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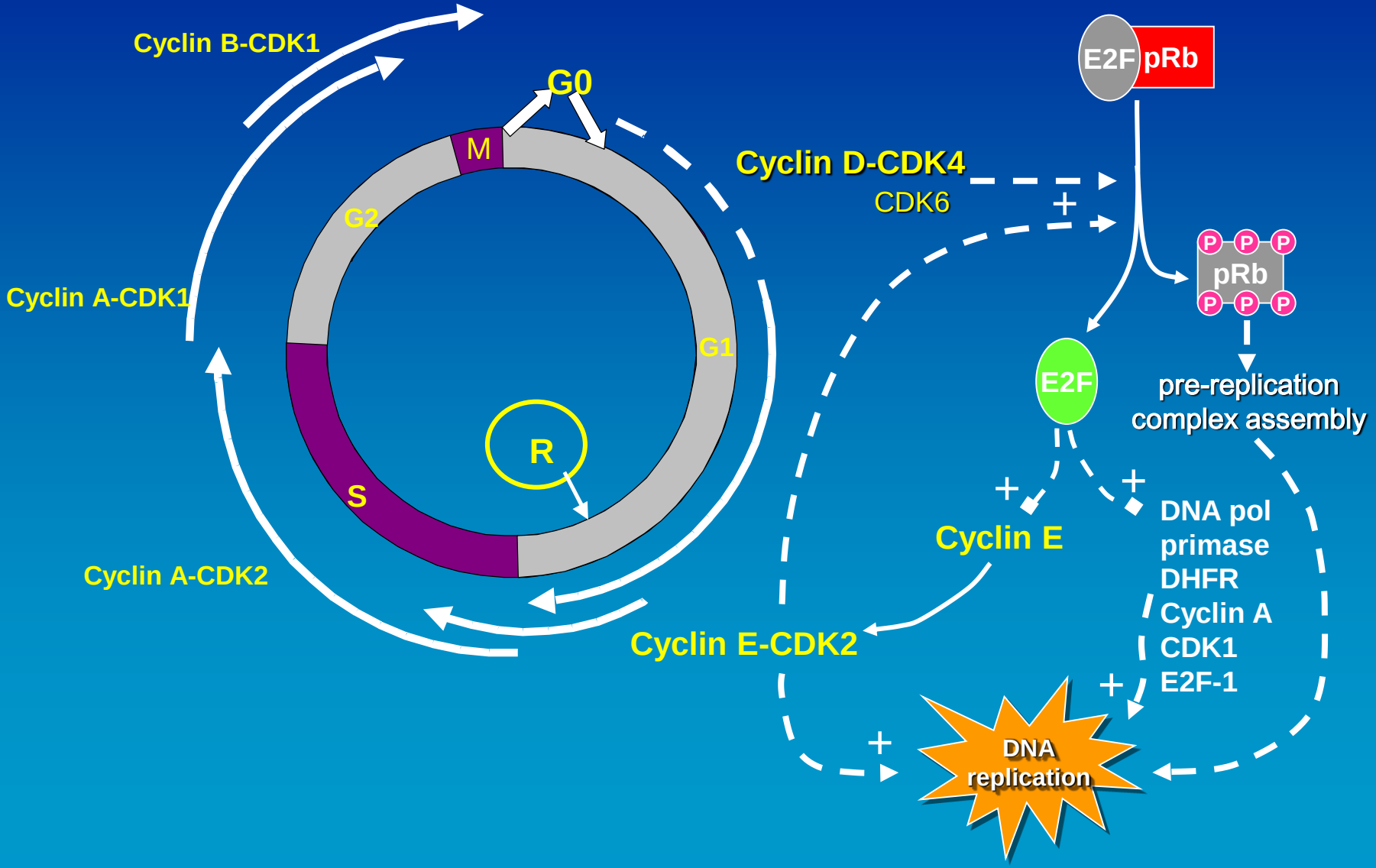
Revisiting post-translational regulation of cell cycle CDKs

télévie

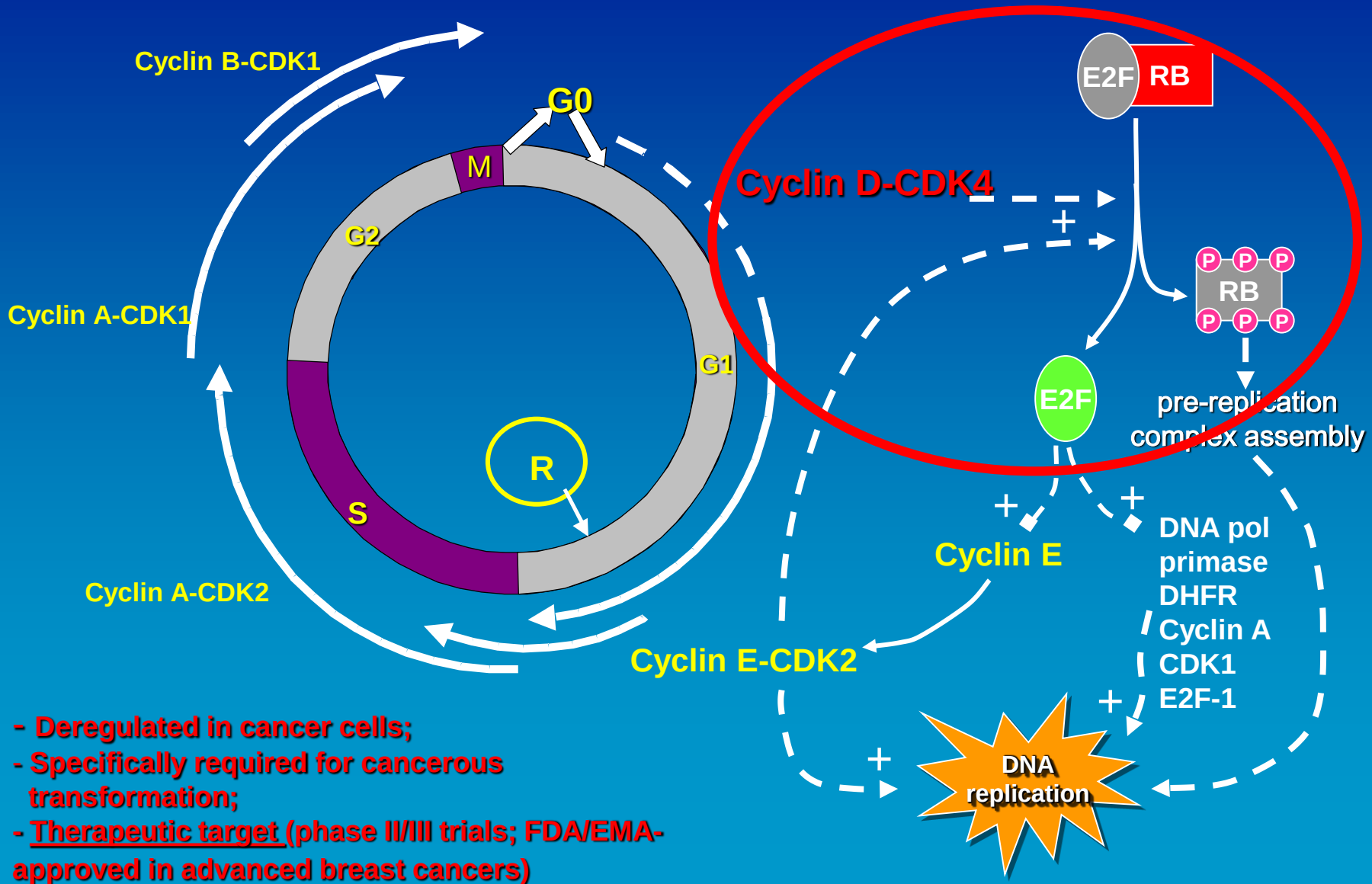
fnrs LA LIBERTÉ DE CHERCHER

Fondation contre le Cancer

Cyclin-CDK complexes during cell cycle

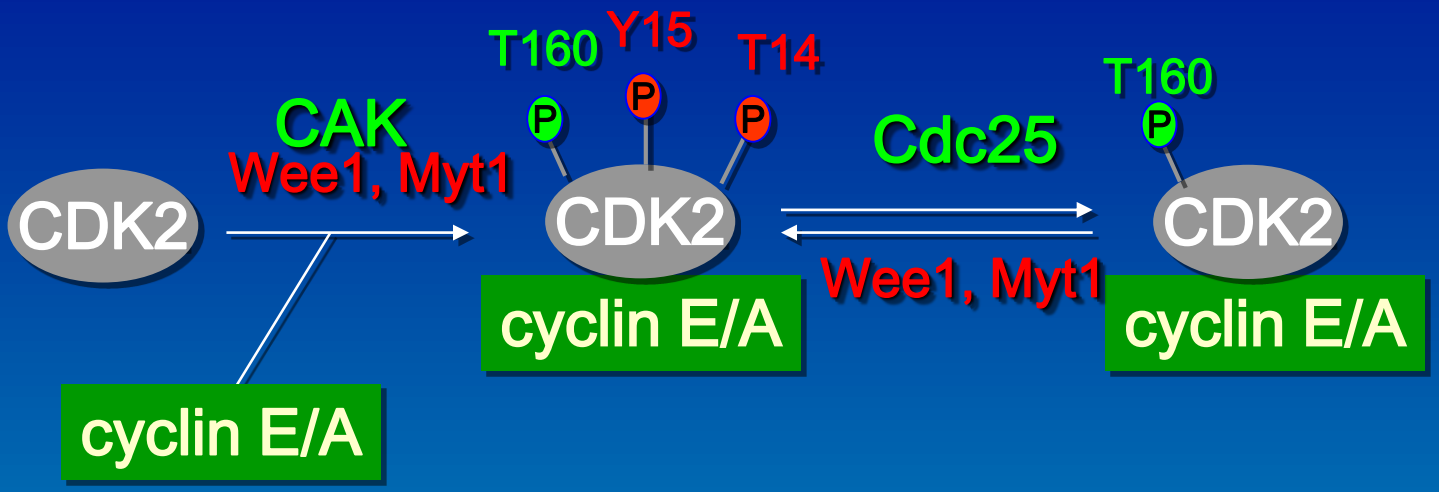


Cyclin-CDK complexes during cell cycle



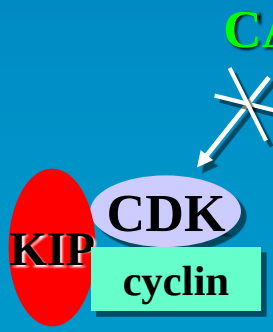
- Deregulated in cancer cells;
- Specifically required for cancerous transformation;
- Therapeutic target (phase II/III trials; FDA/EMA-approved in advanced breast cancers)

Regulations of CDKs



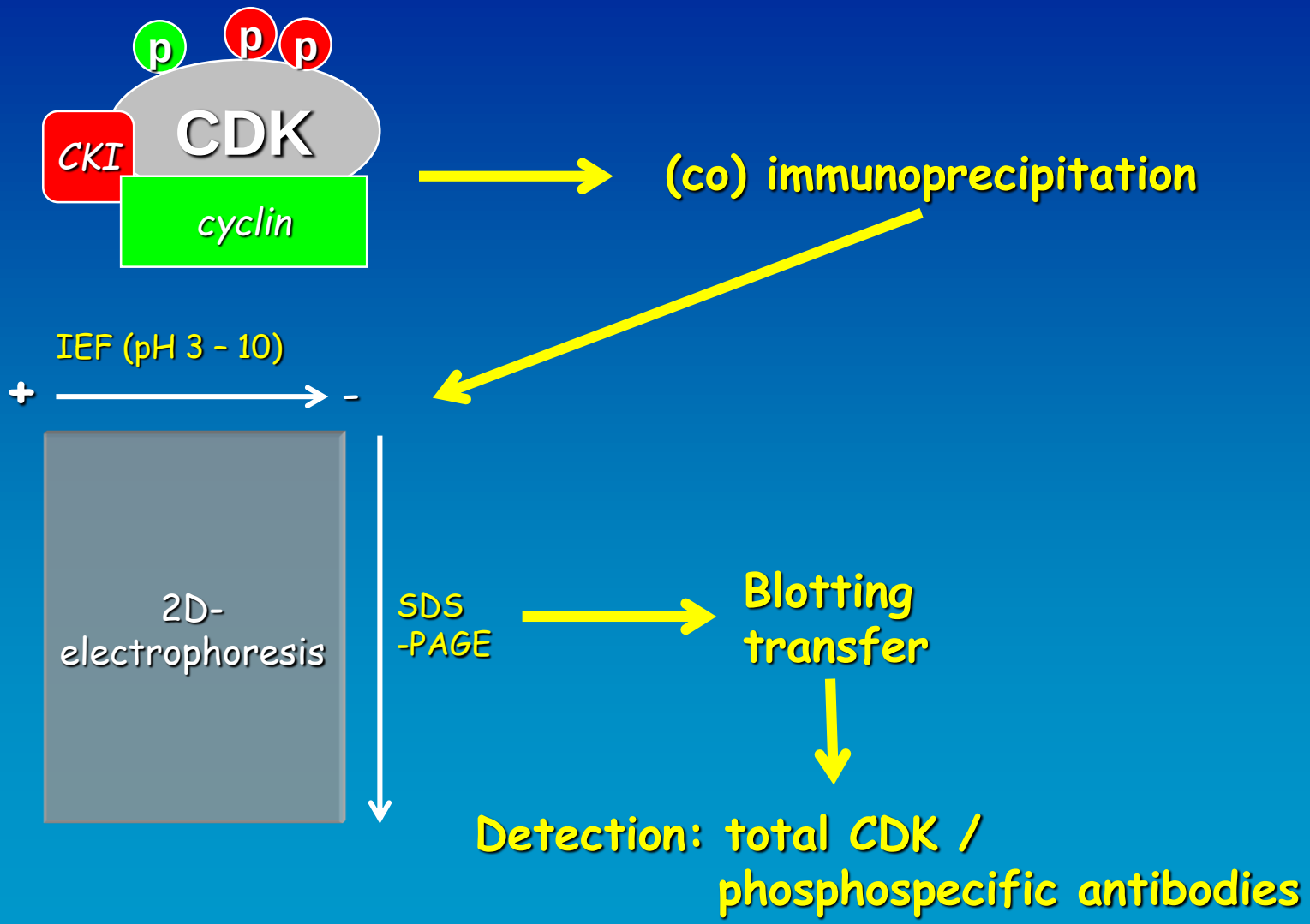
INK4 (p16, p15, p18, p19)

CIP/KIP (p21, p27, p57)



(+insertion in the catalytic cleft)

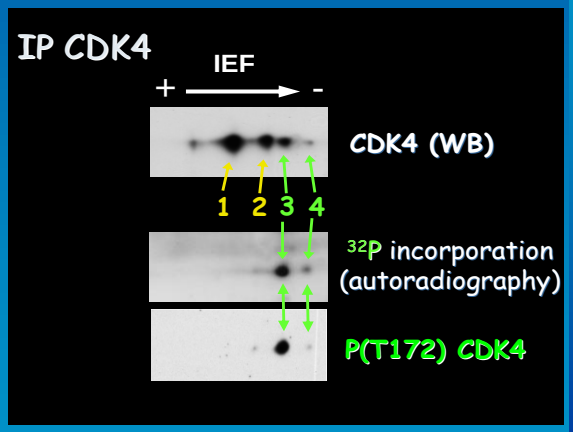
Interrelationship between the different levels of CDK regulation



Compairing post-translational modifications of CDKs

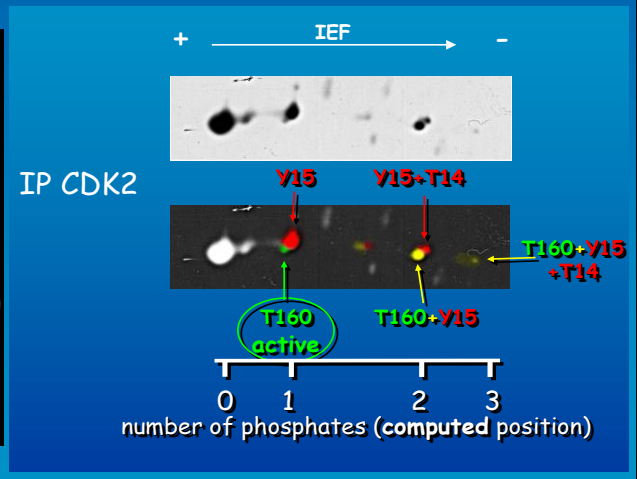
G1 S G2/M

CDK4 CDK2 CDK1

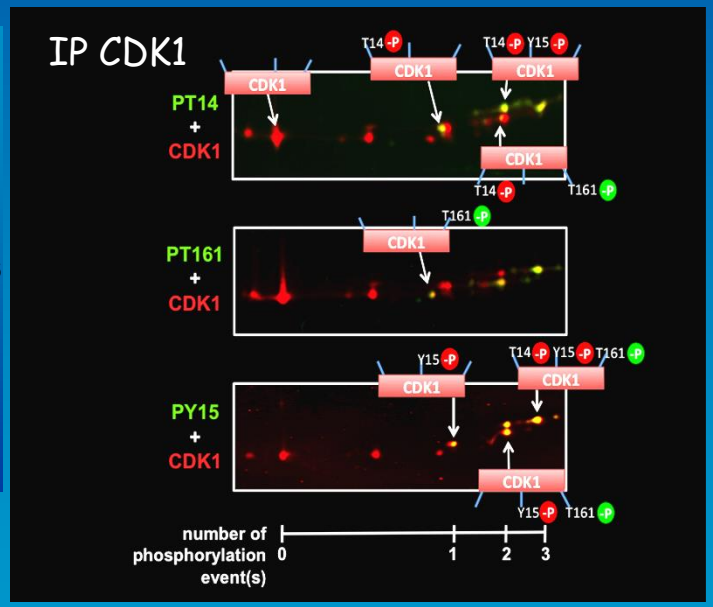


Coulonval, Bockstaele et al
Exp Cell Res 291: 135 (2003)

Bockstaele (Coulonval) et al
Mol Cell Biol 26: 5070 (2006)



Coulonval et al
J Biol Chem 278: 52052 (2003)



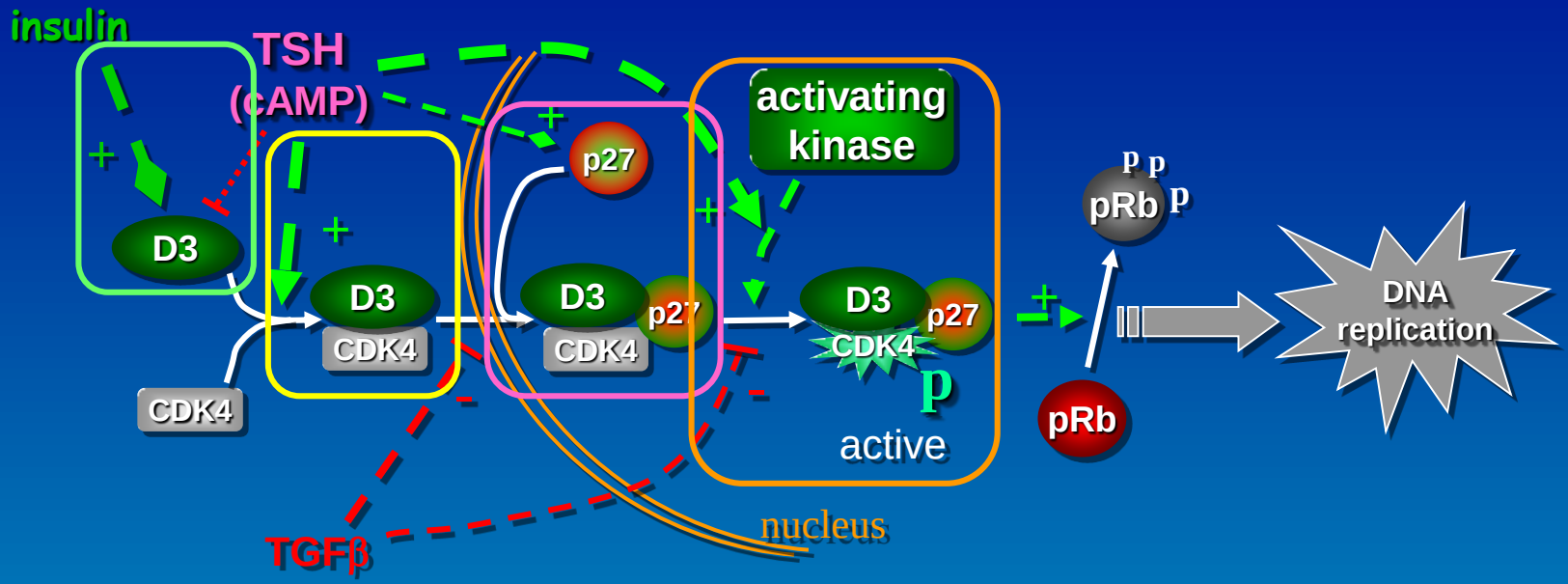
Coulonval et al
Mol Biol Cell 22, 3971 (2011)

CDK4

PATERNOT, S., COULONVAL, K., DUMONT, J.E., ROGER, P.P.
Cyclic AMP-dependent phosphorylation of cyclin D3-bound CDK4 determines the passage through the cell cycle restriction point in thyroid epithelial cells.
J. Biol. Chem. 278, 26533-26540 (2003).

According to current concepts, the cell cycle commitment after restriction (R) point passage requires the sustained stimulation by mitogens of the synthesis of labile d-type cyclins, which associate with cyclin-dependent kinase (CDK) 4/6 to phosphorylate pRb family proteins and sequester the CDK inhibitor p27kip1. In primary cultures of thyroid epithelial cells, the cAMP-dependent cell cycle induced by a sustained stimulation by thyrotropin or forskolin differs from growth factor mitogenic pathways, as cAMP does not upregulate D-type cyclins but increases p27 levels. Instead, cAMP induces the assembly of required cyclin D3-CDK4 complexes, which associate with nuclear p27. In this study, the arrest of forskolin stimulation rapidly slowed down the entry of thyrocytes into S phase and the phosphorylation of pRb family proteins. The pRb kinase activity, but not the formation, of the cyclin D3-CDK4-p27 complex was strongly reduced. Using two-dimensional gel electrophoresis, a phosphorylated form of CDK4 was separated. It appeared in response to forskolin and was bound to both cyclin D3 and p27, presumably reflecting the activating Thr-172 phosphorylation of CDK4. Upon forskolin withdrawal or after cycloheximide addition, this CDK4 phosphoform unexpectedly persisted in p27 complexes devoid of cyclin D3 but it disappeared from the more labile cyclin D3 complexes. These data demonstrate that the assembly of the cyclin D3-CDK4-p27 holoenzyme and the subsequent phosphorylation and activation of CDK4 depend on distinct cAMP actions. This provides a first example of a crucial regulation of CDK4 phosphorylation by a mitogenic cascade and a novel mechanism of cell cycle control at the R point.

cAMP-dependent mitogenesis in thyrocytes: a unique model of multi-step activation of CDK4



CDK4 activation is complex and distinctly regulated at each of its steps:

- Induction of cyclin D does not suffice for CDK4 activation.
- Mechanism of regulated cyclin D3-CDK4 assembly **??**
- Binding to p27 (or p21 in growth factor-stimulated cells) determines the nuclear translocation of cyclin D-CDK4.
- Activating phosphorylation of CDK4 is directly regulated.

(Depoortere JCB 1998; Van Keymeulen Oncogene 1999; Roger ECR 1999; Depoortere MBC 2000; Van Keymeulen Endocrinology 2001; Coulonval ECR 2003; Paternot JBC 2003; Paternot Cell Cycle 2006; Bockstaele MCB 2006; Paternot Mol Endo 2006)

CDK4/CDK6

BOCKSTAELE, L., KOOKEN, H., LIBERT, F., PATERNOT, S., DUMONT, J.E., de LAUNOIT, Y., ROGER, P.P., COULONVAL, K.
Thr172-phosphorylation of CDK4: its relationship with cyclins and CDK “inhibitors”.
Mol. Cell Biol. 26, 5070-5085 (2006).

Cyclin-dependent kinase 4 (CDK4) is a master integrator of mitogenic and antimitogenic extracellular signals. It is also crucial for many oncogenic transformation processes. Various molecular features of CDK4 activation remain poorly known or debated, including the regulation of its association with D-type cyclins, its activating Thr172 phosphorylation, and the roles of Cip/Kip CDK "inhibitors" in these processes. Thr172 phosphorylation of CDK4 was reinvestigated using two-dimensional gel electrophoresis in various experimental systems, including human fibroblasts, canine thyroid epithelial cells stimulated by thyrotropin, and transfected mammalian and insect cells. Thr172 phosphorylation of CDK4 depended on prior D-type cyclin binding, but Thr172 phosphorylation was also found in p16-bound CDK4. Opposite effects of p27 on cyclin D3-CDK4 activity observed in different systems depended on its stoichiometry in this complex. Thr172-phosphorylated CDK4 was enriched in complexes containing p21 or p27, even at inhibitory levels of p27 that precluded CDK4 activity. Deletion of the p27 nuclear localization signal sequence relocalized cyclin D3-CDK4 in the cytoplasm but did not affect CDK4 phosphorylation. Within cyclin D3 complexes, T-loop phosphorylation of CDK4, but not of CDK6, was directly regulated, identifying it as a determining target for cell cycle control by extracellular factors. Collectively, these unexpected observations indicate that CDK4-activating kinase(s) should be reconsidered.

CDK4/CDK6

BOCKSTAELE, L., BISTEAU, X., PATERNOT, S., ROGER, P.P.
Differential regulation of CDK4 and CDK6, evidence that CDK4 might not be activated by CDK7, and design of a CDK6 activating mutation.
Mol. Cell. Biol., 29,4188-4200 (2009).

The homologous cyclin-dependent kinases (CDK) CDK4 and CDK6 integrate mitogenic and oncogenic signaling cascades with the cell cycle. Their activation requires binding to a D-type cyclin and then T-loop phosphorylation at Thr172 and Thr177 (respectively) by the only CDK-activating kinase identified in animal cells, cyclin H-CDK7. At odds with the existing data showing the constitutive activity of CDK7, we have recently identified the Thr172 phosphorylation of cyclin D-bound CDK4 as a crucial cell cycle regulatory target. Here we show that Thr172 phosphorylation of CDK4 is conditioned by its unique proline 173 residue. In contrast to CDK4, CDK6 does not contain such a proline and, unexpectedly, remained poorly phosphorylated and active in a variety of cells. Mutations of proline 173 did not adversely affect CDK4 activation by CDK7, but in cells they abolished CDK4 Thr172 phosphorylation and activity. Conversely, substituting a proline for the corresponding residue of CDK6 enforced its complete, apparently cyclin-independent Thr177 phosphorylation and dramatically increased its activity. These results lead us to propose that CDK4 might not be phosphorylated by CDK7 in intact cells but is more likely phosphorylated by another, presumably proline-directed kinase(s). Moreover, they provide a new model of a potentially oncogenic activating mutation of a CDK.

CDK2

COULONVAL, K., BOCKSTAELE, L., PATERNOT, S., ROGER, P.P.
Phosphorylations of cyclin-dependent kinase 2 revisited using two-dimensional gel electrophoresis.
J. Biol. Chem. 278, 52052-52060 (2003).

To control the G1/S transition and the progression through the S phase, the activation of the cyclin-dependent kinase (CDK) 2 involves the binding of cyclin E then cyclin A, the activating Thr-160 phosphorylation within the T-loop by CDK-activating kinase (CAK), inhibitory phosphorylations within the ATP binding region at Tyr-15 and Thr-14, dephosphorylation of these sites by cdc25A, and release from Cip/Kip family (p27kip1 and p21cip1) CDK inhibitors. To re-assess the precise relationship between the different phosphorylations of CDK2, and the influence of cyclins and CDK inhibitors upon them, we introduce here the use of the high resolution power of two-dimensional gel electrophoresis, combined to Tyr-15- or Thr-160-phosphospecific antibodies. The relative proportions of the potentially active forms of CDK2 (phosphorylated at Thr-160 but not Tyr-15) and inactive forms (non-phosphorylated, phosphorylated only at Tyr-15, or at both Tyr-15 and Thr-160), and their respective association with cyclin E, cyclin A, p21, and p27, were demonstrated during the mitogenic stimulation of normal human fibroblasts. Novel observations modify the current model of the sequential CDK2 activation process: (i) Tyr-15 phosphorylation induced by serum was not restricted to cyclin-bound CDK2; (ii) Thr-160 phosphorylation engaged the entirety of Tyr-15-phosphorylated CDK2 associated not only with a cyclin but also with p27 and p21, suggesting that Cip/Kip proteins do not prevent CDK2 activity by impairing its phosphorylation by CAK; (iii) the potentially active CDK2 phosphorylated at Thr-160 but not Tyr-15 represented a tiny fraction of total CDK2 and a minor fraction of cyclin A-bound CDK2, underscoring the rate-limiting role of Tyr-15 dephosphorylation by cdc25A.

CDK1

COULONVAL, K., KOOKEN, H., ROGER, P.P.
Coupling of T161 and T14 phosphorylations protects cyclin B-CDK1 from premature activation.
Mol. Biol. Cell, 22, 3971-3985 (2011).

Mitosis is triggered by the abrupt dephosphorylation of inhibitory Tyr15 and Thr14 residues of cyclin B1-bound cyclin-dependent kinase (CDK)1 that is also phosphorylated at Thr161 in its activation loop. The sequence of events leading to the accumulation of fully phosphorylated cyclin B1-CDK1 complexes remains unclear. 2D-gel electrophoresis allowed us to discriminate whether Thr14, Tyr15 and Thr161 phosphorylations occur on same CDK1 molecules and to characterize the physiological occurrence of their seven phosphorylation combinations. Intriguingly, in cyclin B1-CDK1, the activating Thr161 phosphorylation never occurred without the Thr14 phosphorylation. This strict association could not be uncoupled by a substantial reduction of Thr14 phosphorylation in response to Myt1 knockdown, suggesting some causal relationship. However, Thr14 phosphorylation was not directly required for Thr161 phosphorylation, because Myt1 knockdown did uncouple them when cyclin B1-CDK1 complexes were prevented to accumulate in cytoplasm by leptomycin B. The coupling mechanism thus depended on unperturbed cyclin B1-CDK1 traffic. The unsuspected observation that the activating phosphorylation of cyclin B1-CDK1 was tightly coupled to its Thr14 phosphorylation, but not Tyr15 phosphorylation, suggests a mechanism protecting from premature activation by constitutively active CDK-activating kinase. This explained opposite impacts of reduced expression of Myt1 and Wee1 with only the latter inducing catastrophic mitoses.

Restriction point passage (1)
(CDK4/CDK2/CDK7/p21)

BISTEAU, X., PATERNOT, S., COLLEONI, B., COULONVAL, K., ECKER, K., DE GROOTE, P., DECLERCQ, W., HENGST, L., ROGER, P.P.
CDK4 T172-phosphorylation is central in a CDK7-dependent bidirectional CDK4/CDK2 interplay mediated by p21 phosphorylation at the restriction point.
PLoS Genetics, , 9(5) e1003546 (2013).

Cell cycle progression, including genome duplication, is orchestrated by cyclin-dependent kinases (CDKs). CDK activation depends on phosphorylation of their T-loop by a CDK-activating kinase (CAK). In animals, the only known CAK for CDK2 and CDK1 is cyclin H-CDK7, which is constitutively active. Therefore, the critical activation step is dephosphorylation of inhibitory sites by Cdc25 phosphatases rather than unrestricted T-loop phosphorylation. Homologous CDK4 and CDK6 bound to cyclins D are master integrators of mitogenic/oncogenic signaling cascades by initiating the inactivation of the central oncosuppressor pRb and cell cycle commitment at the restriction point. Unlike the situation in CDK1 and CDK2 cyclin complexes, and in contrast to the weak but constitutive Thr177 phosphorylation of CDK6, we have identified the T-loop phosphorylation at Thr172 as the highly regulated step determining CDK4 activity. Whether both CDK4 and CDK6 phosphorylations are catalyzed by CDK7 remains unclear. To answer this question, we took a chemical-genetics approach by using analogue-sensitive CDK7(as/as) mutant HCT116 cells, in which CDK7 can be specifically inhibited by bulky adenine analogs. Intriguingly, CDK7 inhibition prevented activating phosphorylations of CDK4/6, but for CDK4 this was at least partly dependent on its binding to p21cip1. In response to CDK7 inhibition, p21-binding to CDK4 increased concomitantly with disappearance of the most abundant phosphorylation of p21, which we localized at Ser130 and found to be catalyzed by both CDK4 and CDK2. The S130A mutation of p21 prevented the activating CDK4 phosphorylation, and inhibition of CDK4/6 and CDK2 impaired phosphorylations of both p21 and p21-bound CDK4. Therefore, specific CDK7 inhibition revealed the following: a crucial but partly indirect CDK7 involvement in phosphorylation/activation of CDK4 and CDK6; existence of CDK4-activating kinase(s) other than CDK7; novel CDK7-dependent positive feedbacks mediated by p21 phosphorylation by CDK4 and CDK2 to sustain CDK4 activation, pRb inactivation, and restriction point passage.

Restriction point passage (2)

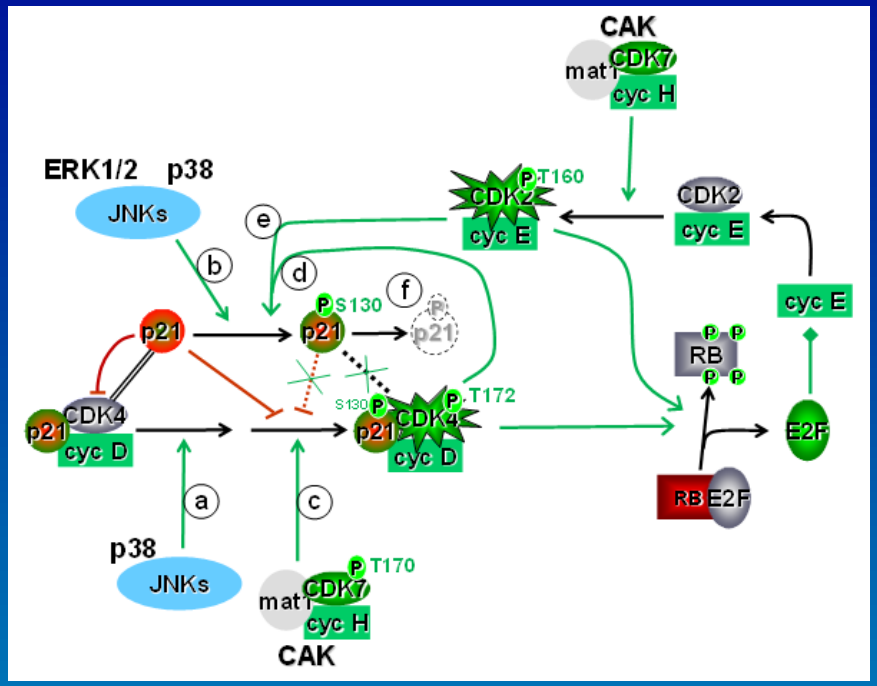
(CDK4/JNKs/p21)

COLLEONI, B., PATERNOT, S., PITA, J.M., BISTEAU, X., COULONVAL, K., DAVIS, R.J., RASPÉ, E. ROGER. P.P.

JNKs function as CDK4-activating kinases by phosphorylating CDK4 and p21.

Oncogene, 36:4349-4361 (2017).

Cyclin D-CDK4/6 are the first cyclin-dependent kinase (CDK) complexes to be activated by mitogenic/oncogenic pathways. They have a central role in the cell multiplication decision and in its deregulation in cancer cells. We identified Thr172 phosphorylation of CDK4 rather than cyclin D accumulation as the distinctly regulated step determining CDK4 activation. This finding challenges the view that the only identified metazoan CDK-activating kinase, cyclin H-CDK7-Mat1 (CAK), which is constitutively active, is responsible for the activating phosphorylation of all cell cycle CDKs. We previously showed that Thr172 phosphorylation of CDK4 is conditioned by an adjacent proline (P173), which is not present in CDK6 and CDK1/2. Although CDK7 activity was recently shown to be required for CDK4 activation, we proposed that proline-directed kinases might specifically initiate the activation of CDK4. Here, we report that JNKs, but not ERK1/2 or CAK, can be direct CDK4-activating kinases for cyclin D-CDK4 complexes that are inactivated by p21-mediated stabilization. JNKs and ERK1/2 also phosphorylated p21 at Ser130 and Thr57, which might facilitate CDK7-dependent activation of p21-bound CDK4, however, mutation of these sites did not impair the phosphorylation of CDK4 by JNKs. In two selected tumor cells, two different JNK inhibitors inhibited the phosphorylation and activation of cyclin D1-CDK4-p21 but not the activation of cyclin D3-CDK4 that is mainly associated to p27. Specific inhibition by chemical genetics in MEFs confirmed the involvement of JNK2 in cyclin D1-CDK4 activation. Therefore, JNKs could be activating kinases for cyclin D1-CDK4 bound to p21, by independently phosphorylating both CDK4 and p21.



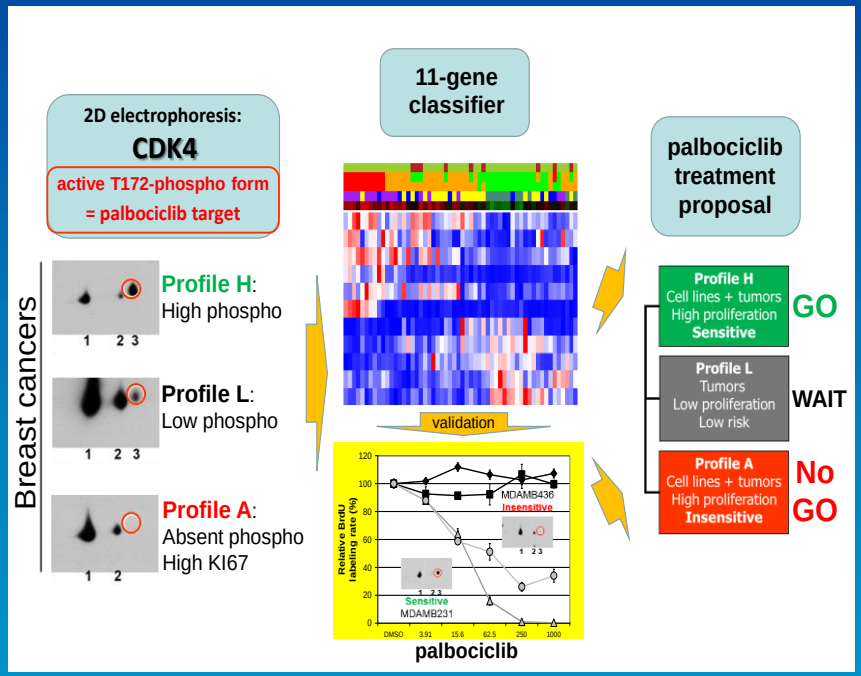
Central involvement of Thr172-phosphorylation of CDK4 at the cell-cycle decision R point (from Bisteau et al PLoS Genetics 2013 and Colleoni et al Oncogene 2017).

This model includes the evidence that p21 stabilizes cyclin D-CDK4 complexes but, at a higher stoichiometry of p21 binding, inhibits their activity. The model also considers that the Thr172 phosphorylation of CDK4 is particularly unstable, requiring a sustained activity of CDK4-activating kinases.

Whereas CAK/CDK7 cannot phosphorylate CDK4 bound to unphosphorylated p21, JNKs and possibly p38 do it and thus are able to initiate the activation of p21-stabilized cyclin D1-CDK4 complexes by direct phosphorylation of CDK4 (**a**). JNKs, p38 and ERK1/2 also phosphorylate p21 on Ser130 (**b**), which could facilitate the CDK7-dependent phosphorylation of CDK4 (**c**). The activation of CDK4 is then amplified and maintained by Ser130 phosphorylation of p21 by other active complexes of CDK4 (**d**) and CDK2 (**e**). As demonstrated by others, Ser130 phosphorylation of p21 subsequently leads to increased degradation of p21 (**f**), which facilitates CDK2 activation. Cooperation between JNKs and other signal transduction kinases (**a,b**), CAK (**c**) and CDKs (**d,e**) would thus initiate, amplify and maintain the activation of CDK4 to permit the passage through the R point. Green/Red colors indicate a final positive/negative influence on R point passage.

CDK4 phosphorylation in human cancers

ERIC RASPÉ, KATIA COULONVAL, JAIME M. PITA, SABINE PATERNOT, FRANÇOISE ROTHÉ, LAURE TWYFFELS, SYLVAIN BROHÉE, LIGIA CRACIUN, DENIS LARSIMONT, VÉRONIQUE KRUYS, FLAVIENNE SANDRAS, ISABELLE SALMON, STEVEN VAN LAERE, MARTINE PICCART, MICHAIL IGNATIADIS, CHRISTOS SOTIRIOU, PIERRE P. ROGER.
CDK4 phosphorylation status and corresponding gene expression profile predict sensitivity to Palbociclib.
EMBO Mol. Med. 9:1052-1066 (2017).



Biomarkers able to predict breast cancer sensitivity to the FDA/EMA-approved CDK4/6 inhibitor palbociclib are still lacking.

- CDK4 T172-phosphorylation was for the first time investigated in human cancer using 2D-gel electrophoresis. Unexpectedly, it is absent in a subset of highly proliferating cancers and cancer cell lines. Cell lines lacking CDK4 phosphorylation are completely insensitive to CDK4/6 inhibitors.
- In frozen breast cancers samples, three distinct CDK4 modification profiles are observed using 2D-gel electrophoresis: profile H, preponderant presence of T172-phosphorylated CDK4 (most Luminal B and HER2-positive cancers); profile L, minor presence of phosphorylated CDK4 (90% of lower risk luminal A tumors); profile A, no phosphorylated CDK4 despite high proliferation index (60% of triple-negative cancers).
- An 11-gene classifier that correctly predicts the three CDK4 modification profiles was generated and optimized using these breast tumors.
- In 4000 breast cancer samples, the CDK4 modification profiles as predicted by the 11-gene classifier are associated with subtype, grade, relapse status and risk profile.
- The 11-gene classifier correctly predicts the observed presence or absence of CDK4 phosphorylation in 24 out of 25 breast cancer cell lines and the observed or reported sensitivity to palbociclib in 50 out of 52 breast cancer cell lines.
- Once transposed to an assay (RT-qPCR, RNAseq,..) compatible with formalin-fixed breast tumor samples, this classifier should predict the potential sensitivity or resistance to CDK4 inhibitors and the risk and grade of tumors.

conclusions

The visualization of the phosphorylation profiles, as resolved using 2D-gel electrophoresis, of CDK4, CDK6, CDK2 and CDK1 has illustrated the different logics of their regulations. Cyclin-CDK1/CDK2 accumulate as a reservoir of inactive complexes containing both activating (Thr161/160) and inhibitory (Tyr15 and Thr14) phosphorylations until their activation by dephosphorylation of the inhibitory residues by Cdc25 phosphatases. On the other hand, CDK4/CDK6 activity could not be generally restricted by substantial Tyr-phosphorylation, but by lack of activating phosphorylation, at variance with the corresponding phosphorylations of CDK2/1 by CAK which are not thought to be rate-limiting.

Only Thr172-phosphorylation of CDK4 is distinctly regulated. We have identified this step as an ultimate regulatory target determining CDK4 activity, pRb phosphorylation and the passage through the G1 phase restriction point. Therefore, major efforts should be devoted to the understanding of mechanisms responsible for this regulation of CDK4 phosphorylation, which remains enigmatic.

We observed that a subset of highly proliferating cancers and cancer cell lines do not harbor the Thr172-phosphorylated active CDK4. These cell lines are completely insensitive to CDK4 inhibitory drugs. Our work in breast cancers provided the proof-of-principle that the relative level of phosphorylated CDK4 and an associated gene expression signature can indeed predict sensitivity or insensitivity to CDK4 inhibitors and their potential clinical benefit.