EXPERT REVIEWS

The role of structural disorder in cell cycle regulation, related clinical proteomics, disease development and drug targeting

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Understanding the molecular mechanisms of the regulation of cell cycle is a central issue in molecular cell biology, due to its fundamental role in the existence of cells. The regulatory circuits that make decisions on when a cell should divide are very complex and particularly subtly balanced in eukaryotes, in which the harmony of many different cells in an organism is essential for life. Several hundred proteins are involved in these processes, and a great deal of studies attests that most of them have functionally relevant intrinsic structural disorder. Structural disorder imparts many functional advantages on these proteins, and we discuss it in detail that it is involved in all key steps from signaling through the cell membrane to regulating transcription of proteins that execute timely responses to an ever-changing environment.

Keywords: cancer • cell-cycle • checkpoint • post-translational modification • protein disorder • signal transduction

Cell cycle: the cornerstone of multicellular life

Every postembryonic eukaryotic cell goes through the distinct phases of cell cycle, G1, S, G2 and M. G1, S and G2 are commonly termed as interphase during which the cells prepare for division in M phase. Progression through these phases is crucial for the stability of the cells, and its correct regulation is the cornerstone of the integrity of the whole organism. The detection and repair of DNA damage are as important part of this as the prevention of uncontrolled cell division.

Cell cycle regulation has been one of the most studied biological processes for decades, and many key molecular mechanisms have been revealed [1,2]. Understanding the structural background of the most important regulatory proteins highlighted the importance of intrinsic protein disorder in the regulation of cell cycle progression [1].

Over the past few decades, there has been increasing awareness that a significant number of proteins are able to fulfill important functions without possessing a stable three-dimensional structure [3,4]. These proteins, termed intrinsically disordered proteins or regions (IDPs/IDRs), participate in many regulatory processes [4,5] and are also the main players in cell cycle regulation. IDPs function either as disordered polypeptide chains (entropic chains) or via molecular recognition (as shorter or longer binding motifs), which entails manifold functional advantages, such as enabling weak but specific binding, rapid interactions, adaptability to the binding partner and subtle regulation by post-translational modifications [1,3,6]. In this article, we take it under scrutiny how regulation of cell cycle benefits from these functional features.

Cell-cycle checkpoints

Checkpoints in the cell cycle serve as means for the cell to detect and possibly repair damaged DNA or other cellular damage before continuing to the next phase. Arrest of cell cycle progression is achieved through the

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activity of the key regulator proteins. These cell cycle checkpoints include the transitions not only from G1 to S and G2 to M but also in S phase and mitosis [7]. The G1-S phase checkpoint is termed restriction point, where the phosphorylated retinoblastoma protein (Rb) releases the transcription factor E2F, which in turn activates many S-phase genes, including those for cyclins D, A and E. The key mediators of Rb phosphorylation are cyclin-dependent kinase (CDK) 4- or 6/cyclin D complexes [8], and while the release of E2F directly leads to entry into S phase, cyclin-E kinase activity contributes to the cell cycle progression signal [9].

As the key regulators of cyclin-CDK activity, CDK inhibitors (CKIs) play prominent role in the integrity of the G1-S checkpoint. Apart from inhibiting CDK activity, they also act as adaptors to promote CDK–cyclin complex assembly, giving them a dual role in cell cycle regulation (moonlighting) and are themselves under close transcriptional control. p21Cip1 is controlled by p53, thus is a key player in DNA damage-induced G1 arrest [7]. p27Kip1, on the other hand, is regulated by DNA-damage-independent pathways, such as growth factor signaling, and is considered intrinsic G1 regulator.

Members of the other CKI family, INK4 (p16INK4a, p15INK4b, p18INK4c and p19INK4d), are specific for CDK4 and CDK6 and have roles in the early G1 phase. p16INK4a acts upstream of the Rb pathway and is frequently inactivated in cancer. P14ARF, a different CKI protein encoded by the same gene as p16INK4a, can induce cell cycle arrest both in G1 and G2 through interaction with MDM2 and the resulting p53 activation [10].

p53 itself is involved in the G1-S checkpoint because it is involved in various signal transduction pathways that lead to cell cycle arrest or apoptosis as a response to DNA damage. p53 also regulates the G2–M checkpoint through induction of 14-3-3σ, a protein that sequesters CDK1 in the cytoplasm. 14-3-3σ is a component of the G2–M checkpoint because its overexpression leads to G2 arrest [11]. Other, p53-independent mechanisms also exist at the G2-M checkpoint, for example, through post-translational modifications of CDK1, keeping it in its inactive form through inhibitory phosphorylation [12].

The mechanisms regulating the S-phase DNA damage checkpoint are less deeply understood and differ from the other cell cycle checkpoints in many important features. Unlike the other checkpoints, it reduces DNA synthesis in the presence of damaged DNA instead of completely halting it and checkpoint activity is not directly related to DNA damage resistance [13]. S-phase DNA damage checkpoint can be divided to two major processes: the inhibition of origin firing and the slowing of replication fork progression. As a response to ionizing radiation, ATM kinase activates Chk2, which phosphorylates Cdc25A promoting its degradation [14]. UV radiation also leads to the rapid degradation of Cdc25A through activation of Chk1 [15], preventing dephosphorylation and activation of the Cdk2-CyclinE complex. In the absence of Cdk2 activity origin, firing is inhibited due to the failed loading of Cdc45 and AND-1/CTF4 on origins [16]. The slowing of the replication fork is more of a local response to DNA damage. The proposed mechanism is that forks do not actually slow down, but their replication is paused at the sites of damage [13], manifesting in an overall slower fork progression. Both ATR and Chk1 are required for the fork slowing response, but additional components are also necessary in vertebrate cells. The timeless-timeless interactingprotein (Tim-Tipin) complex is a Chk1 target, and Tipin is required for UV-induced reduction of fork progression [17]. Checkpoint kinases are active under unperturbed conditions, suggesting a regulatory normal cell cycle [16] and they are essential in embryonic development [18,19].

Accurate chromosome segregation is also monitored by the cells, at the spindle assembly checkpoint (SAC) by controlling for microtubule-kinetochore attachment defects [20]. The key proteins that control SAC are protein kinases Bub1, BubR1 and Mps1. Bub1 recruits several checkpoint components to the kinetochore when checkpoint conditions are unsatisfied, but it is also important for the assembly of the inner centromere. BubR1 is required for the establishment of proper kinetochore-microtubule attachment and chromosome alignment. Together with Bub3, Mad2 and Cdc20, BubR1 forms a part of the mitotic checkpoint complex that inhibits the E3 ubiquitin ligase activity of the anaphase-promoting complex (cyclosome or APC/C) toward securin and cyclinB1. When all checkpoint conditions are fulfilled, APC/C inhibition is released, allowing chromosome separation and mitotic progression [20].

Functional modes of IDPs in signaling and cell cycle regulation

As outlined below, many cell cycle proteins abound in structural disorder. IDPs exist and function in a disordered ensemble state [21,22], which either directly represents their functional state or from which they undergo induced folding upon encountering their binding partner. The combination of these two distinct functional modes imparts many advantages on IDPs, due to which they are frequently used in signaling and regulation in the cell. In terms of their molecular mechanism of action, they often affect the activity of their binding partner (effectors) or assist interaction, localization and assembly of complexes of other proteins (assemblers) [4,23]. We will outline next these functional types of disordered proteins in cell cycle.

Effectors

Due to their key importance in cell cycle regulation, the activity of different Cdk/cyclin complexes is strictly controlled, primarily by CKIs. The most thoroughly studied group of CKIs is the CIP/KIP family that comprises p21Cip1, p27Kip1 and p57Kip2 [24]. The three proteins share a conserved, 60-residue-long N-terminal kinase inhibitory domain and nuclear localization signals within their C-terminal domains [24]. Other, divergent sequential features suggest their distinct functions and regulation. The CIP/KIP proteins are IDPs [2], and although they inhibit multiple Cdk/cyclin complexes, they also facilitate the assembly and nuclear transport of the Cdk 4(6)/cyclin-D complexes [25,26]. The kinase inhibitory domain can be divided

into three subdomains: D1, LH and D2, with D1 binding to the cyclin and D2 to the Cdk subunit. It is the highly flexible LH subunit that confers adaptability to different Cdk/cyclin complexes [27], enabling a wrapping-around type of binding, termed a 'molecular staple' [28]. The first recognition and binding step, when subdomain D1 binds to its cyclin partner through the RXL motif is accelerated by the fly casting [29] mechanism, followed by the slower binding of the D2 subdomain to the Cdk subunit [28].

p16INK4a and p14ARF are different effectors encoded in alternative, but partially overlapping reading frames by the INK4a/ARF locus [30]. While p16INK4a is a globular protein, p14ARF is mostly disordered in solution and its N-terminal disordered region binds Mdm2 in an induced folding process [31]. P14ARF is a potent regulator of the cell cycle that is not expressed in normal cells, but its expression is activated upon exposure to mitogenic signals [32,33]. It binds to Mdm2, inhibits the cytoplasmic transport of p53 and reduces the ubiquitin ligase activity of Mdm2 [31]. Interestingly, the region of Mdm2 recognized by p14ARF is also disordered in the unbound form, that is, the interaction has to proceed by mutual induced folding [31], possibly increasing the specificity of the interaction.

A central player in cell-cycle regulation is p53, which is activated by DNA damage, heat sock and other stress signals [34]. Activation of p53 results in changes of several genes that intervene in the progression of the cell cycle leading to cell cycle arrest and apoptosis [35]. The 393 amino acid-long protein consists of an intrinsically disordered N-terminal DNA-binding domain, a proline-rich domain, a central DNA-binding domain and a C-terminal tetramerization domain. The N-terminal domain is involved in the binding of transcriptional coactivators and corepressors and is essential for binding with Mdm2 [35], by induced folding [36]. The DNA-binding domain contains a disordered loop that is important for p53 function. Flexibility of the linker enables fast dissociation from nontarget DNA sequences and stable complex formation with the target sequences [37].

Assemblers

An important functional property of the IDPs is their large surface available for interaction due to which they are ideal docking platforms for the assembly of large complexes [38]. During the progression of the cell cycle, many such complexes form.

One example of these assemblers is a component of SAC. Proper SAC function requires communication with the kinetochore complex that connects centromeric DNA to microtubules. The structural core of the kinetochore is the KMN (Knl1-Mis12-Ndc80) network, which constitutes a docking platform for the kinetochore recruitment of SAC components [39]. By providing large interaction surfaces and a variety of functional motifs, Knl1 is responsible for the recruitment of kinetochore proteins and proteins implicated in the SAC [40]. Knl1 uses its C-terminal region for its localization to the kinetochore [41] and for the interaction with Nsl1 (a component of the Mis12 complex) [42] but can also bind Zwint, a kinetochore protein [43]. The N-terminal SILK and RVSF motifs of Knl1 recognize and directly bind protein phosphatase PP1 [44] that mediates SAC silencing [45]. PP1 binding supposedly inhibits the recruitment of Bub1 and BubR1 to Knl1 [46]. Through binding with Bub1, Knl1 indirectly mediates the recruitment of PP2A, which is important for stabilizing kinetochore-microtubule binding [47]. Another interaction motif, MELT is found in the N-terminal and middle regions of Knl1 [39], in varying numbers. The MELT motif is phosphorylated by the mitotic checkpoint kinase Msp1, and this modification is necessary for the recruitment of Bub1 and Bub3 to the kinetochore [46]. Bub1 and BubR1 binding localizes to the N terminus of Knl1, through two KI motifs [39]. The C-terminal part of Knl1 contains the structured RWD domain and a coiled coil regions [48] that mediate Nsl1 and Zwint binding, respectively [39].

Mediator of DNA Damage Checkpoint 1 (MDC1) is also a large, mostly disordered protein that functions through interacting with multiple partners to participate in various aspects of DNA damage response. This protein is recruited to the site of DNA double-strand breaks (DSBs), and together with RNF8 is necessary for the recruitment of BRCA1 and 53BP1 [49]. The protein contains an N-terminal FHA domain, which mediates phospho-protein interaction, a central Pro/Ser/Thr-rich repeat domain (PST repeat) and a C-terminal tandem BRCT (tBRCT) domain, implicated in protein–protein interactions [49]. The PST motif, an imperfect repetitive motif of about 41 amino acids, does not appear in other proteins [50] and human MDC1 contains 13 full PST repeats, five more than the murine MDC1; the PST repeats act as protein binding modules [51]. Binding of partners such as γ-H2AX, histone H2AX and 53BP1 is important for the activation of cell cycle checkpoints [49,52]. MDC1 also interacts with several components of the APC/C through the FHA, tBRCT and PST repeat domains of MDC1 [53] Thus, by affecting APC/C activity, MDC1 may have a role in the normal transition of cells from metaphase to anaphase, independent of the DNA damage or SACs [49].

Breast cancer type 1 susceptibility protein (BRCA1) is a 1863 amino acid-long multifunctional protein implicated in DNA DSB repair, transcription coupled repair, cell cycle checkpoint control, centrosome duplication, transcription regulation, DNA damage signaling, growth regulation and the induction of apoptosis [54]. Despite the large size of the protein, only two, relatively small conserved domains, were identified and characterized in its sequence. The N-terminal RING finger domain forms a heterodimer with BARD1, resulting in an active E3 ubiquitin ligase complex [55], and the C terminus contains two tandem BRCT domains [56]. The central region of the protein is shown to be largely disordered [54] and apart from mediating the interaction with many proteins, it contains a number of DNA-damage-induced phosphorylation sites [57]. BRCA1 participates in four major complexes to repair DSBs. The first complex contains RAP80, MERIT40, BRCC36/45 and Abraxas and is involved in G2/M checkpoint arrest after DNA damage. The second, containing TopB1 and BACH1 along with BRCA1 is involved in S-phase checkpoint activation [58]. The third complex formed between BRCA1, Mre11, Rad50 and Nbs1 is involved in DSB

end resection [59], and fourth complex of BRCA1, BRCA2, PALB2 and Rad51 facilitates sister chromatid invasion [60]. Many of these interactions were mapped to the disordered central region of BRCA1, underlining the importance of this region of the protein [54].

Regulation of cell cycle by PTMs: targeting disordered regions

Phosphorylation is the most well-known post-translational modification that participates in the regulation of almost all cellular processes. It has been shown earlier that phosphorylation sites preferentially occur in disordered regions, where they are accessible for the modifying enzyme [61]. An elegant study aiming at the investigation of the dynamics of phosphorylation revealed that the majority of phosphorylation sites that are linked with the cell cycle are found in disordered segments [62]. These segments differ from the ordered segments not only in their accessibility but also a higher evolutionary variability and underline the general importance of protein disorder in the regulation of the cell cycle. Phosphorylation can play a role in the activation of a specific protein, exemplified by the p27Kip1, where the flexibility of the segment that blocks Cdk enables the exposure and subsequent phosphorylation of a tyrosine residue. This leads to partial activation of the Cdk, which can phosphorylate p27 at another residue resulting in its degradation and full activation of Cdk [63]. On the other hand, changes in phosphorylation pattern may increase the sensitivity and robustness of the cellular response and may promote the switch-like behavior [64]. This is achieved by the multiple phosphorylation sites on many proteins, giving rise to the idea that it is the cluster of phosphorylation sites that defines the function [65].

Progression of the cell cycle is an ordered and directional process, meaning that each step occurs in a sequential fashion. This directional course of events is achieved through the sequential activation of the key regulatory proteins of the cell cycle, cyclins and CDKs [2], which form heterodimers to perform phosphorylation of various downstream targets, thus orchestrating coordinated entry into the next phase of the cell cycle. CDKs are constitutively expressed in cells, whereas cyclins are synthesized at specific stages of the cell cycle, in response to various molecular signals. Different Cdk/cyclin complexes control entry into G1 phase (Cdk4 and Cdk6 paired with D-type cyclins) and progression from G1 to S phase (Cdk2 paired with A- and E-type cyclins), and direct experimental evidence suggests the role of structural disorder in cyclin function.

Cdk activity is regulated by the Cip/Kip protein family, also termed as CKIs. The Cip/Kip family members, including p21, p27 and p57, associate with the full repertoire of Cdk/cyclin complexes and regulate their kinase activities at the cell cycle checkpoints [2], and are fully disordered proteins.

IDPs in cell cycle: disorder established

IDPs established in cell cycle

Besides the foregoing examples, structural disorder is established in many other cell-cycle regulatory proteins (Tables 1 & 2).

Securin regulates separase, the protease responsible for the physical separation of sister chromatids [66]. Securin is a disordered, dual function protein [67], which not only holds separase in the inactive state until the onset of anaphase but also acts as a chaperone of separase activity [68,69].

Sic1, the disordered kinase (Cdk) inhibitor of budding yeast, is responsible for setting the timing of cell cycle progression [70]. The interaction of Sic1 with the SCF ubiquitin ligase subunit Cdc4 in yeast is a model example of an ultrasensitive regulatory system based on structural disorder [71]. When Sic1 is phosphorylated on any six (or more) of nine sites, it binds to a WD40 domain in Cdc4, which leads to the ubiquitination and degradation of Sic1 [64], bringing about the development of B-type cyclin–CDK activity and the onset of DNA replication.

Mdm2 is also a central player in the regulation of the cell cycle as mentioned before in various aspects of this review. Its interaction with P14ARF falls to an intrinsically disordered region of Mdm2 and its disorder-to-order transition in the interaction contributes to the specificity of the binding interaction [31].

Viral proteins often take advantage of the specific features of disordered regions to override cell cycle control to enable the transcription of viral genes [1]. By incorporating different binding motifs within its disordered N-terminal region, the adenovirus oncoprotein early region 1A (E1A) [72] can bind numerous cellular proteins and organize them into higher-order complexes that disrupt regulatory networks and reprogram gene expression [1]. Human papillomavirus E7 protein uses its disordered C-terminal region to bind and induce degradation of Rb, forcing infected cells to enter S-phase [73].

Prediction of disorder in cell-cycle associated proteins

While the human proteome contains high levels of disorder, it was previously shown that structural disorder is particularly abundant in certain regulatory processes. In Figure 1, we compare the frequency distribution of proteins with different levels of disorder in the complete human proteome (UniProt/SwissProt human protein dataset, 2015 February, n = 20198) and in proteins annotated to cell cycle (filtered from SwissProt using KW-0131, n = 619). Structural disorder was predicted by using IUPred algorithm [74,75]. Proteins annotated to cell cycle have significantly different frequency distribution from all human protein, with significant difference in the first group (0–20% disorder content), the mostly globular proteins being under-represented, and in second and fourth groups (20–80% disorder content), where the partly disordered proteins are over-represented. This distribution bias shows a strong association of structural disorder with cell cycle. To rationalize this finding, we also extracted the typical functional annotations (based on Gene Ontology functional annotations) for every category among the cell-cycle-related proteins. While the first and fifth categories contain typical functions related to globular (mainly enzymatic functions) and disordered (inhibitory and regulatory) proteins, respectively, the middle categories mostly related to transcription, and adaptor functions.

We also selectively collected the 10 most disordered proteins for G1/S and G2/M transitions (Tables 1 & 2). Highly disordered

Table 1. 10 most disordered protein involved in cell-cycle G1/S transition.

Protein name (SwissProt ID)	Function in the G1/S transition	Size (in residues)	Disorder frequency
Protein phosphatase 1 regulatory subunit 1C (PPR1C_HUMAN)	Inhibitor of protein-phosphatase 1, promotes cell growth and cell cycle progress at the G1/S transition	109	1.000
Cyclin-dependent kinase inhibitor 1B (CDN1B_HUMAN)	Important regulator of cell cycle progression. Involved in G1 arrest	198	0.955
RAD9, HUS1, RAD1-interacting nuclear orphan protein 1 (RHNO1_HUMAN)	Required for the progression of the G1 to S phase transition	238	0.672
Protein NPAT (NPAT_HUMAN)	Required for progression through the G1 and S phases of the cell cycle and for S phase entry. Activates transcription of the histone H2A, histone H2B, histone H3 and histone H4 genes in conjunction with MIZF	1427	0.654
Cyclin-dependent kinase inhibitor 1C (CDN1C_HUMAN)	Potent tight-binding inhibitor of several G1 cyclin/CDK complexes (cyclin E-CDK2, cyclin D2-CDK4, and cyclin A-CDK2) and, to lesser extent, of the mitotic cyclin B-CDC2	316	0.652
Protein BEX2 (BEX2_HUMAN)	Required for the normal cell cycle progression during G1 in breast cancer cells through the regulation of CCND1 and CDKN1A	128	0.640
Growth arrest and DNA damage-inducible proteins-interacting protein 1 (G45IP_HUMAN)	Acts as a negative regulator of G1 to S cell cycle phase progression by inhibiting cyclin-dependent kinases	222	0.617
Cyclin-dependent kinase inhibitor 2A, isoform 4 (CD2A2_HUMAN)	Capable of inducing cell cycle arrest in G1 and G2 phases	132	0.599
Bromodomain-containing protein 7 (BRD2_HUMAN)	Inhibits cell cycle progression from G1 to S phase	651	0.555
Serine/threonine-protein kinase LATS2 (LATS2_HUMAN)	Negatively regulates G1/S transition by down-regulating cyclin E/CDK2 kinase activity	1088	0.546

proteins are involved in the regulation of each transition, but interestingly, their length distribution is different. While the small regulator, inhibitor proteins are abundant among disordered proteins in G1/S, longer proteins with the potential to have adaptor, complex and bridge forming function are dominant among IDPs in G2/M transition.

Diseases, & drug targets

Due to the frequent involvement of IDPs in signaling and regulatory processes [5], impairments of their function are very often causally involved in diseases, such as cancer, inflammation and neurodegenerative diseases [76]. We will next overview the involvement of cell-cycle regulatory proteins in diseases.

Diseases

Seeing the central role of cell-cycle control in the integrity of a multicellular organism, it is of no surprise that its proteins play primary roles in several diseases, the most important of which is cancer. Tumor genesis is promoted by either the aberrant expression of positive regulators, such as cyclins, or the loss of function of negative regulators, such as CKIs [7]. Overexpression of Cyclin D1 and Cyclin E has been found in breast carcinomas and multiple other tumors. Given their crucial role in regulating the activity of cyclins, it is surprising that mutations of CKIs are not frequently found in human tumors [77]. Nevertheless, many human tumors, including breast cancers, colon, gastric and prostate tumors show decreased p27KIP1 protein levels [7].

Rb, the most important CDK target, is often mutated in human retinoblastoma and lung cancer [78]. Loss of function of Rb due to mutation or binding of tumor virus proteins leads to unrestrained cell proliferation [79]. Approximately 90% of human cancers have abnormalities in some component of the Rb pathway [78].

The tumor suppressor p53 is the most prominent and extensively studied example due to its dominating effect on tumor cell growth and proliferation. The p53 gene is the most frequently mutated gene in human cancers [80], and other mechanisms, like binding of viral oncoproteins, can also alter or block p53 function [81]. Overexpression of MDM2, the negative regulator of p53 has also been reported in leukemia, lymphoma, breast carcinoma, sarcoma and glioma [12].

BRCA1 is a major breast and ovarian cancer susceptibility gene, with mutations in this gene predisposing women to a very high risk of developing breast and ovarian tumors [57]. BRCA1 mutations often result in defective DNA repair, genomic instability and sensitivity to DNA damaging agents.

Cohesins mediate sister chromatid cohesion and cellular long-distance chromatin interactions affecting genome

Table 2. 10 most disordered protein involved in cell-cycle G2/M transition.

Protein Mame (SwissProt ID)	Function in the G2/M Transition	Size (in Residues)	Disorder Frequency
Cell division cycle-associated protein 3 (CDCA3_HUMAN)	Acts by participating in E3 ligase complexes that mediate the ubiquitination and degradation of WEE1 kinase at G2/M phase	268	1.000
High mobility group protein HMGI-C (HMGA2_HUMAN)	Plays an important role in chromosome condensation during the meiotic G2/M transition of spermatocytes	109	1.000
Mediator of DNA damage checkpoint protein 1 (MDC1_HUMAN)	Required for checkpoint mediated cell cycle arrest in response to DNA damage within both the S phase and G2/M phases of the cell cycle	2089	0.840
Protein FAM32A (FA32A_HUMAN)	Isoform 1 may induce G2 arrest and apoptosis	112	0.830
Centrosomal protein of 164 kDa (CE164_HUMAN)	Plays a critical role in G2/M checkpoint and nuclear divisions	1460	0.699
Apoptosis-stimulating of p53 protein 2 (ASPP2_HUMAN)	Impedes cell cycle progression at G2/M transition	1128	0.668
Serine/threonine-protein kinase LATS1 (LATS1_HUMAN)	Negatively regulates G2/M transition by down-regulating CDK1 kinase activity	1130	0.640
Breast cancer type 1 susceptibility protein (BRCA1_HUMAN)	Component of the BRCA1-RBBP8 complex which regulates CHEK1 activation and controls cell cycle G2/M checkpoints on DNA damage via BRCA1-mediated ubiquitination of RBBP8	1863	0.620
Cyclin-dependent kinase inhibitor 2A, isoform 4 (CD2A2_HUMAN)	Capable of inducing cell cycle arrest in G1 and G2 phases	132	0.599
Pre-mRNA-splicing regulator WTAP (FL2D_HUMAN)	Regulates G2/M cell-cycle transition by binding to the 3' UTR of CCNA2, which enhances its stability	396	0.599

maintenance and gene expression. Mutations in cohesin's subunits and its regulator proteins result in defects of cellular growth and differentiation, causing different diseases, commonly termed as cohesinopathies [82].

Drug development

As the loss of control over cell cycle is by definition the very essence of cancer, many of the proteins mentioned in the article are involved in cancer as either oncogenes or tumor suppressors. It follows that they are primary drug targets, and actually several of them are under investigation in drug development programs, or are targeted by cancer drugs on the market [83,84]. The preponderance of structural disorder, however, is an apparent impediment to drug development efforts, because our traditional approaches – which brought many successes – cannot effectively target protein–protein interaction interfaces, which are usually large and flat. In accord, most current drugs on the market target the active site of enzymes, such as kinases [85] or ligand-binding sites of receptors, such as G-protein-coupled receptors [86]. This feature is also reflected in the so-called Lipinski rules (or Lipinski rule of 5) [87], which state that successful drug molecules are small hydrophobic chemicals of certain chemical nature (in terms of solubility, charge and the number of H-bonds), which ensure successful cellular delivery and interaction with the partner.

Due to their large flexibility, IDPs are not inherently amenable to bind such small molecules with sufficient affinity and specificity. It explains that no successful drug development has been based on structural disorder, despite IDPs being primary drug targets. Because of the frequent involvement of cell-cycle regulatory IDPs in cancer, and the ensuing interest in developing drugs against them, we outline the recent status of ideas and efforts of drug development against IDPs. Because IDPs most often function by protein–protein interactions, their interfaces might in principle be targeted by small molecules. As suggested, the interfaces of globular proteins are large and flat, due to which most attempts to develop drug molecules that block protein–protein interactions have so far failed [88]. In fact, there are only about eight drugs on the market that target protein–protein interaction interfaces [89,90].

IDPs, however, most often engage in a special type of interaction, mediated by their short recognition elements (preformed structural elements, PSEs [91], molecular recognition features, MoRFs [92], short linear motifs (SLiMs, also termed eukaryotic linear motifs, ELMs [93,94]), which bind in a hydrophobic pocket of the partner molecule, in an interface that resembles receptor–ligand or enzyme–substrate binding. Not surprisingly, four of the eight drugs against interaction interfaces (affecting the complex of BAK/Bcl-xL, p53/MDM2, Tcf/β-catenin and Smac/XIAP [89,90]) actually target a complex that involves a disordered and a structured partner. The conspicuously high incidence of success is very suggestive that the binding partners of IDPs might be successfully targeted by small molecules [95].

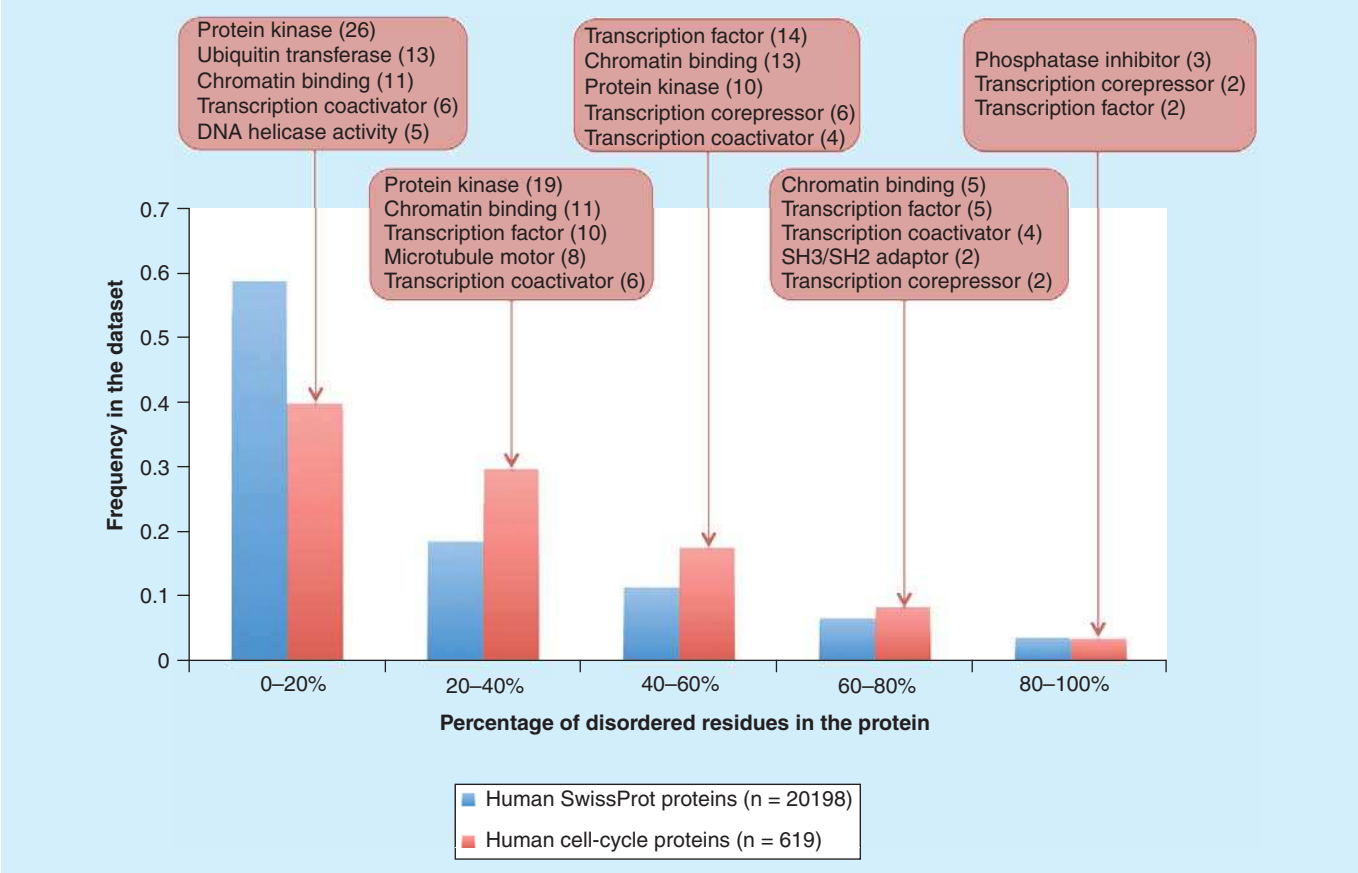

Figure 1. Frequency distribution of proteins with different percentage of disorder among all human and cell-cycle-related proteins. Human proteins were extracted from UniProt/SwissProt database, cell-cycle proteins were filtered by using SwissProt keyword (KW-0131). Disorder propensity and the derived percentage were calculated by using the IUPred algorithm (choosing 0.5 as threshold in IUPred, regions below that were considered as structured, above were considered as disordered protein region). Characteristic cellular functions among cell-cycle-related proteins in different disorder percentage categories are highlighted in boxes (according GO annotations, number of hits indicated).

If there is one example to demonstrate the potency of this concept, one should mention the case of targeting the p53–Mdm2 interaction by a family of small molecules, nutlins [84]. As suggested, p53 is a central player in the stability and integrity of the genome [96]. p53 becomes stabilized upon DNA damage, it level increases and initiates a variety of cellular responses, aimed at repairing DNA and/or delaying cell-cycle progression, or causing apoptosis. The primary regulator of p53 is Mdm2, an E3 ubiquitin ligase that is engaged in a feedback regulatory loop with p53. Mdm2-mediated ubiquitination and subsequent proteasomal degradation ensures low resting levels of the protein [97]. It has been suggested that the physical inhibition of the interaction of the two proteins could reactivate p53 in tumors overexpressing Mdm2 and provide a potent therapeutic strategy in cancer. The complex of p53 and Mdm2 corresponds to the above definitions: Mdm2 presents a deep hydrophobic binding pocket in which an amphipathic short helix of p53 is inserted: the pocket can bind be filled with potent and selective small-molecule antagonists, the Nutlins [84]. Nutlins compete with p53 binding and have the potency to activate the p53 pathway in cancer cells, which can even re-initiate cell-cycle arrest, apoptosis and growth inhibition of human tumor xenografts in nude mice.

Therefore, partner targeting can be an effective strategy to combat diseases caused by IDPs. Because the number of short disorder-related binding motifs in the proteome can be very large [94], this approach can be a general strategy of very broad applicability. Dunker *et al.* have estimated the possible number of such targets [95] in the thousands with interfaces that are ideal drug targets: the IDP engages in weak interaction due to induced folding, which can be competed with a small molecule, the binding element of the IDP is an isolated helical segment, which is likely to fit into a groove or pocket, the amphipathic nature of the helix positions hydrophobic residues on one side, which makes it likely the existence of a complementary concave hydrophobic-binding pocket amenable for targeting by a small molecule. Many of the identified potential targets are involved in cancer, making them linked with cell-cycle regulation.

In principle, IDPs could also be targeted directly by interfering small molecules although their very dynamic nature and extreme structural heterogeneity hamper such efforts. In a few

cases, however, small-molecule interference of IDP function has been achieved. The first system successfully targeted was the c-Myc transcription factor, which is involved in many types of cancer [98,99]. cMyc falls into the basic helix-loop-helix leucine zipper (bHLHZip) transcription factor family, and forms functional heterodimers with Max through a coiled-coil dimerization interface. Formation of c-Myc–Max complexes are attractive targets in oncology, and although their interaction regions are intrinsically disordered prior to heterodimerization, inhibitory small molecules were found in systematic screens. A variety of biophysical experiments (primarily NMR) suggested that binding of the molecules selected is specific to these sites and they preserve the disordered state of c-Myc, thereby inhibiting its heterodimerization with Max [98,99].

Another class of disordered oncogenic targets that might be targeted by small molecules is that of oncogenic fusion protein. These proteins result from chromosomal translocations and show strong correlation with structural disorder [100]. Such fusion event was observed, for example, in Ewing's sarcoma family tumors (ESFTs), which contain a characteristic translocation that leads to the expression of the oncogenic fusion protein EWS-FLI1. EWS-FLI1 is mostly disordered, against which a small molecule binder could be developed, which inhibits its binding to RNA helicase A [101]. This compound induces apoptosis in ESFT cells and reduces the growth of ESFT orthotopic xenografts.

A small molecule binding to IDPs of even more subtle effect has been identified in protein tyrosine phosphatase 1B (PTP1B), which has a positive role in HER2 signaling in breast tumors. In a way similar to kinases, targeting their evolutionarily conserved active site is problematic for specificity, due to cross-reaction with the many other phosphatases in the cell. This problem may now have been circumvented by developing a binder to the long, disordered, non-catalytic C-terminal tail of the enzyme [102]. Binding of the small molecule at this site locks the catalytic domain in an inactive state, antagonizes HER2 signaling, inhibits tumorigenesis in xenografts and abrogates metastasis in a mouse model of breast cancer. The importance of this finding is that small molecule not only can bind to a disordered segment of a protein but also can have an allosteric inhibitory effect. Allosteric drugs have a unique flavor of specificity [103], which, in the case of IDPs, also puts emphasis on subtle allosteric long-range communication in IDPs termed multistery [104].

Clinical proteomics

The potential targets, that is, disorder-related disease-associated proteins can be identified in high-throughput (HTS) proteomic analyses. There are numerous novel techniques with the potential to provide valuable biological information related to IDPs in the physiological or pathological process of cell cycle. *In vitro* kinase assays, like the kinase assay linked with phosphoproteomics technique can be used to determine the substrate specificity and identifying direct substrates of protein kinases. The method was already used to map phosphorylation patterns and kinase-ligand pairs within the Syk and ERK pathways [105,106]. A special rapid purification-linked method combined with on-bead kinase assay (native enzyme–substrate complex kinase assay, NESKA) was developed very recently and was already used in synchronized cells to identify substrates cyclin/CDKs [107]. Kinase substrates can be also labeled using ATP analogues and modified enzymes. Using a mutated Cdk1, Blethrow *et al.* were able to identify more than 70 phosphorylation targets for the Cdk1-cyclin B complex [108].

Affinity purification, a relatively traditional method, has become a HTS method with the combination of modern mass spectrometry. Several studies aimed to target kinases in the process of cell cycle, and identified numerous phosphorylation targets for cyclin–Cdk complexes, and specifically to cyclin E1, A2 and B1 [109,110]. Selective kinase targeting using small-molecule inhibitors can further enhance quantitative phosphoproteomics. With this technique, phosphorylation sites, and possible interactions were identified in the case of Aurora A, Aurora B and kinases of the Plk family [111]; yeast Mec1, Tel1 and Rad53 kinases [112]; ATM and ATR kinases [113]. HTS proteomic studies are always coupled with extended bioinformatics methods to analyze and to store the large amount of output data.

Expert commentary & 5-year view

Recognition of structural disorder some 15 years ago sparked a revolutionary transition in the field of structural molecular biology. It is now ever more appreciated that this phenomenon exists *in vitro* and also *in vivo*, and it plays a critical role in the function of many key proteins in signaling and regulation. The central role of cell cycle in disease makes the study of this phenomenon imperative, by the battery of structural and functional techniques at our disposal. We have quite a detailed view on how IDPs function [4,23], and we have rapidly advancing tools to study them [114]. The ensemble description of IDPs [21,22] suggests that we might be able to extend the structure–function paradigm over the disordered state of proteins, in the cell cycle and other regulatory paradigms, which will provide a much deeper understanding of the regulation of cell cycle.

In many cases, it is already clear how intrinsically disorder contributes to cell-cycle regulation. Its role is most easily interpreted in inhibitors (e.g., securin, p27), which bind and inhibit critical elements of the regulatory circuit. These are usually small proteins or well-defined regions of larger ones, they become ordered in the presence of the partner and can even be crystallized in complex. Their detailed characterization can be approached by technical means already at our disposal.

More challenging and difficult to approach is the assembly of complexes, which are often transient and subject to lots of regulatory inputs and communication. Often, we only know about the involvement of large IDPs in the function of complexes. Many proteins involved in G2/M also fall into this category (Table 2), they are long and disordered, and probably function as spacers and connectors, and raise special problems

for studying in detail. It appears that they are often involved in reaching out for remote partners by fly-casting [29] and are subject to an extremely large number of regulatory inputs by post-translational modifications, primarily phosphorylation.

Fine tuning of function by such multisite post-translational modifications is exemplified by p53, for example. Increasing residual p53 helicity by tuning phosphorylation results in an increasing binding to MDM2, altered dynamics of the protein and impaired target gene expression and lack of arrest of cell cycle [115]. Results of similar multisite phosphorylation devices may have different readouts, such as ultrasensitive threshold response, coincidence detection and rheostat [1], regulatory relations that will be a great challenge to sort out in the future. A key and upcoming method one should be keen on using in this regard is in-cell NMR, which has the power of reporting on structural–functional relations from within a live cell [114].

In all, our message for the future is that disordered proteins and regions of disorder in proteins are functional, and we should not be discouraged to find out what and how they do. Often they link the action of an enzyme on a different target, or add a subtle regulatory module to a functional domain. For example, protein Tyr phosphatase 1B (PTP1B) has a long C-terminal regulatory domain, and this long noncatalytic segment could be targeted by an allosteric inhibitor [102]. It is not unfounded to expect that studies and analyses on structural disorder in cell-cycle regulation will have a golden era just ahead of us, with rich rewards not only in our basic knowledge of the cell but also in our ability in interfering with them in case of disease.

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Key issues

- Progression through the phases of the cell cycle is crucial for the stability of the cells and its correct regulation is the cornerstone of the integrity of the whole organism. Proteins involved in cell cycle regulation play primary roles in several diseases, the most important of which is cancer. Tumor genesis is promoted by either the aberrant expression of positive regulators, such as cyclins, or the loss of function of negative regulators, such as cyclin-dependent kinase (CDK) inhibitors (CKIs).
- Intrinsically disordered proteins or regions (IDPs/IDRs) function without possessing a stable three-dimensional structure and are among the main players in cell cycle regulation. IDPs function either as disordered polypeptide chains (entropic chains) or via molecular recognition (as shorter or longer binding motifs), which entails manifold functional advantages.
- Proteins annotated to cell cycle have significantly different frequency distribution from all human proteins. This distribution bias shows a strong association of structural disorder with cell cycle. Highly disordered proteins are involved in the regulation of G1/S and G2/M transitions, and while the small regulator, inhibitor proteins are abundant among disordered proteins in G1/S, longer proteins with the potential to have adaptor, complex and bridge forming function are dominant among IDPs in G2/M transition.
- The activity of different Cdk/cyclin complexes is strictly controlled, primarily by CKIs. The most thoroughly studied group of CKIs is the CIP/KIP family that comprises p21Cip1, p27Kip1 and p57Kip2. The CIP/KIP proteins are IDPs and although they inhibit multiple CDK/cyclin complexes, they also facilitate the assembly and nuclear transport of the CDK 4(6)/cyclin-D complexes.
- A central player in cell-cycle regulation is p53, which is activated by DNA damage, heat sock and other stress signals. Activation of p53 results in changes of several genes that intervene in the progression of the cell cycle leading to cell cycle arrest and apoptosis. Its disordered N-terminal domain is involved in the binding of transcriptional coactivators and corepressors and is essential for binding with Mdm2.
- Phosphorylation participates in the regulation of almost all cellular processes, and it has been shown that phosphorylation sites preferentially occur in disordered regions where they are accessible for the modifying enzyme. Changes in phosphorylation pattern may increase the sensitivity and robustness of the cellular response and may promote the switch-like behavior of a cellular process.
- The preponderance of structural disorder is an apparent impediment to drug development efforts, but because IDPs most often function by protein–protein interactions, their interfaces might in principle be targeted by small molecules. IDPs most often engage in a special type of interaction, mediated by their short recognition elements, which bind in a hydrophobic pocket of the partner molecule, suggesting that the binding partners of IDPs might be successfully targeted by small molecules. Four of the eight drugs against protein–protein interaction interfaces target a complex that involves a disordered and a structured partner.
- The potential targets, that is, disorder-related disease-associated proteins can be identified in high-throughput (HTS) proteomic analyses. There are numerous novel techniques with the potential to provide valuable biological information related to IDPs in the physiological or pathological process of cell cycle. HTS proteomic studies are always coupled with extended bioinformatics methods to analyze and to store the large amount of output data.

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•• **The authors performed a time-resolved analysis of the in vivo interactors of cyclins E1, A2 and B1. This revealed the temporal dynamics of cyclin function in which networks of cyclin–CDK interactions vary according to the type of cyclin and cell cycle stage. The results explain the temporal specificity of the cell cycle machinery, thereby providing a biochemical mechanism for the genetic requirement for multiple cyclins in vivo, and reveal how the actions of specific cyclins are coordinated to control the cell cycle.**

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