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MYC and RAF: Key Effectors in Cellular Signaling and Major Drivers in Human Cancer

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Abstract

The prototypes of the human *MYC* and *RAF* gene families are orthologs of animal proto-oncogenes that were originally identified as transduced alleles in the genomes of highly oncogenic retroviruses. *MYC* and *RAF* genes are now established as key regulatory elements in normal cellular physiology, but also as major cancer driver genes. Although the predominantly nuclear *MYC* proteins and the cytoplasmic *RAF* proteins have different biochemical functions, they are functionally linked in pivotal signaling cascades and circuits. The *MYC* protein is a transcription factor and together with its dimerization partner *MAX* holds a central position in a regulatory network of bHLH-LZ proteins. *MYC* regulates transcription conducted by all RNA polymerases and controls virtually the entire transcriptome. Fundamental cellular processes including distinct catabolic and anabolic branches of metabolism, cell cycle regulation, cell growth and proliferation, differentiation, stem cell regulation, and apoptosis are under *MYC* control. Deregulation of *MYC* expression by rearrangement or amplification of the *MYC* locus or by defects in kinase-mediated upstream signaling, accompanied by loss of apoptotic checkpoints, leads to tumorigenesis and is a hallmark of most human cancers. The critically controlled serine/threonine *RAF* kinases are central nodes of the cytoplasmic MAPK signaling cascade transducing converted extracellular signals to the nucleus for reshaping transcription factor controlled gene expression profiles. Specific mutations of *RAF* kinases, like the prevalent BRAF(V600E) mutation in melanoma, or defects in upstream signaling or feedback loops cause decoupled kinase activities which lead to tumorigenesis. Different strategies for pharmacological interference with *MYC*- or *RAF*-induced tumorigenesis are being developed and several *RAF* kinase inhibitors are already in clinical use.

Keywords

Protein-protein interactions; Compartmentalized signaling; Oncogene addiction; Metabolic rewiring; Small-molecule inhibition; Binding interface; Off-target effects; Drug resistance; Paradoxical activation; Synthetic lethality

1 Introduction

The original discoveries of several important cancer drivers, including *MYC* and *RAF*, are closely linked to animal retrovirology (Vogt 2012). The first biochemical identification of a

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Dedication This article is dedicated to Peter K. Vogt. His pioneering work has profound and sustained impact on the fields of virology and cancer genetics.

cancer gene (Duesberg and Vogt 1970) – the *v-src* oncogene of Rous sarcoma virus – and the striking proof of its origin from a normal cellular gene (*c-src* proto-oncogene) (Stehelin et al. 1976) were landmark discoveries in molecular cancer research (Bister 2015). They immediately stimulated the search for the transforming principles of other highly oncogenic retroviruses (Vogt 2012; Bister 2015). A specific class of retroviruses, the avian acute leukemia viruses, proved to be a rich source of novel cancer genes, including *myc*, *mil*(*raf*), *erbB*, *erbA*, *myb*, and *ets* (Bister and Jansen 1986). In 1977, specific protein products and nucleic acid sequences of the *myc* oncogene were discovered by biochemical analyses of acute leukemia virus MC29 (Bister et al. 1977; Duesberg et al. 1977). The viral genome was shown to be defective in all replicative genes (*gag*, *pol*, and *env*), to contain a contiguous novel insert unrelated to *src* and later termed *v-myc* (Coffin et al. 1981), and to encode a single protein product, a Gag-Myc hybrid protein (Fig. 1) (Bister et al. 1977; Duesberg et al. 1977; Bister and Vogt 1978; Mellon et al. 1978; Sheiness et al. 1978). Subsequently, *v-myc* alleles were identified in all other members of the MC29 subgroup of acute leukemia viruses, CMII, OK10, and MH2 (Bister and Jansen 1986). Following the *src* paradigm, the cellular origin of the *v-myc* alleles was soon proven and the chicken *c-myc* gene identified (Sheiness and Bishop 1979; Roussel et al. 1979; Robins et al. 1982; Vennström et al. 1982). The *c-myc* gene has been conserved throughout metazoan evolution and may even have pre-metazoan ancestors (Hartl et al. 2010; Young et al. 2011). The discovery of chromosomal translocations of the human *MYC* gene in Burkitt lymphoma cells provided the first evidence for the involvement of the cellular homolog of the *v-myc* retroviral oncogene in human tumorigenesis (Dalla-Favera et al. 1982; Taub et al. 1982). Today, deregulated *MYC* expression is established as an important driving force in the majority of all human cancers (Vogt 2012; Dang 2012; Vogelstein et al. 2013; Stine et al. 2015; Tokheim et al. 2016).

In 1983, biochemical analyses of avian acute leukemia virus MH2 (Mill Hill virus 2) revealed that the viral genome – in addition to *v-myc* – contains a second unique insert termed *v-mil* (Fig. 1) (Jansen et al. 1983a,b; Coll et al. 1983; Kan et al. 1983). The cellular origin of *v-mil* was demonstrated by detailed structural comparisons of cloned MH2 *v-mil* and chicken *c-mil* genes (Jansen et al. 1983b). In the same year, a cell-derived insert (termed *v-raf*) was found in the genome of murine sarcoma virus 3611 (Fig. 1) isolated from mice that had been inoculated with a virus stock obtained by 5-iodo-2'-deoxyuridine induction in chemically transformed mouse cells (Rapp et al. 1983). It was subsequently shown that *v-mil* and *v-raf* were derived from orthologous genes in chicken and mice (Jansen et al. 1984). MH2 induces leukemia and carcinoma in infected fowl and is one of the oldest natural retroviral isolates (Begg 1927). It is an intriguing genetic curiosity that a highly oncogenic chicken virus arose long time ago by transduction of two cellular genes in tandem both of which are recognized today as key drivers in human cancer. Indeed, elucidation of the genetic structure of MH2 provided a direct hint at possible oncogene cooperativity (Jansen et al. 1983b; Bister and Jansen 1986), which was supported by the observation of enhanced oncogenic properties of MH2 *in vitro* and *in vivo* (Graf et al. 1986) or by construction of a murine MH2 analog with a remarkably strong oncogenic potential in mice (Rapp et al. 1985). Today, mutated forms of the human ortholog of *mil/raf* and its paralogs are involved in a variety of human cancers (Wellbrock et al. 2004; Vogt 2012; Holderfield et al. 2014a;

Tokheim et al. 2016), and there is also evidence for synergism of *MYC* and *RAF* genes in tumorigenesis (Tabor et al. 2014; Ratnikov et al. 2017).

In mammalian systems, including man, the gene designation *raf* coined for the murine oncogene is established and will be used henceforth in this article on human genes. Genes and proteins will be designated according to human nomenclature conventions, e.g. *MYC* and *RAF1* for the human orthologs of the avian *c-myc* and avian/murine *c-mil/raf* genes, respectively, and MYC and RAF1 for their protein products. Paralogous members of the human *MYC* gene family are *MYCN* and *MYCL*, encoding the N-MYC and L-MYC proteins. *ARAF* and *BRAF* are paralogs of *RAF1*, and specify the ARAF and BRAF proteins. Also, *MYC* or MYC and *RAF* or RAF will be used as generic terms when no specific member of the gene or protein families is addressed.

2 Protein Structure

2.1 MYC Proteins

The domain structure of the human MYC protein with a total length of 439 amino acids is shown in Fig. 2. MYC belongs to a family of proteins with a characteristic hallmark, a dimerization and DNA binding domain (bHLH-LZ) encompassing a basic region (b) as DNA contact surface, a helix-loop-helix (HLH) and a leucine repeat (zipper) (LZ) region as protein-protein interaction (PPI) domains. The preferred binding partner for MYC is another member of the bHLH-LZ protein family, MAX, and PPI between these proteins leads to formation of a stable MYC:MAX heterodimer. The discovery of MAX and the recognition of MYC:MAX as a sequence-specific DNA binding complex enabled crucial leaps forward in the understanding of MYC biochemistry (Blackwood and Eisenman 1991; Eisenman 2001; Conacci-Sorrell et al. 2014). In the absence of MAX and at physiological concentrations, MYC is monomeric in solution and displays properties of an intrinsically disordered protein (IDP) with isolated regions of dynamic secondary structure elements and helical fraying (Fieber et al. 2001). MAX forms homodimers, albeit with lower stability than that of the MYC:MAX heterodimer. X-ray structures of the bHLH-LZ domains in MAX homodimers (Ferré-D'Amaré et al. 1993) or in MYC:MAX heterodimers (Nair and Burley 2003) revealed the structural details of the dimer-specific selective PPIs between the parallel protein chains (Fig. 2). The α -helical basic regions of MYC:MAX heterodimers bind to specific DNA sequence elements (E-boxes) with the preferred structure 5'-CACGTG-3' by making specific base contacts in the major groove of DNA (Fig. 2) (Blackwood and Eisenman 1991; Nair and Burley 2003). MYC:MAX complexes induce transcription by binding to E-boxes in promoter or enhancer regions of target genes and form PPIs with a variety of other factors.

In addition to the distinctive bHLH-LZ domain and a proximate nuclear localization signal, MYC contains several regions (MYC boxes, MBI-IV) that are highly conserved among members of the MYC protein family (Fig. 2) (Conacci-Sorrell et al. 2014; Thomas et al. 2015). MBI and MBII are located within the transactivation (transcriptional activation) domain (TAD) and their precise biochemical functions are best defined. MBI contains a phospho-degron regulating ubiquitylation and proteolysis of the distinctively unstable MYC protein exhibiting half-lives of only 20-30 min. Phosphorylation at Ser62 by the kinases

ERK or CDK increases MYC stability, whereas phosphorylation at Thr58 by GSK-3 β initiates dephosphorylation at Ser62, ubiquitylation by Fbw7 E3 ligase, and proteasomal degradation (Farell and Sears 2014). MBII interacts with components of histone acetyltransferase (HAT) complexes including TRRAP and other cofactors to stimulate histone acetylation and gene activation. MBII is essential for most biological activities of MYC, and is also an interaction site for the E3 ligase Skp2 (Conacci-Sorrell et al. 2014). In contrast to MBI and MBII, the molecular functions of the other MYC boxes are not completely explored (Conacci-Sorrell et al. 2014; Thomas et al. 2015). However, MBIIIa, also named just MBIII (Conacci-Sorrell et al. 2014), interacts with histone deacetylase 3 (HDAC3) leading to transcriptional repression, the chromatin association of MYC depends in part on the interaction of MBIIIb with the WD40-repeat protein WDR5, and MBIV associates with the conserved transcriptional cofactor HCF-1 (Thomas et al. 2015, 2016). Specific cleavage of MYC at Lys298 by calcium-dependent calpain proteases yields MYC-nick, a cytoplasmic form of MYC that retains MYC boxes I through IIIb and is involved in α -tubulin acetylation and cell differentiation (Conacci-Sorrell et al. 2010; Anderson et al. 2016). Paralogs of *MYC* are *MYCN* and *MYCL* that were originally identified as amplified genes in human neuroblastoma or small cell lung cancer, respectively (Brodeur et al. 1984; Nau et al. 1985). The human N-MYC (464 amino acids) and L-MYC (364 amino acids) proteins share the principal domain topography with MYC (Fig. 2), with the exception that MBIIIa is missing from the shorter L-MYC protein (Conacci-Sorrell et al. 2014; Thomas et al. 2015). Notably, the transduced *v-myc* alleles of avian acute leukemia viruses contain the entire coding region of the chicken *c-myc* proto-oncogene, and the Myc amino acid sequences of the viral Gag-Myc or v-Myc protein products (cf. Fig. 1) differ from the 416-amino acid chicken c-Myc protein sequence only by individual amino acid substitutions and, in the case of MH2, a small internal deletion (Bister and Jansen 1986).

2.2 RAF Proteins

The domain structure of the human RAF1 protein (648 amino acids) is depicted in Fig. 3. The X-ray structure of the RAS binding domain (RBD) of RAF1 in complex with the G domain of HRAS, a member of the RAS protein family of small GTPases (Karnoub and Weinberg 2008; McCormick 2016), is shown below (Fetics et al. 2015). RAF proteins are cytoplasmic serine/threonine-specific protein kinases that share three conserved regions (CR). CR1 is located in the N-terminal auto-inhibitory domain of RAF and comprises the RBD and the cysteine-rich domain (CRD), which are both involved in a GTP-dependent interaction with RAS. The central serine/threonine-rich CR2 is essential for phosphorylation-dependent regulation of RAF, and the C-terminal CR3 encompasses the catalytic kinase domain (Wellbrock et al. 2004; Baccarini 2005; Holderfield et al. 2014a; Lavoie and Therrien 2015; Desideri et al. 2015). Notably, the transduced *v-mil* and *v-raf* alleles in MH2 and 3611-MSV contain only the 3' segment of the coding domains of *c-mil* and *c-raf*, respectively, and hence the Gag-Mil and Gag-Raf hybrid proteins lack the auto-inhibitory N-terminal domain (cf. Fig. 1). RAF proteins are essential effectors within the mitogen activated protein kinase (MAPK) pathway and phosphorylate MEK (Dent et al. 1992; Howe et al. 1992; Kyriakis et al. 1992) which then activates ERK signaling (Lavoie and Therrien 2015; Desideri et al. 2015). They are activated and relieved from intramolecular auto-inhibition by (i) interaction with GTP-loaded RAS proteins (Moodie et

al. 1993; Van Aelst et al. 1993; Vojtek et al. 1993; Zhang et al. 1993), (ii) recruitment to the plasma membrane, and (iii) formation of allosterically regulated homo- and heterodimers (Wellbrock et al. 2004; Baccarini 2005; Lavoie and Therrien 2015; Desideri et al. 2015). These activating interactions are regulated by several phosphorylation and dephosphorylation events at crucial residues (Fig. 3). Phosphorylation of Ser259 in CR2 by PKA generates a binding site for 14-3-3 scaffold proteins and this interferes with RAS binding and membrane recruitment. For RAF activation, Ser259 has to be dephosphorylated by protein phosphatase 2A (Abraham et al. 2000; Desideri et al. 2015). In contrast, several other phosphorylation sites have positive effects on RAF activation. Phosphorylation of the C terminal 14-3-3 binding site Ser621 facilitates RAF dimerization (Lavoie and Therrien 2015; Desideri et al. 2015). Other positive phosphorylation events involve Ser338 and Tyr341 in the N-region (*Negative* charge required for RAF activation) adjacent to CR3, and Thr491 and Ser494 within the activation segment of the kinase domain (Fig. 3) (Wellbrock et al. 2004; Lavoie and Therrien 2015).

The paralogs of *RAF1* are *ARAF* isolated by human and mouse cDNA screening (Huebner et al. 1986) and *BRAF* that was originally identified in human Ewing sarcoma DNA (Ikawa et al. 1988) and at the same time as a transduced *mil*-related oncogene (*v-Rmi1*) in avian retrovirus IC10 (Marx et al. 1988). The human *ARAF* (606 amino acids) and *BRAF* (766 amino acids) proteins exhibit the principal domain architecture of *RAF1* (Fig. 3), with *BRAF* containing an additional N-terminal segment (Wellbrock et al. 2004). Phylogenetic sequence comparisons revealed that the *BRAF* gene is the human paralog that is most similar to the single *Raf* genes in non-vertebrate organisms and that *BRAF* is probably the prototypic RAF kinase (Desideri et al. 2015). The regulation of human *BRAF* shares common features with the other paralogs, but also displays important distinctions. The N-region of *BRAF* is negatively charged independent of regulatory events, with Asp449 corresponding to the position of Tyr341 in *RAF1* and with Ser446 constitutively phosphorylated unlike the equivalent residue Ser338 in *RAF1*. While the N-regions of *RAF1* and *ARAF* have to be negatively charged for activation, the *BRAF* N-region carries a constant negative charge requiring fewer steps in the activation of *BRAF* by RAS (Wellbrock et al. 2004; Baccarini 2005).

3 Cellular Signaling

Mitogenic signaling pathways connect membrane localized receptor activities with cytoplasmic and nuclear effector systems, as shown in Fig. 4 for the MAPK pathway. MYC and RAF proteins occupy apex positions in such signal transduction cascades. Remarkably, several protein effectors in these pathways are encoded by proto-oncogenes originally identified in transduced form as transforming principles in retroviral genomes. This includes the receptor tyrosine kinase (RTK) EGF receptor (EGFR) and the GTPase RAS (Vogt 2012; Bister 2015), in addition to the cytoplasmic serine/threonine-specific protein kinase RAF and the transcription factor MYC (cf. Fig. 1). The most important regulatory functions of MYC will be addressed here, followed by an outline of the upstream cytoplasmic signaling involving RAF kinases.

3.1 The MYC Master Regulator

The broad physiological functions of the master regulator MYC reach to virtually all important cellular activities and compartments: transcriptional activation or repression of target genes, general transcriptional amplification, control of the entire non-coding transcriptome including microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), DNA replication, energy metabolism, cell cycle progression, cell proliferation, apoptosis, differentiation, cell migration, and stem cell biology (Soucek and Evan 2010; Conacci-Sorrell et al. 2014; Stine et al. 2015). This huge and still expanding regulatory network of MYC represents an enormous challenge for exhaustive functional investigations. The canonical function of MYC is that of a transcriptional regulator in a dimeric DNA-binding complex with MAX (cf. Fig. 2). Indeed, most physiological and tumorigenic functions of MYC require dimerization with MAX, although MAX-independent and even cytoplasmic functions of MYC have also been reported. MYC:MAX dimers generally stimulate transcription, but a significant number of transcriptionally repressed target genes have also been identified (Eilers and Eisenman 2008; Conacci-Sorrell et al. 2014). A specific mechanism of transcriptional repression involves association with the zinc-finger transcription factor Miz-1 (Wanzel et al. 2003). The number of transcriptionally activated target genes is huge, and it has been estimated that over 15% of all human genomic loci are bound and possibly regulated by MYC (Conacci-Sorrell et al. 2014). In a global mapping analysis, more than 4,000 MYC binding sites were identified in human B cells, and by gene expression profiling, more than 660 direct MYC-regulated target genes were identified (Zeller et al. 2006). A large number of the upregulated genes are involved in processes related to cell growth and proliferation, like ribosomal and mitochondrial biogenesis, translation, biosynthetic pathways, glucose and glutamine metabolism, and cell cycle progression (Dang 2012, 2013; Stine et al. 2015). In agreement, MYC transcriptional regulation involves not only protein-coding or small RNA specifying genes transcribed by RNA polymerase II, but also genes transcribed by RNA polymerases I and III (Gomez-Roman et al. 2003; Grandori et al. 2005). In contrast to the common view of a gene-specific MYC transcriptional signature, it was proposed that MYC functions non-specifically as a global non-linear amplifier of gene transcription, mainly enhancing the ongoing gene expression program in the cell by stimulating transcriptional elongation (Lin et al. 2012; Nie et al. 2012; Rahl and Young 2014). It has been argued, that this *general amplification* model would not explain specific transcriptional repression by MYC or the MYC-induced specific changes in gene expression profiles in the transitions from resting to proliferating cells or from normal to transformed cells (Dang 2013; Stine et al. 2015). In the *selective amplification* model, the specific selection of MYC targets is largely determined by chromatin accessibility and interaction with other transcription factors to activate or repress gene transcription specifically (Dang 2014; Stine et al. 2015). However, the two models are not mutually exclusive, and MYC may function along both routes, depending on cell and chromatin status (Dang 2014; Wolf et al. 2015). Also, the net outcome of MYC-induced general amplification, including indirect effects on gene expression, can in the end still generate unique MYC transcriptional signatures (Rahl and Young 2014). It is important to note that the MYC:MAX interaction is inseparably embedded in a large network of interacting bHLH-LZ proteins (Conacci-Sorrell et al. 2014; Wilde and Ayer 2015). MYC does not homodimerize under physiological conditions and forms heterodimers exclusively

with MAX. In contrast, MAX forms homodimers and also heterodimers with other bHLH-LZ proteins, like the MXD (formerly MAD) family of proteins (MXD1-4) or MNT and MGA (Ayer et al. 1993; Hurlin et al. 1997, 1999; Conacci-Sorrell et al. 2014). Furthermore, MXD proteins and MNT form heterodimers with MLX, which in turn can undergo complex formation with transcriptional activators of the Mondo protein family, like MondoA or ChREBP (Conacci-Sorrell et al. 2014; Wilde and Ayer 2015). This extensive network of interacting bHLH-LZ proteins facilitates broad adjustments of transcriptional programs in response to mitogenic or metabolic signaling. MYC and the antagonistic MXD proteins compete for heterodimerization with MAX, and MYC:MAX and MXD:MAX compete for E-box binding sites. MYC:MAX heterodimers generally induce transcription, whereas MXD:MAX complexes repress transcription. Transcriptional repression by MXD:MAX involves recruitment of the mSIN3 and histone deacetylase corepressor complex (Conacci-Sorrell et al. 2014; Wilde and Ayer 2015), in contrast to transcriptional activation by MYC:MAX that involves recruitment of TRRAP and histone acetyltransferase GCN5 (McMahon et al. 2000). Heterodimer formation by the various members of the MYC/MAX/MXD network is dependent on the relative abundance of the bHLH-LZ proteins, and also on the specific binding affinities. For instance, MYC:MAX heterodimers have very low dissociation constants and are significantly more stable than MAX:MAX homodimers (Fieber et al. 2001; Nair and Burley 2003). The relative abundance of MYC proteins is tightly controlled at all levels of gene expression, including transcription, translation, and protein degradation (Sears 2004; Liu and Levens 2006; Farrell and Sears 2014). Transcriptional activation of *MYC* is strongly dependent on the cell cycle and differentiation status of the cell. While *MYC* expression is nearly shut off in quiescent cells, it is rapidly induced by mitogenic signals transduced by pathways like RTK-MAPK (Fig. 4) or WNT/ β -catenin (Kelly et al. 1983; He et al. 1998; Liu and Levens 2006; Soucek and Evan 2010). The nuclear effectors of these pathways are members of the E twenty-six (ETS) or T-cell factor/lymphoid enhancer binding factor (TCF/Lef) families of transcription factors and regulate *MYC* expression, among many other factors (Wasylyk et al. 1998; Yochum et al. 2010). Transcriptional regulation of *MYC* is followed by a multitude of post-transcriptional and post-translational control elements including miRNAs, lncRNAs, protein modification and degradation (Lal et al. 2009; Hung et al. 2014; Tseng et al. 2014; Farrell and Sears 2014; Stine et al. 2015).

Some of the earliest transcriptional targets of MYC to be identified encode metabolic enzymes, like lactate dehydrogenase (LDHA) involved in the glycolytic pathway (Shim et al. 1997; Stine et al. 2015). Today, MYC is established as a master regulator of genes involved in ribosomal and mitochondrial biogenesis, glucose and glutamine metabolism, protein synthesis, nucleotide and lipid biosynthesis (Dang 2013; Stine et al. 2015). MYC-induced metabolism in normal cells is under control by mitogenic signaling and nutrient supply, whereas in cancer cells deregulated MYC expression drives metabolic genes constitutively and leads to nutrient addiction, like glucose and glutamine dependency (Dang 2013; Shroff et al. 2015; Stine et al. 2015; Altman et al. 2016). MYC directly regulates specific genes encoding essential factors of metabolic pathways, including glycolytic and glutaminolytic enzymes and transporters. The recognition of MYC as a master regulator of energy metabolism also links current molecular cancer research to one of the first

observations of specific metabolic features of cancer cells, the increased conversion of glucose to lactate even in non-hypoxic conditions, a phenomenon known as aerobic glycolysis or Warburg effect (Warburg et al. 1924; Koppenol et al. 2011). Under hypoxic stress, normal cells activate expression of hypoxia inducible factors (HIFs) that induce genes for adaptation to hypoxia, including nearly all glycolytic genes. There are extensive interactions between the MYC/MAX/MXD network and HIFs, with antagonistic mechanisms in the response of normal cells to hypoxia, in contrast to cooperative functions in hypoxic cancer cells (Dang et al. 2008). In addition to the profound effect of MYC on the regulation of intermediary and energy metabolism, MYC also directly induces cell-cycle progression by transcriptional activation of genes such as CDK4 or cyclin D2 (Hermeking et al. 2000; Bouchard et al. 2001). A non-transcriptional function of MYC involves direct interaction with the pre-replicative complex (pre-RC) controlling the initiation of DNA replication (Dominguez-Sola et al. 2007). In addition, the *CDT1* gene encoding a key component of the pre-RC, the origin licensing factor Cdt1, is transcriptionally activated by MYC (Valovka et al. 2013). Hence, DNA replication is both under transcriptional and non-transcriptional control by Myc. As a huge expansion of its regulatory sphere, MYC also controls virtually the entire non-coding transcriptome (Weinberg et al. 2015). MYC regulates the expression of miRNAs, short RNA molecules that regulate the half-life or translational efficiency of target mRNAs (Mendell 2005, 2008; Croce 2009; Di Leva et al. 2014). One of the best characterized miRNA clusters involved in tumorigenesis, miR-17-92, is activated by MYC, attenuates the function of E2F transcription factors and also has anti-apoptotic effects (He et al. 2005; O'Donnell et al. 2005; Mendell 2005, 2008; Croce 2009; Di Leva et al. 2014). MYC-regulated miRNAs are also involved in embryonic stem cell differentiation (Lin et al. 2009). In addition to miRNAs, MYC also regulates the transcription of lncRNAs (Hart et al. 2014a; Kim et al. 2015). Most of the lncRNAs are not well characterized yet, but some are involved in cell cycle regulation and tumorigenesis (Kim et al. 2015).

In apparent contrast to its pivotal role in cell proliferation, MYC is also critically involved in cell death. In the absence of survival factors, overexpression of MYC leads to apoptosis (Evan et al. 1992; Shortt and Johnstone 2012). Dependent on cellular context, MYC induces apoptosis by the ARF/MDM2/p53 axis, but also by p53-independent interactions of ARF and MYC (Zindy et al. 1998; Qi et al. 2004; Gregory et al. 2005; Boone et al. 2011). Disturbances of the fail-safe balance between the pro-proliferative and pro-apoptotic functions of MYC are critical in tumorigenesis and can be caused by perturbations of the ARF/MDM2/p53 pathway or by cooperative effects of MYC and anti-apoptotic proteins like BCL2 (Beverly and Varmus 2009; Shortt and Johnstone 2012). MYC has also crucial roles in differentiation and development. Targeted disruption of *c-myc* or *N-myc* genes in mice leads to lethality at around day 10 of gestation (Davis et al. 1993; Grandori et al. 2000; Laurenti et al. 2009), and MYC is an important regulator of stem cell self-renewal and differentiation (Lin et al. 2009; Scognamiglio et al. 2016; Kanatsu-Shinohara et al. 2016). Although all functions discussed so far relate to the canonical full-length nuclear MYC protein, cytoplasmic functions have also been described. Proteolytic cleavage of MYC by calpains, a family of Ca²⁺-dependent cysteine proteases, generates a cytoplasmic form of MYC (MYC-nick) that lacks the C-terminal region of MYC beyond residue 298 (cf. Fig. 2)

and promotes α -tubulin acetylation and cell migration (Conacci-Sorrell et al. 2010; Anderson et al. 2016). Interestingly, synergistic functions of MYC and Ca^{2+} -signaling in B cell differentiation, and direct Ca^{2+} -dependent interactions of MYC with the Ca^{2+} -sensor calmodulin have been reported (Habib et al. 2007; Raffeiner et al. 2017).

3.2 The RAF Signaling Node

Interconnected kinase activities are at the heart of signaling networks which transmit and propagate the extracellularly sensed input signal to the nuclear destination for dictating the cell fate (Fig. 4). In response to growth factor, cytokine, or hormone mediated activation of membrane receptors, kinases of the evolutionary conserved MAPK signaling pathway (RAS-RAF-MEK-ERK) take the center stage for spatiotemporally controlled signal transmission (Wellbrock et al. 2004). The simplified illustration in Fig. 4 emphasizes a linear architecture of the transient and adaptive RAS-RAF-MEK-ERK signal response leading to transmission of the signal to the nucleus. The integrity of consecutive phosphorylation events is mandatory for physiological processes such as cellular growth, proliferation, and differentiation (Robinson and Cobb 1997). At the plasma membrane, signal transmission is initiated by receptor stimulation which results in activation of the RAS-GTPase family members HRAS, NRAS, or KRAS linking the receptor sensed input signals with downstream kinases. RAS functions as molecular switch and cycles between GDP-bound inactive and GTP-bound active states. Many external but also internal signals converge on the RAS isoforms to trigger a collection of non-redundant biological functions (Karnoub and Weinberg 2008; Pylayeva-Gupta et al. 2011; McCormick 2016). The critical GDP/GTP exchange during this activation cycle is regulated through guanine nucleotide exchange factors (GEF), while inactivation results from GTP hydrolysis through the intrinsic GTPase activity of the small G-proteins which can be strongly enhanced by GTPase activating proteins (GAP). RAS-GTP signaling involves binary PPIs with central kinases such as RAF but also phosphatidylinositol-3 kinase (PI3K) (Cully et al. 2006; Desideri et al. 2015). The RAF enzymes (ARAF, BRAF, or RAF1) act as initiating kinases for MAPK signaling. Physiological RAF activation depends on PPI with GTP-bound RAS via the RBD of RAF (Desideri et al. 2015; Lavoie and Therrien 2015). The consequences are membrane recruitment, dimerization, and the subsequent release of the auto-inhibitory RAF configuration leading to a shift to the open and active RAF conformation. This involves sequential phosphorylation and dephosphorylation events contributing to the intricate mode of RAF regulation by imposing either positive or negative constraints onto the RAF kinase function (Lavoie and Therrien 2015). Moreover, allosteric activation through side-to-side interaction of kinase domains of RAF homodimers or heterodimers, or interactions with the pseudokinase KSR (kinase suppressor of RAS) are functionally integrated (Brennan et al. 2011; Cseh et al. 2014). Activated RAF phosphorylates the downstream located gatekeeper enzymes MEK1 or MEK2 (Roskoski 2012a; Caunt et al. 2015). MEK1/2, acting as dual specific kinases, phosphorylate ERK1 and ERK2 which in turn phosphorylate a plethora of nuclear and cytoplasmic substrates such as kinases and transcription factors (Fig. 4) (Roskoski 2012b).

Cytoplasmic substrates of ERK are upstream located kinases such as EGFR, RSK2, BRAF, MEK1, and the pseudokinase KSR1 (Lake et al. 2016). In the nucleus, ERK regulates

transcription factors including members of the ternary complex factor (TCF) and ETS families (Wasylyk et al. 1998; Buchwalter et al. 2004). TCF and ETS induce the transcriptional activation of immediate early genes such as *MYC* and *FOS*. Their gene products MYC and FOS but also cyclin D1 are key factors for cell cycle progression, cell survival, cell division, and cell motility (Lito et al. 2013; Murphy and Blenis 2006; Roskoski 2012b). Another mechanism is the direct phosphorylation of transcription factors such as ELK1, FOS, or MYC by ERK (Fig. 4). ELK1 is a member of ETS transcription factors and the most thoroughly studied ERK target (Roskoski 2012b). ELK1 contains a C-terminal transcriptional activation domain which is the target for ERK phosphorylation leading to increased transcriptional activity (Hollenhorst et al. 2011). FOS forms a heterodimer with JUN for the assembly of the activator protein 1 (AP1) transcription factor complex. FOS phosphorylation in the nucleus extends the half-life of the protein to several hours (Okazaki and Sagata 1995). Double phosphorylation of FOS by ERK and Jun kinases (JNK) increases its transcriptional activity (Morton et al. 2003). Another critical transcription factor which is regulated by phosphorylation is MYC. In response to mitogenic signaling the evolutionary conserved MYC residues Ser62 and Thr58 (cf. Fig. 2) are targets for phosphorylation (Sears 2004; Farrell and Sears 2014). Phosphorylation of Ser62 by ERK but also by kinases of the CDK family transiently increases MYC stability. This is antagonized by Thr58 phosphorylation by GSK-3 β which triggers PP2A mediated Ser62 dephosphorylation targeting MYC for the ubiquitin-proteasome system through E3-ligase SCF-Fbw7 (Welcker et al. 2004; Yada et al. 2004). Furthermore, ERK kinases activate additional kinases such as p90 ribosomal S6 kinase or MAPK interacting kinases (MNKs) which are also relevant for the transcriptional control of the RAS-RAF-MEK-ERK cascade transmitted signal to the nucleus. Dependent on the cellular context, diverse biological functions related to cell cycle progression, differentiation, or metabolic cell signaling are initiated. For the precise biological output, the MAPK-initiated adjustments of transcription factor controlled nuclear gene expression programs are crucial. The scheme in Fig. 4 is a very basic diagram of the feedforward regulation of the RAS-RAF-MEK-ERK signaling network, and schematics for the different modes of feedback regulation of the MAPK cascade have not been integrated. The complexity of MAPK dynamics is reflected by the activities of the signaling hubs RAS and ERK which control a large number of effectors. Alterations of functional interactions emanating from these two signaling nodes dramatically affect spatiotemporal MAPK dynamics (Rauch et al. 2016). In many cells, activation of the RAS-RAF-MEK-ERK pathway is transient through a fast negative feedback directly impacting components of the cascade through ERK mediated phosphorylation. On a longer time scale, ERK activities enhance the transcriptional expression of DUSP family phosphatases to counterbalance kinase activities (Santos et al. 2007; Nagashima et al. 2015). These negative feedback loops have been implemented to ensure robustness and precise spatiotemporal control of transient or oscillating MAPK dynamics. The physiological response is determined by the integration and processing of the incoming signal with negative and positive feedback regulation. In particular, positive feedback loops are relevant for switch-like and bistable MAPK signaling responses (Santos et al. 2007; Rauch et al. 2016). An important strategy to achieve MAPK specificity is the organization of subsets of signaling units in space and time. For example, regulation of MAPK activities is ensured through molecular interactions with scaffolding proteins such as the pseudokinase KSR. The RAS-dependent compartmentalization of

several MAPK through the KSR1 scaffold increases both the signaling strength and the fidelity of signal transmission (Yu et al. 1998; Brennan et al. 2011; Matallanas et al. 2011).

4 Tumorigenesis

4.1 Cancer Driver *MYC*

Deregulated *MYC* genes are a driving force in the majority of all human cancers (Vogt 2012; Dang 2012; Vogelstein et al. 2013; Stine et al. 2015; Tokheim et al. 2016), and *MYC* was dubbed the emperor of oncogenes (Weinberg et al. 2015). *MYC*, *MYCN*, and *MYCL* were listed in the census of human cancer genes (Futreal et al. 2004) and classified as driver genes in cancer genome landscapes (Vogelstein et al. 2013). Most importantly, *MYC* and *MYCN* are included in a consensus list of 401 cancer drivers predicted by at least one of the three top performing methods in a stringent re-evaluation of cancer driver identification (Tokheim et al. 2016). In contrast to many other prominent cancer genes, including *RAS* and *RAF* (see below), oncogenic activation of *MYC* genes typically does not require mutations in the protein coding region. Hence, deregulation of *MYC* gene expression and uncontrolled levels of *MYC* protein are the hallmarks of *MYC* oncogenicity. There are several genetic mechanisms that can cause *MYC* deregulation. Retroviral transduction uncouples *c-myc* expression from cellular controls and is the mechanism that led to the original discovery of *MYC* in the form of avian retroviral *v-myc* alleles (cf. Fig. 1). Retroviral insertion in the vicinity of the *c-myc* locus is another mechanism disturbing normal cellular control of *c-myc* expression and was identified in B-cell lymphomas induced by avian leukosis viruses that lack oncogenic (*v-onc*) inserts in their genomes (Hayward et al. 1981; Payne et al. 1982). However, the important mechanisms relevant for human tumorigenesis do not require retroviral involvement and are characterized by major rearrangements or duplications of the *MYC* locus such as chromosomal translocation or gene amplification. Mapping of the human *MYC* locus to chromosome 8q24 and the identification of reciprocal t(8;14) translocations in Burkitt lymphoma promoting *MYC* transcription by juxtaposed immunoglobulin enhancers was the first demonstration of the involvement of *MYC* in human cancer (Dalla-Favera et al. 1982; Taub et al. 1982). Gene amplification of *MYCN* in neuroblastoma strongly correlates with advanced stages of the disease (Brodeur et al. 1984), and *MYCL* but also *MYC* and *MYCN* were found to be amplified in small cell lung cancer (Nau et al. 1985; Vita and Henriksson 2006). In all Burkitt lymphoma cases analyzed, the frequency of *MYC* translocations is 100%, and of *MYC* overexpression 91% (Vita and Henriksson 2006). *MYC* family gene amplification and/or overexpression has been observed at significant frequencies in a broad range of virtually all human cancer types, including myeloma, melanoma, ovarian, cervical, prostate, colon, breast, bladder, lung, and gastric cancers (Vita and Henriksson 2006). Although amplifications and less common translocations are the major genetic alterations leading to deregulation of *MYC* family genes in human tumors, it is important to bear in mind that aberrant *MYC* expression can also be caused by any defects in upstream signaling, like in the Wnt/ β -catenin, Notch, Sonic hedgehog, or MAPK pathways (He et al. 1998; Weng et al. 2006; Knoepfler and Kenney 2006), or by disturbance of post-transcriptional and post-translational control elements, including loss of negative feedback control of signaling pathways.

Oncogenic activation manifested by constitutive elevated *MYC* expression is essential both for cancer initiation and maintenance, and established *MYC*-driven tumors become addicted to constitutive *MYC* expression (Dang 2012; Gabay et al. 2014; Hsieh et al. 2015). Furthermore, based on the broad influence of *MYC* on metabolic regulation, cancer cells with deregulated *MYC* expression are also addicted to continuous nutrient supply, such as glucose or glutamine, whereas *MYC* expression and *MYC*-controlled energy metabolism in normal cells can be shut down by withdrawal of mitogenic signals or insufficient nutrient supply. In cancer cells with runaway *MYC* expression, frequent loss of p53 or ARF tumor suppressor checkpoints prevents the apoptotic loophole and allows uncontrolled cellular growth and proliferation (Stine et al. 2015). In addition to deactivation of tumor suppressors, *MYC*-induced tumorigenesis frequently involves cooperative activation of other oncogenes like *BCL2* or *RAS* (Beverly and Varmus 2009; McFadden et al. 2016). There is also evidence that cooperative interactions between MYC and effectors of the PI3K/AKT/mTOR signaling pathway contribute to oncogenesis and that resistance to therapeutic PI3K inhibition may be mediated by *MYC* amplification (Liu et al. 2011; Hsieh et al. 2015). Furthermore, there are mutual control mechanisms of *MYC* and miRNA expression that adopt important roles in tumorigenesis (Croce 2009; Di Leva et al. 2014). Loss of *MYC* repressive miRNAs such as *let-7*, or *MYC*-mediated transactivation of oncogenic miRNA clusters like miR-17-92 are involved in many cancer types (He et al. 2005; O'Donnell et al. 2005; Kumar et al. 2007; Cairo et al. 2010). Deregulated *MYC* expression has also been implicated in the emergence of chromosomal instability (CIN) (Felsher and Bishop 1999; Louis et al. 2005; Dang 2012). CIN causes aneuploidy, a hallmark of most human cancers, and is possibly involved in tumorigenesis and metastasis (Rajagopalan et al. 2003; Gao et al. 2016; Turajlic and Swanton 2016).

4.2 Oncogenic RAF Signaling

Hyperactivation of many components of the RAS-RAF-MEK-ERK pathway contributes to the initiation or progression of cancer. *HRAS*, *KRAS*, *NRAS*, *BRAF*, and *RAF1* are all included in the stringent consensus list of cancer drivers (Tokheim et al. 2016). In human cancers, recurrent oncogenic point mutations at the RAS codon positions Gly12, Gly13, or Gln61 eliminate the precisely controlled GDP/GTP exchange cycle and cause constitutive RAS activation. This leads to uncontrolled RAS signaling to a multitude of effector molecules and pathways including kinases such as RAF and PI3K (Cully et al. 2006; Karnoub and Weinberg 2008; Stephen et al. 2014; Lavoie and Therrien 2015). Under physiological conditions, the formation of homo- and heterodimers of RAF is a central element for phosphotransferase activation which is attenuated and disrupted by ERK-mediated feedback phosphorylation (Freeman et al. 2013; Lake et al. 2016). Oncogenic mutations in RAF kinases lead to decoupled phosphorylation events. Although the RAF isoforms share substantial sequence similarity, their mode of regulation differs in crucial details and this is important for the predominant role of BRAF in human cancers (Wellbrock et al. 2004; Desideri et al. 2015). In the entire superfamily of kinase genes, *BRAF* has been identified as the most frequently mutated proto-oncogene (Davies et al. 2002; Fleuren et al. 2016). An important structural basis for its paramount cancerogenic role is the unique presence in the BRAF sequence of a phospho-mimetic Asp449 and a constitutively phosphorylated Ser446 conferring a constant negative charge to the functionally crucial N-

region (Lito et al. 2013). Hence, BRAF is poised for activation and single point mutations are sufficient to convert BRAF into a constitutively active kinase. BRAF mutations have been identified in many cancers including malignant melanomas (50-60%), thyroid (30-50%), colorectal (10%) and lung (3%) cancers, hairy cell leukemia (100%), and others (Tiacci et al. 2011; Lito et al. 2013). The predominant oncogenic gain-of-function mutation in BRAF is the substitution of glutamic acid for valine at position 600 (V600E) which is found in 60% of all malignant melanomas (Lito et al. 2013). This mutation is located in the activation segment of the BRAF kinase domain between two regulatory phosphorylation sites (Thr599, Ser602) and apparently acts as a phospho-mimetic substitution. Based on molecular modeling and crystallographic evidence, the V600E mutation also promotes formation of a salt bridge between Lys507 and Glu600, thereby stabilizing the active conformation of the activation segment. Either phosphorylation of the activation segment (Thr599, Ser602) or substitution of charged amino acids (Glu, Lys, Asp, or Arg have been found in tumors) for Val600 convert the kinase into the active conformation. Furthermore, additional phosphorylation events throughout the RAF activation cycle control the activity status of the kinase. Differential RAF activation involves posttranslational modifications of the activation segment, the negatively charged N-region, and the C-terminal 14-3-3 binding site (Lavoie and Therrien 2015). However, the critical role of the phosphorylated N-region is not fully understood. Recent data suggest that it could determine the transactivation direction in RAF dimers (Hu et al. 2013).

So far, nearly 300 distinct missense mutations of BRAF have been identified in tumor samples and cancer cell lines (Forbes et al. 2011; Holderfield et al. 2014a). Most of the mutations occur in the activation (A) loop or in the phosphate-binding (P) loop of the kinase domain. However, the biochemistry of altered BRAF proteins varies substantially. In addition to kinase activating mutations, some amino acid substitutions lead to intermediate or low kinase activity. A subgroup of these mutations endorse MEK-ERK signaling through heterodimerization-mediated activation of RAF1 (Wan et al. 2004; Lito et al. 2013; Desideri et al. 2015). This suggests that independent of the BRAF catalytic function, the dimerization with RAF monomers releases the intrinsic activity of BRAF-bound RAF1. Interestingly, it has been shown that kinase-impaired activities of BRAF mutants coexist with RAS mutations (Andreoli et al. 2012). Analyses of the crystal structures of RAF kinase domains revealed a conserved side-to-side interface (Rajakulendran et al. 2009). Amino acid substitutions in the interface such as R509H impeded RAF dimerization and activity underscoring that side-to-side homo- and heterodimer formation leads to protomer transactivation (Lavoie and Therrien 2015). In addition, the dimerization-dependent RAF activation involves the allosteric reorganization of the N- and C-lobe of the kinase domain to obtain an active-like conformation. The alignment of hydrophobic residues spanning the N- and C-lobes are critical for the catalysis (Taylor and Kornev 2011; Hu et al. 2015; Lavoie and Therrien 2015). However, in contrast to wild-type and kinase impaired BRAF mutants, tumors harboring the BRAF(V600E) mutation show hyperactivated ERK signaling resulting in increased proliferation and evasion of apoptosis, independent of RAF dimerization (Poulikakos et al. 2011; Samatar and Poulikakos 2014). Since BRAF(V600E) is not dependent on dimerization and upstream regulation, it bypasses the inhibitory effect of negative feedback regulation of ERK (Tsavachidou et al. 2004; Lake et al. 2016). ERK-

dependent negative feedback regulation results in low RAS-GTP levels in cancer cells with BRAF mutations (Lito et al. 2013). However, the RTK/RAS-uncoupled BRAF(V600E) mutant acts as a functional monomer, constitutively activating MEK and cytoplasmic and nuclear ERK signaling. MEK1/2 are activated as a result of RAF-catalyzed phosphorylation in the kinase activation loop, in the case of MEK1 at Ser218 and Ser222 (Roskoski 2012a; Caunt et al. 2015). MEK1/2 in turn are the only activators of ERK1/2 and phosphorylate specific residues, such as Thr202 and Tyr204 in ERK1. MEK1/2 serve as critical gatekeeper kinases for ERK1/2 which regulate multiple cellular events through phosphorylation of over 150 substrates in the cytoplasm and nucleus, many involved in cell proliferation and survival (Yoon and Seger 2006; Roskoski 2012b; Caunt et al. 2015; Lake et al. 2016). In contrast to BRAF, activating mutations in MEK1 or MEK2 are only found at low frequency in human tumors (Marks et al. 2008; Murugan et al. 2009).

5 Pharmacological Interference

5.1 MYC Inhibition

In view of the strikingly broad involvement of *MYC* family genes in virtually all human cancers, strategies to interfere with *MYC*-induced oncogenesis, directly or indirectly, are an obviously urgent goal in cancer research and clinical treatment (Soucek et al. 2008; Prochownik and Vogt 2010; Dang 2012; McKeown and Bradner 2014; Stefan et al. 2015). A genetic approach using an engineered dominant negative MYC mutant with altered dimerization specificity provided principal proof of guided MYC inhibition as a means to interfere with cell transformation. The engineered mutant, termed Omomyc, competes with MAX for dimerization with MYC and blocks MYC-dependent transcriptional activation (Soucek et al. 1998; Soucek et al. 2008). In tissue culture and in preclinical mouse models, systemic Omomyc expression interfered with cell transformation and tumorigenesis, with surprisingly tolerable and fully reversible effects on normal tissues (Soucek et al. 2008). In another genetic approach, ectopic expression of the repressed MYC target gene *BASPI* was shown to inhibit MYC-induced cell transformation (Hartl et al. 2009). Obviously, for pharmacological interference and therapeutic applications, the development of small-molecule inhibitors is of highest priority. In view of the striking success in the development of small-molecule inhibitors for the therapy of cancers driven by oncogenic kinases (Holderfield et al. 2014a), MYC also became a target for possible drug development. However, in contrast to well-structured enzymes with defined catalytic clefts, MYC is intrinsically disordered in free form (Fieber et al. 2001) and its biological activities are mainly based on macromolecular interactions, such as PPIs involving large flat surface areas that are difficult to target with small molecules. Nevertheless, several small-molecule inhibitors of MYC:MAX dimerization and MYC-induced cell transformation were isolated from combinatorial chemical libraries using a fluorescence resonance energy transfer (FRET) assay or a yeast two-hybrid approach (Berg et al. 2002; Yin et al. 2003). The compounds displayed diverse specificity and efficacy, but represented an important proof of concept for targeting the MYC:MAX interface. Small-molecule inhibitors of MYC:MAX dimerization with pharmacokinetic properties suitable for possible drug development were isolated from a Kröhnke pyridine library in a fluorescence polarization screen (Hart et al. 2014b; Raffener et al. 2014). The compounds KJ-Pyr-9 (Fig. 5) and KJ Pyr 10 proved to be

highly effective in the nanomolar range and interfered specifically with MYC:MAX complex formation, the MYC-driven transcriptional signature, MYC-induced cell transformation, and the growth of breast cancer MDA-MB-231 xenografts carrying *MYC* amplifications (Hart et al. 2014b; Raffeiner et al. 2014).

The intrinsic difficulties of targeting MYC proteins directly have stimulated various efforts to develop indirect inhibitory strategies, such as attenuating *MYC* transcription. G-quadruplex (G4) regulatory regions in chromatin are implicated in transcriptional regulation and have been identified in several cancer associated genes, including *MYC*. Endogenous G4 structures in promoters were linked to elevated transcriptional activity, whereas G4 stabilization by small molecules was shown to repress *MYC* expression (Balasubramanian et al. 2011; Hänsel-Hertsch et al. 2016). An important approach to target *MYC* transcription involves small-molecule inhibition of members of the bromodomain and extraterminal (BET) subfamily of bromodomain proteins, such as BRD4 (McKeown and Bradner 2014; Shu and Polyak 2017). BET bromodomain proteins are histone modification readers that recognize acetylated lysine residues and facilitate transcriptional activation by recruiting transcriptional cofactors to chromatin. Small-molecule inhibitors (BETi) were developed, such as the very potent thienodiazepine-based JQ1 compound, that compete for the acetyl binding pocket of BET bromodomains causing displacement of BET proteins from chromatin and repression of gene expression (McKeown and Bradner 2014; Shu and Polyak 2017). BETi were shown to efficiently downregulate *MYC* transcription and the MYC-driven transcriptional program (Delmore et al. 2011; Mertz et al. 2011). Several BETi have entered clinical trials and clinical responses have been linked to *MYC* repression (Shu and Polyak 2017). A dual-activity small-molecule compound has been developed that simultaneously inhibits BRD4 and PI3K leading to concomitant decrease of *MYC* transcription and increase of MYC degradation, respectively. In mouse models, the compound effectively blocked tumor growth and metastasis (Andrews et al. 2017). In view of the pivotal role of the MYC master regulator in nearly all fundamental processes in normal cells (see above), the success of inhibitory strategies for clinical treatment of MYC-driven cancers will largely depend on the specificity of targeting the uncontrolled MYC expression and ensuing changes in cancer cells. The addition of many tumors to deregulated *MYC* expression and metabolic reprogramming may offer additional therapeutic opportunities, like targeting key enzymes of *MYC*-driven glucose or glutamine metabolism (Dang 2012; Stine et al. 2015; Altman et al. 2016). In principle, the pharmacological inhibition of genes whose function is specifically required for the survival of *MYC*-driven cancer cells is a promising strategy. Screens for *MYC* synthetic lethality, including genome-wide RNA interference, have identified several potential *MYC* synthetic lethal genes encoding aurora-B kinase, CSNK1e kinase, or the SUMO-activating enzyme SAE1/2 (Yang et al. 2010; Kessler et al. 2012; Toyoshima et al. 2012; Cermelli et al. 2014).

5.2 RAF Inhibition

Kinases have become one of the most intensively pursued classes of drug targets. They are involved in virtually every signal transduction process, and the human genome encodes over 500 different protein kinases (Manning et al. 2002). Despite the high degree of conservation of the ATP-binding pocket in the catalytic kinase domain, a collection of highly selective

ATP-competitive small molecule inhibitors have been developed. So far, more than 25 oncology drugs that target kinases have been approved for cancer therapy (Gross et al. 2015). In the early years of this millennium, the identification of BRAF mutations in a wide variety of human tumor types, most notably in melanoma, colorectal cancer, glioma, and lung adenocarcinoma (Davies et al. 2002; Fukushima et al. 2003), initiated extensive research efforts to develop RAF inhibitors (RAFi). The first general RAFi was Sorafenib acting as a multi-targeted kinase inhibitor. The incomplete specificity of Sorafenib promoted the development of mutation-selective BRAFi such as Vemurafenib (Fig. 5) and Dabrafenib which block the phosphotransferase activity of RAF mutants like BRAF(V600E) (Samatar and Poulikakos 2014). Both BRAFi were approved for clinical use and brought unprecedented clinical benefits to patients with melanomas containing the BRAF(V600E) or BRAF(V600K) mutations (Flaherty et al. 2010; Chapman et al. 2011; Hauschild et al. 2012; Lito et al. 2013). These inhibitors selectively block monomeric BRAF(V600E/K) activities without impacting the activities of the wild-type BRAF dimer. The selective and direct blocking of mutant BRAF-initiated downstream signaling to MEK-ERK is a remarkable feature of BRAFi in a subset of tumors harboring the specific mutations (Solit and Rosen 2011; Holderfield et al. 2014a). The discrimination for mutant BRAF inhibition is partially responsible for the broad therapeutic index of BRAFi which are either FDA-approved (Vemurafenib, Dabrafenib) or currently in clinical trials, for example PLX8394 or Encorafenib (Samatar and Poulikakos 2014; Zhang et al. 2015; Adelman et al. 2016). Of the more than 300 different missense mutations identified in BRAF, BRAF(V600E) is clearly the most common cancer driver mutation in melanoma (Forbes et al. 2011; Holderfield et al. 2014a). However, oncogenic mutations are not restricted to BRAF, and RAF1 mutations have also been found but at a much lower rate (Lavoie and Therrien 2015). Furthermore, BRAF mutations do not always predict favorable responsiveness to BRAFi. In addition to the type of mutation, the cellular context in which BRAFi are used is an important determinant for drug effectiveness. BRAFi are ineffective in BRAF(V600E)-dependent metastatic colorectal carcinoma and thyroid cancer (Holderfield et al. 2014a; Desideri et al. 2015). In these cancer types, a crucial negative feedback inhibition of EGFR signaling is lost upon BRAFi exposure, leading to increased EGFR signaling and intrinsic resistance to the drug (Prahallad et al., 2012). In contrast, melanoma cells express low basal levels of EGFR, evade this mechanism, and show favorable initial response to BRAFi treatment (Lito et al. 2012; Sun et al. 2014; Holderfield et al. 2014a; Desideri et al. 2015). However, the therapeutic benefits of BRAFi in melanoma patients are temporary and limited due to the development of drug resistance. A collection of mechanisms causing primary or acquired drug resistance have been described that limit the application of this approach (Hatzivassiliou et al. 2010; Heidorn et al. 2010; Poulikakos et al. 2010; Lito et al. 2013; Samatar and Poulikakos 2014; Yao et al. 2015). BRAFi resistance involves RTK and ERK reactivation through multiple mechanisms such as increased expression of RTKs, BRAF(V600E) or RAF1 amplification, occurrence of BRAF splice variants, mutational activation of NRAS, MEK mutations, or COT kinase overexpression (Lito et al. 2013; Desideri et al. 2015). Another unwanted consequence of BRAFi treatment is the so-called *paradoxical activation* of ERK signaling in BRAF wild type cells with activated RAS (Hatzivassiliou et al. 2010; Heidorn et al. 2010; Poulikakos et al. 2010; Holderfield et al. 2014b). The mechanism is based on formation of RAF dimers and BRAFi-induced

conformational changes of a protomer leading to transactivation in the dimer of the other non-drug bound RAF isoform (Hatzivassiliou et al. 2010; Heidorn et al. 2010; Poulikakos et al. 2010; Lito et al. 2013; Lavoie et al. 2013; Holderfield et al. 2014a; Lavoie and Therrien 2015). Hence, the drug binding allosterically activates signaling by the drug-free RAF kinase to MEK-ERK thereby enhancing cellular proliferation. This BRAFi-driven mechanism can promote skin cancers like cutaneous squamous-cell carcinoma and keratoacanthomas that often harbor RAS mutations (Chapman et al. 2011; Su et al. 2012). The multiple mechanisms of acquired BRAFi resistance and off-target effects underscore the need to develop improved drugs for cancer therapy. There is rising interest in identifying RAFi with different mechanistic functions, such as paradox breakers, with the ultimate goal to develop inhibitors with improved efficacy and selectivity (Zhang et al. 2015; Okimoto et al. 2016). Moreover, the implementation of poly-pharmacology approaches using a collection of kinase inhibitors may improve clinical drug efficacies by delaying or preventing the onset of drug resistance.

6 Conclusions

By now, four or almost four decades, respectively, have passed since the prototypes of the *MYC* and *RAF* gene families were discovered, initially as avian or murine orthologs transduced in retroviral genomes (cf. Fig. 1). In these past decades, a spectacular amount of detailed knowledge of the structure and function of these genes and their protein products has accumulated. It is one of the most stunning outcomes that *MYC* is apparently involved directly or indirectly in all major signaling and regulatory pathways and hence a true master regulator of cell biology and biochemistry. Furthermore, it is also one of the most prominent cancer driver genes, apparently deregulated and causally involved in the majority of all human cancers. In a way, it is not surprising that unleashing a master regulator like *MYC* profoundly abrogates the normal cellular regime. Accordingly, *MYC* has also broad influence in tumorigenesis, especially when crucial checkpoints designed to guard against uncontrolled cell proliferation are lost simultaneously with *MYC* deregulation. Although the mere complexity of the *MYC* regulatory sphere poses an enormous challenge for biochemical and biomedical research, it may also provide multiple opportunities to interfere with *MYC*-driven cancer growth. Efficient direct targeting of *MYC* PPIs, attenuating the expression of *MYC*, exploiting the unique oncogene and nutrient addiction of *MYC*-driven cancers, or systematic screening for synthetic lethality could all be feasible strategies for therapeutic intervention. As for the *RAF* kinases, pharmacological inhibition initially appeared to be more straightforward, since competitive or allosteric inhibition of enzymes is standard biochemical technology, and the stunning clinical success of several BRAFi is compelling proof. However, the inevitable development of drug resistance and the occurrence of even adverse effects of clinically approved BRAFi caused by perturbation of feedback mechanisms or activation of redundant signaling pathways are severe drawbacks. Here, the development of more specific inhibitors, combinations of inhibitors, or specific targeting of PPIs appear to be promising strategies. Obviously, the paramount medical relevance of deregulated or mutated *MYC* or *RAF* genes is the impulse for intensive research into their role as major cancer driver genes. Similarly, the physiological roles of *MYC* and *RAF* as master regulator of transcription or key cytoplasmic signal transducer,

respectively, are an important source for further research into fundamental principles of regulatory networks in normal cells. Also, the transition from physiological to oncogenic function, particularly in the case of *MYC*, may be gradual and dependent on cellular context and critical threshold levels. Hence, the oncogenic and proto-oncogenic facets of *MYC* and *RAF* will remain in the focus of biochemical and biomedical research.

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Fig. 1.

Discoveries of *MYC* and *RAF*. The genome structures of avian acute leukemia viruses MC29 and MH2, and of murine sarcoma virus 3611 are depicted. The retroviral oncogenes *v-myc*, *v-mil*, and *v-raf* are transduced alleles derived from the chicken *c-myc* and *c-mil* genes, or the mouse *c-raf* gene, respectively. Chicken *c-mil* and mouse *c-raf* are orthologous genes. In the MH2 genome, *v-mil* and *v-myc* alleles are directly adjacent, and their coding regions are separated by *c-myc* intron derived sequences (*small blue box*). The protein products (p) of the retroviral oncogenes are expressed from genome-sized mRNAs as hybrid proteins containing N-terminal Gag sequences, or from a subgenomic mRNA (MH2 *v-myc*).

Fig. 2.

Structure of the human MYC protein and its dimerization partner MAX. The dimerization and DNA binding domains (bHLH-LZ) are indicated. On the MYC protein, conserved MYC boxes (MBI-IV), the transactivation domain (TAD), the nuclear localization signal (NLS), critical phosphorylation sites (Thr58, Ser62), and a calpain cleavage site (Lys298) are depicted. The X-ray structure of a dimer of the MYC and MAX bHLH-LZ domains bound to DNA is shown below (Nair and Burley 2003). The image was created from the PDB entry 1NKP using the PyMOL graphics system.

Fig. 3. Structure of the human RAF1 and HRAS proteins. The conserved regions (CR1-3), the RAS binding domain (RBD), the cysteine-rich domain (CRD), and the kinase domain (KD) of RAF1 are indicated. Critical phosphorylation sites in CR2 (Ser259), in the C-terminal region (Ser621), in the N-region upstream of CR3 (Ser338, Tyr341), and in the activation segment (Thr491, Ser494) are marked. On the HRAS protein, the G domain and the hypervariable region (HVR) are depicted. The X-ray structure of the RAF1 RBD bound to the G domain of HRAS loaded with the GTP analog GppNHp is shown below (Fetics et al. 2015). The image was created from the PDB entry 4G0N using the PyMOL graphics system.

Fig. 4.

Schematic diagram of the mitogen activated protein kinase (MAPK) signaling pathway. Exposure to extracellular ligands leads to dimerization, autophosphorylation, and activation of receptor tyrosine kinases (RTK). This triggers recruitment of adaptor proteins and nucleotide exchange factors (not shown) that enhance GDP/GTP exchange leading to RAS activation (RAS-GTP). RAF, the first MAPK of the cascade, is activated through a multistep process including RAS binding, recruitment to the plasma membrane, conformational change, phosphorylation (P), and dimerization. Activated RAF phosphorylates and activates

the gatekeeper kinase MEK which in turn phosphorylates ERK. Activated ERK directly or indirectly regulates a plethora of cytoplasmic and nuclear substrates including transcription factors (TF), such as transcriptional regulators of *MYC* expression. In addition, ERK directly phosphorylates the transcription factor MYC which enhances its stability. TF-mediated alterations of gene expression profiles are relevant for cell cycle progression and proliferation. The different modes of negative feedback regulation of RAS-RAF-MEK-ERK signaling are not shown.

Fig. 5.

Strategies for MYC and RAF inhibition. *Top:* The MYC:MAX heterodimer is shown, bound to a DNA E-box sequence (*shaded*) and activating transcription. The inhibitor KJ-Pyr-9 (Hart et al. 2014b) interferes with MYC:MAX dimerization, preventing DNA binding and transcriptional activation. KJ-Pyr-9 is effective both *in vitro* and *in vivo* in experimental settings. *Bottom:* The X-ray structure of the BRAF(V600E) kinase domain (KD) in complex with the PLX4032 (Vemurafenib) inhibitor is shown (Bollag et al. 2010). Vemurafenib is in clinical use for treatment of melanoma patients. The image was created from the PDB entry 3OG7 using the PyMOL graphics system.