PROGRAMA DE DOCTORADO EN EN BIOLOGÍA MOLECULAR Y BIOMEDICINA

TESIS DOCTORAL

NUEVO PAPEL DE ERK EN LA REGULACIÓN TRANSCRIPCIONAL DE MYC

PhD THESIS

A NOVEL ROLE FOR ERK ON MYC TRANSCRIPTIONAL REGULATION

AUTORA

MARTA MORANTE EZQUERRA

DIRECTORES

PIERO CRESPO BARAJA

LORENA AGUDO IBÁÑEZ

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Realizada por: **Marta Morante Ezquerra**

Dirigida por: **Piero Crespo Baraja y Lorena Agudo Ibáñez**

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El Dr. PIERO CRESPO BARAJA, Profesor de Investigación del Consejo Superior de Investigaciones Científicas (CSIC) en el laboratorio de Regulación espacial de las señales RAS/ERK en cáncer en el departamento de Señalización celular y molecular ubicado en el Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC), como Tutor/Director de esta Tesis y la Dra. LORENA AGUDO IBÁÑEZ, Investigadora del Consejo Superior de Investigaciones Científicas (CISC) en el mismo instituto, como co-Directora de esta Tesis

CERTIFICAN:

Que MARTA MORANTE EZQUERRA ha realizado bajo su dirección el presente trabajo de Tesis Doctoral en el Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC) titulado:

Nuevo papel de ERK en la regulación transcripcional de MYC

A novel role for ERK on MYC transcriptional regulation

Que consideran que dicho trabajo se encuentra terminado y reúne los requisitos necesarios para su presentación como Memoria de Doctorado al objeto de poder optar al grado de Doctor en Biología Molecular y Biomedicina por la Universidad de Cantabria.

Y para que conste y surta los efectos oportunos, expiden el presente certificado en Santander a 29 de marzo de 2022.

Fdo. Piero Crespo Baraja

Fdo. Lorena Agudo Ibáñez

Esta Tesis ha sido realizada en el Instituto de Biomedicina y Biotecnología de Cantabria (IBBTEC) perteneciente a la Universidad de Cantabria (UC) y al Consejo Superior de Investigaciones Científicas (CSIC), en Santander (Cantabria, España).

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RESUMEN

1. Introducción

Las proteínas quinasas activadas por mitógenos (MAPK) desempeñan un papel fundamental en la transmisión de señales externas generadas por estímulos al núcleo, donde activan los programas genéticos necesarios para la proliferación, diferenciación y supervivencia celular, además de cientos procesos celulares y tejido-específicos (M. J. Robinson & Cobb, 1997). En concreto, en la ruta de señalización Ras-ERK, los factores de crecimiento activan a Ras, una GTPasa que posteriormente induce una cascada de fosforilaciones que activan al resto de componentes de la ruta: Raf, MEK y ERK. Una vez activado, ERK puede quedarse en el citoplasma y actuar sobre sus sustratos citoplasmáticos, o translocarse al núcleo, donde puede regular la expresión génica de una amplia gama de factores de transcripción (Elk-1, CREB) (Whitmarsh, 2007), entre los que se encuentra MYC (Sears et al., 2000a). Sin embargo, en los últimos años se ha demostrado que ERK también puede regular procesos como la expresión génica y la arquitectura de la cromatina independientemente de su actividad quinasa (Rodríguez & Crespo, 2011). Además, se ha publicado que ERK2 se une a promotores de genes activos, uniéndose al DNA con un motivo definido: C/G-AAA-G/C (Hu et al., 2009).

En concreto *MYC* es uno de los genes cuya proteína está regulada por ERK. MYC es un factor de transcripción que, a su vez, regula la expresión de diversos genes (entre 1000 y 4000) y su expresión está altamente controlada por mecanismos transcripcionales, postranscripcionales y pos-traduccionales (Coller et al., 2000; Fernandez et al., 2003; Guo et al., 2000). En tumores, la expresión del gen *MYC* a menudo se desregula a través de diversos mecanismos que incluyen: mutaciones estabilizadoras, translocación cromosómica, amplificación de genes y sobreexpresión de la transcripción (Meyer & Penn, 2008); siendo ésta última la más frecuente. MYC se expresa en células en crecimiento, mientras que en células quiescentes su transcripción se encuentra inhibida (Albert et al., 2001). En cuanto a sus funciones biológicas se encuentran la regulación del ciclo celular, metabolismo, inmortalización, apoptosis, etc.; todo ello dependiendo de si su actividad transcripcional está activada o inhibida (C. Grandori et al., 2000). Sin embargo, a pesar del amplio conocimiento sobre muchas de las funciones de MYC, falta un conocimiento detallado de los procesos que se desarrollan en su promotor, responsable de la regulación de la producción transcripcional de MYC, tanto en entornos fisiológicos como patológicos.

Las proteínas quinasas y fosfatasas determinan el balance de fosforilaciones a nivel celular, lo cual supone un cambio funcional en muchas proteínas, alterando, por ejemplo, su localización celular, su estabilidad o su asociación a otras proteínas. Las quinasas dependientes de ciclina (CDK) son serina-treonina quinasas que funcionan para coordinar múltiples funciones celulares. Dependiendo de su función, podemos dividir las CDKs en aquellas que están involucradas principalmente en la regulación del ciclo celular (CDK1, CDK2, CDK4 y CDK6) o aquellas que juegan un papel en la regulación de la transcripción para influir en la proliferación y supervivencia celular al impulsar la expresión de numerosos genes diana (CDK7-9, CDK11-13, CDK19) (Malumbres et al., 2009).

En concreto, la transcripción de genes es un proceso llevado a cabo por la ARN polimerasa II (Pol II) y está altamente regulado, siendo las CDKs muy importantes en dicho proceso. La transcripción génica está dividida en cuatro partes: inicio, pausa, elongación y terminación (Parua & Fisher, 2020). Poco después del inicio de la transcripción, la ARN Pol II se detiene en la región próxima al promotor del sitio de inicio de la transcripción. Para su reanudación, y regulación de la elongación, el dominio carboxi-terminal (CTD) debe ser hiperfosforilado (Gibbs et al., 2017). Existen dos principales fosforilaciones, llevadas a cabo por ciclinas dependientes de quinasas. La primera la realiza la CDK7 (que fosforila la Ser5) lo cual permite la activación de la Pol II y estimula la transcripción del RNA, pero no su elongación (Akhtar et al., 2009; Glover-Cutter et al., 2009). Posteriormente entran en juego la CDK9 y su correspondiente ciclina T (CycT), promoviendo la transición de la pausa a la elongación transcripcional mediante la fosforilación de la Ser2 (Wong et al., 2014). CDK9 y CycT juntos constituyen el factor de elongación de la transcripción positiva b (P-TEFb), cuya activación es un proceso complejo. En células de mamífero, aproximadamente la mitad de P-TEFb está presente en grandes complejos inactivos. El equilibrio de P-TEFb activo e inactivo está estrictamente controlado y puede cambiar drásticamente en respuesta a señales externas como la estimulación por factores de crecimiento (Q. Zhou & Yik, 2006). Además, otras quinasas también participan en la activación de P-TEFb por mecanismos aún desconocidos. Por ejemplo, se ha demostrado que las señales mediadas por ERK favorecen el ensamblaje de heterodímeros CyclinT1-CDK9, lo que facilita el reclutamiento de P-TEFb a genes transcritos activamente (Fujita et al., 2008). También se ha demostrado que MYC juega un papel importante en el reclutamiento de P-TEFb en promotores de genes para ayudar en el paso de la elongación de la transcripción (Rahl et al., 2010).

Resultados previos obtenidos por nuestros colaboradores del laboratorio de Javier León, observaron que dentro del promotor *MYC* hay varias "ERK boxes" con la secuencia C/G-AAA-C/G que están altamente conservadas en humanos, ratones y ratas. Además, corroboraron mediante ensayos de inmunoprecipitación de cromatina (ChIP) que ERK2 se une al promotor *MYC* humano y de ratón. Dada la importancia de MYC en la promoción de la pausa-liberación de la ARN polimerasa II mediante el reclutamiento de p-TEFb, también realizaron un ChIP de CDK9 y ERK2 en células HeLa y 293T, observando que ERK2 y CDK9 coinciden en la misma región del promotor de *MYC*. Finalmente, otro hallazgo interesante reveló que ERK2 interactúa con CDK9 observado por Proximity Ligation Assays (PLAs).

2. Objetivos

En base a todos los resultados mencionados y a los datos publicados anteriormente, decidimos profundizar en el triángulo ERK2-MYC-CDK9 y nos propusimos los siguientes objetivos:

1. Estudiar el efecto de ERK en la regulación de MYC
2. Caracterizar la interacción de ERK con CDK9 y determinar las consecuencias en su funcionalidad
3. Estudiar si la interacción de ERK-CDK9 juega un papel en la regulación y la expresión de MYC

3. Materiales y Métodos

Para estudiar el papel de ERK2 en la regulación de MYC se utilizaron diversos inhibidores de componentes de la ruta Ras-ERK como el inhibidor de MEK (U0126) o el inhibidor de ERK (SCH772984) y se analizó el efecto de dichos inhibidores en células quiescentes o en crecimiento.

También se han utilizado fibroblastos embrionarios de ratón (MEFs) modificados, los cuales tienen eliminado ERK1 y tienen floxeado ERK2 por lo que, mediante la adición de hidroxi-tamoxifeno, se puede eliminar ERK2. Dichas células se han utilizado para el estudio de los efectos de la eliminación de ERK2 en la regulación y la expresión de MYC.

Mediante transfecciones en diferentes líneas celulares, hemos introducido un plásmido que tiene ERK2 fusionado con una secuencia de localización nuclear, la cual permite a ERK2 translocarse al núcleo en condiciones sin suero y estar constitutivamente activo. De esta manera, nos permite observar el efecto que tiene ERK2 en la regulación de MYC en células que no están proliferando ni están estimuladas por factores mitogénicos. También hemos utilizado plásmidos con mutaciones en diferentes puntos de ERK2, de manera que su actividad catalítica se veía inhibida. Dichos plásmidos, una vez transfectados en células humanas, nos han permitido analizar el efecto quinasa-dependiente o quinasa-independiente de la actividad catalítica de ERK2 sobre MYC o CDK9. Además, otros plásmidos con la región "insert" de ERK2 eliminada, nos han servido para analizar el efecto que tiene dicha región en la regulación de MYC.

Por otro lado, para analizar la especificidad de la interacción ERK-CDK9, se realizaron ensayos de co-inmunoprecipitación, así como pull-down de proteínas purificadas donde diferentes fragmentos de CDK9 fueron generados para ver cuál de ellos interaccionaba con ERK2. Además, se han generado construcciones de CDK9 con diferentes mutaciones en los dominios D y FXF, que son aquellos que permiten interaccionar con ERK. Dichos plásmidos se han utilizado para realizar co-inmunoprecipitaciones y PLAs para comprobar si se produce dicha interacción.

También se han utilizado siRNAs para silenciar CDK9. Dichos siRNAs han sido transfectados junto con ERK-nuclear para ver el efecto que tienen en la fosforilación de la serina 2 del CTD (C-terminal domain) de la ARN polimerasa II. Del mismo modo, se han usado para ver el efecto que tienen en la regulación de MYC comparando con y sin el efecto de ERK2 nuclear en células quiescentes. Además, se han generado partículas lentivirales con shRNAs de CDK9, con los cuales se generaron células estables para el estudio de la expresión de MYC.

Asimismo, se realizaron estudios *in vitro* de actividad quinasa para dilucidar la capacidad de los diferentes mutantes de ERK2 para fosforilar el CTD de la ARN polimerasa II, utilizando CDK9/ciclina K como sustrato.

Por último, generamos una construcción quimérica en la cual fusionamos la secuencia correspondiente a la región "insert" de ERK2 con el extremo C de CDK9 humano. Para

evaluar el efecto de esta quimera, realizamos ensayos de luciferasa que nos permiten ver el efecto que dichos plásmidos tenían en la regulación del promotor de *MYC*.

4. Resultados y Discusión

En este proyecto hemos estudiado cómo la presencia de ERK2 en el promotor *MYC*, donde coincide con CDK9, regula su transcripción de manera quinasa-independiente. Para ello, empezamos analizando el efecto de ERK2 en la síntesis de MYC tras estímulos mitogénicos, donde observamos que tanto la síntesis de mRNA como de proteína se veían afectadas cuando tratábamos células NIH3T3 con diferentes inhibidores de la ruta Ras-ERK, como el inhibidor de MEK (U0126) o el inhibidor de ERK (SCH772984). Además, observamos que, en MEFs con fenotipo "ERK-less", cuando eliminamos por completo ERK los niveles de MYC caían drásticamente, lo que apuntaba a ERK como un regulador directo de MYC.

Sin embargo, dado que ya estaba descrito que MYC es regulado a nivel de proteína por componentes de la ruta, quisimos discernir si el efecto que estábamos viendo era debido a una acción directa de ERK o si eran desencadenados por otros eventos transcripcionales dependientes de ERK. Para ello, utilizamos una construcción ERK2 con una señal de localización nuclear cuya secuencia permite que ERK se transloque al núcleo en ausencia de estímulos mitogénicos y permanezca constitutivamente activo. Gracias a ello, vimos que, en ausencia de suero, ERK2 era capaz de inducir la expresión de *MYC* a nivel de mRNA. Además, corroboramos que dicho efecto era causa directa de ERK y no de otros substratos como puede ser Elk-1. Estos resultados demuestran que la sola presencia de ERK2 nuclear es suficiente para inducir la expresión de *MYC* en condiciones en las que los factores de transcripción dependientes de ERK no están activos. Por lo tanto, apunta a la acción directa de ERK2 con el promotor *MYC* como desencadenante de la expresión de MYC.

Además de lo descrito anteriormente, vimos que el efecto de ERK2 en la expresión de MYC era independiente de su actividad quinasa, ya que observamos que mutantes deficientes en actividad quinasa eran igual de capaces de rescatar la expresión de MYC en células quiescentes cuando llevaban una señal nuclear. Asimismo, hemos determinado que la región responsable de inducir la síntesis de *MYC* se encuentra dentro la región "insert" de ERK2.

Por otro lado, también hemos caracterizado la unión de CDK9 y ERK2. Empezando por determinar que la unión entre ambas proteínas es específica de CDK9 y no otras CDKs como CDK7 o CDK8. Además, hemos visto que dicha interacción también ocurre con ERK1 y con mutantes de ERK2 que no tienen actividad catalítica, lo que indica que dicha interacción es independiente de la actividad quinasa de ERK2. Para poder caracterizar las regiones a través de las cuales ERK2 y CDK9 estaban interaccionando, realizamos diferentes construcciones donde fusionamos a GST diferentes partes de la proteína de CDK9 y con ellos realizamos un pull-down en células 293T donde inmunoprecipitamos ERK2. Con ello determinamos que las regiones comprendidas entre los aminoácidos 1-100 y 264-333 interaccionan con ERK2 y que dentro de dichas secuencias había un dominio de unión a ERK: un dominio D y un dominio FXF respectivamente. Para determinar qué región era crítica en dicha unión, creamos dos plásmidos con mutaciones en dichos dominios y mediante co-inmunoprecipitaciones y PLAs, determinamos que ERK2 y CDK9 se unen a través del dominio D, ya que en aquel plásmido con mutaciones en dicho dominio observamos que CDK9 y ERK2 no interaccionan, mientras que en el que tenía mutaciones para el dominio FXF sí. Además, cuando utilizamos mutantes de ERK2 en los que dichos sitios estaban eliminados o mutados, realizamos la misma observación.

También quisimos saber las consecuencias funcionales de la interacción ERK2 con CDK9. Como la fosforilación de la serina 2 del CTD de la RNA polimerasa por CDK9 es el hallmark de la activación de la elongación de la transcripción, utilizamos siRNAs para silenciar CDK9 a la vez que co-transfectamos ERK2 nuclear. Con ello observamos que es CDK9, y no ERK2, la quinasa que fosforila a la serina 2 del CTD de la RNA Pol II. Además, mediante un ensayo quinasa in vitro, confirmamos que ni ERK2, ni sus diferentes mutantes aumentan la actividad de CDK9 *in vitro*.

Por último, quisimos saber el efecto que la unión CDK9-ERK2 podía tener en la regulación de la expresión de MYC. Para ello, llevamos a cabo un experimento en el que se co-transfectaron células 293T con un siRNA de CDK9 y ERK2 nuclear en condiciones sin suero y vimos que el silenciamiento parcial de CDK9 es suficiente para impedir el aumento de los niveles de ARNm de *MYC* que es inducido por ERK2 nuclear. También vimos que al silenciar CDK9 la inducción de *MYC* provocada por estímulos mitogénicos se veía reducida.

En base a la bibliografía que indica que ERK2 puede unirse al promotor activo de genes y nuestras observaciones previas sobre la región "insert" de ERK2 como la región clave responsable de impulsar la expresión de *MYC*, pensamos que para impulsar la expresión de *MYC*, ERK podría estar sirviendo como link para CDK9 en el promotor *MYC* al unirse al ADN a través de su región insert. Para poner a prueba esta teoría, generamos una construcción quimérica en la que la secuencia correspondiente a la región "insert" de ERK2 se fusionó con el extremo C-terminal de CDK9 humano y se utilizó dicha construcción en un ensayo de luciferasa para determinar el efecto que tenía sobre el promotor de *MYC*. Con ello vimos que con la simple introducción de la secuencia "insert" de ERK2 en CDK9, era suficiente para reforzar la capacidad de CDK9 para inducir la expresión de *MYC* en condiciones de privación de suero.

5. Conclusiones

1. ERK2 regula la expresión de MYC a través de su interacción directa con el promotor de *MYC*, independientemente de otros eventos transcripcionales provocados por ERK.

2. Dicho mecanismo es independiente de la actividad quinasa ERK2.
3. Esta regulación requiere la interacción de ERK con CDK9. En tal complejo, ERK2 interactúa con CDK9 principalmente a través del dominio D. Al mismo tiempo, ERK2 interactúa con el promotor MYC a través de su región "insert".

4. ERK2 evoca la expresión de MYC al reclutar CDK9 en el promotor de MYC. Aunque ERK2 no induce la actividad quinasa de CDK9, sí que facilita la fosforilación mediada por CDK9 de la RNA Pol II en la serina 2 del dominio carboxi-terminal.

i. ABBREVIATIONS

Abbreviations

4HT	4- Hydroxytamoxifen
Ala, A	Alanine
AGO2	Argonaute RISC Catalytic Component 2
Arg, R	Arginine
Asp, D	Aspartate
ATP	Adenosine Triphosphate
BBS	Borate Buffered Saline
BLIMP-1	B lymphocyte-induced maturation protein-1
BRD4	Bromodomain Containing 4
BSA	Bovine Serum Albumin
CAK	CDK-activating kinase
CD	Common domain. MAP kinase D-recruitment domain
CDKs	Cyclin-dependent kinases
cDNA	Complementary DNA
CKM	CDK8 kinase module
CMV	Human cytomegalovirus
Co-IP	Co-ImmunoPrecipitacion
CS	Calf Serum
CTCF	CCCTC-Binding Factor
CTD	Carboxi Terminal Domain
Cys, C	Cysteine
DBD	DNA binding domain
DBP	DEF binding pocket
DMSO	Dimethyl sulfoxide
DSIF	DRB sensivity-inducing factor
DTT	Dithiothreitol
DUSP	Dual Specificity Phosphatase
ECL	Enhanced chemiluminescent system

EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
Elk-1	ETS ((E-twenty six) Like-1
EMT	Epithelial Mesenchymal Transition
Eno1	Enolase 1
ERK	Extracellular Signal-Regulated kinase
FBP	FUSE Binding Protein
FBS	Fetal bovine serum
FIR	FBP interacting Repressor
FXFP	Docking site for ERK
FUSE	Far UpStream Element
Gbr2	Growth Factor Receptor-Bound Protein 2
Glu, E	Glutamate
GTFs	General transcription factors
GTP	Guanosine Triphosphate
HAT	Histone Acetyltransferase
HDACs	Histone deacetylases
HDR	ERK Catalytic loop
HEPES	N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid)
HePTP	Hematopoietic PTP
HEXIM	Hexamethylene bis-acetamide inducible proteins
Hk2	Hexokinase 2
His, H	Histidine
HIV	Human immunodeficiency virus
HRP	Horseradish Peroxidase
HSV	Herpes Simplex Virus
HTLV-1	Human T-lymphotropic virus

IEGs	Immediate early genes
IL-6	Interleukin-6
IMP	E3 ubiquitin ligase
IMP7	Importin7
IPTG	isopropyl-β-d-thiogalactopyranoside
JNKs	c-Jun amino (N)-terminal kinases
Kb	Kilobase
Kd	Dissociation Constant
kDa	Kilodalton
KIM	Kinase Interacting Motif
KO	Knock Out
KSHV	Kaposi's sarcoma-associated virus
KSR1	Kinase Suppressor of Ras 1
LARP7	La-Related Protein
LB	Luria-Bertani Medium
Ldh	Lactate Dehydrogenase
mA	Milliamps
MKPs	MAPK phosphatases
MAPK	Mitogen-Activated Protein Kinases
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MEK	MAPK ERK Kinase
MePCE	Methylphosphatase Capping Enzyme
Met, M	Methionine
MKP-3	Mitogen-activated protein kinase phosphatase 3
NELF	Negative Elongation Factor
NES	Nuclear Export Signal
NGF	Nerve Growth Factor
NLK	Nemo-like kinase
NLS	Nuclear Localization Signal
NTS	Nuclear Translocation Signal
PARP-1	PolyADP-ribose polymerase 1

PBS	Phosphate Buffered Saline
PDGF	Platelet-Derived Growth Factor
PIC	Preinitiation Complex
PKA	Protein kinase A
PTPs	Protein Tyrosine Phosphatases
PPs	Protein Serine/Threonine phosphatases
PP2A	Protein Phosphatase 2A
Rb	Retinoblastoma
rpm	Revolutions per minute
RTK	Tyrosine Kinase Receptor
SDS	Sodium Dodecyl Sulfate
Ser, S	Serine
SHC	SH2-Containg Protein
SKP2	S-Phase Kinase Associated Protein 2
STAT3	Signal Transducer And Activator Of Transcription 3
STEP	Striatum enriched phosphatase
SOS	Son of Sevenless. RAS GEF
TAE	Tris-acetate-EDTA
TBS-T	Tris Buffered Saline-Tween
TCF4	Transcription Factor 4
Thr, T	Threonine
TL	Total lysate
TOP2A	Topoisomerase IIα
TRRAP	Transformation/ transcription domain-Associated Protein
Tyr, Y	Tyrosine
uDBPs	Unconventional DNA Binding Proteins
Val, V	Valine
WB	Western blot
wt	Wild-type

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1. INTRODUCTION

1.1. Mitogen-Activated Protein Kinases

Approximately 2% of the human genome encodes for protein kinases, which are a class of enzymes in charge of catalyzing the phosphorylation of different substrates within the cell. To date, 535 protein kinases and 50 pseudokinases have been identified in humans (KinBase: www.kinase.com). These proteins exert their function by transferring a phosphate group from a donor, typically ATP, to a protein substrate (Wilson et al., 2018). Protein kinases can be classified as protein-serine/threonine kinases or protein-tyrosine kinases, depending on the aminoacid acting as the phospho-acceptor. Protein phosphorylation is a tightly regulated process that controls distinct protein properties like its catalytic activity, stability or its localization (Hanks & Hunter, 1995; Johnson & Lapadat, 2002).

Despite being separated by a membrane from the outer environment, cells are able to respond to external stimuli, such as mitogens and growth factors, and transform those signals into cellular activities. Signaling pathways act as complex networks where proteins interact with each other, thereby transferring signals which regulate multiple cellular processes involved in transcription, differentiation, cell cycle or apoptosis among others.

One of the best characterized protein kinase families is the Mitogen-Activated Protein Kinases (MAPK hereafter), a family of serine-threonine kinases highly conserved throughout evolution (Cargnello & Roux, 2012). The MAPKs can be subdivided in four families with similar features, but involved in different biological processes (figure 1.1), namely: ERK1/2

(Extracellular signal-Regulated Kinases); c-Jun amino-terminal kinases (JNK1/2/3; also called SAPKs); p38 kinases (p38α/β/γ/δ); and ERK5 (Big MAP Kinase). These MAPKs share the characteristic of being activated by a three-tier protein kinase cascade which include: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and finally the MAPK itself. The latter is activated by dual phosphorylation of a tripeptide motif (Thr-X-Tyr) located in the activation loop (T-loop) (Kassouf & Sumara, 2020). While ERK1/2 is usually activated by growth factors, JNK, p38 and ERK5 cascades can also be triggered by stress stimuli (Roberts & Der, 2007; Roskoski, 2012).

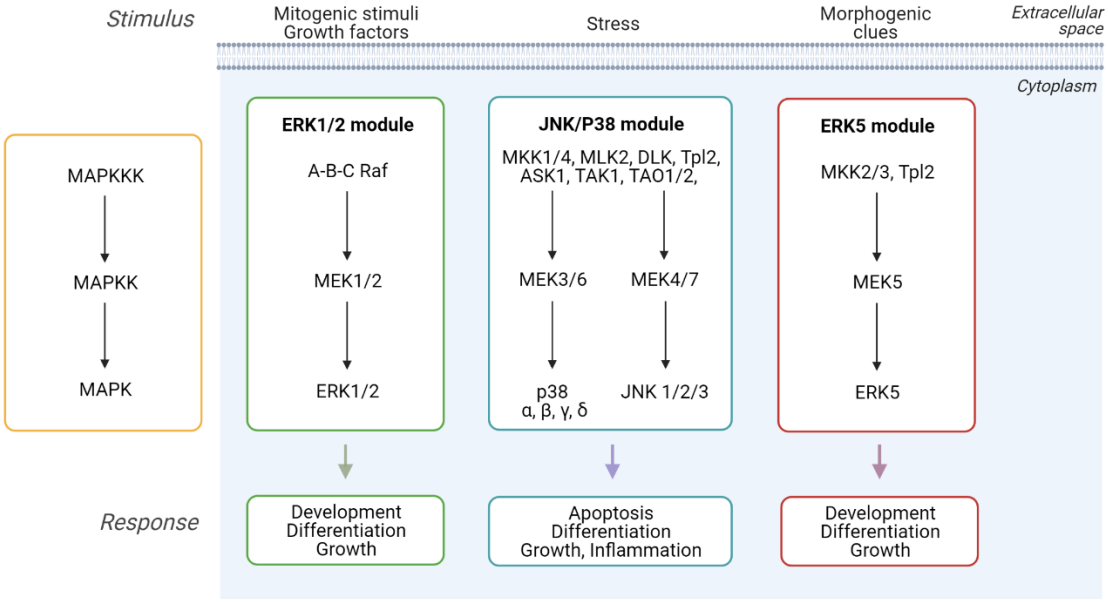


Figure 1.1. Simplified overview of the four classical MAPK. Each cascade is triggered by a stimulus such as growth factors or stress, and ends up provoking a response with different biological outcomes. Generated with BioRender.

In addition to the aforementioned MAPKs, others have been described, named atypical MAPKs. These include ERK3/4, ERK7, and NLK (Nemo-like kinase). In contrast to the conventional MAPKs, these are not structured into classical three-tiered kinase cascades, and there is still a lot of information missing regarding the exact molecular mechanisms involved in their activation and their role (Coulombe & Meloche, 2007).

1.1.1. RAS-ERK signaling pathway

Another common feature for all of the aforementioned cascades is that they are regulated at their origin by small GTPases of the RAS family (Goitre et al., 2014). The pathway connecting RAS to the ERK cascade, the RAS/RAF/MEK/ERK pathway (RAS-ERK hereafter) is, undoubtedly, one of the best studied and characterized signaling pathways within the cell (figure 1.2). This route can be activated by different growth factors such as PDGF (Platelet-Derived Growth Factor), EGF (Epidermal Growth Factor), and NGF (Nerve Growth Factor), and in response to insulin (Boulton et al., 1990). Activation of this pathway is crucial for transmitting signals in order to regulate gene expression, proliferation, development, differentiation or prevent apoptosis (Mebratu & Tesfaigzi, 2009). Therefore, it is not surprising that its dysregulation is associated with many diseases, including cancer (Kim & Choi, 2015).

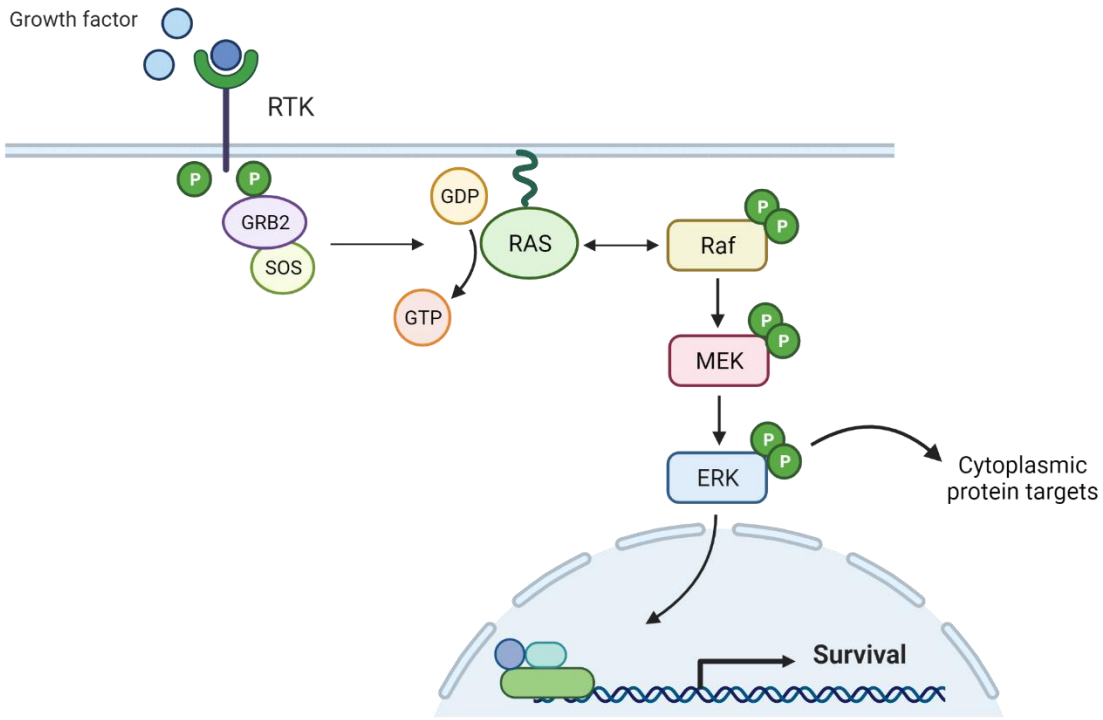


Figure 1.2. Scheme of the RAS-ERK signaling pathway. Upon growth factor stimulation the Grb2-SOS complex is formed, which induces a change in the GTPase RAS from its inactive form RAS-GDP to its active form RAS-GTP. Activated RAS recruits RAF to the plasma membrane where it becomes activated. Then, activated RAF is able to active and phosphorylate MEK, which, in turn, phosphorylates and activates ERK. Finally, once ERK is activated it can exert its kinase function over its cytoplasmic and its nuclear substrates. Generated with BioRender.

As previously mentioned, the cascade is mainly activated by growth factors and other types of ligands via cell surface receptors like RTKs (receptor tyrosine kinase). In response to ligand binding these dimerize inducing the autophosphorylation of tyrosine residues, that provide binding sites for proteins containing phospho-tyrosine binding domains (SH2 domains) including the growth factor receptor-bound protein 2 (Grb2). These adaptor proteins recruit guanine nucleotide exchange factors such as SOS (son of sevenless). SOS is recruited from the cytosol to the plasma membrane as a consequence of its interaction with Grb2 resulting in its activation. Once activated, SOS catalyzes the nucleotide exchange on RAS from GDP to GTP (Waters et al., 1996).

In quiescent conditions, Ras is bound to GDP. Upon activation, the GEFs (Guanine Nucleotide Exchange Factors) induce a conformational change on RAS structure by promoting the exchange from GDP to GTP, allowing RAS activation at the plasma membrane. Activated RAS has seven *bona fide* downstream effectors: PI3K, RalGDS, RIN, TIAM-1, PLCε, RASSF1 and RAF (Herrmann & Nassar, 1996; Nakhaeizadeh et al., 2016). In particular RAF kinases are the MAPKKKs responsible for initiating the ERK phosphorylation cascade. Their exact activation mechanism is still controversial, but it is well known that it requires the activation of RAS at the plasma membrane (Waters et al., 1996); phosphorylation at different residues, carried out by PKA (protein kinase A) (Dhillon et al., 2002) and AKT (Zimmermann & Moelling, 1999); and the dissociation from 14-3-3 proteins that in resting conditions maintain RAF in its inactive form (Zimmermann & Moelling, 1999).

Activated RAF subsequently phosphorylates thereby activates MEK (MAPK ERK Kinase). The MEK family is formed by two members with an aminoacid identity of 80% (Zimmermann & Moelling, 1999), MEK 1 and MEK2, which can also be phosphorylated by other MAPKKKs like Tpl-2 (Zimmermann & Moelling, 1999), MLKs (Marusiak et al., 2014) and PAKs (Frost et al., 1997). MEK1/2 are dual specificity kinases, their phosphorylation occurs in two contiguous serine residues, that in MEK1 are Ser218 and Ser222 (D. R. Alessi et al., 1994). These residues are found within the activation loop, which has the typical MAPKKs motif Ser-Xaa-Ala-Xaa-Ser/Thr. MEK exerts its kinase activity over its only known substrate, ERK1/2, which becomes phosphorylated both in tyrosine and threonine, thereby the dual specificity. However, MEK not only phosphorylates ERK, but also function as an ERK1/2 cytoplasmic anchor (Fukuda et al., 1997).

In resting conditions ERK1/2 is found in the cytoplasm bound to different anchoring proteins, such as MEK. Once the cascade is triggered and MEK is phosphorylated, it can phosphorylate and activate ERK and the ERK interaction with such proteins is broken (Caunt & McArdle, 2010), allowing ERK to exert its kinase activity over its substrates in different ways. On the one hand, activated ERKs can dimerize and regulate multiple cytoplasmic substrates. On the other hand, ERKs monomers can enter into the nucleus where ERK acts over its nuclear substrates such as transcription factors like ELK or c-MYC (B. Casar et al., 2009).

1.1.1.1. ERK1/2 structure

The genes coding for *ERK1* and *ERK2* are found on the chromosomal loci *16p11*.2 and *22q11.21* and have 10 and 9 exons respectively (L. Li et al., 1994; Olea-Flores et al., 2019; Xing, 2013). In mammals, ERK1 and ERK2 cDNAs were cloned in the early 90s and share an 83% aminoacid homology and a 100% similarity regarding the residues involved in catalysis and docking interactions (Boulton et al., 1990, 1991). ERK1 protein has a molecular weight of 44 kDa, whereas the molecular weight of ERK2 is 42 kDa. Both proteins are ubiquitously expressed, with particular high levels in the brain, skeletal muscle, thymus, and heart (Boulton et al., 1991). However, in general, ERK2 is expressed at higher levels than ERK1 (Boucher et al., 2004). Other ERK isoforms derived from alternative splicing ERK1b (Yung et al., 2000), ERK1c (Shaul & Seger, 2006) and ERK2b (Gonzalez et al., 1992) have been described, but these are expressed at very low levels (Boucher et al., 2004). ERKs proteins are very stable, they display very long half-lifes, being 68 h for ERK1 and 53 h for ERK2 (Schwanhüusser et al., 2011).

There are only a few differences between ERK1 and ERK2 regarding their sequence. The former contains 17 extra aminoacids on its N-terminal domain and two more residues on its C-terminal domain, which makes ERK1 a 379 aminoacids protein in comparison to the 360 aminoacids of ERK2 (figure 1.3) (Olea-Flores et al., 2019). However, despite these minor differences it is no clear yet whether ERK1 and ERK2 have specific or redundant functions. For instance, *erk1* knock-out (KO) mice are viable and fertile (Nekrasova et al., 2005; Pagès et al., 1999), whereas the *erk2* KO results in embryonic lethality (Hatano et al., 2003; Saba-

El-Leil et al., 2003), suggesting different biological functions. However, more recent evidence suggest that both proteins could have redundant functions since the phenotype observed in the *erk2* KO mice could be due to the higher expression observed in ERK2, in comparison with ERK1 (Buscà et al., 2015; Frémin et al., 2015). Moreover, this study highlights that the overexpression of ERK1 can compensate the ERK2 deficiency during development, which points out that ERK1 and ERK2 may exert redundant functions in mouse development (Frémin et al., 2015) and that the overall ERK dosage is an important factor. Thus, it is important to take into account the global level of ERK1/2 activity, since ERK2 is generally expressed at higher levels than ERK1.

The protein structure of both ERKs is composed of an N-terminal region, formed by five antiparallel β sheet-strands (β1- β5) and a conserved αC-helix. Between the β1- β2 strands a conserved glycine-rich (GxGxG), ATP-phosphate-binding loop, and a conserved valine (V56/V59 for ERK1 and ERK2 respectively) are found, which will make contact with the adenine of ATP. The β3-strand contains a lysine (K71/54 for ERK1 and ERK2 respectively) responsible for the connection between the α and β phosphate of ATP and a conserved glutamate (E88/71 for ERK1 and ERK2 respectively) of the αC-helix (Roskoski, 2012). This interaction is essential for catalysis to happen as demonstrated by the substitution of the K54 residue of ERK2 by an arginine (K54R), which results in a dead-kinase mutant devoid of catalytic activity (Canagarajah et al., 1997).

Within the ERK C-terminal region there are six conserved α helixes (αD-αI) and four short β-strands (β6-β9), where most of the catalytic residues responsible of the phospho-transfer activity are found. The HRD motif (the first D of K/D/D) found in the β6 strand is where the catalytic loop begins (Roskoski, 2012). The aspartate (D169/149 for ERK1 and ERK2 respectively) located within the HRD motif extracts the proton from the OH group of the protein substrate. This facilitates the nucleophilic attack of oxygen on the γ-phosphorus atom of Mg-ATP (Madhusudan et al., 1994). After the HRD motif, the DFG motif is located (the second D of K/D/D), which is composed of another aspartate (Asp186/169 for ERK1 and ERK2 respectively), and constitutes the first residue of the activation segment. Within this segment, the activation loop (also known as activation lip in ERK1/2) can be found, where the phosphorylatable threonine and tyrosine residues are located (T202/204 in ERK1 and T185/Y187 in ERK2). This motif is known as TEY, due to the glutamate that is located

between them (Canagarajah et al., 1997). Phosphorylation of the TEY motif induces conformational changes that allows a pocket formation, which act as a docking site for the proline in the P+1 position (P-X-S/T-P consensus phosphorylation motif) found in ERKs substrates. Another region of ERK2 known as the MAPK insert (residues 241-272) provides additional functional specificity and is responsible for the binding of substrates with FXF motifs (Whitehurst et al., 2004). Finally, at the end of the C-terminal domain another docking domain is found (CD domain hereafter), involved in ERK interaction with MEK, substrates and phosphatases. Within the CD domain two aspartate residues (316 and 319 in ERK2) are found, essential for such interactions (Lee et al., 2004). This domain shares common features with other MAP kinases, but it is believed that the differences in the CD domains and adjacent residues are responsible for the substrate specificity (Tanoue et al., 2001). From now on I will be referring as ERK when talking about ERK1/2, unless specified otherwise.

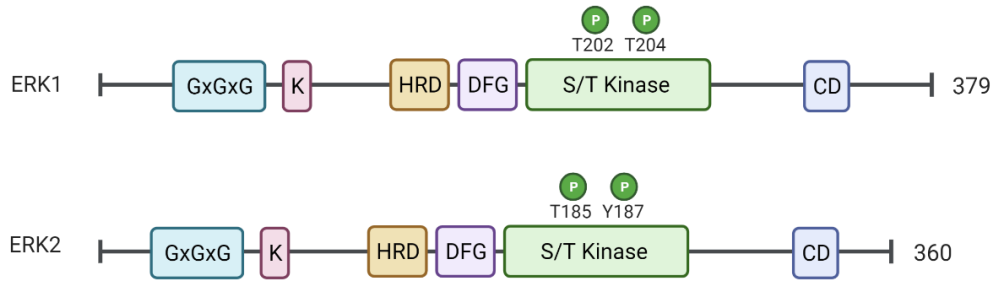


Figure 1.3. Scheme of human ERK1 and ERK2. The N-terminal region is composed of a glycine rich domain (GxGxG) and the lysine 54 (K) important for ERK catalytic activity. Within the C-terminal domain are found the motifs HDR and DFG that corresponds to the ERK catalytic domain and the beginning of the activation segment respectively. Moreover, within the DFG motif is also found the activation lip with the phosphorylatable residues. At the end of this domain is found the CD domain indispensable for ERK interaction with its substrates. Adapted from (Ryan et al., 2015). Generated with BioRender.

1.1.1.2. ERK activation

Upon MEK-mediated phosphorylation at residues T202/204 in ERK1 and T185/Y187 in ERK2 a conformational change in the phosphorylation and the nucleotide binding pocket occurs. These phosphorylations augment ERK kinase activity up to 6 orders of magnitude (F. Zhang et al., 1994). Once activated, ERK can follow two paths: they can dimerize and stay in the cytoplasm, or enter the nucleus in a monomeric form (Berta Casar et al., 2008; Herrero et al., 2015).

ERK2 capability to dimerize when phosphorylated was discovered in Cobb's laboratory (Canagarajah et al., 1997), whereas heterodimers of ERK1 and ERK2 are unstable. Dimers can also be formed by unphosphorylated ERK1/2, but its dissociation constant (Kd) is 3000 times higher in comparison with the phosphorylated ones (Khokhlatchev et al., 1998). Since its discovery, there have been different theories about the role of ERK dimerization. For instance, Philipova and Whitaker proposed that they could help in the maintenance of ERK basal activity (Philipova & Whitaker, 2005), whereas Khokhlatchev and colleagues thought that dimers could be playing a role in ERK nuclear translocation (Khokhlatchev et al., 1998). Nonetheless, data from our laboratory has shown that ERK dimers remain primarily in the cytoplasm, where they can associate with scaffold proteins (Casar et al., 2008). These platforms facilitate the signaling from the pathway helping phosphorylated ERKs to act over its cytoplasmic substrates (Casar et al., 2009).

Approximately half of ERK phosphorylated molecules will remain in the cytoplasm in dimeric form, while the other half will enter the nucleus as monomers. As aforementioned, in resting conditions ERK is anchored in the cytoplasm to proteins like MEK (Fukuda et al., 1997), or others like the scaffold protein KSR1 or the phosphatase DUSP6 (Caunt & Keyse, 2013). Once phosphorylated and activated, monomeric ERK translocates to the nucleus acting over its nuclear substrates. The mechanism of its nuclear translocation is not fully understood and different theories are in place. However, what is certain is that ERK does not contain a nuclear localization signal (NLS), meaning that its translocation does not occur by the canonical NLS and importin machinery, which, upon binding, facilitate the transfer of proteins across the NPC (Nuclear Pore Complex) (Maik-Rachline et al., 2019; Tran & Wente, 2006). Rony Seger's group have elucidated a mechanism whereby ERK binds to importin7

(Imp7), which then escorts ERK to the nucleus via the nuclear pores (Chuderland et al., 2008). This is possible thanks to a SPS (Ser244-Pro245-Ser246) sequence, defined as a nuclear translocation signal (NTS), that once phosphorylated at its serines can bind to Imp7. In the nucleus, the small GTPase Ran dissociates ERK from Imp7, leading to an accumulation of this kinase in the nucleus and the export of Imp7 back to the cytoplasm (Chuderland et al., 2008; Flores & Seger, 2013; Maik-Rachline et al., 2019; Zehorai et al., 2010). Another mechanism of ERK nuclear translocation is via binding with Mxi2 (a p38α isoform). Since this protein can also bind to nucleoporins, Mxi2 is capable of promoting ERK nuclear translocation and accumulation in a stimulus-independent manner (Casar et al., 2007). Recently, another translocation mechanism mediated by the protein kinase CK2 has been reported: while ERK is bound to its cytoplasmic anchors, the NTS cannot be phosphorylated; however, upon stimulation, phosphorylation of the ERK TEY domain releases ERK and allows phosphorylation of its NTS by CK2, thanks to which ERK can bind to importin 7 and translocate to the nucleus (Plotnikov et al., 2019).

1.1.1.3. ERK substrates

Given the importance of ERK signaling in a wide variety of cellular processes, it is not surprising that ERK is capable of phosphorylating more than 400 substrates localized in different cell compartments like the nucleus, cytoplasm or different organelles (Ünal et al., 2017; L. Yang et al., 2019; Yoon & Seger, 2006). As previously highlighted, ERK substrates are phosphorylated in a motif contained on their sequence: Ser/Pro P+/-1 (Canagarajah et al., 1997). However, this phosphorylation motif is not specific for ERK and does not determine ERK binding. A requisite for being an ERK-interacting protein is the presence of docking regions within their sequence, namely the D domain and the DEF domain.

- *D domain*, also known as DEJL site (docking site for ERK or JNK, LXL). This domain consists of two or more basic residues, a short peptide linker, and a cluster of hydrophobic residues (Kallunki et al., 1994) and has the consensus sequence $(K/R)_{1–3}$-$X_{1–5}$-*φXφ*, where *φ* is a hydrophobic residue, typically Leu, Ile, Val, or Met. Some ERK substrates containing this domain are MEK, Elk-1, p90Rsk-1, MKP-3, and caspase-9 (Sharrocks et al., 2000). However, this domain is not an ERK-exclusive

binding site, as it also serves as a docking site for other MAPKs (Dimitri et al., 2005). In particular, the ERK region that interacts with this domain is the CD domain, which has two critical residues (D316 and D319 in ERK2) that, if mutated, impede ERK interaction with its substrates (Lee et al., 2004; Tanoue et al., 2000).

- *DEF domain*, also known as FXFP or docking site for ERK. This domain contains the consensus sequence F/W-X-F/Y/W-$P_{+3,4}$. The F-site is located 6-20 amino acids C-terminal to the phosphorylation site (Sheridan et al., 2008). Some of the ERK substrates bearing this domain are scaffolds like KSR1 or transcription factors such as Elk-1, c-Fos, c-MYC, and JunD (Jacobs et al., 1999; Murphy et al., 2004; Vinciguerra et al., 2004). However, not all ERK substrates contain this domain, which suggest that is a more specific binding site than the D domain (Sharrocks et al., 2000). The DEF domain binds to ERK within the motif DEF binding pocket (DBP) (L198/232/235 and Y231/261 in ERK2) present in ERK insert region (Lee et al., 2004).

1.1.1.3.1. Nuclear substrates

Once stimulated, ERK rapidly translocate into the nucleus and regulates and activates many sets of genes, playing an important role in the regulation of proliferation and also in oncogenic transformation (Plotnikov et al., 2015; Whitehurst et al., 2004). Some of its most important substrates are transcription factors such as Elk-1, c-Fos or c-MYC (for further insight for c- MYC see chapter 1.2.). Elk-1 is a member of the ternary complex factor (TCF) subfamily of ETS-domain transcription factors (Buchwalter et al., 2004) and is simultaneously phosphorylated at 6-9 different sites (Cruzalegui et al., 1999), being the phosphorylation at Ser383 crucial for its activity (Buchwalter et al., 2004). The interaction with ERK occurs through the two docking regions present in Elk-1, the D domain (Sharrocks et al., 2000) and the DEF domain (Jacobs et al., 1999), which makes Elk-1 a widely used model for the study of ERK phosphorylation-dependent regulation of transcriptional activation (Yoon & Seger, 2006). Another nuclear target of ERK is c-Fos, a transcription factor whose activity is essential for the induction of proliferation and also plays roles in in differentiation and oncogenic transformation (Eferl & Wagner, 2003). c-Fos is an immediate early gene (IEGs), meaning that it is expressed within minutes after cellular stimulation. Its

induction is mediated by Elk-1. Unlike c-Fos, which in resting conditions is not expressed, Elk-1 does, and after ERK phosphorylation Elk-1 binds to a transcriptional complex that binds to the c-Fos promoter, inducing its expression (Gille et al., 1992). After that, ERK phosphorylates c-Fos at Ser374 and stabilizes it, augmenting its transforming activity (R. H. Chen et al., 1996; Okazaki & Sagata, 1995). Another difference is that c-Fos lacks a D domain, but its sequence contains a DEF docking domain located between amino acids 343 to 346, which allows its binding with ERK (Murphy et al., 2004). The duration of ERK activity regulates the accumulation of c-Fos, making this protein an important readout mechanism of the ERK signaling (Murphy et al., 2002).

1.1.1.4. Regulation of ERK's activity

Activated ERK must be tightly regulated in order to maintain a proper cell physiological homeostasis and prevent deleterious effects of unnecessary prolonged pathway stimulation. This regulation is carried out by protein phosphatases and negative feedback loops.

1.1.1.4.1. Protein phosphatases

One of the main regulatory mechanisms that modulates ERK activity is based on protein phosphatases, which remove one or both phosphates from the ERK activation lip (Anderson et al., 1990). Phosphatases can be categorized based on their substrate specificity as follows:

- *Protein tyrosine phosphatases (PTPs).* These proteins belong to a family of enzymes capable of dephosphorylate tyrosine residues within the ERK TEY motif. This family includes subfamilies like PTP-SL (Step like PTP), STEP (striatum enriched phosphatase) and HePTP (hematopoietic PTP) based on its protein domain architecture (Alonso et al., 2004). PTPs substrate specificity is determined by their kinase interacting motif (KIM), which binds to ERK through its D-domain (Tanoue et al., 2000).

- *Protein Serine/Threonine phosphatases (PPs).* Usually, the initial dephosphorylation of the tyrosine residue by PTPs is followed by the removal of the second phosphate from the threonine residue by the PPs (Alessi et al., 1995; Yao et al., 2000). PP1A and PP1B are the ones in charge of dephosphorylating the threonine residue of ERK's TEY motif in both the nucleus and the cytoplasm (Dario R. Alessi et al., 1995; H. Sun & Wang, 2012).

- *Dual Specificity phosphatases (DUSPs).* Also known as MKPs (MAPK phosphatases). These are able to dephosphorylate both residues, the threonine and the tyrosine, of ERK's TEY motif. DUSPs can be classified depending on their localization and substrate specificity: class I (DUSP1/MKP-1, DUSP2/PAC-1, DUSP4/MKP-2 and DUSP5), which have a NLS and are induced by ERK; class II (DUSP6/MKP-3,DUSP7/MKP-X and DUSP9/MKP-4), that contain a NES sequence (nuclear export signal) thereby acting in the cytoplasm and are ERK specific; and class III (DUSP8, DUSP10/MKP-5 and DUSP16/MKP-7), more promiscuous and located in both nucleus and cytoplasm (Owens & Keyse, 2007).

1.1.1.4.2. Feedback loops

The RAS-ERK signaling cascade is subjected to extensive feedback regulation, which allows a tight adjustment of path activity (figure 1.4). Feedback loops are mainly based on the suppression of the activity of different upstream components of the pathway and can be subdivided into: events that depend on direct ERK phosphorylation and those that depend on ERK-induced transcriptional activity. The former are short-term, rapid mechanisms involving ERK-catalyzed inactivating phosphorylations of pre-existing components upstream in the pathway, such as SOS (Dong Chen et al., 1996), RAF (Dougherty et al., 2005; Hekman et al., 2005), MEK (Eblen et al., 2004; Sundberg-Smith et al., 2005), and some RTKs. The latter are delayed, but longer-term, mechanisms that require *de novo* protein synthesis and are exemplified by the ERK-induced expression of the DUSPs (Owens & Keyse, 2007), which subsequently dephosphorylate ERK. Another example is the sprouty protein, which prevents the binding of Grb2 with SOS (Mason et al., 2004). Such homeostatic controls are key to an

amplifying cascade of protein kinases in which the magnitude and duration of ERK activation can determine whether cells proliferate, differentiate, or die (Lake et al., 2016).

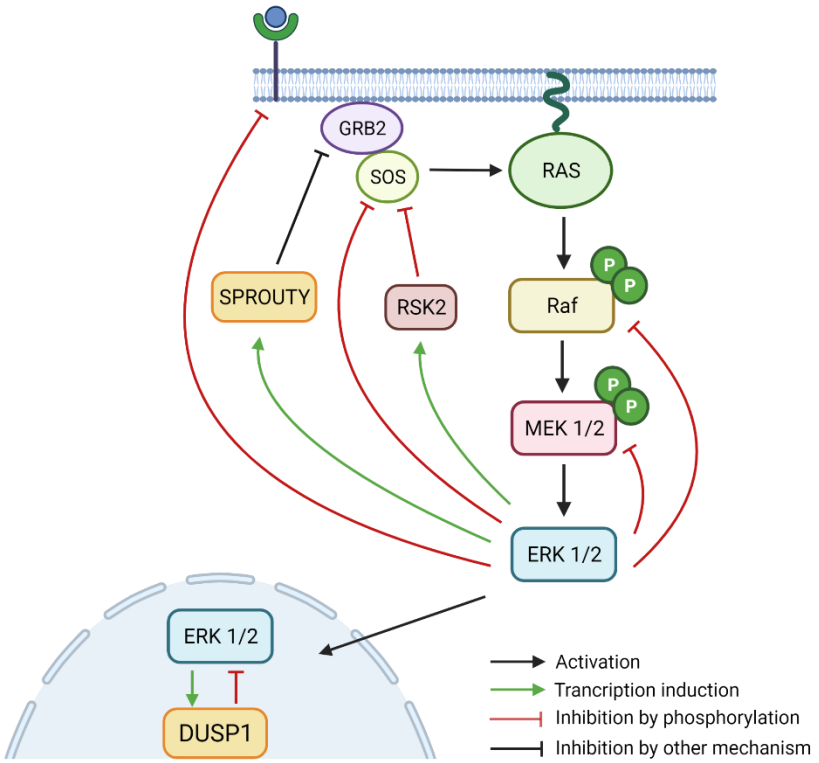


Figure 1.4. ERK-mediated feedback loops. Representation of some of the positive and negative feedback loops mediated by ERK, where ERK is capable of inhibiting almost every component of the RAS-ERK pathway. Adapted from (Lake et al., 2016). Generated with BioRender.

1.1.1.5. ERK kinase-independent activities

During the last decade data has been gathered demonstrating that some ERK functions are independent of its phosphotransfer activity (figure 1.5). These roles vary from chromatin remodeling to DNA transcription or cell cycle regulation and will be described below (Rodríguez & Crespo, 2011).

1.1.1.5.1. Cytoskeletal arrangements

A great amount of ERK interactors are related to the cell cytoskeleton, being tubulin microtubules (Reszka et al., 1995), intermediate filaments (Perlson et al., 2005) or actin

fibers (Leinweber et al., 1999) some examples. ERK interaction with these targets is not affected by agonist stimulation, since this interaction is carried out by a region in the loop 6 (L6) (Reszka et al., 1995). Mutations in the TEY motif of ERK2 have shown to exacerbate cellular flattening, reorientation of actin stress fibers and a radial distribution of microtubules, all of which was followed by an increase in chemotactic cell migration. Moreover, the kinase-dead mutant K52R also recapitulated the aforementioned effects, but without affecting the chemiotaxis. These catalytic-inactive mutants showed that ERK2 is still able to produce alterations in the cytoskeletal architecture in the absence of phospho-transfer activity (Reszka et al., 1997) pointing out to an unconventional ERK activity regardless of its role as a kinase (figure 1.5 A).

1.1.1.5.2. Self-regulation

Another example concerns the dual-specificity phosphatase 6 (DUSP6). As previously explained, DUSPs are in charge of dephosphorylating the threonine and tyrosine residues within the ERK TEY motif and these phosphatases interact with ERK through their KIM motif. Studies in Arkinstall's laboratory unveiled that this interaction is not dependent on the phosphorylated status of ERK, since the effect of the ERK2 kinase-dead K54R mutant had on the activity of DUSP6 was the same as the wild type ERK2. Moreover, they observed that this interaction enhanced the phosphatase activity of DUSP6. This pointed out to the involvement of ERK2 in DUSP6 activity in a kinase independent manner. They also observed that the physiological specificity of DUSP6 for ERK inactivation reflects tight substrate binding by its N-terminal domain (Camps et al., 1998; Muda et al., 1996, 1998). Since then, other studies came out proposing that the catalytic activation of DUSPs results from their binding to ERKs (P. Chen et al., 2001; Dowd et al., 1998; Slack et al., 2001; Q. Zhang et al., 2005). However, despite that in these cases no ERK kinase-deficient mutants were used, they were done in bacteria, where ERK is unphosphorylated. Taken together these data supported the idea that ERK2 kinase activity is unnecessary for its interaction with DUSPs (figure 1.5 B).

1.1.1.5.3. Cell cycle

Another important feature of ERK functions is its ability to regulate cell cycle entry and progression, which includes the transcriptional induction of cyclin D (Lavoie et al., 1996); the stabilization of MYC by phosphorylation (Seth et al., 1991) ; or the regulation of cell cycle inhibitors (Chambard et al., 2007; E Kerkhoff & Rapp, 1997). Additionally, work from our laboratory uncovered a novel kinase-independent role for ERK in cell cycle regulation (Rodríguez et al., 2010a). Lamins, which are part of the nuclear matrix, control many physiological events by interacting with different proteins, being the retinoblastoma (Rb hereafter) one of them. Rb and ERK bind to the same region of lamin A in a mutually exclusive fashion (Ozaki et al., 1994). Once activated ERK1 and ERK2 enter the nucleus they dislodge Rb from its interaction with lamin A. Rb is released to the nucleoplasm and is rapidly phosphorylated and inactivated, leading to cell cycle entry and progression (Rodríguez et al., 2010a). Our work demonstrated that the ERK2 kinase-dead mutant with a nuclear localization signal could, just as wild type ERK2, break these lamin A-Rb complexes, promoting cell cycle entry (Rodríguez et al., 2010a) (figure 1.5 C). In addition, another study in prostate carcinoma cell lines had also found a kinase-independent role of ERK in promoting RAS-ERK-mediated growth arrest, where the ERK2-K52R mutant selectively restored Raf-induced growth inhibitory signaling in ERK1/2-depleted cells (Hong et al., 2009).

1.1.1.5.4. Association with DNA

Once ERK is activated, a portion rapidly translocates to the nucleus, where ERKs can exert different chromatin remodeling activities. One of its targets is the PolyADP-ribose polymerase 1 (PARP-1), which catalyzes the posttranslational modification of nuclear proteins, affecting the interaction between chromatin proteins and DNA-binding proteins (Morales et al., 2014). Upon DNA damage, PARP-1 is activated and binds to DNA breaks. In normal conditions, PARP-1 can be activated by ERK2 (figure 1.5 D). This activation requires phosphorylated ERK2, but does not depend on ERK kinase activity to happen (Cohen-Armon et al., 2007). What's more, through this interaction, PARP-1 not only binds ERK2, but also its

substrate Elk-1. As a consequence, some of the genes regulated by Elk-1, like *c-Fos*, increase their expression (Cohen-Armon et al., 2007).

An additional kinase-independent function of ERK is related to the topoisomerase IIα (TOP2A). Topoisomerases can be divided as: type I topoisomerases, which introduce transient breaks into
DNA single strands, and type II, which introduce transient double-strand breaks (Bush et al., 2015). TOP2A is a subtype II topoisomerase involved in gene transcription, DNA replication and it is crucial for the maintenance of mitotic chromosome structure (Nielsen et al., 2020). TOP2A can be activated by multiple protein kinases, including ERK. Nevertheless, the topoisomerase IIα can be also activated by ERK by a phosphorylation-independent process (figure 1.5 E), whose mechanism is still not well understood (Shapiro et al., 1999). What is known is that it requires phosphorylated ERK and this interaction probably induces a conformational change in TOP2A leading to the unwinding activity of TOP2A (Rauch et al., 2011; Shapiro et al., 1999).

Last but not least, some years ago, Hu and colleagues unveiled a novel ERK feature in DNA regulation, through direct binding to DNA. In their study, a high-throughput profiling of the human protein-DNA interactome in HeLa cells was performed with the intention of identifying unconventional DNA binding proteins (uDBPs). Interestingly, they observed that one of these uDBPs was ERK2, which could bind to DNA thought the consensus sequence G/CAAAG/C (figure 1.5 F). Mutations in this sequence demonstrated that the binding was sequence-specific. Moreover, they determined that this DNA-binding activity was independent of ERK's kinase activity, since the K52R mutation did not affect the DNA-binding properties of ERK2. This study also found the two key residues responsible for the interaction with DNA. These residues (K259 and R261) are located within the ERK2 insert domain (residues 259–277) close to its C-terminus domain. Hu and colleagues also tested the physiological function of their discovery, and observed that ERK2 directly repressed the expression of interferon IFNγ signaling via its DNA binding activity (Hu et al., 2009). After this seminal finding, others have reported ERK2 binding to promoters of active genes and regulating them in human embryonic stem cells (Göke et al., 2013) and mouse embryonic stem cells (Klein et al., 2013; Tee et al., 2014).

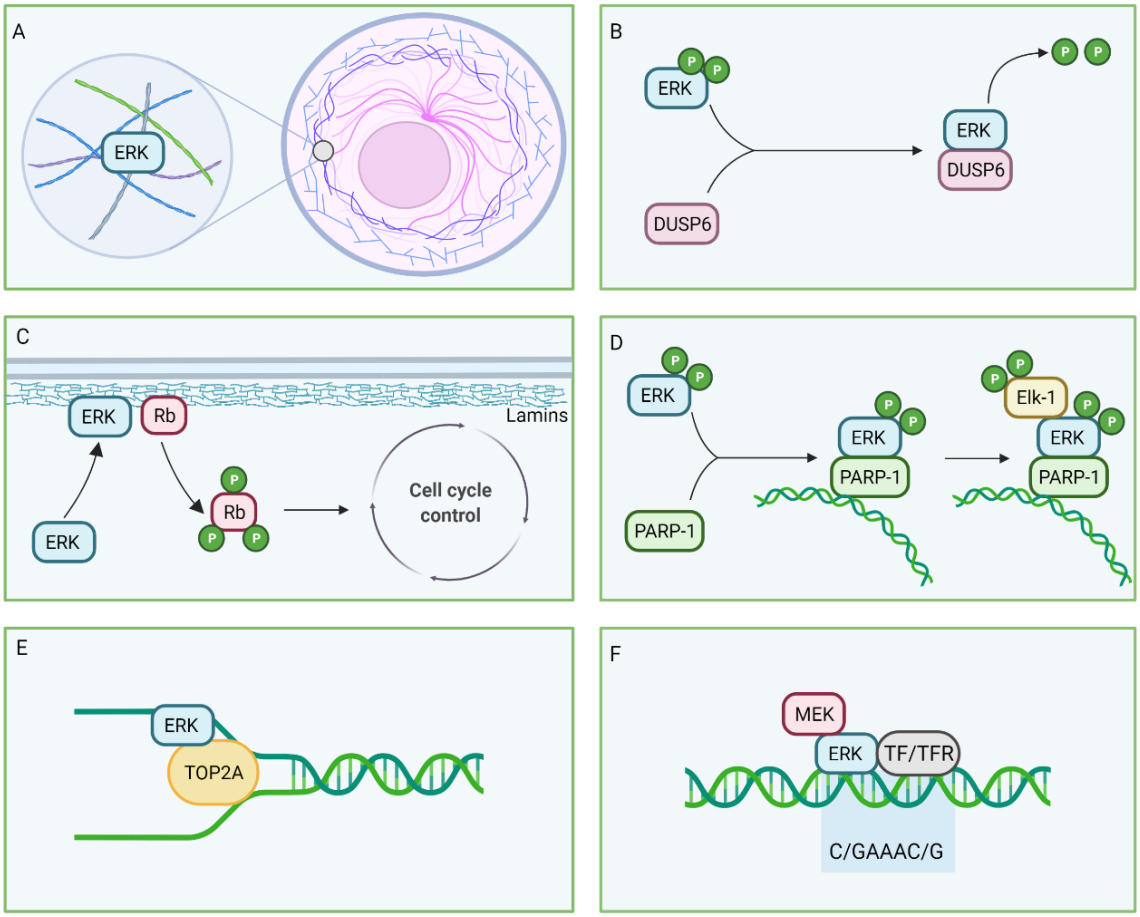


Figure 1.5. ERK kinase independent functions. A) Remodeling of cytoskeletal architecture and cell shape. B) ERK binds to phosphatases and stimulate them in order to regulate its own dephosphorylation. C) ERK regulates cell cycle by disrupting the interaction between RB and lamin A. D) ERK induces PARP-1 activation along with the increase of Elk-1 activity. E) ERK binds and promotes the activation of DNA topoisomerase IIα. F) ERKs can directly bind to a consensus sequence (C/GAAAC/G) at gene promoters to act as transcriptional regulators. Adapted from (Rodríguez & Crespo, 2011). Generated with BioRender.

1.2. MYC family proteins

The oncogene *MYC* was first described in the late seventies in studies with the avian MC29 **my**elo**c**ymatosis transforming virus (first described as *v-myc*) (Sheiness & Bishop, 1979). Some years later, the *MYC* gene was identified in chicken cells (Vennstrom et al., 1982; Vennström & Michael Bishop, 1982) and subsequently in human, mouse and rat (Crews et al., 1982; Dalla-Favera, Bregni, et al., 1982; Sümegi et al., 1983).

MYC family of proteins belong to the superfamily of basic helix-loop-helix leucine zipper (b-HLH-LZ) DNA-binding proteins and is composed of three members: c- MYC, N- MYC and L-MYC, encoded by the genes *c-MYC*, *MYCN* and *MYCL1* respectively (DePinho et al., 1987). While c-MYC is ubiquitously expressed, N-MYC is found mainly in neurons (Kohl et al., 1983) and L-MYC in lungs (Nau et al., 1985). All family members present a nuclear localization and function in a similar manner, but differ in potency (Barrett et al., 1992; Nesbit et al., 1998) and expression patterns (Legouy et al., 1987; Strieder & Lutz, 2002). Unless expressed otherwise, in this thesis we will be referring to c-MYC.

1.2.1. *MYC* gene structure and regulation

Human *MYC* is located within chromosome 8 (*8q24.21*) and is composed of three exons (Dalla-Favera, Bregni, et al., 1982; Dalla-Favera, Gelmann, et al., 1982) whose transcription is regulated by four different promoters: P0, P1, P2 and P3 (figure 1.7). However, most *MYC* mRNAs are transcribed though P1 and P2, being responsible of 25% and 75% of *MYC* transcripts respectively (Wierstra & Alves, 2008). The *MYC* promoter is tightly regulated by transcription factors, signaling pathways and many other regulatory elements, some of which will be described below. However, despite all the time that has passed since the discovery of *MYC*, its promoter regulation is still a “black box”, due to its redundancy, the feedback loops and the cross-regulatory pathways that are involved in such process (Wierstra & Alves, 2008).

MYC expression depends on the proliferative state of the cells, and the regulation of the *MYC* promoter activity rests on intracellular and extracellular cues. Within the *MYC* promoter there is a complex network of transcription factors and other types of regulatory proteins that orchestrate, directly or indirectly, *MYC* expression (figure 1.6) (Wierstra & Alves, 2008).

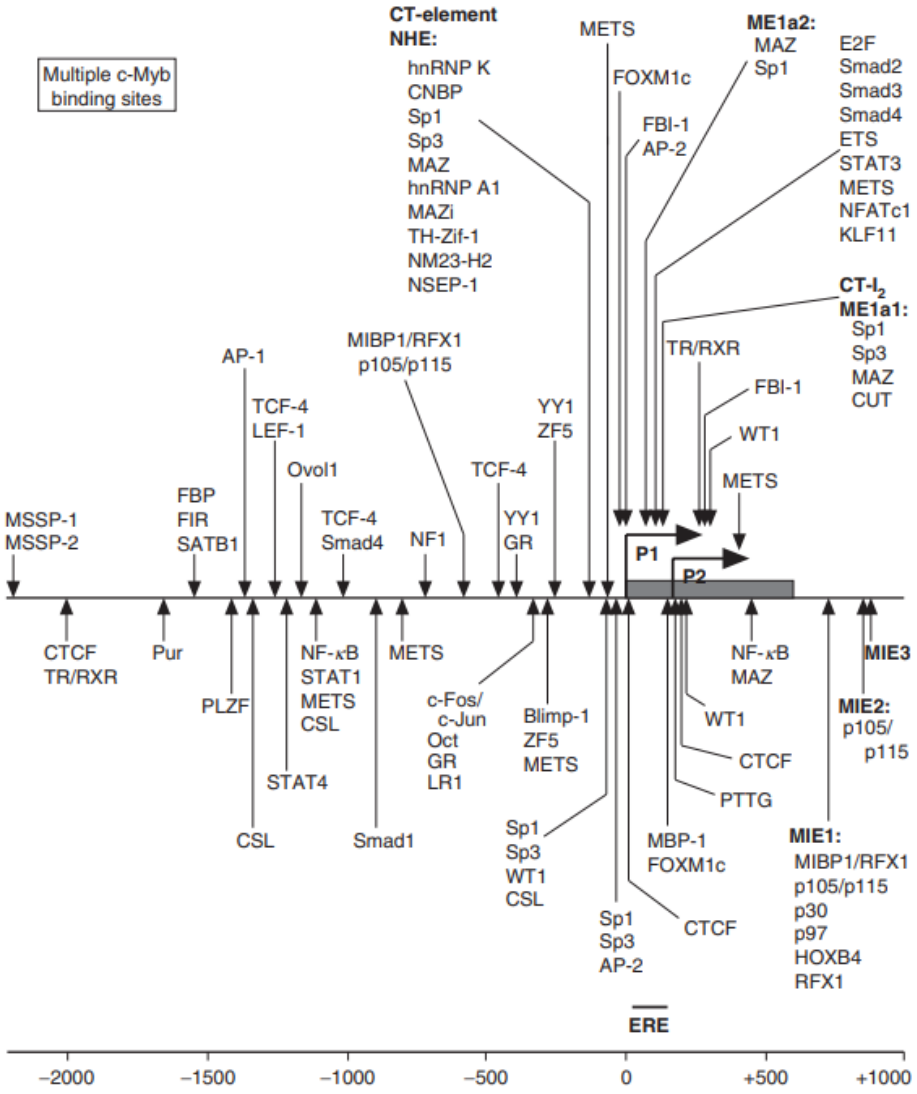


Figure 1.6. Transcription factors binding sites within the MYC promoter. Exon 1 is shown in grey, Promoter 1 and 2 are shown with horizontal arrows, whereas vertical arrows represent one or more binding sites for a particular transcription factor. Adapted from (Wierstra & Alves, 2008).

In quiescent cells *MYC* levels are imperceptible, whereas it becomes highly expressed under mitogenic stimulation. However, during differentiation and growth arrest processes, *MYC* expression levels drop by 90% (Spencer & Groudine, 1991). Under quiescent conditions, different protein actors such as the EF2 family proteins (E2F1, E2F2 and E2F4), histone deacetylases (HDACs) and pocket family proteins (Rb, p107 and p130) are bound to the *MYC* promoter, inhibiting its transcription (Albert et al., 2001). Once cells start to proliferate, the aforementioned complexes are disassembled and MYC expression is induced by the participation of other transcription factors such as FBP, CNBP, TCF3, Sp1 or YY1. Moreover, besides the traditional transcription factors that bind through a conventional double helix, *MYC* can also be induced by non-B DNA structures like single-stranded bubbles-loops of unpaired bases-Z-DNA, quadruplex, and triplex (Hann et al., 1985; Levens, 2010; Wölfl et al., 1997).

Within the *MYC* promoter there is an important sequence named FUSE (Far UpStream Element). The protein FBP (FUSE Binding Protein) is tethered to FUSE, activating *MYC* transcription, whereas the binding of the protein FIR (FBP interacting Repressor) causes its inhibition and the inactivation of *MYC* transcription (L. He et al., 2000; Liu et al., 2006). Binding of FBP to the FUSE sequence in proliferating cells, allows *MYC* to be constantly expressed. Beyond this regulation, MYC is also able to self-suppress its own promoter in a concentration-dependent manner (Facchini et al., 1997; Lucas et al., 1993; Luo et al., 2004). This negative feedback regulation works as a safety mechanism and is missing in tumor cells, contributing to oncogenic *MYC* activation (Facchini et al., 1994; Grignani et al., 1990).

As shown in figure 1.6 there are many other transcription factors that directly bind to *MYC* promoter. For instance, the ones implicated in MYC repression during the differentiation process such as CTCF (Delgado et al., 1999; Torrano et al., 2005), STAT3 (Kiuchi et al., 1999), C/EBP (Kiuchi et al., 1999), BLIMP-1 (Kiuchi et al., 1999), TCF4 (Liu et al., 2006) or METS, which for example, binds to *MYC* promoter in six different sites, acting as a *MYC* repressor (Kiuchi et al., 1999; Wierstra & Alves, 2008).

Regarding *MYC* mRNA stability, diverse mechanisms are known to play a role on its regulation. *MYC* mRNA has a short life of less than 30 minutes, due to the enrichment in A and U on its sequence (Dani et al., 1984). Upon proper stimuli, *MYC* mRNA levels are

increased, reaching its maximum within two hours. When that peak is achieved, *MYC* mRNA remains at lower levels until the completion of the cell cycle (Hann et al., 1985; Thompson et al., 1985). Some of the *MYC* post-transcriptional regulation mechanisms include its regulation by miRNAs such as let-7, miR-34 and miR-145 (Cannell & Bushell, 2010; Christoffersen et al., 2010; Sachdeva et al., 2009). However, it has also been described that *MYC* is regulated by other means, such as the protein AGO2 (Argonaute RISC Catalytic Component 2), whose overexpression stabilizes the *MYC* transcript independently of miRNAs (K. Zhang et al., 2020).

1.2.2. MYC protein structure and regulation

Depending on which start codon the translation is initiated from, three isoforms of c- MYC can be generated: c- MYC-1, c- MYC-2 and a shorter form called c-MYC-S. c-MYC1 is translated from a noncanonical CUG codon and has a molecular weight of 67 kDa, whereas c-MYC-2 is transcribed from a canonical stat point AUG at the beginning of the second exon and has a molecular weight of 64 kDa (Hann et al., 1988; Persson et al., 1984). This isoform is the predominant one. The third isoform, c-MYC-S, is translated from an internal AUG, 100 codons further downstream and lacks most of the N-terminal region that contains the transactivation domains (Spotts et al., 1997; Xiao et al., 1998). Finally, a shorter form of MYC lacking the C-terminal region can be found, known as c-MYC-NICK, which results from a proteolytic cleavage of c-MYC within the cytoplasm. It has been described that this isoform plays a role in differentiation (Conacci-Sorrell et al., 2010). In this thesis we will be referring to c-MYC-2, the most prevalent form.

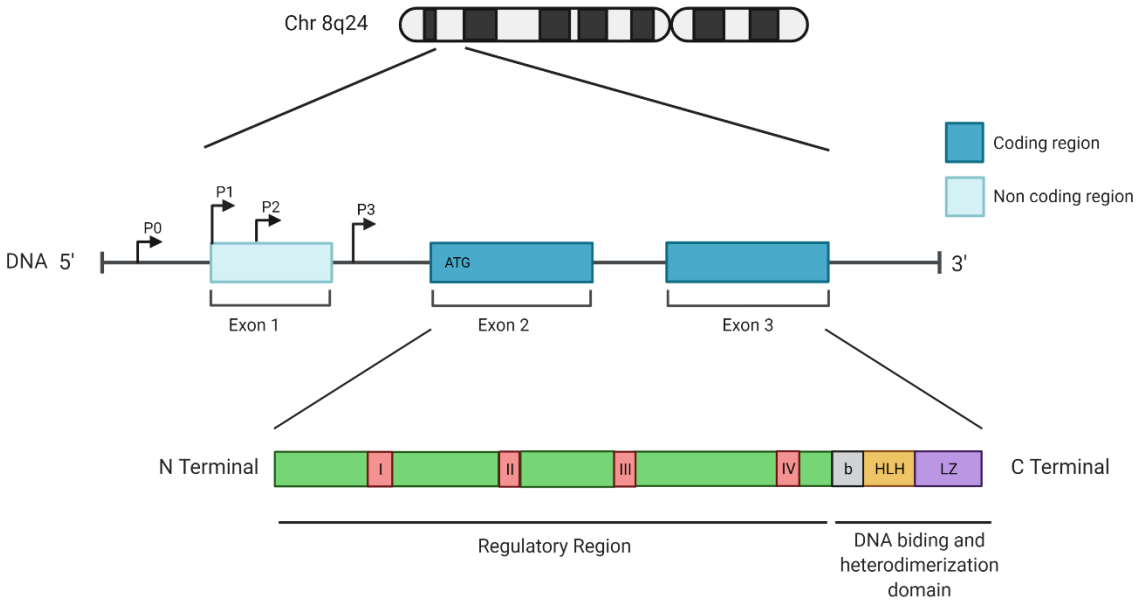


Figure 1.7. MYC gene and protein structure. Human *MYC* is located within the chromosome 8 and is composed of four promoters, three exons and two start codons. The coding regions are represented in dark blue. Four MYC boxes are located within the N-terminal region of the MYC protein (MB-I to MB-IV, red boxes), while the C-terminal region is composed by the bHLH-LZ domain that allows the dimerization with other b-HLH-LZ proteins and the DNA binding motif. Generated with BioRender.

The general architecture of MYC contains multiple domains which are responsible for its different activities (figure 1.7). The N-terminal domain contains the transcriptional activation domain (TAD) that, when fused to a heterologous DNA binding domain (DBD), is responsible for transcriptional activation (Kato et al., 1990). Within this region, four regulatory domains are also found, known as MYC Boxes, (MB-I, MB-II, MB-III AND MB-IV) which are highly conserved among species. MB-I plays a role in MYC protein stability and contains two important phosphorylation sites: Ser62, mainly phosphorylated by ERK/MAPKs pathways, and Thr58, phosphorylated by the PI3K/AKT pathway. Both residues are important for MYC stability and transforming activity (Kapeli & Hurlin, 2011; Sears et al., 2000a; Stone et al., 1987). MB-I is also the site where the interaction with the complex p-TEFb takes place, which confers MYC the ability to stimulate transcriptional elongation (Eberhardy & Farnham, 2001; Kanazawa et al., 2003). Moreover, MB-I seems to have a role in ubiquitin/proteasome-dependent MYC degradation (Kalkat et al., 2018). MB-II is essential for MYC ability to promote cellular transformation *in vitro* (Stone et al., 1987), drive tumorigenesis *in vivo* (McClure et al., 2020) and regulate transcription (L. H. Li et al., 1994; X. Y. Zhang et al., 2006). Through this domain MYC interacts with TRRAP (transformation/

transcription domain-associated protein), recruiting HAT complexes (histone acetyltransferase) that regulate the transcription of MYC-target genes (McMahon et al., 1998; Vervoorts et al., 2003). The other two MYC boxes are less studied and are not localized within the TAD region. MB-III contributes to MYC-mediated transcriptional repression and transformation *in vitro* (Herbst et al., 2005), whereas MB-IV is involved in MYC-mediated apoptosis, transformation and DNA binding (Cowling et al., 2006; Poole & van Riggelen, 2017).

The central region of MYC protein is not well understood. It contains a nuclear localization signal (NLS) (C V Dang & Lee, 1988). However, this signal is not necessary for MYC entry into the nucleus and is missing in the L-MYC isoform (De Greve et al., 1988). Finally, the carboxy terminus of MYC contains the b-HLH-LZ (basic helix-loop-helix-leucine zipper) region. MYC binds to DNA though the basic domain, and the HLZ and LZ domains mediate in the dimerization with other b-HLH-LZ proteins, of which the most important one is MAX (Blackwood & Eisenman, 1991). The MYC-MAX heterodimer recognizes the consensus sequence "CACGTG", known as "Enhancer box" (E-box) (Blackwell et al., 1993), and this interaction is absolutely required for MYC activity. Since MYC does not homodimerize, the MYC-MAX heterodimer plays an essential role in functions as important as transformation, transactivation and apoptosis (B. Amati et al., 1993; Bruno Amati et al., 1992; García-Gutiérrez, Delgado, et al., 2019).

Given the importance of MYC in cellular processes as important as apoptosis, differentiation and transcriptional regulation, it is not a surprise that its protein expression is tightly regulated at multiple levels. MYC post-translation modifications include: acetylation, ubiquitination and phosphorylation. MYC can be stabilized by acetylation in some of its lysine residues by CBP/p300, Gcn5 and Tip60, which results in an increase of its transcriptional activity (Faiola et al., 2005; Patel et al., 2004; Vervoorts et al., 2003). On the other hand, MYC deacetylation by SIRT1 and Sin3a/b has the opposite effect, leading to decreased MYC protein stability (Garcia-Sanz et al., 2014; Yuan et al., 2009).

In a similar way, MYC can also be regulated by ubiquitination, that can either stabilize it, like the ones performed by β-TrCP and HectH9 (Adhikary et al., 2005; Popov et al., 2010), or ubiquitination that provokes MYC destabilization, as performed by ubiquitin ligases like Skp2 (Von Der Lehr et al., 2003), Fbx29 (Koch et al., 2007) or CHIP (Paul et al., 2013).

Finally, MYC protein stability is also regulated by phosphorylation, after activation of several signaling pathways. Among those, the Ras-ERK cascade and the PI3K-Akt pathway, are well studied. The former phosphorylation can be carried out by ERK and/or CDKs in response to stimulatory signals (Haeng et al., 2008; Hydbring et al., 2010; Sears et al., 2000b). These stimuli provoke an increase in *MYC* transcription and production of MYC protein that is phosphorylated at Serine 62, which increases its stability. Moreover, this phosphorylation is also necessary for a second phosphorylation of MYC at Threonine 58, performed by GSK-3β (Gregory et al., 2003). After that, PIN1 (Peptidylprolyl Cis/Trans Isomerase) isomerizes Proline 57, which allows PP2A (protein phosphatase 2A) to dephosphorylate serine 62 (Yeh et al., 2004). The cycle ends when pT58-MYC is recognized by the E3 ubiquitin ligase SCF^{Fbw7}, leading to MYC degradation through the ubiquitin pathway (Arnold & Sears, 2006; Welcker et al., 2004). Recently, it has been reported that the transcriptional and epigenetic regulator BRD4 is also capable of phosphorylating MYC at Threonine 58 leading to MYC ubiquitination and further degradation (Devaiah et al., 2020).

1.2.3. MYC-mediated regulation of transcription

While it is well established that MYC can activate transcription of its target genes, a trans-repression role for MYC has also been described. On the one hand, in order to regulate transcription, MYC must dimerize with MAX. These heterodimers bind DNA at the canonical sequence "CACGTG", although other non-canonical E-boxes have also been described (Allevato et al., 2017; Carla Grandori et al., 1996). In addition, they act as platforms that facilitate the recruitment of other proteins needed for the transcriptional machinery, like TBP, TFII-I or TRRAP (Henriksson & Luscher, 1996; Sakamuro & Prendergast, 1999). MYC transcriptional activity requires binding to E-box elements and the recruitment of coactivators among which are P-TEFb (described in further detail in chapter 1.3.2.3.3.), the Mediator complex (Eberhardy & Farnham, 2002), ATPases (TIP48 and TIP49) (Wood et al., 2000), and very importantly, histone acetyltransferases (CBP, p300, GCN5 and TIP60) (Vervoorts et al., 2003). In particular, the TRRAP protein acts as a scaffold, allowing the indirect interaction of GCN5 and TIP60 with MYC (McMahon et al., 1998). Furthermore, MYC can also exert its activity through other mechanisms, such as binding to the E3 ubiquitin ligase SKP2, which recruits other components needed for the activation of several MYC target genes (Von Der Lehr et al., 2003) (figure 1.8 A).

On the other hand, several studies have reported a role of MYC in transcriptional repression (Herkert & Eilers, 2010; Tu et al., 2018). However, the role of MAX in this feature is controversial and the role played by the MYC-MAX heterodimer is not fully understood (García-Gutiérrez, Delgado, et al., 2019; Mao et al., 2003). The principal mechanism of MYC transrepression activity is based on the interaction with two transcription factors: SP1 and/or MIZ1 which, under normal conditions, are transcriptional activators. Once MYC interacts with them, it displaces SP1 and MIZ1 co-activators (such as p300), turning them into transcriptional repressors (Feng et al., 2002; Jiang et al., 2007; Staller et al., 2001) (figure 1.8 B). Genes encoding for p15INK4B and p21CIP1 are some of the targets of this repression mechanism (Scafuro et al., 2021). Another way of inhibiting transcription is based on MYC regulation of miRNAs clusters, like miR-17-92 (Chang et al., 2008) that targets E2F1 or MYC; or the microRNA cluster *let-7a-1∼let-7d* that play critical roles in development and cancer (Wang et al., 2011).

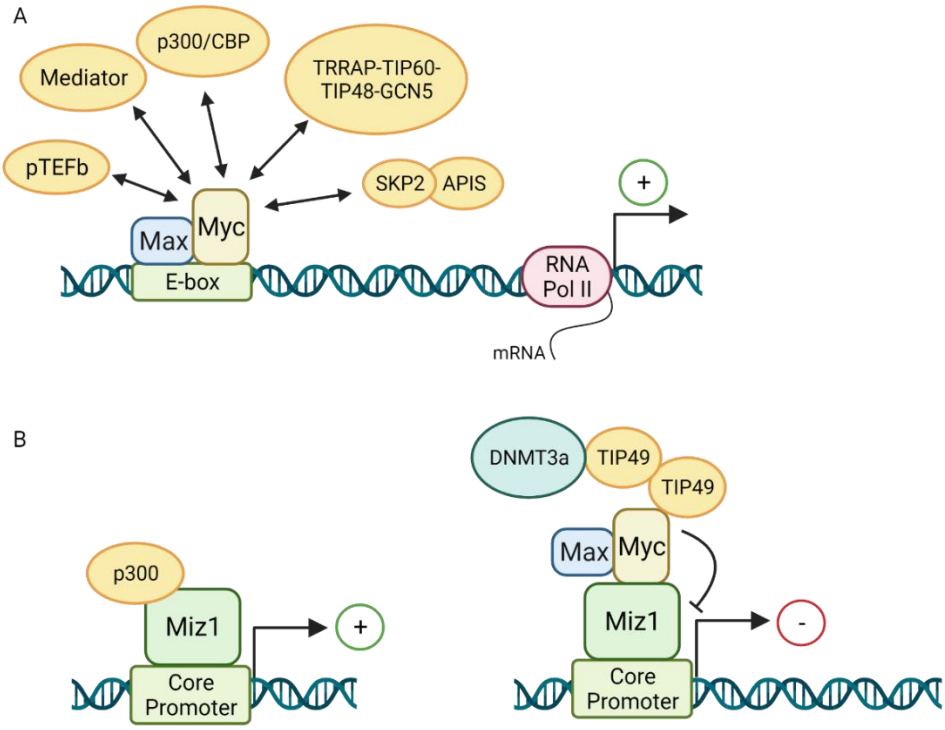


Figure 1.8. MYC-mediated regulation of transcription. A. Transcriptional activation occurs when the heterodimers MYC-MAX bind to E-box elements and recruit coactivators and protein complexes. Some of which are P-TEFb, ATPases or histone acetyltransferases. B. Transcriptional repression mediated by MYC occurs on promoters that are activated by Miz1. In this cases, the MYC–MAX heterodimer blocks transactivation by Miz1, partly through disruption of the interaction between Miz1 and p300, which turns Miz1 in a transcriptional repressor. Adapted from (Adhikary & Eilers, 2005). Generated with BioRender.

1.2.4. Biological functions of MYC

Since MYC binds to about 10-15% of human genes, it is nonot surprising that some of its functions are related to vital processes for the cell. Some of the most important include the regulation of cell cycle progression, proliferation, apoptosis, differentiation, biomass accumulation and, under pathological settings, tumorigenesis (Lüscher & Vervoorts, 2012) (figure 1.9).

1.2.4.1. Cell cycle

MYC influences cell cycle in different ways. Upon mitogenic stimulation, MYC proteins play an important role in cell cycle induction and progression, promoting G_1-S phase transition and augmenting the duration of both G1 and G2 phases (De Alboran et al., 2001; Karn et al., 1989). MYC is considered a potent activator of S-phase entry. It promotes the expression of genes encoding for CDKs and cyclins such as *CDK4* and *CCND2* (Cyclin D2), *CCNE1* (Cyclin E1) and *E2F* (Bouchard et al., 2001; Fernandez et al., 2003; Hermeking et al., 2000), whilst it inhibits cell cycle checkpoints genes such as *gadd45* and *gadd153* and cyclin kinase inhibitors like p15, p21 and p27 (Chi V. Dang, 1999; García-Gutiérrez, Delgado, et al., 2019; C. Grandori et al., 2000). Interestingly, MYC has been described to promote cell cycle progression in cells lacking CDK2, CDK4 and CDK6, thus only relaying on CDK1 (García-Gutiérrez, Bretones, et al., 2019). What's more, it has also been described that MYC can promote transition from G_0 to S phase in a growth stimuli-independent manner (Eilers et al., 1989; Kaczmarek et al., 1985).

1.2.4.2. Cell growth and metabolism

Dividing cells require a high amount of nutrients, energy and proteins. MYC not only is implicated in cell cycle progression, but also it enhances protein synthesis leading to cell mass accumulation (Rosenwald, 1996). When MYC is overexpressed, cells double their size, produce twice as many proteins and have twice the total RNA content (Iritani & Eisenman, 1999; Lin et al., 2012; Nie et al., 2012) in comparison with cells with normal MYC levels. This is possible due to MYC capacity to regulate genes involved in ribosome biogenesis, protein biosynthesis and its ability to activate transcription (S. Kim et al., 2000; Schlosser et al., 2003, 2005). For instance, it was found that the reduction in the number of ribosomes in precancerous cells that overexpress MYC is enough to inhibit the transition of the cell to a tumorigenic state (Barna et al., 2008), evidencing the importance of MYC's ability to regulate biomass production during carcinogenesis.

Besides cell growth, MYC also enhances the metabolic capacity of the cells, and it does so by altering glycolysis and glutamine metabolism. This is no surprising, given the fact that

MYC stimulates the expression of genes involved in energy metabolism, such as *GLUT1*, or glycolytic enzymes like enolase 1 (Eno1), lactate dehydrogenase (Ldh) or hexokinase 2 (Hk2), which makes MYC an important candidate in the contribution to the Warburg effect in tumors (Chi V. Dang, 2010, 2013). What's more, MYC-transformed cells quickly become "glucose addicted", suffering apoptosis due to the lack of glucose or exposure to glucose antimetabolites (Shim et al., 1998).

1.2.4.3. Apoptosis

The induction of apoptosis is a mechanism of the cells to protect themselves against improper proliferation, that may lead to malignant transformation. Many MYC target genes are somehow implicated in apoptosis induction, like: ODC, CDC25A or LDH-A (Chi V. Dang, 1999). MYC overexpression can induce apoptosis both dependently and independently of the p53 pathway (Sakamuro et al., 1995) and in order to induce apoptosis MYC must dimerize with MAX (B. Amati et al., 1993). For instance, the p53-dependent pathway hinge on MYC activating ARF, which results in the inhibition on Mdm2, a p53 inhibitor (Zindy et al., 1998). This inhibition will activate p53 that is able to induce growth arrest or apoptosis (Sakamuro et al., 1995). On the other hand, p53-independent induced apoptosis is based on the murine myeloid leukemia system (Amanullah et al., 2000). In this model, the cells proliferate rapidly, but differentiation can be induced with IL-6 treatment, which provokes a decrease in endogenous MYC expression. Enforced MYC expression impedes this differentiation induction, which ushers cells to undergo apoptosis in a p53-independent manner (McMahon, 2014).

1.2.4.4. Differentiation

Cell differentiation is a complex process where cells gradually change their size, morphology and metabolic activity in order to become specialized in some function. MYC involvement in differentiation is well characterized in many cell types, and this process requires MYC suppression so that the repression that it exerts over differentiation genes is removed (Freytag, 1988; Hoffman-Liebermann & Liebermann, 1991). For instance, some of these

genes are *MXD1* and *GATA1,* responsible of erythroid differentiation (Acosta et al., 2008) or c-*JUN* in the UR61 pheochromocytoma cells, that inhibits Ras-mediated differentiation (Vaqué et al., 2008). However, there are also examples where MYC does not inhibit differentiation. For instance, in hematopoietic stem cells and B lymphocytes (Dolores Delgado & León, 2010; A. Wilson et al., 2004) or in human epidermal stem cells (Gandarillas & Watt, 1997). Normally, in these cases the process is compatible with proliferation, which makes sense, given that proliferation and differentiation are mutually exclusive functions in some models (Leon et al., 2009). Such is the importance of MYC in differentiation that it is considered one of the four factors capable of inducing pluripotent stem cells (Takahashi & Yamanaka, 2006).

1.2.4.5. Tumorigenesis

Given the importance of the cellular processes regulated by MYC, it is no surprise that its deregulation causes human malignancies and is associated with cancer progression (Oster et al., 2002) as a consequence of genomic instability events, uncontrolled cell proliferation, escape from immune surveillance, immortalization and growth factor independence (Vita & Henriksson, 2006) (figure 1.9). Unlike other oncogenes like Ras, whose mutations are drivers of cancer, the main mechanism whereby MYC becomes an oncogene is its elevated expression (Tansey, 2014). In fact, MYC is found deregulated in more than half of human solid tumors and hematopoietic malignancies (Madden et al., 2021). This occurs by several mechanisms such as retroviral transduction, or gross genetic abnormalities that affect its gene. Among the multiple mechanisms that account for MYC deregulation we can find the following:

- *MYC* gene amplification of different kinds: small focal amplifications (Muzny et al., 2012; Northcott et al., 2012), large amplifications (Cher et al., 1996) or double-minute amplifications (Thomas et al., 2004; Yamamoto et al., 2020) that vary among the tumor type, causing gene duplication.
- *MYC* translocations: particularly important in Burkitt's lymphoma (Dalla-Favera, Bregni, et al., 1982), but less common in other cancers. These translocations result

in *MYC* coding sequences are under the control of the immunoglobulin μ heavy chain enhancer, provoking a boost in its mRNA synthesis (Region et al., 2016).

- Transcriptional amplification: where MYC is expressed at high levels and accumulates close to the promoter of active genes, causing an extra production of those transcripts. Due to this, MYC has been considered as a global transcriptional amplifier, enhancing the output of the existing gene expression program (Lin et al., 2012). However, this point is highly controversial. Others argue that MYC is not a general transcriptional amplifier, but instead directly regulates the expression of distinct subsets of genes that lead to the indirect amplification of global transcripts through downstream effects of MYC-induced genes (Alderton, 2014).
- MYC protein stability: MYC stabilization processes due to the deregulation of the signaling pathways that control MYC's protein stability can lead to enhanced MYC activity. For instance, such is the case of the Ras-ERK pathway. As previously mentioned, mutations in different components of this cascade, like Ras or BRAF, lead to a hyperactivation of the signaling, provoking that ERK, its major effector protein, transmits pro-survival and pro-growth signals (Region et al., 2016). Besides many other effects, this constitutively activated kinase causes MYC protein stabilization and MAD1 degradation, which allows the dimer MYC-MAX to remain active over its target genes, increasing MYC transcriptional activity (Jidong Zhu et al., 2008).

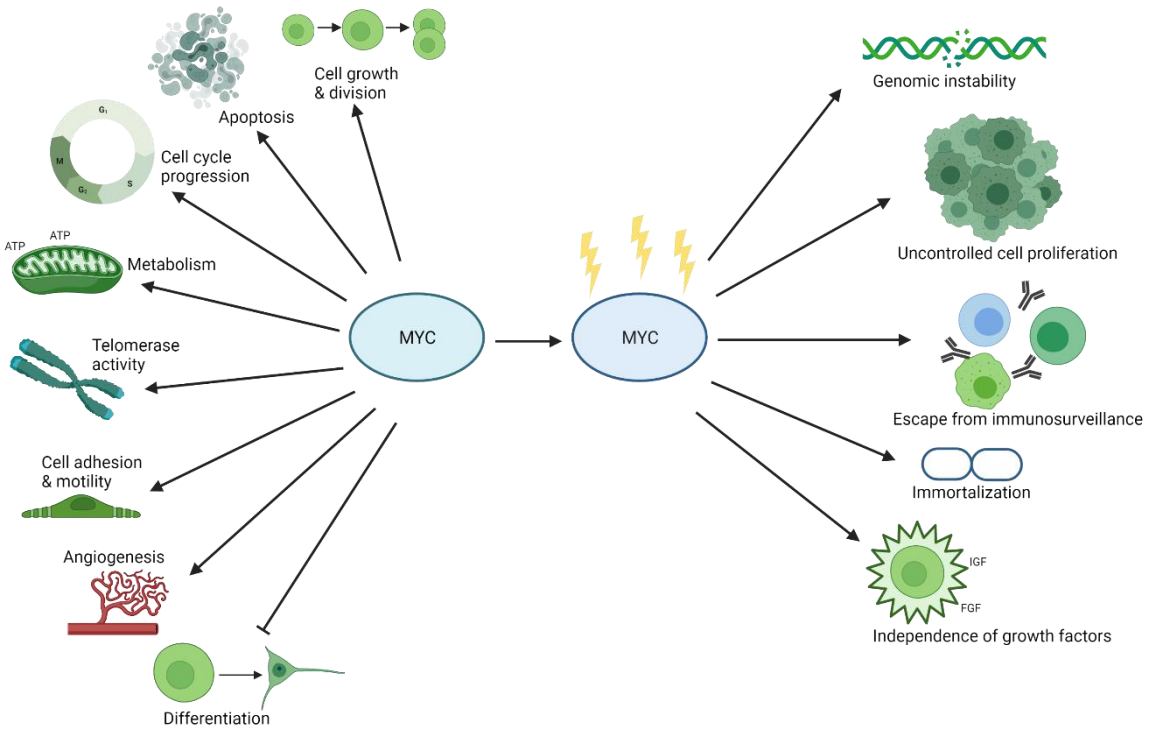


Figure 1.9. Cellular processes influenced by MYC in normal conditions and during tumorigenesis. MYC regulates many biological activities including cell growth and division, cell cycle progression, cell differentiation, cell metabolism, angiogenesis, cell adhesion and motility. MYC deregulation causes genomic instability, uncontrolled cell proliferation, escape from immune surveillance, immortalization and growth factor independence. Adapted from (Vita & Henriksson, 2006). Generated with BioRender.

1.3. Cyclin-dependent kinases

The cyclin-dependent kinases (CDKs) are a family of proteins composed of 20 serine/threonine kinases that are involved in key cellular processes such as cell survival and growth (Malumbres et al., 2009). They can be subdivided into two major classes: the "mitotic" CDKs, implicated in cell cycle regulation (CDK1, CDK2, CDK4 and CDK6) and those that regulate gene transcription (CDK7, CDK8, CDK9, CDK12 and CDK13). In order to be active, CDKs must interact with their regulatory subunits, the cyclins (Pines, 1995). Like CDKs, they can be subdivided into groups depending on whether they regulate cell cycle progression (cyclins A, B, D and E) or gene transcription (cyclins C, H, K, L, Q and T) (Hydbring et al., 2016; Lolli, 2010). In this thesis, we are focused on CDK/cyclin complexes that regulate transcription (figure 1.10).

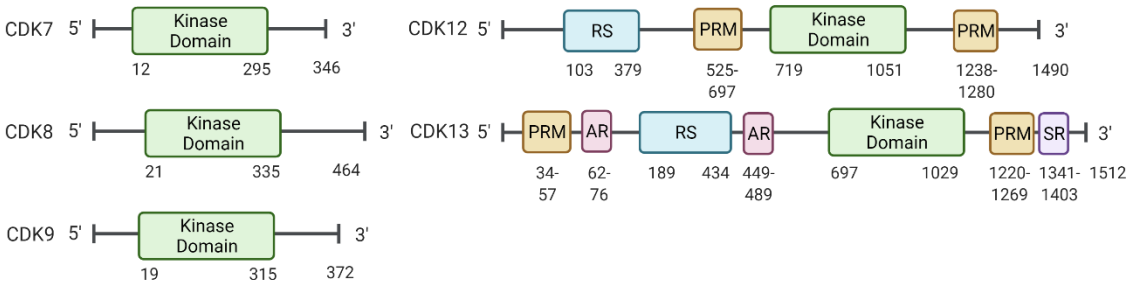


Figure 1.10. Schematic representation of the functional domains of the family of transcription-associated CDKs. While CDK7, CDK8, and CDK9 are mainly composed of a kinase domain, both CDK12 and CDK13 contain other domains besides their central kinase domains. In the case of CDK12, it also has an amino-terminal RS and PRM domain, and a carboxy-terminus PRM domain. On the other hand, CDK13 not only contains an amino-terminal PRM and RS domain, but also two AR domains and a SR domain. Numbers represent the amino acid positions that form the limits of the protein domains. Adapted from (Chou et al., 2020). Generated with BioRender.

1.3.1. Role of CDKs in the regulation of RNA polymerase II-mediated transcription

Normal cell growth and development depends directly on an efficient regulation of gene expression. Many diseases are the result of genetic abnormalities caused by an aberrant gene expression product of the DNA-dependent RNA polymerase II. RNA pol II is responsible of the process of transcription in eukaryotic cells. It constitutes a complex of 550 kDa formed

by 12 subunits (Rpb1-12) (Jiafu Zhu et al., 2018). Besides its catalytic core, the carboxy-terminal domain (CTD) of Rpb1 is where most regulatory signals impinge during transcription. The CTD consists of a heptapeptide with the consensus sequence Tyr1-Ser2-Pro3-Thr4-Ser5-Pro6-Ser7 (Woychik, 1998) (YSPTSPS) repeated 52 times in humans (27 in yeast) and it can be phosphorylated which will result in different behaviors of the RNA pol II throughout the transcription process.

Gene transcription can be divided into four steps: initiation, pause/release, elongation and termination (figure 1.11). While the length of the CTD can determine the efficiency of the RNA Pol II for processing different substrates (Rosonina & Blencowe, 2004), the pattern of phosphorylation of the different aminoacids within the CTD, dictates the transition between the different phases of transcription (Gibbs et al., 2017). Previous to transcription initiation, a complex of 100 proteins called the preinitiation complex (PIC) binds to the gene transcription starting point in order to facilitate the DNA entry into the active site of the RNA Pol II (Plaschka et al., 2016; P. J. Robinson et al., 2016). For the formation of PIC several general transcription factors (GTFs) are required, including TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Buratowski et al., 1989). The last GTF to be recruited by PIC is TFIIH, which has multiple subunits including CDK7, cyclin H and MAT1.

i) Initiation:

CDK7, also known as CDNK7, consists of a 346 amino acid protein that binds cyclin H and the ring-finger protein MAT1 in order to be active (Tassan et al., 1995). MAT1 acts as a helicase (Spangler et al., 2001), promoting the opening of DNA, while CDK7 along with cyclin H form the CDK-activating kinase (CAK) complex that mediates the phosphorylation of Ser5 of the CTD of the RNA Pol II and thereby promoting the promoter clearance and the initiation step (Serizawa et al., 1995; Spangler et al., 2001). Besides, the CAK complex phosphorylates CDK9 at the Threonine-186 leading to its activation (Larochelle et al., 2012). CDK9-mediated regulation of transcription will be explained in detail in the following sections.

CDK8 is a 484 amino acid protein that forms a 600 kDa complex with cyclin C, MED12 and MED13, known as CDK8 kinase module (CKM) (Tsai et al., 2013). CKM interacts through MED13 with the Mediator complex, which is a multimeric complex that communicates regulatory signals from DNA-binding transcription factors (TFs) directly to RNA Pol II (Allen

& Taatjes, 2015). Through this interaction, the CDK8 module promotes activation of transcription. Different studies support the idea that CDK8 has a biphasic effect *in vivo*. Some suggest that it can inhibit transcription by impeding the incorporation of PIC by phosphorylating the CTD (Wong et al., 2014). The CKM can also phosphorylate the complex CDK7/cyclin H, which represses the ability of TFIIH to regulate transcription, and its CTD kinase activity (Akoulitchev et al., 2000). Others propose that CDK8 has a positive regulation of transcription by impeding that a second RNA Pol II immediately restarts the promoter. The phosphorylation carried out by CDK7 causes mediator dissociation, thereby permitting rapid promoter escape of RNA Pol II from the preinitiation complex (Wong et al., 2014). This will allow the activation of RNA Pol II and stimulate RNA transcription, but not elongation.

CDK19, a paralog of CDK8, can also bind cyclin C and associate with the Mediator complex to regulate transcription (Fant & Taatjes, 2019). However, the mechanisms and functions underlying the differences between both CDKs are still under investigation. Recently, a study has identified more than 60 proteins that are phosphorylated by CDK8 and CDK19, whose roles range from chromatin modification to DNA repair (Poss et al., 2016).

ii) Pause/Release:

Shortly after transcription initiation, the DRB sensitivity-inducing factor (DSIF) and the negative elongation factor (NELF) come into play, provoking transcriptional pausing. The purpose of this suspension is not completely clear, but some of the proposals include increasing the accessibility of genes (Wada et al., 1998; Yamaguchi et al., 1999); quality control for coupling elongation (Chou et al., 2020); or to integrate regulatory signals. After that, the complex P-TEFb (CDK9 and cyclin T) along with BRD4 releases RNA Pol II from its paused form. To do so, activated P-TEFb phosphorylates Ser2 at the CTD as well as the SPT5 subunit of DSIF and the RD subunit of NELF (Wong et al., 2014). Thanks to these phosphorylations, NELF is liberated from RNA Pol II and DSIF is transformed into a positive elongation factor (Fujinaga et al., 2004), converting the RNA Pol II in a fully productive elongation complex.

iii) Elongation and Termination:

Besides CDK9, two other CDKs can phosphorylate the Ser2 of the CTD: CDK12 and its homologue CDK13. However, based on studies in *Drosophila* and yeast, it is thought that

CDK9 carries out the phosphorylation in early stages of transcription elongation, and then CDK12 is the CDK in charge of phosphorylating Ser2 at later stages (Ghamari et al., 2013; Qiu et al., 2009).

CDK12, also known as CRKRS, encodes a 1490 amino acid protein that, unlike the previous CDKs, has several domains: the common carboxy-terminal kinase domain, two proline-rich motifs (PRM) involved in protein-protein interactions and transcriptional regulation; and an arginine/serine rich (RS) domain (Ko et al., 2001) that serves as docking site for the assembly of protein complexes. The cyclin involved in its activation is cyclin K (Bösken et al., 2014; S.-W. G. Cheng et al., 2012), although it was initially thought that cyclins L1 and L2 were its cognate cyclins (Poss et al., 2016). The protein contains multiple nuclear localization sequences (NLS) and is mainly detected in the nucleus and nuclear speckles (Ko et al., 2001). CDK12 can also phosphorylate several residues of the CTD, not only phosphorylates Ser2, but also Ser5 and Ser7 (Bösken et al., 2014; Liang et al., 2015)) and its loss of function disturbs the expression of genes related to DNA damage and homologous recombination repair (Woychik, 1998). CDK12 is essential for embryonic development and the maintenance of genomic stability (Juan et al., 2016) and has also been implicated in normal premRNA splicing patterns and termination (Tien et al., 2017).

CDK13, also known as CDC2L5 or CHED, is another CDK, consisting of 1512 amino acids with a 50% of similarity with CDK12 (Kohoutek & Blazek, 2012). Both CDKs are activated by cyclin K and have kinase domains with a 92% shared identity. However, the amino and terminal domains are different, with CDK13 containing a carboxi-terminal serine-rich (SR) domain and two alanine-rich (AR) domains, that are not present in CDK12 (Chou et al., 2020). Despite its similarity, each CDK seems to regulate the expression of different genes (Liang et al., 2015).

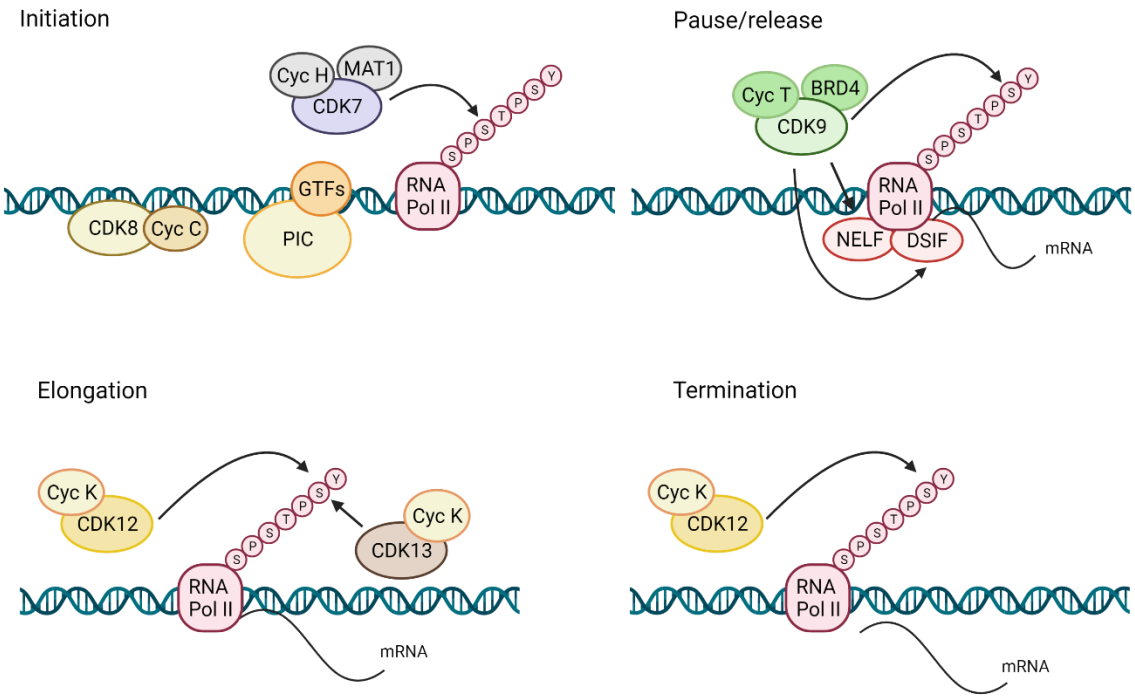


Figure 1.11. Roles of transcription-associated CDKs in the regulation of RNA Pol II. CDK7, CDK9, CDK12 and CDK13 regulate transcription by phosphorylating the CTD of the RNA Pol II on its residues Ser2 and Ser5. Moreover, CDK8 binds to the mediator complex to promote transcription activation and regulates gene expression from super-enhancers. Adapted from (Chou et al., 2020). Generated with BioRender.

1.3.1.1. CDK9 structure

CDK9 is a 372 amino-acid protein localized within the nucleoplasm that can associate with cyclin K or the different subunits of cyclin T (cyclin T1, T2a and T2b) (Peng et al., 1998) to perform its kinase activity. CDK9 was first isolated and named as PITALRE, due to its characteristic amino-acid motif (N. F. Marshall & Price, 1995). Later on, a study of the human immunodeficiency virus (HIV) demonstrated that CDK9 was the kinase component of the P-TEFb (Fujinaga et al., 2004) (positive transcription elongation factor) which phosphorylates the Ser2 residue of the CTD of RNA Pol II and the DRB sensivity-inducing factor (DSIF) and the negative elongation factor (NELF). These phosphorylations release the pause of the RNA Pol II and promote the elongation step.

The gene encoding *CDK9* is located at 9q34.1. There are two isoforms of CDK9 based on its molecular weight: $CDK9_{42}$ and $CDK9_{55}$ (figure 1.12). Both isoforms are transcribed from the same *CDK9* gene. The latter comes from an alternative promoter that is approximately 500

bp upstream of the mRNA transcription start-point. It generates a 55 kDa protein with an extension of 117 aminoacids (Shore et al., 2003) on its N-terminal lobe that is highly expressed in liver and brain. Any mention to CDK9 in this thesis is referred to the isoform $CDK9_{42}$, which is the most highly expressed and best studied in transcription regulation.

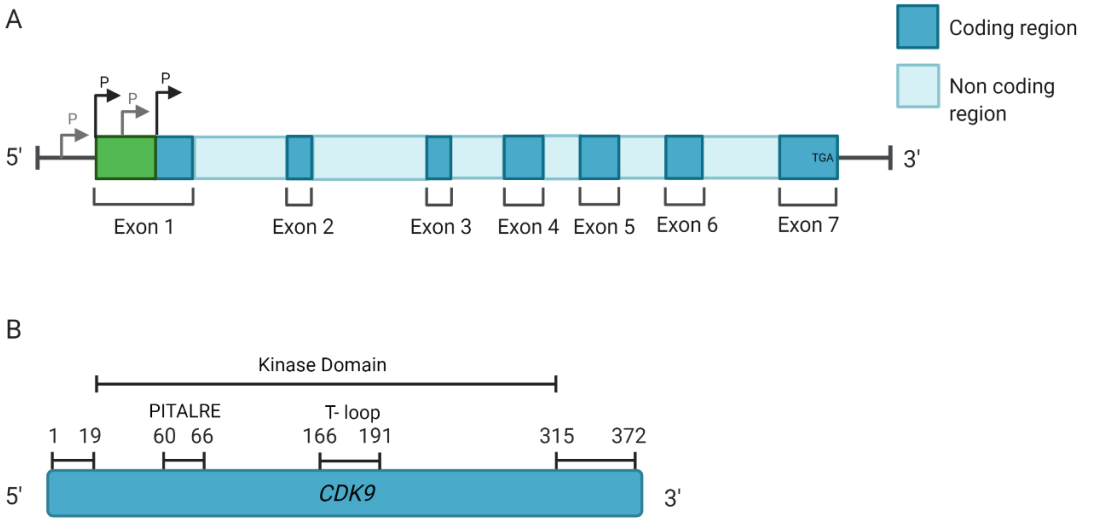


Figure 1.12. Structure of human *CDK9* gene. A. The diagram illustrates the organization of the CDK9 gene intronic (light blue) and exonic (dark blue) sequences. The grey arrows indicate alternative starting points for $CDK9_{55}$ and $CDK9_{42}$ mRNA transcription at the promoters. Black arrows show the positions of start codons (ATG) for each CDK9 isoform. $CDK9_{55}$ isoform has an extension of 117 aminoacids (green). Adapted from (Paparidis et al., 2017). B. Representation of human CDK9 and some of its regions: Kinase domain, PITALRE and the T-loop. Numbers represent the amino acid positions that form the limits of the protein domain. Adapted from (Mandal et al., 2021). Generated with BioRender.

The N-terminal region (residues 16-108) of CDK9 is composed of five β-sheet and one α-helix, while the N-terminal lobe (residues 109-330) has four β structures and seven α-helices (Baumli et al., 2008). The α-helix of the N-terminus contains the motif called PITALRE (Graña et al., 1994) (Pro-Ile-Thr-Ala-Leu-Arg-Glu), a peptide sequence vastly conserved within the CDK family. The interaction between the CDKs and their corresponding cyclins occurs through this domain (Baumli et al., 2008) (figure 1.13).

The adenosine triphosphate (ATP) binding motif is highly conserved among the different CDKs and forms a cleft between the N- and C- terminal domains. The substrate recognition motif is found in the cleft between both lobes and is universal for all CDK members, with some differences regarding the substrate preference. For example, unlike CDK2, CDK9 has

a strict requirement for the substrate consensus motif Ser/Thr-Pro-X-Arg/Lys (Baumli et al., 2008). Another well conserved region among protein kinases is the catalytic loop, where the residues involved in the transphosphorylation reaction (Asp149, Lys151 and Thr191) are found. Finally, CDK9 has also a T-loop. When the corresponding cyclin is not bound, a C-terminal loop blocks the catalytic cleft, forming what is named as the T-loop or activation segment (De Bondt et al., 1993). When the cyclin is bound to its corresponding CDK, it pulls the T-loop outside of the catalytic cleft, exposing a threonine residue (Thr186 in CDK9). When this threonine is phosphorylated, a conformational change in the heterodimer CDK9-cyclin T1 is triggered, exposing the ATP and substrate biding sites. This will stabilize the T-loop in an open position, which will result in the active kinase (Anshabo et al., 2021; Baumli et al., 2008). It has been demonstrated that the mutants T188A and T186D reduce the association of CDK9 with cyclin T1 by 90 and 50% respectively (Mbonye et al., 2013). In order to be fully active and perform its activity in gene transcription, CDK9 must be phosphorylated at Thr186, and form a heterodimer with its activators cyclin T1, T2a, T2b or K. Originally, it was thought that autophosphorylation was the mechanism responsible for the formation of pThr186 (Baumli et al., 2008). However, new evidence supports the idea that CDK7 is responsible for that phosphorylation (Larochelle et al., 2012). Finally, phosphorylation of the Thr29 at the N-terminal reduces CDK9 activity (M. Zhou et al., 2006).

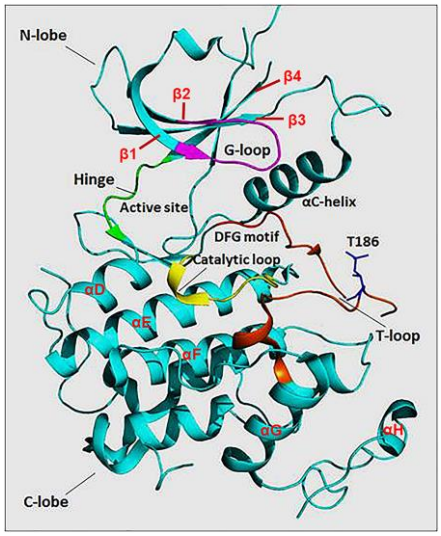


Figure 1.13. Protein structure of CDK9. (Protein Data Bank: 3BLQ). The N-terminal β-sheets 1 to 4 of the bilobal CDK9 are shown as well as the C-terminal α-helices D to H. Both lobes are linked by a hinge region (green). The N-terminus contains a glycine-rich loop (G-loop, purple) and an αC helix which binds ATP and cyclin, respectively. The C-terminus includes the catalytic loop (yellow) and T-loop (brown). The threonine 186 residue (T186), essential for CDK9 activation, is found in the T-loop structure. Adapted from (Anshabo et al., 2021).

1.3.1.2. Regulation of CDK9 activity

1.3.1.2.1. Regulation by sequestration in an inactive complex

Due to the importance of P-TEFb in transcriptional regulation, it is essential that its nuclear activity is tightly controlled. In human cells, more than half of P-TEFb is found in a large catalytically inactive complex, whereas the other half is found in an active state bound to BRD4 (Moon et al., 2005; Taube et al., 2002). However, this percentage varies depending on the cell type. For instance, in growing HeLa cells, 50% of CDK9 is found inactive, whereas in human primary blood lymphocytes this number rises up to 90% (Barboric et al., 2007; Y. K. Kim et al., 2011).

When CDK9 is sequestered in the complex called 7SK snRNP its kinase activity is negatively regulated. This complex if formed by 7SK small nuclear snRNA (RNAP III transcribed, non-coding 7SK snRNA), which acts as a scaffold for the rest of the components of the complex: MePCE (Methylphosphatase Capping Enzyme), LARP7 (La-Related Protein) and HEXIM1/2 (Hexamethylene bis-acetamide inducible proteins) (Michels et al., 2004; Nguyen et al., 2001) (figure 1.14). MePCE and LARP7 are necessary for the integrity and stability of the complex, protecting the 5' and 3' ends of 7SK RNA respectively, from being degraded by exonucleases (N. He et al., 2008; Jeronimo et al., 2007; Krueger et al., 2008). P-TEFb is sequestered by and homo- or heterodimer of HEXIM 1 or 2 that, when bound to the 5' terminal hairpin of 7SK snRNA, unmasks its C-terminal P-TEFb binding domain and interacts with cyclin T1 (Barboric et al., 2005; Q. Li et al., 2005). Meanwhile, cyclin T1 is also in contact with the 3' hairpin of the 7SK snRNA (Egloff et al., 2006). Although the whole structure of the complex has not yet been resolved, it is known that once CDK9 and cyclin T1 are bound to HEXIM1, their substrates cannot access the catalytic site of CDK9 (Pinhero et al., 2004; Schulte et al., 2005). Moreover, it has been widely demonstrated that the CDK9 sequestered in the 7SK snRNP is phosphorylated on its Thr186 residue and, therefore, the P-TEFb is ready to be used (Q. Li et al., 2005). Once P-TEFb is needed, it can be released from the complex under stimuli such as DNA damage, viral infection, transcription blockade or activation of some signaling pathways (Napolitano et al., 2013; Y. Sun et al., 2019; Z. Yang et al., 2001). In addition to these stimuli, other post-translational modifications like cyclin T1 acetylation (Cho et al.,

2009), ubiquitination and phosphorylation (Faust et al., 2018; Mbonye et al., 2015) of HEXIM1 have also shown to contribute to the release P-TEFb from the complex.

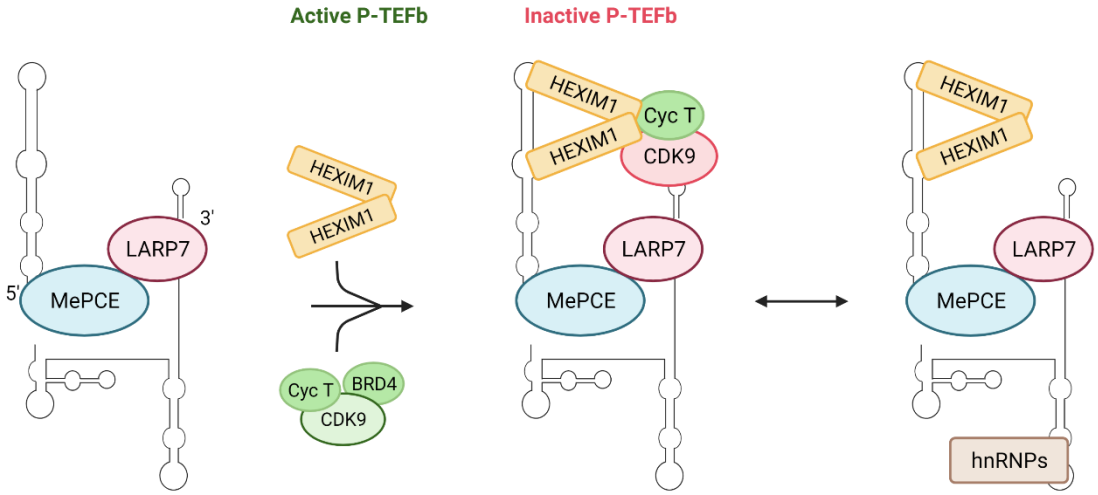


Figure 1.14. Formation of the inhibitory complex 7SK snRNP. Representation of the 7SK RNA with the union of the proteins MePCE and LARP7 to protect its 5' and 3' ends from degradation. HEXIM1 dimers bind to the stable complex and exposes their P-TEFb binding domains, allowing HEXIM1 to bind activated P-TEFb and inhibit its kinase activity. Once CDK9 is needed, P-TEFb is liberated from the complex and 7SK snRNP is stabilized thanks to the binding to heterogeneous nuclear ribonucleoproteins (hnRNPs). Adapted from (Anshabo et al., 2021). Generated with BioRender.

1.3.1.2.2. Regulation by post-translational modifications

The activity of P-TEFb is not only regulated by the inhibitory complex, but also by many post-translational modifications such as phosphorylations, ubiquitination and acetylation. For example, as previously remarked, CDK9 phosphorylation on its Thr186 is necessary for its kinase activity and this phosphorylation is also present once P-TEFb is sequestered in the inhibitory complex (Chen et al., 2004). Moreover, once P-TEFb is released from the inhibitory complex, a phosphorylation on CDK9 Ser175 carried out by CDK7, allows its binding with BRD4 or TAT (Mbonye et al., 2013; Z. Yang et al., 2005). Another important phosphorylation that reduces CDK9 activity occurs on Thr29 and helps to limit CDK9 activity during transcription (M. Zhou et al., 2009). Regarding acetylation, different modifications have been described both in cyclin T1 and CDK9. Acetylation on Lys380, Lys386, Lys390, and Lys404 of cyclin T1 by the HAT (histone acetyl-transferase), p300, have been described and play a role in the dissociation of P-TEFb from the inhibitory complex (Cho et al., 2009). CDK9

is also acetylated by p300 and GCN5 on its residues Lys44 and Lys48. However, different studies differ on whether the effect of these acetylation increases (Fu et al., 2007) or hinders (Blank et al., 2017; Sabò et al., 2008) its kinase activity. Finally, polyubiquitination by the SCF E3 ubiquitin ligase core components, recruited by cyclin T1, facilitates CDK9 degradation by the proteasome (Kiernan et al., 2001).

1.3.1.3. CDK9 involvement in pathology

1.3.1.3.1. CDK9 and viruses

Many viruses depend on P-TEFb for their replication, including human T-lymphotropic virus (HTLV-1), Herpes simplex virus (HSV-1 and HSV-2), human cytomegalovirus (CMV), Epstein-Barr virus (EBV), human adenovirus, Influenza A virus, Dengue virus, Kaposi's sarcoma-associated virus (KSHV) and human immunodeficiency virus (HIV) (Zaborowska et al., 2016). The latter is widely studied and, when it was discovered that P-TEFb plays an essential function on its transcription, many molecular mechanisms implicated in virus replication were elucidated. These viruses sequester P-TEFb by physically interacting through virus-encoded proteins which will promote an efficient transcription of the viral genome. In particular, HIV transcription depends on Tat, a viral transactivator protein that binds to cyclin T1 (Romano et al., 1999). With this interaction, P-TEFb locates at the promoter region of the viral mRNA and stimulates the transcription elongation of the new viral DNA in the same way that it does with cellular genes (Tahirov et al., 2010).

1.3.1.3.2. CDK9 and cardiac hypertrophy

Cardiac hypertrophy occurs as a result of an enlargement of the size of cardiac myocytes caused by an increase in RNA and protein content. Given that some hypertrophic signals lead to the dissociation of the 7SK complex, P-TEFb plays a critical role in the development of this disease. When the complex has been separated, there is a shift in the equilibrium and more P-TEFb is found on its active form (Espinoza-Derout et al., 2007; Sano et al., 2002).

As a result, RNA Pol II transcription is enhanced and protein synthesis and cell enlargement are increased.

The control of the available P-TEFb is critical to regulate the cardiomyocytes development and function. Different studies have demonstrated that cardiac hypertrophy can be recapitulated in mice by activating P-TEFb. For instance, inactivation of 7SK RNA by RNA-i in cardiac myocytes induces abnormal cell growth and CDK9 activation (Sano et al., 2002). Others have deleted HEXIM1 (known as CLP-1 in mice), which results in embryonic lethality and shows signs of cardiac hypertrophy (F. Huang et al., 2004). Another study in zebrafish demonstrates that knocking down LARP7 provokes an increase on Ser2 phosphorylation and cardiomyocyte proliferation (Matrone et al., 2015). All together, these studies evidence the importance of CDK9 as a regulator of the cardiomyocyte transcription program, whose disruption can lead to cardiomyopathies.

1.3.1.3.3. CDK9 and cancer

Given the importance of P-TEFb in transcription regulation and gene expression, it is not surprising that CDK9 contributes to progression and maintenance of many cancer types. Since P-TEFb is required during embryonic development (Hatch et al., 2016), genetic alterations affecting the loci of CDK9 or cyclin T1 are very rare. Therefore, a dysregulation in the activity of CDK9, such as an increase of activity, is the main alteration in cancer (Modur et al., 2018). This happens because many genes whose transcription depends on CDK9 are essential for processes as important as proliferation, development or stress responses.

Certain genes such as *MYC*, *BCL2* (B cell lymphoma 2) and *MCL1* (myeloid cell leukemia 1) are highly dependent on CDK9 activity, since their over-expression drives cell survival (Gabay et al., 2014). It has been well stablished that CDK9 inhibitors can successfully prevent these pro-survival genes (Rong Chen et al., 2005). Within the genome, there are regions known as super-enhancers that can mediate transcriptional control and activate the expression of these genes. In a similar way, BRD4, a partner of CDK9, is frequently found in super-enhancers, including that of *MYC*. This interaction helps to recruit P-TEFb to the enhancer, which directly implicates CDK9 in the regulation of this oncogene. Several studies have

demonstrated that, by inhibiting BRD4, the recruitment of CDK9 to the *MYC* promoter is impaired, *MYC* transcripts levels are reduced and anti-proliferative effects ensue (Delmore et al., 2011; Lovén et al., 2013; Mertz et al., 2011). However, the relationship between CDK9 and MYC goes further. Different studies have revealed a role for MYC in the regulation of the RNA Pol II pause-release by recruiting P-TEFb to its target genes. This interaction is intensified in cells with high MYC levels, magnifying transcript production and ending transcriptional amplification (Rahl et al., 2010). Moreover, when MYC is inhibited, the levels of elongating RNA Pol II are reduced, as well as the levels of phosphorylated Ser2 (Gargano et al., 2007; Larochelle et al., 2012). Therefore, new cancer treatments could combine CDK9 and MYC inhibitors, that would be especially effective in those cases where MYC is overexpressed.

Another example of the involvement of CDK9 in cancer is hepatocellular carcinoma. As previously explained, the $CDK9_{55}$ isoform is predominant in the liver and when cells go through cell cycle the $CDK9_{42}$ isoform increases and becomes dominant (Shore et al., 2005). Since CDK9 is involved in the process of transformation from normal hepatic tissue to hepatocellular carcinoma (HCC), it is believed that the ratio of both isoforms is important in the regulation of hepatocyte maturation. What's more, overexpression of MYC is frequent in HCC, which maintains malignancy by increasing the activity of P-TEFb. This will alter the ratio between both CDK9 isoforms and make the $CDK9_{55}$ prevalent (C. H. Huang et al., 2014). Moreover, the silencing of CDK9 with shRNAs demonstrated its involvement in disease maintenance in those HCC that depends on MYC overexpression. CDK9 silencing provoked anti-tumor effects that correlated with MYC expression levels (C. H. Huang et al., 2014).

Uncontrolled growth can also be caused by abnormal activity of the 7SK snRNP complex. For example, low expression levels of some of its components have been found in cervical, thyroid, breast and gastric cancer (Biewenga et al., 2008; Y. Cheng et al., 2012; Ji et al., 2014). Disrupting the equilibrium of P-TEFb towards its active state, promotes mammary epithelial transformation (N. He et al., 2008) and epithelial mesenchymal transition (EMT), invasion and metastasis in breast cancer cells (Ji et al., 2014).

2. OBJECTIVES

Despite all the accumulated knowledge on the *MYC* promoter, there is still much to be learned about its regulation. As previously highlighted in the introduction, ERK2 can bind to active promoters of genes and regulate them in a kinase-independent manner. In this line, previous results obtained in Dr. Javier León's laboratory revealed that within the *MYC* promoter there are several ERK-Boxes with the sequence C/G-AAA-C/G that are highly conserved in the human, mouse and rat genes. Moreover, using Chromatin Immunoprecipitation assays (ChIP) they observed that ERK2 binds to the human and mouse *MYC* promoters. Furthermore, they also performed ChIPs of CDK9 and ERK2 both in HeLa and 293T cells, observing that ERK2 and CDK9 coincide at the same region of the *MYC* promoter. Finally, another interesting finding revealed that ERK2 interacts with CDK9 observed by Proximity Ligation Assays (PLA). Data published in Andrea Quintanilla's thesis.

Based on all the aforementioned results and the data previously published, we decided to take a deeper insight in the ERK2-MYC-CDK9 triangle and proposed the following aims:

Objective 1. To study the regulation of MYC expression by ERK2

Objective 2. Characterize the interaction of ERK2 with CDK9 and determine its consequences on its functionality

Objective 3. Study if the interaction of ERK2-CDK9 plays a role in the regulation and expression of MYC.

3. MATERIALS AND METHODS

3.1. DNA and RNA analysis

3.1.1. Bacterial transformation and DNA purification

Plasmid DNA was transformed into heat-shock *E. coli* DH5α competent cells (Invitrogen). Once the DH5α cells were thawed, an amount of 500 ng of plasmid DNA was added, mixed and incubated for 30 min at 37 °C. After that, a heat-shock of 1 min at 42 °C was done and then the bacteria were incubated for 5 min in ice. Once this step was completed, 250 µL of LB (Luria-Bertani Broth) growth media was added and the mixture was incubated for 1 h at 37 °C. Finally, 100 µL of bacteria were seeded on an LB plate with antibiotic selection (100 µg/mL ampicillin or 50 µg/mL kanamycin), or added to 5 mL of LB growth media with the antibiotic (for miniprep) or 250 mL (for maxiprep) and incubated overnight (O/N) at 37 °C. In the case of the agar plate, colonies were selected and inoculated in 5 mL of LB growth media with the desired antibiotic and incubated O/N at 37 °C.

After being incubated O/N, bacteria were centrifugated at 3500 rpm 5 min (miniprep) or 6000 rpm for 10 min (maxiprep). DNA was purified following the manufacturer's instructions. For the maxiprep, the Qiagen Plasmid Maxi Kit was used and the DNA was resuspended in 250 µL of distilled deionized water (ddw). For the case of lower volumes, the bacterial culture was processed by GeneJET Plasmid Miniprep Kit (Thermo Fisher) and resuspended in 40 µL of Elution Buffer (10 mM Tris, 1 mM EDTA).

Plasmid DNA was quantify using the NanoDrop 2000 (Thermo Scientific). To further determine the quality of the DNA an electrophoresis was also performed. 2 µL of DNA mixed with loading buffer, with bromophenol blue to monitor the progression, were loaded into a 0.8% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen). To determine the concentration of the DNA, Lambda DNA/HindIII Marker (Thermo Scientific) was used. The electrophoresis was run at 100 V in TAE buffer 1X (0.09 M Tris-acetate, 2mM EDTA) and visualized using a Gel Doc EZ Imager (Bio-Rad).

3.1.2. Plasmids description

All the plasmids used in this thesis are listed in the following table.

Table 3.1 Description of the plasmids used in this thesis

PLASMID	DESCRIPTION
pCEFL	Mammal expression vector. EF-1α Promoter/ bGH poly-A. Empty vector used as a control to normalize the amount of DNA to transfect.
pCEFL HA ERK1 NLS	Mammal expression vector. Encodes the isoform ERK1 fused to HA epitope and a nuclear localization signal (NLS).
pCEFL HA ERK2 APA	Mammal expression vector. Encodes the isoform ERK2 fused to HA epitope. Serines in position 244 and 246 have been substituted by Alanines (A).
pCEFL HA ERK2 EPE	Mammal expression vector. Encodes the isoform ERK2 fused to HA epitope. Serines in position 244 and 246 have been substituted by glutamic acid (E).
pCEFL HA ERK2 NLS	Mammal expression vector. Encodes the isoform ERK2 fused to HA epitope and a nuclear localization signal (NLS).
pCEFL HA ERK2 D316N, D319N NLS	Mammal expression vector. Encodes the isoform ERK2 fused to HA epitope and a nuclear localization signal (NLS). Mutations in position 316 and 319 have been made.
pCEFL HA ERK2 ΔINS NLS	Mammal expression vector. Encodes the isoform ERK2 fused to HA epitope and a nuclear localization signal (NLS). Region between aminoacids 241-272 has been deleted.

pCEFL HA ERK2 DK NLS	Mammal expression vector. Encodes the isoform ERK2 fused to HA epitope and a nuclear localization signal (NLS). Mutation in position K52 for R (K52R) has been made. Kinase-deficient mutant.
pCEFL HA ERK2 AEF NLS	Mammal expression vector. Encodes the isoform ERK2 fused to HA epitope and a nuclear localization signal (NLS). Mutations T185A and Y187F have been made. Non-phosphorylatable mutant.
pCEFL HA ERK2 WT	Mammal expression vector. EF-1α Promoter/ bGH poly-A. Encodes the isoform ERK2 fused to HA epitope. Source: Dr. D. Engelberg
pGEX 4T-3	Bacterial vector for expressing fusion proteins with a thrombin site.
pGEX 4T-3 CDK9 full length (FL)	Bacterial vector. Encodes for CDK9.
pGEX 4T-3 CDK9 1-100	Bacterial vector. Encodes for the aminoacids 1-100 of CDK9.
pGEX 4T-3 CDK9 101-263	Bacterial vector. Encodes for the aminoacids 101-263 of CDK9.
pGEX 4T-3 CDK9 264-333	Bacterial vector. Encodes for the aminoacids 264-333 of CDK9.
pGEX 4T-3 CDK9 334-372	Bacterial vector. Encodes for the aminoacids 334-372 of CDK9.
pCMV Flag CDK9 wt	Mammal expression vector. Encodes for human CDK9 fused to Flag epitope. Purchased from Addgene, ref: 28100.
pCEFL Flag CDK9 FM	Mammal expression vector. Encodes for human CDK9 fused to Flag epitope. Mutations F314A and W316A

	corresponding to the consensus FXF domain have been made.
pCEFL Flag CDK9 FDM	Mammal expression vector. Encodes for human CDK9 fused to Flag epitope. Mutations R39A and K40A corresponding to the consensus D domain have been made.
pLKO.1 control	Lentiviral empty vector, Sigma-Mission®
pLKO.1 shCDK9	TRCN0000039640 Lentiviral shRNA for human CDK9 gene, Sigma-Mission®
pLKO.1 shCDK9	TRCN0000039642 Lentiviral shRNA for human CDK9 gene, Sigma-Mission®
pLKO.1 shCDK9	TRCN0000039655 Lentiviral shRNA for human CDK9 gene, Sigma-Mission®
pCMV-VSV-G	VSV-G gene encoding enveloped lentiviral protein, Addgene
psPAX2	GAG and POL genes enconding packaging lentiviral proteins, Addgene
pRL-null	Renilla sp. luciferase reporter gene regulated by the T7 promoter, Promega
pSG424 G5P2 LUC (MYC P2 promoter)	Contains 5 union sites for GAL4 upstream c-MYC promoter with luciferase expression, Majello et al 1999
siRNA control	sc-4390843 Silencer selective negative control
siRNA against CDK9 (human) 10 µM	sc-29268, Santa Cruz Biotechnology

3.1.3. RNA extraction and purification

RNA samples were purified from cell cultures using the RNA Isolation Kit (Nzytech) following manufacturer's instructions. RNA was resuspended in 50 µL of water free of RNAses and its concentration was measured at 260 nm in a microvolume spectrophotometer (Thermo Scientific NanoDrop 2000).

3.1.4. Reverse transcription and quantitative polymerase chain reaction (RT-qPCR)

To analyze gene expression at a mRNA level a RT-qPCR was performed.

Reverse transcription was carried out with the iScript cDNA Synthesis Kit (Bio-Rad) from 100 ng of RNA, according to manufacturer's instructions in a total volume of 20 µL. The protocol for the reaction was the following:

$$\frac{25\,°}{5\,min};\ \frac{46\,°}{20\,min};\ \frac{95\,°}{1\,min};\ \frac{4\,°}{\infty}$$

Once the cDNA was obtained it was stored at -20 °C until used.

Next, a quantitative PCR (qPCR) was performed. For each sample, the qPCR mix consisted of 10 µL 2X SYBR® Select Master Mix (Applied Biosystems); 0.3 µM of forward and reverse primer mix; 1 µL cDNA sample and up to 20 µL of ultrapure water. Then, the reaction mix was added to a 96-well white PCR plate in duplicates. Reaction mix without cDNA was used as negative control. qPCRs were carried out in a CFX Connect Real-Time PCR Detection System (Bio-Rad) following this protocol:

$$\frac{95\,°}{10\,min};\ \left(\frac{95\,°}{15\,sec},\frac{60\,°}{1\,min}\right)x\ 40;\ \frac{95\,°}{1\,min}$$

The mRNA expression was normalized to the housekeeping gene using the comparative Delta Ct (ΔCt) method:

$$\Delta Ct = 2^{(Ct\ normalizing\ gene - Ct\ gene\ of\ interest)}$$

The next table shows the primers used for the qPCR.

Table 3.2 List of primers sequence

Gene	Primers sequence (5' → 3')
	Human
S14	TATCACCGCCCTACACATCA
	GGGGTGACATCCTCAATCC
MYC	TCGGATTCTCTGCTCTCCTC
	CCTGCCTCTTTTCCACAGAA
	Mouse
GAPDH	ACCACAGTCCATGCCATCAC
	GATGGTATTCAAGAGAGTAGGGAGG
MYC	TAGTGCTGCATGAGGAGACAC
	TCTCCACAGACACCACATCAA

3.2. Tissue culture

3.2.1. Cell lines and maintenance

The cell lines used in this project were grown in DMEM (Dulbecco's Modified Eagle's Medium, Thermo Fisher) supplemented with 10 % of FBS (fetal bovine serum, Gibco) and 1% of Penicillin-Streptomycin antibiotics mix (10000U/mL) (Thermo Fisher). All cells were grown at 37 °C and 5 % CO2. Cell density was maintained under 80 % confluence and the washes were performed with 5 mL of PBS 1X (Phosphate Buffered Saline). Trypsin-EDTA (Gibco) was used to detached the cells.

In the following table are listed all the cell lines uses in this thesis.

Table 3.3 List of cell lines used in this thesis

Cell Line	Culture Media	Cell description
HEK 239T	DMEM + 10 % FBS	Epithelial cells derived from human embryo kidney. Immortalized with SV40 T-antigen
HeLa	DMEM + 10 % FBS	Epithelial cells derived from human cervical carcinoma
MEFs	DMEM + 10 % FBS	Mice embryonic fibroblasts cells
MEFs ERK less (ERK1 $^{-/-}$, ERK2 $^{lox/lox}$)	DMEM + 10 % FBS	Mice Embryonic fibroblasts cells. ERK1 null (ERK1$^{-/-}$) and ERK2 floxed (ERK2 $^{lox/lox}$).
NIH-3T3	DMEM + 10 % FCS	Established from NIH Swiss mouse embryo cultures

Quiescent, serum deprived or starved cells refers to DMEM culture media without FBS.

3.2.2. Drug treatments or other stimuli.

The MEK inhibitor, U0126 (Promega), was used at 10 µM for 1 h.

The ERK inhibitor SCH772984 (Selleckchem), was used at 1 µM for 4 h.

EGF (Epidermal Growth Factor, SIGMA) has been used to stimulate cells at 50 ng/mL final concentration at the indicated times.

4-hydroxitamoxifen (4HT, Sigma) was used at 600 nM at the indicated times to activate the Cre-ER recombinase to eliminate ERK2 in the MEFs ERK less cell line.

*UO126 (Promega), 1mg resuspended in 234 µl of DMSO; stock concentration: 10 mM; stored at -20°C.
*SCH772984 (Promega), 5mg resuspended in 1.7 ml of DMSO; stock concentration: 5 mM; stored at -80°C.
*EGF (Sigma-Aldrich) dissolved in MiliQ water with 2.3 µL of glacial acetic acid and 4 mg of BSA, filtered. Stock concentration: 50 µM/mL, stored at -20 °C.
*4-hydroxitamoxifen (Sigma-Aldrich) dissolved in DMSO; stock concentration: 1 mM; stored at -20°C.

3.2.3. Cell transfection

3.2.3.1. Transfection with Polyethylenimine (PEI)

HEK293T cells were transfected using Polyethylenimine (PEI). PEI condenses DNA into positively charged particles that bind to anionic cell surfaces. In this way, the DNA-PEI complex is endocytosed by the cells and the DNA released into the cytoplasm (Longo et al., 2013).

HEK293T were seeded into p60 plates and were transfected when they reach a 60 % confluence. PEI (Polysciences, Inc.) was used at a concentration of 1 mg/mL in a ratio of 1:3 w/w (DNA:PEI). For a p60 plate, 3 µL of PEI were diluted in 200 µL of Opti-MEM medium

(Gibco) and 1 µg of DNA was diluted in another tube. After 5 min of incubation, the two tubes were mixed, vortexed and incubated again for 10 min at room temperature (RT). Meanwhile, the media of the cells was changed for new one. Finally, the mixture was added to the cells, reaching a final volume of 2.5 mL in the plate. As a final point, cells were harvested for analysis between 36-72 h after transfection.

*PEI (Polysciences, Inc.): dissolved in distilled water with a pH: 2. Once dissolved, pH was adjusted at 7, the solution was filtered with a 0.22 µm pore size sterile syringe filters and stored at -20 °C. Stock concentration: 1 mg/mL.

3.2.3.2. Lipofectamine LTX

Lipofectamine comprises lipids that, in an aqueous environment, can produce liposomes, which capture DNA plasmids. To overcome the electrostatic repulsion of the cell membrane, cationic liposomes form a compound with negatively charged DNA (Cardarelli et al., 2016)

HeLa and NIH 3T3 cells were transfected using Lipofectamine LTX (Invitrogen, Thermo Fisher). Cells were seeded 24 h before transfection to reach 60-70% of confluence at transfection. 8 µL of LTX was diluted in 200 µL Opti-MEM medium (Gibco, Thermo Fisher). 1 µg of DNA was diluted in another tube containing 200 µL Opti-MEM medium with 4 µL of PLUS Reagent. After 5 min of incubation at room temperature, the content of the DNA tube was added to the Lipofectamine LTX Reagent tube and incubated 10 min at RT, the mix DNA-lipid complex was added to the cells. After 24 h, the media was change for new one and cells were collected 48 h post transfection.

3.2.3.3. Lipofectamine RNAi/Max

The small interfering RNA (siRNA) against CDK9 was transfected with Lipofectamine RNAiMAX (Invitrogen. Thermo Fisher). 10 µL of 10 µM siRNA were diluted in 250 µL of Opti-MEM medium and in other tube with 250 µL of Opti-MEM medium 10 µL of Lipofectamine RNAiMAX were added. Both solutions were incubated separately for 5 minutes at room temperature and, then, they were mixed and vortexed. Then, an incubation for 10 minutes at room temperature was performed. After this incubation, the mix was added to the cells,

which were previously washed with 1X PBS. For an optimal efficiency, cells were collected at least 72 hours post-transfection.

3.2.4. Lentivirus infection

Viral transduction was used to infect HeLa cells with a short hairpin RNA (shRNA). The shRNA encodes a specific sequence that can be integrated into the host genome, allowing the creation of stable cell lines or transient expressions. In this thesis, a pLKO shCDK9 vector was utilized to silence CDK9.

3.2.4.1. Lentiviral production

HEK293T cells transfected with PEI were used to produce the lentiviral particles. In order to produce the lentivirus, three different plasmids were used:

- psPAX2, packaging plasmid encoding the HIV gag, pol, rev, and tat genes
- pCMV-VSV-G, envelope plasmid encoding the VSV-G gene
- Transfer plasmid containing the LTR sequences, the psi packaging signal and the cDNA/shCDK9 (pLKO.1 vector).

The cells were transfected at 70% of confluence in 150 mm Ø plate. The mixture of PEI + DNA (50 µg in a proportion of 6:19:25 µg) was added to the cells containing 15 mL of DMEM (without serum or antibiotics). After 12 h, the media was withdrawn and replaced by complete medium. The supernatant containing the lentiviral particles was collected at 48 h and 72 h after transfection, mixed and clarified by centrifugation at 1,500 rpm for 10 min. Then, this supernatant was filtered through a 45 µm pore size sterile syringe filter and stored at 4 °C.

3.2.4.2. Lentiviral concentration

Once the lentiviral-containing supernatants were clarified, Polyethylene Glycol 8000 (PEG8000, Fisher BioReagents) was added at a final concentration of 15 %-PEG8000 and homogenized gently by inversion. An incubation of 12 h or up to 6 days at 4 °C was

performed. After that, the mixture was centrifuged for 30 min at 1,500 x g 4 °C in order to concentrate the lentiviral particles. The obtained pellet was then resuspended in serum-free media (150 µL for each 15 mL), aliquoted and stored at -80 ºC.

*PEG8000 (Fisher BioReagents): dissolved in PBS 1X; stock concentration 40 % (w/v); autoclaved; stored at RT.

3.2.4.3. Lentivirus tittering

In order to determine the lentivirus titer, HeLa cells were seeded in a six-well plate ($2*10^4$/well). Once attached, media was replaced by 1.5 mL of serum-free DMEM and 5 µg/mL of Polybrene (Sigma-Aldrich) was added, together with different volumes of the concentrated lentivirus (normally 10 µL, 5 µL, 1 µL, 0.5 µL and 0.1 µL). After 12 h, 1.5 mL of complete media was added to each well and, 48 h after infection, the selective antibiotic (puromycin for all the viral particles used in this work, at 1 µg/mL) to select the infected cells. Media supplemented with puromycin
was refreshed daily until the colonies had grown and remained separated from each other. After, the plate was washed twice with PBS 1X for posterior staining with crystal violet solution. Finally, the number of colonies was determined by counting, assuming that each one comes from one single cell infected by one single lentiviral particle. The titer was later calculated by:

$$\frac{x\ number\ of\ colonies}{y\ \mu L\ of\ virus} * 10^3 = U.F.C/\ mL$$

*Polybrene (Sigma-Aldrich): dissolved in distilled water; stock concentration: 5 mg/mL; stored at -20 °C.

3.2.4.4. Cell transduction

To infect the cells of interest, $5*10^5$ cells/p60 were plated and once attached, media was removed for 1.5 mL of serum-free media with the corresponding volume of lentiviral particles, together with Polybrene (5 µg/mL) in a 1.5 mL Eppendorf tube. After 12 h, 1.5 mL

of complete media was added. If cell selection was needed, puromycin (1 µg/mL) was added 48 h after infection and the selection extended for the estimated days, depending on the experiment.

*Puromycin (Sigma-Aldrich): dissolved in MilliQ and filtered; stock concentration 25 mg/mL; stored at -20 °C.

3.3. Protein analysis

3.3.1. Western Blot

To determine the protein expression, cells were washed with PBS 1X and harvested with 200 µL of 1% NP-40 lysis buffer by scrapper. In order to completely lysate the cells, protein samples were sonicated using the Bioruptor Plus sonication device (Diagenode) set at high powersetting for 10 cycles (30 s ON, 30 s OFF). After sonication, cell lysates were clarified by centrifuging 20 min at 4 °C at 14000 rpm. The supernatant containing the proteins was collected and transferred to a new tube and stored at -20 °C until used.

Quantification was determined using Bradford method reading the absorbance at 620nm wavelength (Protein assay reagent – Bio-Rad). Final protein concentration was set around 50 µg and mixed with Laemli loading buffer 5X. The mixture was boiled at 95 °C for 5 min for protein denaturalization and kept on ice until used. Then, proteins were loaded for separation in sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE). The SDS gel (8-12 % acrylamide/bis-acrylamide, depending on the molecular weight of the analyzed proteins) was run in a Mini Protean III cuvette (Bio-Rad) with running buffer at 140 V for approximately 2h. “Precision PlusProtein Dual Color Standards” (Bio-Rad) were used to evaluate protein migration and separation during gel electrophoresis. Once resolved, proteins were transferred from the acrylamide gel to a nitrocellulose membrane (Amersham Protran Supported 0.45 NC, GE Healthcare Life Sciences) in a Mini-Trans Blot cell (Bio-Rad) using transfer buffer at constant amperage of 400 mA for 1 hour, depending on the molecular weight of the proteins of interest. After that, the nitrocellulose membranes were blocked with BSA 4 % dissolved in Tris Buffered Saline-Tween (TBS-T) in agitation at RT in

order to avoid unspecific antibody binding during the next steps. The blocking was performed for 1 h at RT. Once blocked, the blots were incubated for 1 h at RT or O/N at 4 °C (depending on the antibody affinity) with the different antibodies (table 3.4) prepared in blocking solution. After the incubation, the membranes were washed 2 times for 10 min with TBS-T and incubated for 1 h at RT with secondary antibodies. Particularly, anti-rabbit Immunoglobulin (Ig) (Bio-Rad) or anti-mouse Ig (Bio-Rad) secondary antibodies conjugated with peroxidase (1:10000) in 4 % milk (GE Healthcare) dissolved in TBS-T. Finally, membranes were washed twice for 5 min with TBS-T and the proteins were detected by chemiluminescence with an enhanced chemiluminescent system (ECL). Konica Minolta films were used as autoradiography to reveal the protein signals.

*1% NP-40 lysis buffer: 50 mM Tris-HCl pH 8, 1 % NP-40 (v/v), 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 0.2 % SDS, and protease inhibitors added just before lysing: 10 µg/mL of aprotinin and 10 µg/mL of leupeptin; all dissolved in ultrapure water; stored at 4 °C.

*5X Laemli loading buffer: 100 mM Tris pH 6.8, 4% SDS, 20% glycerol, 20 mM DTT and 0.005% bromophenol blue.

*Poliacrylamide gels:

- Stacking gel: 4% acrylamide, 125 mM Tris-HCl pH 6.8, 0.4% SDS, 0.1% Ammonium Persulfate (APS) and 0.1% Tetramethylethylenediamine (TEMED).
- Resolving gel: acrylamide (from 8% to 12%, depending on the molecular weight of the protein), 375 mM Tris-HCl pH 8.8, 0.4% SDS, 0.1% APS and 0.1% TEMED.

*Running buffer: 25 mM Trizma pH 8.3, 192 mM glycine and 0.1 % SDS (w/v); stock concentration: 1X; stored at RT.

*Transfer buffer: 25 mM Trizma pH 8.3 and 192 mM glycine; stock concentration: 1X; stored at RT.

*TBS-T: 20 mM Tris-HCl pH 7.5, 137 mM NaCl and 0.05 % Tween 20 (v/v); stock concentration: 1X; stored at RT.

*Enhanced chemiluminescent system (ECL)

- Solution 1: 1 M Tris HCl pH 8.5, 90 mM Coumaric Acid, 250 mM Luminol.
- Solution 2: 1 M Tris HCl pH 8.5, 30% Hydrogen Peroxide.

Table 3.4 List of primary and secondary antibodies used in this thesis

Name	Type	Technique and dilution	Reference
Primary antibodies			
α-Tubulin	Mouse monoclonal	WB (1:5000)	T5168, Sigma
ERK2	Mouse monoclonal	WB (1:1000)	sc-1647, Santa Cruz
FLAG	Mouse monoclonal	WB (1:4000) IP: 0.5 µg IF (1:200)	F1804, Sigma
HA	Mouse monoclonal	WB (1:1000) IP: 1 µg IF (1:100)	probe F-7, sc-7392, Santa Cruz
p-ERK 1/2 (Tyr 204)	Mouse monoclonal	WB (1:1000)	sc-7383, Santa Cruz
GST (B-14)	Mouse monoclonal	WB (1:1000)	sc-138, Santa Cruz
CDK9 (D-7)	Mouse monoclonal	WB (1:1000)	sc-13130, Santa Cruz
CDK7 (MO1)	Mouse monoclonal	WB (1:1000) IP (1:200)	2916, Cell Signaling
FLAG	Rabbit polyclonal	WB (1:5000) IP: 0.5 µg IF (1:200)	F7425, Sigma
HA (SG77)	Rabbit polyclonal	WB (1:1000) IF (1:100)	71-5500, Thermo Fisher
CDK9	Rabbit polyclonal	WB (1:1000) IP (1:100)	2316, Cell Signaling
c-MYC	Rabbit polyclonal	WB (1:3000)	9402, Cell Signaling
c-MYC (Y39)	Rabbit polyclonal	WB (1:3000)	ab32072, ABCAM

pELK (pSer383)	Rabbit polyclonal	WB (1:500)	ab34270, ABCAM
ELK 1	Rabbit polyclonal	WB (1:1000)	9182, Cell Signaling
CDK8	Rabbit polyclonal	WB (1:1000) IP (1:200)	17395, Cell Signaling
RNA Pol II (pSer2)	Rabbit polyclonal	WB (1:1000)	ab5095, ABCAM
RNA Pol II (CTD)	Rabbit polyclonal	WB (1:1000)	sc-899, Santa Cruz
Secondary antibodies			
Anti-Mouse-HRP (Mouse IgG)	Goat	WB (1:10000)	170-5047, Bio-Rad
Anti-Rabbit-HRP (Rabbit IgG)	Goat	WB (1:10000)	170-5046, Bio-Rad
Anti-Mouse-HRP ($F(ab')_2$ fragment)	Goat	WB (1:15000)	JAC-115-036-006, Jackson ImmunoResearch
Anti-Rabbit-HRP ($F(ab')_2$ fragment)	Goat	WB (1:15000)	JAC-111-036-047, Jackson ImmunoResearch
FITC (mouse IgG)	Goat	IF (1:300)	31569 Invitrogen, Thermo Fisher
Alexa 594 (Rabbit IgG)	Goat	IF (1:600)	A32740 Invitrogen, Thermo Fisher
Alexa 647 (mouse IgG)	Goat	IF (1:400)	JAC-115-605-003, Jackson ImmunoResearch

3.3.2. Coomassie blue staining

For quantification and correct visualization of the quality of a protein purification, coomassie blue staining was used. To do so, after running the proteins in a SDS-PAGE gel, coomassie blue solution was added to dye the polyacrylamide gel and incubated for 30 min at RT. Next, gel was de-staining until the protein bands were well-defined. A Bovine Serum Albumin (BSA) was used to calibrate unknown concentrations and determine the concentration of the desired protein.

*Coomassie blue solution: 0.1 g brilliant blue, 10 mL acetic acid, 50 mL methanol, up to 100 mL of water; stored at RT.
*De-staining solution: 30 % Methanol, 10 % Acetic Acid, 60 % water; stored at RT.

3.3.3. Co-immunoprecipitation assay

To study the protein-protein interaction co-immunoprecipitation (Co-IP) assays were performed. The objective of this technique is to immunoprecipitate a protein of interest using a specific antibody anti-epitope and analyze the proteins that are pulled down with it.

Once the proteins had been harvested and lysate with 1% NP-40 lysis buffer as previously described, 40 µg of lysate was separated and loaded with Laemli 5x buffer as a control to measure the total lysate (TL). The rest of lysate was incubated with 1 µg of the desired antibody rocking at 4 °C for 2 h or O/N. After this time, 5 µL of magnetic beads protein G (Dynabeads™ Protein G Immunoprecipitation Kit - Invitrogen) was added for 1h rocking at 4 °C. The protein G bind the immunoglobulins of the primary antibody which allows to precipitate the immunocomplexes (protein-antibody) using a magnetic retainer. The next step was washing the beads two times with NP-40 1% (or one time with NP-40 1% and another one with 1% NP-40 lysis buffer, depending on the experiment). Finally, the beads were resuspended in 25 µL of loading buffer Laemmli 2.5 X and boiled 5 min at 95 °C, together with the total lysate previously reserved. Proteins were loaded and analyzed by SDS-PAGE as previously described, with a difference in the election of the secondary antibody. In this case, anti-rabbit and anti-mouse secondary antibodies were specific and did not recognize the primary antibody heavy and light chain, so that no interference with the protein signals appeared.

3.3.4. Immunofluorescence

The immunofluorescences in this work have been carried out in HeLa cells. The cells were previously grown in a glass cover slip (10 mm Ø), washed twice with PBS 1X and fixed with 4% paraformaldehyde (PFA) during 10 min at RT. Then, cells were washed twice with PBS 1X for 5 min. Subsequently, cells were permeabilized for 10 min and washed twice with 1X PBS for 5 min. Before the addition of the primary antibody, glasses were washed with 0.05 % Tween-20 (Thermo Fisher) PBS 1X solution in order to reduce surface tension. After that, the primary antibody (prepared in filtered PBS 1X) was added as a drop over the glass and incubated for 1 hour in a humidity chamber to prevent drying (primary antibody could also be incubated O/N at 4°C). Primary and secondary antibody dilutions are shown in table 3.4. Next, cells were washed twice for 5 min with PBS 1X and one time with the solution 0.05% Tween-20. Then, a secondary antibody (conjugated with a fluorophore), specific for the primary antibody, was added for 1 hour in the humidity chamber and then removed washing twice with 1X PBS. The cells were incubated for 5 min with Hoescht (Sigma) in order to stain the nucleus and washed again for 5 min. Finally, the glasses were set over a slide in mounting media (ProLong, Thermo Fisher) and sealed with clear nail polish. The cells were examined by fluorescence microscopy (photomicroscope Axiophot, Carl Zeiss). The images were processed using Image J software.

*Permeabilization solution: 0.5% Triton X-100 in PBS 1X

3.3.5. Proximity Ligation Assay (PLA)

Another approximation to measure protein-protein interactions used in this thesis work is the Proximity Ligation Assay (PLA), which allows to detect interactions in vivo. This technique detects interactions between proteins that are closed together (between 0 and 40 nm) through an immunodetection, allowing also to detect the cellular compartment where the interaction is taking place. The signal can be observed as red fluorescent dots in the cellular localization where the interaction is taking place.

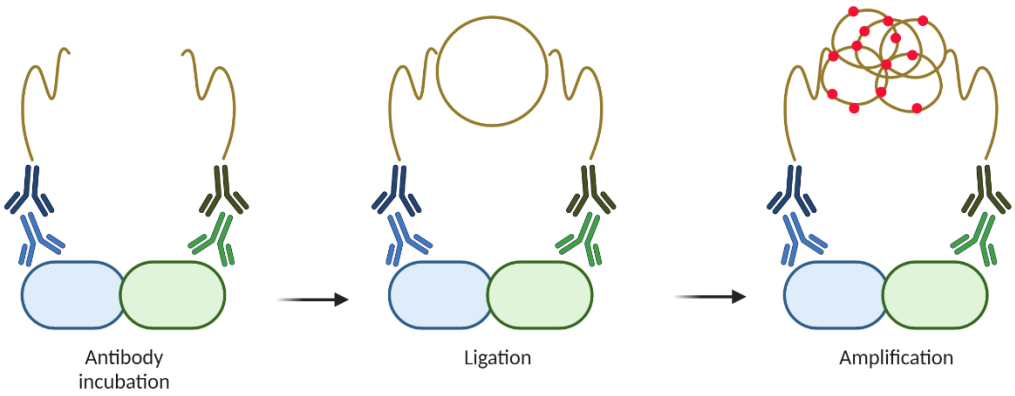


Figure 3.1. Schematic representation of PLA main steps. The first step is the incubation with the primary and the secondary antibodies. Then, a ligation step allows the hybridization of the oligonucleotides. Finally, the amplification phase, where fluorescent probes are introduced for the posterior detection of the interaction as red dots.

The PLA was performed with Duolink *in situ* Red Starter kit Mouse/Rabbit (Sigma-Aldrich®). As the normal immunofluorescence previously described, cells were seeded in cover slips (10 mm Ø), fixed with paraformaldehyde, washed and permeabilized. Afterwards, the samples were incubated with a drop of a solution containing the two antibodies against our proteins of interest, which have to be produced in different species. The incubation was done for 1h (or O/N in a wet chamber at 4 °C) and then the cells were washed twice for 5 min with buffer A. While the primary antibody was incubating, oligonucleotide-conjugated secondary antibodies solution (PLA probe anti-Rabbit PLUS and anti-Mouse MINUS, Sigma-Aldrich) was prepared in the homemade antibody dilution buffer and incubated 20 min at RT. After the cells were washed, the samples were incubated with one drop of the secondary antibody solution for 1 h in a wet chamber at 37 °C. Next, crystals were washed twice with buffer A for 5 min and incubated with the ligation solution for 30 min at 37 °C. This step is necessary for the hybridization of the oligonucleotide probe that will form a DNA circle. Then, another two washes with buffer A for 5 min were done and the amplification solution was added to the samples and incubated for 90 min in a wet and dark chamber at 37 °C. If the proteins are close enough, this step will amplify the DNA through rolling circle DNA amplification, introducing red fluorescent probes. After this step, samples were washed two times with buffer B for 10 min and stored O/N with PBS 1X at 4 °C. The following day, an extra step consisting on adding a secondary antibody (as previously described) against the proteins of interest was performed. Since the experimental cells are transfected, this extra

step allows to determine if the cells are properly transfected and the observed dots corresponds to those cells that are transfected. After this, cells were washed, incubated for 5 min with Hoescht, washed again, mounted and sealed with nail polish for visualization. The final result was checked in a Zeiss IMAGER M1 fluorescence microscope. Images were acquired and analyzed with the ImageJ software.

*Permeabilization and blocking buffer: 1 % (v/v) Triton X-100, 3 % BSA (w/v) in 1X PBS.
*Antibody dilution buffer: 3 % (w:v) BSA, 0.1 % (v:v) Triton X-100 in 1X PBS.
*Buffer A: 0.01 M tris, 0.15 M NaCl, 0.05 % Tween 20 (added after filtration); pH 7.4; filtered through a 0.22 µm filter; stored at 4 °C.
*Buffer B: 0.2 M Tris, 0.1 M NaCl; pH 7.5; filtered through a 0.22 µm filter; stored at 4 °C.

3.4. Recombinant protein purification

GST (Glutatione S-tranferase) is a helpful gene fusion system for purifying proteins from *E. coli* that have been fused with GST from *Schistosoma japonicum*. The pGEX plasmids are designed to achieve high levels of inducible expression of entire or fragmented genes.

In order to obtain purified proteins, the BL21 DE3 strain of *E.Coli* was used. To do so, the different parts of CDK9 were subcloned into pGEX vector in order to obtain recombinant protein GST-tagged at their N-terminus. Once the transformed bacteria were obtained, they were inoculated in 30 mL of LB culture medium, supplied with the specific antibiotic, and incubated O/N at 37 °C shaking. The next day, the bacterial culture was added into 500 mL of LB and re-incubated until 0.6-0.8 OD (optical density) was reached. At that time, protein expression was induced by the addition of 1mM IPTG (isopropyl-β-d-thiogalactopyranoside) for 4 h at 37 °C. IPTG blocks the lac repressor activity; thus, when IPTG is added, the T7 promoter is released allowing the T7 RNA polymerase to transcribe the protein of interest. Once the incubation was completed, bacteria were obtained by centrifugation 30 min at 3000 g. The pellet was then resuspended in 10 mL of lysis buffer and sonicated 3 times (amplitude 80 %, 0.9 cycles) for 10 min. When the bacterial suspension turned viscous (DNA was released) samples were centrifuged at 3500 rpm for 30 min at 4 °C. Once done, the supernatant was saved and 500 µL of glutathione-sepharose beads (GE-Healthcare) were

added. Incubation for 4 h rocking at 4 °C was performed. After that, the beads were washed three times with PBS NP-40 1 % and two times with PBS 1X. Beads were suspended in PBS 1x and stored at 4 °C for short term storage. Quantitation of the obtained protein was checked by running a small amount of the eluted protein in a SDS-PAGE, stained with Coomassie blue as described above.

*Lysis buffer: PBS 1% NP-40, 2 µg/mL aprotinin- leupeptin.

3.4.1. Pull down in vitro assay

The GST purified proteins were incubated with total lysates of HEK293T cells lysed in MLB buffer during 2 hours rocking at 4ºC. After incubation, the precipitated Glutation-Sepharose beads were washed twice with cold 1X PBS, twice with cold 1X PBS 1% NP-40 and, finally, twice with MLB buffer. The beads were resuspended in loading buffer 2X Laemli and loaded in a 12% SDS-PAGE, as previously described.

3.5. In vitro kinase assay

3.5.1. CTD phosphorylation mediated by ERK

To elucidate the ERK2 mutant's ability to phosphorylate the C-terminal domain (CTD) of the RNA polymerase II, an *in vitro* kinase assay was performed, using the CDK9/cyclin K as substrate.

First, transfected cells with the mutants with a HA tagged, were immunoprecipitated as described previously. Kinase assays were performed following the manufacturer's instructions. First, ERK2 mutants and CTD (50 µg each one) were affinity purified using glutathione S-transferase (GST) from *E.coli* BL21. Commercial CDK9/CyclinK active (0.1 µg/µl) was purchased from Sigma. CTD kinase reaction was carried out for 15 min at 30 °C in kinase assay buffer. The kinase assay buffer was diluted 5-fold with a 50 ng/µl BSA solution and then added 0.25 mM DTT, 100 µM ATP and 100 µM γP^{32}ATP to perform de reaction in 40 µl. Kinase reactions were stopped with the addition of 8 µl 5× Laemmli buffer. Samples

were heated at 95 °C for 5 minutes and resolved by 12 % SDS-PAGE, dried and exposed. The gel was dried, using a gel dryer device for 2 h (Bio-Rad), and phosphorylated CTD was detected by autoradiography using Konica films.

*Kinase assay buffer: 25 mM MOPS, pH 7.2, 12.5 mM β-Glycerolphosphate, 25 mM MgCl2, 5 mM EGTA, 2 mM EDTA; stored at 4 °C.

3.6. Promoter analysis

3.6.1. Luciferase reporter assays

Luciferase reporter assays were performed to analyze the *MYC* promoter activation upon different overexpression of the gene of interest. As previously described, cells were plated and transfected with a mix of DNA plasmids specific for each experiment:

- The firefly (Photinus pyralis; 0.5 µg) luciferase MYC reporter vector carrying a 5' transcription regulatory sequence
- "pRL-null" Renilla (Renilla reniformis; 0.25 µg) luciferase gene construct that is constitutively expressed and it is used as control of transfection efficiency
- the specific gene or control constructs that desired to use for each experiment (1 µg)

The assay was performed using the Dual-Luciferase Reporter System (Promega). It started by lysing the cells with 200 µL of PLB (Passive Lysis Buffer) 1X diluted in ultrapure water. The lysates were then frozen at -80 °C for 5 min to increase the lysis efficiency. After that, lysates were clarified by centrifugation at 14000 rpm for 1 min. Supernatants were collected and added to a new 1.5 mL Eppendorf tube.

For each condition, 20 µL of cell lysate were loaded into a 96-well plate (in duplicates) and 100 µL of Luciferase Assay Reagent (LARII) containing the firefly luciferase substrate (luciferin) was added. Luminiscence was measured and then 100 µL of Stop&Glo Reagent was added. This reaction turns off the firefly luciferase reaction, but also contains the Renilla luciferase substrate (coelenterazine) so that starts the second luciferase reaction. After

measuring the Renila with the Glomax Multi-Detection System (Promega), firefly luminescence values were normalized against Renilla luminescence values and final data were relativized against the empty vector (control). Finally, results were represented as Relative Luciferase Units (R.L.U.).

3.7. Bioinformatic analysis

- Statistical analysis: data was processed and analyzed using the GraphPad Prism 7 Software (GraphPad Software, Inc., San Diego, CA).
  In bar graphs data is given as Mean ± SEM and Two tailed unpaired Student's t-test was used to determine differences between data sets and significance (*$p<0.05$, **$p<0.01$, ***$p<0.001$ and **** $p<0.0001$). In the case of group analysis (three or more groups), One-way ANOVA was used instead of Student's t-test.

- Western blot analyses and confocal images processing was carried out and analyzed using Fiji-Image-J Software.

- Bibliography was sorted by Mendeley reference management Software.

4. RESULTS

4.1. ERK2 regulation of MYC

4.1.1. Effect of ERK2 on MYC synthesis upon mitogenic stimulation

As highlighted in the introduction, the Ras-ERK pathway plays a critical role in MYC regulation, particularly by phosphorylating MYC, which allows MYC protein stabilization. Given the previous findings by our collaborators where they observed that ERK binds to the *MYC* promoter, we decided to take a deeper look on the consequences of this union.

First, we check what was the role of ERK on MYC expression in response to mitogenic stimulation. To do so, we analyzed MYC mRNA and protein synthesis in quiescent NIH3T3 cells (a cell line derived from mouse fibroblasts), stimulated with fetal calf serum (FCS) in the presence of the MEK inhibitor U0126. As observed in figure 4.1A, mitogenic stimulation unleashed MYC mRNA synthesis and protein expression, while treatment with the MEK inhibitor completely abolished this effect. Similarly, to acute stimulation, when proliferating NIH3T3 cells were treated with the aforementioned MEK inhibitor or with the ERK inhibitor SCH772984, we also observed a similar drop on *MYC* mRNA levels. Taken together, these results suggested that ERK was implicated on the regulation of MYC expression.

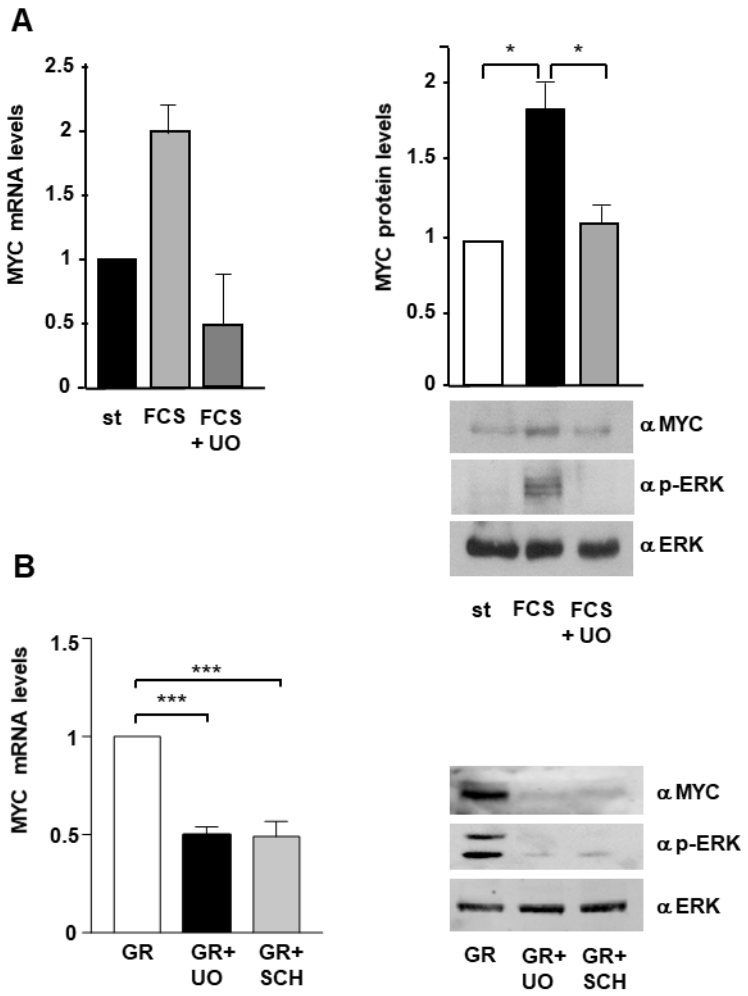


Figure 4.1. Regulation of MYC expression by ERK. A) *MYC* mRNA levels (left panel) were measured by RT-qPCR in NIH3T3 cells starved for 24 h and stimulated with 10% FBS for 45 min. Where indicated, cells were treated with U0126 for 1 h at 10 µM. Right panel corresponds to protein expression levels. B) *MYC* mRNA levels (left panel) were measured by RT-qPCR in proliferating NIH3T3 cells. Cells were treated with U0126 for 1 h at 10 µM and SCH772984 at 1 µM for 4 h. Right panel shows protein expression levels. Data shows mean ± SEM from 3 different experiments.

To further substantiate these findings, we took advantage of a murine embryonic fibroblasts (MEFs) cell line, which is knock-out for *ERK1* and harbors a floxed *ERK2* plus a 4-hydroxy-tamoxifen (4HT)-inducible Cre recombinase (*ERK1*$^{-/-}$; *ERK2*$^{lox/lox}$; Cre-ER) (Drosten et al., 2010). In this cell line the addition of 4HT, gradually yields an "ERK less" phenotype. As shown in figure 4.2, once 4HT has been added to growing MEFs, a drop in ERK2 levels is observed after 5 days, resulting in its complete absence after 9 days. We observed that the drop on ERK levels correlated with a reduction in MYC protein expression (panel A). Likewise, compared to wild-type MEFs a remarkable reduction in *MYC* mRNA synthesis was observed in 4HT-treated cells upon EGF stimulation (panel B). In combination, these results pointed out to ERK2 as a regulator of *MYC* expression under mitogenic stimulation.

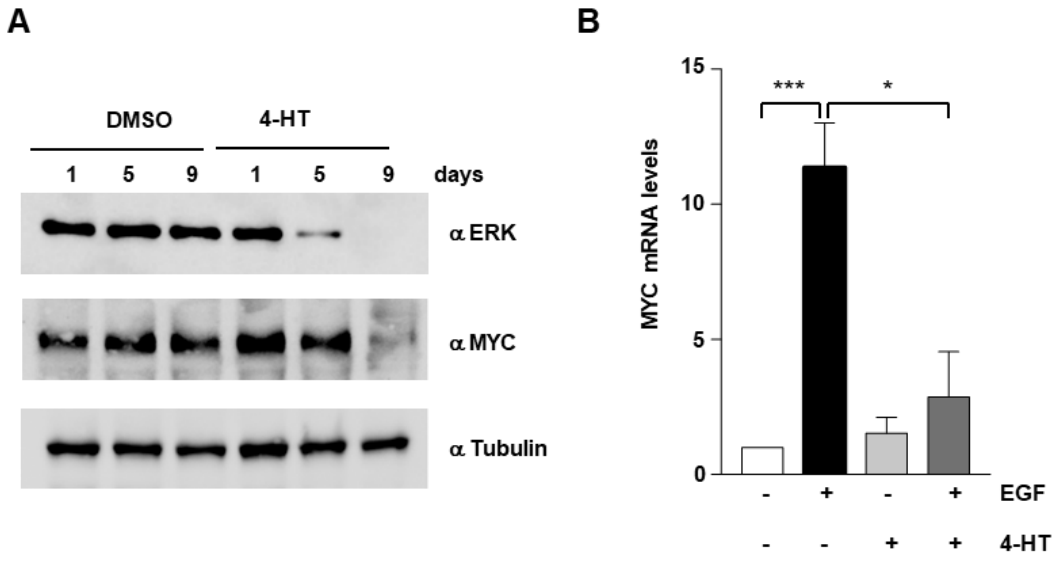
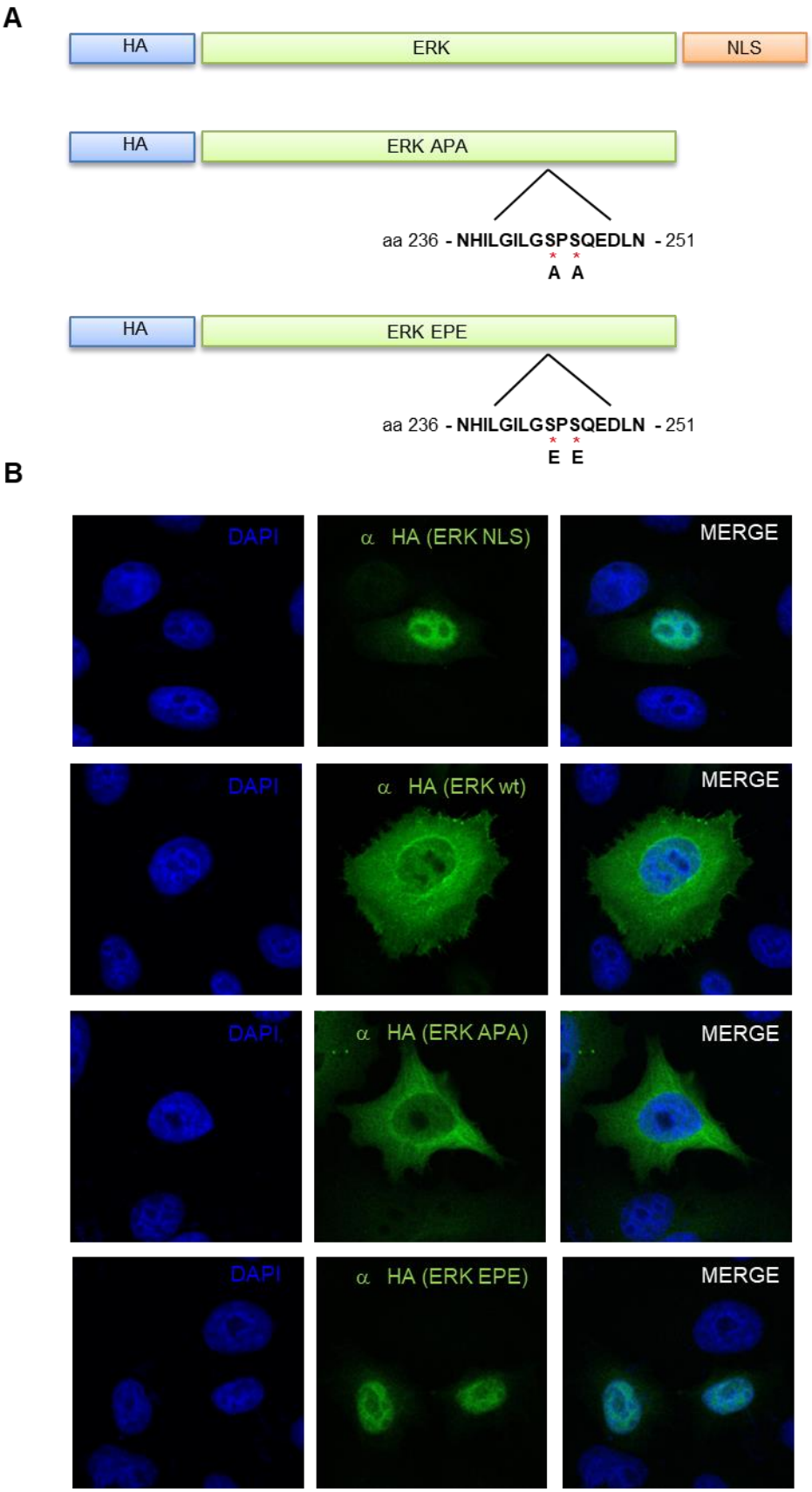


Figure 4.2. Effect of ERK depletion on MYC expression. A) MYC protein expression in response to ERK depletion in growing "ERK-less" MEFs 1, 5 and 9 days after addition of 600 nM 4HT. DMSO was used as control. B) *MYC* mRNA levels in starved ERK-less MEFs wild-type (-) or treated 9 days with 600 nM 4HT, after stimulation (+) with EGF (50 ng/ml; 45 min). Data shows mean ± SEM from 3 independent experiments.

4.1.2. Effect of nuclear ERK2 on MYC expression

Once we had determined that ERK2 plays an important role on MYC expression, we wanted to distinguish if the changes that we see in MYC synthesis was a consequence of ERK nuclear activity or on the contrary it could be elicited by ERK irrespective of its localization. To do so, we utilized an ERK2 construct with a Nuclear Localization Signal (NLS hereafter). This sequence allows ERK to translocate to the nucleus in the absence of mitogenic stimuli. Besides this plasmid, we also used constructs generated in Rony Seger's lab with mutations in the SPS sequence of ERK. Since wild-type ERK2 lacks a nuclear localization signal, one of the proposed mechanisms for its nuclear transport is based on a sequence Ser244-Pro245-Ser 246, (SPS) that is phosphorylated upon stimulation to mediate their nuclear translocation. The phosphorylated sequence within a 19 amino acid stretch acts autonomously by binding to the nuclear translocating protein importin7 (Imp7) and by promoting release from NUPs in active transports (Chuderland et al., 2008). In this publication, they generated two mutants within this sequence. The unphosphorylatable APA mutant, where they replaced both Ser residues by alanine. Thus, such protein cannot translocate into the nucleus and remains in the cytoplasm. And the EPE mutant, which has the two Ser residues in the SPS domain replaced with Glu, thereby acting as a phosphomimetic and shows a constitutive nuclear localization. Therefore, these constructs enabled us to study ERK effects on MYC expression in the absence of other mitogenic stimulation-induced events, which could make interpretations far more complicated (Rodríguez et al., 2010a) (Figure 4.3 A).

HEK293T cells (293T hereafter) were transfected with ERK-NLS, ERK wild-type and the mutant forms for comparative purposes, and then starved for 24 h. It was observed that ERK-NLS induced a potent MYC expression, which was also the case with wild-type ERK and the EPE mutant, but no with the APA mutant form (figure 4.3 C). It must be noticed that wild-type ERK spontaneously translocates to the nucleus if overexpressed (Chuderland et al., 2008) (figure 4.3 B). These results demonstrated that under serum starvation conditions ERK at the nucleus can evokes MYC expression.


A
HA
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A A
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ERK EPE
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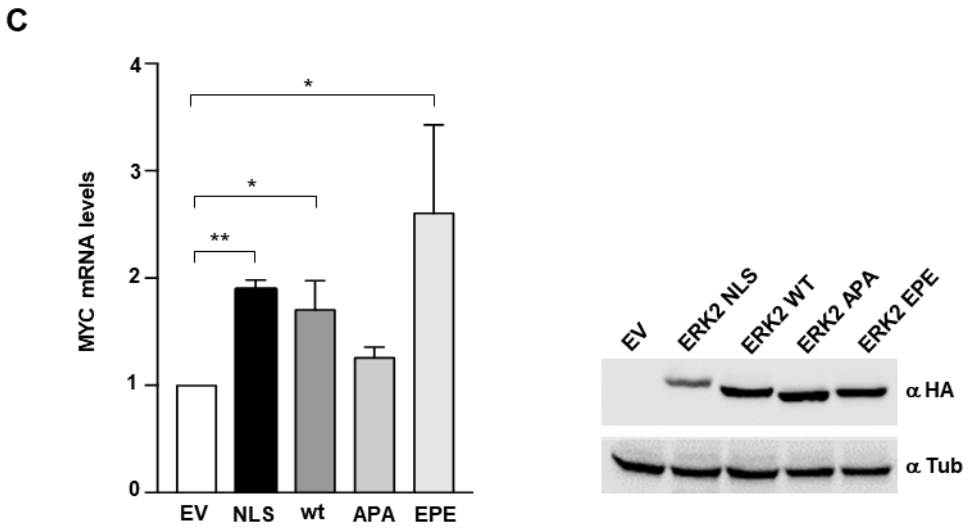


Figure 4.3. Effect of nuclear ERK on MYC expression. A) Schematic representation of the constructs HA ERK2 NLS (up), HA ERK2 APA (middle) and HA ERK2 EPE (down). B) Immunofluorescence showing the different ERK constructs transfected in HeLa cells (2 µg each). C) *MYC* mRNA levels in 293T cells transfected with HA-tagged ERKs (2 µg) after 24 h of serum depravation, measured by RT-qPCR. Protein levels showing transfection efficiency are also shown. Data shows mean ± SEM from 3 independent experiments.

Moreover, in cells expressing ERK2-NLS, MYC expression was observed under conditions of quiescence, in which phosphorylation of the ERK-inducible transcription factor ELK-1 was completely absent, indicating that the up-regulation of MYC was independent of Elk-1 activation (figure 4.4).

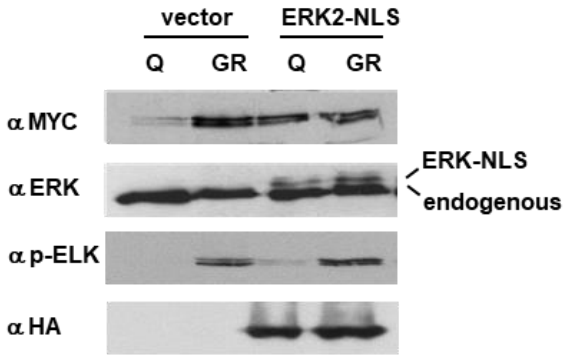


Figure 4.4. Effects of nuclear ERK on ELK-1 phosphorylation. 293T cells transfected with 2 µg of ERK-NLS in quiescent (Q) or growing (GR) conditions. Western Blotting was used to determine the expression of the different proteins.

These results demonstrated that the sole presence of nuclear ERK2 was sufficient to induce *MYC* expression under conditions in which ERK-dependent transcription factors were not active. Therefore, it points to the direct interaction of ERK2 with the MYC promoter as a trigger for *MYC* expression.

4.1.3. Effects of ERK kinase-inactive mutants on MYC expression

Given the previous result, we set up an experiment to determine if the observed effects on MYC upregulation was due to ERK2 catalytic activity or if it was a kinase-independent event. To do so, we took advantage of the already described ERK2 dead-kinase (DK) form, harboring a mutation in lysine 52 for an arginine (K52R) that completely eliminates its kinase activity (M. J. Robinson et al., 1996). In addition, we also used an unphosphorylatable ERK2 form (AEF), with mutations within the TEY motif: a substitution of threonine 185 for an alanine (T185A) and another one in tyrosine 187 for a phenylalanine (Y187F) (Wolf et al., 2001), since this form cannot be phosphorylated, it does not display kinase activity. To these constructs we added an HA tag at the N terminus for detection, and a NLS sequence in its C terminus, which allowed both proteins to translocate to the nucleus in the absence of stimulation (Rodríguez et al., 2010b).

As such, 293T were transfected with ERK-NLS wild-type and the ERK2 mutant's defective for phospho-transfer activity (DK and AEF). We observed that both mutants were able to induce MYC mRNA synthesis (figure 4.5 B) and protein expression (figure 4.5. C) in quiescent conditions as efficiently as the wild-type form. As before, these constructs did not evoke Elk-1 phosphorylation, providing further support to the notion that ERK can induce MYC expression in the absence of its kinase activity (figure 4.5. C).

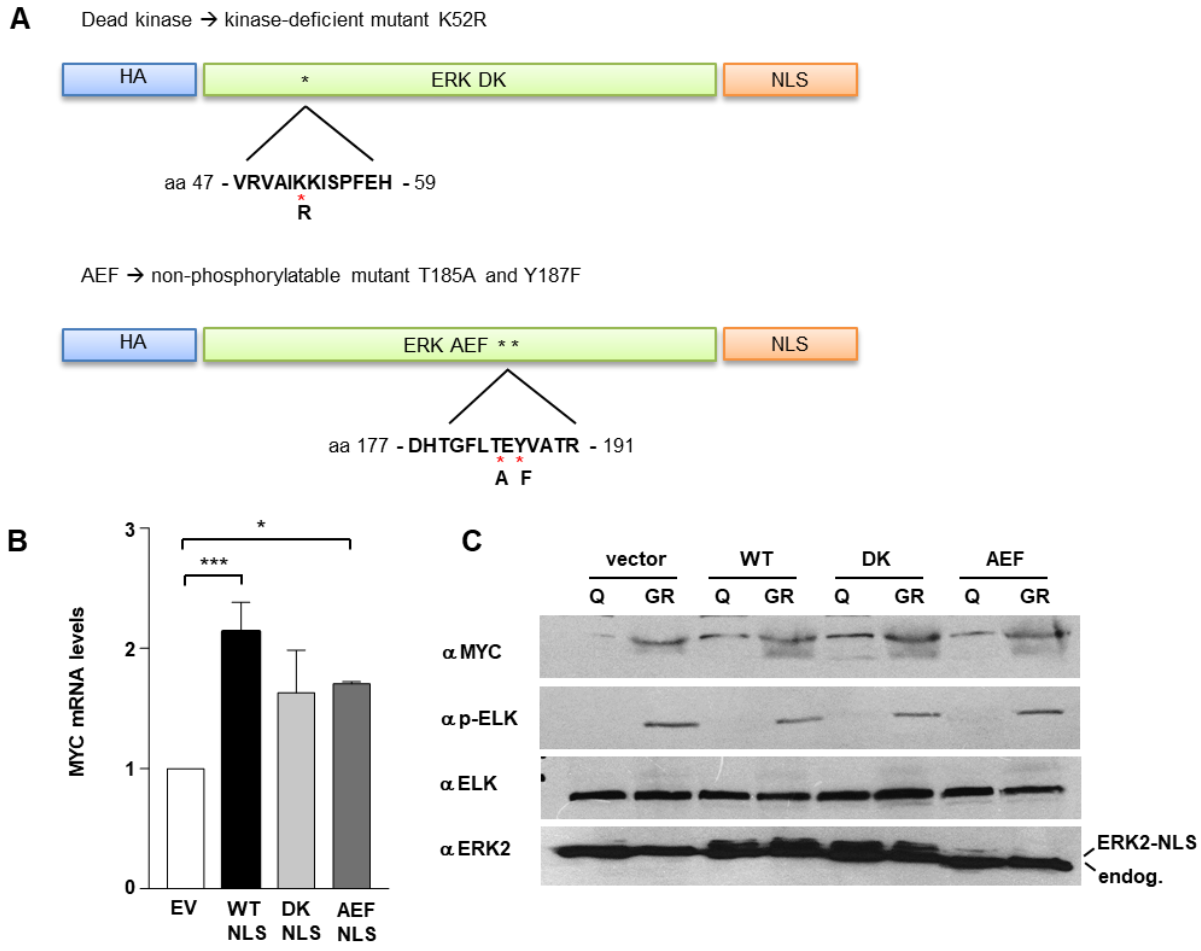


Figure 4.5. Effects of activity-deficient ERK2 mutants on MYC expression. A) Schematic representation of the constructs HA ERK DK NLS (up) and HA ERK AEF NLS (down). B) *MYC* mRNA levels in 293T cells transfected with 1 µg of the different plasmids and starved for 24 h, measured by RT-qPCR. Data shows mean ± SEM from 3 independent experiments. C) MYC protein levels in quiescent 293T cells transfected with the indicated ERK2 mutant forms (1µg each).

4.1.4. Determination of the ERK2 region responsible for driving MYC synthesis

Subsequently we set out to identify the region of ERK2 responsible of unleashing *MYC* expression. As stated in the introduction, Hu and collaborators determined that the residues responsible for ERK2 DNA binding were K259 and R261 (Hu et al., 2009). In order to corroborate if those residues took part in ERK2 capability to drive *MYC* synthesis, we used a construct were the ERK2 insert region spanning residues 241–272, (named as ΔINS) is deleted (Whitehurst et al., 2004), also containing a NLS sequence in the C-terminal domain and tagged at the N-terminal with a HA (generated by Javier Rodríguez). HeLa were transfected with ERK-NLS wild-type and ERK ΔINS NLS and deprived of serum for 24 h. We found that, unlike wild-type ERK, ERK ΔINS was incapable of inducing *MYC* mRNA synthesis. Therefore, this result identified the insert region of ERK2 as being involved in the regulation of *MYC* expression.

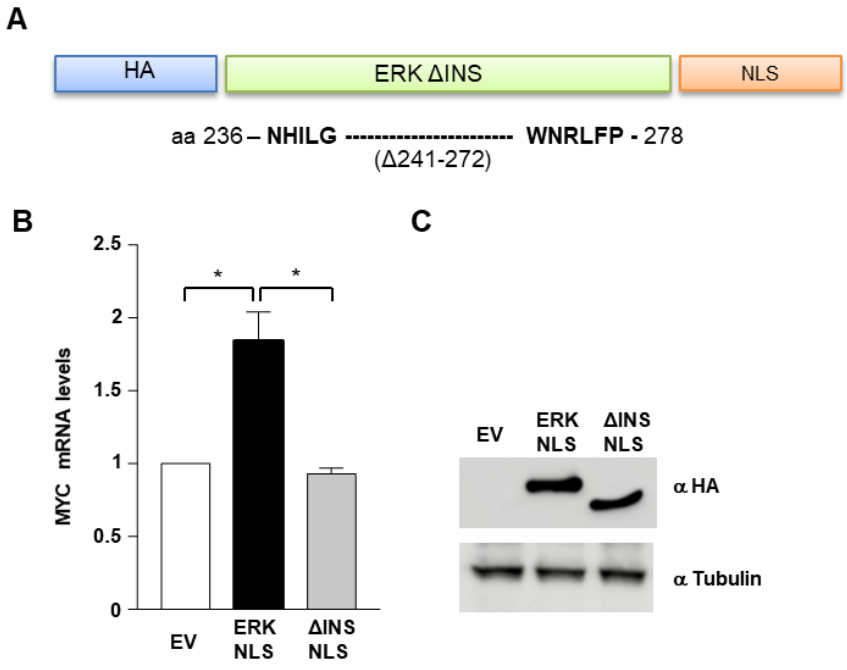


Figure 4.6. Role of ERK insert region on MYC synthesis. A) Schematic representation of the construct HA ERK ΔINS NLS. B) *MYC* mRNA and protein levels in HeLa cells transfected with 2 µg of ERK-NLS or ERK-ΔINS NLS in quiescent cells measured by RT-qPCR. Data shows mean ± SEM from 3 independent experiments.

4.2. Characterization of the interaction ERK2-CDK9 and determination of the consequences on its functionality

Previous results from Andrea Quintanilla using ChIP, showed that ERK2 and CDK9 are both bound to the *MYC* promoter and that both proteins coincide at the same region. Furthermore, using proximity ligation assays (PLA) they obtained results indicating that endogenous ERK2 and CDK9 could interact. Based on these preliminary results and in the search for the mechanism whereby nuclear ERK activated *MYC* expression, we studied in further depth the interaction between ERK2 and CDK9 and its possible implication on the regulation of MYC expression.

4.2.1. Determination of the specificity of the ERK2-CDK9 interaction

We started by investigating if the interaction of CDK9 with ERK2 was specific of this CDK, or if other members of the family like CDK7 or CDK8 were also able to interact with ERK2. To do so, co-immunoprecipitation assays in growing 293T cells were performed expressing nuclear ERK2 to analyze their interaction with endogenous CDK7, CDK8 or CDK9. As shown in figure 4.7. we could only observe an interaction between CDK9 and ERK2. Neither CDK7 or CDK8 could associate to ERK2.

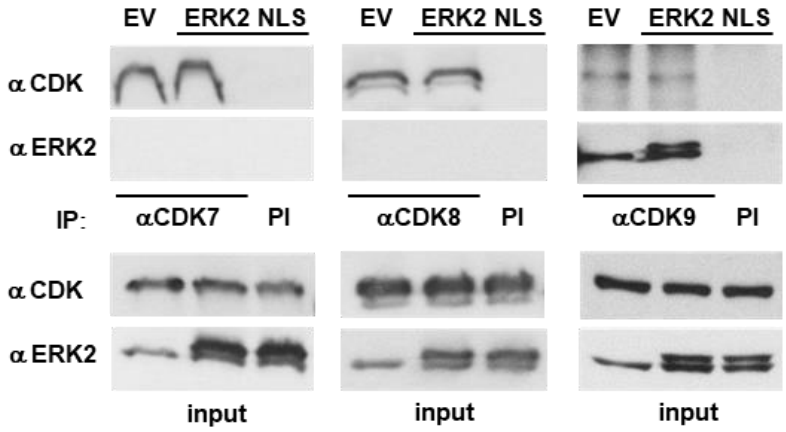


Figure 4.7. ERK2 interaction with CDKs. Co-immunoprecipitation assays were performed in growing 293T cells transfected with 1 μg of ERK-NLS and immunoprecipitated with the corresponding endogenous CDK. Co-immunoprecipitation was exposed with ERK2. Input: total lysates; EV: empty vector; IP: immunoprecipitation; PI: pre-immune IgG used as negative control.

Next, we determined if, in order to interact with CDK9, ERK2 had to be active. To carry out this experiment, the aforementioned HA-tagged ERK2 inactive forms were transfected in 293T cells and an immunoprecipitation against endogenous CDK9 was performed. In the same way, interaction between CDK9 and ERK1 was tested. Remarkably, we found that CDK9 binds just as well to ERK1. Likewise, CDK9 associated to the inactive ERK mutants as efficiently as to the wild-type (figure 4.8), indicating that the interaction between ERK2 and CDK9 is independent of ERK-catalytic activity.

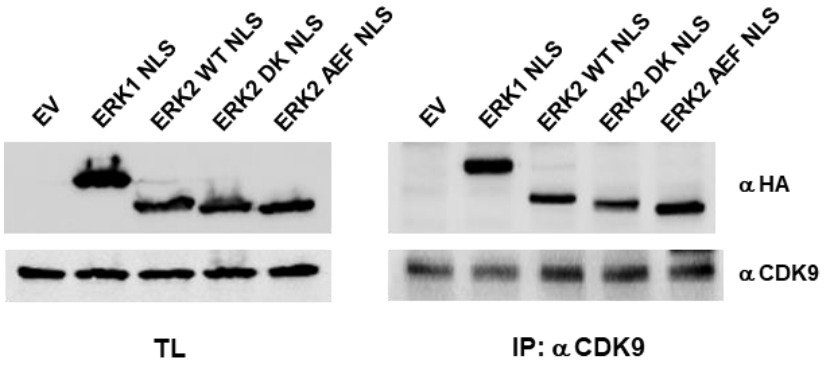


Figure 4.8. CDK9 interaction with non-catalytic ERK2 mutants. Co-immunoprecipitation assays were performed in growing 293T cells transfected with 1 μg of the different constructs and immunoprecipitated (IP) with endogenous CDK9, co-immunoprecipitation was exposed with HA. Empty vector was used as negative control for the co-immunoprecipitation.

4.2.2. Identification of the ERK-binding regions in CDK9

It was of interest to identify the determinants on CDK9 responsible for binding to ERK2. For this purpose, a series of deletion mutants were generated in order to determine which of them were capable of binding to ERK2. To do so, different fragments of human CDK9 were fused to GST (Glutathione S-transferase), a 211 amino acid 26 kDa protein, which would enable their immobilization in glutathione beads. The regions of CDK9 spanned from amino-acids 1-100; 101-263; 264-333; and 334-372, comprising the full length of the protein. Once the plasmids were generated (Javier Rodríguez) and purified from bacteria, they were used as baits to perform a pull-down of ERK from 293T cells lysates.

As observed in figure 4.9 A, it was found that two different regions of CDK9 were interacting with ERK2, particularly those between amino acids 1-100 and 264-333. When we analyzed the primary sequence of those regions it was realized that both regions contained a canonical ERK Binding Domain (EBD). Particularly, the region 1-100 harbored a D-domain, while the region between 264-333 contained an FXF domain (figure 4.9. B).

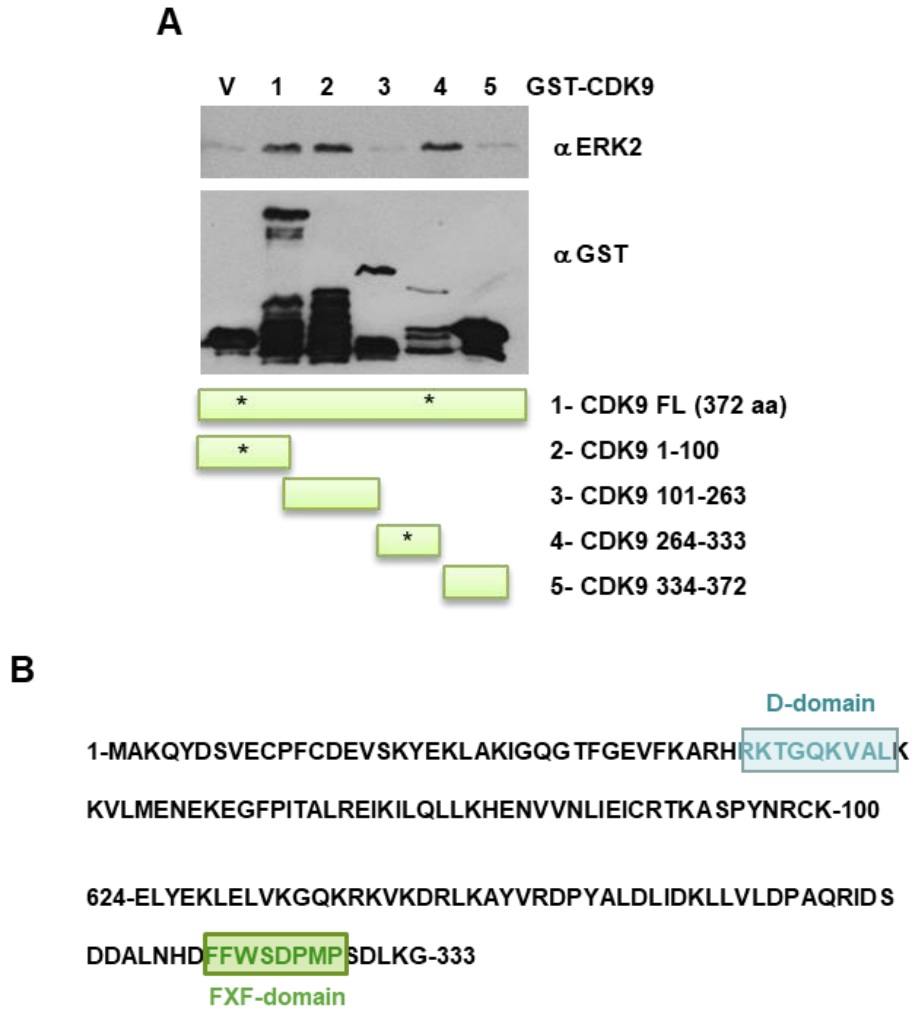
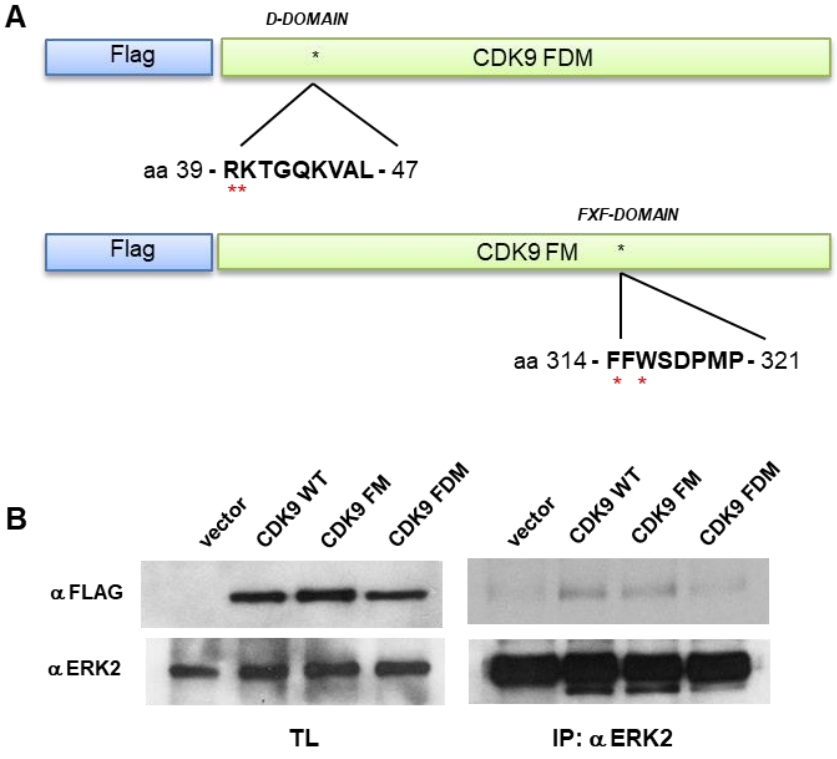


Figure 4.9. Identification of CDK9 ERK-binding regions. A) Pull-down of ERK2 from 293T lysates, using fusion proteins expressing GST plus the indicated CDK9 fragments. Asterisks indicate the presence of canonical ERK binding sites. The different constructs of CDK9 are represented by green boxes. B) CDK9 sequence highlighted in blue and green with the D-domain and FXF-domain respectively.

In light of this observation, we decided to generate ERK-binding defective CDK9 mutants by disrupting the binding domains by substituting critical residues for alanine. A FLAG tag was added for their detection (figure 4.10. A). Once obtained, those constructs were transfected along with FLAG-CDK9 wild-type in 293T cells and immunoprecipitations were performed in order to detect endogenous ERK2 binding. We observed that CDK9 FM, a mutant harboring a non-functional FXF domain, retained its ability to interact with ERK2, while the mutant FDM, with a disabled D-domain, impaired the CDK9-ERK2 interaction (figure 4.10. B). This result demonstrated that the interaction between ERK2 and CDK9 was mediated primarily via ERK2 CD domain-CDK9 D-domain binding.

In order to further corroborate the previous results, a proximity ligation assay was carried out (PLA) in HeLa cells in which the CDK9 mutant forms were co-transfected with nuclear ERK2. Interestingly, as observed in figure 4.10 C, disruption of the FXF and D domains dramatically altered CDK9 cellular sublocalization from nuclear to cytoplasmic. However, despite that issue we were still capable of seeing the interaction of ERK2 with the CDK9-FM mutant, but not with the FDM, corroborating the previous result obtained by the co-immunoprecipitation.



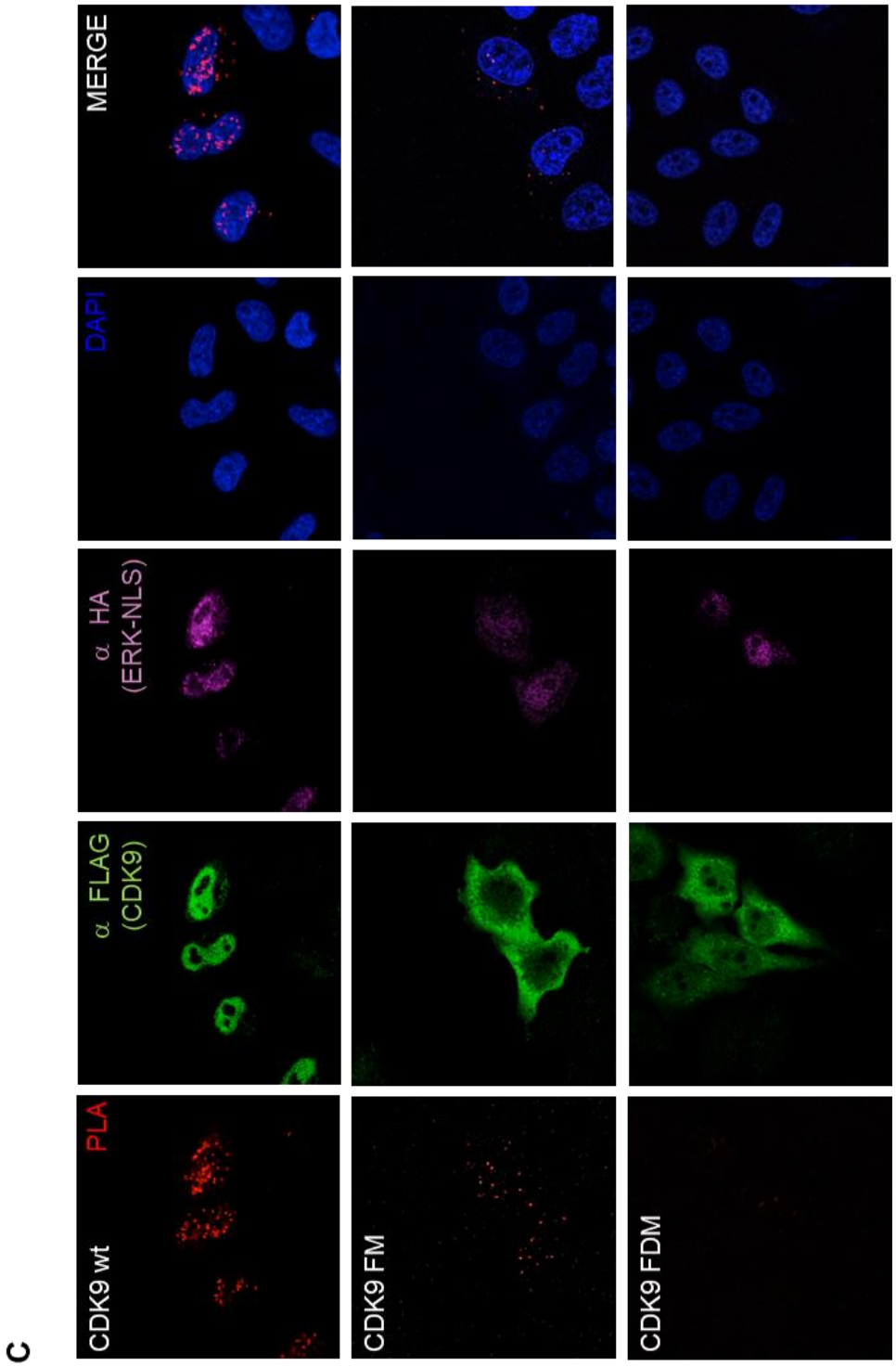


Figure 4.10. Interaction between ERK2 and CDK9 binding-defective mutants. A) Schematic representation of the constructs FLAG CDK9 FDM (up) and FLAG CDK9 FM (down). B) ERK2 co-immunoprecipitation (IP) with the indicated FLAG-tagged CDK9 mutants transfected (1µg each) in growing 293T cells. TL = total lysates. C) PLA assays with the indicated FLAG-tagged CDK9 mutants co-transfected with ERK NLS (1µg each) in growing HeLa cells. Interaction can be spotted as red dots.

In light of the data obtained with the CDK9 ERK-binding mutants, subsequently immunoprecipitation experiments were performed using ERK2 mutants for its binding domains, in order to corroborate the aforementioned results. CDK9 wild-type was co-transfected along with either ERK2 ΔINS or ERK2 D319N. The first has deleted the insert region, through which ERK2 binds FXF domains, while the latter has a mutation on its CD domain that impairs binding to D-domains (Tanoue et al., 2000), both proteins contained a HA tag and an NLS (figure 4.11 A). It was found that the ERK2 deletion mutant comprising its insert region retained its capacity for associating to CDK9, while the mutant D319N, failed to interact with CDK9 (figure 4.11 B). Taken together, all the preceding results demonstrated that ERK binds to CDK9 mainly through the D-domain.

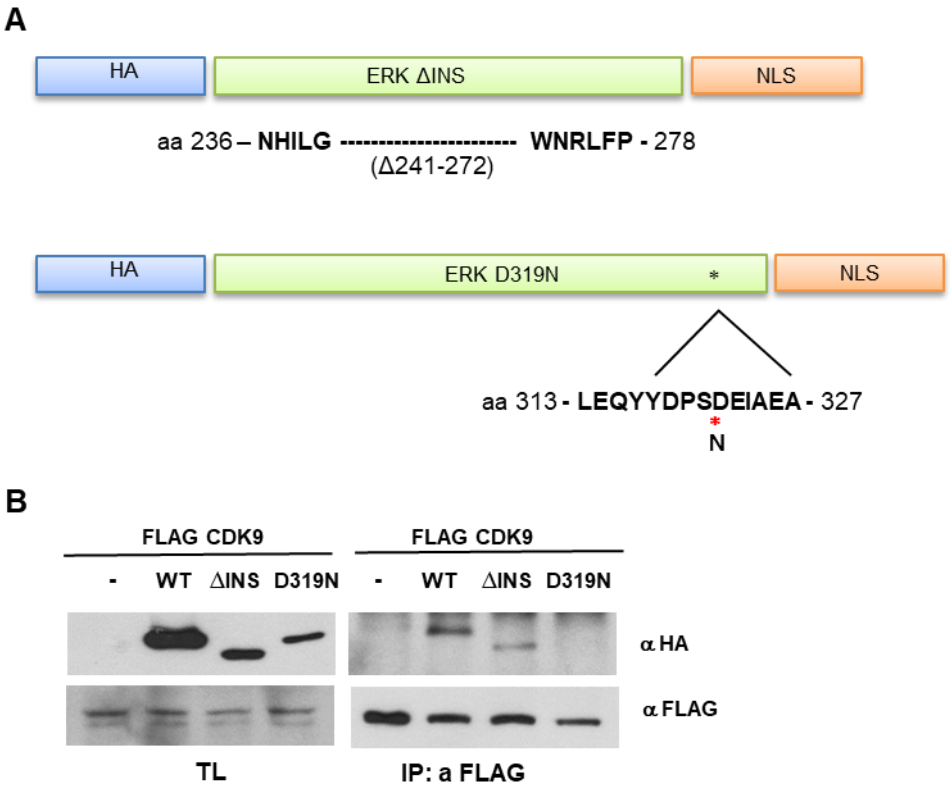


Figure 4.11. CDK9 interaction with ERK2 binding domain mutants. A) Schematic representation of the constructs HA ERK2 ΔINS NLS (up) and HA ERK2 D319N NLS (down). B) FLAG-CDK9 co-immunoprecipitation (IP) co-transfected with the indicated HA-tagged ERK2-NLS or its mutant forms (1μg each) in proliferating 293T.

4.2.3. Functional consequences of ERK2-CDK9 interaction

Once we had characterized the regions responsible of CDK9-ERK2 interaction, we set out to investigate the functional consequences of this binding. As explained in the introduction, CDK9 phosphorylates the CTD of the RNA Pol II at Ser 2, which allows RNA Pol II to continue from pause to the elongation step of gene transcription (Dan Chen & Zhou, 1999). Thus, we evaluated if ERK-CDK9 interaction impacted on Ser 2 phosphorylation. To do so, nuclear ERK was transfected in parental and CDK9-deficient 293T cells and observed that nuclear ERK was capable of evoking CTD phosphorylation in the normal cells, but not in those where CDK9 was down-regulated by siRNA expression (figure 4.12).

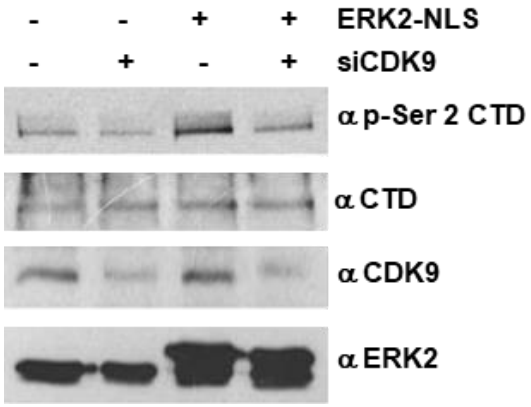


Figure 4.12. Role of CDK9 on nuclear ERK2-evoked CTD phosphorylation. Pol II CTD Ser 2 phosphorylation was measured in starved 293T cells transfected with ERK2-NLS (1μg) where indicated (+), in which CDK9 expression was down-regulated using siRNAs where specified (+).

With the purpose of corroborate this finding, we asked whether ERK2 could be potentiating CDK9 kinase activity. Consequently, an in vitro CDK9 kinase assay was performed, in which we tested the ability of CDK9/cyclin K to phosphorylate CTD, in the presence of ERK2 or its aforementioned mutants. All ERK2 constructs were immunoprecipitated from transfected proliferating 293T cells, and then incubated with CDK9/cyclin K, together with radioactive labelled ATP (γ-32P). It was found that incubation of CDK9 with ERK2, or its mutant forms, did not affect CDK9 capacity for phosphorylating CTD (Figure 4.13) indicating that, at least in vitro, ERK does not stimulate CDK9 kinase activity.

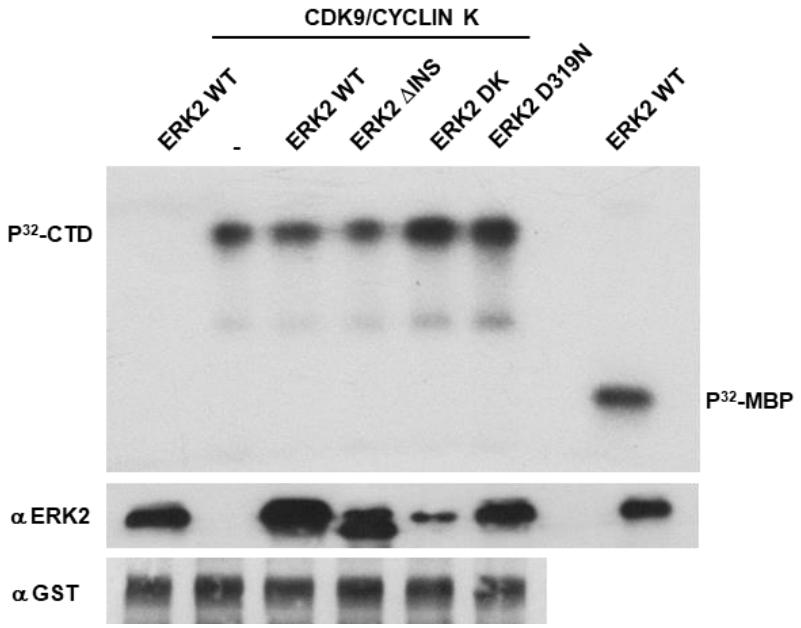


Figure 4.13. Effect of ERK2 on CDK9 kinase activity in vitro. In vitro kinase assay using purified ERK mutant forms in presence of CDK9/Cyclin K complex. GST-CTD and myelin basic protein (MBP) were used as substrates.

4.3. Role of ERK2-CDK9 interaction in the regulation of MYC expression

After characterizing the functional link between ERK and CDK9, we asked if such interaction was required for *MYC* gene expression. To begin with, we asked if ERK required CDK9 interaction in order to induce MYC expression. To do so, MYC expression was analyzed in serum deprived 293T cells expressing nuclear ERK2 and co-transfected with a siRNA for CDK9. As observed in figure 4.14, the partial silencing of CDK9 was sufficient to impede the upregulation of MYC mRNA and protein, as induced by nuclear ERK2 (figure 4.14 B).

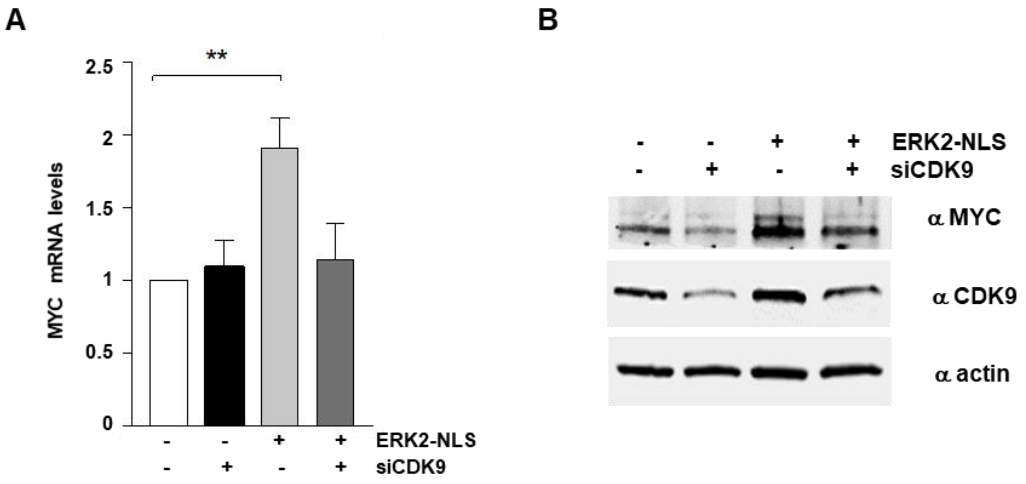


Figure 4.14. Role of CDK9 on nuclear ERK2-induced MYC regulation. A) *MYC* mRNA levels in quiescent 293T cells co-transfected with 1 µg of ERK-NLS and CDK9 siRNAs were indicated (+), measured by RT-qPCR. Data shows mean ± SEM from 3 independent experiments. B) Protein levels corresponding to one of the transfections performed, showing α CDK9 and MYC antibodies. Actin was used as loading control.

Similarly, the upregulation of MYC mRNA levels in HeLa cells in response to EGF treatment was significantly reduced when CDK9 was silenced by means of an siRNA (figure 4.15), further demonstrating the requirement of CDK9 for mitogen-driven MYC expression.

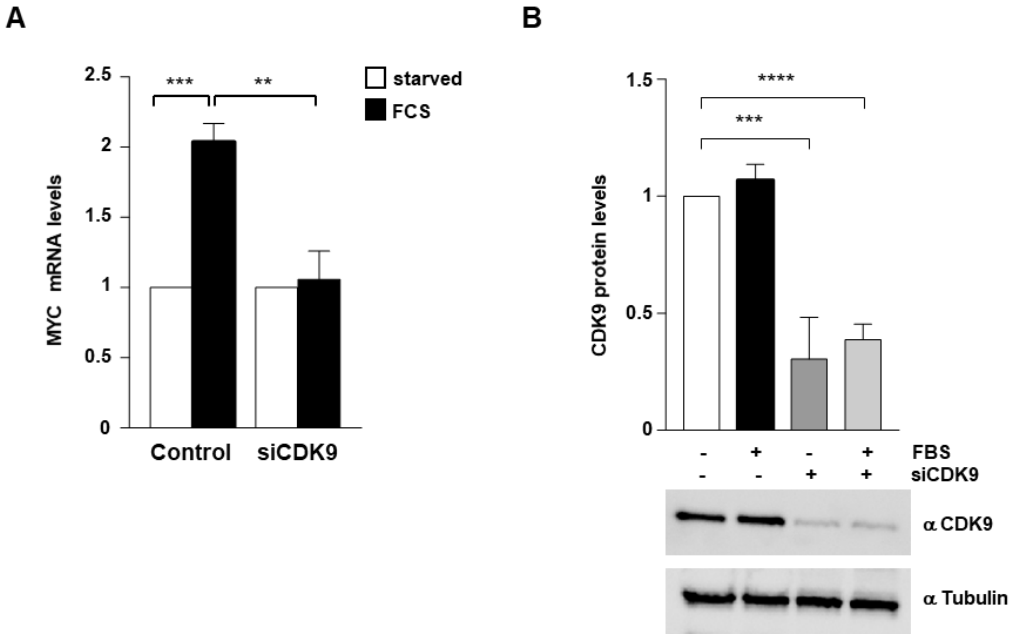


Figure 4.15. Effects of CDK9 silencing on MYC regulation under mitogenic stimulation. A) *MYC* mRNA levels in HeLa cells transfected with CDK9 siRNAs in starved cells or stimulated 45 min with FBS, measured by RT-qPCR. Data shows mean ± SEM from 4 independent experiments. B) Quantification of CDK9 protein levels from 4 different experiments. Tubulin was used for normalization and loading control.

To further substantiate the importance of ERK2-CDK9 interaction for MYC expression CDK9 was stably silenced in 293T by means of lentivirus-transduced shRNAs. In that stable cell line, we tested the extent to which MYC expression could be rescued by the re-expression of CDK9 ERK-binding mutants. We observed that the expression of an ectopic CDK9 wild-type, restored MYC protein expression. This was also the case when cells were transfected with the CDK9 FM mutant. However, when cells were transfected with the CDK9 FDM mutant, unable to interact with ERK2, MYC protein levels remained low (figure 4.16). Pointing to the necessity of a direct interaction between ERK2 and CDK9 in the regulation on MYC expression.

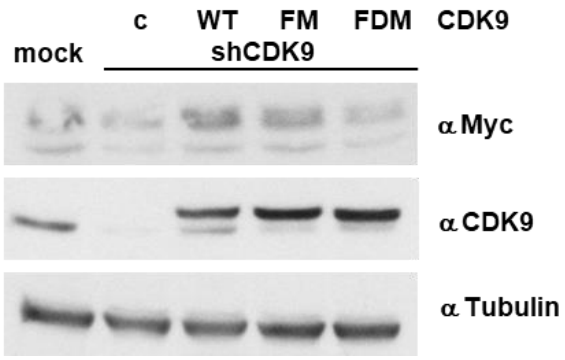


Figure 4.16. Effects of CDK9 binding mutants on MYC expression. 293T stable cell line generated with puromycin selection that have been infected with lentivirus containing shRNAs for CDK9. Then, CDK9 WT, FM and FDM constructs were transfected (1 µg each). Tubulin was used as loading control. The results were reproduced in two independent experiments.

4.3.1. Effect of a chimera with ERK2 insert region fused to CDK9

As previously highlighted, previous results from Dr Javier Leon's laboratory had observed by the use of ChIP assays in 293T and HeLa cells, that ERK2 and CDK9 coincided on the *MYC* promoter around the 1kb region. Such region is essential to drive MYC transcription (figure 4.17, published in Andrea Quintanilla's thesis).

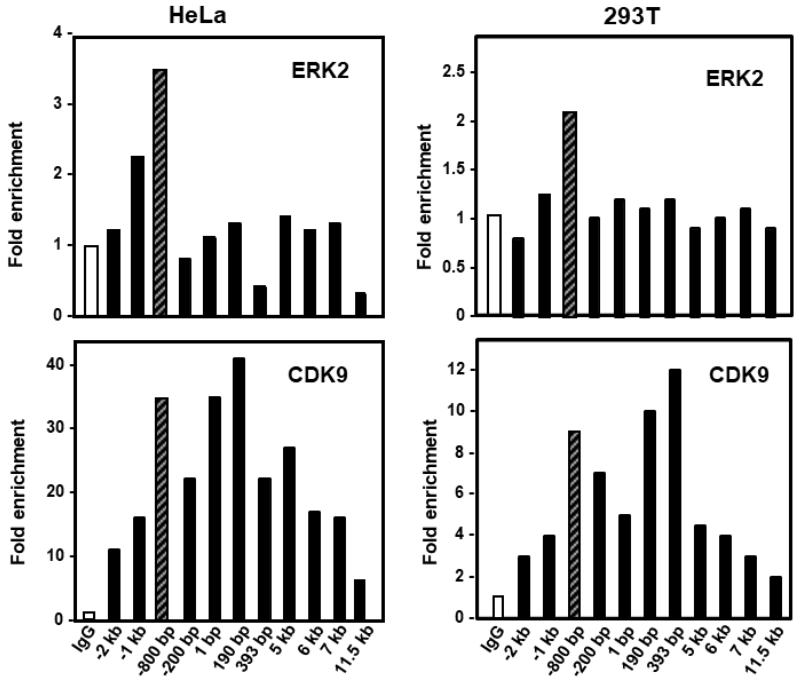


Figure 4.17. ERK2 and CDK9 coincidence at the MYC promoter. ChIP using anti-CDK9 and anti-ERK2 antibodies on the *MYC* gene of HeLa and 293T cells. The immunoprecipitated DNA was subjected to PCR for the indicated regions of *MYC* and the results are expressed as fold enrichment above the signal measured with control anti- IgGs. Figure from Andrea Quintanilla's thesis.

Based on previous data that indicates that ERK2 can bind to active promoter of genes by means of residues present in its insert region (Hu et al., 2009), and our previous observations pointing to ERK2 insert region as an essential region for driving *MYC* expression, we hypothesized that in order to drive MYC expression ERK could be serving as a tether for CDK9 at the MYC promoter by binding to DNA through its insert region and to CDK9 via its CD domain.

To put this theory into test, we generated a chimeric construct were the sequence corresponding to the ERK2 insert region was fused to the C-terminus of human CDK9 (CDK9-INS). In order to assess the effect of this chimera, luciferase assays were performed in which the ability of CDK9 wild-type was compared with CDK9-INS for driving the expression of a MYC promoter reporter in quiescent 293T cells. We found that, unlike wild-type CDK9, the addition of ERK2 insert region to CDK9 was sufficient to bolster its ability for inducing MYC expression to levels similar to those elicited by nuclear ERK. Overall, all of the foregoing findings show that ERK2 elicits MYC expression by promoting CDK9 recruitment to the *MYC* promoter, acting as a kinase-independent tether.

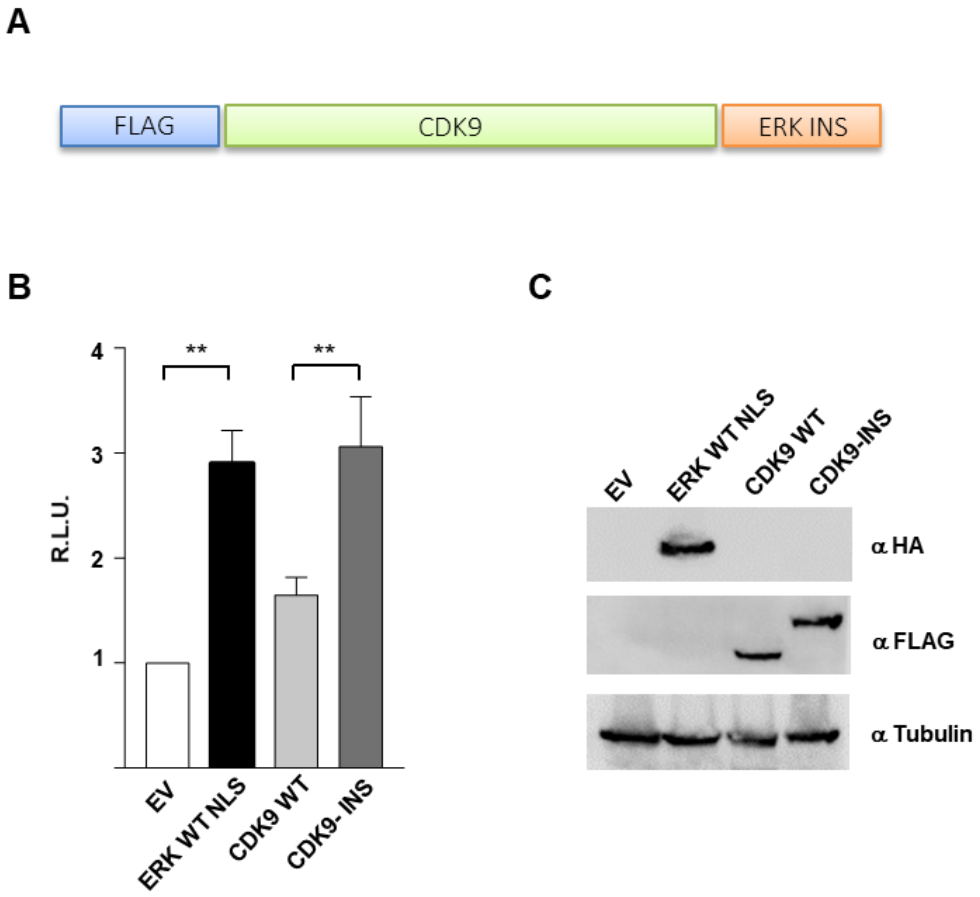


Figure 4.18. Regulation of the MYC promoter by CDK9-INS. A) Schematic representation of the construct FLAG CDK9-INS. B) Luciferase expression under the regulation of the *MYC* promoter in response to the indicated constructs (1µg each) in starved 293T cells. Results are expressed in relative luciferase units (R.L.U.). Data shows mean ± SEM from 4 independent experiments. C) Expression levels of the HA-tagged ERK and FLAG-tagged CDK9 constructs. Tubulin was used as loading control. EV= empty vector.

5. DISCUSSION

During the last thirty years the regulation of MYC expression has been widely studied due to its importance in cellular transformation and its role in critical cellular processes. It has been well established that *MYC* synthesis is induced by growth factor stimulation and, on the contrary, *MYC* mRNA levels remain low in growth-arrested cells (Müller et al., 1984). One of the main pathways implicated in the regulation of MYC expression is the Ras-ERK pathway. Over the years, data has been gathered regarding the role that different components of this pathway play in such regulation. A direct effect of this pathway on MYC induction was discovered by Kerkhoff and collaborators when they observed an effect on *MYC* expression with an inducible Raf-1 cell line (Eugen Kerkhoff et al., 1998). However, the mechanism by which this regulation occurred remained obscure. Later on, others demonstrated that MYC protein stability is directly regulated by the Ras-ERK pathway, throughout the phosphorylation of Ser 62 by ERK, following either Ras activation or serum stimulation (Sears et al., 2000a). Recently, it has been discovered that LIM kinase 1 (LIMK1), a serine/threonine and tyrosine kinase, interacts with phosphorylated ERK, which facilitates its nuclear shuttling (Pan et al., 2021). Once in the nucleus, LIMK1 could bind to the promoter region of *c-MYC* and stimulate c-MYC transcription (Pan et al., 2021). Nevertheless, all of the aforementioned effects have been connected to ERK kinase activity via transcription factors associated with the activation of the pathway. For instance, within the *MYC* promoter, there are binding sites for the Ets family transcription factors (Roussel et al., 1994) which are commonly activated by the Ras-ERK pathway (McCarthy et al., 1997); among other transcription factors such as TCF, SP1, AP1 also known to function downstream of this pathway (Yoon & Seger, 2006).

Besides its capability to phosphorylate and activate different nuclear and cytoplasmic targets, ERK can also exert different functions in a kinase-independent manner. Some of which vary from chromatin remodeling to DNA transcription or cell cycle regulation (Rodríguez & Crespo, 2011). Naturally, in order to translocate to the nucleus ERK must be phosphorylated. However, most of the previous reports about kinase-independent functions of ERK occur in the nucleus. Therefore, ERK kinase-independent activities, like transcriptional activation, could be sustained long after phosphatases come into play, implying that, for a total switch-off of ERK nuclear functions, there must be an active mechanism for re-shuttling ERK to the cytoplasm.

Regarding ERK kinase-independent activities, Hu and collaborators unveiled a novel ERK kinase-independent function in DNA regulation, through direct binding to DNA. Interestingly, they observed that ERK2 could bind to DNA thought the consensus sequence G/CAAAG/C (Hu et al., 2009). Based on that, our collaborators in Dr. Javier León's laboratory have unveiled that within the *MYC* promoter there are several ERK-Boxes with the sequence C/GAAAC/G that are highly conserved in the human, mouse and rat genes. Moreover, using Chromatin Immunoprecipitation assays (ChIP) they observed that ERK2 binds to the human and mouse *MYC* promoters and that CDK9 and ERK2 coincide in the same regions within the *MYC* promoter (results published in Andrea Quintanilla's thesis).

In this work, we have demonstrated a direct effect of ERK on *MYC* expression, by the use of MEK and ERK inhibitors, resulting on the down-regulation of mitogen-induced MYC mRNA and protein levels. We have also observed that inducible ERK1/2 genetic knock-out in MEFs also entails a dramatic reduction on MYC expression. Moreover, we have determined that ERK2 with a nuclear signal localization, that makes it constitutively nuclear-bound, is able to up-regulate *MYC* mRNA levels in serum-deprived conditions. This result has been corroborated by using a phosphomimetic ERK mutant form in which the two Ser residues in the SPS domain have been replaced with Glu resulting in a constitutive nuclear localization (Chuderland et al., 2008).

Active ERK exercise its functions over many targets, many of which are transcription factors like c-fos or Elk-1 (Murphy et al., 2002; Yoon & Seger, 2006). Thus, we have investigated to which extent the effect that we were seeing in *MYC* regulation was a consequence of ERK-related transcriptional events, such as the activation of transcription factors. We have observed that when nuclear ERK2 induces MYC expression in quiescent cells, Elk-1 remains unphosphorylated. In this respect, it is worth mentioning that in Andrea Quintanilla's thesis the ERK boxes at the MYC promoter that were critical for *MYC* induction by nuclear ERK2 were identified. She found that the fragment containing two conserved ERK boxes mapping at -1 Kb was sufficient for full activation of the *MYC* promoter by nuclear ERK2 under quiescence conditions. Noticeably, data released by the ENCODE project (www.encodeproject.org) describes a binding site for ELK1 within this region, suggesting

that ERK-driven MYC expression is independent of ERK-responsive transcription factors such as ELK-1. The fact that the upregulation observed in *MYC* synthesis by nuclear ERK occurred in quiescent conditions, suggests that the effect that we were seeing could be due to an ERK2 kinase-independent mechanism. Indeed, we have observed that mutant forms lacking phospho-transfer activity are as competent as the wild-type protein for driving *MYC* expression, probably by acting directly on the *MYC* promoter.

In this line, we have observed that an ERK2 mutant form of lacking its insert region (residues 241–272) (Whitehurst et al., 2004), is deficient for upregulating *MYC* expression under quiescence conditions. This finding is in full agreement with the data of Hu and colleagues that identify the residues responsible for ERK2 DNA binding as K259 and R261 (Hu et al., 2009), which are included in the insert region.

Previous results obtained in Dr. Javier León's lab indicate that ERK2 and CDK9 colocalize within the *MYC* promoter. In addition, by Proximity Ligation Assays they also observed an interaction between CDK9 and ERK2. CDK9 is a cyclin-kinase that, along with cyclin T or K compose the P-TEFb complex, which is responsible of phosphorylating the CTD of the RNA Pol II to promote transcription elongation (Kwak & Lis, 2013). Previous data from Fujita and colleagues had shown that active ERK up-regulated nuclear CDK9 and CDK9/cyclinT1 dimers, facilitating the recruitment of P-TEFb to genes like *c-fos* (Fujita et al., 2008). They assessed this effect by the use of the MEK inhibitor U0126 and did not investigate a direct interaction. Instead, we have found that ERK2 and CDK9 can interact directly, and that this interaction is specific for CDK9, as ERK2 cannot interact with other CDKs like CDK7 or CDK8. We have also observed that CDK9 can also associate with ERK1, and that neither ERK kinase activity nor being phosphorylated are requisites for such interaction, as an ERK2 kinase-deficient mutant K52R (DK) (M. J. Robinson et al., 1996) and an unphosphorylatable mutant (AEF) (Wolf et al., 2001) can interact with CDK9 as efficiently as wild-type ERK2. This is not the first time that an interaction between a MAPK and a CDK has been observed. Tomás-Loba and colleagues have described a binding interaction between p38γ and CDK2 and that this interaction cooperate in the induction of retinoblastoma phosphorylation and liver proliferation (Tomás-Loba et al., 2019).

Analyzing the ERK2-CDK9 interaction in further detail, we have observed that within the CDK9 sequence there are ERK docking motifs, both FXF and D- domains. Our results indicate that ERK2/CDK9 interaction takes place mainly through CDK9 D-domain binding to ERK2 CD domain; since the corresponding loss-of-function mutant forms, CDK9 FDM and ERK2 D316N, are defective in their mutual association. On the other hand, the disruption of CDK9 FXF domains does not prevent its binding to ERK, even though it dramatically alters its subcellular localization from nuclear to cytoplasmic, through a mechanism that we have not elucidated. This is consistent with our results showing that unphosphorylatable ERK2 can still bind to CDK9, as the hydrophobic DEF/FRS pocket, the part of the insert region involved in binding to FXF domains, is formed only when ERK becomes dually phosphorylated (Roskoski, 2012).

We have also studied the functional consequences of ERK-CDK9 interaction. As previously highlighted, the phosphorylation of Ser 2 of RNA Pol II CTD is the hallmark of transcription elongation (Nick F. Marshall et al., 1996; X. Yang et al., 1996). We have observed that nuclear ERK2 enhances CTD phosphorylation in quiescent conditions, while in the absence of CDK9 this did not occur. This pointed to CDK9 as the kinase phosphorylating CTD Ser2 as evoked by ERK2. This is in line with the results obtained by Fujita and colleagues, showing that within the *c-fos* promoter ERK2 is incapable of directly phosphorylating Pol II CTD (Fujita et al., 2008). As previously revealed, CDK9 interacts not only with wild-type ERK2, but also with its kinase-deficient forms. In this respect, by the use of in vitro kinase assays we have determined that neither ERK2 wild-type nor its mutant forms affect CDK9 capacity for phosphorylating CTD. Also in line with previous findings showing that treatment with U0126 did not alter the catalytic activity of CDK9 (Fujita et al., 2008). Even though ERK does not influence CDK9 catalytic activity, the link between CDK9-ERK2 in the regulation of MYC expression becomes apparent by our experiments in which the partial silencing of CDK9 is sufficient to impede the upregulation of MYC mRNA and protein levels induced by nuclear ERK2. Likewise, we have observed that the upregulation of *MYC* mRNA levels evoked my mitogenic stimuli, is significantly reduced when CDK9 is silenced.

In light of all these data and considering that while the ERK2 ΔIns mutant is able to bind to CDK9, even though it cannot induce *MYC* synthesis, and since ERK2 binds to DNA through two residues, K259 and R261, which are located within its insert region, we propose a model in which, on one hand, ERK would associate to DNA through its insert region, and, on the other hand, it would bind to CDK9, via its CD domain. As such ERK would be acting as a tether that would anchor CDK9 to the MYC promoter and thereby, promoting RNA pol II phosphorylation and *MYC* expression (figure 5.1).

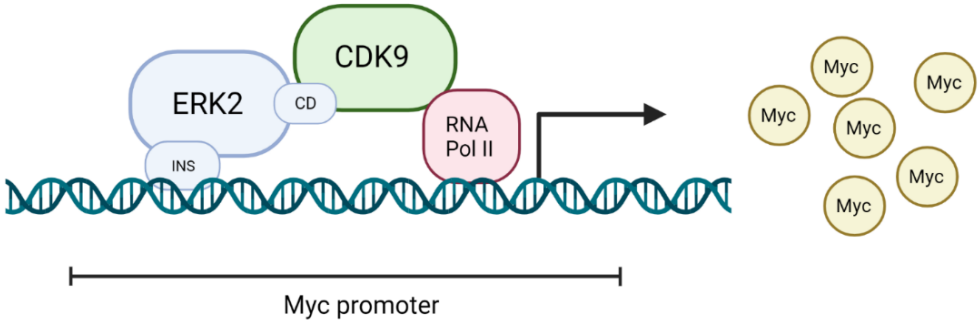


Figure 5.1. Proposed model for CDK9 recruitment to the *MYC* promoter by ERK. ERK attaches to DNA at the MYC promoter through its insert region, anchoring CDK9 therein by connecting with the CDK9 D-domain via the ERK CD-domain. CDK9 kinase activity is not activated by ERK, but it is brought closer to RNA Pol II, which boosts transcription. Generated with BioRender.

To put this theory into test, we generated a chimeric construct were the sequence corresponding to the ERK2 insert region was fused to the C-terminus of human CDK9. By luciferase assays we demonstrated that the addition of ERK2 insert region to wild-type CDK9 is sufficient to bolster the ability of CDK9 for inducing MYC expression under serum starvation conditions, which validated our hypothesis that ERK is serving as a tether for recruiting CDK9 to the MYC promoter.

Overall, data has been gathered for years pointing to ERK's role in gene transcription control by direct interaction with promoters and other regulatory regions. Our findings add to the growing body of data showing that ERK is involved in many cellular processes beyond its role as a kinase, confirming prior findings that kinase-inactive ERK mutants have the ability to bind DNA (McReynolds et al., 2016). Furthermore, evidence has accumulated that CDK9 may

be a therapeutic target in MYC-driven malignancies (Blake et al., 2019; Hashiguchi et al., 2019; C. H. Huang et al., 2014). In this regard, the data provided here support such findings by revealing CDK9's crucial role as a major regulator of MYC expression in conjunction with ERK.

6. CONCLUSIONS

1. ERK2 regulates MYC expression via its direct interaction with the *MYC* promoter, independently of other ERK-evoked transcriptional events.

2. Such mechanism is independent of ERK2 kinase activity

3. This mechanism requires ERK interaction with CDK9. In such complex, ERK2 interacts with CDK9 mainly thought the D-domain. Concomitantly, ERK2 interacts with the MYC promoter via its insert region.

4. ERK2 evokes *MYC* expression by recruiting CDK9 to the *MYC* promoter. While ERK2 does not enhance CDK9 kinase activity, it facilitates CDK9-mediated phosphorylation of RNA Pol II at serine 2 in the carboxy-terminal domain.

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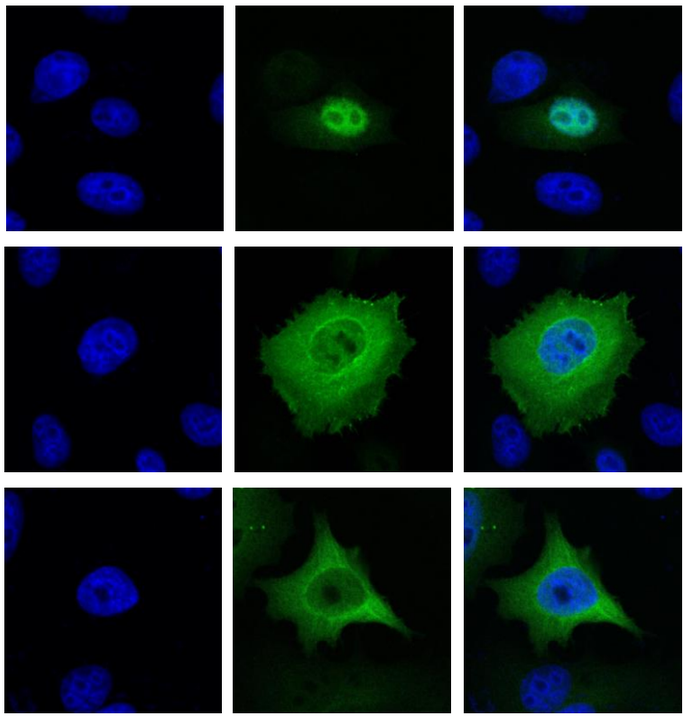
8. PUBLICATIONS

Zaballos MA, Acuña-Ruiz A, Morante M, Crespo P, Santisteban P. Regulators of the RAS-ERK pathway as therapeutic targets in thyroid cancer. Endocr Relat Cancer. 2019 Jun;26(6):R319-R344. doi: 10.1530/ERC-19-0098. PMID: 30978703. https://doi.org/10.1530/ERC-19-0098



En los últimos años se ha demostrado que ERK2 puede unirse directamente a promotores activos de genes y regular su expresión. En este trabajo hemos desvelado un nuevo mecanismo por el que ERK2 puede inducir la expresión de MYC, a través de su interacción directa con el promotor de *MYC*, de forma independiente a su actividad quinasa. Dicho mecanismo requiere la interacción de ERK2 con CDK9, cuya unión se da a través de su dominio D, y es independiente de la actividad quinasa ERK2. Como resultado, nuestros hallazgos revelan una función completamente nueva de ERK2 fuera de sus funciones típicas como una proteína quinasa, actuando como enlace para la activación transcripcional en el promotor *MYC*.

Over the last years, it has been demonstrated that ERK2 can directly bind to active promoter of genes and regulate their expression. In this work we have unveiled a new mechanism by which ERK2 can induce MYC expression, via its direct interaction with the *MYC* promoter, in a kinase-independent manner. Such mechanism requires ERK2 interaction with CDK9 via binding thought its D-domain, and is independent of ERK2 kinase activity. As a result, our findings reveal an entirely new function for ERK outside of its typical kinase functions, acting as a tether for transcriptional activation at the *MYC* promoter.

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