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Minireview

Pathways governing G1/S transition and their response to DNA damage

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Abstract The ability to self-replicate is a fundamental feature of life, reflected at the cellular level by a highly regulated process initiated in G1 phase via commitment to a round of DNA replication and cell division. Here we briefly highlight recent advances in understanding the molecular pathways which govern the decision of mammalian somatic cells to enter S phase, and the so-called cell cycle checkpoints which guard the G1/S transition and S phase progression against potentially deleterious effects of genotoxic stress. Particular emphasis is put on the emerging parallel yet cooperative pathways of retinoblastoma protein (pRB)–E2F and Myc, their convergence to control the activity of the cyclin-dependent kinase 2 (Cdk2) at the G1/S boundary, as well as the two waves of checkpoint responses at G1/S: the rapid pathway(s) leading to Cdc25A degradation, and the delayed p53–p21 cascade, both silencing the Cdk2 activity upon DNA damage.

Key words: G1/S control; Retinoblastoma protein; E2F; Myc; Cyclin E–cyclin-dependent kinase 2; Cdc25A; p53; DNA damage

1. Introduction

Many of the fate decisions of vertebrate somatic cells are taken in the G1 phase of their cell division cycles. Key among such decisions is the question whether or not the cell should proliferate. When the cellular and tissue environments are favourable and the cell does initiate its division cycle, then tight surveillance mechanisms (cell cycle checkpoints) are also imposed to monitor the order and quality of the cell cycle events, with the option to halt the cell cycle at virtually any transition point if a major malfunction or DNA damage are encountered [1]. The necessity to carefully regulate both the cell cycle machinery itself, and the cell cycle checkpoints is apparent from the central role of these mechanisms in development and homeostatic tissue renewal, and the fact that failure to execute these programmes in an error-free manner may lead to devastating consequences such as cancer. The cell's commitment to replicate the genome and eventually divide has been assigned to an operationally defined period in late G1, known as the restriction point, when the cell switches from its mitogen-dependent growth in early G1, to a largely growth factor-independent progression into, and beyond, S phase. Considerable efforts in the cell cycle field have been devoted to explain the restriction point switch in molecular terms, mainly focusing on the retinoblastoma protein (pRB) pathway as a candidate mechanism operating in G1 [2–5]. Recent evidence shows, however, that the RB pathway alone can not account for the G1/S control, and evidence is mounting to implicate the Myc proto-oncogene as a central element of a pathway parallel to, and cooperating with, the pRB–E2F axis [6–11]. Another type of dichotomy, reviewed here, encompasses the emerging basis of the so far elusive, rapid and transient cell cycle checkpoint response at G1/S and in S phase. This initial, acute phase of the G1/S checkpoint operates via silencing Cdc25A (see Section 3.2), and it functions independently of the known, more delayed and sustained G1 arrest due to stabilisation and transcriptional activation of the p53 tumour suppressor [12,13]. Significantly, a feature shared by all these pathways is their convergence on the control of cyclin-dependent kinase 2 (Cdk2), a cyclin E- and A-dependent G1/S kinase whose activity is rate-limiting and essential for DNA replication.

2. Molecular pathways controlling G1/S transition

2.1. The RB–E2F pathway

The functions and regulation of the p16–cyclin D–Cdk4(6)–pRB–E2F cascade, known as the RB pathway, have been reviewed thoroughly in recent years [2–5,14], and here we only address the most recent novel information about this key G1/S controlling mechanism. The growth-suppressive, underphosphorylated form of the pRB 'pocket protein' prevents premature entry into S phase by binding to a number of cellular proteins such as the transcription factors E2F-1–5 which regulate expression of S phase genes. Phosphorylation of a host of pRB's serine and threonine residues in mid-to-late G1 by mitogen-induced cyclin D-dependent kinases cancels the growth-inhibitory effects of pRB, thereby allowing activation of a plethora of cell cycle regulatory genes including those for cyclins E and A. Novel insights have been obtained into the mechanistic aspects of gene expression control via pRB–E2F, such as the roles of local chromatin remodeling through pRB-bound histone deacetylase, E2F acetylation, and the relative balance between direct inhibition of E2F activity by pRB versus active repression of transcription by the pRB–E2F complex [14]. Exploration of non-phosphorylat-

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Abbreviations: pRB, retinoblastoma protein; Cdk, cyclin-dependent kinase; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia related

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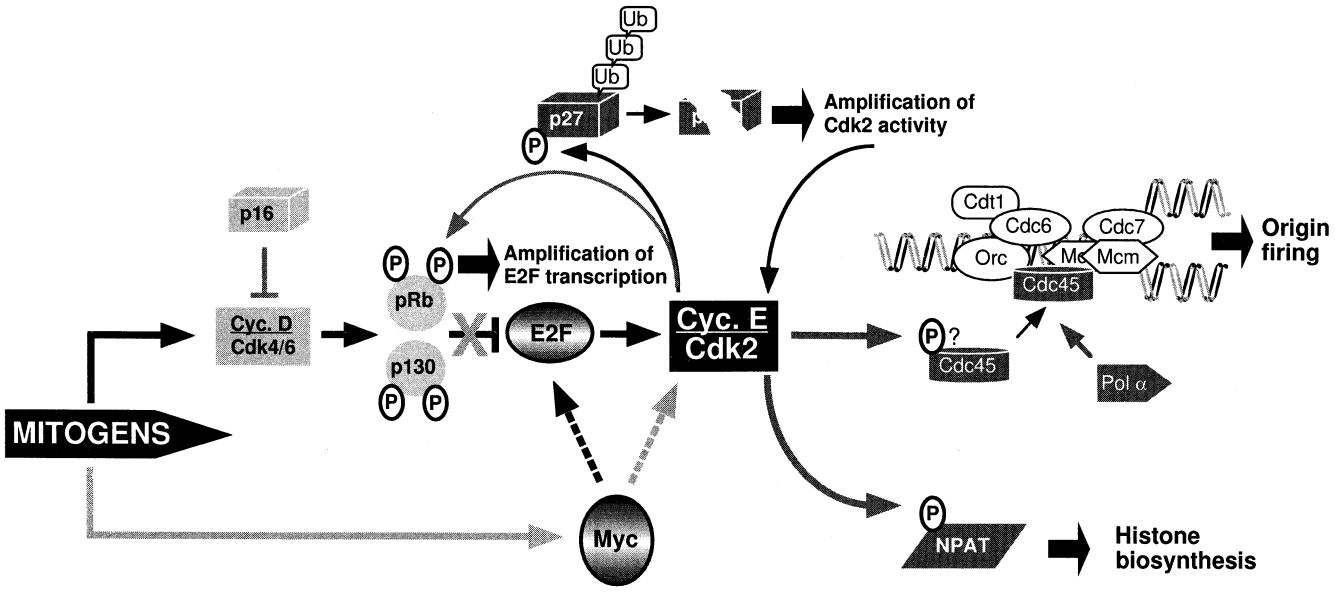

Fig. 1. Cyclin E–Cdk2 as a nodal point integrating signals from the G1/S-promoting pathways. Cyclin E–Cdk2 stimulates DNA synthesis presumably by facilitating origin firing through Cdc45-dependent loading of DNA polymerase α and promoting histone biosynthesis through direct phosphorylation of NPAT, a factor required for histone transcription. In addition, cyclin E–Cdk2, once activated, amplifies the signals from the mitogen-induced G1/S-promoting pathways by phosphorylating and inactivating the E2F repressors pRB and p130, and by targeting the p27 CDK inhibitor for ubiquitination and rapid proteasome-mediated destruction.

able, constitutively active mutants of pRB revealed some unexpected functions of pRB even in the S/G2/M phases of the cell cycle, such as centrosome duplication, or stabilisation of mitotic regulators [15–17]. Together with some conditions when endogenous pRB becomes activated in post-G1 phases of the cell cycle [18,19], these findings indicate why it is critical for cycling cells to maintain pRB in its phosphorylated state (through cyclin E–Cdk2 and cyclin A–Cdk2 activities) from late G1 until mitosis. Most relevant to the focus of this review, the ability of mammalian cells to eventually escape a G1 arrest imposed by such constitutively active mutant pRB strongly suggested the existence of another, pRB–E2F-independent mechanism allowing entry into S phase ([6] and Sections 2.2 and 2.3). Although not commonly regarded as a component of the RB pathway, the pRB-related p130 is the major pocket protein which inhibits E2F in quiescent cells, and it becomes phosphorylated during the G0–G1–S traverse with kinetics similar to that of pRB phosphorylation. Recent identification of 22 phosphorylation sites of p130 targeted in vivo by combined activities of cyclin D–Cdk4, cyclin E–Cdk2 and non-Cdk kinases [20] shows only a surprisingly limited analogy with regulation of pRB. The ability of a phosphorylation-deficient p130 mutant to impose a sustained G1 block, considerably more powerful than the effect of analogous mutant pRB, along with the recently identified cancer-associated p130 mutations, qualify p130 as a candidate tumour suppressor ([20] and references therein). Another incentive to understand p130 stems from the experiments with cells genetically deficient in p130 and p107, the third pocket protein. These p130/p107 double-deficient cells are resistant to p16-mediated G1 arrest, analogous to cells deficient in pRB, thus implying that in addition to pRB, the p130/p107 pocket proteins also provide an essential regulatory function in G1 [21]. While p107 becomes expressed only later during the cell cycle, p130 operates throughout G0–S phase progression, and we suggest that at least p130 should also be adopted as a component of the RB pathway, as schematically outlined in the relevant part of Fig. 1.

2.2. The role of Myc in regulation of G1/S transition

The *c-myc* proto-oncogene is a transcription factor of the helix–loop–helix/leucine zipper protein family, whose endogenous expression is promptly induced by mitogens and whose ectopic expression, like that of E2F [22,23] or cyclin E–Cdk2 [24], is able to induce entry into S phase in quiescent cells [25]. Despite the generally accepted role of Myc in promoting proliferation, the identity of its target genes which mediate the mitogenic effects, and the mechanistic links of Myc with the cell cycle machinery including the RB pathway are only very recently coming to light. Among the downstream target genes of Myc, relevant for G1/S control, are those encoding cyclins D2, D1 and E, and the Cdc25A phosphatase, an essential regulator of S phase entry which strips Cdk2 of its inhibitory phosphates on Tyr 15/Thr 14 ([6] and references therein, [26,27]). In terms of the key activities or mitogenic processes, c-myc has been implicated in at least three distinct, and genetically separable, programmes: regulation of cyclin E–Cdk2 activity, E2F-dependent transcription, and cell growth (i.e. an increase in cell mass) [9,25,28]. The positive effect of Myc on cell growth is becoming apparent from work with both *Drosophila* and mammalian cells [25,28], and from the identity of putative target genes of Myc, a subset of which promote metabolism, in particular protein translation [29,30]. Arguably the best studied of the Myc-induced proliferation-promoting programmes is the activation of cyclin E–Cdk2 kinase. This complex programme apparently includes direct transcriptional effects on cyclin E and Cdc25A expression, and indirect mechanisms such as sequestration of the Cdk inhibitor $p27^{kip1}$ into cyclin D–Cdk4(6) complexes away from cyclin E–Cdk2, and phosphorylation and subsequent ubiquitination and proteasome-mediated degradation of the $p27^{kip1}$ protein at G1/S [6,7,26,27,31,32].

Recent construction of a mutant known as MadMyc capable of actively repressing c-myc target genes [8], allowed examination of cellular effects upon both short-term [8,9] and long-term [6] silencing of endogenous Myc. Transient expression of MadMyc causes G1 arrest independent of functional pRB [8,10], yet mitogen-stimulated cells exposed to MadMyc in long-term experiments eventually enter S phase despite the lack of Myc-mediated transcription [6]. These and other data can be reconciled by a concept of two parallel and cooperating G1/S-governing pathways, regulated by pRB–E2F and Myc, respectively (Fig. 1). While silencing of either cascade only delays but does not completely block entry into S phase, cells with concomitantly silenced E2F and Myc are unable to initiate DNA synthesis despite the presence of growth factors [6]. Not surprisingly, a significant functional cross-talk also exists between these parallel RB–E2F and Myc pathways, for instance through Myc-mediated transcriptional activation of E2F-2, E2F-3 and Id2, three transcriptional regulators of G1/S negatively controlled by pRB [33–35]. Despite their links, however, either the E2F- or the Myc-induced programmes appear sufficiently powerful to drive mammalian cells into S phase, possibly reflecting the fact that either pathway can induce cyclin E–Cdk2 and/or cyclin A–Cdk2 activity (Fig. 1 and Section 2.3). On the other hand, cells selectively deprived of either E2F or Myc activity are unable to reach mitosis [6,9,10,17], strongly suggesting that activities of both the RB and Myc pathways are required for the timely progression through, and successful completion of, the cell division cycle, and thus for continuous proliferation of mammalian somatic cells.

2.3. Cyclin E–Cdk2 as a convergence point of G1/S control

Thus, the concept which is emerging from these new discoveries on the role of the RB–E2F and Myc cascades is the one of two parallel, cooperating and interacting pathways, each targeting a number of downstream genes and cellular functions. A striking feature, also of central importance for this review, is the convergence of both the RB–E2F- and Myc-regulated programmes on the control of abundance and activity of cyclin E–Cdk2, a key G1/S-promoting enzyme which is both rate-limiting and essential for S phase entry [3,4]. This concept is also supported by the ability of ectopically expressed cyclin E and Cdc25A to synergistically rescue the concomitant blockade of both the pRB–E2F and Myc pathways, consistent with cyclin E–Cdk2 inducing DNA synthesis downstream of E2F and Myc [6]. The multiple ways through which Myc stimulates cyclin E–Cdk2 are listed in Section 2.2, and E2F contributes to the timely expression and activation of this key S phase-promoting kinase by transcriptional induction of genes for cyclin E and Cdc25A ([14,36], and references therein).

A fundamental question which follows the realisation of the central role of cyclin E–Cdk2 in the regulation of G1/S transition is the issue of the underlying molecular mechanisms and, thus, the identity of the key physiological substrates phosphorylated by cyclin E–Cdk2. Probably the best known substrate of cyclin E–Cdk2 is pRB and its related pocket proteins p130 and p107 [2–5,14,20]. Cdk2 activity appears to complete and maintain the neutralising phosphorylation of pRB, a process initiated by cyclin D-dependent kinases in G1, thereby preventing unscheduled activation of pRB in S phase, and feeding a regulatory loop which amplifies both E2F and cyclin E–Cdk2 activity (Fig. 1).

Another established target of the cyclin E–Cdk2 kinase is $p27^{kip1}$, whose phosphorylation and subsequent degradation allows the timely elevation of Cdk2 activity necessary for S phase initiation and progression ([31,37], Fig. 1). However, neither of these substrates can account for the powerful S phase-promoting effect of cyclin E–Cdk2 activity in cells arrested in G1 by the non-phosphorylatable pRB [38], clearly indicating that other important substrates of this kinase must exist at G1/S. Two recently identified candidate substrates may explain the so far elusive roles of cyclin E–Cdk2 in histone biosynthesis and initiation of DNA replication. Histones are components of nucleosomes, and must be provided during DNA replication, through a process partly controlled by elevated transcription of the histone genes. Recently, the p220NPAT protein was identified as a substrate associated with, and phosphorylated by, the cyclin E–Cdk2 kinase. In addition, such modification by cyclin E–Cdk2 was shown to be essential for the ability of p220NPAT to cyclically activate the histone gene promoters at the onset of S phase (Fig. 1; [39–41]). Finally, while the licensing of replication origins via sequential binding of the origin recognition complex, Cdc6, and the MCM family of proteins probably does not require cyclin E–Cdk2, the actual firing of the licensed origins appears dependent on loading of Cdc45 which is dependent on Cdk activity and essential for subsequent recruitment of DNA polymerase α into the pre-initiation complex [42–44]. Although other relevant substrates of cyclin E–Cdk2 may well be discovered, the cyclin E–Cdk2-dependent functions of Cdc45 and p220NPAT in the critical steps required for successful DNA replication may provide the long-sought insight into the physiological role of cyclin E–Cdk2 in G1/S control (Fig. 1).

Apart from its essential function in cell proliferation, the strategic role of cyclin E–Cdk2 at the G1/S transition makes this kinase a candidate downstream target of the cell cycle checkpoint responses to DNA damage, a topic which is addressed in the following sections of this review.

3. DNA damage response pathways at G1/S

3.1. The p53–p21 axis

When the genetic material is damaged, a delay in cell cycle progression facilitates DNA repair, thereby avoiding the replication and subsequent propagation of potentially hazardous mutations. The ability of the cell cycle checkpoints, signalling pathways which monitor the integrity and replication status of the genome, to inhibit entry into S phase is intimately associated with the function of the p53 tumour suppressor [12]. The p53 protein is a transcription factor which becomes stabilised and active upon DNA damage, and in turn regulates transcription of a large number of genes, among them the $p21^{Waf1/Cip1}$ Cdk inhibitor capable of silencing the Cdks which are essential for S phase entry [4,12]. Rapid advances in the DNA damage field have recently provided some novel insights into the molecular mechanics of the p53 pathway, particularly the upstream elements of the cascade(s) which eventually modify p53. The emerging scenario indicates an early activation (within a few minutes after DNA damage) of ataxia telangiectasia mutated (ATM) or ataxia telangiectasia related (ATR) (the choice between the two depending on the precise

nature of the DNA lesion), two large kinases from the PI-3 kinase superfamily [1,13]. ATM/ATR then phosphorylate a range of substrates, including p53 (on serine 15) and the checkpoint kinases Chk2 and Chk1 (again dependent on the type of damage) which become activated and subsequently propagate the signal to downstream effectors [1,13]. Recent data [45–47] show that activated Chk2 and Chk1 phosphorylate p53 on serine 20, an event which leads to decreased protein turnover and thus accumulation of p53 (Fig. 2). The high lability of the p53 protein in non-damaged cells depends on Mdm2, which binds to the N-terminus of p53 around Ser20 and targets p53 for ubiquitination and proteasome-mediated degradation. Phosphorylation of Ser20 prevents efficient interaction of p53 with Mdm2 and appears essential for stabilisation of p53 after DNA damage, but other modifications of p53 itself and phosphorylation of Mdm2 by the ATM/ATR and Chk1/Chk2 kinases are likely to be involved as well [13,48,49]. Mdm2 is itself transcriptionally activated by p53, thereby creating a negative feedback loop with p53, and this interplay is further complicated by the accompanying dynamic changes of their subcellular localisation [12,50]. The nuclear import and export of p53 may depend on its modifications, and it has very recently been shown that p53 uses its N-terminus to interact with the microtubules and exploit these motors to aid its nuclear localisation [51]. Stabilised and activated p53 affects the transcription of many genes, and ongoing efforts to assess this response globally using the functional genomic approaches may soon answer whether or not the p53-induced Cdk inhibitor $p21^{Waf1/Cip1}$ is the only critical target downstream of p53 (Fig. 2) required to block the cell cycle machinery in G1 in response to DNA damage [12].

3.2. The rapid response via Cdc25A degradation

Not all aspects of the G1 checkpoint responses to genotoxic stress can be attributed to the p53 pathway, however, since at least transient inhibition of Cdk2 in response to DNA damage occurs even in cells lacking p53 or p21 [13,48]. In addition, cells can also reduce the rate of ongoing DNA synthesis when exposed to DNA damage, and this so-called intra-S phase checkpoint is known to operate in an ATM-dependent, yet p53/p21-independent manner [13]. Reports published within the last several months now suggest that pathways targeting the Cdc25A phosphatase may underlie the molecular events which account for the p53-independent delay of G1/S transition, and likely also the transient intra-S phase checkpoint responses [52–54]. Thus, regardless of the status of p53, the total cellular activity of the Cdc25A phosphatase becomes rapidly decreased when mammalian cells are exposed to UV light or γ radiation, reflecting DNA damage-induced ubiquitination and accelerated turnover of the Cdc25A protein by proteasome [52]. This novel checkpoint pathway (Fig. 2) results in persistent inhibitory phosphorylation of Cdk2 on Thr 14/Tyr 15, and thus inhibition of cyclin E–Cdk2 and cyclin A–Cdk2 kinases and G1/S arrest. Both the involvement of

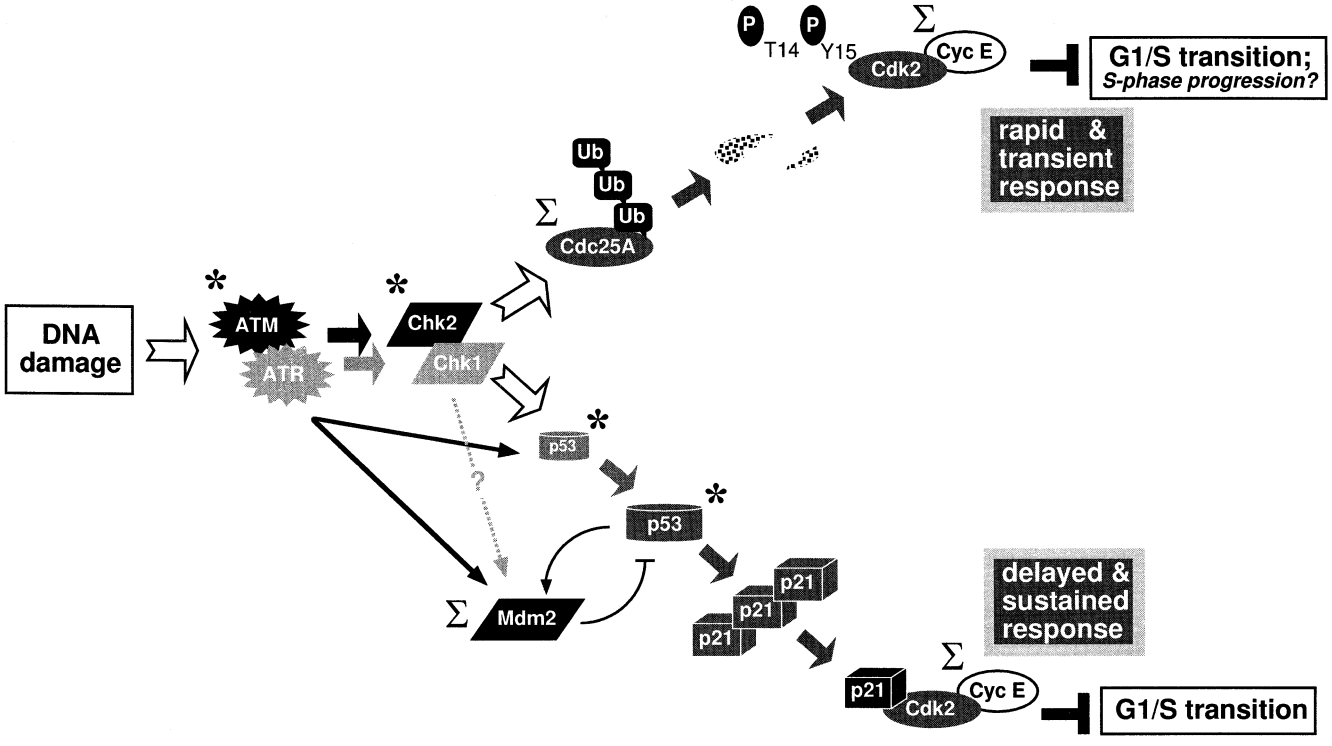

Fig. 2. Cyclin E–Cdk2 integrates checkpoint pathways inducing both rapid and delayed G1/S responses to acute genotoxic stress. DNA damage induced kinase cascades involving ATR/ATM and Chk1/Chk2 target Cdc25A for rapid ubiquitin/proteasome-mediated proteolysis. This event, independent of transcription and de-novo protein synthesis culminates in a rapid inhibition of the S phase-promoting cyclin E–Cdk2 activity through 'locking' it in an inactive, Thr 14- and Tyr 15-phosphorylated state. Activation of the delayed p53-mediated response, on the other hand, requires Chk1/Chk2-dependent stabilisation of p53 itself before it can fully support de-novo synthesis of the p21 CDK inhibitor. In some instances, accumulation of p21 to the threshold sufficient for sustained inhibition of cyclin E–Cdk2 lags behind the Cdc25A-mediated pathway for several hours. As indicated, multiple components of both rapid and delayed mechanisms leading to cyclin E–Cdk2 inhibition in response to DNA damage are deregulated in cancer, either by loss-of-function mutations (*), or by accumulation of the (proto)-oncogenic effector proteins (Σ).

upstream checkpoint kinases such as Chk1 in this pathway, and the fact that enhanced DNA damage and decreased cell survival follow overexpression of Cdc25A which prevents its downmodulation after genotoxic stress, are consistent with this mechanism as a bona fide cell cycle checkpoint [52]. Further supporting the emerging role of Cdc25A in mediating the G1/S and S phase checkpoint responses (Fig. 2), Cdc25A becomes rapidly ubiquitinated and degraded in response to incomplete DNA replication in mammalian cells [53], and in a cell-free model of *Xenopus* egg extracts, the silenced activity of Cdc25A and increased inhibitory phosphorylation of Cdk2 appear instrumental in imposing a checkpoint response to DNA double-strand breaks [54].

3.3. The two-wave checkpoint response at G1/S

Collectively, these recent findings support a concept of two successive waves of cell cycle checkpoint responses at G1/S (Fig. 2). The initial, transient response is very rapid, leading to the inhibition of Cdk2 within 20–30 min, and lasting for only several hours [13,52]. This prompt cell cycle delay is independent of p53 and transcription, and reflects the cascade operating via Cdc25A phosphorylation and degradation, and thus silencing of Cdk2 due to its inhibitory phosphorylation (see Section 3.2). It seems that this early response fits well the purpose of temporarily slowing down the cell cycle progression to provide more time for DNA repair. The second, delayed and considerably more sustained response is carried out by the classical p53/p21 cascade. As the multistep process of p53 modification, accumulation, activation and above all transcriptional induction of the effectors such as p21 requires several hours, this wave of the G1/S checkpoint response is better suited for long-term, and possibly even permanent, elimination of cells whose DNA has been severely damaged [48]. This possibility is also supported by the ability of the p53 pathway to induce apoptosis, another efficient way to remove hazardous, genetically damaged cells from the population. On the other hand, the initial signalling events through ATM/ATR and Chk1/Chk2 kinases appear to be shared and performed in parallel to target both the early and the late branches of the G1/S checkpoint responses (Fig. 2), and it remains to be seen whether these two waves can ever occur independently in cells proficient in either cascade. The latter point is highly relevant to the currently somewhat controversial issue as to what extent the normal mammalian cells with the activated p53 pathway are actually able to recover from this sustained arrest [48,55]. It has also been suggested, that in those cell types expressing cyclin D1, degradation of this cyclin might contribute to the more rapid, p53-independent response to DNA damage in G1, apparently even independently of the upstream checkpoint kinases such as ATM [56]. Despite the time divergence and distinct intermediates of the two major waves of cell cycle checkpoint responses at G1/S, the feature which unifies the p53-independent as well as p53-regulated pathways is their ultimate convergence on silencing the activity of Cdk2, the key effector of both the mitogenic cascades (see Section 2) and the safeguard mechanisms protecting our cells against genotoxic stress (Figs. 1 and 2).

3.4. Defects in G1/S checkpoints, genomic instability and cancer

Deregulated cell proliferation and genetic instability are among the hallmarks of cancer, reflecting defects in cell cycle control, and aberrations in genome integrity checkpoints and DNA repair machinery, respectively. Both the RB and Myc pathways governing G1/S transition (see Section 2) are commonly targeted in oncogenesis, and this field has been reviewed repeatedly [5,57]. Here we will briefly discuss the cancer-predisposing aberrations of the two-wave cell cycle checkpoint response to DNA damage at G1/S, described in Sections 3.1–3.3 and Fig. 2 (where the pathway components which qualify as oncogenes or tumour suppressors are marked). The most common of these defects are those of the p53 tumour suppressor, either deletions or mutations targeting different regions of the p53 gene itself, or the immediate regulators of p53 including the Mdm2 proto-oncogene [12]. Mutations of the p53 gene can also be inherited, such as those predisposing to cancers in diverse organs characteristic of a subset of families with the Li–Fraumeni syndrome. Interestingly, some of the Li–Fraumeni families which do not have germ line mutations of p53 were shown to carry mutations in the gene encoding the Chk2 kinase upstream of p53 in the DNA response pathway ([58], Fig. 2), and occasional sporadic cancer-associated mutations have been detected in both the Chk1 and Chk2 genes [58–60]. Even defects at the very top of the checkpoint cascade, namely mutations of the ATM gene, predispose to cancer and affect the outcome of tumour therapy, in this case particularly the response to radiation [13]. The central component of the rapid G1/S checkpoint pathway, the Cdc25A phosphatase, qualifies as proto-oncogene based on its ability to transform cells in culture and its overexpression in several types of human tumours including carcinomas of the lung, breast and head and neck, where its aberrant elevation correlates with poor prognosis [61–64]. Finally, cyclin E, the activatory subunit of the Cdk2 kinase and a downstream effector targeted by both the rapid and the delayed wave of the G1/S checkpoint response, is a proto-oncogene amplified and/or overexpressed in several types of cancer, and its elevation may both deregulate cell cycle progression and contribute to genomic instability ([65] and references therein).

4. Concluding remarks

Many fundamental phenomena in biology come in pairs, such as the two strings of nucleotides carrying the blueprint of life in the DNA double helix, to name just the most notorious example. Here we provide some new examples of dichotomy in basic biological processes, by presenting the emerging evidence for dual control pathways to govern both the G1/S transition in cycling cells, and the response at G1/S to genotoxic stress. This short review should be regarded as an attempt to help establish a conceptual framework for G1/S transition as a critical period of the cell division cycle, and to inspire further quests for a better explanation of why our cells make the decision to proliferate the way they do. It is our hope that the rapidly accumulating knowledge in this field will also aid in developing more rational cancer treatment strategies in the near future, as exemplified by attempts to develop clinically applicable Cdk inhibitors [66], search for attenuators of checkpoint kinases which might presensitise cancer cells to existing chemotherapy and radiotherapy regimens [67–69], or design activators of the p53 function, either to stimulate wild-type p53 or to re-vitalise its overabundant mutant forms present in many human malignancies [12,70].

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