Phosphorylation-induced unfolding regulates p19\textsuperscript{INK4d} during the human cell cycle

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Cell cycle progression is tightly regulated by cyclin-dependent kinases (CDKs). The ankyrin-repeat protein p19\textsuperscript{INK4d} functions as a key regulator of G1/S transition; however, its molecular mode of action is unknown. Here, we combine cell and structural biology methods to unravel the mechanism by which p19\textsuperscript{INK4d} controls cell cycle progression. We delineate how the stepwise phosphorylation of p19\textsuperscript{INK4d} Ser66 and Ser76 by cell cycle-independent (p38) and -dependent protein kinases (CDK1), respectively, leads to local unfolding of the three N-terminal ankyrin repeats of p19\textsuperscript{INK4d}. This dissociates the CDK6–p19\textsuperscript{INK4d} inhibitory complex and, thereby, activates CDK6. CDK6 triggers entry into S-phase, whereas p19\textsuperscript{INK4d} is ubiquitinated and degraded. Our findings reveal how signaling-dependent p19\textsuperscript{INK4d} unfolding contributes to the irreversibility of G1/S transition.

Results
p19\textsuperscript{INK4d} Phosphorylation at Ser66. p19\textsuperscript{INK4d} is an ankyrin-repeat (AR) protein that harbors five evenly spaced helix-turn-helix motifs: AR1 amino acids 9–29, AR2 amino acids 54–62, AR3 amino acids 77–95, AR4 amino acids 110–128, and AR5 amino acids 142–159 (Fig. 1). AR1 and AR2 mediate inhibitory CDK4/6 binding (17). Phosphorylation of Ser66 and Ser76 within the linker region

Significance
Cell cycle progression is tightly controlled in healthy organisms and often perturbed in human diseases, including, most prominently, many forms of cancers. Cyclin-dependent protein kinases and their inhibitors, such as p19\textsuperscript{INK4d}, regulate the different stages of the cell cycle. Here, we demonstrate how sequential phosphorylation of p19\textsuperscript{INK4d} at two sites first destabilizes and then unfolds the N-terminal half of the protein, which dissociates its cyclin-dependent protein kinase-inhibitory complex and primes p19\textsuperscript{INK4d} for cellular degradation. Our results define a structural mechanism by which phosphorylation-induced protein unfolding controls a key step in cell cycle progression.

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Fig. 1. Schematic showing the function of p19INK4d. (Left) Structure of the CDK6-p19INK4d complex (PDB ID code 1BLX). ARs of p19INK4d are presented as AR1–AR5, regulatory sites Ser66, Ser76, and Lys62 are shown red and the RxL motif in magenta. (Right) The signaling pathways regulated by the INK family members. Red blocks (▲) highlight the cell cycle arrest points used in this study.

connecting AR2 and AR3 is thought to regulate p19INK4d activity, but kinases that modify these sites have not been identified. We expressed and purified full-length human p19INK4d (residues 1–166) from Escherichia coli and added the recombinant protein to lysates that we prepared from various cultured cell lines (e.g., HeLa, U2OS, HEK-293 grown asynchronously, and from Drosophila melanogaster embryos). 32P-incorporation and autoradiography exposure confirmed phosphorylation by endogenous enzymes (Fig. S1A). To identify respective p19INK4d phosphorylation sites, we similarly prepared 15N-isotope–labeled protein that we added to corresponding lysates for 3 h, before we recorded 2D 1H–15N HSQC (heteronuclear single-quantum coherence) and 1D 31P NMR spectra on the resulting lysate mixtures. The NMR resonance assignment of p19INK4d has previously been reported (18) (Fig. S2F). Because NMR chemical shifts are sensitive indicators of residue-specific chemical environments, phosphorylation-induced chemical-shift changes conveniently identify the respective modification sites. One added benefit of detecting protein phosphorylation by NMR is that conformational rearrangements occurring in response to such modifications result in additional chemical-shift changes that are readily interpretable in structural terms as well. Incubation of p19INK4d in each of the four lysates resulted in the phosphorylation of p19INK4d, manifested by pronounced chemical-shift changes of Ser66 resonance signals (Fig. 2A, shown for HeLa cells lysate, and Fig. S1 B–D). Although residues close to Ser66 also displayed slight alterations in their cross-peak positions (red in Fig. 2A, Lower), other p19INK4d serines, including Ser76, were unaffected. These results indicated that all five ARs remained structurally intact upon Ser66 phosphorylation. In the 1D 31P NMR spectrum, phospho-Ser66 gave rise to a characteristic new resonance at −1 ppm, which was clearly offset from the bulk phosphate-buffer signal at 2 ppm (Fig. 2A, Inset). We confirmed the presence of a single protein-phosphate moiety by MALDI-TOF mass spectrometry (Fig. S2 A and B). To verify the identity of Ser66 as the primary phosphorylation site, we repeated these experiments with alanine-substituted, mutant p19INK4d (e.g., S66A). We detected neither 32P incorporation nor the appearance of the 31P-NMR resonance signal at −1 ppm. These data confirmed that endogenous kinases in lysates prepared from asynchronously growing mammalian cells, or from Drosophila embryos, phosphorylated p19INK4d at Ser66.

Stepwise Phosphorylation of Ser66 and Ser76 Induces Local Unfolding. Having preformed the previous set of experiments in lysates of asynchronously growing cells, we were unable to delineate cell cycle-specific contributions to the observed phosphorylation reaction. To our surprise, we also did not detect endogenous p19INK4d in our lysates (Fig. S2C, lane 1). Because the G1/S transition is tightly controlled by CDK4/6 and INK4, we speculated that the kinase targeting p19INK4d might only be active in a fraction of our collected cells, specifically those in S-phase. To test this hypothesis, we subjected HeLa cells to a double thymidine block to arrest them in S-phase (19). We detected abundant amounts of endogenous p19INK4d in these lysates (Fig. S2C, lane 2). Upon thymidine removal and G2 release, p19INK4d levels dropped below the detection limit within 6 h (Fig. S2C, lanes 3 and 4). These findings reaffirmed earlier studies with proliferating cells (20) that

Fig. 2. NMR-detected cell cycle-dependent phosphorylation and local unfolding of p19INK4d. (Upper) Overlays of 2D 1H–15N HSQCs of p19INK4d before (black) and after (red) incubation with (A) asynchronous cell lysate, (B) S-phase HeLa cell lysate, or (C) after incubation of the doubly phosphorylated protein by alkaline phosphatase. (Lower) Spectral changes upon incubation mapped on the p19INK4d structure. The macroscopic helix dipole moments of helices 4 and 6 are depicted by black arrows in A. Mapping of cross-peak on the p19INK4d structure in A and B: red, missing cross-peak; blue, no change; and gray, slight chemical shift or cannot say. Insets show the 1D 31P spectra identifying (A) one (−1 ppm), (B) two (−1 ppm and 4 ppm), and (C) no protein-bound phosphate. The sharp signal at 2 ppm originates from the phosphate buffer (asterisk).
cellular p19INK4d concentrations were highest in S-phase and virtually absent in G1, thus consolidating the notion of a genuine oscillatory behavior.

Next, we sought to investigate p19INK4d modifications in lysates of S-phase–arrested cells. 3P incorporation and autoradiography exposure confirmed p19INK4d phosphorylation (Fig. S2D). The 1D 31P NMR experiments revealed the previously observed phosphor-resonance at ~1 ppm, plus a new signal at 4 ppm, suggesting the presence of a second modified residue under these conditions (Fig. 2B, Inset). MALDI-TOF mass spectrometry indeed confirmed phosphorylation of p19INK4d at two sites (Fig. S2 A and E). To also interrogate possible structural changes of doubly phosphorylated p19INK4d, we recorded 2D 1H–15N HSQC spectra on exogenously added, isotope-labeled protein in lysates of S-phase–arrested HeLa cells (Fig. 2B). Surprisingly, we found all p19INK4d residues of AR1, AR2, and AR3 at new peak positions, narrowly dispersed around 8 ppm along the proton dimension (Fig. 2B and Fig. S2F). Such NMR features are highly characteristic of unfolded protein states. In contrast, residues within AR4 and AR5 exhibited no chemical-shift changes, indicating that these ARs remained folded upon dual p19INK4d phosphorylation. Having established that Ser66 likely constituted one of the modified p19INK4d residues, we set out to identify the second phosphorylation site. To this end, we mutated Ser66 to alanine (S76A) and repeated the S-phase lysate NMR experiment. The 2D 1H–15N HSQC spectra showed that AR1–3 residues, along with those of AR4 and AR5, maintained their original cross-peak positions, hinting toward the structural preservation of all ARs (Fig. 3F and Fig. S2G). Moreover, we clearly detected Ser66 phosphorylation in S76A p19INK4d, suggesting that primary phosphorylation of this site was not affected in the mutant background. Taken together, these results led us to conclude that Ser76 constituted the second p19INK4d modification site in S-phase–arrested cell extracts, that phosphorylation of Ser66 was independent of Ser76 modification, and that double phosphorylation of Ser66 and Ser76 resulted in local unfolding of ankyrin repeats AR1–3.

To confirm that the observed conformational changes were indeed due to phosphorylation, and to test whether local AR1–3 unfolding was reversible, we added a nonspecific alcalinal kinase phosphatase to Ser66-, Ser76-modified p19INK4d. De-phosphorylation resulted in the disappearance of phosphoryl-protein resonances in 1D 31P NMR spectra (Fig. 2C, Inset) and recovered all p19INK4d resonances at their original cross-peak positions in 2D 1H–15N HSQC spectra (Figs. 2C and S3C). These experiments confirmed that (i) double phosphorylation caused unfolding and (ii) that unfolding was fully reversible. They further excluded contributions by other posttranslational protein modifications, which may have remained undetected in lysate NMR experiments.

**p38 and CDK1 Phosphorylate Ser66 and Ser76, Respectively.** We observed Ser66 phosphorylation in lysates of asynchronously growing cells, as well as in lysates of cells arrested in S-phase, which suggested that this modification is mediated by a kinase that is not stringently cell cycle-regulated. In contrast, we only detected Ser76 phosphorylation in S-phase lysates, arguing for an enzyme with cell cycle-specific activity. To identify the respective kinases that modified Ser66 and Ser76 of p19INK4d, we treated S-phase lysates with different kinase inhibitors. Compounds targeting CDK1, CDK4, or protein kinase A (PKA) did not affect Ser66 phosphorylation (Fig. S3 A–C). In contrast, selective inhibition of the constitutively expressed kinase p38 (21–23) abolished its modification (Fig. 3E and Fig. S3D). We further confirmed p38 selectivity and specificity in reconstituted kinase reactions with isolated wild-type and S66A-mutant p19INK4d (Fig. S3E). Furthermore, in an autoradiography experiment, p19INK4d S66A remained silent (Fig. S3E). p38 is a stress-activated MAP kinase protein family and we found Ser66 to get phosphorylated under various conditions not related to the cell cycle. Therefore, p38 may not be the only kinase that phosphorylates Ser66.

Following a similar approach, we found that different CDK1 inhibitors abolished Ser76 phosphorylation, whereas modification of Ser66 was unperturbed (Fig. 3D and Fig. S4, A and B). In earlier studies, CDK2 was found to phosphorylate Ser76 in response to DNA damage (24). The herein used CDK1 inhibitor IV inhibits CDK2 to some extent at elevated concentrations (25), but the specific CDK1 inhibitor III does not (26). Sole inhibition of CDK2 by inhibitor I (Fig. S4C) and, as expected, treating S-phase cell lysates with CDK4 or PKA inhibitors, showed no effects (Fig. S4, D and E). Together, these results suggested that, predominantly, CDK1 mediated Ser76 phosphorylation. Accordingly, we found that CDK1-cyclin B reconstituted kinase reactions modified wild-type but not S76A-mutant p19INK4d (Fig. S5A). CDK1, in complex with cyclins A, B, D, or E, functions as a key cell cycle regulator (27-29) and p19INK4d exhibits several features of a canonical CDK1 substrate (30). It harbors multiple serine or threonine residues followed by a proline (e.g., minimal Ser/Thr-Pro motifs, including Ser66, Ser76, and Thr141), and it contains a classic Arg–X–Leu CDK docking site, which all remain accessible when p19INK4d is bound to CDK4/6 (Fig. 1, Left). To further substantiate the role of CDK1 in Ser76 phosphorylation, we arrested HeLa cells in M-phase, in which CDK1/cyclin B activity is the highest, and prepared lysates to which we added 15N isotope-labeled p19INK4d. The 2D NMR experiments revealed local unfolding of AR1–3, manifested by spectral characteristic that were indistinguishable from results in S-phase–arrested cell extracts (Fig. S3 E–G). Indeed, NMR spectra of isotope-labeled p19INK4d in reconstituted mixtures of isolated p38α and CDK1/cyclin B showed identical features (Fig. S6). In summary, our combined results strongly suggested that p38 and CDK1 constitute the cellular kinases that phosphorylate Ser66 and Ser76 of p19INK4d, respectively.

**p19INK4d Phosphorylation Is a Two Step Process.** Having identified p38 and CDK1 as likely kinase candidates for cellular p19INK4d phosphorylation, we set out to gain mechanistic insights into the Ser66 and Ser76 modification process. Specifically, we asked whether Ser76 phosphorylation required Ser66 modification as a preceding event. We had found that phosphorylation of Ser76 did not occur in the S66A-mutant background (Fig. 3G)
or upon p38 inhibition in S-phase cell lysates (Fig. 3H and Fig. 7A and B), thus raising the possibility that Ser66, Ser76 phosphorylation may constitute a hierarchical, sequential process. Protein phosphorylation sites are generally located in accessible loop and linker regions of folded substrates (30). In p19\textsuperscript{INK4d}, Ser66 is surface-exposed, whereas Ser76 is part of the helical AR3 motif and rather occluded (Fig. 1, Left). As mentioned above, Ser66 phosphorylation led to several NMR chemical-shift changes of residues in its vicinity (Fig. 2A). We hypothesized that these reported on local destabilization of p19\textsuperscript{INK4d} residues surrounding the modification site, probably via repulsive electrostatic interactions of the Ser66 phosphate moiety and the negative charge of the net dipole moments at the C-terminal ends of helices 4 and 6 in AR2 and AR3, respectively (Fig. 2A, and arrows on the corresponding p19\textsuperscript{INK4d} structure). In agreement with this model, residues in these helices displayed the largest chemical-shift changes upon Ser66 phosphorylation (Fig. 2A). To assess the degree of helix destabilization in response to Ser66 modification, we performed proton-deuterium (H-D) backbone amide exchange experiments by NMR. AR3 and AR4 residues in wild-type p19\textsuperscript{INK4d} exhibited protection factors above 10\textsuperscript{3} (Fig. S7A, D-F), which reduced significantly upon phosphorylation (red in Fig. S7A-D). Surprisingly, even though p19\textsuperscript{INK4d} residues within AR1, -4, and -5 did not show chemical-shift changes when Ser66 was phosphorylated, we measured greater solvent-amide proton exchange at these “remote” structural elements, synonymous with lower thermodynamic stabilities in the presence of modified Ser66. Based on these findings, we hypothesized that extended structural destabilization may provide the necessary access for Ser76 phosphorylation by CDK1 (Fig. 1). Local unfolding of AR1–3 may then occur due to additional breaking of hydrogen bonds, such as the one between Ser76 O' and V69 O', for example.

**Phosphorylation Dissociates the CDK6–p19\textsuperscript{INK4d} Complex.** How does p19\textsuperscript{INK4d} phosphorylation correlate with its function as a cell cycle regulator? The crystal structure of the inhibitory CDK6–p19\textsuperscript{INK4d} complex reveals its molecular architecture (17) but it remained unclear how the assembly dissociates and whether p19\textsuperscript{INK4d} modifications affected this process. Therefore, we examined p19\textsuperscript{INK4d} phosphorylation in context of the assembled CDK6–p19\textsuperscript{INK4d} complex. In vitro pull-down assays showed that unmodified p19\textsuperscript{INK4d} specifically bound to GST–CDK6 (31), whereas doubly phosphorylated p19\textsuperscript{INK4d} did not (Fig. 4A, likely because a folded AR1–2 interface is required for CDK6 binding (Fig. 1, Left)). In a second experiment, we followed \textsuperscript{15}N-p19\textsuperscript{INK4d} binding to unlabeled CDK6 by NMR spectroscopy. As we expected from the crystal structure of the complex, p19\textsuperscript{INK4d} AR1 and AR2 residues exhibited pronounced chemical-shift changes upon addition of CDK6 (Fig. 4B and C). No chemical-shift changes were observed when we added CDK6 to doubly phosphorylated p19\textsuperscript{INK4d}, which confirmed the absence of binding and recapitulated our pull-down results. When we treated assembled CDK6–\textsuperscript{15}N-p19\textsuperscript{INK4d} with S-phase–arrested cell lysates, 2D NMR spectra revealed local unfolding of AR1–3, similar to the isolated protein upon Ser66 and Ser76 phosphorylation (Fig. 4D, Middle). Indeed, 1D \textsuperscript{31}P NMR spectra and radioisotope incorporation experiments confirmed the presence of both modifications (Fig. 4D, Bottom). We concluded that p19\textsuperscript{INK4d} double phosphorylation and subsequent local unfolding dissociated the p19\textsuperscript{INK4d}–CDK6 complex.

**Ubiquitination Requires p19\textsuperscript{INK4d} Unfolding.** Many proteins turn into E3 ubiquitin ligase substrates upon phosphorylation, causing them to be degraded by the ATP-dependent ubiquitin/proteasome system (32, 33). Here, we showed that p19\textsuperscript{INK4d} phosphorylation at Ser66 and Ser76 caused local unfolding of AR1–3, which, in turn, released the protein from CDK6. What was the

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**Fig. 4.** Functional implications of p19\textsuperscript{INK4d} phosphorylation. (A) Pull-down assay of p19\textsuperscript{INK4d} using GST–CDK6. The loading in a and b is: lane 1, input (GST–CDK6 beads + p19\textsuperscript{INK4d}); lane 2, supernatant; and lane 3, pellet of pull down. In a, a is doubly phosphorylated p19\textsuperscript{INK4d}, b is p19\textsuperscript{INK4d} as positive control. M, protein molecular mass marker. (B) CDK6–p19\textsuperscript{INK4d} complex formation. Overlaid 2D \textsuperscript{1}H–\textsuperscript{15}N HSQC spectra of \textsuperscript{15}N-labeled p19\textsuperscript{INK4d} before (black) and after addition of unlabeled CDK6 (red). Blue labels indicate residues of p19\textsuperscript{INK4d} that interact with CDK6 via their side chains, as revealed by the crystal structure of the complex (17), but which do not influence the backbone chemical shift of p19\textsuperscript{INK4d}. Red labels indicate the backbone chemical-shift changes upon complex formation both monitored by NMR and reported in the crystal structure. Green labels indicate additional chemical-shift changes observed only by NMR and not reported in the crystal structure. These residues are mapped on the structure of p19\textsuperscript{INK4d} as shown in the C, (D, Top) Image showing autoradiography of: lane 1, untreated p19\textsuperscript{INK4d}; lane 2, p19\textsuperscript{INK4d}, and lane 3, CDK6–p19\textsuperscript{INK4d} complex treated with S-phase cell extract. (Top) The autoradiogram and the corresponding Coomassie brilliant blue stained gel below. (Middle) The overlaid 2D \textsuperscript{1}H–\textsuperscript{15}N HSQC spectra of \textsuperscript{14}N-labeled p19\textsuperscript{INK4d} after the CDK6–p19\textsuperscript{INK4d} complex incubated with extract of S-phase cells (red) and untreated p19\textsuperscript{INK4d} (black). (Bottom) The respective \textsuperscript{31}P NMR spectrum. The asterisk indicates the phosphate buffer signal.
cellular fate of free doubly modified p19INK4d. We reasoned that the locally unfolded protein may become ubiquitinated and eventually degraded, which would explain the rapid disappearance of endogenous p19INK4d in lysates of cells released from S-phase arrest (Fig. S2C). Indeed, this drop of endogenous p19INK4d level upon thymidine removal and G2 release could be prevented by CDK1 inhibition during the S-phase (Fig. 5), confirming the coupling between Ser76 phosphorylation of p19INK4d and its cell cycle-dependent degradation.

In line with this model, we expected AR1–3 unfolding to also expose Lys62, a canonical ubiquitination site of p19INK4d. To test this hypothesis, we performed in vitro ubiquitination assays with S-phase–arrested cell lysates and phosphorylated p19INK4d. We did not detect ubiquitination of unmodified or Ser66-phosphorylated protein. Only Ser66- and Ser76-phosphorylated p19INK4d were evidently modified (Fig. S8). As expected, neither S66A nor S76A-mutant p19INK4d was ubiquitinated.

In summary, these findings (Fig. 6) suggest that cellular ubiquitination of p19INK4d likely depends on its phosphorylation and concomitant structural state. In support of our hypothesis, we conclude that cell cycle-dependent, stepwise phosphorylation of Ser66 and Ser76 induces local unfolding of AR1–3, which, in turn, dissociates p19INK4d from CDK6 and exposes Lys62 for subsequent ubiquitination, which likely serves as the signal for intracellular degradation.

Discussion

To initiate G1/S transition, CDK4/6 phosphatases trigger factors, such as the retinoblastoma Rb protein, which, in turn, release E2F transcription factors required for cell cycle progression (Fig. 1). Unmodified p19INK4d binds CDK4/6 and inhibits its function (34). Multisite phosphorylation of INK4 proteins, such as p16INK4d and p18INK4c, in asynchronously growing cells, has been observed previously (11, 34) and characterized in vitro by Ser-to-Glu substitutions mimicking phosphorylation (35), but the functional consequences of these modifications remained enigmatic. Here, we established that phosphorylation of p19INK4d Ser66 by p38 kinase broadly destabilizes N-terminal ARs, which appears to constitute a necessary priming event for the stepwise modification of Ser76 by CDK1. Double phosphorylation induces local unfolding of p19INK4d AR1–3, which dissociates the inhibitory CDK4/6–p19INK4d complex. In turn, modified, free, and unfolded p19INK4d undergoes ubiquitination, which precedes the protein for cellular degradation. A schematic summary of this process is depicted in Fig. 6. One key feature of this model is the switch-like function that the cell cycle-dependent protein kinase CDK1 imposes on the network. Clearly, CDK1-mediated phosphorylation of p19INK4d Ser76 appears to determine the functional outcome of the entire regulatory hub. In the absence of CDK1 activity, and Ser76 phosphorylation, p19INK4d blocks cell cycle progression by binding to CDK4/6 and, thereby, inhibits their functions. In this regard, the cell cycle-independent, primary phosphorylation of p19INK4d Ser66 maintains the system in a poised state that is primed for activation by CDK1. Once CDK1 activity is triggered, p19INK4d Ser76 phosphorylation frees CDK4/6 for transcriptional activation, cumulating in cell cycle progression. At the same time, doubly phosphorylated p19INK4d undergoes ubiquitination and eventual degradation, which ensures the irreversibility of the process and, by proximity, the directionality of the cell cycle.

Maybe one of the most striking features of this regulatory network is its structural component. Whereas phosphorylation of the primary p19INK4d substrate site (Ser66) destabilizes structured ARs more globally and thereby “enables” secondary site (Ser76) modification, the actual phosphorylation event “executes” the functional reprogramming step, which is largely driven by a local loss-of-structure mechanism. Unfolding not only disrupts the CDK4/6–p19INK4d complex and, thereby, activates these kinases, it also exposes the previously buried p19INK4d Lys62 residue for ubiquitination, which initiates p19INK4d degradation and clearance. At this level of posttranslational modifications, differences between the four INK4 members are evident, which are not related to the cell cycle-dependent mRNA levels (20). P18INK4c declines much more slowly as cells advance through G1/S phase compared with p19INK4d. The major ubiquitin acceptors Lys46 and Lys112 are buried in p18INK4c and, in turn, in p16INK4d and p15INK4b, which structurally well align, for example, the INK4 members INK4c and INK4b, and 53BP2 (18, 40). Ser66 is not conserved in any of the other homologs and is therefore unique as a priming site for p19INK4d degradation. The transcription factor GABPβ subunit contains four ARs and it has been speculated that MAPK/ERK-mediated phosphorylation involves the S/T-P motif that confers nuclear translocalization of KSRP. The S/T-P motif is conserved in the consensus sequence of ARs, which structurally well align, for example, the INK4 members (p19INK4d, p18INK4c, p16INK4d, p15INK4b) with GABPβ, myotrophin, and 53BP2 (18, 40). Ser66 is not conserved in any of the other homologs and is therefore unique as a priming site for p19INK4d degradation. The transcription factor GABP β-subunit contains four ARs and it has been speculated that MAPK/ERK-mediated phosphorylation involves the S/T-P motif in AR2 (Ser39-Pro40) and AR3 (Thr73-Pro74) (41), the latter corresponding to Ser76-Pro77 in p19INK4d. Along the same lines, among the four ARs in myotrophin, only AR3 harbors such a motif at Thr70-Pro71, and its phosphorylation by CDK1 generates Lys62 for ubiquitination. If AR3 kinase inhibition during the S-phase (Fig. 5), confirming the coupling between Ser76 phosphorylation of p19INK4d and its cell cycle-dependent degradation.

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phosphorylation has been discussed during initiation of cardiac hypertrophy (42).

Within these structural homologs, only p19\(^{\text{INK4d}}\) contains one further S/T-P motif at position Thr141-Pro142. This threonine was found to be phosphorylated by PKA during DNA damage response following, for example, UV irradiation or cisplatin treatment of cells (24). A putative priming site is Ser130 between AR5 and AR6, at a structural position homologous to Ser66 of p19\(^{\text{INKA}}\). Destabilization of scaffold repeats AR5 and AR6 (31, 35) might cause the same unfolding of p19\(^{\text{INK4d}}\) and genotoxic stress-induced CDK4/6 release, a hypothesis that has to be experimentally addressed in the future. These examples document that phosphorylation-induced unfolding of ARs, typically involved in protein–protein interactions, and as delineated in molecular detail here for p19\(^{\text{INK4d}}\) might represent an evolutionary conserved principle in cellular signaling.

Materials and Methods

**Protein Expression and Purification.** p19\(^{\text{INK4d}}\) wild-type and mutants were expressed in BL21(DE3) pLysS cells and purified as described previously (31). Additional details are provided in SI Materials and Methods.

Cell Lines and Synchronization. Cervical carcinoma (HeLa), HEC-293 and human osteosarcoma (U2OS) cells were employed and synchronization was achieved by double incubation with thymidine (5-phase) or Nocodazole (M-phase). All details are described in SI Materials and Methods.

**Protein Assays.** The kinase assay was performed by incubating cell lysate (0.5–1 mg/mL total protein concentration) of exponentially growing or synchronizing cells in the presence of \(^{32}\text{P}\) ATP (5 μCi \(^{32}\text{P}-\text{γ}-\text{ATP}\)) and cAMP (5 μM) with 30 μg of p19\(^{\text{INK4d}}\) for 3 h at 37 °C. Aliquots of the mixtures were analyzed by SDS/PAGE and labeled polypeptides were detected by autoradiography. Additional details are provided with the in vitro kinase assay with isolated p38 or CDK2/cyclin B1, the CDK6-p19\(^{\text{INK4d}}\) pull-down assay, and the p19\(^{\text{INK4d}}\) ubiquitination assay are given in SI Materials and Methods.

**NMR Spectroscopy Experiments.** The cells were lysed as mentioned above in the presence of kinase assay buffer, protease inhibitors, and phosphatase inhibitors. The lysate was collected by centrifugation. For the NMR spectroscopy experiment, lysate (15–20 mg/mL) was mixed with 30 μg of \(^{3}N\)-labeled p19\(^{\text{INK4d}}\) or its mutants, 100 μM ATP, and 5 μM cAMP. This reaction mixture was incubated for 3 h at 37 °C. Subsequently, the reaction samples were dialyzed using a 3.5-KDa cut-off membrane in phosphate buffer (20 mM Na\(^{+}\), 25 mM NaCl, 25 mM KCl, and pH 7.4) for 17 h to remove the unincorporated ATP, small molecules, and to minimize buffer effects to facilitate a proper chemical shift analysis of NMR cross-peaks. These samples were also analyzed by MALDI-TOF mass spectrometry. Details about the acquisition of NMR experiments and the \(^1\text{H}^2\text{H}\) exchange detected by NMR are provided in SI Materials and Methods.

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### References