FACULTAD DE MEDICINA
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TRABAJO FIN DE GRADO

CCDC6 en cáncer: funciones y aproximaciones terapéuticas

CCDC6 in cancer: functions and therapeutic approaches

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RESUMEN:

El conocimiento de la biología molecular del cáncer, incluyendo las funciones y alteraciones de genes reguladores de la proliferación y muerte celular, está impulsando el descubrimiento de nuevos biomarcadores. Su análisis permite un diagnóstico más preciso, así como el desarrollo de nuevas terapias dirigidas, que son la base de la medicina personalizada. Este trabajo de revisión se centra en el estudio de CCDC6 y su papel en cáncer. Coiled-coil domain containing 6 (CCDC6) es una proteína codificada por el gen homónimo, que está localizada en el cromosoma 10. Está implicada en la regulación del ciclo celular, la reparación del daño de ADN y el control de la apoptosis, interactuando para ello con otras proteínas como ATM o CREB1. La proteína fue originalmente descubierta fusionada con RET en pacientes con cáncer papilar de tiroides, pero hoy en día se sabe que se fusiona con otras proteínas y que está implicada en otros muchos tumores como los tumores no microcíticos de pulmón. Además, sus mutaciones inducen quimiorresistencia al cisplatino y etopósido; y sensibilizan la célula a los inhibidores de PARP, haciendo a CCDC6 un posible biomarcador para este tratamiento y una nueva diana terapéutica sobre la que actuar.

Palabras clave: CCDC6, cáncer, biomarcador, terapia dirigida

ABSTRACT:

The knowledge of the molecular basis of cancer, including the functions and mutations of regulator genes implicated in proliferation and apoptosis, is driving the discovery of new biomarkers. Their analysis allows a more precise diagnosis and the development of new targeted therapies, that are the basis of personalized medicine. This revision is focused on CCDC6 and its role in cancer. Coiled-coil domain containing 6 (CCDC6) is a protein encoded by a homonymous gene that is located in chromosome 10. It is involved in cell cycle regulation, DNA damage repair and apoptosis, interacting with other proteins such as ATM or CREB1. Originally, the protein was discovered as a partner in fusion with RET in patients diagnosed with Papillary Thyroid Cancer but, nowadays, it is known that it also fuses with other proteins and is implicated in other cancer such as Non-Small Cell Lung Cancer. Besides, its mutations induce chemoresistance to cisplatin and etoposide and sensitize the cell to PARP-inhibitors, making CCDC6 a suitable biomarker for this treatment and a new therapeutic target to act on.

Keywords: CCDC6, cancer, biomarker, targeted therapy

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1 ABBREVIATIONS

- ANK3: Ankyrin 3 or ankyrin G
- AREG: amphiregulin
- ATM: ataxia-telangiectasia mutated
- BER: base excision repair
- CBP: CRE binding protein
- CCDC6: Coiled-Coil Domain Containing 6
- CHK1: checkpoint 1 kinase
- CREB1: cAMP-responsive element-binding protein 1
- CTNNA3: catenin alpha 3
- DSB: double-strand breaks
- EGFR: epidermal growth factor receptor
- EST: expressed sequence tags
- FBXW7: F-Box and WD Repeat Domain Containing 7
- FGFR2: Fibroblast growth factor receptor 2
- HDAC1: histone deacetylase 1
- HR: homologous recombination
- KITLG: KIT ligand
- LIPI: Lipase-1
- MDC-1: mediator of DNA damage checkpoint-1
- NER: nucleotide excision repair
- NHEJ: non-homologous end joining
- NLS: nuclear localization signal
- NSB1: Nijmegen breakage syndrome protein 1
- NSCLC: non-small cell lung cancer
- ORF: Open reading frame
- PARP: Poly(ADP-ribose) polymerase
- PARPi: PARP-inhibitors
- PDGFR-β: Platelet-derived growth factor receptor beta (PDGFR-β)
- PP1: protein phosphatase 1
- PP4c: protein phosphatase 4
- PTC: papillary thyroid cancer
- PTEN: Phosphatase and tensin homolog
- RETi: RET-inhibitors
- RTK: tyrosine kinase receptor
- SSB: single-strand breaks
- SUMO: small-like ubiquitin modifiers
- TKI: tyrosine kinase inhibitors
- TKR: tyrosine kinase receptor
- TST1: Transforming Sequence Thyroid 1
- UBE2D1: Ubiquitin-conjugating enzyme E2 D1
- USP7: ubiquitin specific peptidase 7
- USPi: USP inhibitors
- VPS13B: vacuolar protein sorting 13 homolog B
- γ-H2A: histone H2AX phosphorylated at Ser13

2 INTRODUCTION

According to the American Cancer Society, cancer is defined as a set of illnesses characterized by uncontrolled proliferation and dissemination of abnormal cells that could lead to death. Cancer is considered a genetic disease caused by mutations in genes implicated in the regulation of the cell cycle and many other cellular processes, the loss of tumor suppressor genes or the activation of oncogenes. The knowledge of the molecular basis of cancer, including the functions and mutations of these regulatory genes, allows a more precise diagnosis through biomarkers analysis. Along with the development of new and more specific targeted therapies and the genetic profile analysis, this fact enables the development of personalized medicine.

Coiled-coil domain containing 6 (*CCDC6*), the main subject of this review, is one of these genes implicated in carcinogenesis and its progression. *CCDC6* is a proto-oncogene located on the long arm of chromosome 10 encoding for a protein with the same name that is involved in double-strand damage repair in the presence of genotoxic stress, cell-cycle regulation, proliferation and apoptosis modulation.

CCDC6 was originally identified as a partner gene in fusion with *RET*, forming a chimeric oncogene named *PTC1* that is accountable for developing papillary thyroid cancer (PTC) in the presence of ionizing radiation. Even though it was originally believed that this fusion only induces PTC, it is involved in the development of other carcinomas such as non-small cell lung cancer or colorectal cancer. In the last two decades, there have been found other fusions of CCDC6 with different genes, which are related to several cancer types. Moreover, it was discovered that the loss of CCDC6 activity, because of mutation or modifications in its cellular localization, induces uncontrolled cell proliferation, compromises DNA damage repair and induces chemoresistance to etoposide or cisplatin, reinforcing the idea that CCDC6 alteration is involved in tumorigenesis and cancer progression.

CCDC6 alteration impairs double-strand repair pathways, making cells with damaged DNA a suitable target for new drugs like PARP-inhibitors. Additionally, CCDC6 mutations are associated with chemoresistance to etoposide or cisplatin. CCDC6 is frequently fused to receptor tyrosine kinases like RET, sensitizing cancer cells to specific tyrosine kinase inhibitors, enhancing disease-free interval and survival of the patients.

In this work, it has been made a bibliographic revision of the last years on CCDC6 structure, interactions, post-translational modifications and regulation. Furthermore, this work describes CCDC6 principal functions and how its deregulation induces cell proliferation and tumorigenesis. The most frequent genetic alterations and the most prevalent cancers where CCDC6 has been found implicated are also described. Finally, the most relevant therapeutic approaches related to CCDC6 and the importance to investigate it as a useful biomarker have been exposed in this final degree project.

3 METHODOLOGY

3.1 BIBLIOGRAPHIC REVISION

In this final degree project, a bibliographic revision about CCDC6 and its role in cancer has been carried out. A total of 60 references including research articles, reviews and book chapters have been included in the reference list.

The PubMed database (http://pubmed.ncbi.nlm.nih.govor) was mainly used. PubMed is a public database that contains more than 33 million citations and abstracts of biomedical literature, chemical sciences and bioengineering.

The Mendeley reference manager was used as a tool for the management of documents and bibliographical references.

3.2 ENCODE ANALYSIS

To study the *CCDC6* gene structure, chromosomal localization and phylogenetic conservation, the ENCODE platform was used. The Encyclopedia of DNA Elements (ENCODE) is a public research consortium funded by National Human Genome Research Institute (NHGRI) and it is aimed at identifying all functional elements in human and mouse genomes.

The information showed in several figures and text of this work has been extracted from UCSC GENOME BROWSER (Feb 2009 version) developed by ENCODE. *Homo sapiens coiled-coil domain containing 6 (CCDC6), mRNA (from RefSeq NM_005436)* was the search term used to obtain all the relevant information from UCSC GENOME BROWSER. First, *CCDC6* locus together with introns and exons distribution was analyzed. Then, it was searched for "*Spliced ESTs, dense mode*" in order to see how many isoforms of the protein exist, depending on splicing modifications.

The website where all the browser information is classified and described was used. This website contains CCDC6 mRNA sequence, protein sequence and domains, intracellular location and CCDC6 expression in different tissues, among other information.

3.3 PYMOL

PyMOL is a user-sponsored molecular visualization system on an open-source foundation, maintained and distributed by Schrödinger. PyMOL has been used to create a figure of CCDC6 protein, highlighting the main domains of the protein and showing its tridimensional structure, easing its comprehension.

CCDC6 tridimensional representation was downloaded from AlphaFold Protein Structure Database (AlphaFold Protein Structure Database (ebi.ac.uk)), an AI system designed by Deepmind that predicts a protein's 3D structure from its amino-acid sequence. PyMOL represents the figure and allows its movement in three dimensions, helping to visualize its tertiary structure. The program allows us to change specific aminoacids' form, color and size, in order to represent the different domains of the protein.

3.4 CBIOPORTAL

For the analysis of CCDC6 involvement in cancer, the platform cBioPortal for cancer genomics was used. We choose data from TGCA (The Cancer Genome Atlas) including 32 genomic studies of different types of cancer, and we performed a query for "CCDC6" alterations.

The cBioPortal for Cancer Genomics (http://cbioportal.org) was originally developed at Memorial Sloan kettering Cance Center (MSK) and provides genomic data from large-scale projects. The portal shows different results, such as genomic alteration events (Oncoprint), alterations in different cancer types, mutations and most relevant pathways where CCDC6 is involved that will be presented in Annexe 2.

4 CCDC6 STRUCTURE

4.1 GENE STRUCTURE

Coiled-Coil Domain Containing 6 gene (*CCDC6*) is a gene located in the long arm of chromosome 10 [10q21.2]. *CCDC6* gene was originally identified due to its fusion with *RET*, a protein kinase, giving rise to a chimeric oncogene, *PTC1*, responsible for Papillary Thyroid Carcinoma and initially designated as *H4* (D10S170). It is also known as Transforming Sequence Thyroid 1 (*TST1*), protein H4, *PTC* or *TPC* (1)**.**

CCDC6 gene is composed of 9 exons and 8 introns and its full length is about 113 kb. The CCDC6 transcript contains an open reading frame (ORF) of 475 amino acids with a start site that is localized to a region -100 to -190 bp of the translation initiation site (ATG) (3). Its core promoter is enclosed by the region -259 to +3 (2). Nevertheless, the promoter activity was also detected in the -3 kb fragment (3). The outcoming transcript possesses a molecular length of 3 kb. According to UCSC GENOME BROWSER (Annexe 1, Figure 1), CCDC6 expresses one only spliced EST (expressed sequence tags), therefore, CCDC6 expresses just one isoform of its protein (4). *CCDC6* lacks significant homology to known genes, it is highly phylogenetically preserved and is widely expressed in human tissues (2).

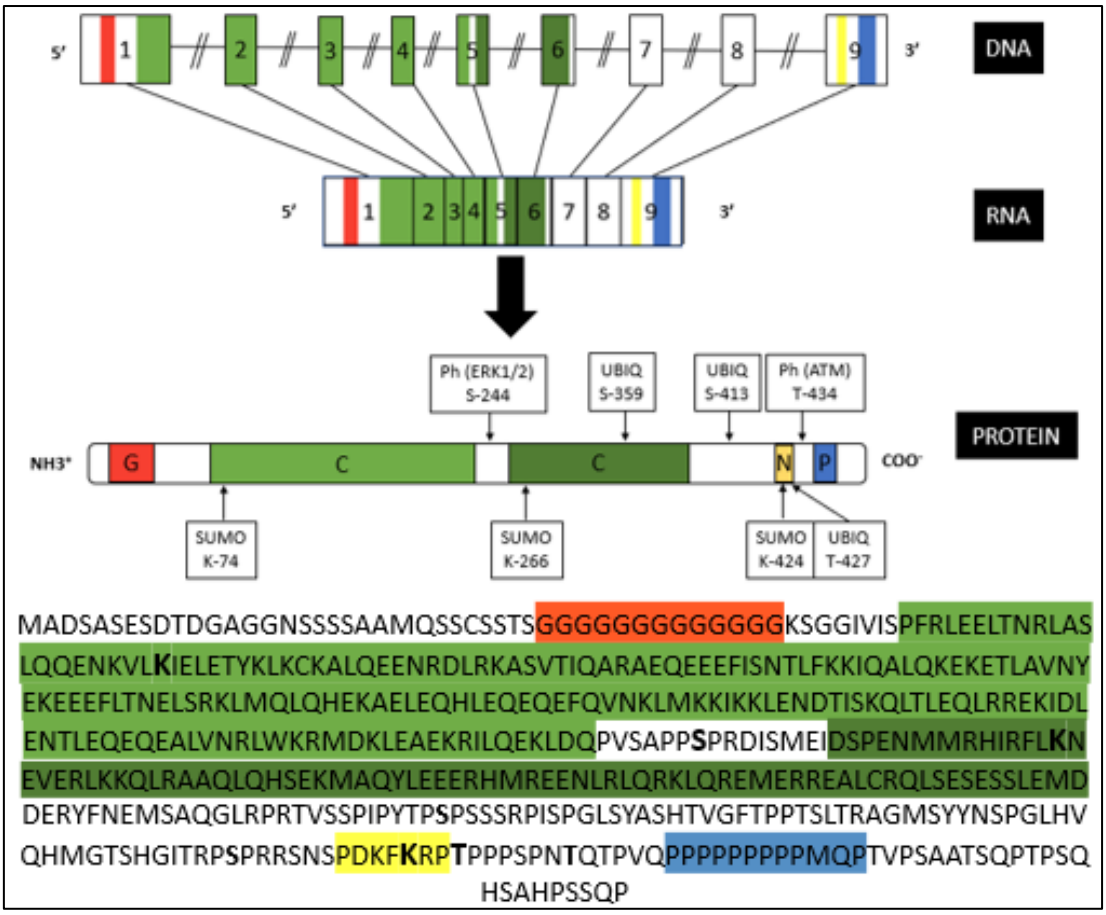

FIGURE 1. CCDC6 structure and principal modifications. This representation shows the 9 exons of the CCDC6 gene, how they turn into RNA, and the resulting protein upon translation. The domains are represented with the same color code than in Figure 2, with the same color code (G, Glycine-rich; C, coiled-coil; N, nuclear localization signal; P, proline-rich). The main post-translational modifications that the protein suffers (SUMO, sumoylation; UBIQ, ubiquitination; Ph, phosphorylation by the indicated kinases) and the modified amino acids are also indicated. Bottom: the complete amino acid sequence, highlighting the different domains of CCDC6. Bold font indicates the modified amino acids.

4.2 PROTEIN STRUCTURE

CCDC6 gene expression results in the CCDC6 protein, constituted by a 475 amino acid chain, with a molecular weight of 65 kDa (2). The protein remains uncrystallized, but a predicted CCDC6 tertiary structure generated with the PYMOL program is shown in Figure 2.

According to the amino acid sequence and the predicted structure, the CCDC6 protein contains several domains:

- Glycine-rich domain: it is located close to the NH_2 terminus, from amino acids 32 to 44, and its function is unknown.
- Coiled-coil domains: they name the protein and gene and are modeled from two or three alpha-helices that are highly amphipathic and bisected at an angle of about 20^o (1). There are two main coiled-coil domains (first one, residues 53-237; second one, residues 253-332). However, besides these two main domains, there might be several shorter coiled-coil domains, which existence may not be clear (5).

- Nuclear localization signal (NLS): it is found between residues 420 and 426 and its sequence is PDKFKRP (Pro-Asp-Lys-Phe-Lys-Arg-Pro). This domain allows the movement of the protein towards the nucleus.

- Proline-rich domain: it encompasses from 440 to 448 amino acids and its function is also unknown, although the proline-rich domains are important for protein-protein interactions.

- In addition, there is a presumed SH3 binding site, located close to the carboxy-terminus (residues 442-451)(5), which may yield an adaptor domain associated with regulatory mechanism, suggesting the idea of physical interactions of CCDC6 with other proteins.

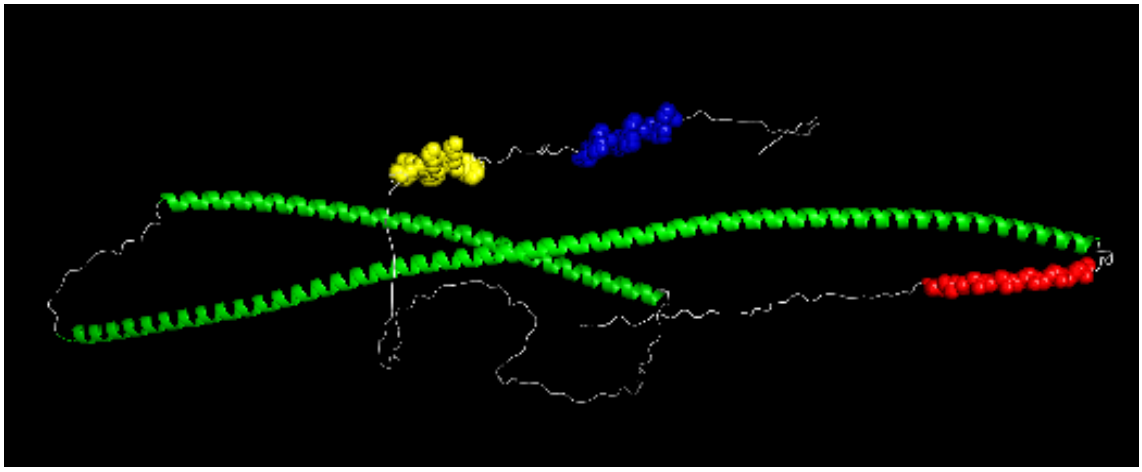

FIGURE 2. CCDC6 tertiary structure and domains. This representation of CCDC6 created using PYMOL shows the main domains of the protein: glycine-rich domain (red), coiled-coil principal domains (green), nuclear localization sequence (yellow), and proline-rich domain (blue).

It has been reported that CCDC6 physically interacts with PP4C (6), CREB1, USP7, MAPK7, ATF2 (7) and FBXW7 (8). CCDC6 also forms homodimers that require the coiled-coil region (2). Additional interactions with RIPK5, TFG, PRDX1, RPS12, HSPA9, and HSPA8 have been described by mass-spec analysis (7). Finally, some proteins involved in the process of DNA repair by homologous recombination such as BTCC3 and BAP1, of the BRCA1 complex have been predicted to interact with CCDC6, based on computational analysis (2).

5 CCDC6 POST-TRANSLATIONAL MODIFICATIONS

The CCDC6 protein can be subjected to several modifications such as phosphorylation, ubiquitylation, SUMOylation, acetylation, and methylation (5). The most relevant are shown in Figure 1 and Table 1.

CCDC6 is a phosphoprotein targeted by different S/T kinases (ATM, ERK1/2, CDK1/2, GSK3) which control the protein stability and translocation to the nucleus upon cellular signals (1). CCDC6 phosphorylation is one of the most important post-translational modifications. For instance, in presence of genotoxic stress (as ionizing radiation), the ataxia-telangiectasia mutated (ATM) kinase route is activated, phosphorylating the threonine 434, and inducing CCDC6 nuclear stabilization (2). Interactions have also been described between CCDC6 and mitotic kinases, such as CDK1 (which phosphorylates the protein at serine 413) and GSK3 (which phosphorylates serine 363). This phosphorylation is needed to ubiquitinate CCDC6 (9). In response to mitogenic stimuli, ERK1/2 phosphorylates CCDC6 at serine 244 (10).

TABLE 1. Phosphorylation, methylation, and acetylation of CCDC6. Modified CCDC6 residues from references (5) (9).

MODIFIED RESIDUES		MODIFIED RESIDUES	
2	N-acetylalanine	359	Ubiquitinserine
52	Phosphoserine	363	Phosphoserine
74	SUMOlysine	367	Phosphoserine
240	Phosphoserine	387	Omega-N-methylarginine
244	Phosphoserine	395	Phosphoserine
249	Phosphoserine	413	Phosphoserine
254	Phosphoserine	413	Ubiquitinserine
266	SUMOlysine	424	SUMOlysine
284	Phosphoserine	427	Ubiquitinthreonine
323	Phosphoserine	431	Phosphoserine
349	Phosphothreonine	434	Phosphothreonine

There are two enzymes involved in ubiquitination: FBXW7 and USP7. FBXW7 is an E3 ubiquitin ligase whose activity is responsible for the stabilization of CCDC6 levels. This protein is involved in CCDC6 ubiquitin-mediated proteasomal degradation, and its deregulation is associated with multiple cancers. CCDC6 is ubiquitinated by FBXW7 at serine 359, serine 413, and threonine 427 residues. USP7, also known as HAUSP, is a de-ubiquitinating enzyme (DUB) that increases CCDC6 levels by reducing its degradation (8).

SUMOylation is a modification consisting of the addition of a covalent supplement of the small-like ubiquitin modifiers (SUMO) peptide which targets lysine residues. It is an important mechanism for CCDC6 subcellular localization, forcing the protein to stay in the cytoplasm. CCDC6 is SUMOylated by SUMO-1 and SUMO-2/3 at lysines 74, 266, and 424. SUMO Specific Peptidase 1 (SENP1) is the enzyme responsible for CCDC6 deSUMOylation (9).

Acetylation occurs in the second alanine, and even though it has not been studied in humans, methylation has been discovered in mice at residue 387, turning it into Omega-N-methylarginine (5).

Apart from these modifications described in the literature, all the reported post-translational modifications of CCDC6 obtained from cBioPortal analysis are shown in Annexe 2, Figure 3.

6 CCDC6 INTRACELLULAR LOCALIZATION AND REGULATION

CCDC6 protein has no transmembrane domain and it can be found in the cytosol and in the nucleus. It was also reported that CCDC6 could be a cytoskeletal protein (5). CCDC6 localization is determined by phosphorylation, ubiquitination and SUMOylation (12).

During the cell cycle, CCDC6 levels and phosphorylation status change cyclically, mediated by post-translational modifications, such as ubiquitination. The intracellular amount of CCDC6 is regulated by post-translational modifications rather than by transcription. CCDC6 levels increase at G1/S, peak at G2, and decrease in mitosis. There are two enzymes involved in this process: FBXW7 and USP7. During the M phase, mitotic kinases (such as CDK1 or GSK3, previously mentioned) and the presence of degron motifs in the CCDC6 protein recruit FBXW7, leading to the reduction of CCDC6 levels. The de-ubiquitinase USP7 is also important for the regulation of CCDC6 stability. Additionally, CCDC6 degradation can be controlled by ATM- or ERK1-mediated phosphorylation, avoiding its translocation to the cytosol (8). SUMOylation is responsible for fast-exporting CCDC6 out of the nucleus and might be related to specific transcriptional process control such as CREB1 activity (see below) (11). Interestingly, fusion proteins found in tumors are located in the cytoplasm and are not able to migrate to the nucleus due to the loss of NLS in CCDC6 protein, as will be described in the following sections (12).

In summary, CCDC6 levels and intracellular localization are highly interconnected and switching in order to regulate CCDC6 functions.

7 FUNCTIONS OF CCDC6

In normal cells, CCDC6 seems to act like a tumor suppressor gene, helping DNA repair and controlling DNA repair checkpoints, being responsible for promoting cell cycle control and maintenance of genomic stability, or leading to apoptosis (2). The most studied cellular function of CCDC6 is related to its role in DNA damage response, although a role in the transcriptional regulation of CREB1 has also been described.

7.1 CCDC6 ROLE IN DNA REPAIR AND ATM-MEDIATED APOPTOSIS

In the presence of genotoxic stress, cells are able to repair the damaged DNA by multiple mechanisms or, in case of irreparable damage, to induce cell death or senescence. The impairment or lack of this DNA damage response might produce an accumulation of genetic aberrations which may induce uncontrolled cellular proliferation. Depending on the number of strands that are involved in the DNA damage we can distinguish between Single-Strand Breaks (SSB) or Double-Strand Breaks (DSB).

On the one hand, there are two kinds of SSB repair mechanisms: Base Excision Repair (BER) and Nucleotide Excision Repair (NER). The first one does not alter the DNA helix and consists of a simple excision-ligation reparation performed by DNA glycosylases (13). NER is the main mechanism of SSB repair, and it is seen in presence of ultraviolet irradiation or other agents that distort DNA helix. There are two major pathways of NER, one which repairs damage anywhere in the genome (global genome NER) and other that only repairs the transcribed strand of active genes (transcriptional-coupled NER) (14).

DSBs are extremely dangerous to the cell because there is no intact DNA strand to ensure accurate repair. Replication errors, reactive oxygen species, or ionizing radiation can produce double-strand damage in the DNA helix. There are two mechanisms responsible for the maintenance of genome integrity in presence of this sort of injury: Non-Homologous End Joining (NHEJ) and Homologous Recombination (HR). In presence of DSB, NHEJ is the most usual reparation mechanism in human cells because of its speed and simplicity. NHEJ consists of resecting the non-matching endings, a compatible nucleotide addition, and their ligation, resulting in losing part of the damaged DNA. On the other hand, HR is more accurate in restoring the original DNA sequence than NHEJ because it uses the homolog chromatid as a template. Hence, HR occurs mainly in the S and G2 phases of the cell cycle, when the two sister chromatids are close enough to each other (14).

The cellular response to DSBs involves highly coordinated and complex events to detect and respond to the damage. In HR, the DSB ends are recognized by the MRN complex, which interacts with ATM kinase, a key component in the regulation of the DNA damage response. Figure 3 shows a simplified scheme of the response to DSBs, highlighting the points where CCDC6 plays a key role.

ATM (ataxia-telangiectasia mutated) is an S/T kinase related to DNA double-strand breaks and involved in the early DNA damage response. In the reaction to this DNA damage, ATM enforces G_1 and G_2 cell cycle checkpoints, inducing a pause in cell proliferation intending to solve DNA injury by NHEJ o HR pathways and protecting genome integrity. Upon exposure to genotoxic stress, ATM kinase is activated by different mechanisms. MRN complex is essential in its recruitment and the following ATM-mediated signaling. Activated ATM then phosphorylates H2AX surrounding the DSBs (see below), which recruits more of the MRN complex to the site (14). ATM phosphorylates many different substrates, giving rise to multiple cellular responses including, apoptosis, cell cycle arrest or DNA damage repair (Figure 3).

Histone H2A is one of the proteins of the histone core in nucleosomes. The histone variant H2AX constitutes 10% of the H2A present in human cells. H2AX not only differs from other variants by some residues but it is also the only variant that is phosphorylated in the presence of DNA damage (16). Upon DNA damage, the H2AXs which surround the DBS are phosphorylated at Ser139 (modification named γ-H2AX) and form part of foci recruiting MDC-1 (mediator of DNA damage checkpoint-1). MDC-1 is stabilized in chromatin and binds to NBS1 (the defective gene responsible for Nijmegen breakage syndrome), a member of the previously mentioned MRN complex, inducing a positive feedback loop between ATM and MRN complex which is essential for efficient signaling.

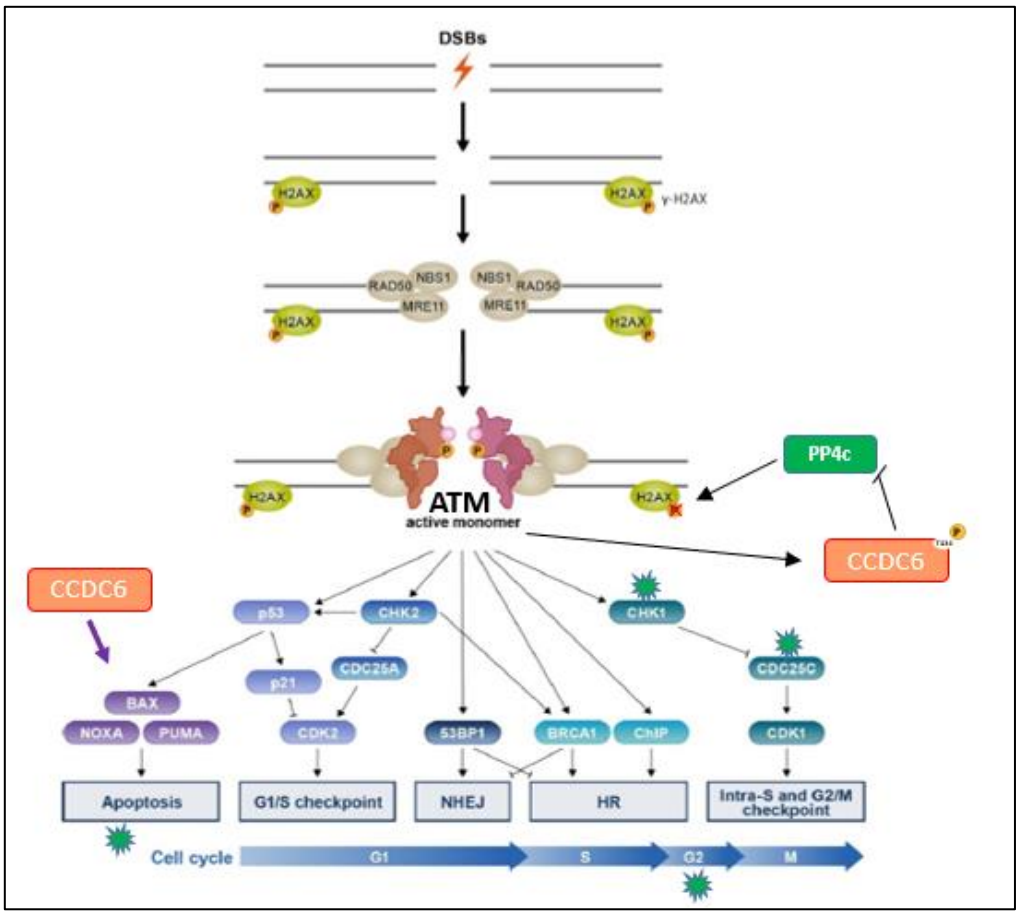

FIGURE 3. DBS repair mediated by ATM and role of CCDC6. When double-strand damage is detected, ATM is recruited by the MRN complex which is mainly composed of RAD50, MRE11, and NBS1. Active ATM phosphorylates numerous substrates leading to different cellular responses. The ATM-mediated phosphorylation of CCDC6 is necessary to avoid γ-H2AX dephosphorylation, performed by PP4c. This positive feedback loop is essential for efficient ATM signaling. Green stars indicate different processes where CCDC6 is involved upon DNA damage. Figure modified from (15).

As mentioned above, CCDC6 is a substrate of ATM. ATM phosphorylates CCDC6 at threonine 434, inducing conformational changes that make it unavailable to interact with the FBXW7 ubiquitin ligase, preventing CCDC6 degradation and keeping it inside the nucleus during DNA damage response (13).

CCDC6 participates in the pathway that relates ATM and H2AX (Figure 3). CCDC6 interacts in the nucleus with the catalytic subunit of the Serin-Threonine Protein Phosphatase 4 (PP4c) that dephosphorylates histone γ-H2AX at Ser139, avoiding DNA repair. CCDC6 acts as a negative regulator of PP4C and γ-H2AX is not dephosphorylated, allowing the correct DNA repair or apoptosis induction (1). Therefore, in the presence of CCDC6 there is an increase in the apoptosis of damaged cells. The loss of CCDC6 limits H2AX phosphorylation at Ser139 and DNA damage is repaired by a faster but less accurate NHJE pathway, which contributes to genomic instability (6).

Additionally, CCDC6 is involved in the cell cycle regulation and is required for the proper intra-S phase checkpoint control. Therefore, the lack of CCDC6 results in an accelerated transition to the G_2 phase and an accumulation of DNA damage that could contribute to the carcinogenic process (18).

7.2 CCDC6 ROLE IN APOPTOSIS

It was initially proposed a role of CCDC6 as a proapoptotic protein. Apoptosis induction by CCDC6 is associated with the upregulation of pro-apoptotic protein BAX and the downregulation of anti-apoptotic protein BCL-2 (Figure 3). The proapoptotic function of CCDC6 requires homodimers composed of the full-length protein (12). ATM-mediated phosphorylation is involved in apoptosis induction by CCDC6 (19). ATM participates in the activation of p53 which directly activates BAX which permeabilizes the mitochondrial outer membrane releasing cytochrome C from the mitochondrial intermembrane space, leading to apoptosis. For its part, BCL-2 is responsible for controlling this process inhibiting BAX (20). CCDC6 also regulates the transcription factor CREB1 (see below) which has been also demonstrated to participate in apoptosis. CREB1 knockdown promotes apoptosis by increasing caspase 3 and BAX expression and downregulating BCL-2 expression (21).

However, recent studies that are trying to figure out the functional consequences of loss of CCDC6 suggest that CCDC6 silencing results in apoptotic cell death at both early and late stages of apoptosis (18). Therefore, the role of CCDC6 in apoptosis is controversial and deserves further studies.

7.3 CCDC6 AS A NEGATIVE REGULATOR OF CREB1 TRANSCRIPTIONAL ACTIVITY

In addition to its role in the DNA damage response, CCDC6 has been described as a negative regulator of the transcriptional activity of CREB1 (cAMP-responsive element-binding protein 1), a transcription factor that regulates multiple cellular responses, including proliferation, survival, and differentiation (11).

CCDC6 decreases CREB 1 transcriptional activity at different levels: i) CCDC6 physically interacts with CREB1; ii) CCDC6 binds CRE element present in target genes and reduces CREB1 binding to these sequences; iii) CCDC6 recruits protein phosphatase 1 and histone deacetylase 1 at the CRE sites of CREB1 target genes (Figure 4).

CCDC6 binds to Protein Phosphatase 1 (PP1) which de-phosphorylates CREB1 at serine 133, reducing its binding to CRE elements. PP1 does not have good substrate specificity, therefore its binding to CCDC6 increases its specificity and catalytic efficiency (7).

On the other hand, CCDC6 interacts with Histone Deacetylase 1 (HDAC1) at the CREB1 promoter, decreasing histone H3 acetylation in this region, which is needed for CREB1 responsive genes transcription. It has been proposed that CCDC6, CREB1, PP1, and HDAC1 form a complex that modulates CREB1-dependent gene expression and, therefore, CREB1-dependent cellular proliferation (7).

The part of the first coiled-coil domain expressed by exon 2 seems to be responsible for the CREB1 activity repression (23). Furthermore, it has been demonstrated the relevance of SUMOylation in regulating CCDC6 action on specific transcriptional responses. SUMOylation is responsible for restraining most of CCDC6 in the cytosol, affecting its functional interaction with CREB1 (6,11).

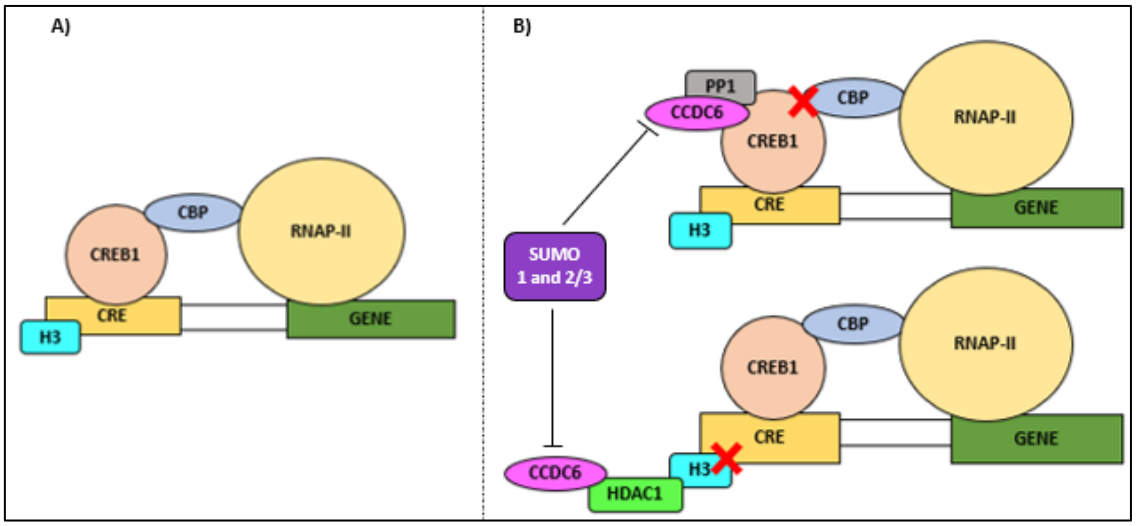

FIGURE 4. CCDC6 negative transcription regulation of CREB1. A) It can be seen how CREB 1 interacts with the CREB-dependent gene, being necessary for its binding to CRE elements (CRE binding protein or CBP is an important transcriptional cofactor) to activate RNA polymerase II. B) Scheme showing the main targets of CCDC6 in its negative transcriptional regulation of CREB 1 and how this interaction is negatively regulated by SUMOylation. Top: CCDC6 binds directly to CREB1 and PP1 phosphatase, which dephosphorylates CREB1, inducing conformational changes that avoid CREB1 binding to CBP. Bottom: CCDC6 recruits HDAC1, which deacetylates histone 3 (H3) hindering CREB1 binding to the promoter. Figure modified from (22).

7.4 EFFECTS OF CCDC6 LACK OF EXPRESSION

Many of the CCDC6 functions described above have been elucidated from investigations using cellular or animal models where CCDC6 was silenced or inactivated. The main effects of CCDC6 lack of expression are summarized below and in Figure 5.

CCDC6 has been shown to be essential for the maintenance of genome stability. Loss or inactivation of CCDC6 releases PP4c action leading to the dephosphorylation of H2AX and, therefore, a non-activation of the positive feedback on ATM which alters the following ATM-depending pathways. For example, it has been demonstrated a reduction of CHK1 (checkpoint 1 kinase) levels in the nucleus and CDC25C phosphatase levels in the cytoplasm upon CCDC6 loss. CHK1 is an activator of the checkpoint pathway and CDC25C is a G_2 checkpoint that should be sequestered in the cytoplasm in the presence of genotoxic stress. Besides, it has been seen a reduced expression of Rad 51 foci related to a decrease in CCDC6 (3). Rad 51 is one of the participating proteins in the Homologue Recombination looking for homology and pairing sister chromatids (15).

Regardless of induced genotoxic stress, cells lacking active CCDC6 show downregulation of 14-3-3σ, a major cell cycle regulator. In presence of genotoxic stress, ATM induces the phosphorylation of p53 at serine 376, which afterward binds to 14-3-3σ, activating p53 and inducing cellular apoptosis (2). However, in the absence of genotoxic stress, the activation of CREB1-dependent genes may be responsible for 14-3-3σ downregulation. CCDC6 impairment increases CREB1 levels because of the lack of its action on PP1 phosphatase and HDAC1, leading to the activation of BCL-2 and downregulation of Caspase 3 and BAX, avoiding apoptosis (12,21).

Besides, CREB 1 levels are related to Cyclin A1, Cyclin B1 and Cyclin D2 expression. These cyclins are involved in the regulation of the cell cycle and their expression is crucial for the progression of the cell cycle. In non-pathological situations, the downregulation of CREB1 decreases the mRNA expression of these previously mentioned cyclins. However, when this downregulation does not exist, the expression of these proteins allows the progression of the cell cycle, altering DNA repair and inducing cell proliferation (20).

CCDC6 alterations has been reported to induce chemoresistance to etoposide and cisplatin. Etoposide, also called *VP-16,* is chemotherapy drug that act specifically on G2 phase forming a complex with topoisomerase II and inhibiting DNA ligation that will eventually produce SSB and DSB and induce apoptosis. In order to complete its action, etoposide requires enough G_2-phase arrest to produce SSB and DSB (24). Cisplatin is a non-specific phase chemotherapist that cross-binds DNA strands affecting its integrity and inducing apoptosis by activating several pathways which release cytochrome-C from the mitochondria that will ultimately trigger a cascade of proteins inducing apoptosis (25). Even though it has been investigated how CCDC6 is involved in ATM-recruitment and BCL-2/BAX regulation and how its mutation induces less arrest of G2 phase in the presence of genotoxic stress, the molecular basis of these processes still remains unknown.

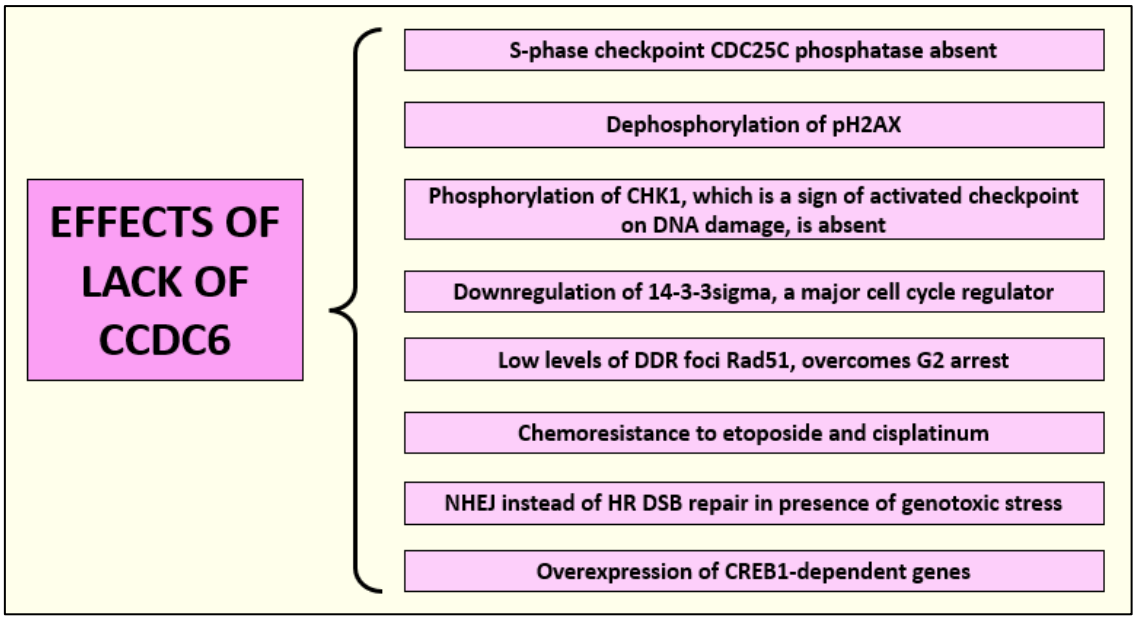

FIGURE 5. Effects of lack of CCDC6 on cell cycle control, DNA repair, and gene expression. Modified from reference (1).

All these alterations explain how CCDC6-depleted cells overcome G_2 arrest and proceed to NHEJ instead of HR and why they are chemoresistant to etoposide or cisplatin. Furthermore, it gives an explanation of how it is induced cell proliferation and uncontrolled growth.

8 CCDC6 ALTERATIONS IN CANCER

In this section, we will describe the major alterations that modify CCDC6's normal functions and have an impact on tumorigenesis. Here we are going to summarize the main findings described in the literature; however, in order to obtain further information on CCDC6 and its role in cancer we have also performed a study using the cBioPortal for Cancer Genomics (https://www.cbioportal.org/) platform. The results of the cBioPortal analysis appear in the Annexe 2 of this work.

Three kinds of alterations have been found that modify CCDC6 expression or its ability to move to the nucleus to perform its functions: i) CCDC6 mutations, ii) low levels of CCDC6 expression, and iii) gene rearrangements giving rise to chimeric fusion proteins (1). Additionally, genetic amplifications of *CCDC6,* that are not described in the literature, were frequently found in the cBioPortal analysis (Annexe 2, Figure 1).

8.1 CCDC6 MUTATIONS

Point mutations may alter the primary protein structure, changing the amino acid chain and the functionality of its regions. It has been found that less than 1% of tumors showing CCDC6 alterations present *CCDC6* gene mutations. Figure 6 shows their most prevalent locations and the frequency of the different types of point mutations. The missense mutations are distributed throughout the entire protein, without specificity affecting a given region; nonsense mutations can lead to dominant-negative isoforms of CCDC6. Even though these mutations are seen in multiple tumors (breast, endometrium, ovary, large intestine, liver, lung, pancreas, prostate, thyroid among others), it is not clear how responsible they are for tumor progression and it has not been found an implicit causal relation. Nowadays, the presence of mutations in tumors that present CCDC6 fusions have not been thoroughly investigated (1). Additional information about *CCDC6* mutations is found in the cBioPortal analysis (Annexe 2, Figures 2 and 3).

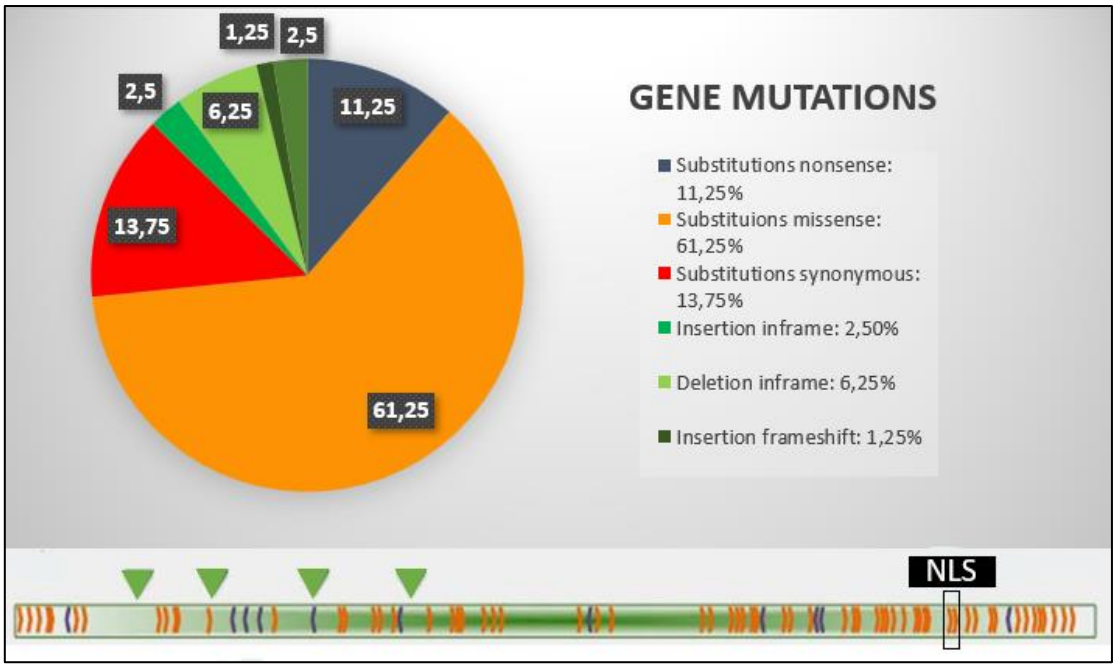

FIGURE 6. CCDC6 gene mutations. The pie chart shows the distribution of different types of mutations in CCDC6: Substitution nonsense, Substitution missense, Substitution synonymous, Insertion inframe, Insertion frameshift, Deletion inframe, Deletion frameshift. The figure below shows, according to the graphic's color code, the location of the most common nonsense mutations (orange), missense mutations (blue), and deletions and insertions (green triangles) in the CCDC6 protein. This last group has been seen to occur more frequently along the first 250 aa of the protein, affecting the minimal dominant-negative region and the first coiled-coil region. Two missenses mutations inside the NLS region are indicated. Modified figure and information from (2).

8.2 LOW LEVELS OF CCDC6 EXPRESSION

CCDC6 protein levels have been found reduced in 20% of non-small and small cell lung cancer and prostate cancer. In 30% of NSCLC, low levels of CCDC6 together with increased aggressiveness and resistance to genotoxic agents have been reported. This downregulation is correlated with the presence of node metastasis and negatively correlated with disease survival (26). Besides, USP-7 is responsible for androgen-dependent deubiquitination of androgen receptors in prostate cancer cells, independently of their castration resistance. The reason why CCDC6 levels are reduced in these tumors remains unknown, but it was detected a reduction in USP-7 levels, which might be a point for future investigations (27).

The majority of tumors bearing rearranged CCDC6 do not express CCDC6 from the normal allele and, in the case that CCDC6 normal protein is produced, the functions of normal CCDC6 are impaired through heterodimerization with the coiled-coil domain of the fusion protein (3,12). As CCDC6 participates in DNA reparation and cell cycle control, its lack of expression or alteration may induce uncontrolled cells proliferation, resulting in cancer. Besides, CCDC6 needs to stay inside the nucleus to perform its functions (1).

8.3 CCDC6 FUSION PROTEINS

CCDC6 was originally identified upon rearrangement with RET, giving rise to a chimeric oncogene in papillary thyroid carcinoma (28). From its discovery, it has been known that *CCDC6* fusion to proto-oncogenes may induce uncontrolled cellular proliferation and the

progression of cancer. Some CCDC6 regions such as the coiled-coil domain provide a hydrophobic surface for oligomerization. Besides, its 101 first amino acids can act as a dominant-negative signal for the presence of CCDC6 full-length protein inside the nucleus, which is needed for its efficient function as a tumor suppressor (1). Figure 7 shows the most relevant proto-oncogenes involved in CCDC6 fusion.

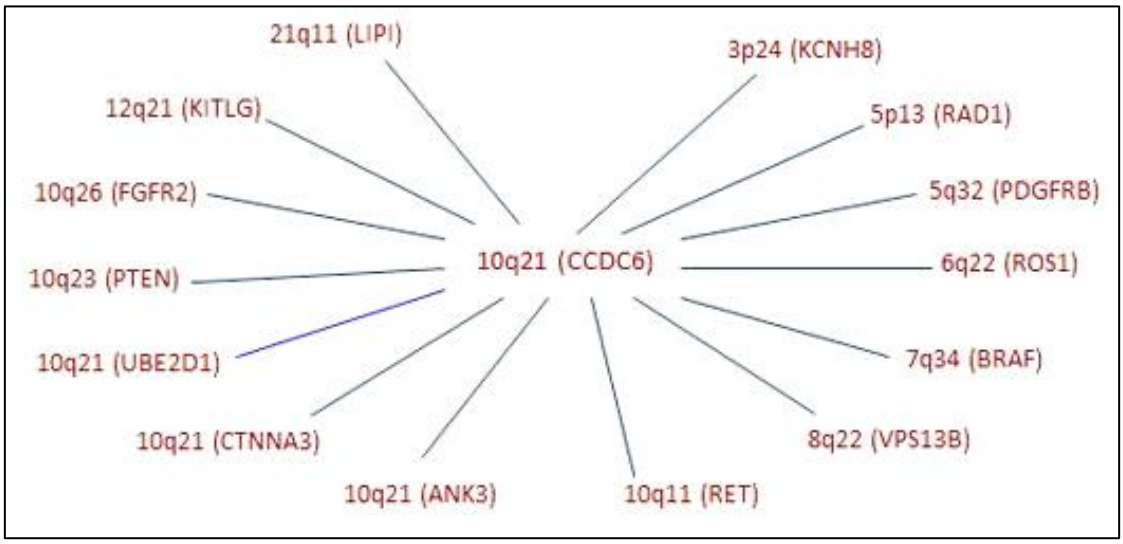

FIGURE 7. Fusions of *CCDC6* with different proto-oncogenes and their loci. This figure shows where are located these genes. Although their loci are usually not close in the same chromosome, rearrangement may occur *(29)*.

8.3.1 CCDC6-tyrosine kinase fusions

Chimeric proteins of CCDC6 fused with receptor tyrosine kinase proteins have been described in different cancers. The protein regions of CCDC6 forming the chimeric oncoproteins always include part of the coiled-coil domain and lack the nuclear localization signal (see Figure 8). In almost all the chimeric proteins, the portion of CCDC6 does not include the FBXW7 phosphodegron (aa S359, S413, T427) which are known to be relevant for the CCDC6 protein stability. Recently, it has been proposed that genetic fusions favor tumorigenesis through degron loss (30). The major CCDC6-tyrosine kinase fusions are:

- **RET**. CCDC6-RET fusion was essential for the discovery of the CCDC6 gene and its biological functions. RET is a receptor tyrosine kinase that is involved in the growth of neural crest-derived cell lineage, kidney, and male germ cells. In most cases, the first 101 amino acids of CCDC6 are present in the chimeric CCDC6-RET protein, while other fusions include the first 150 or 293 amino acids (Figure 8). The N-terminal coiled-coil domain of CCDC6 favours the dimerization of RET, resulting in a non-ligand activation of RET and a constitutively active tyrosine kinase function (12). Furthermore, CCDC6-RET fusion escapes from FBW7 degradation leading to stabilization of the resultant fusion product (30). These findings support the relevance of FBW7 degron loss in CCDC6 fusions. CCDC6-RET fusions were found in different kinds of cancer like papillary thyroid cancer, non-small cell lung cancer, and colorectal cancer (Table 2) (2). This fusion has also been recently found in one case of Adult Acute Lymphoblastic Leukemia (ALL), opening the possibility of therapies with tyrosine kinase inhibitors (31).

- **PDGFR-β.** Platelet-derived growth factor receptor beta (PDGFR-β) is a tyrosine kinase receptor that acts as a cell-surface receptor for homodimers of PDGF-β and PDGF-δ and heterodimers formed by PDGF-β and PDGFR-α. It is important for the regulation of cell proliferation, embryonic development, differentiation, survival, chemotaxis, and blood vessel development by promoting proliferation, migration, and recruitment of pericytes and smooth muscle cells to endothelial cells. Its fusion with CCDC6 was found as a novel translocation in BCR-ABL-negative chronic myeloid leukemia (32). This chimeric protein contains the first 368 amino acids including the coiled-coil domain of CCDC6 and the entire tyrosine kinase and transmembrane domain of PDGFR-β (1). CCDC6-PDGFR fusion has also been reported in non-small cell lung cancer (NSCLC).

- **FGFR2**. Fibroblast growth factor receptor 2 or FGFR2 is a tyrosine kinase which is activated when bound to the FGF ligand, further activating RAS-MAP and PI3K-AKT pathways. FGFR2-CCDC6 fusion (Figure 8) induces oligomerization and produces increased cell growth and decreased apoptosis. It has been found in cholangiocarcinoma, breast cancer, and prostate cancer (1).

- **ROS-1.** ROS proto-oncogene 1 or ROS-1 is a receptor tyrosine kinase related to the growth or differentiation factor receptor. Its fusion with CCDC6 causes non-small cell lung cancer although ROS-1 undergoes genetic rearrangements in other different cancers. When rearranged, it becomes constitutively active inducing deregulated cell proliferation (2).

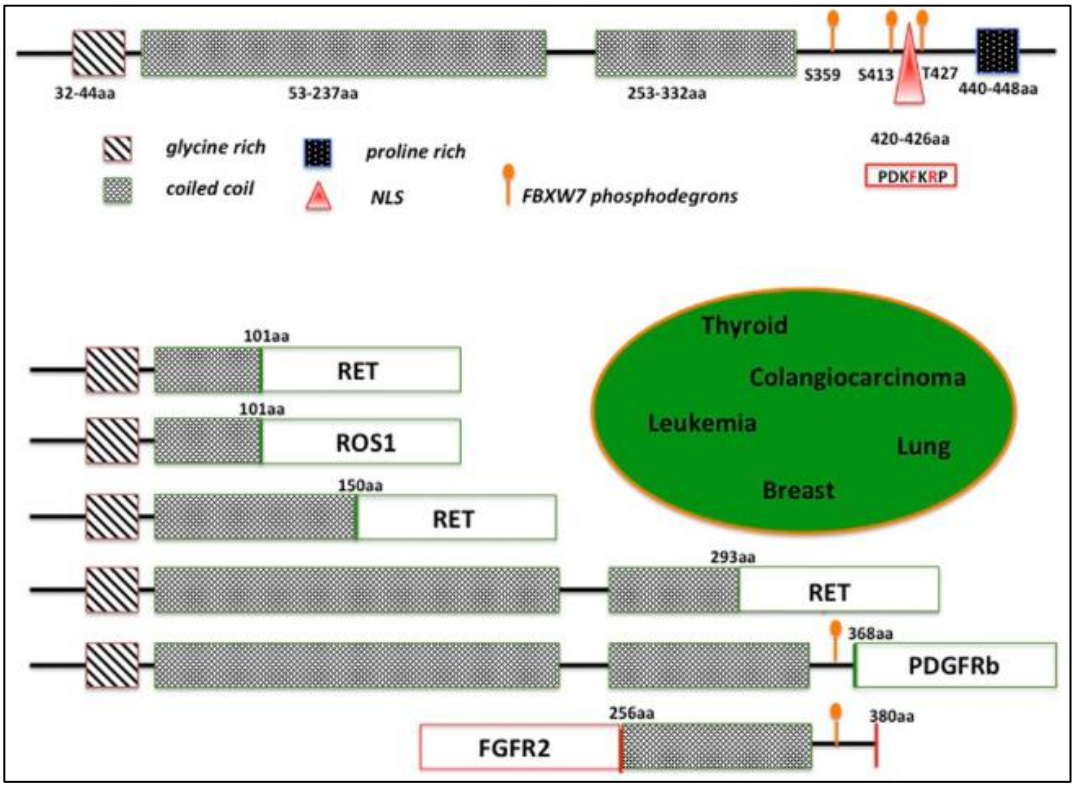

FIGURE 8. CCDC6-tyrosine kinase fusions. The figure shows how the CCDC6 fusion with tyrosine kinases RET, ROS1, PDFGRβ or FGFR2 eliminates the most frequent ubiquitination sites of CCDC6, evading the degradation of this chimeric protein; as well as its NSL site which avoids CCDC6 entrance in the nucleus and, therefore, the performance of its functions. Figure taken from (33).

8.3.2 Other CCDC6 fusions

Apart from its fusion with receptor tyrosine kinase genes, *CCDC6* has been found rearranged with several other genes, involved in different kinds of cancer (1).

- **KITLG**. KIT ligand gene encodes the ligand of the tyrosine kinase receptor KIT. This ligand acts in several tissues such as uterus, germ cells, or blood cells stimulating its development. KITLG or SCF helps in phosphorylating PIK3R1 (a regulatory subunit of phosphatidylinositol 3-kinase) and activating multiple signaling pathways. Fusion of KITLG with CCDC6 is found in lung adenocarcinoma.

- **ANK3**. Ankyrin 3 or ankyrin G is implicated in cell movement, activation, growth, contact, and maintenance of some specific membrane domains. ANK3-CCDC6 fusion is related to breast cancer and epithelial ovarian cancer.

- **LIPI:** Lipase-1 is a gene encoding a phospholipase that breaks down phosphatidic acid transforming it into lysophosphatidic acid. Its rearrangement with CCDC6 may cause non-small cell lung cancer.

- **CTNNA3**. catenin alpha 3 o CTNNA3 is a gene encoding a cell adhesion molecule that helps in the liking of cadherin and actin in the cytoskeleton of muscle cells. Its alteration is detected in non-small cell lung cancer.

- **PTEN**: Phosphatase and tensin homolog or PTEN is a tumor suppressor gene whose resulting protein is a phosphatase that opposes PI3K activity (31). Its fusion with CCDC6 appears in papillary thyroid cancer.

- **VPS13B**. VPS13B or vacuolar protein sorting 13 homolog B is a gene that encodes a transmembrane protein related to vesicle-mediated transport and sorting of protein inside the cell. It is usually found associated with the Golgi apparatus. Its fusion with CCDC6 may develop papillary thyroid carcinoma.

- **UBE2D1**. Ubiquitin-conjugating enzyme E2 D1 (UBE2D1) is a mediator of the ubiquitination of the tumor-suppressor protein p53 (34). Its fusion with CCDC6 produces breast cancer and papillary thyroid carcinoma (1,2).

- **BRAF**. CCDC6-BRAF fusion is present in 0.10% of cases of The AACR Project GENIE Consortium, highlighting its presence in low-grade glioma, pancreatic adenocarcinoma, pilocytic astrocytoma, thyroid gland papillary carcinoma, and astrocytoma.

- **RAD1**. RAD1 is a cell cycle checkpoint DNA Exonuclease and is involved in DNA damage repair. RAD1-associated diseases include Alpha-thalassemia. CCDC6-RAD1 fusion protein has been described in Fusion Gene Annotation DataBase, but the functional consequences of the fusion are yet uncharacterized (35).

- **KCNH8**. *KCNH8* gene encodes the Potassium Voltage-Gated Channel Subfamily H Member 8 and the CCDC6-KCNH8 fusion gene is available in CHIMERKB database but further research is required to determine the role of this fusion.

In addition to these fusion partners of CCDC6 described in the literature, other fusion genes were found in the cBioPortal analysis, including UST-CCDC6 in papillary thyroid cancer, CCDC6-PCBD1 in astrocytoma, CCDC6-RCC1 in serous ovarian cancer, SLCIGAG-CCDC6 in esophageal adenocarcinoma and CCDC6-SLCIGAG in lung adenocarcinoma. Therefore, CCDC6 shows a high susceptibility to form fusion proteins, thus opening a wide range of possibilities.

In agreement with known CCDC6 functions and CCDC6 alterations described in this section, ten cellular pathways were found altered in the cBioPortal analysis: RTK-RAS, cell cycle, HIPPO, MYC, NOTCH, NRF2, PI3K, TGF-beta, TP53 and WNT. The most relevant RTK-RAS (A) and cell cycle (B) pathways are shown in Annexe 2, Figure 4.

9 CCDC6 RELATED CANCERS

As mentioned before, CCDC6 has been found altered in different cancers by several mechanisms including mutations, reduced expression or gene rearrangements. Table 2 summarizes the genetic fusions of CCDC6 found in different types of cancer and the loci of the fusion member. Further information about CCDC6 alteration frequency depending on the cancer type is shown in the cBioPortal (study Annexe 2, Figure 2).

TABLE 2. Proto-oncogene fusions and the most prevalent cancers they produce (1,2,29,31).

FUSION ONCOGENES	TUMOR TYPE
CCDC6 [10q21.2]/RET [10q11.21]	Papillary thyroid carcinoma, non-small cell lung cancer, colorectal cancer and acute lymphoblastic leukemia
CCDC6 [10q21.2]/PDGFR Beta [5q32]	Chronic myeloid leukemia
FGFR2 [10q26.13]/CCDC6 [10q21.2]	Breast cancer, cholangiocarcinoma, and prostate cancer
CCDC6 [10q21.2]/ROS1 [6q22.1]	Non-small cell lung cancer
KITLG [12q21.32]/CCDC6 [10q21.2]	Non-small cell lung cancer
CCDC6 [10q21.2]/ANK3 [10q21.2]	Ovarian epithelial tumor
CCDC6 [10q21.2]/LIPI [21q11.2]	Non-small cell lung cancer
CCDC6 [10q21.2]/CTNNA3 [10q21.3]	Non-small cell lung cancer
CCDC6 [10q21.2]/PTEN [10q23.31]	Papillary thyroid carcinoma, colorectal cancer
VPS13B [8q22.2]/CCDC6 [10q21.2]	Papillary thyroid carcinoma
CCDC6 [10q21.2]/UBE2D1 [10q21.1]	Breast cancer, papillary thyroid carcinoma
CCDC6 [10q21.2]/BRAF [7q34]	Low-grade glioma, pancreatic adenocarcinoma, pilocytic astrocytoma, papillary thyroid carcinoma and astrocytoma

The most studied tumors showing CCDC6 alterations are thyroid cancer and non-small cell lung cancer. Therefore, they will be developed in-depth at this point.

9.1 PAPILLARY THYROID CANCER (PTC)

Thyroid tissue is constituted by follicular cells and parafollicular cells. Follicular cells are the main component of thyroid and their mutation is responsible for 95% of all thyroid cancer cases. Papillary thyroid cancer (PTC), a well-differentiated carcinoma derived from follicular thyroid cells, accounts for 80% of all thyroid cancer patients and it is the most prevalent thyroid cancer subtype in countries with no iodine-insufficient diet. It is usually detected in patients between thirty and fifty years old, affecting more frequently women (2:1), and its incidence increases with the age. Treatment consists of a combination of surgery and radioactive iodine depending on the stage of the tumor (36,37).

The molecular pathogenesis of most thyroid cancers is due to mutations in MAPK and PI3K/AKT signaling pathways (Figure 9). There are at least 13 different forms of RET/PTC rearrangements that have been detected, being the most common RET/PTC1 (CCDC6-RET) and RET/PTC3 (RET-NCOA4) (36). RET/PTC1 mutation is more frequently seen in sporadic cancers and has a better prognosis, in comparison to PTC3 which is usually related to radiation-induced tumors and is associated with more aggressive behavior. It has been postulated that CCDC6 and RET loci are placed at fragile sites of the genome, making them more vulnerable to environmental stress factors that would induce breakage and chromosomal rearrangements (38). It would be interesting to investigate if other CCDC6 fusion partners are also located in fragile sites or if another mechanism is implicated in these mutations.

Less frequently, there have been reported CCDC6 fusions with other proteins like BRAF, PTEN, VSP13 or UBE2D1 that are associated with PTC. PTEN negatively regulates the PI3K pathway, dephosphorylating PIP3 to PIP2. Consequently, it decreases AKT activity, which is implicated in the promotion of cell proliferation, the increase of cellular glycolytic flux and the inhibition of apoptosis (39,40). Therefore, it could be postulated that PTEN-CCDC6 fusion could affect the catalytic region of PTEN, avoiding AKT pathway downregulation and contributing to tumorigenesis and survival.

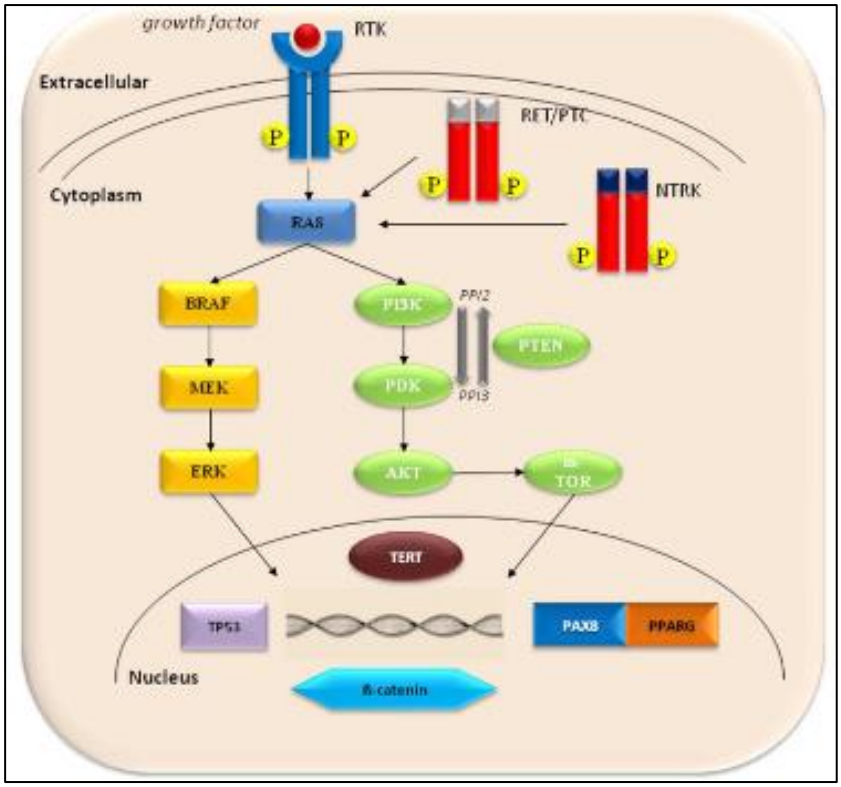

FIGURE 9. Molecular pathogenesis of papillary thyroid cancer. Mutations of BRAF, RAS or RET genes are present in nearly 70% of PTC cases. RET/PTC1 (CCDC6-RET) is responsible for the dimerization of RET without a ligand binding inducing constitutive MAPK pathway activation. Besides, its fusion with CCDC6 produces the deletion of the intracellular juxta membrane domain, causing the relocation of the chimeric mutant into the cytosol and preventing it from interacting with its negative regulators. PTEN-CCDC6 fusion affects PTEN regulation of PI3K/AKT pathway inducing uncontrolled cell proliferation and inhibiting apoptosis. Additionally, BRAF fuses to CCDC6 inducing PTC. Figure taken from (39).

9.2 NON-SMALL CELL LUNG CANCER (NSCLC)

Lung cancer is the fourth most common cancer diagnosed in Spain in both sexes and the deadliest, with a 5-year survival of less than 20%. The most relevant risk factor is tobacco, not only its use, but also the fumes exposition; radon exposure represents the second most common risk factor of lung cancer in Spain. Non-small cell lung cancer (NSCLC) is the most common type. It is derived from epithelial cells and classified into four subtypes: adenocarcinoma, which has turned into the most prevalent lung cancer subtype; squamous cell carcinoma, large cell carcinoma and NSCLC undifferentiated. NSCLC is usually diagnosed in the sixth decade and the median overall survival in the metastatic setting is only 10-12 months despite aggressive treatments (26,41).

Therapeutic approaches include a combination of surgery, radiotherapy, chemotherapy, immunotherapy and molecularly targeted therapy, such as tyrosine kinase inhibitors, depending on the stage, histology, genetic alterations and patient's condition. Platinum is considered the gold standard to treat NSCLC and, in advanced stages, can be combined with the use of tyrosine-kinase inhibitors which target EGFR, ALK or RET benefiting a low percentage of NSCLC patients (42).

Several alterations of CCDC6 have been found in NSCLC, highlighting the importance of CCDC6 in this pathology. CCDC6 is only expressed in about 30% of patients and gene fusions with RET and ROS kinases were detected in about 1% of patients. Additionally,

fusions of CCDC6 with CTNNA3, KITLG and LIPI have been reported (Table 2). CCDC6 point mutations (E227K, S351Y, N394Y and T462A) have also been identified in primary NSCLC. These missense mutations, which are not predicted to affect the coiled-coil domain, represent the 40% of CCDC6 mutations reported in lung (42).

Low levels of CCDC6 protein in NSCLC have been correlated with poor tumor prognosis. It has been proposed that the amount of CCDC6 protein in primary lung tumors may depend on the impairment of the CCDC6 turnover due to altered protein-protein interaction and post-translational modifications, mainly ubiquitination regulated by FBXW7 and USP7 as previously described (8).

As it will be described in section 10, CCDC6 impairment induces chemoresistance and enhanced PARP-inhibitors sensitivity. Hence, the identification of the molecular alterations involving CCDC6 might define predictive biomarkers for personalized treatments in NSCLC.

9.3 OTHER CANCERS

Recent reports highlight the importance of CCDC6 alterations in other cancers like glioblastoma, myeloid/lymphoid neoplasms, acute lymphoid leukemia, malignant pleural mesothelioma, prostate cancer and colorectal cancer (27,31,43–49).

The cBioPortal analysis also shows the genomic alterations in CCDC6 across the 10967 patient samples (Annexe 2, Figure 1) and the frequency of CCDC6 alterations in different cancers (Annexe 2, Figure 2).

10 THERAPEUTIC APPROACHES FOR CANCERS INVOLVING CCDC6

To improve the response of cancer patients to selective inhibitors, personalized treatments are based on genomic biomarkers. The knowledge of CCDC6 functions and alterations made it possible to propose different therapeutic approaches for cancers involving CCDC6.

As it will be exposed below, carcinomas with decreased or truncated CCDC6 can be treated with PARP-inhibitors, given the crucial role of CCDC6 in DNA damage response. Besides this treatment, other drugs targeting CCDC6 regulators such as USP7 or FBXW7 might be used in combination with PARP-inhibitors. Finally, the presence of CCDC6 fusions with tyrosine kinase proteins opens the possibility of new therapies using tyrosine kinase inhibitors, such as RET-inhibitors.

10.1 PARP-INHIBITORS

CCDC6 impairment is considered a “BRCA-like” mutation because its downregulation results in non-effective HR and a defective DSB repair. In the presence of this deficient pathway, if SSB repair pathways got also altered, the cell would start producing major mutations until their amount or severity would be such that apoptosis would be induced.

Poly(ADP-ribose) polymerase-1 and 2 (PARP1/2) are relevant proteins related to single-strand DNA damage response. They belong to the PARP superfamily whose function consists of sensing single-strand breaks and inducing the recruitment by other factors triggering the previously mentioned pathways and repairing these defects (50).

PARPi (PARP-inhibitors) are antineoplastic monoclonal antibodies which bind the catalytic domain of PARP1/2 proteins as antagonists of the PARP cofactor β-NAD^+, avoiding the binding of the real β-NAD^+ molecule necessary to proceed in the process of SSB repair (51). PARPi were the first successful case of targeted therapies utilizing synthetic lethality to kill tumors with DNA-repair deficiency. Synthetic lethality describes the phenomenon where the presence of a single mutation in a pathway is not deleterious, but its combination with other gene mutations simultaneously affects cell viability, causing apoptosis (52). CCDC6 defective cells are BRCA-like cells that have altered DSB repair mechanisms, performing NHEJ instead of HR, which is a less effective pathway for restoring DNA integrity. When a PARPi is administered to these CCDC6-mutated cells, it prevents the SSB repair mechanism, inducing apoptosis in DSB repair-deficient cells (Figure 10) (53).

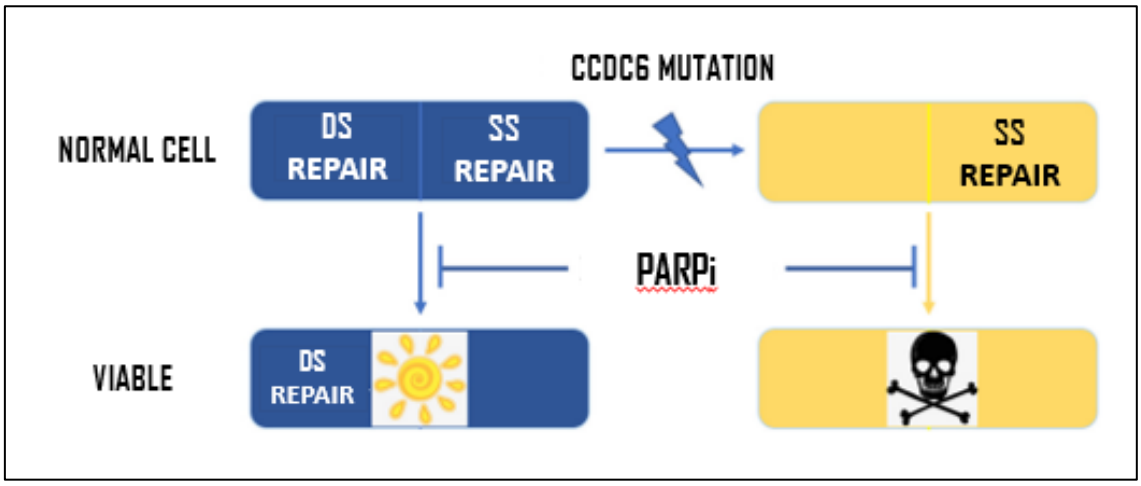

FIGURE 10. Synthetic lethality related to CCDC6 mutation and PARPi administration. Modifications of either DSB or SSB repair mechanisms do not promote cell death or apoptosis. However, when both pathways are ineffective, this situation results deleterious for the cell. Normal cells which do not have any mutation are able to survive to PARPi because their DSB repair pathways remain unaffected. However, when a cell presents non-functional or mutated CCDC6, PARPi will induce apoptosis because of the lack of a proper mechanism to repair SSB and DSB. Figure modified from (54).

There are nine PARPi drugs in the pipeline of drug development, but the US FDA (Food and Drug Administration of US) has approved the use of only five of them for cancer treatment: Talazoparib, Rucaparib, Niraparib, Olaparib and Veliparib (51,54) (Figure 11). Olaparib has been the only PARPi studied in CCDC6 defective tumors, showing a good response in NSCLC, castration-resistant prostate cancer or high-grade urothelial bladder cancer (26,27,46,55). Moreover, the combination of PARPi with conventional chemotherapy seems to be more effective than each agent individually in defective CCDC6 lung cancer. It was demonstrated that Olaparib reestablished cisplatin sensitivity, allowing its combination to treat NSCLC (26). This effect should be studied in other carcinoma tissues showing CCDC6 downregulation.

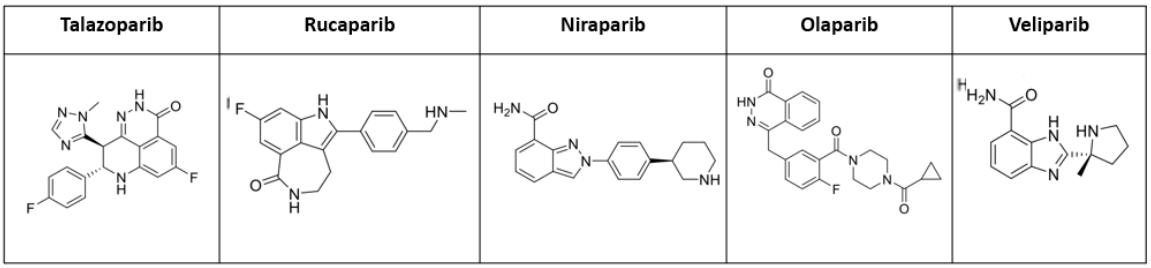

FIGURE 11. PARPi molecules approved by US FDA. In 2018 US FDA approved four of these molecules to treat several cancers. Veliparib was included by FDA (2021) in their recommendations to treat advanced lung squamous cell carcinoma in combination with radiotherapy or chemotherapy (54).

BRCA mutations are, nowadays, the main biomarker for PARPi indication. Other new prognostic markers are platinum response, high levels of PARP1/2 or mutations in genes involved in HR, which are considered "BRCA-like" such as PALB2, CHEK2, RAD51C/D, ATM or Fanconi anemia group. New biomarkers have been studied over the last years, including CDK12, ERCC1 or CCDC6 impairment. The implication of low levels of CCDC6, not only in HR reparation but also in cell proliferation and chemotherapy resistance reflects the importance of including CCDC6 as a PARPi predictive biomarker in the clinical prognosis of several cancers (2,51,55).

10.2 USP7-INHIBITORS AND FBXW7 INDUCTION

As indicated in previous sections, there are two enzymes regulating the stability of CCDC6: FBXW7 ubiquitin ligase involved in CCDC6 proteasomal degradation and USP7 de-ubiquitylating enzyme that increases CCDC6 levels by reducing its degradation.

It has been demonstrated that USP7 targets not only CCDC6 but other proteins related to several carcinomas' progression, such as PTEN. USP7-inhibitors would target USP7, leading to decreasing CCDC6 levels because of its FBXW7-mediated degradation. As CCDC6 overexpression has been related to chemotherapy resistance, using this kind of drug can be relevant to increase the sensitivity to chemotherapy treatment. Besides, they can be used as coadjuvants of PARPi, downregulating CCDC6 levels and improving its efficiency (56).

The first USP7i, called HBX41108, was found in 2009 and, since then, more than 160 molecules have been reported. They present two mechanisms of action: (i) binding to Cys233 in the catalytic domain of USP7 responsible for deubiquitination activity and (ii) allosteric inhibition, interacting with contiguous regions rather than with the catalytic one, preventing the alignment of this catalytic domain and blocking ubiquitin-binding channels (56).

The efficiency of the combination of USP7-inhibitors and PARP-inhibitors has been proved in lung-neuroendocrine tumors, showing that using USP7-inhibitor P5091 reduces CCDC6 half-life and makes the cells sensitive to Olaparib in combination with the standard therapies (57). It has also been studied in high-grade urothelial bladder cancer (UBC) how P5091 sensitizes cells with high levels of CCDC6 to Olaparib and conventional chemotherapy drugs (47). Based on CCDC6 and USP7 expression of urothelial bladder cancer patients, it could be possible to use a combined therapy of PARP-inhibitors and USP7 inhibitor P5091 plus RRx-001, a DNA damage inducer that enhances the sensitivity to PARP-inhibitors (Figure 12).

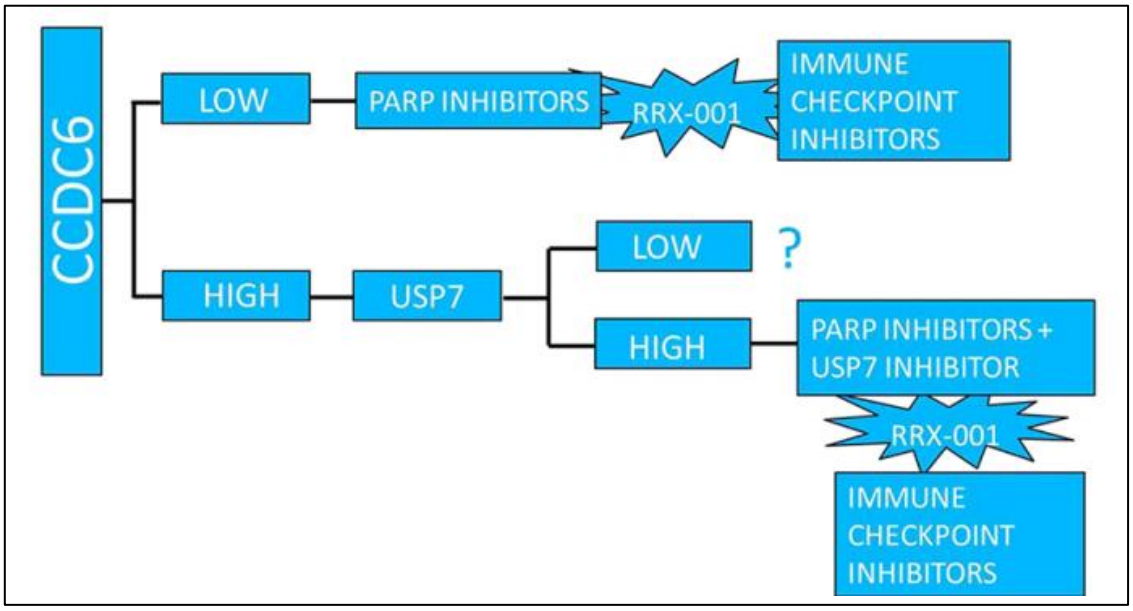

FIGURE 12. Therapeutic approaches depending on CCDC6 and USP7 expression. CCDC6 and USP7 expression levels may suggest a novel therapeutic scheme of personalized treatment in urothelial carcinoma. RRx-001 is an epigenetic and immunomodulatory agent which has been reported to act synergistically with USP-7 inhibitor P5091, enhancing the sensitivity to PARP-inhibitors. This combined effort exposes an additional therapeutic target that should be investigated in future research. Figure taken from (47).

Recent studies have focused on USP7-inhibitors and PARP-inhibitors combination in castration-resistant prostate cancer. They have demonstrated that using USP7-inhibitors not only affects androgen-receptors levels but also reduces CCDC6 levels, sensitizing these cells to PARP-inhibitors alone or in combination with standard radio and chemotherapies (27).

Related to CCDC6 stability regulation, another interesting target that should be explored in future experiments is FBXW7, looking for new therapeutic approaches to increase its activity and, therefore, reduce CCDC6 levels to make cancer cells more susceptible to PARPi (17,26).

A proposed model of CCDC6 alterations and drug sensitivity is shown in Figure 13.

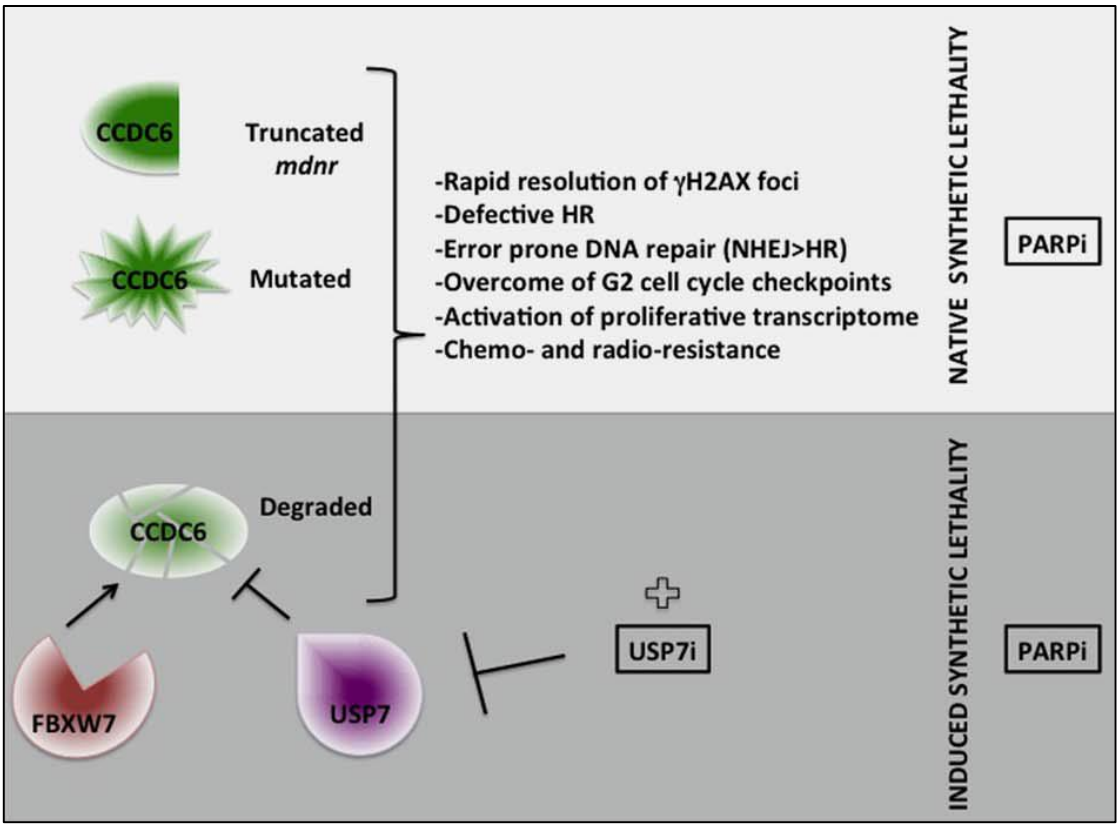

FIGURE 13. CCDC6 and drugs sensitivity. Truncation or inactivating mutations act as a functional dominant negative of wild-type CCDC6 from the residual allele. This native downregulation makes cancer cells sensitive to PARP-inhibitors with a synthetic lethality effect. It can be also induced by USP7-inhibitors, promoting the degradation of the protein, and making the cells sensible to PARP-inhibitors. Figure taken from (1).

10.3 TYROSINE KINASE INHIBITORS

The presence of fusions of CCDC6 with receptor tyrosine kinases (mainly RET and ROS1) in different cancers suggested the use of tyrosine kinase inhibitors (TKI) as specific therapy in those tumors.

Trastuzumab (used as a therapy for metastatic breast cancer, targeting HER2 receptor) and imatinib (targeting BCR-ABL1 in Philadelphia chromosome-positive chronic myelogenous leukemia) were the first examples of gene-based cancer drugs, representing the onset of a new era of target-directed therapies. Tyrosine kinase inhibitors specifically target the ATP-binging site of the intracellular domain of tyrosine kinase receptors, preventing phosphorylation reactions required for signal transduction upon receptor activation (58).

As previously explained, CCDC6 is a negative regulator of the transcriptional activity of CREB1. CCDC6 downregulation induces CREB1 target genes expression, such as the amphiregulin gene (AREG) (59). AREG protein is one of the ligands of the epidermal growth factor receptor (EGFR) family, which has demonstrated its implication in triggering RET-inhibitors resistance (Figure 14).

It was demonstrated that endothelial cells confer resistance to RET inhibitors sunitinib, E7080, vandetanib and sorafenib by activating survival signals of EGFR in NSCLC cell lines

with CCDC6-RET fusion genes. The activation of the EGFR pathway induces the autocrine liberation of AREG, inducing a compensatory survival signaling responsible for tyrosine-kinase inhibitors resistance. Thus, targeting these membrane receptors using specific tyrosine-kinase inhibitors restores cancer cell sensitivity to TK-inhibitors such as RET-inhibitors (RETi) (60). Moreover, detecting CCDC6 impairment might represent an indication for targeting EGFR in cancer therapy.

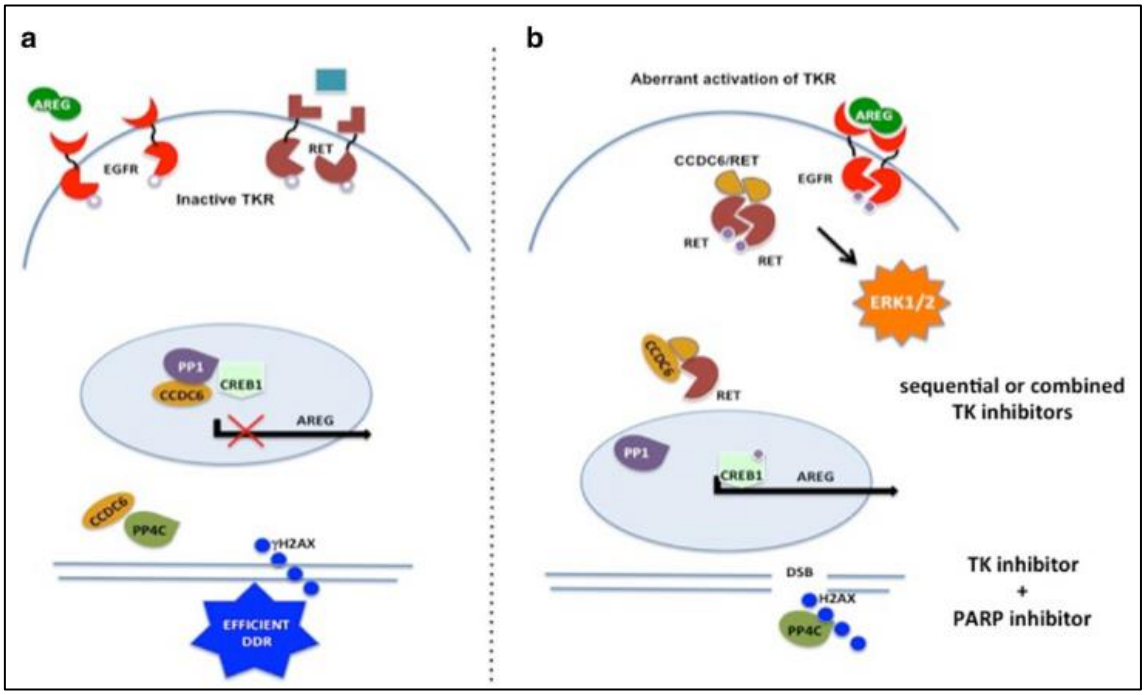

FIGURE 14. Molecular model of cell signaling activated upon CCDC6 fusion to a tyrosine kinase. In cells with unrearranged CCDC6, CREB1 is inactive and AREG is not transcribed, preventing its binding to EGFR. Additionally, CCDC6 inhibits the dephosphorylation of γH2AX by PP4, allowing the correct DNA repair process (a). However, in cells with CCDC6 fused to a TK, such as RET, CCDC6 remains in the cytoplasm and CREB1 promotes the transcription of AREG, which binds to EGFR triggering the MAPK/ERK cascade. Additionally, DNA damage is not repaired correctly (b). Therefore, combined therapy of TKI plus PARPi could be indicated for cancers harboring CCDC6 fused to a tyrosine kinase. Figure taken from (33).

11 CONCLUSIONS

CCDC6 is a protein involved in several pathways related to cellular proliferation, DNA repair and apoptosis regulation, and its downregulation induces chemoresistance, oncogenesis and tumor progression. CCDC6 alteration impairs double-strand breaks repair pathway, making cells with damaged DNA the perfect target to PARP-inhibitor drugs. Additionally, its frequent fusion with tyrosine-kinase proteins allows the potential use of tyrosine-kinase inhibitors as therapeutic approaches in several cancers. Because of this and the information included in this work, it is believed that the presence of CCDC6 fusions or lack of function should be considered as a novel biomarker of prognosis and indication to use PARP-inhibitors. Besides, other therapeutic approaches like USP7-inhibitors should be considered in order to modulate CCDC6 levels and induce PARP-inhibitors sensitivity. In summary, this work highlights the crucial role of CCDC6 deregulation in many types of cancer and the importance of further investigating its role as a potential biomarker or therapeutic target for targeted antitumor therapy.

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13 ANNEXES

ANNEXE 1. CCDC6 gene representation from UCSC genome Browser

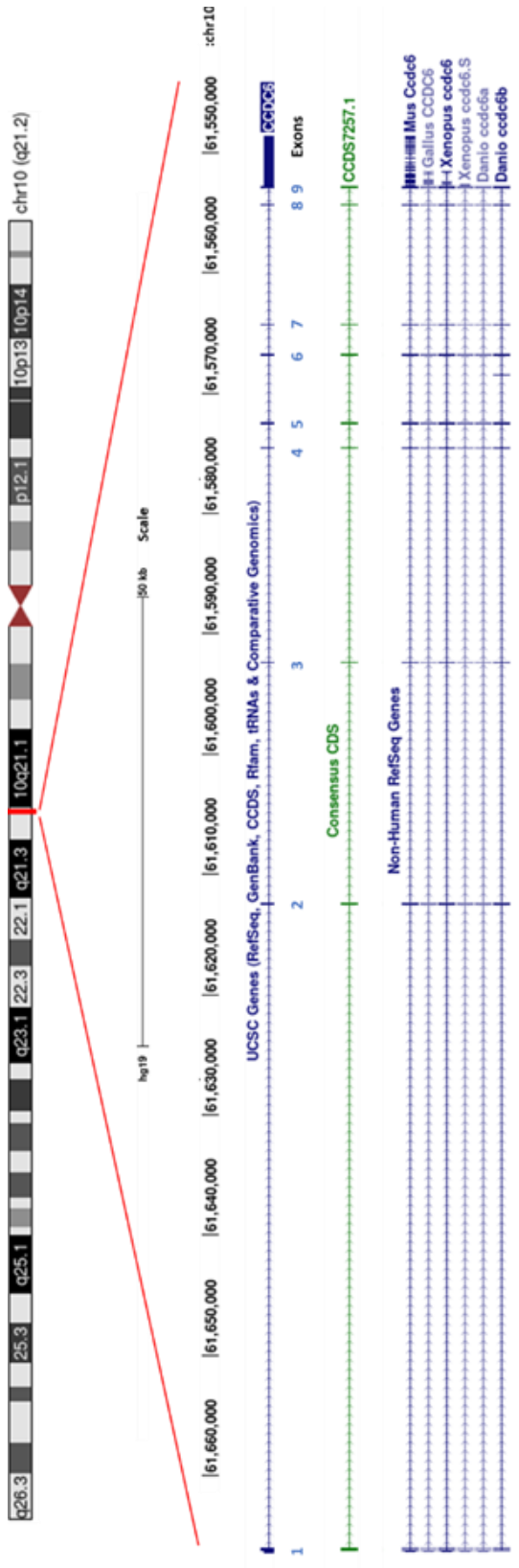


FIGURE 1. CCDC6 gene locus, structure, and phylogenetic conservation. The information showed in this figure has been obtained from UCSC GENOME BROWSER (Feb 2009 version). It shows the CCDC6 gene location, where its 9 exons are placed and the consensus coding sequence (CDS) that is conserved among different species (4).

ANNEXE 2. Analysis of CCDC6 in cancer using the platform cBioPortal for cancer genomics

In order to obtain further information on CCDC6 and its role in cancer we used the platform cBioPortal for Cancer Genomics (https://www.cbioportal.org/). The cBioPortal provides visualization, analysis and download of large-scale cancer genomics data sets.

We choose data from the TGCA (The Cancer Genome Atlas) including 32 genomic studies. After query for "CCDC6", we selected the following tabs:

- OncoPrint: visualize multiple genomic alteration events (Figure 1)
- Cancer Type summary: data of CCDC6 alterations in different cancer types (Figure 2)
- Mutations: analyze CCDC6 mutations as well as post-translational modifications (Figure 3)
- Pathways: the most relevant pathways altered by CCDC6 mutations (Figure 4)

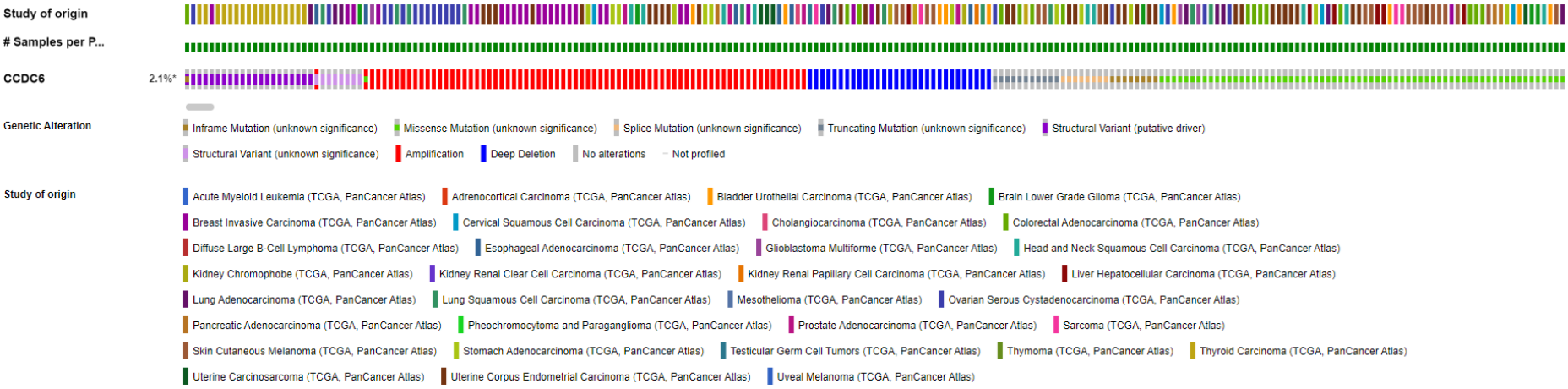


Figure 1. Oncoprint was used to analyze the genomic alteration in CCDC6 across the samples. A total of 10950 samples were analyzed from 32 studies with TGCA (The Cancer Genome Atlas) data. Overall, CCDC6 was altered in 2.1% of queried patients. The most frequent alterations were genetic amplifications (red), missense mutations(green), structural variants (purple), deep deletions (blue). Truncating mutations (dark grey), splice mutations (salmon), and inframe mutations (brown) were also found in some cases.

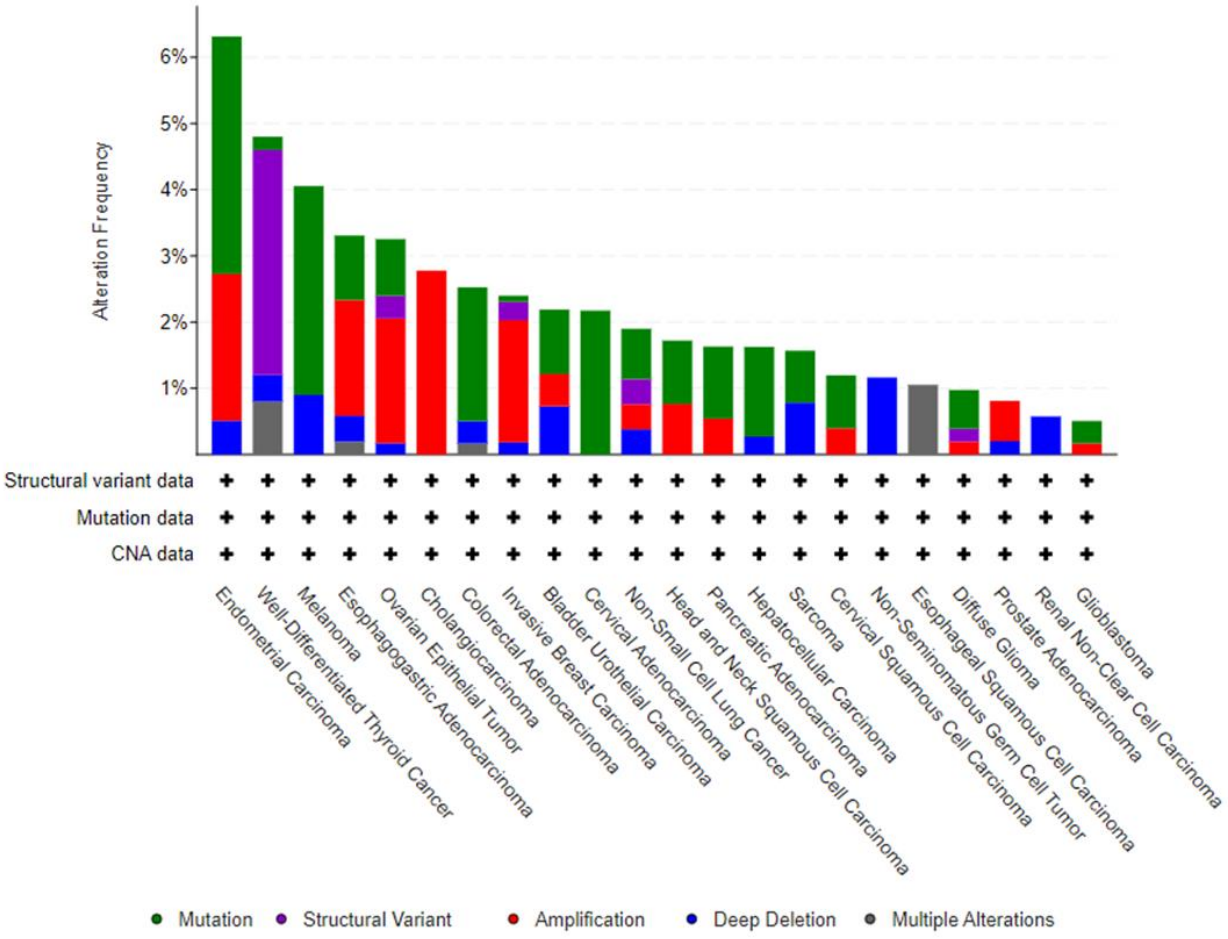


Figure 2. CCDC6 alteration frequency depending on the cancer type.CCDC6 is altered in: 6.31% of endometrial carcinoma, 4.8% of well-differentiated thyroid cancer, 4.05% of melanoma, 3.31% of esophagogastric adenocarcinoma, 3.25% of ovarian epithelial tumor, 2.78% of cholangiocarcinoma, 2.53% colorectal adenocarcinoma, 2.4% of invasive breast carcinoma, 2.19% of bladder urothelial carcinoma, 2.17% of cervical adenocarcinoma, 1.9% of non-small cell lung cancer, 1.72% of head and neck squamous cell carcinoma, 1.63% of pancreatic adenocarcinoma, 1.63% of hepatocellular carcinoma, 1.57% of sarcoma, 1.2% of cervical squamous cell carcinoma, 1.16% of non-seminomatous germ cell tumor, 1.05% of esophageal squamous cell carcinoma, 0.97% of diffuse glioma, 0.81% of prostate adenocarcinoma, 0.57% of renal non-clear cell carcinoma and 0.51% of glioblastoma.

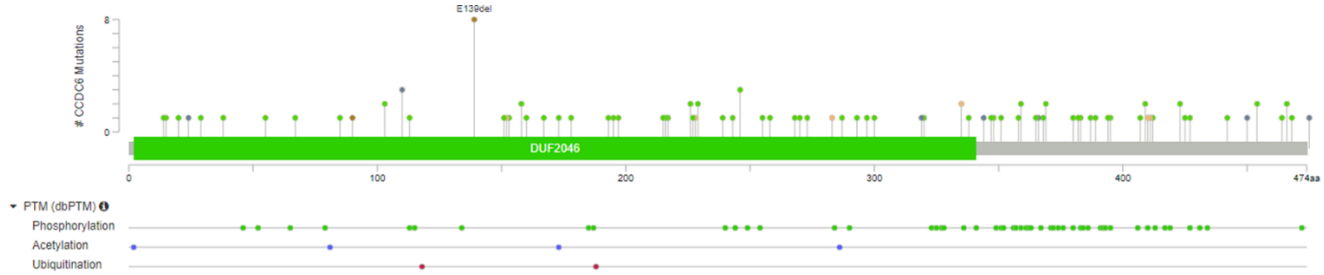
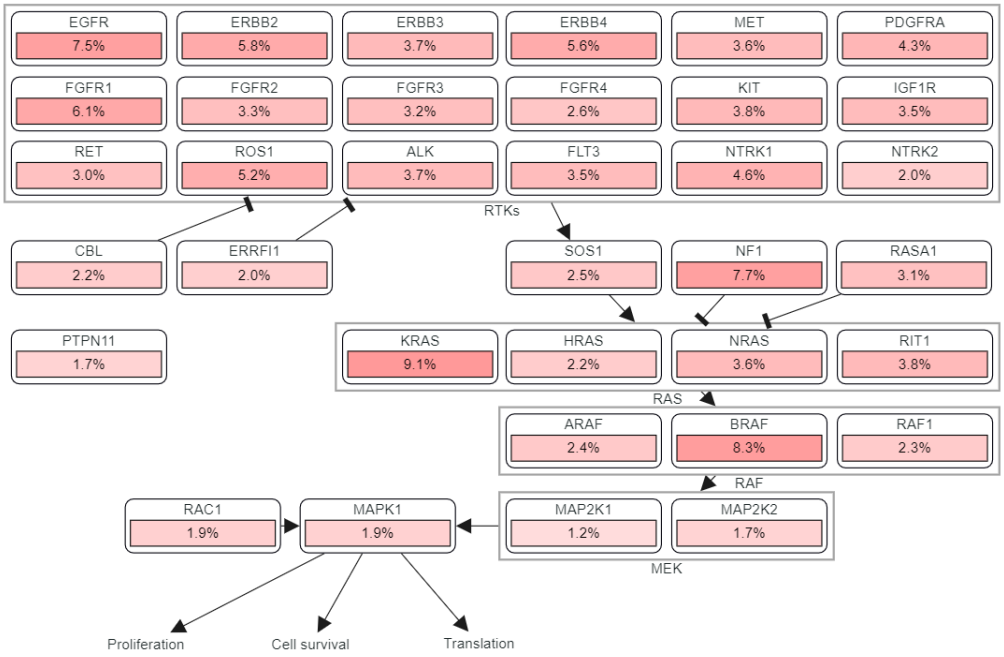


Figure 3. Figure shows the position of the CCDC6 mutations and post-translational modifications. Top. A total of 139 mutations were detected. 75 missense, 11 truncating and 9 inframe mutations were found distributed all along the CCDC6 protein. From the 36 fusions (not shown), 26 were found to be driver mutations. Bottom. Main post-translational modifications were: phosphorylation (green), acetylation (blue) and ubquitination (red).

A) RTK-RAS



B) Cell cycle

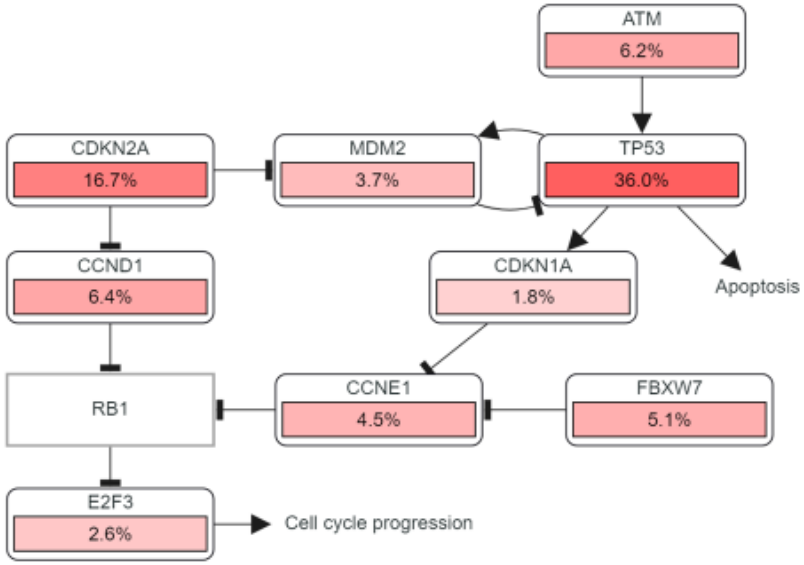


Figure 4. Pathways altered by CCDC6 mutations. From the 10 different pathways found altered, RTK-RAS (A) and cell cycle (B) are shown. Alteration frequencies of selected genes are shown. The other pathways were; HIPPO, MYC, NOTCH, NRF2, PI3K, TGF-beta, TP53 and WNT.