Cdc6 regulation, deregulation, and its role in oncogenesis

Abstract

Cdc6 is a protein that regulates DNA replication in eukaryotic cells and is part of the pre-replication complex (pre-RC). It is tightly regulated in cells, and its deregulation is implicated in a number of cancers, such as prostate, lung, brain and cervical. Both excessive overexpression and underexpression of Cdc6 has been shown to be detrimental to correct cellular proliferation, stressing how important it is that the levels of this protein are tightly regulated at different stages of the cell cycle. This review looks briefly at the different roles of Cdc6, then at the importance of cellular senescence and finally discusses the various Cdc6-related findings in different cancerous tissue, including how various other factors respond to the overexpression of Cdc6 and how Cdc6 can directly influence oncogenesis.
Introduction

Cdc6 is a DNA replication regulator in eukaryotic cells, conserved from *Saccharomyces cerevisiae* to humans. In humans, the *CDC6* gene is found on chromosome 17, and its expression is controlled by E2F/retinoblastoma transcription factors, which regulate genes promoting S-phase\(^1\). The best-studied role of Cdc6 is as a member of the pre-replication complex (pre-RC). In the pre-RC, Cdc6, together with the protein Cdt1, recruits and loads the Mini Chromosome Maintenance (MCM) proteins onto DNA. It is thought that Cdc6-Cdt1 threads DNA through the ring-shaped structure of MCM in an ATP-dependent manner. Thus Cdc6 is vital for the licensing of DNA for replication\(^2\).

Another role for Cdc6 is in the maintenance of replication checkpoints, which occurs after DNA replication. Cdc6 activates the Chk1 checkpoint kinase during S phase to prevent mitosis until all DNA has been replicated: the S-M checkpoint. Cdc6 acts to suppress mitotic catastrophe; its overexpression interferes with progression of the G2 phase and causes extensive delay of mitosis. At the end of G-phase, after the MCM helicase complex and additional factors have been loaded onto DNA, Cdc6 becomes inactivated. This inactivation has been observed both in *S. cerevisiae* and in human cells. Removing and/or degrading Cdc6 after replication has been completed ensures that no re-licensing of DNA occurs.\(^3, \, 4\). However, a portion of Cdc6 remains associated with chromatin, and this pool of Cdc6 acts in checkpoint regulation. During S-phase arrest, Cdc6 anchored to chromatin is stabilized and acts as a receptor for ataxia telangiectasia mutated and Rad-3 related (ATR) kinase and ATR-interacting protein (ATRIP), likely via Mcm7 binding to Cdc6 and recruiting the

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[Fig. 1. Formation of the pre-RC, licensing of DNA and dissociation of the pre-RC. ORC, Cdc6, Cdt1 and MCM bind to chromatin during the G1 phase of the cell cycle. At the G1-S phase transition the pre-RC disassembles, and phosphorylated Cdc6 is either degraded (yeast cells) or exported from the nucleus into the cytoplasm (mammalian cells).\(^2\).]
ATR-ATRIP complex. Cdc6 interacts with ATR in a cyclin dependent kinase (Cdk)-dependent manner\[^5\].

**Regulation of Cdc6**

The regulation of Cdc6 is vitally important for correct cellular proliferation and survival of the organism. There are several mechanisms by which Cdc6 is downregulated upon cell entry into S-phase. In budding yeast, it is either degraded through ubiquitinylation and proteasome action (requiring Cdc28-dependent phosphorylation of Cdc6) or inhibited by forming a complex with Cdc28; Cdc28 is the budding yeast homologue of Cdk1 in humans. In human cells, a portion of Cdc6 is exported from the nucleus after phosphorylation by cyclin A/Cdk2, while the rest is likely degraded\[^6\]. If the mechanisms for Cdc6 regulation become ineffective, Cdc6 tends to accumulate in the cell, and DNA re-replication can occur. This aberration is an important marker of oncogenesis. As will be seen later, Cdc6 overexpression and Cdc6 overproduction are more than simply byproducts of increased proliferation of tumourigenic cells. Cdc6 has been found to be a target by both tumour suppressors and tumour activators, and its overproduction is important for both the cellular senescence pathway and for suppression of anti-cancer gene product expression.

**Controlling DNA replication**

Before delving into the details of Cdc6 oncogenic activity, it is necessary to understand that controlling DNA replication is vital for genomic stability. If DNA replication becomes unregulated, chromosomes can misaggregate and DNA mutations accumulate, all of which can lead to the switching on of oncogenes and to the switching off of tumour suppressor genes and/or loss-of-function of their products. All this can lead to DNA re-replication and thus to “genetic instability”\[^7\]. One of the responses a cell can make in order to halt progression into a cancerous state is to induce senescence. Cellular senescence (aging) is the loss of a cell's ability to divide, and one of the ways that cells achieve this is by degrading Cdc6. Senescence can be induced either via the double strand break-DNA repair pathway (DSB-DDR) or via the expression of tumour-suppressor genes, e.g. p16\[^INK\] and ARF, or, likely, an interplay between the two; tumour suppressors have been found to play a role in DSB-DDR. Progression to a cancerous state correlates with decreasing senescence, stemming from the failure of replication control. Overexpression of CDC6 is one of the cues that leads to activation of tumour-suppressor genes and of the DSB-DDR pathway\[^8\].

In DNA-damaged cells, Cdc6 is targeted for degradation by at least 2 different ubiquitin ligases: ionizing radiation leads to the pathway where Cdc6 is degraded by the Cdh2-associated anaphase-promoting complex (APC/C) and UV/alkylation damage lead to the pathway where the degrading enzyme is HUWE1 (a HECT domain E3 ligase). Which ligase degrades Cdc6 depends on the early signal transducing kinases in the replication stress/checkpoint pathways: ionizing radiation activates ataxia tegalencia-mutated kinase (ATM) while UV/alkylation activate ATR. ATR and ATM phosphorylate Chk1 and Chk2 kinases, respectively;
ATR is responsive to replicative stress and phosphorylates Chk1, while ATM responds to DSBs and phosphorylates Chk2. Also, in early apoptosis, Cdc6 is cleaved by caspase-3[9]. Failure of these mechanisms to reduce the levels of Cdc6 may contribute to oncogenesis.

**Cdc6 and oncogenesis**

Cdc6 can be inferred to have certain oncogenic features from tissue culture experiments: S-phase extracts enriched with Cdc6 cause accelerated G1-S transition in G1 nuclei, Cdc6 cooperates with cyclin E to induce DNA replication in quiescent cells, ectopic expression of CDC6 (together with CDT1) causes DNA over-replication in tumour cells[10]. Even when CDC6 is overexpressed, the level of Cdc6 can be kept at the physiological norm by its regulatory mechanisms. Only if those regulatory mechanisms become ineffective or if the overexpression of the gene is at levels beyond the scope of regulation, Cdc6 overproduction becomes evident.

The expression of the CDC6 gene is deregulated in many cancers. CDC6 overexpression is evident in 55 % of brain tumours[11], and in about half of non-small cell lung cancers, the most common of lung malignancies[12]. Furthermore, the risk of liver cancer is reduced in individuals with a polymorphism in the CDC6 promoter: the C6c6-515A>G allele leads to a lower risk of hepatocellular carcinoma due to tighter binding of nuclear proteins to the promoter and, consequently, the reduced expression of CDC6[13]. Overexpression of CDC6 is also implicated in cervical cancer, and is being investigated as a diagnostic marker. In cervical cancer cells, Cdc6 levels show a positive correlation with dysplasia; i.e. the more cell abnormalities are evident, the greater CDC6 expression. This is illustrated by increasing intensities of Cdc6-positive nuclear staining in dysplastic cells, with highly abnormal lesions and carcinomas showing cytoplasmic staining as well, probably due to the accumulation of cytoplasmic Cdc6 after repeated S phases. Also, Cdc6 mRNA quantification experiments confirm that Cdc6 overproduction increases linearly with increasing severity of dysplasia[14]. However, it was also found that at least in cervical cancer, cells with low abnormalities do not produce elevated amounts of Cdc6, at least detectably. This could be explained by the fact that Cdc6 is a G2/M checkpoint regulator and is degraded during entry into M phase. These findings lead to a conclusion that Cdc6 could be used as a marker for advanced cervical cancer lesions[15]. One possible explanation for increased CDC6 expression in cervical cancer cells is that the human papilloma virus oncoprotein E7 binds to the retinoblastoma protein, which interacts with E2F transcription factors to
stimulate their activity and thus *CDC6* expression\[14\]. Another study found that in a variety of other cancerous tissue (non-small cell lung carcinomas, nonfamilial colon lesions and larynx lesions), *CDC6* overexpression is an early event, and, importantly, that this overexpression is not a result of increased proliferation rates, as there is no correlation with the proliferation marker Ki-67\[16\].

As mentioned in the introduction, the *CDC6* gene is responsive to transcription factors of the E2F family, which are known to be overexpressed in tumours. This one possible way of promoting Cdc6 overproduction\[17\]. Another way that Cdc6 expression becomes uncontrolled is via gene amplification; e.g. in lung cancer tissue, a 2.5-4.5 gene amplification can be observed in nearly half the cases, while no amplification is seen in normal tissue. This was found to be a primary event, i.e. not caused by the amplification of a neighbouring gene\[16\].

There are a number of different factors implicated in regulating Cdc6 levels, as it must be tightly controlled to prevent DNA re-licensing in normal cells. For example, Cdc6 is a target for tumour suppressors; in particular, the p53 pathway. p53 is activated by DNA damage and causes the degradation of Cdc6 by the above-mentioned APC/C complex; this is likely due to the suppressed Cdc6 phosphorylation activity of Cdk2. In cells lacking p53, no decrease of Cdc6 is evident after DNA damage, and, indeed, there is increased cell proliferation\[18\]. This indicates that the control of Cdc6 by the p53 pathway is necessary to prevent DNA replication in damaged cells with possible mutations that could lead to malignancy. Cdc6 is more stable in cells deficient in p53, and is thus likely a driver for the proliferation of tumours. Knocking down Cdc6 in neuroblastoma cells significantly reduces the progression of cancer cells into S-phase and induces apoptosis, with the increase of pro-apoptotic factors such as Bax and the decrease of anti-apoptotic factors such as Bcl-2. Cdc6 actually influences the expression of p53 in neuroblastoma cells, with knockdown of Cdc6 decreasing p53 levels. However, p53 is usually mutated or otherwise non-functional in cancer cells, therefore its decrease has no further effect on these cells. Knocking down Cdc6 leads to a cell fate similar to that when the p53 tumour-suppression pathway is functional\[19\]. Similarly, it was found that in non-small cell lung carcinomas, cells with mutant p53 exhibited greater overexpression of not only Cdc6, but Cdt1 and associated aberrant expression of Geminin as well\[20\]. However, there are some safeguards against non-functional p53: HUWE1, the other ubiquitin ligase responsible for degrading Cdc6 after DNA damage is p53 independent\[9\].

While p53 suppresses Cdc6 activity, other proteins are known to promote *CDC6* expression and thus Cdc6 overproduction. For example, one protein responsible for regulating Cdc6 levels in cancerous cells is YB-1. YB-1 is a protein whose nuclear expression results in poor prognosis in a variety of cancers, including ovarian, lung and breast cancers, as well as soft-tissue malignancies. It was found that in breast and lung cancer cells, knocking out YB-1 reduced the expression of the *CDC6* gene and the number of cancerous cells in S-phase. YB-1 binds to its Y-box consensus sequence on the *CDC6* promoter and plays a role in stimulating transcription\[21\]. Another transcription factor, the androgen receptor (AR), responsible for androgen-dependent cellular proliferation, promotes the expression of Cdc6 in prostate cancer cells by targeting the androgen receptor element (ARE) in the *CDC6* promoter.
Silencing AR in cancer cells reduces Cdc6 expression and androgen-dependent proliferation. In the presence of androgens, AR recruits Mediator and p160/SRC-HAT complexes to induce CDC6 promoter acetylation and to recruit RNA polymerase II. On the other hand, anti-androgen agents repress AR and subsequently CDC6 expression. Oncogenes also stimulate the production of replication licensing factors in order to promote DNA re-replication. H-RasV12 in particular has been implicated in the overproduction of Cdc6, as higher levels of Cdc6 are present upon Ras expression. Initially, the DDR response and tumour suppressors act to degrade the overproduced Cdc6, regardless of what was the stimulus or stimuli for the overexpression of its gene, but if mutations accumulate, these responses eventually become inactive and cells pass into a cancerous state.

Perhaps the most direct way in which deregulated Cdc6 influences oncogenesis is by deactivating the INK4/ARF locus. This locus encodes 3 tumour suppressor genes: p15\(^{INK4b}\), ARF and p16\(^{INK4a}\). This locus has a DNA replication origin on which the replicative machinery, including Cdc6, assembles; this replication origin was also found to have transcriptional activity and was termed regulatory domain, RD\(^{INK4/ARF}\). Experiments using RNA interference-induced heterochromatinization determined that RD\(^{INK4/ARF}\) is necessary for the transcription of the locus. Cdc6, although primarily a replication regulator, also seems to have a role in regulating transcription. High levels of Cdc6, such as found in tumour cells, reduce the expression of the INK4/ARF-encoded genes via the recruitment of histone deacetylases HDAC1 and HDAC2 to promote the silencing of that particular locus. Overexpressed Cdc6 promotes colony formation and takes part, together with oncogenic Ras, in transforming mouse embryo fibroblasts (MEFs). Overexpression of Cdc6 in cells lacking the locus does not promote tumourigenesis, indicating that Cdc6 contributes to oncogenesis via the suppression of INK4/ARF gene products rather than directly\(^{[23]}\). However, it is important to keep in mind that the INK4/ARF gene locus-encoded tumour suppressors are not universally conserved, and that Cdc6 overproduction affects the cell in a variety of ways. One possibility is that Cdc6 overproduction-induced repression of INK4/ARF locus-encoded tumour suppressors causes rapid cell proliferation, which in turn leads to the accumulation of DNA damage and DDR activation. However, this has yet to be widely investigated.

Fig. 3. A summary diagram of the possible ways that Cdc6 deregulation contributes to oncogenesis by interfering with the cellular response to oncogene activation.\(^{[10]}\)
Finally, not all cancers where Cdc6 is implicated show Cdc6 overexpression. Aggressive prostate cancer cells display decreased (25-75 %) expression of Cdc6, likely due to sub-optimal interaction of relevant transcription factors, specifically E2F, whose levels were below normal in aggressive prostate cancer cells, with the promoter of the *CDC6* gene[24]. Keeping in mind that AR promotes Cdc6 production in prostate cancer, a dichotomy arises, where *CDC6* is both under-expressed and overexpressed in the same type of cancer. This inconsistency shows how multi-dimensional cancer proliferation is, and may signify a difference between androgen-dependent and androgen-independent proliferation. That is, in androgen-dependent prostate cancer cells, Cdc6 production is stimulated, and in androgen-independent, it is under-expressed.

**Conclusion**

In conclusion, Cdc6 as been shown to cause DNA damage by licencing re-replication, which in turn causes the breakdown of replication forks. In the early stages of DNA damage, regulated overexpression of *CDC6* allows the cell to try to stop the progression into a cancerous state via tumour-suppression pathways; however, continuous stimulation by elevated levels of Cdc6 favours selection of aggressive cells that have the ability to bypass regulation and proliferate indefinitely. There is likely a switch from regulated to deregulated Cdc6 overproduction in cells, somewhere at the threshold between hyperplasia and dysplasia. Overproduced Cdc6 also plays a direct role in the suppression of anti-tumour effectors encoded by the INK4/ARF locus. The mechanisms and implications of Cdc6 deregulation are far from resolved, and much more research needs to be done in this area.
References
