, G12R, and Q61H) at 7% and MAP2K1 mutations at 3% (K57N and C121S) [80]. In addition, amplifications at EGFR, BRAF V600E, NRAS and KRAS loci, mutations of NRAS, KRAS and MAP2K1, and PTEN loss are often observed in resistant lesions of melanoma and CRC patients with treatment of combined RAF targeted therapies [85-87]. NRAS can enhance BRAF and CRAF interaction to activate ERK signaling in RAFi-refractory cells [83, 88]. Therefore, inhibitors targeting RAF dimers, CRAF or MEK may overcome the resistance.

MEK inhibition resistance. Three independent *in vitro* studies all observed BRAF or KRAS amplification in MEKi resistant CRC and breast cancer cells [89-91]. Since these cells still addict to MAPK signaling, dual inhibition of MEK and BRAF (or ERK) is effective to reverse resistance.

Epigenetic modulators

Writers. HAT1 acetylates newly synthesized free histone H4 in cytoplasm, whose function is controversial in different cancer models [92-95]. HAT1 downregulation is associated with non-responsiveness of BRAFi and MEKi in melanoma patients, and HAT1 depletion drives resistance to BRAFi vemurafenib and dabrafenib by upregulating MAPK via IGF1R in melanoma cells [96]. Histone methyltransferase SMYD3 methylates MAP3K2 at

lysine 260 that blocks PP2A interaction in KRAS mutant PDAC and NSCLC cells, followed by activation of MAPK signaling [97]. Thus, knockout of SMYD3 could potentiate MEKi to impair KRAS-driven pancreatic neoplasia. However, whether HAT1 loss confers BRAFi resistance *in vivo* and whether SMYD3 is a potential therapeutic target to prevent KRASi resistance still need further investigation.

Erasers. Histone deacetylase (HDAC) family members have been extensively studied in therapy resistance [98-101]. Depending on sequence homology and domain structures, HDACs are classified into four classes: I (HDAC1, 2, 3, 8), IIA (HDAC4, 5, 7, 9), IIB (HDAC6, 10), III (sirtuins) and IV (HDAC11). Pan-HDAC inhibitors potentiate the cytotoxic effect of MEKi in human PDAC, uveal melanoma and CRC cells [98-100], and one possible mechanism is that HDAC inhibitors attenuate treatment-induced upregulation of pAKT and YAP1 [100]. Functional studies in PDAC cells reveal that HDAC4 and HDAC6, instead of HDAC1, likely rescue the pro-apoptotic phenotype induced by dual inhibition of HDAC and MEK [99]. However, HDAC class I, HDAC6 or HDAC8 specific inhibitors are less effective for tumor suppression compared to pan-HDAC inhibitors [100], suggesting the functional redundancy among HDACs.

SETD5, a scaffold of HDAC3-G9a co-repressor complex, drives trametinib resistance in PDAC cells by upregulating genes in cytochrome P450 and glutathione metabolism pathways [101]. HDAC5, a scaffold of HDAC3 corepressor complex, is the only HDAC promoting PDAC cells to bypass KRAS dependency [102]. HDAC5-HDAC3 corepressor complex suppresses *Socs3* expression via deacetylation of H3K9 and H3K27, resulting in chemokine reprogramming and followed by TME remodeling. TGFβ provided by tumor associated macrophages (TAMs) is the key driver of the KRAS bypass. In summary, HDACs may play distinct or redundant roles in regulating therapy resistance in a context-dependent manner. Considering that HDAC inhibitors may also affect cells of the TME such as reprogramming TAMs [103], comparing the efficacy of HDAC pan- and specific inhibitors in cancer mouse models *in vivo* is essential.

Readers. Bromo and Extra Terminal Domain (BET) family proteins include BRD2, BRD3, BRD4 and BRDT that recognize acetylated lysine residues for protein-histone association and chromatin remodeling [104]. The tumoricidal activity of BET inhibitors (BETi) varies in solid tumors, while genetic analysis has identified that KRAS G12 mutations are significantly associated with BETi resistance [105]. Synergy of MEKi and BETi is observed in RAS hyperactivated CRC, triple-negative breast cancer (TNBC), multiple myeloma (MM) and PDAC [105, 106], and is explained by suppression of adaptive epigenetic reprogramming [107]. Compared to monotherapy, combinatory treatment widely downregulates mitotic genes, upregulates apoptotic genes, sustains pERK inhibition and causes cell cycle arrest [105]. In TNBC models, BETi JQ1 disrupts enhancer remodeling induced by MEKi trametinib, blocks kinome reprogramming and suppresses tumor growth; in lung cancer models, JQ1 can overcome MEK and TBK1 inhibition resistance via suppressing adaptive activation of YAP1/TAZ and IGF1/IGF1R signaling [108].

Tumor genetic heterogeneity regulates tumor vulnerabilities to dual inhibition of MEK and BET. Homozygous loss of PRC2 components SUZ12 and EED is frequently observed in malignant peripheral nerve sheath tumors (MPNSTs) with NF1 microdeletion [1]. SUZ12 loss amplifies Ras-driven transcription by promoting an epigenetic switch from H3K27me3 to H3K27Ac. JQ1 suppresses PRC2-regulated expression of RAS signature genes and synergizes with MEKi to severely impair MPNST cell growth. In addition, PRC2 dysregulation increases HOXC10 expression in KRAS mutant NSCLC, which coordinates with MEK to enhance the expression of E2F1 targets encoding pre-replication complex proteins [109]. Simultaneous deletion of NF1 and SUZ12 is presented in 24% melanoma and 14% GBM cases and dual inhibition of MEK and BET induces the genetic context-specific replication fork stalling and DNA damage, highlighting the potential therapeutic benefit of the combinatory treatment.

Receptor tyrosine kinases

RAS or RAF oncogenic alterations hyperactivate MAPK signaling regardless of upstream inputs, while pharmacological inhibition of RAS-RAF-MAPK suppresses the negative feedback on RTKs. The preferential upregulation of specific RTKs and their ligands responded to RAS signaling inhibition varies [110-112], resulting in cancer type or subtype-specific combinatory strategies to overcome RAS signaling inhibition resistance.

ErbB receptor family. The ErbB receptor family is composed of four members: ERBB1/EGFR, ERBB2/HER2, ERBB3/HER3 and ERBB4/HER4, which mainly activate MAPK and PI3K-AKT signaling pathways. High EGFR promotes RAFi resistance in CRC, lung, melanoma and thyroid cancer cells by rapid feedback activation of EGFR signaling [113-117], and determines the G12Ci refractory state in KRAS G12C cancers [33, 34, 118-120]. However, EGFR does not seem to be the dominant driver of MEKi resistance

in PDAC. Blocking all activated RTKs, including AXL, PDGFRα, EGFR and HER2, is required to further impair tumor growth with MEKi [121], suggesting functional redundancy of these RTKs in PDAC. In addition, increased autocrine secretion of HER3 ligand Neuregulin-1 and the upregulation of HER3 lead to MAPK reactivation upon RAFi in thyroid cancer cell lines [122]. Targeting HER3-EGFR and HER3-HER2 heterodimers can reverse MEKi non-responsiveness in KRAS mutant NSCLC and CRC cells where MYC activates HER3 [123].

PDGF receptor family. PDGF receptors are engaged by homo- or heterodimers of PDGF-A, -B, -C and -D. PDGFRA and PDGFRB are homo- or heterodimerized depending on bound PDGFs to activate downstream signaling. Upregulation of PDGFRB drives RAFi resistance in a subset of melanoma cell lines which have low RAS and MAPK activities [124]. Another study in PDAC revealed PDGFRA as a driver of MEKi resistance via JAK-STAT3 activation [125].

FGF receptor family. The FGF receptor family comprises FGFR1, 2, 3 and 4 that interact with their ligands to activate signaling pathways including RAS-MAPK. FGFR mediates refractory to G12Ci in PDAC and NSCLC [118]. Depletion of FGFR1 sensitized tumor cells to trametinib in KRAS mutant NSCLC and PDAC, but not CRC [126]. MEKi activates FGFR1 signaling via downregulation of SPRY4 to protect tumor cells from death by elevating AKT survival signal [127].

IGF1 receptor. IGF1R regulates resistance to chemotherapy [128], radiotherapy [129] and EGFR targeted therapy [130]. BRAFi resistant melanoma cells upregulate several RTKs, but only co-targeting IGF1R and MEK exacerbates cell death [131]. Moreover, dual inhibition of IGF1R and KRAS signaling (MEK, mTOR and G12C) markedly enhances anti-tumor effects in CRC and NSCLC cells [132-134]. IGF1R also promotes PI3K activation and KRAS depletion resistance in a group of KRAS mutant CRC cell lines [135].

MET receptor. MET is a proto-oncogene encoding c-Met that binds hepatocyte growth factor (HGF) to activate signaling pathways, including MAPK, PI3K, SRC and STAT [136]. MET amplification is a major resistance mechanism to EGFR inhibition in lung cancer [137]. MET depletion suppresses ERK phosphorylation and overcomes BRAFi resistance in melanoma cells [138]. Moreover, *MET* is recurrently amplified in relapsed tumors after genetic depletion of BRAF in anaplastic thyroid carcinoma models, which elevates MAPK signaling and is essential for resistant cell growth [139].

SHP2/PTPN11. SHP2 is an allosteric protein-tyrosine phosphatase (PTP) that is released from autoinhibition by tyrosine phosphorylation upon cytokine or growth factor stimulation [140]. SHP2 inhibition (SHP2i) blunts GAB1-GRB2-SOS1 complex formation assembled by RTKs, thus interfering with RAS-GTP loading and activation [141]. Increased RAS-GDP occupancy provides a unique vulnerability to KRAS-GDP bound G12Ci, and complete engagement of KRAS G12C by ARS-1620 was observed in 1 hour compared to about 70% basal engagement rate in cells [142]. SHP2i enhances the efficacy of G12Ci, exacerbates MAPK suppression and overcomes G12Ci resistance in various cancers [33, 112, 118, 143-146]. Though RAS/RAF-dependent cancer cells are refractory to SHP2i monotherapy [147], SHP2i blocks EGFR-mediated MAPK rebound and confers sensitivity in BRAFi resistant CRC cells [138, 148], and synergizes with MEKi to suppress PDAC and NSCLC growth [149, 150].

SHOC2. The SHOC2-PP1c holoenzyme is composed of SHOC2 scaffold protein and the catalytic subunit of protein phosphatase 1 (PP1c), an effector of MRAS essential for growth factor-mediated RAS signaling activation [151]. Depletion of SHOC2 blocks RTK-mediated ERK activation and causes synthetic lethal with MEKi in KRAS mutant PDAC and NSCLC cells [111] and with RAFi in BRAF mutant CRC cells [138].

Other kinases

TBK1. TBK1 is an atypical IκB kinase family member that is directly recruited and activated by the RalB/Sec5 effector complex [152]. RalB-TBK1 activation is regulated by RalGEFs with RAS association domain or RAS-independent RalGPS1 [153]. TBK1 promotes cancer cell proliferation via CCL5 and IL-6 mediated STAT3 activation, and inhibition of TBK1 overcomes MEKi resistance in KRAS mutant NSCLC and NRAS mutant melanoma cells [154, 155].

PP2A. PP2A, a serine/threonine phosphatase in the form of trimeric protein complex, is a putative tumor suppressor, inhibition of which facilitates RAS-induced human cell transformation [156]. Depletion of PP2A results in MEK/ERKi resistance in KRAS mutant NSCLC by upregulation of AKT-mTOR signaling and MYC, while PP2A activator DT-061 and MEKi synergistically impair NSCLC growth [157].

CDKs. Targeting KRAS, BRAF or MEK alone mainly exerts cytostatic rather than cytotoxic effects in cancer cells [145, 158-162]. CDK7/12 inhibition attenuates MEKi induced transcriptional and enhancer remodeling, resulting in enhanced cytotoxicity and tumor regression in NSCLC, melanoma, and gastric cancer models [163]. In

addition, CDK4/6 inhibitors exhibit synergistic cytotoxicity with G12Ci across multiple cancer types [33, 34, 118, 143].

SRC family kinase. Src family kinases, which are often overexpressed in cancers, regulate various biological events including gene transcription, cell adhesion, invasion, proliferation, survival and angiogenesis [164]. Src inhibitor dasatinib synergizes with trametinib to induce cell cycle arrest and apoptosis by downregulating TAZ in KRAS-mutant cancer cells, but not in wild type KRAS or EGFR mutant cancer cells [165].

COT/TPL2. COT kinase is encoded by MAP3K8 that activates MAPK signaling independent of RAF. Enforced expression or amplification of *MAP3K8* promotes BRAFi and MEKi resistance in melanoma cells, and MAP3K8 upregulation is observed in a group of disease-progressed melanoma patients on BRAFi or MEKi treatment [166]. As required for resistant melanoma cell survival, MAP3K8 is a potential therapeutic target in this specific setting.

Aurora A kinase. Aurora A belongs to a family of mitotic serine/threonine-protein kinases that regulates cell division [167]. AURKA is upregulated in G12Ci resistant cells, depletion of which augments the antiproliferative effect of ARS-1620 [158]. Aurora A interacts with KRAS G12C-CRAF complex in lung cancer cells, and dual inhibition of G12C and aurora A dissociates the complex resulting in a long-term MAPK signaling inhibition and the prevention of tumor relapse.

Signaling pathways

PI3K/AKT/mTOR. MEKi reactivates PI3K in PDAC models [121, 161]. Though ineffective alone, targeting AKT synergizes with MEKi to suppress PDAC growth [121, 161, 168]. Conversely, forced PI3K and AKT hyperactivation promotes KRAS bypass in PDAC [121, 169]. Clinical data analysis reveals that ~4% BRAFi unresponsive melanoma patients gain nonsynonymous mutations of PTEN, PI3K and/or AKT [80]. In CRC, elevated AKT signaling is associated with tumor intrinsic MEKi resistance [170, 171]. In addition, PI3K inhibitor could shift IC50 of ARS-1620 to over 2-fold lower compared to monotherapy, overcome G12Ci resistance and retard tumor growth in NSCLC [110, 143]. Consistently, mTOR inhibitor enhances tumoricidal effect of adagrasib [33].

JAK-STAT. STATs activate genes related to cell proliferation, differentiation, and apoptosis, providing the basis for regulating tumor response towards drug treatments. A pilot study of 38 NSCLC lines identified that JAK-STAT activation is correlated with resistance to AZD6244 [172]. STAT3 inhibition enhances AZD6244-induced cell apoptosis by downregulation of miR-17 and induction of BIM, while activation of STAT3 elicits AZD6244 resistance. The autocrine activation of STAT3 upon MEKi is mediated by FGFR-PI3K and JAK kinases in KRAS mutant NSCLC but not in CRC or KRAS wild-type NSCLC [173], suggesting the context-dependent engagement of STAT3 feedback loop.

Wnt. Both canonical and non-canonical Wnt pathways regulate cancer stem cells (CSCs), which are often accounted for treatment refractory and tumor recurrence [164]. Elevated WNT5A is associated with disease progression and acquired resistance in a subset of melanoma cases under BRAFi [174]. Mechanistically, WNT5A binds to its receptors RYK and FZD7 to activate PI3K-AKT signaling. WNT5A also promotes KRAS bypass in PDAC cells by stimulating YAP1 nuclear translocation [175]. On the other hand, KRAS mutant CRC cells with high activation of canonical Wnt-β-catenin signaling pathway are refractory to MEKi, inhibition of which overcomes resistance and induces cell apoptosis [176].

Other mechanisms

YAP1. YAP1 acts as a transcriptional coactivator or corepressor downstream of Hippo pathway that plays an oncogenic role in various cancers [177]. YAP1 is negatively correlated with tumor response to BRAFi or RAF/MEK dual inhibition in melanoma patients [178], is recurrently amplified in KRAS-independent relapsed PDAC tumors [179], and is nuclear-localized and activated after KRAS suppression in relapsed lung tumors [180]. YAP/TEAD gene signature is also enriched in dormant human NSCLC cells under dual inhibition of EGFR and MEK [181]. Functionally, *YAP1* is required for resistance to KRAS signaling inhibition and promotes KRAS independent tumor cell growth [179, 180]. Mechanistically, YAP1 cooperates with TEAD2 to activate cell cycle and DNA replication [179], or with the AP-1 transcription factor *FOS* to regulate epithelial-to-mesenchymal transition (EMT) modulators such as *SLUG* [180]. Depletion of YAP1 abolishes *SLUG*-mediated repression of pro-apoptotic BMF, leading to enhanced cell apoptosis in combination with MEK and EGFR inhibition [178, 181].

HSP90. HSP90, a molecular chaperone, usually plays a critical oncogenic role because many of its clients are signaling transducers and cellular stress responders, and oncogenes hijack its functions to prevent aberrantly expressed or mutant oncoproteins from misfolding or degradation. HSP90 inhibition alone attenuates MAPK signaling and cell growth in RAS or RAF mutant MM cells. Dual inhibition of RAS signaling and HSP90 impairs AKT and ERK activity

and amplifies the pro-apoptotic effects [143, 182, 183].

Deubiquitinases. Loss of USP28 deubiquitinase stabilizes and upregulates BRAF protein via FBW7, a substrate recognition subunit in the SCF ubiquitin ligase complex, which results in ERK activation and RAFi resistance in melanoma [184]. USP21 deubiquitinase not only regulates cancer cell stemness but also drives KRAS bypass in PDAC [185, 186]. USP21 can elevate macropinocytosis in KRAS-depleted PDAC cells via deubiquitinating MARK3 to support amino acid homeostasis and activate mTOR signaling pathway.

Metabolic regulators. Pyruvate dehydrogenase kinase 4 (PDK4), a gatekeeper of TCA cycle, is downregulated in MEKi resistant NSCLC cells [187], yet the role of metabolic regulators in driving KRAS targeted therapy resistance are still under exploited.

Salvage pathway activation

Protective autophagy

Autophagy is a fine-tuned catabolic program that degrades and recycles damaged proteins and organelles to protect cells from stress induced apoptosis. MEKi, ERKi and KRAS ablation strongly elevate protective autophagy flux via LKB1-AMPK-ULK1 axis, inhibition of which sensitizes tumor cells to KRAS signaling inhibition in PDAC, melanoma and CRC models [162, 188, 189], implying autophagy as a common surviving mechanism.

Macropinocytosis

Oncogenic KRAS hijacks macropinocytosis to support the high amino acid demand by PDAC cells [190, 191]. KRAS-depleted PDAC cells opt out cell cycle, reduce macropinocytosis and encounter severe metabolic stress. USP21 can partially elevate macropinocytosis by modulating microtubule dynamics, which supports KRAS-independent PDAC cell growth [186].

Oxidative Phosphorylation

KRAS drives PDAC tumorigenesis and maintains malignancy partially through regulating glucose uptake and flux into biosynthesis pathways [192]. KRAS ablation in PDAC leads to decreased glycolysis and cell death, while a small population of CSCs persist depending upon active oxidative phosphorylation (OXPHOS) [162]. OXPHOS inhibitors have been shown to effectivity eliminate the residual surviving cells after KRAS ablation.

BCL-2 family proteins

BCL-2 family proteins regulate apoptosis commitment via induction of mitochondrial outer membrane permeabilization and release of pro-apoptogenic factors such as cytochrome c [193]. BCL-2 proteins are composed of 3 functionally distinct groups: pro-survival proteins including BCL-2 and BCL-XL, pro-apoptotic pore-formers including BAX and BAK, and pro-apoptotic BH3-only proteins including BIM and PUMA. Adaptive downregulation of BIM and PUMA is observed in MEK and PI3K inhibition resistant NSCLC cells, and triple inhibition of BCL-XL/BCL-2, MEK and PI3K induces cell apoptosis [194].

CDKN2A

CDKN2A depletion is recurrently acquired by 7%-28% progressed melanoma patients on treatment with BRAFi or BRAF/MEKi [80, 85], which facilitates tumor cells to overcome cell cycle arrest and subsequent apoptosis.

Phenotypic dynamics

EMT

EMT plays a pivotal role in regulating conventional and targeted therapy resistance in GBM, head and neck squamous cell carcinoma, pancreas, lung, prostate and ovarian cancers [195]. The expression of EMT transcription factors (EMT-TFs) and the mesenchymal phenotype are inversely correlated with therapeutic outcome. Induction of EMT by TGFβ overcomes KRAS-MEK dependency in NSCLC through activation of FGFR1 signaling [127], bypasses KRAS dependency in PDAC via activation of MYC and replication-regulatory genes [102] and drives G12Ci resistance by activation of IGFR-IRS1-PI3K pathway in NSCLC [146]. EMT-TF *SNAIL*, *SLUG* and *ZEB1* are required for KRAS signaling independence in several NSCLC models [180, 196]. Thus, understanding and targeting the EMT program is meaningful for prevention of KRASi resistance.

Reversible cytostatic state

Instead of acute cell death, inhibition of KRAS-MAPK signaling in NSCLC, PDAC and melanoma models sequesters a subgroup of tumor cells in a plastic, quiescent, and CSC-like state that enables some of them to gain adaptive changes and become resistance [158, 159, 162, 197]. Incomplete KRAS suppression only decreases PDAC cell proliferation in 2-D culture or induces quiescence in 3-D culture while these cells are still able to form tumors *in vivo* [197]. Pseudo-time analysis reveals three trajectories representing initial, inhibited, and adapting cell states in G12Ci-treated NSCLC cells, and an elevated quiescent gene signature is observed in most cells [158].

Quiescent G12Ci resistant tumor cells are able to

reactivate KRAS signaling and resume proliferation by increasing the synthesis of active KRAS-GTP or stimulating upstream regulators such as EGFR, SHP2 and AURKA [158]. OXPHOS is highly relied upon by quiescent PDAC CSCs for energetic metabolism [162]. Thus, eliminating quiescent cells by blockage of addicted survival pathways may prolong progression free survival. RAFi induced quiescent melanoma cells express neural crest markers such as NGFR, suggesting a dedifferentiated adaptive state, while inhibition of JUN-FAK-Src axis and BET could efficiently prevent the clonal evolution from quiescence to proliferation [159].

Intratumoral support

Paracrine signals

Tumor cell-cell interaction. Inhibiting KRAS-RAF-MAPK signaling causes secretome remodeling [102, 198, 199]. An elegant design using cell admixture of resistant and sensitive melanoma cells reveals that BRAFi resistant cells only proliferated in tumors with mixed cells, but not in homogenous tumors, suggesting that the paracrine signals from sensitive cells stimulate the outgrowth of resistant cells in response to treatment [198]. BRAFi-induced *Fosl1* downregulation remodels the secretome in sensitive cells that stimulates resistant cells to grow, infiltrate and migrate partially via AKT pathway activation. Growth factors such as IGF1, EGF, ANGPTL7 and PDGFD are mediators of the intercellular interaction.

Tumor-stroma cell interaction. Tumor associated macrophages are not only sufficient but also essential for KRAS-independent tumor growth [102], providing the first evidence that immune cells play a critical role in regulating KRAS targeted therapy resistance. KRAS-depleted PDAC cells recruit macrophages via CCL2/7-CCR2 axis and reprogram them to an immature, M2-like state. Macrophages, in turn, served as the major source of TGFβ in TME that activated SMAD3/4-dependent canonical TGFβ signaling in tumor cells.

Matrix support

An interesting work investigating MEKi response in PDAC organoid reveals that, while cells in the interior layers underwent apoptosis, cells in the outer layer could sustain proliferation [200], The β1 integrin is required for cell-Matrigel interaction, depletion of which triggers cell death in both layers and overcomes MEKi resistance. In addition, interaction with fibroblasts desensitizes melanoma cells to BRAFi, while depletion of fibronectin in tumor cells suppresses NRG and HGF-mediated AKT activation [201]. Moreover, proliferative MEKi resistant melanoma cells tend to colocalize with bundled collagen [202], implying that matrix interaction provides tumor cells with survival signals.

Major challenges of KRAS targeted therapy and future perspectives

Great progresses in the past decade have changed the paradigm from "undruggable RAS" to “RAS is druggable”. Though G12Ci has shown promising results in clinical trials, development of inhibitors targeting other KRAS mutants remains challenging. As we have discussed above, other KRAS inhibitory methods have their own technical uncertainties, and thus only clinical trials will judge their feasibility. Another challenge to target KRAS is to prevent therapy resistance. Given that various molecular mechanisms and factors have been identified, significant knowledge gaps remain to be filled to overcome the common problem for targeted therapy. Here, we discuss six precedent research areas:

(1) While KRAS is frequently mutated in human cancers, the much higher tumor response rate to G12Ci in NSCLC patients versus CRC patients implies that the dependency on KRAS signaling pathway is distinct between various cancer types. It has been shown that activation of RTKs especially EGFR accounts for the reactivation of MAPK signaling pathway and the non-responsiveness of KRASi in CRC [119]. Another possibility of the lack of responsiveness in CRC patients may be due to the mosaicism of wildtype and mutant KRAS in tumors that the continued growth of subclones with wildtype KRAS takes over the tumors under KRASi treatment. Mouse model studies revealed that conditional null alleles of *Apc* and *Trp53* are essential and sufficient to drive colonic tumorigenesis, while KRAS is only required for metastasis [203, 204]. Inactivation of KRAS in autochthonous tumors transiently suppressed CRC growth followed by tumor recurrence. In addition, the gut microbiota is distinct in KRAS mutated and wildtype CRC groups [205], which may regulate tumor response to KRASi as well. Thus, understanding the difference between cancer types in responding to KRASi by comprehensive analysis of accumulated clinical data and employing genetic cancer models are crucial for resistance mechanism dissection and development of cancer type specific treatment approaches.

(2) Given that the genetic heterogeneity affects tumor response to KRAS targeted therapy, our knowledge on the roles of common cancer genetic alternations in regulating therapy resistance remains largely unknown. Early-stage clinical trial of adagrasib suggested that NSCLC patients harboring mutated *STK11* (aka *LKB1*), a recurrent tumor

suppressor in 13% lung cancer patients, may respond better than wildtype ones (KRYSTAL-1 study), though none of *TP53*, *STK11* and *KEAP1* mutational status has a clear association with NSCLC patient response to sotorasib in another clinical trial [37]. *USP21*, an oncogene amplified in 4.5% NSCLC patients and 3.3% PDAC patients (TCGA PanCancer Atlas), has been shown to promote pancreatic cancer cells to bypass the dependency of KRAS [186]. In addition, depletion of tumor suppressor *SMAD4* prevents TGFβ-driven KRASi resistance in pancreatic cancer models [102]. Taken together, comprehensive studies using functional genomics approaches, genetically engineered mouse models and patient-derived xenograft models are needed to understand the function of oncogenes, tumor suppressors and other frequently altered genes, miRNAs and lncRNAs in human cancers upon targeting KRAS and elucidate the molecular mechanisms leading to therapy hypersensitivity or refractoriness. The dependency of KRAS signaling in KRASi refractory tumor cells and how the residual tumor cells in a quiescent state survive, re-proliferate, and eventually form a relapsed tumor need to be determined for effective combination therapy.

(3) KRAS signaling inhibition remodels tumor microenvironment that can be exploited to enhance tumoricidal effect. Dual inhibition of MEK and CDK4/6 induces cell senescence in PDAC and remodels cell secretome that promotes tumor vascularization, which in turn enhances drug delivery and CD8+ T cell infiltration [199]. As expected, chemotherapy drug gemcitabine caused significant tumor shrinkage in combination with MEK and CDK4/6 inhibitors, and blockage of tumor vascularization by VEGFR antibody neutralized the effect. Moreover, PD-1 blockade that leverages the infiltrated CD8+ T cells further suppressed tumor growth relative to dual inhibition of MEK and CDK4/6. Another example is G12Ci treatment in CRC mouse models [34, 206]. KRASi treatment increases immune cell infiltration including CD8+ T cells, macrophages, and dendritic cells, and stimulates IFN and chemokine expression, providing a pro-inflammatory tumor microenvironment suitable for immune therapy combinations. Indeed, though monotherapy shows only moderate tumoricidal effect, PD-1 immune checkpoint blockade synergized with sotorasib or adagrasib to significantly prolong mouse survival. It is critical to delineate the intercellular crosstalk and identify the paracrine signals within tumor milieu under KRAS targeted therapy.

(4) Oncogene addiction is a process in which cancers dependent on one or several genes for maintenance and survival. Accumulated evidence suggests that tumor cells are prone to reactivate the addicted oncogene and corresponding signaling pathways to sustain tumor growth once it gets inhibited, providing the rationale to induce collateral lethality by targeting the vertical signaling pathway. ATP-competitive RAF kinase inhibitors effectively inhibit ERK signaling in BRAF mutant tumor cells, but they activate ERK signaling in tumor cells with wildtype BRAF or mutant RAS [207-212]. The paradoxical effect is linked with conformational changes of RAF kinase domain when bound with inhibitors [209]. Mutant BRAF as monomer activates ERK independent of RAS, but drug-inhibited BRAF transactivates drug-free protomer of CRAF homodimers (CRAF-CRAF) or heterodimers (CRAF-BRAF) to elevate MAPK signaling in a RAS-dependent manner [207-209]. In addition, RAFi can relieve the negative feedback of Spry proteins on RTK-RAS signaling, followed by potentiation of mitogenic signaling from growth factors [213]. Consistently, several growth factors such as EGF, HGF and NRG1 potentiality drive BRAFi resistance in BRAF V600E melanoma models, and combined inhibition of BRAF and MEK nearly completely blocked the rebound of ERK signaling [210, 213]. Moreover, MEKi enhances the cytotoxicity of G12Ci sotorasib and impairs tumor growth [34]. Taken together, vertical pathway activation is the major resistance mechanism to targeted therapy, which has already been extensively studied. The challenge is to identify the key node in KRAS signaling pathway to prevent activation of most surrogate factors and develop therapeutics with acceptable AEs.

(5) Inhibition of KRAS-MEK-ERK cascade causes increased autophagosome formation and mitochondrial stress in PDAC cells [162, 186, 188, 189], indicating the imbalance of metabolic homeostasis upon treatment. KRAS mutant PDAC cells have high demand of amino acids, thus scavenger pathways such as macropinocytosis and autophagy as well as *de novo* amino acid synthesis pathways are upregulated. The elevation of autophagy upon KRAS signaling inhibition compensates the downregulation of macropinocytosis and amino acid synthesis activity to salvage the cells, while autophagy inhibitors are efficient to enhance tumoricidal effect of KRAS signaling inhibitors. KRAS-depleted PDAC cells also switch from glycolysis to OXPHOS to fulfil their energy requirement, inhibition of which impairs cell survival. These studies imply a new direction to prevent resistance by exacerbating the metabolic imbalance induced by KRAS targeted therapy. Except for amino acid and energy homeostasis, whether other metabolites involving in key biological events such as epigenetics and lipid metabolism are

deregulated or contributed to therapy resistance need to be determined by comprehensive metabolomics analysis.

(6) Complete response cases are all alike, every relapsed tumor is relapsed in its own way. The heterogeneity is the elephant in the room that we have to not only explore the possible common adaptive mechanisms of KRAS targeted therapy resistance but also think about how to manage tumor evolution. EMT and tumor associated macrophages are correlated with resistance to targeted therapy, chemotherapy, radiotherapy, and immunotherapy in various cancers, implying that they may be common drivers of therapy resistance. In addition, vertical pathway activation is another major resistance mechanism to targeted therapy as aforementioned above. Further studies need to be performed to dissect the molecular mechanism how EMT reprograms oncogene addiction, how tumor associated macrophages nourish tumor cells under KRAS targeted therapy, and how the vertical pathway is reactivated. On the other hand, given that treatment induced tumor evolution seems inevitable, elimination of the dormant cancer stem cells survived under oncogene targeted therapy may prevent tumor cell evolution and recurrence, which needs to be comprehensively explored in genetically engineered mouse models.

Collectively, there is a growing consensus that combination therapy rather than KRASi monotherapy is essential to achieve favorable clinical benefits. Approaches on different combinations, including vertical pathway inhibition, immune checkpoint blockade and chemotherapeutic regimens, are actively being investigated in clinical trials. It is noted that the various targeting KRAS methods have distinct working mechanisms, thus unique combination strategies may be required to prevent resistance. Alternatively, KRASi-induced reprogramming of tumor cell intrinsic dependency and TME remodeling provides opportunities to exploit the treatment-dependent cancer vulnerabilities. In summary, the central goal in the coming decade is to enhance tumoricidal effect of KRAS targeted therapy by leveraging the advantages of different therapeutics and finding the optimal combinatory approaches.

Abbreviations

PDAC: pancreatic ductal adenocarcinoma; CRC: colorectal cancer; NSCLC: non-small cell lung cancer; GEM: genetically engineered mouse; GEFs: guanine nucleotide exchange-factors; GAPs: GTPase-activating proteins; RTKs: receptor tyrosine kinases; GPCRs: G-protein coupled receptors; TCGA: Cancer Genome Atlas; TME: tumor microenvironment; AEs: adverse events; G12Ci: KRAS G12C inhibitors; ORR: objective response rate; PFS: progression-free survival; SOC: standard of care; KRASi: KRAS inhibitors; TILs: tumor-infiltrated lymphocytes; TCR: T cell receptor; PBL: peripheral blood lymphocytes; mTCR: murine TCR; RBD: RAS-binding domain; MEKi: MEK inhibitors; RAFi: RAF inhibitor; HDAC: histone deacetylase; BET: Bromo and Extra Terminal Domain; BETi: BET inhibitors; TNBC: triple-negative breast cancer; MM: multiple myeloma; MPNSTs: malignant peripheral nerve sheath tumors; HGF: hepatocyte growth factor; PTP: protein-tyrosine phosphatase; SHP2i: SHP2 inhibition; PP1c: catalytic subunit of protein phosphatase 1; CSCs: cancer stem cells; EMT: epithelial-to-mesenchymal transition; PDK4: pyruvate dehydrogenase kinase 4; OXPHOS: oxidative phosphorylation; EMT-TFs: EMT transcription factors; Ph: Phase; mPDAC: mNSCLC and mCRC: metastatic PDAC: NSCLC and CRC; Pro: pro-resistance; anti: anti-resistance; ATC: anaplastic thyroid cancer.

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Competing Interests

The authors have declared that no competing interest exists.

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