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REGULATION OF THE RP-MDM2-P53 PATHWAY BY SUMO

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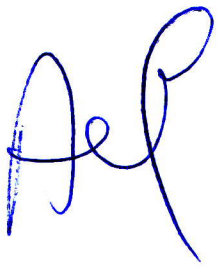

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D. Manuel Collado Rodríguez, investigador Miguel Servet del SERGAS e investigador principal del grupo "Células madre en cáncer y envejecimiento" del Instituto de Investigaciones Sanitarias de Santiago de Compostela,

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REGULATION OF THE RP-MDM2-P53 PATHWAY BY SUMO

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Declaro no tener ningún tipo de conflicto de intereses, ni ninguna relación económica, personal, política, interés financiero ni académico que pueda influir en este trabajo.

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ABBREVIATIONS

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ActD	Actinomycin D
ARF	Alternate reading frame protein product of the CDKN2A
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CDKN2A	cyclin-dependent kinase inhibitor 2A gene
CHX	Cycloheximide
DAPI	4′,6-diamidino-2-phenylindole
DBA	Diamond-Blackfan anemia
DMEM	Dulbecco´s modified Eagle´s medium
dNTP	deoxynucleotides triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GA	Ginkgolic acid
GFP	Green fluorescent protein
GFP-F	Farnesylated GFP
GST	Glutathione S-transferase
h	Hours
His	Histidine
IB	Western-blot
IF	Immunofluorescence
IP	Immunoprecipitation
IPTG	Isopropyl β-D-1-thiogalactopyranoside
K0	lysine less
kDa	Kilodalton
MDM2	Mouse double minute 2 homolog
min	Minutes
NAE	NEDD8 activating enzyme
NEDD8	Neural Precursor Cell Expressed, Developmentally Down-Regulated 8
NEDP	NEDD8-specific protease
NEM	N-Ethylmaleimide
NoLS	nucleolar localization signal

NPM	Nucleophosmin
OD	optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
RPL11	Ribosomal protein L11
RPL23	Ribosomal protein L23
rpm	round per minute
rRNA	Ribosomal RNA
SAE	SUMO activating enzyme
SDS-page	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SENP	Sentrin-specific protease
siC	scramble siRNA
siRNAs	Small interfering RNA
siUbc9	Ubc9 siRNA
SUMO	Small ubiquitin-like modifier
TOR	Target of Rapamycin
TTF-I	Transcription Termination Factor 1
U	unit
WCL	Whole protein extracts
WT	wild-type

SUMMARY

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La síntesis de ribosomas es un componente clave para regular la síntesis proteica general y el crecimiento celular, por lo que requiere una regulación estricta. La alteración de la síntesis de ribosomas puede ser causada por diversas razones tales como (i) el agotamiento de nutrientes (Bhat, Itahana, Jin y Zhang, 2004); (ii) agentes químicos o radiaciones que perturban la producción del ARN ribosómico (ARNr) o que inducen la degradación de proteínas ribosomales como la Actinomicina D, un fármaco anticancerígeno que a bajas concentraciones reprime la actividad de la ARN Polimerasa I y afecta a la transcripción del ARNr (Fumagalli et al., 2009; Golomb, Volarevic y Oren, 2014; X. Zhou, Liao, Liao, Liao y Lu, 2015; Bhat et al., 2004; Iapalucci-Espinoza & Franze -Fernández, 2018; Perry y Kelley, 2018) o (iii) a la deficiencia o fallo en el funcionamiento de algunas proteínas ribosómicas como resultado de alteraciones genéticas que generan estrés ribosómico (también llamado estrés nucleolar) (Daftuar, Zhu, Jacq y Prives, 2013; Fumagalli et al., 2009; Fumagalli, Ivanenkov, Teng y Thomas., 2012).

Las mutaciones más frecuentes de los genes asociados a la síntesis de ribosomas que causan estrés ribosómico son aquellas asociadas con mutaciones en genes de proteínas ribosómicas tales como RPL11, RPL5, RPL35A, RPS19, RPS24 y RPS17. Una de las enfermedades hereditarias causadas por mutación en alguna de estas proteínas es la anemia de Blackfan-Daimond (DBA) (Narla & Ebert, 2010), caracterizada por niveles elevados de p53. Estas patologías destacan la importancia de comprender el mecanismo molecular involucrado en la activación de p53. Además, las células tumorales necesitan una maquinaria ribosómica más activa en comparación con las células somáticas normales, lo que sugiere que pueden ser más sensibles al estrés ribosómico. Por lo tanto, la inducción de estrés ribosomal se ha propuesto como una estrategia terapéutica contra el cáncer (Brighenti, Treré, y Derenzini, 2015). En consecuencia, la caracterización de los mecanismos moleculares que regulan este proceso es fundamental para este objetivo.

El punto de control activado como respuesta a una alteración en la biogénesis de los ribosomas se basa en la capacidad que poseen algunas proteínas ribosómicas para translocarse del nucleolo al nucleoplasma donde pueden ejecutar sus funciones independientes del ribosoma (Fumagalli et al., 2009). Sin embargo, no está claro si todas estas proteínas ribosómicas tienen un papel esencial en la activación de la vía MDM2-p53 ante el estrés ribosómico ya que también se ha publicado que solo se requieren dos proteínas ribosómicas, RPL11 y RPS5, para

la activación de p53 en respuesta al estrés ribosomal (Fumagalli et al., 2012). La liberación de proteínas ribosómicas del nucleolo no es un proceso pasivo y, aunque se han identificado algunos reguladores, el mecanismo molecular implicado en la translocación del nucleolo al nucleoplasma de las proteínas ribosómicas no está claro.

La proteína ribosomal RPL11 es una de las proteínas ribosómicas más estudiadas debido a su conexión con la enfermedad hereditaria Daimond-Blackfan anemia (DBA) y las vías oncogénicas (Robledo et al., 2008). RPL11 es un componente de la subunidad 60S pero, además de la función ribosomal, RPL11 libre de ribosomas juega un papel importante en la respuesta celular frente a diferentes tipos de estrés como el estrés ribosomal (Fumagalli et al., 2009, Havel, Li, Cheng, Peng, y Fu, 2015; Lohrum et al., 2003; Y. Zhang et al., 2003) o el estrés oncogénico (Nishimura et al., 2015). Cuando las células están expuestas a dicho estrés, RPL11 se transloca del nucleolo al nucleoplasma. Aquí, la proteína RPL11 ya no forma parte de los ribosomas y se une a MDM2 (Mouse double minute 2), inhibiendo su capacidad de poliubiquitinar p53, induciendo, por tanto, la estabilización y activación de p53 (Dai y Lu, 2004; Lohrum et al., 2003). Por ello, RPL11 se considera un supresor tumoral, lo que explica que los pacientes afectados por DBA tengan una propensión a desarrollar tumores (Fumagalli & Thomas, 2011).

La acumulación de RPL11 en el nucleoplasma es el mecanismo clave para la inactivación de MDM2 y la activación de p53 (Kruse & Gu, 2009; Marine y Lozano, 2009). Se han identificado varios reguladores de la translocación de RPL11 del nucleolo al nucleoplasma y entre ellos se encuentra la conjugación de RPL11 a NEDD8 que estabiliza RPL11 y retiene RPL11 dentro del nucleolo (Sundqvist, Liu, Mirsaliotis, y Xirodimas, 2009). Por el contrario, ARF parece favorecer la interacción de RPL11 con MDM2 y de esta forma contribuye a la activación de p53 inducida por ARF y a la inducción de arresto del ciclo celular (Dai et al., 2012), un punto de conexión entre el estrés oncogénico y el estrés ribosómico.

p53 es el gen más frecuentemente mutado en cáncer (Hainaut y Hollstein, 1999; Vogelstein, Lane y Levine, 2000). Este supresor de tumores, también llamado "el guardián del genoma" (Lane, 1992), es un regulador transcripcional que coordina las respuestas celulares a distintos tipos de estrés (Marchenko y Moll, 2007; Vogelstein et al., 2000; Vousden y Lane, 2007). La regulación de p53 es un proceso complejo. En las células normales, la proteína p53 se mantiene en niveles bajos gracias principalmente a la actividad de MDM2, la ubiquitina ligasa de p53 que induce la ubiquitinación y degradación de la proteína (Haupt, 1997; Reiko

Honda, Tanaka y Yasuda, 1998; Kubbutat, Ludwig, Ashcroft, & Vousden, 1998). En respuesta a distintos tipos de estrés, p53 se estabiliza y se activa por un mecanismo dependiente de estímulo (Hofseth et al., 2004; Manfredi, 2003; Meek, 2004; Riley et al., 2008; Sengupta & Harris, 2005; Vogelstein et al., 2000; Vousden & Lane, 2007; Vousden y Lu, 2002). En el caso de estrés ribosómico, p53 se estabiliza debido a la inhibición de la ubiquitinación y degradación de p53 mediada por MDM2 (Dai et al., 2004; Donati, Peddigari, Mercer, y Thomas, 2013; Fumagalli et al., 2009, 2012; Golomb et al. al., 2014; Narla y Ebert, 2010; X. Zhou et al., 2015). Además de ubiquitinarse, p53 puede regularse a través de su conjugación a otras proteínas de tipo ubiquitina, como SUMO (Santiago, Li, Zhao, Godsey y Liao, 2013) o NEDD8 (Xirodimas, Saville, Bourdon, Hay y Lane., 2004).

Como mencionamos anteriormente, MDM2 es la ubiquitina ligasa de p53 encargada de inducir su poliubiquitinación y degradación (Kubbutat et al., 1998). Además de la ubiquitinación de p53, MDM2 también puede inducir la conjugación de p53 con otras proteínas de tipo ubiquitina, como NEDD8 (Oliner, Kinzler, Meltzer, George y Vogelstein, 1992) y SUMO (Stindt, Carter, Vigneron, Ryan y Vousden, 2011). Su actividad se regula a muchos niveles. Por un lado, MDM2 es una diana transcripcional de p53, un mecanismo que favorece el control de la activación de p53. MDM2 puede también regularse a través de modificaciones post-traduccionales, como la acetilación (X. Wang, Taplick, Geva y Oren, 2004) o SUMOilación (Xirodimas, Chisholm, Desterro, Lane y Hay, 2002). Además, la actividad de MDM2 está regulada por el supresor tumoral ARF. ARF interactúa con MDM2 e inhibe el control de MDM2 sobre p53 (Pomerantz et al., 1998; JD Weber, Taylor, Roussel, Sherr, & Bar-Sagi, 1999; Y. Zhang, Xiong, y Yarbrough, 1998).

El supresor tumoral ARF es uno de los productos de transcripción del gen CDKN2A (inhibidor de quinasa dependiente de ciclina 2A) y es la segunda proteína supresora de tumores mutada con mayor frecuencia en cáncer (Sharpless y Depinho, 1999; Sherr, 2001).

La proteína nucleolar ARF se regula a través de mecanismos transcripcionales y post-traduccionales que pueden diferir dependiendo del origen de la proteína. Así, la expresión de la proteína ARF de ratón se induce por estrés oncogénico, como por ejemplo tras la sobreexpresión de los oncogenes Ras, c- myc o E2F1 (Delin Chen et al., 2010; Palmero, Murga, Zubiaga y Serrano, 2002). Sin embargo, la transactivación de la proteína ARF humana tiene lugar en respuesta a sobreexpresión de c-myc, radiación o estrés genotóxico (Delin Chen et al., 2013; Lo et al., 2015). A pesar de que la proteína ARF humana carece de residuos de lisina (la

proteína de ratón tiene una), puede ser poliubiquitinada en su región N-terminal y esta ubiquitinación conduce a su degradación (Kuo, den Besten, Bertwistle, Roussel, & Sherr, 2004).

ARF funciona como un sensor de diferentes tipos de estrés como por ejemplo el estrés oncogénico (Delin Chen, Shan, Zhu, Qin y Gu, 2010; Gjerset, 2006; Liontos, Pateras, y Gorgoulis, 2012; Matheu, Maraver, y Serrano, 2008; Olson, 2004). Su activación puede conducir a la parada del ciclo celular, inducción de apoptosis o senescencia, a través de vías moleculares dependientes o independientes de p53. Un mecanismo molecular por el cual ARF activa p53 es a través de la interacción con MDM2 bloqueando la ubiquitinación mediada por MDM2 y la degradación de p53 (Y. Zhang et al., 1998). Además ARF puede inducir la estabilización de p53 mediante la relocalización de MDM2 dentro del nucleolo (JD Weber et al., 1999). Una de las actividades de ARF independientes de p53 es su capacidad de inducir la SUMOilación de proteínas con las que interacciona como por ejemplo p53 (L. Chen y Chen, 2003), MDM2 o nucleofosmina (Xirodimas et al., 2002). El mecanismo molecular por el cual ARF induce SUMOilación es desconocido hasta ahora. Sin embargo, dado que ARF interactúa con Ubc9 (Helen Rizos et al., 2005), se ha propuesto que ARF podría facilitar la interacción con Ubc9 y la transferencia de SUMO a las proteínas de unión a ARF.

SUMO es una proteína pequeña de 11 kDa con una estructura similar a la ubiquitina (Chan et al., 2008; Rabut y Peter, 2008; Xirodimas et al., 2008). A pesar de que se han descrito, hasta el momento, cinco isoformas de SUMO (SUMO1, 2, 3, 4 y 5), las isoformas que mejor se conocen son SUMO1, 2, y 3. La secuencia de aminoácidos de SUMO2 y SUMO3 es casi idéntica, mientras que su homología con SUMO1 es de alrededor del 45%. SUMO modifica la proteína sustrato a través de una interacción covalente reversible, también llamada SUMOilación, un proceso enzimático similar a la ubiquitinación. Las proteínas SUMO se sintetizan como precursores inmaduros que con activados mediante la acción de peptidasas específicas de SUMO (SENP). Las proteínas SUMO maduras se activan después a través de la acción de la enzima heterodímera E1 SAE1/SAE2 de una manera dependiente de ATP. El SUMO activado se transfiere luego a la cisteína del sitio activo de la enzima de conjugación E2, Ubc9. Finalmente, SUMO se une a un residuo de lisina específico localizado en la proteína diana, un proceso que generalmente requiere una E3 SUMO ligasa (Wilkinson y Henley, 2010).

La SUMOilación modula las interacciones proteína-proteína lo que puede resultar en una regulación de la localización, estabilidad o actividad subcelular del sustrato. La

modificación por SUMO de numerosas proteínas celulares convierten esta modificación en un mecanismo crucial para el control de la progresión del ciclo celular, apoptosis, respuesta inmune, reparación del daño en el ADN, etc y una alteración en los procesos de SUMOilación se correlaciona con diversas enfermedades tales como el cáncer (de la Cruz-Herrera et al., 2014; Domingues et al., 2015; Enserink, 2015; C. Guo & Henley, 2014; Saitoh & Hinchey, 2000; Šramko, Markus, Kabát, Wolff, y Bies, 2006; W. Zhou, Ryan y Zhou, 2004). Varios sustratos de SUMO tienen un papel relevante en la respuesta celular a diferentes tipos de estrés, incluido el estrés ribosómico, aunque su regulación es muy compleja. Así, se ha descrito que tanto dos moduladores positivos de p53 como son RPL11, ARF como el modulador negativo de p53 MDM2, inducen la SUMOilación de p53 (Stindt et al., 2011). Además, ARF también puede inducir la SUMOilación de MDM2 (L. Chen y Chen, 2003; Xirodimas et al., 2002).

Otra proteína tipo ubiquitina, la proteína NEDD8 también juegan un papel relevante en la activación de p53 en respuesta a distintos tipos de estrés. NEDD8 es una proteína que comparte un 60% de identidad de secuencia de aminoácidos con ubiquitina (Kumar, Yoshida y Noda, 1993) y la modificación de un sustrato por NEDD8 o NEDDilación es un proceso similar a la ubiquitinación. La proteína NEDD8 se activa por la acción de una enzima activadora de NEDD8 E1 (heterodímero UBA3-APPB1) a través de un proceso dependiente de ATP. NEDD8 activado es transferido a la enzima conjugadora de NEDD8 E2 o UBC12 y finalmente la E3 ligasa de NEDD8 transfiere NEDD8 al sustrato mediante un enlace covalente isopeptídico entre el extremo carboxilo del último residuo de glicina en la proteína NEDD8 y el aceptor ε amino de un residuo de lisina localizado en la proteína diana (Rabut y Peter, 2008). Hasta la actualidad se han identificado muy pocos sustratos de NEDD8. NEDD8 se conjuga a la proteína culina, núcleo del complejo "Cullin-RING ubiquitin ligase (CRL)", responsable de la ubiquitinación de varias proteínas con un papel importante en cáncer (Deshaies, Emberley y Saha, 2010). Además, también se ha demostrado la conjugación de NEDD8 a proteínas ribosómicas (Chan et al., 2008; Rabut & Peter, 2008; Xirodimas et al., 2008). Otro sustrato de NEDD8 es p53, conjugación que inhibe su actividad transcripcional (Xirodimas et al., 2004). De hecho, se ha descrito que MDM2 actúa como una E3 ligasa de NEDD8 promoviendo la NEDDilación de p53, otro mecanismo a través del cual MDM2 inhibe la actividad de p53 (Xirodimas et al., 2004). En los últimos años, se ha propuesto la inhibición de NEDD8 como una estrategia terapéutica contra el cáncer. Se han descubierto varios inhibidores de la NEDDilación, y la actividad de algunos de ellos como el compuesto MLN4924 están siendo evaluados en ensayos clínicos (Deshaies et al., 2010).

El objetivo de este estudio es evaluar si RPL11 se regula a través de la conjugación de SUMO y cómo esta modificación afecta a la vía RP-MDM2-p53.

Nuestros resultados demuestran que RPL11 se modifica por SUMO1 y SUMO2 *in vitro* e *in vivo*. Intentamos crear un mutante de RPL11 incapaz de ser SUMOilado con el fin de determinar la función de la SUMOilación de RPL11. Sin embargo, nuestros resultados revelaron que una proteína RPL11 en la que se habían mutado todos los residuos de lisina a arginina, seguía SUMOilándose, lo que sugiere la existencia de un nuevo mecanismo de SUMOilación, independiente de la presencia de lisinas en el sustrato. Ya se ha descrito previamente procesos de conjugación de ubiquitina de forma independiente de residuos de lisina (Kuo, den Besten, Bertwistle, Roussel y Sherr, 2004); sin embargo, hasta ahora, esta es la primera descripción de una conjugación de SUMO de forma independiente de residuos de lisina.

Previamente se había demostrado que la proteína RPL11 puede regularse a través de su conjugación con la proteína NEDD8 y que se requiere la mutación de todos los residuos de lisina en RPL11 para detectar una reducción en la NEDDilación de la proteína (Sundqvist et al., 2009). De hecho, en este trabajo observamos que, al igual que ocurre con SUMO, NEDD8 se conjuga a la proteína RPL11 carente de residuos de lisina. Por lo tanto, especulamos que NEDD8 y SUMO podrían competir por la conjugación a RPL11. Los experimentos de competencia sobreexpresando SUMO y NEDD8, así como el tratamiento con inhibidores de NEDDilación o de SUMOilación, revelaron que SUMO regula negativamente la NEDDilación de RPL11. También observamos que la SUMOilación de RPL11 se veía favorecida por la inhibición de NEDDilación, lo que sugiere que NEDD8 también regula negativamente la SUMOilación de RPL11. Sin embargo, no observamos una clara inhibición de la SUMOilación de RPL11 tras la sobreexpresión de NEDD8. Dado que la sobreexpresión de NEDD8 estabiliza y promueve la localización de RPL11 en el nucleolo, estos resultados pueden deberse a la estabilización de RPL11 o a que su retención en el nucleolo favorezca la SUMOilación de RPL11. La competencia entre SUMO y NEDD8 para conjugarse a RPL11 podría ser una competencia directa por unirse a los mismos residuos de RPL11 o podría ser un efecto más general, como por ejemplo una modulación del proceso de NEDDilación por SUMO o de SUMOilación por NEDD8. Nuestros resultados revelaron que SUMO y NEDD8 ya no competían para unirse al mutante de RPL11 en el que se había mutado todas las lisinas, lo que sugiere que existe una competencia entre SUMO y NEDD8 para unirse a las mismas lisinas en

RPL11, aunque no se puede descartar que también exista una interacción SUMO-NEDD8 más compleja.

Previamente se había demostrado que NEDD8 retiene a RPL11 dentro del nucleolo en células no sometidas a estrés y que en respuesta al estrés ribosómico RPL11 se deconjuga de NEDD8 y se desplaza fuera del nucleolo (Sundqvist et al., 2009). Nuestros resultados indicaron que el estrés ribosómico promueve la modificación de RPL11 por SUMO2, lo que de nuevo apoya la existencia de una competición entre ambas modificaciones. Además, también observamos que la sobreexpresión de SUMO2 induce la translocación de RPL11 del nucleolo al nucleoplasma, de acuerdo con una relación antagonista entre NEDDilación y SUMOilación de RPL11. Asimismo, observamos que la enzima conjugadora de SUMO Ubc9 era necesaria para la estabilización y activación de p53 en respuesta a la sobreexpresión de RPL11. En conjunto estos resultados nos llevan a proponer que la SUMOilación de RPL11 favorece su salida del nucleolo, su inhibición de MDM2 y la activación de p53.

Aunque RPL11 se conoce principalmente como una proteína clave en el control de la activación de p53 en respuesta al estrés ribosomal, existen estudios que demuestran que RPL11 también es necesaria para la activación de p53 en respuesta al estrés oncogénico (Nishimura et al., 2015) y para la activación de p53 por ARF (Dai et al., 2012). Se desconocen los mecanismos moleculares subyacentes a la activación de p53 mediada por RPL11 en respuesta al estrés oncogénico. Una hipótesis propuesta es que el aumento en los niveles de ARF resultante del estrés oncogénico, induce estrés ribosómico que resulta en la inhibición de MDM2 a través de RPL11 (Dai et al., 2012; Nishimura et al., 2015). La demostración de que RPL11 se regulaba a través de su conjugación a SUMO nos llevó a evaluar la posibilidad de que SUMO tuviera un papel en la activación de p53 mediada por RPL11 en respuesta al estrés oncogénico. Numeroso artículos han demostrado la capacidad de ARF para inducir la SUMOilación de proteínas celulares con las que ARF interacciona y para promover la conjugación global de SUMO (Alagu et al., 2018, Tago, Chiocca, y Sherr, 2005; Wang et al., 2015). Nuestros resultados demuestran que RPL11 es otra de las proteínas cuya SUMOilación se ve favorecida por ARF. Estos resultados nos llevan a proponer que la promoción de la SUMOilación de RPL11 por ARF, que a su vez conduce a la translocación de RPL11 al nucleoplasma, podría servir de enlace molecular entre el estrés oncogénico y la activación de p53.

La identificación de SUMO como un regulador de RPL11 nos llevó a preguntarnos si esta modificación podría estar regulando a otras proteínas ribosomales. En este caso estudiamos

la proteína RPL23, una proteína ribosomal capaz de interaccionar con MDM2 y de activar p53 en respuesta a RAS (Meng et al., 2016), a pesar de que existe cierta controversia acerca de su papel en respuesta al estrés ribosomal (Dai y Lu, 2004; Dai et al., 2004; Jin, Itahana, O'Keefe, Y Zhang, 2004). Nuestros resultados demostraron que RPL23 también puede ser modificado por SUMO y por NEDD8. Además, los experimentos de competencia revelaron que SUMO regula negativamente la NEDDilación de RPL23, como se observó para RPL11. Además, mostramos que ARF regula negativamente la NEDDilación de RPL23 mientras que aumenta su SUMOilación. No conocemos el efecto de SUMO ni de la conjugación de NEDD8 en RPL23. Sin embargo, la evaluación de la localización subcelular de la proteína después de la sobreexpresión de SUMO reveló que SUMO promueve la translocación de RPL23 al nucleoplasma, como ocurre con RPL11. Si esta translocación conduce a la activación de p53 y si esta modificación se ve modulada en respuesta a distintos tipos de estrés son cuestiones que requieren de nuevos estudios.

El mecanismo por el cual ARF aumenta la SUMOilación no se conoce claramente. El estudio de la relación entre NEDD8 y SUMO nos llevó a evaluar si ARF tenía algún efecto sobre la NEDDilación de RPL11. Nuestros resultados mostraron que ARF no sólo modulaba negativamente la NEDDilación de RPL11 sino que también ejercía un efecto negativo sobre la NEDDilación de RPL23 y sobre la conjugación de NEDD8 global, lo que se correlaciona con el aumento de SUMOilación, pero si el aumento en la SUMOilación y la disminución en la NEDDilación inducidos por ARF están relacionados no lo sabemos. Para conocer mejor la interacción entre estas dos modificaciones post-traduccionales, decidimos estudiar la posible existencia de cadenas mixtas SUMO-NEDD8. No pudimos detectar ninguna NEDDilación de la proteína SUMO o de Ubc9. Sin embargo, observamos que SUMO tiene un efecto sobre NEDD8. Si este efecto consiste en la SUMOilación de NEDD8 o si SUMO promueve la formación de cadenas de NEDD8 está aún sin resolver. Nuestros resultados también revelaron que el tratamiento con el inhibidor de la NEDDilación MLN4924 aumenta los niveles de Ubc9 y la SUMOilación global, lo que nos lleva a plantear la hipótesis de que SUMO podría estar implicado en la actividad antitumoral ejercida por este agente quimioterápico.

Se ha propuesto que ARF podría estar mejorando la SUMOilación de sus interactores gracias a su capacidad de promover la interacción de los mismos con Ubc9 (Rizos, Woodruff y Kefford, 2005). La interacción de ARF con Ubc9 y nuestros resultados que revelan que SUMO puede conjugarse a residuos que no sean lisina nos llevaron a plantear la hipótesis de

que la proteína ARF pueda ser un sustrato de SUMO. La evaluación de dicha hipótesis reveló que, de hecho, ARF puede SUMOilarse *in vitro* e *in vivo.* Estudiar las consecuencias de dicha SUMOilation será uno de nuestros principales objetivos en el futuro.

En resumen, mostramos aquí que SUMO, a través de su interacción covalente con RPL11, es un regulador clave de la respuesta celular al estrés nucleolar y en respuesta al estrés oncogénico. Esta no es la única función de SUMO en el nucleolo ya que al menos otro componente del ribosoma, la proteína RPL23 y el supresor de tumores ARF también pueden ser moduladas por SUMO. Además, este estudio nos ha llevado a avanzar en el conocimiento de las modificaciones post-traduccionales por proteínas de tipo ubiquitina ya que revela que SUMO se puede unir a un sustrato de manera independiente de residuos de lisina y que existe una interacción muy compleja entre SUMOilación y NEDDilación.

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INTRODUCTION

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1. RIBOSOMAL STRESS

Ribosome biogenesis is a key component to regulate overall protein synthesis and cell growth, and requires tight regulation. Alteration of ribosome biogenesis can be caused by (i) nutrient depletion (Bhat, Itahana, Jin, & Zhang, 2004); (ii) chemical agents or radiation that perturbs ribosomal RNA (rRNA) production or induces ribosomal protein degradation (Fumagalli et al., 2009; Golomb, Volarevic, & Oren, 2014; X. Zhou, Liao, Liao, Liao, & Lu, 2015) or (iii) some ribosomal proteins malfunction or deficiency resulting from genetic alterations. This alteration results in ribosomal stress (also called nucleolar stress) (Daftuar, Zhu, Jacq, & Prives, 2013; Fumagalli et al., 2009; Fumagalli, Ivanenkov, Teng, & Thomas, 2012).

Nutrient depletion leads to the serine-threonine kinase TOR (target of rapamycin) signaling pathway inhibition perturbing the ribosome biogenesis at different levels (Cardenas, Cutler, Lorenz, Di Como, & Heitman, 1999; Claypool et al., 2004; Fumagalli et al., 2009; Hannan et al., 2003; Mayer, Zhao, Yuan, & Grummt, 2004; Mayer & Grummt, 2006; Meyuhas, 2001; Zaragoza, Ghavidel, Heitman, & Schultz, 1998). The chemical agents can alter the ribosome biogenesis by different mechanisms. Some examples of ribosomal stress induced by a chemical agent are (i) Actinomycin D, an anticancer drug that at low concentrations (less than 10 nM) repress RNA Pol I activity impairing rRNA transcription (Bhat et al., 2004; Iapalucci-Espinoza & Franze-Fernández, 2018; Perry & Kelley, 2018) or (ii) DNA damage-inducing drugs that inhibit rRNA transcription or rRNA processing (Burger et al., 2010; Ghoshal & Jacob, 1994; Jordan & Carmo-Fonseca, 1998; Rubbi & Milner, 2003, 2003). The most frequent mutations of ribosome biogenesis-associated genes causing ribosomal stress are those mutations in ribosomal protein genes such as RPL11, RPL5, RPL35A, RPS19, RPS24, and RPS17, producing the inherited disease Diamond-Blackfan Anemia (DBA) (Narla & Ebert, 2010) or the deletion of the RPS14-encoding gene, causing the 5q- syndrome (Barlow et al., 2010; Ebert et al., 2008). These pathologies, characterized by elevated levels of p53 caused by mutation of ribosomal proteins, underscore the importance of understanding the molecular mechanism involved in p53 activation.

The checkpoint elicited as a response to an alteration in ribosome biogenesis is due to the ability of some ribosomal proteins to translocate from the nucleolus to the

nucleoplasm where they can execute their ribosome-independent functions (Fumagalli et al., 2009). The activity of some free ribosome proteins can induce transformation, as shown after overexpression of RPS3A protein (Naora, Takai, Adachi, & Naora, 1998) or RPS13 (X. Guo et al., 2011; Shi et al., 2004). However, other free ribosome proteins exert a tumor suppressor activity by either inactivating oncoproteins or activating tumor suppressors (de las Heras-Rubio, Perucho, Paciucci, Vilardell, & LLeonart, 2014). Thus, sixteen ribosomal proteins have been reported to regulate the mouse double minute 2 (MDM2)-p53 pathway in response to ribosomal stress, inhibiting p53 ubiquitination and degradation and, consequently, leading to cell cycle arrest: RPL5 (Dai & Lu, 2004; Marechal, Elenbaas, Piette, Nicolas, & Levine, 1994), RPL11 (Lohrum, Ludwig, Kubbutat, Hanlon, & Vousden, 2003; Zhang et al., 2003), RPS3 (Yadavilli et al., 2009), RPS15 (Yuan et al., 2005), RPS20 (Yuan et al., 2005), RPS25 (Zhang et al., 2012), RPS27 (Xiong, Zhao, He, & Sun, 2011), RPS27a (Sun, DeVine, Challagundla, & Dai, 2011), RPL6 (Bai, Zhang, Xiao, & Zheng, 2014), RPL26 (Takagi, Absalon, McLure, & Kastan, 2005), RPS7 (D Chen et al., 2007; Fumagalli et al., 2009), RPS14 (X. Zhou, Hao, Liao, Zhang, & Lu, 2013), RPS26 (Cui et al., 2013), RPS27L (Xiong et al., 2011, 2014), RPL23 (Dai et al., 2004; Jin, Itahana, O'Keefe, & Zhang, 2004) and RPL37 (Llanos & Serrano, 2010). Whether all these ribosomal proteins have an essential role in the activation of the MDM2-p53 pathway upon ribosomal stress is not clear since it has been also reported that only two ribosomal proteins, RPL11 and RPS5, are required for the activation of p53 in response to ribosomal stress (Fumagalli et al., 2012).

The release of ribosomal proteins from the nucleoli is not a passive process and, although some regulators have been identified, the molecular mechanism involved in the nucleolar to nucleoplasmic translocation of the ribosomal proteins is not clear. Tumor cells need more ribosome machinery compared with normal somatic cells, suggesting that they may be more sensitive to ribosomal stress. Therefore, induction of ribosomal stress has been proposed as an anti-cancer therapy strategy (Brighenti, Treré, & Derenzini, 2015). Consequently, characterization of the molecular mechanisms regulating this process is also critical to this aim.

2. RPL11

The ribosomal protein RPL11 is one of the most studied ribosomal proteins due to its connection with the inherited disease Diamond Blackfan Anemia (DBA) and oncogenic pathways (Robledo et al., 2008). RPL11 is a component of the 60S subunit and it has been reported that RPL11 silencing reduces the ribosomal synthesis (Dai et al., 2012). In addition to the ribosomal function, ribosome-free RPL11 plays an important role in the cell response to different types of stress such as ribosomal stress (Fumagalli et al., 2009; Havel, Li, Cheng, Peng, & Fu, 2015; Lohrum et al., 2003; Zhang et al., 2003) or oncogenic stress (Nishimura et al., 2015). In fact, it has been reported that RPL11 together with RPL5 are the only two essential proteins for the ribosomal stress response (Dai & Lu, 2004; Lohrum et al., 2003). When cells are exposed to conditions that perturb ribosome biogenesis, RPL11 translocates outside the nucleoli resulting in ribosome-free RPL11 which binds to MDM2 and inhibits p53 polyubiquitination and, consequently, induces the stabilization and activation of p53 (Dai & Lu, 2004; Lohrum et al., 2003). MDM2-RPL11 interaction also promotes the ubiquitination of the negative regulator of p53 MDMX in a MDM2-dependent manner (Li & Gu, 2011) and it is required for the recruitment of p53 transcriptional transactivators p300/CBP at the p53 promoter (Mahata, Sundqvist, & Xirodimas, 2011). In addition, ribosome-free RPL11 binds to Myc and inhibits its transcriptional activity (Dai, Arnold, Sun, Sears, & Lu, 2007) and promotes mir-24/miRISC-mediated c-Myc RNA degradation (Challagundla et al., 2011). Therefore, RPL11 is considered a tumor suppressor, explaining why in patients affected by DBA, a pathological condition characterized by heterozygous loss of function mutation in ribosomal protein genes such as RPL11, have a propensity to develop tumors later in life (Fumagalli & Thomas, 2011).

The accumulation of RPL11 in the nucleoplasm is the key mechanism for MDM2 inactivation and p53 activation. Several regulators of the translocation of RPL11 from the nucleolus to the nucleoplasm to drive p53-mediated response to stress have been identified. Thus, it has been reported that the oncoprotein PICT1 sequesters RPL11 inside the nucleolus preventing its association with MDM2, leading to p53 inactivation (Sasaki et al., 2011). In addition, conjugation of RPL11 to the ubiquitin-like protein neural precursor cell expressed, developmental downregulated (NEDD8)

stabilizes RPL11 and also retains RPL11 inside the nucleolus (Sundqvist, Liu, Mirsaliotis, & Xirodimas, 2009). Activation of the RPL11-MDM2-p53 pathway upon treatment with the NEDDylation inhibitor MLN4924 also supports a negative modulation of the RPL11-MDM2-p53 pathway by NEDD8 (Bailly et al., 2015). However, NEDD8 together with MDM2 have also been reported to be required for the recruitment of RPL11 at the p53 promoter (Mahata et al., 2011). These authors also demonstrated that MDM2 promotes NEDDylation of RPL11, whereas ribosomal stress induces deNEDDylation of RPL11(Sundqvist et al., 2009; Xirodimas et al., 2008). It has been also proposed that the interaction of RPL11with RPL5 and 5S rRNA may stabilize RPL11 in the nucleoplasm (Bailly et al., 2015; Sloan, Bohnsack, & Watkins, 2013).

Other protein that interacts with RPL11 is the tumor suppressor ARF. Interestingly, overexpression of ARF upregulates the levels of ribosome-free RPL11 which in turn enhances the ARF-induced p53 transcriptional activity and cell cycle arrest (Dai et al., 2012), supporting a connection between oncogenic stress and ribosomal stress (Figure 1)

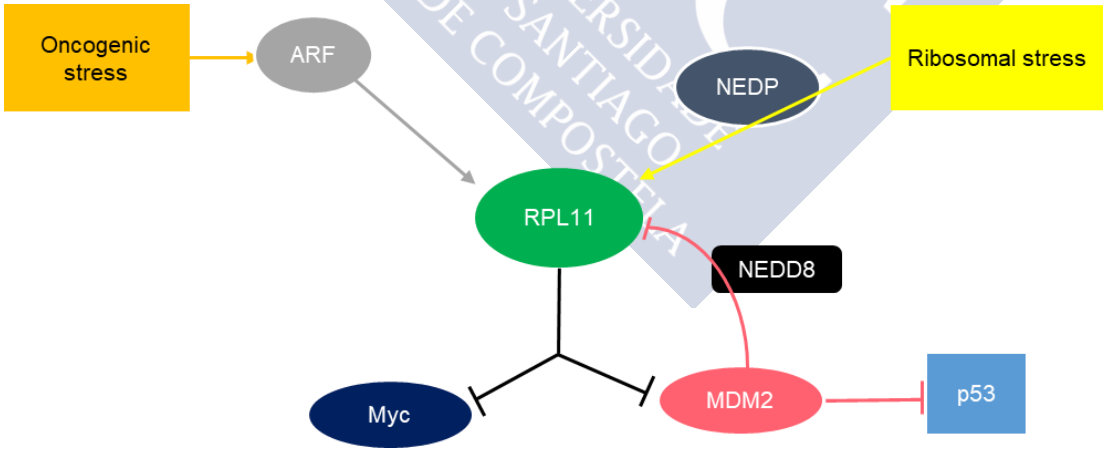

Figure 1. RPL11 can activate p53 in response to both ribosomal and oncogenic stress

In response to oncogenic stress, ARF promotes the RPL11- MDM2 interaction and p53 activation. Ribosomal stress induces the deNEDDylation of RPL11, resulting in the translocation of RPL11 outside of the nucleolus, its binding to MDM2 and the activation of p53. MDM2 in turns induces the NEDDylation of RPL11 resulting in sequestering RPL11 inside the nucleolus. RPL11 can also suppress the oncogenic activity of Myc independently on p53.

3. P53

p53 is the most frequently mutated gene in human cancer (Hainaut & Hollstein, 1999; Vogelstein, Lane, & Levine, 2000). The tumor suppressor p53, also called "the guardian of the genome" (Lane, 1992) is a sequence-specific DNA binding protein that regulates transcription and coordinates the cellular responses to a variety of stress factors (Marchenko & Moll, 2007; Vogelstein et al., 2000; Vousden & Lane, 2007). Although the role of p53 is not limited to that of a tumor suppressor. Thus, p53 participates also in glucose metabolism (Gomes, Ramos, Soares, & Saraiva, 2018; Madan et al., 2011, 2011), mitochondrial oxidation(Yadavilli et al., 2009), antiviral response (Lazo & Santos, 2011; Sato & Tsurumi, 2012) or the response to alterations in ribosome biogenesis(Zhang & Lu, 2009). Activated p53 can induce cell cycle arrest, apoptosis, senescence, DNA repair (Hofseth, Hussain, & Harris, 2004; Manfredi, 2003; Meek, 2004; Riley, Sontag, Chen, & Levine, 2008; Sengupta & Harris, 2005; Vogelstein et al., 2000; Vousden & Lane, 2007; Vousden & Lu, 2002) or autophagy (Levine & Abrams, 2008; White, 2016).

Regulation of p53 is a complex process. In normal cells, p53 protein is maintained at low levels mainly by the activity of MDM2, the p53 ubiquitin ligase that promotes p53 ubiquitination and degradation (Haupt, 1997; Reiko Honda, Tanaka, & Yasuda, 1998; Kubbutat, Ludwig, Ashcroft, & Vousden, 1998). Upon different types of stress, p53 is stabilized and activated by a stimuli-dependent mechanism (Hofseth et al., 2004; Manfredi, 2003; Meek, 2004; Riley et al., 2008; Sengupta & Harris, 2005; Vogelstein et al., 2000; Vousden & Lane, 2007; Vousden & Lu, 2002). Thus, in response to DNA damage, p53 is phosphorylated, a modification that inhibits its interaction with MDM2 and stabilizes the protein (Muller & Vousden, 2014; Shieh, Ikeda, Taya, & Prives, 1997). However, upon ribosomal stress p53 is stabilized due to the inhibition of MDM2-mediated p53 ubiquitination and degradation with no p53 phosphorylation (Dai et al., 2004; Donati, Peddigari, Mercer, & Thomas, 2013; Fumagalli et al., 2009, 2012; Golomb et al., 2014; Narla & Ebert, 2010; X. Zhou et al., 2015). Another modification of p53 that promotes its activation is the acetylation (Yamaguchi et al., 2009). In addition, p53 can be modified by different ubiquitin-like proteins such as small ubiquitin-like modifiers (SUMOs) (Santiago, Li, Zhao, Godsey, & Liao, 2013) or NEDD8 (Xirodimas, Saville, Bourdon, Hay, & Lane, 2004).

Importantly, MDM2 is also one of the downstream p53 target genes. p53 induces the transcription of its negative modulator MDM2 which in turns promotes the degradation of p53 and quenches p53 activity (Michael & Oren, 2003; Piette, Neel, & Maréchal, 1997; Prives, 1998). Therefore, the use of inhibitors of MDM2 has been explored as a putative therapeutic strategy to reactivate p53 (Vu et al., 2013; Zhao, Aguilar, Bernard, & Wang, 2015)

4. MDM2

MDM2 is an E3 ubiquitin ligase that induces p53 polyubiquitination and proteasomal degradation (Kubbutat et al., 1998). In addition to the ubiquitination of p53, MDM2 can also induce the conjugation of p53 to other ubiquitin-like proteins such as NEDD8 (Oliner, Kinzler, Meltzer, George, & Vogelstein, 1992) and SUMO (Stindt, Carter, Vigneron, Ryan, & Vousden, 2011). As a consequence of the negative regulation of p53 by MDM2, inactivation of MDM2 leads to p53 upregulation and activation.

MDM2 contains three domains: the N-terminal region that interacts with and inhibits p53 (Kussie et al., 1996); the C-terminal RING finger domain with ubiquitin ligase activity that can interact with MDMX and mediate the ubiquitination of MDMX, MDM2 and p53 (R Honda & Yasuda, 2000); and a central region that interacts with ARF (Sherr, 2006b), RPL11 (Zhang & Lu, 2009) and other ribosomal proteins (Zhang et al., 2003).

As described for p53, MDM2 can be regulated through post-translational modifications such as acetylation (X. Wang, Taplick, Geva, & Oren, 2004) or SUMOylation (Xirodimas, Chisholm, Desterro, Lane, & Hay, 2002). In addition, MDM2 levels are transcriptionally upregulated by p53 (Michael & Oren, 2003; Piette et al., 1997; Prives, 1998) and its activity is regulated by the tumor suppressor ARF. ARF interacts with MDM2 and inhibits MDM2´s control of p53 (Pomerantz et al., 1998; J. D. Weber, Taylor, Roussel, Sherr, & Bar-Sagi, 1999; Zhang, Xiong, & Yarbrough, 1998) (Figure 2).

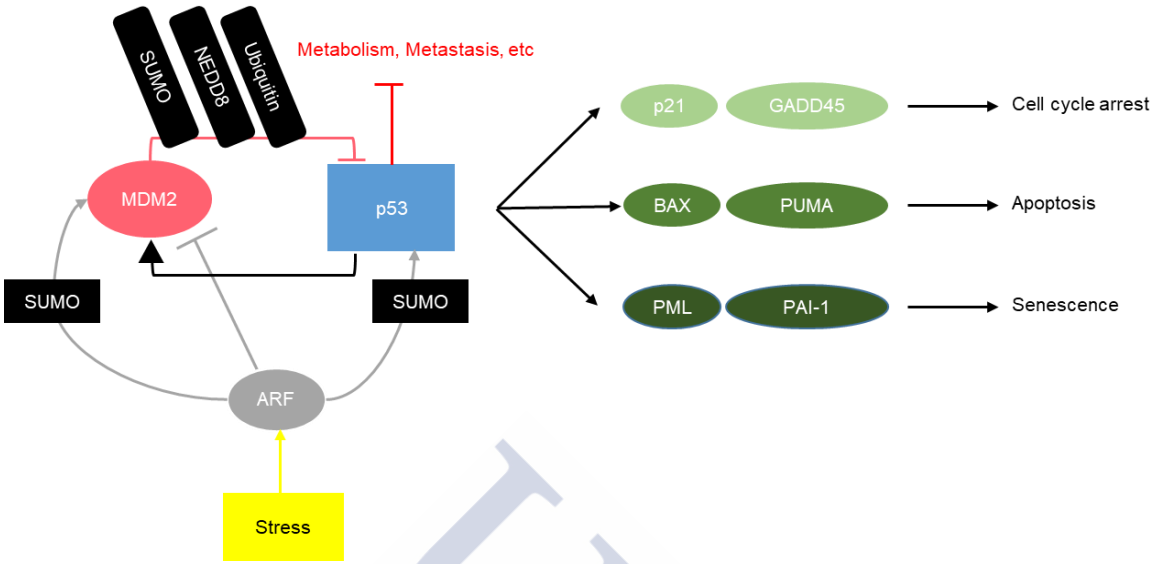

Figure 2. MDM2-p53 pathway regulation

MDM2 is transcriptionally upregulated by p53. MDM2 protein is SUMOylated and its ubiquitin-ligase activity is inhibited by ARF. MDM2 induces the ubiquitination, NEDDylation and SUMOylation of p53, and induces its degradation. Interaction between ARF and MDM2 as well as ARF levels are regulated in response to stress.

5. ARF

The tumor suppressor ARF is a nucleolar protein of 14 kDa and one of the transcript products from CDKN2A (cyclin-dependent kinase inhibitor 2A) gene. ARF is the second most frequently mutated tumor suppressor protein in cancer (Sharpless & Depinho, 1999; Sherr, 2001).

ARF regulation is not completely understood. ARF does not have recognizable structural motifs and it has been proposed that its folding may require the interaction with partners. Both mouse and human ARF proteins contain only an internal methionine residue that led to the translation of a shorter version of the protein, that after overexpression is detected in the mitochondria (Reef et al., 2006; van Oosterwijk, Li, Yang, Opferman, & Sherr, 2017). Interestingly, human ARF does not have any lysine residue in nature (mouse protein has one), however, the protein can be polyubiquitinated in its N terminus region in a p53 and MDM2-independent manner

(Kuo, den Besten, Bertwistle, Roussel, & Sherr, 2004). Although ubiquitination of ARF induces its degradation, an *in vitro* degradation of ARF by the 20S proteasome in a ubiquitin-independent manner has also been reported (Pollice, Vivo, & Mantia, 2008). The nucleolar localization of the mouse protein depends on one nucleolar localization signal (NoLS) (J. D. Weber et al., 2000). The human protein contains two NoLSs (H Rizos, Darmanian, Mann, & Kefford, 2000). The first one is required for the anti-proliferative activity of the protein (H Rizos et al., 2000) and the second one is required for inducing SUMOylation of its partner proteins (Xirodimas et al., 2002). Interaction with nucleophosmin (NPM) also contributes to the nucleolar localization and stability of ARF (Korgaonkar et al., 2005).

ARF works as a sensor of different types of stress (Delin Chen, Shan, Zhu, Qin, & Gu, 2010; Gjerset, 2006; Liontos, Pateras, & Gorgoulis, 2012; Matheu, Maraver, & Serrano, 2008; Olson, 2004). The expression of mouse ARF protein is induced by oncogenic stress such as after overexpression of the oncogenes Ras, c-myc, or E2F1 or in response to virus infection (Delin Chen et al., 2010; García et al., 2006; Palmero, Murga, Zubiaga, & Serrano, 2002). However, human ARF is upregulated in response to overexpression of the oncogene c-myc, radiation, or genotoxic stress (Delin Chen et al., 2013; Lo et al., 2015).

Several activities of ARF can contribute to its tumor suppressor and antiviral functions: induction of p53-dependent or independent cell cycle arrest and apoptosis (Radfar, Unnikrishnan, Lee, DePinho, & Rosenberg, 1998; H. O. Weber, Samuel, Rauch, & Funk, 2002; Zindy et al., 1998), decreasing rRNA transcription and processing (Lessard et al., 2010) or activation of DNA damage response (Gjerset, 2006; Liontos et al., 2012; Stott et al., 1998). In addition, ARF can also alter the mitochondrial membrane potential regulating autophagy (Balaburski, Hontz, & Murphy, 2010; Pimkina & Murphy, 2009; Sherr, 2006a) and promotes SUMOylation of its binding partners such p53 (L. Chen & Chen, 2003), MDM2 (Xirodimas et al., 2002) or NPM (Tago, Chiocca, & Sherr, 2005). The molecular mechanism by which ARF induces SUMOylation is unknown. However, since ARF interacts with Ubc9 (Helen Rizos, Woodruff, & Kefford, 2005), it has been proposed that ARF may facilitate the transfer of SUMO to the ARF-binding proteins.

One molecular mechanism by which ARF activates p53 is through the interaction with MDM2, blocking the MDM2-mediated ubiquitination and degradation of p53 (Zhang et al., 1998) or promoting stabilization of p53 by relocalizing MDM2 inside the nucleoli (J. D. Weber et al., 1999). In addition, ARF can promote the interaction of RPL11 with MDM2 (Figure 3).

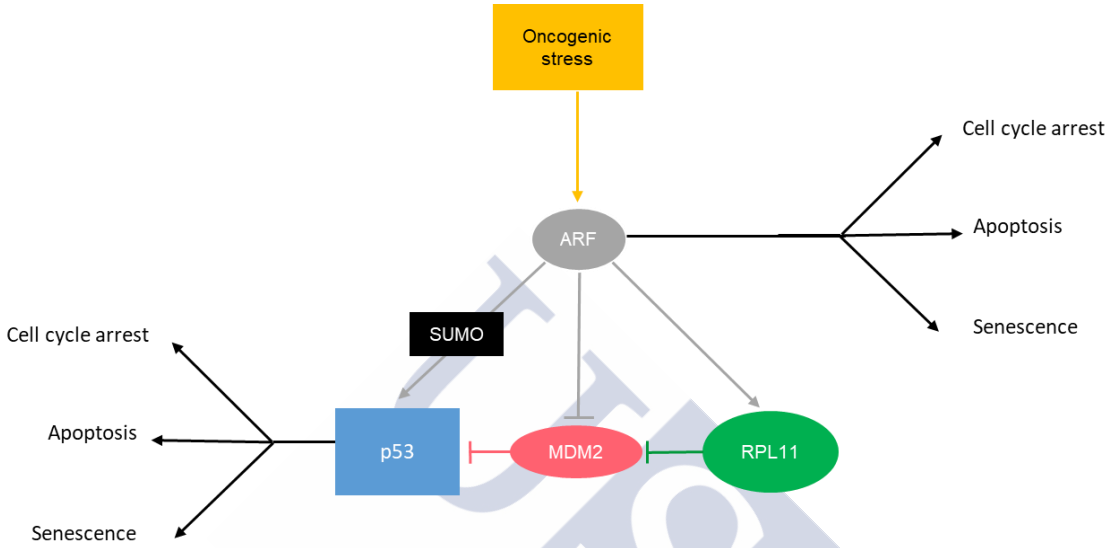

Figure 3. Tumor suppressor activities mediated by ARF

In response to oncogenic stress, ARF promotes the interaction between RPL11 and MDM2; interacts with MDM2 and inhibits the ubiquitination of p53 mediated by MDM2. ARF also induces the SUMOylation of MDM2 and p53. ARF can also induce cell cycle arrest, apoptosis, and senescence in a p53-independent manner.

As mentioned above, ARF can also restrain cell growth by p53-independent mechanisms. Thus, ARF sequesters the c-myc or E2F-1 oncogenes in the nucleoli or prevents the recruitment of transcriptional activators, inhibiting its transcriptional activity in a p53-independent manner (de Stanchina et al., 1998). ARF can also inhibit the nucleolar import of the RNA polymerase transcription factor TTF-I, inhibiting ribosome biogenesis (Lessard et al., 2010).

6. UBIQUITIN

Ubiquitin is a small protein of 8.5 kDa that is attached to substrates by a three-step enzymatic cascade involving an E1 ubiquitin activating enzyme, an E2 conjugating enzyme and a variety of E3 ubiquitin ligating enzymes. Ubiquitin modifications are removed by deubiquitinating enzymes (Pickart & Eddins, 2004). ubiquitination can

occur in lysine residues or in the N-terminus (Kuo et al., 2004). In addition, the ubiquitin protein itself can be ubiquitinated on seven lysine residues or in the N-terminus, it can be SUMOylated (Nie & Boddy, 2016) NEDDylated (Hjerpe, Thomas, & Kurz, 2012) acetylated (Caron, Boyault, & Khochbin, 2005) or phosphorylated (Nguyen, Kolch, & Kholodenko, 2013). Each modification can potentially provide additional regulation in the ubiquitin system and alter the signaling outcome. In addition to cooperation between modifications, crosstalk between modifiers can involve a competition to bind to the same residue. Although ubiquitin conjugation may have non-proteolytic functions, ubiquitination is sometimes the signal required for degradation (Glickman & Ciechanover, 2002), a process that plays a crucial role in cell-cycle regulation, DNA repair, cell growth, etc.

Since the discovery of ubiquitin and ubiquitination as a form of post-translational modification, different ubiquitin-like modifiers have been identified, including SUMO, NEDD8, FAT10, ATG8, FUB1, ISG15 or UBL5 (Cajee, Hull, & Ntwasa, 2012).

7. SUMO

SUMO is a small protein of 11 kDa with a structure similar to ubiquitin (Wilkinson & Henley, 2010). So far, five isoforms have been identified, which are SUMO1, 2, 3, 4 and 5. The best well-known SUMO isoforms are SUMO1, 2, and 3. The amino acid sequence of SUMO2 and SUMO3 are almost identical whereas SUMO1 shows 45% homology to SUMO2/3. SUMO2/3 have been reported to form SUMO chains (Mullen, Das, & Brill, 2011), while SUMO1 has not, although SUMO1 may act as a chain terminator of SUMO2/3 polymers (Ulrich, 2008).

SUMO modifies the substrate protein through reversible covalent interaction, also called SUMOylation, an enzymatic process similar to ubiquitination. SUMO precursors are cleaved by SUMO-specific peptidases (SENP) to expose a C-terminal di-glycine motif. Matured SUMO is then activated by the E1 heterodimer enzyme SAE1/SAE2 in an ATP dependent manner. The activated SUMO is then transferred to the active site cysteine of the E2 conjugating enzyme, Ubc9. Finally, SUMO is attached to a specific lysine residue located in the target protein, a process that usually requires an E3 ligase (Wilkinson & Henley, 2010). De-modification of SUMO is then carried

out by specific proteases SENP (Müller, Hoege, Pyrowolakis, & Jentsch, 2001) (Figure 4). So far, the SENP family contains six SENPs: SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7. They differ in the subcellular localization and SUMO isoform preference (Y. Wang & Dasso, 2009). The consensus SUMOylation site is "Ψ-K-x-E" where Ψ is a hydrophobic residue, K is the lysine residue, x is any amino acid, and E is an acidic residue (Müller et al., 2001). However, SUMOylation of proteins in non-consensus SUMOylation sites has been extensively reported (Kamitani et al., 1998; Wilkinson & Henley, 2010).

SUMOylation modulates protein-protein interactions in order to modulate subcellular localization, stability or activity and has emerged as a crucial mechanism involved in the regulation of cell cycle progression, apoptosis, immune responses, DNA damage repair, etc. Moreover, the SUMO2/3 modification has a crucial role in the cellular response to different types of stress. Therefore, alteration in SUMOylation can lead to the development of a number of diseases, including cancer (de la Cruz-Herrera et al., 2014; Domingues et al., 2015; Enserink, 2015; C. Guo & Henley, 2014; Saitoh & Hinchey, 2000; Šramko, Markus, Kabát, Wolff, & Bies, 2006; W. Zhou, Ryan, & Zhou, 2004).

Several SUMO substrates have a relevant role in the cellular response to different types of stress, including ribosomal stress and a complex regulation of their modification has been reported. Thus, two positive modulators of p53 (RPL11 and ARF), and one negative modulator, MDM2, have been reported to induce the SUMOylation of p53 (L. Chen & Chen, 2003; Stindt et al., 2011). In addition, ARF can also induce the SUMOylation of MDM2 (Xirodimas et al., 2002).

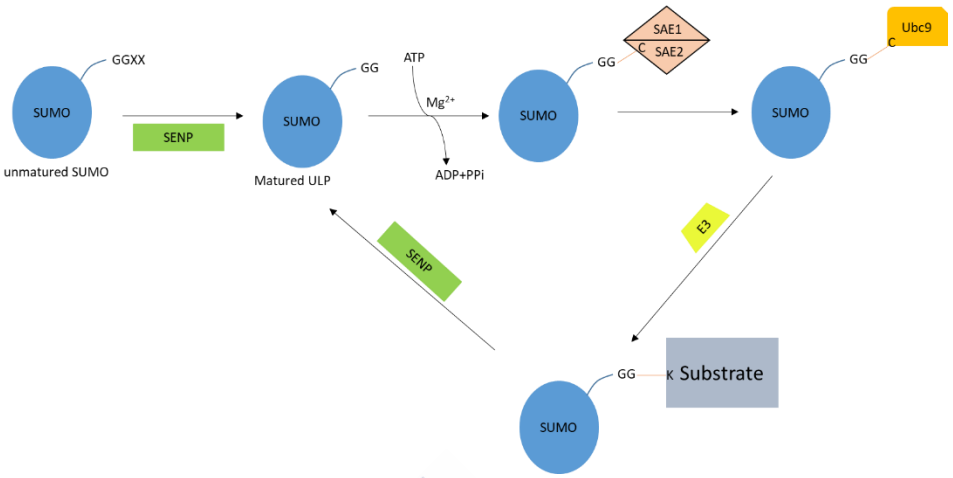

Figure 4. Illustration of SUMO modification process

Precursor proteins are cleaved by proteolytic enzymes (SENP). Matured protein is then activated by the heterodimer SAE1/2 enzyme in an ATP dependent manner. The activated protein is then transferred to the active site cysteine of the Ubc9 conjugating enzyme. Finally, the modifier is attached to a specific lysine residue located in the target protein, a process that usually requires an E3 ligase. SUMO is deconjugated from the substrate protein by the action of SENPs.

8. NEDD8

NEDD8 is a ubiquitin-like protein with 9 kDa molecular weight that shares around 60% amino acid sequence identity to ubiquitin (Kumar, Yoshida, & Noda, 1993). Modification of a substrate by NEDD8 or NEDDylation is a process similar to ubiquitination. The NEDD8 precursor is matured by the action of a NEDD8-activation enzyme, NAE. Matured NEDD8 then conjugates to E1 (UBA3–APPBP1 heterodimer) in an ATP dependent manner. NEDD8 then binds to the E2 enzyme or UBC12 and finally NEDD8 is transferred by E3 ligase to bind to the substrate through an isopeptide covalent bond between the carboxyl terminus of the last glycine residue in NEDD8 protein and the acceptor ε amino of a lysine residue located in the target protein (Rabut & Peter, 2008) (Figure5).

So far few proteins have been reported to be NEDDylated. NEDD8 conjugates to the core cullin protein of Cullin-RING ubiquitin ligase (CRL) complexes, responsible for the ubiquitination of several proteins with an important role in cancer, playing a critical role in its activation (Deshaies, Emberley, & Saha, 2010). NEDD8

conjugation to ribosomal proteins has been also demonstrated (Chan et al., 2008; Rabut & Peter, 2008; Xirodimas et al., 2008). In addition, NEDD8 can also conjugate to p53, inhibiting its transcriptional activity (Xirodimas et al., 2004). Interestingly, MDM2 acts as NEDD8 E3 ligase promoting the NEDDylation of p53, another mechanism by which MDM2 inhibits the activity of p53 (Xirodimas et al., 2004).

In the last years, NEDD8 inhibition has been proposed as a therapeutic strategy against cancer. Several NEDDylation inhibitors have been reported, and some of them, like MLN4924, are actually under clinical trials. *In vitro* results using tumor cells indicate that MLN4924 induces apoptosis (Swords et al., 2018) likely due to the inhibition of cullin NEDDylation (Soucy, Dick, Smith, Milhollen, & Brownell, 2010).

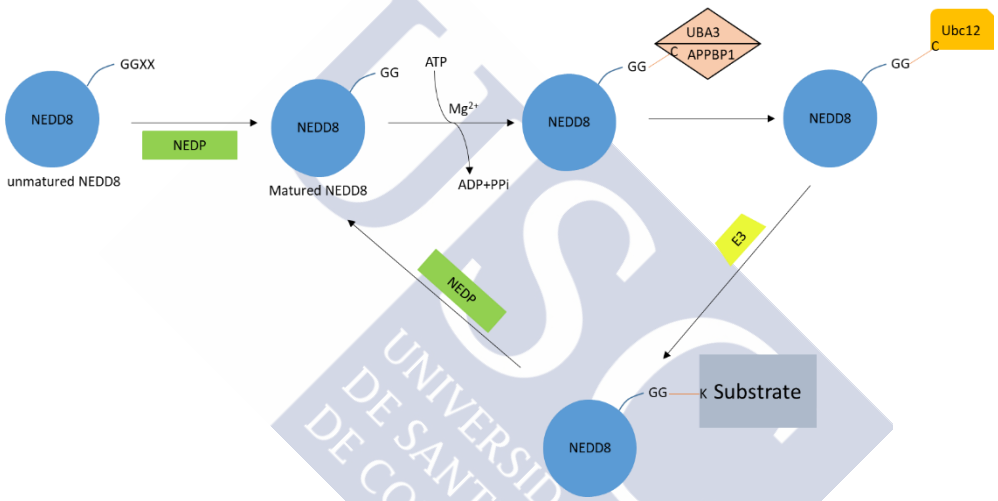

Figure5. Illustration of NEDD8 modification

Precursor NEDD8 is cleaved by NEDP peptidase. Matured NEDD8 is then activated by the NAE1 heterodimer enzyme in an ATP dependent manner. The activated protein is then transferred to the active site cysteine of the Ubc12 conjugating enzyme. Finally, NEDD8 is attached to a specific lysine residue located in the target protein, a process that usually requires an E3 ligase.

9. RPL23

The ribosomal protein L23 (RPL23) is a component of the 60s large ribosomal subunit. Overexpression of RPL23 has been reported to act as a negative regulator of MDM2 ubiquitin E3 ligase function to p53 (Dai et al., 2004). Although RPL23, similar to the RPL11 protein, binds to the central acidic domain in MDM2, the specific binding site of RPL23 on MDM2 differs from the site of RPL11 binding to MDM2 since the

zinc finger mutant MDM2^{C305F} cannot bind to RPL11, but it still can bind to RPL23 (Dai et al., 2004). However, RPL23 can bind to RPL11 and form a ternary structure with MDM2, inhibiting its activity (Lindström, Jin, Deisenroth, White Wolf, & Zhang, 2007). Both ribosomal proteins, RPL11 and RPL23, are differentially regulated. Thus, upon ribosomal stress RPL11 is stabilized while RPL23 is destabilized (Jin et al., 2004). In addition, oncogenic stress mediated by RAS overexpression has been reported to increase mRNA levels of RPL23 (correlating with the induction of p53) but it does not alter RPL11 levels (Meng et al., 2016). All these differences in the regulation suggest the existence of divergences in the mechanism of action of each ribosomal protein which may be a protective mechanism if any of them fails.

OBJECTIVES

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Objective 1. To study whether RPL11 is regulated through SUMO conjugation

1.1. To determine whether RPL11 is modified by SUMO.

1.2. To study the function of SUMOylated RPL11.

1.3. To evaluate the impact of both oncogenic and ribosomal stress on RPL11-SUMOylation.

Objective 2. To study the overall role of SUMO in the activation of the RP-MDM2-p53 pathway.

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MATERIALS AND METHODS

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1. CELL CULTURE

1.1 CELL LINES USED

HEK-293T	Human Embryo Kidney
H1299	Human non-small cell lung carcinoma
HeLa	Human cervix epitheloid carcinoma
MCF-7	Human Caucasian breast adenocarcinoma
PC-3	Human Caucasian prostate adenocarcinoma

1.2 CELL CULTURE MEDIUM

HEK-293T, H1299, HeLa, MCF-7, PC3, and U2OS cells were cultured in Dulbecco′s Modified Eagle′s Medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (Thermo Fisher), 1% penicillin/streptomycin (Sigma) and 1% L-Glutamine (Sigma). Cells were passaged upon reaching 80-90 % confluence.

1.3 CELL TRANSFECTION

1.3.1 DNA PLASMID TRANSFECTION

Cells were transfected with DNA plasmids using 1mg/ml PEI (Polysciences, Inc, Hirschberg an der Bergstrasse, Germany). The appropriate concentration of DNA plasmid and the optimal volume of PEI (ratio 3:1 PEI: total DNA) were diluted in serum and antibiotic-free DMEM in two sterile tubes. The diluted PEI was added to the DNA solution and the resulting mixture was incubated 30 min at room temperature and added drop by drop to cells.

The amount of DNA and PEI used in the transfection were:

Culture plate diameter (mm)	15.6	34.8	100	150
PEI (µL)	1.3	7.5	30	90
DNA (µg)	0.3125	1.25	5	15
Media volume per tube (µL)	25	100	500	1500

1.3.2 SIRNA TRANSFECTION

Cells were transfected with siRNAs using Xtreme-GENE siRNA (Roche, Barcelona, Spain). The appropriate concentration of siRNA and the optimal volume of Xtreme-GENE siRNA were diluted in serum and antibiotic-free DMEM in two sterile tubes. The diluted Xtreme was added to the siRNA solution and the resulting mixture was incubated 10 min at room temperature and added drop by drop to cells.

The amount of siRNA and Xtreme used in the transfection were

Culture plate diameter (mm)	15.6	34.8	100	150
Xtreme-GENE siRNA (µL)	1	4	16	42
Media volume per tube (µL)	25	100	500	1500

2. BACTERIAL TRANSFORMATION AND COMPETENT CELLS

2.1 COMPETENT BACTERIA PRODUCTION

First, Escherichia coli strains DH5α, STLB3, BL21 or C41 were grown at 37°C overnight on LB plates. The next day a single colony was picked and grown in 3 ml LB culture medium overnight at 28ºC with shaking (225 rpm). On the third day, the cells were refreshed into 200 ml of LB and were grown until reached Optical Density (OD)

600 nm. Then, the cells were harvested by centrifugation (4500 x g for 20 min at 4ºC) and washed with cold $CaCl_2$ (0.1M). Finally, the cells were resuspended in 6 ml of a solution containing $CaCl_2$ (0.1M) and glycerol (50 %), aliquoted and stored at -80ºC.

2.2 BACTERIAL TRANSFORMATION

Competent bacteria were thawed on ice, the plasmid DNA was mixed with chilled cells and incubated on ice for 30 min. The plasmid-cell mixture was then heated for 40 s at 42ºC. The mixture was placed back on the ice for 3 min. The bacteria were then grown at 37ºC for one hour with shaking (500 rpm) in 1ml of LB. Finally, the bacteria were harvested by centrifugation (4500 x g for 5 min), resuspended in 100 microliters of LB and plated on LB plates containing appropriate antibiotic.

2.3 ISOLATION OF PLASMID DNA

The isolation of plasmid DNA was performed using different commercial kits:

Kit	Miniprep	Midiprep	Maxiprep
Brand	GeneJET	Genomed, Eurogold and QIAGEN	Genomed
Bacterial culture volumes	3 ml	200 ml	200 ml

Briefly, a colony was picked and grown in LB medium supplemented with the appropriate antibiotic overnight at 37ºC with shaking (225 rpm). The cells were then harvested by centrifugation (6800 x g for 30 min at 4ºC), and resuspended in resuspension buffer, lysed with a lysis buffer and neutralized with a neutralization buffer. The cell debris was then pelleted by centrifugation and the plasmid-containing supernatant was loaded onto a silica column. Columns were washed with washing buffer, plasmid DNA was eluted using elution buffer, and precipitated using Isopropanol. Finally, DNA was washed with 70% ethanol and resuspended in sterile milliQ H_2O.

2.4 GST-TAGGED PROTEINS PRODUCTION AND PURIFICATION

First, the plasmid encoding for the GST-tagged protein was introduced into C41or BL21 competent cells, which were plated onto LB agar plates supplemented with the appropriate antibiotic, and incubated overnight at 37ºC. The next day one single colony was picked and grown overnight in 3ml of LB medium at 28° C with shaking (225 rpm). On the third day, the 3 ml of bacteria were transferred to 200 ml of LB medium and were grown until reached OD (600 nm). We then added IPTG to a final concentration of 1mM and the bacterial culture was incubated for another 4 h at 37ºC and 225 rpm. The bacteria were then harvested by centrifugation (20 min at 4500 x g) at 4ºC, washed with cold PBS and resuspended in 10 ml of PBS containing 0.5 M NaCl, 1 mM PMSF and 2 mM of benzamidine. The resuspended bacteria were lysed with 1 % of Triton X-100 and sonication. The bacterial lysate was clarified by centrifugation at 20000 rpm for 2h at 4ºC. The supernatant was then incubated with glutathione sepharose beads overnight at 4ºC in the presence of 1 mM of DTT. On the next day, after washing the beads 4 times, the GST-fused proteins were eluted from the beads using 50 mM of Tris (pH 9.5) and 10 mM of reduced glutathione. The eluted proteins were then dialyzed to get rid of the glutathione, concentrated if necessary using Amicon Ultra columns, supplemented with 10 % glycerol and aliquoted (Some GST- fused proteins were kept directly at -80ºC after adding 10% of glycerol without elution from the beads).

To produce and purify SUMO machinery recombinant proteins (SUMO1, SUMO2 and Ubc9) we used a similar protocol to the above described with some modifications: the bacterial culture was scaled up to 1000 ml. After washing the glutathione beads previously incubated with the cell lysate, the beads were incubated with 100 U of Thrombin (Sigma) overnight at room temperature. Later on, buffer exchange was performed using dialysis against 50 mM Tris pH 7.5 and 5 mM beta-mercaptoethanol overnight at 4ºC.

The dialyzed SUMO samples were incubated with prewashed Q-Sepharose for 1 h at room temperature and the dialyzed Ubc9 sample was incubated with prewashed S-Sepharose for 4 h at 4ºC. The columns were then washed three times, eluted using NaCl gradient concentrations and each collected fraction was evaluated by Coomassie staining.

Only the fractions containing the recombinant protein were mixed and dialyzed against 50 mM Tris pH 7.5, 5 mM beta-mercaptoethanol. Finally, glycerol was added to a 10 % final concentration and aliquots were done and kept at -80ºC.

3. PLASMIDS

3.1 PLASMIDS USED IN THIS WORK

GFP-C3	Plasmid expressing GFP vector (Clontech)
GFP-C3-p14 ARF	Plasmid expressing GFP tagged p14 ARF protein. Kindly provided by Susana Llanos (Kim, Mitchell, Fujii, Llanos, & Peters, 2003)
Myc-RPL23	Plasmid expressing Myc-tagged RPL23 protein. Kindly provided by Susana Llanos.
p14 ARF 3XHA	Plasmid expressing HA-tagged p14 ARF protein was purchased from Addgene (Addgene plasmid 78764)(Ko et al., 2012)
pcDNA3.1	Empty vector (Invitrogen)
pcDNA3.1-v5-Ubc9	Plasmid expressing SUMO conjugating enzyme Ubc9 fused to V5 tag. Kindly provided by Manuel S Rodríguez (Rodriguez, Dargemont, & Hay, 2001)
pcDNA3-Flag-RPL11*	Plasmid expressing Flag-tagged RPL11 protein. Kindly provided by Dimitris Xirodimas (Sundqvist et al., 2009)
pcDNA3-Flag-RPL11-3CCDKR mutant (3)*	Plasmid expressing a Flag-tagged RPL11 mutant protein with no lysine residues in the central domain or the C-terminus. Kindly provided by Dimitris Xirodimas (Sundqvist et al., 2009)

pcDNA3-Flag-RPL11-3CKR mutant (8)*	Plasmid expressing a Flag-tagged RPL11 mutant protein with no lysine residues in the C-terminus. Kindly provided by Dimitris Xirodimas (Sundqvist et al., 2009)
pcDNA3-Flag-RPL11-3NCDKR mutant (2)*	Plasmid expressing a Flag-tagged RPL11 mutant protein with no lysine residues in the N-terminus or the central domain. Kindly provided by Dimitris Xirodimas (Sundqvist et al., 2009)
pcDNA3-Flag-RPL11-3NLSKR mutant (5)*	Plasmid expressing a Flag-tagged RPL11 mutant protein with no lysine residues in the nuclear localization sequence. Kindly provided by Dimitris Xirodimas (Sundqvist et al., 2009)
pcDNA3-Flag-RPL11-4N3CKR mutant (9)*	Plasmid expressing a Flag-tagged RPL11 mutant protein with no lysine residues in the N- or the C- terminus. Kindly provided by Dimitris Xirodimas (Sundqvist et al., 2009)
pcDNA3-Flag-RPL11-4N9CDKR mutant (4)*	Plasmid expressing Flag-tagged RPL11 protein. Kindly provided by Dimitris Xirodimas (Sundqvist et al., 2009)
pcDNA3-Flag-RPL11-4N9CDKR mutant (7)*	Plasmid expressing a Flag-tagged RPL11 mutant protein with no lysine residues in the N-terminus or the central domain. Kindly provided by Dimitris Xirodimas (Sundqvist et al., 2009)
pcDNA3-Flag-RPL11-4NKR mutant (1)*	Plasmid expressing a Flag-tagged RPL11 mutant protein with no lysine residues in the N-terminus. Kindly provided by Dimitris Xirodimas (Sundqvist et al., 2009)

pcDNA3-Flag-RPL11-6CD3CKR mutant (10)*	Plasmid expressing a Flag-tagged RPL11 mutant protein with no lysine residues in the central domain or the C-terminus. Kindly provided by Dimitris Xirodimas (Sundqvist et al., 2009)
pcDNA3-Flag-RPL11-9CDKR mutant (6)*	Plasmid expressing a Flag-tagged RPL11 mutant protein with no lysine residues in the C-terminus. Kindly provided by Dimitris Xirodimas (Sundqvist et al., 2009)
pcDNA3-Flag-RPL11-GIGA mutant	Plasmid expressing a cytoplasmic Flag-tagged RPL11 mutant protein. Kindly provided by Dimitris Xirodimas (Sundqvist et al., 2009)
pcDNA3-myc3-RPL11	Plasmid expressing Myc-tagged RPL11 protein. Kindly provided by Susana Llanos (Zhang et al., 2003) (Addgene plasmid 20936).
pcDNA3-p14-ARF	Plasmid expressing untagged p14 ARF protein. Kindly provided by Susana Llanos (Stott et al., 1998)
pcDNA-His6-NEDD8	Plasmid expressing Histidine-tagged NEDD8 protein. Kindly provided by Manuel S Rodríguez (Xirodimas et al., 2004) .
pcDNA-His6-SUMO1	Plasmid expressing Histidine-tagged SUMO1 protein. Kindly provided by Manuel S Rodríguez (Rodriguez et al., 1999)
pcDNA-His6-SUMO2	Plasmid expressing Histidine-tagged SUMO2 protein. Kindly provided by Manuel S Rodríguez (Vertegaal et al., 2006)

pcDNA-SUMO1	Plasmid expressing untagged SUMO1 protein (Ling et al., 2004)
pcMV5-HA-RPL11	Plasmid expressing HA-tagged RPL11 protein. It was generated by PCR amplification of RPL11 and cloning into the pcMV5-HA vector as described below

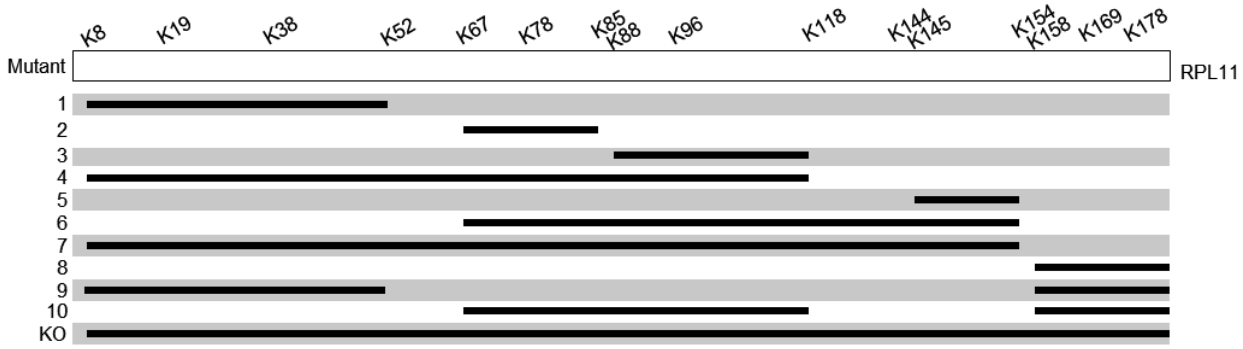

***Figure1. Scheme showing the RPL11 lysine residues that have been changed to arginine in each mutant (marked with a bold line)**

3.2 CLONING

3.2.1 HA-RPL11 WT AND HA-RPL11 KO

RPL11 was amplified by PCR using the following primers, containing sites for restriction enzymes BglII and PstI:

HA-RPL11-F-BglII- 5' GGGAGATCTGCGCAGGATCAAGGTGAA 3'

RPL11-R-PstI- 5' GGGCTGCAGTTATTTGCCAGGAAG 3'

The PCR program used was:

Initial strand separation	98ºC	2 min	
Denaturation	98ºC	10 sec	25 cycles
Annealing	64ºC	30 sec	
Extension	72ºC	3:30 min	

Final elongation	72ºC	10 min	1 cycle

The PCR product was then purified from an agarose gel, digested with fast digest BglII and PstI for 20 min at 37ºC, and purified from an agarose gel using GeneJET Gel Extraction Kit (Thermo Scientific).

The digested insert was then incubated with the digested pcMV5-HA vector and T4 DNA ligase for one hour at room temperature. Finally, competent bacteria were transformed with the ligation reaction. The resulting colonies were analyzed by restriction enzyme treatment and sent to sequencing for confirmation.

3.2.2 T7-RPL11 WT and T7-RPL11 K0

RPL11 was amplified by PCR using the following primers:

T7-RPL11-F 5'GGATCCTAATACGACTCACTATAGGAGCCACCATGGCGCA

GGATCAAGGTGAAAAGG 3'

RPL11-R - 5' GGGCTGCAGTTATTTGCCAGGAAG 3'

And using the same PCR program used as in HA-RPL11 cloning.

The PCR product was then purified from an agarose gel, and the purified DNA was applied directly to *In vitro* translation using TNT system.

3.3 MUTAGENESIS

3.3.1 RPL11K158R

The mutant RPL11 K158R was performed using Thermo Scientific Phusion High-Fidelity DNA Polymerase and the following PCR Mix:

300 ng of myc-RPL11 WT as DNA template

500 nM of RL11K158R-F oligonucleotide (5'CCAAACACAGAATCAGCAGAGAGG

AGGCCATGCG 3')

500 nM of RL11K158R-R oligonucleotide (5'CGCATGGCCTCCTCTCTGCTGATT

CT GTGTTTGG 3')

10 µL of 5X Phusion HF Buffer*

1µL of DMSO

1 µL of dNTP mix

0.5 µL of DNA polymerase (2.5 U/µl)

milliQ H2O to a final volume of 50 µl

The PCR program was:

Initial strand separation	98ºC	30 s	
Denaturation	98ºC	10 s	35 cycles
Annealing	68ºC	30 s	
Extension	72ºC	3:30 min	
Final elongation	72ºC	10 min	1 cycle

The PCR product was then treated with 1 µl of Dpn1 and incubated at 37ºC for 2 h. The mutagenesis product was then introduced into competent bacteria as previously described. The resulting colonies were grown, the plasmid DNA was purified using Miniprep Kit (Thermo Fisher) and the DNA plasmid was sent for sequencing.

4. ANTIBODIES USED IN THIS WORK

Antibody	Characteristics	Company
Anti 6x-His Tag	(H-5) Mouse monoclonal	Invitrogen
Anti c-Myc	(D84C12) Rabbit monoclonal	Cell Signaling

Anti-Flag (Oct-A)	(H-5) Mouse monoclonal	Santa Cruz
Anti GAPDH	(6CS) Mouse monoclonal	Santa Cruz
Anti GAPDH	(GA1R) Mouse monoclonal	Thermo fisher
Anti GFP	(B34) Mouse monoclonal	Biolegend
Anti HA	(16B12) Mouse monoclonal	Biolegend
Anti HA- tag	(16B12) Mouse monoclonal	Biolegend
Anti MDM2	(SMP14) Mouse monoclonal	Santa Cruz
Anti Myc tag	(71D10) Rabbit monoclonal	Cell Signaling
Anti Myc tag	(9B11) Mouse monoclonal	Cell Signaling
Anti Myc tag	(9E10) Mouse monoclonal	Santa Cruz
Anti NEDD8	(Y297) Rabbit monoclonal	Abcam
Anti p14 ARF	(14P02) Mouse monoclonal	Neomarkers
Anti p14 ARF	(4C6/4) Mouse monoclonal	Cell Signaling
Anti p53	(DO-1) Mouse monoclonal	Santa Cruz
Anti RPL11	Rabbit polyclonal	Invitrogen
Anti RPL11	Rabbit polyclonal	Abcam

Anti SUMO1	Rabbit polycolonal	Cell Signaling
Anti SUMO2/3	(18H8) Rabbit monoclonal	Cell Signaling
Anti Ubc9	(4786) Rabbit monoclonal	Cell Signaling
Anti V5	(7/4) Mouse monoclonal	Biolegend
Anti β-tubulin	Rabbit polyclonal	Cell Signaling

5. ELECTROPHORESIS AND WESTERN-BLOT

Purified proteins or cell extracts were boiled for 5 min in sample buffer and loaded in polyacrylamide gels containing SDS (SDS-page). The gels were then transferred to nitrocellulose membranes and blocked in 5% non-fat dry milk in TTBS (150 mM NaCl, 10 mM Tris pH 8.0, 0.1 % v/v Tween20) for 30 min. The blocked membranes were incubated with the primary antibody diluted in 5% non-fat dry milk in TTBS or in 5% BSA in TTBS (following manufacturer's indications) at 4ºC overnight with shaking. The membranes were then washed 3 times (10 min each) with TTBS and incubated with the secondary antibody diluted in 5% non-fat dry milk in TTBS for 1 h at room temperature with shaking. Finally, the membranes were washed four times with TTBS (10 min each), incubated with ECL solution, and exposed to X-ray film.

6. HISTIDINE-TAGGED PROTEINS PURIFICATION

Cells were washed twice with PBS, scraped and recovered in PBS. 10 % of the cell suspension was recovered to an Eppendorf, centrifuged at 1000 xg for 5 min and the cell pellet was boiled in Sample buffer for 5 min at 100ºC (input). The remaining cells were centrifuged and the cell pellet was lysed in buffer G (6 M guanidine HCl, 0.932 M Na_2HPO_4, 6.8 mM NaH_2PO_4, 25 mM Tris HCl pH 8). To break the DNA, the samples were passed through a 0.3 X 13 mm needle several times. The protein extracts were then incubated with TALON® Nickel Affinity Resin (Clontech) and rotated for 2 hours at room temperature. The nickel beads were then washed 4 times with 1 ml of

buffer U (8 M Urea, 100 mM Tris HCl pH=8, 93.2 mM Na_2HPO_4, 6.8 mM NaH_2PO_4) and finally, the Histidine-purified proteins were eluted by adding sample buffer and boiling for 5 min at 100ºC.

7. IMMUNOPRECIPITATION UNDER DENATURING CONDITIONS

Cells were washed twice with PBS, scraped and recovered in PBS. 10 % of the cell suspension was recovered to an Eppendorf, centrifuged at 1000 xg for 5 min and the cell pellet was boiled in sample buffer for 5 min at 100 C (input). The remaining cells were resuspended lysis buffer (50mM Tris, pH 7.5, 70 mM beta-mercaptoethanol pre-boiled), then the samples were boiled for 10 m at 100 ºC, diluted with 4X volumes of BC100 buffer (20 mM Tris, pH 8, 0.1 M KCl, 10% Glycerol, 1 mM EDTA, 1 mM DTT, 0.1 % NP-40 (supplemented with Protease inhibitors cocktail (Roche) and SUMO protease inhibitor NEM), incubated with the desired antibody and rotated overnight at 4ºC. On the next day, the samples were incubated with recombinant Protein G – Sepharose beads (Thermo Fisher), previously washed with the lysis buffer and rotated for another 2 hours at 4ºC. Finally, the beads were washed 4 times with the same lysis buffer and the captured proteins were eluted by adding Sample buffer and boiling for 5 min at 100ºC.

8. IMMUNOFLUORESCENCE ASSAY

Cells were grown on glass coverslips inserted in wells of 24 well-plates and transfected as indicated in each experiment. At the indicated time after transfection, cells were washed with PBS and fixed with 2 % paraformaldehyde in PBS for 20 min at room temperature. After fixation, cells were washed 4 times (5 min each) with PBS, permeabilized with Triton 0.25 % in PBS for 20 min, washed again with PBS and blocked by incubating in BSA 2% in PBS for another 30 min. The cells were then incubated with the indicated primary antibody overnight at 4ºC. On the next day, the cells were washed with PBS and incubated with Alexa 488-conjugated, and Alexa 594-conjugated secondary antibodies for 1 h at room temperature in dark. After washing with PBS four times, the cells were incubated with DAPI for 5 min at room temperature and washed again with PBS. Finally, the coverslips were mounted over slides using ProLong™ Diamond Antifade Mountant (Invitrogen) and analyzed using a confocal laser microscope (Leica TCS SP5) or an Olympus fluorescence microscope.

9. *IN VITRO* TRANSLATION

TNT Quick Coupled Transcription/translation system (Promega) was used to *in vitro* translate the desired proteins following the manufacturer´s instructions. Briefly, the reticulocytes were thawed on ice and then they were incubated with DNA and S^{35}-labelled or unlabelled methionine for 90 min at 30ºC. At this time, we took 1 microliter of the reticulocyte mixture to be analyzed by SDS-PAGE. The remaining protein was kept at -80ºC until further use.

10. *IN VITRO* SUMOYLATION ASSAY

0.3 µg of E1/2 (Biomol, Enzo Life Sciences), ATP 2 mM, Tris pH 7.5 50 mM, $MgCl_2$ 5 mM, Creatine Phosphate 10 mM, Creatine Kinase 3.5 U/ml, Inorganic pyrophosphate 0.6 U/ml, 600 ng of Ubc9, 10 µg of SUMO1 or SUMO2, and the previously *in vitro* translated protein (the volume depends on the translation efficiency) were incubated at 37ºC for 90-180 min. The reaction was then stopped by adding sample buffer and boiling for 5 min at 100ºC. Finally, the samples were loaded in SDS-page and analyzed by autoradiography or Western-blot.

11. SOFTWARE

To predict the SUMOylation sites we used GPS (http://sumosp.biocuckoo.org/online.php)

The pictures were processed using Adobe Photoshop CS4

The Western-blot was quantified bi ImageJ software

12. STATISTICAL ANALYSIS

Differences between samples were evaluated by using Student's t test

RESULTS

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1. RIBOSOMAL RPL11 PROTEIN IS MODIFIED BY SUMO

1.1 RIBOSOMAL RPL11 PROTEIN IS MODIFIED BY SUMO *IN VITRO*

To evaluate if RPL11 is SUMOylated, we performed *in vitro* SUMOylation assays using [^{35}S] methionine-labelled RPL11 *in vitro* translated protein as a substrate. We detected the unmodified myc-RPL11 protein as a band of around 28 kDa molecular weight, as expected (Figure 1A, left panel). We observed a higher molecular weight band of around 43 kDa when the reaction was incubated with SUMO1 (Figure 1A, left panel) and two additional higher molecular weight bands of around 43 kDa and 58 kDa molecular weight when SUMO2 was added to the reaction (Figure 1A, left panel). The intensity of the SUMO-modified bands depends on several factors, including the batch of reticulocytes used for *in vitro* translation, since they may contain different levels of specific E3 SUMO ligases. To further demonstrate that the observed higher molecular weight bands correspond to RPL11-SUMO conjugates, we performed a deSUMOylation assay. The RPL11-SUMO1 or RPL11-SUMO2 proteins were incubated with the recombinant SUMO-specific protease SENP1. The high molecular weight bands detected when the RPL11 protein was incubated with SUMO1 or SUMO2 almost disappeared after incubation with SENP1 (Figure 1A, right panel). Altogether, these results indicate that RPL11 is modified by SUMO1 and SUMO2 *in vitro*.

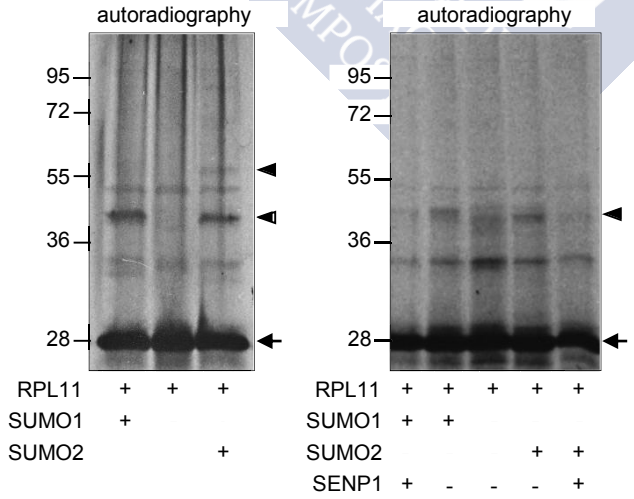

Figure 1A. RPL11 is modified by SUMO1 and SUMO2 *in vitro*.

In vitro translated [^{35}S]-labelled RPL11 was subjected to *in vitro* SUMOylation assay in the presence of SUMO1 or SUMO2 (left panel). SUMO1 or SUMO2-conjugated RPL11 protein was then incubated in the presence or absence of SENP1 as described in Material and Methods (right panel). Proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Arrows and arrowheads indicate the unmodified and SUMO conjugated RPL11 protein, respectively.

1.2 RPL11 IS MODIFIED BY SUMO *IN VIVO*

In order to evaluate whether RPL11 is also SUMOylated *in vivo*, we co-transfected HEK-293 cells with myc-RPL11 together with pcDNA3, Ubc9 and His6-SUMO1 or Ubc9 and His6-SUMO2. At 48 h after transfection, the cells were harvested, lysed in denaturing conditions and the histidine-tagged proteins were purified using nickel affinity beads. The whole cell lysates and the histidine-tagged purified proteins were then analyzed by Western-blot using anti-myc antibody. After analysis of the purified extracts, we detected bands of the expected size corresponding to RPL11-SUMO1 or RPL11-SUMO2 only in those cells co-transfected with His6-SUMO1 and His6-SUMO2, respectively. These results indicated that RPL11 is modified by SUMO1 and SUMO2 in transfected cells (Figure 1B).

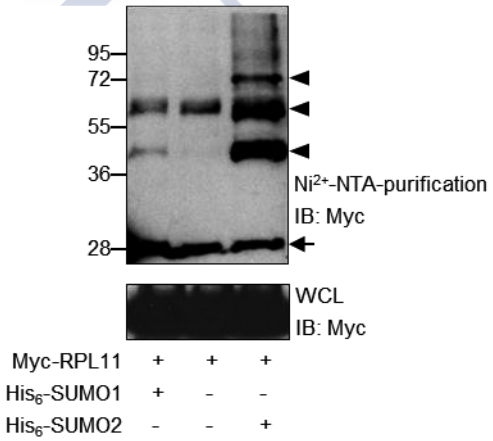

Figure 1B. RPL11 is modified by SUMO1 and SUMO2 *in vivo*.

HEK-293 cells were co-transfected with myc-RPL11 together with pcDNA, Ubc9 and His6-SUMO1 or Ubc9 and His6-SUMO2. Whole protein extracts (WCL) and histidine-tagged purified proteins (Ni^{2+}-NTA-purification) were analyzed by Western-blot (IB) using anti-myc antibody. Arrow and arrowheads indicate the unmodified and SUMO conjugated RPL11 protein, respectively.

1.3 SUMOYLATION OF RPL11 IS CELL LINE- AND TAG-INDEPENDENT

In order to determine whether the SUMOylation of RPL11 is cell line- and tag-independent, we performed *in vivo* SUMOylation assays similar to that described in point 1.2, in U2OS, MCF7 and HEK-293 cell lines and using HA- or Flag-tagged RPL11 expression constructs. We observed SUMOylation of RPL11 in all the studied cell lines and independently of the RPL11 plasmid transfected (Figure 1 C). Altogether these results indicate that the SUMOylation of RPL11 is cell line and tag- independent.

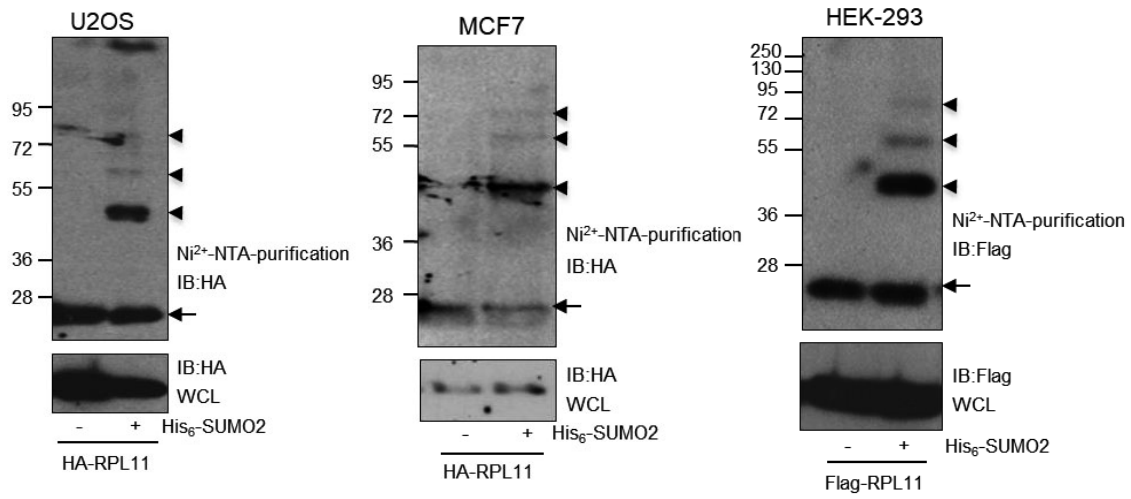

Figure 1C. SUMOylation of RPL11 is cell line and tag-independent.

U2OS (left panel), MCF-7 (middle panel), and HEK-293 (right panel) cells were co-transfected with HA- or Flag-RPL11 plasmid together with pcDNA or Ubc9 and His6-SUMO2. Whole protein extracts and histidine-tagged purified proteins were analyzed by Western-blot using anti-HA or anti-Flag antibodies. Arrow and arrowheads indicate the unmodified and SUMO conjugated RPL11 proteins, respectively.

1.4 RPL11 IS SUMOYLATED AT THE ENDOGENOUS LEVELS

We then decided to evaluate the SUMOylation of endogenous RPL11. HEK-293 cells were transfected with pcDNA3, Ubc9 and His6-SUMO1 or Ubc9 and His6-SUMO2. At 48 h after transfection, the cells were harvested, lysed in denaturing conditions and the histidine-tagged proteins were purified using nickel affinity beads. The whole cells lysates and the histidine-tagged purified proteins were analyzed by Western-blot using anti-RPL11 antibody. We detected bands of the expected size corresponding to RPL11-SUMO1 or RPL11-SUMO2 exclusively in the purified extracts of those cells transfected with His6-SUMO1 and His6-SUMO2, respectively. These results indicate that endogenous RPL11 can be modified by SUMO1 and SUMO2 (Figure 1D, upper panel). Altogether these results demonstrate that RPL11 is modified by SUMO1 and SUMO2 *in vitro* and that both endogenous and transfected RPL11 are also modified by SUMO1 and *SUMO2 in vivo*.

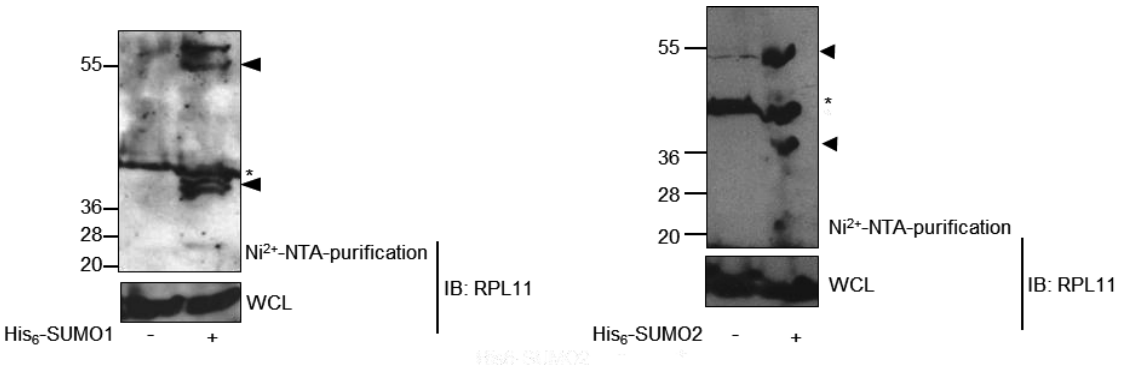

FIGURE 1D. RPL11 IS SUMOYLATED AT THE ENDOGENOUS LEVELS.

HEK-293 cells were transfected with pcDNA, Ubc9 and His6-SUMO1 or Ubc9 and His6-SUMO2. Whole protein extracts and histidine-tagged purified proteins were analyzed by Western-blotting using anti-RPL11 antibody. Arrowheads indicate the SUMO conjugated RPL11 protein. The position of a nonspecific band is indicated by an asterisk (upper panels).

2. SUMO IS CONJUGATED TO A NON-LYSINE RESIDUE IN RPL11

2.1 A MUTANT OF RPL11 IN THE LYSINE RESIDUE K158 IS STILL SUMOYLATED

To identify the amino acid residue that conjugates to SUMO in RPL11 we first performed *in silico* analysis using the GPS prediction program. The result of the analysis pointed to lysine residue K158 in RPL11 as a putative SUMO conjugating site. Therefore, we decided to replace the lysine residue K158 in RPL11 by arginine. Once we generated the RPL11K158R mutant, we performed *in vivo* SUMOylation assay. HEK-293 cells were co-transfected with myc-RPL11 WT or the RPL11 K158R mutant plasmid together with pcDNA3 or Ubc9 and His6-SUMO2. At 48 h after transfection, the cells were harvested, lysed in denaturing conditions and the histidine-tagged proteins were purified using nickel affinity beads. Whole cell lysates and histidine-tagged purified proteins were then analyzed by Western-blot using anti-myc antibody. Analysis of the purified extracts revealed bands of the expected size corresponding to RPL11-SUMO2 protein in both, myc-RPL11 WT and myc-RPL11K158R transfected cells (Figure 2A). These results indicated that the K158R mutant RPL11 protein is modified by SUMO2, indicating that SUMO can conjugate to other lysine residues in RPL11.

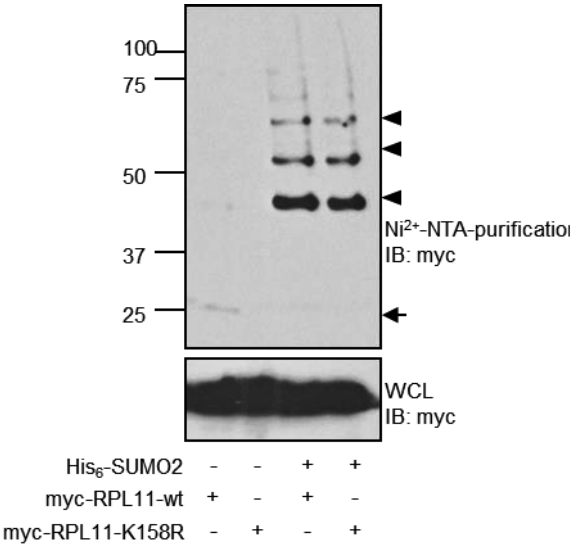

Figure 2A. SUMOylation of the RPL11K158R mutant protein.

HEK-293 cells were co-transfected with myc-RPL11-WT or myc-RPL11K158R together with pcDNA or Ubc9 and His6-SUMO2. Whole protein extracts and histidine tagged purified proteins were analyzed by Western-blot using anti-myc antibody. Arrow and arrowheads indicate the unmodified and SUMO2 conjugated RPL11 protein, respectively.

2.2 SUMO IS CONJUGATED TO A NON-LYSINE RESIDUE IN RPL11 *IN VITRO*

In order to identify the amino acid residues in RPL11 that can conjugate to SUMO, in collaboration with Dr. Dimitris Xirodimas (CNRS, Montpellier, France), we obtained different RPL11 constructs in which several lysine residues were mutated to arginine (Figure 1, materials and methods). In addition, we also obtained a lysine less RPL11 construct (a plasmid with all lysine residues mutated to arginine, RPL11 KO). We then performed *in vitro* SUMOylation assays with SUMO1 and using the different RPL11 mutants as a substrate. Surprisingly, we did not observe differences between the SUMO1 conjugation of wild-type and the mutants of RPL11 (Figure 2B), suggesting that SUMO can conjugate to a non-lysine residue in RPL11

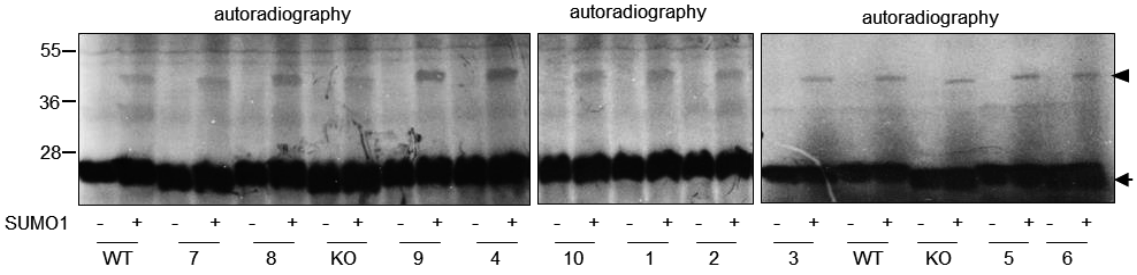

Figure 2B. SUMO conjugates to a non-lysine residue in RPL11 *in vitro*.

In vitro translated [^{35}S]-labelled Flag-RPL11 WT or the indicated mutant proteins were subjected to *in vitro* SUMOylation assay in the presence of SUMO1. Proteins were resolved using SDS-PAGE and visualized by autoradiography. Arrow and arrowheads indicate the unmodifiedand SUMO1 conjugated RPL11 proteins, respectively.

2.3 SUMO IS CONJUGATED TO A NON-LYSINE RESIDUE IN RPL11 *IN VIVO*

To determine whether the SUMOylation of RPL11 also occurs in a non-lysine residue in cells, we co-transfected HEK-293 cells with Flag-RPL11 WT or the indicated Flag-tagged RPL11 mutants together with pcDNA3 or Ubc9 and His6-SUMO2 and, at 36 h after transfection cells were treated with 10 μM MG132 for 4 h to inhibit the degradation of some mutant proteins. Whole protein extracts and histidine-tagged proteins purified under denaturing conditions were analyzed by Western-blot with anti-Flag antibody. We observed that SUMO2 covalently conjugates to all the RPL11 mutants tested (Figure 2C), suggesting that RPL11 can be SUMOylated in a non-lysine residue.

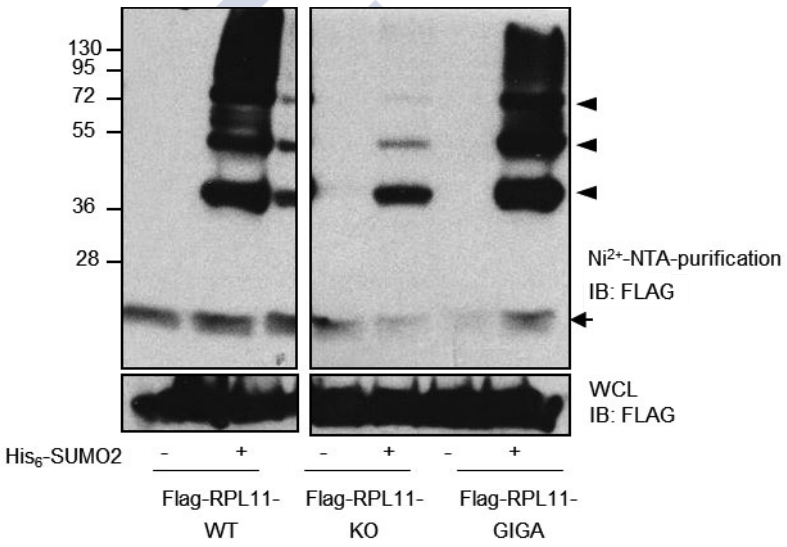

Figure 2C. SUMO2 conjugates to a non-lysine residue in RPL11 *in vivo*.

HEK-293 cells were co-transfected with the indicated mutants together with pcDNA or Ubc9 and His6-SUMO2. 36 hours after transfection cells were treated with MG132 for 4 hours. Whole protein extracts and histidine-tagged purified proteins were then analyzed by Western-blot using anti-Flag antibody. Arrow and arrowheads indicate the unmodified and SUMO2 conjugated RPL11 protein, respectively.

2.4 SUMOYLATION OF RPL11-KO IS TAG-INDEPENDENT *IN VIVO*

Since the Flag tag contains two lysine residues, and in order to avoid a putative conjugation of SUMO to the Flag tag, we cloned the coding sequence of both wild-type and lysine-less (K0) RPL11 fused to HA tag, which does not have any lysine residue, and repeated the *in vivo* SUMOylation assay, as described above. We co-transfected HEK-293 cells with HA-RPL11 WT or HA-RPL11 K0 together with pcDNA3 or Ubc9

and His6-SUMO2. At 48 h after transfection, the cells were harvested, lysed in denaturing conditions and the histidine-tagged proteins were purified using nickel affinity beads. Whole cells lysates and histidine-purified proteins were then analyzed by Western-blot using anti-HA antibody. We detected the bands of the expected size corresponding to RPL11-SUMO2 in the purified extracts of both WT and K0 RPL11 transfected cells, confirming that SUMO conjugates to a non-lysine residue in RPL11 (Figure 2D).

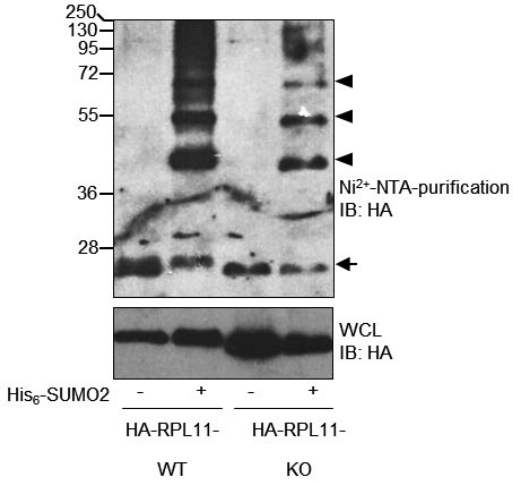

Figure 2D. SUMOylation of RPL11-KO is tag-independent *in vivo*

HEK-293 cells were co-transfected with HA-RPL11-WT or HA-RPL11-K0 together with pcDNA, or Ubc9 and His6-SUMO2. Whole protein extracts and histidine tagged purified proteins were analyzed by Western-blot using anti-HA antibody. Arrow and arrowheads indicate the unmodified and SUMO2 conjugated RPL11 protein, respectively.

2.4 SUMOYLATION OF RPL11-K0 IS TAG-INDEPENDENT *IN VITRO*

To confirm the SUMOylation of RPL11 K0 *in vitro* we also carried out an *in vitro* SUMOylation/deSUMOylation assay using untagged WT (Figure 2E, left panel) or K0 (Figure 2E, right panel) RPL11 proteins as a substrate. To synthesize untagged RPL11 WT or RPL11 K0 protein we first synthesize DNA encoding for RPL11 WT or RPL11 K0 proteins fused to T7 promoter and we then used this DNA to in vitro transcribe/translate the proteins, in the presence of [^{35}S]methionine, using rabbit reticulocytes. After *in vitro* SUMOylation assay we observed the appearance of bands corresponding with SUMOylated WT and K0 RPL11 proteins that disappeared after incubation with SENP (Figure 2E). Altogether, these results indicate that SUMOylation of RPL11 is lysine- and tag- independent.

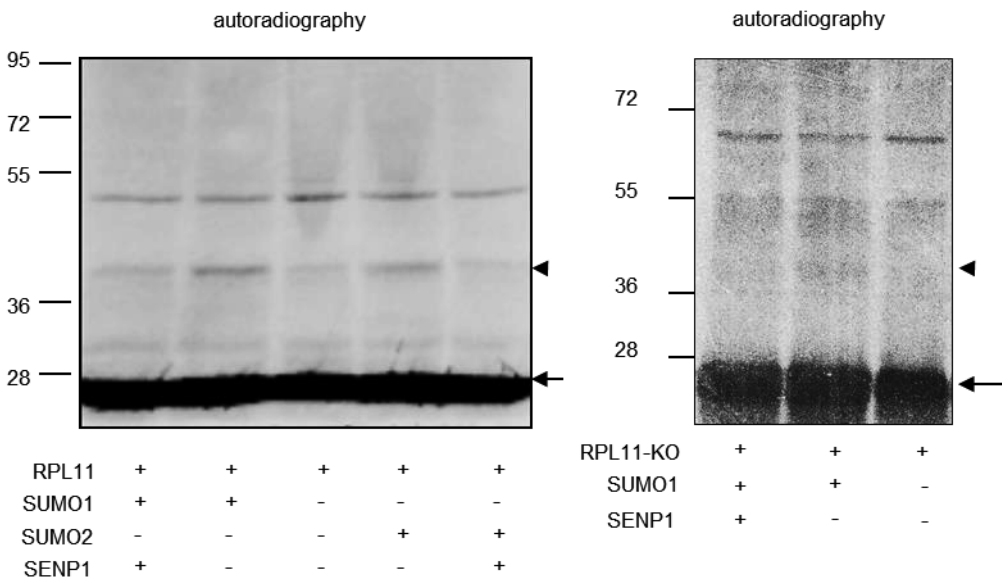

Figure 2E. SUMOylation of RPL11-K0 is tag-independent *in vitro*

In vitro translated [^{35}S]-labelled untagged WT (left panel) or K0 (right panel) RPL11 proteins were subjected to *in vitro SUMOylation assay in the* presence of SUMO1 or SUMO2, as indicated. SUMOylated RPL11 protein was then incubated in the presence or absence of SENP1, as indicated. Proteins were resolved by SDS-PAGE and visualized by autoradiography.

3. INTERPLAY BETWEEN SUMO AND NEDD8 CONJUGATION TO RPL11

3.1 SUMO1 DOWNREGULATES NEDD8 CONJUGATION TO RPL11

It has been previously reported that RPL11 can be conjugated to the ubiquitin-like protein NEDD8 and that only after mutation of all the lysine residues in RPL11 a reduction in NEDDylation could be observed (Sundqvist et al., 2009). Our results indicated that SUMO can conjugate to RPL11 in a lysine residue-independent manner. Therefore, we hypothesized that SUMO might compete with NEDD8 for RPL11 conjugation. To evaluate this hypothesis, we first co-transfected HEK-293 cells with myc-RPL11 together with pcDNA, His6-NEDD8 and pcDNA or His6-NEDD8 and untagged SUMO1. At 48 h after transfection, the cells were harvested, lysed in denaturing conditions and the histidine-tagged proteins were purified using nickel affinity beads. Whole cell lysates and histidine-purified proteins were then analyzed by Western-blot using anti-myc antibody. Analysis of the histidine-tagged purified proteins revealed the appearance of bands corresponding to RPL11-NEDD8 protein only in the His6-NEDD8 transfected cells. Interestingly, we observed a clear reduction in the levels of NEDDylated RPL11 protein in the lane corresponding to SUMO1 co-

transfected cells, suggesting that upregulation of SUMO1 may downmodulate the NEDDylation of RPL11 (Figure 3A).

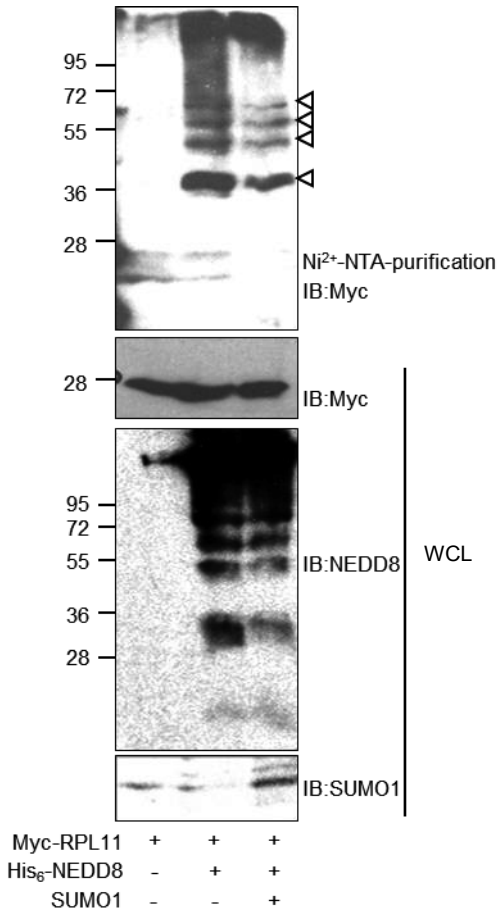

Figure 3A. SUMO1 downregulates NEDD8 conjugation to RPL11.

HEK-293 cells were co-transfected with myc-RPL11 together with pcDNA3, His6-NEDD8 or His6-NEDD8, Ubc9 and untagged SUMO1. At 48 h after transfection, histidine-tagged purified proteins were analyzed by Western-blot using anti-myc antibody. Open arrowheads indicate the NEDD8 conjugated RPL11 protein. Whole protein extracts were analyzed with the indicated antibodies.

3.2 SUMO2 DOWNREGULATES NEDD8 CONJUGATION TO RPL11

To evaluate a putative competition between SUMO2 and NEDD8 to conjugate to RPL11, we co-transfected HEK-293 cells with myc-RPL11 together with pcDNA, His6-NEDD8 and pcDNA, Ubc9, His6-SUMO2 and pcDNA or Ubc9, His6-SUMO2 and His6-NEDD8, and 48 h after transfection, histidine-tagged proteins were purified under denaturing conditions. Western-blot analysis of the histidine-tagged purified proteins using anti-myc antibody revealed the appearance of bands corresponding to RPL11-SUMO2 and RPL11-NEDD8 in cells transfected with His6-SUMO2 or His6-NEDD8, respectively. When both proteins His6-SUMO2 and His6-NEDD8 were overexpressed together we observed a clear reduction in the levels of the NEDDylated RPL11 protein (Figure 3B), suggesting that upregulation of SUMO2 downregulates the conjugation of RPL11 to NEDD8.

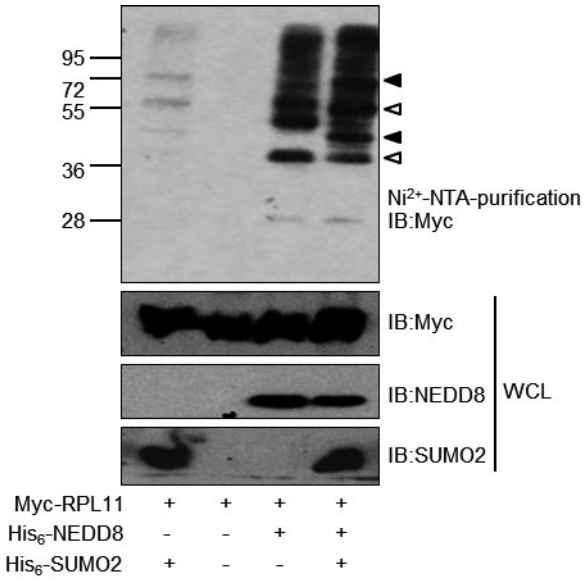

Figure 3B. SUMO2 downregulates NEDD8 conjugation to RPL11

HEK 293 cells were co transfected with myc RPL11 together with pcDNA3, His6 NEDD8, Ubc9 and His6 SUMO2 or His6 NEDD8, Ubc9 and His6 SUMO2. At 36 h after transfection, histidine tagged purified proteins were analyzed by Western blot using anti myc antibody Open and solid arrowheads indicate the NEDD8 and the SUMO2 conjugated RPL11 protein, respectively. Whole protein extracts were analyzed with the indicated antibodies.

3.3 TREATMENT OF CELLS WITH THE SUMOYLATION INHIBITOR GINKGOLIC ACID (GA) POTENTIATES NEDD8 CONJUGATION TO RPL11

To further study the SUMO2-NEDD8 competition to conjugate to RPL11, we decided to evaluate the NEDDlation of RPL11 after treatment with the SUMOylation inhibitor ginkgolic acid (GA). We co-transfected HEK-293 cells with myc-RPL11 together with pcDNA, His6-NEDD8 or Ubc9 and His6-SUMO2, and 36 h after transfection cells were treated or not with GA for 4 h. Whole protein extracts were then analyzed by Western-blot with anti-myc antibody. We observed a decrease in the levels of SUMOylated RPL11 protein after treatment with ginkgolic acid, as expected (Figure 3C, left panel). In contrast, we detected an increase in the levels of the NEDDylated RPL11 protein in those cells treated with the SUMOylation inhibitor (Figure 3C, right panel). These results suggested that SUMOylation inhibition upregulates NEDD8 conjugation to RPL11

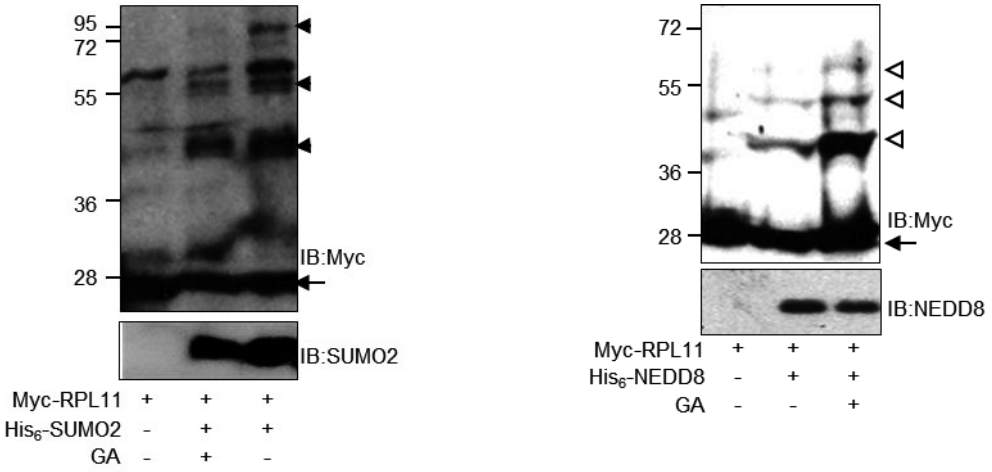

Figure 3C. TREATMENT WITH GA UPREGULATES NEDD8 CONJUGATION TO RPL11

HEK-293 cells were co-transfected with myc-RPL11 together with pcDNA3, His6-NEDD8 or Ubc9 and His6-SUMO2. At 36 h after transfection, cells were treated or not with the SUMOylation inhibitor GA (25 μM). At 4 h after treatment, whole protein extracts were analyzed by Western-blot using the indicated antibodies. Arrows indicated the unmodified RPL11 protein. Open and solid arrowheads indicate the NEDD8 and the SUMO2 conjugated RPL11 protein, respectively.

3.4 TREATMENT WITH THE NEDDYLATION INHIBITOR MLN4924 POTENTIATES SUMO CONJUGATION TO RPL11

Our results suggested that SUMO can downmodulate the NEDDylation of RPL11. However, whether NEDD8 can downmodulate the SUMOylation of RPL11 was not clear. To further study this possibility, HEK-293 cells were transfected with HA-RPL11 together with pcDNA, His6-NEDD8 or Ubc9 and His6-SUMO2, and 36 h after transfection, cells were treated or not with the NEDDylation inhibitor MLN4924 for 4 h. At 48 h after transfection, the cells were harvested, lysed in denaturing conditions and the histidine-tagged proteins were purified using nickel affinity beads. Whole cells lysates and histidine-tagged purified proteins analyzed by Western-blot using anti-HA antibody revealed the appearance of bands corresponding with NEDDylated-RPL11 in the lanes corresponding with His6-NEDD8 transfected cells, whose intensity clearly decreased in the cells treated with MLN4924 (Figure 3D), as previously reported (Soucy et al., 2009). In contrast, treatment of cells with MLN4929 induced an increase in the levels of the RPL11-SUMO2 protein (Figure 3D). These results suggested that NEDD8 may compete with SUMO2 to conjugate to RPL11.

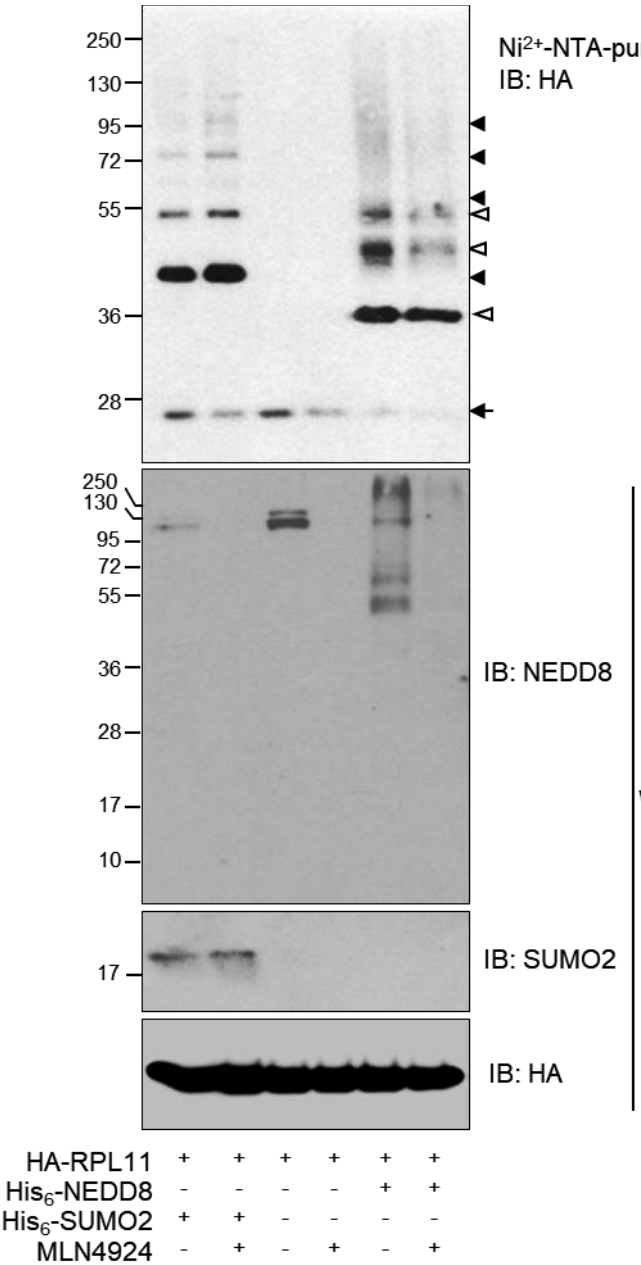

Figure 3D. TREATMENT WITH THE NEDDylation Inhibitor MLN4929 upregulates SUMO conjugation to RPL11

HEK-293 cells were co-transfected with HA-RPL11 together with pcDNA3, His6- NEDD8 or Ubc9 and His6-SUMO2. At 36 h after transfection, cells were treated or not with the NEDDylation inhibitor MLN4924 (1 μM). At 4 h after treatment, histidine-tagged purified proteins were analyzed by Western-blot using anti-HA antibody. Open and solid arrowheads indicate the NEDD8 and the SUMO2 conjugated RPL11 protein, respectively.

3.5 COMPETITION BETWEEN ENDOGENOUS SUMO AND NEDD8 TO CONJUGATE TO RPL11

Finally, we also tested whether endogenous SUMO can compete with endogenous NEDD8 to conjugate to RPL11. For that, HEK-293 cells were transfected with Ubc9 siRNA (siUbc9) or scramble siRNA (siC) and at 48 h cells were transfected with myc-RPL11. At 24 h after transfection cells were harvested, lysed in denaturing conditions and protein extracts were subjected to immunoprecipitation with anti-myc antibody. Western-blot analysis of the immunoprecipitated proteins using anti-NEDD8 antibody showed a band of the expected size corresponding to RPL11-NEDDylated protein whose intensity clearly increased in those cells transfected with siUbc9 (Figure

3E), indicating that endogenous SUMO can compete with endogenous NEDD8 to conjugate to RPL11.

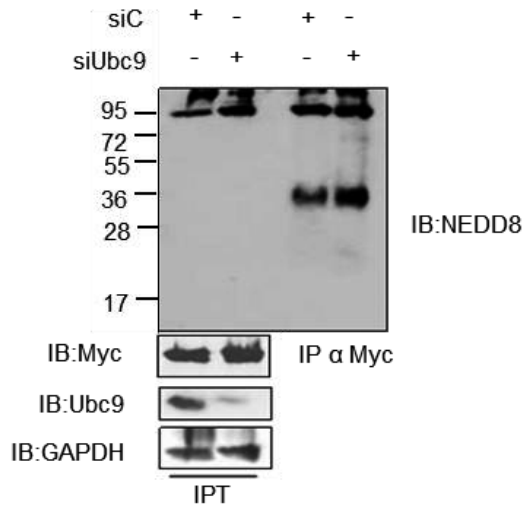

Figure 3E. Endogenous SUMO and NEDD8 compete to conjugate with RPL11

HEK-293 cells were transfected with Ubc9 siRNA (siUbc9) or scramble siRNA (siC). At 48 h after transfection, the cells were transfected with myc-RPL11 and at 24h total protein extracts and immunoprecipitated transfected RPL11 protein using anti-myc antibody were analyzed by Western blot using the indicated antibodies

3.6 COMPETITION BETWEEN SUMO AND NEDD8 TO CONJUGATE TO RPL11 REQUIRES LYSINE RESIDUES TO OCCUR

Our results show that SUMO and NEDD8 compete to conjugate to RPL11 and that SUMOylation of RPL11 can occur in a lysine residue-independent manner. Therefore, we decided to evaluate whether SUMO and NEDD8 compete to conjugate to RPL11 K0. HEK-293 cells were co-transfected with HA-RPL11-WT or HA-RPL11-K0 together with pcDNA, His6-NEDD8 and pcDNA, Ubc9, His6-SUMO2 and pcDNA or Ubc9, His6-SUMO2 and His6-NEDD8. At 48 h after transfection, histidine-tagged proteins were purified under denaturing conditions. Western-blot analysis of the purified proteins using anti-HA antibody revealed the appearance of bands corresponding to RPL11-WT-SUMO2 and RPL11-WT-NEDD8, as expected, and the negative impact of SUMO2 transfection on RPL11-WT NEDDylation, as previously observed (Figure 3F). However, we did not observe a decrease in the levels of the NEDDylated RPL11-K0 protein after overexpression of SUMO2 (Figure 3F). These results suggest that SUMO and NEDD8 can conjugate to different residues on RPL11-K0.

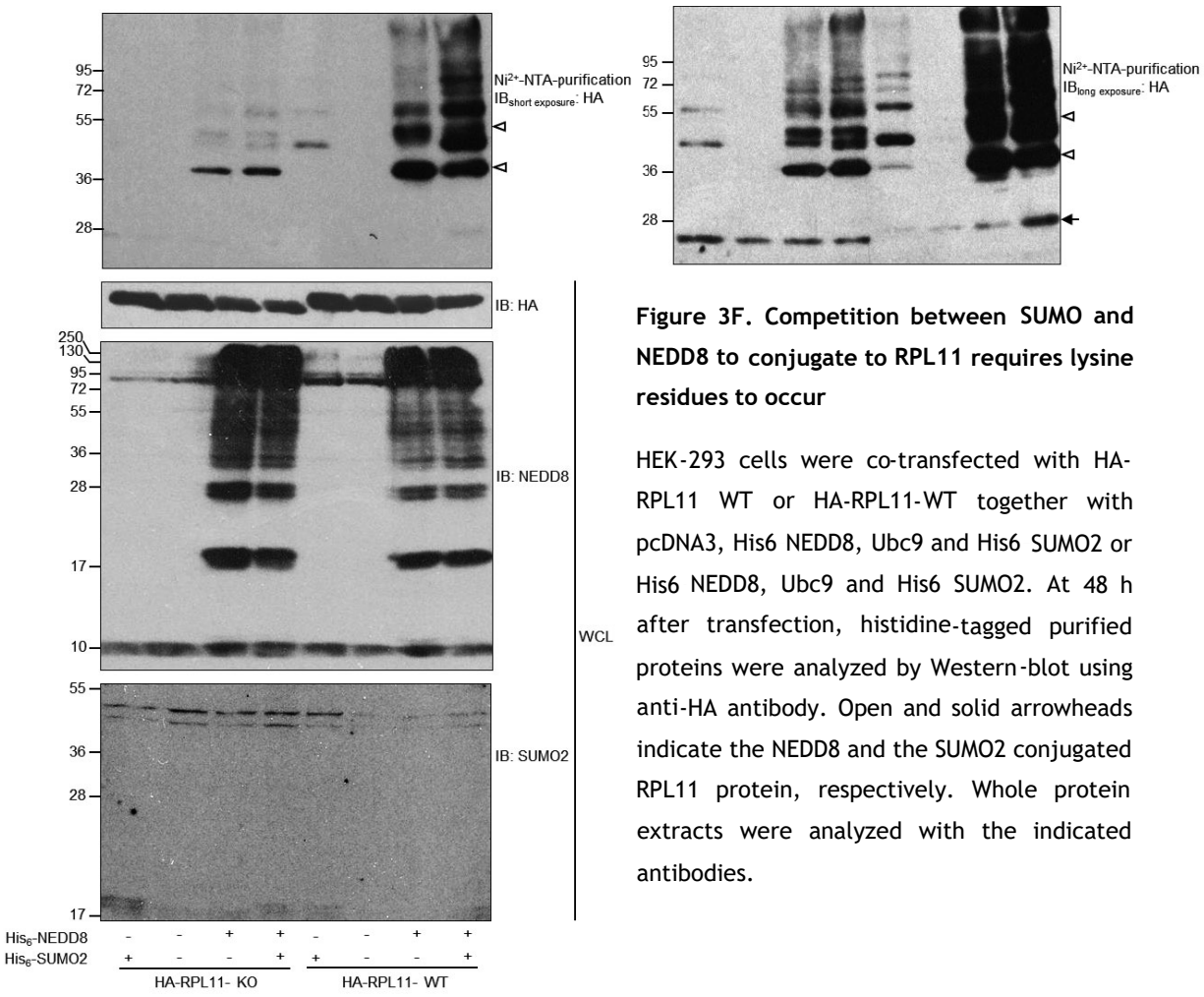

Figure 3F. Competition between SUMO and NEDD8 to conjugate to RPL11 requires lysine residues to occur

HEK-293 cells were co-transfected with HA-RPL11 WT or HA-RPL11-WT together with pcDNA3, His6 NEDD8, Ubc9 and His6 SUMO2 or His6 NEDD8, Ubc9 and His6 SUMO2. At 48 h after transfection, histidine-tagged purified proteins were analyzed by Western-blot using anti-HA antibody. Open and solid arrowheads indicate the NEDD8 and the SUMO2 conjugated RPL11 protein, respectively. Whole protein extracts were analyzed with the indicated antibodies.

4. OVEREXPRESSION OF SUMO2 PROMOTES THE NUCLEOLUS TO NUCLEOPLASM TRANSLOCATION OF RPL11 AND THE SUMO CONJUGATION ENZYME UBC9 IS REQUIRED FOR THE ACTIVATION OF P53 IN RESPONSE TO RPL11 UPREGULATION.

4.1 OVEREXPRESSION OF SUMO2 PROMOTES THE NUCLEOLUS TO NUCLEOPLASM TRANSLOCATION OF RPL11

SUMO conjugation can alter the subcellular localization, stability or activity of the substrate protein. To study the consequences of the conjugation of SUMO to RPL11, we started by evaluating the effect of SUMO in the subcellular localization of RPL11. We co-transfected MCF7 cells with myc-RPL11 together with pcDNA or His6-SUMO2 and 48 h after transfection, cells were fixed, permeabilized and analyzed by immunofluorescence using anti-myc and anti-SUMO2 antibodies. RPL11 was detected

in the nucleolus of those cells co-transfected with pcDNA, as it has been previously reported (Havel et al., 2015) (Figure 4A). However, RPL11 was detected in the nucleoplasm in those cells transfected with SUMO2, suggesting that SUMO2 promotes the release of RPL11 from the nucleolus to the nucleoplasm.

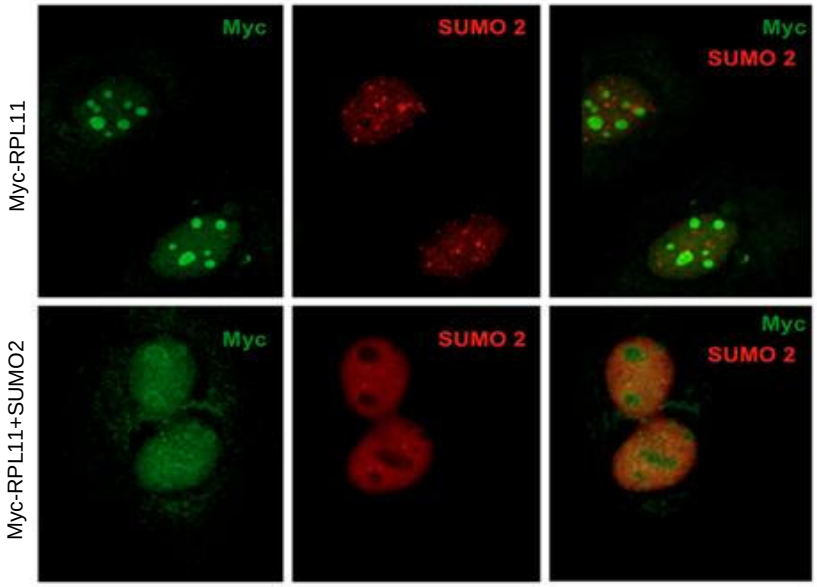

Figure 4A. SUMO overexpression promotes the translocation of RPL11 from the nucleolus to the nucleoplasm

MCF7 cells were co-transfected with myc-RPL11 and pcDNA3 or SUMO2, as indicated. At 48 h after transfection cells were immunostained with anti-myc and anti-SUMO2 antibodies. Subcellular localization of the expressed proteins was analyzed under a confocal microscope. Images were processed with Adobe Photoshop.

4.2 SUMO CONJUGATION IS REQUIRED FOR THE STABILIZATION OF P53 MEDIATED BY RPL11

Interestingly, the effect of SUMO on the subcellular localization of RPL11 is the opposite effect of NEDD8 conjugation, which promotes the nucleolar localization of RPL11. NEDD8 has also been reported to inhibit the stability of p53 mediated by RPL11(Sundqvist et al., 2009). Therefore, we decided to evaluate the effect of SUMO on the stability of p53, and consequently on the cell cycle arrest mediated by RPL11. We transfected U20S cells (p53 WT) with siRNA Ubc9 or scramble siRNA (siC), and 48 h after transfection, cells were transfected with pcDNA3 or myc-RPL11. At 24 h after transfection, cells were treated with cycloheximide (CHX) and at the indicated times after treatment whole protein extracts were analyzed by Western-blot with the

indicated antibodies. We did not observe significant differences in the stability of p53 between the cells transfected with siRNA Ubc9 or siC in cells transfected with pcDNA (Figure 4B, upper panel). Overexpression of RPL11 clearly increased the stability of p53, as it has been previously reported (Lohrum et al., 2003; Zhang et al., 2003). However, the p53 stability was clearly reduced after silencing Ubc9 (Figure 4B, upper panel). Altogether these results indicate that SUMO conjugation is required for the stability of p53 mediated by RPL11.

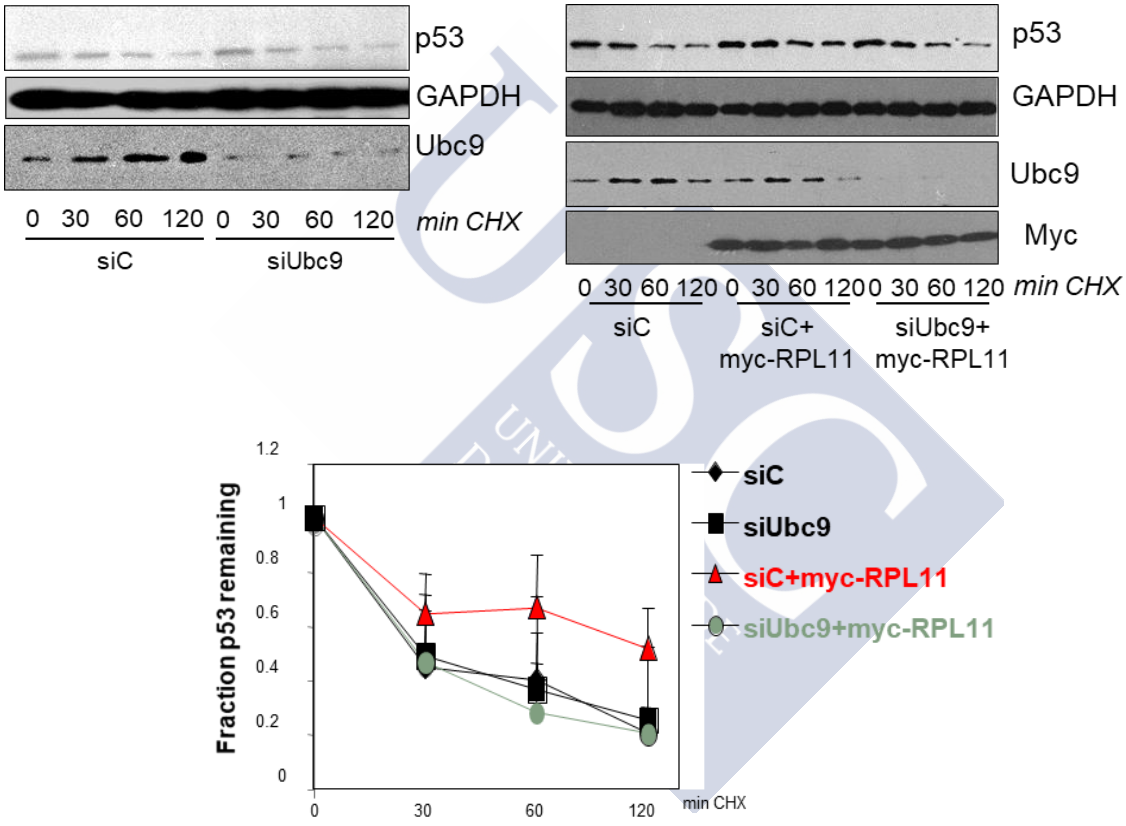

Figure 4B. SUMO conjugation is required for the stabilization of p53 mediated by RPL11.

U2OS cells were transfected with siC or siUbc9 and 48 h after transfection the cells were transfected with myc-RPL11 or pcDNA3. At 24 h cells were treated with cycloheximide (CHX) (100 µg/ml). At the indicated times after CHX treatment protein extracts were recovered and analyzed by Western-blotting using the indicated antibodies (upper panels). The levels of p53 remaining at each time were quantified from three different experiments using ImageJ and values were normalized to time zero samples. The average fraction of p53 remaining and their standard deviations are shown (lower panel).

4.3 SUMO CONJUGATION IS REQUIRED FOR THE CELL CYCLE ARREST MEDIATED BY RPL11

Overexpression of RPL11 induces upregulation of p53 and cell cycle arrest. Therefore, we decided to evaluate the effect of Ubc9 downmodulation on the cell cycle arrest induced in response to RPL11 overexpression. U2OS cells were transfected with siUbc9 or siC and 48 h after transfection, cells were co-transfected with pcDNA or myc-RPL11 together with a plasmid expressing farnesylated GFP (GFP-F) (ratio 5:1). At 24 h after transfection, GFP positive cells were gated and the cell cycle distribution was evaluated by flow cytometry analysis. We did not observe significant differences in the percentage of cells in S-phase between cells transfected with siRNA Ubc9 or siC in those cells co-transfected with pcDNA. We detected a significant decrease in the percentage of cells in S-phase in the cells co-transfected with siC and RPL11 relative to the percentage detected in the cells co-transfected with siC and pcDNA (Figure 4C), as expected (Zhang et al., 2003). However, a significant reduction in the percentage of cells in S phase induced by RPL11 was not observed after silencing Ubc9. Altogether these results indicate that SUMOylation is required for the activation of p53 in response to RPL11 overexpression.

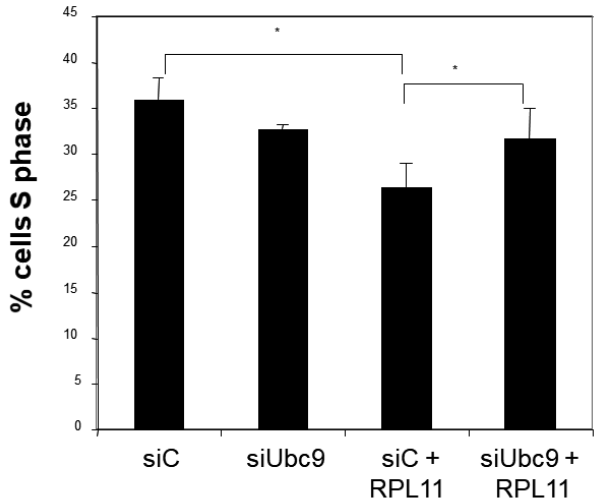

Figure 4C. SUMO conjugation is required for the cell cycle arrest mediated by RPL11.

U2OS cells were transfected with siC or siUbc9 for 48 h. Then, cells were co-transfected with farnesylated GFP (GFP-F) and myc-RPL11 or pcDNA3. At 24 h after transfection, cells were harvested, fixed, permeabilized and stained with propidium iodide. GFP positive cells were gated for cell cycle analysis. Mean percentage of cells in S phase from triplicates is shown. Error bars are standard deviation of triplicates. *P< 0.005, Student's test.

5. NUCLEOLAR STRESS PROMOTES THE MODIFICATION OF RPL11 BY SUMO2

Our results indicate that SUMO and NEDD8 compete to conjugate to RPL11, and this competition may have an impact on the activation of p53, an essential protein in the cellular response to stress. Moreover, it has been reported that RPL11 is de-NEDDylated upon nucleolar stress (Sundqvist et al., 2009). Therefore, we decided to to study the SUMOylation of RPL11 in response to nucleolar stress. U2OS cells were co-transfected with myc-RPL11 and pcDNA or Ubc9 and His6-SUMO2. At 36 h after transfection, cells were treated with low doses of Actinomycin D (5nM) and at different times after treatment whole protein extracts and histidine-tagged proteins purified in denaturing conditions were analyzed by Western-blot using anti-myc antibody. We observed an increase in the levels of RPL11-SUMO2 protein in response to Actinomycin D, indicating that nucleolar stress promotes SUMO2 modification of RPL11.

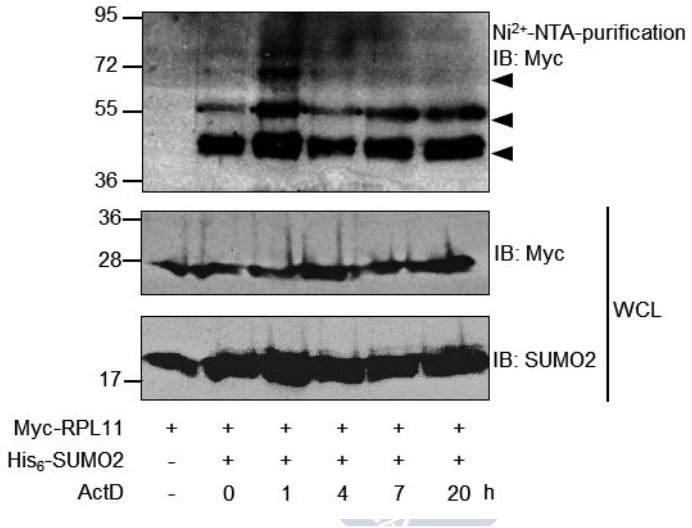

Figure 5. Nucleolar stress promotes the modification of RPL11 by SUMO2

U2OS cells were cotransfected with myc-RPL11 together with pcDNA or Ubc9 and His6-SUMO2. At 36 h after transfection, cells were treated with low concentration of Actinomycin D (5 nM). At the indicated times after treatment, histidine-tagged purified proteins were analyzed by Western-blot using anti-myc antibody. Whole protein extracts were analyzed with the indicated antibodies. Arrowheads indicate the SUMO2 conjugated RPL11 protein.

6. ARF PROMOTES THE SUMO2 MODIFICATION OF RPL11

6.1 ARF MODULATES THE SUMOYLATION OF RPL11

The tumor suppressor ARF promotes the SUMOylation of different interactors (L. Chen & Chen, 2003; Xirodimas et al., 2002). It has been also reported that p14ARF

interacts with RPL11, and that RPL11 is a mediator in ARF regulated p53 activation (Dai et al., 2012; Zhang et al., 2003). Therefore, we decided to evaluate whether ARF can also promote RPL11 SUMOylation. U2OS cells (ARF null) were co-transfected with myc-RPL11 together with pcDNA or Ubc9 and His6-SUMO2, in presence or absence of GFP-ARF. At 48 h after transfection, the cells were harvested, lysed in denaturing conditions and the histidine-tagged proteins were purified using nickel affinity beads. The whole cell lysates and the histidine-purified proteins were then analyzed by Western-blot using anti-myc antibody. As shown in Figure 6A, left panel, overexpression of ARF dramatically upregulated the levels of RPL11-SUMO2 protein. To evaluate if the upregulation in RPL11 SUMOylation induced by ARF is p53 dependent, we carried out the same experiment in the p53-null H1299 cell line. We observed similar results to those detected in U2OS cells (Figure 6A, right panel), indicating that the effect of ARF on RPL11-SUMOylation is p53 independent.

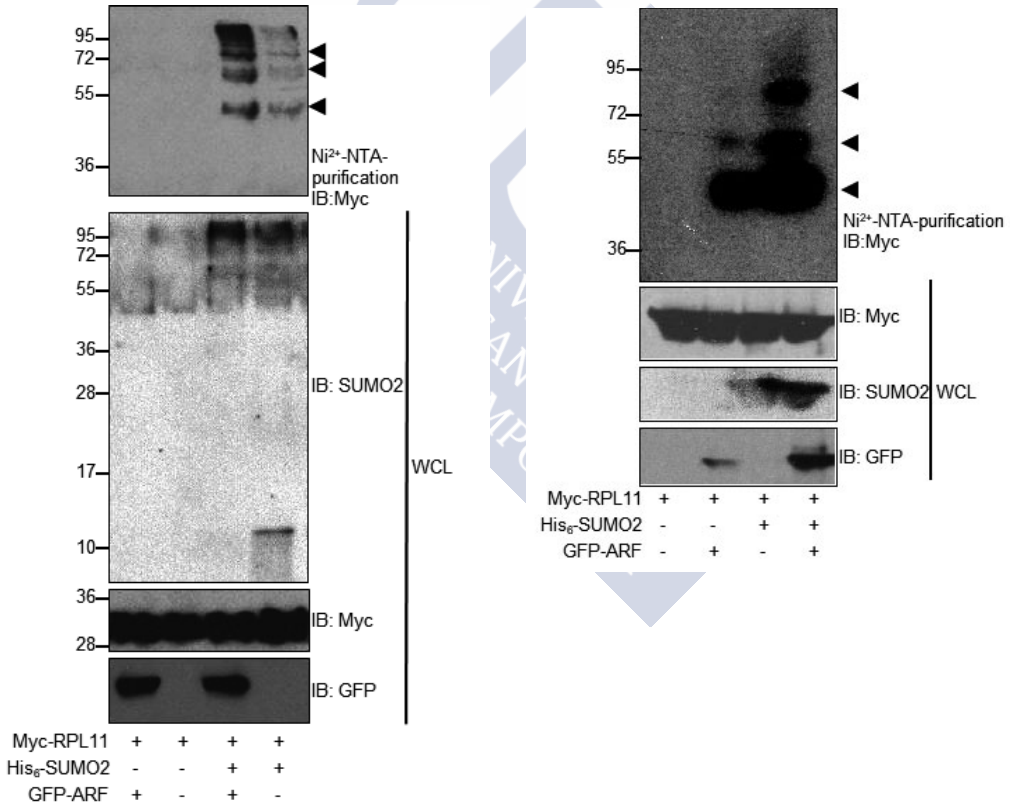

Figure 6A. ARF promotes RPL11 SUMOylation

U2OS cells (left panel) or H1299 cells (right panel) were co-transfected with myc-RPL11 together with pcDNA3 or Ubc9 and His6-SUMO2 and in the presence or absence of GFP-ARF. At 48h after transfection, histidine-tagged purified proteins were analyzed by Western-blot using anti-myc antibody. Solid arrowheads indicate the SUMO2- conjugated RPL11 protein. Whole protein extracts were analyzed with the indicated antibodies.

6.2 ARF DOWNREGULATES RPL11 NEDDYLATION

We showed here that an increase in RPL11 SUMOylation correlated with a decrease in RPL11 NEDDylation. Therefore, we decided to study the effect of ARF overexpression on the NEDDylation of RPL11. U2OS cells were co-transfected with myc-RPL11 and pcDNA or His6-NEDD8, in presence or absence of GFP-ARF. At 48 h after transfection cells were harvested, lysed in denaturing conditions and the histidine-tagged proteins were purified using nickel affinity beads. The whole cells lysates and the histidine-purified proteins were analyzed by Western-blot using anti-myc antibody. We observed that RPL11 NEDDylation was clearly reduced after ARF overexpression (Figure 6B). Interestingly, a global downmodulation of NEDD8 conjugation was also observed when we performed Western-blot analysis using anti-NEDD8 antibody, suggesting that NEDD8 and SUMO may compete to conjugate to other proteins (Figure 6B).

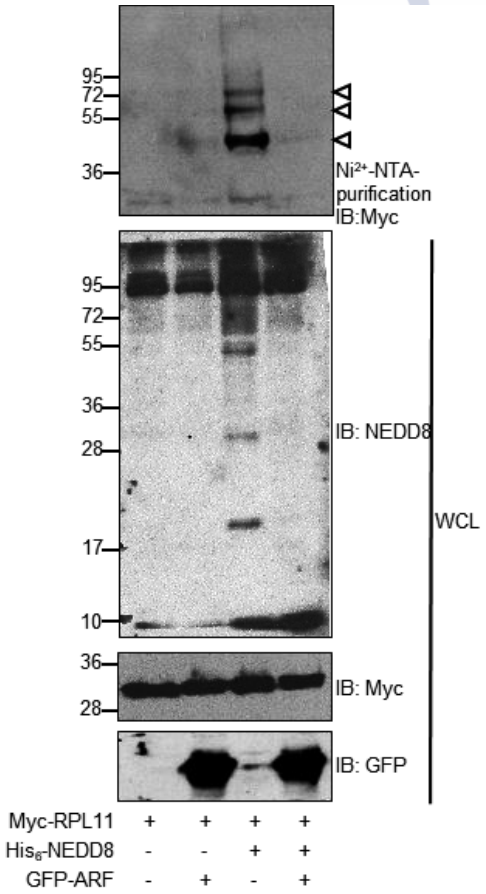

Figure 6B ARF downregulates RPL11 NEDDylation

U2OS cells were co-transfected with myc-RPL11 together with pcDNA3 or His6-NEDD8 and in the presence or absence of GFP-ARF. At 48 h after transfection, histidine-tagged purified proteins were analyzed by Western-blot using anti-myc antibody. Open arrowheads indicate the NEDD8-conjugated RPL11 protein. Whole protein extracts were analyzed with the indicated antibodies.

7. THE RIBOSOMAL RPL23 PROTEIN CAN BE MODIFIED BY SUMO AND NEDD8

7.1 THE RIBOSOMAL RPL23 PROTEIN IS MODIFIED BY SUMO *IN VITRO*

Our data revealed that the activity of the ribosomal RPL11 protein is modulated by SUMO and NEDD8 conjugation. Therefore, we wonder whether these post-translational modifications may also regulate other ribosomal proteins such as RPL23. So far, no SUMO or NEDD8 conjugation to RPL23 has been reported. Then, we first evaluated whether RPL23 is SUMOylated *in vitro*. We performed *in vitro* SUMOylation assay using [^{35}S] methionine-labelled RPL23 *in vitro* translated protein as a substrate. We detected the unmodified myc-RPL23 protein as a band of around 22 kDa molecular weight, as expected (Figure 7A). We observed a higher molecular weight band of around 40 kDa, corresponding to RPL23-SUMO protein when the reaction was incubated with SUMO1 and a fainter band when it was incubated with SUMO2 (Figure 7A). In addition, we also observed that the intensity of the 40 kDa band clearly decreased after the incubation with the SUMO protease SENP1 (Figure 7A), indicating that RPL23 is modified by SUMO1 and SUMO2 *in vitro*.

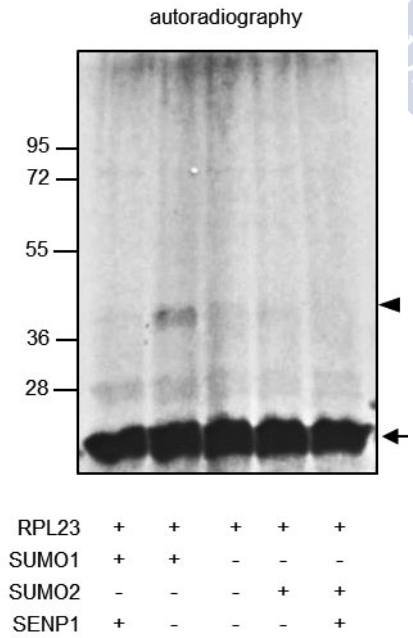

Figure 7A. The ribosomal protein RPL23 can be modified by SUMO *in vitro*

In vitro translated [^{35}S]-labelled RPL23 was subjected to *in vitro* SUMOylation assay in the presence of SUMO1 or SUMO2. SUMOylated protein was then incubated in the presence or absence of SENP1 as described in Material and Methods. Proteins were resolved by SDS-PAGE and visualized by autoradiography. Arrows and arrowheads indicate the unmodified and SUMO conjugated RPL23 protein, respectively.

7.2 RPL23 IS MODIFIED BY SUMO *IN VIVO*

In order to evaluate whether RPL23 is also SUMOylated *in vivo*, we co-transfected HEK-293 cells with myc-RPL23 together with pcDNA3 or Ubc9 and His6-SUMO2. At 48 h after transfection, the cells were harvested, lysed in denaturing conditions and the histidine-

tagged proteins were purified using nickel affinity beads. The whole cell lysates and the histidine-tagged purified proteins were then analyzed by Western-blot using anti-myc antibody. We detected bands of the expected size corresponding to RPL23-SUMO2 only in the purified extracts of those cells co-transfected with His6-SUMO2 (Figure 7B). These results indicated that RPL23 is modified by SUMO2 in transfected cells (Figure 7B).

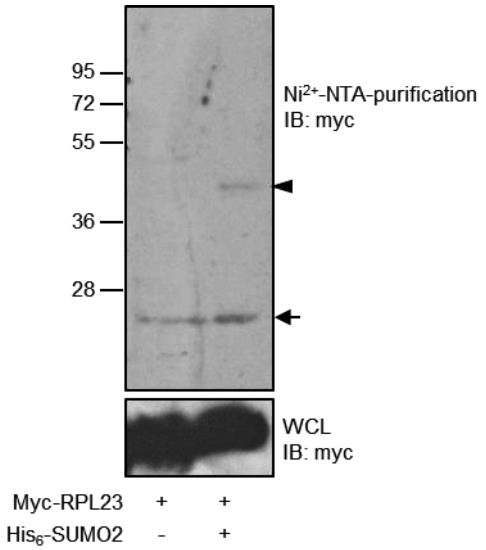

Figure 7B. The ribosomal protein RPL23 can be modified by SUMO2 *in vivo*

HEK-293 cells were co-transfected with myc-RPL23 together with pcDNA or Ubc9 and His6-SUMO2. Whole protein extracts and histidine tagged purified proteins were analyzed by Western-blot using anti-myc antibody. Arrow and arrowheads indicate the unmodified and SUMO conjugated RPL23 protein, respectively.

7.3 RPL23 IS MODIFIED BY NEDD8

In order to evaluate whether RPL23 is modified by NEDD8 *in vivo*, we co-transfected HEK-293 cells with myc-RPL23 together with pcDNA3 or His6-NEDD8. At 48 h after transfection, the cells were harvested, lysed in denaturing conditions and the histidine-tagged proteins were purified using nickel affinity beads. The whole cells lysates and the histidine-tagged purified proteins were then analyzed by Western-blot using anti-myc antibody. Analysis of the purified extracts revealed the appearance of higher molecular weight bands of around 34 and 44 kDa, corresponding to RPL23-NEDD8 protein, only in those cells co-transfected with His6-NEDD8, indicating that RPL23 is modified by NEDD8 in transfected cells (Figure 7C).

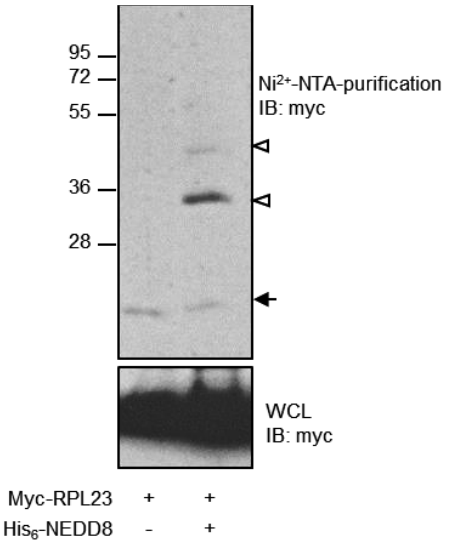

Figure 7C. RPL23 can be modified by NEDD8 in transfected cells

HEK-293 cells were co-transfected with myc-RPL23 together with pcDNA or His6 NEDD8. Whole protein extracts and histidine tagged purified proteins were analyzed by Western-blot using anti-myc antibody. Arrow and open arrowheads indicate the unmodified and NEDD8 conjugated RPL23 protein, respectively.

8. SUMO2 PROMOTES THE TRANSLOCATION OF RPL23 OUTSIDE OF THE NUCLEOLUS

To study the consequences of the conjugation of SUMO to RPL23, we first evaluated the effect of SUMO in the subcellular localization of RPL23. We co-transfected U2OS cells with myc-RPL23 together with pcDNA or His6-SUMO2 and 36 h after transfection, cells were fixed, permeabilized and analyzed by immunofluorescence using anti-myc and anti-His antibodies. After confocal analysis we observed that RPL23 was detected in the nucleolus of those cells co-transfected with pcDNA (Figure 8), as it has been previously reported (Degenhardt & Bonham-Smith, 2008). However, RPL23 was detected outside of the nucleolus in those cells transfected with SUMO2, suggesting that SUMO2 promotes the release of RPL23 from the nucleolus to the nucleoplasm.

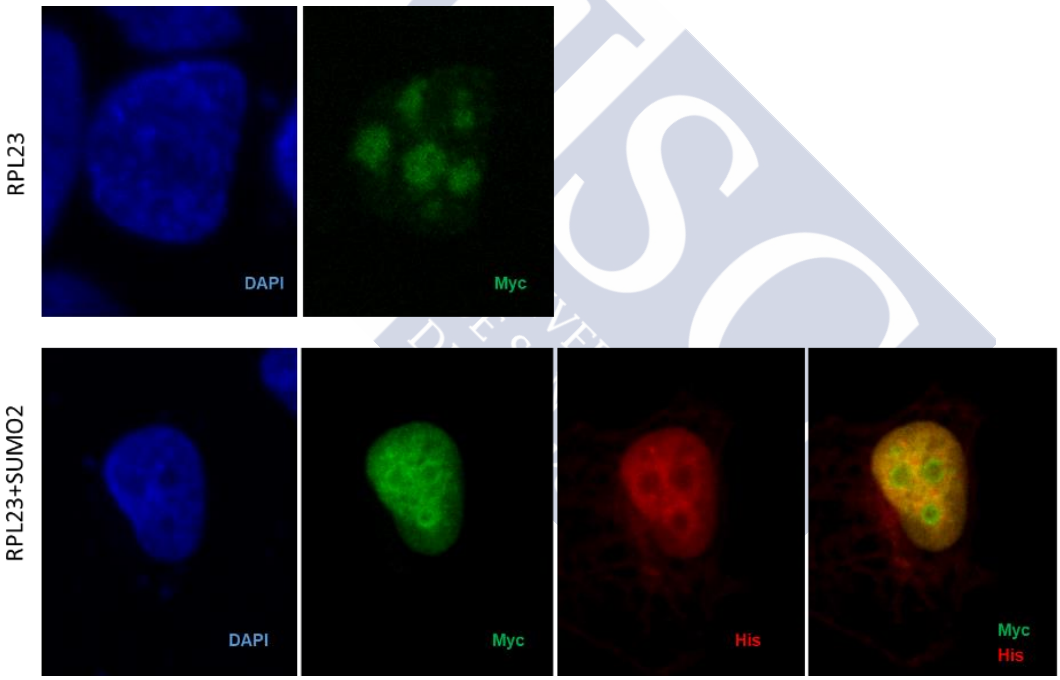

Figure 8. SUMO promotes the translocation of RPL23 outside of the nucleolus

U2OS cells were co-transfected with myc-RPL23 together with pcDNA or His6-SUMO2, as indicated. At 36 h after transfection cells were immunostained with anti-myc and anti-His antibodies. Subcellular localization of the expressed proteins was analyzed under a confocal microscope. Images were processed using Adobe Photoshop.

9. INTERPLAY BETWEEN SUMO AND NEDD8 CONJUGATION TO RPL23

In order to evaluate whether SUMO2 and NEDD8 also compete to conjugate to RPL23, we co-transfected HEK-293 or U2OS cells with myc-RPL23 together with pcDNA, His6-

NEDD8 and pcDNA, Ubc9, His6-SUMO2 and pcDNA or Ubc9, His6-SUMO2 and His6-NEDD8, and 36 h after transfection, histidine-tagged proteins were purified under denaturing conditions. Western-blot analysis of the histidine-tagged purified proteins using anti-myc antibody revealed the appearance of bands corresponding to RPL23-SUMO2 and RPL23-NEDD8 in cells transfected with His6-SUMO2 or His6-NEDD8, respectively both in HEK-293 (Figure 9, left panel) and in U2OS (Figure 9, right panel) cells. We observed a clear reduction in the levels of the NEDDylated RPL23 protein when both proteins His6-SUMO2 and His6-NEDD8 were overexpressed (Figure 9), suggesting that upregulation of SUMO2 downregulates the conjugation of RPL23 to NEDD8.

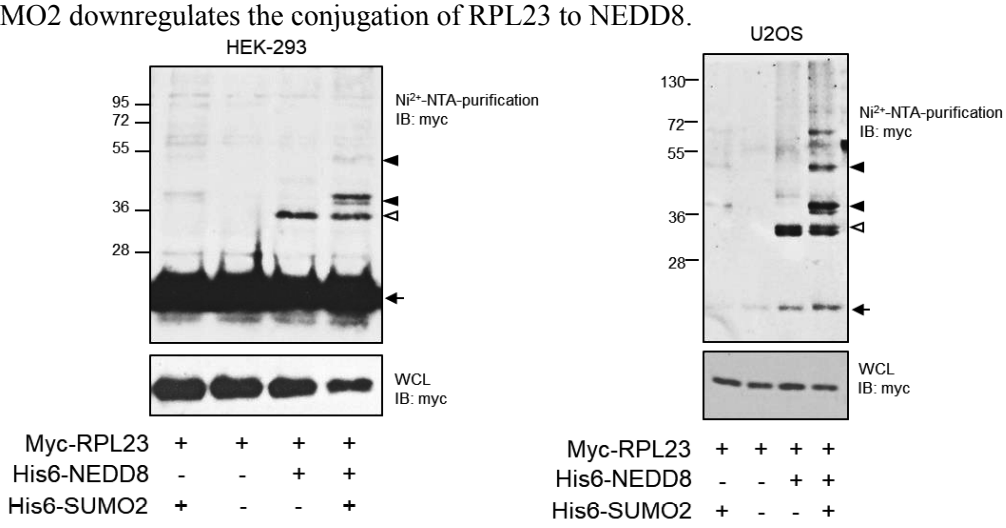

Figure 9. SUMO2 downregulates NEDD8 conjugation to RPL23

HEK-293 (left panel) or U2OS (right panel) cells were co-transfected with myc-RPL23 together with pcDNA3, His6-NEDD8, Ubc9 and His6-SUMO2 or His6-NEDD8, Ubc9 and His6-SUMO2. At 36 h after transfection, histidine-tagged purified proteins were analyzed by Western-blot using anti-myc antibody. Arrows indicate the unmodified RPL23, and open and solid arrowheads indicate the NEDD8 and the SUMO2 conjugated RPL23 protein, respectively.

10. ARF REGULATES SUMO2 AND NEDD8 MODIFICATION OF RPL23

Our data revealed that ARF modulates the SUMOylation and NEDDylation of RPL11. We then decided to evaluate whether ARF can also modulate RPL23 SUMO2 and NEDD8 conjugation. U2OS cells (ARF null) were co-transfected with myc-RPL23 together with pcDNA, Ubc9 and His6-SUMO2 or His6-NEDD8 and pcDNA, in presence or absence of GFP-ARF. At 48 h after transfection the cells were harvested, lysed in denaturing conditions and the histidine-tagged proteins were purified using nickel affinity beads. The whole cells lysates and the histidine-purified proteins were then analyzed by Western-blot using anti-myc antibody.

Western-blot analysis of the histidine-tagged purified proteins revealed that overexpression of ARF upregulated the levels of RPL23-SUMO2 protein. In contrast, we observed that RPL23 NEDDylation was clearly reduced after ARF overexpression (Figure 10). Altogether, these results suggested that ARF promotes the SUMOylation of RPL23 and downmodulates the NEDDylation of RPL23.

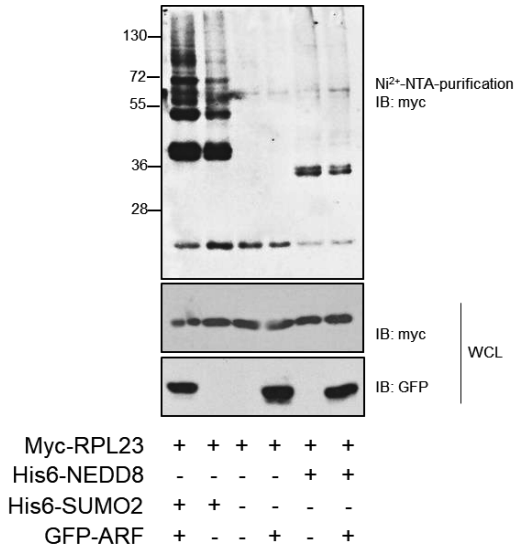

Figure 10. SUMO2 and NEDD8 modification of RPL23 are regulated by ARF.

U2OS cells were co-transfected with myc-RPL23 together with pcDNA3, Ubc9 and His6-SUMO2 or pcDNA and His6-NEDD8, and in the presence or absence of GFP-ARF. At 48h after transfection, whole protein extracts and histidine-tagged purified proteins were analyzed by Western-blot using the indicated antibodies.

11. ARF PROMOTES THE SUMOYLATION OF P53 BUT DOWNMODULATES ITS NEDDYLATION

Here we showed that SUMO and NEDD8 compete to conjugate to RPL11 and RPL23 and that ARF modulates this competition. Therefore, we wonder whether ARF can modulate the SUMOylation and NEDDylation of other proteins. We then decided to evaluate the effect of ARF on the SUMOylation and NEDDylation of p53. HEK-293 cells were co-transfected with Ubc9 and His6-SUMO2 or His6-NEDD8, and in the presence or absence of GFP-ARF. At 48 h after transfection whole protein extracts and histidine-tagged proteins purified under denaturing conditions were evaluated by Western-blot using anti-p53 antibody. Overexpression of ARF clearly upregulated the levels of p53-SUMO2 (Figure 11, left panel), as previously reported (L. Chen & Chen, 2003). We also observed that ARF overexpression led to a decrease in the levels of p53-NEDD8 protein (Figure 11, right panel).

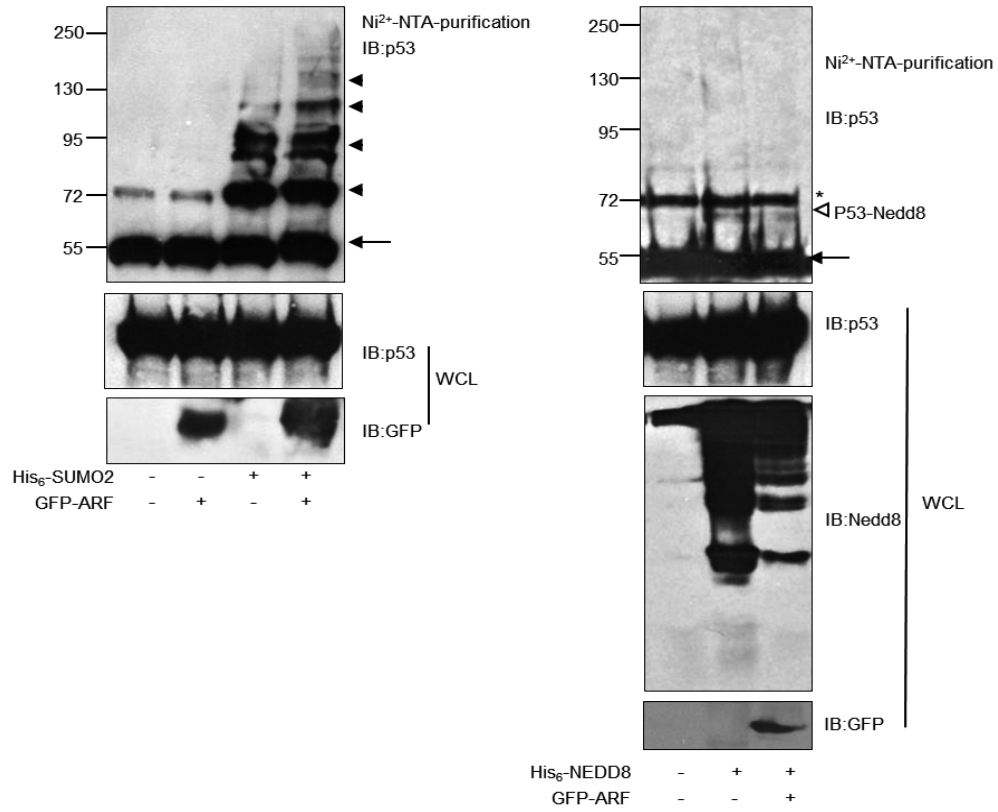

Figure 11. ARF promotes the SUMOylation of p53 but downmodulates its NEDDylation.
Left panel, HEK-293 cells were co-transfected with pcDNA3 or Ubc9 and His6-SUMO2, and in the presence or absence of GFP-ARF. At 48 h after transfection, histidine-tagged purified proteins were analyzed by Western-blot using anti-p53 antibody. Arrowheads indicate the SUMO2-conjugated p53 protein. Whole protein extracts (WCL) were analyzed with the indicated antibodies. Right panel, HEK-293 cells were co-transfected with pcDNA3, His6-NEDD8 and pcDNA or His6-NEDD8 and GFP-ARF. At 48 h after transfection, histidine-tagged purified proteins were analyzed by Western-blot using anti-p53 antibody. Open arrowhead indicates NEDD8-conjugated p53 protein. Whole protein extracts (WCL) were analyzed with the indicated antibodies. The position of a nonspecific band is indicated by an asterisk.

12. NEDD8-SUMO INTERPLAY

12.1 *IN VITRO* SUMOYLATION ASSAY USING NEDD8 PROTEIN AS A SUBSTRATE

Our results suggested that SUMO and NEDD8 may compete for binding to the same residues in RPL11-WT. In addition, we showed that ARF negatively regulates the NEDDylation of RPL11, likely as a consequence of the SUMO-NEDD8 competition to bind to RPL11. However, we also observed that upregulation of ARF downmodulated the conjugation of NEDD8 to RPL23 or to the tumor suppressor p53, modification that has been reported to occur in a lysine residue in p53 different to which SUMO is conjugated (Rodriguez et al., 1999; Xirodimas et al., 2004). Therefore, we

speculated that SUMO may regulate the capability of NEDD8 conjugation to substrates by modifying NEDD8 or NEDDylation enzymes.

To evaluate this hypothesis we first decided to analyze whether NEDD8 protein can be a substrate for SUMO conjugation. First, we performed *in vitro* SUMOylation assay using [^{35}S] methionine-labelled *in vitro* translated NEDD8 protein as a substrate. We detected the unmodified NEDD8 protein as a band of around 10 kDa molecular weight, as expected (Figure 12A). When the reaction was incubated with SUMO1 or SUMO2, we observed the appearance of higher molecular weight bands of around 17, 25 and 40 kDa (Figure 12A), suggesting that (i) NEDD8 protein can be modified by SUMO or that (ii) SUMO is inducing the conjugation of NEDD8 to a substrate *in vitro*.

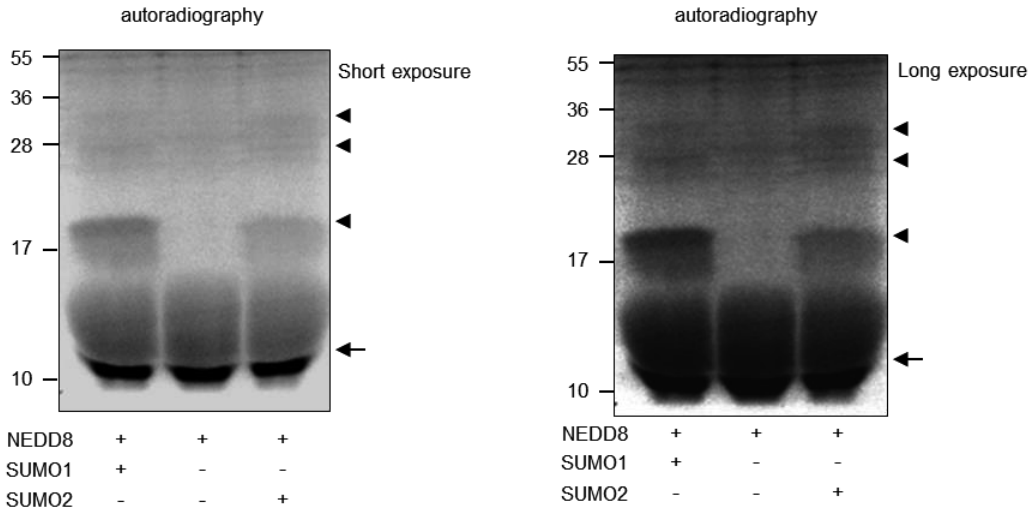

Figure 12A. *In vitro* SUMOylation assay using NEDD8 protein as a substrate

In vitro translated [^{35}S] methionine-labelled NEDD8 was subjected to *in vitro* SUMOylation assay inthe presence of SUMO1 or SUMO2. Proteins were resolved by SDS-PAGE and visualized by autoradiography. Arrows indicate the free NEDD8 protein. Arrowheads indicate NEDD8-SUMO or NEDD8-modified proteins.

12.2 NEDD8-SUMO INTERPLAY IS ALTERED BY MUTATION OF THE LYSINE RESIDUES IN NEDD8

Trying to discern whether SUMO is binding to a lysine residue in NEDD8, we carried out an *in vitro* SUMOylation assay using a lysine less NEDD8 protein (NEDD-K0) as a substrate (generated by our collaborator Dimitris Xirodimas at CNRS, Montepellier, France). We detected the unmodified [^{35}S] methionine-labelled NEDD8

WT or NEDD8-K0 protein as a band of around 10 kDa molecular weight, as expected (Figure 12B). We observed the appearance of the three higher molecular weight bands of around 17, 25 and 40 kDa when the reaction using NEDD8-WT protein as a substrate was incubated with SUMO1 or SUMO2 (Figure 12B). However, we only detected the 17 kDa band in those reactions using NEDD8-K0 as a substrate (Figure 12B), indicating that NEDD8-SUMO interplay requires the lysine residues in NEDD8. Altogether, these results suggest that SUMO can play a role in the regulation of NEDD8 function.

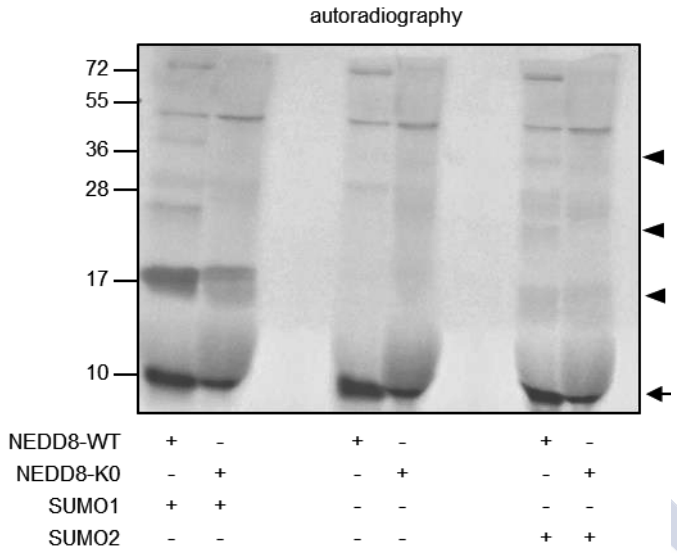

Figure 12 B. NEDD8-SUMO interplay is altered by mutation of lysine residues in NEDD8 protein. *In vitro* translated [^{35}S] methionine labelled WT or K0 NEDD8 proteins were subjected to *in vitro* SUMOylation assay in the presence of SUMO1 or SUMO2. Proteins were resolved by SDS-PAGE and visualized by autoradiography. Arrows indicate the free NEDD8 protein. Arrowheads indicate NEDD8-SUMO or NEDD8-modified proteins.

13. P14ARF IS MODIFIED BY SUMO

13.1 P14ARF IS MODIFIED BY SUMO *IN VITRO*

Our data indicated that ARF can modulate both SUMO and NEDD8 conjugation, suggesting it can regulate the NEDD8-SUMO interplay. Therefore, we speculated that ARF itself may be a substrate of SUMO and/or NEDD8. To evaluate this hypothesis we first decided to analyze whether ARF protein can be a substrate for SUMO conjugation. We performed an *in vitro* SUMOylation/deSUMOylation assay using *in vitro* translated p14ARF-HA protein as a substrate. Samples were evaluated by Western-blot using anti-HA antibody. We detected the unmodified ARF protein as a band of around 20 kDa molecular weight, as expected (Figure 13A). When the reaction was

incubated with SUMO2 we observed the appearance of two higher molecular weight bands of around 34 and 50 kDa (Figure 13A), suggesting that p14ARF is modified by SUMO2 *in vitro*. When the SUMOylated protein was incubated with the recombinant SUMO-specific protease SENP1 we observed a clear decrease in the intensity of both higher molecular weight bands (Figure 13A). Altogether these results indicate that p14ARF is modified by SUMO2 *in vitro*.

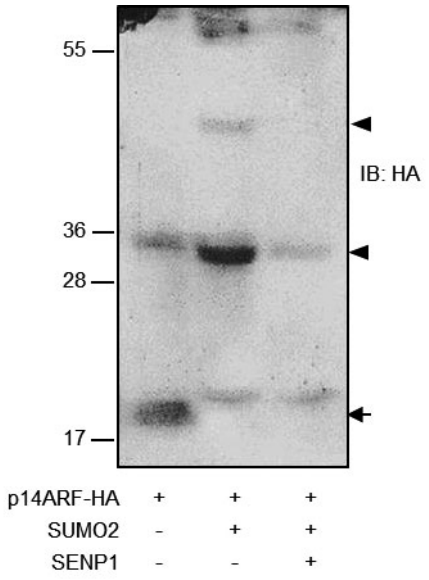

Figure 13A. ARF is modified by SUMO *in vitro*
In vitro translated p14-ARF-HA was subjected to *in vitro* SUMOylation assay in the presence of SUMO2. Proteins were resolved by SDS-PAGE and evaluated by Western-blot analysis using anti-HA antibody. Arrows and arrowheads indicate the unmodified and SUMO conjugated ARF protein, respectively.

13.2 P14ARF IS MODIFIED BY SUMO *IN VITRO* IN A TAG-INDEPENDENT MANNER

To further confirm that p14ARF protein can be SUMOylated *in vitro*, we also carried out *in vitro* SUMOylation (Figure 13B) assay using untagged p14ARF protein as a substrate. We detected the unmodified ARF protein as a band of around 15 kDa molecular weight, as expected (Figure 13B). When the reaction was incubated with SUMO2 we observed the appearance of two higher molecular weight bands of around 28 and 42 kDa (Figure 13B), indicating that p14ARF is modified by SUMO1 and SUMO2 *in vitro* in a tag-independent manner.

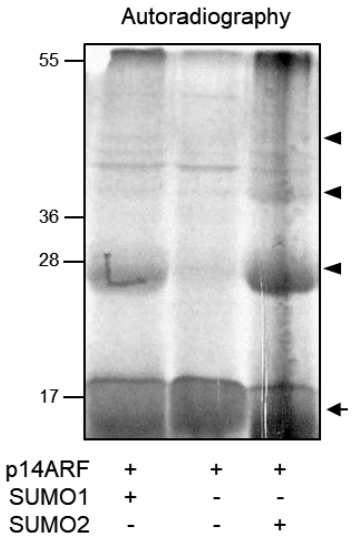

Figure 13B. SUMOylation of ARF is tag-independent *in vitro*

In vitro translated [^{35}S] methionine labelled untagged ARF was subjected to *in vitro* SUMOylation assay in the presence of SUMO1 or SUMO2. Proteins were resolved by SDS-PAGE and visualized by autoradiography.

13.3 P14ARF IS MODIFIED BY SUMO *IN VIVO*

In order to evaluate whether ARF is also SUMOylated *in vivo*, we co-transfected HEK-293 cells with GFP-ARF together with pcDNA3 or Ubc9 and His6-SUMO2. At 48 h after transfection the cells were harvested, lysed in denaturing conditions and the histidine-tagged proteins were purified using nickel affinity beads. The whole cell lysates and the histidine-tagged purified proteins were then analyzed by Western-blot using anti-GFP antibody. We detected bands of the expected size corresponding to ARF-SUMO2 only in in the purified extracts of those cells co-transfected with His6-SUMO2 (Figure 13C). These results suggested that p14ARF is modified by SUMO2 in transfected cells (Figure 13C).

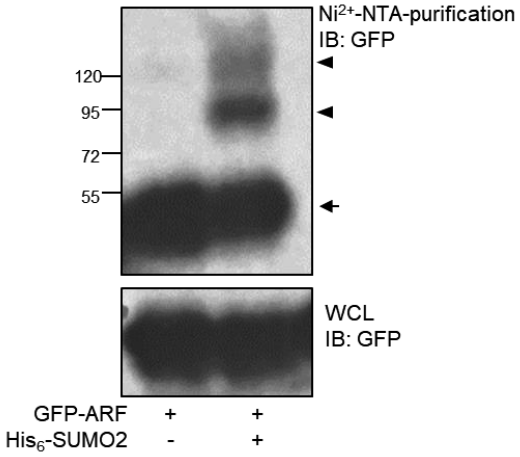

Figure 13C. ARF is modified by SUMO *in vivo*

HEK-293 cells were co-transfected with GFP-ARF together with pcDNA or Ubc9 and His6-SUMO2. Whole protein extracts and histidine-tagged purified proteins were analyzed by Western-blot using anti-GFP antibody. Arrow and arrowheads indicate the unmodified and SUMO conjugated ARF protein, respectively.

13.4 *IN VIVO* SUMOYLATION OF 14ARF IN A TAG-INDEPENDENT MANNER

Since the GFP tag contains 20 lysine residues, and in order to avoid a putative conjugation of SUMO to the GFP tag in GFP-ARF, we decided to analyze the *in vivo* SUMOylation of p14ARF-HA. We co-transfected HEK-293 cells with p14ARF-HA together with pcDNA3 or Ubc9 and His6-SUMO2. At 48 h after transfection the cells were harvested, lysed in denaturing conditions and the histidine-tagged proteins were purified using nickel affinity beads. Whole cells lysates and histidine-purified proteins were then analyzed by Western-blot using anti-HA antibody. We detected the unmodified ARF protein as a band of around 20 kDa molecular weight, as expected (Figure 13D, left panel). When the reaction was incubated with SUMO2 we observed the appearance of two higher molecular weight bands of around 34 and 50 kDa (Figure 13D, left panel), suggesting that p14ARF is modified by SUMO2 *in vivo*. Finally, we also carried out an *in vivo* SUMOylation assay using untagged p14ARF. We co-transfected HEK-293 cells with p14ARF together with pcDNA3, Ubc9 and His6-SUMO1 or Ubc9 and His6-SUMO2. At 48 h after transfection the cells were harvested, lysed in denaturing conditions and the histidine-tagged proteins were purified using nickel affinity beads. Whole cell lysates and histidine-purified proteins were then analyzed by Western-blot using anti-p14ARF antibody. Bands of the expected size corresponding to p14ARF-SUMO1 or p14ARF-SUMO2 were detected in the purified extracts, confirming that SUMO conjugates to ARF (Figure 13D, right panel). Altogether confirming that ARF is SUMOylated *in vitro* and *in vivo*.

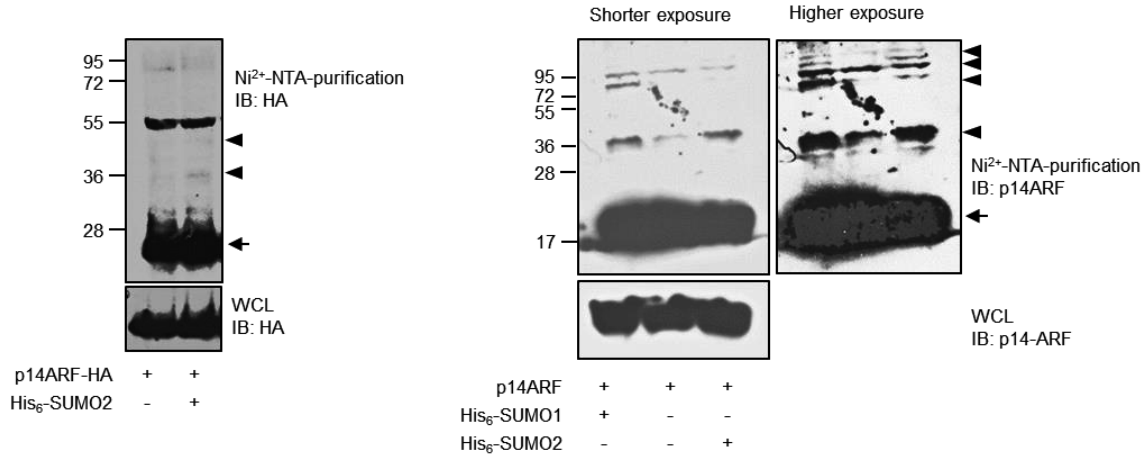

Figure 13D. SUMO conjugation to p14ARF *in vivo* is tag-independent

HEK-293 cells were co-transfected with ARF-HA (left panel) or untagged ARF (right panel) together with pcDNA, Ubc9 and His6 SUMO2 or Ubc9 and His6 SUMO1, as indicated. Whole protein extracts and histidine-tagged purified proteins were analyzed by Western-blot using the indicated antibodies. Arrow and arrowheads indicate the unmodified and SUMO conjugated p14ARF protein, respectively.

14. NEDD8 INHIBITOR MLN4924 TREATMENT INDUCES THE UPREGULATION OF UBC9 LEVELS AND INCREASES GLOBAL SUMOYLATION

Our results revealed the existence of an interplay between SUMO and NEDD8 and we hypothesized that SUMO may play a role in the regulation of NEDD8 function**.** Treatment with the chemotherapeutic drug MLN4924 has been reported to inhibit NEDDylation and to trigger apoptosis or decrease cell poliferation and migration of different types of cancer (Bhatia et al., 2016; Soucy et al., 2010; Swords et al., 2018; Tong et al., 2017). We then spectulated that SUMO may have a role in the activities of the NEDD8 inhibitor. To test this hypothesis, we evaluated the global NEDDylation and SUMOylation in cells treated or not with the NEDDylation inhibitor. The prostate cancer cell line PC3 was treated with MLN4924 (10 μM) for three days and then the whole cell lysate was analyzed by Western-blot using anti-NEDD8, anti-SUMO2, anti-Ubc9 or anti-GAPDH antibodies. We observed a clear reduction in the global NEDDylation, as expected. We also observed a clear increase in Ubc9 levels and in global SUMOylation (Figure 14A), suggesting that SUMO may play a role in the activities of the NEDD8 inhibitor.

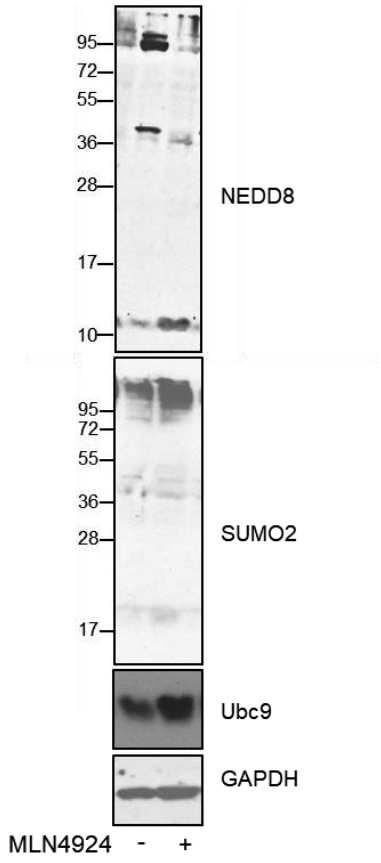

Figure 14A. NEDD8 inhibitor upregulates UBC9 levels and increases global SUMOylation
PC3 cells were treated or not with MLN4924 (10 μM) for three days. Whole cell lysate were then analyzed by Western-blot using the indicated antibodies

The result showing an increase in Ubc9 levels in response to NEDD8 inhibitor led us to speculate whether Ubc9 protein could be NEDDylated. In order to evaluate NEDDylation of Ubc9, we co-transfected HEK-293 with V5-Ubc9 together with pcDNA or His6-NEDD8 and 36 h after transfection, histidine-tagged proteins were purified under denaturing conditions. We could not detect bands corresponding to NEDDylated Ubc9 in the purified extracts (Figure 14B). Altogether these results suggest that the upregulation of Ubc9 levels upon treatment with NEDDylation inhibitor may not be related with a downmodulation in Ubc9 NEDDylation.

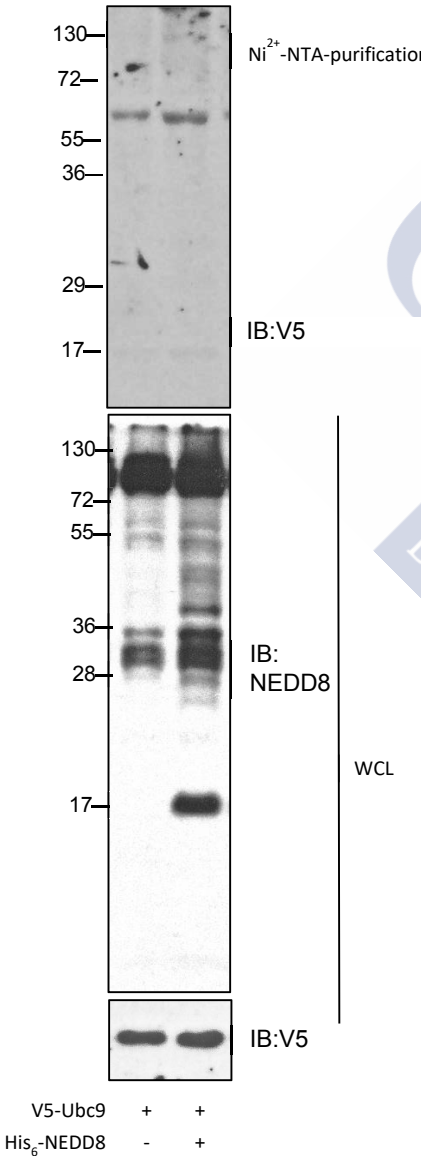

Figure 14B. Evaluation of the NEDDylation of Ubc9 *in vivo*

HEK-293 cells were co-transfected with V5-Ubc9 together with pcDNA3 or His6-NEDD8. At 36 h after transfection, histidine-tagged purified proteins were analyzed by Western-blot using the indicated antibodies.

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DISCUSSION

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Ribosome biogenesis is a key component to regulate overall protein synthesis and cell growth, requiring tight regulation. Alterations in ribosome biogenesis can be induced via multiple mechanisms in response to a variety of stress signals. The checkpoint elicited as a response to these conditions is due to the ability of some ribosomal proteins to be released from the nucleolus to the nucleoplasm where they bind to MDM2 and inhibit MDM2-mediated p53 ubiquitination and degradation, resulting in the activation of p53 and cell cycle arrest (Dai & Lu, 2004; Horn & Vousden, 2008; Lohrum et al., 2003; Sundqvist et al., 2009; Zhang et al., 2003). The importance of these findings to human pathology has been underscored in ribosomopathies and developmental defects caused by ribosome biogenesis alterations that result from a mutation in ribosomal proteins.

One of the ribosomal proteins required for the p53 activation in response to ribosomal stress is RPL11 (Dai & Lu, 2004; Horn & Vousden, 2008; Lohrum et al., 2003; Sundqvist et al., 2009; Zhang et al., 2003). Translocation of RPL11 outside the nucleolus was though initially to be a passive event caused by alterations in nucleolar structure associated with the different stresses (Yuan et al., 2005). However, later on, it was demonstrated that the nucleolar structure was not altered, indicating that it is a regulated event (Fumagalli et al., 2009). How the translocation of RPL11 from the nucleolus to the nucleoplasm is regulated is unclear. Here we decided to evaluate whether SUMO plays a role in this process. First, we demonstrated that RPL11 can be modified by SUMO1 and SUMO2 *in vitro* and *in vivo*. We attempted to create an RPL11 SUMOylation mutant to evaluate RPL11-SUMOylated function. However, our results revealed that even mutation of all the lysine residues in RPL11 did not abolish SUMOylation, suggesting the existence of a novel non-canonical SUMOylation pathway. Conjugation of ubiquitin in a lysine residue-independent manner has been already reported (Kuo et al., 2004); however, so far, this is the first description of SUMOylation in a non-lysine residue. We have purified a SUMOylated lysine-less mutant RPL11 (RPL11-K0) protein from transfected cells and we have sent the samples for mass spectrometer analysis. Although RPL11 peptides were found, confirming the SUMOylation of the RPL11-K0 mutant protein, we were not successful in the identification of the SUMOylation site.

It has been previously reported that RPL11 protein can be regulated by conjugation to the ubiquitin-like protein NEDD8 and that mutation of all lysine residues in RPL11 was required to detect a reduction in the NEDDylation of the protein (Sundqvist et al., 2009). NEDDylation assay carried out with RPL11-K0 revealed that RPL11 can be also NEDDylated in a lysine residue independent-manner. Therefore, we speculated that NEDD8 and SUMO might compete for conjugation to RPL11. Competition experiments, as well as treatment with NEDDylation or SUMOylation inhibitors, revealed that SUMO negatively regulates the NEDDylation of RPL11. Although SUMOylation of RPL11 was positively modulated after NEDDylation inhibitor treatment, suggesting that NEDD8 also negatively regulates the SUMOylation of RPL11, this modification was not reduced by NEDD8 overexpression. We speculate that the RPL11 stabilization or nucleolar localization promoted by NEDD8 (Sundqvist et al., 2009) may have a positive impact on RPL11 SUMOylation. Different points of crosstalk may occur between SUMOylation and NEDDylation, including competition for the same amino acid residue in a substrate or regulation of the NEDD8 conjugation machinery by SUMO. We did not detect a clear competition between SUMO2 and NEDD8 to conjugate to RPL11-K0, suggesting that the SUMO-NEDD8 interplay on RPL11 requires lysine residues in RPL11 to occur.

It has been previously reported that NEDD8 conjugation to RPL11 retains the ribosomal protein inside the nucleolus in unstressed cells and that the protein is deNEDDylated in response to ribosomal stress (Sundqvist et al., 2009). We observed that ribosomal stress promoted the SUMO2 modification of RPL11. Moreover, we also observed that overexpression of SUMO2 promoted the translocation of RPL11 from the nucleolus to the nucleoplasm, supporting the existence of an antagonistic relationship between NEDDylation and SUMOylation on RPL11. As mentioned before, nucleoplasmic RPL11 binds MDM2 and promotes p53 activation (Dai & Lu, 2004; Horn & Vousden, 2008; Lohrum et al., 2003; Sundqvist et al., 2009; Zhang et al., 2003). Our data showed that the SUMO ligase Ubc9 was required for the stabilization and activation of p53 in response to RPL11 overexpression. However, SUMO has been previously shown to modulate several components of the RPL11-MDM2-p53 pathway, including p53 (L. Chen & Chen, 2003; Rodriguez et al., 1999; Santiago et al., 2013; Stindt et al., 2011) or MDM2 (Xirodimas et al., 2002). Therefore, we cannot exclude that the negative effect of Ubc9 downmodulation on the stability or activity of p53 we

observed, is due to SUMOylation inhibition of other factors and not of RPL11. In addition, it has been reported that RPL11 can associate with RPL5 via 5S rRNA and that this preribosomal complex is essential for p53 activation upon impairment of ribosome biogenesis (Donati et al., 2013; Horn & Vousden, 2008). It will be interesting to evaluate then how SUMO affect the relationship of RPL11 with RPL5 and 5SRNA and to determine whether SUMO can also regulate the other components of the preribosomal complex.

Although RPL11 is mainly known as a key protein in the control of p53 activation in response to ribosomal stress, some reports demonstrate that RPL11 is also required for oncogenic or replicative stress-induced activation of p53 (Nishimura et al., 2015) and for activation of p53 by ARF (Dai et al., 2012). The molecular mechanisms underlying the RPL11 mediated p53 activation upon replicative or oncogenic stress are not known. One proposed explanation is that the increase in ARF levels resulting from replicative or oncogenic stress induces ribosomal stress resulting in RPL11 suppression of MDM2(Dai et al., 2012; Nishimura et al., 2015). We explored here whether the ability of ARF to trigger SUMOylation of its interactors and to enhance global SUMO conjugation (Alagu et al., 2018; Tago et al., 2005; S. Wang et al., 2015) play a role in the RPL11-mediated activation of p53 induced by ARF. We show here that upregulation of ARF triggers SUMO2 modification of RPL11, leading us to propose that promotion of RPL11 SUMOylation by ARF which in turns leads to RPL11 translocation to the nucleoplasm may be a molecular link between the oncogenic or replicative stress and the activation of p53. Also, we showed that ARF overexpression downmodulated RPL11 NEDDylation and global NEDDylation. A negative effect of ARF on global NEDDylation lead us to propose the existence of a complex interplay between SUMOylation and NEDDylation and to suggest that ARF may be a key regulator of SUMO-NEDD8 crosstalk. We then wondered whether the interplay between SUMO and NEDD8 and its modulation by ARF can also affect other ribosomal proteins. Here we show that RPL23 can be modified by SUMO and NEDD8. Competition experiments also revealed that SUMO negatively regulates the NEDDylation of RPL23 and that ARF downregulates RPL23 NEDDylation while promotes its SUMOylation, as we observed for RPL11. We do not know the effect of SUMO or NEDD8 conjugation on RPL23. However, evaluation of the subcellular localization of the protein after SUMO overexpression revealed that SUMO promotes

RPL23 translocation to the nucleoplasm, as shown for RPL11. RPL23 can bind to MDM2 but if this binding results in the stabilization of p53 in response to ribosomal stress is not clear (Dai & Lu, 2004; Dai et al., 2004; Jin et al., 2004). While RPL23 has been reported to work as a molecular link between RAS signaling to p53 activation (Meng et al., 2016), RPL23 was not required for the p53 activation in response to ribosomal stress (Bhat et al., 2004). Additional studies to uncover the regulation of the RPL23 SUMOylation and to determine the consequences of the translocation of RPL23 outside of the nucleoli are needed.

The mechanism by which ARF increases SUMOylation is not clearly known. Here we show that upregulation of ARF leads also to a decrease in RPL11, RPL23 or p53 NEDDylation and, importantly, in global deNEDDylation, correlating with an increase in general SUMOylation. Downmodulation of NEDD8 conjugation by ARF may result from the upregulation in the SUMOylation mediated by the tumor suppressor. Trying to clarify the interplay between this two post-translational modifications, we studied the putative existence of mixed SUMO-NEDD8 chains. We did not observe NEDDylation of SUMO or Ubc9 proteins. Although *in vitro* SUMOylation assays using NEDD8 as a substrate revealed the appearance of higher molecular weight bands only after incubation with SUMO1 or SUMO2, the molecular weight of some of the bands did not correspond with the expected size of NEDD8-SUMO2 protein. Therefore, we cannot conclude whether NEDD8 protein can be SUMOylated or whether SUMO induces the NEDDyylation of NEDD8. However, our data reveals a complex SUMO-NEDD8 interplay and suggest that SUMO may have an impact on NEDDylation. In addition, the increase in the levels of Ubc9 and global SUMOylation as a response of the treatment of PC3 human prostate cancer cell line with the NEDD8 inhibitor led us to hypothesize that SUMO may play an important role in the anti-cancer effect exerted by the NEDD8 inhibitor.

It has been proposed that ARF may enhance the SUMOylation by promoting the interaction of the SUMO substrates with Ubc9 (Helen Rizos et al., 2005). This suggestion and our results revealing that SUMO may covalently conjugate to a non-lysine residue led us to hypothesize that ARF itself may be a SUMO substrate. Our data revealed that, indeed, ARF can be SUMOylated *in vitro* and *in vivo*. However, we consistently observed low levels of SUMOylated ARF protein *in vivo,* suggesting that

it may require a specific stimulus or a SUMO ligase. Deciphering the stimuli or the SUMO ligase which triggers ARF SUMOylation and the consequences of SUMOylation on ARF activities will be one of our main objectives in the future.

In summary, we show here that SUMO is a key regulator of RPL11 protein-driven p53-mediated responses to nucleolar stress and it may be also an essential player in the induction of the RPL11-MDM2-p53 pathway in response to oncogenic stress. This is not the unique role of SUMO in the nucleolus since at least another ribosome component, the RPL23 protein, and the nucleolar tumor suppressor protein ARF can be also modulated by SUMO. In addition, this study has led us to advance in the knowledge of post-translational modifications by ubiquitin-like proteins demonstrating that SUMO can bind to a substrate in lysine independent manner and that there is an interplay between SUMOylation and NEDDylation.

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CONCLUSIONS

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1. Ribosomal RPL11 protein is modified by SUMO1 and SUMO2 *in vitro* and *in vivo*.
2. SUMO is conjugated to a non-lysine residue in RPL11.
3. SUMO and NEDD8 compete to conjugate to RPL11.
4. SUMO2 promotes nucleolus to nucleoplasm translocation of RPL11
5. Ubc9 is required for the activation of p53 in response to RPL11.
6. Nucleolar stress promotes the conjugation of RPL11 to SUMO2.
7. ARF upregulates the SUMO2 modification of RPL11 and downmodulates the NEDDylation of RPL11.
8. The ribosomal protein RPL23 can be modified by SUMO and NEDD8.
9. SUMO2 promotes the nucleolus to nucleoplasm translocation of RPL23.
10. SUMO and NEDD8 compete to conjugate to RPL23.
11. ARF promotes the SUMO2 modification of RPL23 and downmodulates the NEDDylation of RPL23.
12. There is an interplay between SUMO and NEDD8 conjugation.
13. p14ARF tumor suppressor protein is modified by SUMO *in vitro* and *in vivo*.
14. Inhibition of NEDDylation regulates global SUMOylation.

1. La proteína ribosómica RPL11 se modifica por SUMO1 y SUMO2 *in vitro* e *in vivo*.
2. SUMO se conjuga a un residuo que no es lisina en RPL11.
3. SUMO y NEDD8 compiten para conjugarse con RPL11.
4. SUMO2 promueve la translocación de RPL11 desde el nucleolo al nucleoplasma.
5. Ubc9 es necesaria para la activación de p53 en respuesta a RPL11
6. El estrés nucleolar favorece la modificación de RPL11 por SUMO2.
7. ARF promueve la modificación de RPL11 por SUMO2 y disminuye la NEDDilación de RPL11.
8. La proteína ribosomal RPL23 se modifica por SUMO y por NEDD8.
9. SUMO2 promueve la translocación de RPL23 desde el nucléolo al nucleoplasma.
10. SUMO y NEDD8 compiten para modificar RPL23.
11. ARF promueve la SUMOilación e inhibe la NEDDilación de RPL23.
12. Existe una compleja relación entre la conjugación de SUMO y NEDD8.
13. El supresor de tumores ARF es modificado por SUMO *in vitro* e *in vivo*.
14. La inhibición de la NEDDilación regula la SUMOilación global.

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