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Emerging links between E2F control and mitochondrial function

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Abstract

The family of E2F transcription factors is the key downstream target of the Retinoblastoma tumor suppressor protein (pRB), which is frequently inactivated in human cancer. E2F is best known for its role in cell cycle regulation and triggering apoptosis. However, E2F binds to thousands of genes and, thus, could directly influence a number of biological processes. Given the plethora of potential E2F targets, the major challenge in the field is to identify specific processes in which E2F plays a functional role and the contexts in which a particular subset of E2F targets dictates a biological outcome. Recent studies implicated E2F in regulation of expression of mitochondria-associated genes. The loss of such regulation results in severe mitochondrial defects. The consequences become evident during irradiation-induced apoptosis, where E2F-deficient cells are insensitive to cell death despite induction of canonical apoptotic genes. Thus, this novel function of E2F may have a major impact on cell viability, and it is independent of induction of apoptotic genes. Here, we discuss the implications of these findings in cancer biology.

Introduction

The E2F/DP heterodimeric transcription factors (referred to as E2F) are best known for their ability to regulate the G1-to-S transition (1). The transcriptional activity of E2F is inhibited by the retinoblastoma protein (pRB), which blocks S-phase entry and, in response to anti-proliferative signals, promotes cell-cycle exit. While control by pRB and E2F is important during normal development their prominent roles in cancer initiation explains the continuous interest in these proteins. It is generally accepted that pRB is functionally inactivated in most tumor cells. Thus, tumor cells are not only unable to respond to cell cycle exit cues, but are also driven into inappropriate proliferation by unrestrained E2F activity (2).

This classical view of E2F is so appealing because it provides a simple and elegant explanation of why inactivation of the Rb pathway is an obligatory event in cancer. However, E2F has other roles beyond cell-cycle control. For example, the E2F1 member of the E2F family has been linked to apoptosis induction. This is not an indirect consequence of abnormal cell proliferation since E2F1 induces cell death by a distinct transcriptional program that includes canonical apoptotic genes such as *APAF1*, *p73*, caspases *CASP3 and CASP7*, and *CDKN2A*^{ARF} (3). This property of E2F1 is thought to be important in triggering apoptosis in irradiated cells in response to DNA damage.

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Besides cell cycle and apoptotic genes, genome-wide location studies have revealed that E2F binds to promoters of thousands of genes (4) and, thus, could directly influence a number of biological processes (Figure 1). This highlights the major challenge in determining whether E2F binding to its putative targets is important to impact a specific function. Combining genome-wide data with downstream genetic analysis using model organisms is one way to address this question. In this respect, *Drosophila* is particularly advantageous due to high conservation yet relative simplicity of the RB pathway (5). Unlike large multi-gene mammalian E2F and DP families, there are only two E2F genes, *dE2f1* and *dE2f2*, and one DP gene, *dDP*, in *Drosophila* genome. Both dE2f1 and dE2f2 heterodimerize with dDP and require dDP to bind to DNA. Thus, inactivation of *dDP* phenocopies the loss of both *dE2f1* and *dE2f2*. Interestingly, in flies, loss of E2F control is permissive for most of animal development since *dDP* single mutant or *dE2f1 dE2f2* double mutants develop normally throughout embryonic and larval stages. Therefore, the phenotype of *dDP* mutant larva reflects the results of E2F inactivation without unwanted consequences of developmental defects.

E2F regulates mitochondrial function in *Drosophila*

An important clue to understanding the plethora of E2F functions came from integrated bioinformatics (6). The authors identified generation of energy, protein transport and metabolism as non-canonical ontology categories for genes with E2F binding sites. Several recently published reports support this idea. For example, E2F targets were shown to significantly overlap with genes that are transcriptionally regulated by key factors of mitochondrial biogenesis: nuclear respiratory factors NRF1 and NRF2/GABP (7,8) and by HCF1-Ronin (9). E2F4 binds to genes with a function in electron transport chain, *COX8*, *CYB5-M*, *CYP51A1*, *FDXR* and *SUCLG1* (7). Intriguingly, E2F1 directly interacts with NRF2-β in yeast two-hybrid (9). Additionally, E2F may influence mitochondrial function by forming repressive complexes with pRB, which is known to affect the transcription level of NRF1/2 and PGC-1β.

The functional link between E2F and mitochondria was initially incited by the unexplained resistance of *dDP* mutant animals to irradiation-induced apoptosis. In response to irradiation, flies, like mammals, trigger a DNA damage checkpoint to halt cell cycle progression and induce an apoptotic transcriptional program. Previous genetic data implicated dE2f1 as an important factor in triggering cell death in irradiated cells. Using a *dDP* mutation to inactivate dE2f1, Moon et al. found that in response to irradiation, *dDP* mutants fail to trigger apoptotic cell death even though the DNA damage checkpoint is properly induced (10). As dE2f1 directly regulates apoptotic genes, the simplest explanation is that the loss of E2F control prevents the induction of apoptotic genes in irradiated cells. This turned out to be not the case since gene expression microarrays revealed that the DNA damage-induced apoptotic transcriptional program is properly activated in the irradiated *dDP* mutants (11). Furthermore many of the apoptotic genes were expressed at a higher level in *dDP* mutants than in control animals prior to irradiation and induced to an even greater level following irradiation. Thus, in contrast to the prevalent view in the field, the resistance of E2F-deficient cells to cell death was not due to an inability to induce the apoptotic gene expression program.

What then prevents cell death in *dDP* mutants? As a first step towards addressing this question, genes that are differentially expressed in response to irradiation only in *dDP* mutants but not in wild-type animals were subjected to gene set enrichment analysis. High enrichment was found only among downregulated genes in the irradiated *dDP* mutants, and all enriched gene sets were related to mitochondria-associated categories. Intriguingly, a significant number of mitochondria-associated genes were already downregulated in the *dDP* mutants even prior to irradiation. This is not a circumstantial result of the loss of *dDP* because many of these genes are direct E2F targets as evident by ChIP-seq and by ChIP-qPCR.

An obvious implication inferred by these data is that the reduced expression of mitochondria-associated genes in *dDP* mutants may lead to mitochondrial defects. Two lines of experiments support this idea (11). Firstly, immunofluorescence and electron microscopy of *dDP* mutants revealed a highly fragmented and reduced mitochondrial network with mitochondria appearing to be more globular and swollen. These abnormalities were accompanied by reduced mitochondrial membrane potential and reduced ATP generation. Secondly, down-regulation of several of mitochondria-associated E2F targets by RNAi *in vivo* was sufficient to phenocopy the fragmented mitochondrial phenotype in *dDP* mutants. Thus, reduced expression of these genes in *dDP* mutants has a causative effect on mitochondrial function.

The observation that the loss of E2F leads to mitochondrial dysfunction is significant because it may help explain the resistance of *dDP* mutants to DNA damage-induced apoptosis. There is an increasing body of evidence that mitochondria play an important role in triggering cell death in *Drosophila* (12,13). Although the precise molecular mechanism is not fully elucidated, it was shown that the pro-apoptotic proteins Hid and Rpr are localized to mitochondria and that this was a critical event in apoptosis induction. Furthermore, these studies showed that altering mitochondrial dynamics influences efficiency of apoptosis. Thus, the simplest explanation is that mitochondrial defects prevent DNA damage-induced apoptosis in irradiated *dDP* mutants. To test this, RNAi approach was used to knockdown mitochondria-associated E2F targets and this was shown to ultimately result in altered apoptotic response to irradiation (11). Significantly, in each case, the severity of the mitochondrial defects induced by the RNAi correlated well with the degree/level of apoptotic response to irradiation. These data suggest that downregulation of mitochondria-associated dE2F/dDP target genes is sufficient to induce both the dysfunctional mitochondrial phenotype and protect against irradiation-induced cell death. Both of these features are the hallmarks of the *dDP* mutant phenotype.

Conservation of E2F regulation of mitochondrial function in mammalian cells

What is the relevance of these findings to mammalian E2Fs? To address this question, E2F1 occupancy was studied at the promoters of human orthologs of *Drosophila* mitochondria-associated dE2F/dDP targets (11). ChIP-qPCR analysis showed that E2F1 was bound to these promoters in human osteosarcoma SAOS-2 cells. Importantly, the binding was highly specific, because it was lost when DP1 and DP2, the heterodimeric partners of E2F1, were

depleted by siRNA. Furthermore, expression of these genes was reduced when E2F1 was inactivated by expression of a dominant-negative form of E2F1. This result is consistent with another study that linked *E2F1* inactivation to misregulation of genes implicated in mitochondrial biogenesis and function (14). Significantly, like in *dDP* mutant flies, E2F inactivation in SAOS-2 cells resulted in strikingly similar mitochondrial defects and protection from DNA damage induced apoptosis (11).

Intriguingly, mammalian E2F1 was shown to play an important role in regulation of glycolysis and fatty acid metabolism in response to DNA-damaging agents. *E2f1* loss resulted in improved utilization of glucose and insulin responsiveness (15). One of the plausible mechanisms is induction of pyruvate dehydrogenase kinase 4 (PDK4). The mitochondrial PDK enzymes are “gatekeeper” inhibitors of the passage of pyruvate from glycolysis to the TCA cycle. PDK4 level is increased in obesity and diabetes and becomes acutely induced during fasting and starvation. *E2f1* deficient mice displayed protection from high-fat diet-induced diabetes, consistent with PDK downregulation (16).

Taken together, these results strongly argue that the loss of E2F/DP leads to remarkably similar phenotypes in flies and mammalian cells, indicating that the role of E2F/DP in the regulation of mitochondrial function is highly conserved. This idea parallels and expands on the previous studies in mammals that linked the RB pathway to mitochondrial biogenesis (7,17) and to the regulation of oxidative phosphorylation genes during the adaptive metabolic response (18).

Role of E2F in cellular functions that are highly dependent on intact mitochondria

Although the significance of E2F inactivation on mitochondrial function at first might be viewed as being restricted to cellular response to DNA damage, responses to other signals may be affected as well. Such perturbations could arise from the impact of mitochondrial dysfunction in E2F deficient cells on the levels of specific metabolites, oxygen consumption, reactive oxygen species (ROS) production or apoptosis. One anticipates that tissues that are particularly dependent on fully functional mitochondria would be especially sensitive to E2F inactivation and therefore the main challenge would be to identify such specific contexts. It is conceivable that cell cycle aspect of E2F control may not be important in these contexts as has been shown in muscle (19,20), adipose (21) and pancreatic β–cells (16). This could be because E2F is no longer able to activate cell cycle genes in these contexts. For example, in differentiated cells many *bona fide* E2F1 targets become hypermethylated at CpG islands in promoter regions, which will make them refractory to activation by E2F1. Additionally, a switch from an activator to a repressor, which was reported for E2F1-3 to occur during transition from a progenitor cell, may occur only on the subset of cell cycle genes (22); in contrast, the subset of metabolic genes may still be responsive to E2F1-mediated activation. This type of regulation will fulfill heavy demands on ATP generation and muscle mass growth concurrently with the cease of cell proliferation.

Studies of Duchenne muscular dystrophy (DMD) further point to such a non-canonical role of E2F1. DMD is associated with an increased level of E2F1, while genetic ablation of E2f1 in the mouse model of the DMD provided protection from muscular dystrophy (20). The effect was specifically linked to the improved phenotype of slow-twitch fibers that are characterized by oxidative metabolism. Intriguingly, the glycolytic muscle gastrocnemius in *E2f1/2* knockout mice was shown to undergo a switch to oxidative metabolism that is characterized by increased mitochondrial content and mitochondrial activity (21).

Another group of diseases where E2F regulation of mitochondrial function may play a causal role are neurodegenerative disorders such as Alzheimer's and Parkinson's diseases. The brains of these patients show both an increase in expression of cell-cycle proteins and oxidative stress damage (23). As there is currently no effective treatment for these diseases, finding the drug targets represents the main challenge in brain research. Given the neuroprotective effect of drugs inhibiting CDKs in experimental models, targeting the cell cycle re-entry and DNA damage holds promise as a common strategy in neurodegenerative diseases. Another example is a situation where excessive E2f1-dependent apoptosis is causal to the disease and leads to deafness in a mouse model. A recent study showed that inner ear cells undergo E2f1 dependent apoptosis as the result of mitochondrial stress (24).

Thus, accumulating evidence suggests that E2F has pathological roles, bringing interest in targeting RB/E2F in muscular and neurodegenerative diseases and in reducing mitochondrial stress during progressive hearing loss. Relating other cases of mitochondrial dysfunction to altered E2F activity may help in development of in future targeted cell therapeutics in other diseases.

Where do E2F and pRB converge on regulation of mitochondrial function?

A number of events, G1-to-S transition, differentiation, cold induction and fasting condition, are accompanied by pRB hyperphosphorylation that frees E2F. On the contrary, uncoupled electron transport results in pRB hypophosphorylation and formation of RB/E2F complexes (25). There is scarce evidence of consequences of such pRB/E2F dynamics, however, it might represent another important checkpoint for metabolic cycle and ROS production that is established through the RB pathway (26).

Progression to differentiation, when pRB forms protein complexes that can activate or repress transcription, affecting cell cycle exit, cell survival and expression of differentiation genes, might be particularly dependent on RB/E2F function in mitochondria. First, pRB was shown to couple mitochondrial biogenesis with cell cycle exit during erythropoiesis, and *Rb1* deficiency interfered with erythroid development (17). Second, pRB forms complexes with histone demethylase KDM5A/RBP2 during monocytic differentiation (27), and KDM5A directly binds to and represses promoters of genes encoding mitochondrial components (28). Decrease in KDM5A level in *RB* negative cells improves mitochondrial network, suggesting that pRB regulates KDM5A activity that impacts mitochondrial function. Third, studies of myoblasts induced for differentiation showed that in the absence of pRB family members, their survival was compromised, and could have been rescued

through increase in mitochondrial biogenesis, such as by treating with the pan-PPAR agonist bezafibrate (29).

pRB was shown to form repressive complexes with E2F1 in muscle and brown adipose tissue (21), but whether these complexes regulate mitochondrial genes is unknown. In *Drosophila*, dE2F directly binds the promoters of apoptotic genes *hid* and *rpr* and their increased expression in irradiated *rbf* mutants was dependent on dE2f1 (10). Notably, this correlated with increased sensitivity of *rbf* mutants to DNA damage-induced apoptosis. However, the loss of E2F did not prevent induction of the apoptotic transcriptional program. While this is unexpected result, it is in line with observations in human cells where potent response to DNA damage required pRB recruitment to promoters of apoptotic genes and formation of activating RB/E2F1 complexes (30). As pRB controls transition to differentiation in an E2F-independent manner (31), it needs to be determined under which cellular condition pRB and E2F converge on regulation of mitochondrial function.

Exploring the link between E2F and mitochondria for chemotherapy

As dE2f1 may boost expression of apoptotic genes in specific settings such as in *rbf* mutants after irradiation (10), the key question is to what extent is this mechanism conserved in mammalian cells? This is an important topic in cancer biology because conventional chemotherapy operates by induction of DNA damage and subsequent activation of the mitochondrial cell death pathway. There is a great range in the efficiency of activation of the mitochondrial cell death pathway among different cell types. Varied response to chemotherapy can be correlated with the extent to which mitochondria are “primed” to undergo apoptosis: cells with a higher threshold for apoptosis induction are more resistant to chemotherapy and visa versa (32). In several cancers, the mitochondrial priming correlates with clinical response to chemotherapy and thus, can help to explain tumor chemoresistance. Since inactivation of the RB pathway is an obligatory event in cancer, the impact of deregulated E2F activity on mitochondrial function, and ultimately on apoptosis induction, should be considered when evaluating the tumor response to chemotherapy.

Exploiting a novel link between E2F and mitochondria may help in devising strategies to sensitize tumors to drug combination. This idea is especially relevant given recent success of a small molecule CDK4/6 inhibitor PD0332991 in clinical trials (33). Phosphorylation of pRB by CDK4/6 releases the inhibitory effect of pRB on E2F and this results in activation of the E2F-dependent transcriptional program. Relevance of this program to metabolic control was shown in studies of mice with constitutively active CDK4 (Blanchet1146), where pRB phosphorylation was required for repression of mitochondrial regulators PGC-1α, TFAM and SDHA. Therefore, inhibition of CDK4/6 by PD0332991 prevents dissociation of pRB from E2F, thus, rendering E2F inactive. PD0332991 has been efficient in several cancer models and is currently in clinical trials in patients with mantle cell lymphoma (MCL) (34) and other malignancies (available from www.clinicaltrials.gov). MCL is a B-cell lymphoma that is frequently caused by the t (11; 14)(q13;q32) translocation that involves the *CCND1* gene encoding Cyclin D1. The translocation causes sustained Cyclin D1 production, resulting in CDK4/6 activation. A recent study showed that targeting mitochondria in MCL through E2F and its targets readily induces cell death, thus

representing an effective therapeutic strategy (35). Inhibiting E2F1 through PD0332991 may inadvertently affect mitochondrial function and promote cell survival. Although the impact of PD0332991 on mitochondria was not thoroughly investigated it is intriguing that pre-treatment with PD0332991 protects breast cancer cells from cell death induced by DNA damage agents (36). One of the approaches would be to modulate mitochondrial function in order to sensitize the cells to cytotoxic agents. Given that the effect of E2F inactivation on mitochondrial morphology is highly conserved in flies it would be appealing to use the strength of the *Drosophila* model system to rescue these mitochondrial defects in order to restore the sensitivity to apoptosis.

Implications and future directions

E2F regulates mitochondrial function in both flies and mammalian cells and this role of E2F is essential in DNA damage-induced apoptosis (Figure 1). It is important to note that the decreased apoptosis following E2F inactivation was not specific to irradiation as the type of DNA damage (11). Thus, other DNA damage-inducing treatments would have a similar outcome, and this should be considered in designing a therapy. Intriguingly, regulation of mitochondrial function also involves pRB that controls cell cycle exit and survival after exposure to differentiation condition. Very similar mechanisms may operate after exposure to other exogenous stimuli, including nutrition, physical exercise and various types of stress. Not surprisingly, activation of multiple growth factor receptor pathways, Myc, Ras, PI3K/Akt, Hippo, Wnt, Jak/STAT, in one way or another converge on RB/E2F pathway. This ensures that the increased cellular demands for energy production, new nucleotide, protein and lipid syntheses will be met. Our current knowledge about RB pathway is limited by identification of E2F targets in cancer cells and embryonic stem cells. There is much to be learnt on E2F genome occupancy in cells exposed to irradiation and in different cell types. Identification of tissues and contexts where the E2F-dependent control of mitochondrial function is particularly important will be helpful for development of new cytotoxic therapies targeting E2F activities.

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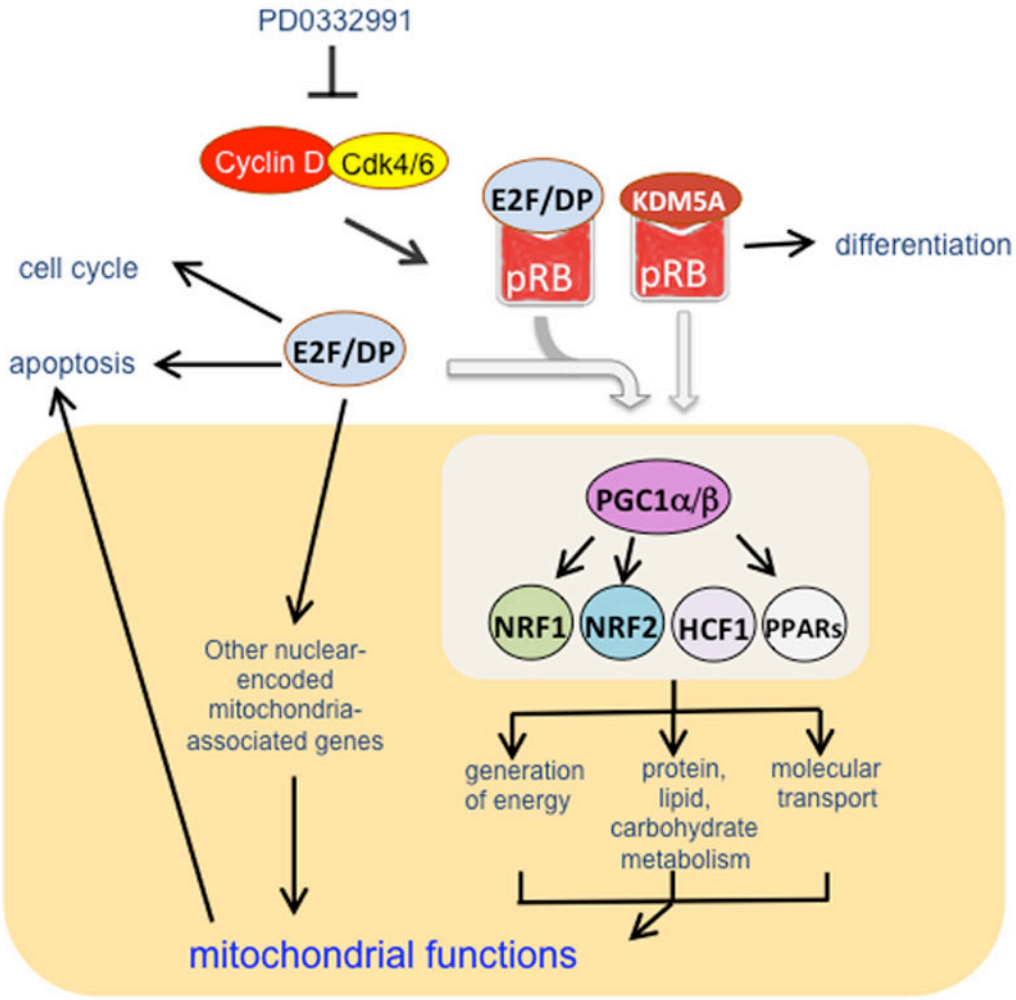

Figure 1. Emerging role of E2F in regulation of mitochondrial functions

Cyclin D/Cdk4/6 phosphorylation disrupts E2F/pRB complexes and results in release of free E2F. E2F is best known for its regulation of cell cycle and apoptosis. Recent studies revealed that E2F/DP influences efficiency of apoptosis *via* regulation of nuclear-encoded mitochondria-associated genes (11). Bioinformatics approach uncovered multiple new roles of E2Fs (shown in yellow area) (6,7). Accumulating evidence suggests that E2F regulates genes involved in mitochondrial functions through direct binding to their promoter regions (left) and through interactions with the NRF1, NRF2, HCF1 and PPAR, key regulatory factors of mitochondrial biogenesis (right). pRB exerts a major effect on mitochondrial biogenesis during differentiation, which ultimately converges on the PGC-1α/β transcriptional axis. pRB/E2F complexes may repress transcription levels of genes encoding PGC-1s and NRFs. A small molecule PD0332991 inhibits CDK4/6 that eventually prevents the release of E2F from inhibition by pRB.