



## Journals Club

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### Review of: The inhibitor of cyclin-dependent kinase 4a/alternative reading frame (INK4a/ARF) locus encoded proteins p16INK4a and p19ARF repress cyclin D1 transcription through distinct cis elements

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#### Abstract of the original article

The Ink4a/Arf locus encodes two structurally unrelated tumour suppressor proteins, p16(INK4a) and p14(ARF) (murine p19(ARF)). Invariant inactivation of either the p16(INK4a)–cyclin D/CDK–pRb pathway and/or p53–p14(ARF) pathway occurs in most human tumours. Cyclin D1 is frequently over-expressed in breast cancer cells contributing an alternate mechanism inactivating the p16(INK4a)/pRb pathway. Targeted over-expression of cyclin D1 to the mammary gland is sufficient for tumorigenesis, and cyclin D1 [minus sign]/[minus sign] mice are resistant to Ras-induced mammary tumours. Recent studies suggest cyclin D1 and p16(INK4a) expression are reciprocal in human breast cancers. Herein, reciprocal regulation of cyclin D1 and p16(INK4a) was observed in tissues of mice mutant for the Ink4a/Arf locus. p16(INK4a) and p19(ARF) inhibited DNA synthesis in MCF-7 cells. p16(INK4a) repressed cyclin D1 expression and transcription. Repression of cyclin D1 by p16(INK4a) occurred independently of the p16(INK4a)–CDK4-binding function and required a cAMP-response element/activating transcription factor-2-binding site. p19(ARF) repressed cyclin D1 through a novel distal cis element at [minus sign]1137, which bound p53 in chromatin-immunoprecipitation assays. Transcriptional repression of the cyclin D1 gene through distinct DNA sequences may contribute to the tumour suppressor function of the Ink4a/Arf locus.

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## Review

Cyclin D1 is the regulatory subunit of the cyclin-dependent kinase (CDK) 4/6 holoenzymes, protein kinases whose activity is strictly controlled during the cell cycle and represents a limiting step for G1–S transition, mainly acting upon the retinoblastoma tumour-suppressor gene product pRb [1]. Activation of *CCND1*, the gene encoding cyclin D1, is a crucial step for G1 phase progression and completion. All the mitogenic pathways activated by both extra-cellular and intra-cellular signals, including female sex steroids, increase cyclin D1 levels, a required step for cell transition through the G1–S restriction point [2–4]. The cyclin D1 gene is over-expressed in 50% of breast cancers and its activity is required for breast carcinogenesis [5–6] and responsiveness of breast epithelia to ovarian steroids [7]. Nonetheless, *CCND1* amplification occurs only in 13–15% of breast cancer specimens [8], indicating that over-expression of this gene in breast cancer is mainly due to dysfunctions of its transcriptional and post-transcriptional controls.

In their recent report, D'Amico *et al.* [9] highlight a novel mechanism for *CCND1* gene regulation by the two proteins encoded by the *CDKN2A* (cyclin-dependent kinase inhibitor 2A) tumour suppressor locus: p16<sup>INK4a</sup> and p19<sup>ARF</sup>. These two cell cycle inhibitors are translated from the same mRNA but from different reading frames, depending upon alternative splicing of exon 1, and share the sequence encoded in exons 2 and 3 [10]. Starting from the observation that in human breast cancers cyclin D1 expression is reciprocal to that of p16<sup>INK4a</sup>, the authors went on to observe an increased proliferation rate of embryonic fibroblasts (MEFs) from *INK4a/ARF*<sup>-/-</sup> mutant mice, with respect to their normal counterparts, concomitant to cyclin D1 over-expression. On the other hand, they found that high levels of either p16<sup>INK4a</sup> or p19<sup>ARF</sup> hamper G1–S transition in human MCF-7 breast cancer cells and, most notably, reduce cyclin D1 expression. Considering the possibility that the *CCND1* gene itself might be targeted by the two tumour suppressor proteins, resulting in transcriptional repression, the authors demonstrated by transient transfection assays in MCF-7 cells that over-expression of either one of the two proteins indeed induces inhibition of *CCND1* promoter activity. Molecular and genetic analysis of the mechanisms underlying this effect of the inhibitors revealed that p16<sup>INK4a</sup>-dependent gene suppression requires a functional CRE (cAMP Response Element) in the *CCND1* promoter, and is probably mediated by interference with DNA-bound CREB–ATF-2 complex interactions with components of the basal transcription machinery, while promoter silencing by p19<sup>ARF</sup> might depend on an atypical p53 response element, thereafter named ARE (ARF Response Element).

Cyclin-dependent kinase inhibitors play a fundamental role in regulating cellular proliferation and often show tumour suppressor actions. The two proteins encoded by the *CDKN2A* locus are able to block cellular proliferation in two different ways and in response to different stimuli. p16<sup>INK4a</sup> inhibits the kinase activity of the cyclin D1-CDK4/6 complexes, blocking E2F1 release from pRb suppression and thereby acting as a modulator of the G1–S transition; the observation that it also prevents *CCND1* transcription unveils a novel mechanism for interfering with the cyclin/retinoblastoma protein pathway, that is, prevention of cyclin D1 synthesis. On the other hand, p19<sup>ARF</sup> is activated by cellular stresses and enhances p53 stability by inhibiting MDM2-dependent degradation. As a consequence, the two products of the *CDKN2A* locus influence the activity of two main cell cycle regulators: pRb and p53. Furthermore, the observation that both proteins inhibit *CCND1* transcription through distinct cis-acting genetic elements

highlights an additional point of intersection between the INK4a–cyclin D/CDKs–pRb and the ARF–MDM2–p53 pathways, and underlines the importance of *CCND1* regulation for cellular proliferation control. p16<sup>INK4a</sup> and p19<sup>ARF</sup>, in fact, appear to be effectors of growth inhibitory circuits also independently from their ability to inhibit CDKs, exerting each in its own way a direct control on *CCND1* promoter activity.

The *CCND1* gene, thus, emerges once again as a true cellular sensor, capable of integrating the complexity of extra-cellular (growth factors and growth inhibitors, hormones, inter-cellular signals, etc.) and intra-cellular (cell stress, in its many variants, etc.) signals onto functional changes of the cell cycle machinery. Interestingly, also cyclin D1 can control transcription of its own gene, suggesting a way for signal amplification. In human breast cancer cells, *CCND1* activation by female sex steroids occurs stepwise. During early G1, gene activation in response to these hormones occurs via direct interaction (tethering) of the oestrogen and progesterone receptors with the AP-1 complex bound to the distal *CCND1* regulatory region, resulting in recruitment of the basal transcriptional machinery to the promoter; later in the cell cycle sustained gene transcription rate is supported, instead, by recruitment of cyclin D1 to a separate regulatory site of the promoter [11].

These observations, indicating that three main regulators of the ‘cell cycle clock’, namely INK4a, ARF and cyclin D1, converge on *CCND1* confirms the importance of transcriptional regulation of this gene in breast cancer cells, both for signal integration and maintenance of specific cellular phenotypes, including aberrant cell proliferation and survival.

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