The role of E2Fs in proliferation during development

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1. Cell cycle regulation

1.1 Cell-cycle control system

All cells undergo DNA replication and cell division in a controlled manner in order to proliferate. During the embryonic development of multicellular species, cells need to undergo many cell cycles to produce a new fully functioning organism. There are even proliferating cells in the adult body to replace cells that undergo apoptosis or get lost in a different way. The main task of cell division is to give genetic information to the daughter cells. A cell-cycle control system has evolved in eukaryotic cells to ensure that this information is given in a correct manner. This control system consists of several phase-specific check points, which arrest the cell in a specific phase of the cell cycle until everything is in order (Figure 1). For example, entry into mitosis, or M-phase, is prevented if DNA synthesis is not yet completed. These check points use intrinsic and extrinsic signals to control the cell cycle (1).

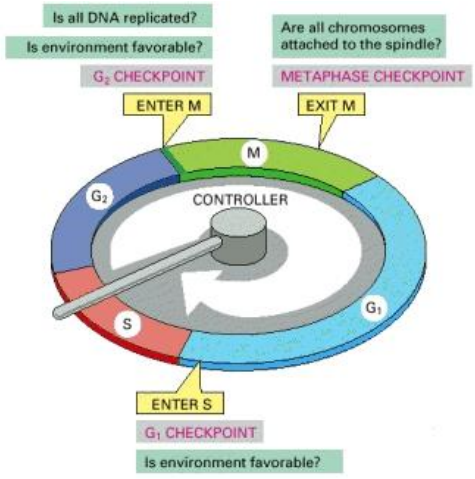

Figure 1: The check points of the cell cycle.
Intrinsic and extrinsic signals are registered by the cell-cycle control system and can be a cause for the system to arrest the cell cycle at explicit checkpoints (1).

1.2 Cyclins and Cyclin Dependent Kinases

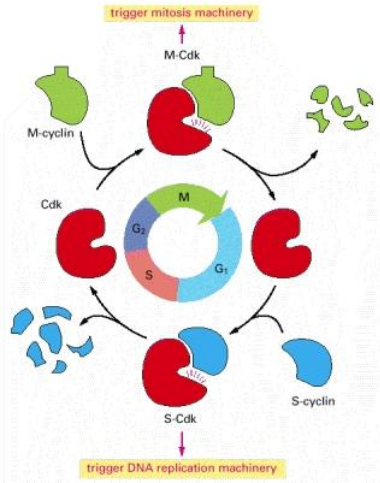

Figure 2: Cyclins are synthesized and degraded every cell cycle.
CDK binds to different cyclins to start specific events during the cell cycle. Here, only S-cyclin and M-cyclin are shown (1).

The main players of the cell-cycle control system are the protein kinase family of cyclin-dependent kinases (CDKs) and the family of cyclins. The activity of CDKs oscillates during the cell cycle. These fluctuations change the phosphorylation state of proteins that are important for the events during the cycle, namely DNA replication, mitosis and cytokinesis. The cyclical change of CDK activity is controlled by many enzymes and proteins, but the most important CDK regulators are cyclins. CDKs only have protein kinase activity when the kinase is bound to a cyclin. Cyclins are synthesized and degraded every cell cycle (Figure 2). The changes in cyclin levels are the cause of the oscillating activity of cyclin-CDK complexes (1).

The main control of CDK activity is in the rise and fall of cyclin levels during the cell cycle, but there are also other important mechanisms that adjust CDK activity at specific phases of the cell cycle. Cyclin-CDK complexes

can be inhibited by phosphorylation of the active site of the CDK, which is the used method to fine-tune the control of M-CDK. Another method to regulate the CDK activity is by binding of CDK inhibitor proteins (CKIs). Most CKI proteins are important in the control of the G_1- and S-phase. A third way to inactive CDKs is by the inhibition of cyclin gene transcription (1).

1.3 Rb-E2F pathway

An important family of gene regulatory proteins to regulate the transition from G_1- to S-phase is the E2F family. E2Fs bind specific DNA sequences in the promoter region of many genes that encode proteins that are required for S-phase entry. The classical view is that the retinoblastoma protein (Rb), an inhibitor of cell cycle progression, is in control of E2F function (Figure 3).

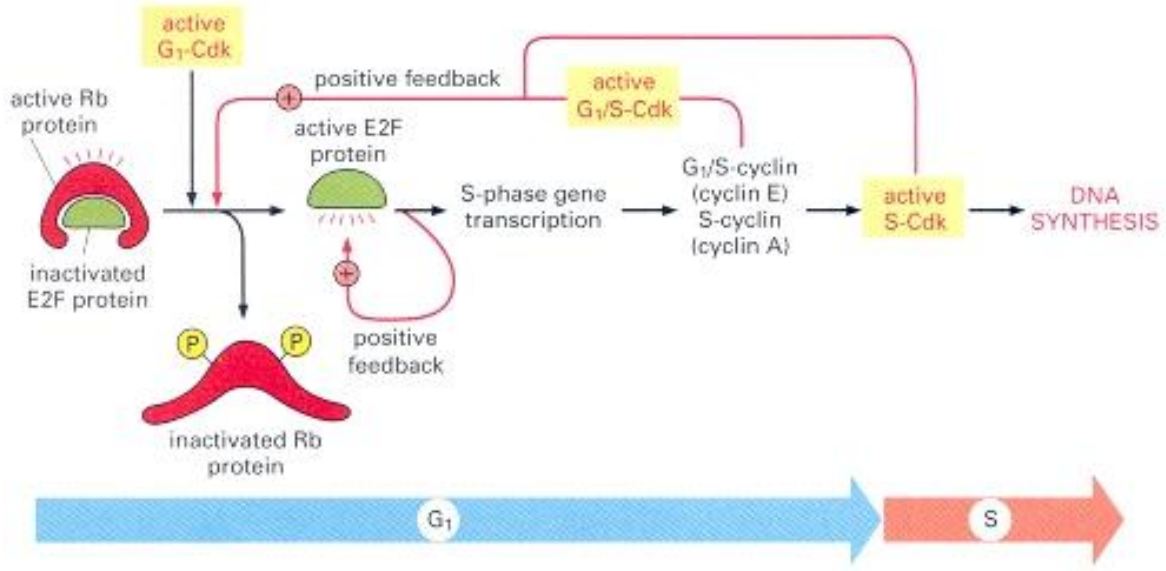

Figure 3: The Rb-E2F pathway controls the progression from G_1- into the S-phase of the cell cycle. Rb binds to E2F during G_1-phase. When the cell progresses through this phase, active G_1-CDK will accumulate and phosphorylate Rb. This inactivates Rb and leads to dissociation from E2F. Upon release, free active E2F will activate S-phase genes, such as G_1/S-cyclin. This will form a positive feedback loop that will sharpen the G_1-to-S transition (1).

Rb is bound to E2F during G_1 and inhibits the expression of S-phase genes. As the cell progresses through G_1, the active G_1-CDK accumulates and Rb becomes more and more phosphorylated. This phosphorylation leads to a decreased affinity for E2F and dissociation from the protein. This gives active free E2F, which activates S-phase gene expression. There are multiple positive feedback loops in this control system to tighten the G_1-to-S transition. Free active E2F stimulates its own gene transcription and E2F dependent transcription of G_1/S-cyclin and S-cyclin increases the activity of G_1-CDK, which increases the phosphorylation of Rb and increases release of E2F (1; 2).

2. The E2F transcription factor family

2.1 The E2F transcription factor family

The E2F transcription factor family is a family of proteins that share a related DNA-binding domain and bind to overlapping sets of target gene promoters. This family is conserved in many plant and animal species. Most E2F proteins form heterodimers with a DP protein and bind to a specific DNA sequence. This binding controls the temporal

expression of many genes that are required during the cell cycle. Depending on the formed complex, E2Fs can either activate or repress transcription. The repressor complexes inhibit transcription in quiescent cells, in differentiated cells and during the G_1-phase. These repressor complexes are replaced by E2F activator complexes when cells start to proliferate. E2F genes act redundantly and the proteins can compensate for each other. This makes it more difficult to study each family member individually (3).

The mammalian E2F family consists of eight E2F transcription factor genes. The E2F proteins are divided into different classes based on biochemical studies. However, this classification is not yet validated *in vivo* (Figure 4). E2F1, E2F2 and E2F3 mainly act as activators to promote cell proliferation. E2F4, E2F5, E2F6, E2F7 and E2F8 act as repressors of their target genes. The repressors are artificially divided in to typical (E2F4, E2F5, E2F6) and atypical (E2F7, E2F8) repressors. All family members contain a DNA binding domain (DBD), E2F7 and E2F8 even have two DBDs. The first six members (E2F1-E2F6) have conserved dimerization domains (LZ and MB) and form heterodimers with DP proteins before binding to specific DNA sequences. E2F7 and E2F8 lack these DP-binding dimerization domains. They form homo- or heterodimers with each other to suppress the transcription of a part of the E2F-regulated genes. Rb can bind to E2F1, E2F2 and E2F3 in the transactivation (RB) domain. E2F4 and E2F5 interact with Rb related pocket proteins, like p130 and p107. E2F6, E2F7 and E2F8 lack this transactivation domain, this suggests that they have a repressing function independent of Rb (2; 3).

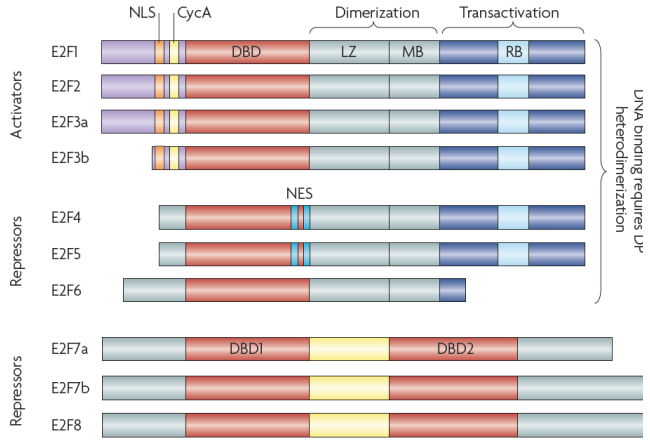

Figure 4: The mammalian E2F family.
The mammalian E2F family consists of eight family members. E2F1, E2F2 and E2F3 act as activators. E2F4, E2F5, E2F6, E2F7 and E2F8 act as repressors. All members have a DNA binding domain (DBD). The first six members (E2F1-E2F6) have dimerization (leuzine zipper, LZ, and marked box, MB) domains. Rb binds to E2F1, E2F2 AND E2F3 in the transactivation (RB) domain. E2F4 and E2F5 bind to Rb related pocket proteins. E2F6, E2F7 and E2F8 have no RB domain, which suggests that they perform their repressing function independently of Rb or related pocket proteins. The repressors are artificially divided into typical (E2F4, E2F5, E2F6) and atypical (E2F7, E2F8) repressors. E2F7 and E2F8 have two DBDs (2).

2.2 E2F activators

The E2F activators E2F1, E2F2 and E2F3 are important for the progression into S-phase to start DNA replication. Their expression is cell cycle regulated and is highest during the G_1-to-S transition (Figure 5). These factors have a redundant function during proliferation. It has been shown *in vitro* that mouse embryonic fibroblasts (MEFs) deficient for E2F1 or E2F2 could still proliferate, but additional loss of E2F3 affected proliferation severely. The loss of all three activators stopped proliferation completely (4).

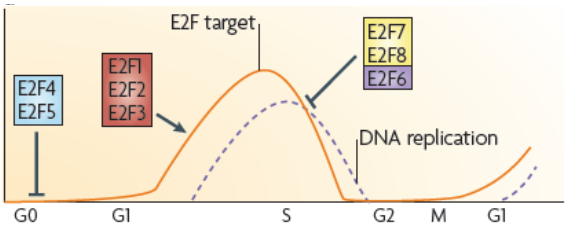

Figure 5: The nuclear expression and activity of E2f family members during the cell cycle.
E2F4 and E2F5 repress E2F target genes in quiescent cells (G_0). Upon mitogenic stimulus, E2F1, E2F2 and E2F3 get activated late in G_1-phase. This stimulates the expression of E2F target genes and drives the cells into S-phase. E2F6, E2F7 and E2F8 are active during S-phase. They repress E2F target genes to limit its expression to S-phase to prevent ectopic DNA replication (2).

Other findings show that E2F1, E2F2 and E2F3 have functions in transcription activation and repression *in vivo*. E2F1, E2F2 and E2F3 are used to activate the expression of target genes in dividing progenitor cells, when Rb is inactivated by hyperphosphorylation. Cells become gradually more committed to a differentiated state during development. Rb becomes dephosphorylated and forms a complex with E2F1, E2F2 and E2F3. The suggestion is made that this complex is not just formed to repress the activating activity of E2F1, E2F2 and E2F3, but that it is a necessary first repressor complex to help cells to stop proliferating. Other, more stable, E2F repressor complexes take over after this to give a more permanent repression (5).

2.3 Typical E2F repressors

The typical repressors E2F4 and E2F5 are continuously synthesized in cells, but they are only localized in the nucleus in quiescent cells. Here, they inhibit E2F target genes so that the cells have no ectopic expression of S-phase genes (Figure 5). The repressors are actively exported, through CRM1-mediated transport, to the cytoplasm during the cell cycle to relieve the cells of their inhibition. Other E2F family members, like E2F1, E2F2 and E2F3, are constitutively nuclear localized (6; 7).

The other typical repressor, E2F6, is regulated in a cell cycle specific manner (Figure 5). This regulation is mediated by E2F1. The activity peak of E2F6 is during G_1- and S-phase. The induction of E2F1, E2F2 and E2F3 occurs just before that of E2F6, which suggests that these E2F members act upstream of E2F6. This is a possible feedback mechanism to limit the expression of E2F target genes to the S-phase to prevent ectopic DNA replication (2; 8).

2.4 Atypical E2F repressors

E2F7 and E2F8 are classified as atypical repressors because of the duplication of the DBD and the lack of the RB domain and the dimerization domain (Figure 4). This duplicated DBD can both bind DNA and dimerize to form homo- and heterodimers with the atypical members itself. The preferred dimerization state of these members is E2F7 homodimerization. E2F8 homodimers are the least preferred form (2; 9; 10; 11). It is not known if this E2F7 homodimerization is also the most frequently occurring dimerization *in vivo*, since these states are based on biochemical experiments and affinities. It is possible that different dimers are formed in different tissues and thereby have tissue-specific functions. The lack of the RB domain suggests that E2F7 and E2F8 act as repressors in an Rb-independent manner. It is possible that they are in competition with E2F activators for the same E2F-binding sites (9).

The expression of E2F7 and E2F8 is cell-cycle regulated and mediated by E2F1. The transcription starts at the G_1-to-S transition and peaks during S-to-G_2 (Figure 5). This expression profile fits with the role of E2F7 and E2F8 as repressors of gene expression. E2F7 and E2F8 limit the expression of E2F1, a target gene, to the S-phase, to prevent ectopic DNA replication. The majority of E2F7 and E2F8 target genes are involved in cell-

cycle control, especially during the G_1-to-S transition. This indicates that these atypical members have a role in cell-cycle control, particularly during proliferation (9; 10; 12).

E2F7 and E2F8 are highly expressed in adult mice in skin, thymus, liver and testis. There is little or no expression in brain, muscle and stomach. This tissue specific expression pattern of E2F7 and E2F8 is in agreement with their role in proliferation, since these tissues keep proliferating during adulthood (10; 12).

3. E2Fs during development

E2F genes regulate the expression of many genes that are involved in cell cycle-related events, such as proliferation, differentiation and apoptosis. These processes are essential to embryonic development, but the role of E2F factors is still unclear (13). It seems that the three activator transcription factors play a redundant role during embryonic development. E2F3 has a central role during development, since $E2F1^{-/-}E2F2^{-/-}$ mice were viable and could develop to adulthood, but $E2F1^{-/-}E2F3^{-/-}$ and $E2F2^{-/-}E2F3^{-/-}$ animals died early during embryonic development (4; 14). However, inactivation or overexpression of E2F1 and E2F2 does lead to tumor formation in adulthood.

E2F7 and E2F8 have a redundant role during embryonic development. E2F7 or E2F8 single knockout mice are viable and show no phenotype during their lifetime. However, $E2F7^{-/-}E2F8^{-/-}$ mice die between E9.5 and E11.5 because of widespread apoptosis, vascular defects and hemorrhaging (11). These varying phenotypes suggest that E2F7 and E2F8 have specific roles in different tissues, such as the vascular system but more research needs to be done to specify which transcription factor has which role and where.

Another model organism that is used to investigate the role of E2Fs is the chick wing. The budding wing is easy to access and to manipulate. There are no major cell movements during the development, so almost only proliferation and apoptosis contribute to development. The proliferation and apoptosis patterns are well documented in wing bud development. E2F1, E2F2 and E2F3 are expressed in the early proliferative phase of development. E2F4 and E2F7 are expressed at later stages, when differentiation takes place. All other E2F members are also expressed during the development and are related to the ongoing developmental events (13). The expression of E2F family members at the same time suggests that they have overlapping roles during development.

4. Cell culture or model organisms?

4.1 *In vitro*

Most studies on proliferation and E2Fs use cell cultures to find answers to their research questions. There are many *in vitro* techniques to investigate cell cycle progression and proliferation. A relatively easy method to measure proliferation is a growth curve of wild type and specific mutant cells. Changes in the proliferation rate of these mutant cells can be attributed to the altered expression of the mutated protein in the mutant cells (4). It is also possible to treat cells with compounds that affect cell growth and measure the difference in response in cell types (15).

Often used techniques to analyze the cell cycle in more detail are FACS (fluorescence activated cell sorting), BrdU (Bromodeoxyuridine) incorporation and pH3 (phosphohistone H3) staining. To determine in which phase of the cycle a cell is, DNA is labeled fluorescently and analyzed by FACS (Figure 6). With this analysis, proliferating cells can be divided into three categories, namely cells without duplicated DNA, which are cells in G_1 phase; cells that have completed DNA duplication, which are in G_2 and M phases; and cell that undergo DNA duplication during S phase (1).

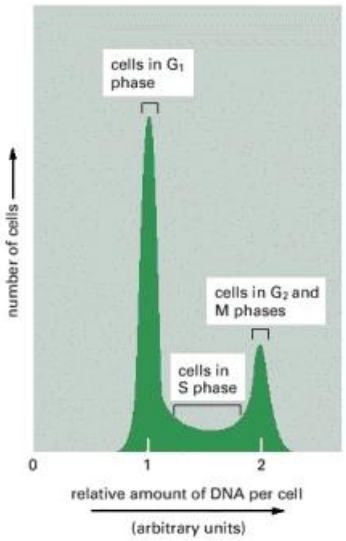

Figure 6: FACS analysis of DNA content.
A typical example of a FACS analysis of a non-synchronous proliferating cell population has different categories. Three classes can be made, namely: cells in G_1 phase, with a relative DNA amount of one; cells in G_2 and M phases, with completely duplicated DNA (relative amount of two); and cells in S phase, those are undergoing DNA duplication (1).

A method to examine whether cells are in S phase is by measuring BrdU incorporation. This is an artificial thymidine analog that can be incorporated into newly synthesized DNA when this is added to the cell culture medium. The integration can be visualized and quantified with an antibody staining for BrdU (1).

A specific staining for cells in G_2- and M phase is pH3. Histone H3 starts to get phosphorylated during the late G_2 phase and is fully phosphorylated during mitosis. It is possible to visualize this phosphorylation by using an antibody specifically for phosphorylated histone H3 (1). Every phase of the cell cycle can be visualized if BrdU staining and pH3 staining are combined, because all four phases can be discriminated. Cells in G_1 phase have no staining, cells in S phase are BrdU positive, cells in G_2 phase have a punctuated pH3 staining and cells in M phase have a dense uniform pH3 staining.

Many aspects of proliferation can be investigated *in vitro*, but there are some disadvantages. It has been shown that some regulators, such as E2F1, E2F2 and E2F3, have different functions in different cell types. It is even possible that these regulators have specific and unique functions at different time points in different tissues during development (5). This is very difficult to prove in cell culture studies.

Another feature of the E2F family is a non-cell autonomous function. This is also very difficult to assess *in vitro*. It has been shown previously that Rb has a cell autonomous function in the cell cycle and a non-cell autonomous function in suppressing apoptosis and inducing differentiation (16). As E2Fs are involved in all these processes, it is probable that E2F and Rb work together and therefore E2Fs may have non-cell autonomous functions. It is possible that the E2F proteins are transported from the producing cell to the cell where they perform their function. However, since the E2Fs have nuclear localization signal sites and work on DNA, it is more likely that the non-cell autonomous function is indirect via their target genes.

4.2 *In vivo*

Most E2F properties and roles in proliferation regulation have been investigated *in vitro* and with biochemical assays, but *in vivo* studies have also been performed, especially on the developmental functions of the family members. For this type of research, chick wing development and embryonic mice development have been used. The budding chick wing

is used for proliferation research because it is easily accessible and the proliferation and apoptosis patterns are well documented. Another reason is the lack of major cell movements during development, so almost only proliferation and apoptosis contribute to development (13).

The most often used mammalian model organism for embryonic development is the mouse. A problem with the E2F research is redundancy of the factors. Single knockout mice show no or a very mild phenotype and double ($E2F7^{-/-}$ $E2F8^{-/-}$) or triple ($E2F1^{-/-}$ $E2F2^{-/-}$ $E2F3^{-/-}$) knockout mice are embryonic lethal (11; 14). It is possible to create conditional knockout mice to overcome this embryonic lethality and investigate tissue specific roles of different family members. Another used method to investigate regulator roles is harvesting embryonic stem cells or embryonic fibroblasts from mutant mice and go back to *in vitro* studies.

Whole-mount *in situ* hybridization is used to visualize the mRNA expression pattern of a specific regulator, a proliferation marker or a tissue marker. This technique can be used to gain some understanding about the spatiotemporal expression of E2F family members. However, it is possible that the mRNA is transported to another cell and that the protein is produced in this different cell. It is also possible that the protein performs a non-cell autonomous function (11).

Microarray data can be useful to discover in which pathways a regulator is involved and which proteins are affected when this regulator is taken away. This technique is used regularly in whole mice embryos, which can give answers but can also generate more complications if proteins have antagonizing functions in varying tissues (11). To overcome this issue, it is possible to sort the cells by FACS when transgenic mice have tissues with a fluorescence tag and perform the microarray on a specific tissue.

It is possible to perform BrdU and pH3 staining on developing mice embryos, but the embryos first need to be fixed to do these staining because they are based on antibody binding. This makes it difficult to follow a process over time in mice. Another disadvantage is that the embryonic development occurs *in utero*.

Another model organism that is relatively new in the proliferation and E2F field is the zebrafish. The different stages of embryonic development are well described. Advantages over mice embryos are the external development and transparency of the embryos (17). The transparency gives the opportunity to follow the same embryo over a period of time in detail under a microscope. Many transgenic fish are available with fluorescence tags which make time-lapse imaging much more efficient. Most often, GFP (green fluorescent protein) and its derivatives are used for this purpose. These proteins are expressed in the whole embryo or in specific tissues, depending on the used promoter.

A transgenic zebrafish protein that can be used in proliferation research is the H2A.F/Z:GFP fusion protein. During DNA replication, histone H2 is replaced with histone H2A. This way, the mitotic divisions can be visualized by using the fusion protein (18). GFP can be used in this construct because histone H2A is transcribed and translated constantly, but the maturation of GFP is too long to study the dynamics of the cell cycle in more detail. It is a good possibility that the process has finished by the time GFP becomes detectable. Another complication that can arise if multiple derivatives of GFP are transfected into one zebrafish line are mixtures in emission colors, which can lead to intramolecular fluorescence resonance energy transfer (19). Other fluorescent proteins

that mature faster than GFP and its derivatives are the monomeric versions of Azami-Green (mAG) and Kusabira-Orange (mKO). These proteins are better equipped to study spatiotemporal dynamics of the cell cycle (20; 21).

A new system is developed with the antiphase oscillating proteins Cdt1 and Geminin in the cell cycle and mAG and mKO as fluorescent labels to study the dynamics of this process. The SCF complex and APC complex are part of the cell cycle regulation (Figure 7). These complexes are E3 ligases that regulate each other by negative feedback via ubiquitination. Cdt1 and Geminin are direct substrates of these complexes and show the same oscillating expression pattern. Cdt1 and Geminin are fused to mKO and mAG to label individual G_1 phase nuclei in red and $S/G_2/M$ phase nuclei in green. The system is named Fucci (Fluorescent Ubiquitination-based Cell Cycle Indicator), or zFucci in zebrafish. This system works in cell culture, in mice and in zebrafish. An advantage of this system in zebrafish is that the cell cycle progression can be monitored by time-lapse microscopy (22; 23).

5. Outstanding questions on E2F regulation

Many aspects of the regulation of proliferation by E2Fs remain unclear, especially during embryonic development. Focusing on the atypical E2Fs, the most important questions include the mechanisms by which E2F7 and E2F8 repress gene transcription, the involved co-factors in the E2F complex, the spatiotemporal expression pattern during development and the *in vivo* dimerization preferences. It is also still unclear what the target genes of the different atypical E2Fs are. It is possible that different dimers or complexes have a different subset of target genes (9).

The vascular phenotype in $E2F7^{-/-}E2F8^{-/-}$ double knockout mice suggests that these transcription factors play part in the development of endothelial cells (11). DKO zebrafish embryos also have vascular defects. *In situ* hybridization data of E2F7 and E2F8 in zebrafish shows that the factors are not expressed in endothelial cells, implying that they have a non-cell autonomous function on these cells. An option to investigate this role is by driving the zFucci system (Figure 7) under an endothelial specific promoter in $E2F7^{-/-}$ $E2F8^{-/-}$ DKO zebrafish embryos to explicitly follow the cell cycle over time in endothelial cells. It is possible to see differences in the length of the cell cycle or in proliferation when DKO and wild type embryos are compared. This might shed some light on the vascular phenotype.

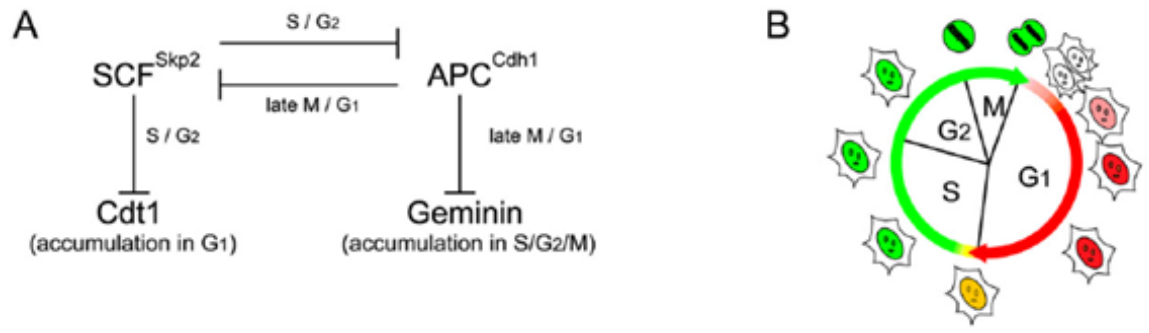

Figure 7: Control of the cell cycle by SCF and APC is used for visualization of the cycle.
A) The SCF complex and APC complex regulate each other by negative feedback. Cdt1 and Geminin are direct substrates of these complexes and show the same oscillating expression. Cdt1 accumulates during G1 and Geminin accumulates during S/G2/M phases. B) Cdt1 and Geminin are used to create fusion proteins with mKO and mAG to label individual G_1 phase nuclei in red and $S/G_2/M$ phase nuclei in green (22).

A disadvantage of ISH is that the sample needs to be fixated before the technique can be used because it is based on antibody binding. The expression patterns of E2F7 and E2F8 can also be followed while the embryos are still alive with fluorescent tagging. This is done by driving a fluorescent protein (like GFP, mAG or mKO) behind the E2F7 or E2F8 promoter. A disadvantage of this technique is that it is not possible to investigate the dynamics of the transcription factors, since degradation signals are generally not located in the promoter region. With this technique, it is possible to visualize if the mRNA stays in the cell that produces it or if it is transported to another compartment of the cell or to another cell. It can also give insight in tissue specific expression of E2F7 or E2F8.

Fusion proteins are needed to research the expression dynamics in more detail. An option is to make fusion proteins for E2F7 and E2F8 with mAG and mKO, respectively. This way, the degradation signals remain present in the protein, so the fluorescent tag is degraded simultaneously with the protein. This might show an oscillating expression of both transcription factors. It is even possible to discriminate between E2F7 and E2F8 to see if they have a slightly differing expression.

Expression of both E2F7 and E2F8 in the same tissue does not necessarily mean that they always form heterodimers. The biochemical data showed a preference for E2F7 homodimers, but this has not been shown *in vivo* yet (11). A way to picture the dimerization state is by using a proximity ligation *in situ* assay (24). This technique can monitor interactions of endogenous proteins in individual cells and tissues (Figure 8). First, the sample is incubated with target specific primary antibodies that are raised in two different species (Figure 8A). After this incubation, proximity ligation assay (PLA) probes PLUS and MINUS are added to the sample. Each PLA probe recognizes one species specific primary antibody. If the proteins have formed a complex, the PLA probes can be

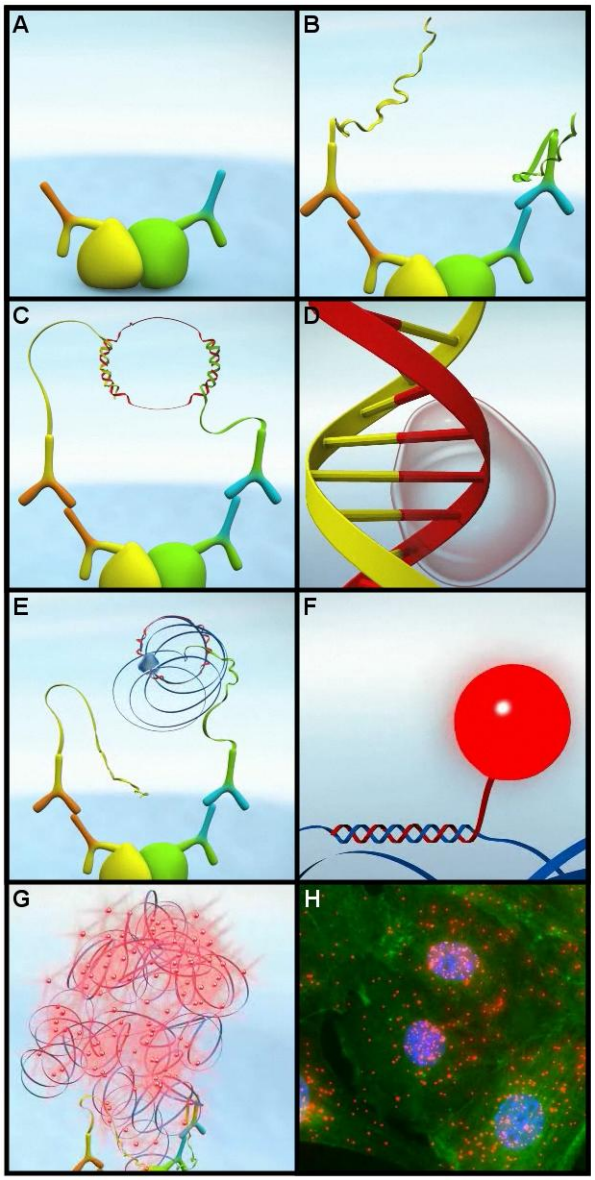

Figure 8: Individual endogenous protein complexes can be observed *in situ* by proximity ligation.
A) The sample, cells or tissue, is incubated with target specific primary antibodies raised in two different species. B) The proximity ligation assay (PLA) probes (PLUS and MINUS) are added to the sample. Each probe recognizes one or the other primary antibody. C) The PLA probes are hybridized with connector oligos if the probes are in close proximity. D) A ligation is done form a complete circularized oligo. E) The oligo is amplified by rolling circle amplification. F-G) The fluorescent detection probes are hybridized to the amplified oligos. H) Each red spot is an individual endogenous protein complex (pictures are stills from movie at http://www.olink.com/movie.php).

hybridized with connector oligos and a circularized oligo can be formed (Figure 8B-D). This oligo is amplified by rolling circle amplification (Figure 8E). The last step is to visualize the amplified oligo. This is done by hybridization of fluorescent detection probes (Figure 8F, G). Each fluorescent spot that can be seen under a fluorescent microscope represents an individual endogenous protein complex (Figure 8H).

It is possible that different protein complexes, E2F7-E2F7; E2F7-E2F8; E2F8-E2F8, have a subset of different target genes. It is also possible that the complexes have tissue specific target genes. Once it is discovered which dimer is formed in which tissue, this knowledge can be used to investigate the complex-specific subsets of target genes. Specific tissues can be isolated and microarrays can be done to find the target genes of each complex. Comparing the datasets can show overlap or specificity of target genes.

Answering these questions about E2F7 and E2F8 will give deeper insight into these atypical transcription factors, but will also help to gain understanding about the whole E2F transcription factor family. Once the developmental role becomes more clear, the acquired knowledge will help in cancer research, where proliferation is often aberrant.

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