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DIFFERENT ROUTES IN WHICH PROTEIN P27^{KIP1} IS INVOLVED IN ONCOGENESIS

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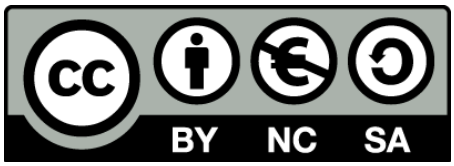

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1. Summary

The $p27^{Kip1}$ protein is a regulator of the cell cycle in eukaryotic cells, and it belongs to the Cip/Kip family of cyclin-dependent kinase inhibitors (CKIs). A characteristic feature is that it is an unstructured and multifunctional protein and its loss is involved in many types of tumours. The first function described is to regulate cell cycle progression by inhibiting cyclin-dependent kinases (CDKs). In addition to regulating cell proliferation, it has now been shown that p27Kip1 is involved in various biological processes such as transcriptional regulation, cell migration, regulation of metabolism, apoptosis, and autophagy. All these capacities give p27Kip1 an important role in the initiation and development of cancer, although depending on its subcellular location, it can act as a tumour suppressor or as an oncogenic protein. Cancer is one of the most complex pathologies and exists many different types of it. Due to the complexity of the tumour development, since the year 2000, common aspects have been studied in different types of tumours and have been grouped into a series of characteristic biological processes known as "**Hallmarks of Cancer**", which consist of different capabilities acquired by cells during the development of tumours. This work describes how $p27^{Kip1}$ is involved in several of these hallmarks: regulating cell proliferation, self-sufficiency in growth signals, preventing apoptosis, angiogenesis and tissue invasion. For all these reasons, $p27^{Kip1}$ could be considered an important target for new cancer treatments.

Resum

La proteïna $p27^{Kip1}$ és un regulador del cicle cel·lular en les cèl·lules eucariòtiques que pertany a la família Cip/Kip dels inhibidors de les quinases dependents de ciclina (CKIs). Un aspecte característic és que és una proteïna desestructurada i multifuncional i la seva pèrdua esta implicada en molts tipus de tumors. La primera funció descrita és la de regular la progressió del cicle cel·lular a través d'inhibició de les quinases dependents de ciclines (CDKs). A banda de la regulació de la proliferació cel·lular, actualment, s'ha vist que $p27^{Kip1}$ esta implicada en diferents processos biològics com la regulació transcripcional, la migració cel·lular, la regulació del metabolisme, l'apoptosi i l'autofàgia. Totes aquestes capacitats li donen a la $p27^{Kip1}$ un paper important en la iniciació i desenvolupament del càncer, tot i que depenent de la seva localització cel·lular pot actuar com un supressor de tumors o com un proteïna oncogènica. El càncer és de les patologies més complexa i n'existeixen de molts tipus diferents. Degut a aquesta complexitat del desenvolupament tumoral, des de l'any 2000, s'han estudiat aspectes comuns en diferents tipus de tumors i s'han agrupat en una sèrie de "processos biològics característics" coneguts com a ***Hallmarks* del càncer**, que consisteixen en diferents capacitats biològiques adquirides per les cèl·lules durant el desenvolupament dels tumors. En aquest treball es descriu com $p27^{Kip1}$ participa en varis d'aquests *Hallmarks:* Regulació de la proliferació cel·lular, auto-estimulació del creixement, evasió de l'apoptosi, angiogènesi i invasió de teixits. Per totes aquestes raons, la $p27^{Kip1}$ podria considerar-se com una diana important d'estudi per a nous tractaments del càncer.

2. Integration of different academic fields in this project

Cellular Biology is the main field integrated and discussed within this project. Most of the mechanisms discussed are involved in different types of cells, either in the nucleus or cytoplasm, or in the extracellular environment: extracellular matrix and surrounding tissues. Also, to understand and analyse all the different cellular functions and processes talked about in this project such as cell cycle, transcriptional regulation, proteolysis or autophagy, proper knowledge of cell biology is needed.

Biochemistry and Molecular Biology. There are many pathways and processes discussed here that involve very specific molecules and chemical interactions between them, for example, $p27^{Kip1}$ phosphorylation leading to a partial activation of CDKs and its ubiquitin-proteasome-dependent degradation which allows progression of the cell cycle, or its role in metabolism regulation. In addition, analysing the molecular structure and corresponding domains of $p27^{Kip1}$, as well as its interactions with other proteins, explain possible cellular functions in which $p27^{Kip1}$ is involved.

Physiology and Physiopathology. In relation to this field, cancer and its relationship with $p27^{Kip1}$ is discussed. Cancer is a very complex pathology that involves many different molecular mechanisms to achieve its malignant characteristics, which are reported in this project through the Hallmarks of Cancer.

Pharmacology & Therapeutics. The main goal in studying the role of $p27^{Kip1}$ in oncogenesis is to further review new or existing targets for cancer treatment. Targets can range from $p27^{Kip1}$ itself to its many regulators, through which we are able to regulate $p27^{Kip1}$ levels and thus alter pathways where $p27^{Kip1}$ participates, such as for instance cell proliferation or apoptosis.

3. Sustainable Development Goals (SDG)

Within the 17 SDG, there are two to which I believe this project contributes to achieve its global goal of peace and prosperity by 2030. The first one being goal number three, "**Good health and well-being**", since I discuss and learn about what role $p27^{kip1}$, a cell cycle regulator protein, plays in oncogenesis. Cancer is one of the leading causes of death and is a very complex disease requiring very specialized knowledge to understand its many different mechanisms. Further reviewing, learning, and investigating oncogenesis contributes to a better understanding of it and a step closer to developing better treatments, especially in the early stages of cancers.

The second one is number nine, **"Industry, innovation and infrastructure"**, with special emphasis on innovation. Research and development (R&D) is an integral part of the pharmaceutical industry which allows, most importantly, discovering and testing new drugs and conducting clinical trials for safety and efficacy purposes. Innovation is crucial in this industry, either in development of a new drug, new dosages, or existing mechanisms of an already known drug, and the testing of these additional indications. All these lead to new possible and improved treatments for patients, improving their quality of life. In this project I have reviewed many studies and reviews that talk about $p27^{Kip1}$ research and its possible role within many routes, focusing on oncogenesis.

4. Introduction and contextualisation of the project

Cancer is one of the main leading causes of death in the world, which can greatly impact the lives of those affected and their loved ones. According to the World Health Organization (WHO), cancer is not among the 10 main causes of death globally, however, in more developed countries, specific types of cancer are included among the top 5 leading causes of death, behind cardiovascular diseases (ischemic heart disease) and neurodegenerative diseases (Alzheimer's and Parkinson's disease). In general, the highest incidence of cancer is found in developed countries, with high life expectancy, education, and standard of living. As reported by the WHO, it is expected that in 2040 the number of new cancer cases a year will significantly increase as well as the number of deaths caused by it.

In individuals who have undergone cancer treatment successfully, recurrence of the cancer is always a possibility, even in different locations from the original one. Whether the treatment is surgery, chemotherapy, or radiotherapy we can still not be sure of permanent elimination, especially in cancers that have already metastasized.

The main reason for this chance of recurrence is that within a tumour there are different cancer cell subpopulations with different genetic alterations, and consequently different characteristics and capabilities. Therefore, after a given treatment, many of these tumorous cells can be eliminated, but a few of them can still survive giving way to unregulated proliferation and a recurrence. For that reason, new cancer therapies are currently under development aiming to reduce these subpopulations within the tumour as much as possible. The current idea is to develop combined therapies including synthetic drugs, immunotherapies, and vaccines, aiming to block many of the different tumour cells in order to eliminate these subpopulations and thus avoid future recurrences. These tumour characteristics are defined within the Hallmarks of Cancer.

This project deals with protein $p27^{Kip1}$, a CDK inhibitor that is involved in cell cycle regulation, however it can also participate in the regulation of other cellular functions such as transcription, cell migration, apoptosis, etc. which are also discussed in this review, and how it relates to the Hallmarks of Cancer. It is seen that the decrease of $p27^{Kip1}$ in tumour cells could play a pivotal role in cancer development and may become a new molecular target for cancer therapies.

5. Hypothesis and objectives

Within the last 20 years D. Hanahan and R.A. Weinberg have defined a number of common characteristics that define most tumours. These are characteristics that "normal" cells acquire in order to achieve a tumour phenotype, known as the Hallmarks of Cancer.

$p27^{Kip1}$ regulates the activity of cyclin/CDK complexes which regulate transition between different phases of the cell cycle. The protein p27 acts as a tumour suppressor, however, it has recently been observed that it has other cellular functions where it plays an oncogenic role. This duality leads to the belief that $p27^{Kip1}$ could be an essential protein involved in initiation and development in oncogenesis.

The hypothesis I propose is:

"$p27^{kip1}$ regulates many routes involved in oncogenesis and participates in the acquisition of different Hallmarks of Cancer"

To research my hypothesis, the following objectives are tabled:

1. To briefly describe the various *hallmarks of cancer* and its evolution during the last 20 years.
2. To conduct a bibliographic search on the characteristics of $p27^{Kip1}$: protein structure, regulation, and cellular localization.
3. To define its role as a cell cycle regulator.
4. To describe other cellular functions, in which $p27^{Kip1}$ is involved, implicated in cancer development.
5. To argument how $p27^{Kip1}$ would be implicated within the different hallmarks of cancer.

6. Methodology and design of the project

With the goal of organising the development of this project, I contacted my tutor to elaborate an outline defining the hypothesis and targets, and we determined the most important topic points to be included. During the semester, I have regularly sent updates on project progress so I could get input and opinions, and we have also regularly had meetings to discuss it. The research has been done using i) scientific articles accessed through the ***Pubmed*** (https://pubmed.ncbi.nlm.nih.gov/) and ***Mendeley*** databases (https://www.mendeley.com), ii) books specializing in Cell Biology, and iii) different websites.

During this project, I have become familiar with databases like:

- ***GeneCards:*** *The Human Gene Database* (https://www.genecards.org)
- ***UniProt*** (https://www.uniprot.org)
- ***Gene Ontology Resource*** (*http://geneontology.org*)
- ***Kyoto Encyclopedia of Genes and Genomes*** (*https://www.genome.jp/kegg*)
- ***PyMOL*** (*https://pymol.org*)

For the bibliography I have used Mendeley Cite, using Vancouver as the citation style.

7. Results

7.1. Hallmarks of Cancer

Cancer is a very complex and diverse disease, whose diagnosis and treatment demands a very sound and fundamental understanding of its underlying mechanisms. The concept of Hallmarks of Cancer is defined as a tool that provides a better understanding of the fundamental principles that define neoplastic disease and help to rationalise its complexity. Hallmarks of Cancer comprises several biological characteristics acquired during the multistep development of human tumours. They were first introduced in 2000 by D. Hanahan and R.A. Weinberg in the: "Hallmarks of Cancer" review which has become widely accepted as a comprehensive conceptualization of the complex cancer biology (Figure 1.A). Since then, more hallmarks have been defined as cancer becomes more properly understood. In this first review, they defined six hallmarks or "functional capabilities" that allow tumours cells to survive, proliferate and migrate to colonize other tissues (1):

- **Self-sufficiency in growth signals**: Normal cells grow and only proliferate through a highly regulated mechanism, in response to external signals such as growth factors and mitogens. Nevertheless, one of the characteristics of cancer cells is their continuous ability to proliferate. These cells contain deregulated oncogenes and tumour suppressor genes, thus sustaining chronic proliferation by mimicking the previously mentioned external signals.

- **Evading apoptosis**: Programmed cell death, commonly known as apoptosis, is a process in which unwanted or abnormal cells are eliminated, and is regulated by an intracellular proteolytic cascade. Apoptosis is triggered in different situations such as in response to cellular stress or DNA damage. The tumour suppressor p53 is one of the most relevant proteins in this process. Tumour cells can limit or evade apoptosis by a variety of different mechanisms, the most common being the loss of the p53 function.

- **Sustained angiogenesis:** Angiogenesis, the formation of blood vessels, is a transitory and tightly regulated process. Cell survival is dependent on the oxygen and nutrients supplied by the vasculature. Tumour cells can induce and sustain angiogenesis. This capability seems to be acquired during the early to mid-stage of human cancer development.

- **Limitless replicative potential**: Normal cells have a limitation on how many growth-and-division cycles they can go through, either by going into senescence, a state where the cell remains viable but is no longer able to proliferate, or cellular stress in which cell death is induced. In contrast, tumour cells have the ability to proliferate without limits. This enabling of replicative immorality is linked to the ability of tumour cells to maintain telomere lengths that avoid activating senescence or a crisis state.

- **Tissue invasion & metastasis:** All previously mentioned hallmarks are needed for invasion and metastasis to take place. Metastatic or colonization processes begin with local invasion of adjacent tissue by tumour cells, followed by their

penetration into the blood and lymphatic vessels. Later, extravasation occurs followed by micro-metastases development.

- **Insensitivity to anti-growth signals:** Growth-inhibitory signals are essential to maintain tissue homeostasis and cellular quiescence. In order to proliferate, cancer cells are able to bypass these anti-growth signals.

Over a decade later, in 2011, with new research and developments, two new hallmarks of cancer or "**Emerging Hallmarks**" were defined (Figure 1.B) (2):

- **Deregulating cellular energetic**s: Metabolic adaptation is needed to sustain neoplastic cell growth and division. Cancer cells are capable of altering their glucose metabolism, mainly prioritizing glycolysis causing a state of aerobic glycolysis.

- **Evading immune destruction:** The immune system functions as a notable barrier to tumour formation and progression. Therefore, if a tumour is formed, it means that it must have managed to evade its eradication by the immune system.

Furthermore, the acquisition of these capabilities is made possible by "**Enabling Characteristics"** that are pivotal to the development of the aforementioned hallmarks (Figure 1.B) (2):

- **Genome instability and mutation**: DNA defects are controlled by genome maintenance systems ensuring that spontaneous mutations are overall very low in each cell generation. Due to their ineffectiveness or loss of DNA repairing mechanisms, cancer cells have increased sensitivity to mutagenic agents, and in consequence, are more prone to accumulate mutations.

- **Tumour promoting inflammation**: Multiple evidence indicates that immune inflammatory cells can actively be tumour promoting, which bring growth-stimulating factors to help tumour proliferation, facilitating neoplastic progression.

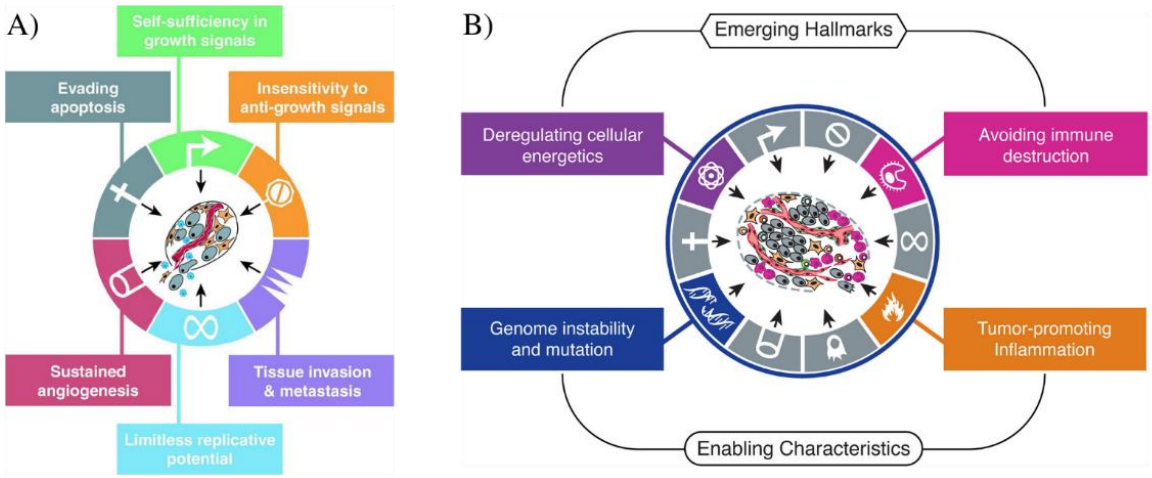

Figure 1. A) The first six acquired functional capabilities defined as the hallmarks of cancer in the year 2000 (1). B) New enabling characteristics and emerging hallmarks within the already defined ones, defined in 2011 (2).

On the other hand, in 2021, S.S. Senga and R.P. Grose proposed, in addition to those already described by Hanahan, four novel hallmarks (3):

- **Dedifferentiation and transdedifferentation**: The ability of the cells to regress from a specific specialized state.
- **Epigenetic dysregulation:** Which can affect gene expression.
- **Altered microbiome**: Normal population of microorganisms in human body are altered in cancer patients, thus helping tumour progression.
- **Altered neuronal signalling**: Innervation of tumours is indispensable for growth and survival of tumour cells.

Recently, as of January 2022, the latest review for Hallmarks of Cancer by Hanahan has been released adding another two new "**Emerging Hallmarks"** (4):

- **Unlocking phenotypic plasticity:** Cell differentiation constitutes a barrier to uncontrolled cell proliferation. Normal progenitor cells undergo cellular differentiation, increasing specialization of function and leading to specialized cells. These cells stop growing by a process named terminal differentiation, limiting their phenotypic plasticity (5). Neoplastic cells are able to avoid this limitation and evade terminal differentiation.

- **Non-mutational epigenetic reprogramming**: Genome mutations can be found in practically every type of human cancer. However, mutations are not the only cause of genome reprogramming, as epigenetic changes also play a very important role in altering the genome, referred to as non-mutational epigenetic reprogramming.

In addition, in this latest review two new **"Enabling Characteristics"** where defined:

- **Senescent cells:** Cell senescence is a state characterized by a stable cell cycle arrest, it also induces changes in cell metabolism and morphology and, the activation of a senescence-associated secretory phenotype which involves a release of various bioactive proteins. This state has been largely known as a neoplastic protective mechanism; however, recent studies have shown that in certain situations senescent cells can evoke tumour development and malignant progression.

- **Polymorphic microbiomes**: Growing evidence supports that the microbiome ecosystems which consist of resident bacteria and fungi have a significant impact on health and disease. Polymorphic variability in these microbiomes between individuals can have an important effect on cancer phenotypes.

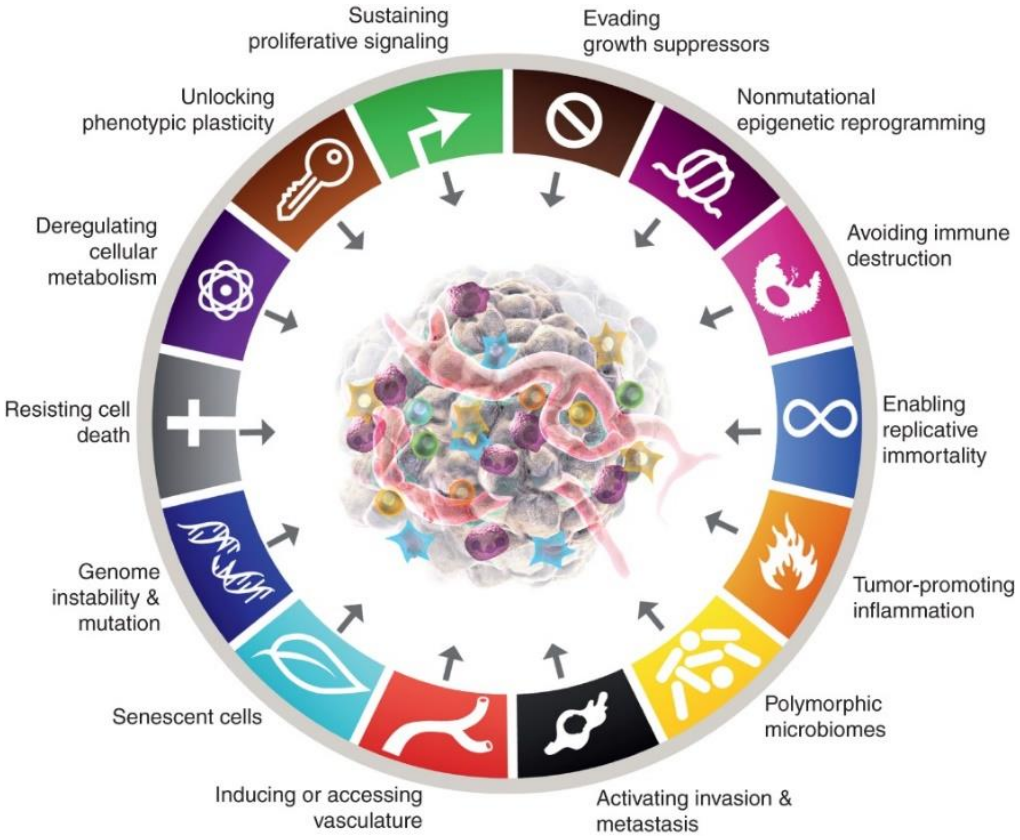

Figure 2. The Hallmarks of Cancer as of 2022, including the recently discovered new emerging hallmarks and enabling characteristics (4).

During recent years there have been many investigations directed to deciphering the molecular events that may be involved in the acquisition of the different Hallmarks of Cancer and consequently to enable cells to develop a tumour. Recently, different type of studies (expression microarrays, Chip-on chip, Chip-seq, etc.) have connected the protein $p27^{Kip1}$ (a classical regulator of the activity of CDKs) with the acquisition of different Hallmarks of Cancer and for this reason, this work aims to describe the putative important role of this protein in cancer development.

7.2. The Protein $p27^{Kip1}$

The protein $p27^{Kip1}$ (hereinafter reported as p27) is a member of Cip/Kip family of CKIs, that also include $p21^{Cip1}$ (p21) and $p57^{Kip2}$ (p57). For a long time, its main function was associated with cell cycle progression, through CDK inhibition, causing cell cycle arrest. (6) However, recently, it has been shown that p27 is an intrinsically unstructured and multifunctional protein, with various functions regardless of its role as an inhibitor of cell proliferation (7). For this reason, it can play both tumour suppressive and oncogenic roles.

7.3. Structure of p27

The human p27 gene (CDKN1B) is located in the chromosome 12p13. It contains three exons that encode for a protein of 198 amino acids (Figure 3.A) with a molecular mass of 22073 Da.

The **N-terminal** region of p27 (aa 1-109) include a K*inase Inhibitory Domain* (KID, aa 25-90) that consists of three subdomains: the cyclin-binding subdomain (D1, aa 29-51), the CDK-binding subdomain (D2, aa 51-90) and the linker helix subdomain (LH) that joins D1 and D2. Thus, p27 inhibits CDK activity by forming a trimeric complex with the cyclin and the CDK. However, phosphorylation of Y74 and Y88, by Src kinases allows a partial activation of the CDK in the trimeric complex. Thus, p27 have a dual regulator role of CDK-cyclin complexes by inhibiting their activity when Y74 and Y88 are not phosphorylated and allowing a partial activation when these residues are phosphorylated (8).

In addition, this region contains a PRD domain (*Proline Rich Domain*, aa 91-96) which can interact with proteins containing *Src homology 3* (SH3) domains. The PRD of p27 interacts with a number of proteins that play important roles as for instance in cell-cycle control (Grb2), transcriptional regulation (PCAF), and nuclear transport (NPAP60). Finally, this region also presents a *Nuclear Export Signal* (NES, aa 32-46) (8).

In the **C-terminal** region of p27 (aa110-198) is located a *Nuclear Localization Signal* (NLS, aa152-168) sequence and a *Scatter-domain* (aa118-158). Through this *Scatter-domain*, p27 interacts with Rac, RhoA and stathmin, which play relevant cellular roles, independently of its CDK-regulatory activity, like modulating cellular migration by regulating acto-myosin remodelling and microtubule dynamics (Figure 3.B) (9).

A)

MSNVRVSNGS PSLERMDARQ AEHPKPSACR NLFGPVDHEE LTRDLEKHCR DMEEASQRKW
NFDFQNHKPL EGKYEWQEVE KGSLPEFYYR PPRPPKGACK VPAQESQDVS GSRPAAPLIG
APANSEDTHL VDPKTDPSDS QTGLAEQCAG IRKRPATDDS STQNKRANRT EENVSDGSPN
AGSVEQTPKK PGLRRRQT

B)

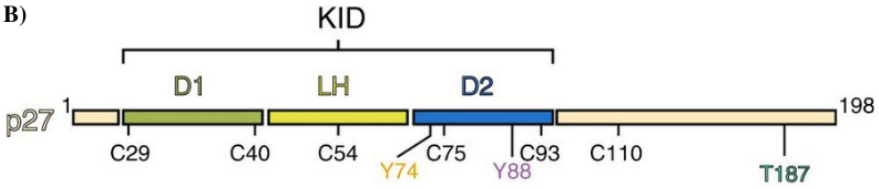

Figure 3. A) Amino acid sequence of p27. **B)** Interaction domain of cyclin/CDK complexes (9).

p27 is defined as an intrinsically unstructured (or disordered) protein (IUP or IDP), meaning that it partially lacks a stable three-dimensional tertiary structure when isolated under physiological conditions, and not bound to other proteins (8). Figure 4.A shows a predicted 3-dimensional structure of p27 using AlphaFold, measuring the per-residue model confidence in pLDDT, using different colours.

The “very low” category is a reasonably strong predictor of disorder, thus, regions that fall under this category are considered unstructured regions in isolated physiological conditions. This, can also be applied to a lesser degree to the regions under the “low” category. However, regions under the “very high” and “confident” categories are predicted to be structured regions, with the former indicating a higher structured region than the latter. (Figure 4.A) (10–12).

Disordered proteins adopt an ordered tertiary structure when they specifically interact with other biomolecules such as proteins or nucleic acids through the process of folding-upon-binding which is how most IDPs perform their biological function (8). To perform this process, p27 interacts with a cyclin through its cyclin-binding (D1) subdomain inducing a conformational change in the linker helix (LH) subdomain (Figure 4.B) (9).

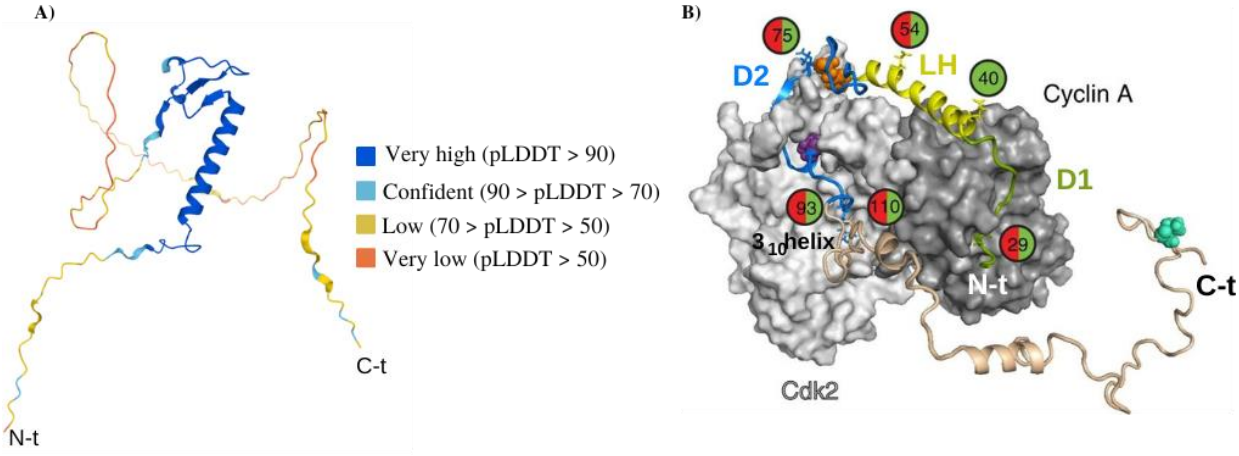

Figure 4. A) Predicted 3-dimensional structure of p27 using AlphaFold. Per residue confidence is measured in pLDDT (10–12). **B)** p27 binding with cyclin A /CDK2 complex (9).

7.4. Regulation of the cellular levels of p27

Cellular levels of p27 are regulated by both transcriptional and post-transcriptional processes. Proper positive and negative regulation of p27 levels play a very important role in regulating the cell cycle, where as a loss of a single p27 allele would be enough to increase tumour incidence via CDK mediation. Moreover, reduced p27 levels are observed in human cancers in association with aggressiveness and a poor clinical prognosis. Mostly, low p27 levels are produced by post-transcriptional mechanisms that induce its degradation via the ubiquitin-proteasome pathway (13).

7.4.1. Transcriptional Regulation

Transcriptional regulation of p27 occurs via the interaction of transcription factors (TFs) with the CDKN1B promoter gene. Different TFs have been identified as transcriptional activators of p27, (AFX, Sp1, E2F1, FKHR-L, Menin and FOXO proteins), therefore inhibiting or activating these TFs will directly regulate p27 expression. FOXO1a, FOXO3a and FOXO4 activates p27 expression, thus inducing cell arrest at G0. Various pathways such as PKB phosphorylation, 14-3-3 protein binding, cytoplasmic

sequestration and the proto-oncogene PIM-1 and serine/threonine kinase (PIM1) down-regulate these TFs (7).

Protein kinase B (PKB or AKT) is a serine/treonine protein kinase that is a key regulator of a wide variety of cellular functions including cell proliferation, survival, metabolism, angiogenesis, and apoptosis in both normal and malignant cells (14). PKB phosphorylates and inhibits FOXO3a. Its phosphorylation at Thr 32, Ser 253 and Ser 315, facilitates its binding with the protein 14-3-3 causing FOXO3a cytoplasmic accumulation, and a reduction of p27 transcription. PKB is activated by phosphorylation, meaning that depending on PKB phosphorylation or dephosphorylation, it will indirectly regulate p27 transcription (7).

In contrast, c-myc, Id3 and Ap1 are transcriptional repressors. The most studied negative regulator of p27 expression is c-myc, a proto-oncogene that can directly inhibit p27 expression by binding to its promoter. Nevertheless, the role of transcriptional regulation in the cellular levels of p27 is quite limited, being the control of the stability of the p27 protein, by degradation via proteasome in the nucleus or cytoplasm, the main mechanism controlling p27 cellular levels.

7.4.2. Translational regulation

p27's translation during the cell cycle reaches its maximum at G_0 and early-G_1 phases and decreases during the transition to S phase (15).

p27's 5'UTR contains an internal ribosomal entry site (IRES) and an upstream open reading frame (uORF), which are used to initiate translation in a cap-independent manner. Regulators of IRES or uORF activity will therefore affect p27 translation. There are several p27 IRES regulators including: The *long Intergenic Non-Protein Coding RNA 2303 (LINC02303) p53-inducible lncRNA (TRMP),* (16), the ELAV/Hu proteins (17), the Dyskerin Pseudouridine Synthase 1 (DKC1) (18), the Polypyrimidine tract-binding protein (PTB), a multifucional RNA-binding protein (19), and the Poly (RC) Binding protein 1 (PCBP1) (18).

As examples, the protein PTB, increases p27 mRNA IRES activity, and consequently increases p27 expression. TRMP is a p53-inducible lncRNA that suppresses IRES-dependent p27 translation by binding to polypyrimidine tract binding protein 1 (PTBP1) which regulates posttranscriptional gene expression. TRMP-PTBP1 binding alters the PTBP1 interaction with p27 5'UTR, decreasing p27 expression (16). ELAV/Hu RNA binding proteins HuD and HuR repress expression of p27 by also inhibiting the protein's IRES activity. HuR has been found over-expressed in malignant brain tumours and colon cancer (17). PCBP1 binds to p27's 3'UTR through its KH1 domain, stabilizing it and enhancing its translation. Recent studies have shown that PCBP1 loss is involved in a variety of carcinogenesis (20). Studies on mice pituitary gland showed DKC1 reduced p27 IRES-dependent translation. Deregulation of DKC1 can be found in various human cancers such as breast carcinomas and prostate cancers (18).

7.4.3. Post-translational modifications and stability

The regulation of p27 levels is mainly performed by post-translational modifications and its degradation via de ubiquitin-proteasome pathway is the main mechanism to regulate p27 levels in the cell (Figure 5).

The KID domain of p27 contains three tyrosine residues Y74, Y88 and Y89, that can be phosphorylated by different tyrosine kinases as JAK2, BCR-Abl, c-Src or Lyn. These phosphorylations regulate post-translational modifications and degradation of p27 via the ubiquitin-proteasome pathway (7). Y88 is a conserved tyrosine in the Cip/Kip family of CDKIs and its phosphorylation is preferential among the three tyrosine residues mentioned above. Degradation of p27 begins in the early-G_1 phase with the phosphorylation of Y88. This phosphorylation triggers a conformational change leading to a partial activation of the CDK activity in the p27-cyclin-CDK complexes. A second phosphorylation takes place in T187 of p27 by the activated CDK, allowing recognition of p27 by the SCF-Skp2 complex and thus initiating ubiquitin-proteasome-dependent degradation of the protein (21,22).

The protein p27 can move between the cytoplasm and the nucleus and this movement is regulated by two domains in the protein: the Nuclear Export Signal (NES) and the Nuclear Localization Signal (NLS). This transport is regulated by several kinases like PKB, RSK1/2 and ERK1/1 (23). PKB can phosphorylate the S10 residue of p27, thus facilitating the association of the p27 KID domain to the CRM1/Ran-GTP exporter complex, leading to its translocation from the nucleus to the cytoplasm. PKB can also phosphorylate and inactivate the mammalian target of rapamycin (mTOR) inhibitor, and the serum and glucocorticoid-induced kinase 1 (SGK1), and in turn phosphorylate p27 impairing its nuclear import causing cytoplasmic accumulation (24).

Under glucose deficiency conditions, the human kinase interacting with stathmin (hKIS) induces S10 phosphorylation of p27 leading to its translocation to the cytoplasm and its consequent degradation (25).

The protein Myc, an oncogene found activated in many human cancers, can regulate p27 levels by post-translational mechanisms. Myc when activated, promotes the formation of CDK2 complexes with cyclins A and E, becoming activated. Then, CDK2 can phosphorylate T187 of p27 triggering its ubiquitin-dependent degradation (7).

It has also been reported that at early G1, the acetyltransferase PCAF, a transcriptional co-activator, directly binds to p27 (aa91-120) through its HAT catalytic domain (24). PCAF acetylates K100 of p27, inducing its ubiquitylation and subsequent degradation through the proteasome (26). Thus, an overexpression of PCAF induces p27's degradation, however, knockdown of PCAF stabilizes p27 (27).

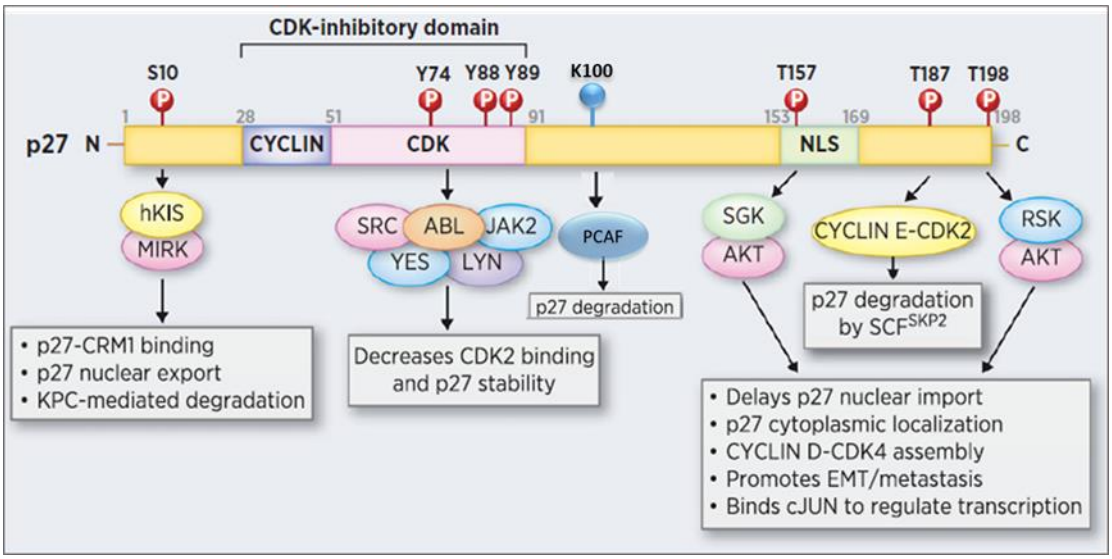

Figure 5. p27 post-translational modifications depicting phosphorylation sites (in red) and acetylation of K100 by PCAF (in blue) (26) which has been added to the original figure (22).

7.5. Cellular functions of p27

As previously mentioned, p27 is essential to properly regulate cell cycle progression, by modulating cyclin-CDKs activities. However, modulating CDK activity isn't its only relevant function since p27 also plays a role in regulating other relevant functions such as: cell migration, metabolism, transcription, and cell death (7).

7.5.1. p27 and cell proliferation

a) The cell division cycle

The mammalian cell division cycle is an essential process by which a given cell duplicates generating to daughter cells. Both daughter cells are genetically identical. This process is regulated by a variety of complex mechanisms to keep the cell functioning and dividing properly, at a steady rate. Tight regulation of this process is very important since uncontrolled proliferation of cells can lead to cancer, one of the highest leading causes of death in the world.

The cell cycle consists of two main phases that are the Interphase and Mitosis (28). The interphase initiates as soon as a daughter cell is formed. The interphase consists of the **G1 phase**, where the cells integrate various external stimuli that activate different metabolic and signalling pathways producing the accumulation of macromolecules and substrates necessary for cellular growth and cell division. A relevant molecular process during G1 is the hyper-phosphorylation of the proteins of the retinoblastoma family (pRB, p130 and p107) that specifically takes place in late G1. These phosphorylations trigger the release of E2F transcription factors that activate the expression of genes essential for the progression from G1 to the later phases of the cell cycle. The next step of the interphase is the **S phase.** During this phase, the cellular genetic material is duplicated. Later, after

DNA replication, cells enter in a short transition phase named **G2** (also included in the interphase) and finally cells move into mitosis (**M phase).**

In the M phase, the cell goes through drastic changes, including mitotic spindle formation, sister chromatids separation (mitosis) and cytokinesis where the cytoplasm separates into two parts that will form two independent daughter cells. It worth mentioning that many different cells from adult organisms (neurons, hepatocytes….) once emerged from the cell cycle, remain in a quiescent state known as G0.

The levels of p27 as well as its subcellular localization vary throughout the cell cycle. Cells in G0 and during the first half of G1 have high levels of this protein in the nucleus. However, as G1 progresses, p27 translocate into the cytoplasm and its amount decreases until it is almost undetectable in the S phase. This suggests that besides being a direct modulator of the CDKs activities during cell proliferation, in the nucleus of quiescent cells it may be performing other functions relevant for cell cycle progression (29).

Progression through the cell cycle is finely regulated by specific **CDK complexes** that are activated and deactivated sequentially at different times of this process. These complexes are composed of a catalytic subunit with kinase activity (CDK) and a regulatory subunit (cyclin). The CDKs are only active in association with their cyclins. The following complexes control each of the cell cycle phases (29):

- G_1-CDK complex: Cyclin D/CDK4, 6
- G_1/S-CDK complex: Cyclin E/CDK2
- S-CDK complex: Cyclin A/CDK2
- M- CDK complex: Cyclin A/CDK1 and Cyclin B/CDK1

b) Cyclin-dependent-kinases (CDKs) and their regulation

As mentioned above, cell cycle progression is mainly regulated by complexes containing two subunits: CDKs and cyclins. CDKs are a family of serine/treonine kinases that regulate different cellular functions. Their association with the regulatory subunits (cyclins) is essential for their activity and the formation of specific **cyclin-CDK complexes** is performed at specific moments during cell cycle (30). Up to now, there have been found 20 CDKs in human cells (from CDK1 to CDK20), at least six of which are directly involved in cell cycle progression: CDK1, CDK2, CDK3, CDK4, CDK6 and CDK7 (31).

Cyclins are another family of cell cycle regulator proteins that vary their concentrations in a cyclical pattern during the cell cycle. These oscillations drive the cyclic assembly and activation of the cyclin-CDK complexes. Once cyclin-CDK complexes are activated, these will help trigger various cell cycle events such as entry into the S phase or M phase. (32) In mammalian cells there are at least 16 families of cyclins (from cyclin A to L and cyclins O, P, T and L), with each family containing one or more members.

Cyclin-CDK complexes play a key role in the cell cycle control system which regulates proper cell cycle progression transition points: **G_0/G_1; G_1/S; G_2/M phase and M phase-**

exit. The cell cycle control system ensures that the different phases of the cycle will be operated in a defined sequence and the next phase will not start if the current one is not finished.

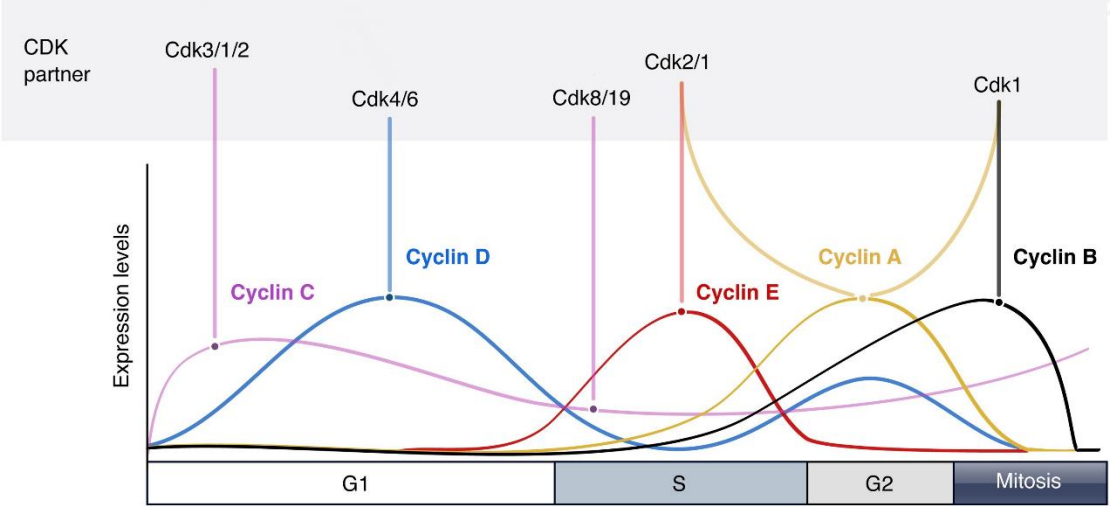

Figure 6. Representation of different cyclin levels during cell cycle and their respective kinase partners (32).

Different mechanisms have been reported to be involved in the regulation of the activity of cyclin-CDK complexes:

i) The formation of the cyclin-CDK complex. Specific CDKs associated with their corresponding cyclins (Figure 6).

ii) Activating and inhibitory phosphorylations and dephosphorylations. CAK and Wee1 kinases activating and inhibiting respectively; also, the activating Cdc25 phosphatase.

iii) Association with members of the **CKIs** protein family (Figure 7). There are two sub-families of CKIs: the INK4 sub-family (p15, p16, p18 and p19), and the CIP/KIP sub-family (p21, p27 and p57). The INK4 sub-family prevents the interaction of Cdk4 and 6 with members of the D-type of cyclins, thus regulating G0/G1 transition. The CIP/KIP sub-family are inhibitors of most cyclin-CDK complexes (cyclin D/CDK4, 6, cyclin E/CDK2, cyclin A/CDK2, cyclin A/CDK1 and cyclin B/CDK1).

iv) Ubiquitin ligase complex (KPC, SCF^{SKP2} and APC) mediates proteolysis of cyclins and CKIs, and also modulates CDK activity.

v) Subcellular localization. The specific roles of the different cyclin-CDK complexes also depend on their specific subcellular localization.

c) The role of p27 in the regulation of cell proliferation

The p27 protein, as a modulator of cyclin-CDK activities, plays a key role in the progression of the cell cycle by mainly regulating the G1/S transition (7).

In quiescent cells, levels of p27 are high and mainly located in the nucleus. After proliferative activation, it is translocated to the cytoplasm where it is progressively degraded by the ubiquitin-proteasome pathway. Moreover, after proliferative activation, cyclin D is synthesized and the formation of cyclin D/CDK4, 6 complexes is promoted.

Interestingly, p27 helps to keep together the two subunits of the complex, although it initially remains inactive. Then, p27 is phosphorylated by the tyrosine kinases of the SRC family in residues Y74, Y88 and Y89. These phosphorylations allow cyclin D/CDK4,6 complexes to be partially active. Subsequently, active cyclin D/CDK4,6 complexes start to phosphorylate the protein p130 and pRB (member of the retinoblastoma family) allowing transcription of genes involved in cell cycle progression, among them cyclin E. Cyclin E can then associate with CDK2 that becomes activated. Finally, cyclin E-CDK2 additionally phosphorylates p130 and pRB inducing transcription of a lot of genes involved in the entry into the S phase and the subsequent progression to the M phase. Additionally, active cyclin E-CDK2 complexes phosphorylate p27, inducing its degradation (33).

Therefore, p27 acts as a dual regulator of the cell cycle during the first half of G1, allowing the formation of complex CDKs but keeping them inactive. Subsequently, once p27 is phosphorylated into specific residues, these complexes can be activated and therefore can phosphorylate their substrates and induce the progression of the cell cycle. Low levels of p27 in tumour cells, are involved in the tumorigenesis process and associated with poor prognosis (33).

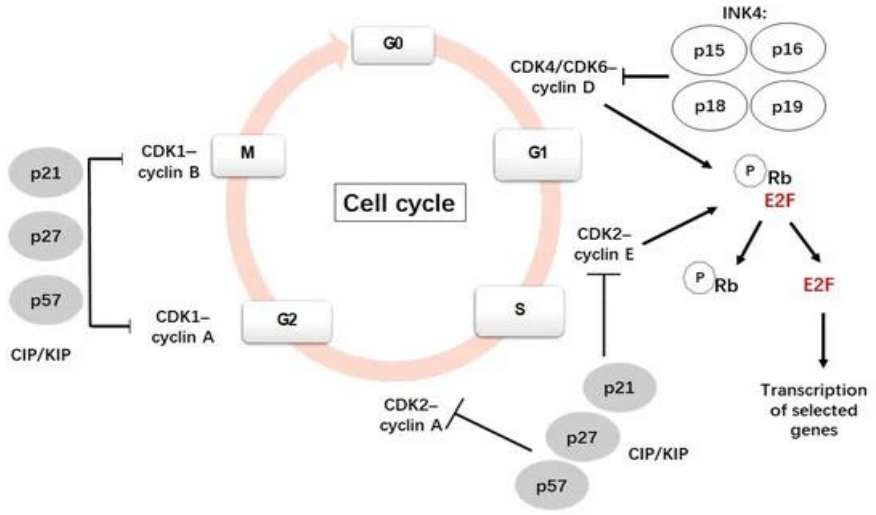

Figure 7. Cell cycle and its main regulatory mechanisms by CKIs (33).

7.5.2. p27 as a transcriptional regulator

During quiescence and at early G1 phase of the cell cycle, normal cells have high levels of p27 in the nucleus. When cells are induced to proliferate, nuclear p27 translocates into the cytoplasm where it is degraded. Since it has been seen that proliferating cells need low levels of p27, the presence of high levels of p27 in the nucleus of quiescent cells, suggests that p27 plays an important role in the nucleus related to cell cycle inhibition. The identification of chromatin-associated proteins as p27-binding proteins, strongly suggests that p27 can be associated with chromatin as a transcriptional regulator. (26). There is no evidence that p27 binds directly to DNA, thus the binding to chromatin must be via intermediate TF and co-regulator proteins (Figure 8). It has been described that p27 binds to chromatin regions containing interaction motifs to TFs such as **E2F4**, and

members of the **ETS** family (Ets1). The direct interaction of p27 with these TFs has been further confirmed by affinity chromatography. Thus, p27's association with these TFs in promoter regions of specific genes indicates a specific role of p27 in transcriptional regulation (34). Chip-on-chip, Chip-seq and IP analysis has revealed a number of transcriptional regulators that could associate with p27, including c-JUN, STAT3, MYOD, AHR, PAX4, PAX5, AHR, PCAF1, HDAC1, Ap2α, Ap2γ, SIN3A, NGN2, E2F4 and p130. (22,35–37).

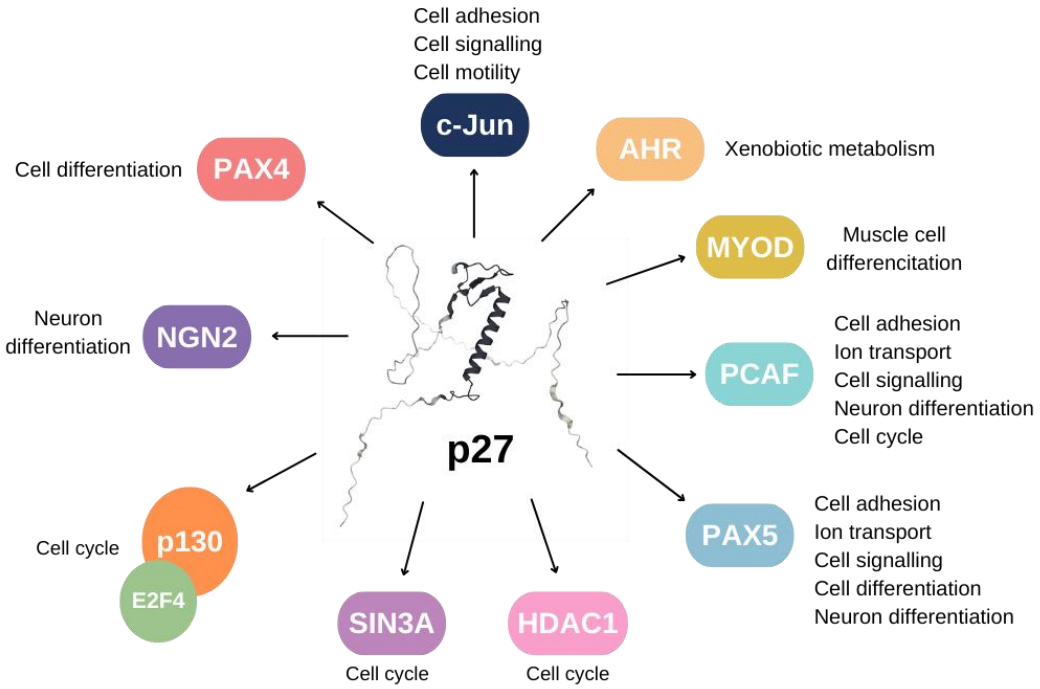

Figure 8. Regulation of transcriptional activity by p27 is mediated by its association with different TFs and co-regulators. Adapted from Figure 1.B of (22) and Figure 4 of (26).

Expression microarray analyses performed in quiescent mouse embryonic fibroblasts (MEFs) from WT and p27 knock out (p27KO) animals indicated that p27 regulates a great diversity of transcriptional programs including cell cycle, cell motility, neuronal differentiation, cell adhesion and axon guidance among many others (Figure 9). Interestingly, in addition to these transcriptional programs, cells lacking p27 also present deregulation of genes involved in various relevant pathologies like cancer and several neurodegenerative diseases such as Huntington's disease, Alzheimer's disease, and Parkinson's disease. These results suggest a role of p27 in the development of these different pathologies. Moreover, cells lacking p27 also show the deregulated expression of a significant number of TFs. This observation is very relevant since it indicates that in the absence of p27, in addition to the initial deregulation of gene expression mediated directly by p27, several other waves of gene expression mediated by these deregulated TFs may be produced. The expression of some of the deregulated genes observed in p27KO cells was found to be down-regulated, but also, the expression of many other genes was up-regulated, indicating that p27 plays a role as a transcriptional repressor but also as a transcriptional activator (26).

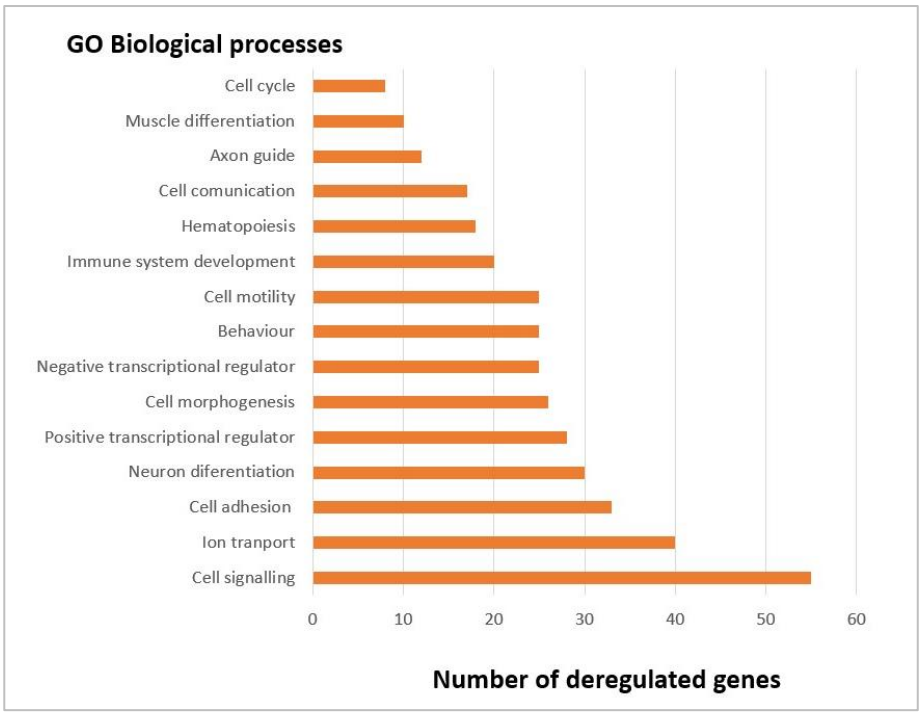

Figure 9. **Gene Ontology (GO) analysis of the protein coding with p27 binding sites in their proximity.** The Database for Annotation, Visualization, and Integrated Discovery (DAVID) program was used to determine biological processes enriched in the 852 protein coding genes (36).

Several subsequent studies have led to description of different signalling pathways transcriptionally regulated by p27 associated with different TFs and transcriptional co-regulators (Figure 10).

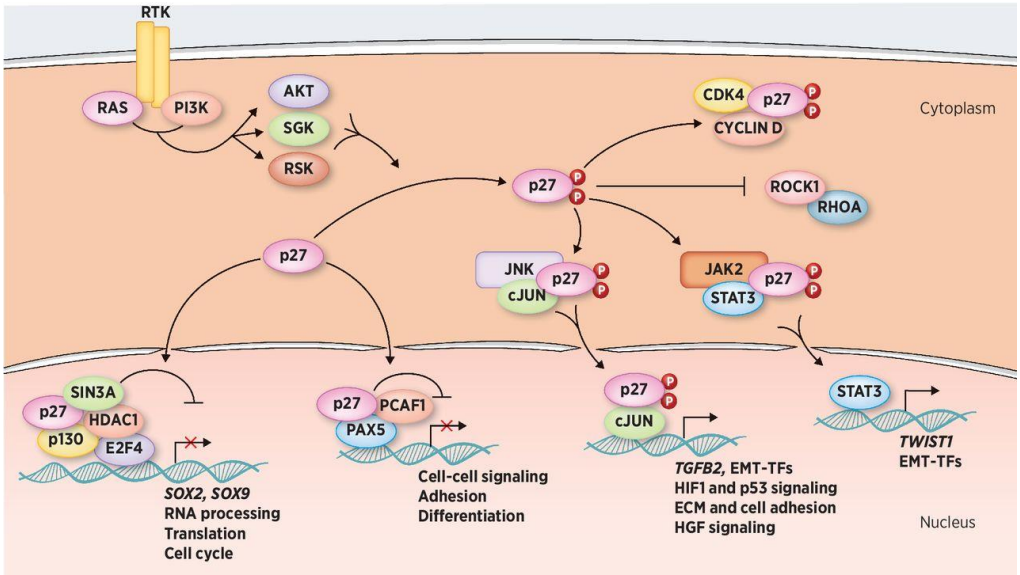

Figure 10. Some signalling pathways transcriptionally regulated by p27 and interacting with TFs and transcriptional co-regulators and their role in biological processes (22).

a) p27 as a transcriptional regulator of the cell cycle

As described above, p27 regulates the cell cycle progression by modulating the activity of the cyclin/CDK complexes, but it can also regulate proliferation by regulating the expression of essential genes for cell cycle progression. The fact that p27 is an IUP makes it possible to interact with different proteins, including TFs and transcriptional co-regulators.

The mechanism by which p27 regulates transcription in collaboration with E2F4 has been extensively described. In quiescent cells, E2F4 associates with p130 and p27 in the promoters of genes involved in the cell cycle, to form a transcriptional repressor complex (p27 / p130 / E2F4). Additionally, other co-repressors such as HDACs and mSin3A can also associate to these complexes. Therefore, in the G0 state, the genes involved in DNA replication and the subsequent phases of the cycle are repressed, and only are activated when the cells enter and progress into the cell cycle.

During G1 progression, hyperphosphorylation of p130 and pRB by cyclin-CDK complexes results in loss of this interaction and activation of E2F4-dependent gene expression. P27 has been shown to directly interact with E2F4, p130, HDACs, and mSin3A. Specifically, p27 binds to E2F4 / p130 through its C-terminal region, thus, participating in the transcriptional regulation of E2F4-depending genes (34).

On the other hand, the N-terminal region of p27 interacts with cyclin-CDK complexes. This indicates that at early G1, p27 acts as a scaffold protein, holding the repressor complex together with cyclin/CDK. When p27 is phosphorylated at residues Y74 and Y88 by Src kinases, the cyclin/CDK complexes are activated which then phosphorylate p130 and consequently, the expression of genes involved in cell proliferation is activated (38).

Furthermore, p27 indirectly regulates the transcription of $p21^{cip1}$ (another member of the Cip/Kip family of CKIs) repressing the expression of the transcription factor Pitx2 (an activator of p21 expression). P27 associates with a regulatory region of this gene together with an E2F4 repressive complex. In this way, the decrease of p27 levels at the end of G1 or in cells lacking p27, would activate the expression of Pitx2 and consequently that of p21 (39).

7.5.3. p27 as a cytoskeleton regulator and its implication in cell migration

p27's cytoskeleton regulation relies on its interaction with different proteins through its C-terminus region (23). Through this mechanism it can regulate both actin filaments and microtubule cytoskeleton. Unlike p27's regulatory roles in cell proliferation, these roles are conducted in a CDK-independent manner (34).

Rho GTPases are essential actin cytoskeleton and cell migration regulators. Specifically, RhoA and its effector ROCK1 *(Rho-dependent kinase 1*) plays an essential role in the regulation of actin filaments and hence affecting cell migration (23). RhoA activates

ROCK1 through the LIM kinase, which phosphorylates the actin-binding protein cofilin. Phosphorylated cofilin cannot associate to monomeric actin thus stabilising actin filaments and blocking cell migration. Moreover, ROCK1 also phosphorylates the Myosin light chain (MLC) by modulating the ATPase activity of Myosin and induces the generation of stress fibres and blocking cell motility (40).

It has been reported that p27 can regulate RhoA activity. Specifically, p27 inhibits RhoA activity by blocking RhoA's binding to its activating factors, these being guanine nucleotide-exchange factors (GEFs) (40). Thus, inactive RhoA favours actin depolymerization and promotes cell migration. Interestingly, phosphorylation of p27's T198 by different kinases also induces its association to RhoA and in consequence favours cell migration (41). To summarize, cytosolic p27 induces cell migration by blocking RhoA activity.

Several reports also indicate that cytosolic p27 stabilizes microtubules through different mechanisms. Studies using murine cortical neurons showed that p27 C-terminal domain directly interacts with α-tubulin-acetyltransferase1 (α-TAT1), a key enzyme involved in α-tubulin acetylation, affecting its stability. This interaction prevents αTAT1's proteosome-mediated degradation and modulates microtubule acetylation and axonal transport (23,42).

A direct binding between protein regulator of cytokinesis 1 (PRC1) and p27 has also been reported. PRC1 is mainly localized in the nucleus and is involved in cytokinesis. p27 interaction with PRC1 prevents the formation of perinuclear thick bundled MTs observed in PRC1 overexpression (23). Its expression is increased in a variety of different cancers, and it acts as an oncogene, as for instance, in bladder cancer it promotes cancer cell proliferation, apoptosis inhibition and carcinogenic progression.

P27 also stabilizes MTs by associating with Stathmin, an MT-destabilizing protein. Specifically, Stathmin-p27 binding decreases the Stathmin ability to bind to MTs, therefore decreasing its MT-destabilizing activity (23).

P27 can also regulate cell migration through its role as a transcriptional regulator. For instance, it has been shown that p27KO cells present the up-regulation of several chemokines such as CXCL1 and CXCL5 that are involved in angiogenesis, invasion, and metastasis (26).

7.5.4. Role of p27 in metabolism regulation

Evidence indicates that p27 may play a role in regulation of energetic metabolism. A KEGG pathway analysis of data from an expression microarray analysis performed in MEFs-p27KO unveiled several metabolic pathways seemingly regulated by p27, these being glutathione metabolism, pyruvate metabolism, purine and pyrimidine metabolism, fatty acid metabolism and amino acid metabolism (26).

As previously mentioned, p27 can regulate transcription by associating with TFs such as E2F4. Several E2F-target genes have been described to be involved in glycolysis and lactate synthesis (43). Specifically, it has been reported that E2F1 can activate the

expression of phosphofructokinase (pfk-1) and pyruvate kinase 2 (pkm2) (44), indicating that p27 regulates the glycolytic flow and the levels of pyruvate and lactic acid. Thus, the loss of p27 induces a reduction in pyruvate levels and an increase in lactic acid levels.

The other hand, p27 positively regulates the expression of the NdufB9 and NdufS3 subunits of complex I (CI) of the respiratory chain. That is, the loss of p27 induces a decrease of these subunit levels and consequently decreases oxidative phosphorylation. This regulation is mediated by the transcriptional complexes p130/E2F4.

It has also been shown that p27 regulates the expression of transcriptional coactivators (PGC-1α, PGC-1β, and PRC) that regulate the expression of mitochondrial genes, present in the nucleus and mitochondria, involved in the integrated metabolic response to intracellular bioenergy demands. Therefore, suggesting that p27 is also involved in the regulation of mitochondrial biogenesis.

7.5.5. Role of p27 in apoptosis and autophagy

p27 plays a significant role in apoptosis. Studies suggest that apoptosis occurs in the G1 phase of the cell cycle, and that late G1 or S phase arrest can accelerate it. Since cell cycle is regulated by cyclins/CDK complexes, these can play an essential role in apoptosis. The most important ones are cyclin D/CDK4,6 and cyclin E or A/CDK2 which are assembled during G1, G1/S and S phase of the cell cycle. As p27 is a regulator of cyclin/CDK activity, thus it may affect apoptosis by regulating the activity of these complexes. Even though overexpression of p27 can have a protecting effect from apoptosis, mainly through maintaining CDK2 inactivity, other studies have reported that it also has pro-apoptotic effects (7).

Studies in cultured human umbilical vein endothelial cells (HUVEC), show that apoptosis in these cells is related to the up-regulation of cyclin A/CDK2 and cyclin E/CDK2 complexes. The C-Ter region cleavage of p27 during apoptosis by the cysteine protease (CPP32) and CPP32-like caspase alters its intracellular localization and leads to its separation from cyclin A/CDK2 and cyclin E/CDK2 complexes, and thus, inducing a substantial increase of CDK2 activity (7,45). Another study has shown that under stress states like growth factor deprivation, reduced or absent p27 levels causes an increase of cyclin A/CDK2 activity but not cyclin E-CDK2 activity, which causes an unscheduled increase in CDK2 activity inducing cell cycle exit through apoptosis. However, when in presence of growth factors, reduced or absent p27 levels increase activity in cyclin A/CDK2 and cyclin E/CDK2, favouring cell cycle progression and proliferation (46).

Research on lncRNA urothelial carcinoma associated 1 (UCA1) on cardiomyocytes support that up-regulation of p27 in heart injury has been associated with an increase of cell apoptosis. Reduction of UCA1 plays a pro-apoptotic role on cardiac cells through increased p27 expression (47).

In $A\beta_{42}$ induced neuronal apoptosis, p27 has been reported to have a pro-apoptotic role. This mechanism of apoptosis is mediated by an aberrant activation of the MEK-ERK pathway, leading to cyclin D1 overexpression. This overexpression prevents negative

regulation of the pathway through p35/CDK5 due to cyclin D separating CDK5 from p35. p27 stabilizes cyclin D1/CDK5 but not p35/CDK5, hence promoting $A\beta_{42}$ induced neuronal apoptosis (48).

Autophagy is another essential process in which p27 is involved. It is a cell self-degradative process that allows degradation and recycling of intracellular components allowing to maintain cellular homeostasis under stress or nutrient depletion. In some circumstances, autophagy can lead to cell death (49).

mTOR is a crucial regulator of cell growth, keeping a balance between anabolic and catabolic metabolism. It has been shown that it negatively regulates cell autophagy. In situations of nutrient depletion, energy/nutrient-sensing kinases LKB1 and AMPK phosphorylate p27 at different residues causing cytoplasmic retention. Cytoplasmic p27 was discovered to be a pro-autophagic regulator of basal and induced autophagy. p27 negatively affects mTOR signalling on inhibiting the Regulator Complex Assembly, thus promoting autophagy in conditions of prolonged amino acid-depravation (50).

8. Discussion of p27 within the Hallmarks of Cancer

Several lines of evidence indicate a role of p27 in cancer development. It has been shown that p27KO mice show gigantism and multiorgan hyperplasia, as well as pituitary tumours (51). Moreover, haploinsufficiency of p27 (p27+/-) in mice has been shown to be sufficient for tumour generation after exposure to γ radiation or chemical carcinogens. In addition, mice with a p27 mutation that incapacitates them to bind to CDKs (p27CK-) show spontaneous tumorigenesis in different organs (52).

Different types of alterations relevant for cancer development have been shown to significantly reduce p27 levels. For instance, Src kinase that shows an increased activity in many tumours and has been related to the general decrease in p27 levels (53). Moreover, increased expression of different ubiquitin ligases such as Skp2 or Pirh2 in several tumours has also been linked to the widespread decrease in p27. Increases in c-myc reduce p27 levels by various mechanisms are common in different cancers (54). The PI3K/PKB axis is also increased in many types of cancers and has been linked to the nuclear delocalization of p27 in different tumours and with a higher metastatic potential (55).

It has been described that in many different types of human tumours, a decrease in p27 or its cytoplasmic localization is associated with an increase in malignancy and worse prognosis. The mutations of p27 in tumours are infrequent, hence its decrease or its cytoplasmic localization are the main alterations related to cancer development (26).

As previously stated, in addition to its role as a **cell cycle regulator**, p27 has many other cellular functions. This functional diversity gives to p27 an important role in tumour formation and development. The duality of p27 in acting either as an oncogene or as a tumour suppressor depending on its **subcellular localization** is crucial in its role in cancer development (7). It is especially relevant in its role as a **transcriptional regulator**. The

great variety of transcriptional programs regulated by p27 reveal that it is involved in different Hallmarks of Cancer, which is discussed below.

a) Role of p27 in "Sustaining proliferative signalling"

According to its role as a **CDK inhibitor**, decrease of p27 levels induced an increase in activity of cyclin-CDK complexes. Thus, as mentioned above, many different tumours, including lung, colon, prostate, breast, esophageal carcinomas, head and neck cancers, melanomas, gliomas/astrocytomes, Barret's associated adenocarcinomas and haematological tumours show decreased levels of p27. These reduced levels are mainly regulated by post-transcriptional mechanisms primarily through the up-regulation of p27's degradation, which is frequently associated with increase of Skp2 and Cks1 activity (56). In addition, reduction of p27 promotes (**as a transcriptional regulator**) increased expression of genes involved in DNA replication (under the control of repressor complexes p130/E2F4) (26). The expression of other genes, related to cell proliferation (growth factors, growth factor receptors MAPK, G- proteins), or that participate in different signalling pathways (Wnt and Notch) are also up-regulated in the absence of p27.

b) Role of p27 in "Tissue invasion and metastasis"

This is another well-known hallmark in which p27 has a significant role. Osteosarcoma (OS) is a type of cancerous bone tumour and is most frequently diagnosed in children and adolescents. Its leading cause of death is the development of pulmonary metastasis. Studies show that cytoplasmic p27 is associated with metastasis and poorer prognosis in OS patients. A possible model explaining this association is via cytoplasmic p27-mediated p21 activated kinase 1 (PAK1) phosphorylation (57). PAK1 is a crucial effector linking Rho-GTPases to cytoskeleton reorganization and nuclear signalling to mainly regulate cell motility and morphology (58). Its overexpression can be associated with many types of cancer, including OS. Cytoplasmic p27 can bind with PAK1 causing a conformational change of PAK1 inducing auto-phosphorylation. Subsequently, phosphorylated PAK1 initiates actin polymerization and promotes tumour cell motility and metastasis (57).

Tumour microenvironment has an important role in metastasis as well as tumour initiation. In gallbladder carcinoma, studies found that under nutrient starvation, stathmin1, a microtubule-binding-protein, is overexpressed and promotes migration and metastasis (59). Recently, new evidence suggested that in glucose deficiency, the hKIS kinase induced phosphorylation at Ser10 of p27, inducing its degradation in the cytoplasm. This fact induces up-regulation of E2F1 to promote stathmin1 expression inducing cell migration and invasion (25).

Reports have linked lymph node metastasis in thyroid cancer with over-expressed cyclin D1 and under-expression of p27. Most of papillary thyroid carcinomas show under-expressed levels of 27, which also gives them a higher incidence of metastasis. The mechanism in which cyclin D1 is over-expressed in the thyroid is not yet known, but

higher cytoplasmic levels of the protein were linked with higher incidence of metastasis (60).

Another aspect in which p27 can regulate invasion and metastasis by tumour cells would be by up-regulating the expression of different cytokines and chemokines. Thus, p27 reduction induces the expression of the chemokines CXCL1 and CXCL5, that act through binding to the CXCR2 receptor that has been reported to be involved in invasion and metastasis (26,61).

Several proteins such as tenascin C, periostin and Procollagen-Lysine, 2-Oxoglutarate 5-Dioxygenase 2 (Plod2) facilitate the survival of tumour cells disseminated in pre-metastatic niches are under transcriptional repression by p27 (26). Therefore, low levels of p27 would increase the expression of these genes thus facilitating the survival of these cells and, consequently, the induction of metastasis.

c) Role of p27 in "Sustained angiogenesis"

As mentioned above, p27 regulates the expression of different cytokines and chemokines (CXCL1 and CXCL5). Thus, p27 reduction induces the expression of these two chemokines, which through their binding to the CXCR2 receptor are involved in inducing angiogenesis, in addition to their role in invasion and metastasis (62,63).

d) Role of p27 in "Tumour promoting inflammation"

It has been described that most tumour processes are accompanied by inflammatory reactions in which an increase of different chemokines (CXCL1, CXCL5) and their receptors would be involved. So, deficient p27 levels would also be related to this hallmark due to its capacity to regulate the expression of these chemokines (64).

e) Role of p27 in "Deregulating cellular energetics"

The analysis of data from expression microarrays and chip on chip performed in p27KO cells versus p27WT cells showed that a significant number of genes involved in oxidative phosphorylation and belonging to different complexes of the respiratory chain were down-regulated in absence of p27.

Specifically, at least 18 genes involved in oxidative phosphorylation and belonging to different respiratory chain complexes were found to be down-regulated in p27KO cells. Interestingly, seven of these genes belong to complex I. It is worth mentioning that decreased respiration is a key mechanism leading to aerobic glycolysis, a feature of tumour cells.

In addition, alterations in a variety of metabolic enzymes have also been observed, meaning oxidative stress cannot be efficiently controlled in p27KO cells as there is a significant decrease in at least 15 enzymes involved in glutathione metabolism (37).

f) Role of p27 in "Evading apoptosis"

A very important physiological mechanism to avoid tumour formation and progression is apoptosis, which tumour cells can limit or evade (1).

Tyrosine kinase Src regulates key processes in tumour development including apoptosis, angiogenesis, and invasion. Src activity is found altered in various human cancers. Research done in malignant mesothelioma (MM) suggests that Src hyperactivation plays an essential role in its development which also correlates with a more advanced pathologic stage and metastasis. Src activation can accelerate p27 proteolysis by phosphorylating Tyr74 and Tyr88, thus reducing the protein's nuclear levels. This reduction has been related to adverse clinical outcomes in several tumour types. Src has been an important molecular target for MM treatment in the form of Src inhibitors. Further research tested Src inhibitor pyrazolo[3,4-*d*]pyrimidine in MM cell lines expressing active Src, which suggest that Src inhibition promotes p27 stability and therefore inducing apoptosis (65).

In other studies, using squamous cell carcinoma cells, increased p27 stabilization induced by proteasome inhibition also promoted apoptosis, and treatment with antisense p27 oligonucleotide was able to block apoptosis induced by proteasome inhibition, which further supports the proapoptotic role of p27 (66).

HOXA5, a member of the homebox gene (HOX) family, has an important role in tumour development, and is found down-regulated in several malignant tumours including cervical cancer, which its mortality rate is the highest among all female reproductive tract tumours. Analysis report that HOXA5 induces cell apoptosis, and that a reduction of its expression has been associated with cell proliferation and invasion. It was determined that HOXA5 overexpression causes up-regulation of p27 via PKB modulation and induces apoptosis in cervical cancer cells (67).

In summary, as shown in Figure 11, a decrease of p27 levels or its cellular relocation in tumorous cells could participate in increased malignancy due to deregulating a number of genes involved in the acquisition of at least five Hallmarks of Cancer and one Enabling Characteristic.

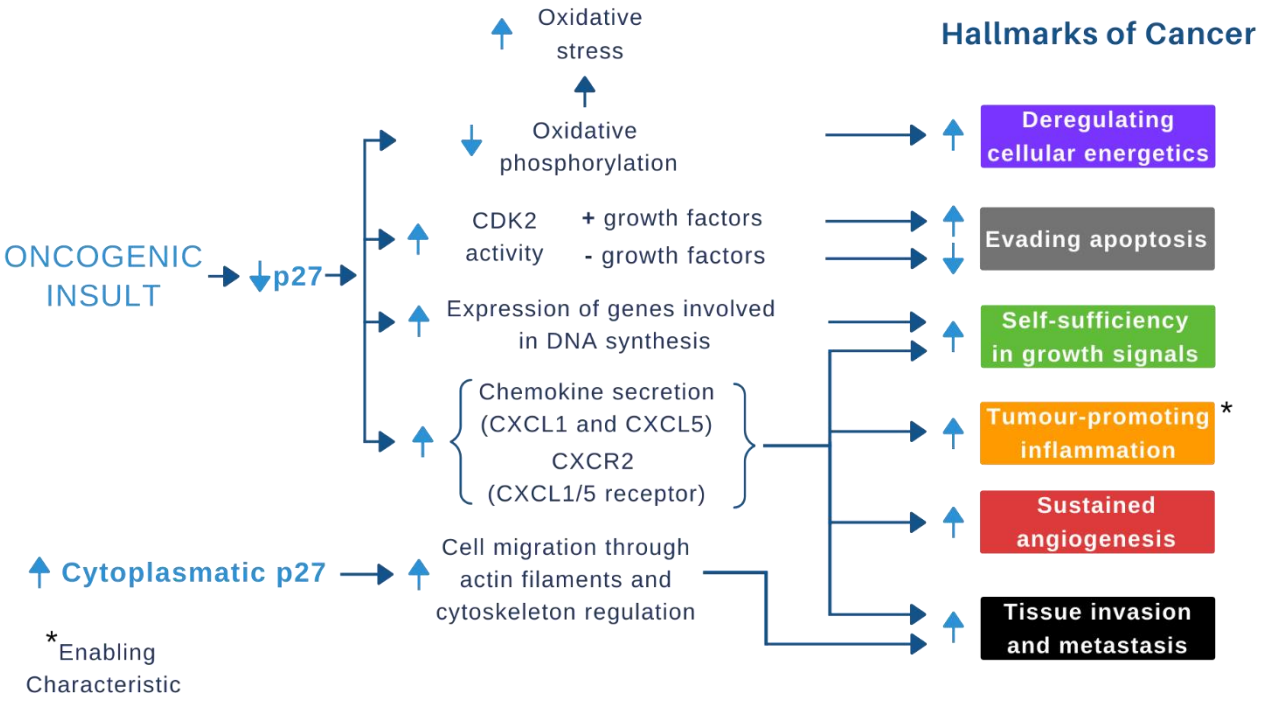

Figure 11. Summarized regulatory functions of p27 within the Hallmarks of Cancer under decreased p27 levels due to an oncogenic stimulus. The low p27 levels observed in many human tumours and their participation in tumour progression and malignancy. Adapted from Figure 6 of (26).

9. Conclusions

All the compelling data reviewed indicates the many functions in which p27 participates aside from its role in cyclin/CDK modulation during the cell cycle. The other roles where p27 is involved are transcriptional regulation, metabolism regulation, angiogenesis, apoptosis and autophagy, and cell migration. In order to perform its function as a transcriptional regulator, p27 needs TFs to interact with chromatin, since there is no data that suggests that it can bind directly to DNA.

1. p27 is an IUP, which adopts an ordered tertiary structure when it binds to cyclin/CDK complexes to perform its function as a CDK inhibitor. Its N-terminal region contains a KID domain which consists of three subdomains, D1, LH and D2. In D2 we find phosphorylation sites (Y74 and Y88) which allow partial activation of the trimeric complex. The C-terminal region contains a scatter domain which allows p27 to participate in roles in a CDK-independent manner.

2. During the cell cycle p27 acts as a dual regulator. In quiescent "normal" cells p27 acts as a repressor of transcriptional genes involved in the cell cycle. During the first half of G1 phase, it allows the formation of CDK complexes but keeps them inactive, then once p27 becomes phosphorylated in specific residues, these complexes become active which in turn are able to phosphorylate their substrates and induce cell cycle progression. In transcriptional regulation, p27 plays as a transcriptional repressor, but also as a transcriptional activator.

3. The Hallmarks of Cancer is a heuristic tool to better understand and conceptualize neoplastic disease and its complexities. These hallmarks consist of several biological capabilities that are acquired during human tumour development, and have been updated throughout the last 20 years, also including Enabling Characteristics which are biological characteristics that allow the acquisition of these hallmarks.

4. Evidence indicates that p27 plays an important role in oncogenesis, and it varies depending on its subcellular localization. Its cytoplasmic localization is associated with oncogenesis and increased malignancy, and on the other hand, nuclear localization is associated with tumour suppression. p27 participates in at least 6 Hallmarks of cancer which are: "Self-sufficiency in growth signals", "Sustained angiogenesis", "Evading apoptosis", "Deregulating cellular energetics", and "Tissue invasion and metastasis", and "Tumour-promoting inflammation".

Thus, ***p27 could be an important molecular target in cancer treatment as evidenced by its participation in several Hallmarks of Cancer. There is still a lot more to learn about this protein, but it remains clear that it has great potential as a prognostic factor and for further possible cancer treatments.***

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