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Review

Oncogenic *KRAS* signaling and YAP1/β-catenin: Similar cell cycle control in tumor initiation

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ABSTRACT

Why are YAP1 and c-Myc often overexpressed (or activated) in KRAS-driven cancers and drug resistance? Here, we propose that there are two independent pathways in tumor proliferation: one includes MAPK/ERK and PI3K/A kt/mTOR; and the other consists of pathways leading to the expression (or activation) of YAP1 and c-Myc. *KRAS* contributes through the first. *MYC* is regulated by e.g. β-catenin, Notch and Hedgehog. We propose that YAP1 and ERK accomplish similar roles in cell cycle control, as do β-catenin and PI3K. This point is compelling, since the question of how YAP1 rescues K-Ras or B-Raf ablation has recently captured much attention, as well as the mechanism of resistance to PI3K inhibitors. The similarity in cell cycle actions of β-catenin and PI3K can also clarify the increased aggressiveness of lung cancer when both K-Ras and β-catenin operate. Thus, we propose that the two pathways can substitute one another – or together amplify each other – in promoting proliferation. This new understanding of the *independence* and *correspondence* of the two pathways in cancer – MAPK/ERK and PI3K/Akt/mTOR; and YAP1 and c-Myc – provide a coherent and significant picture of signaling-driven oncogenic proliferation and may help in judicious, pathway-based drug discovery.

Contents

Abbreviations: YAP1, Yes; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; eIF4E, eukaryotic translation initiation factor 4E; PI3K, phosphatidylinositide-3-kinase; WNT, Wingless-type mouse mammary tumor virus integration site family member; GPCRs, G protein-coupled receptors; TNF, tumor necrosis factor; mTOR, mammalian target of rapamycin; TLR, Toll-like receptor; JAK, Janus kinase; STAT, signal transducer and activator of transcription; CDK, Cyclin-dependent kinase; pRb, retinoblastoma; CaM, calmodulin; CaMKII, Ca^{2+}-dependent protein kinase II; TCF, transcription factor; RTK, receptor tyrosine kinase; HVRs, hypervariable regions; cSH2, c-Src homology 2; APC, adenomatous polyposis coli; EGFR, Epidermal growth factor receptor.

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1. Introduction

Have we reached a point where we can merge the literature reports toward a coherent picture of pathway-driven cell proliferation? Here we propose that this could be the case. The body of available data – including recent discoveries that YAP1 and β-catenin are an integral part of cell cycle control [1,2] as is MAPK/ERK, that overexpressed YAP1 promotes proliferation of cells treated with MAPK inhibitors [3,4], that overexpression of proteins upregulating *MYC* (e.g. β-catenin Refs. [5–7], Notch [8,9], Hedgehog [10–12], and eIF4E [13–16]) promote proliferation of cells treated with PI3K inhibitors – can provide the blueprints for this highly significant aim. Ultimately, when tested and completed it could yield protocols for drug resistance treatments, mitigating the long-standing enigma of which pathways would be up- (or down)-regulated in drug resistance.

K-Ras, and in particular its splice variant K-Ras4B, is observed with very high frequencies in pancreatic, colorectal and non-small cell lung carcinoma (95%, 45%, and 35% respectively) but not in some others, like melanoma, head and neck or even brain cancer [17]. Recent observations that overexpression of YAP1 counteracts K-Ras4B inhibition [1–4,18–20] beg the question of how? Despite decades of research, the hallmarks of physiological and oncogenic signaling networks in specific cells are still enigmatic and the question of what decides which oncogenic isoform among the four canonical H-Ras, N-Ras, K-Ras4A and K-Ras4B will dominate the disease phenotype in specific tissues still poses a major challenge. Isoform expression levels vary; but they do not resolve these questions. This question has been augmented by recent findings related to the occurrence of oncogenic K-Ras4A splice variant [21]. Surprisingly, this isoform was observed to be associated with both colorectal and lung cancers, which are K-Ras4B cancers, and leukemia, an N-Ras cancer [22]. The significance of insight into what makes a certain oncogenic isoform drive cancer in distinct cells and tissues are vast, as it may spawn new classes of cancer type-specific drugs with lessened toxicity. Insight into 'redundant' signaling is equally crucial since drug resistance inevitably ensues [23–26]. The emergence of overexpressed, transcriptionally-active YAP1 and β-catenin as a survival rescue strategy of K-Ras4B-inhibited cells [1–4,18–20] has further become a key concern since it can offset K-Ras4B therapeutics.

Here we suggest that even though the Hippo, WNT, MAPK, and PI3K pathways respond to different cues – correspondingly cell-cell contact/mechanical strain and growth factor/hormone stimulated signaling – their functions in cell cycle control and tumor initiation are analogous [13,27,28].

Under normal physiological conditions, mechanical strain controls E-cadherin-dependent YAP1 and β-catenin activation. Cell-cell contact activates the Hippo pathway [29] preventing proliferation. In contrast, downregulation of these pathways exits the cell quiescence state—just as upregulation of the Raf/MAPK and PI3K/Akt pathways does (Akt is protein kinase B). These comparable cell cycle responses can explain why overexpressed YAP1 and β-catenin can bypass K-Ras4B inhibition. Drug resistance to blocking PI3K can arise through the analogous (e.g. β-catenin) cell cycle action via transcriptional regulation on translational control [14,15]. In line with the comparable MAPK vs. YAP1 cell cycle actions, the nucleotide sequences of the DNA response elements of the downstream transcription factor complexes modulated by these two cellular pathways are similar [13,27,28].

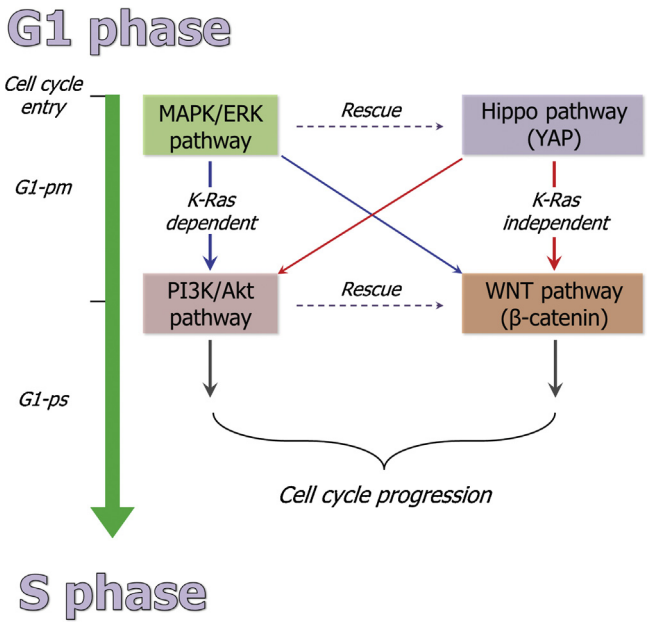

Fig. 1. The concept described in this work. It is inspired by recent landmark reports (e.g. Refs. [1–4,18]) merged with earlier ones and an increasing body of new experimental and clinical observations. Combined, the data essentially point to two independent pathways in proliferation: MAPK and PI3K (via phosphorylation), and YAP1 and c-Myc (β-catenin, Notch, Hedgehog, etc. through direct transcription regulation). The four combinations of the two – Ras-dependent and Ras-independent – oncogenic pathways can remove the imposed controls on cell cycle regulation at the G1 to S restriction point. The figure depicts the four combinations of the pathways: MAPK + PI3K; YAP1 + β-catenin; MAPK + β-catenin; and PI3K + YAP1 which may take place in drug resistance. A single combination can dysregulate the cell cycle, thus sustaining proliferative signaling. Overexpression of YAP1 and β-catenin (or broadly Myc) is able to rescue tumor cells in Ras drug resistance because they act in ways analogous to MAPK/ERK and PI3K/Akt in cell cycle regulation. Both act *consecutively* in the G1 phase through the G1 → S cell cycle restriction point.

Fig. 1 outlines the general concept described in this work. It illustrates the two independent pathways in proliferation: MAPK and PI3K (via phosphorylation), and YAP1 and c-Myc (β-catenin, Notch, Hedgehog, etc. through direct transcription regulation). Even though here we focus on K-Ras, stimulated receptors can signal through the MAPK pathway (e.g. GPCRs and TNF) and PI3K/Akt/mTOR (TLR, JAK-STAT, and more) bypassing the oncogenic K-Ras protein. The figure depicts the four combinations of the pathways (MAPK + PI3K; YAP1 + β-catenin; MAPK + β-catenin; PI3K + YAP1) which may take place in drug resistance. A single combination can result in cell cycle dysregulation, thus sustaining proliferative signaling. Fig. 2 illustrates the two corresponding and independent pathways in the framework of the cell.

2. G1 cell cycle deregulation in proliferation

The cell cycle [30] is sequential (Fig. 3) with five states, G1-pm (Gap 1-postmitotic), G1-ps (G1-pre-S), S (Synthesis), G2 (Gap 2), and M (Mitosis). The cell commits to complete its division cycle in the G1 phase, making it a key regulatory restriction point. This determination for cell proliferation takes place at the transition from the G1 to the S phase; deregulation promotes oncogenesis [30–34]. There are two exit states from the cell cycle, quiescence and senescence. Quiescence (G0) is induced by a prolonged absence of growth signals. It is reversible and maintained by high

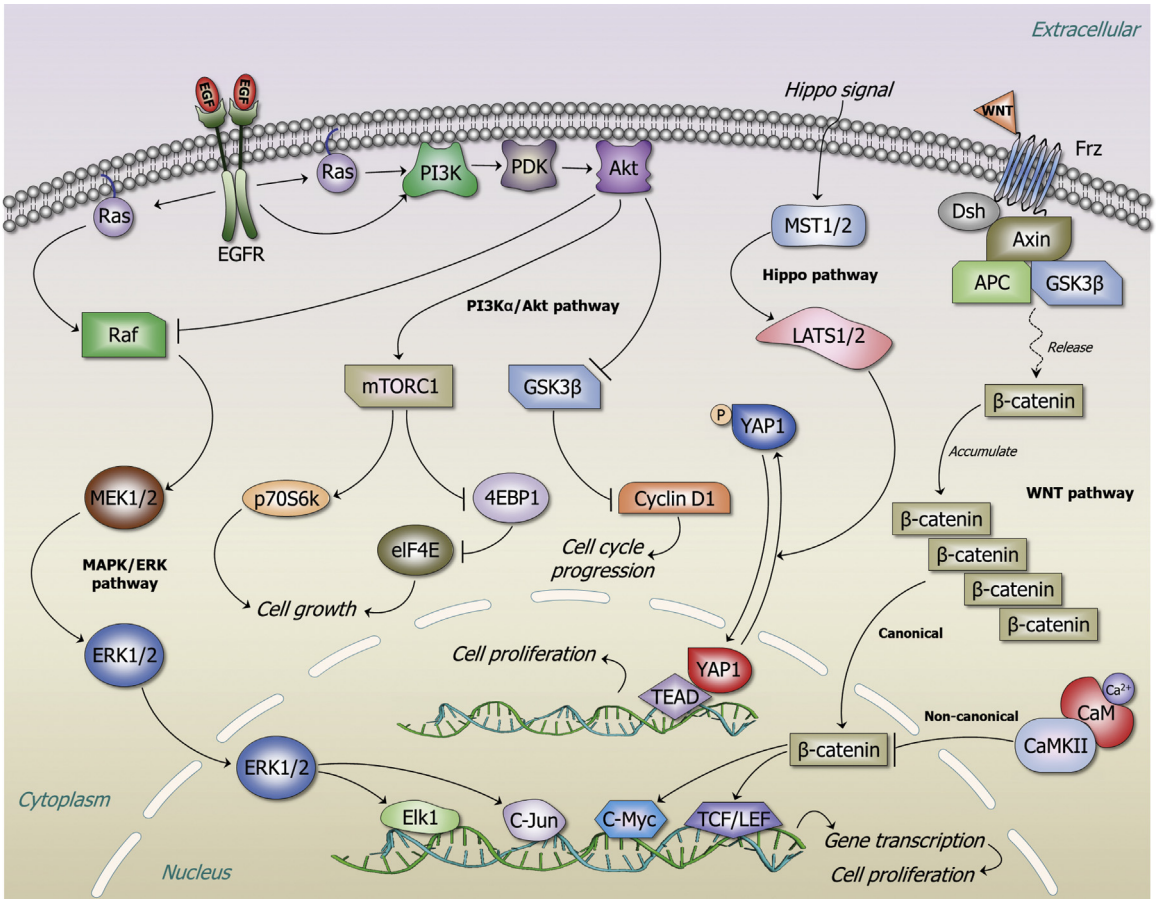

Fig. 2. The two corresponding and independent pathways in cell proliferation: the first is MAPK and PI3K (on the left hand-side of the figure) through phosphorylation, and the second is YAP1 and β-catenin (or Notch, Hedgehog, etc.) through transcription regulation (on the right hand-side). Ras signaling contributes through the first. MAPK activates transcription factors such as Elk1 and c-Jun. The Hippo pathway regulates the phosphorylation and thus degradation of YAP; signaling through the WNT pathway translocates β-catenin to the nucleus where it binds to TCF/LEF transcription factors, dysregulating c-Myc. β-catenin upregulates c-Myc. Together they act to enhance proliferation. The correspondence of the two pathways at the cell cycle level (MAPK/ERK with YAP1; PI3K/Akt with β-catenin or other c-Myc promoting factor, see Fig. 1) can explain drug resistance in Ras pathways-driven cancers. Drug resistance to Raf can take place by high levels of YAP1, and resistance to PI3K via c-Myc.

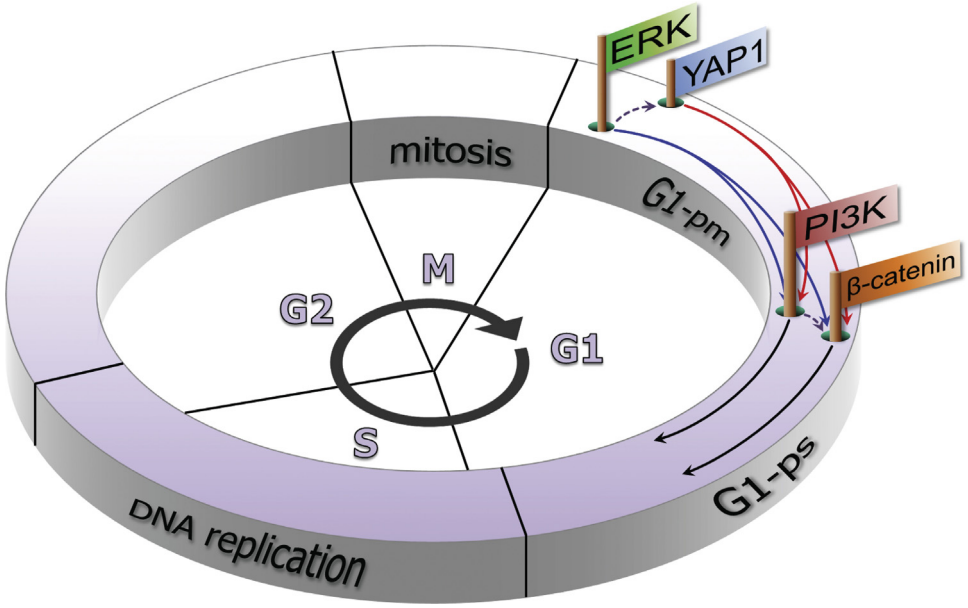

Fig. 3. An overview of the cell cycle phases and the steps where ERK and YAP1, and PI3K and β-catenin, correspondingly act. ERK and YAP1 regulate the G1 phase early, whereas PI3K and β-catenin late. To go through the critical G1 → S cell cycle restriction point after which DNA synthesis takes place, passing through both early and late G1 stages is a requirement. ERK and YAP1, and PI3K and β-catenin, correspond to each other because they act in the *same* cell cycle stage; MAPK and PI3K cell cycle actions are consecutive as are those of YAP1 and β-catenin. As Fig. 1 shows, any one of the four combinations of the two pathways can work. This model can explain and forecast drug resistance in the Ras signaling pathway. Drugging Ras itself can promote resistance through Hippo/YAP1 and/or WNT/β-catenin. G1-pm is post-mitosis; G1-ps pre-synthesis.

levels of CDK inhibitor p27 [35–37]; senescence is irreversible. Oncogene-induced senescence, which can take place in unbalanced Ras signaling (e.g. MAPK but not PI3K/Akt) is one example. The two states differ in the pRb, a tumor suppressor protein, phosphorylation status and the abundance of the associated CDK inhibitors. A bistable switch allows the cell to switch states at certain *RB-E2F* concentrations [38–40] and constitutes the cell cycle restriction point. Once passed, the cell commits to complete the division cycle even in the absence of growth signals. Via its major pathways K-Ras signaling can regulate G1 cell cycle progression at two *consecutive* stages [41]: the early 'growth factor-dependent' and the late 'cell growth' [42]. The early 'growth factor-dependent' cue is transmitted by the MAPK/ERK pathway; the late 'cell growth' signal is transmitted via the PI3K/Akt/mTOR pathway. Both act through specific Cyclin:CDK complexes [43]. The MAPK/ERK pathway leads to CyclinD:CDK4/6 phosphorylating the hypophosphorylated pRb in the early G1-pm (or G1E) phase; the hyperphosphorylated pRB releases the E2F transcription factors. The bistable pRb/E2F transition at the peak of the restriction point in G1-ps is through the activation of CyclinE:CDK2 by the PI3K pathway. Via CyclinE:CDK2 the PI3K pathway acts in the late phase G1-ps (or G1L) phase committing the proliferating cell to complete cell cycle progression.

Ras signaling pathways impact cell cycle progress and regulation [44–46], particularly at the G1 phase [47]. YAP corresponds to ERK, and β-catenin (or Myc) corresponds to PI3K [48] (Fig. 1). Thus, oncogenic modulation of the Hippo and WNT pathways to overexpress YAP and β-catenin and activation of Ras pathways lead to the same cell cycle outcome with analogous actions. YAP1 and β-catenin are integral parts of cell cycle regulation in cells with encoded contact inhibition. Accordingly, YAP is required for proliferation in endothelial cells but apparently not HeLa cells [1].

3. K-Ras-specific signaling

We draw attention to two significant points in K-Ras4B related signaling: (i) we point out that it may vary in different cells. In CaM/Ca^{2+}-rich tissues signaling may preferentially take place through MAPK/ERK and PI3K/Akt/mTOR pathways; in contrast, in tissues with abundant accessible CaM/CaMKII it may act by suppressing the non-canonical WNT pathway [49] (Fig. 2). Mechanistically, in the first scenario it acts by modulating MAPK and fully activating the PI3Kα/Akt/mTOR pathways. In the second, in cells with high levels of CaMKII expression, as in hippocampal neurons (estimated to be from 1 to 2% of the total protein [50]), or where the available CaM/Ca^{2+} is relatively low, CaMKII activity is reduced suppressing non-canonical WNT signaling, thus increasing β-catenin/TCF activation [49]. The interaction of K-Ras4B with CaM/Ca^{2+} is unstable and to date no crystal structure is available despite years of effort, which is not the case for CaMKII-CaM/Ca^{2+} [51]; thus competitive CaM binding would shift the equilibrium toward CaMKII-CaM/Ca^{2+} unless other currently unknown cellular factors play a role. (ii) YAP1 and β-catenin transcriptional regulators act and confer drug resistance to targeted K-Ras4B by controlling the G1 to S restriction point in the cell-cycle progression. ERK's action corresponds to that of YAP1 and PI3Kα to β-catenin (Fig. 1).

4. ERK and YAP, and PI3K and β-catenin, can act independently and additively in tumor initiation

Thus, here we propose that there are two major *corresponding* and *independent* pathways in cell cycle control in K-Ras driven cancer which may act in an additive manner in tumor initiation: one is MAPK – PI3K, and the other Hippo – WNT. Both act at the same cell cycle restriction point; consequently YAP1 and β-catenin can correspondingly substitute for ERK and PI3K. These corresponding actions suggest how YAP1 can rescue Ras or B-Raf ablation. Combined, oncogenic ERK and PI3K, and YAP1 and β-catenin signaling can result in more aggressive cancer as indeed shown recently [52]. In oncogenic fibroblasts signaling through non-canonical WNT to overexpress β-catenin, either ERK or YAP1 can amplify tumor emergence.

YAP can rescue B-Raf therapeutic ablation, whereas β-catenin can rescue K-Ras/PI3K but not B-Raf. ERK induces cell cycle reentry at the G1 phase; PI3K prompts progression through G1 into S (G1 → S) phase (Fig. 3). These steps are correspondingly also executed by YAP and β-catenin. Thus, to avoid senescence both YAP and β-catenin are required for cell cycle entry and progression. Here our thesis is that regardless of which signaling pathway is invoked, the outcome is unchanged: cell cycle reentry—by ERK or YAP—and progression through G1 into S phase—by PI3K or β-catenin. Only the signaling cue varies: hormone/growth factor in RTK-Ras signaling, or mechanical cell-cell contact controlled by Hippo and WNT. ERK and YAP lead to comparable consequences as do PI3K and β-catenin, and that this is why K-Ras4B drug resistance takes place by commandeering YAP and β-catenin [1,2,14,15]. In cells/tissues with high levels of free CaMKII as in fibroblasts or low CaM/Ca^{2+}, CaMKII suppresses the non-canonical WNT pathway, which results in high β-catenin expression [49]. Combined with high levels of YAP1 or ERK can result in tumor initiation.

The independent cell cycle roles of PI3K and β-catenin at the G1 into S cell cycle phase are supported by recent colorectal cancer cells studies: Tankyrase, a Golgi-associated mitogen-activated protein kinase substrate, inhibition blocks the WNT/β-catenin pathway. When combined with PI3K/Akt inhibitors and applied to the treatment of resistant colorectal cancer, tumor growth is repressed [53]. In line with this, β-catenin confers resistance to PI3K and Akt inhibitors [54], and β-catenin inhibitors block β-catenin-dependent transcriptional activity and synergize with K-Ras inhibitor in colon cancer cells driven by WNT and K-Ras—but not in cells carrying B-Raf mutations [55]. B-Raf mutations would correspond to YAP.

High YAP activity expands progenitor cell populations in the liver, intestine, nervous system, and skin, although not in the heart [56] or hematopoietic system [57].

5. CaM interacts with K-Ras; but not with N- and H-Ras

In ductal tissues *KRAS*-driven cancers are the most highly expressed as compared with other Ras isoforms. Oncogenic K-Ras4B and depalmitoylated K-Ras4A [22] are the only isoforms that may exploit CaM to modulate MAPK and fully stimulate the PI3Kα/Akt/mTOR pathway (Fig. 4). The CaM/Ca^{2+}-rich ductal tissue environment may explain why PI3K/Akt/mTOR signaling is reinforced in these *KRAS*-driven cancers [58] for passing through the G1/S restriction point.

Why only K-Ras binds CaM? The sequences of H-Ras, N-Ras, K-Ras4A and K-Ras4B are highly similar in the catalytic domains but differ in the C-terminal HVRs [59] resulting in differential interactions of the HVRs with the membrane and CaM. The HVRs of H-Ras and N-Ras are only weakly positively charged (+2 and +1, respectively); that of the K-Ras4A is most similar to K-Ras4B with both being highly positively charged although that of the K-Ras4B is more so (+6 and +9, respectively). In addition, H-Ras has two palmitoyl groups and a farnesyl, N-Ras one palmitoyl and farnesyl. K-Ras4A also has one palmitoyl and farnesyl, and K-Ras4B only a farnesyl. Palmitoyl can be cleaved thus is reversible; farnesyl is not. K-Ras4A may thus be bimodal with two functional states [22]: In the first it has only farnesyl (state 1); in the second palmitoyl and farnesyl (state 2). With a polybasic region and only farnesyl K-

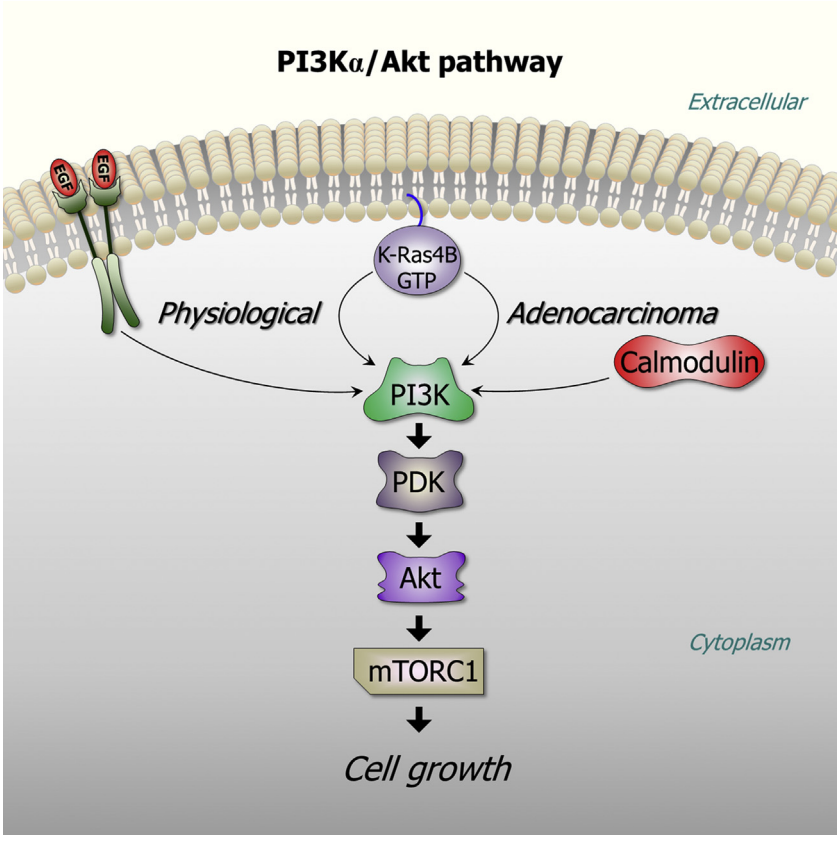

Fig. 4. The mechanism through which calmodulin and calcium can promote KRAS-driven adenocarcinomas. *KRAS*-driven cancers are the most highly expressed in ductal tissues where CaM/Ca^{2+} are abundant as compared with other Ras isoforms. Oncogenic K-Ras4B and depalmitoylated K-Ras4A [22] are the only isoforms that can stably bind CaM/Ca^{2+} due to their highly positively charged C-terminal HVRs. Calmodulin acts by fully activating PI3Kα, substituting the missing EGFR signal in oncogenic K-Ras signaling, thus stimulating the PI3Kα/Akt/mTOR pathway. The left hand-side pathway depicts normal physiological signaling, with PI3Kα activation resulting from GTP-bound K-Ras4B and an EGFR cue. On the right, in the absence of the EGFR signal, CaM interacts with the SH2 domains of the p85 subunit of PI3Kα, both unlocking the inhibitory action of nSH2 and allosterically activating the p110 catalytic subunit through its binding to the cSH2 domain. The CaM/Ca^{2+}-rich environment on the right hand-side clarifies why PI3K/Akt signaling is amplified in these *KRAS*-driven cancers [58] for passing through the G1 → S restriction point [74].

Ras4A resembles K-Ras4B; with farnesyl and palmitoyl it resembles N-Ras. The relative proportions of the two states in different tissues are unknown; we expect that state 1 will be observed with higher levels in CaM/Ca^{2+}-rich tissues. The positively charged HVR is able to associate with a negatively charged inner leaflet of the plasma membrane. The farnesyl prefers to insert into liquid ordered phase membrane, where unsaturated fatty acids are abundant [60]. K-Ras4B and to a lesser extent depalmitoylated K-Ras4A is the only isoforms with a strongly positively charged HVR which can interact with the negatively charged CaM linker surface, with electrostatics being the major driving force to the (nonspecific) CaM/K-Ras interaction. The farnesyl group docks into CaM further stabilizing the complex. An added palmitoyl may sterically interfere with CaM binding.

CaM's binding to the GTP-bound K-Ras4B differentially affects its two key effector pathways. The Raf/MAPK pathway stimulates a proliferative effect by inducing the expression of G1/S-specific cyclin D1. Cyclin D1 interacts with CDK4, which phosphorylates and inhibits the pRb thereby regulating G1/S phase transition and cell cycle progression. Since Ras anchoring in the membrane is required for Raf's activation and CaM's binding to K-Ras shifts the equilibrium toward its membrane-free form, CaM's binding to K-Ras4B-GTP temporally downregulates Raf modulating MAPK signaling. It stimulates the PI3Kα/Akt pathway, which propels the cell cycle and cell migration. To understand how CaM stimulates PI3Kα, we modeled the K-Ras4B-GTP/CaM/PI3Kα ternary complex (Fig. 4). We used the G-domain of K-Ras, full length CaM and the p110 catalytic and p85 regulatory subunits of PI3K as target proteins [58]. Using a powerful template-based protein-protein complex structure prediction algorithm (PRISM) [61,62] we built models for the binary interactions between K-Ras4B and PI3Kα, and CaM and PI3Kα, then constructed the ternary complex based on the binary interactions and available literature data. Two molecules of CaM can bind to PI3Kα in this complex: one to the cSH2 domain of the p85 regulatory subunit, the other to the nSH2 domain. The CaM-cSH2 interaction is an integral part of the K-Ras4B/PI3Kα/CaM trimer. However, for full PI3Kα activation both CaM's binding events are required. CaM's binding to cSH2, which takes place along with its binding to membrane-free K-Ras4B, allosterically stimulates p110 catalysis; CaM's binding to nSH2 relieves nSH2 autoinhibitory action on p110 in a manner similar to that executed by the RTK's pYXXM peptide under physiological conditions.

An alternative pathway involving CaM/K-Ras specific interaction may work by depleting available CaM for the interaction with CaMKII [49]. In this K-Ras-specific pathway the free CaMKII depresses the non-canonical WNT, which results in overexpression of β-catenin. Since the affinity of CaMKII to CaM is much higher than to K-Ras, tissues harboring this pathway may have higher levels of available CaMKII. Whichever pathway is adopted the outcome is unchanged: YAP and β-catenin and the corresponding ERK and PI3K can act independently or additively.

6. Discussion

Recent landmark discoveries revealed that YAP1 and β-catenin are an integral part of cell cycle regulation in cells with encoded

contact inhibition [1,2]; in parallel, recent striking reports indicated the ability of overexpressed YAP1 to offset MAPK inhibition [3,4]. These remarkable findings combine with an increasing body of compelling observations consistently indicating that overexpression of YAP1, and of proteins upregulating *MYC* (such as β-catenin [5–7], Notch [8,9], Hedgehog [10–12], and eIF4E [13–16]) correspondingly promote proliferation of cells treated with MAPK or PI3K inhibitors. Notably, MAPK/ERK and PI3K/Akt/mTOR pathways are well-established to act at the G1 phase cell cycle restriction [63–67]. We propose that the emerging picture from these experimental and clinical data point to oncogenic *KRAS* – and YAP1 and β-catenin – playing similar roles in cell cycle control in tumor initiation. Thus, our major thesis is that overexpression of YAP1 and β-catenin (or broadly c-Myc) is able to rescue tumor cells in Ras drug resistance—because they act *consecutively* in the G1 phase through the G1 → S cell cycle restriction point just like MAPK/ERK and PI3K/Akt do (Fig. 3). We suggest that this explains why mutations in YAP1 and β-catenin (or in the Hippo and WNT pathways), are often observed in *KRAS*-driven cancers. Further, the *independence* and *correspondence* of the two pathways – MAPK and PI3K; and YAP1 and c-Myc – also explain the clinical data of why when combined they can result in more aggressive tumors. Thus, for example, mutations in APC, a tumor suppressor that plays a critical role in the turnover of cytosolic β-catenin, and in K-Ras, may collaborate in promoting cancer stem cell phenotypes and in drug resistance. On the other hand, K-Ras^{G12D}-driven leukemogenesis – where cell contact plays no role – may not require β-catenin [68].

Going forward, the implications of our new understanding could be significant. They argue that to deter drug resistance in *KRAS*-driven cancers [69,70], MAPK and PI3K/Akt/mTOR, and the Hippo and WNT (or other c-Myc promoting pathway combinations, Figs. 1 and 3)—should be co-targeted. Currently, multiple strategies against *Ras*-driven cancers are investigated; some are broad (e.g. Refs. [71,72]; reviewed in Refs. [73]); others might lead to K-Ras4B specific therapeutics [58,74]. Promising powerful interventions are also tested against mutant Ras pathways proteins, such as Raf, ERK, and PI3K. However, even if successful, drug resistance will inevitably take place. Forecasting alternate pathways that can be involved—and the reason for this involvement—can be expected to open new horizons. Here we propose blueprints toward this critically-important goal: the four equivalent combinations of the components of the two major pathways through which drug resistance can emerge.

Working out the critical details – pathway linkages, combinations and actions, specific proteins to target and toxicity – is an immense challenge. Our model indicates that oncogenic signaling in cancer initiation can be organized into two pathways; it also points to their equivalences in cell cycle roles, and is able to suggest an explanation for the drug resistance. However, to paraphrase W. Somerset Maugham, we, as a community, still have a long and arduous road to travel to decipher the cell's immense complexity and treat its oncogenic breakdown.

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