How Cell Cycle Transcription Factors Inhibit Neural Proliferation

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Abstract

Adult neurons are incapable of cellular proliferation due to their abnormal cell cycle. Upon maturation, post-mitotic neurons face terminal cell cycle arrest at the G1/S checkpoint. If cell cycle re-entry is attempted, neurons trigger pro-apoptotic pathways that kill the cell before any proliferative progress can be made (Figure 1). These processes inhibiting neural replication are highly influenced by cell cycle transcription factors, which face neuro-specific regulations to induce such unique cellular events. This paper examines how the regulations and differential functions of neural cell cycle transcription factors facilitate neurons' inability to proliferate. Specifically, we review how BHLH, E2F, SMAD, FOXO1, SP1, c-MYC, p53, and Brn-3a regulate cell cycle arrest, and how E2F1, FOXO1, C-JUN, and p53 facilitate neural apoptosis upon attempted cell cycle re-entry.

Keywords

Neuron, Cell cycle arrest, Transcription factor, Apoptosis, G0 Phase, Anti-proliferation, CDK inhibition

1. Introduction:

In the vertebrate central nervous system (CNS), neurons are a unique cell type that lack proliferative action due to their abnormal cell cycle.

The general cell cycle consists of 4 main phases: G1, S, G2, and M. In G1, the first phase after a new cell is formed, cells grow, replicate organelles, and acquire nutrients. Upon replicative stimuli, cells transition from G1 to S. This process is highly regulated by a G1/S checkpoint that ensures cells possess all S phase prerequisites. After passing this checkpoint, cells move into the S phase where DNA is completely replicated. They then transition into G2, a final growth phase, where they prepare for division. Finally, cells prepared to replicate pass through a final G2/M checkpoint into the M phase where they undergo mitosis (division into two identical daughter cells). This process then repeats in proliferating cells, contributing to organismal longevity by allowing tissues to grow and regenerate upon inevitable damage (1).

Transitions between cell cycle phases are highly regulated by cyclins, proteins that rise and fall throughout the cell cycle to signal activation of key events. As cyclic levels rise, they bind to and activate cyclin-dependent kinases (CDKs), which phosphorylate proteins that activate cell cycle progression genes. Transduction of these genes is also highly dependent on the activity of cell cycle transcription factors (TFs). TFs are proteins that control DNA transcription rate and are essential for the progression of the cell cycle at proper times and locations in proliferating cells (2).

In neurons, the activity of cell cycle TFs has the exact opposite effect. While post-mitotic neurons undergo terminal differentiation, cell cycle TFs inhibit rather than activate the G1/S checkpoint. This action arrests the neural cell cycle in a terminal state of G1 termed G0. Neural TF's also prevent cell cycle re-entry by inducing apoptosis (programmed cell death) when stimulation of the G1/S checkpoint is attempted. By these means, TF's prevent neurons from pursuing any proliferative or regenerative action (Figure 1).

Figure 1: Graphic Abstract

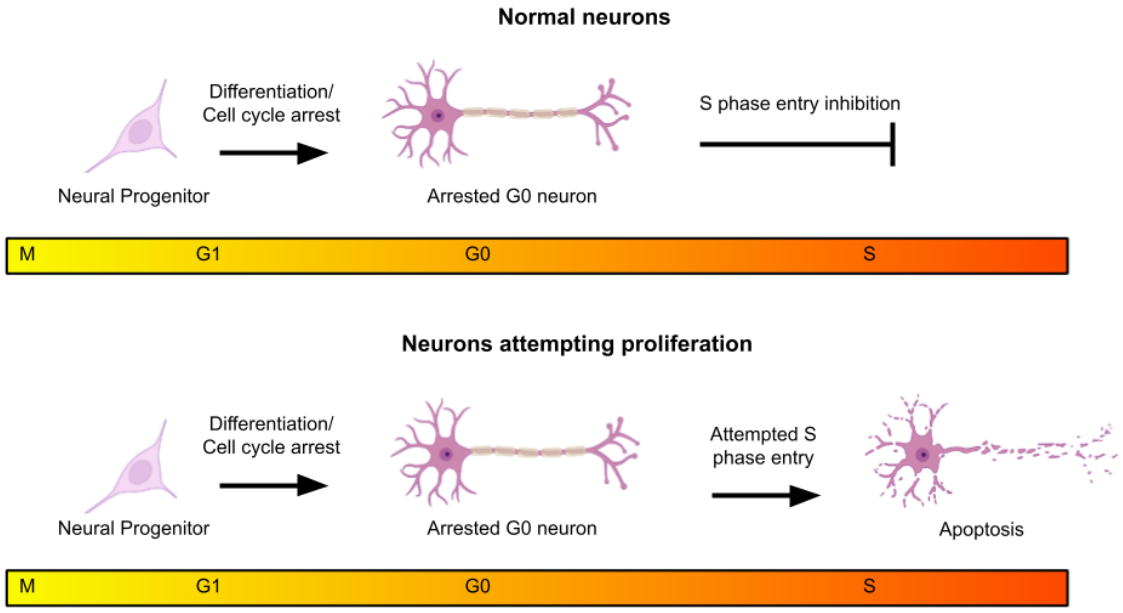


Figure 1: Neurons are one of the only cells in the vertebrate body which cannot replicate. This is because upon initial stem cell mitosis, neurons undergoing differentiation exit the cell cycle into a terminal G0 state and are inhibited from entering S phase. Most neurons live in G0 until they die. Upon attempted S phase entry, neurons have pathways that stimulate apoptosis (cell death), preventing their cell cycle progression and proliferation. Created using BioRender.com.

This paper examines how the specific regulations of cell cycle TFs in neurons inhibits their proliferation. Particularly, we consider how CDK inhibitors facilitate terminal G0 entry, and how these factors are regulated by BHLH, E2F, SMAD, FOXO1, SP1, c-MYC, p53, and Brn-3a TFs. We then review the mechanisms by which the G0 state is maintained, and how TFs E2F, FOXO1, C-JUN, and p53 facilitate apoptosis upon attempted G0 exit, securing neuron's anti-proliferation.

2. How TF's induce cell cycle arrest by producing CDK inhibitors

2.1 How CDK inhibition induces cell cycle arrest:

CDKs, in all cell types, act as proliferative factors that progress the cell cycle. For cell cycle arrest to be achieved in neurons, CDKs 4/6 and 2 face inhibition at the G1/S phase checkpoint such that S phase entry is prevented. Two classes of cyclin kinase inhibitors (CKIs) that contribute to cell cycle arrest by repressing CDKs 4/6 and 2 are found in neurons. The first is the INK4 family, consisting of p15INK4b (p15), p16INK4a (p16), p18INK4c (p18), and p19INK4d (p19), which inhibit CDK 4/6 by impairing its interactions with type D cyclins. The second is the Cip/Kip family, including p21Cip1 (p21), p27Kip1 (p27), and p57kip2 (p57), which inhibit both CDK 4/6 and 2 by forming a ternary complex. The Cip/Kip family are the main regulators of cell cycle arrest in neurons, whereas INK4 proteins appear to play a larger role in G0 maintenance (discussed later). P27 is suspected to be the main CKI facilitating neural cell cycle arrest, however, p21 and p57 also contribute immensely to the process. As neurons proliferate and prepare for G0, all three Cip/Kip CKIs accumulate. Correspondingly, a decrease in CDK 4/6 and 2 activity results as cells near maturation. Upon peak Cip/Kip expression, complete CDK inhibition occurs, blocking the G1/S transition and arresting the cell cycle. To facilitate G0 entry by means of CKI production, three major pathways dependent on cell cycle

TFs must take place: the Cyclin D/RB induced pathway, the TGFβ induced pathway, and the p53/Brn-3a pathway (3, 4).

2.2 The Cyclin D/ RB dependent pathways: utilizing TFs BHLH and E2F:

The first TF-dependent neural G0 pathway is stimulated by a rise of cyclin D1. In proliferating cells, cyclin D1 rises in response to inducers of cell cycle progression and promotes the G1/S transition. In neurons, cyclin D1 elevates as a result of inducers of differentiation and cell cycle arrest. As cyclin D1 rises in neurons, CDK 4/6 experiences increased activity in the early stages of G0 entry. However, in order for cell cycle arrest to proceed, CDK 4/6 deviates from its proliferative function to instead stimulate production of refractory Cip/Kip CKIs. This negative feedback cycle represses activity of both CDKs 4/6 and 2 until a G0 status is achieved (3).

The Retinoblastoma (RB) family of proteins is responsible for initiating CKI transduction in a CDK-dependent manner. The RB family, including pRB, p130, and p107, regulate the cell cycle in all vertebrate cell types. In neurons, RB's role is specific to cell cycle arrest. RB's arresting function is mediated by cyclin D1. As cyclin D1 binds to and activates CDKs, RB proteins (major substrates of neural CDK 4/6) face hypophosphorylation their on serine (Ser) and threonine (Thr) residues and subsequent activation. Hypophosphorylated RB proteins can directly induce pro-G0 p27. However, their larger role is in regulating two distinct growth arrest pathways mediated by TFs BHLH and E2F (Figure 2) (3, 5).

Figure 2: Visual of Cyclin D/RB pathway

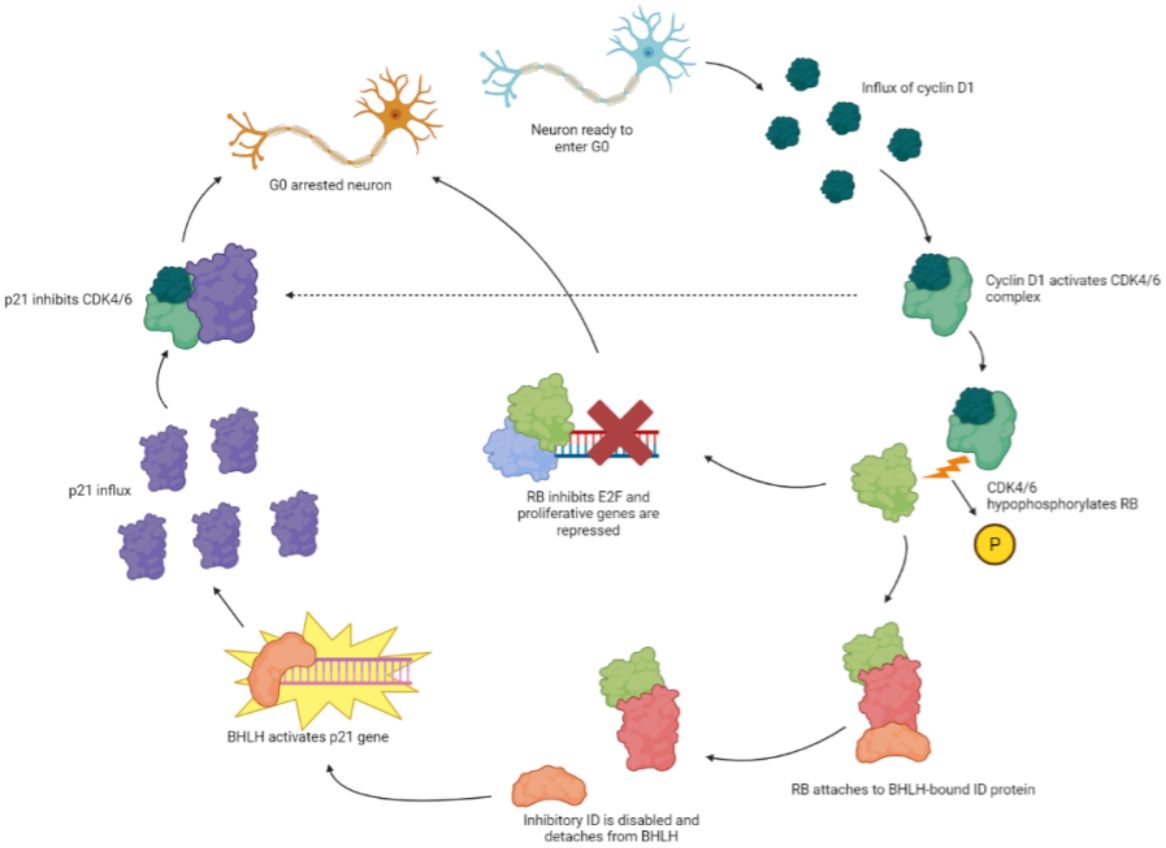


Figure 2: As cyclin D1 rises in neurons ready to enter G0, it binds to and hypophorsphorylates RB proteins. Hypoposphorylated RB inhibits proliferative E2F factors and releases pro-G0 BHLH from inhibitory ID proteins. Both of these actions lead to cell cycle arrest.

The first RB-regulated mechanism of cell cycle arrest involves the basic helix-loop-helix (BHLH) family of TFs. In proliferating cells, BHLH members control cell cycle progression, cell lineage commitment, and cell differentiation. Specifically in neurons, they drive cells from a proliferative to a terminally differentiated and arrested state at the proper times and locations. Two distinct classes of BHLH proteins regulate neural cell cycle arrest. The first is proneural BHLH. Proneural BHLH promotes the onset of G0 by up-regulating CKI expression and repressing certain proliferative factors (Table 1). The second class of BHLH consists of ID proteins. The ID family, consisting of Id1, Id2, Id3, and Id4, prevents G0 onset by working against proneural BHLH. ID proteins inhibit the rest of the BHLH family by withdrawing essential E proteins from BHLH factors. Neural cell cycle arrest requires a coordinated increase in proneural BHLH and decrease in ID proteins. Reduced ID activity is achieved in an RB-dependent pathway. As RB is hypophosphorylated upon G0 stimuli, it binds to and inhibits all ID members, allowing proneural BHLH to exert its anti-proliferative function (3, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15).

Table 1: Proneural BHLH types/functions

BHLH Family	Atonal family	Nato family	Oligo family	NeuroD family	Neurogen family	Achate-scute family	Nscl family
Member proteins (vertebrates)	Math1 and Math5	Nato3	Beta3, Beta4, Olig1, Olig2, and Olig3	NeuroD, Ndrf, Math3, and Math2/nex	amphioxNgn, Ngn1, Ngn2, and Ngn3	Cash4, Mash1, Mash2, and Xash3	Nscl1 and Nscl2
Anti-proliferative function	Derepresses CKIs p27 and p57	Upregulates neurogen BHLH and represses proliferative HES proteins which inhibit p21 expression	Induces p27	Induces p27, p21, p57, and p16	Induces p27 and p57	Induces p21 and p27	Induces p21

Table 1: Proneural BHLH types/functions. Proneural BHLH promote cell cycle arrest through induction of Cip/Kip CKIs and repression of proliferative genes. The NeuroD family appears to be the most significant proneural BHLH in regulating neuro-specific cell cycle arrest. (7, 8, 9, 10, 11, 12, 13, 14, 15)

A second RB-regulated G0 pathway involves TF E2F. The E2F family, consisting of E2F1-6, strongly activates transcription of several genes involved in differentiation and cell cycle progression. E2F1, E2F2, E2F3, and E2F6 act mainly as inducers of cell cycle progression, whereas E2F4 and E2F5 are more important during differentiation. In developing neurons, E2F1, E2F2, and E2F5 are detected. E2F1 and E2F2 are active preceding G0, allowing cells to grow and prepare for maturation. Upon differentiation, E2F5 expression increases as well. As differentiation proceeds, the corresponding onset of G0 stimuli dissipates E2F expression through RB hypophosphorylation. Hypophosphorylated RB proteins inhibit E2F-gene expression in two ways. The first is by directly binding to and blocking E2F's transactivation domain at its carboxyl terminus, inhibiting E2F gene activation. The second is by recruiting chromatin-modifying proteins such as histone deacetylases (HDACs), methyltransferases, and Polycomb group proteins, to E2F binding sites, making E2F genes epigenetically inaccessible to transcription altogether. E2F1 and E2F2 have a stronger affinity for pRB inhibition, whereas E2F5 binds more strongly to p107 and p130. Upon G0 stimuli, rising levels of (p107/p130)/E2F5

complexes slow differentiation until a mature phenotype is concrete. Meanwhile, the onset of pRB/(E2F1/2) complexes initiates cell cycle arrest as proliferative genes are repressed. Combined with its activation of BHLH, these efforts conclude the RB-dependent cell cycle arrest pathways in neurons (3, 5, 16, 17).

2.3 The TGFβ pathway: utilizing TFs SMAD, FOXO1, and c-MYC

A second TF-reliant neural cell cycle arrest pathway involves the stimulus of cytokine transforming growth factor beta (TGFβ). TGFβ signaling influences the activity of certain TFs to facilitate an array of biological processes in proliferating cells, including cell fate decisions, tissue patterning, and cell death. In neurons, TGFβ elevates in cells ready to enter G0 and stimulates cell cycle arrest by interacting with TFs SMAD, SP1, FOXO1, and c-MYC to enhance pro-G0 CKI production (Figure 3) (6, 18).

Figure 3: Visualization of TGFβ signaling pathway

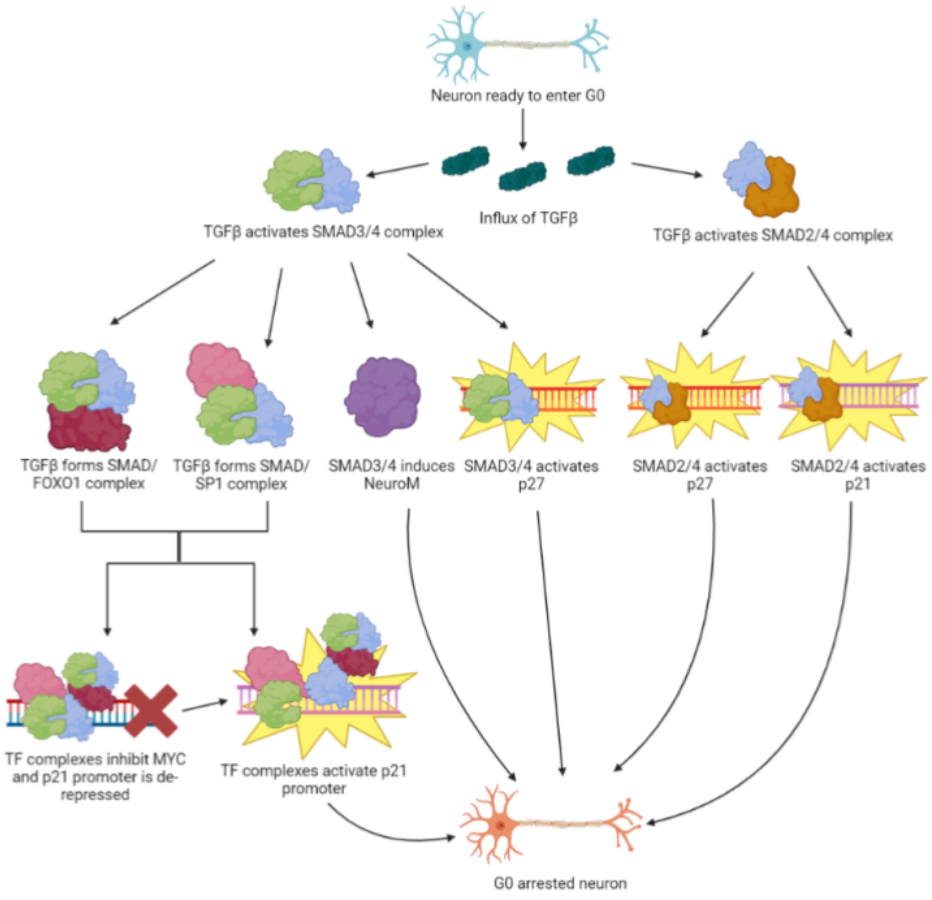


Figure 3: The influx of TGFβ witnessed in neurons ready to enter G0 activates a variety of cell cycle arrest pathways, all of which involve the SMAD family of transcription factors. SMADs 2,3, and 4 can activate pro-G0 CKI genes on there own, however SMADs 3 and 4 go on to further induce CKI expression by interacting with TFs FOXO1 and SP1. The SMAD/FOXO1 and SMAD/SP1 complexes that TGFβ forms both induce the p21 gene and repress the p21-inhibitory MYC gene. Collectively, these actions induce cell cycle arrest.

The main receptor of TGFβ signaling is the mothers against decapentaplegic (SMAD) family of TFs. In proliferating cells, SMADs have numerous functions and face type-dependent

induction by various TGFβ ligands (Table 2). In neurons, R-SMADs 2/3 and Co-SMAD4 regulate G0 entry. In preparation for cell cycle arrest, TGFβ facilitates SMAD2/3/4 nuclear localization and phosphorylation. All three of these SMADs significantly induce the expression of CKIs p27 and p21. SMAD 3 and 4 also reduce expression of neural ID1 and ID2 and induce proneural BHLH factor neuroM. SMADs' efforts alone exert a strong anti-proliferative force on developing neurons (6, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27).

Table 2: SMAD family members and function

SMAD Family	SMAD1	SMAD2	SMAD3	SMAD4	SMAD5	SMAD6	SMAD7	SMAD8/9
Method of activation	BMP pathway	TGFβ/activin responses	TGFβ/activin responses	Formation of a transcriptional complex with other SMAD family members	BMP pathway	BMP pathway and TGFβ/activin responses	TGFβR1	BMP pathway
Subtype	R-Smad	R-Smad	R-Smad	Co-Smad	R-Smad	I-Smads	I-Smads	R-Smad
Proliferative cell function	Cell growth, apoptosis, morphogenesis, development and immune responses	Cell proliferation, apoptosis, and differentiation	Regulating gene activity and cell proliferation	Shuttles other SMAD family members between the nucleus and cytoplasm	Angiogenesis	BMP signal inhibitor	General TGFβ signal inhibitor	BMP/TGFβ signal transducer
Neural function	Neuron development (pre-G0)	Cell cycle arrest	Cell cycle arrest	Cell cycle arrest	Neuron development (pre-G0)	Differentiation	Neuron proliferation	Neuron development (pre-G0)

Table 2: SMAD family members and function. There are three subtypes of SMAD proteins: receptor-regulated SMADs (R-Smads), common partner SMADs (Co-SMADs), and inhibitory SMADs (I- SMADs). The BMP pathway and TGFβ/activin responses activate SMAD-phosphorylation membrane receptor serine/threonine kinases (receptors type I and II). TGFβ mediated activation of SMADs increases their affinity for a common SMAD (SMAD4), an essential component for the assembly of transcriptional complexes and for the generation of specific SMAD responses. (20, 21, 22, 23, 24, 25, 26, 27)

However, SMADs 3 and 4 also interact with forkhead box protein O1 (FOXO1) and SP1 TFs to further induce cell cycle arrest. In proliferating cells, FOXO1 and SP1 act as tumor suppressors and maintain organismal longevity. In neurons, these factors modulate cell cycle arrest. TGFβ initiates the nuclear translocation of FOXO1 and SP1 proteins and forms SMAD3/SMAD4/FOXO1 and SMAD3/SMAD4/SP1 complexes. TGFβ then recruits these complexes to multiple sites of the p21 promoter, where they activate p21 expression and facilitate G0 entry as such. (18, 19).

SMAD3/SMAD4/FOXO1 and SMAD3/SMAD4/SP1 complexes further contribute to cell cycle arrest by repressing c-MYC. The myelocytomatosis oncogene (MYC) family of TFs regulates cell proliferation and differentiation in multiple cell types, including neurons. c-MYC proteins promote cellular growth and proliferation during early neural development by inhibiting CKIs, however, their down-regulation is essential for differentiation and cell cycle arrest. TGFβ recruits SMAD3/SMAD4/FOXO1 and SMAD3/SMAD4/SP1 complexes to the c-MYC promoter, which inhibit c-MYC transcription. TGFβ then administers c-MYC cytoplasmic localization, removing all existing c-MYC factors from the nucleus. Complete annihilation of c-MYC sufficiently de-represses the p21 promoter such that it can initiate G0 entry. There is rising evidence that c-MYC inhibition may also de-repress the p15 and p27 CKI promoters, its repression being potentially even more significant to G0 entry (3, 18).

Combined, as TGFβ activates SMAD2/3/4, FOXO1, and SP1 and represses c-MYC, cell cycle arrest is induced by p21, p27, and the pathways involving proneural BHLH factor neuroM.

2.4 The p53/Brn-3a TF pathway:

A third and final pathway crucial to neural cell cycle arrest involves TFs p53 and Brn-3a. P53 is most well known for its pro-apoptotic function in most cell types, including neurons. However, through differential regulation, p53 also promotes cell cycle arrest and differentiation in neural progenitors. The difference between p53's pro-apoptotic phenotype (discussed later) and cell cycle arrest phenotype relies heavily on neuro-specific post-translational modifications (PTM). p53 has highly variable amino and carboxyl termini, which allow it to face a variety of PTMs, including phosphorylation, acetylation, sumoylation, neddylation, and ubiquitination. Depending on which PTM's p53 receives, its protein-protein interactions and subsequent function differ. P53's cell cycle arrest phenotype is mediated primarily by mechanisms of acetalization by histone acetyltransferases (HATs) (Figure 4). P53 acetylation occurs in response to a variety of G0 events including TGFβ signaling, RB hypophosphorylation, and the stimuli associated with various steps of neural differentiation. These modifications prevent p53 from binding to pro-apoptotic genes and allow it to activate the p21 promoter, inducing G0 entry. However, p53's shift from a pro-apoptotic phenotype to a pro-G0 phenotype also relies on the neuro-specific TF Brn-3a. Upon differentiation, p53 and Brn-3a form a complex that inhibits p53 from activating pro-apoptotic genes and increases its affinity for the p21 promoter. Combined, PTMs and Brn-3a help p53 adopt pro-G0 functionality. P53 then regulates G0 entry by activating p21 transcription, inhibiting proliferative MYC transcription, and activating the growth arrest and DNA damage (GADD45) gene (inductive of p21 and p27) (3, 18, 28, 29, 30).

Figure 4: Mechanisms of p53 acetalization to induce G0.

HAT mediating acetalization	CBP/p300	P/CAF
Target amino acid(s)	Lys 370, Lys 372, Lys 373 and Lys 382	Lys 320
Effects of acetylation	Contributes to p53 mediated differentiation and nuclear localization	Allows p53 to transcriptionally activate the p21 promoter and signals for p53 nuclear localization

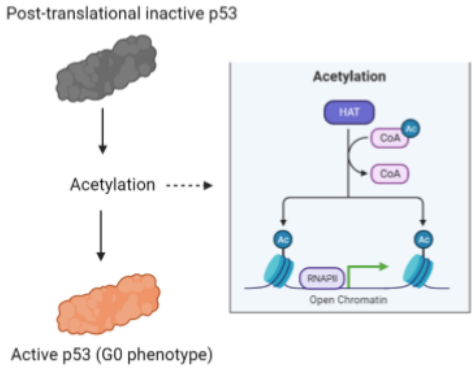


Figure 4: P53 acetylation at its carboxyl terminus regulates its ability to induce cell cycle arrest by increasing it's protein stability, allowing it to induce pro-G0 genes, and inhibiting its ability to bind to pro-apoptotic genes. Created with BioRender.com.

2.5 Summary of TF-dependent neuron cell cycle arrest:

Cell cycle arrest is achieved by inhibition of CDKs through production of CKIs. In neurons, this process is highly regulated by several TF-dependent pathways. The discussed mechanisms of TF-dependent neural cell cycle arrest can be summed up as follows: upon the stimulus of cyclin D1 in developed neurons ready to enter G0, CDK 4/6 is initially activated and hypophosporylates RB proteins. Hypophosphorylated RB inhibits proliferative ID proteins to promote activity of pro-G0 BHLH TFs while also directly repressing proliferative E2F TFs. Meanwhile, the onset of TGFβ in neurons prepared to enter G0 induces TF SMAD-dependent cell cycle arrest. TGFβ activated SMAD proteins promote expression of proneural BHLH and CKIs. However, SMADs also interact with TFs FOXO1 and SP1 to induce CKI expression and

inhibit proliferative c-MYC TFs. Finally, both hypophosphorylated RB and TGFβ, alongside other G0 stimuli, activate TF p53. P53 interacts with TF Brn-3a to further induce CKI expression. As CKIs rise to substantial enough levels through the pathways discussed, they inhibit the CDKs which induced their very onset. CDK inhibition blocks expression of proliferative S phase genes, leaving the cell cycle arrested in G0.

3. How TFs induce neural apoptosis upon attempted cell cycle re-entry:

3.1 G0 maintenance/ Apoptosis upon cell cycle re-entry:

After initial withdrawal from the cell cycle, the G0 state is maintained CKIs, necdin proteins, and nerve growth factors (NGF). In these components' absence, neural cell cycle re-entry is attempted but thwarted by a lack of proper S phase machinery. To prevent abnormal and potentially cancerous S phase onset, neurons have developed TF-dependent pro-apoptotic pathways in response to attempted proliferation. Discussed are the mechanisms by which CKIs, necdin, and NGF contribute to G0 maintenance, and how their withdrawal triggers neural apoptosis (16).

CKIs, by inhibiting CDKs, are essential to G0 maintenance. Cip/Kip CKIs p21, p27, and p57 are upregulated preceding cell cycle arrest and continue to be expressed throughout the G0 lifespan. Neural INK4 CKIs p15, p16, and p19 also face increased expression upon G0 entry (Figure 5). Collectively, these two classes of CKIs maintain neuronal mitotic quiescence by inhibiting CDK 4/6 and 2. Upon Cip/Kip and INK4 CKI withdrawal, CDK-derepression-dependent apoptosis is triggered. Specifically, CDK 4/6 plays the largest role in regulating several neural apoptosis pathways (3, 5, 16, 19).

Figure 5: CKI expression in neurons.

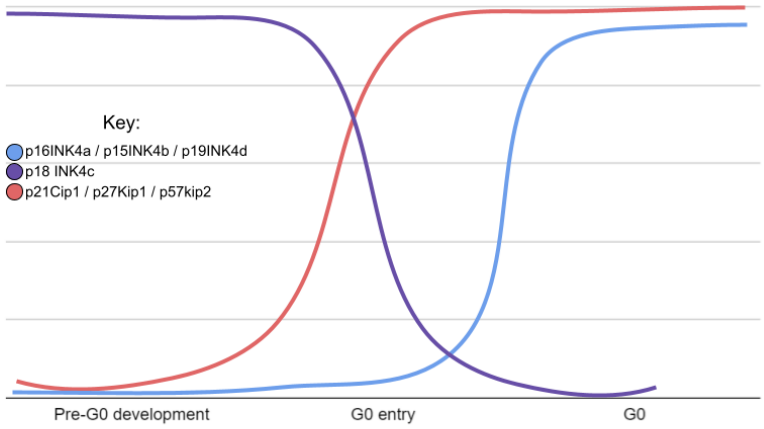


Figure 5: P18 is the only CKI expressed during pre-G0 development. Before G0 entry, Cip/ Kip CKIs p21, p27, and p57 rise preliminarily and facilitate cell cycle arrest. Upon G0 entry in neurons, p18 expression diminishes and is replaced with p19, p16, and p15. P21, p27, p57, p19, p16, and p15 expression remains high while neurons rest in G0 to maintain its non proliferative state. Note: data is relative not numerical.

The neuron-specific protein necdin is also essential to G0 maintenance by functionally substituting for RB proteins pRB and p107 after their degradation post-differentiation (Figure 6). Necdin fills the essential niche of these RB members by binding to the transactivation domain of proliferative E2F1 and repressing E2F-dependent transcription. While RB and necdin are functionally similar, the two are structurally divergent (Figure 7). Structural differences allow necdin to also bind to the amino-terminal transactivation domain of p53 and inhibit

p53-dependent transcription. It does not counteract p53-induced cell cycle arrest but does repress p53-dependent apoptosis, further contributing to the maintenance of the postmitotic state of neurons. In necdin's absence, both p53 and E2F1's pro-apoptotic functions are activated and cell death ensues (16, 31, 32).

Figure 6: RB/Necedin expression in neurons.

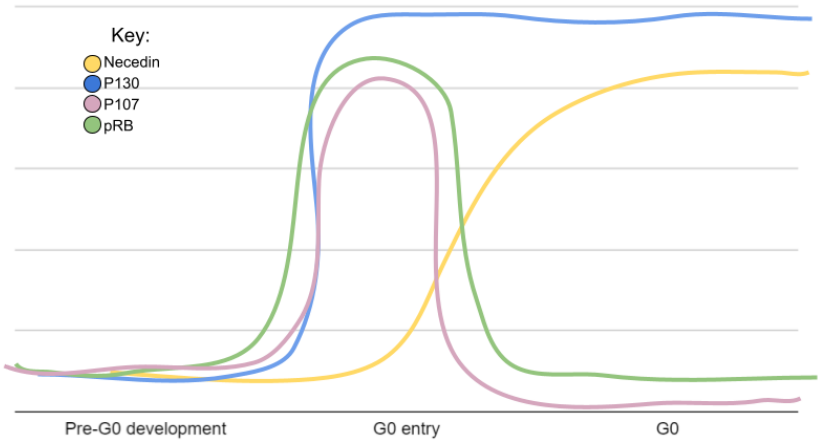


Figure 6: All RB expression is negligible while neurons are in a developing pre-G0 state. Upon G0 entry, all three RB protein expression levels rise. During terminal differentiation, RB members pRB and p107 are rapidly downregulated in neurons and replaced with protein necdin, while p130 expression remains high. Note: data is relative and not numerical.

Figure 7: RB1 vs. Necdin structural comparison.

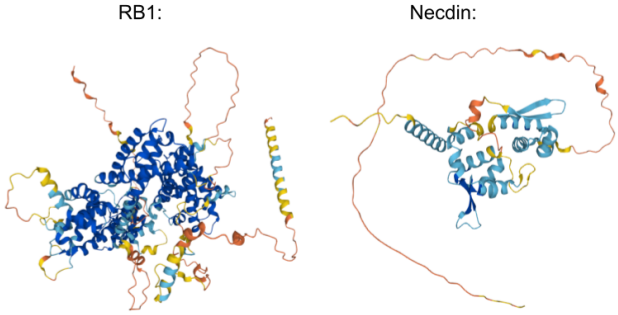


Figure 7: While necdin is functionally similar to Rb, the two are structurally dissimilar: in the mouse CNS, necdin has 325 amino acids whereas RB1 has 921. Since necdin is so much smaller than RB1, it binds to T-antigen at the amino-terminal region of the Rb-binding domain to suppress E2F-driven transcription. It's reduced size is also what allows necdin to bind to p53. (31,32)

Finally, NGF is also vital to the maintenance of the G0 state because it activates essential survival pathways. NGF promotes pathways that allow cells to thrive in G0 and represses pro-apoptotic pathways and molecules such as the p75 neurotrophin receptor. Deprivation of NGF results in direct de-repression of these pro-apoptotic machinery. NGF's absence also triggers CDK-dependent apoptosis by promoting the expression of cyclin D1. Like CKI withdrawal, this method of apoptosis relies heavily upon CDK 4/6 (5, 19, 33, 34).

Combined, the presence of CKIs, necdin, and NGF helps neurons maintain a stable G0 state. In these factors' absence, pro-apoptotic pathways which rely on CDK 4/6 and cell cycle TFs are activated. Here, we discuss how CDK 4/6 interacts with RB proteins to trigger multiple

E2F1-dependent pro-apoptotic pathways. Specifically, we observe E2F1's interactions with FOXO1, C-JUN, and p53 TFs, which collectively facilitate neural cell death.

3.2 Interactions between CDK4/6 and RB: spurring a pro-apoptotic excess of E2F1:

The majority of TF-dependent pro-apoptotic pathways in neurons also rely on CDK 4/6. In cells experiencing a temporary G0 state, CDK 4/6 activation causes cells to re-enter the cell cycle and proceed to S phase through modulation of proliferative genes. However, since S phase entry is recognized as malignant in neurons, neural CDK 4/6 signals have instead evolved to facilitate apoptosis through RB-mediated mechanisms. CDK 4/6's interactions with RB differ upon apoptotic stimuli from those witnessed upon cell cycle arrest (Table 3). Ultimately, CDK 4/6 hyperphosphorylates and subsequently inactivates p130, the only RB member remaining post-G0 entry. This initiates neural apoptosis through p130's interactions with E2F1 (Figure 8) (5, 16, 19).

Table 3: CDK interactions with RB in cell cycle arrest vs. apoptosis in neurons

	Cell cycle arrest	Apoptosis
CDKs initiating RB interaction	CDKs 4/6 and 2	CDK 4/6
RB's facing phosphorylative modulation	pRB, p130, and p107	p130
CDK action upon RB	hypophosphorylation (activation)	hyperphosphorylation (inactivation)

Table 3: CDK interactions with RB in cell cycle arrest vs. apoptosis in neurons. During cell cycle arrest CDKs hypophosphorylate and subsequently activate RBs. During apoptosis, CDKs hyperphosphorylate and inactivate RBs. All three RB members, pRB, p130, and p107, are prevalent in the mechanisms regulating cell cycle arrest. However, upon G0 entry, neural pRB and p107 are downregulated and replaced by necdin. Correspondingly, neither pRB nor p107 yield any regulative effect regarding neural apoptosis upon cell cycle re-entry, whereas p130 (remaining upregulated) controls massive strain-dependent neuronal death.

Figure 8: Visual of E2F1 de-repression

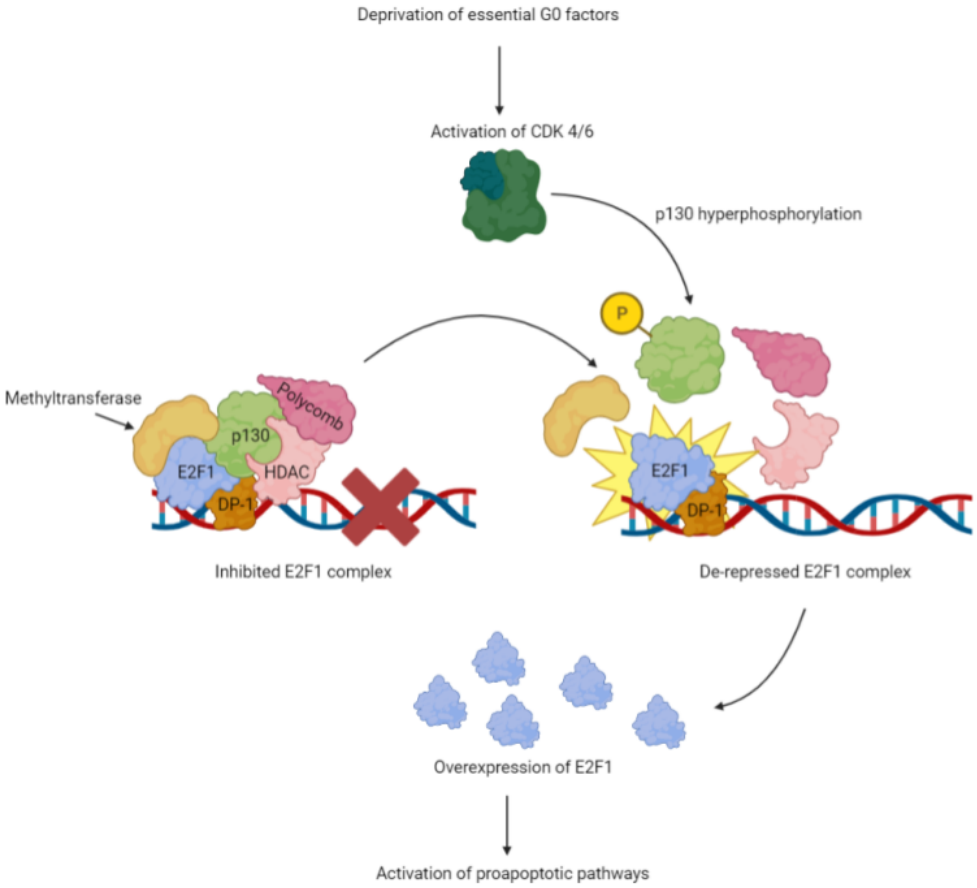


Figure 8: Upon deprivation of essential G0 factors, the pathway towards neural apoptosis is initiated by an influx of activated CDK 4/6. CDK4/6 de-represses the E2F1 inhibitory complex by hyperphosphorylating p130, leading to an overexpression and nuclear accumulation of E2F1. Free E2F1 then activates multiple TF-dependent pro-apoptotic pathways.

Upon G0 entry, E2F1 is degraded by various post-mitotic mechanisms (Figure 9) such that its activity is negligible in G0 neurons. Hyperphosphorylated p130 reverses E2F1 inhibition in cells destined for apoptosis. There are two major consequences of CDK-mediated hyperphosphorylation of p130. The first is the release of p130 from the E2F1 transactivation domain, promoting E2F1-dependent gene induction. The second is the dissolution of E2F1-chromatin modifying complex tethered to p130 (Figure 10), promoting transcriptional de-repression of E2F1-regulated genes. It is this latter mechanism of de-repression that facilitates E2F1-mediated apoptosis. In other words, it is not the expression of the E2F-modulated genes themselves that transduces E2F1-regulated neural apoptosis, but the fact that the inhibitory complex blocking E2F1 activity is absent. Loss of the necdin E2F- inhibitory complex is another mechanism by which this de-repression could occur. In either case, E2F1 de-repression transduces an over-amplified positive feedback cycle of E2F1 production. As E2F1 is overexpressed beyond the capacity of DNA binding sites, an excess accumulates in the nucleus. This free E2F1 then activates multiple TF-dependent pro-apoptotic pathways, including the CDK1/FOXO1 pathway, the JNK/C-JUN pathway, and the p53 pathway (5, 16, 35).

Figure 9: Mechanisms of post-mitotic E2F1 degradation.

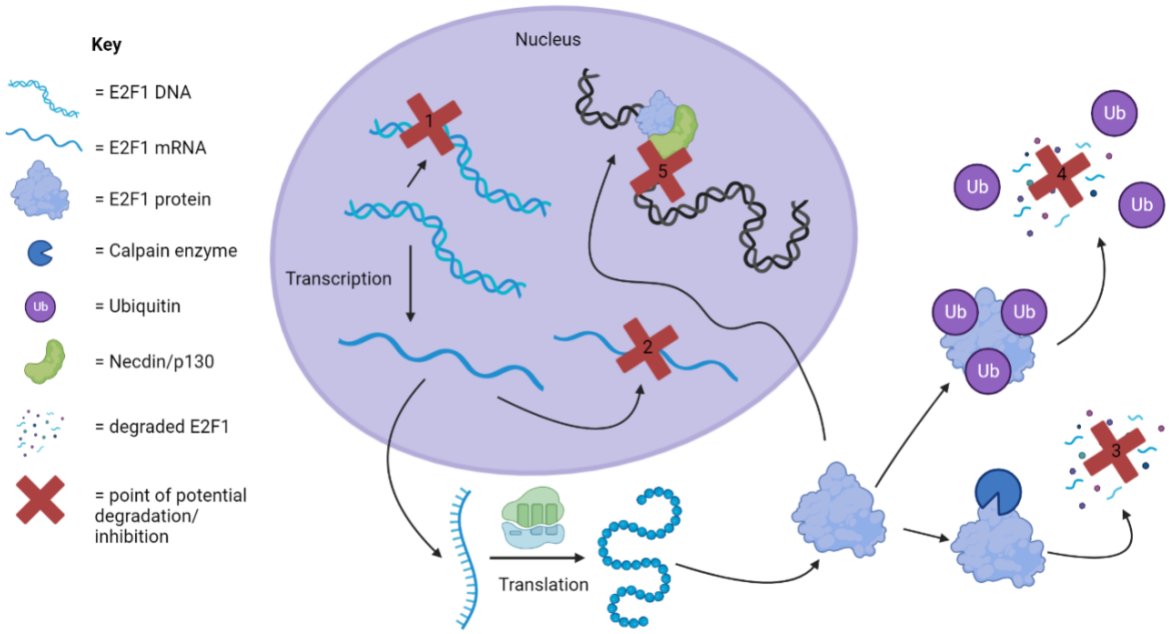


Figure 9: Created with BioRender.com. After the initial increase of E2F1 expression during neural differentiation and cell cycle arrest, E2F1 expression and activity decreases to undetectable levels in post mitotic neurons. This is achieved in the following mechanisms:

1. Transcriptional repression of E2F1 DNA by neural transcriptional mediators
2. Degradation of E2F1 mRNA during RNA processing: potential mechanisms of siRNA or miRNA degradation could inhibit E2F1 expression post-transcriptionally.
3. Post translational cleavage: there is potential evidence supporting post-transcriptional cleavage of E2F1 proteins by calpains. This would degrade E2F1 before it reaches the nucleus
4. Ubiquitin-proteasomal pathway: E2F1 is degraded post-translationally in G0 neurons by the Ubiquitin-proteasomal pathway. As ubiquitin proteins covalently attach to E2F1, they recruit a proteasome complex that degrades E2F1
5. Repression of E2F1 expression by Necedin and p130 proteins

Figure 10: E2F1/RB/HDAC complex.

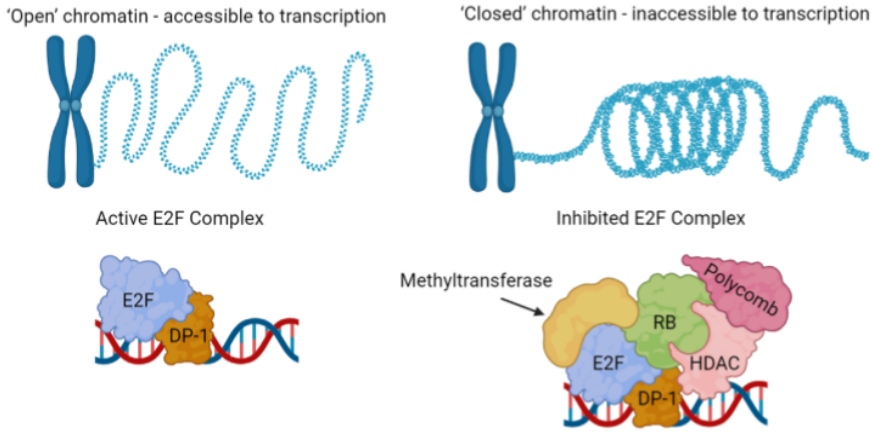


Figure 10: When RB binds to DNA-bound E2F, it recruits HDACS and methyltransferases to form a histone/chromatin modifying complex that represses transcription of target genes. Upon RB hyperphosphorylation, the chromatin modifying complex tethered to RB dissipates, causing a transcriptional derepression of E2F regulated DNA. Created with BioRender.com.

3.3 FOXO1 pro-apoptotic pathway: facing induction by E2F1 Interactions with CDK1

The first TF-dependent cell death pathway detonated by free E2F1 first involves CDK1. CDK1 is associated with neural cell death triggered by membrane activity deprivation and various neurodegenerative diseases such as Alzheimer's, frontotemporal dementia, and progressive supranuclear palsy. Excess free E2F1 regulates CDK1 in all of these contexts by activating the CDK1 promoter.

Activated CDK1 regulates apoptosis in two ways. The first is by directly phosphorylating and activating the pro-apoptotic BAD gene. CDK1 phosphorylates Bcl-2 family member BCL2 Associated Agonist Of Cell Death (BAD) at its Ser128 site, dissociating BAD from inhibitory 14-3-3 proteins and activating its transcriptional activity. The second is by inducing the TF FOXO1. While in non-neuronal cells, FOXOs initiate transcription of genes involved in DNA damage repair, protection against reactive oxygen species, and maintenance of organismal longevity, neural FOXO1 facilitates cell death. CDK1 binds to and phosphorylates FOXO1 on Ser249 in a mechanism resemblant of its activation of the BAD gene. Upon phosphorylation, FOXO1 faces nuclear accumulation and subsequent transcriptional activity, activating the pro-apoptotic Bcl-2 Interacting Mediator of cell death (BIM) gene. BIM gene induction, with the help of the BAD gene, is ultimately what facilitates apoptosis in a CDK1 and FOXO1 context (16, 19, 34).

3.4 C-JUN pro-apoptotic pathway: facing induction by E2F1 Interactions with GCK

The second E2F1-mediated pro-apoptotic pathway involves transduction of the C-JUN TF. E2F1 directly activates the MAP4K germinal center kinase (GCK). Transcriptional induction of GCK induces a phosphorylation cascade (Figure 11) that ultimately activates the JNK (Jun N-terminal kinases) family of MAP kinases. The JNK family consists of JNK1, JNK2 and JNK3. All three JNKs phosphorylate and activate the C-JUN protein of the activator protein-1 (AP-1) TF. In most proliferating cells, C-JUN functions in preventing apoptosis. However, in neurons, it does the exact opposite. C-JUN activation by JNKs causes neural cell death by activating the pro-apoptotic BIM, PUMA, and dp5/hrk family genes. These efforts combined facilitate C-JUN-induced neural apoptosis (34, 36, 37, 38).

Figure 11: E2F1/MAP4K phosphorylation cascade.

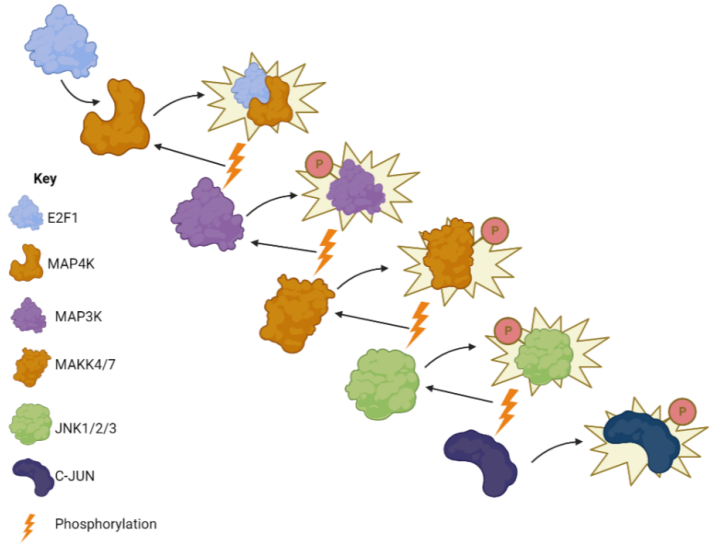


Figure 11: E2F1 is a direct substrate of the MAP4K germinal center kinase (GCK). Transcriptional induction of GCK by E2F1 induces it to phosphorylate MAP3Ks, which then phosphorylate the MAP2K isoforms MKK4 and MKK7. MKK4 and MKK7 phosphorylate and activate JNKs (Jun N-terminal kinases). The JNK family of MAP Kinases consists of JNK1, JNK2 and JNK3. A major target of all three JNKs is the c-Jun protein which is a part of the activator protein-1 (AP-1) TF. Created with BioRender.com.

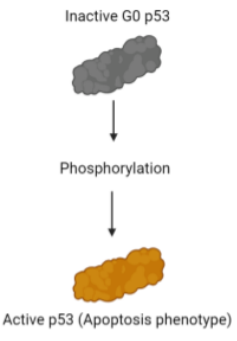
3.5 p53 pro-apoptotic pathway: directly modulated by free E2F1

A third and final TF-dependent neural apoptosis pathway mediated by free E2F1 involves the TF p53. p53 is notorious for its pro-apoptotic function in many cell species in response to a plethora of stimuli. In neurons, its activity is observed in response to NGF withdrawal, CKI inhibition, DNA damage, and a number of neurodegenerative disorders such as Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease. P53 has a heightened relevance to neuro-specific apoptosis over other TFs. Overexpression of p53 alone can spur cell death even in the presence of NGF, as all other pro-apoptotic pathways remain dormant. Furthermore, apoptosis is prevented in p53 deficient cells even in the absence of NGF and activation of all other pro-apoptotic pathways. Conclusively, p53 is a determinant factor in whether neurons will live or die upon G0 exit.

P53's pro-apoptotic function requires highly specific PTMs to distinguish it from its cell cycle arrest pathways. Specifically, to induce its pro-apoptotic phenotype, p53 faces several rounds of phosphorylation on its amino terminal region. These modifications induce p53's nuclear localization, activation of specific pro-apoptotic genes, and inhibition of proliferative genes (Figure 12). Specific phosphorylation of the Ser46 site by free E2F1 also inhibits p53's ability to bind to the p21 promoter. This action prevents the counteractive p53 cell cycle arrest pathway that would otherwise prevent p53-mediated apoptosis. P53 PTMs modulated by other TF pathways are also of note. Specifically, the pro-apoptotic JNK/C-JUN pathway mediates Thr81 phosphorylation on the p53 amino terminal. This action allows p53 to activate the pro-apoptotic Bcl-2 Associated X-protein (BAX) gene. The dissolution of necdin proteins from the p53 complex is another neuro-specific PTM required for p53 apoptotic regulation. Necdin proteins inhibit p53 at its amino terminal transactivation domain. A pathway outlining the release of necdin from p53 required for apoptosis has yet to be outlined. (5, 16, 28, 29, 33, 37, 39, 40, 41, 42, 43, 44).

Figure 12: Mechanisms of p53 phosphorylation to induce apoptosis.

Site of phosphorylation	Ser 15	Ser 20	Ser 33	Ser 46	Ser366	Ser392	Thr 18	Thr 81	Thr 304	Thr 77	Thr 387
Mechanism of phosphorylation	MAPK–p38 signaling	PLK3 and Chk2	MAPK–p38 signaling	Free E2f1, MAPK–p38 signaling, ATM signaling	CHK1	CK2	MAPK–p38 signaling and VRK1/2	JNK/C-Jun activity	LRRK2	LRRK2	CHK1
Pro-apoptotic consequence of phosphorylation	Prevents p53 nuclear export and triggers phosphorylation cascade of other p53 C/N terminal sites	Prevents p53 nuclear export and activates pro-apoptotic Fas, PUMA, IGFBP-3, Noxa, Bax and PIG3 genes	*	Induces p53API1 and inhibits binding to P21 promoter	Induces proapoptotic BAX gene	*	Prevents p53 nuclear export and activates pro-apoptotic Fas, PUMA, IGFBP-3, Noxa, and PIG3 genes	Induces pro-apoptotic BAX gene	*	*	Induces proapoptotic BAX gene



* The precise effects of the phosphorylation of this target site are unclear.
* P53 is also known to activate pro-apoptotic genes Bid, Bad, APAF1, and p73 in neurons, as well as repress proliferative genes Bcl-2, Bcl-xL, and Mcl-1. The PTMs allowing P53 to modulate these sites are unclear.

Figure 12: P53's pro-apoptotic phenotype is mediated primary by various rounds of phosphorylation on it's N and C terminals. Created with BioRender.com. (37, 39, 40, 41, 42, 43, 44)

3.6 Summary of TF-dependent neuron apoptosis:

When neurons attempt to exit G0, multiple pro-apoptotic pathways involving cell cycle TFs are activated. All of the pathways we discuss rely on the activation and de-repression of CDKs 4/6 by various apoptotic stimuli. These CDKs hyperphosphorylate RB protein p130, which de-represses the E2F1 gene by releasing its attached inhibitory complex. De-repression of E2F1 corresponds with a nuclear excess of free E2F1. Free E2F1 triggers three main TF-dependent pathways. The first involves E2F1's activation of CDK1. CDK1 directly phosphorylates the FOXO1 TF. FOXO1 then regulates the pro-apoptotic BIM gene, resulting in cell death. The second E2F1-dependent pathway relies on its activation of GCK which, through a phosphorylation cascade, ultimately activates TF C-JUN. C-JUN is a regulator similar to FOXO1 in its transcriptional activation of BIM, but also functions in promoting many other pro-apoptotic genes. Finally, E2F1 overexpression directly activates transcription factor p53. The group of pathways promoted by p53 is vast and likely the main stimulus of TF-induced neural apoptosis. Combined, these TF pathways facilitate cell death in neurons upon attempted proliferation, preventing their re-entry into the cell cycle.

4. Conclusion:

The inhibition of neural proliferation requires a complex collection of pathways that arrest cells in G0, maintain a stable G0 state, and prevent cell cycle re-entry by means of apoptosis. Cell cycle TFs contribute to all of these processes by functioning in a neuron-specific anti-proliferative manner. Regarding the initial entry into G0 by CDK inhibition through CKI production, three major TF-dependent pathways emerge. The first involves hypophosphorylated RB proteins which induce the activity of proneural BHLH and repress proliferative E2F. The second involves the stimulus of TGFβ, which induces the activity of pro-G0 SMAD, FOXO1, and SP1 while repressing proliferative c-MYC. The third pathway involves activation of pro-g0 p53 and Brn-3a. Combined, these efforts facilitate entry into the G0 phase which is maintained by essential CKIs, necdin proteins, and NGF. Upon deprivation of these elements, cell cycle progression is attempted but impeded by apoptotic pathways that facilitate cell death before any significant proliferative progress can be made. The TF-dependent pathways regulating this process all rely on the hyperphosphorylation of RB and subsequent overexpression of E2F1. The presence of excess free E2F1 activates three main pathways of relevance. The first involves E2F1 activation of CDK1 which induces pro-apoptotic FOXO1 TFs. The second entails E2F1 induction of GCK which ultimately facilitates the pro-apoptotic function of the C-JUN TF. Finally, the last involves E2F1's activation of pro-apoptotic p53 TFs, which appear to play the greatest role in facilitating TF-dependent cell death.

These efforts of neural cell cycle transcription factors induce cell cycle arrest and prevent its re-entry. This keeps neurons in a terminally unreplicable state that inhibits them from reproducing or repairing upon damage, disease, or degradation with age. The malignant consequences of this system are especially evident in the cases of significant injury, DNA damage, or degenerative disease. It remains speculative why neurons, in all their importance to organismal functionality, have evolved to possess such vulnerability to these threats compared to other cells. However, a growing body of research is unveiling potential solutions to neurons' anti-proliferation, giving hope for a future in which their aversion to replication can be diluted or potentially even annihilated.

Abbreviations:

BHLH - Basic helix–loop–helix
CDK - Cyclin dependent kinase
CKI - Cyclin dependent kinase inhibitor
CNS - Central nervous system
FOXO: Forkhead box protein O
HDAC - Histone deacetylases
JNK - Jun N-terminal kinase
MYC - Myelocytomatosis oncogene
NGF - Nerve growth factor
PTM - Post-translational modification
P15 - p15INK4b
P16 - p16INK4a
P18 - p18INK4c
P19 - p19INK4d
P21 - p21Cip1
P27 - p27Kip1
P57 – p57kip2
RB - Retinoblastoma
Ser - Serine
SMAD - Mothers against decapentaplegic
TF - Transcription Factor
Thr - Threonine
TGFβ - Transforming growth factor-beta

Acknowledgements
I would like to thank Danielle Clark, 5th year PhD student from the immunology department at Duke University, for acting as my mentor and guiding me through the writing process. I would also like to acknowledge the Polygence program and team for giving me this opportunity and helping me along the way.

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