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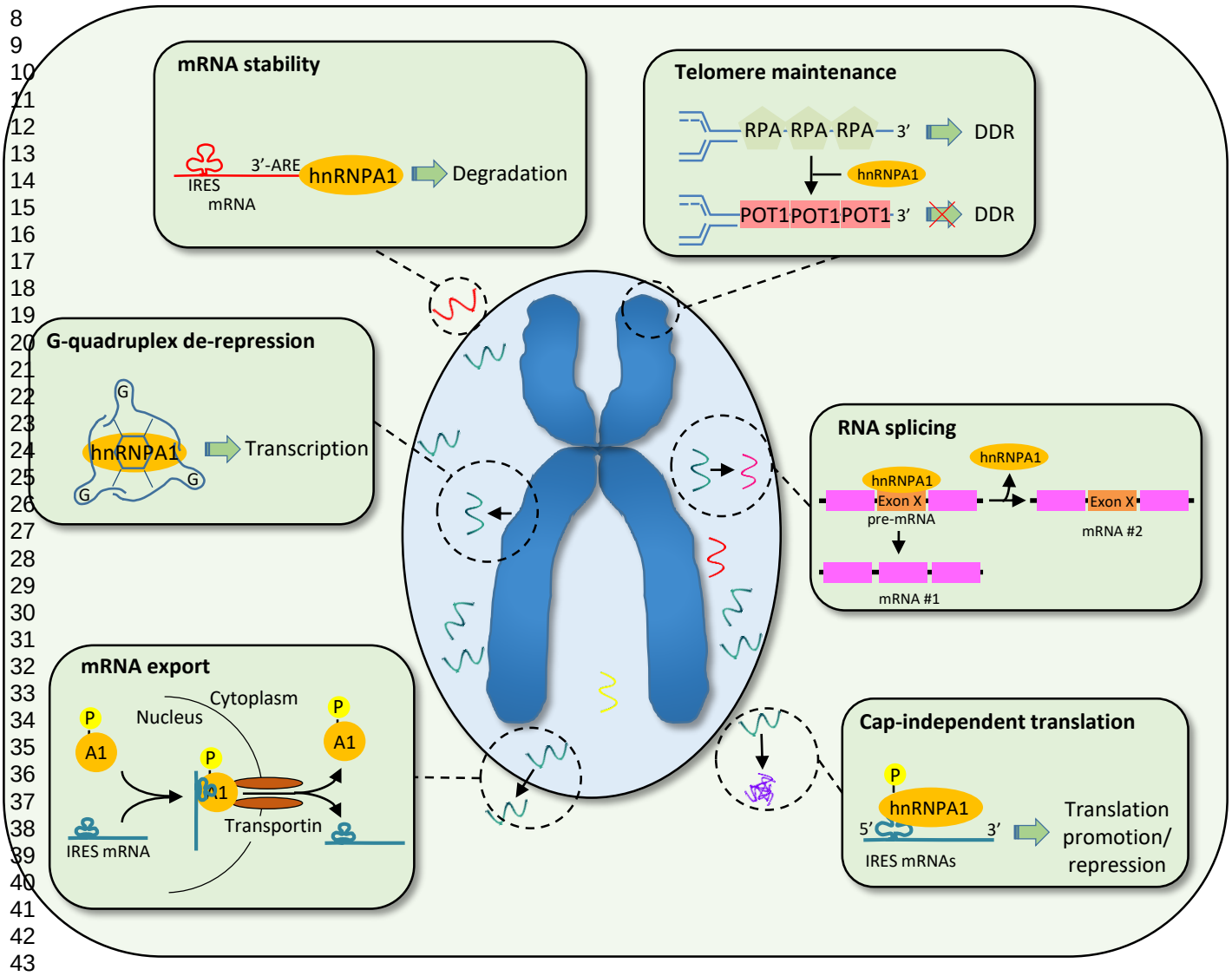
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IRES
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hnRNPA1
Degradation
Telomere maintenance
RPA RPA RPA 3'
DDR
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POT1 POT1 POT1 3'
DDR
G-quadruplex de-repression
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Transcription
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IRES mRNA
Cap-independent translation
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IRES mRNAs
Translation promotion/ repression

Emerging roles of hnRNPA1 in modulating malignant transformation

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ABSTRACT

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are RNA-binding proteins associated with complex and diverse biological processes such as processing of heterogeneous nuclear RNAs (hnRNAs) into mature mRNAs, RNA splicing, transactivation of gene expression and modulation of protein translation. hnRNPA1 is the most abundant and ubiquitously expressed member of this protein family and has been shown to be involved in multiple molecular events driving malignant transformation. In addition to selective mRNA splicing events promoting expression of specific protein variants, hnRNPA1 regulates the gene expression and translation of several key players associated with tumorigenesis and cancer progression. Here, we will summarise our current knowledge of the involvement of hnRNPA1 in cancer, including its roles in regulating cell proliferation, invasiveness, metabolism, adaptation to stress and immortalisation.

INTRODUCTION

Heterogeneous nuclear ribonucleoproteins (hnRNP) are a family of RNA-binding proteins (RBPs) comprising 20 members in humans, labelled A1 to U [1, 2]. In addition, several hnRNP genes generate multiple isoforms through alternative splicing. Hence, the total number of functional hnRNPs is hard to determine as low-abundant variants still evade characterisation. These hnRNPs are known to be highly conserved throughout evolution highlighting their functional significance. Historically, hnRNPs have been defined as proteins that associate with nascent transcripts (namely, pre-mRNAs or hnRNAs), precursors of the functional, protein coding mRNAs [3]. Initial studies indicated that nascent RNA polymerase II (RNAPII) transcripts were packaged with six "core" hnRNP proteins (A1, A2, B1, B2, C1 and C2) in a bead-on-a-string structure that resembles the organization of DNA nucleosomes [4]. Their functions in cellular nucleic acid metabolism include alternative splicing, mRNA stabilization and transcriptional regulation [5]. As such, they play a fundamental role in the regulation of gene expression through maturation of nascent transcripts, modulation of the capping, splicing, polyadenylation, nuclear export, stability rates and translation of cellular messenger RNAs (mRNAs) by binding specific sequences or secondary structures within these transcripts [5].

All hnRNPs share a common modular structure consisting of one or more highly conserved RNA-binding domains (RBDs), also known as RNA recognition motifs (RRMs). RRMs are approximately 90 amino acids-long and participate in both general and specific interactions with nucleic acids[6 7]. As well as binding nucleic acids, the RRM of certain hnRNPs, such as, hnRNPA1, are involved in protein–protein interactions. In addition to RRMs, hnRNPs contain auxiliary domains, including glycine-rich, acidic or proline-rich domains, which also mediate protein-protein interactions, subcellular localization and functional specificity. Although predominantly nuclear, hnRNPs may be transported out of the nucleus together with cargo mRNAs and be sequestered to various cellular compartments [5, 8, 9]. hnRNPs undergo several post-translational modifications, including phosphorylation, sumoylation, ubiquitination, acetylation and methylation, which may play crucial roles in regulating

their activity. Amongst hnRNPs, hnRNPA1 is the best studied member of the family. Its role in controlling RNA splicing is well established but other functions of hnRNPA1 in mediating cellular signalling are only now being appreciated. As such, hnRNPA1 has been found overexpressed in various tumour types, including lung cancer [10], Burkitt lymphoma [11], multiple myeloma [12] and leukemia [13] where it is thought to regulate tumorigenesis through a variety of mechanisms that impact multiple hallmarks of cancer. Indeed, hnRNPA1 is involved in telomere length maintenance through the activation of telomerase, which initiates malignant transformation [14]. It then promotes cell cycle progression [15, 16] and aerobic glycolysis to support tumour growth [17, 18]. Finally, our failure to cure patients suffering from various malignancies is associated with the development of drug resistant and metastatic disease, processes in which hnRNPA1 plays crucial roles. For instance, hnRNPA1 controls the nucleo-cytoplasmic shuttling and translation of anti-apoptotic mRNAs [9, 19, 20], to promote tumour maintenance and therapy resistance. hnRNPA1 also regulates the splicing of genes such as CD44 and SRSF1 to promote metastatic dissemination of cancer cells [21-23]. Consequently, hnRNPA1 supports various stages of tumour progression and this review will attempt to condense our current knowledge of the molecular mechanisms involved.

AN OVERVIEW OF hnRNPA1 STRUCTURE, POST-TRANSLATIONAL MODIFICATIONS AND BINDING SPECIFICITY

Structure

hnRNPA1 is one of the most abundant and ubiquitously expressed nuclear proteins and plays a major role in mRNA biogenesis. While there are several reported coding and non-coding transcripts of hnRNPA1, only two isoforms are well characterized: A1-B (372 aa, 38 kDa, ENSEMBL ID ENST00000340913), and A1-A (320 aa, 34 kDa, ENSEMBL ID ENST00000546500), with the smaller isoform being 20 times more abundant than the larger one [24]. The N-terminus of the protein contains two RBDs, referred to as RRM1 and RRM2, which mediate interactions with target RNAs

(Figure 1). In addition, a flexible Glycine rich C-terminal region consisting of Arg-Gly-Gly tripeptide repeats (RGG), interspersed with aromatic (Phe, Tyr) residues to form RGG boxes provide both protein and RNA binding capabilities to hnRNPA1. Despite a high degree of homology between the two RRMs, these domains operate as functionally distinct entities. Two conserved Phe residues in each of the RRMs appear to be involved in specific RNA-protein interactions and are essential for modulating alternative splicing but not for general pre-mRNA binding or RNA annealing activity [25]. While the precise regulation of the RGG boxes is unknown, they were deemed essential for alternative splicing activity, stable RNA binding and optimal RNA annealing activities [25]. However, more recently, a truncated form of the protein, lacking one of the RBDs and the RGG-box region, has been shown to still be able to regulate splicing of a reporter mini-gene and down-regulate replication of the HIV-1 virus [26]. Downstream from the RGG boxes, the Gly rich domain also harbours the M9 sequence that is involved in the bi-directional cytoplasmic-nuclear shuttling of hnRNPA1 [27] in response to specific stimuli [28, 29]. As such, M9 is distinct from other known nuclear localisation signals as it mediates both the nuclear import and export of hnRNPA1. This is achieved through the direct interaction of M9 with the receptors Transportin 1 and 2, of the β-Karyopherin family.

Post-translational modifications

hnRNPA1 has been shown to be post-translationally modified via phosphorylation, methylation, ubiquitination and sumoylation: Arginines in the RGG motifs can be methylated to regulate RNA binding activity [30-32], Serines present at both N- and C-termini are phosphorylated by kinases such as Protein Kinase C (PKC), MAPKs and S6-kinases [9, 29, 33, 34] and at least one sumoylation/ubiquitination site is contained within the RRM2 [28 35]. These post-translational modifications have been shown to control the activity of hnRNPA1 in response to upstream signals. For instance, phosphorylation and sumoylation regulate the ability of hnRNPA1 to bind RNAs and shuttle between nucleus and cytoplasm [8, 28]. Sites known to incur post-translation modifications are highlighted in Figure 1.

Binding specificity

hnRNPA1 was initially discovered as a DNA-binding protein [36, 37] whereby its binding to hormone response elements resulted in vitamin D-resistant rickets [37]. Subsequently, its RNA-binding capabilities were established and consensus binding sequences identified. Various reports suggested that hnRNPA1 specifically binds AUUUA-rich sequences or UAGGGA(U)-motifs present in the 3'-UTR region of transcripts [38, 39]. Later, a number of publications also suggested that the UAGA(G) motif found in many transcripts can also bind hnRNPA1 [40-42]. Therefore, hnRNPA1 may bind transcripts through various motifs and it may be hypothesised that post-translational modifications impact the sequence specificity of hnRNPA1 binding. This hypothesis is supported by our own findings showing that S6K2-mediated phosphorylation on Ser 4 and 6 promotes binding of hnRNPA1 to IRES-containing mRNAs over mRNAs devoid of this structure [9]. In addition, Ser4/6 phosphorylation did not equally impact the binding to all IRES-containing mRNAs as interaction of hnRNPA1 to the cIAP1 mRNA was left unchanged by this post-translational modification while that to the BCL-XL and XIAP mRNAs was increased [9]. Hence, this suggests that additional cues in the sequence or structure of mRNAs may be involved in modulating interaction with hnRNPA1.

REGULATION OF CELL SURVIVAL

Tumour cells develop under stress conditions, including reduced nutrients and growth factors availability and increased exposure to oxidative damage. In order to thrive, they must overcome death-triggering signals, particularly those leading to apoptosis (programmed cell death). Resistance to apoptosis in cancer is commonly achieved through the over-expression of specific anti-apoptotic genes. Of particular interest are members of the inhibitor of apoptosis (IAP) and Bcl-2 families of proteins that interfere with multiple steps of the apoptotic process. X chromosome-linked inhibitor of apoptosis (XIAP) is the most potent member of the IAP family. It directly interacts with and inhibits caspases 3, 7, and 9 and is therefore a key regulator of apoptosis [43]. In contrast, BCLXL controls apoptosis by maintaining mitochondrial membrane homeostasis [44] to prevent

cytochrome c release thereby blocking the formation of the apoptosome complex in the cytoplasm. Interestingly, both the XIAP and BCLXL mRNAs contain an internal ribosome entry site (IRES) that drives their translation by a cap-independent mechanism in situations where cap-dependent translation is inhibited (eg. cellular stress) [45-47]. In contrast, under normal physiological conditions, these IRESs limits the translation of these mRNAs to tightly control the expression of these pro-survival proteins. Although the mechanism regulating IRES-mediated translation is still poorly understood, it is now clear that it requires the binding of some of the canonical initiation factors as well as that of additional proteins termed IRES-trans acting factors (ITAFs) [48]. hnRNPA1 is such an ITAF and could harbour both stimulatory and repressive activity on IRES-mediated translation. Indeed, while it enhances IRES-mediated translation of *Basic Fibroblast Growth Factor (FGF2)* [49], *CYCLIN D1* and *cMYC* mRNAs [50, 51], it represses that of key apoptosis regulatory molecules such as *XIAP*, *BCLXL* and *APAF1* [9, 19, 52].

Five different isoforms of FGF2 exist, each having distinct functions in cell survival, immortalization and transformation. Except for the one coding the largest isoform, all FGF2 mRNAs contain an IRES element in their 5'- untranslated region (UTR) [53, 54]. *FGF2* mRNAs contain several alternative translation start sites and hnRNPA1 preferentially promotes translation initiation events at a given codon [49]. In addition, hnRNPA1 promotes translation initiation events at internal start codons through its binding to the IRES. The role of FGF2 in tumorigenesis has been largely attributed to its function in cancer cell survival, proliferation and angiogenesis [9, 55]. Interestingly, it has been demonstrated that the p53 tumour suppressor protein inhibits FGF2 translation through its direct binding to the FGF2 mRNA [56, 57], revealing important links between tumorigenesis and the control of FGF2 mRNA translation. The control of FGF2 mRNA translation may represent the first identified of many such interplays between p53 and hnRNPA1 in controlling the expression of proteins important to tumour progression and this warrants further investigation. Moreover, the translational

regulation of FGF2, and subsequent auto/paracrine FGF-2 signalling, may be the priming step in promoting a selective translational program that promotes tumour progression.

In this regard, our own work provided a detailed mechanistic insight into how FGF2 can enhance the translation of specific mRNAs necessary for cell survival and drug resistance [9]. A schematic of the mechanism is described in Figure 2. Stimulation of lung cancer cells with physiologically-relevant concentrations of FGF2 activates the kinase S6K2 that binds and phosphorylates hnRNPA1 on the N-terminal Ser4/6 residues. This phosphorylation event increases the association of hnRNPA1 with BCLXL and XIAP mRNAs to promote their nuclear export. Once in the cytoplasm, phospho-Ser4/6-hnRNPA1 dissociates from these mRNAs, de-repressing their IRES-mediated translation. The dissociation of hnRNPA1 from cargo mRNAs correlates with the phosphorylation-dependent association of hnRNPA1 with the scaffolding proteins, 14-3-3σ and 14-3-3θ, leading to sumoylation on K183 and re-import of hnRNPA1 into the nucleus.

The above FGF-2-stimulated cycling of hnRNPA1 in and out of the nucleus partly explains how this protein, in concert with various interacting partners, enhances translation of specific mRNAs. Also, , while hnRNPA1 has previously been reported to be a translation repressor [58, 59], this cycle demonstrates that post-translational modifications can over-rule this repression to promote the translation of IRES-containing mRNAs. Hence, our findings reconcile the over-expression of hnRNPA1 seen in many cancers with the need for enhanced translation of pro-survival proteins in this disease. Indeed, elevated levels of BCLXL in cancer biopsies correlate with increased hnRNPA1 nuclear location and increased S6K2 expression [9], consistent with the fact that knockdown of hnRNPA1 leads to apoptosis in various cancer cells [60].It is also noteworthy that the serum levels of FGF2 are highly increased in patients suffering from a variety of carcinoma [61], so that the hnRNPA1 cycle described above may represent a conserved mechanism for pro-tumorigenic mRNA translation in various cancers.

cIAP1 is another member of the IAP family that is involved in the regulation of cell survival in lung [62, 63] and other cancers [62].While the 5'UTR of cIAP1 also harbours an IRES element [64], the export of this mRNA is not promoted by FGF2-mediated hnRNPA1 Ser4/6 phosphorylation[9]. Instead, hnRNPA1 has been shown to bind an AU-rich element (ARE) within the 3'-UTR of the cIAP1 mRNA rather than its IRES, leading to altered mRNA stability [9, 65]. Indeed, accumulation of hnRNPA1 in the cytoplasm following UV irradiation leads to destabilization of cIAP1 mRNA. This reduces cellular cIAP1 protein levels, activating both canonical and non-canonical NF-κB signalling, via receptor interacting protein (RIP1) and NF-kB inducing kinase (NIK), respectively [65-67]. There are 3 ARE sequences in the 3'-UTR of cIAP1 and hnRNPA1 binds only two of these sequences [65]. It has been recognized that the binding of proteins to AREs is dependent on both the primary sequence and sequence context, and that the presence of AUUUA in itself does not guarantee a functional ARE [68]. Our data suggest that a similar scenario could be envisaged for IRES elements. The selectivity of hnRNPA1 for particular IRESs is the subject of further studies in our laboratory and preliminary data suggest that it is regulated by the specific sites-specific post-translational modifications on hnRNPA1 elicited by individual upstream signals.

ALTERATION OF CELL CYCLE

cMYC is a cellular proto-oncogene associated various human cancers and is strongly implicated in the control of cellular proliferation, programmed cell death, and differentiation [69]. The expression of the *cMYC* gene is closely correlated with growth, and removal of growth factors at any point in the cell cycle results in its prompt downregulation [70]. Loss of cMyc causes a profound growth defect manifested by the lengthening of the G1 phase of the cell cycle [71]. The transition from G1/G0 to S phase is controlled by a series of sequential regulatory events in which, D-type cyclins along with their binding partners, cyclin-dependent kinases (Cdks) Cdk4 and Cdk6, play a major role [72]. The major targets of the cyclin D-CDK complexes are the retinoblastoma (Rb) proteins, and their phosphorylation activates the E2F family of transcription factors and the downstream expression of

S-phase-specific genes such as Thymidine kinase and DNA Polymerase α [73-75]. Knockdown of hnRNPA1 has been shown to inhibit proliferation of lung adenocarcinoma through arrest at the G0/G1 phase of the cell cycle [16]. Interestingly, translation of both cyclin D1 and *cMYC* mRNAs occur via IRES-mediated initiation under conditions of reduced cap-dependent translation and Akt activity [76]. hnRNPA1 binds and promotes translation through these IRESs both *in vitro* and in intact cells [50]. AKT regulates this by inducing phosphorylation of hnRNPA1 on serine 199, leading to normal binding of hnRNPA1 to IRESs but preventing IRES-mediated translation *in vitro*. The small-molecule compound, Rapamycin, inhibits the mammalian target of rapamycin protein (mTOR) kinase resulting in the global inhibition of cap-dependent protein synthesis, a blockade in ribosome component biosynthesis, and G_1 cell cycle arrest [77, 78]. Hypersensitivity to mTOR inhibitors has been demonstrated in cells having elevated levels of AKT kinase activity, whereas cells containing quiescent AKT activity are relatively resistant [76]. The adaptive response of tumour cells to mTOR inhibitors through stimulation of the cap-independent translation of critical cell cycle proteins may constitute a mechanism of cellular resistance to these drugs. In particular, tumours with relatively little dependence on the phosphatidylinositol 3-kinase/AKT/mTOR signaling cascade (*i.e.* low AKT activity) could markedly increase the hnRNPA1 dependent cap-independent synthesis of cyclin D1 and cMYC following mTOR inhibition. Further studies into the mechanisms through which AKT-mediated phosphorylation of hnRNPA1 inhibits translation of IRES-containing nRNAs may propose synthetically lethal combination with mTOR inhibitors. Also, these data suggest that the serine 199 phosphorylation status of hnRNPA1 may be an effective response biomarker to mTOR inhibitors.

TELOMERE MAINTENANCE

Telomeres are repetitive elements at eukaryotic chromosomal termini that protects linear ends of chromosomes from being recognised as broken DNA ,thereby evading DNA damage response (DDR) [79]. Maintenance of telomeres and the end-capping structure and function they provide, is imperative for preserving genome integrity and stability [80]. Recent evidence has highlighted the role of

hnRNPA1 in the regulation and maintenance of telomere length [81, 82]. A ribonucleoprotein enzyme complex, known as telomerase, maintains telomere length in cancer cells by adding TTAGGG repeats onto telomeric ends, compensating for the normal telomeres shortening that occurs in all dividing cells [80]. Telomere shortening strongly correlates with an increased risk of cancer and chronic disease associated with aging as the loss of telomere capping function contributes to chromosomal instability [83]. hnRNPA1 has been shown to bind telomeric sequences and plays a critical role in telomere biogenesis [84] and maintenance. Indeed, it promotes telomerase activity and telomere length extension, facilitating removal of replication protein A (RPA) from single-stranded telomeric DNA and participating in telomeric end-capping following replication [85-88]. The sensitivity of telomeric regions to unrepaired DSBs has also been proposed as an important contributor to chromosome instability in human cancer. Other proposed functions of hnRNPA1 that may contribute to telomere replication include its ability to unwind G-quadruplexes [87], and to interact with human telomerase [86] as well as telomerase RNA [89]. Telomeric G-rich RNA has been shown to form G-quadruplex structures with telomeric DNA [90] and unwinding of these G-quadruplexes can stimulate telomere elongation, a phenomenon associated with malignant transformation [91].

Telomeres were believed to be heterochromatic in nature until they were shown to be transcribed into heterogeneous, non-coding transcripts, known as telomere repeat-containing RNA (TERRA) [92, 93]. A variety of functions have been proposed for TERRA, including regulation of telomerase activity possibly via interaction with the complementary telomerase RNA template, hTR [93, 94], regulation of telomeric heterochromatin formation [95], promotion of telomere replication [96] and facilitation of end-capping function [88]. Chromatin-bound TERRA specifically localizes at telomeres and *in vitro* structural studies have demonstrated that telomeric RNA can specifically associate with telomeric DNA via formation of hybrid parallel G-quadruplex structures [90]. *In vitro*, naked TERRA molecules are efficient inhibitors of human telomerase, base-pairing via their 5'-UUAGGG-3' repeats with the template sequence of telomerase RNA, in addition to contacting the telomerase reverse

transcriptase protein subunit [97]. *In vivo*, TERRA is partially associated with hnRNPA1 and hnRNPA1 binding to TERRA alleviates its inhibition of telomerase [98]. However, when in excess over TERRA, hnRNPA1 becomes itself an inhibitor of telomere extension following binding of the telomeric DNA substrate [98]. Therefore, telomerase-induced telomere extension may require balanced local levels of TERRA and hnRNPA1 (Figure 3) that function as a bimolecular switch for telomerase activity.

This switch appears to be further regulated through post-translational modification of hnRNPA1. Indeed, hnRNPA1 has been shown to be directly phosphorylated on Ser 95 by the DDR protein DNA-PKcs, a member of the phosphoinositide 3- kinase-like kinase (PI3KK) family [87] and this phosphorylation was stimulated in presence of hTR [14] (Figure 4). Inhibition of DNA-PKcs kinase activity, or silencing of hnRNPA1, led to significant accumulation of TERRA at telomeres and associated increased frequency of fragile telomeres [81]. Fragile sites represent specific chromosomal regions that challenge replication, especially under conditions of limiting nucleotide pools or partial inhibition of DNA polymerases [99]. The idea that common fragile sites represent regions where replication forks stall and collapse is consistent with the increased rate of recombination at these loci [100]. Indeed, common fragile sites are hotspots for deletions and chromosome rearrangements in cancer [101]. hTR/DNA-PKcs and hnRNPA1 interactions at telomeres contribute to the removal of chromatin bound TERRA, thereby facilitating efficient replication of telomeres and effective end-capping [81].

Furthermore, DNA-PKcs mediated phosphorylation of hnRNPA1 could also promote the switch from RPA to Protection of telomeres 1 (POT1) binding at single-stranded telomeric DNA sites (Figure 4). One of the critical issues of telomere maintenance is the transition between DNA replication and re-establishment of the capping by shelterin complex at the single-stranded 3'-overhangs. Replication Protein A (RPA) is the predominant single-stranded DNA binding entity and is essential for both DNA replication and damage repair [102]. When replication forks stall, the extension of single-stranded DNA

and the coating by RPA triggers the activation of ataxia-telangiectasia and Rad3-related (ATR) kinase and DDR [103, 104]. Thus, it is critical to displace RPA from the newly replicated telomeric 3'-overhangs to prevent unnecessary activation of the ATR signalling pathway at telomeres. POT1, one of the shelterin components, binds to the single-stranded telomeric 3' overhang and is required for suppression of ATR-dependent DDR activity [105, 106]. However, POT1 alone cannot out-compete RPA for the binding of single-stranded telomeric DNA but requires additional support from hnRNPA1 for the RPA-to-POT1 switch. TERRA, which traps and modulates the availability of hnRNPA1 to telomeric DNA therefore plays a crucial role in orchestrating this to achieve completion of telomere capping [88]. In short, DNA-PKcs-mediated phosphorylation of hnRNPA1 is critical for the protective capping of newly replicated telomeres to prevent the accumulation of telomeric aberrations.

REGULATION OF METASTASIS

Around 90% of cancer deaths are attributable to metastasis; the spread of disease from one organ, or part of, to another not directly connected with the primary tumour site. A better understanding of the molecular drivers of tumour dissemination may provide strategies to block this process or to efficiently target metastatic lesions in the clinic. The metastatic cascade is a multi-step process which includes local tumour cell invasion, entry into the vasculature followed by the exit of carcinoma cells from the circulation and colonization at the distal sites [107]. During cancer progression, subpopulations of tumour cells often undergo morphological changes that promote metastatic dissemination, including the epithelial to mesenchymal transition (EMT) [108]. hnRNPA1, through its regulation of alternative splicing, regulates the expression of genes involved in promoting invasive behaviour [21-23].

Specific splice variant of the transmembrane protein CD44 has been demonstrated to regulate metastasis [109]. CD44 splice variants reported in various cancer types are depicted in Figure 5. CD44 is a member of a large family of cell adhesion molecules that mediate the communication and

adhesion between adjacent cells and between cells and the extracellular matrix. CD44 also directs intracellular signalling for growth and motility. The *CD44* gene has 20 exons. Exons 1–5 (c1–c5) and 16–20 (c6–c10) are constant while alternate splicing within exon 6 through 15 generates 10 splice variants numbered CD44v1-v10 [110]. CD44v6 and v9 were found to be expressed specifically in a metastatic pancreatic carcinoma cell line, but not in the parental tumour [109]. Indeed, the expression of these variants in cells derived from the parental tumour was sufficient to render them metastatic [111] [109]. Interestingly, overexpression of hnRNPA1 was shown to promote invasion of hepatocellular carcinoma cells by upregulating expression of CD44v6 [22].

In breast cancer cells, CD44 has been shown to have conflicting functions [112]. Splicing patterns of the CD44v6 variants in invasive breast cancer cells (MDA-MB-231) was different from that of non-invasive breast cancer (MCF7) and normal breast cells (MCF10A). Invasive MDA-MB-231 cells expressed the c5v6c6 and c5v6v8v9v10c6 isoforms, whereas the MCF7 and MCF10A did not [21]. In contrast, the c5v6v7v8v9v10c6 isoform was expressed in all three cell lines. Knockdown of hnRNPA1 induced a significant decrease in the c5v6v7v8v9v10c6 and c5v6v8v9v10c6 isoforms, and an increase in the c5v6c6 isoform [21]. Therefore, it is likely that hnRNPA1 and/or its interaction partners fine-tune these complex splicing events. Defining these regulatory pathways could provide clues to the exact involvement of hnRNPA1 in regulating cell invasion through modulating CD44 splicing.

The hnRNPA1-mediated control of CD44 splicing may also be regulated by upstream oncogenic signalling pathways and associated post-translational modification of hnRNPA1. Indeed, Matter et al., using a mouse carcinoma model, demonstrated that inclusion of CD44 exon v5 was repressed by hnRNPA1 [113]. The inhibitory effect was abolished in the presence of activated Ras and by dominant active MEKK1 or Cdc42, two molecules that mediate signalling downstream of Ras. Interestingly, Cdc42 has been demonstrated to be necessary for Ras transformation in fibroblasts and to induce invasive cell growth [114], although a direct link to hnRNPA1 was not drawn.

More recently, epidermal growth factor (EGF) has been shown to modulate the splicing activity of hnRNPA1 via ubiquitination [35]. Indeed, EGF signalling upregulates an E3 ubiquitin (Ub) ligase, SPRY domain-containing SOCS box protein 1 (SPSB1), which recruits Elongin B/C-Cullin complexes to poly-ubiquitinate hnRNPA1 on K183. This post-translational event impacts on the splicing of RAC1, a member of the RAS superfamily involved in cytoskeletal rearrangement and cell migration [115]. Production of the Rac1b splice isoform, found upregulated in colon, breast and lung cancers [116] [115], promotes invasiveness through increased levels of reactive oxygen species and matrix metalloproteinase-3 (MMP-3)-induced EMT [117]. Binding of hnRNPA1 to a silencer element in *RAC1* exon 3b normally hinders production of Rac1b by preventing exon 3b inclusion [118] (Figure 6). However, ubiquitinated hnRNPA1 shows low binding affinity to the RAC1 mRNA, leading to de-repression RAC1b production [35] (Figure 6), a fact likely to promote cellular invasiveness.

During EMT, epithelial cancer cells undergo a series of gene expression changes to acquire mesenchymal features that facilitate their dissemination, including the ability to move as single cells through the extracellular matrix [119]. SRSF1, an oncoprotein upregulated in many human tumours, has been shown to regulate the splicing of the receptor tyrosine kinase and proto-oncogene, *RON* [120, 121]. Through the skipping of exon 11 of RON, SRSF1 promotes the production of ΔRON, a constitutively active isoform that favours EMT [121]. This process is controlled by two adjacent regulatory elements, a splicing silencer (SS) and enhancer (EE) located in exon 12 [122]. hnRNPA1 regulates the silencer by antagonizing the binding of SRSF1 and thereby preventing exon skipping . This leads to inhibition of ΔRON production, activating the reversal program (aka. mesenchymal-to-epithelial transition) once tumour cells have reached their final metastatic sites. This activity may be crucial for the malignant process and the formation of secondary tumours because re-differentiation from a mesenchymal to an epithelial phenotype is required for the colonization of distant organs by carcinoma [119].

hnRNPA1-MEDIATED REGULATION OF CELLULAR ENERGETICS

The deregulation of cellular energetics is now a recognised *hallmark of cancer*, with cancer cells favouring aerobic glycolysis with associated changes to mitochondrial metabolism that better supports their biosynthetic needs [123]. Here, glucose metabolism is diverted from ATP production towards the synthesis of cellular building blocks (nucleotides, amino acids, and lipids) to support cellular proliferation [124]. This metabolic shift is partly achieved by a switch in the splicing of the glycolytic enzyme, pyruvate kinase. Although normal cells express the pyruvate kinase M1 isoform (PKM1), tumour cells predominantly express the M2 isoform (PKM2). These two isoforms are produced through the mutually exclusive alternative splicing of the PKM pre-mRNA, with inclusion of either exon 9 (PKM1) or exon 10 (PKM2). hnRNPA1, hnRNPA2 and polypyrimidine tract binding protein (PTB) bind repressively to sequences flanking exon 9, resulting in preferential exon 10 inclusion [17, 125] (Figure 7). Switching from PKM1 to PKM2 promotes aerobic glycolysis and provides a selective advantage to tumour formation [17]. Indeed, PKM2, but not PKM1, is regulated through binding of tyrosine phosphorylated proteins, that releases the allosteric activator fructose-1-6-bisphosphate to inhibit pyruvate kinase activity [126]. This may enable growth factor signalling to channel glycolytic intermediates into biosynthetic processes. The tight coupling of PKM2 expression and cell proliferation suggests that expression of PKM splicing regulatory proteins might be under the control of a proliferation-associated mechanism. Indeed, *cMYC* has been demonstrated to bind the *hnRNPA1*, *hnRNPA2* and *PTB* promoters [127] and upregulate the expression of all three genes [128, 129]. As such, *cMYC* directly impacts on PKM splicing, with *cMYC* silencing increasing the PKM1/PKM2 ratio [125]. Furthermore, expression of the constitutively active variant of epidermal growth factor receptor (EGFR), EGFRvIII, upregulates hnRNPA1 expression in gliomas [130].This contributes to the alternative splicing of MYC-associated factor X (*MAX*) to form ΔMAX and promote expression of glycolytic genes, such as Hexokinase (*HK2*), Glucose transporter (*GLUT1* and *3*) and pyruvate dehydrogenase kinase (*PDK1*) [130, 131]. Hence, in association with cMYC, hnRNPA1 profoundly affects cancer cell metabolism to support cell proliferation and tumorigenesis.

CONCLUDING REMARKS AND PERSPECTIVE

hnRNPA1 is a multifunctional protein that regulates the splicing, transcription, nucleo-cytoplasmic shuttling and translation of mRNAs as well as telomere maintenance. As such, it is involved in key hallmarks of cancer such as increased cell growth, survival and metastasis, modified cellular energetics and immortalisation. In this review, we have attempted to summarise our current knowledge of the molecular mechanisms regulating the functions of hnRNPA1 through post-translational modifications and interaction with cofactors. However, many questions still remain unanswered.

An important gap in our knowledge is how post-translational modifications impacts on the structure and biological activity of hnRNPA1. For instance, our understanding of how changes in upstream signalling modulates its selectivity is still very poor. Indeed, while we found that specific AGC kinases phosphorylate separate residues on hnRNPA1, how this impacts on selectivity towards target mRNAs remains unclear. Moreover, the interaction of hnRNPA1 with IRESs or 3'UTR regions, may, for the same target mRNA, lead to different biological outcomes and the role of specific post-translational modifications in guiding this choice is presently unknown. Also, whether the nature of post-translational modifications on the same residue of hnRNPA1 (ie. sumoylation versus ubiquitination of Lys183) leads to different biological outcomes is undetermined.

Moreover, when hnRNPA1 participates to several steps of the same biological process, it is difficult to assess whether one of them actually is rate limiting. This is true for the dual involvement of this protein in the regulation of mRNA transport and translation or in its involvement in the RPA/POT1 switch and unwinding of G-quadruplex structures at the telomeres.

Conversely, many of the roles proposed for hnRNPA1 are somehow anecdotal and the extent to which they can be generalised is unclear. For instance, it is currently unknown whether the binding of hnRNPA1 to IRES structures is relevant to all IRES-containing mRNAs or whether only a restricted number of targets, such as BCLXL and XIAP mRNAs, are so regulated. Similarly, while hnRNPA1 has been shown to bind the G-quadruplex structures present in the promoters of *cMYC* and *KRAS* to de-repress their expression [132, 133], it remains unknown whether this regulation extends to all other G-quadruplex-controlled genes.

Finally, our understanding of hnRNPA1 function is more than often not based on studies conducted in disease models such as cancer cells. Hence our understanding of the functions of hnRNPA1 in normal cells is mostly lacking and the role of this protein in homeostasis may differ significantly from what has so far been revealed. Future work will hopefully shed light on these gaps in our knowledge and may lead to novel therapeutic or diagnostic possibilities for cancer.

CONFLICT OF INTEREST

Authors declare no conflict of interests.

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FIGURE LEGENDS

Figure 1

hnRNPA1 structure and function: hnRNPA1 has two RRMs on its N-terminal, each about 80 nucleotides long. The C-terminal Glycine-rich domain contains an RGG (Arg-Gly-Gly) box and a nuclear targeting sequence (M9). M9 is responsible for both nuclear import and export. Post-translational modifications regulate hnRNPA1's RNA-binding ability, protein-protein interaction and subcellular localization.

Figure 2

hnRNPA1 regulates cell survival by modulating translation of anti-apoptotic proteins: Upon activation, S6K2 binds and phosphorylates hnRNPA1 on its Ser4/6 site. The phosphorylated hnRNPA1 binds the IRESs in XIAP and BCLXL mRNAs. The hnRNPA1/mRNA complexes are then exported to the cytoplasm where the mRNAs undergo cap-independent translation. This coincides with phospho-hnRNPA1 being recognized by 14-3-3 proteins, resulting in recruitment of the sumoylation machinery and sumoylation of hnRNPA1 on Lys183. This enables the re-import of hnRNPA1 into nucleus. hnRNPA1 can also bind the 3'-ARE in the cIAP1 mRNA rather than the IRES site. UV irradiation results in the accumulation of hnRNPA1 in the cytoplasm and increased of this binding with subsequent mRNA degradation and activation of the NFκB pathway.

Figure 3.

Telomere extension requires balanced expression of hnRNPA1 and TERRA: When the levels of hnRNPA1 are significantly lower than those of TERRA, excess TERRA binds to hTR through base pairing, inhibiting telomerase from approaching 3' telomeric DNA overhangs. When the amount of hnRNPA1 is higher than that of TERRA, excess hnRNPA1 binds directly to the telomeric sequence, preventing the access of telomerase and subsequent inhibition of telomere extension. Balanced levels of the two allows hnRNPA1 to bind TERRA in an inert complex allowing telomerase to access the 3'-telomeric overhangs and mediate efficient telomere maintenance.

Figure 4.

Phosphorylated hnRNPA1 facilitates the RPA-POT1 switch at telomeric sequence: Stalling of replication fork could result in RPA-coated single strand DNA triggering a DNA damage response (DDR) downstream of ATR signalling. DNA-PKs-mediated phosphorylation of hnRNPA1 on Ser95, stimulated in the presence of hTR, allows hnRNPA1 to bind telomeric sequences and remove RPA.

POT1, sequestered from the shelterin complex, then replace hnRNPA1 on the exposed overhang and prevent unnecessary DDR.

Figure 5

CD44 splicing variants and their association to cancer: Blue and red boxes represent constant and alternatively spliced exons, respectively

Figure 6.

hnRNPA1 promotes cancer metastasis by regulating the alternative splicing of RAC1: Under normal conditions, hnRNPA1 binds to a silencer component in Exon 3b of the RAC1 mRNA, preventing inclusion of this exon and promoting expression of full-length RAC1 that controls cytoskeletal rearrangement and cell migration. EGF stimulation facilitates the formation of the SPSB1- Elongin B/C-cullin complex that poly-ubiquitinates hnRNPA1 on Lys183. This results in decreased affinity of hnRNPA1 towards the silencer component and inclusion of exon3b in mRNAs. The resulting RAC1b protein mediates increase in cellular ROS and EMT.

Figure 7.

hnRNPA1, hnRNPA2 and PTB modulate metabolism in cancer cells through alternative splicing of PKM: In normal cells, inclusion of exon10 is repressed leading to generation of PKM1. In tumour cells, cMYC can upregulate expression of hnRNPA1, hnRNPA2 and PTB. These then bind and repress exon9, resulting in the inclusion of exon10 and generation of PKM2. The switch from PKM1 to PKM2 potentiates aerobic glycolysis to support tumour growth.

FIGURE 1

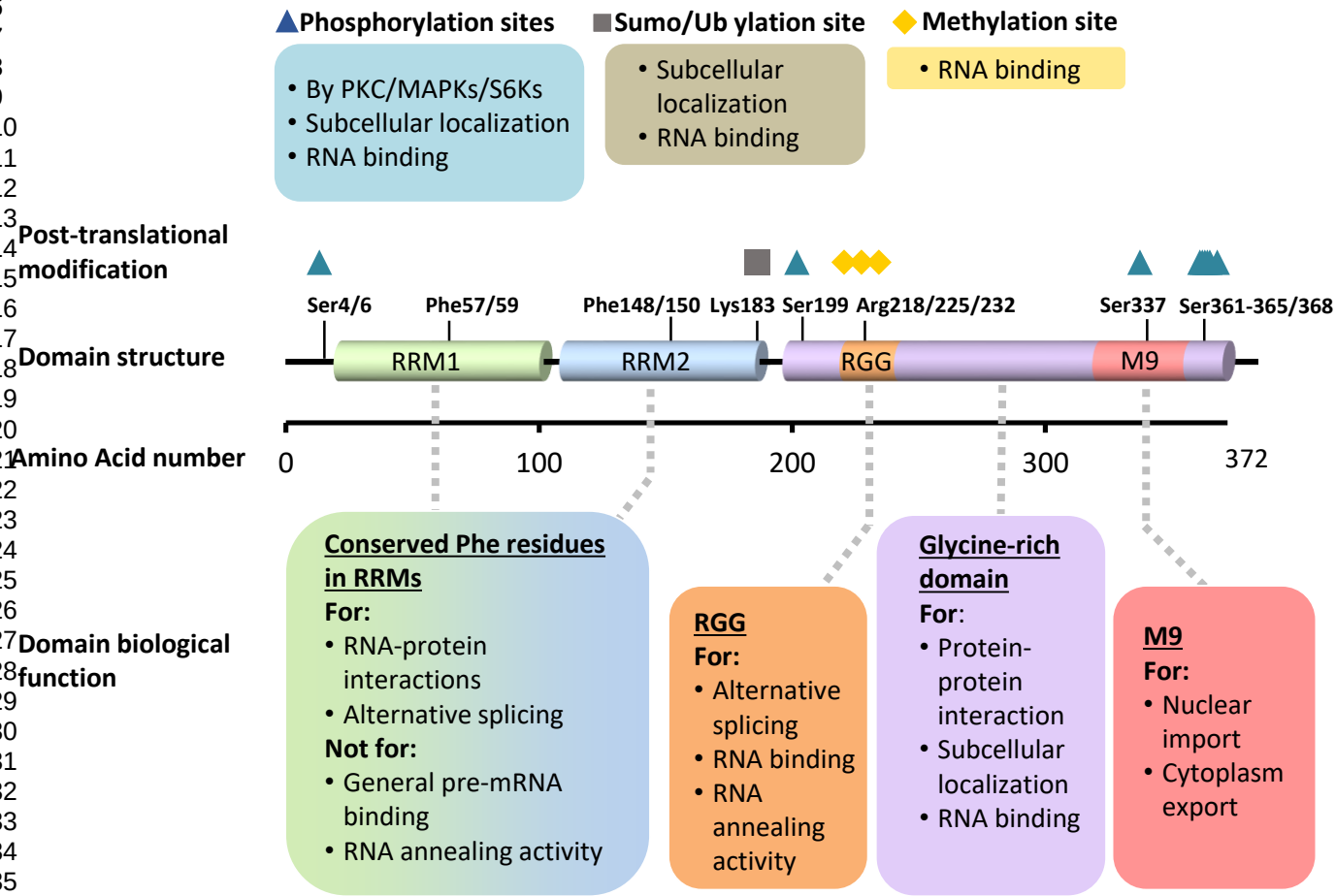

Phosphorylation sites
• By PKC/MAPKs/S6Ks
• Subcellular localization
• RNA binding
Sumo/Ub ylation site
• Subcellular localization
• RNA binding
Methylation site
• RNA binding
Post-translational modification
Ser4/6
Phe57/59
Phe148/150
Lys183
Ser199
Arg218/225/232
Ser337
Ser361-365/368
Domain structure
RRM1
RRM2
RGG
M9
Amino Acid number
0
100
200
300
372
Conserved Phe residues in RRMs
For:
• RNA-protein interactions
• Alternative splicing
Not for:
• General pre-mRNA binding
• RNA annealing activity
RGG
For:
• Alternative splicing
• RNA binding
• RNA annealing activity
Glycine-rich domain
For:
• Protein-protein interaction
• Subcellular localization
• RNA binding
M9
For:
• Nuclear import
• Cytoplasm export
Domain biological function

FIGURE 2

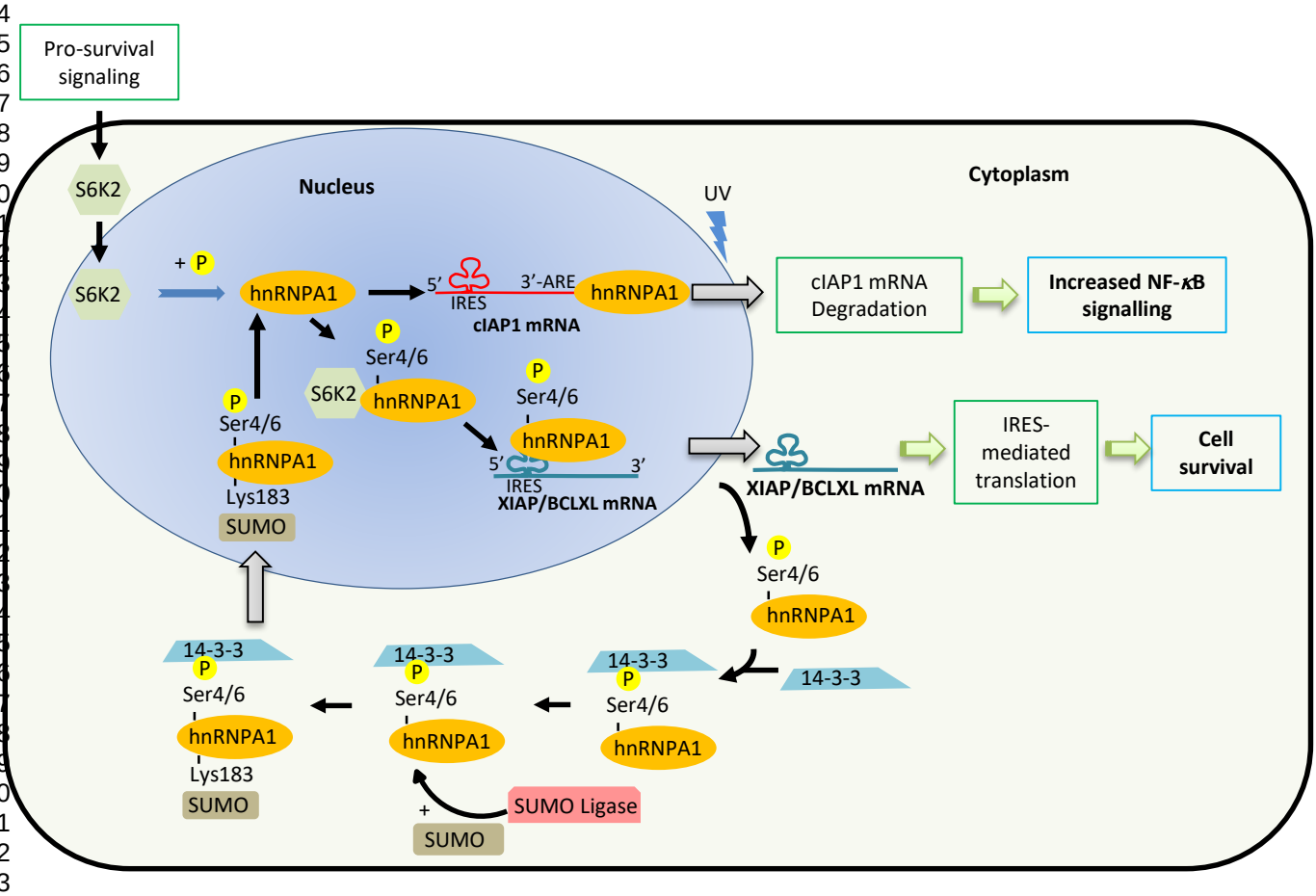

Pro-survival signaling
S6K2
S6K2
Nucleus
Cytoplasm
UV
+ P
hnRNPA1
5′
IRES
3′-ARE
hnRNPA1
cIAP1 mRNA
cIAP1 mRNA Degradation
Increased NF-κB signalling
P
Ser4/6
S6K2
hnRNPA1
P
Ser4/6
hnRNPA1
Lys183
SUMO
P
Ser4/6
hnRNPA1
5′
IRES
3′
XIAP/BCLXL mRNA
XIAP/BCLXL mRNA
IRES-mediated translation
Cell survival
P
Ser4/6
hnRNPA1
14-3-3
14-3-3
P
Ser4/6
hnRNPA1
14-3-3
P
Ser4/6
hnRNPA1
Lys183
SUMO
14-3-3
P
Ser4/6
hnRNPA1
+
SUMO
SUMO Ligase

FIGURE 3

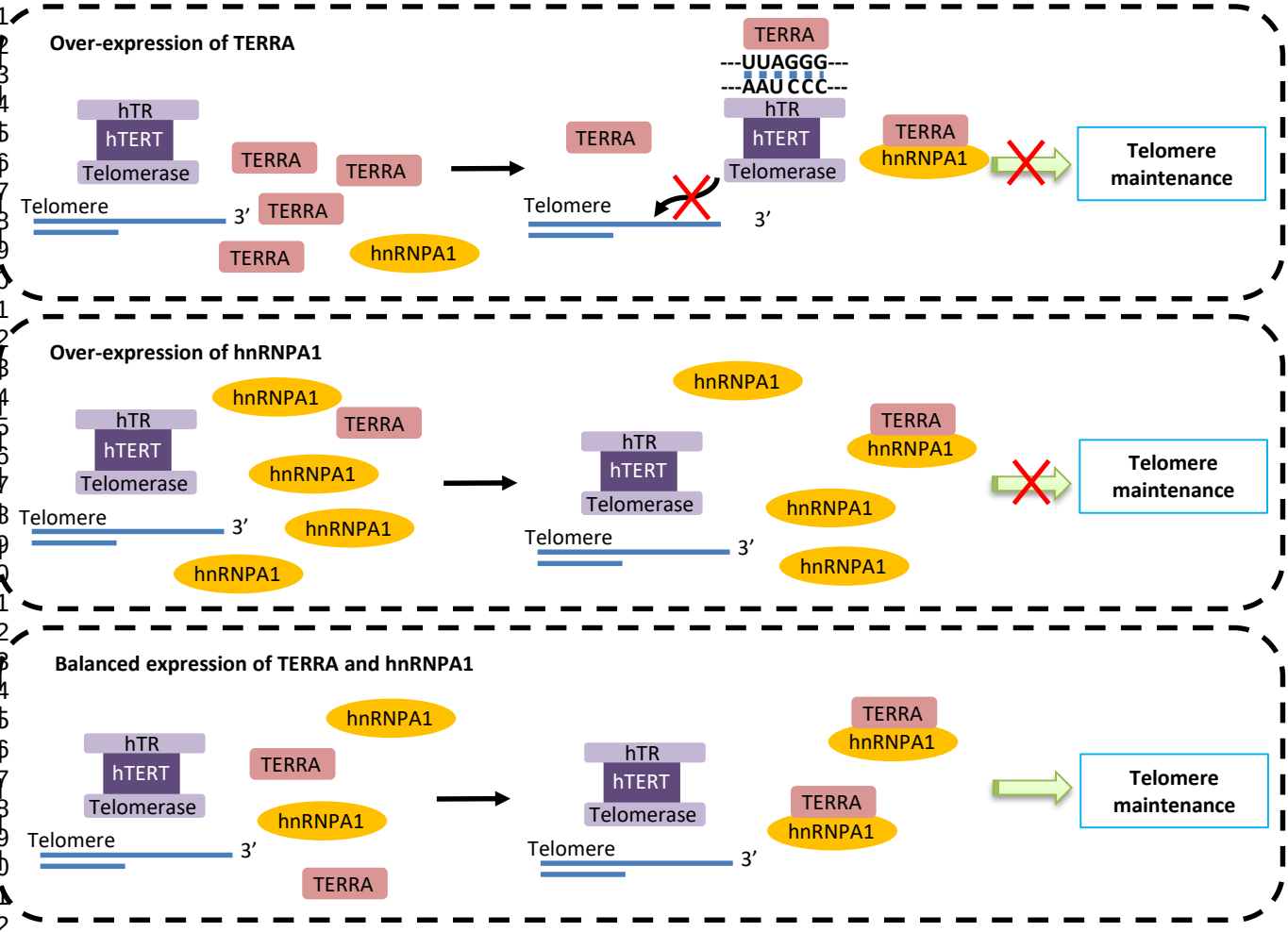

Over-expression of TERRA
hTR
hTERT
Telomerase
TERRA
TERRA
Telomere
3′
TERRA
TERRA
hnRNPA1
TERRA
---UUAGGG---
---AAU CCC---
hTR
hTERT
Telomerase
TERRA
Telomere
3′
TERRA
hnRNPA1
Telomere maintenance
Over-expression of hnRNPA1
hnRNPA1
hnRNPA1
TERRA
hTR
hTERT
Telomerase
hnRNPA1
Telomere
3′
hnRNPA1
hnRNPA1
hnRNPA1
hTR
hTERT
Telomerase
TERRA
hnRNPA1
Telomere
3′
hnRNPA1
hnRNPA1
Telomere maintenance
Balanced expression of TERRA and hnRNPA1
hnRNPA1
hTR
hTERT
Telomerase
TERRA
hnRNPA1
Telomere
3′
TERRA
TERRA
hnRNPA1
hTR
hTERT
Telomerase
TERRA
hnRNPA1
Telomere
3′
Telomere maintenance

FIGURE 4

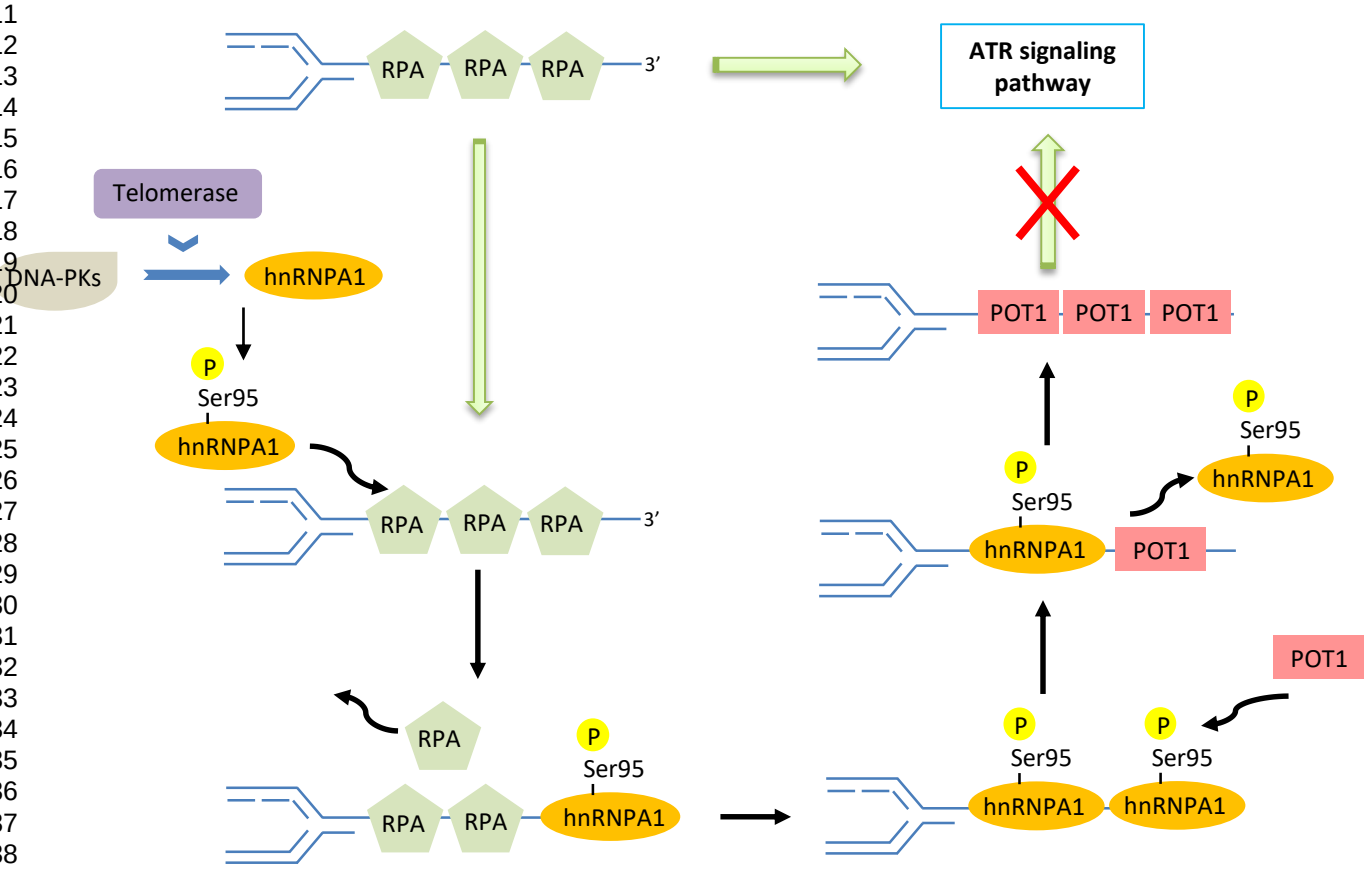

RPA
RPA
RPA
3'
ATR signaling pathway
Telomerase
DNA-PKs
hnRNPA1
P
Ser95
hnRNPA1
POT1
POT1
POT1
RPA
RPA
RPA
3'
P
Ser95
hnRNPA1
POT1
RPA
P
Ser95
hnRNPA1
RPA
RPA
hnRNPA1
POT1
P
Ser95
hnRNPA1
P
Ser95
hnRNPA1

FIGURE 5

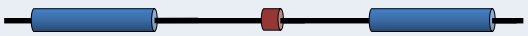
CD44	Mediate communication and adhesion between cells and between cells and the extracellular matrix	
CD44 V5	• Inhibited by hnRNPA1 by repressing exon 10 • De-repressed by activated RAS and dominant active MEKK1/Cdc42	
CD44 V6	**Pancreatic carcinoma cell line:** • Expressed specifically in metastasizing cell line • Not in parental cell line **Hepatocellular carcinoma cell line:** • Overexpressed by increasing hnRNPA1 , promote invasion ability **Breast cancer cell line:** • Expressed in metastatic cell line • Overexpressed by knock down of hnRNPA1	
CD44 V9	**Pancreatic carcinoma cell line:** • Expressed specifically in metastasizing cell line • Not in parental cell line	
CD44 V6V7V8V9V10	**Breast cancer cell line:** • Decreased as a result of hnRNPA1 knock down	
CD44 V6V8V9V10		

FIGURE 6

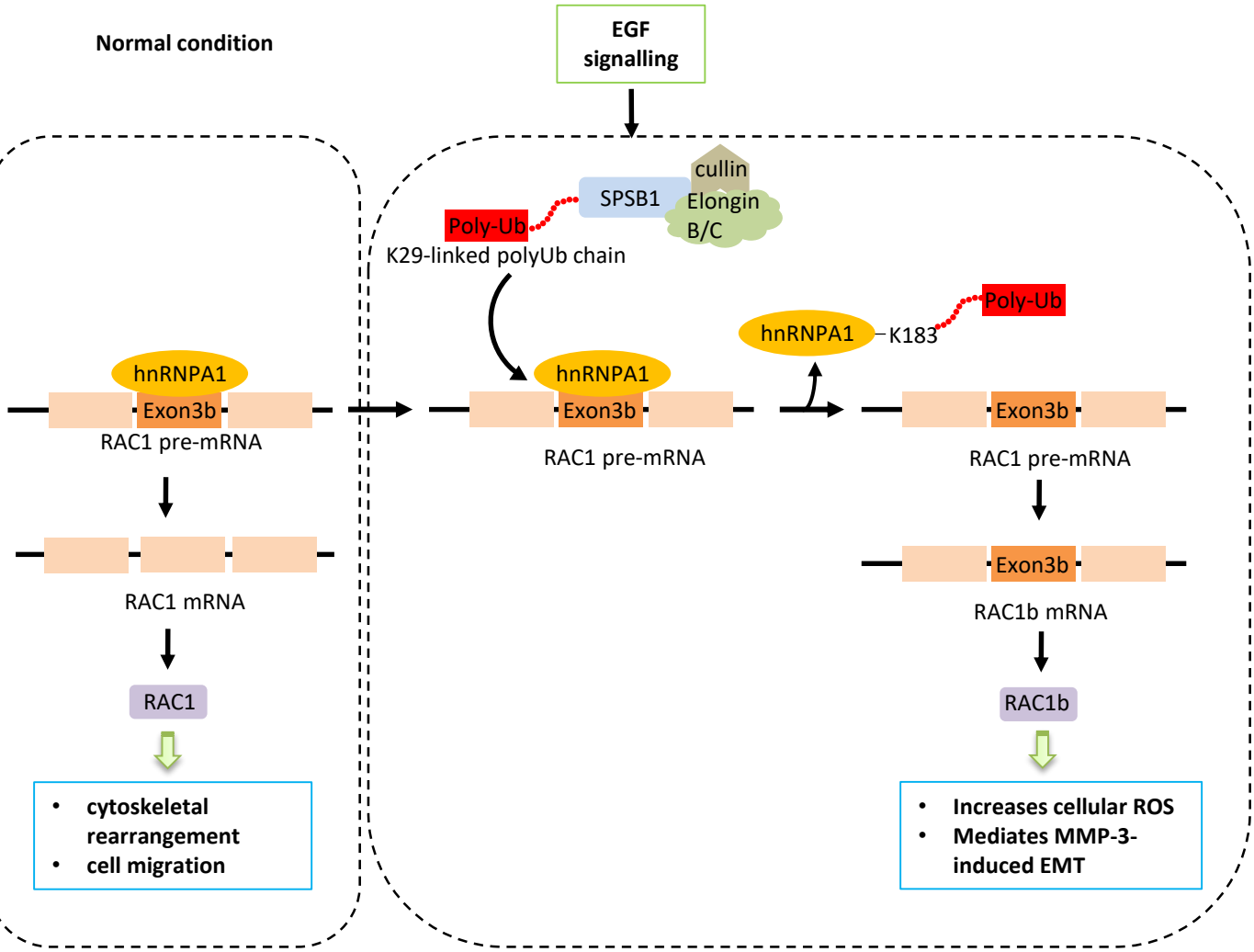
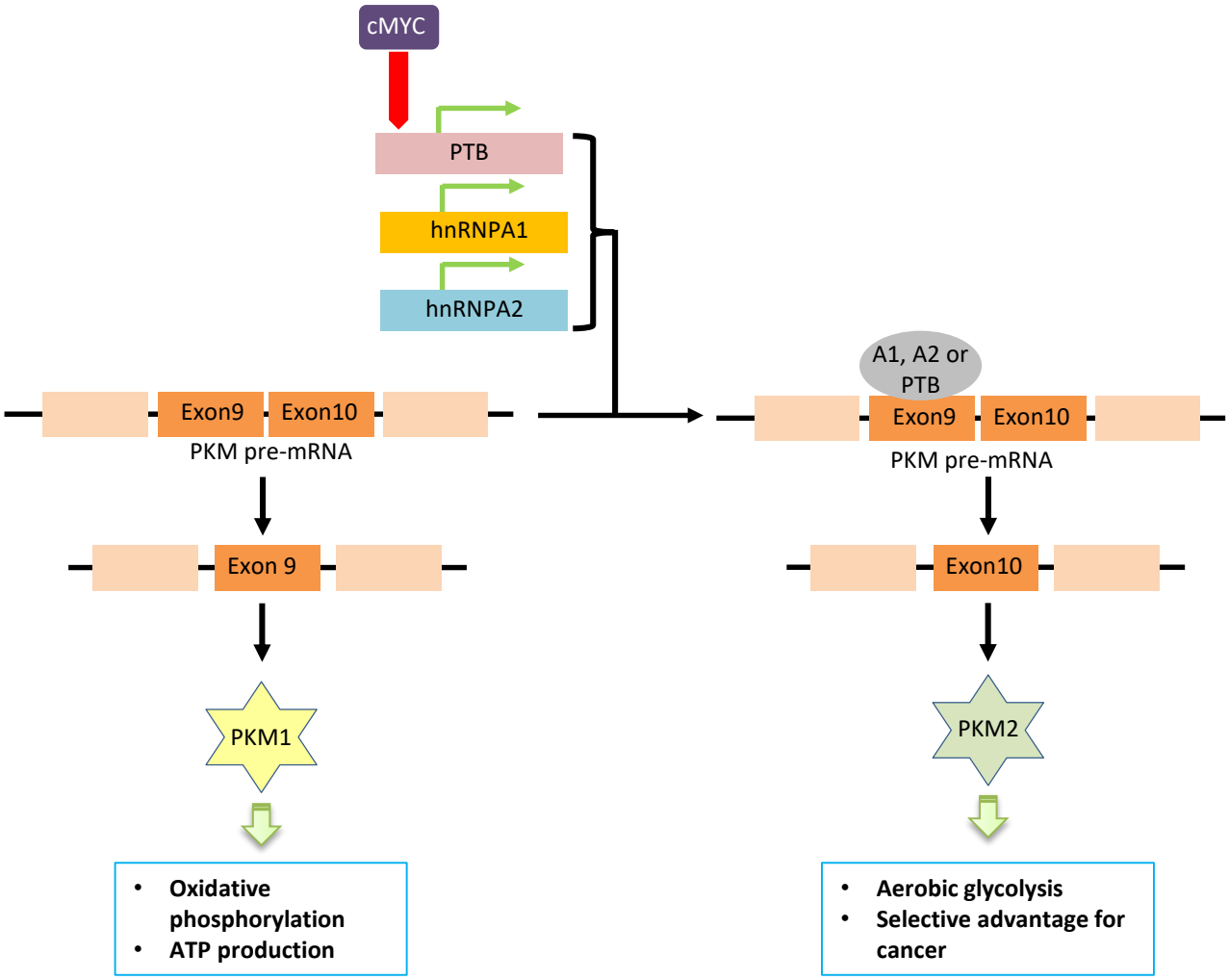

Normal condition
EGF signalling
cullin
SPSB1
Elongin B/C
Poly-Ub
K29-linked polyUb chain
hnRNPA1
Exon3b
RAC1 pre-mRNA
RAC1 mRNA
RAC1
cytoskeletal rearrangement
cell migration
hnRNPA1
Exon3b
RAC1 pre-mRNA
hnRNPA1
K183
Poly-Ub
Exon3b
RAC1 pre-mRNA
Exon3b
RAC1b mRNA
RAC1b
Increases cellular ROS
Mediates MMP-3-induced EMT

FIGURE 7


cMYC
PTB
hnRNPA1
hnRNPA2
Exon9
Exon10
PKM pre-mRNA
Exon 9
PKM1
Oxidative phosphorylation
ATP production
A1, A2 or PTB
Exon9
Exon10
PKM pre-mRNA
Exon10
PKM2
Aerobic glycolysis
Selective advantage for cancer