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Neomorphic mutations create therapeutic challenges in cancer

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Abstract

Oncogenesis is a pathologic process driven by genomic aberrations, including changes in nucleotide sequences. The majority of these mutational events fall into two broad categories: inactivation of tumor suppressor genes (hypomorph, antimorph or amorph) or activation of oncogenes (hypermorph). The recent surge in genome sequence data and functional genomics research has ushered in the discovery of aberrations in a third category: gain-of-novel-function mutation (neomorph). These neomorphic mutations, which can be found in both tumor suppressor genes and oncogenes, produce proteins with entirely different functions from their respective wild-type proteins and the other morphs. The unanticipated phenotypic outcomes elicited by neomorphic mutations imply that tumors with the neomorphic mutations may not respond to therapies designed to target the wild-type protein. Therefore, understanding the functional activities of each genomic aberration to be targeted is crucial to devising effective treatment strategies that will benefit specific cancer patients.

Introduction

Cancer development involves a complex, yet specific series of genetic events that must be defined in order to truly advance cancer biology and develop novel therapies. The current explosion of genomic data presents an unprecedented opportunity to identify genomic aberrations as molecular targets or markers for cancer therapies. However, the translation of data into clinical practice is not straightforward. Only a small fraction of mutations in a tumor cell are functional drivers that might be paired with therapeutic options. The remainder are neutral passengers that do not confer growth advantages and/or are not targetable in the clinic. Over 2 million different coding region mutations have been identified across patients with only a small fraction of these being characterized functionally. Broadly, the majority of characterized drivers at the nucleotide level are either (1) hypomorph,

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antimorphic or amorphic resulting in decreased or complete loss of protein activity or expression, or (2) hypermorphic, resulting in enhanced activity or expression. Accumulating reports now support the prevalence of a third important category of driver mutations in cancer: neomorph.

Neomorph was first described in *Drosophila* by the Nobel Prize winning geneticist, H.J. Muller in 1932. Neomorph represents a “change in the nature of the gene at the original locus, giving an effect not produced, or at least not produced to an appreciable extent, by the original normal gene...giving a gene that produces a new effect, foreign to the original gene, and not competing with the latter.”¹ Essentially, unlike other morphs that result in either gain or loss of the activity mediated by the wild-type (WT) protein, neomorphs carry new and unanticipated functions through inducing unexpected alterations of cellular signaling pathways and networks. The neomorphic effects can alter the response to therapeutic targeting, even potentially converting a drug that inhibits the functional consequences of the WT protein into a drug that increases the outcomes driven by the neomorphic mutation. It is important the note that hypermorphic mutations by increasing the duration and magnitude of signaling or by being insensitive to feedback regulatory events can rewire intracellular networks in unexpected manners. This pseudo-neomorphic activity may contribute to unexpected lack of response to drugs targeted pathways normally activated by the parent WT molecule.

This review explores the increasingly appreciated role of neomorphic mutations in cancer promotion. These neomorphic mutations can be recurrent or infrequent mutations in cancer genes and are primarily discovered by experimental characterization. However, suggestions of neomorphic activity can frequently be gleaned by determining associations of specific mutations with unexpected transcriptional profiles or proteomic readouts in patient samples or cell lines. We also discuss the mechanisms engendered by an example set of neomorphic activities and the potential strategies to target tumors bearing these neomorphic mutations.

IDH1 and IDH2

Isocitrate dehydrogenases (IDHs) are key enzymes of the Krebs cycle, catalyzing the oxidative decarboxylation of isocitrate to α -ketoglutarate (α -KG) with concomitant reduction of NAD⁺ or NADP⁺ to NADH or NADPH respectively.^{2–4} There are two NADP(+)-dependent IDH isoforms in eukaryotic cells. IDH1 localizes to the cytoplasm while IDH2 is in the mitochondria. α -KG represents a key substrate in the Krebs cycle and also provides building blocks for the synthesis of fatty acids and amino acids. The products of IDH isoforms regulate dioxygenase-mediated hydroxylation reactions. The NADPH generated during the reaction by IDH1 or IDH2 fuels lipid biosynthesis and protects cells against oxidative damage.^{4, 5} Under hypoxic conditions, the reaction catalyzed by IDH1/2 is reversed and α -KG is converted to isocitrate which can in turn be converted to acetyl-CoA to support lipid metabolism.^{6, 7}

To date, *IDH1* mutations have been identified in up to 82% of grade II-III gliomas and secondary glioblastomas in multiple cohorts.^{8–11} *IDH1* mutations have also been detected in other cancers including acute myeloid leukemia (AML; 10%) and prostate cancer (1–3%).^{9, 10, 12, 13} The frequency of *IDH2* mutations is much lower than that of *IDH1* in glioma (0–

7%^{9–11}) but is slightly higher in AML (15%^{10, 14, 15}). The most common *IDH1* mutation occurs at R132, which is located in the enzyme active site and forms hydrogen bonds with the α - and β -carboxylic groups of isocitrate.¹⁶ Initial studies suggested that *IDH1* R132H was an antimorph (dominant negative mutant) by inhibiting the activity of WT *IDH1* because R132H led to reduction in the affinity for isocitrate and α -KG production.¹⁷ Interestingly, however, a study by Dang et al. suggested that R132H is a neomorph that alters binding affinity for substrates.¹⁸ The authors first analyzed metabolites from R132H-expressing glioblastoma cells using liquid chromatography-electrospray ionization-mass spectrometry. The cells displayed elevated level of R(-)-2-hydroxyglutarate (2-HG), a molecule structurally similar to α -KG that thereby functions as a competitive inhibitor of α -KG-dependent enzymes.¹⁸ Analysis of the crystal structure of *IDH1* suggested that R132H caused a shift in the active site resulting in decreased binding affinity of the enzyme for isocitrate and increased affinity for NADPH.¹⁸ Together, this promoted NADPH-dependent reduction of α -KG to 2-HG (Figure 1).

The neomorphic function of *IDH1* R132H was further extended to the mitochondrial homolog *IDH2* by Ward and colleagues, who observed elevated 2-HG in a number of AML patients who did not have *IDH1* mutations.¹⁴ Some of the tumors that lacked *IDH1* mutation carried *IDH2* R172K or R140Q mutation.^{9, 11, 14} *IDH2* R172K is an analogue of *IDH1* R132H and is the most frequent *IDH2* hotspot mutation. These studies suggested that the production of the oncometabolite 2-HG may be a common consequence of the *IDH1* and *IDH2* hotspot mutations seen in cancers. Due to the functional redundancy of *IDH1* and *IDH2*, mutations of the two gene were mutually exclusive.¹⁰ All reported *IDH1* or *IDH2* mutations are heterozygous, therefore the conversion of isocitrate into α -KG can still be fulfilled by the retained WT *IDH1* or *IDH2* allele. Intriguingly, these *IDH1* or *IDH2* neomorphic mutations tended to co-occur with *TP53* mutation but demonstrate mutual exclusivity with aberrations of *PTEN*, *EGFR*, *CDKN2A*, or *CDKN2B*.⁹ The interactions of the *IDH1* or *IDH2* neomorph with the function of these genes remain to be investigated.

Several mechanisms underlying the oncogenic activities of the *IDH1* and *IDH2* neomorphs have been proposed. Specifically, 2-HG competes with α -KG for binding to the active sites of dioxygenases that use α -KG as a co-substrate for catalysis. Recent studies have identified at least 3 classes of α -KG-dependent dioxygenases that are targeted by 2-HG (Figure 1).^{19, 20} The first class includes prolyl hydroxylases (PHDs) which promote the degradation of HIF-1 α , an essential transcription factor in response to hypoxia.²¹ *IDH1* R132H upregulates HIF-1 α and increases HIF-1 α -inducible genes including VEGF, presumably by competing with α -KG and preventing PHD-mediated degradation of HIF-1 α .^{17, 22} The second class involves jumonji C domain-containing histone demethylases (JHDMs) which target methylated lysine residues on histones thereby regulating gene expression. 2-HG competitively inhibits JHDMs including JMJD2A.^{23–25} The *IDH1/2* neomorphs therefore inhibit histone demethylation, which in turn blocks the differentiation of non-transformed lineage-specific progenitor cells.²⁴ Thirdly, *IDH1/2* neomorphs inhibit TET2, a DNA demethylase enzyme through catalyzing 5-methylcytosine hydroxylation, thereby leading to global hypermethylation and epigenetic alterations.^{26, 27} The latter two mechanisms have broad implications for gene regulation.

The identification of 2-HG as a point of convergence for IDH1 and IDH2 neomorphic effects allows for the potential use of 2-HG as a clinical biomarker of *IDH1/2* mutations. In fact, Ward et al. used 2-HG to screen AML patients for *IDH1/2* mutations leading to the discovery of the function of *IDH2* R140Q and R172K,¹⁴ demonstrating the potential of 2-HG measurement for prediction of *IDH1/2* mutation status. Moreover, *in vivo* detection of 2-HG in body fluids could potentially be used as a surrogate for more invasive and less frequently obtained histopathologic assessments. Using proton magnetic resonance spectroscopy, 2-HG levels were detected in glioma and AML patients and correlated with the presence of *IDH1/2* mutations.^{15, 28–31} *IDH1/2* mutations also remain exciting therapeutic targets because the altered active sites are potentially selectively targetable by small molecule inhibitors, the mutations are stable even as the cancer progresses and *IDH1/2* neomorphic mutations appear relatively early in cancer development.^{9, 32} The overall objectives would be to either inhibit the mutant enzymes to prevent 2-HG production, restore normal IDH1/2 function, deplete 2-HG and/or replace α -KG. Multiple preclinical studies have shown that *IDH1/2* mutant-specific inhibitors could reverse the activity of the mutants and 2-HG accumulation.^{33–38} Remarkably, the growth inhibitory effect of the inhibitor was specific to neomorph-bearing cells but not to cells with WT *IDH1/2*.³⁶ Indeed, some of these inhibitors have entered clinical trials and are showing encouraging results in patients with AML.³⁹ Inhibitors that target dioxygenases have also been developed.⁴⁰ Targeting the downstream targets of 2-HG presents may provide another opportunity. For example, blocking VEGF function in glioma patients with *IDH1* neomorphic mutations resulted in improved progression free survival and overall survival.⁴¹

PIK3CA

Phosphatidylinositol-3-kinases (PI3Ks) are lipid kinases that catalyze the phosphorylation of phosphoinositides at the 3'-hydroxyl group generating second messengers that activate AKT and other pleckstrin homology domain-containing proteins to regulate diverse cellular activities.^{42–45} Among the three classes of PI3Ks, class I, particularly the 1A subclass, is most widely studied for its role in oncogenesis. Class 1A PI3Ks are obligate heterodimers composed of a catalytic subunit (p110 α , p110 β , or p110 δ) and a regulatory subunit p85 (p85 α , p85 β , p85 γ and 4 additional splice variants). p85 stabilizes and inhibits p110 activity under quiescent conditions but the inhibition is relieved upon activation of growth factor signaling.^{46–49}

PIK3CA, which encodes the p110 α catalytic subunit, is frequently aberrant in human cancers with more than 5000 reported mutations.¹⁰ *PIK3CA* is within the 4th most amplified region across 11 cancer types⁵⁰ and is the 2nd most frequently mutated gene and most commonly mutationally activated gene across 12 cancer types in the Cancer Genome Atlas (TCGA).⁵¹ *PIK3CA* was shown to be amplified in 1999,⁵² which was followed by demonstration of mutations in glioblastoma, and colon gastric, breast and lung cancers in 2004.⁵³ *PIK3CA* mutations have subsequently been identified in other neoplasms including cancers of the endometrium, ovary, liver, stomach, and brain.^{54, 55} Although somatic mutations are scattered throughout *PIK3CA*, the majority of mutations cluster in three hotspots: E542 and E545 in the helical domain encoded by exon 9 and H1047 on exon 20 which encodes the kinase domain. E542 and E545 are commonly substituted with lysine

while H1047 is often changed to arginine. These mutants are thought to be hypermorphs due to altered interaction with p85 or conformational changes of the activation loop, leading to constitutive low level activity of the E542 and E545 variants and increased sensitivity of the H1047 mutant to ligand activation and thus increased tumorigenicity.^{56–59}

Intriguingly, a recent study by Hao et al. demonstrated a neomorphic activity of the E545K mutant.⁶⁰ Using mass spectrometry to identify interacting partners of the pulled-down wild-type p110 α and E545K mutant, E545K was found to associate with insulin receptor substrate 1 (IRS1) independent of its canonical partner p85 (Figure 2). Indeed, p85 might compete with IRS1 for binding to E545K mutant p110 α . Instead of solely relying on bound p85 for stabilization and membrane localization, the interaction with IRS1 stabilized E545K mutant p110 α and enhanced the membrane association of E545K p110 α , resulting in activation of this p110 α neomorph and the PI3K pathway. The experiments were performed in serum-depleted conditions, suggesting a growth factor independent activation of neomorph signaling. Interestingly, other hot-spot *PIK3CA* mutations in the helical domain, including E542K, E545A, E545G, and Q546K, also gained interaction with IRS1 suggesting a generalizable activity. In stark contrast, this neomorphic effect was absent with the hot-spot mutations in the kinase domain (H1047R, H1047L, and G1049R).⁶⁰ Two other independent studies showed that cell lines with E542K or E545K had lower phosphorylated AKT levels compared to those with kinase domain mutation or PTEN protein loss.^{61, 62} Instead, E542K or E545K induced robust activation of PDK1, which could be a consequence of enhanced membrane association of PDK1 driven by E542K or E545K.⁶¹ PDK1 promoted anchorage independent growth of breast cancer cells through the activity of downstream substrate SGK3.⁶¹ Whether this induction of PDK1 signaling is linked to the neomorphic p110 α -IRS1 interaction warrants investigation. Further, some relatively rare *PIK3CA* mutations (so-called *PIK3CA* tail mutations) increased the phosphorylation of MEK1/2 (upstream kinase of ERK1/2),⁶³ suggesting potential neomorphic activities of these mutations although this remains to be verified with future mechanistic studies.

As these recurrent *PIK3CA* mutations are present in a large number of cancer patients, understanding the neomorphic functions of the helical domain mutants is required to capitalize on additional and perhaps more facile and effective targets for cancer therapy that are not associated with the H1047R mutation. Hao et al. alluded to these possibilities with a hydrocarbon-stapled mutant peptide that disrupted the neomorphic interaction between IRS-1 and the E545K mutant.⁶⁰ Strikingly, the peptide inhibited AKT phosphorylation and xenograft tumor growth of cancer cells with the neomorph.⁶⁰ This exciting result points to a potential therapeutic approach allowing cancer patients carrying the neomorph to be treated with a small molecule drug that inhibits this interaction and that may have a high therapeutic index as they do not inhibit WT *PIK3CA*. Small molecule inhibitors (e.g. Akt/PDK dual inhibitors, PI3K/mTOR dual inhibitors, and MAPK inhibitors) targeting the downstream signaling pathways activated by the *PIK3CA* neomorphs could also represent potential therapeutic approaches.

PIK3R1

PIK3R1 is the 11th most commonly mutated gene across cancer lineages in TCGA.¹⁰ Somatic mutations in *PIK3R1* are particularly prevalent in endometrial cancer (20–34%),^{55, 64} metastatic prostate cancer (11.5%),¹⁰ glioblastoma (11%),⁶⁵ and colon cancer (4.5–10%).^{10, 66} *PIK3R1* encodes p85 α , which binds and regulates the p110 catalytic subunit as described above. To be more specific, the nSH2 and iSH2 domains of p85 α bind to the helical and C2 domain of p110 respectively, to prevent p110 from thermal degradation and to inhibit its catalytic activity.^{67, 68} Approximately two-thirds of *PIK3R1* mutations are located in the iSH2 domain of p85 α . Driver mutations within this domain, including E439del, KS459delN, DKRMNS560del, D560Y, N564D, Q572*, R574fs, T576del, and QYL579L, are hypomorphs that have lost the ability to inhibit the catalytic activity of p110, thereby allowing constitutive, ligand-independent activation of the PI3K pathway.⁶⁹ Other mutations at the N-terminal domains, such as the BH domain, which does not interact with p110, activate PI3K signaling through mechanisms independent of p110. p110 α -unbound WT p85 α forms homodimers via intermolecular interactions mediated by the SH3 and BH domains.^{55, 70, 71} The p85 α homodimer binds to and stabilizes PTEN, resulting in inhibition of the PI3K pathway. Cancer patient-derived *PIK3R1* mutations in this region (e.g. I133N, E160*, and I177N) are hypomorphs or antimorphs that disrupt the formation of p85 α homodimers leading to PTEN destabilization and subsequent PI3K signaling activation.⁷¹

The hypomorphic or antimorphic mutations described above exclusively target PI3K pathway activation albeit through altering functions of different targets: p110 and PTEN. We have recently discovered a subset of *PIK3R1* truncation mutations that activate MAPK pathways independent of p110.⁷² This group of neomorphic mutations, which clusters around the nSH2 domain and lacks intact SH2 domains, is found in cancers of the breast, colon, uterus, and ovary. Among these mutations, R348* is one of the most frequent recurrent *PIK3R1* mutations (about 10% of all *PIK3R1* mutations)¹⁰ and was first described as a driver mutation because of its ability to transform the interleukin 3 (IL3)-dependent murine myeloid BaF3 cells into IL3-independence.⁵⁵ Subsequently, screening of the R348*-expressing cells against a library of “informer” therapeutic compounds targeting several major signaling pathways revealed increased sensitivity to AKT inhibition and unexpectedly to multiple inhibitors of MEK and JNK.⁷² Pathway activation profiling by reverse-phase protein arrays revealed that R348* and the neighboring mutation, L370fs, increased phosphorylation of members of the ERK and JNK pathways in addition to members of the PI3K pathway.⁷² In contrast, WT p85 α or other mutants (E160*, DKRMNS560del, R574fs, and T576del) had no effect on MAPK pathway activation or sensitivity to MAPK inhibitors. The activation of MAPK signaling was independent of the canonical role of p85 α in regulating PI3K or of its ability to regulate PTEN.⁷² This neomorphic role correlates with the production of truncated proteins that lack the p110-interacting SH2 domains. The activation of ERK occurs in the cytosol and involves upstream kinases including MEK1, MEK2 and BRAf, whereas the activation of JNK is a nuclear event mediated by the kinases MKK7 and MLK3. The intact BH domain and the absence of SH2 domains in the p85 α neomorphs appear to provide the key to nuclear localization. The BH domain of the truncation protein interacts with Rho GTPases family members Cdc42 and Rac1, which facilitates translocation of the mutant from the cytosol to the nucleus (Figure 3).⁷² In the

nucleus, the truncated p85 molecule acts as a scaffold for the tethering of signaling molecules along the JNK pathway, thereby promoting JNK activation. The activities of Cdc42 and Rac1 are also required for the activation of the ERK and JNK pathways.

Co-activation of both the MAPK and PI3K pathways can act as a compensatory survival mechanism to bypass the inhibition of either the MAPK or PI3K pathway.^{73, 74} Thus combinatorial treatment against both the MAPK and PI3K pathways may be required for optimal patient benefit in the context of the p85 neomorph.⁷⁵ Indeed, dual blockade of the MAPK and PI3K pathways demonstrated enhanced anti-tumor effects in cancer cell lines and preclinical mouse models with *KRAS* mutation or co-mutations in both MAPK and PI3K pathways.^{76, 77} This approach is currently being explored in ongoing clinical trials.^{75, 78, 79} Whether *PIK3RI* neomorphic mutations could be potential biomarkers predictive of clinical outcomes and response to pathway inhibitors awaits investigation.

PTEN

PTEN, a lipid and protein phosphatase, is one of the most frequently aberrant bona-fide tumor suppressors across a wide spectrum of tumor types. *PTEN* is the 9th most significantly deleted gene⁵⁰ and 3rd most frequently mutated gene across cancer lineages⁵¹ in TCGA. WT PTEN is primarily a phosphoinositide 3-phosphatase with the key target being the PI3K pathway wherein PTEN opposes the action of PI3K by dephosphorylating the signaling lipids PIP(3,4,5)P3 to PI(4,5)P2 and PI(3,4)P2 to PI(4)P (Figure 4).⁸⁰ In addition, PTEN possesses protein phosphatase activity that inhibits cancer cell invasion, however, whether this is a critical function of PTEN suppressor activity remains controversial.^{81–83} The tumor-suppressing activities of PTEN also extend to the nucleus, where PTEN, independent of its catalytic activity, induces p53-independent apoptosis and maintains genomic stability through preserving heterochromatin structure.^{84, 85} Hypomorphic mutants that lead to the inactivation of these diverse functions of PTEN are frequent in cancers.^{82, 84–86} Mutations leading to reduced PTEN expression have been reported. The C-terminus of PTEN regulates PTEN stability and cancer patient-derived truncated mutants that lack the C-terminus were unstable resulting in loss of PTEN expression.^{87, 88} WT PTEN activities are also suppressed by antimorphic PTEN mutants that bind to and inhibit the WT PTEN protein.⁸⁷

Intriguingly, a recent study has demonstrated neomorphic activity of a *PTEN* mutation detected in a prostate cancer patient.⁸⁹ This neomorphic mutation, A126G, is not frequently observed and was only identified in one breast cancer case across all TCGA samples.¹⁰ The amino acid residue A126 is in close proximity to the catalytic C124 and R130 residues. These residues together constitute the phosphatase signature motif (referred to as the catalytic “P-loop”) at the bottom of the active site pocket. Interestingly, A126G results in an amino acid sequence identical to the family of voltage-sensing phosphatases, which are typical phosphoinositide 5-phosphatases. In silico modeling of the substrate-protein complex using available crystal structures of PTEN predicted a change in enzymatic specificity of A126G and this was confirmed by *in vivo* activity assays.⁸⁹ In contrast to WT PTEN which dephosphorylates 3' phosphoinositides, the mutant demonstrated a shift in specificity toward 5' phosphoinositides, resulting in the production and accumulation of PI(3,4)P2 from

PI(3,4,5)P3 (Figure 4). Consistent with a previous independent study⁸⁶, the mutant dephosphorylated PI(3,4,5)P3 at a reduced rate relative to WT PTEN. Moreover, the neomorphic activity is unique to A126G as mutation of A126 to several other amino acid residues (A126P, A126V and A126S) revealed no neomorphic effect. PTEN-null prostate cancer cells PC-3 transfected with A126G displayed increased AKT and S6 phosphorylation as well as cell migration compared with those expressing WT PTEN.⁸⁹ These consequences could be due to a decrease in catalysis of PI(3,4,5)P3 compared with WT PTEN. Alternatively, the neomorphic product, PI(3,4)P2, can also bind to and activate a subset of PH domains, including that of AKT.^{90, 91}

Accumulating evidence has shown that tumors with aberrations of PTEN including PTEN protein loss or *PTEN* mutation are more susceptible to treatment with PI3K pathway inhibitors.^{29, 92, 93} Further, PTEN aberrations can dictate the dependency on individual Class IA PI3K isoforms.^{29, 94} It would therefore be instructive to examine whether A126G-bearing cells are sensitive to PI3K pathway inhibition and particularly to any isoform-specific inhibitors of PI3K. Along this line, A126G-induced migration of PC-3 cells was abrogated by a PI3K α inhibitor (BYL-719) and a PI3K β inhibitor (AZD6482).⁸⁹ A broader array of PI3K pathway inhibitors and cell models will bring us closer to an answer to this question and to potential clinical applications.

TP53

TP53 encodes p53, which has been extensively studied for its role in tumorigenesis. *TP53* is the most frequently mutated gene in human cancers (with the three most frequent hotspot mutations at R175, R248 and R273).^{50, 95} p53 was initially identified in complex with simian virus 40 large T-antigen and was increased in tumors relative to normal cells.^{96–99} Subsequently, many studies described the tumorigenic properties of p53,^{100, 101} only later to realize that these experiments had actually used mutated p53 isolated from tumor cells.^{102–104} It was demonstrated in 1989 that greater than half of colorectal tumors had loss of heterozygosity at the *TP53* locus.¹⁰⁵ Additional experiments proved that WT p53 overexpression was sufficient to suppress tumorigenesis and thereafter *TP53* was considered as a tumor suppressor gene.^{104, 106–108}

Cellular stress activates and stabilizes p53, which is present at very low levels in non-stressed normal cells. Activated p53 functions as a sequence specific tetrameric transcription factor that induces cell cycle arrest, apoptosis, senescence, DNA repair, and alters metabolism.^{109–112} As with most tumor suppressors, loss of p53 expression can result from frameshift or nonsense mutations leading to truncated or deleted proteins.¹¹³ However, most *TP53* mutations in cancers are missense mutations resulting in single-base pair changes that still allow for the translation of full-length protein. These missense mutations, including the vast majority of hotspot mutations, generally cluster within the highly conserved DNA binding domain.^{10, 114, 115} Some of these missense mutations are antimorphs impairing the DNA-binding activity of the WT p53 by binding to WT protein.,^{116, 117} The idea that some *TP53* mutations are not simply an antimorphs was suggested by Dittmer et al. in 1993 when p53 mutants (including R175H and R273H) were expressed in p53-null cells, resulting in a

more aggressive tumorigenic phenotype compared with parental cells.¹¹⁸ Since then, evidence supporting *TP53* mutations as neomorphs continues to accumulate^{108, 119}.

p53 neomorphs can interact with a number of transcription factors and modulate their transcriptional output. These transcription factors include the p53-related proteins p63 and p73. Despite a remarkable structural similarity among the three family members (about 60% identity in the DNA-binding domain), whether p63 or p73 act as tumor suppressor genes or as oncogenes remains controversial.¹²⁰ Remarkably, while p73 and p63 can regulate p53 target genes, p63 and p73 also have a number of unique target genes that are distinct from p53.^{121, 122} p63 and p73 form heterotetramers through the oligomerization domain but WT p53 does not interact with p63 or p73.^{123, 124} However, neomorphic mutations of p53 cause conformational changes in the p53 protein that allow interaction of mutant p53 with p63 and p73.^{125, 126} This physical interaction restrains p63 and p73 from binding to DNA and thereby blocks the activation of p63/p73 target genes leading to chemoresistance, migration, invasion, and metastasis (Figure 5a).^{125, 127–131} Neomorphic p53 mutants may also selectively recruit other cofactors into the transcriptional machinery resulting in aberrant gene regulation. WT p53 and p53 neomorphs (R175H, R273H and D281G) bind the transcription factor NF-Y but different cofactors are recruited (Figure 5b).¹³² WT p53 recruits cofactor histone deacetylase HDAC1, whereas p53 neomorphs recruit histone acetyltransferase p300. The p53 neomorph/NF-Y/p300 complexes lead to aberrant transcription of NF-Y target genes including the cell cycle control genes *CCNA*, *CCNB*, *CDK1*, and *CDC25C*.¹³² In contrast, the recruitment of HDAC1 by WT p53 represses transcription of these cell cycle genes.¹³³ Thus, analogous to IDH1/2, WT and neomorphic mutant p53 can exert diametrically opposite effects. Further, p53 neomorphs can interact with a number of proteins that are not transcription factors. For example, the nuclease Mre11 interacts with two hotspot p53 neomorphs (R248W and R273H) but not WT p53 (Figure 5c).¹³⁴ Mre11 is a component within the Mre11-Rad50-NBS1 (MRN) complex that acts as a sensor of DNA double-strand breaks (DSB) and is responsible for the activation of ataxia-telangiectasia mutated (ATM) kinase involved in DSB repair and other cellular functions.^{135, 136} The physical interaction between the p53 neomorphs and Mre11 impairs the recruitment of MRN to DNA damages sites and decreases ATM activation, thereby contributing to genetic instability.¹³⁴

WT p53 is maintained at low cellular levels through a negative feedback loop involving the E3 ubiquitin ligase Mdm2, however, p53 mutants are stabilized and are expressed at high levels in tumor cells.¹³⁷ R175H binds to heat shock protein 70 (HSP70) or HSP90, which inhibits the ability of Mdm2 to promote ubiquitination of the p53 mutant.^{138, 139} Therefore, one therapeutic strategy has been to destabilize the p53 neomorphs by either directly targeting HSP proteins or indirectly inhibiting upstream regulators of HSPs such as histone deacetylases (HDACs), which positively regulate HSP.¹⁴⁰ SAHA, a FDA-approved HDAC inhibitor, selectively downregulates p53 neomorphs (R175H and R273H) but not WT p53.¹⁴¹ Inhibition of HSP90 through inhibiting HDAC6 releases HSP90 from p53 neomorphs, which then become susceptible to Mdm2-mediated degradation.¹⁴¹ HDAC inhibitors may also suppress expression of p53 neomorphs by decreasing the expression of the *TP53* transcriptional activator HoxA5.^{141, 142} Other approaches that lead to the destabilization of p53 neomorphs may also provide effective antitumor strategies. For

example, p53 neomorphs including R175H are positively regulated by acetylation.¹⁴³ Deacetylation of the mutants through activating deacetylase sirtuins (SIRT6) reduces p53 neomorph levels and inhibits proliferation of breast cancer cell lines carrying *TP53* neomorphs.¹⁴⁴ Another elegant therapeutic strategy utilizes the mutant p53 structure to develop peptides or small molecules that restore WT p53 protein folding and activities.¹⁴⁵ Alternatively, given the broad range of effects resulting from interaction of p53 neomorphs with p63 and p73, efforts to disrupt this interaction include a screen that identified RETRA (reactivation of transcriptional reporter activity) as being able to block interactions. RETRA disrupted the p53 neomorph:p73 complex reconstituting p73-mediated transcription, however it is unclear whether eliminating the neomorphic effect of the p53:p73 complex will be equivalent to restoring WT p53 function.¹⁴⁶ A number of small molecule drugs including ZMC1, PRIMA1 or APREA-246 and COT12 have been proposed to allow folding of unfolded p53 mutant proteins thus allowing reconstitution of p53 function including reconstitution of DNA binding and transcriptional activity.¹⁴⁷ Understanding the interaction between the p53 neomorphs with their binding proteins will shed light on more approaches that target the activity of the mutants.

MYOD1

Myogenic Differentiation 1 (MYOD1) and c-Myc are both members of the large basic helix-loop-helix (bHLH) family of transcription factors, in which a 12–15 amino acid segment containing basic residues comprises a DNA-binding domain adjacent to a HLH domain that mediates dimerization to enhance DNA binding.^{148–150} Nearly 25 years ago, Van Antwerp et al. mutated amino acids in the basic domain of MYOD1 to analogous amino acids in c-Myc to identify residues mediating DNA-binding specificity.¹⁵¹ Electrophoretic mobility-shift assays revealed that three of six mutants with a single amino acid mutated were neutral, having no impact on DNA binding activity.¹⁵¹ One mutant was hypomorphic with reduced capacity for DNA binding and most interestingly, one mutant (L122R) gained an unexpected ability to bind to the c-Myc recognition site (5'-CACGTG-3') but was still able to bind to the consensus MYOD1 site (5'-CACCTG-3'),¹⁵¹ suggesting that L122 of MYOD1 is critical to maintaining DNA specificity. Indeed, L122 is conserved in all myogenic bHLH transcription factors and is analogous to arginine in the MYC family, providing an explanation for the neomorphic activity of L122R. Remarkably, the mutant also competed with WT MYOD1 for the DNA binding site without leading to target gene transactivation.¹⁵¹ L122R may therefore serve as both an antimorph and a neomorph.

Because MYOD1 is a key player in muscle differentiation,¹⁵² it was postulated that L122R would lead to inhibition of differentiation and promotion of proliferation, especially in skeletal muscles. This postulate was confirmed by Kohsaka et al in 2014 wherein whole-exome sequencing and Sequenom analysis revealed an overall 10% (10/104) prevalence of *MYOD1* L122R in 104 embryonal rhabdomyosarcoma (ERMS) tumors.¹⁵³ ERMS tumors with *MYOD1* L122R demonstrated decreased survival with histology revealing increased cellularity and mitotic activity.¹⁵³ *In vitro*, mouse myoblast C2C12 cells with L122R were less differentiated and displayed increased anchorage-independent growth compared to those with WT MYOD1.¹⁵³ Strikingly, chromatin immunoprecipitation sequencing (CHIP-Seq) and gene expression microarrays also demonstrated a genome-wide shift towards a Myc

transcriptional program.¹⁵³ No *MYOD1* mutation was found in patients with alveolar rhabdomyosarcoma, the other major subtype of rhabdomyosarcoma which is driven by fusion genes comprised of the anchor gene forkhead box protein O1 (*FOXO1*) and fusion partners including paired box 3 (*PAX3*), paired box 7 (*PAX7*) or fibroblast growth factor receptor 1 (*FGFR1*).^{154, 155} *MYOD1* mutations could be detected in other cancer types but L122R was found exclusively in ERMS. Interestingly *MYOD1* L122M was found in one lung cancer case.¹⁰ Whether L122M exhibits the same neomorphic activity as L122R remains unknown.

Aberrations along the PI3K pathway including hotspot mutations in *PIK3CA* or *PTEN* gene deletion occurred exclusively with *MYOD1* L122R in ERMS.^{153, 156} Co-expression of p110α H1047R and *MYOD1* L122R had an additive effect on *in vitro* and *in vivo* tumorigenicity,¹⁵³ implicating cooperativity of the two aberrations and suggesting that inhibiting their activities may be a potential anti-tumor strategy for this molecularly distinct and high risk cohort of patients. Further mechanistic characterization of *MYOD1* L122R is imperative to identify actionable targets associated with this neomorph.

YY1

Yin Yang 1 (YY1) is a ubiquitous zinc-finger transcription factor involved in cell proliferation, apoptosis, cell cycle control, differentiation and hematopoiesis.^{157, 158} Depending on interacting partners, YY1 can either activate or repress gene expression and hence the name “Yin Yang”. YY1 regulates gene transcription through direct binding to gene promoters, by acting as a transcription cofactor, by interfering with other transcription factors or by altering chromatin structure.^{158, 159}

Our understanding of the role of YY1 in tumorigenesis is evolving. It is overexpressed in diverse cancer types.^{160–166} YY1 modulates the expression or transcriptional activity of a number of key players in the pathogenesis of malignancies, for example, *MYC* and *ERBB2*.^{167–169} Also, YY1 binds to the silencer region of the *FAS* promoter, thereby repressing *FAS* gene expression and conferring resistance to Fas-mediated apoptosis.¹⁷⁰ Moreover, YY1 is a negative regulator of p53, disrupting the interaction between p53 and its coactivator p300.¹⁷¹ YY1 interacts with p53 and inhibits transcription of a subset of p53 target genes regulating cell cycle arrest and apoptosis.^{172, 173} Overexpression of YY1 stimulates p53 ubiquitination and degradation through assembly of a p53-Mdm2 complex.^{173, 174} Intriguingly, YY1 overexpression does not mediate the same clinical effects in every tissue. In ovarian cancer, YY1 overexpression correlates with improved overall survival, whereas decreased survival was observed in breast cancers where YY1 protein expression was associated with *ERBB2* expression.^{161, 162, 166}

Insulinoma is a type of pancreatic neuroendocrine tumor that inappropriately secretes insulin independent of serum glucose, thereby resulting in hypoglycemia. Whole exome sequencing of ¹¹³ sporadic insulinomas revealed a recurrent *YY1* mutation at T372R in 30% of tumors.¹⁷⁵ This recurrent mutation was also detected in an independent tumor cohort consisting of benign and malignant insulinomas, suggesting a role of the mutation in early carcinogenesis.¹⁷⁶ The crystal structure of DNA-bound YY1 revealed that T372 locates within a highly conserved zinc finger domain that makes direct contact with DNA. Intriguingly, using CHIP-

Seq, Cromer et al. demonstrated that T372R alters the DNA motif bound by YY1 and therefore transcription factor specificity.¹⁷⁶ In contrast to the consensus DNA sequence 5'-GCCATNTT-3' bound by WT YY1, T372R recognized 5'-CCATC-3'. Gene expression microarray analysis using cells with WT YY1 or T372R identified 149 genes that were differentially expressed by at least 10 fold. Two genes, *ADCY1* (adenylyl cyclase 1), which catalyzes cAMP synthesis, and *CACNA2D2*, which encodes the α -2- δ -2 auxiliary (pore-forming) subunit of a high-voltage gated calcium channel, are not targets of WT YY1 but were highly expressed in cells with T372R. The mechanism underlying this neomorphic effect is unknown. *ADCY1* and *CACNA2D2* are involved in cAMP and calcium signaling; both processes are important for insulin secretion. Concomitant expression of these genes in pancreatic β -cell lines increased insulin release. Further, *YY1* T372R was not found in pancreatic adenocarcinomas or malignant primitive neuroectodermal tumors that did not secrete insulin.^{177, 178} Consistently, although *YY1* mutations were detected across tumor types in the TCGA including cancers of endometrium, head and neck, stomach, prostate breast and melanomas, none of these mutations was T372R.¹⁰ These data together suggest that the neomorphic mutation is specific to insulinomas. The molecular pathology underlying insulinomas remains unclear, and therefore whether targeting the YY1 neomorph and its associated signaling effects will provide an effective treatment is unknown. However, understanding the pathogenesis of insulinoma development allows for the possibility of eventually inducing insulin secretion in the β -cells of patients with diabetes.

Conclusion

To date, most mutations are commonly reported as hypomorphs or hypermorphs; however, for some genes, like *TP53*, neomorphic mutations appear to be more frequent. Herein, we have presented a series of examples of neomorphic mutations, some of which engender therapeutic responses distinct from WT and other types of morphs. Whether subsets of hypermorphic mutations, through high level activity or activity that is insensitive to feedback loops rewire cell signaling sufficiently to activate pathways not normally activated by WT proteins (pseudo-neomorphs) remains a distinct possibility. This underscores the significance of functional characterization of mutations to distinguish whether they are hypomorphic, hypermorphic, or neomorphic. It is likely that the examples we described represent only a subset of the neomorphic mutations that exist. While computational prediction and experimental transformation assays help differentiate between functional drivers and passengers, several experimental approaches can be appreciated for their robustness in the functional characterization of neomorphs we have discussed. The first involves the ability to create and express large numbers of mutant open reading frames to allow evaluation of their activities. CRISPR approaches allow the creation of specific mutations in the endogenous gene and provide an opportunity to characterize the effect of mutations in their normal expression context. Subsequent profiling of gene expression levels, protein levels, and metabolites as well as sensitivity to informer drug libraries or genomic interaction screens can facilitate the identification of signaling outcomes of the aberrations and provides insight regarding potential therapeutic approaches. While similar profile approaches in human tumors can give hints of neomorphic activities, many of the neomorphic mutations are sufficiently rare as to challenge this approach and thus require

careful experimental validations. Additional strategies to determine the functional consequences of neomorphic mutations include analyses of available crystal structures or prediction modeling from conserved protein features. Characterization of protein binding partners as well as DNA binding sites can also provide evidence for neomorphic activities. We expect that more tools geared towards unraveling the functional impact of aberrations will become available. For example, a kinome data-driven systematic approach facilitated the identification of the amino acid residues that govern peptide specificity towards substrates or interacting proteins.^{179, 180} Based on this analysis, PKC γ mutants were shown to gain the capacity to phosphorylate de novo substrates.¹⁷⁹ Further, mutations leading to neomorphic phosphorylation sites were also discovered.¹⁷⁹ In these modeling studies, the description of neomorphs was extended from the concept presented in this review of aberrant downstream consequences of a mutational event to include altering the spectrum of upstream signals or environmental cues that activate specific kinases. Under this definition, the number of neomorphic mutations are likely much higher than presented herein.

All oncogenic neomorphic mutations identified to date are substitution or truncation mutations. Insertion or deletion mutations leading to alteration of protein domains and gene fusion resulting in hybrid genes represent other interesting opportunities for future investigation. Indeed, whether the *BCR-ABL* fusion that alters the location of Abl activation is a neomorph should be considered. Our enhanced understanding of these deviant pathways and players provides us with new targets for rational drug design, novel biomarkers with which to assess therapeutic effects, and the ability to make molecularly-based prognostic classifications. Most importantly, the potential for neomorph-bearing tumors to be insensitive or to potentially bypass therapeutic agents active against the WT molecule or other types of morph raises an urgent need to determine which of the over 2 million point mutations that have been identified in cancer patients function as neomorphs. As genomic data continues to be generated and analyzed, we believe that the neomorphic mutations described in this review will represent just a few of the many more waiting to be identified.

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Figure 1. IDH1 R132H and IDH2 R172K/R140Q reduce α-KG to the oncometabolite 2-HG. WT IDH2 and IDH2 reduce NADP⁺ and convert isocitrate into α-KG, which is a co-substrate of α-KG-dependent enzymes including PHD, TET2, and JHDM to regulate HIF-1α stability and epigenetic modifications. IDH1 R132H and IDH2 R172K/R140Q do not catalyze the conversion of isocitrate into α-KG but instead reduce α-KG to 2-HG. Competition of 2-HG with α-KG for binding to the α-KG-dependent enzymes results in DNA/histone methylation, HIF-1α stabilization and subsequent gene transcription (e.g. VEGF) to promote stem cell maintenance and angiogenesis.

Figure 2. p110α helical domain neomorphs bind to IRS1 for stabilization and activation independent of its canonical partner p85.

Upon RTK activation, p85 binds to phosphorylated IRS1 that thereby activates p110α. p110α neomorphs in the helical domain directly associate with IRS1 independent of p85 and growth factor stimulation. This association stabilizes and activates p110α, thereby promoting PI(3,4,5)P₃ production and *in vivo* tumor growth through AKT signaling. Another independent study showed that these mutants increase membrane localization and activation of PDK1, which in turn promotes anchorage-independent growth through SGK3 activation. The detailed mechanism underlying the activation of PDK1 and whether PDK1 activation is a consequence of neomorphic activity remain to be revealed. RTK, receptor tyrosine kinase.

Figure 3. *PIK3R1* neomorphs activate PI3K and MAPK signaling.

p6α neomorphs activate ERK and JNK signaling pathways to promote cell growth, survival, invasion and inhibit apoptosis. Binding of neomorphs to Cdc42 and Rac1 leads to phosphorylation of Braf, MKK1/2 and ERK1/2 in the cytoplasm. Cdc42 and Rac1 also facilitate the translocation of p85α neomorphs from the cytoplasm to the nucleus, where the neomorphs act as scaffolds for a MLK3/MKK7/JNK1/JNK2 complex that activates nuclear JNK signaling. The neomorphs also activate AKT but the mechanism is unknown. RTK, receptor tyrosine kinase. GPCR, G protein-coupled receptor.

Figure 4. PTEN A126G has altered enzymatic activity.

WT PTEN inhibits the activation of PI3K/AKT signaling by dephosphorylating PI(3,4,5)P₃ and PI(3,4)P₂ to PI(4,5)P₂ and PI(4)P respectively. PTEN A126G, when compared to WT PTEN, dephosphorylates PI(3,4,5)P₃ at reduced rate. Moreover, PTEN A126G displays a shift in enzymatic specificity toward 5' phosphoinositides, resulting in the production of PI(3,4)P₂ rather than PI(4,5)P₂ which is produced by WT PTEN. The accumulation of PI(3,4,5)P₂ and PI(3,4)P₂ together leads to AKT and S6 phosphorylation and ultimately cell motility.

Figure 5. p53 neomorphic mutants gain novel interacting partners leading to pro-oncogenic activities.

(a) p6 and p73 form homotetramers or heterotetramers that activate transcription of genes promoting cell apoptosis and sensitivity to chemotherapeutic drugs. p63 or p73 do not interact with WT p53. Neomorphic p53 mutants bind to p63 and p73 homotetramers or heterotetramers and inhibit p63-/p73-mediated gene transcription leading to increased chemoresistance, cell migration and invasion. (b) WT p53 binds to the transcription factor NF-Y and recruits the histone deacetylase HDAC1 as a co-factor to inhibit the transcription of NF-Y target genes involved in cell cycle progression such as *CCNA*, *CCNB*, *CDK1*, and *CDC25C*. Neomorphic p53 mutants recruit histone acetyltransferase p300, an opposing histone modifying enzyme, to promote the transcription of NF-Y target genes. (c) Neomorphic p53 mutants, but not WT p53, bind to Mre11. The interaction impairs the recruitment of Mre11 to double-strand DNA breaks and inhibits the activation of ATM thereby preventing DNA repair.