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Post-Transcriptional Regulation of E2F Transcription Factors: Fine-Tuning DNA Repair, Cell Cycle Progression and Survival in Development & Disease

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1. Introduction

Cells are continually exposed to genotoxic stresses. Upon DNA damage, the cell activates a coordinated and complex series of responses (Levitt and Hickson, 2002). Multiple factors are implicated in each of these responses. Recently, it has become apparent that various transcription factors play important roles in cellular responses to genotoxic stress. In particular, E2F transcription factors are key for the activation of genes involved in these processes.

E2F family comprises two subfamilies, termed E2F and DP, and includes orthologs expressed across many species, from plants to higher vertebrates (McClellan and Slack, 2007). In mammals, multiple E2F (E2F-1 through -8) and DP (DP-1 through -4) genes have been identified. E2F-1, -2 and -3 are associated with DNA synthesis and cell cycle progression, and function as heterodimers with a DP member (McClellan and Slack, 2007). E2F-4 and -5 also require association with a DP protein, but often function to halt cell cycle progression associated with terminal differentiation or reversible entry into quiescence (McClellan and Slack, 2007). E2F-1 through -5 can mediate transcriptional activation when found as "free" E2F/DP dimers, but can also act as transcriptional repressors if they are associated with a member of the retinoblastoma (pRb) family of proteins (Hallstrom and Nevins, 2009). In contrast, E2F-6 lacks both transcriptional activation and pRb-binding domains, and functions as a constitutive transcriptional repressor. The most divergent members of the E2F family are E2F-7 and -8, which bind neither DP nor pRb-family proteins, and also function as transcriptional repressors to mediate cell cycle arrest (Lammens et al., 2009). To regulate gene expression, E2F factors bind GC-rich elements on proximal promoters, which can conform to either a consensus 5' -TTTC[CG]CGC-3' element, or to non-consensus sequences (Judah et al., 2010; Rabinovich et al., 2008). Considerable efforts have been directed to investigate whether different E2F proteins exhibit target selectivity. Genome-wide screens for E2F targets have revealed considerable overlap in the ability of individual E2F proteins to regulate their targets, although a few promoters activated by specific E2F forms have been identified (Cao et al., 2011).

In spite of the vast similarities in the activities of distinct E2F proteins and their ability to bind potential target Genes, to-date E2F1 is the principal E2F member shown to participate in cellular responses to DNA damage (Bracken et al., 2004). The role of E2F-1 upon DNA damage depends on cellular context. E2F-1 can either induce pro-apoptotic or anti-apoptotic outcomes. During the latter, E2F-1 can play roles to induce cell cycle arrest and upregulate DNA repair, by directing expression of multiple genes. These genes are involved in mismatch repair (MSH2, MLH1), nucleotide excision repair (DDB2, RPA), homologous recombination repair (RAD51, RAD54, RECQL), base excision repair (UNG, APE) & non-homologous end joining (Chang et al., 2006; Ishida et al., 2001; Polager and Ginsberg, 2008; Prost et al., 2007).

In humans, E2F-1 is a 437 amino acid protein, which shows constitutive and rapid nucleocytoplasmic shuttling in a variety of cells (Ivanova et al., 2007). E2F-1 stimulates cell proliferation by positively modulating transcription of genes necessary for DNA synthesis and cell cycle progression (Ivanova et al., 2005). In an apparently paradoxical manner, E2F-1 can also induce cell cycle arrest when associated with pRb, or apoptosis, by activating expression of pro-apoptotic genes (Polager and Ginsberg, 2008). The breadth of E2F-1 targets mediates the distinct biological activities of this transcription factor, which encompass both oncogenic and anti-oncogenic properties, as well as positive modulation of tissue regeneration after injury (D'Souza et al., 2002; Field et al., 1996).

2. E2F-1 and the DNA damage response

Genotoxic stress in cells activates the DNA damage response, and can occur as a result of a variety of insults. The latter include DNA double- strand breaks and single-strand damage. DNA damage can result from exogenous agents (e.g. radiation, exposure to reactive and mutagenic chemicals), or from endogenous products of cell metabolism (Shiloh, 2003). In response to DNA damage, cells activate multiple pathways that result in apoptosis or in DNA repair, cell cycle arrest, changes in gene expression, as well as in protein synthesis and degradation.

Cells require efficient response mechanisms to genotoxic stress, as this is a life-threatening event because it can significantly alter their genetic material. Multiple mechanisms have evolved to repair damage induced by genotoxic stress, including activation of a global signalling network termed the DNA damage response (DDR), which is capable of detecting distinct types of DNA damage, coordinating appropriate responses. The latter include transcriptional activation, cell cycle arrest, apoptosis, senescence and DNA repair (Shiloh, 2003). The DNA damage response plays a critical role in cell survival when damage occurs during DNA replication. In addition, there are specialized processes, including base-excision repair (BER), nucleotide-excision repair (NER) & nonhomologous end-joining, which recognize and repair specific types of lesions (Shiloh, 2003). Central to transduce signals that indicate DNA damage and initiate appropriate cellular responses are two related protein kinases, termed ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3-related). ATM can associate with its regulator, the MRN (Mre11-Rad50-NMS1) complex, when double-strand breaks (DSB) are generated (Levitt and Hickson, 2002). On the other hand, ATR forms complexes with its regulator ATRIP (ATR-interacting protein), which senses single-strand DNA (ssDNA) breaks generated by processing of double-strand breaks, as well as single-strand DNA which arises from stalled replication forks (Shiloh, 2003). These two kinases also phosphorylate E2F-1, thus initiating transcriptional activation of its target DNA repair genes.

2.1 Identification of E2F targets involved in DNA damage repair

Central to understanding the role of the E2F family of transcription factors in DNA repair has been the identification of a large number of putative and demonstrated E2F target genes. Although E2F proteins were originally characterized as important regulators of cell cycle progression, genome-wide screens have demonstrated much broader roles in a variety of primary and immortalized cell types. For example, E2F-1 and E2F-3 bind to the promoters of apurinic/apyrimidinic endonuclease (APE) and other repair enzymes in human primary epidermal keratinocytes, irrespective of their differentiation status (Chang et al., 2006). Similarly, in the GM06990 lymphoblastoid cell line, non-biased genome-wide screening has identified a large number of putative E2F-4 targets involved in responses to DNA damage (Lee et al., 2011). E2F targets important for DNA repair have also been identified in neoplastic cells following therapeutic intervention. For example, treatment of prostate cancer cells with histone deacetylase inhibitors reduces their ability to repair DNA damage induced by radio- and chemotherapy, thus reducing tumour mass (Kachhap et al., 2010). The impaired ability to repair DNA of treated cells was due, at least in part, to decreased recruitment to and activation by E2F-1 to the promoters of key DNA repair genes. Hence, the importance of E2F factors in DNA repair encompasses not only events during carcinogenesis, but also the potential impact of various therapies.

2.2 Role of E2F-1 in responses to DNA damage induced by UV radiation

UV radiation induces severe DNA damage, which is the principal cause of skin carcinogenesis in humans (Brash et al., 1996). UV-B radiation induces formation of cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP), which would result in loss of DNA integrity and genetic instability if left unrepaired. This type of damage to DNA triggers activation of the nucleotide-excision repair pathway, and can occur *via* one or more streams. Such DNA repair streams include (i) global genome repair (GGR), which repairs damage from the entire genome, (ii) transcription-coupled repair (TCR), which generally repairs damage on actively transcribed DNA strands & (iii) transcription domain-associated repair (DAR), which deals with repairing both strands of actively transcribed regions (Nouspikel, 2009).

Normal responses of the epidermis to UV damage are critically dependent on E2F-1 expression. Indeed, increased levels of epidermal apoptosis upon UV-B irradiation have been reported in E2F-1-null mouse epidermis, whereas repair of UV-B-induced DNA photoproducts is more efficient in keratinocytes that overexpress E2F-1 (Berton et al., 2005). UV-induced DNA damage results in stabilization of E2F-1 protein, which stimulates nucleotide excision repair (Berton et al., 2005; Pediconi et al., 2003; Wikonkal et al., 2003). The mechanisms involved include phosphorylation of E2F-1 on Ser31 by ATR and/or ATM kinases (Lin et al., 2001). This modification facilitates E2F-1 recruitment to sites of double-strand breaks or UV-induced DNA damage. Under these conditions, E2F-1 interacts with two key proteins involved in DNA repair: TopBP1 and GCN5 histone acetyltransferase (Guo et al., 2010a; Guo et al., 2010b). Formation of these E2F-1 complexes is necessary for efficient recruitment of factors involved in nucleotide excision repair. Importantly, the association of E2F-1 with TopBP1 and GCN5 occurs at the expense of the E2F-1-induced expression of pro-apoptotic p73, thus ensuring that DNA repair, rather than apoptosis, takes place (Berton et al., 2005; Pediconi et al., 2003; Wikonkal et al., 2003). In mouse embryo fibroblasts, UV-C irradiation results in the formation of both CPD and 6-4PP. In these cells, nucleotide excision repair is activated through pathways that involve activation of xeroderma pigmentosum

(XPC) gene expression by E2F-1 *via* increased binding to the XPC promoter (Lin et al., 2009). XPC is an essential mediator of DNA damage recognition during global genomic repair, and this phase of repair is actually more efficient in pRB-deficient cells, likely because lack of pRb increases E2F-1 activity.

The importance of E2F in repair of DNA damage induced by UV radiation is further demonstrated by the conservation of this pathway through evolution. For example, in *Arabidopsis* and in maize, MSH2 and MSH6, which are two genes that belong to the mismatch repair system, are targets of E2F transcriptional activation following DNA damage by UV-B radiation (Lario et al., 2011).

2.3 E2F is a key factor to maintain the balance between cell cycle arrest and expression of DNA repair genes following DNA damage

Given the key roles that pRb family proteins play in the regulation of E2F activity, it is not surprising that they also modulate the function of E2F factors following DNA damage. For example, the zinc finger-containing transcriptional repressor ZBRK1 is an important modulator of GADD45A transcription. The latter is involved in induction of cell cycle arrest in response to DNA damage (Siafakas and Richardson, 2009). E2F-1, but not other E2F proteins, binds to the ZBRK1 promoter, together with pRb, CtIP and CtBP, forming repressor complexes that interfere with ZBRK1 expression (Liao et al., 2010). In pRb-deficient cells, increased susceptibility to DNA damage induced by UV radiation or methylating agents occurs, partly as a result of abnormal cell cycle arrest and DNA repair. In a similar manner, E2F-1 is essential for normal expression of XRCC1 (x-ray repair cross-complementation group 1), which participates in the repair of single-strand breaks, thus ensuring efficient repair following DNA damage induced by methylating agents (Chen et al., 2008).

In contrast, loss of pRb can improve DNA repair in other circumstances, such as those involving activation of DDB2. Mutations in the *DDB2* gene, which encodes a protein involved in global genomic repair and repair of CPDs, gives rise to xeroderma pigmentosum, a disorder associated with increased risk of cutaneous and ocular tumours (Bennett and Itoh, 2008). DDB2 expression is positively regulated by E2F-1 and E2F-3. Further, deletion of pRb increases DDB2 mRNA and protein levels, together with ability of these cells to repair DNA damage. The latter is associated with more efficient CPD removal relative to that in pRb-expressing cells (Prost et al., 2007).

Solid tumours frequently exhibit hypoxic cores, which contribute to genetic instability within the tumour microenvironment (Bindra et al., 2005). This is partly due to decreased expression of DNA mismatch genes (MLH1 and MSH2), as well as repair genes (RAD51 and BRCA1). E2F factors can also be involved in the downregulation of some of these repair genes, in apparent contrast to their pro-repair roles in other circumstances. Specifically, hypoxic conditions result in the dephosphorylation of the pRb family member p130, which then associates with E2F-4 in the nucleus. This complex can efficiently bind to E2F sites on the RAD51 and BRCA1 promoters, thus interfering with their transcription (Bindra et al., 2005). Thus, E2F factors can positively or negatively regulate DNA repair, depending on cellular context. Given that E2F-4/p130 complexes are also important for cell cycle exit, a balance must exist between these two outcomes, which is essential to avoid increased genetic instability in transformed cells and their clonal expansion.

2.4 Role of E2F-1 in senescence-associated DNA damage

Senescence is defined as irreversible cell cycle arrest, which occurs both in cultured cells and *in vivo* (Lanigan et al., 2011). Senescence has been recognized as a key mechanism that acts as a barrier to tumour formation and progression. Thus, in spite of any DNA damage that may exist in a long-lived cell, if this cell is senescent it will not undergo clonal expansion to generate daughter cells with altered DNA. A number of molecular mechanisms control cellular senesce, and the E2F/pRb pathway is a key component (Lanigan et al., 2011). Under normal circumstances, the frequency of DNA mutations increases with age. DNA mismatch mutation repair is very efficient in mesenchymal cells from young individuals, as well as in embryonic fibroblasts (Chang et al., 2008). In contrast, these mechanisms are less efficient in senescent cells, in which MSH2 expression is decreased. Associated with these abnormalities is the inhibition of E2F-1 transcriptional activity, which leads to repression of MSH2 gene transcription. Thus, E2F-1 activity is essential to maintain normal capacity of cells to repair mismatch mutations. Whether the reduced activity of E2F-1 also increases the risk of transformation in senescent cells probably depends on cell context, extent of DNA damage, and presence of other oncogenic stimuli.

2.5 Role of E2F/DP interactions in DNA repair

The interactions between E2F-1 through -6 and their partner DP proteins are essential for normal transcriptional activity, and can also contribute to abnormal regulation of DNA repair. Again, depending on the exact context, E2F/DP interactions can positively or negatively modulate DNA repair. For example, following DNA damage by a variety of agents, including doxorubicin, etoposide and UV radiation, the abundance of DP-4 protein is substantially increased, replacing other DP proteins in E2F-1-containing complexes (Ingram et al., 2011). As a result, the capacity of E2F-1 to bind target promoters is strongly reduced, which can result in downregulation of cell cycle regulatory and/or DNA repair genes.

A positive modulatory role in DNA nucleotide excision repair through inhibition of repressor E2F complexes has been recently attributed to p14[Arf] (Dominguez-Brauer et al., 2009). Specifically, DNA damage induces p14[Arf] expression, which directly binds to DP-1, disrupting its interactions with E2F-4. As a result, repressive E2F-4/p130 complexes lose their ability to bind promoters of genes such as XPC, resulting in upregulation of their expression.

To-date, multiple mechanisms that regulate E2F-1 activity at the post-transcriptional level have been identified, although only a handful has been studied in the context of DNA repair. These forms of regulation of E2F-1 activity can have important consequences on its ability to modulate DNA damage responses, as discussed below.

3. Role of miRNAs in E2F regulation of cell growth and DNA repair

MicroRNAs (miRNAs) are short nucleotide sequences (~21-24nt) that pair with the 3'-untranslated regions of target mRNAs. They negatively regulate gene expression by mediating degradation of the target mRNA, or by inhibition of protein translation (Almeida et al., 2011). Small miRNAs regulate many cellular processes, such as apoptosis, differentiation, and proliferation. They are upregulated in many human disorders, including cancer and neurological diseases (Almeida et al., 2011). To-date, approximately 800 miRNAs have been identified in humans. A single miRNA can target multiple mRNAs (Griffiths-

Jones, 2004). Consistent with their role in cancer, miRNAs control cell proliferation by regulating E2F factors and, thereby, expression of genes that are important for cell cycle progression.

The E2F signalling pathway is regulated by many different types of miRNA clusters, including *miRNA-17-92, miRNA-106b-25, miRNA-34, miRNA330-3p, miRNA-128, miRNA-195, miRNA-37 and miRNA-193a*, as described below.

3.1 Growth-promoting miRNAs

O' Donnell et al. were the first to provide evidence that E2F is a target for miRNAs (O'Donnell et al., 2005). They showed that miRNA-17 and miRNA-20a decrease E2F-1 translation efficiency. This type of regulation prevents uncontrolled activation of E2F-1 during normal cell cycle progression. Disruption of miRNA-17 and miRNA-20a leads to improperly timed expression of E2F-1, resulting in the accumulation of DNA double strand breaks (Pickering et al., 2009).

An auto-regulatory loop between E2F-1 and E2F-3 and the miRNA-17-92 clusters has been demonstrated. E2F-1 and E2F-3 bind to and upregulate the transcription of the miRNA-17-92 cluster. In turn, the miRNA-17-92 cluster downregulates expression of these two transcription factors (Sylvestre et al., 2007; Woods et al., 2007). This negative feedback loop is important to prevent the accumulation of E2F-1 and E2F-3, thereby allowing proper progression of the cell cycle, preventing apoptosis. Another negative feedback loop has been observed between the miRNA-106b-25 clusters and E2F-1 (Petrocca et al., 2008). miRNA106b and miRNA93 downregulate E2F-1 expression. Reciprocally, transcription of these miRNAs is activated by E2F-1. In this manner, properly timed expression of E2F-1 during the G1/S transition is maintained, as the presence of these miRNAs prevents continuous E2F-1 expression throughout the cell cycle, which would induce apoptosis.

3.2 Tumor suppressor miRNAs

The E2F signalling pathway is also regulated by the miRNA-34 family of clusters (Tazawa et al., 2007). miRNA-34b decreases E2F-1 and E2F-3 transcript levels in a p53-dependent manner, inhibiting cell proliferation and inducing senescence in tumour cells. This demonstrates that miRNAs can function as tumor suppressors. A similar role has been suggested for miRNA-195 (Xu et al., 2009), miRNA-128 (Cui et al., 2010), miRNA-330-3p (Lee et al., 2009) and miRNA193a (Kozaki et al., 2008).

Overexpression of miRNA-195 causes cell cycle arrest at the G1/S boundary, by interfering with the expression of cell cycle regulatory proteins, such E2F-3, Cyclin D1 and cyclin-dependent kinase 6 (CDK6). As a result, pRb remains hypophosphorylated, allowing activation of E2F-dependent target genes (Xu et al., 2009). Exogenous expression of miRNA-127 in glioma cells represses E2F-3a translation, thereby decreasing cell proliferation (Cui et al., 2010). Similarly, in oral squamous cell carcinoma, miRNA193a significantly represses cell growth and down-regulates E2F-6 translation (Kozaki et al., 2008).

3.3 Role of miRNAs in modulation of DNA repair by E2F-1

Several miRNA clusters, including *mir17-92, mir-106a-92* and *mir106b-25,* are downregulated by p53 via E2F-dependent mechanisms. This leads to decreased proliferation and/or promotes senescence in normal and transformed cells (Brosh et al., 2008). In addition, in response to mitogenic stimulation, E2F-1 activates transcription of the miRNA clusters *let-*

7a-d, *mir-15b-16-2* and *mir-106b-25* during the G1/S transition (Bueno et al., 2010). These miRNAs, in turn, regulate E2F-1 activity. In their absence, E2F-1 induces entry into S phase, but also DNA damage. Indeed, E2F-1 and other oncogenes can induce stalling and collapsing of DNA replication forks, leading to the formation of DNA double-strand breaks (Halazonetis et al., 2008). Thus, *let-7a-d*, *mir-15b-16-2* and *mir-106b-25* play key roles in prevention of DNA damage and replicative stress associated with abnormal regulation of E2F-1 (Zhang et al., 2011).

4. Regulation of E2F-1 by post-translational modifications

Another mode of E2F regulation that fine-tunes cell cycle progression and DNA repair occurs at the post-translational level. Post-translational modifications identified in E2F-1 include phosphorylation, acetylation, methylation & ubiquitination. These modifications can exert either activating or inhibitory effects on E2F-1 transcriptional activity.

4.1 Acetylation

E2F-1 is acetylated at three highly conserved lysine residues (K117, K120 and K125) by the p300/CREB-binding protein (CBP) or by p300/CBP-associated factor (P/CAF) acetyltransferase (Martinez-Balbas et al., 2000; Marzio et al., 2000). P/CAF directly interacts with E2F-1 through its adenosine deaminase 2 (ADA2) binding domain (Martinez-Balbas et al., 2000). Acetylation of E2F-1 allows for marked stabilization and significant increase in E2F-1 protein levels. This leads to an increase in transcriptional activation of E2F-1 target genes (Farhana et al., 2002; Martinez-Balbas et al., 2000).

Increases in E2F-1 protein levels upon DNA damage are partly due to cell type-specific acetylation (Blattner et al., 1999; Meng et al., 1999; Zhu et al., 1999). For example, adriamycin-mediated treatment induces E2F-1 acetylation in human glioblastoma T98G cells (Pediconi et al., 2003) , but not in HeLa cells (Ozaki et al., 2009). In response to DNA damage, E2F-1 switches to activate pro-apoptotic gene expression, rather than cell cycle progression. This change requires E2F-1 acetylation and recruitment to promoters of pro-apoptotic target genes, such as p73 (Pediconi et al., 2003). P/CAF, but not p300, is required for E2F-1 stabilization upon DNA damage by doxorubicin (Ianari et al., 2004). On the other hand, overexpression of p300 can be sufficient for acetylation and stabilization of E2F-1 in cells treated with camptothecin, a drug that causes double strand break during DNA replication (Galbiati et al., 2005). The distinct actions of these two acetyltransferase can thus determine the outcome of cellular responses by modulating cellular DNA damage checkpoints (p300) or apoptotic events (P/CAF). The stabilization of E2F-1 by acetylation could also allow it to directly interact with activating signal cointegrator-2 (ASC-2), a mitogenic transcription factor co-activator that regulates cellular proliferation and cell cycle progression (Kong et al., 2003).

4.2 Phosphorylation

E2F-1 is phosphorylated on several residues, giving rise to modifications that can alter different functional aspects. E2F-1 was first identified as a substrate for phosphorylation in a cell-free system (Bagchi et al., 1989). This post-translational modification interfered with E2F-1 DNA binding activity. Consistent with these observations, E2F-1 and E2F-3 showed decreased DNA binding capacity upon phosphorylation by cyclin A-activated cyclin-dependent kinase 2 (cdk2) (Dynlacht et al., 1997; Krek et al., 1995). Complexes containing

cyclin A, cdk2, E2F-1, and DP-1 are formed during Late S-phase to terminate E2F-dependent DNA binding and transcription, and enable orderly S-phase progression (Krek et al., 1995). In the absence of cyclin A-cdk2 activity, there is decreased E2F-1 phosphorylation and increased DNA binding activity (Li et al., 1997). This results in S-phase delay and/or arrest, by mechanisms that involve transcriptional activation of E2F-dependent cell cycle checkpoint genes. Together, these data demonstrate that E2F-1 phosphorylation is essential for timely activation of E2F-1 function and orderly cell cycle progression and survival. A second proline-directed kinase, c-Jun N-terminal protein kinase (JNK1), can phosphorylate E2F-1 in response to stress stimuli mediated by tumor necrosis factor-alpha, decreasing its ability to bind DNA and activate target gene transcription (Kishore et al., 2003).

Following DNA damage, Chk2 and ATM phosphorylate E2F-1 on Ser364 and Ser31, respectively (Lin et al., 2001). E2F-1 phosphorylated on Ser31 subsequently interacts with 14-3-3τ (Wang et al., 2004). This interaction prevents E2F-1 association with the **S**KP1-**C**ullin-**F**-box/ S-phase kinase-associated protein 2 (SCF^{Skp2}) ubiquitin ligase. As a result, E2F-1 is not ubiquitinated and is protected from degradation. The net result of phosphorylation of E2F-1 at Ser31 and Ser364 after DNA damage is activation of the pro-apoptotic gene p73, as well as accumulation of p53 through upregulation of p19^{ARF} expression. The latter protein inhibits ubiquitination and degradation of p53, inducing apoptosis (Weber et al., 1999). In additon, the ATM and Chk2 promoters are activated by E2F-1, thereby forming a positive feedback pathway that promotes apoptosis (Berkovich and Ginsberg, 2003) .

The phosphorylation of E2F can also affect its ability to interact with other proteins. In *Drosophila melanogaster*, phosphorylation of E2F-1 and E2F-2 enhances their ability to interact with the SCF^{slmb} ubiquitin ligase complex, targetting it for degradation during S phase (Heriche et al., 2003). *In vitro*, E2F-1 is phosphorylated at Ser337 by complexes containing p34^{cdc2} and cyclin B (Dynlacht et al., 1997). The significance of this finding is not clear, as E2F-1 phosphorylation on these residues occurs during late G1 phase, and is mediated by cyclin D-cdk4 complexes (Mann and Jones, 1996). Phosphorylation of E2F-1 at Ser332 and Ser337 enhances E2F-1 interactions with the adenovirus E4 protein, simultaneously attenuating its ability to bind pRb (Fagan et al., 1994). Upon adenovirus infection, the enhanced interaction between E2F-1 and E4 increases the efficiency of E2A transcription, which is required for viral DNA replication (Hardy et al., 1989).

Changes in phosphorylation status also modulate the activity and subcellular localization of E2F-4 and E2F-5, although these changes are unlikely to be mediated by cyclin A-dependent cdk activity (Dynlacht et al., 1997). Regulation of E2F-4 and E2F-5 by phosphorylation is important during entry into quiescence associated with cell differentiation, but varies depending on the cell type. For example, hypophosphorylated forms of E3F-4 efficiently associate with p130 in the nucleus, forming transcriptional repressor complexes associated with growth arrest in muscle cells (Shin et al., 1995). In contrast, in human intestinal crypt cells, hypophosphorylated E2F-4 is imported into the nucleus in response to mitogenic stimuli or inhibition of p38 MAP kinase, where it activate genes necessary for S phase entry (Deschenes et al., 2004).

E2F-5 is phosphorylated by cyclin E/cdk2 complexes on Thr251 in the transcriptional activation domain, stimulating cell cycle progression (Morris et al., 2000). This modification stabilizes E2F-5 interaction with the co-activator p300/CBP, resulting in transcription of genes required for DNA synthesis. Significantly, phosphorylation of E2F-5 at Thr251 does not affect its DNA binding activity, intracellular localization or ability to interact with pRb family proteins.

In epidermal keratinocytes, E2F-1 is tightly regulated during normal proliferation and differentiation (Ivanova and Dagnino, 2007; Ivanova et al., 2009; Wong et al., 2003). E2F-1 is localized in the nucleus in undifferentiated keratinocytes, but differentiation induces its export to the cytoplasm, where it is degraded. The signaling pathways involved in E2F-1 turnover in differentiating keratinocytes involve activation by Ca^{2+} of protein kinase C eta and delta, followed by activation of p38β. The latter appears to phosphorylate E2F-1 at Ser403 and Thr433. Once E2F-1 is phosphorylated, it is exported from the nucleus in a CRM1-dependent fashion, and degraded in the proteasome. This sequence of events involving E2F-1 phosphorylation, ubiquitination, nuclear export and subsequent degradation is required for proper keratinocyte differentiation (Ivanova et al., 2006; Ivanova and Dagnino, 2007; Ivanova et al., 2009).

E2F-1 degradation subsequent to phosphorylation also occurs in HeLa cells. Specifically, phosphorylation of E2F-1 at Ser403 and Thr433 by TFIIH-cdk7 targets E2F-1 for degradation during S phase (Vandel and Kouzarides, 1999). Phosphorylation at Ser403 is also induced upon DNA damage (Real et al., 2010). Ser403 and Thr433 in E2F-1 are also phosphorylated by glycogen synthase kinase-3β (GSK3β) in HEK293T cells (Garcia-Alvarez et al., 2007). In U2OS osteosarcoma cells treated with doxorubicin, Ser403 is phosphorylated, but is not a substrate of either p38 MAP or GSK3β kinases (Real et al., 2010). Under these conditions, phosphorylation of Ser403 results in changes in E2F-1 target selectivity. Thus, the mechanisms and consequences of E2F-1 phosphorylation on Ser403 appear to be cell-type and context dependent (Ivanova et al., 2009).

4.3 Methylation

Lysine methylation plays critical regulatory roles for histones and non-histone proteins (Huang et al., 2008). The consequences of methylation on E2F-1 activity are controversial at present. E2F-1 is methylated by Set9, a histone H3 methyltransferase, at Lys185, both *in vitro* and in cultured cells (Kontaki et al., 2010, Xie et al., 2011). It has been reported that DNA damage in p53-deficent H1299 lung carcinoma cells is associated with loss of E2F-1 methylation by the lysine-specific demethylase 1(LSD1). Demethylation stabilizes E2F-1, allowing its upregulation of p73. Importantly, methylation of E2F-1 at Lys185 impairs its acetylation and phosphorylation on Ser364, targeting E2F-1 for ubiquitination and degradation in doxorubicin-treated cells (Kontaki et al., 2010). In stark contrast, methylation of E2F-1 at Lys185 by Set9 in U2OS and HCT116 cells treated with adriamycin resulted in E2F-1 stabilization and cell apoptosis (Xie et al., 2011). The reasons for these pronounced discrepancies are not clear.

4.4 Ubiquitination

Many studies have shown that the expression of E2F-1 is regulated by the ubiquitin proteasome pathway, and that E2F-1 is protected from degradation by binding to pRb (Campanero and Flemington, 1997; Hateboer et al., 1996; Hofmann et al., 1996). In mammalian and plant cells, E2F-1 is regulated at the S/G2 phases of the cell cycle through ubiquitination by the SCF[SKP2]-dependent pathway (del Pozo et al., 2002; Marti et al., 1999). *In vitro*, ROC-cullin ligase ubiquitinates E2F-1 in a Skp2-independent manner. Further, phosphorylation of E2F-1 by cyclin A/cdk complexes does not affect E2F-1 ubiquitination (Ohta and Xiong, 2001). Another E3 ubiquitin ligase complex, the anaphase-promoting complex or cyclosome (ACPC/C), also regulates E2F-1 stability during late S phase (Peart et

al., 2010). The presence of multiple E3 ligases that interact with and mediate degradation of E2F-1 enables orderly control of E2F-1 expression under multiple circumstances.

5. Regulation of E2F activity by protein-protein interactions

The first type of protein-protein interactions shown to modulate E2F transcriptional activity included association with the retinoblastoma family of proteins (pRB, p107 and p130). pRB is a key regulator of E2F-1, -2 and -3 activity and G1/S-phase transition (Weintraub et al., 1995). The importance of pRb regulation of E2F is evidenced by the fact that a majority of human tumours exhibit inactivating alterations in the pRb pathway (Nevins, 2001). Subsequent studies have revealed thet E2F forms complexes with a multitude of additional proteins, underlining the levels of complexity of E2F regulation.

Protein-protein interactions also appear to assist or provide target specificity to E2F under certain conditions. This effects appear to involve cooperative interactions between E2F and other transcription factors, mediated by binding to neighbouring consensus sites on target promoters. Consensus binding sites for various transcription factors have been identified in the promoters of a subset of E2F target genes. These sites are generally adjacent to the E2F binding sites, and include recognition sequences for YY1, TFE3, and C/EBPα (Schlisio et al., 2002; van Ginkel et al., 1997). These sites possess biological significance, and assist E2F in binding to its consensus sequence. This determines the specific phase of the cell cycle in which E2F activates such promoters. In addition, as these other transcription factors do not interact equally well with all E2F members, they constitute a mechanism of activation of individual E2F factors (Giangrande et al., 2003; Schlisio et al., 2002).

5.1 Retinoblastoma family proteins

pRb binds predominantly to E2F-1, E2F-2, and E2F-3, blocking their transactivation domains (Flemington et al., 1993; Xiao et al., 2003). Under certain circumstances, such as during responses to transforming growth factor-beta in certain cell lines, pRb also binds E2F-4 and represses transcription (Yang, et al. 2008). The pRb family of proteins can also repress transcription of E2F target genes by recruiting other factors, such as histone deacetylases, thus creating transcriptional repressor complexes (Dick, 2007; Morrison et al., 2002; Herrera et al., 1996). pRb is, in turn, regulated by cyclin and cyclin-dependent kinases (Cdk),which deactivate pRb through phosphorylation. Specifically, Cyclin D/Cdk4 and Cyclin E/Cdk2 complexes phosphorylate pRb in the G1 phase of the cell cycle, allowing E2F-1, E2F-2 and E2F-3 to activate target genes (Connell-Crowley et al., 1997; Smith et al., 1996). The other pRb family proteins, p107 and p130, generally bind to E2F-4 and E2F-5, and function to modulate their nucleocytoplasmic shuttling during different periods of the cell cycle. Specifically, E2F-4 and E2F-5 translocate into the nucleus outside of the G1 and S-phases, and act as transcriptional repressors in complexes containing p107 and p130 (Ginsberg et al., 1994; Moberg et al., 1996) (Hijmans et al., 1995) (Guo et al., 2009).

5.2 DP proteins

Optimal binding of E2F to DNA requires cooperative interactions with a member of the other subfamily of E2F proteins, the DP (Dimerization Partner) family. In fact, with the exception of E2F-7 and -8, all functional E2F complexes identified contain a member of the E2F family associated with a DP protein. The DP family is composed of three known members, DP-1 (with isoforms DP-1α and DP-1β), DP-2 (and its mouse orthologue DP-3),

and DP-4 (Helin and Harlow, 1994; Milton et al., 2006; Ormondroyd et al., 1995). Different DP proteins have distinct modulatory effects on E2F. For example, DP-1β can mediate E2F translocation to the nucleus, whereas DP-1α, which shows reduced affinity for E2F, participates in E2F nuclear export and translocation to the cytoplasm. In this manner, DP-1α indirectly represses the ability of E2F-1 to activate transcription (Ishida et al., 2005). DP-4 can mediate transcriptional repression as well (Milton et al., 2006). Furthermore, a growing body of evidence shows that other proteins that interact with DP factors, such as C/EBP, TRIP-Br and SOCS3, can modulate E2F activation of gene transcription (Masuhiro et al., 2008; Zaragoza et al., 2010).

5.3 C/EBP

CCAAT/Enhancer Binding Protein (C/EBP) factors are generally characterized as effectors of cellular growth arrest. Within the C/EBP family, C/EBPα has been shown to associate with and repress E2F-1 (Wang et al., 2007). This interaction has been demonstrated through co-immunoprecipitation assays and is independent of pRb family proteins. Rather, it requires the presence of DP-1 or DP-2 (Zaragoza et al., 2010).

The effect of C/EBP repression on E2F activity has been demonstrated in multiple tissues. In primary murine keratinocytes, C/EBPα and β are upregulated as these cells differentiate and move from the basal to the suprabasal layers of the epidermis. Further, the repression of E2F target genes via the action C/EBP is necessary for proper differentiation (Lopez et al., 2009). Interactions between C/EBP and E2F also play important roles during senescence. Indeed, C/EBPα and HDAC1 are recruited to hepatic DNA from older, but not young, mice (Wang et al., 2008). Recruitment of these two factors is accompanied by decreased transcription of E2F target genes.

In mouse 3T3-L1 preadipocytes, C/EBPα, but not C/EBPβ, disrupts E2F-p107 and induces E2F-p130 complexes, leading to decreased proliferation, likely involved in preadipocyte differentiation (Timchenko et al., 1999).

In mouse hepatocytes devoid of C/EBPβ, E2F target genes are repressed and DNA synthesis is severely impaired. In these cells, C/EBP β interacts with E2F-1, facilitating recruitment of CBP and p300 to E2F target genes. The recruitment of these multiprotein complexes results in upregulation of E2F targets involved in cell proliferation (Wang et al., 2007). C/EBPβ is also required for expression of E2F-3 and S-phase progression in uterine epithelial cells (Ramathal et al., 2010). In primary epidermal keratinocytes, C/EBPα interferes with DNA synthesis in response to DNA damage (Johnson, 2005). However, the mechanisms involved are not fully undertood. It has been proposed that C/EBPα functions with E2F/pRb complexes to repress transcription of S-phase genes. In neuroblastoma cells, C/EBP is involved in induction of apoptotic gene transcription by E2F-1 (Marabese et al., 2003).

5.4 SOCS3

The Suppressor of Cytokine Signaling (SOCS) family of proteins act as negative feedback regulators of the JAK-STAT pathway. Recently, SOCS factors have also been shown to associate with DP-1 and DP-3. SOCS3 inhibits transcriptional activation of E2F target genes and cell cycle progression. The mechanisms involved in this repression include SOCS3 inhibition of E2F/DP dimerization, thus preventing the formation of the E2F DNA-binding complexes (Masuhiro et al., 2008).

5.5 TRIP-BR1

The Transcriptional Regulator Interacting with the PHD zinc finger and/or the Bromodomain-1 (TRIP-Br1) protein (also known as p34) is a transcriptional modulator that directly interacts with DP-1, as well as with the co-activators p300/CBP and KRIP1(Hsu et al., 2001). As such, TRIP-Br co-activates E2F responsive genes, such as B-myb, in U2OS osteosarcoma cells, an ability potentiated by KRIP1. This effect is impaired by pRb. TRIP-Br1 also interferes with deactivation of Cyclin D/Cdk4 complex by p16^{INK4}, effectively activating E2F by inhibiting pRb (Sim et al., 2004).

5.6 p110 CUX1

Cut homeobox 1 (CUX1) proteins are transcription factors that can either activate or repress transcription. In particular, the CUX1 isoform p110 can stably interact with DNA and promote entry into the S-phase of the cell cycle (Truscott et al., 2008). P110 CUX1 interacts with E2F-1 or E2F-2, stimulating their recruitment to the DNA polymerase α gene promoter, in a manner that requires ability of E2F to bind DNA. Further, common targets for E2F and p110 CUX1 include genes involved in cell cycle progression, DNA repair and replication (Truscott et al., 2008).

5.7 YY1

The transcriptional repressor YY1 can bind to target sites adjacent to E2F binding elements in the promoters of genes such as *Cdc6* (Schlisio et al., 2002). In addition, the YY1 accessory protein Ring1 and YY1 binding protein (RYBP) can interact with E2F-2, -3 and -4 to synergistically enhance binding of E2F-2 and -3 (but not of E2F-1). In this manner, YY1 and RYBP not only enhance the binding and transcription of E2F to certain promoters, but also add specificity.

5.8 TFE3

Studies of the p68 promoter have shown that transcription factor E3 (TFE3) operates in a similar manner to YY1. Thus, the E Box bound by TFE3 and the E2F consensus sequence occur in close proximity in the *p68* promoter. TFE3 and E2F-3 bind to those sites cooperatively (Giangrande et al., 2003; Giangrande et al., 2004). This interaction requires E2F-3, but not TFE3 binding to DNA. Although a direct interaction between these two proteins was not be demonstrated, these two factors likely work together in a larger protein complex, or interact temporarily to recruit one another to the *p68* promoter.

6. Regulation of E2F activity by viral oncoproteins

Viruses work by hijacking the cellular machinery of their host cell, to facilitate their replication. Hence, it is not surprising that constitutive activation of E2F, which induces cell transition into a state of DNA replication (S-phase), is a critical step in the viral modification of infected cell functions.

6.1 Human papillomavirus protein E7

The human papillomaviruses (HPV) are commonly known oncoviruses. This notoriety is due to their ability to activate E2F proteins, causing rapid and unregulated progression through the cell cycle (Lee et al., 1998). HPV couples this action with deactivation of

pathways that act as fail-safe mechanisms for E2F activity, such as p53-mediated apoptosis (Moody and Laimins, 2010). The key HPV viral protein involved in activating E2Fs is E7. This protein carries an LXCXE domain characteristic of proteins that associate with pRb family proteins (Lee et al., 1998). In this manner, E7 proteins bind to pRb, p107 and p130, dissociating them from E2F factors. The mechanisms involved in this effect include blockade by E7 of the pRb-E2F binding domain (Lee et al., 1998). As a result, E2F species bind to and activate target genes without the possibility of repression. E7 also induces pRb proteasomal degradation, by increasing its ubiquitination (Moody and Laimins). Furthermore, there is evidence to indicate that E7 also binds to p300/CBP, allowing this acetyltransferase to facilitate and rapidly increase the transcription of E2F target genes (Bernat et al., 2003).

6.2 SV40 large-T antigen

The simian virus 40 (SV40) genome encodes a protein that shares some characteristics with HPV E7, termed large T-antigen. Similar to HPV E7, large-T antigen has a LXCXE domain, which can bind all three pRb family proteins, leading to release of free E2F and expression of its target genes (DeCaprio, 2009). In addition, large-T antigen binds preferentially to the hypophosphorylated form of pRb, present during the G1-phase of the cell cycle (Ludlow et al., 1989). The characterization of the interactions between large-T antigen and complexes containing p130 or p107 and E2F-4 has been central to understanding the mechanisms involved in deactivation of pRb family proteins by this viral factor (Sullivan et al., 2000). Dissociation of p107 or p130 from E2F also requires Large-T antigen interactions with the J type of chaperone protein Hsc70 and ATP.

Similar to HPV E7, large-T antigen binds to p300/CBP through its C-terminus (Eckner et al., 1996). This interaction is likely involved in histone acetylation and transcriptional activation of E2F target genes. Significantly, mutations in the C-termimus of large-T antigen impair its ability to bind p300/CBP, but are without effect on its capacity to disrupt pRb binding to E2F (Nemethova et al., 2004).

6.3 Adenovirus E1A

Adenovirus protein E1A functions in a similar manner to HPV E7 and SV40 large-T antigen. E1A interacts with multiple cellular proteins, including the pRb family and p300/CBP (Raychaudhuri, 1991; Liu, 2007). X-ray crystallographic characterization of E1A has revealed that its N-terminal domain competes with the transactivation domain of E2F for binding to pRb. This induces a decrease in E2F binding to pRb by competition (Liu, 2007). Similar to other viral oncoproteins, E1A also has an LXCXE domain that binds to pRb, p107 and p130 (Dyson, 1992). E1A also binds the 400-kDa protein p400, which mediates further interactions with TRRAP/PAF400, along with the DNA helicase TAP54α/β). Together, these proteins form a chromatin remodeling complex, which contributes to cell transformation and activation of E2F target genes that mediate viral DNA replication (Liu, 2007).

6.4 Human parvovirus NS1

Human parvovirus B19 (B19V) is the only pathogenic human parvovirus, and it targets cells of the erythroid lineage, expecially erythroid progenitors (Wan et al., 2010). The B19V protein NS1 (nonstructural protein 1) interacts with E2F-4 and E2F-5, inducing their nuclear accumulation and G2 arrest, necessary for viral replication (Wan et al., 2010). Simultaneously, NS1 also decreases expression of E2F-1, E2F-2, and E2F-3, resulting in

transcriptional repression of genes necessary for the G2/M transition. Thus, B19V targets cells for arrest in the G2 phase by altering E2F activity, indicating the importance of this family of transcription factors in all phases of the cell cycle and multiple aspects of cell cycle progression and DNA replication and repair.

7. Conclusions

A large body of work has been focused on identifying the mechanisms that regulate E2F activity and its consequences on induction of DNA repair. As a result, it has become apparent that E2F activity is complex, and is regulated at multiple levels, including transcription, post-translational modifications and protein-protein interactions. However, understanding of how different post-translational modifications modulate E2F interactions with other proteins, allowing it to form transcriptional activator or repressor complexes is in its infancy.

The biological roles of the various modes of E2F modulation go well beyond normal development and cell differentiation, implicate mechanisms of DNA repair as a central function, and are involved in the genesis of multiple pathologies.

Although pRb family proteins form the central backbone of E2F regulation, they are only one component. Studies of HPV proteins have shown that, in addition to E7, the proteins E5 and E6 are critical for the functional transformation of a cell. In the case of HPV, these proteins serve to deactivate the p53 pathway, preventing the pro-apoptotic responses normally switched on with abnormal activation of E2F. Other viruses encode proteins that serve a similar function. The identification and study of these proteins may provide key insights into the function of these viruses and the pathways that regulate E2F during normal tissue development and homeostasis, and affect DNA repair mechanisms to ensure viral replication.

8. Acknowledgement

9. References

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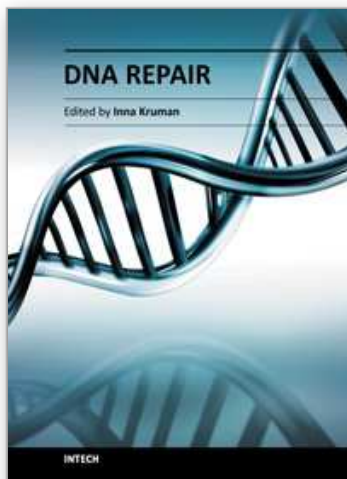
DNA Repair

Edited by Dr. Inna Kruman

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