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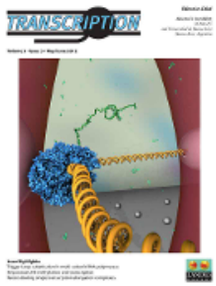

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Cdc6

A multi-functional molecular switch with critical role in carcinogenesis

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Research in the last decade revealed an additional role for the Replication Licensing Factor Cdc6 in transcriptional regulation. This novel function has been linked to human cancer development. Here, we summarize all the findings arguing over a role of Cdc6 as a transcriptional repressor and shed light toward new research directions for this field.

Keywords: origin of replication, chromatin remodeling, human cancer, histones, heterochromatinization, chromosomal instability, insulator

Abbreviations: CDC6, cell division cycle 6; ORC, origin recognition complex; MCM, mini-chromosome maintenance; RLFs, replication licensing factors; NSCLCs, non-small cell lung carcinomas; EMT, epithelial to mesenchymal transition; RD, regulatory domain

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Identification of *CDC6*

CDC6 was initially identified in a genetic screen aimed at finding mutations that arrested the budding yeast cell cycle.[1] It took many years to clone the corresponding gene and identify its homologs in fission yeast and human cells.[2] Actually, *CDC6* is conserved in every eukaryotic organism. There is a homolog in Archaea as well. The first evidence for the participation of *CDC6* in DNA replication stems from the analysis of *Cdc6-1*, a temperature-sensitive yeast *CDC6* mutant that arrested cells at the G_1-S transition.[2]

CDC6 belongs to the AAA$^+$ family of ATPases with chaperone-like activities.[3] All family members contain matches to the conserved bipartite ATP binding and hydrolysis motifs described by Walker and colleagues.[4] The Walker A-motif is required for ATP binding, whereas the Walker B is important for ATP hydrolysis.[3] At least two functions of Cdc6 rely on its ATPase activity. The first one, which so far has only been demonstrated in yeast, concerns the ability of Cdc6 to influence the specificity with which ORC associates with the DNA,[5] whereas the second is the actual loading of MCM proteins. Three convergent studies in budding yeast establish that *CDC6* is a key factor for replication origin licensing due to its responsibility for the loading of MCM proteins onto origins.[6-8] Mini-Chromosome Maintenance (MCM) proteins together with Origin Recognition Complex (ORC) form large structures called pre-Replication Complexes (pre-RCs) and can be visualized by genomic footprinting at origins of replication during G_1.[9] MCMs function as a helicase machinery required to separate the two DNA strands during S phase. Six of them are essential for both initiation and elongation of DNA replication.[10]

The human *CDC6* gene is located at chromosome 17q21.3 and its expression is controlled by the E2F family of transcription factors that regulate S-phase-promoting genes.[2] Shortly after its identification, it was confirmed that human *CDC6* was also essential for initiation of DNA replication. When hCdc6 levels were downregulated in G_1 cells by antibody microinjection, cells could not progress into S phase. Later on, it was found that Cdc6 downregulation by RNA interference (RNAi) prevented cell proliferation and promoted apoptosis.[2]

For a cell, it is important that replication happens only once per cell cycle. To ensure this, cells inactivate the processes for pre-RC formation once S phase is initiated to prevent re-licensing and re-initiation.[11] After mitosis, the pre-RC machinery is de-repressed so that origins

can be licensed again for the next cell cycle. Due to their critical role as licensing factors, the majority of the mechanisms that mammalian cells have developed to prevent re-replication, impinge on the regulation of Cdc6 and Cdt1. In both cases, a ubiquitin-dependent proteolytic degradation mechanism is involved. Moreover, Cdt1 is also regulated by its inhibitor, Geminin.[11]

Replication Factors Involved in Regulation of Transcription and Chromatin Remodeling

It is very interesting to note that several factors involved in DNA replication, have additional cellular roles, such as regulators of transcription and chromatin remodeling. The role of a number of them in heterochromatin silencing and epigenetic inheritance is very nicely reviewed by Li et al.[12] Among them, the ORC, the MCMs and Cdc6 have been suggested to have a direct role in transcription as well.

Regarding MCM proteins, there is evidence implicating their role in transcription, such as association with the C-terminal domain (CTD) of RNA polymerase II (RNA PolII), purification of heterodimer Mcm3-Mcm5 in a complex with STAT1a, a transcription factor stimulated by gamma-interferon, and inhibition of transcription, in vitro, by antibodies against Mcm2. Intriguingly, increased levels of Mcm5 in cells correlate with increased levels of transcriptional activation. Moreover, Mcm7 has also been connected with several transcription regulators, such as Mcm1 and Rb.[13]

The origin recognition complex (ORC) is another example of replication factor linked to transcriptional regulation. Quite early after its identification, it was shown that ORC2 apart from the role in DNA replication has an additional function in transcriptional silencing at the yeast silent mating-type loci.[14] Furthermore, in Drosophila, a position-effect variegation of gene expression occurs when chromosomal rearrangements bring genes in proximity to heterochromatin. These activities involve the binding of HP1 to ORC1.[15] Moreover, HB01, a histone acetyl-transferase responsible for chromatin remodeling followed by transcriptional regulation, binds to ORC1.[16] Finally, Takayama et al.[17] reveal that ORC1 competes with SNF5, a component of chromatin remodeling complex SNF/SWI, for binding to the C-terminal region of c-Myc. As a result of this interaction, the E-box motif-dependent transcriptional activity of c-Myc is inhibited, proposing a direct repressive role for ORC1. It is interesting to note that, Dominquez-Sola and colleagues identified a critical function for c-Myc in DNA replication, suggesting a novel mechanism for its normal and oncogenic functions.[18] This is another finding strongly supporting the bidirectional relationship between DNA replication and transcription.

Cdc6 in Human Cancer

It is more than 10 years now since Cdc6 was also linked to transcriptional regulation. In parallel to their previously mentioned study on ORC1,[17] Hiroyoshi Ariga's group published similar studies for Cdc6.[19] In particular, Cdc6 was also shown to bind to the C-proximal region of c-Myc, thereby competing with Max and changing c-Myc/Max heterodimer to a Max/Max homodimer. As a consequence, the E-box motif-dependent transcriptional activity of c-Myc was abrogated, suggesting a role for Cdc6 as a transcriptional suppressor of c-Myc.

The last few years, though, an intriguing hypothesis has emerged linking transcriptional regulation by Cdc6 to human cancer development. This was first evident when overexpression of Cdc6 was shown to lead to transcriptional repression of *INK4/ARF* locus.[20] Inactivation of this locus is one of the most frequent events in human cancer, since it encodes three important tumor suppressor genes: $p16^{INKa}$ and $p15^{INKb}$, both activators of the retinoblastoma pathway, and ARF, an activator of p53.[21] The mechanism of Cdc6-dependent repression involves recruitment of histone de-acetylases and heterochromatinization of the *INK4/ARF* locus. Interestingly, Cdc6 in cooperation with oncogenic Ras promotes cellular proliferation and neoplastic transformation activity.

Further evidence for the oncogenic role of Cdc6 in human was demonstrated in two recent reports from our group. The status of the two Replication Licensing Factors (RLFs), hCdc6 and hCdt1, in non-small cell lung carcinomas (NSCLCs) was initially studied and we revealed that overexpression of these proteins had synergistic effect with mutant p53 over tumor growth and chromosomal instability.[22] We, then, used epithelial lesions covering progressive stages of hyperplasia, dysplasia and full malignancy, coming mostly from the same patients, in order to examine how early during cancer development de-regulation of these factors occurs.[23] Abnormal accumulation of both proteins occurred early from the stage of dysplasia, caused in a significant proportion of cases due to gene amplification. Moreover, overexpression of hCdc6 and hCdt1 in tissue culture cells promoted re-replication and generated a DNA damage response, which activated the antitumor barriers of senescence and apoptosis. Continuous stimulus of de-regulated proteins induced genomic instability, by over-coming the p53 antitumor barrier, favoring clonal expansion of cells with more aggressive properties. Interestingly, pre-malignant, non-tumorigenic, mouse epithelial cells were challenged and exhibited features of epithelial to mesenchymal transition (EMT), a process contributing to cancer invasion and metastasis.[23] The same effect of overexpressed Cdc6 was also observed in human cells.[24] This phenotype was reversed when Cdc6 expression was shut down. Notably, flow cytometric analysis in mouse (P1) and human (A549) cells overexpressing Cdc6, revealed a significant increase of cells expressing the $CD24^{low}/CD44^{high}$ antigen phenotype (**Fig. S1A**), a configuration associated with stem-like features, thus supporting the proposed link between EMT and the gain of stem cell properties.[25]

Following this observation, we were triggered to elucidate the mechanism by which Cdc6 induces EMT. Consistent with that, we found that the phenotypic changes of mouse and human epithelial cell lines are accompanied by loss of the tumor suppressor E-cadherin (*CDH1*), a hallmark of EMT.[24] Intriguingly, though Cdc6 acts in a similar manner to Snail1, Snail2/Slug and the ZEB family members, ZEB1 (δEF1) and ZEB2 (SIP1),

which represent the major transcription factors known to negatively regulate E-cadherin,[26] our data suggest that the observed suppression of *CDH1* in cells overexpressing Cdc6 is not associated with upregulation of Snail1 and Zeb1 (**Fig. S1B**). The status of Snail2 and Zeb2 remains to be studied. Moreover, we noticed that human and mouse epithelial cells overexpressing Cdc6 display a significant growth benefit over their mock counterparts, in contrast to what has been previously described for the potent EMT inducers Snail1 and Twist.[24,27,28] These data point toward a newly discovered and independent process of EMT, regulated by Cdc6.

We then discovered that Cdc6 represses *CDH1* gene transcription by binding to E-box motif (CANNTG) located at its promoter. One E-box motif is also included in the regulatory domain (*RD*) found at the promoter of *INK4/ARF*.[20] Consistent with the presence of a putative DNA replication origin proximal to *RD* at the *INK4/ARF* locus, we also identified nascent DNA products in a region adjacent to E-boxes of *CDH1* promoter.[24] Interestingly, overexpression of Cdc6 in mouse epithelial cells activated this origin. Moreover, we revealed that Cdc6-dependent repression of *CDH1* involves dissociation of chromosomal insulator CTCF, displacement of histone variant H2A.Z and promoter heterochromatinization, similar to the *INK4/ARF* promoter.[24]

These data demonstrate that Cdc6 acts as a molecular switch at the *E-cadherin* locus, linking transcriptional repression to activation of replication and provide a telling example of how replication licensing factors could usurp alternative programs to fulfill distinct cellular functions.

Future Vision

The above intriguing data have shed light on a new molecular pathway involved in human cancer development and progression. They have also highlighted the versatility of proteins which were thought to have a role only in one of the major cellular processes, such as DNA replication. It is more and more obvious that the cell has developed mechanistic plasticity, in order to utilize the same factors for different jobs, depending on their abundance and the environmental signals.

In this review, we have summarized the data arguing for an essential role of Cdc6 as a transcriptional regulator. As a result, a number of very interesting questions have now raised; Which other genes are directly regulated by Cdc6? What is the specificity of Cdc6 recruitment to DNA? How does Cdc6 exhibit its repressive function?

Based on all the evidence, someone would predict that most Cdc6-regulated target genes would be either cell cycle inhibitors or genes involved in differentiation. In this mode, "oncogenic" Cdc6 would be expected to switch-off genes that restrain cell proliferation and at the same time activate replication initiation. Therefore, it would be interesting to address whether the Cdc6 transcriptional program interferes with other transcriptional pathways. Toward this direction, we have performed gene expression-array analysis. Preliminary results of the mouse DNA-microarray data (overexpressed Cdc6 vs. Control), utilizing the Gene Set Enrichment Analysis method,[29] suggest that there are several protein groups such as proteins involved in Cell adhesion, Signal transduction, Cell migration, Cell proliferation and many others (**Table S1**), which have been significantly impacted (in terms of gene expression-level of deregulation). As expected, our data confirmed the suppression of *E-cadherin* and upregulation of *Vimentin*, that are markers for EMT. Whether, Cdc6 directly regulates the expression of all the identified de-regulated genes or not is currently under investigation.

Another very interesting issue is the specificity of Cdc6 recruitment to DNA. A common feature of the promoter of *INK4/ARF* and *CDH1*, which are both regulated by Cdc6, is the presence of three types of DNA elements; E-box, CTCF-binding site and origin of replication.[20,24] Cdc6 was shown to bind E-box motif in vitro and in vivo, suggesting that this might be the mechanism of Cdc6 recruitment.[20,24] However, the six-nucleotide E-box sequence (CANNTG) is very common across the entire genome. In fact, by performing bioinformatics analysis we have shown that there is one E-box every approximately 200 bp, suggesting that the presence of E-box alone cannot be responsible for Cdc6 recruitment to DNA. Therefore, someone could speculate that the presence of CTCF-binding sites and/or replication origins would be the restrictive factor for the specificity of Cdc6 DNA binding.

An interesting study recently showed that occupied CTCF binding sites are surrounded by 20 well positioned nucleosomes, covering a region of approximately 2 kb from each side.[30] These nuclosomes are enriched for H2A.Z and 11 histone modifications (in descending order of enrichment): H3K4me3, H3K4me2, H3K4me1, H3K9me1, H4K20me1, H3R2me1, H3K27me1, H3K36me1, H2BK5me1, H3R2me2 and H3K79me1. Eight of those modifications (the ones underlined), together with H2A.Z enrichment are features of gene promoters previously correlated with active transcription.[31] Intriguingly, our group have demonstrated that Cdc6 recruitment to DNA results in CTCF and H2A.Z displacement at the *CDH1* locus.[24] Therefore, one possible hypothesis could be that Cdc6 has affinity for E-boxes surrounded by nucleosomes enriched for H2A.Z, due to the presence of bound CTCF. One or more of the histone modifications enriched at nucleosomes flanking CTCF-binding sites, might also contribute to recruitment of Cdc6. In order to examine this idea, we performed a combination of the previously mentioned Gene Set Enrichment Analysis of the microarray data with CTCF binding site Enrichment Analysis, run on the promoters of the microarray-genes. Preliminary data, suggest that 15 of the groups represented in **Table S1** (which were found statistically significant deregulated by Cdc6), also show statistically significant enrichment for CTCF-binding sites at the promoters of their genes (**Fig. 1**). This fact has been observed at both, group-level and individual-promoter level. More specifically group-level means that these groups present a significantly larger-than-expected number of proteins whose promoters contain CTCF binding sites, while the individual-promoter level means that the promoters of these proteins were additionally found to be significantly enriched with CTCF binding sites per se. However, there are genes, either repressed

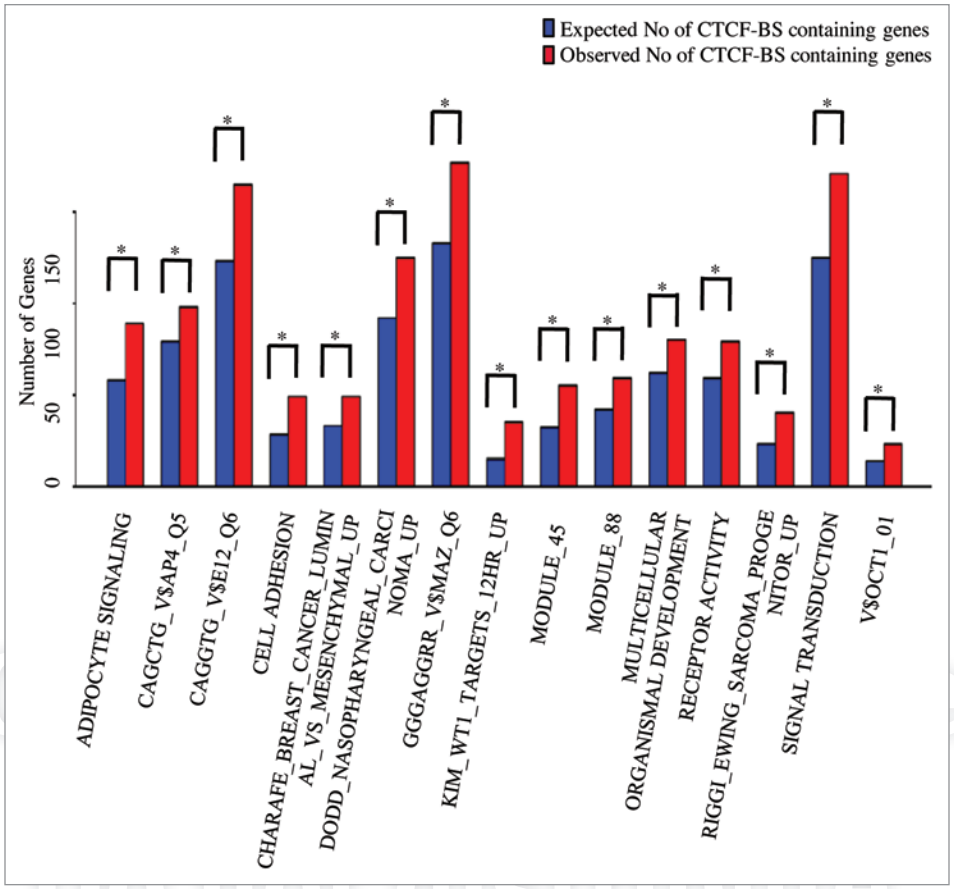

Figure 1. Barplot of group of genes which were found, by microarray analysis, de-regulated on a Cdc6-dependent manner and also show statistically significant enrichment ($p \leq 0.05$) for CTCF binding sites (CTCF-BS) at their promoters. *p-values were calculated based on observed vs. expected number of CTCF-BS containing genes.

or activated due to overexpression of Cdc6, which do not have CTCF sites within their promoters. In case these genes are directly regulated at transcription level by Cdc6, this suggests that Cdc6 is recruited to those promoters by a different mechanism. This possibility is currently under investigation.

The molecular mechanism of Cdc6-dependent transcriptional repression and activation of replication is another exciting area of investigation. Regarding this field of research, studies in budding yeast showed no global correlation between transcriptional activity and replication timing.[32] In the complex genomes of higher eukaryotes, however, things appear to be different. In Drosophila, a strong correlation between the early replication timing of a gene and the likelihood of it being actively transcribed was revealed.[33] Interestingly, this connection is not absolute: 30% of the earliest replicating genes were found to be inactive, and 30% of the latest replicating genes were actively transcribed, suggesting that, despite showing a high degree of positive correlation with active genes, early replication is unlikely to be a general requirement for transcription. Similar microarray studies in the human genome also revealed that early-replicating regions contain more active genes.[34-36] Finally, the imperfect correlation between replication timing and transcription is also supported by developmental studies. In particular, whereas during lymphocyte differentiation only a small number of genes appear to change replication timing upon activation, development-specific changes in DNA replication timing that coincide with gene activation are well described in the case of the β-globin.[37-39]

In an attempt to address the correlation between Cdc6-dependent transcriptional repression and activation of replication, Agherbi and colleagues, showed, recently, that in young cells Cdc6 interacts and recruits BMI1, a member of Polycomb group proteins, to the *INK4/ARF* locus, resulting in transcriptional repression and replication during late S-phase.[40] Upon senescence, the histone de-methylase Jmjd3 is overexpressed and the MLL1 protein is recruited to the locus provoking the dissociation of Polycomb with subsequent transcriptional activation and replication during early S-phase.

Based on all the above information, it is tempting to propose a generic speculative model for Cdc6-dependent transcriptional repression, using our knowledge for *CDH1* and *INK4/ARF* genes (**Fig. 2**); Upon a signal leading to overexpression of Cdc6, the protein is recruited to genomic loci, where E-boxes, CTCF binding sites and/or origins of replication are localized. Through direct binding with Cdc6, members of Polycomb group proteins, such as BMI1, might also recruited. Furthermore,

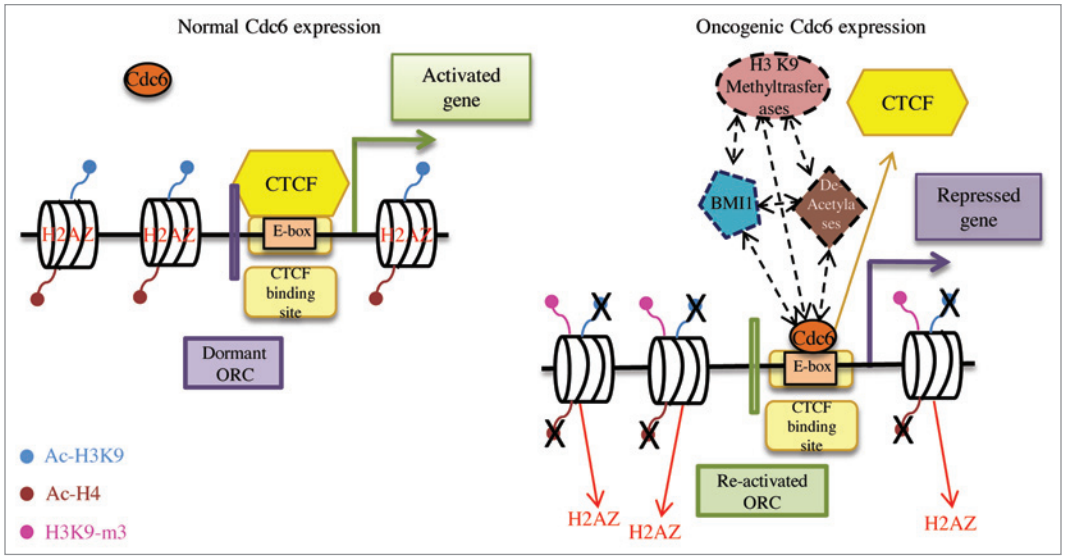

Figure 2. Speculative model for Cdc6-dependent transcriptional repression.

co-recruited histone de-acetylases and histone methyl-transferases modify the chromatin resulting in transcriptional silencing and heterochromatinization of the locus. The verification of this model is currently under investigation. Moreover, two other issues with great interest are the exploration of a possible CTCF-independent mechanism for Cdc6 recruitment, as well as the identification of protein complexes participating in Cdc6-dependent activation of genes.

Overall, studies from our and other laboratories have opened up a new field of transcriptional regulation linked to cancer development. Cdc6, the replication licensing factor, has been shown to regulate the expression of two loci, critical for the development of human cancer, the *INK4/ARF*, expressing three tumor suppressor genes, and the *CDH1*, expressing E-cadherin. Unraveling to which extend Cdc6 regulates cancer-related genes at transcriptional level and identifying the mechanism of its action, will be of enormous importance for the future of studies in cancer treatment and therapy.

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Supplemental Materials

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